Enzyme Studies in Variegate Porphyria

by

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A thesis submitted for the degree *Doctor of Philosophy (Medicine)*

University of Cape Town

March 1990

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MT 616.399 MEI
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Dedication

To Renèe, Bruce and Robyn

With love and admiration

Acknowledgements

Numerous people have contributed unstintingly, and sometimes unknowingly, to all that has gone into the production of this thesis. Initially I would therefore like to express my gratitude to all these people as a whole, lest anyone be omitted.

Special gratitude must go to my supervisor *Professor Ralph Kirsch* whose unbounded enthusiasm and vision has often encouraged me to examine my results more deeply than I would naturally have done so. It is an added pleasure to work with someone who understands both work- and family-dedication and who is an unashamed supporter and friend of his students and workers.

Also to *Ed Sturrock*, who has been a close and necessary friend of my work and myself and has given much time to some of the illustrations in this dissertation. And to *Richard Hift* whose ability to learn and begin to "think porphyria" has been a tremendous personal encouragement over the last two years. Words cannot express my appreciation of the editorial help I have received from him.

I am deeply indebted to the following people with whom I have had the pleasure of working and who have helped guide my path through the Porphyria laboratories of the Department of Medicine at the University of Cape Town. *Bob Day*, who first challenged and inspired me to "think porphyrins"; *Gerry Blekkenhorst*, who demonstrated to me the power of laboratory and scientific method; *Peter Disler*, who first introduced me to protoporphyrinogen oxidase and *Michael Moore* who, while on sabbatical in Cape Town in 1983, broadened my vision and gave me much opportunity. In the same sense I must also acknowledge the indirect, but nevertheless very real influence that *Professor Len Eales* has had on my development.

My thanks also goes to the team of people in my immediate work environment. These are *Jean Sutherland, Brandon Davidson,* and *Abduragman Damon* whose meticulous work in the Porphyria laboratories has contributed enormously to this thesis.

Anne Corrigall facilitated the PBG deaminase purification with admirable skill and patience. Her help in this and other respects, and her friendship, is gratefully acknowledged.

Paul Adams gave invaluable insight and helped me with the kinetic analyses while *Ingrid Baumgarten* spent much time assisting with the culturing of Epstein-Barr virus transformed lymphoblasts.

Others who have contributed useful discussion and comments and who have given practical help are members of the MRC/UCT Liver Research Centre: *Yakov Baruch, Isabel Batho, Helen Botes, Edmund Brice, Chick Campbell, Bethwell Daki, Celeste De Jager, Mario Ehlers, Bill Franks, Lesley Frith, Val Hancock, Michael Klaas, Johnny Kruskal, Dennis Maeder, Dave Marais, Leslie Martin, Fadiel Parker, Lavinia Petersen, Claudette Rabinowitz, Simon Robson, Mogamat Samuels, Rachel Saunders, Trevor Scott, Mike Voigt,* and *Mick Wells*. Better colleagues could no one wish for.

The folk of the Medical Graphics department gave much support and encouragement. In particular, *Susan Abraham* performed wonders with some of the illustrations.

Marietjie Levey and her staff in D9 Renal Laboratory were most helpful and suppotive, particularly during my "early days". They happily performed all the urinary creatinine measurements.

I also wish to record my appreciation of those who have counselled and helped me in the choice and development of my career path as a medical scientist. Notably, *Professors Wieland Gevers* and *Eric Harley* who have given me much to reflect on and to my school science teacher *Ted Warren* whose keen interest and guidance was a personal joy and inspiration to me during my final three years at the South African College High School.

On a purely personal level I wish to express a deep gratitude to my *friends* who have played an invaluable part and given so much humour to my work.

Jock and Val Schoeman, my parents-in-law, and their family, have supported me unconditionally, for which I'm truly grateful.

My brother *Fritz* has been my "academic mentor" throughout my life. For this I thank him, as well as for the help he has given with the statistical analyses in this thesis.

My *parents* have been a constant source of help and perseverance as well as a model of rationality and wisdom. My mother, Sister Doreen Meissner, has been of the utmost help as her 25 years of experience and current activity in the Porphyria Service has naturally rubbed off on me. She has also been responsible for the thankless task of proofreading this thesis. Thank you.

Finally, I must express my sincere appreciation to three special people. My wife *Renèe* and my children *Bruce* and *Robyn*. They have made many sacrifices to enable me to complete this project and have encouraged me with patience, love and prayers all along the line - and this has meant the world to me.

To Ponder...

"Nature is but an effect
Whose cause is God"

William Cowper (1731-1800) British poet
The Task

"Laboratorium est oratorium"
The place where we do our scientific work is a place of prayer

Joseph Needham (1900-) British biochemist
The Harvest of a Quiet Eye (AL Mackay)

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Variegate Porphyria - Review

Abstract

ENZYME STUDIES IN VARIEGATE PORPHYRIA

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This thesis investigates a mechanism for the potential accumulation of δ-aminolaevulinic acid (ALA) and porphobilinogen (PBG) observed during the acute attack of variegate porphyria (VP). The hypothesis that a decrease in the activity of protoporphyrinogen oxidase results in an accumulation of distal porphyrin intermediates which in turn inhibit PBG deaminase is examined.

PBG deaminase activity was measured in Epstein-Barr virus transformed lymphoblasts (lymphoblasts) from subjects with quiescent VP and found to be decreased by approximately 25%. Additional evidence of partially inhibited PBG deaminase activity in VP was provided by finding a small but significant increase in urinary ALA and PBG concentrations in a series of 221 VP patients when compared to a series of normal controls.

A kinetic comparison between control and VP lymphoblast PBG deaminase using the Hill equation was performed. Results revealed that control lymphoblast PBG deaminase had a hyperbolic substrate-velocity curve which obeyed Michaelis-Menten kinetics whereas that of VP lymphoblasts yielded a sigmoidal substrate-velocity curve which did not.

Secondly, addition of protoporphyrinogen-IX to control lymphoblast PBG deaminase transformed the hyperbolic substrate-velocity curve to a sigmoidal form similar to that exhibited by PBG deaminase in VP lymphoblasts. Coproporphyrinogen-III had a similar but less potent effect. In contrast, uroporphyrinogen-III, protoporphyrin-IX, coproporphyrin-III and uroporphyrin-III had no effect on PBG deaminase.

Sephadex (G25) chromatography was used to separate PBG deaminase from accumulated porphyrin(ogen)s in VP lymphoblasts. This restored normal kinetic behaviour to PBG deaminase in VP lymphoblasts.

Similar kinetic alterations to those described above were observed using purified erythrocyte PBG deaminase with and without addition of protoporphyrinogen-IX.

VP lymphoblasts appeared to be a valid model of VP tissue as they exhibited an increase in protoporphyrin-IX and, to a lesser extent, coproporphyrin-III when compared to normal control cells.

These findings lend support to the potentially rate-limiting nature of PBG deaminase in the haem biosynthetic pathway and thus provide a possible mechanism for the accumulation of ALA and PBG in VP. The finding that uroporphyrinogen-III exerted no influence on PBG deaminase is in keeping with the hypothesis since PCT is a non-acute porphyria and ALA and PBG never appear to be elevated in this condition.

Chapter One

General Overview

This section aims at providing an introduction to haem biosynthesis, the structural chemistry of porphyrins and the porphyrias. Conventions and nomenclature used in this dissertation are defined (see Appendix 15 for abbreviations). While pertinent literature is reviewed and some specific comment made additional detailed literature review is provided in further chapters. Some historical perspectives are included since these might allow useful insight into the processes which have resulted in the development of knowledge of haem biosynthesis, the porphyrias in general, and more specifically, in variegate porphyria (covered in detail in chapter 2).

Essential structures and nomenclature

Figure 1.1 depicts a macrocycle consisting of four pyrrole rings joined by methene (-CH=) bridges. This tetrapyrrole is termed the porphin nucleus (Smith, 1975[b]). *Porphyrins* are derived from porphin by substitution of some or all of the peripheral positions with various side-chains (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1980). Thus the different types of porphyrins found

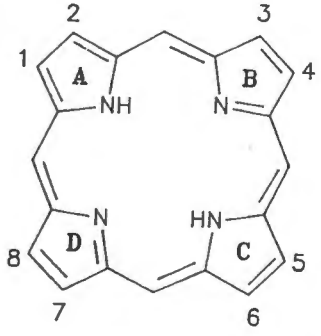

Figure 1.1: *The tetrapyrrolic porphin nucleus, the "backbone" of all porphyrin(ogen) structures. The conventional Fischer nomenclature is indicated.*

in nature, or produced synthetically in the laboratory, derive their differences from the composition of the side-chains attached to the tetrapyrrole ring. Earlier reviews cover detailed aspects of the structural and physical chemistry of porphyrins (Burnham, 1968; Marks, 1969; Adler, 1973; Smith, 1975[a]; Dolphin, 1979[a]).

The classical (Fischer) system of nomenclature numbers the peripheral positions from 1 to 8 and the interpyrrolic carbon bridges (sometimes termed the *meso* carbons) are designated α, β, γ and δ (Fischer and Orth, 1934). The individual pyrrole rings are named A, B, C and D (Falk, 1964) (figure 1.1). Typically, and perhaps understandably, trivial rather than systematic names are normally used. Table 1.1 (Smith, 1975; Eales, 1979; Kappas *et al*, 1983) lists the trivial names and structures of those naturally occurring porphyrins relevant to this dissertation. Most of these are derived from mammalian haem biosynthetic pathways. *Haem* (ferroprotoporhyrin) is a porphyrin macrocycle with a centrally bound iron atom.

Table 1.1: *Trivial names and side-chain substituents of porphyrins pertinent to this dissertation. Abbreviations as follows:*
A: -CH_2COOH, M: -CH_3, V: -$CH=CH_2$, P: -CH_2CH_2COOH, E: -CH_2CH_3

	Substituent on positions 1-8							
PORPHYRIN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Uroporphyrin-I	A	P	A	P	A	P	A	P
Uroporphyrin-III	A	P	A	P	A	P	P	A
Heptacarboxylic porphyrin	A	P	A	P	A	P	P	M
Hexacarboxylic porphyrin	M	P	A	P	A	P	P	M
Pentacarboxylic porphyrin	M	P	M	P	A	P	P	M
Coproporphyrin-I	M	P	M	P	M	P	M	P
Coproporphyrin-III	M	P	M	P	M	P	P	M
Isocoproporphyrin-III	M	E	A	P	M	P	P	M
Harderoporphyrin-IX	M	V	M	P	M	P	P	M
Protoporphyrin-IX	M	V	M	V	M	P	P	M
Deuteroporphyrin-IX	M	H	M	H	M	P	P	M
Mesoporphyrin-IX	M	E	M	E	M	P	P	M

The hexahydro-reduced form of the porphyrins (ie. six additional hydrogen atoms, two attached to the "pyrrolic" nitrogens and one to each of the four *meso*-carbons resulting in four methylene groups) are termed the *porphyrinogens* (Mauzerall, 1979) (figure 1.2). Porphyrinogens are important since they form the immediate and obligatory precursors of all but the penultimate step of haem biosynthesis (Rimington, 1937; Bissell, 1985).

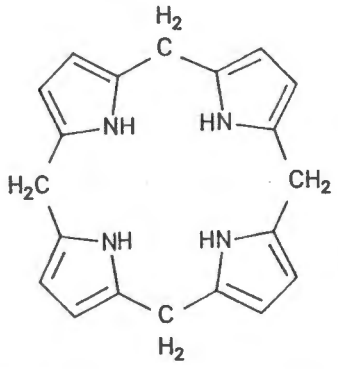

Figure 1.2: *Essential structure of the porphyrinogens, the hexahydro-reduced form of the porphyrins.*

Some general aspects of the chemistry of the porphyrins and porphyrinogens

Metal binding

Porphyrins readily complex with metals, such as iron to form haem, magnesium to form the chlorophylls or cobalt to form vitamin B_{12} via the corrins (Scott *et al*, 1972; Battersby and McDonald, 1975, Jones, 1976; Bissell and Schmid, 1987). These metalloporphyrins are widely distributed throughout the plant and animal kingdoms where they are essential for photosynthesis, oxygen transport, electron transport, the reduction of molecular oxygen and various hydroxylation reactions. Free porphyrins serve no known biological function in man (Bissell, 1985). Haem itself is only biologically functional when bound to various proteins to form the "*haemoproteins*". These include all the cytochromes, various catalases, peroxidases, mono- and dioxygenases and the cytochrome P_{450} series central to a variety of biological hydroxylation reactions (Dolphin, 1979). Thus for many organisms

oxygen transport, storage, reduction and activation are mediated by haemoproteins.

Aromaticity

Porphyrins, "the pigments of life" (Battersby *et al*, 1980), are highly coloured compounds. Their main absorption bands have very high extinction coefficients (up to $4X10^5M$), and they have intense "Soret" bands, at wavelengths in the region of 400nm (Smith, 1975[b]). They also exhibit red fluorescence when irradiated with ultraviolet light of wavelength approximately 400nm. These properties are indicative of a high degree of conjugation of the macrocycle. There are 22 π-electrons, but only 18 of these are included in any one delocalisation pathway; this is consistent with Hückel's $4n+2$ rule for aromaticity. Porphyrins are thus considered to be highly aromatic with the -electron clouds lying above and below the planar macrocycle, rather "like a ring doughnut" (Bonnett, 1981).

The porphyrinogens, on the other hand, are relatively non-aromatic (ie. aliphatic) compounds; the macrocycle exists in an unconjugated state since the methene bridges are replaced by four methylene bridges. This results in colourless, nonfluorescent compounds, with no "Soret" absorption band and with less rigid, more "floppy" structural characteristics (Smith, 1975[b]).

Isomeric forms

Where dissimilar side-chains occur on the particular pyrrole rings of the tetrapyrrole different isomeric forms are possible. Thus, in the case of an octasubstituted porphyrin or porphyrinogen with two types of side-chain, such as uroporphyrin and coproporphyrin, there are four possible isomers (Fischer and Orth, 1934). These are termed isomers I, II, III and IV (Falk, 1964). When there are three different side-chains, as in protoporphyrin, fifteen isomers are possible. In nature only the type III and I isomers have been found to exist. In mammals only the type III uroporphyrin(ogen) through to type III

coproporphyrin(ogen) and the type IX protoporphyrin(ogen) are used in the synthesis of haem.

Unless otherwise stated in this dissertation the terms uro-, hepta-, hexa-, penta- and copro- porphyrins or porphyrinogens will refer to the type III isomer and in the case of protoporphyrin(ogen), the type IX isomer.

Haem biosynthesis

General description

(Meyer and Schmid, 1974; Tait, 1978; Elder, 1980; Kappas *et al*, 1983; Moore *et al*, 1987)

Haem biosynthesis (figure 1.3) consists of a series of chemical reactions and modifications catalysed and linked by a number of enzymes forming an irreversible, unbranched metabolic pathway. The haem synthetic enzymes are compartmentalised within the cell, the first step and the last three steps occurring in the mitochondrion and the intermediate steps in the cytosolic compartment.

Haem synthesis commences with the condensation of *succinyl CoA* (formed by the citric acid cycle) and the amino acid *glycine* to form *δ-aminolaevulinic acid* (ALA). This takes place in the mitochondrion and is catalysed by the enzyme ALA synthetase. Succinic acid and glycine, two relatively small but readily available molecules, can thus be viewed as providing the constituent carbon and nitrogen atoms for haem. ALA leaves the mitochondrion and once in the cytosol, ALA dehydratase catalyses the condensation of two molecules of ALA to form the pyrrole subunit of the porphyrin ring, *porphobilinogen* (PBG). Next, four molecules of PBG are assembled by PBG deaminase to form an "unrearranged" bilane, *hydroxymethylbilane*. Working concurrently, uroporphyrinogen cosynthetase performs the necessary intramolecular rearrangement and ring closure of the bilane to form

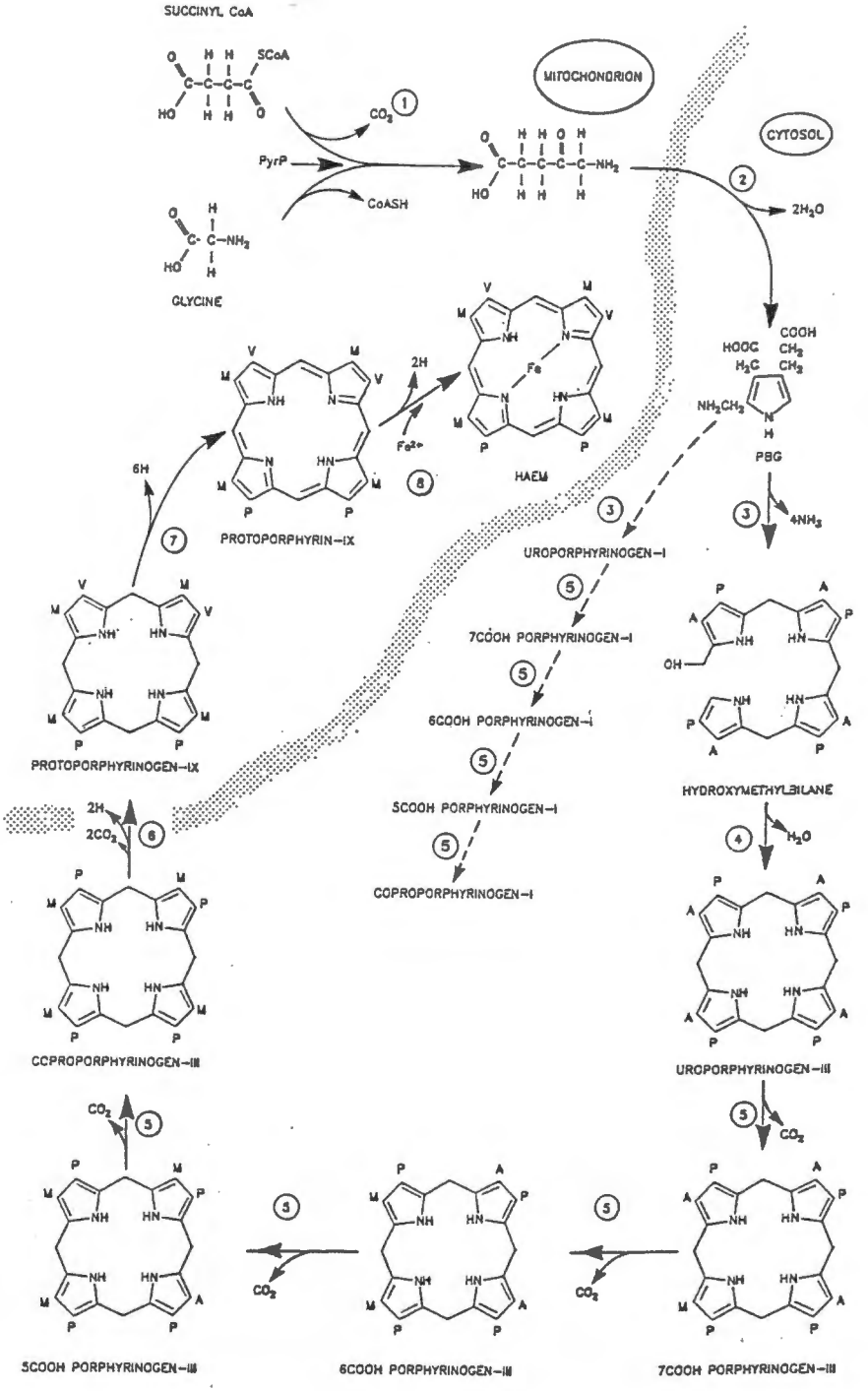

Figure 1.3: *The haem biosynthetic pathway. The numbers refer to the enzymes of haem biosynthesis as follows: 1, ALA synthetase; 2, ALA dehydratase; 3, PBG deaminase; 4, Uroporphyrinogen cosynthetase; 5, Uroporphyrinogen decarboxylase; 6, Coproporphyrinogen oxidase; 7, Protoporphyrinogen oxidase; 8, Ferrochelatase.*

uroporphyrinogen, the first of a series of porphyrinogens. In the absence of uroporphyrinogen cosynthetase hydroxymethylbilane spontaneously undergoes chemical cyclisation to form the type I uroporphyrinogen isomer which is apparently biologically functionless. Uroporphyrinogen decarboxylase catalyses the stepwise decarboxylation of uroporphyrinogen, which has eight carboxylated side-chains, through *hepta-*, *hexa-* and *penta-carboxylic porphyrinogen* intermediates to form the tetracarboxylic porphyrinogen, *coproporphyrinogen*. The process returns to the mitochondrion where coproporphyrinogen oxidase produces the dicarboxylic porphyrinogen, *protoporphyrinogen* by oxidative decarboxylation of the propionate side-chains in peripheral positions 2 and 4 to vinyl groups. Protoporphyrinogen is enzymatically oxidised by protoporphyrinogen oxidase to give *protoporphyrin* and the final step of the pathway is the chelation of the protoporphyrin ring structure with ferrous iron (Fe^{2+}) to form *haem*.

Historical aspects of the study of porphyrins and haem biosynthesis

Porphyrin research began with the acid treatment of haemoglobin to produce "iron-free" haematin (Berzelius, 1840; Scherer, 1841; Mülder, 1844). This was later purified and characterised by Thudichum who noted its fluorescence which he described as "a splendid blood-red colour" (Thudichum, 1867). The terms "haematoporphyrin", "phylloporphyrin" (chlorophyll) and indirectly "porphyrin" were introduced by Hoppe-Seyler in the 1870's (Hoppe-Seyler, 1871 and 1879). Around this time the first description of the appearance of "porphyrin" pigment in the urine of a human subject appeared (Schultz, 1874; Baumstark, 1874) (see *Historical aspects of the porphyrias*). In 1883 Soret discovered the highly distinctive spectroscopic 400nm wavelength absorption band in haemoglobin (Soret, 1883). Further studies on the nature of the pigments found in urines of the rather rare patients who excreted abnormal amounts of porphyrins ensued (Stokvis, 1889; Hammarsten 1891[a,b]; Salkowski, 1891; Garrod, 1892, 1893, 1896; Günther, 1911[a])

but it was not until 1912 that the correct tetrapyrrolic nature of the pigments was predicted by Küster (Küster, 1912). At that time it was thought that such a large ring would be unstable, and this structure was not accepted by Hans Fischer, the father of contemporary porphyrin chemistry, until much later. However, Fischer did have a hand in the final proof, which was provided by his total synthesis of "koproporphyrin" and "protohaem" in 1929 (Fischer *et al*, 1929; Fischer and Zeile, 1929). Fischer had been active in porphyrin chemistry since around 1915 (Fischer, 1915 and 1916), and continued to be so for approximately 30 years. His studies were both broad and deep. He inspired many pupils, including Watson and Waldenström (see Chapter 2, *Historical aspects of the porphyrias*). His profound contribution to knowledge was recognised by the award of the Nobel prize for Chemistry in 1930.

In the 1930's and 1940's a young chemist named Claude Rimington shed important new light on the porphyrins. His early work included studies on the urinary porphyrins excreted by cattle with congenital porphyria (Rimington, 1936), work which he was able to apply to human congenital porphyria (Rimington, 1937), and on the flight feathers of the South African Turaco (Rimington, 1939). Later, he did research to elucidate the chemistry and structure of the monopyrrole, PBG (Cookson and Rimington, 1954) and described many important analytical and separative techniques for the investigation of porphyrins (Rimington, 1943; Nicholas and Rimington, 1949; Rimington and Sveinsson, 1950).

Important contributions to knowledge of haem biosynthesis during the 1940's and '50's are generally ascribed to the radioisotope experiments of Shemin, and Neuberger although it must be remembered that many others such as Watson and his colleagues made significant contributions using similar techniques. These studies led to the description of the incorporation of ^{15}N glycine into haem in both humans and animals (Shemin and Rittenberg, 1946; Grinstein *et al*, 1949; Radin *et al*, 1950; Muir and Neuberger, 1950; Gray and Neuberger, 1950; Gray *et al*, 1950; Grinstein *et al*, 1950; Shemin and Wittenberg, 1951; Gray, 1952; Shemin *et al*, 1955). It was thus established that glycine and succinate supplied all the carbon and

nitrogen atoms of haem. Importantly, it was realised that ALA (Shemin and Russell, 1953; Neuberger and Scott, 1953) and PBG (Falk *et al*, 1953) were the precursors of all the porphyrins and of haem.

Simultaneously the necessary "tool" with which to study and confirm the synthetic steps of haem production came to hand with the discovery of experimental porphyria in animals by Schmid and Schwartz (Schmid and Schwartz, 1952), who used the drug *Sedormid* to induce porphyria in rabbits. Shortly after this the drug allylisopropylacetamide (AIA) was shown to have a similar effect (Goldberg, 1954) and thereafter followed the description of a number of other drug-related experimental porphyrias (Granick, 1953; De Matteis, 1967). Using these models, the sequence of haem biosynthesis was clearly established by the mid-1950's. It is of interest to observe that Lemberg and Legge had predicted virtually the correct sequence in 1949 (Lemberg and Legge, 1949).

The next major period of porphyrin biochemistry saw the investigation of the enzymes responsible for the catalysis of the haem biosynthetic pathway. This began in 1953 with the description of an enzyme facilitating conversion of PBG to porphyrins (Bogorad and Granick, 1953). This step was well studied and attributed to two distinct enzymes, namely PBG deaminase and uroporphyrinogen cosynthetase by 1958 (Bogorad, 1958). ALA dehydratase was described in 1954 (Granick, 1954), ALA synthetase (Kikuchi *et al*, 1958; Gibson *et al*, 1958) and uroporphyrinogen decarboxylase (Granick and Mauzerall, 1958) in 1958, ferrochelatase in 1959 (Goldberg, 1959) and coproporphyrinogen oxidase in 1961 (Sano and Granick, 1961). The last haem synthetic enzyme to be described was protoporphyrinogen oxidase in 1974 (Jackson *et al*, 1974; Poulson and Polglase, 1975). Much productive work on the enzymes of haem biosynthesis continues today.

The most recent areas of porphyrin research involve the application of the techniques of molecular biology to the study of the control and synthesis of the haem synthetic enzymes and the disturbances which result from disease.

Thus evolved the current era of porphyrin chemistry/biochemistry. A more detailed examination of each enzymatic step of the pathway follows.

Enzymes of haem biosynthesis

Formation of ALA by ALA synthetase (EC 2.3.1.37)

ALA synthetase is a pyridoxal phosphate requiring enzyme that promotes the condensation of succinyl CoA and glycine to form ALA (Kikuchi *et al*, 1958; Granick and Sassa, 1971; Jordan and Shemin, 1972). A sulphydryl group associated with initial binding of the pyridoxal phosphate co-factor has been identified at the catalytic site in rat liver ALA synthetase (Scholnick *et al*, 1972). The proposed catalytic mechanism involves initial condensation of glycine and succinate (activated as succinyl CoA) via a Schiff's base mechanism with formation of α-amino-β-ketoadipic acid as an enzyme-bound unstable intermediate, followed by decarboxylation of the glycyl carboxyl group (with release of CO_2) and release of ALA from the enzyme (Zaman *et al*, 1973; Akhtar *et al*, 1976).

In mammalian systems ALA synthetase appears to have a widespread tissue distribution (Kappas *et al*, 1983). It is present in high concentrations in immature erythrocytes where it is required for the synthesis of haemoglobin and in the liver where it is involved in the synthesis of a number of haemoproteins including cytochrome P_{450}, peroxisomal catalase and tryptophan pyrrolase.

Subcellularly it is found mainly in the mitochondria (Whiting and Elliott, 1972; Batlle, 1986). Smaller amounts of ALA synthetase have been detected in the cytosol. The latter is presumed to be newly synthesised ALA synthetase *en route* to the mitochondrion (Hayashi *et al*, 1969; Patton and Beattie, 1973). Indeed, in the rat liver ALA synthetase is synthesised on the cytoplasmic polysomes of the rough endoplasmic reticulum as a larger molecular weight complex than the

fully functional, processed, translocated mitochondrial form (Hayashi *et al*, 1970; Whiting and Elliott, 1972; Yamauchi *et al*, 1980) (see below). Within the mitochondrion it may be loosely associated with the inner mitochondrial membrane (McKay *et al*, 1969) but submitochondrial localisation studies in mitoplasts (intact inner membranes containing matrix) using established marker enzymes show it to be an enzyme of the mitochondrial matrix (Scotto *et al*, 1983). *In vitro* studies of rat liver ALA synthetase also suggest that is localised in the mitochondrial matrix (Ohashi and Sinohara, 1978).

The purification of ALA synthetase has led to discrepant (and confusing !) reports of its properties, contingent on the source and conditions of preparation. Its propensity to form aggregates both with itself and other proteins (Ohashi and Kikuchi, 1977; Batlle, 1986), explains some of these varied results. 3,5-Dicarbethoxy-1,4-dihydrocollidine (DDC) induced rat liver mitochondrial ALA synthetase has a reported molecular weight of 77000. The molecular weight of its cytosolic unprocessed form is 178000 (Whiting and Elliott, 1972). In contrast, the uninduced rat liver mitochondrial ALA synthetase has a molecular weight of 126000 (Paterniti and Beattie, 1979). Rat liver cytosolic ALA synthetase has also been reported to consist of a trimeric unit with an active subunit of molecular weight 110000, and two inactive subunits of 150000 and 60000 (Ohashi and Kikuchi, 1972). Mitochondrial ALA synthetase purified from chick liver embryos gave molecular weights in the presence of sodium dodecyl sulphate (SDS) of 49000 (Whiting and Granick, 1976) and more recently, 68000 (Pirola *et al*, 1983) and 65000 (Watanabe *et al*, 1983). The unprocessed ALA synthetase accumulating in the cytosol of chick liver has a molecular weight (in SDS) of 73000 (Watanabe *et al*, 1983).

Molecular biological approaches to ALA synthetase have thus far produced complementary DNA (cDNA) (Borthwick *et al*, 1984; Borthwick *et al*, 1985) and genomic clones to chick liver ALA synthetase (Maguire *et al*, 1986) and cDNA clones to rat liver ALA synthetase (Srivastava *et al*, 1988). Using this approach it was shown that rat ALA synthetase mRNA was identical in all tissues examined (liver, heart, kidney, erythroid spleen, brain and testis) and that only a

single species existed (Srivastava *et al*, 1988). This is consistent with a report that erythroid ALA synthetase in mouse spleen was coded for by the same gene as that in mouse liver (Schoenhaut and Curtis, 1986), but contrary to the proposal favouring the existence of a multigene family for ALA synthetase with different mRNA species in different tissues (Yamamoto *et al*, 1985). Further studies involving this approach should be most enlightening.

Hepatic ALA synthetase has a short half-life of 60-70min for the mitochondrial form in mammalian systems (Marver *et al*, 1966; Hayashi *et al*, 1969; Barnes *et al*, 1971) and is highly inducible (Granick and Sassa, 1971), responding rapidly to haem requirement. The amount of active ALA synthetase is also very sensitive to induction by a wide variety of compounds such as allyl isopropyl acetamide (AIA) and phenobarbital (Granick, 1966). These facts fit well with the concept that the formation of ALA by ALA synthetase is the rate-limiting step of haem synthesis. This is supported by the finding that ALA synthetase has a lower activity than any of the other enzymes involved in haem synthesis (Elder, 1982; Kappas *et al*, 1983). ALA synthetase is thus widely regarded as the flux controlling step in haem biosynthesis. Further aspects of its role in the regulation of haem production will be considered below.

Finally, mention must be made of the intriguing observation that the precursor of ALA formation in plant systems, 4,5-dioxovaleric acid (DOVA) (Beale *et al*, 1975) is present in bovine liver extracts (Vartikovski *et al*, 1980) and that there is an enzyme (DOVA transaminase) mediating its conversion to ALA which can be used for haem production (Morton *et al*, 1981). The significance and role of this alternative metabolic route of ALA formation in mammalian mitochondrial systems is obscure (Bissell and Schmid, 1987; Batlle, 1986; Moore *et al*, 1987).

Formation of PBG by ALA dehydratase (EC 4.2.1.24)

Purified ALA dehydratase is a relatively large protein complex with reported molecular weights of the order of 280000 (Kappas *et al*, 1983;

Batlle, 1986). It has been shown to consist of eight subunits, probably identical, which combine to form an active complex residing in the cytosolic compartment (Wu *et al*, 1974; Shemin, 1975; Anderson and Desnick, 1979). The gene encoding the human enzyme has been shown by linkage and by somatic cell hybridisation and specific immunoassay to reside on chromosome 9 (Eiberg *et al*, 1983; Beaumont *et al*, 1984[a]).

Zinc (Zn^{2+}) is required for maximal activity (Abdulla and Haeger-Aronsen, 1971; Finelli *et al*, 1984), each ALA dehydratase octamer binding up to eight ions (Wu *et al*, 1974; Tsukamoto, 1980). Zn^{2+} may play a structural role, allowing the enzyme to adopt the correct conformation for activity (Hasnain *et al*, 1985). However, there is also evidence to suggest that zinc may protect the active site by preventing autoxidation of essential sulphydryl groups to a disulphide bond (Tsukamoto, 1980). Other metals such as aluminium are also activators of ALA dehydratase (Shemin, 1972; Elder, 1980). The enzyme is inactivated by sulphydryl blocking agents and its activity *in vitro* may be maximised by addition of sulphydryl-reducing agents such as -mercaptoethanol, cysteine, reduced glutathione or dithiothreitol (Kappas *et al*, 1983; Batlle, 1986). ALA dehydratase is very sensitive to inhibition by lead (Pb^{2+})(Gibson and Goldberg, 1970; Granick *et al*, 1973). Pb^{2+} can replace the intrinsic Zn^{2+} stoichiometrically (Finelli *et al*, 1975) probably by occupying the same, or a nearby, site (Tsukamoto, 1980). Ethanol (Moore *et al*, 1971) and succinylacetone (accumulation of which may occur in hereditary tyrosinaemia) may also exert inhibitory effects on ALA dehydratase (Sassa *et al*, 1981). Succinylacetone, is structurally similar to ALA in that it also has a keto group in the gamma position relative to the carboxyl carbon (figure 1.4).

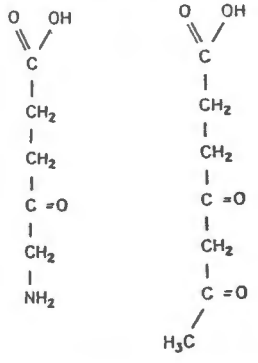

Figure 1.4: *Structural similarity of ALA (left) and succinyl acetone (right), a competitive inhibitor of the enzyme ALA dehydratase.*

Mechanistically ALA dehydratase (from *Rhodopseudomonas spheroides*) catalyses a series of reactions (Nandi and Shemin, 1967; Shemin, 1976) which result in the condensation of two identical substrate (ALA) molecules to form the monopyrrole PBG (figure 1.3) with the removal of two molecules of water. One molecule of ALA is covalently bound to the enzyme through the formation of a Schiff's base between the -amino group of a lysine residue and the keto group of ALA. The ALA molecule initially bound to the enzyme has been shown to provide the propionate side-chain of PBG (Jordan and Seehra, 1980).

ALA dehydratase has a relative activity many times higher than that of ALA synthetase (Kappas *et al*, 1983). Thus ALA dehydratase is thought not to have any significant role in the regulation of haem biosynthesis.

Formation of uroporphyrinogen by PBG deaminase (EC 4.3.1.8) and uroporphyrinogen cosynthetase (EC 4.2.1.75)

The formation of uroporphyrinogen from PBG has been a subject of interest and debate since the earliest days of porphyrin biosynthetic chemistry. It has been referred to as "the type III problem in porphyrin synthesis" (Battersby *et al*, 1979) and only of late have most of the clues to the solution of the "problem" been assembled into a coherent picture (Battersby, 1988). In essence the problem revolves around the fact that the symmetrical type I uroporphyrinogen isomer which one would think to be the easiest and most logical isomeric form to synthesise, cannot be metabolised further than to coproporphyrinogen-I and is thus not a source of haem. Although uroporphyrinogen-I has recently been shown to be a substrate for the methylases of the vitamin B_{12} pathway (Müller *et al*, 1986; Müller *et al*, 1987), it is generally accepted that Nature demands the series III isomer for haem production in man.

Production of the series III isomer is facilitated as follows: Ingenious single turnover experiments with labelled substrate have shown that PBG deaminase operates as the assembly enzyme by catalysing the

head-to-tail assembly of four units of PBG, starting with ring A and ending with ring D (Battersby *et al*, 1979; Seehra and Jordan, 1980). The product of this action was shown by ^{13}C nuclear magnetic resonance (NMR) spectroscopy to be the linear (ie. uncyclised, open chain) tetrapyrrole hydroxymethylbilane* (Battersby *et al*, 1976; Burton *et al*, 1979) (figure 1.3). The structure of this product has been confirmed by total *in vitro* synthesis (Battersby *et al*, 1982). Clearly, PBG deaminase is not a ring-closing enzyme; its function is to build the hydroxymethylbilane and release it for subsequent processing.

Were spontaneous chemical cyclisation to occur at this stage (as happens in the absence of uroporphyrinogen cosynthetase) the arrangement of the pyrrole side-chains would be such that the series I isomer is formed (figure 1.3).

Hydroxymethylbilane is the substrate for uroporphyrinogen cosynthetase which effects cyclisation and concomitant inversion of the terminal D ring by an intramolecular process (Jordan *et al*, 1979). Thus the asymmetric series III isomer of the cyclic tetrapyrrole uroporphyrinogen is formed. There is evidence that formation of a *spiro* system is the means by which Nature performs the "conjuring trick" of converting the hydroxymethylbilane into uroporphyrinogen (Jordan *et al*, 1979; Battersby *et al*, 1980; Stark *et al*, 1985). This is supported by elegant experiments (Stark *et al*, 1986) involving the synthesis of two isomeric, very close analogues of the proposed spirane-structured intermediate. PBG deaminase and uroporphyrinogen cosynthetase can thus be viewed as acting concertedly and concurrently to form uroporphyrinogen-III firstly by assembly of four monopyrroles, followed by intramolecular rearrangement and ring closure (Battersby, 1988).

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* One mole of ammonia is released for every mole of PBG assembled, hence the designation PBG deaminase. The alternative name hydroxymethylbilane synthase is perhaps more appropriate; however the older, more commonly recognised name PBG deaminase is used in this thesis.

PBG deaminase has been purified from a wide variety of sources (Sancovich *et al*, 1969; Davies and Neuberger, 1973; Jordan and Shemin, 1973; Anderson and Desnick, 1980; Fumagalli *et al*, 1985). It is a cytosolic enzyme, appears to be monomeric and reported molecular weights have varied between 34000 and 44000. The gene coding for PBG deaminase in *E. coli* (*hem C*) has been cloned and sequenced (Thomas and Jordan, 1986). In man the gene is thought to reside on chromosome 11 (Meisler *et al*, 1980, 1981; Wang *et al*, 1981).

PBG deaminase has an unusual property in that it is able to form relatively stable intermediate complexes, sharing one, two, three or four pyrrole units (substrate) bound to the catalytic site (Anderson and Desnick, 1980; Berry *et al*, 1981). It has also been established that a covalent bond is formed between the enzyme and the first bound substrate (Jordan and Berry, 1981; Battersby *et al*, 1983; Evans *et al*, 1986). This linkage is remarkable in that it appears that an enzyme-bound cofactor, in the form of a dipyrromethane, is responsible for "binding of the substrate and for directing the synthesis of the tetrapyrrole at the catalytic site" (Jordan and Warren, 1987; Scott *et al*, 1988). The dipyrromethane cofactor is itself formed from two molecules of PBG, the suggestion being that the PBG required for this is incorporated into the apoenzyme before folding (Scott *et al*, 1988). It is thought to be anchored to the enzyme via the second cysteine residue found in human and *E. coli* PBG deaminase (Evans *et al*, 1986; Scott *et al*, 1988).

All facts considered, the putative mechanism of PBG deaminase is shown in figure 1.5 (Scott *et al*, 1988). The process begins with the dipyrromethane cofactor covalently bonded to the enzyme cysteine-SH residue via its free, terminal -pyrrole carbon. Thereafter, the first (kinetic) encounter with substrate involves attachment of a single molecule of PBG (with loss of ammonia) to the dipyrromethane to form the so-called ES_1 complex. This process is repeated until the "tetra PBG" (ES_4) complex is formed. The latter can best be envisaged as a "growing oligopyrrolic chain". Once the final hexapyrrole chain has been formed the complex is cleaved (site-specifically) to yield a linear tetrapyrrole and the remaining bound dipyrromethane (figure 1.5). The tetrapyrrole is in the form of an azafulvene bilane which may

either become the substrate of uroporphyrinogen cosynthetase, or, in the absence of this enzyme, may be stereospecifically hydrated to hydroxymethylbilane at pH 12, or cyclised chemically to uroporphyrinogen-I at pH 8.

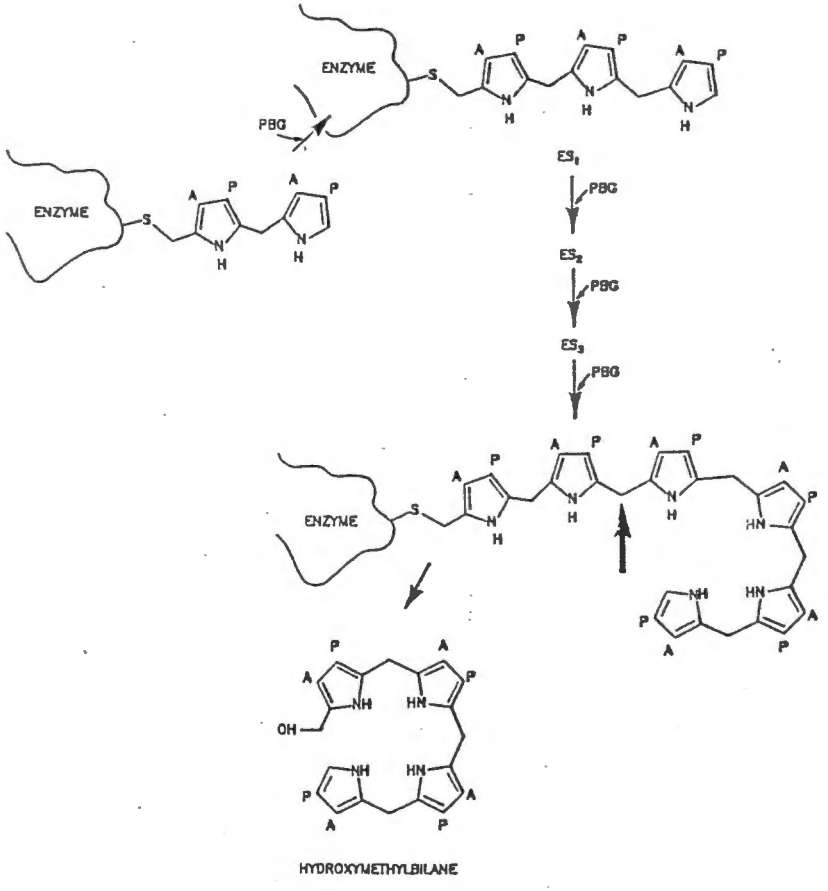

Figure 1.5: *Detail of action of PBG deaminase involving the dipyrromethane cofactor and formation of ES_1, ES_2, ES_3, and ES_4 intermediates prior to site-specific cleavage (marked with arrow) to give hydroxymethylbilane.*

Kinetically PBG deaminase has been described as a relatively simple enzyme displaying classical Michaelis-Menten kinetics (Frydman and Frydman, 1970; Anderson and Desnick, 1979; Sassa *et al*, 1978; Hart *et al*, 1984; Scott *et al*, 1988). There are reports from one group describing more thorough kinetic investigations (Sancovich *et al*, 1969; Llambias and Batlle, 1970; Fumagalli *et al*, 1985). These report inhibition of the enzyme at PBG concentrations above 400μM, and that PBG deaminase may possess two cooperative substrate binding sites per molecule of enzyme. The latter finding is based on the observation of non-linear double reciprocal substrate-velocity plots and Eadie plots (velocity/[substrate] versus velocity), and by application of the Hill equation (Segel, 1975). Eadie plots are particularly useful for demonstrating deviation from Michaelis-Menten behaviour and the Hill equation yields a Hill coefficient (n) which indicates the number of cooperatively interacting ligand binding sites on the protein. Although no specific inhibitors or allosteric effectors of PBG deaminase have been described it is conceivable, particularly in the case of an enzyme with multiple cooperative binding sites that inhibition may occur through interaction at one or other, or all of the sites.

Uroporphyrinogen cosynthetase is also a cytoplasmic enzyme. Purified uroporphyrinogen cosynthetase from *Euglena gracilis* has been shown to have a molecular weight of between 30000 and 40000 (Hart and Battersby, 1985). It appears to be present in relative excess to the deaminase which, under normal circumstances ensures that PBG is completely metabolised to uroporphyrinogen-III (Bissell and Schmid, 1987)

Formation of coproporphyrinogen by uroporphyrinogen decarboxylase (EC 4.1.1.37)

After the formation of the basic tetrapyrrole ring, uroporphyrinogen decarboxylase successively decarboxylates the four acetyl side-chains to yield coproporphyrinogen. It has been established that decarboxylation of the octacarboxylic uroporphyrinogen commences

on the asymmetric D ring and proceeds through rings A, B and C to give heptacarboxylic, hexacarboxylic and pentacarboxylic porphyrinogen intermediates (Mauzerall and Granick, 1958; Jackson *et al*, 1976) (figure 1.3). These intermediates are not merely transient enzyme-substrate complexes, but relatively stable individual porphyrinogen species and are detectable *in vivo*. Both the series III and I isomer uroporphyrinogens are suitable substrates for uroporphyrinogen decarboxylase but the series III isomer is decarboxylated more rapidly than the series I isomer (Granick and Mauzerall, 1958; Smith and Francis, 1981), which appears to be decarboxylated in any order (Jackson *et al*, 1977). The dissimilarity presumably results from the difference in symmetry offered by the series III isomer.

The erythrocytic form of the enzyme has been purified to apparent homogeneity from human (De Verneuil *et al*, 1983; Elder *et al*, 1983) and chicken erythrocytes (Kawanishi *et al*, 1983). Chicken erythrocyte studies suggest that uroporphyrinogen decarboxylase exists as a dimer consisting of two subunits, each with a molecular weight of approximately 40000 (Kawanishi *et al*, 1983) whereas the human erythrocyte enzyme appears to exist as a catalytic unit of molecular weight reportedly ranging between approximately 40000 (Elder *et al*, 1983) and 46000 (De Verneuil *et al*, 1983) in the presence of SDS. It is not entirely clear whether this is a monomer or a dimer.

Specific antibodies to the human erythrocyte enzyme have been generated and quantitative immunoprecipitation and crossed immunoelectrophoresis indicate that the erythrocyte and liver enzymes are similar but not immunochemically identical (Elder *et al*, 1983). The gene encoding human uroporphyrinogen decarboxylase has been localised to chromosome 1 (De Verneuil *et al*, 1984; Dubart *et al*, 1986) and the cDNA sequence determined (Romeo *et al*, 1986).

At least one sulphydryl group appears to be involved in the decarboxylation process and titration of the purified uroporphyrinogen decarboxylase with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) suggests the presence of five free sulphydryl groups in the protein structure as a whole (De Verneuil *et al*, 1983). The enzyme

is sensitive to inhibition by sulphydryl blocking agents and by certain divalent metals such as Cu^{2+}, Hg^{2+} and Pt^{2+} (Batlle, 1986; Woods and Fowler, 1987). Because of clinical evidence that iron promotes the expression and aggravates the biochemical features of conditions related to uroporphyrinogen decarboxylase deficiency, it has been suggested that the essential sulphydryl group may render uroporphyrinogen decarboxylase susceptible to inhibition by iron. However, iron (Fe^{2+} and Fe^{3+}) has variously been reported to stimulate (Blekkenhorst *et al*, 1979), to have no effect on (Woods *et al*, 1981), or to inhibit uroporphyrinogen decarboxylase (De Verneuil *et al*, 1983; Smith and Francis, 1983; Mukerji *et al*, 1984). The different effects may depend on the specific enzyme preparation and on the conditions under which the experiments are carried out (opinion).

Formation of protoporphyrinogen by coproporphyrinogen oxidase (EC 1.3.3.3)

The next step in haem synthesis is the decarboxylation and oxidation (dehydrogenation) of the propionyl side-chains of rings A and B of coproporphyrinogen to vinyl groups yielding protoporphyrinogen (figure 1.3). This transformation is catalysed by coproporphyrinogen oxidase (Batlle *et al*, 1965). This enzyme is reported to be located in the intramitochondrial space (mitochondrial matrix) (Batlle *et al*, 1965; Sano and Granick, 1961; Poulson and Polglase, 1971) although more recent evidence shows it to be an enzyme residing in the intermembrane space of the mitochondrion (Elder and Evans, 1978; Grandchamp *et al*, 1978). It appears not to be bound to the mitochondrial membrane. It requires molecular oxygen as a hydrogen acceptor (Sano and Granick, 1961; Batlle *et al*, 1965). The gene encoding human coproporphyrinogen oxidase has been assigned to chromosome 9 (Grandchamp, 1983). Coproporphyrinogen oxidase purified from bovine liver has been shown to exist in a monomeric form and has a molecular weight approximately 71000 (Yoshinago, 1980). Rat liver coproporphyrinogen oxidase has a similar molecular weight (Batlle, 1965).

The enzyme accepts the III and IV isomers of coproporphyrinogen as substrate, but not the I or II isomers (Sano and Granick, 1960; Porra and Falk, 1964; Batlle *et al*, 1965). Thus any uroporphyrinogen-I formed at an earlier step will be metabolised only as far as coproporphyrinogen-I. Nature thus ensures that only the correct isomeric form of protoporphyrinogen, the series IX form, necessary for haem production, enters the final stage of haem biosynthesis. Because two decarboxylations are involved formation of an intermediate tricarboxylic, monovinyl porphyrinogen might be anticipated. This appears to be the case (Jackson *et al*, 1980) although under normal circumstances it is difficult to detect. It has been shown that this tricarboxylic porphyrinogen (harderoporphyrinogen) consistently bears its vinyl group in position 2 of the A ring, rather than that of the 4 position in ring B (Jackson *et al*, 1978; Yoshinago and Sano, 1980). Thus enzymatic conversion of the propionate side-chains is envisaged as occurring in a step-wise manner from positions 2 to 4, with the formation of tricarboxylic (-hydroxypropionate) porphyrinogen as an obligatory intermediate (Sano, 1966).

Formation of protoporphyrin by protoporphyrinogen oxidase (EC 1.3.3.4)

The next step, oxidation of protoporphyrinogen to protoporphyrin, is catalysed by the enzyme protoporphyrinogen oxidase (Jackson *et al*, 1974; Poulson and Polglase, 1975; Poulson, 1976). This was the last of the haem synthetic enzymes to be "discovered" and its existence was doubted for some time. Since the simple oxidation of protoporphyrinogen required to facilitate iron insertion into the tetrapyrrole ring to produce haem is a spontaneous chemical reaction proceeding rapidly and unaided *in vitro*, the necessity for enzymatic assistance was initially not appreciated (Moore *et al*, 1987). However, in the highly-reducing environment of the mitochondrion spontaneous oxidation is not possible and an enzyme is required to remove six hydrogen atoms from protoporphyrinogen to form protoporphyrin.

The reported characteristics of this inner mitochondrial membrane (Deybach *et al*, 1985), oxygen-requiring enzyme (Poulson and Polglase, 1975; Poulson, 1976) are discrepant in some respects. Reported molecular weights are 57000 (bovine), 35000 (rat) and 65000 (murine) (Siepker *et al*, 1987; Poulson, 1976; Dailey and Karr, 1987 [respectively]). In all reports the enzyme does, however, appear to be present as a single subunit. It has been suggested that sulphydryl groups are functionally important in rat liver protoporphyrinogen oxidase (Poulson, 1976); yet the lack of inhibition of activity by *N*-ethylmaleimide or iodoacetamide, or stimulation by β-mercaptoethanol or dithiothreitol in murine protoporphyrinogen oxidase argue against catalytically important sulphydryl groups (Dailey and Karr, 1987). Whereas recent studies point to a flavin adenine dinucleotide (FAD) prosthetic group associated with bovine protoporphyrinogen oxidase (Siepker *et al*, 1987) initial work on murine protoporphyrinogen oxidase showed no evidence of a chromophoric cofactor such as a flavoprotein. Subsequently, however, the use of a more rapid purification procedure for murine protoporphyrinogen oxidase has resulted in detection of a flavin mononucleotide (FMN) chromophoric cofactor (Proulx and Dailey, 1989).

VP family studies demonstrating linkage between the alpha-1-antitrypsin gene and the gene locus for VP suggest that protoporphyrinogen oxidase is encoded on chromosome 14 (Bissbort *et al*, 1988). There is evidence to suggest significant homology between bovine protoporphyrinogen oxidase and ferrochelatase. This is based on the demonstration of common epitopes in antibodies raised to the two enzymes and of common peptides resulting from chymotryptic digestion of the two enzymes (Siepker *et al*, 1987). Others, however, argue against sequence homology between the two enzymes, based on the dissimilar specific functions of each of the two enzymes.

The properties of this enzyme are thus not yet fully elucidated. The precise mechanism whereby the oxidation of protoporphyrinogen is accomplished remains to be clarified.

Formation of haem by ferrochelatase (EC 4.99.1.1)

The final step of haem biosynthesis is the insertion of ferrous iron (Fe^{2+}) into protoporphyrin to form ferroprotoporphyrin (ie. haem) (figure 1.3) (Goldberg *et al*, 1956; Rimington, 1958). Ferrochelatase is found on the inner mitochondrial membrane (Jones and Jones, 1969; McKay *et al*, 1969). Lipids appear to be necessary for activity, indicating that it may exist as a lipoprotein complex *in vivo* (Sawada *et al*, 1969; Yoneyama *et al*, 1969). Lipid moieties may be necessary for transferring iron from an aqueous to the nonaqueous membrane environment; for maintaining solubility of protoporphyrin which is relatively hydrophobic, or for both of these functions (Moore *et al*, 1987). An intramitochondrial pool of iron, not associated with cytochromes or iron-sulphur proteins, has been postulated as the source of iron (Tangeras, 1985). It is probable that the reduction of ferric iron (Fe^{3+}) to Fe^{2+} takes place in close proximity to ferrochelatase (Moore *et al*, 1987).

Purified bovine ferrochelatase has a molecular weight of approximately 40000 (Dailey and Fleming, 1983). It is of low substrate-specificity and will insert Fe^{2+} into other dicarboxylic porphyrins such as meso- and deuteroporphyrin (Dailey *et al*, 1986). Indeed, it is not specific for Fe^{2+} either, and will effect the chelation of several divalent metals such as Zn^{2+} and Co^{2+} (Johnson and Jones, 1964; Jones and Jones, 1969; Elder, 1980).

Ferrochelatase is activated by sulphydryl containing agents. This suggests that sulphydryl group(s) may be involved at the catalytic site (Goldberg *et al*, 1956; Dailey *et al*, 1986). It also suggests that sulphydryl agents may be necessary to maintain the substrate iron in its reduced form (Porra and Jones, 1963[a,b]) and may also be necessary to protect the enzyme and substrate against phospholipid peroxidation (Petersen *et al*, 1980; Dailey and Fleming, 1986)

Tissue localisation and activities of haem biosynthetic enzymes

All cells requiring haem are presumed able to synthesise this compound (Elder, 1980; Bissell, 1985). Thus all tissues capable of aerobic metabolism synthesise haem. Erythrocyte precursors are able to synthesise haem to meet their requirement for haemoglobin production during the early stages of their differentiation. The concentration and turnover rates of the many haem-requiring proteins differ significantly. Similarly individual tissue requirement for haem synthesis is equally varied.

The liver, which via enzyme systems such as the cytochrome P_{450} family*, plays a major role in the detoxification and metabolism of a wide range of endogenous and exogenous compounds, is one of the organs most active in haem synthesis (Bloomer and Straka, 1988). It is estimated that of all the ALA synthesised by the liver, approximately 65% is used as the prosthetic group of the various cytochrome P_{450}'s, 15% for peroxisomal catalase, 8% for cytochrome b_5 and 6% for mitochondrial cytochromes (Sassa and Kappas, 1981). While the actual amount of haem contained in the cytochrome P_{450}'s in the liver is small in comparison with the amount contained in the body's haemoglobin pool, they have a short half life compared to that of haemoglobin (hours as opposed to many days) (Bissell, 1985). Thus a significant proportion of total haem production in the body is required by the liver for the cytochrome P_{450} enzymes.

A detailed discussion of the family of enzymes which constitute cytochrome P_{450} is beyond the scope of this dissertation. A recent review (Waterman *et al*, 1986) once again stresses the fact that the synthesis of the cytochrome P_{450}'s is highly inducible and appears to be related to metabolic needs of the cell. A large number of endo- and

* The term cytochrome P_{450} used in this dissertation embodies all the haemoproteins of the cytochrome P_{450}-P_{448} series.

xenobiotics are capable of inducing cytochrome P_{450}'s. These include the barbiturates and gonadal steroids.

Thus the haem requirement varies and haem synthesis appears to be regulated to meet this need. Under normal circumstances this regulation is highly efficient and is discussed below.

Haem formation by erythroblasts accounts for approximately 85% of total body haem (Jones *et al*, 1971; Berk *et al*, 1976; Elder, 1980) and can thus be considered a major, if not the major, site of haem synthesis. Erythroblast haem synthesis is apparently effected by the same pathway described above, yet little is known about the effect of erythroblast differentiation on the activity and regulation of haem biosynthesis in erythropoietic tissue (Moore *et al*, 1987).

Within developing erythroid cells haem may be required, not only as the prosthetic group in haemoglobin, but also for other purposes. Free intracellular haem may play a role in control of globin gene expression (Harrison, 1984), may regulate iron uptake (by controlling the release of iron from transferrin (Ponka *et al*, 1974) and/or by regulating the number of transferrin receptors on differentiating erythroid cells (Pelicci *et al*, 1982)), and may play a role in the overall coordination of erythroblast metabolism and differentiation (Glass *et al*, 1975; Ibrahim *et al*, 1982; Monette *et al*, 1984).

In considering the nett flux through a metabolic pathway it is of interest to compare the activity of each enzyme with the others. Such data has been collated previously in descriptions of haem biosynthesis (Elder, 1980, 1982; Kappas *et al*, 1983; Moore *et al*, 1987) and is summarised in table 1.2. Inter-laboratory variation in absolute enzyme activities and the varied sources and methods of preparation of tissue for assay make comparison difficult, yet one point is clear. When all activities are calculated as nmol of ALA equivalents per h per g of tissue or per g of protein (see table 1.2) and expressed relative to ALA synthetase activity, the activities of all the other enzymes are well in excess of that of ALA synthetase. The exception is PBG deaminase, which has a marginally greater activity than ALA synthetase. This observation is crucial to the work described in this thesis.

Table 1.2: *Control activities of haem biosynthetic enzymes. (**A**, Activity; **RA**, Relative Activity). Units of activity are nmol of ALA or ALA equivalents produced or utilised/h/g of liver tissue or /g of fibroblast protein. To obtain relative activity all activities were divided by the ALA synthetase activity. The values are extracted from the cited references and represent work collated from many laboratories. References 1 and 3: Elder, 1986; Reference 2: Bishop and Desnick, 1982*

	Fibroblasts (Reference 1)		Rat liver (Reference 2)		Human liver (Reference 3)	
ENZYME	**A**	**RA**	**A**	**RA**	**A**	**RA**
ALA synthetase	36	1	41	1	22	1
ALA dehydratase	-	-	3300	80	±12850	±584
PBG deaminase	95	2.6	110	2.7	150	6.8
Uro'gen cosynthetase	-	-	6160	150	-	-
Uro'gen decarboxylase	8900	247	2544	62	11000	500
Copro'gen oxidase	4300	119	1176	29	13000	591
Proto'gen oxidase	26400	733	1200	29	-	-
Ferrochelatase	200	5.6	17560	450	16600	754

Regulation of haem biosynthesis

The finding that some cells produce more haem than others suggests the existence of regulatory mechanisms for haem production in each tissue. The precision, specificity and efficiency of this system *in vivo* is shown by the close coupling of the rates of synthesis of haem and of the various apoproteins to which it is bound. It is also demonstrated by the extremely small losses of haem intermediates occurring from the pathway under normal circumstances (Bissell, 1985); in other words, the amount made is closely matched to the amount required. These losses consist of those porphyrinogens transported out of cells, and those intracellularly oxidised to porphyrins. The proportion of ALA entering the pathway which is lost in this way is estimated to be of the order of 1 - 2.5% (Neuberger, 1980; Elder, 1982).

The mechanism by which haem biosynthesis is regulated appears to vary from one tissue to another (Elder, 1980); and it is thus difficult to generalise. The following description is an *outline* of some of the

putative mechanisms by which haem biosynthesis may be regulated; not all will necessarily be operative in a specific tissue or cell type.

Regulation by ALA synthetase

The chief means by which the rate of haem biosynthesis in mammalian systems is controlled is through regulation of ALA synthetase (Granick and Urata, 1963; Granick, 1966; Granick and Sassa, 1971; Meyer and Schmid, 1978; Kappas *et al*, 1983). Thus alterations in the rate of haem synthesis are accompanied by corresponding alterations in ALA synthetase activity. This is consistent with other pathways of intermediary metabolism where the enzyme catalysing the first committed step of an unbranched, irreversible pathway frequently controls the flux of substrate down the pathway (Newsholme and Crabtree, 1979; Kacser and Burns, 1979). In broad terms this regulation is achieved by feedback regulation of ALA synthetase by haem.

The first direct evidence for such feedback was the observation that in chick embryos, the induction of hepatic ALA synthetase by drugs was prevented by the simultaneous administration of haem (Granick, 1966). The mechanism by which this inhibition is achieved has remained a central question ever since. Much research has generated conflicting data and the mechanism is still not entirely clear. It was originally suggested that haem and inducing drugs compete for a site on a gene-controlling protein. This appears not to be so. Most of the available evidence indicates that repression of ALA synthetase by haem appears to be the sole mechanism controlling ALA synthetase (Srivastava *et al*, 1980). Thus the induction of ALA synthetase by drugs is currently thought to be a consequence of haem removal and not a direct effect of the drug (May *et al*, 1986). Subsequently some workers have suggested that haem influences ALA synthetase at a translational level, while others have proposed an effect at the transcriptional level (Yamamoto, 1982; May *et al*, 1986). Recently probes derived from a cDNA clone for rat liver ALA synthetase were used to examine control of the production of ALA synthetase-coding messenger RNA

(mRNA) (Srivastava, 1988). This work provided strong evidence that control of ALA synthetase by haem was exerted, at least in rat liver, at the transcriptional level. In this case haem regulated ALA synthetase mRNA levels predominantly by inhibiting transcription of the ALA synthetase gene.

The primary translation product of ALA synthetase mRNA is a cytosolic protein which undergoes further processing to a smaller functional unit while it is translocated to the mitochondrion (Hayashi *et al*, 1970; Whiting and Elliott, 1972; Ohashi and Kikuchi, 1978; Yamauchi *et al*, 1980). Thus, interference with the post-translational processing of newly synthesised enzyme, or its transit from cytosol to mitochondrion, may well be a control point of haem biosynthesis. Indeed, there is evidence that haem lowers the rate of transfer of cytosolic ALA synthetase to the mitochondrion (Yamauchi *et al*, 1980; Hayashi *et al*, 1983).

Thirdly, haem synthesis might be retarded by direct inhibition of ALA synthetase activity by haem. This has been demonstrated using partially purified enzyme (Scholnick *et al*, 1969). Haem caused a 50% inhibition of ALA synthetase activity at a concentration of $2X10^{-5}M$ (Aoki *et al*, 1971) which is approximately 200 times greater than the $10^{-7}M$ concentration required to produce a 50% repression of synthesis of the enzyme in chick embryo liver (Granick *et al*, 1975; Sassa *et al*, 1979). This is, however, a less efficient mechanism for the regulation of ALA synthetase activity. That direct inhibition of ALA synthetase by haem is physiologically significant is uncertain. ALA synthetase in isolated intact rat liver mitochondria was not inhibited at rates of heme formation estimated to be approximately 75 times that occurring in vivo (Wolfson *et al*, 1979).

In summary, haem modulates ALA synthetase activity by feedback control and this appears to be mediated principally by repression of synthesis of the enzyme at the transcriptional level (Srivastava *et al*, 1988). Control by varying the rate of transfer of cytosolic ALA synthetase to the mitochondrion and by direct inhibition of enzyme activity may constitute subsidiary mechanisms (Bloomer and Straka, 1988). The circumstances under which these latter mechanisms

become important are unknown. It is possible that they may operate as "back-up" mechanisms, particularly in the presence of unusually high concentrations of haem (personal opinion).

The rôle of haem

It is clear that haem plays a central part in the regulation of its own synthesis. This has led to the need for a hypothetical "regulatory pool of free haem" (Meyer and Schmid, 1978; Kappas *et al*, 1983; Bissell and Schmid, 1987). Although no free haem can be detected under normal circumstances (Elder, 1980,1982; Tschudy and Lamon, 1980), it has been estimated that in cultured rat hepatocytes 20% of newly formed haem is directly converted to bile pigment without being incorporated into haemoproteins (Grandchamp *et al*, 1981). The pool would thus consist of newly-synthesised haem, and would be in equilibrium with haemoprotein-associated haem (Bissell, 1985). In addition, the "pool" would be small and have a rapid turnover. Any factor affecting the concentration of haem in this "pool" would either increase (up-regulate) or decrease (down-regulate) haem biosynthesis as a result of feedback derepression or repression of ALA synthetase activity via the mechanisms discussed above (Bloomer and Straka, 1988).

The data from several experiments are consistent with this theory. Free haem can be increased by administering exogenous haem. This has been shown to reduce ALA synthetase activity in experimental animals. Free haem is decreased by the induction of haem-requiring proteins (such as apo-cytochrome P_{450}), with consequent incorporation of haem into newly-synthesised haemoproteins. There is a concomitant, presumably resultant, increase in ALA synthetase activity (Marver *et al*, 1969; Bissell and Schmid, 1987; Correia and Meyer, 1975; Rajamanickam *et al*, 1975). Decreases in hepatic haem levels could also be mediated by induction of haem oxygenase, an enzyme catalysing the first step of haem degradation (Tenhunen *et al*, 1970; Bissell and Hammaker, 1976) or by stimulation of lipid peroxidation (which decreases cytochrome P_{450} levels) (De Matteis,

1970). It is not clear what the nature of the relationship between haem synthesis and degradation is. Studies have demonstrated reciprocal oscillations in hepatic ALA synthetase and haem oxygenase activity *in vivo* following intravenous haemin administration (Schaiter, 1979), but it is not known whether haem oxygenase can be stimulated independently and not only by the presence of excessive amounts of hepatic haem (Bissell, 1985).

The rôle of glycine

The availability of substrate, and in particular of glycine, may play a rôle in regulation of synthesis of haem but this has not received much attention in the past. ALA synthetase has a low affinity for glycine with reported K_m's ranging from 5 to 19mM (Kappas *et al*, 1983). The intracellular concentration of glycine is variable but, at least in the liver, lies just below 1mM (Cowtan *et al*, 1973). Thus the intracellular concentration of glycine could be a rate-determining factor in hepatic haem synthesis (Neuberger, 1980). That glycine availability may regulate haem biosynthesis is borne out by studies showing that the administration of compounds which complex glycine, such as benzoate or *para*-aminobenzoate, greatly reduce the formation of cytochrome P_{450} previously stimulated by phenobarbital (Piper *et al*, 1973). However, in the absence of such pharmacological manipulation glycine is unlikely to become depleted and the mechanism must therefore remain a theoretical possibility.

Regulation of haem biosynthesis in erythroid tissue

The regulation of haem biosynthesis in erythroid tissue is not well understood. There are contradictory reports of induction (Granick and Sassa, 1978) and inhibition (Beaumont *et al*, 1984[b]) of erythroblast ALA synthetase activity by haem. These inconsistent results may, in part, reflect the wide variety of erythroid systems that have been used for haem regulatory studies. These include chick embryo blastoderm (Levere and Granick, 1965), foetal mouse hepatic erythroblasts

(Freshney and Paul, 1971), Friend cells (Sassa, 1976), human erythroleukemia cells (Hoffman *et al*, 1980), and more recently, mammalian bone marrow cultures (Ibrahim *et al*, 1982). Understanding of erythroid ALA synthetase regulation is complicated by the concurrent influences exerted by the processes of cell division and differentiation. It is possible that erythropoietin, hypoxia and some steroid metabolites (Meyer and Schmid, 1978; Tschudy, 1980), act by promoting differentiation rather than by directly increasing ALA synthetase activity *per se* (Sassa, 1976).

Haem has, however, clearly been shown to inhibit the activity of ALA synthetase in reticulocytes (Karibian and London, 1965; Aoki *et al*, 1971). In contrast to other rat tissues studied by Srivastava *et al*, the erythroid elements of rat spleen did not show repression of ALA synthetase-mRNA synthesis by haem (Srivastava *et al*, 1988). It therefore appears that erythroid ALA synthetase is subject to feedback control by haem. It is therefore unclear whether haem acts directly by inhibiting ALA synthetase activity or represses mRNA transcription.

The Porphyrias

Since the activity of the haem biosynthetic pathway is governed by haem requirement and the pathway is extremely efficient with less than 2% wastage due to unused intermediates, the finding of easily measurable concentrations of porphyrins or porphyrin precursors in tissue or excreta implies a disturbance of haem synthesis. These disturbances are associated with specific clinical syndromes which are collectively called the *porphyrias*. The main clinical features are twofold: *photocutaneous sensitivity* and the *acute attack*. The latter consists of mild to severe abdominal and neuropsychiatric symptoms and may be fatal.

Historical aspects of the porphyrias

The first cases of porphyria were described in the second half of the 19th century. In 1874, Schultz described a case diagnosed as "atypical leprosy" (Schultz, 1874). Urinary pigments from this patient were analysed by Baumstark (Baumstark, 1874) who described them as having similar absorption spectra to that of iron-free haematin (haematoporphyrin), a compound which had been characterised some years previously (Hoppe-Seyler, 1871). Baumstark's interpretation of the origin of the urinary pigments in Scultz's patient was brilliant in its foresight. He believed they arose from an error of haemoglobin biosynthesis rather than from an error in its degradation. Some 50 years were to pass before this idea was re-established, this time with "modern" scientific data (Lemberg and Legge, 1949).

Towards the end of the 19th century there was an outbreak of a new, apparently acquired disease following the introduction of the hypnotic, *Sulphonal*. In 1889 Stokvis reported a haematoporphyrin-like pigment in the urine of a woman who had taken a few grams of *Sulphonal*, and subsequently died (Stokvis, 1889). This appears, in fact, to be the first description of the acute attack of porphyria. There were other case reports around this time, the most penetrating of which (With, 1976) were published in 1891 by Geill and Fehr who gave clinical descriptions of what they termed "*Sulphonal* poisoning" (Harley, 1890; Geill, 1891; Fehr, 1891; Bresslauer, 1891; Friedenreich, 1892). Simultaneously chemical studies revealed that the urinary pigment in "*Sulphonal* poisoning" was, as Stokvis had indicated, broadly similar but not identical to haematoporphyrin (Hammarsten, 1891[a,b]) and in 1916 Hans Fischer showed conclusively that the pigment was a unique one - "urineporphyrin" (from whence comes the term uroporphyrin) (Fischer, 1916).

The first serious attempt to classify and define the porphyrias was by Günther in 1911. He described a group of patients with skin sensitivity, who excreted large quantities of porphyrin-type pigments in their urine and appeared to suffer from a hereditary condition (Günther, 1911[a,b]). He subdivided porphyria into "congenital", "acute toxic", "acute genuine", and "chronic" forms. Remarkably, considering the

dearth of knowledge of porphyrin biochemistry at the time, this subdivision is not all that dissimilar to that in use today. Early workers generally used the term *haematoporphyria* to describe what we now refer to as the porphyrias.

In the 1920's and 30's a distinct type of porphyria, characterised by the excessive excretion of the Ehrlich's aldehyde-reacting porphyrin precursor PBG and manifesting clinically with acute neuropsychiatric attacks but not by skin disease was recognised (Sachs, 1931: Waldenström, 1937). The term PBG, which replaced the older term "chromogen" was coined in 1939 by Waldenström, one of the fathers of modern porphyrinology (Waldenström and Vahlquist, 1939). Waldenström was largely responsible for the clinical and genetic description in Swedish families of what we now term acute intermittent porphyria. His data showed that the condition was inherited as a Mendelian autosomal dominant trait. In addition he documented the fact that unaffected relatives of clinically symptomatic patients excreted excess urinary PBG. This he described as "*latent*" porphyria (Waldenström, 1937). Waldenström also drew attention to the rôle of barbiturates in precipitating acute attacks. Subsequent reports described porphyrias with only cutaneous symptoms or "acute" symptoms or both (Schmid *et al*, 1954; Waldenström, 1957). The "mixed" type of porphyria (Watson, 1960) subsequently became known as variegate porphyria which is dealt with in more detail later in part of this dissertation.

The efforts of Waldenström cannot be documented without mentioning the work of another pioneer of porphyrinology, Watson. Watsons' group were largely responsible for establishing some order out of descriptive chaos. Indeed, it was in a paper by his colleague Schmid that the concept of hepatic and erythropoietic porphyrias emerged for the first time (Schmid *et al*, 1954). In addition, Watsons' group were the first to document the existence of latency in all types of porphyria: "It seems quite clear that latent cases may be observed in any form of porphyria, but latency is much more frequent and prolonged in the hepatic type" (Schmid *et al*, 1954; page 187).

The period 1930-1960 witnessed the elucidation of the haem synthetic pathway and its disorders. The ability to correlate events in the haem biosynthetic pathway with the clinical features of the various porphyrias, allowed these "disturbances of haem metabolism" to be identified in terms of specific enzyme deficiencies. Simultaneously the elucidation of the factors regulating haem biosynthesis greatly improved the understanding of the porphyrias as a specific family of diseases.

Enzyme deficiencies and the classification of the porphyrias

Each specific type of porphyria is associated with a characteristic enzyme deficiency (Brodie *et al*, 1977[a]; Elder, 1982; Kappas *et al*, 1983; Moore *et al*, 1987; Bloomer and Straka, 1988). In the *inherited* porphyrias as a rule the enzyme defects should be, and in many cases have been shown to be, present in all haem-synthesising tissue (assuming a single enzyme coded for by a single gene). There are a few exceptions to this, however, and this has led to the notion that while the defect is universal, its expression may be variable. Of course expression can only occur in cells able to synthesise haem. Thus fully differentiated erythrocytes, which lack mitochondria, cannot be used to assess defects related to coproporphyrinogen oxidase, protoporphyrinogen oxidase or ferrochelatase activity.

Most of the enzyme deficiencies may be identified by the pattern of overproduction of haem pathway intermediates. The pattern is specific for each type of porphyria and generally reflects accumulation of the intermediates immediately prior to the defective enzyme (Elder, 1982).

Table 1.3 lists the various porphyrias and presents some aspects of the older classifications, while table 1.4 gives a current classification of the porphyrias in terms of the primary enzyme deficiency (Moore *et al*, 1987 - with modification). The main advantage of the latter classification is that it remains clinically relevant. It is clear in its prediction of whether acute attacks are to be expected or not (*acute* or *non-acute*), while at the same time allowing a differential diagnosis

to be made based on a combination of clinical and biochemical features.

While each each type of porphyria should have a unique pattern of porphyrin accumulation a number of the porphyrias are exceedingly variable and subtle in their biochemical (and clinical) expression. Thus the porphyrin excretion patterns may be equivocal and thorough laboratory investigation is essential.

The laboratory investigation of the porphyrias has been fully reviewed previously (Moore, 1983; Bissell, 1982; Moore *et al*, 1987) and is outside the scope of this dissertation. Suffice to say that most diagnostic laboratories employ high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) for obtaining *quantitative chromatographic profiles* of porphyrin excretion. Where the diagnosis is equivocal, it is wise to determine activity of specific enzymes. However, at present only assays for PBG deaminase (for AIP), ALA dehydratase (useful for differentiating a true porphyria from lead poisoning or hereditary tyrosinaemia) and uroporphyrinogen decarboxylase (for PCT) are generally available.

Quantitative porphyrin profiles give two vital pieces of diagnostic information; which porphyrin species are present and in what proportion or amount they are present. This is certainly of greater value than simple comparison of total concentrations of porphyrins with normal values (Day *et al*, 1978). The profile obtained in urine, stool, plasma and erythrocytes is most often, though not always, characteristic in each type of porphyria, almost like a "fingerprint" of the porphyria. Thus where fully quantitative data is not available but a good profile is, it may be possible to make an accurate diagnosis. Indeed, even when total porphyrin concentrations lie within the normal range, as in latent porphyria, alterations in the chromatographic profiles may suggest a diagnosis to the experienced porphyrinologist (Lim *et al*, 1983).

With this background to the study of porphyrins and porphyrias, the disease central to this thesis, VP, will be considered.

Table 1.3 : *The various types of porphyria, including some historical references to their classification and description. Note that only a selection of the older classifications are included.*

Porphyria	Historical classification and comment
Acute intermittent porphyria (AIP)	Günther (1911) *Haematoporphyria acuta (and acuta "toxica")* Waldenström (1937) *Porphyria acuta* (noted "latent" form and degrees of severity of the acute attack) Schmid et al (1954) *Porhyria hepatica ("intermittent acute" type)* Waldenström (1957) *Acute intermittent porphyria* Watson (1960) *Porphyria hepatica (Hereditary acute intermittent - dominant)* Goldberg and Rimington (1962) *Acute intermittent porphyria*
Variegate porphyria (VP)	Schmid *et al* (1954) *Porphyria hepatica ("Mixed" type)* Watson (1960) *Porphyria hepatica (Hereditary, mixed or "variegate" group)* Goldberg and Rimington (1962) *Cutaneous hepatic porphyria (Hereditary form - Porphyria variegata)*
Hereditary coproporphyria (HCP)	First described in 1955 (Berger and Goldberg, 1955) Difficult to establish when included as a distinct disease entity. Possibly included as "Porphyria hepatica, mixed group" (Waldenström, 1957) and "Cutaneous hepatic porphyria, hereditary form, mixed type" (Goldberg and Rimington, 1962)
Plumboporphyria (PLP)	Recently described (Bird *et al*, 1979; Doss *et al*, 1979)

Table 1.3: *Continued*

Porphyria	Historical classification and comment
Porphyria cutanea tarda (PCT) - *Familial*	Waldenström (1957) *Porphyria cutanea tarda hereditaria* Watson (1960) *Porphyria hepatica (Hereditary cutaneous)* Goldberg and Rimington (1962) *Cutaneous hepatic porphyria (Porphyria cutanea tarda)*
PCT - *Acquired* (Sporadic and Toxic)	Günther (1911) *?Haematoporphyria chronica* Waldenström (1937) Porphyria cutanea tarda Schmid et al (1954) *Porhyria hepatica ("Cutanea tarda" type)* Waldenström (1957) *Porphyria cutanea tarda symptomatica* Watson (1960) *Porphyria hepatica* (Different "causes" such as chemicals, systemic disease, hepatoma etc. made allowance for, including Turkish epidemic) Goldberg and Rimington (1962) *Cutaneous hepatic porphyria* (Acquired forms: symptomatica, "Bantu", "Turkish", porphyrin producing adenoma)
PCT-*Acquired* (Chronic renal failure)	Recently described (Korting, 1975)
PCT -Hepatoerythropoietic porphyria (HEP)	First described in 1969 (Pinol Aquade *et al*, 1969) Characterised as *?homozygous PCT* (Elder *et al*, 1981)

Table 1.3: *Continued*

Porphyria	Historical classification and comment
Erythropoietic protoporphyria (EPP)	First recognised and characterised as a distinct porphyria in 1961 (Magnus *et al*, 1961)
Congenital erythropoietic porphyria (CEP)	Günther (1911) *Haematoporphyria congenita* Waldenström (1937) *Porphyria congenita* Schmid *et al* (1954) *Porphyria erythropoietica* Waldenström (1957) *Congenital porphyria* Watson (1960) *Porphyria erythropoetica (recessive)* Goldberg and Rimington (1962) *Congenital (erythropoietic) porphyria*

Table 1.4 : *A current classification of the porphyrias. The terms acute and non-acute are used in the sense of whether the acute attack of porphyria is likely to be encountered or not. Synoptic comment is included. Abbreviations in Table 1.3.*

	Porphyria	Affected Enzyme	Cutaneous Involvement
A.	**Acute porphyrias**		
	Mainly hepatic expression		
1.	AIP	PBG deaminase	No
	Comment Diminished PBGD activity shown in erythrocytes[1], liver[2], lymphocytes[3], fibroblasts[4] and amniotic cells[5]. Dominant inheritance. Genetically heterogeneous[6]. Usually manifests postpubertally and degree of clinical expression highly variable and dependant on additional precipitating factors. Enzyme deficiency detectable from birth. Clinical syndrome more frequent in females[7]. Majority of carriers are clinically latent throughout adult life[8]. Excessive PBG and to a lesser extent ALA and uroporphyrin in the urine. PBG, ALA and porphyrin levels very high in the acute attack.		
2.	VP	Protoporphyrinogen Oxidase	Yes
	Comment Discussed in detail in Chapter 2.		
3.	HCP	Coproporphyrinogen Oxidase	Yes
	Comment Diminished CO activity shown in leukocytes[9], liver[10], cultured fibroblasts[11] and lymphocytes[12]. Dominant inheritance,homozygous form[13], and a variant form with 10% CO activity (harderoporphyria)[14] have been described. Majority of carriers are clinically latent throughout adult life. Photocutaneous skin lesions occur rarely in the absence of acute attacks. Characterised by excessive excretion of coproporphyrin in the stool and to a lesser extent in the urine.		
4.	PLP	ALA Dehydratase	No
	Comment Diminished ALAD activity in erythrocytes[15], due to enzyme structural alterations[16]. Inherited recessively[17], homo- and heterozygotes exist[18]. Presents prepubertally[19]. Similar to lead poisoning - characterised by elevated urinary ALA and coproporphyrin		
B1.	**Non-acute porphyrias**		
	Mainly hepatic expression		
1.	PCT	Uroporphyrinogen Decarboxylase	Yes
	Comment Many forms (see below). All characterised by deficient UROD activity[20]. Most common form of the porphyrias world-wide[21]. Also referred to as the cutaneous hepatic porphyrias[22]. Main biochemical features are elevated urinary and plasma uro- and heptacarboxlic porphyrin. Lesser elevations of hexa-, pentacarboxylic and coproporphyrin. Stool porphyrins similarly raised in addition to a striking elevation of isocoproporphyrin.		

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1. Strand et al, 1972
2. Miyagi, 1970
3. Sassa et al, 1978
4. Meyer, 1973
5. Sassa et al, 1975
6. Anderson et al, 1981
7. Stein and Tschudy, 1970
8. Kappas et al, 1983
9. Brodie et al, 1977b
10. Hawk et al, 1978
11. Elder et al, 1976
12. Grandchamp and Nordmann, 1977
13. Grandchamp et al, 1977
14. Nordmann et al, 1983
15. Doss et al, 1982
16. Doss et al, 1983
17. Labbe and Bird, 1985
18. De Verneuil et al, 1978
19. Thunell et al, 1985
20. Mascaro et al, 1986
21. Pimstone, 1982
22. Moore et al, 1987

Table 1.4: *Continued*

Porphyria	Affected Enzyme	Cutaneous Involvement
PCT continued	Uroporphyrinogen Decarboxylase	Yes

(a) Familial

Comment
50% decreased UROD in liver[23] and erythrocytes[24]. Dominant inheritance.

(b) Acquired
- Sporadic

Comment
Appears as an unusual response to alcohol abuse, sex steroids and some forms of iron overload including "Bantu" siderosis[21].

- Toxic

Comment
Appears in response to certain toxic chemicals, particularly polyhalogenated hydrocarbons[25], includes "Turkish" porphyria[26].

- Chronic renal failure

Comment
Occurs in a small number of patients with CRF on haemodialysis[27]. Must be ditinguished from "pseudoporphyria" which resembles PCT clinically but there is no evidence of UROD deficiency[22]

(c) HEP

Comment
UROD activity approximately 10% of normal. Probably represents homozygous familial PCT[28]. Appears to be genetically heterogeneous. Severe photosensitivity from infancy. Elevated erythrocyte Zn protoporphyrin is a feature[29]

B2. Non-acute porphyrias

Mainly erythropoietic expression

2. EPP — Ferrochelatase — Yes

Comment
Diminished FE activity found in all tissue types[30]. Dominant inheritance (probably), partial penetrance[31]. Clinical expression highly variable[32]. Manifest in infancy. Homozygous state exists[33]. Elevated erythrocyte free protoporphyrin is the outstanding biochemical feature.

3. CEP — Uroporphyrinogen Cosynthetase — Yes

Comment
Decreased UC activity (10-30%) has been demonstrated in erythrocytes[34] and skin fibroblasts[35]. Recessive inheritance[36]. Manifest from infancy. Severely photomutilating[37]. Characterised by accumulation of uro- and coproporphyrinogen-I in the erythrocytes (and plasma).

REFERENCES:
23. Elder et al, 1978
24. De Verneuil et al, 1978
25. Silbergeld and Fowler, 1987
26. Schmid, 1960
27. Day and Eales, 1980
28. Elder et al, 1981
29. Smith, 1986
30. Bloomer, 1982
31. Went and Klasen, 1969
32. Poh-Fitzpatrick, 1986
33. Deybach et al, 1986
34. Romeo and Levin, 1969
35. Romeo et al, 1970
36. Darocha et al, 1971
37. Nordmann and Deybach, 1986.

Chapter Two

Variegate porphyria - Review

The following is a review of VP based not only on the literature, but also on the knowledge systematically accumulated over approximately 35 years by the Porphyria Service of the Department of Medicine, University of Cape Town and Groote Schuur Hospital. It is appropriate to refer to the outstanding contribution to the clinical biochemistry and medicine of the porphyrias made by Professor Lennox Eales who headed this unit from 1957 till his retirement in 1983. Indeed, his review of VP, based on some 300 index cases (Eales et al, 1980), remains one of the most comprehensive descriptions to date. It is of interest that the first South African case report of porphyria was that of Eales, who, in 1939, as a young 5th year medical student, wrote a brilliantly perceptive description of what he called *acute idiopathic porphyria*, now presumed to be a case of AIP (Eales and Chait, 1939). Shortly thereafter a further case of acute porphyria was reported in the South African medical literature (Kooy, 1939).

VP in South Africa

The origins of VP in South Africa

An unusually high incidence of porphyria was noted in South Africa during the late 1940's and 1950's (Barnes, 1945; Barnes, 1951; Dean, 1953; Dean and Barnes, 1955; Dean and Barnes, 1959) and by the early 1960's it was realised that the incidence of porphyria in South Africa was amongst the highest, if not *the* highest in the world (Dean and

Barnes, 1959; Sweeney, 1963; Eales, 1963). Further investigation resulted in fascinating genealogical studies in which the unusually high prevalence of VP, particularly amongst white Afrikaner and coloured (mixed-descent) South Africans was ascribed to a founder effect (Dean, 1971) amongst the early settlers in South Africa. The term "variegate", which refers to the presence of either, neither or both skin lesions and acute attacks, arose from early case reports from South Africa (Dean and Barnes, 1959) though the first description of VP was probably in 1937 (Van der Bergh and Grotepass, 1937). VP is inherited as an autosomal dominant trait (Dean and Barnes, 1955; Eales *et al*, 1971) and is thought to have been imported to the Cape Colony in 1688 by a Rotterdam orphan, Ariaantjie Ariens, who was part of a contingent of young women sent to the Cape as potential wives for the Free Burghers, or early Dutch settlers. She married Gerrit Jansz van Deventer. Which of these two carried the VP gene is not proven, but approximately 50% of their offspring appear to have inherited the trait (Dean, 1971). For the next two centuries, the disease appears to have been relatively benign, perhaps because the absence of potent pharmacological agents limited the number of acute attacks. Thus the founder effect was sufficient to result in widespread distribution of the trait.

VP on a world-wide basis

Interestingly, although the VP gene in South Africa almost definitely originated from the Netherlands, no direct family link between Dutch VP families and South African patients has been found (te Velde and Noordhoek, 1970; Eales *et al*, 1980). VP is found in other parts of the world, but it is infinitely less common than it is in South Africa. Most other cases have been reported from Europe, especially Finland and Great Britain, and the USA (Barnes *et al*, 1965; Hamnström *et al*, 1967; Baxter and Permowicz, 1967; Cochrane and Goldberg, 1968; Freinkel and Ashman, 1974; Fowler and Word, 1975; Wechsler, 1975; Mustajoki and Koskelo, 1976; Fromke *et al*, 1978; Corey *et al*, 1980; Muhlbauer *et al*, 1982; Kostrzewska and Gregor, 1982; Martasek *et al*, 1983; Day, 1986). A few patients have been described in India (Bhargova and

Gupta, 1970; Handa *et al*, 1975) and Taiwan (Tu *et al*, 1970). VP is also known to exist in Australia (personal communication, Potgieter K). There are also individual cases described in a Curacao Negroid woman (Van der Sar and Den Ouden, 1976) and an American black man (Hughes and Davis, 1983) but both are of unspecified ancestry. Whether VP occurs in any subjects of pure Negroid descent is unclear. Homozygous VP has been described in Europe (Kordac *et al*, 1984; Murphy *et al*, 1986) but not conclusively in the South African VP population to date (Cartwright *et al*, 1978; Kramer, 1980).

Current experience in South Africa

Two important misconceptions have arisen in South Africa: that VP is a disease of Afrikaners, and that it is limited to white South Africans. Both are dangerous generalisations which give rise to both missed and mis-diagnoses. In the early days of the Cape Colony interracial marriage was common and this led to a founder effect amongst coloured South Africans. Furthermore, many so-called "English" South African families have had the gene for several generations so that the apparent lack of an Afrikaner connection cannot be used to exclude the disease.

Estimates of the incidence of VP based on genealogical data have been as high as 3 per 1000 within the white Afrikaner population alone (Dean and Barnes, 1955; Dean and Barnes, 1958). Current estimates put the total figure of affected individuals between 10000 and 20000 (Eales *et al*, 1980; Day, 1986). No accurate figure exists. Many subjects with the disease remain asymptomatic throughout their lives and, like AIP, VP is only rarely manifest before puberty (Eales and Dowdle, 1968; Mustajoki and Koskelo, 1976). Even the most characteristic biochemical feature, elevated faecal protoporphyrin may not be unequivocally raised on occasion (Eales, 1960; Hamnström *et al*, 1967; Mustajoki, 1978; Day, 1986). The biochemically latent carrier is impossible to detect at present. These major limitations make accurate determination of gene prevalence impossible.

Figures representing the number of new cases of porphyria diagnosed by our Centre (situated in the Western Cape, population ±3.5 million) per year (figure 2.1) show that there has been an increase in the number of VP cases diagnosed since 1978 when fully quantitative TLC porphyrin measurements of urine, stool and blood replaced the original solvent extraction technique, with more new cases of VP diagnosed in 1988 than in any other year during this period.

Since an adaptation of the fluorometric TLC method was introduced in the Northern Transvaal region of South Africa in 1985, there has been a dramatic increase in the number of VP patients diagnosed there too (Day, 1986; Personal communication, Hitzeroth H). There could thus be a substantial pool of undiagnosed VP carriers in large areas of South Africa where less accurate methods of diagnosis are in use.

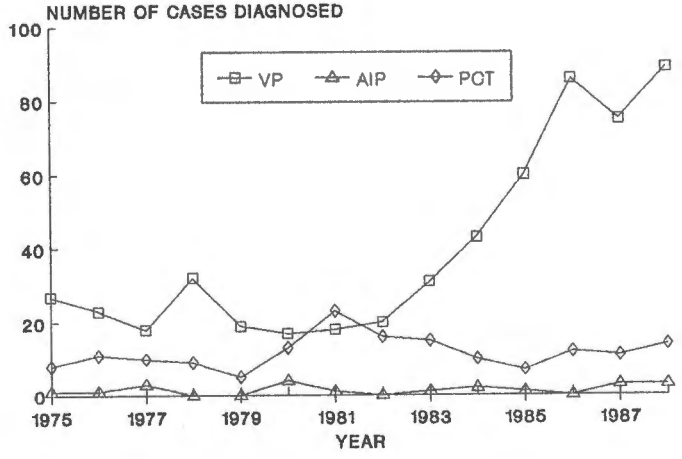

Figure 2.1 : *Number of individuals with VP diagnosed per year since 1975 (new cases). The number of AIP and PCT cases are included for comparison.*

The acute attack of VP

Clinical features of the acute attack

This description is based on data from more than 160 acute attacks of VP documented in our Centre. Some of this data has formed the basis of previous publications (Eales *et al*, 1980; Disler and Moore, 1985; Day 1986; Meissner *et al*, 1987). Studies of the acute attack in AIP and HCP have shown a similarity of the acute attack for all of the acute porphyrias (Bloomer, 1976; Meyer and Schmid, 1978; Mustajoki, 1978; Kappas *et al*, 1983). In our studies acute attacks occurred three times more frequently in women than men, 70% occurred in the 20-40 year age group; and repeated attacks in the same patient were relatively common. Although the acute attack is thought to occur more frequently in AIP than in VP it is an ever-present risk in both (Eales *et al*, 1980). The acute attack consists of varying combinations of abdominal, cardiovascular, psychiatric and neurological features.

The incidence of the main symptoms and signs are given in table 2.1. Abdominal pain (present in 93% of cases) was the presenting complaint in most of the Cape Town series. The pain was "often severe but accompanied by rather unimpressive signs". It was usually continuous, deep-seated and gnawing with fluctuations in intensity which at times became intolerable, "leading to incessant pleas for medicinal relief" (quotes from Eales *et al*, 1980). The pain was most frequently situated in the upper abdomen, especially in the epigastrium, but could also affect the central area or the right or left iliac fossae. Palpation usually evoked complaints of severe tenderness but did not reveal true rigidity or rebound tenderness. Gastrointestinal disturbance resulting in constipation, vomiting and ileus was fairly common. Diarrhoea was rare.

Autonomic instability resulting in hypertension and tachycardia frequently occurred during the early stages of the acute attack. These features were occasionally associated with marked orthostatic changes in blood pressure. A severe anxiety state was also invariably present.

Table 2.1: *Clinical presentation of the acute attack of VP. Based on Eales et al, 1980, Disler and Moore, 1985 and Meissner et al, 1987.*

Symptoms	%	Physical Findings	%
Abdominal pain	93	Tachycardia	75
Vomiting	70	Hypertension	55
Pain in limbs	50	Motor neuropathy	53
Pain in back	50	Pyrexia	38
Constipation	43	Leucocytosis	20
Confusion	32	Bulbar involvement	18
Urinary frequency	30	Sensory loss	15
Dysuria	28	Proteinuria	8
Abnormal behaviour	23	Cranial nerve involvement	2
Seizures	12		
Diarrhoea	5		
Stupor	3		

This, plus a tendency to overreact and to hyperventilate, often led to the patient being labelled as hysterical.

Neurological signs included encephalopathy, possibly with generalised seizures, and paraesthesia which often preceded the onset of a predominantly motor peripheral neuropathy which occasionally involved the respiratory muscles. Psychiatric disturbances included confusion and abnormal behaviour, in addition to the anxiety state already mentioned.

Acute attacks may be associated with leucocytosis and an elevated erythrocyte sedimentation rate. The most frequent abnormal routine biochemical finding was hyponatraemia which was present in 95% of cases (Disler and Moore, 1985). In half these attacks sodium concentration was less than 120mmol/l. Dehydration was also present in some cases. Hypokalaemia, hypomagnesaemia, hypocalcaemia, hypochloraemia and alkalosis were recorded on a few occasions.

In VP in particular, patients appear to be subject to milder versions of the acute attack than in AIP, complaints of pain, nausea or malaise often being the only symptoms. Furthermore, we have noted a marked decrease in the incidence of central nervous system involvement over the last decade.

Pathogenesis of the acute attack

The symptoms of the acute attack can be explained almost entirely on a neurogenic basis (Moore *et al*, 1987). Despite many studies however, the pathogenesis of the neurologic manifestations remains uncertain. Intermittent ischaemia, abnormal myelin or heavy metal metabolism, impaired synthesis of acetylcholine, and depletion of essential substrates or cofactors such as pyridoxal phosphate, zinc and glycine have all been considered as mechanisms (Bonkowsky and Schady, 1982; Bloomer and Straka, 1988). The two hypotheses most favoured are first; the *direct neurotoxic effects of ALA and/or PBG*, the concentrations of which are always grossly elevated during the acute attack (see below), and second; the possibility of a *haem deficiency* in neural tissue, or indirectly in liver. These two hypotheses are not necessarily mutually exclusive. For example neurons with a partial defect in haem synthesis could be uniquely susceptible to ALA toxicity, alternatively ALA might potentiate the effect of neural haem deficiency (Bonkowsky and Schady, 1982; Moore *et al*, 1987).

Neurotoxicity of ALA and/or PBG

Neuropsychiatric symptoms of porphyria are always associated with increased excretion of ALA and PBG. This has led to the belief that these compounds are neurotoxic. PBG is thought by some to be able to inhibit synaptic transmission (Feldman *et al*, 1971); however, this claim has not been universally supported (Goldberg *et al*, 1954; Loots *et al*, 1975; Nicoll, 1976).

ALA is a more likely to be neurotoxic. Neurological abnormalities reminiscent of acute porphyria are found in other disorders in which ALA excretion is increased. These include lead poisoning (Moore and Goldberg, 1985) and hereditary tyrosinemia (Gentz *et al*, 1969; Lindblad *et al*, 1977). Furthermore, ALA is neurotoxic when injected into the brain ventricles of experimental animals (Shanley *et al*, 1975; McGillion *et al*, 1975). In crayfish neuromuscular preparations short-term exposure to $2X10^{-5}M$ ALA, a concentration seen in the plasma of porphyric patients during an acute attack, resulted in a

reversible misfiring of electrically stimulated neurons (Bonkowsky and Schady, 1982). ALA also inhibits brain Na^+,K^+-ATPase *in vitro* (Becker *et al*, 1971; Russell *et al*, 1983), though relatively high concentrations of ALA (10^{-4} and 10^{-3}M) were required for this effect. ALA is a partial agonist of and is structurally similar to gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter. It is possible that ALA may mimic or antagonise the effects of GABA, depending on the circumstances: At relatively high concentrations (1 to $5X10^{-5}$M) ALA may compete for GABA-receptors of rat brain synaptosomes (Muller and Snyder, 1977; Brennan *et al*, 1980). At lower concentrations (10^{-4} to 10^{-6}M) ALA inhibits K^+-stimulated release of GABA from loaded synaptosomes (Brennan and Cantrill, 1979).

Despite these findings, the major difficulty in ascribing the neurological symptoms to ALA is the poor correlation of the severity of acute symptoms with serum and urinary ALA concentrations. Some patients remain asymptomatic despite excreting large amounts of ALA. Furthermore, ALA is not consistently present in the cerebrospinal fluid of patients during acute attacks of porphyria (Bloomer, 1982).

Haem deficiency (and haematin administration)

Neuronal haem deficiency has been suggested as a cause of the acute attack. Such deficiency could conceivably limit the formation of important haemoproteins thereby influencing normal nerve functioning (Shanley *et al*, 1977).

Brain tissue appears to be capable of synthesising haem, although it is much less active than in hepatic and erythropoietic tissue. ALA synthetase has been identified in rat brain (Barnes *et al*, 1971; Paterniti, 1978[b]; Percy and Shanley, 1979; De Matteis *et al*, 1981) and in cultured dorsal root ganglia (Sassa *et al*, 1979). Low levels of ALA and PBG, total porphyrins and cytochrome P_{450} are present in brain tissue. Indeed, brain tissue has been shown to be able to perform certain haemoprotein-dependent functions (Cohn *et al*, 1977; Paul *et*

al, 1977; Sassa *et al*, 1979). It is not clear, however, whether regulation of haem synthesis in neural tissue is subject to negative feedback control by haem. Furthermore it is not certain whether brain ALA synthetase is induced by known inducers of hepatic ALA synthetase (Paterniti *et al*, 1978[b]; Percy and Shanley, 1977; De Matteis, 1981). It is possible that neural tissue haem may, at least in part, be supplied from other sources such as the liver. This supply could be impaired during an acute attack when intrahepatic haem is depleted (Bissell, 1985). However, it is currently unclear whether inherited defects of haem biosynthesis actually result in haem depletion in neural tissue.

Another theoretical mechanism by which hepatic haem deficiency may lead to altered neurological function concerns the requirement that tryptophan pyrrolase (the rate-controlling enzyme for the degradation of tryptophan) has for haem. Tryptophan pyrrolase activity is reduced when free haem availability is decreased (Druyan and Kelly, 1972). This has been linked to the regulatory free haem "pool" and to the effects of porphyrinogenic drugs (Badawy and Evans, 1973; Badawy and Morgan, 1980; Badawy *et al*, 1981). Decreased activity of tryptophan pyrrolase results in elevation of plasma levels of tryptophan, increased tryptophan uptake by the brain, and increased levels of neuroactive metabolites such as 5-hydroxytryptamine (serotonin) (Litman and Correia, 1983). It is significant that serotonin administered to humans results in abdominal pain, psychomotor disturbances and other symptoms reminiscent of the acute attack of porphyria (Douglas, 1980).

Haematin has been used successfully in the treatment of acute porphyric symptoms (Bonkowsky *et al*, 1971; Watson *et al*, 1973; Dhar *et al*, 1975; Watson *et al*, 1978). The efficacy of administration of haematin is consistent with any of the above hypotheses. Administered haem suppresses ALA synthetase, reducing overproduction of ALA and PBG (McColl *et al*, 1979); it could supply the nervous system directly with haem and, in repleting hepatic haem, it may restore tryptophan pyrrolase to full activity thereby "normalising" serotonin levels (Litman and Correia, 1983).

Factors precipitating the acute attack

Most patients with VP go through life without ever experiencing an acute attack (Eales, 1963; Eales *et al*, 1980; Day, 1986). Indeed, as illustrated by the relatively benign nature of the disease prior to the introduction of powerful pharmacological agents during the early part of this century, haem biosynthetic enzyme deficiencies only become hazardous when some factor or factors further increase ALA synthetase activity. Known precipitating factors include many drugs, synthetic steroid hormones, alcohol, physiological hormone fluctuations, infection, fasting and stress (Eales, 1971; Tschudy, 1978; Kappas *et al*, 1983; Disler and Moore, 1985). Many Porphyria Units have drug information available in the form of "*Porphyria Drug Lists*" which are normally composite lists compiled from reported, anecdotal and experiential information. An extract from a porphyria information booklet compiled by our Centre (Hift *et al*, 1989[a]) is included as Appendix 2 and comprises such a drug list. Spontaneous acute attacks precipitated by hormonal fluctuations related to the menstrual cycle (McColl *et al*, 1982) appear to be more of a problem in AIP than in VP.

Several different mechanisms may be involved in the induction of an acute attack. All appear ultimately to result in either direct or indirect induction of ALA synthetase activity. The most obvious mechanism is an increase in the production of cytochrome P_{450} synthesis resulting in depletion of the haem "pool" and thus derepression of ALA synthetase synthesis (Kappas *et al*, 1983). Other agents may exert their effect by inhibiting enzymes of the haem biosynthetic pathway resulting in decreased free haem concentration (Moore and Disler, 1983). Alcohol possibly acts via both routes (Moore *et al*, 1984). The exact mechanism is complex and beyond the scope of this dissertation. Similarly, the precise mechanisms by which hormones, fasting, stress and infections precipitate the acute attack remains to be shown (Moore *et al*, 1987).

Incidence of the acute attack

Figure 2.2 shows the incidence of acute attacks admitted to the Groote Schuur group of hospitals (which covers the Western Cape) between the years 1957 and 1988. Note the dramatic lowering of the incidence of VP acute attacks from 63 in the 10 years spanning 1957-66 to 11 in the 12 years, 1977-88. Acute attacks of AIP are included for comparison and have remained constant. The falling incidence of acute attacks of VP may reflect both identification and education of patients, or a change in the prescribing habits of South African doctors. However, the observation that the number of admissions of patients with AIP has not decreased suggests that currently-used drugs still precipitate acute attacks. While 21 patients died at Groote Schuur Hospital as a result of acute attacks of porphyria between 1954 and 1973, there have been no deaths due to porphyria recorded in our hospital in the last 16 years, though we are aware of deaths in the wider community.

With increasing awareness of porphyria, the major acute attack has definitely become rarer, and those acute attacks seen are milder than those cared for in our unit 20 to 30 years ago.

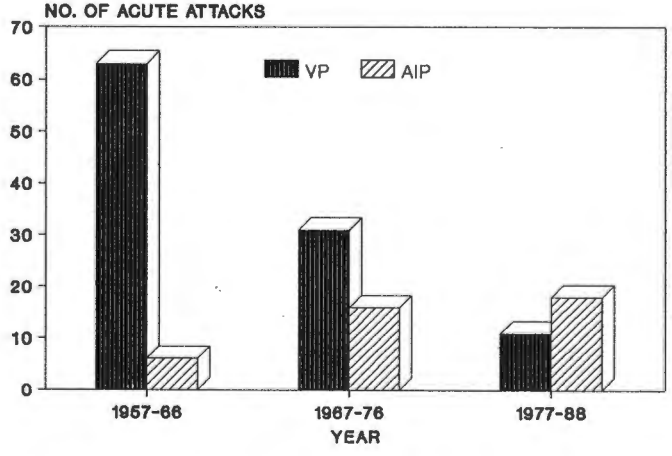

Figure 2.2 : *Incidence of the acute attack of VP and AIP in the Groote Schuur group of hospitals over the last 32 years.*

Treatment of the acute attack

A guideline on the management of the acute attack is given in Appendix 3. The approach described is that practised by our unit and has been extracted from our porphyria information booklet (Hift *et al*, 1989[a]).

The outcome of the acute attack is greatly influenced by the time taken before the correct diagnosis is made (Meissner *et al*, 1987). The principles of management are to remove and subsequently avoid any precipitating factors (Moore *et al*, 1987), to alleviate symptoms (Disler and Moore, 1985) and to apply specific measures aimed at decreasing porphyrin production by decreasing ALA synthetase activity.

Cutaneous manifestations of VP

Clinical features

(Eales, 1963, 1980; Mustajoki, 1978; Corey *et al*, 1980; Day, 1986, Moore *et al*, 1987)

The cutaneous features of VP are characterised by photosensitivity and increased fragility of the skin in sun-exposed areas, especially the face, the neck and the back of the hands. Minor trauma leads to blistering and to well-defined eroded areas. The lesions tend to be superficial and although they may heal without scarring, they are inclined to leave the patient with hyper- or hypopigmentation, atrophy and a disfigured skin. With repeated trauma and sun-exposure some patients develop pseudoscleroderma of the hands. Hypertrichosis is not uncommon. In females in particular, growth of a downy hair on the sides of the face may be noticed. Characteristically patients demonstrate a combination of lesions ranging from fresh blisters through pigmented and depigmented scars. Photocutaneous involvement in VP is identical to that observed in PCT (Bickers *et al*,

1979; Mascaro *et al*, 1986) and much of our understanding of the pathogenesis of the cutaneous lesions of VP comes from studies of the identical lesions associated with PCT.

Pathogenesis of the photocutaneous symptoms

(Spikes, 1975; Poh-Fitzpatrick, 1985; Day, 1986)

Although there is evidence to suggest that the photoactivity of excess porphyrins in the plasma and/or skin are involved in all types of cutaneous porphyria, the precise underlying mechanisms are not fully understood. The generally accepted hypothesis revolves around the absorption of photons of near ultraviolet light (400nm) by porphyrin molecules to produce a singlet-excited state. Excited porphyrin molecules in the singlet state are thought to react directly with biomolecular targets via a free radical mechanism. Alternatively singlet-excited porphyrin molecules may convert to the metastable triplet-excited state, allowing transfer of the excitation energy to molecular oxygen, producing potentially destructive singlet oxygen (oxygen free radical).

Other hypotheses follow on a report of the activation of the complement system by porphyrins plus appropriate wavelengths of ultraviolet light (Lim *et al*, 1984; Torinuki, 1985). This system is the most probable mechanism for endothelial damage in porphyria. The various hypotheses are not mutually exclusive. Indeed different mechanisms may act synergistically to amplify tissue injury (Meurer *et al*, 1985).

A puzzling aspect of the cutaneous lesions of VP is the lack of correlation between circulating plasma porphyrin concentrations and cutaneous symptoms in VP and further, that the porphyrin profiles measured in skin biopsies from VP subjects did not resemble the plasma porphyrin profile (Day *et al*, 1978). Uro- and heptacarboxylic porphyrin rather than copro- and protoporphyrin were the predominant porphyrin species present in the skin (Day, 1986). It has

therefore been suggested that excess porphyrins may be synthesised locally, in the dermis. It has also been suggested that the pattern of porphyrin accumulation may be a result of porphyrin catalysed photoinactivation of uroporphyrinogen decarboxylase by uro- and coproporphyrin (Batlle, 1986; Day, 1986)

Incidence of cutaneous features

The incidence and severity of the photocutaneous features of VP seem to vary with the geographic location. The South African experience suggests that men and women are similarly affected, 60-70% of our cases exhibiting cutaneous lesions (Eales, 1963; Eales *et al*, 1980). This figure is not truly representative in that it excludes subjects who in all probability have VP but do not show an unequivocal porphyrin excretory pattern. The number of entirely asymptomatic patients could therefore be large (Eales, 1961; Mustajoki, 1978; Day, 1986). Indeed, a systematic study of 45 patients in Finland revealed that half had no skin disease at all (Mustajoki and Koskelo, 1976). On the other hand the South African figure of approximately 70% with skin involvement may be an accurate figure and may simply be higher because of more frequent and severe sun exposure. Of the Finnish group the half that did exhibit cutaneous involvement were only mildly affected, almost certainly because of the lower exposure to sunlight than that experienced by South African patients (Mustajoki, 1978; Muhlbauer *et al*, 1982).

Treatment of the skin lesions

Treatment of the cutaneous lesions in VP is generally of a prophylactic nature. Patients should be advised to minimise skin trauma and to avoid sun exposure wherever possible, particularly in the middle part of the day. This includes the use of protective clothing, hats etc. and application of opaque sun barrier creams containing zinc oxide or titanium dioxide. Good skin care must be vigorously encouraged. More recently, activated charcoal has proved to be of value in the

treatment of the cutaneous manifestations of congenital erythropoietic porphyria (Pimstone *et al*, 1987). However, a twelve week trial designed to study the effect of charcoal treatment specifically in VP , failed to elicit a favourable response in seven VP patients with active skin lesions (Hift *et al*, 1989[b]).

Biochemical description of VP

Porphyrin and porphyrin precursor excretion

Detailed description of porphyrin and porphyrin precursor excretion patterns in urine, and porphyrin patterns in stool, blood and other tissues has led to recognition of a characteristic biochemical profile of VP (Mustajoki, 1978; Day, 1986). This typical porphyrin excretory profile of VP is summarised in table 2.2 and examples of TLC tracings from VP patients are shown in figure 2.3.

The most striking feature is an elevated faecal concentration of protoporphyrin and, to a lesser extent, of coproporphyrin. (Other helpful diagnostic markers are illustrated in figure 2.3). It is important to stress that since only the reduced porphyrinogens are metabolized in haem synthesis (until the formation of protoporphyrin by protoporphyrinogen oxidase), it can be assumed that excesses of protoporphyrinogen and coproporphyrinogen must exist intracellularly. It is probably only once these intermediates are *being* excreted or *have been* excreted by the cell that spontaneous oxidation to their porphyrin form is facilitated. The environment within the cell is such as to facilitate reduction; that within the mitochondrion, where excess protoporphyrinogen would first be formed, even more so. Indeed, porphyrinogens have been measured in excreta of porphyric patients (Bloomer and Straka, 1988) confirming that porphyrinogens do accumulate in at least some of the porphyrias.

The above features are present both during remission and in the acute attack. During the acute attack the concentrations of proto- and coproporphyrin are further increased and are accompanied by

Table 2.2: *Main biochemical features in VP as generally experienced Reference 1, Moore et al, 1987; Reference 2, Bloomer and Straka, 1988. Various authors place differing emphases on these features as well as on other characteristic details not presented in this table.*

	REFERENCE 1	REFERENCE 2	
		Remission	*Relapse*
URINE			
ALA	Raised in attack	Normal	Moderately increased
PBG	Raised in attack	Normal	Moderately increased
Uroporphyrin	Usually raised in attack	Normal	Moderately increased
Coproporphyrin	Usually raised in attack	Normal or mildly increased	Markedly increased
STOOL			
Coproporphyrin	Raised	Mildly increased	Moderately increased
Protoporphyrin	Raised	Moderately increased	Markedly increased

increases in the more proximal porphyrin intermediates, in particular uroporphyrin. In addition the acute attack is invariably associated with grossly elevated concentrations of ALA and PBG in the urine.

The varied clinical and biochemical findings in VP have given rise to terms such as "latent VP", "silent VP", "prepubertal VP" and "dual porphyria" (Day, 1986). Two terms are used in this thesis, "quiescent VP" and "acute VP". These require definition. *Quiescent VP* will be used to describe subjects in whom porphyrin metabolic abnormalities

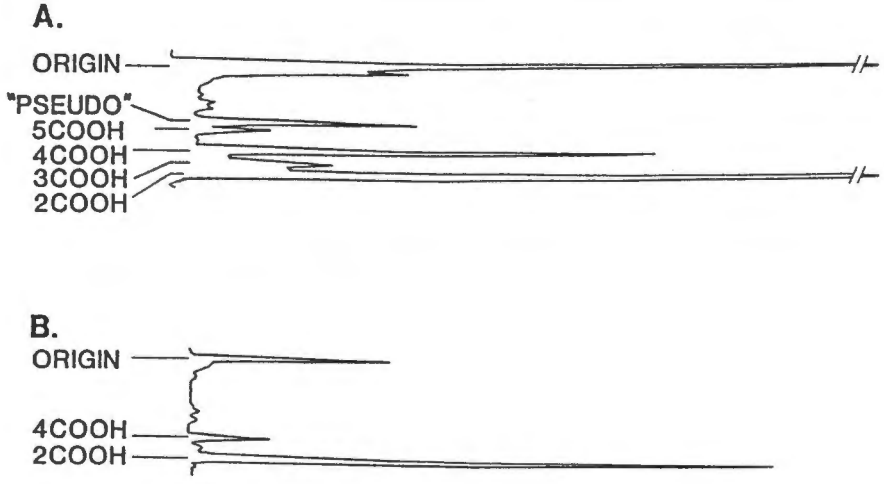

Figure 2.3: *Typical TLC tracing obtained in the stool from a quiescent VP patient (A). A porphyrin excretion profile representing the normal population (B) is included for comparison. Protoporphyrin level in A calculated to 1474 nmol/g dry weight and coproporphyrin to 207 nmol/g dry weight. B's levels were 181 and 13 nmol/g dry weight respectively. The peak marked "pseudo" (pseudo-pentacarboxylic porphyrin) is a useful diagnostic marker of VP. It appears to be present in approximately 83% of VP cases in our laboratory and is thought to be an hydroxylated dicarboxylic porphyrin (Day, 1986 and personal communication, Smith SG, Day RS, Moore MR). 8COOH, uroporphyrin; 7COOH, heptacarboxylic porphyrin; 6COOH, hexacarboxylic porphyrin; 5COOH, pentacarboxylic porphyrin; 4COOH, coproporphyrin; 3COOH, harderoporpyhrin; 2COOH, protoporphyrin.*

are present in the stool, plasma and sometimes urine; photocutaneous clinical features may be present but there is no clinical evidence of an acute attack. *Acute VP* will be used to describe patients in whom there are clinical features of an acute attack accompanied by pronounced increases in urine, stool and plasma porphyrin concentrations as well as marked elevation of urinary ALA and PBG.

Further detailed comment on our experience of porphyrin excretion patterns is given in Appendices 13 and 14 (to which the reader will be referred in Chapter 7).

The primary enzymatic "block" in VP

Most studies indicate that a deficiency of protoporphyrinogen oxidase activity is the primary enzyme defect in VP. Although a 50% reduction in ferrochelatase activity in VP was reported and could explain the preponderance of stool protoporphyrin in this condition (Viljoen *et al*, 1979), other workers have reported normal ferrochelatase activity in a variety of tissues from VP patients (Pimstone *et al*, 1973; Becker *et al*, 1977; Siepker and Kramer, 1985). In addition, a deficiency in ferrochelatase is unequivocally associated with EPP, a porphyria which is biochemically and clinically distinct from VP (Bonkowsky *et al*, 1975; De Goeij *et al*, 1975; Bottomley *et al*, 1975).

In contrast, an approximately 50% decrease in activity of protoporphyrinogen oxidase has consistently been reported in all VP tissues studied to date. These include fibroblasts (Brenner and Bloomer, 1980[a]), lymphocytes (Deybach *et al*, 1981), Epstein-Barr virus transformed lymphoblasts (Meissner *et al*, 1986) and leukocytes (Viljoen *et al*, 1983; Boyle *et al*, 1986) in VP. A defect in protoporphyrinogen oxidase would result in accumulation chiefly of protoporphyrinogen which would be evident in the stool in its oxidised form, protoporphyrin. The elevation of coproporphyrin can be ascribed to "backing up" of the substrates immediately prior to protoporphyrinogen. It is of interest that cultured fibroblasts from VP patients have been shown not to accumulate protoporphyrin, purportedly as a result of the "exclusive" hepatic expression of VP, yet protoporphyrinogen oxidase has been shown to be decreased in fibroblasts (Brenner and Bloomer, 1980[a]).

Other haem biosynthetic enzymes in VP

The activities of the more proximally situated haem synthetic enzymes have not been systematically investigated in any large series of VP patients. Some information on ALA synthetase and PBG deaminase activity in VP is nevertheless available.

Leucocyte ALA synthetase activity was significantly raised in six cases of quiescent VP whereas erythrocyte PBG deaminase activity was normal (Moore *et al*, 1980). Another study has reported raised hepatic ALA synthetase activity in one patient with quiescent VP (Dowdle *et al*, 1967). Erythrocyte PBG deaminase activities have been reported to be normal in patients with quiescent VP. Close examination of these reports reveal that PBG deaminase levels lie towards the lower limit of the quoted normal range (Magnusson *et al*, 1974; Brodie *et al*, 1977[a]; Doss and Von Tiepermann, 1978) and, in some cases, slightly below the normal range of PBG deaminase activity (Mustajoki, 1976; McColl *et al*, 1985). PBG deaminase activity is most certainly never raised.

In the light of the above it is apparent that VP is one of the lesser studied porphyrias, no doubt due to its relative rarity. The remainder of this thesis is concerned with the formulation and testing of two linked hypotheses aimed at understanding the underlying mechanisms which may result in elevated concentrations of ALA and PBG associated with the acute attack of VP.

Chapter Three

Formulation of the hypothesis

One of the most fascinating areas in VP is the increase in ALA and PBG concentrations associated with the acute attack. Indeed, all three of the acute porphyrias have elevated ALA and PBG concentrations during acute attacks. The increase in ALA and PBG is easily explained in AIP (where there is diminished PBG deaminase activity) but not in HCP and VP where the enzyme defects (coproporphyrinogen oxidase and protoporphyrinogen oxidase) involve the distal portion of the haem biosynthetic pathway. The consistent finding of elevated concentrations of ALA and PBG in the acute porphyric attack plus the evidence suggesting that these precursors may be involved in the pathogenesis of the acute attack underline the need for understanding why they should be increased during the acute attack of VP. This chapter reviews the current explanations for the increased ALA and PBG found during the acute VP attack and formulates the hypothesis which forms the basis of this thesis.

Current explanations

Damming up of intermediates proximal to the enzyme block

This theory is based on the assumption that precursor excretion in VP results from a single distally situated, inherited enzyme block at the level of protoporphyrinogen oxidase. It therefore implies that there is sequential damming up of intermediates prior to the block, which, during the acute attack extends to include ALA and PBG.

There are a number of arguments against this. Increased concentrations of ALA and PBG are not found in PCT where large amounts of uroporphyrinogen, the haem intermediate only one step distal to PBG, accumulate as a result of a defect in uroporphyrinogen decarboxylase (Pimstone, 1982; Mascaro *et al*, 1986). Secondly, there is little precedent for this in other pathways of intermediary metabolism, the effects of a partial block in a pathway normally only resulting in build up of intermediates one to two, and never to six steps behind the block.

PBG deaminase as a second rate limiting enzyme

There is universal agreement that ALA synthetase has the lowest relative activity and is rate limiting for the haem biosynthetic pathway. PBG deaminase appears to exhibit the second lowest activity in the pathway and clearly differs from all the other haem synthetic enzymes which possess far greater activities than either ALA synthetase or PBG deaminase (see table 1.2).

ALA synthetase, however, is highly "inducible", at least in the liver. Under these circumstances PBG deaminase becomes rate limiting. Thus induction of ALA synthetase would result in accumulation of ALA and PBG.

It appears logical that ALA synthetase should be slightly derepressed at all times in all so-called "haem deficiency" porphyrias since provision of additional substrate might be expected to maintain haem synthesis. Indeed, ALA synthetase activity has been shown to be increased in all types of porphyria (Brodie *et al*, 1977[a]; Meyer and Schmid, 1978; Moore *et al*, 1980; Elder, 1982). Since PBG deaminase is probably rate limiting in all of the "haem deficiency" porphyrias any further decrease in its activity might be expected to have a profound influence on ALA and PBG concentrations.

It is of course important to bear in mind that haem synthetic regulation differs in various tissues. While the primary enzyme deficiency in the inherited porphyrias is thought to be present in all cells types, the compensatory changes in ALA synthetase activity and substrate

overproduction may be restricted to certain tissues (Elder, 1982). Although the excess haem synthetic intermediates observed in VP are believed to originate mainly in the liver, it is interesting to note that increased ALA synthetase activity in quiescent VP has been measured in both liver (Dowdle *et al*, 1967) and in leucocytes (Brodie *et al*, 1977[a]; Moore *et al*, 1980). Thus excess intermediates could conceivably originate from extrahepatic tissue. Also, there is evidence to suggest that excess intermediates in quiescent and acute VP are synthesised in the kidney (Day *et al*, 1981; Day and Eales, 1982) while the excess stool porphyrins may originate in the intestinal tract (Day *et al*, 1980). Thus the regulation of haem biosynthesis and the resultant substrate accumulation in various tissues is complex and is not well understood.

If PBG deaminase is important in explaining the increased ALA and PBG seen in the acute porphyrias, the absence of such accumulations in PCT, EPP and CEP also requires attention. In these porphyrias, PBG deaminase never appears to be rate-limiting in spite of reported increases in leucocyte ALA synthetase in all three (Moore *et al*, 1980) and hepatic ALA synthetase in clinically "active" PCT (Dowdle *et al*, 1967). In EPP and CEP the explanation could involve their erythroid expression. But PCT, an "hepatic" porphyria, remains anomalous in its behaviour, assuming the haem pathway is subject to the same synthetic demands as in the acute porphyrias. Some workers have suggested that PBG deaminase is increased in these conditions but such increases are small and not present in all patients. Alternatively differences in permeability of the hepatocyte to different haem substrates has been suggested (Elder, 1982). If true, a particular substrate could be confined to a small space so that a relatively small increase could drive the cycle and restore/maintain haem synthesis.

The Hypothesis

The hypothesis which this thesis sets out to examine is that the primary defect in VP, a decrease in the activity of protoporphyrinogen oxidase, results in an accumulation of distal haem pathway intermediates which

in turn inhibit PBG deaminase. When the pathway is relatively quiescent this inhibition is marginal. However, any further increase in ALA synthetase activity would increase the accumulation of distal pathway intermediates and have an increased effect on PBG deaminase until a point is reached where ALA and PBG accumulate.

Thus *PBG deaminase activity should be slightly decreased in quiescent VP* and secondly, *the obligate haem substrates proto- and coproporphyrinogen which accumulate in VP should inhibit PBG deaminase activity.* Equally important, the substrate predominantly increased in PCT (ie. uroporphyrinogen) should not effect PBG deaminase.

Chapter Four

Protoporphyrinogen oxidase and Porphobilinogen deaminase in Variegate porphyria

This chapter describes the studies which examine the first part of the hypothesis outlined in the previous chapter.

Objectives of the study

- ☐ To confirm that VP is characterised by a reduction of protoporphyrinogen oxidase activity.
- ☐ To test the hypothesis that PBG deaminase activity is also reduced in VP.

Choice of a tissue system in which to assay protoporphyrinogen oxidase and PBG deaminase

While it would obviously be preferable to study the haem synthetic enzymes in hepatocytes from normal and VP subjects, this would involve invasive procedures which are clearly unacceptable. However, since VP is likely to arise from a defect encoded by a single gene and has previously been shown to be expressed in tissues other than the liver (see Chapter 2), a tissue culture system should afford a convenient opportunity for pursuing the above objectives.

Protoporphyrinogen oxidase activity has been measured in lymphocytes and leucocytes and has been shown to be decreased in

these cell types in VP patients (Deybach *et al*, 1981; Boyle *et al*, 1986). PBG deaminase has been measured in many different tissue types, including Epstein-Barr virus transformed lymphoblasts derived from control and AIP subjects (Sassa *et al*, 1978). We therefore considered it reasonable to investigate the use of Epstein-Barr virus transformed lymphoblasts as a "model" tissue. The use of transformed tissue, however, required validation in order to be certain that the transformation process did not alter the relative expression of the enzymes of interest in this study.

Methods

Preparation and culture of Epstein-Barr virus transformed lymphoblasts and choice of VP patients

Approximately $18X10^6$ lymphocytes were isolated from 20ml of heparinised blood from each subject by centrifugation over a ficoll gradient ("Histopaque", Sigma Chemical Company). These were incubated in a medium containing Epstein-Barr virus to effect transformation according to previously described procedures (Nilsson, 1971; Bird *et al*, 1981; Van der Westhuizen *et al*, 1984). Transforming medium was prepared from semi-confluent cultures of the Epstein-Barr virus producing marmoset cell line B95/8 (Miller and Lipman, 1973). Full details on the transformation and maintenance of the cell lines in culture is given in Appendix 6. Once a secondary culture and sufficient numbers of viable cells were available, protoporphyrinogen oxidase and PBG deaminase could be assayed. Generally this point was reached when 60ml of suspension culture containing approximately $5X10^6$ cells/ml of 80% viability, as determined by trypan blue exclusion, were available.

Epstein-Barr virus-transformed lymphoblasts are hereafter referred to as "lymphoblasts".

Successful Epstein-Barr virus transformations were obtained in 86% of cultures. Failures were ascribed to super-infection, to a failure to maintain cultures at a density appropriate for successful growth and in three cases the virus just did not "take". Optimal growth rate occurred at a cell density of 1-5X10^6 cells/ml. Healthy lymphoblasts could be distinguished by their characteristic clustering (figure 4.1); they often appeared to have "tails" and a greenish tinge when viewed under a phase contrast microscope. Protein concentration was determined in a 1.5ml sonicate by the Lowry method (Lowry *et al*, 1951), cell density by a Coulter counter and viability using a trypan blue exclusion test in all cultures (Williams and Wilson, 1981). The protein concentration, cell density and viability immediately (1h) prior to preparation showed no appreciable variation from the sample mean (Table 4.1). Comparison of these parameters between the control and VP group showed no significant difference using the Students two-tailed T-test (Bailey, 1981) ($p = 0.191$, 0.194 and 0.692 respectively)

Table 4.1: *Mean protein concentration (mg/ml), cell density (cells/ml) and viability (%) as determined in lymphoblast cultures 1h prior to preparation for protoporphyrinogen oxidase or PBG deaminase assay. SD, standard deviation.*

	Protein concentration *(mg/ml)*	**Cell density** *(X106 cells/ml)*	**Viability** *(%)*
Controls (N = 27)			
Mean	0.71	5.27	89
Median	0.71	5.26	89
Range	0.64-0.80	4.78-5.89	83-96
SD	0.04	0.33	4.1
VP (N = 27)			
Mean	0.70	5.25	89
Median	0.69	5.24	90
Range	0.59-0.79	5.32-6.01	83-95
SD	0.05	0.35	3.4

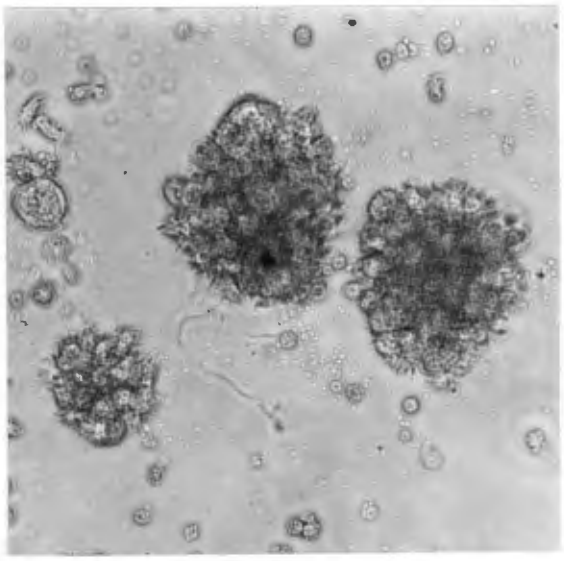

Figure 4.1: *Photomicrograph showing typical Epstein-Barr virus transformed lymphoblasts in suspension culture. Growing medium was Ham F-10 (with L-glutamine) containing 10% foetal calf serum. Cells were best maintained at a density of approximately 1X10^6 cells/ml.*

Lymphocytes from 33 normal control subjects and from 30 quiescent VP subjects yielded 54 successful cultures, 27 in each group. The diagnosis of VP was based on the clinical and family data and on the demonstration of the characteristic "VP" pattern of porphyrins in stool, urine and plasma using a quantitative TLC technique (Day *et al*, 1978[a]). This technique is described in detail in Appendix 4). ALA and PBG concentrations were assayed using an ion-exchange method (Davis and Andelman, 1967) available in "kit" form (see Appendix 5). The main inclusion criterion was a stool protoporphyrin of concentrations greater than 200nmol/g dry weight. In most cases elevated concentrations of coproporphyrin and pentacarboxylic porphyrin and/or a porphyrin species running very closely to pentacarboxylic porphyrin which we have termed "pseudo-pentacarboxylic" porphyrin, were present (see figure 2.3). Urinary ALA and PBG concentrations indicated that none of the patients were in an acute attack.

Assay of protoporphyrinogen oxidase

Protoporphyrinogen oxidase was assayed using an adaptation of a previously described fluorometric assay (Poulson, 1976; Brenner and Bloomer, 1980[b]; Jacobs and Jacobs, 1982; Dailey and Karr, 1987). The adaptation was necessary to facilitate measurement of protoporphyrinogen oxidase in lymphoblasts. The optimization of pH,

the time course of the reaction and of reducing agents and detergents in the assay system are discussed below. Full details of the assay method are given in Appendix 7 and 8.

In brief: Protoporphyrinogen solution (produced by treating protoporphyrin with 4% sodium amalgam) was added at 5 different concentrations, to a lymphoblast sonicate, and reaction buffer. The reaction was allowed to proceed in the dark for 1h at 37°C. Protoporphyrin concentrations were measured by direct fluorometry. Non-enzymatic production of protoporphyrin was controlled for by measuring the amount of protoporphyrin produced under similar conditions but without active sample solution. The rate of non-enzymatic oxidation of protoporphyrinogen was subtracted from the enzymatic rate. Apparent V_{max} and K_m* values for protoporphyrinogen oxidase were determined from double-reciprocal Lineweaver-Burk plots. All assays were performed in duplicate.

Optimal conditions for protoporphyrinogen oxidase

The influence of pH was studied using potassium phosphate, Tris-HCl and sodium borate buffers in 50mM concentrations ranging from pH 6.8 to 9.4. Activity was maximal around pH 8.6 and was affected minimally by using different buffers (figure 4.2).

The linearity of the rate of enzymatic protoporphyrin production was ascertained by measuring activity every 15min up to 60min and at 30min intervals up to 150min. There was good linearity between 15 and 90 minutes after a slight "lag" in the initial production of protoporphyrin (figure 4.3). Non-enzymatic auto-oxidation of substrate (protoporphyrinogen) never exceeded 18% of the total measured oxidation of protoporphyrinogen (figure 4.3).

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* The term "apparent" is used as the enzymes' V_{max} and K_m determined in sonicated preparations of lymphoblasts may not reflect the true V_{max} and K_m of the enzyme under investigation. The true V_{max} and K_m would only be reflected were the enzyme in a pure state. Although the term "apparent" will not be used hereafter, it is implied.

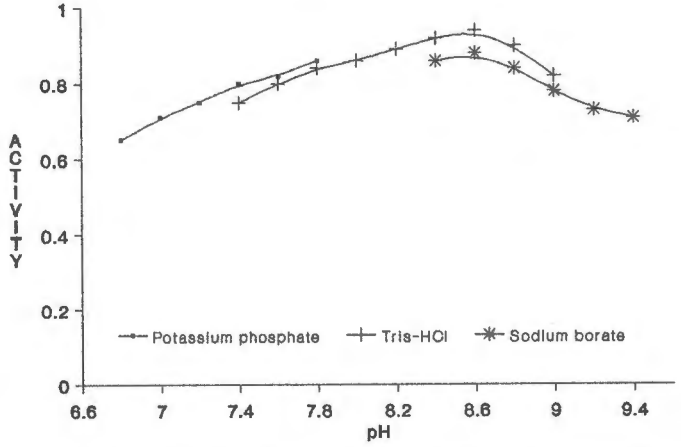

Figure 4.2: *Effect of pH on human lymphoblast protoporphyrinogen oxidase. Activity (nmol protoporphyrin/mg protein/h) was determined as described over a pH range of 6.8 to 9.4 using 50mM potassium phosphate, Tris-HCl or sodium borate buffers. Individual data points represent the mean activities assayed in 3 normal control lymphoblast cultures.*

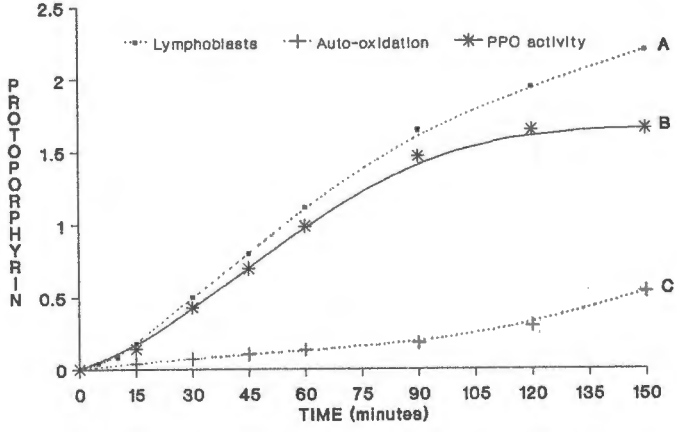

Figure 4.3: *Time course of protoporphyrinogen oxidation by lymphoblast protoporphyrinogen oxidase (PPO). Protoporphyrin production (nmol/mg protein) was linear with respect to time to 90min after a "lag" phase of approximately 15min. Curves A and C are included to illustrate how PPO activity (curve B) was arrived at. Curve C represented protoporphyrin production by the non-enzymatic controls. This was considered as auto-oxidation of the protoporphyrinogen substrate and subtracted from the total protoporphyrin produced by the sonicated lymphoblasts (curve A) to give enzymatic production of protoporphyrin due to PPO. Data points represent the mean of 3 iidentical experiments.*

Table 4.2: *Effects of various reducing agents on the enzymatic and non-enzymatic formation of protoporphyrin from protoporphyrinogen. Standard assay conditions were used except for the variation in type and concentration of reducing agent. Values are the result of the mean of identical runs in 2 normal control cultures.*

REDUCING AGENT	PROTOPORPHYRIN FORMATION *(nmol)*	
	Enzymatic	Non-enzymatic
Control	1.16	0.15
Glutathione (1mM)	1.19	0.15
Glutathione (5mM)	1.15	0.12
Dithiothreitol (1mM)	1.17	0.14
Dithiothreitol (3mM)	1.17	0.08
Dithiothreitol (5mM)	0.91	0.05
ß-Mercaptoethanol (3mM)	1.15	0.11

The effects of various concentrations of glutathione, dithiothreitol and ß-mercaptoethanol on the enzymatic and non-enzymatic formation of protoporphyrin were determined (table 4.2). The most effective of these reducing agents was 3mM dithiothreitol; auto-oxidation of the substrate was minimised and neither inhibition nor stimulation of the enzyme was apparent in the presence of 3mM dithiothreitol.

The amount of fluorescence emitted from protoporphyrin in solution was found to depend on the amount of tissue present, the greater the amount added, the more fluorescence was emitted (table 4.3). This was independent of the amount of porphyrin present.

Table 4.3: *Enhanced fluorescence quantum yield accompanying increasing amounts of sample protein. Fluorescence readings were performed using 1 and 10 μM protoporphyrin solutions in the standard reaction mixture, including 1% Brij 35.*

AMOUNT OF LYMPHOBLAST PROTEIN ADDED *(mg)*	FLUORESCENCE UNITS *(on a X10 and a X1 scale)* [Protoporphyrin]	
	1 μM	10 μM
0.25	46	44
0.50	61	64
1.00	91	92
2.00	148	150

It has been reported that this effect can be minimised and the fluorescence quantum yield increased by addition of a detergent, such as Tween-20, to the reaction mixture (Brenner and Bloomer, 1980[b]). It has been suggested that this enhancement of fluorescence may occur because protoporphyrin is maintained in monomeric form by the detergent (Brenner and Bloomer, 1980[b]). We therefore compared Triton X100, Tween 20 and Brij 35 by adding each to the assay mixture to yield a final 1% detergent concentration. Brij-35 was the most beneficial as it increased fluorescence quantum yield by approximately 4-fold (figure 4.4).

Figure 4.4 also shows that the measured fluorescence was proportional to the protoporphyrin concentration up to 12μM a concentration which includes the entire range of protoporphyrin concentrations actually encountered in the assay.

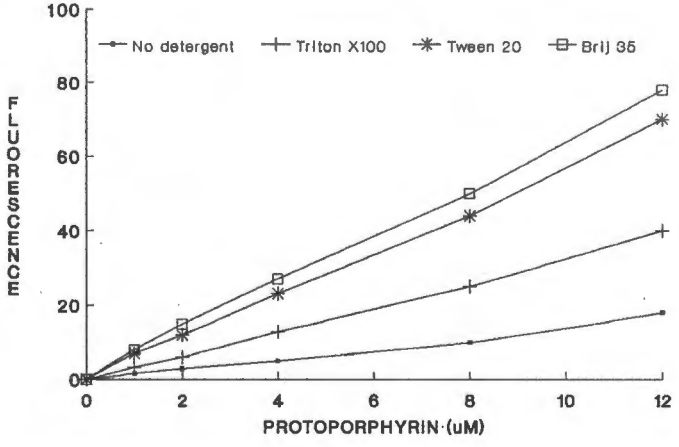

Figure 4.4: *The effect of different detergents (in a 1% concentration) on fluorescence of protoporphyrin (measured at excitation wavelength of 405nm, emission of 630nm). 1% Brij 35 maximised the fluorescence quantum yield. Data points represent the mean of 3 identical experiments.*

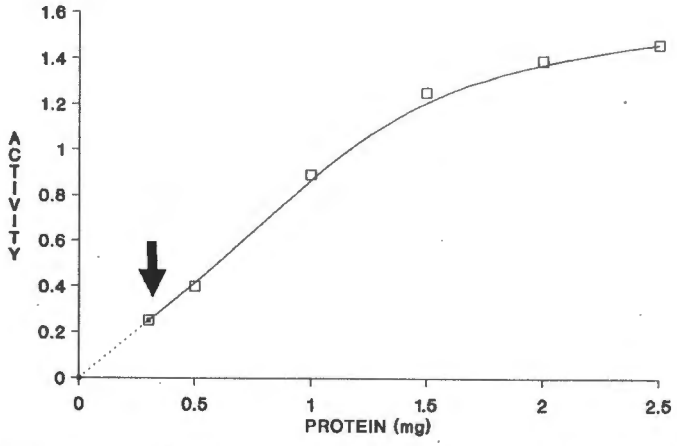

Figure 4.5: *Protoporphyrinogen oxidase activity as a function of the amount of lymphoblast protein. Activity is expressed as nmol of protoporphyrin generated enzymatically per h. Assays were performed under standard conditions. Data points represent the mean of 2 identical experiments in triplicate. The lower limit of reproducibility is indicated by an arrow.*

The rate of protoporphyrin production by lymphoblast protoporphyrinogen oxidase was linear with respect to the amount of lymphoblast protein present in the assay reaction mixture up to approximately 1.5mg/assay tube *ie.* approximately 3mg/ml (figure 4.5). The lower limit of reproducibility was approximately 0.3mg of protein/assay tube. This was established by running two identical experiments which measured protoporphyrinogen oxidase activity with amounts of protein ranging between 0 and 2.5mg/assay tube, in triplicate.

Assay of porphobilinogen deaminase

PBG deaminase activity in lymphoblasts could be measured by minimal adaptation of previously described methods for measuring erythrocyte and lymphocyte PBG deaminase activity under conditions of substrate excess (Piepkorn *et al*, 1978; Sassa *et al*, 1978; Ford *et al*, 1980; Anderson and Desnick, 1982). Appropriate conditions for the

enzyme in our system were examined. The method, like that of protoporphyrinogen oxidase, is a fluorometric one. The rate of production of uroporphyrinogen from PBG can be determined by incubating lymphoblast sonicates in the presence of excess PBG at 37°C in the dark, stopping the reaction with trichloroacetic acid (TCA), allowing oxidation of the uroporphyrinogen to uroporphyrin, and measuring fluorescence directly. Full detail of the method is given in Appendix 9. All assays were performed in duplicate. Since this method has been used in lymphoblasts before, in depth studies used for optimisation of the assay were not necessary. Studies measuring PBG deaminase activity as a function of pH, of time and of protein concentration were however performed in order to confirm that the method worked in our system.

Optimal conditions for PBG deaminase

The influence of pH was examined using 0.1M citrate phosphate, potassium phosphate, Tris-HCl or sodium borate buffer over a pH range from 6.0 to 9.4. The pH optimum was from 8.2-8.4, no activity was measured below pH 6.2 (figure 4.6) and there was slight loss of activity in the presence of sodium borate buffer.

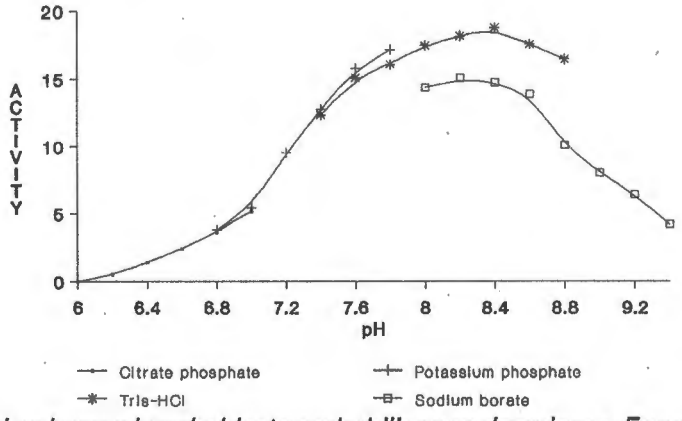

Figure 4.6: *Effect of pH on human lymphoblast porphobilinogen deaminase. Enzymatic activity (pmol uroporphyrin/mg protein/h) was determined as described in the methods section except that the pH was varied between 6.8 to 9.4. Individual data points represent the mean activities assayed in 2 control lymphoblast sonicates.*

PBG deaminase activity was measured at 15min intervals from 0 to 120min. The assay was linear from approximately 5 to 75min at 37°C (figure 4.7). Activity was apparent at 20°C but was reduced to 19% of that measured after 1h at 37°C.

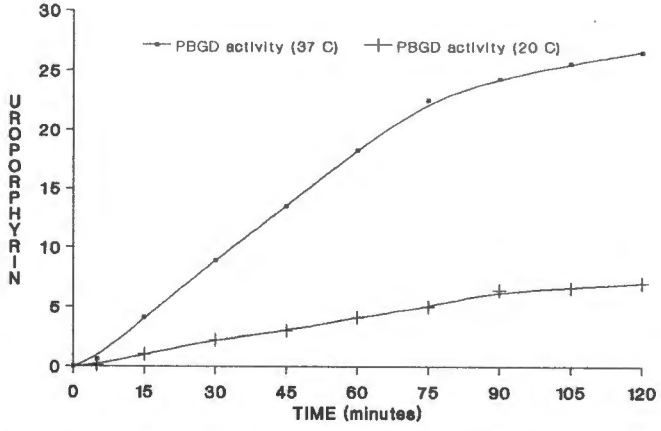

Figure 4.7: *Linearity of PBG deaminase assay with respect to time at 37°C and at 20°C. Uroporphyrin production (pmol/mg protein) was linear between 5 and 75min at 37°C and until approximately 90min at 20°C.*

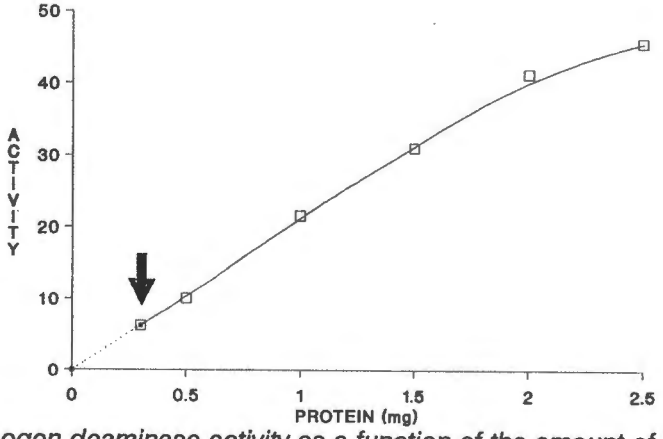

Figure 4.8: *Porphobilinogen deaminase activity as a function of the amount of lymphoblast protein. Activity is in pmol of uroporphyrin generated enzymatically per h. Assays were performed under standard conditions. Data points represent the mean of 3 identical experiments. The lower limit of reproducibilty is indicated by an arrow.*

The rate of uroporphyrin production by lymphoblast PBG deaminase was directly proportional to the amount of lymphoblast protein present in the assay reaction mixture up to concentrations of approximately 2.0mg/assay tube ie. approximately 2.2mg/ml (figure 4.8). The lower limit of reproducibility, as in the case of protoporphyrinogen oxidase, was approximately 0.3mg of protein/assay tube.

Evaluation of transformed lymphoblasts for assay of protoporphyrinogen oxidase and PBG deaminase

Having established that both lymphoblast protoporphyrinogen oxidase and PBG deaminase activities were within the linear portion of the assays as described, the suitability of lymphoblasts as a model in which to study protoporphyrinogen oxidase and PBG deaminase was assessed.

Firstly four normal control lymphoblast lines had protoporphyrinogen oxidase and PBG deaminase assayed at three 10 day intervals to assess the variation in activity in culture over a fairly long period (table 4.4). Both enzymes showed remarkably constant activity over this period. Reproducibility of the assays was verified by measuring the enzymes in triplicate, in the same tissue batch on two separate occasions separated by 2h. Proteolysis was assumed to be minimal over this time period. The results did not differ by more than 14% (table 4.5).

Table 4.4: *Protoporphyrinogen oxidase (PPO) and porphobilinogen deaminase (PBGD) activities in culture was constant. Activity was assayed at three 10 day intervals in 4 control lymphoblast cultures by the methods described. The mean result (±SD) is given as all 4 behaved similarly.*

	PPO *(nmol/mg/h)*	**PBGD** *(pmol/mg/h)*
Day 30 (ie. 30 days post-transformation)	0.95 (±0.1)	21.2 (±1.4)
Day 40	0.91 (±0.1)	22.4 (±1.2)
Day 50	0.94 (±0.1)	21.9 (±1.1)

Table 4.5: *Reproducibility of the protoporphyrinogen oxidase (PPO) and porphobilinogen deaminase (PBGD) assays.*

	PPO (nmol/mg/h)	PBGD (pmol/mg/h)
Time 0 (Tissue batch L74)	0.95	21.2
	0.92	20.4
	0.98	21.8
Time +2 (Tissue batch L74, 2h later)	0.84	19.6
	0.94	20.8
	0.90	19.9

The effect of transformation on protoporphyrinogen oxidase and PBG deaminase activity

Secondly, any stimulatory or inhibitory effects of the transformation process on protoporphyrinogen oxidase and PBG deaminase were assessed by comparing activities of these two enzymes after 3, 4, 5, 7, 14, 21 and 28 days after initiation of the transformation process. These results (obtained from identical experiments in five control cell lines) are shown in figure 4.9.

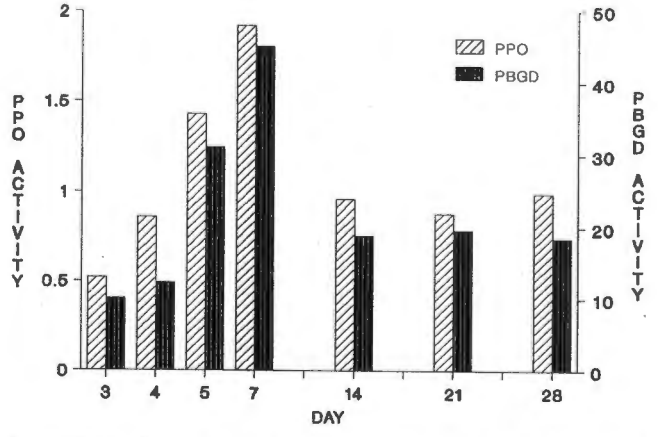

Figure 4.9: *Effect of transformation on protoporphyrinogen oxidase activity (nmol/mg protein/h) and PBG deaminase activity (pmol/mg protein/h). Assays were carried out at 3, 4, 5, 7, 14, 21 and 28 days after initiating transformation. After an initial maximal stimulation both enzymes displayed a consistent level of activity.*

Both protoporphyrinogen oxidase and PBG deaminase activity appeared to fluctuate similarly over this period. Maximal activities were measured at 7 days (4-fold increases). Thereafter both enzyme activities dropped to a steady level approximately twice the baseline value. This stimulatory effect of transformation is probably due to a general increase in metabolic activity in the transformed cells.

Evidence that the assumed VP defect in protoporphyrinogen oxidase activity is carried through into culture

Finally, in a comparative experiment designed to validate the lymphoblast cultures as a model of the assumed inherited deficiency in VP, protoporphyrinogen oxidase was assayed in fresh lymphocytes isolated from 500ml of blood from 3 normal controls and 3 VP subjects. Epstein-Barr virus transformed lymphoblasts from the same controls and VP subjects had previously been established and were sufficiently populous for the assay. Thus protoporphyrinogen oxidase V_{max} and K_m of transformed lymphoblasts and lymphocytes originating from the same subjects could be compared. The results are shown in table 4.6. The VP protoporphyrinogen oxidase defect appeared to have been maintained through the transformation procedure and into secondary culture.

Table 4.6: *Presence of an approximate 50% decrease in protoporphyrinogen oxidase V_{max} activity (nmol/mg protein/h) was apparent both prior to and after transformation of lymphoblasts originating from the same VP subject. There was no significant variation in K_m (μM) between the two groups. Values given represent the mean of 3 subjects in each group.*

	LYMPHOCYTES		TRANSFORMED LYMPHOBLASTS	
	V_{max}	K_m	V_{max}	K_m
Controls	0.28	0.98	0.79	1.01
VP	0.14	1.06	0.35	1.02
% Difference	-50%	-	-55.7%	-

Data analysis

Units of expression for protoporphyrinogen oxidase activity were nmol of protoporphyrin/mg protein/h and for PBG deaminase, nmol uroporphyrin/mg protein/h. Where applicable data are presented as mean ±standard deviation (SD). Enzyme activities of transformed lymphoblasts from the group of VP patients were compared to the results obtained in normal controls and statistical significance was assessed with the Student T-test for comparing the means of two population samples (Bailey, 1981). Significance was gauged from the probability (p) value.

Results

Protoporphyrinogen oxidase activity in VP

The protoporphyrinogen oxidase mean V_{max} activity for the normal control group of cultures was 0.82±0.10nmol protoporphyrin/mg protein/h (N=27) and that for the VP cultures 0.39±0.08nmol protoporphyrin/mg protein/h (N=27) (figure 4.10). This represented a 52% decrease in mean activity which was statistically significant ($p<0.001$). The mean protoporphyrinogen oxidase apparent K_m for the normal control cultures was 1.06±0.28μM and that for the VP cultures 1.00±0.27μM which was not significantly different ($p=0.505$).

PBG deaminase activity in VP

The mean activity of PBG deaminase in cultures from the normal control subjects was 19.4±0.14pmol uroporphyrin/mg protein/h and that in the cultures from the VP subjects 15.0±0.18pmol uroporphyrin/mg protein/h (figure 4.11). This represents a 24% decrease in activity and is statistically significant ($p<0.001$).

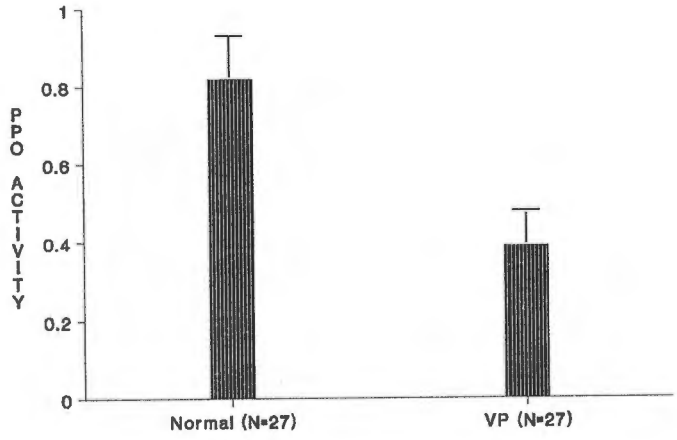

Figure 4.10: *Human lymphoblast protoporphyrinogen oxidase V_{max} (nmol/mg protein/h) was decreased by an average of 52% in a group of 27 VP lymphoblast cultures. K_m values were not significantly different. The "T" bars represent one standard deviation.*

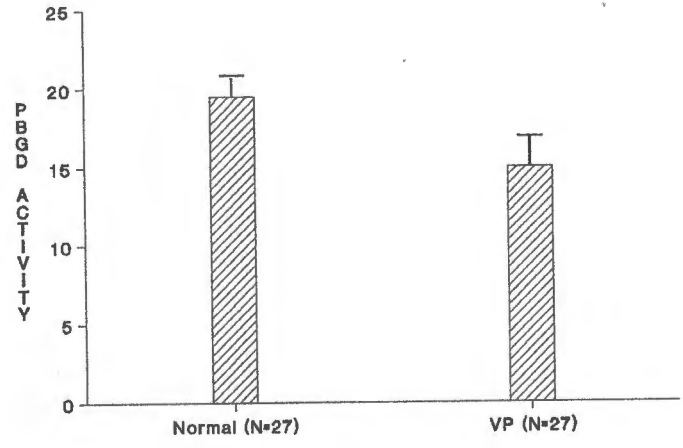

Figure 4.11: *Human lymphoblast porphobilinogen deaminase apparent V_{max} (pmol/mg protein/h) was decreased by an average of 24% in a group of 27 VP lymphoblast cultures. The "T" bars represent one standard deviation.*

Family study

Enzyme activity was measured in affected and non-affected members of 3 generations of a VP family. The data obtained from these 14 subjects are presented as a simplified family tree in figure 4.12. All subjects in whom protoporphyrinogen oxidase activity was decreased by more than 2 standard deviations from the control mean activity also exhibited a decrease in PBG deaminase activity greater than 2 standard deviations below control mean activity. One asymptomatic and biochemically normal subject had a protoporphyrinogen oxidase activity of 0.57nmol/mg/h which lay between the porphyric and the control group. His PBG deaminase was normal.

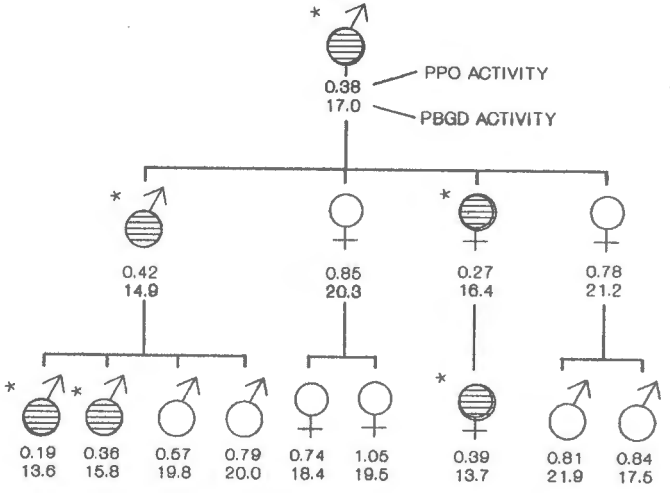

Figure 4.12: *Pedigree of the VP family studied. Protoporphyrinogen oxidase and PBG deaminase activities are given in nmol/mg protein/h and pmol/mg protein/h respectively. Hatched symbols denote decreased protoporphyrinogen oxidase and PBG deaminase activity by more than 2 standard deviations below the mean control activity. Asterisks denote the presence of typical VP porphyrin excretory profiles. All patients with unequivocally decreased protoporphyrinogen oxidase appeared to demonstrate typical porphyrin profiles as well.*

Discussion

Firstly, some comment regarding the use of lymphoblasts to examine protoporphyrinogen oxidase and PBG deaminase in VP is appropriate.

Lymphoblast cultures

The use of Epstein-Barr virus transformed lymphoblasts in the study of haem biosynthetic enzymes was attractive since it appeared to offer several advantages. Cells could be obtained with little inconvenience to the donor subjects (10-20ml of peripheral blood is required) and this compared favourably to more invasive procedures such as skin or liver biopsy. A long-lived cell line is produced which can be stored with minimal loss of viability (Bird *et al*, 1981; Van der Westhuizen, 1984 and our results). This is of particular advantage where large surveys are undertaken. Thus the cultures allow for simultaneous assay of relatively large numbers of subjects but are also available for repeat assays, further experimentation and inter-laboratory transport. Finally large numbers of cells can be produced when required.

Protoporphyrinogen oxidase and PBG deaminase assay in lymphoblasts

Assays of both PBG deaminase and protoporphyrinogen oxidase in the lymphoblast preparations proved to be relatively easy using the methods described. Because of the time required to produce sufficient cells, transformation followed by enzyme measurement is not recommended as a routine diagnostic laboratory procedure. It is important to use cultures with more than 80% viability. We found that our results became inconsistent and irreproducible when the proportion of viable cells diminished to below this level. We found (figures 4.2-4.8 and tables 2 and 3) that the conditions previously described for the assay of both of these enzymes in other tissues were

applicable to our lymphoblasts. Thus, those laboratories with existing assays could easily adapt these for use in lymphoblast cultures.

The characteristics of protoporphyrinogen oxidase in our system were similar to those reported (Brenner and Bloomer, 1980[b]; Jacobs and Jacobs, 1982). The pH optimum of 8.6 (figure 4.2) is similar to that reported for rat liver mitochondria (Brenner and Bloomer, 1980[b]), and for purified murine (Dailey and Karr, 1987) and bovine (Siepker *et al*, 1987) liver protoporphyrinogen oxidase. In keeping with other reports we found it difficult to obtain good activity at substrate concentrations above 30μM and concentrations in excess of 50μM were inhibitory (Camadro *et al*, 1985; Siepker *et al*, 1987). The initial lag in the generation of protoporphyrin (figure 4.3) has also previously been documented (Brenner and Bloomer, 1980[b]) although the reasons for this remain unclear. However, after 15min, generation of protoporphyrin was linear for up to 90min. The V_{max} activity of protoporphyrinogen oxidase in lymphoblasts was about a third of that reported in untransformed human lymphocytes (Deybach *et al*, 1981). The reason is unclear but since the results were so consistent, this was felt to be acceptable. The use of a "kinetic" as opposed to an end-point assay allowed us to determine V_{max} and K_m values. Though these are "apparent" V_{max} and K_m values, they are nevertheless valid for making direct comparisons between control and VP groups.

The characteristics of PBG deaminase in our system are also similar to those reported previously (Anderson and Desnick, 1982). The activity of lymphoblast PBG deaminase was within the range reported elsewhere for human lymphoblasts and lymphocytes (Sassa *et al*, 1978; Anderson and Desnick, 1982).

The effect of transformation on protoporphyrinogen oxidase and PBG deaminase

Mitogen-stimulated lymphocytes demonstrate striking increases in metabolic activities related to DNA, RNA and protein synthesis. It was thus likely that enzymes in lymphoblasts would also show a degree of increased activity when compared to unstimulated precursor

lymphocytes (Sassa *et al*, 1978). Sassa *et al* previously measured the activities of ALA synthetase, ALA dehydratase, uroporphyrinogen synthetase (PBG deaminase) and catalase in phytohemagglutinin (PHA)- and pokeweed mitogen (PWM)-stimulated lymphocytes from normal and AIP subjects (Sassa *et al*, 1978). ALA synthetase, ALA dehydratase and catalase activities were increased two- to three-fold by mitogen treatment for 4 days in all cells, whereas a 10-15 fold induction of PBG deaminase was observed in control (normal) mitogen-stimulated lymphocytes and an approximately 30-fold induction in mitogen-stimulated AIP lymphocytes.

Our results show that control lymphoblast cultures in the presence of Epstein-Barr virus demonstrate an approximately 4-fold increase in PBG deaminase activity after 7 days (figure 4.9). After 4 weeks, however, PBG deaminase activities levelled off at mean levels of 1.5 to 2 times those found in the untreated lymphocytes. Protoporphyrinogen oxidase behaved almost identically (figure 4.9).

Our data would therefore appear to show no difference in the relative response of PBG deaminase and protoporphyrinogen oxidase. While this may appear to differ from the previously mentioned study (Sassa *et al*, 1978) it is important to note that that study did not measure protoporphyrinogen oxidase while ours did not measure ALA synthetase or ALA dehydratase. Furthermore the two studies included certain methodological differences including the method of stimulation and the times at which PBG deaminase activity was determined. It is conceivable that we would have found significantly more stimulation of PBG deaminase activity (and possibly protoporphyrinogen oxidase as well) at an earlier point in the transformation process. This would have explained our apparently discrepant results. Whatever the explanation, in our hands lymphoblasts (after 4 weeks in culture) showed similar increases in PBG deaminase and protoporphyrinogen oxidase.

Confirmation of the protoporphyrinogen oxidase activity defect in VP

In this study of 27 lymphoblast cultures from quiescent VP subjects, lymphoblast protoporphyrinogen oxidase V_{max} activities were found to be decreased by approximately 50% (figure4.10). This data is similar to that obtained by others (Brenner and Bloomer, 1980[a]; Deybach *et al*, 1981; Viljoen *et al*, 1983; Boyle *et al*, 1986) and suggests that decreased protoporphyrinogen oxidase activity may be the underlying defect in VP.

The similar K_m of protoporphyrinogen oxidase in lymphoblasts in control and VP lymphoblasts appears to favour the suggestion that protoporphyrinogen oxidase in VP is not structurally mutant; only activity from the enzyme produced by the normal allele is being measured. Either the abnormal inherited allele is producing no enzyme or it is producing an enzyme with a markedly increased K_m. It must however be emphasised that we have determined an apparent K_m using unpurified enzyme. Thus caution must therefore be exercised in basing any conclusions regarding protoporphyrinogen oxidase structure on our data.

Perpetuation of protoporphyrinogen oxidase activity through transformation into culture.

VP lymphoblasts exhibited the same diminution of protoporphyrinogen oxidase activity as untransformed lymphocytes derived from the same VP patient when compared to the equivalent normal control cultures (table 4.6). The deficiency in protoporphyrinogen oxidase activity thus appeared to be carried through the transformation process and into secondary culture.

Evidence that PBG deaminase activity is also decreased in lymphoblasts from subjects with VP

This was a most significant finding in terms of the development of the hypothesis outlined in Chapter 3 of this thesis; *the mean lymphoblast PBG deaminase activity in the VP cultures, including those from the 6 affected members of the VP family, was reduced by approximately 25% ($p<0.001$)* (figure 4.11). The significance of this difference has not been fully documented previously. This is probably due in part to the fact that most studies measuring PBG deaminase in VP have used erythrocytes (Magnusson *et al*, 1974; Brodie *et al*, 1977[a]; Doss and Von Tiepermann, 1978) and in part to the small numbers invloved in these studies. In one larger study (16 VP patients) a modest decrease of erythrocyte PBG deaminase was apparent although neither statistical comparisons nor comment was made (Mustajoki, 1976).

The significance of diminished PBG deaminase activity in VP

We would like to suggest that the association of a low PBG deaminase activity with diminished protoporphyrinogen oxidase activity may be significant. This we believe might explain some of the features characteristic of VP. Thus the elevation in later (distal) porphyrin intermediates found in VP (mainly protoporphyrin and coproporphyrin) is in keeping with the decreased protoporphyrinogen oxidase activity and the increased concentrations of ALA and PBG found under conditions of increased haem synthetic drive, namely during acute attacks, could be due to the decrease in PBG deaminase activity which we have observed. Presumably the flux through the pathway in the quiescent phase of VP is not sufficient to stress the modest diminution in PBG deaminase activity.

Why is PBG deaminase activity diminished in VP?

The cause of the decreased porphobilinogen deaminase activity in VP is not obvious. While the exact site of the gene coding for

protoporphyrinogen oxidase is unknown there is evidence that it is encoded for on chromosome 14 (Bissbort *et al*, 1988), far removed from that encoding PBG deaminase on chromosome 11 (Meisler *et al*, 1980, 1981; Wang *et al*, 1981). There is thus little evidence or precedent to favour the genes for these haem synthetic enzymes being close to one another. Thus, assuming the enzymes are on different chromosomes, an inherited mutation of PBG deaminase should be passed on independently of any defect in protoporphyrinogen oxidase and our family study should have revealed some subjects with one or the other deficiency alone. Yet none were detected. Thus simple genetic linkage would appear not to be an explanation and it might therefore be more appropriate to consider the decreased PBG deaminase activity as a secondary phenomenon linked in some way to the primary inherited protoporphyrinogen oxidase defect. This is the suggestion examined in the following chapter.

Conclusions

- *Epstein-Barr virus transformed lymphoblasts appear to be a valid model in which to study protoporphyrinogen oxidase and PBG deaminase.*

Epstein-Barr virus transformed lymphoblasts appear to retain their haem synthetic enzyme activity in that both protoporphyrinogen oxidase and PBG deaminase are present and can be assayed. (Previous workers have also demonstrated active ALA synthetase and ALA dehydratase in Epstein-Barr virus-stimulated lymphocytes). Although the transformation process results in a mild stimulation of porphobilinogen deaminase and protoporphyrinogen oxidase activities, neither appears to be selectively stimulated. Finally, decreased activity of protoporphyrinogen oxidase was demonstrated in lymphocytes from the same VP subjects before and after transformation with Epstein-Barr virus.

- *There is a 50% decrease in protoporphyrinogen oxidase activity lymphoblasts from VP subjects.*

This study represents the largest series of VP subjects to have protoporphyrinogen oxidase activities measured to date.

- *Lymphoblasts from VP subjects demonstrate a modest but significant decrease in PBG deaminase activity.*

This decrease of roughly 25% appears to coexist with the decrease in protoporphyrinogen oxidase activity. We suggest that the porphyrin and porphyrin precursor accumulation observed in VP can be explained on the basis of the enzyme alterations described above. In particular, our data suggest an explanation for the elevations of ALA and PBG seen in the acute phase of VP. A constriction in the form of partially diminished PBG deaminase activity may well contribute to the elevations of proximal haem intermediates in acute VP. The idea of a second "constriction" or control point in haem biosynthesis (Brodie *et al*, 1977[a]; Moore *et al*, 1980; Doss, 1978) would thus appear to offer a potential explanation for elevated concentrations of ALA and PBG in VP. We suggest that the decrease in PBG deaminase activity is not due to a separate autosomal dominant mutation since genetic linkage seems improbable.

The possibility that the reduction in PBG deaminase activity is a consequence of the protoporphyrinogen oxidase defect is examined in the following chapter.

Chapter Five

PBG Deaminase kinetics in VP and kinetic effects of haem intermediates on PBG deaminase

The data presented thus far point to intriguing questions regarding the cause of the diminished PBG deaminase activity in VP. Having dismissed the simultaneous genetic transmission of two enzyme defects (Chapter 4) we turn to the second part of the hypothesis outlined in chapter 3. Our hypothesis suggested that PBG deaminase is directly influenced by an "external" factor. Because PBG deaminase activity appears to be linked to the presence of decreased protoporphyrinogen oxidase activity it would seem logical to include those intermediates which accumulate as a result of the decreased protoporphyrinogen oxidase activity in the list of putative inhibitors of PBG deaminase. Further, if the mechanism envisaged is to be more generally operative and hence to be extrapolated to HCP too, it must in some way be linked to a deficiency in coproporphyrinogen oxidase as well. Conversely any explanantion would have to account for the lack of accumulation of ALA and PBG in PCT, EPP and CEP.

We thus hypothesise that raised concentrations of proto- and coproporphyrinogen but not of uroporphyrinogen act as negative allosteric effectors* resulting in inhibition of PBG deaminase activity. Thus conditions such as VP and HCP, where elevated concentrations

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* The term allosteric is used in the sense that PBG deaminase is inhibited by certain factors other than the catalytically active substrate (Atkinson, 1970).

of proto- and coproporphyrinogen are assumed to exist intracellularly, could well be expected to demonstrate decreased PBG deaminase activity, or at the least a PBG deaminase that would not be able to increase its activity to cope with the haem synthetic demands under certain conditions. On the other hand conditions such as PCT in which uroporphyrinogen accumulate should not be associated with a decrease in PBG deaminase activity.

Objectives

- To compare the basic kinetic profiles of PBG deaminase in VP and control lymphoblasts.
- To examine the effects of various porphyrinogens and porphyrins on PBG deaminase activity and its kinetic behaviour.
- To establish whether normal V_{max} activity of the enzyme in VP lymphoblasts could be restored by removal of any endogenously occurring allosteric inhibitors.

Study design

- PBG deaminase activity was assayed in sonicates of Epstein-Barr virus transformed lymphoblasts obtained from each of 12 patients with quiescent VP and 12 normal control subjects. The kinetic characteristics of PBG deaminase in each group were compared.
- The effects of different concentrations of added porphyrinogens and porphyrins on PBG deaminase were assessed. Here protoporphyrin, protoporphyrinogen, coproporphyrin, coproporphyrinogen, uroporphyrin and uroporphyrinogen were added to sonicates of lymphoblasts from both the control and VP group.

- In a further set of experiments endogenous porphyrinogens and porphyrins were removed from VP lymphoblast preparations prior to the kinetic assay of PBG deaminase.

Methods

Lymphoblasts

Lymphoblasts were prepared from both VP and control subjects as described previously (see Chapter 4, *Methods* and Appendix 6 and 7).

PBG deaminase assay

PBG deaminase activity was assayed using the method described in Chapter 4 (*Methods*) and Appendix 9 with the following modification: Each assay was performed with substrate (PBG) concentrations of 1.25, 2.5, 5, 10 and 20μM and a substrate versus velocity curve obtained. PBG concentrations were selected to straddle the reported K_m of PBG deaminase.

Each experiment was controlled for non-enzymatic porphyrin production (which is normally negligible), by subtracting the fluorescence produced in matching assay tubes containing all reaction ingredients except the PBG deaminase, from the fluorescence obtained in equivalent tubes containing enzyme.

Protein concentrations were determined in all cases using Biorad protein dye solution (see Appendix 12) according to the "Bradford" technique for microgram quantities of protein (Bradford, 1976).

Potential difficulties associated with assaying PBG deaminase in the presence of additional porphyrins or porphyrinogens

1. PBG deaminase assay against a high background of porphyrin fluorescence

We were concerned that the PBG deaminase assay might be confounded by the addition of exogenous porphyrins, which might themselves provide too much fluorescence to allow accurate determination of that resulting from the enzymatic production of uroporphyrin from PBG. Similarly added porphyrinogens, once oxidised, might also result in a high background fluorescence. We evaluated this potential problem as follows:

The assay was performed in six control lymphoblast lines in the presence of an added mixture resulting in 10μM uroporphyrin, coproporphyrin and protoporphyrin in the incubation mixture. This created a fluorescence background even higher than that anticipated in the proposed experiments. The fluorescence ultimately resulting from enzymatic production of uroporphyrin and the added porphyrins was determined by total fluorescence determination of a 1/10 dilution (in order to exclude the effects of fluorescence quenching) of reaction mixture by direct fluorometry using an excitation wavelength of 405nm and an emission wavelength of 595nm. Fluorescence in control assay tubes containing all reaction ingredients except the PBG deaminase was subtracted from fluorescence in the enzyme-containing tubes. This resulted in positive readings. Thus some additional porphyrin, presumed to be uroporphyrin, had been produced from PBG by PBG deaminase.

We verified that the increased fluorescence was indeed due to uroporphyrin by performing TLC analysis of all porphyrin species present before and after assay in a parallel experiment using aliquots of the same six control lymphoblast preparations (Table 5.1). This served a useful dual purpose by yielding further data with which to

Table 5.1: *Integrated peak fluorescence readings of porphyrins present in TLC traces of reaction mixture pre- and post-PBG deaminase assay. Added porphyrins were contrived to give reaction mixture concentrations of 10μM for each of uro-, copro- and protoporphyrin. Results are expressed in arbitrary fluorescence units and represent the mean (±SD) of 6 control lymphoblast cell lines, each performed in duplicate. Note that the quantum fluorescence yield of proto<copro<uroporphyrin; hence equivalent quantities of these 3 porphyrins give correspondingly variable readings on fluorscanning. (*Significantly different, p<0.001).*

	Pre-assay *(Fluorscence Units)*	**Post-assay** *(Fluorescence Units)*
Uroporphyrin	124 (±18)	*169 (±20)
Coproporphyrin	116 (±19)	110 (±15)
Protoporphyrin	95 (±15)	93 (±16)

calculate PBG deaminase activity and which could be compared with results obtained using the direct "total fluorescence" method.

The latter experiments were accomplished by setting up and running the assays as described. After 30min, however, the reaction was terminated by addition of 3ml of ethyl acetate:acetic acid (3:1, v/v) instead of TCA. Any protein precipitate was removed by centrifugation (10000Xg, 10min) and any porphyrinogens present (including reaction product, uroporphyrinogen) oxidized to their porphyrin form by exposure to longwave ultraviolet and white fluorescent light for 30min. Any porphyrins present in the assay mixture at this stage (endogenous, reaction product or additions) were analysed on TLC by esterification, extraction, separation and quantitation as already described (Appendix 4).

The first method of assay (direct fluorometry of a 1/10 dilution of reaction mixture) yielded a mean PBG deaminase V_{max} of 26.2(±4.1)pmol/mg protein/h and the second method (extraction and quantitative porphyrin TLC) gave a mean V_{max} of 28.0(±2.1)pmol/mg protein/h. A comparison of these two methods showed a marginally

lower V_{max} with a slightly larger scatter using the first method although the two results were not statistically different ($p = 0.230$, $N = 6$). Furthermore, TLC analysis indicated similar quantities of copro- and protoporphyrin both before and after assay, whereas the amount of uroporphyrin was increased post-assay (Table 5.1). It was concluded that the assay as described, using a single fluorescence reading on a diluted aliquot of reaction mixture was acceptable as uroporphyrin product was able to be ascertained even against a high background of porphyrin fluorescence. Thus the potentially more accurate (certainly more visible), but tedious, alternative extraction and TLC procedure offered no significant advantage and was thus only used to verify the assay described above.

2. Preparation and stability of porphyrinogens

The second potential problem related to the known instability of porphyrinogens. Because testing of the hypothesis demanded that porphyrinogens be investigated it was necessary to examine the stability of various porphyrinogens under assay conditions. An experiment to ascertain the relative stability of proto-, copro- and uroporphyrinogen under PBG deaminase assay conditions was performed; 5ml of a 50μM solution of the appropriate porphyrin was reduced to the porphyrinogen using 4% sodium amalgam as described for the preparation of protoporphyrinogen (Chapter 4 and Appendix 8). The reduced solution was filtered and the pH adjusted to 8.2 with glacial acetic acid. 2ml of this was added to 8ml of PBG deaminase assay buffer and the solution incubated in a 37°C shaking water bath in the dark for 2h. A 1ml aliquot was removed every 15min and its fluorescence measured in a fluorometer. In this way the likely rate of re-oxidation of porphyrinogens under assay conditions was determined. After 2h any porphyrinogen present in the remaining volume was re-oxidised by adding 50μl of a 0.005% (w/v) aqueous iodine solution and a small crystal of cysteine added to decolourise the excess iodine (Fuhrhop and Smith, 1975). This confirmed that porphyrinogens were still present after 2h and that there was no

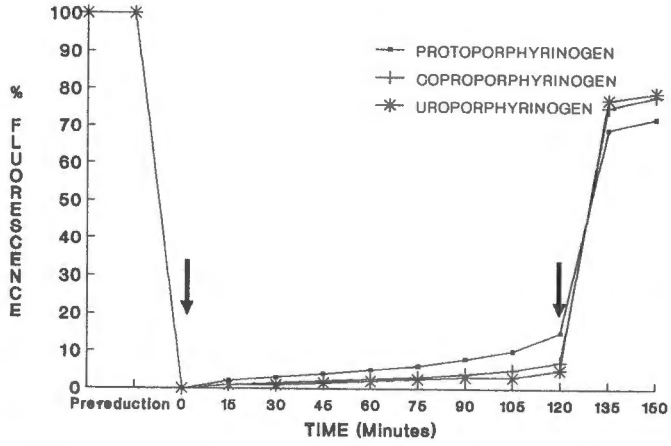

Figure 5.1: *Rate of auto-oxidation of 10μM proto-, copro- and uroporphyrinogen under standard assay conditions over a 2h period. At time zero the porphyrinogens were 100% reduced as judged by the complete absence of fluorescence. Auto-oxidation was followed by fluorescence readings every 15 minutes. After 2h chemical re-oxidation of any remaining porphyrinogens was stimulated by addition of iodine.*

significant porphyrin destruction/modification due to the reduction process.

The results of this experiment are shown in figure 5.1. Oxidation of these porphyrinogens as judged by reappearance of porphyrin fluorescence was negligible over the first 30min and very slight over the next 90min, the rates of re-oxidation of the 3 porphyrinogens being slightly different. Protoporphyrinogen was the least stable, uroporphyrinogen most stable. It was concluded that the stability of the porphyrinogens under investigation was acceptable under the proposed assay conditions.

3. Oxidation of porphyrinogens

It was important to ensure that porphyrinogens could be completely oxidised to the corresponding porphyrins since it is these that are measured. Thus VP lymphoblasts were sonicated, 100μM proto-,

Table 5.2: *Three experiments illustrating re-oxidation of 100μM proto-, copro- and uroporphyrinogen in the presence of lymphoblast sonicates using exposure to ultraviolet light and vigorous shaking in air. These conditions encouraged spontaneous re-oxidation of the porphyrinogens and was judged over 2h by appearance of porphyrin fluorescence. Results were expressed as a % of total porphyrin fluorescence measured prior to reduction.*

	Protoporphyrinogen *(100μM)*	**Coproporphyrinogen** *(100μM)*	**Uroporphyrinogen** *(100μM)*
TIME *(min)*	Relative Fluorescence	Relative Fluorescence	Relative Fluorescence
Pre-oxidation	100	100	100
0	0	0	0
15	35	27	26
30	56	50	48
45	67	63	62
60	75	72	70
75	82	80	77
90	88	86	83
105	92	90	89
120	94	91	90

copro- or uroporphyrinogen added and the preparation exposed to longwave ultraviolet and white light while being vigorously shaken. Porphyrin fluorescence was monitored every 15min for 2h. Table 5.2 indicates that exposure to these oxidising conditions for 2h resulted in 94% re-oxidation of protoporphyrinogen, 91% re-oxidation of coproporphyrinogen and 90% of uroporphyrinogen.

Analysis of kinetic data comparing VP and control lymphoblast PBG deaminase

Data obtained from assays of PBG deaminase reaction velocity at different substrate concentrations using lymphoblasts derived from 12 quiescent VP and 12 control subjects were initially treated by application of the Michaelis-Menten equation (Bergmeyer, 1978) in

order to obtain V_{max} and K_m. Data which did not conform to the Michaelis-Menten rate law were examined using the Hill equation (Van Holde, 1971). This equation is normally used to analyse cooperative interactions. A non-linear, least squares grid search procedure computed the following parameters from the Hill equation which were used for comparative purposes: V_{max}, $K_{0.5}$ and the Hill coefficient, n. V_{max} is the maximal catalytic rate (velocity) for PBG deaminase, $K_{0.5}$ is the substrate concentration at which half-maximal velocity is achieved. The Hill coefficient describes the extent of cooperativity for ligand binding processes, or the extent to which multiple ligand-binding sites interact. The coefficient, n, is always less than the actual number of ligand-binding sites (Hammes, 1982). Thus for single site substrate binding processes displaying perfect hyperbolic Michaelis-Menten behaviour the Hill coefficients will approximate to 1. For hyperbolic behaviour K_m, as obtained from the Michaelis-Menten equation is identical to $K_{0.5}$. For processes not following Michaelis-Menten behaviour the K_m *per se* is meaningless and an equivalent parameter, in this case termed $K_{0.5}$, is more informative. Appendix 10 gives detail of the Hill equation. A Hill plot, $\log v/(V_{max} - v)$ versus log[S] (where v is the initial velocity at the given substrate concentration and [S] is the substrate concentration), will have a slope of approximately 1 where the binding is independent and a slope approaching n where there is cooperativity. However, the binding is rarely so highly cooperative as to yield a straight line with slope $n1$ over a wide range of log[S]. A more detailed analysis shows that the graph will approach a slope of unity near the extremes and have a maximum slope of $<n$. Numerically the Hill coefficient is always less than the actual number of ligand (substrate) binding sites.

Comparisons were assessed for statistical significance using the Student's T-test.

Addition of porphyrins and porphyrinogens

Varying concentrations of proto-, copro- and uroporphyrin and proto-, copro- and uroporphyrinogen were added to control lymphoblasts and

to VP lymphoblasts. Data were analysed using the Hill equation and compared to those obtained in control or VP lymphoblasts without added test substances. The porphyrinogens were produced by reduction with sodium amalgam (Appendix 8) immediately prior to use. All test substances were added to the incubation mixture in final concentrations of 1, 5 and 10μM 5min prior to the start of the assay. The effect of varying the time between the addition and initiation of the PBG deaminase reaction was also examined by pre-incubating with porphyrins and porphyrinogens for times ranging from 5 to 60min. There was no significant change in the value of any of the determined parameters between 5 and 60min (see *Results* and figures 5.7 and 5.9).

Separation of porphyrin(ogen)s from PBG deaminase in VP lymphoblasts

1. Sephadex G25 chromatography

After sonication of VP lymphoblasts Sephadex G25 chromatography was used to separate the low molecular weight endogenous porphyrin(ogen)s from fractions containing PBG deaminase activity. Fractions containing PBG deaminase were used for subsequent kinetic studies.

In order to validate the separation of porphyrins and PBG deaminase a mixture of 0.5mM uro-, copro- and protoporphyrin was added to 10000Xg supernatants of control lymphoblast sonicates. Additional porphyrins were added to ensure visible porphyrin fluorescence in the resulting porphyrin containing fractions eluted from the column. PBG deaminase activity was found to elute in the G25 void while porphyrins eluted at 3.2-3.4X the void volume (figure 5.2).

Next supernatants (10000Xg) of sonicated VP lymphoblasts were applied to a 1.5X10cm Sephadex G25 column, equilibrated with 0.1M Tris-HCl buffer (pH 8.2) and eluted, with the same buffer, at a flow rate of 40ml/h. Fractions containing PBG deaminase activity were pooled, concentrated on Centricon-30 (Amicon) columns through a

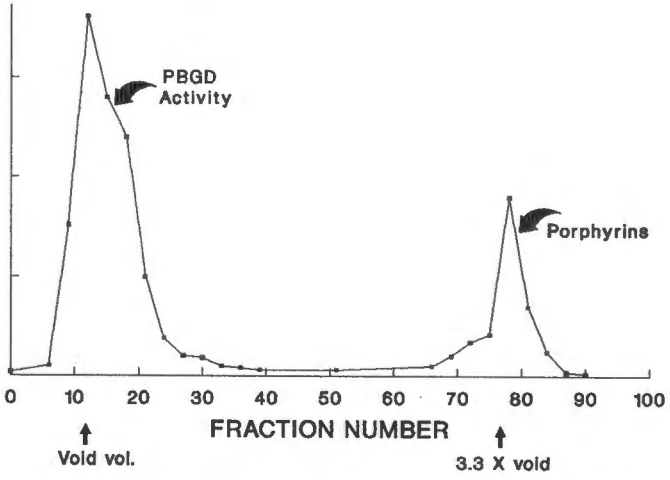

Figure 5.2: *Elution profile of a mixture of porphyrins and lymphoblast PBG deaminase on Sephadex G25. Those fractions containing PBG deaminase activity were clearly separated from those containing porphyrins.*

P30 membrane and assayed for protein content prior to PBG deaminase assay.

2. Oxidation of intracellular porphyrinogens

If porphyrinogens are exerting an effect on PBG deaminase then their "removal" should be facilitated by subjecting lymphoblast preparations to oxidising conditions. VP lymphoblast sonicates were thus subjected to the "oxidising" conditions described above for 2h and PBG deaminase assayed in an aliquot both before and after porphyrinogen oxidation.

Results

PBG deaminase kinetics in control and VP lymphoblasts

Lymphoblast sonicate PBG deaminase from the 12 control subjects gave a mean V_{max} of 25.2(±1.7)pmol/mg protein/h and a mean Michaelis constant (K_m) of 8.1(±0.7)μM when fitted to the Michaelis-Menten equation and evaluated by double-reciprocal substrate versus velocity (Lineweaver-Burk) plots. Linearity of the Lineweaver-Burk plot and of the rate/substrate concentration versus rate (Eadie) plot confirmed the adherence of "control" PBG deaminase to the Michaelis-Menten rate law (figure 5.3). Application of the Hill equation to the same data gave mean values for V_{max} of 28.7(±1.8)pmol/mg protein/h and $K_{0.5}$ of 8.5(±0.8)μM which were not significantly different from those obtained using the Michaelis-Menten equation. As expected, the Hill coefficient of 0.83 was close to 1.

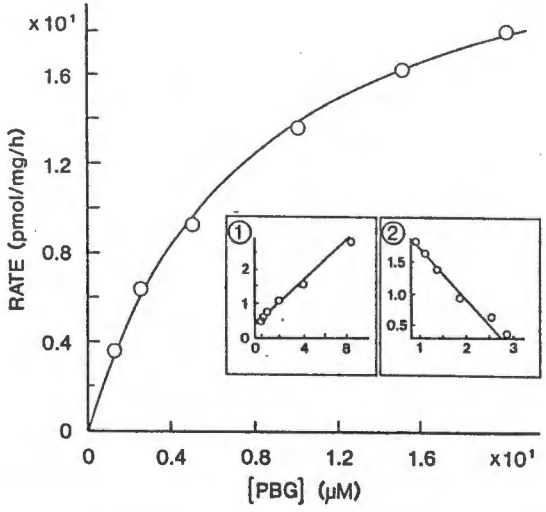

Figure 5.3: *Substrate-velocity plot of PBG deaminase in control lymphoblast sonicates (mean of 12 sonicates). Michaelis-Menten kinetics were obeyed as illustrated by the straight lines obtained in the Lineweaver-Burke plot (inset 1) and the Eadie plot (inset 2).*

Identical experiments in sonicates of lymphoblasts from 12 VP subjects yielded substrate-velocity data that did not conform to Michaelis-Menten kinetics. Non-hyperbolic behaviour was demonstrated by failure of the data to produce a straight line with either the Lineweaver-Burk or Eadie plots (figure 5.4). (The Eadie plot is particularly suitable for showing lack of adherence to Michaelis-Menten kinetics.) Hence V_{max} and K_m could not be assessed by this method. The Hill equation, however, did allow these determinations. Control and VP results are contrasted table 5.3. Lymphoblast V_{max} values were decreased by a mean of 26% in the VP group, a figure similar to the decrease in V_{max} observed in the conventional substrate excess assay described in the Chapter 4. The mean substrate-velocity plot of the group of VP lymphoblasts was sigmoidal (figure 5.5). The Hill plot had a maximum slope equal to the Hill coefficient but approached unity near the extremes.

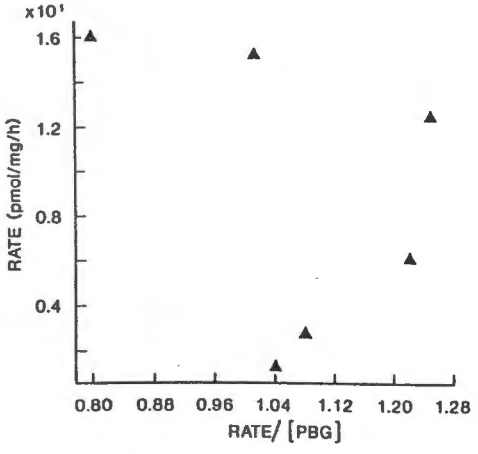

Figure 5.4: *PBG deaminase in VP lymphoblast sonicates (mean of 12 sonicates) showed lack of conformity to the Michaelis-Menten rate equation as demonstrated by non-linearity of the Eadie plot.*

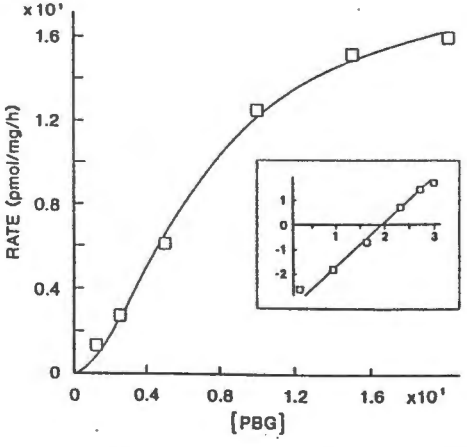

Figure 5.5: *Sigmoidal substrate-velocity plot of VP lymphoblast PBG deaminase. The curve was drawn by fitting the data to the Hill equation. The Hill plot is shown in the inset.*

Table 5.3: *Kinetic parameters for control and VP lymphoblasts when analysed by the Hill equation. Vmax was significantly decreased in the VP cells. $K_{0.5}$ values were not significantly altered but the Hill coefficients of the 2 groups were different.*

	V_{max} (pmol/mg/h)	$K_{0.5}$ (μM)	Hill coeffcient
Controls	28.7 ±1.8	8.5 ±0.8	0.83 ±0.07
VP	21.2 ±2.0	7.4 ±0.7	1.78 ±0.17
% Difference	26% ($p<0.001$)	Not Significant ($p=0.424$)	

Addition of porphyrins to control lymphoblast sonicates

The addition of 1, 5, or 10μM proto-, copro- or uroporphyrin had no effect on the kinetic behaviour of PBG deaminase in 12 control lymphoblast sonicates. It was hoped that this range would approximate that found in the various phases of VP *in vivo*. Figure 5.6 shows the

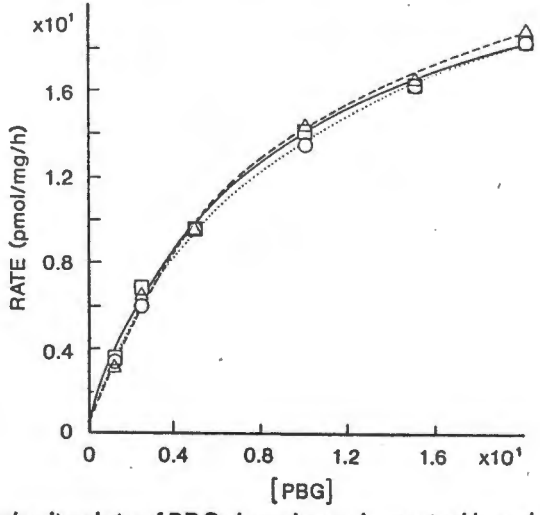

Figure 5.6: *Substrate-velocity plots of PBG deaminase in control lymphoblast sonicates after 5min pre-incubation in the presence of 10μM protoporphyrin (□—□), 10μM coproporphyrin (○····○) and 10μM uroporphyrin (△--△). There were no differences between these plots and that obtained without additional porphyrins (figure 5.3). Kinetic data derived from these curves are given in table 5.4.*

mean substrate-velocity plots of lymphoblast PBG deaminase after the addition of 10μM proto-, copro- and uroporphyrin which were pre-incubated for 5min before substrate (PBG) was added. Table 5.4 lists the results obtained with 1, 5 and 10μM concentrations of the corresponding porphyrin series.

Pre-incubating lymphoblast preparations with 10μM proto-, copro- and uroporphyrin for periods of time up to 1h before assaying PBG deaminase (figure 5.7) shows that these longer periods of pre-incubation did not alter PBG deaminase behaviour.

Table 5.4: *Control PBG deaminase kinetic parameters in the absence and in the presence of 1, 5 and 10μM proto-, copro- and uroporphyrin. The porphyrins were added to the incubation mixture 5min prior to the start of the assay. Results were obtained by fitting substrate-velocity data to the Hill equation and are expressed as the mean of the 12 control lymphoblast preparations used in the study (±SD). No systematic or significant trends or differences were observed.*

	V_{max} *(pmol/mg/h)*	$K_{0.5}$ *(μM)*	**Hill coeffcient**
Control lymphoblasts			
No additions	28.7 ±1.8	8.5 ±0.8	0.83 ±0.07
+1μM Proto	28.4 ±2.9	8.4 ±1.1	0.89 ±0.10
+5μM Proto	28.4 ±1.7	8.3 ±0.9	0.90 ±0.09
+10μM Proto	29.3 ±1.6	8.0 ±1.0	0.87 ±0.10
+1μM Copro	30.2 ±2.1	8.8 ±1.9	0.86 ±0.10
+5μM Copro	31.2 ±1.3	8.9 ±1.2	0.89 ±0.05
+10μM Copro	30.8 ±1.6	9.5 ±0.9	0.88 ±0.07
+1μM Uro	28.8 ±1.3	9.0 ±1.0	0.95 ±0.11
+5μM Uro	34.5 ±3.3	8.5 ±1.3	0.78 ±0.16
+10μM Uro	30.9 ±2.0	8.7 ±1.3	0.82 ±0.08

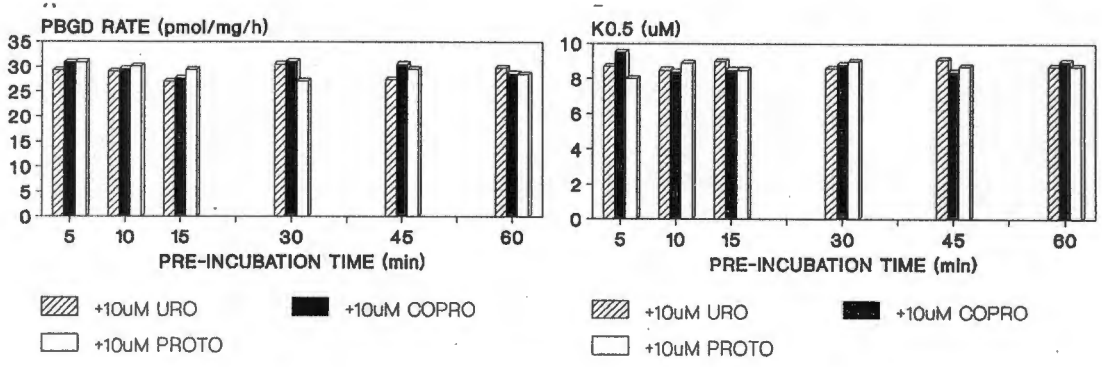

Figure 5.7: *Effect of length of pre-incubation period in the presence of 10μM proto-, copro- and uroporphyrin on PBG deaminase rate (A) and $K_{0.5}$ (B). No systematic or significant changes are observed.*

Addition of porphyrinogens to control lymphoblast sonicates

1, 5 and 10μM concentrations of protoporphyrinogen all displayed a striking ability to alter the kinetic behaviour of control lymphoblast PBG deaminase, resulting in reduced V_{max} activity and sigmoidal substrate-velocity plots resembling those observed in VP lymphoblasts. Figure 5.8 illustrates these effects and table 5.5 summarises the data obtained from the Hill equation in each case.

Compared to the values obtained in the absence of additional porphyrinogen, V_{max} was decreased by an average of 41% ($p<0.001$) while the $K_{0.5}$ was unaltered ($p = 0.361$). V_{max} tended to decrease as the concentration of protoporphyrinogen increased, but this did not achieve statistical significance. The $K_{0.5}$ obtained in the presence of 10μM protoporphyrinogen was greater than that obtained with 5μM protoporphyrinogen which was in turn greater than that in the presence of 1μM protoporphyrinogen. As in the case of V_{max}, variation of $K_{0.5}$ within the group didn't achieve significance.

As the concentration of protoporphyrinogen was increased over a ten-fold range, the Hill coefficient increased to approach a value of 4 in the limit.

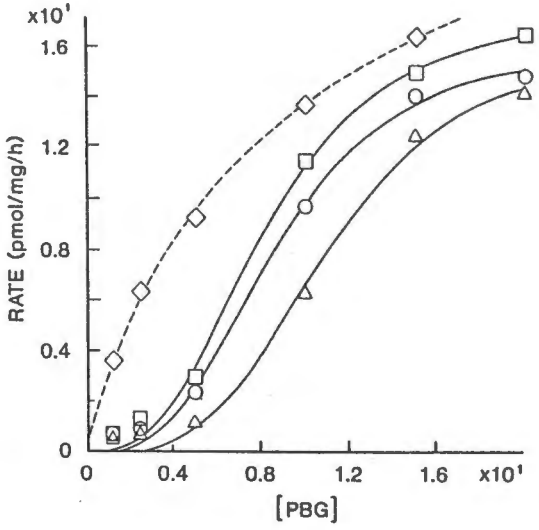

Figure 5.8: *Substrate-velocity plots of PBG deaminase in control lymphoblast sonicates after 5min incubation in the presence of 1(□—□), 5(○—○) and 10μM(△—△) protoporphyrinogen. There were striking differences between these curves and that obtained with no addition (◇---◇). Each data point on each curve represents the mean of observations carried out in 12 control lymphoblast preparations. Kinetic data corresponding to these curves are given in table 5.5.*

Table 5.5: *Control PBG deaminase kinetic parameters in the absence and in the presence of 1, 5 and 10μM protoporphyrinogen. Results were obtained by fitting substrate-velocity data to the Hill equation and are expressed as the mean of the 12 control lymphoblast preparations used in the study (SD).*

	V_{max} *(pmol/mg/h)*	$K_{0.5}$ *(μM)*	Hill coeffcient
Control lymphoblasts			
No additions	28.7 ±1.8	8.5±0.8	0.83 ±0.07
+1μM Proto'gen	17.7 ±1.3	7.7 ±1.1	2.89 ±0.43
+5μM Proto'gen	16.4 ±1.7	8.9 ±1.1	3.05 ±0.47
+10μM Proto'gen	16.1 ±1.6	10.9 ±2.1	3.61 ±0.45

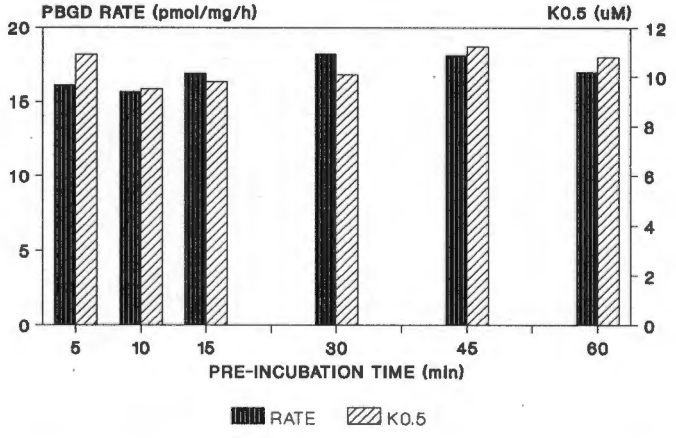

Figure 5.9: *Effect of period of pre-incubation in the presence of 10μM protoporphyrinogen on PBG deaminase rate and $K_{0.5}$. The kinetic changes as determined by the Hill equation apparent at 5min were not further enhanced or diminished over 1h.*

Longer periods of pre-incubation in the presence of protoporphyrinogen appeared to exert no further alterations on the kinetic behaviour of control lymphoblast PBG deaminase (figure 5.9). The effect of pre-incubation times under 5min were not determined.

Addition of 1, 5 and 10μM concentrations of coproporphyrinogen had a similar but less impressive effect on control lymphoblast PBG deaminase. V_{max} was decreased by an average of 27% ($p<0.001$) and the Hill coefficients appeared to approach 3 (in the limit) as coproporphyrinogen concentrations were increased over a ten-fold range. The mean of the $K_{0.5}$ values between the lymphoblasts with additions and those without were not significantly different ($p = 0.247$). The distinctive sigmoidal substrate-velocity plot was once again observed as were the trends in V_{max} and $K_{0.5}$ (figure 5.10 and table 5.6). Longer periods of pre-incubation in the presence of coproporphyrinogen showed no further changes to those measured after 5min of pre-incubation (data not shown).

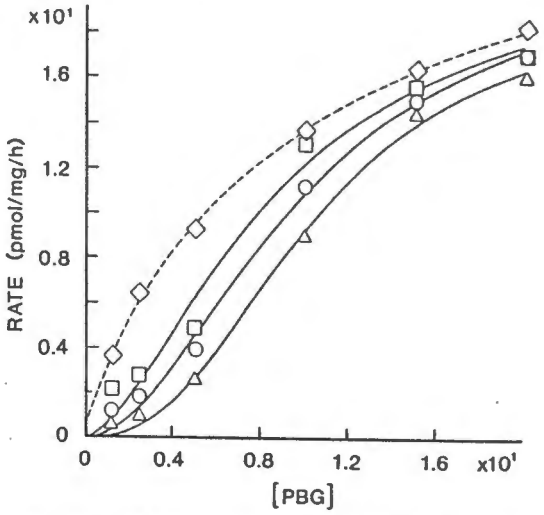

Figure 5.10: *Substrate-velocity plots of PBG deaminase in control lymphoblast sonicates after 5min incubation in the presence of 1(□—□), 5(○—○) and 10μM(△—△) coproporphyrinogen. There were significant differences between these curves and that obtained with no addition (◇--◇). Each data point on each curve represents the mean of observations carried out in 12 control lymphoblast preparations. Kinetic data corresponding to these curves are given in table 5.6.*

Table 5.6: *PBG deaminase kinetic parameters in the absence and in the presence of 1, 5 and 10μM coproporphyrinogen. The experiments were performed and results obtained exactly as for the protoporphyrinogen additions.*

	V_{max} *(pmol/mg/h)*	$K_{0.5}$ *(μM)*	Hill coeffcient
Control lymphoblasts			
No additions	28.7 ±1.8	8.5 ±0.8	0.83 ±0.07
+1μM Copro'gen	21.8 ±1.8	8.5 ±2.3	1.67 ±0.51
+5μM Copro'gen	21.5 ±2.2	9.8 ±1.3	1.96 ±0.33
+10μM Copro'gen	19.5 ±1.9	10.3 ±1.4	2.51 ±0.38

In contrast to the striking effects noted on the addition of proto- and coproporphyrinogen, uroporphyrinogen failed to influence the kinetic behaviour of control lymphoblast PBG deaminase. There were no significant differences in V_{max}, $K_{0.5}$, the Hill coefficient ($p>0.05$ in all cases) or in the shape of the substrate-velocity plot (figure 5.11 and table 5.7).

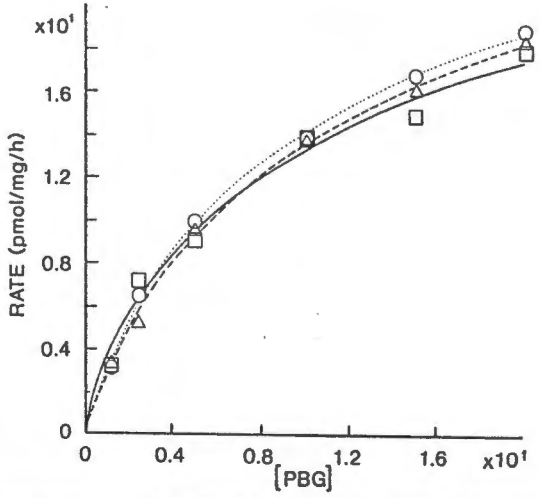

Figure 5.11: *Substrate-velocity plots of PBG deaminase in control lymphoblast sonicates after 5min incubation in the presence of 1(□—□), 5(○····○) and 10μM(△- -△) uroporphyrinogen. Each data point on each curve represents the mean of observations carried out in 12 control lymphoblast preparations. Kinetic values in table 5.7.*

Table 5.7: *PBG deaminase kinetic values in the absence and in the presence of 1, 5 and 10μM uroporphyrinogen. The experiments were performed and results obtained exactly as for the protoporphyrinogen additions.*

	V_{max} *(pmol/mg/h)*	$K_{0.5}$ *(μM)*	Hill coeffcient
Control lymphoblasts			
No additions	28.7 ±1.8	8.5 ±0.8	0.83 ±0.07
+1μM Uro'gen	30.1 ±1.5	7.8 ±1.2	0.79 ±0.26
+5μM Uro'gen	28.7 ±2.4	8.7 ±1.0	0.93 ±0.11
+10μM Uro'gen	29.0 ±1.6	8.4 ±1.1	0.89 ±0.13

An identical series of experiments was then carried out using lymphoblast sonicates from 12 VP subjects.

Addition of porphyrins to VP lymphoblast sonicates

The addition of 1, 5, or 10μM proto-, copro- or uroporphyrin had no additional effect on the already anomalous kinetic behaviour of PBG deaminase in 12 VP lymphoblast sonicates. Figure 5.12 shows the mean substrate-velocity plots of VP lymphoblast PBG deaminase after the addition of 10μM proto-, copro- and uroporphyrin while table 5.8 details these results and the results obtained with 1 and 5μM concentrations of the same porphyrin series. Longer periods of pre-incubation in the presence of the various porphyrin additions did not result in any additional effects on VP PBG deaminase behaviour (data not shown).

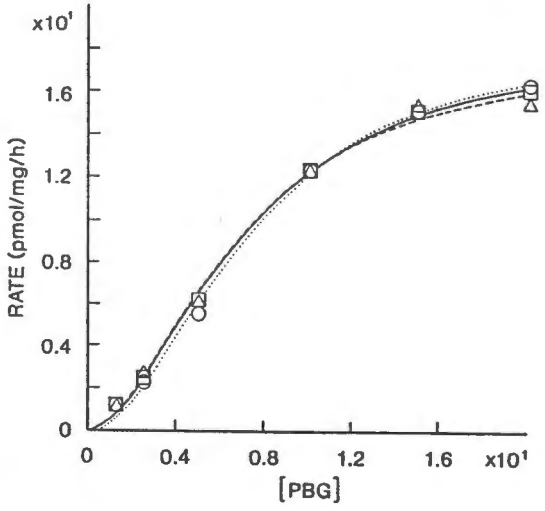

Figure 5.12: *Substrate-velocity plots of PBG deaminase in VP lymphoblast sonicates after 5min incubation in the presence of 10μM protoporphyrin (□—□), 10μM coproporphyrin (○···○) and 10μM uroporphyrin (△– – –△). There were no apparent differences between these plots and that obtained in VP lymphoblasts without additional porphyrins (figure 5.5). See table 5.8 for kinetic data.*

Table 5.8: *VP PBG deaminase kinetic parameters in the absence and in the presence of 1, 5 and 10μM proto-, copro- and uroporphyrin. Experiments were carried out as for the addition of porphyrins to control lymphoblasts. There was no systematic variation or significant differences in any of the values.*

	V_{max} (pmol/mg/h)	$K_{0.5}$ (μM)	Hill coeffcient
VP lymphoblasts			
No additions	21.2 ±2.0	7.4 ±0.7	1.78 ±0.17
+1μM Proto	19.2 ±2.8	10.1 ±1.2	1.97 ±0.20
+5μM Proto	20.0 ±1.6	9.2 ±1.9	1.91 ±0.21
+10μM Proto	18.8 ±1.4	7.6 ±2.2	1.82 ±0.16
+1μM Copro	19.8 ±1.8	7.5 ±1.6	1.83 ±0.25
+5 μM Copro	19.3 ±2.2	8.5 ±1.8	1.72 ±0.18
+10μM Copro	21.1 ±1.9	8.4 ±1.7	1.93 ±0.21
+1μM Uro	17.9 ±1.5	7.4 ±1.4	1.87 ±0.30
+5μM Uro	20.4 ±2.3	7.1 ±1.0	1.92 ±0.31
+10μM Uro	18.1 ±1.5	8.9 ±1.3	1.88 ±0.29

Addition of porphyrinogens to VP lymphoblast sonicates

Addition of proto- and coproporphyrinogen to VP lymphoblast sonicates (figures 5.13 and 5.14, table 5.9) resulted in an exaggeration of the kinetic changes already present. Once again protoporphyrinogen appeared to have a greater effect than coproporphyrinogen. V_{max} values were not decreased further than the decreased levels induced by addition of equivalent amounts of proto- or coproporphyrinogen to control lymphoblasts. Similarly, the Hill coefficients never tended to a value greater than 4 in any of the cases examined.

As in the control lymphoblasts, uroporphyrinogen failed to exert any additional effect on VP PBG deaminase kinetic behaviour (figure 5.15 and table 5.9).

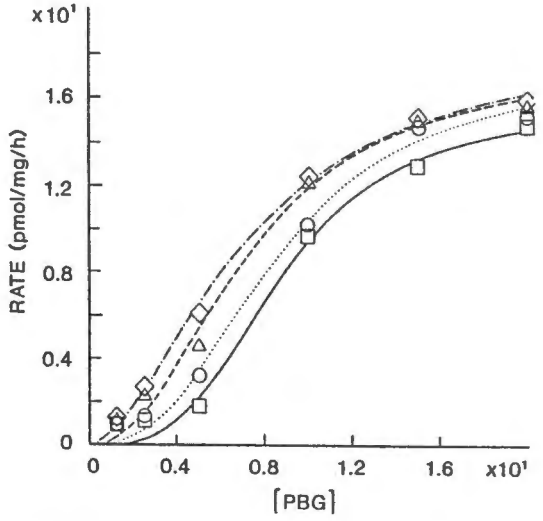

Figure 5.13: *Substrate-velocity plots of PBG deaminase in VP lymphoblast sonicates in the presence of 1(△–––△), 5(○·····○) and 10μM(□——□) protoporphyrinogen. Each data point on each curve represents the mean of observations carried out in 12 VP lymphoblast preparations. The curve obtained with no additions is included for reference (◇–·–·–◇). The kinetic alterations observed in VP lymphoblasts were further enhanced by addition of protoporphyrinogen.*

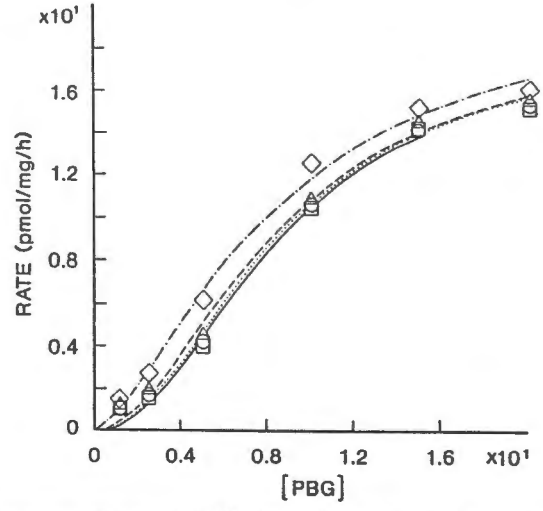

Figure 5.14: *Substrate-velocity plots of PBG deaminase in VP lymphoblast sonicates in the presence of 1(△– –△), 5(○···○) and 10μM(□—□) coproporphyrinogen. Each data point on each curve represents the mean of observations carried out in 12 VP lymphoblast preparations. The curve obtained with no additions is included for reference (◇–◇). Kinetic values in table 5.9.*

Table 5.9: *VP PBG deaminase kinetic parameters in the absence and in the presence of 1, 5 and 10μM proto-, copro- and uroporphyrinogen. Kinetic anomalies already present in the VP lymphoblasts were further enhanced by addition of*

	V_{max} (pmol/mg/h)	$K_{0.5}$ (μM)	Hill coefficient
VP lymphoblast			
No additions	21.2 ±2.0	7.4 ±0.7	1.78 ±0.17
+1μM Proto'gen	17.7 ±1.8	7.2 ±1.0	2.25 ±0.40
+5μM Proto'gen	17.3 ±1.5	8.5 ±1.2	2.64 ±0.49
+10μM Proto'gen	16.6 ±1.3	8.7 ±1.1	3.22 ±0.42
+1μM Copro'gen	19.0 ±1.5	8.5 ±1.1	1.88 ±0.31
+5μM Copro'gen	18.5 ±2.0	7.9 ±2.2	2.01 ±0.33
+10μM Copro'gen	18.3 ±1.8	8.9 ±1.4	2.05 ±0.31
+1μM Uro'gen	20.4 ±1.4	7.5 ±1.6	1.75 ±0.19
+5μM Uro'gen	19.5 ±1.3	9.4 ±1.8	1.75 ±0.18
+10μM Uro'gen	21.3 ±2.2	7.6 ±0.	1.83 ±0.20

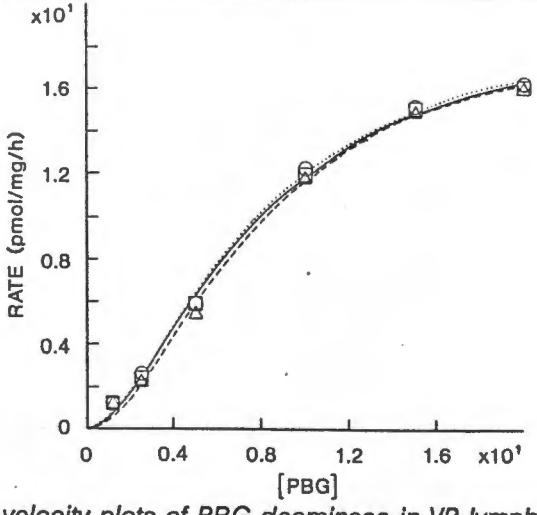

Figure 5.15: *Substrate-velocity plots of PBG deaminase in VP lymphoblast sonicates after 5min incubation in the presence of 1(□—□), 5(△- - -△) and 10μM(○····○) uroporphyrinogen. These curves were not significantly different to those obtained from VP lymphoblasts in the absence of uroporphyrinogen additions. Kinetic values corresponding to these curves are given in table 5.9.*

Sephadex G25 chromatography

Sephadex G25 allowed separation of fractions containing PBG deaminase activity and those containing porphyrins (and presumably porphyrinogens) (see *Methods*, figure 5.2). Application of control lymphoblast preparations to this column resulted in the same kinetic profile before and after Sephadex G25 chromatography (data not shown). In striking contrast, application of VP lymphoblast preparations to this column resulted in fractions containing PBG deaminase activity which after pooling and concentrating displayed kinetic characteristics identical to those of control lymphoblasts (figure 5.16 and table 5.10).

Oxidation of intracellular porphyrinogens

The results of an experiment designed to convert any endogenous porphyrinogens to their corresponding porphyrin forms and thus to restore normal kinetic behaviour to VP lymphoblast PBG deaminase are summarised in table 5.11. The kinetic profile of VP PBG deaminase was unchanged after exposure to oxidising conditions for 2h.

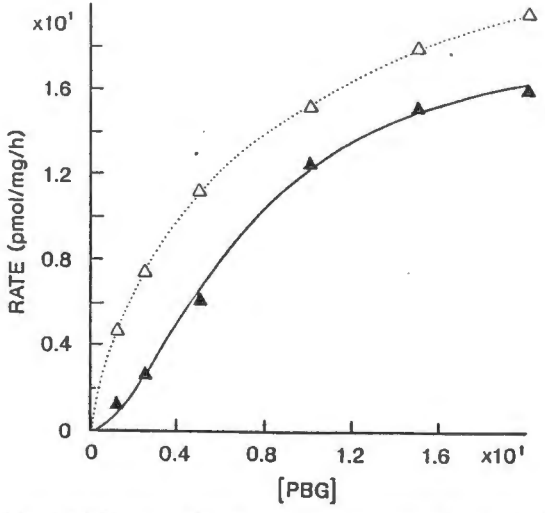

Figure 5.16: *VP lymphoblast PBG deaminase substrate-velocity plots before (▲——▲) and after (△·····△) Sephadex G25 chromatography. "Normal" kinetic behaviour was apparently restored to PBG deaminase.*

Table 5.10: *Kinetic parameters for VP lymphoblast PBG deaminase before and after Sephadex G25 chromatography. All parameters as determined by the Hill*

	V_{max} (pmol/mg/h)	$K_{0.5}$ (μM)	Hill coefficient
VP: Pre-Sephadex	19.9 ±1.9	7.9 ±1.2	1.78 ±0.21
VP: Post-Sephadex	30.8 ±1.6	6.7 ±2.9	0.82 ±0.33

Table 5.11: *Effect of 2h of oxidising conditions on VP lymphoblast PBG deaminase. Kinetic values determined by the Hill equation appeared unaffected by this treatment. The data quoted represents the mean of 4 separate experiments using 4 different VP cell lines.*

	V_{max} (pmol/mg/h)	$K_{0.5}$ (μM)	Hill coefficient
VP: Pre-oxidation	21.0 ±1.7	7.3 ±1.5	1.84 ±0.30
VP: Post-oxidation	20.1 ±1.6	7.5 ±1.6	1.79 ±0.29

Discussion

Validity of methods

Substrate-velocity data for PBG deaminase were obtained by measuring the rate of formation of uroporphyrinogen at 5 substrate concentrations. Ideally these concentrations should fall both above and below the K_m for the enzyme (Van Holde, 1971) so as to cover a wide range of the V_{max}. The literature reports K_m values ranging between 6 and 22μM (Sancovich *et al*, 1969; Sassa *et al*, 1978; Anderson

and Desnick, 1980, 1982; Fumagalli, 1985) and our data indicated a K_m (or the equivalent $K_{0.5}$ using Hill analysis) of approximately 8μM for lymphoblast PBG deaminase originating from healthy control subjects. Thus the choice of PBG concentrations of 1.25, 2.5, 5, 10 and 20μM appears to be justified since these substrate concentrations cover a range from 13% to 73% of V_{max}.

Analysis of the data obtained in control lymphoblasts by the Michaelis-Menten equation yielded a V_{max} of 25.2 ±1.7 pmol/mg/h and a K_m of 8.1 ±0.7 μM. There are only two previous reports of the measurement of PBG deaminase activity in lymphoblasts. One (Anderson and Desnick, 1982) gives an activity of 11pmol/mg protein/h for human lymphoblast lines but it is unclear whether these were Epstein-Barr virus-transformed lymphoblast lines, and another (Sassa *et al*, 1978) 40 and 68pmol/mg protein/h for PHA or PWM stimulated lymphocytes and Epstein-Barr virus transformed lymphoblasts respectively. It must be noted that reported erythrocyte PBG deaminase activities show a large range which underscores the need to establish control values for each individual laboratory and method (Kreimer-Birnbaum *et al*, 1980). Our control lymphoblast results appear to be of the same order as those in other reports and were thus considered a valid standard for the purposes of this study.

We were concerned that addition of exogenous porphyrins to the assay system would result in inaccuracies in the "standard" PBG deaminase assay. We therefore examined this possibility by quantifying all porphyrins, including reaction product, with an analytical TLC technique. This enabled us to gauge precisely the types and quantities of porphyrin present at time zero and at the completion of the assay reaction. The rate of uroporphyrin production determined using the "standard" assay (26.2 ±4.1 pmol/mg protein/h) agreed favourably with that obtained by the TLC approach (28.0 ±2.1 pmol/mg protein/h) although it gave marginally less consistent results (SD = 4.1 as opposed to 2.1 using the TLC approach). It therefore appeared satisfactory to continue using the "standard" PBG deaminase assay method. (Note, however, that we did dilute aliquots of the assay mixture tenfold prior to fluorometric determination to reduce any possible quenching.)

The stability of the porphyrinogens used in the study was also an area of concern. Had significant auto-oxidation occurred during the pre-incubation and assay periods it would have been difficult to ascribe any change in PBG deaminase behaviour to the presence of porphyrinogen. Fortunately all 3 porphyrinogens used displayed less than 6% re-oxidation over 1h under conditions of the PBG deaminase assay (figure 5.1). Protoporphyrinogen was the most labile and approximately 18% had re-oxidised by 2h. This agrees with the experience of other workers (Brenner and Bloomer, 1880[b]; Jacobs and Jacobs, 1982; Personal communication, Camadro JP).

To assess whether there was any appreciable degradation or inter-conversion of porphyrins as a result of the reduction process, we assessed the recovery rate following chemical re-oxidation at 2h. After iodine treatment there was virtually complete recovery of the original fluorescence of uro- or coproporphyrin and approximately 90% that of protoporphyrin (figure 5.1). The slight loss of protoporphyrin might result from reduction of the vinyl side-chains by sodium amalgam (Fuhrhop and Smith, 1975) or by iodine attack on the vinyl groups (Sano and Granick, 1961). However, we were satisfied that virtually complete reduction of the porphyrins could be achieved without damage to the porphyrin side-chains of copro- and uroporphyrin, and little, if any, to those of protoporphyrin.

Differences in kinetic behaviour of VP and control lymphoblast PBG deaminase

Comparison of kinetic behaviour of VP lymphoblast PBG deaminase with that of control lymphoblasts yielded the unprecedented observation that whereas normal (control) PBG deaminase exhibited kinetic properties associated with a hyperbolic substrate-velocity curve involving enzyme substrate interaction at a single ligand-binding site, VP PBG deaminase did not.

While the mechanism of action of PBG deaminase remains a subject of research activity and debate (Jordan and Berry, 1981; Jordan and Warren, 1987; Battersby, 1988; Scott *et al*, 1988. See Chapter 1), it is

generally assumed that in most cases the appearance and rate of production of uroporphyrinogen from PBG by the enzyme follows the Michaelis-Menten rate law. There are, however, reports of non-Michaelian behaviour from one group (Sancovich *et al*, 1969; Llambias and Batlle, 1970; Fumagalli *et al*, 1985. See Chapter 3).

In our study, in impure preparations, PBG deaminase from *control* lymphoblasts exhibited classic Michaelis-Menten behaviour. This was borne out by the linearity of the Lineweaver-Burk plot (1/Rate versus 1/[Substrate]) and the Eadie plot (Rate/[Substrate] versus Rate) (figure 5.3).

The Hill equation (Von Holde, 1971 and Appendix 10) can also be used for determining kinetic data. Under conditions where there is a hyperbolic substrate-velocity relationship, the Hill coefficient approaches 1 and should yield V_{max} and $K_{0.5}$ values equivalent to the Michaelis-Menten V_{max} and K_m. This was the case for control lymphoblast PBG deaminase.

The Hill equation is typically used to analyse the number of ligand-binding sites and their interdependence of macromolecular species such as proteins. The macromolecule as a whole may be either multimeric or monomeric, but with more than one possible ligand-binding site. A Hill coefficient close to 1 (in this case 0.83) implies that the enzyme has only one binding site or that multiple sites, if present, are identical and that the binding at any site is independent of the occupation of other sites. It is generally accepted that PBG deaminase exists in monomeric form and, that there is only one substrate (ligand)-binding site (Sassa *et al*, 1978; Anderson and Desnick, 1979; Jordan and Berry, 1981. See Chapter 3). However, closer kinetic examination of the pure enzyme in one case (Llambias and Batlle, 1970; Fumagalli *et al*, 1985) suggests that there may be two.

The substrate-velocity data for *VP* lymphoblast PBG deaminase does not fit the Michaelis-Menten equation (figure 5.4). The most striking difference was a *sigmoidal* substrate-velocity plot. However, the data fitted the Hill equation well.

Using the Hill equation, V_{max} in the VP cells gave a mean value of 21.2 ±2.0 pmol/mg protein/h, a significant 26% decrease over that of control cells (table 5.3). This is consistent with our earlier observation of decreased PBG deaminase activity in VP lymphoblasts when we used a single saturating substrate concentration for the assay (figure 4.11). Comparison of the $K_{0.5}$ values obtained in the VP and control groups showed no significant alterations. This implies that there was no major difference in the thermodynamics of ligand binding by PBG deaminase in the two groups. It is thus unlikely that the different behaviour of the two groups is due to a major structural defect.

A sigmoidal substrate-velocity curve implies the presence of more than one cooperative ligand-binding site. The substrate-velocity plot of VP lymphoblast PBG deaminase yielded a Hill coefficient of 1.78 ±0.17 (*ie.*<2). The data thus suggest that PBG deaminase is converted from a form displaying independent substrate binding in the control lymphoblasts to one which displays multiple cooperative binding sites in the VP lymphoblasts. In this case, there appears to be two interacting substrate-binding sites. Furthermore, this form of PBG deaminase exhibits a reduced V_{max}.

These findings may support the suggestion that monomeric PBG deaminase possesses more than one substrate-binding site only one of which is available under normal circumstances for binding, while the others are blocked either by some controlling factor or by conformational exclusion. Alternatively PBG deaminase may possess only one ligand-binding site, but in VP a number of PBG deaminase monomers are somehow brought into close proximity to form a cooperative multimeric structure with decreased catalytic ability.

Addition of porphyrinogens to control lymphoblast PBG deaminase preparations

In contrast to the various porphyrins which failed to influence PBG deaminase kinetics, addition of proto- and coproporphyrinogen to control lymphoblasts produced a marked alteration in the PBG deaminase substrate-velocity behaviour. Indeed, addition of proto-

and coproporphyrinogen resulted in PBG deaminase kinetics which closely resemble those observed in "native" VP lymphoblasts.

1. Addition of protoporphyrinogen

Figure 5.8 illustrates both a decreased V_{max} and a change in the form of the substrate-velocity plot to a sigmoidal form when protoporphyrinogen was added. V_{max} was decreased most by 10μM protoporphyrinogen and least by 1μM protoporphyrinogen (table 5.5). Although there was an inverse relationship between V_{max} and protoporphyrinogen concentration this did not appear to be directly proportional. Possibly the effect is already maximal at a concentration of 5 or 10μM, or the impurity of PBG deaminase in the lymphoblast preparations is such that small trends in activity are not accurate reflections on the true behaviour.

Secondly, there was a uniform increase in the Hill coefficient which ranged between 2.89 and 3.61. This implied that, in response to additional protoporphyrinogen, PBG deaminase exhibits up to four ligand-binding sites, and not just the two observed in "native" VP lymphoblasts. Either protoporphyrinogen mediates the aggregation of up to four monomeric units, or that of alternative schemes such as two dimeric units, or it in some way removes blocking factors present on other, hitherto unsuspected, binding sites.

Thirdly, addition of protoporphyrinogen resulted in a small, although statistically insignificant, trend in $K_{0.5}$, its value increasing with an increase in the concentration of protoporphyrinogen. As in VP PBG deaminase this indicates no major change to the overall thermodynamics of ligand-binding by PBG deaminase.

2. Addition of coproporphyrinogen

Addition of coproporphyrinogen to control lymphoblast preparations produced effects similar to those observed after addition of protoporphyrinogen. The changes in shape of the substrate-velocity curve and the diminution V_{max} activity were less marked than those

produced by protoporphyrinogen. For 1 and 5μM coproporphyrinogen the Hill coefficient approached 2 and for 10μM coproporphyrinogen, 3. Presumably coproporphyrinogen influenced PBG deaminase in the same way as protoporphyrinogen, but is less "potent" an inhibitor. This may be due to the difference in side-group composition.

3. Addition of uroporphyrinogen

Thus far the data supports the thesis that proto- and coproporphyrinogen, presumably present in higher intracellular concentrations in VP lymphoblasts than in control lymphoblasts, alter PBG deaminase in such a way that there is a partial inhibition of activity and the appearance of cooperativity with multiple substrate-binding sites. Further support of this thesis is lent by the failure of uroporphyrinogen to alter the kinetic behaviour of PBG deaminase. Though it is highly likely that intracellular concentrations of uroporphyrinogen are elevated in PCT, there is no evidence to suggest altered PBG deaminase functioning; ALA and PBG concentrations are never elevated in PCT and certainly no acute attacks have ever been described for this condition. The lack of effect of uroporphyrinogen on PBG deaminase may explain this (figure 5.11 and table 5.7).

Addition of porphyrinogens and porphyrins to VP lymphoblast PBG deaminase preparations

Analysis of PBG deaminase substrate-velocity data in VP lymphoblast sonicates by the Hill equation yielded Hill coefficients (table 5.3). Greater degrees of change were induced in control lymphoblasts to which 1, 5 and 10μM concentrations of proto- and coproporphyrinogen were added. It should therefore be possible to enhance the anomalous behaviour of VP lymphoblast PBG deaminase by addition of further proto- and coproporphyrinogen. Similarly, the addition of uroporphyrinogen, proto-, copro- and uroporphyrin would be expected to be without effect. This proved to be true.

1. Addition of protoporphyrinogen

Addition of 1, 5 and 10μM protoporphyrinogen to VP lymphoblast preparations did indeed enhance the kinetic changes already noted (figure 5.13). The data obtained (table 5.9) indicated a similar minimum level of PBG deaminase activity and similar $K_{0.5}$ values to those observed in control lymphoblasts to which proto- or coproporphyrinogen were added. The Hill coefficients, however, differed. 1 and 5μM protoporphyrinogen yielded Hill coefficients between 2 and 3, and 10μM protoporphyrinogen gave a Hill coefficient of 3.22 (compare to table 5.5). The Hill coefficients in treated VP lymphoblasts never exceeded 4. Thus the addition of protoporphyrinogen to VP lymphoblasts indeed appeared to aggravate an existing alteration in the behaviour of PBG deaminase.

2. Addition of coproporphyrinogen

Addition of coproporphyrinogen to VP lymphoblast preparations resulted in changes similar to those described above (figure 5.14). However, the coproporphyrinogen data differ from the addition of protoporphyrinogen in one respect. Whereas protoporphyrinogen induced increasing degrees of enhancement with increasing concentration, 1, 5 and 10μM coproporphyrinogen all produced a very similar degree of enhancement (figure 5.14 and table 5.9). It thus appeared that the kinetic changes induced in VP lymphoblasts by coproporphyrinogen were already at a maximum when 1μM additional coproporphyrinogen was present. This was not so in control lymphoblast PBG deaminase.

3. Addition of uroporphyrinogen and porphyrins

Uroporphyrinogen, like the porphyrins, failed to influence the kinetics of PBG deaminase in VP lymphoblasts (figure 5.15, table 5.9 and figure 5.12, table 5.8). This was consistent with the finding in control lymphoblasts.

Further comment on the data thus far discussed

Decreasing the number of carboxyl groups on the porphyrinogen appeared to enhance the effect of the porphyrinogen on PBG deaminase: Uroporphyrinogen had no effect, while the effect of coproporphyrinogen was less than that of protoporphyrinogen. This suggests that steric and/or hydrophobic effects may be import in the phenomenon observed. Secondly, the lack of influence of porphyrins on PBG deaminase may be due to the strongly aromatic nature of these compounds, in contrast to the unconjugated macrocyclic ring system of the porphyrinogens. In the porphyrins the conjugated π-electron systems lie above and below the whole tetrapyrrolic macrocycle and may shield the porphyrin from potential interaction with the enzyme molecule. The porphyrinogens, on the other hand are aliphatic and the aromaticity is confined to the individual pyrrolic "corners" (Bonnett, 1981). This means that the interpyrrolic methylene groups are more accessible.

These data give no direct clue as to the nature of the interaction between proto- and coproporphyrinogen and PBG deaminase. It is thus only possible to theorise: Presumably the binding may involve hydrogen bonding, hydrophobic interaction or covalent attachment. The maximum of 4 possible ligand-binding sites would imply that up to 4 molecules of PBG deaminase (assuming PBG deaminase to possess a single substrate-binding site) may be brought together by the porphyrinogen. This may result in a randomly stacked complex or perhaps an ordered complex arranged around a single molecule of porphyrinogen. Thus the porphyrinogen, being a *tetra*pyrrole, may possess 4 similar areas of potential interaction and may therefore bind 4 molecules of PBG deaminase, resulting in a Hill coefficient, $n<4$ However, a random stacking arrangement might allow a larger number than 4 PBG deaminase molecules to stack together in a multimeric, or even polymeric, unit. The Hill data suggest that this does not happen, possibly because of thermodynamic instability, or because the association is not via a random stacking.

Furthermore, PBG deaminase requires a covalently bound dipyrromethane cofactor (Jordan and warren, 1987; Scott *et al*, 1988)

which facilitates binding of substrate (PBG) and directs synthesis of the linear tetrapyrrole hydroxymethylbilane. The porphyrinogens and dipyrromethane exhibit structural similarity; in very broad terms 1 porphyrinogen = 2X dipyrromethane. This may lead to the PBG deaminase binding to an analogous structural area in the porphyrinogens rather than to the dipyrromethane cofactor. There are, however, several objections to this speculation. The side groups possessed by dipyrromethane are exactly analogous to those on uroporphyrinogen, and less to those on copro- or protoporphyrinogen (table 1.1 and figure 1.5). Yet uroporphyrinogen has no effect on PBG deaminase. It has also been suggested that the dipyrromethane cofactor binds before folding of the PBG deaminase polypeptide takes place, and that it remains resident for "life" (Scott *et al*, 1988). It must therefore be stressed that the above suggestion is purely speculative.

That elevated intracellular concentrations of proto- and coproporphyrinogen lead to altered behaviour of PBG deaminase appears therefore to be true, at least in the lymphoblast system used in this study. This would be further supported if the removal of protoporphyrinogen and/or coproporphyrinogen from VP lymphoblasts resulted in restoration of normal function to PBG deaminase.

Removal of porphyrin(ogen)s from VP lymphoblasts restored a normal kinetic profile to PBG deaminase

One method of "removing" porphyrinogens is by their conversion to the corresponding porphyrins, which do not inhibit PBG deaminase. However, when VP lymphoblast sonicates were treated in oxidising conditions (light, atmospheric oxygen) for up to 2h prior to the assay, there was no significant change in the substrate-velocity behaviour or V_{max} (table 5.11), even though in a separate experiment, it was shown that approximately 100μM proto- copro- and uroporphyrinogen could be almost fully oxidised under identical conditions (table 5.2). There are several possible explanations: PBG deaminase might be irreversibly affected by the oxidation, the porphyrinogens interacting

with PBG deaminase were "locked" into position, 2h was not long enough for the effect to be reversed, or the observed phenomenon was not due to porphyrinogens at all.

Thus a different approach was taken. VP lymphoblast cytosolic preparations were subjected to molecular sieving by Sephadex G25 chromatography. This was intended to remove all porphyrin(ogen)s from the solution containing PBG deaminase. This might allow for the dissolution of any bound porphyrinogens from PBG deaminase because of the resultant negative equilibrium. In a pilot experiment it was established that fractions containing PBG deaminase activity and those containing porphyrins were well separated by Sephadex G25 chromatography (figure 5.2). It was assumed that porphyrinogens would be equally well separated. Single passage of VP lymphoblast cytosolic preparations down a Sephadex G25 column resulted in a restoration of "normal" kinetic behaviour to PBG deaminase. Although it was possible that Sephadex G25 had removed some other factor(s) responsible for alteration of PBG deaminase behaviour the removal of protoporphyrinogen and, possibly coproporphyrinogen, appeared the most likely cause as previous experiments had shown them to be directly implicated in the alteration of PBG deaminase kinetic behaviour.

Conclusions

- ☐ *VP lymphoblast PBG deaminase exhibits different kinetic properties to that of normal lymphoblast PBG deaminase.*

The kinetic profile of control lymphoblast PBG deaminase is consistent with that of a monomeric Michaelis-Menten type enzyme with a single substrate-binding site whereas that in VP lymphoblasts is consistent with an allosteric, cooperative, multimeric structure.

- *Addition of proto- and coproporphyrinogen in contrast to uroporphyrinogen or any of the porphyrins resulted in a striking transformation of the "normal" kinetics to a kinetic profile resembling that displayed by PBG deaminase in VP lymphoblasts.*

Addition of these porphyrinogens to VP lymphoblasts further enhanced the observed anomalous kinetic behaviour.

- *Sephadex G25 chromatography resulted in restoration of "normal" kinetics to VP lymphoblast preparations.*

This was presumed to be due to removal of proto- and coproporphyrinogen from the VP cells.

The above observations lend considerable support to the hypothesis that the "defect" in PBG deaminase observed in VP is linked to the primary defect of protoporphyrinogen oxidase and that the haem synthetic intermediates likely to accumulate in response to this block, proto- and coproporphyrinogen, may exert a negative allosteric effect on PBG deaminase in VP. This concept is strengthened by the occurrence of acute attacks in HCP, where elevated concentrations of coproporphyrinogen may occur, and the lack of acute attacks in PCT where excessive concentrations of uroporphyrinogen predominate. It may be possible to extrapolate the concept to EPP and CEP as well, but the predominant erythropoietic expression of these diseases could require a different explanation for the lack of acute attacks in these conditions.

A further point of definite interest would be to establish whether the behaviour of *purified* PBG deaminase in the presence of these haem pathway intermediates is similar to that of the unpurified preparations. This is explored in the following chapter.

Chapter Six

Kinetic effects of protoporphyrin(ogen) on purified PBG deaminase

This section sets out to examine whether the inhibition of PBG deaminase by proto- and coproporphyrinogen is due to a direct interaction with the enzyme, or is mediated by an indirect mechanism such as an interaction with some endogenous factor present in the cell? Purified PBG deaminase was used in order to address this question.

Objectives

- ☐ To purify human PBG deaminase.
- ☐ To compare the kinetic profile of purified PBG deaminase with that of unpurified lymphoblast PBG deaminase.
- ☐ To determine whether the addition of proto- or coproporphyrinogen to the pure enzyme would result in similar anomalous behaviour to that observed in lymphoblasts.

This chapter describes the purification of PBG deaminase and the determination of kinetic data as determined in the presence and absence of porphyrinogen or porphyrin.

Methods

Purification of PBG deaminase

There are a number of reported methods for the purification of PBG deaminase (Sancovich *et al*, 1969; Frydman and Frydman, 1970; Davies and Neuberger, 1973; Jordan and Shemin, 1973; Higuchi and Bogorad, 1975; Miyagi *et al*, 1979; Anderson and Desnick, 1980; Fumagalli *et al*, 1985). Many use erythrocytes as a convenient source of the enzyme. Since there appears to be good inter-tissue homology of PBG deaminase we chose to use erythrocyte PBG deaminase in our study.

The purification procedure was adapted from two previous methods (Anderson and Desnick, 1980; Fumagalli *et al*, 1985). Briefly, erythrocytes were lysed and haemoglobin was removed by specific denaturation. The cytosol was subjected to ammonium sulphate fractionation and heat treatment, Sephadex G25 and G100 chromatography, DEAE cellulose chromatography, chromatofocusing over a 7 to 4 pH gradient and finally hydrophobic interaction chromatography on a phenyl-Sepharose column. The method is outlined in figure 6.1 and full detail is given in Appendix 11 together with related methodologies. Activity was followed throughout the purification by assaying 100μl aliquots of sample for PBG deaminase activity as described in Appendix 9. Protein concentrations were determined using the "Bradford" technique of protein-dye binding (Bradford, 1976 and Appendix 12).

Addition of porphyrinogen and porphyrin

Substrate-velocity data were obtained over a 1.25-20μM PBG concentration range (as used in the previous experiments), at protoporphyrinogen concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5 and 10μM. Protoporphyrin was assessed for its effects in 0.1, 1 and 10μM concentrations.

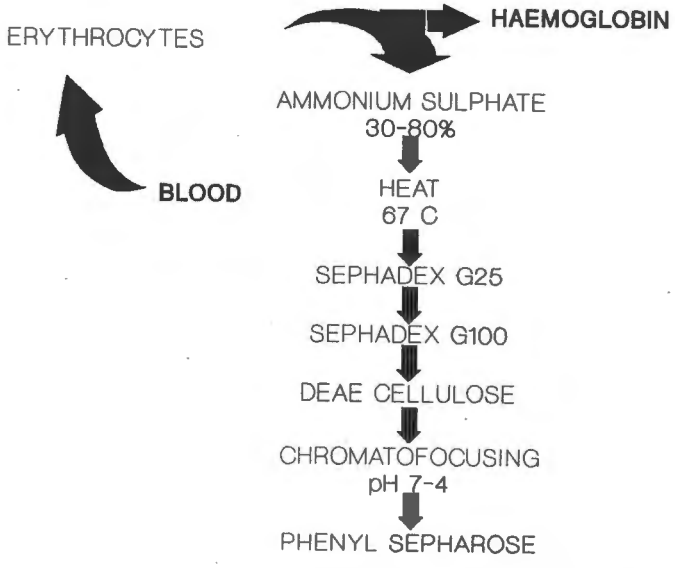

Figure 6.1: *Main steps of the purification procedure for PBG deaminase. Using this procedure 460ml of blood yielded approximately 100μg of PBG deaminase purified 7115-fold.*

The activity of PBG deaminase in the presence of added protoporphyrinogen or protoporphyrin was assayed at a single, substrate saturating concentration using the methods described previously (see Chapter 4 and Chapter 5, *Methods* and Appendix 9). All enzyme assays were performed in duplicate.

Kinetic analysis

All substrate-velocity data shown below represents the mean of three experiments. All substrate-velocity data were analysed with the Hill equation (see Chapter 5, *Methods* and Appendix 10) and V_{max}, $K_{0.5}$ and the Hill coefficient values were obtained for comparative purposes.

Results

Purification of PBG deaminase

Table 6.1 summarises the purification of human erythrocyte PBG deaminase from 460ml of blood. The enzyme was purified approximately 7000-fold.

The elution traces (protein absorbance at 280nm and corresponding PBG deaminase activity) for the Sephadex G100 and chromatofocusing runs are shown in figures 6.2 and 6.3. The peak of activity for the final phenyl-Sepharose was sharp and well defined, but a reliable continuous protein absorbance trace was not obtained since the ethylene glycol in which it was run interfered with this. Protein concentrations were measured only in the regions containing PBG deaminase activity. An SDS-PAGE of an aliquot of the final post-phenyl Sepharose PBG deaminase fractions (after concentration and exchange into 0.05M Tris-HCl buffer, pH 7.4) is shown in figure 6.4. The clear single protein band obtained with silver staining was estimated to have a molecular weight of 41200 (±2300).

This solution (pure PBG deaminase in Tris-HCl buffer) served as the source of PBG deaminase for all subsequent kinetic experiments.

Table 6.1: *Purification of PBG deaminase from 190ml of human erythrocytes. Protein concentration, mg/ml; Volume, ml; Specific activity, nmol of uroporphyrin fromed/mg protein/h; Purification, -fold.*

Step	[Protein]	Volume	Specific activity	Purification
1. Erythrocytes	231	190	0.034	1
2. Supernatant from dialysis	22	90	0.94	28
3. Post Sephadex G25	17	106	1.11	33
4. Post Sephadex G100	38.5	22.5	1.20	35
5. Post DEAE cellulose	0.88	7.5	20.80	614
6. Post chromatofocusing	0.058	6	150.00	4425
7. Post phenyl Sepharose	0.018	4	241.20	7115

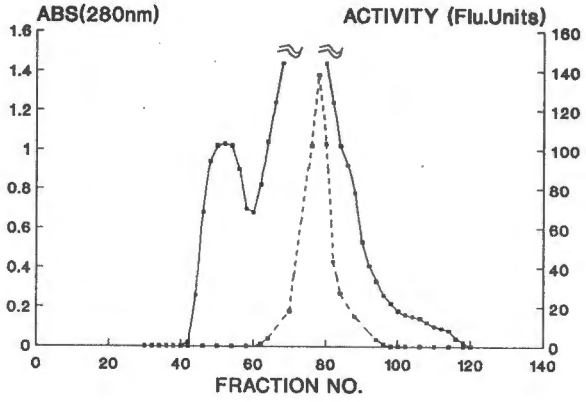

Figure 6.2: *Elution profile from Sephadex G100 column (2.5X100cm), the second of the chromatography steps in the purification procedure followed. The column was equilibrated and the protein eluted with 0.05M Tris-HCl, pH7.4. The dashed peak corresponds to PBG deaminase activity and is expressed as arbitrary fluorescence units/50µl aliquot of the fractions tested. The main protein peak has been broken at the points marked (~). PBG deaminase eluted from this column represented an approximate 35-fold purification.*

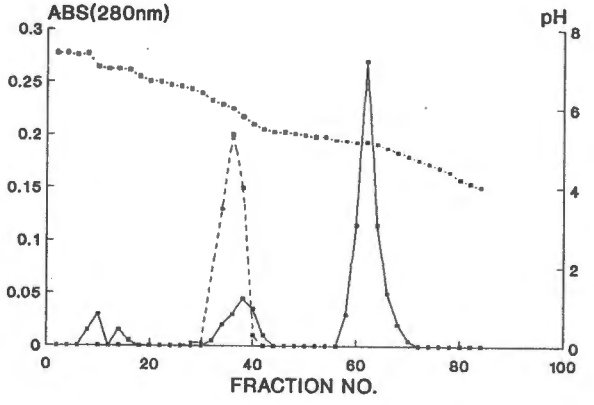

Figure 6.3: *Elution profile from chromatofocusing on PBE 94 column (0.9X30cm). The column was equilibrated with 0.025M imidazole-HCl, pH7.4 and the protein eluted with a 7.4 to 4 pH gradient using "Polybuffer 74". The dashed peak corresponds to PBG deaminase activity and is expressed as arbitrary fluorescence units/50µl aliquot of the fractions tested (axis not shown). The pH gradient is also shown. PBG deaminase eluted from this column represented an approximate 4400-fold purification.*

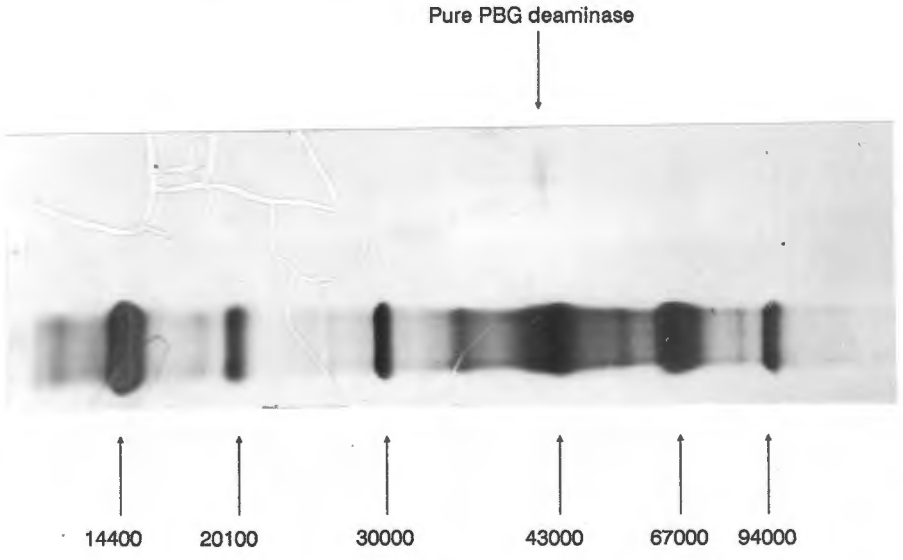

Figure 6.4: *SDS-PAGE of PBG deaminase post phenyl-Sepharose stained with silver stain. PBG deaminase appeared as a single protein band of molecular weight 41200 (±2300) when compared to molecular weight markers indicated below. Molecular weights are given in Daltons.*

Kinetics of pure PBG deaminase

A simple substrate-velocity plot (figure 6.5) of pure PBG deaminase yielded a hyperbolic curve with a V_{max} of 249 (±36) nmol/mg/h, a $K_{0.5}$ of 8.9 (±1.5) μM and a Hill coefficient of 0.93 (±0.14). Lineweaver-Burk and Eadie plots yielded linear plots.

Addition of 0.1, 1 and 10μM concentrations of protoporphyrin had no effect on pure PBG deaminase. This is illustrated by the substrate-velocity plots in figure 6.6 and the kinetic data given in table 6.2. Remarkably consistent curves were obtained at all protoporphyrin concentrations.

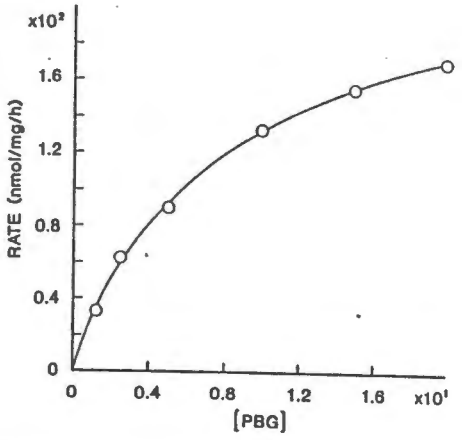

Figure 6.5: *Hyperbolic behaviour of substrate-velocity data of pure PBG deaminase. Data points represent the mean of three experiments.*

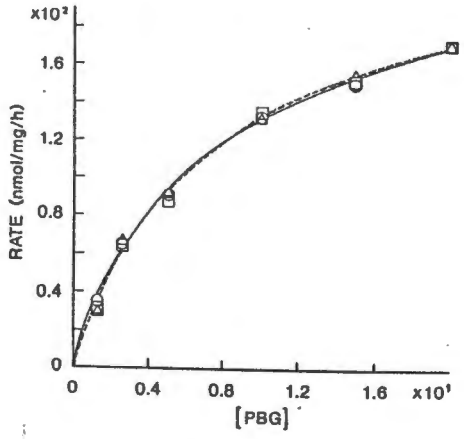

Figure 6.6: *Addition of 0.1, 1 and 10μM protoporphyrin had no effect on the kinetics of purified PBG deaminase.*

Table 6.2: *Addition of protoporphyrin had no effect on the kinetics as ascertained by application of the Hill equation. Figures represent the mean of three experiments in each case. Proto, protoporphyrin.*

	V_{max} *(nmol/mg/h)*	$K_{0.5}$ *(μM)*	Hill coefficient
Purified control PBG deaminase			
No additions	249 ±36	8.9 ±1.5	0.93 ±0.14
+0.1μM Proto	264 ±48	8.2 ±1.1	0.85 ±0.12
+1μM Proto	246 ±44	8.6 ±1.2	0.95 ±0.16
+10μM Proto	235 ±41	8.1 ±1.0	0.96 ±0.17

Addition of protoporphyrinogen over a 1000-fold range of concentrations (0.01 to 10μM) resulted in the striking transformation of the hyperbolic substrate-velocity curve obtained in the absence of protoporphyrinogen to sigmoidal curves similar to those displayed by impure PBG deaminase in the presence of 1, 5 and 10μM proto- and

coproporphyrinogen (figure 6.7, table 6.3). 0.01μM protoporphyrinogen had a negligible effect on pure PBG deaminase. There was an inverse relationship between V_{max} and the protoporphyrinogen concentration, the lowest V_{max} values being recorded at the highest protoporphyrinogen concentration. V_{max} in the presence of 10μM protoporphyrinogen was 169 (±28) nmol/mg/h, which was 32% lower than that in the absence of protoporphyrinogen. The Hill coefficients appeared to approach 4 in the limit and $K_{0.5}$ was essentially unaltered. This was unlike the behaviour of impure PBG deaminase where there was a small non-significant systematic variation.

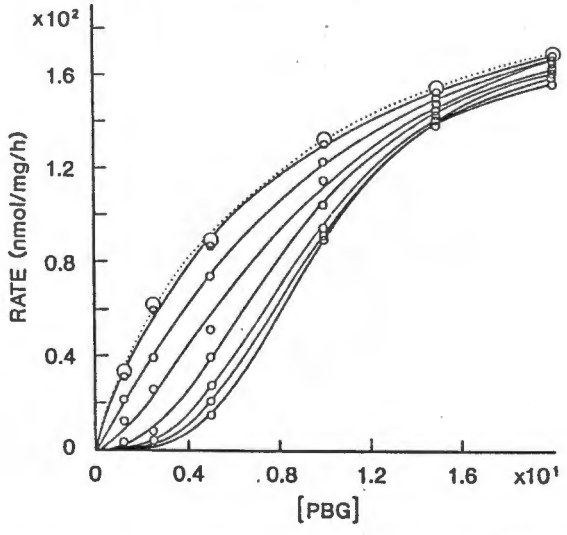

Figure 6.7: *Addition of 0.01 to 10μM protoporphyrinogen to pure PBG deaminase resulted in sigmoidal substrate-velocity plots with decreased V_{max} activity and a Hill coefficient that approached 4 in the limit.*

A plot of V_{max} and the Hill coefficient versus protoporphyrinogen concentration (figure 6.8) shows a single exponential growth relationship in the case of the Hill coefficient and a single exponential decay for V_{max}. Single exponential analysis reveals that the protoporphyrinogen concentration at which the Hill coefficient is half its estimated maximum is 0.2μM and the concentration at which V_{max} is half its estimated maximum inhibition is 0.19μM.

Table 6.3: *Addition of various concentrations of protoporphyrinogen resulted in altered PBG deaminase kinetics as ascertained by application of the Hill equation. Figures represent the mean of three identical experiments in each case. Proto'gen, protoporphyrinogen.*

	V_{max} *(nmol/mg/h)*	$K_{0.5}$ *(μM)*	**Hill coeficicient**
Purified control PBG deaminase			
No additions	249 ±36	8.9 ±1.5	0.93 ±0.14
+0.01μM Proto'gen	246 ±37	8.9 ±1.0	0.96 ±0.09
+0.05μM Proto'gen	235 ±42	9.5 ±1.5	1.19 ±0.14
+0.10μM Proto'gen	221 ±40	9.8 ±1.1	1.58 ±0.20
+0.50μM Proto'gen	190 ±48	9.1 ±1.9	2.27 ±0.16
+1.00μM Proto'gen	186 ±46	9.7 ±1.6	2.66 ±0.16
+5.00μM Proto'gen	178 ±39	9.7 ±1.4	3.02 ±0.11
+10.0μM Proto'gen	169 ±28	9.7 ±1.6	3.49 ±0.19

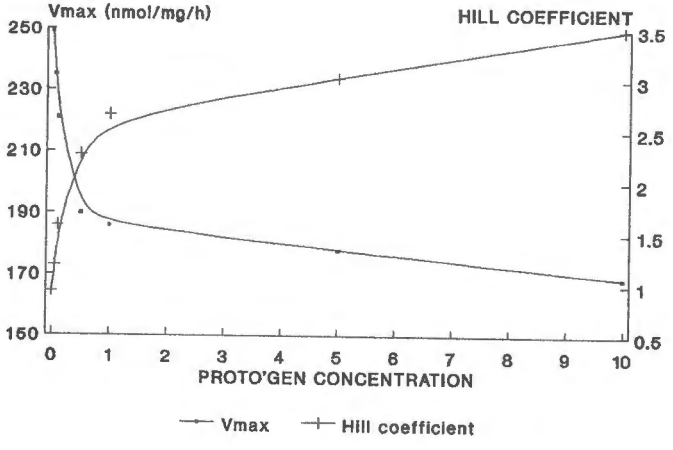

Figure 6.8: *Relationship between added protoporphyrinogen (μM) and V_{max} and the Hill coefficient.*

Discussion

Our purification procedure, judged by silver staining of SDS-PAGE, yielded apparently homogeneous PBG deaminase (figure 6.4). The final specific activity of 241nmol uroporphyrin formed/mg/h is intermediate between the approximately 2300nmol/mg/h (Anderson and Desnick, 1980) and the 68nmol/mg/h (Fumagalli *et al*, 1985) reported by the two groups on whose methods our method was based. This represented an approximately 7100-fold purification which, again lies between the 3400-fold (Fumagalli *et al*, 1985) and 42000-fold (Anderson and Desnick, 1980) reported by the two methods followed.

The product ran on SDS-PAGE as a single unit of molecular weight 41200 (±2300). We did not detect the five distinct peaks reported to correspond to native enzyme and its four enzyme-substrate intermediates, the mono-, di-, tri- and tetrapyrroles (Anderson and Desnick, 1980). These intermediates are normally observed when a final step based on charge-separation (such as DEAE cellulose chromatography) is employed.

Our purification procedure itself behaved similarly to the other reported methods, except for the DEAE cellulose chromatography where we found that PBG deaminase eluted as a single peak in the latter part of the breakthrough peak, rather than well into the sodium chloride gradient.

Although ethylene-glycol is effective in eluting PBG deaminase from a phenyl-Sepharose column it is difficult to remove (by dialysis) and this resulted in substantial losses. Other methods of elution were tried without much success, hence we continued to employ this method.

The kinetic experiments carried out in the presence of protoporphyrinogen are most illuminating. Firstly, purified PBG deaminase, in the absence of added porphyrinogen, behaved as a Michaelian enzyme with a single substrate binding site (figure 6.6, table 6.2). We could find no evidence that purified erythrocyte PBG deaminase from control subjects possessed more than one binding site in its "native" state.

Secondly, the purified PBG deaminase responded to protoporphyrinogen in a similar fashion to the non-pure lymphoblast PBG deaminase preparation. The substrate-velocity curve became sigmoidal, V_{max} was decreased and the Hill coefficient approached 4 (figure 6.8, table 6.3). Thus, once again, PBG deaminase appeared to be transformed into a cooperatively behaving unit, with up to 4 interacting binding sites. Both V_{max} and the Hill coefficient were maximally affected at 10μM protoporphyrinogen and tenuously affected at 0.01μM. Single exponential analysis of both V_{max} and the Hill coefficient against protoporphyrinogen concentration (figure 6.8) revealed that the concentrations at which the effects were half "maximal" were almost identical. It can therefore be concluded that the property of protoporphyrinogen which causes inhibition of V_{max} appears also to be that which causes the increase in cooperativity.

There was no significant variation in $K_{0.5}$. The "mini trend" apparent in non-pure PBG deaminase preparations was absent. The lack of change in $K_{0.5}$ is strong evidence that protoporphyrinogen exerts a direct effect on PBG deaminase and that the disturbed kinetics in the VP PBG deaminase are not due to a structurally mutant enzyme. This suggests that PBG deaminase is normal in VP subjects, but exhibits anomalous behaviour in the presence of protoporphyrinogen.

Addition of protoporphyrin had no effect on the kinetics of PBG deaminase. This is wholly consistent with the observations in the lymphoblast sonicates. Kinetic experiments have not yet been performed in the presence of copro- and uroporphyrin(ogen), but it is probably fair to extrapolate the findings in the lymphoblasts to the purified enzyme since the effects of porphyrinogen and protoporphyrin are entirely concordant.

Conclusions

- *The kinetic profile of pure erythrocyte PBG deaminase is analogous to that of the non-pure enzyme in lymphoblast sonicates.*

- *Addition of protoporphyrinogen but not of protoporphyrin to pure PBG deaminase results in anomalous kinetics with an inhibited Vmax and apparent cooperativity with respect to substrate binding.*

It appears therefore, invoking Occam's razor*, that neither endogenously occurring blocking or stimulatory agents (other than proto- and coproporphyrinogen), nor complex *in vivo* effector mediated mechanismsn nor the presence of a structurally mutant enzyme are required to explain the non-hyperbolic, cooperative kinetics of VP PBG deaminase. Protoporphyrinogen (and coproporphyrinogen) appear to exert a direct allosteric effect on PBG deaminase which, in their absence, is capable of functioning normally.

* William of Occam's (1280-1349) dictum - *Entia non sunt multiplicanda practer necessitatem*.

Chapter Seven

Urinary ALA and PBG concentrations in VP

The data presented in chapters 4 to 6 appear to favour the hypothesis under examination in this thesis. Firstly, partially diminished PBG deaminase activity has been measured in lymphoblasts and secondly it has been shown that both protoporphyrinogen and, to a lesser extent coproporphyrinogen, exert negative allosteric effects on PBG deaminase, resulting in decreased activity and sigmoidal substrate-velocity behaviour.

If the hypothesis is true it may be argued that VP patients who exhibit elevated concentrations of protoporphyrin, and often coproporphyrin as well, will have decreased PBG deaminase activity and should demonstrate mildly elevated levels of PBG (and possibly ALA) during both the quiescent and acute phases of VP.

Indeed a few authors have noted urinary ALA and PBG concentrations to be mildly elevated in quiescent VP. However, this has attracted little attention (Mustajoki, 1976; Muhlbauer, 1982). We therefore considered it important to examine the concentration of ALA and PBG in the urine of unequivocal cases of quiescent VP and to compare these with those observed in normal subjects.

Objectives

- ☐ To characterise a large group of quiescent VP subjects with respect to urinary ALA and PBG concentrations.

- To confirm the diagnosis of quiescent VP by measuring the levels and patterns of porphyrin excretion in the stool, urine and plasma.

Methods

Porphyrin precursor and porphyrin quantitation

The method used for determination of urinary ALA and PBG is given in Appendix 3.2. This method for quantifying urinary ALA and PBG appeared to be accurate at both high and low concentrations of ALA and PBG (table 7.1). This was verified by measuring standard solutions of known precursor concentration ranging from 0.5 to 1000μmol/l. The lower limit of reliable determination (based on repeat experiments) for ALA lay between 1 and 5μmol/l and that of PBG was approximately 1mol/l. Urine samples subjected to repeated testing over a period of two days showed a 6% variability in ALA concentration and an 8% variability in PBG concentration. Storage of urines (at 4°C, in the dark) for a period not exceeding two days thus appeared acceptable.

Table 7.1: *Comparison of standard ALA and PBG concentrations to measured concentrations using "ALA Test kits" (Bio-Rad). IRR: Irreproducible.*

ALA concentration (μmol/l)		PBG concentration (μmol/l)	
Real	**Measured** (Mean of 3)	**Real**	**Measured** (Mean of 3)
0.5	1.2 (IRR)	0.5	0.6
1.0	1.4	1.0	1.1
5.0	6.2	5.0	5.2
10.0	13.3	10.0	10.5
50.0	57.8	50.0	49.1
100.0	105.0	100.0	96.3
500.0	491.0	500.0	486.0
1000.0	950.0	1000.0	911.0

For diagnostic purposes porphyrins were analysed and quantified by the TLC method referred to in Chapter 4 (*Methods*) and described in detail in Appendix 3.1. The method was also assessed for reliability and sensitivity. Stool porphyrins measured on repeated occasions demonstrated a 19% variability in total porphyrin concentration. The lower limit of reproducible sensitivity in stool was approximately 0.5nmol/dry g although concentrations down to 0.1nmol/dry g were detectable. These are approximate figures as the limits varied according to the precise type of porphyrin in question, the fluorescence quantum yield of the different porphyrin species varying over an approximate twofold range. Similar experiments in urine demonstrated a 15% variability; the lower limit of reproducible sensitivity was approximately 0.9nmol/l though, once again concentrations considerably less than this could be detected. Although the limits of sensitivity should allow determination of most plasma porphyrin concentrations, as verified in a previous report (Day, 1978[a]), accurate figures were difficult to obtain as readings often fell at the lowest levels of sensitivity. Furthermore, TLC was often hampered by the presence of unknown factor(s) that retarded the migration of the porphyrins on the plate and appeared to interfere with the fluorometric readings.

Any process involving extraction of porphyrins can be expected to involve some losses. Using the system described standard porphyrin mixtures of known concentrations (0.5-1000nmol/l) were tested for loss after esterification and extraction. Recovery ranged from 96% (low concentrations) to 83% (concentrations 500nmol/l).

We have used TLC in this study as the method has been successfully employed in our laboratories for some 15 years. We have found the method to be as sensitive as any HPLC method we have experimented with. HPLC methods for separation and quantitation of porphyrin esters tried by us (Seubert and Seubert, 1982; Chiba and Sassa, 1982; Lim *et al*, 1983) appear not to be as reproducible as TLC owing, in large part, to quantities of porphyrin sticking to the column, in spite of experimentation with different solvent mixtures. These methods were inconsistent and a "noisy" baseline resulted in inaccuracies and loss of

identification of small porphyrin peaks. This may be due to unknown perturbing factors present in biological samples.

Diagnosis of VP

All urinary, stool and plasma porphyrin data of the patients included in the study are tabulated and further comment given in Appendices 6.1 and 6.2. These results, representing a large number of patients, are presented in order to add useful information to the overall descriptive profile of VP, apart from giving confirmatory evidence of the diagnosis of VP in these patients.

Subjects were considered to have VP where typical VP porphyrin excretory profiles were evident. All patients had elevated stool protoporphyrin of concentrations greater than 200nmol/dry g, the mean stool protoporphyrin concentration being 992nmol/dry g, and elevated concentrations of coproporphyrin greater than 50nmol/dry g in 89% of cases.

"Secondary" diagnostic criteria were also used and were as follows: Approximately 85% of the stool analyses revealed pentacarboxylic porphyrin and/or a porphyrin species running very closely to pentacarboxylic porphyrin which we have termed "pseudo-pentacarboxylic" porphyrin. It was not always possible to differentiate the two, or to assign a single peak to one or the other. The significance of "pseudo-pentacarboxylic" porphyrin is that it is an extremely useful marker of VP; elevated porphyrins in the pentacarboxylic porphyrin retention region appearing extremely infrequently in normal stools or in other types of porphyria (Day, 1986; Personal experience). "Pseudo-pentacarboxylic" porphyrin has previously been identified by two-dimensional TLC as a hydroxylated dicarboxylic porphyrin and presumably originates from spontaneous conversion of excess protoporphyrin or protoporphyrinogen (Moore *et al*, 1987; Personal communication Smith SG, Day RS and Moore MR).

Secondly, a common feature of abnormal porphyrin metabolism in the stool of VP patients was the presence of porphyrin fluorescence

remaining at the origin after the TLC plate was developed (92% of the VP cases). Previous workers have termed this fraction porphyrin X. Porphyrin X is thought to consist of hydrophilic porphyrin-peptide complexes and has been the subject of much investigation and speculation (Rimington *et al*, 1968; Grosser *et al*, 1971 and 1972; Moore *et al*, 1972; Elder *et al*, 1974). While not considered diagnostic of VP any longer (Eales *et al*, 1980; Day, 1986) it yielded useful confirmatory evidence of VP in most cases.

Unfortunately data describing the exact incidence of clinical symptoms in the complete group of VP patients can not be forthcoming because adequate clinical information was not supplied for all patients.

Patients and samples

Laboratory data obtained over a 5 year period (April 1984 - April 1989) of all patients who were unequivocally diagnosed as having VP were included in the ALA and PBG analysis. Random urine samples, 30-50ml, 5-10g stool samples and where possible 10ml of heparinised blood had been sent to the laboratory for full porphyrin and porphyrin precursor analysis when a diagnosis of porphyria had been considered. The subjects were drawn from the Cape Province of South Africa and Namibia and comprised all sections of the Southern African social and ethnic spectrum. Samples were received within 24h and were usually wrapped in opaque material to exclude light. All specimens were initially screened for the presence of PBG and porphyrins, quantitative urinary ALA and PBG concentrations determined immediately where possible (or stored for no longer than 2 days in the dark at 4°C) and TLC analysis of porphyrin profiles performed thereafter.

In the final analysis, urinary ALA and PBG concentrations in 225 VP and 93 control subjects were compared. ALA and PBG data were not available in 24 of the original 249 urines from subjects with the unequivocal diagnosis of VP over the 5 year period because of technical problems in the laboratory on two occasions. Often only urine and stool were received for investigation. In 34% of cases we

received plasma as well. Where a stool specimen was not received, the urine was only included in the analysis if the patient had previously been proven to have VP. 4 of the 225 patients were diagnosed as being in an acute attack at the time of investigation; ALA and PBG levels were grossly elevated and there were definitive clinical symptoms of the acute attack. These 4 were analysed separately from the quiescent VP subjects.

Control data was obtained by analysis of 93 normal subjects demonstrating no evidence of porphyria.

Data recording and statistical analysis

Urinary ALA and PBG concentrations are expressed as mol/10mmol creatinine and porphyrins as nmol/10mmol creatinine. This takes into account variability in urinary concentration and renders urine results comparable. Plasma concentrations are expressed as nmol/l of plasma and stool porphyrins as nmol/g of dry stool.

The data were recorded and entered into a computer spreadsheet (Lotus 123) to facilitate statistical analysis. Where applicable, the significance of the difference between the mean of the VP and control group was calculated using the Wilcoxon rank sum test for two non-parametric, independent population groups (Bailey, 1981). Significance was gauged from the probability (p) value where necessary.

Results

Urinary ALA and PBG concentrations in quiescent VP

In this group (N = 221) there was a statistically significant increase in the mean ALA and PBG concentrations when compared to the normal control group (N = 93, $p<10^{-6}$). The means were increased 2.5- and

4.6-fold respectively. Table 7.2 gives the mean, the standard deviation and the range, and probability (p) value obtained from the statistical comparison of the two groups. Figure 7.1 depicts them graphically. Approximately 50% of the ALA and PBG values were more than two standard deviations above the mean of the control group (table 7.2, last column). *It must be emphasised that these increases were noted despite the absence of any symptoms suggestive of the acute attack.*

Urinary ALA and PBG concentrations in acute VP

In this group (N = 4) there was a dramatic increase in the mean ALA and PBG concentrations by 30-fold and 135-fold respectively (table 7.2 and figure 7.1). These patients were exhibiting acute attack symptoms at the time of sampling.

Table 7.2: *Urinary porphyrin precursor data. Units of expression are μmol/10mmol creatinine. The small number of data points in the acute group disallows meaningful statistical comparisons.*

	Mean	SD (Range)	p	Proportion (%) >2 SD's above normal mean
CONTROLS (93)				
ALA	12.85	5.7 (0-35.8)	-	5.5
PBG	3.4	2.3 (0-15.4)	-	4.4
QUIESCENT VP (221)				
ALA	32.4	26.3 (3.2-158.4)	$<10^{-3}$	49.7
PBG	15.5	23.6 (0.2-170.4)	$<10^{-3}$	52.5
ACUTE VP (4)				
ALA	390.3	248.5 (192-669)	-	100
PBG	463.8	286.9 (203-771)	-	100

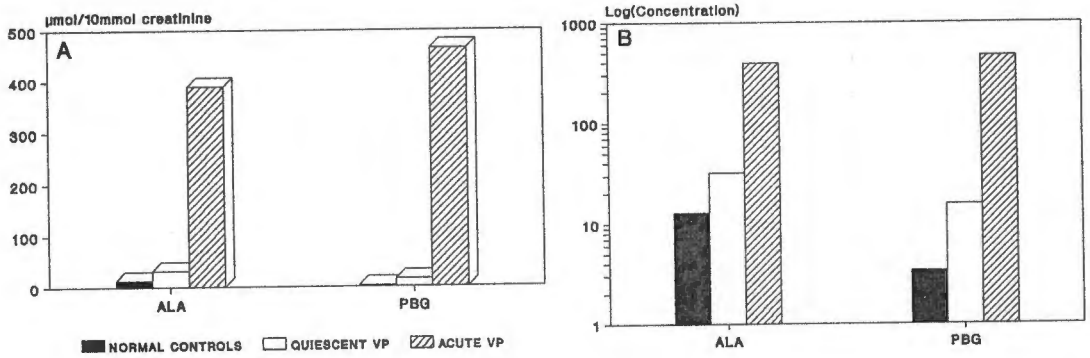

Figure 7.1: *Mean ALA and PBG concentrations in urine samples from 93 normal controls, 221 non-acute VP's and 4 acute VP's. Refer to table 7.2 for detailed values and comparative parameters. A: [Concentration], B: Log[Concentration].*

Discussion

Urinary ALA and PBG concentrations

Not much import appears to have been attached to the few reports of mildly elevated ALA and PBG in quiescent VP. Typical of the statements on this point are: "In VP the presence of elevated PBG is usually limited to the period of the acute attack" (Fromke et al, 1978). This type of statement implies that porphyrin precursors *may* be elevated during quiescent VP (Taddeini and Watson, 1968). Indeed, other reports are slightly more specific stating, for example: "The urinary porphyrin and precursor levels in this phase (quiescent VP) are usually within the normal ranges although the precursors (*ie*. ALA and PBG), coproporphyrin, and, less frequently, the uroporphyrin levels may all become mildly elevated with no concomitant symptoms of the acute attack." (Day, 1986). Some have presented data showing normal precursor excretion in just about all quiescent VP cases (Dean and Barnes, 1959; Hamnström, 1967; Eales 1963; Eales *et al*, 1980; Day, 1986). Others have described data in which precursors are

definitely raised in quiescent VP (Mustajoki and Koskelo, 1976; Muhlbauer, 1982), or at least, in certain individual patients (Eales *et al*, 1966; Wetterberg *et al*, 1968; Mustajoki, 1978). Values of ALA and PBG in relatively small VP populations ((cases) have been given, and, although overlapping with the given normal ranges of values, have mean values raised approximately twofold over the normal population (Mustajoki and Koskelo, 1976; Muhlbauer, 1982). Because of the apparently variable precursor excretion in patients with quiescent VP, which is therefore considered of dubious value in establishing a VP diagnosis (Muhlbauer, 1982), very little discussion about these observations has ensued. Thus, most reports suggest that ALA and PBG concentrations are generally normal in VP except during the acute attack.

This study shows clearly that increased porphyrin precursor output does occur in quiescent VP as well as in the acute attack. There were highly significant increases in both ALA and PBG concentrations by factors of 2.5 and 4.6 respectively in the urines of quiescent VP patients (figure 7.1, table 7.2). This increase appeared to be more consistent than is generally recognised. ALA fell more than two SD's above mean of normal in 110 of the 221 cases (49.7%). The corresponding figure for PBG was 116 (52.5%). A further point of interest was that PBG was proportionately more increased than was the uroporphyrin concentration. (The mean uroporphyrin values were increased 2.8-fold (see Appendix 6.1)). This observation lends additional indirect evidence of a "constriction" in the haem biosynthetic pathway at the level of PBG deaminase.

Conclusion

- *ALA and PBG in VP can be elevated to varying degrees, the highest concentrations being recorded during the acute attack. VP urine, however, often demonstrated significant increases in precursor concentrations without any accompanying clinical signs of the acute attack.*

Indeed, mean ALA and PBG levels in the non-acute VP group were increased 2.5- and 4.6-fold respectively. This is good supporting evidence of partially decreased PBG deaminase activity in quiescent VP.

Chapter Eight

Analysis of porphyrins present in Epstein-Barr virus transformed lymphoblasts derived from VP patients

In Chapter 4 we documented the suitability of lymphoblasts as a "model" VP tissue for the measurement of protoporphyrinogen oxidase and PBG deaminase activities. While the transformation process resulted in a mild stimulation of the activity both these enzymes, neither appeared to be selectively affected. Indeed, the 50% reduction in protoporphyrinogen oxidase activity present in lymphocytes from VP subjects persisted after transformation with Epstein-Barr virus.

The presence of decreased protoporphyrinogen oxidase activity in VP lymphoblasts suggests that these cells should contain increased concentrations of intermediates immediately prior to the block. If PBG deaminase is indeed decreased by increased concentrations of proto- and coproporphyrinogen as our hypothesis demands, levels of these intermediates or their oxidised products should be elevated in VP lymphoblasts. Although cultured fibroblasts from VP patients have been shown not to accumulate protoporphyrin (Brenner and Bloomer, 1980[a]), purportedly as a result of the "exclusive" hepatic expression of VP, we considered it justified to investigate the existence of altered porphyrin profiles in VP lymphoblasts.

The feasibility of this study was supported by the fact that mitogen-stimulated and Epstein-Barr virus-transformed lymphocytes

have been previously shown to produce quantifiable amounts of porphyrin (Sassa *et al*, 1978).

Objective

- To determine whether the lymphoblast system used in this study exhibited evidence of increased concentrations of intermediates similar to that found in VP.

We thus set out to determine whether native porphyrin production in lymphoblasts could be measured, both in the cells "resting" state and after incubation in the presence of ALA, which might stimulate porphyrin production (Sassa *et al*, 1978). Secondly, the effects of transformation on porphyrin production were assessed by comparing porphyrin concentrations (with and without ALA) at various stages during and after transformation. Finally, evidence of altered porphyrin production in the VP cultures both before and after ALA addition was sought by measuring porphyrin profiles and comparing those obtained for VP cells to those obtained from normal controls.

Methods

Control and VP lymphoblasts were cultured as previously described (Chapter 4, *Methods* and Appendix 3.3). To prepare the cells for determination of intracellular porphyrin content lymphoblasts were spun down at 1000Xg for 15min and washed twice in Hanks Balanced Salt Solution. 10ml of 5% sulphuric acid in methanol was added to the final cellular pellet, the mixture was vigorously agitated and heated, in the dark, at 55°C for 8h. This ruptured the cells and esterified any porphyrin present. The mixture was centrifuged (2000Xg, 15min), the supernatant neutralised, the porphyrin esters extracted and quantitatively analysed by TLC and fluoroscanning as described in a previous section (Chapter 4, *Methods* and Appendix 3.1).

Stimulation of porphyrin production by ALA was evaluated as follows: The lymphoblasts were centrifuged down (1000Xg, 15min) and washed twice in serum-free F10 medium. This medium has been shown to be more effective than a serum-containing medium in supporting the formation of porphyrins and inhibiting release of porphyrins into the growth medium (Sassa *et al*, 1974; Sassa *et al*, 1978). Cells were then suspended in serum-free Ham F10 medium containing 0.6mM ALA and incubated for 24h at 37°C in a 5% CO_2 atmosphere. At the end of the incubation period the cells were spun down (850Xg, 10min) and the porphyrins present esterified, extracted and analysed as described above.

Results

Porphyrin measurement in lymphoblasts

Initial attempts to quantify porphyrins in the transformed lymphoblasts in their "resting" state were unsuccessful. It appeared that too few cells were present in these early attempts. When much larger numbers of viable cells were cultured, as in later experiments, porphyrins could just be detected using the TLC fluoroscanning technique described in the previous section. At least $500X10^6$ cells (500ml of $1X10^6$ suspension culture!) were required for reliable detection of porphyrins in our system. Figure 8.1 shows the total porphyrin level as a function of cell number and indicates the proportion of each different type of porphyrin species present. Results indicate that there was approximately 1.1pmol of total porphyrin/10^6 cells.

Porphyrin measurement in lymphoblasts after addition of ALA

As shown in a previous study (Sassa *et al*, 1978), the amount of endogenous porphyrin produced could be significantly stimulated by

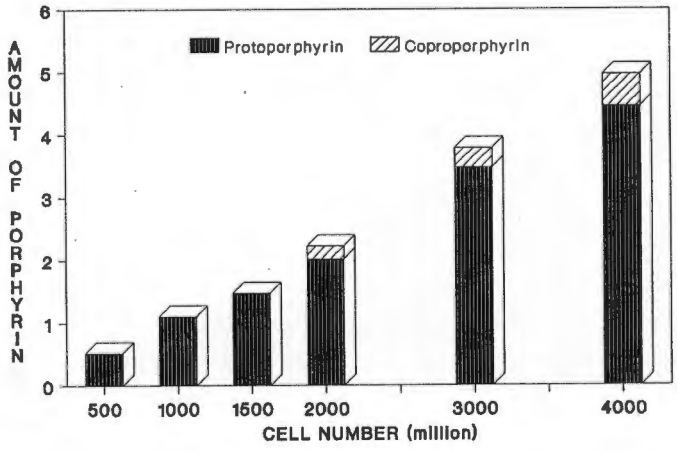

Figure 8.1: *Total amounts (nmol) of different porphyrins present in "resting" lymphoblasts. By far the most abundant porphyrin species was protoporphyrin. Porphyrin quantitation below 0.2nmol was difficult and inaccurate, small amounts of coproporphyrin presumably present in the lower cell numbers were therefore undetectable. Results represent the mean of porphyrin estimations in 5 control lymphoblast cultures for experiments involving $2000X10^6$ or less than $2000X10^6$ cells and the mean of 2 control cultures for experiments involving more than $2000X10^6$ cells.*

adding 0.6mM ALA to a serum-free Ham F10 medium in which the cells were maintained and incubating for 24h before extraction of endogenous porphyrin and analysis on TLC. Total porphyrin concentrations were increased 20-fold on average with this concentration of ALA (figure 8.2).

The effect of transformation on porphyrin production

Porphyrin determination in lymphoblasts was possible only when the cultures contained sufficient cells; that is, on days 7, 14, 21 and 28. It was necessary to stimulate porphyrin production by addition of ALA for 24h prior to measurement, as described above, in order to obtain meaningful results. Results obtained from identical experiments in five control cell lines are shown in figure 8.3. As with

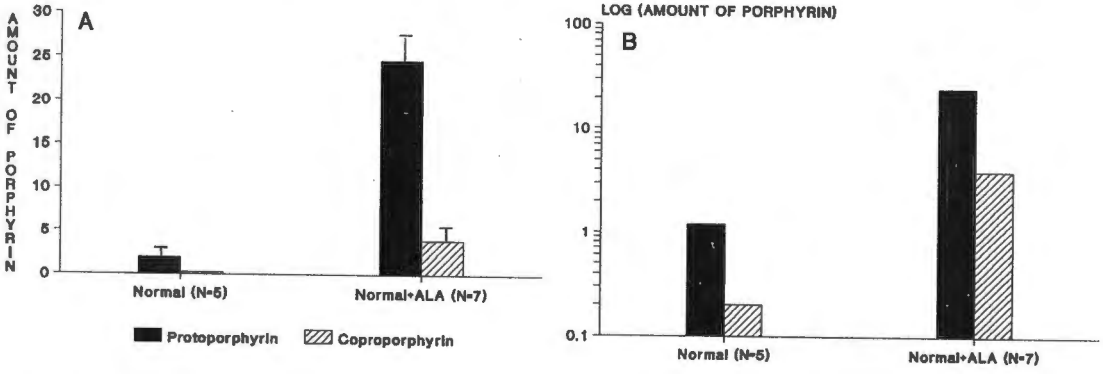

Figure 8.2: *Effect of 24h incubation in the presence of 0.6mM ALA on porphyrin production in control lymphoblasts. Porphyrin production was stimulated approximately 20-fold when ALA was added to the growing medium. Amount of protoporphyrin is given as pmol/mg of lymphoblast protein. Note the LOG scale in (B).*

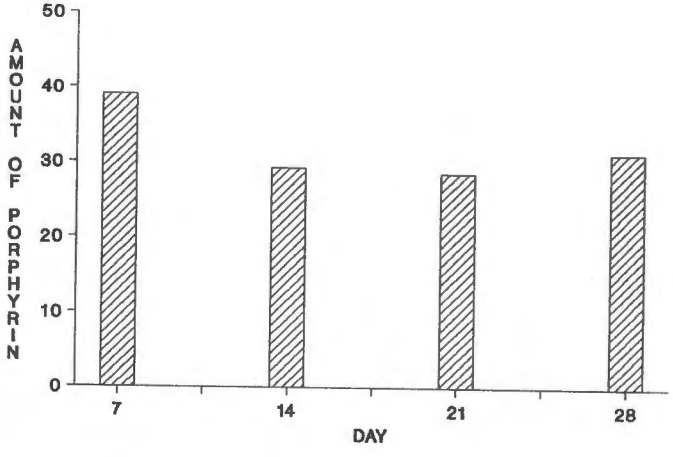

Figure 8.3: *Effect of transformation on total porphyrin levels on days 7, 14 and 21 (ie. once the cells had proliferated sufficiently). Stimulation of porphyrin production was high on day 7 but achieved a steady level over the ensuing 3 week period. Measurement of amount of total porphyrin was achieved by removing an aliquot of cells 24h prior to the due time and incubating in the presence of 6mM ALA.*

protoporphyrinogen oxidase and PBG deaminase activities there was some fluctuation in porphyrin levels; the highest levels were measured on day 7 (see figure 4.9), but steady levels were measured over the ensuing 3 week period.

Porphyrin production in VP cell lines

Figure 8.4(A) contrasts the porphyrin measurements in five normal control lymphoblasts with those in seven VP lymphoblasts. There was a significant increase of 20% ($p<0.005$) of the mean endogenous protoporphyrin in the VP cells; but copro- or uroporphyrin were not significantly increased, though there appeared to be a trend to a slight increase in coproporphyrin.

When VP and control cell porphyrin productions were stimulated by addition of ALA and compared, the porphyrin profile of VP lymphoblasts differed similarly. After ALA stimulation, protoporphyrin was elevated by 30% ($p<0.001$) and coproporphyrin by 14% ($p<0.005$) in the VP lymphoblasts (figure 8.4(B)).

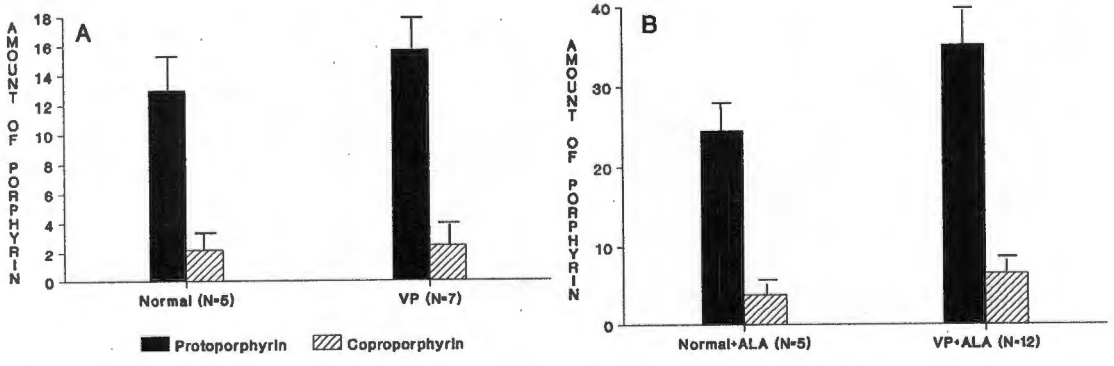

Figure 8.4: *Differences in porphyrin production in control (normal) and VP lymphoblasts.*
(A) With no added ALA, protoporphyrin levels (pmol/mg lymphoblast protein) were significantly raised in a group of 7 VP cultures when compared to a group of 5 control cultures. In order to obtain these figures approximately 1000ml of a 1-2X10^6 cell/ml suspension culture had to be prepared.
(B) After ALA stimulation, protoporphyrin and coproporphyrin levels (pmol/mg lymphoblast protein) were significantly raised by 30% and 14% respectively in a group of 12 VP cultures when compared to a group of 5 normal control cultures. "T" bars represent one standard deviation.

Discussion

Porphyrin production in lymphoblasts

Few previous studies have used transformed lymphoblasts for the study of porphyric disorders in man. One report shows that lymphocytes are capable of generating porphyrins from ALA and that 96h of incubation in the presence of phytohemagglutinin (PHA) considerably increased porphyrin production (Saillen *et al*, 1969). Another study showed that porphyrins are produced by human lymphocytes when incubated with ALA for 48h and that PHA had no effect on porphyrin production up to 72h after stimulation (Josephson *et al*, 1972). Porphyrin measurements were not performed after 72h. The ability of transformed lymphoblasts to produce porphyrins from added ALA in lymphocytes transformed by PHA as well as pokeweed mitogen (PWM) and Epstein-Barr virus has been more recently confirmed (Sassa *et al*, 1978). Our studies corroborate these findings and also show that lymphoblasts retain these characteristics in long term culture (figures 8.1 and 8.3). We are not aware of any previous report describing porphyrin production in Epstein-Barr virus transformed lymphoblasts at later stages of culture nor, indeed, in the absence of ALA stimulation. These results, in lymphoblasts well established in culture, show porphyrin and haem pathway activity both in the "resting" cells and after pathway stimulation by addition of 0.6mM ALA (figure 8.2).

Evidence of altered porphyrin metabolism in VP lymphoblasts

It is of interest to compare porphyrin production in control lymphoblasts with that in VP lymphoblasts. VP cells showed a significant 20% increase in endogenous protoporphyrin concentrations (figure 8.4(A)). When porphyrin production was stimulated by addition of ALA to the growing medium both protoporphyrin and coproporphyrin rose in the VP group, by 30% and 14% respectively (figure 8.4(B)). The similarity between the porphyrin

profile obtained in these cells and that found in the stool of VP patients is notable.

This increase in concentrations of distal porphyrin intermediates in lymphoblasts from VP subjects has not previously been documented, and contrasts with the porphyrin production reported in VP fibroblasts, where no elevation of protoporphyrin was found (Brenner and Bloomer, 1980[a]). The authors of that study state that it is not clear why abnormal porphyrin metabolism is not apparent in fibroblasts despite a demonstrated decrease in protoporphyrinogen oxidase activity. They suggest that this may reflect varying activities of the haem biosynthetic pathway in different tissues and that it is only in the liver that protoporphyrinogen oxidase activity is sufficiently reduced for protoporphyrinogen to accumulate. Another study, however, points out that in 9 liver biopsy specimens from VP subjects no elevation of protoporphyrin was evident either (Day *et al*, 1980[a]). This would therefore appear to be a contentious issue. The accumulation of porphyrin in tissues would appear to depend on flux through the haem pathway and presumably may not be evident if haem synthesis was well within the V_{max} activities of haem synthetic enzymes. Our data suggest that in transformed lymphoblasts the flux through the pathway may be high enough for a defect in protoporphyrinogen oxidase to declare itself by the accumulation of substrate.

Conclusions

- *Porphyrin production is apparent in transformed lymphoblasts and it is possible to stimulate this by incubation for 24h in the presence of ALA.*

- *There are alterations of porphyrin presence in lymphoblasts from VP subjects when compared to control lymphoblasts.*

The pattern of altered porphyrin production in VP lymphoblasts was consistent with a partial block in the distal portion of the haem biosynthetic pathway, as would be expected with a deficiency of protoporphyrinogen oxidase.

Whatever the reason for demonstrably altered porphyrin concentrations in VP lymphoblasts, this data supports the validity of lymphoblasts as a "model" VP tissue on which to base conclusions pertaining to the hypothesis under examination in this dissertation.

Chapter Nine

Overview and implications of the present study

In the wake of the experimental work presented and discussed in this thesis, all that remains is to provide a concise synthesis of the data that has emerged from the present study and to examine its contribution to the established framework of knowledge concerning porphyria.

Overview

This study has concerned itself primarily with the study of PBG deaminase in VP.

We have observed a decrease in PBG deaminase activity in lymphoblasts derived from subjects with quiescent VP. In contrast to normal lymphoblast PBG deaminase substrate-velocity curves which are hyperbolic and appear to obey Michaelis-Menten kinetic laws, VP lymphoblast PBG deaminase substrate-velocity curves are sigmoidal and do not conform to Michaelis-Menten kinetics. Addition of *proto-* and *coproporphyrinogen* but not of *uroporphyrinogen* nor any of the *porphyrins* tested to control lymphoblasts resulted in substrate-velocity curves reminiscent of VP. Experiments designed to remove porphyrin(ogen)s from PBG deaminase derived from VP lymphoblasts restored PBG deaminase kinetics to normal.

Similar kinetic alterations were observed using purified PBG deaminase with and without addition of proto- and

coproporphyrinogen. Uroporphyrinogen nor proto-, copro- or uroporphyrin failed to influence PBG deaminase.

Further work showed that VP lymphoblasts appeared to exhibit altered porphyrin profiles with an increase in protoporphyrin (and sometimes coproporphyrin) as compared to control cells.

Additional evidence of partially inhibited PBG deaminase activity in VP was provided by finding a small but significant increase in urinary ALA and PBG concentrations in a large series of VP patients when compared to a series of normal controls.

Implications

The finding that proto- and coproporphyrinogen exert negative allosteric effects on PBG deaminase lend support to those who have observed that PBG deaminase is potentially rate-limiting in the haem biosynthetic pathway. In this regard our study provides a possible mechanism for the accumulation of the early haem precursors ALA and PBG during the acute attack of VP.

In addition this study suggests a mechanism by which VP and HCP may be grouped with AIP as causes of "acute" porphyria. Our data would suggest that these are the only porphyric conditions in which PBG deaminase is an "affected" enzyme. In AIP, PBG deaminase deficiency is inherited. In VP and HCP the intermediates accumulating as a result of the inherited enzyme defect partially inhibit PBG deaminase. This we suggest results in accumulation of ALA and PBG when there is increased flux through the haem pathway. The finding that uroporphyrinogen exerted no influence on PBG deaminase is in keeping with our hypothesis since PCT is a non-acute porphyria and concentrations of ALA and PBG are not elevated in this condition.

Future work

Firstly the work suggests that the Epstein-Barr virus lymphoblast may be a useful model system for the study of haem synthesis. At least some of the haem synthetic enzymes are able to be assayed in this system. Furthermore, any true inherited factors/deficiencies should be perpetuated thus providing a ready source of material for the study of inherited porphyrias in general. Epstein-Barr virus transformed lymphoblasts we believe should be considered as potential model system in which to study porphyria. Further, this system may provide a good model for studying the potential effects of drugs on haem biosynthesis in cells derived from porphyric subjects.

If elevated intracellular concentrations of proto- and coproporphyrinogen do inhibit PBG deaminase then attempts to decrease the concentrations of these intermediates might have potential therapeutic benefits. Although no such methods are currently available the present study should give added impetus to efforts to achieve a reduction in porphyrin(ogen) accumulation, for example by the use of sorbent therapy such as the use of oral activated charcoal.

This work might also be of use in the treatment of the acute attack *per se*. Again removal of excessive proto- and coproporphyrinogen by some means should provide a way of normalising apparent PBG deaminase activity to cope with the "switched-on" haem synthetic machinery and so avoid porphyrin precursor accumulation.

Because VP is traditionally considered as being mainly of hepatic expression, further work should be performed to study whether hepatic haem synthesis is subject to the same potential mechanism of ALA and PBG build-up. This type of work could be feasible using cultured hepatocytes in which kinetic experiments on PBG deaminase similar to those described here could be performed.

Afterword

Many questions still surround the understanding of the porphyrias and in particular the pathogenesis of the acute attack of porphyria. While the present study has not been without its frustrations it has proved an exciting venture for me personally. In the words of the immunologist Peter Medawar: "Scientific discovery is a private event, and the delight that accompanies it, or the despair of finding it illusory does not travel"*.

The apparently successful application of a basic science, such as *enzyme kinetics*, to a "clinically biochemical problem" has nevertheless provided much joy. It is my sincere hope that the new developments presented in this work will serve to advance the understanding of not only VP but the porphyrias generally.

* *Hypothesis and Imagination* (Peter Medawar, 1915-1987)

Appendices

and

Bibliography

Appendix 1

Materials and equipment

Materials

- Unless otherwise stated all chemicals and reagents were of analytical grade and obtained from Sigma Chemical Co., St. Louis, Missouri, USA and Merck, Darmstadt, West Germany respectively.
- All porphyrins, porphyrin esters and porphyrin chromatographic markers were from Porphyrin Products, Logan, Utah, USA.
- Acetic acid, acrylamide, ammonium persulphate, ammonium sulphate, bromophenol blue, ethanol, glutathione (reduced), glycerol, methanol and trypan blue were from BDH Chemicals Ltd, Poole, England.
- Hanks balanced salt solution, HAM F-10 culture medium and foetal calf serum were from Gibco Ltd, Paisley, Scotland.
- All column chromatographic media used for the purification of PBG deaminase and Sephadex G25 were from Pharmacia Fine Chemicals, Uppsala, Sweden.
- ALA and PBG were quantified using Bio-Rad ALA columns (Bio-Rad, Munich, West Germany).
- All tissue culture flasks and disposable pipettes were from Falcon Inc, Cockeysville, Maryland, USA.
- Thin layer chromatographic plates were from Merck, Darmstadt, West Germany.
- Centricon 30 microconcentrators were from Amicon, Danvers, Massachusetts, USA.
- 0.45 and 0.22μm Millex HA filter units were from Millipore Corporation, Bedford, Massachusetts, USA.

Equipment

- A Vitatron TLD 100 scanning densitometer (Dieren, The Netherlands) interfaced to a Spectra Physics SP4270 recording integrator unit (San Jose, California, USA) was used for all quantitative fluoroscanning.
- A Perkin Elmer 204A fluorescence spectrophotometer (Connecticut, USA) was used for all fluorometry.
- A Sorvall RC5B refrigerated centrifuge (Du Pont Instruments, Connecticut, USA) and a Sigma 3E-1 benchtop centrifuge (Osterode, West Germany) were used for all centrifugation purposes.
- Sonication was performed by a Heat Systems Ultrasonic Model W-10 sonicator (Plainview, New York).
- Absorbance spectrophotometry was performed on a Zeiss DM4 dual beam spectrophotometer (Carl Zeiss, Oberkochen, West Germany).
- pH meter was a Radiometer AutoCal Model PHM83s (Radiometer, Copenhagen, Denmark).
- All tissue culture work was carried out in a laminar flow hood (Laminaire, Bino Instrumentation, Cape Town) and a water jacketed, CO_2 incubator (Forma Scientific, Ohio, USA) was used for maintaining growth of cell cultures.
- Where necessary data were analysed using a Bondwell AT IBM compatible computer system using the following software: DBase 3-Plus®, Lotus 123®, Epistat©, Enzfitter©.
- This thesis was produced on the above system using a Kyocera laser printer and the following software: Microsoft Word® (Version 5), Harvard Graphics® and Ventura® (Release 2).

Appendix 2*

DRUG SAFETY IN PORPHYRIA

INTRODUCTION

Drugs are the most common factor precipitating the acute attack of porphyria. Thus identification of potentially unsafe drugs in porphyria is vital.

The list included here has been presented in two ways. Firstly, all commonly prescribed drugs on which there is information regarding that drug's *porphyrinogenicity* (ability to provoke an acute attack of porphyria) are listed alphabetically (by generic name). Secondly, the same drugs have been listed according to their specific therapeutic use.

The information presented here has been obtained by combining and comparing data from several porphyria drug lists obtained from both local and overseas sources. The data regarding a drug's porphyrinogenicity is based upon experimental evidence (either in tissue culture systems, animal models or human leukocytes), or upon the personal experience of doctors managing porphyric patients. Such information is by no means valid under all circumstances, and the list needs to be interpreted with caution.

SUGGESTIONS FOR THE DOCTOR OR PHARMACIST

We suggest strongly that porphyric patients do not receive medication unless it is felt to be absolutely vital. Most of the drug-related problems we encounter have involved medication which was not strictly necessary in the first place. Do not allow the patient to extract "a pill

* Extracted from Hift *et al*, 1989. Drug list and preamble compiled by Meissner PN

for every ill" from you. Secondly, avoid compound formulae - particularly analgesics: though the paracetamol and codeine in them may be acceptable, often one of the "lesser" ingredients, such as *meprobamate*, is not. Thirdly, remember that these lists are only a guide. It is sometimes necessary to use a drug which is listed as *possibly safe*, *probably unsafe* or even *unsafe*, though, wherever possible, a safer alternative should be chosen. Even such potentially fatal agents as *phenobarbitone* have been used without precipitating an attack. However, such a step is never lightly undertaken. In such cases, our practice is to warn the patient what we are doing; to advise him to stop the drug and report back *immediately* he experiences any abdominal discomfort; to begin with low doses; and, where particular concern exists (as with the antituberculous agents or anticonvulsants) to monitor porphyrin and precursor production daily. If in doubt, do not hesitate to contact us for advice. Finally, always take a complaint of abdominal pain in a porphyric subject seriously, and regard any medication he may be taking as the cause until disproven.

Note that this list is not exhaustive; also that new drugs are constantly being introduced. We have listed here only those drugs which are at all likely to be used at present. However, information is available from our Centre on a number of other agents (older, experimental or uncommonly used) which have been omitted in the interests of brevity. You should therefore note that absence of a drug from this list does not necessarily mean that no information is available on it, still less that its safety can be assumed. Any patient given an agent where any doubt exists should be warned of the possible effects and instructed to stop it *immediately* any abdominal pain is experienced.

Always remember that you, as the prescribing doctor, will bear the responsibility for a resultant acute attack. Therefore in every case weigh up the potential benefit of the drug against the harm which will result should an acute attack arise.

SUGGESTIONS FOR THE PERSON WITH PORPHYRIA

Avoid medication wherever possible. Do *not* force your doctor or pharmacist to prescribe something for every symptom you may experience: this may result in you becoming very ill with porphyria. Do not use any medication yourself except for *paracetamol* (Panado). Should you at any stage, while on medication, experience abdominal pain or feel that your condition is deteriorating, stop your treatment *immediately* and check with your doctor. This is *very* important.

USE OF THESE LISTS

The following are the safety "status" terms that are used in the lists, together with a fuller explanation of their meaning:

☐ S:	Safe:	Thought to be safe
☐ U:	Unsafe:	Thought to be unsafe
☐ C:	Contentious:	Drugs for which there is conflicting evidence
☐ PS:	Possibly Safe:	There is conflicting evidence, but the drug is more likely to be safe than unsafe.
☐ PU:	Probably Unsafe:	There is conflicting evidence, but the drug is more likely to be unsafe than safe.

Although every effort has been made to ensure that these lists are correct, responsibility cannot be taken for errors or for any contrary experience that may be encountered.

ALPHABETICAL LISTING

Acetazolamide . . . . . .S
Actinomycin D . . . . . .S
Acyclovir . . . . . .S
Adrenaline . . . . . .S
Alcuronium . . . . . .U
Allopurinol . . . . . .PS
Alpha-methyldopa . . . .U
Alprazolam . . . . . .U
Amethocaine . . . . . .S
Amiloride . . . . . .S
Aminocaproic acid . . . .S
Aminoglycosides . . . . .S
Aminophylline . . . . . .U
Amiodarone . . . . . .U
Amitriptyline . . . . . .PU
Amoxycillin . . . . . .S
Amphetamines . . . . . .PU
Amphotericin . . . . . .S
Ampicillin . . . . . .S
Androgens . . . . . .PU
Ascorbic acid . . . . . .S
Aspirin . . . . . .S
Atenolol . . . . . .S
Atropine . . . . . .S
Auranofin . . . . . .U
Aurothiomalate (gold) . .U
Azathioprine . . . . . .S

Barbiturates . . . . . .U
Beclomethasone . . . . . .S
Bendrofluazide C
Benoxaprofen . . . . . .U
Benzothiazide C
Beta-blockers . . . . . .S
Beta-carotene . . . . . .S
Biguanides . . . . . .S
Bromocriptine . . . . . .U
Bumetanide . . . . . .S
Bupivacaine C
Buprenorphine . . . . . .S
Busulphan . . . . . .U

Captopril . . . . . U
Carbamazepine . . . . . U
Carbimazole . . . . . S
Cefuroxime . . . . . PU
Cephalexin . . . . . PU
Cephalosporins . . . . . PU
Cephradine . . . . . PU
Chloral hydrate . . . . . S
Chlorambucil . . . . . U
Chloramphenicol . . . . U
Chlordiazepoxide . . . . U
Chlormethiazole C
Chlormezanone . . . . . U
Chloroform . . . . . U
Chloroquine C
Chlorothiazide . . . . . PS
Chlorpheniramine . . . . S
Chlorpromazine . . . . . S
Chlorzoxazone . . . . . U
Cimetidine . . . . . PU
Cinnarizine . . . . . U
Cisplatin . . . . . S
Clofibrate . . . . . S
Clomiphene citrate . . . S
Clomipramine . . . . . U
Clonazepam C
Clonidine . . . . . U
Cloxacillin . . . . . S
Cocaine . . . . . PU
Codeine . . . . . S
Codeine phosphate . . . S
Colchicine . . . . . S
Corticosteroids . . . . . PS
Corticotrophin (ACTH) S
Co-trimoxazole . . . . . U
Coumarin . . . . . S
Crystal violet . . . . . U
Cyclizine . . . . . S
Cyclopenthiazide . . . . S
Cyclophosphamide . . . U
Cyclopropane . . . . . S

Cycloserine . . . . . .U
Cyclosporin . . . . . .U
Cyproterone acetate . . .PU

Dexamethasone . . . . .S
Dextropropoxyphene . . .U
Dextrose . . . . . .S
Diamorphine . . . . . .S
Diazepam C
Diazoxide . . . . . .S
Diclofenac . . . . . .U
Dicyclomine . . . . . .S
Diethyl ether . . . . . .S
Diflunisal . . . . . .S
Digitalis preparations . . .S
Digoxin . . . . . .S
Dihydralazine . . . . . .U
Dihydrocodeine . . . . . .S
Dihydroergotamine . . .U
Diltiazem . . . . . .PU
Dimenhydrinate . . . . . .U
Dimethicone . . . . . .S
Dinoprost . . . . . .U
Diphenhydramine . . . .PU
Dipyrone . . . . . .U
Disopyramide . . . . . .PS
Domperidone . . . . . .S
Dothiepin . . . . . .PS
Doxorubicin . . . . . .S
Doxycycline . . . . . .U
Droperidol . . . . . .S
Dydrogesterone . . . . . .U

Enflurane . . . . . .PU
Ergometrine . . . . . .U
Ergot compounds . . . .U
Ergotamine maleate . . .U
Ergotamine tartrate . . .U
Erythromycin . . . . . .PU
Ethacrynic acid . . . . . .S
Ethambutol . . . . . .S
Ethanol . . . . . .U
Ethinyloestradiol C
Ethionamide . . . . . .U
Ethosuximide . . . . . U
Etomidate . . . . . U

Fenfluramine . . . . . U
Fenoprofen . . . . . S
Fentantyl . . . . . S
Flucloxacillin . . . . . PU
Flucytosine . . . . . S
Flufenamic acid . . . . . U
Flunitrazepam . . . . . U
Flurazepam . . . . . U
Flurbiprofen . . . . . S
Fluroxene . . . . . U
Folic Acid . . . . . S
Fructose . . . . . S
Frusemide . . . . . PU
Fusidic acid . . . . . PS

Gentamicin . . . . . S
Glipizide . . . . . S
Glucagon . . . . . S
Glucose . . . . . S
Glyceryl trinitrate . . . . S
Griseofulvin . . . . . U
Guanethedine . . . . . S

Haloperidol . . . . . S
Halothane C
Haem arginate . . . . . S
Heparin . . . . . S
Hydantoins . . . . . U
Hydralazine . . . . . U
Hydrochlorothiazide . . PU
Hydrocortisone . . . . . PS
Hydroxyzine . . . . . U
Hyoscine C
Hyoscine butylbromide . U

Ibuprofen . . . . . S
Imipramine . . . . . U
Indomethacin . . . . . PS
Insulin . . . . . S
Iron preparations . . . . S
Isoniazid . . . . . PU

Ketamine . . . . . .PS
Ketoprofen . . . . . .S

Labetalol . . . . . .S
Licorice . . . . . .S
Lignocaine . . . . . .U
Lithium salts . . . . . .S
Lofepramine . . . . . .U
Loperamide . . . . . .S
Lorazepam . . . . . .PS
Lysuride maleate . . . . .U

Magnesium sulphate . .S
Mebendazole . . . . . .PS
Mefenamic acid . . . . . .PU
Megestrol acetate . . . .U
Melphalan . . . . . .PS
Mepivacaine . . . . . .U
Meprobamate . . . . . .U
Mercaptopurine . . . . . .U
Mestranol . . . . . .U
Metformin . . . . . .S
Methadone . . . . . .S
Methamphetamine . . . .PU
Methohexitone . . . . . .U
Methotrexate . . . . . .U
Methotrimeprazine . . .PS
Methoxyflurane . . . . . .U
Methsuximide . . . . . .U
Methylphenidate . . . . .S
Methyluracil . . . . . .S
Methysergide . . . . . .U
Metoclopramide . . . . .PS
Metoprolol Tartrate . . .S
Metronidazole . . . . . .U
Metyrapone . . . . . .U
Mianserin . . . . . .PS
Miconazole . . . . . .U
Midazolam . . . . . .PS
Minoxidil . . . . . .U
Morphine . . . . . .S

Nadolol . . . . . .S
Nalidixic acid . . . . . .U
Naproxen . . . . . S
Neostigmine . . . . . S
Nifedipine . . . . . U
Nitrazepam . . . . . U
Nitrofurantoin . . . . . PU
Nitrous oxide . . . . . S
Nortriptyline . . . . . PU

Oestrogens . . . . . U
Oral contraceptives . . . PU
Orphenadrine . . . . . U
Oxazepam . . . . . PU
Oxybuprocaine . . . . . S
Oxycodone . . . . . U
Oxymetazolone . . . . . U
Oxyphenbutazone . . . U
Oxytetracycline . . . . U
Oxytocin . . . . . S

Pancuronium . . . . . PS
Paracetamol . . . . . S
Paraldehyde . . . . . S
Penicillamine . . . . . S
Penicillin . . . . . S
Pentazocine . . . . . U
Pentobarbitone . . . . . U
Perhexiline . . . . . U
Pethidine . . . . . S
Phenacetin . . . . . U
Phenelzine . . . . . U
Phenformin . . . . . S
Phenoxybenzamine . . . U
Phentolamine . . . . . S
Phenylbutazone . . . . . U
Phenytoin . . . . . U
Pirenzipine . . . . . S
Piroxicam . . . . . U
Pivampicillin . . . . . U
Prazosin . . . . . PS
Prednisolone . . . . . S
Prilocaine C
Primaquine . . . . . S
Primidone . . . . . U
Probenecid . . . . . PU

Probucol . . . . . .S
Procainamide . . . . . .S
Procaine . . . . . .S
Prochlorperazine . . . . .S
Progestogens . . . . . .PU
Promazine . . . . . .S
Promethazine . . . . . .PU
Propanidid . . . . . .PU
Propantheline bromide .PS
Propofol . . . . . .PS
Propoxyphene C
Propranolol . . . . . .S
Propylthiouracil . . . . . .S
Prostigmine . . . . . .S
Pseudoephedrine . . . . .S
Pyrazinamide . . . . . .U
Pyridoxine . . . . . .S
Pyrimethamine . . . . . .PS

Quinidine . . . . . .S
Quinine . . . . . .S

Ranitidine C
Reserpine . . . . . .S
Rifampicin . . . . . .U

Salbutamol . . . . . .S
Senna . . . . . .S
Spironolactone . . . . . .U
Stanozolol . . . . . .U
Streptomycin . . . . . .S
Succinyl Choline . . . . .S
Sulphadoxine . . . . . .U
Sulindac . . . . . .S
Sulphacetamide . . . . . .U
Sulphamethoxazole . . .U
Sulphasalazine . . . . . .U
Sulphonamides . . . . . .U
Sulphonylureas . . . . . .U
Suxamethonium . . . . .S

Talampicillin . . . . . .S
Tamoxifen . . . . . .U
Temazepam . . . . . .S

Tetracyclines . . . . . PS
Theophylline . . . . . U
Thiazides C
Thiopentone . . . . . U
Thioridazine . . . . . U
Thyroxine . . . . . S
Tiaprofenic acid . . . . . S
Tilidine . . . . . U
Timolol . . . . . S
Tocopherol acetate . . . S
Tolbutamide . . . . . U
Tranexamic acid . . . . . S
Tranylcypromine . . . . U
Triamterene . . . . . S
Triazolam C
Trifluoperazine . . . . . S
Trimethoprim . . . . . U
Trimipramine . . . . . PU
Tubocurarine . . . . . S

Valproate . . . . . PU
Verapamil . . . . . U
Vincristine . . . . . PS
Vitamins . . . . . S

Warfarin . . . . . S

Zinc preparations . . . S
Zinc sulphate . . . . . S

LISTING BY USE

☐ Cardiovascular

Adrenaline . . . . . .S
Amiodarone . . . . . .U
Atropine . . . . . .S
Digitalis preparations . . .S
Digoxin . . . . . .S
Diltiazem . . . . . .PU
Disopyramide . . . . . .PS
Glyceryl trinitrate . . . .S
Nifedipine . . . . . .U
Perhexiline . . . . . .U
Procainamide . . . . . .S
Quinidine . . . . . .S
Verapamil . . . . . .U

☐ Diuretics

Acetazolamide . . . . . .S
Amiloride . . . . . .S
Bendrofluazide C
Benzothiazide C
Bumetanide . . . . . .S
Chlorothiazide . . . . . .PS
Cyclopenthiazide . . . . .S
Ethacrynic acid . . . . . .S
Frusemide . . . . . .PU
Hydrochlorothiazide . . .PU
Spironolactone . . . . . .U
Thiazides C
Triamterene . . . . . .S

☐ Antihypertensives (including Beta-blockers)

Alpha-methyldopa . . . .U
Atenolol . . . . . .S
Beta-blockers . . . . . .S
Captopril . . . . . .U
Clonidine . . . . . U
Diazoxide . . . . . S
Dihydralazine . . . . . U
Guanethedine . . . . . S
Hydralazine . . . . . U
Labetalol . . . . . S
Metoprolol . . . . . S
Minoxidil . . . . . U
Nadalol . . . . . S
Phenoxybenzamine . . . U
Phentolamine . . . . . S
Prazosin . . . . . PS
Propranolol . . . . . S
Reserpine . . . . . S
Timolol . . . . . S

☐ Anticoagulants and Fibrinolytics

Aminocaproic acid . . . S
Coumarin . . . . . S
Heparin . . . . . S
Tranexamic acid . . . . . S
Warfarin . . . . . S

Asthma

Aminophylline . . . . . U
Beclamethasone . . . . . S
Hydrocortisone . . . . . PS
Salbutamol . . . . . S
Theophylline . . . . . U

☐ Antihistamines

Chlorpheniramine . . . . S
Cyclizine . . . . . S
Dimenhydrinate . . . . . U
Diphenhydramine . . . PU
Hydroxyzine . . . . . U

☐ Sedatives and Major Tranquillisers

Alprazolam . . . . . .U
Chloral hydrate . . . . . .S
Chlordiazepoxide . . . . .U
Chlormethiazole C
Chlormezanone . . . . . .U
Chlorpromazine . . . . . .S
Diazepam C
Droperidol . . . . . .S
Flunitrazepam . . . . . .U
Flurazepam . . . . . .U
Haloperidol . . . . . .S
Lorazepam . . . . . .PS
Meprobamate . . . . . .U
Methadone . . . . . .S
Methotrimeprazine . . .PS
Midazolam . . . . . .PS
Nitrazepam . . . . . .U
Oxazepam . . . . . .PU
Paraldehyde . . . . . .S
Pentobarbitone . . . . . .U
Promazine . . . . . .S
Promethazine . . . . . .PU
Temazepam . . . . . .S
Thioridazine . . . . . .U
Triazolam C
Trifluoperazine . . . . . .S

☐ Antidepressants

Amitriptyline . . . . . .PU
Clomipramine . . . . . .U
Dothiepin . . . . . .PS
Imipramine . . . . . .U
Lithium salts . . . . . .S
Lofepramine . . . . . .U
Mianserin . . . . . .PS
Nortriptyline . . . . . .PU
Phenelzine . . . . . .U
Tranylcypromine . . . . .U
Trimipramine . . . . . PU

☐ Anticonvulsants

Barbiturates . . . . . U
Carbamazepine . . . . . U
Clonazepam C
Ethosuximide . . . . . U
Hydantoins . . . . . U
Magnesium sulphate . . S
Methsuximide . . . . . U
Phenytoin . . . . . U
Primidone . . . . . U
Valproate . . . . . PU

☐ CNS Stimulants and Anorectics

Amphetamines . . . . . PU
Fenfluramine . . . . . U
Methamphetamine . . . PU
Methylphenidate . . . . S

☐ Migraine

Ergot compounds . . . . U
Ergotamine maleate . . . U
Ergotamine tartrate . . U
Lysuride maleate . . . . U
Methysergide . . . . . U

☐ Analgesics

Buprenorphine . . . . . S
Codeine . . . . . S
Codeine phosphate . . . S
Dextropropoxyphene . . U
Diamorphine . . . . . S
Dihydrocodeine . . . . . S
Dipyrone . . . . . U
Fentantyl . . . . . S
Morphine . . . . . S

Oxycodone . . . . . .U
Paracetamol . . . . . .S
Pentazocine . . . . . .U
Pethidine . . . . . .S
Phenacetin . . . . . .U
Propoxyphene C
Tilidine . . . . . .U

☐ Ear, Nose and Throat

Oxymetazolone . . . . . .U
Pseudoephedrine . . . . .S

☐ Non-steroidal Anti-inflammatory

Aspirin . . . . . .S
Benoxaprofen . . . . . .U
Diclofenac . . . . . .U
Diflunisal . . . . . .S
Fenoprofen . . . . . .S
Flufenamic acid . . . . .U
Flurbiprofen . . . . . .S
Ibuprofen . . . . . .S
Indomethacin . . . . . .PS
Ketoprofen . . . . . .S
Mefenamic acid . . . . . .PU
Naproxen . . . . . .S
Oxyphenbutazone . . . .U
Phenylbutazone . . . . . .U
Piroxicam . . . . . .U
Sulindac . . . . . .S
Tiaprofenic acid . . . . . .S

☐ Arthritis and Gout

Allopurinol . . . . . .PS
Auranofin . . . . . .U
Aurothiomalate (Gold) . .U
Colchicine . . . . . .S
Penicillamine . . . . . .S
Probenecid . . . . . .PU

☐ Antimicrobial Agents

Acyclovir . . . . . S
Aminoglycosides . . . . S
Amoxycillin . . . . . S
Amphotericin . . . . . S
Ampicillin . . . . . S
Cefuroxime . . . . . PU
Cephalexin . . . . . PU
Cephalosporins . . . . . PU
Cephradine . . . . . PU
Chloramphenicol . . . . U
Chloroquine C
Cloxacillin . . . . . S
Co-trimoxazole . . . . . U
Cycloserine . . . . . U
Dapsone . . . . . U
Doxycycline . . . . . U
Erythromycin . . . . . PU
Ethambutol . . . . . S
Ethionamide . . . . . U
Flucloxacillin . . . . . PU
Flucytosine . . . . . S
Fusidic Acid . . . . . PS
Gentamicin . . . . . S
Griseofulvin . . . . . U
Isoniazid . . . . . PU
Metronidazole . . . . . U
Miconazole . . . . . U
Nalidixic Acid . . . . . U
Nitrofurantoin . . . . . PU
Oxytetracycline . . . . . U
Penicillin . . . . . S
Pivampicillin . . . . . U
Primaquine . . . . . S
Pyrazinamide . . . . . U
Pyrimethamine . . . . . PS
Quinine . . . . . S
Rifampicin . . . . . U
Streptomycin . . . . . S
Sulphadoxine . . . . . U
Sulphacetamide . . . . . U
Sulphamethoxazole . . . U
Sulphonamides . . . . . U

Talampicillin . . . . . .S
Tetracyclines . . . . . .PS
Trimethoprim . . . . . .U

☐ Gastro-intestinal and Antispasmodics

Cimetidine . . . . . .PU
Dicyclomine . . . . . .S
Hyoscine C
Hyoscine butylbromide . .U
Loperamide . . . . . .S
Pirenzipine . . . . . .S
Propantheline bromide .PS
Ranitidine C
Senna . . . . . .S
Sulphasalazine . . . . . .U

☐ Anti-emetics

Cinnarizine . . . . . .U
Domperidone . . . . . .S
Metoclopramide . . . . .PS
Prochlorperazine . . . . .S

☐ Endocrine

Bromocriptine . . . . . .U
Carbimazole . . . . . .S
Dinoprost . . . . . .U
Metyrapone . . . . . .U
Propylthiouracil . . . . . .S
Thyroxine . . . . . .S

☐ Antidiabetic Agents

Biguanides . . . . . .S
Glipizide . . . . . .S
Metformin . . . . . .S
Phenformin . . . . . .S
Sulphonylureas . . . . . .U
Tolbutamide . . . . . U

☐ Steroids and Hormonal Preparations

Androgens . . . . . PU
Beclomethasone . . . . . S
Clomiphene citrate . . . S
Corticosteroids . . . . . PS
Corticotrophin (ACTH) S
Cyproterone acetate . . PU
Danazol . . . . . U
Dexamethasone . . . . . S
Dydrogesterone . . . . . U
Ethinyl oestradiol C
Glucagon . . . . . S
Hydrocortisone . . . . . PS
Insulin . . . . . S
Megestrol acetate . . . . U
Mestranol . . . . . U
Oestrogens . . . . . U
Oral contraceptives . . . PU
Oxytocin . . . . . S
Prednisolone . . . . . S
Progestogens . . . . . PU
Stanozolol . . . . . U

☐ Cytotoxics and Immunosuppressives

Actinomycin D . . . . . S
Azathioprine . . . . . S
Busulphan . . . . . U
Chlorambucil . . . . . U
Cisplatin . . . . . S
Cyclophosphamide . . . U
Cyclosporin . . . . . U
Doxorubicin . . . . . S
Melphalan . . . . . PS
Mercaptopurine . . . . . U
Methotrexate . . . . . U
Methyluracil . . . . . S

Tamoxifen . . . . . .U
Vincristine . . . . . .PS

☐ Dermatology

Beta-carotene . . . . . .S
Dimethicone . . . . . .S

☐ Drugs used in Anaesthesia

Alcuronium . . . . . .U
Chloroform . . . . . .U
Cyclopropane . . . . . .S
Diethyl ether . . . . . .S
Enflurane . . . . . .PU
Etomidate . . . . . .U
Fluroxene . . . . . .U
Halothane C
Ketamine . . . . . .PS
Methohexitone . . . . . .U
Methoxyflurane . . . . . .U
Neostigmine . . . . . .S
Nitrous oxide . . . . . .S
Pancuronium . . . . . .PS
Propanidid . . . . . .PU
Propofol . . . . . .PS
Prostigmine . . . . . .S
Succinyl Choline . . . . .S
Suxamethonium . . . . .S
Thiopentone . . . . . .U
Tubocurarine . . . . . .S

☐ Local Anaesthetics

Amethocaine . . . . . .S
Bupivacaine C
Cocaine . . . . . .PU
Lignocaine . . . . . .U
Mepivacaine . . . . . .U
Oxybuprocaine . . . . . .S
Prilocaine C
Procaine . . . . . .S

☐ Nutrients and Haematinics

Ascorbic acid . . . . . S
Dextrose . . . . . S
Ethanol . . . . . U
Folic acid . . . . . S
Fructose . . . . . S
Glucose . . . . . S
Iron preparations . . . . S
Pyridoxine . . . . . S
Tocopherol acetate . . . S
Vitamins . . . . . S

Appendix 3*

MANAGEMENT OF THE ACUTE ATTACK

At the outset, it must be stressed again that *any patient known or thought to have VP (or AIP) should have a complaint of abdominal discomfort taken seriously.* All medication should immediately be stopped and should only be restarted once an incipient acute attack has been confidently ruled out.

Secondly, one should realise that the acute abdomen of porphyria often mimics the surgical acute abdomen. As a rule, the porphyric with an acute abdomen is more likely to be suffering from an acute attack than a coincidental surgical emergency. In VP, the use of Ehrlich's reagent will quickly and reliably confirm an acute attack, and the temptation to perform an exploratory laparotomy "just in case" should be resisted. This decision is more difficult in AIP, where the Ehrlich's test may be positive even in remission.

Guidelines

- ☐ Admit the patient. Remove any precipitating factors, particularly drugs.

- ☐ Correct dehydration and electrolyte abnormalities. Potassium, calcium and magnesium supplements are often required. Once dehydration has been corrected, fluid balance should be handled with great care. Inappropriate ADH secretion (SIADH) commonly occurs during acute attacks and may result in severe hyponatraemia. Excessive fluid administration (often intravenously) is a common error in management. Hyponatraemia

* Extracted from Hift *et al*, 1989. Co-authored by Meissner PN.

may be *prevented* as well as *treated* by fluid restriction and only rarely is it necessary to correct hyponatraemia by the cautious use of hypertonic saline.

- ☐ Carbohydrate loading suppresses porphyrin production, though its mechanism of action is unknown. This is at present standard therapy. The aim is to administer 2000 kCal per day *enterally* (using agents such as oral Hycal). Intravenous IV dextrose can cause fluid expansion and aggravate hyponatraemia. If intravenous administration cannot be avoided, our practice is to administer one litre of a 50% glucose solution (2000 kCal/l) per day. This must be given via a central venous line. An equivalent amount of carbohdrate given as 10% glucose would require the administration of 5l of fluid, which is unacceptable.

- ☐ Treat the symptoms with non-porphyrinogenic drugs. Suitable agents are:

  - *Pain*: dihydrocodeine, morphine, pethidine
  - *Vomiting*: prochlorperazine, promazine, chlorpromazine
  - *Psychosis*: promazine, chlorpromazine, trifluoroperazine
  - *Convulsions*: clonazepam, lorazepam. The use of diazepam is contentious, but may be necessary if no other drug is available.

Beta adrenergic blockers may have some anti-porphyrinogenic effect in themselves and are thus particularly useful for the control of hypertension and tachycardia.

- ☐ A further form of therapy is a *haematin* infusion. Haematin is believed to inhibit the porphyrin pathway by negative feedback of ALA synthetase. At present haematin is derived from pooled plasma and therefore carries the risk of viral transmission. Its use may be complicated by local thrombophlebitis, and it may precipitate disseminated intravascular coagulation. The usual dose is 4-8 mg/kg/day for 2-3 days. Newer formulations such as lyophilised haematin and haem arginate are superior to the older

agents. In an emergency, information on their availability can be obtained from the Porphyria Service of the the University of Cape Town.

- ☐ All patients with acute attacks should be intensively monitored for the development of paralysis and respiratory failure, and facilities for ventilation should be available. Transfer severe cases to a centre with intensive care facilities and expertise in porphyria if necessary. The neuropathy of porphyria is reversible and continued ventilation is justified in all cases.

Appendix 4

Porphyrin determination by TLC and quantitative fluoroscanning

A highly sensitive TLC assay for porphyrins in blood (plasma and erythrocytes), based on quantitative fluoroscanning, has previously been described, well characterized and standardized (Day *et al*, 1978[a]). The adaptation of these methods to stool and urine was achieved by simply substituting erythrocyte or plasma starting material. The method is described in full detail below.

Sample preparation

3ml of urine or plasma, or approximately 0.3g of stool, were esterified in 30ml of a 5% sulphuric acid/methanol solution (v/v) overnight, at room temperature in the dark. In the case of stool, the wet weight of the sample used was determined and noted. A further aliquot of the same stool sample was weighed, allowed to dry overnight at 60°C, and the dry weight determined, thus allowing determination of a wet weight/dry weight ratio.

Extraction

All procedures were performed in a darkened room. The stool and plasma esterification mixtures were centrifuged at 800Xg for 15 minutes. The supernatants of plasma or stool mixtures or the urine esterification mixture itself were transferred into separating funnels and the pH neutralized with 17% ammonia solution. The porphyrin esters were then extracted into chloroform. Plasma was extracted into a total volume of 10ml of chloroform (2 X 5ml aliquots), urine into a total of 20ml (2 X 10ml aliquots) and stool into a total of 45ml (3 X 15ml aliquots). Where a gross excess of porphyrin was suspected as suggested by the initial screening for porphyrins, proportionately more chloroform was used in the extraction procedure.

Quantitative TLC

The volume of the chloroform extract was noted and precise aliquots of each extract were applied in spot form, in semi-dark conditions, to TLC plates (Merck Silica gel-60, without fluorescent indicator) using a microsyringe (Hamilton). Care was taken to adjust the amount of porphyrin esters spotted so that they fell within the linear portion of the fluorescence concentration curve. Generally, 30-50μl of stool extract was spotted (the exact amount was suggested by to the results of an initial screening procedure), 100-300μl of the urine extract, and 50-100μl of the plasma extract, after it had been evaporated to dryness and an exact 1ml volume of chloroform added. Evaporation of the chloroform during the spotting was aided by a constant stream of warm air blown over the working area by a fan. Each plate accommodated 9 spots spaced at 2cm intervals, 2 of these lanes being reserved for porphyrin ester standards. Standards containing mixtures of known amounts of uro-, heptacarboxylic-, hexacarboxylic-, pentacarboxylic-, copro- and mesoporphyrin methyl esters were spotted in these two lanes. Mesoporphyrin behaves similarly to protoporphyrin on TLC.

Plates were developed for 45-60 minutes in a solvent system consisting of carbon tetrachloride/ dichloromethane/ethyl acetate/ethyl propionate in a 2:2:1:1 (v/v) ratio. Each plate was then dipped in an

enhancing system consisting of chloroform/dodecane/ hexadecane (18:1:1, v/v/v) in order to increase the fluorescence quantum yield. This allowed the quantitation of very small amounts of porphyrin ester which would otherwise remain undetected. After drying, the plates were scanned on a fluoroscanning photodensitometer connected to an integrator/recorder unit. Two interference filters were used, the incident at 399nm and the fluorescence filter at 620nm. A trace showing the peaks of porphyrin ester fluorescence and their integrated areas was thus produced. Individual species of porphyrin ester could be identified by direct comparison of the retention times of the unknown samples and the standards. Examples of such traces are given in figure 2.3.

Calculation of porphyrin concentration

The integrated peak areas of the various porphyrin esters separated on the TLC plates were compared directly with those of the standards on the same plate. The porphyrin concentrations were calculated according to the following formulae (adapted from Day *et al*, 1978[b]):

Urine and plasma

Concentration (*nmol/l*) = [(SA/SV)/(PA/PV)] * [(VF*C)/IV]

Stool

Concentration (*nmol/ dry g*) = [(SA/SV)/(PA/PV)] * [(VF*C)*(WW/DW)/WS]

where

SA = peak area of sample porphyrin spot
PA = peak area of relevant standard porphyrin spot
SV = volume, in μl, of sample applied to TLC plate
PV = volume, in μl, of relevant porphyrin stand applied to TLCplate
VF = final volume of chloroform extract from sample

IV = initial sample volume of urine
IW = initial sample wet weight of stool
C = relevant porphyrin ester standard concentration in nmol/l
WW = wet weight of stool sample in g
DW = dry weight of stool sample in g
SW = wet weight of stool sample esterified in g

Appendix 5

Urinary ALA and PBG determination

ALA and PBG concentrations were assayed according to an established ion-exchange method (Davis and Andelman, 1967) available in "kit" form (Bio-Rad, Munich, West Germany). The manufacturers instructions were followed. Reaction of the eluted PBG and ALA (after conversion to PBG) with freshly prepared Ehrlich's aldehyde produced a colour response which could be gauged spectrophotometrically by measuring absorbance at 553nm.

Appendix 6

Establishment of Epstein-Barr virus transformed lymphoblast cell lines

Preparation of transformation medium

Medium containing Epstein-Barr virus was prepared from semi-confluent cultures of the Epstein-Barr virus producing marmoset cell line B95/8 (Miller and Lipman, 1973). Fresh Ham F10 medium containing 15% foetal calf serum was added to the cells and removed 4 days later. Any B95/8 cells in this medium were removed by centrifugation followed by passage through a 0.45μm membrane filter (Millipore). The medium was mixed with an equal volume of Ham F10 medium containing 15% foetal calf serum. This mixture constituted the transformation medium and was effective for at least 3 months when stored at 4°C.

Establishment of transformed lymphoblasts

20ml of blood was taken into heparin from each subject and diluted with 1/3rd volume of Ham F-10 nutrient medium. The lymphocytes were isolated by layering 10ml aliquots of diluted blood onto 5ml of Histopaque solution (Sigma) and centrifuging at 1500Xg for 20 minutes. The resulting white cell band so formed on the Histopaque/blood interface was gently removed by pipette, washed twice in Ham F-10 and resuspended in 3ml of transformation medium. These cells were then set up for transformation by incubating at 37°C in a 5% carbon dioxide environment in a 25cm^2 tissue culture flask. Small volumes (0.5-1ml) of medium were added, generally on alternate days, until rapid multiplication of transformed lymphoblasts allowed passage into new flasks. This took from 7-21 days and growth progress was monitored microscopically. At this stage secondary culture could be routinely maintained as a suspension in Ham F10

containing 10% foetal calf serum at densities between 1 and $5X10^6$ cells/ml. In each case a proportion of transformed lymphoblasts underwent step-wise freezing to -180°C and were stored in liquid nitrogen. Frozen lymphoblast cell lines could be thawed and grown up in culture for repeat assays or further investigations up to 4 years later.

Appendix 7

Preparation of Epstein-Barr virus transformed lymphoblasts for assay

To prepare cells for assay the lymphoblast suspension was centrifuged at 850Xg for 15min and washed twice in Hanks Balanced Salt Solution (HBSS) to remove any residual protein in the medium. The cells were counted on a Coulter counter and their viability measured using a trypan blue exclusion test. Only cultures of 80% viability were used and cell densities were adjusted by addition of a final small volume of assay buffer (see below) to yield a density of $250\text{-}350X10^6$ cells/ml (generally 1-2ml). Lymphoblasts and lymphoblast mitochondria were ruptured by sonication at full power (Heat Systems Ultrasonic Inc, Model W-10) for 6X30sec periods, the preparation being cooled intermittently on ice. Protein concentrations (used ultimately for expressing enzyme activities/mg of protein) were determined in an aliquot by the Lowry method (Lowry *et al*, 1951). Protein standard curves were constructed using a 1mg/ml stock solution of human serum albumin (HSA) and diluted in appropriate buffer to yield concentrations ranging from 0.01 to 1mg/ml.

Appendix 8

Lymphoblast protoporphyrinogen oxidase assay

The assay for enzymatic oxidation of protoporphyrinogen to protoporphyrin is based on the fluorescent properties of protoporphyrin and the non-fluorescent properties of protoporphyrinogen. The product of the reaction (protoporphyrin) can thus be quantified by direct fluorometry with good specificity and sensitivity. Our method was based on the description of such a fluorimetric assay previously established and characterized for yeast cells, rat liver mitochondria, rat liver homogenates, human skin fibroblasts and purified mouse protoporphyrinogen oxidase (Poulsen, 1976; Brenner and Bloomer, 1980[b]; Jacobs and Jacobs, 1982; Dailey and Karr, 1987).

Preparation of substrate

Good substrate is essential for successful assay of protoporphyrinogen oxidase as it is extremely unstable and spontaneously undergoes autoxidation to protoporphyrin. Time was thus spent in attaining a good, reproducible method for substrate production in high yield. The method used was as follows:

An approximately 30μM solution of protoporphyrin in degassed 10mM potassium hydroxide containing 20% ethanol (v/v) was reduced by vigorous shaking in a stoppered boiling tube, preflushed with nitrogen, using 4% sodium amalgam that had been crushed to a hard, grainy consistency, in the dark. Sodium amalgam was produced fresh and according to well described methods (Vogel, 1974; Fuhrhop and Smith, 1975; Brenner and Bloomer, 1980[b]), 2g of the amalgam per ml of protoporphyrin solution was used. When reduction was complete, as judged by complete disappearance of fluorescence when viewed under ultraviolet light, the resultant strongly alkaline solution was filtered through a 0.45μm filter (Millipore) and the pH adjusted to 8.5 by addition of glacial acetic acid. If reduction was not complete within

5 minutes it was assumed that full reduction was not attainable and the procedure restarted using fresh amalgam and solutions. Experiments involving fluorescence measurements before and after reduction showed that reduction was always more than 96% complete and the concentration of protoporphyrinogen was thus taken as being equal to that of the protoporphyrin starting solution. The precise concentration of protoporphyrin starting solution was determined spectrophotometrically by diluting 0.2ml into 4ml of 2.7M hydrochloric acid and measuring absorbance at 554nm using the extinction coefficient of E_{mM} of 13.5 (Jacobs and Jacobs, 1982). Substrate was always produced immediately prior to use. As long as the final protoporphyrinogen solution was kept in the dark, protected from the atmosphere by either evacuation or under nitrogen, and not shaken vigorously, spontaneous reoxidation was negligible for up to 3hr after its production.

Protoporphyrinogen oxidase activity

The apparent V_{max} activity of protoporphyrinogen oxidase in each lymphoblast cell line was determined by measuring the velocity (directly proportional to the amount of fluorescence produced) over a range of 5 substrate concentrations. The substrate concentrations were chosen after an idea of the apparent K_m had been obtained for the lymphoblast enzyme in a series of substrate concentration versus velocity experiments. Initial indications were that the K_m was in the order of 1μM. Hence substrate concentrations ranging from 0.75 to 2.5μM were used. The detail of the method is as follows:

Different volumes of substrate, freshly prepared as detailed above, were added to corresponding volumes of reaction buffer (50mM Tris-HCl pH8.5, 1mM EDTA, 3mM dithiothreitol and 1% Brij-35) to give 5 final concentrations ranging between 0.75 and 2.5μM. The reaction tubes all contained 0.2ml of sonicated sample preparation in reaction buffer and the final volume of reaction mixture was 0.5ml. In addition 5 corresponding control tubes were prepared, the only difference being the substitution of sample preparation with 1mg/ml bovine serum albumen fraction V (BSA).

BSA was used as a non-enzymatic control instead of the previously described heated tissue preparation (Brenner and Bloomer, 1980[b]; Jacobs and Jacobs, 1982) as we experienced difficulty in producing a non-turbid solution in the latter. In a separate experiment we compared BSA (1mg/ml) with a preparation of lymphoblasts heated at 90°C for 15 minutes and were able to show no significant differences in the rate of non-enzymatic autoxidation of protoporphyrinogen to protoporphyrin under the described conditions. Turbidity in the case of heated tissue preparation was minimized by sonication (Brenner and Bloomer, 1980[b]).

The substrate was added rapidly but at controlled time intervals and the reaction was allowed to proceed for 1hr, in the dark, at 37°C. Fluorescence was measured at zero time and after 1hr using a spectrofluorimeter (Perkin Elmer, Model 204A) by injecting a 150μl aliquot into a flow cell of 60μl capacity and set at an excitation wavelength of 402nm and an emission wavelength of 630nm.

Use of a single measurement of protoporphyrin produced after 1hr was validated in a time-course experiment where protoporphyrinogen oxidase activity was shown to be linear for up to 90min, although an approximate 10min "lag" phase was observed.

The amount of fluorescence produced enzymatically was calculated by subtracting both the amount of fluorescence at time zero (if any) and the amount produced non-enzymatically in the controls from the final fluorescence readings. A standard solution of known protoporphyrin concentration was prepared as a means of relating fluorescence units to the actual amount of protoporphyrin present. The relationship between substrate concentration and protoporphyrin production could be plotted graphically, a double-reciprocal Lineweaver-Burk plot giving V_{max} and K_m values in each case.

Appendix 9

Lymphoblast PBG deaminase assay

Assay of PBG deaminase activity was accomplished by measuring the rate of formation of product, uroporphyrinogen, after its oxidation to uroporphyrin. The method has been previously described and characterised and, as in the hands of the originators of this particular assay (Anderson and Desnick, 1982), the "assay of PBG deaminase by measurement of uroporphyrin production was found to be a sensitive, reliable and reproducible assay." A similar assay has been applied to the measurement of cultured human lymphoblast PBG deaminase (Sassa *et al*, 1978). The assay was linear with time between approximately 3 and 75min. Linear ranges of porphyrin fluorescence versus uroporphyrin concentration were established and used. The pH optimum was 8.2 and the activity obtained in our system was similar to that reported for human lymphoblasts and lymphocytes elsewhere (Sassa *et al*, 1978; Anderson and Desnick, 1982). The detail of the method is as follows:

Lymphoblasts were prepared for assay in reaction buffer (0.1M Tris-HCl pH 8.2 and 0.1mM DTT added fresh) as described above, the PBG deaminase being released by rupture of the cell membrane by sonication. The sonicates were centrifuged at 30000Xg for 45min and the supernatants used for assay. The reaction mixture contained 100μl of sample and 600μl of reaction buffer. A 0.5mM PBG solution was made up fresh immediately prior to assay and 200μl of this solution added to start the reaction. The reaction was allowed to proceed in the dark, at 37°C in a shaking water bath. The reaction was stopped after 30min by addition of 100μl of 50% TCA after 30min and any precipitated protein present (normally invisible) was pelleted by centrifugation at 10000Xg for 20min. The assay tubes were exposed to long wave ultraviolet light for 30min in order to oxidize uroporphyrinogen formed to its corresponding fluorescent porphyrin form. Fluorescence using an excitation wavelength of 405nm and an

emission wavelength of 595nm was read in a fluorometer. Activities were reported as pmol of uroporphyrin formed/mg of sample protein/h.

Appendix 10

Form and derivation of the "Hill" equation

The Hill equation is a simplified velocity equation for allosteric enzymes. For a comprehensive treatment of the derivation of a basic velocity (v/V_{max}) equation and the modification of this equation to take into account more than one possible ligand/substrate binding site by use of so-called *"interaction factors"*, readers are referred to Chapter 7 of Segel's superb book, Enzyme Kinetics (Segel IH, 1975). A brief summary follows.

Consider an enzyme with *n* equivalent ligand binding sites. If the cooperativity in ligand binding is very marked (*ie.* interaction factors *a, b, c,* and so on, are very small numbers), then the concentrations of all enzyme-substrate complexes containing less than *n* molecules of substrate will be negligible at any [S] (where [S] is the substrate concentration) that is appreciable to K_S (the intrinsic dissociation constant of the enzyme-substrate "reaction"). The velocity equation under these circumstances reduces to a general form:

$$v/V_{max} = [S]^n / (K^* + [S]^n)$$

where *n* is the number of binding sites per enzyme molecule and K^* is a constant comprising the interaction factors *a, b, c,* and so on, and the intrinsic dissociation constant, K_S. This is known as the *Hill equation which is a simplified velocity equation that can be converted to a useful linear form as shown below.*

$$\nu/V_{max} = [S]^n / (K^* + [S]^n)$$

Thus $V_{max}[S]^n = \nu K^* + \nu[S]^n$

Thus $[S]^n(V_{max}-\nu)/\nu = K^*$

Thus $n\log[S] + \log(V_{max}-\nu)/\nu = \log K^*$

Which can be written $\log(\nu/(V_{max}-\nu) = n\log[S] - \log K^*$

Thus a plot of $\log\nu/(V_{max}-\nu)$ versus log[S] is a straight line with a slope of n. When $\nu/(V_{max}-\nu) = 1.0$, then $\nu = 0.5V_{max}$ and the corresponding [S] gives $[S]_{0.5}$. In this dissertation $[S]_{0.5}$ has been termed $K_{0.5}$.

Appendix 11

Purification of PBG deaminase

Purification procedure

We were unable to purify PBG deaminase to homogeneity using the method described by Fumagalli *et al* (Fumagalli *et al*, 1985). We therefore modified this method by incorporating chromatographic steps described in another method (Anderson and Desnick, 1980) and we added a chromatofocusing step.

460ml of fresh heparinised blood was obtained from a normal healthy volunteer. Hereafter all procedures were carried out at 4°C unless otherwise specified. Erythrocytes were obtained by centrifugation (2000Xg, 15min), the plasma and buffy layer removed and the erythrocytes washed twice with cold 0.9% normal saline and the pH adjusted to 8.0 with concentrated ammonia.

One of the major problems in purifying proteins from erythrocytes is their separation from haemoglobin. To this end, haemoglobin was specifically denatured using an organic solvent mixture and step-wise cooling, a method reported to remove 97% of haemoglobin (Scott, 1976; Bustos *et al*, 1980). Cold chloroform/*n*-butanol (0.4:1, v/v) was added to the erythrocytes in a ratio of 0.2 volumes of chloroform mixture to 1 volume of erythrocytes in three stages with vigorous stirring throughout. The three stages consisted of 30min each at the following temperatures: Stage I, 4 to 6°C; Stage II, 4 to -10°C and Stage III, -10 to -20°C. The mixture was then allowed to stand in a 20°C water bath for 1h. Centrifugation (24000Xg, 20min) was followed by dialysis of the supernatant against 200 volumes of 0.01M potassium phosphate buffer at pH 6.8 for 24h.

The dialysate was centrifuged (20000Xg, 20min) and the supernatant fractionated by ammonium sulphate precipitation. The 0-30% precipitate was discarded and the 30-80% precipitate collected by centrifugation (20000Xg, 15min) and dissolved in a minimal volume (36ml) of 0.05M Tris-HCl buffer (pH 7.4) (buffer A).

This was loaded onto a Sephadex G25 column (2.5X100cm, equilibrated with buffer A) at a flow rate of 38ml/h. 8min (ie. 5.06ml) fractions were collected. The fractions containing activity (as identified by PBG deaminase assay) were pooled and subjected to heat treatment (Fumagalli *et al*, 1985) which is designed to separate any uroporphyrinogen cosynthetase from the deaminase. The pooled fractions were heated to 67°C for 15min. They were then cooled in an ice bath, centrifuged (10000Xg, 10min), and the precipitate discarded. The supernatant was concentrated by 80% ammonium sulphate precipitation. The pellet was spun down (20000Xg, 15min) and redissolved in a minimal volume of buffer A (30ml).

This was applied to a Sephadex G100 column (2.5X100cm, equilibrated with buffer A), the column was run at a flow rate of 28ml/h and 8min (3.7ml) fractions collected.

The fractions showing PBG deaminase activity were pooled and concentrated in an Amicon ultrafiltration cell through a PM10 membrane down to a volume of 22.5ml. The solution was applied to a DEAE Spectra Gel M (Spectrum Medical Industries Inc., Los Angeles) column (1.6X20cm, equilibrated with buffer A) and run at a flow rate of 31ml/h while 8min (4.1ml) fractions were collected. Although linear gradient of 0-0.12M sodium chloride over 12h is recommended to elute the protein and was used here (Anderson and Desnick, 1980; Fumagalli *et al*, 1985), we found that PBG deaminase eluted in the latter portion of the breakthrough peak, before the gradient was run. The fractions containing activity were pooled and concentrated using an ultrafiltration cell and a PM10 membrane. A buffer change to 7.5ml of 0.025M imidazole-HCl, pH 7.4 (buffer B) was simultaneously effected.

This was applied to a PBE 94 chromatofocusing column (0.9X30cm, equilibrated with buffer B) (Pharmacia, Sweden). The column was run at a flow rate of 35ml/h and 4min (2.3ml) fractions collected. Proteins were eluted by a pH gradient with Polybuffer 74 (diluted 1:8 with water, pH 4.0) (Pharmacia, Sweden).

The fractions containing PBG deaminase activity were pooled, concentrated and the buffer exchanged using the ultrafiltration cell with a PM10 membrane for 0.02M potassium phosphate buffer (pH 8.0) containing 0.12M sodium chloride (buffer C). The 6ml so obtained was applied to a phenyl Sepharose CL 4B column (0.9X14cm, equilibrated with buffer C) (Pharmacia) and run at a flow rate of 25ml/h. 8min (3.3ml) fractions were collected. The column was then washed with 53ml of buffer C followed by 47ml of buffer C without sodium chloride. Finally PBG deaminase was eluted by a linear gradient of 0-80% (v/v) ethylene glycol over 12h. The pH of the alkaline ethylene glycol was adjusted to pH 8.0 with 0.05M phosphoric acid. Finally, the fractions containing PBG deaminase activity were pooled, concentrated and the buffer exchanged in the ultrafiltration cell through a PM10 membrane into buffer A to yield 72ml of PBG deaminase in solution at a concentration of 0.018mg/ml.

All protein determinations were measured using Biorad protein dye solution (See Appendix 12).

The purified enzyme could be stored with minimal loss of activity over a period of 4 weeks in buffer A at 4°C.

Analytical sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

This is based on a previously described method (Laemmli, 1970). Buffers and solutions were mixed as follows:

Acrylamide/bis-acrylamide solution (A-bis-A) consisted of 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide in water, filtered and stored in a dark bottle at 4°C.

Two resolving gel solutions were prepared, one a 17.5% resolving solution and the other a 7.5% resolving solution. The former consisted of 7ml of 1M Tris-HCl, pH 8.8 containing 30% glycerol, 12ml of A-bis-A, 0.3ml of 10% SDS (w/v) made up to 20ml with water. The latter consisted of 7ml of 1M Tris-HCl, pH 8.8 containing 7.5% glycerol, 5ml of A-bis-A and 0.3ml of 10% SDS made up to 20ml with water.

Spacer gel was composed of 0.125M Tris-HCl pH 6.8.

PAGE tank buffer consisted of 0.025M Tris-HCl/0.2M glycine at pH8.8 and containing 0.1% SDS.

Silver staining reagents were mixed according to manufacturers instructions ("Biorad Silver Stain Kit" GL1804, Biorad Laboratories, Richmond, California).

Samples for protein analysis were prepared by boiling for 5min with an equal volume of 2% SDS, 0.2% ß-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue in 0.125M Tris-HCl, pH6.8.

A 3.6% stacking gel in 0.125M Tris-HCl, pH6.8 and a 7.5-17.5% linear polyacrylamide gradient gel in 0.35M Tris-HCl, pH 8.8 were prepared as follows:

To each resolving solution was added 0.1ml of freshly prepared 5% ammonium persulphate (w/v) and 0.01ml of TEMED immediately before use.

16ml of the 17.5% solution was poured into a gradient mixer. The 7.5% solution was pumped into the gradient mixer at half the rate at which the solution in the mixer was pumped into the "slab space" between two upright glass plates (160X180X3mm) mounted in a gel pouring stand. Once the gel had been cast a small volume of water was layered onto the top of the gel solution using a syringe.

Once the resolving gel had set the water was poured off and the spacer solution containing 15% ammonium persulphate (freshly prepared) and 0.01ml of TEMED was poured onto the top of the gel and a spacer comb with facility for creating 10 sample bays was inserted. The spacer gel was allowed to set.

The gel was then assembled in the vertical slab gel electrophoresis unit and the tank filled with tank buffer.

Prepared samples were introduced using a microsyringe (Hamilton) into the sample bays.

Gels were run at a constant voltage of 50-60V for 18h or until the tracker dye advanced to within 1cm of the lower end of the gel.

After completion of the electrophoresis the glass plates were separated and the gel fixed and stained by following the instructions of the "Biorad Silver Staining Kit" (Biorad Laboratories, Richmond, California).

The stained gel was destained in 5% acetic acid (volume/volume) for approximately 24h, washed in deionised water and finally dried by sandwiching between blotting paper and cellophane, using heat and vacuum.

Molecular weight estimation using SDS-PAGE

Molecular weight was estimated by comparison with standards of known molecular weight, namely phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000) soybean trypsin inhibitor (20100) and -lactalbumin (14400) (Pharmacia Electrophoresis Calibration kit, Uppsala, Sweden). The relative migration values (R_f) were calculated for each standard using the formula R_f=(distance protein has migrated from the top)/(distance from origin to reference point). The protein which had travelled the furthest was used as the reference point and the top of the resolving gel was taken as the origin. The logs of the standard

molecular weights were plotted against these values to obtain a straight line. This line was used to determine the molecular weight of the purified protein.

Appendix 12

Protein determination using the "Bradford" technique

Protein "micro"-assay

Protein standards were prepared from a 0.1mg/ml stock solution of HSA. Six standards were prepared, ranging from 1 to 16μg/ml, by diluting 0.01, 0.016, 0.04, 0.08, 0.12 and 0.16ml of the HSA stock solution up to 0.8ml with buffer A. The blank consisted of 0.08ml of buffer A alone.

Samples were prepared by diluting 0.002 to 0.2ml of protein samples up to 0.8ml with buffer A. 0.2ml of Biorad dye concentrate (Bio-Rad Laboratories, Richmond, California) was added to all standards, blanks and samples, and mixed thoroughly by vortex mixing. The solutions were allowed to stand for 5min at room temperature and the absorbance against the reagent blank at 595nm determined. Protein concentrations were established by comparison of unknowns to the standard curve as determined by linear regression analysis.

Appendix 13

Porphyrin data of VP patients

Stool porphyrins

The mean concentrations of all stool porphyrins in the complete series of 221 VP and normal subjects used to establish the range of ALA and PBG concentrations encountered in VP patients are contrasted in figure App.1. Table App.1 details the actual range and occurrence of the parameters within the VP group.

Porphyrin fluorescence remaining at the origin after the TLC plate was developed (porphyrin "X" - see Chapter 7, *Diagnosis of VP*) was noted in 92% of the VP cases and in 19% of the normal controls, but was not quantified.

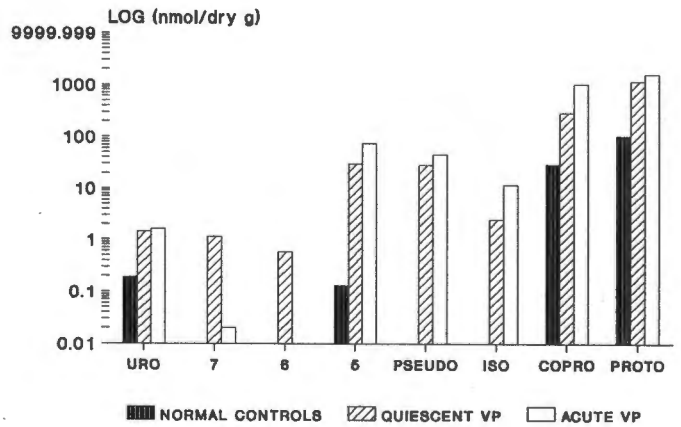

Figure App.1: *Log of the mean porphyrin concentrations in stool samples from the normal controls and 221 quiescent VP's and 2 acute VP's used as unequivocally diagnosed VP patients in the study. Abbreviations as follows: URO, Uroporphyrin; 7, Heptacarboxylic porphyrin; 6, Hexacarboxylic porphyrin; 5, Pentacarboxylic porphyrin; PSEUDO, Pseudo-pentacarboxylic porphyrin; ISO, Isocoproporphyrin; COPRO, Coproporphyrin; PROTO, Protoporphyrin.*

Table App.1: *Analysis of stool porphyrin data. Porphyrin concentrations are in nmol porphyrin/dry g. For the acute group the two values given in the standard deviation (SD) column are the values obtained for each of the two cases. The small number of data points in this group does not allow meaningful statistics.*

	Mean	SD (Range)	p	Proportion (%) >2 SD's above normal mean
NORMAL CONTROLS (88)				
Uroporphyrin	0.19	0.35 (0-1.74)	-	5.9
7COOH porphyrin	Trace	- (0-0.10)	-	1.1
6COOH porphyrin	0	-	-	0
5COOH porphyrin	0.13	0.52 (0-3.30)	-	4.8
Pseudo 5COOH	Trace	- (0-0.50)	-	2.3
Isocoproporphyrin	0	-	-	0
Coproporphyrin	28.3	25.8 (0.1-65.8)	-	0
Protoporphyrin	104.4	64.5 (2.1-232)	-	3.4
QUIESCENT VP (221)				
Uroporphyrin	1.41	4.52 (0-47.90)	0.5	21.0
7COOH porphyrin	1.14	4.35 (0-35.80)	0.048	15.4
6COOH porphyrin	0.57	2.86 (0-25.6)	0.364	6.6
5COOH porphyrin	29.2	16.9 (0-261)	$<10^{-3}$	83.3
Pseudo 5COOH	27.8	47.1 (0-298)	$<10^{-3}$	46.1
Isocoproporphyrin	2.43	12.73 (0-161)	0.131	11.0
Coproporphyrin	286.7	300.0 (22.5-1773)	$<10^{-3}$	76.3
Protoporphyrin	1136	992 (207.4-5776)	$<10^{-3}$	95.6

Table App.1: *Continued*

	Mean	SD (Range)	p	Proportion (%) >2 SD's above normal mean
ACUTE VP (2)				
Uroporphyrin	1.6	1.38; 1.76	-	100
7COOH porphyrin	0.02	0.01; 0.03	-	100
6COOH porphyrin	Trace	-; .01	-	50
5COOH porphyrin	72.6	62.5; 82.7	-	100
Pseudo 5COOH	45.7	23.0; 68.4	-	100
Isocoproporphyrin	11.3	8.0; 14.6	-	100
Coproporphyrin	1007	769; 1245	-	100
Protoporphyrin	1545	1469; 1621	-	100

(A) Quiescent VP

Significant elevations of proto-, copro-, pseudo- pentacarboxylic and pentacarboxylic porphyrin were present in this group.

There was a small shift in favour of protoporphyrin excretion in the VP population as evidenced by the change in mean protoporphyrin: coproporphyrin ratio from 3.7:1 in the normal group to 4.0:1 in the non-acute VP group. This is very similar to earlier published figures for TLC analysis in VP patients in the Cape Province (Eales *et al*, 1980; Day, 1986). Elevated isocoproporphyrin was present in 11% of the quiescent VP cases. While the mean was significantly raised, it cannot be considered diagnostic of VP as it is of PCT. Nevertheless it is a good index of porphyrin metabolic "activity" as it was almost always found in those patients with high levels of faecal and urinary porphyrin excretion.

An important feature of VP stool porphyrin profiles was the presence in most cases of either pentacarboxylic- (46.1% of cases) or pseudo-pentacarboxylic porphyrin (83.3% of cases). Although there

may be difficulty in distinguishing these two porphyrins, the observation of a peak of porphyrin fluorescence in the pentacarboxylic porphyrin "retention area" appears to be of great importance in the diagnosis of VP as it appears extremely infrequently in normal stools and seldom in other types of porphyria. Indeed, pseudopenta- or pentacarboxylic porphyrin were more frequently significantly elevated than there was coproporphyrin (83.3 versus 76.3%).

(B) Acute VP

The profile of the two patients in the acute VP group was similar to that seen in the quiescent VP group, though the porphyrin concentrations were exagerrated (values shown in table App.1 and figure App.1). The mean stool porphyrin values were significantly higher than the mean of the quiescent group but fell comfortably within the same range. Reliable statistics were not possible as there were only two data points in this group. However, there appears to be no clear marker of the acute attack in the stool porphyrin profiles, though highly elevated isocoproporphyrin was noted in both.

Urinary porphyrins

Figure App.2 contrasts the mean urinary porphyrin values in the same series of VP patients. Table App.2 details the analyses.

(A) Quiescent VP

Urinary porphyrin excretion in the quiescent VP group was highly variable and ranged from completely normal patterns and concentrations, to profiles exhibiting marked elevations of all porphyrins. There were statistically significant elevations in the means of all porphyrin intermediates. However, since the excretion of hepta-, hexa- and pentacarboxylic porphyrin intermediates in normal subjects is zero, or near-zero, an elevation in a few VP patients will suffice for the means to differ significantly. This is shown in the last column of table App.2 which indicates the proportion of values falling more than two SD's above the mean of the normal group. The most *consistent* changes were mild elevation in copro- and uroporphyrin, the means

of which were increased by a factor of 3.7 and 2.8 respectively. Both were increased to amounts more than two standard deviations above the mean of the normal group in approximately 30% of cases (table App.2, last column).

(B) Acute VP

In this group there were elevations in all porphyrin species present.

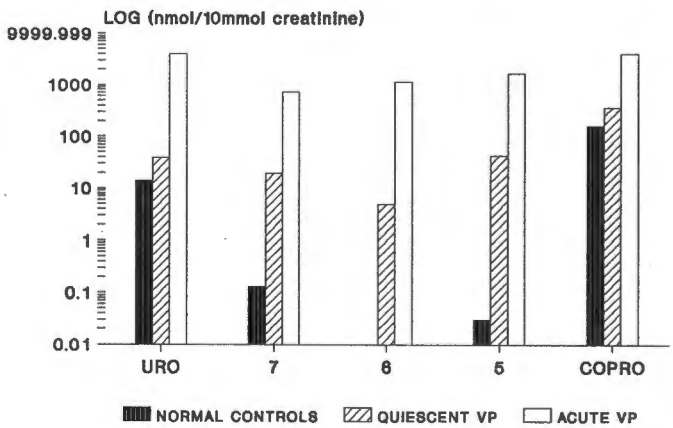

Figure App.2: *Log of the mean porphyrin concentrations in urine samples from 93 normal controls, 221 quiescent VP's and 4 acute VP's.*

Table App.2: *Analysis of urinary porphyrin data. Porphyrin concentrations are given in nmol/10mmol creatinine. The small number of data points in the acute group does not allow meaningful statistical comparisons.*

	Mean	SD (Range)	p	Proportion (%) >2 SD's above normal mean
NORMAL CONTROLS (93)				
Uroporphyrin	14.1	10.9 (0-57.9)	-	5.4
7COOH porphyrin	0.13	0.59 (0-4.62)	-	3.2
6COOH porphyrin	0	-	-	0
5COOH porphyrin	0.03	0.33 (0-3.14)	-	2.2
Coproporphyrin	100.3	106.2 (0.1-543.5)	-	4.3
QUIESCENT VP (221)				
Uroporphyrin	39.3	60.8 (0-508.5)	$<10^{-3}$	33.8
7COOH porphyrin	19.2	65.3 (0-640)	$<10^{-3}$	21.3
6COOH porphyrin	4.8	26.7 (0-369)	$<10^{-3}$	25.0
5COOH porphyrin	42.7	149.1 (0-1751)	$<10^{-3}$	21.3
Coproporphyrin	367.4	750.0 (8.9-6698)	$<10^{-3}$	32.4
ACUTE VP (4)				
Uroporphyrin	3972	681 (3105-4769)	-	100
7COOH porphyrin	726	636 (267-1627)	-	100
6COOH porphyrin	471	457 (131-1144)	-	100
5COOH porphyrin	1659	961 (642-2950)	-	100
Coproporphyrin	4039	1886 (2009-6553)	-	100

Plasma porphyrins

Results of the plasma analyses in quiescent VP where performed (see below) are summarized in table App.3. Uro- and protoporphyrin were elevated in comparison to normal values extracted from the literature (Moore *et al*, 1987). Elevated coproporphyrin occurred as a complex termed "PU" (Peak Unknown (Day *et al*, 1978[a])) in 88% of the 85 samples; the significance of this is discussed below.

Plasma porphyrin analysis by TLC was found to be troublesome although it did, in some cases, yield useful descriptive and diagnostic information. It was often impossible to determine the identity or quantity of a particular porphyrin species precisely. This may be because endogenous factors present in the plasma survive the extraction procedure, bind to the porphyrins and alter (to varying degrees) their behaviour on TLC.

Nevertheless enough information is available to be able to comment on a previous detailed analysis of plasma porphyrins in VP (Day *et al*, 1978[a]). These workers described a dominant complex of

Table App.3: *Plasma porphyrin data obtained from the analysis of 85 quiescent VP cases. All data is quoted as nmol/l of plasma. PU, "Peak Unknown". Normal ranges from Moore et al, 1987.*

	Mean	SD	Normal ranges	Proportion(%) >2 SD's above normal mean
QUIESCENT VP (85)				
Uroporphyrin	2.4	3.6	<1.6	50.6
7COOH porphyrin	0	-	-	0
6COOH porphyrin	0	-	-	0
5COOH porphyrin	Trace	0.2	-	1.2
PU	8.9	9.2	-	88.2
Coproporphyrin	0.4	1.7	<2.2	7.1
Protoporphyrin	19.1	30.1	<3.0	82.4

coproporphyrin and a membrane phospholipid constituent (possibly cholesterol) which constituted the predominant plasma porphyrin in a series of VP subjects. This complex had the same retention time as coproporphyrin in our TLC system but produced a distinctive sharp line instead of the more usual diffuse spot and is termed "PU". In our series of 85 plasma analyses, PU did not appear quite as ubiquitous in our VP population as in the previous study, appearing in 75 of the 85 (88.2%), but, when present, was unmistakable. It is therefore principally of use as a confirmatory marker of VP, making a valuable contribution to the biochemical "fingerprint" of VP.

Appendix 14

General comment on Porphyrin analyses

In the light of the porphyrin data presented in Appendix 13, one factor is clear. *Data obtained from TLC or HPLC porphyrin analyses is highly informative and, in the absence of haem synthetic enzyme assays, should be considered necessary, for the accurate differential diagnosis of the porphyrias.* Indeed, the information derived from the distribution of porphyrin types alone may be more valuable in diagnosis than a mere comparison of porphyrin concentrations with normal values. There are some instances where total porphyrin content in a sample lies within or just outside control ranges, as is the case with some patients with poorly expressed biochemical features. In these cases it is still possible to make a diagnosis of porphyria on the basis of a distinctive chromatographic pattern.

The usefulness of this type of biochemical data must not, however, be overestimated. We would suggest that it is extremely useful in identifying VP (except in the case of *silent VP*) and further in classifying the porphyria as *acute* or *quiescent* VP. It also allows a diagnosis of

latent VP (clear biochemical features but clinically asymptomatic) which is of great value in identifying otherwise healthy (possibly unsuspecting) individuals at risk.

Although more efficient and precise than simple screening tests and the older solvent-extraction techniques, "quantitative porphyrin profiling" still results in a number of equivocal results. Because VP is highly variable in its expression interpretation of these results is difficult.

Thus determination of the true incidence of the disease and its sub-categories awaits the development of more precise tests such as would be afforded by a reliable, practical protoporphyrinogen oxidase activity assay and the techniques of molecular biology.

Appendix 15

Abbreviations

g	gram	pmol	picamole
mg	milligram	sec	second
μg	microgram	min	minute
l	litre	h	hour
ml	millilitre	cm	centimetre
μl	microlitre	mm	millimetre
M	molar (moles/litre)	μm	micrometre
mmol	millimole	%	percent
μmol	micromole	°C	degrees centigrade
nmol	nanomole		

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