THE METABOLIC BASIS FOR PORPHYRIA CUTANEA TARDA:

CORRELATION BETWEEN THE HUMAN DISEASE AND HEXACHLOROBENZENE-INDUCED RAT PORPHYRIA.

By

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A T H E S I S

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TO NOELENE

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ABBREVIATIONS USED IN THIS THESIS

AIA	-	Allylisopropylacetamide
AIP	-	Acute Intermittent Porphyria
ALA	-	δ-Aminolaevulinic Acid
ALA-S	-	δ-Aminolaevulinic Acid Synthetase
ATP	-	Adenosine Triphosphate
CoA	-	Coenzyme A
COPRO	-	Coproporphyrin
COPROGEN	-	Coproporphyrinogen
EDTA	-	Ethylenediamine Tetra-acetic Acid
HCB	-	Hexachlorobenzene
NAD	-	β-Nicotinamide Adenine Dinucleotide
NADP	-	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	-	Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form.
PBG	-	Porphobilinogen
PCT	-	Porphyria Cutanea Tarda
PP	-	Protoporphyria
PROTO	-	Protoporphyrin
PROTOGEN	-	Protoporphyrinogen
SGOT	-	Serum Glutamate Oxalacetate Transaminase
TLC	-	Thin Layer Chromatography
tris	-	tris - (Hydroxymethyl) - Aminomethane
URO	-	Uroporphyrin
UROGEN	-	Uroporphyrinogen
VP	-	Variegate Porphyria
4-COOH	-	Tetracarboxylic
5-COOH	-	Pentacarboxylic
6-COOH	-	Hexacarboxylic
7-COOH	-	Heptacarboxylic

CONTENTS

CHAPTER 1

Introduction

South Africa has the highest world prevalence of porphyria cutanea tarda (PCT)[70], a disorder of haem metabolism. The disease presents as a purely cutaneous disorder, with excessive fragility of the skin, which results in considerable disability especially in manual workers. PCT is one of the hepatic porphyrias, as classified in recent reviews[193,219], and occurs in many thousands of patients in South Africa[74]. Thus in spite of relatively infrequent occurrence on a world-wide basis[193], this disorder constitutes a major health problem in this country.

The disease occurs mostly as an unusual accompaniment of common hepatic disorders e.g. alcoholic liver disease. However, there have been reports of epidemics of PCT in groups of people when they were exposed to porphyrogenic hepatotoxins[21,57,251]. One of these toxins is hexachlorobenzene (HCB), which caused an outbreak of PCT in Turkey when seed wheat treated with a fungicide containing HCB was used for human consumption[57]. The disturbance of haem metabolism in PCT biochemically manifests itself as a unique pattern of porphyrin overproduction and excretion. In particular, there is a marked increase of urinary URO and heptacarboxylic porphyrin excretion[74], which reflects the accumulation of these porphyrins in the liver[23,64,65]. In addition, there is a significant quantity of porphyrin P_1 fraction (isocoproporphyrin and de-ethylisocoproporphyrin) excreted in the faeces[79].

The main purpose of this thesis is to study the derangement of haem metabolism in PCT. In order to amplify necessarily limited data from humans, experimental data was obtained from rats treated with HCB, so that data from both sets of observations could be correlated. It has been shown that experimental porphyria develops in rats treated with HCB[210, 252,253,255], and furthermore,there are certain similarities between idiosyncratic PCT and HCB-induced rat porphyria. Firstly, there is the characteristic urinary pattern of porphyrin excretion both in PCT and in HCB-treated rats, including large amounts of porphyrins with 8,7,6 and 5 carboxyl groups[74,210,246]. Secondly, the isocoproporphyrins are present in the faeces[77,78,79], and thirdly, livers are morphologically abnormal in both situations[143,144,186,250,253, 307,311]. Differences between PCT and HCB-induced rat porphyria are principally in the isomer composition of excreted porphyrins and liver porphyrins, these being mainly of the III series in the rat[246], whereas in PCT URO is mainly of the isomer I series, with other porphyrins being mixtures of both series of isomers[65].

Because of the biochemical similarities between human PCT and HCB-induced rat porphyria, it was decided to determine whether biochemically the HCB-rat "model" resembled the human disease on more detailed studies. If this were to be borne out, then some basic aspects of the metabolic disorder in the HCB-intoxicated rat might point the way to finding a similar defect in PCT which may allow for a more rational approach to the understanding and management of the latter.

CHAPTER 2

THE PORPHYRIAS - WITH SPECIAL REGARD TO PCT AND HCB-INDUCED PORPHYRIA

2.1 Definition and Classification of the Porphyrias

The porphyrias may be defined as a group of acquired or inherited clinical illnesses, characterised by porphyrin and/or porphyrin-precursor overproduction, accumulation and excretion. Classification of the porphyrias is based on clinical manifestations and unique patterns of porphyrin and porphyrin-precursor excretion in urine and faeces. (Fig. I).

Porphyrins are haem precursors[38], haem being the prosthetic group of haemoproteins, whose major functions are transport and utilisation of oxygen. The major sites of haemoprotein synthesis in mammals are the liver and bone marrow, so that in the porphyrias, metabolic derangement of haem biosynthesis manifests principally in the liver or bone marrow or both. In 1954, Schmid et al[257] proposed a classification of the porphyrias into 2 groups, viz. hepatic and erythropoietic. A third group, the erythrohepatic porphyrias, was proposed[83,219] because of increasing evidence of liver involvement in erythropoietic protoporphyria. A working classification of the porphyrias is given in Table 1.

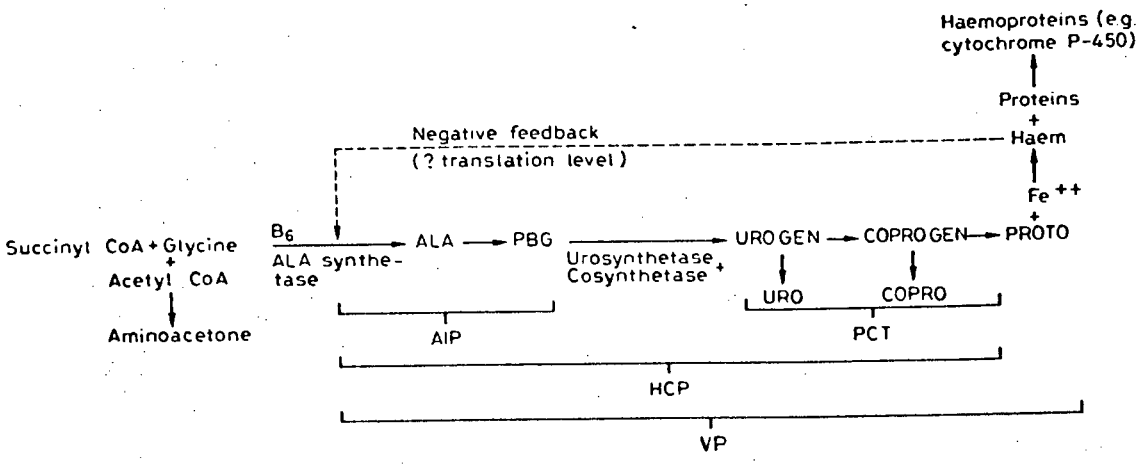

Figure 1 Biosynthetic pathway of haem illustrating the pattern of precursor excretion in the hepatic porphyrias and the regulation of ALA-S by the end product, haem. ALA = δ-Aminolaevulinic acid; PBG = porphobilinogen; URO'GEN = uroporphyrinogen; COPRO'GEN = coproporphyrinogen; URO = uroporphyrin; COPRO = coproporphyrin; PROTO = protoporphyrin IX; AIP = acute intermittent porphyria; PCT = porphyria cutanea tarda; HCP = hereditary coproporphyria; VP = variegate porphyria.

TABLE 1

Classification of the Porphyrias, together with Synonyms

I. Erythropoietic Porphyria
   Congenital erythropoietic porphyria (congenital photosensitive porphyria, Gunther's disease).

II. Hepatic Porphyria
   A. Genetic autosomal dominant porphyrias
      1. Acute intermittent porphyria (intermittent acute porphyria, acute porphyria, Swedish porphyria, pyrroloporphyria).
      2. Variegate porphyria (porphyria variegata, South African genetic porphyria, mixed hepatic porphyria, porphyria cutanea hereditaria, protocoproporphyria).
      3. Hereditary coproporphyria.

   B. Porphyria cutanea tarda (symptomatic porphyria, acquired porphyria, constitutional porphyria, idiosyncratic porphyria, toxic porphyria, uro-coproporphyria, porphyria cutanea tarda symptomatica, porphyria cutanea chronica, Bantu porphyria).
      1. Idiosyncratic
      2. Toxic
      3. Porphyrin-producing hepatic neoplasms.

III. Erythrohepatic protoporphyria (erythropoietic protoporphyria, protoporphyria).

A secondary type of coproporphyrinuria can occur in other conditions and does not in itself produce symptoms or classic signs of cutaneous or acute porphyria[219].

A number of reviews on the porphyrias have appeared[16,57,68, 70 72,83,107,193,219,293,303] in which the clinical manifestations and diagnosis of the different types of porphyria have been well documented.

2.2 PCT - Historical Considerations

Gunther[118] in 1911 described for the first time in the literature the disease now known as PCT. At the time the disease was designated "haematoporphyria chronica", although he later considered this disease a mild and late form of porphyria congenita[119], now known as congenital (erythropoietic) porphyria. Waldenström[315] introduced the term "porphyria cutanea tarda" (PCT) in place of Gunther's "chronic porphyria" to describe a group of patients who developed a cutaneous form of porphyria after puberty. This term clearly differentiated "chronic porphyria" from congenital erythropoietic porphyria, which was present from birth, and from acute intermittent porphyria, in which cutaneous symptoms do not occur. It was established by reports subsequent to that of Waldenström that PCT was principally a disease of men over the age of 40, who frequently gave a history of alcoholism and showed evidence of impairment of liver function. Furthermore, these patients suffered no attacks of acute porphyria and a family history of porphyria was uncommon[35, 36, 292].

Much confusion has arisen out of the term "porphyria cutanea tarda". The term implies a slow or late onset of cutaneous porphyria, although cases of PCT have been reported in children[11,70], and furthermore, the outbreak of PCT in Turkey was primarily a disease of children and adolescents[57]. Until 1958, the hereditary porphyria now known as variegate porphyria was included with PCT[132,188]. Thus in earlier reports[42,63,113,132,188,239,319,325] cases of PCT were described where skin lesions and acute attacks occurred but the patients were, in general, younger than 40, and there was familial evidence of porphyria. In the United States of America these cases

were described as "mixed porphyria"[320] and the term "porphyria cutanea tarda" was used only for cutaneous "middle-aged alcoholic" porphyria. Waldenström[316] in 1957 recognised that the disease that had hitherto been referred to as "porphyria cutanea tarda" was in fact a mixed group of two diseases. Patients with skin lesions and acute attacks were reclassified as "porphyria cutanea tarda hereditaria". Those patients in whom the disease manifested by skin lesions only and in whom there was an onset of porphyria later in life, were reclassified "porphyria cutanea tarda symptomatica". Dean and Barnes[58] in 1958 described cases of "porphyria cutanea tarda hereditaria" in South Africa as "porphyria variegata" because of variety of presentation of this disease. Porphyria variegata or variegate porphyria was shown to have an autosomal dominant pattern of inheritance[56,69]. It was later shown[75,288] that the porphyrin excretion patterns in "porphyria variegata" and "porphyria cutanea tarda symptomatica" were quite distinct, so that the existence of a purely cutaneous form of hepatic porphyria became established.

In recent years, the condition has been called "symptomatic cutaneous hepatic porphyria"[83] or "symptomatic porphyria[69]. With[327] has proposed "porphyria cutanea chronica" for the disease. I propose to use "porphyria cutanea tarda" (PCT) because I feel there has been no general agreement on terminology to describe the disease. However, the term porphyria cutanea tarda (PCT) is to be construed in its strictest sense, i.e. to describe a purely cutaneous form of porphyria, usually with evidence of liver disease, and clinically with no clear-cut pattern of inheritance and with attendant biochemical abnormalities.

2.3. PCT - Clinical Considerations

Manifestations of PCT appear as a sporadic reaction to a variety of liver diseases. PCT presents as a purely cutaneous disorder, indistinguishable clinically from purely cutaneous variegate porphyria (VP) in remission, and excessive fragility of sun-exposed skin is characteristic in both. Even slight trauma leads to erosion formation and the development of blisters or bullae[67, 70, 219]. Patients with PCT develop no acute attacks with accompanying abdominal pain and neurological complications however, and reaction to drugs, which in patients with VP or acute intermittent porphyria can precipitate acute attacks, is normal[293]. Further, there is almost invariably evidence of hepatocellular disease, and on analysis a unique pattern of porphyrin excretion is found. There appears to be no evidence of PCT being a hereditary disease.

As shown in Table 1, PCT can be subdivided into 3 categories:

(a) Idiosyncratic PCT occurs as an unusual accompaniment of liver disease, frequently alcoholic liver disease with siderosis. PCT may follow Bantu siderosis, ingestion of oral contraceptives and immunopathic disorders usually involving connective tissue. The fact that only a minority of patients with these relatively common liver ailments have coexisting PCT might be indicative of an occult genetic abnormality[321]. However, there is little unequivocal evidence that idiosyncratic PCT is a purely inherited form of porphyria[74,219].

Idiosyncratic PCT in South Africa occurs mainly in the Bantu population[9,70, 164] but there are many cases in the Cape Coloured and White population groups[69]. PCT manifests mainly after the age of 45, but Eales[70] has reported two unrelated children of ages 8 and 15 years with the disease. Alcohol appears to play an important role in the genesis of PCT in about 90% of the

cases[70], but other factors need to be taken into account, viz. iron overload, malnutrition and protein deficiency[74]. A factor of great importance, and one which should be taken into account when considering the development of PCT in the Bantu of South Africa, is that members of this population group frequently partake of illicitly brewed "Kaffir Beer" which can contain porphyrogenic toxins[151].

In a study of 42 patients with PCT, histopathology revealed changes associated with alcoholic liver disease[311]. These changes reflected the whole range of alcoholic liver disease, with liver damage in the mildest form being fatty change. Siderosis was found to be invariably present. This finding has been substantiated by other authors[143, 144, 186, 250, 307]. Timme[298] has described lesions, thought to be typical of PCT, on electron microscopy of liver from patients with this disease.

Oral contraceptives have been implicated in the precipitation of idiosyncratic PCT[168,193], but although liver damage is present on histological examination, siderosis is unusual in these cases.

Immunopathic diseases which occur in association with PCT have been well described[219].

(b) Toxic PCT results usually from exposure to chemicals which can cause porphyria. The first clear instance of toxic PCT in man was the Turkish "epidemic" which has been well documented by Schmid[251] and Dean[57]. This disease affected several thousand individuals, predominantly children and adolescents, in 3 south-eastern provinces in Turkey, between 1955 and 1959. The children had blisters and sores on the hands and face, with dark pigmentation and particularly pronounced hairiness of the face. Hepatomegaly was present, and liver function tests showed impairment of liver function. Urine was dark red or brown, containing ether-soluble and ether-insoluble porphyrins, but no porphobilinogen (PBG) was present[57]. These findings were virtually identical to those in Bantu PCT[10,44]. Epidemiological studies indicated that the toxin causing the PCT was a fungicide

containing HCB, used in the treatment of wheat[57,251]. No new cases of porphyria have been reported from Turkey since 1960, which coincides with the HCB-treated wheat having been withdrawn in 1959. An additional factor which pointed to the HCB being the toxic agent was that rats when treated with this toxin developed experimental porphyria [210,252,253,255].

Toxic PCT has been reported in workers in a chemical factory where the herbicide 2,4,5-trichlorophenoxyacetic acid was manufactured[21]. The most likely causative agent in this outbreak of PCT would have been 2,3,7,8-tetrachlorodibenzo-p-dioxin[223a], a porphyrogenic contaminant which is frequently formed during the synthesis of 2,4,5-trichlorophenoxyacetic acid.

(c) A porphyrin-producing hepatic neoplasm, associated with a PCT-like syndrome has been reported by Tio et al[300]. The tumour fluoresced, and appeared to be the source of the increased porphyrin excretion, since removal of the neoplasm resulted in remission of the syndrome in the patient.

2.4. PCT - Biochemical Considerations

As has been stated, the porphyrias can be distinguished biochemically by virtue of the fact that in each of the porphyrias there is a characteristic pattern of porphyrin excretion (Fig. I). In patients with idiosyncratic PCT, there is a markedly increased urinary excretion of ether-insoluble porphyrins, principally URO (8-COOH) and heptacarboxylic porphyrin (7-COOH) and smaller amounts of hexa- and penta-carboxylic porphyrins (6-COOH and 5-COOH)[74]. Urinary quantities of δ-aminolaevulinic acid (ALA) and PBG in this condition are normal[9, 69, 164]. Faecal porphyrins are normal or moderately increased, but generally the COPRO (4-COOH) fraction exceeds

the PROTO (2-COOH) fraction[69,73,75,83,288]. Probably as much porphyrin is excreted by this route as in the urine[288]. URO is a minor component of faecal porphyrins in PCT and is of little diagnostic value[74]. Elder[77] showed that much of the faecal COPRO fraction in PCT consisted of a mixture of tetracarboxylated porphyrins, designated as P1, P2 and P3. Tentative identification showed P1 was a mixture of isocoproporphyrin and de-ethylisocoproporphyrin, P2 was hydroxyisocoproporphyrin and P3 was a hydrophillic conjugate of isocoproporphyrin[78]. The main porphyrins of this type present in the faeces were found to be isocoproporphyrin and de-ethylisocoproporphyrin[79], i.e. the P1 fraction.

Of the urinary porphyrins excreted in PCT, URO is about 70% isomer I, 7-COOH and 6-COOH porphyrins almost all isomer III, with 5-COOH porphyrin and COPRO 50% isomer I[65]. The large quantity and the isomer composition of URO in PCT is in contrast to that found normally, where URO I is produced and excreted in very small amounts.

In addition to the excretion of URO and 7-COOH porphyrin, there is an accumulation of these porphyrins in the liver, as might be expected. Liver biopsy samples from patients with PCT, viewed under ultra-violet light, show an intense red fluorescence[88,311]. On microscopic study, the fluorescence attributable to the porphyrins was found in the cytoplasm of the liver cells, but in a freely diffusible form[88]. An interesting, but unexplained phenomenon is that increased amounts of porphyrin can persist within the hepatocyte after complete clinical and biochemical remission[88]. On analysis of porphyrins present in PCT liver, mainly URO and 7-COOH porphyrin are found[23,64,65], with URO principally in the isomer I form. The large quantity of porphyrin present in the liver,

especially URO, underlies the unique response of patients with PCT to chloroquine administration. In therapeutic doses, chloroquine produces transitory liver damage associated with nausea, vomiting and fever. This is accompanied by massive uroporphyrinuria[291] because chloroquine forms a water soluble complex with URO, which is readily excreted in the urine[261].

2.5. PCT - Treatment

Treatment resulting in clinical and biochemical remission of this condition has been well documented[83,219]. Withdrawal of the offending agent in the specific PCT syndromes may lead to clinical and biochemical improvement although the syndrome may persist in toxic PCT and in alcoholic liver disease with idiosyncratic PCT, if underlying liver disease has been established. The removal of storage iron by repeated venesection over a period of a few months produces a clinical and biochemical remission in the majority of patients with idiosyncratic PCT.

2.6 HCB Porphyria in the Rat

As has been stated in section 2.3.(b), experimental porphyria developed in rats treated with HCB[210,252,253,255]. Experimental porphyria has been induced also with HCB in guinea pigs, mice, rabbits[62], in chick embryo liver cultures[108] and in birds[286].

Rats treated with HCB for a prolonged period of time (4 - 8 weeks) have hepatomegaly, tremor, ataxia, weakness and paralysis[290], and show a high mortality rate[210]. There is a marked increase in the excretion of urinary porphyrins in these rodents[210]. The relative urinary excretory pattern of porphyrins in rats treated

with HCB is URO>COPRO>7-COOH>5-COOH>6-COOH, with these porphyrins being of the isomer III series. There is accumulation of porphyrin in the livers, the major porphyrins are URO III and 7-COOH porphyrin [246]. Faeces have been shown to contain the porphyrin P1 fraction i.e. isocoproporphyrin and de-ethylisocoproporphyrin[78].

Livers of rats that have been treated chronically with HCB, show fluorescence in islands of tissue surrounding the central veins, when viewed under a fluorescent microscope[290]. Liver damage is apparent on light microscopy, with focal necrosis and increased size and number of Kupffer cells[253]. An increase in smooth endoplasmic reticulum is shown on electron microscopy[290]. Marked induction of hepatic cytochrome P450 has been reported in rats after treatment with HCB[23,175,231,282,290,308].

CHAPTER 3

3.1. Aims of the Study

The aims of this investigation were threefold:

(a) To study disordered haem biosynthesis in patients with PCT. Parameters studied were:

(i) Hepatic ALA-synthetase (ALA-S) activity.

(ii) Hepatic cytochrome P450 levels.

(iii) Hepatic cytochrome P450-dependent enzyme systems which metabolise aminopyrine and 3,4-benzpyrene.

(iv) Liver ultrastructure by electron microscopy.

(v) Degree of liver damage and liver fluorescence by light and ultraviolet microscopy.

(b) To study disordered haem biosynthesis in HCB-induced rat porphyria. Parameters studied were:

(i) Urinary, faecal and hepatic porphyrins.

(ii) Hepatic cytochrome P450 levels.

(iii) Hepatic cytochrome P450-dependent enzyme systems which metabolise aminopyrine and 3,4-benzpyrene.

(iv) Hepatic uroporphyrinogen (UROGEN) I decarboxylase.

(v) Effects of iron on hepatic UROGEN I decarboxylase and the effects on faecal and hepatic porphyrins.

(c) To study the activity of UROGEN I decarboxylase in red blood cells from patients with PCT and their relatives to determine whether measurement of this enzyme provides a sensitive means of testing any genetic predisposition for development of PCT.

3.2. Rationale for the Study

Haem formation is regulated (Fig. 1) by the activity of the first and rate-limiting enzyme in the haem biosynthetic pathway, ALA-S[108,111,192]. This is repressed by haem, the end product, probably at a level of translation[248,284,309]. The unique porphyrin excretory pattern in PCT (Fig. 1) suggests impaired de-carboxylation of UROGEN to PROTO The increased porphyrin production in PCT is not clearly understood and conflicting data has been published relating to the activity of hepatic ALA-S[66,149,168,204,283,285,328]. For this reason, the activity of this enzyme was re-investigated, using a highly specific method of assay.

Further to the suggestion that the biochemical defect in PCT would relate to a partial block in the conversion of UROGEN to PROTO in the haem biosynthetic pathway, a more direct assessment of hepatic haem synthesis in this syndrome was attempted by measurement of levels of hepatic cytochrome P450. Cytochrome P450 is a rapidly turning-over microsomal haemoprotein, and its synthesis would be expected to require a considerable quantity of haem and its precursors. Thus, by measurement of hepatic cytochrome P450 in patients with PCT, it might be anticipated that any partial block in haem synthesis would be reflected by the levels of this haemoprotein. Furthermore, it was important to measure hepatic cytochrome P450 in PCT in view of its regulatory role in haem biosynthesis (Fig. 1, Section 4.5.).

Evidence obtained early in this investigation indicated that an alternative form of cytochrome P450 might be present in the livers of patients with PCT. Cytochrome P450 is an integral component of the microsomal drug metabolising system, which catalyses a series of widely diverse reactions[105, 190]. It has been shown that in rats

there are at least 2 forms of cytochrome P450, one form inducible with phenobarbital and the other, cytochrome P448, inducible with 3-methylcholanthrene[156,189]. The administration of phenobarbital to rats has been reported to enhance almost all reactions catalysed by cytochrome P450, including the metabolism of ethylmorphine, hexabarbital, aminopyrine, acetanilide and 3,4-benzpyrene[105]. However, administration of 3-methylcholanthrene enhanced relatively few reactions; the metabolism of acetanilide and 3,4-benzpyrene was enhanced, but not that of ethylmorphine, hexabarbital and aminopyrine[105]. An investigation of the functional capacity of hepatic cytochrome P450-dependent enzyme systems to metabolise aminopyrine and 3,4-benzpyrene in patients with PCT may therefore reveal the presence of a different type of cytochrome P450, if the situation as regards cytochrome P450 and cytochrome P448 with respect to microsomal drug metabolism in rats were extrapolated to man. It was considered impractical to determine spectrally whether another form of cytochrome P450 was present in liver of patients with PCT, owing to the limited quantity of tissue available.

Data obtained and published during the course of this study indicated that levels of hepatic cytochrome P450 were significantly elevated in PCT[222] but not in other hepatic porphyrias or in non-porphyrics with alcoholic liver disease[22]. It was therefore important to attempt to correlate levels of hepatic cytochrome P450 seen in PCT with liver ultrastructure in the same tissue. Light and fluorescent microscopic observations were included in the investigation to corroborate findings of other authors[88,143,164, 279,299, 307,311].

A correlative investigation was attempted in HCB-induced rat porphyria in view of the similarities to PCT. Urinary, faecal and hepatic porphyrins were investigated in HCB-treated rats to verify the porphyric state of the animals and to corroborate published findings[80,210,246,252,253,255]. Levels of hepatic cytochrome P450 and the ability of the hepatic microsomal drug metabolising system to metabolise aminopyrine and 3,4-benzpyrene were determined. Furthermore, an attempt was made by spectral measurements to ascertain whether a different form of cytochrome P450 might be present in the livers of rats treated with HCB.

Accumulation of URO and 7-COOH porphyrin in the livers of rats made porphyric with HCB may be indicative of a decrease in the activity of UROGEN-I decarboxylase, the enzyme which catalyses the stepwise decarboxylation of 4 acetate side chains of UROGEN III to give COPROGEN III. The possibility of a decrease in activity of this enzyme in HCB-treated rats was investigated by study of UROGEN-I decarboxylase. The UROGEN-I decarboxylase system was selected for this investigation since it has been shown that the kinetics for UROGEN-I and UROGEN III as substrates are virtually identical, using a decarboxylase from mouse spleen[242].

Hepatic iron appears to play an important role in the genesis of the porphyric process, especially in PCT complicating alcoholic liver disease[143,144,161,164,186,250,265,307,311]. This prompted an investigation as to the effects of iron in HCB-induced rat porphyria. The in vivo effect of iron on UROGEN-I decarboxylase and on urinary and hepatic porphyrins was studied by treating rats with HCB and iron simultaneously. Further, the in vitro effect of divalent iron on UROGEN-I decarboxylase was investigated.

There has been speculation as to the possibility of PCT being a genetically determined disease. There has been no documented evidence of an hereditary basis for PCT[219]. However, investigations as to this possibility have been directed to the study of urinary and faecal porphyrins of patients with PCT and those of their families[70]. Data has been obtained which suggests that diminished UROGEN I decarboxylase activity is the basic metabolic defect in PCT[158,159]. Reduced levels of this enzyme have been demonstrated in hepatic tissue [158,159, 160]and in red blood cells [158,160] of patients with PCT. Furthermore, diminished red cell UROGEN I decarboxylase has been recorded in clinically non-porphyric relatives of these patients, suggesting that the enzyme deficiency was inherited as an autosomal dominant trait [160, 158]. It was considered pertinent to determine the activity of red cell UROGEN I decarboxylase in patients with PCT and their families to determine whether an autosomal dominantly inherited defect in this enzyme does indeed play a role in the genesis of PCT in South Africa.

CHAPTER 4

HAEM BIOSYNTHESIS AND THE RELATIONSHIP OF CYTOCHROME P450 TO THIS BIOSYNTHETIC PATHWAY

The biosynthesis of haem for haemoprotein formation has been extensively reviewed by a number of authors [14,68,71,83,193,302]. It is my intention to give a broad overview of haem biosynthesis, pinpointing some uncertainties in this pathway, then to discuss in greater depth those areas which form the core of this work.

4.1. Haem Biosynthesis

The general pathway for haem biosynthesis is shown in Fig. 2. Initially, ALA is formed as a result of the combination of glycine and succinyl CoA which is derived from the citric acid cycle. This reaction, which occurs in the presence of pyridoxal phosphate as co-factor, is catalyzed by the enzyme ALA-S, after which ALA dehydratase catalyzes the condensation of 2 molecules of ALA to form the mono-pyrrole PBG. The enzymes UROGEN synthetase and UROGEN III cosynthe-tase, catalyze the formation of 1 molecule of UROGEN III from 4 mole-cules of PBG. UROGEN III subsequently undergoes a stepwise decarboxy-lation through 7-,6- and 5-COOH porphyrinogens to COPROGEN III, the enzyme involved being UROGEN III decarboxylase. Coproporphyrinogenase catalyses the formation of PROTOGEN IX from COPROGEN III, then PROTOGEN IX is oxidised to PROTO IX by protoporphyrinogen oxidase. Haem is formed from PROTO IX and Fe^{2+}, this reaction being catalyzed by ferro-chelatase. The haem thus formed can combine with various apoproteins

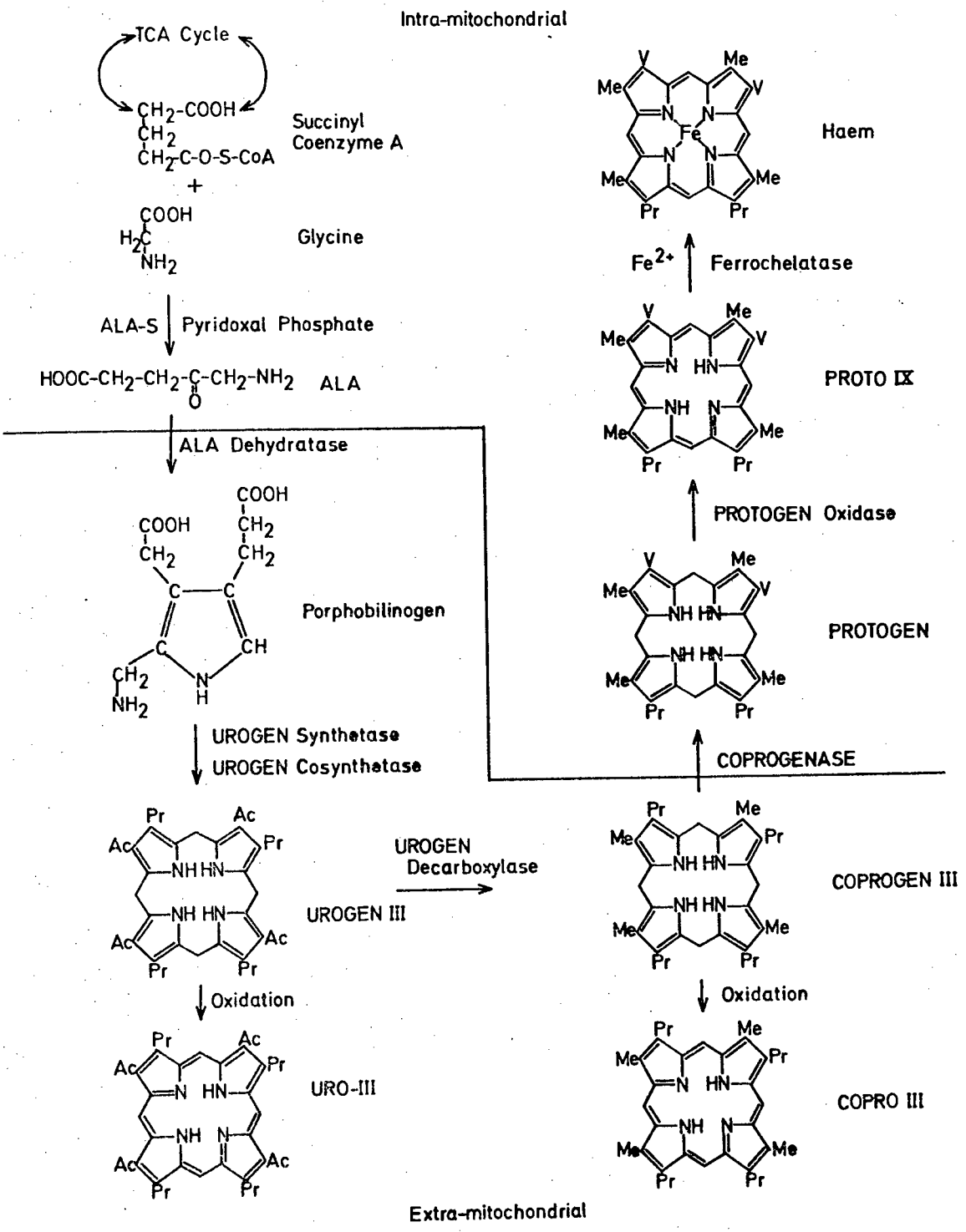

Figure 2 The biosynthetic pathway of haem. Ac = $-CH_2COOH$; Pr = $-CH_2CH_2COOH$; Me = $-CH_3$; V = $-CH{=}CH_2$.

to yield haem proteins, including haemoglobin, catalases and cytochromes.

Only those porphyrinogens of type III isomer are intermediates in the biosynthesis of haem. In the pathway UROGEN III can undergo oxidation to result in URO III, which is a side product and does not serve as a substrate for UROGEN decarboxylase. Further,it has been demonstrated that UROGEN III can be oxidised to URO III by photocatalytic auto-oxidation, a reaction accelerated by the product[110]. In addition, COPROGEN III may be oxidised to COPRO III, which again cannot be utilised in haem synthesis. Since the amount of porphyrins excreted by normal healthy individuals is small when compared to the overall magnitude of haem synthesis, it may be reasonable to assume that the fraction of porphyrinogens which is oxidised is relatively insignificant. In addition, the presence of antioxidants such as glutathione and cysteinein the cells, as well as the exclusion of light, are factors which are important in keeping most of the tetrapyrroles in a reduced form[198].

Another side reaction of importance in the haem biosynthetic pathway is the production of UROGEN I (Fig. 3). This can undergo oxidation to URO I, which cannot be further utilised, or may undergo an enzymatic decarboxylation through 7-, 6-, and 5-COOH porphyrinogens to COPROGEN I. COPROGEN I does not serve as a substrate for coproporphyrinogenase, but can be oxidised to COPRO I. As a result of this side pathway, normal human urine will contain URO and COPRO of both type I and type III isomers, but the ratio varies considerably (48,323).

There is uncertainty as to the mechanism for the formation of UROGEN III. It has been demonstrated that when PBG is incubated together with enzyme systems obtained from either Chlorella[31],

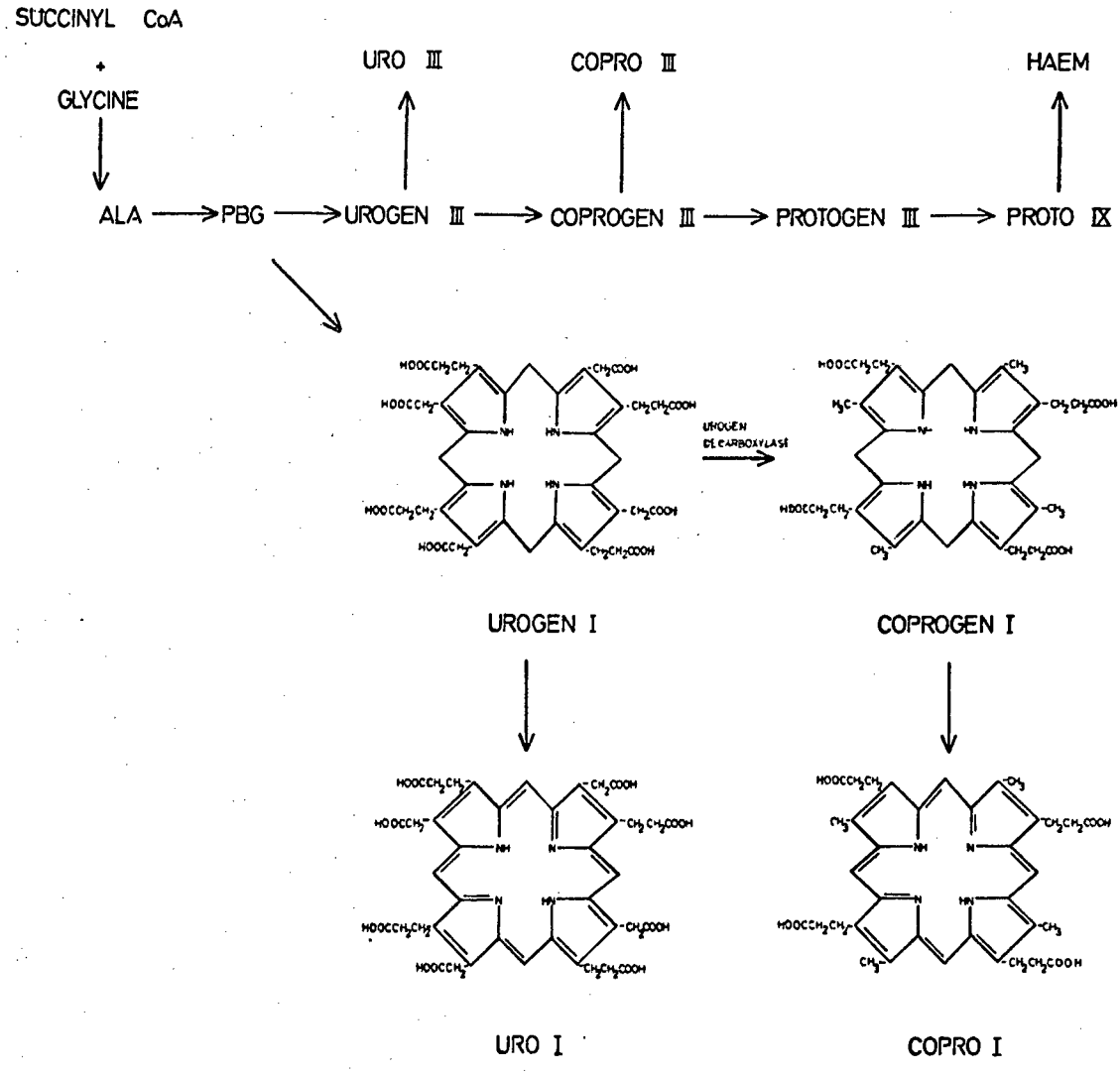

Figure 3 The production of UROGEN I and COPROGEN I, which may be oxidised to URO I and COPRO I, as side products of haem synthesis.

R. spheroides[125], red blood cell haemolysates[32,176] or mouse spleen[169, 170], porphyrins of the type III isomer series are obtained. However on heating the enzyme systems to 60°C it was found that only type I isomers were formed[30,32], which indicated that at least two enzymes were involved in the formation of UROGEN III. Two separate enzyme fractions, viz. UROGEN I synthetase, which is relatively heat stable, and UROGEN III co-synthetase, which is heat-labile, were prepared from plants[26,27] and bacteria[129], and more recently UROGEN III cosynthetase was prepared from haematopoietically active mouse spleen[169]. It was found that UROGEN I synthetase alone converted PBG to UROGEN I [26], that UROGEN I synthetase together with UROGEN III cosynthetase converted PBG to UROGEN III[27] but UROGEN III cosynthetase alone did not react with either PBG or UROGEN I.

More than 20 different mechanisms have been proposed to explain the formation of UROGEN III, where the acetate and propionate substituents are reversed in ring D as compared with UROGEN I. One attractive proposal is the "zipping up" theory of Frydman et al[95]. It is envisaged that UROGEN I synthetase and UROGEN III cosynthetase act as a dual enzyme system. Four units PBG can be polymerised head-to-tail to yield UROGEN I on the surface of the UROGEN I synthetase, or the way in which PBG condenses can be modified if there is an association between the synthetase and cosynthetase. In this latter case, a head-to-head condensation of 2 PBG units occurs to yield finally UROGEN III. In this way UROGEN III cosynthetase acts as a "specifier protein" of UROGEN I synthetase.

Until recently, uncertainty existed over the conversion of COPROGEN III to PROTO IX. It was unclear as to whether coproporphyrino-

genase was responsible for the entire conversion of COPROGEN III to PROTO IX. However, there is mounting evidence that the conversion of PROTOGEN IX to PROTO IX is a separate enzymatic process[14, 230].

4.2. ALA-S

By 1952, from the work of Shemin and Rittenberg[269] and Shemin and Kumin[268], it became clear that the primary precursors for haem synthesis were glycine and succinyl coenzyme A. It was subsequently deduced that ALA might be a further intermediate in haem biosynthesis and it was shown that {5-^{14}C} ALA yielded protohaem with an identical labelling pattern to that of {2-^{14}C} glycine, when these substrates were incubated with haemolysates of duck red blood cells[270]. It was further demonstrated that glycine condensed with succinyl coenzyme A to form ALA in vitro using preparations from avian red blood cells [167,270] and from mammalian liver[111]. However, this condensation did not occur when mature mammalian erythrocytes were used [167, 177]. The condensation reaction is catalyzed by the enzyme ALA-S.

The formation of ALA in the intact mammalian cell occurs within the mitochondria [111,167,306] since it is here where the major fraction of ALA-S is located[111,122], and where succinyl coenzyme A is generated. ALA-S has also been detected in the cytosol fraction in the livers of rats treated with AIA[122,211]. It has been suggested that the cytosol ALA-S, which has a higher molecular weight than the mitochondrial enzyme[123,153] may be converted to the smaller mitochondrial-type ALA-S and subsequently transferred into the mito-

chondrion. However, the physiological mechanism by which the cytosol ALA-S is converted to the mitochondrial ALA-S is as yet unknown.

ALA-S has been purified from bacterial and mammalian sources (122,123,153,211,260,318) but whatever the source of enzyme, there is an absolute requirement for pyridoxal phosphate for the reaction to proceed (84,259,262). From various studies it appears likely that this cofactor is bound to the ALA-S, and furthermore, kinetic studies have shown that pyridoxal phosphate is always bound more strongly to the enzyme than succinyl coenzyme A or glycine (92,100,152,167,259).

A scheme for the mechanism of formation of ALA has been suggested by Battersby and McDonald (14) (Fig. 4). It is postulated that the ALA-S, glycine and pyridoxal phosphate form a complex. An anion generated from this complex undergoes acylation by succinyl coenzyme A. It is not known whether succinyl coenzyme A acylates a functional group on the enzyme, which then transfers the acyl group, or whether the thiol ester is bound to ALA-S in a non-covalent manner. It might be expected that the initial product of the enzymatic reaction would be 2-amino-3-oxoadipic acid, which undergoes spontaneous decarboxylation, since it is known that this compound is extremely labile (100,166). Alternatively the decarboxylation may be enzyme catalized. Analysis of the sterochemistry of {5 - ^{3}H} ALA produced enzymatically from the 2S {2 - ^{3}H} glycine enantiomer (1) would seem to support this. However, it is not known whether there is a specific 2-amino-3-oxoadipate decarboxylase present in the cell, or whether ALA-S itself is responsible for the decarboxylation.

The activity of ALA-S is regulated in both bacterial and animal cells (41,146). Thus there exists a control mechanism determining the intracellular levels of haem, which will be discussed in greater depth

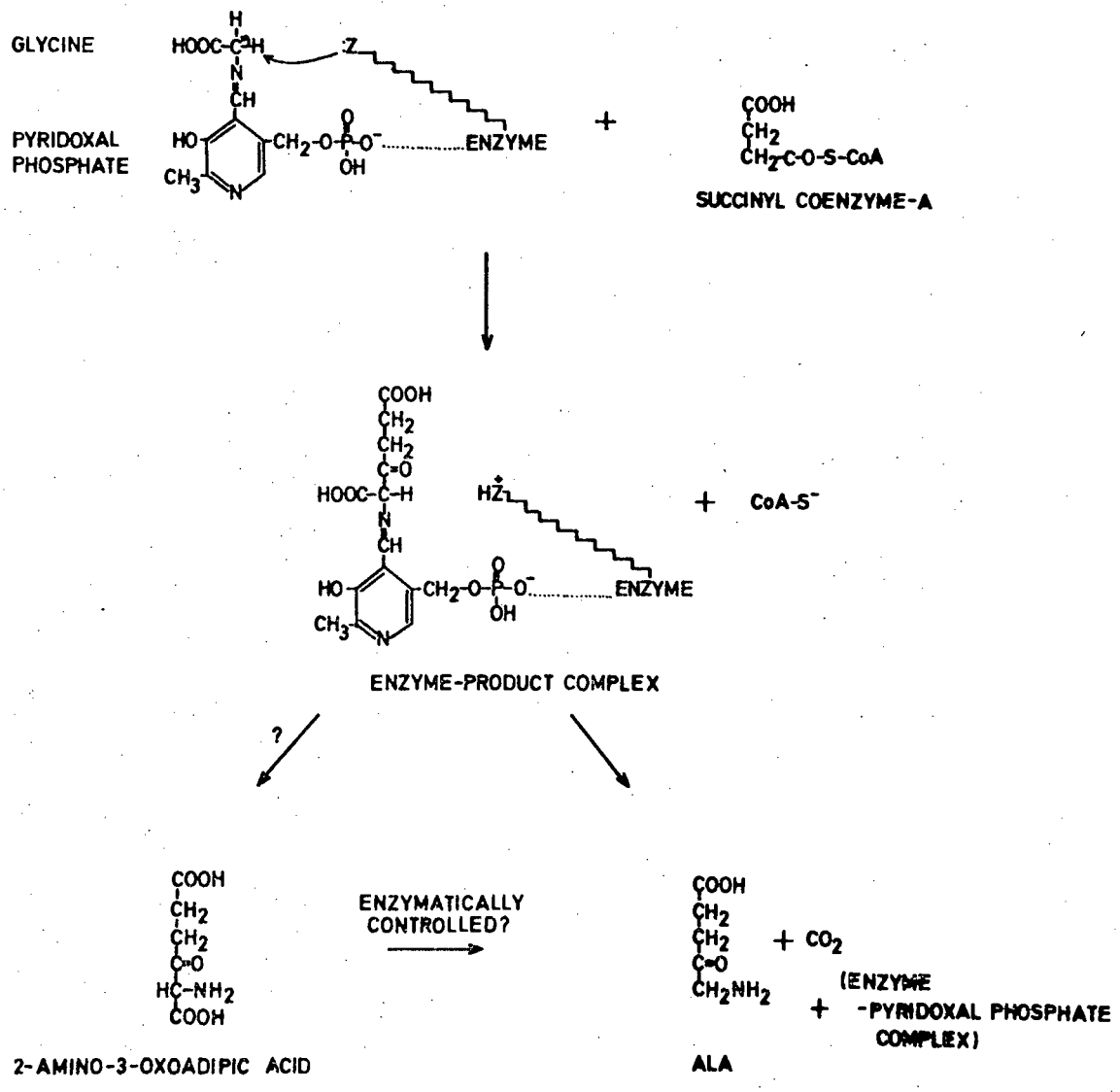

Figure 4 Proposed mechanism for the biosynthesis of ALA from glycine and succinyl coenzyme A, with the reaction catalysed by ALA-S. ENZYME = ALA-S.

in section 4.5.

4.3. UROGEN Decarboxylase

UROGEN decarboxylase catalyzes the decarboxylation of the four acetic acid side chains of UROGEN to yield COPROGEN. This enzyme has been identified in extracts of bacteria[125], avian red cells[301], mammalian reticulocytes[198], mouse spleen[242], rat liver[23, 245], pig liver[162], human erythrocytes[22,23,158] and human liver[159]. The decarboxylation is a stepwise process so that intermediate porphyrinogens with 7-, 6-, and 5-carboxyl groups are formed[15,25,96, 136,198,240,244]. It appears that a single enzyme rather than several closely related enzyme systems is responsible for the four successive decarboxylations[96, 301] and as such can utilise as substrates porphyrinogens which have fewer than 8 carboxyl groups. URO cannot replace UROGEN as a substrate for UROGEN decarboxylase and neither are ALA and PBG affected by the enzyme[110].

It was thought originally that the removal of four carboxyl groups from UROGEN III was a random process, but this does not appear to be the case. From the studies of Jackson et al[136] and Smith et al[276], UROGEN III is decarboxylated sequentially in a clockwise direction starting at pyrrole ring D via rings A and B to ring C (Fig.5). These workers chemically synthesised a number of isomers of 7-, 6-,[136] and 5-[276] carboxylated porphyrinogens and investigated the conversion of these isomers to PROTO using chicken erythrocyte haemolysates. All these isomers were converted to PROTO, but at different rates. The conclusion drawn was that the findings were in agreement with a postulated preferred clockwise route of decarboxylation

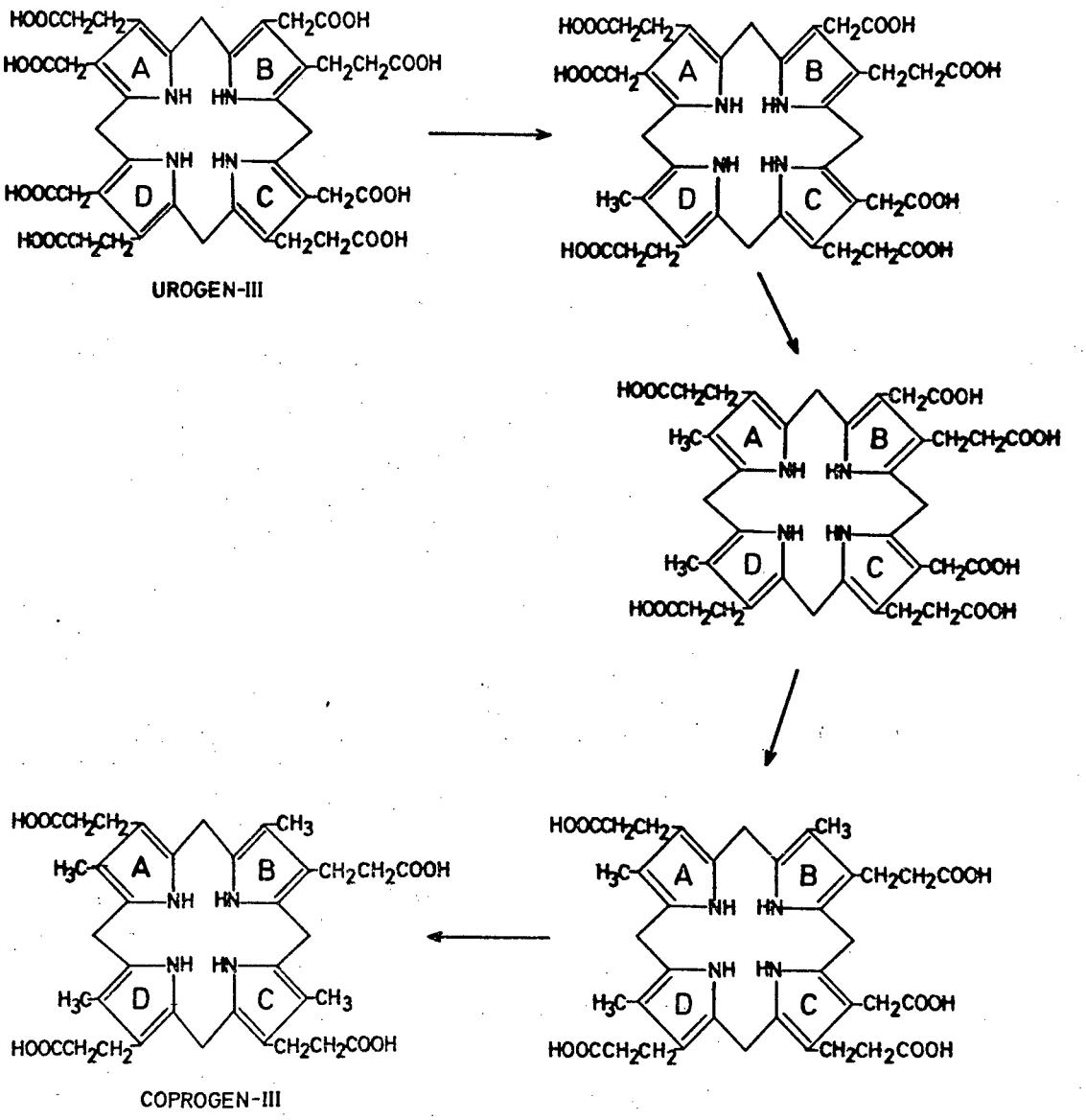

Figure 5 The clockwise sequence of decarboxylation of UROGEN-III to COPROGEN III, starting at ring D, via rings A and B to ring C.

as well as another slow route, where the sequence of decarboxylation was starting at ring B, through C and D and finishing at ring A, to yield COPROGEN III.

Under normal physiological circumstances, only very small concentrations of polycarboxylated intermediates are found in the body fluids, tissues or excreta, which can be attributed to the rapid transformation of these intermediates to COPROGEN through PROTO IX to haem. This is in accordance with the reported low K_m ($< 5 \times 10^{-6}$M) and a high turnover number of UROGEN decarboxylase [198]. In contrast, relatively large amounts of 7-, 6- and 5-carboxylated intermediates are present in the excreta of PCT patients[65] and rats treated with HCB[246].

The UROGEN decarboxylase enzyme is capable of converting the I, II, III and IV isomers of UROGEN to COPROGEN[14]. Kinetic studies on mouse spleen UROGEN decarboxylase have revealed that the K_m and V_{max} values for UROGEN I and UROGEN III are virtually identical[242]. This seems to indicate that the same decarboxylase enzyme is responsible for the decarboxylation of both naturally occurring isomers.

Studies on UROGEN decarboxylase from avian erythrocytes[96] indicate that the elimination of the first carboxyl group from UROGEN III is not rate-limiting in the multiple decarboxylation because large amounts of 7-carboxyl porphyrinogen accumulated. The decarboxylation of UROGEN III to COPROGEN III by UROGEN decarboxylase could thus be regarded as occurring in two stages, firstly the elimination of the first carboxyl group and secondly the removal of the further three carboxyl groups. It was further shown[96] that UROGEN III and 7-carboxyl porphyrinogen concentrations affected the

UROGEN decarboxylase in that UROGEN III inhibited its own decarboxylation, that 7-carboxyl porphyrinogen inhibited its own decarboxylation, that 7-carboxyl porphyrinogen inhibited the decarboxylation of UROGEN III, and that UROGEN III might inhibit the decarboxylation of 7-carboxyl porphyrinogen.

It has been demonstrated that mercury, copper, manganese and oxygen inhibit UROGEN decarboxylase[28,198]. However, oxygen probably enhances the auto-oxidation of UROGEN to URO. When iron was added to mitochondria-free crude extracts of normal porcine liver, the activity of UROGEN decarboxylase was inhibited[162].

4.4. Cytochrome P450 and its Relationship to Haem Synthesis and Drug Metabolism

Cytochrome P450 is a rapidly turning-over microsomal haem protein. Measurement of the rate of turnover of cytochrome P450 shows a biphasic curve with apparent half-lives of 8 and 40 hours [171]. If this is taken to be indicative of two forms of cytochrome P450, then it might be expected that considerable quantities of haem and its precursors would be required in the synthesis of the cytochrome, and in particular in the synthesis of the cytochrome with the shorter half life[193].

Cytochrome P450 was originally reported as a carbon monoxide binding pigment by Klingenberg[154] and Garfinkel[97] and evidence for its haemoprotein nature came from the work of Omura and Sato[212]. Cytochrome P450 is so-called because the carbon monoxide compound of the reduced pigment has an intense absorption band at 450 nm. It

was later established[50,90,214] that cytochrome P450 was an integral component of a nonspecific enzyme system, located in the endoplasmic reticulum, which metabolises a wide variety of drugs and steroids[33,51,190].

The haemoprotein is found not only in abundance in hepatic microsomes but also in the microsomes and mitochondria of the adrenal cortex, where it is involved in the metabolism of steroids [90,214]. It is found also in the kidney and intestinal mucosa, although less abundantly[190]. The presence of cytochrome P450 has been detected in liver microsomes in several types of mammals, a bird[201], a frog and a fish[190], as well as in bacteria[148] and bacteroids[7].

The enzymes catalyzing oxidative reactions in the hepatic endoplasmic reticulum are frequently called "mixed function oxidases" according to the nomenclature of Mason[197] or "monooxygenases" according to that of Hayaishi[124]. It was shown, using ^{18}O-labelled oxygen that during oxidation reactions in the hepatic endoplasmic reticulum, the first atom of oxygen was incorporated into the substrate, and the second was reduced to water[197]. The mixed function oxidases of the hepatic endoplasmic reticulum catalyse widely diverse reactions: deamination; O-, N- and S-dealkylation; hydroxylation of alkyl and aryl hydrocarbons; epoxidation; formation of alkyl derivatives; N-hydroxylation; N- and S-oxidation; and dehalogenation [190]. It was pointed out by Brodie and associates[34] that these reactions could be visualised as hydroxylations. It was suggested[34] that NADPH reduced the component in liver microsomes which reacted with molecular oxygen to form an "active oxygen" complex, which in turn hydroxylated the various sub-

strates by a group of nonspecific enzymes. The reaction was formulated by Gillette[102] as follows:

1. $NADPH + A + H^+ \rightarrow AH_2 + NADP^+$
2. $AH_2 + O_2 \rightarrow$ "Active oxygen" complex.
3. "Active oxygen" complex + drug → oxidised drug + $A + H_2O$

The overall reaction can be considered as:

$$NADPH + O_2 + \text{drug} \rightarrow NADP^+ + H_2O + \text{oxidised drug.}$$

It was established[50] that cytochrome P450, a carbon monoxide-sensitive haem protein, was the moiety reduced by NADPH. Further evidence indicated that NADPH-cytochrome C reductase was responsible for the reduction of cytochrome P450 by NADPH[106].

The spectral characteristics of cytochrome P450 and its derivative, cytochrome P420, have been extensively investigated and documented[190,212,213,214,235]. Hepatic cytochrome P450 appears to be very strongly bound to the endoplasmic reticulum, and early attempts to isolate the haemoprotein resulted in loss of both spectral characteristics and activity[213]. Later[180,201], it was shown that a solubilised form of cytochrome P450 could be obtained with retention of spectral and enzymatic properties.

The first stage in the oxidative metabolism of various drugs by liver microsomes is the formation of a complex of the drug and oxidised cytochrome P450[238], resulting in changes in the difference spectrum i.e. the absorbance spectrum of liver microsomes in the pre-

sence of substrate (sample cuvette) minus the spectrum of liver microsomes in the absence of substrate (reference cuvette). It became clear that drugs and other foreign compounds combine with cytochrome P450 to produce difference spectra of two general types, type I and type II[133,238]. Compounds giving type I spectra with hepatic microsomes, exemplified by hexobarbital, result in a difference spectrum with an absorption maximum in the general range of 385 to 390 nm and an absorption minimum in the general range 418 to 427 nm. Compounds giving type II spectra, for example aniline, give a difference spectrum with an absorption maximum at 425 to 435 nm and an absorption minimum at 390 to 405 nm[272]. The compounds which result in type II spectra usually contain nitrogen, and it is thought that the spectral change is due to ferrihaemochrome formation caused by direct interaction between the basic nitrogen and the haem iron[130]. Furthermore, there is no evidence that a type II spectral change reflects the formation of a metabolically active cytochrome P450-substrate complex[130]. Aminopyrine and 3,4-benzpyrene, which were used as substrates for cytochrome P450 mediated reactions in the present work, both combine with cytochrome P450 to result in type I spectra[238,247,271,272].

Variable enzyme activities attributed to liver microsomal cytochrome P450 after treatment of animals with various inducing agents has suggested that more than one form of cytochrome P450 may be involved in microsomal drug hydroxylations[49,98,103,104]. However, kinetic data obtained using microsomal suspensions[243, 297,314] and with a reconstituted enzyme system[182] showed that a number of substrates act as mutually competitive inhibitors, indicating that they may be acted upon by a single enzyme. Spectral

evidence was reported which suggested that two forms of cytochrome P450, induced either by phenobarbital or polycyclic aromatic hydrocarbons, were present in liver microsomes[6,128,139,207,274]. The genetic regulation also supported the involvement of a separate enzyme system for aryl hydrocarbon hydroxylation[101]. Fractionation procedures have been applied to liver microsomes following the administration of different inducing agents to animals[47], and it has been shown that different substrate specificity, as tested in reconstituted systems, is found in the different cytochrome fractions[179,180,208]. Multiple forms of cytochrome P450 have been separated and purified from rabbit liver microsomes and it has been demonstrated that these different forms vary in their catalytic properties[121]. It was suggested that the individual forms of hepatic P450 could bind many or all of the potential substrates but there may be a difference in their relative efficiencies in the hydroxylation of such compounds[121].

The hydroxylation mechanism by the cytochrome P450 enzyme system of liver microsomal membranes is only partly understood. A mechanism has been postulated by Nordblom et al[209] as shown in Fig. 6. As envisaged by these workers, the following occurs:- The substrate initially reacts with the oxidised form of cytochrome P450. Under anaerobic conditions, two electrons are transferred from NADPH to the cytochrome P450 substrate complex via NADPH cytochrome reductase, one electron to the iron atom and the other to an as yet unidentified acceptor[8,116]. Molecular oxygen is then bound to the ferrocytochrome and together with protonation, a ternary peroxide complex is formed[91,117,135]. Electron transfer to the oxygen occurs with the production of an "activated oxygen" capable of attacking the substrate, with the possible involvement of superoxide[287]. The

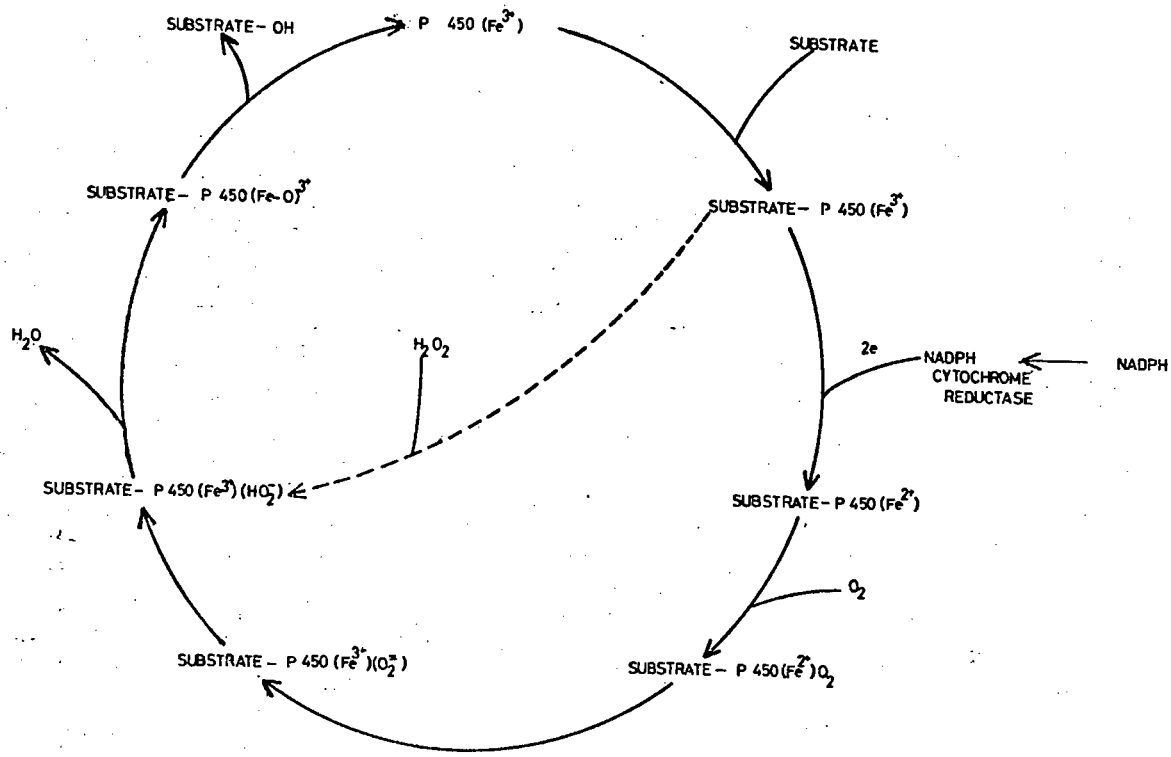

Figure 6 The proposed mechanism for the catalytic action of liver microsomal cytochrome P450.

ternary peroxide complex undergoes further protonation, and the subsequent loss of a molecule of water leaves an activated oxygen species which rapidly inserts an oxygen atom in a favourably positioned C-H bond of the substrate. The hydroxylated product then dissociates with regeneration of the free oxidised cytochrome P450.

It has been further shown that in cytochrome P450 mediated reactions, molecular oxygen, NADPH and NADPH cytochrome reductase could be replaced by hydrogen peroxide, alkyl hydroperoxides or peracids[209].

A number of compounds are capable of inducing microsomal haemoproteins with an associated proliferation of hepatic microsomes, when administered to experimental animals. Furthermore, the pretreatment can result in a stimulation of the metabolism of steroids and foreign compounds bythe mixed function oxidase system of the hepatic endoplasmic reticulum[45,49,87,93,140,141,142,215,236]. The mechanism by which induction of drug metabolising enzyme systems occurs has been reviewed in detail by Gelboin[99]. Conney[49] has divided the inducers into two major groups, firstly a small group of polycyclic hydrocarbons such as 3-methylcholanthrene which stimulates the metabolism of only a limited number of compounds, and secondly, a large group, exemplified by phenobarbital, which is characterised by its ability to stimulate the metabolism of a large variety of compounds. The inducers of drug metabolism can affect any of the components of the mixed function oxidases of the hepatic endoplasmic reticulum. Thus phenobarbital increases the amounts of NADPH cytochrome reductase and cytochrome P450[127,215,237]. In contrast, 3-methylcholanthrene increases the amount of a spectrally distinct

haemoprotein, cytochrome P448[156,189], but not the activity of NADPH cytochrome reductase[127]. It has been reported that pregnenolone - 16 - α - carbonitrile pretreatment of rats resulted in an increase in cytochrome P450 content and NADPH-cytochrome reductase in the hepatic microsomes, yet the specificity of the induction effect on the oxidative metabolism of benzphetamine, ethylmorphine and 3,4-benzpyrene differed from the specificity of either phenobarbital or 3-methylcholanthrene[181]. It was suggested that the pregnenolone - 16 - α - carbonitrile-induced cytochrome P450 might be catalytically different from phenobarbital induced cytochrome P450, this being responsible for the varying substrate specificities[181].

There is a wide variation in the metabolism of drugs among animal species, strains and individuals, and in rats and mice between the sexes. Furthermore, drug metabolism may be altered by the removal of various endocrine glands[105]. The demonstration that multiple forms of cytochrome P450 exist,may explain the specific effects of enzyme inducers on catalytic activities as well as species, strain and sex differences observed in drug metabolism[172].

4.5. Regulation of Haem and Haemoprotein Biosynthesis

The control of haem and haemoprotein biosynthesis has been discussed in recent reviews[193,219] and at international symposia [226,227,228]. However controversy exists over some aspects of the regulatory mechanism, in particular the role of cytochrome P450, and close examination is warranted. The primary and rate-controlling enzyme in haem biosynthesis in the liver is ALA-S[193]. Haem, the

end product of the pathway, appears to regulate the activity of this enzyme possibly through a negative feedback mechanism[259]. That is, if the model of Jacob and Monod[138] for bacterial cells in invoked, haem would react with an aporepressor to form a repressor which in turn impedes the synthesis of m-RNA coding for ALA-S. This model will not necessarily hold true for animal cells and the mechanism of regulation of ALA-S by haem is still an open question[20]. However it has been shown that when haem is administered to rats[194, 322] or added to the incubation medium of cultured chick embryo liver cells [108,248,284], the induction of ALA-S by agents such as AIA or phenobarbital is blocked. The biologic half life of hepatic ALA-S is of the order of 72 min.[192] so that a relatively sensitive control mechanism is provided for primary regulation of this enzyme at the level of its synthesis. If haem is indeed involved in the regulation of the rate of synthesis of ALA-S, its effect is thought to be mediated at the level of translation[248, 284,309].

It has been proposed that a "pool" of hepatic haem exists which regulates the activity of ALA-S[59,191,200,304,322]. However there have been a number of inconsistences associated with this hypothesised regulatory pool of haem. For example, presumably the regulatory pool is identical with the precursor pool for hepatic haemoprotein synthesis. However, exogenous haem, although it interferes with the induction of ALA-S, may not be available for incorporation into haemoprotein[254]. Thus the question arises as to how endogenous and exogenous haem together can constitute a single regulatory hepatic haem pool.

The size of the regulatory pool would be affected by the rate of endogenous haem synthesis and exogenously administered haem. Bissell and Hammaker[20] have suggested that cytochrome P450 dissociates into haem and apoprotein with the haem entering a "free" haem pool, prior to its degradation. If it is considered that more than 50% of available haem in the liver is utilised in the synthesis of cytochrome P450[19], then dissociation of haem from cytochrome P450 would make a substantial contribution to the "free" or regulatory haem pool. It was shown that endotoxin, like haem, appears not only to block the induction of ALA-S by AIA in rats, but also to accelerate the catabolism of cytochrome P450 haem[20]. From the evidence presented[20] it appears that the haem moiety of cytochrome P450, in addition to being a constituent of cytochrome P450, is metabolically important. It was postulated[20] that the regulatory haem pool is fed by endogenous synthesis as well as by dissociation of haem from cytochrome P450, and that there is a dynamic equilibrium of haem between the regulatory pool and apocytochrome P450, providing the fine control mechanism for haem synthesis i.e. a mechanism which is sensitive to changes in the metabolism of cytochrome P450.

In recent years another model has been proposed for the regulation of ALA-S, that is, that apocytochrome P450 (or a closely related protein) regulates ALA-S by positive control at the translational levels, and that haem binding to this apoprotein abolishes this positive effect[216]. Other evidence has been obtained which suggests that apocytochrome P450 may regulate haem synthesis. By reconstitution experiments, it was shown that in rats treated with phenobarbital and cobalt, i.e. a situation where protein synthesis

is enhanced by the drug and haem synthesis is partially blocked by cobalt, apocytochrome P450 accumulated[52]. Furthermore, an induction of ALA-S was observed when this occurred[52] and observations suggest that under conditions of maximal induction of cytochrome P450, haem synthesis appears to lag behind apoprotein synthesis, consistent with the hypothesis that apoprotein synthesis is the primary and rate-limiting event in the control of cytochrome P450 formation. Rajamanickam et al[232] have shown that by following the incorporation of {^{3}H} leucine and {2-^{14}C}glycine into, respectively, the protein and haem moieties of cytochrome P450 in phenobarbital-treated rats, an increased rate of apocytochrome P450 synthesis precedes the induction of ALA-S activity, which in turn precedes an increase in the rate of haem synthesis. Only with a significant increase in the rate of haem synthesis was there any measurable increase in levels of cytochrome P450. Thus the primary effect of phenobarbital is to increase the rate of apocytochrome P450 synthesis at the level of translation as determined by studies with cyclohexamide[232].

The concept that apocytochrome P450 regulates the rate of haem synthesis is open to question. It might be argued that the availability of haem is ratelimiting for cytochrome P450 synthesis [12,13,108,191]. Under conditions where there is increased apocytochrome P450 synthesis, an increase in the levels of cytochrome P450 might result when the apoprotein draws haem from the regulatory pool. Consequently in an attempt to maintain homeostasis there might be increased degradation of the haemoprotein, thus diminishing the regulatory haem pool. At this time the ALA-S synthetic mechanism may become derepressed, resulting in an induction of the activity of this enzyme.

Increased ALA-S activity alone cannot readily explain the different patterns of porphyrin accumulation and excretion as seen in the various hepatic porphyrias (Fig. 1). It has been shown that partial blocks at specific steps in haem biosynthesis can lead to decreased haem formation, with resultant derepression of ALA-S synthesis and haem precursor overproduction in a pattern which is consistent with the block[3,60,61,218,229,284]. From the data of De Matteis[60] it becomes apparent that normal adaptive fluctuations in the rates of haem biosynthesis may become exaggerated when haem repression is altered in any way. In the hepatic porphyrias in man, reduced enzyme activity, possibly due to the presence of inhibitors, reduced enzyme synthesis or structural change of the enzyme (i.e. a genetically determined enzyme activity deficiency), is indicative of a defective step in haem biosynthesis[219]. Furthermore, loss of intermediates due to changes in membrane permeability may occur,[155] or the irreversible oxidation of porphyrinogens to porphyrins could effectively impair haem biosynthesis[219]. Thus in cases of acute intermittent porphyria, which are characterised by an elevated excretion of urinary ALA and PBG, a partial block is indicated in the conversion of PBG to UROGEN III. The activity of UROGEN I synthetase, which catalyses this reaction, was examined and found to be reduced by more than 50% in the livers of patients with acute intermittent porphyria[202,283].

It is possible that in variegate porphyria (VP) (Fig. 1) the reported increase in activity of hepatic ALA-S[66,283] may be due to a defect in the mitochondrial enzyme ferrochelatase, to the extent that, owing to diminished haem synthesis, the mechanism for synthesis of ALA-S becomes derepressed. In rats, 3,5-diethoxycarbonyl-1,

4-dihydrocollidine (DDC) inhibits ferrochelatase and produces experimental porphyria, which resembles VP in that an increase in ALA-S and porphyrin excretion is observed[60]. On the premise that in a genetically determined hepatic porphyria, the enzymatic deficiency could be detected in extrahepatic tissue, measurements of skeletal muscle ferrochelatase have been made in patients with VP in remission[222]. However, no difference was recorded in activity of these patients and in age-matched controls[222]. It may be possible that the enzymatic defect in variegate porphyria is due to a decreased activity of PROTOGEN oxidase, the suggested site of glucose repression of haem biosynthesis[230].

Finally, it is important to note that the overall pathway for haem biosynthesis may be additionally regulated by the location of the enzymes within the cell[193]. For example, the close proximity of ALA-S and ferrochelatase in the mitochondria might result in ALA-S being subjected to high local concentrations of haem, which has been shown to inhibit this enzyme[259]. In addition, the conversion of cytosol ALA-S to mitochondrial ALA-S and subsequent transfer into the mitochondria[123,153] may be important regulatory events in the biosynthesis of haem.

CHAPTER 5

EXPERIMENTAL DESIGNS AND TECHNIQUES

Experimental Design will explicitly indicate the collection of patients studied, the source of experimental rats, the experimental designs and the procurement of various tissues e.g. liver and red blood cells. Following this section, Experimental Techniques will be detailed with appropriate discussion.

I. EXPERIMENTAL DESIGNS

A. Human Patients Studied

Liver tissue for this study was provided by 19 patients with PCT and 38 control patients.

For the PCT patients, the clinical diagnosis was made by physicians of the Porphyria Service at Groote Schuur Hospital, Cape Town. The disease was confirmed biochemically by the Porphyrin Diagnostic Laboratory, Groote Schuur Hospital, Cape Town in 2 ways:-

(1) Quantitative assay of 24 hour urine and stool porphyrins and porphyrin precursors using standard techniques[132, 241, 289].

(2) Qualitative examination by extraction and esterification of porphyrins present in excreta, followed by thin layer chromatography and study of the porphyrin profile thus obtained using a Vitatron VLD 100 densitometer. In cases which were difficult to distinguish, the presence of porphyrin P1 (an isocoproporphyrin) in the stool, was taken as indicative of PCT[78,79].

PCT is almost invariably associated with liver disease; usually with hepatomegaly being present, and in addition, liver

function as judged by serum proteins, serum SGOT and bromsulphthalein excretion are abnormal in more than 50% of cases[72]. Thus for diagnostic purposes, it was essential that liver histology also be examined.

Control patients who supplied liver tissue were admitted to Groote Schuur Hospital suffering from alcoholic liver disease, cirrhosis and other liver diseases. These patients required liver biopsy for diagnostic purposes. All control patients exhibited no biochemical abnormalities of porphyrin metabolism.

Procurement of Liver

Patients were admitted to Groote Schuur Hospital after clinical diagnosis in the Outpatients Department. Many of the patients with PCT admitted to excessive alcohol intake, and one patient had recently taken oral contraceptives. Non-porphyric patients included those with a history of excessive alcohol intake. The patients were not told to alter their drinking habits prior to admission into the hospital wards. Percutaneous liver biopsy for diagnostic purposes was performed in the hospital wards by Dr. N.R. Pimstone, and in all cases within 48 hours of the patient being admitted.

Procurement of Blood

10 ml venous blood was drawn from 9 patients with PCT; from 16 random patients; from 2 families where the father was the propositus of PCT; from 8 members of a family who showed no clinical or biochemical manifestations of PCT; and from the author of this thesis.

B. Rats Studied

The rats used in all the experiments described were female

Wistar rats of a strain originating from 6 breeding pairs obtained from Professor G. Berlyne, Seroka Medical Centre, P.O. Box 151, Beersheba, Israel.

For study of the various parameters rats weighing from 160g to 200g were used.

In all experiments the rats were starved 24 hours prior to being sacrificed, but water was allowed ad libitum. In those experiments where urinary and faecal porphyrins were determined, the animals were kept in stainless steel metabolic cages during the 24 hour starvation period, and urine and faeces were collected.

(i) Control rats

These were untreated animals, maintained on a diet of Epol cubes (Vereeniging Mills, Maitland, Cape).

(ii) HCB-treated rats

Groups of rats were treated with 0,2% HCB, mixed with finely powdered Epol cubes. The diet was allowed ad libitum, then at various time intervals up to 60 days, the rats were sacrificed after the 24 hour starvation period.

(iii) HCB and Iron treatment

In addition to HCB treatment, some groups of rats received 25 mg elemental iron/kg twice weekly. The iron as Jectofer, an iron-sorbitol-citrate complex was injected intramuscularly into the upper hind leg of the animals

(iv) Other Treatment Schedules

Rats were rendered haemosiderotic by injection of iron as Jectofer into the upper hind leg at a dose of 25 mg elemental iron/kg twice weekly.

Allylisopropylacetamide (AIA) was injected intraperitoneally at a dose of 300 mg AIA in physiological saline/kg, after the rats had been starved for 24 hours. Sixteen hours later the rats were killed.

Phenobarbital was administered intraperitoneally as Gardenal (sodium salt) at a dose of 80 mg/kg daily for 5 days. Rats were starved during the last 24 hours of treatment before killing.

C. Preparation of Tissue

1. Human Liver

(a) Initial handling

The liver specimen was treated in 5 different ways immediately following biopsy:

(i) A portion of 5 - 10 mg was placed in 10% formalin for light microscopy following staining.

(ii) A portion of 5 - 10 mg for fluorescence microscopy was placed in a dry tube covered with aluminium foil, i.e. the sample was kept in total darkness, before further processing.

(iii) A portion of 5 - 10 mg was taken for electron microscopy. This was placed on a sheet of Glassine paper together with a drop of 5% phosphate-buffered glutaraldehyde. This sample was very finely chopped with a blade, then transferred into a vial containing fresh 5% phosphate-buffered glutaraldehyde.

All the work pertaining to light, fluorescence and electron microscopy was performed by a histopathologist, Dr. B.L. Webber, of the Department of Pathology, University of Cape Town Medical School.

(iv) Approximately 10 mg liver was placed in a dry test tube covered with aluminium foil, for the determination of the porphyrin profile by the Porphyria Diagnostic Laboratory, Groote Schuur Hospital.

(v) The remainder of the biopsy material, weighing from 30 to 70 mg was further prepared for determinations of ALA-S activity, cytochrome P450 levels; and aminopyrine-N-demethylation and 3,4-benzpyrene hydroxylation kinetics. The material was placed in a vial containing 10 ml ice cold KCl:tris buffer (4 parts 1,15% KCl, 1 part 0,25 M tris:HCl, pH 7,5) and transported to the laboratory under ice, with weighings and homogenisations being performed within 2 hours of obtaining the biopsy material.

(b) Preparation of human liver homogenates

(i) For ALA-S assay

After drying between 2 sheets of tissue paper, approximately 4 to 7 mg of the biopsy material was weighed out and homogenised 1:9 w/v with ice cold 0,9% NaCl solution which was 0,5 mM in respect of EDTA and 0,01M in respect of tris:HCl, pH 7,4. The homogenisation was carried out under ice using a Potter type homogeniser of 1 ml capacity fitted with a Teflon pestle (No. 0678, Thomas, Philadelphia, U.S.A.) Homogenates were stored under ice for a maximum period of 2 hours before assay for ALA-S.

(ii) For cytochrome P450 determinations, aminopyrine-N-demethylation and 3,4-benzpyrene hydroxylation.

The remainder of the biopsy material was dried between tissue paper and weighed. This was homogenised 1:10 w/v with fresh KCl:tris (4 parts 1,15% KCl, 1 part 0,25M tris:HCl, pH 7,5) under ice, again using an homogeniser of 1 ml capacity. The homogenate was stored under ice for a period of not longer than 2 hours before the start of any of the determinations.

2. Human Blood

Venous blood was collected in heparinised tubes and kept under ice for the minimum period of time i.e. less than 2 hours, before further manipulations were commenced.

(a) Preparation of haemolysates from human blood for assay of red blood cell UROGEN-I decarboxylase

Each blood sample was poured into a 50 ml polyethylene centrifuge tube and approximately 30 ml of ice cold normal saline was added. This was gently mixed and centrifuged at 500xg for 10 min. using a Sorvall RC 2 centrifuge at $4^{o}C$. The supernatant and buffy layer were gently aspirated off, then the remaining red cells were washed twice more with 30 ml cold normal saline. After the last wash, the packed red cells were suspended in an equal volume of 0,1M tris:HCl,pH 6,8. The cells were sonicated for 20 sec. using an Ultrasonics Rapidis 300 sonicator fitted with a 3 mm tip, at power setting 3. During the sonication the tube was kept in an ice bath. Cells were twice frozen and thawed, using liquid nitrogen for freezing and a water bath at $37^{o}C$ for thawing. After the last thaw, the haemolysed cells were centrifuged at 32000xg for 45 min. at $4^{o}C$ in a Sorvall RC-2 centrifuge. The supernatant was decanted and used as a source for UROGEN-I decarboxylase.

3. Rat Liver

When whole liver homogenates were to be prepared, or when porphyrin contents of the liver were to be examined, rats were killed by decapitation, the livers excised and dried superficially with tissue paper. In those experiments where rat liver microsomes were prepared, rats were killed by decapitation, then the liver was exposed. The liver was thoroughly perfused in situ with ice cold 0,9% saline, excised, and dried superficially with tissue paper.

(a) Preparation of rat liver homogenates:

(i) For ALA-S assay

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(i) For ALA-S Assay

One g liver was weighed out and placed under ice cold homogenising medium (0,9% sodium chloride solution which was 0,5mM in EDTA and 0,01 M in tris:HCl, pH 7,4). The liver was cut with scissors into small pieces and after swirling the medium was decanted. Fresh homogenising medium was added to result in a total volume of 10 ml and the liver was homogenised under ice in a 50 ml capacity glass homogeniser fitted with a motor-driven Teflon pestle. The homogenate was stored under ice for a maximum of 2 hours before the assay.

(ii) For cytochrome P450 determinations; aminopyrine-N demethylation and 3,4-benzpyrene hydroxylation kinetic studies

One g liver was treated in exactly the same way as described in the previous section, but the homogenising medium in this case was KCl:tris buffer (4 parts 1,15% KCl, 1 part 0,25 tris:HCl, pH 7,5). Again the homogenate was stored under ice for not longer than 2 hours before commencement of any of the determinations.

(iii) For UROGEN-I decarboxylase assay

One g liver was again treated as described for the preparation of homogenate for ALA-S assay, but the homogenising medium was 0,1 M tris:HCl buffer, pH 6,8. The homogenate was centrifuged at 32000xg at 4°C for 45 min. in a Sorvall RC-2 refrigerated centrifuge. The supernatant was decanted and used as a source of UROGEN-I decarboxylase. This post mitochondrial supernatant was kept under crushed ice for a maximum of 2 hours before the start of the assay.

(iv) Preparation of liver for determination of porphyrin profile

Approximately 1 g liver was weighed, placed into a test tube covered with aluminium foil to protect porphyrins from being degraded by light. The tissue was kept in a deep freeze at -20°C until the time of assay for the porphyrins, usually about 2 weeks later.

(b) Preparation of rat liver microsomes

Microsomes were always prepared from livers pooled from 3 rats. 3g of perfused liver from each of 3 rats were weighed, pooled and homogenised 1:3 w/v with KCl:tris buffer (4 parts 1,15% KCl, 1 part 0,25M tris:HCl, pH 7,5), under ice using an homogeniser fitted with a motor driven Teflon pestle. The homogenate was centrifuged at 9000 x g for 20 minutes at 4°C in a Sorvall RC-2 refrigerated centrifuge. Microsomes were sedimented by centrifugation of the supernatant in a Beckman Model L3-50 Ultracentrifuge at 105000xg using a 40 Ti fixed angle rotor. After discarding the supernatant, the microsomal pellets were resuspended with 2 ml aliquots of ice cold 0,25 M potassium phosphate buffers at pH 6,0; 6,5; 7,0; 7,5;8,0. A Potter Elvejhem homogeniser of 5 ml capacity was found to be convenient for resuspending the microsomes. The microsomal suspensions were kept under ice and used within 8 hours of preparation for cytochrome P450 determinations. Following this the suspensions could be stored, deep-frozen for subsequent protein determinations.

II. EXPERIMENTAL TECHNIQUES

Introduction

Methods used for the investigation of the following parameters will be discussed:

1) Hepatic ALA-S activity, with a subsection on the determination of bacterial succinyl coenzyme A synthetase activity.
2) Levels of hepatic cytochrome P450.
3) Kinetics i.e. K_m and V_{max} of aminopyrine-N-demethylation by the liver in vitro.
4) Kinetics i.e. K_m and V_{max} of 3,4-benzpyrene hydroxylation by the liver in vitro.

5) Red blood cell and hepatic UROGEN-I decarboxylase activity.

6) Porphyrin levels in liver, urine and faeces.

Other methods used, viz. haemoglobin determination, protein determination and total hepatic iron determination will also be described.

Results from light, fluorescence and electron microscopy formed an integral part of this project. This work was performed by a histopathologist, Dr. B.L. Webber of the Department of Pathology, University of Cape Town Medical School, and methods used in these studies will be described briefly.

Each of the experimental techniques used will be presented in the following format:

(a) Principles of the particular assay will be discussed

(b) A detailed account of materials and methods will be given.

(c) The assay procedure will be succinctly described.

(d) Comments on any particular assay will be made when necessary.

(e) Ancillary procedures to any particular assay will be described.

Of the methods to be described, the radiochemical micromethod used for assay of ALA-S activity is the most complex. The assay is successful only if meticulously performed. For this reason, the section on materials and techniques will be presented at length, with scrupulous attention being given in particular to the finer detail.

The methods for calculation of results from the experimental data as well as statistical methods are given in Appendix A.

A list of all reagents and specialised equipment together with the names of suppliers will be found in Appendix B.

All light absorbance readings and spectral recordings of solutions were made on a Pye Unicam SP 1700 Ultraviolet Spectrophotometer fitted with a Unicam AR 25 Recorder. In addition the readings were always made at room temperature.

5.1. Hepatic ALA-S Activity

(a) Principles of Assay

The radiochemical micromethod used for the assay of this enzyme was essentially the method of Strand et al[285] with modifications as described by Pimstone et al[222].

ALA-S catalyses the condensation of glycine and succinyl-CoA in the presence of pyridoxal-5-phosphate to form ALA[267].

The assay method, in principle, is the measurement of incorporation of ^{14}C-succinate into ALA in the presence of the enzyme source viz. liver tissue homogenate, CoA, and succinyl CoA synthetase with subsequent isolation of ^{14}C-ALA by employment of a triple column technique.

Using liver homogenates as enzyme source for the ALA-S assay to prevent the ^{14}C-succinate being metabolised to other compounds via the tricarboxylic acid cycle, inhibitors are used[134]. Antimycin A together with malonate are present to inhibit succinic dehydrogenase, the malonate being a specific competitive inhibitor with the antimycin A present as an inhibitor of electron transport, blocking electron flow via the cytochromes b and c. Malate is present to trap any ^{14}C-malate produced which is subsequently removed by a cation exchange column. Arsenite will inhibit the α-oxoglutarate oxidase system, since Granick and Urata[111] showed that mitochondria are able

to synthesise ALA with α-oxoglutarate as a source of succinyl CoA. It is necessary in this assay to prevent succinyl-CoA formation from α-oxoglutarate since this will be unlabelled and will compete with ^{14}C - succinyl - CoA. Inhibition of ALA dehyratase is effected with EDTA[195].

A succinyl CoA generating system i.e. succinyl CoA synthetase, succinyl CoA and ATP is included in the assay system, together with magnesium to activate succinyl CoA synthetase. It has been shown that methods which rely on endogenous succinyl CoA synthetase[76,94] will underestimate total homogenate ALA-S activity[122,259].

The effects of the reagents used in the assay have undergone extensive investigation[76, 134, 195, 285].

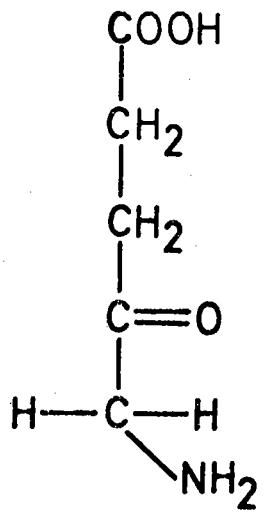
(i) Triple Column Technique

Ion exchange column chromatography in 3 sequential steps is used to separate the ^{14}C-ALA from succinate and other metabolic products after incubation and termination of the reaction. Initially the pH of the incubation mixture is adjusted to 7,0 which is passed over Dowex-1-Acetate at pH7, then the eluate is passed over Dowex-50 H^+ at pH2. The Dowex-50 H^+ is washed with 0,1N HCl and subsequently eluted with 1M sodium acetate. The ALA is converted to 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole by condensation with acetyl acetone according to the Knorr reaction shown in Fig. 7.

The pyrrole is applied to a Dowex-1-acetate column at pH 4,6 and eluted with methanol/glacial acetic acid 2:1.

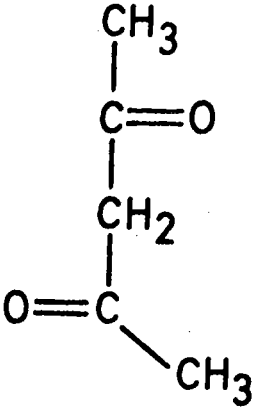
(ii) Resin Flow

Table 2 lists the values for pK_1 and pK_2 at 25^oC in aqueous solution of the acids present in the incubation system for the ALA-S assay.

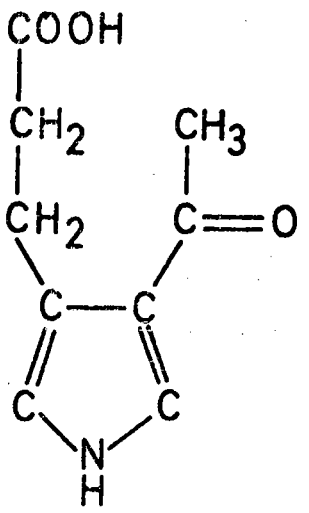


ALA

+



acetylacetone



+ $2H_2O$

2-methyl-3-acetyl-4(3-propionic acid) pyrrole

Figure 7 The condensation of ALA with acetylacetone.

TABLE 2

IONISATION CONSTANTS FOR THE ACIDS PRESENT IN THE ALA-S INCUBATION SYSTEM[18]

Acid	pk_1	pK_2
Succinic Acid	4,21	5,64
ALA	4,05	8,90
Malic Acid	3,46	5,05
Malonic Acid	2,86	5,70
Oxaloacetic Acid	2,56	4,37
Fumaric Acid	3,02	4,38

Dowex-1 is a strongly basic anion exchange resin composed of quaternary ammonium exchange groups attached to a styrene divinylbenzene polymer lattice. For use in the first ion exchange column in the isolation of ALA it is converted to the acetate form and used at pH 7. At this pH the succinate will be in anionic form and will replace acetate as will the other carboxylic acids. However, ALA will not be exchanged by the column since it has a net positive charge at pH 7.

Dowex -50, which is a strongly acidic cation exchange resin composed of nuclear sulphonic acid exchange groups attached to a styrene divinylbenzene polymer lattice, is used in the second chromatography step in the acid form at pH 2.

At this pH, ALA will be retained by the resin, probably with greater affinity than remaining succinate and other dicarboxylic acids, since they have relatively lower constants for dissociation.

Washing the resin with 0,1N HCl will displace remaining succinate and dicarboxylic acid cationic groups. Sodium acetate 1M (pH 8,4) will displace the ALA retained on the resin; the eluate is collected and reacted with acetyl acetone to form a pyrrole. The sodium acetate acts by

(a) decreasing the cationic NH_3^+ charge

(b) cationic competion.

The pyrrole formed by condensation with acetylacetone has a propionic acid residue, which permits a further purification by an anionic exchange column. This column contains Dowex-1 in the acetate form, at pH 4,65. At this pH the pyrrole exists in the anionic form, and will strongly bind to the resin.

Any residual dicarboxylic acids and succinate will also be bound to the resin, but with the ionisation constants from 2,5 to 5 will not be predominantly in the salt form. These are eluted with 1M acetic acid, followed by elution with methanol, which will remove any "aminoacetone pyrrole-like material" that may have been formed. The aminoacetone may have been formed in the incubation mixture originally by condensation of glycine with acetyl-CoA. The final elution step with glacial acetic acid and methanol will displace the ALA pyrrole, methanol being included since it is a good solvent for the pyrrole.

(iii) Specificity of the Assay

The radiochemical assay method for determination of ALA-S activity is extremely sensitive, and measures specifically newly formed ALA. ALA-S activities are normally measured by conversion of the produced ALA to a pyrrole by condensation with acetylacetone, the pyrrole being measured colorimetrically with Ehrlich's reagent. Aminoacetone as well as ALA is produced by the liver and other cells[85,86,114], and both will give

rise to pyrroles. Thus it is necessary to separate ALA and aminoacetone in ALA-S activity measurements, and methods used have been described where resin columns are used either before[310] or after[196] conversion to pyrroles. The radiochemical ALA-S assay, however, excludes aminoacetone synthesis.

(b) Materials and Methods

(i) Incubation Conditions

(1) An inhibitor/cofactor solution was made up freshly for the assay as described below and kept under ice. Except where otherwise indicated, the reagents were made as stock solutions, and stored at $4^{o}C$. The stock solutions were discarded after 3 months. All reagents were made up in 0,125M tris:HCl buffer, pH 7,4.

2 parts each of:-
- (a) EDTA 0,025M
- (b) Glycine 2,5M
- (c) Sodium arsenite 0,25M
- (d) Antimycin A 6,25mg/100ml. (Freshly made). The antimycin A, which is not readily soluble in an aqueous medium was dissolved in 5 ml ethanol with the resulting solution being made up to 100 ml with 0,125M tris: HCl buffer, pH 7,4,which contained 0,1% bovine serum albumin.
- (e) Sodium d-l malate 0,125M
- (f) Adenosine triphosphate 0,625M (Freshly made).
- (g) Dithiothreitol 0,025M which was 0,010625M in respect of coenzyme A. (Freshly made).

1 part each of:-
- (h) Magnesium chloride 1,0M
- (i) Pyridoxal-5-phosphate 0,05M (Freshly made).

4 parts of:-
- (j) Malonic acid 1,25M

The pH of this solution was adjusted to 7,4 with 1 N NaOH.

(2) To a vial containing 250 µCi {1,4 - ^{14}C} succinic acid,

0,5 ml distilled H_2O was added, the resulting solution neutralised with NaOH 0,1M, then made up to 1 ml with H_2O. This solution was stored at 4^oC.

(3) A fresh solution containing approximately 100 U of activity per ml of succinyl - CoA synthetase was made up with 0,125 M tris:HCl pH 7,4, and kept under ice.

For assay of hepatic ALA-S, the incubation mixture consisted of 0,020 ml cofactor/inhibitor solution, 0,010 ml neutralised {1,4 - ^{14}C} succinic acid, and 0,010 ml succinyl - CoA synthetase. At the start of the reaction 0,010 ml liver homogenate, corresponding to 1 mg liver tissue (wet weight), was added. The mixture was incubated at 37^oC for 30 min. with shaking in a Dubnoff Metabolic Shaking Incubator at 100 r.p.m. The reaction was stopped by adding 0,020 ml 25% trichloracetic acid. 0,050 ml of carrier solution (4,19 mg ALA. HCl and 0,675 g sodium succinate per 10 ml H_2O) was added, then reaction tubes were stored frozen at -20^oC until isolation of the radioactive ALA by the triple column method took place.

(ii) Chromatography - Preparation of Resin

Dowex - 1 Cl^- was converted to the acetate form in the following way: Approximately 300 g of the resin was added to 2 litres 1N NaOH and stirred gently with a magnetic stirrer and bar for 1 hr. The resin was filtered using a Buchner filter flask, and washed with a total of 3 litres distilled water. After suspension in 2 litres 3M sodium acetate and stirring for 1 hr, the resin was again filtered and washed with 2 litres distilled water. This last step was repeated twice more. Two-thirds of the resin was then further prepared for use in the first column, by initial suspension in 1 litre 1M acetate buffer pH 7, followed by filtration and washing with the same buffer. Resin was then suspended in 1 litre 0,1M acetate buffer pH 7,0, again filtered and washed and finally suspended in approximately 500 ml 0,025M acetate, pH 7,0. Slurry was poured into the polyethylene columns to give a final column height of 4 cm. Columns were washed with 0,025M sodium acetate, pH, 7,0

until the pH of the wash fluid was 7,0. This final step required approximately 100 ml buffer to achieve.

The remaining third of Dowex-1 was prepared for use in the third (anion) exchange column exactly as above, except that the sodium acetate buffers were at pH 4,6, and the final slurry suspension was in 0,05M sodium acetate pH 4,6. The slurry was poured to result in a column height of 1,5 cm and washed with 0,05M sodium acetate at pH 4,6 until the pH of the wash was 4,6 - this usually required 30 ml of the buffer.

Dowex - 50 H^+ was prepared for use by suspension of approximately 300 g resin in 2 litres 1N HCl, and stirring gently for 1 hr. This was followed by filtration and washing with distilled water. Resin was suspended in, and subsequently washed with 0,5N HCl, followed by suspension and washing with 0,1N HCl (2 litres). Finally, the resin was suspended in 500 ml 0,01 N HCl, the slurry then being poured into columns to give a resin bed height of 4 cm. The resin in the columns was equilibrated to pH 2 with 0,01N HCl. This step required approximately 100 ml.

The resin in the columns was at all times kept under the various equilibration solutions by blocking off the flow with plastic caps, the tops of the columns being covered. It was found possible to store the resin columns in this way for up to 6 months, although it was always necessary to equilibrate the resin to the required pH with about 20 ml of equilibrating solution before use.

(iii) Chromatography - Isolation of ^{14}C-ALA

A three-step sequential ion-exchange procedure was used.

(1) The pH of the incubation mixture was adjusted to pH 7 with 0,1N NaOH after addition of 4 ml H_2O, then passed over a Dowex-1-acetate column (1 x 4 cm) equilibrated to pH 7,0 with 0,025 M sodium acetate. The column was washed through with 20 ml H_2O, and the total eluate from the column was collected.

(2) The total eluate from the first column was charged to a 1 x 4cm Dowex - 50 H^+, column equilibrated to pH 2 with 0,01 N HCl. The column was washed with 20 ml 0,1 N HCl followed by 3 ml 1M Na acetate. The ALA was eluted with a further 10 ml 1M Na acetate and collected in a large boiling tube. Conversion to 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole was achieved by heating this eluate together with 0,2 ml acetylacetone in a boiling water bath for 20 min., after placing a very loose fitting stopper on the boiling tube.

(3) After being allowed to cool, the pyrrole was applied to a Dowex-1-acetate (1 x 1, 5 cm) column equilibrated to pH 4,6 with 0,05M Na acetate. The column was washed with 15 ml H_2O, 5 ml 1N acetic acid, then with 1 ml methanol. The ALA pyrrole was eluted with 10 ml methanol glacial acetic acid (2:1 v/v). The volume of this final eluate was recorded, then 3 ml was added to 12 ml Instagel and counted in a Beckman L.S. scintillation counter. Samples were again counted after the addition of ^{14}C-n-hexadecane for determination of counting efficiency.

(iv) Spectrophotometric Determination of Recovery of ALA

The recovery of ALA was determined spectrophotometrically using Ehrlich mercury reagent[187]. This reagent was always freshly made up before use and consisted of p-dimethylaminobenzaldehyde 1g; 10 ml of a solution of $HgCl_2$ in glacial acetic acid (16g/L); 9 ml perchloric acid (70%), the solution being made up to 50 ml with glacial acetic acid.

2 ml of the eluted pyrrole from the third column was added to 2 ml of Ehrlich mercury reagent, and absorbance determined at 556 nm after 20 min.

A standard was made by reacting 0,050 ml of a solution of ALA, HCl (4,19mg/10ml H_2O) in 1 ml 1 M Na acetate with 0,2 ml acetylacetone at 100°C for 20 min; then after cooling methanol glacial acetic acid (2:1) was added to result in a

final volume of 10 ml. A 2 ml aliquot of the standard pyrrole was reacted with 2 ml Ehrlich's mercury reagent as above, and the absorbance determined at 556 nm. For determination of efficiency of recovery, the absorbance reading for the pyrrole was converted to an absorbance reading which would be obtained if the volume of eluate from the third column was 10 ml. This value was then compared directly to the value obtained for the standard ALA-pyrrole.

(c) Assay Procedure

The incubations were carried out at 37°C for 30 min. aerobically with shaking in a Dubnoff Metabolic Shaking incubator at 100 r.p.m. The incubation mixture contained in a total of 0,05ml: EDTA (disodium salt) 0.05 µmol; glycine 5 µmol; sodium arsenite 0,5 µmol; magnesium chloride 1 µmol; antimycin A 0,125 µg; sodium d-l malate 0,25 µmol; malonic acid 5 µmol; pyridoxal-5-phosphate 0,05 µmol; ATP 1,25 µmol; coenzyme A 0,021225 µmol; dithiothreitol 0,05 µmol; succinic acid including {1,4 - ^{14}C} succinic acid 0,01225 µmol; purified bacterial succinyl coenzyme A synthetase (E.C. 6.2.1.5.) 0,89 units; tris:HCl 3,75 µmol. The pH of the reaction mixture was adjusted to 7,4 and reaction started with 0,010 ml liver homogenate (100 mg/ml). The reaction was terminated with 0,020 ml 25% trichloracetic acid, and sodium succinate 12,5 µmol and ALA.HCl 0,125 µmol were added as carriers.

The pH of the incubation mixture was adjusted to 7,0 with NaOH and passed over Dowex 1 acetate (1 x 4 cm) equilibrated to pH 7,0 with 0,025 M Na acetate. The column was washed through with 20 ml water and the total eluate was applied to Dowex 50 H^+

(1 x 4 cm) equilibrated to pH 2 with 0,01 N HCl. The Dowex 50 H^+ column was washed with 20 ml 0,1N HCl, then with 3 ml 1M Na acetate. The ALA containing fraction was eluted with 7 ml 1M Na acetate, then the ALA was converted to 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole by boiling with 0,2 ml acetylacetone. This pyrrole was applied to Dowex 1 acetate (1 x 1,5 cm) equilibrated to pH 4,6 with 0,05M Na acetate buffer, followed by washing with 15 ml water, 5 ml 1N acetic acid and 1 ml methanol. The pyrrole was eluted with 10 ml methanol/glacial acetic acid (2:1).

3 ml of the eluate was added to 12 ml Instagel and counted in a Beckman liquid scintillation counter. Samples were re-counted after addition of ^{14}C-n-hexadecane as internal standard for determination of counting efficiency. Recovery of ALA was determined spectrophotometrically with Ehrlich mercury reagent.

(d) Comment on ALA-S Activity Determinations

The assay was always performed in triplicate where possible. In each run, blanks were always included and consisted of the entire reaction mixture with the exception of liver homogenate, this being replaced by 0,125 M Tris:HCl buffer, pH 7,4.

When a batch of columns was prepared, the efficiency of 3 sets of triple columns selected at random was determined prior to chromatographic isolation of ALA from incubated samples. The procedure followed was that detailed in (b) (iii) and (iv) above but the entire incubation mixture was replaced by 0,050ml carrier solution (4,19 mg ALA:HCl and 0,675 g sodium succinate per 10 ml H_2O). The Dowex 1 and Dowex - 50 was reprocessed as detailed in (b) (ii) above if the efficiency of ALA recovery as determined spectrophotometrically was less than 50%. Usually the recovery

of ALA from the triple columns ranged from 50% to 80%. The columns were never allowed to run dry during the charging or washing procedures.

(e) Bacterial Succinyl-CoA Synthetase Activity

(i) Principles of Assay

The purified bacterial succinyl coenzyme A synthetase was assayed essentially according to the method of Kaufman[150]. The reaction involved is as follows:-

$$\text{Succinate + ATP + Coenzyme A-SH} \xrightarrow[\text{synthetase}]{\text{succinyl CoA}} \text{Succinyl-Coenzyme A + ADP + P}$$

In the presence of hydroxylamine, the succinyl coenzyme-A reacts to form succinohydroxamic acid, with coenzyme A being constantly regenerated. The ferric chloride reagent of Lipmann and Tuttle[174] is used to determine the succinohydroxamic acid at 540 nm. One unit of activity of the enzyme is defined as the amount of enzyme which catalyses the formation of 1 µmol of succinohydroxamic acid in 30 min at 37°C.

(ii) Materials and Methods

A "substrate mixture " was made up consisting of 2,5 ml 1,0M tris, 5 ml 0,1M $MgCl_2$ and 5 ml 1,0M disodium succinate The pH was adjusted to 7,4 with HCl and the volume was made up to 17,5 ml with water. A"cofactor mixture" was made up with 2 ml 0,2M ATP, 1 ml coenzyme A (10mg/ml) and neutralized before adding water to result in a total volume of 6,5 ml. Hydroxylamine was prepared by mixing equal volumes of 4M KOH and 4M $NH_2OH.HCl$, the pH subsequently being adjusted to 7,4 by the addition of either reagent dropwise. It was important that this solution be freshly made before use. For the assay 0,35 ml "substrate mixture", 0,1 ml "cofactor mixture" and 0,1 ml hydroxylamine were pipetted into test tubes. Purified bacterial succinyl CoA synthetase (10mg/ml H_2O) and H_2O were

added to result in a final volume of 1 ml at the start of the reaction. Incubation was at 37°C for 30 min. The reaction was terminated by addition of 1 ml of a solution of 10g ferric chloride hexahydrate and 3,3g trichloracetic acid in 100ml 0,66 N HCl. After addition of 2 ml H_2O, the solutions were centrifuged at 2000 r.p.m. for 20 min. on a Roto-Uni bench centrifuge. The absorbance of the supernatant was determined at 540 nm using as reference a sample tube to which no enzyme preparation had initially been added. Standards were prepared which contained the substrate and cofactor mixtures and 1 µmol succinohydroxamic acid. (Succinohydroxamic acid was prepared by making a 10mM solution of succinic anhydride in neutralised hydroxylamine). The total volume of the standard solutions was made up to 1ml with water, then after the addition of 1ml ferric chloride reagent and 2 ml water, the absorbance was determined at 540nm. Absorbance readings were compared directly to those for the standard for calculation of activity of the enzyme preparation.

5.2. Hepatic Cytochrome P450 Determinations

I. Cytochrome P450 Levels in Whole Liver Homogenate with Carbon Monoxide as Ligand.

(a) Principles of Assay

Cytochrome P450 is a microsomal enzyme system and under normal circumstances is measured in microsomal preparations. However, due to the limited amount of tissue available from human liver biopsies, a micromethod was adapted from Schoene et al[258] as described previously[222] for whole liver homogenates.

Omura and Sato[212] indicated that cytochrome P450 was a reducible pigment and only the reduced form could bind CO. A difference spectrum

could be obtained between cytochrome P450:Fe^{2+}: CO and cytochrome P450:Fe^{2+} with a peak at 450 nm after reduction of all cytochromes and possibly flavins in both spectrophotometer cuvettes with sodium dithionite and subsequently bubbling the sample cuvette with CO. However, in homogenates, haemoglobin will interfere in the assay since the difference spectrum between haemoglobin: Fe^{2+}: CO (418nm) and haemoglobin: Fe^{2+} (430 nm) will overlap the cytochrome P450 peak. Further, the 30 fold excess of haemoglobin over cytochrome P450[258] would cause so much overlap in the difference spectrum that quantitative measurements of the cytochrome P450 by this procedure would be useless.

By modification of this method to measure difference spectra between cytochrome P450: Fe^{2+}: CO and cytochrome P450:Fe^{3+}, which can be achieved by passing CO through both spectrophotometer cuvettes, with subsequent reduction of only the sample cuvette, haemoglobin will not interfere in the assay for cytochrome P450 in liver homogenates. In this case the haemoglobin which is almost all present in the reduced form [234] will combine with CO to form CO-haemoglobin, the absorbances due to this balancing out in the difference spectrum.

(b) Materials and Methods

A sample of human or rat liver homogenate (100 mg/ml) was diluted with KCl: Tris buffer (4 parts 1,15% KCl and 1 part 0,25M Tris:HCl pH 7,5) to result in a solution containing from 2,5 to 10 mg wet weight liver per ml, in a total of 2 ml. This solution was bubbled with CO at room temperature for 60 seconds, then divided between 2 semi-microcuvettes. These were placed in the "second

sample position" of the spectrophotometer, i.e. closest to the photomultiplier tube to minimise light scattering errors due to turbidity of the samples. In those cases where the hepatic cytochrome P450 levels were very low, e.g. in non-porphyric patients, recorder amplification was such that a full scale deflection corresponded to a change in extinction of 0,04 O.D. units.

A baseline was recorded from 410 nm to 500 nm then the suspension in the sample cuvette was reduced by the addition of about 1 mg sodium dithionite. After a time interval of 2 to 3 min. the difference spectrum was recorded from 410 nm to 500 nm.

(c) Comment on the Assay Method for Cytochrome P450 in whole Liver Homogenate

Bubbling of the sample with CO was gentle, at approximately 10 bubbles/sec. The following criteria were applied for acceptability of results:- In the baseline the change in absorbance readings between 490 and 450 nm should be less than 0,006, from 490 to 430 nm less than 0,0150 and from 490 to 470 nm, less than 0,004. Occasionally after addition of dithionite precipitation of sulphur in the cuvette caused rapid changes of absorbance with time. Results from experiments where this occurred were considered meaningless. The absorbance at 490 nm was monitored after addition of dithionite until the change in absorbance was less than 0,002 per min. before scanning the spectrum. This time interval was usually between 2 and 3 min. The peak of absorbance at 450 nm requires this amount of time to develop after the addition of the reducing agent, which is in contrast to the almost immediate maximum found with the addition of CO in the method of Omura and Sato[212].

II. Cytochrome P450 Levels in Rat Liver Microsomes with Carbon Monoxide as Ligand

(a) Principles of Assay

The method used for the determination of cytochrome P450 levels in microsomes was similar to that used by Omura and Sato[212]. The difference spectrum between cytochrome P450: Fe^{2+} : CO and cytochrome P450: Fe^{2+} was recorded.

(b) Materials and Methods

0,6 ml of a microsomal suspension in 0,25M potassium phosphate buffer, pH 7,5 was diluted with 5,4 ml of the same buffer. The sample was equally divided between a sample and reference cuvette, then a baseline of equal light intensity was recorded from 410 nm to 500 nm. The contents of both the reference and sample cuvettes were reduced with approximately 1 mg sodium dithionite, then only the sample cuvette was gassed gently with CO for 1 min. The difference spectrum between the two cuvettes was recorded from 410 nm to 500 nm.

(c) Comments on Assay Method for Determination of Microsomal Cytochrome P450 with Carbon Monoxide.

The baselines always showed a difference in absorbance readings between 450 nm and 490 nm of less than 0,005. Cuvettes contained from 0,8 mg to 1,5 mg protein per ml.

III. Cytochrome P450 Determinations in Rat Liver Microsomes with Ethyl Isocyanide as Ligand

(a) Principles of Assay

In 1966, Imai and Sato[133] suggested the existence of two forms of cytochrome P450 in rabbit liver microsomes. Using ethyl isocyanide rather than carbon monoxide as the ligand for reduced haemoprotein (since ethyl isocyanide combines reversibly with haemoglobin and myoglobin to give characteristic spectra[317], they observed Soret peaks at 430 and 455 nm. It was further found that the relative heights of the two peaks in respect of absorbance at 500 nm was pH dependent.

The use of ethyl isocyanide as a ligand for reduced P450 haemoprotein is not quantitative but qualitative, in that a change in the ratio of 455 to 430 nm peak heights at a particular pH suggests the existence of a different haemoprotein from that which in its reduced form combines with CO to result in a peak at 450 nm in the difference spectrum[274].

(b) Materials and Methods

0,6 ml of each microsomal suspension in 0,25 M potassium phosphate buffer at pH 6,0; 6,5; 7,0; 7,5 and 8,0 was diluted with 5,4 ml of the corresponding buffer. Samples were divided between sample and reference cuvettes (3 ml capacity), and a baseline of equal light absorbance was recorded between 400 and 510 nm. A few grains of sodium dithionite (about 1 mg) were added to both cuvettes, then 0,6 µl ethyl isocyanide was added to the sample cuvette. Absorbance was again recorded between 400 and 510 nm.

(c) Comment on P450 Assay with Ethyl Isocyanide

Baselines of equal light intensity always showed a difference in absorbance readings at 430 nm and 455 nm of less than 0,004. The amount of protein per cuvette ranged from 2,2 mg to 3,3 mg, at the different pH's.

(d) Ancillary Procedure - Synthesis of Ethyl Isocyanide according to Jackson and McKusick[137].

Ethyl isocyanide is not commonly synthesised hence the procedure is presented in detail.

The reactions involved are as follows:

$$C_2H_5I + Ag\ CN \rightarrow C_2H_5\ NC.AgI$$

$$C_2H_5NC.AgI + 2\ KCN \rightarrow C_2H_5NC + K\ Ag\ (CN)_2 + KI.$$

(The recommended procedure was scaled down to one/tenth). Silver cyanide 45,4g (0,34 mol) was added with stirring to 53g (0,34 mol) ethyl iodide in a 500 ml round-bottomed flask with 2 necks and containing a magnetic stirrer bar. One neck was then fitted with a reflux condenser, the other neck was closed. The flask was lowered into a beaker containing boiling water on a magnetic stirrer fitted with a heating plate. The reagents were vigorously stirred with the magnetic stirrer whilst in the boiling water bath, until a viscous homogeneous brown liquid resulted (about 2 hours). The boiling water bath was removed and 30 ml water was added through the condenser. Potassium cyanide 61g (0,937 mol) and 26 ml H_2O was added through the second neck of the flask, and the reaction mixture was stirred without further heating for 10 minutes. Stirring was stopped, and the reflux

condenser was replaced with a distillation condenser. A thermometer was placed through the second neck of the flask into the aqueous layer. A receiving flask was put into an ice bath, and the reaction mixture was heated with an electric mantle. When the temperature of the residual mixture in the reaction flask was 115°C, the distillation was discontinued. Sodium chloride 0,7g was dissolved in the aqueous layer contained in the receiving flask, then the ice-cold mixture was transferred to a separating funnel. The aqueous layer (lower phase) was separated and discarded. The crude ethyl isocyanide was washed with two 5 ml portions of ice cold saturated sodium chloride solution, then dried overnight with 1 g anhydrous magnesium sulphate. After decanting, the material was distilled, and the product which boiled off between 77 and 79°C was collected in an ice bath.

Caution was exercised throughout the synthesis, since ethyl isocyanide has been known to explode. Ethyl isocyanide has a vile odour and the entire procedure was carried out in an efficient fume hood, equipped with a thick glass front. Furthermore, aqueous cyanide is extremely toxic, and was disposed of by flushing down a drain together with copious quantities of water.

The pure ethyl isocyanide was stored in a sealed container at 4°C.

5.3. Hepatic Aminopyrine-N-Demethylation

(a) Principles of Assay

The hepatic microsomal mono-oxygenase system metabolises a wide variety of structurally dissimilar xenobiotics such as therapeutic agents, food additives, insecticides and chemical carcinogens[49]. The usual in vitro assay of activity of a drug-

metabolising enzyme is achieved by incubation of the hepatic post-mitochondrial supernatant or microsomal fraction with a substrate and cofactors which include NADPH or an NADPH generating system, then measuring either substrate disappearance or metabolite formation. A widely used marker enzyme assay is oxidative demethylation [102] of drugs, whereby the formaldehyde formed is quantitated spectrophotometrically after conversion to a chromogen in the Hantzsch reaction[46,206]. However, due to a limited sensitivity (10^{-7} to 10^{-8} moles of formaldehyde) using this method, a radiochemical method was developed by Poland and Nebert[224] which enabled oxidative N-demethylation to be measured in vitro in small samples of human liver biopsy material.

Aminopyrine is metabolised to 4-aminoantipyrine and formaldehyde in two successive oxidative N-demethylations [163] as shown in fig.8, the reaction being catalysed by the hepatic microsomal oxygenase system.

For the radiochemical assay, the substrate used was 4- {$^{14}CH_3$}2-amino-2,3 dimethyl - 1 - phenyl - 3 - pyrazolin - 5 - one. Unreacted lipophilic substrate can be separated from the hydrophilic ^{14}C-formaldehyde after reaction. In the course of reaction the ^{14}C - formaldehyde reacts with semicarbazide present in the assay mixture to form a semicarbazone:

$$H^{14}CHO + NH_2NHCONH_2 \rightarrow H_2{}^{14}CN : NHCONH_2$$

Formaldehyde Semicarbazide Formaldehyde Semicarbazone.

The hydrophilic carbazone is easily separable from the hydrophobic aminopyrine, 4-monomethylaminopyrine and 4-aminoantipyrine.

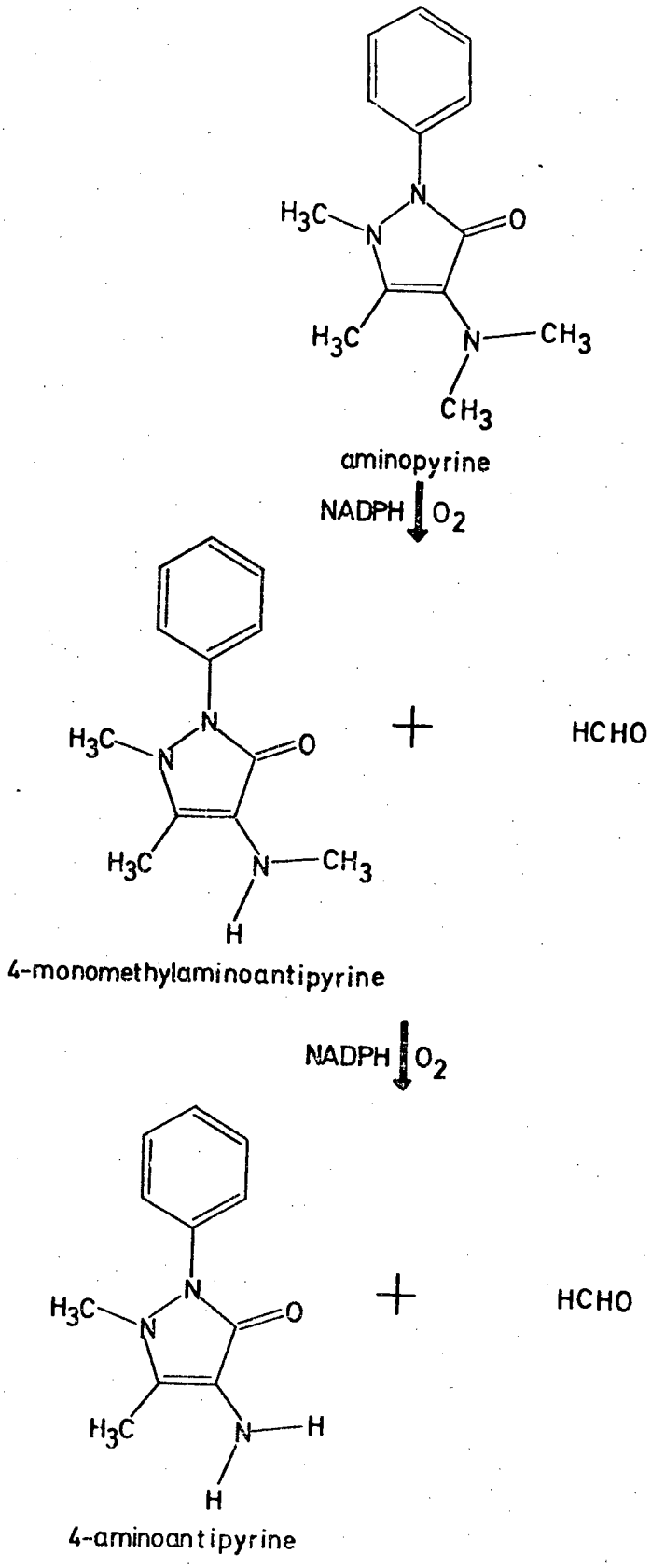


Figure 8 Oxidative N-demethylation of aminopyrine by the hepatic mixed function oxidase system.

(b) Materials and Methods

(1) A "cofactor" solution was freshly prepared as follows before the assay and kept under ice. All reagents were made up in 40 mM potassium phosphate buffer, pH 7,4.

1 part each of:	a.	Semicarbazide Hydrochloride 0,5 M (stock solution)
	b.	Nicotinamide 0,5M (stock solution)
	c.	Magnesium Chloride 0,25 M (stock solution)
	d.	NADP 0,05M
10 parts of:	e.	Glucose-6-phosphate 0,15M

The pH of this solution was adjusted to 7,4 with NaOH.

(2) a. The substrate solution consisted of aminopyrine 0,05 M in 40 mM potassium phosphate buffer, pH 7,4, kept under ice.

b. {dimethylamino ^{14}-C} Aminopyrine stock solution was prepared by the addition of 5 ml 40 mM potassium phosphate buffer to a vial containing 250 µCi radioactivity, with specific activity of 9,6 mCi/mmol.

Degradation of the stock solution of {dimethylamino ^{14}-C} aminopyrine occurred on storage at 4°C. To overcome this problem, just prior to assay, the stock solution was extracted with 20 ml chloroform, then the chloroform was evaporated using a water bath at 80°C. The radioactive aminopyrine was redissolved in 40 mM potassium phosphate buffer, pH 7,4.

(3) Glucose-6-phosphate dehydrogenase solution was made up in 40 mM potassium phosphate buffer, such that 0,005 ml contained 0,5 Kornberg units of activity.

For assay of aminopyrine demethylation, the incubation mixture consisted of 0,14 ml "cofactor" solution, varying amounts of substrate solution, ranging from 0,005 ml to 0,05 ml; and 0,020 ml {dimethylamino ^{14}-C } aminopyrine. The volume of the incubation mixture was adjusted

to 0,445 ml with 40 mM potassium phosphate buffer, pH 7,4.

At the start of the reaction, 0,005 ml glucose-6-phosphate dehydrogenase and 0,050 ml liver homogenate, (corresponding to 5 mg wet weight liver) were added to the incubation tube. Incubation was for 20 min. at 37°C, in a shaking water bath.

The reaction was terminated by the addition of the incubation mixture to 8 ml cold chloroform contained in a 50 ml round bottomed glass test tube fitted with a ground glass stopper. One ml of 0,1 N NaOH was added for alkalinity and to facilitate sample handling. The tube was stoppered, and mixed using a Vortex mixer. The substrate was by this step extracted into the chloroform phase, with the formaldehyde semicarbazone remaining in the aqueous phase. After centrifugation at 500 g for 20 minutes, 1,0 ml of the aqueous phase was reextracted with 8 ml chloroform, and the centrifugation was repeated. The radioactivity of the formaldehyde semicarbazone was determined by counting 0,5 ml of aqueous phase with 10 ml Instagel in a Beckman LS scintillation counter.

In addition, the radioactivity of the {dimethylamino ^{14}-C} aminopyrine used for each set of assays was determined by counting 0,010 ml of a 1:100 dilution of the radioactive aminopyrine with 10 ml Instagel in the liquid scintillation counter.

Blanks were always assayed, and consisted of the entire reaction mixture to which liver homogenate had been added after incubation and addition to chloroform.

For determination of ^{14}C - formaldehyde recovery, ^{14}C-formaldehyde was added to the reaction mixture containing rat liver homogenate but lacking {dimethylamino ^{14}-C} aminopyrine. The percentage recovery

of the ^{14}C- formaldehyde semicarbazide in the aqueous phase after extraction was determined.

All samples were recounted after the addition of 0,010 ml ^{14}C-n-hexadecane to determine counting efficiencies.

(c) Assay Procedure

In a total incubation volume of 0,5 ml contained in a small glass test tube 0,05 ml liver homogenate (100 mg wet weight liver/ml) was incubated with NADP, 0,5 µmol; glucose-6-phosphate 15 µmol; glucose-6-phosphate dehydrogenase, 0,5 Kornberg units; magnesium chloride 2,5 µmol; potassium phosphate, 20 µmol; semicarbazide hydrochloride 5 µmol; nicotinamide, 5 µmol; and varying amounts of aminopyrine (0,25 µmol to 2,5 µmol) containing 2 to 8 x 10^5 dpm radioactive aminopyrine. Incubation was for 20 min at 37°C, at pH 7,4.

The reaction was terminated by addition of the incubation mixture to 8 ml cold chloroform, then 1 ml 0,1 N NaOH was added. The unreacted lipophilic substrate was extracted into the chloroform. The aqueous phase was re-extracted with a further 8 ml cold chloroform, then 0,5 ml of the aqueous phase was counted with 10 ml Instagel in a Beckman scintillation counter. Samples were again counted after addition of ^{14}C-n-hexadecane as internal standard.

5.4. Hepatic 3,4-Benzpyrene Hydroxylation

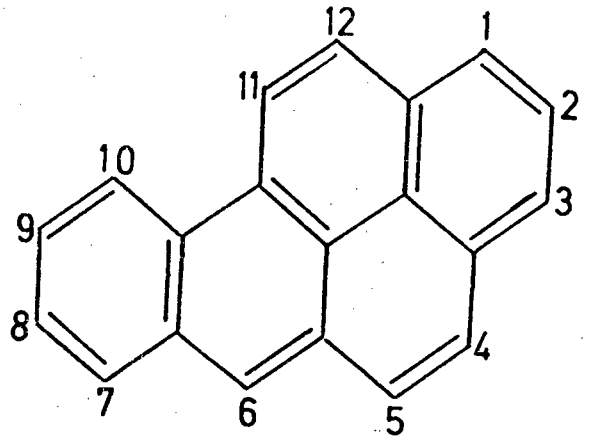
(a) Principles of Assay

Another widely used marker enzyme assay for the hepatic

microsomal mono-oxygenase system is the hydroxylation of 3,4-benzpyrene. The assay method followed was the spectrofluorometric method of Kuntzman et al[157] as modified by Alvares et al[5] as described previously[22]. Small amounts of tissue may be used in the assay system, since the product of the hydroxylation reaction, viz. hydroxy-3,4-benzpyrene is highly fluorescent, thus small amounts of product can be determined.

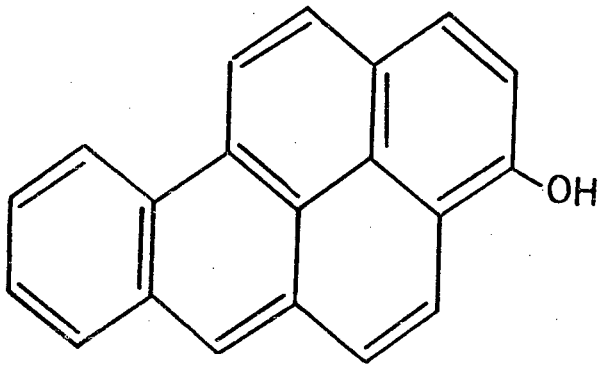
Under the conditions of the assay, 3,4-benzpyrene is converted to a variety of phenolic and other hydroxylated products [131,264]. However the activation and fluorescence spectra of the hydroxylated metabolites are largely identical to that of 3-hydroxy-3,4- benzpyrene, one of the major products, and thus used as a standard to calculate the amount of hydroxy-3,4-benzpyrene formed. The reaction involved is shown in fig. 9. The 3,4-benzpyrene and its metabolites are extracted into an organic phase, then the hydroxylated products are extracted into sodium hydroxide solution in which they are more soluble. The 3-hydroxy-3,4-benzpyrene can then be determined spectrofluorophotometrically.

By addition of albumin to the incubation system, linear reaction rates could be obtained with microsomes from small amounts of liver (less than 10 mg) [5]. Two possibilities for the mechanism of action for albumin were proposed: a. that albumin or a high concentration of microsomes could solubilise the 3,4-benzpyrene, or b. an inhibitor e.g. a heavy metal, in the cofactor mixture, inhibits the enzyme system at low enzyme concentrations. Added albumin could bind the inhibitor and prevent its access to enzymatic sites in the microsomes. Thus to ensure that the reaction was linear for the low tissue homogenate concentrations available from human liver



3,4-benzpyrene

NADPH O_2



3-hydroxy-3,4-benzpyrene

+

other hydroxy- and
quinone derivatives

Figure 9 Hydroxylation of 3,4-benzpyrene by the hepatic mixed function oxidase system.

biopsy samples, albumin was always included.

(b) Materials and Methods

(1) A"cofactor"solution was freshly prepared as detailed below and kept under ice. All reagents were made up in 0,1M potassium phosphate buffer, pH 7,4.

1 part each of:	a. NADP 0,005 M
	b. NAD 0,006 M
	c. ATP 0,01 M
	d. Potassium chloride 2 M (stock solution)
	e. Magnesium chloride 0,1 M (stock solution)
2 parts each of:	f. Nicotinamide 0,06 M (stock solution)
	g. Glucose - 6 - phosphate 0,03 M

The pH of this solution was readjusted to 7,4 with NaOH.

(2) A substrate solution was freshly prepared, and consisted of 50 µg 3,4-benzpyrene dissolved in 1 ml acetone.

(3) Glucose - 6 - phosphate dehydrogenase solution was made up in 0,1 M potassium phosphate buffer, pH 7,4, such that 0,005 ml contained 5 Kornberg units of activity.

(4) Albumin was dissolved in 0,1 M potassium phosphate buffer to a concentration of 300 mg/l.

For assay of 3,4-benzpyrene hydroxylation, the incubation medium consisted of 0,9 ml cofactor solution, 2 ml 0,1M potassium phosphate buffer containing 0,6 mg albumin and 0,020 ml liver homogenate (corresponding to 2 mg liver wet weight). This incubation system was preincubated at 37^{o}C for 5 min. The reaction was initiated by the addition of 0,005 ml glucose - 6 - phosphate dehydrogenase solution and varying quantities from 0,020 ml to 0,100 ml of the 3,4-benz-

pyrene substrate. A further quantity of acetone was added so that the total incubation volume was 3,025 ml.

Incubation was for 20 min at 37^{o}C in a shaking water bath, in very dim light. 3 ml cold acetone was added to terminate the reaction, followed by the addition of 9 ml n-hexane. 3,4-benzpyrene and its hydroxylated metabolites were extracted into the organic phase by mixing on a Vortex mixer. An 8 ml aliquot of the organic layer was shaken with 5 ml 1,0 N NaOH for 1 min, and the fluorescence of the extracted hydroxylated metabolites of 3,4-benzpyrene in alkaline solution was determined exactly 10 min. later using an Aminco-Bowman Spectrophotofluorometer at an activating wavelength of 382 nm and emission wavelength 510 nm, after standardisation of the instrument with quinine sulphate. The solvent extractions were performed in very dim light.

In order to standardise the Aminco-Bowman Spectrophotofluorometer, adjustments were made so that the reading was 33% when the meter multiplier was set on 0,1 for quinine sulphate solution (300 ng/ml) in 0,1 N sulphuric acid, at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. When a 100 ng/ml solution of 3-hydroxy-3,4-benzpyrene in 1 N NaOH was measured spectrophotofluorometrically at an excitation wavelength of 382 nm and an emission wavelength of 510 nm, without further adjustment of the machine after standardisation with quinine sulphate, the reading obtained was 51.5%. Thus each division on the scale corresponded to 1,942 ng 3-hydroxy-3,4-benzpyrene. Because of the limited supply of 3-hydroxy-3,4-benzpyrene, quinine sulphate was routinely used for standardisation.

Blanks were always included for each set of assays and consisted

of the entire reaction mixture to which 3,4-benzpyrene substrate was added only after the addition of 3 ml cold acetone.

In order to determine the recovery of hydroxylated metabolites from the incubation system by the solvent extraction procedure described above, 50 ng 3-hydroxy-3,4-benzpyrene contained in 0,050ml acetone was added to a complete reaction mixture including liver and 3,4-benzpyrene and extracted immediately.

(c) Assay Procedure

The reaction mixture for the assay in a total volume of 3,025 ml contained 0,02 ml liver homogenate (100 mg/ml); glucose-6-phosphate, 6 µmol; NADP, 0,5 µmol; NAD, 0,6 µmol; ATP 1 µmol; nicotinamide, 12 µmol; potassium chloride, 200 µmol; magnesium chloride, 10 µmol; glucose-6-phosphate dehydrogenase, 5 Kornberg units; potassium phosphate, 300 µmol;bovine serum albumin 0,6 mg; and from 4 to 20 nmol 3,4-benzpyrene (added in 0,1 ml acetone). Reactions were initiated with glucose-6-phosphate dehydrogenase and 3,4-benzpyrene after pre-incubation for 5 min. at 37°C. The mixture was incubated for 20 min. at 37°C in a shaking water bath in the dark. The reaction was terminated by the addition of 3 ml cold acetone, then the 3,4-benzpyrene and its hydroxylated products were extracted with 9 ml n-hexane. 8 ml of the organic phase was extracted with 5 ml 1,0 N NaOH for 1 min.,then the fluorescence of the extracted 3-hydroxy-3,4-benzpyrene in the alkaline aqueous phase was determined in an Aminco-Bowman spectrophotofluorometer (activation 382 nm; fluorescence 510 nm) exactly 10 min. later.

(d) Comment

The entire assay procedure for 3,4-benzpyrene hydroxylation was performed in very dim light because the decomposition of the hydroxylated metabolites of 3,4-benzpyrene is accelerated by light. Fluorometric measurements were speedily executed after addition of 1,0 N NaOH, since the fluorescence of the metabolites in alkaline solution decreases with time.

5.5. Red Blood Cell and Hepatic UROGEN-I Decarboxylase

(a) Principles of Assay

UROGEN III is converted to the tetracarboxylic COPROGEN-III in an intermediate step in the biosynthesis of haem[165]. Enzyme preparations from bacteria[125], avian red cells[301], mammalian reticulocytes[198], erythropoietic murine spleen[242] and porcine liver[162] have all been shown to catalyse the sequential decarboxylation of the four acetic acid side chains. A single enzyme is thought to be responsible for the removal of all four carboxyl groups, although the first decarboxylation proceeds more readily than the subsequent three[39].

UROGEN-I was chosen as the substrate for the decarboxylase assay, since it was shown that UROGEN decarboxylase from mouse spleen [242] and a pig liver extract as enzyme source[162] decarboxylated both I and III isomers at the same rate. The radiochemical assay used was essentially that of Kushner et al[158,162] with further modifications described hereunder. In essence, ^{14}C-PBG is converted to UROGEN-I at pH 7,65 using a bacterial preparation of UROGEN-I-synthetase. No UROGEN cosynthetase is present, thus only the isomer I will be formed. The ^{14}C-UROGEN-I formed is used directly at pH 6,8 as substrate for the decarboxylase present in an haemolysate or liver

post-mitochondrial supernatant. At this pH no further UROGEN I is generated. By a stepwise decarboxylation the ^{14}C-UROGEN-I is converted to COPROGEN-I as shown in Fig. 10.

The substrate and products of the reaction, which include all the intermediate porphyrinogens, viz. 7,6,5 and 4 carboxyl porphyrinogens are firstly converted to the porphyrins and subsequently to methyl esters, which are easily separable by thin layer chromatography. The ^{14}C-labelled porphyrin methyl esters can then be isolated from the thin layer chromatography plates, and counted in a liquid scintillation counter.

(b) Materials and Methods

(i) Incubation Conditions

The usual incubation system contained 0,15 ml 0,00885 M ^{14}C-PBG of specific activity 0,12 mCi/mmol; 0,1 ml 0,066M L-cysteine; and 0,1 ml of a 7 mg/ml solution of bacterial UROGEN-I synthetase. The solutions were made up in 0,1M tris:HCl, pH 7,65, and the volume of the incubation mixture was adjusted to 0,5 ml with the same buffer.

In some of the experiments, a modification was made in that ^{14}C-UROGEN-I was generated from ^{14}C-ALA in the presence of a mixture of bacterial ALA-dehydratase and UROGEN-I synthetase. In this case, the assay mixture consisted of usually 0,05 ml 0,02994M ^{14}C-ALA of specific activity 0,42 mCi/mmol; and 0,1 ml 0,066 M L-cysteine and 0,1 ml of a 7 mg/ml solution of ALA dehydratase and UROGEN-I synthetase. Again,the solutions were made up in 0,1 M tris:HCl buffer, and the volume of the incubation system was adjusted to 0,5 ml with the same buffer.

Reactions were initiated by the addition of the enzyme solutions. After an initial incubation in the dark for 30 min at 37^{o}C with shaking, 0,1 ml 0,4 M KH_2PO_4 was added before the addition of either 0,4 ml haemolysate or

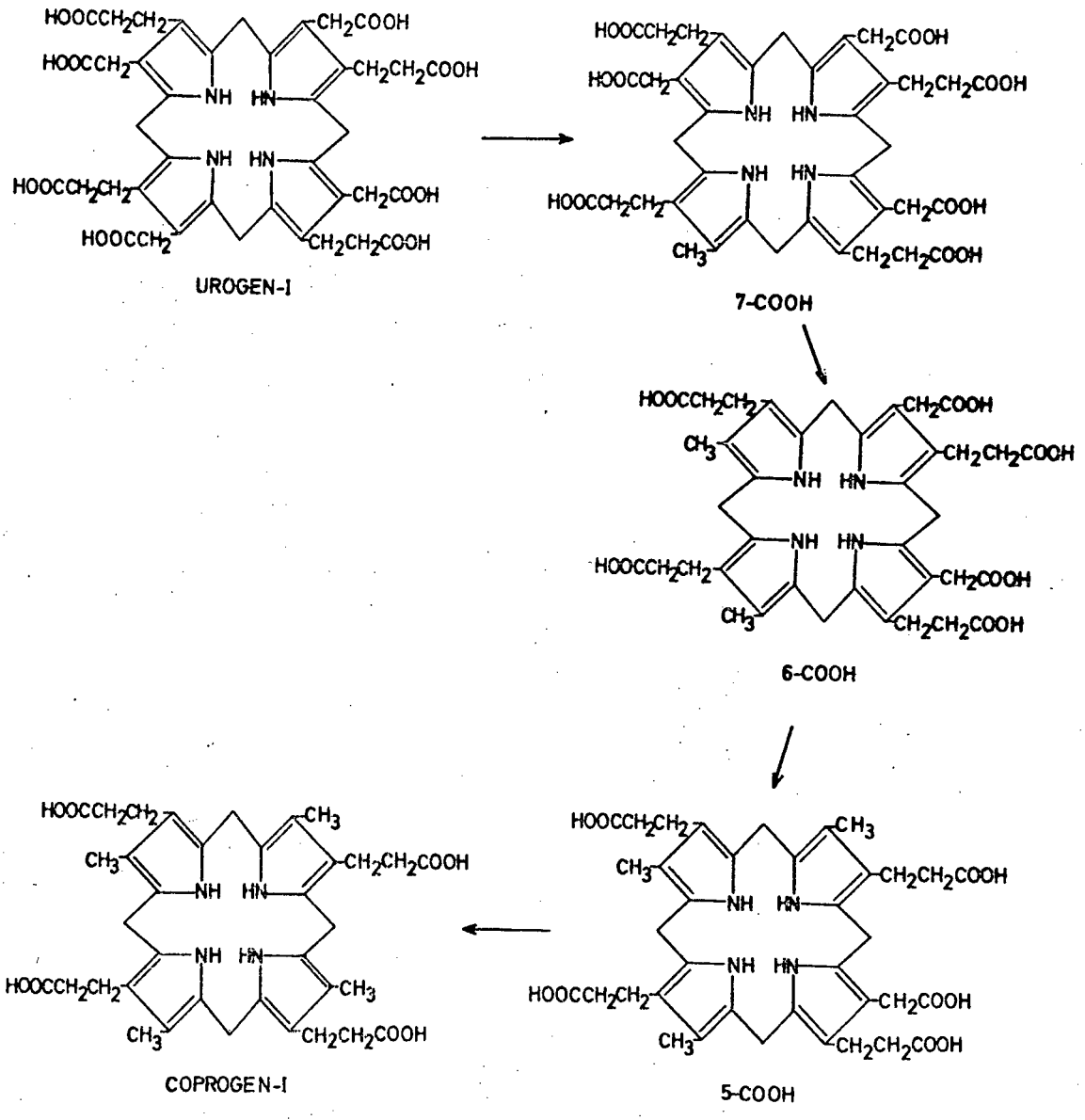


Figure 10 The stepwise decarboxylation of UROGEN-I through 7-, 6- and 5-carboxyl porphyrinogens to COPROGEN-I, catalysed by UROGEN decarboxylase.

0,2 ml liver post mitochondrial supernatant. The final incubation volume was adjusted to 1 ml with 0,1 M tris:HCl, pH 6,8 then the sample was incubated for a second period of 30 min. at 37°C, with shaking, in the dark. The reaction was terminated by the addition of 1 ml 3 N HCl.

(ii) Extraction and Esterification of Porphyrins

The sample was centrifuged on a Roto-Uni bench centrifuge, and after transfer of the supernatant to a second centrifuge tube, the pH was adjusted to 3,1 with saturated sodium acetate. Approximately 1g talc was added to adsorb and oxidise the porphyrinogens. The sample was allowed to stand in the dark for 1 hr with occasional stirring using a Vortex mixer. After centrifugation on a bench centrifuge and discarding the supernatant, the talc was dried by washing with 2 ml methanol. The porphyrins were converted to their methyl esters on the talc with 3 ml 5% H_2SO_4 in methanol, the sample being left overnight in the dark.

(iii) Extraction of Porphyrin Methyl Esters

The talc was repeatedly washed with 2 ml aliquots of 5% sulphuric acid in methanol until no further fluorescence was detectable in the wash under ultraviolet light. The porphyrin esters in the pooled washes were extracted into $CHCl_3$ by addition of $CHCl_3$ equal to half the volume of methanol/sulphuric acid, and 7% NaCl, resulting in a final volume 3 times that of the methanol/sulphuric acid. After washing of the $CHCl_3$ layer with an equal volume of 10% NH_4OH, followed by washing with an equal volume of 7% NaCl, it was filtered through $CHCl_3$-wetted filter paper (Whatman no. 1) into a beaker.

(iv) Chromatography of Porphyrin Esters

The chloroform was evaporated by directing a stream of air from a hairdrier into the beaker containing the

porphyrin ester solution. The esters were redissolved in 0,5 ml fresh chloroform and either an aliquot of 0,2 ml was applied to a silica gel TLC aluminium backed plate as a spot, or the entire sample was applied as a band, in both cases using a 100 µl Hamilton syringe to apply the porphyrin ester solution. Applications to TLC plates were made with a stream of air from a hair-drier blowing onto the plate. To each plate URO- COPRO- and PROTO- esters were applied as chromatographic markers. Plates were eluted with a solvent system consisting of carbon tetrachloride, dichloromethane, ethyl acetate and ethyl propionate in the ratio 2:2:1:1 (263).

Development of the plate in the solvent system was allowed to proceed until the solvent front had migrated 12 to 16 cm from the origin.

(v) Scintillation Counting

After drying,the TLC plate was exposed to ultraviolet light and the positions of 8-COOH, 7-COOH, 6-COOH, 5-COOH and 4-COOH porphyrin esters were marked. Each of the fluorescent spots or bands was scraped off, the scrapings being placed in a counting vial. 10 ml Instagel was added to the scrapings and samples were counted in a Beckman scintillation counter.

(vi) Control Assay

A blank assay for UROGEN-I-decarboxylase was always performed in duplicate witheach set of assays. Assay was done exactly as described previously, but no enzyme source for the decarboxylase was added prior to the second incubation, the volume being made up to 1 ml with 0,1 M tris:HCl, pH 6,8. The apparent UROGEN-I-decarboxylase activity observed in these samples (due to the possible presence of UROGEN-I-decarboxylase as a contaminant in the enzyme preparations) was then applied as a correction factor in the calculation of UROGEN-I-decarboxylase activity in red blood cells or liver.

(c) Assay Procedure

In a total volume of 0,5 ml, the reaction mixture contained PBG including ^{14}C-PBG, 1,33 µmol; L-cysteine, 6,61 µmol; UROGEN-I synthetase (sufficient to produce 25 - 30 nmol UROGEN-I); tris:HCl 50 µmol; pH 7,65. For preparation of the ^{14}C-UROGEN-I substrate from ^{14}C-ALA, the reaction mixture contained ALA including 4-^{14}C-ALA, 1,497 µmol; L-cysteine, 6,61 µmol; a mixture of ALA-dehydratase and UROGEN-I synthetase (sufficient to produce 25 - 30 nmol UROGEN-I); tris:HCl 50 µmol, pH 7,65. Reactions were always initiated with the enzyme preparation.

After incubation for 30 min. at 37^{o}C in the dark with shaking, KH_2PO_4, 40 µmol; haemolysate containing from 30 to 60mg haemoglobin or a post-mitochondrial liver supernatant containing 1,5 to 2,5 mg protein; and 0,1 M tris:HCl pH 6,8 were added to result in a total volume of 1 ml. Following a further incubation of 30 min. at 37^{o}C in the dark with shaking, reactions were terminated by addition of 1 ml 3 N HCl. After centrifugation, the pH of the supernatant was adjusted to 3,1 with saturated sodium acetate, and the porphyrins adsorbed on talc and esterified overnight in 5% H_2SO_4 in methanol. The porphyrin methyl esters were transferred into chloroform, and identified chromatographically on aluminium backed silica gel TLC plates, developed with carbon tetrachloride, dichloromethane, ethyl acetate and ethyl propionate in the ratio 2:2:1:1[263]. The silica gel at the fluorescent spots was scraped off into Instagel and the radioactivity determined..

(d) Comment

In each series of assays for UROGEN-I decarboxylase, the amount of enzymatically produced UROGEN-I substrate was determined as detailed in (e)(i) below. In one series of experiments, the effects on UROGEN-I decarboxylase of ferrous ammonium sulphate, ferritin and 1,10-phenanthroline added to the assay medium were assessed. Solutions were made up in 0,1M tris:HCl, pH 7,65 and either 0,05 ml 0,020M ferrous ammonium sulphate or 0,005 ml of a 1:1 dilution of commercial ferritin in .15 M NaCl or 0,15 ml 0,020M 1,10-phenanthroline, were added at the start of incubations. The volume of 0,1M tris:HCl, pH 7,65 was adjusted to result in an initial incubation volume of 0,5ml. The assay was performed as described in (b) and (c) above using rat liver as the source of UROGEN-I decarboxylase. Further, the quantity of UROGEN-I generated in the presence of each of these additives was determined as described in (e)(i) below.

In another experiment, the effect of increasing concentrations of ferrous iron in the incubation medium on UROGEN-I decarboxylase was investigated. From 0,002 ml to 0,05 ml 0,005 M ferrous ammonium sulphate in 0,1M tris:HCl, pH 7,65 for the lower concentrations and from 0,02M to 0,10 ml 0,0200 ferrous ammonium sulphate in the same buffer for the higher concentrations, was added at the start of incubation. Suitable adjustments in the volume of 0,1M tris:HCl buffer, pH 7,65 again were made, to result in an initial incubation volume of 0,5 ml. The assays were performed as described previously with rat liver as the source of UROGEN-I decarboxylase. Again, the quantity of UROGEN-I substrate generated at each concentration of Fe^{2+} was determined.

In some of the later experimental work, an alternative method was utilised for the oxidation of the porphyrinogens produced in the

the UROGEN-I-decarboxylase assay, and a modified method was used for the esterification and extraction of porphyrins. These modifications will be described in Section 6.12.

(e) Ancillary Procedures to UROGEN-I-Decarboxylase Activity Determinations

(i) Determination of UROGEN-I Substrate Formed

The determination was performed in duplicate concurrently with each group of assays for UROGEN-I-decarboxylase. The incubation mixture was identical to that described in section 5.5.(b) for UROGEN-I-decarboxylase assay, as were the incubation conditions. However, no enzyme source for UROGEN decarboxylase was added prior to the second 30 minute incubation, this beingreplaced by the appropriate volume of 0,1 M tris:HCl, pH 6,8. At the end of the second 30 min. incubation period, the UROGEN-I which had been formed was converted to URO-I by the dropwise addition of 0,2 M iodine in 0,3 M potassium iodide to the assay tube until a straw yellow colour was obtained. This was immediately followed by the addition of a crystal of sodium thiosulphate to reduce any excess iodine.

The absorbance of the solution was determined immediately at 560 nm [29]. The reference cuvette contained all reagents used in the assay including UROGEN-I synthetase or a mixture of ALA-dehydratase and UROGEN-I synthetase, added just prior to spectrophotometry.

To determine the degree of autooxidation of UROGEN-I under the conditions of assay, porphyrin content was determined both before and after treatment with iodine.

(ii) The Extinction Coefficient for URO-I at 560 nm in the Incubation Mixture for UROGEN-I-Decarboxylase.

The millimolar extinction coefficient for URO at 560 nm has been determinedas 7,59 $cm^{-1}mM^{-1}$ in 0,1 M tris, pH 8,2, using a Cary spectrophotometer[29]. It was felt necessary to check this figure, since UROGEN-I determinations (equivalent to URO-I determinations assuming complete oxidation of

the porphyrinogen) described in the previous section were made at pH 6,8 using a Pye Unicam SP 1700 Ultra-violet Spectrophotometer, in the presence of UROGEN-I synthetase.

Approximately 10 mg chromatographically pure URO-I was weighed and dissolved in 0,1M tris:HCl, pH 7,65 to result in a solution which was 200 µM in respect of URO-I. Varying quantities of this up to 0,14 ml were added to solutions containing 0,15 ml 8,85 mM solution of ^{14}C-labelled PBG, 0,1 ml 0,066 M L-cysteine and 0,1M tris:HCl, pH 7,65 to result in a volume of 0,4 ml. In each case, 0,1 ml 0,4M KH_2PO_4 and 0,4 ml 0,1M tris:HCl, pH 6,8 were added.

0,2M iodine in 0,3M KI was added dropwise to each sample until a straw yellow colour was obtained, followed immediately by the addition of a crystal of sodium thio-sulphate. Optical densities of the solutions at 560nm (i.e. the maximum absorbance in the region 550 nm to 565 nm) were determined after the addition of 0,1 ml of a 7 mg/ml solution of UROGEN-I synthetase to each solution. A solution containing the above reagents, with the exception of URO-I, which was replaced by 0,1M tris:HCl, pH 7,65, was used in the reference cuvette.

(iii) Establishment of Substrate and Enzyme Concentrations for Generation of Sufficient UROGEN-I for the Assay of UROGEN-I-Decarboxylase.

In most of the studies where the activity of UROGEN-I-decarboxylase in human red blood cells and in the livers of rats was determined, UROGEN-I synthetase and ^{14}C-PBG were used in the generation of ^{14}C-UROGEN-I. It was necessary at an early stage to determine the concentrations of enzyme and substrate, which, under the conditions of incubation, would result in the formation of the 25 - 30 nmol UROGEN-I required as substrate for the UROGEN-I decarboxylase. Thus in an initial experiment, incubations were performed as described in (i) above, but where an arbitrary and convenient

amount of enzyme, 0,1ml of a 7 mg/ml solution was incubated in the presence of varying volumes of a 2 mg/ml ^{14}C-PBG solution.

In some of the later studies, a mixture of ALA dehydratase and UROGEN-I synthetase was used to generate ^{14}C-UROGEN-I from ^{14}C-ALA. In the initial experiment to determine the enzyme and substrate concentrations required for the generation of 25 - 30 nmol UROGEN-I, incubations were performed exactly as described above, but 0,1 ml of a 7 mg/ml solution of the ALA dehydratase/UROGEN-I synthetase mixture was incubated in the presence of various quantities of a 5 mg/ml (0,02994M) ^{14}C-ALA solution.

5.6. Porphyrin Determinations in Liver, Urine and Faeces of Rats

I. Liver

(a) Principles of Assay

After excision of the livers from rats,weighed amounts of liver are homogenised with 5% H_2SO_4 in methanol. In this way the porphyrins present are directly esterified, the esters subsequently being extracted into chloroform. Aliquots of the chloroform extract are chromatographed on thin layer plates, then the plates are scanned using a Vitatron VLD 100 densitometer, fitted with an integrating recorder. By comparison of areas under each peak with the areas under the peaks of known quantities of porphyrin ester standards, the quantity of each porphyrin in the liver can be calculated.

(b) Materials and Methods

From 40 to 80 mg liver (in the case of HCB-treated animals) and from 200 to 250 mg liver (in the case of control animals) were accurately weighed. The liver samples were homogenised with 5 ml 5% H_2SO_4 in methanol. After allowing the porphyrins to esterify

overnight in the dark, the methyl esters were extracted into $CHCl_3$ by addition of 5 ml $CHCl_3$ and 5 ml 7% NaCl. The $CHCl_3$ layer was then washed successively with 10% NH_4OH and 7% NaCl, followed by filtration through $CHCl_3$-wetted Whatman no. 1 filter paper. After evaporation of the $CHCl_3$ as described in Section 5.5(b), the esters from the porphyric livers were redissolved in 1 ml $CHCl_3$. A 5 µl aliquot of the ester solution was applied as a spot with a Hamilton syringe to an aluminium-backed thin layer chromatography plate. The porphyrin esters from the control livers were redissolved in 0,5 ml $CHCl_3$, with 50 µl being applied as a spot to the thin layer chromatography plate.

To each plate, URO- COPRO- and PROTO- esters in $CHCl_3$ with respective concentrations of .006361 mg/ml, 00832 mg/ml and .005553 mg/ml were applied to the same spot. (The concentrations of the standard solutions was checked periodically by measuring the absorbance at 380nm, the absorbance maximum between 395 and 410 nm and at 430 nm). The plates were eluted with a solvent system consisting of 2:2:1:1 carbon tetrachloride, dichloromethane, ethyl acetate and ethyl propionate. After development and drying, each spot was scanned in a Vitatron VLD 100 densitometer, fitted with a 399 nm activating filter and 620 nm emission filter. The area under each peak was recorded with an integrating recorder.

II. Urine

(a) Principles of Assay

The quantitative assessment of urine by thin layer chromatography is not satisfactory, but this method can be used for qualitative work. The method of choice for quantitating URO and

COPRO is the solvent extraction method of Rimington and Sveinsson[241]. Using this classical technique, COPRO is extracted from urine with glacial acetic acid and ether, and URO is extracted with cyclohexanone at pH 1,5. However, it should be realised that the 7-, 6-, and 5 carboxylated porphyrins will contaminate both fractions.

(b) Materials and Methods

(i) Qualitative Assessment

The pH of 5 ml of pooled rat urine was adjusted to 3,1 with saturated sodium acetate. Approximately 1g talc was added to adsorb the porphyrins, then the sample tube was allowed to stand in the dark for 1 hr with occasional stirring using a Vortex mixer. The tube was centrifuged on a Roto-Uni bench centrifuge, and the supernatant was discarded. The talc was dried by washing with 2 ml methanol, then the porphyrins were esterified overnight in the dark by the addition of 5 ml 5% H_2SO_4 in methanol until no further fluorescence under a Woods lamp was detectable in the wash. The esters were extracted into chloroform as described in Section 5.5.(b)(iii), washed, filtered and evaporated. After reconstitution with 1 ml chloroform, an aliquot of the porphyrin ester solution was applied as a spot to a thin layer chromatography plate - sufficient being applied to be easily visible as a fluorescent red spot under ultra-violet light. Standard URO-, COPRO-, and PROTO- esters were applied to each plate as markers. The plates were eluted and scanned as in section 5.6.I(b), without recording of the areas under the peaks.

(ii) Quantitative Assessment

Urinary "URO" and "COPRO" fractions were quantitated according to Rimington and Sveinsson[241]. A 5 ml aliquot of pooled urine from control rats or an 0,5-1,0 ml aliquot of pooled urine from HCB-treated rats was taken for analysis. The urine was acidified with 0,1 volumes

of glacial acetic acid and extracted with 20 ml aliquots of ether until no further fluorescence was extractable. The ether extracts were pooled, and washed with 20 ml 0,5% sodium acetate, 20 ml 0,005% iodine and 10 ml distilled water. All aqueous phases from the ether extraction were pooled for "URO" determination.

The ether solution was extracted with repeated 2 ml volumes 1,5 N HCl until no further fluorescence could be extracted. The HCl extracts were combined for "COPRO" determination and volume recorded.

The pH of the residual urine and combined aqueous washes was adjusted to 1,5 with 10% HCl. The solution was extracted twice with 40 ml volumes of cyclohexanone, then 80 ml petroleum ether was added to the pooled cyclohexanone. "URO" was extracted with repeated 2 ml volumes of 2% HCl until no further fluorescence was extractable, and the HCl extracts combined for URO determination. The volume of the HCl pooled extracts was recorded.

The absorbances for "COPRO" and "URO" against 1,5N HCl and 2% HCl respectively at 380 nm, the absorbance maximum between 400 and 410 nm, and at 430 nm were determined.

III. Faeces

(a) Principles of Assay

Because the object in analysing faecal porphyrins was to detect the presence of the porphyrin P_1 fraction, this being indicative of PCT or a PCT-like syndrome[78,79], it was necessary only to do a qualitative analysis. This was achieved by thin layer chromatography of the porphyrin methyl esters.

(b) Materials and Methods

Faeces were pooled and mixed with a pestle and mortar. To 2g of this mixture was added 5 ml 1,5 N HCl after which it was stirred for 5 min. with a glass rod. The faecal residue was separated by

centrifugation in a Roto-Uni bench centrifuge, and after decanting, the pH of the supernatant solution was adjusted to 3,1 with saturated sodium acetate. The porphyrins were adsorbed onto talc, esterified and the methyl esters analysed as detailed in Section 5.6(II)(b)(i).

5.7. Other Methods Used in the Experimental Work

I. Haemoglobin Determination in Haemolysates

The haemoglobin concentration in haemolysates was determined by the cyanmethaemoglobin method, using Aculute Diluent Pellets and Acuglobin Haemoglobin Standard (Ortho Diagnostics, Rariton, N.J.) Usually 20 µl of haemolysate was added to 5 ml Aculute Diluent, and the absorbance determined after 10 min. at 540 nm against Aculute Diluent as a blank. Readings were compared to that obtained for Acuglobin Haemoglobin Standard against Aculute Diluent, for calculation of haemoglobin concentrations.

II. Protein Determinations

These were made by the method of Lowry et al[178] using bovine serum albumin, fraction V as standard. All protein determinations were made so that the amounts measured ranged from 0,05 mg to 0,25mg thus falling within the linear range of concentration as measured spectrophotometrically at 750 nm.

III. Determination of Total Hepatic Iron

(a) Principles of Assay

Ferrozine has been used by Carter[43] to spectrophotometrically determine serum iron, at the submicrogram level. Furthermore, the

method has been adapted slightly by Montgomery et al[203] to measure microsomal iron. In this study, the method had been extended to the measurement of total hepatic iron. Ferrozine, or the disodium salt of 3-(2-pyridyl) -5,6-bis-(4-phenylsulphonic acid)-1,2,4-triazine is a bidentate ligand, and with iron forms a stable magenta coloured, water soluble iron II complex, which is spectrophotometrically determined at 562 nm.

(b) Assay Procedure

Rat liver homogenate, 0,1 ml was oxidised at 100°C for 24 hours with 1.9 ml 10% perchloroacetic acid and 15% hydrogen peroxide. 0,1 ml of the oxidised-homogenate was added to 0,4 ml deionized water in a centrifuge tube and to this solution was added 0,5 ml of a reducing solution consisting of 20 mg ascorbic acid and 1,67 ml concentrated HCl per 100 ml. The solution thus obtained was allowed to stand at room temperature for 5 to 10 min., then 0,5 ml of an 11,3% trichloracetic acid solution was added to precipitate the proteins. After centrifugation in a Roto-Uni Bench centrifuge for 5 min. at 1500 rpm, 1,0 ml of the clear supernatant was transferred to another tube. To this was added 0,4 ml 10% NH_4COOH, and 0,1 ml of colour reagent. The colour reagent was made by dissolving 75 mg ferrozine and 25 mg neocuproin in 25 ml deionized water containing one drop of concentrated HCl. The colour was allowed to develop for 5 min. before the absorbance was determined at 562 nm against water.

A blank and a standard were prepared with each set of unknowns where the oxidised liver homogenate was respectively substituted by deionized water and 0,5 ml of a 1:100 dilution of standard solution. This consisted of $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$, 0,7 0 2g per liter, made up

to contain 0,5 ml concentrated sulphuric acid.

IV. Microscopic Studies

It was an important part of the experimental work to correlate microscopic studies with biochemical findings. The microscopic studies and their interpretation were performed by Dr. B.L. Webber of the Department of Pathology, University of Cape Town Medical School.

(a) Fluorescence microscopy

An International Harris Cryostat (Model CTD) was used to obtain frozen sections. These sections were examined in an Olympus FLMP 2 fluorescence microscope, with a mercury vapour lamp as the light source with a UV excitor and O-54 barrier filters. The samples were exposed to artificial lighting for the minimum period of time, i.e. less than 10 min.

(b) Light Microscopy

Tissues fixed in formalin were examined after staining with haemotoxylin and eosin[53]. Tissue was stained also for reticulin by the method of Gordon and Sweet[53], as well as for haemosiderin, using Perls Prussian Blue stain with heated reagents[53].

(c) Electron Microscopy

Tissues that were fixed immediately after biopsy in 5% phosphate buffered glutaraldehyde were post-fixed in Palade's osmium fixative. The tissues were embedded in Spurr's resin and cut on an LKB automatic ultramicrotome with glass knives. Sections were stained with uranyl acetate and lead citrate, then examined on a GEC-AEI 6B electron microscope at 60 kV.

CHAPTER 6

RESULTS

The 38 patients selected as controls for biochemical studies admitted to excessive alcohol intake and had clinical, biochemical or histologic evidence of ethanolic liver damage. They exhibited no biochemical evidence of deranged porphyrin metabolism, as assessed by screening tests for urinary and faecal porphyrins[75]. The screening tests, which were performed in the Porphyrin Diagnostic Laboratories, Groote Schuur Hospital, proved to be negative in each case.

Sixteen of the 19 patients with clinical symptoms of PCT admitted to excessive alcohol intake. In one patient, H.G., there was no history of alcohol, and another was taking oral contraceptives. In patient B.H., aged 8, there was no history of alcohol; nor was there any evidence that the patient had ingested any porphyrogenic toxin. On liver biopsy, the only abnormality was fatty change. Hepatic siderosis could not be demonstrated.

6.1.(a) Porphyrin Excretion and Accumulation in PCT

Quantitative analysis by the Porphyrin Diagnostic Laboratory of porphyrins present in the excreta of the 19 patients with clinical symptoms of PCT confirmed the diagnosis (Table 3).

TABLE 3

Porphyrin Excretion in 19 Patients with PCT

	Urine (µg/liter)		Faeces (µg/g)	
	URO	COPRO	COPRO	PROTO
Mean	2356	569	129	91
Range	262-5279	86-1262	11-597	11-253

In all cases the quantity of urinary URO was elevated above that of urinary COPRO. The separation of urinary porphyrins as their methyl esters by thin layer chromatography yielded a profile similar to that shown in Fig. 11 for all patients with PCT. The faecal porphyrin profiles invariably revealed the presence of the porphyrin P_1 fraction, i.e. isocoproporphyrin and de-ethylisocoproporphyrin (Fig. 11). Liver biopsy samples fluoresced strongly under ultra-violet light, and the characteristic hepatic porphyrin profile, i.e. almost exclusively URO and 7-COOH porphyrin, was noted in every case (Fig. 11).

(b) Porphyrin Excretion and Accumulation in HCB- and HCB plus * Jectofer-treated Rats.

(i) Urinary Porphyrins

Urinary excretion of URO and COPRO at 15 day intervals up to 60 days in HCB- and HCB plus Jectofer-treated rats is shown in Fig. 12a,b.

At 15 days, both groups showed no substantial increase in porphyrin excretion. A rise in the output of URO was manifested at 30 days in the HCB plus Jectofer-treated animals which increased to a mean value of 80,94µg/ml

* An iron-sorbitol-citric acid complex

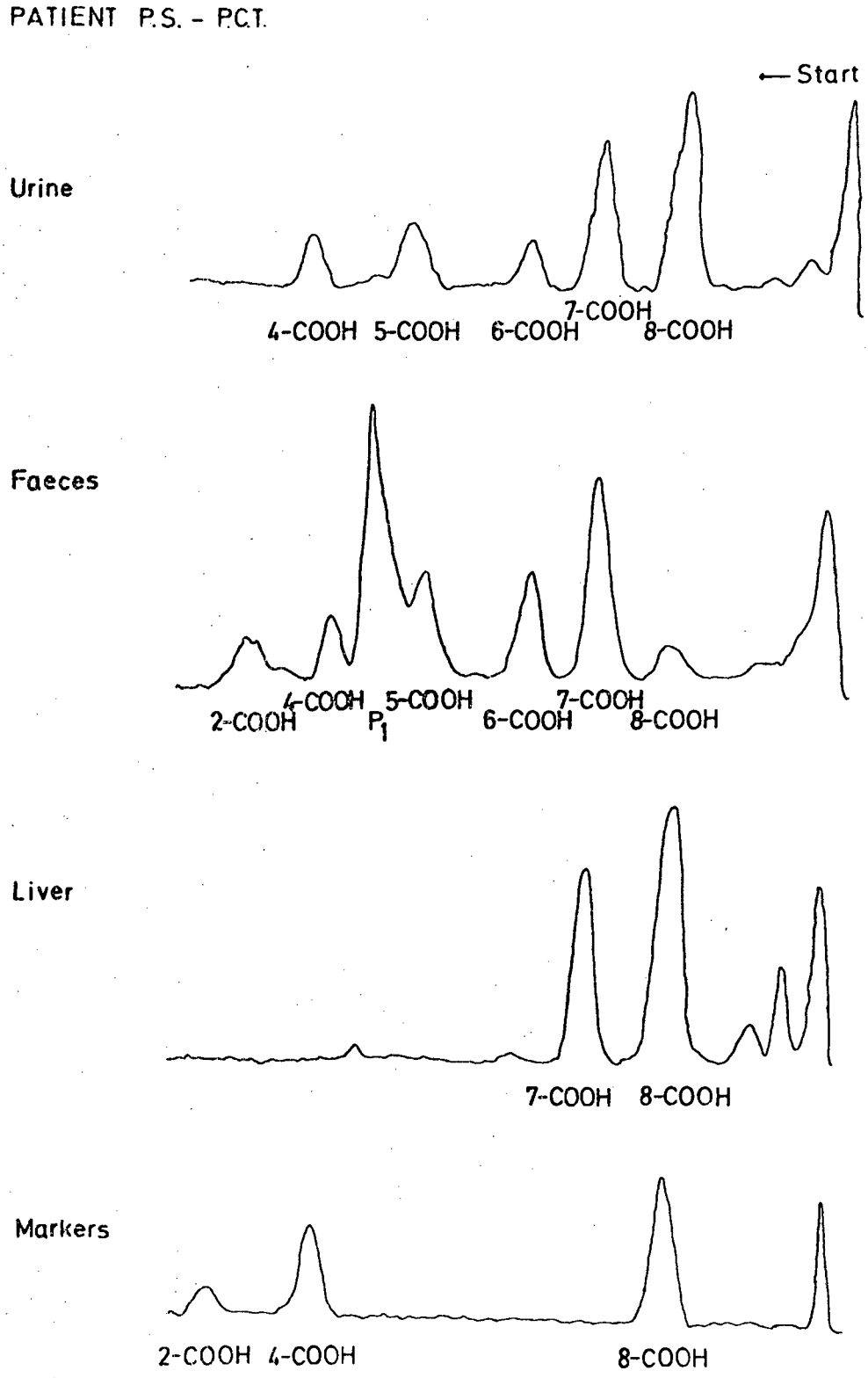


Figure 11 Porphyrin composition in urine, faeces and liver in a patient with PCT. Porphyrins were extracted, esterified, chromatographed, and the fluorometric scans obtained using a Vitatron VLD 100 densitometer are shown.

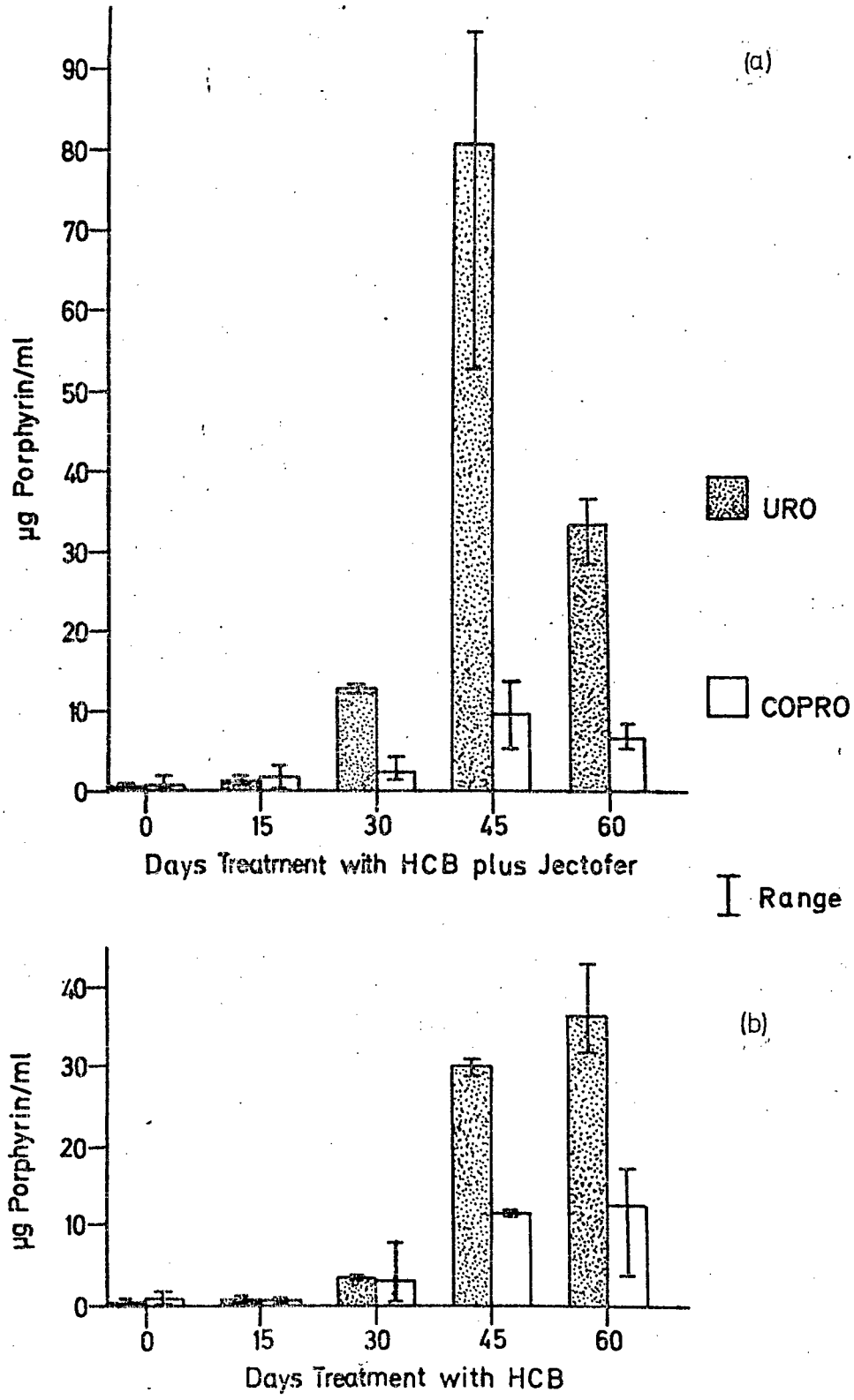


Figure 12 Urinary excretion of URO and COPRO as determined by the method of Rimington and Sveinsson (241) at 15 day intervals in rats treated simultaneously with HCB and Jectofer and in rats treated with HCB alone. The height of the bars is the mean value of porphyrin excretion observed in 3 rats: (a) simultaneously treated with HCB and Jectofer; (b) treated with HCB.

at 45 days, then dropped to a mean value of 33,94µg/ml at 60 days. The mean level of COPRO in this group did not rise appreciably at 30 days, reaching a mean of 9,69µg/ml at 45 days, and decreasing slightly to a mean of 6,55µg/ml at 60 days. The rats treated with HCB alone did not show significant porphyrinuria at 30 days but at 45 days both URO and COPRO excretion was elevated to mean values of 30,35µg/ml and 11,55µg/ml respectively. At 60 days mean URO excretion was 36,67µg/ml and mean COPRO excretion was 12,67µg/ml.

Thin layer chromatographic analyses of the methyl esters of extracted urinary porphyrins from rats on both treatment schedules are shown in Fig. 13. The trend of urinary porphyrin excretion in both groups followed that indicated in Fig. 12 a,b. At 30 days treatment with HCB plus Jectofer there was a shift from mainly COPRO excretion to URO excretion, as judged by the areas under the peaks, and the presence of 7-, 6- and 5-COOH porphyrins was detected. Note that at 30 days treatment with HCB alone, the shift from COPRO to URO was less evident. The shift towards the higher carboxylated porphyrins became increasingly pronounced at 45 and 60 days for both treatments, at which times the intermediate 7-, 6- and 5-COOH porphyrins were present in significant quantities.

(ii) Faecal Porphyrins

Qualitative assessment by thin layer chromatography of methyl esters of extracted faecal porphyrins from rats treated for 45 days with HCB showed the presence of a significant quantity of the porphyrin P_1 fraction (Fig.14). No porphyrin P_1 was detected in the faeces of untreated rats.

(iii) Hepatic Porphyrins

Hepatic porphyrins were not measurable in the livers of control animals (Figs. 15,16). After 15 days treatment of rats with either HCB or HCB plus Jectofer, small increases in the levels of URO and COPRO were observed (Figs. 15,16) with small quantities of 7-COOH porphyrin being de-

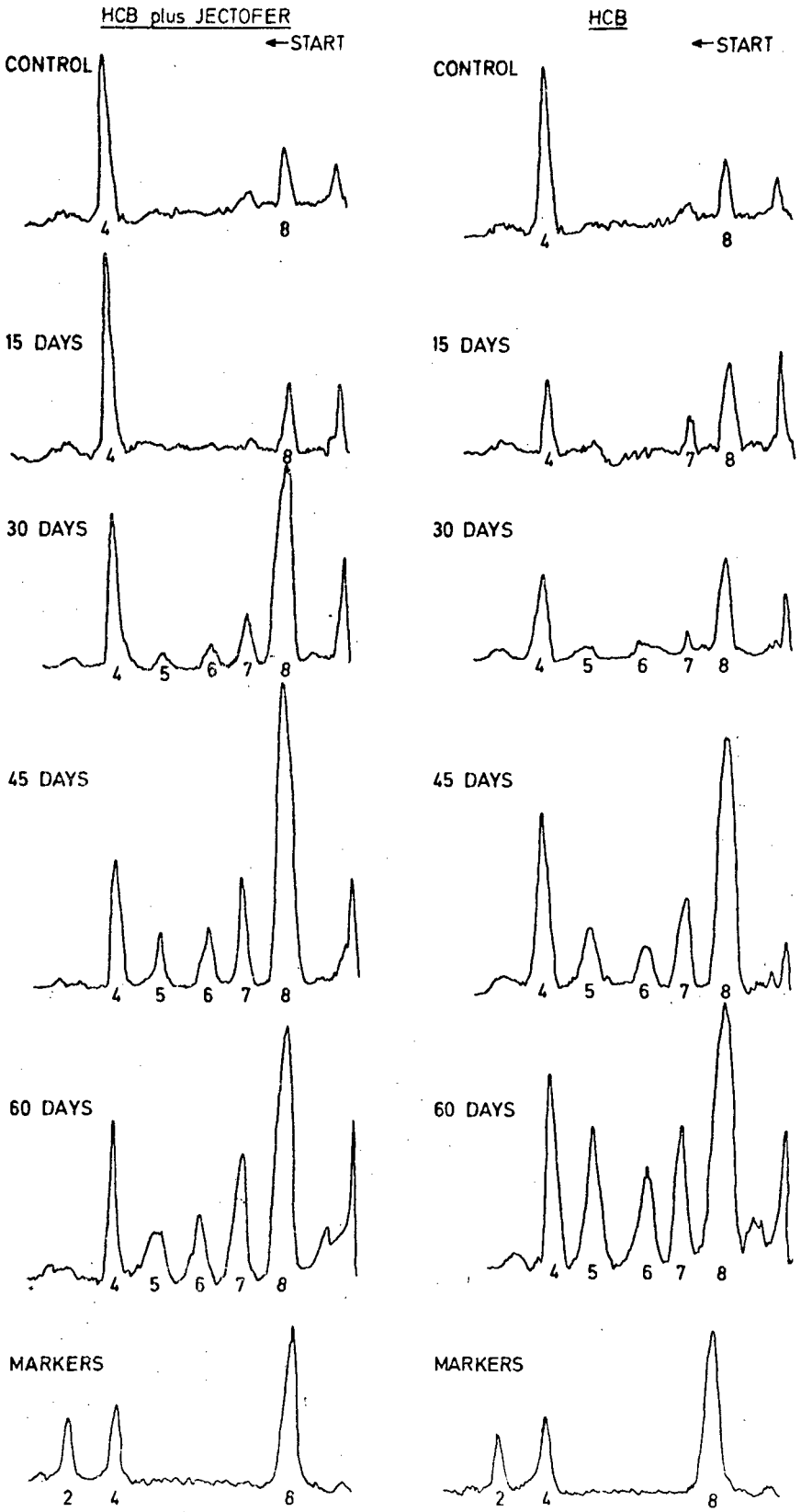


Figure 13 Urinary porphyrin composition at 15 day intervals in rats treated simultaneously with HCB and Jectofer and in rats treated with HCB. Porphyrins were extracted, esterified, chromatographed, and the fluorometric scans obtained using a Vitatron VLD 100 densitometer are shown.

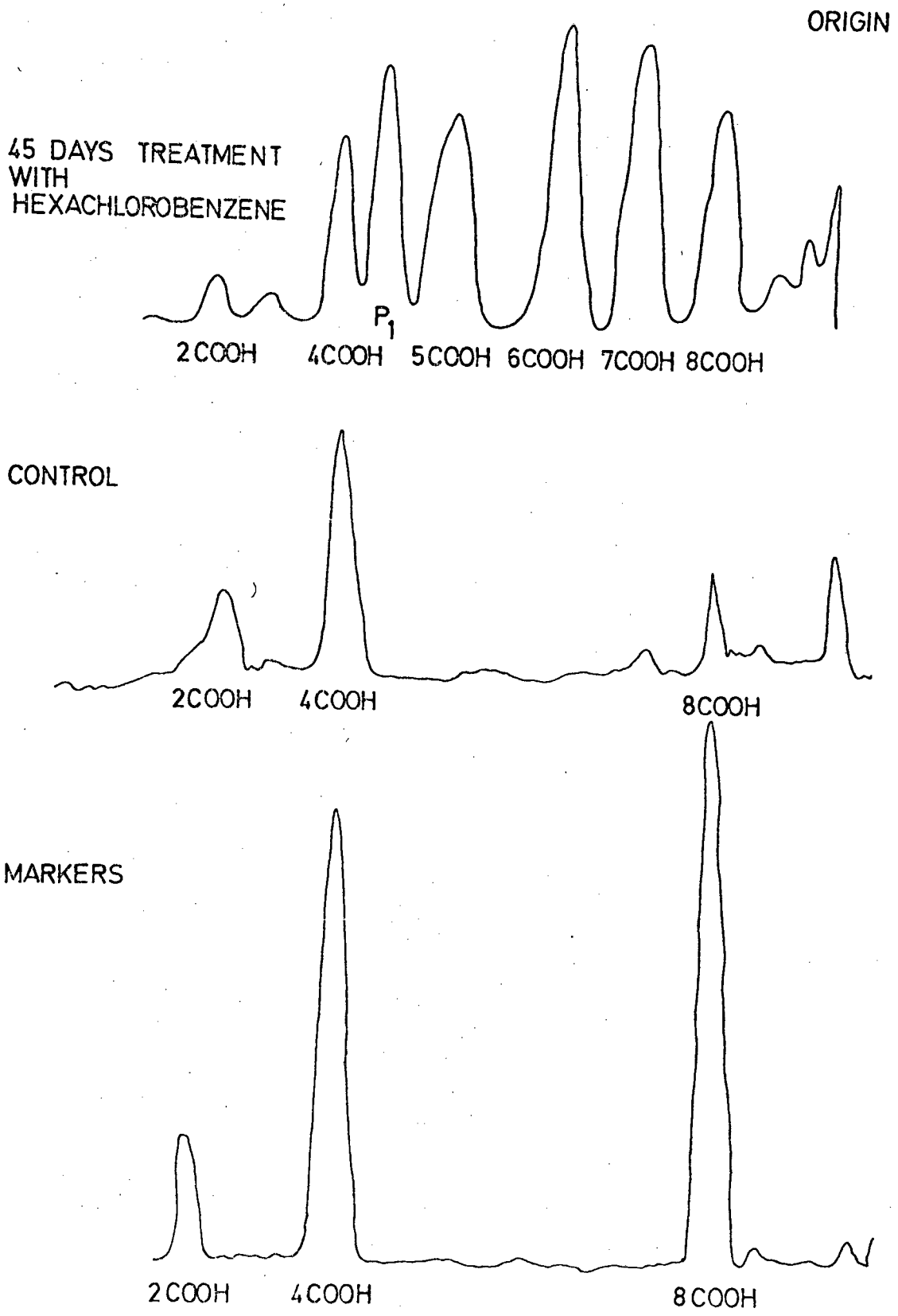


Figure 14 Faecal porphyrin composition in a rat treated for 45 days with HCB, compared to that in an untreated rat. Porphyrins were extracted, esterified, chromatographed, and the fluorometric scans obtained using a Vitatron VLD 100 densitometer are shown.

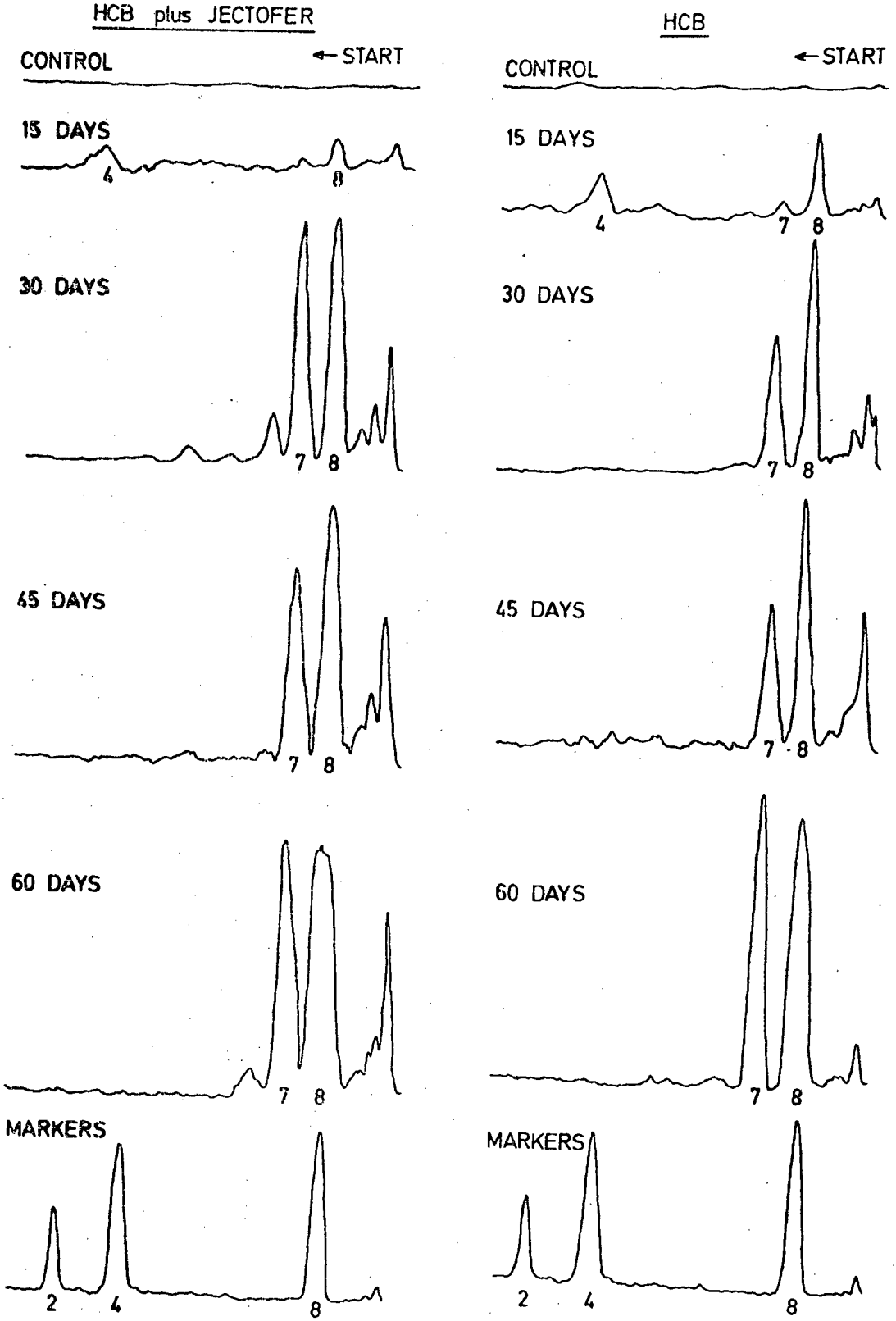


Figure 15 Hepatic porphyrin composition at 15 day intervals in rats treated simultaneously with HCB and Jectofer and in rats treated with HCB. Porphyrins were extracted, esterified, chromatographed and the fluorometric scans obtained using a Vitatron VLD 100 densitometer are shown.

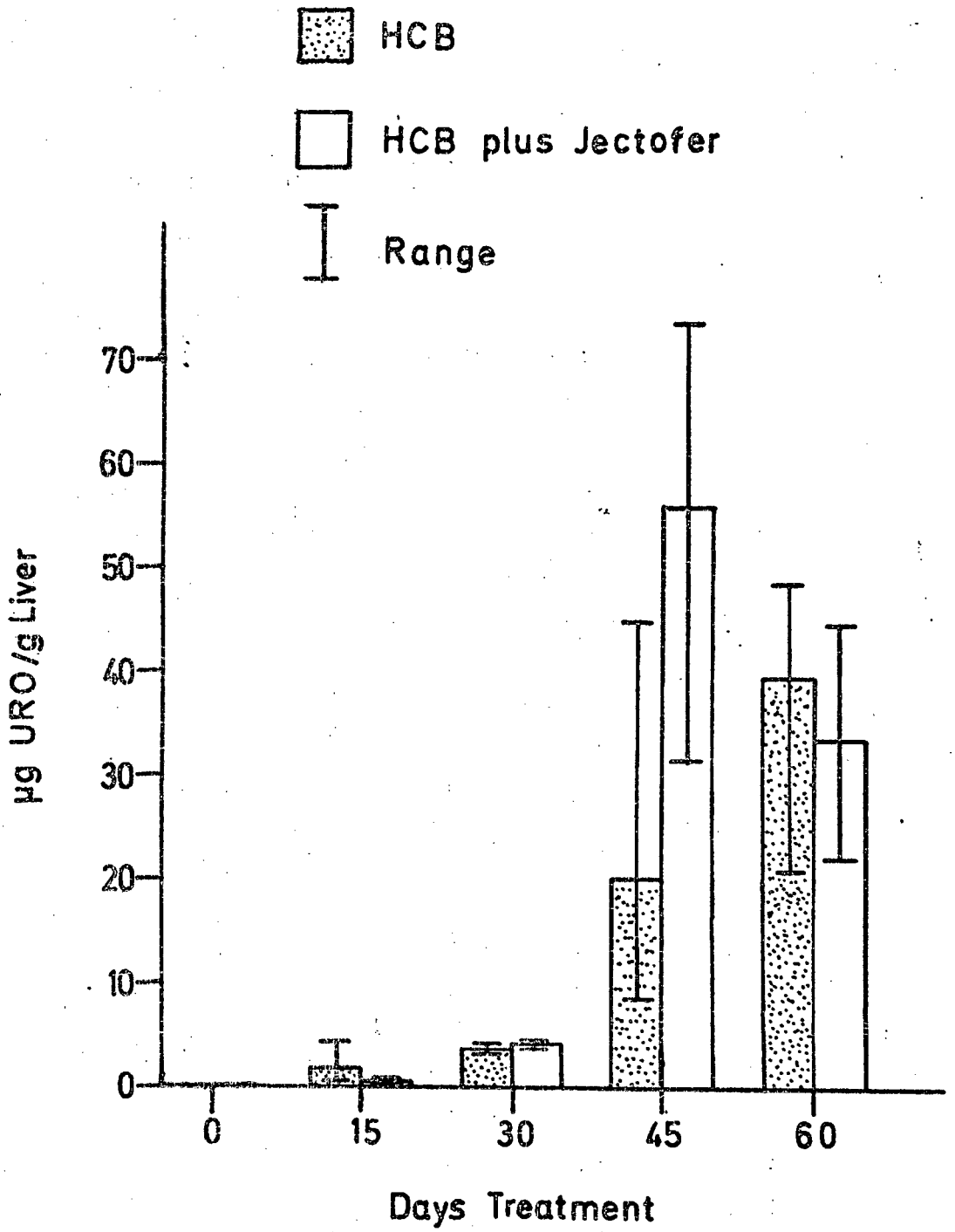


Figure 16 Hepatic URO content at 15 day intervals in rats treated with HCB alone and in rats treated simultaneously with HCB and Jectofer. Liver porphyrins were directly esterified, chromatographed and scanned fluorometrically using a Vitatron VLD 100 densitometer fitted with an integrating recorder. URO was quantitated by comparison of the area under each peak corresponding to URO with that of a standard quantity of URO III octamethyl ester applied to the thin layer chromatography plate. The height of the bars is the mean value of URO content in the liver of 3 rats.

tected. At 30 days, the excess hepatic porphyrins in both groups were almost entirely URO and 7 -COOH porphyrin (Fig. 15), the mean URO levels at this time were 3,74µg/g liver and 4,12µg/g liver for the HCB- and HCB plus Jectofer-treated groups respectively (Fig. 16). A dramatic increase was observed in the amount of URO to a mean value of 55,92 µg/g liver at 45 days simultaneous treatment with HCB and Jectofer, which diminished somewhat to 33,45µg/g liver at 60 days (Fig. 16). In the HCB-treated group increased levels of URO were noted at 45 days, the mean being 20,01 µg/g liver, but after 60 days treatment there was a further increase to a mean value of 39,27µg URO/g liver (Fig. 16).

When there was increased URO storage in both groups of animals, a concomitant increase in 7-COOH porphyrin was noted (Fig. 15). The quantity of 7-COOH porphyrin at these times was approximately 40% of the total porphyrins. This observation held true for both treatment schedules (Table 4).

TABLE 4

Mean Percentage Composition of Liver Porphyrins in Rats Treated with HCB and HCB plus Jectofer

(Mean values for 3 observations in each case)

Days Treatment	HCB		HCB plus Jectofer	
	% 8-COOH	% 7-COOH	% 8-COOH	% 7-COOH
15	83	17	87	13
30	60	40	55	45
45	63	37	61	39
60	60	40	55	45

Following 30, 45 and 60 days treatment with either HCB or HCB plus Jectofer, livers fluoresced strongly when viewed under ultraviolet light.

(c) Total Hepatic Iron in HCB- and HCB plus Jectofer-treated Rats

HCB treatment did not significantly affect total liver iron during the course of the experiment (Fig. 17). Simultaneous treatment with HCB and Jectofer resulted in an increase at 15 days to a mean value of 5,32 µg iron/mg protein. There was no appreciable change from this increased value at 30, 45 and 60 days administration (Fig. 17).

6.2. Validity of ALA-S Activity Determinations by the Radiochemical Microassay

(a) Choice of Radioactive Label for ALA Isolated by the Triple Column Technique

The complete incubation mixture for assay of ALA-S, including 0,010 ml rat liver homogenate (100 mg/ml), but where radioactive succinate was replaced by either {^{14}C} - ALA or {^{3}H} - ALA, was subjected to the triple column technique for isolation of ALA, after addition of 0,020 ml trichloracetic acid and 0,050 ml ALA/succinate carrier solution. As shown in Table 5 recoveries of radioactivity were very much lower in the case of {^{3}H} - ALA when compared to {^{14}C} - ALA. This low recovery may be caused by {^{3}H} exchanging with the various resins, washes and solutes.

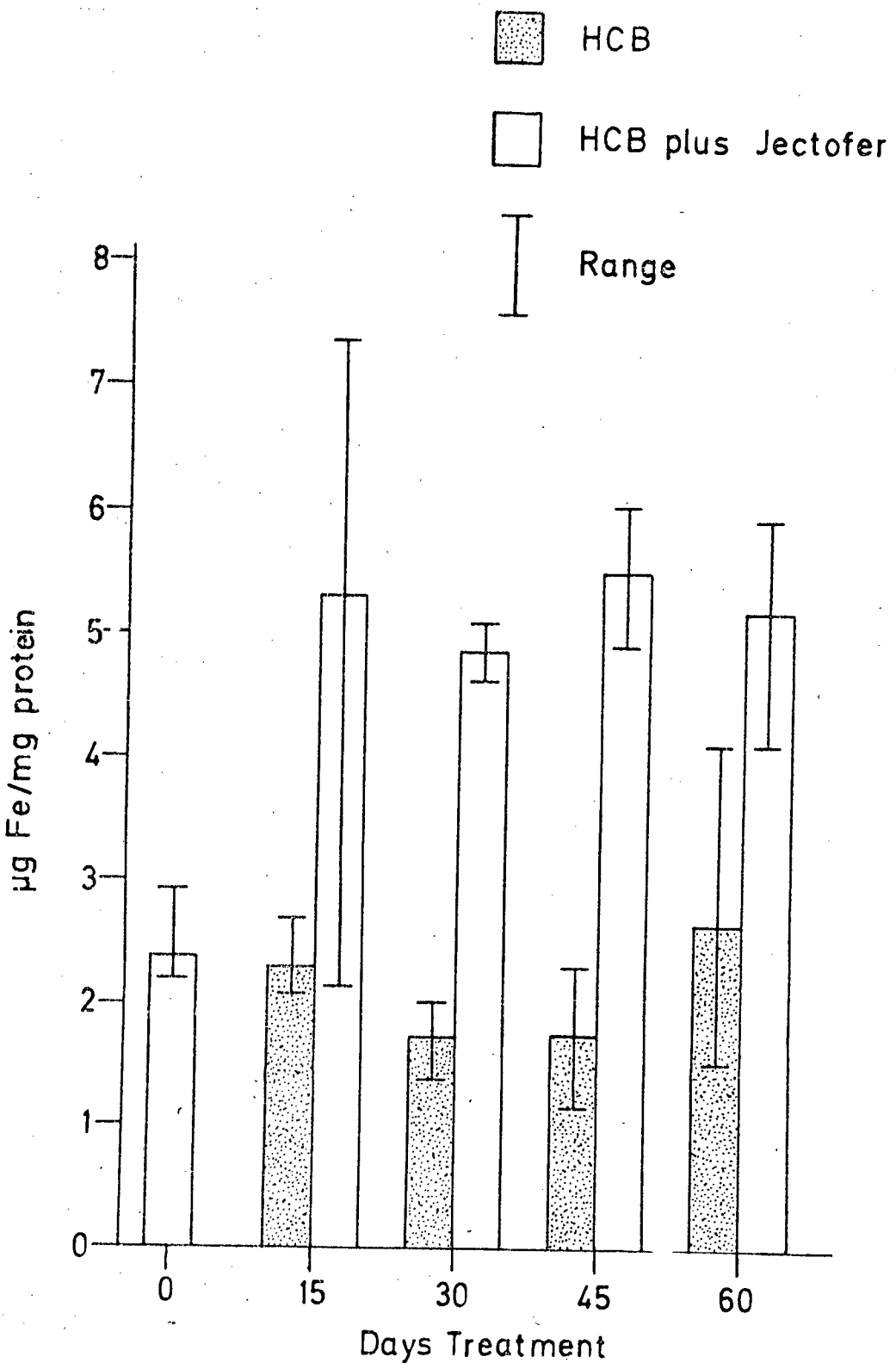


Figure 17 Total hepatic iron content at 15 day intervals in rats treated with HCB alone and in rats treated simultaneously with HCB and Jectofer. The height of the bars is the mean value of total liver iron in three rats.

TABLE 5

Recoveries of Radiolabelled ALA from Triple Columns

Label	Total D.P.M. Applied to Columns ($\times 10^{-6}$)	Total D.P.M. Recovered in Final Eluate($\times 10^{-6}$)	% Recovered
^{14}C-ALA	6,61	4,60	69
	6,61	4,77	72
	6,76	4,55	67
	6,76	5,34	79
		Mean	72
^{3}H-ALA	7,64	1,36	18
	7,64	0,63	8
	11,1	1,32	12
	11,1	1,58	14
		Mean	13

In view of these results, ^{14}C was used as a label in all ensuing experiments for determination of ALA-S activity.

(b) Determination of Activity of Bacterial Succinyl-CoA Synthetase Used in the ALA-S Assay

Succinohydroxamic acid formation, when determined as the ferric complex at 540 nm, is not proportional to enzyme concentration except at very low enzyme concentrations [37,40]. For this reason, the least amount of enzyme which gave an absorbance reading at 540 nm in the range 0,13 to 0,20 was used for calculation of the activity[40]. The single preparation of purified, freeze-dried bacterial succinyl-CoA synthetase used in the ALA-S assays over six months showed no significant change in

activity, as shown in Table 6.

TABLE 6
Activity of Purified Bacterial Succinyl-CoA Synthetase

Date of Assay	Vol. Enzyme (10 mg/ml) per Assay	Absorbance at 540 nm	µmol Succinyl-CoA formed/ 30 min. (Units succinyl-CoA Synthetase)
Aug.1972	0,005	,095	0,60
	0,005	,105	0,67
	0,010	,146	0,93
	0,010	,133	0,85
Jan.1973	0,005	,104	0,65
	0,005	,076	0,48
	0,010	,150	0,96
	0,010	,130	0,83

(c) ALA-S Activity Determination by the Radiochemical Assay Method in AIA-treated Rats

It has been shown by a number of investigators that AIA stimulates the activity of hepatic ALA-S in the rat[2,20,191,249], with maximal activity of the enzyme reached 8 hours after injection with AIA. It was further demonstrated that the activity of ALA-S remained significantly elevated for at least 24 hours[191]. The activity of ALA-S in the liver of each of 6 control rats and 6 AIA-treated rats was determined using the radiochemical microassay for this enzyme. As shown in Fig. 18, the hepatic ALA-S activity, expressed as nmol ALA formed per gram liver per hour, was more than 3-fold greater 16 hours after treatment with AIA, being 719 $\pm$ S.E. 131,8 against 221 $\pm$ S.E. 66,0 in untreated animals. The increase is significant with $P<0,005$.

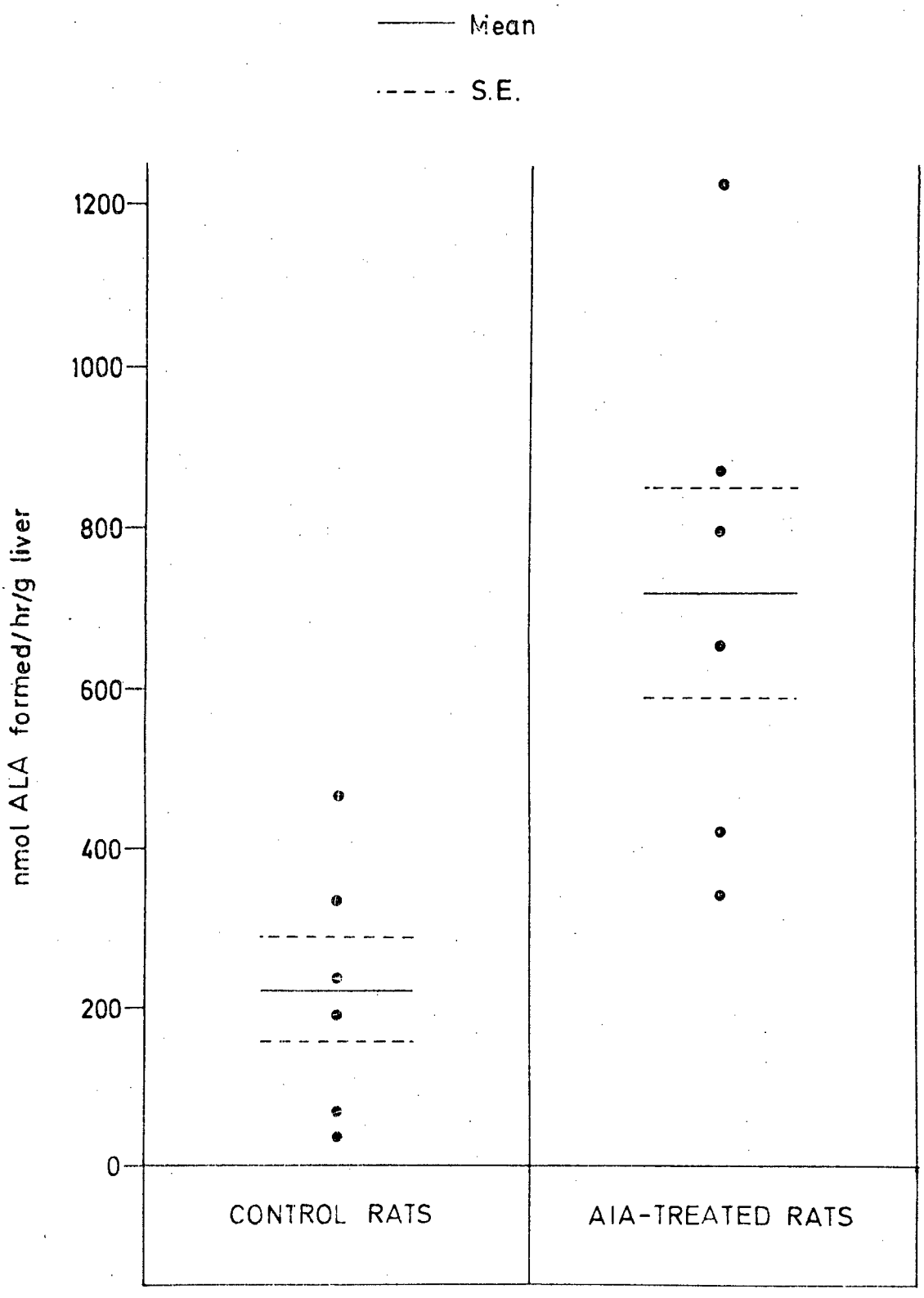


Figure 18 Hepatic ALA-S activity in untreated rats and rats treated with 300 mg/kg AIA. Rats were starved for 24 hours, injected intraperitoneally with AIA, then killed 16 hours later. Control rats were starved for 40 hours before killing. The standard assay was performed as described in Section 5.1.

(d) Linearity of the Radiochemical Assay Method for ALA-S

Homogenates containing 50, 75, 100, 150 and 200 mg liver/ml were prepared from the liver of an untreated rat and the activity of ALA-S in ,010 ml of each of these preparations was determined. From Fig. 19 it can be seen that ALA production was linear when up to 2,0 mg liver was used in the microassay.

6.3. Hepatic ALA-S Activity in PCT and in Non-Porphyrics

No significant difference was found in the activity of hepatic ALA-S between patients with active PCT and non-porphyric patients (Fig. 20). The mean activity of the enzyme, expressed as nmol ALA formed per g liver per hour was 136 $\pm$ S.E. 16 in the PCT group and 107 $\pm$ S.E. 14 in the control group.

6.4. Hepatic Cytochrome P450

(a) Linearity of the Assay Method for Cytochrome P450 in Liver Homogenates

A 100 mg/ml liver homogenate from one untreated rat was diluted to concentrations between 2 and 10 mg/ml. An increase in the concentration of cytochrome P450 proportional to the concentration of liver in the samples was noted (Fig.21).

(b) Hepatic Cytochrome P450 in Humans

During the course of this investigation the opportunity arose to measure cytochrome P450 in whole liver homogenates not only in the livers of 38 non-porphyric patients and 19 patients with PCT, but also in those of 6 patients with VP and of 4 patients with protoporphyria. As shown in Table 7

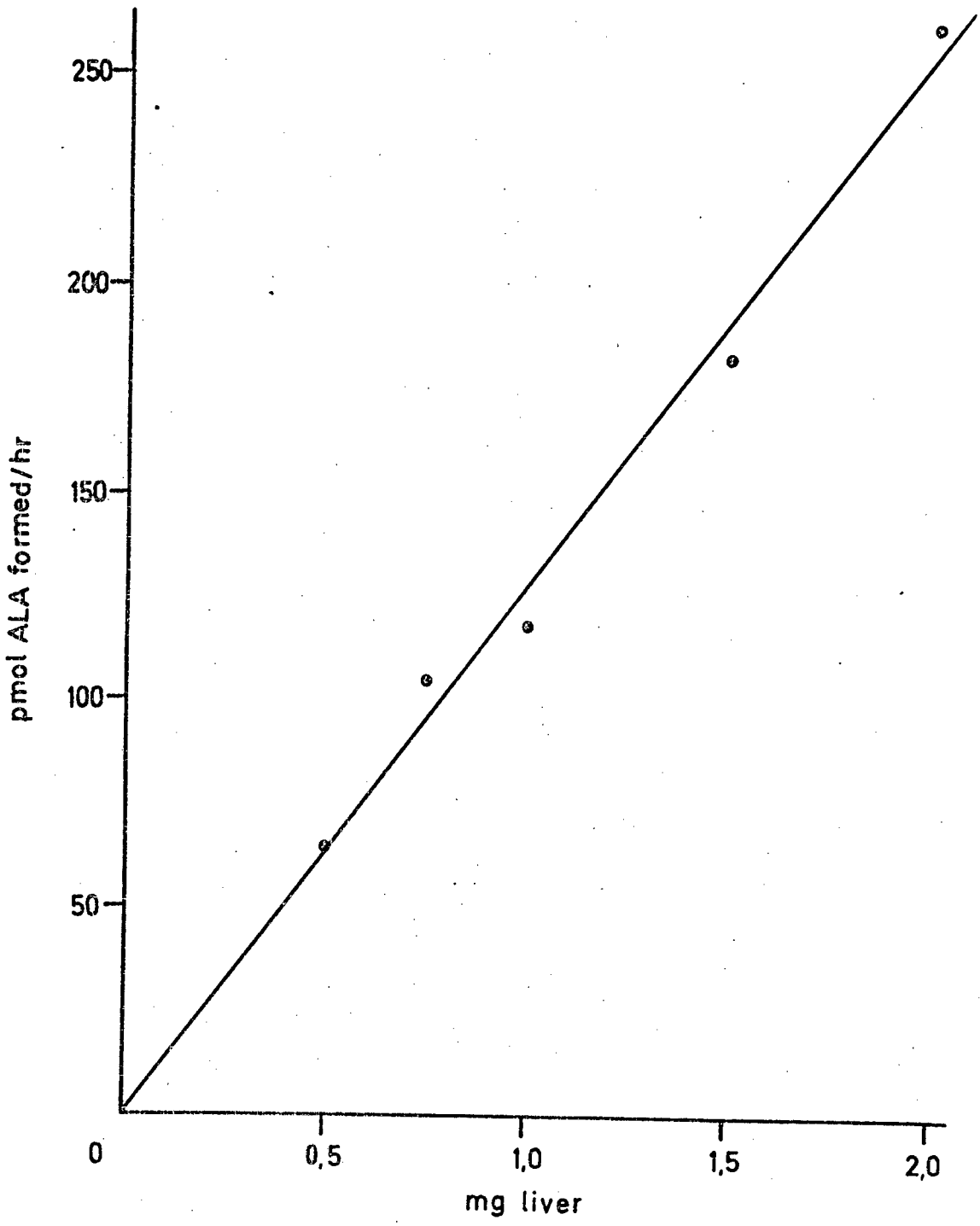


Figure 19 Relationship of ALA formation to the amount of liver tissue present. Assay was performed as described in Section 5.1., using homogenates containing 50; 75; 100; 150 and 200 mg rat liver/ml as source for ALA-S.

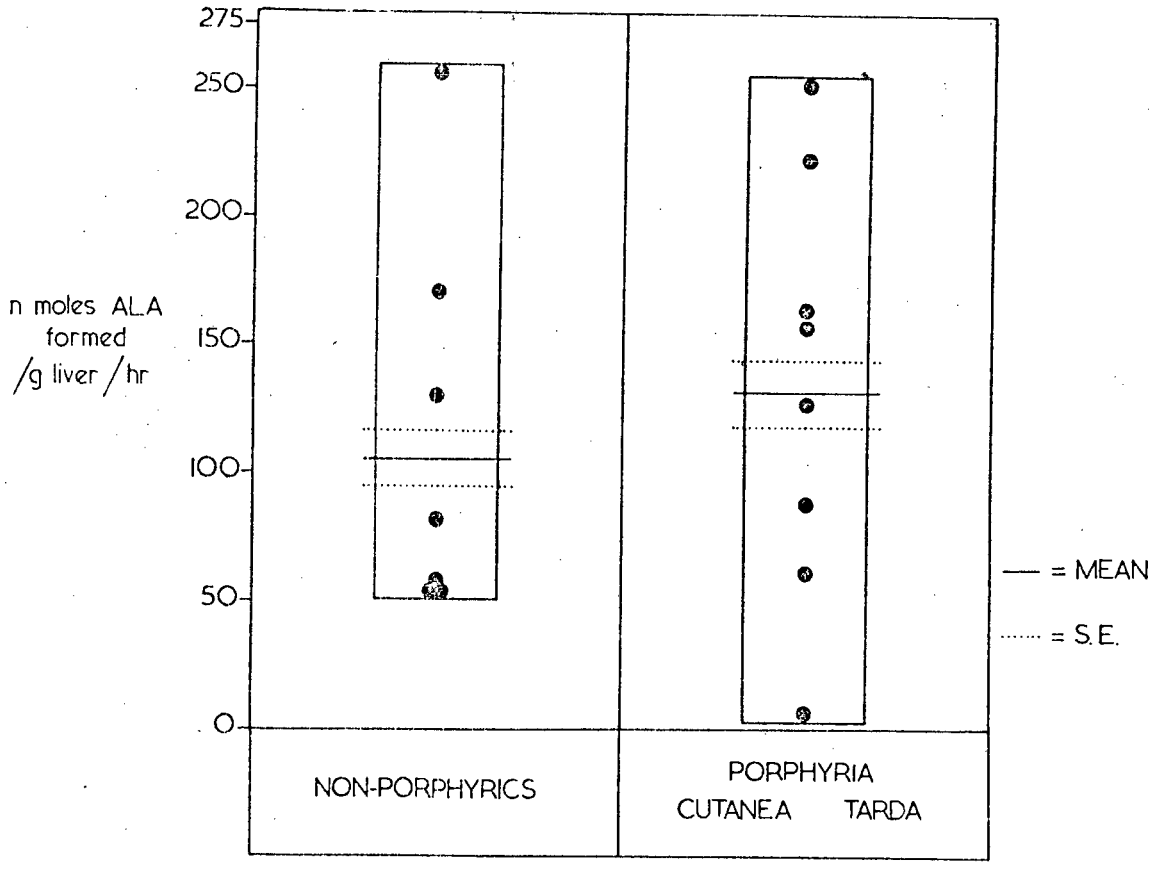


Figure 20 Hepatic δ-aminolaevulinic acid synthetase (ALA-S) activity as determined by a radiochemical assay in PCT and in matched non-porphyrics (patients with normal porphyrin metabolism). — = Mean; ..... = S.E. Each point represents the mean of triplicate determinations which agreed to within 10%.

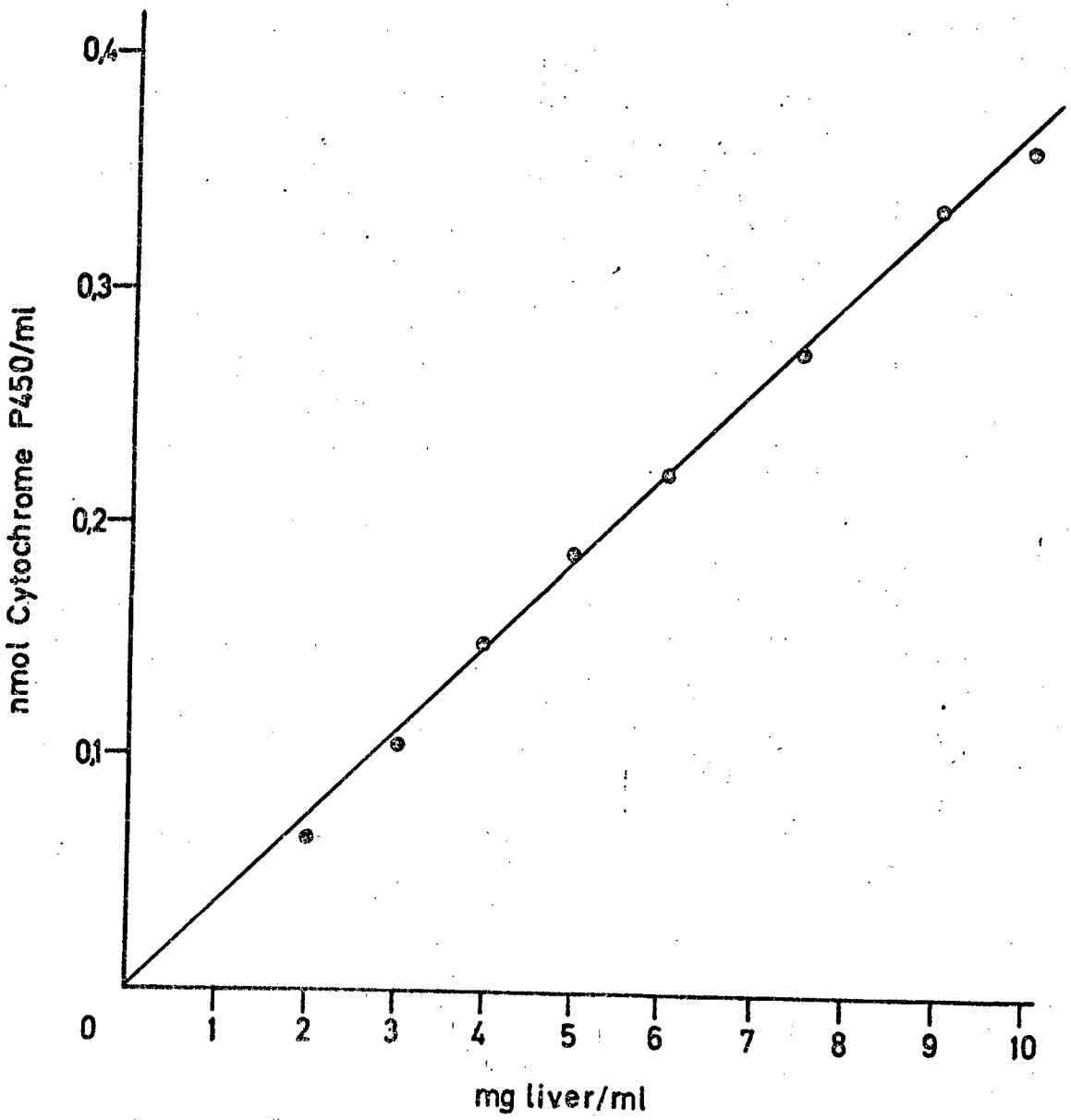


Figure 21 Relationship of rat hepatic cytochrome P450 to content of liver wet weight per millilitre homogenate assayed.

and in Fig. 22, the levels of hepatic P450 were increased only in the PCT group. The 4-fold increase in cytochrome P450 found in this group when compared to the non-porphyric group was significant, with $P<0,001$.

TABLE 7

Levels of Hepatic Cytochrome P450 in Controls, Variegate Porphyria, Protoporphyria and PCT. Results are expressed ± S.E.

	Hepatic cytochrome P450 (nmol/g liver)	No. of observations
Controls (non-porphyric)	15,5 ± 1,3	38
Variegate Porphyria	21,7 ± 4,0	6
Protoporphyria	20,6 ± 5,6	4
PCT	63,1 ± 9,3	19

(c) Hepatic Cytochrome P450 in HCB-treated Rats

Hepatic levels of cytochrome P450, measured in whole liver homogenates, in rats treated with HCB up to 60 days is shown in Fig. 23. After 20 days treatment an increase was noted in the hepatic cytochrome P450 levels to a mean value of 57,5 nmol/g liver, compared with a mean of 33,6 nmol/g liver in untreated rats. A further increase was noted after 30 days treatment to a mean value of 68,5 nmol/g liver. The levels of cytochrome P450 were found to be increased by a factor of approximately 2,5 after 45 days, the mean value being 81,6 nmol/g liver at this time. Sixty days after commencement of HCB-treatment the mean hepatic cytochrome P450 content was found to be 183,0 nmol/g liver.

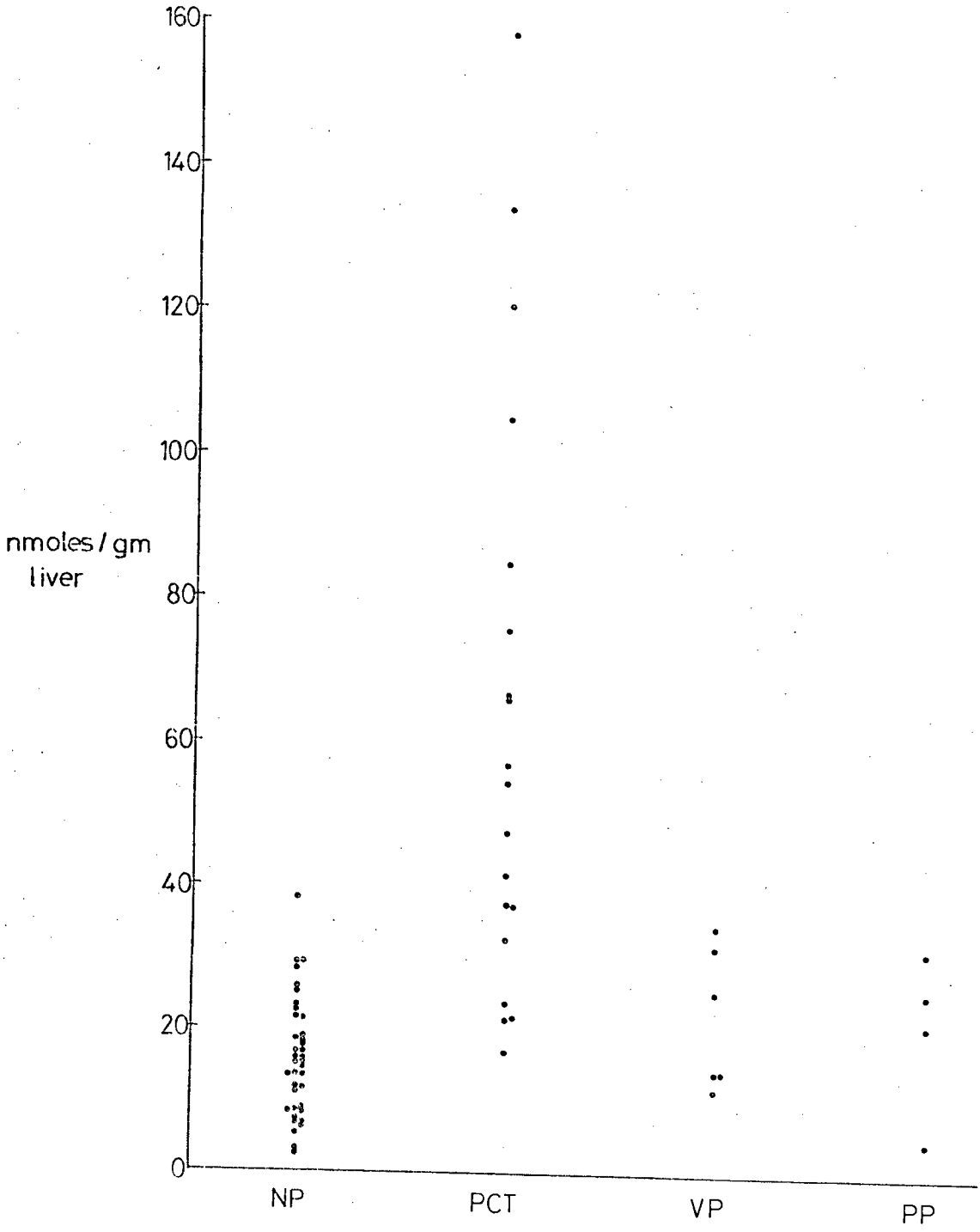


Figure 22 Levels of hepatic cytochrome P450 in non-porphyric controls (NP) and in patients with PCT, VP and PP.

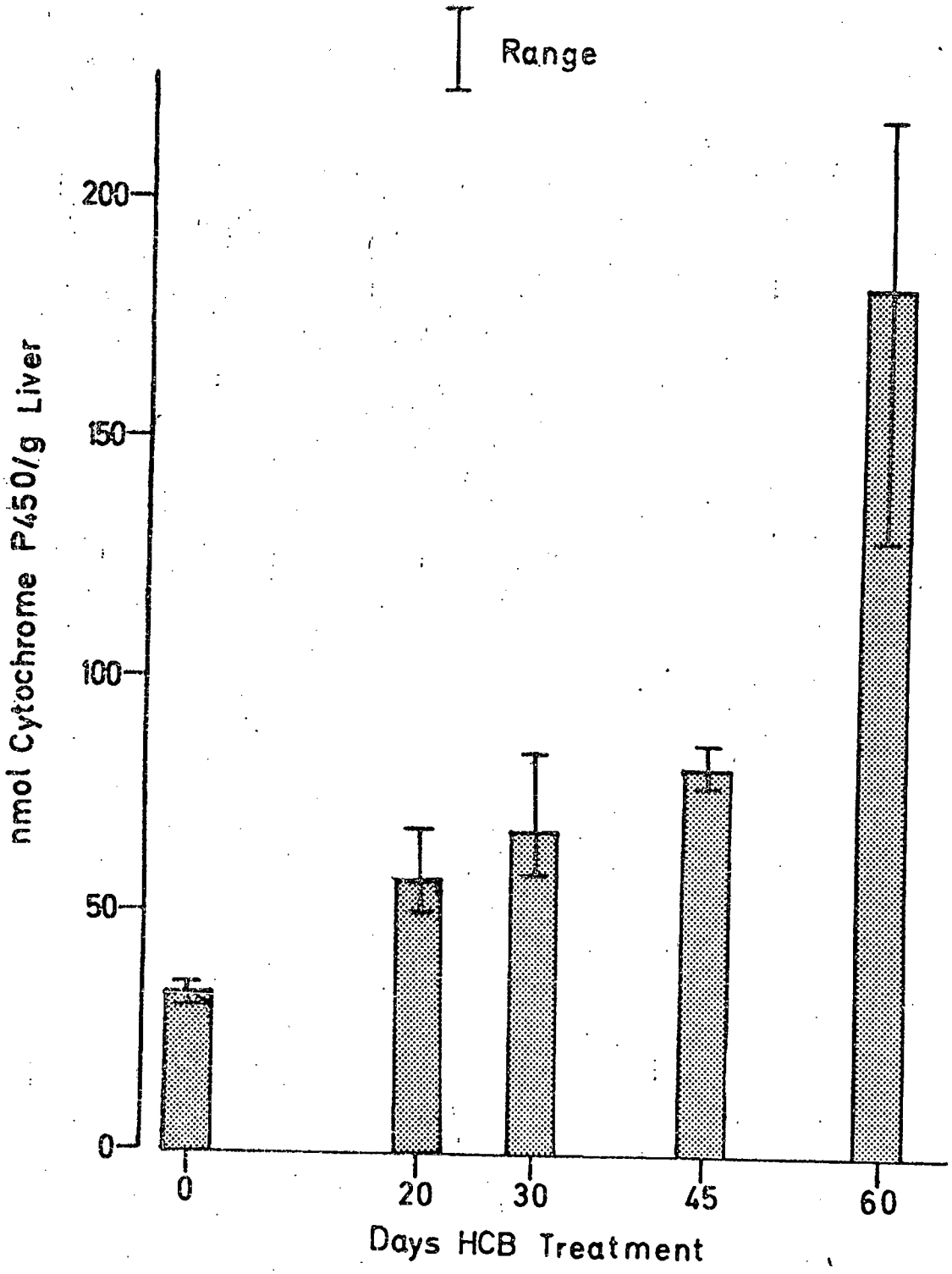


Figure 23 Levels of hepatic cytochrome P450 at various times in rats treated with HCB. The mean values together with the range obtained for the levels of the haemoprotein in 3 animals is shown in case.

(d) Siderotic Rat Liver Cytochrome P450 Determined in the Presence of Excess URO III

To test whether excess URO I could be bound to excess hepatic iron to form a compound with spectral characteristics similar to that of cytochrome P450, the levels of this cytochrome were measured in 10 mg/ml homogenates of liver from rat treated for 60 days with Jectofer alone, the homogenising fluid containing from 0 to 5µg/ml URO I. Hepatic cytochrome P450 measured in the presence of the various concentrations of URO I was not found to be significantly different from the value of 38,0 nmol/g liver found in the absence of URO I. Histological examination of the liver after staining with Prussian blue showed massive deposition of haemosiderin throughout the sample, confirming that the rat used in this experiment was indeed siderotic.

(e) Cytochrome P450 Determinations in Liver Homogenates and in Liver Microsomes of Untreated Rats, HCB-treated Rats and Phenobarbital-treated Rats.

Hepatic cytochrome P450 was measured in groups of untreated rats, rats treated for 45 days with HCB and phenobarbital-treated rats using the whole homogenate technique (Section 5.2.I.) and the microsomal technique (Section 5.2.II.). A group of animals that had been treated with phenobarbital was included, since it has been shown that this pretreatment increased cytochrome P450 in rat hepatic microsomes[127,215,237]. In each experiment cytochrome P450 was measured in liver homogenates and microsomes prepared from the pooled livers of the same 3 animals. The levels of hepatic cytochrome P450 as determined by both methods in the three groups of animals are compared in Table 8.

TABLE 8

Hepatic Cytochrome P450 in Untreated Rats, HCB-treated Rats and Phenobarbital-treated Rats as Measured in Whole Homogenates and Microsomes

Treatment	Experiment no.	Homogenate Cytochrome P450 nmol/g liver(wet weight)	Microsomal Cytochrome P450 nmol/mg microsomal protein
None	1	10,99	0,64
	2	12,09	0,66
	3	17,58	0,72
		(13,55)	(0,67)
HCB (45 days)	4	30,08	1,39
	5	29,25	1,20
	6	37,60	1,26
		(32,31)	(1,28)
Phenobarbital	7	30,77	1,02
	8	21,10	0,96
	9	21,90	0,99
		(24,59)	(0,99)

(Mean values are given in parentheses)

As shown in Table 8, significant increases in the levels of homogenate cytochrome P450 were found in the groups of rats treated with

HCB and phenobarbital, when compared with the control group. These increases were reflected in the levels of microsomal cytochrome P450 in the HCB- and phenobarbital-treated groups.

From Table 8 it will be observed that consistently low values for the amount of hepatic cytochrome P450 were obtained when compared to those shown in Fig. 23. This may be due to a different batch of sodium dithionite being used as a reducing agent, which may have undergone a certain amount of decomposition, thus affecting the determination of the levels of the haemoprotein.

6.5. The Type of Hepatic Cytochrome P450 Present in PCT and in HCB-treated Rats

(a) CO Difference Spectra

It was shown in Section 6.4.(b) that levels of hepatic cytochrome P450 were increased about 4-fold in patients with PCT. Further, it was shown in Section 6.4.(c) that cytochrome P450 levels were increased by a factor of about 2,5 in rats which had been treated with HCB for 45 days. Examples of the difference spectra obtained by the homogenate method of measurement of cytochrome P450 are shown in Fig. 24. In the non-porphyric control patient L.M. the peak at 450 nm is separate from the cytochrome b5 peak at 428 nm (Fig. 24). In the case of patient R.M. who had active PCT, an approximate 5-fold increase in the height of the peak attributable to cytochrome P450 can be seen. A slight shoulder can be observed in the range 420 to 430 nm which may reflect the incorporation of the cytochrome b5 spectrum into that of cytochrome P450. The spectral peak of the CO-binding pigment in patients with PCT was shifted to about 448 nm, which is similar to the shift which occurs in rodents after treatment with 3-methylcholanthrene[6]. In the difference spectrum shown for an untreated rat (Fig. 24), the spectral

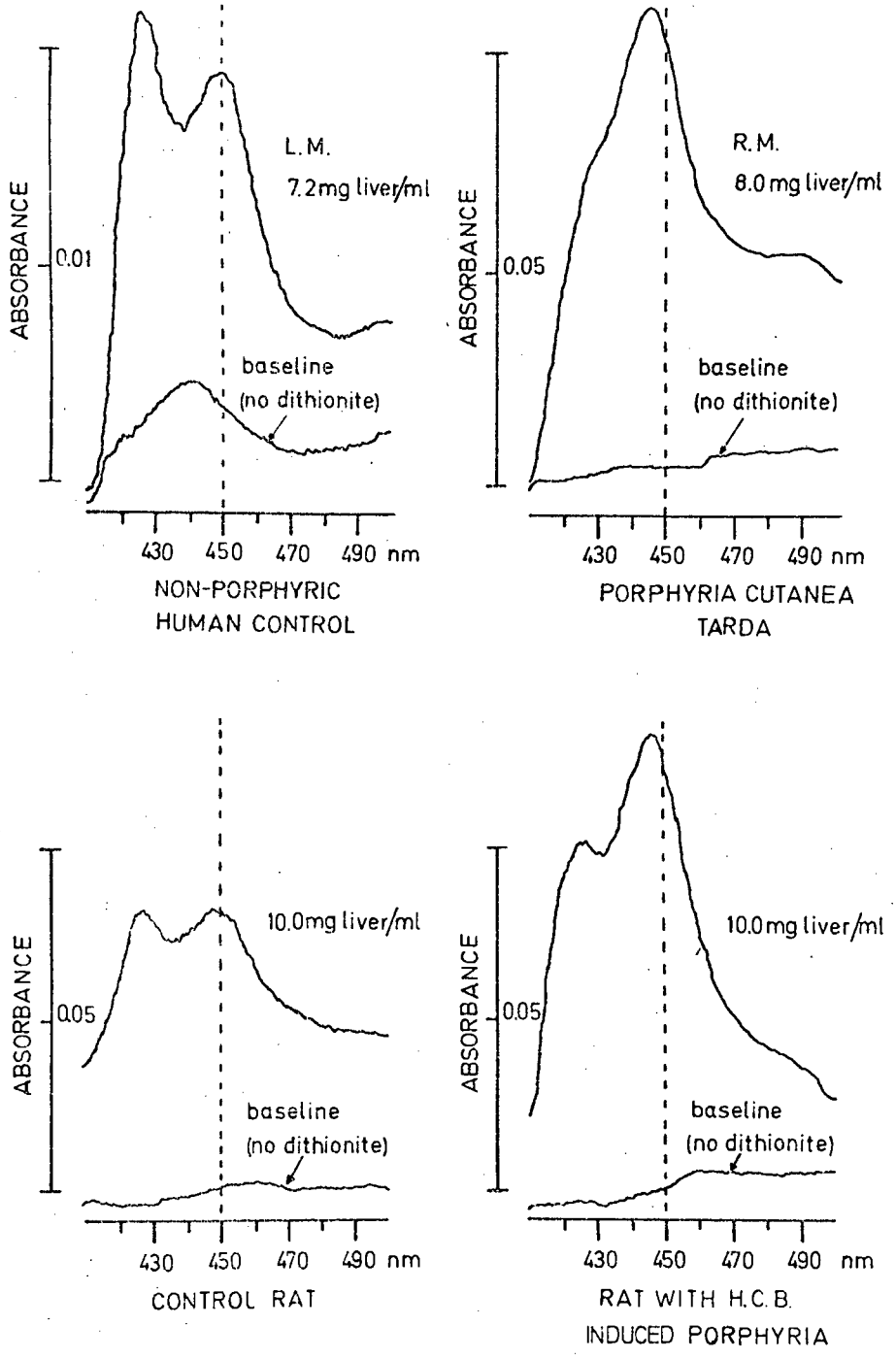


Figure 24 Cytochrome P450 levels in human and rodent liver. Difference spectra (liver homogenate plus carbon monoxide plus sodium dithionite) minus (liver homogenate plus carbon monoxide), using approximately the same concentration of liver, are shown. The carbon monoxide-binding pigment (cytochrome P450), when compared to non-porphyric controls, is increased in PCT and in the rat with HCB porphyria. In the porphyric situation the spectral peak occurs at a lower wavelength than 450 nm.

peak is at 450 nm. After 45 days treatment with HCB, at which time urinary, faecal and hepatic porphyrin analysis indicate that the rats are porphyric (Figs 12b, 13,14,15,16), there is an approximate 2,5 fold increase in the level of cytochrome P450 when compared to untreated rats, and furthermore there is a shift of the peak in the difference spectrum to about 448 nm (Fig. 24). From the difference spectra it becomes apparent that there is similarity in the spectral characteristics of hepatic cytochrome P450 in non-porphyric states in man and rat, and in porphyria, both in PCT and in the HCB-intoxicated rat.

(b) Ethyl Isocyanide Difference Spectra

The ethyl isocyanide-binding haemoprotein of liver microsomes from untreated rats and rats treated with HCB for 45 days, was examined according to the method of Imai and Sato[133]. Three experiments were performed in each case, using microsomal preparations derived from 3 groups of 3 animals. When the ratio of the heights of 455 nm peaks to the heights of the 430 nm peaks were plotted against pH of the microsomal suspension (Fig. 25), the ratio was found to be 1 (i.e. the heights of the two peaks were equal) at a mean pH value of approximately 7,52 for untreated rat liver microsomes and at a mean pH value of approximately 7,28 for HCB-treated rat liver microsomes. The lower pH i.e. 7,28, at which the heights of the 430 nm and 455 nm peaks were equal in microsomes from rats treated with HCB for 45 days, is interpreted to indicate the presence of a form of cytochrome P450 with a different pH dependent equilibrium constant to that seen with normal microsomes.

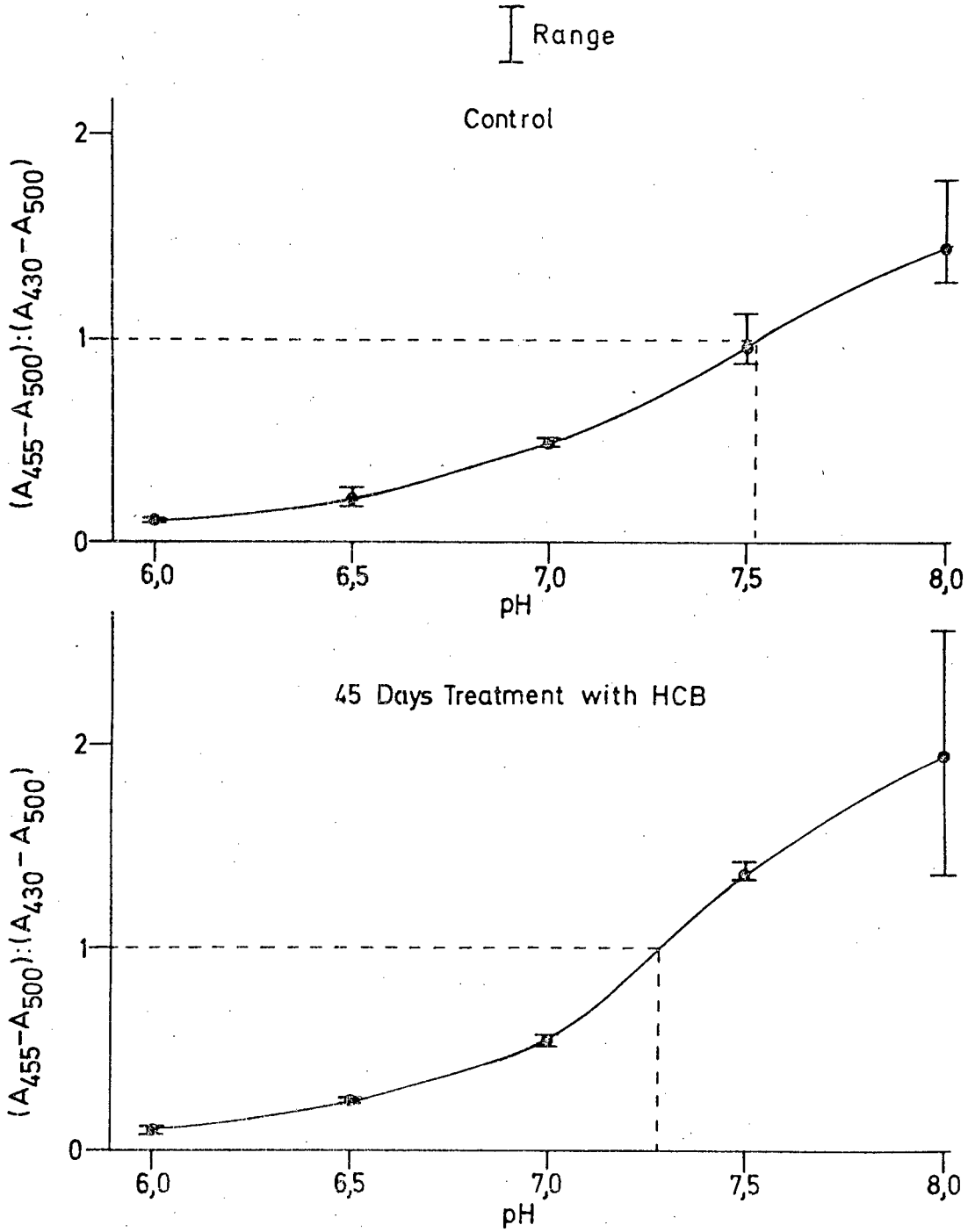


Figure 25 Dependence of the ratio of the height of the 455nm peak to the height of the 430nm peak on pH in the ethyl isocyanide difference spectrum using microsomes from untreated rats and microsomes from rats which had been treated for 45 days with HCB. Each point is the mean of 3 determinations, each determination having been made using microsomes prepared from livers pooled from 3 rats.

6.6. The Functional Capacity of Cytochrome P450 as Assessed by Determination of Aminopyrine N-Demethylation and 3,4-Benzpyrene Hydroxylation

(a) Product Recoveries and Linearity of Product Formation

(i) Aminopyrine N-Demethylation

In a series of 12 experiments, the percentage recovery of radioactivity from incubation mixtures containing ^{14}C-formaldehyde but no {dimethylamino-^{14}C} aminopyrine was determined to be 55,34 ($\pm$ S.E.M. 2,75).

As shown in Fig. 26a, where increasing quantities of homogenates from an untreated rat were used as enzyme source, and aminopyrine including {dimethylamino-^{14}C} aminopyrine concentration was kept constant at 1,052 mM, formaldehyde formation increased linearly when between 3 and 7 mg liver was present in the assay mixture.

(ii) 3,4-Benzpyrene Hydroxylation

In 6 experiments, 50 ng 3-hydroxy-3,4-benzpyrene contained in 0,05 ml acetone was added to complete assay mixtures and immediately extracted. By comparison of spectrophotofluorometer readings to that obtained for a 10 ng/ml solution of 3-hydroxy-3,4-benzpyrene in 1N NaOH, the percentage recovery was 17,65 $\pm$ S.E.M. 3,82.

It was demonstrated that 3-hydroxy-3,4-benzpyrene formation was linear when from 1 to 8 mg liver from an untreated rat was used as enzyme source (Fig. 26b). Substrate concentration was 6,61 x 10^{-5}M (0,1 ml of a 500µg/ml solution of 3,4-benzpyrene per assay).

(b) Kinetics of Aminopyrine N-Demethylation and 3,4-Benzpyrene Hydroxylation in PCT and Non-Porphyrics

Data obtained for cytochrome P450-mediated N-demethylation of aminopyrine and hydroxylation of 3,4-benzpyrene by liver tissue from 9 patients with PCT and from 13 non-porphyric patients, were plotted according to the method of Lineweaver and Burk[173] as shown in Fig. 27. Data

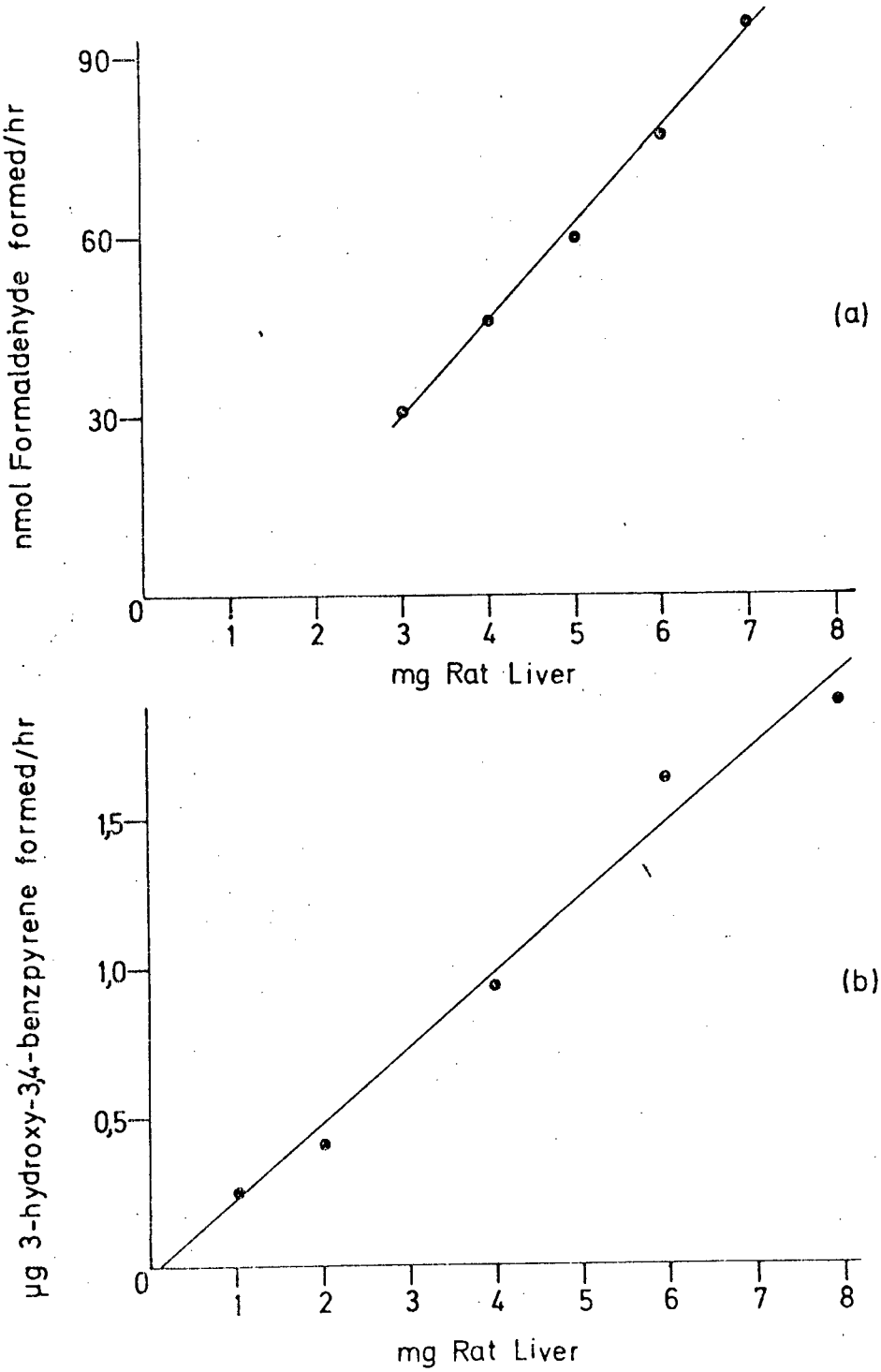


Figure 26 Relationship of the formation of the products of aminopyrine N-demethylation and 3,4-benzpyrene hydroxylation to the quantity of rat liver present in the respective incubation systems. Liver from an untreated rat was used as a source of the mixed function oxidase system in each experiment: (a) Aminopyrine N-demethylation; (b) 3,4-benzpyrene hydroxylation.

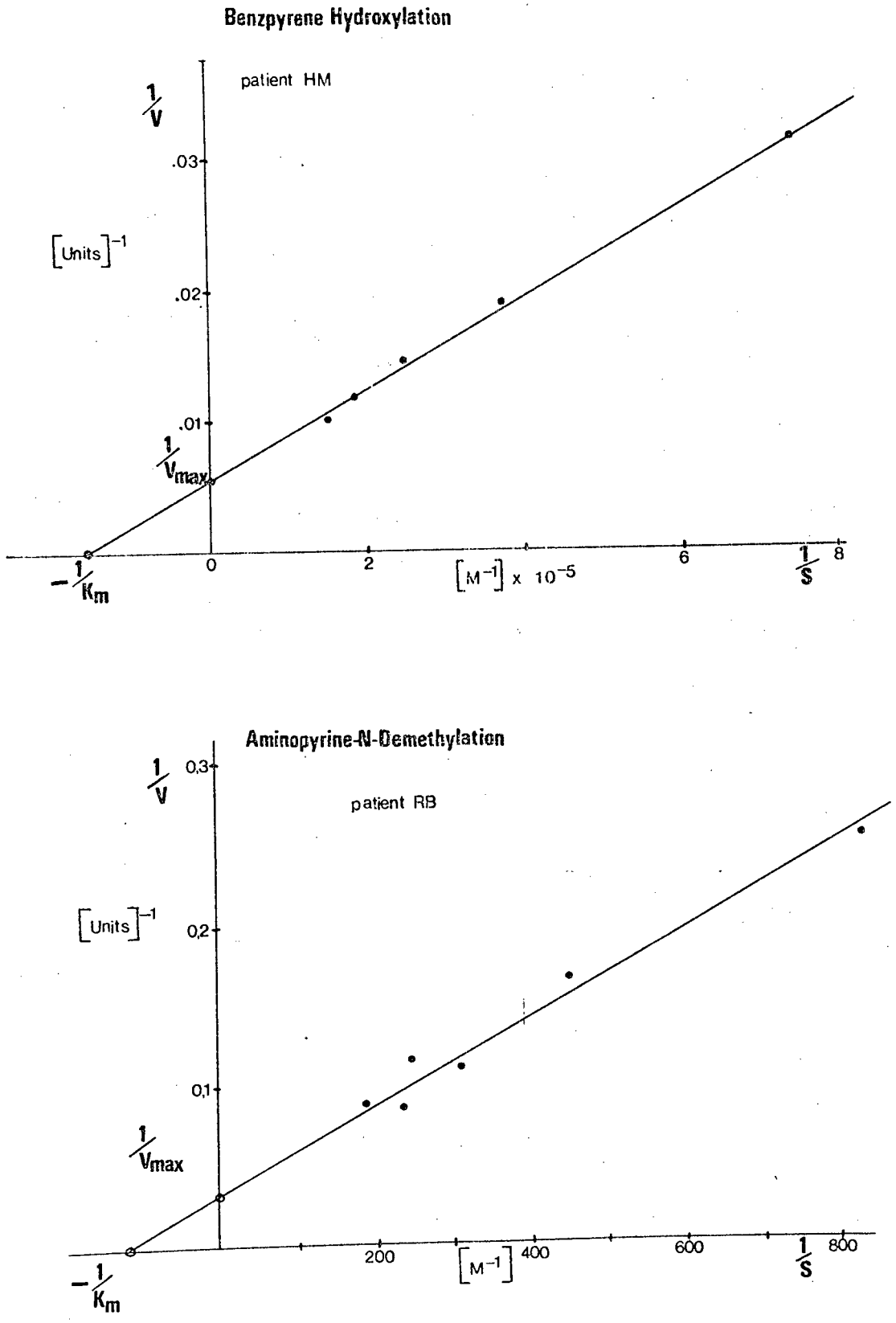


Figure 27 Plots of $\frac{1}{v}$ versus $\frac{1}{s}$ for the hydroxylation of 3,4-benzpyrene and the N-demethylation of aminopyrine. Velocities are given as nanogram 3-hydroxy - 3,4-benzpyrene formed per milligram liver and nanomoles formaldehyde formed per hour per µg liver; substrate concentrations are in moles per litre. Lines were fitted by calculation of linear regression coefficients.

points in the plots of $\frac{1}{v}$ vs$\{\frac{1}{s}\}$ followed an essentially linear relationship, and linear regression curves were fitted by calculation of the liver regression coefficient a and b in the equation $y = a + bx$, using a Hewlett Packard 97 programmable calculator. The coefficient of determination r^2, was determined in each case (Table 9). The apparent K_m and V_{max} values were derived from the calculated linear regression curves (Table 9). As shown in Figs. 28 and 29 and Table 9, there were no significant differences in the apparent K_m and V_{max} values for both aminopyrine N-demethylation and 3,4-benzpyrene hydroxylation between the two groups of patients studied, when related to levels of hepatic cytochrome P450.

(c) Kinetics of Aminopyrine N-demethylation and 3,4-Benzpyrene Hydroxylation in HCB-treated and Control Rats.

The apparent K_m and V_{max} values for aminopyrine N-demethylation and 3,4-benzpyrene hydroxylation by pooled liver homogenates from 3 groups of 3 rats treated with HCB for 45 days and by pooled liver homogenates from 3 groups of 3 untreated rats were calculated as described in section 6.6.(b) above (Table 10). Further plots constructed of $\frac{1}{s}$ vs $\frac{1}{v}_{mean}$ aminopyrine N-demethylation and 3,4-benzpyrene hydroxylation by liver from both groups of animals, as shown in Figs. 30, 31, where each point represents the mean value from 3 different experiments, and with each experiment performed on pooled liver homogenate from 3 rats. The mean apparent K_m for aminopyrine N-demethylation in liver of rats treated with HCB for 45 days (3,01mM) was about 3-fold increased above that obtained in livers from untreated rats (1,04 mM), whilst the mean maximal velocity was increased about 2-fold after 45 days treatment with HCB (Fig. 30).

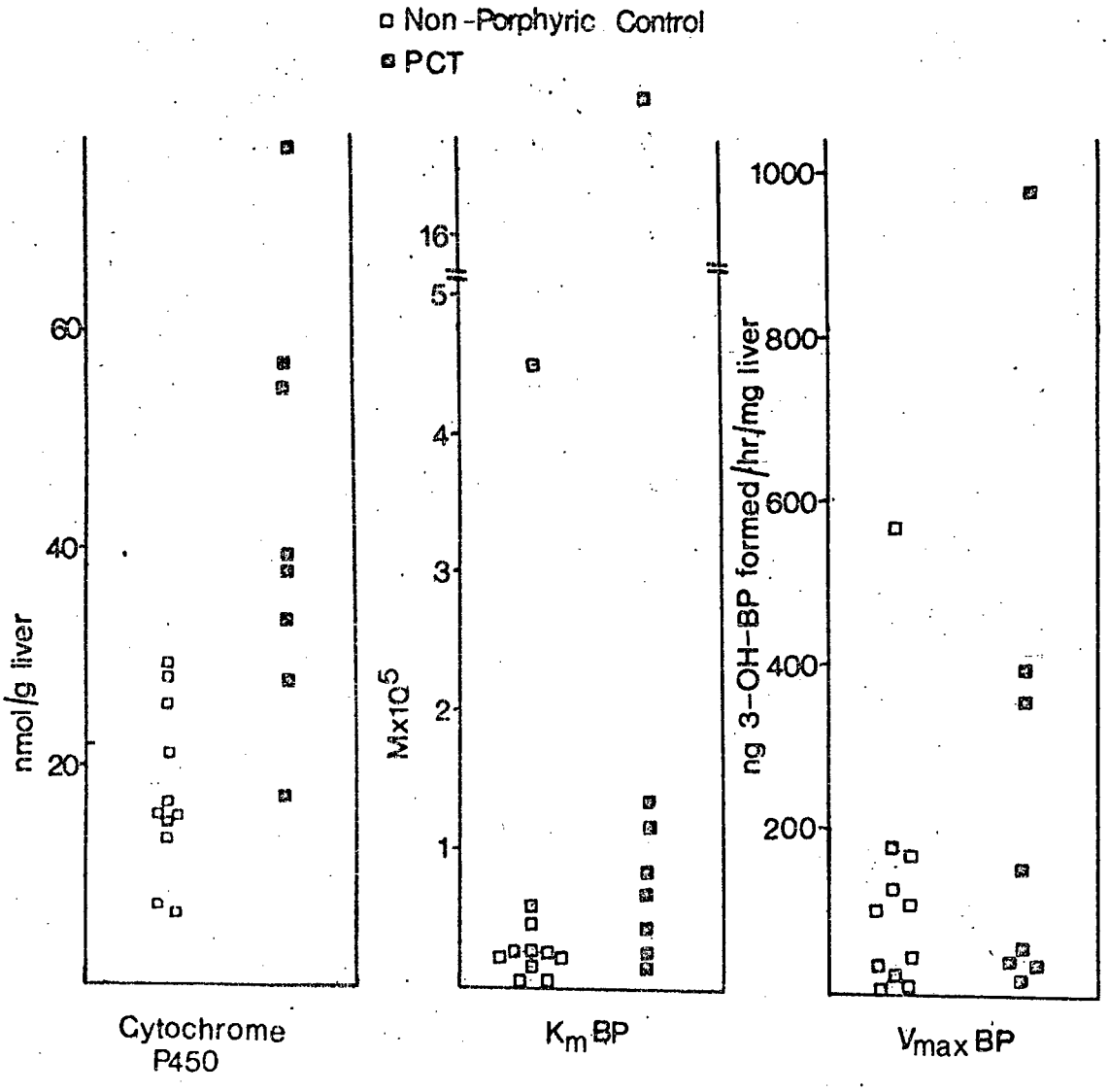
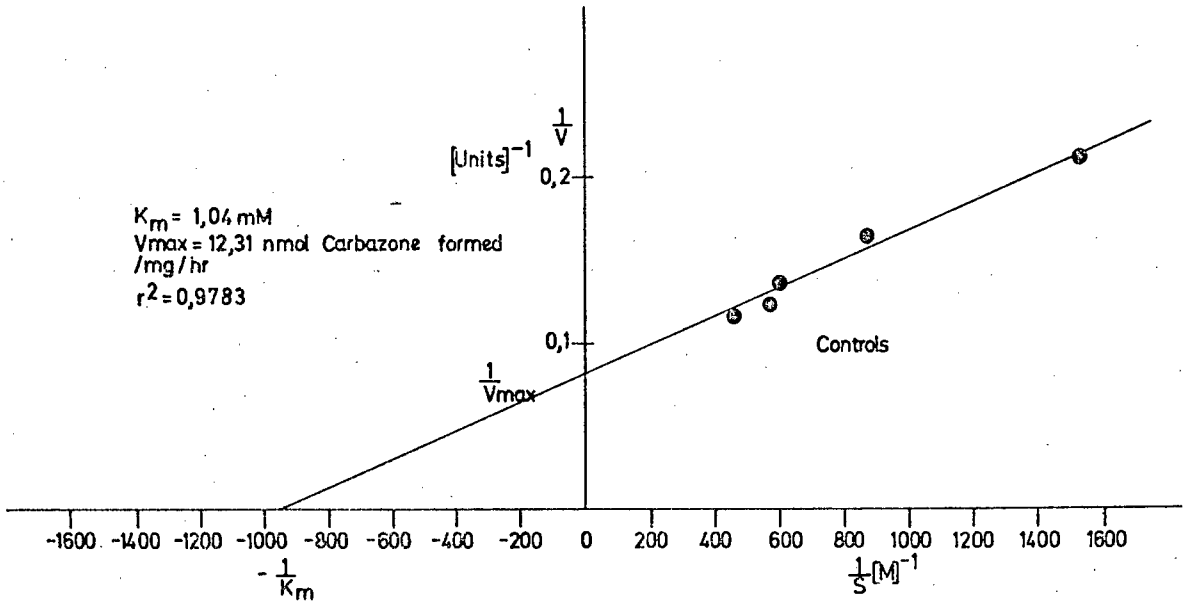


Figure 29 Comparison of the hepatic levels of cytochrome P450 and K_m and V_{max} for 3,4-benzpyrene hydroxylation in liver tissue obtained from non-porphyric controls (open squares) and patients with PCT (closed squares). K_m BP and V_{max} BP respectively are the K_m and V_{max} values for 3,4-benzpyrene hydroxylation, with V_{max} expressed as nanogram 3-hydroxy-3,4-benzpyrene formed per hour per milligra liver.



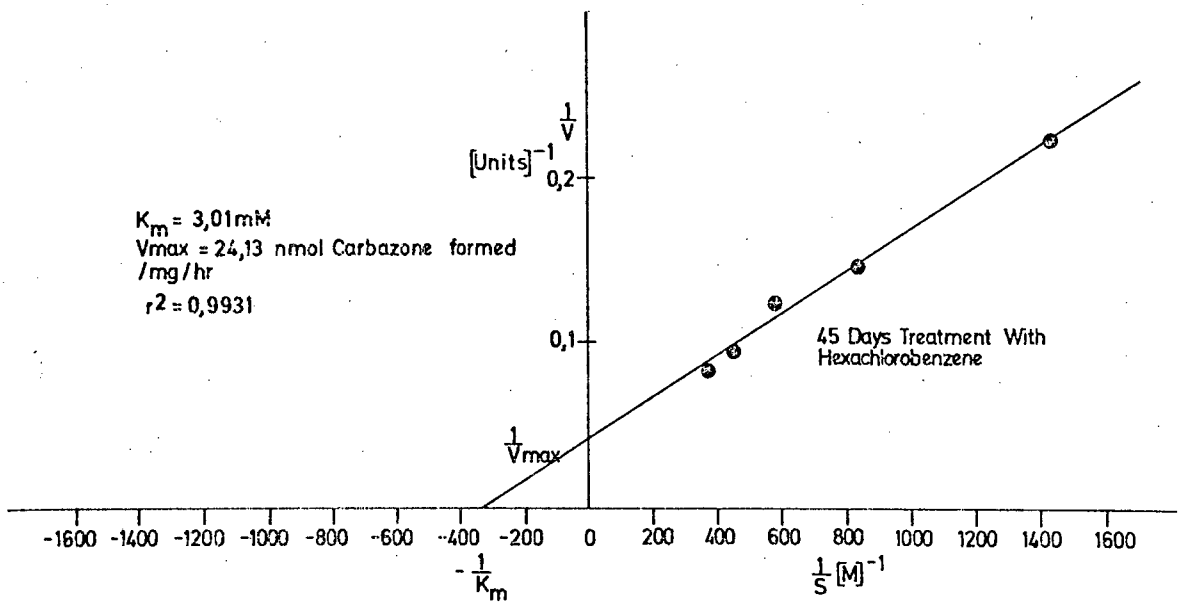


Figure 30 Plots of $\frac{1}{V}$ versus $\frac{1}{S}$ for the N-demethylation of aminopyrine by liver from untreated rats and rats treated for 45 days with HCB. Velocities are given as nmol formaldehyde formed per hour per mg liver; substrate concentrations are in moles per litre. Each point represents the mean value of three different experiments. Each experiment was performed using pooled liver homogenates from three rats. Lines were fitted by calculation of linear regression coefficients.

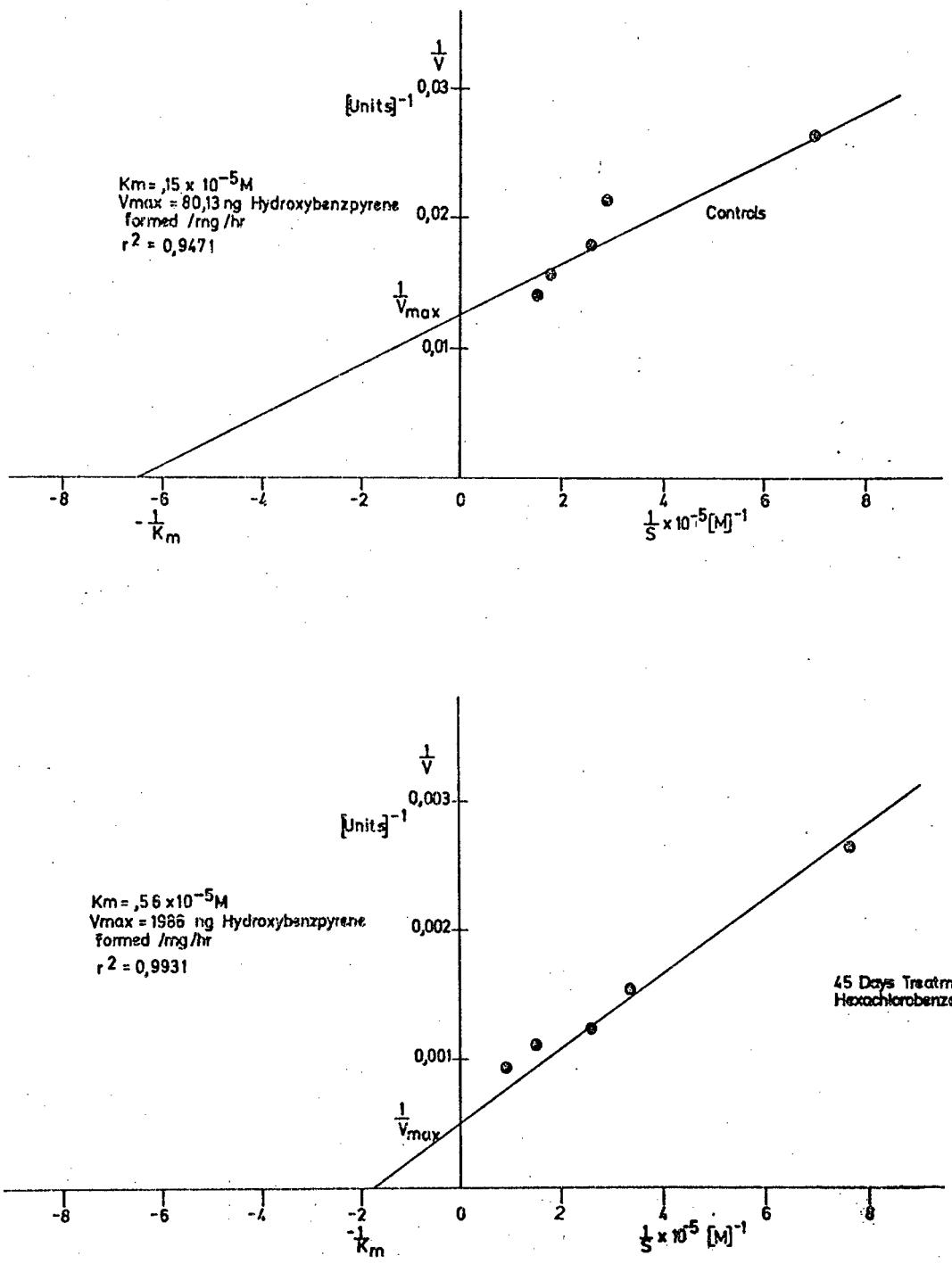


Figure 31 Plots of $\frac{1}{v}$ versus $\frac{1}{s}$ for the hydroxylation of 3,4-benzpyrene by liver from untreated rats and rats treated for 45 days with HCB. Velocities are given as ng 3-hydroxy-3,4-benzpyrene formed per hour per mg liver; substrate concentrations are in moles per litre. Each point represents the mean value of three different experiments. Each experiment was performed using pooled liver homogenates from 3 rats. Lines were fitted by calculation of linear regression coefficients.

The mean apparent K_m for 3,4-benzpyrene hydroxylation was found to be 0,15 x 10^{-5}M in untreated rats, whereas a value of 0,56 x 10^{-5} M was obtained in the case of rats that had been treated with HCB for 45 days (Fig. 31). An almost 25 fold increase in the mean maximal velocity of 3,4-benzpyrene hydroxylation was observed in the case of rats treated with HCB for 45 days ($V_{max(mean)}$ = 1986 ng 3-hydroxy-3,4-benzpyrene formed/hr/mg liver) when compared to the mean maximal velocity observed in the case of the control animals ($V_{max(mean)}$ = 80 ng 3-hydroxy-3,4-benzpyrene formed/hr/mg liver) (Fig. 31).

Table 9/...

TABLE 9

Apparent K_m and V_{max} Values for Aminopyrine N-demethylation and 3,4-Benzpyrene Hydroxylation in Non-Porphyric Controls and Patients with PCT

	Patient	AMINOPYRINE N-DEMETHYLATION			3,4-BENZPYRENE HYDROXYLATION		
		K_m {mM}	V_{max} (nmol semicarbazone formed/hr/mg liver)	r^2	K_m ($M \times 10^{-5}$)	V_{max} ng 3-hydroxy-3,4-benzpyrene formed /hr/mg liver	r^2
Non-Porphyric	M.K.	3,497	0,86	0,8514	4,500	572	0,9856
	R.B.	8,400	31,11	0,9805	0,243	108	0,8628
	H.M.	0,763	8,64	0,9877	0,636	182	0,9965
	M.G.	1,761	0,44	0,9801	-	-	-
	A.L.	3,010	4,90	0,9877	0,475	100	0,9932
	P.O.	0,916	4,98	0,8602	0,160	127	0,9922
	J.P.	-	-	-	0,062	41	0,8763
	A.S.	24,460	51,63	0,9943	0,062	22	0,8418
	A.Si.	-	-	-	0,220	37	0,7973
	P.T.	4,961	32,93	0,9908	0,273	169	0,9584
	C.P.	1,049	38,29	0,9966	0,262	9	0,8967
	E.Y.	2,833	12,32	0,9844	0,291	6	0,8052
	B.S.	2,150	7,79	0,9142	-	-	-
PCT	D.S.	0,156	4,12	0,8183	0,135	25	0,8984
	S.M.	-	-	-	0,686	42	0,9152
	T.B.	1,578	2,38	0,9822	16,940	984	0,9774
	H.L.	-	-	-	1,367	393	0,9937
	P.S.	5,253	2,63	0,9625	0,465	352	0,8210
	H.P.	3,011	1,08	0,9847	-	-	-
	N.E.	-	-	-	1,180	17	0,8762
	J.C.	2,993	10,77	0,9437	0,269	59	0,9221
	J.H.	19,092	62,07	0,9937	0,761	156	0,9578

(The apparent K_m and V_{max} were derived from plots of $\frac{1}{s}$ vs $\frac{1}{v}$ where linear regression curves were fitted to the data. The coefficient of determination of the computed linear regression curve is given by r^2).

TABLE 10

Apparent K_m and V_{max} Values for Aminopyrine N-Demethylation and 3,4-Benzpyrene Hydroxylation in Untreated Rats and Rats Treated with HCB for 45 Days

		AMINOPYRINE N-DEMETHYLATION			3,4-BENZPYRENE HYDROXYLATION		
Treatment of Rats	Experiment no.	K_m {mM}	V_{max} (nmol semicarbazone formed/hr/mg liver)	r^2	K_m ($M \times 10^{-5}$)	V_{max} ng 3-hydroxy-3,4-benzpyrene formed /hr/mg liver	r^2
Controls	1	1,914	18,63	0,9721	0,178	97,69	0,9781
	2	0,507	9,54	0,6370	0,113	72,12	0,7054
	3	0,916	10,23	0,9291	0,174	74,90	0,9437
45 Days with HCB	4	2,322	18,07	0,9185	1,263	3205	0,9778
	5	3,597	26,55	0,8726	0,486	1824	0,9948
	6	3,492	31,74	0,9871	0,283	1535	0,9670

(In each experiment homogenates from each of 3 rats were pooled. The apparent K_m and V_{max} were derived from plots $\frac{1}{s}$ vs $\frac{1}{v}$ where linear regression curves were fitted to the data. The coefficient of determination of the computed linear regression is given by r^2).

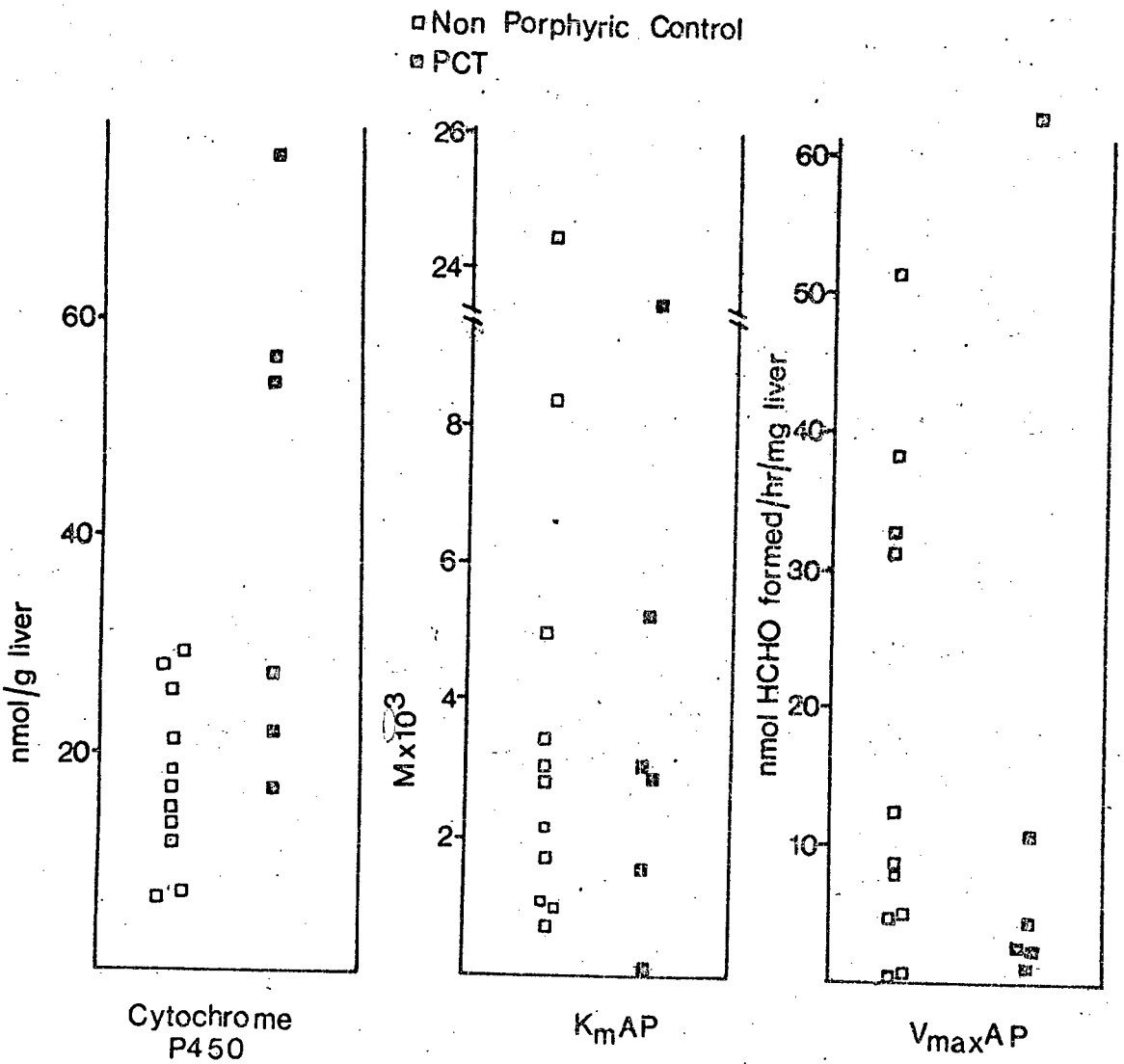


Figure 28 Comparison of the hepatic levels of cytochrome P450, and K_{max} and V_{max} for aminopyrine N-demethylation in liver tissue obtained from non-porphyric controls (open squares) and patients with PCT (closed squares). K_m AP and V_{max} AP respectively are the K_m and V_{max} values for aminopyrine N-demethylation.

6.7. Histological Findings

Light, fluorescence and electron microscopy was performed by a histopathologist, Dr. B.L. Webber of the Department of Pathology, University of Cape Town and the findings have been reported in detail elsewhere[23].

(a) Human Hepatic Tissue

Light microscopy revealed significant abnormalities of hepatic morphology in all patients with PCT. Haemosiderin was demonstrable in 18 of the PCT patients. Fluorescence microscopy showed fluorescence to be patchy and diffuse in all the samples examined from PCT patients. On electron microscopy of liver biopsy samples, there appeared to be no significant increase in hepatic smooth endoplasmic reticulum in cases of PCT when compared to cases of VP and protoporphyria. This might have been expected in view of increased levels of cytochrome P450 found in PCT and not in VP or protoporphyria (Section 6.4.(b)). An important finding from electron microscopic studies was that the quantity of smooth endoplasmic reticulum varied greatly from one cell to another. For the interested reader, electron micrographs obtained by Dr. Webber of PCT liver and protoporphyric liver may be found in previously published papers [23,223].

(b) Rat Hepatic Tissue

No stainable iron in the form of haemosiderin (Prussian blue staining) was present in the livers of rats that had been treated with HCB and Jectofer simultaneously for 30 days. In a control group given Jectofer without HCB, at the same dosage schedule, abundant stainable iron was present on histological examination.

6.8. UROGEN-I Decarboxylase - Preliminary Experiments

(i) Extinction Coefficient for URO-I at 560 nm under the Conditions of Assay for UROGEN-I Decarboxylase

Enzymatically generated UROGEN-I substrate for the decarboxylase was measured as URO-I in all the experiments by determination of the absorbance of band II shown in Fig. 32. The absorbance of varying concentrations of URO-I was measured at 560 nm in the presence of the incubation mixture for UROGEN-I decarboxylase (Fig. 33). A linear increase in absorbance with increasing URO-I concentrations was observed. From the slope of the calculated linear regression curve, whose coefficient of determination was 0,997, the millimolar extinction coefficient for URO-I at 560 nm was 7,554 $cm^{-1}mM^{-1}$, under the conditions of assay.

(ii) Enzymatic Generation of UROGEN-I Enzyme and Substrate Conditions for the Generation of 25 - 30 nmol UROGEN-I

The quantity of UROGEN-I generated from various quantities of a 2 mg/ml solution of the ^{14}C-PBG preparation purchased from Porphyrin Products by UROGEN-I synthetase in a total volume of 1 ml is shown in Table 11.

TABLE 11

Formation of ^{14}C-UROGEN-I by UROGEN-I Synthetase from ^{14}C-PBG

Sample Number	Volume of 2 mg/ml ^{14}C-PBG Solution	nmol ^{14}C-UROGEN-I formed
1	0,015	6,06
2	0,030	8,04
3	0,060	13,18
4	0,090	20,02
5	0,120	25,69
6	0,150	27,67

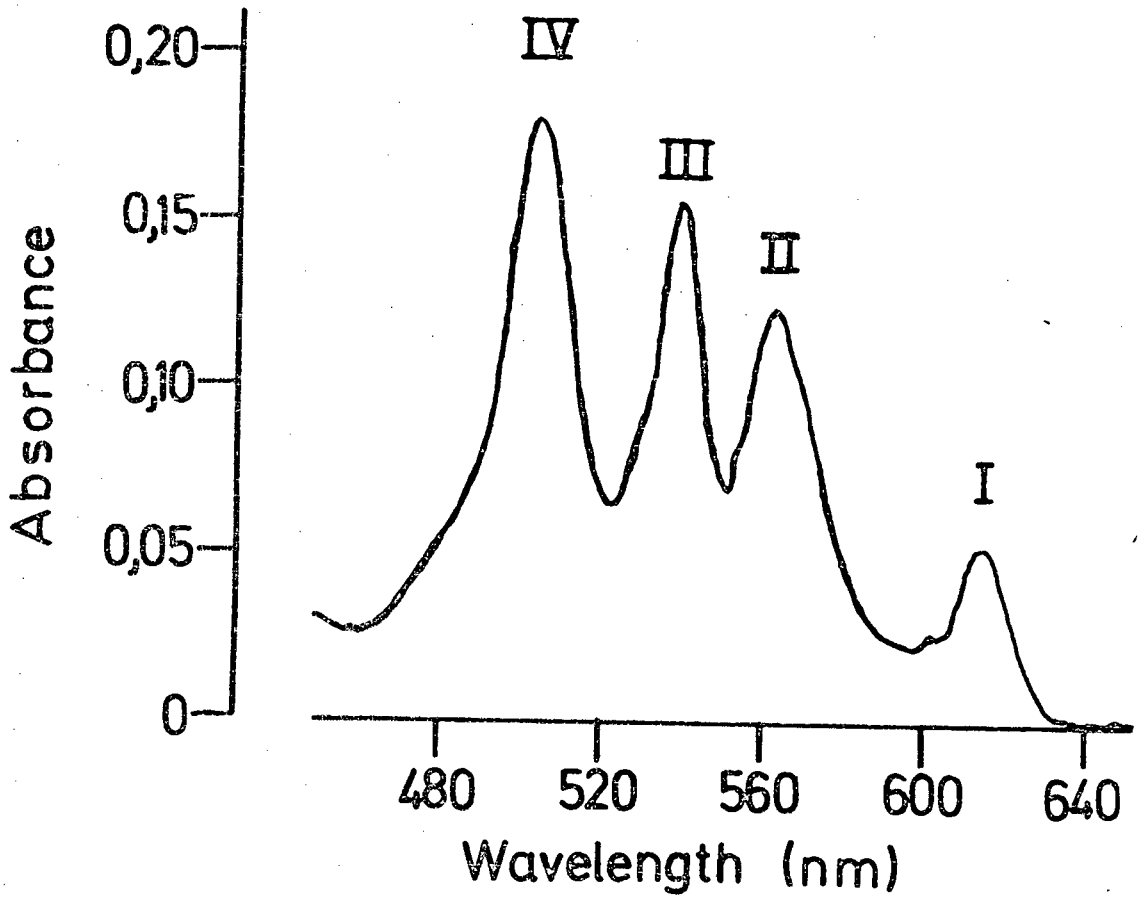


Figure 32 Absorption spectrum (Soret band omitted) of URO-I (20μM) in the assay mixture used for the determination of UROGEN-I decarboxylase activity.

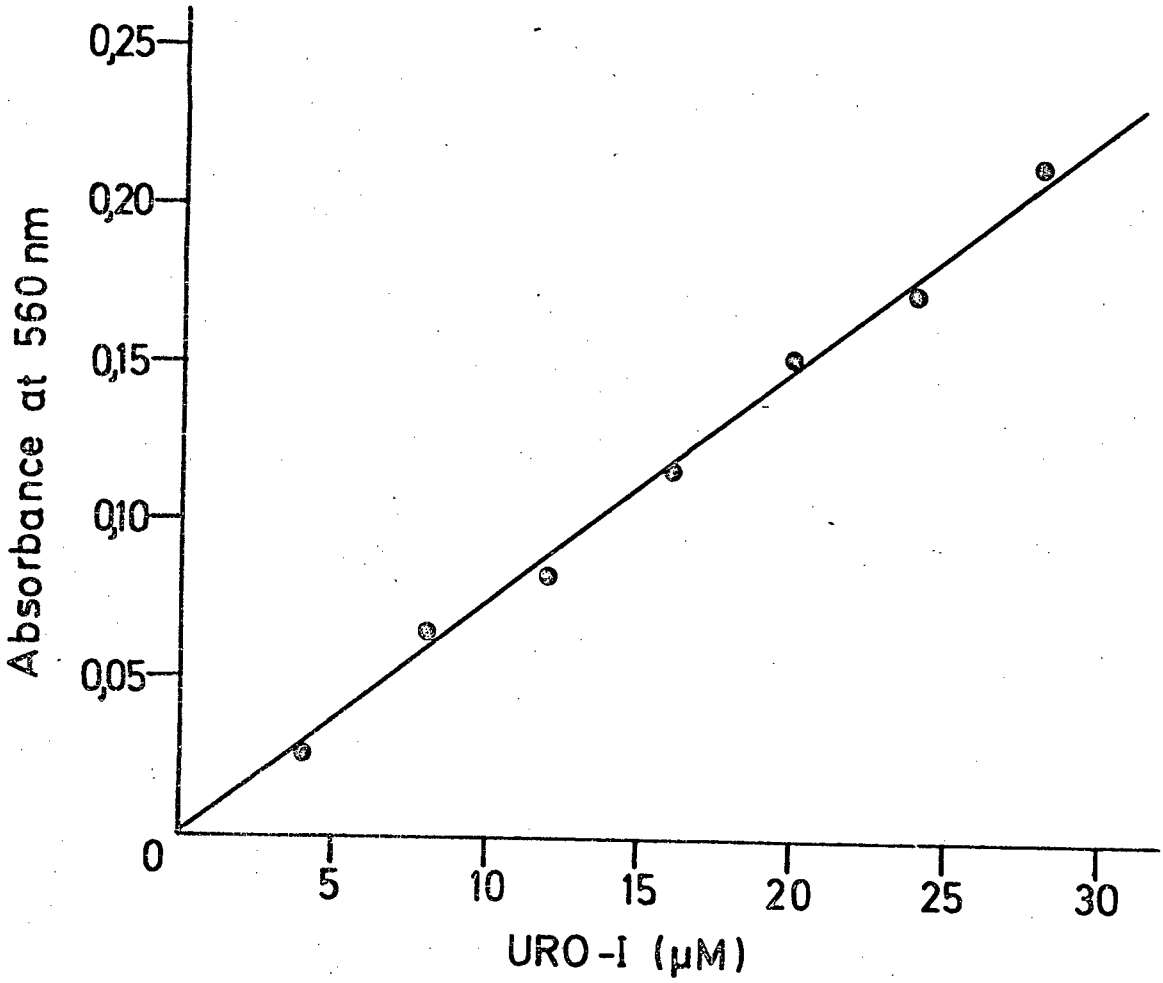


Figure 33 Relationship of the absorbance of URO-I at 560nm in the incubation mixture for UROGEN-I decarboxylase to the concentration of URO-I. The line was fitted by calculation of linear regression coefficients.

(Each sample contained ^{14}C-PBG, 0,1ml 0,066M L-cysteine, 0,1 ml 7 mg/ml UROGEN-I synthetase and 0,1M tris:HCl pH 7,65 to a total volume of 0,5 ml. Samples were incubated in the dark for 30 min. at 37°C, at which time 0,1 ml 0,4M KH_2PO_4 and 0,4 ml 0,1 M tris:HCl were added. After a further incubation period of 30 min. at 37°C in the dark, the UROGEN-I formed was oxidised to URO, and absorbance at 560 nm determined. URO concentration was calculated using ϵ_{mM} = 7,554 $cm^{-1}mM^{-1}$).

From the result of this experiment, in the ensuing experiments, 0,15 ml of a 2 mg/ml solution of ^{14}C-PBG in 0,1M tris:HCl, pH 7,65 and 0,1 ml of a 7 mg/ml solution of UROGEN-I synthetase in 0,1M tris:HCl, pH 7,65, were present in the assay mixture.

The amount of UROGEN-I generated from various quantities of a 5 mg/ml solution of ^{14}C-ALA by a preparation of bacterial ALA dehydratase and UROGEN-I synthetase in a total volume of 1 ml is shown in Table 12.

TABLE 12

Formation of UROGEN-I by a mixture of ALA Dehydratase and UROGEN-I synthetase from ^{14}C-ALA

Sample number	Volume of 5 mg/ml solution ^{14}C-ALA	nmol ^{14}C-UROGEN-I formed
1	0,02	20,28
2	0,03	24,88
3	0,04	26,77
4	0,05	27,31
5	0,10	29,32
6	0,15	27,30

(Each sample contained ^{14}C-ALA, 0,1ml 0,066M L-cysteine, 0,1 ml 7mg/ml ALA dehydratase/UROGEN-I synthetase and 0,1M tris:HCl pH 7,65 to a total volume of 0,5 ml. Incubation conditions and UROGEN-I determinations were as for those described for Table 11).

In the experiments where UROGEN-I was generated by this latter method, 0,05 ml of a 5 mg/ml solution of ^{14}C-ALA in 0,1M tris:HCl, pH 7,65 and 0,1 ml of a 7 mg/ml solution of the ALA dehydration/UROGEN-I synthetase preparation were used in the assay mixture.

It was found, using either method for production of UROGEN-I substrate, that from 7 to 10% of the UROGEN-I produced was autooxidised under the incubation conditions by spectrophotometric determination before and after oxidation with iodine.

6.9. UROGEN-I Decarboxylase in Human Red Blood Cells.

In all the assays for determination of the activity of red blood cell UROGEN-I decarboxylase, UROGEN-I substrate was generated from ^{14}C-PBG by UROGEN-I synthetase. The amount of haemoglobin present per assay ranged from 23,44 mg to 66,00 mg.

Red cell UROGEN-I decarboxylase activity in 3 large families is shown in Fig. 34. There appeared to be no obvious genetically-determined deficiency of this enzyme in the progeny of the 2 patients with PCT. Furthermore, no significant differences were noted in the activity of red cell UROGEN-I decarboxylase between male or female patients with PCT and sex-matched non-porphyric controls (Fig. 35). By application of the students t test, P values between non-porphyric males and PCT males are >0,05; P values between non-porphyric females and PCT females are >0,2. These differences were not considered significant.

6.10 UROGEN-I Decarboxylase in Livers of Rats Treated with HCB and HCB plus Jectofer

The activity of hepatic UROGEN-I decarboxylase expressed as nmol UROGEN-I decarboxylated/hr/mg protein at 15 day intervals up to 60 days in HCB-and HCB plus Jectofer-treated rats, is shown in Fig. 36.

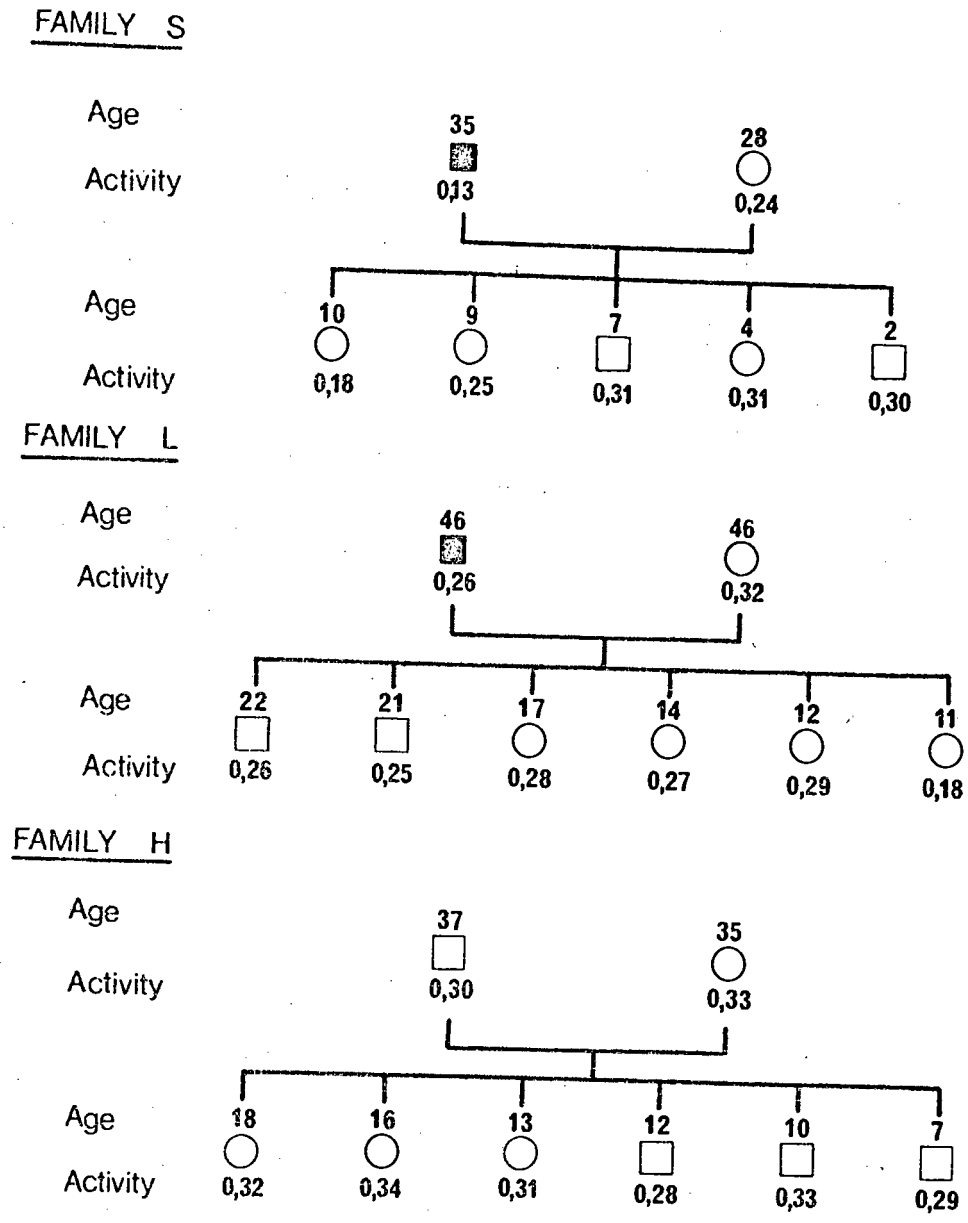


Figure 34 Red blood cell UROGEN-I decarboxylase activity in three unrelated families. In two families the father had overt PCT. The activity of UROGEN-I decarboxylase is expressed as nanomoles UROGEN-I decarboxylated per hour per milligram.
■ = Propositi; □ = males; ○ = females.

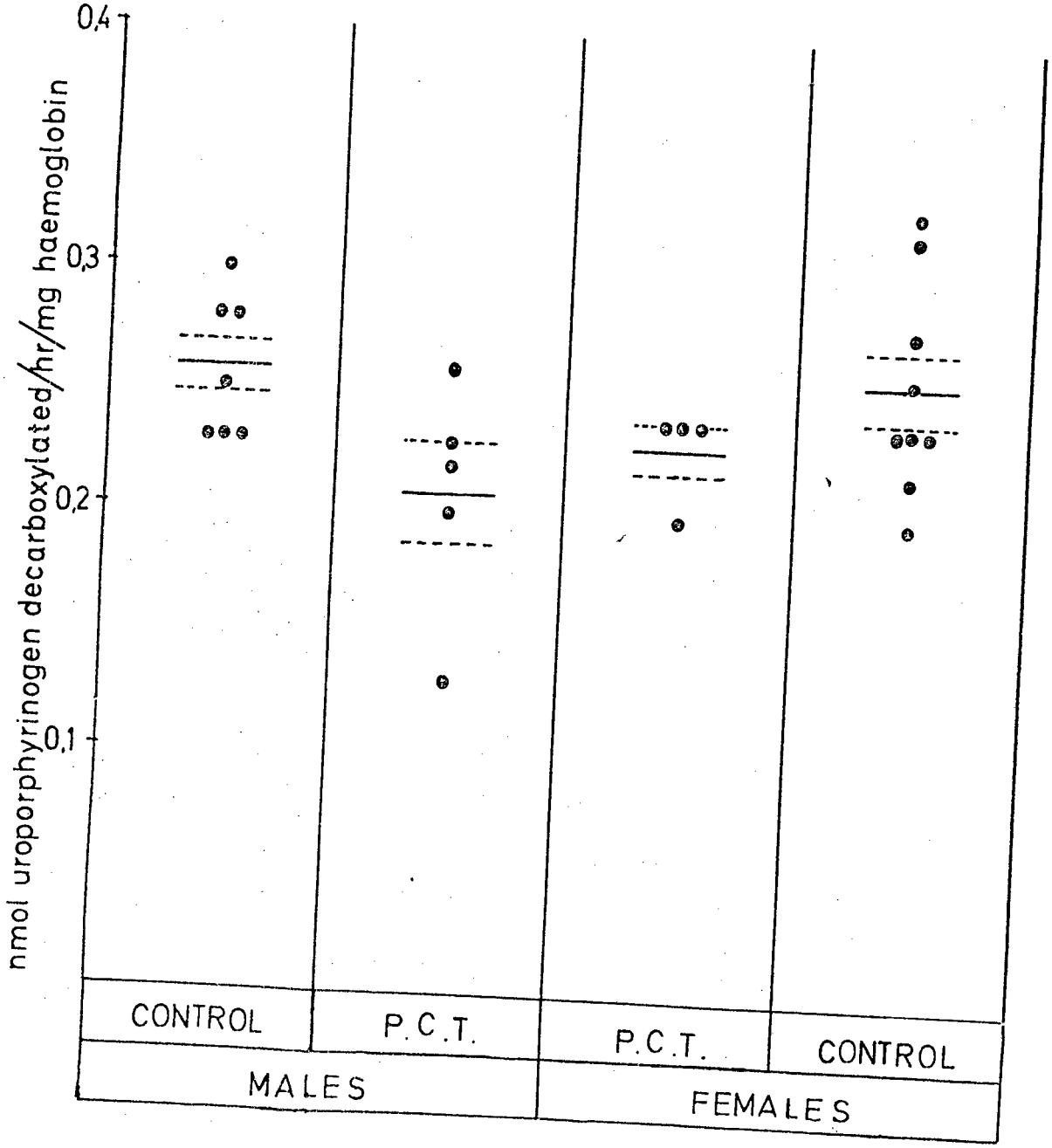


Figure 35 Red blood cell UROGEN-I decarboxylase activity in unrelated non-porphyric controls and in unrelated PCT patients. — = Mean ----- = S.E.

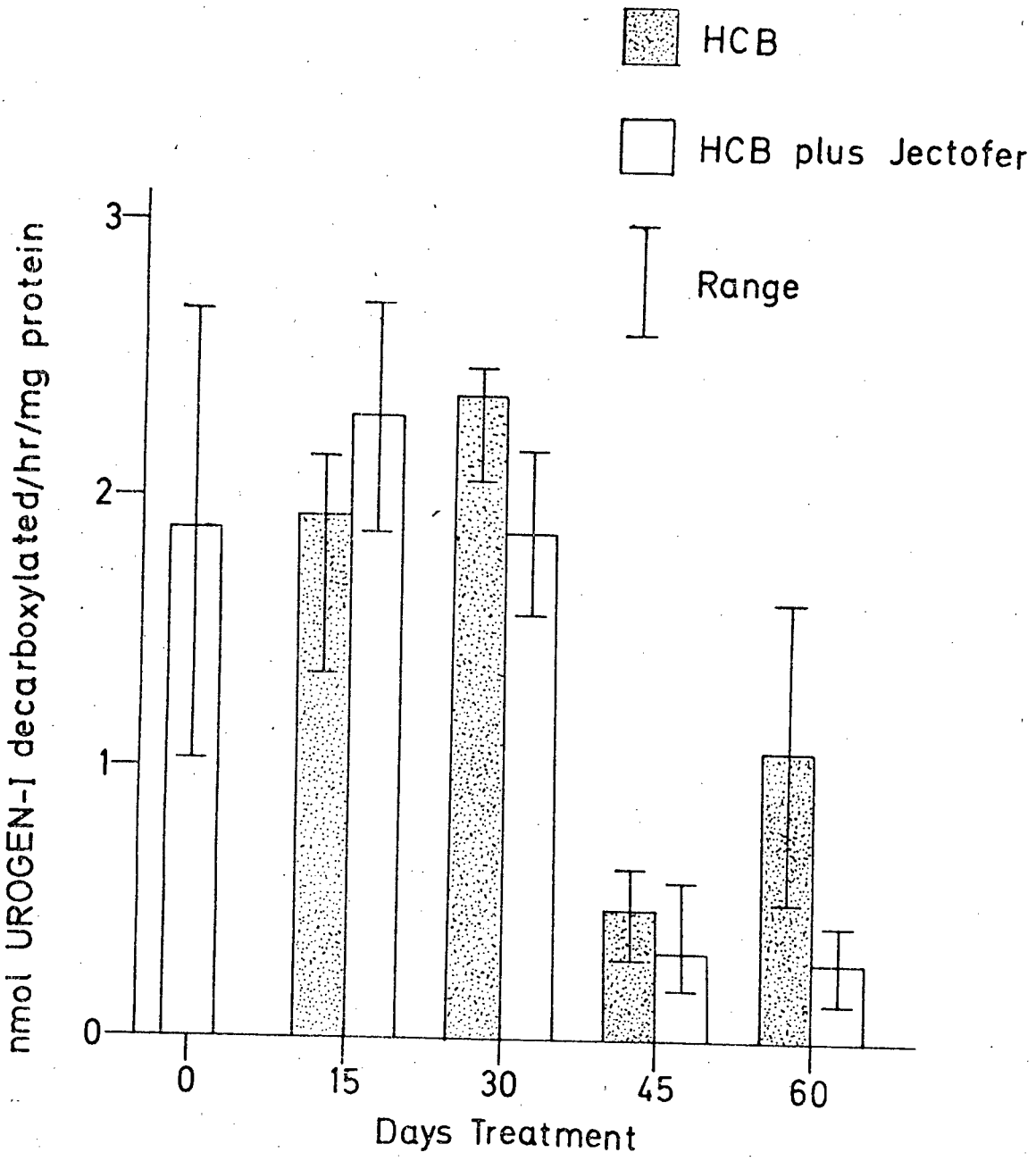


Figure 36 Hepatic UROGEN-I decarboxylase activity at 15 day intervals in rats treated with HCB alone and in rats treated simultaneously with HCB and Jectofer. The heights of the bars represent the mean activity of the enzyme in the livers of 3 rats.

For up to 30 days, the activity of the enzyme did not appear to be affected by either treatment schedule. However, after 45 days treatment, the mean activity of the enzyme decreased to 0,45 in the case of the HCB-treated rats, and to 0,33 in the case of the HCB plus Jectofer-treated rats. This decrease in the activity was highly significant, with $P<0,001$ in both cases, when compared to the activity in untreated rats where the mean activity was 1,88. At 60 days, hepatic UROGEN-I decarboxylase activity increased somewhat to a mean value of 1,07 in the group treated with HCB alone, but this activity was still significantly different from that of the untreated group, with $P<0,05$. In rats treated with HCB together with Jectofer, a slight decrease in the activity of the enzyme to a mean value of 0,30 was noted, and again the difference was significant when compared to the control group with $P<0,001$.

6.11. Effect of in vitro Addition of Iron on UROGEN-I Decarboxylase in Rat Liver.

The effects of in vitro additions of iron as ferrous ammonium sulphate and ferritin, and an iron chelator, 1,10-phenanthroline, on hepatic UROGEN-I decarboxylase in untreated rats and rats treated for 45 days with HCB are shown in Table 13. As demonstrated in Section 6.10, a significant diminution of the activity of the enzyme was again noted in HCB-treated rats when compared to the untreated group of animals. The addition of ferrous ammonium sulphate to the assay mixture for UROGEN-I decarboxylase resulted in a significant increase in the activity both in control and HCB-treated rat liver, whereas ferritin had no such effects (Table 12). 1,10-Phenanthroline did not significantly influence the hepatic UROGEN-I decarboxylase.

TABLE 13

UROGEN-I Decarboxylase Activity in Rat Liver. Effects of In Vitro Additions of Iron (Ferrous Ammonium Sulphate and Ferritin) and 1,10-Phenanthroline

Additives	nmol UROGEN-I Decarboxylated/hr/mg Protein	
	Controls (no treatment)	HCB (45 days)
None	4,9 (4,4 - 5,7)	0,8 (0,4 - 1,4)
Fe^{++} (1mM)	10,1 (8,4 - 12,4)	2,1 (1,4 - 2,7)
Ferritin(1mM Fe)	3,8 (2,9 - 4,6)	0,5 (0,2 - 0,9)
1,10 Phenanthroline(3mM)	6,4 (5,7 - 7,1)	1,8 (1,2 - 2,4)

(Three animals were used in each study and values are expressed as a mean with the range for individual data in parenthesis. It was assumed that 23% of the ferritin was iron).

The in vitro effect of increasing concentrations of ferrous iron (as ferrous ammonium sulphate) up to 0,25mM on rat liver UROGEN-I decarboxylase is shown in Fig. 37. It was found that maximal activity was manifested at concentrations of ferrous iron from 0,10 mM. Further, the activity remained elevated at this level up to the highest concentration used, viz. 2mM (Table 14).

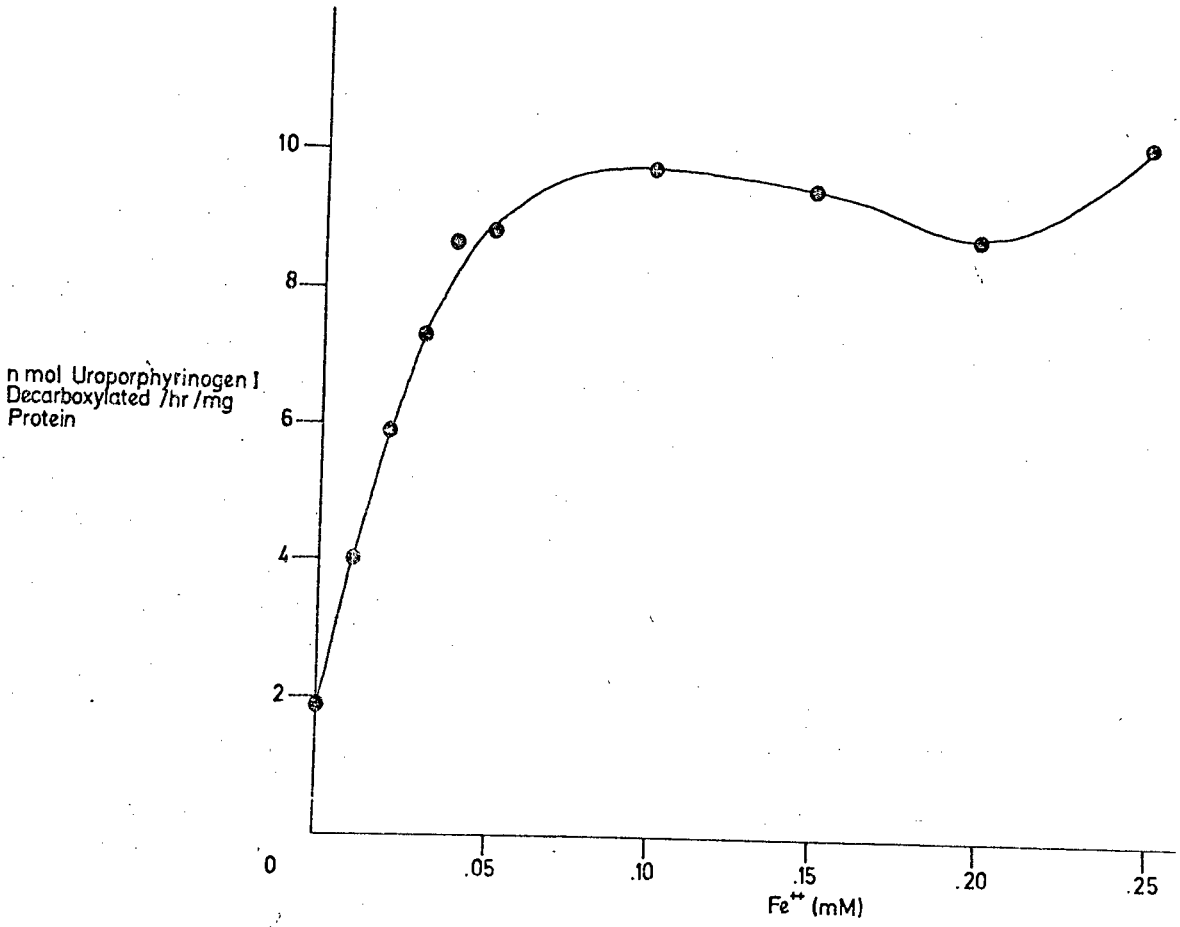


Figure 37 The effect of added in vitro iron as ferrous ammonium sulphate on the activity of hepatic UROGEN decarboxylase. The 32000xg supernatant of a liver homogenate from an untreated rat was used as enzyme source. Each point is the mean of two observations.

TABLE 14

Effect of In Vitro Addition of Ferrous Ammonium Sulphate on Rat Liver UROGEN-I Decarboxylase

Fe^{2+} (mM)	nmol UROGEN-I Decarboxylated /hr/mg Protein
0	1,9
0,01	4,1
0,02	5,9
0,03	7,4
0,04	8,8
0,05	9,1
0,10	10,0
0,15	9,8
0,20	9,1
0,25	10,3
0,40	9,9
0,80	10,6
1,20	10,3
1,60	9,4
2,00	10,6

(The mean value for two determinations of UROGEN-I decarboxylase activity at each concentration of Fe^{2+} is given. Liver from an untreated rat was used as enzyme source).

6.12. UROGEN-I Decarboxylase Assay - Extraction of Reaction Products.

The activity of UROGEN-I decarboxylase is calculated from the radioactivity of the decarboxylated porphyrin esters expressed as a fraction of total radioactivity of the URO and 7-,6-,5- and 4-COOH porphyrin esters (Appendix A). If different proportions of the porphyrin esters are extracted by the method described in Section 5.5.(b) and (c), either from the talc or from the esterification mixture, a true value for the activity of the enzyme will not be obtained. An experiment was thus designed to test the possibility of differential extraction of reaction products. With the pooled post-mitochondrial supernatants from the livers of 3 rats as enzyme source, UROGEN-I decarboxylase activity was determined in triplicate for increasing protein concentrations in two parallel experiments where two methods were used for the extraction of the reaction products.

In both sets of determinations, incubations and termination of the reactions were performed as in Section 5.5.(b) and (c), with ^{14}C-ALA and a mixture of ALA dehydratase and UROGEN-I synthetase used to generate UROGEN-I substrate. The reaction products in one set of assay tubes were adsorbed onto talc, esterified, extracted and chromatographed as detailed in Section 5.5.(b) and (c).

In an attempt to overcome the possibility of differential desorbtion of porphyrin esters from talc, a different method was used to oxidise and esterify the reaction products from the second set of assay tubes. After termination of the reactions the samples were left in the light, but screened from direct sunlight, for 1 hr to allow oxidation of the porphyrinogens to porphyrins[162]. The porphyrins were converted to their methyl esters by addition of 50 ml 5% H_2SO_4 in methanol, and left overnight at room temperature in the dark.

A modified procedure was used to extract the porphyrin esters from

the esterification mixture. The esterification mixture was neutralised with 10% NH_4OH and extracted repeatedly with 10 ml aliquots of $CHCl_3$, until no further fluorescence was visible in the $CHCl_3$ layer when viewed under ultraviolet light. The $CHCl_3$ extracts were pooled, washed twice with distilled water, then filtered through $CHCl_3$-wetted filter paper (Whatman no. 1) into beakers. Chromatographic analysis and scintillation counting of the porphyrin esters was as described in Section 5.5.(b) and (c).

The above method was used for the extraction of porphyrin esters from the esterification mixture since it was found in a preliminary experiment, performed in triplicate, where 4,7 nmol URO III octamethyl ester and 6,3 nmol COPRO-III tetramethyl ester were added to 50 ml aliquots of 5% H_2SO_4 in methanol and extracted by this method, the recoveries of URO-III ester and COPRO-III ester (determined spectrophotometrically as in Section 5.6.I(b)) were 89.6% (Range 88,3 - 91,1) and 90,8% (Range 88,7 - 93,4) respectively. In a similar experiment, where the esters were recovered as described in Section 5.5.(b) and (c), recoveries were 61,7% (Range 58,5 - 64,2) and 82,4% (Range 81,9 - 83,2) respectively for the methyl esters of URO and COPRO.

Fig. 38 compares the activities of rat liver UROGEN-I decarboxylase with increasing protein concentration obtained where the two methods were used to extract the products of the reaction. The activities as determined where the reaction products were adsorbed and oxidised on talc, esterified and extracted as in Section 5.5.(b) and (c) were 39% greater than the activities as determined where the reaction products were oxidised by light, directly esterified and extracted from a neutralised esterification mixture with $CHCl_3$. This result indicates that a differential exists in the extractability of the products of the UROGEN-I

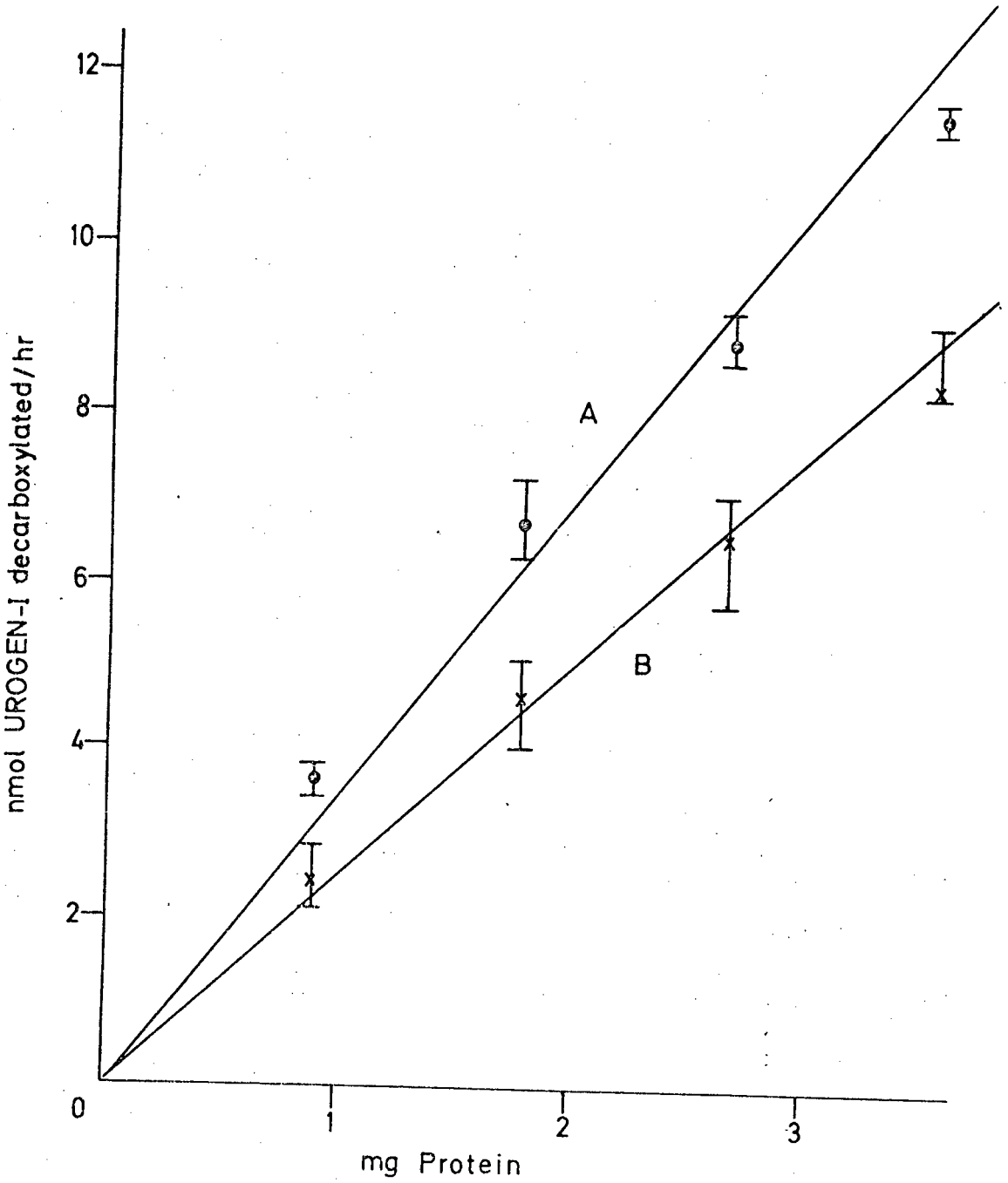


Figure 38 Relationship of the activity of rat liver UROGEN-I decarboxylase to the quantity of protein present in the assay. Each point is the mean of 3 observations. Pooled 32000xg supernatant prepared from the livers of three untreated rats was used as enzyme source. Incubation conditions were as described in Section 5.5. Line A was obtained when the reaction products were adsorbed on talc, esterified, extracted and chromatographed as detailed in Section 5.5. Line B was obtained when the reaction products were oxidised by light, directly esterified, exhaustively extracted into chloroform at neutral pH and chromatographed, as described in Section 6.12. Lines were fitted by calculation of linear regression coefficients.

decarboxylase assay when these are extracted by the method as described in Section 5.5.(b) and (c). By this method of extraction, it appeared that URO-I as URO-I octamethyl ester was the most incompletely extracted ester, with a consequent overestimation of the activity of UROGEN-I decarboxylase.

A further finding was that 17857 to 27373 dpm remained in the talc after extraction with 5% H_2SO_4 in methanol in those assay samples extracted as described in Section 5.5.(b) and (c). By calculation, if the specific activity of ^{14}C-ALA is 0,42 µCi/µmol, the specific activity of UROGEN-I formed will be 8 x 0,42 = 3,36 µCi/µmol. In the experiment, 26,3 nmol UROGEN-I was generated, corresponding to 194410 dpm. Thus 9,18% to 14,08% of the radioactive porphyrin esters remained on the talc.

The above findings do not invalidate the results obtained for the activity of UROGEN-I decarboxylase where the reaction products were extracted as described in Section 5.5.(b) and (c) for 3 reasons:

(i) Using either method of extraction, reproducibility to within 15% was obtained (Fig. 38)

(ii) Using both methods of extraction, there was an essentially linear increase of activity with increasing protein concentration to 3,5 mg protein (Fig. 38)

(iii) The results obtained for the activity of UROGEN-I decarboxylase have been used in comparative studies only

6.13. Effect of Haemoglobin on UROGEN-I Decarboxylase Activity.

(a) Activity of Human Red Blood Cell UROGEN-I Decarboxylase with Increasing Concentration of Enzyme Source.

The activity of UROGEN-I decarboxylase was determined in human red blood cells using as enzyme source from 0,1 to 0,4 ml haemolysate derived

from the author of this thesis. ^{14}C-UROGEN-I substrate was generated from ^{14}C-PBG by UROGEN-I synthetase, and reaction products of the decarboxylation were extracted by the method as described in Section 5.5.(b) and (c). A linear increase in the activity was not observed with increasing amounts of haemolysate as shown in Fig. 39.

(b) Activity of Rat Liver UROGEN-I Decarboxylase in the Presence of Increasing Concentrations of Haemoglobin.

With a constant quantity of post-mitochondrial supernatant prepared from a 1:5 w/v liver homogenate from an untreated rat as enzyme source, the activity of UROGEN-I decarboxylase was determined in the presence of increasing concentrations of electrophoretically pure human haemoglobin prepared by Mr. J. Rees of the Provincial Blood Grouping Laboratories, University of Cape Town Medical School, Cape Town. ^{14}C-UROGEN substrate was generated from ^{14}C-ALA using a mixture of ALA dehydratase and UROGEN-I synthetase. At the start of the second 30 min. incubation, from 10 to 56 mg haemoglobin contained in 0,3 ml 0,1M tris:HCl buffer, pH 6,8 were added together with 0,1 ml 0,4M KH_2PO_4 and 0,1 ml post-mitochondrial supernatant. After termination of the reaction, the products were isolated by the alternative method described in Section 6.12. The activity of UROGEN-I decarboxylase relative to the protein concentration of the 32000 x g supernatant was not found to be constant in the presence of haemoglobin (Table 15).

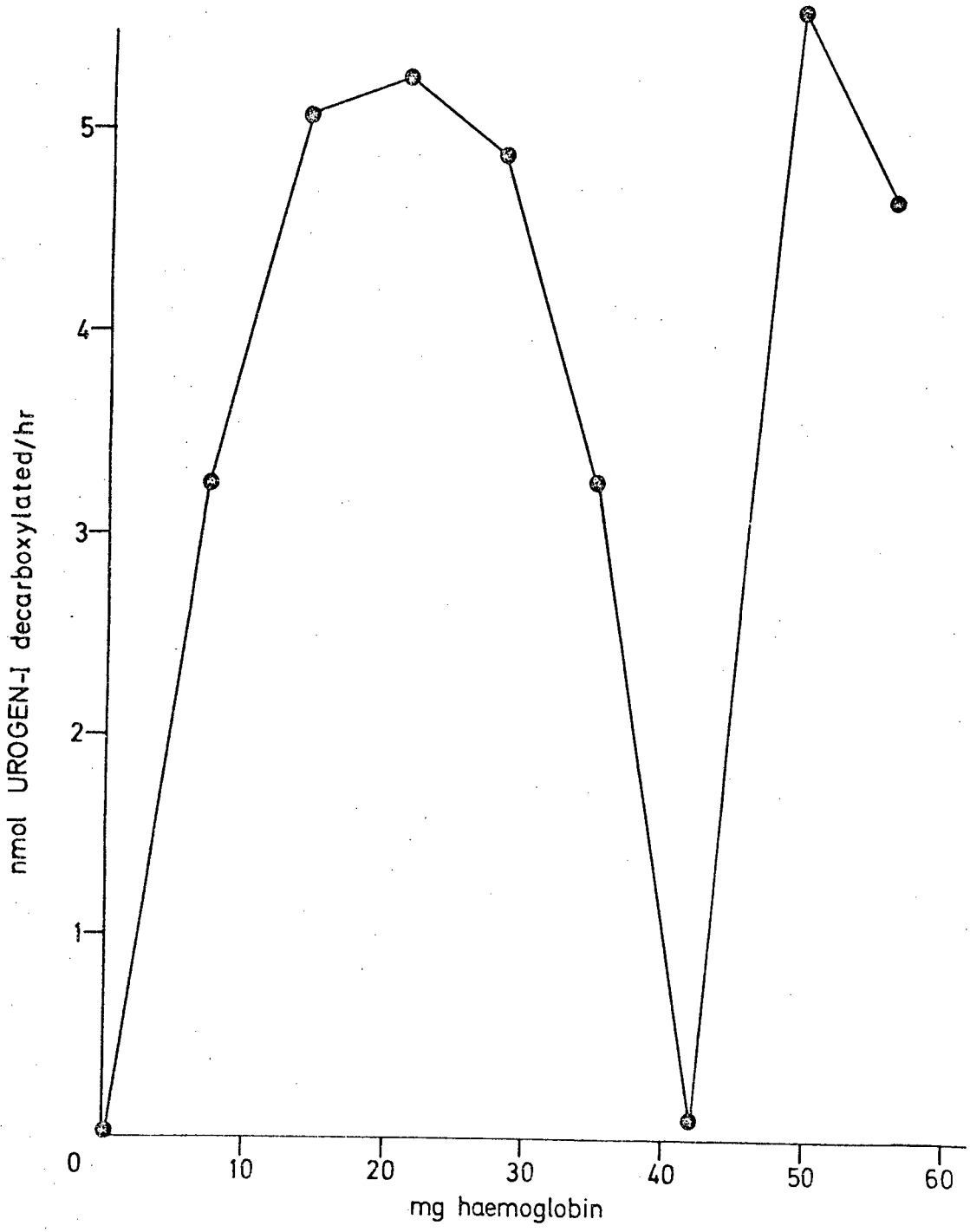


Figure 39 Relationship of the activity of human red blood cell UROGEN-I decarboxylase to the quantity of haemoglobin present in the assay. The 32000 xg supernatant of an haemolysate prepared from red blood cells of a non-porphyric subject was used as enzyme source.

TABLE 15

Effect of Added Haemoglobin on the Activity of Rat Hepatic UROGEN-I Decarboxylase

Quantity Haemoglobin added to Reaction Tube(mg)	nmol UROGEN-I decarboxylated /hr/mg Protein
-	1,2 (1,1 - 1,3)
10	0,5
19	0,1
37	0
56	0,4

(The activity in the absence of haemoglobin is given as the mean of four determinations, with the range given in parenthesis. In view of the limited quantity of electrophoretically pure haemoglobin available for this experiment, all other values given are from single observations).

(c) Activity of Rat Red Cell UROGEN-I Decarboxylase with Increasing Concentration of Enzyme Source

Blood was obtained from an untreated anaesthetised female rat by cardiac puncture and an haemolysate was prepared as for human red blood cells. The activity of UROGEN-I decarboxylase was determined in rat erythrocytes using as enzyme source 0,02, 0,07 and 0,13 ml

haemolysate, with ^{14}C-UROGEN-I substrate generated from ^{14}C-PBG by UROGEN-I synthetase. Reaction products were extracted as detailed in Section 5.5.(b) and (c).

A linear increase in the activity was observed with increasing amounts of haemolysate containing up to 4 mg haemoglobin as shown in Fig. 40. Rat red blood cell haemolysates were found to have a low haemoglobin content when compared to human red cell haemolysates due to the fact that rat haemoglobin can be crystallised at pH 6,8[19] which is the pH of the buffer added before haemolysis.

The observations detailed in (a),(b) and (c) immediately preceeding indicate that high concentrations of haemoglobin may interfere non-specifically in the assay for human red cell UROGEN-I decarboxylase activity. It is recognised that no definite conclusions can be made from merely 3 points. However the proportional increase in enzyme activity observed with increasing amounts of rat red blood cell haemolysate indicates that it may be possible to overcome the non-specific interference by haemoglobin on human red blood cell UROGEN-I decarboxylase by determination of the activity of the enzyme in haemolysates where haemoglobin concentration is low.

(d) Human Red Blood Cell UROGEN-I Decarboxylase Activity in Haemoglobin-Free Haemolysate.

Haemoglobin-free haemolysates were prepared essentially according to the method of Hennessey et al[126].

An haemolysate was prepared from 20 ml of the author's blood as detailed in Section 5.IC2.but the buffer used was 0,003M potassium phosphate, pH 7,0. The stroma-free haemolysate was divided equally among four 50 ml polyethylene centrifuge tubes. The following procedure

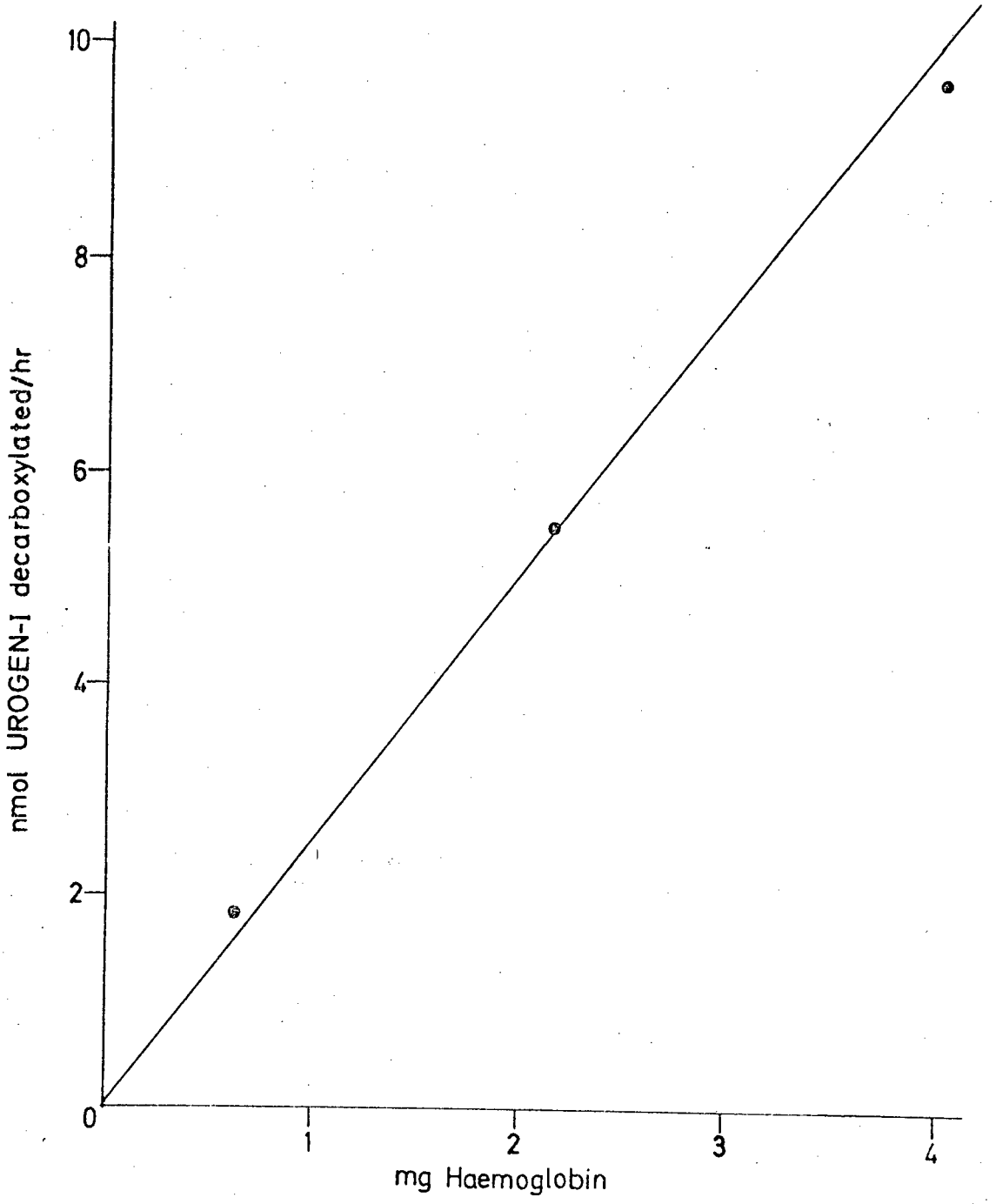


Figure 40 Relationship of the activity of rat red blood cell UROGEN-I decarboxylase to the quantity of haemoglobin present in the assay. The 32000xg supernatant of an haemolysate prepared from red blood cells of an untreated rat was used as enzyme source.

represents the treatment of one of the 5 ml samples carried out at $4^{o}C$. To 5 ml of haemolysate was added 5 ml of an aqueous suspension of Whatman DE 52 (diethylaminoethyl cellulose anion exchange resin, 40g/liter, pH 7,0), and the mixture was allowed to stand at $4^{o}C$ for 20 min., with manual stirring approximately every 4 min. The unadsorbed material in the supernatant,principally haemoglobin which has an isoelectric point near neutrality, was separated by centrifugation for 15 min at 5000 x g, and residual haemoglobin was removed by washing the adsorbant 8 times with a total of 200 ml 0,003M phosphate buffer, pH 7,0. The final wash was colourless.

The enzyme protein fraction was desorbed from the DE 52 resin by adding 10 ml 0,5M KCl and gently stirring the mixture magnetically for 1 hr in an ice bath. The supernatant fluid was collected by centrifugation at 5000 x g for 15 min. The adsorbant was washed twice more with 5 ml aliquots of 0,5M KCl. The combined supernatant from all three washes was dialysed overnight against 0,1M tris:HCl, pH 6,8, following which the dialysate was concentrated to about 5 ml by placing the dialysis bag in concentrated polyethylene glycol 6000 solution.

The activity of human red blood cell UROGEN-I decarboxylase was measured using as enzyme source from 0,1 to 0,4 ml of each of the four haemoglobin-free preparations. The ^{14}C-UROGEN-I substrate was prepared by the action of a mixture of ALA dehydratase and UROGEN-I synthetase on ^{14}C-ALA. Analysis of the reaction products was performed by the alternative method described in Section 6.12. As shown in Table 16, considerable variation in the activity of red blood cell UROGEN-I decarboxylase, expressed as nmol UROGEN-I decarboxylated/hr/mg protein, was observed, both within a group where the same haemolysate preparation was used as enzyme source, and between the groups where different haemo-

globin-free enzyme preparations from the same original haemolysate were used as enzyme source. The former variation may be due to loss of enzyme stability in the dilute haemoglobin-free preparations, the protein concentration of which was found to range from 0,3 to 0,5 mg/ml in the 4 preparations. The variation between groups might be due to varying recoveries of UROGEN-I decarboxylase from the anion exchange resin.

TABLE 16

Human Red Blood Cell UROGEN-I Decarboxylase Activity as Determined in Haemoglobin-Free Haemolysate

Haemolysate Preparation No.	Volume of Haemolysate per Assay(ml)	UROGEN-I Decarboxylase Activity (nmol UROGEN-I Decarboxylated/hr/mg protein).
1	0,1	6,2
	0,2	8,2
	0,3	5,5
	0,4	4,2
2	0,1	5,7
	0,2	3,6
	0,3	3,1
	0,4	5,7
3	0,1	10,5
	0,2	9,6
	0,3	8,2
	0,4	8,5
4	0,1	12,5
	0,2	7,1
	0,3	7,5
	0,4	8,4

CHAPTER 7

DISCUSSION

In each of the 19 patients with clinical symptoms of PCT, investigations of their urinary, faecal and hepatic porphyrins fulfilled all the established diagnostic criteria for a positive unequivocal diagnosis of PCT[23,64,65,69,73,75,77,78,79,83,288]. The controls who all had clinical and histological evidence of ethanolic liver disease, similar to that present in patients with PCT, had negative screening tests for urinary and faecal porphyrins, and quantitation tests were therefore not performed.

HCB is a known porphyrogenic compound which has produced an acquired PCT syndrome in man[57,251], and an experimental porphyria in rats[210,252,253,255] which biochemically resembles PCT. The development of HCB porphyria in female rats over a period of 60 days at 15 day intervals was studied by analysis of urinary, faecal and hepatic porphyrins.

Female rats were used throughout the study, since it has been shown that these are more susceptible to HCB than male rats[112,246].

It was found that after 30 days treatment with HCB, the animals showed no significant porphyrinuria (Fig. 12b). After 45 days treatment it was found that there was a significant increase in the excretion of urinary porphyrins, and furthermore, at this stage, the level of URO was about 3-fold greater than the level of COPRO. This finding corroborates that of Taljaard et al[294], but is at variance with that of both Elder et al[80] and Ockner and Schmid[210], who found that URO excretion exceeded that of COPRO only after approximately 60 days. The disparity may be caused by differences in the strain of rat, a different treatment

schedule or the age of the rat. In the studies of Ockner and Schmid[210] a male rat was used which may account in part for the difference between their findings and those reported in this thesis.

Hepatic URO accumulation in HCB-treated rats over the 60 day treatment period reflected the urinary URO excretion pattern. After 30 days treatment with HCB, an increase was noted in the amount of URO accumulated in the livers, with further increases observed at 45 and 60 days. Concomitantly, increases in hepatic 7-COOH porphyrin content was observed, such that at these times the excess porphyrins were almost entirely URO and 7-COOH porphyrin.

Hepatic porphyrins were not measurable in untreated rats by the methods used to quantitate hepatic porphyrins in the porphyric animals, but by using larger quantities of liver tissue, an indication of the pattern and quantity of hepatic porphyrins present in untreated rats might be obtained.

Analysis of faecal porphyrins after 45 days treatment with HCB, i.e. at a time when the rats were porphyric, with the urinary porphyrin excretion pattern and the hepatic porphyrin accumulation pattern closely resembling that found in human PCT, indicated the presence of a significant quantity of the porphyrin P_1 fraction, which is in agreement with the findings of Elder[78,79].

Iron appears to play a central role in the genesis of the PCT syndrome, since hepatic siderosis is an almost invariable finding in PCT[143,144,161,164,186,250,307,311]. Moreover, porphyrin excretion is decreased when removal of storage iron by venesection is used to effect clinical remission[89,185,307]; this can be prevented[185] or reversed[89,184] by oral iron ingestion. In the experimental porphyrias, iron has been observed to influence porphyrin biosynthesis at different levels

in the biosynthetic pathway. Iron as ferric citrate administered orally exerts a synergistic effect on AIA induction of ALA-S in rodent porphyria[281], emphasising the importance of iron in the regulatory control of porphyrin biosynthesis. Kushner et al[161] have shown that in vitro ferrous iron inhibits UROGEN cosynthetase, which has been regarded as a possible explanation for the excess production and excretion of URO I in patients with PCT.

Experimental hepatic siderosis in the rat due to dietary iron overload has been shown to result in urocoproporphyrinuria[266] which was further aggravated by concurrent drinking of ethanol. Further, rats previously rendered siderotic by intraperitoneal administration of iron-Dextran developed HCB-induced hepatic porphyria more rapidly than rats fed HCB alone[294,295] and the porphyrin profile in the liver and urine resembled that seen in PCT.

In the work presented here, a study was made of the effect of simultaneous treatment of rats with HCB and iron on the development of HCB porphyria. It was found after 30 days simultaneous treatment there was already significant uroporphyrinuria, but at this time the level of hepatic URO was very similar to that found in rats treated with HCB alone (Figs. 12a, 16). After 45 days treatment with HCB and iron, the large increase in hepatic URO was mirrored by a large increase in urinary URO. The increase in urinary and hepatic URO at this time was more than twice that seen in rats treated with HCB alone. Kalivas et al[145], found that a single intraperitoneal injection of iron-dextran either before or after the onset of porphyria in HCB-treated rats did not result in porphyrinuria of greater degree than that found in HCB-fed uninjected animals, although the rats were siderotic

after approximately 56 days treatment with the toxin. Similar observations to those reported in this thesis as regards the effect of HCB and iron on urinary porphyrin excretion have been made by Taljaard et al[294].

A puzzling feature was noted in that in rats treated with HCB and iron simultaneously for 30 days, there was no stainable iron in the liver at a time at which rats given iron alone are markedly haemosiderotic on light microscopy. A similar observation was made by Taljaard et al[294] who showed that in rats rendered siderotic before HCB feeding, there was a uniform distribution throughout the liver lobules, whereas following HCB ingestion, hepatic iron was depleted in the centrilobular regions which are the first areas to show intense red porphyrin fluorescence. Further, it was observed in the present study that after 30 days total hepatic iron was increased more than two-fold in rats treated simultaneously with HCB and iron when compared with untreated rats, with no change from the increased level up to 60 days treatment. A similar increased level of hepatic iron was observed in a rat treated with iron alone. No significant increase in total hepatic iron content was observed in rats treated with HCB alone, which is at variance with the findings of Saunders et al[250], who reported that HCB feeding resulted in the accumulation of iron in the liver.

There appear to be two possible explanations to account for the absence of stainable iron in the livers of rats treated simultaneously with HCB and iron, at a time when the total hepatic iron content is elevated. Firstly, it is possible that HCB or HCB metabolites interfere with the stain. The possible interference of HCB could be checked in vitro by addition of the staining reagents to a suspension of HCB in

aqueous Tween 20 containing ferric iron. Interference by HCB metabolites would be more difficult to ascertain, since it is quite possible that these may be strongly bound in the liver. Secondly, hepatic iron may be in the form of ferritin and thus not detected after staining with Prussian blue. This could be checked by subjecting the liver to electron microscopy, which might reveal the presence of ferritin. This second possibility lends itself to an attractive hypothesis to explain the absence of haemosiderin when total hepatic iron is elevated in HCB and iron-treated rats. Ferritin appears to be the precursor of haemosiderin that is formed when the rate of iron deposition exceeds the rate of apoferritin formation[225]. HCB or a metabolite might act in two ways, firstly to increase the rate of synthesis of apoferritin such that no haemosiderin is deposited, and secondly to increase mobilisation of stainable iron. The finding of Taljaard et al[294] of stainable hepatic iron depletion in the centrilobular areas in the HCB-fed rats previously rendered siderotic, might well be explained on this basis if some further considerations are taken into account. It was demonstrated by Burger and Herdson[17] that in the case of phenobarbital-induced rat liver, the centrilobular hepatocytes were the first to be affected by the drug, with the characteristic cellular changes consisting of proliferation of the smooth endoplasmic reticulum progressively involving more peripheral cells, such that after 3 days phenobarbital administration, the inner half of the liver lobule is profoundly transformed whereas the outer half is virtually unchanged[199]. HCB has been shown to cause an increase in smooth endoplasmic reticulum in rats treated with this toxin[290]. If the induction of microsomal proteins such as hepatic cytochrome P450 is taken to be indicative of proliferation

of smooth endoplasmic reticulum[45, 49,87,93,140,141,215, 236] then HCB exerts its inductive effect more slowly, if it is considered that only after 20 days intoxication is there any marked increase in the levels of this haemprotein, as shown in Section 6.4(e) of this thesis. Thus it might be expected that if HCB or a metabolite acted in such a way as to increase the mobilisation of stainable iron in the liver, the centrilobular areas would be the first areas to show a depletion in the quantity of stainable iron i.e. the quantity of haemosiderin, once the more readily available stored iron as ferritin became utilised in haem synthesis. It is recognised that in the treatment of rodents simultaneously with HCB and iron in the long term, a maximal rate of synthesis of apoferritin might be attained, such that iron deposition as haemosiderin might occur. It is suggested that in view of the above considerations, further research into the effect of HCB on iron metabolism is warranted in order to understand more completely the mode of action of the toxin.

The excessive hepatic synthesis and urinary excretion of URO and 7-COOH porphyrin in the HCB-treated rat is highly indicative of an impaired ability to decarboxylate UROGEN to COPROGEN, a reaction catalysed by UROGEN decarboxylase. Previously, Taljaard et al[295,296] found no diminution in the activity of this enzyme in HCB-treated rats, whereas in rats previously rendered siderotic with iron-Dextran, 55 days feeding almost abolished hepatic UROGEN decarboxylase activity. In the present investigation, it was found that 30 days treatment with HCB or HCB simultaneously with iron did not significantly affect the activity of the enzyme, but at 45 days and at 60 days there was a significant decrease in the activity of UROGEN-I decarboxylase in both

groups of rats when compared to control animals. Recently, other workers have similarly found a decrease in HCB-fed rats[80,81,245]. Elder et al[80,81] have reported a progressive decrease in the activity of UROGEN decarboxylase during feeding with HCB, using as substrate 5-COOH porphyrinogen on the assumption that a single enzyme is responsible for the 4 successive decarboxylations of UROGEN[96,301].

In the present investigation, on comparing the activity of UROGEN-I decarboxylase (Fig. 36) with urinary porphyrin excretion (Figs. 12a,b), it appears that the activity of the enzyme is inversely related to porphyrin excretion. Furthermore, if a single enzyme is responsible for the stepwise decarboxylation of UROGEN to COPROGEN, as the activity of UROGEN decarboxylase decreases, it was found in both the HCB- and HCB plus iron-treated rats, the porphyrins corresponding to the substrate for, and intermediates of the reaction catalysed by the enzyme become more prominent in the urine (Fig. 13). From these results, it is suggested that the HCB-induced porphyria is due to the decrease in the activity of this enzyme.

The failure of Taljaard et al[295,296] to demonstrate a decrease in UROGEN decarboxylase in rats treated with HCB for 55 days is at variance with the results presented in this thesis. These workers emphasised the focal nature of early HCB porphyria and suggested that the normal enzyme activity they observed in HCB-treated rats might represent the sum of decreased activity in porphyric hepatocytes and compensatory increased activity in surrounding unaffected cells[143]. Alternatively, the differences between the present findings and those of Taljaard et al[295,296] may be related to differences in sensitivity of the methods employed for the measurement of UROGEN-I decarboxylase. Furthermore, these workers were unable to measure the activity of the

enzyme in their HCB and iron-treated rats[295,296] whereas the present investigation revealed reduced enzyme activity. This may well be due to the fact that in the method used for the assay of UROGEN-I decarboxylase in this study, reaction products other than COPRO can be demonstrated, viz. 7-, 6- and 5-COOH porphyrinogens.

It was found in this study that large amounts of URO and 7-COOH porphyrin accumulated in the livers both of rats treated with HCB and those treated simultaneously with HCB and iron. A decrease in the activity of hepatic UROGEN-I decarboxylase would be expected to lead to an accumulation of porphyrinogens. If the rate and pattern of urinary and faecal porphyrin excretion is compared to the rate and pattern of hepatic porphyrin accumulation in the HCB-treated rat, then it becomes apparent that the liver selectively retains a proportion of the more hydrophilic porphyrins. Thus, the maximum combined rate of hepatic URO and 7-COOH accumulation found in this study was approximately 28µg/g liver/15 days, or about 19µg/24 hours (Fig.16, Table 4), if the weight of rat liver is taken to be 10g. If the method used for the recovery of URO and 7-COOH porphyrin from the liver wereonly 10% efficient, instead of the 60% efficiency of recovery of URO found (Section 6.12), the maximum combined rate of accumulation of these porphyrins would be about 190µg/24 hours. Elder et al[80] have indicated that in HCB-treated rats, total urinary and faecal porphyrin excretion amounts to some 1,3 to 1,9 mg/24 hours, which includes large quantities of 4-, 5- and 6-COOH porphyrins, as well as URO and 7-COOH porphyrin. Thus it is clear that the porphyrins stored in the liver do not represent the pattern of overproduction of intermediates in HCB-induced porphyria in the rat. Moreover, storage of porphyrin is compatible with initial accumulation as porphyrinogens

followed later by oxidation.

On the basis of the observation that there is little difference in the decreased activity of hepatic UROGEN I decarboxylase after 45 and 60 days of treatment with either HCB alone or HCB simultaneously with iron (Fig. 36), it appears unlikely that iron accelerates the onset of porphyria in the rat if the manifestations of HCB porphyria are attributed to a decreased activity of UROGEN decarboxylase. It is suggested that iron potentiates the effect of HCB as evidenced by increased urinary porphyrin excretion and increased hepatic porphyrins in rats treated simultaneously with HCB and iron, when compared to rats treated with HCB alone (Figs. 12, 16). Yet there are other considerations to be taken into account. Kushner and associates[161] have reported that when iron is added to mitochondria-free crude liver extracts in vitro, an increased amount of porphyrin is synthesised, and in a later investigation, Kushner et al[162] found the activity of hepatic UROGEN decarboxylase to be decreased in liver extracts preincubated with ferrous iron. Further, the findings of Shanley et al[266] of urocoproporphyrinuria in experimental siderosis in the rat is indicative of increased porphyrin production. As shown in the present study, there appears to be a delay before the onset of porphyria in the HCB-fed rat. This might represent the time taken to accumulate a toxic dose of HCB, if this is indeed the porphyrogenic agent, or alternatively, the time taken for the formation of porphyrogenic metabolites of HCB[183] if it is considered that HCB appears to be a slowly metabolised toxin which has been reported to be virtually inert in rabbits[217]. It is possible then that at an early stage of HCB intoxication in rats treated simultaneously with iron, a very low concentration of HCB metabolite(s) may potentiate the effect of iron on

porphyrin synthesis in vivo, and conversely, iron might potentiate the porphyrogenic effect of very low concentrations of HCB metabolite(s). If the liposome model of Kószó et al[155] is invoked, where HCB increases membrane permeability, loss of intermediates in haem synthesis might occur, so that initially on treatment with HCB together with iron, increased excretion of porphyrin might be observed when compared to the case of rats treated with HCB alone.

If the manifestations of HCB porphyria are direct consequences of decreased UROGEN decarboxylase activity, it may be important to establish the mechanism whereby HCB or porphyrogenic metabolites exert their effects. The simplest explanation of the results presented here would be that a metabolite of HCB directly inhibits the activity of UROGEN decarboxylase.

Reports have been published which suggested that overproduction of ALA is the primary defect accounting for the biochemical abnormalities in all the hepatic porphyrias[109,147,205,305]. Thus, it was possible that diminished haem biosynthesis, with derepression of the rate-limiting enzyme ALA-S, might account for the elevated levels of URO and COPRO excreted in patients with PCT[219]. However, it may also be expected that a modest, barely discernable increase in ALA-S activity would similarly account for porphyrin overproduction in PCT, according to the deductions of Kaufman and Marver[149], in which case another enzymatic step may become rate-limiting, i.e. at the UROGEN synthetase level. The activity of hepatic ALA-S in patients with PCT has been measured by a number of investigators[66,149,168,204,283,285,328] but conflicting results have been published. However, in some of these studies[66,168,204,328], the enzyme assay used was limited by the small quantity of liver obtained, resulting in an inability to separate enzymatically

generated ALA quantitatively from another enzymatically generated aminoketone, aminoacetone. Further characterisation of ALA is crucial since normal and porphyric liver mitochondria contain aminoacetone synthetase, with the product of the reaction, aminoacetone, giving the same colour reaction with Ehrlichs reagent as ALA[305,310]. Moreover, under the conditions of the assay used, human liver produces about five times more aminoacetone than it does ALA [305], and it has been found that in nonporphyric subjects there is a substantial variation in the activity of aminoacetone synthetase[305]. It is possible then, that under certain physiological or pathological conditions, the activity of this enzyme may be elevated. It is for these reasons that on measuring the activity of ALA-S in liver, it is absolutely imperative that ALA and aminoacetone are separated before quantitation.

In view of the contradictory results obtained for the activity of hepatic ALA-S in PCT, a highly specific radiochemical assay which discriminates ALA from other aminoketones like aminoacetone, was adapted from the method of Strand et al[285] to measure ALA-S activity in the livers of patients with PCT and nonporphyrics. Two modifications to the method of Strand et al[285] were made. Firstly, dithiothreitol was used in place of glutathione in the incubations since better reproducibility was obtained with the former. It is possible that the commercial glutathione preparation used in some of the preliminary experiments contained an impurity which interfered in the assay. Secondly, a lower concentration of succinate including {1,4 - ^{14}C} - succinate was employed, resulting in a high specific activity succinate as substrate. It is recognised that this may not be the optimal substrate concentration for maximal incorporation of radioactive succinate into ALA[134,285], but use of a lower specific activity substrate under the conditions of the assay employed in this study resulted in radioactivity recovered as

^{14}C-ALA being only slightly greater than that found in the succinate blanks, using both human and rat liver as sources for ALA-S. In a comparative study, such as that in the present investigation, low radioactive counts may have resulted in data that might have been difficult to interpret. The assay method used in this work was valid on three counts: Firstly, the production of ^{14}C-ALA increased linearly with increasing amounts of rat liver up to 2 mg. Secondly, reproducible results are obtained as evidenced by triplicate analysis in close agreement (within 10%). Thirdly, using the radiochemical microassay, it was demonstrated that the activity of ALA-S was increased in rats treated with AIA, which is in agreement with the findings of other investigators[2,20,191,249] who in general measured ALA production from approximately 250 mg liver using colorimetric methods.

The activity of hepatic ALA-S was not found to be significantly different in PCT and nonporphyric controls. This supports the data of Strand et al[283,285] and Kaufman and Marver[149], but is at variance with the results of Dowdle et al[66], Levere[168], Moore et al[204] and Zail and Joubert[328]. Three possible explanations for the disparity of results have been considered. Firstly, the difference may be due to the fact that different methods of assay were used: in this study, those of Strand et al[283,285] and Kaufman and Marver[149] ALA was discriminated from aminoacetone. In other reports, total aminoketone production was assumed to represent ALA. The selection of nonporphyric controls may offer a second explanation for the disparity as the majority of patients in this group had alcoholic liver disease. Alcohol has been shown to stimulate ALA-S activity in rodents [265,266], which may explain in part why the hepatic ALA-S activity in the non-porphyric patients was greater than that observed by other

investigators. Indeed, Shanley et al[265] have intimated that ALA-S activity may be normal in patients with PCT if they have not recently imbibed alcohol. Finally, a modest increase in hepatic ALA-S, not significantly greater than normal, may adequately account for increased porphyrin excretion in PCT, according to the calculations of Kaufman and Marver[149].

An unexpected finding in PCT was the highly significant elevation of the levels of hepatic cytochrome P450, when compared to those found in nonporphyric controls and in other hepatic porphyrias such as variegate porphyria and protoporphyria. It may have been anticipated that a "block" in PROTO and haem biosynthesis might account for the pattern of porphyrin excretion in this condition. PCT is the only pathological condition in which the levels of hepatic cytochrome P450 are significantly elevated.

There appear to be three possible explanations to account for the elevated levels of cytochrome P450 in PCT. Firstly, the result may be due to an artefact. Secondly, increased amounts of cytochrome P450 may reflect increased hepatic synthesis of haem and cytochrome P450 apoprotein, or increased formation of cytochrome P450 apoprotein with diversion of an already depleted haem pool to apoprotein. The third possibility is that there may be stabilisation of cytochrome P450 with decreased degradation.

It appears unlikely that the elevated levels of cytochrome P450 found in PCT are due to an artefact. If the increase in the levels of the haemoprotein was due to the formation of a complex which resembled cytochrome P450 in spectral properties, then it might be surmised that in view of the hepatic accumulation of, principally, URO[23,64,65] and the almost invariable finding of hepatic siderosis in PCT[143,144,161, 164,186,250,307,311], URO might combine with haemosiderin to yield

that complex. However on testing this possibility by measuring cytochrome P450 levels in siderotic rat livers in the presence of URO I, such that the URO I concentration corresponded to up to 500µg/g liver, the levels of this cytochrome were not increased.

The possibility that the measurement of levels of hepatic cytochrome P450 in whole liver homogenates, the technique used in the studies on PCT and nonporphyric livers, does not give a true reflection of microsomal levels of this haemoprotein, was discounted by a number of observations. In the first place, levels of hepatic cytochrome P450 were compared between patients with PCT and nonporphyric controls, using approximately the same concentrations of liver in the determinations. Secondly, using rat liver homogenates, a linear increase in levels of the haemoprotein were found with increasing concentrations of liver up to 10 mg. All determinations of levels of hepatic cytochrome P450 were made within this linear range. Thirdly, consistent results using liver homogenates were obtained. Finally, it has been reported that the levels of cytochrome P450 are elevated in the hepatic microsomes of rats treated with HCB[175,231,282,290,308] and in those of rats treated with phenobarbital[127,215,237]. In the present investigation, levels of hepatic cytochrome P450 as measured in microsomes and in homogenates from the same rats which were either untreated, treated with HCB for 45 days, or treated with phenobarbital, were compared. Significant increases in the levels of the haemoprotein in rats treated with HCB and phenobarbitone when compared to untreated rats were found in both microsomes and in homogenates and furthermore, these increases were of the same order of magnitude by both methods of determination (Table 8).

It was observed that in rats treated with HCB, levels of cytochrome

were significantly increased after 20 days with an increase after 30 days, then further increases were noted at 45 and 60 days treatment. Stonard and Nenov[282] have reported an initial fourfold rise in levels of cytochrome P450 after 10 days HCB treatment followed by a decrease with the levels increased about twofold above that of the controls at 15 days. The cytochrome P450 content remained at this level up to 30 days. Rajamanickam et al[231] showed a striking increase in the amount of cytochrome P450 during the initial 3 days of HCB treatment with a smaller increase over and above this after 15 days. These workers found that the level of the haemoprotein remained constant for the remainder of the treatment period, i.e. up to 29 days. Lissner et al[175] have studied the effect of HCB-feeding for up to 100 days on hepatic cytochrome P450 and have observed similar results to those reported in this thesis, i.e. an initial increase, a plateau where the levels remained relatively constant, followed by a further increase in the levels of the haemoprotein.

In this investigation it was observed that 45 days feeding with HCB resulted in elevated levels of cytochrome P450 (Fig. 23), decreased UROGEN decarboxylase activity (Fig. 36), elevated urinary porphyrin excretion, with more URO than COPRO excreted by this route (Fig. 12), significant levels of the porphyrin P_1 fraction in the faeces (Fig. 14) and the accumulation of virtually exclusively URO and 7-COOH porphyrin in the liver (Fig. 15). These data are consistent with the findings of increased hepatic cytochrome P450 (Fig 22, Table 7), the porphyrin excretory and accumulation pattern (Fig. 11, Table 3), and the diminished hepatic UROGEN-I decarboxylase activity as reported by Kushner et al [159,160] in human PCT. The observations that levels of hepatic cytochrome P450 were increased in both PCT and in HCB-treated rats despite

the deficiency in the liver of UROGEN-I decarboxylase[159,160,this thesis] suggest that the haem pool in these conditions is initially not rate limiting in the formation of the microsomal haemoprotein. This would correlate well with data[52] which imply that the primary and rate-limiting event during induction of cytochrome P450 is the induction of its apoprotein, and moreover that apo-cytochrome P450 may control the rate of haem biosynthesis under these conditions[219]. Thus in a situation in which apo-cytochrome P450 might have accumulated, haem precursor overproduction and excretion could be expected to accompany accelerated formation of cytochrome P450 when haem synthesis is impaired.

Yet there are other data that should be considered. It has been shown in this thesis that the activity of hepatic ALA-S in PCT was not significantly different from that found in nonporphyric controls, although levels of cytochrome P450 were significantly elevated in PCT. There have been reports which have indicated conditions where amounts of cytochrome P450 increase without any change in ALA-S activity: in neotatal rats administration of phenobarbital increases the levels of cytochrome P450 without affecting ALA-S activity[278]; in fed adult rats, phenobarbital brings about a similar effect[24]. Further, Rajamanickam et al[231] have reported that initially on feeding rats with HCB, the rate of cytochrome P450 synthesis is greater than that of total haem synthesis, which indicates that even under conditions where an enhanced rate of total haem synthesis is not discernable, there may be a significant channelling of the available haem towards cytochrome P450. It was suggested that the primary effect of HCB was to enhance the rate of apo-cytochrome P450 synthesis[231]. The above

situations where the levels of cytochrome P450 increase without any change in ALA-S activity were in rats that were non-porphyric. When the animal becomes porphyric, it may be expected that the liver is required to synthesise more porphyrins to achieve the same level of haem, and a modest increase in ALA-S activity might be anticipated. Rajamanickam et al[231] have found this to be the case and have reported a small increase in the activity of ALA-S, as HCB porphyria in the rat develops.

If in both PCT and HCB-porphyria there is accumulation of apocytochrome P450, a situation might arise where thereis an increase in the levels of cytochrome P450 as haem is drawn from the regulatory haem pool. If the postulates of Bissell and Hammaker[20] are considered, where the regulatory hepatic haem pool is fed by both endogenous haem synthesis and dissociation of haem from cytochrome P450 prior to its degradation, then initially there may be derepression of ALA-S, leading to an increase in the activity of the enzyme. This increase need be only modest to account for increased haem synthesis. With enhanced haem synthesis the regulatory haem pool would become augmented and further haem would be provided for cytochrome P450 synthesis. The overall result would be a further increase in cytochrome P450, assuming there is still excessive apocytochrome P450 present. The haem moiety of cytochrome P450 would feed the regulatory haem pool with concomittant repression of ALA-S activity. The possibility exists that in PCT and HCB-porphyria there may be continual induction of apocytochrome P450, so that the pattern outlined above is repetitive, which may account for the increased cytochrome P450 levels noted in PCT and the continual increment of the haemoprotein observed in HCB-treated rats.

It is unlikely that as suggested recently by Elder et al[81], the fall in UROGEN decarboxylase activity in HCB-treated rats initially limits the rate of haem synthesis to decrease hepatic haem concentrations, so that there is a derepression of ALA-S activity, with consequent increased production of haem precursors to maintain haem synthesis at the required level. The increasing levels of cytochrome P450 during HCB-feeding found in this study and that of Lissner et al[175] suggests that there is in fact accelerated haem synthesis. It is suggested that in both PCT and HCB-porphyria, the decrease in UROGEN decarboxylase activity is an event which follows accelerated haem synthesis. Thus, under these conditions there may be overproduction of the initial and intermediate substrates for UROGEN decarboxylase to maintain the rate of COPROGEN formation - and hence haem synthesis - at an elevated level when the activity of UROGEN decarboxylase is decreased. Recently, Elder et al[82] have emphasised the importance of the consideration of the probable relative activities of the enzymes of haem biosynthesis. These workers have suggested that in hereditary coproporphyria, where the activity of COPROGEN oxidase is decreased, that normal rates of hepatic haem synthesis would be maintained if the concentration of substrate i.e. COPROGEN III, normally lower than the apparent K_m value, is increased, so that the rate of formation of product approached its maximum. The required increase in COPROGEN III concentration may be achieved at the expense of increased loss of this intermediate from the pathway. It is suggested that in this situation the activity of UROGEN-I synthetase may become rate limiting, if it is considered that the activity of the enzyme is so low in human liver (0,018 nmol porphyrin formed/h/mg protein[283] compared with the basal rate of haem synthesis of 0,016 nmol/h/mg protein[82]) that the rate of

UROGEN formation prevents compensation of the COPROGEN oxidase defect by substrate accumulation when the demand for haem synthesis is increased. It has been suggested[82] that three factors determine whether an enzyme defect distal to UROGEN synthetase can be compensated by an increase in substrate concentration when hepatic haem synthesis is stimulated: the activity of the defective enzyme, the relationship between the substrate concentration and the K_m value, and the extent to which substrate concentration can be increased without enhancing loss from the pathway. If it is considered that in PCT the activity of the defective enzyme, UROGEN decarboxylase, is high (0,2 - 1,0 nmol porphyrin formed/h/mg protein[159,160]) relative to the requirement for haem synthesis, with a slow rate of loss of UROGEN from the hepatocyte[273], increases in substrate concentration can occur, so that the rate of formation of product is increased.

On spectral analysis, the carbon monoxide-binding haemoprotein in both PCT and HCB-porphyria showed apparent peaks at 448 nm (Section 6.5.), which is similar to the microsomal cytochrome induced by the polycyclic aromatic hydrocarbon 3-methylcholanthrene[6,274]. Further definitive spectral characterisation of the haemoprotein in PCT was hampered by the limited amount of liver tissue available for study. Study of the ethyl isocyanide binding spectra of hepatic microsomes from untreated and HCB-treated rats revealed that the 455 nm and 430 nm peaks were of equal height at pH 7,52 for the untreated rats and at pH 7,28 for the HCB-treated rats. Lui et al[183] have reported a value of pH 7,52 in phenobarbital-treated rodents, and pH 7,42 in rats treated with HCB for 35 days. The results obtained in the present study are interpreted to indicate a greater admixture of a spectrally distinct haemoprotein, cytochrome P448, which is formed during induction

with HCB. The carbon-monoxide difference spectra in PCT suggest the possibility of a similar greater admixture of P448.

The functional nature of the increased cytochrome P450 was explored by examination of the ability of liver tissue from patients with PCT and rats treated with HCB for 45 days, a time at which HCB porphyria in the rodents closely resembled biochemically, human PCT (Figs. 11, 12b,13,14,15,16,22,23,24) to metabolise drugs in vitro. The metabolism of aminopyrine and 3,4-benzpyrene were studied, since it has been shown that cytochrome P450, which is induced by phenobarbital, enhances the metabolism of both aminopyrine and 3,4-benzpyrene, whereas cytochrome P448 which is induced by 3-methylcholanthrene, enhances the metabolism of 3,4-benzpyrene but not that of aminopyrine[105]. In vitro drug metabolism studies were performed using liver homogenates in place of hepatic microsomes as the source of the hepatic mixed function oxidase system. These studies were performed with quantities of liver which were within the range where the rate of product formation increased linearly with increasing liver concentration (Section 6.6.(a)).

In rats fed with HCB for 45 days, the mean apparent K_m for aminopyrine N-demethylation was about 3-fold with an approximate 2-fold increase in the maximal velocity, when compared to that observed in untreated animals. The apparent K_m for 3,4-benzpyrene hydroxylation was found to be increased about 4-fold and the maximal velocity increased approximately 25-fold in the HCB-treated rats when compared to the control animals. Alvares et al[5] have reported that in 3-methylcholanthrene-treated rodents, the apparent K_m for 3,4-benzpyrene hydroxylation by liver microsomes is decreased, whereas the maximal velocity for the reaction rises about 3-fold when compared to the values obtained

in control animals. The lower K_m obtained by these workers[5] suggests that the induced cytochrome P448[105] has a higher affinity for 3,4-benzpyrene, thereby differing from the naturally occurring cytochrome P450. The greater K_m values found for both aminopyrine N-demethylation and 3,4-benzpyrene hydroxylation in HCB-treated rats suggests the presence of an induced haemoprotein which has a lower affinity for both substrates. However, the K_m values are apparent K_m values and may be influenced by non-specific binding to proteins [102], which may be an important consideration as it has been shown [308] that in HCB-treated rats hepatic microsomal protein levels are increased. Moreover, nonspecific binding may in part account for the lower K_m for 3,4-benzpyrene hydroxylation found in this study in untreated animals when compared to that found by Alvares et al[5]. These investigators performed determinations using liver microsomes, whereas in the present investigation, liver homogenates were used. The increased maximal velocity values for both aminopyrine N-demethylation and 3,4-benzpyrene hydroxylation found in HCB-treated rats are not inconsistent with the induction of both cytochromes P450 and P448. The latter might be induced to a greater extent to account for the greater elevation in the maximal velocity for 3,4-benzpyrene hydroxylation in these animals and furthermore, studies of ethyl isocyanide-binding to the haemoprotein in HCB-fed rats indicate a greater amount of the spectrally distinct cytochrome P448.

An alternative possibility which might explain the kinetics of hepatic aminopyrine N-demethylation and 3,4-benzpyrene hydroxylation in HCB-fed rats may be that the toxin is responsible for the induction of a different form of cytochrome P450, which has different catalytic properties to those of either cytochrome P450 or cytochrome P448.

This mechanism has been proposed to account for the inductive effect of pregnenolone-16-α-carbonitrile pretreatment of rats[181].

In the studies on PCT liver tissue, however, no significant differences were observed in K_m on maximal velocity for hepatic 3,4-benzpyrene hydroxylation and aminopyrine N-demethylation when compared to similar in vitro data in non-porphyric controls (Figs. 28,29). The interindividual differences in enzyme kinetics exhibited a wide range, which is consistent with other studies[55,312,313]. Large differences in the rates of oxidation of ethylmorphine by human liver microsomes derived from different patients were reported by Davies et al[55]. It was further suggested by these investigators[55] that interindividual differences in the plasma half-lives of lipid soluble drugs such as antipyrine, phenylbutazone and oxyphenbutazone may be due at least in part to the varying activities of the cytochrome P450-dependent drug oxidising systems. Vesell and Page[312, 313] observed larger differences in the metabolic dispositions of phenylbutazone and antipyrine in fraternal twins when compared to that seen in monozygotic twins. Thus, genetic factors might make it impossible to distinguish small yet significant correlations between hepatic cytochrome P450 levels and drug metabolism in patients with PCT, variegate porphyria and protoporphyria. Indeed, Alvares et al[4] were unable to detect the expected retardation of the metabolism of phenylbutazone or antipyrine in lead-poisoned children, where haemsynthesis is inhibited, when compared to randomly selected controls. This was demonstrable only when the patients were used as their own controls i.e. by removal of the genetically determined variables. Treatment which reversed lead inhibition of haem synthesis resulted in an increase in the rate of plasma disappearance of the two drugs.

From the above observations it becomes evident that when studies are performed in a heterogeneous, unrelated group of people, difficulty is encountered when relating in vitro drug metabolism to increased hepatic levels of, or a different form of, cytochrome P450. It might be possible to use patients with PCT as their own controls to correlate drug metabolism with levels of cytochrome P450 by investigation of the parameter before and after phlebotomy, when no disturbance of porphyrin metabolism is evident. However, repeated biopsy cannot be justified under any circumstances in patients who have recovered and do not require liver biopsy for diagnostic purposes.

It might be expected that in view of the elevated levels of hepatic cytochrome P450 found in patients with PCT, proliferation of the smooth endoplasmic reticulum would be observed, since in experimental animals, induction of microsomal haemoproteins by HCB, phenobarbital, progesterone or other compounds, is associated with a marked proliferation of liver microsomes[45, 87, 93, 140,141,142,215, 236,290]. However, on comparing the amounts of smooth endoplasmic reticulum in the hepatocytes of patients with PCT with those found in variegate porphyria and protoporphyria, where there was no elevation in the levels of hepatic cytochrome P450 (Table 7, Fig. 22), no significant difference was observed[22,23]. The finding that the quantity of hepatic smooth endoplasmic reticulum in PCT varied from cell to cell, suggests that differences in the amounts found in this syndrome and in variegate porphyria and protoporphyria might become more apparent by electron microscopic mapping of large sheets of cells.

Light microscopy of the livers of patients with PCT showed similar damage to that which has been previously described[164,298,299,311]. Fluorescence microscopy indicated a patchy and diffuse distribution of

porphyrins in the liver samples, which confirms the findings of other investigators[88,143,279].

In the literature, there appears to be little evidence that PCT may be a purely inherited form of porphyria. The family study by Holti and associates[132], referred to in a review by Beattie and Goldberg[16] represents on closer scrutiny a typical instance of variegate porphyria and thus cannot be quoted to support the existence of hereditary PCT. It was suggested by Redeker[233] that cutaneous symptoms could be hereditary but this was discounted by Schmid in the same discussion on the basis of stool porphyrin analysis not being available. Later, Welland et al[324] described PCT in only one member of a pair of homozygous twins. Haegar-Aronson[120] has reported PCT in 3 members of one family, and another family in which 4 members were affected. Investigations by Eales[74] of 133 relations of 22 PCT patients revealed only one case of familial PCT involving a father and daughter, both alcoholics, and prior to this survey only 4 other instances of familial involvement have been documented in South Africa [74]. Of note in these investigations was the fact that no more than 2 members of one family were cases of PCT.

In 1974, it was suggested by Kushner[158] that idiosyncratic PCT might be an overt manifestation of an autosomal dominantly inherited defect in UROGEN decarboxylase activity. Diminished levels of activity of this enzyme were observed in hepatic tissue[159] and in red blood cells[158] of patients with PCT. In a more recent report, Kushner et al[160] have extended their studies in that multiple examples of decreased erythrocyte UROGEN decarboxylase activity were detected in asymptomatic members of families of patients with PCT. In addition, these workers[160] reported that the mean erythrocyte enzymatic activity

was greater in males than in females in both patients and in normal subjects. In the present investigation using similar assay conditions to those of Kushner et al[158,160] it was found that red blood cell UROGEN-I decarboxylase was not significantly different in non-porphyric controls when compared with patients with active PCT (Fig. 35). In the two family studies presented in this thesis where the propositi had overt PCT, there did not appear to be definitive evidence of a genetically determined deficiency of UROGEN decarboxylase (Fig. 34). In addition, the activity of the enzyme in erythrocytes did not appear to be influenced by the sex of the patients (Fig. 35).

In the assay for red blood cell UROGEN decarboxylase the activity of the enzyme is measured in the presence of a large excess of haemoglobin. Hennessey et al[126] have found an approximate 20-fold excess of haemoglobin over other soluble proteins in human red blood cell haemolysates. Thus, the excess of haemoglobin over UROGEN decarboxylase will be many-fold greater than this. It was considered that haemoglobin might interfere with the determination of red cell UROGEN decarboxylase. This possibility was checked in two ways. Firstly, the effect of increasing quantities of haemolysate,derived from the red blood cells of the author of this thesis, on the activity of erythrocyte UROGEN-I decarboxylase was investigated. A linear increase in enzyme activity was not observed (Fig. 39). Secondly, the effect of increasing quantities of electrophoretically pure human haemoglobin on the activity of rat liver UROGEN-I decarboxylase was investigated using a constant quantity of post-mitochondrial supernatant as enzyme source. The activity of the enzyme was not found to be constant under these conditions (Table 15). Thus it is suggested that under the conditions of assay for red blood cell UROGEN-I decarboxylase, there is non-specific interference by haemoglobin, and in view

of this both the results presented here and those of Kushner et al[160] should be interpreted with this in mind. Idiosyncratic PCT appears to occur sporadically in patients with common hepatic disorders[219] so that the proposal by Kushner et al[160] based on studies of the activity of erythrocyte UROGEN-I decarboxylase in the presence of an excess of haemoglobin, that PCT may be due to an inherited defect in UROGEN decarboxylase requires confirmation.

An attempt was made to measure human red blood cell UROGEN-I decarboxylase in haemolysates in which the haemoglobin was removed by the method of Hennessey et al[126]. However the results obtained were not consistent (Table 16). It must further be pointed out that when comparative studies of UROGEN-I decarboxylase activity are to be carried out in PCT and in nonporphyric patients on haemoglobin-free 32000xg supernatants, there may be varying recoveries of UROGEN decarboxylase after separation, so that definitive conclusions as to the activity of the enzyme may be difficult to reach. The observation, albeit on only 3 points, that the activity of rat red blood cell UROGEN-I decarboxylase increased in a linear fashion with increasing amounts of haemolysate (Fig. 40) indicates that the activity of the enzyme could be measured in highly diluted haemolysates. The haemoglobin content of rat liver haemolysates was low, owing to the fact that rat haemoglobin largely crystallizes out at pH6,8[19], the pH of the buffer added before haemolysis. If human red blood cell UROGEN-I decarboxylase were to be measured in diluted haemolysates using ^{14}C-UROGEN-I as substrate and in a range where the activity increased linearly with increasing enzyme concentration, the initial substrate concentration should be similar to that used in the present work, i.e. 25 - 30 µmol/litre. Dilution of the haemolysate

would result in very much reduced decarboxylation of UROGEN-I, so that decarboxylation in reagent blanks would of necessity have to be as close to zero as possible to detect small yet significant decarboxylation of the substrate by the erythrocyte UROGEN decarboxylase. Furthermore, in order to detect the anticipated reduced level of decarboxylation, the use of substrate of much greater specific activity would be necessitated. It is suggested that it might be possible to measure the enzyme activity in diluted haemolysates with no interference by haemoglobin, and moreover, to detect significant changes in activity in different situations by using pure synthetically prepared ^{14}C-UROGEN-I of high specific activity as substrate, rather than to generate ^{14}C-UROGEN-I just prior to use.

The augmentation of rat liver UROGEN-I decarboxylase activity in vitro by ferrous iron from concentrations of 0,01 to 2,00 mM has not been previously described (Fig. 37, Table 14). In addition, an increase in the activity of enzyme in the presence of 1,00 mM ferrous ammonium sulphate of a similar order of magnitude was observed in the livers of rats treated for 45 days with HCB, i.e. a situation where the activity of UROGEN-I decarboxylase is decreased (Table 13). The observed increase in enzyme activity was real, since corrections were made in each observation for the effects of the same concentrations of ferrous iron on non-enzymatic decarboxylations and on the production of ^{14}C-UROGEN-I substrate.

It is probable that ferrous iron added in vitro directly activates UROGEN-I decarboxylase. Neither the biologically occurring iron compound, ferritin, nor 1,10-phenanthroline, a ferrous chelator of high specificity[275], had any significant effect on the activity

of rat liver UROGEN-I decarboxylase both in untreated rats and rats treated for 45 days with HCB.

The finding that added iron in vitro enhances the activity of hepatic UROGEN-I decarboxylase is at variance with the findings of Kushner et al[162] who have reported inhibition of the enzyme in porcine liver extracts preincubated with ferrous ammonium sulphate. The disparity between the results reported here and those of Kushner et al[162] may be due to a different methodological approach since in the present work, ferrous iron was added at the beginning of the assay, and furthermore, these investigators[162] did not indicate whether further substrate generation occurred during the course of incubation with crude liver extracts in the presence of ferrous iron.

Results obtained during the course of investigations into the activity of hepatic UROGEN-I decarboxylase indicated that the measured activity when reaction products are extracted onto talc does not yield an absolute value for the activity of the enzyme, owing to a differential in the extractability of porphyrin esters from talc. In the alternate method described in section 6.12 where the reaction products of UROGEN decarboxylation were first oxidised by light, followed by esterification with a large volume of 5% sulphuric acid in methanol with subsequent exhaustive extraction of the porphyrin methyl esters from the esterification mixture of chloroform, two assumptions were made. First, it was assumed that equal proportions of each porphyrinogen were oxidised to the corresponding porphyrin by the action of light. Second, it was assumed that equal proportions of the porphyrins in aqueous samples were esterified in the presence of a twenty-four-fold excess of 5% sulphuric acid in methanol. Day[54]

in PCT[158,159] was not confirmed by investigation of the activity of the enzyme in red blood cells; neither was a deficiency in the activity of the enzyme detected in red blood cells of patients with PCT. However, the assay system for the determination of UROGEN-I decarboxylase activity in red blood cells suffers a serious shortcoming in that the large excess of haemoglobin interferes in the assay in a non-specific manner. It is suggested that the activity of the enzyme should be reinvestigated in PCT, either in haemoglobin-free haemolysates, or in diluted haemolysates along the lines suggested previously,or in other extra-hepatic tissue e.g. skin fibroblasts, before any definitive statements can be made about an inherited defect.

The role of iron in the genesis of PCT is a complicated one. It is possible that in PCT,as suggested from the studies on the development of porphyria in the rat treated with HCB and iron, the effect of iron may be to potentiate the porphyric process. Hepatic iron may interfere with UROGEN cosynthetase activity, and additionally more UROGEN might be synthesised, if the in vitro findings of Kushner et al[161] are extrapolated to the in vivo situation. It is further possible that the effect of mobilisation of storage iron by venesection may be to remove this effect and additionally increase the concentration of ferrous iron. The latter might activate UROGEN decarboxylase which has been found to be decreased in PCT[159,160], if the in vitro finding of augmentation of the activity of the enzyme by ferrous iron reported in this thesis is extended to man, such that biochemical remission occurs. It may be worthwhile to investigate the effect of bleeding on the activity of hepatic UROGEN decarboxylase and porphyrin overproduction in rats treated simultaneously with HCB and intramuscular

iron to test this possibility . Additionally, if hepatic iron inhibits UROGEN cosynthetase activity[161], the overproduction of predominantly the isomer series I porphyrins would be expected, so that it may be important to establish the nature of the isomer type in siderotic rats treated with HCB.

The development of PCT should be viewed in relation to the levels of hepatic cytochrome P450, which are increased in this disease[this thesis, 22,23,220,221,222]. It is an interesting possibility that the primary event in the genesis of the syndrome may be the induction of apo-cytochrome P450 which may be consequential to liver insult or injury. This would lead to increased cytochrome P450 formation and increased haem synthesis, as haem from the regulatory pool is channelled towards cytochrome P450. Moreover, there may be an over-response for the increased demand for haem, resulting in haem precursor overproduction and excretion. Spectral studies on the binding of carbon monoxide to the induced hepatic cytochrome P450 tentatively suggest that both in PCT and in HCB-treated rats a similar alternative form of the haemoprotein may be induced. Further definitive studies were possible only in the HCB-intoxicated rat, where ethyl isocyanide-binding studies indicated a greater admixture of a spectrally distinct haemoprotein. If the same form of cytochrome P450 is induced in PCT as in the HCB-fed rat, then by inference the induction of a different apoprotein to that found in untreated rats and in control patients, is indicated. It is interesting to speculate that this induced apocytochrome may directly inhibit UROGEN decarboxylase or inhibit its synthesis, to result in the porphyrin overproduction pattern seen in PCT. Hepatic iron may serve to potentiate porphyrin overproduction to produce the overt symptoms.

If the manifestations of PCT are considered secondary to the induction of a different form of apocytochrome P450, then it is possible that only in some susceptible patients will this apoprotein be induced when there is injury or insult to the liver. This may form a basis for explanation of the sporadic occurrence of PCT.

Finally, it is hoped that this thesis has contributed towards the understanding of the metabolic basis for PCT, and will stimulate further research in this intriguing field.

APPENDIX A

METHODS OF CALCULATION

1. Hepatic ALA-S Activity

$$\text{nmol ALA formed/h/g liver} = \left[\frac{abg}{1{,}1 \times 10^5 \, (c-a) \, efh} - k \right] \frac{1}{j} \times 10^6$$

Where
- a = counts per min in sample without internal standard
- b = disintegrations per min of added internal standard
- c = counts per min in sample after addition of internal standard
- e = volume (ml) of final eluate taken for counting
- f = absorbance at 556 nm of final eluate with Ehrlich mercury reagent
- g = absorbance at 556 nm of standard ALA-pyrrole with Ehrlich mercury reagent
- h = specific activity of {1,4 - ^{14}C} - succinic acid
- j = mg liver tissue present in assay
- k = $\frac{abg}{1{,}1 \times 10^5 (c-a) efh}$ for succinate blanks

2. Hepatic Cytochrome P450 Levels with Carbon Monoxide as Ligand

(i) $$\text{nmol P450/g liver} = \frac{(A_{450} - B_{450}) - (A_{490} - B_{490}) \times 10^6}{91 \times a}$$

(ii) $$\text{nmol P450/mg protein} = \frac{(A_{450} - B_{450}) - (A_{490} - B_{490}) \times 10^3}{91 \times b}$$

Where
- A_{450} = absorbance at 450 nm in the difference spectra
- B_{450} = absorbance at 450 nm in the baseline
- A_{490} = absorbance at 490 nm in the difference spectra
- B_{490} = absorbance at 490 nm in the baseline
- a = concentration of liver in cuvettes (mg/ml)
- b = concentration of protein in cuvettes (mg/ml)

3. Hepatic Aminopyrine N-Demethylation

nmol formaldehyde semicarbazone formed/hr/mg liver

$$= \left[\left(\frac{a\ b\ d}{e(c - a)} \right) - f \right] \quad 4{,}9282 \times 10^{-3} \quad \left[\frac{(h + g)}{g\ j\ k} \right]$$

Where a = counts per min in sample without internal standard
b = disintegrations per min of added internal standard
c = counts per min in sample after addition of internal standard
d = volume (ml) of aqueous phase
e = volume (ml) of aqueous phase taken for counting
$f = \left(\frac{abd}{e\ (c-a)} \right)$ for sample blank
g = nmol {dimethylamino - ^{14}C} aminopyrine in incubation mixture
h = nmol non-radioactive aminopyrine in incubation mixture
j = specific activity of {dimethylamino - ^{14}C} aminopyrine
k = mg liver tissue present in assay

4. Hepatic 3,4-Benzpyrene Hydroxylation

ng 3-hydroxy-3,4-benzpyrene formed/hr/mg liver

$$= \frac{165{,}0171\ (T_1 - T_2)}{\omega}$$

Where T_1 = transmission reading for sample on 0,1 scale
T_2 = transmission reading for sample blank on 0,1 scale
ω = mg liver tissue used in assay

5. UROGEN-I Decarboxylase Activity

(i) nmol UROGEN-I decarboxylated/hr/mg haemoglobin

$$= \left(\frac{b}{a+b} - \frac{d}{c+d} \right) \frac{2e}{f}$$

(ii) nmol UROGEN-I decarboxylated/hr/mg protein

$$= \left(\frac{b}{a+b} - \frac{d}{c+d} \right) \frac{2e}{g}$$

Where a = counts per min in URO ester spot

b = total counts per min in 7-, 6-, 5- and 4- COOH porphyrin ester spots

c = counts per min in URO ester spot for blank

d = total counts per min in 7-, 6-, 5- and 4-COOH porphyrin ester spots for blank

e = nmol UROGEN-I produced during assay = $\frac{\text{Absorbance at 560nm}}{.007554}$

f = mg haemoglobin present in assay

g = mg protein present in assay

6. Concentration of Porphyrin Esters in Chloroform

$$\text{g porphyrin ester/liter} = \frac{\text{Dcorr} \times \text{M.W.}}{k_i \times \varepsilon}$$

Where Dcorr = $2 A_{max} - (A_{380} + A_{430})$ with A_{max} the absorbance maximum between 400 and 410 nm, A_{380} the absorbance at 380 nm and A_{430} the absorbance at 430 nm.

M.W. = molecular weight of porphyrin ester

k_i = absorbance correction factor

ε = molar extinction coefficient

Values used for M.W., k_i and ε are given in Table 17 for the esters in chloroform[289].

TABLE 17

FACTORS USED FOR CALCULATION OF PORPHYRIN ESTER CONCENTRATION IN CHLOROFORM

Ester	M.W.	k_i	ε
URO	943	1,91	$2,15 \times 10^5$
COPRO	711	1,496	$1,8 \times 10^5$
PROTO	590	1,4043	$1,71 \times 10^5$

7. Urinary COPRO and URO (289)

(i) $\mu g\ COPRO/ml = \left[2\ A_{max} - (A_{380} + A_{430}) \right] \times \frac{0,932a}{b}$

(ii) $\mu g\ URO/ml = \left[2\ A_{max} - (A_{380} + A_{430}) \right] \times \frac{0,837c}{b}$

Where

A_{max} = absorbance maximum between 400 and 410 nm

A_{380} = Absorbance at 380 nm

A_{430} = Absorbance at 430 nm

a = total volume(ml)of 1,5N HCl extracts

b = volume (ml) of urine taken for analysis

c = total volume (ml) of 2% HCl extracts

8. Statistical Methods (277)

(i) The mean $(\bar{x})$ is given by

$$(\bar{x}) = \frac{\Sigma x}{n}$$

Where

Σx = sum of all observations in the sample population

n = number of observations

(ii) Standard deviation (s) is given by

$$s = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n - 1}}$$

Where Σx^2 = the sum of square of all observations in the sample population

$(\Sigma x)^2$ = square of the sum of all observations in the sample population

(iii) Standard Error (S.E.) is given by

$$S.E. = \frac{s}{\sqrt{n}}$$

(iv) The significance of difference between two mean values was ascertained using Student's t in a two-tailed test, with $n_1 + n_2 - 2$ degrees of freedom.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where $\bar{x}_1$ = mean value of observations in first sample population

$\bar{x}_2$ = mean value of observation in second sample population

S_1 = standard deviation for first sample population

S_2 = standard deviation for second sample population

n_1 = number of observations in first sample population

n_2 = number of observations in second sample population

The value of P was ascertained from tables.

(v) The coefficients of regression (a and b) in the regression line whose equation is $y = a + bx$ and the coefficient of determination (r^2) are given by

$$b = \frac{\Sigma xy - \frac{\Sigma x \, \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

$$a = \frac{\Sigma y}{n} - b\frac{\Sigma x}{n}$$

$$r^2 = \frac{\left[\Sigma xy - \frac{\Sigma x \, \Sigma y}{n}\right]^2}{\left[\Sigma x^2 - \frac{(\Sigma x)^2}{n}\right]\left[\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right]}$$

APPENDIX B

Chemical Reagents and Special Items Used in the Experimental Work, together with Suppliers

(All reagents were of the highest grade available, except where otherwise stated).

Afrox, Maitland, Cape:

Carbon Monoxide

Beckman Instruments:

Semi-microcuvettes, 1,5 ml capacity

Biorad Laboratories, 32nd and Griffin Avenue, Richmond, California:

Disposable polyethylene columns, 5,5 cm x 1,0 cm inside diameter, with reservoir 3,5 cm x 1,9 cm inside diameter.

British Drug Houses (B.D.H.), Poole, England.

Acetyl acetone
Ammonium acetate
L-Cysteine
Dichloromethane
EDTA (ethylene diamino tetra-acetic acid, disodium salt)
Ether (Laboratory Reagent)
Ethyl Acetate (Laboratory Reagent)
Ferric Chloride
Glycine
Mercuric Chloride
Petroleum Spirit (Petroleum ether) (Laboratory Reagent)
Quinine Sulphate
Sodium Arsenite
Sodium Thiosulphate
Talc fine powder
Tris (Tris(hydroxymethyl)methylamine)

Eastman Kodak Company, Rochester, N.Y. 14650 U.S.A.

Hexachlorobenzene (practical grade)

Hach Chemical Company, P.O. Box 907, Ames, Iowa, 50010, U.S.A.

Ferrozine

Hopkin and Williams Ltd., Chadwell Heath, Essex, England:

DL-Malic Acid

May and Baker, Dagenham, England:

- Acetic Acid (Glacial)
- Acetone
- Ammonia Solution S.G. 0,9 27% w/w Ammonia (Laboratory Reagent)
- Ascorbic Acid
- Carbon Tetrachloride (Laboratory Reagent)
- Chloroform
- Cyclohexanone (Laboratory Reagent)
- Ethyl Iodide
- Ferrous Ammonium Sulphate Hexahydrate
- Gardenal (Phenobarbitone Sodium)
- n-Hexane (Laboratory Reagent)
- Hydrochloric Acid
- Hydrogen Peroxide
- Hydroxylamine Hydrochloride
- Magnesium Chloride
- Methanol (Laboratory Reagent)
- Perchloric Acid
- Potassium Chloride
- Potassium Cyanide
- Potassium Hydroxide
- Silver Cyanide
- Sodium Acetate
- Sodium Chloride
- Sodium Dithionite
- Sodium Hydroxide
- Sulphuric Acid (Laboratory Reagent)
- Trichloracetic Acid

E. Merck, Darmstadt, Germany:

- p-Dimethylaminobenzaldehyde
- Dipotassium Hydrogen Phosphate
- Ethyl Propionate
- Neocuproin
- Potassium Dihydrogen Phosphate
- Potassium Iodide
- TLC Aluminium Sheets, Silica Gel 60, Layer Thickness 0,2 m.m. (without fluorescence indicator)

Miles Laboratories, Epping, Cape:

Bovine Serum Albumin (fraction V)

Coenzyme A

Nutritional Biochemicals Corporation, Cleveland, Ohio, 44128, U.S.A.:

Antimycin A

Ortho Diagnostics, Rariton, New Jersey, 08869, U.S.A.:

Aculute Diluent Pellets

Acuglobin Hemoglobin Standard

Packard Instrument Company, Inc., 2200 Warrenville Road, Downers Grove, Illinois 60515, U.S.A.:

Instagel Scintillator Fluid

Porphyrin Products, P.O. Box 31, Logan, Utah, 84321, U.S.A.:

Coproporphyrin III tetramethyl ester

^{14}C-Porphobilinogen

Protoporphyrin IX dimethyl ester

Uroporphyrin I

Uroporphyrin III octamethyl ester

Bacterial Uroporphyrinogen I synthetase

Mixture of Bacterial Uroporphyrinogen I synthetase and ALA Dehydratase

The Radiochemical Centre, Amersham, Buckinghamshire, England:

δ- {4 - ^{14}C} Aminolaevulinic Acid Hydrochloride

{dimethylamino - ^{14}C} Aminopyrine

{^{14}C} Formaldehyde

{^{14}C} n-Hexadecane Reference Standard

{1,4 - ^{14}C} Succinic Acid

Riedel de Haën, Seelze bei Hanover, Germany

Iodine

Saphar ML Laboratories (Pty) Ltd., Stephen Road,Ophirton,Johannesburg:

Jectofer

Sodium Chloride Injection B.P. 0,9% (m/v) (Normal Saline)

Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, 63178,U.S.A.:

δ-Aminolevulinic Acid Hydrochloride

ATP

3,4-Benzpyrene

Dithiothreitol (Clelands Reagent)

Dowex - 1 x 8 400 Chloride Form

Dowex - 50 x 4 200 Acid Form

Ferritin (100 mg/ml in 0,15M NaCl)
Glucose-6-Phosphate (disodium salt)
Glucose-6-Phosphate Dehydrogenase (Torula Yeast XI)
Malonic Acid (disodium salt)
N A D
NADP
Nicotinamide
1,10-Phenanthroline
Pyridoxal-5-Phosphate
Semicarbazide Hydrochloride
Succinic Acid

Aminopyrine was a gift from Mr. N.M. Shapiro, Groote Schuur Hospital and 3-hydroxy-3,4-benzpyrene was a gift from Dr. H.V. Gelboin, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 20014, U.S.A. Dr. Bruce Burnham supplied a gift of purified bacterial succinyl coenzyme A synthetase. Allylisopropyl-acetamide was a gift from Roche Products, 4 Brewery Street, Isando, Transvaal.

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