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MONOPYRROLES IN PORPHYRIA AND RELATED DISORDERS

by

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Being a thesis submitted for the degree of Doctor of Philosophy to the University of Glasgow.

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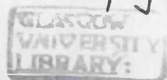

PREFACE

I am deeply indebted to Professor A. Goldberg for the offer of the post of Research Assistant at Stobhill General Hospital in 1974. From him also I have derived my interest in the porphyrias, a field which he has so ably explored.

During the period of the study I have become fascinated by the nature of the subject and, although my work has primarily been concerned with but a small area of the subject, I have derived great pleasure from the interplay with other areas and disciplines. As a result I have gained invaluable insight into other realms, especially that of the clinician.

The nature of the work has made collaboration with these other disciplines a prerequisite for its satisfactory completion. Recognition of this fact is made in the acknowledgements. Parts of the work have been published and reprints of such work are enclosed with this thesis.

Except where indicated, the work described has been carried out by me or under my supervision. The writing of this thesis has been carried out solely by myself.

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The study of the psychiatric patients could not have been carried out without the help of the appropriate ward staff and physicians in Stobhill General Hospital, the Southern General Hospital and Gartnaval Royal Hospital, especially that of Dr. R. McCreadie, whose interest has been much appreciated. The study of lead workers was carried out with Dr. Peter Meredith. The expertise in the ethological study was provided by Dr. M. Cutler and Miss F. Ewart of the Department of Biological Sciences, Glasgow College of Technology. Thyroid function

tests were carried out by Dr. J. Ratcliffe and staff of the Biochemistry Department, Glasgow Royal Infirmary and the free hormone concentrations were assayed by Dr. P. Yeo of the Endocrine Unit, Royal Victoria Infirmary, Newcastle.

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SUMMARY

The hereditary porphyrias are a group of disorders in which the genetic defect results in an abnormality in the biosynthetic pathway leading to the respiratory pigment haem. The characteristic result of this is excessive synthesis and excretion of the tetrapyrrolic porphyrins and, in the acute porphyrias, their precursors δ-aminolaevulinic acid (ALA) and porphobilinogen.

The acute attacks of acute porphyria show certain similarities to psychiatric disorders and the finding of a pyrrolic metabolite in the urine of patients with acute intermittent porphyria (AIP) and certain psychiatric disorders was the common factor that implicated this metabolite in the aetiology of certain manifestations of these disorders. Identification of the metabolite proved difficult but it was eventually unambiguously identified as 3-ethyl-5-hydroxy-4,5-dimethyl-Δ^3-pyrrolin-2-one (OHHPL). The work contained in this thesis describes clinical and biochemical investigations into the occurrence and properties of this compound.

Initially a qualitative method was used to detect OHHPL and it was shown to be present in the urine of patients with acute porphyria in attack and remission but to be undetectable in patients who were latent for the disease.

Using a gas-liquid chromatography method developed for the quantitative assay of OHHPL, it was shown that in carriers of the AIP gene defect there was no significant difference in OHHPL excretion in latency, attack and remission, but that levels in these patients were significantly greater than in control subjects. No correlation could be shown between OHHPL excretion and clinical condition either in the group of patients or in multiple estimations in single patients. However, a significant association was found between the excretion of OHHPL and that of ALA and PBG and it was also shown that in the porphyric patients ALA and PBG excretion were related in a linear fashion.

Quantitation of OHHPL in groups of psychiatric patients showed that there was a significant elevation of OHHPL excretion in psychotic patients who were undergoing drug therapy and also in groups of patients with schizophrenia and organic brain disease. In the patients with schizophrenia there was a negative correlation between the clinical condition of the patients as assessed on the Hamilton-Lorr rating scale and urinary OHHPL.

In an attempt to examine further the relationship between OHHPL and PBG and ALA in the patients with porphyria a group of patients, industrial lead workers, with sub-clinical lead poisoning were studied. These patients had raised blood lead and increased urinary excretion of ALA resulting from an

inhibition by lead of the enzyme δ-aminolaevulinic acid dehydratase (ALA.D). OHHPL and PBG concentrations in the urine of these workers were within the normal range. It was found that there was a negative association between OHHPL and ALA levels which suggested that the ALA-OHHPL association in the porphyric patients was purely a result of the ALA-PBG relationship.

In patients with liver disease raised levels of OHHPL were found in the urine of a proportion of patients with cirrhosis while all patients with hepatitis alone had normal OHHPL excretion.

To study the biochemical properties of OHHPL and other pyrroles a group of alkyl pyrroles and some of their oxidised derrivatives (including OHHPL) were administered to rats. Porphyrin and porphyrin precursor excretion and hepatic porphyrin levels were monitored and, in the case of OHHPL and its $\beta\beta'$ isomer (OHKPL), the activities of the enzymes of haem biosynthesis and that of haem oxygenase (HAEM.O), the haem degrading enzyme, were measured. Microsomal haem and cytochrome levels were also measured in the latter two cases.

It was found that treatment with each of the pyrroles caused increased porphyrin synthesis and excretion. Results from OHHPL and OHKPL treated animals suggested that this was due to an increase in δ-aminolaevulinic acid synthase (ALA.S), the rate controlling enzyme of haem biosynthesis, and that

this in turn was due to a reduction in microsomal levels of haem. Microsomal cytochrome P-450 levels were also depressed by this treatment.

Neither OHHPL nor OHKPL had an effect on the enzymes of haem biosynthesis in vitro. However, it was found that at high concentrations OHHPL caused an inhibition of HAEM.O.

The proportion of the porphyrin intermediates produced in response to monopyrrole treatment in vivo was studied by high performance liquid chromatography (HPLC). It was shown there were inconsistent changes in the proportion of individual porphyrins but that OHHPL increased porphyrin excretion without markedly altering the proportion of each porphyrin present. Increasing the dosage of a single non-toxic monopyrrole was associated with changes in the excretion of porphyrins, the proportion of 7- and 8-carboxylic porphyrins increasing with dose.

The finding in four patients with AIP of an attack associated increase in thyroid hormone levels, was followed by an attempt to show an effect of OHHPL or experimental porphyria on thyroid hormone excretion in the rat and an effect of thyroid hormone on the induction of ALA.S by OHHPL. Both of these experiments gave negative results.

Finally, the behavioural pharmacology of OHHPL was studied in two systems used to demonstrate behavioural changes in response to ALA. OHHPL was found to have no effect on the

spontaneous activity of rats or on the social and non-social behaviour of mice.

ABBREVIATIONS

AIA	Allylisopropylacetamide
AIP	Acute intermittent porphyria
ALA	δ-Aminolaevulinic acid
ALA.D	δ-Aminolaevulinic acid dehydratase
ALA.S	δ-Aminolaevulinic acid synthase
BPb	Blood lead
CHP	Cutaneous hepatic porphyria
CP	Congenital porphyria
COPRO.O	Coproporphyrinogen oxidase
DMO	5,5-Dimethyloxazolidine-2,4-dione
EADC	Ethyl-3-acetyl-2,4-dimethylpyrrole-5-carboxylate
EDTA	Ethylenediaminetetra-acetic acid
EPP	Erythropoietic protoporphyria
FERRO.C	Ferrochelatase
FTI	Free thyroxine index
FT3	Free triiodothyronine
FT4	Free thyroxine
HAEM.O	Haem oxygenase
HC	Hereditary coproporphyria
Hepta	Hepta-carboxylic porphyrin
Hexa	Hexa-carboxylic porphyrin
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate, oxidised.

NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
OHHPL	3-Ethyl-5-hydroxy-4,5-dimethyl-Δ^3-pyrrolin-2-one
OHKPL	4-Ethyl-5-hydroxy-3,5-dimethyl-Δ^3-pyrrolin-2-one
PBI	Protein bound iodine
PBG	Porphobilinogen
PBG.O	Porphobilinogen oxygenase
Penta	Penta-carboxylic porphyrin
PROTO.O	Protoporphyrinogen oxidase
rT3	Total reverse triiodothyronine
TBG	Thyroxine binding globulin
TRH	Thyrotrophin releasing hormone
TSH	Thyroid stimulating hormone
T3	Total triiodothyronine
T4	Total thyroxine
URO.COS	Uroporphyrinogen cosynthetase
URO.D	Uroporphyrinogen decarboxylase
URO.S	Uroporphyrinogen-1-synthetase
VP	Variegate porphyria

Liver Function tests :

Alb.	Serum albumin
Alk.Phos.	Serum alkaline phosphatase
ALT	Serum alanine transaminase
AST	Aspartate transaminase
Bil.	Serum bilirubin
LD	Serum lactate dehydrogenase

C H A P T E R   1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

1.1. HISTORY AND BACKGROUND OF THE PORPHYRIAS

The porphyrias are a rare group of genetic disorders first reported in the 19th century, although the first classification of the diseases in terms of disordered porphyrin metabolism was not made until 1922 by Gunther. The diseases are characterised by excessive excretion of porphyrins and their precursors due to a genetically defined metabolic defect. The porphyrins, a class of heterocyclic compounds, play essential roles in the formation of the respiratory pigments in animals, plants and microorganisms, the most important of which is haem (Figure 1).

The work of the Nobel Laureate Hans Fischer laid the foundations of the chemistry and biochemistry of porphyrins and it was he who introduced the term 'porphyria'. Subsequent work by Waldenstrom formed the basis of the modern classification of the porphyrias and has led to the general acceptance of the term. The porphyrias can be subdivided according to the site of the production of the bulk of the excreted porphyrins and precursors (Schmid et al, 1954); hepatic porphyria where the excess porphyrin is produced by the liver and erythropoietic porphyria where the bone marrow is the site of the overproduction.

Although the incidence of porphyria is low (about 1.5 cases per 100,000 for acute intermittent porphyria), interest has been great because of the ways in which the diseases manifest themselves and the similarity to other non-hereditary disorders such as lead

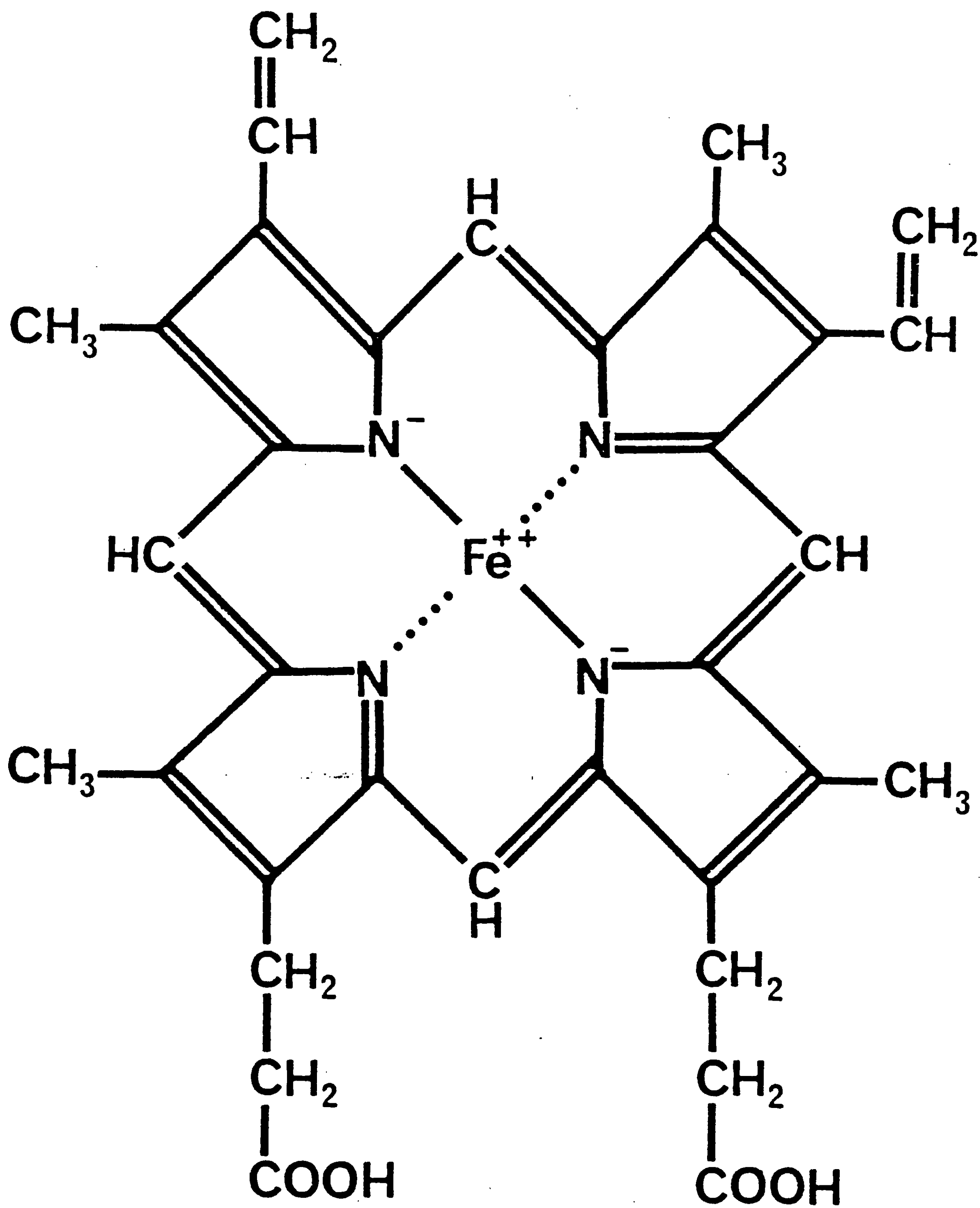


FIGURE 1 THE STRUCTURE OF HAEM

poisoning and anaemia, or more so in the cases of toxic porphyria where the condition is indistinguishable from a form of hereditary porphyria.

It is now generally believed that each of the porphyrias is primarily the result of an abnormality in the biosynthetic pathway of haem and that the symptomatology associated with each of the porphyrias may be ascribed to the overproduction of intermediary metabolites seen in that porphyria.

Our knowledge of the particular effects of individual metabolites has progressively increased and, with the improvements brought with modern research technologies, will continue to do so.

1.2. HAEM BIOSYNTHESIS

The mechanisms involved in the biosynthesis of haem have been the subject of many investigations since the elucidation of the biosynthetic pathway (Figure 2) following the work of Shemin (1955). As a part of the work in this thesis is concerned with abnormalities in the pathway, whether naturally occurring or experimentally produced, a resume of the present knowledge will be presented here. The enzymes are the active principals of the pathway and it will therefore be described in terms of their actions.

As can be seen from Figure 2, the enzymes of this pathway are located both in the cell sap and in the mitochondria necessitating translocation of pathway intermediates between these compartments. As yet, little is known of the processes involved in such transport; whether they are active or passive. These mechanisms represent a possible controlling area which will not be reviewed.

As haem catabolism plays an active role in the maintenance of haem levels, the initial enzyme of haem degradation will also be mentioned in the final sub-section.

1.2.1. δ-AMINOLAEVULINIC ACID SYNTHASE
(ALA.S; Succinyl CoA : Glycine C Succinyltransferase (decarboxylating); E.C. 2.3.1.37)

ALA.S, the initial enzyme, catalyses the condensation of glycine and succinyl CoA forming δ-aminolaevulinic acid (ALA) (Figure 3)

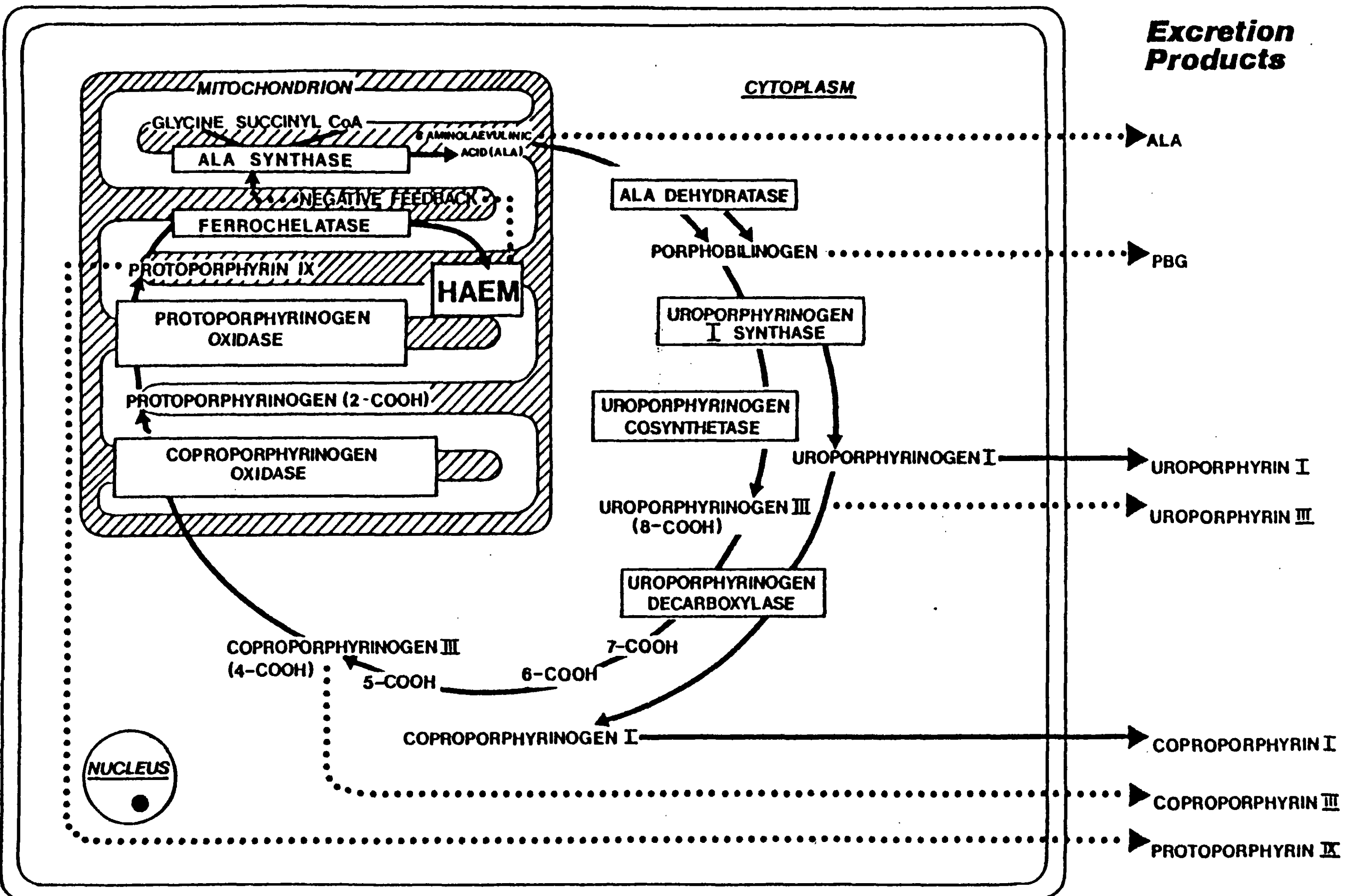


FIGURE 2 THE HAEM BIOSYNTHETIC PATHWAY

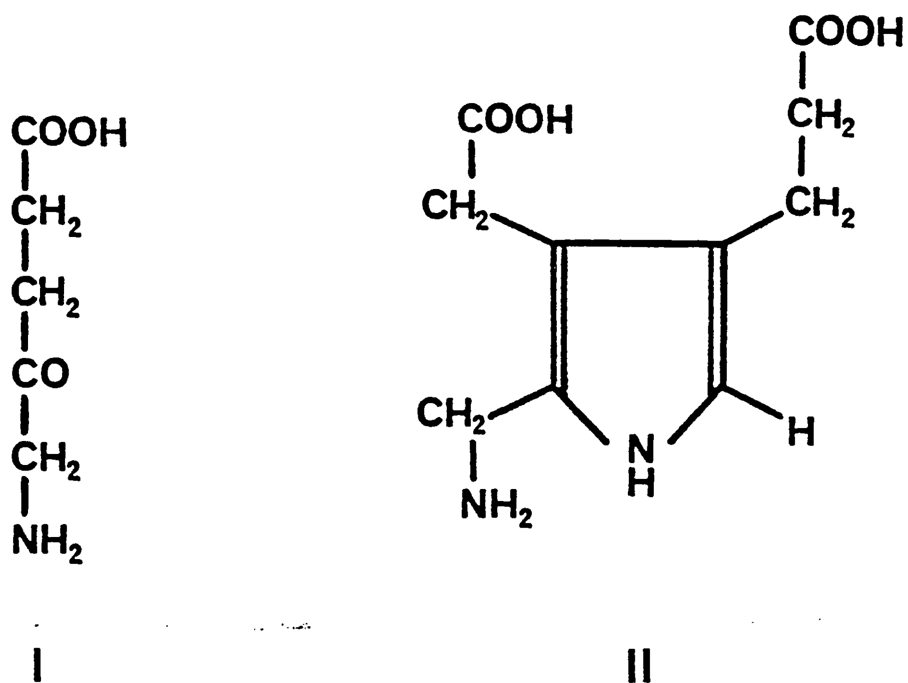


FIGURE 3 THE PORPHYRIN PRECURSORS

I δ-Aminolaevulinic acid (ALA)

II Porphobilinogen (PBG)

via the transient intermediate α-amino-β-ketoadipic acid (Granick and Urata, 1963). This enzyme, active in the mitochondrion, is rate controlling (Lascelles, 1964; Levere and Granick, 1965) and requires as cofactors pyridoxal phosphate (Schulman and Richert, 1956) and ferrous iron (Brown, 1958).

The control of ALA.S activity is mediated mainly by feedback regulation by the end product haem, which appears to act at a translational level (Sassa and Granick, 1970; Padmanaban et al, 1973; London et al, 1976). However, there is evidence to suggest that certain steroids can act in a permissive role at a transcriptional level (Matsuoka et al, 1968). The turnover of ALA.S is also a regulatory factor, the enzyme having a half-life of 60 minutes, (Marver et al, 1966).

Haem has also been shown to exert a direct inhibitory effect on ALA.S (Burnham and Lascelles, 1963; Whiting and Elliot, 1972), although nonphysiological concentrations are required for this effect using a partially purified form of ALA.S (Schachter et al, 1976).

ALA.S is synthesised on cytoplasmic ribosomes and undergoes translocation to the mitochondria (Hayashi et al, 1970) with a concomitant decrease in molecular weight. This process can also be inhibited both in vivo (Kurashima et al, 1970; Hayashi et al, 1972) and in vitro (Ohashi and Kikuchi, 1977) by exogenous haem although the physiological significance of these results is uncertain.

The activity of ALA.S can be altered by treatment with a great number of drugs and xenobiotics of which allylisopropylacetamide (AIA) and 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC) are two classic examples. The majority of these effects however are thought to be mediated by changes in intracellular haem levels. Possible mechanisms important in this respect are utilisation for haemoprotein synthesis, degradation by haem oxygenase or direct chemical degradation.

The mechanisms involved in the regulation of ALA.S activity, described above, apply mainly to hepatic tissue. There is evidence that the factors involved in such regulation differ from tissue to tissue. For example, it has been shown that ALA.S in adrenal tissue is refractory to treatment with AIA and DDC which markedly increase the activity of the hepatic enzyme and that the activity of the liver enzyme does not respond to treatment with adrenocorticotrophin (ACTH) which increases the activity of the adrenal enzyme (Condie et al, 1976).

Finally it should be noted that ALA.S can be demonstrated in vitro in bacteria, yeasts, avian and mammalian tissues, but not in plants. It would appear that in plants the immediate precursors of ALA are not glycine and succinyl CoA, hence the inability to show ALA.S activity, but that α-ketoglutarate and glutamate directly provide the carbon skeleton of ALA (Jurgenson et al, 1976).

1.2.2. δ-AMINOLAEVULINIC ACID DEHYDRATASE

(ALA.D; porphobilinogen synthase; 5-aminolaevulinate hydrolase (adding 5-aminolaevulinate and cyclizing); E.C. 4.2.1.24)

This, the second enzyme of the pathway, catalyses the condensation of two molecules of ALA to form the monopyrrole porphyrin precursor porphobilinogen (PBG) (Figure 3) (Shemin, 1976) The enzyme is cytoplasmic and activity of the enzyme from various sources requires metallic cations (Wu et al, 1974). ALA.D purified from bovine liver is an octomer with a molecular weight of about 30,000 daltons (Wu et al, 1974).

Human erythrocyte ALA.D is very sensitive to inhibition by inorganic lead. The inactive enzyme species, however, can be reactivated by incubating with reagents providing sulphydryl (-SH) groups (Hapke and Prigge, 1973) suggesting that lead binds to the enzyme at -SH groups.

1.2.3. UROPORPHYRINOGEN-I-SYNTHASE/UROPORPHYRINOGEN-III-COSYNTHASE

(URO.S; porphobilinogen deaminase; porphobilinogen ammonia-lyase (polymerising); E.C. 4.3.1.8/URO.CoS; no E.C. number)

The third enzymic step of the pathway is essentially at a branch point. Acting alone, URO.S catalyses the formation of the functionally inactive symmetrical uroporphyrinogen I (Figure 4) from four molecules of PBG. Uroporphyrinogen I is not

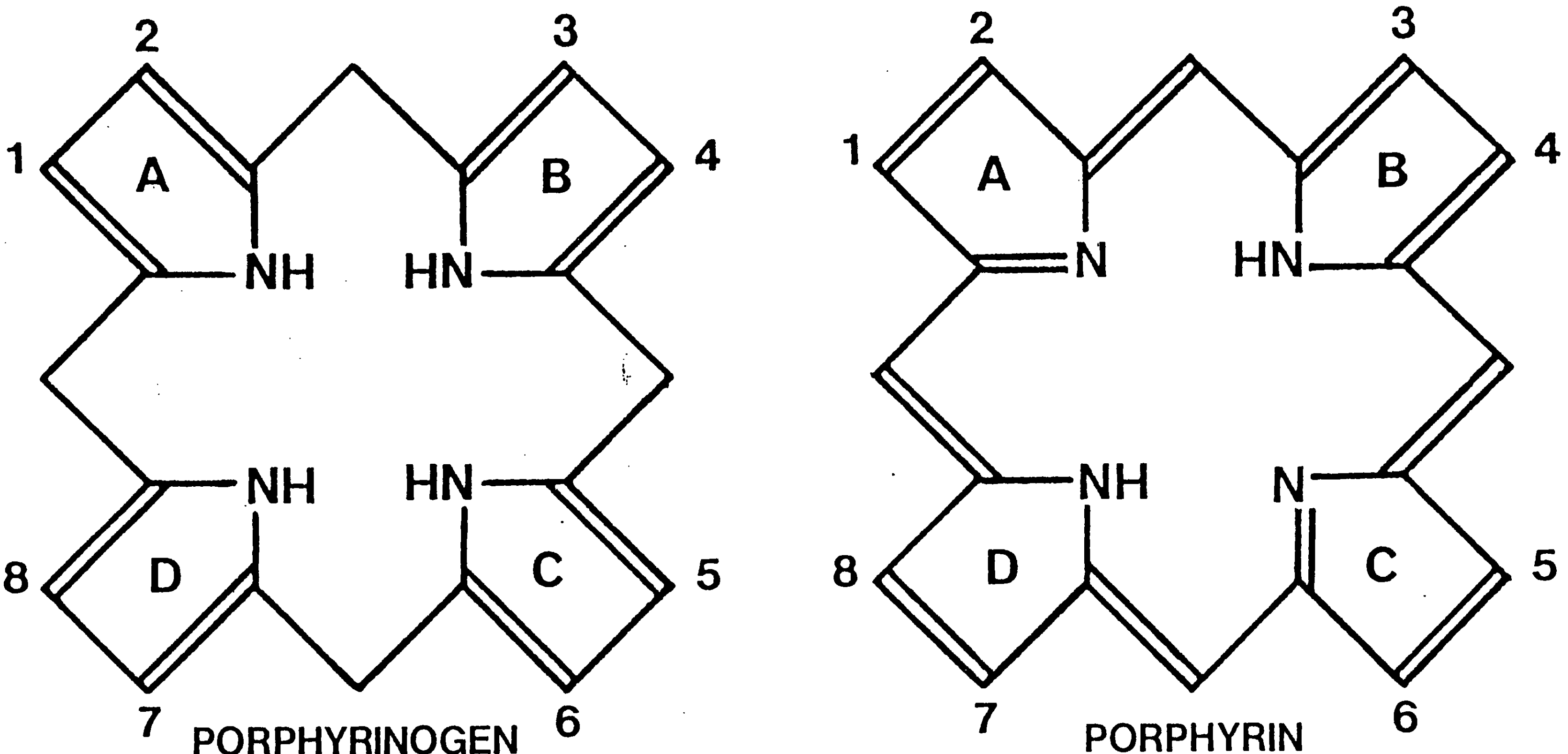


FIGURE 4 THE STRUCTURES OF INDIVIDUAL PORPHYRINS AND PORPHYRINOGENS

Position		1	2	3	4	5	6	7	8
Uroporphyrin(ogen)	I	Ac	P	Ac	P	Ac	P	Ac	P
	III	Ac	P	Ac	P	Ac	P	P	Ac
Coproporphyrin(ogen)	I	Me	P	Me	P	Me	P	Me	P
	III	Me	P	Me	P	Me	P	P	Me
Harderoporphyrin		Me	V	Me	P	Me	P	P	Me
Protoporphyrin IX		Me	V	Me	V	Me	P	P	Me
Mesoporphyrin		Me	P	P	Me	Et	Me	Et	Me

Ac = Acetyl
Et = Ethyl
Me = Methyl
P = Propionyl
V = Vinyl

incorporated into haem and is excreted in its oxidised form as uroporphyrin I or as coproporphyrin I.

The formation of the functional isomer, the asymmetrical uroporphyrinogen III, (Figure 4) requires the concerted action of both URO.S and URO.CoS. The mechanism by which this is effected is still unclear, although it is thought that in the presence of URO.CoS the primary reaction is as proposed by Russell (1974), namely the condensation of two molecules of PBG in a 'head to head' configuration forming an enzyme bound dipyrrylmethane (Frydman et al, 1976; Battersby and McDonald, 1976). Uroporphyrinogen III is then completed by the sequential condensation of two further molecules of PBG.

1.2.4. UROPORPHYRINOGEN DECARBOXYLASE
(URO.D; uroporphyrinogen carboxy-lyase; E.C. 4.1.1.37)

URO.D catalyses the ordered sequential decarboxylation of uroporphyrinogens I and III although the specificity of the reaction is much in favour of the III isomer (Jackson et al, 1976). As a by-product of the reaction porphyrinogens with 7, 6 and 5 carboxyl groups are produced as well as the product coproporphyrinogen (Figure 4). This effect is exaggerated in some pathological conditions (Doss et al, 1971) and under in vitro conditions (Kushner et al, 1976). There is evidence to suggest (Tomio et al, 1970) that all the decarboxylations take place on the same enzyme protein, although it can be shown that

hepta-, hexa- and penta-carboxylic porphyrinogens act as substrate for the enzyme (Elder and Tovey, 1977) showing that any proposed mechanism need not require the binding of the porphyrinogen throughout the four decarboxylations.

1.2.5. COPROPORPHYRINOGEN OXIDASE

(COPRO.O; coproporphyrinogen : oxygen oxidoreductase (decarboxylating) ; E.C. 1.3.3.3)

COPRO.O unlike the preceding three enzymes is mitochondrial. It catalyses the conversion of coproporphyrinogen III in a two step process to protoporphyrinogen IX (Figure 4) (Akhtar et al, 1976). As with the decarboxylations from uroporphyrinogen to coproporphyrinogen, the enzyme appears to act in a specific manner (Jackson et al, 1976) decarboxylating the propionic acid on the A ring before that on the B ring. The intermediate tricarboxylic porphyrinogen, harderoporphyrinogen (Figure 4) does not accumulate to any extent but is found in quantity in the harderian gland of certain rodents.

1.2.6. PROTOPORPHYRINOGEN OXIDASE

(PROTO.O; protoporphyrin IX dehydrogenase; No E.C. number)

The final step in porphyrin synthesis involves the removal of six hydrogen atoms from protoporphyrinogen IX forming protoporphyrin IX (Figure 4). Under aerobic extraction procedures protoporphyrin readily autooxidises and it was originally thought that this occurred

in vivo. The isotope labelling studies of Jackson et al (1974) provided definite evidence in favour of an enzymic mechanism for the conversion.

The enzyme responsible (PROTO.O) has been shown to be bound to mitochondrial membranes (Poulson and Polglase, 1975) and would appear to contain active sulphydryl groups. Cyanide, 2,4-dinitrophenol and azide inhibitors of mitochondrial respiration do not affect the activity of the enzyme while Cu^{2+}, Co^{2+} haem and haemin all cause inhibition.

1.2.7. FERROCHELATASE
(FERRO.C; haem synthetase; protohaem ferro-lyase; E.C. 4.99.1.1)

The final step in haem synthesis is the chelation of ferrous ions (Fe^{2+}) with protoporphyrin, a reaction catalysed by the enzyme ferrochelatase. The enzyme, particulate in nature, is attached to the inner mitochondrial membrane (McKay et al, 1969). It is not specific for protoporphyrin but can also insert Fe^{2+} into both mesoporphyrin (Llambias, 1976) and deuteroporphyrin (Figure 4) (Bonkowsky et al, 1975). The mechanism has not been fully elucidated, although the overall reaction is complex involving the penetration of substrate into the mitochondrial matrix and the transport and reduction of Fe^{3+} to give the cosubstrate Fe^{2+}.

1.2.8. HAEM OXYGENASE
(HAEM.O; E.C. 1.14.99.3)

Haem catabolism in mammalian systems is catalysed by the microsomal enzyme haem oxygenase which cleaves the tetrapyrrole ring at the α-methene bridge, with concomitant oxidation, to form biliverdin IXα. Intracellular free haem levels control the activity of haem oxygenase by substrate mediated induction (Pimstone et al, 1971).

The enzyme which was originally thought to be of the mixed function oxidase type (Tenhunen et al, 1972) is induced by certain trace metals (Maines and Kappas, 1974, 1976a and b; Kappas and Maines, 1976). More recent studies have shown that haem oxygenase activity can be dissociated from cytochrome P-450 (Maines and Kappas, 1974). Maines and Kappas (1977) have proposed a mechanism by which haem substrate is bound to the enzyme forming a transient haemoprotein which catalyses the oxidation of its own prosthetic group. Evidence in favour of this mechanism has still to be described.

1.3. THE PORPHYRIAS

As has been mentioned, the porphyrias are a group of six hereditary disorders (cutaneous hepatic porphyria may also be acquired) characterised by excessive production and excretion of the intermediates in the haem biosynthetic pathway. They can be classified according to whether the bulk of the excessive porphyrins and precursors is produced in the liver or the bone marrow (Table 1) or according to the nature of the symptoms observed as acute or non-acute.

The acute porphyrias, acute intermittent porphyria (AIP), hereditary coproporphyria (HC) and variegate porphyria (VP) all exhibit sporadic acute attacks which can be induced by endogenous or exogenous influences. Accompanying the acute attack, the common biochemical finding is the excretion in the urine of the porphyrin precursors ALA and PBG in increased amounts. As well, each of the acute porphyrias shows an individual pattern of porphyrin excretion. Both HC and VP can present in sub-acute phases with the solar sensitivity characteristic of the non-acute porphyrias.

The non-acute porphyrias, cutaneous hepatic porphyria (CHP), congenital porphyria (CP) and erythropoietic protoporphyria (EPP) all show solar sensitivity which has been shown to be due to the presence of porphyrins in the skin. As with the acute porphyrias, each of the non-acute porphyrias exhibits a characteristic pattern of porphyrin excretion which is never associated with increased porphyrin precursor excretion.

TABLE 1 CLASSIFICATION OF THE PORPHYRIAS

Porphyria		
ACUTE INTERMITTENT PORPHYRIA	ACUTE PORPHYRIA	HEPATIC PORPHYRIA
HEREDITARY COPROPORPHYRIA		
VARIEGATE PORPHYRIA		
CUTANEOUS HEPATIC PORPHYRIA	NON-ACUTE PORPHYRIA	
ERYTHROPOIETIC PROTOPORPHYRIA		ERYTHROPOIETIC PORPHYRIA
CONGENITAL PORPHYRIA		

It has long been suspected that each of the porphyrias was the result of a single enzymopathy and the recent accumulation of evidence, reviewed recently by Gajdos (1976) and Brodie et al (1977a) has supported this point of view. It would appear, however, that the disease process is influenced by many extraneous factors, as can be seen from the following review of each of the porphyrias.

1.3.1. ACUTE INTERMITTENT PORPHYRIA (AIP)

Acute intermittent porphyria, clinically the most severe of the porphyrias is, in most areas, the most prevalent (Goldberg and Rimington, 1962). The disease has been shown to be transmitted as an autosomal dominant disorder and it is surprising to find that in its acute form it affects more women than men. One of the main features of the disease is the intermittency of acute attacks and it can be distinguished from other forms of porphyria by the dominance of gastrointestinal and neuropsychiatric features in the total absence of solar photosensitivity. AIP has been the subject of several reviews in the literature (Waldenstrom, 1937; Goldberg and Rimington, 1962; Tschudy et al, 1975; Boucherat, 1977).

Clinical Manifestations

The clinical features associated with AIP are summarised in Figure 5. Most of these can be explained by lesions in various areas of the nervous system (Goldberg, 1959), although the

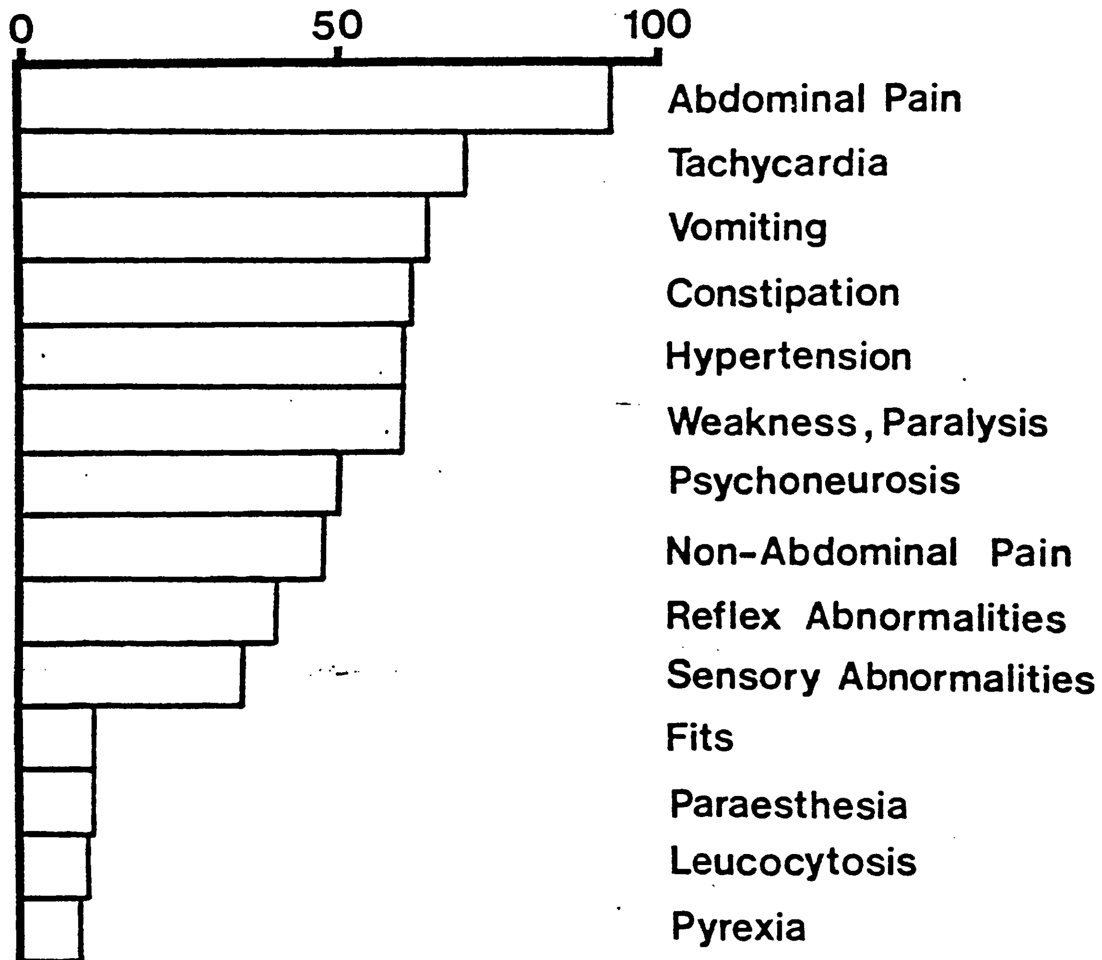


FIGURE 5 CLINICAL MANIFESTATIONS OF ACUTE INTERMITTENT PORPHYRIA

distribution of the lesions and hence the symptomatology can vary from patient to patient. The disease can exist in latency though the fundamental abnormality is still present. Precipitation of acute attacks can be caused by endogenous and exogenous factors and the possible mechanisms of this will be discussed below.

Abdominal pain, probably an automomic neuropathy (Tschudy et al, 1975) is the presenting symptom in about 85% of acute attacks and almost always precedes a peripheral neuropathy. Other autonomic manifestations which may be prominent in the acute attack include tachycardia, postural hypotension, labile hypertension, retinal artery spasm and urinary incontinence or retention.

The pattern and rate of progression of the attacks may vary greatly. The peripheral neuropathy which may accompany or follow abdominal pain may be sensory or motor. Motor neuropathies can be symmetrical or localised and may progress over a period of weeks or months or can appear explosively resulting in complete quadraplegia. Sensory signs and symptoms include paraesthesia, numbness and objective evidence of sensory impairment such as loss of pinprick sensation.

The pathological nature of the neuropathic lesions is uncertain although patchy demyelination has been found at all levels of the neuraxis including the autonomic nervous system (Ten Eyck et al, 1961). Recently it has been proposed that these findings are part of a dying-back neuropathy, the toxic effect

being exerted at the cell body.

A small percentage of patients may exhibit epilepsy and abnormal electroencephalograms have been recorded both in patients with and without epilepsy. More common, however, is the presence of psychiatric aberrations (Wetterberg, 1967) in the form of simple neuroses, endogenous depression, schizophrenia and various psychoses. Although these symptoms are occasionally severe enough to have warranted mental certification, they do not appear to last beyond the period of the somatic illness.

The disease is rarely fatal, the most common cause of death being respiratory failure, or associated complications.

Biochemical Abnormalities

The most important biochemical finding in AIP is the excessive urinary excretion of the porphyrin precursors ALA and PBG (Haeger, 1958). This has been shown in latent subjects as well as subjects in attack and in remission. Alternatively though, patients in remission or latent subjects can have normal or near normal excretion. Patients in attack have also been shown to excrete abnormally high amounts of uroporphyrin.

The abnormality originally thought to be fundamentally responsible for the increased ALA and PBG excretion was an increase in the activity of the rate controlling enzyme of haem biosynthesis - ALA.S (Tschudy et al, 1965). Later work, however, has shown this to be secondary to a decrease in hepatic URO.S - probably the primary enzymatic defect in AIP (Nakao et

al, 1966; Dowdle et al, 1967; Strand et al, 1970).

Brodie et al (1977b) have demonstrated decreased URO.D in the peripheral blood of patients with AIP providing a possible reason for abnormal uroporphyrin excretion which was originally thought to be due to non-enzymic polymerisation of PBG.

The observed increase in hepatic ALA.S activity is likely to be due to decreased haem formation as a result of the partial blockage at the level of URO.S. This in turn would lead to derepression of ALA.S. Evidence for depressed haem levels has been shown by decreased drug metabolism in patients with AIP who showed excessive excretion of ALA and PBG (Song et al, 1974; Anderson et al, 1976). Latent patients who showed normal porphyrin precursor excretion had normal drug metabolism. It appears that in the asymptomatic conditions of latency and remission the diminished level of URO.S is partially or completely compensated by an increase in ALA.S which results in adequate, if not normal, haem formation.

The level of ALA and PBG excretion cannot be correlated with ALA.S or URO.S activities and the normal excretion of these products in some latents with raised ALA.S and lowered URO.S remains to be explained.

Other biochemical abnormalities have been described although patients with AIP, essentially a hepatic disorder, usually show normal liver function tests. Possibly the most relevant finding has been the demonstration of increased excretion of certain

steroid metabolites (Goldberg et al, 1969; Moore et al, 1973; Paxton et al, 1974) and a deficit in steroid 5βH reductase activity in AIP (Kappas et al, 1972a,b; Bradlow et al, 1976). About 40% of patients with AIP have hypercholesteraemia (Taddeini et al, 1964) and an even greater percentage have hyperbetalipoproteinaemia (Lees, 1970). These findings would seem to suggest a common control mechanism for porphyrin and lipid metabolism.

Precipitation of the Acute Attack

Acute intermittent porphyria can be present in the latent form for an indefinite period and the genetic defect remains innocuous until acted upon by superimposing factors. These factors, not all exogenous, can be divided into four main catagories :-

1. Drugs and xenobiotics
2. Steroids
3. Dietary factors
4. Infections

One of the first cases of porphyria ever recorded was a fatal attack precipitated by sulphonal one year after its introduction in 1888. Since then many drugs have been implicated in the precipitation of an acute attack. One of the most important groups of drugs in this respect is the barbiturates (Tschudy et al, 1975).

Experimental studies in animals and cell culture systems have shown that a number of drugs known to precipitate the acute porphyric attack can induce ALA.S or an experimental form of porphyria in these systems (Granick, 1966; Moore et al, 1970; Tschudy and Bonkowsky, 1972; Rifkind et al, 1973). Many of these drugs have also been shown to alter microsomal cytochrome P-450 levels (Rifkind et al, 1973) and it is thought that the drugs increase ALA.S activity by altering a regulatory haem pool causing derepression of ALA.S and that this is the mechanism by which they precipitate the acute attack. Direct evidence for this is still lacking. The experimental systems, however, have proved useful in the screening of drugs for use in porphyria, although it has not been proved conclusively that drugs which stimulate ALA.S in experimental systems will precipitate an acute attack.

Steroid hormones and related steroids comprise the second group of precipitating factors. Clinical findings provided circumstantial evidence in favour of this; the disease is rarely manifest before puberty (Marver and Schmid, 1972) and there is an overall predominance of woman sufferers (Goldberg, 1959), some of whom experience regular pre-menstrual attacks (Perlroth et al, 1965) or attacks associated with pregnancy.

Exogenous steroids in the form of the contraceptive pill have been shown to be responsible for the precipitation of an acute attack, although in some patients oral contraceptives

have been shown to be beneficial in the prevention of attacks related to menstruation.

Work in experimental systems has shown that certain steroids can increase the activity of ALA.S (Granick, 1966) especially those steroids with the 5βH configuration (Granick and Kappas, 1967a,b; Kappas and Granick, 1968). This, considering the abnormal excretion of steroid metabolites and steroid metabolism in the porphyrias (see previous section) suggested a role for 5βH steroids in the pathogenesis of the disease (Kappas et al, 1972a,b). Recent evidence demonstrating the equipotency of 5αH and 5βH steroids in inducing ALA.S (Edwards and Elliot, 1975; Stephens et al, 1977) would cast doubts on a specific role of 5βH steroids. However, it remains clear that certain steroids have a porphyrin inducing effect related to precipitation of the acute porphyric attack.

The role of dietary factors is less clear cut than that of drugs or steroids. Acute attacks have been associated with water diets or carbohydrate restriction. Conversely high carbohydrate intake has been shown to have beneficial effects (Welland et al, 1964; Bonkowsky et al, 1976; Brodie et al, 1977b). This effect reflects those in experimental animals where high carbohydrate diet can prevent induction of ALA.S by chemicals (Tschudy et al, 1964; Bonkowsky et al, 1973).

The mechanism of this co-called 'glucose effect' is unknown, although there is speculation that it may be due to

inhibition of RNA formation and thus similar to bacterial catabolite repression, or to a blocking of glucocorticoid effects (Goldberg, 1974) resulting in altered formation of RNA specific for ALA.S and lowering levels of this enzyme.

The final category in this section, infections, is the least well understood. Precipitation of the attack may be by both bacterial or viral infection (Goldberg and Rimington, 1962; Tschudy, 1974), although the mechanism is unknown. The effect may be mediated by changes in hormone levels due to stress.

Possible Biochemical Origin of the Neuropathy of AIP

As has been said the majority of the clinical features of the acute porphyric attack can be explained on the basis of an autonomic or peripheral neuropathy. It is surprising that, although the fundamental defect in AIP and the exacerbation of the acute attack are now well understood, the biochemical mechanism of the production of the neuropathy remains a mystery. There are a number of theories on the possible mechanisms giving rise to the acute attack, but there are two of special note because of their simplicity. Firstly, that the partial block in haem synthesis is present in neural tissue and gives rise to a deficiency of haem which results in degeneration and death of the nerve cells or, secondly, that the partial block results in elevated circulating

levels of the porphyrin precursors and that either ALA or PBG (or a derivative of them) exerts a direct toxic effect on the nerves.

Early studies on the pharmacological properties of the porphyrin precursors suggested that they had little or no activity (Goldberg et al, 1954; Jarrett et al, 1956). Clinical evidence in agreement with these results was provided by the existence of latent subjects and patients with porphyria in remission who excreted large amounts of the porphyrin precursors without any adverse effects. In individual patients, however, excretion is generally raised during an attack falling again with remission.

More recent studies have shown that both ALA and PBG have significant behavioural and pharmacological properties. McGillion et al (1973) have shown that ALA causes significant changes in the spontaneous activity of mice. It has also been shown that ALA can inhibit brain and red cell Na^+ K^+ dependent ATPase (Becker et al, 1971) and that both ALA and PBG can affect neuromuscular transmission and motor end-plate potentials (Feldman et al, 1971; Loots et al, 1975). Doubt, however, has been cast on the significance of these results by the recent studies of Percy and Shanley (1977) who have shown that ALA and PBG levels in the cerebrospinal fluid (CSF) are much lower than those in plasma and well below the levels shown to exert effects on neural tissue in vitro.

There is no direct evidence in favour of the alternative hypothesis, that the neuropathy of the acute attack is due to a deficiency of haem in neural tissue. It is apparent, however, that raised ALA.S activity observed in liver and leucocytes (Brodie et al, 1977b) is due to reduced haem levels in these tissues and that exacerbation of the acute attack by most exogenous agents is due to further depletion of a regulatory haem pool. The hypothesis requires that this be true also for nerve tissue. Exacerbation of the acute attack by steroids, thought to act at a transcriptional level, would possibly provide evidence to the contrary. Unless haem levels are also lowered by steroids an increase in ALA.S due to steroids would be expected to be beneficial. A beneficial effect of steroids has, however, been demonstrated in a small number of cases (Perlroth et al, 1965).

It remains virtually impossible to differentiate between the two possible mechanisms based on present knowledge. A decrease in haem will almost invariably be associated with increased ALA.S activity and increased porphyrin precursor excretion. The two therapeutic regimens at present used in the treatment of acute phases, high carbohydrate intake and haematin infusion both appear to produce beneficial effects associated with decreases in porphyrin precursor excretion (Bonkowsky et al, 1976; Brodie et al, 1977b; Watson et al, 1977). As yet nothing is known about the effects of these treatments on tissue haem levels.

One alternative not yet mentioned is that there is another substance, possibly a metabolite of ALA or PBG, which is produced during the acute attack possibly resulting in the neuropathy. As the quantitation of such a substance forms a major part of this thesis discussion of this will take place in a later section.

1.3.2. HEREDITARY COPROPORPHYRIA (HC)

Hereditary coproporphyria, the second acute hepatic porphyria, is characterised as its name suggests by excessive excretion of coproporphyrin. Like AIP it is transmitted as an autosomal dominant. It has been the subject of a recent review (Brodie et al, 1977c).

Clinically, the disease usually presents during an acute attack which remarkably resembles that of AIP (Figure 6), except that there is also a high incidence of solar photosensitivity which presents as a bullous eruption and skin fragility on exposed parts.

The laboratory findings in HC in latency or remission show elevated excretion of coproporphyrin in faeces and usually urine, with excretion of porphyrin precursors and other porphyrins mainly within the normal ranges. During the acute attack, however, urinary excretion of coproporphyrin is massively elevated as is that of uroporphyrin in a majority of cases. The excretion of the porphyrin precursors also becomes grossly elevated in these

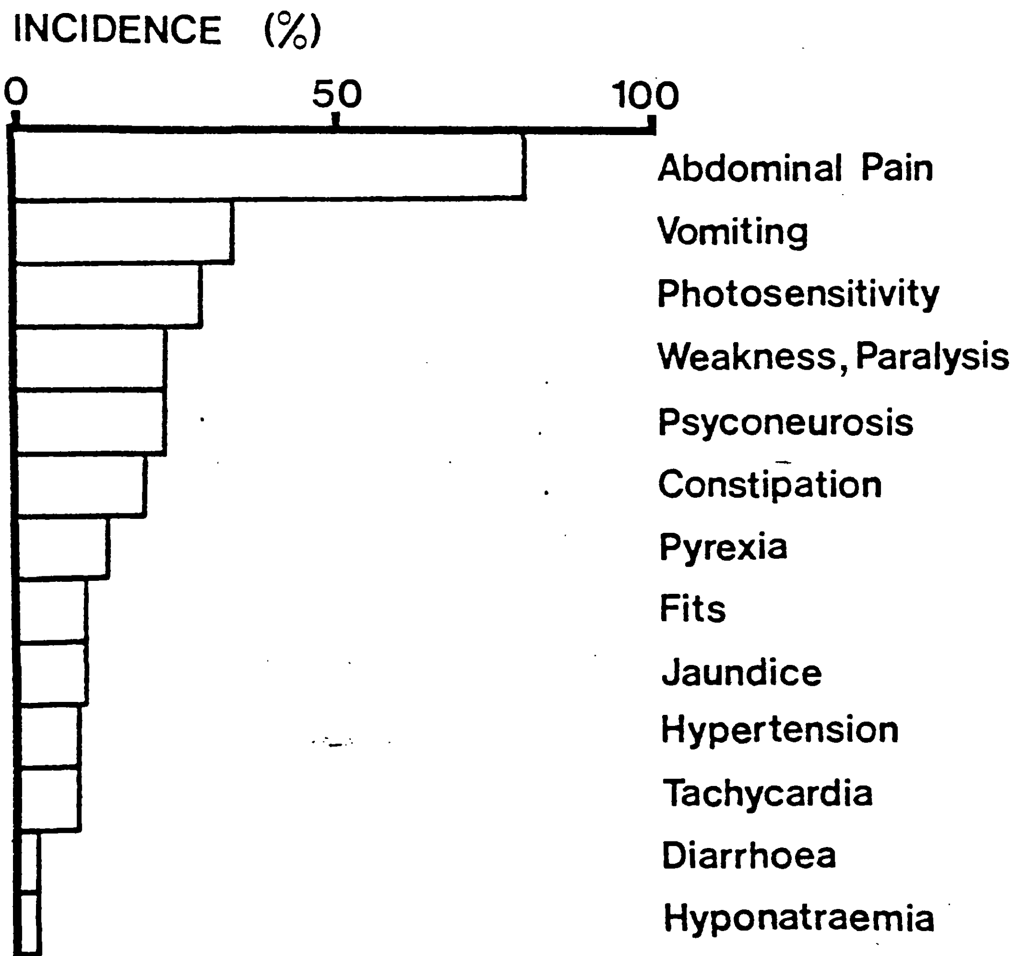


FIGURE 6 CLINICAL MANIFESTATIONS OF HEREDITARY COPROPORPHYRIA

subjects (Brodie et al, 1977c). Enzymatic studies have shown that the main abnormality is a decreased activity of COPRO.O (measured in leucocytes, lymphocytes and skin fibroblasts) (Elder et al, 1976; Brodie et al, 1977c; Grandchamp and Nordmann, 1977). Secondary to this there is an increase in the activity of hepatic ALA.S (Kaufman and Marver, 1970; McIntyre et al, 1971; Brodie et al, 1977c).

As has been found with AIP, there are a number of reports of acute episodes being precipitated by the administration of drugs (including barbiturates), or steroids, or by calorific restriction. This would suggest that the precipitation occurs by the same mechanism as in AIP which in turn suggests that the development of the acute manifestations common to both disorders occur in the same manner. The excretion of ALA and PBG in the acute attack in the absence of a deficiency in PBG.D remains to be explained. It has been proposed that in the attack situation, where there is induction of ALA.S, the flow through the pathway becomes so great (reflected by massive excretion of coproporphyrin) that the activity of URO.S becomes limiting, resulting in excretion of ALA and PBG (Brodie et al, 1977d).

The occurrence of photosensitivity, probably due to the presence of porphyrin in the skin, will be discussed under erythropoietic protoporphyria where it is the main presenting feature.

1.3.3. VARIEGATE PORPHYRIA (VP)

Variegate porphyria, like the other hepatic porphyrias, is transmitted as an autosomal dominant. In South Africa, where the disease is most common, the genetic trait can be traced for almost 300 years. The disease can present with an acute attack, which is similar to those of AIP or HC, or with solar photosensitivity without any gastrointestinal involvement.

The biochemical picture in latency and remission is of minor increases in the urinary excretion of uroporphyrin and coproporphyrin with major elevations in faecal coproporphyrin, protoporphyrin and a characteristic porphyrin-peptide conjugate (X-porphyrin) (Goldberg, 1971).

Acute attacks are invariably associated with increases in the excretion of ALA and PBG along with massive porphyrinuria. Severe bouts of photosensitivity have been reported, in which faecal excretion of porphyrins is reduced, with elevation of urinary uroporphyrin and coproporphyrin (Dean, 1965).

Dowdle et al (1967) have reported an increase in hepatic ALA.S in VP and the porphyrin excretion pattern would suggest a partial block in haem synthesis in conformity with the other acute porphyrias. Evidence for this has been provided by Becker et al (1977) who have shown a decreased activity of ferrochelatase in bone marrow lysates from patients with VP in agreement with earlier work in two subjects by Brodie et al (1976).

It is clear that VP can be provoked into an acute attack

similar to those of AIP and HC (Lyberatos et al, 1976; Percy and Shanley, 1977) by misuse of certain drugs. However, the acute photosensitive bout, which is free from gastrointestinal features (Dean, 1965), may be associated with gross abnormalities in liver function and in that case be more similar to the non-acute type of porphyria, cutaneous hepatic porphyria (section 1.3.4.).

1.3.4. CUTANEOUS HEPATIC PORPHYRIA (CHP)

Known also as porphyria cutanea tarda (PCT) or symptomatic porphyria, CHP is perhaps the most perplexing of the porphyrias. It is apparent that the disease can be caused by toxic liver damage as seen in an outbreak in Turkey following ingestion of hexachlorobenzene contaminated wheat (Schmid, 1960), or it may also be hereditary (Dehlin et al, 1973; Kushner et al, 1976).

The main biochemical findings are faecal excretion of coproporphyrin and protoporphyrin during remission and excretion of uroporphyrin and to a lesser extent coproporphyrin in the urine during acute phases.

The acute phases which present with solar photosensitive skin lesions are precipitated in the vast majority of cases by alcohol intake (Pimstone, 1975), although oral contraceptive steroids (Haberman et al, 1975) or barbiturates (Ziprkowsky, 1966) have been implicated. Liver disease is invariably associated with the manifest disease as is siderosis which has

been implicated in the derangement of haem biosynthesis (Kushner et al, 1975).

Enzymatic studies have given inconsistent results. However, Kushner et al (1976) and Elder and Tovey (1977) have demonstrated decreased hepatic activity of uroporphyrinogen decarboxylase which may account for the porphyrin excretion pattern in acute phases. ALA.S has been shown to be raised (Mocre et al, 1972) providing evidence in favour of a partial block as in the other hepatic porphyrias.

It remains clear that liver disease and siderosis are important cofactors in the relapse of the disease involving diversion of formed porphyrins into the systemic circulation resulting in photosensitivity. Remission can be achieved by the treatment of the siderosis by venesection (Ippen, 1961).

1.3.5. ERYTHROPOIETIC PROTOPORPHYRIA (EPP)

EPP, the more common of the two erythropoietic porphyrias, is, like the hepatic porphyrias, transmitted as an autosomal dominant with variable penetrance.

The main biochemical findings are of raised faecal protoporphyrin excretion with high protoporphyrin content in circulating erythrocytes. The enzymatic defect has been traced to ferrochelatase, decreased activity being found in liver and cultured skin fibroblasts (Bonkowsky et al, 1975). More recently Becker et al (1977) have shown that ferrochelatase in

bone marrow lysates is unstable, differentiating the abnormality from VP (section 1.3.3) where the ferrochelatase is probably inactive.

The main clinical feature is solar photosensitivity due to protoporphyrin in the blood (Piomelli et al, 1975) and hence skin. In later stages protoporphyrin deposition in the liver can result in progressive liver disease (Barnes et al, 1968; Donaldson et al, 1971). The photosensitivity which is manifest as burning, local oedema, pruritus, and erythema in most cases, can also show skin pain and residual scarring (de Leo et al, 1976).

Experimental studies into the mechanism of the photoreaction have revealed that light of 400nm is most active, a wavelength close to the Soret maximum of porphyrin absorption (Magnus et al, 1961). Laboratory models have shown that photosensitivity is an oxygen dependent process involving singlet oxygen ($^1O_2$) (Fugimori et al, 1966); thus the effect is probably due to porphyrin sensitised photooxidation of cell components. This role of singlet oxygen is also suggested by the protection in experimental systems afforded by treatment with α-tocopherol which quenches singlet oxygen (Moshell and Bjornson, 1977).

The treatment of choice in EPP is with β-carotene which quenches singlet oxygen as well as absorbing light at 400nm.

1.3.6. CONGENITAL PORPHYRIA (CP)

Congenital porphyria, mentioned for fullness, is the most rare of the porphyrias and, in contrast to the others, is transmitted in a recessive fashion. The characteristic finding of excessive excretion of the biologically inactive series I porphyrin isomers has led to the belief that the genetic abnormality in this condition is a deficiency or a relative deficiency of uroporphyrinogen cosynthetase (Miyagi et al, 1976; Brodie et al, 1977a).

1.4. MONOPYRROLES IN PORPHYRIA AND OTHER DISORDERS

Interest in the association of monopyrroles with acute porphyria arose with the discovery by Cookson and Rimington (1953) of the pyrrolic nature of one of the abnormal metabolites in AIP, namely porphobilinogen. The occurrence of PBG has been fully documented following the establishment of a quantitative assay for it (Mauzerall and Granick, 1956).

Evidence for the excretion of a second pyrrolic metabolite in AIP was provided in 1972 (Irvine and Wetterberg; Huszak et al). This metabolite, originally discovered by Irvine (1961), was termed "mauve factor" due to its colour reaction with Ehrlich's reagent. The identification of "mauve factor" proved difficult due to its sensitivity to mild treatment. However, it was shown that it was not artifactual in origin (Sohler et al, 1970).

Prior to its discovery in the urine of porphyric patients an association between "mauve factor" excretion and psychiatric illness had been made (O'Reilly et al, 1965; Ellman et al, 1968). Typically the factor was shown to be present in up to 50% of endogenous psychoses (Hoffer and Osmond, 1963); 60 - 70% in acute schizophrenia (Sohler et al, 1967) and 60% of schizophrenics who showed excessive porphyrin excretion (Huszak et al, 1972). As well as being found in AIP mauve factor excretion has also been shown in patients with CHP (Huszak et al, 1972) and VP (Irvine and Wilson, 1976).

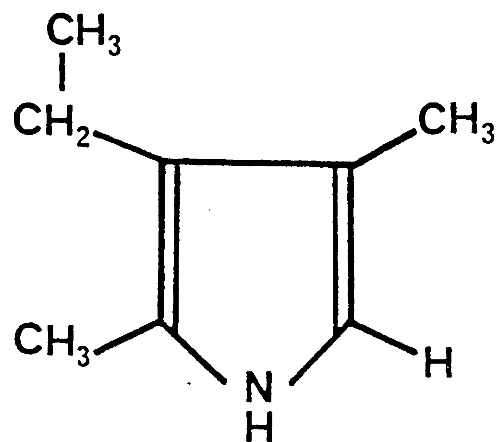
As much of the work of this thesis is concerned with the

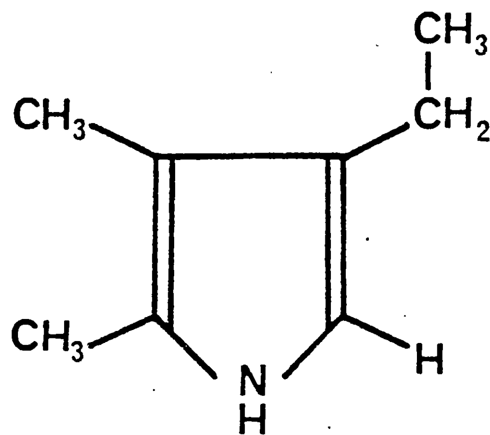
quantitation of the mauve factor and with biochemical and pharmacological effects of this and related substances, work leading to its eventual identification will be covered in some detail here. Also covered will be previous pharmacological and behavioural studies which have been reviewed recently (Irvine, 1974).

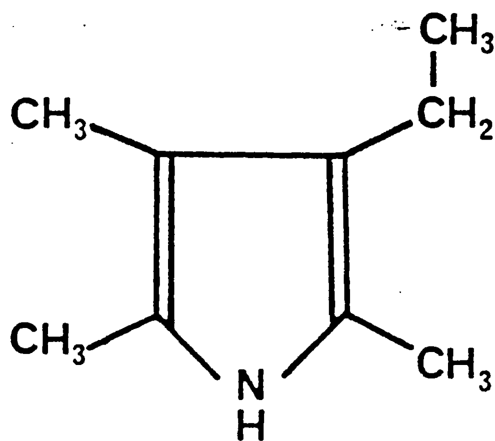
1.4.1. IDENTIFICATION OF MAUVE FACTOR

Mauve factor was originally discovered by Irvine in the urine of schizophrenic patients using a chromatographic method that suggested it was an alkyl pyrrole. Using a more powerful chromatographic method following charcoal precipitation Irvine et al (1969) were able to resolve the mauve factor into five separate entities which if eluted and reprocessed would again give rise to all components. Mass spectrometric analysis of a single component spot showed a molecular ion that was suggestive of a mono-ethyl dimethyl pyrrole. This led to the processing of one such alkyl pyrrole, kryptopyrrole (2,4-dimethyl-3-ethyl-pyrrole) (Figure 7), which produced a pattern of spots identical to that of the natural product. These findings led to the hypothesis that the "mauve factor" was essentially due to kryptopyrrole, an observation made independently by Sohler et al (1970).

In an attempt to identify the component substances, the oxidation products of kryptopyrrole were synthesised (Lightner







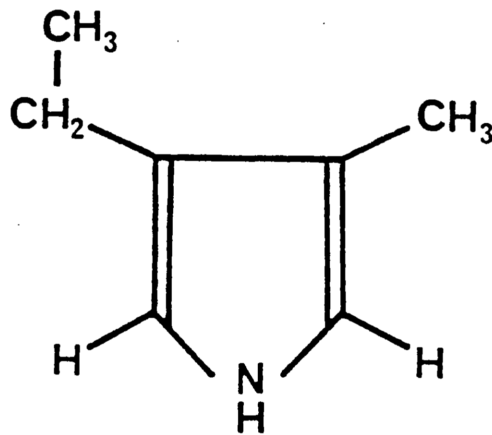


FIGURE 7 HETEROLOGOUS ALKYL PYRROLES

I 2,4-Dimethyl-3-ethylpyrrole (Kryptopyrrole)

II 2,3-Dimethyl-4-ethylpyrrole (Haemopyrrole)

III 4-Ethyl-2,3,5-trimethylpyrrole (Phyllopyrrole)

IV 3-Ethyl-4-methylpyrrole (Opsopyrrole)

and Crandall, 1973) (Figure 8). The chromatographic processing of these compounds showed that all of the five component spots could be produced from compounds II and III, and that interconversion was due to acid used in the chromatographic separation (Irvine et al, 1973). It was also shown that II could give rise to III when methanol was used for the extraction of the charcoal. This suggested that compound II, 4-ethyl-5-hydroxy-3,5-dimethyl-Δ^3-pyrrolin-2-one OHKPL was the primary compound and that the others were by-products produced during extraction or chromatography. This conclusion was supported by the work of Jacobson et al (1975) who were unable to demonstrate the presence of the parent pyrrole, kryptopyrrole, in the urine of schizophrenic patients using a sensitive gas-liquid chromatography - mass-spectrometry method.

As was said, however, the original mass spectrometric evidence suggested a mono-ethyl dimethyl pyrrole and, as has since been recognised, the subsequent methodology was not specific enough to differentiate between the oxidation products of kryptopyrrole and those of the ββ' isomer of kryptopyrrole, haemopyrrole (2,3-dimethyl-4-ethyl pyrrole) (Irvine and Wetterberg, 1972) (Figure 7).

It was not until recently that the development of improved extraction and chromatographic methods allowed unambiguous differentiation of the two isomeric forms (Irvine and Wilson, 1976). Comparison of the synthetic oxidation products of

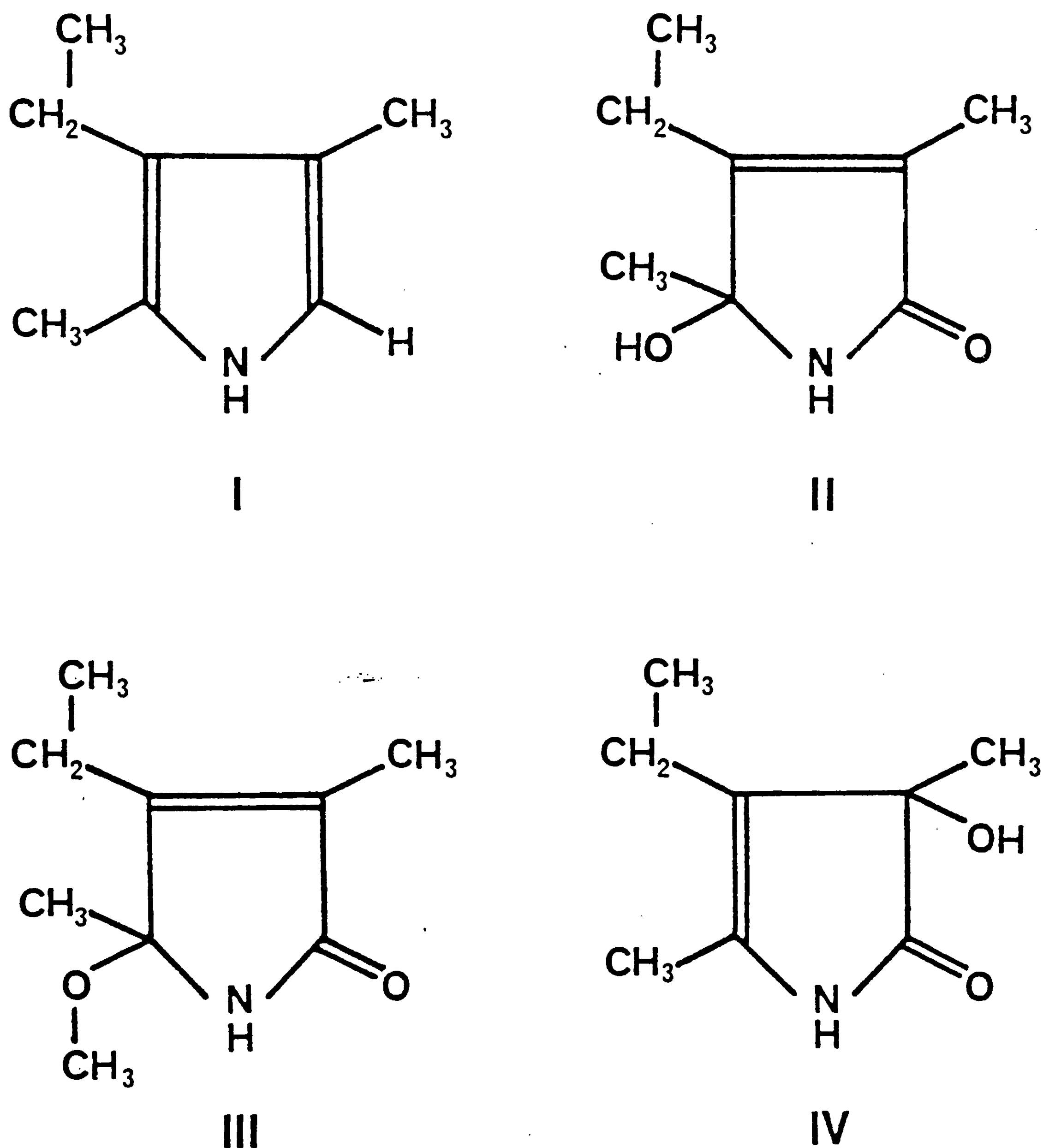


FIGURE 8 KRYPTOPYRROLE AND ITS OXIDATION PRODUCTS

I 2,4-Dimethyl-3-ethylpyrrole (Kryptopyrrole)

II 4-Ethyl-5-hydroxy-3,5-dimethyl-Δ^3-pyrrolin-2-one (OHKPL)

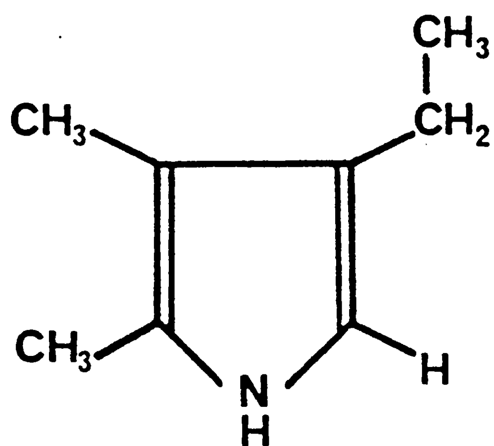
III 4-Ethyl-5-methoxy-3,5-dimethyl-Δ^3-pyrrolin-2-one

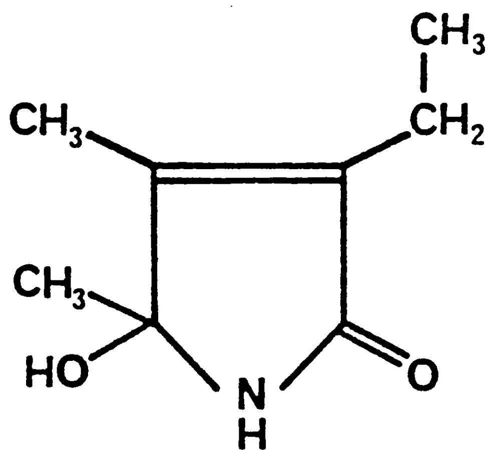
IV 4-Ethyl-3-hydroxy-3,5-dimethyl-Δ^4-pyrrolin-2-one

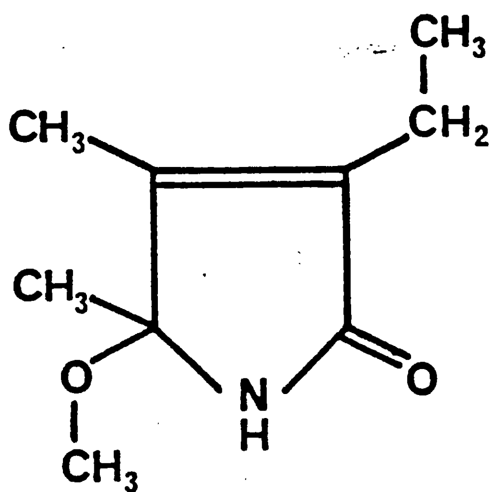
kryptopyrrole and haemopyrrole and the natural product has allowed the final structural assignation to be made as 3-ethyl-5-hydroxy-4,5-dimethyl-Δ^3-pyrrolin-2-one (Figure 9), the oxidised form of haemopyrrole. In the work that is covered in this thesis this will be the substance referred to as the natural product usually abbreviated to OHHPL (see nomenclature section).

1.4.2. PHARMACOLOGY AND BEHAVIOURAL PHARMACOLOGY OF MONOPYRROLES RELATED TO 3-ETHYL-5-HYDROXY-4,5-DIMETHYL-Δ^3-PYRROLIN-2-ONE (OHHPL)

It was not until recently that the mauve factor was finally identified and so most of the work in this area has been concerned with the effects of kryptopyrrole and, to a lesser extent, oxidation products of kryptopyrrole. There is a very close structural similarity between the oxidised forms of kryptopyrrole and the natural metabolite and it may not be unjustified in suggesting that they have properties in common. Furthermore, it has been shown that kryptopyrrole is metabolised oxidatively by microsomal enzymes in the rat, the sole product being the ββ' isomer of OHHPL, namely OHKPL (Irvine, 1974). This would imply that, where experiments are carried out with kryptopyrrole over an extended period using living animals, the possibility exists that the effects may be due to the metabolite and not the kryptopyrrole itself.







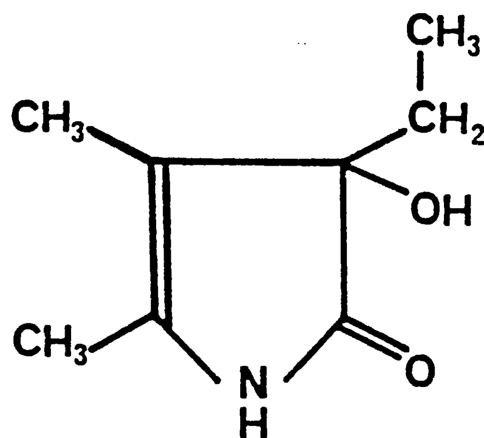


FIGURE 9 HAEMOPYRROLE AND ITS OXIDATION PRODUCTS

I 2,3-Dimethyl-4-ethylpyrrole (Haemopyrrole)

II 3-ethyl-5-hydroxy-4,5-dimethyl-Δ^3-pyrrolin-2-one (OHHPL)

III 3-ethyl-5-methoxy-4,5-dimethyl-Δ^3-pyrrolin-2-one

IV 3-ethyl-3-hydroxy-4,5-dimethyl-Δ^4-pyrrolin-2-one

The toxicity of kryptopyrrole has been studied extensively and it has been found to be abnormally high among a series of related pyrroles (Moffett, 1968). Toxic effects were most obvious in the central nervous system, the lung and pleural cavity and the eye and orbital region. It has been found to have anticonvulsant properties in experimental animals, both in electrically provoked seizures in rats (Moffett, 1968) and in photically induced seizures in Fayoumi fowl, a species susceptible to seizures (Irvine et al, 1973).

Neuroelectrical studies have shown that in non-toxic doses kryptopyrrole causes profound changes in electroencephalograph (EEG) patterns and activity, which bear resemblance to the EEG patterns seen with psychotomimetic agents such as LSD (Walker, 1975).

One significant pharmacological effect of kryptopyrrole is its ability to induce hypothermia in experimental animals. A recent study using the oxidised forms of kryptopyrrole, compounds III and IV (Figure 8) (Wetterberg and Formgren, 1976) showed that compound IV elicited a greater hypothermic effect than kryptopyrrole and was at least as toxic as kryptopyrrole, while compound III showed a lesser hypothermic effect and lower toxicity. The number of animals (mice) used in this study was, however, very small.

The behavioural effects shown with kryptopyrrole are a reduction in exploratory behaviour and general activity

associated with huddling.

Other behavioural studies designed to look at the effects on established performance; fixed ratio responding to food reinforcement (Irvine and Zdanivsky, 1971) and discriminated avoidance (Zdanivsky and Irvine, 1972), revealed that kryptopyrrole showed effects similar to LSD in the former test, while in the latter showed effects intermediate between amine depletors (e.g. p-chorophenylalanine) and psychotomimetics (e.g. LSD, mescaline). In long term studies using the second test, kryptopyrrole showed effects that were identical to psychotomimetics.

1.4.3. IMPLICATIONS OF THE EXCRETION OF OHHPL IN THE PORPHYRIAS.

As has been discussed in the foregoing sections, the origin of the psychiatric syndrome of the acute porphyric attack is as yet unknown, although it is suspected that it is due to a lesion in the CNS, just as other features of the attack are due to lesions elsewhere in the nervous system. Attempts to relate the biochemical abnormalities to the clinical condition have all but failed. The only biochemical finding common to the three acute porphyrias during an attack is the excessive excretion of the porphyrin precursors ALA and PBG and up to date there have been no conclusive findings which would implicate either of these compounds in the

generation of the neural lesion responsible for the psychiatric disturbance.

The finding that there is excessive excretion of a second monopyrrole in acute porphyria, a substance also excreted in a high percentage of cases of psychoses, presents the possibility that this substance is the materia peccans responsible for the clinical features of acute porphyria. As yet this is purely hypothetical as evidence in favour is completely lacking. In the past investigations have been carried out using related substances and extrapolation of results may prove fallacious. As there has been no satisfactory method available for the quantitation of OHHPL, its role in the porphyrias remains to be determined. With this in mind the work covered in this thesis was designed with two objectives :-

1. To develop a satisfactory method for the quantitation of OHHPL in biological material in order to investigate any relationships between these results and clinical findings.

2. To investigate selected biochemical and pharmacological effects of OHHPL and related compounds in experimental situations.

It is hoped that these studies will be able to give some insight into the possible role of OHHPL in the aetiology of the manifestations of the porphyrias and in particular of the psychiatric syndrome.

CHAPTER 2

MATERIALS AND GENERAL METHODS

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1. MATERIALS

Radiolabelled chemicals, 4-^{14}C ALA and 2-^{14}C glycine were obtained from the Radiochemical Centre, Amersham, Bucks., England. ^{59}Fe was obtained from the radionuclide dispensary, Glasgow Western Infirmary, Glasgow, Scotland. Non-labelled ALA was supplied by Koch Light Laboratories Ltd., Colnbrook, Bucks., England. Kryptopyrrole was supplied by the Aldrich Chemical Company, Gillingham, Dorset, England. Silica gel from the Merck and Woelm ranges was obtained through BDH., Poole, Dorset and Koch Light Laboratories respectively.

A sample of OHHPL was a kind gift from Dr. D.G. Irvine, University Hospital, Saskatoon, Saskatchewan, Canada, while porphyrin standards were either obtained from the Sigma Chemical Company, London, England, or prepared and kindly donated by Mr. G.G. Thompson, Department of Materia Medica, University of Glasgow, Scotland.

Supplies of chemicals, packings, supports, spares etc. for the gas-liquid chromatography work were obtained from Field Instruments Ltd., Richmond, Surrey, England.

Enzymes and co-enzymes were obtained from BDH and other chemicals and reagents of the appropriate grade were obtained from BDH, Aldrich Chemical Company or Koch Light Laboratories.

Experimental animals were supplied by the Tuck Animal Breeding Centre, Battlesbridge, Essex, England.

2.2. NOMENCLATURE OF MONOPYRROLES

The systematic naming of the pyrroles encountered in this thesis is, in most cases, long and unwieldy and unfortunately not all have well known trivial names.

One of the most important biological pyrroles, 2-amino-methyl-4-2'-carboxyethyl-3-carboxymethyl pyrrole, is universally known as porphobilinogen, a name created by Waldenstrom (Waldenstrom and Wahlquist, 1939) prior to the elucidation of its structure by Cookson and Rimington (1953).

The four heterologous pyrroles (Figure 7), haemopyrrole, kryptopyrrole, phyllopyrrole and opsopyrrole, were all isolated as major reduction products of natural substances such as haemin, bilirubin or green chlorophyll (Sidgwick, 1966). The four trivial names reflect their isolation from these substances.

Trivial names for the oxidised forms of kryptopyrrole and haemopyrrole (Figures 8 and 9) are not arrived at with such ease, but tend to reflect the expediency of normal usage. Those oxidised forms with a ketone group at position 2 are essentially lactams as the adjacent secondary amine allows lactam - lactim tautomerism. Compounds II and IV in Figures 8 and 9 are then hydroxy-lactams of kryptopyrrole and haemo-pyrrole respectively. Compounds III are methoxy lactams. The 5-hydroxy compounds, the compounds of interest, will thus be denoted as OHHPL and OHKPL (hydroxy-haemopyrrole lactam etc).

Where the 3-hydroxy or 5-methoxy species arise in the text, it will be made clear to which compound reference is being made. This will be done using the semi-systematic forms 5-hydroxy-haemopyrrolin-2-one (OHHPL), 3-hydroxyhaemopyrrolin-2-one, 5-methoxyhaemopyrrolin-2-one etc. or by reference to Figures 8 and 9.

2.3. SYNTHESIS OF MONOPYRROLES

The unavailability of a suitable commercial supply for certain pyrroles, or in the case of porphobilinogen a suitable radioactive form, necessitated the production of such material by organic syntheses or biosynthetic methods.

2.3.1. BIOSYNTHETIC PREPARATION OF PORPHOBILINOGEN

Porphobilinogen was produced enzymically from ALA using a purified preparation of ALA.D from bovine liver.

The product was isolated, purified and crystallised from the assay mixture by the method of Cookson and Rimington (1954).

Radiolabelled PBG was produced in an identical manner by spiking the substrate ALA with specifically labelled 4-^{14}C ALA.

Purification of ALA.D

A whole bovine liver was obtained from the abattoir as soon as possible following slaughter. Portions were diced and homogenised, using a domestic blender, in 0.15M potassium chloride buffered with 0.02M potassium phosphate (pH 6.8) containing 1mM 2-mercaptoethanol. The homogenate was then spun at 2,500g for 45 minutes.

The supernatant was then heated to 60°C for five minutes with constant stirring, followed by rapid cooling on ice. The solution was then spun at 2,500g for 20 minutes and the resultant deep red supernatant retained.

24.7g of ammonium sulphate was then added per 100ml and any resultant precipitate removed by centrifugation. A further 10.6g of ammonium sulphate was then added per 100ml and the resultant precipitate spun down and retained (may be stored at -25°C).

The precipitate was dissolved in 0.05M potassium phosphate (pH 6.8) containing 1mM 2-mercaptoethanol to give a protein concentration of 20 - 30mg/ml. This solution was then heated to 72 - 74°C for 3 minutes, then chilled on ice. The supernatant obtained following centrifugation (2,500g) was dialysed for 15 hours in 0.05M potassium phosphate (pH 6.8) containing 1mM 2-mercaptoethanol. The inactive precipitate was then spun down (10 minutes, 2,500g) leaving a slightly turbid green enzyme solution.

Enzyme Incubation

The protein concentration of the purified enzyme preparation was determined and adjusted to 20mg/ml with sodium phosphate buffer, pH 6.0, 0.05M. 1.6ml of this solution was then added to 6.6ml of sodium phosphate buffer, pH 6.8, 0.1M, containing ALA and L-cysteine both in a final concentration of 0.625M. The reaction mixture in a lead-free Thunberg tube was then degassed, the tube evacuated and the reaction incubated for 17 hours.

Isolation of Porphobilinogen

The reaction mixtures were decanted into flasks containing mercuric acetate solution (15% $^{w}/v$) and brought to ph 4 - 4.5 with acetic acid. Further mercuric acetate was added until all porphobilinogen was precipitated (negative Ehrlich reaction of the supernatant). The precipitate was collected on a Buchner funnel and washed with mercuric acetate (1% $^{w}/v$). The precipitate was then suspended in water and decomposed by bubbling with hydrogen sulphide gas. After filtration and aeration, excess of lead acetate (10% $^{w}/v$) was added. The deeply pigmented precipitate was removed and washed repeatedly with lead acetate solution (1% $^{w}/v$). Silver nitrate solution (20% $^{w}/v$) was added to the combined filtrate and washings until precipitation was complete. After filtration and washing of the precipitate with silver nitrate solution (1% $^{w}/v$) the combined filtrate and washings were treated, without prior removal of lead or silver ions, with a slight excess of mercuric acetate solution (15% $^{w}/v$). This mercury precipitate, almost white, was collected by centrifugation and decomposed by bubbling with hydrogen sulphide gas without the addition of further fluid. Mercuric sulphide was easily removed by centrifugation from the pale yellow supernatant, which was freed from hydrogen sulphide by aeration and then adjusted to pH 4 with aqueous ammonia. PBG crystallised immediately in almost colourless, well formed, crystals. After chilling,

the solution was filtered and the crystalline PBG washed first with water containing a little acetic acid, and then with acetone. Further quantities could usually be obtained by the same method by extracting the final mercuric sulphide precipitate with a little water. Yields were about 60%.

Radiolabelled Porphobilinogen

Radiolabelled labelled PBG was produced by the same method except that 300µCi of 4-^{14}C ALA was added to 1.2g ALA over 40 reaction tubes. The product obtained (100mg; 56%) had a specific activity of 2.08 x 10^6 cpm/mg or 0.508 x 10^6 cpm/µmol. The purity was checked by paper chromatography on Whatman 3MM paper using the upper layer of a butanol:acetic acid:water (4:1:5) mixture for development. A second method of analysis was the estimation of the PBG content of a solution of the product by the standard PBG assay (section 2.4.2). In both cases the purity was found to be greater than 96.5%.

2.3.2. PREPARATION OF ALKYL PYRROLES

Haemopyrrole (2,3-dimethyl-4-ethylpyrrole)

Haemopyrrole was synthesised essentially by the method of Rapoport (1976) following the scheme in Figure 10. The starting material ethyl propionylacetate was synthesised according to Ellis et al (1964).

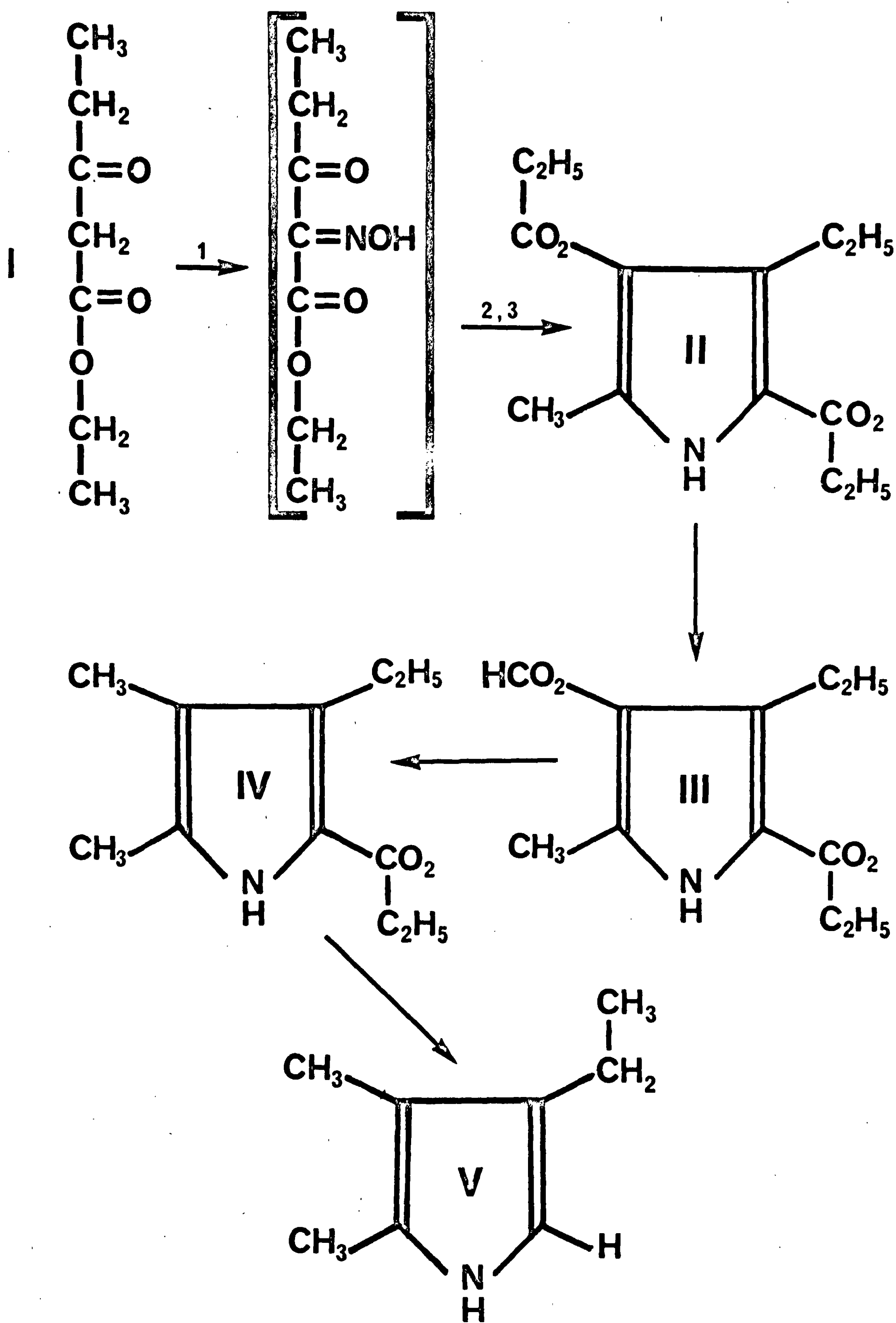


FIGURE 10 ORGANIC SYNTHESIS OF HAEMOPYRROLE

1. HNO_2; 2. Zn, HOAc; 3. $CH_3COCH_2CO_2C_2H_5$

Diethyl Propionylmalonate - Magnesium turnings (67g), ethanol (67ml), carbon tetrachloride (3ml) and 80ml of a solution of diethyl malonate (410g) in ethanol (205ml) were warmed gently until the evolution of hydrogen began. The remainder of the diethyl malonate was added slowly to maintain the reaction rate. 1350ml of dry ether was added when the reaction mixture had cooled and heating restarted till the magnesium had all dissolved. Propionyl chloride (250ml) in dry ether (270ml) was added slowly, then the mixture heated under reflux for 1 hour. The cooled magnesium complex was then decomposed carefully with excess ice-cold dilute sulphuric acid. Ether extraction and distillation gave diethyl propionylmalonate (400g; 72%), bp. 199°C/760mm.

Ethyl Propionylacetate (I) - Diethyl propionylmalonate (285g) was hydrolysed by boiling with water (855ml). 600ml of water was then distilled off over 4.5 hours. The organic fraction in the residue was separated and the two aqueous fractions combined and extracted with ether (2 x 200ml). The organic fractions were combined (with product) and washed with saturated sodium bicarbonate (50ml), water (4 x 50ml), saturated sodium carbonate (4 x 25ml), water (3 x 25ml), dilute sulphuric acid (2 x 25ml) and finally with water (4 x 50ml). Evaporation and distillation gave ethyl propionylacetate (150g; 79%), bp. 100 - 120°C/20 - 30mm.

Diethyl 3-ethyl-5-methylpyrrole-2,4-dicarboxylate (II) - Sodium nitrite (75g) in water (135ml) was added slowly to ethyl propionyl acetate (150g) in acetic acid (380ml) with cooling so that the temperature was maintained below 5°C. When addition was complete the temperature was allowed to rise to room temperature overnight. This solution was then run slowly with stirring into ethyl acetoacetate (135g) and acetic acid (380ml), a mixture of zinc dust (175g) and anhydrous sodium acetate (180g) being added simultaneously at 60 - 80°C. When addition was complete the temperature was raised to 100°C for an hour then the mixture was decanted into ice and water (10L). The product was filtered, water washed and recrystallised as needles from ethanol (106g; 47%), mp. 113 - 115°C.

Ethyl 3-ethyl-4-carboxy-5-methylpyrrole-2-carboxylate (III) - In a 1L 3-necked flask fitted with a nitrogen bubbler-capped reflux condenser and magnetic stirring bar, were added 182ml of concentrated sulphuric acid (96 - 98%) and 40ml of H_2SO_4.20% SO_3. When the temperature fell to 30°C diethyl 3-ethyl-5-methylpyrrole-2,4-dicarboxylate (110g) was added over 10 minutes. The flask was then heated to 40°C for 20 minutes. The contents were then decanted into ice (3L) and left overnight. The resultant brown precipitate was purified by twice boiling with 1L of methanol giving 55g; (56%), mp. 250 - 255°C (dec.).

Ethyl 3-ethyl-4,5-dimethylpyrrole-2-carboxylate (IV) - To a 1L 3-necked flask with a nitrogen bubbler capped precision addition funnel containing ca. 150ml redistilled diglyme was added 10.5g of sodium borohydride. The solution was stirred then cooled in an ice bath. Slowly over 3 hours 42.5g of methyl iodide was added and 30 minutes after the addition was complete 22.5g of ethyl 3-ethyl-4-carboxy-5-methylpyrrole-2-carboxylate was added in one portion with ice cooling. After 120 minutes methanol was added to the reaction mixture till no more hydrogen was evolved. The solvent was then removed overnight by freeze drying. The residue obtained was partitioned between ether and water. The aqueous fraction was washed twice with ether (100ml) and the ether fractions combined, washed with water, dried with magnesium sulphate, filtered and evaporated to give crude product which was recrystallised from ethanol/water to give 7g (35%), mp. 96 - 97°C.

2,3-Dimethyl-4-ethylpyrrole (Haemopyrrole) (V) - To a 250ml round bottomed flask with nitrogen bubbler capped reflux condenser was added 175ml triethylene glycol (trigol), 5.6g potassium hydroxide and 7g ethyl 3-ethyl-4,5-dimethylpyrrole-2-carboxylate. The flask was heated at 165°C for 18 hours in the dark. When the reaction was complete the mixture was cooled on ice, extended with 75ml deoxygenated water, then extracted (x 4) with 75ml diethyl ether under a safe light. The combined ether extracts were washed with 30ml aliquots of

deoxygenated water till neutral. The ether was evaporated under reduced pressure to ca. 10ml and the product crystallised at -25°C, (4g; 90%).

Phyllopyrrole (2,3,5-trimethyl-4-ethylpyrrole)

Phyllopyrrole was prepared by a method analogous to that for haemopyrrole. Acetylacetone was condensed with butanedione monoxime, in a reaction similar to the formation of II above (Knorr pyrrole condensation) giving 2,3,5-trimethyl-4-acetyl-pyrrole. The product was formed by the standard Huang-Minlon modification of the Wolff-Kishner reduction (Vogel, 1970). The pyrrole ketone was refluxed in digol with 3 equivalents of hydrazine hydrate for 1 hour to form the hydrazone. Water and excess hydrazine was then distilled off until a temperature of 170 - 190°C was attained and reflux continued for a further 3 - 5 hours. The product was collected by ether extraction from the cooled reaction mixture.

Opsopyrrole (3-ethyl-4-methylpyrrole)

This was again prepared by a method similar to the preceding two. The product, however, proved rather unstable and was unsuitable for extended storage and subsequent animal experiments and was therefore omitted from the series.

2.3.3. SYNTHESIS OF OXIDISED ALKYL PYRROLES

The purposes of study required the synthesis of two oxidised

pyrroles, oxidised forms of kryptopyrrole and haemopyrrole. These compounds, the 2-keto-5-hydroxy forms (OHKPL and OHHPL) were synthesised by photooxidation by methods based on those of Lightner and Crandall (1973) and Lightner et al, 1974.

4-ethyl-5-hydroxy-3,5-dimethyl-Δ^3-pyrrolin-2-one (OHKPL)

A 0.1% ($^v/v$) solution of kryptopyrrole in methanol containing 3.5mg/100ml Rose Bengal ($^1O_2$ sensitizer) was illuminated in a glass cylinder maintained at 15°C by water cooling. The light source utilized was three 100w tungsten bulbs situated at 10cm from the apparatus. A slow stream of oxygen was bubbled through the mixture during the oxidation procedure. The process was stopped when aliquots no longer gave a rapid positive reaction with an Ehrlich reagent (2% p-aminodimethylbenzaldehyde in methanol : HCl (9:1)). At this point the solvent was removed by rotary evaporation below 40°C. The residue was taken up in a small volume of chloroform and column chromatographed on silica gel, (column: 2.5cm x 35cm; silica gel 40, E. Merck, Darmstadt, 70 - 230 mesh ASTM) using chloroform, diethyl ether, ethyl acetate and acetone. A more complete separation of the eluted material in the ethyl acetate fraction was obtained by partition thin layer chromatography (tlc) on silica gel (Silica gel F, M. Woelm, Eschwege, 1mm, diethyl ether) to give three main components (Figure 8); II (R_f 0.11), III (R_f 0.45) and IV (R_f 0.32).

These could be visualised if required by spraying with Ehrlich reagent and heating to 60°C. The required product was recovered from the tlc plates by scraping off the required region, eluting it with acetone, evaporating the solvent and recrystallising from ethyl acetate.

The yields of products obtained were variable. However, OHKPL (II) was always the major photo-product. Typical values were II 25 - 35%, III 8 - 12%, IV 5 - 15%.

The eluted fractions from the chromatography column were also analysed by gas-liquid chromatography. Five microlitres were injected on to the column and the fractions containing components II, III or IV retained for further purification. The purity of the final product was also checked by glc on the same column and found to be in excess of 99%.

<u>3-ethyl-5-hydroxy-4,5-dimethyl-Δ^3-pyrrolin-2-one (OHHPL)</u>

This was produced essentially by the same method as used for OHKPL using haemopyrrole as starting material. The residue from photooxidation was taken up in a small volume of ethyl acetate for column chromatography on silica gel (column 2.5 x 35cm; silica gel 70 - 230 mesh, M. Woelm, Eschwege) using ethyl acetate and ethyl acetate/acetone (60 : 40). The eluted fractions were again examined by glc for the main oxidation products (Figure 9; II, III and IV) and further purification carried out by partition thin layer chromatography

(silica gel F, M. Woelm, Eschwege, 1mm, diethyl ether); II (R_f 0.09) III (R_f 0.25) and IV (R_f 0.43). Yields obtained were very similar to those obtained in the photooxidation of kryptopyrrole.

2.4. PORPHYRIN PRECURSORS AND PORPHYRINS

2.4.1. δ-AMINOLAEVULINIC ACID

ALA was measured in the urine by the method of Mauzerall and Granick (1956).

ALA was removed from a PBG-free urine fraction by retention on an acidic cation exchange resin. After elution from the resin the ALA was condensed with acetylacetone and the resultant pyrrole complexed with p-dimethylaminobenzaldehyde. The resultant coloured complex was measured spectrophotometrically.

Reagents

1. Dowex 2-X8, 200 - 400 mesh. Following the removal of fines by repeated suspension and sedimentation in water with removal of slow sedimenting particles, the resin was converted to the acetate form by washing on a column with 3M sodium acetate till chloride-free (as tested with silver nitrate). The resin was then washed with water until neutral (litmus paper). The resin in its acetate form could be stored for 3 - 4 months at room temperature in about twice its volume of water.

2. Dowex 50-X8, 200 - 400 mesh. Fines were removed as for Dowex 2. The resin was then converted to the sodium form by storage for 20 hours in twice its volume of 2M sodium hydroxide after which it was washed with water until neutral. It was then

reconverted to the acid form by successive treatment with 1 volume of 4M hydrochloric acid and 6 volumes of 2M hydrochloric acid. The resin was then stored for up to 4 months in twice its volume of 1M hydrochloric acid.

3. Ehrlich reagent II - 1g of p-dimethylaminobenzaldehyde was dissolved in ca. 30ml of glacial acetic acid. 8ml of 70% perchloric acid (specific gravity 1.7) were then added and the volume made up to 50ml with glacial acetic acid.

Method

For this analysis custom-made glass columns (1cm x 20cm for Dowex 2, 1cm x 40cm for Dowex 50) plugged with cotton wool were packed by sedimentation with each ion exchange resin to a height previously found to be optimum (2 - 3cm).

Before use the columns were well flushed with water and kept wet at all times. 1ml of urine, adjusted to between pH4 and 7 if necessary, was placed on a Dowex 2 column for the retention of PBG and the column washed after the passage of the urine with water (2 x 2ml). The total eluted material including the washings was quantitatively transferred to a Dowex 50 column and after passage was washed through with 30ml of water to remove urea. The column then was washed with 3ml sodium acetate solution (1M). A further 7ml of 1M sodium acetate was then passed through the column and the eluate collected in a 10ml graduated test tube (with stopper) 0.2ml

of acetyl acetone was then added to the tube and the volume made up to 10ml with sodium acetate buffer, 1M pH 4.6. The tube was then stoppered and placed in a heating block at 100°C for 10 minutes.

When cooled, 2ml of this solution was mixed with 2ml of Ehrlich reagent II and the extinction at 553mm read in a 1cm cell exactly 15 minutes later. The blank consisted of 7ml of 1M sodium acetate treated in the same manner as the Dowex 50 eluate.

A standard curve was constructed by the addition of known amounts of ALA to known volumes of urine. The standards were then analysed according to the method given.

Results were expressed as nmol ALA/L of urine.

2.4.2. PORPHOBILINOGEN

PBG was measured in urine by the method of Mauzerall and Granick (1956).

The PBG was removed from urine by retention on a Dowex 2 anion exchange resin. After elution the PBG was complexed with p-dimethylaminobenzaldehyde and quantitated by a method similar to ALA (previous section).

Reagents

1. Dowex 2-X8, 200 - 400 mesh. See previous section.

2. Ehrlich reagent I. A 2% (^{w}/v) solution of p-dimethylamino-benzaldehyde in 6N hydrochloric acid.

Method

The Dowex 2 resin containing the PBG (see previous section) was eluted successively with 2ml 1M acetic acid and 2ml 0.2M acetic acid. The total eluate was collected and diluted to 10ml with distilled water. 2ml of this was mixed with 2ml Ehrlich reagent I and the extinction read at 555nm after 5 minutes. The blank was 2ml distilled water plus 2ml Ehrlich reagent I.

Results were calculated by a method analagous to that for ALA and expressed as nmol PBG/L of urine.

2.4.3. PORPHYRINS IN URINE

The main porphyrins in urine are uroporphyrin and coproporphyrin, although in certain cases the intermediate penta-, hexa- and hepta- carboxylic porphyrins are also found. Porphyrinogens which also occur were oxidised to the corresponding porphyrin before estimation.

Porphyrins were measured by one of two methods.

1. Solvent extraction followed by fluorimetric or spectrophotometric quantitation. This method, based on that of Rimington (1971), has drawbacks in that hexa- and hepta-carboxylic porphyrins are measured with uroporphyrin and penta-carboxylic porphyrin is measured with coproporphyrin.
2. High performance liquid chromatography (HPLC). This has the advantage of being less time consuming and has the ability to

measure the intermediate porphyrins individually. Based on the method of Gray et al (1976), this method will be covered in a separate section.

Method (solvent extraction)

25ml of urine were placed in a separating funnel and extracted twice with a mixture of 10ml glacial acetic acid and 100ml diethyl ether. The ether extracts were combined and washed with successive 25ml portions of sodium acetate solution (3% $^{w}/v$ hydrated salt) until the aqueous phase no longer showed red fluorescence under ultra violet (UV) light. The organic phase was used for the extraction of coproporphyrin while the combined aqueous phases contained the uroporphyrin.

Coproporphyrin: The combined organic phase was washed firstly by shaking for one minute with 20ml of iodine solution (0.005%, freshly prepared) and secondly with 25ml of distilled water. Coproporphyrin was then extracted, by shaking, from the ethereal solution, with successive 5ml portions of 5% hydrochloric acid until the extract no longer showed red fluorescence under UV light. The total volume of these extracts was recorded, the solution filtered and the fluorescence measured in a broad-band fluorimeter (Locarte Company Ltd.).

Uroporphyrin: The combined aqueous phases were adjusted to pH 1.5 - 2.0 and then extracted twice with 25ml of cyclohexanone.

The combined cyclohexanone fractions were then thoroughly mixed with 100ml diethyl ether and an aqueous phase allowed to separate. This fraction was removed, volumed and mixed with an equal volume of 10% hydrochloric acid. The organic phase was then extracted with successive 5ml portions of 5% hydrochloric acid until no fluorescence showed under the UV light. The acid extracts were combined, volumed and the extinction measured in a 1cm cell at 380 and 430nm and at the Soret maximum (about 406nm).

Calculations

The coproporphyrin concentration in urine was given by :

$$\left[(F.U. \times 0.717) - 0.031\right] \times \frac{A.V}{100} \frac{V}{v} \frac{1}{0.654}$$

= nmol coproporphyrin/24hrs or /L

where F.U. = Fluorimetry units

A.V = Acid volume

V = 24hr urine volume (1000 gives results as per litre)

v = volume of urine extracted

0.717) = Factors relating to standard graph
0.031) (gradient and intercept)

0.654 = Conversion factor for S.I. units

Uroporphyrin concentration was given by :

$$\left[2 \times Emax - (E430 + E380)\right] \times 0.832 \times A.V \times 1.205 \times \frac{V}{v}$$

= nmol uroporphyrin/24hrs or /L

where Emax = Extinction at Soret maximum

E430 = Extinction at 430nm

E380 = Extinction at 380nm

A.V = Acid volume

V = 24hr urine volume (1000 gives results as per litre)

v = Volume of urine extracted

0.832 = "Extinction coefficient"

1.205 = Conversion factor for S.I. units

2.4.4. PORPHYRINS IN FAECES

The main porphyrins found in faeces are protoporphyrin and coproporphyrin. However, in certain pathological conditions the excretion of other porphyrins may become substantial (Gray et al, 1976). As with urinary porphyrins, HPLC has become the method of choice for the measurement of the excretion of individual porphyrin species. Solvent extraction methods, however, provide useful screening tests for excessive porphyrin excretion.

The solvent extraction method for the estimation of coproporphyrin and protoporphyrin is very similar to that for uroporphyrin and coproporphyrin in urine, and is described below. The HPLC method will be covered in a separate section.

Method (solvent extraction)

0.5g and 1g of faeces were taken for estimation of porphyrin and faecal dry weight respectively. The faecal sample for porphyrin estimation was homogenised in ca. 10 ml of glacial acetic acid and then 30 - 40ml of diethyl ether was added and mixed thoroughly. The suspension was then centrifuged at 400g for 3 - 4 minutes, then the supernatant was removed and viewed under U.V light. The extraction was repeated until no fluorescence was seen in the supernatant.

The combined ether-acetic fractions were extracted twice with sodium acetate solution (3% $^{w}/v$ hydrated salt) to remove traces of uroporphyrin, then with 20ml iodine solution (0.005% $^{w}/_{v}$), then 20ml of distilled water. The resultant organic phase contains the coproporphyrin and protoporphyrin.

Coproporphyrin: The organic phase was extracted with successive 5ml portions of 0.1% hydrochloric acid until no fluorescence was present in the aqueous fraction. The acid fractions were combined, volumed and filtered and a portion used for fluorescence estimation of coproporphyrin.

Protoporphyrin: The protoporphyrin was extracted with successive 5ml portions of 5% hydrochloric acid and the acid fractions combined and treated as for coproporphyrin.

Calculations

The coproporphyrin concentration in terms of faecal dry weight was given by :

$$\left[(F.U. \times 0.63) + 0.063\right] \times \frac{A.V}{100} \times \frac{w.w}{d.w} \times \frac{1}{W} \times 1.529$$

= n moles coproporphyrin/g dry wt.

where F.U. = Fluorimetry units

A.V = Acid volume

w.w = Wet weight of faeces

d.w = Dry weight of faeces

W = Weight of faeces used for estimation

0.63)
0.063) = Factors relating to standard graph

1.529 = Conversion factor for S.I. units

for protoporphyrin :

$$\left[(F.U. \times 1.42) - 0.175\right] \times \frac{A.V.}{100} \times \frac{w.w.}{d.w.} \times \frac{1}{W} \times 1.779$$

= n moles protoporphyrin/g dry wt.

Abbreviations are as for coproporphyrin above.

2.4.5. MEASUREMENT OF PORPHYRINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Porphyrins extracted from urine and faeces were quantitated by HPLC on a μ-Porasil column (3.9mm (i.d.) x 30cm) (Waters Associates Inc., U.S.A.). Solvent delivery was made by a dual head reciprocating pump (Model M 6000A, Waters Associates Inc.) capable of delivering 10ml per minute at up to 6000 psi.

Porphyrin esters were detected and quantitated using a variable wavelength U.V. monitor (model CE212 Cecil Instruments Ltd., U.K.) in conjunction with a flatbed pen recorder (model P.M. E.202, Philips). Porphyrin concentrations were estimated by a peak height method or by peak areas as calculated by a digital integrator. Porphyrins in liver tissues were measured by the method for faecal porphyrins following homogenisation of the tissue in the esterification mixture.

Method - Urinary Porphyrins

25ml of urine was mixed with 300ml of a mixture of methanol and sulphuric acid (specific gravity 1.84) (95:5) to esterify the porphyrins. This was left in the dark at room temperature for 24 hours. 2.5 nmoles of mesoporphyrin-IX-dimethyl ester were then added in chloroform to each sample as internal standard and the porphyrin methyl esters extracted with chloroform until no fluorescence showed under U.V. light.

The chloroform extract was then evaporated to dryness under a stream of nitrogen gas (below 40°C). The residue was taken up in 1 - 2ml of chloroform and filtered through a 0.7μm glass microfibre filter and the filtrate blown down to small volume (ca. 0.1ml). About 30μl of this was then taken for chromatography.

The solvent used for the separation of urinary porphyrins was a mixture of 2,2,4-trimethyl pentane (isooctane) and methyl acetate (2:1). The solvent mixture was filtered and degassed

prior to use by passing through a 0.3μm glass microfibre filter under vacuum. It was found that a solvent flow rate of 4ml/minute gave the best separation of porphyrin esters in the shortest time possible (Figure 11).

Calculations

Porphyrin concentrations were calculated from the ratio of peak height (or peak area) with respect to the internal standard. Response factors were obtained from a standard injection containing porphyrins in known concentrations.

Results for individual porphyrins were given by the equation :-

$$\text{nmol IS} \times \frac{\text{PH IS}}{\text{PH X}}(\text{std}) \times \frac{\text{Conc.X}}{\text{Conc.IS}}(\text{std}) \times \frac{\text{PH X}}{\text{PH IS}}(\text{test}) \times \left(\frac{1000 \text{ or } 24\text{h}}{\text{V}}\right)$$

$$= \text{nmol porphyrin/L or /24 hours}$$

where IS = Internal standard mesoporphyrin ester

PH X= Peak height of individual porphyrin

V = Volume of urine extracted

24h = 24 hour urine volume

Method - Faecal Porphyrins

The porphyrin in 0.5g (wet wt.) of faeces was esterified overnight in 50ml of methanol/sulphuric acid mixture (95:5). 2.5 nmoles of protoporphyrin-IX-diethyl ester was added as internal standard and the porphyrin esters extracted with chloroform. Protoporphyrin diethyl ester was produced by

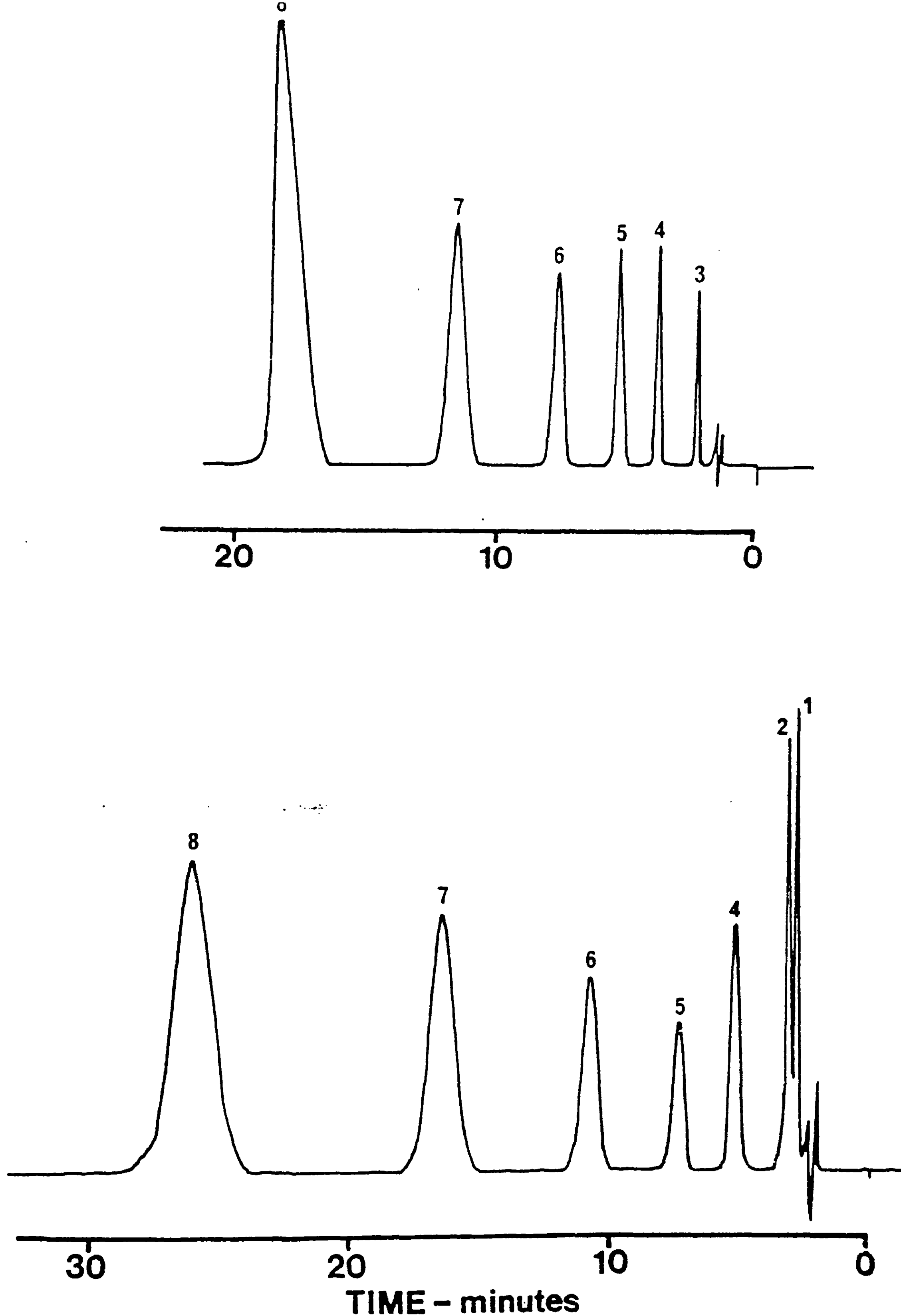


FIGURE 11 SEPARATION OF PORPHYRIN ESTERS BY HPLC

1. Protoporphyrin diethyl ester
2. Protoporphyrin dimethyl ester
3. Mesoporphyrin dimethyl ester
4. Coproporphyrin tetramethyl ester
5. Penta-carboxylic porphytin methyl ester
6. Hexa-carboxylic porphyrin methyl ester
7. Hepta-carboxylic porphyrin methyl ester
8. Uroporphyrin octa methyl ester

hydrolysis (6M HCl, 16 hrs) and re-esterification (ethanol/ sulphuric acid (95:5), 16 hrs) of protoporphyrin dimethyl ester .

The combined chloroform extracts were evaporated to dryness and the residue taken up in 1ml dichloromethane for preparative column chromatography. Columns (0.8 cm i.d. x 10cm) were packed with silica gel (Woelm 70 - 230 mesh) suspended in dichloromethane/methanol (96:4), flushed with 30ml of the same solvent mixture and the porphyrin extract applied. The porphyrins were then quantitatively eluted using the above solvent. The porphyrins in a fast moving band were identified under UV light and were collected in the first 20ml of eluate. Dark brown contaminating pigments remained on the column. The porphyrin containing eluate was evaporated to 0.5ml and a portion of this used for HPLC without prefiltering.

The solvent system used for this separation was n-heptane/methyl acetate (80 : 45) with a solvent flow of 1.5ml/min. A typical chromatogram is shown in Figure 11.

Calculations

Calculation of individual porphyrin concentrations was performed as for urinary porphyrins.

2.5. ENZYMES OF HAEM BIOSYNTHESIS

The enzymes of haem biosynthesis were assayed in rat liver homogenates prepared from livers which had been perfused, in situ, via the portal vein with 25ml ice cold saline (0.15M sodium chloride) prior to excision. Homogenates were prepared in saline using an ultrasonic-mechanical homogeniser (Polytron) at strengths described in the methods for each assay.

2.5.1. δ-AMINOLAEVULINIC ACID SYNTHASE

Assay of the activity of ALA.S was performed by a modification of the methods of Dowdle et al (1967) and Freshney and Paul (1970).

Reagents

Reaction mixture: Phosphate buffer (0.05M, pH 6.8) containing glycine (0.5mM), sodium malate (0.5mM), magnesium chloride (2mM) and pyridoxal phosphate (0.4mM).

Homogenisation mixture: Phosphate buffer (0.12M, pH 7.0) containing glycine (1mM), sucrose (0.5M), magnesium chloride (0.04M), mercaptoethanol (8mM) and EDTA (0.02M).

Method

0.1ml of liver homogenate (1:1 in saline) was mixed with 0.1ml of homogenisation mixture, then 0.2ml of reaction mixture added containing 2-^{14}C glycine (12.5μCi/ml). This was then incubated, stoppered, at 37°C for 1 hour on a shaking water bath.

The reaction was stopped by adding 0.2ml of the total reaction mixture to 40µl carrier ALA solution (1.5M trichloroacetic acid containing ALA (1.9M)). Precipitated protein was spun down at 4°C and 10µl of the resultant clear supernatant spotted on Whatman 3MM paper for electrophoresis.

The ALA and glycine were then separated by electrophoresis in phthalate buffer (0.05M, pH4) run at 54v/cm for 2 hours in water-cooled high voltage electrophoresis apparatus (Locarte).

After electrophoresis the ALA spot was visualised with ninhydrin spray (5% in butanol), excised and burnt in a sample oxidiser (Intertechnique) prior to β counting in a liquid scintillation counter (Packard). Enzyme activity was calculated from the amount of ALA produced and expressed as nmol /ALA produced/g protein/hour.

2.5.2. δ-AMINOLAEVULINIC ACID DEHYDRATASE

This was measured by modification of the standardised EEC method for blood (Berlin and Schaller, 1974).

Reagent

Special Ehrlich reagent: 2.5g p-dimethylaminobenzaldehyde was dissolved in 50ml of glacial acetic acid and 24.5ml of perchloric acid (specific gravity 1.7) added. When cooled the volume was made up to 100ml with glacial acetic acid.

Method

0.2ml of homogenate (1:3 in saline) was diluted with 1.3ml of water and placed in a water bath at 37°C along with an ALA solution (0.01M in sodium phosphate buffer, 0.1M, pH 7.4) for 10 minutes to equilibrate. After the equilibration time 1ml of the ALA solution was added to each reaction tube, the tubes stoppered, mixed and replaced in the bath in total darkness. The assays were run in duplicate with a zero time that was stopped with 1ml of 10% trichloroacetic acid.

After an incubation of 1 hour each tube was stopped with 1ml trichloroacetic acid and the tubes centrifuged to remove the protein precipitate. The supernatant was decanted and respun then 2ml removed and added to 2ml of special Ehrlich reagent. The extinction of the sample was then read after 5 minutes at 555nm against a blank of 2ml trichloroacetic acid and 2ml special Ehrlich reagent.

Results were calculated by using an extinction coefficient of $113mM^{-1}\ cm^{-1}$ for the PBG-Ehrlich complex and results expressed as nmol PBG formed/g protein/hour.

2.5.3. UROPORPHYRINOGEN-I-SYNTHASE

This was assayed by a modification of the method of Frydman and Feinstein (1974).

Method

1ml of homogenate (1:3 saline or 0.15M potassium chloride) was incubated with 0.1ml of PBG solution (0.82mM in sodium phosphate buffer 83mM, pH 7.65) and 1ml of reduced glutathione (3.2mM in phosphate buffer). This mixture was incubated for 1 hour at 37°C in the dark and the reaction stopped by the addition of 9ml of an ethyl acetate/glacial acetic acid mixture (2:1). This was centrifuged at 2000g to remove the precipitate which was then re-extracted with 5ml aliquots of ethyl acetate/ glacial acetic acid until no more porphyrin fluorescence was observed. The total volume of extract was noted and 0.4ml mixed with 5ml of 0.5N hydrochloric acid. The fluorescence of this mixture was measured spectrofluorimetrically to measure the formation of uroporphyrin. Uroporphyrin was estimated as for urinary uroporphyrin (section 2.4.3) and the results expressed as nmol uroporphyrin formed/g protein/hour.

2.5.4. UROPORPHYRINOGEN DECARBOXYLASE

This assay was carried out essentially by the method of Frydman and Feinstein (1974) which involves production of uroporphyrinogen substrate in situ, for further conversion to coproporphyrin.

Method

The assay was set up as for URO.S and the incubation carried out for 1 hour. Thereafter 0,2ml of potassium dihydrogen

orthophosphate solution (0.4M) was added. This changed the pH of the reaction mixture halting the URO.S reaction and allowing the URO.D reaction, which had previously been inhibited, to proceed. The reaction was then allowed to continue for a further hour when it was stopped with 9ml of the ethyl acetate/glacial acetic mixture (2:1) and extractions continued as before until no further porphyrin fluorescence could be extracted from the precipitate. The combined extract was then back-extracted with a saturated solution of sodium acetate until all the uroporphyrin had been extracted into the aqueous phase. The ethyl acetate/glacial acetic acid phase containing the synthesised coproporphyrin was then treated as for the faecal coproporphyrin estimation described earlier (section 2.4.3). The results of the assay were expressed as nmol coproporphyrin produced/g protein/hr.

2.5.5. COPROPORPHYRINOGEN OXIDASE

This assay was performed using a modification of the method of Battle et al (1965).

Method

Coproporphyrinogen was prepared from coproporphyrin by reduction with sodium amalgam (3% $^{w}/v$) under nitrogen in the dark. The coproporphyrinogen was then separated by filtration through a sintered tube into a Buchner flask containing 0.13ml

sodium thioglycollate (2M) and subsequently brought to pH 7.4 with phosphoric acid (40% $^{w}/v$). Before use, this solution was diluted 1 : 1 with Tris buffer (0.01M, pH 7.4). 0.2ml of this solution of coproporphyrinogen were added with 2.5ml of Tris buffer and 1ml of the liver homogenate (1 : 3 in saline or 0.15M potassium chloride) to a 25ml Erlenmeyer flask which was closed with a cotton wool stopper. This was incubated with shaking at 37°C in the dark for 1 hour. The reaction was stopped with 10ml of ethyl acetate/glacial acetic acid mixture (4 : 1), mixed and left to stand in daylight for 30 minutes to convert the residue of coproporphyrinogen to coproporphyrin. Protoporphyrin produced by this reaction was extracted as before and quantified by the method described earlier for protoporphyrin estimation. Results were expressed as nmol protoporphyrin produced/g protein/hour.

2.5.6. FERROCHELATASE

FERRO.C was assayed by the method of Goldberg et al (1956).

Method

A solution of protoporphyrin dimethyl ester was made up (1.7 µmol in 2ml of 7M hydrochloric acid) and left for 18 hours in the dark to hydrolyse. The resulting solution of protoporphyrin was freeze dried and redissolved immediately before use in 10ml sodium hydrogen carbonate solution (0.14M). Incubation was

carried out in Thunberg tubes "in vacuo" at 37°C for 1 hour. Into each tube was placed 0.2ml of reduced glutathione solution (10mM) in Tris buffer (0.45M, pH 8.2) and 0.2ml of ferric chloride solution (16μM) also in Tris buffer, containing $^{59}FeCl_3$ (2μCi). The reaction was started by the addition of 0.2ml of homogenate (1 : 3 in saline or 0.15M potassium chloride) and stopped by the addition of 4ml of haem carrier, prepared by the haemolysis of packed red cells which had been centrifuged to remove cell debris. The whole solution was carefully mixed and the protein and haem precipitated and water removed by the addition of 10ml anhydrous acetone. The mixture was then centrifuged and the supernatant discarded. Haem was then extracted from the precipitate by successive extraction with 4 x 20ml portions of acetone/glacial acetic acid solution (4 : 1). The combined solutions of haem were heated in a water bath at 90°C until all the acetone had evaporated and the resultant solution was left overnight for haem to crystallise. The haem crystals were washed twice with glacial acetic acid, water, absolute ethanol and ether in succession. The crystals were then dried in hot-block at 100°C, weighed and counted in a γ counter. The recovery of haem was calculated from the known haemoglobin concentration and the results expressed as % ^{59}Fe incorporation into haem/mg protein/ hour.

2.6 QUALITATIVE DETECTION OF "KRYPTOPYRROLE"

This method is based on the method of Irvine (1961) for the detection of what was then thought to be kryptopyrrole but as has been stated (section 1.4.1.) is now known to be OHHPL. As will be seen the method is very non-specific and is of little use other than as a screening test.

Fresh urine (100ml) was mixed with 2g of activated charcoal, allowed to stand for 2 hours and then filtered in a Buchner funnel. After the charcoal was washed with 80ml of distilled water the "kryptopyrrole" was eluted with 100ml of acetone, which was evaporated in vacuo at 30°C. The residue was taken up in water and extracted three times with 3.5ml of peroxide-free diethyl ether. Acetone (2.2ml) was then added and the mixture extracted twice with 6.6ml of ether. Both ether fractions were flash-evaporated at 30°C, the residue being dissolved in 2ml of acetone and then applied to Whatman 3MM chromatography paper. A standard of synthetic kryptopyrrole (400 nmol) was applied at the same time and ascending chromatography run for 12 hours in the solvent system propan-2-ol/water/ammonia solution (sp. gr. 0.88) (20:2:1). The chromatograms were dried in cool air and the "kryptopyrrole" was visualized as a purple-blue spot (R_f value 0.9) by development in Ehrlich's reagent (1g of p-dimethylaminobenzaldehyde dissolved in hydrochloric acid/ methanol, 1:9, $^v/v$) followed by heating at 55°C.

The gas-liquid chromatography method for the quantitation of OHHPL will be covered in a later section.

2.7. MICROSOMES AND MICROSOMAL HAEM AND CYTOCHROMES

2.7.1. PREPARATION OF MICROSOMES

Tissue for the preparation of microsomes was kept on ice after removal until it was homogenised in a 1:1(^{v}/w) volume of ice cold 0.15M potassium chloride using an ultrasonic mechanical homogeniser (Polytron). All further procedures were carried out at 4°C. Cell debris, nuclei etc. were first removed by centrifugation for 15 minutes at 900g. The resulting supernatant was then spun at 17,500g for 15 minutes on a preparative ultracentrifuge to remove mitochondria. The post-mitochondrial supernatant was spun at 105,000g for 1 hour to remove the microsomes, the supernatant being discarded.

The microsomal pellet was washed and resuspended in 0.15M potassium chloride using a Potter-Elvehjem homogeniser and again centrifuged at 105,000g for 1 hour. The final washed microsomal pellet was resuspended in 0.15M potassium chloride (ca. 7ml) and a protein estimation carried out on the suspension. This suspension was then used for the estimation of microsomal haem and cytochromes.

2.7.2. ESTIMATION OF MICROSOMAL HAEM

Microsomal haem was measured by the pyridine haemochromogen method of Paul et al (1953).

Method

2ml of the final microsomal suspension was taken and to it was added 1ml pyridine and 2ml sodium hydroxide solution (0.25M) and the mixture shaken thoroughly. The resultant solution was then divided equally between two glass cuvettes and 5mg of sodium dithionite added to the test cuvette and mixed. The difference spectrum was then recorded in a dual beam spectrophotometer (SP 8000, Pye Unicam Ltd.) between 550 and 575nm.

Calculation

The haem concentration is calculated using an extinction coefficient of $32.4 mM^{-1} cm^{-1}$ as follows :-

$$\frac{\Delta E \times 77.16}{a} = \text{nmol/mg protein}$$

ΔE = difference in extinction between 557 and 575nm

a = protein concentration of microsomal suspension

2.7.3. ESTIMATION OF MICROSOMAL CYTOCHROME P-450 AND CYTOCHROME b_5

Cytochrome P-450 and Cytochrome b_5 were measured by the method of Omura and Sato (1964).

Method

4ml of potassium phosphate buffer (0.15M, pH 7.0) was added to 2ml of microsomal suspension to give a final protein concentration

of approximately 2mg/ml. This mixture was then split equally between two glass cuvettes.

50µl of a 12mM NADH solution in phosphate buffer was then added to the test cell to reduce cytochrome b_5 and the difference spectrum recorded in the range 400 - 500nm.

Using the same cells, carbon monoxide was bubbled through the test cell for 15 seconds. 5mg of sodium dithionite was then added to both the test and reference cells and carbon monoxide again bubbled through the test cell for 15 seconds. The cytochrome P-450 difference spectrum was then recorded in the range 400 - 500nm.

Calculation

Cytochrome b_5 concentration was expressed in terms of the difference in extinction between 423 and 500nm per unit of protein :-

$$\frac{\Delta E\ (423 - 410)}{\text{protein concentration}} \quad mg^{-1}$$

Cytochrome P-450 concentration was expressed as follows :-

$$\frac{\Delta E \times 3000}{\varepsilon \times d \times c} \quad \text{nmol Cyt.P-450/mg microsomal protein}$$

where ΔE = difference in extinction between 450 and 490nm of the difference spectrum.

ε = molar extinction coefficient of cytochrome P-450 between 450 and 490nm on the difference spectrum = $91 cm^{-1}\ mM^{-1}$

d = light path = 1cm

c = protein concentration of microsomal suspension

2.8. MICROSOMAL OXYGENASES

2.8.1. PORPHOBILINOGEN OXYGENASE

The oxidation of PBG by microsomal enzymes was measured essentially by the method of Frydman et al (1973) and Tomaro et al (1973) using an unpurified enzyme preparation.

Reagent

Reaction mixture: This was 0.1M sodium phosphate buffer, pH 7.4 containing porphobilinogen (0.26mM) and either sodium dithionite (2mM) or NADPH (0.8mM) plus a NADPH-regenerating system (nicotinamide (0.1M), glucose-6-phosphate (10mM), glucose-6-phosphate dehydrogenase (5μg/ml) and magnesium chloride (20mM)).

Method

The final microsomal pellet was resuspended in sodium phosphate buffer (0.1M, pH 7.4) to give a final protein concentration of 10 - 20mg/ml. 1ml of this suspension was mixed with 1ml of reaction mixture and the incubation performed at 37°C for 1 hour. When NADPH plus the regenerating system was used the reaction mixture was preincubated for 10 minutes before the addition of microsomal suspension. Blanks were run simultaneously omitting dithionite or microsomal suspension.

The reaction was stopped by the addition of 1ml of 0.3M trichloroacetic acid and the precipitated protein spun down.

Substrate consumption was assayed by the addition of 1ml of Ehrlich reagent II followed by measurement of the extinction at 553nm 15 minutes later. Porphobilinogen was estimated with reference to a standard graph drawn up with known PBG concentrations and results were expressed as nmoles PBG utilised/g protein/hour.

When the radiochemical assay was used the unlabelled substrate PBG was substituted with the radiolabelled PBG described in section 2.3.1. The assay was incubated and stopped as before but on removal of the protein precipitate the pH of the reaction mixture was adjusted to between 4 and 7 using saturated sodium bicarbonate solution. The PBG and reaction products were then removed from the reaction mixture by retention on Dowex 2 ion exchange resin as for urine samples. The eluted material was then frozen and lyophylised.

The dried material was then taken up in a small volume of ethanol : acetone (1:1) and applied to thin layer chromatography plates (precoated microcrystalline cellulose, 0.5mm) and run using the upper layer of a butanol : acetic acid : water mixture (4:1:5) as developer. PBG and reaction products (Figure 12) were visualised by spraying with an Ehrlich reagent (2% p-dimethylaminobenzaldehyde in methanol : HCl (9:1)). PBG, purple R_f 0.63; 2-keto-PBG, orange R_f 0.51; 5-hydroxy-2-keto-PBG, yellow R_f 0.39. The formation of each product could be quantitated as follows. For each compound the area of the plate

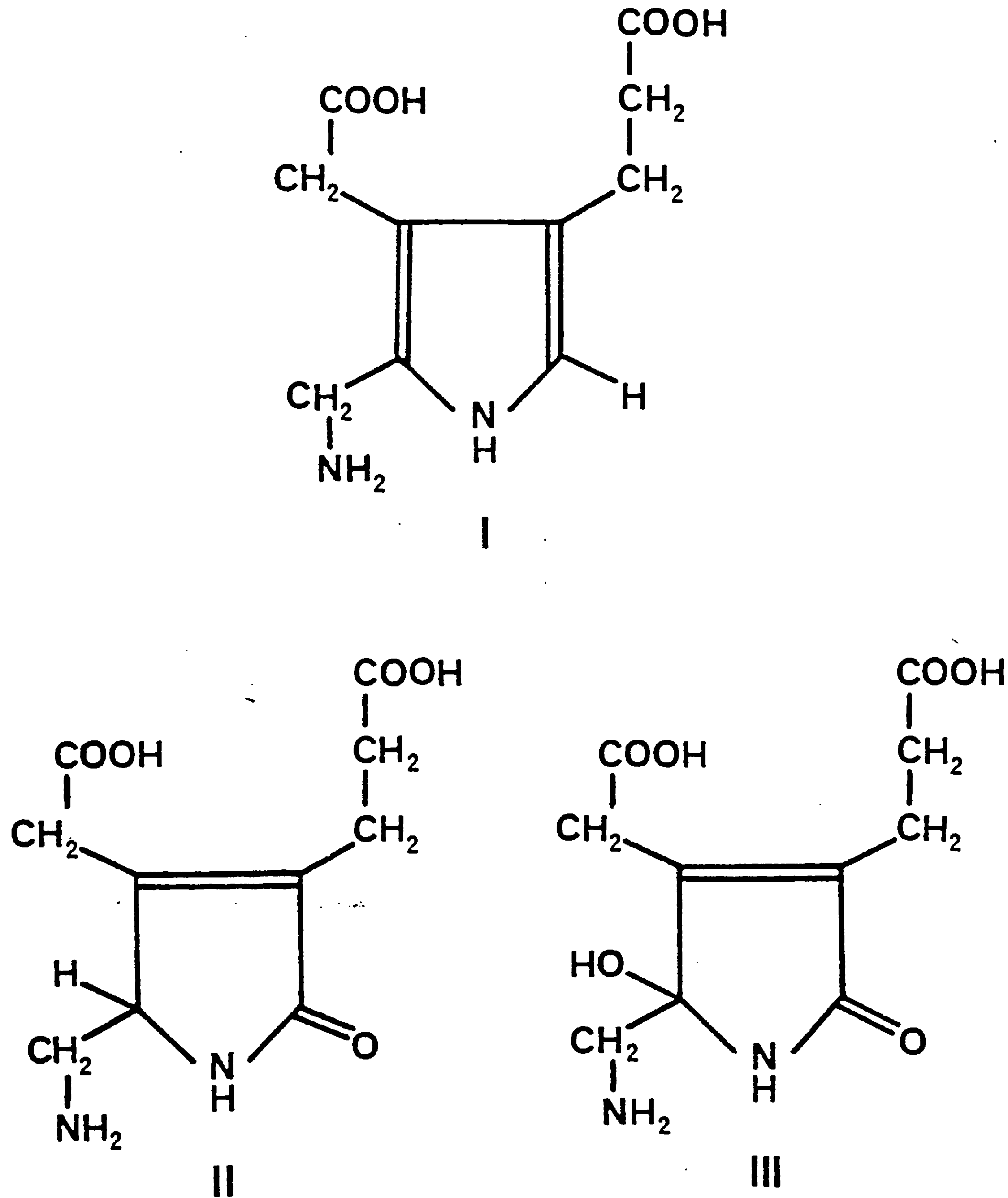


FIGURE 12 PORPHOBILINOGEN AND ITS OXIDATION PRODUCTS

1. Porphobilinogen
2. 2-ketoporphobilinogen
3. 2-keto-5-hydroxyporphobilinogen

was removed, placed in a polycarbonate combustion capsule and burnt in a sample oxidiser followed by β-counting. The amount of product could be calculated from the ratio of counts in the product and PBG spots knowing the original PBG concentration.

2.8.2. HAEM OXYGENASE

The activity of haem oxygenase was measured by a modification of the method of Maines and Kappas (1975).

Method

Reaction mixture: Potassium phosphate buffer (0.11M, pH 7.4) containing glucose-6-phosphate (1.3mM) magnesium chloride (3mM), glucose-6-phosphate dehydrogenase (7.5μg/ml) and haem (25μM).

The final microsomal pellet was resuspended in phosphate buffer (0.1M, pH 7.4) to give a final protein concentration of 10 - 20mg/ml. 4ml of reaction mixture and 1ml of microsomal suspension were preincubated for 5 minutes at 37°C. Thereafter the mixture was divided into two equal aliquots. To the test aliquot NADP (final concentration 0.8mM) in bicarbonate solution (1% $^{w}/v$) was added whilst to the reference incubation mixture sodium bicarbonate alone was added. The incubations were carried out for 15 minutes after which period the reaction was stopped by the addition of 2ml of the reaction mixture to a

tube containing 2ml of 0.25M NaOH and 1ml of pyridine. Haem consumption was then measured by the method of Paul et al (1953) (section 2.7.2).

The results were expressed as nmol haem oxidised/mg microsomal protein/hour.

2.9. THYROID FUNCTION TESTS

Serum total thyroxine (T4) and triiodothyronine (T3) were determined by semi-automated radioimmunoassay methods (Challand et al, 1975) employing a second antibody separation (Ratcliffe et al, 1974). The free thyroxine index (FTI) was calculated from the result of the total T4 and the thyroid hormone binding capacity (Thyopac-3, Radiochemical Centre, Amersham, U.K.) (Clark and Brown, 1970). 3,3',5'-triiodothyronine (reverse T3) was assayed in unextracted serum (Ratcliffe et al, 1976) while serum thyroid stimulating hormone (TSH) was measured by a method based on that of Hall et al (1971). Free thyroid hormone levels (FT3, FT4) were measured directly by radioimmunoassay of a serum dialysable fraction (Yeo et al, 1977a). Normal values of these parameters are listed in Table 2. Free thyroid hormone concentrations in pregnancy were taken from Yeo et al (1977b). A standard thyrotrophin releasing hormone (TRH) test was employed (Ormston et al, 1971).

TABLE 2 NORMAL RANGES OF THYROID FUNCTION TESTS

Test	Abbreviation	Range	Units
Total Thyroxine	T4	55 - 144	nmol/L
Total Triiodothyronine	T3	0.9 - 2.8	nmol/L
Free Thyroxine index	FTI	50 - 145	-
Total Reverse Triiodothyronine	rT3	0.15 - 0.42	nmol/L
Thyroid stimulating hormone	TSH	0 - 8	mU/L
Free Thyroxine (non-pregnant)	FT4	4.1 - 16.7	pmol/L
Free Thyroxine (pregnant)	FT4	5.6 - 12.0	pmol/L
Free Triiodothyronine (non-pregnant)	FT3	4.5 - 15.7	pmol/L
Free Triiodothyronine (pregnant)	FT3	2.1 - 11.8	pmol/L

2.10. ADDITIONAL METHODS

2.10.1. PROTEIN ESTIMATION

Protein estimations were made by the method of Lowry et al (1951).

2.10.2. URINARY CREATININE

Urinary creatinine was determined by routine auto-analysis.

2.11. STATISTICAL METHODS

Where applicable the results were calculated and expressed as mean ± standard deviation (S.D.), the standard deviation being calculated from the expression :-

$$\text{S.D.} = \sqrt{\frac{\sum(x - \bar{x})^2}{N - 1}}$$

where x was any of the values measured, $\bar{x}$ the mean value, and N the number of observations.

The significance of the results was calculated by Students t test using the formula :-

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

where s was an estimate of the combined standard deviation of both groups calculated from :-

$$s^2 = \frac{\sum x - \bar{x}_1)^2 + \sum(x - \bar{x}_2)^2}{N_1 + N_2 - 2}$$

the degrees of freedom $F = N_1 + N_2 - 2$

$\bar{x}_1$ and $\bar{x}_2$ were the means of both groups and N_1 and N_2 the number of observations in each group.

In some studies where a normal distribution was not assumed the non-parametric Mann-Whitney U test was used to examine significance. This test was based on the formula :-

$$U = N_1N_2 + \frac{N_1(N_1 + 1)}{2} - R_1$$

or $$U^1 = N_1 N_2 + \frac{N_2(N_2 + 1)}{2} - R_2$$

where R_1 = the sum of the ranks assigned to the group whose sample size was N_1 and R_2 = the sum of the ranks assigned to the group whose sample size was N_2. Of the two values obtained from these equations it was the smaller value of U that was used to test significance.

The parametric least squares regression analysis of Pearson was used to analyse linear relationships. With n pairs of associated observations represented by (x, y) the true regression line for the regression of y on x is :-

$$y = A + Bx$$

$$B = \frac{\sum(x - \bar{x})\ (y - \bar{y})}{\sum(x - \bar{x})^2}$$

$$A = \bar{y} - \bar{x}\ B$$

and the correlation coefficient r :-

$$r = \frac{(x - \bar{x})\ (y - \bar{y})}{\sqrt{\left[\sum(x - \bar{x})^2\right]\ \left[\sum(y - \bar{y})^2\right]}}$$

Non-parametric correlation analysis was carried out using the two ranking tests of Spearman and Kendall.

In both tests the values of x and the values of y were ranked separately. In the Spearman test the difference in the value of the ranks of x and y (d) for each of the N x, y pair was summed ($\sum d$). Thus the equation for the Spearman rank correlation

coefficient was as follows :-

$$r_s = 1 - \frac{6\sum d^2}{N^3 - N}$$

In the Kendall rank correlation test the ranks of x were arranged in natural order (i.e. 1, 2, 3 ....N). The values of the ranks of y were arranged under their corresponding value of x. The value of S was then determined by starting at the first value of the rank of y on the left and counting the number of ranks to its right which were larger and subtracting the number of ranks to its right which were smaller. This was done for all ranks of y and the sum of the results corresponded to S. Given S the value of the Kendall rank correlation coefficient was :-

$$\tau = \frac{S}{0.5N\ (N - 1)}$$

The probability associated with r_s could be found directly from tables. However for τ values for $N > 10$, Z was calculated as follows :-

$$Z = \frac{\tau}{\sqrt{\frac{2(2N + 5)}{9N\ (N - 1)}}}$$

and the probability associated with values of Z obtained from the appropriate tables.

Significance was assumed in both of these tests if the probability of an event occurring on the basis of the null hypothesis was less than 5 in 100 ($p < 0.05$).

Where analyses involved large numbers of observations the ranking tests, Mann-Whitney U, Spearman and Kendall, were carried out on the Northumbrian Universities Multiple Access Computer (NUMAC). Examples of each are shown in Appendix 1.

CHAPTER 3

QUANTITATIVE DETERMINATION OF 3-ETHYL-5-HYDROXY-4,5-DIMETHYL-Δ^3-PYRROLIN-2-ONE BY GAS-LIQUID CHROMATOGRAPHY

CHAPTER 3

QUANTITATIVE DETERMINATION OF 3-ETHYL-5-HYDROXY-4,5-DIMETHYL-Δ^3-PYRROLIN-2-ONE BY GAS-LIQUID CHROMATOGRAPHY

3.1. INTRODUCTION

Since the discovery of the mauve factor, even before its unequivocal identification, attempts have been made to quantitate it. Most of these previous attempts have involved reaction of the pyrrolic metabolites with Ehrlich's aldehyde reagent. Irvine in 1971 made semiquantitative estimations of each of the oxidised components after reaction with Ehrlich reagent, using integrating densitometry. This method was applied after two dimensional chromatography, a process which itself did not maintain the integrity of the urinary metabolite (see section 1.4.1.).

Other attempts have involved integrating ion current mass spectral quantitation (Boulton and Majer, 1970) and the complexing of "kryptopyrrole" with Ehrlich's aldehyde or ^{14}C-formaldehyde (Wetterberg and Formgren, 1976). Neither of these methods have provided consistent results.

It was obvious that the successful establishment of a suitable method for the quantitation of OHHPL would allow, and was essential for, a greater in-depth investigation into the natural occurrence than had before been possible.

The method of quantitation chosen, gas-liquid chromatography (glc) reflects the availability of apparatus as well as

suitability considering the physio-chemical properties of the compounds in question. It combines the required sensitivity and specificity with proven capability and precision.

A method is described for the quantitation of OHHPL in the urine of human subjects using a simple solvent extraction method. This method was found to be inadequate for plasma. However, a similar method was used for the quantitation of OHHPL in the serum and plasma of experimental animals treated with OHHPL and consequently had very high levels. In addition OHKPL could be measured using OHHPL as an internal standard also in experimental animals.

3.2. METHOD

3.2.1. APPARATUS

Gas liquid chromatography was performed on a Pye 104 series, dual channel gas chromatograph (Pye Unicam Ltd., Cambridge, U.K.) equipped with dual flame ionisation detectors and injection port heaters.

Output from the glc amplifier was connected to a Philips PM 8000 flatbed recorder.

The carrier gas used was oxygen-free nitrogen, used without pre-treatment and the detectors were supplied with high purity hydrogen and compressed air.

Glass columns either 1.7m or 2.8m long x 4mm i.d. were supplied by Pye Unicam and injections were made with a standard 5µl glass syringe (S.G.E. Ltd., Melbourne, Australia).

Packing of Columns

The stationary phase chosen for chromatography was Carbowax 20M (polyethylene glycol 20M) and the support was Gas Chrom. Q, 100 - 120 mesh. Column packings were either bought pre-coated or prepared as follows : For the coating of 25g support 100ml (4 x 25) of a solution of Carbowax in chloroform was prepared at the same concentration required on the final packing. The support was then rapidly added to the solution and dispersed with a minimum of stirring. The mixture was then

vacuum filtered on a Buchner funnel and the coated support removed and oven dried.

Columns were pre-treated with dimethyldichlorosilane in toluene (5% $^{w}/v$) for 24 hours. Prior to packing they were washed with methanol and air dried. This process ensured the removal of 'active sites' on the interior glass surface that might otherwise reduce the efficiency of the column. The column was packed under suction and the packing settled with a vibrating spatula (Mettler). The open column end was then plugged with glass wool which had been previously acid washed and silanised.

Before use the columns were conditioned by heating to 250°C for 24 hours in the chromatograph without connection of the distal end to the detector. This ensured the removal of volatiles that may have otherwise contaminated the detector.

3.2.2. STANDARDS

As a safeguard against variable recovery during extraction and to eliminate the need for accurate aliquot measurement during the extraction procedure, use was made of an internal standard added to the urine or plasma before extraction. For this purpose one of two compounds was used.

Standard A: OHKPL. This was produced and purified as in section 2.3.3.

Standard B: 5,5-dimethyloxazolidine-2,4-dione (DMO) (Figure 13).

This was produced and kindly donated by Dr. F. Rowell (Sunderland Polytechnic, Sunderland).

Both of these compounds fulfilled the requirements necessary for use as an internal standard. These were :-

1. Linearity of detector response over the range used.
2. Conservation of internal standard/OHHPL concentration ratio throughout the extraction.
3. Ease of separation from OHHPL by glc.
4. Absence of contaminating peaks on glc.
5. Similarity of structure to OHHPL.

The obvious choice for internal standard was OHKPL (Standard A) the ββ' isomer of the natural metabolite. The likeness in structure that ensured the identical behaviour during the extraction also however made the separation of the two compounds by glc difficult. Separation could, however, be effected using lower temperatures and longer columns. This inevitably resulted in cycle times of around 30 minutes.

Cycle times could be reduced by the use of DMO (Standard B) as internal standard. This was resolved using shorter columns and higher temperatures, with cycle times of about 10 - 12 minutes. For this reason the majority of urine analyses were performed using DMO as internal standard. Occasional urines were encountered with interference in the region of the chromatogram at which DMO was eluted. These could usually be analysed if required using OHKPL as standard. Plasma assays all required the use of OHKPL as internal standard.

3.2.3. STABILITY

The stability of OHHPL in urine was found to be poor although this was pH dependent. The half-life of the compound in urine was found to be in the order of 10 - 12 hours.

The variability of stability of OHHPL in urine precluded the use of 24 hour specimens and spot samples were used.

Degradation of OHHPL or either of the internal standards during the assay procedure was negligible.

3.2.4. EXTRACTION PROCEDURE

Urines

Fresh urine was used immediately or deep frozen (-25°C) until use, when 10ml were taken and thoroughly mixed with 10ml of ethyl acetate containing the internal standard (25 nmol standard A, or 700 nmol standard B). The samples were then extracted twice with 20ml of peroxide free diethyl ether, and the combined organic phases evaporated to dryness (below 40°C, under nitrogen). The residue was taken up in 50µl of ethanol for chromatography.

Experimental Plasmas and Serum

These were samples from animals which had been treated with OHHPL and had large amounts of OHHPL in the plasma. Serum or plasma was de-proteinised with an equal volume of

propanol containing OHKPL as internal standard, the samples centrifuged to remove the precipitate and the supernatant lyophylised. The residue was extracted with a small volume of ethanol and a portion taken for glc.

3.2.5. CHROMATOGRAPHY

For chromatography 1 - 5 µl of the final ethanolic extract was injected on to the glc columns.

The optimum conditions for sensitivity and resolution of standards were as follows :-

i) For Standard A (2.8m column), column temperature $190^{\circ}C$ (isothermal); injection port temperature $230^{\circ}C$; carrier gas flow rate 100ml/min.

ii) For Standard B (1.7m column), column temperature $230^{\circ}C$ (isothermal); injection port temperature $250^{\circ}C$; flow rate of carrier gas (oxygen-free nitrogen) 50ml/min.

For both, detector gas flow rates: air 360ml/min, hydrogen 75ml/min.

The amplifier attenuation setting usually employed was x 200 (detector response of 2×10^{-10} amp f.s.d.), although this could be altered instantaneously as conditions required.

3.2.6. CALCULATION

From standard chromatograms the response factor (peak height/quantity injected) of OHHPL and the internal standard were calculated.

The concentration of OHHPL in each test is then given by :

$$\frac{\text{PH OHHPL}}{\text{PH IS}} \text{(test)} \times \frac{\text{RF IS}}{\text{RF OHHPL}} \times \frac{a}{V} \times \frac{1000}{155} = \mu\text{mol/L}$$

where PH IS = Peak height of the internal standard

RF = Response factor

a = Amount of internal standard added (μg)

V = Volume of urine or plasma extracted

Results for urines were expressed in terms of creatinine concentration by dividing the above result by the creatinine result (mol/litre).

3.3. EVALUATION OF THE METHOD

Prior to the use of the assay for the determination of OHHPL in biological fluids an evaluation of its reliability was made. It had to be shown that the requirements made of the choice of internal standard (section 3.2.2) were fulfilled and also that in its final form the assay had adequate specificity, precision and accuracy for the purposes for which it was intended.

3.3.1. ASSESSMENT OF INTERNAL STANDARDS

Similarity in Structure

The structures of OHKPL and OHHPL are shown in Figures 8 and 9 respectively and that of DMO is shown in Figure 13. The similarity between the two pyrroles is obvious but the similarity between DMO and OHHPL is not so readily so. The two compounds do, however, have certain features in common. They are both heterocyclic with a ring nitrogen. They also have two substituent oxygen functions and substituent alkyl groups. The DMO has an extra ring oxygen not present in the pyrroles.

Linearity of Detector Responses

This was checked by the injection of varying amounts of each compound, amounts extending far beyond the range expected to be encountered. The detector response, guaged as peak height,

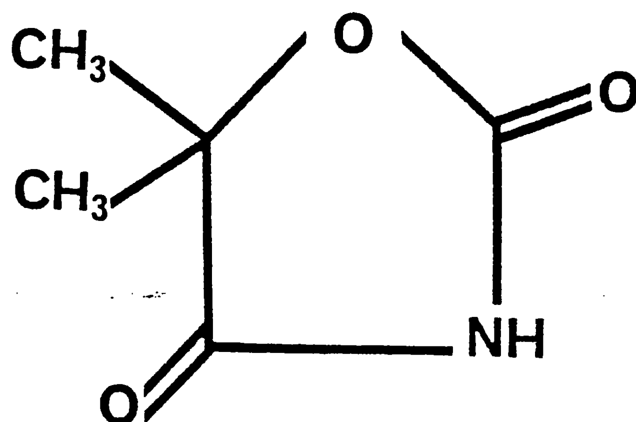


FIGURE 13 5,5-Dimethyloxazolidine-2,4-dione,
INTERNAL STANDARD FOR GLC

was shown to be linear over the range used for all three compounds (Figure 14). As would be expected the responses to OHHPL and OHKPL were very similar while that to DMOD was about 30-fold less. This was partly due to the peak shape of DMO , which for equivalent injections was broader and thus for equivalent area responses should be shorter. This, however, was not enough to account for the large differences observed in this parameter.

Conservation of OHHPL/Internal Standard Ratio

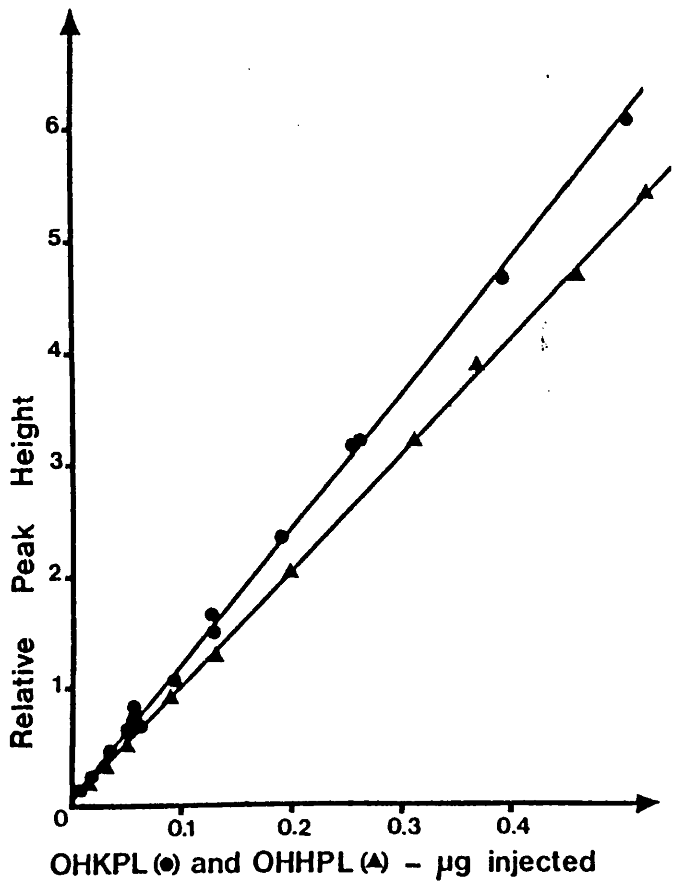
The extraction procedure for urines, basically a single solvent extraction, make the requirements placed upon the internal standard less stringent due to its simplicity. As both DMO and OHHPL are extracted in excess of 95% the errors arising from variations in the ratio at this stage are minimal. The extraction properties of OHHPL and OHKPL in all systems used are essentially identical.

Ease of Separation

Figure 15 shows the separation achieved for both OHKPL and DMO . In both cases are these adequate.

Absence of Contamination

This was assessed by the same methods used to test the specificity of the assay (section 3.3.2). Occasional samples were encountered with interference at the points interest on



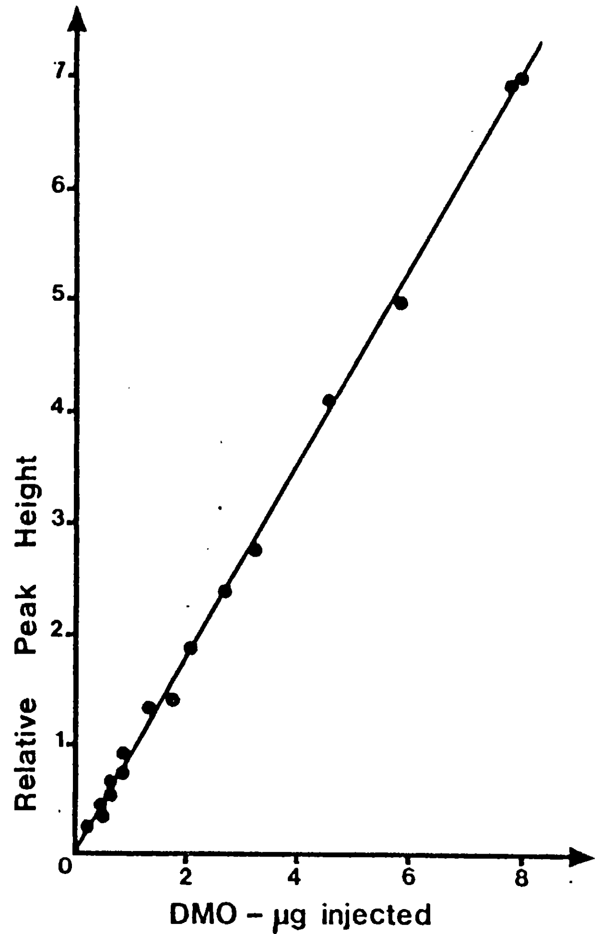


FIGURE 14 DETECTOR RESPONSE LINEARITY FOR OHKPL, OHHPL AND DMO

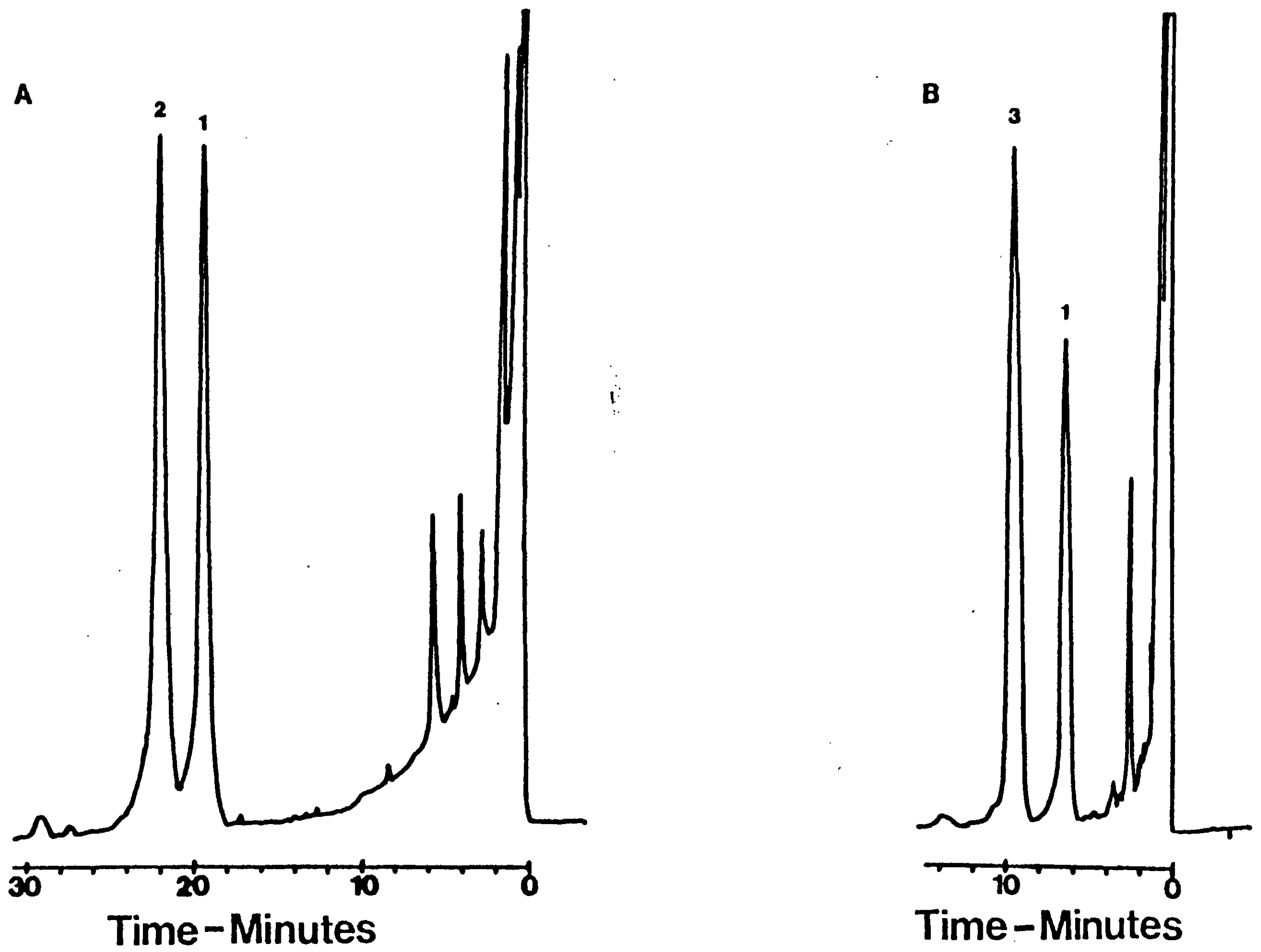


FIGURE 15 STANDARD GLC CHROMATOGRAMS FOR THE SEPARATION OF OHHPL (1) and OHKPL (2) OR OHHPL AND DMO (3) FROM URINE EXTRACTS

the chromatogram. For the most part this could be avoided by the use of the alternative internal standard.

3.3.2. SPECIFICITY

The specificity of the method depends on the extraction of OHHPL and standards free from compounds of similar chromatographic behaviour. Evidence for the lack of interference from solvents and reagents was obtained by the analysis of distilled water containing added OHHPL. The final glc traces of these extracts showed no extraneous peaks either from solvent or from breakdown of standards during the procedure.

The absence of peaks interfering with the OHHPL on the test chromatograms was checked by on-column methylation of these components, observing quantitative migration of the test peak. When OHHPL was added to a specimen which showed an OHHPL peak no new peaks were found, only an increase in the OHHPL peak height was observed. Omission of internal standards failed to show any other components with identical retention times.

Occasional samples were encountered with unknown peaks incompletely resolved from the OHHPL or standards. In the case of interference with internal standard, the sample could be re-analysed using the alternative standard. If this occurred with the OHHPL peak the estimation had to be discounted. The occurrence of this was low and could not be correlated with

any group of patients. The random occurrence of this allowed the omission of the subject from any investigative series.

3.3.3. PRECISION

The overall precision of the method was determined by the replicate extractions (10) of the same blank urine or plasma (i.e. urine in which OHHPL was undetectable or plasma from control animals) to which synthetic OHHPL had been added. The coefficients of variation for the assays for each standard are shown in Table 3.

The same assays allowed calculations of recovery of each of the compounds from urine or plasma. Results of these are also shown in Table 3.

3.3.4. SENSITIVITY

The sensitivity of the assays as they stand was 0.1 μmol/L for urine and 25 μmol/L for experimental plasma. This was gauged from the peak height twice the background noise.

TABLE 3 PRECISION AND RECOVERY OF OHHPL ASSAY FOR URINE AND PLASMA (EXPERIMENTAL)

		Internal standard used	
		OHKPL	DMO
Urine	Coefficient of variation	5.1%	7.4%
	Recovery	94-97%	92-95.5%
Plasma	Coefficient of variation	5.4%	-
	Recovery	84-93%	-

3.4. NORMAL VALUES

The evaluation of the assay was followed by a study of normal subjects. These were either healthy members of laboratory or ward staff, or normal siblings of porphyric patients.

A total of 82 subjects were studied of whom 16 were rejected because of administration of the contraceptive pill. Although mean values or urinary OHHPL excretion in these subjects was greater than matched controls (0.59 ± 0.46µmol/L compared with 0.34 ± 0.33µmol/L) the difference was not statistically significant. The remaining 66 were made up of 38 females (age 17 - 58) and 28 males (age 22 - 64).

Urinary excretion of OHHPL in these subjects is shown in Figure 16. Values were expressed in terms of creatinine concentration because of the use of spot urine samples. The distribution found by plotting OHHPL concentrations in terms of urine volume was almost identical. The group, 0 - 33µmol/mol creatinine, (32 subjects) includes 15 subjects in whose urine OHHPL was undetectable.

The normal range of OHHPL excretion taken as 95% of the normal distribution was 0 - 1.7µmol/L or 0 - 217µmol/mol creatinine.

There were no significant sex-related or age differences in excretion, although group numbers in the latter analysis were small and there was some variation in mean values (Table 4).

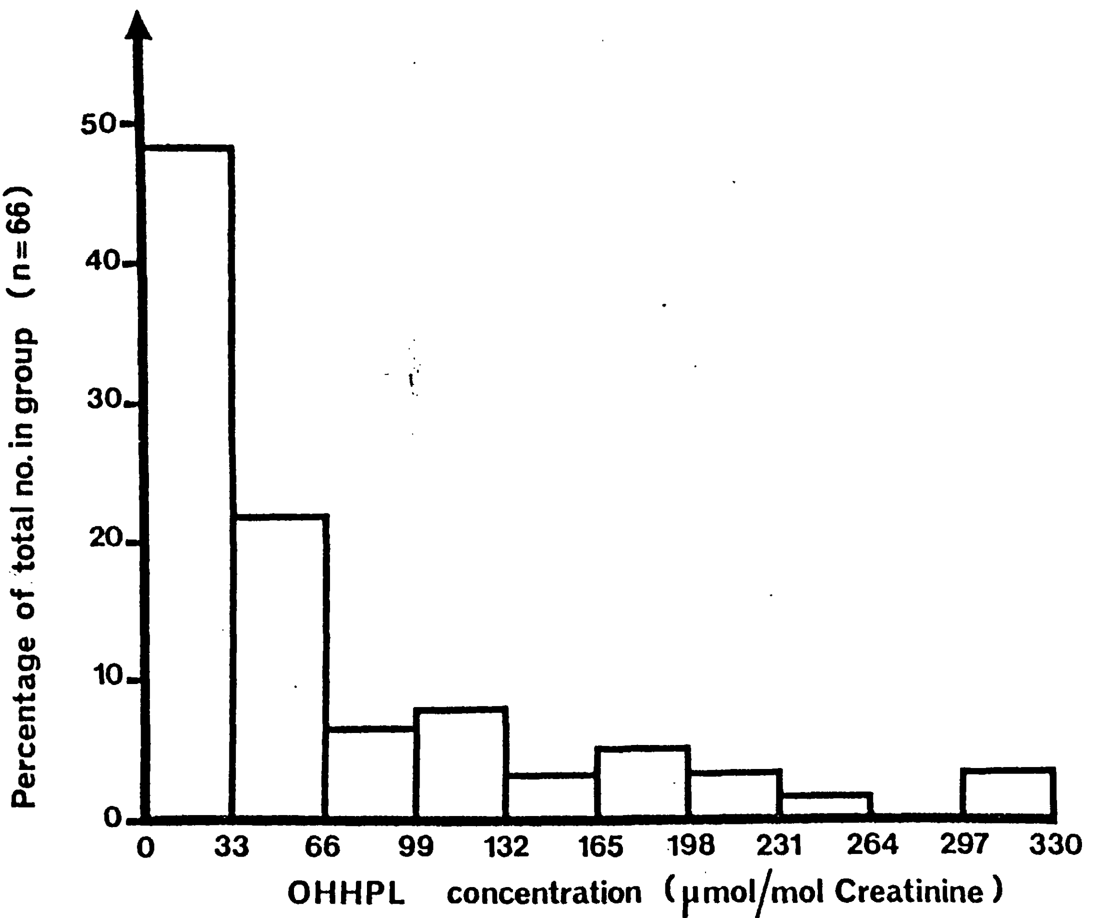


FIGURE 16 DISTRIBUTION OF URINARY OHHPL LEVELS IN NORMAL SUBJECTS

The group 0 - 33 contains 15 subjects in whose urine OHHPL was undetectable

TABLE 4 AGE AND SEX VARIATIONS IN URINARY OHHPL EXCRETION

			AGE GROUP				
		Total	0-20yrs	21-30yrs	31-40yrs	41-50yrs	>51 yrs
Male	mean	0.53	0.45	0.51	0.54	0.63	0.37
	S.D.	0.59	0.18	0.63	0.57	0.47	0.42
	No. in group	28	2	10	10	4	2
Female	mean	0.61	0.88	0.55	0.63	0.57	0.27
	S.D.	0.56	1.03	0.52	0.41	0.55	0.2
	No. in group	38	4	16	11	5	4

3.5. DISCUSSION

The major problem encountered in the development of the assay for urine was the instability of OHHPL under certain conditions. Extraction procedures had to avoid acidic conditions or the presence of methanol with which the OHHPL could be methylated. Cold storage (-20°C) overcame any instability in urine while the solvent extraction method reduced the possibility of losses through degradation. The simplicity of the extraction methods, however, limit the sensitivity which can be attained by the assay. Gas liquid chromatography has the potential for much greater sensitivity than that used in the assays. Increased sensitivity in this case would require a much greater purification of the substances of interest. This was particularly true in the case of the plasma assay. When human plasma was extracted there was gross contamination at the areas of interest on the chromatogram. This could not be overcome by variations in the chromatographic conditions such as temperature programming and any other purification procedures have the attendant risks of degradation. It may be that a method with more specific detection capabilities such as glc with nitrogen detection or glc coupled to mass spectrometry may be required to achieve the required sensitivity and specificity.

The method used for serum or plasma from experimental animals had low sensitivity because there was little concentration

of OHHPL during extraction. This however meant that there was little interference from other substances. It allowed satisfactory quantitation of OHHPL and OHKPL in animals given relatively high doses of these substances.

As it stands, the urine method allows, for the first time, the absolute quantitation of OHHPL and is suitable for investigation into those pathological conditions in which its excretion is abnormally raised.

CHAPTER 4

3-ETHYL-5-HYDROXY-4,5-DIMETHYL-Δ^3-PYRROLIN-2-ONE IN PORPHYRIA AND RELATED DISORDERS

CHAPTER 4

3-ETHYL-5-HYDROXY-4,5-DIMETHYL-Δ^3-PYRROLIN-2-ONE IN PORPHYRIA AND RELATED DISORDERS

4.1. INTRODUCTION

The acute porphyrias exhibit certain similarities to some overt psychiatric disorders. The psychiatric syndrome of the porphyrias is invariably associated with the expression of the genetically defined biochemical abnormality but no gross abnormalities in haem biosynthesis have been shown in psychiatric illness. A proportion of schizophrenics, however, do show mild disturbances in porphyrin metabolism. The demonstration of excretion of OHHPL in both the porphyrias and some psychiatric disorders was the common factor that was the basis of the suggestion that OHHPL played a role in the aetiology of the psychiatric syndrome of the porphyrias. Pharmacological and behavioural studies carried out using like substances, especially kryptopyrrole, did not provide evidence contrary to this view.

The failure of previous attempts to quantitate OHHPL has hindered attempts to investigate this association further. The following chapter deals with the quantitation of OHHPL in porphyria, although the first sections cover earlier studies using a qualitative method.

OHHPL excretion has also been studied in several other disorders because they exhibit certain common features. These are psychiatric illness, lead poisoning and liver disease.

4.2. QUALITATIVE INVESTIGATION OF MONOPYRROLE EXCRETION IN HEPATIC PORPHYRIA

Prior to the development of the glc method for the quantitation of OHHPL, a qualitative survey was undertaken using a modification of Irvine's method (section 2.6). Using this method excretion of OHHPL, then thought to be kryptopyrrole, could only be assessed as positive or negative.

It was originally puzzling that two such compounds, kryptopyrrole and OHHPL, differing as they do in polarity and hence chromatographic properties, could possibly be confused. The matter however was resolved by the following findings.

1. When OHHPL was run in the chromatographic system it migrated to a position with an R_f of 0.9, identical to that of the kryptopyrrole standard. Development of colour with Ehrlich reagent required heating to 50 - 60°C.
2. When a kryptopyrrole standard was run and Ehrlich reagent applied there was an immediate colour reaction of a component with R_f 0.95 with a more reddish colour than that of the OHHPL complex. Heating of the chromatogram to 50 - 60°C resulted in the visualisation of a second component, R_f 0.9, an identical colour to the OHHPL complex, with concomitant fading of the kryptopyrrole spot. An example of a test chromatogram is shown in Figure 17.

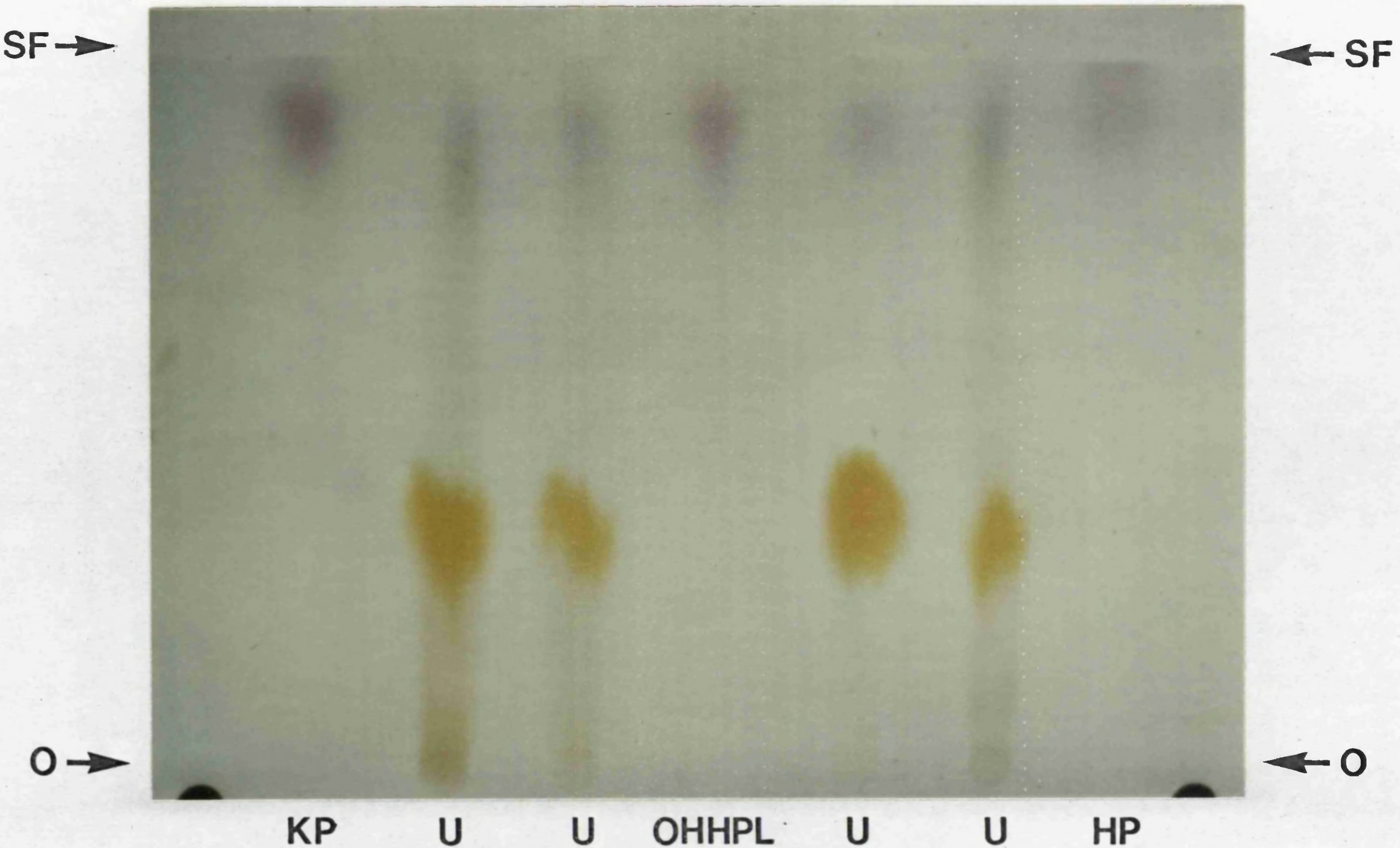


FIGURE 17 EXAMPLE OF CHROMATOGRAM USED FOR THE DETECTION OF 'KRYPTOPYRROLE' BY THE METHOD OF IRVINE

The chromatogram shows 4 urine extracts (U) (all positive) and standards of kryptopyrrole (KP), OHHPL and haemopyrrole (HP). Both HP and KP spots show components on the solvent front and components isographic with OHHPL and the urinary metabolite.

SF = solvent front   O = origin

These results indicated that the chromatographic system was unable to adequately resolve kryptopyrrole from its oxidation products or in fact from these of haemopyrrole. Processing of kryptopyrrole in this system causes oxidation of a large proportion of the applied sample producing the component with R_f 0.9. Development of colour with test urine samples required heating indicating that it was an oxidised pyrrolic species. The unequivocal identification of this is described in the introductory section 1.4.1.

In this section the presence or absence of the mauve spot only will be described.

4.2.1. PATIENTS

A group of seventeen patients with the three acute forms of porphyria (AIP, HC, VG) were studied. Of these four were in attack, four in remission and nine were latent for the conditions. Urine from ten healthy volunteers was also screened.

Urine specimens were first morning samples analysed within an hour of voiding and full porphyrin and precursor analyses were carried out.

4.2.2. RESULTS

The age, sex, type of porphyria, porphyric status, porphyrin and precursor excretion and the result of the kryptopyrrole test are outlined in Table 5.

TABLE 5 "KRYPTOPYRROLE" EXCRETION, URINARY PORPHYRINS AND PRECURSORS AND FAECAL PORPHYRINS IN PATIENTS WITH PORPHYRIA

Case Number	Sex	Age	Type of Porphyria	Porphyric status	KP	Urinary Porphyrins (nmol/L) and Precursors (μmol/L)				Faecal Porphyrins nmol/g dry wt.	
						ALA	PBG	COPRO	URO	COPRO	PROTO
1	F	30	AIP	Attack	+ve	438	594	1139	3242	-	-
2	M	42	AIP	Attack	+ve	47	93	692	410	208	701
3	F	26	AIP	Remission	+ve	122	172	191	302	54	273
4	M	42	AIP	Remission	+ve	160	221	429	219	-	-
5	F	51	AIP	Remission	+ve	13	19	92	38	-	-
6	F	43	HC	Remission	+ve	18	5	241	28	-	-
7	F	35	VG	Attack	+ve	44	7	430	48	809	2265
8	F	26	HC	Attack	+ve	33	11	7724	341	1556	167
9	F	54	HC	Latent	-ve	18	4	128	0	1233	138
10	M	24	HC	Latent	-ve	18	1	368	0	600	195
11	F	20	HC	Latent	-ve	128	44	3112	429	2751	90
12	F	43	HC	Latent	-ve	31	13	354	0	2901	99
13	M	19	HC	Latent	-ve	24	9	195	0	345	37
14	M	17	HC	Latent	-ve	27	9	258	0	382	178
15	F	24	AIP	Latent	-ve	75	42	729	131	7	20
16	M	40	VG	Latent	-ve	14	1	97	22	152	257
17	F	69	VG	Latent	-ve	13	6	84	16	205	623
NORMAL VALUES						<44	<25	<227	<22	<76	<200

URO = Uroporphyrin COPRO = Coproporphyrin PROTO = Protoporphyrin KP = "Kryptopyrrole"

In all patients in attack and remission "kryptopyrrole" could be demonstrated in the urine while a negative test result was obtained in urine from latent subjects. No "kryptopyrrole" could be detected in the urine from any of the normal subjects.

The detection limit of the test was difficult to estimate. The lower limit of detection of the Ehrlich test for kryptopyrrole itself is approximately 3nmoles. However, for the oxidised pyrrolic forms which were originally thought not to react with Ehrlich's aldehyde, and do so only with heating, the detection limit is about 100-fold less. Assuming 100% recovery from urine, the lower level of detection was about 3μmol/L, although this is a very rough estimate.

4.3. HEPATIC PORPHYRIA

4.3.1. PATIENTS

Eighteen carriers of the AIP gene defect were studied of whom 10 were latent (3 male, 7 female). All of the remaining patients (all female) were studied when totally asymptomatic and four were studied during an acute episode. A brief resume of the history of the non-latent porphyrics is given in Table 6, while fuller case histories of those studied in attack are given in Appendix 2.

Two patients with hereditary coproporphyria (HC) (both female) and four with cutaneous hepatic porphyria (CH), (3 male, 1 female) were also studied. The patients with HC were asymptomatic at the time of study and those with CHP were undergoing monthly venesection. None of these patients showed overt signs of porphyria at the time of study.

4.3.2. RESULTS

When urinary OHHPL was measured, estimation of urinary ALA, PBG and creatinine was also made. It was not possible, however, to perform porphyrin estimations in all of the samples but estimations were carried out on 24 hour specimens from some of the patients using solvent extraction methods.

Where appropriate, statistical evaluation of differences in OHHPL concentrations has been made using the non-parametric

TABLE 6 CLINICAL DETAILS OF PATIENTS WITH ACUTE INTERMITTENT PORPHYRIA STUDIED IN ATTACK AND/OR REMISSION

Case No.		Sex	Born	Diagnosed	
1.	M.A.	F	1952	1971	Has had about 6 acute attacks since diagnosed most of which have been associate with menstruation although one was associated with pregnancy.
2.	S.C.	F	1922	1951	Diagnosed following severe attack associated with menstruation which resulted i peripheral neuropathy. Was given methyl testosterone which prevented menstruation associated attacks. Over the years attacks have become less frequent or severe and disease is now largely "burnt-out."
3.	J.C.	F	1955	1976	First attack precipitated by the contraceptive pill. Has suffered several seve attacks associated with menstruation involving autonomic and peripheral neuropathy. Has also had attack associated with dietary restriction. (Case history - Appendix 2)
4.	M.McA.	F	1950	1976	Had acute attack following mebhydrolin. Normal appendix removed at laparotomy when anaesthesia was induced with thiopentone sod. causing severe exacerbation. Since then well with occasional postural hypotension. (Case history-Appendix 2)
5.	J.McM.	F	1955	1976	Diagnosed during extremely severe attack during pregnancy. Main features of attack were abdominal pain, quadriplegia, diaphragmatic palsy, tachycardia and obstipation. Satisfactory recovery was achieved after 12 weeks hospitalisatior Has had subsequent attacks associated with urinary tract infection and menstruation. (Case history - Appendix 2)
6.	I.M.	F	1944	1967	Normally well but has had acute attack associated with dietary restriction.
7.	P.W.	F	1948	1969	Diagnosed following severe puerperal attack resulting in residual neuropathy. Since then has had frequent pre-menstrual attacks many of which require hospita isation. Takes large doses of oral dextrose prior to menstruation with some amelioration of symptoms. (Case history - Appendix 2)
8.	E.W.	F	1932	1953	Complained of abdominal pain and nausea following a missed menstrual period. C admission to hospital examination showed loss of muscle power and tachycardia. Diagnosis of AIP was made. She was lost to follow-up when she defaulted from t ante-natal clinic where pregnancy was confirmed. Since then she has had 6 heal children and has taken the contraceptive pill for 14 years. (Mother of Case 5)

Mann-Whitney U test while non-parametric correlations were made using the Spearman or Kendall ranking tests.

Where possible urinary estimations were performed on early morning spot samples. However, there was no consistent diurnal variation observed in in-patients. Control values used were those of age and sex matched normals chosen at random from the normal group.

Urinary excretion of OHHPL in the patients with AIP in attack, remission and latency is shown in Figure 18. Where multiple estimations have been carried out on a single patient median values have been used. Compared with control values excretion is significantly raised in all three groups (latents $p < 0.05$; remission $p = 0.001$, attack $p < 0.05$). Expression of the results in terms of creatinine concentration (Figure 19) did not alter the significance of these findings. There was no significant difference in the excretion of OHHPL in the three test groups. OHHPL excretion in the patients with HC and CHP is shown in Table 7. Except for the values indicated these lie within the normal range.

OHHPL excretion was followed in patient 3 through an acute attack (see case history). Daily OHHPL, ALA and PBG excretion during the attack is shown in Figure 20. There was little apparent correlation between the clinical condition and OHHPL excretion, while that of ALA and PBG showed reasonably good correlation including rebound excretion after the discontinuation of laevulose therapy.

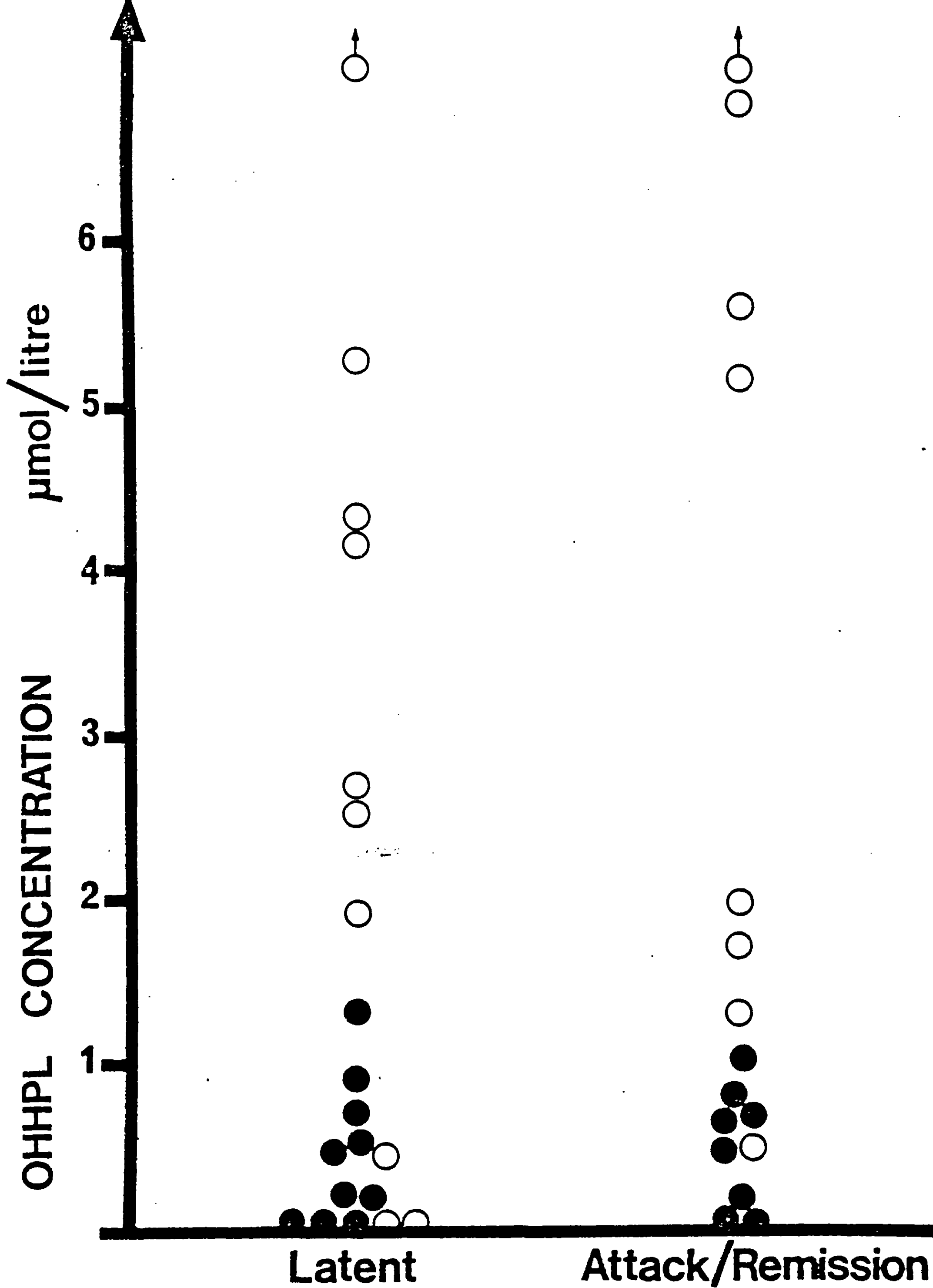


FIGURE 18 URINARY OHHPL EXCRETION IN AIP

Excretion is shown in terms of urine volume

○ = subjects with AIP

● = age and sex matched control values

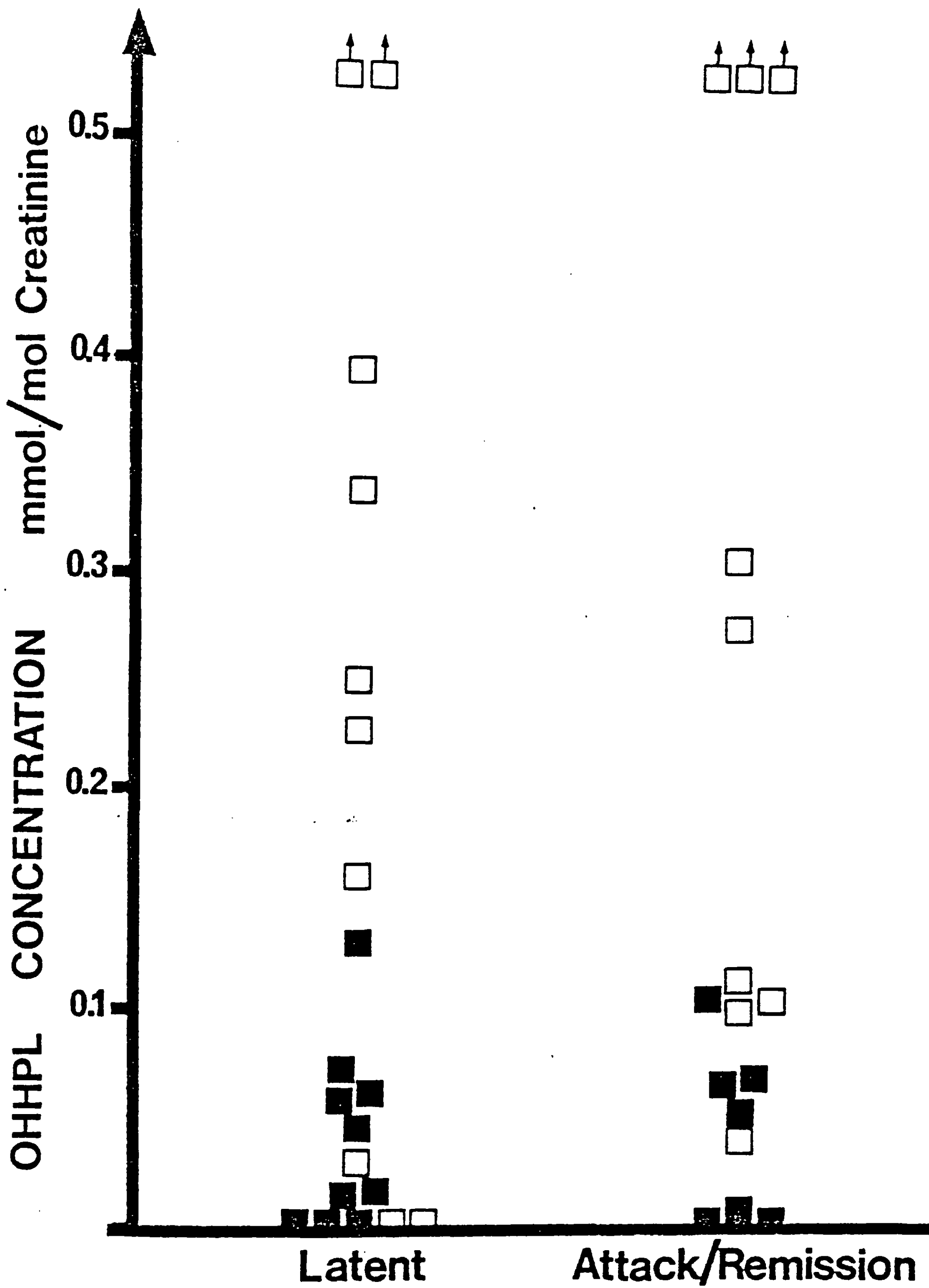


FIGURE 19 URINARY OHHPL EXCRETION IN AIP

Excretion is shown in terms of urinary creatinine concentration.

□ = subjects with AIP

■ = age and sex matched control values

TABLE 7 URINARY OHHPL EXCRETION IN PATIENTS WITH HEREDITARY COPROPORPHYRIA AND CUTANEOUS HEPATIC PORPHYRIA

HEREDITARY COPROPORPHYRIA

Case	Date	OHHPL μmol/L
1 A.M.	27.5.77	0.81
2 J.B.	27.5.77	2.1 *

CUTANEOUS HEPATIC PORPHYRIA

Case	Date	OHHPL μmol/L
1 C.B.	5.8.77	0.17
2 W.W.	15.4.77	2.9 *
	2.9.77	0.97
	18.3.77	1.5
3 J.W.	20.5.77	ND
	5.8.77	ND
	2.9.77	0.45

ND = Not detectable * Outwith normal range

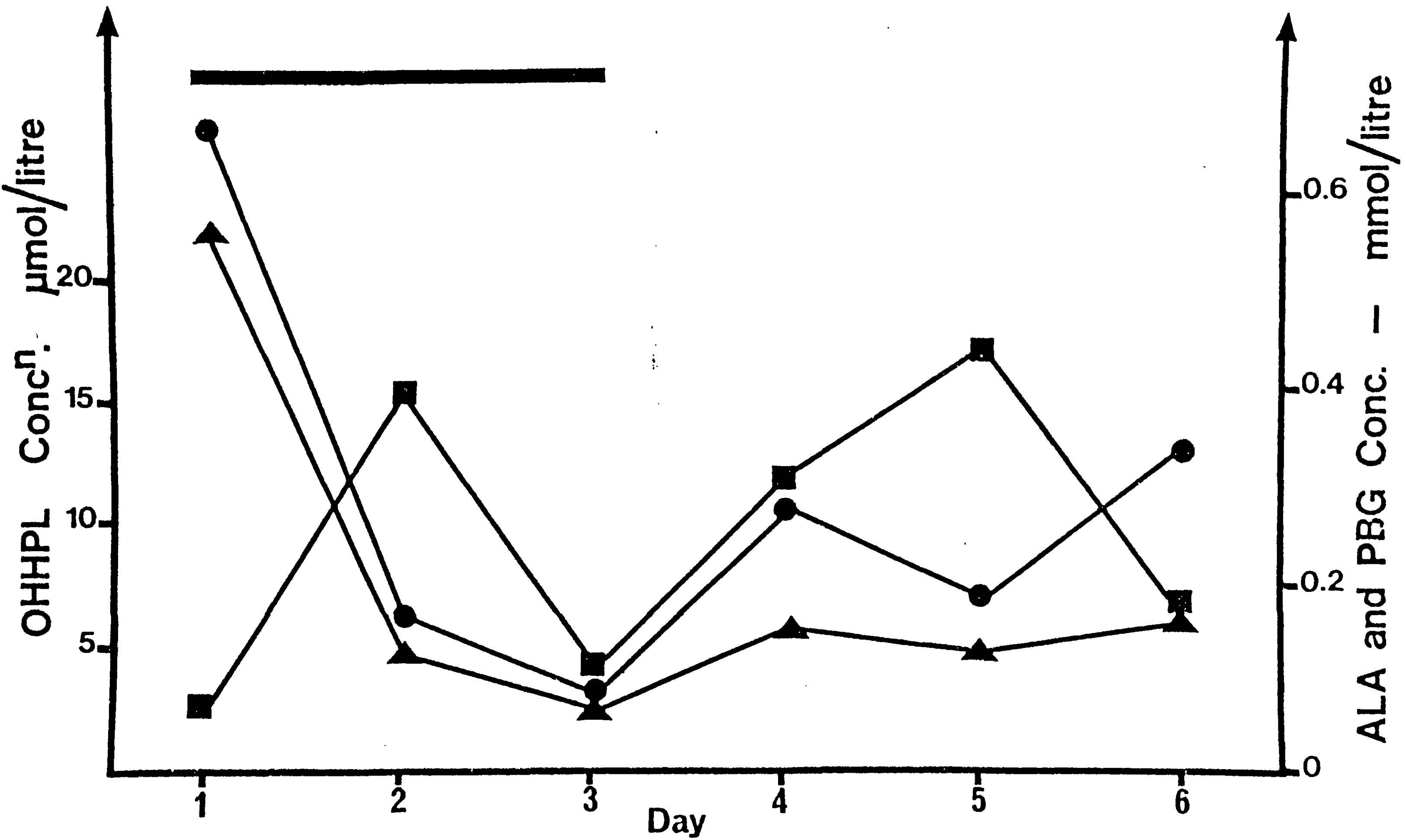


FIGURE 20 URINARY OHHPL, PBG AND ALA FOLLOWED IN CASE 3 THROUGHOUT AN ACUTE ATTACK

■ = OHHPL ● = PBG ▲ = ALA ▬▬ denotes laevulose infusion

Two patients (cases 3 and 5) suffered from frequent severe attacks. OHHPL estimations were carried out on several occasions on each patient. Results of these estimations are shown, along with the patient's condition in Table 8.

A significant association was found in all subjects with AIP between the excretion of OHHPL and ALA (Figure 21) ($p < 0.01$) and an almost identical correlation between PBG and OHHPL (Figure 22) ($p < 0.05$). The similarity of these associations was not surprising in the light of the demonstration of a highly significant linear correlation ($p < 0.001$) between urinary ALA and PBG (Figure 23). There was no such correlation in normal subjects.

TABLE 8 MULTIPLE ESTIMATION OF URINARY OHHPL IN TWO PATIENTS WITH AIP

	Date	OHHPL(μmol/L)	Condition
Case 3	4.11.76	26.6	Asymptomatic
	18.3.77	36.8	Mild abdominal pain
	29.4.77	2.68	Acute attack (see also Figure 20 and text)
	30.4.77	15.3	
	1.5.77	4.2	
	2.5.77	12.1	
	3.5.77	17.3	
	28.6.77	1.8	Abdominal pain, constipation
	29.6.77	2.3	Mild abdominal pain, postural hypotension
	13.9.77	5.2	Tachycardia, postural hypotension
	20.9.77	9.3	Abdominal and limb pain, tachycardia, agitation
Case 5	11.3.77	12.9	Mild abdominal pain
	15.4.77	6.9	Asymptomatic
	27.5.77	3.85	Intermittent abdominal pain
	12.7.77	8.4	Asymptomatic
	3.8.77	1.05	Asymptomatic
	30.8.77	2.7	Abdominal pain
	31.8.77	1.8	Abdominal pain

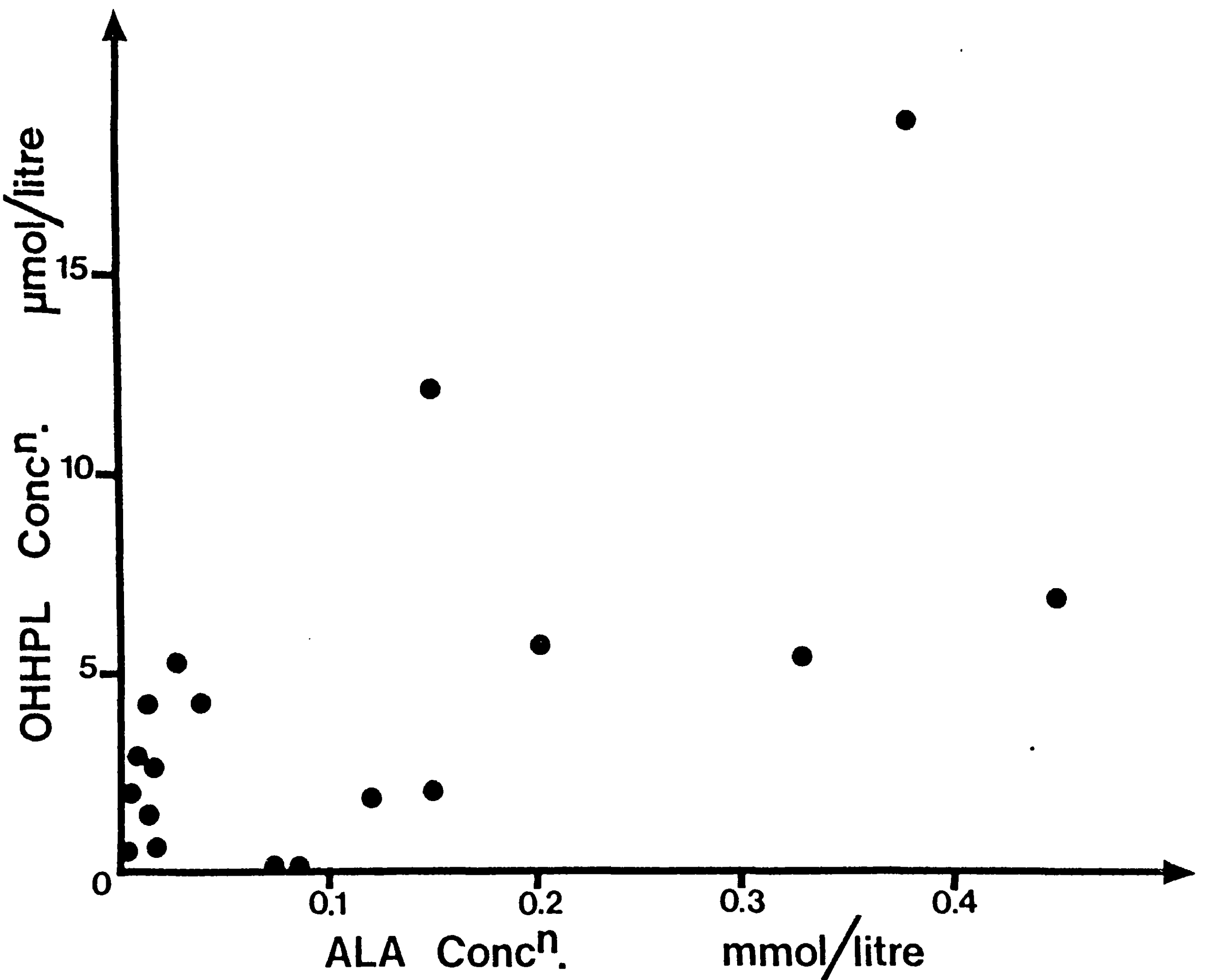


FIGURE 21 ASSOCIATION BETWEEN URINARY OHHPL AND. ALA IN SUBJECTS WITH AIP

The association is statistically significant, $p < 0.01$

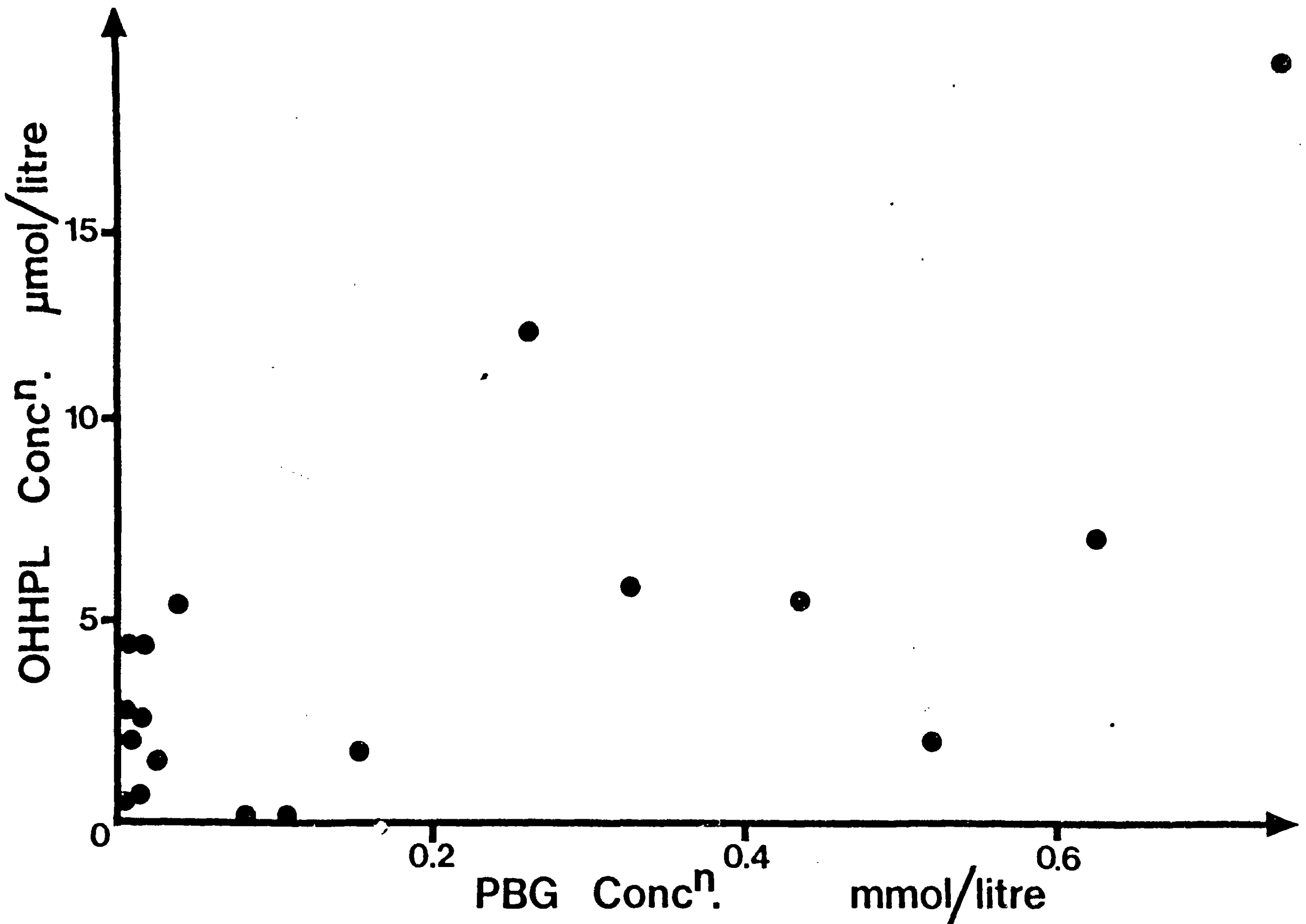


FIGURE 22 ASSOCIATION BETWEEN URINARY OHHPL AND PBG AND SUBJECTS WITH AIP

The association is statistically significant $p < 0.05$

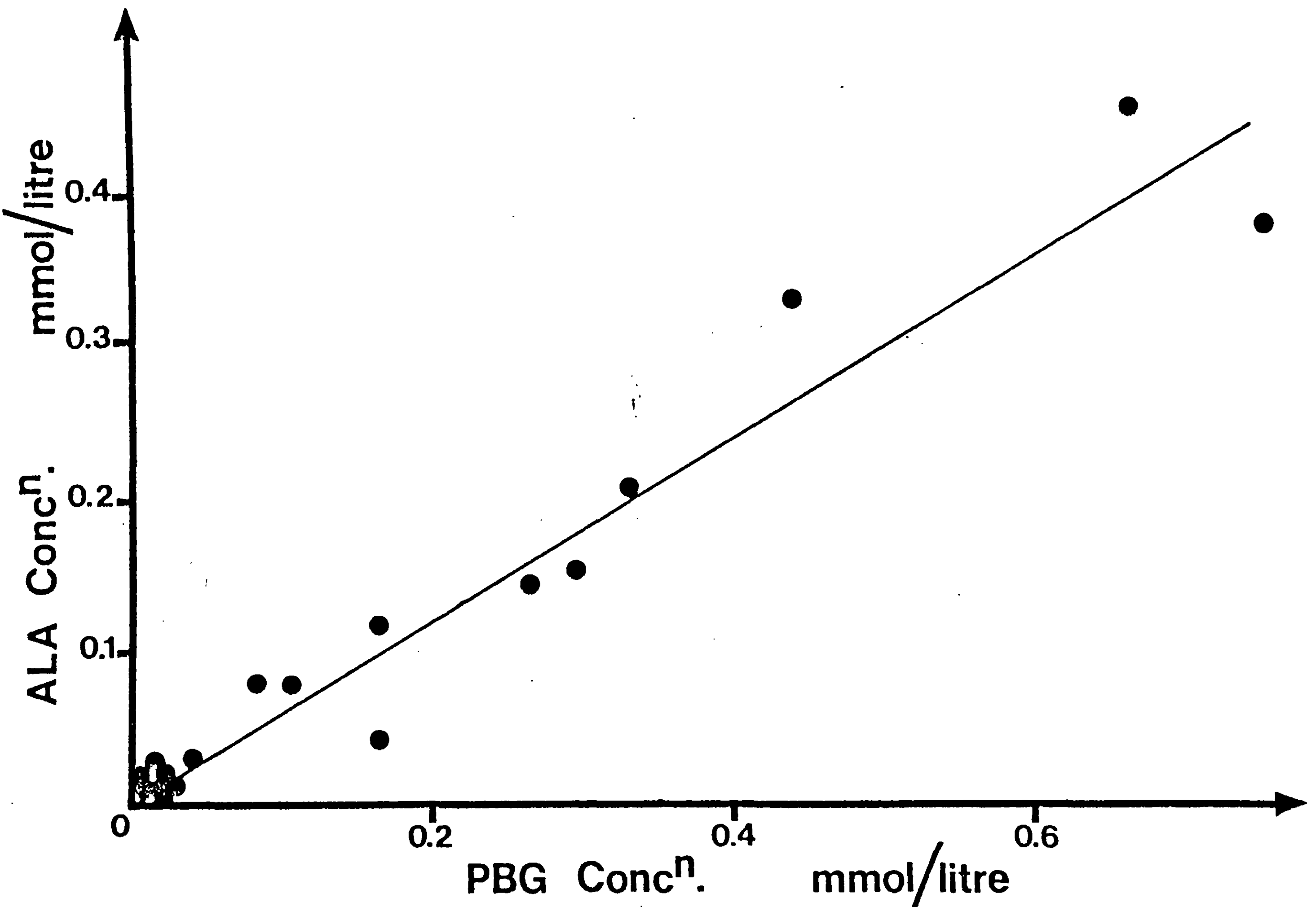


FIGURE 23 LINEAR RELATIONSHIP BETWEEN URINARY ALA AND PBG IN SUBJECTS WITH AIP

The relationship is statistically significant, $p < 0.001$

4.4. PSYCHIATRIC DISORDERS

OHHPL was measured in the urine of patients with various psychiatric disorders as an extension of previous studies by other workers who had shown increased incidence of OHHPL in certain types of psychiatric illnesses.

4.4.1. PATIENTS

The major difficulty encountered in this study was in accumulating adequate numbers of patients within the various classifications of psychiatric disease. There were also large variations in the clinical status of patients within a particular group. The clinical diagnosis of each patient was made by the consultant psychiatrist and in some cases Feighner criteria (Feighner et al, 1972) were used for confirmation. Where a quantitative assessment of the severity of a patient's condition was made the patients were rated using the Hamilton-Lorr rating scale (Hamilton et al, 1960). An example of this is shown in Appendix 3.

Initially classifications were made broadly to give larger samples for analysis. These were :-

1. Psychosis: schizophrenia, endogenous depression, manic depression.
2. Neurosis: neurotic depression, anxiety depression, obsessional neurosis.

3. Organic brain disorder: This was principally organic brain damage resulting from alcoholism, syphilis or physical trauma.

There were gross variations in clinical conditions even within the sub-groups, and it was virtually impossible to obtain homogeneity with significant numbers.

The group of patients studied initially was a group of in-patients from the psychiatric wards of Stobhill General Hospital and of the Southern General Hospital, Glasgow. These patients fell mainly into the schizophrenia or endogenous depression groups. There were 24 subjects (19 female, 5 male) aged between 24 and 78 years old, of whom 22 could be broadly classed as psychotic (5 schizophrenia, 17 endogenous depression). At the time of study all of these patients were undergoing drug therapy for their psychiatric condition and/or non-associated illnesses. Table 9 shows the drugs in use with these patients. Liver function tests were carried out in all of the patients from the Southern General Hospital. In every case these were normal.

The possibility of drug-mediated alterations in OHHPL excretion made it desirable to obtain a similar group of patients not on drugs. This group of patients was obtained from the Gartnaval Royal Hospital, Glasgow, and consisted of 22 patients (all male) of whom 8 were psychotic (7 schizophrenia) and 9 had an organic brain syndrome almost always

TABLE 9 THERAPEUTIC AGENTS GIVEN TO PSYCHOTIC SUBJECTS STUDIED

NEUROLEPTICS

Thioridazine	Haloperidol	Chlorpromazine
Perphenazine	Trifluoperazine	Fluphenazine

MINOR TRANQUILLISERS

Nitrazepam	Lorazepam	Pimozide
Chlordiazepoxide		

ANTIDEPRESSANTS

Imipramine	Phenelzine	Amitryptyline
(L-Tryptophan)	Lithium Carbonate	

MISCELLANEOUS

Propranolol	Dextropropoxyphene	Frusemide
Carbimazole	Oxprenolol	Digoxin
Aminophylline	Orphenadine	Alcohol

associated with alcoholism. Liver function tests were carried out on the patients with alcoholism. Most of the patients had a raised serum alkaline phosphatase; one had raised bilirubin and one had raised alanine transaminase (ALT) activity. None of these patients had been on drug therapy for at least four months.

Three patients were studied with an organic brain syndrome not related to alcoholism and 6 others with miscellaneous disorders were also studied.

4.4.2. RESULTS

The results of OHHPL estimations in the urine of psychotic patients on drugs is shown in Figure 24. Although a number of the values are within the normal range when treated as a group, excretion is significantly greater than that of the age and sex matched control values ($p < 0.01$). There was no significant difference between the schizophrenic patients and those with endogenous depression. Furthermore no correlation could be shown between OHHPL levels and the urinary excretion of ALA and PBG. Elevated excretion showed no association with any particular drug being prescribed for the patients and the large variety of drugs involved meant that analysis of the effect of single drugs could not be looked at.

OHHPL estimations in patients with schizophrenia and alcoholic organic brain syndrome who were not on drugs showed differences

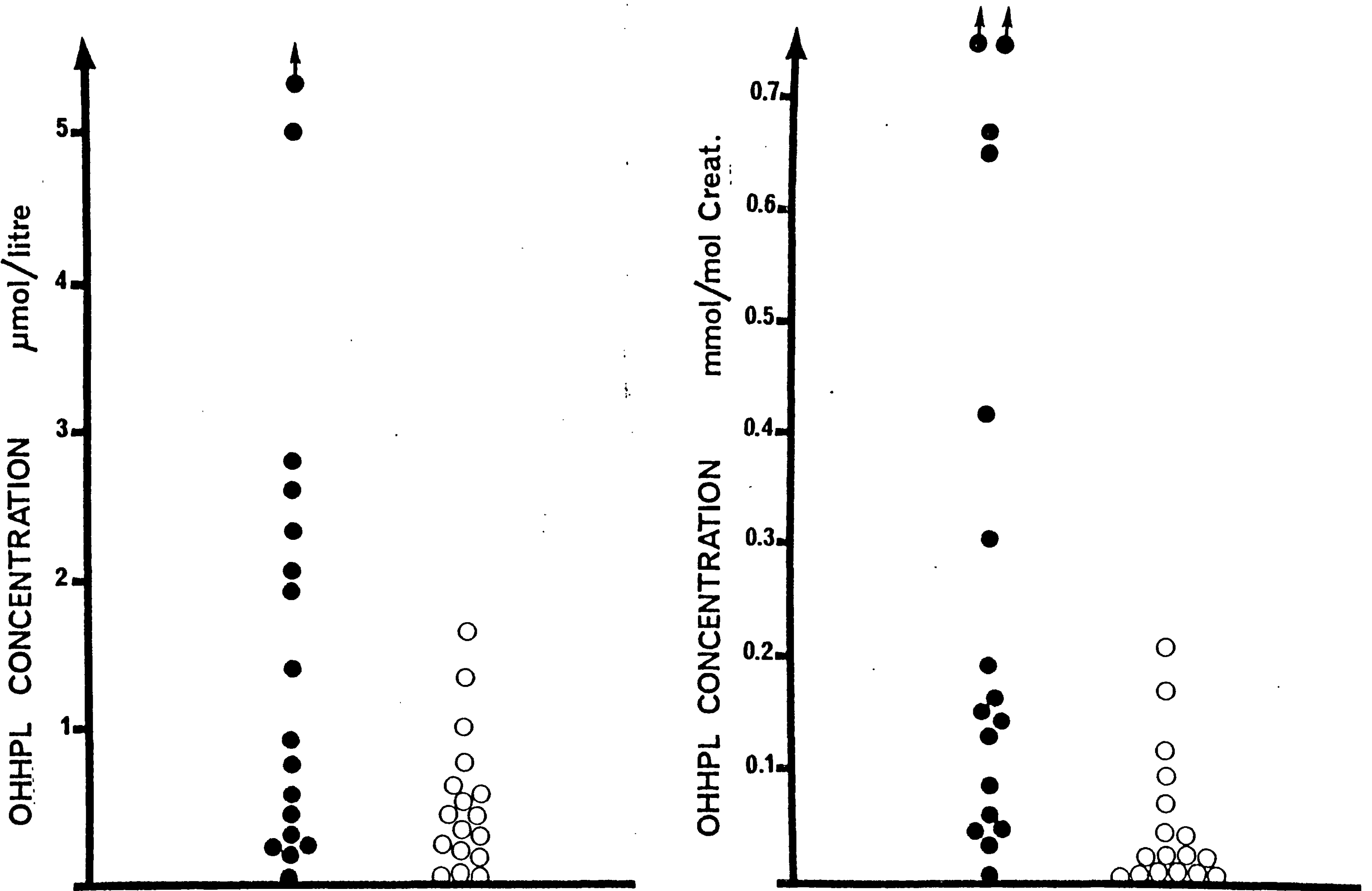


FIGURE 24 URINARY OHHPL IN PSYCHOTIC SUBJECTS UNDERGOING DRUG THERAPY

Concentrations are expressed both in terms of urine volume and urinary creatinine

● = psychotic subjects ○ = age and sex matched controls

when expressed in terms of creatinine or urine volume (Figure 25). The significance of this could not be determined. It is clear, however, that several of the patients in each group showed raised OHHPL excretion. In all cases urinary ALA and PBG levels were within the normal range and there were no correlations or associations with OHHPL levels. The Hamilton rating scores of the patients with schizophrenia are shown in Table 10. The scores in each of the P and N sub-scales are shown as well as the total score. OHHPL levels in terms of creatinine and urine volume are also shown. Table 11 shows the results of statistical analyses of possible correlations between each of the Hamilton rating scores and OHHPL levels. When expressed in terms of urine volume there is a tendency towards a negative correlation. However, when levels are expressed in terms of urinary creatinine, there are significant negative correlations between OHHPL and the HRS(T) and HRS(P) values.

HRS values were not obtained for the patients with organic brain disorders. Abnormal OHHPL excretion showed no statistical correlation with abnormal liver function tests.

TABLE 10 HAMILTON RATING SCORES (HRS) AND URINARY OHHPL LEVELS IN SCHIZOPHRENIC PATIENTS

The values of the total score (T) and the scores on the P and N subscales are given (see Appendix 3)

Case	HRS(T)	HRS(P)	HRS(N)	OHHPL µmol/L	OHHPL µmol/mol creat.
1	25	25	0	5.5	443
2	2	2	0	1.26	851
3	49	43	6	0.46	36.5
4	0	0	0	0.78	-
5	26	12	14	1.46	198
6	32	28	4	0.55	102
7	5	3	2	0.69	259

TABLE 11 SPEARMAN RANK CORRELATION COEFFICIENTS (r_s) FOR THE DATA SHOWN IN TABLE 10

Significance of results: * $p < 0.05$ ** $p < 0.01$

	OHHPL µmol/L	OHHPL µmol/mol creat.
HRS(T)	-0.43	-0.94 **
HRS(P)	-0.39	-0.83 *
HRS(N)	-0.32	-0.77

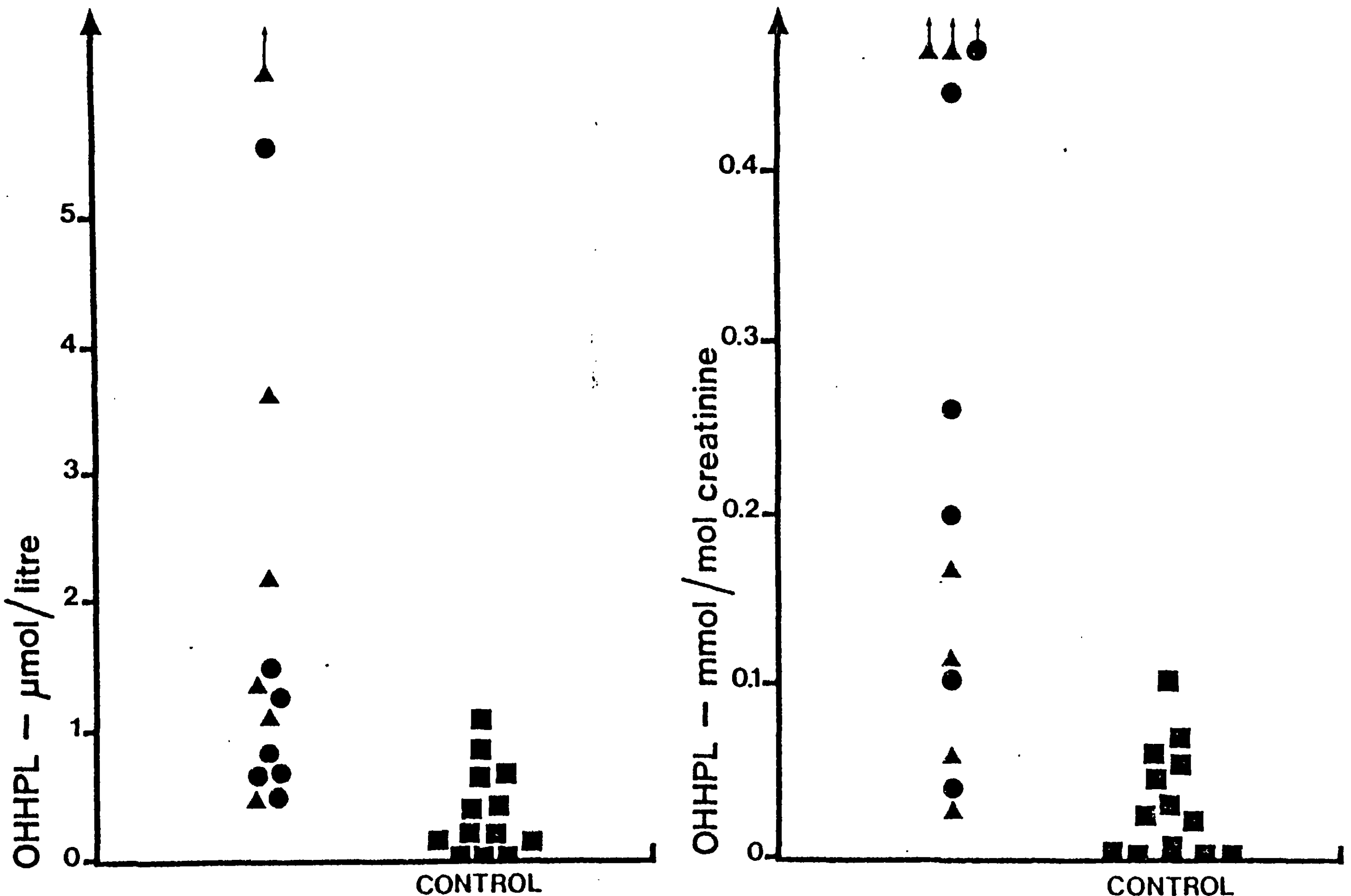


FIGURE 25 URINARY OHHPL IN DRUG FREE PSYCHIATRIC PATIENTS

Patients were receiving no drugs at the time of the study or in at least a four month period preceding the study.

▲ = Organic brain syndrome; ● = Schizophrenia; ■ = Age and sex matched controls

4.5. LEAD POISONING

Lead poisoning was included in the series because it has long been known that lead burden causes disruption of haem biosynthesis (Garrod, 1892). Lead has been shown to affect the activities of several of the enzymes of haem biosynthesis and in fact inhibition of ALA.D by lead (Hernberg and Nikkanen, 1972) has been used in the past as an index of the severity of lead poisoning. Lead also causes a decrease in FERRO.C and URO.D activities (Boyett and Butterworth, 1962; Schmidt et al, 1973) and a concomitant increase in the activity of ALA.S (Maxwell and Meyer, 1976). Accompanying these enzyme changes is increased urinary excretion of ALA and porphyrins but not of PBG (Haeger, 1957).

4.5.1. SUBJECTS

Frank lead poisoning is now a rare occurrence. The study was thus limited to subjects with sub-clinical lead toxicity. The subjects studied were a group of industrial lead workers none of whom showed any outward signs or symptoms of lead poisoning, although all had blood lead levels outwith the normal ranges.

4.5.2. RESULTS

Urinary OHHPL, urinary ALA and PBG, blood lead levels and erythrocyte ALA.D activity were measured in 15 subjects. The

results are shown in Table 12. All subjects had blood lead levels (BPb) outwith the normal range and all had depressed erythrocyte ALA.D activity. ALA excretion was raised in a high percentage of the cases but urinary OHHPL and PBG were within normal ranges.

It was noticed that high ALA excretion was associated with lower OHHPL values and non-parametric analysis revealed a significant negative association between OHHPL and ALA ($p < 0.05$) (Figure 26) and also between OHHPL and ALA.D ($p < 0.05$). There were also significant correlations between the parameters of lead poisoning, ALA, ALA.D and BPb ($p < 0.001$ in all cases).

TABLE 12 OHHPL, ALA AND PBG IN URINE, ERYTHROCYTE ALA.D ACTIVITY AND BLOOD LEAD (BPb) LEVELS IN INDUSTRIAL LEAD WORKERS

Case	OHHPL μmol/L	ALA μmol/L	PBG μmol/L	BPb μmol/L	ALA.D nmol ALA/min/ ml rbc
1	1.2	31.3	8.4	2.3	11.3
2	1.26	27.8	5.5	3.4	6.8
3	1.84	47.3	6.3	3.3	4.2
4	0.21	30.5	8.3	2.1	13.2
5	0.12	199.0	7.2	4.4	0.9
6	0.01	50.6	5.5	4.3	1.8
7	0.59	78.7	20.4	3.9	0.7
8	0.41	61.8	4.9	3.7	8.4
9	0.22	193.0	7.2	4.8	1.3
10	0.22	28.1	3.3	3.2	8.6
11	0.84	33.1	3.8	3.5	7.0
12	0.75	85.2	0.01	3.9	2.8
13	0.66	35.5	2.7	3.9	2.9
14	0.13	60.1	5.0	3.3	8.1
15	0.01	210.0	0.55	5.0	0.7
Normal range	0-1.7	0-43	0-25	0-2	16-38

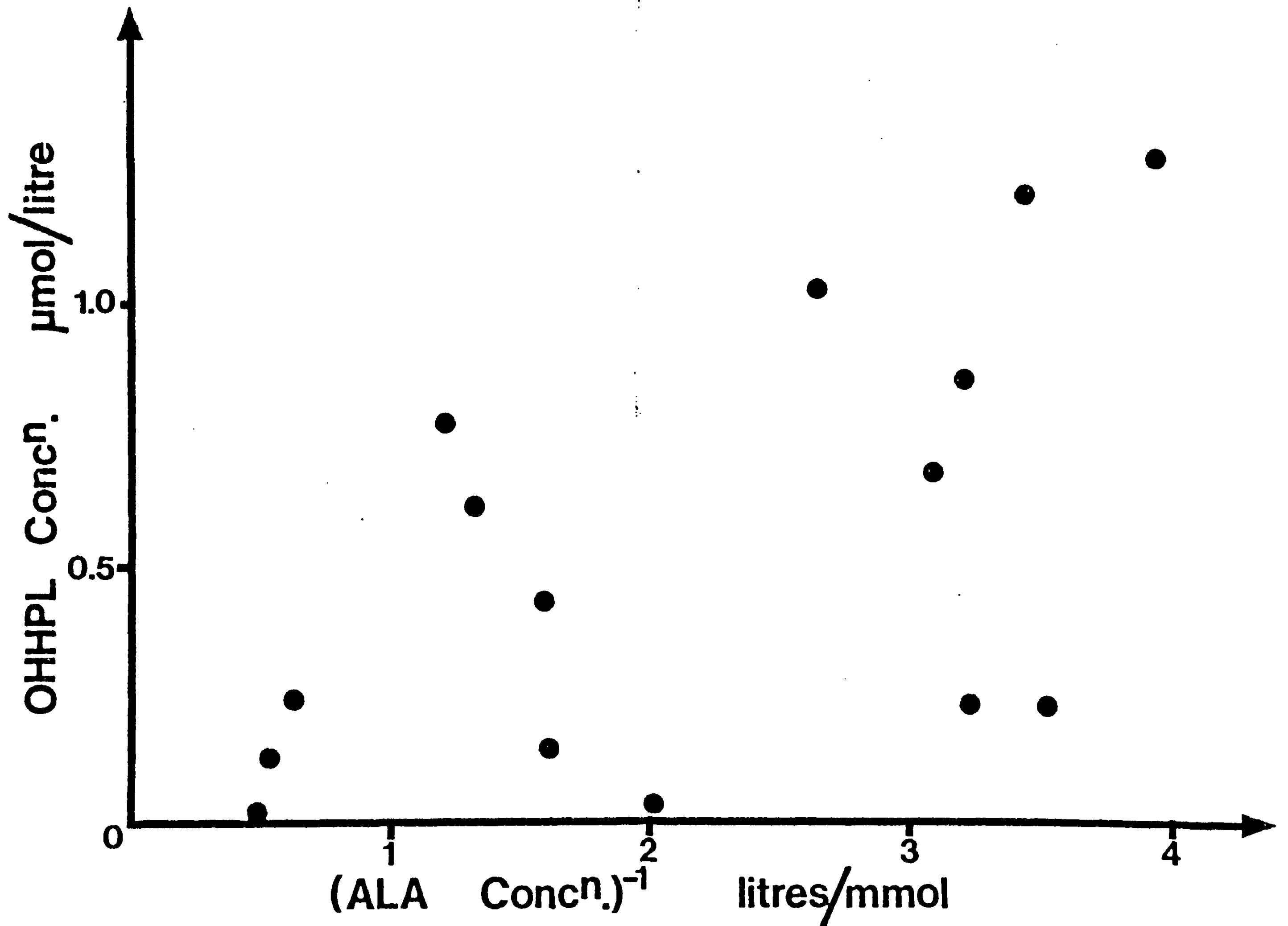


FIGURE 26 ASSOCIATION BETWEEN URINARY OHHPL AND THE RECIPROCAL OF ALA CONCENTRATION IN LEAD WORKERS

The association is statistically significant, $p < 0.05$

4.6. LIVER DISEASE

In hepatic porphyria there can be changes in liver function that varies from near normality in AIP to gross dysfunction in CHP and in each porphyria the degree of dysfunction bears some relationship to the activity of the disease. On the other hand porphyrinuria has been associated with abnormal liver function of origin other than porphyria. Excessive urinary excretion of coproporphyrin has been found in hepatitis, cirrhosis, jaundice and alcoholic liver disease, (Lamon, 1977). In the case of cirrhosis depressed ALA.D activities have also been found (Allain et al, 1977).

This tentative connection between liver disease and porphyria suggested that it may have been worthwhile to look at the excretion of OHHPL in a group of patients with liver disease. The following sections describe such a study.

4.6.1. PATIENTS

The patients studied were all attending the out-patient clinic of Stobhill General Hospital. They fell into two main groups: chronic active hepatitis and cirrhosis. Of the patients with cirrhosis, 3 had primary biliary cirrhosis, 5 had alcoholic cirrhosis and 1 patient had cirrhosis of unknown origin (cryptogenic).

All of the patients with hepatitis were undergoing steroid therapy at the time of study while the patients with cirrhosis

were receiving various regimens consisting mainly of vitamin supplementation.

4.6.2. RESULTS

Relevant clinical data, liver function tests and urinary OHHPL, ALA and PBG in the patients studied are shown in Tables 13 and 14. In all cases of hepatitis, urinary ALA and PBG were normal as were OHHPL levels, except for one patient with accompanying cirrhosis. Likewise in the cirrhosis group porphyrin precursor excretion was largely normal but several cases showed increased OHHPL excretion. This latter finding could not be correlated with liver function tests, the severity of their clinical condition or ALA and PBG levels.

TABLE 13 CLINICAL AND BIOCHEMICAL DATA ON PATIENTS WITH HEPATITIS

		Sex	Age	Diagnosis	Drugs	LIVER FUNCTION TESTS								
						Alb. g/L	Bil. mmol/L	Alk.Phos. IU/L	AST IU/L	ALT IU/L	LD IU/L	Ca++ µmol/L	OHHPL µmol/L	ALA µmol/L
1	J.W.	F	72	ACH	P,S	41	14	45	18	82	497	2.48	ND	19.2
2	G.McK.	F	45	ACH	nil	37	8	80	75	113	437	2.41	0.19	9.2
3	E.McD.	F	48	ACH	P,A,S,T	30	47	360	180	156	841	2.27	ND	45.6
4	A.F.	F	58	ACH	P,A	46	12	152	33	34	275	2.45	ND	15.4
5	C.C.	F	69	ACH	P	37	10	112	33	34	347	2.31	0.31	12.4
6	R.A.	M	53	ACH	P	30	27	58	50	38	973	2.24	0.37	6.2
7	M.L.	F	60	ACH	P	38	8	57	33	43	412	2.30	ND	13.9
8	E.M.	F	56	ACH,C,E	P,S	35	97	125	61	52	251	2.64	2.42	23.2
Normal Values						36 - 52	5 - 20	40-115	13-42	11-55	250-520	2.2-2.40	0-1.7	0-43

ACH = Active chronic hepatitis
C = Cirrhosis
E = Encephalopathy

P = Prednisolone
S = Spironolactone
A = Azothioprine
T = Thyroxine

N.D. = Not detectable

TABLE 14 CLINICAL AND BIOCHEMICAL DATA ON PATIENTS WITH CIRRHOSIS

		Sex	Age	Diagnosis	Drugs	LIVER FUNCTION TESTS Alb. g/L	Bil. mmol/L	Alk.Phos. IU/L	AST IU/L	ALT IU/L	LD IU/L	Ca^{++} µmol/L	OHHPL µmol/L	ALA	PI µmol/
1.	A.M.	F	30	PBC	Nil	42	73	360	109	109	444	2.42	3.9	44.4	6.
2.	M.M.	F	30	PBC	V	35	19	370	112	168	365	2.36	ND	9.5	NI
3.	M.McL.	M	67	PBC	V	33	79	1190	144	84	250	2.2	5.67	16.9	7.
4.	E.McL.	F	73	CC	F,K,V	30	15	218	45	21	943	2.13	ND	2.0	5.
5.	W.McC.	M	61	AC	nil	39	13	84	33	14	396	2.4	ND	3.2	NI
6.	T.C.	M	46	AC,PA,E.	N.K M.V	27	40	90	47	28	896	2.31	1.9	59.2	1.
7.	H.C.	F	49	AC	F,K,Fo.V	36	73	154	47	23	335	2.46	ND	7.7	NI
8.	D.W.	M	44	AC	nil	27	348	128	89	53	416	2.0	0.73	5.6	2
9.	W.A.	M	41	AC	nil	44	23	102	31	37	278	2.5	10.2	23.1	1.
				Normal Values	-	36-52	5-20	40-115	13-42	11-55	250 -520	2.2 - 2.4	0-1.7	0-43	0-

PBC = Primary Biliary Cirrhosis
CC = Cryptogenic Cirrhosis
AC = Alcoholic Cirrhosis
PA = Portocaval Anastomosis
E = Encephalopathy

F = Frusemide
K = Potassium
N = Neomycin
M = Methionine
Fo = Folic Acid
V = Vitamin injections

4.7. DISCUSSION

Qualitative chromatographic methods have been used to show an association between the excretion of OHHPL and psychosis and various forms of porphyria (CHP, VP and AIP) (Irvine and Wilson, 1976). The same workers were also able to demonstrate the excretion of OHHPL in 12% of normal subjects. The results presented in this chapter also show that OHHPL is excreted in HC.

One interesting feature arising from the qualitative study (section 4.2.) was the absence of chromatographic evidence for the excretion by latent porphyria cases. Although this is most certainly due to the insensitivity of the method, it provided some clues to excretion levels in each of the porphyrias studied. The main shortcoming of the study was the lack of numbers in each group and it may be erroneous to try and draw too many conclusions from it.

The establishment of the quantitative method has allowed the measurement of OHHPL in all but 22% of normals and from the normal distribution some estimate of the sensitivity of the qualitative methods can be gained. The maximum sensitivity of the method described in section 2.6., which could not detect OHHPL in normals, is about 2.1 μmol/l while that of the method used by Irvine and Wilson (1976) is greater than 1.5 μmol/l.

The results of the quantitative assay on urine from the patients with AIP are similar to earlier studies which were able to

show excretion in up to 90% of the cases (Irvine and Wilson, 1976).

No significant differences were found in attack, remission or latency. This, along with the findings in repeated analyses in single patients in attack and remission would appear to cast doubt on a possible role for OHHPL in the aetiology of any of the manifestations of AIP. Any attempt to correlate OHHPL with the psychiatric status of patients with acute porphyria was frustrated by the lack of patients presenting with a psychiatric syndrome.

It may be that urinary excretion does not provide a good enough index of plasma or tissue levels and variations in plasma levels may be masked by gross disturbances in urinary levels. This is also probably the case with ALA and PBG excretion and plasma levels, where again it is much easier to measure urinary concentrations of ALA and PBG than plasma levels and significant changes in plasma levels may never be reflected in urine.

One interesting finding was the association between OHHPL and ALA and PBG levels. The linear relationship between ALA and PBG requires that OHHPL excretion be related to only one of ALA and PBG for both of the above associations to arise. That is, if for example OHHPL excretion was a factor of PBG excretion, the association of ALA with OHHPL would arise automatically. The findings in lead workers may shed some light on this. The absence of any correlation between the raised ALA levels seen in these

subjects would suggest that in AIP the ALA-OHHPL association is a secondary result.

This finding in turn raises the obvious question, 'is OHHPL formed from PBG ?'. Figure 27 shows a comparison between OHHPL and PBG and it can be seen that decarboxylation (x 2) and deamination of PBG would give the required carbon skeleton while PBG.O has been shown to insert the required oxygen functions (Frydman et al, 1973).

There are other compounds which could theoretically give rise to OHHPL. Reviewed recently by Irvine and Wilson (1976), these include dicarboxylic porphyrins with the correct 'haemo' configuration in the D ring, tetrapyrrolic bile pigments of the IX-α on IX-β series, again with the 'haemo' configuration or certain bilirubic acids or propentdyopents. Irvine and Wilson have proposed that the most likely of these are certain porphyrins of the isocoproporphyrin series which are produced in excess in CHP but this would not account for OHHPL excretion in AIP, where these porphyrins do not occur, unless all of the isocoproporphyrin produced in AIP is metabolised via OHHPL. It would thus appear that PBG may be the most likely precursor of OHHPL.

The findings in the psychiatric patients again reinforce earlier results by other workers. There are significant increases in urinary OHHPL levels in patients with schizophrenia, endogenous depression and alcoholic dementia (organic brain syndrome) but again there was no positive correlation with excretion and patient's condition. The negative correlation between the Hamilton

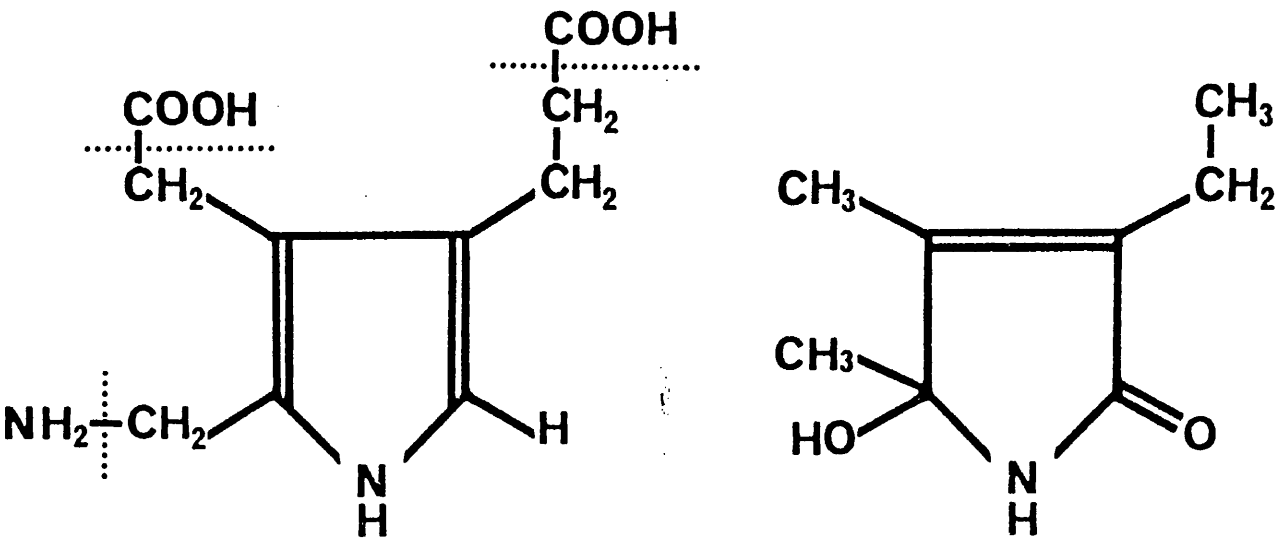


FIGURE 27 STRUCTURAL COMPARISON OF OHHPL AND PBG

The enzyme PBG.O is known to introduce the required oxygen functions. No mechanism is known for decarboxylation and deamination.

rating scores of the schizophrenics and OHHPL would in fact suggest that exacerbation of the psychiatric illness is connected with a decrease in OHHPL excretion. However, verification of this finding would require the accumulation of greater numbers and again plasma levels.

Increased excretion in patients with alcohol induced organic brain syndrome may be a result of abnormal liver function, although abnormalities shown in liver function tests were not great. There may be some similarity in these cases to cirrhotic liver disease (including alcohol induced) where some patients showed increased excretion. The fact that both in the psychotic patients and those with liver disease, OHHPL excretion is increased in the absence of PBG, may be contrary to the hypothesis of PBG as a precursor. It may be that, in these cases, increased activity of the OHHPL synthesising enzyme system (if there is one) is due to deranged liver metabolism as compared to substrate induction in the porphyrias.

So, in none of the conditions studied can raised OHHPL excretion be associated with exacerbation of the clinical condition and these findings must cast doubt on any role for OHHPL in the aetiology of the manifestations in these disorders. It may be that knowledge of plasma levels will allow a definite statement to be made in this respect.

CHAPTER 5

MONOPYRROLES AND HAEM BIOSYNTHESIS

CHAPTER 5

MONOPYRROLES AND HAEM BIOSYNTHESIS

5.1. INTRODUCTION

Qualitative studies, following the discovery of OHHPL excretion in schizophrenics, showed that excretion of OHHPL was also found in two porphyrin producing disorders; porphyria (Irvine and Wilson, 1976) and porphyrin producing psychotic states (Huszak et al, 1972). Qualitative work described in section 4.2. was suggestive of a role for OHHPL in the precipitation of the acute porphyric attack, but the more recent quantitative studies proved contradictory in this respect. These studies did provide evidence, however, that OHHPL excretion was directly associated with the abnormalities in haem biosynthesis seen in the porphyrias.

The following studies were prompted by the earlier work and were designed to investigate the possibility that OHHPL excretion was not the result of abnormal porphyrin metabolism but was a factor in the aetiology of such abnormalities. It was known that the haem biosynthetic pathway is sensitive to treatment with a wide variety of agents and this work was designed to investigate a possible direct effect of OHHPL and related compounds on haem biosynthesis.

The substances used in the study were those originally

thought to be the natural product; kryptopyrrole and OHKPL, as well as OHHPL itself. For comparison, three other pyrrole species were used. These were :-

1. Haemopyrrole: The synthetic precursor of OHHPL and ββ' isomer of kryptopyrrole.
2. Phyllopyrrole: An alkyl pyrrole with the same substituents as kryptopyrrole and haemopyrrole but with a methyl group on the reactive 5-position.
3. Ethyl 3-acetyl-2,4-dimethylpyrrole-5-carboxylate (EADC), an intermediate in the synthesis of kryptopyrrole with carboxy-ethyl and acetyl substituents replacing the α-methyl and β-ethyl groups of phyllopyrrole.

The latter two compounds were found to lack the extreme toxicity of kryptopyrrole and haemopyrrole.

Biochemical studies were designed to look at the effects of OHHPL, the natural compound, in particular, although selected properties of the other compounds were studied. Effects on the following aspects of haem metabolism were looked at :-

1. In vivo porphyrin and precursor excretion.
2. The activities of the enzymes of haem biosynthesis in vivo and in vitro.
3. Hepatic porphyrin levels.

To guage the effects on haem utilisation and breakdown, the activity of hepatic haem oxygenase (HAEM.O), the haem

degrading enzyme, and microsomal haem and cytochromes P-450 and b_5 were also measured.

Attempts were also made to assay porphobilinogen oxygenase (PBG.O) one of an ill-defined group of oxygenases capable of oxidising monopyrroles such as PBG and krypto-pyrrole. Although not described in humans, induction of this enzyme has been shown to reverse experimentally produced porphyria in animals (Frydman et al, 1975).

5.2. PORPHYRIN SYNTHESIS AND EXCRETION

The following set of experiments were designed as a preliminary investigation into the effects of monopyrroles on porphyrin excretion in rats. Hepatic levels were also measured to monitor synthesis. In one set of experiments ALA supplementation was given to look at the effects of these compounds on an already overloaded pathway.

Dose responses were studied using EADC which was in plentiful supply and was non-toxic and thus allowed administration in increased doses.

5.2.1. METHODS

In all experiments Sprague-Dawley rats weighing between 150 - 200g were used. Animals were housed in metabolic cages and given food and water ad libitum.

Animals were injected (intraperitoneally) with the compounds of interest. The doses of kryptopyrrole and haemopyrrole were limited by their extreme toxicity. Table 15 shows the doses normally employed: for kryptopyrrole and haemopyrrole these were the maximal non-lethal doses, for OHHPL and OHKPL these were the minimum effective doses and for EADC and phyllopyrrole arbitrary non-toxic effective doses.

Compounds were dissolved either in propylene glycol or 50% ($^{v}/v$) ethanol and control animals received propylene glycol

TABLE 15 DAILY DOSES OF MONOPYRROLES AND ALA FOR *in vivo* STUDIES

Unless stated otherwise these were the daily doses used

	DOSE	
	mmol/kg	mg/kg
Kryptopyrrole	0.65	80
OHKPL	0.65	100
Haemopyrrole	0.41	50
OHHPL	0.65	100
Phyllopyrrole	0.65	90
EADC	0.96	200
ALA (HCl)	0.60	100

or 50% ethanol alone. Where ALA supplements were added these were dissolved in saline and the pH of solution brought to pH7 with saturated sodium bicarbonate.

Urine and faeces collections were made daily and on sacrifice livers were removed for porphyrin estimation. Normally porphyrin estimations were made by solvent extraction methods, although HPLC analyses of the porphyrin products of some experiments were carried out to verify the identity of the extracted porphyrin and to gain information on the individual porphyrin species present.

Urinary porphyrin and precursor excretion were expressed as nmol/rat/24h while faecal excretion and hepatic concentrations were given as nmol/g dry wt. and nmol/g wet wt. respectively.

5.2.2. RESULTS

Using the doses of monopyrroles shown in Table 15 all of the compounds were found to increase porphyrin excretion and hepatic porphyrin levels. ALA and PBG excretion was unaltered by treatment in all cases.

Urinary excretion and faecal levels are shown in Table 16. In all cases total urinary excretion of porphyrins is raised, and in each case, except EADC, coproporphyrin is by far the major fraction. HPLC analyses of the individual porphyrins in the urine of animals treated with OHHPL, haemopyrrole and EADC are shown in Figure 28.

TABLE 16 URINARY AND FAECAL PORPHYRINS IN RATS TREATED WITH MONOPYRROLES

In each test group n = 6 while for the control group n = 24

	Urinary excretion nmol/24h/rat)		Faecal levels nmol/g dry wt	
Group	Uroporphyrin	Coproporphyrin	Coproporphyrin	Protoporphyrin
Control	0	2.8	6.0	31.5
Kryptopyrrole	0.5	16.6	9.0	39.6
OHKPL	0.5	11.5	6.4	35.0
Haemopyrrole	4.1	12.3	5.2	35.9
OHHPL	0.3	14.3	3.5	38.1
Phyllopyrrole	0	7.8	7.9	28.7
EADC	4.7	5.1	2.2	23.3

There is no apparent consistent change in the proportion of each porphyrin. Monopyrrole treatment caused a decrease in the proportion of coproporphyrin while EADC also reduced the proportion of penta. OHHPL and haemopyrrole increased the proportion of uroporphyrin and penta while EADC increased uroporphyrin and hepta. Treatment with OHHPL only marginally changed the proportion of each porphyrin.

Faecal levels show little change from control values although EADC treatment causes a decrease in faecal porphyrin excretion. HPLC analysis revealed no major change in the proportional make-up of the total porphyrin. The coproporphyrin contained a small percentage of penta and this was uniform throughout.

Hepatic levels are shown in Table 17. In all cases protoporphyrin concentration is significantly raised. However, treatment with OHHPL and OHKPL results in no significant elevation of coproporphyrin levels.

HPLC analysis of the hepatic porphyrins showed that penta was only present in one case (haemopyrrole) and then as a very small percentage of the total porphyrin.

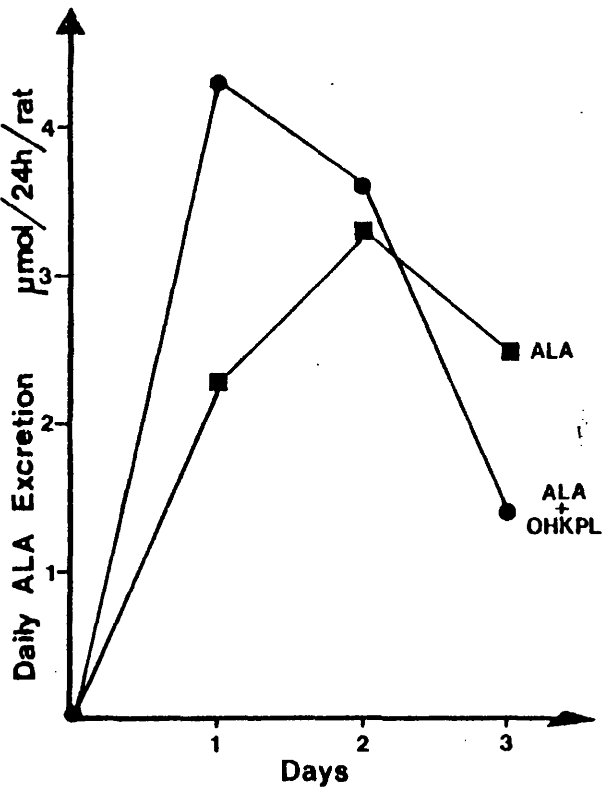
Two experiments were carried out to study the effects of ALA loading in pyrrole treated animals. For this, OHKPL and OHHPL were used. The results with both pyrroles were almost identical. Those for OHKPL are shown in Figures 29, 30 and 31. Treatment with ALA alone increased urinary excretion of ALA,

TABLE 17 HEPATIC PORPHYRIN CONCENTRATIONS IN RATS TREATED WITH MONOPYRROLES

n = 6 in test groups and n = 24 in the control group

Significance of difference from control values :
* $p < 0.005$ ** $p < 0.001$

Group	Hepatic concentrations (nmol/g wet wt)	
	Coproporphyrin	Protoporphyrin
Control	0.15 ± 0.07	0.37 ± 0.13
Kryptopyrrole	0.27 ± 0.06**	0.72 ± 0.23*
OHKPL	0.18 ± 0.07	0.76 ± 0.22**
Haemopyrrole	0.38 ± 0.18*	1.47 ± 0.39**
OHHPL	0.17 ± 0.09	0.97 ± 0.35**
Phyllopyrrole	0.48 ± 0.13**	3.51 ± 0.7**
EADC	0.33 ± 0.11**	1.81 ± 0.52**



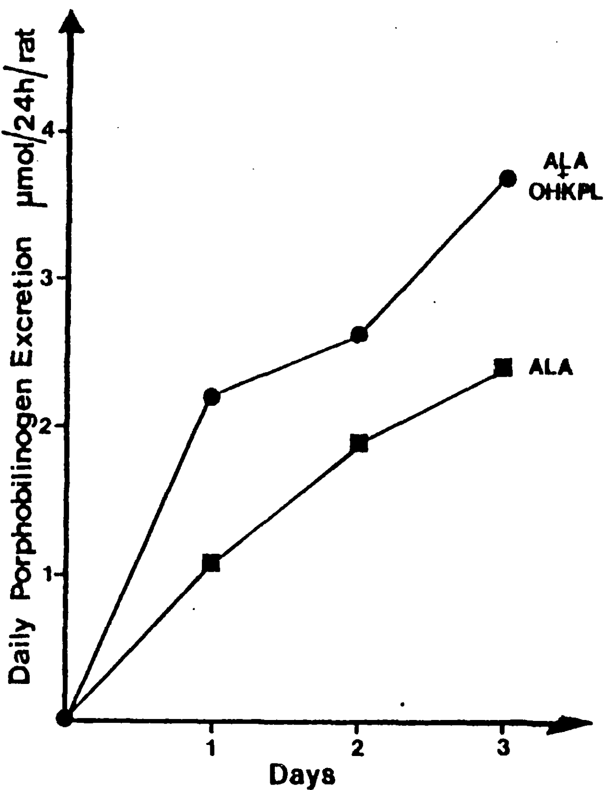


FIGURE 29 DAILY URINARY EXCRETION OF ALA AND PBG IN ANIMALS TREATED DAILY WITH ALA OR ALA AND OHKPL

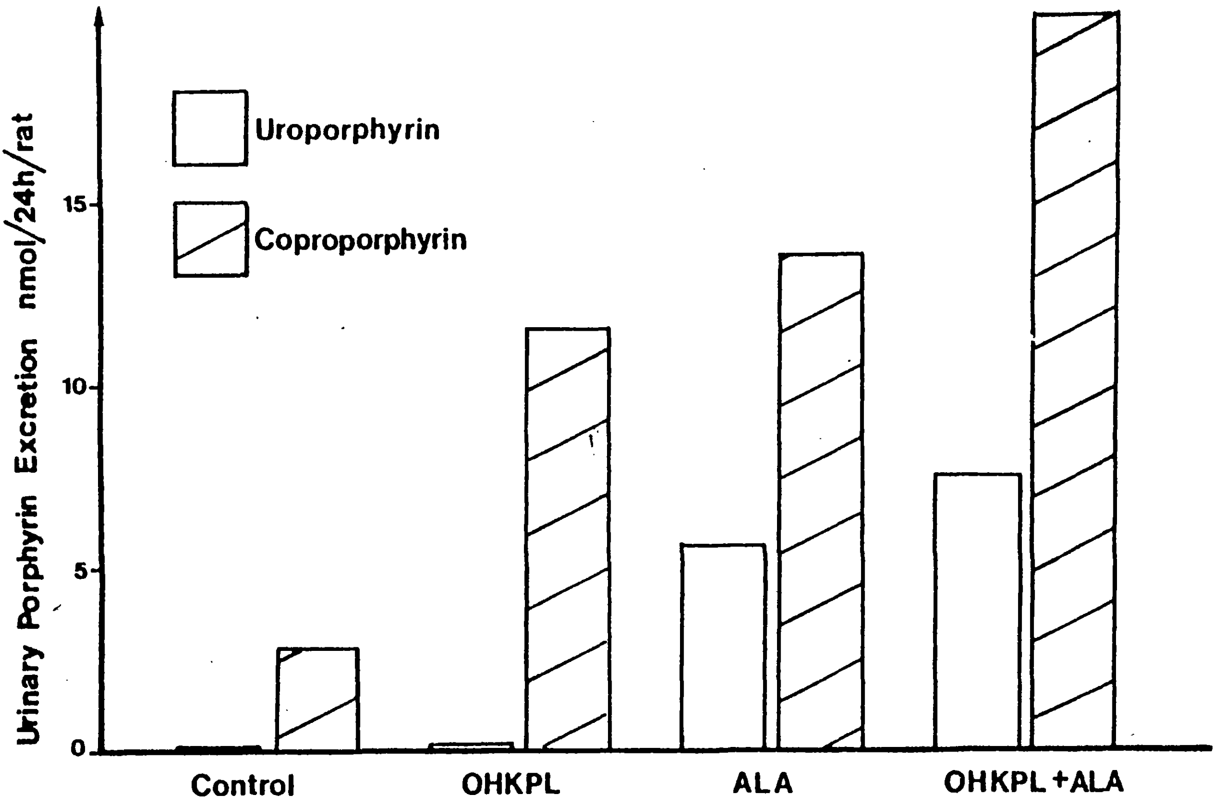


FIGURE 30 PEAK URINARY PORPHYRIN EXCRETION (DAY 3) IN ANIMALS TREATED WITH OHKPL AND/OR ALA

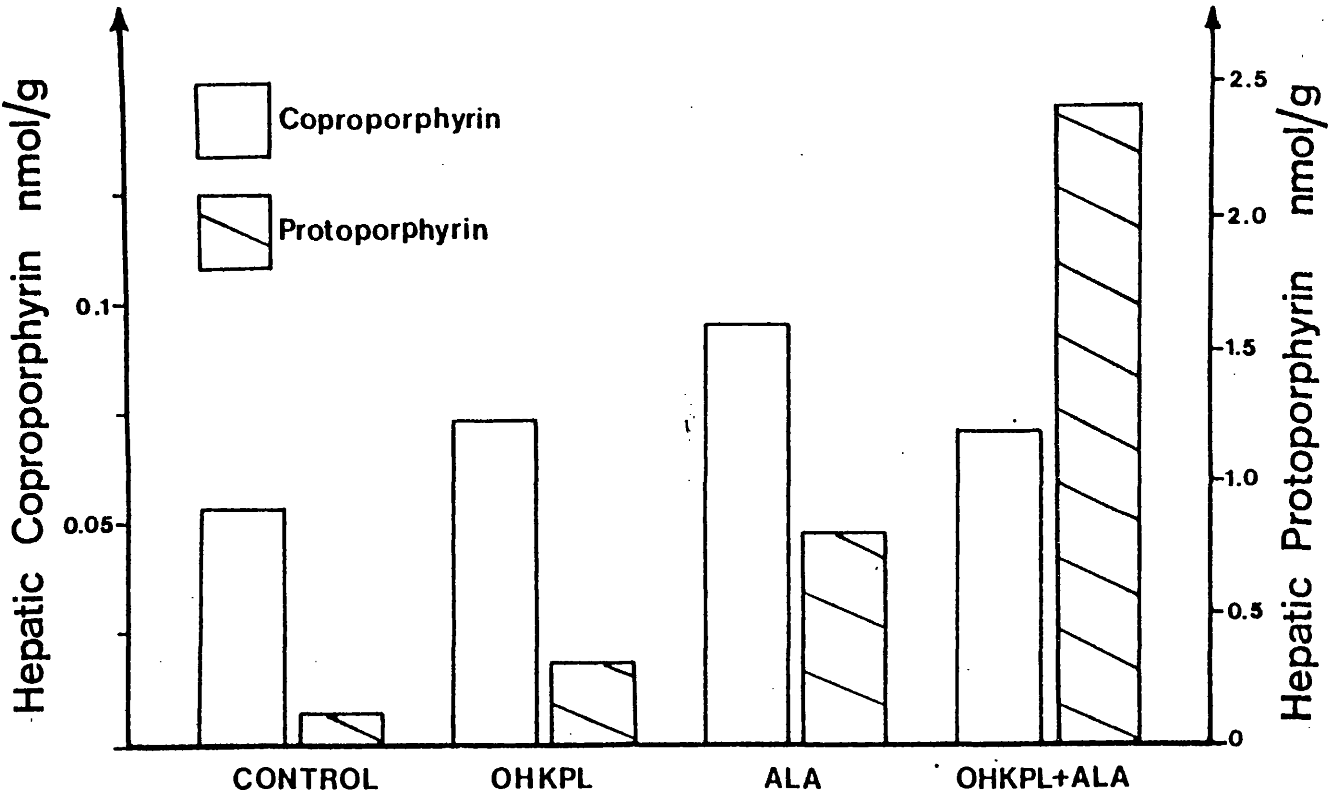


FIGURE 31 HEPATIC PORPHYRIN LEVELS IN ANIMALS TREATED WITH OHKPL AND/OR ALA

PBG, coproporphyrin and uroporphyrin, faecal levels of copro-porphyrin and protoporphyrin and hepatic levels of coproporphyrin and protoporphyrin. OHHPL treatment alone had no effect on ALA or PBG excretion but increased urinary and hepatic porphyrins. When given together the effects appeared to be synergistic with most parameters being increased above those of ALA treated animals.

The dose response of porphyrin excretion to increasing monopyrrole dosage was studied using EADC. Total daily excretion in animals given daily doses of 0.48, 0.96, 1.44 and 1.92 mmol/kg is shown in Figure 32. The maximum response was shown at 1.44 mmol/kg. Peak excretion was on either the second or third day, although the animals at the lowest dose showed no peak of excretion over the period studied.

Animals receiving the three highest doses showed a marked diuresis which was also greatest at 1.44 mmol/kg and at the peak urine volumes were up to four times that of control animals. The increased excretion of porphyrin was not entirely due to the diuresis as porphyrin levels in the urine were also raised.

Figure 33 shows the individual porphyrins in the urine of each group of animals at the peak excretion point (day 2 or 3). With increasing dosage there is a decrease in the proportion of coproporphyrin with a concomitant increase in the formation of hepta then uroporphyrin.

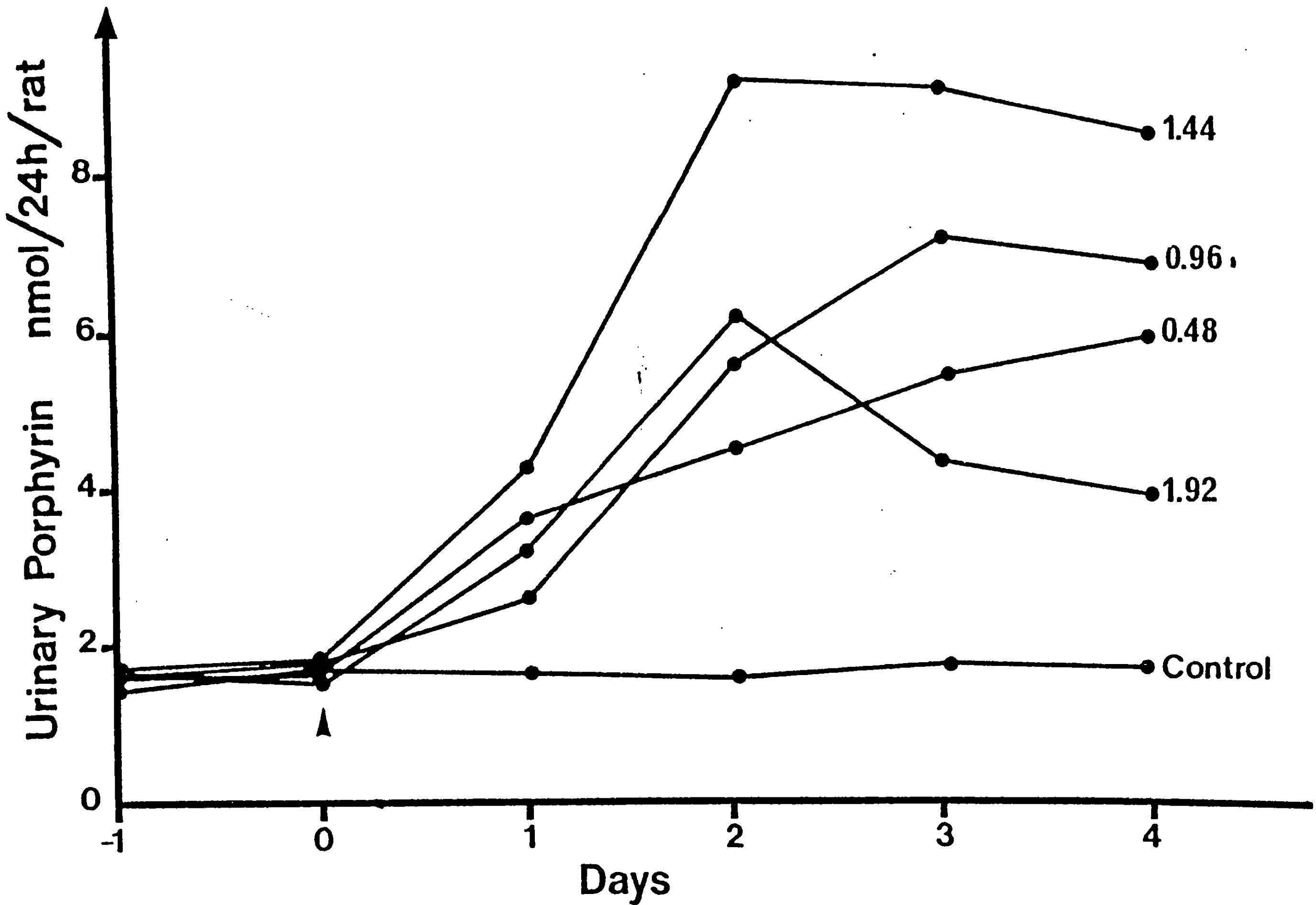


FIGURE 32 TOTAL DAILY URINARY PORPHYRIN EXCRETION IN RATS GIVEN EADC IN THE DOSES SHOWN (mmol/kg)

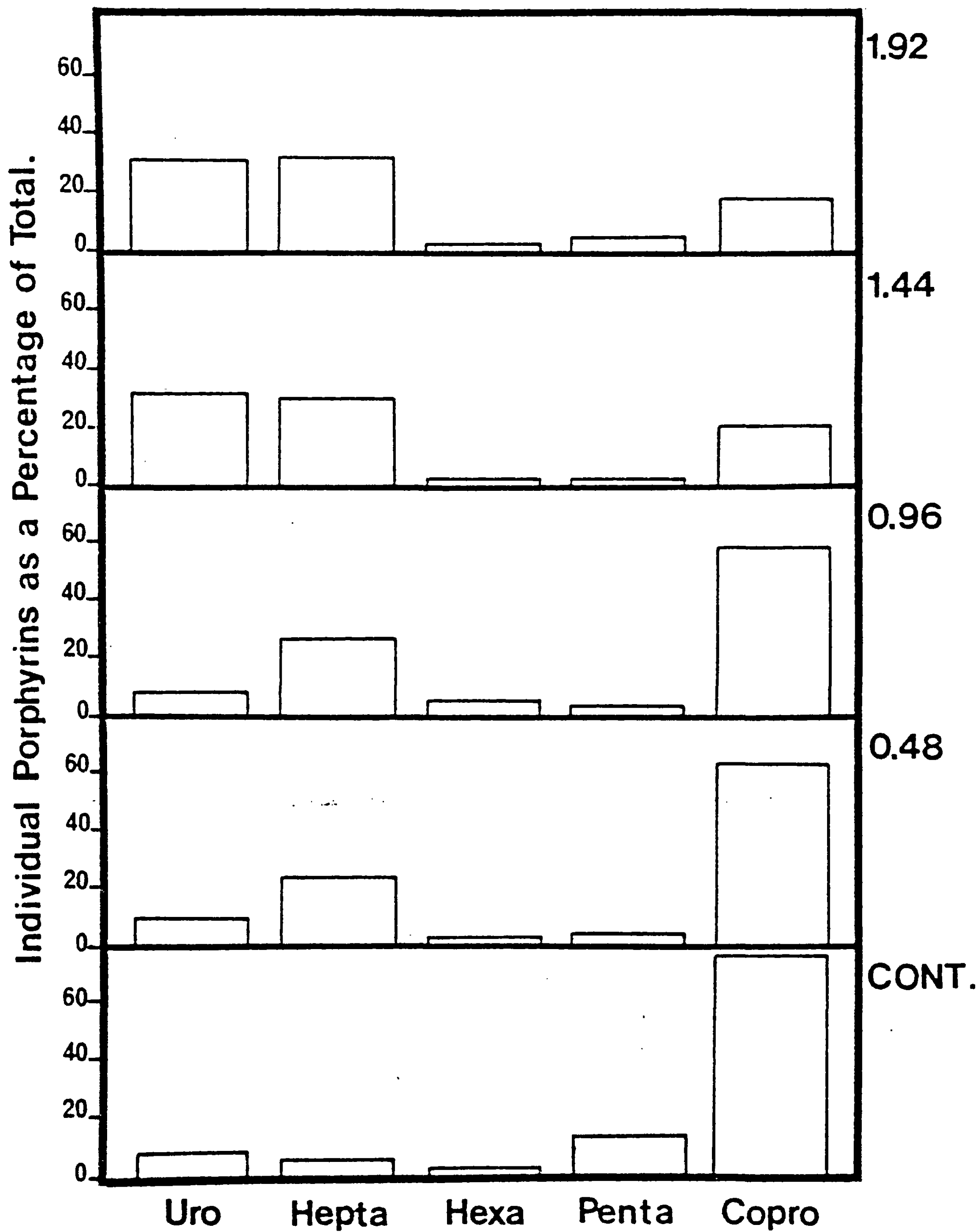


FIGURE 33 INDIVIDUAL URINARY PORPHYRINS IN ANIMALS TREATED WITH EADC IN DOSES SHOWN (mmol/kg)

Uro = Uroporphyrin Copro = Coproporphyrin

Hepta, Hexa, Penta = Heptacarboxylic porphyrin etc.

5.3. ENZYMES OF HAEM BIOSYNTHESIS

This set of experiments were carried out with OHKPL and OHHPL only. The effects of these compounds on each enzyme activity was measured in vivo and in vitro.

5.3.1. METHODS

In the in vitro study assays of each of the haem enzymes were carried out in the presence of OHHPL or OHKPL at differing doses calculated to span the projected plasma levels in animals given the porphyrinogenic dose of each compound and maximal plasma levels in humans. The final concentration of OHHPL and OHKPL in the assay mixtures was 161, 32, 6.5 and 1.3 µmol/L. Assays were carried out as described in the method section and OHHPL or OHKPL were added in one component of the assay mixture to give these final concentrations. Pooled liver homogenate from 3 rats was used.

For the in vivo experiment animals were treated as in the preceding section and were killed at the time of maximum porphyrin excretion (normally 48 hours after the first injection). Livers were perfused in situ with ice cold saline, excised and homogenates used for enzyme assay.

5.3.2. RESULTS

The results of the enzyme assays with added OHHPL are shown in Table 18. In no case was there a significant difference in

TABLE 18 IN VITRO ENZYME ACTIVITIES IN THE PRESENCE OF OHHPL

All enzyme activities are expressed as nmol product formed/g protein/hour

	Final OHHPL conc. (μmol/L)				
Enzyme	161	32	6.5	1.3	0
ALA.S	32	28	31	29	33
ALA.D	2.81	2.81	2.85	3.48	3.35
URO.S	11.28	13.29	14.05	15.05	12.06
URO.D	2.68	5.98	2.64	0.16	1.55
COPRO.O	57.2	61.6	59.0	58.5	55.0
FERRO.C	0.42	0.49	0.49	0.47	0.46

any of the treated groups. This was also found for OHKPL.

In vivo results are shown in Table 19. With OHKPL the only significant difference was in the raised activity of ALA.S There was very large variation in the activities of URO.D and COPRO.O and these were not assayed in the animals treated with OHHPL. In these animals raised ALA.S was again the only difference.

In a separate experiment haemopyrrole also was shown to cause an increase in ALA.S activity in rat liver.

TABLE 19 ENZYME ACTIVITIES IN ANIMALS TREATED WITH OHKPL AND OHHPL

* Significantly different from control $p < 0.005$

† As percentage of ^{59}Fe into haem/mg of protein/hour incorporation

Enzyme	Activity (nmol product formed/hour per g protein)			
	Control	OHKPL	Control	OHHPL
ALA.S	72.2 ± 16.5	195.8 ± 65.6*	79.5 ± 10.4	132.4 ± 24.1*
ALA.D	10.1 ± 2.4	8.9 ± 2.1	14.3 ± 3.7	15.2 ± 4.2
URO.S	21.9 ± 7.7	26.1 ± 7.9	37.8 ± 2.5	41.5 ± 2.9
URO.D	16.5 ± 24.2	6.5 ± 6.4	-	-
COPRO.O	92.2 ± 70.0	194 ± 89.0	-	-
FERRO.C†	1.81 ± 0.95	1.7 ± 0.66	2.6 ± 0.19	2.6 ± 0.358

5.4. MICROSOMAL HAEM AND CYTOCHROMES

To investigate further the increased activity of ALA.S described in the foregoing section, microsomal levels of haem and cytochrome P-450 and b_5 were measured in the livers of animals treated with OHKPL and OHHPL.

5.4.1. METHOD

Animals were treated with OHHPL and OHKPL as before. Microsomes were prepared and cytochromes measured by the methods described in section 2.7.

5.4.2. RESULTS

The results are shown in Table 20.

Both OHHPL and OHKPL cause a significant decrease in the hepatic microsomal levels of haem and cytochrome P-450. In neither case do the observed changes in cytochrome b_5 levels achieve significance.

TABLE 20 HEPATIC MICROSOMAL LEVELS OF HAEM AND CYTOCHROMES P-450 AND b_5 IN ANIMALS TREATED WITH OHHPL AND OHKPL

The numbers of animals in each group is given in parenthesis

* Significantly different from control values $p < 0.01$

	Haem	Cytochrome P-450	Cytochrome b_5
	nmol/mg protein		ΔA/mg
Control	1.84 ± 0.24 (17)	1.17 ± 0.18 (17)	0.087 ± 0.064 (17)
OHHPL	1.07 ± 0.12* (6)	0.53 ± 0.17* (6)	0.061 ± 0.032 (6)
OHKPL	1.22 ± 0.38* (6)	0.55 ± 0.25* (11)	0.056 ± 0.040 (11)

5.5. HAEM OXYGENASE

Haem oxygenase (HAEM.O) the haem degrading enzyme was assayed in animals treated with OHHPL in an attempt to explain the haem deficiency that results from treatment with these compounds. Tlc of a chloroform extract of whole liver after methylation was also carried out to look for the formation of green pigments which have been associated with apparently non-enzymic loss of liver haem (Unseld and De Matteis, 1976; De Matteis and Unseld, 1976). In vitro effects of OHHPL were also studied using a microsomal preparation from animals treated with stannous chloride to induce HAEM.O as the basal level was too low to detect any depression of activity by the method employed.

5.5.1. METHODS

In the in vivo studies rats were injected with OHHPL at two dose levels: 0.65 mmol/kg and 1.3 mmol/kg. Groups of six animals were killed at 16 and 40 hours and the livers removed without perfusing.

On removal a macroscopic examination of the livers was made for green discolouration. A portion was then taken for the preparation of microsomes and the remainder was pooled in test and control groups and esterified overnight in a methanol/ sulphuric acid mixture (95:5). The methanolic mixture was extracted with chloroform, the chloroform fractions evaporated

and the residue applied to silica gel plates and chromatographed in a system consisting of chloroform/kerosene/methanol (20:5:3). On development examination was carried out for the appearance of green bands at positions below that of a haem standard.

Microsomal pellets were also examined for green colouration before resuspension for the assay of HAEM.O (Section 2.8.2).

The in vitro effects of OHHPL were studied using a high activity preparation of HAEM.O obtained by treating animals with stannous chloride (0.25 mmol/kg in saline) 16 hours before the preparation of the microsomal fraction. This produced an HAEM.O activity of 40 - 50 times basal. The effects of OHHPL were measured by adding OHHPL to the reaction mixture as for the haem enzyme although higher concentrations were used.

5.5.2. RESULTS

All attempts to demonstrate the presence of green pigments proved fruitless. Colourations of the microsomal pellets and esterified liver extracts from test animals were indistinguishable from those from control animals. Tlc of the liver extract showed no green fractions.

Microsomal HAEM.O activity in animals treated with OHHPL is shown in Table 21. There was no significant difference in haem oxygenase in any of the groups.

TABLE 21 MICROSOMAL HAEM.O ACTIVITY IN THE LIVER OF OHHPL TREATED ANIMALS

Haem.O activity was measured in microsomal preparations at 16 hours following a single injection or at 40 hours following injections of OHHPL at 0 and 24 hours.

	Haem.O activity (nmol haem used/ mg protein/hour)	
Group	16 hours	40 hours
Control	1.58 ± 0.55	1.49 ± 0.72
OHHPL (0.65 mmol/kg)	1.47 ± 0.75	1.61 ± 0.87
OHHPL (1.3 mmol/kg)	1.45 ± 0.58	1.51 ± 0.75

The effect of OHHPL on HAEM.O from pooled induced livers is shown in Figure 34. It can be seen that at the highest concentration used (16μM) there was a 30% decrease in the activity of HAEM.O.

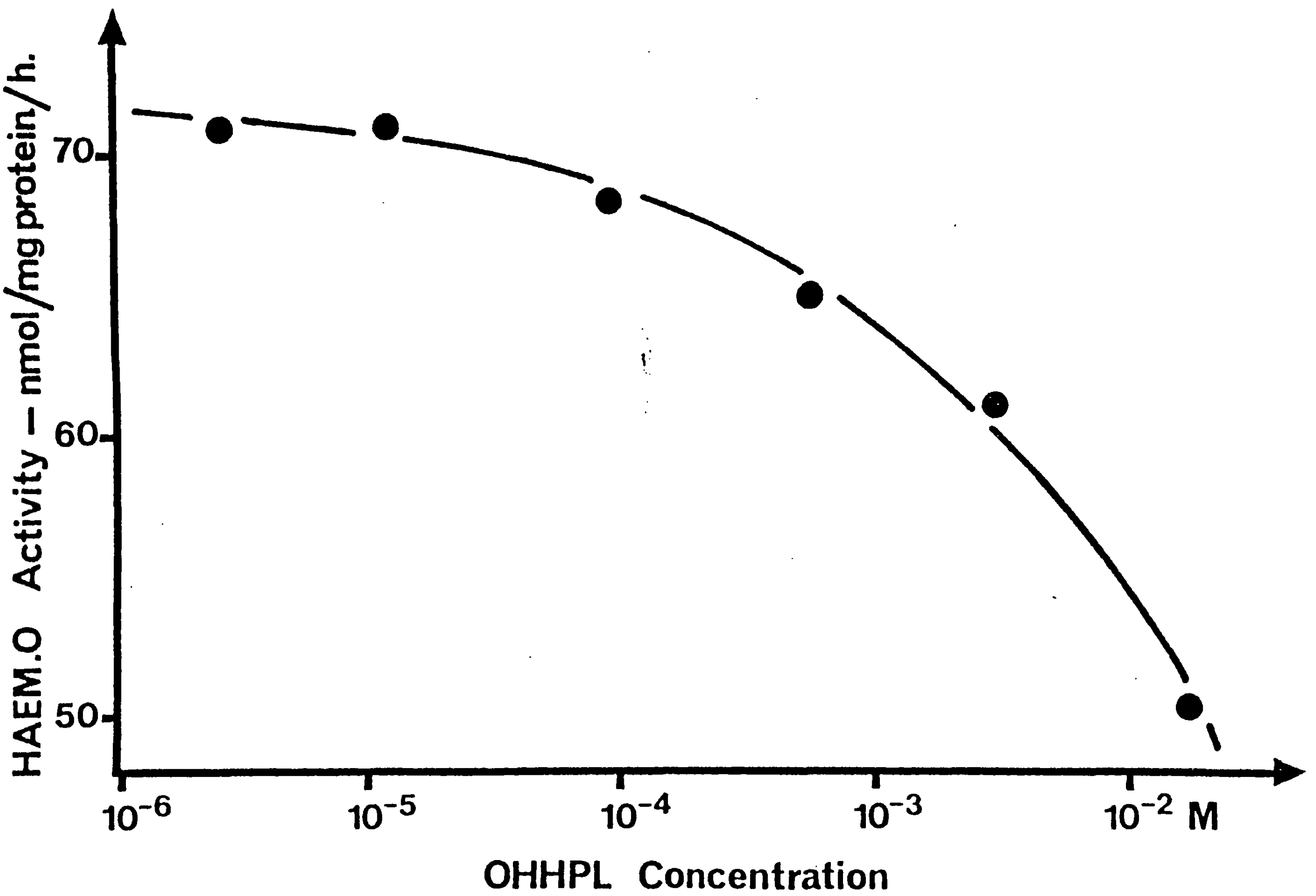


FIGURE 34 THE EFFECT OF OHHPL ON MICROSOMAL HAEM.O FROM RATS PRE-TREATED WITH $SnCl_2$

5.6. PBG OXYGENASE

Measurement of PBG.O by the method in section 2.8.1 proved extremely problematical. The activity in the microsomes from control animals was so low that it could not be measured using the colorimetric method. It has been suggested that this low activity is due to the presence of an inhibitor of the enzyme under normal circumstances.

Using the radiochemical method the reaction products 2-keto-PBG and 5-hydroxy-2-keto-PBG (Figure 12) could be isolated and quantitated. However, it was found that identical processing of blank reaction mixtures also yielded both of the reaction products indicating that autooxidation of the PBG was occurring during the extraction process.

Processing of PBG solutions in the same way showed that the extent of autooxidation was extremely variable and could not be eliminated by alteration of the extraction procedure without drastically affecting the yield of enzymatically produced oxidation products.

Using the enzyme assay, as described, the enzymic production of oxidation products was about 20% of non-enzymically produced material and it was impossible to detect any alteration in activity following treatment with haemopyrrole reported as substrate for the same or a similar enzyme (Irvine, 1974). Animals treated with either kryptopyrrole or haemopyrrole showed the appropriate oxidation products in their

urine but it is conceivable that these also could be produced non-enzymically.

Human placental tissue was used to produce microsomes in which the activity of PBG.O could be detected. However, the poor quality of the assay precluded any comparative studies although the findings were indicative of the presence of this system in human tissue.

Purification of the enzyme from any of the sources was not carried out as a quantitative assay of the activity of the enzyme could not then be obtained. A more sensitive assay will probably be required before comparative experiments are possible, although it may be that levels are very low in the uninduced state.

5.7. DISCUSSION

Prior to the initiation of these studies the sole report of porphyrinogenesis due to a monopyrrole was the report by De Matteis that the pyrrole derivative 4-methyl-2-methoxymethyl-5-carbethoxypyrrole-3-propionic acid methyl ester could cause porphyrin accumulation in chick embryo liver cell cultures (De Matteis, 1968). The ability of other pyrroles, reported in the foregoing chapter, to cause increases in porphyrin synthesis and excretion in rats is suggestive that this may be a property of pyrroles as a class of compounds.

It is extremely unlikely, considering the structures of the pyrroles involved, that the increased porphyrin formation caused by administration of these compounds is due to direct incorporation of the compounds into porphyrins. Evidence in support of this has been obtained in bacterial systems. Kryptopyrrole, which causes increased porphyrin accumulation in the medium of Bacillus subtilis cultures, cannot support the growth of mutant strains of the bacteria lacking the initial enzymes of haem biosynthesis, ALA.S and ALA.D (Berek et al, 1975).

It is also equally unlikely that each of the pyrroles used is metabolised to a common intermediate, although it is feasible that haemopyrrole and kryptopyrrole exert their porphyrin inducing effects through the oxidised forms OHHPL and OHKPL. Thus using bacterial cultures Durko et al (1975) have shown that the effects of kryptopyrrole are enhanced if oxidation products are present

although these include compounds other than OHKPL.

The mechanism of action of the compounds in this study is not clear although it is probable that in each case the mechanism is the same as that of OHHPL and OHKPL the compounds studied in greatest depth. It is clear that the increased porphyrin synthesis is due to elevated ALA.S activity and that this is probably due to derepression resulting from a decrease in a regulatory haem pool as evidenced by decreased microsomal haem levels.

Depletion of the regulatory haem pool, or free haem, could occur by one of several mechanisms. Increased enzymic or chemical degradation, increased utilisation, or decreased formation could all lead to depletion of free haem levels. In liver the major fraction of haem is utilised in cytochrome P-450 formation and as microsomal cytochromes are also depleted there would appear to be no increase in utilisation. There is also no evidence of increased degradation of haem. No green pigments, indicative of abnormal haem breakdown, were found nor was there any change in HAEM.O activity in vivo.

Oxidised forms of kryptopyrrole have been found to bind to haem in vitro (Durko et al, 1976) and this effect may be responsible for the inhibition of haem oxygenase at high OHHPL concentrations. It is unlikely that in vivo concentrations become great enough to cause any significant effect on the enzyme.

The most likely reason for the decrease in haem formation is an inhibition at some point in the biosynthetic pathway. Evidence

for this is given by the accumulation of porphyrins in the liver and increased excretion of porphyrins. It is feasible that pyrroles, due to their similarity in structure to the porphyrins, could cause competitive inhibition in the pathway enzymes or transport processes. No effect was shown, however, in the enzymes of the pathway studied, in vivo or in vitro, although the assays of URO.D and COPRO.O may have lacked the necessary sensitivity or precision to detect any changes.

Little is known of the processes involved in the transport of pathway intermediates between the cytoplasm and the mitochondria and it is possible that the inhibition occurs there or in the penultimate enzyme of haem biosynthesis, protoporphyrinogen oxidase for which there is no established assay.

One final possibility is that the monopyrroles exert their effects by altering the availability of cofactors or secondary substrates for the pathway enzymes such as the iron required for the ferrochelatase reaction. The latter may be possible if like kryptopyrrole the other monopyrroles are able to complex iron (Fischer and Orth, 1934). No evidence in favour of this was obtained from the studies with FERRO.C, but it is not inconceivable that the situation in vivo is quite different to that in the in vitro assay and that in the in vivo situation the concentration and availability of iron are critical.

Although no reports have been made of inhibition of haem biosynthesis in this manner, the porphyrin excretion pattern is

not inconsistent with this. At lower doses the main finding is of increased coproporphyrin in the urine with increased hepatic levels of coproporphyrin and protoporphyrin. This is consistent with the findings of Durko et al (1975) who found increased coproporphyrin, penta and uroporphyrin (all III isomer) in culture medium of B. subtilis given kryptopyrrole.

The effect of EADC in increasing doses is similar to the findings in rats treated with hexachlorobenzene (HCB)(Doss et al, 1976). Prolonged treatment with HCB results in uroporphyrin and hepta being the major urinary porphyrins with coproporphyrin next. With EADC the effect was much more rapid. The relative increase in the proportion of these two porphyrins at the higher doses may reflect progressively greater liver dysfunction and the associated diuresis may be also due to this or renal damage.

The effects of these compounds were not entirely related to their toxicity, although the most toxic, haemopyrrole, was the most effective in increasing porphyrin excretion. The extreme toxicity of haemopyrrole and kryptopyrrole appeared to be associated with the vacant α-position as substitution with an oxygen function or methyl group greatly reduced the toxicity. In addition, increasing the number of oxygen functions and thus polarity and water solubility, decreased the toxicity without abolishing the effects on porphyrin metabolism.

It would appear then that further investigations are required before fuller understanding of the properties and mechanism of action of this group of compounds is obtained and it may be that the use of the non-toxic EADC may provide a model system for the action of the others.

CHAPTER 6

BEHAVIOURAL EFFECTS OF 3-ETHYL-5-HYDROXY-4,5-DIMETHYL-Δ^3-PYRROLIN-2-ONE

CHAPTER 6

BEHAVIOURAL EFFECTS OF 3-ETHYL-5-HYDROXY-4,5-DIMETHYL-Δ^3-PYRROLIN-2-ONE

6.1. INTRODUCTION

In attempts to investigate further the possibility that the porphyrin precursors ALA and PBG were involved in the aetiology of the manifestations of the acute porphyrias, a large number of pharmacological studies have been carried out. This is especially true of ALA the more readily available compound. Recent work reviewed by Becker and Kramer (1977) has shown that ALA and PBG both have potent pharmacological effects on the nervous system. However, the physiological significance of these findings is uncertain.

As well as pharmacological activity, ALA has been shown to exert behavioural effects including impairment of voluntary movement (McGillion et al, 1973; McGillion, 1975) and effects on social behaviour in mice (Cutler and Moore, 1978).

The association of the excretion of OHHPL with porphyria and psychiatric disorders has led to the testing of the pharmacological and behavioural effects of certain pyrroles, especially kryptopyrrole originally thought to be the natural metabolite. Although kryptopyrrole and its oxidised form (OHKPL) were found to have both pharmacological and behavioural properties (see Section 1.4.2.) OHHPL has not been tested in any

system. The following sections describe experiments designed to look at the effects of OHHPL in two systems in which ALA was found to be active. These were designed to look at spontaneous voluntary activity in rats and social behaviour in mice. Both of the methods allow quantitation of any changes in behaviour.

6.2. METHODS

The spontaneous voluntary activity of rats treated with OHHPL was measured by the method of McGillion et al (1973).

A group of six adult male rats were injected with OHHPL (0.65 mmol/kg in ethanol (50% $^{v}/v$)) and six control animals received 50% ethanol alone 7 minutes later. Ten minutes following the injection each group of animals was placed in an activity monitoring box and the activity registered for five minutes.

The activity monitoring system comprised a wooden glass-fronted box (60cm x 60cm x 60cm) into the roof of which two ultrasonic pressure transducers were mounted. One of the transducers, acting as a transmitting element, was driven by an oscillator unit at 40 KHz, setting up a standing wave pattern within the test area. The second transducer acted as a receiver and changes in the standing wave pattern caused by the movement of animals within the box produced the output of the unit. The demodulated and amplified output from the receiver was used to drive a mechanical counter and the count registered on it was proportional to the activity monitored by the receiver.

By using a pair of counter/amplifiers both attached to the same receiver it was possible to differentiate between gross and fine movements by manipulating the sensitivity of each. The counter set at greater sensitivity picked up fine and gross

movements while that at lower sensitivity picked up gross movements only. A measure of fine movement could be obtained from the difference of the two values.

The spontaneous activity of each group of animals was measured for 5 minutes every 15 minutes and measurements were continued for 1.5 hours. The whole experiment was carried out under a 'safelight' and the animals were observed for any gross abnormalities in behaviour.

The second set of experiments involved the direct observation of the behavioural acts and postures during social interaction between mice. The method entailed the placing of two unfamiliar mice together and recording the acts and postures during the ensuing encounter. The postural elements and behavioural acts forming the basis of the study were outlined by Grant and Mackintosh (1963) and were grouped into the categories of non-social behaviour, social investigation, aggression and flight. The subdivisions of each category are shown in Table 22.

In practice, adult male mice of the CWF strain were injected with OHHPL in trigol/saline (1:8) at two dose levels (0.975 mmol/kg and 1.95 mmol/kg). Control animals received trigol/saline. Each treatment group was divided in two with animals in each half drawn from different stock cages. At 50 minutes to 1 hour after injection two mice, one from each half of the same treatment group, were placed in a clear transparent

TABLE 22 BEHAVIOURAL CATEGORIES AND INDIVIDUAL ELEMENTS OBSERVED IN THE ETHOLOGICAL STUDY OF THE EFFECT OF OHHPL IN MICE (Grant and MacKintosh, 1963)

1. NON-SOCIAL BEHAVIOUR

Explore	Dig	Scratch
Scan	Push-dig	Crawl-over
Wash	Eat	on-bars
Self-groom	Jump	off-bars

2. SOCIAL INVESTIGATION

Attend	Nose	Groom
Investigate	Follow	Stretch-attend
Sniff	Push-under	Crouch

3. FLIGHT

Flag	Upright posture	Defensive sideways
Evade	Defensive upright	Retreat

4. AGGRESSION

Bite

5. IMMOBILITY (No individual elements)

cage between two observers. The behaviour shown by each animal was recorded by the observers for five minutes and the frequency and duration of each category listed. Following observation animals receiving the higher dose level were anaesthetised with ether and blood removed by heart puncture for estimation of plasma OHHPL levels. They were then killed before recovery.

6.3. RESULTS

The results of the spontaneous activity measurements are shown in Table 23. When summed over the times of the experiment each day there was no significant differences between test and control group. Within each group and category there was a large variation in values and this may reflect an effect of the injection itself.

In the ethological study there were no apparent differences in any of the categories in either of the test groups. The combined results are shown in Tables 24, 25 and 26. In no category was there any significant difference in mean frequency of any of the elements in each category or the sum of the individual elements of a category. The total mean duration of all elements of each category showed no differences between groups.

The mean plasma OHHPL level in the test group receiving OHHPL at 1.95 mmol/kg was 0.31 ± 0.11mmol/L.

TABLE 23 SPONTANEOUS ACTIVITY IN ANIMALS GIVEN OHHPL AND CONTROLS

Activity was measured every 15 minutes and the animals were injected on two consecutive days.

	Total counts in each category					
	Test (n = 6)			Control (n = 6)		
DAY 1	Gross	Gross+Fine	Fine	Gross	Gross+Fine	Fine
10min	10076	12801	1725	5554	11427	5873
25min	5412	10485	5073	6110	11029	4919
40min	3238	7658	4420	4403	8696	4293
55min	2968	6594	3526	4032	8689	4646
70min	2337	4581	2244	2900	6433	3533
85min	2895	10298	4403	6337	10595	4258
Mean	4987	8736	3490	4889	9478	4587
S.D.	±2865	±3001	±1460	±1338	±1895	±748
DAY 2						
10min	4311	7795	3484	3988	7786	3799
25min	3323	6960	3637	2842	6490	3648
40min	4415	8550	4135	5401	10383	4982
55min	1460	3382	1922	2271	5563	3292
Mean	3377	6671	3295	3625	7556	3930
S.D.	±1369	±2287	±956	±1382	±2093	±733

TABLE 24 RESULTS OF OBSERVATIONS OF NON-SOCIAL BEHAVIOUR IN ANIMALS TREATED WITH OHHPL

NON-SOCIAL BEHAVIOUR	Mean frequency of individual elements	
	Control (n = 28)	Test (n = 32)
Explore	57.2	55.69
Scan	48.9	49.34
Wash	3.5	2.25
Self-groom	2.57	1.88
Dig	7.50	6.63
Push dig	2.50	3.34
Eat	1.35	1.10
Jump	1.47	0.75
Scratch	0.57	0.41
Crawl-over	0.04	0
on-bars	0.47	0.12
off-bars	0	0.06
Overall mean frequency	126.57	121.5
Overall mean duration	227.23	224.75

TABLE 25 RESULTS OF OBSERVATIONS OF SOCIAL INVESTIGATION IN ANIMALS TREATED WITH OHHPL

SOCIAL INVESTIGATION	Mean frequency of individual elements	
	Control (n = 28)	Test (n = 32)
Attend	18.18	16.1
Investigate	6.39	7.88
Nose	6.04	4.97
Sniff	0.75	1.81
Follow	0.75	0.91
Push under	1.0	1.0
Groom	0.29	0.13
Stretch-attend	0.04	0
Crouch	0.43	0.28
Overall mean frequency	33.9	33.1
Overall mean duration	46.3	49.3

TABLE 26 RESULTS OF OBSERVATIONS OF FLIGHT, AGGRESSION AND IMMOBILITY IN ANIMALS TREATED WITH OHHPL

	Mean frequency of individual elements	
	Control (n=28)	Test (n=32)
FLIGHT		
Flag	0.61	0.38
Evade	0.07	0.06
Upright posture	0	0.03
Defensive upright	0	0.1
Defensive sideways	0.04	0.07
Retreat	0	0.03
Overall mean frequency	0.71	0.67
Overall mean duration	0.73	1.4
AGGRESSION		
Bite	0	0.07
Overall mean frequency	0	0.07
Overall mean duration	0	0.21
IMMOBILITY		
Mean frequency	5.55	5.38
Mean duration	25.26	23.9

6.4. DISCUSSION

Methods which involve observation or measurement of changes in the behaviour of laboratory animals have been used in the past to look at the pharmacological properties of certain compounds. Section 1.2.4 outlines experiments carried out in the past to test the behavioural activity of certain monopyrroles, either thought at the time to be the natural excretion product in psychoses and porphyria or structurally related to such compounds. The work described in this chapter was designed to investigate the properties of OHHPL, the natural product, in two experimental systems which had already been used to show behavioural properties of ALA.

The first method, that of McGillion et al (1973), was used to show that ALA treatment caused changes in the spontaneous activity of mice. In an identical system, using rats, OHHPL caused no significant changes in activity although the dose used was sufficient to cause increased hepatic porphyrin synthesis and porphyrinuria. Temporal variations were observed in both treated and untreated animals but these may just reflect changes due to the trauma of the injection procedure or to disturbances in the surrounding environment.

The second experiment utilised a technique which has been shown to provide an highly sensitive index of drug induced behavioural changes (Chance and Silverman, 1964). It has also been shown that certain drugs can elicit quantitative dose-dependent changes in some of the behavioural categories (Cutler

et al, 1975). Cutler and Moore (1978) have shown using this system that there is an increase in immobility in mice treated with ALA with a concomitant decrease in non-social behaviour. OHHPL was found to be totally inactive in this system at each of the doses used.

Contrary to the above findings of Cutler et al (1975) it has been shown that certain categories of behaviour only undergo drug-induced changes in a narrow dose range (Krsiak, 1974) and the lack of activity shown by OHHPL at the two doses used may not preclude activity at lower, or even higher concentrations. EADC, for example (used in porphyrinogenicity testing) was found to have sedative and hypnotic effects at doses of 0.96, 1.44 and 1.92 mmol/kg.

The behavioural effects elicited by ALA are probably direct effects on the central nervous system as ALA has been shown to cross the blood brain barrier (BBB) at the concentrations used. However, no information is available on the ability of OHHPL to cross the BBB although it is clear that high plasma levels are attained following injection.

As was said above, it may be that the failure to show effects of OHHPL may reflect the inadequacy of the dose range used. It is, however, difficult to project doses in animals equivalent to the human situation. The maximum human plasma level was estimated to be 10% of the maximum urinary level, i.e. 4 µmol/l and the plasma level encountered in the final experiment

was many times this. It is recognised however that in the case of certain drugs (e.g. barbiturates) the effective dose in rodents can be at least forty times the dose for humans.

It remains to be seen therefore whether the dose levels of OHHPL and OHKPL bear any relationship to the human situation but it must be said that the doses used produced plasma levels probably far in excess of those in man. These experiments therefore provide no evidence of a behavioural activity of OHHPL of physiological significance.

CHAPTER 7

THYROID FUNCTION AND PORPHYRIA

CHAPTER 7

THYROID FUNCTION AND PORPHYRIA

7.1. INTRODUCTION

The clinical suspicion and later biochemical confirmation of thyrotoxicosis in a patient (Appendix 2, Case 5) during an acute attack of AIP led to this study of thyroid function in AIP.

The acute porphyric attack shows many common features with acute thyrotoxicosis including tachycardia, vomiting, weight loss and a neuropsychiatric syndrome (Hall et al, 1974). Although only one case of hyperthyroidism associated with AIP has been reported (Mann and de Nardo, 1965), serum protein-bound iodine (PBI) (Hellman et al, 1963) and total thyroxine may also be raised in AIP (Hollander et al, 1967). These latter findings may be related to a rise in thyroxine-binding globulin (TBG) (Hollander et al, 1967). The rise in total thyroxine was thought to be independent of the clinical status of the patient (Hollander et al 1967).

Biochemical studies on the effects of thyroid hormone on haem biosynthesis have shown that in rats T3 can potentiate the induction effects of allylisopropylacetamide (AIA) on hepatic ALA.S (Matsuoka et al, 1968). More recently Morgan et al (1976) have demonstrated that although inactive by themselves T3 and T4 can also stimulate AIA induced porphyrin biosynthesis in serum-free chick embryo liver cell cultures.

As well as looking at thyroid function in AIP this study looks at some possible effects of thyroid hormone on porphyrin synthesis and also at thyroid hormone levels in experimentally produced porphyria.

7.2. CLINICAL STUDIES

7.2.1. PATIENTS

Thyroid function of a group of patients with AIP was studied. Four patients were studied during an acute attack (Appendix 2: Cases 3, 5, 7 and 9) and in three of these (Cases 3, 5 and 7) sequential measurements of thyroid function were made during further acute attacks. Seven patients in remission and six latent for the disease were also studied.

7.2.2. RESULTS

Methods employed are outlined in Section 2.9 and normal values of thyroid function tests are shown in Table 2.

In all patients with AIP in remission or latency, T3, T4, FTI, T3 and TSH values were within the normal range. In two patients in remission TSH response to the standard TRH test was normal. Serum T3, T4, FTI in patients 3, 5 and 7 are shown in Figures 35, 36 and 37. Where levels of free T3 and T4 were measured, these are also shown.

In Case 3, with one exception, acute attack was accompanied by a rise in T3, T4 and FTI (Figure 35) and on one occasion T3 was outwith the normal limits. Basal rT3 and TSH in attack and remission were normal.

Case 7 showed similar trends (Figure 36) and on one occasion T4 was above the upper limit of normal. Again basal rT3 and TSH were normal throughout as were TRH tests.

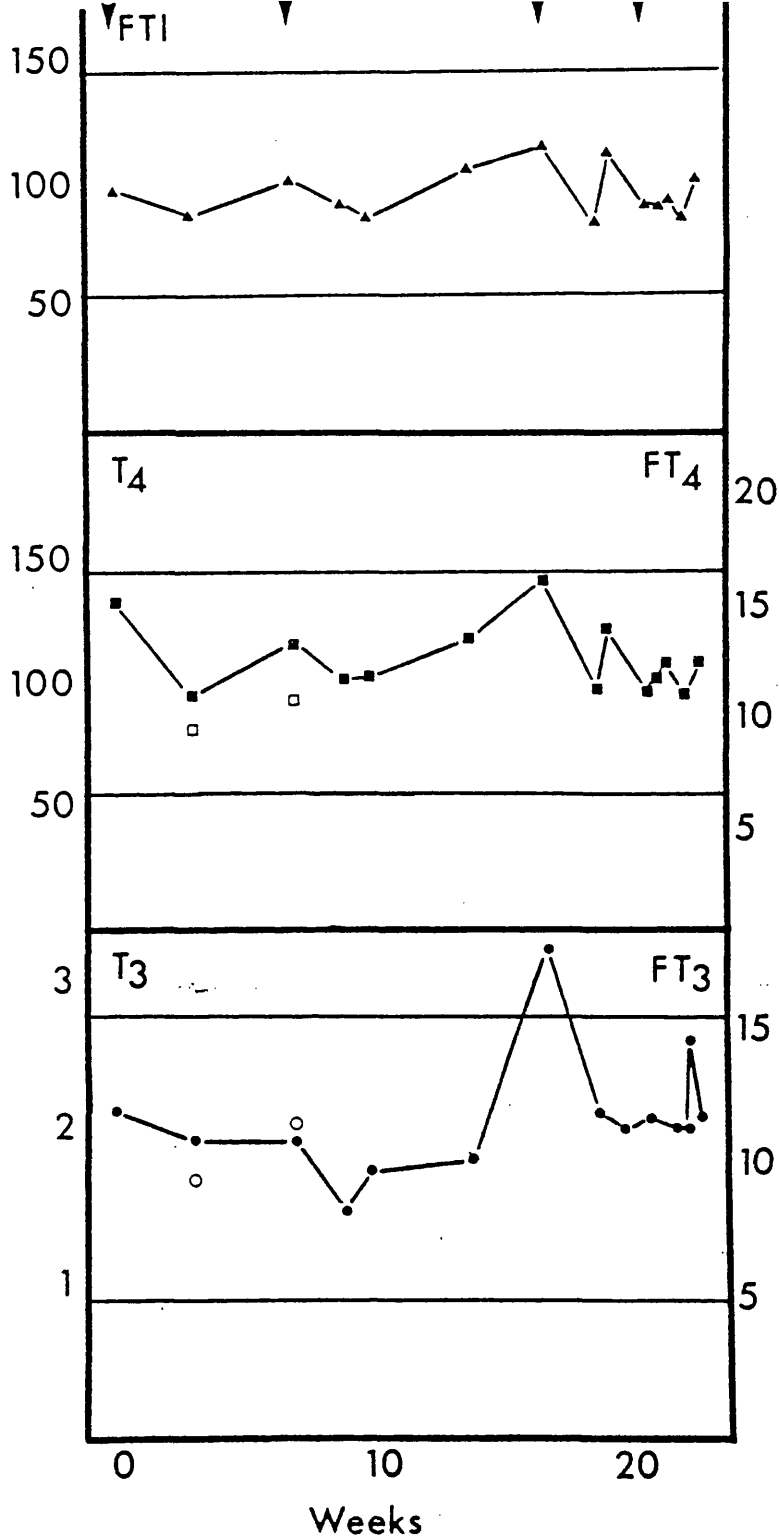


FIGURE 35 SEQUENTIAL MEASUREMENTS OF TOTAL (T3, T4) AND FREE (FT3, FT4) THYROID HORMONE CONCENTRATIONS AND FREE THYROXINE INDEX (FTI) IN CASE 3

Normal ranges are denoted by the solid lines

▼ = FTI A = Acute attack
■ = T4 nmol/litre □ = FT4 pmol/litre
● = T3 nmol/litre ○ = FT3 pmol /litre

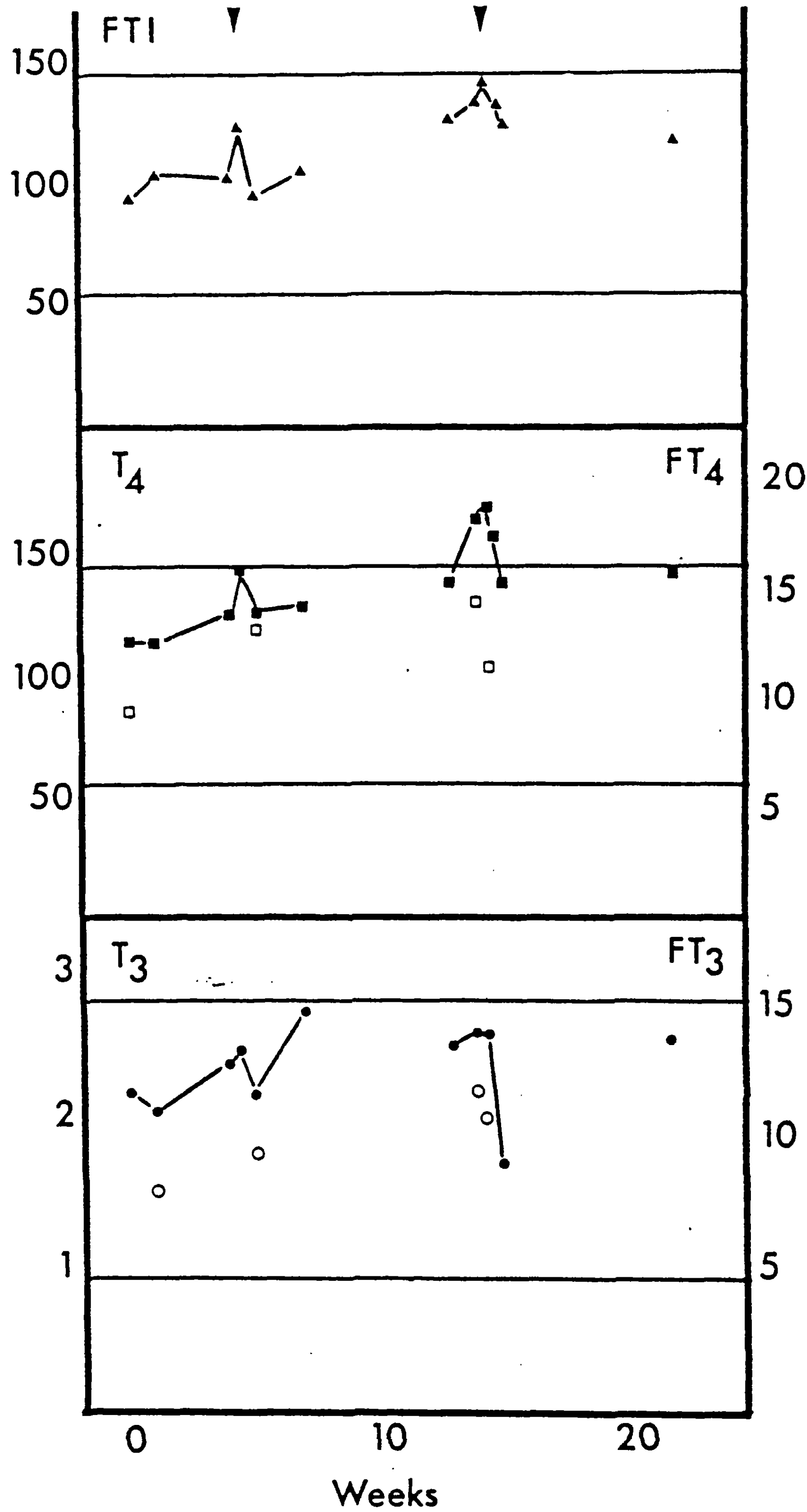


FIGURE 36 SEQUENTIAL MEASUREMENTS OF TOTAL (T3, T4) AND FREE (FT3, FT4) THYROID HORMONE CONCENTRATIONS AND FREE THYROXINE INDEX (FTI) IN CASE 7

Normal ranges are denoted by the solid lines

▼ = FTI A = Acute attack
■ = T4 nmol/litre □ = FT4 pmol/litre
● = T3 nmol/litre ○ = FT3 pmol/litre

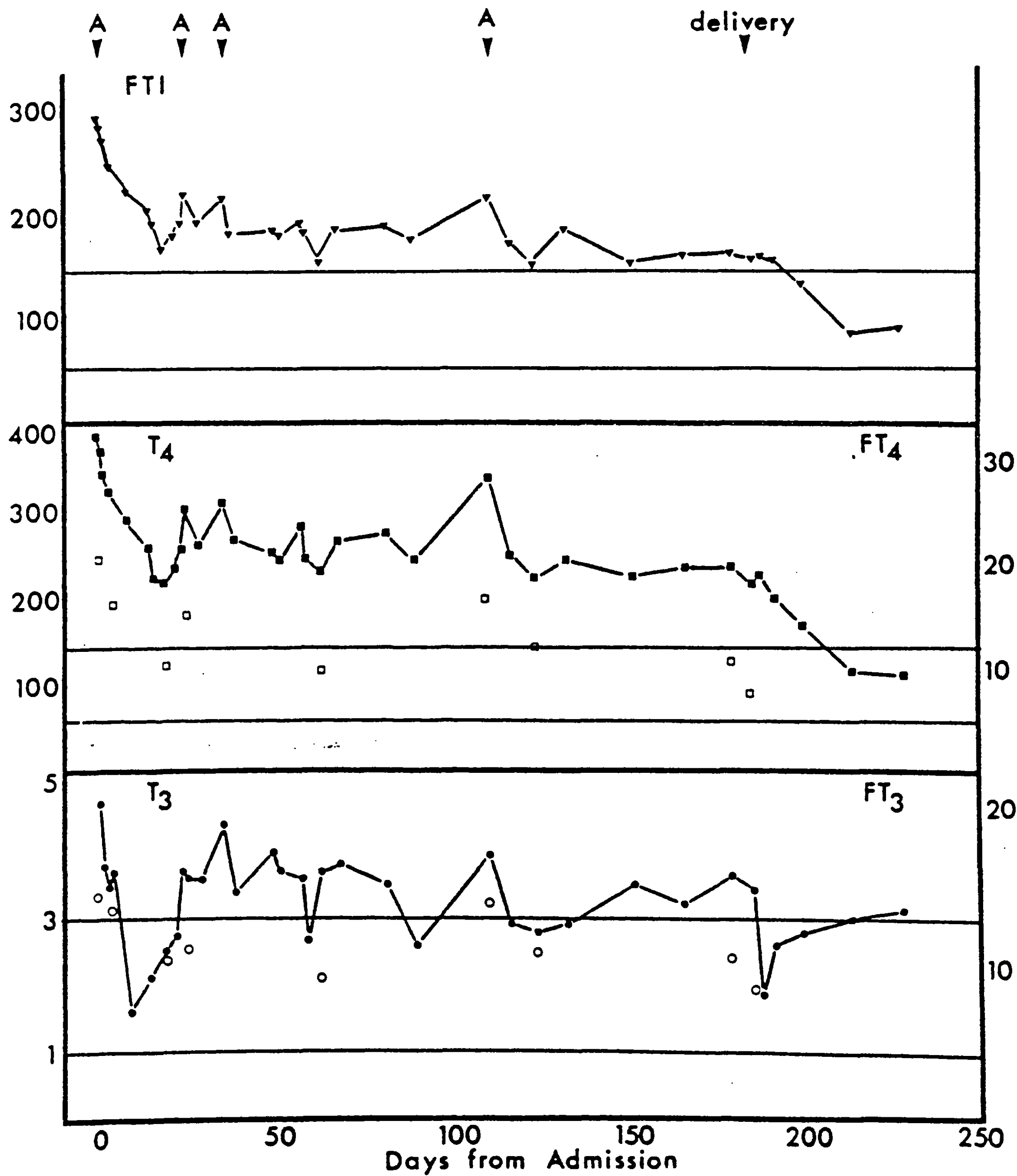


FIGURE 37 SEQUENTIAL MEASUREMENTS OF TOTAL (T3, T4) AND FREE (FT3, FT4) THYROID HORMONE CONCENTRATIONS AND FREE THYROXINE INDEX (FTI) IN CASE 5

Normal ranges are denoted by the solid lines

▼ = FTI
■ = T4 nmol/litre
● = T3 nmol/litre
A = Acute attack
□ = FT4 pmol/litre
○ = FT3 pmol/litre

Case 5 was followed throughout her pregnancy (Figure 37). There was a marked, consistent increase in T3, T4 and FTI throughout the pregnancy, the levels being higher when she was symptomatic ($p < 0.025$). rT3 values were also raised but showed no correlation with clinical condition. Levels of T3, T4, rT3 and the FTI fell to normal levels on delivery. Free T4 levels were above the upper limit of normal for pregnant women (Yeo et al, 1977a) and T3 levels were outwith the normal range on all but one occasion. TSH values were normal throughout and did not vary with clinical condition.

Thyroid function tests were normal in cord serum and in the baby at three days.

Case 9, when studied in attack showed a raised T4 (165 nmol/L) and FTI (147) with a normal T3. These parameters were all within the normal range one month later when the subject was asymptomatic.

In all four subjects urinary ALA and PBG levels were raised and showed little correlation with acute episodes. Similarly in cases 3, 5 and 7 urinary OHHPL levels, when measured, showed no correlation with clinical condition or thyroid hormone levels.

7.3. BIOCHEMICAL STUDIES

These studies fell into two sections. Firstly in an attempt to assess the possible effects of porphyria, or more specifically the metabolites produced in porphyria, rats were made "porphyric" by the administration of ALA or OHKPL and thyroid hormone measurements carried out. Secondly, following the report of the potentiating effects of T3 and T4 on drug-induced porphyrinogenesis, the effect of T3 on the induction of ALA.S by OHHPL was investigated.

7.3.1. METHODS

Groups of 8 and 6 Sprague-Dawley rats were treated with ALA and OHKPL respectively. Dosage was as described in Table 13. The animals given ALA were housed in metabolic cages and urine and faeces collected for porphyrin precursor and porphyrin measurements. After 48 hours both groups of animals were killed by stunning followed by exsanguination. In all animals serum total T4 measurements were performed and in the OHKPL group hepatic ALA.S activity was measured.

In the second experiment six groups of 6 animals were treated as follows: group 1, control (1ml 50% ethanol); group 2, OHHPL 0.13mmol/kg; group 3, OHHPL 0.13mmol/kg + T3 0.77μmol/kg; group 4, OHHPL 0.65mmol/kg; group 5, OHHPL 0.65mmol/kg + T3 0.77μmol/kg; group 6, T3 0.77μmol/kg. Animals were injected at 0 and 24 hours and killed at 48 hours for the estimation of hepatic ALA.S activity.

7.3.2. RESULTS

The results of T4 estimations in animals treated with ALA and OHKPL are shown in Table 27 along with porphyrin precursor and porphyrin results for the ALA group and ALA.S activities in the OHKPL group.

Treatment with ALA caused large increases in the excretion of porphyrins and porphyrin precursors and presumably circulating levels of ALA, PBG and to a lesser extent coproporphyrin as seen in the urinary excretion. Treatment, however, had no effect on serum T4 levels in these animals.

In the OHKPL group, treatment markedly raised hepatic ALA.S activity but again there was no effect on serum T4 levels.

ALA.S activities in the second set of experimental animals are shown in Table 28. Treatment with OHHPL was only effective in raising ALA.S activity at the higher dose and in none of these groups did T3 have an effect on ALA.S activity.

TABLE 27 THE EFFECT OF ALA AND OHKPL ON SERUM T4 LEVELS IN THE RAT

Group	Serum T4 levels		Urinary Excretion				Faecal excretion		ALA.S
	Mean ± S.D.	Range	ALA	PBG	URO	COPRO	COPRO	PROTO	nmol ALA/g protein/hour
			nmol/24h/rat				nmol/g dry wt.		
Control (n = 8)	71.5 ± 5.2	63 - 77	25	0	0	3	8	22	-
ALA (n = 8)	68.5 ± 8.4	59 - 84	14375	2337	0	12	38	143	-
Control (n = 6)	48 ± 15	26 - 68	-	-	-	-	-	-	72.2 ± 16.5
OHKPL (n = 6)	49 ± 42	15 - 119	-	-	-	-	-	-	195.8 ± 65.6

URO = Uroporphyrin COPRO = Coproporphyrin PROTO = Protoporphyrin

TABLE 28 THE EFFECT OF OHHPL AT T3 ON ALA.S ACTIVITY

* Significantly different from control values $p < 0.001$

Group (n=6)	Treatment	ALA.S nmol/g protein/hour
1	Control	58.2 ± 6.9
2	OHHPL 0.13 mmol/Kg	65.8 ± 7.2
3	OHHPL 0.13 mmol/kg T3 0.77 µmol/kg	63.1 ± 5.2
4	OHHPL 0.65 mmol/kg	158.7 ± 27.2 *
5	OHHPL 0.65 mmol/kg T3 0.77 µmol/kg	148.2 ± 25.0 *
6	T3 0.77 µmol/kg	59.6 ± 6.1

7.4. DISCUSSION

A single case of AIP has been reported with associated hyperthyroidism which was thought to have a deleterious effect on the porphyric neuropathy (Mann and de Nardo, 1965). In this patient the signs of both diseases improved with propylthiouracil therapy. Other studies have shown increased PBI (Hellman et al, 1963), total thyroxine and TBG (Hollander et al, 1967), although in these studies no correlation was drawn with clinical condition.

In the present study TBG was found to be raised only during an attack. In the three patients in whom sequential sampling was possible, total serum T4 levels and the FTI was increased in the acute attack. In patients 3 and 7, who presented regularly with mild pre-menstrual attacks, T4 levels were raised on 5 out of 6 occasions (Figures 35 and 36), although levels were within the normal limits. On the other hand there is little doubt that patient 5 became acutely thyrotoxic during an attack (Figure 37). Raised free hormone levels correlated well with attacks, although total hormone levels were difficult to interpret because of the pregnancy.

Any mechanism proposed for the increases in thyroid hormone levels can only be hypothetical. Abnormal synthesis and metabolism of other hormonal agents, for example steroid hormones, have been shown to be associated with the acute porphyric attack (Moore et al, 1973; Bradlow et al, 1976) and

carrier proteins other than TBG have been found to be raised in AIP (Stein and Tschudy, 1970). Although there have been reports of abnormal hypothalamic function in AIP (Perlroth et al, 1967; Stein and Tschudy, 1970) consistently low TSH levels suggest that pituitary driven thyroid stimulation is unlikely and no evidence obtained from TRH tests would indicate abnormalities in the hypothalamic-pituitary axis. It would seem then that the increased thyroid activity must be the result of a direct effect on the thyroid or reduced hormone clearance, although this would require that feedback mechanisms also be altered. Present knowledge does not allow unequivocal differentiation of these possibilities. There is an accumulating body of evidence of direct neural stimulation of the thyroid in man (Melander et al, 1976). Direct stimulation of sympathetic input to the thyroid has been shown to increase thyroid hormone secretion without affecting TSH levels (Melander et al, 1976). As the major clinical features of AIP can be explained on the basis of a generalised autonomic neuropathy (Goldberg, 1959), it may be that the hyperthyroidism seen in the patients with AIP may be neurogenic in origin.

The neuropathy in the acute porphyrias is probably due to neural demyelination itself due either to a haem deficiency or excessive metabolite production. As no animal model has yet been found that mimics the pathological changes found in AIP, it is not surprising to find normal thyroid function in the simplistic

animal systems studied. The experiments with ALA and OHKPL would, however, appear to rule out the possibility of a direct pharmacological effect of these or other metabolites on the thyroid in the rat. However, extrapolation to the human may not be valid.

It has been proposed (Matsuoka et al, 1968; Morgan et al, 1970) that the effects of T3 and T4 on AIA-induced porphyrin synthesis may be of physiological significance in AIP where there may be increased levels of these hormones. The possibility arises of a cyclical effect whereby the porphyric neuropathy increases free thyroid hormone levels which in turn may potentiate the induction of ALA.S.

AIA is thought to act primarily by destruction of the haem moiety of cytochrome P-450 following conversion of its allyl group to an epoxide group (Doedens, 1971). T4 has been shown to increase microsomal oxidases (Kato and Gillette, 1965) and it is possible that T3 and T4 enhance the effect of AIA by increasing the rate of epoxide formation. If this was so it would be expected that these hormones would also enhance the effects of other porphyrinogenic allyl compounds such as allyl-containing barbiturates, or other compounds requiring oxidative activation.

The lack of any effect, found in these studies of T3 on OHHPL induction of ALA.S suggests that OHHPL acts by another mechanism and does not require prior oxidation.

It has also been suggested that thyroid hormones act by increasing ALA.S-specific messenger RNA (Matsuoka et al, 1968).

If this was the case treatments which decrease intracellular haem, thought to repress ALA.S at a translational level (Sassa and Granick, 1970), would be expected to be enhanced by T3 or T4 treatment. In this case both AIA and OHHPL effects would be enhanced.

It is obvicus that greater knowledge of the mechanism of action of thyroid hormones is required before a full evaluation of the consequences of increased thyroid hormone levels in AIP can be made.

C H A P T E R   8

GENERAL DISCUSSION

CHAPTER 8

GENERAL DISCUSSION

Insight into the biochemical abnormalities in disease can be gained using analytical techniques to quantitate body substituents and it has been stated in the past,

> "It seems reasonable to assume that if one were able to identify and determine the concentrations of all compounds (of interest) inside the human body including high and low molecular weight substances, one would probably find that almost every known disease would result in characteristic changes of the biochemical composition of the cells and body fluids."
>
> (Jellum et al, 1973)

Although this view is futuristic, its relevance in the porphyrias is obvious. In the porphyrias, in particular, knowledge of the fundamental defect and of plasma metabolite levels has not as yet allowed unambiguous identification of the cause of the acute manifestations. There is little doubt that such complete knowledge would soon result in the understanding of the disease process.

Part of the work contained in this thesis is an extension of present knowledge of the occurrence of OHHPL, a metabolite of interest in the porphyrias. It was designed to investigate the possibility that OHHPL may play a role in the manifestations of acute porphyria.

Restriction of the quantitation of OHHPL to urine means that certain assumptions must be made in any discussion of plasma levels or of the effects of this compound. It may be erroneous to project plasma levels from urinary levels but the use of urinary creatinine levels for 'normalising' the urinary concentration of OHHPL may allow better comparison of results. If it is assumed that these levels to some extent reflect plasma levels (a totally random relationship between urine and plasma would be unexpected) then the results of quantitations provide no evidence for an acute role for OHHPL in the acute porphyrias. However, it may be that the urinary levels do not adequately reflect the plasma levels and that these in turn do not adequately reflect the tissue levels. This also holds true for the other metabolites which could arguably be the peccant substances in the acute porphyrias.

The finding and quantitation of OHHPL in psychoses is in confirmation of earlier studies by other workers. Again there was no evidence of a direct role for OHHPL in the generation of any of the clinical features of these disorders, although there was some evidence that OHHPL excretion was in some way related to the remission of symptoms in patients with schizophrenia.

The excretion of OHHPL in porphyria and psychoses may be unrelated but knowledge of the biosynthetic mechanism for formation of OHHPL is required for clarification of this point. It is

unlikely that OHHPL production in psychoses is a fundamental defect and it is more likely that it is a result of generally deranged metabolism. Other abnormalities that have been found in psychoses, schizophrenia in particular, include abnormalities in carbohydrate, amino acid and steroid metabolism (Tourney and Hatfield, 1972; Benjafield and Rutler, 1973).

The evidence from the patients with liver disease and industrial workers, taken along with the other results, is suggestive of an hepatic origin for OHHPL and that PBG could be a likely precursor. Both these possibilities require substantiation. Determination of plasma levels and of the biosynthetic origin of OHHPL should provide much greater insight into the possible roles of OHHPL in these disorders.

Determination of plasma levels will also allow a better assessment of the possible physiological significance of the finding that the group of monopyrroles studied, including OHHPL, were all porphyrinogenic. Most significantly these compounds caused a decrease in microsomal haem and an increase in the activity of ALA.S in rats.

Under normal circumstances any increased demand for haem can be met by a modest increase in ALA.S activity (De Matteis and Gibbs, 1972). However, in the porphyrias, the partial block in haem biosynthesis results in diminished haem formation and concomitant induction of ALA.S. Without the

superimposition of other factors a steady state is reached whereby the metabolic block is partially compensated by a raised ALA.S activity in turn maintained by a decreased haem level. A direct result of this is that any mechanism which puts an extra demand on haem will have a greater effect on this partially induced system than on a normal uninduced one. Such an example is the precipitation of acute porphyric attacks by drugs which are innocuous in this respect in normal subjects.

It would be impossible to predict the physiological significance of these results. If it is accepted that OHHPL is a direct product of PBG or any of the subsequent pathway products it would be extremely rare and unusual for a biological substance to cause an increase in the activity of the pathway forming it. The reverse is almost always the case. With this in mind it would be expected that any effect of OHHPL on patients with porphyria must be minimal although OHHPL may play a role in the maintenance of raised ALA.S activity.

More significant perhaps is the finding of raised thyroid hormone levels in patients in attack. The biochemical studies suggest that the raised hormone levels per se may not affect haem biosynthesis and any effect shown previously is due to the increased formation of active metabolite (for example activation of AIA). It is clear, however, that in one case studied the patient became thyrotoxic and there is little

doubt that her clinical condition suffered adversely. Considering the similarities between thyrotoxicosis and the acute porphyric attack, it is possible that in patients in attack raised thyroid hormone levels may be detrimental where the neuropathy of the attack pre-exists. This of course is purely hypothetical.

In conclusion then it can be said that, although OHHPL and related compounds have been found to have certain interesting effects on haem biosynthesis, no evidence has been obtained which points directly to OHHPL as the offending metabolite in either porphyria or psychosis. It is clear that further studies on its natural occurrence and pharmacological properties are required before any definitive conclusions are arrived at. These studies will certainly be expedited by the recent synthetic method (Wooldridge and Lightner, 1977) for its preparation. It will hopefully not be long before a conclusion can be reached on the role of this interesting compound, which for so long has frustrated researchers and ironically was originally misidentified as kryptopyrrole - 'the hidden pyrrole'.

APPENDICES

APPENDIX 1. Computer printouts of typical non-parametrical analyses of results using the Mann-Whitney U test and the Spearman and Kendall correlation analyses.

```
&RUN *BMDP
#EXECUTION BEGINS
BMDP3S

PROGRAM CONTROL INFORMATION

&CONTINUE WITH LGDC(1,10)+DGDOMIS(23,24)+(67,88)
          PROBLEM TITLE IS 'M.W.U.-DMMPL'./
          INPUT VARIAB=2
          CASE=44
          FORMAT IS '(F3.4,16X,F3.0)'./
          VARIAB NAMES ARE 'DMMPL','GROUP'.
          VARIAB GROUP=2./
          GROUP NAMES ARE 'PSYOFFDR','PSYNODR'./
          TEST TITLE IS 'DATA FOR KRUSKAL WALLIS H TEST'.
          KRUWAL./
          END./

PROBLEM TITLE . . . . . . .M.W.U.-DMMPL

NUMBER OF VARIABLES TO READ IN. . . . . . . . . .         2
NUMBER OF VARIABLES ADDED BY TRANSFORMATIONS. .           0
TOTAL NUMBER OF VARIABLES . . . . . . . . . . .           2
NUMBER OF CASES TO READ IN. . . . . . . . . . .          44
CASE LABELING VARIABLES . . . . . . . . . . . .
LIMITS AND MISSING VALUE CHECKED BEFORE TRANSFORMATIONS
BLANKS ARE. . . . . . . . . . . . . . . . . . .       ZEROS
INPUT UNIT NUMBER . . . . . . . . . . . . . . .           5
REWIND INPUT UNIT PRIOR TO READING. . DATA. . .          NO

INPUT FORMAT
       (F3.4,16X,F3.0)


VARIABLES TO BE USED
            1 DMMPL        2 GROUP
GROUPING VARIABLE . . . . . . . . . . . . . . . . .GROUP
PERFORM KRUSKAL-WALLIS H TEST

NUMBER OF CASES READ. . . . . . . . . . . . . . .       44



KRUSKAL-WALLIS ONE WAY ANALYSIS OF VARIANCE TEST RESULTS


  VARIABLE      1 DMMPL
   GROUP           FREQUENCY      RANK
  NO. NAME                         SUM
   1   PSYOFFDR       22         524.0
   2   PSYNODR        22         466.0
  KRUSKAL-WALLIS TEST STATISTIC =    0.46345
  LEVEL OF SIGNIFICANCE = 0.4960 ASSUMING CHI-SQUARE DISTRIBUTION
  WITH 1 DEGREES OF FREEDOM

  MANN-WHITNEY TEST STATISTIC =     271
  LEVEL OF SIGNIFICANCE = 0.2480 ASSUMING NORMAL DISTRIBUTION


#EXECUTION TERMINATES.
```

```
$RUN *BMDP
#EXECUTION BEGINS
 BMDP3S

   PROGRAM CONTROL INFORMATION

 $CONTINUE WITH DGDC(1,9)+DGDOMMS(23,24)+(26,36)+(38,40)+(42,44)
          PROBLEM TITLE IS 'OHHPL-ALA-PBG CORR. SPEAR/KEND'./
          INPUT VARIAB=3.
          CASE=19.
          FORMAT IS '(F8.4,F8.4,F8.4)'./
          VARIAB NAMES ARE 'OHHPL','PBG','ALA'./
          TEST TITLE IS 'SPEAR/KEND CORR.'.
          SPEAR.
          KENDAL./
          END./


   PROBLEM TITLE . . . . . . . .OHHPL-ALA-PBG CORR. SPEAR/KEND

   NUMBER OF VARIABLES TO READ IN. . . . . . . . . .          3
   NUMBER OF VARIABLES ADDED BY TRANSFORMATIONS. .            0
   TOTAL NUMBER OF VARIABLES . . . . . . . . . . .            3
   NUMBER OF CASES TO READ IN. . . . . . . . . . .           19
   CASE LABELING VARIABLES . . . . . . . . . . . .
   LIMITS AND MISSING VALUE CHECKED BEFORE TRANSFORMATIONS
   BLANKS ARE. . . . . . . . . . . . . . . . . . . .      ZEROS
   INPUT UNIT NUMBER . . . . . . . . . . . . . . .            5
   REWIND INPUT UNIT PRIOR TO READING. . DATA. . .           NO

   INPUT FORMAT
          (F8.4,F8.4,F8.4)


   VARIABLES TO BE USED
               1  OHHPL          2  PBG            3  ALA

   COMPUTE KENDALL RANK CORRELATION COEFFICIENT(S)
   COMPUTE SPEARMAN RANK CORRELATION COEFFICIENT(S)

   NUMBER OF CASES READ. . . . . . . . . . . . . .           19

  KENDALL RANK CORRELATION COEFFICIENTS

                 OHHPL       PBG        ALA
                     1          2          3

 OHHPL       1     1.0000
 PBG         2    -0.0539     1.0000
 ALA         3    -0.2242     0.1934     1.0000


  SPEARMAN RANK CORRELATION COEFFICIENTS

                 OHHPL       PBG        ALA
                     1          2          3

 OHHPL       1     1.0000
 PBG         2    -0.0319     1.0000
 ALA         3    -0.3224     0.3131     1.0000

#EXECUTION TERMINATES.
```

APPENDIX 2. Case histories of patients with acute intermittent porphyria studied during an acute attack.

CASE 3 J.C. Born 1955 Diagnosed 1976

This 21 year old woman was admitted to Stobhill General Hospital in March 1976 with intermittent epigastric and limb pain, constipation and severe depression of six weeks duration. Her urinary porphyrins had been estimated on 21 separate occasions in an investigation of abdominal pain in view of a strong family history of porphyria. All of these estimations were within normal limits. Prior to her admission she had been prescribed pentazocine for dysmenorrhoea, discontinued because of nausea, and amitryptyline in view of her depression. Two weeks prior to admission she was begun on an oral contraceptive agent.

On admission she was anxious and tremulous and there was slight epigastric tenderness. Pulse and blood pressure were normal and there were no abnormal motor or sensory signs. Urinary porphyrin excretion was consistent with an acute porphyric attack. Urinary OHHPL levels were followed in this patient throughout the acute phase of this attack and were also measured on several subsequent occasions.

Abdominal pain was treated with pethidine; constipation with neostigmine and bizarre behavioural states controlled with promazine. Laevulose infusion was started to increase caloric intake and was discontinued after four days when the patient was asymptomatic. Rebound epigastric pain was controlled with dihydrocodeine and she was discharged 16 days after admission.

In July she had a mild attack with transient abdominal pain and depression associated with menstruation and a further mild attack some months later due to caloric restriction.

Throughout 1977 she had several minor attacks usually associated with menstruation, with abdominal pain, postural hypotension and tachycardia. In October 1977 she was admitted suffering from severe hypotension and nausea and was diagnosed as suffering from an acute attack of which these were the only symptoms. She responded slowly to therapy and since then has remained well.

CASE 4 M.McA. Born 1950 Diagnosed 1976

This lady was admitted as an emergency to the surgical wards of Glasgow Royal Infirmary suffering from colicky lower abdominal pain of two days duration which was increasing in severity and accompanied by vomiting.

Prior to admission she had developed an urticarial eruption that was treated with mebhydrolin.

On examination it was decided that she required an exploratory laparotomy and a normal appendix was removed under anaesthesia induced using thiopentone sodium. Following surgery her pain was very severe and unrelieved with morphine. Consequently the diagnosis of porphyria was suggested and confirmed.

She was then transferred to Stobhill General Hospital where she was found to be suffering from abdominal pain and constipation. There was however no evidence of tachycardia, hypertension or neuropsychiatric upset. Urinary OHHPL measurement was made at this time.

Her recovery was uneventful and she has remained well save for occasional postural hypotension. OHHPL estimations were repeated when she was asymptomatic.

CASE 5 J.McM. Born 1955 Diagnosed 1976

This 20 year old married woman was admitted to Stobhill General Hospital in April 1976 with a 3 week history of abdominal and joint pain, generalised weakness and anorexia. She had been amenorrhoeic for three months. In the previous year prescription of an oral contraceptive agent was discontinued after four months because of severe abdominal pain.

On examination she was thin and looked pale and unwell. Her eyes were prominent and hyperthyroidism was suspected. There was slight abdominal pain, tachycardia and hypertension. Early pregnancy was confirmed by pelvic examination.

Biochemical and haematological examinations revealed abnormal liver function tests (raised alkaline phosphatase, aspartate transaminase (AST) and alanine transaminase (ALT)), raised serum urea and deficient serum iron with low serum iron saturation. Haemoglobin was decreased with a raised

E.S.R. ALA and PBG levels in urine were greatly elevated consistent with an acute attack of porphyria.

Her condition progressively deteriorated and she was transferred to the intensive care unit. Limb weakness increased to almost complete paralysis with a loss of tendon reflexes and muscle wasting. She also developed difficulty in swallowing and a diaphragmatic palsy. She became incontinent of urine and aphonic.

Therapy was initiated with 20% laevulose; hypertension and tachycardia were treated with propranolol and oral prednisolone was given empirically. Obstipation was treated with neostigmine and restlessness with promazine. In addition she received high tension oxygen therapy for 3 days. A week after admission she was transfused four units of packed red cells because of persistent anaemia.

Improvement was slow but consistent. A right basal bronchopneumonia was treated with ampicillin. She had an isolated grand mal seizure two weeks after admission. As soon as swallowing improved a high protein and carbohydrate diet was begun. Muscle power and tendon reflexes gradually improved and laevulose therapy was discontinued three weeks after admission. Eight weeks after admission there was some return of voice power, full urinary retention, normal ability to swallow and she was able to walk unaided. The pregnancy progressed uneventfully and she was discharged 12 weeks after admission.

A mild attack precipitated by a urinary tract infection responded to laevulose, propranolol and promazine. Despite this muscle power improved continuously and she delivered a healthy male child after induction of labour with intravenous sintocinon.

Since then she has suffered several attacks usually associated with menstruation and urinary OHHPL levels have been measured on several occasions, in attack and in remission.

CASE 7 P.W. Born 1948 Diagnosed 1969

This woman was diagnosed as having AIP in 1969 following an attack during her second pregnancy. Her first pregnancy three years earlier had resulted in the spontaneous delivery of a stillborn infant at 28 weeks gestation. Following the delivery of a full term healthy infant the patient developed a severe attack of porphyria which left her with a residual peripheral neuropathy.

Since then the patient has had frequent attacks invariably occurring in the week prior to menstruation. These attacks are characterised by severe abdominal pain, vomiting, acute depression, hypertension and tachycardia. Each treatment course lasted 2 - 3 days consisting of laevulose with pethidine, promazine and propranolol for pain, neuropsychiatric disturbance and hypertension respectively.

This patient was one of the four cases in which urinary OHHPL was measured in attack and remission.

CASE 9 A.O. Born 1950 Diagnosed 1970

This 26 year old woman became ill in August, 1976 in Yugoslavia, with vomiting, abdominal pain, constipation and the passage of dark urine. She was flown back to Great Britain and became confused and hyponatraemic. At this time she was receiving phenobarbitone for epilepsy and had been taking the oral contraceptive pill since February, 1976. These were discontinued when the diagnosis of AIP was made. Following this she developed grand mal seizures, severe postural hypotension and proximal upper limb weakness. She was treated with intravenous laevulose, sodium valproate and clonazepam and slowly improved although proximal upper limb weakness remains.

APPENDIX 3. Hamilton rating scale for assessment of psychiatric patients with N and P subscales.

Dr McCreadie **PIM 110** Gartnavel Royal Hospital

PHYSICIAN'S RATING SCALE

SUBJECT'S NAME	WARD	PATIENT NUMBER
RATER'S NAME	DATE	

P.1. How direct and relevant are his responses to questions or to the topic discussed?

0 Direct and relevant
1 Somewhat rambling and tangential
2 For the most part irrelevant
3 Wholly irrelevant

P.2. Does he assume or maintain peculiar, unnatural or bizarre postures?

0 None
1 For short periods
2 Throughout most of the interview
3 Throughout the entire interview

P.3. Are his thoughts consistent with his mood, or is there a discernible lack of harmony between them?

0 Consistent
1 A little disharmonious
2 Distinctly disharmonious
3 Appeared totally unrelated

P.4. Does he exhibit any repeated peculiar gestures, grimaces or mannerisms?

0 None
1 Occasionally
2 Fairly frequently
3 Throughout the interview

N.5. Does he tend to suspect or to believe on slight evidence or without good reason that people and external forces are trying to or now do influence his behaviour and control his thinking?

0 No unjustified suspicion
1 Inclined to suspect
2 Believes others are trying to control him
3 Believes he is influenced or controlled

P.6. Are the elements of his speech logically consistent and connected by some idea or relationship, or do they tend to be inconsistent and disconnected? (Rate what is most representative during the interview.)

0 Coherent and consistent
1 Slightly incoherent and inconsistent
2 Distinctly incoherent and inconsistent
3 Conspicuously scattered, disconnected or incoherent

N.7. Does he bear little hostility or a high degree of ill will, resentment, bitterness or hate?

0 No hostility
1 Slight hostility
2 Moderate hostility
3 Much hostility

P.8. Does he have any insight into his hallucinations? (Score 0 for no evidence of hallucinations.)

0 Full insight
1 Possibly full insight
2 Some insight
3 No insight

P.9. How frequently does he speak, mutter or mumble to himself, seemingly to carry on conversations with hallucinatory voices?

0 Not at all
1 Occasionally
2 Fairly frequently
3 Throughout the interview

P.10. Is there any evidence that the patient has auditory hallucinations?

0 None
1 Doubtful
2 Probable
3 Certain

P.11. Does the patient ever glance up as if listening to auditory hallucinations?

0 Not at all
1 Doubtfully or occasionally
2 Fairly frequently
3 Throughout the interview

P.12. Does he repeat certain words or phrases in a meaningless, stereotyped or mechanical fashion?

0 Never
1 Occasionally
2 Fairly frequently
3 Almost constantly

P.13. Is his speech irregularly interrupted, halted or blocked for varying periods of time because of difficulty in finding words for his thoughts?

0 No speech blocks
1 A few interruptions
2 Many interruptions and conversation very difficult
3 Patient is mute or almost mute

N.14. Does he have an exaggeratedly high opinion of himself, or an unjustified belief or conviction of having unusual ability, knowledge, power, wealth or status)

0 No exaggerated high opinion of himself
1 An exaggeratedly high opinion
2 Conviction of unusual power, wealth, etc.
3 Conviction of grandiose or fantastic power, wealth, etc.

N.15. Does he tend to suspect or to believe on slight evidence or without good reason that some people are against him (persecuting, conspiring, cheating, depriving, punishing) in various ways?

0 No unjustified suspicions
1 Inclined to suspect
2 Inclined to believe
3 Has firm conviction

N.16. Does he tend to suspect or to believe on slight evidence or without good reason, that some people talk about, refer to, or watch him?

0 No unjustified suspicions
1 Inclined to suspect
2 Inclined to believe
3 Has firm conviction

N.17. Is there evidence of false ideas or beliefs? If present, are these ideas or beliefs (a) sufficiently plausible as to be accepted by a normal person uninformed as to the facts, (b) implausible but not impossible, (c) impossible or bizarre (e.g. mind controlled by radio waves, heart removed or dead)?

0 No evidence of false beliefs
1 Plausible to the uninformed
2 Implausible
3 Impossible or bizarre

P.18. Does the patient's mood and emotional response show blunting?

0 Not at all
1 Slight blunting
2 Severe blunting
3 Complete apathy

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