EXPERIMENTAL STUDIES ON BILIRUBIN AND HAEM BIOSYNTHESIS

by

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SUMMARY

It has recently been shown that patients with unconjugated hyperbilirubinaemia due to Gilbert's syndrome have reduced activity of the enzyme protoporphyrinogen oxidase in circulating leucocytes. This may be explained by the further observation that unconjugated bilirubin competes with protoporphyrinogen for binding to protoporphyrinogen oxidase. In order to further investigate the effect of unconjugated hyperbilirubinaemia on porphyrin metabolism and haem biosynthesis studies have been performed in the Gunn rat which has unconjugated hyperbilirubinaemia and raised plasma bilirubin concentrations. Protoporphyrinogen oxidase activity was found to be reduced in the liver of the Gunn rat compared to heterozygous controls with normal plasma bilirubin concentrations. This reduction of protoporphyrinogen oxidase activity was not accompanied by any increase in the activity of the initial and rate-controlling enzyme of the pathway delta-aminolaevulinic acid synthase. In contrast to hepatic tissue, the activities of both protoporphyrinogen oxidase and delta-aminolaevulinic acid synthase were normal in renal tissue of the Gunn rat. Porphyrin excretion was reduced in the Gunn rat compared to that in heterozygous controls.

Further studies were performed to investigate the effects of unconjugated hyperbilirubinaemia on haem biosynthesis in the brain of the Gunn rat. This was studied as the major effect of hyperbilirubinaemia is brain damage and the mechanism by which it occurs is unknown. Inherited partial enzyme deficiencies in haem biosynthesis are known to produce neurological damage.

Therefore the possibility that bilirubin induced brain damage might be due to inhibition of protoporphryinogen oxidase activity and hence deficient neuronal haem synthesis, was investigated. Optimal conditions for the measurement of protoporphyrinogen oxidase activity were determined in the brain and found to be identical to those which provided optimal activity in hepatic tissue. The activity of protoporphyrinogen oxidase in brain tissue homogenates from Gunn rats was similar to that in heterozygous rats with normal bilirubin concentrations. This was also the case in neonatal Gunn rats in whom kernicterus had been induced by the displacement of bilirubin into the brain by sulphonamide treatment. Delta-aminolaevulinic acid synthase activities were also similar in the homozygous Gunn rat and heterozygous controls. These findings make it unlikely that the brain damage induced by hyperbilirubinaemia is due to inhibition of protoporphyrinogen oxidase activity and impaired neuronal haem biosynthesis.

The above studies concerned the effect of disturbed haem catabolism on haem biosynthesis. The latter part of the studies extended the theme of the investigation by examining the effect of the administration of Tin-protoporphyrin, an inhibitor of haem biosynthesis, on haem biosynthesis in acute porphyria. The effect of administering Tin-protoporphyrin and haem arginate alone and in combination on succinyl acetone induced porphyria in rats was studied. This animal model proved to be unsatisfactory as the haem arginate given alone did not significantly reduce the over-production of the porphyrin

precursor, delta-aminolaevulinate. This may be explained by inhibition of aminolaevulinic acid dehydratase by succinyl acetone so increasing aminolaevulinic acid overproduction in most body tissues. The effect of the haem arginate is largely confined to the liver. Studies in 1 patient during clinical attacks proved more encouraging. They showed that the co-administration of Tin-protoporphyrin with haem arginate prolonged both the biochemical response and clinical remission induced by haem arginate therapy. These findings indicate that co-administration of Tin-protoporphyrin and haem arginate may be used as prophylactic therapy in patients experiencing recurrent attacks of acute porphyria.

Except where acknowledged, all the work performed which forms this thesis was carried out by myself.

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PUBLICATIONS

Graham A.M., McColl K.E.L. & Moore M.R. (1989): Haem Biosynthesis in the Unconjugated Hyperbilirubinaemias: Observations in the Gunn Rat.
Clinical Biochemistry 22 177-179 .

ABSTRACT

Dover S. B., Moore M.R., Graham A. & McColl K.E.L. : Tin Protoporphyrin combined with haem arginate: an improved treatment for acute porphyria. (in press)

CONTENTS

ABBREVIATIONS

AIA	allylisopropylacetamide
AIP	acute intermittent porphyria
ALA	5-aminolaevulinic acid
ALA-D	5-aminolaevulinic acid dehydratase
ALA-synthase	5-aminolaevulinic acid synthase
BBB	blood brain barrier
BSP	sulphobromophthalein
CO	carbon monoxide
Co^{2+}	cobalt ions
COPRO	coproporphyrin
COPRO'gen	coproporphyrinogen
COPRO-O	coproporphyrinogen oxidase
CP	congenital porphyria
cyt.P450	cytochrome P450
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
EDTA	ethylene diamine tetra-acetic acid
EPP	erythropoietic protoporphyria
Fe^{2+}	ferrous iron atom
Fe^{3+}	ferric iron atom
G.I. tract	gastro-intestinal tract
GSH	glutathione
HC	hereditary coproporphyria
HCl	hydrochloric acid

HMB	hydroxymethylbilane
HPLC	high performance liquid chromatography
Mg	magnesium atom
$MgCl_2$	magnesium chloride
mMolar (mM)	millimolar, millimoles per litre
Mn^{2+}	manganese ions
ODS	octadecylsilane
PBG	porphobilinogen
PBG-D	porphobilinogen deaminase
PCT	porphyria cutanea tarda
p-DMAB	para-dimethylaminobenzaldehyde
PROTO	protoporphyrin IX
PROTO'gen	protoporphyrinogen IX
PROTO-O	protoporphyrinogen oxidase
SAX	strong anion exchange
Sn-PROTO	tin protoporphyrin
succinyl CoA	succinyl coenzyme A
TCA	trichloroacetic acid
UDP	uridine diphosphate
UDP-GT	uridine diphosphoglucuronyl transferase
URO	uroporphyrin
URo'gen	uroporphyrinogen
URO-D	uroporphyrinogen III decarboxylase
VP	variegate porphyria

CHAPTER 1

INTRODUCTION

1.1 THE BIOSYNTHESIS OF HAEM

The pathways of biosynthesis and degradation of haem have been of major interest for many years. Haem consists of a porphyrin molecule with a ferrous iron atom in its nucleus (see Figure 1). Iron is only one of the metals with which this tetrapyrrole can combine, magnesium being the other most common one. These metalloporphyrins are found throughout nature in plants, yeasts, bacteria, avian and mammalian tissues and are central to a large number of biological reactions. Synthesis of haem has been identified in virtually all mammalian tissues (Granick & Urata, 1963 ; Bottomley & Smithee, 1968; Barnes et al, 1971; Briggs et al, 1976; Paterniti et al, 1978) with the liver and bone marrow being the richest sources. As the prosthetic group of various proteins, haem is involved in many biological oxidation reactions. Haemoglobin transports oxygen in the red cell, catalase prevents accumulation of toxic hydrogen peroxide and various cytochromes are involved in several aspects of cellular respiration. The predominant one in liver, cytochrome P450 (cyt P450), has the ability to metabolise a wide range of xenobiotics and potentially toxic endogenous compounds.

Haem biosynthesis consists of eight well characterised steps (see Figure 2), elucidated from work with isotopic tracers initiated 35 years ago (Neuberger & Scott, 1953; Shemin, 1955) and involves both mitochondrial and cytosolic enzymes. From the readily available precursors succinyl coenzyme A (succinyl CoA) produced in the citric acid cycle and the amino acid glycine, the first intermediate in the pathway, 5-aminolaevulinic acid (ALA) is formed. Condensation of two ALA molecules

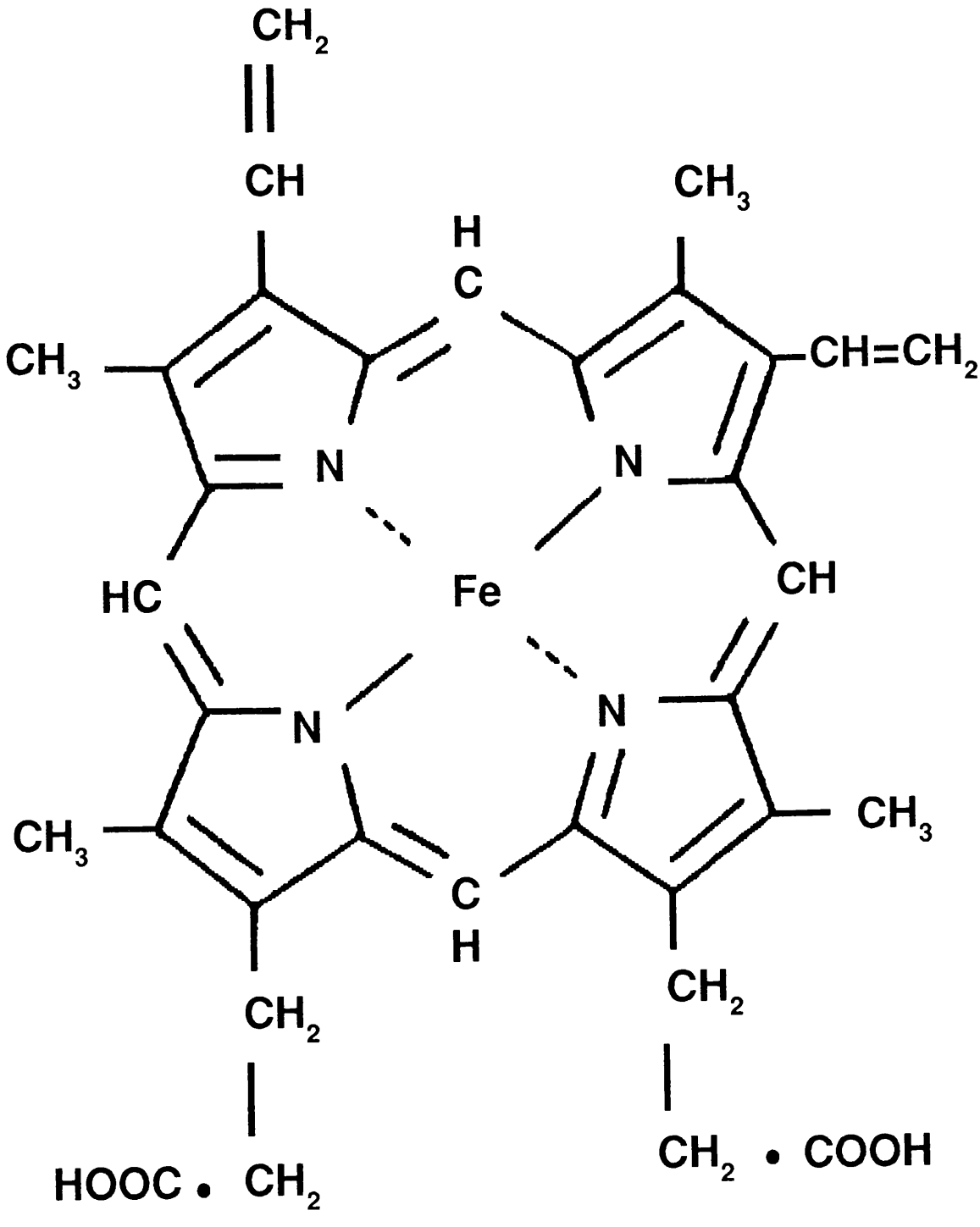


FIGURE 1 : THE MOLECULAR STRUCTURE OF HAEM

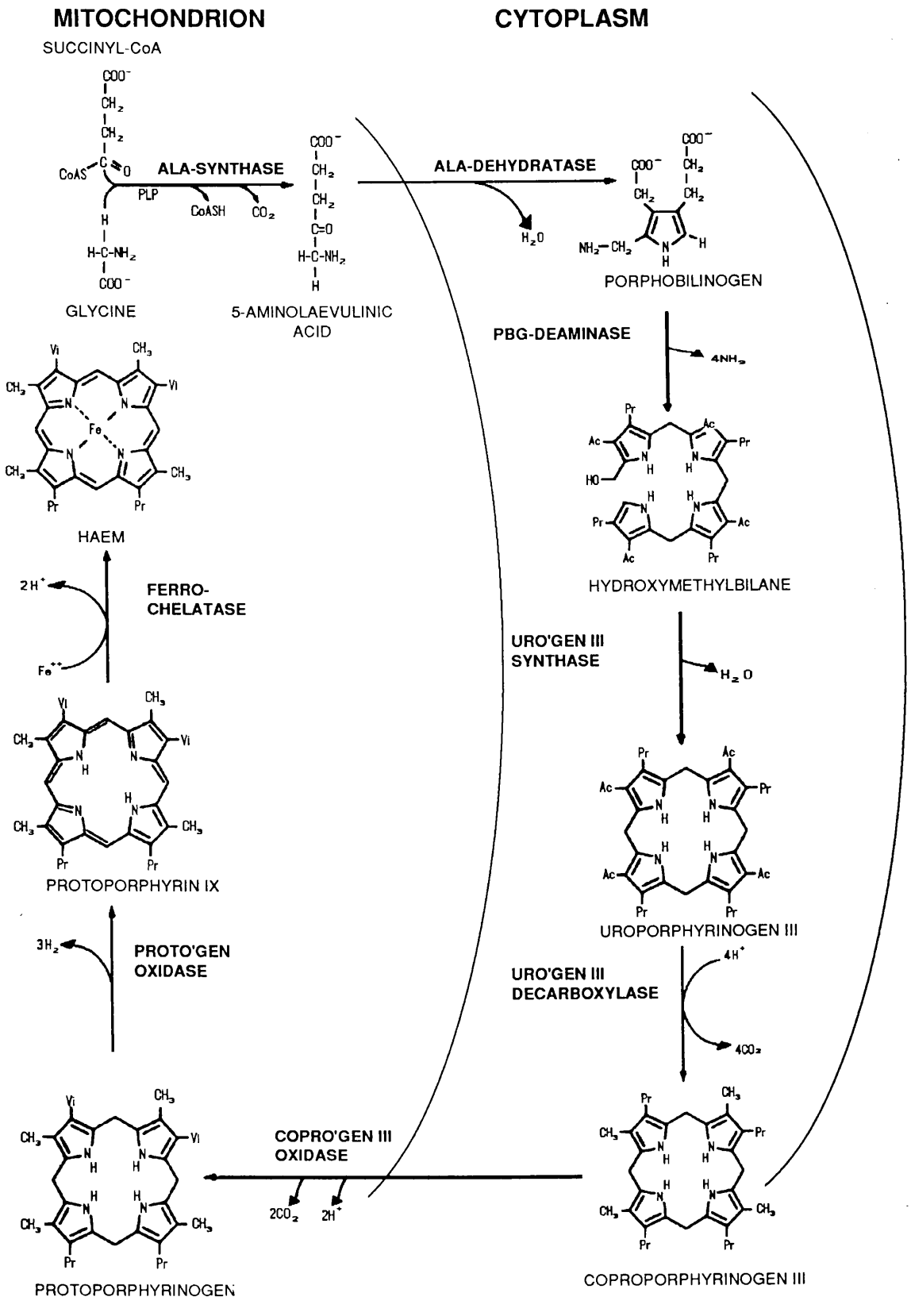


FIGURE 2 : THE HAEM BIOSYNTHETIC PATHWAY

produces porphobilinogen (PBG) a monopyrrole which cyclises to form uroporphyrinogen, the first of the colourless porphyrinogens, the reduced intermediates of the porphyrins. Stepwise decarboxylation and oxidation then leads to the deep red coloured protoporphyrin IX (PROTO) via coproporphyrinogen and protoporphyrinogen. The final step involves incorporation of Fe^{2+} to form haem. The enzymes of haem biosynthesis have been closely studied and a brief summary of current knowledge of each of these follows. Particular attention will be paid to 5-aminolaevulinate synthase (ALA-synthase), the first and rate limiting enzyme of the pathway and protoporphyrinogen oxidase (PROTO-O), the penultimate and most recent enzyme to be described. These enzymes are especially of interest as the effects of bilirubin on protoporphyrinogen oxidase and any resultant effects on ALA-synthase will be assessed in this thesis.

1.1.1 5-Aminolaevulinic Acid Synthase (E.C. 2.3.1.37)

ALA-synthase catalyses the condensation of succinyl CoA and glycine to form aminolaevulinic acid (ALA). Pyridoxal phosphate (vitamin B6) acts as a cofactor in the reaction (Schulman & Richert, 1956) which is thought to proceed via reaction of glycine with enzyme bound pyridoxal phosphate to form a stable Schiff base carbanion. The stable reaction product can then react with succinyl CoA producing an αamino-βketo adipic acid and losing CoA. On decarboxylation ALA is produced (Figure 3). In a secondary role pyridoxal phosphate helps prevent inactivation of ALA-synthase (Beattie et al,

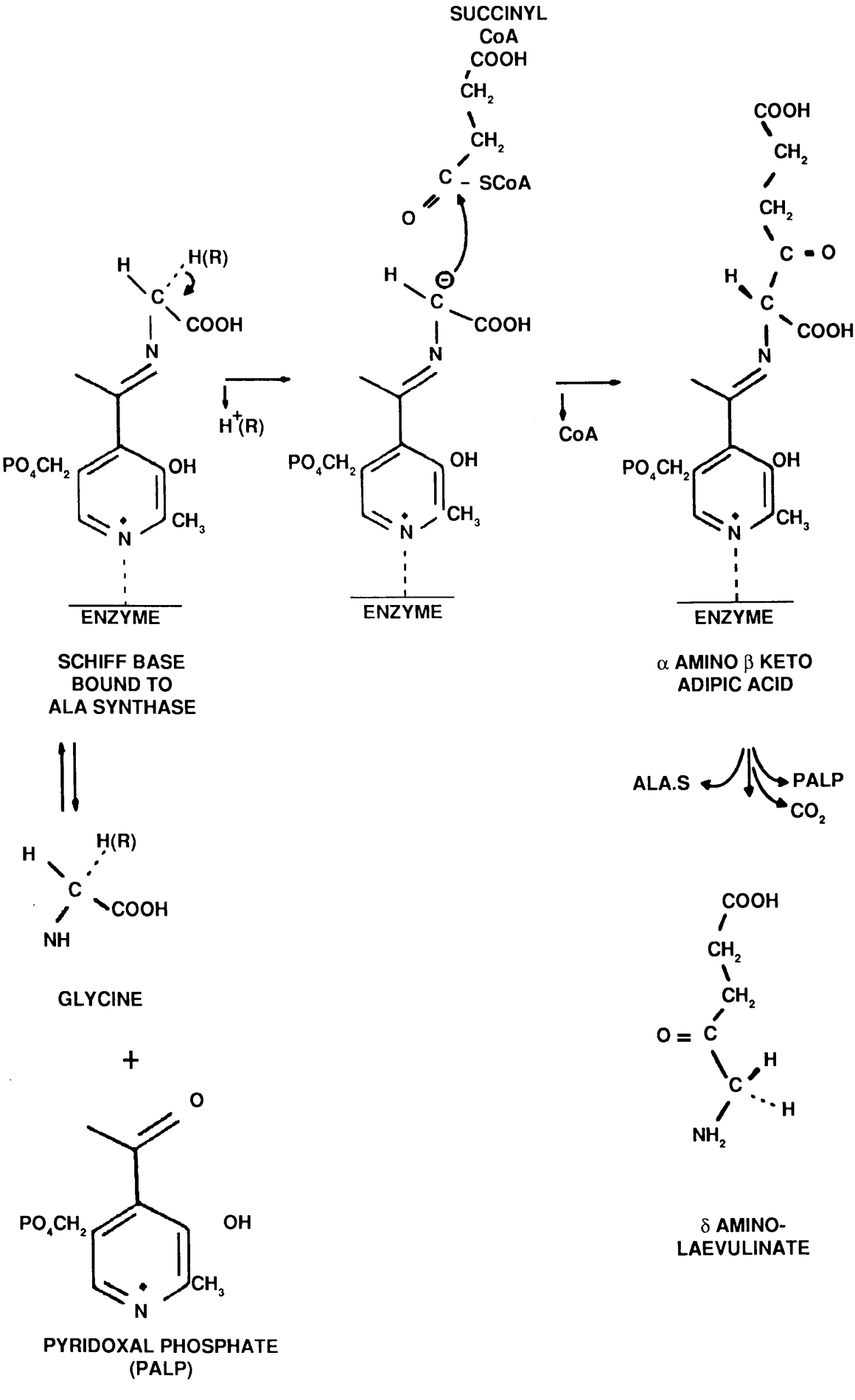


FIGURE 3 : THE MECHANISM OF FORMATION OF ALA BY ALA-SYNTHASE

1985). ALA-synthase activity has been studied in many tissues including the heart (Briggs et al, 1976), adrenal gland (Condie et al, 1976), spleen (Ebert et al 1970), kidney (Barnes et al, 1971) and brain (Paterniti et al, 1978). Most of the earlier work concentrated on defining the hepatic activity and it was quickly established that ALA-synthase controlled the rate of the reaction. It is now well documented that ALA-synthase is important in the regulation of the haem biosynthetic pathway in hepatic tissue as detailed in section 1.3.2. However in recent years the role of this enzyme in the control of haem biosynthesis, in erythroid tissue has been disputed and contradictory evidence has implicated ALA-synthase, ferrochelatase and PBG-deaminase (Beru & Goldwasser, 1985) in regulatory roles - see section 1.3.3. Genetic and other advances have allowed closer investigation of the possibility that the enzymes have different roles to play depending on tissue. It is now widely believed that tissue specific control of haem biosynthesis does exist and this is supported by several pieces of evidence. The structure of ALA-synthase is thought to vary slightly in different tissues, and it has been shown that the enzymic proteins display a different molecular mass in liver and erythroid tissue (Grandchamp et al, 1987). Certain properties of the enzyme have also been shown to vary according to the tissue source. These pieces of evidence are in support of tissue specific roles for ALA-synthase. This may also prove to be the case for the other enzymes. The hepatic enzyme is inducible by a large variety of endogenous and exogenous factors while this is not

generally the case for the erythroid form of the enzyme. Studies of brain ALA-synthase have also shown that this enzyme has unique properties and these are further discussed in Chapter 3.

ALA-synthase is located within the mitochondrion, loosely bound to the inner membrane (Patton & Beattie, 1973). Cytosolic activity has been recorded, but is probably due to the presence of newly formed enzyme since synthesis of ALA-synthase occurs on the rough endoplasmic reticulum in the cytoplasm before translocation to the mitochondrion (Hayashi et al, 1970). Cytoplasmic ALA-synthase has a higher molecular weight than the mitochondrial form with slight variations depending on the source of tissue and species studied. Using various purification techniques on rat liver cytosolic ALA-synthase was shown to consist of three subunits molecular weights 51 000, 79 000 and 120 000 daltons (Ohashi & Kikuchi, 1979). The two larger subunits detected in cytosol are thought to be catalytically inactive. The active form is thought to be a dimer of the 51 000 dalton subunit and it appears this is the only subunit transported into the mitochondrion (Nakakuki et al, 1980; Ohashi & Kikuchi, 1979).

The half-life of the enzyme is very short with values of 34 minutes recorded in young rats (Woods, 1973; Woods & Murthy, 1974) and 60-70 minutes recorded in adult rats (Marver et al, 1966a; 1966b) and this is one of the criteria which initially made it likely that the enzyme catalysed the rate controlling step of the pathway. At the molecular level the half-life of the mRNA for ALA-synthase in rats is thought to be of similar

duration to that of the enzyme itself (Tschudy et al, 1965; Marver et al, 1966b).

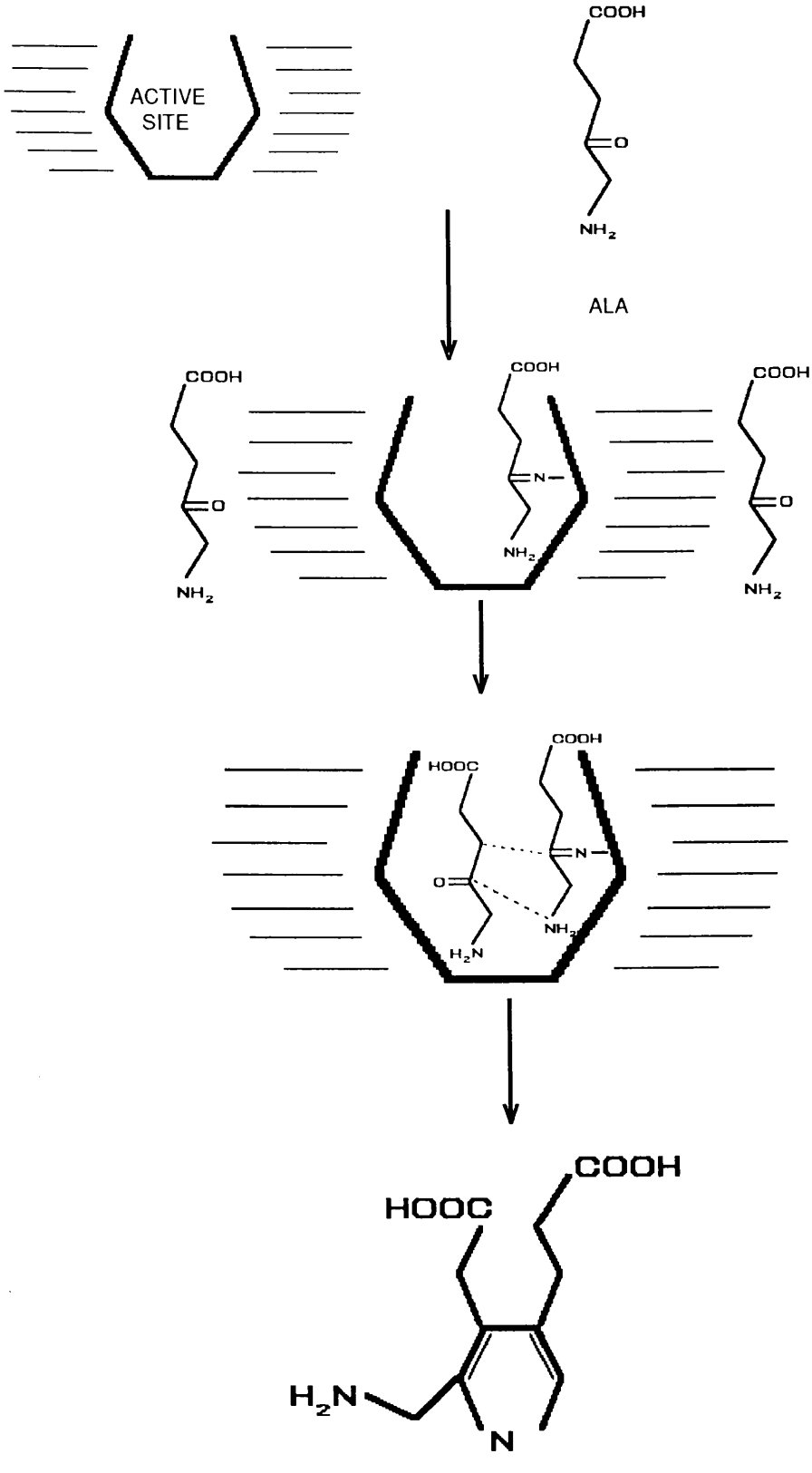
Measurement of hepatic ALA-synthase activity has shown a range from 20 - 80 nmoles ALA formed/g liver/hour in humans (Strand et al, 1970; Sweeney et al, 1970) to 200-800 pmol/mg protein/hour in rats. From studies with purified enzyme in rats, development of both hepatic and brain ALA-synthase activities appear to follow a similar pattern with the highest activity recorded in the first three weeks of life, tapering off to the adult values already listed (De Matteis, 1981; Woods, 1974). The cytochromes are formed in these early weeks of life and it has been suggested that haem requirement will be at a peak during this period. This would explain the necessity for higher ALA-synthase activity. Hepatic ALA-synthase is an inducible enzyme and its activity is affected by many foreign substances (Granick, 1966; Marver et al, 1966a), in addition to haem requirements (Moore et al, 1987). The role of the enzyme in control of the pathway of haem biosynthesis will be more fully discussed in section 1.3.

1.1.2 Aminolaevulinic Acid Dehydratase (E.C. 4.2.1.24)

ALA-dehydratase (ALA-D) is the first cytosolic enzyme in the pathway of haem biosynthesis, and catalyses the condensation of two molecules of ALA to form PBG, eliminating two molecules of water (Shemin, 1972). The structure of the enzyme has been closely studied in various mammalian tissues and purification of the bovine liver form revealed a quaternary cube-like structure consisting of eight subunits each with molecular weight 35 000

daltons and thought to be identical (Wu et al, 1974). The catalytic process involves subsequent binding of ALA molecules to the enzyme (Shemin, 1976). The presence of two highly active sulphydryl groups in the active site is thought to be one important contribution to the enzyme's activity. Initially an ALA molecule binds to one side of the active site of the enzyme forming a Schiff base. This later becomes the part of PBG bearing the propionic acid side chain (Jordan & Gibbs, 1985). The presence of further substrate allows another ALA molecule to enter the active site and eventually leads to formation of the PBG molecule shown in Figure 4. EDTA inhibits ALA-D activity, and this loss can be restored by addition of zinc (Wilson et al, 1972). More recently the importance of zinc has been emphasised (Finelli et al, 1975) as an activator of ALA-D activity effective at the site of synthesis of the enzyme. A high affinity binding site has been identified on each subunit of the enzyme and occupation may result in protection of the sulphydryl groups in the active site. Most other metals inhibit ALA-D (Wilson et al, 1972; Chiba & Kikuchi, 1978) with the greatest sensitivity to non-competitive inhibition by lead (Gibson & Goldberg, 1970). These effects are again counteracted by zinc (Finelli et al, 1975). The mechanism appears to be competition which occurs between lead and zinc binding to the enzyme, with lead inhibiting and zinc activating.

Other reagents inhibit ALA-D. Important ones to be considered are ethanol and succinyl acetone. Ethanol is thought to cause oxidation of sulphydryl groups (Moore et al, 1971) while



PORPHOBILINOGEN
(PBG)

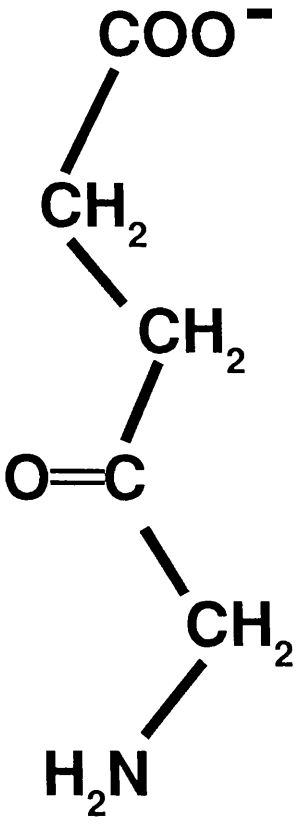
FIGURE 4 : THE MECHANISM OF FORMATION OF PORPHOBILINOGEN BY ALA-DEHYDRATASE

succinyl acetone blocks the site of formation of the Schiff base (Tschudy et al, 1981). The similarity in structure of this inhibitor to native ALA demonstrated in Figure 5 is thought to explain the mechanism of inhibition. Succinyl acetone causes irreversible inhibition and in severe cases can interfere with haem biosynthesis.

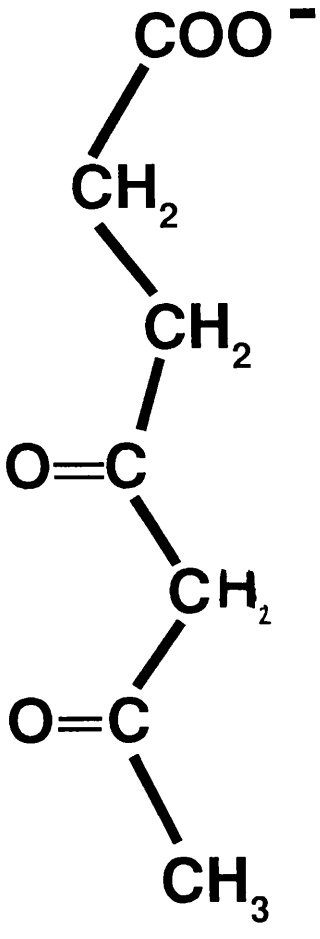
1.1.3 Porphobilinogen Deaminase (E.C. 4.3.1.8) & Uroporphyrinogen III Cosynthase (E.C. 4.3.1.75)

The polymerisation and cyclisation of four molecules of PBG to form the first porphyrinogen is carried out in the cytoplasm by a two enzyme system comprising PBG-deaminase (uroporphyrinogen-I-synthase, hydroxymethyl bilane synthase) and uroporphyrinogen-III-cosynthase. The product which goes on to form haem, uroporphyrinogen-III (URO'gen III), is asymmetrical and can only be formed in the presence of URO'gen-III-cosynthase. The action of PBG-D alone forms URO'gen I, an uncommon molecule which cannot be converted to haem and is present in the disease states which are covered in section 1.4. The complex reaction is started by PBG-deaminase which catalyses a head to tail condensation of four PBG molecules to form hydroxymethylbilane (HMB) or preuroporphyrinogen. This linear tetrapyrrole is the substrate for URO'gen-III-cosynthase to form the asymmetrical URO'gen III (Jordan et al, 1979), see Figure 6.

PBG-deaminase has been purified from human, bacterial and plant sources with the molecular weight of the human enzyme estimated at 38 000 daltons (Anderson & Desnick, 1980). URO'-gen-III-cosynthase has been purified from bacteria with molecular weight between 30 000-40 000 daltons (Hart &



5-AMINOLAEVULINIC ACID



SUCCINYL ACETONE

FIGURE 5 : SIMILAR STRUCTURES OF 5-AMINOLAEVULINIC ACID & SUCCINYL ACETONE

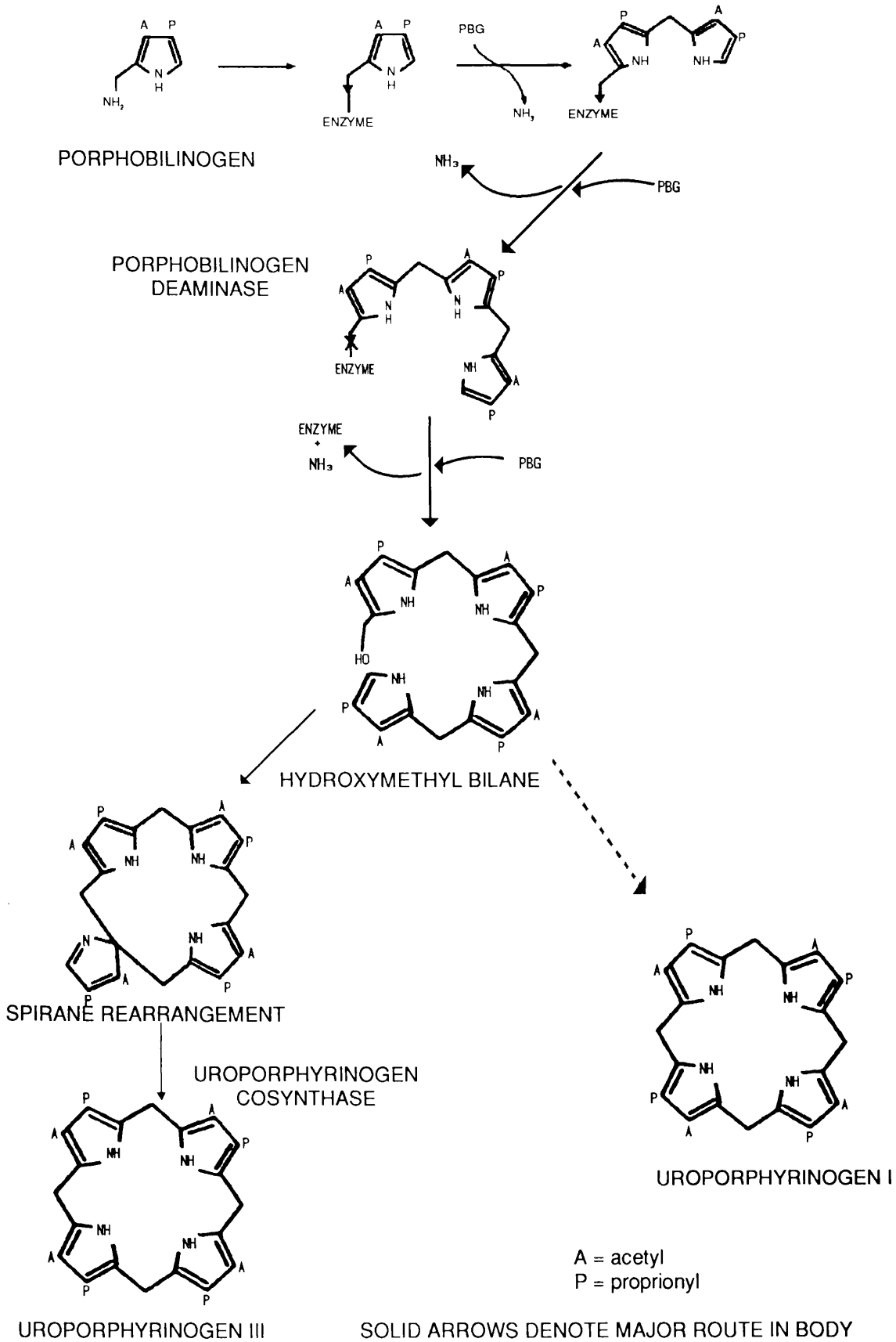


FIGURE 6 : FORMATION OF UROPORPHYRINOGENS BY PORPHOBILINOGEN DEAMINASE AND UROPORPHYRINOGEN COSYNTHASE

Battersby, 1985) and from rat liver, molecular weight 42 000 daltons (Kohashi et al, 1984).

PBG-deaminase has lower activity than most of the haem biosynthetic enzymes excepting ALA-synthase and so has been proposed as a secondary control point of haem biosynthesis (Brodie et al, 1977). In support of this, activity has been shown to increase in an attempt to compensate for a block in haem synthesis (Piper et al, 1986). Inhibitors reported include sulphonamides which are reversible and non-competitive in vitro (Peters et al, 1980), haem precursors especially PROTO'gen (Meissner et al, 1990) and bilirubin (Piper et al, 1986) possibly because of its similarity in structure to HMB and PROTO'gen IX.

1.1.4 Uroporphyrinogen Decarboxylase (E.C. 4.1.1.37)

Uroporphyrinogen decarboxylase (URO-D) catalyses the last cytosolic step in the biosynthesis of haem, sequentially removing four carboxymethyl substituents from URO'gen to form coproporphyrinogen (COPRO'gen). In sequence, ring D reacts first and most quickly and is followed by clockwise decarboxylation of rings A, B and C (Jackson et al, 1976) see Figure 7.

There is disagreement over the specificity of URO-D. Several authors have reported URO III is preferentially decarboxylated (Elder, 1977, Elder & Tovey, 1977) but Rasmussen and Kushner (1979) found no evidence for a preferred substrate although they did find differences in accumulation of intermediates.

URO-D has been purified from bovine liver, molecular weight

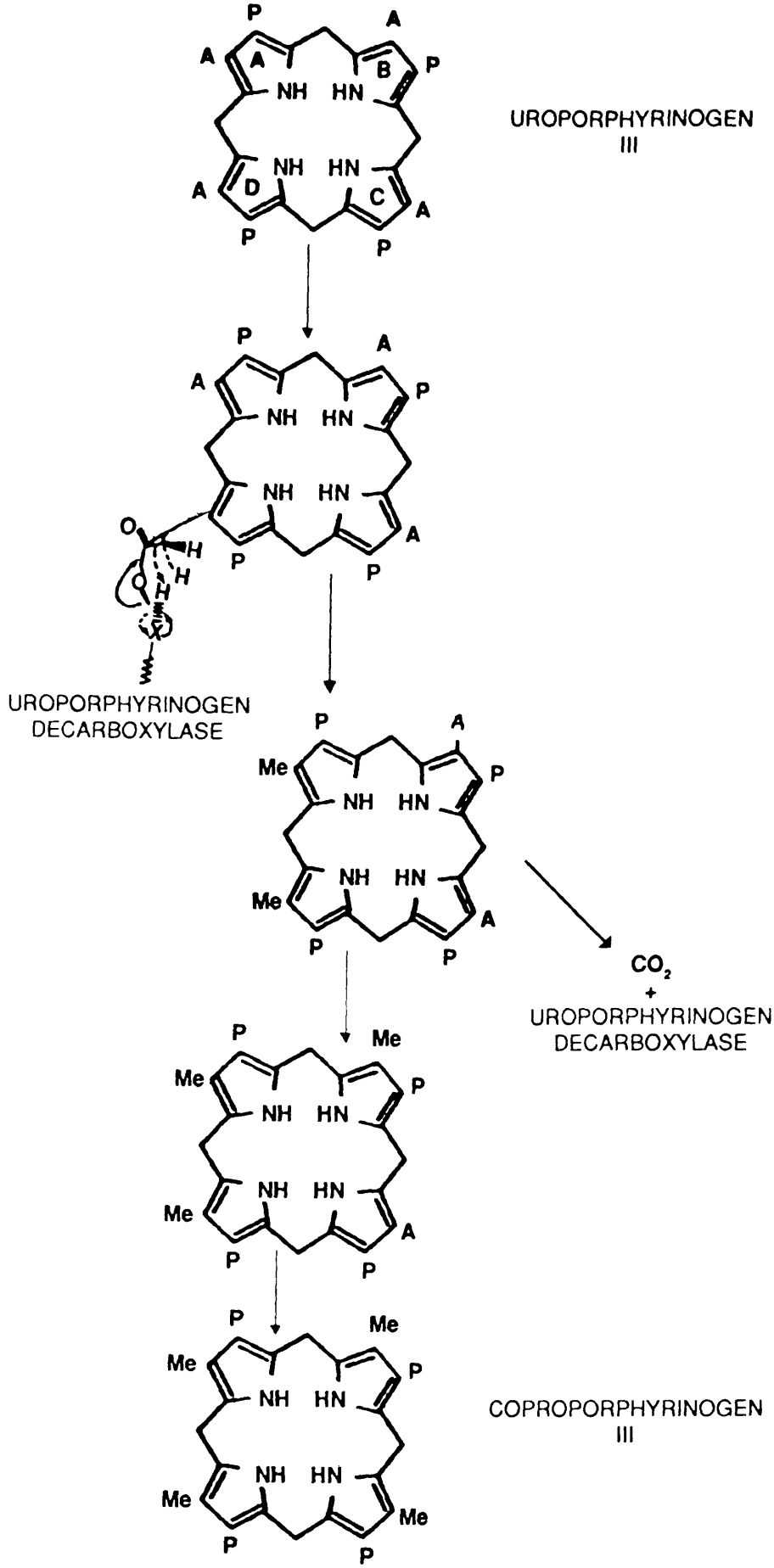


FIGURE 7 : ENZYMIC REACTION CATALYSED BY UROPORPHYRINOGEN DECARBOXYLASE

57 000 daltons (Straka & Kushner, 1983) and human erythrocytes, molecular weight 46 000 (De Verneuil et al, 1983a). From kinetic data the enzyme seems to have at least two separate active sites (De Verneuil, 1983a)

The enzyme is sensitive to sulphydryl modificatiion and is inhibited by copper, mercury and platinum ions. Inactivation occurs on exposure to polycyclic hydrocarbons eg 3-Me-cholanthrene (Sinclair et al, 1984) and, in vitro, iron (Smith & Francis, 1983).

1.1.5 Coproporphyrinogen Oxidase (E.C. 1.3.3.3)

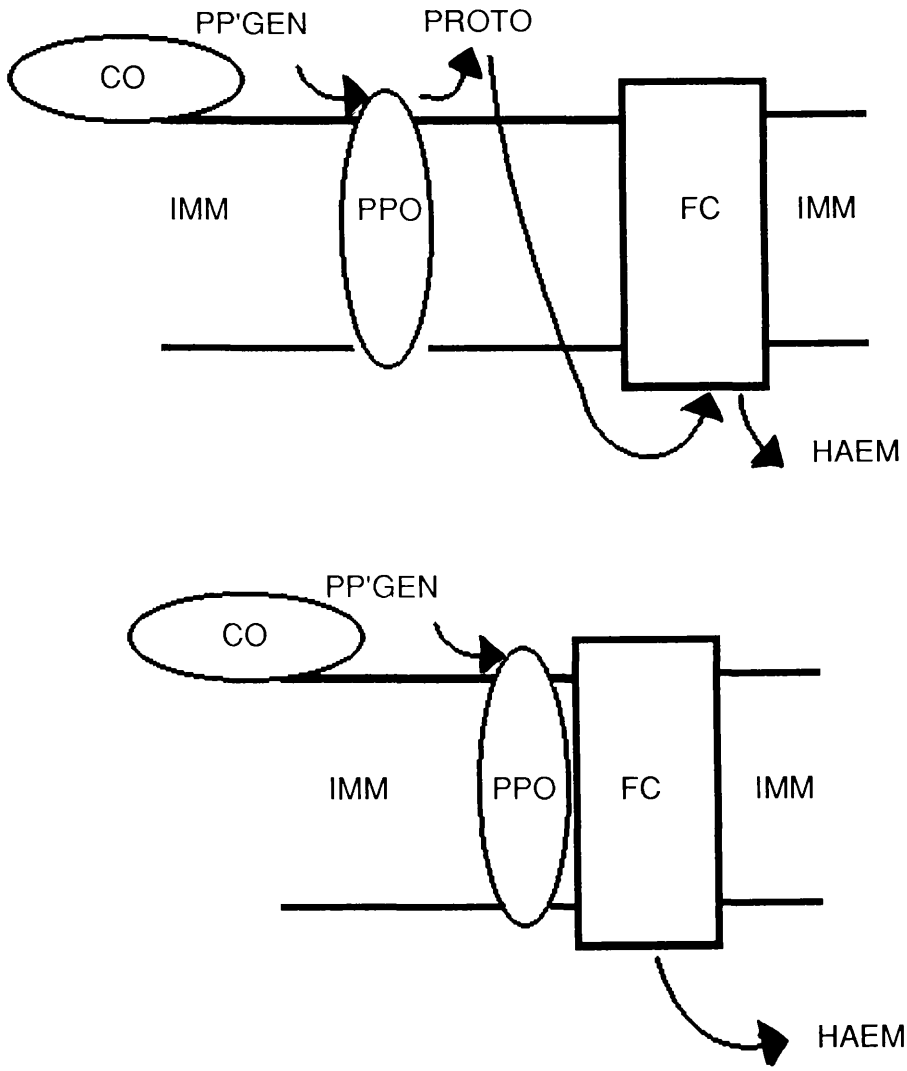
Conversion of COPRO'gen III to protoporphyrinogen IX (PROTO'gen IX) is catalysed by the mitochondrial enzyme copropo-rphyrinogen oxidase (COPRO-O). The enzyme is very specific for COPRO'gen III, so that any COPRO'gen I formed at the previous step cannot proceed along the pathway of biosynthesis of haem (Batlle et al, 1965). The reaction combines oxidation and decarboxylation of propionyl residues on rings A and B respecti-vely to produce vinyl groups (Ahktar et al, 1976). Molecular oxygen is required as a hydrogen acceptor (Sano & Granick, 1961).

The enzyme is located in the intermembrane space of the mitochondrion and may be loosely bound by the membrane (Elder & Evans, 1978),

1.1.6 Protoporphyrinogen Oxidase (E.C. 1.3.3.4)

Protoporphyrinogen oxidase (PROTO-O), the penultimate enzyme of the pathway of haem biosynthesis, is the most recently identified and was proven to exist after Jackson and coauthors (Jackson et al, 1974) showed with isotopic markers that all six hydrogen atoms in COPRO'gen are removed from the same side of the porphyrin ring. Further work by Poulson and Polglase (Poulson & Polglase 1975; Poulson, 1976) localised the enzyme activity to the mitochondrial fraction. In all eukaryotic cells studied oxygen is required as an electron acceptor before the reaction can proceed. More recent work has localised PROTO-O activity to the inner mitochondrial membrane (Deybach et al, 1985), as an intrinsic protein oriented towards the cytosolic side of the membrane (Ferriera et al, 1988) and evidence exists supporting the presence of a membrane complex with some type of interaction between the terminal three enzymes of the pathway occurring. A model for organization of the terminal portion of the pathway has been proposed (Ferreira et al, 1988) and is demonstrated in Figure 8.

PROTO-O has been purified and partially characterised from mouse liver mitochondria (Dailey & Karr, 1987), rat liver mitochondria (Kolarov et al, 1983), bacterial and plant sources (Jacobs & Jacobs, 1981; 1984; 1987). The murine and yeast PROTO-O enzymes have reported molecular weights of 65 000 daltons but the plant enzyme, isolated from barley organelles, has a reported molecular weight of 36 000 daltons (Jacobs & Jacobs, 1987) along with several different properties to the non-plant enzymes. These differences in molecular weights have



FC = FERROCHELATASE, PPO = PROTOPORPHYRINOGEN OXIDASE,
CO = COPROPORPHYRINOGEN OXIDASE
PP'GEN = PROTOPORPHYRINOGEN, PROTO = PROTOPORPHYRIN
IMM = INNER MITOCHONDRIAL MEMBRANE

FIGURE 8 :MODELS FOR A POSSIBLE MEMBRANE COMPLEX OF THE TERMINAL 3 ENZYMES OF HAEM BIOSYNTHESIS

not yet been explained.

Inhibition of PROTO-O activity has been demonstrated by various peroxidising herbicides (Matringe et al, 1989) and by bilirubin *in vitro* (Ferreira & Dailey, 1988). In humans several disease states lead to reduced activity of this enzyme. Variegate porphyria patients have PROTO-O activities reduced to around 50% of normal, and lower than normal levels of activity have been recorded in patients with Gilbert's syndrome, a benign state of hyperbilirubinaemia affecting around 5% of the population (McColl et al, 1985). It has been suggested that the reduced PROTO-O activity recorded in such patients may be induced by an inhibitory effect of bilirubin on the penultimate enzyme of haem biosynthesis. This proposal will be further investigated in this thesis.

1.1.7 Ferrochelatase (E.C. 4.99.1.1)

Ferrochelatase or haem synthase catalyses the last step in the biosynthesis of haem (Goldberg et al, 1956) by inserting ferrous iron into the centre of the protoporphyrin IX molecule releasing two protons. As with PROTO-O, ferrochelatase is embedded in the inner mitochondrial membrane with the active site facing into the mitochondrial matrix (Jones & Jones, 1969; Honeybourne et al, 1979). Bovine and rat liver mitochondrial forms have been purified and molecular weights of around 40 000 daltons recorded (Dailey & Fleming, 1983; Taketani & Tokunaga, 1981). The presence of lipid is essential for enzyme activity (Sawada et al, 1969; Taketani & Tokunaga, 1981) and may act by increasing solubilization of the porphyrin

intermediate. Additionally the carboxylic groups on fatty acids may provide the optimal conditions for approach of the porphyrin to the enzyme active site. Another requirement for activity is the presence of sulphydryl groups (Labbe & Hubbard, 1961). It has been postulated that the oxidation of PROTO'gen could be linked inextricably with the action of ferrochelatase, the terminal enzyme of the pathway using the available reducing equivalents to drive the reduction of iron (Moore et al, 1987). Ferrochelatase is not specific for either of the two substrates it combines and so other divalent metal ions and porphyrin analogues can be utilised (Jones & Jones, 1969; Labbe & Hubbard, 1961; Honeybourne et al, 1979). However several metals such as cobalt, zinc and lead inhibit activity of the enzyme (Taketani & Tokunaga, 1981). Inhibition also occurs with high concentrations of both substrate and product, and has been induced in a severe form in experimental animals with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). Transmethylation from DDC to a nitrogen on PROTO IX causes stereochemical alteration of one of the porphyrin rings preventing binding of the iron molecule (Ortiz de Montellano, 1981).

1.2 DEGRADATION OF HAEM

As already mentioned haem is an extremely versatile molecule and has many functions. To maintain intracellular haem content constant formation and degradation occurs, with old haem being removed from the haemoproteins, iron removed for reuse and the porphyrin moiety broken down for excretion. Biodegradation of haem occurs via a much shorter pathway than the synthetic one

with either one or two steps in animals. In birds, reptiles and amphibia the enzyme haem oxygenase cleaves the tetrapyrrolic ring and the resulting linear tetrapyrrole, biliverdin, can be excreted. In mammals however, a further step catalysed by the enzyme biliverdin reductase produces bilirubin. The pathway of biodegradation of haem is shown in Figure 9.

Labelling studies in man have showed appearance of active bilirubin in three distinct pools. By far the largest fraction of body haem is incorporated into haemoglobin in the erythrocyte and consequently bilirubin from around 80% of haem is seen at the expected time of red cell breakdown (approximately 120 days). The remaining fraction of bilirubin is detected much earlier in a double peak which is thought to be hepatic in origin. Turnover of haemoproteins such as cyt. P450 and catalase occurs much more rapidly than that of haemoglobin and explains the majority of the peak. This is described as early labelled bilirubin. However a small proportion can be detected even earlier, within minutes of administration of labelled bilirubin. It is widely believed that this portion arises from breakdown of newly formed haem in the so called free haem pool. The free haem pool is a small and constantly turning over source of haem in the body which has an important role to play in regulation of haem in vivo. The concept of the free haem pool is more fully discussed in section 1.3.1. A more detailed discussion of the biological and chemical degradation of haem and its end product, bilirubin follows.

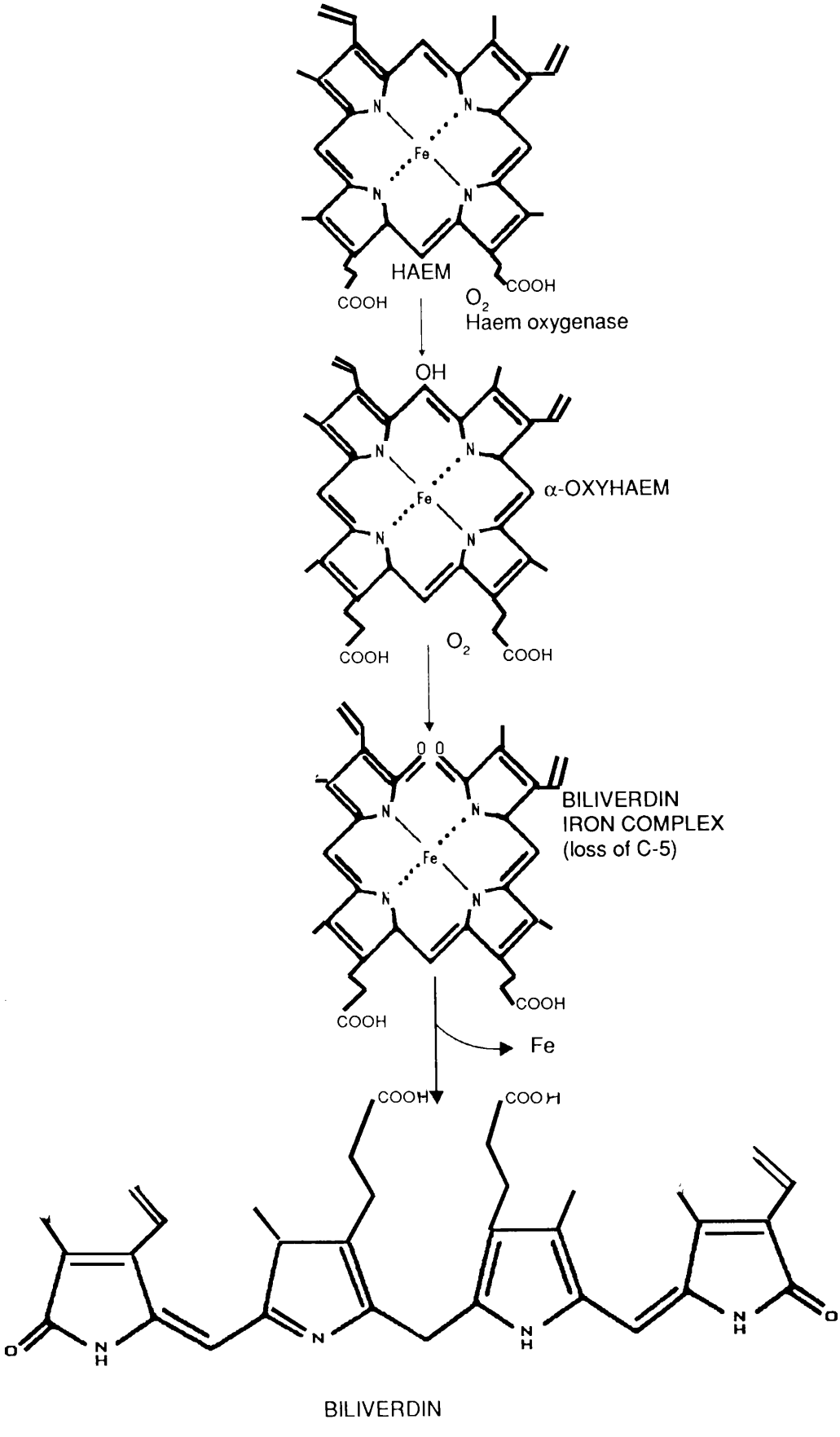


FIGURE 9 : THE REACTION CATALYSED BY HAEM OXYGENASE

1.2.1 Haem Oxygenase (E.C. 1.14.99.3)

Biological degradation of haem occurs by oxidative cleavage of the ∝-methene bridge of the molecule to form equal amounts of biliverdin IXα, carbon monoxide (CO) and iron. This reaction is catalysed by the microsomal enzyme system haem oxygenase (Tenhunen et al, 1968; 1972) and has an absolute requirement for a reducing equivalent and molecular oxygen (King & Brown, 1978) (see Figure 9). NADPH is the best reducing agent although NADH has been shown to be active in the rat liver system (Hino et al, 1979). Immunochemical studies demonstrated the flavoprotein NADPH-cytochrome c reductase is necessary to the reaction (Schacter, 1972) and that interactions with haem oxygenase and biliverdin reductase were occurring (Yoshinaga et al, 1982). The detailed mechanism of conversion of haem to biliverdin is not totally clear but free haem is known to be the preferred substrate, so haemoprotein cleavage does not appear to be involved. The mechanism by which the apoprotein and haem are split requires further research. Biliverdin formation is thought to occur through several intermediates and one possible mechanism is schematically represented in Figure 9. This theory supposes that haem binds to haem oxygenase forming an intermediate with spectral properties similar to cytochrome P450. This is followed by reaction with oxygen to form ∝-hydroxyhaem which can then split leaving the enzyme-biliverdin-Fe complex and carbon monoxide. The final step is release of iron and biliverdin from the enzyme. In total three moles of molecular O_2 are required for each molecule of haem broken down. This mechanism has not been confirmed and the role of haem

oxygenase after cleavage of the ∝methene bridge is not certain. (For review see Schacter, 1988 or Brown et al, 1989).

Protohaem is the best substrate for the enzyme, but several other haem compounds can react with the haem oxygenase system, including mesohaem, deuterohaem, coprohaem, and some synthetic analogues of protohaem eg haemin III (Frydman et al, 1979). Some of these compounds even have higher affinity for the enzyme than the natural substrate. The absence of iron, as in the porphyrins, produces an unsuitable substrate. The presence of certain other metals in the porphyrin nucleus such as tin, zinc and manganese result in metalloporphyrins which bind more tightly to the enzyme than the native substrate and so inhibit enzyme activity. Tin protoporphyrin has been proposed as a useful agent in reducing the hyperbilirubinaemia associated with certain conditions (Simionatto et al, 1985; Anderson et al, 1986; Kappas et al, 1988). In contrast cobalt-protoporphyrin administration results in increased enzyme activity. Control of haem metabolism by synthetic metalloporphyrins has been recently studied (Drummond, 1987). The effects of tin protoporphyrin (Sn-PROTO) on the biosynthesis and catabolism of haem will be examined later in this chapter and in more detail in Chapter 8.

The enzyme protein was initially isolated and partially purified from rat liver microsomes, SDS polyacrylamide gel electrophoresis revealing two bands of molecular weights 68 000 and 34 000 (Maines et al, 1977). Further work on spleen enzymes from porcine and bovine sources have demonstrated molecular weights around the 30 000 mark (Yoshida et al, 1974;

Yoshinaga et al, 1982a).

Haem oxygenase is another inducible enzyme making it an important factor in the control of intracellular haem levels as will be discussed in section 1.3. Various chemical and biological agents can induce haem oxygenase. The presence of cobalt, cadmium, silver, tin and nickel induce activity (Maines & Kappas 1974; 1976a; 1976b). Simultaneous administration of manganese and zinc complexes are reported to prevent the induction by the listed metal ions (Drummond & Kappas, 1980). These observations add to the complex nature of the effect of metals on haem degradation since these same metal ions combined with the PROTO ring lead to inhibition of the enzyme. A review of the effect of metals on haem oxygenase has been recently published (Sunderman, 1987). Other experimental manipulations which result in increased haem oxygenase activity include administration of chemicals eg phenylhydrazine (Maines & Veltman, 1984), fasting, administration of insulin, glucagon, or cyclic AMP. The effects of non-metal agents on haem oxygenase activity are described more fully elsewhere (Bakken et al, 1972).

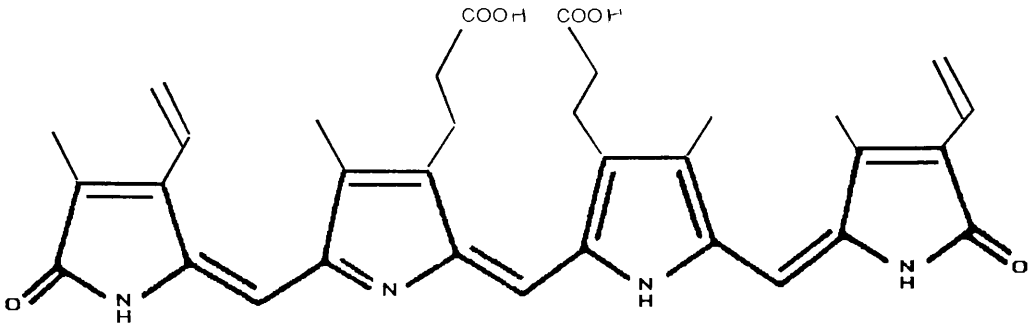
Recently genetic evidence has revealed that two isoenzymes of haem oxygenase exist, only one of which is inducible (Maines et al, 1986). The physiological significance of these isoforms has yet to be determined.

1.2.2 Biliverdin Reductase (E.C.1.3.1.24)

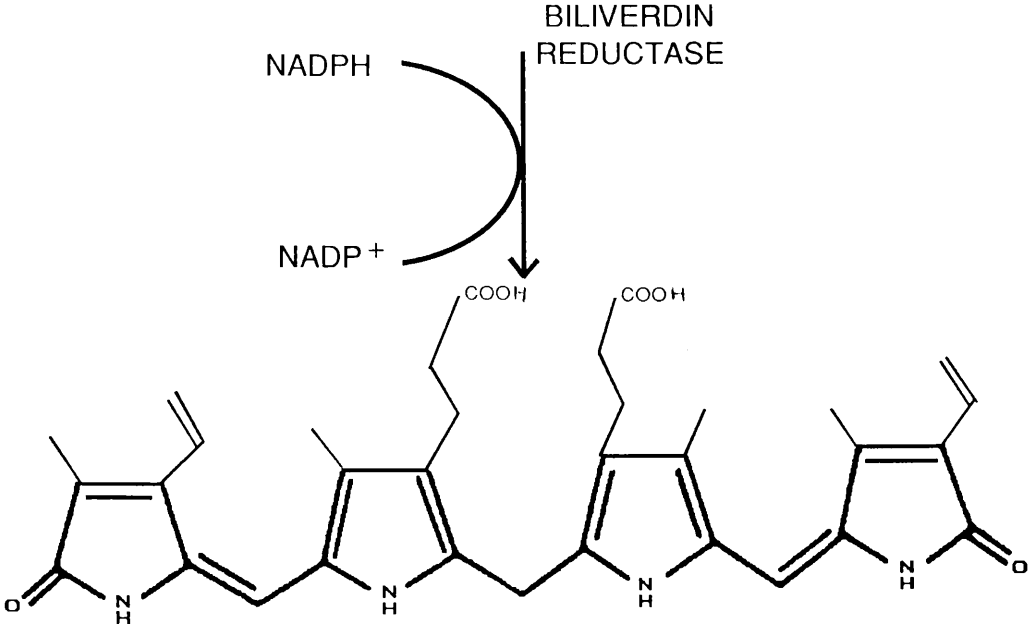
In mammalian tissues biliverdin is further reduced to bilirubin, structure shown in Figure 10, by the enzyme biliverdin reductase. NADPH binds to the enzyme creating high affinity binding sites for biliverdin, which on contact with the enzyme can then be converted to bilirubin (Tenhunen et al, 1970). Biliverdin reductase is found in the cytosol of many tissues and has been purified from rat liver (Noguchi et al, 1979) with molecular weight of 32 000 daltons. Predominantly, conversion of biliverdin IXα occurs, but the biliverdin reductase activity is not specific and the presence of other isomers of biliverdin leads to production of the corresponding isomer of bilirubin. However the conversion of β, γ and δ isomers of biliverdin is unlikely to be a functional role of the enzyme since haem oxygenase specifically cleaves the α methene bridge leading almost exclusively to bilirubin IXα(Tenhunen et al, 1968). The presence of excess activity of this enzyme (Tenhunen, 1972), implicates the lower enzyme activity of haem oxygenase as the rate limiting step in the breakdown of haem.

1.2.3 Excretion of Bilirubin

Bilirubin is the end product of physiological degradation of haem. Although the structure of bilirubin is represented as a linear tetrapyrrole in Figure 10 this arrangement is not the preferred structure in the body. Addition of two H^+ ions allows intramolecular bonding to occur between the slightly negatively charged carboxylic acid oxygens and the two adjacent amino groups. The result is a rigid structure shown in Figure



BILIVERDIN IX α



BILIRUBIN IX α

FIGURE 10 : THE REACTION CATALYSED BY BILIVERDIN REDUCTASE

11 as determined by X-ray crystallography. This is virtually insoluble in water (Bonnett et al, 1976). Only the trans form of bilirubin IX can undergo intramolecular bonding and it is this property which makes bilirubin potentially toxic to the body. The toxic effects of bilirubin are more fully discussed in section 1.5.2 with reference to kernicterus.

On release of bilirubin from the reticuloendothelial system to the circulation, the body is protected from any adverse effects of the bile pigment by the tight binding to albumin which occurs in plasma. The equilibrium which exists between free and albumin bound bilirubin leads to only a tiny percentage of free biliburin under normal circumstances. Bilirubin is removed from the circulation by the hepatocyte and evidence supports the theory that transport across the plasma membrane is carrier mediated. The exact nature of this process is still under investigation. Dissociation of bilirubin from albumin on contact with the hepatocyte membrane does not seem to be followed by instantaneous equilibration between the bound and free forms and simple diffusion into the cell (Sorrentino et al, 1987). In vitro studies have shown that dissociation of bilirubin from albumin is too slow to account for the rate of uptake of bilirubin which occurs in vivo (Wolkoff et al, 1979). However, addition of uncomplexed albumin seems to competitively inhibit uptake (Weisiger et al, 1982). The attractive concept of a specific albumin receptor has not been backed up by experimental evidence and suggestions have been made that non-equilibrium binding of bilirubin occurs

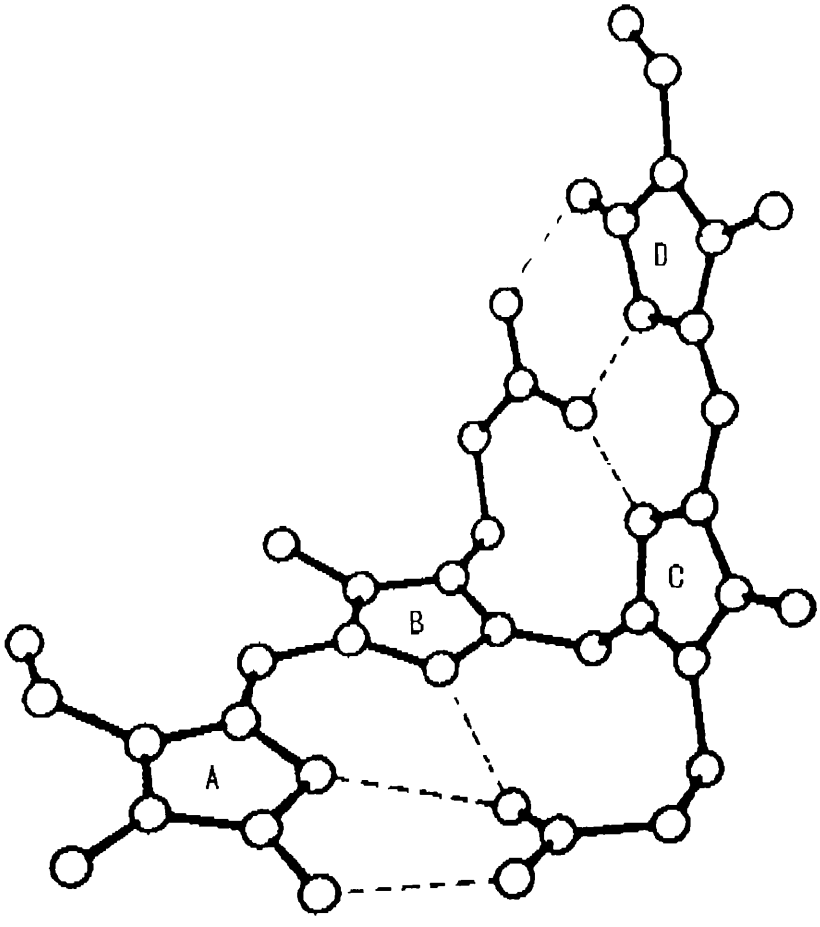


FIGURE 11 : THE INTRAMOLECULARLY BONDED FORM OF BILIRUBIN IXα

(Weisiger, 1985) or that the hepatocyte has certain peculiar characteristics which would facilitate uptake (for review see Sorrentino et al, 1987). After entry to the liver cell intracellular binding to a specific bilirubin binding protein called ligandin (or Y protein) (Kamisaka et al, 1975) occurs, and this prevents efflux back into the circulation.

Intracellularly bilirubin disrupts normal cellular processes and can under certain circumstances kill cells. The mechanisms by which bilirubin exerts this effect are not well understood. Bilirubin is known to uncouple oxidative phosphorylation (Cowger et al, 1965; Diamond & Schmid, 1967). More detailed analysis has revealed one aspect of this is inhibition of some mitochondrial linked dehydrogenases (Noir et al, 1972) and aminotransferases, including enzymes responsible for production of ATP (McLoughlin & Howell, 1987). Inhibition of RNA synthesis, protein and carbohydrate synthesis in the brain have been reported (Katoh et al, 1975; Nandi Majumdar et al, 1974; Greenfield, 1974). Bilirubin has also been shown to bind to membrane lipids (Mustafa & King, 1970) and it has been proposed from *in vitro* studies that incorporation into synaptosomal membranes and subsequent crystallisation may affect membrane function and contribute greatly to toxicity of bilirubin in the brain (Vazquez et al, 1988). However, no particular mechanism has been proven (for review see Sarnat, 1984).

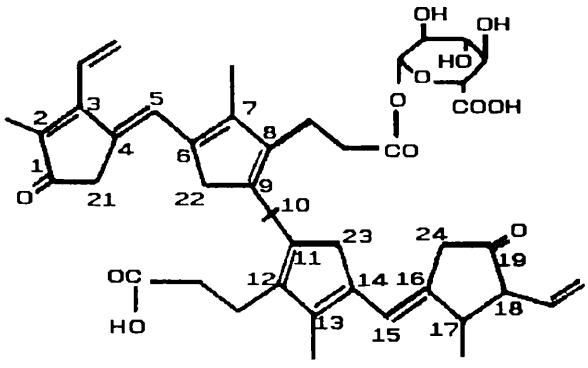
Excretion of bilirubin is facilitated by conjugation with uridine diphosphate glucuronic acid producing either bilirubin monoglucuronide or bilirubin diglucuronide. The enzyme UDP

glucuronyl transferase (UDP-GT), present in the microsomal fraction of the hepatocyte (Dutton, 1966), catalyses glucuronidation as shown in Figure 12. The monoglucuronides and diglucuronide (the main product) are water soluble and can enter the biliary system. From the bile ducts and gall bladder bilirubin glucuronides are secreted into the gastro-intestinal tract where intestinal bacteria cause degradation to urobilinogen and related products. A small amount of the urobilinogen is reabsorbed from the G.I. tract but the majority is oxidised to urobilin and then excreted in the faeces.

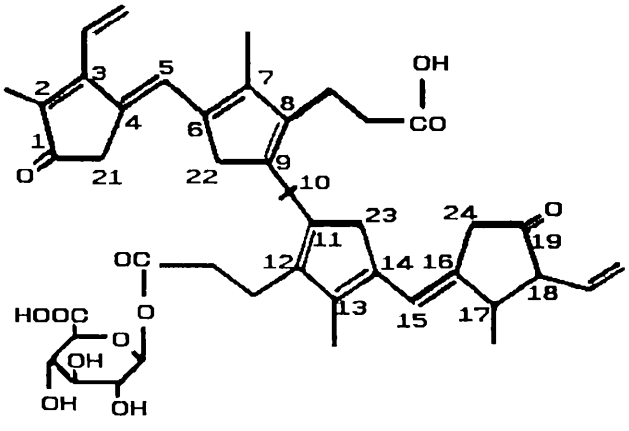
1.2.4 Non-Biological Degradation of Haem

Most of the information available on the biodegradation of haem has been determined by the use of models of biodegradation. The most common model used is that of coupled oxidation. Addition of a strong reducing agent in the presence of O_2 causes rapid breakdown of haem with simultaneous oxidation of the reductant. Common reducing agents used are ascorbate and hydrazine (Lemberg, 1935). Haemoproteins can be directly oxidised using this system and evidence shows that the protein moiety appears to be influential in determining which methene bridge comes under attack (Brown et al, 1989).

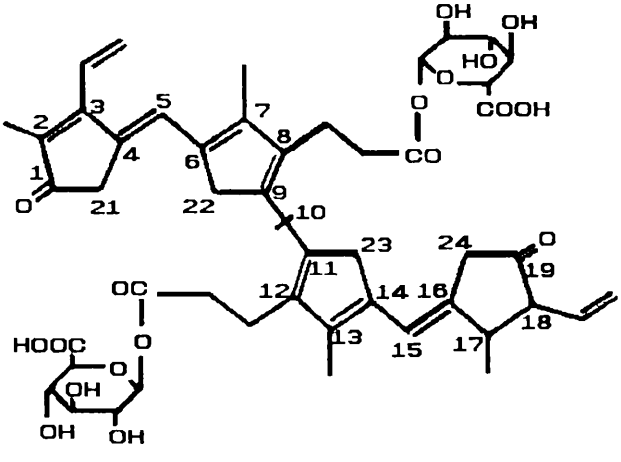
Treatment of experimental animals with drugs such as allylisopropylacetamide (AIA) leads to accumulation of green pigments in the liver. This type of drug causes suicide inactivation of cyt. P450 haem leading to production of N-alkylated porphyrins, compounds which are of interest in the study of cyt. P450 system (Ortiz de Montellano, 1983).



BILIRUBIN MONOGLUCURONIDE (C-8)



BILIRUBIN MONOGLUCURONIDE (C-12)



BILIRUBIN DIGLUCURONIDE

FIGURE 12 : STRUCTURES OF THE GLUCURONYL CONJUGATES OF BILIRUBIN

Haem and porphyrin derivatives were characterised by methods using strong oxidising agents such as hydrogen peroxide (Rudiger, 1968). This type of oxidation produced fragments of the haem ring but there is no evidence to confirm that this is comparable to the physiological system of breakdown.

1.3 REGULATION OF THE HAEM METABOLIC PATHWAY

Production of haem is regulated mainly by the intracellular levels of haem itself (Granick, 1966; Marver et al, 1966; Marver et al, 1968). The presence of haem results in negative feedback on the first enzyme of the biosynthetic pathway, ALA-synthase, to reduce the rate of haem synthesis. At the same time haem induces the haem oxygenase system so increasing breakdown. Simultaneously production of the protein molecules which combine with haem such as the cyt. P450 apoprotein or globin, is stimulated. Conversely haem deficiency leads to stimulation of haem production while inhibiting breakdown by negative feedback type mechanism. The fine balance achieved between these processes efficiently regulates haem production and prevents accumulation or deficiency of haem under normal circumstances.

This mechanism has been well characterised in the liver. This tissue is the main site of haem production for haemoprotein formation and accounts for formation of more than 15% of total body haem. In the erythroid tissue where the largest proportion of body haem (around 80%) is formed, the situation is not as well defined, mainly due to the difficulty in obtaining sufficient amounts of tissue for study.

ALA-synthase possesses characteristics of a regulatory enzyme. Activity is normally very low, but can be affected by requirement for the end product of the pathway, haem. Turnover is more rapid than that of most other mitochondrial proteins (Beattie & Struchel, 1970).

The best known role of haem, that of oxygen transportation, is carried out by haemoglobin in the red cells and this haem is formed in the bone marrow. Hepatic haem becomes incorporated into haemoproteins such as catalase, tryptophan pyrrolase, cytochrome b5 and the major one, cytochrome P450. Multiple forms of cyt. P450 exist, unsurprising because of the varied role of the cytochrome in drug metabolism. Cyt. P450 is responsible for detoxification of xenobiotics ranging from drugs to environmental pollutants, which would be harmful to the body under prolonged exposure. Some naturally occurring steroids are also metabolised by this system. The cytochrome P450 system has an extraordinary ability to adapt and react with a huge variety of drugs and chemicals even on first exposure. On exposure cyt. P450 content increases resulting in a higher proportion of newly synthesised haem being incorporated into the cytochrome. To prevent haem deficiency, biosynthesis is stimulated. Thus in hepatic tissue the haem biosynthetic pathway is finely tuned to accomodate increased requirement for haem.

Erythroid tissue however has little of the ability of biotransformation present in liver since the majority of haem produced is for incorporation into haemoglobin. In contrast to

the liver increased requirement for haem tends to be met by an increased rate of cellular differentiation, producing more cells.

It is clear that study of these tissues has revealed fundamental differences in the control of haem biosynthesis. In support of this recent genetic evidence has shown that genetic sequences which produce hepatic and erythroid ALA-synthase are coded for by separate genes (Riddle et al, 1989) with the erythroid form highly specific to this tissue. The genes for the two different isoforms are located on different chromosomes (Bishop et al, 1990). Differences in haem biosynthetic control will now be considered in more detail.

1.3.1 Control in the Hepatocyte

ALA-synthase possesses characteristics of a regulatory enzyme and, as will now be discussed, much evidence supports the theory of this enzyme as the rate controlling enzyme in liver cells. The very low activity of hepatic ALA-synthase is still sufficient to maintain adequate production of haem under normal circumstances, however increased haem requirement triggers induction of the enzyme. This ultimately leads to more haem being produced, since the intermediate enzymes of the pathway are normally present in excess. The mechanism of induction is via de novo synthesis of ALA-synthase (Granick & Urata, 1963). The trigger for induction is thought to be a depletion of the end product of the pathway, haem. This is usually caused by greater than normal demands for the final haemoprotein product, mainly cyt. P450.

Production of cyt. P450 increases, so using more haem, on exposure to a wide range of substances some of which have already been mentioned. Several mechanisms by which haem causes ALA-synthase induction and regulates its own synthesis have been proposed. These all rely on the existence of free haem pools which appear to consist of so far unmeasured, small amounts of haem. The sources of the free haem pools are thought to be newly synthesised haem before incorporation into haemoprotein and haem split from haemoprotein sources before breakdown (Granick & Sassa, 1971; Yannoni & Robinson, 1975; Grandchamp et al, 1981). It is assumed the free haem pools exist in all subcellular fractions of the cell so that an equilibrium exists between mitochondrial endoplasmic reticulum and cytosolic free haem in the pool similar to the situation shown in Figure 13. This haem is assumed to turn over rapidly. The possible mechanisms by which haem may regulate its own synthesis follow.

Possible Mechanisms of End Product Regulation

a) Haem produced may directly inhibit the activity of ALA-synthase.

b) Haem may repress translation of the messenger RNA (mRNA) coding for the enzyme.

c) Haem may have an adverse effect on transcription thus decreasing availability of mRNA coding for ALA-synthase.

d) Haem may inhibit translocation of the mature enzyme to mitochondria.

It has been reported that haemin directly inhibits the enzyme (Aoki et al, 1971) consistent with a), but studies with exogenous haemin have shown that ALA-synthase activity is

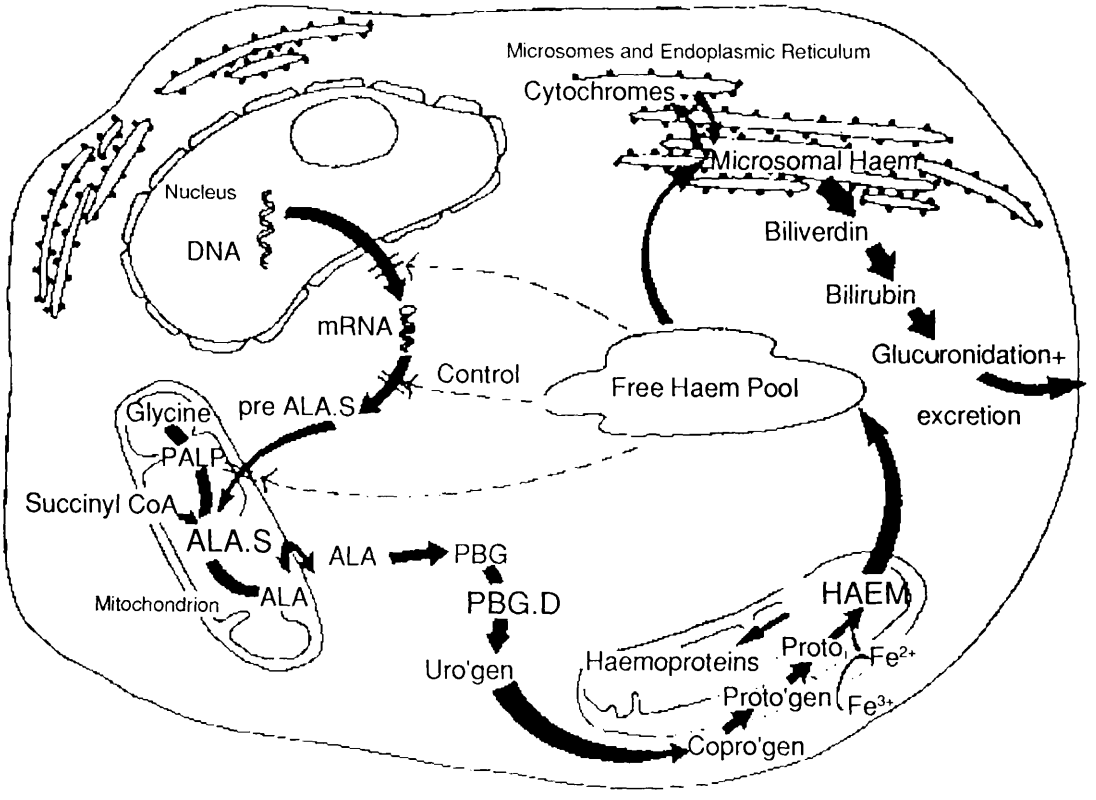


FIGURE 13 : INTERRELATIONSHIP OF THE FREE HAEM POOL IN CONTROL OF HAEM BIOSYNTHESIS

supressed at haem concentrations far below those which would be necessary for a direct effect when calculated from the K_i constant of 1-2 $\times 10^{-8}$ (Sinclair & Granick, 1976; Srivastava et al, 1980). Haem levels in the mitochondria did not appear to be high enough to inhibit enzyme activity directly but might result in inhibition of synthesis of ALA-synthase (Wolfson et al, 1979). This is one area which merits further study. It has been shown that haem represses translation of the mRNA of ALA-synthase (Tyrell & Marks, 1972) and that transcription is abnormal (Whiting, 1976). Thus it appears that a combination of various effects of haem result in the control of ALA-synthase activity. Induction of ALA-synthase is modified by various factors. In chick embryo hepatocytes cultured under controlled conditions, insulin is required for induction (Sinclair et al, 1979). The presence of glucocorticoids is conducive to ALA-synthase induction. Marver and others showed that induction of ALA-synthase is less marked in adrenalectomised rats although these compounds do not by themselves exert an inducing effect. This effect has been shown to directly involve the liver (Bock et al, 1971). In rats and other mammals, diet is an important factor with a large intake of glucose and protein repressing induction. In contrast fasting enhances the induction of ALA-synthase.

The rate limiting reaction of haem biosynthesis can also be controlled by other factors. Concentration of both substrates must be in excess for the reaction to proceed and haem biosynthesis has been controlled by limiting the amount of glycine

available under experimental conditions. Similarly absence of pyridoxal phosphate, an essential cofactor to the enzyme activity, prevents ALA formation and so results in haem deficiency.

1.3.2 Control in Erythroid Tissue

Where regulation of hepatic haem biosynthesis has many well defined aspects, the situation in the erythroid tissue is much less clear. This has been largely due to the difficulty in obtaining sufficient numbers of mammalian cells for study. Early work using chick blastoderm led to the belief that regulation of the pathway was by a similar mechanism to that in liver (Levere & Granick, 1967). Subsequently virtually all recent studies have used cell culture techniques to observe the haem biosynthetic pathway and the results obtained present a more complex picture than that of hepatic control. Studies using erythroleukemic cell cultures of murine and human origin and normal bone marrow colonies in vitro suggest that regulation of the pathway in erythroid tissue is not through feedback control on ALA-synthase activity. It has been shown that dimethylsulphoxide (DMSO) administration leads to increases in ALA-synthase activity, globin, mRNA, ALA-D, PBG-D, and consequently results in increased production of haem in the T3-Cl-2 clone of Friend cells (Ross et al, 1972; Sassa, 1976). A similar pattern has been observed in other clones of Friend cells (Sassa, 1980; Rutherford et al, 1979). Certainly it appears that ALA-synthase alone is not the rate limiting enzyme for formation of haem in erythroid cells. In fact evidence

suggests that ferrochelatase may be more important in control since the increased levels of haem observed above occur only after activity of ferrochelatase is raised. In addition, in contrast to the situation in the liver it has been reported that administration of hemin leads to increased ALA-synthase activity in erythroid tissue (Hoffman, 1980). Thus the situation in erythroid tissue is very different to that in liver with induction of all enzymes in the pathway occurring before more haem is produced. The likely role of ferrochelatase as a rate controlling enzyme also demonstrates the differences in the mechanism of control of haem biosynthesis in this tissue when compared to the liver.

Further evidence of tissue specific regulation has been obtained from murine Harderian gland (Margolis, 1971), cardiac tissue (Briggs et al, 1976) and lymphocytes and skin fibroblasts of patients with acute intermittent porphyria (Bonkowsky et al, 1975).

1.4 THE PORPHYRIAS

The eight steps of the pathway of haem biosynthesis have already been discussed. Abnormalities could conceivibly occur at any of these stages, resulting in disease. Porphyria is a general term encompassing several distinct diseases of porphyrin metabolism, usually due to a hereditary enzymatic defect at some stage in the pathway of haem biosynthesis. When the clinical symptoms and biochemical defects are considered, the porphyrias are classified as either acute or non-acute. Alternatively the porphyrias can be classified according to the tissue of origin

of the enzymatic defect thus defining them as either hepatic or erythropoietic. In addition certain porphyrias can be acquired. A number of endogenous and exogenous factors may result in disturbances in porphyrin metabolism and these are more fully discussed in section 1.4.3.

1.4.1 The Acute Porphyrias

As the title suggests each of these porphyrias are characterised by the occurrence of an acute attack followed by a period of remission. The attacks are of variable length and are usually precipitated by exogenous factors such as drugs. Links have also been made with endogenous compounds such as steroid hormones. Discrete types of compounds can cause an acute porphyric attack. The three most common diseases, acute intermittent porphyria (AIP), hereditary coproporphyria (HC) and variegate porphyria (VP) all result in overproduction of the early haem precursors ALA and PBG. In HC and VP overproduction of precursors formed later in the pathway occurs and often leads to cutaneous lesions.

In attack patients suffer clinical symptoms including abdominal pain, peripheral neuropathy and autonomic neuropathy. Photosensitivity can develop in HC and VP, due to the overproduction of porphyrins.

AIP is the commonest form of acute porphyria with the activity of PBG-D, the enzyme responsible for condensing PBG molecules, reduced to around 50% of normal levels (Meyer et al, 1972). This reduction in activity has been observed throughout the body in liver, erythrocytes, cultured skin fibroblasts,

cultured amniotic cells and lymphocytes (Moore et al, 1987). Despite the enzyme deficiency being commonly identified in many family members of sufferers only a small fraction of heterozygous patients present with clinical symptoms of the disease. The remainder, around 90%, are described as latent cases. Attacks of AIP occur after puberty and are more common in women than men. This has suggested a link with the sex hormones (Kappas et al, 1982). In hereditary coproporphyria (HC) COPRO-O activity is lower than normal and this has been demonstrated in leukocytes, cultured skin fibroblasts, lymphocytes and hepatic biopsies (Brodie et al, 1977b; Elder et al, 1976; Grandchamp & Nordmann, 1971; Hawk et al, 1978). Homozygous sufferers have been identified with virtually no COPRO-O activity and these patients display very severe clinical symptoms (Grandchamp et al, 1977; 1980). In variegate porphyria there has been controversy as to the source of the enzyme defect with both PROTO-O and ferrochelatase implicated. In erythroid cells and fibroblasts of some VP patients 50% of normal ferrochelatase activity has been recorded (Viljoen et al, 1979) In contrast normal activity has been observed by other groups (Siepker & Kramer, 1985), and reports have described a similar percentage reduction in PROTO-oxidase activity (Brenner & Bloomer, 1980; Deybach et al, 1981). Patients with virtually no PROTO-oxidase exist supporting the theory that the defect is in the penultimate enzyme of the pathway (Kordac et al, 1984; Murphy et al, 1986). Biochemically high amounts of protoporphyrin are excreted in the stool of these patients and this is often accompanied by a slight increase in coproporphyrin

excretion. Clinical symptoms of an attack of acute porphyria include severe abdominal pain, peripheral neuropathy and autonomic neuropathy. Additionally in HC and VP cutaneous lesions often develop. This is thought to be due to porphyrin deposition near the surface of the skin. The photosensitivity which sometimes develops intensifies on exposure to sunlight. Exacerbation of the clinical symptoms occurs as the negative feedback normally operated by haem on ALA-synthase activity is absent. The resulting increase in ALA-synthase activity intensifies the build-up of ALA, PBG and porphyrinogens.

More recently a deficiency in ALA-D has been identified in a small number of cases (Bird et al, 1979; Doss et al, 1979). Symptoms are identical to those seen in AIP with extensive peripheral and central nervous system neuropathy without photosensitivity (Brandt & Doss, 1981). The structure of the enzyme is known to be abnormal (De Verneuil et al, 1985) with activity of around 50% normal in heterozygotes and almost absent in homozygotes. This condition has been termed plumboporphyria.

Genetically the enzyme deficiencies which produce acute porphyria are transmitted in autosomal dominant fashion. Investigations into HC patients have showed a mutant gene product exists and this is thought to have virtually no activity (Grandchamp & Nordmann, 1977).

All diagnosed porphyrics are advised to avoid a large number of therapeutic drugs since administration often precipitates an attack. In addition the link of the disease to sex hormones is strengthened by many women sufferers having attacks at certain

stages of the menstrual cycle. From human and animal evidence a druglist of recommended safe drugs and drugs most commonly producing attack (unsafe drugs) has been compiled (Moore & McColl, 1988). This publication also lists drugs which have been shown to be safe for administration to porphyric patients.

1.4.2 The Non-Acute Porphyrias

Three separate diseases are known as the non-acute porphyrias. These are cutaneous hepatic porphyria (CHP) also known as porphyria cutanea tarda, erythropoietic protoporphyria (EPP) and congenital erythropoietic porphyria (CP). Generally these diseases have more serious consequences for the patients with the condition gradually worsening with increasing age.

PCT or cutaneous hepatic porphyria exists in two forms. In both, activity of URO-D is reduced. In congenital PCT reduced activity is seen in the liver (Kushner et al, 1976), erythrocytes and skin fibroblasts (Felsher et al, 1978). In these fairly rare cases the reduced activity is thought to result from an alteration to enzyme structure leading to decreased substrate affinity. Higher susceptibility to Fe^{2+} inhibition has also been shown (Mukerji et al, 1985). The more common form of the diseaseis acquired in later life by patients who have no family history of porphyria. Alcoholic liver disease is prevalent among these patients, but the condition has also been linked with states of iron overload and the contraceptive pill. Unlike the familial form hepatic URO-D activity alone is reduced. A small number of cases of CHP are a direct result of exposure to polyhalogenated hydrocarbons, for example hexachlorobenzene but

little information is available on enzyme activities in sufferers. Liver dysfunction is seen in most patients but it is unclear whether this is due to an underlying metabolic defect or simply a result of the aquired factors which resulted in production of the disease (Topi & D'Alessandro, 1976).

Erythropoietic protoporphyria (EPP) is an inherited porphyria where ferrochelatase activity is reduced to less than 50% of normal. Erythrocytic protoporphyrin is greatly increased and there is increased excretion in faeces. EPP also has a hepatic component (Gray et al, 1964; Barnes et al, 1968) especially in later years, when build-up of protoporphyrin in this tissue and the hepatobiliary system can lead to dysfunction which may be fatal (Poh-Fitzpatrick, 1985). Most of the excess protoporphyrin is probably derived from bone marrow erythroblasts and hepatocytes (Gray et al, 1964) with a small contribution from other tissues.

Congenital porphyria (CP) or Gunther's disease is the rarest type of porphyria and has a severe prognosis, with no patients surviving middle age and a high death rate in early life. Enzymatic defects occur primarily in URO'gen III cosynthase (Romeo & Levin, 1969; Moore et al, 1978) and leading to production of high amounts (greater than 80%) of series I total porphyrins which can proceed no further in the pathway and are excreted in urine. Faecal porphyrin excretion is also increased and increased activity of PBG deaminase has been reported (Nordmann & Deybach, 1982). URO-decarboxylase activity is also diminished (Hofstad et al, 1973). The excretory pattern of porphyrins can be explained by any of the above hypotheses.

Observation of erythroblasts in the bone marrow showed apparently two distinct types of cell, one of which fluoresced strongly under UV light, the other being normal in appearance (Schmid et al, 1954). However more recent work suggests that all developing erythroblasts carry the allele for CP and fluorescence is more easily detected in some cells than others.

Patients who have non-acute porphyria all suffer from varying degrees of photosensitivity, caused by accumulation of the later intermediates of haem biosynthesis. In all cases excretion of ALA and PBG remains virtually normal. The absence of psychiatric symptoms lends support to the theory that accumulation of these early precursors of the pathway is involved here. PCT generally develops later in life and the main clinical symptom is increased skin fragility, with skin lesions occurring on light exposed areas which are loaded with porphyrin. EPP patients experience intense burning sensations on exposure to sunlight from an early age, and scarring quickly occurs. In later years, skin thickens and ages prematurely and changes in texture of skin (orange peel) is occasionally observed. In later years build-up of PROTO in the liver and hepatobiliary system can lead to dysfunction which may be fatal (Poh-Fitzpatrick, 1985). The most severe photosensitivity reactions are seen in CP with exposure to light causing development of large blisters which can burst and ulcerate leading to secondary infection. Scarring is widespread on exposed skin and often appendages become deformed or even lost in later stages of the disease. Haemolytic anaemia is usually present

and splenomegaly results from the need to rid the body of the high proportion of abnormal red cells.

1.4.3 Experimental Porphyria

As has already been discussed in section 1.4 porphyria can be induced in man when certain drugs are administered. Similarly, induction can occur in other animals and experimental porphyria in the rodent has provided an extremely useful animal model for the study of disturbances in haem biosynthesis (Smith & De Matteis, 1980). In non-susceptible humans i.e. those with homozygous normal genes, and normal rats there are very few classes of drug which cause porphyria-like symptoms, namely some derivatives of branched aliphatic acetamide or barbiturate e.g. 2-allyl-2-isopropylacetamide (AIA) (Goldberg & Rimington, 1955), or 5,5-diallylbarbituric acid respectively, DDC or griseofulvin, or related chemicals (De Matteis, 1978). Within a few hours of administration, porphyrin precursors are excreted in excess in a pattern dependant on the drug used. On treatment with AIA, ALA-synthase activity is induced, a mixture of porphyrins are produced and the liver assumes a greenish tinge. In the liver AIA initially stimulates formation of cyt. P450. Oxidation of the drug by the cyt. P450 system then leads to destruction of the protein (Ortiz de Montellano & Correia, 1983). Thus haem content is further reduced and the pathway is accelerated to try and compensate. This combination has been proposed as a general mechanism of action for allyl-containing drugs which induce porphyria (Moore et al, 1987).

Metabolism of DDC by the liver produces a metabolite which

strongly inhibits ferrochelatase (De Matteis et al, 1980a, 1980b, 1980c). This analogue of protoporphyrin, N-methyl protoporphyrin IX is formed as part of the suicide inactivation of DLC by cyt. P450 and results in a block of the pathway.

As has already been discussed, halogenated hydrocarbons cause a toxic form of cutaneous porphyria in humans and this condition can be reproduced in rats by adding hexachlorobenzene (HCB) to the diet (Salamanca & Salamanca, 1986) although the mechanism of action of production of the porphyria is uncertain.

1.5 DISORDERS OF HAEM DEGRADATION

The breakdown pathway of haem involves only two intermediates, biliverdin and bilirubin and has been discussed in section 1.2. Generally mammalian disease states involve the end product of the pathway, bilirubin. Of the total bilirubin formed three quarters results from breakdown of haemoglobin and the remainder from degradation of other haemoproteins. In nature bilirubin is usually found in the IXα isomer form, however traces of non-α isomers do exist, formed by the action of biliverdin reductase on various biliverdin isomers.

Clearance of bilirubin from the body by conjugation with glucuronic acid has already been discussed in section 1.2.3. Hereditary disorders of the metabolism of bilirubin lead to abnormal levels of either unconjugated or conjugated forms of bilirubin and are termed hyperbilirubinaemias. States of hyperbilirubinaemia may occur at any of the four stages of bilirubin metabolism which follow.

a) uptake from the circulation by the hepatocyte may be impaired.

b) intracellular storage may be abnormal.

c) conjugation may be impaired or totally absent.

d) biliary excretion may be abnormal

When either a), b) or c) occurs the result is unconjugated hyperbilirubinaemia. Hereditary conjugated hyperbilirubinaemia does occur but is rare. Much more common are disorders where haemolysis causes the elevated bilirubin. Under these conditions bilirubin production is increased but this type of hyperbilirubinaemia is considered to be secondary to other disorders and is discussed elsewhere (Fevery et al, 1980).

1.5.1 Hereditary Conjugated Hyperbilirubinaemia

The conjugated hyperbilirubinaemias are two very rare inherited diseases. The mechanisms by which the hyperbilirubinaemia occurs are distinct and are described below.

Dubin-Johnson Syndrome

Dubin-Johnson syndrome was first identified in 1954 (Dubin & Johnson, 1954) and is characterised by chronic non-haemolytic jaundice resulting from conjugated hyperbilirubinaemia. The liver tissue is black in colour due to a pigment which is thought to be related to, but distinct from, melanin (Sonnet et al, 1969; Swartz et al, 1979). Liver function is predominantly normal but analysis of porphyrin excretion in urine shows that the main coproporphyrin product is the series I isomer (over 80%) instead of the series III isomer which is observed in normal urine (Kondo et al, 1976). Heterozygous Dubin-Johnson

sufferers also have increased coproporphyrin I excretion, but to a lesser extent with around 40% remaining as the series III isomer (Ben-Ezzer et al, 1973) The syndrome is inherited as an autosomal recessive characteristic (Edwards, 1975).

Rotor's Syndrome

Rotor's syndrome is an extremely rare form of conjugated hyperbilirubinaemia which was at one time thought to be related to Dubin-Johnson syndrome but is now recognised as a distinct condition (Wolkoff et al, 1976). In Rotor's syndrome transport of the organic anion sulphobromophthalein (BSP) is impaired by an unknown mechanism and liver pigmentation is normal. Urinary COPRO excretion is much higher than normal and a greater percentage than normal is of the Type I isomer (Wolkoff et al, 1976). This increase may well be explained simply by reduced biliary excretion of COPRO leading to increased excretion of COPRO by the kidney.

1.5.2 Unconjugated Hyperbilirubinaemia

The term unconjugated hyperbilirubinaemia covers a range of diseases. Two of these, kernicterus and Gilbert's syndrome, have a fairly common incidence. Unconjugated hyperbilirubinaemia occurs when large amounts of free bilirubin present in the circulation are unable to bind to albumin. This can be due to an inherited complete or partial defect in conjugation as in Crigler-Najjar syndrome and Gilbert's syndrome or to increased production of bilirubin.

Jaundice is produced and this can develop into the potentially

Footnote: One important cause of hyperbilirubinaemia is rhesus incompatibility. The jaundice occurs in premature infants and is severe.

life threatening condition of kernicterus. Under normal circumstances bilirubin is tightly bound to albumin in the serum. More than one binding site on albumin has been identified but in the body only one has high affinity for bilirubin. Consequently, when the bilirubin to albumin ratio exceeds one, bilirubin is allowed access to the body cells where it is potentially toxic. Animal models exist for some of these conditions i.e. the mutant Southdown sheep and the Gunn rat. The Gunn rat and its suitability for use as a model for various human unconjugated hyperbilirubinaemias will be considered in Chapter 4 of this thesis. As an alternative to naturally occurring models which are rare, hyperbilirubinaemia can be induced in situations where bilirubin is displaced from albumin. Several drugs, including certain sulphonamides have this property. The mechanism by which displacement occurs is not thought to be via direct competition for the same site. Instead it appears that by binding to another site on "spare albumin" the drug causes a conformational change to occur. This conformation is unable to bind bilirubin and so the concentration of free bilirubin increases. The resultant free unconjugated bilirubin is then free to diffuse into body cells where it can exert a toxic effect.

Neonatal Kernicterus

By far the most common form of hyperbilirubinaemia is the jaundice associated with newborn infants. This does occur in term babies but tends to be most severe in premature infants where it can develop into the life-threatening condition known

as kernicterus. Kernicterus produces brain damage of varying severity, caused by displacement of unconjugated bilirubin from the circulation into brain tissue. On post-mortem, yellow staining of the tissue can be clearly seen (Mollinson & Cutbrush, 1949; Friede, 1975). In the neonate there is less albumin than in older children and adults. This, combined with the underdeveloped UDP-glucuronyl conjugating system (Brown & Zueler, 1958), means that the infant has no adequate means of disposal of circulating bilirubin which is then free to form the potentially toxic acid form and deposit in tissue. Although the serious brain damage which was common at one time is rare nowadays, minor neurological impairment is thought to occur often, and not identified until later. Areas of the brain most often affected (presenting with intense yellow colour at post-mortem) are the basal ganglia, hippocampus and cerebral nucleus (Isherwood & Lathe, 1982). The specific mechanism by which bilirubin enters the brain is still unclear. One theory is that free bilirubin can cross the blood brain barrier, enter neuronal cells and cause damage (Brodersen, 1979; Hansen et al, 1979). From in vitro and animal studies this would be a suitable explanation (Brodersen & Anderson, 1976; Diamond & Schmid, 1966; Odell et al, 1970; Nelson et al, 1974). Not concurrent with the free bilirubin theory, very high levels of bilirubin occasionally do not produce kernicterus (Chen et al, 1965) and analbuminaeic rats do not exhibit the condition (Esumi et al, 1980). To explain this, some recent work has produced evidence that albumin bound bilirubin enters the brain when the blood-brain barrier is disrupted (Levine et al, 1982). These

authors suggest that perturbation of the blood brain barrier is an important consideration as to whether kernicterus can occur. In support of this EEG patterns of experimental rats which received albumin-bilirubin were only abnormal when the blood brain barrier was disrupted. The large variations in susceptibility of these rats to brain damage merits further investigation (Wennberg & Hance, 1986). Burgess and his coworkers suggested that the deposited bilirubin results from deposition of both free and bound forms (Burgess et al, 1985). After entry of bilirubin into the brain the role of the complex as opposed to the free bilirubin in toxicity has yet to be determined.

The mechanisms by which bilirubin exerts its toxic effect have been studied but no clear conclusions can yet be drawn. Originally it was thought that bilirubin could dissolve in lipid membranes and this was proposed as a mechanism of toxicity to the lipid rich CNS. It is now known that bilirubin acid is not lipophilic but attaches to the phospholipid molecules present in membranes (Brodersen, 1981; Vazquez et al, 1988). A popular theory is that mitochondrial damage is the primary event (Jew & Williams, 1977; Schutta et al, 1970)) but much evidence has been presented to the contrary (Katoh et al, 1975; Schutta & Johnson, 1971). There is no doubt that bilirubin is toxic to the CNS but the exact mechanism by which the toxicity occurs needs further study.

On identification of the hyperbilirubinaemic state the therapies used to prevent kernicterus are usually quite effective. Originally neonates were treated with an exchange

blood transfusion but this treatment has been superseded by the advent of phototherapy. Phototherapy relies on the fact that under illumination with visible light bilirubin "disappears". In fact when phototherapy is employed bilirubin is metabolised in two ways. Photodegradation occurs when bilirubin is oxidised reaction to pyrrole fragments which are water soluble and can be identified in urine (Porto, 1970). At the same time photo-solubilisation occurs, a process where geometrical isomerisation of bilirubin leads to production of more hydrophilic products known as photobilirubins (McDonagh et al, 1979). A further step where either of the vinyl groups cyclise leads to even more stable water soluble products (the photobilirubins II) which can be measured in both serum and bile of icteric infants. This is thought to constitute the major pathway of bilirubin 'removal' in the body (Onishi et al, 1986) and is represented in Figure 14. However, the main problem in treatment of this hyperbilirubinaemia is on deciding when the bilirubin will cause kernicterus. Elevation of serum bilirubin followed by displacement and development of acute symptoms can occur very quickly, and from a relatively low serum bilirubin level. Serum bilirubin levels have not been shown to be a good predictor of when this will occur. One new type of treatment which has been successfully used in treatment of hyperbilirubinaemia of the new born is administration of tin-PROTO (Simionatto et al, 1985; Anderson et al, 1986; Kappas, 1988). This synthetic metalloporphyrin binds to haem oxygenase but is not degraded. Inhibition of haem oxygenase activity occurs and is prolonged (Landaw et al, 1987). No severe side effects have

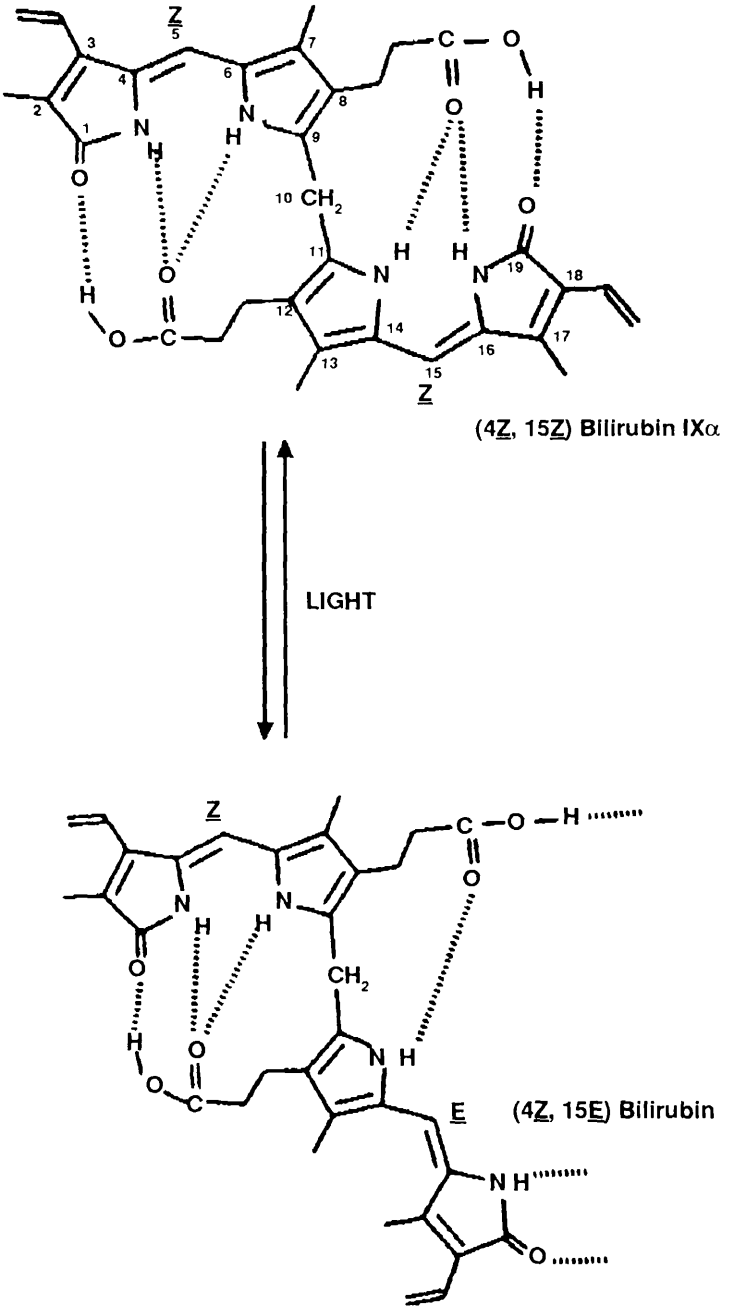


FIGURE 14 : A PROPOSED MECHANISM FOR THE PHOTOSOLUBILISATION OF BILIRUBIN

been reported with mild transient photosensitivity the only danger.

Crigler-Najjar syndrome Type I

This is a very rare disorder first described by Crigler and Najjar in which there is total absence of hepatic bilirubin UDP-GT (Crigler & Najjar, 1952). Severe hyperbilirubinaemia develops within a few hours of birth, and prognosis is severe. In the past few sufferers survived the neonatal period however modern prompt treatment with phototherapy has allowed patients to reach childhood or early adulthood. Unfortunately these patients are likely to succumb to the disease later in life. All serum bilirubin is unconjugated in patients with the Type I syndrome and it has been shown that the syndrome is transmitted as an autosomal recessive trait (Szabo & Ebrey, 1963).

Crigler-Najjar syndrome Type II

The phenotype of Crigler-Najjar Type II is similar to that of the Type I syndrome with the activity of the enzyme UDP-GT affected. Activity of the enzyme however is reduced rather than absent and so in contrast to the Type I the condition is usually clinically benign with unconjugated bilirubin levels higher than normal (around 10-20 mg/dl) (Berk et al, 1975) but not life threatening. Increases can occur however on fasting or during illness (Gollan et al, 1975; Gordon et al, 1976). Although levels of UDP-GT activity are barely measurable (Gordon et al, 1976) conjugation does occur with monoconjugates as the major fraction (Fevery et al, 1977). The reason for this is

unknown. It has been suggested that Crigler-Najjar syndrome Type II and Gilbert's syndrome may be identical or that Gilbert's syndrome may constitute a milder form of the same disease (Smith et al, 1967).

Gilbert's syndrome

Gilbert's syndrome is a benign condition characterised by mild but chronic unconjugated hyperbilirubinaemia in the absence of haemolysis. Clinically there are no symptoms so this condition may go unobserved, and is often discovered during other tests. Patients have a reduced UDP-GT activity (Black & Billing, 1969; Felsher et al, 1973). This does not show a negative correlation with the level of serum bilirubin (Metreau et al, 1978), indicating a defect in hepatic bilirubin uptake also exists (Berk et al, 1970; Martin et al, 1976) in addition to the defect in conjugation. Gilbert's syndrome has been reported as a heterogeneous condition on the basis of morphology and bilirubin turnover. Electron micrographs of the hepatocyte in patients with Gilbert's syndrome show two distinct sub-populations, one with normal morphology and the other showing changes in the structure of the smooth endoplasmic reticulum (Dawson et al, 1979a; Dawson et al, 1979b). Zeneroli and her colleagues sub-divided the syndrome by differences in bilirubin turnover with one group presenting with decreased hepatic uptake and conjugation but normal turnover and the other group showing normal uptake, reduced conjugation and increased turnover (Zeneroli et al, 1982). Simultaneous studies of the two parameters has not yet been undertaken. In most patients

other liver function tests are normal and no treatment is necessary, but it has been shown that treatment with phenobarbital results in a decrease in serum unconjugated bilirubin (Kawasaki et al, 1982) presumably by affecting UDP-GT activity although this has not been proven (Felsher et al, 1973). In rats the mutant Gunn rat displays a defect in conjugation of bilirubin and it has been suggested that this is analagous to Crigler-Najjar syndrome Type I. The congenital hyperbilirubinaemia which the rat suffers from does provide a useful model for study of hyperbilirubinaemia in an in vivo system.

1.6 AIMS OF THE THESIS

Unconjugated bilirubin is potentially toxic within the body as has been shown in the introduction of the thesis. In infants, excess bilirubin can gain access to the brain where it is preferentially retained and results in brain damage. *In vitro*, bilirubin has been shown to cause disruption of mitochondrial reactions of cells and is known to adversely affect membrane function. In brain, cerebellar Purkinje cell hypoplasia has frequently been observed in the presence of bilirubin but it is still unclear whether this is a direct effect of deposition of the bile pigment. In fact the mechanisms by which bilirubin exerts its toxic effects are still not proven.

Recent *in vitro* evidence has shown bilirubin is a competitive inhibitor of the enzyme PROTO-O. A similar *in vivo* role of the bile pigment is supported by the finding of a reduced leucocyte PROTO-O activity in humans suffering from the mild unconjugated hyperbilirubinaemia of Gilbert's syndrome. The significance of bilirubin as a competitive inhibitor of PROTO-O activity has not been assessed.

My intention was to study the effects of the presence of bilirubin *in vivo* on the haem biosynthetic pathway particularly with reference to any effects on PROTO-O activity. Use of an animal model allowed study of the enzyme activities within body tissues. The ultimate aim was to determine whether bilirubin in the brain of these animals may be disrupting normal haem biosynthesis in this tissue.

This involved several steps:-

1) Finding a suitable animal model of hyperbilirubinaemia.

2) Characterising haem biosynthesis in accessible body tissues of this animal to establish if bilirubin has any effects on haem biosynthesis.

3) Development of accurate assays for the haem biosynthetic enzymes of interest in the brain and a sensitive method for measurement of brain levels of bilirubin.

4) Production of a state of kernicterus in the hyperbilirubinaemic animal model.

5) Measurement of bilirubin content in the brain of these icteric animals and measurement of PROTO-O and ALA-synthase activities in this tissue in order to determine if disruption of the haem biosynthetic pathway in the brain occurs as a result of this condition.

It was hoped that these studies would provide an insight into one particular mechanism by which bilirubin causes brain toxicity.

In addition to these studies manipulation of haem degradation by disrupting bilirubin synthesis was studied within an unrelated system. From the literature, bilirubin formation has been shown to be inhibited over long periods of time with Sn-PROTO, and this has been discussed in the introduction. Sn-PROTO has previously been used in control of hyperbilirubinaemia. Relating to the haem biosynthetic pathway, prevention of bilirubin formation would increase haem content. This would provide a novel way of treating disease states where haem deficiency occurred ie the porphyrias, and I decided to study this in addition to the other work as my interest had been stimulated. Investigations were undertaken with an animal model to determine the effectiveness of Sn-PROTO as a therapeutic agent in acute hepatic porphyria, both alone and in combination with the traditional haem arginate therapy.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

This chapter contains a description of the materials and methods used to construct the thesis. Two sections are included, the first describing the materials and equipment used and the second the methodology. All the methods used are explained, at least in summary, with those adapted from original references described in full.

2.1 MATERIALS AND EQUIPMENT

2.1.1 Materials

Radioisotopes: [2,3-^{14}C] succinic acid, [4-^{14}C] ALA, [2-^{14}C] glycine were obtained from Amersham International plc, Amersham, U.K.

Biochemicals: ALA, antifoam concentrates A and C, ascorbic acid, bilirubin, coenzyme A, coproporphyrin III tetramethyl-ester, dimethylsulphoxide, 4,6-dioxo-heptanoic acid, EDTA, ethyl acetate, glutathione, glycine, 1-heptanesulphonic acid, human albumin fraction V, magnesium chloride, mercaptoethanol, potassium chloride, potassium hydrogen phthalate,potassium phosphate (monobasic and dibasic), protoporphyrin IX dimethyl ester, pyridoxal phosphate, sodium acetate, sodium bicarbonate, sodium malate, sodium nitrite, succinate, succinic thiokinase, sulphanilic acid, Trizma hydrochloride, Trizma base, trichloroacetic acid were all obtained from Sigma. Haem arginate was obtained from Leiras. Di-n-octylamine was obtained from Aldrich. Ethylacetoacetate was from Fluka-Garantie. Mercury and sodium were from BDH. Sn protoporphyrin, coproporphyrin and porphyrin methyl esters were obtained from Porphyrin Products. Para-dimethylaminobenzaldehyde (p-DMAB) was from Merck.

Thin layer chromatography plates were obtained from Merck.

2.1.2 Equipment Used in Analyses

Colorimetric Analyses: Spectrophotometric measurements were made on a Pye Unicam SP8-200 UV/vis double beam spectrophotometer, spectrofluorometric detection was carried out on a Perkin Elmer 3000 Fluorescence spectrometer.

Blood Measurements: A zinc haematofluorimeter was used to measure blood porphyrins. A microhaem centrifuge plus haematocrit measurement apparatus was used to measure haematocrit.

HPLC: Two systems were used. For gradient bilirubin separation and porphyrin separation a Hewlett Packard system consisting of a 1084B Liquid Chromatograph, a 79831A injector, a 79850B LC terminal and a Gilson Holochrom detector was used. For isocratic bilirubin separation and ALA pyrrole measurement the system consisted of either a BIO-RAD pump or a Waters pump, a Waters U6K injector or a rheodyne injector, a Gilson holochrom detector and a Gilson fraction collector.

Homogenisers: Where preservation of an enzyme was important a glass Potter Elvehjam vessel and motor powered teflon pestle were used. The alternative method used a polytron homogeniser and included sonication in the homogenisation process.

Centrifuges: For preparation of tissues a refrigerated MSE Mistral 4L, followed by either an MSE Superspeed 75 ultracentrifuge or a Beckman L2-65B ultracentrifuge was used. To extract plasma or serum from blood an eppendorf centrifuge or refrigerated Heraeus Minifuge GL was used depending on volumes.

Liquid Scintillation Counting: a Canberra Packard Tricarb scintillation counter was used.

2.2 METHODS FOR PREPARATION OF TISSUE FOR ASSAY

Rats were used in all experiments. Adult rats were defined as those 2 months of age or older. The young rats were aged 15 days in most cases although variations of 1 or 2 days did sometimes occur.

2.2.1 Strains of Rat Used

Either Wistar or Sprague Dawley rats were used in development of enzyme assays, and in some cases as controls. Gunn rats were used as test animals. These were either bred in the departmental animal house (see Chapter 3) or obtained from the Royal Free hospital, London by kind permission of Professor B. Billing. Gunn x Wistar cross rats were bred in the departmental animal house from Gunn male and Wistar female parents, and these rats were used as control animals in the majority of the experiments.

2.2.2 Blood Sampling

Serum bilirubin levels were determined in live Gunn and control rats. Animals were anaesthetised by inhalation of ether until unconscious. The tail vein was heated and a small nick made at the end of the tail. Blood was removed into either a capillary tube for determination of whether the rat was a homozygous or heterozygous Gunn or an eppendorf tube when measurement of the serum bilirubin level was to be made.

2.2.3 Sacrifice of Animals and Blood and Tissue Removal

All animals were deeply anaesthetised by inhalation of ether.

(i) Blood Removal: the abdominal cavity was opened and blood removed from the hepatic vein in adult animals. In young animals after opening the abdominal cavity the diaphragm was cut to expose the descending aorta, from which blood could be removed.

(ii) Liver: after opening of the abdominal cavity the liver was excised and perfused with ice cold saline via a syringe inserted into the junction of the hepatic vein and liver. In the case of young animals perfusion was still carried out, despite difficulties in keeping vein and arteries intact under pressure of perfusion.

(iii) Kidneys: these were removed after the liver and as before, perfused with ice-cold saline using a needle in adult and young animals.

(iv) Brain: in these animals the ribcage was opened and the descending aorta clamped. The right atrium was punctured with a needle. Perfusion was achieved by a catheter inserted into the left ventricle flushed with ice-cold saline. The saline then penetrated the brain via the blood vessels. When the solution running from the right side of the heart was clear (approx 1ml per gram body weight) perfusion was complete. After exposing the skull the neck was snapped with brain clippers and the skull chipped away between the hemispheres of the brain. Care was taken not to damage the tissue. The whole brain could then be eased out with a needle and kept on ice until use.

All tissues were kept at 0-4^{o}C until enzyme assays could be

carried out.

2.2.4 Tissue Preparation for Enzyme Assays

(i) Homogenates: tissue in ice cold buffer was diced with scissors and homogenised using a polytron homogeniser for 30 seconds (includes sonication). The homogenate was then ready for use in assays.

(ii) Mitochondria: tissue in an appropriate ice cold buffer was homogenised using a Potter Elvehjem glass homogenising vessel with motor powered teflon pestle. The resulting homogenate was then centrifuged at 1000xg at 4°C for 10 minutes to remove tissue debris and the supernatant decanted into ultracentrifuge tubes to be centrifuged at 25 000xg at 4°C for 20 minutes. The supernatant from this spin was discarded and the pellet containing mitochondria kept on ice until it could be resuspended in assay buffer* using a Potter-Elvehjem homogeniser with teflon pestle. Volume for resuspension varied from tissue to tissue, with volume equal to weight favoured for liver and kidney and volume half original weight used for brain. Enzyme assays were then carried out after sonication of resuspended samples for 3 bursts of 5 seconds at 15 microns.

* In some cases tissues were prepared in 0.25Molar sucrose. Otherwise tissues were prepared in the appropriate buffer for the enzyme assay. These are listed under the methods for each enzyme assay.

(iii) Microsomes: samples were prepared exactly as for mitochondria but in 0.25 Molar sucrose as buffer. After centrifugation at 25 000xg the supernatant containing the microsomes was decanted into clean ultracentrifuge tubes on ice and centrifuged at 108 000xg at 4°C for 60 minutes. The resulting pellet contained the microsomes and could be resuspended in the appropriate assay buffer.

(iv) Preparation of Enzyme Inactivated Tissue: a batch of animals were sacrificed and tissues removed as before. Homogenates or mitochondria were prepared as previously noted. Deterioration of enzyme activity was achieved by either of two means:-

a) Heat Denaturation: prepared tissue was heated in a water bath to temperatures of 65 to 75°C. This caused denaturation of the enzyme without altering protein conformation, and largely prevented non-enzymic oxidation of PROTO'gen to PROTO in the PROTO-O assay.

b) Freeze Thaw Denaturation: prepared tissue was repeatedly subjected to -20°C and mild heat (50°C).

Tissue treated in either of the above two ways was then stored at -20°C in aliquots and thawed for use as tissue blanks in enzyme assays. The method of choice was that of heat denaturation since the freeze thaw technique seemed to be less effective at destroying enzyme activity.

2.2.5 Collection of Urine and Faeces

Individual rats were kept in metabolic cages for 24 hour periods. Over this period urine and faeces were collected and kept dark to prevent any deterioration of haem precursors which occurs on exposure to light.

2.3 MEASUREMENT OF ENZYME ACTIVITIES

2.3.1 5-Aminolaevulinic Acid Synthase (E.C.2.3.1.37)

Two assay methods were used to measure ALA-synthase in both tissue homogenates and mitochondrial suspensions. Both of these were radiochemical assays where a radioactive precursor was incubated under optimal conditions for production of ALA, so generating a radioactive product. The radiolabelled ALA product could then be quantitated.

i) [2-^{14}C] glycine was used as substrate. The method was adapted from that of Freshney and Paul (Freshney & Paul, 1970).

Tissue obtained was homogenised in 4 volumes of a buffer made up of two parts saline to one part "incubation buffer" containing 500mM sucrose, 25mM magnesium chloride, 0.2mM EDTA, 8mM mercaptoethanol, 1mM glycine, and made up in a 50mM potassium phosphate buffer to pH7.0. 200µl was then added to 200µl [2^{14}C] glycine dissolved in a buffer containing 50mM glycine, 500mM sodium malate, 2mM magnesium chloride and 0.4mM pyridoxal phosphate in a 50mM potassium phosphate buffer pH6.8 (contains 2.5µCi per tube). The 400µl sample was incubated without shaking at 37^{o}C for one hour and the reaction stopped by adding one tenth (40µl) 40% TCA containing 6mMolar non-

radioactive or "cold" ALA. After sonication for 15 seconds at 15 microns the samples could be stored at -20^{o}C until high voltage electrophoresis which was always within 2 days of assay.

The ALA formed was separated from contaminants by high voltage electrophoresis. Samples were spotted onto chromatography paper and dried. The paper was soaked in 5mM potassium hydrogen phthalate and blotted. High voltage electrophoresis was for 30 minutes at 3000 volts. After drying the ALA spot was identified using ninhydrin spray, isolated and counted in a β counter either after combustion with a sample oxidiser and combination with scintillant or by direct addition of the ALA containing spot to scintillant. ALA formed was calculated per protein, using the DPM's in the glycine spot as a standard.

ii) [2,3^{14}C] succinic acid was used as substrate. The conditions used in the original method by Fitzsimons (Fitzsimons et al, 1984) were checked for optimal brain ALA-synthase activity (see chapter 3).

Brain homogenates or mitochondria were prepared as in section 2.2.3 in ALA-synthase buffer containing 250mM sucrose, 5mM $MgCl_2$, 45mM glycine, 40mM tris base, 2mM EDTA in distilled water. For optimum pH, 50mM potassium dihydrogen orthophosphate was added to half the buffer and this used to pH the rest to pH 7.5 at 37^{o}C.

When liver or kidney tissue ALA-synthase activity was to be measured, the ALA-synthase buffer was exactly as that for brain measurement except for concentration of glycine which was raised to 100mM.

400µl of tissue in ALA-synthase buffer was added to 50µl 2mM 4,6-dioxoheptanoic acid in ALA-synthase buffer plus 2.5 µCi [2,3^{14}C] succinic acid dissolved in 20mM succinate solution. After vortex mixing the samples were incubated for 1 hour at 37^{o}C without shaking. Tissue blanks were regularly run. The reaction was stopped with 250µl ice-cold TCA (10%) and the sample cooled in ice. 4mM ALA (50µl) was then added for identification of the ALA pyrrole peak followed by 500µl 1M sodium acetate. After mixing the samples were spun in an eppendorf centrifuge. The supernatant was removed with a pasteur pipette and the pellet discarded. For formation of the pyrrole, 50µl ethylacetoacetate was added. Samples were loosely capped and heated to 100^{o}C for 20 minutes. After cooling in ice 2.5ml distilled water was added to dilute the sample for purification. This ALA-pyrrole could be stored overnight if necessary in the dark at 4^{o}C.

Determination of recovery in the samples was necessary due to the extensive manipulation and this was achieved using [4^{14}C] ALA. 2.5µCi radioactive ALA was added to incubation buffer, cold ALA, TCA and sodium acetate as before. The pyrrole was made by addition of 150µl ethylacetoacetate.

Sample blanks contained either no tissue (buffer only) or tissue after inactivation of enzyme, and all components of the samples.

The pyrrole was isolated from radioactive and structural contaminants by Sep-pak ODS cartridges (Waters). Cartridges were activated with acetonitrile then methanol. Addition of

water provided a favourable environment for application of ALA pyrrole. The pyrrole is preferentially retained. After drying the pyrrole was eluted with methanol. The samples were then dried down under N_2 and resuspended in 1:1 methanol:water (100µl). Half of this was injected into a ODS reverse phase HPLC column. The system was isocratic with a mobile phase consisting of 37:63 parts methanol to water and containing the base pairing agent heptane sulphonic acid at a concentrationof 5mM dissolved in 20ml per litre 1N acetic acid. Solvent was always filtered and degassed before use. On elution from the HPLC column fractions of the peak and those surrounding were collected. After addition of scintillant these were counted. Recovery was assessed by using [4-^{14} C] ALA which had been under identical conditions to the samples.

Calculation: Background DPM's were subtracted from the fractions containing the peak. The resulting figure was corrected for recovery (calculated from [^{14}C] ALA sample) and total volume of sample. To convert DPM to pmoles ALA in the sample the specific activity of the [2,3^{14}C] succinic acid and concentration of cold succinate had to be considered.

$$\text{pmoles ALA formed per hour} = \frac{\text{corrected DPM in ALA pyrrole}}{\text{DPM per nmol [}^{14}\text{C] substrate}}$$

enzyme activity could then be calculated per milligram protein.

Method i) was the first method to be used and the activity of ALA synthase was measured in liver and kidney samples, both in homogenates and mitochondria. However it soon became clear

that this method was not producing reproducible results for brain tissue, and therefore the Fitzsimons method was tried and provided an alternative which gave comparable results in both liver and brain as is shown in Chapter 3. This method was adopted for all tissues. All results were reported in pmol ALA produced/mg tissue/hour.

2.3.2 5-Aminolaevulinic Acid Dehydratase (E.C. 4.2.1.24)

Measurement of ALA-D activity is achieved by quantitation of the product of the reaction, PBG. Tissue preparations containing the enzyme activity were incubated with exogenous ALA for one hour and the PBG produced reacted with para-dimethylbenzaldehyde (p-DMAB) to produce a pink compound which is measured spectrophotometrically at 555nm.

For rat liver samples 2g of tissue was homogenised in 6ml 100mM sodium phosphate buffer pH 6.8 using polytron homogeniser (with sonication). 200µl was then added to three identical tubes containing 1.3ml water at 37°C. After equilibrium at this temperature was reached the reaction was started by addition of 1ml of 10mM ALA dissolved in sodium phosphate buffer as before. Immediately a zero time sample was taken by stopping the reaction in one of the triplicate samples with 10% TCA. The remaining duplicate samples were run for 1 hour and then the reaction stopped with 1ml of 10% TCA as for the zero time. The precipitated protein was centrifuged at 1 500xg for 15 minutes to remove turbidity and the clear supernatant containing PBG decanted and added to an equal volume of

Ehrlich's reagent (p-DMAB)*. Development of colour took 5 minutes and the colour in the samples read against zero times. Activity was expressed in μmoles PBG/g protein/hour.

Calculation

$$\mu\text{mol PBG/g protein/h} = \frac{\text{OD} \times 18.81 \times 60}{\text{protein (mg/ml)}}$$

ALA-D activity is greatly inhibited in the presence of lead. To prevent the possibility of any contamination all glass was acid washed and the assays were carried out in plastic tubes.

* Ehrlich's Reagent: 2.5g pDMAB was added to 30ml glacial acetic acid. 24.5ml perchloric acid was added and the solution made up to 100ml with more glacial acetic acid. The solution was always made up fresh, on the day of use and used within one hour usually.

2.3.3 Coproporphyrinogen Oxidase (E.C.1.3.3.3)

Measurement of the enzyme COPRO-O is achieved by quantitating COPRO produced fluorimetrically. In this reaction COPRO'gen substrate is added to the tissue preparation containing enzyme. The method used was modified from that of Batlle (Batlle et al, 1965). Conversion of COPRO to COPRO'gen requires the action of a strong reducing agent. For these experiments a 3% sodium mercury amalgam was used. 3g non-oxidised sodium was diced and heated under toluene until the sodium appeared as silver liquid spheres. Mercury (2.5ml) was added slowly with careful mixing and the amalgam allowed to cool. The toluene was decanted and amalgam washed with petroleum ether (40-60^{o}C). After carefully drying the solid block was ground to a fine powder with a mortar and pestle and stored in a sealed glass boiling tube. Amalgam prepared in this way was active for one to two weeks, longer if stored under nitrogen. The pH of amalgam is very high (pH >11) and so before addition to tissue amalgam is filtered out from the substrate using a sinter glass funnel and the pH returned to the range of the assay with 40% and 10% MOPS acid.

For each COPRO-O assay tissue homogenate or mitochondria were prepared and resuspended in 0.15M KCl. 400ul of the resuspended mitochondria were added to a 10ml conical flask containing 1ml 100mM Tris buffer pH 7.4. After mixing, the solution was placed in a water bath at 37^{o}C. Coproporphyrinogen solution was prepared by diluting stock coproporphyrin standard, kept at 4^{o}C in 10mM KOH, with more 10mM KOH to a final concentration of 100mM. This solution was added to sodium amalgam and shaken

for a few minutes until no fluorescence was visible under a UV light ie COPRO'gen had been formed. After correction of the pH to approx pH 7, 1ml of this substrate was added to the diluted tissue preparation in the water bath and a one hour incubation carried out in the dark. The final concentration of substrate in the reaction was 50mM. The reaction was stopped by addition of 5ml 4:1 ethyl acetate/acetic acid. Samples were stored at $-20^{o}C$ until extraction of coproporphyrin could be carried out.

Extraction of Coproporphyrin: The contents of the conical flasks were transferred to separating funnels and after addition of more 4:1 ethyl acetate/acetic acid to rinse the flask, washed with saturated sodium acetate. All porphyrins were extracted using 15% HCl, and after returning the pH to neutral with sodium acetate, ether was added. Water was then added to remove any remaining acid and the coproporphyrin extracted from the organic phase using 0.1N HCl. Protoporphyrin was extracted using 5% HCl. The acid extractions were filtered and read on a fluorometer set at wavelengths 395nm excitation and 595nm emmission against a coproporphyrin standard.

Calculation

$$\frac{FU2 * 4.2 * V2}{Protein * V3}$$

where FU2 = fluorescence reading of acid extracted volume
V2 = acid volume obtained
V3 = volume of tissue homogenate added (in ml)

The results were expressed as nmol PROTO/mg protein/hour

2.3.4 Protoporphyrinogen Oxidase (E.C.1.3.3.4)

The principle of the PROTO-O assay is similar to that of the COPRO-O assay. The substrate PROTO'gen is produced using a strong reducing agent then added to tissue preparation containing enzyme activity and formation of the product PROTO quantitated. One important difference is the high level of non-enzymic conversion of PROTO'gen to PROTO which occurs on exposure to light which means the assay has to be carried out in darkness. Levels of product are measured directly fluorometrically. Immediately after addition of substrate at 37°C in the dark, an aliquot is removed and used as a zero time. At ten minute intervals further aliquots are removed and the change in fluorescence is proportional to the amount of PROTO produced, providing a measure of PROTO-O activity when related to protein content.

All assays were carried out on mitochondria which had been resuspended in the appropriate volume of 0.15M KCl which resulted in protein contents of not greater than 2mg/ml. It was found that addition of 20µl/ml of the detergent Tween 20 before sonication minimised the effect of different concentrations of protein. Mitochondrial suspensions of tissue were sonicated immediately before addition of 400µl to 1.1ml 50mM Tris buffer pH 9.2 for liver, kidney and brain. The reaction vial at 37°C contained 0.3mMolar glutathione (GSH), a reducing agent, to help prevent autooxidation of the substrate. A reagent blank was set up containing 400µl Tris buffer instead of tissue suspensions to measure residual non-enzymic oxidation of protoporphyrinogen to protoporphyrin (PROTO). A tissue

blank was also used, containing killed tissue see section 2.2.3 part iv). This accounted for any PROTO produced by remaining oxidising agents present in the tissue preparation.

The substrate, PROTO'gen was prepared by adding 0.4mM PROTO in N/7 sodium bicarbonate (approximately 5ml) to 6g sodium amalgam on ice in the dark and shaking for between 2 and 5 minutes, until no fluorescence was visible under UV illumination. The clear PROTO'gen was then filtered using a glass sinter funnel and the pH brought down to approx pH 9.0 using either 10% or 40% MOPS acid. 100µl of the substrate was then added to the tissue /buffer mixtures at 37°C and the tubes were vortexed. The concentration of substrate was determined to be above the Km for PROTO'gen. Immediately a 50µl aliquot was diluted with 3.1ml 50mM Tris buffer again containing GSH. This gave a manageable reading on the fluorometer. Fluorescence in this solution was determined in a spectrofluorometer at wavelengths Ex 405nm Emm 635nm against a COPRO standard. The fluorometer contained a red filter to help in sensitivity of determining the colour of the forming PROTO. Slits were set at 10nm. The 50µl sampling procedure was repeated after 10 minutes in brain tissue and 20 minutes in liver and kidney mitochondria. The change in fluorescence readings reflected the PROTO'gen oxidase activity.

Generation of PROTO for substrate: protoporphyrin methyl ester is the most stable form of PROTO. 1mg aliquots were hydrolysed with 10 drops of a 70% solution of HCl overnight in the dark in

a sealed container. The resultant PROTO acid was freeze dryed and storing at -20°C. Prior to the assay, one aliquot was thawed in the dark and N/7 sodium bicarbonate solution was added to dissolve the PROTO. This gave a concentration of approximately 0.4mMolar. The dark red PROTO was then added to amalgam and converted to PROTO'gen as described earlier in the section.

Calculation

a) (FU7 * .0328 * 1450/50) - (FU6 * .0328 * 1500/50) represents the change in fluorescence in the sample. FU7 = final fluorescence value and FU6 = initial fluorescence with .0328 a constant for the standard and 1500/50 and 1450/50 representing the volume of the reaction vessel in ul before and after sampling.

b) (Q6E * .0328 * 1450/50) - (Q6F * .0328 * 1500/50) represents the change in fluorescence in the blank sample (autooxidation). Q6E = final fluorescence value and Q6F = initial fluorescence value. The other constants are as above.

b) was subtracted from a) and a correction applied for
The result was divided by (.4 * T6 * 562.7 * protein) where .4 = volume of homogenate added, T6 = time of incubation and 562.7 is the formula weight for PROTO.

The final result was expressed as nmol PROTO/mg protein/hour.

2.4 PORPHYRIN AND PRECURSOR MEASUREMENTS

2.4.1 5-AMINOLAEVULINIC ACID IN URINE

Measurement of ALA levels in urine was carried out using kits supplied by BIO-RAD. The details of the method are available in the BIO-RAD Laboratories Technical Bulletin No.4208 (1979). In principal, urine at pH 5-6 (altered with glacial acetic acid) which has had PBG removed was dripped onto a column containing a cation exchange resin. ALA was then retained on the column while urea and other contaminants were washed through with water. Elution of ALA followed by a period of condensation with acetylacetone resulted in a pyrrole derivative which produced a coloured compound on reaction with pDMAB (Ehrlich's reagent for urine)**. Quantitation was carried out on a spectrophotometer, with results being calculated from the molar extinction coefficient of the complex at 555nm (mM=62), and reported as either nmoles ALA excreted per litre for spot samples or pmol ALA per 24 hours for 24 hour collections of urine.

** Ehrlich's Reagent for Urines:

1mg pDMAB was added to 8ml perchloric acid and the volume made up to 50ml with glacial acetic acid. This solution was prepared fresh on the day of assay and used within 3 hours.

2.4.2 Porphobilinogen in Urine

PBG was measured in urine by kits from BIO-RAD, with details provided by BIO-RAD Laboratories Technical Bulletin No.4208 (1979). Acidified urine (pH 5-6) was passed through an anion exchange column and PBG was retained. After elution Ehrlich's reagent for urines (see previous page) was added and the coloured compound produced quantified as in 2.4.1. Results were reported as nmoles PBG excreted per litre or pmol PBG per 24 hours as appropriate.

2.4.3 Total Porphyrin in Urine

Total porphyrin was measured in urine using kits from BIO-RAD Laboratories. The procedure followed was adapted from BIO-RAD Laboratories Technical Bulletin No.4003 (1981). Columns were shaken to resuspend the ion exchange resin and allowed to drain. After the resin had settled the columns were rinsed with 10ml distilled water. After draining, 1ml urine (0.5ml if total porphyrin was expected to be very high and so saturation of the column was likely to occur) was added and allowed to drain before 10ml distilled water was drained through the column to remove any interfering chemicals. Porphyrins were eluted with 2 aliquots of 2ml 3M HCl and the fluorescence of the sample determined at wavelengths 396nm excitation and 594nm emission. The concentration of total porphyrin was calculated against a coproporphyrin standard of known concentration. Results were reported as µg porphyrin excreted per litre or 24 hours as appropriate.

2.4.4 Porphyrin Excretion by HPLC

By using highly sensitive HPLC techniques it is possible to separate the different porphyrins which are excreted in urine - URO, hepta, hexa, and penta-carboxylic porphyrins, coproporphyrin and protoporphyrin. Two types of assay have been developed, one using free porphyrins and the other relying on derivatisation of the porphyrins to their corresponding methyl esters. The free method requires less preparation but run times are lengthy and preparation of a reliable set of standards difficult. For these reasons, only the porphyrin ester method was employed in measurement of porphyrins in rat excreta.

i) Preparation of rat urine

20ml of rat urine from pooled 24 hour urine collections was adjusted to approximately pH 6 with glacial acetic acid. 1g of talc was added and the mixture left to stir in the dark for 15 minutes. Talc absorbed the porphyrin and was then dried firstly in a Buchner funnel and then more thoroughly by baking at 90^{o}C for 1 hour. Addition of 15ml methanol/conc. sulphuric acid mixture (95/5 v/v) stripped the porphyrins from the talc into solution. Both the porphyrin containing solution and talc were left in the dark at room temperature for 16 hours. This allowed formation of porphyrin esters. Any residual porphyrin ester remaining on the talc was removed by addition of 2 x 5ml aliquots of chloroform. These were added to the original methanol/sulphuric acid solution followed by 10ml water to separate the organic and aqueous phases. The aqueous phase was discarded and the water wash repeated. 3% sodium bicarbonate

was then added and the solution washed with another two aliquots of water. The resulting chloroform phase was shaken with anhydrous sodium sulphate (0.1g) and filtered before evaporating to dryness under air. The porphyrin ester residue was dissolved in 20µl chloroform and 800µl mobile phase for HPLC added. After a final filtration step through 0.2µm filters (Gelman Sciences) a portion of the sample was injected. Sample injection volumes varied with the type of sample. For Gunn rats 50µl injections were required while control rat porphyrin esters could be resolved after injection of only 10µl. Samples were quantitated against porphyrin methyl ester standards (Porphyrin Products, Utah, USA).

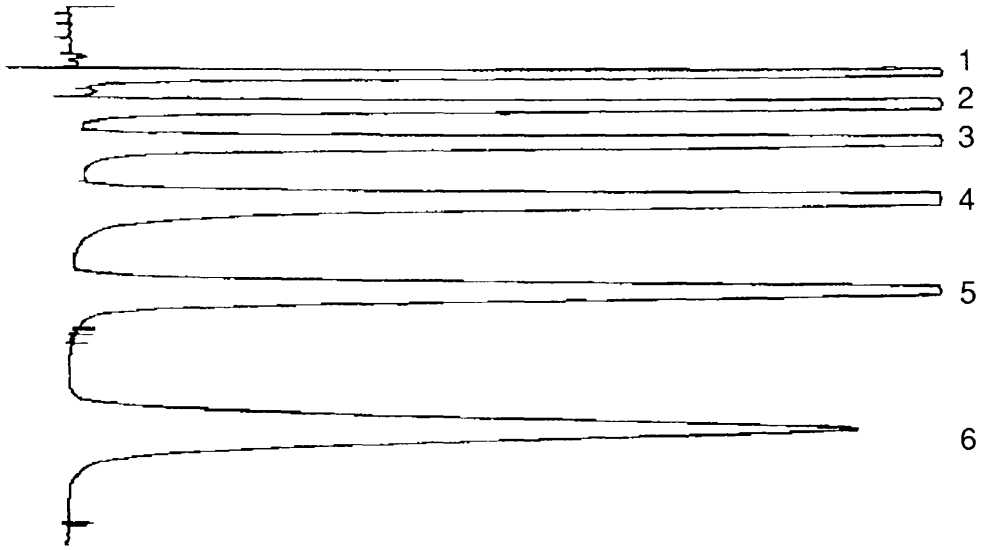
ii) HPLC of porphyrin methyl esters

Chromatography was performed on the Hewlett Packard system described in section 2.1.2. with a silica column (200 x 46mm) and solvent containing n-heptane/ethylacetate/chloroform/methanol (60:25:12.5:2.5) at a flow rate of 1.2 ml/min. Detection was by fluorescence at Ex. 405nm Emm. 625nm. An example of a chromatogram from a standard run is shown in Figure 15.

2.5 PORPHYRIN MEASUREMENT IN FAECES

Rats were confined to metabolic cages for 24 hours and the collections of faeces kept dark. Faecal matter was homogenised and porphyrins were extracted with acid. Faecal coproporphyrin and protoporphyrin were quantitated against a coproporphyrin standard of known concentration at Emm. 594nm with Ex. set at 396nm.

After a 24 hour period of collection the total amount of



1 = PROTO, 2 = COPRO, 3 = PENTA, 4 = HEXA, 5 = HEPTA, 6 = URO (MESO)

FIGURE 15 : TYPICAL HPLC TRACE OF STANDARD SET OF PORPHYRIN ESTERS

faeces was weighed and homogenised in approximately half the w/v water with the polytron homogeniser. Porphyrins were then extracted from a 1-2g aliquot. A small proportion was removed for wet weight/dry weight estimation. This was weighed then baked for at least 6 hours at 100^{o}C and reweighed.

Glacial acetic acid, 5-10ml, was added to the aliquot for extraction and then this made up to a 10% solution with ether. After thorough mixing deposits were removed by centrifugation at 1000xg for 10 minutes. Porphyrin present could then be identified under UV illumination in the supernatant and the pellet was discarded. The suspension was transferred to a separation flask for extraction of porphyrins. The solution was washed twice with 3% sodium acetate, the first wash being used to rinse the discarded container minimising porphyrin loss and the aqueous phase discarded each time. 20ml of a 0.005% solution of iodine (freshly made up) was added to oxidise any PROTO'gen and again the aqueous phase discarded. After a further wash with water, porphyrins were extracted from the remaining organic phase with acid. Faecal coproporphyrin was extracted using 10ml aliquots of 0.1N HCl (until the final aliquot showed no fluorescence) and protoporphyrin was extracted from the remaining solution with 5% HCl (5ml aliquots). Both acid fractions were volumed, filtered with Whatmans No 1 filter paper and read against a coproporphyrin standard at the wavelengths already mentioned.

HPLC of faecal samples

A known weight of faeces was added to excess methanol/-sulphuric acid (95:5 v/v). This mixture was stirred to extract porphyrins and then left standing to enable esters to form. The resultant mixture was filtered as for the urine and the residue washed with chloroform. The porphyrin esters were then prepared and applied to an HPLC column in a manner identical to that of urine.

2.6 CYTOCHROME P450 MEASUREMENT

In the liver cytochrome P450 is the major product of haem formed. Measurement of this haemoprotein is likely to detect any disturbances in haem synthesis in hepatic tissue. Measurement of cyt. P450 was carried out in microsomal preparations of rat hepatic tissue by a modification of the method of Omura and Sato (Omura & Sato, 1964). Identification of cyt. P450 was by the difference spectrum recorded at 450nm on reaction of the microsomal preparation with carbon monoxide (CO).

Microsomes were prepared in 0.15M KCl to minimise haemoglobin adsorption as described in section 2.2.4. Pelleted microsomes were resuspended in potassium phosphate buffer using a Potter-Elvehjem glass homogeniser with teflon pestle to a solution containing between 2-4mg/ml protein. This solution was split evenly between two glass cuvettes path length 1cm. The test cuvette was bubbled with CO for 10 seconds then 5mg sodium dithionite added to both the test and blank cuvettes and mixed. The test cuvette was then bubbled with CO for a further 10 seconds and mixed and the difference spectrum recorded in a

double beam spectrophotometer over the wavelength range 400nm to 500nm.

Calculation

$$\frac{\Delta E_{450-495} * 3.2 * 1000}{91 * 1 * \text{protein content (mg/ml)}}$$

where Δ E is the difference in optical density between the peak at 450nm and the trough at 495nm

2.7 DETERMINATION OF CONJUGATED AND TOTAL BILIRUBIN IN SERUM/PLASMA

Several laboratory methods have been developed which measure bilirubin in serum. These are all variations of the Van den Bergh reaction where bilirubin is coupled to diazotized sulphanilic acid to form azobilirubin. This compound is strongly coloured in strong acid or alkali and adaptations produced the two most common laboratory methods which allowed colorimetric quantitative analysis, the Malloy and Evelyn method and the Jendrassik and Grof method. Two colorimetric reactions occurred, the first in aqueous solution was known as direct bilirubin and the second which only coloured after addition of alcohol was called indirect bilirubin. After the discovery that bilirubin is excreted as a water soluble conjugate the direct bilirubin was identified as the diconjugate and the indirect bilirubin as the unconjugated form. Research has shown the Jendrassik and Grof method to be the method of choice in the laboratory (Tietz, 1976) and this was the laboratory method adopted. Plasma samples were also analysed by

autoanalyser to determine bilirubin content. These results were expressed as total bilirubin content. Investigation into the various forms of bilirubin in plasma was also investigated by HPLC methods. Four fractions of bilirubin have been identified in plasma diconjugate, monoconjugate of bilirubin, the unconjugated fraction and a fraction tightly bound to albumin (Lauff et al, 1981). Bilirubin is light sensitive and up to 50% decrease in the pigment can be seen within one hour of exposure to direct sunlight. For this reason, where possible plasma bilirubin determination was carried out soon after blood sampling, and when the plasma/serum samples had been kept dark. Samples are stable in the dark at 4^{o}C for one week and for 3 months if kept at -20^{o}C. All samples were assayed for bilirubin content within this time period.

2.7.1 Plasma Bilirubin Measurement by Autoanalyser

Blood from Gunn and control rats was removed and the plasma removed by centrifugation. Plasma samples were then stored in the dark at -20^{o}C and sent in batches for autoanalysis in another department.

2.7.2 Serum Bilirubin Determination by Jendrassik & Grof Method

Non-haemolysed fresh or frozen serum or plasma was analysed for conjugated (direct) and unconjugated (indirect) bilirubin content. For conjugated bilirubin measurement the sample was added to acid (HCl) then diazo reagent (diazotized sulphanilic acid) added. Only conjugated bilirubin can react under these conditions. The reaction was terminated by ascorbic acid which destroyed excess diazo reagent and the solution made alkaline by

addition of tartrate solution. The strong blue colour was then measured at 600nm in a double beam spectrophotometer against a blank containing caffeine reagent instead of acid and conjugated bilirubin quantitated. For total bilirubin instead of acid solution the sample was added to caffeine reagent which acted as an accelerator for coupling of bilirubin with diazotized sulphanilic acid. The reaction was allowed to proceed for 10 minutes before addition of alkaline tartrate solution and determination of optical density at 600nm against the blank as before.

i) Direct Bilirubin

For rats with elevated serum bilirubin 100µl serum/ plasma was analysed, diluted with 150µl water. For rats with serum-/plasma bilirubin in the normal range up to 250µl could be taken. The plasma was added to 1ml 0.05M HCl, and 250µl fresh diazo reagent added. This was made up and used within 30 minutes and consisted of 10 ml of a solution of sulphanilic acid - 1g in 3ml concentrated HCl, per 200ml added to 0.25ml of a 72mM sodium nitrite solution (ie 1 in 40 v/v). After 1 minute 50µl 0.23M fresh ascorbic acid was added followed immediately by 500µl of a solution containing 100g/l sodium hydroxide and 350g/l sodium tartrate.

The absorbance of this cuvette was measured against a blank containing 1ml caffeine reagent before addition of plasma instead of HCl and sulphanilic acid solution described above instead of diazo reagent.

Total Bilirubin

For total bilirubin 100µl sample was diluted with water to make 250µl total and added to caffeine reagent as for the blank. Fresh diazo reagent was added as for conjugated bilirubin and the azobilirubin product made alkaline for quantitation with tartrate solution. Total bilirubin was quantitated against a calibration curve of concentrations of 0,2,5,10,15,20mg/100ml bilirubin in rat albumin fraction V shown in Figure 16.

For the standards to make up the calibration curve, commercial bilirubin 20mg was dissolved in 1ml DMSO, then 2ml 0.1M sodium carbonate added and the solution made up to 80ml with 4% rat albumin fraction V pH 7.4. This was neutralised with 2ml 0.1M HCl and made up to 100ml.

2.7.3 Serum Bilirubin Determination by HPLC

The laboratory method of determination of bilirubin has proved useful in determining the fractions of conjugated and unconjugated bilirubin in serum and plasma samples. In more recent years it has become evident that there are several bilirubin conjugates, the diconjugate with glucuronide is the most abundant in serum but isomers of monoconjugated bilirubin (glucuronidation at position 8 or position 12) are also present. In addition to the mono and di-conjugated forms of bilirubin, recently a form has been isolated which is covalently bound to albumin. This is known as the delta fraction.

As a consequence of these discoveries more detailed methods capable of determining all the forms of bilirubin in the blood have been developed. HPLC techniques have provided the tool

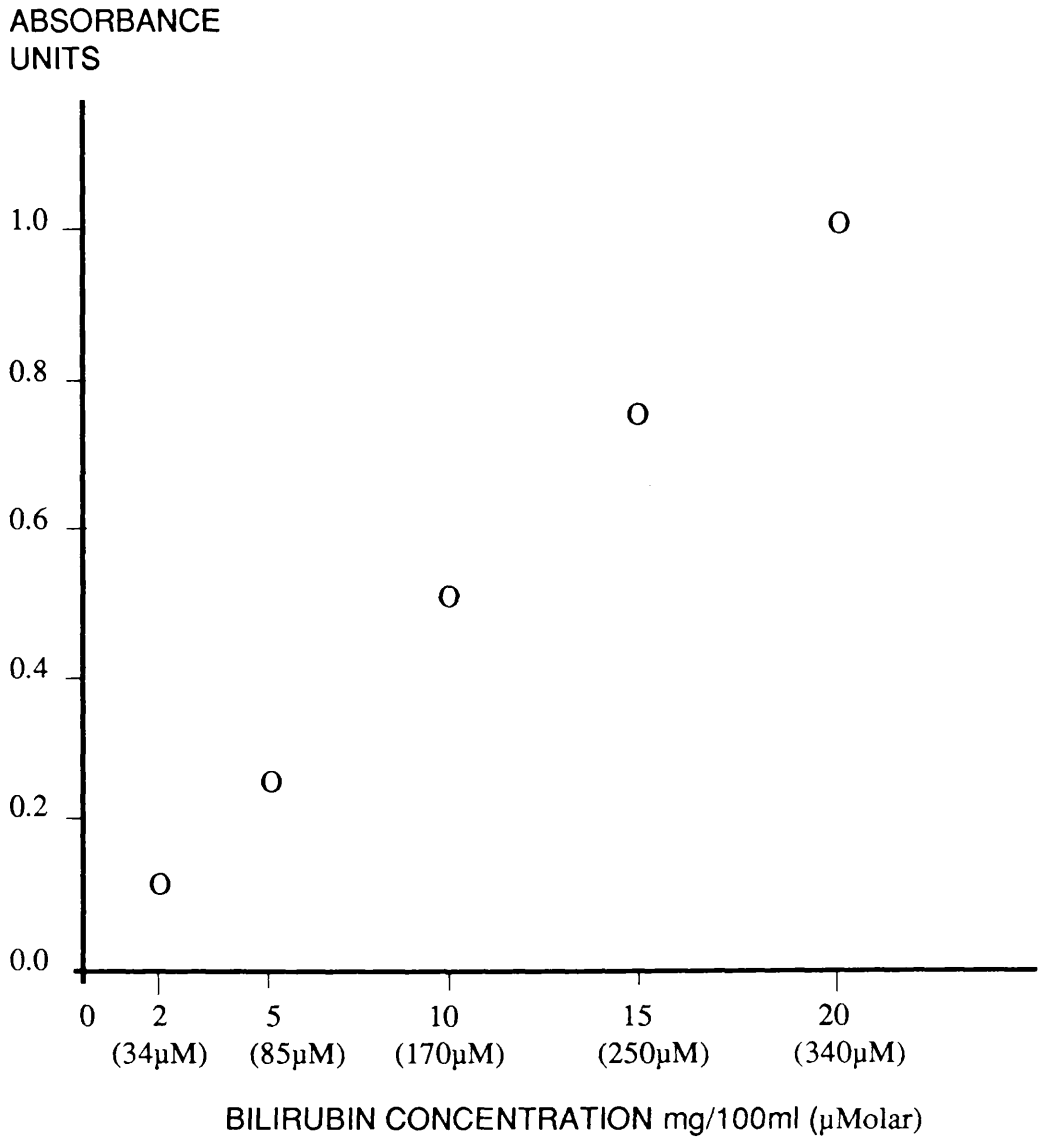


FIGURE 16 : CALIBRATION CURVE FOR DETERMINATION OF SERUM BILIRUBIN

for separation of these forms of bilirubin known as α,β, and γ bilirubin. Two HPLC separation methods were used in these experiments.

a) Gradient Separation of Bilirubin Species

The initial method used was that of Lauff (Lauff et al, 1981), a gradient separation on a reversed phase column (octyl silane, 10μm) maintained at 41^{o}C. Detection of bilirubin species was carried out on a UV/Visible wavelength detector at 450nm. Both components of the mobile phase contained phosphoric acid. The initial mobile phase was aqueous and contained 0.05Molar of 2Molar phosphate buffer and 5% methoxyethanol, in distilled water at pH 2 (adjusted with phosphoric acid). The organic mobile phase contained 5% 2-methoxyethanol in 95% isopropyl alcohol acidified with 2.5% phosphoric acid. A linear gradient was efficient in separating the various forms and the bilirubins were eluted over 16 minutes at a flow rate of 1.4ml/minute. The delta fraction eluted last at 80% organic phase. This high percentage of organic mobile phase was held for 8 minutes to ensure all components had eluted from the column. A 7 minute equilibration period with 100% aqueous mobile phase followed before application of the next sample.

Preparation of Samples

Application of diluted serum/plasma directly onto the column resulted in loss of resolution due to irreversible adherence of large molecular weight protein onto the column so the Yeoman method of deproteination was used. 0.25ml serum/plasma was added to 3.5ml of a 27.7g/l sodium sulphate solution (pH7) at

37°C and shaken for 5 minutes. This caused precipitation of proteins with molecular weight greater than 10^6 daltons. The solution was then passed through a 0.45μm filter (MILLEX) into a 5ml volumetric flask containing 0.5ml 10% ascorbic acid solution prepared in 1:5 of a 2 Molar phosphate buffer pH 5.8. Distilled water made up the volume and the samples were stored at -20°C in the dark or immediately applied to HPLC. Bilirubin standards were serial dilutions of a 20mg/dl bilirubin dissolved in 1ml DMSO, with 2ml 0.1M sodium bicarbonate in 4% human serum albumin (HSA) fraction V pH7.4. The pH was adjusted using 0.1M HCl. Rat and human serum albumin were found to be interchangeable giving similar results.

For application onto the column 0.25ml diluted standard was prepared with sodium sulphate solution as with the samples above

This method allowed clear separation of the various species of bilirubin in Gunn rat serum.

Isocratic Separation

A method has recently been developed for isocratic separation of the species of bilirubin found in serum (McDonagh, personal communication). In this method a small volume of serum is mixed with the mobile phase, which leads to precipitation of protein. After centrifugation to remove the residue, the supernatant is then applied directly onto the octadecyl silane (C18) column. The column temperature is maintained at 31°C. The organic mobile phase consisted of 24.15g/l di-N octylamine mixed with methanol with 6.005g/l glacial acetic acid added. The flow rate was 1ml/minute, and

bilirubin eluted after 8-10 minutes. Addition of a small percentage of water gave better resolution of the different species of bilirubin but with longer run times.

2.8 IDENTIFICATION OF BILIRUBIN BY THIN LAYER CHROMATOGRAPHY

The true nature of bilirubin in brain homogenate samples or in serum samples was determined by thin layer chromatography.

Bilirubin was organically extracted as described in section 2.9.1. The chloroform fraction was concentrated under N_2 gas in subdued light and then developed on a silica gel H plate (Merck) with a chloroform:acetic acid solvent (McDonagh & Assisi, 1971). The Rf values of resultant bands were compared to standard bilirubin from a commercial source.

The silica gel plates were firstly activated by incubating at 120^{o}C for 60 minutes, and allowed to cool before use. The solvent consisted of 1% glacial acetic acid in chloroform. Rf values found for commercial bilirubin had a corresponding band on the samples thought to contain bilirubin in the brain.

2.9 EXTRACTION AND DETERMINATION OF BRAIN BILIRUBIN

2.9.1 Organic Extraction of Brain Bilirubin

Rats were sacrificed as described in section 2.2.3 and the brains perfused and removed. Brains were kept cold and exposure to light was limited as much as possible. Tissue was homogenised in exactly 4 volumes of 0.25 Molar sucrose using a polytron homogeniser. The homogenates were kept in the dark at -80^{o}C until analysis which was always within 3 months of animal sacrifice.

Bilirubin was extracted from homogenates using the method of Katoh (Katoh et al, 1975). 1 volume brain homogenate in sucrose was added to 18 volumes chloroform: methanol: water mixture (2:5:2), and the bilirubin extracted using 10 strokes of a PotterElvehjem glass homogeniser with a teflon pestle. 8 volumes water was then added, the solution vortexed and the layers separated by centrifugation at 3000xg for 15 minutes. The lower chloroform phase, containing bilirubin, was removed and evaporated to dryness under a stream of N_2 gas. The residue was resuspended in mobile phase for injection into HPLC.

2.9.2 Quantitation of Bilirubin By Isocratic HPLC

Coumarin 6 (Kodak) was used as internal standard. 100ng coumarin 6, dissolved in DMSO was added to bilirubin standards and brain homogenates. A standard curve was constructed as in Chapter 6. Standardisation of measurement of bilirubin in brain homogenates was with a bilirubin standard of 1.7nmol/g wet weight. This was also regularly run between samples to act as a quality control mechanism. Wavelength used was 400nm.

The lower limit of detection was 0.12μg/g wet weight - equivalent to 0.2 nmol/g wet weight.

2.10 DETERMINATION OF PROTEIN CONTENT OF SAMPLES

The enzyme assays which form a large part of this thesis determine enzyme activity with regard to the amount of protein contained in each sample. Therefore a reproducible method of measuring protein content was required. The most popular method for measurement of protein content in biological tissue

preparations is the one published by Lowry (Lowry et al, 1951). In small volume samples with low concentrations of protein or when it was unsuitable to use the Lowry method for other reasons a more sensitive method developed recently (BIORAD) was used.

2.10.1 Protein Estimation by Lowry Method

A range of protein standards 25-200µg/ml were prepared using bovine serum albumin (BSA). Samples containing unknown protein concentrations were diluted to within this range. Samplesand standards were reacted with Protein reagent, a solution containing Cu^{2+} ions in mild alkali. This was followed by addition of Folin and Ciocalteu's reagent. This combination reaction detects the presence of peptide bonds and is very sensitive. After 30 minutes a deep blue colour developed, the intensity of which was proportional to protein content. The absorbance of the solution was measured at 750nm. Protein content of samples in µg was determined from a standard curve prepared from absorbance values of the BSA standards. Samples were run in duplicate and the results were corrected for the dilution, averaged and expressed in mg/ml.

2.10.2 BIORAD Protein Estimation

This method depends on the colour change of a dye (Coomassie Blue) in response to concentration of protein. A range of standards were prepared at concentrations 5 to 20ug/ml BSA. Samples with unknown protein concentrations were diluted into this range. BIORAD protein assay dye reagent was diluted 1:1 with water, added and the intensity of colour obtained measured at 595nm by spectrophotometer. This was proportional to

protein content and after construction of a standard curve protein content of samples could be determined as in 2.10.1. Dilution factors were again taken into account.

2.11 STATISTICAL METHODS

For all the analyses undertaken the Mann Whitney U test was employed. This non-parametric test was chosen to compare two independent sample groups mainly because of the large variations and abnormal distributions which arose in the animal experimental work.

CHAPTER 3

DEVELOPMENT OF THE 5-AMINOLAEVULINIC ACID SYNTHASE AND PROTOPORPHYRINOGEN OXIDASE ASSAYS IN RAT BRAIN TISSUE

3.1 INTRODUCTION

ALA-synthase has been well characterised as the initial and rate controlling step of haem biosynthesis in liver tissue and the mechanisms of the reaction have been outlined in section 1.1.1.

Although the pathway in mammals has been studied in most detail in hepatic tissue this has been mostly due to availability of this tissue. Studies of ALA synthase have been undertaken in many tissues, including the brain. Developmental aspects, the effects of ageing and the various effects ofadministration of exogenous agents known to affect ALA-synthase activity in the traditionally studied hepatic tissue in mammals and bacterial systems have all been examined. The results of these studies revealed that the brain enzyme has several unique properties.

In contrast PROTO-O, the penultimate enzyme of haem biosynthesis and the most recently discovered enzyme has not been investigated so fully. Severe difficulties in isolation and measurement of the enzyme from the most abundant tissue sources have hindered detailed study in a large number of different tissues. Published observations of PROTO-O activity appear to be exclusive to hepatic tissue in mammals with properties of the purified enzyme only available in some of the recent literature (Siepker et al, 1987; Dailey & Karr, 1987). None of the previous interest in PROTO-O has focused on defining activity in the brain. Brain haem biosynthesis studies have concentrated on mechanisms of control of the pathway without much consideration of the intermediate steps of the reaction. Thus PROTO-O activity in the brain has never been identified or

quantitated previously. In order to analyse any effects of bilirubin on the haem biosynthetic pathway of the Gunn rat, development of an enzyme assay for PROTO-O in rodent brain is required. From various publications it is clear that adaptation of an existing assay to new tissue involves several important considerations. All aspects of buffer content and concentration need investigation as well as optimal pH.

An existing assay of ALA-synthase developed for bone marrow activity measurement (Tikerpae et al, 1981; Fitzsimons et al, 1984) appears to present a more sensitive quantitation technique than the traditional one which derivatises ALA to a pyrrole and separates the product by column chromatography. The suitability of this assay for brain tissue is investigated in the first sections of this chapter. Following the production of a successful assay for brain ALA-synthase an appropriate assay for brain PROTO-O will be developed enabling sensitive measurement of the early and late steps of haem biosynthesis in Gunn rat brain and comparison with activities in control rats.

3.2 BRAIN ALA-SYNTHASE

A large amount of work has focused on the function and properties of ALA-synthase in the brain. Purification of the enzyme from rodent brain has allowed study of the pattern of development. From a very low level immediately after birth activity rises, peaking at around 15 days and falling off to adult levels at 6 to 8 weeks (De Matteis et al, 1981). This is similar to the situation in liver and has been related to the increased haem demand while cytochromes are being formed. This

is in support of a regulatory role for ALA-synthase in brain and contrasts with the age dependent maturation of mitochondrial cytochromes and respiratory activity in brain where a slow steady rise from birth to adult levels is observed (Chepelinsky & Arnaiz, 1970; Bull et al, 1979; Land et al, 1977). Adult levels of ALA-synthase activity are maintained throughout life even up to an age of two years (Paterniti et al, 1978).

ALA-synthase is located within the mitochondrion in brain tissue as in liver (Granick & Sassa, 1971) and synthesis is similarly thought to occur in the cytosol before translocation to the mitochondria, since use of a succinyl CoA generating system has demonstrated a cytoplasmic form of the enzyme exists (Paterniti et al, 1978). Within brain mitochondria it is likely that the enzyme will be loosely bound to the inner mitochondrial membrane, facing the matrix, with some free in the matrix (Scotto et al, 1983) as has been reported for liver (McKay et al, 1969)

Within cranial tissue regional ALA-synthase activity has been studied. Highest activity was located in cerebellum (Percy & Shanley, 1979; Maines, 1980) but the cerebral cortex and midbrain regions also had relatively high activity (Maines, 1980). Much lower but detectable activities were found in the striatum, septum, hypothalamus, thalamus, amygdala and hippocampus (Percy & Shanley, 1979; Maines, 1980). Measurement of total porphyrin and haem content did not vary greatly although less haem was found in the cerebellum (Maines, 1980). Haem oxygenase activity appeared to be constant throughout the

regions studied (Maines, 1980).

The regulatory role of ALA-synthase in liver is well documented, with activity of the enzyme under negative feedback control by haem as has been discussed in section 1.3.1. In the acute porphyrias, a partial deficiency of a particular enzyme of the pathway results in block to some extent of the production of haem. Impairment of nervous function is a common symptom and it has been postulated that disturbances in haem biosynthetic pathway in the brain may account for this (De Matteis et al, 1981). It remains unclear whether this type of disturbance is due to depletion of haem or related to the increased levels of haem precursors which result from the enzymatic block.

In order to try and determine if ALA-synthase has a regulatory role in brain as postulated by De Matteis and coworkers, the effect of various agents on brain ALA-synthase activity have been studied to assess inducibility. Early workers who treated rats with haematin, an exogenous form of haem, found no effect on the brain enzyme but this was thought to be due to lack of access to the brain through intravenous administration (De Matteis et al, 1981). Haematin intraventricularly produced a drop in ALA-synthase activity (De Matteis et al, 1982), an effect which would be expected for the regulatory enzyme in the presence of excess haem. In addition administration of succinyl acetone, a very effective competitive inhibitor of ALA-D, caused marked reduction of brain ALA-D in parallel to that seen in liver of both rats and mice (De Matteis & Ray, 1982; Kang et al, 1987). Also in support of a regulatory role of ALA-synthase, when ALA incorporation into haem was reduced in

rat brain following administration of succinyl acetone to deplete haem content, a compensatory increase in ALA-synthase activity was observed (De Matteis & Ray, 1982). In mice, despite the successful inhibition of ALA-D no increase in ALA-synthase could be detected and total brain haem levels were unaffected (Kang et al, 1987). Kang and coworkers were also unable to pinpoint any behavioural disturbances as a result of the increased concentration of ALA but the actual levels were not measured so this does not discount the possibility that increased levels of ALA may contribute to neurological dysfunction.

Manipulation of brain ALA-synthase activity is clearly possible but this particular form of the enzyme is not affected by the wide range of factors which are well known inducers of the liver enzyme. The enzyme is refractory to induction by AIA, DDC, ethanol, and nutritional states (Paterniti et al, 1978). Some divalent metal ions, especially cobalt and manganese (Mn^{2+}) are well known inhibitors of ALA-synthase activity in hepatic tissue. A corresponding induction of haem oxygenase (Sunderman et al, 1987) . . is likely to result in a marked reduction in liver haem content. In brain, Co^{2+} ions were ineffective but Mn^{2+} ions caused inhibition of ALA-synthase in various regions of the brain (Maines, 1980; De Matteis et al, 1981). The mechanism is not well established, and no corresponding effect was observed in haem oxygenase activity. This suggests the effects of metal ions in the brain are distinct from those seen in the liver. In vitro studies failed to show

any effect of the Mn^{2+} ion on the enzyme and so it has been postulated that the lowering of activity seen in vivo occurs via a disruption of enzyme turnover. Metal ions are known to inhibit protein synthesis by electrostatic interactions with DNA (Eichorn & Shin, 1968) and Mn can substitute for Mg as the metal activator of DNA polymerase so causing errors in nucleotide incorporation (Sirover & Loeb, 1976). Experimentation with various manganese containing compounds has shown that only the free metal ion is effective (Maines et al, 1984).

Decreased ALA-synthase activity has also been observed when rats are treated with cycloheximide, a compound which prevents stimulation of hepatic ALA-synthase after administration of inducers (Maines et al, 1976), or when the methyl ester of ALA is given. Conflicting reports exist as to the effect of ALA itself on brain haem biosynthesis. De Matteis and coworkers (De Matteis et al, 1981) reported a reduction in ALA-synthase activity in the brain when ALA was administered intravenously. This is in support of a regulatory role for brain ALA-synthase as for the liver form. Levels of ALA-synthase activity in rat brain homogenates were found to be around one fifth of those in liver when assessed per milligram wet weight tissue, with values of around 40 - 80pmoles ALA formed/hour/milligram protein in adult rats (De Matteis et al, 1981; Paterniti et al, 1978) with higher values of around 120 to 180pmol/hour/ milligram protein found in mitochondrial preparations. In immature animals the peak activity has been reported to be 3 to 4 times that seen in mature animals. The highest activity was found in the cerebellum (Percy & Shanley, 1979), with the cerebral cortex

and midbrain also having comparatively high values (Maines, 1980). Much lower activities were found in the striatum, septum, hypothalamus, thalamus, amygdala and hippocampus.

3.3 BRAIN ALA-SYNTHASE ASSAYS

Measurement of ALA-synthase activity has been possible since 1963 when ALA produced by enzyme activity was converted to pyrrole by condensation with acetyl acetone. Colorimetric analysis could then be achieved by addition of Ehrlich's reagent (Granick & Urata, 1963).

3.3.1 Colorimetric Assay of ALA-synthase

The sensitivity of the colorimetric method is relatively low and is only of practical use when large amounts of tissue with fairly high activity are available. Although endogenous activity of rat liver is detectable (Marver et al, 1966) the spectrofluorimetric analysis of ALA-synthase activity was not considered for brain tissue as both amounts of tissue and activity of the enzyme are low.

3.3.2 Fluorometric Assay of ALA-synthase

A fluorometric assay for ALA-synthase has been developed which measures ALA production at low levels, but this method is indirect. Since ALA does not itself fluoresce the assay is coupled to ALA-D and PBG-D assays and formation of oxidised to uroporphyrin I is quantitated. Use of this assay was rejected since intermediates can under some circumstances be elevated so giving falsely high values for ALA-synthase activity.

3.3.3 Radiochemical Assay of ALA-synthase

Other sensitive methods for assay of ALA-synthase activity involve incorporation of a radiochemical precursor into ALA and quantitation of the activity in the product. Radiolabelling of the direct substrates is more easily achieved by [2^{14}C] glycine since [^{14}C] succinyl CoA is unstable for storage and so would have to be manufactured on site, an expensive process.

[2-^{14}C] glycine has been used as the labelled precursor and the radioactive product separated from contaminants by thin layer chromatography (t.l.c.) (Freshney & Paul, 1970). In Friends cells the time required for separation has been reduced by use of high voltage electrophoresis followed by oxidation of the resolved ALA into scintillation cocktail for determination of DPM content (Rutherford et al, 1979). This method has proved successful for measuring ALA-synthase activity in leucocytes (McColl et al, 1982) and was investigated for use on liver and brain tissue for this thesis.

Alternatively either [^{14}C] α ketoglutarate or [2,3^{14}C] succinate can be used. Although not direct substrates for the enzyme both of these compounds result in production of labelled succinyl CoA through the citric acid cycle. Most commonly in animal preparations [2,3^{14}C] succinate is used. In assay of each tissue many of the essential requirements and cofactors in the incubation medium are similar but actual concentrations often vary. In addition some specialised factors may be necessary to enable measurement of ALA-synthase activity in certain tissues another reference to the tissue specific nature of the enzyme. This emphasises the necessity for optimising the

conditions of assay for each new tissue studied. In brain [^{14}C] succinic acid has previously been most commonly used and it was decided to use this substrate.

Separation of [^{14}C] ALA

In the published assays of measurement of ALA-synthase activity using [2,3^{14}C] succinate the most common difference is in the method for isolation of the [^{14}C] ALA product. In murine liver and spleen homogenates [^{14}C] ALA has been directly determined after separation on a Dowex 50 chromatography column (Ebert et al, 1970). A three step process of ion exchange resulted in better recovery (Strand et al, 1972). In brain these separation techniques proved insufficient to separate [^{14}C] ALA from radioactive contaminants (De Matteis et al, 1981). Conversion of the labelled ALA to a pyrrole allowed adequate separation either by extraction and colorimetric analysis (Maines, 1980) or ion exchange chromatography (De Matteis et al, 1981). In bone marrow a successful HPLC technique has been used to isolate [^{14}C] ALA produced.

3.3.4 Choice of ALA-synthase Assay for Brain

For these experiments initial studies on activity of ALA-synthase in brain homogenates and mitochondria were carried out using the [2^{14}C] glycine method described in section 2.3.1. i) Unfortunately the high voltage electrophoresis of [^{14}C] ALA did not produce clear separation of the labelled ALA from radioactive contaminants. This problem was encountered in previous assays of brain ALA-synthase using the [2,3^{14}C] succinate as

substrate. To resolve the labelled ALA the pyrrole of ALA with ethylacetoacetate {2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole} was formed but separation still proved to be a problem. Reverse phase HPLC, adapted from the method used by Tikerpae and coworkers (Tikerpae et al, 1981) was insufficient to separate the radioactive contaminant from the ALA peak, so rendering [^{14}C] ALA production unquantifiable.

The unsuitability of [2^{14}C] glycine prompted attempts to use [2,3-^{14}C] succinate, an indirect substrate which has been successfully resolved by HPLC. Conditions for brain assay using [2,3-^{14}C] succinate have been optimised, as have conditions for separation of the labelled ALA for the bone marrow preparations but the two have not yet been combined.

3.4 CONDITIONS FOR ASSAY OF BRAIN ALA-SYNTHASE

Brain tissue homogenates and mitochondrial preparations were prepared as described under methods section 2.3.3. The best tissue preparation was first determined on rats with ALA-synthase activities within the normal range (Wistar or Sprague Dawley adult animals) then components of the enzyme assay system optimised.

3.4.1 Determination of the Best Tissue Fraction for Brain ALA-synthase Activity Measurement

In brain, ALA-synthase has been studied in both homogenates and mitochondria but most frequently tissue homogenates have been used. Comparisons were carried out between activity of brain ALA-synthase in brain homogenate and mitochondrial preparations and the results are presented in Table 1. The assay

ALA-SYNTHASE ACTIVITY
pmoles ALA formed/mg protein/hour

HOMOGENATE	MITOCHONDRIA
14.1	70.9
12.8	57.7
18.1	43.1
20.0	34.0
14.2	30.5
15.2	34.0
22.6	43.0
10.5	
8.6	

$p<0.001$ by Mann Whitney U test

TABLE 1: MEASUREMENT OF ALA-SYNTHASE ACTIVITY IN BRAIN HOMOGENATE VERSUS MITOCHONDRIAL PREPARATIONS

medium used was that found to produce optimum activity of ALA-synthase. Each figure in the table is the mean of duplicate determinations on rats of similar age and weight. The results clearly show that ALA-synthase activity recorded from mitochondrial preparations is significantly higher than the results using homogenised brain (25% w/v) ($p<0.001$ by Mann Whitney U test). This result is the expected one for the subcellular location of the enzyme, and it was decided to carry out all subsequent determinations of ALA-synthase activity on mitochondrial preparations.

3.4.2 Inclusion of Sucrose

In previous methods for brain ALA-synthase assay, tissue has been homogenised in sucrose buffer which maintains the osmotic stability of the enzyme, then added to an incubation medium containing all the requirements for the assay. In assay of bone marrow where very small amounts of tissue are available the tissue sample is prepared directly in an incubation buffer containing sucrose. Since the ultimate aim of this work was to concentrate on brain tissue of immature rats with a limited amount of brain tissue 0.25 Molar sucrose was included in the incubation buffer, and all tissues were prepared in this medium.

3.4.3 Choice of Buffering System

Tris HCl is the most common buffer used in the assay of brain ALA-synthase activity but sodium phosphate has also been used (Maines,1980). A combination of Tris HCl and phosphate buffer has proved successful when HPLC is used to quantitate ALA-pyrrole (Tikerpae et al, 1981) but the exact concentrations

of Tris and phosphate are unknown and may vary slightly in each batch of buffer. The use of phosphate buffer alone was discounted by these authors because extraction into ether formed an emulsion. Liquid/liquid extraction was not adopted in this thesis and the optimum pH for formation of the pyrrole is above pH 6 so a 50mMolar potassium phosphate buffering system, at pH 7.5 was measured against the combined buffer and a Tris buffer system and the results presented in Table 2 show that the buffer combining Tris HCl and phosphate consistently gave better results. These results were also the tightest with a wider spread being observed on use of the other two buffer systems. Thus a mixture of Tris and potassium dihydrogen phosphate was used in the buffering system.

3.4.4 Glycine

As one of the substrates for ALA-synthase, sufficient glycine is required to saturate the enzyme. 50 - 100 millimolar quantities have been previously used for brain tissue assays. The optimal concentration, 50 millimolar, has been determined for brain homogenates but not mitochondria (Paterniti et al, 1978). Studies were undertaken to determine the optimum concentration of glycine in brain mitochondrial preparations and results are presented in Figure 17. Adult Wistar male rats, fasted for 24 hours, were used in each case. Each point represents the mean of three determinations. 45 millimolar glycine was subsequently included in all brain assays of ALA-synthase activity.

	ALA-synthase activity pmol/mg protein/hour
TRIS HCl BUFFER	38.9 ± 14.5
KH_2PO_4 BUFFER	36.4 ± 9.5
TRIS HCl AND KH_2PO_4 BUFFER	47.6 ± 8.3

Results are expressed as mean ± SD

n=6

TABLE 2: THE OPTIMAL BUFFERING MEDIUM FOR MEASUREMENT OF ALA-SYNTHASE ACTIVITY IN BRAIN MITOCHONDRIA

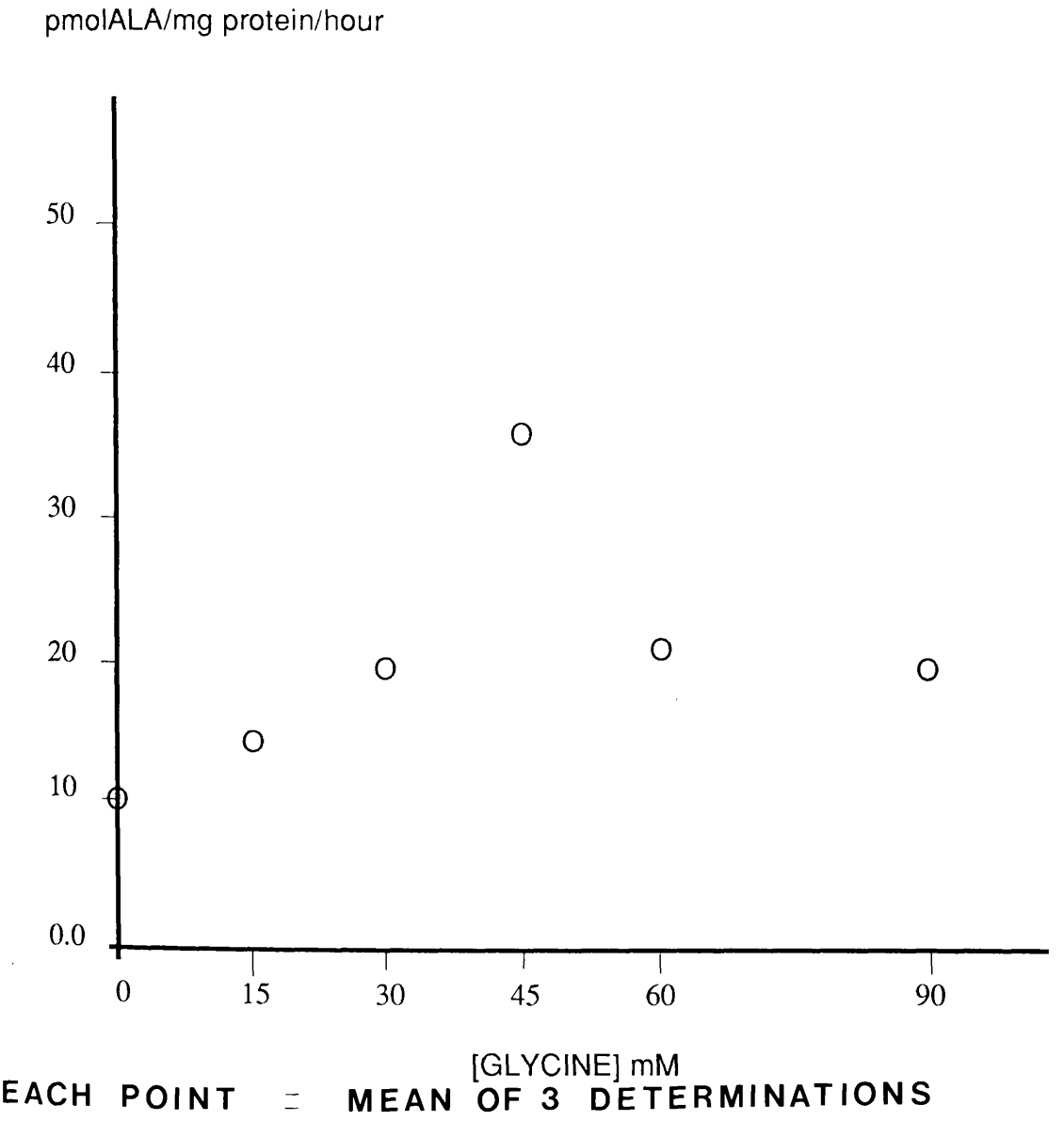


FIGURE 17 : THE EFFECT OF DIFFERENT CONCENTATIONS OF GLYCINE ON RAT BRAIN ALA-SYNTHASE ACTIVITY

3.4.5 Inclusion of Magnesium Chloride ($MgCl_2$)

Magnesium ions are essential for succinate thiokinase activity and so are necessary for conversion of [2,3-^{14}C] succinate to the radiolabelled succinyl CoA. $MgCl_2$ is necessary when an external succinyl CoA generating system is added to the assay medium, and has been included in incubation media for several published brain ALA-synthase assays. The use of a succinyl CoA generating system is investigated in section 3.4.9 and $MgCl_2$ at a concentration of 5mMolar was routinely included in the incubation medium.

3.4.6 Inclusion of EDTA

In hepatic systems EDTA is reported to inhibit ALA-D activity and prevent formation of aminoacetone, an intermediate which may interfere with measurement techniques and make quantitation of the true amount of ALA formed more difficult. The presence of EDTA in brain preparations assayed for ALA-synthase appears to have no effect on activity (Paterniti et al, 1978) but no reduction in activity was observed by these authors and so a low concentration (2mMolar) was included in the assay medium.

3.4.7 Optimum Concentration of Succinate

[2,3-^{14}C] succinic acid had specific activity of 45mCuries/mmole. 2.5 μCi was included in each assay. [2,3-^{14}C] succinic acid powder was dissolved in a solution of 20mMolar "cold" succinate. The optimum concentration for succinate has been determined as 3mMolar, the Km for succinate being much lower than that for glycine and so this ensured excess succinate

was present to allow the reaction to proceed.

3.4.8 Concentration of Pyridoxal Phosphate

Pyridoxal phosphate is well documented as an essential cofactor for ALA-synthase activity. A concentration of 0.4mMolar is generally used to give optimum measurement of activity. Several concentrations of pyridoxal phosphate were added to the incubation medium and results agreed well with those of other authors. The results are presented in Figure 18 with each point the mean of three determinations. The concentration of 0.4mMolar was the value chosen since this was on the plateau. Formation of ALA was linear with respect to time (Figure 19).

3.4.9 Effect of an External Succinyl-CoA Generating System on ALA-synthase Activity

In brain preparations there is dispute over whether addition of an external succinyl CoA generating system will produce higher ALA-synthase activities. Homogenates appear to supply enough intermediates but some improvement has been seen in mitochondria after addition of external GTP and succinate thiokinase. Results of the comparison of ALA-synthase activities with and without incorporation of the external succinyl CoA generating system in homogenate and mitochondria are shown in Table 3.

In fresh tissue, whether mitochondria or homogenate preparations were used, the succinyl CoA generating system did not give higher results for ALA-synthase enzyme activity.

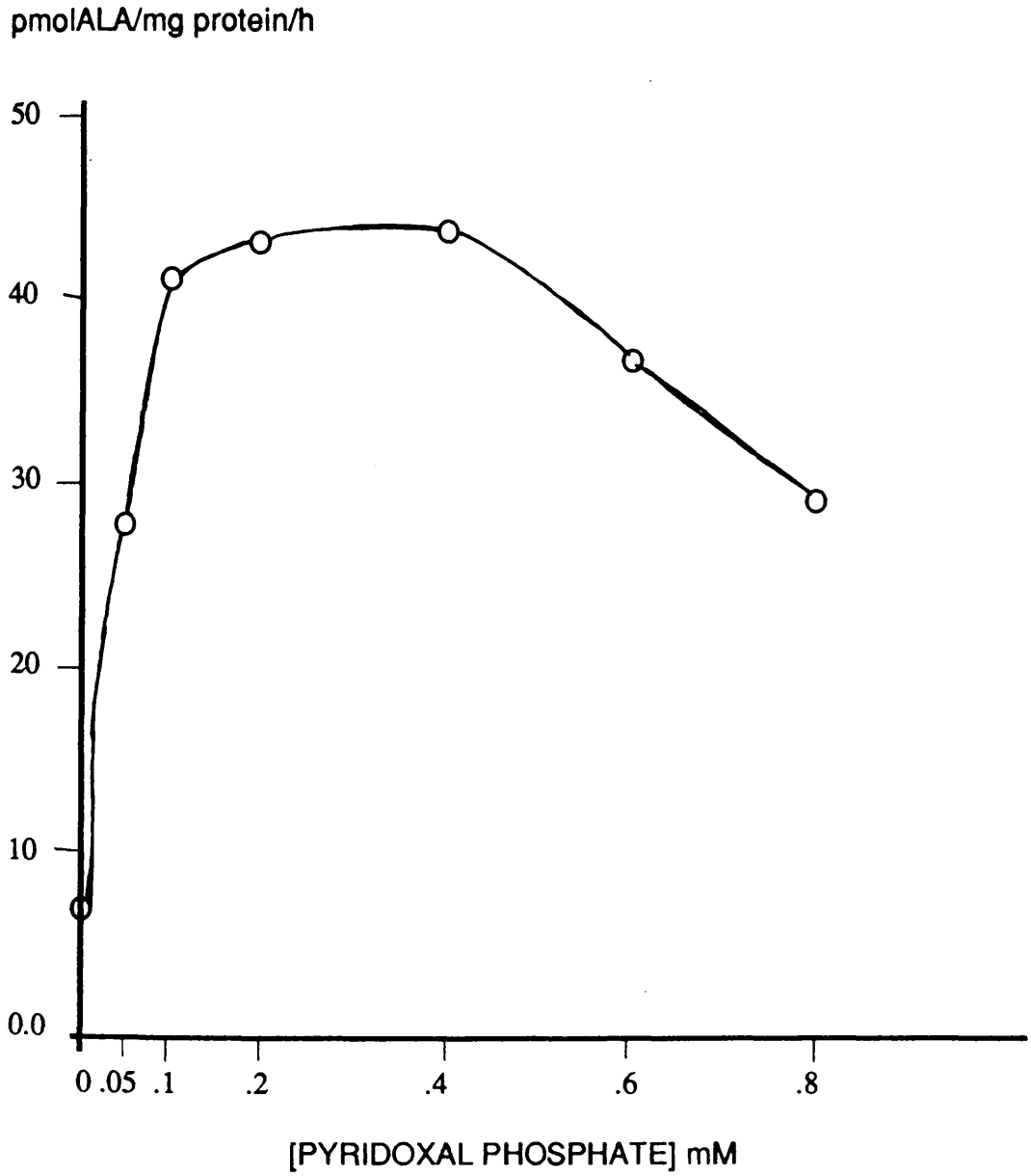


FIGURE 18 : THE EFFECT OF DIFFERENT CONCENTRATIONS OF PYRIDOXAL PHOSPHATE ON RAT BRAIN ALA-SYNTHASE ACTIVITY

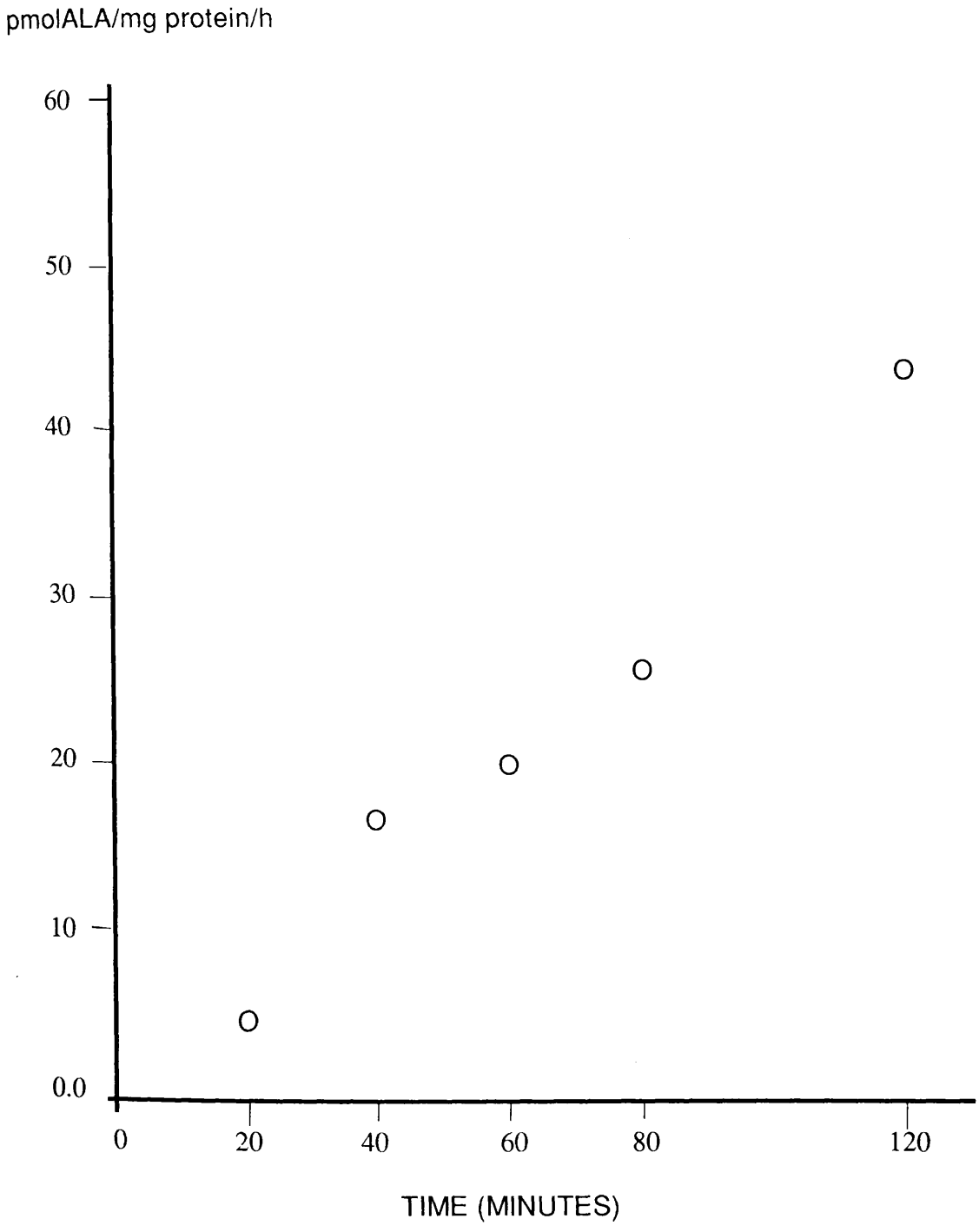


FIGURE 19 : FORMATION OF ALA WITH RESPECT TO TIME

ALA-SYNTHASE ACTIVITY pmol ALA formed/mg protein/hour	
MINUS SUCCINYL CoA GENERATING SYSTEM	PLUS SUCCINYL CoA GENERATING SYSTEM
13.4	14.1
12.2	12.8
17.4	18.1
32.9	20.0

TABLE 3: THE EFFECT OF AN EXOGENOUS SUCCINYL CoA GENERATING SYSTEM ON ALA-SYNTHASE ACTIVITY IN BRAIN HOMOGENATES

3.5 ISOLATION OF THE ALA PRODUCT FROM BRAIN ALA-SYNTHASE ASSAY

3.5.1 Pyrrole Formation

Direct measurement of [^{14}C] ALA produced in the brain ALA-synthase assay has not proved possible due to a contaminating factor which co-elutes with ALA on chromatography (Paterniti et al, 1978; Percy & Shanley, 1979; De Matteis et al, 1981). Conversion of ALA to a pyrrole derivative allows separation and quantitation. Pyrrole derivatives have been formed with acetyl-acetone and ethylacetoacetate see Figure 20. Ethylacetoacetate is preferred when HPLC is used to separate the [^{14}C] ALA pyrrole. Cold ALA (4mMolar) was added to each sample prior to pyrrole formation to enable easy quantification of the ALA-pyrrole. Purification of the derivatised mixture was achieved using solid phase sample preparation.

3.5.2 Reverse Phase HPLC of ALA-pyrrole

High performance liquid chromatography (HPLC) is a fast efficient method of separation of biological molecules with a wide range of applications. The main advance from traditional chromatography is the use of much smaller molecules within the column (10 microns or less) resulting in much larger surface areas for interaction with samples. Separation of molecules can be based on size (molecular sieve chromatography) or charge (ion exchange chromatography) but the most common form in separation of small biological molecules is based on the hydrophobic or hydrophilic properties of the molecules.

Although these types of separation have different principles the system used in each case is essentially the same. A

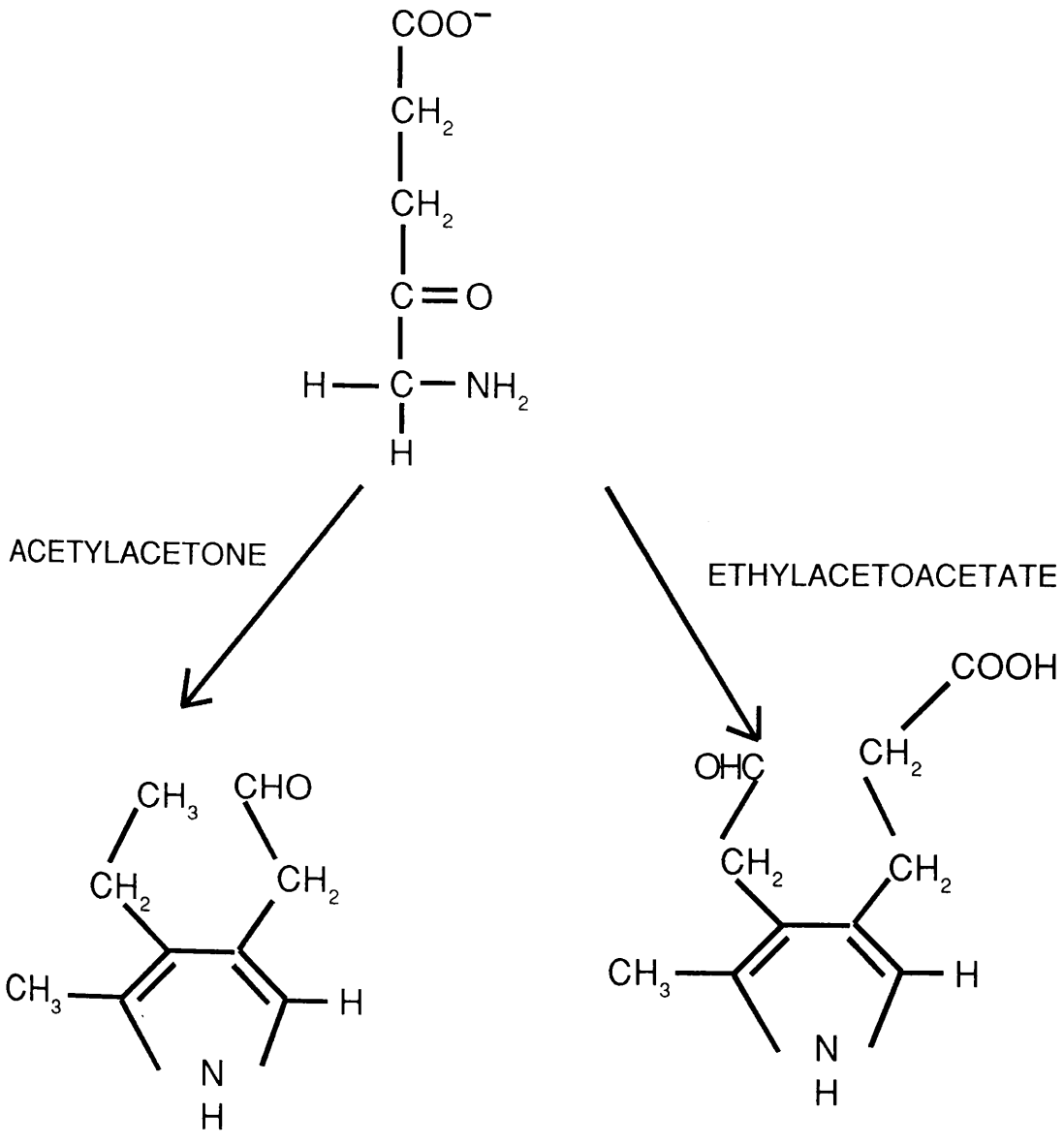


FIGURE 20 : FORMATION OF ALA - PYRROLE DERIVATIVES

solution containing the sample is dissolved in liquid (mobile phase) and pumped through a column consisting of small particles (stationary phase) where separation occurs. The size and tight packing of the column necessitates use of a high pressure pump to push the sample through the column. On elution a detector measures a peak and this is reported and can then be quantitated.

Normal and reverse phase HPLC are especially useful for the separation of small molecules. In reverse phase chromatography the stationary phase is composed of silica with hydrocarbon chains covalently bound to their surface. Increasing the length of the hydrocarbon chains makes the stationary phase more hydrophobic. The mobile phase is a hydrophilic medium so that more hydrophilic components of the sample will remain dissolved in this medium and will pass through the column while lipophilic components can interact with the hydrocarbon chains on the column and are retained.

The range of molecules which can be separated is widened by addition of varying amounts of an organic component such as methanol or acetonitrile to the mobile phase. For separation of several similar molecules from complex mixtures a gradient type of elution, where the organic content of the mobile phase is gradually increased, can be employed. Interactions of molecules with the stationary phase can be altered to improve separation in other ways. In some cases a change in the pH of the mobile phase improves resolution. The presence of charged groups on the molecule of interest can prevent maximum interaction with the stationary phase by reducing solubility so

limiting contact. Alternatively the reaction can be modified by addition of an ion pairing agent. These compounds may complex with the molecule of interest resulting in an alteration of the reaction with the stationary phase. However interactions of the ion pairing agents with the stationary phase or both sample and stationary phase may be responsible for improved separation. Thus weak acids can be made more hydrophobic by acidifying the mobile phase and similarly, for weak bases, raising the pH increases interaction with the stationary phase.

For resolution of ALA, 1-hepatanesulphonic acid, an ion pairing agent, was added to aqueous mobile phase containing 37% methanol. The stationary phase, C18 bound silica, was contained in a 250 x 4.5mm column. In HPLC of ALA-pyrrole from bone marrow samples the same ion pairing agent, but in the form of PIC B7 (Waters) was used. When 0.005 Molar 1-hepatanesulphonic acid was dissolved in 20ml/litre 1Molar acetic acid and added to the mobile phase no loss of resolution resulted. Mobile phase containing this solution was made up fresh daily.

Flow rate is an important factor in determining sharpness of peaks. Low flow rates can result in peak broadening but increasing the rate too much prevents maximum interaction with the stationary phase lowering resolution. Optimum flow rate for separation of ALA was 1.5 ml/minute. ALA was eluted at approximately 8 to 9 minutes after injection, but retention time could be decreased by addition of a slightly higher percentage of methanol (up to 40%).

The column eluent was collected in 0.5 minute fractions

around and during the ALA peak and the fractions were added to scintillant for counting. Background DPMs were subtracted and the results calculated as in the Methods, section 2.3.1.

Before application to the HPLC column samples were purified using solid phase sample preparation. This increased column life and reduced background DPMs.

3.5.2 [^{14}C] ALA-pyrrole Purification

Liquid/liquid extraction of ALA has previously been used in brain assays but the volumes obtained would require much concentration before application to a HPLC method could be achieved. Recently solid phase sample preparation has been used to detect ALA pyrrole in bone marrow samples (Fitzsimons et al, 1986). The sample is applied to a small disposable column which preferentially retains the molecule of interest, in this case ALA pyrrole, while other components which are more soluble in the application buffer pass through in a similar manner to the situation on HPLC. Elution is achieved by changing to a solvent which has higher affinity for the molecule than the column. A Sep-pak cartridge (Waters) containing C18 bound silica binds non-polar molecules. ALA pyrrole, applied in a slightly acidic aqueous solution will bind to the column allowing more polar components of the sample to pass through. The cartridge can then be dried before application of methanol which strips the column of the pyrrole. Prewetting of the column with a water miscible solvent, in this case acetonitrile, is necessary to activate the column when samples are applied in aqueous solution. This "clean-up" step allowed many more

samples to be injected into each column and simultaneously removed any contaminating radioactivity.

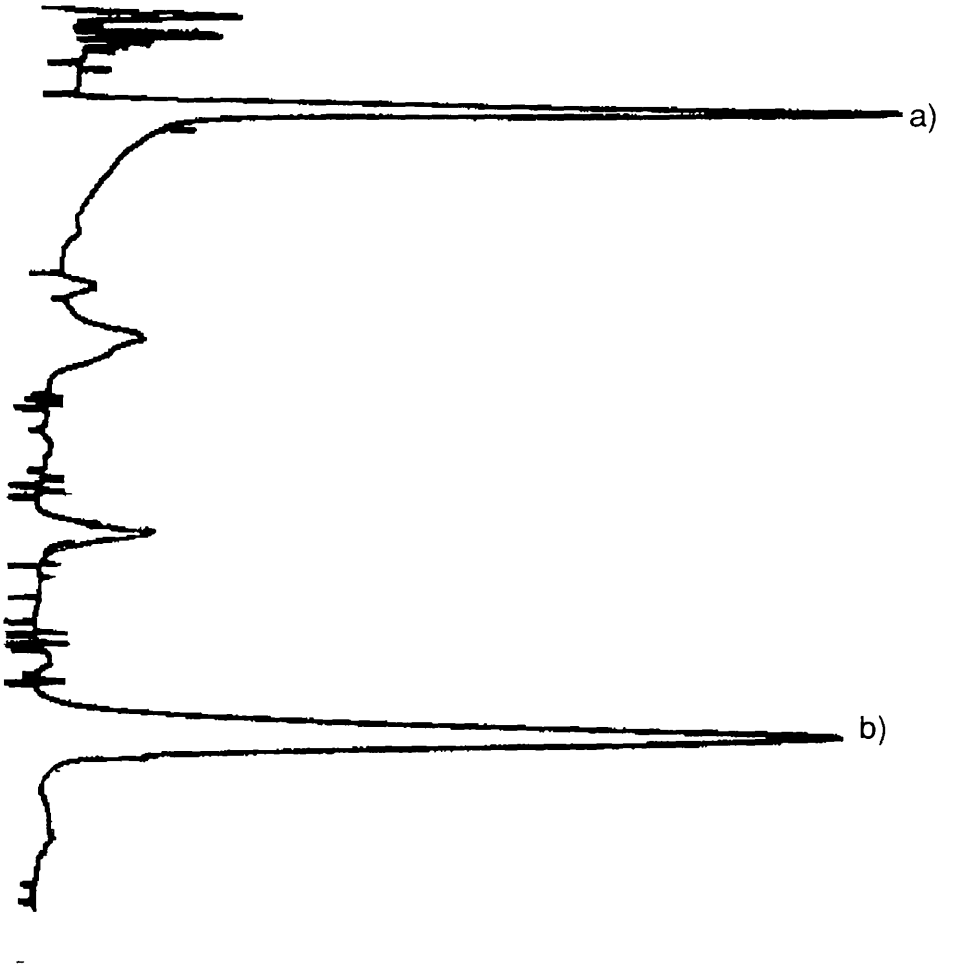
3.5.4 Results of HPLC of ALA-pyrrole

An example of an HPLC trace of ALA-pyrrole is included in Figure 21. The peak of interest is clearly identified and cannot be mistaken for any other peak on the chromatogram. The full method used to measure ALA-synthase activity in rat brain is quoted in the methods section 2.3.1. The results obtained for rat brain are within a similar range to those previously obtained (Paterniti et al, 1978).

ALA-synthase activities in liver and brain mitochondria were both measured and compared. The brain ALA-synthase activities were found to be significantly lower as previously determined in the literature. Examples of liver and brain ALA-synthase activities are demonstrated in Table 4.

3.6 PROTO-OXIDASE IN RAT BRAIN

As stated earlier PROTO-O is the most recently identified enzyme of the haem biosynthetic pathway, the concrete evidence for its existence as a separate entity emerging only in the last 15 years. (Jackson et al, 1974; Poulson & Polglase, 1975). The enzyme has now been isolated, purified and measured in hepatic tissue of mouse, (Dailey & Karr, 1987) and ox (Siepker et al, 1987) but no work has concentrated on other tissue sources of the enzyme. Thus existing assays have been developed for hepatic sources of enzyme in animals and for plant



a = solvent front

b = Peak due to ALA-pyrrole

FIGURE 21 : TYPICAL HPLC TRACE OF ALA-PYRROLE PEAK

	ALA-SYNTHASE ACTIVITY pmol ALA/mg protein/hour
LIVER	356 ± 46
BRAIN	49.1 ± 8.3

Results are expressed as mean ± SD

n=9

TABLE 4: ALA-SYNTHASE ACTIVITIES IN BRAIN AND LIVER MITOCHONDRIA DETERMINED WITH ^{14}C SUCCINATE AS SUBSTRATE

and bacterial sources. Even many of these assays list several problems with their existing PROTO-O assays. It is likely that modifications to these assays will have to be carried out in order to adequately measure the enzyme in the brain.

3.6.1 Choice of PROTO-O Assay

Two types of PROTO-O assay exist at present. Both quantitate appearance of the strongly red-coloured PROTO from the enzymatic substrate, straw coloured PROTO'gen. PROTO has a well defined spectrum in the visible range and this has been measured spectrophotometrically. In addition PROTO fluoresces and this provides another method of quantitating the emergence of enzyme product. In both assays an accurate result depends on a linear relationship existing between the concentration of PROTO in the reaction mixture and the absorbance/fluorescence. Evidence has been obtained that at low protein concentrations the linearity of the relationship between absorbance and PROTO concentration may be compromised (Jacobs & Jacobs, 1982). The assay of choice for brain PROTO-O must be useful at low protein concentrations as the source of brain tissue is limited. As the ultimate aim of the project is to examine PROTO-O activities in neonatal Gunn rats and compare enzyme activities with matched control and drug treated rats availability of all tissues will be low, necessitating the use of the fluorometric assay.

Reliability of the PROTO-O assay depends on production of a relatively pure substrate for the enzyme. Problems can occur when full reduction of PROTO does not occur. Significant levels of PROTO leads to product inhibition of the enzyme

activity (Jacobs & Jacobs, 1982). A strong reducing agent is therefore necessary. Auto-oxidation is another major problem, with PROTO'gen reverting to PROTO spontaneously on exposure to light. This necessitates all steps after conversion of PROTO to enzyme substrate having to be carried out under conditins which as far as possible exclude light. Even with these precautions a reagent blank containing buffer and substrate is included in the assay and any increase in fluorescence subtracted from all results obtained.

In the fluorometric assay mitochondria are resuspended in PROTO-O buffer, Tween 20 added, the solution sonicated and an aliquot diluted and GSH added to prevent auto-oxidation. This solution is then allowed to reach 37^{o}C before addition of the freshly prepared substrate. This is the reaction mixture, and small aliquots are removed at pre-determined times and diluted further into measuring tubes before the fluorescence is determined.

3.6.2 Choice of Buffering System

The most common choice of buffering system previously used for the PROTO-O assay has been a Tris buffer. The pH values found to be optimal for measurement of PROTO-O activity have varied according to the source of tissue, but in general this has been above neutral, ranging from pH 7.45 in yeast (Poulson & Polglase, 1975) to pH 8.7 in rat liver (Jacobs & Jacobs, 1982) The concentration of Tris used has been fairly constant at around 100mmoles/litre. From previous experience in these laboratories pH 9.2 was discovered to be optimal for PROTO-O

assay in rat liver mitochondria. The optimum pH for assay of rat brain mitochondria was determined with 100mmolar Tris HCl. The results of these determinations are presented in Figure 22. Each point represents the mean of 3 duplicate determinations. The optimal pH for assay of rat brain PROTO-O was found to be at pH 9.2. The PROTO-O buffer was also saturated with air so that there would be no shortage of oxygen to accept the electrons which are lost from PROTO'gen.

The optimal concentration of Tris HCl for assay of the brain enzyme was also determined. The best results were obtained with 100mMolar Tris HCl as shown in Figure 23.

EDTA has previously been included in assays of PROTO-O. Its presence helps prevent chelation of other metals, especially zinc which can lead to a block of incorporation of iron into haem (Jacobs & Jacobs, 1982). EDTA at a level of 1mmol/l was routinely included in all PROTO-O assay buffers of brain and other tissues.

3.6.3 Presence of a Reducing Agent

Auto-oxidation has been mentioned as a considerable problem in attempts to measure PROTO-O activity. The presence of a reducing agent in the assay medium has been shown to significantly reduce the rate of auto-oxidation (Jacobs & Jacobs, 1982). 5mmol/l glutathione (GSH) was added to both reaction vials and measuring tubes. Auto-oxidation still occurred at a significant rate and this change in fluorescence not attributable to PROTO-O activity was subtracted from true activity.

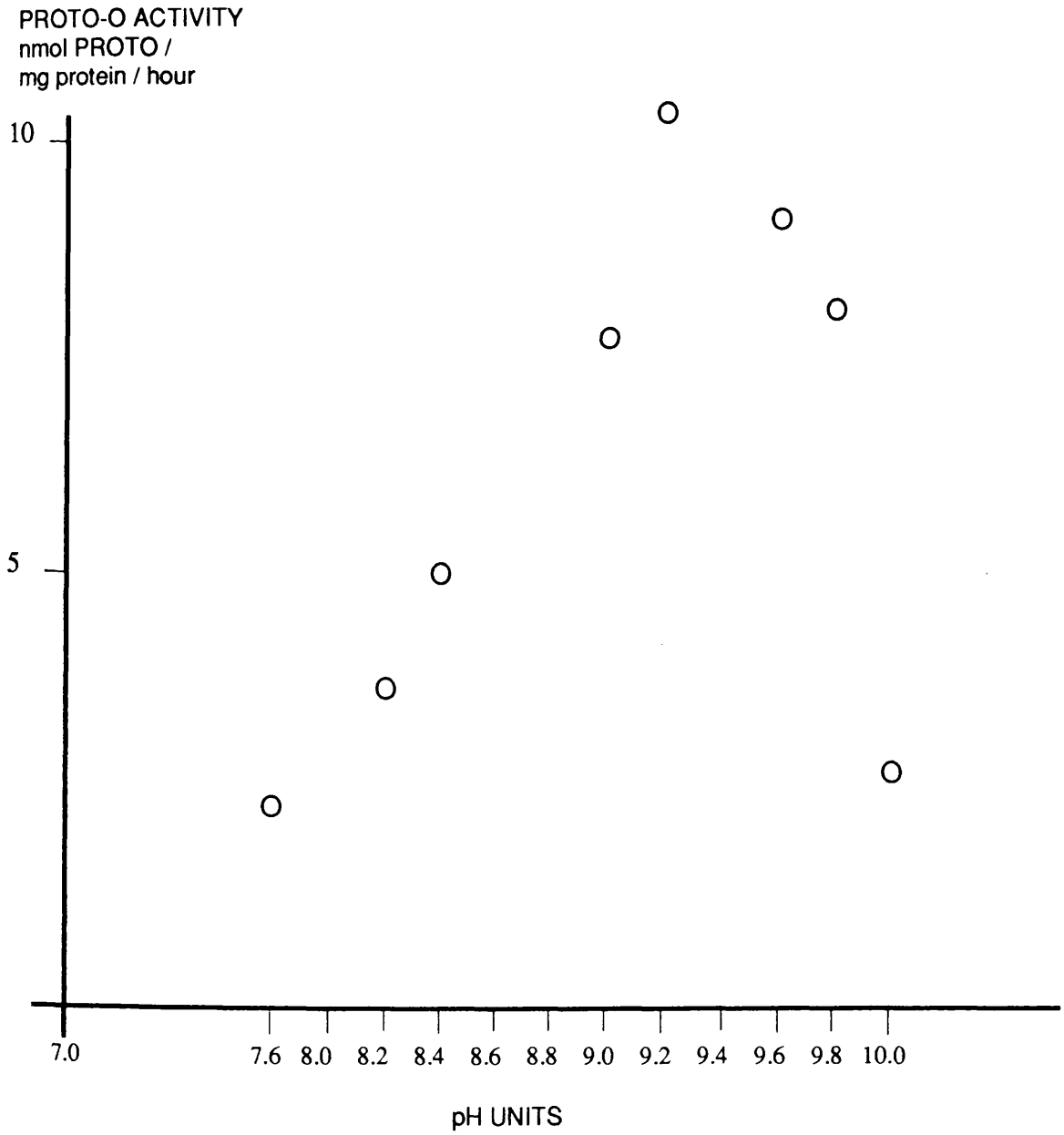
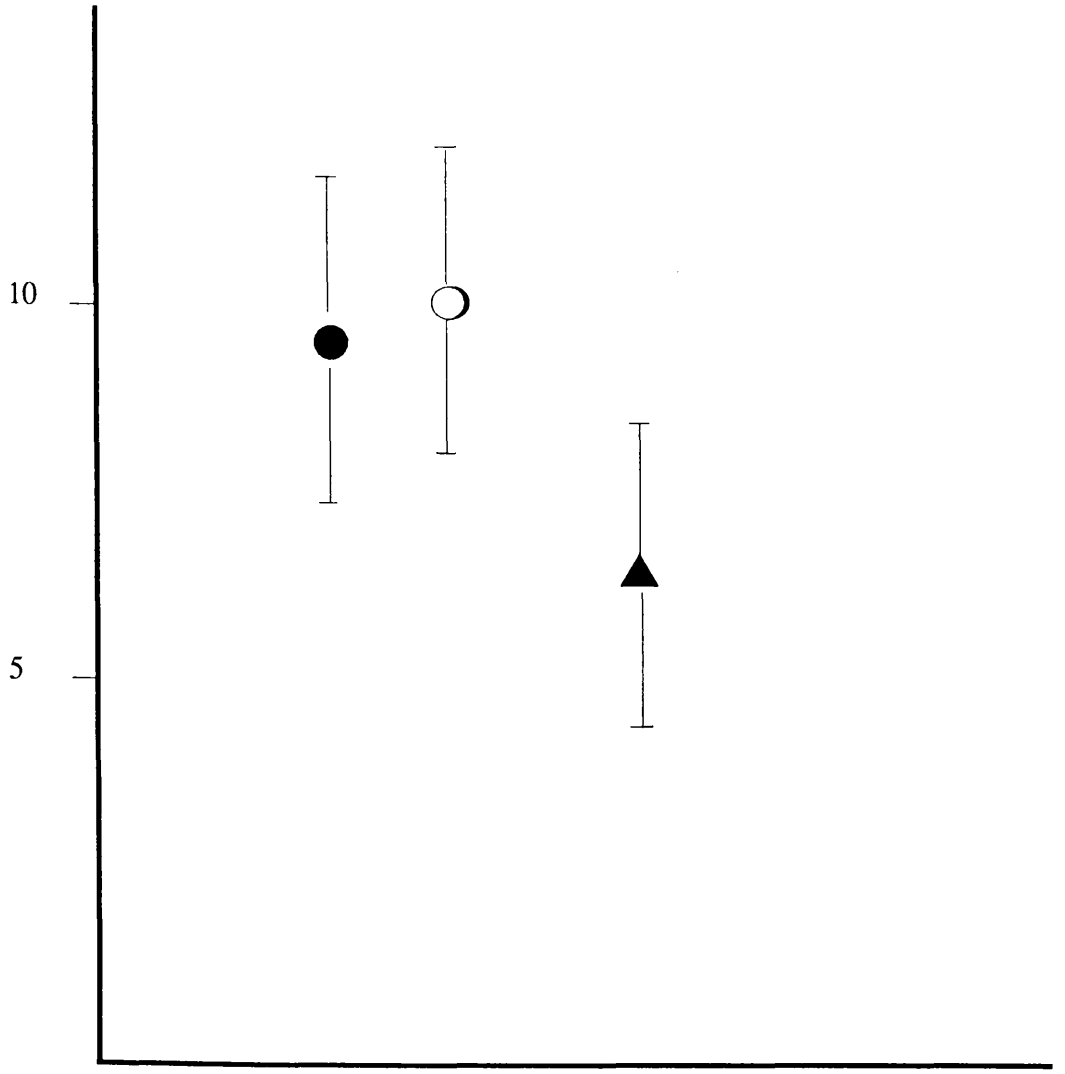


FIGURE 22 : OPTIMUM pH OF ANALYSIS OF PROTOPORPHYRINOGEN ACTIVITY IN RAT BRAIN



● PROTO-O ASSAY AT 100 mmol/l TRIS Cl

○ PROTO-O ASSAY AT 50 mmol/l TRIS Cl

▲ PROTO-O ASSAY AT 25 mmol/l TRIS Cl

Results are expressed as mean ± SD

FIGURE 23 : OPTIMAL BUFFERING CAPACITY FOR PROTOPORPHYRINOGEN IX OXIDASE ACTIVITY IN BRAIN

3.7 PRODUCTION OF PROTOPORPHYRINOGEN SUBSTRATE

Reduction of PROTO to substrate for the enzyme, PROTO'gen, has proved difficult. Previous authors have noted a lack of reproducibility of results with the assay. Each batch of PROTO'gen substrate appears to result in a slightly different rate of oxidation in mitochondrial preparations (Jacobs & Jacobs, 1982). This heterogeneity of substrate leads to a high variability of rates of formation of PROTO and it would be useful to try and remove this variability.

On contact with sodium/mercury amalgam, the PROTO reacts violently. This leads to production of a large amount of foam resulting in a low volume of PROTO'gen. Addition of a larger ratio of PROTO to amalgam simply leads to partial conversion with a visible proportion of PROTO present. If the violence of the reaction could be reduced more substrate would be produced. Changesfrom classical dissolution agents for PROTO and addition of antifoaming agents were attempted.

3.7.1 Preparation of PROTO for Reduction

PROTO methyl ester was hydrolysed overnight as described in the methods section 2.3.4., freeze dried and stored at -20^{o}C until use. PROTO was allowed to reach room temperature in the absence of light then dissolved and reduced to PROTO'gen immediately. The classical dissolution agent of 10mmolar KOH containing 20% ethanol was compared to N/7 sodium bicarbonate. The results showed no significant differences in production of substrate but the reaction with bicarbonate was slightly less violent. Quicker dissolution also occurred on use ofthis

solution and N/7 sodium bicarbonate was chosen as an agent of choice to dissolve PROTO in all experiments.

3.7.2 Addition of an Antifoam Agent

An antifoaming agent, a silicon based product, is manufactured by Sigma. It was hoped that addition of a small volume of this type of preparation to the PROTO solution before addition to amalgam would reduce the amount of foam produced so maximising the amount of PROTO'gen generated.

Initial experiments on the fluorescence properties of the antifoam agent were first carried out. The spectrophotometric profile of PROTO and PROTO'gen, with and without antifoam were analysed and found to be identical. Solutions of PROTO had 1%, 2%, and 5% Antifoam A added, before addition to amalgam. The experiment was successful in reducing the amount of foam produced by the reaction while having no effect on the spectrophotometric profile of the substrate. Fluorometric analysis of PROTO'gen prepared in the presence of antifoam showed that a higher blank value was obtained. This was thought to reflect a higher proportion of unconverted PROTO in the solution, but when substrate produced in the presence of antifoam was reacted with mitochondrial preparations the rate of conversion of PROTO'gen to PROTO appeared to be greatly increased. Unfortunately this also proved to be true for the tissue blank sample, indicating that this result was a false positive, likely to be due to increased auto-oxidation. Addition of an antifoaming agent as an aid to production of improved character of PROTO'gen substrate was therefore abandoned.

3.7.3 Purification of PROTO'gen by Solid Phase Sample Preparation

It has been previously been recorded in the literature that contaminating PROTO can be removed from converted PROTO'gen by solid phase sample preparation (Li et al, 1987). A strong anion exchange column (SAX, manufactured by Bond-Elut) could be conditioned with buffer and converted PROTO'gen applied such that any unconverted PROTO would adhere to the column, producing a purer solution of PROTO'gen for use as substrate in the PROTO-O assay. The column was conditioned using successively methanol, water and 0.25 Molar Tris buffer pH 7.2. Reduced substrate was added to an equal volume of Tris buffer pH 7.2 and passed through the column. The resulting solution was used in a PROTO-O assay but it was discovered that no PROTO-O activity could be recorded. On conversion with acid very little PROTO was measured. It appeared that PROTO'gen was also being retained by the SAX column. This step was therefore not used prior to assay.

3.7.4 Adopted Method of Production of PROTO'gen Substrate

1mg PROTO methyl ester was hydrolysed overnight with 70% HCl as in methods section 2.3.4. Prior to assay the freeze dried PROTO was suspended in the absence of light with N/7 sodium bicarbonate to give a working solution of concentration approximately 400μMolar. The resuspended PROTO was added to 6g sodium/mercury amalgam in a boiling tube and the solution shaken on ice in the absence of light until no fluorescence was visible under UV illumination. PROTO'gen was filtered through a fine porosity glass sinter funnel under pressure and the pH

lowered to approximately pH 9 by titration with 1 molar 3-(N-morpholino)propane-sulphonic acid (MOPS acid), which has a more favourable pka (7.2) than phosphoric acid and is less likely to cause overshoot of pH into the acidic range (Dailey & Karr, 1987). 100µl of the PROTO'gen was added to the reaction vial containing 1.5ml enzyme/ buffer mixture and the reaction started.

3.8 ASSAY OF BRAIN PROTO-O ACTIVITY

Brain mitochondria were prepared as in the methods section and resuspended in 0.15 Molar KCl to a final protein concentration not exceeding 5mg/ml. 0.4 ml was then added to a reaction vial at 37°C containing 1.1ml Tris buffer (100mmol/l, pH 9.2), 1mmol/l EDTA and 5mmol/l GSH. The temperature was allowed to reach 37°C then freshly prepared PROTO'gen added. On mixing 50µl of the sample was removed and used as a zero time, to determine fluorescence of tissue before reaction occurs. After 10 and 20 minutes further 50µl aliquots were removed and the fluorescence determined. Auto-oxidation was determined by subtracting the change in fluorescence over the same time period by a tissue sample containing PROTO-O enzyme inactivated by heat.

PROTO-O activities in rat brain were found to be comparable with the levels detected in liver with Gunn-Wistar and Wistar rats showing values of 8.2 $\pm$ 2.9 nmol PROTO formed/mg protein/ hour (mean $\pm$ SD). These levels are very similar to the activities recorded in liver by other authors (Poulson, 1976; Brenner & Bloomer, 1980). It was consistently found that auto-

oxidation was lower in brain tissue than in liver. This was thought to reflect a cleaner preparation.

3.9 SUMMARY

Accurate and specific assays for both ALA-synthase and PROTO-O activities in the brain have been developed. From the existing ALA-synthase assay slight changes have optimised the method for brain tissue. With respect to PROTO-O activity, the optimal assay method has proved to be identical to that which has previously been used in other tissues. The lack of specificity required for the PROTO-O assay may reflect that the enzyme is homologous with that in other tissues. This is already known not to be the case for ALA-synthase which seems to have tissue specific properties. The erythroid and hepatic forms of the enzyme are distinct, with different genes coding for the two enzyme proteins (Riddle et al, 1989; Bishop et al, 1990). Brain ALA-synthase activity is dependent on stage of development (De Matteis et al, 1981) and certain other factors such as the presence of certain metals (Maines, 1980; Maines et al, 1984) or succinyl acetone (De Matteis & Ray, 1982) but no such information is available on activities of PROTO-O in the brain. More information on PROTO-O activity is hoped to be obtained from the studies in this thesis.

CHAPTER 4

THE GUNN RAT AS A MODEL OF HYPERBILIRUBINAEMIA

4.1 INTRODUCTION

Hyperbilirubinaemia in humans has been discussed in Chapter 1.5 but man is not the only species to suffer from the effects of elevated bilirubin. Mutant forms of various different species exist, and these can display either conjugated or unconjugated hyperbilirubinaemia. These mutant species have been very useful to the research carried out on this range of conditions and have provided answers to many of the important questions in this area which can then be related to the human disease states.

Only one popular animal model exists for hereditary conjugated hyperbilirubinaemia and this animal, the mutant Corriedale sheep, displays a condition which compares well to Dubin-Johnson syndrome (Cornelius et al, 1965). Whether the metabolic basis for this disease is the same as that in humans remains to be determined.

The various hereditary unconjugated hyperbilirubinaemias provide more than one animal model system with another sheep, the mutant Southdown sheep, arguably a model for the moderate unconjugated hyperbilirubinaemia of Gilbert's syndrome. The Gunn rat has a defect in hepatic conjugation (Carbone & Grodsky, 1957; Schmid et al, 1958) and this has been compared to the defect in Crigler-Najjar syndrome. The homozygous rat is unable to form bilirubin conjugates and in this way is similar to Type I of the syndrome

On closer study the mutant Southdown sheep has reduced clearance of bilirubin and various other organic anions which

are removed from the circulation by the same system (Cornelius et al, 1968; Gronwall, 1970) and has been compared with the situation in Gilbert's syndrome (Mia et al, 1970). In contrast to Gilbert's syndrome much of the unconjugated bilirubin in the plasma appears to efflux from liver, with no reduction in glucuronidation rates obvious. Thus the basis behind the hyperbilirubinaemia in the mutant Southdown sheep is thought to differ from that in Gilbert's syndrome.

The two sheep models have been useful but work on live animals of this size is difficult, and in most situations occurs at veterinary establishments. Much of the animal work which has led to our present understanding of states of hyperbilirubinaemia has been carried out on the Gunn rat, a mutant of the Wistar strain which suffers from congenital unconjugated hyperbilirubinaemia.

4.2 THE GUNN RAT

The Gunn rat was first identified in 1938 (Gunn, 1938). On observation these rats were suffering from congenital jaundice which within a few hours of birth resulted in the skin displaying a yellow tinge. Growth in the Gunn rat was consistently stunted when compared to Wistar counterparts, resulting in a lower adult body weight. Most Gunn rats displayed nervous symptoms and brain damage was evident from defects in balance often described as "wobbly gait". Serum bilirubin levels were found to be elevated when compared to normal and values range from 3 to 20 mg/dl or 51 to >300μmolar (Wolkoff et al, 1982). When it was established that excretion

of bilirubin was achieved by glucuronidation to more water soluble conjugates the hepatic conjugating system of the Gunn rat was investigated. A severe defect in this pathway was consistently demonstrated in these rats (Schmid et al, 1958) and it was quickly confirmed that Gunn rats were unable to conjugate bilirubin in the normal manner. Despite this, the rats did not die as a result of high bilirubin levels. Instead withi a few weeks of birth the serum bilirubin levels stabilised and circulating bilirubin, though remaining elevated evened out. Thus bilirubin was still excreted in these animals at virtually normal rates but the unconjugated bilirubin was metabolised by unknown routes. It was discovered that unusual derivatives were formed and the mechanisms leading to these compounds have been investigated.

Phenobarbital is a well known inducer of hepatic mono-oxygenase and UDP-GT systems in normal animals (Remmer, 1972). Effects of this drug on Gunn rats have been studied and results showed it to be ineffective in increasing glucuronidation of bilirubin (Vainio & Hietanen, 1974). In accordance with this, recent genetic evidence has showed that the UDP-GT enzyme protein responsible for conjugating bilirubin is absent (Scragg et al, 1985). Evidence suggests that a number of isoenzymes of UDP-GT exist and the presence of UDP-GT activity towards phenol in Gunn rats supports the evidence for existence of isoenzymes. Activity is lower than that found in control animals but the defect is incomplete and this form of the enzyme remains inducible (Harding et al, 1989). The decreased

activity towards phenol is thought to result from a mutation which affects a number of the isoenzymes of UDP-GT (Harding et al, 1989) including the bilirubin UDP-GT system. Gunn rats do retain the ability to excrete bilirubin but the route used leads to production of unusual derivatives of bilirubin. This pathway has been linked to another of the mixed function monooxygenase enzyme systems. Administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin which is known to stimulate the microsomal P448-dependent monooxygenases, led to increased bilirubin catabolism and reduced total serum bilirubin levels (Kapitulnik & Ostrow, 1977). Conjugation was not stimulated but production of excretable polar derivatives of bilirubin was increased.

The heterozygous Gunn rat has reduced activity of the UDP-GT enzyme system but does retain enough activity to maintain serum bilirubin levels within the normal range seen in Wistar rats (Schmid et al, 1958). It has been suggested that this heterozygote may provide a model for Gilbert's syndrome (Carbone & Grodsky, 1957)

For many years yellow staining has been observed in the brain tissue of human fatalities resulting from kernicterus and in Gunn rats. The mechanism by which the neurotoxicity occurs after deposition of bilirubin however is poorly understood. In the homozygous Gunn rat there is marked cerebellar hypoplasia (Schutta & Johnson, 1967) with degeneration of the Purkinje cells occurring postnatally (Mikoshiba et al,1980). There is reduced production of neurotypic and gliotypic proteins and this reduction is consistent with the extent of morphological change and behavioural impairment (O'Callaghan & Miller, 1985). It

has been documented that the Purkinje cells are most sensitive to bilirubin damage when rats were aged between 5 and 7 days old (Takagishi & Yamamura, 1987), but reports show that this is prior to the period in which bilirubin is preferentially deposited in the cerebellum (Yamamura et al, 1974; Aono et al, 1989). This is a discrepancy which merits further investigation.

Although there is little doubt that bilirubin is present in the central nervous tissue of both Gunn rats and icteric infants there is great dispute over its route of entry. It is still unclear whether bilirubin bound to albumin crosses the blood brain barrier or if only the circulating unconjugated form is able to do so. Certainly it seems that the unconjugated form is responsible for the neurological damage. If the blood brain barrier of infants is immature then bilirubin may enter the brain more readily (Bakay, 1953; Ernster et al, 1957). Studies which showed that bilirubin deposition in the brain occurred less frequently in adult animals, and resulted in less marked staining, were concurrent with the belief that free bilirubin was able to enter the brain (Robinson & Rapoport, 1987), and that this was likely to occur when the blood brain barrier was immature . However Davis and Yeary showed similar uptake of bilirubin into brain by newborn and adult Gunn rats and so the increased sensitivity of the neonatal model is in question (Davis & Yeary, 1975; Robinson & Rapoport, 1987).

Under certain conditions, levels of unconjugated bilirubin in Gunn rats may be elevated even further allowing observation

of a condition similar to severe kernicterus. This situation can occur naturally, for example when infection which leads to increased bilirubin production occurs, or it can be experimentally induced. Experimental kernicterus is usually achieved by injection of a competitive agent for bilirubin binding to albumin. The common one used is sulphadimethoxine or a related sulphonamide (Rose & Wisniewski, 1979; Davis & Yeary, 1975). When these drugs were first administered to neonates a much higher incidence of kernicterus resulted and these drugs are now contraindicated in infants. In the newborn the bilirubin conjugating system is immature and is unable to cope with the increased level of unconjugated bilirubin which is released from albumin during treatment putting the infant at risk of kernicterus. On administration to Gunn rats signs of neurotoxicity are observed within hours (Rose & Wisniewski, 1979; Davis & Yeary, 1975) and death often follows.

4.3 DEVELOPMENT OF A GUNN RAT COLONY

Gunn rats are very difficult to breed. When this study was undertaken there were only two known breeding centres in Britain. The first batches of animals were received from Dundee and these were in a successful breeding colony originally started from rats obtained from the other breeding centre, "The Royal Free Hospital" and run by Professor B. Billing. Primarily it was intended to breed Gunn rats on site since ultimately it was our aim to look at kernicterus induced by sulphonamides in 14 day old rats. This task proved alternately easy and difficult with some of the breeding pairs producing two or three

litters and some not breeding at all.

Within the period of this project the intensely inbred colony at Dundee died out and subsequently all rats were received from The Royal Free Hospital.

4.3.1 Breeding Stock

Gunn rats were caged in pairs. This was in contrast to the harem system used by other breeding centres. The animals were subjected to a twelve hour light and dark cycle with minimal disturbance. The breeding pairs were fed a special diet with high lipid content. A high content of lipid in the diet of Gunn rats has been shown to lower serum bilirubin levels (Gollan et al, 1975). This has not been attributed to increased excretion but to altered distribution of the bilirubin and deposition in tissues (Gollan et al, 1979).

Under these conditions pregnancy did occur but unreliably with only two or three litters being produced at most, over a period of more than a year. Pregnant female rats were fed with a supplement of dried milk made up to 250ml containing an egg yolk. By the time the infant rats were weaned at approximately four weeks of age the mothers' special diet had been gradually reduced and exchanged for water.

After the lack of success of this system male Gunn rats were teamed with two female Gunn rats. Unfortunately this did not produce any improvement in breeding.

4.3.2 Gunn Rat Litters

The Gunn rat litters produced in our department were invariably small with at most three or four siblings. Often one or more of the infants would die before they could be weaned. In addition the female Gunn rats made very bad mothers. For this reason in other colonies a heterozygous Gunn female is often used as the mother but this experiment was not tried in our breeding programme. The size and sickly manner of the Gunn rat infants suggested that serum bilirubin levels would be high but the high mortality rate was disappointing. This combined with the unreliability of the breeding of two homozygous Gunn rats led most of the work to be carried out on imported rats.

Gunn rat females either pregnant or with young litters (less than a week old) were transported from the Royal Free Hospital and allowed to settle before the offspring were used.

4.3.3 Control Rats

Commonly Sprague Dawley rats have been used in this department as controls for the experimental work. In these experiments it was decided that Wistar rats would be used, since the Gunn rat is a mutant of the Wistar strain. It was hoped use of this control would minimise any cross strain differences which could affect results. The Wistar rats provided a better match genetically for the Gunn rat but wide variations in levels of urinary porphyrin excretion have been recorded in these animals and it has been suggested that two separate populations of rats may exist with one population having lower porphyrin

excretion than the other (Gartzke & Burck, 1986). The urinary porphyrin excretion in Gunn and control rats will be compared in the experiments in Chapter 5.

Since the ultimate aim of the experimental work was to establish the effects of hyperbilirubinaemia on haem biosynthesis the merits of the heterozygous Gunn-Wistar cross rat as a control were also considered.

The heterozygous Gunn rat has lower than normal activity of the enzyme UDP-GT but enough activity is present to produce serum bilirubin levels within the normal range. Thus interference of bilirubin with any of the haem biosynthetic enzymes in these animals is unlikely. In addition it proved extremely easy to breed heterozygous Gunn rats from a female Wistar and male Gunn parentage. This produced the F_2 generation, one hundred percent heterozygous.

The difficulty in homozygous breeding programme led us to take this generation one step further and produce the F_3 generation by breeding heterozygotes of different parentage. The F_3 generation produced both homozygous and heterozygous rats see Figure 24. Theoretically one quarter should be homozygous for the hyperbilirubinaemia gene one half will be heterozygous and not display hyperbilirubinaemia since the gene is recessive and the remaining quarter will be genetically true Wistar. The results of comparison of the serum bilirubin levels in the F_3 generation showed this to be true with ratios of 1 homozygous for hyperbilirubinaemia to 3 with normal serum bilirubin levels. It is likely that one third of the rats with normal serum bilirubin levels are true Wistar but this was not exclusively

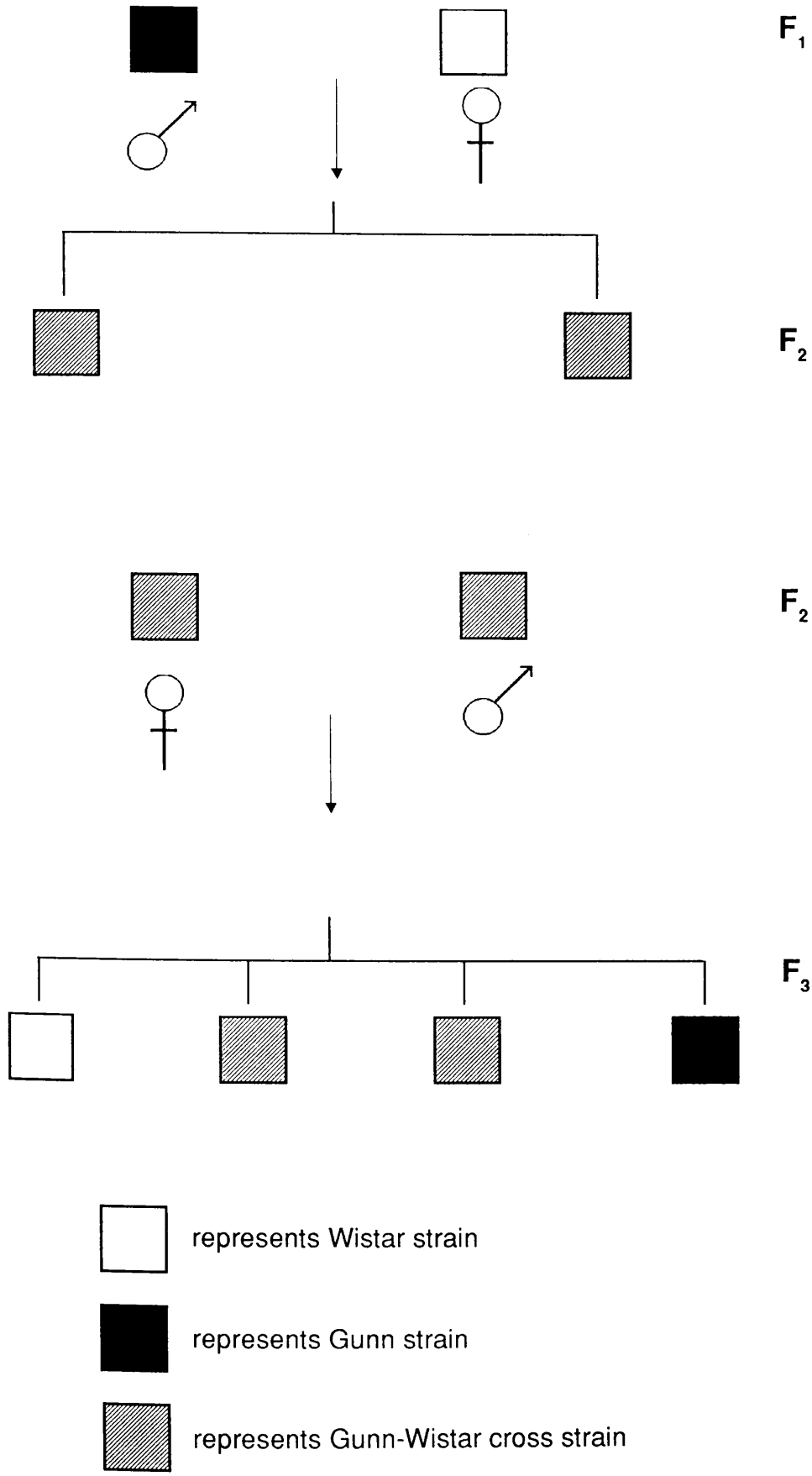


FIGURE 24 : PRODUCTION OF GUNN, WISTAR, AND GUNN-WISTAR CROSS LITTERMATES

proven as coat colour was found not to be linked to the expression of hyperbilirubinaemia ie occasionally a white coated animal was found to have high levels of unconjugated bilirubin in serum. This breeding programme provided the advantage of exactly age matched test and control rats. Differentiation between homozygous and heterozygous animals was carried out at the same time as weaning by extracting a small amount of tail vein blood for centrifugation. In homozygous Gunn rats the plasma was clearly yellow when compared to the relatively clear plasma of heterozygotes.

As previously mentioned the Gunn rats grew more slowly than either the heterozygous animals or Wistar animals and their adult body weight remained lower than in control animals by an average of 25%. This led to some deliberation over whether controls for each experiment should be age or weight matched. It was felt that there were advantages and disadvantages in each case. Enzyme activities, in the case of ALA-synthase at least, can be raised during the first few weeks of life (Paterniti et al, 1978) and so for experiments carried out on young animals (less than two months of age) where enzyme activities were being determined, age matching was considered the better alternative.

Excretion of intermediates of the haem biosynthetic pathway in Gunn rats and control rats was also measured. For this work the merits of age and weight matching were compared and are presented in Chapter 5 on the haem biosynthetic pathway of adult Gunn rats.

Within both the department bred Gunn rat colony and imported

Gunn rats, serum bilirubin values were obtained. This gave an indication of the severity of hyperbilirubinaemia suffered by each rat and the serum bilirubin levels which constitute a normal range of hyperbilirubinaemia. Serum bilirubin levels in control rats were also determined for comparison to the levels obtained in Gunn rats and to establish a normal range in non-jaundiced animals.

4.4. PLASMA BILIRUBIN LEVELS IN GUNN RATS AND CONTROLS

As stated in the introduction the serum unconjugated bilirubin level in the mutant Gunn rat is much higher than in the native Wistar strain. Gunn/Wistar cross rats bred from male Gunn and female Wistar rat still retain some UDP-GT activity. This is sufficient for conjugation to occur such that serum bilirubin levels are in the same range as Wistar rats.

Measurement of serum bilirubin in rats was carried out under light ether anaesthesia by nicking the tail vein after heating to dilate the tail vessels. Whole blood was collected in a heparinised eppendorf tube and maintained at $4^{o}C$ until it could be centrifuged at 2000g for 10 minutes to separate plasma. Plasma bilirubin levels were determined by autoanalyser. For comparison the diazo method of Jendrassik and Grof was employed in the lab as described in the methods section 2.7.2. Results from the two methods were comparable with no significant difference as determined by the Mann Whitney U test.

Haemolysis was a problem which could be largely avoided when samples of whole blood were kept cool and mixed gently.

The original colony of Gunn rats in the department consisted of 46 Gunn rats obtained from the colony in Dundee. The average serum bilirubin value from autoanalyser determination was 58.5 $\pm$ 16.7umol/l (mean $\pm$ SD). By Jandrassik and Grof method values of 65.2 $\pm$ 19.0 (mean $\pm$ SD) were obtained. The range of serum bilirubin levels in Gunn rats is represented in Figure 25 and is very wide and values tended to be lower than those previously recorded (Davis & Yeary, 1975; Gollan et al, 1975; Gollan et al, 1979). Autoanlyser values were for total bilirubin.

The lab method was not always sensitive enough to detect plasma bilirubin levels in Wistar and Gunn/Wistar cross rats and so the results quoted in Table 5 for these animals are calculated by autoanalyser. Both these groups of control rats had total plasma bilirubin levels in the same range (< 5umol/l). In general, this was in the region of one tenth or less of those values recorded for Gunn rats and is demonstrated in Table 6.

4.5 SUMMARY

The Gunn rats used in all experiments in this thesis have statistically higher serum bilirubin levels when compared to Wistar or Gunn-Wistar counterparts ($p < 0.001$ by Mann Whitney U test). Gunn-Wistar cross rats were chosen as controls in the majority of the experimental work as these rats while maintaining normal serum bilirubin levels appeared to be most closely matched to the Gunn rats. No overlap in serum bilirubin levels was seen at any time between the Gunn rats and the chosen controls.

The nature of haem biosynthesis in Gunn rats has not been

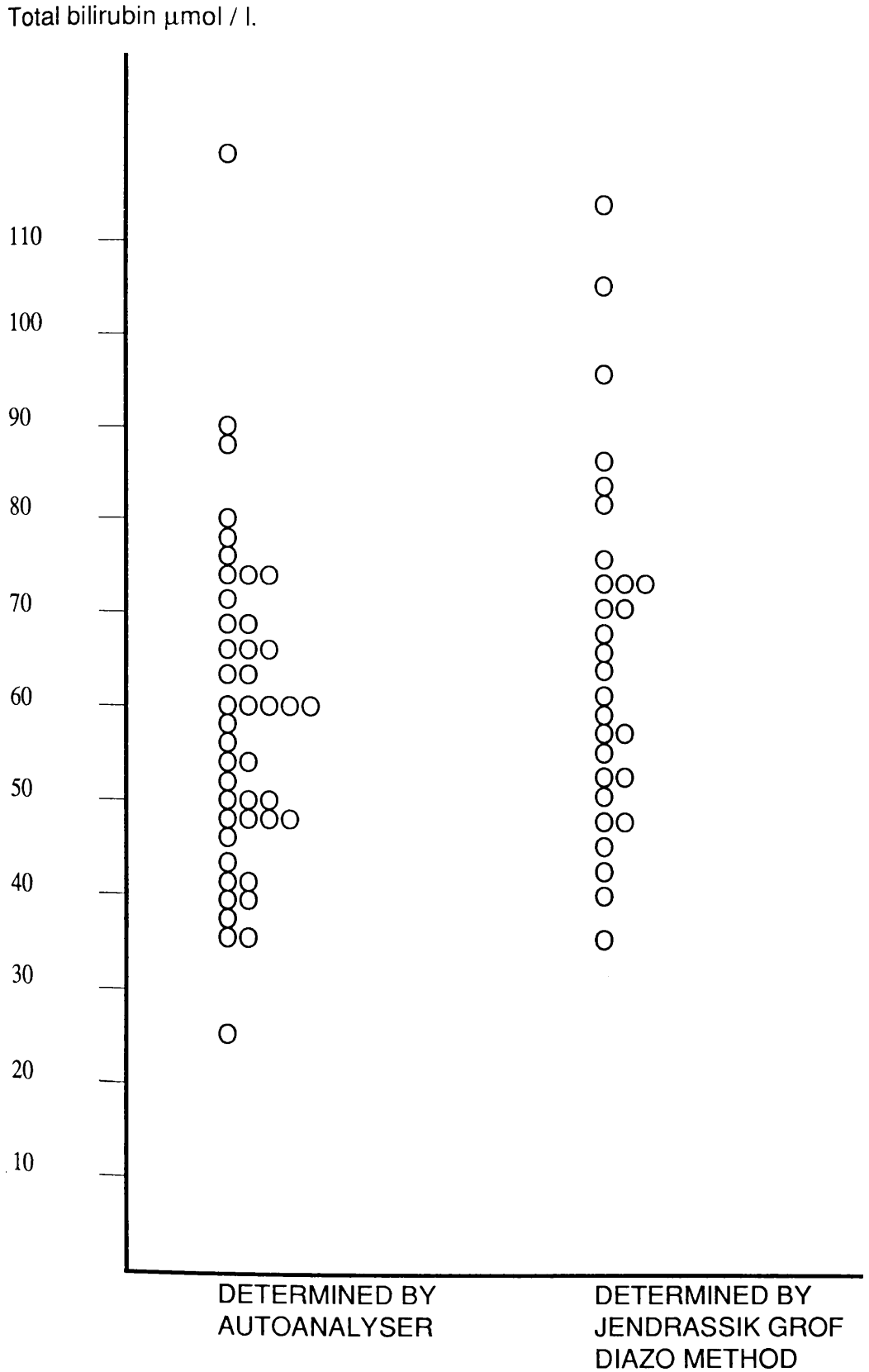


FIGURE 25 : DETERMINATION OF PLASMA BILIRUBIN LEVELS IN GUNN RATS BY AUTOANALYSER AND LABORATORY JENDRASSIK GROF METHOD

PLASMA BILIRUBIN LEVEL μmol/l	
WISTAR	GUNN/WISTAR CROSS
1	6
2	3
2	2
1	1
3	2
8	6
4	2
6	1
1	

TABLE 5: PLASMA BILIRUBIN LEVELS IN CONTROL RATS DETERMINED BY AUTOANALYSER

	PLASMA BILIRUBIN LEVEL µmol/l
WISTAR	3.1 ± 2.3
GUNN/WISTAR CROSS	2.9 ± 1.9
GUNN	55.1 ± 4.7

In the Wistar group n=9, for the Gunn/Wistar cross group n=8 and in the Gunn group n=7.

Gunn rats have significantly higher plasma bilirubin levels ($p<0.001$)

Results are expressed as mean ± SD

TABLE 6: PLASMA BILIRUBIN LEVELS IN GUNN RATS AS COMPARED TO CONTROLS

closely studied and so hepatic enzyme activity and blood porphyrin levels along with porphyrin excretion patterns will be studied prior to examining brain haem biosynthesis in detail.

CHAPTER 5

HAEM BIOSYNTHESIS IN THE GUNN RAT

5.1 INTRODUCTION

Although the Gunn rat is a recognised model of hyperbilirubinaemia most previous studies have concentrated on characterisation of the defect in the conjugating system for bilirubin and the resultant effect on bilirubin at certain intracellular sites such as respiratory cytochromes or *in vivo*. Little work has concentrated on the haem biosynthetic pathway and how it may be affected in these animals.

Recent work *in vitro* has shown that bilirubin is a competitive inhibitor of the penultimate enzyme of the haem biosynthetic pathway, PROTO-O (Ferriera & Dailey, 1988). In support of this, the enzyme also appears to have reduced activity in humans presenting with the mild hyperbilirubinaemia of Gilbert's syndrome (McColl et al, 1986). The mechanism by which bilirubin inhibits PROTO-O has not been fully determined but the suggestion is that the similarity in structure to PROTO'gen allows access of bilirubin to the active site of the enzyme where it competes with the substrate. PROTO-O activity in Gunn rats has not been so far been measured. Since these rats display a more severe hyperbilirubinaemia than that of Gilbert's syndrome patients, it may be expected that enzyme activity would be reduced when compared to controls.

Additionally, activity of the rate controlling enzyme ALA-synthase, was measured in these rats. A large reduction in PROTO-O activity could lead to partial block of the haem biosynthetic pathway resulting in lower than normal haem production. In variegate porphyria a hereditary partial deficiency of PROTO-O activity occurs, and leads to further

disruption of the haem biosynthetic pathway at the rate controlling step. Thus increased activity of ALA-synthase as seen in porphyric states may be expected. Any block in the pathway would also be likely to affect the excretion pattern of haem precursors. Initially study of the porphyrin excretion patterns of Gunn and control rats was undertaken.

5.2 PORPHYRIN PROFILES IN GUNN RATS AND CONTROLS

The diagnostic tests for human porphyria consist of determination of porphyrin and porphyrin precursor excretion. ALA, PBG and porphyrins are measured in urine, porphyrin levels are measured in faeces and these figures are combined with blood porphyrin measurements to give a complete picture of the extent of porphyrin and precursor accumulation in the body and their excretion. Porphyric patients have abnormally high excretion of porphyrins in a pattern unique to the type of porphyria suffered, and this depends on the enzyme which is affected. Porphyrin profiles in Gunn rats have not been determined. The pattern of porphyrin excretion in these rats was observed and compared to the excretion pattern in control rats. Blood porphyrin levels in Gunn rats and control animals were also determined and compared. At present there is some controversy over whether the presence of high levels of circulating bilirubin may affect the accuracy of blood porphyrin measurement by an automated method using a zinc haematofluorimeter (Buhrmann et al, 1978). As the Gunn rats have high serum bilirubin levels blood porphyrin measurements were undertaken by liquid/liquid extraction as well as by haematofluorimeter.

5.2.1 Blood Porphyrin Levels

Routinely erythrocyte protoporphyrin measurement in rat and human samples was carried out in the lab using a zinc haematofluorimeter. The main advantages of this method are the speed by which a large number of samples can be analysed and the lack of specialised reagents required. After calibration of the machine 50µl blood was spotted onto a coverslip and oxygenated before a reading was obtained. This value could be related to erythrocyte protoporphyrin concentration. As noted earlier, some controversy has arisen over whether the presence of bilirubin interferes with the results obtained by this automated method. The alternative and more traditional extraction method separates porphyrins from bilirubin in the plasma and allows determination of both COPRO and PROTO content of whole blood as shown in Table 7. In general only protoporphyrin values were considered, as the amount of COPRO was very small in comparison. To assess the potential source of error of the zinc haematofluorimeter, blood porphyrin levels from control and Gunn rats were measured by extraction and the automated method.

The results obtained when both extraction and haematofluorometric measurement was carried out in Gunn-Wistar cross rats and Wistar rats were comparable as shown in Figure 26. For Gunn rats the levels of blood protoporphyrin were consistently higher (1058 ± 219) (mean $\pm$ SD) than those obtained by extraction (659 ± 229) (mean $\pm$ SD) - see Figure 26. These results agree with the hypothesis of Buhrmann and coworkers (Buhrmann et al, 1978) who maintain that the presence of bilirubin interferes with

	COPRO	PROTO
GUNN-WISTAR CROSS	26 ± 6	609 ± 106
GUNN RAT	32 ± 4	678 ± 93

Results were determined from liquid/liquid extraction in nmol/l and are expressed as mean ± SD

n=6

TABLE 7: BLOOD PORPHYRIN LEVELS IN RATS

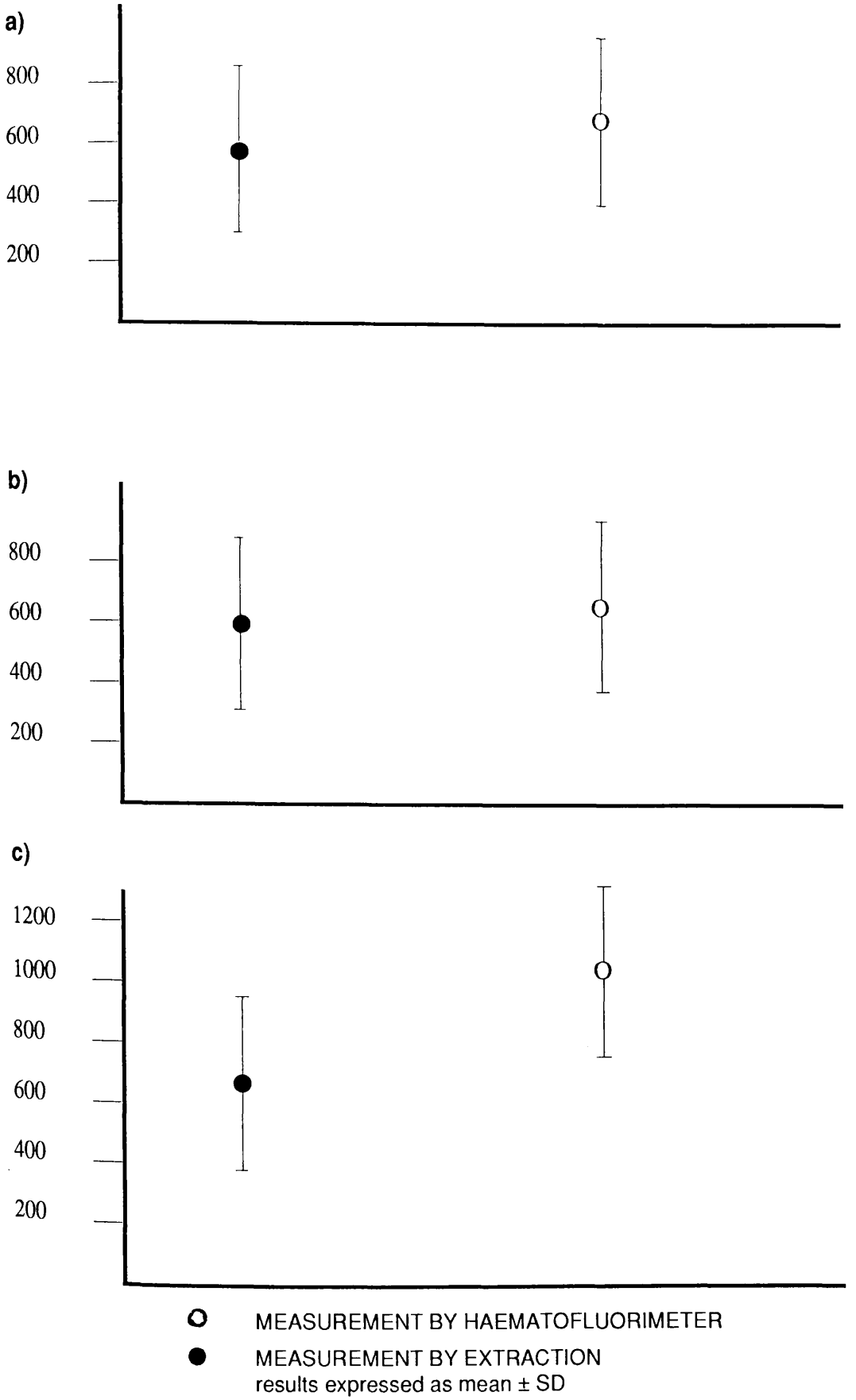


FIGURE 26 : BLOOD PROTOPORPHYRIN LEVELS IN CONTROL AND GUNN RATS
A COMPARISON OF EXTRACTION & HAEMATOFLUORIMETER MEASUREMENT

accurate measurement of blood porphyrins by haematofluorimeter. Consequently blood protoporphyrin levels in Gunn rats were compared to those in controls by using the extraction method. As can be seen in Table 8, when extraction techniques were applied there was no significant difference in the protoporphyrin content of Gunn rat blood when compared to Gunn-Wistar cross controls when assessed by Mann Whitney U test.

5.2.2 Urinary Porphyrin and Porphyrin Precursor Excretion

ALA, PBG and porphyrin excretion patterns in the Gunn and control rats were investigated. In urine the main excretory products were ALA and PBG although some porphyrins were detected and these were expressed as total porphyrin.

i) Column Determination of Urinary Intermediates

Gunn rats of approximately two months of age and Gunn-Wistar rats aged one month were confined to metabolic cages and urine collected over a 24 hour period. Collections were not exposed to light. These groups of rats, although different ages, were in the same weight range (140-180g). Excretion was also compared to that of 5 week old Wistar rats, weight 140-180g. The results of the urinary excretion studies in Gunn rats and controls are presented in Table 9 as mean results $\pm$ SD. ALA excretion in the Wistar rat of 191 $\pm$ 41.7 nmol ALA/24h was significantly higher than in either Gunn, 46.4 $\pm$ 32.1nmol ALA/24h - ($p<0.001$) or Gunn-Wistar animals, 61.3 $\pm$ 13.2nmol ALA/24h ($p<0.001$) by Mann Whitney U test. When ALA excretion in Gunn-Wistar and Gunn rats was compared, excretion in the control rats was significantly higher ($p<0.05$). There was no

	BLOOD PROTOPORPHYRIN LEVELS nmol/l
WISTAR	670 ± 270
GUNN WISTAR CROSS	659 ± 229
GUNN	647 ± 199

Results are expressed as mean ± SD

For Wistar n=7, Gunn n=8 and cross n=9

TABLE 8: RELATIVE BLOOD PROTOPORPHYRIN LEVELS IN GUNN RATS AS COMPARED TO CONTROLS WHEN EXTRACTED

TYPE OF RAT	EXCRETION		
	ALA nmol/24h	PBG nmol/24h	TOTAL PORPHYRIN µg/24h
WISTAR	191 ± 41.7	32.5 ± 29.3	4.5 ± 3.7
GUNN/WISTAR	61.3 ± 13.2	39.6 ± 20.8	8.0 ± 4.3
GUNN	46.4 ± 32.1	43.7 ± 50.5	1.1 ± 1.0

Results are expressed as mean ± SD

For Wistar rats n=9 for all groups. For Gunn/Wistar controls n=6 for ALA excretion, n=4 for PBG excretion, n=9 for total porphyrin excretion. For Gunn rats n=6 for ALA excretion, n=5 for PBG excretion and n=9 for total porphyrin excretion

TABLE 9: DAILY URINARY PORPHYRIN EXCRETION IN GUNN RATS AS COMPARED TO CONTROLS

significant difference in PBG excretion between any of the three groups of rats.

Total porphyrin excretion was highest in Gunn-Wistar control rats with 8.0 $\pm$ 4.3µg total porphyrin excreted in 24 hours. Wistar rats excreted significantly less porphyrin (4.5 $\pm$ 3.7µg/24 hours) ($p<0.05$). Porphyrin excretion in Gunn rats was even lower with mean levels of 1.1 $\pm$ 1.0µg/24 hours, a more significant reduction when compared to heterozygous rats at $p<0.001$ by Mann Whitney U test. With Wistar rats as controls the reduction was significant compared to Gunn rats at $p<0.01$. From the results obtained for ALA and PBG excretion in Table 9 these Wistar rats exhibited high ALA and total porphyrin excretion. It has been previously described that Wistar rats appear to excrete porphyrin in a distribution pattern which is bimodal (Gartzke & Burck,1986). In contrast, investigation of the distribution of other parameters i.e. hippuric acid and creatinine, showed normal distribution. The Gunn rat is a mutant of the Wistar strain. Possibly the low porphyrin excretion observed in these animals is a result of the animal from which the mutant Gunn animal arose belonging to the group of Wistar animals with low porphyrin excretion. The greater amount of porphyrin excreted by the Gunn Wistar cross rats would be consistent with the crossing of a Gunn rat with low porphyrin excretion with a Wistar rat which excreted porphyrin at the top end of the distribution. Any possibility that the lower levels of porphyrin excreted in the Gunn and Gunn-Wistar groups of animals was due to day to day variation were discounted since 24

hour collections on Gunn and Gunn-Wistar cross rats on three consecutive days showed no significant changes - see Table 10. Animals were weight matched and excreted similar volumes of urine in 24 hours. Differences due to age were discounted by checking excretion patterns in Gunn-Wistar cross rats of a similar age to the Gunn rats in this study (2 months of age). No significant differences were found in excretion of these animals when compared to the one month old animals if results were calculated from a spot sample of urine.

The colorimetric method used to measure total porphyrins in urine is not sensitive enough to determine the pattern of porphyrin excretion. Details of the exact composition of the urine with respect to porphyrins can be obtained by HPLC techniques. To examine whether the changes in porphyrin excretion in Gunn rats reflected a different composition of urine from these animals compared to controls, HPLC was applied to pooled urine.

ii) HPLC of pooled urine

Due to the small volumes of urine collected from Gunn rats and Gunn/Wistar cross rats over a 24 hour period, urine from 72 hours of collection were pooled and derivatised to the reciprocal porphyrin esters. HPLC was then applied to the esterified porphyrins as in the methods section 2.4.4. By this sensitive method the relative amounts of each porphyrin intermediate could be determined. The results are shown in Table 11. In each of the different classes of rats the highest amount of porphyrin was excreted as COPRO. The pattern of

	GUNN	GUNN/WISTAR CROSS
DAY 1	2.5 ± 1.4	14.3 ± 1.0
DAY 2	2.7 ± 1.2	15.7 ± 1.3
DAY 3	2.0 ± 1.2	12.0 ± 5.7

Results are expressed in μg per 24 hours.

Each figure (mean ± SD) is taken from porphyrin excretion in three animals.

TABLE 10: LACK OF DAILY VARIATION IN TOTAL PORPHYRIN EXCRETION IN GUNN AND GUNN/WISTAR CROSS RATS

PORPHYRIN INTERMEDIATE	WISTAR	GUNN/WISTAR CROSS	GUNN
PROTO	4.5 ± 1.8	2.2 ± 1.8	4.6± 0.1
COPRO	48.3 ± 5.5	77.9 ± 4.1	43.1 ± 4.6
PENTA	5.7 ± 1.6	4.1 ± 0.9	8.4 ± 1.6
HEXA	2.0 ± 1.7	2.2 ± 0	10.7 ± 2.9
HEPTA	9.3 ± 2.3	5.9 ± 1.6	10.8 ± 0.3
URO	10.0 ± 7.2	6.3 ± 1.5	10.0 ± 7.0

Results are expressed as mean ± SD

TABLE 11: RELATIVE PERCENTAGES OF VARIOUS PORPHYRIN ESTERS EXCRETED IN URINE OF GUNN AND CONTROL RATS

excretion in Gunn and Wistar rats appeared to be similar while the Gunn-Wistar cross rat excreted a much higher percentage of COPRO in urine than Gunn rats. Despite the similarities in percentages of excretion between the groups of rats, the actual amount of total porphyrin excreted was significantly lower in Gunn rats compared to controls as larger volume of urine from the mutant rats had to be applied to the HPLC column to give results comparable to control rats. This is consistent with the Gunn rats originating from a Wistar rat with low porphyrin excretion and was expected after the results obtained in Table 9. Each percentage quoted is from chromatography of urine from 4 groups of rats (mean $\pm$ SD).

5.2.3 Faecal Porphyrin Excretion

i) Extraction of faecal porphyrins

The faecal porphyrin excretion was examined in detail in Gunn rats and control rats. The major constituents COPRO and PROTO were extracted from faecal matter as described in the methods section 2.5. Daily excretion for all groups is presented in Table 12. The results showed that COPRO excretion in faeces of Gunn rats was significantly reduced when compared to either Wistar or Gunn-Wistar cross controls ($p<0.001$ in both cases). PROTO excretion in Gunn rats was significantly reduced when compared to Wistar controls ($p<0.05$) and there was a trend towards lower PROTO excretion as compared to heterozygous controls although this did not quite reach significance. From these results it is likely that the lower faecal porphyrin excretion is a consequence of the type of Wistar rat which the

TYPE OF RAT	EXCRETION	
	COPRO nmol/24h	PROTO nmol/24h
WISTAR	236 ± 150	426 ± 104
GUNN/WISTAR CROSS	163 ± 26.9	290 ± 82.1
GUNN	41.8 ± 18.4	157± 69.5

Results are expressed as mean ± SD

In each group n=6

TABLE 12: DAILY EXCRETION OF FAECAL PORPHYRINS IN GUNN AND CONTROL RATS AS DETERMINED BY EXTRACTION

mutant Gunn rat arose from.

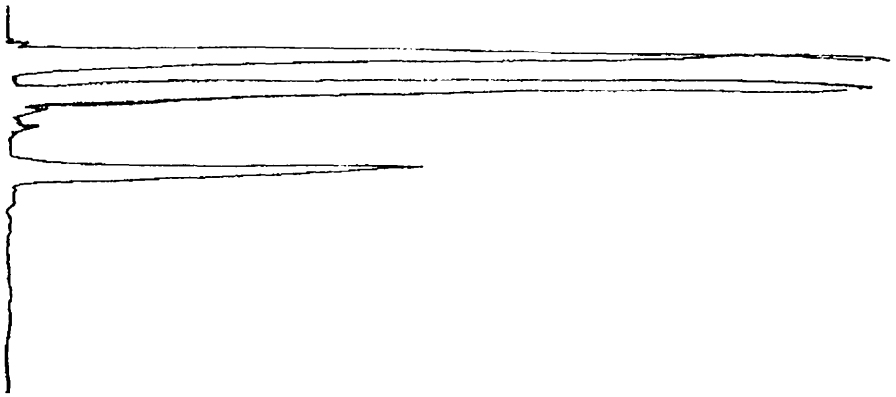
ii) HPLC of faecal porphyrins

Analysis of faecal samples was also carried out by a similar HPLC method to that used for urine (see methods section 2.5). The results obtained emphasised the reduction in COPRO excretion which occurs in the Gunn rat, with a ration of PROTO:COPRO of 3:1 in control heterozygous rats and 5:1 in homozygous Gunn rats. Typical chromatograms are shown in Figure 27.

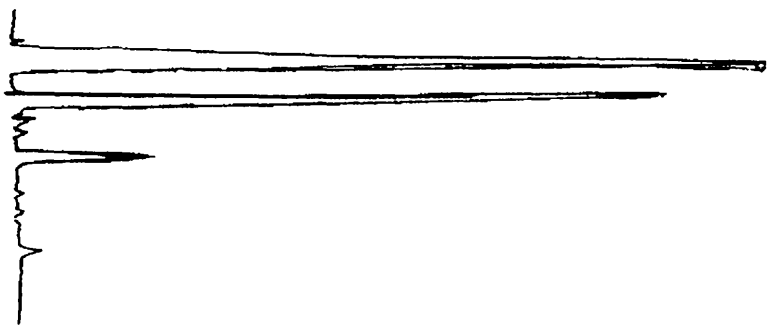
5.3 HAEM BIOSYNTHETIC ENZYMES IN THE GUNN RAT

In contrast to previous intracellular studies on bilirubin toxicity my aim was to study the interaction of bilirubin with enzymes of the haem biosynthetic pathway in the brain. The inhibition of PROTO-O activity previously observed may be a cause of the brain damage which results when kernicterus is induced. This involved study of the haem biosynthetic enzymes in rat brain. Before these studies were initiated the hepatic and renal activities of the enzymes of interest were determined in adult Gunn rats and control rats. Because of the known effect of bilirubin in reducing PROTO-O activity (Ferriera & Dailey, 1988; McColl et al, 1986) this enzyme activity was measured. ALA-synthase activity was measured to determine if disruption of the pathway at the penultimate step was causing induction of the rate controlling step of the reaction. These experiments were valuable in identifying the normal enzymic pattern of the haem biosynthetic pathway in the Gunn rat. It was hoped that any enzyme abnormalities due to bilirubin would be recognised in the easily accessible hepatic and renal

a)



b)



a) represents faecal porphyrin pattern from Gunn-Wistar cross rat

b) represents faecal porphyrin pattern from homozygous Gunn rat

Peak 1 = solvent front
Peak 2 = PROTO
Peak 3 = COPRO

FIGURE 27 : TYPICAL HPLC TRACES OF FAECAL PORPHYRINS FROM GUNN AND CONTROL RATS

tissues.

As has already been seen from the excretion results, no increases in ALA or PBG excretion are evident. However these results do not exclusively prove that there is no effect on ALA-synthase activity in these animals. It has been reported that ALA-synthase activity is raised compared to normal in leucocytes of patients with acute porphyria even when porphyrin excretion patterns are in the normal range (McColl et al, 1982). Thus ALA-synthase activity was studied in Gunn rat and control rat tissues. Activity of the enzyme COPRO-O was also determined to act as a marker enzyme. Measurement of hepatic cyt. P450 was carried out to assess whether hepatic haemoprotein production in Gunn rats was abnormal as compared to controls.

5.3.1 PROTO-O Activity in Adult Gunn Rat Hepatic and Renal Tissue

Hepatic PROTO-O activity proved easier to measure than the activity in renal tissue due perhaps to a combination of the small amount of renal tissue available and a lower intrinsic activity in the kidney. Groups of Gunn and control rats were starved for 24 hours, anaesthetised in ether and the liver and kidneys removed and perfused free of blood as in the methods section 2.2.3 The results of the hepatic and renal PROTO-O measurements are presented in Table 13. In the liver both groups of controls had similar activities with Wistar rats recording values of 6.6 $\pm$ 2.2 nmol proto formed/mg liver/hour (mean $\pm$ SD) and Gunn-Wistar cross rats activities of 7.1 $\pm$ 3.7 nmol proto formed/mg protein/h. PROTO-O activities were significantly lower in the homozygous Gunn rats (4.2 $\pm$ 2.2nmol

	WISTAR	GUNN-WISTAR CROSS	GUNN
HEPATIC	6.6 ± 2.2	7.1 ± 3.7	4.2 ± 2.2
n	6	10	11
RENAL	6.6 ± 2.1	7.0 ± 2.8	5.8 ± 2.4
n	6	6	6

Results are expressedas nmol proto formed/mg mitochondrial protein/hour and as the mean ± SD

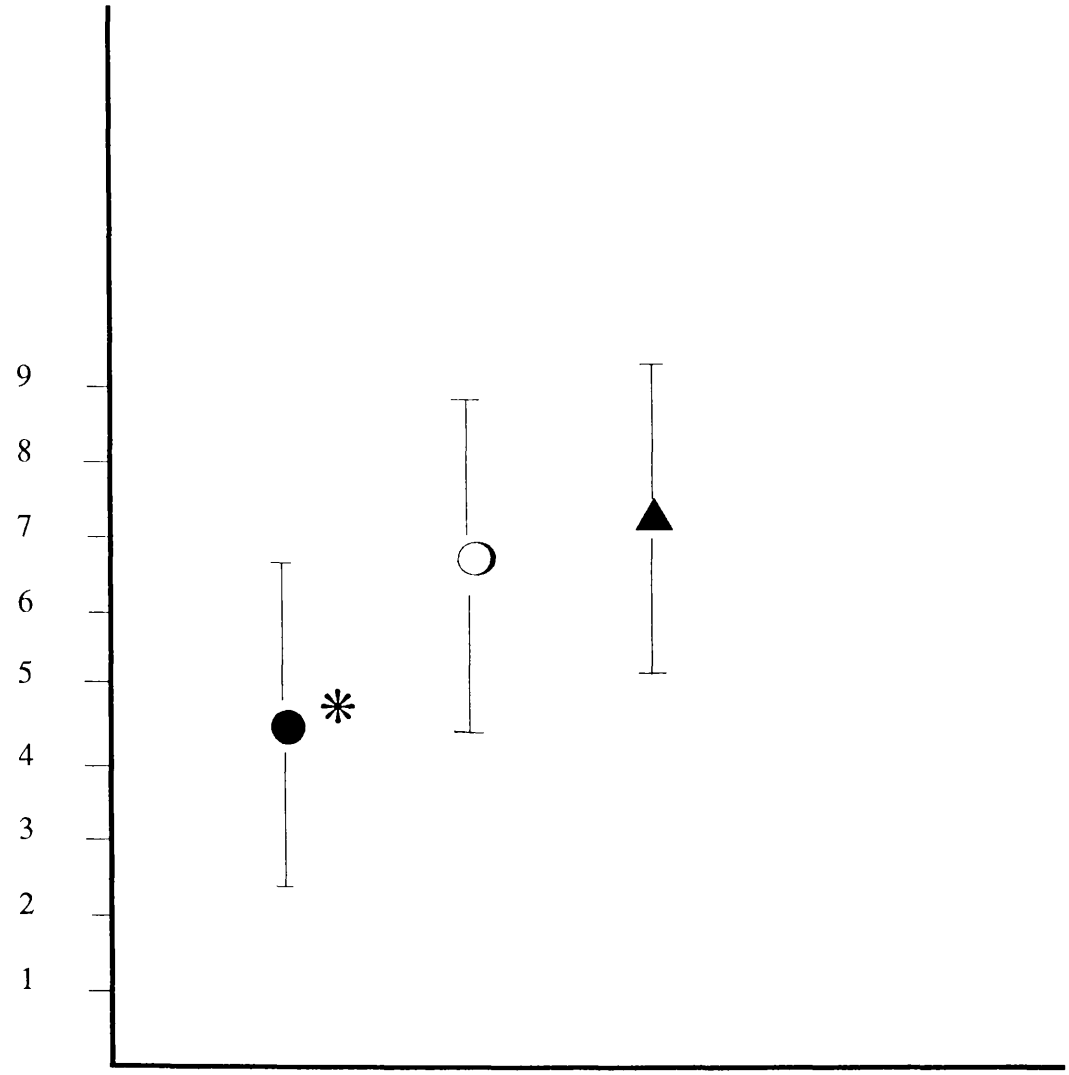
Gunn rat hepatic PROTO-O activity is significantly lower ($p < 0.05$) by Mann Whitney U test when compared to cross rats.

TABLE 13: HEPATIC AND RENAL PROTO-O ACTIVITIES IN GUNN RATS COMPARED TO CONTROLS

PROTO/h) (mean ± SD) - $p < 0.05$ when compared to activities in heterozygotes by Mann Whitney U test, but no significant differences were found in the PROTO-O activities recorded for Wistar rats compared to the other groups. The mean activities ± SD in hepatic tissue are shown in Figure 28. The Gunn Wistar cross rat was chosen as the best control in these and all other experiments as it was decided it most closely resembled the Gunn rat strain. In contrast to hepatic findings, renal PROTO-O activities were found to be similar in all three groups of rats with mean values of 6.6 ± 2.1 in Wistar, 7.0 ± 2.8 in Gunn-Wistar cross rats and 5.8 ± 2.4 nmol PROTO/mg protein/hour in Gunn rats (means ± SD). The mean activities shown in Figure 28 and in Table 13 have a large standard deviation. However this is due to the difficulty in producing a consistently pure PROTO'gen substrate. Variations in production of PROTO by the enzyme reaction occur on an inter assay basis. When comparisons were made on the same day control rat hepatic activities were found to be consistently higher.

5.3.2 COPRO-O Activity in Adult Gunn Rat Hepatic and Renal Tissue

Activity of COPRO-O, the enzyme which catalyses conversion of coproporphyrinogen to protoporphyrinogen was also measured. No evidence has so far been obtained showing that activity of this enzyme is affected by the presence of bilirubin and so measurement of COPRO-O activity was chosen as a marker for



● represents Gunn rats n = 11

○ represents Wistar rats n = 6

▲ represents Gunn-Wistar cross rats n = 10

Units are in nmol protoporphyrinogen IX / mg protein / hour

Results are expressed as mean ± SD

* significantly different to Gunn-Wistar cross ($p<0.05$) by Mann Whitney U test

FIGURE 28 : HEPATIC PROTOPORPHYRINOGEN OXIDASE ACTIVITES IN GUNN AND CONTROL RATS

enzyme activity in the Gunn and control rats. The results obtained are shown in Table 14. In all cases the COPRO-O activity of hepatic tissue was significantly higher than that of renal tissue. In liver the mean activity was 135.7 ± 54.6nmol PROTO/g protein/hour (mean ± SD) in Wistar rats. No significant difference was found between these activities and those in Gunn-Wistar cross rats (103.8 ± 40.2nmol PROTO/g protein/hour), or Gunn rats (117.4 ± 43.8nmol PROTO/g protein/h). The renal COPRO-O activities were also similar in all three groups of rats, Wistar, cross and Gunn rats having COPRO-O activities of 72.2 ± 33.3, 59.5 ± 27.8 and 90.5 ± 40.4nmol COPRO/g protein-/hour respectively.

5.3.3 ALA-synthase Activity in Adult Gunn Rat Hepatic and Renal Tissue

Hepatic and renal activity of the initial, rate controlling step of haem biosynthesis was measured in adult Gunn rats and compared to controls. The method used was the one quoted in the methods section 2.3.1 part ii) for liver. After sacrifice the liver and kidneys were quickly excised and perfused with ice-cold saline. Tissue was homogenised in ALA-synthase buffer and mitochondria prepared. All mitochondria were washed to remove bilirubin from the solution before ALA-synthase activity was measured. Results were expressed in pmolALA formed/mg protein/h. The hepatic ALA-synthase activities were all within the same range with 374 ± 70.0, 386 ± 67.4 and 307 ± 83 pmol ALA/mg protein/hour recorded in Wistar, Gunn-Wistar cross and Gunn rats respectively. Renal activities were significantly lower than hepatic levels ($p < 0.001$) and no significant differe-

	WISTAR	GUNN-WISTAR CROSS	GUNN
HEPATIC	135.7 ± 54.6	103.8 ±40.2	117.4 ± 43.8
n	7	14	12
RENAL	72.2 ± 33.3	59.5 ± 27.8	90.5 ± 40.4
n	8	8	6

Results are expressed in nmol PROTO/g protein/ and as mea ± SD

TABLE 14: HEPATIC AND RENAL COPRO-O ACTIVITIES IN GUNN RATS COMPARED TO CONTROLS

nces in activities were evident between the Gunn rat group (138 ± 29.9 pmol ALA/mg protein/h) and the control groups (148 ± 26.1 for Wistar and 124 ± 23.8pmol ALA/mg protein/h for Gunn-Wistar cross rats). The mean results ± SD are shown in Table 15.

5.4 DISCUSSION AND SUMMARY

Several aspects of haem biosynthesis have been assessed in this chapter. Blood porphyrin levels in Gunn rats were found to be similar to those in either group of control rats when porphyrin was extracted by the traditional method. My experiments clearly showed that the zinc haematofluorimeter was unsatisfactory for measurement of blood porphyrin when bilirubin is present in significant quantities.

Porphyrin patterns showed unique patterns in the different groups of rats with a significant lack of uniformity being found in the two control groups. Wistar rats excreted the highest amount of ALA but Gunn-Wistar rats excreted correspondingly higher porphyrin. Gunn rats excreted the lowest amount of precursors and porphyrin and this can not be explained by a lower body weight since weight matched rats were compared. Chromatographic separation of the various porphyrins present in urine did not provide any explanation of the lower excretion in Gunn rats as similar proportions of most of the porphyrins PROTO through to URO were found. The most likely explanation of the lower porphyrin excretion in Gunn rats is relating to the recently discovered bimodal distribution of excretion of porphyrin in Wistar rats. Two populations of this strain of rat exist when porphyrin excretion in urine is considered (Gartzke &

	WISTAR	GUNN-WISTAR CROSS	GUNN
HEPATIC	374 ± 70	386 ± 67	307 ± 83
n	8	11	11
RENAL	148 ± 26.1	124 ± 23.8	138 ± 29.9
n	6	6	6

Results are expressed in pmol ALA/mg protein/hour, and as mean ± SD

TABLE 15: HEPATIC AND RENAL ALA-SYNTHASE ACTIVITIES IN GUNN RATS COMPARED TO CONTROLS

Burke, 1986). One population excretes a much lower amount of porphyrin than the other. Since the Gunn rat is a mutant of the Wistar strain it is possible that the original parentage from which the mutant rat developed was from mating two Wistar rats with porphyrin excretion in the lower range. This would explain the levels of porphyrins recorded. COPRO excretion in Gunn-Wistar rat urine was proportionally higher than in the other two groups of rats. Measurement of faecal porphyrin excretion showed that the COPRO excretion was reduced in Gunn-Wistar heterozygotes compared to Wistar rats, not unexpected as more COPRO had been excreted in the urine of these animals. Thus the balance of excretion of COPRO between urine and faeces appeared to be different in Wistar rats compared to the cross rats. The balance appeared to be regained when faecal excretion was taken into account.

PROTO-O activity in Gunn rat hepatic tissue was lower than in the control rats. It is reasonable to suggest that this is due to an effect of bilirubin competitively inhibiting the enzyme activity. The reduction seen was not in the region recorded in vitro by Ferriera and Dailey (1988) but the mitochondrial preparations which were used in these experiments were thoroughly washed to remove excess bilirubin from the surrounding medium. This ensured that no direct effects of bilirubin could be seen on the fluorescence values recorded for PROTO-O activity. No similar reduction was observed in the renal tissue of these rats and this was more unexpected but may be due to a lower residual content of bilirubin in this tissue compared to liver where bilirubin is retained in the hepatocyte bound to

ligandin before the conjugation process occurs. The mitochondrial washing process in kidney may also have been more efficient at removing the bile pigment as it had no intracellular binding site equivalent to ligandin. This theory is consistent with bilirubin inhibiting PROTO-O activity in a competitive manner as removal by efficient wash of kidney mitochondria would result in a return to normal PROTO-O activities. It is impossible to predict the results of studies on PROTO-O activity in brain tissue as yellow staining signifying bilirubin deposition has been frequently reported and so the bile pigment once deposited may remain to cause competitive inhibition.

Measurement of ALA-synthase activity in the three groups of rats showed no significant differences in liver or kidney. This would suggest that the inhibition of PROTO-O assumed to occur in the liver in vivo is insufficient to result in a block in the pathway of haem biosynthesis in the way which is observed in variegate porphyria. ALA-synthase activity was found to be within the ranges previously observed by other workers (Strand et al, 1972; Marver et al, 1966) showing that the method employing HPLC to quantitate the ALA pyrrole produced in the assay (Fitzsimons et al, 1986) was satisfactory. COPRO-O activities in all three groups of animals and in both tissues were similar and this was expected as this enzyme activity is not inhibited by bilirubin and is not a point of control of the pathway of haem biosynthesis.

CHAPTER 6

BILIRUBIN LEVELS IN GUNN RAT BRAIN

6.1 INTRODUCTION

Bilirubin, the end product of haem degradation, is known to be toxic. As has been described in Chapter 4 the Gunn rats used in these studies have a congenital defect in bilirubin conjugation and consequently have elevated circulating unconjugated bilirubin. The increased bilirubin load leads to deposition in tissues. Liver, kidney, and fat deposits under the skin are generally stained yellow. As they age the Gunn rats fall into one of two groups - either suffering from disturbances in movement, or behaviourally remaining relatively normal although of a lower body weight and appearing hyperexcitable compared to control animals of the same age. The wobbly gait and behavioural disturbances observed are thought to result from brain damage caused by toxicity of bilirubin in the brain in a manner similar to the brain damage observed in kernicterus. However, as in patients with kernicterus, there appears to be a lack of correlation between serum bilirubin levels and the extent of the neurological symptoms at least in young rats (Sato & Semba, 1978).

This lack of predictability in development of kernicterus has caused serious difficulties in diagnosis of the condition since bilirubin levels can rise very quickly causing brain damage. The mechanism by which bilirubin exerts its toxic effect is still unclear although many putative suggestions have been made and these have been summarised elsewhere in the thesis (chapter 1.2.3).

Although bilirubin deposits have been found in many tissues, these appear to be most concentrated in the brain.

This tissue has high lipid content and so is a favourable environment for lipophilic bilirubin. Brain bilirubin levels have been previously measured and levels appear to be fairly low, in the range of 1 to 3 nmoles/ gram wet weight tissue (Katoh et al, 1975; Aono et al, 1989). The recent discovery that bilirubin *in vitro* inhibits the penultimate enzyme of haem biosynthesis, PROTO-O (Ferriera & Dailey, 1988) prompted interest on the situation *in vivo*. Depression of PROTO-O activity in leucocytes of patients with Gilbert's syndrome (McColl et al, 1986) supports the theory that bilirubin may have an effect on haem biosynthesis *in vivo*. The results presented in Chapter 5 on the levels of haem biosynthetic enzymes in Gunn rat liver and kidney show that in hepatic tissue PROTO-O activity in Gunn rats is reduced as compared to control Gunn-Wistar cross rats, while renal activity of the enzyme is similar in both groups. From these results it is impossible to predict whether PROTO-O activity in the brain of Gunn rats will be reduced or normal. The lipophilic nature of bilirubin, the high lipid content of brain tissue and the obvious yellow staining, signifying bilirubin deposition suggests that enough of the pigment may be present to compete with PROTO'gen and cause inhibition of PROTO-O activity.

The work in Chapter 5 on the hyperbilirubinaemic Gunn rats constitutes the first investigation into the effect of bilirubin on the haem biosynthetic pathway in tissues *in vivo*. This chapter continues the work by developing a method to accurately measure bilirubin content in the brain of the Gunn rat and so

determine whether a correlation between these levels and the levels in serum exists.

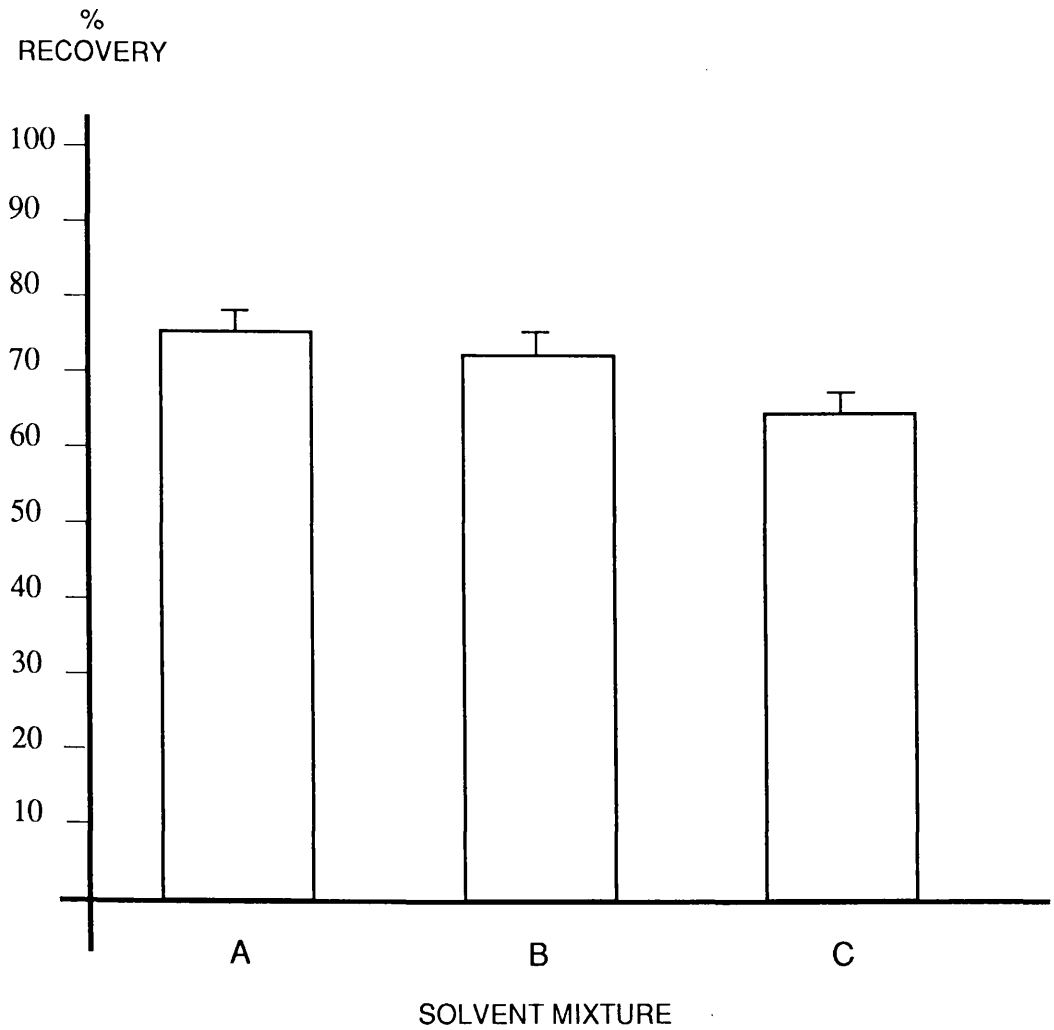
6.2 DEVELOPMENT OF METHODS FOR MEASUREMENT OF BRAIN BILIRUBIN

Measurement of brain bilirubin levels has up until now proved difficult with the most commonly used technique consisting of an organic extraction of bilirubin from the tissue followed by direct spectrophotometric quantitation. However this method may not be sensitive enough to detect slight differences in bilirubin content between various Gunn rats and additionally does not take into account losses of bilirubin which are likely to occur on extraction thus failing to provide a quantitative measure of brain bilirubin levels. Initially it was decided in our laboratory to try and determine the level of bilirubin in the brain of Gunn rats by using a combination of an extraction method similar to that previously employed (Katoh et al, 1975), and a modification of the Jendrassik-Grof diazo method which is generally used to calculate serum bilirubin and one of the methods used to determine serum bilirubin levels in this thesis.

6.2.1 Extraction of Bilirubin from Brain Tissue

Bilirubin is a lipophilic molecule, and is soluble in organic solvents. A mixture of methanol, chloroform and water has provided a successful method of isolation of bilirubin in previous work (Bratlid & Winsnes, 1971) with the bilirubin containing organic layer separating out under centrifugation. Rat brains were perfused and removed from deeply anaesthetised animals as described in the methods section 2.2.3 part iv).

Brains were homogenised in exactly 4 volumes 0.25 Molar sucrose and frozen at -80°C in the dark until measurement. Various proportions of chloroform : methanol : water mixtures were used as described in Figure 29. The optimum combination of chloroform : methanol : water was determined. From Figure 29 the best recovery of bilirubin from the organic phase of the mixture was obtained with 2:5:2 v/v chloroform : methanol : water mixture similar to that described by Katoh (Katoh et al, 1975). One volume of brain in sucrose was added to 18 volumes of the 2:5:2 v/v mixture already described. The bilirubin was then extracted using ten strokes of a Potter Elvehjem glass homogeniser with teflon pestle and a further 8 volumes of distilled water added. This step resulted in a milky suspension being formed. In samples spiked with high concentrations of bilirubin a yellow colour could be clearly seen at the bottom of the homogeniser tube. However in brains from Gunn rats no obvious yellow colour was observed at this stage. Separation of the organic and aqueous phases was achieved by centrifugation at 3000g for 15 minutes. The centrifugation step produced two clear phases with a white disc at the interface. To account for any loss of bilirubin into this interface, after removal of the lower organic phase the extraction process was repeated three times until the interface was clear. Brain homogenates spiked with bilirubin, {1mg/l or 2mg/l} were extracted with chloroform : methanol : water mixture as in the methods section 2.9.1 and the optical density measured at 450nm after one extraction and then multiple extractions.



SOLVENT A : 2:5:2 CHLOROFORM: METHANOL : WATER, 18 PARTS TO 1PART
1:5 HOMOGENATE FOLLOWED BY 8 PARTS DISTILLED WATER

SOLVENT B : 5:12:4 CHLOROFORM: METHANOL : WATER, 21 PARTS TO 1 PART
1:5 HOMOGENATE FOLLOWED BY 8 PARTS DISTILLED WATER

SOLVENT C : 3:5:2 CHLOROFORM: METHANOL : WATER, 18 PARTS TO 1 PART
1:5 HOMOGENATE FOLLOWED BY 8 PARTS DISTILLED WATER

FIGURE 29 : RECOVERY OF BILIRUBIN FROM ORGANIC EXTRACTION

Table 16 clearly shows this step did not lead to any significant increase in the amount of bilirubin extracted but did significantly increase the volume of the organic phase containing bilirubin. The lack of significant increase in recoveries of bilirubin from the aqueous and interface phases may have been due to the presence of bound fractions of bilirubin where the biomolecule attached was insoluble in the organic phase. Degradation of bilirubin which occurs slowly but significantly on exposure to light must be considered as a negating factor when time consuming extraction processes are considered. It was therefore decided to discard this further step in future experiments.

6.2.2 Identification of the Extract as True Bilirubin

The above spectrophotometric method of measurement of the yellow coloured extract from rat brain showed that in Gunn rats the product had the same spectrum as commercial bilirubin extracted from gall stones (Sigma). The identity of the extract as bilirubin was confirmed using thin layer chromatography. The organic phase prepared as in the methods section 2.9.1 was concentrated under a stream of N_2 gas to a volume of 200µl. Simultaneously commercial bilirubin was prepared as in the methods in both brain homogenate and in human serum albumin (the standard used for serum bilirubin determination). The concentrated extracts were developed on a silica gel H plate (MERCK) with 1% glacial acetic acid in chloroform solvent (McDonagh & Assisi, 1971). The results obtained showed Rf values which were very similar for commercial bilirubin and

NUMBER OF EXTRACTIONS	MEAN % RECOVERY	% RECOVERY RANGE
x 1	76	72-79
x 2	78	70-79
x 3	72	68-74

Results were the mean of 6 determinations

TABLE 16: THE EFFECT OF REPEATED EXTRACTIONS ON RECOVERY OF BILIRUBIN FROM BRAIN HOMOGENATES

bilirubin extracted from samples and confirm that bilirubin is extracted from the Gunn rat brain while no band comigrated from Gunn-Wistar cross brain extracts. Slower migration was observed for bilirubin in 4% HSA and is likely to be due to the different medium of dissolution.

6.2.3 Determination of the Nature of Brain Bilirubin

Determination of the nature of the bilirubin content of brain tissue has not previously been carried out. Bilirubin has simply been identified and assumed to consist of the unconjugated pigment. It was decided to check whether any conjugated pigment was also present in the brain. The method initially used to measure the conjugated and unconjugated fractions was based on the Jendrassik and Grof method used to measure serum bilirubin. All brain tissue was perfused free of blood prior to homogenising in sucrose as described in methods section 2.9.1. Extraction of bilirubin was by the method previously described in this section of the methods. After the organic layer containing bilirubin had been separated, the solution was evaporated to dryness and the resulting residue resuspended using the same medium for preparing standards for serum bilirubin determination. Brain bilirubin levels were measured and serum bilirubin levels were read off a calibration curve constructed as in the methods section 2.7.2. The results of serum and brain bilirubin levels of 3 Gunn animals and age matched Gunn-Wistar controls are presented in Table 17. Brain total bilirubin concentration was calculated from the optical density and initially reported in mg/ml. This was then

RAT	SERUM BILIRUBIN			BRAIN BILIRUBIN
	TOTAL µmolar	CONJUGATED µmolar	UNCONJUGATED µmolar	UNCONJUGATED nmol/gram wet wt.
GUNN 1	122	10	112	143
GUNN 2	109	8	101	129
GUNN 3	100	11	89	126
CONTROL 1	12	6	6	116
CONTROL 2	10	4	6	112
CONTROL 3	4	2	2	29

TABLE 17:
SERUM AND BRAIN BILIRUBIN LEVELS IN GUNN RATS COMPARED TO CONTROLS BY MODIFIED JENDRASSIK GROF METHOD

corrected for the 1 in 4 dilution of brain tissue with sucrose and the final result converted to nmol/g wet weight from µg/gram wet weight tissue. These results clearly show several things; serum total bilirubin levels in Gunn rats are elevated far above that of controls and the raised fraction is predominantly unconjugated pigment. This method for measurement of brain bilirubin appears to show that brain bilirubin content of control rats is almost as high as that of Gunn rats. This is in contrast to the results obtained by t.l.c. and use of direct spectrophotometric measurement has previously revealed little or no bilirubin content of control rat brain. In addition, the observation of whole brain where yellow staining can be clearly seen in Gunn rats and is absent in controls. The high final results obtained for all the rats suggests that the high absorbances recorded are due to another component of brain which is being resuspended along with bilirubin. It is also worth noting that on resuspension of the residue before quantitation it is difficult to obtain a non-turbid solution suitable for spectrophotometric analysis.

Separation of bilirubin from the component of brain causing the artificially high absorbances was attempted by several methods.

a) Detergent

Addition of detergent (Triton X-100) at concentration 500µl/ml at the homogenisation stage only resulted in turbidity which could not be sufficiently removed for accurate spectrophotometric measurement.

b) Filtration

Filtration, using Millipore filters (0.45μm, material) prior to diazo-conjugation resulted in a tenfold reduction in apparent total bilirubin as measured by Jendrassik Grof method. However it is likely that a large proportion of the bilirubin content of the homogenate was also removed on filtration. Filtration by the same system after diazo conjugation also reduced the apparent brain bilirubin level but to a lesser extent supporting this theory and this is shown in Table 18. Unfortunately this method of filtration failed to include an assessment of recovery of bilirubin and so was not pursued as a useful method of purifying bilirubin extracted from brain tissue.

6.2.4 Purification of Brain Bilirubin and Gradient HPLC

At this point the validity of attempting to use a modification of the method of Jendrassik-Grof was reassessed. Measurement had so far been unsuccessful due largely to turbidity of solution and lack of solubility of the successfully extracted bilirubin from brain tissue in the reagents used for diazo measurement. Filtration and separation techniques so far tried were unhelpful.

One of the most powerful tools of separation is HPLC. These techniques have been previously applied to bilirubin in serum and are described in the methods section 2.7.3. Because of the failure of the above techniques to adequately isolate and quantitate bilirubin it was decided to change the direction of the work and concentrate on trying to produce a method for

BILIRUBIN LEVELS
nmol/gram wet weight tissue

	TOTAL	CONJUGATED	UNCONJUGATED
A	700	210	490
B	60	-	60
C	320	10	310

A: Gunn rat brain prepared as in methods with no filtration

B: Gunn rat brain prepared as in methods, filtered prior to diazo-reaction

C: Gunn rat brain prepared as in methods, filtered after diazo reaction prior to absorbance measurement

All samples were prepared from one original rat brain

TABLE 18: EFFECT OF FILTRATION ON DETERMINATION OF BRAIN BILIRUBIN LEVELS

measurement of brain bilirubin by HPLC. As described in the methods sections 2.7.3 two methods for serum bilirubin measurement were successfully used in the construction of this thesis to qualitatively analyse bilirubin. The hope was that either or both of these methods could be adapted as necessary to quantitatively measure bilirubin in brain. As in the experiments in secton 6.2.2, brain homogenate from control rat brain spiked with a known concentration of bilirubin was used to develop a method for isolation of bilirubin with adequate recovery for application to an HPLC column. For preservation of the octadecyl (C18) column the preparations had to be as free from contaminating proteins and lipids which are endogenous to brain as possible. Several attempts were made before the optimal clean-up procedure for brain bilirubin was determined.

a) Protein Precipitation

i) TCA Precipitation

Brain tissue homogenised in sucrose as described in methods 2.9.1 was added to an equal volume of 10% TCA. The mixture was vortex mixed and then centrifuged at 3000 xg to remove precipitated protein. The pH of this solution was then raised to neutral, compatible with mobile phase for injection into HPLC.

This crude method of protein removal was unsuccessful since the majority of bilirubin remained in the precipitate. Consequently only a very small peak attributable to bilirubin was detectable by HPLC.

ii) Sodium Sulphate Precipitation

A near saturated solution of sodium sulphate has been used to successfully precipitate serum proteins and so extend column life by Lauff (Lauff et al, 1981) and a micro method has been used to identify serum bilirubin fractions in chapter 4 of the thesis. For brain 0.5ml of homogenate in sucrose was added to 7ml sodium sulphate solution (27.7g/100ml) at 37°C and the solution occasionally shaken for 5 minutes. The resulting suspension was then filtered (MILLEX, 0.45μm) into a 20ml volumetric flask containing 1ml ascorbic acid and made up to volume with more sodium sulphate solution. The resulting solution was not as observed for serum a clear solution suitable for injecton into HPLC. This was thought to be due to the presence of non-protein entities, in addition to proteins outwith the molecular weight range precipitated by this molarity of sodium sulphate solution. Thus use of protein precipitating methods suitable for serum is incompatible with removal of contaminating molecules in brain.

c) Solid Phase Extraction

One method which has proved successful in purifying various biological compounds has been that of solid phase extraction. This has already been employed elsewhere in this thesis, in isolation of ALA-pyrrole in the HPLC assay for ALA synthase.

The chemical structure of bilirubin shown in figure 10 (Introduction) means there are several sites which may be charged, and these could form temporary bonds under suitable conditions with the right solid medium. After isolating

so releasing the more concentrated bilirubin into solution for quantitation. The strongest charges on bilirubin are those of the carboxyl groups of the unconjugated form. These groups are strongly anionic (COO^-) in alkali. The strong anion exchanger (SAX,1cm^3 BOND-ELUT) was a suitable solid phase. The SAX column was activated with methanol and primed with buffer consisting of $CHCl_3$:MeOH:H_2O 2:5:2 at pH 11. Methanolic KOH was used for raising pH of solutions.

Brain spiked with bilirubin was extracted in the normal way and the pH of the organic phase raised with methanolic KOH to approximately pH 11. The solution was then applied to the SAX column where it bound in a tight band. The column was thoroughly dried to remove all traces of buffer. Various combinations of buffer were tried to remove the bound bilirubin. The most successful one was a mixture of chloroform, methanol and HCl. 3:5:5 v/v $CHCl_3$:MeOH:HCl (1 Molar) removed the highest proportion of bilirubin but there were still large losses of bilirubin which remained irreversibly bound to the column. Use of alternative alcohols with higher polarity eg isopropyl alcohol resulted in conversion of the yellow colour to a green colour which did not separate on HPLC to give the same retention time as bilirubin. It appeared the bilirubin had been converted or degraded by mixing with this alcohol.

Due to the large volumes of buffer required to recover substantial bilirubin from the SAX column, concentration of the solution for application to HPLC was tedious and likely to result in losses due to exposure to light. Solid phase sample

preparation as a means of purification of bilirubin before application to HPLC by this method were abandoned.

None of the above attempts used to try and isolate bilirubin and make measurement easier were successful more often resulting in such poor recoveries that little or no peak could be detected on HPLC using the gradient system of HPLC used for qualitatively identifying serum bilirubin. More success was achieved using the isocratic method of HPLC quoted in the methods section 2.7.3.

6.2.5 Isocratic Reversed Phase HPLC of Bilirubin

An isocratic method for measurement of bilirubin isomers in serum was developed by McDonagh (McDonagh, unpublished observations) and the conditions are described in the methods section 2.7.3 part ii). For serum determination, either serum or plasma is added to mobile phase directly, the resulting suspension centrifuged by an eppendorf centrifuge and the supernatant directly injected onto the column. Resolution of bilirubin isomers is achieved by addition of a percentage of distilled water to the organic mobile phase.

In our laboratory through trial and error using spiked serum bilirubin standards and serum from human and rat sources the optimum proportion of water was determined to be 5%. This allowed resolution of the bilirubin isomers in approximately 12 to 13 minutes, with the unconjugated peak having a retention time of between 8 and 10 minutes. A further increase to 8% water led to doubling of the retention times with significant loss of resolution and peak sharpness.

Brain tissue homogenate was directly added to mobile phase in the proportion 1:4 as recommended for serum. After centrifugation in an eppendorf centrifuge, the resulting supernatent was injected onto the column. As might have been expected from previous attempts to measure bilirubin without extraction no bilirubin was observed emphasising the need for removal of bilirubin from heterogeneous medium of brain.

After organic extraction with $CHCl_3$:MeOH:H_2O the chloroform phase was concentrated under N_2 and mixed with mobile phase. A peak attributable to unconjugated bilirubin was seen with a retention time of 8.6 minutes. This method of concentration under gas followed by mixing with mobile phase was not ideal for quantitation and so the solution was evaporated to dryness and then resuspended in a known volume of mobile phase. These samples were still fairly cloudy and so a guard column was used to extend column life. This method still failed to accurately quantitate bilirubin content in brain since losses incurred on extraction were not accounted for. Thus the losses on extraction were taken into account by use of an internal standard.

6.2.6 Identification of an Internal Standard for HPLC of Bilirubin

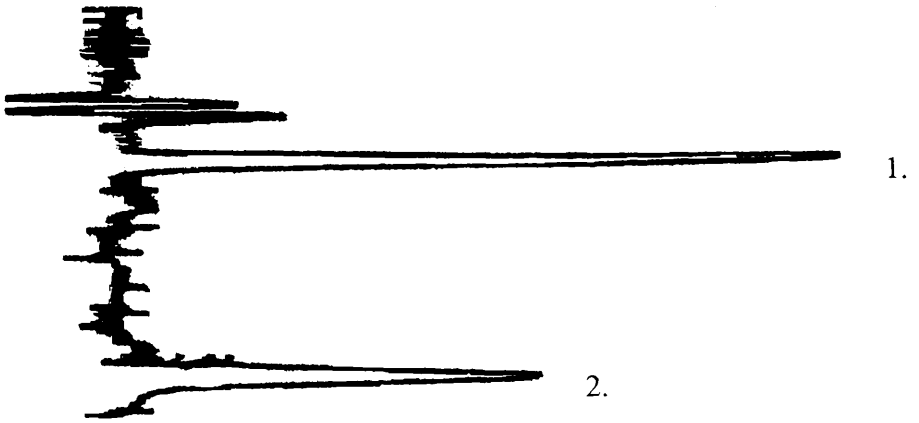
Losses of bilirubin extracted from brain tissue by organic solvents were high as described in Figure 29. The easiest way to compensate for these losses was to identify an internal standard for addition at the homogenisation stage of rat brain. Study of coloured compounds which would be detected at the same wavelength as bilirubin revealed a laser dye Coumarin 6 (Kodak)

with a similar spectrum. This was one of a range of laser dyes which have often been used as internal standards for HPLC.

Coumarin 6 was found to dissolve easily in organic solvents, including the mobile phase for this HPLC method to give a highly fluorescent yellow/green colour. On injection, retention time was found to be between 2 and 3 minutes, early enough to be totally separate from the unconjugated bilirubin peak and so likely to be useful as an internal standard. DMSO was chosen to dissolve Coumarin 6 since it exhibited no peak on HPLC and was a component used to dissolve commercial bilirubin used as standard. Stock Coumarin 6 was made up as described in the methods and 100ng was added in 20µl aliquots to brain homogenates. This gave a peak in the same range as bilirubin extracted from brain tissue and is shown in Figure 30.

6.2.7 Quantitation of Brain Bilirubin

A standard curve for quantitation of bilirubin in brain was set up using brain samples spiked with commercial bilirubin and extracted. The standard curve obtained is shown in Figure 31. Quality control samples containing 1.7nmol/g wet weight commercial bilirubin in brain homogenate, extracted as with the samples were regularly run. Results were corrected for bilirubin recovery by the presence of internal standard.



Peak 1 is internal standard (Coumarin 6, 100ng) - retention time 2.74 minutes

Peak 2 is brain bilirubin - retention time 7.30 minutes

FIGURE 30 : A TYPICAL HPLC TRACE FOR BRAIN BILIRUBIN

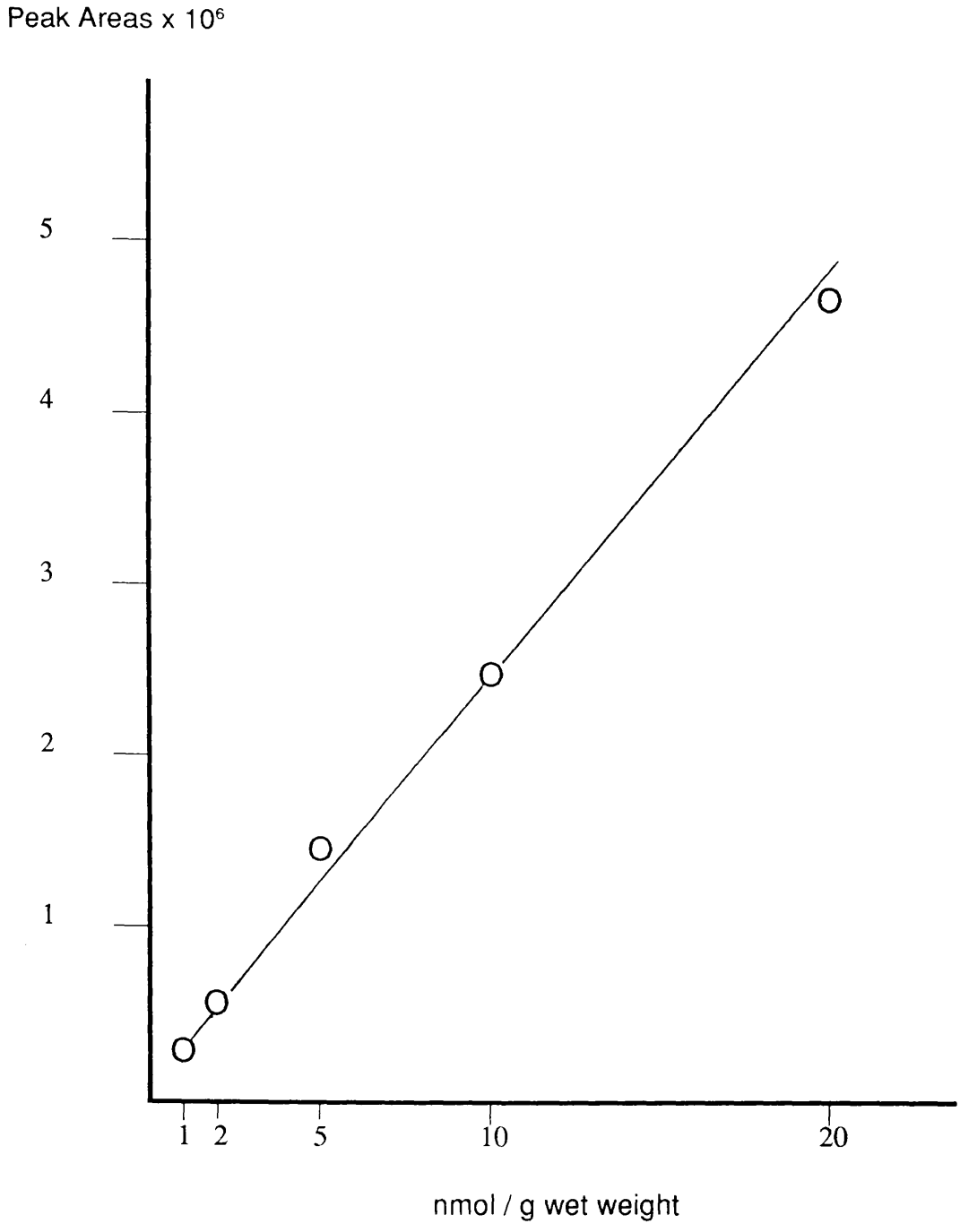


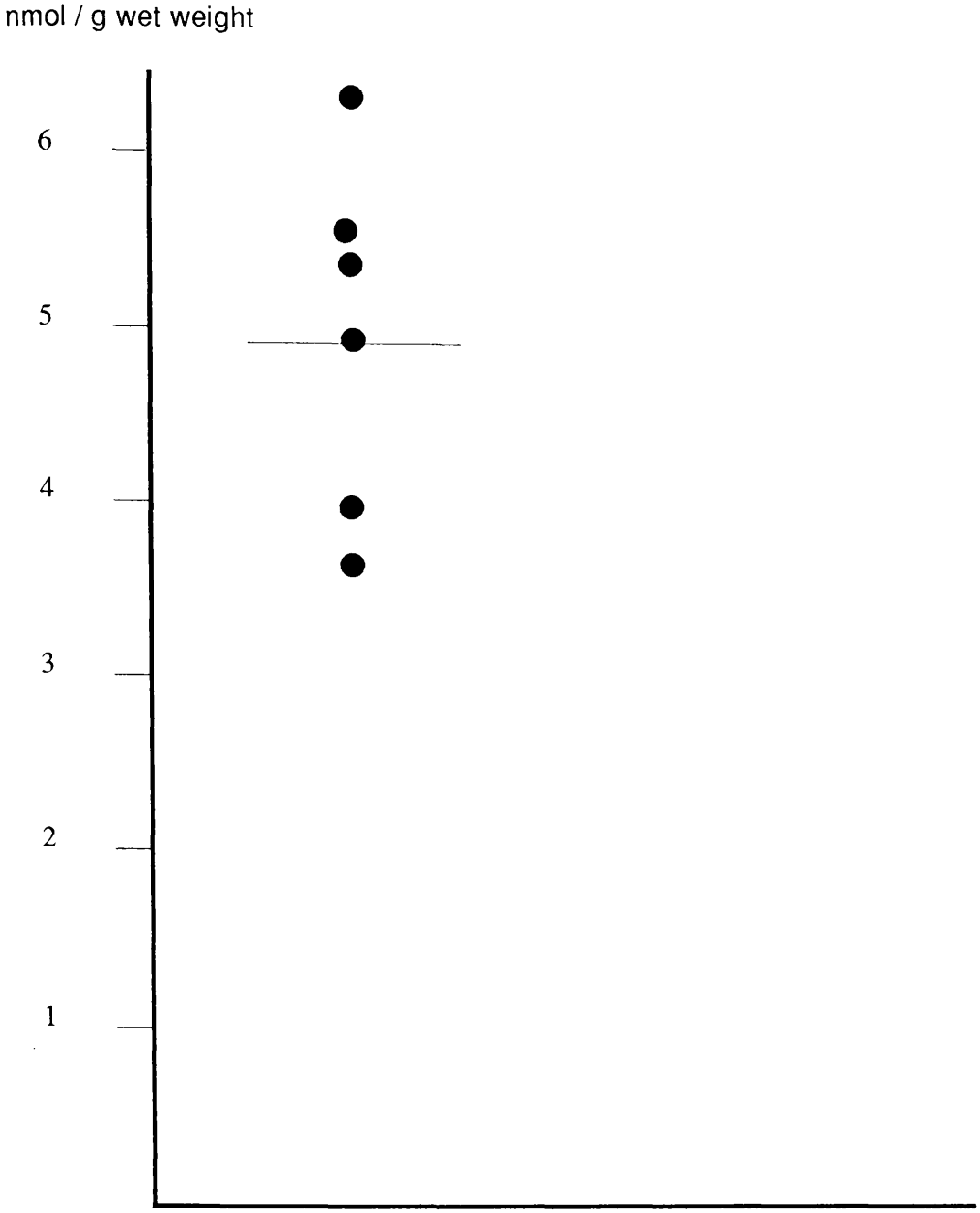
FIGURE 31: CALIBRATION CURVE FOR BRAIN BILIRUBIN LEVELS

6.3 BRAIN BILIRUBIN LEVELS IN ADULT GUNN RATS COMPARED TO CONTROLS

Adult Gunn rats were anaesthetised using ether and the brains perfused through the heart with 0.9% NaCl as described in section 2.2.3. Brain tissue free of blood was removed and homogenised in sucrose. Internal standard (Coumarin 6, 100ng) was added. Bilirubin was extracted using the method in section 2.9.1, resuspended in 100µl mobile phase for isocratic determination of bilirubin and injected onto the column. The results of measurement of brain bilirubin in 6 adult Gunn rats and 6 control rats are presented in Figure 32. Levels in Gunn rat brain of 2.9 $\pm$ 0.5 µg/gram wet weight tissue or 5.0 $\pm$ 0.9 nmol/gram wet weight tissue are within the same range as those values in the literature (Katoh et al, 1975; Sawasaki et al, 1976; Aono et al, 1989). The total lack of recording of bilirubin in control Gunn-Wistar cross rats also agrees with the literature which quotes no spectrophotometric peak for bilirubin (Sawasaki et al, 1976). No peak was seen on the HPLC trace.

6.3.1 Effect of Anaesthetic Used on Brain Bilirubin in Gunn Rats

The ether used up until now in these experiments was as an inhaled drug which induced anaesthesia. Any possibility that ether may cause partition of bilirubin to tissues other than the brain was discounted by comparing the bilirubin content of Gunn rat brains anaesthetised with ether to that of rats injected with a barbiturate anaesthetic. In these experiments sacrifice by cervical dislocation was impractical as an intact CNS capillary circulation was required to allow perfusion of the



——— represents the mean of Gunn rat brain bilirubin determination. Levels in Gunn-Wistar cross rats were not measurable.

FIGURE 32: BRAIN BILIRUBIN LEVELS IN GUNN RATS

brain free of blood which would contain contaminating bilirubin and may give falsely high results. There was no significant difference in the bilirubin content of rat brains by either method of anaesthesia. Bilirubin content of Gunn rat brain anaesthetised with ether was 3.1 $\pm$ 0.4 μg/g wet weight tissue while comparative content of Gunn rat brain using barbiturate anaesthesia was 2.9 $\pm$ 0.4 μg/g wet weight tissue. These were the results of 3 experiments repeated in duplicate.

6.3.2 Sex Difference in Bilirubin Content of Gunn Rat Brain

Using the isocratic HPLC method previously described the bilirubin content of Gunn rat adult male brain tissue was compared to that of female Gunn rats of similar age. The results are presented in Table 19. The rats used were 10 months old. Adult female Gunn rats had significantly lower body weights (275g) than their male counterparts (325g). Bilirubin content in male rat brain was significantly higher than that of the female. Interestingly no significant difference was found in the weights of Gunn rat female and male brains, 1.94 $\pm$ 0.21g in male rats as compared to 1.81 $\pm$ 0.32g. It is postulated that the different hormonal balance may have some bearing on bilirubin deposition in Gunn rat brain. Additionally lipid content of female peripheral body cells is likely to be higher than their male counterparts, as a result of hormone balance. The lower bilirubin content of brain may reflect higher deposition of bilirubin in peripheral tissues. The fact that there was no significant difference in the serum bilirubin levels between male and female rats is in agreement

	GUNN RAT MALE	GUNN RAT FEMALE	
AGE (MONTHS)	10	10	
MEAN WEIGHT (g)	326 ± 24	274 ± 26	*
WEIGHT RANGE (g)	300-375	250-320	
[a]TOTAL SERUM BILIRUBIN μmol/litre (mean ± SD)	66.2 ± 15.8	70.7 ± 8.3	
[a]BRAIN BILIRUBIN nmol/g wet wt (mean ± SD)	4.9 ± 0.8	2.7 ± 0.7	**
n	6	6	

a - each result is the mean of duplicate determinations

* $p < 0.01$ by Mann Whitney U Test

** $p < 0.001$ by Mann Whitney U Test

TABLE 19: BILIRUBIN LEVELS IN ADULT MALE GUNN RAT BRAIN AS COMPARED TO FEMALE RATS

with the citing in the literature that little correlation exists between serum total bilirubin levels and brain bilirubin content.

6.4 DISCUSSION AND SUMMARY

A method has been developed for accurate measurement of bilirubin content in Gunn rat brain.* This method utilises the separation abilities of HPLC to provide a more sensitive method than the previous spectrophotometric ones. Brain bilirubin levels in adult Gunn rats in these experiments were discovered to be in the same range as reported in the literature. The use of ether as an anaesthetic for the studies proved to have no different effect on the bilirubin levels found in Gunn rat brain when compared to a barbiturate as anaesthetic. Study of the sex differences of brain bilirubin levels showed that female rats appeared to have less deposition of bilirubin centrally than their male counterparts. Even with this method which had a lower limit of detection of 0.2 nmol/g wet weight of tissue no bilirubin could be detected in the brains of control Gunn-Wistar cross rats or Wistar rats. It is possible that levels in these animals are lower than the limit of detection of this method.

*see appendix 1

CHAPTER 7

THE EFFECT OF BILIRUBIN ON BRAIN HAEM BIOSYNTHESIS

7.1 INTRODUCTION

From the details given in previous chapters and from the literature the Gunn rat appears to be the animal model which most closely resembles the human clinical condition of kernicterus in infants. The behaviour of adult Gunn rats is not entirely normal when compared to the heterozygous Gunn-Wistar cross or Wistar rats. The rats are prone to excitability especially when disturbed and this is attributed to the neurological damage caused by hyperbilirubinaemia. However the extent of the brain damage suffered is thought to be mild. In contrast to infants with kernicterus the rats lead fairly normal and active lives. These animals can suffer from more severe symptoms but these tend to die within the first few days of life. The haem biosynthetic pathway in the brain of the Gunn rat has not previously been studied. The results in Chapter 5 clearly show that the presence of bilirubin can lead to inhibition of PROTO-O activity in an *in vivo* model of hyperbilirubinaemia. In this section of the thesis haem biosynthesis in the brain of these animals with elevated bilirubin will be examined.

7.2 HAEM BIOSYNTHESIS IN THE ADULT GUNN RAT BRAIN

Adult Gunn and control (Gunn-Wistar) rats were starved for 24 hours and sacrificed. Blood was removed for serum bilirubin determination. Brain tissue was perfused through the heart, quickly removed and evenly divided between the hemispheres. Tissue was kept on ice after removal from the animal. One half of the brain was homogenised in 0.15 Molar KCl and mitochondria

were prepared for PROTO-O assay as in the methods section 2.3.4. The other half was homogenised in 0.25 Molar sucrose and homogenates stored at -20°C until extraction of brain bilirubin could be carried out. Bilirubin was extracted as in section 2.9.1 and assayed using the isocratic HPLC method described in section 2.9.2.

In agreement with the results obtained in previous chapters serum bilirubin and brain bilirubin levels were significantly higher in Gunn rats as compared to controls when statistically compared by Mann Whitney U test ($p<0.001$). PROTO-O activities in Gunn rat brain tissue were comparable to those in control rat brain (8.7 $\pm$ 2.7nmol PROTO/mg protein/hour as compared to 9.2 $\pm$ 3.0nmol PROTO/mg protein/hour). All these results are shown in Table 20. Therefore the levels of bilirubin found in the brain of adult Gunn rats appears to be insufficient to cause disruption of the haem biosynthetic pathway.

It is possible that the bilirubin levels in Gunn rat brain, while elevated far above normal levels as recorded both in Chapter 6 and in this chapter are still not in parallel with the levels which result in human kernicterus and that the brain damage observed is of a far milder form. If this is the case then a truer model of kernicterus may result if brain bilirubin levels in Gunn rats were further elevated.

	GUNN	GUNN-WISTAR CROSS
ALA-synthase activity	54.7 ± 9.3	46.9 ± 8.9
PROTO-O activity	8.7 ± 2.7	9.2 ± 3.0
BRAIN BILIRUBIN	5.4 ± 1.4 *	-
SERUM BILIRUBIN	65 ± 13 *	4.0 ± 2.0

Results are expressed as mean ± SD

n=5 in all cases

* significantly higher ($p<0.001$) by Mann Whitney U test.

ALA-synthase activity is in pmol ALA/mg protein/h, PROTO-O activity is in nmol PROTO/mg protein/h. Brain bilirubin is expressed in nmol/g wet weight tissue and serum bilirubin is expressed as μmol/l.

TABLE 20: HAEM BIOSYNTHETIC ENZYME ACTIVITIES AND BILIRUBIN LEVELS IN ADULT GUNN RAT BRAIN COMPARED TO CONTROLS

7.3 ELEVATION OF BRAIN BILIRUBIN IN GUNN RATS

The lack of inhibition of the penultimate enzyme of haem biosynthesis in brain by bilirubin as was observed in liver of adult Gunn rats was disappointing. It appears levels of bilirubin in Gunn rat brain require to be increased further before effects of a similar nature to those in human kernicterus are seen. The brain damage observed in Gunn rats can be increased. Several mechanisms of producing symptoms resembling kernicterus have been employed. Use of the neonatal animal is popular (Sawasaki et al, 1976). The popularity of this model was thought to lie in the immaturity of the blood brain barrier which made access of bilirubin to the brain easier, but the validity of this observation is now disputed (Davis & Yeary, 1975). The most common method of observation of an icteric state has been by administration of a displacing agent for bilirubin from albumin (Katoh et al, 1975; Rose & Wisniewski, 1979; Aono et al, 1989). Displacement of bilirubin from albumin in the circulation greatly increases the proportion of free bilirubin, and this would then have access to the tissues. Several classes of drugs themselves bind tightly to albumin so causing release of bilirubin from albumin and potentially allowing free access to brain tissue for deposition. In the literature sodium buculome (Aono et al, 1989), novobiocin (Katoh et al, 1975) and a range of sulphonamides have been used to stimulate release of bilirubin. The most common sulphonamide used has been sulphadimethoxine (Davis & Yeary, 1975; Rose & Wisniewski, 1979). Another model which has become popular more recently is one where the blood brain barrier is disrupted in

some way (Levine et al, 1982; Wennberg & Hance, 1986), allowing free access of both the free form of bilirubin and the albumin-bound form. As previously discussed in section 1.5.2 several authors believed that the increased toxicity of bilirubin in newborns was a result of an immature blood brain barrier. Recent observations are more of the opinion that entry to the brain increases under conditions where the blood brain barrier has been disrupted and rendered open by hypoxia, hypertension or other abnormal states. Certainly in adult rats where the BBB is intact even treatment with high doses of bilirubin displacing drugs does not appear to cause the extensive brain damage seen in icteric infants.

Much evidence is consistent with the theory that free bilirubin is the form which can enter the brain and cause the brain damage (Diamond & Schmid, 1966; Maisels et al, 1981; Cashore & Oh, 1982). Despite these reports conclusive proof on a role of free bilirubin as the cause of the neurological damage is absent from the literature (Levine, 1979; Levine et al, 1982). A recent report suggests that both the state of BBB and free bilirubin level are important (Wennberg & Hance, 1986).

For the remainder of the experiments to determine the effect of bilirubin on haem biosynthesis in the brain it was elected to use neonatal Gunn rats at 15 days old. It was elected to produce symptoms of kernicterus by displacing bilirubin from albumin in these animals. The combination of these two methods has been successful in the past (Katoh et al, 1975; Rose &

Wisniewski, 1979; Aono et al, 1989). One of the most successful drugs has been sulphadimethoxine. This sulphonamide was used to raise free bilirubin levels in the experiments using neonatal Gunn rats to determine effects of bilirubin on brain haem biosynthesis. The intention was to study the possible effects which bilirubin might have on the haem biosynthetic pathway in the brain. Assays for measurement of ALA-synthase and PROTO-O activity have been worked up for brain tissue. Bilirubin in serum and brain has also been measured. From the results obtained in Chapter 5 of the effect of bilirubin on PROTO-O activity in hepatic tissue it was hoped study of a potential effect of bilirubin on the brain haem biosynthetic pathway may be another aspect of the toxicity to the CNS suffered in kernicterus. Groups of age matched Gunn and Gunn-Wistar control rats were compared. Several characteristics of the rats were considered.

7.3.1 The Effect of Bilirubin on Physical Characteristics of Neonatal Gunn Rats

The physical characteristics, body weights and tissue weights of 15 day old Gunn rats were compared to matched Gunn-Wistar controls.

Body weights of Gunn rats aged 15 days were compared to body weight of Gunn-Wistar cross rats of the same age. The Gunn rats were from the same litter. The cross rats were from one litter but were not littermates of the Gunn animals. As Table 21 clearly shows Gunn rats were significantly lighter than their cross counterparts of the same age - $p<0.001$ by Mann Whitney U test. In all cases cross rats were at least 150% the weight of

		GUNN	GUNN-WISTAR CROSS
a	MEAN PHYSICAL WEIGHT	18.1 ± 4.6	27.5 ± 4.6*
	RANGE	10.0 - 25.0	20.0 - 35.0*
a	MEAN LIVER WEIGHT	0.51 ± 0.11	0.86 ± 0.21 *
a	MEAN KIDNEY WEIGHT	0.16 ± 0.02	0.31 ± 0.04 *

a = results expressed as mean ± SD

* = significantly higher ($p<0.001$) by Mann Whitney U test.

n= 10 in all cases

TABLE 21: PHYSICAL WEIGHTS AND TISSUE WEIGHTS OF 15 DAY OLD GUNN RATS COMPARED TO CONTROLS

the Gunn animals. As might be expected from these observations the tissue weights from Gunn animals were also significantly lower - $p < 0.01$ by Mann Whitney U Test.

7.3.2 Haem Biosynthesis in Hepatic and Renal Tissue of 15 Day Old Gunn Rats Compared to Controls

Haem biosynthesis in hepatic and renal tissue of Gunn and age matched Gunn-Wistar cross rats were compared to establish that the pattern was similar to the one seen in adult rats.

15 day old Gunn and Gunn-Wistar cross rats were anaesthetised in ether, blood removed for serum bilirubin determination and livers and kidneys excised and perfused as in section 2.3.2. Tissues were then equally divided and half was homogenised in buffer for ALA-synthase assay while the remainder was homogenised in KCl for PROTO-O assay as previously described (2.3.1. and 2.3.4.). Both assays were carried out after preparation of mitochondria. Tissue weights were small and tissues from two animals were pooled wherever possible to provide enough tissue for all analyses.

Hepatic and renal ALA-synthase levels in Gunn and Gunn-Wistar cross rats were not significantly different in the two groups with activities of 365 $\pm$ 74 and 305 $\pm$ 81pmol ALA/mg protein/h respectively, mean $\pm$ SD seen in liver and 107 $\pm$ 42 and 74 $\pm$ 28pmol/mg protein/h in kidney. These results are shown in Table 22. The adult pattern of a lower activity in kidney in both groups of animals was maintained. From these results the enzyme activities in liver and kidney of neonatal rats are in the same range as those seen in adults demonstrated in Table 15

	GUNN	GUNN-WISTAR CROSS
HEPATIC pmol ALA/ mg protein/h	365 ± 74	305 ± 81
n	7	8
RENAL pmol ALA/ mg protein/h	107 ± 42	74 ± 28
n	6	8

Results are expressed as mean ± SD

TABLE 22: ALA-SYNTHASE ACTIVITIES IN HEPATIC AND RENAL TISSUE OF 15 DAY OLD GUNN RATS AND CONTROLS

of Chapter 5. Similarly PROTO-O activities in the same groups of Gunn and Gunn-Wistar cross rats were compared and the activities in hepatic tissue of the Gunn rat group were found to be significantly lower than those in the heterozygous group ($p<0.05$ by Mann Whitney U test) as is demonstrated in Table 23.

7.4 TREATMENT OF 15 DAY OLD GUNN AND CONTROL RATS WITH SULPHADIMETHOXINE AND DETERMINATION OF BRAIN ENZYME ACTIVITIES

15 day old Gunn rats, where possible from the same litter were divided into two groups. One group of animals were injected ip with a dose of 50mg/kg body weight sulphadimethoxine and these constituted the drug treated animals. The remainder were injected with carrier only and were the control Gunn group. Concurrently, age matched Gunn-Wistar cross rats were similarly divided into control and drug treated groups. After administration of the drug or placebo the animals were separated from their dams and maintained under a heat lamp for 5 hours to enable the drug to displace bilirubin and for deposition in the brain to occur. After the 5 hour period, rats were sacrificed, blood was collected for bilirubin estimation, and body tissues perfused free of blood. The brain was then excised, weighed and divided such that bilirubin content, PROTO-O and ALA-synthase activities could be measured. Due to the small amounts of tissue available in most cases the brains of two animals were pooled. Brain tissue was halved between the hemispheres, and the halves from different rats combined. Before homogenisation in the appropriate buffer a small portion was removed from each of the combined brains and prepared for brain bilirubin estimation as detailed in section 2.9.

	GUNN	GUNN-WISTAR CROSS
HEPATIC	12.3 ± 3.5	17.7 ± 1.5
RENAL	18.2 ± 1.8	12.6 ± 0.9

n=8 in all cases

Results are expressed as mean ± SD and in nmol PROTO/mg protein/hour

TABLE 23: PROTO-O ACTIVITIES IN HEPATIC AND RENAL TISSUE OF 15 DAY OLD GUNN RATS COMPARED TO CONTROLS

7.4.1 Bilirubin Levels in Serum and Brain of Treated and Untreated Rats

FromTable 24 serum bilirubin levels in 15 day old Gunn rats which have been untreated are significantly higher than those of their Gunn-Wistar cross controls ($p<0.001$ by Mann Whitney U test) as was expected since these rats exhibit the congenital defect in bilirubin conjugation. Serum bilirubin levels in the Gunn-Wistar cross rat group did not overlap with those levels seen in Gunn rats (11.7 $\pm$ 1.0, 12.6 $\pm$ 1.2μmol/l compared to 148 $\pm$ 12.9, 68.3 $\pm$ 4.8μmol/l) (mean $\pm$ SD) but the Gunn-Wistar serum bilirubin levels were higher than those observed in adult rats. This may be explained by the immaturity of the bilirubin conjugating system in these young rats. There was no significant change in the serum bilirubin levels of cross rats after treatment with sulphadimethoxine. In contrast there was a significant decrease in serum bilirubin level of Gunn rats after treatment with the drug - demonstrated in Table 24 and Figure 33. This shows that the drug is successfully displacing bilirubin from albumin. The significant rise in brain bilirubin from 4.2 $\pm$ 0.1 nmol/g wet weight to 9.8 $\pm$ 0.4 nmol/g wet weight after administration of the drug reflects successful entry of bilirubin into this tissue. Since there is little free bilirubin in cross rats no bilirubin was detected in the brain of these rats either before or after sulphadimethoxine treatment. Since the lower limit of detection of the adopted brain bilirubin assay was 0.1μg/g wet weight tissue the levels of bilirubin in the brain of these rats must have been below

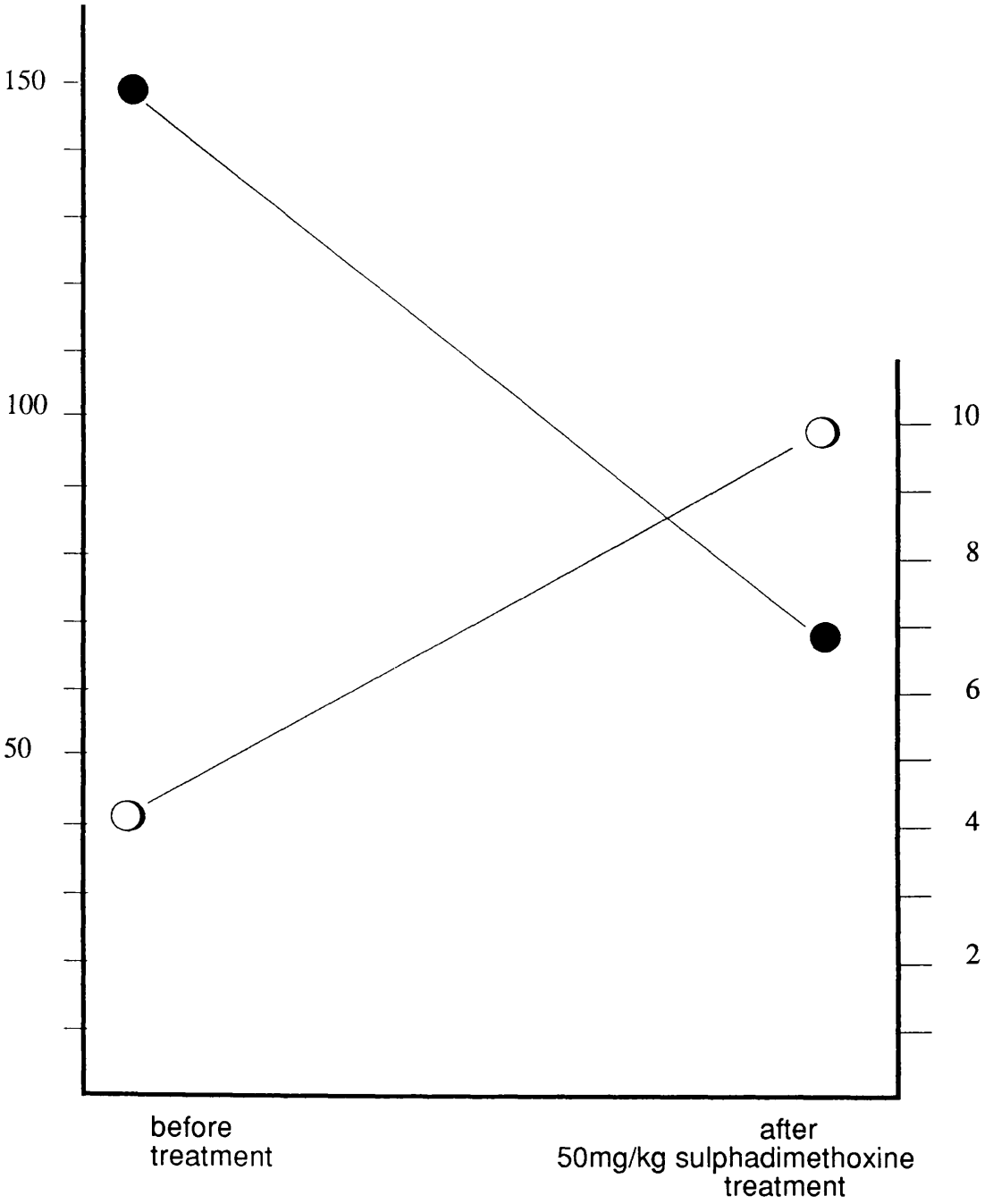
	BILIRUBIN LEVEL	
	SERUM μmol/l	BRAIN nmol/g wet wt
CROSS RATS UNTREATED	12.6 ± 1.2	-
CROSS RATS TREATED	11.7 ± 1.0	-
GUNN RATS UNTREATED	148 ± 13	4.2 ± 0.1
GUNN RATS TREATED	68.3 ± 4.8	9.8 ± 0.4

n = 8 in all groups

Results are expressed as mean ± SD

Treated refers to administration of sulphadimethoxine 50mg/kg four hours prior to sacrifice.

TABLE 24: BILIRUBIN LEVELS IN SERUM AND BRAIN OF TREATED AND UNTREATED RATS



The left ascending axis shows µmol / l for serum bilirubin
The right ascending axis shows nmol / g wet weight for brain bilirubin

● represents serum bilirubins of homozygous Gunn rats before and after treatment

○ represents brain bilirubins of homozygous Gunn rats before and after treatment

Results are mean of 8 experiments, calculated in duplicate.

FIGURE 33 : EFFECT OF SULPHADIMETHOXINE ON GUNN RAT SERUM AND BRAIN BILIRUBIN LEVELS

this level.

For completeness the weights of the brains of Gunn rats and Gunn-Wistar cross rats were measured, both on treatment with sulphonamide and without. Treatment with sulphadimethoxine made no difference to the weights of the tissue, but there was a significant difference in the brain weights of Gunn rats compared to control Gunn-Wistar rats. Gunn rat brains were consistently of a lower weight than cross rat brain - $p<0.01$ by Mann Whitney U test see Table 25.

7.4.2 Activity of Brain Haem Enzymes in 15 Day Old Gunn Rats With Elevated Brain Bilirubin Levels

In a similar group of experiments to those in section 7.3, groups of 15 day old Gunn rats and Gunn Wistar rats were divided to give four groups. Generally eight animals were used. The Gunn rats were divided into two groups of two, and one group was treated with 50mg/kg sulphadimethoxine while the other group were administered with carrier only (saline). An identical regime was carried out with the Gunn-Wistar cross rats. All animals were separated from their dams and allowed free movement for five hours to allow equilibration between the drug and albumin in the system and maximal displacement of bilirubin. The rats were then sacrificed, blood removed for serum bilirubin determination, brains perfused and removed on ice for enzymatic measurement (ALA-synthase and PROTO-O activities) and bilirubin determination. Brain tissue from two animals was pooled for each determination.

The mean ($\pm$ SD) results obtained for enzyme activities are represented in Table 26 and in Figures 34 & 35. Even with

	BRAIN WEIGHT (g)
CROSS RATS UNTREATED	1.12 ± 0.07
CROSS RATS TREATED	1.11 ± 0.11
GUNN RATS UNTREATED	0.90 ± 0.08
GUNN RATS TREATED	0.92 ± 0.05

Results are expressed as mean ± SD

Treated refers to administration of sulphadimethoxine 50mg/kg four hours prior to sacrifice.

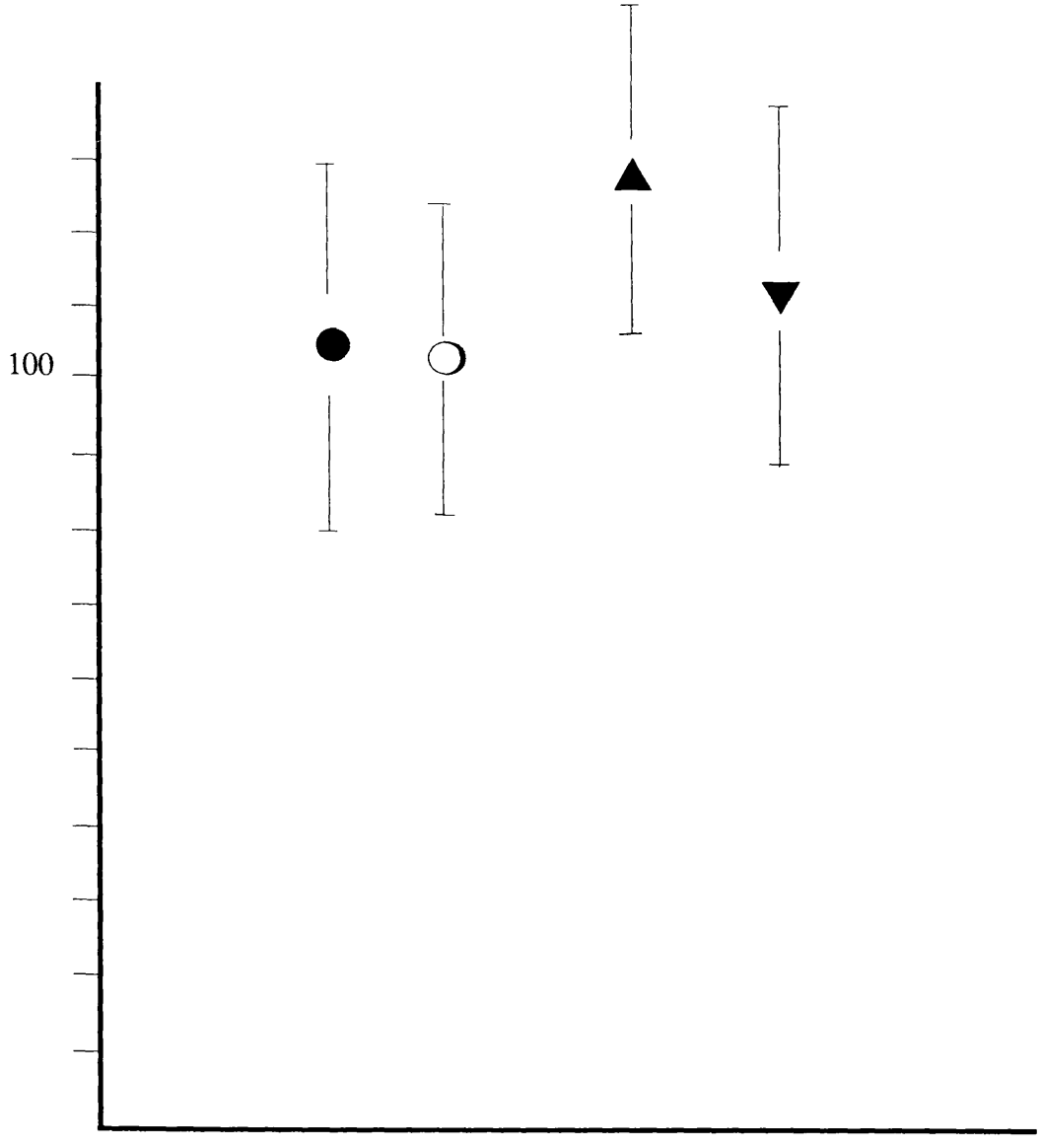
TABLE 25: BRAIN WEIGHTS IN 15 DAY OLD GUNN AND CONTROL RATS

	ENZYME ACTIVITIES	
	PROTO-O ACTIVITY nmol PROTO/mg protein/h	ALA-synthase ACTIVITY pmol ALA/mg protein/h
CROSS RATS UNTREATED	18.3 ± 4.3	108 ± 35.6
n	10	11
CROSS RATS TREATED	20.5 ± 5.4	100 ± 27.0
n	8	8
GUNN RATS UNTREATED	16.7 ± 3.3	130 ± 46.2
n	8	8
GUNN RATS TREATED	16.3 ± 4.1	115 ± 29
n	9	9

Treated refers to administration of sulphadimethoxine 50mg/kg four hours prior to sacrifice.

Results are expressed as mean ± SD

TABLE 26: ACTIVITY OF BRAIN HAEM ENZYMES IN SULPHADIMETHOXINE TREATED RAT BRAIN AND CONTROL RAT BRAIN OF 15 DAY OLD GUNN AND GUNN-WISTAR CROSS RATS



Units are pmol ALA / mg protein / hour

● represents untreated Cross rat brain tissue

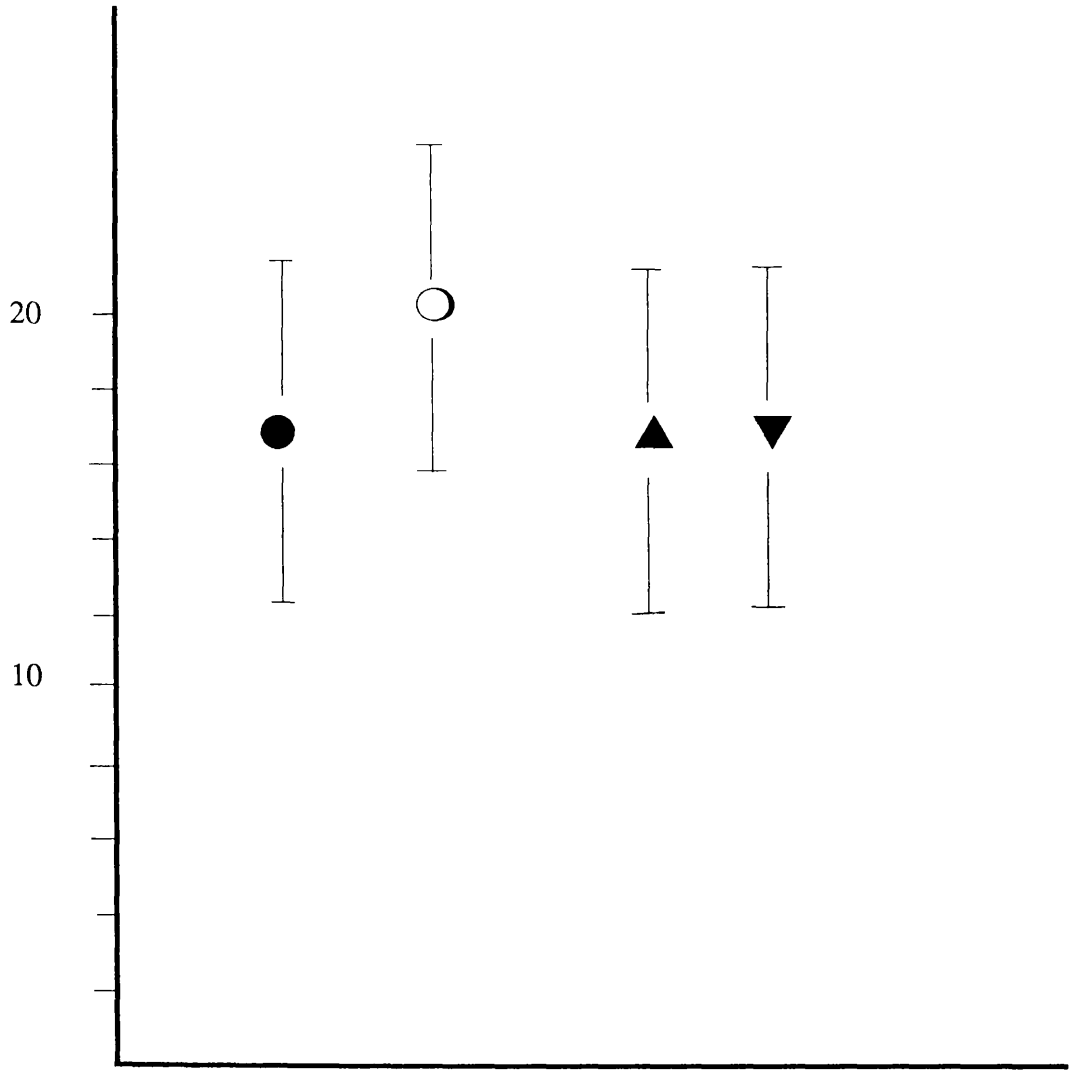
○ represents Cross rat brain tissue, in rats treated with 50mg/kg sulphadimethoxine

▲ represents untreated Gunn rat brain tissue

▼ represents Gunn rat brain tissue, in rats treated with 50mg/kg sulphadimethoxine

Results are expressed as mean ± SD

FIGURE 34 : ALA-SYNTHASE ACTIVITIES IN GUNN RAT BRAIN AND CROSS RAT BRAIN, WITH AND WITHOUT BILIRUBIN DISPLACEMENT



Units are nmol PROTO / mg protein / hour

● represents untreated Cross rat brain tissue

○ represents Cross rat brain tissue, in rats treated with 50mg/kg sulphadimethoxine

▲ represents untreated Gunn rat brain tissue

▼ represents Gunn rat brain tissue, in rats treated with 50mg/kg sulphadimethoxine

The results are mean activities ± SD

FIGURE 35 : PROTOPORPHYRINOGEN OXIDASE ACTIVITES IN GUNN RAT BRAIN AND CROSS RAT BRAIN WITH AND WITHOUT BILIRUBIN DISPLACEMENT

the elevation of bilirubin in brain clearly seen from Table 24 and Figure 33 no significant differences could be detected in either PROTO-O activity or ALA-synthase activity of Gunn rats. In untreated Gunn rats PROTO-O activity was recorded at 16.7 $\pm$ 3.3nmol PROTO/mg protein/hour (mean $\pm$ SD), and this was not significantly different to the values obtained in Gunn-Wistar cross rats (18.3 $\pm$ 4.3nmol PROTO/mg protein/hour). By increasing brain bilirubin levels to greater than twice those seen in untreated 15 day old Gunn rats the PROTO-O activities seen were virtually identical with a mean of 16.3 $\pm$ 4.1 nmol PROTO/mg protein/h. The absence of any depression in PROTO-O activity seen meant that it was unsurprising that no significant differences were recorded in ALA-synthase activity of brain of these icteric Gunn rats.

7.5 DISCUSSION AND SUMMARY

Initial experimentaldata obtained on the activity of the haem biosynthetic enzymes of interest in the brain of adult Gunn rats showed that PROTO-O activity was similar to those activities recorded in control, Gunn-Wistar cross rats. From the previous data obtained on the activity of PROTO-O in other tissues of Gunn rats this was a disappointing result, as the brain is known to be a major site of deposition of bilirubin. However the levels of bilirubin found in this tissue are still far below those reported in the liver, where bilirubin is sequestered for excretion and where *in vitro* inhibition of PROTO-O activity has previously been measured. The brain

damage which occurs during kernicterus seems only to be produced in young mammals under normal conditions. In adult humans kernicterus does not develop and this is thought to be due to the efficiency of the excretion routes which are then fully developed. Icteric states have been induced in adult animals but these require extensive disruption of the blood brain barrier, after administration of bilirubin displacing agents, to allow access of large amounts of free bilirubin to the brain. No similar type of condition is observed in humans.

The lack of successful inhibition found in adult Gunn rat brain, the fact that kernicterus develops in the newborn only and the success of displacing agents for bilirubin in mimicking the symptoms of kernicterus in rat models led to the decision to use neonatal Gunn rats and to elevate brain bilirubin levels by displacement of bilirubin from its binding to albumin in plasma with sulphadimethoxine.

After treatment with sulphadimethoxine bilirubin was successfully raised in the brain of Gunn animals. Despite this increase no inhibition of PROTO-O activity by bilirubin was detected. One aspect observed was the increase in both ALA-synthase activity and PROTO-O activity in the neonatal animal as compared to the adults. High ALA-synthase activities during early animal development is well documented (Paterniti et al, 1978) and these results show that a similar increase in PROTO-O activity is present in the first few weeks of life.

With respect to bilirubin as an inhibitor brain haem biosynthesis the results obtained here show that no inhibitory effect can be demonstrated even after elevation of brain

bilirubin levels to the same range as those previously shown to produce symptoms of kernicterus. The Gunn rats displayed abnormal behaviour after treatment with sulphadimethoxine, consistent with observations in other models of the disease. Thus these experiments are not consistent with the hypothesis that a component of the brain damage observed in kernicterus may be due to disruption of the haem biosynthetic pathway in this tissue, similar to the neurological disturbances which develop in porphyria.

The reasons for this lack of inhibition of PROTO-O activity in the brain, despite observation in other tissues, must be considered. Efficient washout of bilirubin from the tissue was not thought to contribute to these results as the tissues were prepared in a similar way to liver tissue where the reduction in PROTO-O activity found in adults was seen. The in vitro studies which demonstrated effective inhibition of PROTO-O activity showed a mean inhibitory concentration of 25uM (Ferriera & Dailey, 1988). In these experiments the highest concentration of bilirubin recorded in brain after treatment with sulphadimethoxine was in the region of 10μM. Because of the lower brain concentration less inhibition than that observed by Ferriera's group would be expected. However no percentage decrease could be seen in brain tissue of these animals. This was despite the fact that the concentrations obtained were associated with clear manifestations of brain dysfunction.

Brain bilirubin is known to deposit unevenly in the brain, with highest concentrations in the cerebellum (Sawasaki et al,

1976). All the measurements of brain haem biosynthetic enzymes were carried out in whole brain mitochondrial preparations. The manifestations of brain damage seen are associated with dysfunction of this particular area of the brain and it is therefore a possibility that on entry to the brain bilirubin preferentially deposits in this area. Further experiments where dissection of the brain and measurement of PROTO-O activity in the various regions would be interesting and may point to a local effect. One problem with these type of experiments would be the number of animals from which tissue would have to be pooled to provide enough tissue for accurate assay. There is scope for producing a micro method for measurement of PROTO-O activity for this type of work.

CHAPTER 8

THE EFFECT OF TIN PROTOPORPHYRIN ON HAEM METABOLISM IN PORPHYRIA

8.1 INTRODUCTION

The work so far contained in this thesis has concentrated on the haem biosynthetic pathway in the brain. The experimental data obtained has shed little light on the mechanism by which bilirubin exerts its toxic influence on neurological tissue in kernicterus. It appears that disruption of haem biosynthesis in the brain may not be a factor in the neurological damage sustained in this condition. As detailed in the introduction the porphyric disease states are a result of disruption of the haem biosynthetic pathway. The acute porphyrias are hereditary diseases where a genetic lesion occurs resulting in a defect in enzyme activity. Neurological manifestations are common in attack, in addition to the abdominal pain and photosensitivity reactions suffered in some of these conditions. One of the most common forms of porphyria is acute intermittent porphyria, where activity of the enzyme PBG-D is greatly reduced compared to normal. On exposure to certain precipitating factors such as drugs which increase the requirement for cyt.P450, the haem produced by the inhibited biosynthetic pathway is insufficient to meet demand and the resultant haem deficiency leads to derepression of synthesis of the rate controlling enzyme and a consequent increase in activity. This is in an attempt to increase haem production and overcome the block but simply exacerbates the condition as porphyrins and precursors build up prior to the blocked step. Acute intermittent porphyria and its effects are discussed more fully in section 1.4.1.

Treatment of porphyria has always been difficult.

Administration of haematin (haem dissolved in alkali) to replete the intracellular free haem pool has been moderately successful (Bonkowsky et al, 1971). Some patients find no improvement in their symptoms however in most cases the biochemical profile returns to levels approaching normal during therapy only to rebound to pretreatment levels after stopping therapy. The haematin solution is known to be unstable (Mendenhall, 1984) and may decompose *in vivo* before full uptake to liver can occur. This is the probable cause of variability in effectiveness recorded in various studies (Bissell et al, 1988; Granick & Sassa, 1978). To combat this a recent advance has been the introduction of a more stable form of haem, bound to the amino acid arginine (Tenhunen et al, 1987). Haem arginate has been used to return induced ALA synthase activities to normal in AIA induced porphyria in animals (Tenhunen et al, 1987; Tokola et al, 1987). This treatment has also been successful to some extent in the treatment of acute hepatic porphyria (Mustajoki et al, 1986). However the improvements in the symptoms of porphyria seen are often shortlived. Thus, even increased stability of the haem compound for intravenous administration is not a satisfactory solution. A major disadvantage is that administration of exogenous haem leads to induction of haem oxygenase resulting in enhanced metabolism of a percentage of the administered haem. Therefore duration of therapy is usually short (over 4 days) but induction still occurs.

A number of metals, including tin

molecule,inserted in the nucleus of porphyrins produce effective inhibitors of haem oxygenase. These metalloporphyrins, such as Sn-PROTO inhibit haem oxygenase with stronger affinity for the enzyme than haem itself. This inhibition of haem oxygenase leads to a reduction in the production of bilirubin and CO (Simionatto et al, 1985; Milleville et al, 1985). This property of Sn-protoporphyrin (Sn-PROTO) led to investigations into the use of this compound as a treatment in lowering hyperbilirubinaemia in various conditions. Initial work on animals was promising with umolar doses effectively reducing bilirubin levels in bile duct ligated rats (Simionatto et al, 1985; Anderson et al, 1986), neonatal rats (Drummond & Kappas, 1982) and those with induced hyperbilirubinaemia (Drummond & Kappas, 1984). Biochemical studies then showed that this synthetic haem analogue had low affinity for haem binding proteins in the circulation (Breslow et al, 1986). This explained the low half-life of Sn-PROTO in the plasma (less than 4 hours in rats). Sn-PROTO was found to deposit in tissues and act as an effective long term inhibitor of haem oxygenase with one dose resulting in reduction of serum bilirubin levels for a minimum of four days (Berglund et al, 1988). This metalloporphyrin appeared to be fairly harmless with no toxic effects seen at doses 100 times those in the therapeutic range in animals. Even after long periods of dosage no toxic effects have been seen in animals (Sassa et al, 1985). Hyperbilirubinaemia has been safely reduced in neonates with Sn-PROTO (Kappas et al, 1988). The only recorded side effect in some cases has been a transient

photosensitivity (Kappas et al, 1988). In response to this, manipulations of the PROTO ring structure have been carried out and Sn-mesoporphyrin, with the vinyl groups at C2 and C4 reduced to ethyl groups, was found to be a more effective inhibitor of haem oxygenase with potentially less of a photosensitizing effect (Drummond et al, 1987). This raises the possibility of designing metalloporphyrins which would not cause photosensitivity (Delaney et al, 1988).

This approach to treatment of hyperbilirubinaemia is a novel one since it treats the condition by reducing production of the potentially toxic bilirubin rather than altering disposal of the preformed pigment. Thus the haem degradative pathway is being manipulated. The reduction in the amount of bilirubin produced leads to an increase in the free haem pool, evidenced by saturation of the tryptophan pyrrolase enzyme within 1 hour of dosing (Kappas et al, 1985). This leads to excretion of small amounts of haem in the bile, urine and faeces. The increased content of haem in the liver during this treatment has led to investigations into whether Sn-PROTO would be a useful drug in other conditions.

Studies on the effect of Sn-PROTO in experimental porphyria in animals showed that this synthetic metalloporphyrin effectively prevented the induction of ALA-synthase in rats treated with AIA (Galbraith et al, 1985). In porphyric patients Sn-PROTO significantly reduced the hyperexcretion of ALA, PBG and porphyrins (Galbraith & Kappas, 1989). The mechanism of action of Sn-PROTO is still unclear

but it is thought that the structural similarity of the compound to haem allows entry of Sn-PROTO to the active site where binding occurs, leading to inhibition of the enzyme (Simionatto et al, 1985). Alternatively, or additionally, the potent inhibition of Sn-PROTO on haem oxygenase may lead to a sufficient increase in endogenous haem to allow repression of ALA-synthase to occur. As documented earlier in this thesis no adverse effects have so far been seen on administration of Sn-PROTO. Thus, use of the drug in porphyria should cause no adverse effects.

This section compares the metabolic effect of Sn-PROTO with that of haem arginate in induced porphyria in rats. In addition, we report the effect of Sn-PROTO in combination with haem arginate in the treatment of acute intermittent porphyria. Administration of an exogenous haem source, such as haem arginate, when combined with a block in the degradative pathway of haem is potentially a very powerful tool in repleting haem content of tissues while preventing the accompanying induction of haem oxygenase and increased production of bilirubin. It would be advantageous if Sn-PROTO and haem arginate combined had a synergistic effect in reducing the porphyrin excretion and returning the disrupted enzymes to normal levels allowing the dose of haem arginate required to be reduced. The Sn-PROTO might also prolong the beneficial effect of the administered haem.

8.2 PRODUCTION OF INDUCED PORPHYRIA IN RATS

This combination therapy has never been clinically applied so initial studies were carried out on rats with induced porphyria. Sprague Dawley rats 200-250g body weight were injected intra-peritoneally (i.p.) with various doses of 4,6-dioxoheptanoic acid (succinyl acetone) in saline solution. This compound competitively inhibits ALA-D activity (Tschudy et al, 1981) effectively leading to a block of the haem biosynthetic pathway. The most common acute hepatic porphyria is AIP where PBG-D activity is reduced. Succinyl acetone was chosen to mimick an enzymatic block early in the pathway. This is one step earlier than the PBG-D defect and as such was thought to be most similar to this porphyria. Similar increases in the excretion of the precursor ALA to those seen in AIP are observed without any increase in PBG excretion. Urinary ALA excretion and hepatic ALA-D levels were measured and an optimum dose of succinyl acetone was obtained (Table 27). The results in Table 27 show the enzyme activities and ALA excretion levels at doses of 10, 20 and 50mg/kg body weight per day for 2 days sacrificing the animals on day 3. The animals were injected twice daily (b.d.) and results compared to controls. It can be clearly seen that each of the two higher doses of succinyl acetone successfully inhibited ALA-D activity and caused large increases in excretion of ALA in urine, a measure of induced ALA-synthase activity. The dose of 20mg/kg body weight per day was used in two 10mg/kg i.p. injections. The main reasons for this were to limit any

	HEPATIC ALA-D ACTIVITY μmol ALA/g protein/h	URINARY ALA EXCRETION nmol/24 hours	
		DAY 1	DAY 2
CONTROL RAT	13.4 ± 3.2	16.5 ± 3.4	21.5 ± 13.9
SUCCINYL ACETONE TREATED RAT 10mg/kg/day	8.18 ± 4.78	161 ± 44.2	146 ± 36.6
SUCCINYL ACETONE TREATED RAT 20mg/kg/day	4.42 ± 1.78	2301 ± 84.9	3499 ± 97.9
SUCCINYL ACETONE TREATED RAT 50mg/kg/day	3.35 ± 1.51	4419 ± 101	4603 ± 118

Results are expressed as mean ± SD

n=4 in all cases

TABLE 27: THE EFFECT OF SUCCINYL ACETONE ON RAT HEPATIC ALA-D ACTIVITIES AND URINARY ALA EXCRETION

toxic effects which may then be seen on treatment with two therapies for this induced porphyria and also to more accurately mimic the type of block seen in hereditary porphyria where the enzyme activity is reduced to around 50% of normal activity. Subsequent studies used a dose of 20mg/kg/day of succinyl acetone to induce experimental porphyria in these rats.

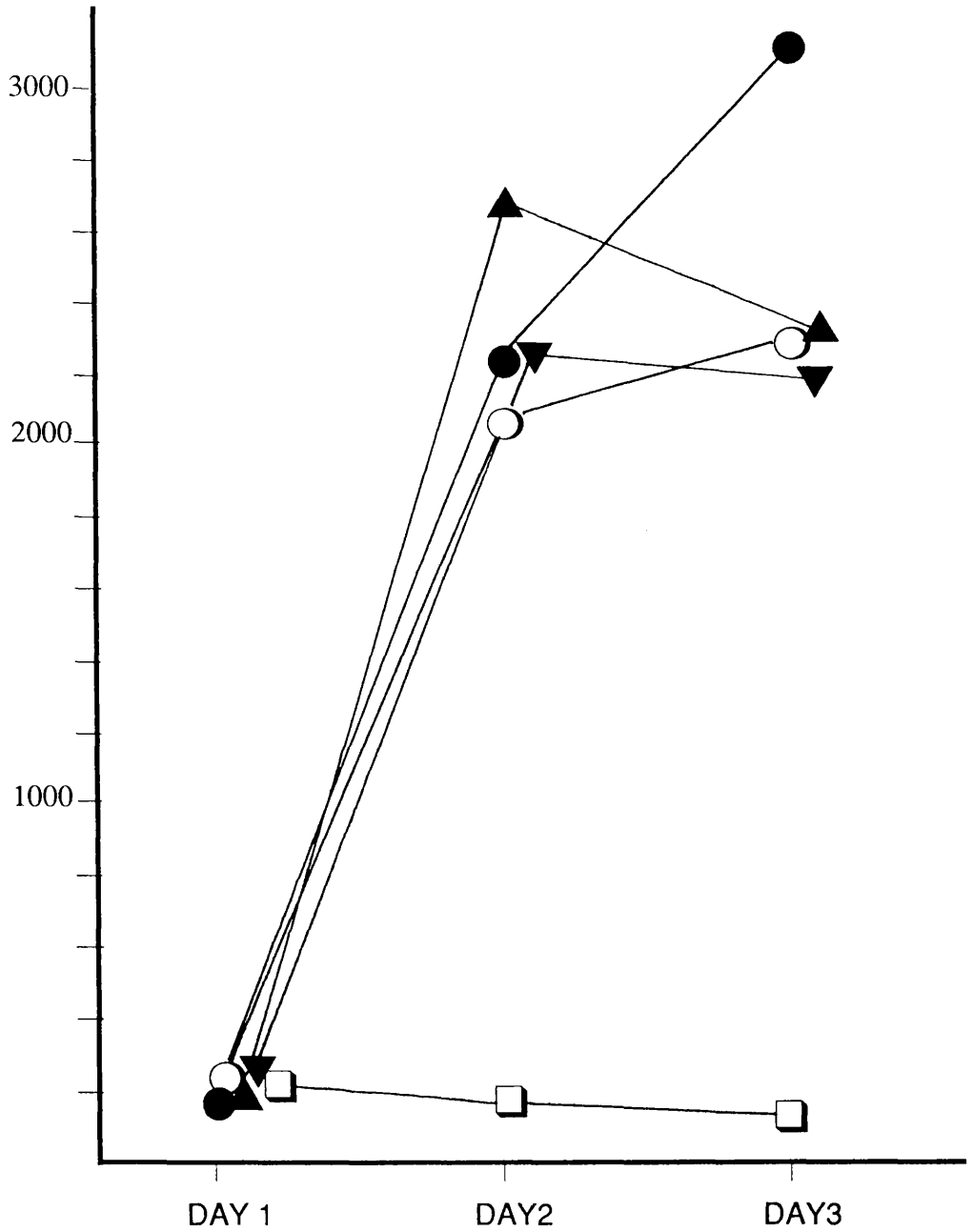
8.3 CONTROL OF INDUCED PORPHYRIA WITH VARIOUS TREATMENT REGIMENS

A protocol was devised for confining Sprague Dawley rats to metabolic cages, measuring their urinary ALA excretion, then inducing porphyria with succinyl acetone administration (i.p.). Successful induction was shown by greatly increased ALA excretion levels. For the first day all rats were confined to these cages and urine collected so that baseline urinary ALA levels could be determined. This constituted Day 1 of the experiment. A control group was set up which received placebo, injections containing only the carrier solutions for the drugs. On the next 2 consecutive days the remaining four rats received succinyl acetone at the pre-determined dose of 20mg/kg. Of the rats in which porphyria was induced, within a few hours of the first dose, each received a separate treatment to alter the biochemical profile of the porphyria and precursor excretion. One rat in each group received no further therapy, and was considered to be the porphyric rat. Another of the rats was treated with haem arginate alone, one was treated with Sn-PROTO alone and the final one was treated with a combined therapy of Sn-PROTO

followed by haem arginate. Dosage was carried out twice daily for 2 days as for the succinyl acetone. In all cases succinyl acetone, was administered first (i.p.). This was followed after a 10 minute break by Sn-PROTO subcutaneously and this after a further 20 minutes by haem arginate i.p. The rationale for this order of therapeutic drugs was that earlier injection of Sn-PROTO which causes inhibition of haem oxygenase activity should be administered first to initiate the block in haem degradation. Thus haem arginate should be unable to cause any induction of haem oxygenase. The rats were sacrificed early on the fourth day of the experiment. Doses of the drugs for administration to the rats were determined both from previous rat studies and human work and based on the fact that rats metabolise drugs approximately 2.5 times faster than humans.

8.3.1 Effect of Succinyl Acetone on Hepatic Haem Biosynthesis and ALA Excretion

Sprague Dawley rats were treated with succinyl acetone to induce a porphyric state at the predetermined dose of 20mg/kg body weight/day for 2 consecutive days. Urinary ALA excretion was measured and is recorded in Table 28 and Figure 36. ALA excretion was significantly higher in rats treated with succinyl acetone, with 20 times the amount of precursor excreted compared to control rats ($p < 0.001$ by Mann Whitney U Test). Hepatic ALA-synthase and ALA-D activities and cytochrome P450 levels were measured and are reported in Tables 29 and 30 and Figures 37,38 and 39. ALA-D activities



● represents porphyric rat (succinyl acetone only) n=8 DAYS 1 & 2, n=13 DAY 3

○ represents porphyric rat treated with haem arginate n=9 DAYS 1 & 2, n=12 DAY 3

▲ represents porphyric rat treated with SN-PROTO n=9 DAYS 1 & 2, n=12 DAY 3

▼ represents porphyric rat on combination therapy n=8 DAY 1 n=9 DAY 2, n=13 DAY 3

❑ represents control rat n=9 DAYS 1 & 2, n=12 DAY 3

Units are in nmol ALA excreted / 24 hours

FIGURE 36 : URINARY EXCRETION OF ALA IN PORPHYRIC AND TREATED RATS

	URINARY ALA EXCRETION nmol/24 hours		
	DAY 1 (no treatment)	DAY 2	DAY 3
CONTROL	274 ± 165	178 ± 184	152 ± 131
n	9	9	12
PORPHYRIC	144 ± 65	2291 ± 1231	3190 ± 1999
n	8	8	13
PORPHYRIC + HAEM ARGINATE	177 ± 146	2034 ± 1681	2385 ± 1288
n	9	9	12
PORPHYRIC + Sn-PROTO	188 ± 121	2766 ± 1514	2362 ± 2003
n	9	9	12
PORPHYRIC + COMBINED THERAPY	236 ± 165	2296 ± 1026	2092 ± 861
n	8	9	13

Combination therapy consisted of 30umol/kg body weight Sn-PROTO followed twenty minutes later by 6mg/kg haem arginate twice daily. These were identical to the doses in monotherapy with haem arginate and Sn-PROTO.

Results are expressed as mean ± SD.
Statistical analysis is in the text.

TABLE 28: EXCRETION OF ALA IN SPRAGUE DAWLEY RATS WITH TREATED PORPHYRIA, UNTREATED PORPHYRIA AND ON PLACEBO

	ALA-D ACTIVITY µmol PBG/g ptn/h	ALA-S ACTIVITY pmol ALA/g ptn/h
CONTROL	13.8 ± 6.1	203 ± 112
PORPHYRIC	4.9 ± 2.4	703 ± 457
PORPHYRIC + HAEM ARGINATE	5.3 ± 3.6	377 ± 154
PORPHYRIC + Sn-PROTO	4.2 ± 2.9	270 ± 207
PORPHYRIC + COMBINED THERAPY	4.9 ± 2.9	290 ± 168

For ALA-D activity n=8 for the control and porphyric groups and n=9 for the three treated groups.

For ALA-synthase activity n=10 for the porphyric group and n=11 for all other groups.

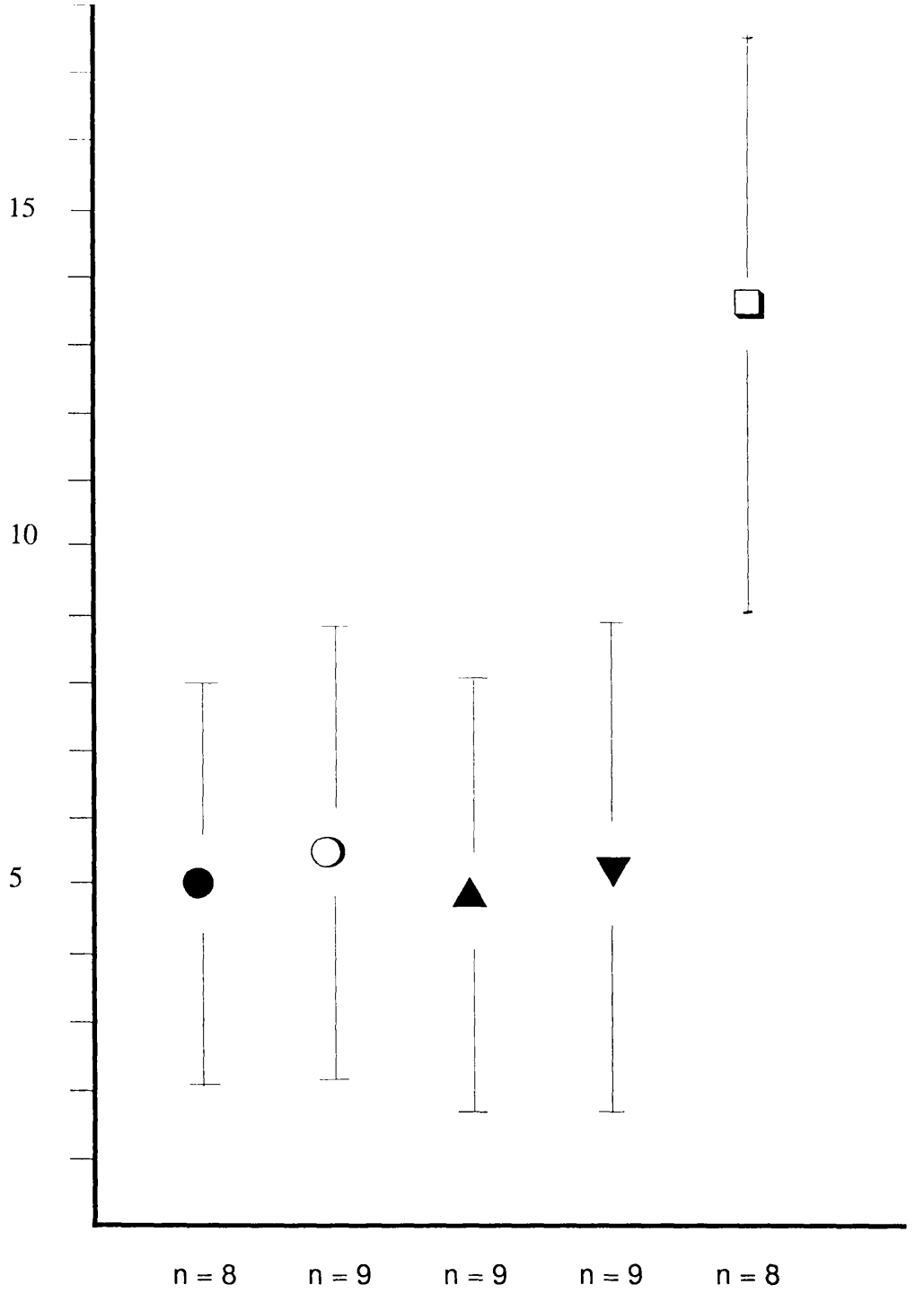
Results are expressed as mean ± SD

TABLE 29 : HEPATIC ALA-D AND ALA-SYNTHASE ACTIVITIES OF SPRAGUE DAWLEY RATS WITH TREATED PORPHYRIA, UNTREATED PORPHYRIA AND ON PLACEBO

	(n)	HEPATIC CYT. P450 LEVELS nmol cyt.P450/mg microsomal protein
CONTROL	6	1.04 ± 0.36
PORPHYRIC	7	1.58 ± 0.72
PORPHYRIC + HAEM ARGINATE	8	1.30 ± 0.68
PORPHYRIC + SN-PROTO	6	0.96 ± 0.37
PORPHYRIC + SN-PROTO + HAEM ARGINATE	5	0.87 ± 0.46

Results are expressed as mean ± SD.

TABLE 30: HEPATIC CYT. P450 LEVELS IN SPRAGUE DAWLEY RATS, RATS WITH INDUCED PORPHYRIA AND IN RATS WITH TREATED INDUCED PORPHYRIA



● represents porphyric rat (succinyl acetone only)

○ represents porphyric rat treated with haem arginate

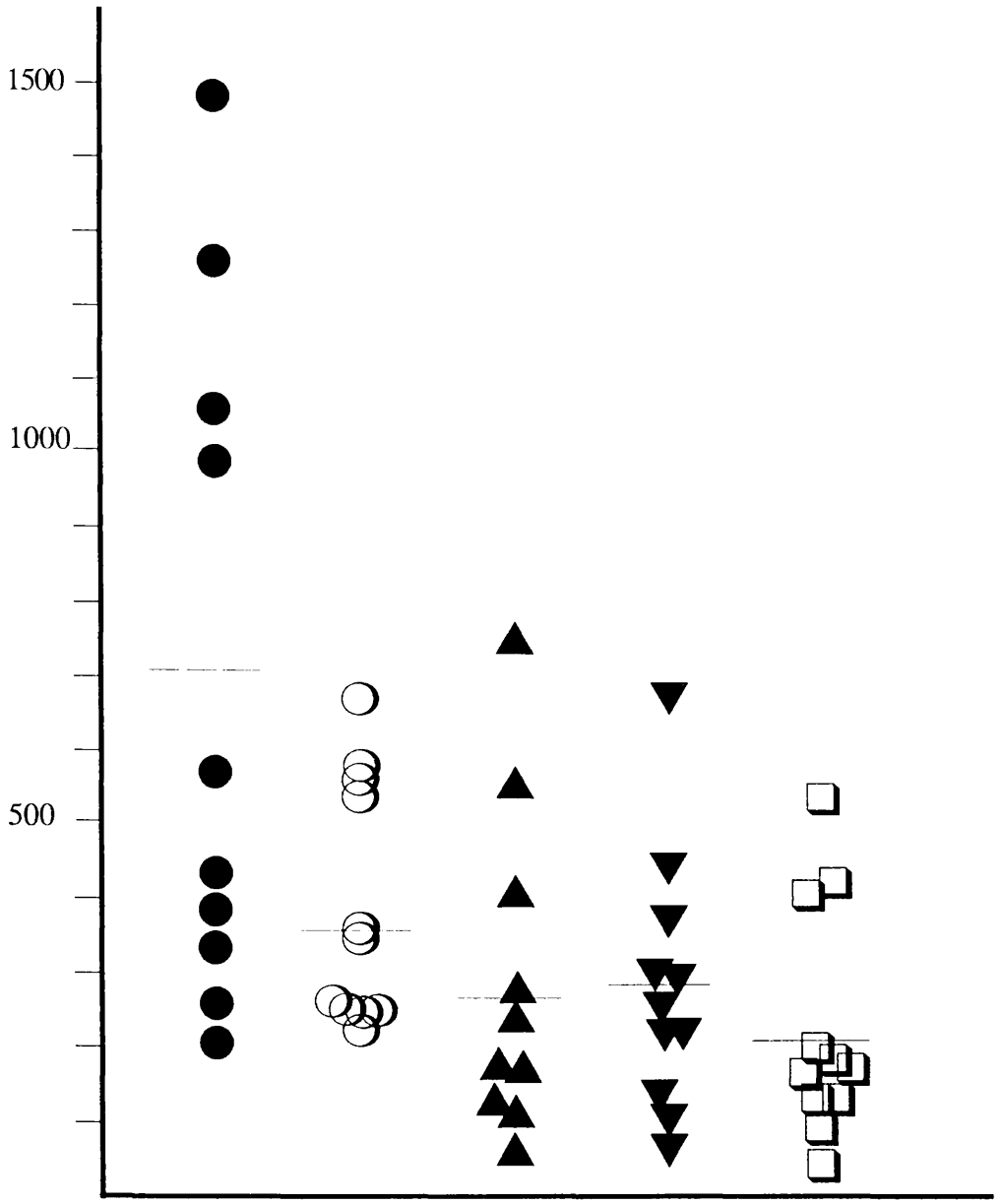
▲ represents porphyric rat treated with SN-PROTO .

▼ represents porphyric rat on combination therapy

❑ represents control rat

Figures represent mean ± SD
Units are in µmol ALA / g protein / hour

FIGURE 37 : HEPATIC ALA-DEHYDRATASE ACTIVITY IN INDUCED PORPHYRIA, AND AFTER VARIOUS TREATMENTS



● represents porphyric rat (succinyl acetone only)

○ represents porphyric rat treated with haem arginate

▲ represents porphyric rat treated with SN-PROTO

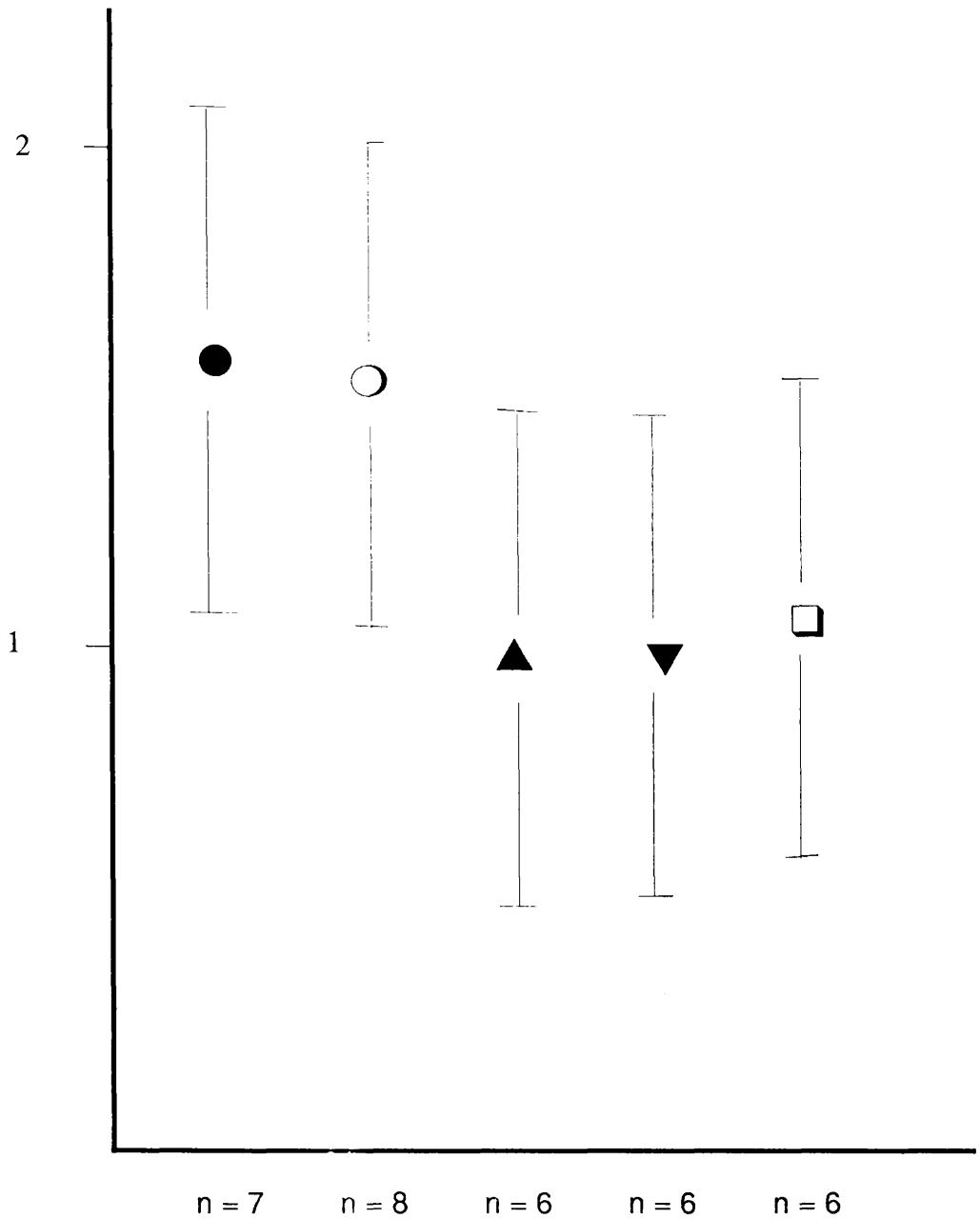
▼ represents porphyric rat on combination therapy

❑ represents control rat

—— represents the means

Units are in pmol ALA / mg protein / hour

FIGURE 38 : HEPATIC ALA-SYNTHASE ACTIVITY IN INDUCED PORPHYRIA, AND AFTER VARIOUS TREATMENTS



● represents porphyric rat (succinyl acetone only)

○ represents porphyric rat treated with haem arginate

▲ represents porphyric rat treated with SN-PROTO

▼ represents porphyric rat on combination therapy

❑ represents control rat

Units are in nmol cyt. P450 / mg microsomal protein

FIGURE 39: CYTOCHROME P450 CONTENT IN LIVER OF PORPHYRIC RATS AND PORPHYRIC RATS ON VARIOUS FORMS OF TREATMENT

for the porphyric group of rats were significantly lower than the controls with values of 4.9 ± 2.4μmol PBG/g protein/hour (mean ± SD) in porphyric rats compared with 13.8 ± 6.1μmol PBG/g protein/hour in control rats, ($p < 0.005$ by Mann Whitney U Test). No significant differences could be found between the ALA-D activities recorded in the three groups of animals on the various different therapies and the porphyric group. This was still true for the rats treated with haem arginate after succinyl acetone treatment ($p < 0.05$) showing that the succinyl acetone was still effective in reducing the enzyme activity.

ALA-synthase activities in the porphyric group had a mean of 703 ± 457pmol ALA/mg protein/hour (± SD) as compared to 203 ± 112pmol ALA/mg protein/hour (± SD). The porphyric animals ALA-synthase activities were significantly higher than the controls ($p < 0.005$). Therefore, a sufficient block in the pathway had been achieved at the step catalysed by ALA-D to cause induction of the rate controlling enzyme.

8.3.2 Control of Porphyria with Haem Arginate

A group of animals were treated with succinyl acetone as in 8.3.1. Thirty minutes after each succinyl acetone injection haem arginate was administered i.p. at a dose of 12mg/kg body weight daily. As with the animals in section 8.3.2, ALA excretion, cyt. P450 in liver and hepatic ALA-D and ALA-synthase activities were measured. ALA-D activity remained low (5.3 ± 3.6μmol PBG/g protein/hour, significantly lower than in the control group ($p < 0.005$). This was

evidence that the block in haem biosynthesis was effective.

Hepatic ALA-synthase activities in rats with induced porphyria which had been treated with haem arginate were found to be 377 $\pm$ 154 pmol ALA/mg protein/h. This was significantly lower than in the group with untreated porphyria (703 $\pm$ 457 pmol ALA/mg protein/h) at $p < 0.05$. These haem arginate treated results remained higher than those recorded in the control, non-porphyric animals (203 $\pm$ 112 pmol ALA/mg protein/h).

8.3.3 Control of Induced Porphyria with Sn-PROTO Therapy

As described in section 8.3.1 and 8.3.2, a group of Sprague Dawley rats were confined to metabolic cages and porphyria was induced. Some of the rats were then treated with Sn-PROTO to try and control their induced porphyria.

On day one the rats were untreated to determine baseline urinary ALA excretion. On days two and three the rats were dosed with succinyl acetone followed after 10 minutes by Sn-PROTO at a dose of 30µmol/kg. SnPROTO was made up in saline from powder after addition of alkali to dissolve the porphyrin (0.2ml of 0.5M NaOH). The solution was neutralised with 0.3M HCl. This solution was made up in subdued light immediately prior to use and kept dark using aluminium foil. Ten minutes after each injection of succinyl acetone, 30µmol/kg Sn-PROTO was injected subcutaneously (s.c.) into the back of the neck. After repeating this treatment regime for two days (days 2 and 3 of experiment), on day 4 animals were sacrificed and hepatic ALA-synthase activity,

ALA-D activity and cyt. P450 content were determined. The results are also presented in Tables 29 and 30 and in Figures 37,38, and 39.

The rats were successfully made porphyric as shown by the significantly lower ALA-D activities recorded (4.2 $\pm$ 2.9 µmol PBG/g protein/hour) compared to the control levels already quoted ($p < 0.005$). Treatment with Sn-PROTO successfully lowered the induced ALA-synthase levels from 703 $\pm$ 457pmol ALA/mg protein/h in the porphyric group to 270 $\pm$ 107pmol ALA/mg protein/h in the treated group ($p < 0.01$). These ALA-synthase activities were not significantly different to those recorded for control animals.

Despite the effective normalisation of ALA-synthase activity, no significant reduction of ALA excretion was observed in these animals as is clear from Table 28. This apparent discrepancy will be discussed in section 8.5.

8.3.4 Control of Induced Porphyria with Combination Therapy

In parallel with rats given succinyl acetone to induce porphyria and control rats, a group of rats were injected with succinyl acetone to induce porphyria then treated with a combination of haem arginate and Sn-PROTO to assess any synergistic effect the two drugs may have on further improving the symptoms of porphyria.

The doses of haem arginate and Sn-PROTO were used identical to those in sections 8.3.2 and 8.3.3 respectively. Rats were initially dosed with succinyl acetone, followed

after 10 minutes by Sn-PROTO (s.c. into the neck) then a further 20 minutes later by haem arginate i.p. Treatment was again for 2 consecutive days: ALA excretion in urine measured over the two 24 hour periods and hepatic ALA-synthase, ALA-D activities and cyt.P450 content were also measured.

A significant reduction in ALA-D activity ($p < 0.05$) was again seen with a value of 4.9 $\pm$ 2.9 μmol PBG/g protein/h (mean $\pm$ SD) recorded.

ALA-synthase activity in the group of rats treated with combination therapy was significantly lower (290 $\pm$ 112 pmol ALA/mg protein/h) than that of the porphyric group (703 $\pm$ 457pmol ALA/mg protein/h) ($p < 0.05$). The levels recorded were not significantly different to those seen in controls suggesting that the biochemical profile of porphyria was successfully being treated by the combination of the two drugs. The only piece of information which was inconsistent with this was the ALA excretion values obtained. Urinary ALA excretion was found to be significantly higher than control excretion ($p < 0.001$) and not significantly reduced when compared to levels in porphyric rats. The mean excretion levels ($\pm$ SD) are shown in Table 28 and the means represented on Figure 36.

8.3.5 Hepatic Cytochrome P450 Levels in Porphyric Rats and Rats on Treatment for Induced Porphyria

The hepatic cyt.P450 content of Sprague Dawley rats was compared to that of rats in the same age and weight range with succinyl acetone induced porphyria and then to rats with treated induced porphyria using the treatment regimes

already described. The findings are presented in Figure 39. There was no significant difference in the cyt.P450 content in any of the groups of rats. This suggests that haemoprotein content in liver of these animals is not being significantly altered by the induction of porphyria by succinyl acetone or by any of the treatment regimens used.

8.3.6 Comparison of the Three Different Treatment Regimes as Successful Therapies for Induced Porphyria

Statistical comparison of the treatments by haem arginate alone, Sn-PROTO alone and the combination therapy showed that all the treatments were successful in reducing ALA-synthase activity with the most significant improvements seen with Sn-PROTO treatment alone ($p < 0.01$). The less marked effect seen with haem arginate was surprising since the treatment has been shown to be effective previously in rat models of porphyria. Combination of Sn-PROTO and haem arginate still produced a significant reduction in ALA-S activity in the liver when compared to porphyric controls. This is still not as good as the result found with Sn-PROTO therapy alone. The most consistent reduction was seen in these animals. There was less variation in ALA-synthase activities as reflected by the lower standard deviation. Unfortunately, these results are not in support of an additive effect of Sn-PROTO and haem arginate as a combined therapy over either of the two treatments alone. The lack of a significant reduction in ALA-synthase activity on haem arginate therapy is in contrast to the result seen in patients where this treatment results in normalised activities

and ALA excretion. It is possible that the two day treatment regime may not be long enough to see a significant effect.

8.3.7 Finding the Optimum Dose of Haem Arginate to Successfully Treat Porphyria Induced with Succinyl Acetone

Two groups of rats were treated with a dose of succinyl acetone of 10mg/kg/day in two doses. One group of these rats were the porphyric rats and administered placebo in addition to succinyl acetone (saline). The other group received haem arginate at the dose already described of 12mg/kg/day for two days. The ALA excretion levels in urine were determined. It was found that the excretion in the porphyric group was 2538 $\pm$ 804 nmol ALA/24 hours and in the haem arginate treated group was 2340 $\pm$ 1438 nmol ALA/24 hours (means $\pm$ SD). The reduction seen was not significant and the results are shown in Table 31.

In a similar experiment the original dose of succinyl acetone at 20mg/kg/day was maintained and the dose of haem arginate was raised to 24mg/kg/day. The porphyric animals recorded ALA excretion levels in urine of 3817 $\pm$ 1240 nmol ALA/24 hours while the treated group excreted 2800 $\pm$ 881 nmol ALA/24 hours. This excretion was significantly lower than the porphyric group. It was then hoped to increase the improvement seen by a combination of reduction of succinyl acetone dose and increase of the haem arginate dose. Groups of rats were treated with 10mg/kg/day succinyl acetone and compared with a group of animals on the same dose with haem arginate at a dose of 24mg/kg/day after 30 minutes i.p. The increase in ALA excretion seen in the succinyl acetone treated

	PORPHYRIC	PORPHYRIC + HAEM ARGINATE 12 mg/kg {24 mg/kg}
SUCCINYL ACETONE 10mg/kg (n=6)	2583 ± 804	2340 ± 1438
SUCCINYL ACETONE 20mg/kg (n=6)	3817 ± 1240	2800 ± 881
SUCCINYL ACETONE 10mg/kg (n=8)	2678 ± 1428	{2138 ± 872}

Results are expressed as mean ± SD and the doses of drugs are per day.

TABLE 31: MANIPULATION OF DOSES OF HAEM ARGINATE ON TREATMENT OF PORPHYRIA INDUCED WITH SUCCINYL ACETONE

animals was to 2678 ± 1428 nmol ALA/24 hours and this was not significantly reduced by the higher dose of haem arginate (2138 ± 872nmol ALA/24hours).

The lack of effectiveness of haem arginate in porphyria induced with succinyl acetone appears to exist even after manipulation of the doses of both agents. From these results haem arginate is not effective in reducing ALA excretion in these animals. The ALA-synthase activity was not measured in these rats but would have been unlikely to throw light on why the treatment is so ineffective. I conclude that the administration of succinyl acetone produces a state of altered haem biosynthesis which is not responsive to haem control.

8.4 TREATMENT OF HUMAN ACUTE HEPATIC PORPHYRIA WITH A COMBINATION OF HAEM ARGINATE AND SN-PROTO THERAPY

A female patient who was regularly admitted suffering from acute attacks of AIP was chosen to compare the effects of Sn-PROTO, haem arginate alone and haem arginate in combination with Sn-PROTO. Sn-PROTO was administered in a dose of 1 μmol/kg body weight, intravenously in 100ml normal saline over 15 minutes and haem arginate in a dose of 3mg/kg body weight, intravenously in 100ml normal saline over 15 minutes. When combination treatment was used, the two drugs were given in the above doses with the Sn-PROTO being administered 30 minutes before haem arginate. Each treatment was given over three consecutive days. The patient had four attacks treated with placebo, four with haem arginate, four with haem arginate and Sn-PROTO and one attack with Sn-PROTO

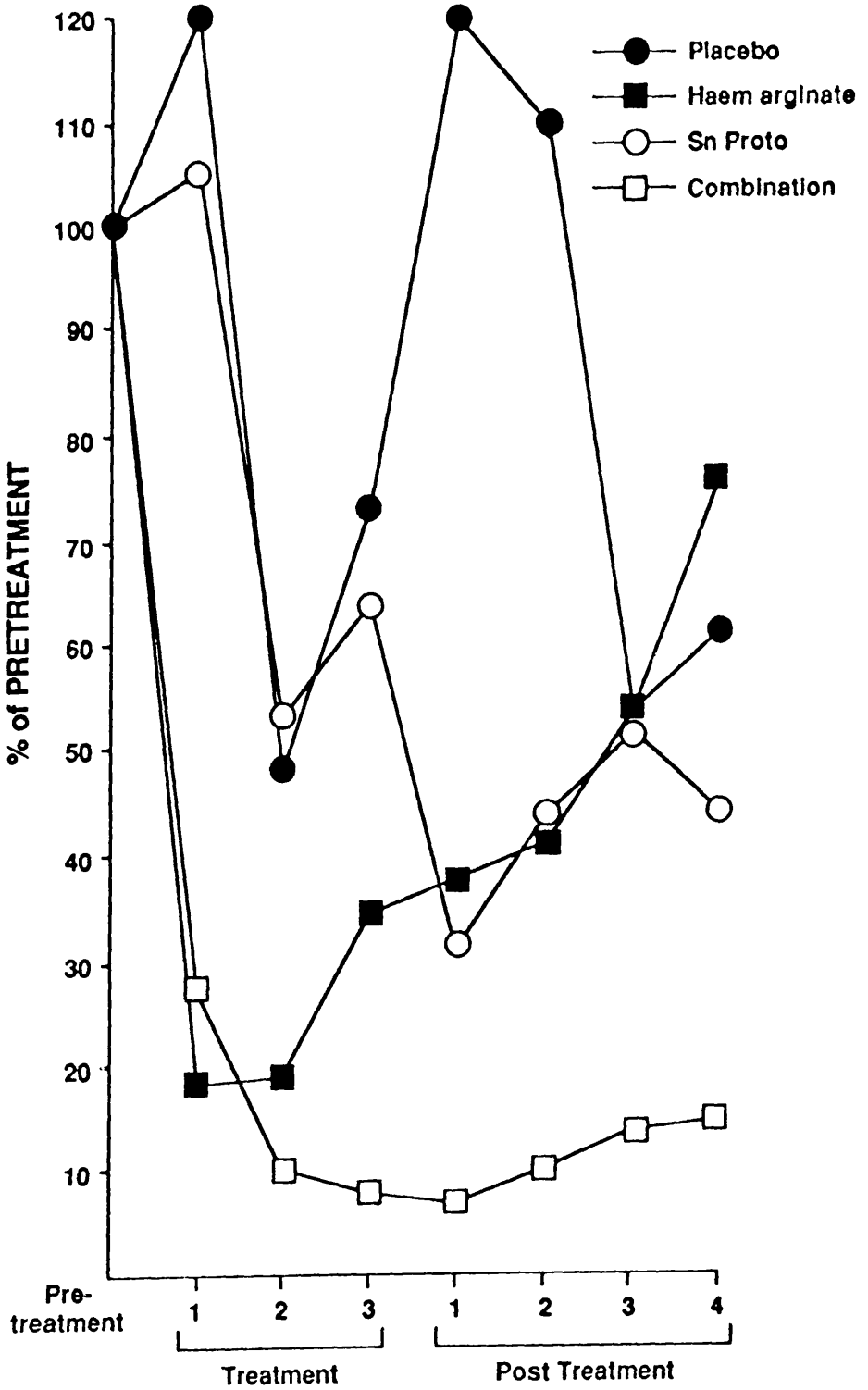
alone.

8.4.1 Biochemical Responses

Urinary excretion of the porphyrin precursors ALA and PBG is greatly increased during an attack of acute porphyria. Haem arginate alone or combined with Sn-PROTO resulted in a marked reduction in porphyrin precursor excretion to near normal levels. The degree of depression of porphyrin precursor production during the three days of treatment was similar with haem arginate alone or used in combination with Sn-PROTO (Table 32 for ALA excretion and Table 33 for PBG excretion. Sn-PROTO on its own did not reduce porphyrin precursor excretion to a significant extent. Following cessation of treatment the urinary excretion of porphyrin precursors rapidly rose with haem arginate alone reaching 80% of pretreatment values by the fourth day post-therapy (Figure 40, Tables 32 and 33). In contrast, when haem arginate was co-administered with Sn-PROTO the excretion remained depressed being only 15% of pretreatment levels at the fourth day post-therapy (Figure 40, Tables 32 and 33). This indicates that the Sn-PROTO prolongs the biochemical efficacy of haem arginate.

8.4.2 Clinical Response

Information was available on the clinical outcome of 4 attacks treated with placebo, 23 with haem arginate, 3 with combination therapy and 1 with Sn-PROTO alone. There was no significant difference between the four treatments with regard to duration of admission. However, there was evidence



n=3 for placebo, haem arginate and combination treatments
n=1 for Sn-PROTO treatment alone

FIGURE 40: THE EFFECTS OF VARIOUS DIFFERENT TREATMENTS ON URINARY ALA EXCRETION DURING PORPHYRIC ATTACK

	PERCENTAGE OF MEAN PRETREATMENT VALUES	
	Day 3 treatment (last day)	Day 5 Post-therapy
PLACEBO (n=3)	74 (61-89)	80 (41-119)
HAEM ARGINATE (n=3)	25 (13-45)	113 (82-164)
Sn-PROTO (n=1)	63	44
HAEM ARGINATE + Sn-PROTO (n=3)	8 (16-13)	18 (16-20)

TABLE 32: URINARY ALA EXCRETION OF ONE PATIENT ON VARIOUS TREATMENT REGIMES

	PERCENTAGE OF MEAN PRETREATMENT VALUES	
	Day 3 treatment (last day)	Day 5 Post-therapy
PLACEBO (n=3)	80 (53-102)	82 (58-98)
HAEM ARGINATE (n=3)	39 (24-57)	112 (23-251)
Sn-PROTO (n=1)	88	24
HAEM ARGINATE + Sn-PROTO (n=3)	12 (6-21)	25 (14-45)

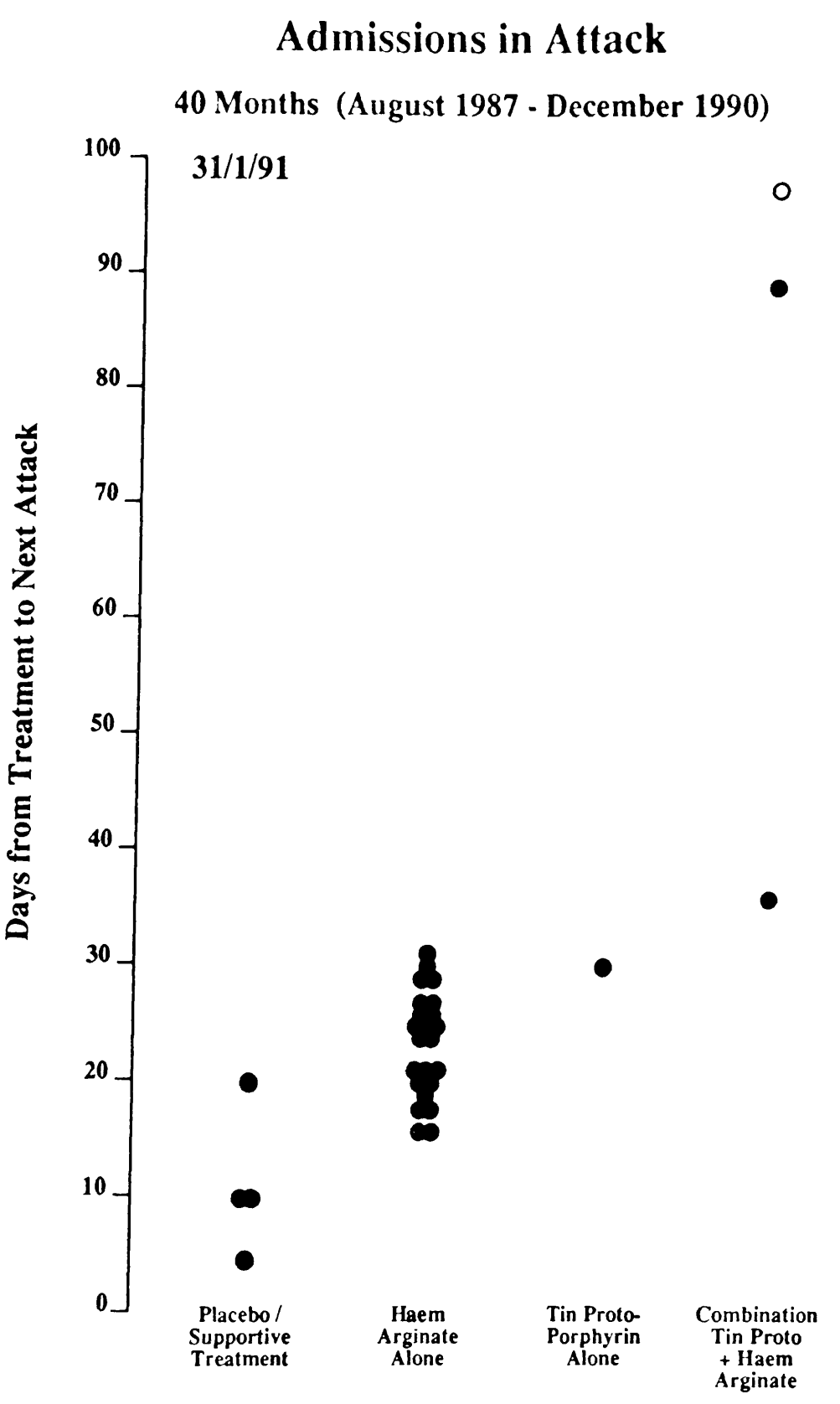
TABLE 33: URINARY PBG EXCRETION OF ONE PATIENT ON VARIOUS TREATMENT REGIMES

indicating that the different treatments influenced the duration of clinical remission achieved following the treatment. With placebo treatment the mean time from commencement of treatment to readmission in next attack was 11 days (range 4-20); with haem arginate it was 23.4 days (range 16-31); with SnPROTO it was 30 days and with the combination of haem arginate and Sn-PROTO it was > 74 days (range 36- >97) (Figure 41). These results indicate that the co-administration of Sn-PROTO with haem arginate prolongs the clinical as well as the biochemical remission. Following treatment with Sn-PROTO alone or in combination with haem arginate, patients noted mild cutaneous photosensitivity which persisted for up to 3 months after treatment.

8.5 DISCUSSION AND SUMMARY

Production of a porphyria-like state in rats proved effective using succinyl acetone, administered twice daily for a two day period. ALA excretion was dramatically raised as compared to normal with levels around 20 times those in the normal range and ALA-D activities effectively a third of control activities.

Treatment of this induced porphyria with haem arginate was not very effective as shown by the results in this chapter. Hepatic ALA-synthase activities remained higher than normal. In contrast, in the rats which were treated with Sn-PROTO, hepatic ALA-synthase activity normalised very quickly which was taken to indicate control of the hepatic porphyria. Treatment with a combination of the



The open circle denotes the patient has remained out of hospital until date shown.

FIGURE 41: LENGTH OF REMISSION FROM ACUTE PORPHYRIC ATTACK AFTER VARIOUS TREATMENTS

two drugs showed hepatic ALA-synthase activities in the normal range. This was likely to be due to the effect of Sn-PROTO, with haem arginate not providing any supplementary improvement. Measurement of the excretion levels in the rats which had been treated with various drugs were inconsistent with the biochemical improvement seen. ALA excretion remained very high in all of the treated groups of rats as well as in the porphyric group. The possibility that this maintained increase was due to some adverse effect on kidney function was discounted as the creatinine levels in the groups of rats were determined and these were found to be within the same range for all of them. These results indicated that kidney function was normal and that ALA excretion levels recorded were a true reflection of ALA produced in the rats.

Haem arginate is known to be taken up by the liver where it restores the haem deficiency produced by a block in the haem biosynthetic pathway. The lack of significant reduction in ALA excretion was unexpected in these rats. Injections of the haem arginate were carried out i.p. and this is not the same as in humans (i.v.) (Tokola, 1986, 1987; Tokola et al, 1986). However, i.p. administration has been used in rats when porphyria has been induced with the chemical AIA (Tokola et al, 1987b). It is possible that the dose of 12mg/kg/day haem arginate was not high enough to be effective in reducing the porphyric like state induced by succinyl acetone, but this dose of haem arginate has previously been shown to be effective in AIA induced porphyria

in rats (Tokola, 1987b).

In order to see if the dosing regime was optimal, the effects of reductions in the succinyl acetone dose and increases in the haem arginate dose were studied. These results showed that ALA excretion was not efficiently reduced in the animals even when this action was taken. Unfortunately, this was the only parameter assessed in these animals. Enzyme activities would have been a useful guide to liver haem biosynthesis.

The results presented in this chapter show that there are some inconsistences between the effects of treatment of an acute hepatic porphyric attack and the situation which occurs on induction of porphyria in rats using succinyl acetone. The choice of this drug to provide the animal model has already been discussed and was mainly due to the ability of this drug to block the haem biosynthetic pathway in these animals at a similar stage to that in AIP where PBG-D activity is reduced in the liver.

The fact that haem arginate treatment seemed to provide a poorer treatment for succinyl-acetone induced porphyria was not expected. By way of explanation, it is possible that administration of succinyl acetone to these animals may result in overproduction of ALA in all tissues and not just in the liver. The role of haem in controlling its synthesis is specific to the hepatic or housekeeping form of ALA-synthase and would not be expected to be effective in erythroid synthesis of ALA. The control of the ALA production by the liver appears to be occurring as shown by the normalised ALA-

synthase activities but production in other tissues such as the bone marrow may not be controlled by the therapeutic regimes used. Thus, excess ALA would still be in evidence, and would be excreted in the urine.

The above information suggests that succinyl acetone administration to produce an animal model of porphyria is unsatisfactory and that it would be useful to pursue one of the other available animal models of induced porphyria where haem arginate therapy has been shown to be effective (Tokola et al, 1987b).

The results of the clinical studies comparing the effect of the coadministration of haem arginate and Sn-PROTO were more encouraging. The degree of suppression of excretion of ALA and PBG was similar during the first 48 hours of treatment with haem arginate alone and in combination with Sn-PROTO. However with the combination treatment the duration of the suppression of overproduction was prolonged. This would be consistent with Sn-PROTO inhibiting the breakdown of the exogenously administered haem by haem oxygenase. The increased time between clinical attacks also indicated that coadministration of Sn-PROTO with haem arginate considerably prolongs clinical remission. These results indicate that co-administration of these two drugs may have a prophylactic role in acute porphyria. The observation of cutaneous photosensitivity lasting for several months after the administration of Sn-PROTO indicates that it remains in the tissues for a long time. This persistence of the Sn-PROTO

for months is consistent with its ability to prevent attacks even several months after its administration. It may be that much smaller doses of haem arginate will be equally effective when given in combination with Sn-PROTO. Indeed, it is even possible that a single injection of haem arginate/Sn-PROTO solution could achieve the same results. Further investigations of this attractive combination treatment are required.

CHAPTER 9

DISCUSSION AND CONCLUSIONS

9.1 DISCUSSION

The results contained in chapters 4, 5, 6 and 7 provide information on haem biosynthesis and porphyrin metabolism in an animal model of hyperbilirubinaemia, the Gunn rat (Gunn, 1938). These rats exhibit a congenital defect in bilirubin conjugation which leads to high levels of unconjugated bilirubin in the plasma. Bilirubin deposits in the tissues and has been especially noticeable in the brain. This has resulted in the animal being used to provide an animal model of kernicterus. The investigations have shown that blood porphyrin levels in Gunn rats are similar to those in control rats. In contrast, both urinary and faecal excretion of porphyrins is lower in the Gunn rat than in control rats. As explained fully in section 5.4, this may be a reflection of the type of porphyrin excretion observed in the Wistar animal from which the mutant Gunn originated.

The effect of bilirubin on the enzymes of the haem biosynthetic pathway was studied in various tissues of the Gunn rat. In the adult Gunn rat the activity of the penultimate enzyme of haem biosynthesis, PROTO-O was reduced in hepatic tissue. This is consistent with reports that bilirubin may act as a competitive inhibitor of this enzyme (Ferriera & Dailey, 1988; McColl et al, 1985). The inhibition of this enzyme activity was not accompanied by any compensatory increase in the rate controlling enzyme, ALA-synthase. This suggests that the degree of inhibition of PROTO-O activity was not sufficient to impair hepatic haem biosynthesis and thus result in derepression of the rate controlling step of the pathway. The absence of

overproduction of the porphyrin precursors of haem prior to the PROTO-O step is also in support of normal activity of ALA-synthase being present in the Gunn rat. In human variegate porphyria. PROTO-O activity is reduced by approximately 50% yet overproduction and increased excretion of porphyrins and their precursors is seen in only a proportion of patients.

Within the kidney of the Gunn rat bilirubin appeared to have no effect on PROTO-O activities. The reason for this is unknown but may be due to a much lower concentration of bilirubin in this tissue compared with that of hepatic tissue. The presence of much lower concentrations of bilirubin in this tissue is not surprising since the kidney does not play a role in the excretion of bilirubin and no sequestering protein, such as ligandin, is present.

The main objective of the studies was to determine whether bilirubin had any inhibitory effect on haem biosynthesis in the brain. The mechanism by which bilirubin causes the brain damage observed in kernicterus are unclear with reports stating adverse effects of the bile pigment on many intracellular processes ranging from disruption of various enzyme systems to integration into membranes. Abnormalities in neurological histology have frequently been observed. The effect of bilirubin on the brain haem biosynthetic pathway has not previously been assessed. In porphyria where the hepatic haem biosynthetic pathway is disrupted by a congenital defect in one of the enzymes, neurological dysfunction is frequently observed. This was the basis for investigation of the effect of bilirubin

on brain haem biosynthesis; to determine if disruption of the pathway may contribute to the neurological dysfunction which results from kernicterus.

The results of Chapter 7 provide the first measurements of brain PROTO-O activity. In the adult Gunn rat brain activity was found to be similar to the liver enzyme, although ALA-synthase activity was much lower in this tissue compared to levels seen in hepatic mitochondria. In the brain ALA-synthase is the point of control for haem biosynthesis. This lower activity corresponds with a much lower haem content of this tissue and probably reflects the fact that less haem is necessary to these cells. Since all other enzymes of the pathway except ALA-synthase are thought to be in excess it is unsurprising that PROTO-O activity in the brain is not lower than in hepatic tissue. The results suggest that the brain enzyme is identical to the form found in hepatic tissue. Future studies on the genetic loci of PROTO-O will determine if the PROTO-O enzyme is identical in brain and liver.

Bilirubin levels in Gunn rat brain were consistently measurable at approximately 4nmol/g wet weight tissue, and these levels were comparable regardless of whether ether or a barbiturate anaethetic were used on the animal. Cervical dislocation was avoided to enable removal of brain tissue undamaged. The presence of bilirubin in the brain of the adult Gunn rat did not produce any inhibition of PROTO-O activity. The literature states that kernicterus cannot be induced in the adult animal without disruption of the blood brain barrier. Thus the lack of PROTO-O inhibition observed in adult animals (section 7.2)

does not show whether PROTO-O inhibition is involved in the brain damage observed in kernicterus. To produce a more satisfactory animal model of kernicterus, bilirubin was displaced into the brain of neonatal rats by the drug sulphadimethoxine. The effects closely resembled symptoms of kernicterus. Despite the successful increase in brain bilirubin produced in these animals, no inhibitory effect of bilirubin on brain PROTO-O activity could be observed. This result does not support the theory that inhibitionof the penultimate stage is one of the causes of brain damage in kernicterus.

The bilirubin levels which were found in the brain were lower than the levels of the bile pigment which resulted in inhibition of PROTO-O activity *in vitro*. This may help to explain the lack of inhibition observed in the experiments carried out in the thesis. The *in vitro* work used a purified form of the enzyme and so may not accurately compare to the *in vivo* situation. The levels of bilirubin which were measured in brain in these experiments were up to 10nmol/g wet weight, still much lower than the concentrations of bilirubin (8-34μmolar) used by Ferriera and Dailey (1988) to induce a 50% inhibition of the purified hepatic enzyme *in vitro*. Therefore it is probable that the levels of bilirubin which were produced in these experiments in Chapter 7 were insufficient to cause inhibition of the enzyme provided that the protein is identical to that in liver. Development of symptoms which are consistent with kernicterus were observed in these animals tends to suggest that

bilirubin inhibition of haem biosynthesis is not a factor to be considered as a possible cause of the toxicity.

Another factor which may be important is the regional distribution of bilirubin within the brain. The cerebellum is known to contain the highest amount of bilirubin. All measurements of bilirubin content and enzyme activity were carried out in whole brain preparations, not in specific brain regions, and the results may reflect this. Local inhibitory effects of bilirubin on the activity of PROTO-O cannot be discounted.

The possibility that inhibition of PROTO-O activity may occur locally cannot be discounted. Regions of brain containing the highest amount of bilirubin, especially the cerebellum, could be selectively dissected and assayed for PROTO-O activity. In the future it would be interesting to examine the effect of bilirubin on purified preparations of brain tissue. This would determine whether bilirubin can inhibit the brain enzyme, and the concentrations of the bile pigment required. Certainly if the enzyme is identical to the hepatic form, inhibition would be expected.

Production of kernicterus in another model may also be useful. Lowering the plasma pH such that acidosis occurs appears to increase the risk of bilirubin encephalopathy (Kim et al, 1980). Alternatively disruption of the blood brain barrier in order that bilirubin can enter the brain would be yet another effective way to induce kernicterus.

It may be that the brain damaging effects of bilirubin act selectively on neuronal tissue, the bile pigment being

preferentially sequestered in nerve cells, so affecting their function. Effects observed on mitochondrial function may be secondary in this instance.

Analysis of the literature provided much information on haem degradation. In addition to stimulating interest in bilirubin toxicity, interest in the treatment of hyperbilirubinaemia developed. One recent and successful aspect has been the administration of Sn-PROTO to inhibit haem oxygenase activity and so decrease bilirubin production. The effects of Sn-PROTO have already been discussed, both in the introduction and in Chapter 8. This synthetic metalloporphyrin has been used to reduce hyperbilirubinaemia. As a consequence of the inhibitory action of action Sn-PROTO on haem breakdown, haem content of cells increases. The departmental interest in porphyria allowed access to information which suggested that this treatment might be beneficial to acute porphyria patients. Sn-PROTO has been previously applied to treat an acute porphyric attack, but only as monotherapy. The approach which I employed and presented in Chapter 8 was to combine haem arginate, to replace depleted haem, with Sn-PROTO which would prevent the induction of haem oxygenase. Induction of haem oxygenase activity after haem arginate administration limits the effectiveness of therapy by leading to increased haem breakdown. From the experimental data obtained, the animal model which was used in these experiments did not appear to adequately mimic hepatic porphyria. Both ALA and PBG excretion was elevated as compared to normal within a few hours of administration of

succinyl acetone for induction of porphyria. This was consistent with a useful model of acute hepatic porphyria. In contrast to the patients with the disease, animals treated with haem arginate, the traditional first line therapy, showed no reduction in precursor excretion. This was thought to be due to persisting overproduction of porphyrin precursors in tissues other than the liver. Thus the falling levels of ALA and PBG excreted from the liver would be masked. Induction of ALA-synthase by either AIA or DDC would be appropriate alternatives.

More encouraging results were obtained when the effects of the co-administration of Sn-PROTO and haem arginate were studied in patients experiencing acute attacks of porphyria. The addition of Sn-PROTO prolonged the reduction in porphyrin precursor overproduction produced by haem arginate and also prolonged the clinical remission. The co-administration of Sn-PROTO therefore provides an exciting new way of increasing the effectiveness of haem arginate therapy of acute porphyria. The only side-effect found with the Sn-PROTO was cutaneous photosensitivity which persisted for several months. Hopefully further inhibitors of haem oxygenase will be developed which do not have this side effect.

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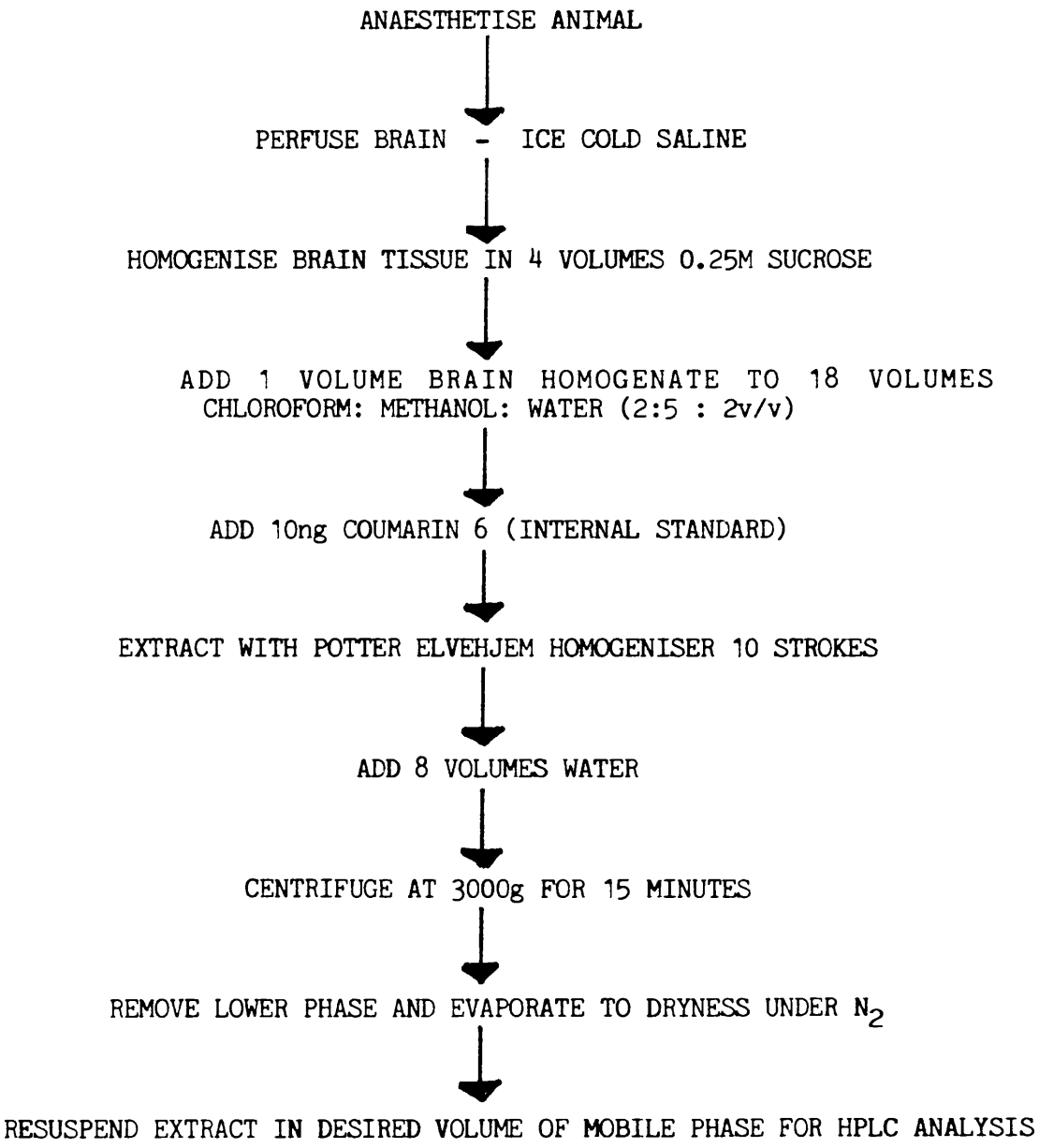
APPENDIX 1


ANAESTHETISE ANIMAL
PERFUSE BRAIN - ICE COLD SALINE
HOMOGENISE BRAIN TISSUE IN 4 VOLUMES 0.25M SUCROSE
ADD 1 VOLUME BRAIN HOMOGENATE TO 18 VOLUMES CHLOROFORM: METHANOL: WATER (2:5 : 2v/v)
ADD 10ng COUMARIN 6 (INTERNAL STANDARD)
EXTRACT WITH POTTER ELVEHJEM HOMOGENISER 10 STROKES
ADD 8 VOLUMES WATER
CENTRIFUGE AT 3000g FOR 15 MINUTES
REMOVE LOWER PHASE AND EVAPORATE TO DRYNESS UNDER N_2
RESUSPEND EXTRACT IN DESIRED VOLUME OF MOBILE PHASE FOR HPLC ANALYSIS