HAEM BIOSYNTHESIS IN ISOLATED HUMAN ERYTHROBLASTS

by

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DECLARATION

Except where acknowledged, the work presented has been carried out by myself.

The writing of this thesis is entirely my own work.

COMMUNICATIONS

1. Tracey Houston, Edward J. Fitzsimons, Ivor Cavill, Kenneth E.L. McColl, George G. Thompson, Ariane Herrick, Michael R. Moore and Sir Abraham Goldberg (1988).
   Biochemical investigation of hepatoerythropoietic porphyria-homozygous porphyria cutanea tarda.
   Biochemical Society Transactions, 16: 829-830.

2. Tracey Houston, Edward J. Fitzsimons and Michael R. Moore (1988).
   A simple reversed-phase high-performance liquid chromatographic (RPHPLC) method for the separation of haem, protoporphyrin and iron.
   Biochemical Society Transactions, 16: 831-832.

3. Tracey Houston, M.R. Moore and E.J. Fitzsimons (1990).
   5-Aminolaevulinic acid synthase activity in haem deficient human erythroblasts.
   Molecular Aspects of Medicine, 11: 98 (Abstract).

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The Biochemical Society, Sheffield, 1988.
The Tetrapyrrole Discussion Group, Leeds, 1988.
Biochemical Investigation of hepatoerythropoietic porphyria - homozygous porphyria cutanea tarda.
Tracey Houston, Edward J. Fitzsimons, Ivor Cavill, Kenneth E.L. McColl, George G. Thompson, Ariane Herrick, Michael R. Moore and Sir Abraham Goldberg.

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5-Aminolaevulinic acid synthase activity in haem deficient human erythroblasts.
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Human erythroblast ALA synthase activity: The effect of haem.
E.J. Fitzsimons, T. Houston, M.R. Moore.

SUMMARY

Hepatic haem biosynthesis has been well characterised. The first enzyme of the haem biosynthetic pathway, 5-aminolaevulinic acid (ALA) synthase, is rate-limiting and under negative feedback control by haem. Liver is, however, a relatively minor site of haem synthesis as 80% of haem is formed in the erythroid cells of the bone marrow.

Within developing erythroid cells haem is required both for its specific complexing with globin and for the coordinate regulation of erythroblast metabolism and differentiation. The study of erythroid haem synthesis has been hampered by the heterogeneous nature of the marrow cell population, the various differentiation stages of the erythroblast population, the small sample sizes available and the lack of sensitive enzyme assays. A variety of models and techniques have been used to study erythroid haem synthesis. Accordingly, the results have been inconclusive.

This work describes human bone marrow fractionation to provide four purified age-matched erythroblast cell populations. Myeloid (white) cells were removed from the sample by monoclonal antibody mediated cell lysis. The remaining cells were then fractionated by equilibrium density gradient centrifugation. These 'in vivo' human erythroblasts were used to examine the effects of normal and abnormal erythroid differentiation on the first and last enzymes of the haem pathway, ALA synthase and ferrochelatase. A sensitive radiochemical assay for ALA synthase was adopted and improved. This was then used to examine the temperature-dependent inactivation of erythroid ALA synthase. A novel radiochemical assay for ferrochelatase was developed. Both assays utilise HPLC for isolation of the radioactive product and are capable of detecting picomoles of activity.

The pattern of ALA synthase and ferrochelatase activities during

normoblastic erythropoiesis was established. ALA synthase activity was maximal in the most immature erythroid cells and diminished as the cells matured. Ferrochelatase activity was maximal in the intermediate erythroid cells. Hence, the development of enzyme activity appears to be sequential rather than simultaneous. On a quantitative basis, however, it is uncertain whether the development of ferrochelatase activity is limiting for haem formation.

The effects of iron and haem deficiency on haem enzyme activity were studied. In iron deficient erythroblasts ALA synthase activity was significantly reduced, particularly in the most immature erythroid cells. This enzyme reduction is likely to result from consequent haem deficiency rather than iron deficiency per se as an iron replete patient with haem deficiency associated with hepatoerythropoietic porphyria also showed reduced enzyme activity. This suggests that erythroid haem synthesis is controlled in a different manner to hepatic haem synthesis - a view compatible with the recent identification of specific genes and tissue-specific isoenzymes for ALA synthase.

ALA synthase and ferrochelatase activities were measured in patients with sideroblastic anaemia (SA). Haem synthesis is impaired in SA and ALA synthase is generally believed to be the site of the primary defect. ALA synthase activity was markedly impaired in 3 patients with congenital SA (CSA) and 7 patients with primary acquired SA (PASA). One patient with secondary SA (SSA) demonstrated normal activity. Ferrochelatase activity was measured in six patients (1 CSA, 5 PASA) and was found to be significantly reduced.

The latter 6 patients (1 CSA, 5 PASA) were treated with haem arginate in an attempt to correct the haem deficiency and to stimulate haem enzyme activity. Haem arginate was clinically ineffective (Hb

levels did not change in any of the patients). However, the study demonstrated that exogenous haem is able to enter erythroblasts and influence haem enzyme activity. In two patients (1 CSA, 1 PASA), both ALA synthase and ferrochelatase activities returned to normal. This indicates a potential role for haem arginate in the treatment of haematological disorders other than SA which may respond to stimulated haem biosynthesis. The results also suggest that the primary abnormality in these cases of CSA and PASA is not ALA synthase (or ferrochelatase) deficiency. Furthermore, the stimulatory effect of exogenous haem on enzyme activity and the reduced ALA synthase activity in haem deficiency provide strong evidence of independent regulatory mechanisms for haem biosynthesis in erythroid and hepatic tissue.

ABBREVIATIONS

AIA	2-allyl-2-isopropylacetamide
AIP	acute intermittent porphyria
ALA	5-aminolaevulinic acid
ATP	adenosine triphosphate
AUFS	absorbance units full scale
BFU-E	erythroid burst-forming units
BPA	burst promoting activity
CEP	congenital erythropoietic porphyria
CFU-E	erythroid colony forming units
CFU-S	spleen colony forming units
CoA	coenzyme A
cpm	counts per minute
CSA	congenital sideroblastic anaemia
CSF	colony stimulating factor
C.V.	coefficient of variation
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DMAB	dimethylaminobenzaldehyde
DMSO	dimethylsulphoxide
DNAase	deoxyribonucleotidase
DOVA	δ -δ - dioxovalerate
dpm	disintegrations per minute
E_1	early basophilic and pro erythroblasts
E_2	intermediate polychromatic erythroblasts
E_3	late orthochromatic erythroblasts
EDTA	ethylenediaminetetra-acetic acid
EEF	erythroblast enhancing factor
Epo	erythropoietin
EPP	erythropoietic protoporphyria

FEL	Friend erythroleukaemia cells
GTP	guanosine triphosphate
Hb	haemoglobin
HCP	hereditary coproporphyria
HCR	haemin-controlled translational repressor
HEP	hepatoerythropoietic porphyria
HIA	humoral inhibitory activity
HPLC	high performance liquid chromatography
HSA	humoral stimulating activity
INH	isoniazid
LSM	lymphocyte separation medium
MDS	myelodysplastic syndromes
M:E	myeloid: erythroid
MEM	minimum essential medium
M.W.	molecular weight
ODS	octa decyl silane
PASA	primary acquired sideroblastic anaemia
PBG	porphobilinogen
PCT	porphyria cutanea tarda
PL	pyridoxal
PLP	pyridoxal 5-phosphate
PN	pyridoxine
RPHPLC	reversed-phase high performance liquid chromatography
R. Spheroides	Rhodospeudomonas Spheroides
SA	sideroblastic anaemia
S.D.	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSA	secondary sideroblastic anaemia
STK	succinate thiokinase

$T^1/_2$	half life
TBAHS	tetrabutylammonium hydrogen sulphate
TCA	trichloroacetic acid
TIBC	total iron binding capacity
VP	variegate porphyria
$\bar{X}$	mean value

CHAPTER 1

INTRODUCTION

I INTRODUCTION

1.1 THE STRUCTURE AND PROPERTIES OF PORPHYRINS

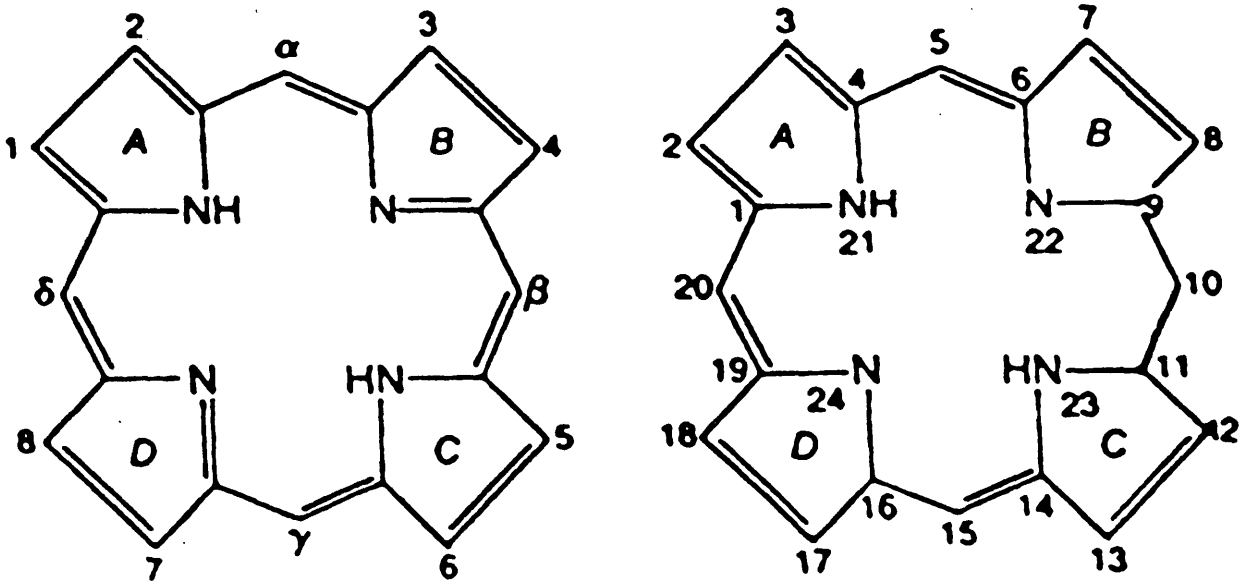
The tetrapyrrolic porphyrin ring is a unique biological structure, the term "porphyrin" being taken from "haematoporphyrin" first used by Hoppe Seyler (1871) to describe the major constituent of an iron-free preparation of blood. Porphyrins and metalloporphyrins are found ubiquitously in nature and are used for many different biological functions. Due to their complex ring structure and available ligand-binding sites, porphyrins are capable of binding many metals. The most common metals bound are iron and magnesium to form haem and the chlorophylls respectively. Haem is central to all biological oxidations while chlorophylls are central to solar energy utilisation in the biosphere by harnessing the energy of the sun to the synthesis of carbohydrates and production of oxygen in green plants. The porphyrin structure of haem and chlorophyll was described by Hans Fischer who in 1930 was awarded the Nobel medal for unveiling the secrets of nature in showing that "despite her extravagant diversity she had been sufficiently economical to use the same building material when constructing two substances so greatly different in appearance and occurrence" (Soderbaum, 1930). This building material, 5-aminolaevulinic acid (ALA), a 5-carbon aminoketone, is the first intermediate in the porphyrin biosynthetic pathway.

The basic porphyrin nucleus consists of four pyrrole rings linked by four methylene bridges to form the macrocycle (Figure 1). This represents a rigid planar structure to which eight side chains may be affixed at positions 1 to 8. It is the type of side chain which determines the physical characteristics of

the particular porphyrin in question. The four pyrrole rings are designated A, B, C and D and the four methylene bridges α, β, γ and δ. The normal biological intermediate is not this highly conjugated biologically active compound, but the hexahydroporphyrin, the porphyrinogen, in which each methylene bridge is found in the reduced form. Porphyrinogens are colourless and unconjugated as compared with the conjugated and highly coloured porphyrins (for review of porphyrin chemistry see Smith, 1975).

Porphyrin nomenclature was developed by Fischer and the terminology employed has since been used widely (Figure 1). More recently the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) issued new recommendations concerning the nomenclature of tetrapyrroles (IUPAC-IUB Joint Commission of Biochemical Nomenclature, 1980) (Figure 1). Nevertheless, the traditional (Fischer) system remains the one used by principal workers in this field.

Porphyrins display a distinctive absorption spectrum allowing them to be readily identified in biological fluids or tissues. This spectrum consists of the Soret band, a major absorption band in the 400nm region, and four smaller absorption bands at longer wavelengths between 500nm and 630nm, with decreasing intensity towards the red. The Soret band generally has a 10-15 times greater absorption (molar extinction coefficient of $2\text{-}5 \times 10^5$) than the next major band at 500nm. With the formation of a porphyrin dication in aqueous HCl or the formation of the metal complex of a porphyrin (Falk,



NOTE:
Porphin (Fischer nomenclature) or porphyrin (IUPAC nomenclature) is the basic structure of biologically occurring ring tetrapyrroles.

FIGURE 1
THE STRUCTURE AND NOMENCLATURE OF THE PORPHYRIN MACROCYCLE

1964; Williams, 1956) the four bands in the longer wavelength region are replaced by two bands known as α and β.

As metal-free porphyrins emit an intense red fluorescence upon excitation by long wavelength ultraviolet light (~400nm) spectrofluorometry provides a far more sensitive means of detecting porphyrins than visible light absorption spectrophotometry (Fuhrhop and Smith, 1975). Porphyrin chelates with those metals which have no unpaired electrons (e.g. Mg, Zn, Sn) also display strong fluorescence upon illumination with long wavelength ultraviolet light. Thus, while all free porphyrins and the porphyrin chelates with diamagnetic metals fluoresce, porphyrin chelates with paramagnetic metals do not fluoresce e.g. the Mg-porphyrin chelate chlorophyll, fluoresces, whereas iron-protoporphyrin IX i.e. haem, does not.

Although free porphyrins occur in nature in small quantities, no known function has been assigned to them. In contrast, haem, chlorophyll and corrin (which are Fe-, Mg- and Co- chelates of porphyrins or porphyrin derivatives respectively) carry out crucial biological functions.

1.2 THE BIOLOGICAL ROLE OF HAEM

Haem (ferroprotoporphyrin IX) (Figure 2) is the prosthetic group for many haemoproteins which serve important metabolic functions. These haemoproteins include myoglobin and haemoglobin (Hb) which carry out oxygen binding or transport; mitochondrial cytochromes a, a_3, b, c and c_3, which are involved in electron transfer; microsomal cytochrome P_{450}, which catalyzes mixed function oxidations; catalase, which

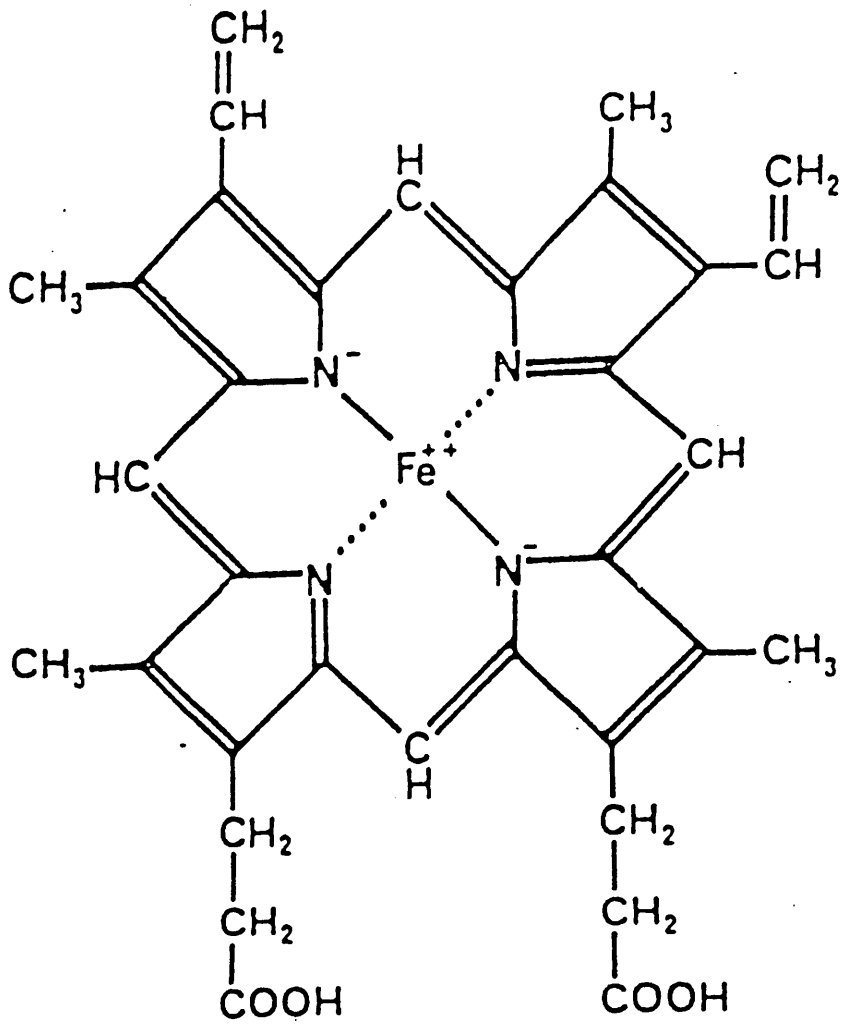


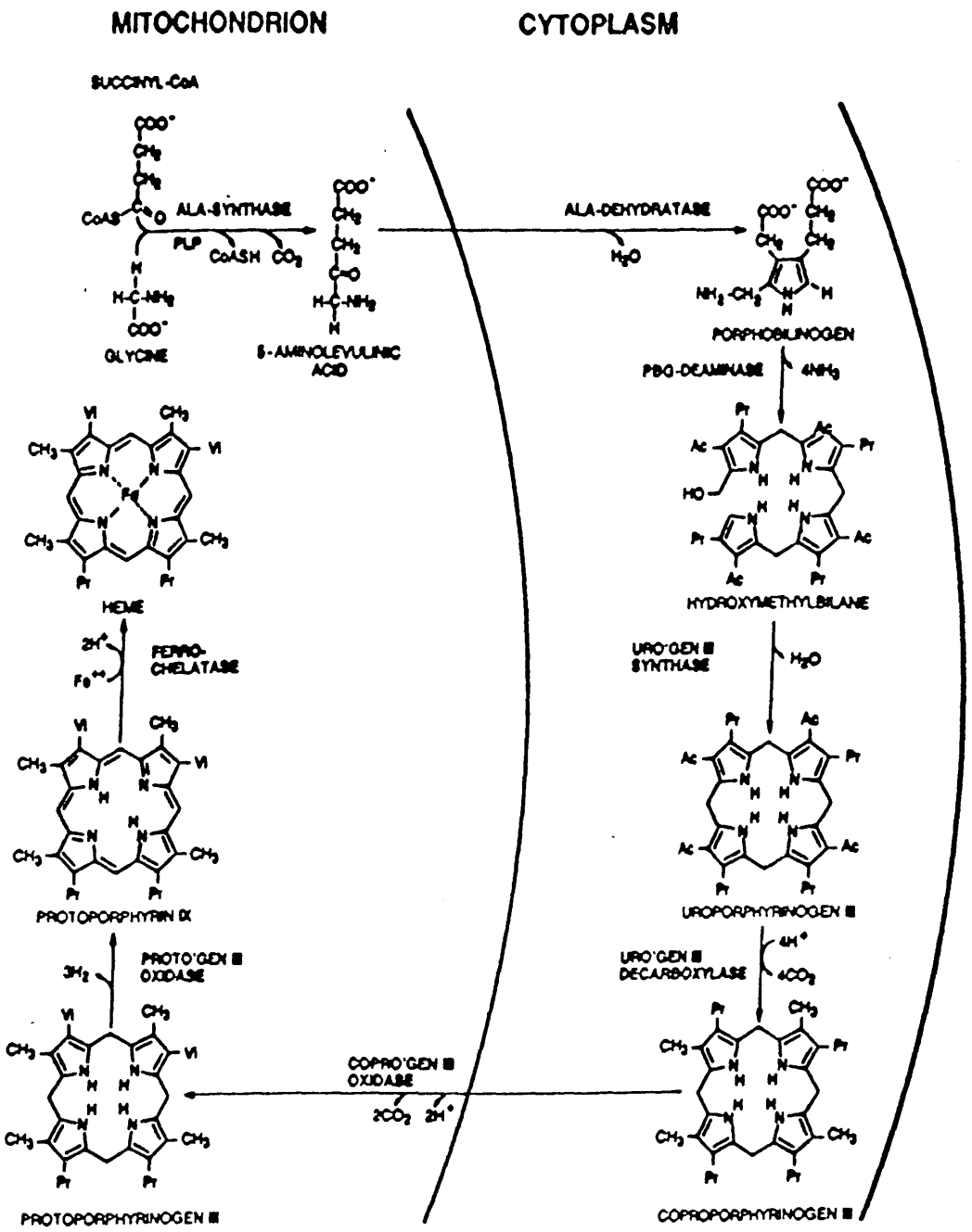
FIGURE 2

THE CYCLICAL TETRAPYRROLE STRUCTURE OF HAEM (FERROPROTOPORPHYRIN IX)

decomposes H_2O_2; peroxidase, which activates H_2O_2; and tryptophan pyrrolase, which catalyzes the oxidation of tryptophan. Beyond its role in Hb production haem is also of central importance in erythropoiesis (i.e. red blood cell formation) by playing a major role in the regulation of erythroid cell metabolism (Sections 1.10, 1.11 and 1.12).

1.3 THE BIOSYNTHESIS OF HAEM

Haem biosynthesis occurs via an unbranched pathway consisting of a series of irreversible reactions (Figure 3). Each molecule of haem requires 8 moles of glycine and 8 moles of succinyl CoA and a total of 8 enzymes are involved in the biosynthetic process. The first enzyme, 5-aminolaevulinic acid (ALA) synthase, and the last three enzymes (coproporphyrinogen III oxidase, protoporphyrinogen III oxidase and ferrochelatase) are located within the mitochondria while the intermediate enzymes (ALA dehydratase, porphobilinogen deaminase, uroporphyrinogen III cosynthetase and uroporphyrinogen III decarboxylase) are located in the cytosol. The first intermediate of the pathway is 5-ALA, a 5-carbon aminoketone which is formed by the condensation of glycine activated by pyridoxal-5-phosphate (PLP) and succinyl CoA (Shemin and Russell, 1953). Two molecules of ALA are converted to porphobilinogen (PBG), which is a monopyrrole. PBG is, in turn, condensed to form porphyrins, consuming four PBG molecules per molecule of porphyrin. Uroporphyrinogen III is the first porphyrin formed, the side chains of which are then modified by a series of decarboxylation reactions to give



NOTE:
PLP = pyridoxal-5'phosphate; URO'GEN = uroporphyrinogen;
COPRO'GEN = coproporphyrinogen; PROTO'GEN = protoporphyrinogen;
Ac = acetate; Pr = propionate; Vi = vinyl.

FIGURE 3

THE PATHWAY OF HAEM BIOSYNTHESIS

protoporphyrinogen IX. Protoporphyrinogen IX is then rapidly oxidised to protoporphyrin IX into which Fe^{2+} is inserted to form haem.

The principal site of mammalian haem biosynthesis is in the erythroid cells of the bone marrow, with lesser sites of haem biosynthesis in the liver, spleen and lymph nodes. However, due to the biological importance of haem and its vital role in mitochondrial function, all cells must synthesise haem, the difference being only that of extent.

1.4 THE ENZYMES OF HAEM BIOSYNTHESIS

1.4.1 5-Aminolaevulinic Acid (ALA) Synthase (EC.2.3.1.37)

ALA synthase catalyzes the formation of ALA via the condensation of glycine and succinyl CoA in a reaction which requires PLP as cofactor (Shemin and Russell, 1953). Although this enzyme is known to be rate-controlling in hepatic haem biosynthesis (Section 1.8) its role in erythroid tissue is less certain. The study of this enzyme is of central importance to this work and as such, this step and ALA synthase measurement in erythroid tissue are discussed in depth in Chapter 3.

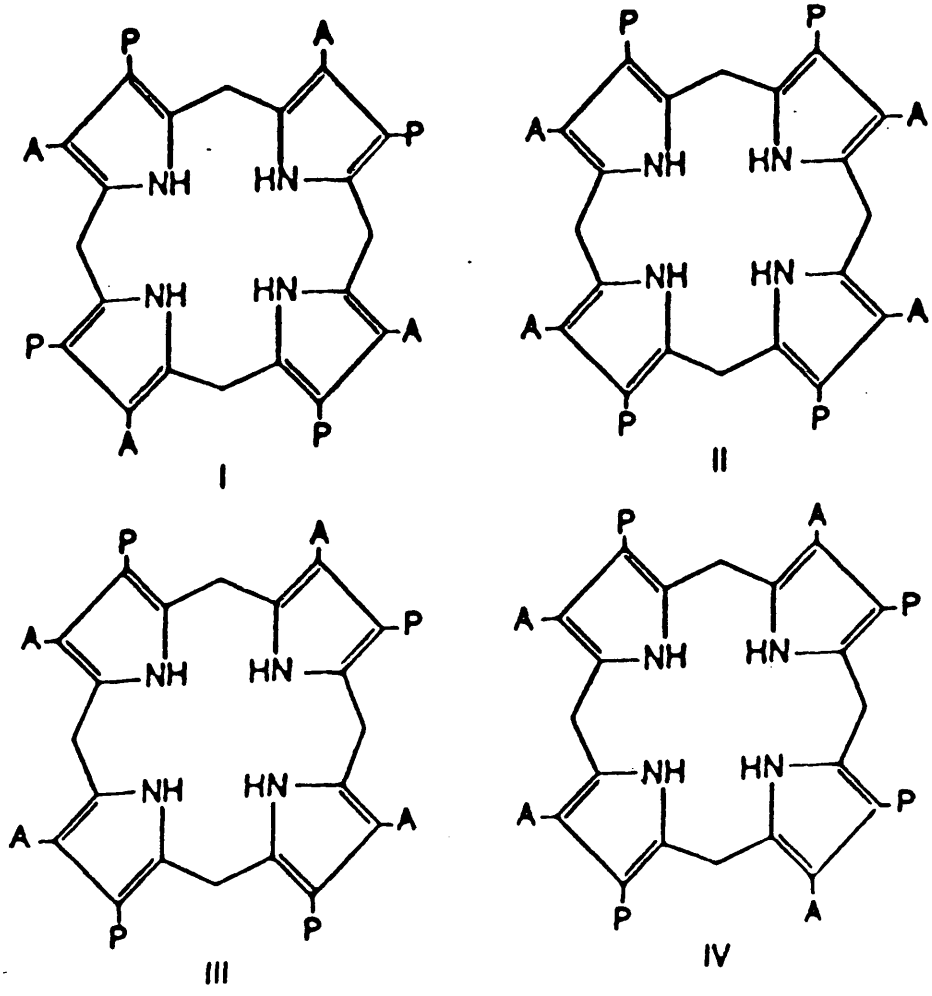
1.4.2 5-Aminolaevulinic Acid (ALA) Dehydratase (EC.4.2.1.24)

ALA dehydratase catalyzes the combination of two molecules of ALA to form porphobilinogen (PBG). This takes place in several stages (Shemin, 1976). Firstly, one molecule of ALA forms a Schiff base via combination of its keto group with an amino group of the enzyme. This ALA molecule then combines with a second ALA molecule as part of a Knorr reaction involving an intramolecular condensation of the carbanion of

the Schiff base with the carboxyl group on the other ALA molecule with the loss of one water molecule (Nandi and Shemin, 1968). The free amino grouping of the second ALA molecule displaces the amino group of the enzyme by transaldimination to form PBG. The ALA molecule initially bound to ALA dehydratase becomes the propionic acid substituent of PBG (Jordan and Seehra, 1980). The purified enzyme obtained from bovine liver and human erythrocytes is yellowish in colour and spectral analysis and kinetic studies suggest that PLP may be bound to the enzyme (Anderson and Desnick, 1979). PLP competitively inhibits human erythrocyte ALA dehydratase (Anderson and Desnick, 1979) but its precise role in the enzyme reaction is not known.

1.4.3 Porphobilinogen Deaminase (EC.4.3.1.8) and Uroporphyrinogen III Cosynthase (EC.4.2.1.75)

Although chemical condensation of four molecules of porphobilinogen (PBG) can yield four possible uroporphyrinogen isomers (Cookson and Rimington, 1954; Mauzerall, 1960) (Figure 4), enzyme catalyzed reactions yield only type I and type III isomers of uroporphyrinogen. In the presence of PBG deaminase only uroporphyrinogen I is formed from PBG, whereas when both PBG deaminase and uroporphyrinogen III cosynthase are present uroporphyrinogen III is also formed. In the type III isomer one of the PBG molecules (ring D) is reversed (Figure 4) and it is only this structure that may proceed past the stage of coproporphyrinogen to form haem. PBG deaminase has frequently been referred to as uroporphyrinogen I synthase. However, the enzyme does not directly produce uroporphyrinogen, but first



NOTE:

The only isomers found in nature are types I and III; and only isomer III can proceed to the formation of haem.

A = $-CH_2COOH$; P = $CH_2\ CH_2\ COOH$.

FIGURE 4

THE FOUR ISOMERS OF UROPORPHYRINOGEN

forms a straight chain tetrapyrrole intermediate, hydroxymethylbilane (Battersby et al, 1979; 1979).

The way in which PBG deaminase and uroporphyrinogen III cosynthase interact in the assembly of uroporphyrinogens has been one of the most puzzling problems in the biochemistry of this field. It has been shown using ^{13}C nuclear magnetic resonance spectroscopy and specifically labelled [^{13}C]-PBG (Battersby et al, 1976) that PBG deaminase catalyzes the head to tail joining of four PBG molecules to create a linear tetrapyrrole with the release of four molecules of ammonia. In the absence of uroporphyrinogen III cosynthase this product is released from the enzyme as hydroxymethylbilane, which then cyclizes spontaneously to form uroporphyrinogen I. In the presence of the cosynthase as well as the deaminase, the hydroxymethylbilane is rapidly transformed into uroporphyrinogen III (Levin, 1968). Rearrangement of ring D to form the asymmetrical uroporphyrinogen III occurs after formation of the linear tetrapyrrole just prior to closure of the macrocycle ring. A dipyrromethane resident at the enzyme active site provides the attachment site for the covalent binding of the substrate (Jordan and Warren, 1987; Warren and Jordan 1988a). An association or complex of the deaminase and cosynthase molecules accounts for the rapid enzymatic formation of uroporphyrinogen III (Frydman and Feinstein, 1974; Higuchi and Bogorad, 1975; Rossetti et al, 1980).

1.4.4 Uroporphyrinogen Decarboxylase (EC.4.1.1.37)

At this stage the basic porphyrin structure has been produced and all that remains prior to iron insertion is a

series of decarboxylations and oxidations to form the protoporphyrin nucleus. Uroporphyrin decarboxylase catalyzes the sequential removal of the four carboxyl groups of the acetic acid side chains in uroporphyrinogen to yield coproporphyrinogen (Granick and Mauzerall, 1958). The enzyme decarboxylates all four isomers of uroporphyrinogen, but the naturally most abundant type III isomer is decarboxylated most rapidly, followed by types IV, II and I in decreasing order (Granick and Mauzerall, 1958; Cornford, 1964; Smith and Francis, 1979). There is no evidence that there is more than one enzyme for the four successive decarboxylation steps (Elder and Tovey, 1977; De Verneuil et al, 1978). In fact, the reaction catalyzed by uroporphyrinogen decarboxylase yields from uroporphyrinogen, hepta-, hexa-, penta- and tetracarboxylate porphyrinogens.

1.4.5 Coproporphyrinogen Oxidase (EC.1.3.3.3)

Coproporphyrinogen oxidase removes the carboxyl group and two hydrogens from the propionic acid groups of pyrrole rings A and B of coproporphyrinogen to form vinyl groups at these positions. The reaction therefore yields a divinyl compound, protoporphyrinogen IX (Granick and Mauzerall, 1958). The enzyme requires molecular oxygen for activity and can act on isomers III and IV of coproporphyrinogen, but not on types I or II (Sano and Granick, 1961; Batlle et al, 1965; Porra and Falk, 1964). Decarboxylation proceeds only at the β-carbon atom of the propionate side chains, with a stereospecific loss of one hydrogen atom (Zaman et al, 1972; Battersby et al, 1972). The

reaction involves removal of a hydride ion with simultaneous decarboxylation, yielding β-hydroxypropionate as an intermediate (Sano, 1966). Studies using a chemically modified coproporphyrinogen oxidase or synthetic porphyrin substrates have suggested (Yoshinaga and Sano, 1980) the involvement of a tyrosine residue at the active site and that formation of a vinyl group from a β-hydroxypropionate side chain is faster at position 2 than position 4. An intermediate 3-carboxylate porphyrinogen formed in the decarboxylation of hydroxypropionic porphyrin to protoporphyrin IX has been isolated by HPLC and characterised by mass spectroscopy as harderoporphyrinogen. The corresponding porphyrin was originally isolated from the Harderian gland of rodents (Jackson et al, 1980).

1.4.6 Protoporphyrinogen Oxidase (EC.1.3.3.4)

The penultimate step in the haem biosynthetic pathway is the oxidation of protoporphyrinogen IX. Although chemical oxidation of protoporphyrinogen to protoporphyrin occurs rapidly at neutral pH, this does not happen normally in cells. The existence of protoporphyrinogen oxidase was suggested from indirect observations, but it was the partial purification of this enzyme (Poulson and Polglase, 1975) that confirmed its existence.

In the conversion of protoporphyrinogen to protoporphyrin IX, six electrons are removed with molecular oxygen as the electron acceptor (Poulson and Polglase, 1975; Poulson, 1976). The enzyme exhibits relatively high substrate specificity since it will only use mesoporphyrinogen IX, protoporphyrinogen XIII and 2(4) vinyl-4(2) hydroxyethyl deuteroporphyrinogen, although

at lower rates than protoporphyrinogen IX (Jackson et al, 1978; Poulson and Polglase, 1975). Coproporphyrinogen I and III and uroporphyrinogen I and III are not substrates.

There is currently insufficient data to postulate a model for catalysis or substrate binding. Since the enzyme uses only protoporphyrinogen and mesoporphyrinogen IX, it is likely that the enzyme binding site for porphyrinogen recognises not only the vinyl groups at positions 2 and 4 on the A and B ring, but also the propionates at positions 6 and 7 on the C and D ring (Dailey, 1989). Those features involved in actual catalysis remain unknown, although the possibility of a protein tyrosyl residue involvement as with coproporphyrinogen oxidase has been proposed (Yoshinaga and Sano, 1980b).

1.4.7 Ferrochelatase (Protohaem Ferrolyase; Haem Synthetase) (EC.4.99.1.1)

Ferrochelatase catalyzes the final step in the haem biosynthetic pathway i.e. the insertion of iron into protoporphyrin IX to form haem with the loss of two protons (Goldberg et al, 1956). The study of this enzyme is fraught with difficulties and constitutes one of the major topics of the work described in this thesis. Its role in haem biosynthesis is examined in depth in Chapter 4. Chapter 5 then describes the stepwise development of a novel highly sensitive radiochemical assay suitable for the measurement of erythroid ferrochelatase activity in the small sample sizes available from human erythroid tissue.

1.5 ALA FORMATION VIA A PATHWAY NOT INVOLVING GLYCINE AND SUCCINYL CoA

With few exceptions, ALA synthase activity has not been found in plant cells. Instead, the incorporation of [^{14}C]-glutamate or α-ketoglutarate into ALA has been shown to occur (Beale and Castelfranco, 1974). In this reaction C-1 of glutamate becomes C-5 of ALA via the formation of an intermediate, δ-δ-dioxovalerate (DOVA) (Beale et al, 1975). Transamination of DOVA to ALA then occurs as has been shown in Chlorella (Gassman et al, 1968), bean leaves (Gassman et al, 1966) and R. spheroides (Neuberger and Turner, 1963). It is uncertain whether chlorophyll formation is exclusively associated with the DOVA pathway (Porra and Grimme, 1978) since in green algae both the classical ALA synthase reaction and the DOVA pathway have been demonstrated (Klein et al, 1980).

A reaction similar to that catalyzed by DOVA transaminase has also been found in mammalian cells (Varticovski et al, 1980). Total DOVA transaminase activity was much greater than that of ALA synthase in bovine liver, suggesting that this enzyme may also have a role in ALA formation in mammalian cells. DOVA transaminase in mammalian liver and kidney is associated with alanine: glyoxylate transaminase activity (Noguchi and Mori, 1981). The activity of the latter is much greater than the former and the reaction favours glycine formation.

The role of DOVA transaminase in the regulation of haem formation in animal tissues remains to be elucidated but it is unlikely that this pathway has any direct role in mammalian haem biosynthesis.

1.6 THE ENZYMES OF HAEM BIODEGRADATION

1.6.1 Haem Oxygenase (EC.1.14.99.3)

The catabolic sequence of haem degradation starts with the reductive breakdown of the haem macrocycle (Figure 5). The cycle is most open to oxidative attack at the carbon bridges linking the pyrrole rings, resulting in the formation of linear tetrapyrroles, referred to collectively as the bile pigments or biliverdins (Heirwegh and Brown, 1982). Since there are four different ring positions $-\alpha, \beta, \gamma$ and $\delta-$ at which the haem ring may be cleaved, haem oxygenase can potentially produce four biliverdins (O'Carra, 1975; Tenhunen, 1976). However, the reaction is specific for one position i.e. cleavage between rings A and B to produce biliverdin IXα (Frydman et al, 1981). The haem oxygenase reaction requires NADPH and oxygen and produces biliverdin and carbon monoxide. Either NADPH cytochrome c reductase or NADPH cytochrome P_{450} reductase is also required and protohaem IX is the best enzyme substrate. In the intact cell, haem must be proteolytically split from its associated haemoproteins before it may be metabolised (Berk et al, 1974). Haem oxygenase is rate-controlling for haem degradation. Carbon monoxide inhibits the enzyme as does light of wavelength 450-470nm. Studies which have shown enzyme induction with cobalt and other metal ions suggest that the enzyme has a sulphydryl-active regulatory component (Maines and Kappas, 1974; 1976; Maines, 1981; Maines et al, 1982).

1.6.2 Biliverdin Reductase (EC.1.3.1.24)

No biological role has been found for the biliverdin produced by haem oxygenase. Mammals transform this harmless

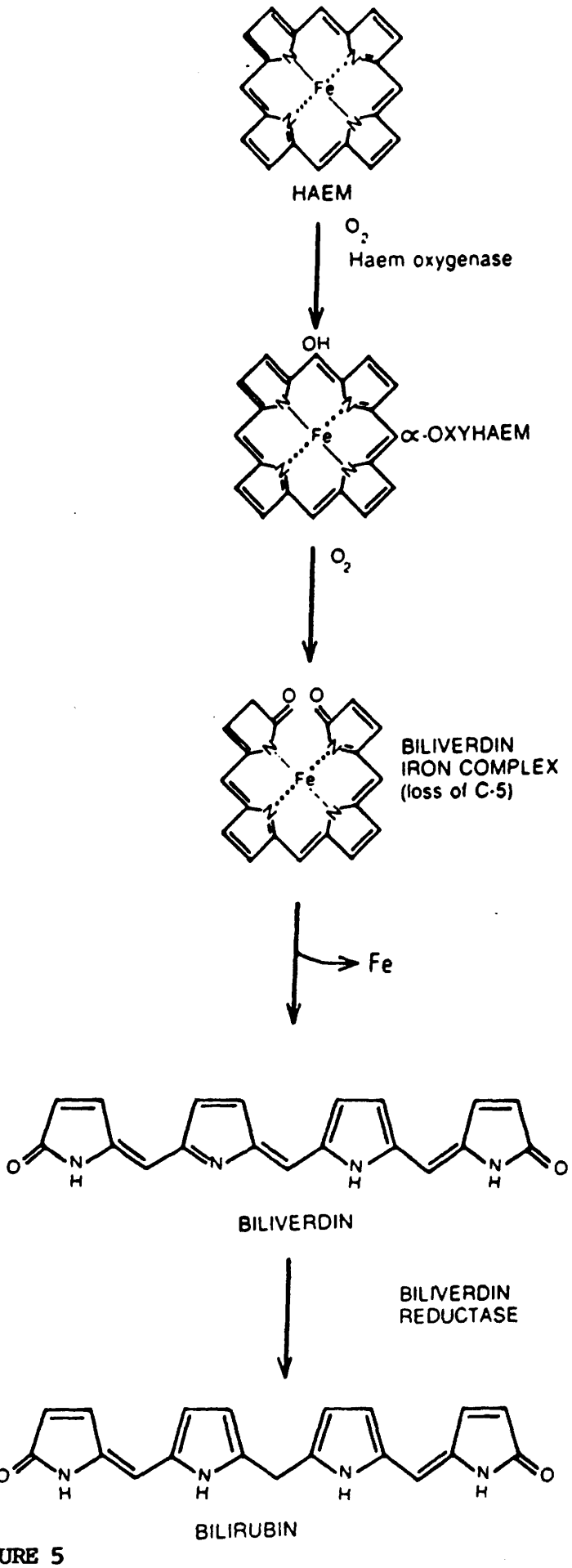


FIGURE 5
THE PATHWAY OF HAEM BIODEGRADATION

compound, which is readily disposable, into the toxic and highly insoluble compound bilirubin by a reductive reaction requiring NADPH and catalyzed by biliverdin reductase. This compound is then conjugated with glucuronic acid to form more soluble bilirubin glucuronides. These are excreted in bile into the intestines and thereafter in the faeces, normally following transformation to mesobilirubinogen, stercobilinogen, stercobilin and urobilin (O'Carra, 1975). Bilirubin may also be oxidised to dipyrroles by a mitochondrial enzyme bilirubin oxidase, the importance of which remains to be assessed (Brown and Troxler, 1982).

1.7 DISORDERS OF PORPHYRIN METABOLISM

The Porphyrias

The porphyrias are inherited and acquired disorders in which there are partial defects in the haem enzymes. They are classified as either hepatic or erythroid in origin depending on the principal site of expression of the gene defect (for review see Kappas et al, 1982). In all of the inherited human porphyrias, environmental factors play a vital role in determining clinical expression of the gene abnormality. The porphyrias are characterised by defects in specific haem enzymes; increased accumulation and excretion of chemical intermediates, and a variety of clinical manifestations among which neurological abnormalities and cutaneous photosensitivity dominate. There are certain naturally occurring animal porphyrias of genetic origin as well as experimental porphyrias that can be induced 'in vivo' by a variety of chemicals in normal animals and 'in vitro' in certain tissue preparations,

such as cultured liver cells. These animals and experimental models of the porphyrias have provided important insights into the regulatory mechanisms by which cellular porphyrin and haem metabolism are controlled. Indeed, in few other categories of metabolic and genetic disorders has there been such a close parallel between the human condition and the laboratory model nor such direct relevance of information obtained from experimental systems to the pathogenesis and therapy of the human disease.

1.7.1 Congenital Erythropoietic Porphyria (CEP)

This autosomal recessive disorder is extremely rare. The porphyrins which are overproduced are primarily of the type 1 series with the genetic defect most probably being a deficiency of uroporphyrinogen III cosynthase.

CEP is the most striking of the porphyrias in terms of the marked severity of cutaneous photosensitivity and scarring which it may produce. Anaemia due to haemolysis may occur which serves as a further stimulus for increased porphyrin production in the bone marrow. Avoidance of sunlight, hypertransfusion (Piomelli et al, 1986) and treatment of secondary infections of the skin are important in the management of the disease.

1.7.2 Erythropoietic Protoporphyria (EPP)

EPP is an autosomal dominant disorder due to a deficiency of ferrochelatase. The disease is characterised by mild to moderate photosensitivity, but unlike CEP haemolytic anaemia is insignificant (Turnbull et al, 1973). Excess protoporphyrin is

found in erythroid cells, plasma, bile and faeces. Progressive hepatic damage associated with marked protoporphyrin deposition in the liver is a severe and often fatal complication. Gene expression at the clinical level is highly variable, such that some carriers of the genetic defect have normal red cell porphyrin levels and no photosensitivity. Avoidance of sunlight is important in the prevention of cutaneous symptoms.

1.7.3 Acute Intermittent Porphyria (AIP)

This hepatic porphyria is inherited in an autosomal dominant fashion and is characterised by a deficiency of porphobilinogen (PBG) deaminase activity. PBG deaminase activity is approximately 50% of normal in all tissues examined (i.e. red blood cells, skin, cultured fibroblasts, mitogen-stimulated lymphocytes, amniotic cells and liver). Porphyrin overproduction is relatively minor and hence there is no photosensitivity. AIP is latent before puberty and the degree of clinical expression after puberty is variable despite the fact that PBG deaminase deficiency is the same in latent and expressed AIP. The clinical syndrome is most frequent in females. Hormonal, drug and nutritional factors predispose to full expression of the disease probably by inducing hepatic ALA synthase. Ninety per cent of individuals who inherit the gene defect for AIP remain clinically latent throughout adult life. Subtle endocrine abnormalities, including a demonstrated deficiency of hepatic steroid 5α-reductase activity in clinically expressed AIP, may underly disease expression in the 10% of AIP gene carriers who develop clinical symptoms. The symptoms of AIP are largely neurovisceral in character and may

be due to pharmacologic effects of porphyrin precursors on nervous tissue. Clinical management includes symptomatic therapy during acute attacks, a high carbohydrate intake, intravenous glucose, prevention of exposure to harmful drugs, and screening of family members to detect gene carriers of AIP. Haematin may be administered during the acute attack to reduce hepatic ALA synthase activity but there is no statistical evidence of its clinical efficacy in this disorder.

1.7.4 Hereditary Coproporphyria (HCP)

HCP is a milder disorder than AIP, but may cause similar neurovisceral symptoms. It is associated with overproduction of coproporphyrinogen III and excretion of the porphyrin precursors ALA and PBG. The genetic defect of HCP is reflected in a 50% deficiency of coproporphyrinogen III oxidase inherited as an autosomal dominant trait. Clinical expression of HCP as with AIP is dependent upon metabolic, chemical and environmental factors with clinical management similar to that for AIP. In HCP, coproporphyrinogen III accumulates in the liver and is also found in plasma and skin accounting for the photosensitivity observed in some patients.

1.7.5 Variegate Porphyria (VP)

VP has been recognised in many populations but is most common in South African whites. Inheritance is autosomal dominant and the underlying genetic defect causes a deficiency in the activity of either protoporphyrinogen oxidase or ferrochelatase. As is the case in AIP and HCP, hormones, drugs and metabolic factors influence clinical expression of the gene

defect. Clinically expressed VP is characterised by neurovisceral symptoms as in AIP. Photosensitivity due to the accumulation of porphyrins in plasma and skin is more common than in HCP. Clinical management is similar to that for AIP and HCP.

1.7.6 Porphyria Cutanea Tarda (PCT)

PCT is the most common form of porphyria. It usually begins in middle or late adult life with males affected most frequently. Patients with PCT have mild to severe photosensitivity and often liver disease. Alcohol, oestrogens and hepatic siderosis are predisposing factors. A deficiency of hepatic uroporphyrinogen (URO) decarboxylase activity characterises all cases of PCT. In PCT patients with the less common genetic (familial) form of this disease, the enzyme deficiency can also be demonstrated in erythrocytes, lymphocytes and cultured skin fibroblasts. This form of PCT is autosomal dominant. In most cases of PCT there is no family history of the disease (sporadic). Some studies have suggested that there is an inherited URO decarboxylase defect expressed in red cells as well as hepatic cells in all PCT patients, while others have reported that patients with sporadic PCT have abnormal enzyme activity only in the liver. The deficiency of hepatic URO decarboxylase in PCT results in a complex pattern of porphyrin excretion which includes excess production of the isocoproporphyrin series, a distinctive feature of this disease.

A second type of porphyria, believed to be related to PCT, has been termed "hepatoerythropoietic porphyria". Only 15

cases have been reported in the world literature to date and HEP is thought to represent a homozygous form of familial PCT (see Chapter 7).

1.8 THE REGULATION OF HEPATIC HAEM BIOSYNTHESIS

Hepatic haem synthesis has been well characterised, facilitated by the homogeneous nature of hepatic tissue and the detailed study of the porphyrias. However, liver is a relatively minor site of haem biosynthesis producing approximately 15% of total body haem (Granick and Sassa, 1971; Hutton and Gross, 1970) with the remainder produced in the bone marrow (Jones et al, 1971; Berk et al, 1976). Hepatic haem is utilised as the prosthetic group for a number of apoproteins including mitochondrial cytochromes (16%), catalase (16%), cytochrome P_{450} (50%), cytochrome B_5 (16%) and tryptophan pyrrolase (5%) (Badaway, 1979; Marver and Schmid, 1972). Many of these haemoproteins undergo rapid turnover thereby necessitating a continuous high rate of hepatic haem synthesis. The ability of the liver to rapidly synthesise haem is particularly dramatised by cytochrome P_{450} which is of critical importance in the detoxification and biotransformation of many drugs and chemicals. Thus, in liver cells, control of haem synthesis must be finely regulated in order to cope with insidious adverse changes in the environment.

Control of hepatic haem synthesis occurs at the first step of the haem biosynthetic pathway i.e. the condensation of succinyl CoA and glycine to form ALA catalyzed by ALA synthase (Granick and Urata, 1963; Granick and Beale, 1978; Meyer and

Schmid, 1978; Tait, 1978). Under basal conditions, hepatocyte ALA synthase activity is low but sufficient to maintain an adequate supply of microsomal haem (Hutton and Gross, 1970; Sassa and Kappas, 1981). Many compounds can induce ALA synthase activity in avian and mammalian liver including various lipophilic chemicals and drugs and some natural and synthetic steroids (Granick, 1966). For example, certain chemicals such as 2-allyl-2-isopropylacetamide (AIA) (De Matteis, 1970; 1971; 1978) destroy cytochrome P_{450} haem, leading to the accumulation of abnormal "green" pigments in the liver; 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or griseofulvin cause a rapid inhibition of ferrochelatase (De Matteis et al, 1973; Rifkind, 1979; Anderson, 1978) leading to decreased haem formation; while phenobarbital reduces the concentration of regulatory "free" haem (in the range 10^{-8}-10^{-7}M) by increased synthesis of apocytochrome P_{450} (Baron and Tephly, 1970; Rajamanickam et al, 1975). Experimental depletion of haem by these methods causes ALA synthase induction, while conversely, excess cellular haem inhibits hepatic ALA synthase activity. Enzyme regulation by haem is achieved in several different ways:-

(1) Direct inhibition of ALA synthase activity (Tait, 1978)

(2) Inhibition of mRNA synthesis for ALA synthase (Whiting, 1976)

(3) Inhibition at a post-transcriptional step (Sassa and Granick, 1970)

(4) Inhibition of the enzyme transfer from the cytosol to the mitochondrial inner membrane (Hayashi et al, 1972)

The concept of a **regulatory haem pool** (0.1μM) was first suggested by Granick et al (1975) and its existence is now

generally accepted. This haem pool has two major regulatory effects, each of which results in a net decrease of its concentration. One effect is inhibition of functional mitochondrial ALA synthase and the other is induction of haem oxygenase. ALA synthase and haem oxygenase therefore exhibit a reciprocal relationship in hepatic tissue.

Haem synthesis may also be affected by other factors, for example, the rate of ALA formation may be limited by lack of substrate or cofactor. ALA synthase has a high Km value for glycine and experimental depletion of hepatic glycine reduces the effect of ALA synthase (Tephly et al, 1973). Enzyme activity may also be decreased by pyridoxine deficiency or inhibitors of pyridoxine metabolism because PLP is an essential cofactor for ALA synthase activity (Shemin and Russell, 1953).

1.9 THE ROLE OF HAEM IN HAEM BIODEGRADATION

Multiple factors influence haem catabolism, including haem, Hb, sex steroids, heavy metals, hormones and drugs (Pimstone et al, 1971; Tenhunen et al, 1970; 1984; Gemsa et al, 1973; Kikuchi and Yoshida, 1983; Bakken et al, 1972). Induction of haem oxygenase by haem or Hb has been reported in macrophages, the arachnoid and choroid plexes of the brain, the kidney and the liver. Spleen has the highest activity, followed by bone marrow then liver (Tenhunen et al, 1970).

The inter-relationship between hepatic haem synthesis and catabolism has never been fully elucidated, although studies have shown reciprocal oscillations in hepatic ALA synthase and

haem oxygenase in vivo following intravenous haem administration (Schacter et al, 1976). This is explained as an initial repression of ALA synthase mRNA by haemin, then a succeeding induction of haem oxygenase followed by decreased cellular haem and derepression of ALA synthase (Bissell and Hammaker, 1976; Ibraham et al, 1979).

The effect of haem on haem oxygenase activity in other tissues, such as haemopoietic bone marrow cells, remain uncertain.

1.10 ERYTHROPOIETIC HAEM BIOSYNTHESIS

Less extensive investigations have characterised haem biosynthesis in erythropoietic tissue as contrasted with the liver. Many mysteries therefore remain with regard to our understanding of bone marrow erythroid haem synthesis, yet it is the major haem-forming tissue (Berk et al, 1976) synthesising 80% of total body haem. The principal difficulties which have hampered the study of haem biosynthetic and degradative pathways in the immature erythron are the association of bone marrow erythroid cells with a variety of other cells; the dynamic state of red cell precursors as a continuously developing tissue; the various stages of differentiation and the lack of sensitive specific enzyme assays. It is, however, thought that the regulation of haem synthesis is closely linked to erythropoiesis and may be different from that in liver.

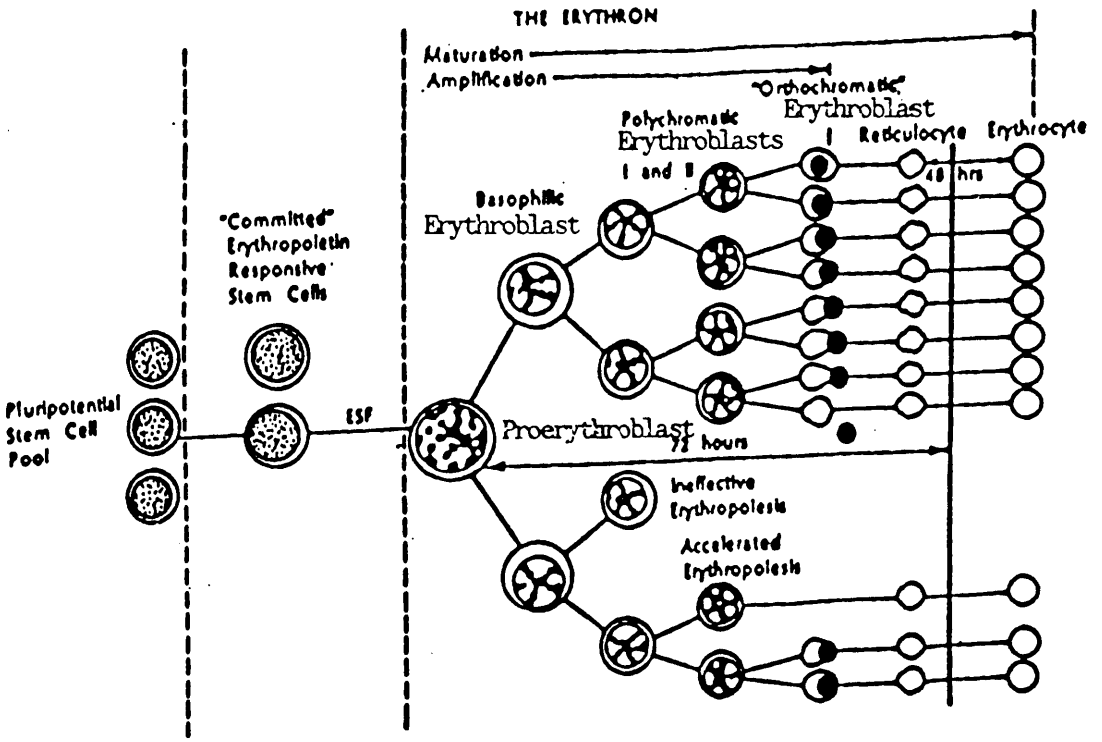
1.10.1 Erythropoiesis

Mature erythrocytes arise from a series of differentiation

and maturation steps starting from the pluripotent stem cell (Figure 6). The pluripotent stem cell has the capacity for self replication as well as for differentiation into granulocyte, monocyte, platelet or erythroid cell lines. The most striking molecular event during erythroid differentiation is the accumulation of Hb. While other proteins accumulate progressively, e.g. catalase, the haem enzymes accumulate during early maturation and diminish progressively (Freshney and Paul, 1972). The loss of nuclear material and mitochondria occurs late in the maturation sequence just prior to reticulocyte formation. The study of morphologically recognisable marrow erythroid progenitors has provided much information about the terminal steps of both normal and disordered erythropoiesis and the associated haem synthesis. However, early differentiation and maturation events occur in cells which are not morphologically identifiable and this has been a major limitation with regard to gaining a full understanding of the earliest events in erythropoiesis. We are still to a large extent in the dark regarding the control, organisation and sequence of biological change responsible for the conversion of the round nucleated haematopoietic stem cell to the mature anucleate disc-shaped erythrocyte.

1.10.2 Systems Available for the Study of Erythropoiesis

Workers have employed a variety of techniques and models to study erythropoietic haem synthesis. The range of cells and tissues is wide and includes chick blastoderm (Levere and Granick, 1965), foetal mouse liver (Freshney and Paul, 1971),



NOTE:

Figure 6 depicts the erythron and maturation and amplification of the red cell series. Pluripotent stem cells are recruited into the pool of committed erythropoietin-responsive stem cells. Under the stimulus of erythropoietin and other factors (Section 1.11.1) committed cells differentiate into proerythroblasts. Normal maturation occurs by four mitotic divisions during which the proerythroblast matures to the reticulocyte, passing through three stages: the basophilic (early) erythroblast, polychromatophilic erythroblasts 1 and 2 (intermediate), and the orthochromatic (late) erythroblast which by ejection of its nucleus emerges as a reticulocyte. Amplification results from maturational divisions with each erythroblast producing 16 reticulocytes in a process lasting 72 hours. In the ensuing 48 hours, the circulating reticulocyte matures into an erythrocyte. Ineffective erythropoiesis, with failure of erythroblast maturation and intramedullary cell destruction, and accelerated erythropoiesis, with skipped maturational divisions are also depicted.

FIGURE 6:
THE ERYTHRON

erythroid leukaemia cell lines, FEL (Sassa, 1976; Beaumont et al, 1984) and K562 cells (Hoffman et al, 1980), rabbit (Ponka and Schulman, 1985) and human reticulocytes (Gardner and Cox, 1988) and 'in vitro' bone marrow cultures (Ibrahim et al, 1982). However, techniques recently developed now allow the study of purified, age matched human bone marrow erythroid cells (Section VI).

Functional 'in vivo' and 'in vitro' progenitor cell assays, erythroid marrow cultures and established erythroid cell lines have permitted the study of early differentiation steps and have proved useful in the study of haem synthesis.

Progenitor Cell Assays

(1) 'In Vivo' Systems

'In vivo' systems involve manipulation of intact experimental animals with drugs, radiation, bleeding or hypertransfusion. This is followed by 'in vitro' erythroid progenitor cell assays to assess the commitment of tripotent marrow stem cells to erythroid development. The **erythroid burst forming units (BFU-E)** develop from the stem cells and then mature further to cells from which the **erythroid colony forming units (CFU-E)** develop.

The original 'in vivo' assay for progenitors capable of giving rise to differentiated haematopoietic cells was the spleen colony-forming assay (Till and McCulloch, 1961). When normal mouse marrow cells were injected intravenously into lethally-irradiated syngeneic hosts, macroscopic colonies of haematopoietic cells appeared in the spleens of the recipients by 10-14 days. Irradiated animals, not rescued by marrow

cells, died of haematopoietic failure. It was later demonstrated that these complex colonies, which contained all of the usual elements found in the blood, arose from a single multipotent progenitor termed the **spleen colony-forming unit (CFU-S)** (Becker et al, 1963). Successful 'in vitro' growth of specific murine erythroid colonies (Stephenson et al, 1971) required the addition of various factors. This was later extended to human erythroid cultures to provide comparable information.

(ii) 'In Vitro' Assays

'In vitro' assays are of two types. The usual assay involves cultures of haematopoietic tissues in semi-solid media containing methylcellulose (Iscove et al, 1974) or in plasma clots (McLeod et al, 1974). These assays provide conditions which allow terminal differentiation of cells already committed to differentiation rather than allow initiation of differentiation in the multipotent stem cell. The phenotype of the erythroid progenitor is "fixed" at the stage of maturation at which they existed in the marrow or peripheral blood when they were removed for culture.

A second type of 'in vitro' assay, a continuous liquid culture method, depends upon an adherent cell matrix (Eastment et al, 1982) and permits a measure of number, replication and maturation of even earlier progenitors. This system developed by Dexter and associates (Dexter and Lajtha, 1974; Testa and Dexter, 1977) is useful in studies of murine erythroid maturation.

Erythroid Cell Lines/Cultures

(i) Friend Erythroleukaemia (FEL) Cells

Friend leukaemia virus-infected erythroid precursor (FEL) cells are an immortalised cell line of mouse origin which grow in suspension culture as undifferentiated haemocytoblasts, but undergo erythroid differentiation when treated with dimethylsulphoxide, (DMSO) (Friend et al, 1971), other aprotic solvents (Sassa et al, 1975; Tanaka et al, 1975), butyric acid (Leder and Leder, 1975) or haem (Friend et al, 1971). DMSO-treated FEL cells show the characteristics of erythroid cells as judged by the histochemical staining of Hb in the cytoplasm (Friend et al, 1971), the incorporation of ^{59}Fe into haem (Friend et al, 1971), the fluorometric quantitation of haem (Sassa et al, 1975), the absorption spectra of the cell extract (Friend et al, 1971) and the characteristic electrophoretic migration pattern of mouse Hb on polyacrylamide gels (Scher et al, 1971). DMSO-treated FEL cells form a mouse erythrocyte membrane antigen by the 4th day of culture (Sugano et al, 1973) and become agglutinable with phytohaemagglutin (Fujinami et al, 1973). These murine erythroleukaemia cells thus represent a useful model of erythroid differentiation in tissue culture which cannot be achieved using cells of normal haematopoietic origin.

There is, however, a serious limitation to the use of FEL cells as a model of normal erythroid differentiation. In nonerythroid cells, transferrin receptors are correlated with cell proliferation (Larrick and Creswell, 1979; Sutherland et al, 1981; Trowbridge and Omary, 1981). Cultured transformed cells (Neckers, 1984) and malignant cells 'in vivo' (Faulk et

al, 1980; Aulbert et al, 1980) have high numbers of transferrin receptors. Furthermore, malignant cells contain high levels of ferritin (Drysdale et al, 1977) which probably results from an increase in transferrin-receptor-mediated iron uptake. Therefore, the basal level of iron transport and the amount of iron stored in uninduced FEL cells may be considerably higher than that found in the normal physiological equivalent.

(ii) K562 Cells

K562 cells are often referred to as the human equivalent of FEL cells (Schulman et al, 1981; Frazier et al, 1982; Klausner et al, 1983). Most of the iron taken up by these cells is incorporated into ferritin and only 10% is recovered in haem (Schulman et al, 1981). Although K562 cells are inducible for Hb synthesis with haemin they are ill-defined cells originating from a patient with chronic myeloid leukaemia (Lozzio and Lozzio, 1975). Moreover, Hb production following haemin addition is entirely of embryonic or foetal types (Rutherford et al, 1979) and ALA dehydratase induced in K562 cells is immunologically distinct from the enzyme of normal adult or foetal erythrocytes (Chang and Sassa, 1985). K562 cells are not therefore an ideal model of normal erythroid differentiation with regard to the regulation of haem synthesis.

(iii) Reticulocytes

Reticulocytes do not contain DNA, but may contain some mitochondrial fragments, ribosomes and Golgi bodies. They are

the only system of pure erythroid cells readily available and easily obtained from peripheral blood. However, since they are anucleated they can only be used to investigate the posttranscriptional aspects of haem synthesis and iron transport. Nevertheless, reticulocytes have been extensively used to study erythroid haem regulation despite the fact that at the reticulocyte stage of development erythroid cells have all but stopped synthesising haem and Hb.

(iv) Isolated Bone Marrow Erythroblasts

A major obstacle to the study of human erythropoiesis has been an inability to achieve the physical separation of erythroid cells at different stages of development, both from one another and from cells of different types. Separation must also provide sufficient cell numbers to permit accurate biochemical analysis. To date, the most satisfactory method of cell separation is density sedimentation whereby separation depends on the continual decrease in cell size and corresponding increase in density during erythroid development. This has allowed the fractionation of bone marrow cells into distinct cell populations of similar differentiation status (Ali et al, 1982, 1983; May et al, 1982). However, this stage alone does not provide highly purified erythroid populations without white cell removal. Prior to fractionation, bone marrow cells may be treated with a monoclonal antibody (TG-1), cytotoxic for myeloid (white) cells (Beverely et al, 1980), which results in an increase in erythroid purity from approximately 20% to 80%. Fractionation of the remaining cells then produces erythroid subpopulations with erythroid purity of

up to 95% (Chapter 2).

1.11 THE REGULATION OF ERYTHROPOIESIS

Erythropoiesis is a complicated process which is influenced by hormones, metabolites, growth factors and cellular interactions. Erythroid development and haem biosynthesis are inextricably linked. To maintain a suitable perspective on the role of haem in erythropoiesis, the regulation of erythropoiesis in a more general sense must first be considered as it is unlikely that any one factor has an overiding effect on the development of erythroid cells.

The differentiation sequence of committed erythroid stem cells has been identified by 'in vitro' culture techniques. Although CFU-E cells themselves are not morphologically identifiable, each class of progenitor can be separated by physical and functional properties (Ogawa et al, 1977a).

Stem cell → primitive BFU-E → mature BFU-E → CFU-E → proerythroblast.

Further development then takes place producing the first morphologically identifiable erythroid cell (the proerythroblast).

Proerythroblast → early erythroblast → intermediate erythroblast → late erythroblast → reticulocyte → erythrocyte.

One view of stem cell differentiation sees the microenvironment as having an instructive role in causing a stem cell to become committed to a particular blood cell

lineage (Trentin, 1970; Wolf, 1978) through mechanisms such as cell-cell interactions or short range 'inducers'. Critics of this microenvironment theory have shown that the way in which a stem cell proliferates or becomes committed can be explained in terms of a purely random genetic process (Till et al, 1964; Humphries et al, 1987). It has been argued that the various haematopoietic tissue environments influence blood cell production purely by facilitating the proliferation of particular kinds of committed precursor cells.

The question as to how the haematopoietic microenvironment facilitates the proliferation and/or differentiation of particular cell types remains open. Both the earliest precursor cells and the various intermediate cells however require growth factors to sustain their proliferation and differentiation. Such factors are often produced by other haematopoietic or stromal cells in the tissue environment and it is clear that the regulation of haematopoiesis involves complex interacting cell networks. Purified growth factors cannot mimic the local effects of the microenvironment indicating that cell-cell contact is necessary.

1.11.1 Factors Influencing Erythropoiesis

Erythropoiesis is influenced by systemic factors such as **erythropoietin** and by local factors such as **burst promoting activity (BPA), colony stimulating factor (CSF)** and **cell-cell interaction.**

The hormone **erythropoietin (Epo),** a glycoprotein produced in the juxtaglomerular cells of the kidney, has a major influence on erythropoiesis. It is essential to erythroid

development and differentiation and binds to specific cell surface receptors. Erythroid progenitors show increased sensitivity to Epo with increasing maturity (Ogawa et al, 1977a). The haem enzymes in committed cells are also affected by Epo, in particular ALA synthase and PBG deaminase (Abraham et al, 1989), as are the globin genes (Sahr and Goldwasser, 1985). Expression of haem metabolic enzymes has been shown to be a prerequisite for erythroid cell development and differentiation in both FEL cells (Sassa, 1976) and human bone marrow (Brown et al, 1988). **Burst promoting activity (BPA)** acts with Epo to induce the maturation of tripotent stem cells to immature erythroid progenitors (Eliason, 1982; Iscove, 1978). **Colony stimulating factor (CSF)** has a suppressive effect on BFU-E and is competitive with respect to Epo (Van Zant and Goldwasser, 1977a; Van Zant and Goldwasser, 1979). A variety of hormones, including β-adrenergic agonists, thyroid hormones, growth hormone and androgenic/nonandrogenic steroids interact with Epo to enhance the cloning efficiency of BFU-E and CFU-E from a variety of species. Only the 5β-steroids may act independently of Epo. A common phenomenon of 5β-steroids on CFU-E formation in rodent (Singer et al, 1976) and human (Urabe et al, 1979) bone marrow is the induction of ALA synthase with a subsequent increase in haem (Granick and Kappas, 1967; Levere and Gidari, 1974). On a molar basis steroid metabolites are 100 fold more active in stimulating erythroid colony formation from human bone marrow cells than they are in inducing ALA synthase or haem formation in avian liver cells. This sensitivity of bone marrow cells to steroids

including glucocorticoids extends to rats (Singer et al, 1976), rabbits (Fischer et al, 1978) and man (Gold et al, 1976) suggesting an intrinsic biological characteristic of these cells not evident in hepatic cells. Differences of erythrocyte count, Hb concentration and haematocrit exist between men and women and may result from the effect of gonadal steroid hormones (Kappas and Palmer, 1963) as well as differences in body iron content. The presence of such hormones can reduce the requirement for Epo in culture by up to 90% (Udupa et al, 1986).

The mechanism by which various hormones have their effect is mediated by mature accessory cells also present in the bone marrow such as T lymphocytes, monocytes and macrophages. Although an apparent effect of T cells on erythroid growth in culture can be demonstrated (Zanjani and Kaplan, 1979), further clarification is hampered by the lack of adequate techniques to remove other interacting cell populations. In addition to having a regulatory role in normal erythropoiesis, activated T cells (Nedwin et al, 1985) and T cell-derived lymphokines (Zoumbos et al, 1985; 1985) have been implicated in various types of bone marrow failure. T cells produce interleukins (Burdach and Levitt, 1987) and other lymphokines e.g. interferons (Broxmeyer et al, 1985) and tumour necrosis factor (Broxmeyer et al, 1985). Monocytes and macrophages are at least one source of CSF (MacVittie and Walker, 1978). BPA, humoral stimulating and humoral inhibitory activity (HSA/HIA) (Burke and Karp, 1987; Blackburn and Patt, 1980; Toksoz et al, 1980) and erythroblast enhancing factor (EEF) (Krystal, 1983) are derived from the stromal cell population. The role of such

factors in erythropoiesis is currently under investigation. Lithium has been implicated in the control of erythrocyte production (Barr et al, 1987) and may act via inhibition of suppressor T lymphocytes (Bille et al, 1975; Chatelain et al, 1983).

Although animal models are useful in the study of mammalian erythropoiesis the further development of existing techniques for the study of human bone marrow erythropoiesis is necessary. Separation of erythroblasts into age-matched populations of equivalent differentiation status would permit such studies and allow analysis of haem enzyme activities, haem formation and iron incorporation. The regulation of erythropoiesis involves many complicated interactions and a multiplicity of factors. Deregulation of erythropoiesis may result from alterations in one or more of the components of the regulatory system. As haem synthetic activity is dependent upon the stage of erythroblast development, any factor that affects the control of erythropoiesis will then also affect haem synthesis. Conversely, while haem is known to have a regulatory role in erythropoiesis (Section 1.13), factors that influence haem synthesis then must also influence erythropoiesis.

1.12 THE REGULATION OF ERYTHROID HAEM BIOSYNTHESIS

Haem is produced by erythroid cells primarily to complex with globin as Hb. This process is finely coordinated such that free haem controls the initiation of globin gene expression (Harrison, 1984). Haem complexed with globin is preserved in circulating erythrocytes for 120 days. As such,

it does not require the same sensitive control that regulates hepatic haem production with a $T^1/_2$ for haem of only a few days (Robinson, 1975). Haem synthesis in erythroid cells also differs from that in hepatic tissue in being inextricably linked to tissue differentiation (Fitzsimons et al, 1986, 1988). During differentiation mitochondria and hence mitochondrial haem enzymes are lost. At the stage of reticulocyte formation few cells have intact mitochondria (Section 1.10). It must then be stressed that it is difficult to distinguish primary effects on haem biosynthesis from effects that are primarily linked to differentiation. Hb synthesis in erythroid cells is dependent on iron uptake (Ponka and Schulman, 1986) and the co-ordinate regulation of haem and globin biosynthesis. The very structure of haem as Fe-protoporphyrin demands close links between iron uptake, intracellular iron transfer and the haem biosynthetic pathway.

1.12.1 The Effect of Haem on Erythroid ALA Synthase

It would seem as if erythroid ALA synthase activity is controlled in a different manner to the hepatic enzyme. This control has two separate components : the development of enzyme activity as haem synthesis becomes established during differentiation and the short term regulation of activity in cells that are actively synthesising haem. Exogenous haem may have contradictory effects on two types of erythroid cells studied. In undifferentiated erythroid cells, haem has been shown to induce ALA synthase (Granick and Sassa, 1978; Elder, 1981; Abraham and Levere, 1990) whereas in differentiated cells e.g. reticulocytes, haem appears to inhibit ALA synthase

(Bruns and London, 1965; Ibrahim et al, 1978). The evidence is not yet however clear as even within the same cell type e.g. FEL cells, both positive and negative effects of haem on ALA synthase in early erythroid precursors have been demonstrated (Beaumont et al, 1984; Lu and Broxmeyer, 1983). Therefore, unlike liver cells, feedback inhibition of haem biosynthesis by haem at the level of ALA synthase may not be the major control mechanism in erythroid cells (Elferink et al, 1988).

This difference may in part be explained by the existence of specific liver and erythroid isoenzymes of ALA synthase as have been identified in guinea pig (Bishop et al, 1981), chicken (Watanabe et al, 1983) avian cells (Riddle et al, 1989) and man (Bishop, 1990). The concept of tissue-specific isoenzymes for ALA synthase had been disputed by various workers (Elferink et al, 1987; Schoenhaut and Curtis, 1986). They instead favoured the view that tissue specific regulation of a single gene could be achieved by use of a separate promoter in each cell type (Shaw et al, 1985) or that transcription could occur from two different but highly homologous genes differing only at the 5' end of the RNA or in untranscribed or untranslated regions. However, the recent publication of reliable genetic data has now confirmed the existence of tissue-specific isoenzymes in hepatic and erythroid tissues (Chapter 3, Section 3.3). The hepatic ALA synthase gene has been localised to chromosome 3 (Sutherland et al, 1988) and the erythroid ALA synthase gene to chromosome X (Cox et al, 1990).

1.12.2 The Role of Other Haem Enzymes in Erythroid Haem Control

It has been shown that during erythroid differentiation the activity of not only ALA synthase but also of the other enzymes in the haem biosynthetic pathway increases. It has been demonstrated in FEL cells treated with DMSO and in normal human bone marrow cells treated with Epo (Sassa, 1976; 1980) that a sequential induction of enzymes of the haem biosynthetic pathway occurs. It has therefore been suggested that ferrochelatase, the final enzyme of the pathway, may be rate limiting for haem formation. Whether ferrochelatase was truly rate-limiting in these circumstances remains debatable as an enzyme may only be considered to be rate limiting if the enzyme pathway is intact and fully developed. In this case, however, it was the late induction rather than regulation of ferrochelatase activity that was thought to limit haem production. A second group have also obtained similar results using FEL cells (Rutherford et al, 1979). Both these results have however been contradicted (Beaumont et al, 1984) by the demonstration of simultaneous rather than sequential induction of the haem enzymes in DMSO-treated FEL cells. This study suggested that iron availability rather than delayed appearance of ferrochelatase may limit haem synthesis. However, several workers have reported ferrochelatase inhibition by haemin in a variety of tissues including Rhodopseudomonas spheroides (Jones and Jones, 1970; Dailey, 1982), Spirillum itersonni (Dailey, 1977) and mammalian liver (Koller and Romslo, 1977; Dailey and Fleming, 1983) indicating a possible regulatory function of this enzyme in haem biosynthesis. This theory is examined more fully in Chapter 4 of this thesis.

Beru and Goldwasser (1985) demonstrated in rat bone marrow cells, Epo induced synthesis of PBG deaminase without any discernible effect on the other enzymes. Cells prior to the addition of Epo already contained levels of each haem enzyme sufficient to maintain haem synthesis in the subsequent few days. Activity of PBG deaminase increased linearly with Epo concentration and this increase was proportional to the increase in haem synthesis. The molecular biology of human PBG deaminase has since been investigated (Mignotte et al, 1988; 1990; Chretien et al, 1988; Raich et al, 1989) leading to the discovery of two promoter sequences preceding the PBG deaminase gene. The additional promoter sequence is used only in erythroid tissue and has been shown to have sequence homology to the promoter sequence for the β-globin chain gene (Mignotte et al, 1990). This suggests that PBG deaminase and β-globin chain production may be coordinated. It has also been shown that different isoforms of PBG deaminase are encoded by distinct tissue-specific mRNAs in human liver and erythroid tissue (Grandchamp et al, 1987). Both are, however, transcribed from the same gene (Raich et al, 1986). Hence, separate promoters of a single gene account for the tissue-specific and tissue-nonspecific mRNA transcripts and their respectively encoded enzymes. In erythroid tissue PBG deaminase and PBG oxygenase, which oxidises PBG to 5-oxo-pyrrolines, exhibit a reciprocal relationship (Frydman et al, 1986). PBG oxygenase may therefore represent a counter-regulation of PBG for haem synthesis. It is of interest that this enzyme contains one haem and two molecules of non-haem iron (Frydman et al, 1988).

1.12.3 The Role of Haem on Iron Uptake

The relationships between intracellular haem, haem synthesis and iron uptake are also unclear. It has been suggested that the regulation of erythroid haem biosynthesis may be dependent upon the effects of haem on transferrin iron release (Ponka and Schulman, 1985) or on the development of transferrin receptors on the erythroid cell membrane (Wilczynska et al, 1984; Pelicci et al, 1982). Iron overload has also been shown to increase bone marrow ALA synthase, reduce ALA dehydratase and increase haem oxygenase activity (Abraham et al, 1985). Erythroid iron uptake is increased by inhibitors of haem synthesis such as isoniazid (INH), an antipyridoxal agent (Bottomley, 1982) and succinylacetone (Ponka et al, 1982), an inhibitor of ALA dehydratase (Tsudy et al, 1981; Sassa and Kappas, 1983). However, the theory that haem controls haem synthesis by regulating the release of iron from transferrin has not been completely accepted and has been challenged by several workers who have demonstrated normal iron uptake under conditions of reduced haem synthesis (Ibrahim et al, 1978; May et al, 1982a, 1982b). The proposed inhibition of iron uptake by haem also seems to occur only in erythroid cells. It has been observed in reticulocytes (Ponka and Neuwirt, 1969; 1971; Ponka et al, 1974; Schulman et al, 1974; Fielding and Speyer, 1975; Garrick et al, 1978; Ponka and Schulman, 1986), foetal mouse liver erythroblasts (Malik et al, 1979), human erythroblasts (May et al, 1982) and Hb-synthesising FEL cells (Granick and Sassa, 1978; Malik et al, 1979) but <u>not</u> with uninduced FEL cells (Malik et al, 1979),

transformed lymphoblastoid cell lines of human origin (Schulman et al, 1981), uninduced K562 cells (Schulman et al, 1981) and rat hepatocytes.

It has also been argued that "free" intracellular haem (not complexed with globin) may limit haem synthesis by regulating the availability of iron to protoporphyrin (Neuwirt et al, 1972; Ponka et al, 1974). At present, however, we know little about the mechanisms within the cell which ensure that iron is presented to protoporphyrin as Fe^{2+} ions and not Fe^{3+} ions (Chapter 4, Section 4.5.4).

In summary, it would appear that the regulation of erythroid haem synthesis may be different from that known to operate in hepatic tissue. Hepatic ALA synthase is the rate controlling enzyme of the haem biosynthetic pathway and is under negative feedback control by haem. However, in erythroid tissue both positive and negative effects of haem on ALA synthase activity have been observed. Negative feedback by haem at ALA synthase may not therefore be the major control mechanism in erythroid haem synthesis. This may be explained by the existence of tissue specific isoenzymes for erythroid and hepatic ALA synthase. In erythroid cells other enzymes of the pathway apart from ALA synthase may be induced in response to factors which stimulate haem synthesis. Possible roles for the enzymes ferrochelatase and PBG deaminase in the regulation of erythroid haem biosynthesis have been suggested. Other workers have supported a role for haem in the regulation of its own synthesis via control over iron acquisition and not via any

direct effect on haem enzymes.

It is apparent that further investigation into the mysteries of erythroid haem biosynthesis is required. For the moment, however, many questions must remain unanswered and open to speculation.

1.13 THE ROLE OF HAEM IN ERYTHROPOIESIS

Haem is known to affect the erythroid differentiation process and has been reported to accelerate haemoglobinisation of erythroblasts in bone marrow cell cultures (Bonanou-Tzedaki, 1981). Haem is necessary for globin synthesis in reticulocytes and erythroblasts (Bruns and London, 1965), for the binding of globin mRNA to ribosomes and for the initiation of globin synthesis in intact cells and cell-free systems (Zucker and Schulman, 1968; Adamson et al, 1969). It has been shown to decrease DNA synthesis and cell division in both mature and immature erythroblasts and to induce globin mRNA synthesis in the immature precursors. Haem has also been shown to enhance the 'in vitro' growth of primitive erythroid progenitor cells. Several workers have reported the greatest effect on BFU-E (Monette and Holden, 1982; Lu and Broxmeyer, 1983) while others report the major effect to be on CFU-E (Porter et al, 1979; Ibrahim et al, 1982). The conflicting results may however simply be a reflection of the different assay systems used.

A haemin-induced specific enhancement of both marrow BFU-E numbers and cell cycling within 6 hours of haemin administration has been demonstrated 'in vivo' (Monette et al, 1984). No effect was observed on CFU-E. Similar results have also been supported by other workers (Iscove, 1977; Suzuki and

Axelrad, 1980; Adamson et al, 1978). It has been suggested that neither haemin, Epo nor BPA can substitute for each other and all appear necessary for optimal BFU-E derived colony formation (Lu and Broxmeyer, 1983). This view has, however, been disputed (Holden et al, 1982).

A haemin-controlled translational repressor (HCR) system is a well established process for the regulation of protein synthesis in reticulocytes (Gross, 1980) but has not been demonstrated in immature erythroid cells. The HCR inhibits the translation of globin protein. Haemin inactivates the HCR and thus permits Hb synthesis to occur and enhances the synthesis of other erythrocyte proteins (cytoplasmic and membrane) (Deutsch and Blumenfield, 1977). Likewise, protein synthesis in FEL cells is augmented by haemin as is the synthesis of globin mRNA (Dabney and Beaudet, 1978; Ross and Sautner, 1976; Hoffman and Ross, 1980). This suggests that haemin may act at both translational and transcriptional levels.

The role of haem biosynthetic and degradative enzymes in erythroid colony development and the effects of haemin have been examined (Ibrahim, 1982). The increase in haem enzymes induced by haemin was suppressed by the addition of cycloheximide suggesting that the translation of mRNA for the haem enzymes is necessary for erythroid differentiation. Furthermore, a reduction in haem oxygenase activity and hence increased availability of haem acts as a trigger for CFU-E development and differentiation. The well recognised reciprocal increase in ALA synthase activity in liver cells as a result of haem oxygenase induction (Ibrahim et al, 1974) is

not applicable in bone marrow erythroid cells. These results demonstrate that decreased haem oxygenase activity and increased intracellular haem are associated with the development and differentiation of erythroid-colony cultures. They also show that the mechanisms which control ALA synthase and haem oxygenase in marrow are different from those in liver.

1.14 HAEM BIOSYNTHESIS IN OTHER TISSUES

Further observations in other non-hepatic tissues have been limited, but so far imply that the control mechanism of haem biosynthesis may again be different from that in liver. For example, neither potent inducers of hepatic ALA synthase nor haemin influence ALA synthase activity in the harderian gland in mice (Margolis, 1971); heart (Briggs et al, 1976), adrenal gland (Condie et al, 1976), testis (Tofilon and Piper, 1980) and spleen (Wada et al, 1967) of rat or in cultured human haemin-treated amniotic cells (Sassa et al, 1974). Alternatively, however, specific hormones increase ALA synthase activity in non-hepatic target tissues. For example, adrenocorticotropic hormone increases ALA synthase activity in the adrenal gland (Condie et al, 1976); human chorionic gonadotropin increases the enzyme activity in the testis (Tofilon and Piper, 1980); and erythropoietin increases ALA synthase in the spleen (Wada et al, 1967). In addition, cobalt chloride treatment produces a marked decrease in cardiac ALA synthase in rats that is of longer duration than that seen with liver (Maines and Kappas, 1977; Sedman and Tephly, 1980). Fasting increases ALA synthase in the adrenal gland, but decreases the enzyme activity in the heart (Condie et al,

1976). Special features of ALA synthase have also been reported in brain (Paterniti et al, 1978). Significantly, ALA synthase activity is normal in mitogen-stimulated acute intermittent porphyria (AIP) lymphocytes (Sassa et al, 1978) and skin fibroblasts (Meyer, 1973; Bonkowsky et al, 1975).

1.15 DISORDERS OF ERYTHROPOIESIS

Erythropoietic disorders are traditionally divided into two groups: (1) anaemia and (2) polycythaemia. Although this division is based on the presence of either too few or too many red cells, anaemia is functionally best characterised by a reduced Hb concentration and polycythaemia by a raised haematocrit. In anaemia the patient suffers from tissue hypoxia, the consequence of a low oxygen-carrying capacity of the blood. In polycythaemia the clinical manifestations are related to increased whole blood viscosity and increased blood volume, both consequences of a high haematocrit.

On the basis of determinations of the red cell mass, anaemia can be classified as:-

(i) Relative (i.e. dilutional)

Relative anaemia is characterised by a normal total red cell mass together with an increase in plasma volume.

(ii) Absolute

Absolute anaemia corresponds to a decreased red cell mass and may be divided as follows: (1) decreased red cell production; (2) increased red cell destruction; and (3) blood loss i.e. increased red cell loss.

Anaemia caused by decreased red cell production can be subdivided into (1) reduced erythropoiesis (hypoplasia); (2)

ineffective erythropoiesis characterised by increased erythropoietic activity in the bone marrow but reduced release of mature erythrocytes and reticulocytes into the circulation.

For the work described in this thesis, two particular disorders of erythropoiesis were chosen for study and their effects on erythroid haem synthesis examined.

1.15.1 Iron Deficiency Anaemia

Severe iron-deficiency anaemia is characterised haematologically by a reduced Hb concentration and red cell count in peripheral blood. The erythrocytes are microcytic due to the reduced Hb content. Bone marrow examination shows a moderate degree of normoblastic hyperplasia while developing red cell precursors show poor haemoglobinisation. The anaemia is associated with minor degrees of ineffective erythropoiesis (Robinson, 1969) and reduced red cell life-span (McKee et al, 1968).

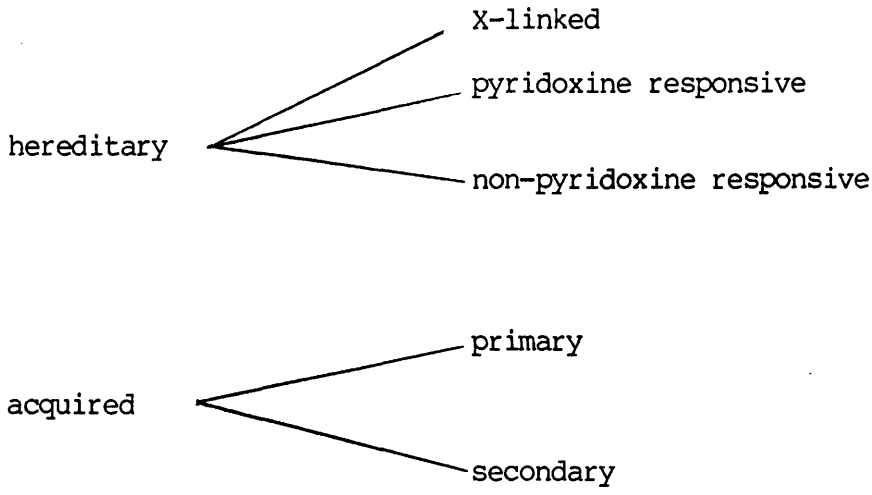
Iron deficiency gives rise to defective synthesis of haem enzymes in many tissues and may result in impaired activity of other iron-dependent systems. As yet, no consistent pattern of depletion for specific enzymes or for particular tissues have emerged (Dagg et al, 1967; Beutler, 1964). In iron deficiency there is abnormal accumulation of both free coproporphyrin and protoporphyrin within erythrocytes (Pagliardi et al, 1959). The normal balance between haem and globin production is disturbed resulting in a relative excess of globin which is broken down by red cell protease activity (Barr et al, 1972).

1.15.2 **Sideroblastic Anaemia**

Refractory anaemia, associated with hyperplastic bone marrow has been recognised for many years. These anaemias have been collectively grouped as **Myelodysplastic Syndromes (MDS)** (Bennett et al, 1981) in which marrow activity is increased but ineffective thereby leading to the association of hypercellular marrow with reduced formed elements in blood.

The sideroblastic anaemias (SA) are members of the MDS group in which the abnormality both in blood and marrow is predominantly erythroid in nature. Platelet and white cell formation are better preserved in SA than in the other MDS groups. SA is characterised by defective haemoglobinisation of red cells, ineffective erythropoiesis and an abnormal accumulation of iron in the maturing erythroblasts distributed in the form of a perinuclear ring (see photographic plate 6). This type of anaemia may be inherited or acquired. Acquired SA may either be primary or secondary, with primary acquired (PASA) the most frequent type. Secondary types of SA may be induced by drugs or alcohol or may be associated with various diseases. Anaemias of this type are similar in that the erythroblasts have an inability to utilise iron and to synthesise haem. It is widely thought that abnormal haem production is the primary abnormality in SA (Hines and Grasso, 1970).

Classification of SA:



It has, however, been suggested from studies using age-matched human erythroblasts that the primary defect in SA may be an abnormality of mitochondrial iron metabolism resulting in reduced haem synthesis. Iron has been shown to accumulate in the cell stroma and mitochondria and has been found in the cell cytoplasm as ferritin aggregates. Further studies involving the incubation of sideroblastic human bone marrow cells with radioactive iron bound to purified human transferrin (May et al, 1982) have shown normal iron uptake per erythroblast, increased iron incorporation into stroma, normal iron incorporation into ferritin but decreased iron incorporation into haem.

Examination of human bone marrow erythroblasts in PASA by electron microscopy has revealed that the accumulation of iron is located within the erythroblast mitochondria and is frequently associated with (1) a depression of RNA synthesis and (2) a more marked depression of protein synthesis (Wickramasinghe and Hughes, 1978). Aoki (1980) in a study of 69 patients with various types of PASA reported impaired

mitochondrial enzyme function in both erythroblasts and granulocytes. Mitochondrial iron loading was, however, only seen in the erythroblasts. In patients with pyridoxine-responsive anaemia, ALA synthase was the only mitochondrial enzyme found to have diminished activity, but this was found in both erythroblasts and granulocytes.

It has been shown that virtually all cases of PASA have decreased ALA synthase activity and many workers believe that this is the primary defect which leads to the accumulation of iron in the mitochondria as a consequence of decreased haem synthesis (Cartwright and Deiss, 1975; Aoki et al, 1974). Genetic evidence has indicated that in cases of congenital SA (CSA), which follows X-linked inheritance (Lee et al, 1968; Bottomley, 1982; McKusick, 1986), the defect in ALA synthase activity may be due to a mutation of the ALA synthase gene now known to be located on chromosome X (Cox et al, 1990). Nevertheless, at present it remains unclear as to whether reduced ALA synthase activity is the primary cause of reduced haem synthesis in cases of CSA or whether it is secondary to some other haematological abnormality. However, the response of CSA to pyridoxine may be seen as strong evidence in favour of decreased ALA synthase activity contributing to the anaemia since PLP is the essential cofactor for ALA synthase. Aoki (1978) has suggested that pyridoxine may exert a stabilising effect on ALA synthase in CSA by preventing a temperature dependent susceptibility of the apoenzyme to a controlling protease in erythroid cells. Alternatively, he argues that whereas CSA may be caused by increased ALA synthase breakdown, PASA is due to reduced ALA synthase formation.

1.16 AIMS OF THE WORK

Little is known of erythroid haem synthesis in the bone marrow, yet it is the major haem synthesising tissue of the body. Thus, erythroid haem biosynthesis demands further examination to define the role that haem plays in erythroblast metabolism and differentiation and the role that impaired haem synthesis plays in the pathogenesis of sideroblastic and other anaemias.

Studies of human erythropoietic haem biosynthesis have been hampered by the heterogeneity of the bone marrow cell population, the small sample sizes available and the lack of suitably sensitive assays for the haem enzymes. Previous workers have employed a variety of cells and models to represent erythropoiesis and a variety of techniques to measure the enzymes of haem biosynthesis. Accordingly, the results have been inconclusive.

It is the aim of this work to employ recently described techniques or to develop new ones to study human erythroid haem synthesis "in vivo" in both normal and abnormal purified age-matched human erythroblasts in an attempt to gain insight into the control mechanisms which operate.

The work involves the study of the two haem enzymes perhaps most likely to be involved in the regulation of erythroid haem synthesis, ALA synthase and ferrochelatase. ALA synthase, the first enzyme of the haem biosynthetic pathway, is the rate controlling enzyme of haem biosynthesis in hepatic cells but its role in erythroid tissue remains to be established. Ferrochelatase, the terminal enzyme of the haem biosynthetic pathway, is examined as this too has been

implicated in the control of erythroid haem synthesis (Sassa, 1976; Jones and Jones, 1970; Dailey, 1982, 1977; Koller and Romslo, 1977; Dailey and Fleming, 1983).

The work presented three major technical challenges:

1 Methods of erythroblast separation had to be tested and a reproducible technique adopted for regular use.

2 Sensitive assays of haem enzyme activity were required for use with isolated human erythroblasts. In particular, it was intended to adopt and further improve the sensitivity of the described radiochemical assay for ALA synthase activity (Fitzsimons et al, 1986) and to develop a novel highly sensitive radiochemical assay for ferrochelatase activity.

3 Bone marrow samples were required from a sufficient number of suitable patients.

It was proposed to examine haem synthetic enzyme activity in isolated age-matched human erythroblasts obtained from patients with:

(a) normoblastic marrow

(b) iron deficiency

(c) sideroblastic anaemia

and then to use the information obtained:

(1) to establish the order in which ALA synthase and ferrochelatase appear during erythroid differentiation and so resolve the conflict as to whether the enzyme activities appear simultaneously (Beaumont et al, 1984) or sequentially (Sassa, 1976). Furthermore, to determine whether ALA synthase (Levere and Granick, 1965) or ferrochelatase (Sassa, 1976) is rate limiting for erythroid haem biosynthesis.

(2) to examine the effects of iron deficiency on the activities of these mitochondrial enzymes during erythroid differentiation. The effects of haem deficiency on erythroblast ALA synthase activity would be compared with the inducing effect that haem deficiency is known to have on hepatic ALA synthase.

(3) to examine the effect of erythroblast iron overload (ring sideroblastic) on erythroid haem biosynthesis.

(4) to further examine ALA synthase in the hope of explaining the temperature-dependent inactivation of the enzyme as has been observed in hepatic mitochondrial matrix fraction (Beattie et al, 1985) and intact human erythroblasts (Fitzsimons et al, 1986).

(5) to examine the effect of haem therapy on ALA synthase and ferrochelatase enzyme activities in individuals with sideroblastic (and hence haem deficient) erythropoiesis.

1.17 LAYOUT OF THESIS

The early chapters of the thesis describe and discuss the methodology involved in cell separation and the measurement of haem enzyme activity. The later chapters deal with the patient studies.

Chapter 2 details the preparation of human bone marrow samples into distinct highly purified age-matched erythroid subpopulations following the removal of the myeloid cell population.

Chapter 3 describes the further improvement of an existing highly sensitive specific radiochemical assay optimised for erythroid ALA synthase activity. The stability of ALA synthase is investigated and the role of PLP as cofactor examined in an

attempt to prevent the consistently observed temperature-dependent inactivation (Beattie et al, 1985; Fitzsimons et al, 1986). The assay is applied for the first time to the measurement of ALA synthase in tissues other than erythroblasts.

Chapter 4 describes the structure and properties of ferrochelatase and discusses its possible regulatory role in erythroid haem synthesis. Chapter 5 describes in stepwise fashion the complete development of a novel highly sensitive specific radiochemical assay for the measurement of ferrochelatase activity in human erythroid cells. This work comprises the major experimental component of the thesis.

The latter chapters of the thesis are the more clinical and describe the application of these experimental techniques to the study of haem biosynthesis during normoblastic erythropoiesis and during various disease states associated with impaired haem biosynthesis.

Chapter 6 describes the pattern of ALA synthase and ferrochelatase activities during normoblastic erythropoiesis and examines the relationship between the two enzymes.

Chapter 7 describes the effects of iron and haem deficiency on ALA synthase activity in developing erythroid cells from individuals with iron deficiency. A unique opportunity arose during the time of study to examine a patient with hepatoerythropoietic porphyria (HEP). As such, the effects of haem deficiency caused by HEP on both erythroid and hepatic ALA synthase enzymes are reported in this chapter.

Chapter 8 examines ALA synthase activity during sideroblastic anaemia, a disease generally believed to be due

to an abnormality of haem synthesis, but in which the primary abnormality is unknown.

Finally, Chapter 9 details a trial of haem arginate (Normosang) as possible therapy in sideroblastic anaemia. For the first time the effects of this drug on both ALA synthase and ferrochelatase activities are examined in developing erythroid cells in sideroblastic patients. This provides an opportunity to further examine the possible control mechanisms of erythroid haem biosynthesis as exogenous haem arginate behaves in the same manner as physiological haem (Tenhunen et al, 1987).

Each individual chapter contains a substantial introduction followed by experimental details and finally a discussion of the results. In the final chapters of the thesis (6-9) particular emphasis is paid to the enzymatic responses to haem deficiency/excess in erythroid cells and this is contrasted with the information which is currently available for hepatic tissue. Further studies are also suggested.

Chapter 10 highlights the conclusions from this work and discusses future perspectives for the study of erythroid haem synthesis.

CHAPTER 2

PREPARATION OF HIGHLY PURIFIED AGE-MATCHED HUMAN ERYTHROBLASTS

...ein and Lewis, 1963).

...mmalian cells apart from mature red ...se been, but bone marrow is by far the ...g and most proliferative tissue in the body ...ever, although the erythroid cells are ...ajority of bone marrow, the myeloid ce... are also rich in haem containing enzym... ...e, notably the mitochondrial cytochrome...

...NGES IN WHOLE UNFRACTIONATED HUMAN FOET...

2 PREPARATION OF HIGHLY PURIFIED AGE-MATCHED HUMAN ERYTHROBLASTS

2.1 THE NATURE OF HUMAN BONE MARROW

Bone marrow is a dynamic and continuously developing tissue composed of cells from different lineages, at different stages of differentiation. The major cell lines are myeloid (white cells with cytoplasmic granules) and erythroid (red cells) with lesser numbers of lymphocytes, plasma cells, monocytes, histiocytes and megakaryocytes. The ratio of myeloid: nucleated erythroid cells is known as the M:E ratio and in normal bone marrow this ranges from 2.5 - 15:1 (Dacie and Lewis, 1975). In the presence of erythroid hyperplasia there is a reduction or a reversal of the normal M:E ratio.

In practice, bone marrow samples may be obtained by fine needle aspiration from the anterior/posterior iliac crests or from the sternum in relatively small volumes. Larger volumes are more difficult to obtain since aspiration of more than 0.3ml leads only to dilution of the sample with peripheral blood (Dacie and Lewis, 1984).

All mammalian cells apart from mature red blood cells synthesise haem, but bone marrow is by far the major iron utilising and haem producing tissue in the body (Berk et al, 1976). However, although the erythroid cells are responsible for the majority of haem synthesis, the myeloid cells in bone marrow are also rich in haem containing enzymes such as peroxidase, catalase and mitochondrial cytochromes.

2.2 HAEM SYNTHESIS IN WHOLE UNFRACTIONATED HUMAN BONE MARROW

Techniques for human bone marrow fractionation and

cellular isolation into distinct age matched erythroid cell populations were first described in 1983 (Ali et al, 1983). Until then most studies of human bone marrow haem biosynthetic activities had been confined to the detection of enzyme activity in unfractionated bone marrow in normal and pathological states. In these studies haem enzyme activities were assumed to reflect only that present in bone marrow erythroblasts and did not take into consideration the contribution made by the myeloid cell population. The enzyme activity was related to the sample erythroblast content and the activities detected in different samples were compared irrespective of any differences in their M:E ratios. Furthermore, in some pathological conditions the erythroblast population is more immature than that found in normal marrow i.e. left shifted. Despite evidence from a variety of alternative erythroid systems of the effect of differentiation (i.e. maturity) on erythroblast haem enzyme activities this was not considered in those studies using unfractionated bone marrow samples (Bottomley et al, 1973; Aoki et al, 1974; Konopka and Hoffbrand, 1979; Manabe et al, 1982).

2.3 CONTRIBUTION OF THE MYELOID CELL POPULATION

Measurements of haem enzyme activities in unfractionated bone marrow are not a reliable index of erythroblast enzyme activity since myeloid cells in both normal and primary acquired sideroblastic anaemia (PASA) bone marrow samples contribute almost 50% of the ALA synthase activity found in unfractionated bone marrow (Fitzsimons et al, 1986).

Previous studies of ALA synthase activity had assumed that

myeloid cells made no significant contribution towards unfractionated bone marrow enzyme activity (Takaku and Nakao, 1971; Bottomley et al, 1973; Aoki et al, 1974; 1979; Aoki, 1980; Konopka and Hoffbrand, 1979; Fraser and Schacter, 1980; Tikerpae et al, 1981; Manabe et al, 1982). This assumption may have originated from the failure to detect ALA synthase activity in normal and leukaemic leucocytes (Walters et al, 1967) and is of course further supported by the visual evidence from light microscopy that clearly shows erythroblasts to be the most active haem forming cells in bone marrow. However, the work of Walters et al (1967) was carried out prior to the development of a sensitive radiochemical assay for ALA synthase and subsequently other workers have produced contradictory evidence suggesting that myeloid cells may contribute significantly to the overall haem biosynthetic capacity of the bone marrow:-

(1) Immature, leukaemic myeloid cells can synthesise haem in significant quantities (Handler and Handler, 1972).

(2) Polymorphonuclear leucocytes contain haem-containing enzymes in particular myeloperoxidase which constitutes more than 5% of the dry weight of the cells (Suzuki et al, 1983).

As such, measurement of ALA synthase activity and other haem enzymes in whole unfractionated bone marrow may only be taken as a crude indicator of erythroblast enzyme activity. Such measurements are insensitive to the contribution made by myeloid cells and to differences in enzyme activity which occur in relation to erythroid cell differentiation.

In 1986 ALA synthase activity was measured in whole and

fractionated human bone marrow (Fitzsimons et al, 1986). That study examined haem synthesis in highly purified age matched 'in vivo' erythroblasts and now serves as the take off point from which this work has started.

In Summary

A critical feature to further progress in the study of human erythroid haem synthesis and its disorders is the ability to successfully isolate erythroid cells from the heterogeneous cell population of the bone marrow. Moreover, since the ability of erythroid cells to actively synthesise haem is chiefly dependent upon the differentiation status of the cell, it is further necessary to separate the erythroid population into smaller distinct subpopulations of similar differentiation status. Methods described to remove white cells and separate erythroblasts have now been adopted and optimised for use in this work. These methods are described in detail in this chapter and comprise of a combination of two techniques:

(1) myeloid cell lysis by monoclonal antibody

(2) separation of erythroblasts into four distinct age-matched cell populations by percoll equilibrium gradient density centrifugation.

2.4 PREPARATION OF BONE MARROW CELLS

Bone marrow cells were obtained (1-2ml) and placed directly into collecting medium (10ml, 4^{o}C) (Appendix 1). The cells were dispersed by sequential repeated aspiration through wide and fine gauge needles (microlance 19G2, 1.1 x 50;

microlance 21G, 0.8 x 40) then washed (x 3) in cold culture medium (Appendix 1). Supernatant was removed at each wash following centrifugation at 4°C at 3000 rpm for 5 minutes (Mistral 4L refrigerated centrifuge). Accurate cell counts were made using a coulter counter with channelyzer attachment (Appendix 1).

2.5 MYELOID CELL LYSIS

Monoclonal antibody, TG-1 has been identified as an IgM complement fixing cytotoxic antibody specific for myeloid cells from promyelocytes to polymorphs (Beverley et al, 1980). It has been used as a myeloid cell lysing agent to produce highly purified preparations of erythroid cells from human bone marrow (Ali et al, 1983). The method for myeloid cell lysis requires incubation of the bone marrow cells with monoclonal antibody TG-1 for 30 minutes at 4°C to allow antibody fixation to the myeloid cell surface. Thereafter, samples are incubated at 37°C in the presence of complement to allow cell lysis.

Hybridoma cells obtained from Dr. Peter Beverley, M.R.C. Tumour Immunology Group, University College Hospital, London, were cultured with the assistance of the Beatson Institute for Cancer Research, Glasgow. TG-1 released into the hybridoma supernatant was stable for several months at -80°C.

The lysis technique was modified (Fitzsimons et al, 1986) from the method described by Ali et al (1983). Bone marrow cell pellets (50 x 10^6 cells) were suspended in TG-1 (2.5ml) by repeated aspiration through a fine gauge needle (microlance, 21G, 0.8 x 40) then placed on ice for 30 minutes. Fresh autologous plasma (2.5ml) was added as a source of complement

containing deoxyribonucleotidase (DNAse, EC 3.1.21.1) (DN-25, Sigma Chemical Company) to a final concentration of 1mg per ml. The sample was then incubated in a shaking water bath (60 rpm) at 37°C for 30 minutes. DNAase was added to digest the DNA liberated from lysed cells and so prevent gel formation and aggregation of viable erythroid cells by the released DNA. The cells were washed (x 3) in culture medium (4°C) to remove myeloid cell debris. By this method the erythroid purity of the sample rose from approximately 20% to 80%.

2.6 BONE MARROW FRACTIONATION

The erythroid enriched cell population obtained following myeloid cell lysis was fractionated to produce four distinct age-matched highly purified erythroid cell populations. This was achieved using preformed percoll density gradients.

Between 1965 and 1970 the development of isopycnic (buoyant density) and velocity sedimentation methods resulted in numerous reports of useful cell separations. The theory of sedimentation has since been reviewed (Pretlow et al, 1975). Since density separation is effected on the basis of respective cell densities, and the densities of many cell types overlap broadly, the application of this method has some limitations (Pretlow et al, 1982). However, in the case of erythroid cells the increase in cell density as the proerythroblast sequentially matures through early, intermediate and late erythroblast stages of development is very marked, although some degree of overlap inevitably exists.

2.6.1 Percoll Density Gradients

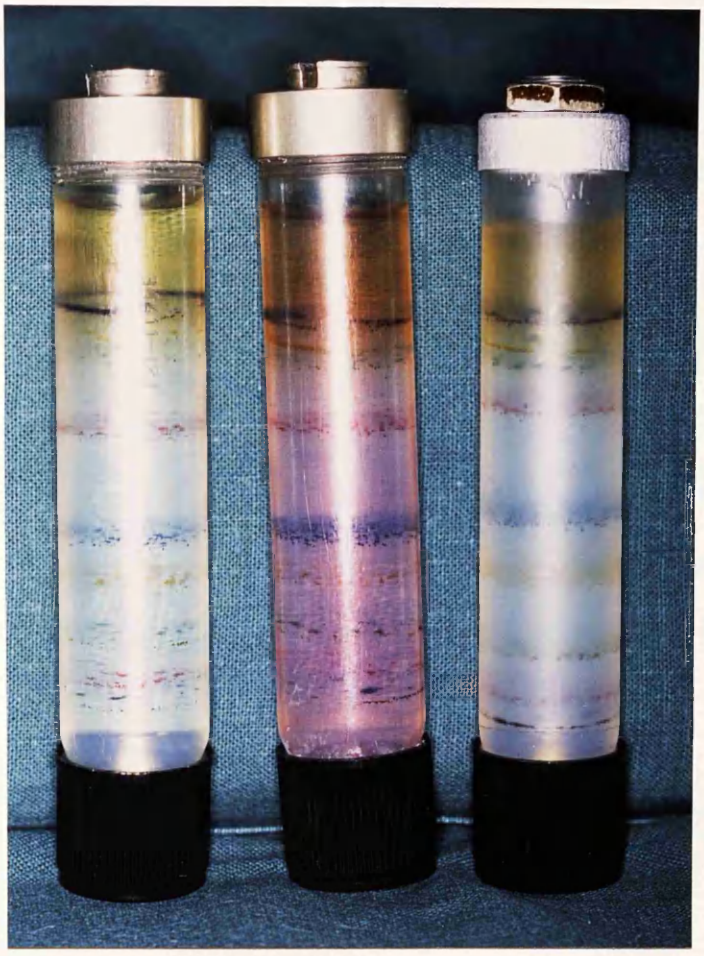
54ml of percoll (Pharmacia) was made isotonic by the addition of 6ml 8g/dl sodium chloride and was prepared to a specific density of 1.083g/l by the addition of 32ml culture medium (Appendix 1). Density gradients were prepared in 14ml capacity capped polycarbonate centrifugation tubes by spinning in a refrigerated ultracentrifuge (MSE superspeed 75) with the brake off in an angle-head rotor (MSE-25) for 45 minutes at 20000 rpm.

Bone marrow erythroblasts were washed (x 3) in culture medium (4^{o}C) following TG-1 myeloid cell lysis. The cells were suspended in heat inactivated filtered (Gelman Acrodisc filter, 0.2 micron) foetal calf serum (1.0ml) to a concentration of 50 x 10^{6} cells per ml. The erythroblast suspension was layered onto each preformed density gradient maintained at 4^{o}C. The gradients were then spun in a refrigerated centrifuge (MSE, model 4L) at 2,400 rpm for 15 minutes at 4^{o}C.

Percoll gradients were calibrated by the addition of percoll density marker beads (Pharmacia) added to a preformed percoll gradient which was then centrifuged as described (photographic plate 1). Erythroblasts were removed from four preset density fractions identified by the marker beads. The erythroblasts were then washed in culture medium (x 3) at 4^{o}C to ensure complete removal of percoll. Cell counts were obtained and cytospin preparations made for each fraction.

2.6.2 The Erythroblasts in the Percoll Fractions

Each cytospin preparation was examined by light microscopy and erythroblast maturity assessed by differential cell



NOTE:

Gradients were calibrated by the addition of 10-15ul of density marker beads in distilled H_2O followed by centrifugation at 2400 rpm for 15 min.

Colour of bead:		density (g/ml)
Orange	:	1.034
Blue/green	:	1.048
Red	:	1.062
Blue/purple	:	1.076
Yellow	:	1.087
Green	:	1.100
Pink	:	1.118

PLATE 1

PERCOLL DENSITY GRADIENT CALIBRATED BY DENSITY MARKER BEADS

counting of 400 cells. Each fraction was scored for its content of pro and basophilic early erythroblasts (E_1), polychromatic intermediate erythroblasts (E_2) and orthochromatic late erythroblasts (E_3) (photographic plates 2-5).

Separation on the basis of density produced successful fractionation of the erythroid cell population into subpopulations of similar differentiation status. Those density steps which provided the most satisfactory separation of E_1 cells from E_2 cells from E_3 cells were similar to those identified by Fitzsimons et al (1986).

	Density (g/ml)	Volume Removed (ml)
Fraction 1	1.062 - 1.070	1.0
Fraction 2	1.070 - 1.080	1.0
Fraction 3	1.080 - 1.100	1.5
Fraction 4	1.100 - 1.118	1.0

2.6.3 Haem Synthesis in Developing Erythroblasts

The progressive accumulation of Hb is clearly visible in the developing erythroid cells of the bone marrow and is inextricably linked to erythropoiesis itself. Hb is not evident in the early basophilic erythroblasts (percoll fractions 1 and 2), becomes evident in the intermediate polychromatic erythroblasts (percoll fraction 3), while by the stage of the late orthochromatic erythroblasts (percoll fraction 4) it packs the cytoplasm as if to squeeze the small densely packed nucleus from the cell.

2.6.4 Erythroblast Differentials

Differential counting of erythroblasts is a subjective procedure liable to interobserver variation. All differential cell counting was performed by Dr. Edward Fitzsimons, project supervisor. Differential cell counts on the four percoll fractions were made on 400 cells. The stage of erythroid development was assessed by its content of E_1, E_2 and E_3 erythroblasts. In general, the intermediate erythroblasts of percoll fraction 2 were less mature than those of percoll fraction 3 (see photographic plates 2-5).

It has been shown with velocity centrifugation (Denton and Arnstein, 1973) that the most effective erythroblast separation was obtained from marrows showing pronounced erythroid hyperplasia. The work produced similar results for density centrifugation. Normal marrows lacking erythroid hyperplasia contained very few pro and early basophilic erythroblasts; as such, the least dense percoll fractions contained very few immature erythroid cells but large numbers of contaminating lymphocytes and monocytes. As a result, only marrows showing erythroid hyperplasia were suitable for study.

Within equivalent fractions from different bone marrows examined, the erythroblasts were found to be at a comparable level of maturity thus permitting the comparison of haem enzyme activity between age-matched erythroblasts from both normal and pathological bone marrow samples. The mean erythroblast differential counts in the percoll fractions were similar for the majority of bone marrows studied. Pro and early basophilic (E_1) erythroblasts were most prominent in fraction 1. These immature cells were also present in fraction 2, but to a lesser

extent and were outnumbered by intermediate (E_2) polychromatic erythroblasts. Percoll fraction 3 contained more intermediate than late (E_3) erythroblasts with the reverse being the situation in fraction 4 (see Table 1).

2.6.5 Other Methods of Bone Marrow Fractionation

Different methods have been used for fractionation of developing bone marrow erythroblasts to provide distinct age-matched populations of similar differentiation status. Erythroblasts may be synchronised in animals by injections of actinomycin D and harvested at intervals thereafter (Konijn et al, 1979). The treatment is, however, toxic and few animals survive. Varying degrees of success have been achieved using the physical separation techniques of velocity (Denton and Arnstein, 1973) and density (Borsook et al, 1969) centrifugation for the fractionation of rabbit and mouse erythroblasts (Glass et al, 1975b). Human erythroblast fractionation using monoclonal antibody TG-1 and percoll equilibrium density centrifugation has the advantages that the centrifugation step is short and refrigerated and that high percentage cell recoveries are achieved (Ali et al, 1983) so allowing its use with small cell numbers.

The density of developing erythroblasts is determined primarily by the nuclear size and Hb content (Borsook et al, 1969). As these characteristics do not change synchronously in every cell some erythroblast heterogeneity in the percoll fractions was unavoidable. However, the four fractions chosen for use produced reproducible erythroblast fractionation and

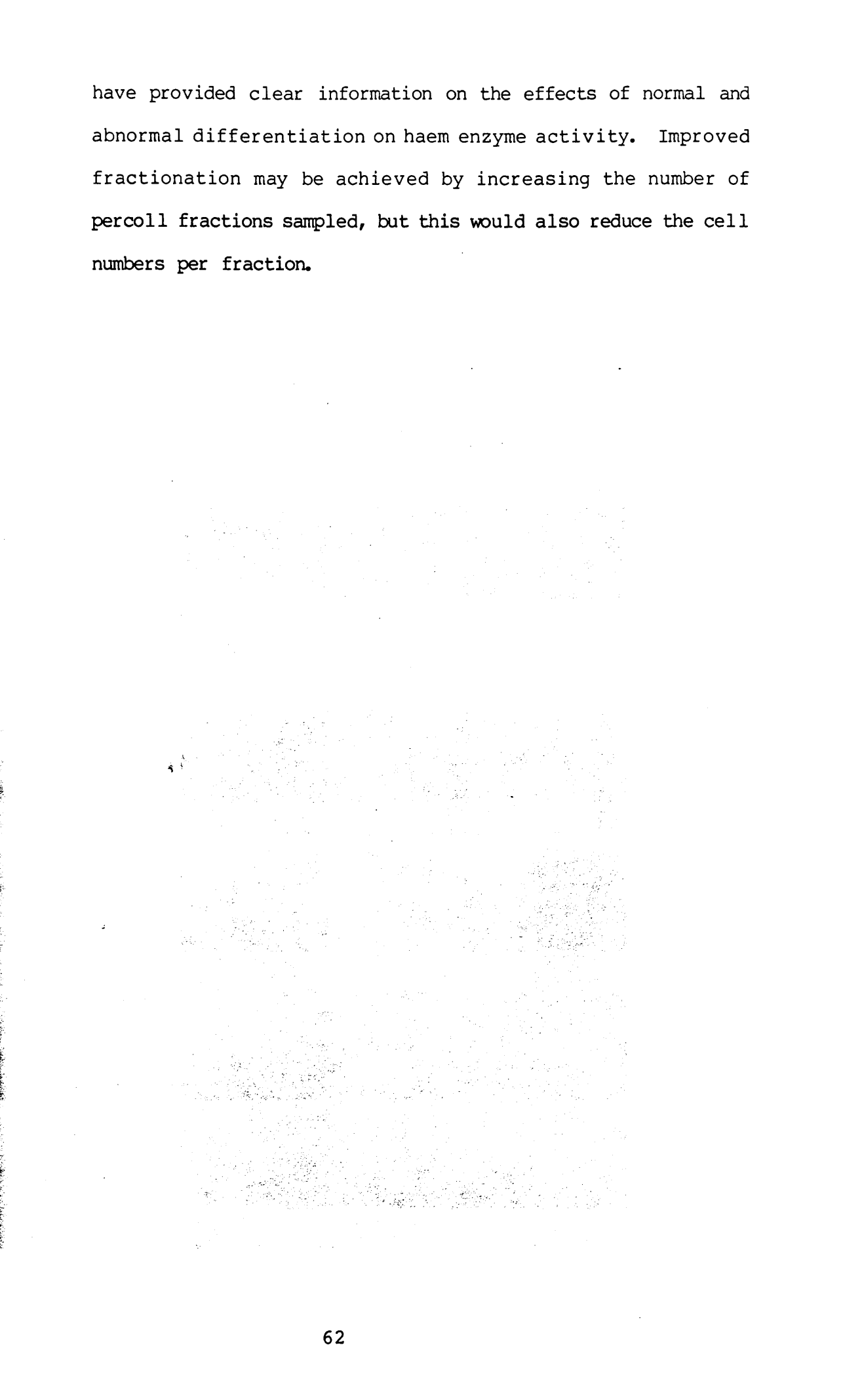
Sample	E_1 %	E_2 %	E_3 %
NORMOBLASTIC (n=10)			
Percoll 1	45	45	10
Percoll 2	21	59	20
Percoll 3	10	63	27
Percoll 4	3	35	62
IRON DEFICIENT (n=10)			
Percoll 1	43	40	17
Percoll 2	43	45	12
Percoll 3	13	60	27
Percoll 4	-	35	65
SIDEROBLASTIC (n=10)			
Percoll 1	33	46	21
Percoll 2	32	59	9
Percoll 3	19	62	19
Percoll 4	3	44	53

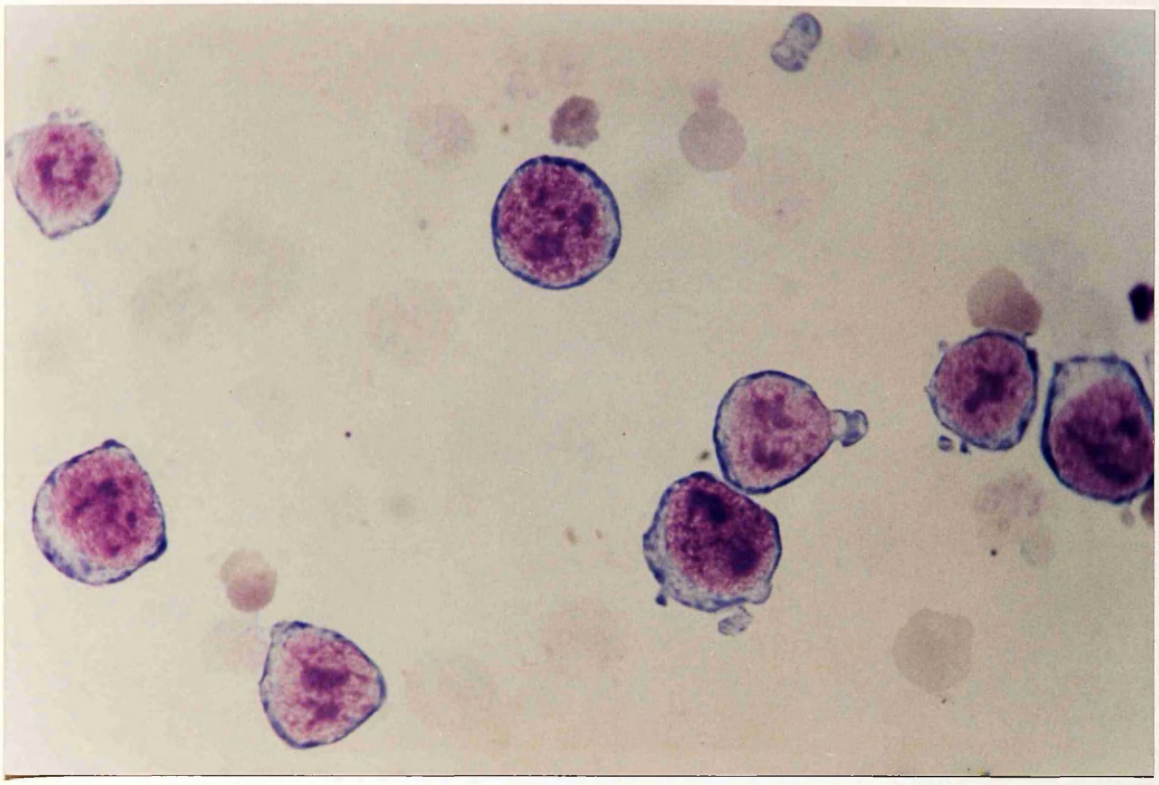
Note: The erythroblast differential cell counts were made from the examination of 400 cells.

TABLE 1

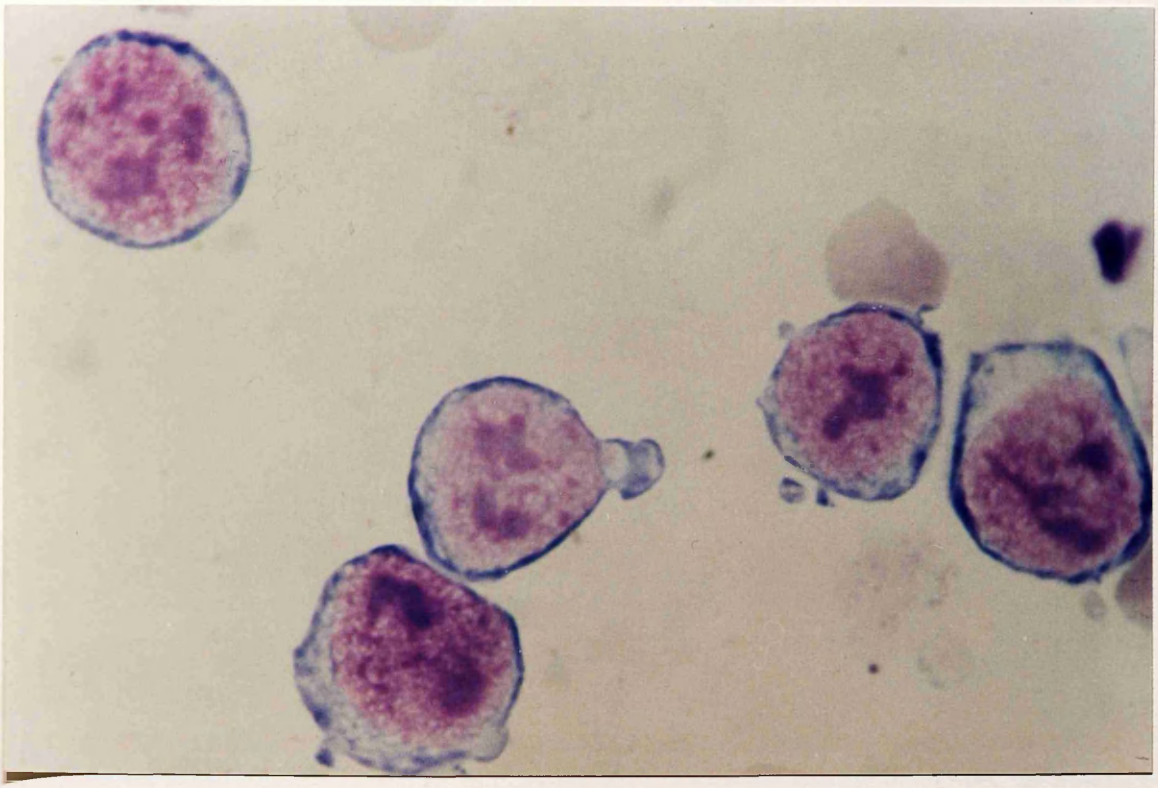
MEAN ERYTHROBLAST DIFFERENTIAL CELL COUNTS FROM THE PERCOLL GRADIENTS

have provided clear information on the effects of normal and abnormal differentiation on haem enzyme activity. Improved fractionation may be achieved by increasing the number of percoll fractions sampled, but this would also reduce the cell numbers per fraction.





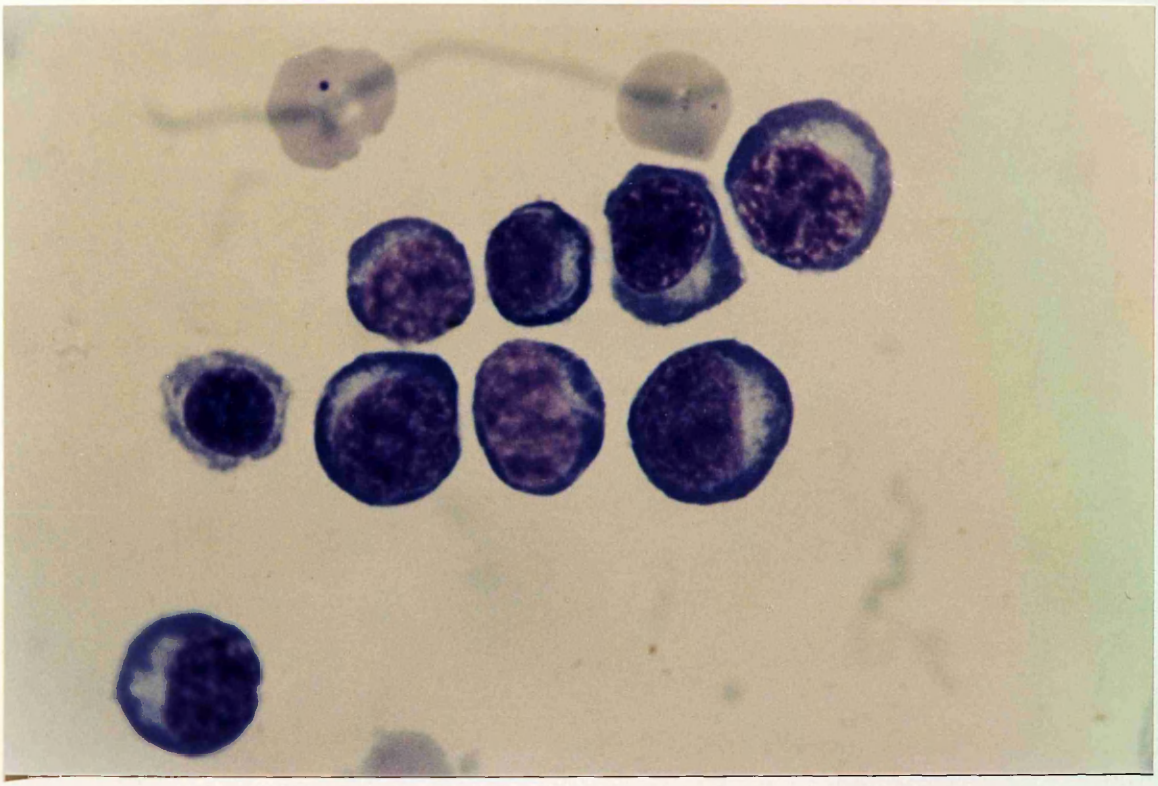
(a) x 800



(b) x 1200

PLATE 2 (a), (b)

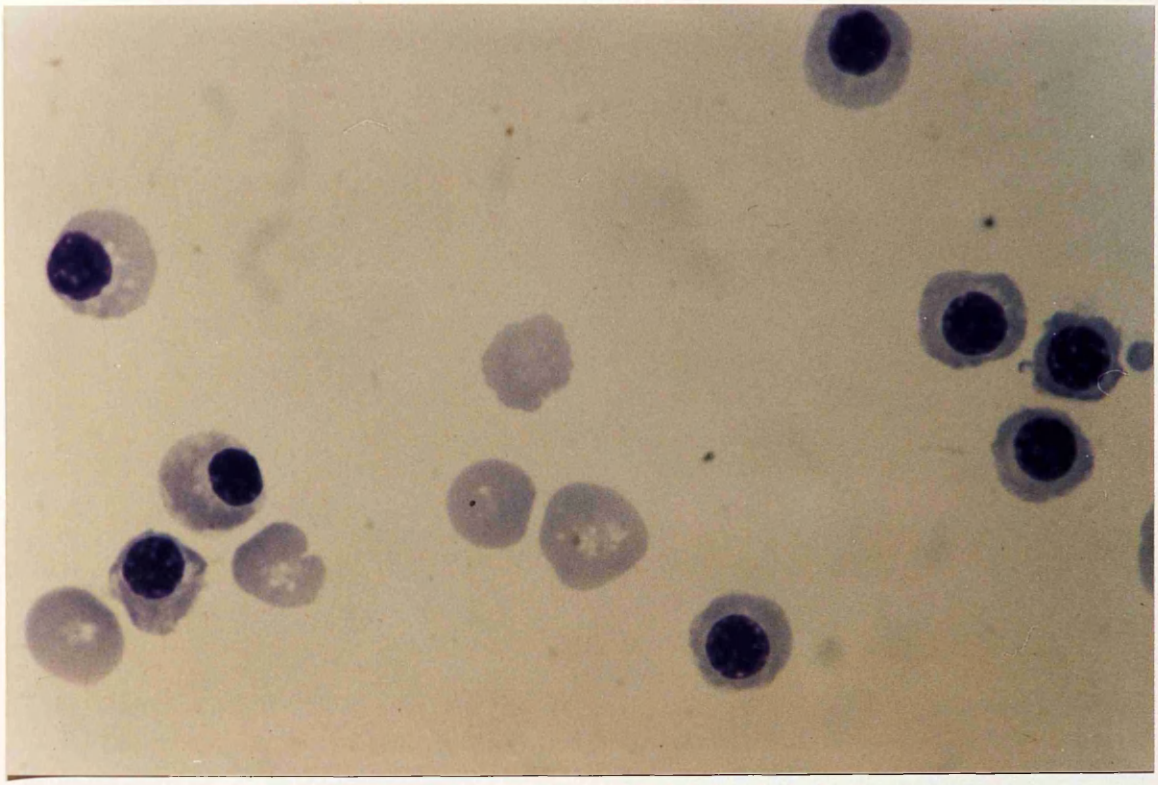
PROERYTHROBLAST (PERCOLL FRACTION 1)



x 800

PLATE 3

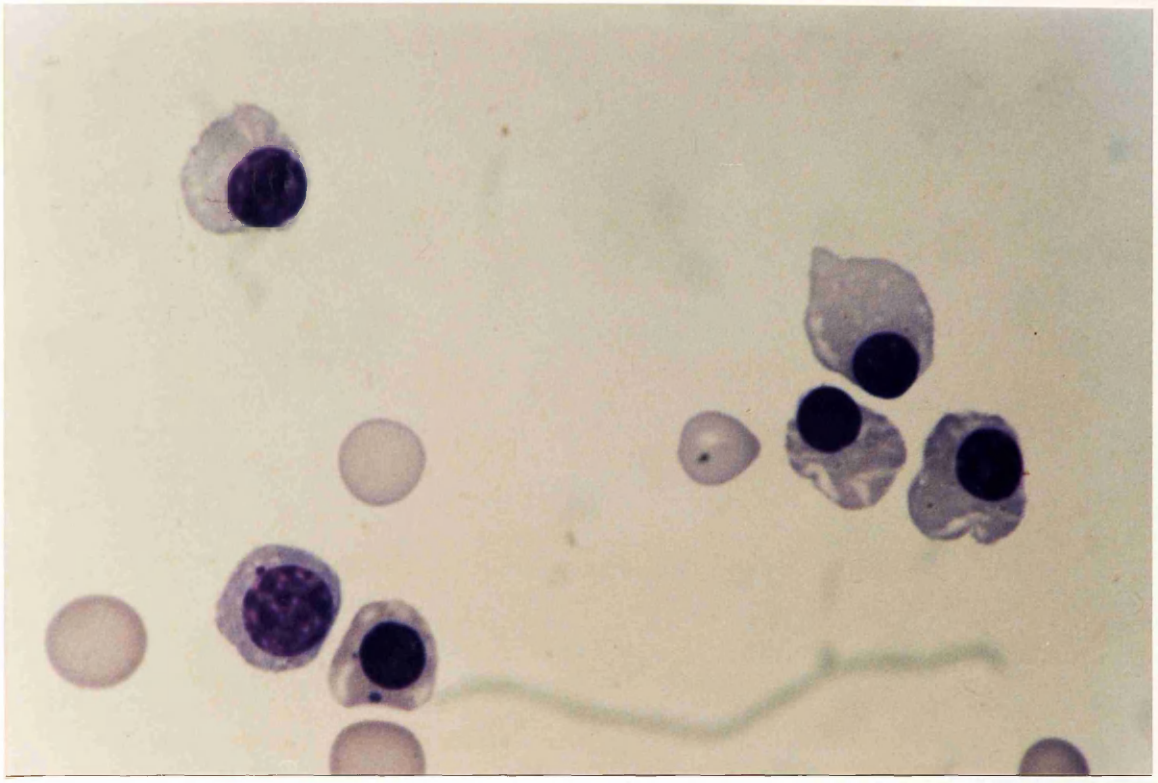
BASOPHILIC ERYTHROBLAST (PERCOLL FRACTION 2)
(Early and intermediate erythroblasts)



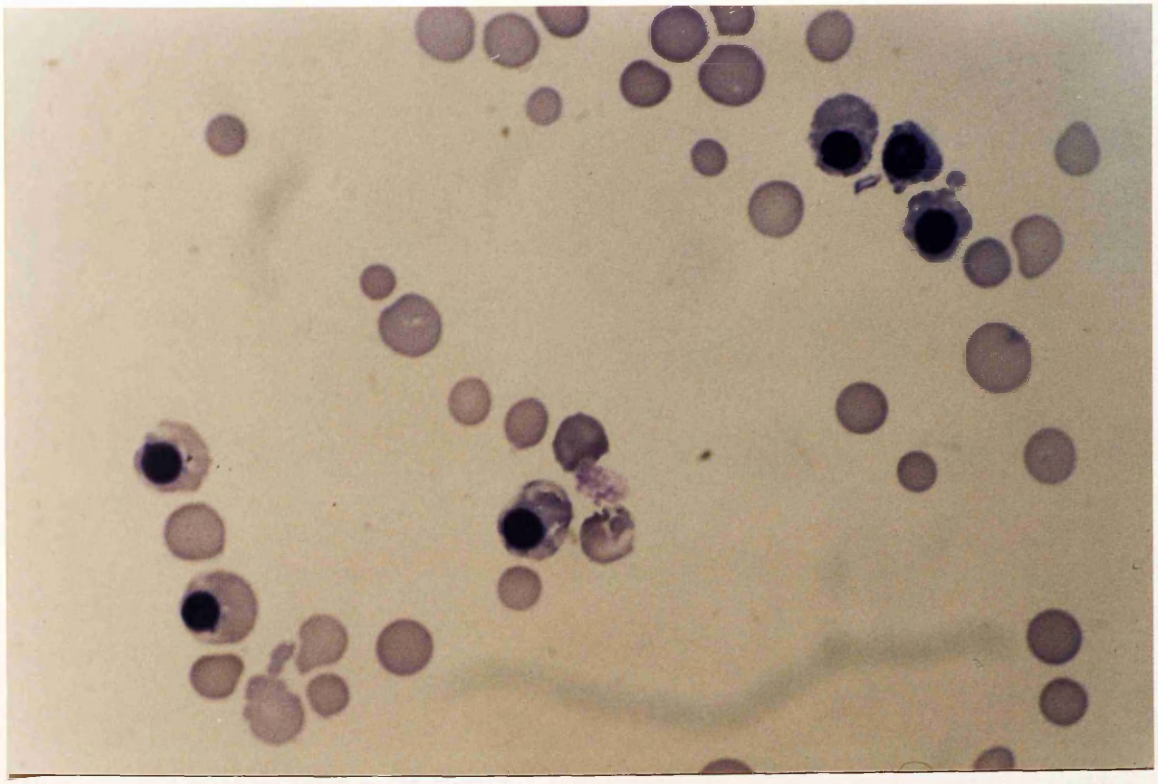
x 800

PLATE 4

POLYCHROMATOPHILIC ERYTHROBLAST (PERCOLL FRACTION 3)
(Intermediate and late erythroblasts)



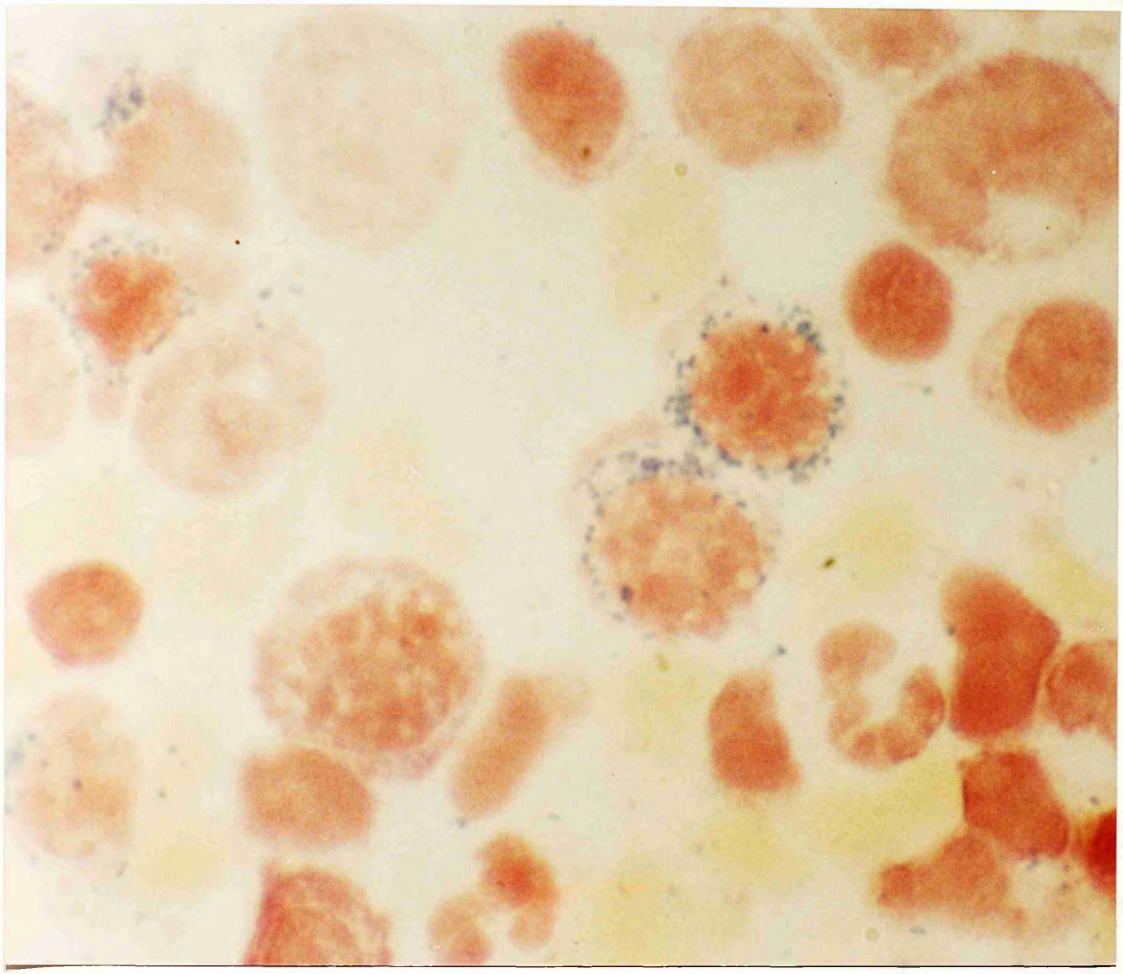
(a) x 800



(b) x 600 (Note the small pyknotic nucleus about to be extruded from the cell)

PLATE 5 (a) (b)

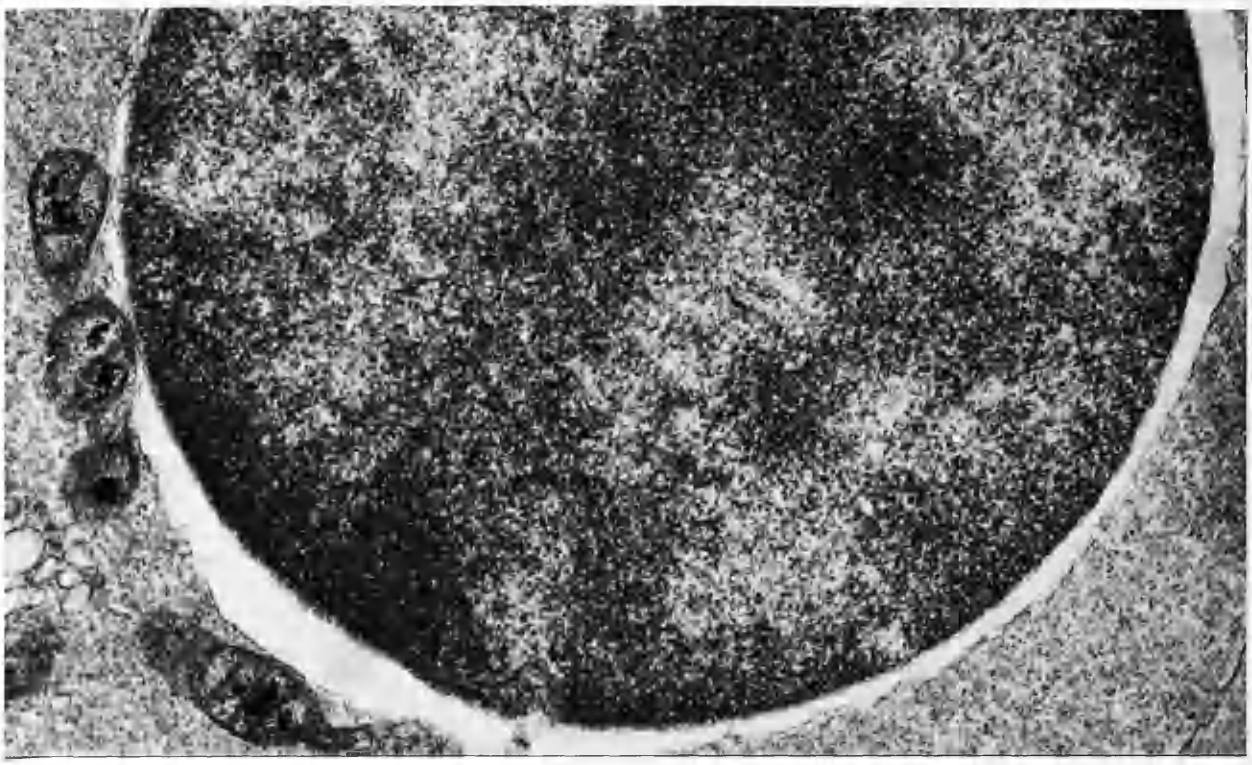
ORTHOCHROMATIC ERYTHROBLAST (PERCOLL FRACTION 4)
(Late and intermediate erythroblasts)



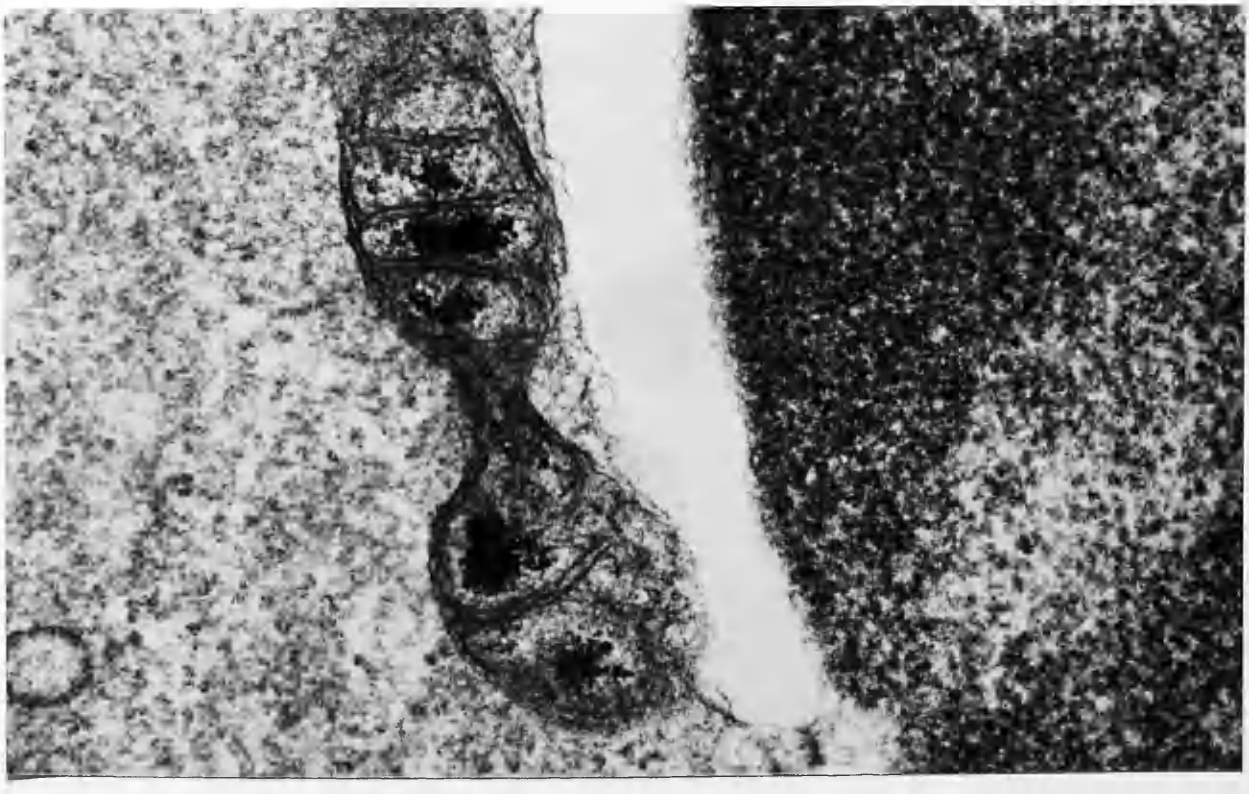
x 1000 Iron granules (Perl's stain) are located in the perinuclear position

PLATE 6 (a)

RING SIDEROBLASTS



(b) Lower power electron micrograph depicts iron loaded mitochondria located alongside the nuclear membrane



(c) High power electron micrograph shows view of an iron-loaded mitochondrion.

PLATE 6 (b), (c)

RING SIDEROBLASTS

CHAPTER 3

5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN HUMAN BONE MARROW

3 5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN HUMAN BONE MARROW

3.1 THE REACTION CATALYZED BY 5-AMINOLAEVULINIC ACID SYNTHASE (ALA SYNTHASE) [SUCCINYL CoA: GLYCINE C-SUCCINYL TRANSFERASE, (EC.2.3.1.37)]

The first reaction in the haem biosynthetic pathway is the condensation of glycine and succinyl CoA to form ALA. This step is catalyzed by the mitochondrial enzyme, ALA synthase. The enzymatic reaction was first identified in the photosynthetic bacterium Rhodopseudomonas spheroides (Kikuchi et al, 1958; Gibson et al, 1958) and requires PLP as enzyme cofactor. Subsequently, the enzymatic activity was demonstrated in a variety of species and tissues including microorganisms (Tait, 1972); yeast (Porra et al, 1972); avian (Granick, 1966) and mammalian liver cells (Granick and Urata, 1963; Marver et al, 1966; 1966); mammalian erythroid cells (Takaku, 1968); mouse harderian gland (Margolis, 1971); rat kidney (Sardesai, 1972); heart (Condie and Tephly, 1978); brain (Paterniti, 1978) and insects (Brattsten and Wilkinson, 1975).

3.2 THE LOCATION AND MOLECULAR PROPERTIES OF ALA SYNTHASE

The purification of ALA synthase is difficult primarily because the enzyme is present in minute quantities and is very unstable. However, the dramatic induction of hepatic ALA synthase by drugs such as AIA and DDC has greatly facilitated the molecular and genetic characterisation of this enzyme. Drug-induced mitochondrial ALA synthase has been purified to apparent homogeneity from embryonic and adult chicken liver (Borthwick et al, 1983; Watanabe et al, 1984) and from adult rat liver (Srivastava et al, 1982). Glutaraldehyde cross-linking studies (Borthwick et al, 1983) and direct electron

microscopic analysis of enzyme preparations (Pirola et al, 1984) have indicated that the solubilised native enzyme exists as a homodimer. The estimated molecular weights (M.W) of the subunits as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) range from 65,000 (adult chicken liver) to 70,000 (adult rat liver).

Electron microscopic localisation of ALA synthase in rat liver using gold-labelled antibodies has indicated that the enzyme is attached to the matrix surface of the inner mitochondrial membrane (IMM) (May et al, 1986). Most nuclear-encoded mitochondrial proteins are synthesised in the cytoplasm as a larger precursor and then posttranslationally imported to the mitochondria where they are processed to the mature form (Hay et al, 1984). In general, precursors contain aminoterminal extensions of 20-70 amino acids which target the protein to the mitochondria and participate in intramitochondrial sorting (Schatz, 1987). The presequence is then removed by a protease. Both chicken and rat hepatic ALA synthase have been shown to be synthesised in the cytosol on membrane-free polyribosomes as larger precursors which undergo proteolytic maturation during transport to the mitochondria (Whiting, 1976; Brooker et al, 1980; Ades and Harpe, 1981; Srivastava et al, 1982; 1983a; Hayashi et al, 1983; Watanabe et al, 1983). Molecular weight estimates for the chicken precursor range from 70,000 (Whiting, 1976; Brooker et al, 1980) to 75,000 (Ades and Harpe, 1981) and the rat precursor has been estimated at 76,000 (Srivastava et al, 1982). The relationship between the cytosolic and mitochondrial forms of

the enzyme has been established both 'in vivo' by pulse-labelling experiments (Srivastava et al, 1982; 1983a) and 'in vitro' by demonstrating the direct posttranslational transfer and processing of the primary translation product into isolated mitochondria (Hayashi et al, 1983).

The cloning of ALA synthase from chicken liver has facilitated the isolation of homologous cDNA from rat (Srivastava et al, 1988; Yamamoto et al, 1988); human (Bawden et al, 1987; Bishop, 1990) and mouse liver (Young and Dierks, 1990). The ALA synthase cDNA isolated from rat and human liver mRNA encode preproteins of identical length which exhibit a high degree of sequence homology with chicken liver ALA synthase. Alignment of the predicted amino acid sequences of these proteins with that of the chicken enzyme has suggested that each preprotein consists of a presequence containing 56 amino acids followed by 586 amino acids of the mature enzyme. The amino acid homology of the mature proteins range from 78% (human to chicken) to 83% (human to rat).

3.3 GENE STRUCTURE AND EXPRESSION OF ALA SYNTHASE

It is now recognised that ALA synthase is encoded by two distinct genes: a housekeeping gene which is expressed in all tissues and cell types that have been examined (including erythroid cells), and an erythroid-specific gene which is dedicated to the management of ALA production during the terminal stages of erythroid cell differentiation. The genetic origin of erythroid ALA synthase has been one of the most controversial areas of research in recent years. Some investigators have favoured the view of separate genes encoding

hepatic and erythroid ALA synthase, while others have supported the concept of alternative promoters and/or splicing patterns within a single gene.

Several lines of evidence have led to the identification of two distinct genes for ALA synthase.

3.3.1 Evidence for a Housekeeping Gene

Borthwick et al (1984) cloned chick embryonic liver ALA synthase mRNA which was later demonstrated to be encoded by a single nuclear gene (Elferink et al, 1987). Northern blot hybridization analyses indicated that the gene encoded a single 2.2kb mRNA which was found in all tissues examined, including liver, heart, brain and reticulocytes (Elferink et al, 1987). The structure of the reticulocyte transcript was identical to that found in liver; primer extension analyses indicated that it had the same 5' terminus as liver mRNA; and RNase protection experiments failed to detect any differences in internal sequence organisation. The size of the reticulocyte enzyme, as determined by SDS-PAGE was also identical to that of the liver (Elferink, 1987).

Srivastava et al (1988) reported similar results from a study in rat, using probes derived from rat liver cDNA. In agreement with the pattern in chicken, a single 2.3kb mRNA was detected in brain, heart, liver, kidney, testes and spleen with the structure of the transcripts identical in each tissue. Treatment of rats with AIA which selectively induces ALA synthase in liver, kidney and testes resulted in a 7-8 fold increase in steady-state transcript levels in each of these

tissues, but not in any of the others.

These results appeared to demonstrate that a single "hepatic" ALA synthase gene was expressed at low levels in a variety of tissues, including those of erythroid origin. This gene responded in liver, kidney and testes to tissue-specific stimuli that induced ALA synthase expression. Thus, it seemed likely that this gene was active in all mammalian cells and was responsible for the maintenance of normal housekeeping function.

3.3.2 Evidence for an Erythroid-Specific Gene

Despite the convincing evidence that the "hepatic" ALA synthase gene encoded identical mRNAs in a variety of tissues, including erythroid tissue, several workers maintained that different hepatic and erythroid ALA synthase isoenzymes were expressed in the two tissues. This was first suggested by Bishop et al (1981) from studies on guinea pig.

In contrast to the results of Elferink et al (1987), Watanabe et al (1983) had found that ALA synthase in chicken liver and reticulocytes differed in size both as mature proteins and as precursors when examined using polyclonal antibodies raised against purified hepatic ALA synthase. In liver mitochondrial extracts, the enzyme had a M.W. of 65,000 which agreed well with M.W. estimates of the purified hepatic enzyme (63,000-68,000) and the calculated M.W. of the mature protein. However, the only cross-reacting proteins in erythroid mitochondrial extracts had a M.W. of 53,000. When the sizes of the preproteins were estimated for each cell type, liver polysomes synthesised a precursor of M.W. 73,000

(calculated M.W. 70,209) while erythroid polysomes produced a protein of M.W. 55,000. Although the accuracy of the estimated M.W.'s for the hepatic forms of the enzyme were undisputed, the size estimates of the erythroid forms were the subject of debate. Since hepatic ALA synthase was known to be susceptible to proteolysis and easily converted to smaller polypeptides (M.W. 49,000, 51,000 in chicken and rat) which retained enzymatic activity (May et al, 1986) Elferink et al (1987) suggested that the smaller erythroid forms detected by Watanabe et al (1983) resulted from proteolysis of the mature and precursor forms of the same protein that was expressed in liver. However, nucleic acid analyses suggested that this was not the case. Yamamoto et al (1985) used the polyclonal antiserum described by Watanabe et al (1983) to identify several putative ALA synthase cDNA clones in a reticulocyte cDNA expression library. One cDNA hybridized selectively to a reticulocyte mRNA encoding the expected preprotein (M.W. 55,000). This cDNA hybridized to a unique 2kb mRNA and was expressed only in reticulocytes.

It was then concluded that the erythroid primary translation product (M.W. 55,000) detected by Watanabe et al (1983) was not encoded by the hepatic ALA synthase gene, but by a distinct gene which was differentially expressed only in erythroid cells. The protein encoded by this gene was later identified as an erythroid-specific form of ALA synthase following isolation and analysis of a cDNA copy of the erythroid ALA synthase mRNA (Riddle et al, 1989). Sequence analysis showed that it encoded a preprotein (M.W. 54,800, 518

amino acids) which shared 56% amino acid sequence homology with hepatic ALA synthase. Genomic DNA blotting experiments and RNase protection assays further showed that this cDNA was encoded by a distinct ALA synthase gene expressed only in erythroid cells. In agreement with Elferink et al (1987) it was also shown that the hepatic ALA synthase gene is coexpressed in erythroid cells, but at a level that is only 1-2% of the erythroid gene.

Similar confusion existed concerning the number of mouse genes encoding ALA synthase. Schoenhaut and Curtis (1986) employed a novel genetic complementation technique to isolate cDNA encoding ALA synthase in BALB/c mouse. The results indicated that ALA synthase was encoded by a single gene. However, using a different approach to isolate ALA synthase cDNA from DBA/2 mouse Young and Dierks (1990) found that the enzyme was encoded by two distinct genes which were differentially regulated in erythroid and nonerythroid cells. This more recent data suggested that the approach used by Schoenhaut and Curtis (1986) introduced a strong bias for the recovery of clones encoding the erythroid-specific form of the enzyme, thereby creating the false impression that drug-inducible hepatic ALA synthase mRNA was the same as that expressed at high levels in differentiating erythroid cells.

The conflict has only recently been resolved with the localisation of housekeeping and erythroid ALA synthase genes to chromosome 3 (Sutherland et al, 1988) and chromosome X (Cox et al, 1990) respectively.

3.4 REGULATORY MECHANISMS IN HEPATIC CELLS

3.4.1 Induction and Repression of ALA Synthase in Hepatic Cells

As discussed in Chapter 1 (Section 1.8) the rate controlling enzyme of haem biosynthesis in hepatic tissue is ALA synthase. A large number of compounds are capable of inducing ALA synthase activity in liver including various lipophilic chemicals and drugs and some natural and synthetic steroids (Granick, 1966). Experimental depletion of haem induces ALA synthase while the presence of excess cellular haem inhibits ALA synthase in four possible ways:-

1. Direct inhibition of ALA synthase activity (Tait, 1978).
2. Inhibition of ALA synthase mRNA synthesis (Whiting, 1976).
3. Inhibition at a post transcriptional step (Sassa and Granick, 1970).
4. Inhibition of enzyme transfer from the cytosol to the inner mitochondrial membrane (Hayashi et al, 1972).

Granick (1975) proposed the now widely accepted concept of a **regulatory haem pool** suggesting that the rate of haem synthesis in hepatic tissue was regulated by the intracellular 'free haem' concentration (0.1μM). Increases in 'free haem' concentration resulted in inhibition of functional ALA synthase and induction of haem oxygenase, while decreases in concentration had the opposite effect. A reciprocal relationship therefore exists between these two enzymes which is influenced by intracellular 'free haem' concentration.

Mitochondrial ALA synthase partially purified from rabbit, rat and guinea pig liver has Km values for glycine, succinyl CoA and PLP in the ranges 5 to 19 mM, 60 to 200 μM and 1 to 10

μM respectively (Whiting and Elliot, 1972; Whiting and Granick, 1976; Aoki et al, 1971; Scholnick et al, 1972; Woods and Murthy, 1975). The relatively high Km (i.e. low affinity) of the enzyme for glycine has raised the possibility that the enzyme activity may further be regulated by the physiological concentration of available glycine in liver (Sinclair and Granick, 1972).

3.4.2 Turnover of Hepatic ALA Synthase

The $T^1/_2$ of mitochondrial ALA synthase is short (i.e. 70min in adult rat liver (Marver et al, 1966; Hayashi et al, 1969; Tschudy et al, 1965)). ALA synthase has one of the most rapid turnover rates of the proteins in mitochondria as compared with the average $T^1/_2$ of mitochondrial proteins of 5 days (Druyan et al, 1969). Control over the rate of enzyme synthesis in liver is believed to be the major regulator of enzyme activity and hence hepatic haem biosynthesis.

3.5 REGULATORY MECHANISMS IN ERYTHROID CELLS

3.5.1 Induction and Repression of ALA Synthase in FEL Cells

In DMSO-treated FEL cells (Chapter 1, Section 1.10.2(i)) Hb accumulation begins during erythropoietic development and results in a 50-fold increase in cellular haem levels (Sassa, 1976). This is accompanied by relatively small increases in the activities of most, if not all, of the haem enzymes (Sassa, 1976; Rutherford et al, 1979; Beaumont et al, 1984). ALA synthase activity reaches a maximum of 3-5 times that of the preinduced level by 72-96 h. However, the extent of ALA synthase induction can be altered by manipulation of haem

availability in differentiating cells (Beaumont et al, 1984). Addition of haemin (10μM) prior to the induction procedure markedly inhibited the differentiation-dependent increase in ALA synthase activity. Conversely, the addition of succinylacetone (4,6-dioxoheptanoic acid), a potent inhibitor of ALA dehydratase, with DMSO led to a 6 fold increase in ALA synthase activity at 96 h compared with that achieved with DMSO alone. Haemin (10μM) added at 72 hour after DMSO inhibited the 'superinduction' of ALA synthase and reduced activity to the level achieved with DMSO alone. Thus, ALA synthase in differentiating FEL cells appeared to be sensitive to intracellular haem balance.

Two possible models are likely for the induction of erythroid gene expression.

1. A derepression model similar to that in hepatic cells whereby ALA synthase gene expression is regulated solely by haem. This would suggest that erythroid gene activation occurs during the differentiation process in response to the reduced haem levels which result from the rise in globin chain production and Hb formation.
2. Alternatively, ALA synthase may be induced by a developmental signal early in the differentiation sequence. In this case, the feedback mechanism(s) may act to coordinate the magnitude of this induction response with other aspects of differentiation.

These theories have been examined by manipulating haem availability in both undifferentiated and differentiated cells then assaying erythroid ALA synthase gene expression (Young and

Dierks, 1990).

While the level of housekeeping gene transcripts remained unchanged during differentiation, the number of erythroid ALA synthase mRNAs per cell increased dramatically to levels 50-75 times above baseline by 96 h of induction with DMSO (Fraser and Curtis, 1987; Young and Dierks, 1990). Most of this increase could be attributed to higher rates of ALA synthase gene transcription (Fraser and Curtis, 1987). If erythroid ALA synthase was controlled by deregulation then erythroid gene expression would be activated in the absence of differentiation by cellular haem depletion. This was examined by culturing undifferentiated FEL cells in the presence of succinylacetone (Young and Dierks, 1990). Under these conditions, the cells entered a state of growth arrest which was both preventable and restorable by haemin addition (10μM). The level of ALA synthase transcripts was identical to that in cells grown either in the absence or presence of succinylacetone and haemin (10μM). Thus, expression of the erythroid gene cannot be activated in undifferentiated cells by haem depletion. Similar experiments in cells induced to differentiate with DMSO produced results in agreement with Beaumont et al (1984) whereby succinylacetone caused a haemin-reversible inhibition of Hb formation and superinduction of ALA synthase activity. However, the kinetics of erythroid ALA synthase transcript accumulation were unaffected by succinylacetone or haemin.

It was therefore concluded that the induction of erythroid ALA synthase gene transcription is developmentally regulated and largely unaffected by intracellular haem balance.

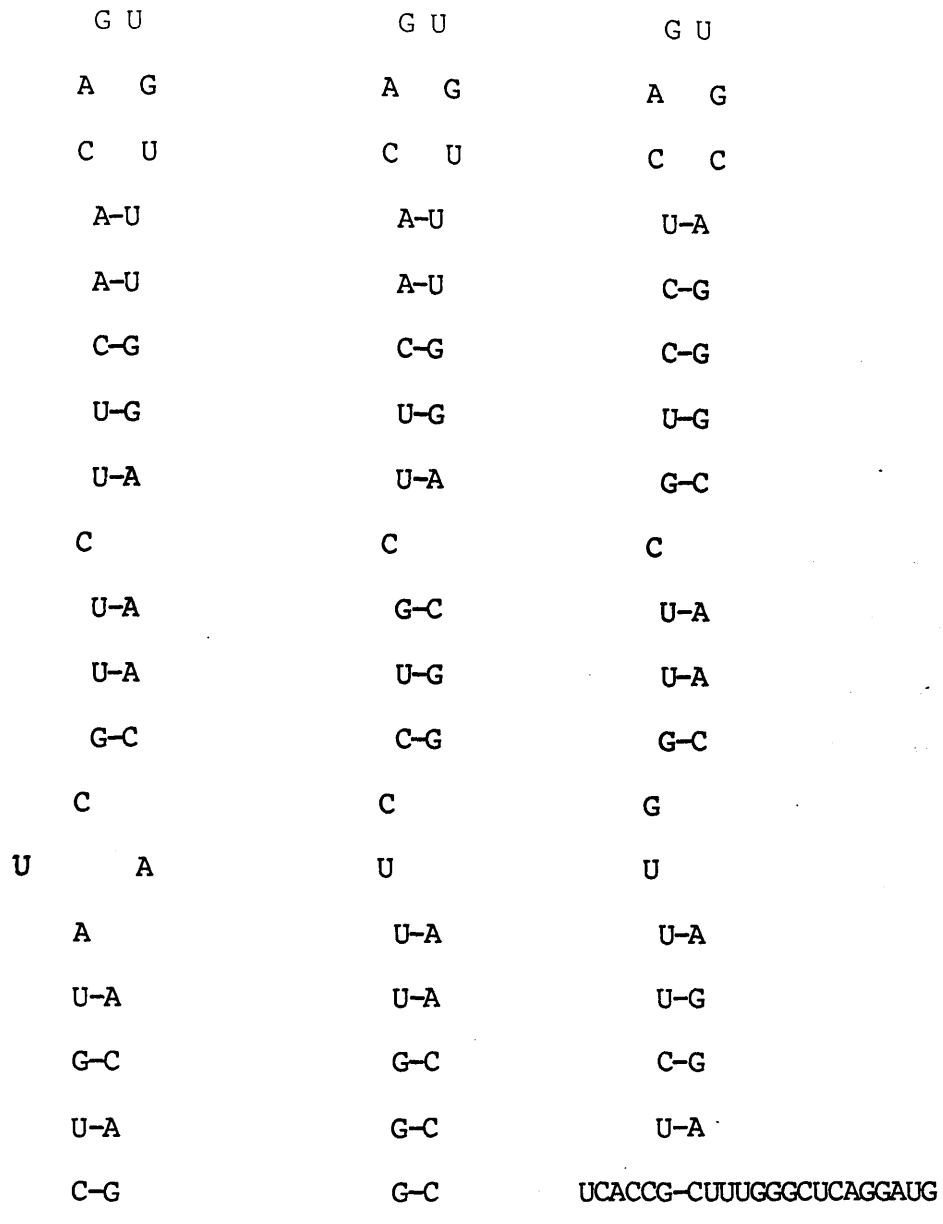
Furthermore, the extent of ALA synthase mRNA induction during differentiation (50-75 fold) was greater than the normal 5 fold increase in enzyme activity. Hence, it would appear that enzyme activity is influenced by post-transcriptional mechanisms sensitive to the presence of succinylacetone or haemin.

3.5.2 Possible Mechanisms of Post-transcriptional Control

An explanation of the opposing effects of succinylacetone and haemin on ALA synthase activity in FEL cells may be that the rate of translation and/or transport of ALA synthase is under negative control by haem. However, the structure of erythroid ALA synthase mRNA suggests that more than one level of posttranscriptional control may be involved. Residues 6 to 40 in the 5' untranslated region (UTR) have been shown to exhibit striking structural and sequence similarity to the cis-acting iron responsive elements (IRE) which coordinate production of ferritin subunits (Aziz and Munro, 1987; Hentze et al, 1987) and synthesis of the transferrin receptor (Casey et al, 1988) with the availability of iron. Casey et al (1988) have suggested that these elements are characterised by:

1. a 6-nucleotide loop containing the sequence CAGUGX;
2. an upper stem of five paired bases;
3. an unpaired 5' C residue separated from the loop by 5 bases;
4. a lower stem of variable length.

All of these elements are present in the putative IRE of the 5' UTR of mouse erythroid ALA synthase mRNA (Schoenhaut and Curtis, 1986) (Figure 7) while no such elements are present in



UTR = untranslated region

FIGURE 7

THE 5' UNTRANSLATED REGION OF ERYTHROID ALA SYNTHASE mRNA SHOWS STRUCTURAL AND SEQUENCE SIMILARITY TO THE IRON RESPONSIVE ELEMENTS OF RAT AND HUMAN FERRITIN SUBUNIT mRNA

any of the housekeeping ALA synthase genes which have been examined to date.

Synthesis of the iron-storage protein ferritin is regulated at the translational level by the availability of iron. The mRNAs for both ferritin subunits (A and H) are stored as inactive ribonucleoprotein particles, which are translated when iron enters the cell (Rogers and Munro, 1987; Zahringer et al, 1976; Aziz and Munro, 1986). The redistribution of these mRNAs into polysomes in the presence of iron is mediated by cis-acting sequences (the IRE) found in the 5' UTR of both subunit mRNAs. It has been proposed that when iron levels are low a cytoplasmic protein selectively binds to the 5'-IRE of ferritin mRNA and blocks translation (Liebold and Munro, 1988). The presence of iron disrupts these complexes allowing access of the 5' UTR to the ribosomes and mRNA translation then occurs. In contrast to ferritin, the synthesis of transferrin receptors is stimulated when intracellular iron levels are low (Casey et al, 1988) presumably as a means of increasing iron acquisition. It is believed that the same type of protein interactions which control the rate of ferritin mRNA translation may also occur with the IREs in the 3'UTR of transferrin receptor mRNA (Casey et al, 1988).

In view of these observations, it is also possible that translation of erythroid ALA synthase mRNA is coupled to iron availability. In addition to causing increased globin and haem biosynthesis, DMSO treatment of FEL cells produces a 6-fold increase in the expression of erythroid transferrin receptors (Wilczynska et al, 1984). Furthermore, the induction of

transferrin receptor expression in FEL cells is blocked by haem (Wilczynska et al, 1984). The effect of increasing the rate of iron transport during differentiation has been examined by circumventing the transferrin receptor-dependent iron transport system with a more effective iron delivery system using the synthetic iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) (Laskey et al, 1986). With Fe-SIH as the iron source, the rate of incorporation of [2-^{14}C]-glycine into haem in DMSO-treated FEL cells increased 3.6 fold (Laskey et al, 1986). Addition of ALA to DMSO-treated FEL cells had no effect on the rate of ^{59}Fe incorporation into haem when ^{59}Fe-transferrin was used as the iron donor. This then indicates that it is the rate of iron transport, rather than the synthesis of ALA which limits haem production in differentiating FEL cells. It has been suggested (Dailey, 1989) that the strong transcriptional response of erythroid ALA synthase may be attenuated at the level of translation by the availability of iron as a means of coordinating the rate of protoporphyrin IX synthesis with the rate of iron transport. This further suggests that the 'superinduction' of ALA synthase by succinylacetone may not reflect a reduction in haem levels, but rather increased levels of iron due to the inhibition of protoporphyrin IX production. This **iron-sensitive feedback loop** at the level of translation may account for the effects of succinylacetone. It is more difficult to explain the inhibition of ALA synthase expression by haem. There is currently no data to indicate the function of this feedback control or how it may operate.

3.6 REACTION MECHANISM FOR ALA SYNTHASE

The following mechanism has been elucidated for the reaction catalyzed by ALA synthase purified from R. spheroides (Zaman et al, 1973; Akhtar et al, 1976; Abboud et al, 1974) (see figure 8)) -

1. Glycine forms a Schiff base with PLP which is bound to the enzyme yielding a stable carbanion.
2. A proton (R-configuration) is removed from the methylene carbon atom of glycine.
3. Succinyl CoA condenses to the carbanion to form α-amino-β-ketoadipic acid with the loss of CoA.
4. α -amino-β-ketoadipic acid is decarboxylated to yield ALA.

The proton on the methylene carbon atom of glycine lost at step 1 is specifically that of the R configuration while the one having the S-configuration is incorporated into ALA (Zaman et al, 1973). Succinyl CoA is not required for the proton removal reaction (Laghai and Jordan, 1976). Haem, an allosteric effector of the enzyme activity, has, however, no effect on the loss of the proton from glycine (Laghai and Jordan, 1976). Decarboxylation of α-amino-β-ketoadipic acid to ALA occurs on the enzyme and then ALA is released into the medium from the pyridoxal-enzyme complex (Abboud et al, 1974).

3.7 THE ASSAY OF ALA SYNTHASE ACTIVITY

3.7.1 Colourimetric Assay of ALA Synthase Activity

ALA synthase activity is generally determined in tissue homogenates or in isolated mitochondrial fractions by the use of a colourimetric method (Granick and Urata, 1963; Marver et

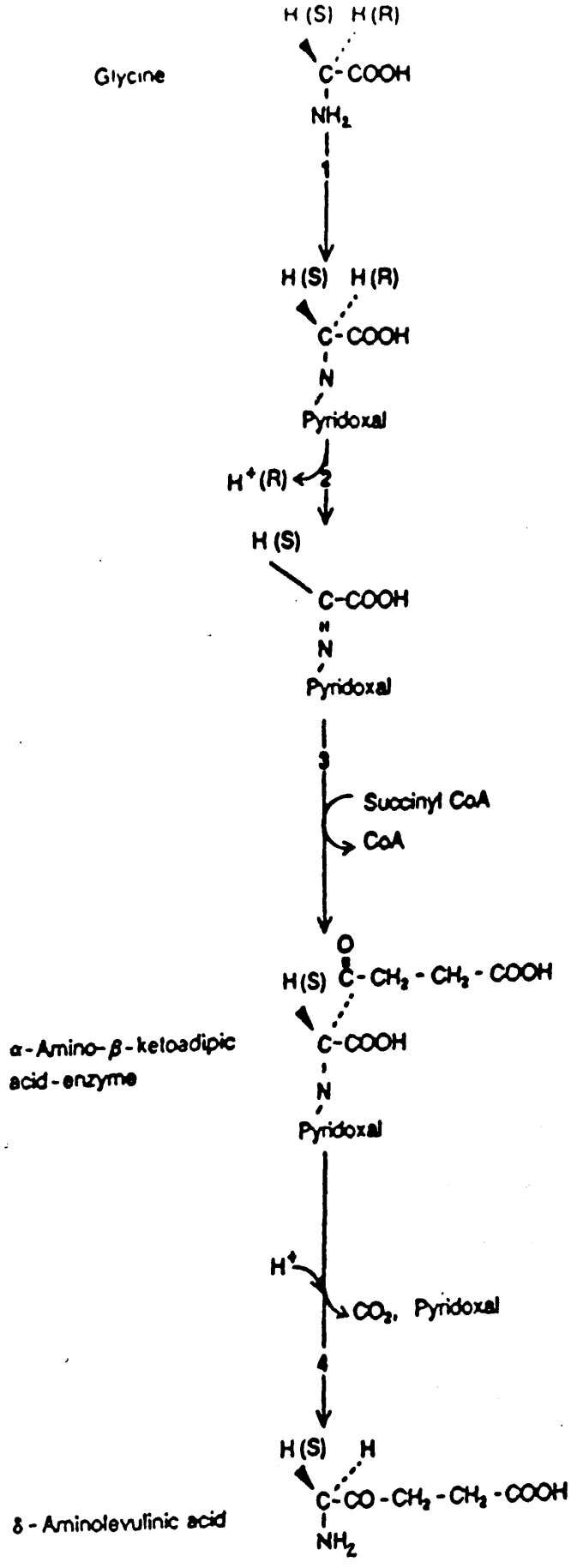


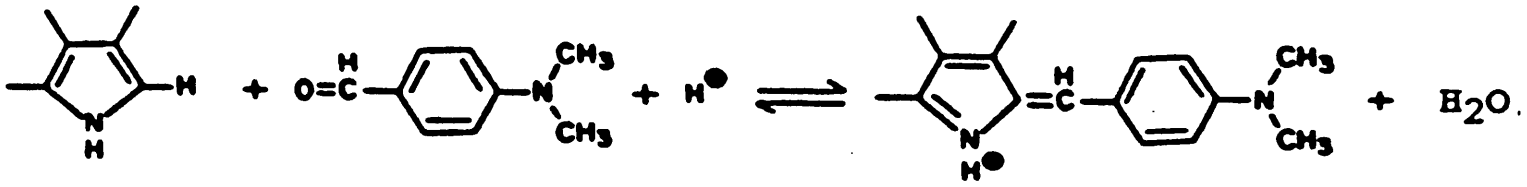
FIGURE 8

THE REACTION MECHANISM FOR ALA FORMATION

al, 1966; Hayashi et al, 1969; Sassa et al, 1979). The original colo rimetric assay (Mauzerall and Granick, 1956) involved the conversion of ALA to a pyrrole by adding either acetylacetone or ethylacetoacetate. ALA pyrrole may then be reacted with modified Ehrlich reagent and quantified by spectrophotometry at 555nm. In this reaction a coloured salt is formed by the reaction of the free α-hydrogen on the pyrrole with the dimethylamino-benzaldehyde (DMAB) in the Ehrlich reagent (Lien and Beattie, 1982) (Figure 9). The colour is not stable and must be read at 15 minutes to avoid the reaction of the coloured salt with a second molecule of pyrrole to form a colourless compound.

The major limitation of this assay method for ALA formation is the lack of sensitivity. The limit of detection with colo rimetric assays is approximately 3 nmoles ALA/ml (Falk, 1964). Such assays are not then suitable for use with tissues containing low levels of ALA synthase activity, or with tissues which are only available in very small quantities such as bone marrow.

The formation of aminoacetone pyrrole which also reacts with Ehrlich reagent may present a significant problem. Aminoacetone is formed by the condensation of glycine with acetyl CoA, catalyzed by aminoacetone synthase, or by the oxidation of threonine (Lien and Beattie, 1982). Separation of ALA pyrrole and aminoacetone pyrrole can be achieved by Dowex ion exchange chromatography (Mauzerall and Granick, 1956; Marver et al, 1966) or alternatively by solvent extraction (Granick, 1966; Sassa et al, 1979).



Note:

P = pyrrole;
DMAB = dimethylaminobenzaldehyde;
E = coloured salt.

FIGURE 9

Colourimetric Measurement of ALA Formation

3.7.2 Fluorometric Assay of ALA Synthase Activity

A highly sensitive fluorometric assay for ALA synthase activity has been described (Bishop et al, 1982). Although this assay was capable of detecting ALA synthase activity in the pmole/ml range it was not able to detect enzyme activity in bone marrow lysates (Bottomley and Moore, 1982).

3.7.3 **Radiochemical Assay of ALA Synthase Activity**

Radiochemical assays of ALA synthase activity have been shown to be the most sensitive means of detection and are sensitive to the level of 10-100 pmoles ALA/ml (Irving and Elliot, 1969). Such methods have been favoured in the detection of ALA synthase activity in liver (Irving and Elliot, 1969), human bone marrow (Bottomley et al, 1973), 'in vitro' cultured cell lines (Ebert and Ikawa, 1974), 'in vitro' bone marrow cultures (Ibrahim et al, 1982), peripheral blood leucocytes (Brodie et al, 1977) fractionated human bone marrow erythroblasts (Fitzsimons et al, 1986), adrenal gland (Condie et al, 1976), heart (Briggs et al, 1976; Israelis et al, 1974), kidney (Barnes et al, 1971), brain (Barnes et al, 1971) and testes (Tofilon and Piper, 1980).

For the purposes of the work described here a highly sensitive specific radiochemical assay suitable for the measurement of ALA synthase activity in the small cell numbers available from human bone marrow was required. Such a method (Fitzsimons et al, 1986) was adopted and modified to further optimise the conditions for the measurement of erythroid ALA synthase activity.

3.8 MEASUREMENT OF ALA SYNTHASE ACTIVITY IN HUMAN BONE MARROW

ALA synthase activity in human bone marrow was measured by the radiochemical method described by Fitzsimons et al (1986). This method utilises [^{14}C]-succinate and glycine as enzyme substrates and employs a means for the isolation of the radioactive product [^{14}C]-ALA which is more sensitive and specific than that offered by other assays. The method involves firstly the conversion of [^{14}C]-ALA product to the more stable compound [^{14}C]-ALA pyrrole . The pyrrole is then partially isolated by sorbent extraction prior to complete isolation from radioactive contaminants by reversed phase high performance liquid chromatography (RPHPLC). The RPHPLC isolation of ALA pyrrole was based on the method first described in the assay of rat liver ALA synthase activity (Tikerpae et al, 1981) and when combined with a radiochemical assay offers sensitivity double that of the next most sensitive assay.

3.8.1 Problems with Radiochemical Assays of ALA Synthase Activity

There are three major difficulties with regard to radiochemical assays of ALA synthase activity in human bone marrow.

1. Choice of suitable radioactive substrate (Section 3.8.2).
2. Isolation of radioactive ALA (Section 3.8.3).
3. Loss of radioactive ALA during enzyme incubation known as **ALA utilisation** (Section 3.8.10(iv)).

3.8.2 Choice of Suitable Radioactive Substrate

The direct substrates of ALA synthase are succinyl CoA and

glycine. The assay described by Fitzsimons et al (1986) utilises [^{14}C]-succinyl CoA as substrate as opposed to [^{14}C]-glycine (Freshney and Paul, 1970) because the Km of ALA synthase for glycine is high at 4.2 mmol/l (Freshney and Paul, 1970), 2.5-7.5 mmol/l (Nakakuki et al, 1980) and approximately 160 times the Km for succinyl CoA (Aoki et al, 1974). Thus, [^{14}C]-glycine is not a practical substrate (Bishop and Wood, 1977).

The Km for succinyl CoA is low at 11 µmol/l (Nakakuki et al, 1980). Succinyl CoA is unstable both at -20°C and during incubation at 37°C (Aoki et al, 1974). It is unavailable commercially and is normally prepared from [^{14}C]-ketoglutarate or [^{14}C]-succinate. [^{14}C]-succinate provides higher sensitivity than [^{14}C]-ketoglutarate in both assays of hepatic (Ebert et al, 1970) and erythroid (Fitzsimons et al 1986) ALA synthase and was therefore chosen for use in conjunction with a succinyl CoA generating system in the work described here.

3.8.3 Isolation of Radioactive ALA

A major problem in the assay of ALA synthase activity is the specific isolation of [^{14}C]-ALA from other radioactive compounds found in the reaction mixture (Patton and Beattie, 1973). Most radiochemical methods to date have employed solvent extraction of ALA pyrrole by ethylacetate followed by single column ion-exchange chromatography (Irving and Elliot, 1969). This method was later simplified (Ebert et al, 1970) resulting in a rapid and sensitive radiochemical assay capable of detecting low levels of activity. The subsequent

development of a sequential multicolumn ion exchange procedure (Strand et al, 1972) improved specificity, but resulted in a laborious procedure which produced significant variability in ALA recoveries from duplicate samples (Schacter et al, 1976). In addition, complete separation of [^{14}C]-ALA from other radioactive compounds has never been achieved by this means in assays using mitochondria or crude liver homogenates (Bishop and Wood, 1977; Managa et al, 1978; Wolfson et al, 1980). Separation has, however, been marginally improved using a Beckman amino acid analyser (Bishop and Wood, 1977). Methods have also been developed to extract impurities from [^{14}C]-succinate which may contaminate [^{14}C]-ALA (Condie et al, 1976; Bishop and Wood, 1977; Brooker et al, 1982). Sodium dodecyl sulphate (SDS) has been used to replace TCA at reaction termination to inhibit the extraction of contaminants along with ALA. However, the presence of SDS resulted in decreased enzyme activity and variable recovery of the internal standard (Scotto et al, 1983). When [^{14}C]-glycine is used as substrate (Freshney and Paul, 1971) in place of [^{14}C]-succinate high voltage paper electrophoresis can effect separation of [^{14}C]-ALA from [^{14}C]-glycine (Rutherford et al, 1979), although this is incomplete. This method is not suitable for the separation of [^{14}C]-ALA from [^{14}C]-succinate.

The use of RPHPLC for the specific isolation of ALA pyrrole was first described by Tikerpae et al (1981). This method has since been modified (Fitzsimons et al, 1986) to enable the specific isolation of [^{14}C]-ALA pyrrole from [^{14}C]-succinate.

3.8.4 Principles of HPLC

Liquid chromatography is a chemical separation method based on the interactions between solutes, liquid mobile phase and solid stationary phase. Separation takes place in a column containing the chromatographic material (the stationary phase) in the form of small particles. The solvent (i.e. eluent or mobile phase) is pumped through the particle bed enabling sample components dissolved in the mobile phase to reversibly interact with the stationary phase in a continuous manner. Separation is achieved on the basis of the relative affinities of the sample components for the stationary and mobile phases. Components with a strong affinity for the stationary phase elute later than those with a greater affinity for the mobile phase.

The HPLC system consists of a pump and a solvent reservoir, with an injection valve for applying a precise volume of sample prior to the column. A solvent delivery system is connected to the column in order to feed it with mobile phase. The pump is capable of operating under high pressure (6000 psi) and the system is optimised to increase speed, resolution and sensitivity. After passing through the column the separated sample components are monitored by a detector then fractionated in a fraction collector.

The Detector

Samples may be detected using a variable wavelength spectrophotometer sensitive to the nanogram level of detection.

3.8.5 Principles of RPHPLC

Reversed-phase HPLC (RPHPLC) utilises solubility properties of the sample. Partitioning of the sample components between a hydrophilic and a lipophilic solvent depends on their respective solubility characteristics. Less hydrophobic components attach to the hydrophilic phase and more hydrophobic ones to the lipophilic phase. In RPHPLC, silica particles with chemically bonded hydrocarbon chains represent the lipophilic phase while an aqueous mixture of an organic solvent (surrounding the particles) represents the hydrophilic phase. The whole process, therefore, depends on the extractive power of the hydrophilic phase. This may be altered by the addition of an organic solvent to the hydrophilic phase. A high concentration of organic solvent will increase the extractive power for hydrophobic compounds.

The ability of a stationary phase to discriminate between two components i.e. the selectivity can be influenced in three different ways -

1 The choice of stationary phase

2 The addition of an organic solvent to the mobile phase

3(a) Changing the pH to alter the hydrophobicity of components containing acid and/or basic groups

(b) By the addition of an ion-pairing agent to render ionic components hydrophobic.

(i) <u>The Stationary Phase</u>

The retention of hydrophobic components is influenced by the thickness of the lipid layer. A C18 layer is more hydrophobic than a C8 layer which in turn is more hydrophobic than a C2

layer. HPLC columns are available prepacked with silica particles chemically bonded with these hydrocarbon chain lengths. C18 packing provides maximum resolving power for hydrophobic compounds.

(ii) The Mobile Phase

The mobile phase is an aqueous solution of an organic solvent, the type and concentration of which determines the extractive power.

(iii) Hydrophobicity of the Sample

The hydrophobicity of the sample may be increased by neutralising charged groups on the molecule by the incorporation of an ion-pairing substance in the mobile phase. The ion-pairing molecule is a bulky molecule which forms a complex with a charged sample component due to electrostatic interaction. The ion-ion bond is shielded by the rest of the molecule. Similarly, hydrophobic molecules may be made less so by the addition of a hydrophilic ion-pairing agent.

ALA pyrrole may be detected by HPLC/spectroscopy at a measuring wavelength of 252 nm. ALA pyrrole elutes from a C18 column between 7 and 8 minutes using a mobile phase of methanol : water (37 : 63 v/v) containing heptanesulphonic acid (0.005M) as ion pairing agent and set at a flow rate of 1.5ml min^{-1} (Fitzsimons et al, 1986).

This experimental work describes the assay of ALA synthase activity in normal human bone marrow and details:

1. Improvements made to the assay with respect to the composition of the reaction mixture.

2. Marrow cell preparation prior to enzyme assay.
3. The choice of labelled enzyme substrate.
4. Further study of the temperature-dependent inactivation of ALA synthase (Beattie et al, 1985; Fitzsimons et al, 1986).
5. The suitability of the assay for use with non-erythroid tissue.

3.8.6 Materials

The following chemicals and enzymes were purchased from Sigma Chemical Company: succinic acid, disodium salt, hexahydrate; trizma base, reagent grade; 5-aminolaevulinic acid, hydrochloride; succinylacetone (4,6-dioxoheptanoic acid); succinic thiokinase (succinyl CoA synthetase, EC.6.2.1.4), partially purified lyophilized powder from porcine heart; coenzyme A, sodium salt; guanosine 5' triphosphate, sodium salt type III; pyridoxal 5-phosphate (codecarboxylase).

Radiochemicals were purchased from Amersham International: 5-amino[4-^{14}C]-laevulinic acid hydrochloride, freeze-dried, sealed under nitrogen; [2,3-^{14}C]succinic acid, freeze-dried, sealed under nitrogen.

All other chemicals were purchased from BDH Chemicals Limited (reagent grade): sucrose, hexahydrate; magnesium chloride, hexahydrate; glycine (aminoacetic acid); ethylenediaminetetra-acetic acid, disodium salt; potassium dihydrogen orthophosphate; sodium acetate, trihydrate; trichloroacetic acid.

HPLC grade chemicals were purchased from the following companies: acetonitrile and methanol (Rathburn Chemicals); PIC B7 (ion pairing agent, heptane sulphonic acid) (Waters Associates).

3.8.7 HPLC EQUIPMENT

The following equipment was purchased from Waters Associates: dual piston pump (model M 6000 A); variable wavelength spectrophotometer (model 481); integrator (model 740, data module); sep-pak sorbent extraction columns (C18).

The following equipment was purchased from Canberra Packard: liquid scintillation analyser 2000 CA (model A2020); scintillation vials (pony vials); scintillation fluid (picofluor 40).

All other materials were purchased from the following companies: sample injector (Rheodyne, model 7125); reversed phase HPLC column-Apex C18 ODS, 5μM particle size (4.6 x 150mm) (Jones Chromatography); fraction collector (Gilson, model 203); sample concentrator (Techne dri block, model D8-3).

3.8.8 Preparation of Bone Marrow Cells

ALA synthase activity can be measured in mitochondrial fractions, partially purified preparations of the enzyme or in crude tissue homogenates. However, due to the loss of mitochondria that occurs during preparation of mitochondrial fractions from small amounts of tissue (as is the case for bone marrow samples), most workers have chosen to work on crude tissue preparations (De Matteis and Hollands, 1982).

The method of tissue preparation described and optimised for use in human bone marrow (Fitzsimons et al, 1986) was employed. Cell pellets were ice cooled then sonicated in incubation medium using an ultrasonic disintegrator (MSE, 150KW) fitted with a $^1/_8$" titanium probe set to 15 microns in air (3 pulses of 5 seconds duration). In the work described

here, the volume of incubation medium added to each cell pellet was reduced from 1.50ml to 0.85ml without loss of enzyme activity. As sonication was shown to reduce ferrochelatase activity (Chapter 5, Section 5.10.1) freeze-thawing was also examined as an alternative means of tissue preparation (Section 3.9.3).

3.8.9 Enzyme Assay Incubation Medium

Initial experiments took place in incubation medium prepared from distilled deionised H_2O containing sucrose (250mM), glycine (50mM), magnesium chloride, $MgCl_2$ (20mM), tris base (40mM), ethylenediaminetetra-acetic acid, EDTA (5mM) pH'd to 7.4 with potassium dihydrogen orthophosphate (KH_2PO_4) (50mM). This was the basic incubation medium to which all other components required for the enzyme reaction were added fresh on the day of assay. This was stable at -20°C for several months.

3.8.10 Function of Incubation Medium Components

(i) Sucrose

Sucrose is commonly present in the incubation medium (Rutherford et al, 1979; Wolfson et al, 1980; Tikerpae et al, 1981) and provides osmotic stability for ALA synthase during tissue preparation.

(ii) Glycine

Glycine is the immediate enzyme substrate and erythroid ALA synthase has been shown to be saturated with 25mM glycine (Bottomley et al, 1973). A concentration of 50mM glycine has

been used in most studies (Irving and Elliot, 1969; Tikerpae et al, 1981; Ibrahim et al, 1982).

(iii) Magnesium

Magnesium ions, Mg^{2+}, are required as cofactor for succinate thiokinase activity which catalyzes the activation of [^{14}C]-succinate to [^{14}C]-succinyl CoA (Ramaley et al, 1967). [^{14}C]-Succinyl CoA is the direct substrate of ALA synthase. The optimum Mg^{2+} concentration was established (Section 3.9.2).

(iv) Ethylenediaminetetra-acetic Acid (EDTA, disodium salt)

EDTA inhibits ALA dehydratase, the second enzyme of the haem biosynthetic pathway, and so prevents the condensation of ALA formed during the enzyme incubation to the monopyrrole, porphobilinogen (i.e. ALA utilisation). Enzyme activity is inhibited by EDTA via the chelation of Zn^{2+} ions (Bevan et al, 1980). Failure to inhibit this enzyme leads to loss of ALA via ALA utilisation in assays of both liver and bone marrow ALA synthase. A concentration of 5mM EDTA was found to completely abolish ALA utilisation in the assay of erythroid ALA synthase only when used in conjunction with succinylacetone (Section viii) (Fitzsimons et al, 1986) while EDTA alone completely abolishes ALA utilisation in the assay of hepatic ALA synthase (Ebert et al, 1970; Strand et al, 1972).

Incubation medium containing these stable components was prepared (Appendix I) and stored at -20^{o}C. On the day of assay further, less stable components were added.

(v) Pyridoxal Phosphate (PLP)

PLP is an essential cofactor for ALA synthase (Section 3.6).

However, exogenous PLP may not be necessary for the detection of maximum enzyme activity in normal human bone marrow (Bottomley et al, 1973). In tissues in which it has been required for maximum enzyme activity 0.1mM PLP was saturating (Irving and Elliot, 1969; Bishop et al, 1982). For this study 0.4mM PLP was chosen for use.

(vi) Coenzyme A (CoA)

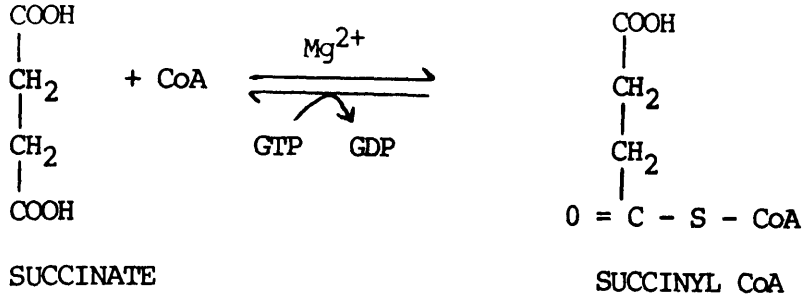
CoA is required as substrate by succinate thiokinase (STK) for the formation of succinyl CoA, the direct substrate of ALA synthase. The quantity of exogenous CoA required for maximum ALA synthase activity is dependent upon the nature of the tissue under study. For example, crude liver homogenate does not require exogenous CoA (Ebert et al, 1970) while crude deep frozen bone marrow sonicates require exogenous CoA at a concentration of 1.35mM (Fitzsimons et al, 1986).

(vii) Succinylacetone (4,6-dioxoheptanoic acid)

Succinylacetone is a profound and specific inhibitor of hepatic and erythroid ALA dehydratase (Tschudy et al, 1981; Sassa and Kappas, 1983). In the assay of human bone marrow ALA synthase a combination of succinylacetone (2mM) and EDTA (5mM) completely inhibits ALA utilisation (Fitzsimons et al, 1986).

(viii) Succinic Thiokinase (STK) (Succinyl CoA Synthetase; EC 6.2.1.4)

STK catalyzes the reversible conversion of succinate to succinyl CoA in a reaction which requires both Mg^{2+} ions and triphosphate as cofactors.



SUCCINATE

SUCCINYL CoA

The optimum concentration in the assay of bone marrow ALA synthase activity is 1 unit per ml (Fitzsimons et al, 1986).

(ix) Guanosine Triphosphate (GTP)

Commercial STK is GTP dependent for maximal activity. In normal human bone marrow the optimum GTP concentration was 5mM (Fitzsimons et al, 1986).

(x) [^{14}C] Succinate

When [^{14}C]-succinate is used as substrate for ALA synthase adequate quantities of [^{14}C]-succinyl CoA must be generated by the bone marrow cells themselves or alternatively by an exogenous succinyl CoA generating system. Such a system may be provided by the addition of STK, GTP, Mg^{2+} ions and CoA. Succinate (2mM, specific activity 3.0Ci/mol) is saturating for marrow ALA synthase and provides suitable sensitivity.

In summary, the incubation medium contains all the reagents required for the conversion of [^{14}C]-succinate and glycine to [^{14}C]-ALA at saturating concentrations. This ensures optimal ALA synthase activity. In addition, the [^{14}C] ALA formed during the reaction is preserved and its further metabolism to PBG by ALA dehydratase inhibited.

3.8.11 Experimental Procedure for the Measurement of Erythroid ALA Synthase

The experimental procedure for ALA synthase measurement is described:

3.8.12 Incubation Conditions

On the day of assay coenzyme A (1.35mM), GTP (5.0mM) and PLP (0.4mM) were added to incubation medium (20ml). This complete incubation medium (0.85ml) was added to each cell pellet (approximately 20 x 10^6 cells) prior to sonication.

The assay was carried out in duplicate in eppendorf tubes (1.5ml capacity) containing: [^{14}C]-succinate (0.05ml), 2mM succinylacetone (0.025ml), sonicated sample (0.40ml) and 0.5 units of succinate thiokinase (0.025ml)

The contents were mixed, then incubated at 37^{o}C for 1 hour in a shaking water bath (60 rpm).

3.8.13 Enzyme Reaction Termination

The enzyme reaction was terminated by the addition of 0.25ml ice cold TCA (10% w/v). A known quantity (0.05ml) of ALA (4mM) was added as internal standard to enable ALA recovery to be calculated for each sample. An aliquot (0.5ml) of sodium acetate (1M) was added to bring the pH to 6.5 and so optimise pyrrole formation.

3.8.14 ALA Pyrrole Formation

Samples were then spun in a microcentrifuge at 1200g (3 min) and the supernatant transferred to glass boiling tubes. Ethylacetoacetate (0.05ml) was added. Each tube was capped

with a marble then heated to 100°C for 20 minutes. This resulted in 100% conversion of ALA to ALA pyrrole, (Section 3.8.20). Distilled water was added to 5.0 ml.

3.8.15 Partial Isolation of [^{14}C]-ALA Pyrrole by Sorbent Extraction

Sorbent extraction is commonly used for sample clean-up prior to HPLC. 'Sep-pak' sorbent extraction columns containing packing material of C18 hydrocarbon chain length were used for the partial separation of [^{14}C]-ALA from the remaining [^{14}C]-succinate and other contaminants. The principles of sorbent extraction are broadly similar to that of RPHPLC and are discussed in Chapter 5, (Section 5.7.1).

(i) Activation of 'Sep-pak' Column

Columns were activated by the sequential addition of acetonitrile (10ml), H_2O (5ml), MeOH (5ml) and H_2O (10ml) and were reusable up to five times when reactivated between samples.

(ii) Application of Sample

Samples containing ALA pyrrole were applied to each activated column at a flow rate of 1.5ml per minute. Retention of ALA pyrrole was determined at 100% (Section 3.8.20). Columns were then dried on a bench top vacuum pump.

(iii) Elution of ALA Pyrrole

ALA pyrrole was eluted by the sequential addition of three aliquots of MeOH (1.0ml) at a flow rate of 1ml min^{-1}.

(iv) Sample Concentration

Samples were concentrated by solvent evaporation under N_2 at

37°C in a sample concentrator.

(v) Sample Reconstruction

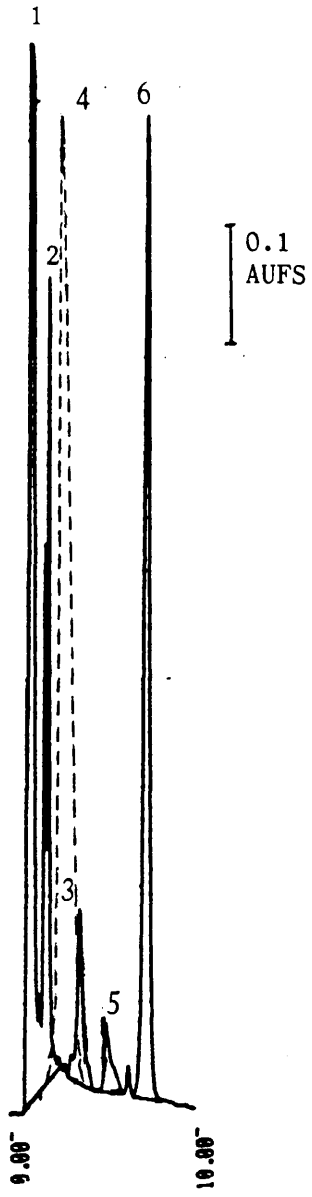
Samples were resuspended in 0.1ml of 50% aqueous methanol (v/v) and an aliquot taken for RPHPLC (0.05ml).

3.8.16 RPHPLC Isolation of [^{14}C] ALA PYRROLE

The HPLC pump was set at a flow rate of 1.5ml per minute. Mobile phase, 37% aqueous methanol (v/v) containing 0.005M heptane sulphonic acid (PIC B7) as ion-pairing agent was filtered (micropore 0.45mm filter) before use. The HPLC column was an Apex C18 ODS column, 5μM particle size (4.6 x 150mm). A variable wavelength spectrophotometer detected ALA pyrrole at 252nm and 1.0 AUFS. Column eluate was collected in 30 second fractions which were counted (dpm, β scintillation counting, 10 min) following the addition of picofluor 40 scintillant (3.0 ml).

ALA pyrrole eluted between 7 and 8 minutes following sample injection as a single sharp peak (Figure 10). Any free [^{14}C]-succinate which had been nonspecifically retained on the sep-pak was eluted by 3 minutes with no radioactive contamination of the ALA pyrrole peak. Other unidentified radioactive peaks eluted within 5 minutes of sample injection but did not interfere with isolation of ALA pyrrole and were probably due to the presence of other radioactive cellular metabolites.

The peak area of ALA pyrrole was directly proportional to the pyrrole content and was used to calculate ALA recovery per sample (Section 3.8.17).



NOTE:

Peak 1 = solvent front
Peak 2 = solvent front
Peak 3 = cellular metabolite
Peak 4 = [^{14}C]-succinate
Peak 5 = cellular metabolite
Peak 6 = [^{14}C]-ALA pyrrole

See text for further details

FIGURE 10

THE ISOLATION OF [^{14}C]-ALA PYRROLE USING REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.8.17 ALA Recovery

To calculate the ALA pyrrole peak area equivalent to 100% ALA recovery a known number of [^{14}C]-ALA dpm's were added to recovery samples containing: 0.50ml fresh incubation medium with [^{14}C]-ALA (0.025uCi).

These samples were treated in the same manner as those containing cells i.e. TCA, carrier ALA and sodium acetate were added prior to pyrrole formation and sep-pak application. From the recovery samples five aliquots (0.05, 0.04, 0.03, 0.02 and 0.01ml) were injected into the HPLC and fractions collected. From the recorded dpm in the original 0.025uCi [^{14}C]-ALA (corrected for the purity of the [^{14}C]-ALA, Section 3.8.20) recovery was calculated as the percentage dpm recovered in the ALA pyrrole peak. The graph relating percentage recovery to pyrrole peak area was linear and the peak area corresponding to 100% recovery could be read off. The average recovery of ALA pyrrole was 70%. The results for each sample were then corrected to 100% recovery.

3.8.18 Control Tubes

The dpm in the ALA pyrrole peak resulting from contaminating [^{14}C]-succinate or radioactive metabolites were determined for each batch of samples. Control tubes contained [^{14}C]-succinate incubated with bone marrow cells either for zero time and then treated as previously described, or for 1 hour at 37^{o}C but without the subsequent addition of ethylacetoacetate required for pyrrole formation. Control dpm were always low (less than 100 dpm; background 80 dpm).

3.8.19 Enzyme Activity Calculation

For each bone marrow sample assayed, the dpm in the ALA pyrrole peak was corrected for background and for 100% recovery. The ALA synthase activity in each sample was then calculated from the formula:

$$\text{pmol ALA/eppendorf tube/h} = \frac{\text{corrected dpm in ALA pyrrole}}{\text{dpm/pmol } [^{14}C]\text{-substrate}}$$

Enzyme activity was related to the erythroblast content of each sample.

3.8.20 Purity of Commercial [^{14}C]-ALA

The purity of commercial [^{14}C]-ALA was assessed at 4-6 weekly intervals since [^{14}C]-ALA slowly degrades during storage at -20^{o}C over a period of months. This enabled an accurate value for the recovery of [^{14}C]-ALA pyrrole to be calculated. The method used was first described by Fitzsimons (M.D. Thesis, 1986).

Method

1.2 nmol [^{14}C]-ALA is added to 0.5ml incubation medium containing 200 nmol cold ALA (identical to that used as internal standard). ALA pyrrole is formed and the volume made to 5.0 ml with water. From this solution 0.5ml is diluted 1 in 5 with water and reacted with an equal volume of modified Ehrlich reagent. The O.D. at 555 nm is then determined (0.32). From the formula:

$$\text{CONC(M)} = \frac{\text{O.D.}}{\text{Extinction coefficient (M)}}$$

it can be confirmed that 100% conversion of cold ALA to ALA

pyrrole occurs under these conditions. 1.0 ml of this solution is taken for scintillation counting and the remainder injected through an activated C18 Sep-pak cartridge. No ALA pyrrole can be detected in the sep-pak eluate by reaction with Ehrlichs reagent thereby indicating that all ALA pyrrole has been retained by the Sep-pak. 1.0ml of eluate is then taken for scintillation counting.

Despite the absence of detectable ALA pyrrole in the eluate, a significant proportion of the original dpm may be recovered in this fraction. 1.0ml of this fraction was reheated to 100% for 20 min with 0.1 ml ethylacetoacetate. On cooling, no ALA pyrrole can be detected with Ehrlich reagent. Therefore, these dpm do not represent unconverted [^{14}C]-ALA but rather contaminating dpm from [^{14}C]-ALA degradation.

ALA pyrrole may be eluted from the Sep-pak with methanol (3.5ml). Complete ALA pyrrole recovery can be confirmed with modified Ehrlich's reagent and the purity of the dpm retained by the sep-pak as [^{14}C]-ALA confirmed by RPHPLC.

It was found that after 6 months storage at -20^{o}C the purity of the [^{14}C]-ALA was reduced from 98% to approximately 90%. The purity of the [^{14}C]-ALA was therefore monitored every 4-6 weeks.

3.9 IMPROVEMENTS TO THE EXISTING ALA SYNTHASE ASSAY

Initial assays detected less ALA synthase activity in whole bone marrow than that reported by Fitzsimons et al (1986). The source of water used to prepare assay incubation medium was identified as the factor responsible for this reduced enzyme activity.

3.9.1 Effect of Water Purity in Incubation Medium on ALA Synthase Activity

Initial assays took place in incubation medium prepared from distilled deionised H_2O. The effect of the purity of the H_2O on ALA synthase activity was tested using three batches of incubation medium prepared from different H_2O sources:

1. distilled, deionised
2. distilled
3. sterilised, bottled.

Results

The results are expressed in Table 2 as dpm in the ALA pyrrole peak. Each value was corrected to 100% recovery.

Conclusions

1. ALA synthase activities in samples assayed in incubation medium prepared from sterilised bottled H_2O and from distilled deionised H_2O were 83% and 58% respectively, relative to the enzyme activity in incubation medium prepared from distilled H_2O.

   It was suspected that water deionisation may have removed an ion required for maximal enzyme activity. Ferrous ions in particular may be involved in the biosynthesis of ALA in chicken erythrocyte preparations (Brown, 1958; Morrow et al, 1969) and were included (10^{-6}M) in two of each of the triplicate assays.

2. The addition of Fe^{2+} ions (10^{-6}M) had the greatest effect on those samples suspended in incubation medium prepared from distilled deionised H_2O. The presence of Fe^{2+} ions at this concentration increased enzyme activity by 24%. Nevertheless,

Source of H_2O for Incubation Medium	Fe^{2+} ions (10^{-6} M)	Corrected Average dpm in ALA-P	Increase in Activity (%) With Fe^{2+}
Double distilled, Deionized H_2O	-	2051	24
	+	2552	
Distilled H_2O	-	3550	N.S.
	+	3768	
Sterilized, Bottled H_2O	-	2963	N.S.
	+	2934	

Each point represents the mean of duplicate observations
NS - not significant

TABLE 2

EFFECT OF THE SOURCE OF THE H_2O IN INCUBATION MEDIUM ON ALA SYNTHASE ACTIVITY

enzyme activity remained 28% below that obtained in incubation medium prepared from distilled H_2O.

3. The addition of Fe^{2+} ions (10^{-6}M) to samples incubated in incubation medium prepared from distilled H_2O or sterilised, bottled H_2O had no effect on enzyme activity.

The results indicate that deionisation of the incubation medium leads to a reduction in ALA synthase activity. The addition of Fe^{2+} ions at 10^{-6}M partially prevented the loss of enzyme activity. The reduction in enzyme activity resulting from the use of sterilised bottled water may relate to the presence of minute quantities of detergent remaining in these bottles. The assay is known to be sensitive to the presence of detergent during incubation (Fitzsimons et al, 1986) and the addition of Fe^{2+} ions (10^{-6} M) did not improve enzyme activity.

Distilled H_2O was used routinely to prepare ALA synthase assay incubation medium (as opposed to the distilled deionised H_2O used previously). This almost doubled the sensitivity of the assay. The effects of altering the concentration of EDTA and $MgCl_2$ in the incubation medium were then examined.

3.9.2 Effect of Altering the Concentration of Components of the Incubation Medium

EDTA (10mM) and $MgCl_2$ (20mM) had previously been used in the assay of rat liver ALA synthase activity (Tikerpae et al, 1981) and were chosen without further experimentation in the assay of human bone marrow enzyme activity (Fitzsimons et al, 1986). Since both EDTA and Mg^{2+} ions are essential components of the assay, EDTA as an inhibitor of ALA dehydratase and Mg^{2+} ions as cofactor for STK, the optimum concentrations of these

components were determined. The need for an exogenous succinyl CoA generating system was also confirmed.

Results

The results are shown in Table 3. Corrected dpm in [^{14}C]-ALA pyrrole was taken as a measure of ALA synthase activity.

Conclusions

1. A reduction in the concentrations of EDTA and $MgCl_2$ from 10mM and 20mM to 2mM and 5mM respectively had no significant effect on ALA synthase activity.
2. In those assays which contained EDTA (10mM) and $MgCl_2$ (20mM) the addition of STK (1 unit/ml) to provide an external succinyl CoA generating system resulted in an increase in detectable ALA synthase activity of 29%.
3. In assays containing EDTA (2mM) and $MgCl_2$ (5mM) the addition of STK (1 unit/ml) produced an increase in detectable ALA synthase activity of 59%.

It is unclear why the presence of STK should apparently produce a larger (x 2) increase in enzyme activity in the presence of lower concentrations of EDTA and $MgCl_2$. Nevertheless, it is obvious that an exogenous succinyl CoA generating system is required for the assay of bone marrow ALA synthase activity in cells which have been stored at -80^{o}C prior to assay.

The conclusions from this work led to alterations in the incubation medium by reducing the concentration of EDTA and $MgCl_2$ to 2mM and 5mM respectively. This is similar to the concentrations chosen in ALA synthase assays which utilise 2-ketoglutarate as substrate (Tikerpae et al, 1981). This

[EDTA] mM	[Mg^{2+}] mM	$\pm$ STK 1 unit/ml^{-1}	Mean Corrected dpm in [^{14}C] ALA-P
10	20	-	1846
10	20	+	2389
10	20	-	1671
2	5	+	2982
2	5	-	1845
2	5	+	2940
2	5	-	1881

Each point represents the mean of duplicate observations

TABLE 3

EFFECT OF ALTERING THE CONCENTRATION OF EDTA, Mg^{2+} IONS AND SUCCINATE THIOKINASE ON ALA SYNTHASE ACTIVITY

improved the sensitivity of the assay possibly via an effect on STK with increased availability of succinyl CoA.

3.9.3 Optimum Procedure for Sample Preparation Prior to Assay

The optimum procedure for bone marrow sample preparation prior to assay of ALA synthase activity (Fitzsimons et al, 1986) was shown to be sonication of bone marrow cells in incubation medium using a $^{1}/_{8}$" titanium probe set to 15 microns in air (3 x 5'). Work described later in this thesis (Chapter 5, Section 5.10.1) determined that optimum ferrochelatase activity is achieved by freeze-thawing (x 3) in incubation medium : glycerol (80:20, vol/vol). The effect of freeze thawing on ALA synthase activity was therefore determined. ALA synthase activity was determined following different sample preparation methods.

Sample Preparation	p moles ALA/10^6 erythroblasts/hr
Sonication (3 x 5')	612
Freeze thawed (x 3) in incubation medium	390
Freeze thawed (x 3) in incubation medium: glycerol (80:20, vol/vol)	275 235

(Each point represents the mean of duplicate observations).

The results confirmed sonication (Fitzsimons et al, 1986) to be the best means of sample preparation prior to measurement of bone marrow ALA synthase activity.

3.9.4 Effect of Using [^{14}C]-Glycine as Labelled Substrate

ALA synthase activity was assayed in human bone marrow using [^{14}C]-glycine as labelled substrate as an alternative to

[^{14}C]-succinate.

Incubation medium was prepared with a reduced glycine concentration of 1mM, as used by Freshney and Paul (1972). On the day of assay all other components of the incubation medium were added but with [^{14}C]-glycine (2.5μCi) in 1mM glycine substituted for [^{14}C]-succinate. The assay was then carried out in the normal manner.

Several attempts to measure ALA synthase activity in bone marrow (10 x 10^6 cells) by this method failed due to an inability to isolate [^{14}C]-ALA using RPHPLC. Although most (80%) of the dpm in commercial [^{14}C]-glycine eluted at 4 mins following sample injection several additional radioactive peaks containing significant dpm were also detected between 0 and 10 minutes and the dpm attributable to [^{14}C]-ALA alone could not then be determined. A known quantity of [^{14}C]-glycine was injected into the HPLC. The pattern of dpm elution was identical to that previously described indicating that the [^{14}C]-glycine was impure and that these impurities rather than radioactive metabolites were the cause of contamination. The separation of [^{14}C]-ALA and [^{14}C]-glycine by high voltage electrophoresis (Freshney and Paul, 1970) may therefore also be subject to error due to the presence of radioactive contaminants in [^{14}C]-glycine. In view of the high Km value for glycine and this clear evidence of contaminating impurities [^{14}C]-glycine is not a suitable substrate for measurement of erythroid ALA synthase.

3.9.5 Stability of [^{14}C] Succinate

The stability of [^{14}C]-succinate during storage at -20°C

was examined.

Several identical bone marrow samples were assayed for ALA synthase activity using either freshly prepared [^{14}C]-succinate solution or [^{14}C]-succinate preparation which had been stored at -20^{o}C for several months. The results were corrected for the specific activity of the [^{14}C]-succinate preparation and to 100% recovery of ALA pyrrole. No deterioration in the effectiveness of the [^{14}C]-succinate preparation as enzyme substrate was observed.

	[^{14}C] Succinate Preparation Stored at -20^{o}C	[^{14}C] Succinate Preparation Freshly Prepared
dpm in [^{14}C]-ALA pyrrole (x)	14863	14649
(S.D.)	52	158
(n)	3	3

3.9.6 Stability of Basic Incubation Medium

The stability of the basic incubation medium during storage at -20^{o}C was examined.

Incubation medium was routinely prepared (Appendix I) then stored in 20ml aliquots at -20^{o}C. At each fresh preparation of incubation medium an assay in stored incubation medium (-20^{o}C, 6 months) was included alongside assays in freshly prepared incubation medium. This enabled the assessment of any inter-batch variation in the effectiveness of the incubation medium.

Incubation medium could be stored for up to 1 year without losing its effectiveness in the assay of ALA synthase activity.

3.10 STABILITY OF ERYTHROID ALA SYNTHASE

The stability of erythroid ALA synthase was investigated. Studies of hepatic ALA synthase have shown that incubation at 37°C of the mitochondrial matrix fraction from either control or AIA-induced rats resulted in a significant loss of enzyme activity (50-70%) after 30 min (Beattie et al, 1985). However, addition of 0.1mM PLP, to the preincubation medium completely prevented this loss. The loss was temperature dependent as a 55% loss of ALA synthase activity occurred following incubation at 30°C (60 min) while the enzyme was completely stable at 22°C. ALA synthase inactivation did not involve proteolytic digestion since the addition of a wide range of protease inhibitors in the absence of PLP did not protect against inactivation. The theory has been put forward (Beattie et al, 1985) that the cofactor, PLP, may dissociate from the enzyme during preincubation resulting in irreversible inactivation of the apoenzyme at temperatures above 22°C.

A loss of enzyme activity during preincubation at 37°C would be particularly significant to the work described here because it was intended to study enzyme activity in fractionated erythroblasts (Chapter 2). This fractionation and white cell removal requires a 30 min period of incubation at 37°C.

In this study of bone marrow erythroid ALA synthase activity a temperature-dependent inactivation was consistently observed. Attempts were made to prevent this loss of activity with PLP and associated metabolites.

3.10.1 Pyridoxine Metabolism

Pyridoxine has several well-documented functions in normal cellular chemistry. In amino acid metabolism it is required as a coenzyme in such reactions as decarboxylation, deamination, transamination, transulphuration and desulphuration. It is also required for the normal cellular transport of amino acids and with regard to the biosynthesis of haem, it is essential as a coenzyme for the formation of 5-aminolaevulinic acid.

Pyridoxine exists in three biologically active forms; pyridoxine, pyridoxamine and pyridoxal. Anderson et al (1971) have partly clarified the mechanism for conversion of pyridoxine to the metabolically active form, pyridoxal, in mature red cells (Figure 11). The mature cell is the only major site for this conversion.

Nutritional pyridoxine deficiency is rare in man. It is manifest by glossitis, dermatitis and neurological abnormalities. Mice maintained on a pyridoxine-deficient diet develop a hypochromic anaemia and abnormal sideroblasts in the bone marrow after 6 weeks. Typical ring sideroblasts are usually absent, but can be produced if the animals are given additional iron or the pyridoxine antagonists cycloserine and isoniazid (Harris et al, 1965). In swine, experimental pyridoxine deficiency results in a microcytic, hypochromic anaemia, a raised serum iron and saturated TIBC (Harris et al, 1965). The bone marrow shows an increase in sideroblasts, indicating that pyridoxine deficiency may have a role to play in sideroblastic anaemia (see Section 1.15.2). However, in man, the effects of pyridoxine deficiency vary between subjects. This is well illustrated by the reports of two pairs

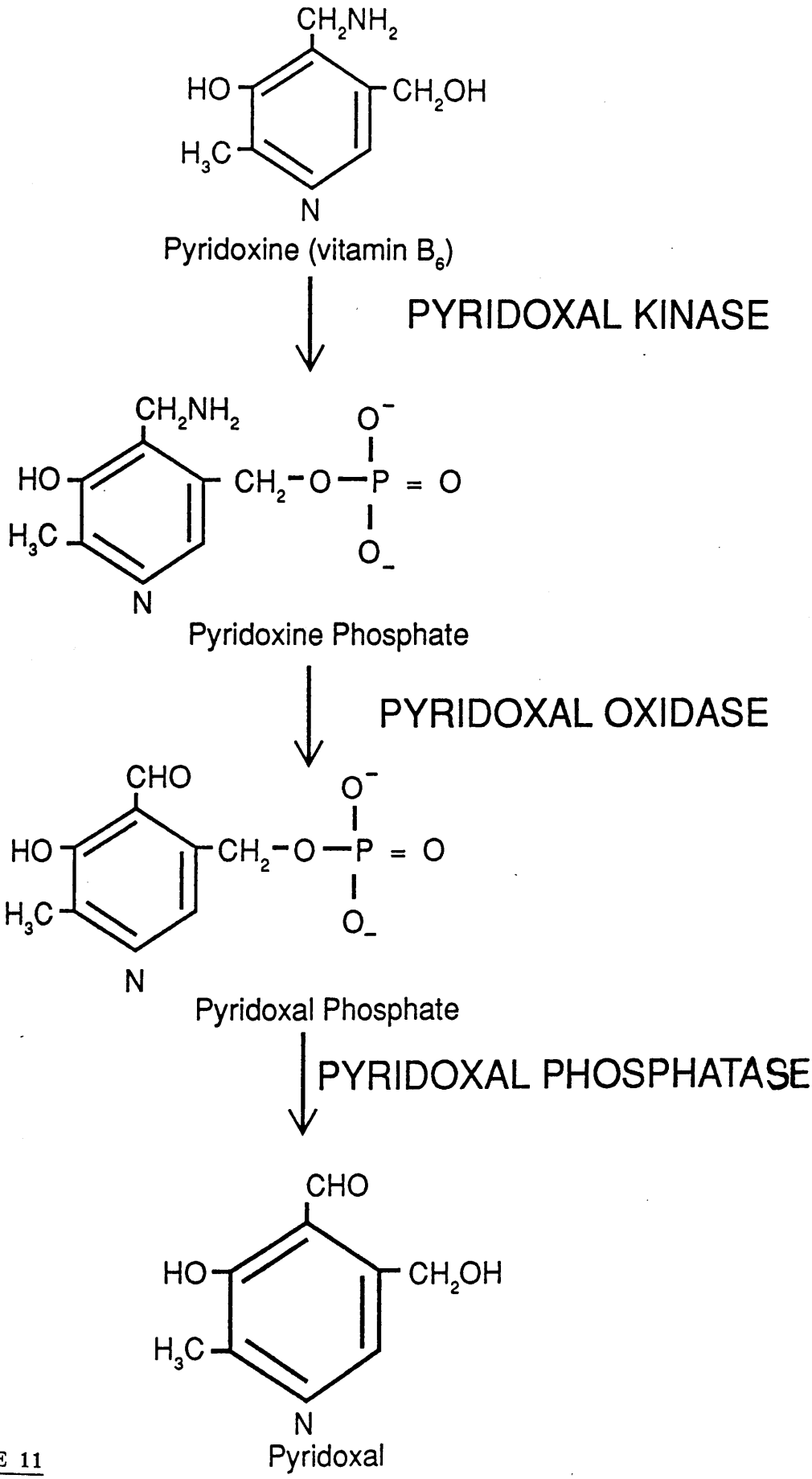


FIGURE 11

THE CONVERSION OF PYRIDOXINE TO PYRIDOXAL WITHIN THE MATURING RED CELL

of brothers who were deficient in pyridoxine. While one brother developed neurological abnormalities, the other only became anaemic (Gehrmann, 1965; Snyderman et al, 1953).

3.10.2 Pyridoxal Phosphate as Cofactor for ALA Synthase Activity

Almost all features of pyridoxine-responsive anaemia can be ascribed to the disturbance of erythroblast haem synthesis (Aoki et al, 1974; Heilmeyer, 1966; Hines and Grasso, 1970; Weintraub et al, 1966; Vogler and Mingioli, 1965). The main defect is believed to lie at ALA synthase. It is generally considered that decreased Hb synthesis and decreased erythrocyte production due to ineffective erythropoiesis are caused by reduced ALA synthase activity in erythroblasts, and that improvement of Hb synthesis and RBC production in the bone marrow by treatment with pyridoxine results from the increase in ALA synthase activity.

Erythroblast ALA synthase has also been shown to be subject to a temperature sensitive inactivation. ALA synthase activity was measured in bone marrow cells following preincubation with PLP and associated metabolites in an attempt to correct for this temperature-dependent inactivation.

Experimental

(i) Stability of Erythroid ALA Synthase at -80°C

Erythroid ALA synthase was found to be stable at -80°C for up to one year.

(ii) Stability of Erythroid ALA Synthase at 4°C

Erythroid ALA synthase was found to be stable in intact cells

maintained at 4°C for up to eight hours.

(iii) Stability of Erythroid ALA Synthase at 37°C

A temperature-dependent inactivation of erythroid ALA synthase was consistently observed following a 30 min incubation at 37°C in culture medium. In a series of six experiments the average fall in ALA synthase activity in whole human bone marrow was 45% (± 14.8).

3.10.3 The Effect of Pyridoxal Phosphate, Pyridoxine and Pyridoxal on ALA Synthase Activity

The effects of pyridoxine metabolites were examined for their ability to protect ALA synthase activity in incubated (37°C) erythroid tissue.

Materials

Pyridoxal 5-phosphate (PLP), pyridoxine (PN) monohydrochloride and pyridoxal (PL) hydrochloride were purchased from Sigma Chemical Company.

3.10.4 The Effect of Pyridoxal Phosphate on the Temperature-Dependent Loss of ALA Synthase Activity

PLP (0-5mM) was added to intact normal human bone marrow cells suspended in 0.5ml serum, serum:culture medium (50% v/v) and incubation medium. The samples were mixed then preincubated at 37°C for 30 min. ALA synthase activity was measured in duplicate for each sample and the results were expressed as corrected dpm in ALA pyrrole (Tables 4(a)-(d)). Each table represents results obtained using bone marrow from a different individual.

A large decrease in ALA synthase activity was observed

(a)

Incubation time at 37°C (mins)	Serum (1ml) + PLP (mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	0	4096	-
30	0	2477	-39.5
30	0.2	1783	-56.4
30	0.5	1952	-52.3
30	1.0	2688	-34.4
30	2.0	2789	-31.9

(b)

Incubation time (mins) at 37°C (mins)	Serum (1ml) + PLP (mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	0	7047	-
30	0	3673	-47.9
30	0.1	2862	-59.4
30	0.2	3776	-46.4
30	0.5	5296	-24.8
30	1.0	4325	-38.6
30	2.0	3776	-46.4

Each point represents the mean of duplicate observations.

TABLES 4 (a), (b)

THE EFFECT OF PLP (0-2mM) ON THE TEMPERATURE-DEPENDENT LOSS OF ALA SYNTHASE ACTIVITY IN INTACT CELLS

(c) Incubation time (mins) at 37°C (mins)	Serum: culture medium (50% v/v) + PLP (mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	0	2398	-
30	0	1022	-57.4
30	1.0	965	-59.8
30	2.0	1132	-52.8
30	5.0	655	-72.7

(d) Incubation time (mins) at 37°C (mins)	Incubation Medium (1ml) + PLP (mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	0	2268	-
30	0	766	-66.2
30	0.5	1125	-50.4
30	1.0	1420	-37.4
30	2.0	1134	-50.0
30	5.0	973	-57.1

Each point represents the mean of duplicate observations.

TABLES 4 (c), (d)

THE EFFECT OF PLP (0-5mM) ON THE TEMPERATURE-DEPENDENT LOSS OF ALA SYNTHASE ACTIVITY IN INTACT CELLS

following preincubation which could not be prevented by PLP over the concentration range 0-5mM. The loss of activity was least in serum (39.5%) and greatest in incubation medium (66.2%). All incubations were carried out at pH 7.4.

Sonicated Cells

Sonication of cells prior to preincubation led to a greater temperature-dependent loss of ALA synthase activity than that observed in whole cells. This could however be prevented by 5-10mM PLP (Tables 5(a)-(c)).

The ability of 5mM PLP to prevent the temperature-dependent loss of enzyme activity in sonicated cells but not in intact cells suggests that extracellular PLP cannot reach intracellular ALA synthase. It would then appear that PLP either cannot traverse the erythroid cell membrane or the mitochondrial membrane. Since PLP is thought to freely pass through the erythrocyte membrane (Hamfelt, 1967) it is likely that PLP can enter the erythroblast but cannot penetrate the mitochondrial membrane to reach the site of the active enzyme.

3.10.5 The Effect of Pyridoxine on the Temperature-Dependent Loss of ALA Synthase Activity

Pyridoxine (PN) under normal circumstances in the mature erythrocyte is readily metabolised to the biologically active form, PLP (Anderson et al, 1971). The experiments described in Section 3.10.4 were repeated in triplicate with PN in place of PLP to determine whether PN could traverse the erythroid/mitochondrial cell membrane.

It was found that PN (0-5mM) could not protect against the

(a)	Incubation time at 37°C (mins)	Incubation medium (1ml) + PLP (mM)	Average corrected dpm	Change in ALA synthase activity (%)
	0	0	436	-
	0	0	456	-
	30	0	193	-51.6
	30	0	239	-45.2
	30	1	337	-24.4
	30	2	438	N.S.

Each point represents the mean of duplicate observations.

N.S. = not significant

TABLE 5 (a)

THE EFFECT OF PLP (0-2mM) ON THE TEMPERATURE-DEPENDENT LOSS OF ALA SYNTHASE ACTIVITY IN SONICATED CELLS

(b)

Incubation time at 37°C (mins)	Incubation medium (1ml) + PLP (mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	0	2268	-
30	0	840	-62.9
30	0.5	735	-67.6
30	1.0	1135	-50.0
30	2.0	1536	-32.3
30	5.0	2149	N.S.

(c)

Incubation time at 37° (mins)	Incubation medium (1ml) + PLP (mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	0	10778	-
30	0	1540	-85.7
30	5	12247	+13.6
30	5	13052	+21.1
30	10	10529	N.S.
30	10	12418	+15.2

Each point represents the mean of duplicate observations.
N.S. - not significant.

TABLES 5 (b), (c)

THE EFFECT OF PLP (0-10mM) ON THE TEMPERATURE-DEPENDENT LOSS OF ALA SYNTHASE ACTIVITY IN SONICATED CELLS

temperature-dependent inactivation of ALA synthase in either intact or sonicated cells (Tables 6(a),(b)).

3.10.6 The Effect of Inhibition of Haem Synthesis on the Temperature-Dependent Loss of ALA Synthase Activity

It was noted that the temperature-dependent loss of ALA synthase activity was greater in incubation medium than in serum or culture medium. Since incubation medium contained EDTA (2mM), an inhibitor of haem synthesis, it was speculated that the excessive loss in the presence of EDTA may have resulted from inhibition of haem synthesis. The effects of two inhibitors of haem synthesis i.e. EDTA and succinylacetone on ALA synthase activity during preincubation were examined. As previously discussed, both EDTA and succinylacetone are inhibitors of ALA dehydratase.

3.10.7 The Effects of EDTA on the Temperature-Dependent Loss of ALA Synthase Activity

Intact and sonicated bone marrow cells (10×10^6 cells) were preincubated in incubation medium both in the presence and absence of EDTA (2mM).

The loss of enzyme activity was equally high in the presence or absence of EDTA (2mM) indicating that EDTA did not protect against the temperature-dependent loss of enzyme activity. The loss of activity observed in sonicated cells (> 90%) (Table 7(a)) was greater than that observed in intact cells (54%) (Table 7(b)) again suggesting that ALA synthase becomes less stable following sonication. In accordance with the earlier results (Section 3.10.4) the addition of PLP (5mM) abolished the loss of enzyme activity in sonicated cells, but not in intact cells.

(a) Incubation time at 37°C (mins)	Incubation medium (1ml) PLP (5mM)	EDTA (2mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	-	+	2351	-
30	-	-	179	-92.4
30	-	+	206	-91.2
30	+	-	2162	-8.0

(b) Incubation time at 37°C (mins)	Incubation medium (1ml) PLP (5mM)	EDTA (2mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	-	+	2351	-
30	-	-	1075	-54.3
30	-	+	1185	-49.6
30	+	-	1437	-38.9

Each point represents the mean of duplicate observations.

TABLE 7 (a), (b)

THE EFFECT OF EDTA (2mM) ON THE TEMPERATURE-DEPENDENT LOSS OF ALA SYNTHASE ACTIVITY IN (a) SONICATED AND (b) INTACT CELLS

3.10.8 The Effect of Succinylacetone, EDTA and Pyridoxal Phosphate on the Temperature-Dependent Loss of ALA Synthase Activity

Intact bone marrow cells were preincubated in incubation medium both in the presence and absence of succinylacetone (2mM).

The results (Table 8) demonstrated that succinylacetone (2mM) alone could partially prevent the temperature-dependent loss of ALA synthase activity by decreasing the loss from 62% to 32%. In the presence of both succinylacetone (2mM) and PLP (5mM) the loss of activity was further reduced to 21%. Once again, the presence or absence of EDTA (2mM) had no effect on enzyme activity.

This protective effect of PLP and SA was investigated in a series of further experiments (Table 9). The results demonstrated that succinylacetone (2mM) and PLP (5mM) together prevented the temperature-dependent inactivation of ALA synthase. PL (5mM) alone could not protect against the loss of enzyme activity, but in the additional presence of succinylacetone (2mM) the loss was reduced from 58% to 12%.

3.10.9 The Effect of Pyridoxal Phosphate and Succinylacetone on the Temperature-Dependent Loss of ALA Synthase Activity

The addition of succinylacetone (2mM) to the incubation medium during preincubation of sonicated cells led to a very surprising loss of the protective effect of PLP (5mM) on ALA synthase activity (Table 10). This indicates that the combination of PLP and SA will protect the enzyme in intact cells but not in sonicated cells.

Incubation time at 37°C (mins)	Incubation medium (1ml) PLP (5mM)	Succinyl acetone (2mM)	EDTA (5mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	-	-	-	13748	-
30	-	-	-	5206	-62.1
30	-	+	-	9282	-32.5
30	+	-	+	6025	-56.2
30	+	+	-	10864	-21.0

Each point represents the mean of duplicate observations.

TABLE 8

THE EFFECT OF SUCCINYLACETONE, EDTA AND PLP ON THE TEMPERATURE-DEPENDENT LOSS OF ALA SYNTHASE ACTIVITY IN INTACT CELLS

Incubation time at 37°C (mins)	Culture medium (1ml) EDTA (2mM)	S.A. (2mM)	PLP (5mM)	PL (5mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	-	-	-	-	2351	-
30	-	-	-	-	999	-57.5
30	+	-	-	-	1135	-51.7
30	-	+	-	-	1518	-35.4
30	-	-	-	+	1267	-46.1
30	+	-	-	+	1213	-48.4
30	-	+	-	+	1813	-22.9
30	-	-	+	-	1603	-31.8
30	+	-	+	-	2219	-5.6
30	-	+	+	-	2075	-11.7

Each point represents the mean of duplicate observations
S.A. = succinylacetone

TABLE 9

THE EFFECT OF EDTA, SUCCINYLACETONE, PLP AND PL ON THE TEMPERATURE-DEPENDENT LOSS OF ALA SYNTHASE ACTIVITY IN INTACT CELLS

(a) Incubation time at 37°C (mins)	Incubation medium (1ml) + succinyl acetone (2mM) + PLP (mM)	Average corrected dpm	Change in ALA synthase activity (%)
0	0	2268	-
30	0	840	-62.9
30	0.5	775	-65.8
30	1.0	1062	-53.2
30	2.0	1354	-40.3
30	5.0	1597	-29.6

Each point represents the mean of duplicate observations.

TABLE 10

THE EFFECT OF PLP AND SUCCINYLACETONE ON THE TEMPERATURE-DEPENDENT LOSS OF ALA SYNTHASE ACTIVITY IN SONICATED CELLS

3.10.10 Summary of Results

A temperature-dependent loss of ALA synthase activity (approximately 45%) was consistently observed during preincubation of bone marrow cells in serum, serum:culture medium (50:50 v/v) or in incubation medium at 37°C for 30 min prior to assay of ALA synthase. Neither PLP nor PN (0-5mM) when added to intact bone marrow cells could protect against the loss of enzyme activity. However, when the cells were sonicated prior to preincubation to disrupt cellular membranes, PLP at 5mM concentration or greater provided complete protection against this loss. PN at the same concentration did not. It was therefore concluded that PLP cannot traverse either the cell or mitochondrial membrane and so is unable to protect ALA synthase. Cell membrane rupture by sonication allowed PLP to gain access to the enzyme and protect against the loss of enzyme activity. Under the same conditions PN (5mM) was ineffective, probably due to an inability of sonicated cells to metabolise PN to the biologically active form of PLP.

The effects of two inhibitors of haem synthesis on ALA synthase activity during preincubation were examined. EDTA (2mM) was found to have no effect on ALA synthase activity in both intact and sonicated cells while succinylacetone (2mM) was found to partially prevent the loss of activity in intact cells. Succinylacetone (2mM) and PLP (5mM) added together to intact cells almost totally abolished the loss of enzyme activity. It is postulated that succinylacetone facilitated the transport of PLP across the cell/mitochondrial membrane. Conversely, when succinylacetone (2mM) and PLP (5mM) were

added together to sonicated cells, the ability of PLP to protect against the temperature dependent loss of ALA synthase activity was greatly reduced. EDTA did not interfere with the protective action of PLP in sonicated cells.

3.10.11 Discussion

The loss of ALA synthase activity in the absence of exogenous PLP apparently results from a temperature-dependent inactivation of the enzyme. The protection afforded by the cofactor, PLP, suggests that the irreversible inactivation at 37°C may occur following removal of the cofactor from the enzyme. However, at 4°C the enzyme is stable following removal of the cofactor and may be reconstituted upon the addition of PLP. This contrasts with the behaviour of many other PLP-containing enzymes which become unstable even at 4°C after removal of PLP (Dzelzkalns et al, 1982). A possible explanation of the observed rapid inactivation of ALA synthase at 37°C is that this process may be a specific event which forms an important part of the intracellular regulation of ALA synthase. It has been suggested that ALA synthase may be subject to certain rigid controls such as the loss of the cofactor, PLP, influencing its sensitivity to proteolytic enzymes in the matrix (DeLoskey and Beattie, 1984; Beattie et al, 1984).

Previous reports (Aoki, 1978; Katunuma and Kominami, 1977; Srivastava et al, 1983; Nakakuki et al, 1980) had indicated that ALA synthase was sensitive to various proteases. Nevertheless, the lack of protection against enzyme

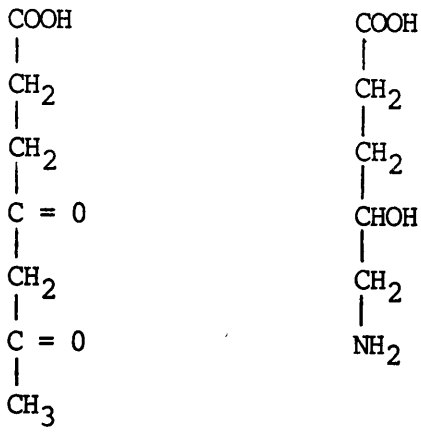
inactivation at 37°C afforded by a wide spectrum of protease inhibitors suggests that the primary event in the loss of activity does not involve proteolysis (Beattie et al, 1984). Furthermore, it has been suggested (Beattie et al, 1984) that initial "tagging" of the enzyme at 37°C prevents reassociation with PLP, thus preventing catalysis. Consequently, the enzyme may then become subject to proteolytic digestion. This type of two-step processing has been reported for many enzymes (Fucci et al, 1983) and it has been speculated that the inactivation of an enzyme may mark the protein for proteolytic digestion and thus may play a major role in the regulation of protein turnover.

It has been suggested that erythroid ALA synthase activity may be susceptible to a controlling protease located on the inner mitochondrial membrane of bone marrow cells (Aoki, 1978). An inverse relationship has been demonstrated between ALA synthase activity and the protease activity in erythroblasts. However, it has since been demonstrated that this protease was similar to, but not identical with, the group specific protease(s) described previously for PLP and PN-dependent enzymes (Katunuma and Kominami, 1977). In addition, both purified and active forms of ALA synthase have been shown to be sensitive to thiol proteases (Srivastava et al, 1983; Nakakuki et al, 1980). Thus the role of any erythroid-specific protease remains to be assessed.

The aim of the work described here was to find a possible means of preventing the temperature-dependent loss of erythroid ALA synthase activity. This was of considerable relevance to the work described in this thesis as much of the experimental

work was to be carried out on fractionated bone marrow erythroblasts. The fractionation process requires that the myeloid cell population be removed via cell lysis by monoclonal antibody TG-1. This requires a 30 minute incubation at 37^{o}C, prior to density gradient centrifugation (Chapter 2) and so presents a possible source of loss of ALA synthase activity.

The results suggest that the loss of enzyme activity during the 30 minute preincubation period could only be prevented in intact cells by the combined presence of PLP (5mM) and succinylacetone (2mM). The reason for the success of this combination is unclear, but it may be speculated that PLP and succinylacetone form a complex which is more readily transported across the cell membrane than PLP alone. Succinylacetone is structurally similar to ALA which exists as an ALA-PLP complex during the final stages of ALA formation (Section 3.6). Although succinylacetone lacks the amino group responsible for Schiff base formation between ALA and PLP it is possible that a third molecule may intervene e.g. glycine. On entering the cell succinylacetone must dissociate from this complex possibly via reaction with ALA dehydratase since the affinity of succinylacetone for ALA dehydratase is approximately 1000 times that of the natural substrate, ALA. PLP may then enter the mitochondria and interact freely with ALA synthase.



SUCCINYLACETONE ALA

In sonicated cells succinylacetone appears to interfere with the ability of PLP to protect against the temperature-dependent loss of ALA synthase activity. This is possibly due to an inability of the succinylacetone-PLP complex to dissociate in the ruptured cell. It is interesting to note that both succinylacetone and PLP are present in the incubation medium used to measure ALA synthase activity and the presence of succinylacetone in the incubation mixture has been shown not to affect erythroid ALA synthase activity (Fitzsimons et al, 1986). The reason why this does not present a problem during assay may be that succinylacetone is present in much lower concentrations (0.1mM).

This theory could be examined experimentally by assessing the effect of using ALA, known to form a complex with PLP, in place of succinylacetone and in conjunction with 5mM PLP. The effects of this combination on ALA synthase activity in intact and sonicated cells could then be observed.

The experiments described here were conducted over a 2 year period. As the protective effect of succinylacetone and

PLP (2mM and 5mM respectively) on ALA synthase activity in intact cells was not realised until the end of this period the fractionation experiments described in subsequent chapters were completed without PLP and succinylacetone.

In order to ensure the consistent loss of ALA synthase activity during myeloid cell lysis, a control sample of whole unfractionated bone marrow was incubated at 37°C alongside the TG-1 treated samples. Enzyme activity was then assayed and compared with that in an identical sample maintained at 4°C prior to assay. The loss was consistent at approximately 45% ± 14% (n = 6) in 30 mins.

3.11 APPLICATION OF THE ALA SYNTHASE ASSAY TO OTHER TISSUES

The assay described has been optimised for the measurement of ALA synthase activity in bone marrow. It was demonstrated for the first time here that this assay could successfully be employed in the measurement of ALA synthase activity in other tissues i.e. rat liver and brain. Working in collaboration with other workers in the University Department of Medicine, this method was used to measure ALA synthase activity in rat liver and brain.

Experimental

Eight fully grown Sprague Dawley rats were treated in the following manner, then sacrificed:-

4 controls:- untreated

4 test :- injected with phenobarbitone, a known inducer of hepatic ALA synthase.

Both liver and brain were then assayed for ALA synthase

activity. In addition, enzyme activity in crude tissue homogenate was compared with that in mitochondrial preparation.

The results are expressed as picomoles of ALA formed per gram of protein per hour for rat liver (Table 11(a)) and for rat brain (Table 11(b)).

Results

(i) ALA Synthase Activity in Rat Liver

ALA synthase activity was successfully measured in rat liver using this assay method (Table 11(a)). The activity detected in crude homogenate was broadly similar to that detected in mitochondrial preparations indicating that crude tissue homogenate was an adequate method of sample preparation. Phenobarbitone produced an increase in detectable enzyme activity of approximately 25%. Significantly, when the same tissue preparations were assayed for ALA synthase activity using the method of Freshney and Paul (1970) no increase in enzyme activity in response to phenobarbitone treatment could be detected (results not shown). This highlights the inadequacies of the method of Freshney and Paul (1970) as discussed in Section 3.9.4.

The results reported here are comparable with those reported by other workers (Brooker et al, 1982; Wolfson et al, 1980; Strand et al, 1972).

(ii) ALA Synthase Activity in Rat Brain

ALA synthase activity was successfully measured in rat brain using this assay method (Table 11(b)). In this case the

(a)

ALA SYNTHASE ACTIVITY (p moles ALA/g protein/hr)

Homogenate		Mitochondria	
Control	Phenobarbitone	Control	Phenobarbitone
386,000	463,330	303,670	420,590
278,160	487,450	318,510	422,740

(b)

ALA SYNTHASE ACTIVITY (p moles ALA/g protein/hr)

Homogenate		Mitochondria	
Control	Phenobarbitone	Control	Phenobarbitone
65,270	71,350	599,080	427,600
76,520	65,960	336,190	392,200

TABLES 11(a),(b)

THE MEASUREMENT OF ALA SYNTHASE ACTIVITY IN (a) RAT LIVER AND (b) RAT BRAIN: EFFECT OF PHENOBARBITONE

activity detected in mitochondrial preparations was several fold greater than that detected in crude tissue homogenates suggesting a requirement for a cleaner tissue preparation. Phenobarbitone treatment had no effect on brain ALA synthase activity.

The results reported are comparable with those reported by other workers (Paterniti et al, 1978).

CHAPTER 4

THE ENZYME FERROCHELATASE

4 THE ENZYME FERROCHELATASE

4.1 THE REACTION CATALYZED BY FERROCHELATASE

It was first concluded from the study of avian erythrocytes (Granick and Gilder, 1946; Granick, 1954), that the terminal step in haem biosynthesis, the chelation of iron by protoporphyrin, occurs enzymatically (Granick, 1954; Goldberg et al. 1956; Krueger et al. 1956). The enzyme activity of the cell extract was heat labile, temperature dependent and exhibited a pH optimum of 8.0. Since haem formation was maximal under nitrogen and reducing conditions it was concluded that haem was formed from Fe(II) and porphyrin. Subsequent studies on avian erythrocytes (Nishida and Labbe, 1959; Labbe, 1959; Labbe and Hubbard, 1960; 1961) using classical methods of cell fractionation and protein purification yielded fractions with increased specific activity which were non-dialyzable and heat labile suggesting that the catalytic activity was dependent upon protein. The partially purified fractions also required the presence of a cofactor which could be satisfied by glutathione (GSH) or ascorbic acid (Goldberg, 1959; Lochhead and Goldberg, 1959). The activity was inhibited by mercurials leading to the conclusion that the observed catalysis was dependent on a sulphydryl group.

Numerous publications have since described the preparation and properties of ferrochelatase from a variety of sources (Porra and Jones, 1963; 1963; Yoneyama et al, 1965; Neuberger and Tait, 1964; Porra and Ross, 1965; Jones, 1968; Goldin and Little, 1969; Camadro and Labbe, 1982; Bloomer and Morton, 1982; Dailey and Fleming, 1982; 1986).

The enzyme ferrochelatase (haem synthase; iron-

protoporphyrin chelating enzyme; protohaem ferrolyase, EC 4.99.1.1) catalyzes the final step in the haem biosynthetic pathway i.e. the insertion of iron (II) into protoporphyrin IX to form protohaem with the loss of two protons (Figure 12).

Protoporphyrin IX + Fe^{2+} ——→ protohaem + $2H^{+}$.

Enzyme activity is maximal when ferrochelatase is surrounded by a lipid environment (Sawada et al, 1969; Simpson and Poulson, 1977; Taketani and Tokunaga, 1981).

4.2 THE LOCATION AND MOLECULAR PROPERTIES OF FERROCHELATASE

Ferrochelatase is located 'in vivo' as an intrinsic membrane protein which spans the inner mitochondrial membrane (Ferreira et al, 1988) with its active site present on the matrix side of the membrane (Harbin and Dailey, 1985). The enzyme is enriched in lysine (11%) and hydrophobic amino acid residues (48%) (Taketani and Tokunaga, 1981) and has a reported M.W. of 40,000 as determined by SDS-PAGE (Dailey and Fleming, 1983; Hanson and Dailey, 1984). However, there is still disagreement regarding the m.w. of the native enzyme as values of 200,000 and 240,000 have also been reported (Taketani and Tokunaga, 1981; 1982). One explanation for this discrepancy is that the dialysis used in some preparations may lead to aggregation of the membrane protein. However, although the enzyme is now generally believed to be a monomer, the possibility of a multisubunit structure exists and resolution of this uncertainty must await determination of the 'in situ' structure of the membrane protein. For the present, most workers have accepted a M.W. of 40,000 for mammalian ferrochelatase.

4.3 THE SYNTHESIS OF FERROCHELATASE

Like ALA synthase, ferrochelatase is synthesised in the cytoplasm and must be imported into the mitochondria to reach its functionally active site. Such proteins are usually synthesised as precursors on cytoplasmic ribosomes and contain N-terminal extensions which target the protein to the mitochondria. Insertion and translocation into the mitochondrial inner membrane requires an electrical membrane potential and, on entering the mitochondria, the proteins are processed by a proteinase which cleaves off the N-terminal extension. Both 'in vivo' and 'in vitro' studies of mouse hepatic and erythroid enzyme (Karr and Dailey, 1988) have demonstrated that ferrochelatase is synthesised as a larger precursor of m.w. 43,000 and is then translocated and processed to a mature-sized protein of m.w. 40,000 in an energy-requiring step. Prior to this study there had been no report of the synthesis and membrane insertion of ferrochelatase. The low abundance of ferrochelatase in cells, its poor immunogenicity, and the presence of a blocked N-terminus had limited such work.

4.4 THE PROPERTIES OF THE PURIFIED ENZYME

Ferrochelatase has been purified from rat (Taketani and Tokunaga, 1981), beef (Dailey and Fleming, 1983; Taketani and Tokunaga, 1982) mouse (Dailey et al, 1986) and human liver (Mathews-Roth et al, 1987) and from chicken erythrocytes (Hanson and Dailey, 1984). Although the bovine enzyme is best characterised, the enzymes from all of these sources share many common characteristics but differ substantially from bacterial ferrochelatases (Dailey, 1982; 1977).

Studies of kinetic parameters have indicated that protoporphyrin IX, the natural substrate, has the lowest measured Km and Vmax (Table 12, Review, Dailey, 1989). However, there are large quantitative variations in the data from various laboratories (Table 12). This may be explained by several factors:

1. A variety of different tissues and animal species have been used as a source of the enzyme, therefore interspecies variations are expected.

2. Differences are likely to exist with regard to assay techniques.

3. The purity of the porphyrins and the manner in which they have been prepared may significantly affect enzyme activity.

4. The nature of the enzyme preparation, i.e. membrane-bound versus solubilised and purified, is also likely to have a significant effect on the enzyme activity.

The most reliable comparisons are either qualitative or quantitative but from a single laboratory.

Only dicarboxylic porphyrins of the IX isomer are substrates of ferrochelatase. Porphyrins with 2,4 substitutions that are charged or larger than vinyl groups are competitive inhibitors of the enzyme with respect to the porphyrin substrate (Honeybourne et al, 1979; Dailey and Smith, 1984).

The enzyme is strongly inhibited by metal ions such as Mn^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+} and Cu^{2+} (Dailey and

ENZYME SOURCE	IRON	PROTO-PORPHYRIN	HEMATO-PORPHYRIN	MESO-PORPHYRIN	DEUTERO-PORPHYRIN	REFERENCE
Human Liver	0.5	0.35	-	-	-	Camadro et al (1984)
Mouse Liver	-	9.0	9.0	156.0	247.0	Dailey et al (1989)
Rat Liver	33.1	28.5	-	26.7	-	Taketani and Tokunaga (1981)
Bovine Liver	80.0	11.0	22.0	34.0	47.0	Dailey and Smith (1984) Dailey and Fleming (1983)
	46.0	54.0	55.0	46.0	36.0	Taketani and Tokunaga (1982)
Sheep Liver	-	0.8	-	1.9	4.0	Honeybourne et al (1979)
Chicken Erythrocytes	166.0	37.0	-	51.0	80.0	Hanson and Dailey (1984)
Duck Erythrocytes	70.0	80.0	-	-	-	Yoneyama et al (1962)

TABLE 12

THE APPARENT Km's (μM) FOR EUKARYOTIC FERROCHELATASES as taken from Dailey, H.A. (1989) in Biosynthesis of Heme and Chlorophylls, McGraw-Hill Publishing Company, Page 136

Fleming, 1983; Fadigan and Dailey, 1987; Tokunaga and Sano, 1972; Labbe and Hubbard, 1961) which are competitive with respect to ferrous iron. Assuming a M.W. of 40,000 per mole of enzyme, inactivation data show that one mole of N-methylprotoporphyrin binds and one mole of N-ethylmaleimide inactivates one mole of enzyme (Dailey and Fleming, 1986). Should then the enzyme exist as multiple subunits each subunit is able, at least 'in vitro', to catalyze the insertion of iron. Reported values for the Km of iron have also varied enormously (Table 12) again for the reasons previously described. Strong enzyme inhibitors such as mercury have a Ki similar to the Km for iron (Dailey, 1989).

4.5 THE RELATIONSHIP BETWEEN IRON METABOLISM AND FERROCHELATASE

The presence of adequate quantities of ferrous iron is an absolute requirement for optimal ferrochelatase activity. However, the details of the acquisition and utilisation of iron for haem synthesis remain unclear.

The iron compartments in man may be separated on the basis of anatomic distribution, chemical characteristics and function into six compartments (Table 13). The largest of the compartments is Hb iron, containing approximately 3g iron.

4.5.1 Storage Iron:

Iron in the storage compartment exists as either ferritin or hemosiderin.

Ferritin is a water soluble complex of ferric hydroxide and a protein, apoferritin. Apoferritin forms a shell within which ferric ions (up to 2,000), hydroxyl ions and oxygen are

COMPARTMENT	IRON CONTENT (mg)	TOTAL BODY IRON (%)
Haemoglobin iron	2,500	67
Storage iron (Ferritin hemosiderin)	1,000	27
Myoglobin iron	130	3.5
Labile pool	80	2.2
Other tissue iron	8	0.2
Transport iron	3	0.08

NOTE:

These values represent estimates for an average person i.e. 70kg, 177cm in height. They are derived from data in several sources.

TABLE 13

THE IRON COMPARTMENTS IN NORMAL HUMAN BEINGS (Haematology Chapter 18, page 168, (1977) W.J. Williams, E. Beutler, A.J. Erslev, R.W Rundles (eds).

dispersed in a lattice-like arrangement (Harrison et al, 1967; Brady et al, 1968; Hoy et al, 1974). This permits a semi-crystalline structure to form. Ferritin is normally found in trace amounts in plasma and in most, if not all, cells of the body. Multiple molecular forms of ferritin or isoferritins are found in several tissues. The terms "anabolic ferritin" and "catabolic ferritin" have sometimes been attached to erythroblast and reticuloendothelial ferritin respectively. It has not however been shown that "anabolic ferritin" is directly used for haem synthesis (Cartwright and Deiss, 1975) or that "catabolic ferritin" is derived from Hb catabolism. The concentration of Fe^{3+} ions in or near the cell enhances apoferritin formation both 'in vitro' (Drysdale and Shafritz, 1975) and 'in vivo' (Bernier et al, 1970). Apoferritin exhibits enzymatic properties in the oxidation of Fe^{2+} to Fe^{3+}, thus facilitating the uptake of iron by the cell and the incorporation of iron into ferritin (Bryce and Crichton, 1973).

Hemosiderin is mainly found in cells of the reticuloendothelial system and, under pathological conditions, it may accumulate in large quantities in most tissues of the body. Hemosiderin may represent partially denatured, partially deproteinised insoluble ferritin aggregates (Sturgeon and Shoden, 1964).

The iron content of the storage compartment varies both in normal circumstances and in disease. Iron depletion occurs when iron loss exceeds iron absorption. The mobilisation of storage iron involves iron release from intracellular ferritin as Fe^{2+}.

4.5.2 The Labile Iron Pool

The labile iron pool is a concept derived from studies of iron kinetics (Greenberg and Wintrobe, 1946; Pollycove and Mortimer, 1961; Hosain et al, 1967). Iron leaves the plasma and enters the interstitial and intracellular fluid compartments. Here it may be bound to cell membranes or to intracellular proteins for a brief period prior to incorporation into haem or storage compounds. A widely distributed intracellular protein may be responsible for the short-term binding and release of iron and therefore may represent the labile iron pool. It has been postulated that an acetate-extractable ferroprotein (AEP) with a m.w. of 12,000 represents such a protein. The Fe^{3+} binding kinetics of AEP exhibits an exponential curve with a $T^1/_2$ of 30 hr, similar to that proposed for iron in the labile pool (Boulard et al, 1972).

4.5.3 Transport Iron

The transport compartment is the smallest of the iron compartments, yet kinetically it is the most active because the iron in it is normally "turned over" at least 10 times per 24 hr. This compartment represents the intermediate pathway through which iron may be interchanged between compartments. Transport iron is bound to the specific protein **transferrin**, an elongated β-globulin of m.w. 80,000. At each end of the molecule globular glycoprotein moieties can bind one trivalent iron atom. Iron binds to these two sites in a random manner. Yet, the two sites may not be identical as evidence suggests that one of the sites, when occupied by iron, has higher

affinity for the erythroblast membrane than does the other site (Zapolski et al, 1974; Fletcher and Huehns, 1967; 1968). Normally, one third of the plasma transferrin iron binding sites are occupied. This is subject to large diurnal fluctuations and also varies under different physiological and pathological conditions.

Ferritin may also serve as a mechanism of iron transport. It enters plasma mainly as a result of Hb catabolism and is rapidly removed from plasma by the liver. The plasma concentration of ferritin is normally small (100ng/ml). However, due to the rapid turnover of plasma ferritin, up to 20mg of ferritin iron may be transported through the plasma daily (Siimes and Dallman, 1974).

(i) Iron Requirements

To provide sufficient iron for normal synthesis of Hb and other iron proteins, the body absorbs small amounts of iron through the intestinal mucosa to balance the small amount (1mg) excreted per day. The average daily dietary intake of iron is 10-20mg, thereby making dietary causes of iron deficiency uncommon in the Western World.

(ii) Transport of Iron

Iron within the body is virtually in a closed system in which it cycles from the plasma to the developing erythroblasts, then into the circulating blood for about 120 days, and finally to the phagocytic reticulum cells. Here it is removed from the Hb and released back into the plasma to repeat the cycle. The transport of iron in the plasma is of

central importance to this process.

Transferrin provides the means of moving iron through the plasma and interstitial fluids to the erythroblasts of the bone marrow, hepatocytes and other cells. Reticuloendothelial cells do not have transferrin receptors but acquire iron from the catabolism of Hb in the red cells that they destroy. The binding of transferrin and release of iron are active processes which can be abolished by enzyme inhibitors (Sly et al, 1975). Evidence supporting this concept comes from studies of reticulocytes; although Hb synthesis is nearly complete by this stage of erythrocyte maturation, the membrane of a reticulocyte can still bind 25,000 to 50,000 iron-laden transferrin molecules per minute (Van Bockxmeer and Morgan, 1979).

4.5.4 Iron in the Erythroblast

Molecules of transferrin iron appear to enter the erythroblast by a process of endocytosis, where the iron-transferrin complex is bound to a "carrier" molecule of m.w. 20,000 (Jandl and Katz, 1963; Sly et al, 1975; Morgan and Appleton, 1969; Martinez-Medellin and Schulman, 1972; Sullivan et al, 1976). It is, however, uncertain as to whether transferrin itself ever penetrates the outer cell membrane (Barnes et al, 1972; Loh et al, 1977). It has been proposed that iron, as the iron-transferrin-carrier complex, is transported to the mitochondria where haem synthesis occurs. The fate of transferrin has not yet been resolved completely. Electron microscopy can visualise iron in mitochondria as amorphous aggregates, called ferruginous micelles. When haem synthesis is impaired, as in lead poisoning or in the

sideroblastic anaemias, the mitochondria accumulate excessive amounts of these amorphous iron aggregates.

The importance of transferrin in the delivery of iron to the erythroblast is emphasised from the observation that when transferrin is congenitally absent, iron accumulates mainly in the liver. Little gets into the bone marrow and a severe hypochromic microcytic anaemia results (Drysdale and Shafritz, 1975).

(i) Release of Iron From Transferrin

Iron is released from transferrin only at specific sites on receptor cells. The mechanism of iron release from transferrin is not known but may involve reduction of the iron or of the binding groups, chelation by an external ligand or displacement of bicarbonate. Egyed et al (1980) have suggested that intracellular haem proteins may play a role in this release process, while more recent work suggests that pyrophosphate may act as a ligand for iron release and subsequent delivery to mitochondria (Nilsen and Romslo, 1984; 1984; 1985).

Maximum iron uptake occurs in early and intermediate erythroblasts and further maturation of the erythroid cells is associated with a decrease in the number of transferrin binding sites (Nunez et al, 1977; Van Bockxmeer and Morgan, 1979). The transferrin receptor has now been identified as a glycoprotein of m.w. 350,000 consisting of two unequal subunits (Leibman and Aisen, 1977; Sullivan and Weintraub, 1978).

Although the process by which iron is mobilised for incorporation into haem remains unclear, this process must

deliver iron in the divalent state to protoporphyrin.

(ii) Iron Reduction

Since ferrochelatase has an absolute requirement for ferrous iron, and because biological iron complexes such as transferrin and ferritin chelate ferric iron, a mechanism for ferric iron reduction must exist in the cell. It has been demonstrated that ferric chloride can be reduced to ferrous iron by mitochondria with NADH or succinate serving as reductants (Barnes and Jones, 1973; Barnes et al, 1972). However, ferritin or transferrin iron was not reduced. The ferrous iron generated in this fashion was used by ferrochelatase for haem biosynthesis when porphyrin was also supplied. The reduction was shown to be coupled to the electron transport chain at the level of NADH and succinate dehydrogenases.

More recently, Taketani et al (1985, 1986) have examined this problem by a different approach. They fractionated the bovine heart mitochondrial electron transport chain into complexes I, II and III and then assayed these complexes for ferrochelatase and iron reduction activity. Complex I contained both ferrochelatase activity and an NADH-dependent ferric-iron-reducing system which was strongly stimulated by FMN.

The ferric iron reduction system is currently then poorly understood. Data suggests that this system is sensitive to inhibition by lead at lower concentrations than ferrochelatase indicating that lead may not directly inhibit ferrochelatase but rather may reduce the availability of the Fe^{2+} substrate.

4.6 THE REACTION MECHANISM FOR FERROCHELATASE

Although the precise nature of the reaction mechanism for ferrochelatase remains to be elucidated in detail, recent advances have been made. Three main areas have been specifically examined:

(i) Protein Amino Acid Resides Involved In Iron Binding

Reaction of a single sulphydryl residue per monomer of purified ferrochelatase results in the total loss of activity (Dailey, 1984). However, reversible inhibition by sodium arsenite as well as by Hg^{2+}, strongly suggests that two vicinal sulphydryl groups are involved in catalysis. Ferrochelatase is protected against inactivation by N-ethylmalemide in the presence of ferrous iron, but not in the presence of porphyrin. This supports a model where vicinal sulphydryl residues are involved in ferrous iron binding by the enzyme (Dailey, 1984).

(ii) Protein Residues Associated with Porphyrin Binding

Since the second substrate of ferrochelatase is a dicarboxylate porphyrin there is a possible role for protein cationic residues in porphyrin binding (Dailey and Fleming, 1986). Bovine ferrochelatase is rapidly inactivated by the arginyl reagents butanedione, cyclohexanedione and comphorquinone 10-sulphonate, but not by lysyl specific reagents. The enzyme is protected against inactivation by porphyrin but not by ferrous iron. The kinetics of the modified enzyme support the model for arginyl involvement in the binding of porphyrin but not of iron.

The current data support a model whereby iron binding occurs via vicinal sulphydryl groups prior to porphyrin binding

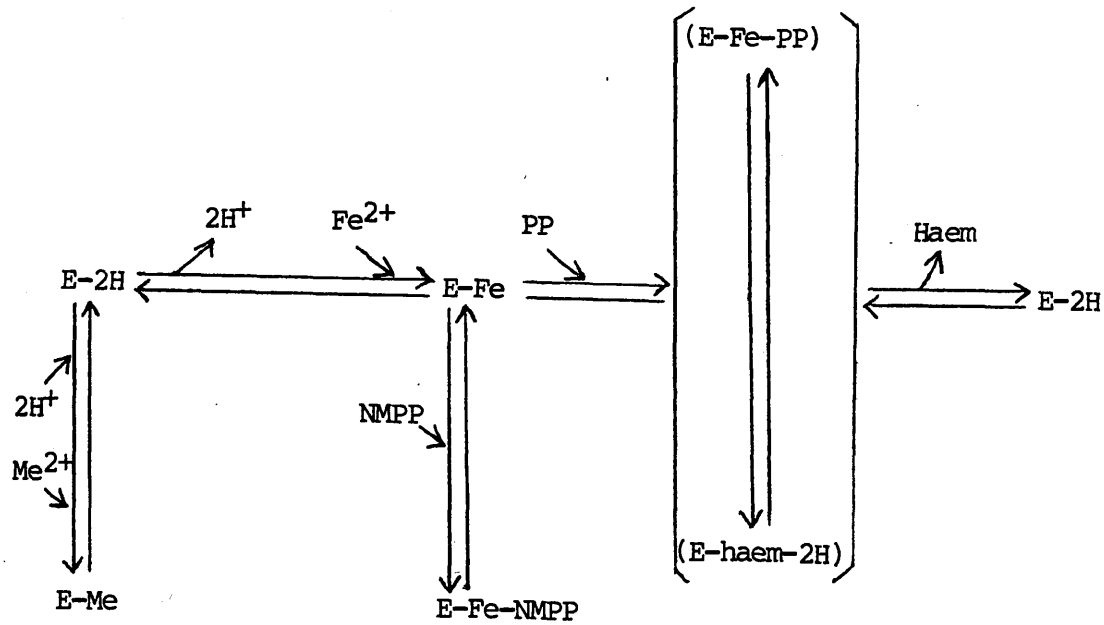
which involves protein arginyl residues(s).

(iii) Enzyme Kinetic Studies

An enzymatic kinetic mechanism for bovine ferrochelatase has been proposed based upon substrate and inhibitor studies (Figure 13) (Dailey and Fleming, 1983). The reaction consists of two substrates, porphyrin and ferrous iron, and two products, protohaem and two protons. The enzyme is proposed to bind initially the divalent iron with the concomitant release of two protons. The binding of protoporphyrin then follows. Iron insertion into the porphyrin macrocycle occurs with the exchange of the two pyrrolic nitrogen protons to the enzyme, thereby, displacing the ferrous iron chelate. Ferro-protohaem is released, completing the cycle. Elimination of the second product, the two protons, occurs only upon initiation of a new catalytic cycle. Hence, ferrochelatase has a sequential Bi-Bi mechanism.

4.7 THE RELATIONSHIP WITH PROTOPORPHYRINOGEN OXIDASE

It has been suggested that 'in vivo' protoporphyrinogen oxidase, the antipenultimate enzyme of the haem biosynthetic pathway, and ferrochelatase are arranged in a complex whereby the product of one enzyme i.e. protoporphyrin IX is directly channelled to the next without being diluted in the phospholipid matrix (Ferreira et al, 1988). The isolated enzymes have calculated Km's in the μM range; a concentration that would not normally be approached in the cell. In a multiprotein complex substrate channelling would eliminate the release of intermediates and obviate the need to justify



NOTE:

E = ferrochelatase
PP = protoporphyrin
NMPP = n-methylprotoporphyrin
Me^{2+} = metal dication

FIGURE 13

THE PROPOSED KINETIC MECHANISM FOR MAMMALIAN FERROCHELATASE (adopted from Dailey and Fleming, 1983).

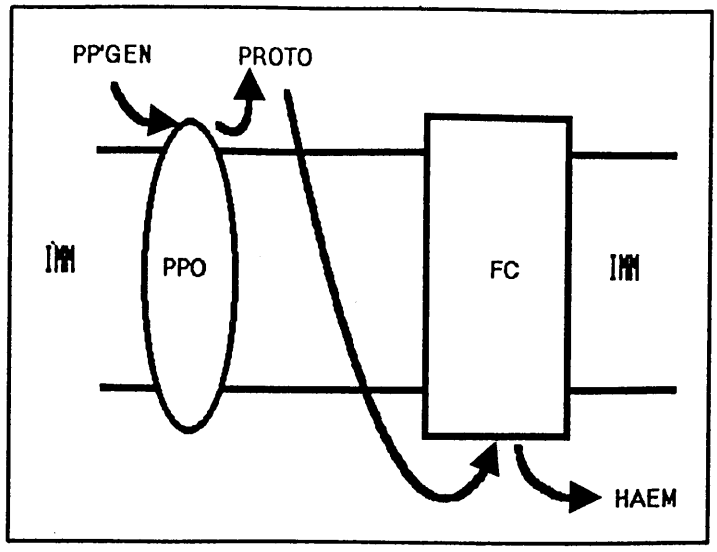
seemingly high experimentally determined Km's. Both enzymes are integral membrane proteins and the exact nature of the interaction between them is currently under investigation (Figure 14).

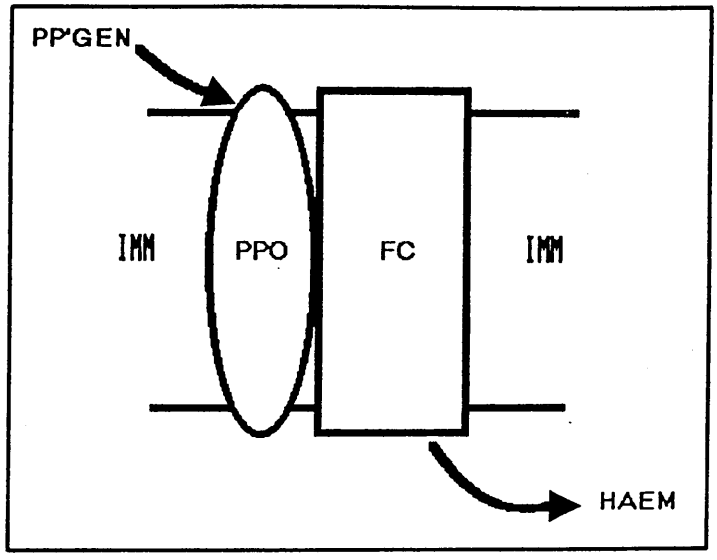
4.8 THE PHYSIOLOGICAL ROLE OF FERROCHELATASE

Since the chelation of iron into protoporphyrin also occurs nonenzymatically (Kassner and Walchak, 1973; Taketani and Tokunaga, 1984) the necessity for an enzyme has been questioned. The demonstration that humans and cattle with protoporphyria have defects in ferrochelatase activity (Becker et al, 1976; Bloomer, 1980; 1980; Bloomer et al, 1976; Bonkowsky et al, 1975; Bottomley et al, 1975; Brenner and Bloomer, 1980; Brodie et al, 1977; de Goeij et al, 1975; Ruth et al, 1977) causing excessive accumulation and excretion of protoporphyrin indicates that the enzyme is necessary for haem formation to proceed normally under physiological conditions. It is, however, surprising that individuals demonstrating deficiency of ferrochelatase to 10-30% of normal activity do not usually develop anaemia (Turnbull et al, 1973).

4.9 THE IMPORTANCE OF FERROCHELATASE: ROLE IN HAEM SYNTHESIS AND ERYTHROPOIESIS

The most striking molecular event during erythroid differentiation is the accumulation of Hb. Haem synthesis is central to this process, being inextricably linked to the control of erythroid maturation, to the promotion of globin synthesis and many other complex processes within the cell (Chapter 1, Section 1.13). Yet, strangely, in contrast to the





FC = Ferrochelatase ; PPO = protoporphyrinogen oxidase
PP'GEN = protoporphyrinogen ; PROTO = protoporphyrin ;
IMM = inner mitochondrial membrane

FIGURE 14

POSSIBLE MODELS FOR THE INTERACTIONS OF THE TERMINAL TWO ENZYMES OF THE HAEM BIOSYNTHETIC PATHWAY (AS SUGGESTED BY FERREIRA ET AL [1988])

information available for hepatic cells, the control of haem synthesis in erythroid cells is not fully understood. Studies of erythroid haem synthesis have been carried out on various cell types using a variety of techniques and the results have inevitably contrasted sharply. Human erythroid haem synthesis has received relatively little attention but clearly demands further investigation.

It is the aim of the work in Chapter 5, 6, 8 and 9 to focus on the terminal stage of haem synthesis, in particular, the reaction catalysed by ferrochelatase, and its role in haem synthesis in the maturing erythroid cell.

There is evidence that ferrochelatase may exercise a limiting role for haem synthesis in erythroid cells:-

1. Hb formation does not occur in FEL cells treated with DMSO until ferrochelatase activity increases. Other enzymatic activities of the haem pathway increase sequentially and considerably earlier than ferrochelatase (Sassa, 1976; Rutherford et al, 1979). In addition, the study of differentiation of induced FEL cells has shown that total haem production is affected by protoporphyrin only after 48 hr, indicating that iron insertion into protoporphyrin is limiting for haem formation (Fadigan and Dailey, 1987).

2. Haemin, but not ALA, PBG or protoporphyrin, can increase Hb concentration in undifferentiated Friend cells (Granick and Sassa, 1978; Ross and Sautner, 1976; Dabney and Beaudet, 1977).

3. A mutant clone of FEL cells with normal induction responses of ALA synthase, ALA dehydratase and PBG deaminase, does not make

Hb after DMSO treatment (presumably due to an enzyme block after PBG deaminase), but does so after DMSO plus haemin addition (Sassa et al, 1978; Eisen et al, 1978).

4. Treatment of a wild type clone of FEL cells with haemin can increase not only the synthesis of Hb but also levels of ALA synthase, ALA dehydratase, PBG deaminase and ^{59}Fe incorporation into haem (Hoffman et al, 1980).

The increase in ALA synthase activity induced by haemin treatment of erythroid cells represents a striking contrast to haemin induced suppression of the hepatic enzyme. A similar finding of haemin-mediated induction of haem pathway enzymes, including that of ALA synthase, has been reported in human erythroleukaemia cells K562 (Hoffman et al, 1980). Furthermore, haemin, but not ALA, stimulates Hb formation in normal mouse bone marrow cultures (Porter et al, 1979).

However, in view of the fact that haemin induces differentiation of FEL cells, it is possible that this results in secondary stimulation of ALA synthase activity.

5. The ferrochelatase deficiency in erythropoietic protoporphyria (EPP) appears to be expressed in terms of protoporphyrin accumulation mainly in erythroid tissue, although ferrochelatase is presumably deficient in all tissues, suggesting that deficient ferrochelatase may become limiting in erythroid cells but not in non-erythroid tissues. This, however, does not cause anaemia.

On the other hand, Beaumont et al (1984) have suggested that there is a simultaneous induction of the enzymes of haem

biosynthesis in DMSO-treated FEL cells. In this study, appearance of ferrochelatase activity did not significantly differ from that of the earlier enzymes of the pathway implying that late induction of ferrochelatase activity was not rate-limiting for haem formation.

Further evidence for a possible regulatory function of haem on ferrochelatase activity in erythroid cells has been presented from various studies and inhibition of ferrochelatase by haemin has been reported in different tissues:-

1. With Co^{2+} and deuteroporphyrin as substrates ferrochelatase activity of Rhodopseudomonas spheroides was effectively inhibited by haemin in a non-competitive manner with respect to both substrates (Jones and Jones, 1970). Furthermore, the inhibitory action of haemin on ferrochelatase purified from Spirillum intersonni and also R. spheroides has been reported (Dailey, 1977; 1982).

2. Inhibition of ferrochelatase activity by haemin has also been demonstrated in rat liver (Koller and Romslo, 1977). In addition, the study of purified bovine hepatic ferrochelatase with Fe^{2+} and protoporphyrin as substrates demonstrated that haemin was a non-competitive inhibitor with respect to Fe^{2+} (Dailey and Fleming, 1983).

3. Two groups working with rat liver mitochondria have suggested that the local concentration of haem formed within the mitochondrial matrix space may be high enough to inhibit ferrochelatase activity and thereby partially control its own synthesis (Koller and Romslo, 1977; Jones and Jones, 1970).

The inhibition of ferrochelatase by haemin may be a possible site for regulation of the haem biosynthetic pathway. In erythroid cells, however, other enzymes of haem synthesis together with the acquisition of iron from transferrin have also been implicated in this control (Chapter 1, Section 1.12).

It was intended that the use of cell separation techniques previously described (Chapter 2) coupled with the development of a highly sensitive radiochemical assay for ferrochelatase activity, would provide hitherto unavailable information regarding the relative activities of ALA synthase and ferrochelatase in maturing erythroid cells. These techniques would then facilitate the study of normal and abnormal human erythroid haem synthesis.

4.10 DISEASES ASSOCIATED WITH DECREASES IN FERROCHELATASE ACTIVITY

Reduction in ferrochelatase activity of the bone marrow of occasional patients with sideroblastic anaemia has previously been reported (Valentine, 1983), while it is also known that ferrochelatase is depressed in erythropoietic protoporphyria and lead intoxication (Schwartz et al, 1971).

CHAPTER 5

DEVELOPMENT OF AN ASSAY TO DETERMINE FERROCHELATASE ACTIVITY IN HUMAN BONE MARROW

5 DEVELOPMENT OF AN ASSAY TO DETERMINE FERROCHELATASE ACTIVITY IN HUMAN BONE MARROW

5.1 PRINCIPLE OF ENZYME ACTIVITY ASSAYS

The quantity of enzyme activity in a given tissue is normally defined as the amount of product formed from substrate per unit time under specified conditions (Mahler and Cordes, 1969). Enzymes are assayed under test conditions in which the pH is optimum and the substrate concentration is above saturation level, such that the initial reaction rate is zero-order for substrate. For a catalyzed reaction, the rate is proportional to enzyme concentration alone when the reaction is zero-order for substrate and cofactors (Lehninger, 1972).

5.2 THE ASSAY OF FERROCHELATASE ACTIVITY

The measurement of ferrochelatase activity is fraught with difficulties. However, several methods are currently available, each varying in sensitivity and suitability for specific tissues.

5.2.1 Measurement of Porphyrin Disappearance by Spectrophotometric or Fluorometric Methods

Ferrochelatase activity may be assayed by measuring the consumption of porphyrin substrate, spectrophotometrically or fluorometrically (Labbe, 1959; Porra et al, 1967; Jones and Jones, 1969; Camadro and Labbe, 1982). However, loss may occur by reactions other than ferrochelatase e.g. by photocatalyzed oxidation reactions. Moreover, sufficient substrate concentrations must be provided to maintain a linear rate of product formation throughout the incubation and ideally greater than 90% of the substrate should remain at the end of the

incubation. The presence of such a large excess of porphyrin in the incubation mixture results in a lack of accuracy and sensitivity for these methods.

5.2.2 Measurement of Haem Formation by the Pyridine Haemochrome Procedure

Haem formation can also be measured by the pyridine haemochrome procedure (Porra and Jones, 1963; McKay et al, 1969; Porra, 1976; Dailey et al, 1986). Haem concentrations are calculated from the reduced minus oxidised spectrum of pyridine haemochromogens over a wavelength range of 500-600 nm. The sensitivity of these methods is also low as the presence of endogenous haem may cause a high background against which any small increase would be difficult to detect. Greater sensitivity is achieved using mesoporphyrin as substrate (Porra, 1976) since alkaline pyridine mesohaemochromogen has an absorption peak distinct from that of background endogenous protohaemochromogen.

5.2.3 Measurement of Haem Formation by Radiochemical Methods

Radiochemical methods with $^{59}Fe^{2+}$ ions as substrate (Krueger et al, 1956; Goldberg et al, 1956; Goldberg, 1959; Bottomley, 1968; Bloomer and Morton, 1982; Dailey and Fleming, 1986) potentially provide much higher sensitivity than other methods, but suffer from difficulty in separating labelled haem from free $^{59}Fe^{2+}$ ions. In addition, the assays are tedious and great care must be taken to ascertain the purity and recovery of the radioactive haem product.

5.2.4 The Need for a Sensitive Assay

Most of the above methods have been primarily used to study tissues which contain high levels of ferrochelatase activity e.g. liver. Many are not sensitive enough to measure the levels of activity that occur in some mammalian tissues. In the case of human bone marrow, although unit activity may be high, very limited cell numbers are available and endogenous haem is present in large enough quantities to make the detection of newly synthesised haem difficult. It was therefore necessary to develop a highly sensitive radiochemical method suitable for the measurement of bone marrow ferrochelatase activity which could overcome the problem of product isolation. The remainder of this chapter describes in stepwise fashion the experimental progression towards this goal.

5.3 A RADIOCHEMICAL ASSAY FOR FERROCHELATASE ACTIVITY

5.3.1 Choice of Incubation Medium

Tris buffer (50mM) was suitable incubation medium for the assay of ferrochelatase activity. This has been the chosen buffer of several workers (Dailey et al, 1986; Bloomer and Morton, 1982).

As enzyme activity is almost universally measured between pH 7.2 and 7.6, initial experiments were carried out at pH 7.4. Later work confirmed that this was the most suitable pH for enzyme measurement in human bone marrow (Section 5.8.4). The need for individual constituents in the enzyme reaction mixture has been examined (Section 5.8).

5.3.2 Choice of Substrates

Ferrochelatase activity can be studied with compounds other than the natural substrates, iron and protoporphyrin IX.

(i) Porphyrin

In addition to protoporphyrin IX, other 2-carboxylate porphyrins e.g. deutero and mesoporphyrin serve as good substrates for ferrochelatase and provide activity that is several times greater than protoporphyrin (Jones and Jones, 1969; Johnson and Jones, 1964; Porra and Jones, 1963). However, protoporphyrin IX is the natural substrate for ferrochelatase and was chosen for use in the work described here for several reasons:-

(1) Enzyme activity determined with one type of porphyrin or metal substrate may differ from that determined with another substrate (Tephly, 1978).

(2) Solvent extraction, the usual technique for isolating haem, is unsuitable when mesoporphyrin or deuteroporphyrin is used as substrate because of low extraction efficiency (Dailey et al, 1986).

(3) Protoporphyrin IX is the natural substrate and it is not known whether in disease states enzyme activity differs for different substrates.

(ii) Radioactive Metal

Only the reduced form of iron i.e. ferrous iron (Fe^{2+}) is incorporated into protoporphyrin IX by ferrochelatase. Ferric iron (Fe^{3+}) cannot act as substrate (Porra and Jones,

1963). Co^{2+} ions and Zn^{2+} ions are more efficient enzyme substrates than Fe^{2+} ions (Johnson and Jones, 1964). However, for reason (1) stated above Fe^{2+} ions were chosen as substrate.

(a) Choice of ^{59}Fe Product

Several preparations of ^{59}Fe are commercially available, varying significantly both in suitability and cost. The choice of product is dependent on several factors - (1) those previously chosen by other workers in the assay of ferrochelatase activity; (2) the availability and (3) the cost.

Two types of ^{59}Fe products were tested:

(1) $^{59}Fe^{2+}$ as $FeSO_4$ in 0.05M H_2SO_4 (New England Nuclear) (Bloomer and Morton, 1982). This was supplied in a minimum quantity of 1mCi at a cost of £200.

(2) $^{59}Fe^{3+}$ as $FeCl_3$ in 0.10M HCl (Amersham International). This was used under conditions which favoured the conversion of $^{59}Fe^{3+}$ ions to $^{59}Fe^{2+}$ ions (Section 5.8.2) and was supplied in small quantities of 100μCi at a cost of £70.

Initial experiments were carried out using $^{59}FeSO_4$. Thereafter $^{59}FeCl_3$ was chosen in preference to $^{59}FeSO_4$.

(b) The Storage of Radioactive Iron

Many problems exist with regard to the storage of radioactive iron.

Firstly, when large quantities of iron are stored over a period of weeks or months (as with $^{59}FeSO_4$) insoluble iron hydroxide may form (Li et al, 1988). To avoid this, iron

should be purchased regularly in small quantities. Safety precautions must also be taken. No more than 100μCi of ^{59}Fe is permitted to be held in the laboratory at any one time and should be shielded by a lead screen to block the high energy gamma rays of the ^{59}Fe isotope.

Secondly, as the half life ($T^1/_2$) of ^{59}Fe is only 44.7 days, regular purchasing is necessary to maintain the specific activity and hence the sensitivity of the assay.

For these reasons, $^{59}FeCl_3$ was a more suitable choice of substrate than $^{59}FeSO_4$.

5.4 ISOLATION OF THE HAEM PRODUCT

A critical feature of the assay of ferrochelatase activity is the ability to isolate and quantify haem, free from porphyrin substrate and non-haem radioactive metal. This presents a considerable problem in radiochemical assays. Other workers have used solvent extraction alone as a means of isolating haem (Bloomer et al, 1983; Camadro and Labbe, 1982). However, this was not ideal as work described here has now shown significant 'carry-over' of free ^{59}Fe along with ^{59}Fe-labelled haem (Section 5.6.4).

The first step in the development of a method to measure ferrochelatase activity in human bone marrow, was to set up a procedure to isolate and collect the haem product.

5.4.1 Methods Currently Available for the Isolation of Porphyrins

As only small cell numbers can be obtained by human bone marrow aspiration, a very sensitive method of measurement, such

as that offered by radiochemical assay, was required for this work. This had to be coupled to a sensitive and efficient method for the isolation of the labelled-haem product.

The use of RPHPLC for the isolation of haem was first demonstrated by Tangeras (1984) in the assay of rat liver ferrochelatase activity. Free porphyrin carboxylic acids can be separated by HPLC using either ion-exchange, normal-phase, reversed-phase or reversed-phase ion-pair chromatographic systems (Evans et al, 1975; Longas and Poh-Fitzpatrick, 1980; Englert et al, 1979; Bonnett et al, 1978). A reversed-phase ion-pair system was reported to be the most reproducible method for the analysis of tetrapyrroles. Tangeras (1984) used RPHPLC to isolate haem in the assay of ferrochelatase activity by a non-radiochemical method. In the work described here, a modification of this RPHPLC method was coupled with a radiochemical assay in an attempt to improve the sensitivity and accuracy of previous assays. The principles of HPLC have been outlined (Chapter 3, Section 3.8.4).

5.5 RPHPLC SEPARATION OF HAEM, PROTOPORPHYRIN AND IRON

5.5.1 Materials

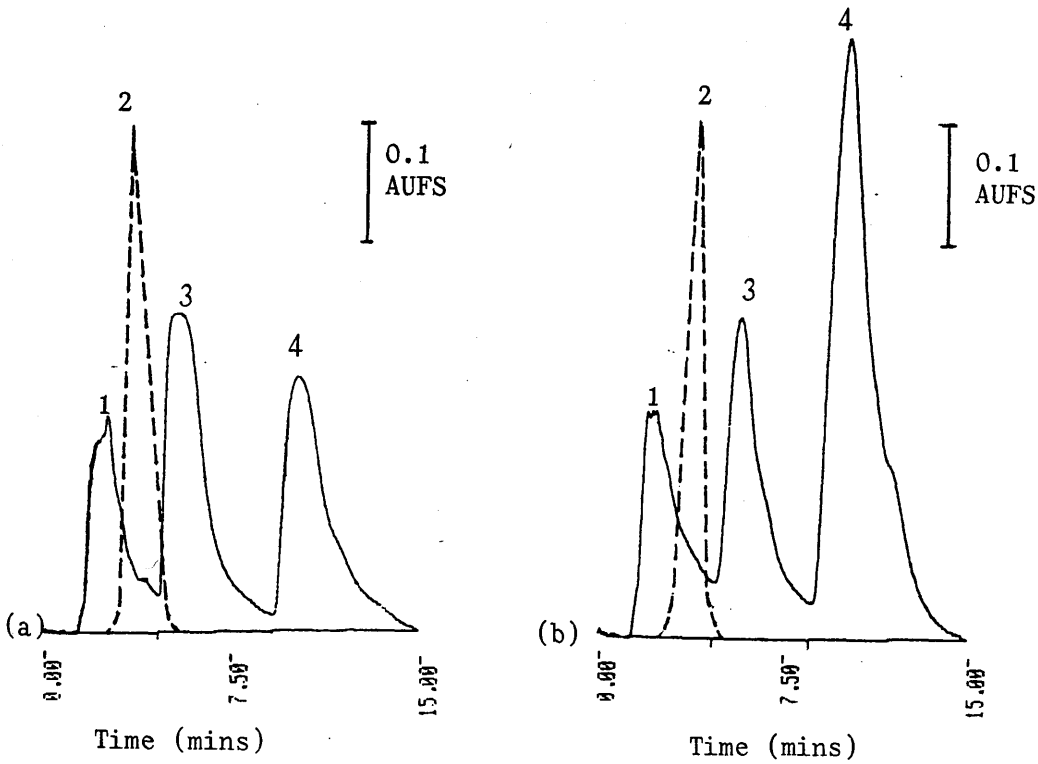
The separation of haem, protoporphyrin and iron was achieved by RPHPLC using an HPLC pump (Waters, Model 510), a valve loop injector of 50µl capacity (Rheodyne, Model 7125) and a variable wavelength spectrophotometric detector (Waters, Model 484) set to 398nm (cell pathlength, 1cm) connected to a data module recording integrator (Waters, Model 740). The column used was a reversed-phase silica supported pre-packed

Apex C18 column (15cm x 4.6I.D., particle size 5μM) (Crawford Scientific) and samples were collected from the column using a fraction collector (Gilson, Model 203). Activity was counted on a gamma counter (Packard Minaxi gamma, 5000 series, Model A5550).

Tetrabutylammonium hydrogen sulphate (TBAHS), the ion-pairing agent, haem and protoporphyrin IX were purchased from Sigma Chemical Co. and HPLC grade methanol obtained from Rathburn Chemicals. Samples were applied to the column in ammonium acetate (1M), pH 7.0 (BDH). (See section 5.7.2 for solubility of haem).

5.5.2 Methods

The mobile phase recommended by Tangeras (1984) i.e. MeOH:H_2O (97:3), TBAHS (1mM), acetic acid (10mM), produced good separation of haem (100μM) and protoporphyrin IX (100μM) when injected in 0.05ml of ammonium acetate (1M), pH 7.0 at a flow rate of 1ml min^{-1}. However, these conditions did not separate haem and iron (0.1μCi, $^{59}FeSO_4$) which eluted simultaneously approximately two minutes after sample injection. Alterations to the flow rate did not improve separation and prevented satisfactory peak resolution. The water content of the mobile phase was therefore increased. At a MeOH: H_2O ratio 83: 17 satisfactory separation of haem, protoporphyrin and iron was achieved, but the chromatography deteriorated and both haem and protoporphyrin peaks were less well defined. This was corrected by increasing the concentration of the ion-pairing agent, TBAHS, from 1mM to 2mM (Figure 15).



NOTE:

Peak 1 = solvent front
Peak 2 = ^{59}Fe ions
Peak 3 = haem/[^{59}Fe]-haem
Peak 4 = protoporphyrin

Mobile phase (MeOH:H_2O, 83:17; Acetic acid, 10mM) contained the ion-pairing agent, tetrabutylammonium hydrogen sulphate (TBAHS), at concentrations of (a) 1mM and (b) 2mM. See text for further details.

FIGURE 15

THE SEPARATION OF RADIOACTIVE IRON, HAEM AND PROTOPORPHYRIN USING REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The mobile phase chosen for routine use in the assay of ferrochelatase activity comprised MeOH:H_2O (83:17), TBAHS (2mM), acetic acid (10mM). TBAHS and acetic acid were added to the water prior to the addition of methanol. The pH was adjusted with ammonium hydroxide (5M) to 6.5 at ambient temperature. The addition of methanol altered the final pH of the mobile phase to 7.0.

Under the described conditions, (flow rate 1.0ml min^{-1}, wavelength 398nm) the retention times of Fe^{2+} ions, haem and protoporphyrin were 3.0, 5.5 and 9.5 minutes respectively, with haem eluting over a 2-3 min period (Figure 15).

Approximately 25,000 cpm were injected into the HPLC as radioactive ferrous sulphate ($^{59}FeSO_4$). ^{59}Fe was detected in 1 min fractions. Iron elution was complete at 4 minutes and subsequent fractions contained background counts of approximately 60cpm. ^{59}Fe-labelled haem was successfully isolated at 5.5 minutes.

5.5.3 RPHPLC of Haem

A linear relationship was demonstrated between the peak area of haem as determined by RPHPLC and the injection volume (and hence haem content) (Figure 16).

5.5.4 Stability of Haem

The quality of haem chromatography was maintained following overnight storage at 4°C under nitrogen in dried form or in ammonium acetate (1M), pH 7.0. Storage at 4°C for longer periods resulted in peak splitting and shoulder formation due to the oxidation of haem and/or the formation of haem aggregates.

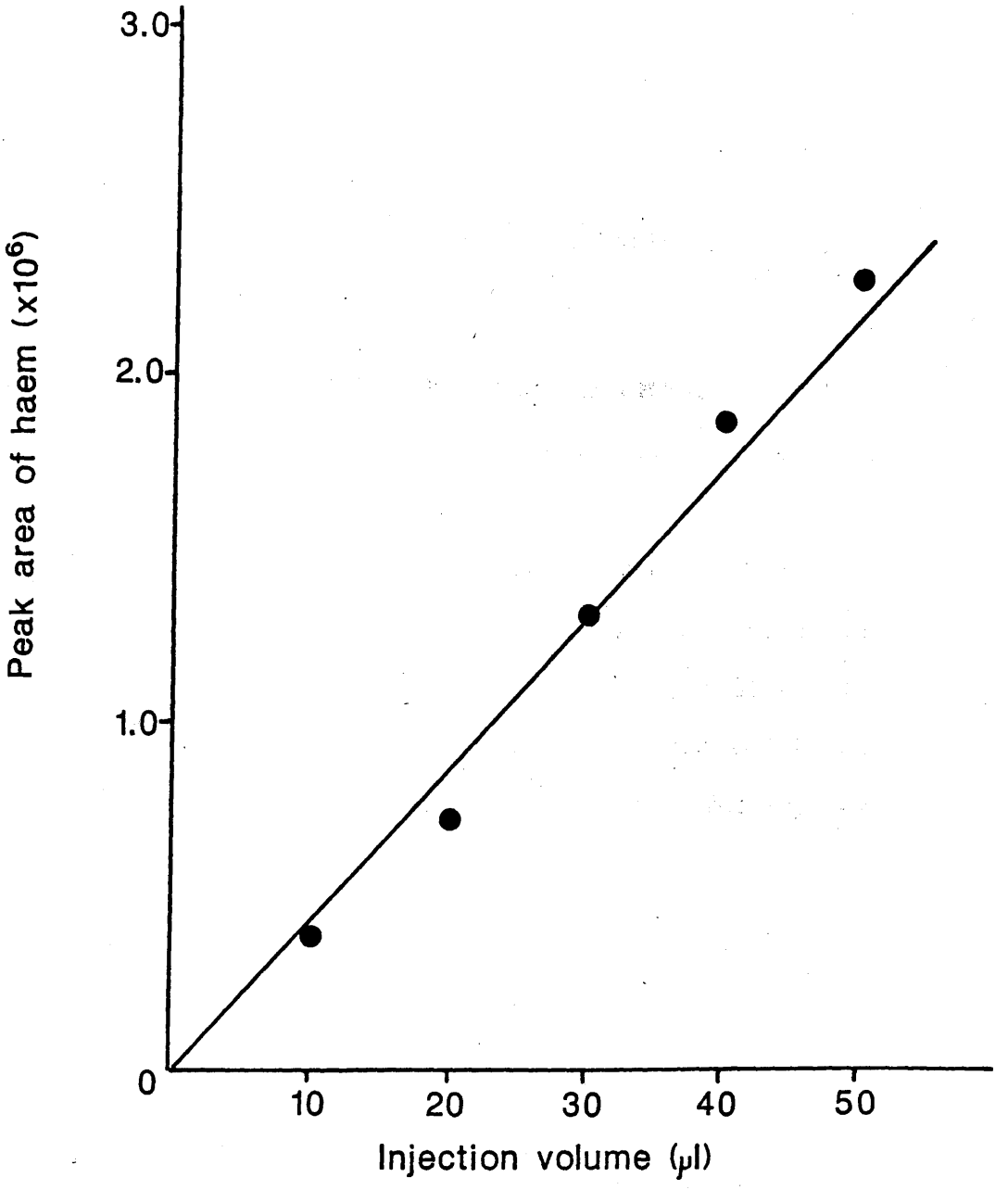


FIGURE 16

THE RELATIONSHIP BETWEEN INJECTION VOLUME AND PEAK AREA OF HAEM AS DETERMINED BY RPHPLC.

Thus, a highly effective RPHPLC method was developed for the isolation of haem from free iron and protoporphyrin. The next stage was to try to develop a method for the removal of haem from the bone marrow sample prior to RPHPLC and to compare this RPHPLC method of product isolation with those used by other workers.

5.6 EXTRACTION OF HAEM FROM THE INCUBATION MEDIUM

Porphyrin extraction from biological samples may be achieved with organic solvents such as ethylacetate: acetic acid (4 : 1 vol/vol) (Bloomer and Morton, 1982) or acidified methylethyl ketone (butan-2-one) (Dailey, 1986). Such extraction has frequently been assumed to effect 100% haem extraction (Bloomer et al, 1983; Camadro and Labbe, 1982; Fadigan and Dailey, 1987) and is essential for the removal of cellular debris.

The effectiveness and consistency of haem extraction using organic solvent was assessed.

Experimental

A mixture of haem (50μM), protoporphyrin IX (100μM) and iron (100μM) as $FeCl_2$ was prepared in tris buffer (50mM), pH 7.4, to mimic ferrochelatase assay conditions. This was divided into 12 aliquots of 1.0ml; 6 were extracted with 3ml ethylacetate : acetic acid (4 : 1 vol/vol) and 6 were extracted into 3ml acidified methylethyl ketone. The samples were evaporated to dryness at 37°C under N_2 then reconstituted in 1ml ammonium acetate (1M) (Section 5.7.2). A portion of the reconstituted sample (0.05ml) was then taken for RPHPLC (Section 5.5).

5.6.1 **Porphyrin Extraction with Ethylacetate: Acetic Acid**

1. Ethylacetate : acetic acid (4 : 1 vol/vol) (1.5ml) was added to incubation medium (1.0ml) containing haem, protoporphyrin and iron. The contents were mixed thoroughly (1 min) and the phases separated by centrifugation at 3,000 rpm (5 min).
2. The organic layer was removed.
3. A further 1.0ml of ethylacetate : acetic acid (4 : 1 vol/vol) was added per tube and Step 1 repeated.
4. The organic layer was removed and added to that removed in Step 2.
5. Steps 3 and 4 were repeated.

5.6.2 **Porphyrin Extraction with Acidified Methylethyl Ketone (Butan-2-one)**

1. HCl (0.5M, 0.5ml) was added to incubation medium (1.0ml) containing haem, protoporphyrin and iron.
2. Methylethyl ketone (1.5ml) was added to each sample. The contents were mixed thoroughly (1 min) and the phases separated by centrifugation at 3,000 rpm (5 min).
3. The organic layer was removed.
4. A further 1.0ml of methylethyl ketone was added per sample and Step 2 repeated.
5. The organic layer was removed and added to that removed in Step 3.
6. Steps 4 and 5 were repeated.

The following method was used to calculate the percentage recovery of haem.

Haem (50μM), identical to that used in each experiment, was dissolved in ammonium acetate (1M). Aliquots of haem

solution (0.05ml) were then assessed for haem peak area by RPHPLC (n = 6) and the mean peak area was taken to represent 100% recovery. Linearity of peak area with volume injected had been established (Section 5.5.3). The peak area following extraction was related to the peak area prior to extraction and thereafter was corrected to 100% recovery.

(i) Acidification of the Organic Solvent

Acidity was of critical importance during the extraction of haem into methylethyl ketone. The reaction mixture was acidified with HCl (0.5ml). At concentrations of $\leq$ 0.2M HCl, haem was not extracted. At concentrations > 0.2M HCl the extraction efficiency varied between 73-86% and could not be improved by increasing the acidity to 5M HCl ($\bar{x}$ = 78%, S.D. = 4.6%, C.V. = 5.9%, n = 6).

HCl Molarity (M)	% Haem Extracted	Peak Area of Protoporphyrin
1	75	2 761 150
1	76	2 233 313
2	78	488 099
2	80	452 155
5	73	62 900
5	86	—

Protoporphyrin recovery varied significantly and was greatly reduced when extracted in the presence of 5M HCl. A reduction in protoporphyrin extraction allowed sample extracts to pass more easily through the sorbent extraction column (section 5.7) prior to RPHPLC. Nevertheless, since acid may damage the HPLC column, 1M HCl was chosen for routine use. The presence of protoporphyrin and/or iron did not affect the extraction efficiency for haem.

(ii) Effect of Acid on Haem

Other workers have avoided acidic conditions when extracting the metal porphyrin from biological samples, believing that this may release the metal from the porphyrin ring (Scobie et al, 1981). RPHPLC analysis of extracted haem showed that this did not occur under the described conditions since no protoporphyrin was detected following acid treatment of haem solutions.

5.6.3 Choice of Organic Solvent for the Extraction of Haem

Extraction of haem was equally efficient ($\bar{x}$ = 78%, n = 6) with ethylacetate : acetic acid (4 : 1, vol/vol) or acidified methylethyl ketone and was consistent (C.V. = 6%). Acidified methylethyl ketone was chosen for routine use since this was very volatile, with 3.0ml evaporating to dryness under N_2 at 37°C in approximately 20 minutes in comparison to the 40 minutes required to evaporate ethylacetate : acetic acid (4 : 1, vol/vol).

5.6.4 Haem Extraction by Organic Solvent as a Means of Haem Isolation

Haem was extracted into acidified methylethyl ketone from a further 6 samples. Each sample (pH 7.4) also contained 0.1μCi of ^{59}Fe (25,000 cpm) as $FeCl_3$ in 0.1M HCl. This quantity of activity was approximately one tenth of that required in the assay of ferrochelatase but was chosen to avoid contamination of the HPLC equipment with large quantities of ^{59}Fe.

Results

Approximately 15% of the original ^{59}Fe ions were carried

over into organic solvent and isolated by HPLC as a radioactive ^{59}Fe peak distinct from that of haem.

Organic solvent extraction alone was not therefore adequate for isolating ^{59}Fe-labelled haem as had been suggested by other workers (Bloomer et al, 1983; Camadro and Labbe, 1982). RPHPLC represented a suitable further step for the complete isolation of ^{59}Fe-labelled haem from free ^{59}Fe ions. However, in the assay of ferrochelatase activity a vast excess of ^{59}Fe ions would be present in the incubation mixture (> 250,000 cpm, i.e. greater than a 10-fold increase in the quantity used here). Hence, an intermediate step between haem extraction by organic solvent and RPHPLC was required to significantly reduce the quantity of free ^{59}Fe ions. This reduction would enable successful isolation of ^{59}Fe-labelled haem from free Fe^{2+} ions without undue contamination of HPLC equipment with free ^{59}Fe.

5.7 PARTIAL ISOLATION OF HAEM PRIOR TO RPHPLC

RPHPLC is highly sensitive to the presence of biological contaminants in organic extracts which may interfere with the effective separation of haem, protoporphyrin and iron. It was therefore necessary to remove these contaminants. In addition, the presence of excess free ^{59}Fe ions, carried over during extraction of haem into organic solvent, would result in equipment contamination with high ^{59}Fe background counts. It was thus necessary to reduce ^{59}Fe cpm prior to HPLC.

Sorbent extraction using disposable solid phase columns containing packing material similar to that of the HPLC column

is commonly used as sample preparation prior to RPHPLC. Sorbent extraction was examined as a possible means of achieving partial isolation of ^{59}Fe-labelled haem prior to RPHPLC.

5.7.1 Sorbent Extraction

Sorbent extraction is a physical extraction process that involves a liquid and a solid phase. The solid phase has a greater attraction for the isolate than the solvent. The specific properties of a given bonded silica sorbent result from the functional group covalently bonded to the silica substrate and a variety of different bonded silicas are commercially available, offering a wide range of selective properties for extraction.

(i) Properties of Bonded Silicas

Bonded silicas are formed by the reaction of organosilanes with activated silica. The product is a sorbent with the functional group of the organosilane attached to the silica substrate through a silyl ether linkage. Another reaction called endcapping may be performed subsequently to inactivate remaining silanol groups on the silica and so minimise interactions with the silica substrate.

Bonded silicas equilibrate rapidly to new solvent conditions and allow many solvent changes to be performed rapidly. Methanol is an effective solvating agent because it can interact with both the silanols on the silica and the carbon atoms of the bonded functional group.

(ii) Choice of Sorbent Extraction Columns

Sorbent extraction interactions can be grouped into one of four

classes:

(1) Non-polar

(2) Polar

(3) Ion-Exchange

(4) Covalent

For the isolation of haem a non-polar interaction is most suitable whereby interactions occur between the C-H bonds of the sorbent functional groups and those of the isolate. The most commonly used sorbent for non-polar interactions is octadecyl silane (C18), a non-selective sorbent. Various other hydrocarbon chain lengths are also commercially available i.e. C2, C8. Two columns, Sep-pak and Bond Elut, were examined for use in the partial isolation of haem prior to RPHPLC:-

(1) 'Sep-pak' Disposable Sorbent Extraction Columns (Waters)

Packing Material: Hydrocarbon chain length C18.

'Sep-pak' columns are uncapped and retention is by both hydrophobic and ionic interactions

(2) 'Bond-Elut' Disposable Sorbent Extraction Columns

(Analytichem)

Packing Material: Hydrocarbon chain lengths C2, C8, C18.

'Bond-Elut' columns are capped and retention is mainly by hydrophobic interaction.

Retention of isolates by non-polar interactions is facilitated by polar solvents. Elution of isolates from non-polar sorbents is facilitated by solvents of sufficient non-polar character to disrupt the non-polar substrate/sorbent

interactions.

Several experiments were carried out to determine the most suitable solid phase column for the retention and subsequent elution of haem. The combined behaviour of haem, protoporphyrin and Fe^{2+} ions was examined on such columns.

5.7.2 Partial Isolation of Haem by Sorbent Extraction

(i) The Solubility of Haem

Haem dissolves relatively well either at extreme alkaline pH (i.e. > 11) or extreme acidic pH (< 3) but not in neutral solvent. Haem must be dissolved initially in acid or alkali and the solution then pH'd to neutrality.

The method developed for the preparation of stock haem solution (prepared fresh daily) was as follows:-

100μM Haem

1mg of haem was dissolved by the sequential addition of 0.3ml of ammonium hydroxide (5M), 14.55ml of ammonium acetate (1M), pH 7.0 and the solution was brought to neutrality with 0.15ml of glacial acetic acid.

(ii) Activation of Sorbent Extraction Columns

Columns were activated with methanol (2ml), followed by ammonium acetate (1M), pH 7.0 (5ml).

(iii) Retention of Haem by Sorbent Extraction Columns

1ml of haem (50μM) in ammonium acetate (1M), pH 7.0 was applied at a flow rate of approximately 1.0ml min^{-1} to four of each of the following columns.

Sorbent Extraction Columns

	Hydrocarbon Chain Length	Packing Volume
'Sep-pak'	C18	1000mg
'Bond-Elut'	C2, C8, C18	300mg

The haem solution had an initial absorbance at 400nm of 0.101 while the A_{400nm} of the eluate in each case was zero. The A_{400nm} of the eluate after washing the columns with ammonium acetate (1M), pH 7.0 was also zero.

The retention of haem was 100% on all columns and washing with ammonium acetate (1M), pH 7.0 did not elute any haem. All tested columns were therefore suitable for the retention of haem. The retention pattern of iron on the columns was later examined (Section XI).

(iv) Choice of Eluting Solvent

The eluting solvent had to satisfy two criteria.

(1) Haem had to be readily soluble in the chosen eluting solvent to achieve maximal elution in minimum volume.

(2) The eluting solvent had to be volatile so that haem could be concentrated by solvent evaporation prior to HPLC.

Five solvents were examined:

1. Methanol
2. Methanol: hydrochloric acid (5M) (99:1, pH 3.0)
3. Methanol: sodium hydroxide (1M) (99:1, pH 9.0)
4. Methanol: ammonium acetate (1M) (90:10, pH 6.64)
5. Methanol: ammonium hydroxide (5M) (99:1, pH 10.5)

Each eluting solvent was applied (3 x 1ml) to four columns of each type and the eluate collected. Excepting methanol,

haem eluted well and was visible as a dark brown colour. A_{400nm} readings showed that most haem eluted in the first ml. The A_{400nm} for haem was similar for each eluting solvent but was greater than in the original ammonium acetate (1M) solvent. Haem recovery was not determined at this stage.

Methanol: ammonium acetate (1M) was chosen as the eluting solvent for haem in subsequent experiments because:-

1. Ammonium acetate (1M) was the solvent in which haem was applied to the sorbent extraction column.
2. Ammonium and acetate ions were present in the HPLC mobile phase.

Since C2, C8 and C18 columns retained haem equally well, the use of the more expensive C18 columns was discontinued.

A further three solvents comprising of methanol: ammonium acetate (1M), were examined for their ability to effect haem elution from C2 and C8 'Bond-Elut' columns.

MeOH : ammonium acetate (1M) (v/v)	pH
70 : 30	6.30
80 : 20	6.46
90 : 10	6.64

1.0ml haem (50μm) in ammonium acetate (1M), pH 7.0, (A_{400nm} of 0.184), was applied to each column type. The columns were washed with ammonium acetate (1M) and haem eluted in 2.0ml of each eluting solvent (Table 14). The experiment was carried out in triplicate. The results showed that:-

1. The recovery of haem was higher (15 - 40%) from C2 than from C8 columns.

C2 'Bond-Elut'

	A_{400nm}					
MeOH:AA	70:30		80:20		90:10	
Column	(a)	(b)	(a)	(b)	(a)	(b)
Elution (1) (1ml)	0.159	0.140	0.409	0.483	0.736	0.801
Elution (2) (1ml)	0.588	0.591	0.359	0.324	0.053	0.011
Total A_{400nm}	0.747	0.731	0.768	0.807	0.789	0.812

C8 'Bond-Elut

	A_{400}^{nm}					
MeOH:AA	70:30		80:20		90:10	
Column	(a)	(b)	(a)	(b)	(a)	(b)
Elution (1) (1ml)	0.399	0.420	0.571	0.425	0.625	0.650
Elution (2) (1ml)	0.141	0.123	0.063	0.289	0.046	0.033
Total A_{400nm}	0.540	0.543	0.634	0.714	0.671	0.683

NOTE:

A.A. = Ammonium Acetate (1M);
(a) and (b) are duplicate columns.

TABLE 14

THE ELUTION OF HAEM FROM SORBENT EXTRACTION COLUMNS (C2 AND C8) USING VARIOUS COMBINATIONS OF METHANOL:AMMONIUM ACETATE (1M) AS ELUTING SOLVENT

2. With both C2 and C8 columns MeOH: ammonium acetate (1M) (90:10) was more efficient at eluting haem than both 80:20 and 70:30 combinations.

3. Recovery was slightly improved using MeOH:ammonium acetate (1M) (95:5)) as the eluting solvent (results not shown).

Methanol : ammonium acetate (1M) (95:5), pH 6.9 was chosen as eluting solvent for routine use.

(v) Choice of Sorbent Extraction Column

Columns with packing material of short hydrocarbon chain length i.e. C2 or C8 may have an advantage over columns with longer hydrocarbon chain packing as impurities may more readily elute at the ammonium acetate wash. In theory, haem may also elute more efficiently because it will bind less strongly to hydrocarbons of shorter chain length. This has been demonstrated experimentally in the previous experiment as haem eluted more readily and with greater percentage recovery from C2 columns than from C8 columns. All subsequent experiments were carried out using C2 'Bond-Elut' columns.

(vi) Optimum Packing Volume

The packing volume of C2 'Bond-Elut' columns (100mg or 300mg) made no material difference to the retention or elution of haem and haem recoveries were consistent to within 5%. C2 'Bond-Elut' columns with 100mg of packing material were chosen for routine use.

(vii) Optimum Elution Volume

The aim was to elute haem from the C2 'Bond-Elut' column in the smallest volume that would permit maximum haem recovery.

Using methanol : ammonium acetate (1M) (95:5), pH 6.9, the optimum elution volume was three volumes of 1ml. The eluted sample was evaporated to dryness under N_2 at 37°C in 40 minutes.

(viii) Consistency of Haem Recovery

Earlier work (Section 5.7.2 (vi)) suggested that haem recovery was highly consistent. To verify this 6 identical samples of haem were applied to 6 activated C2 'Bond-Elut' columns. The A_{400nm} of the original haem solution (3.0ml) was 0.101 and the average A_{400nm} of the eluted haem solution (3.0ml) was 0.446 (S.D. = 0.020, C.V. = 4.5%, n = 6). This confirmed earlier results that the optical density was higher in the eluting solvent than in ammonium acetate (1M) and again demonstrated recoveries consistent to within 5%.

(ix) Viability of C2 'Bond-Elut' Columns for Re-Use

The C2 'Bond-Elut' columns were examined for repeated use by applying five haem samples consecutively to one column. The column was reactivated between samples with methanol (2ml) followed by ammonium acetate (1M) (5ml). The average A_{400nm} of the eluted haem solution was 0.422 (S.D. = 0.006, C.V. = 2.7%, n = 5) indicating that the 100mg C2 'Bond-Elut' columns could be re-used up to 5 times without affecting haem recovery.

(x) Percentage Recovery of Haem From C2 'Bond Elut Columns

The retention of haem on C2 'Bond-Elut' columns in ammonium acetate (1M), pH 7.0, was 100% (Section 5.6.2). The A_{400nm} of haem is dependent on the nature of the solvent. Elution in MeOH : ammonium acetate (1M), pH 6.9 (95:5) resulted

in higher absorbance readings than for haem in the original solution of ammonium acetate (1M). To calculate the percentage recovery of haem from the column a reference graph was constructed relating the A_{400nm} of known concentrations of haem in ammonium acetate (1M), pH 7.0, to the A_{400nm} in MeOH : ammonium acetate (1M), pH 6.9, (95:5) (Figure 17). This was carried out in triplicate [due to difficulty experienced in dissolving dried haem directly in MeOH: ammonium acetate (1M), (95:5)] over the concentration range 0.6μm - 600μm. A straight line reference graph was obtained over the range 0.6μm - 450μm. At concentrations exceeding this, linearity was not maintained presumably due to the propensity of porphyrins to aggregate and change absorbance maxima in the 400nm region with increasing concentration (Gallacher and Elliot, 1973). The Beer-Lambert relationship between absorbance and concentration could, however, be applied over the linear portion of the graph -

$$A = Ecl$$

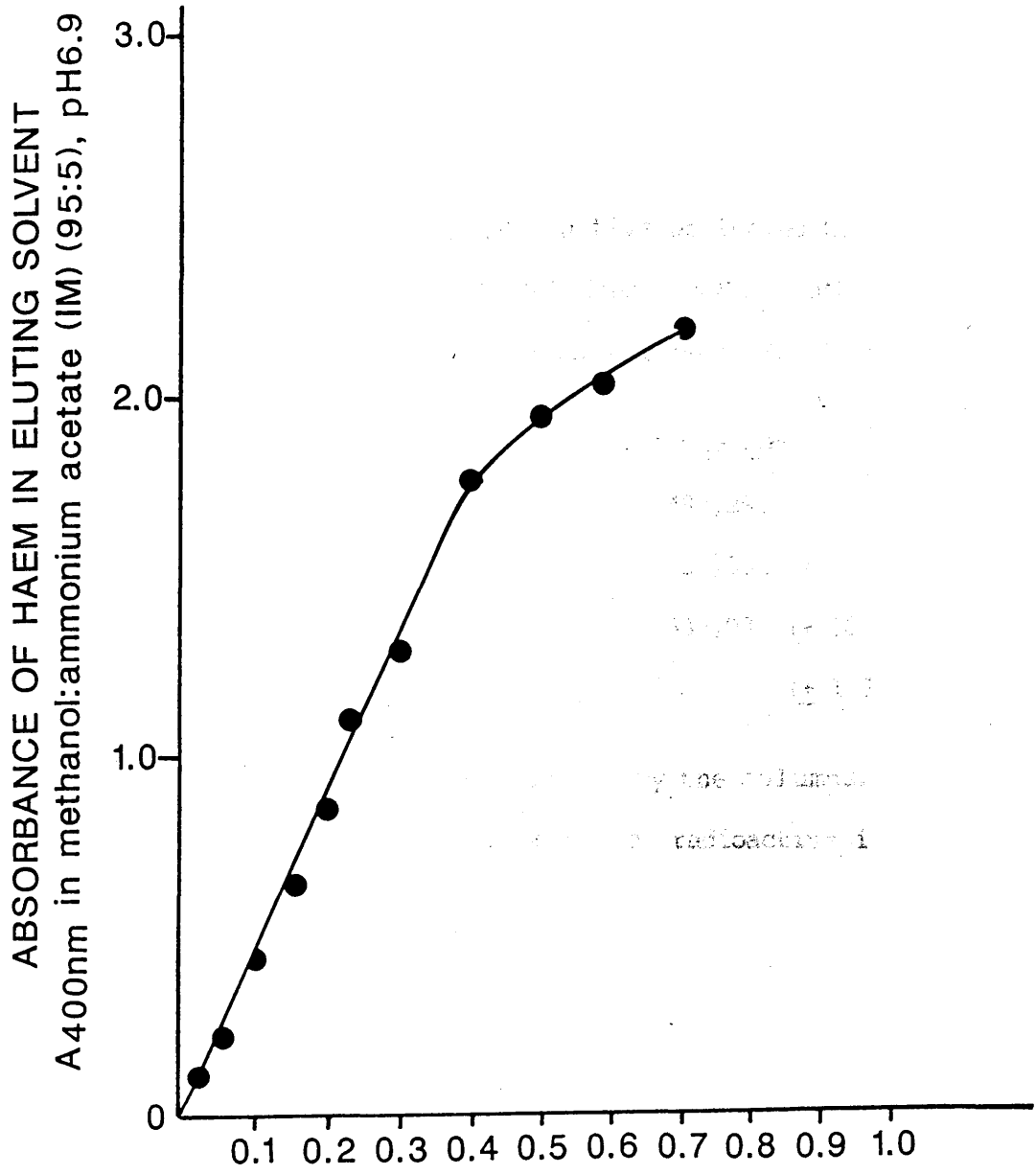
A = absorbance units E = molar extinction coefficient

c = concentration l = pathlength

Since E and l are both constant, the absorbance, A is proportional to the concentration c. Using the reference graph, it was established that the recovery of haem from the C2 'Bond-Elut' column, using MeOH: ammonium acetate (1M), pH 6.9 (95:5) as the eluting solvent, consistently fell within the range 95-100%.

(xi) Partial Separation of Haem and ^{59}Fe by Sorbent Extraction

Further experiments were designed to investigate whether



NOTE :
Each point represents the mean of three observations.
Assay conditions are described in the text.

FIGURE 17

REFERENCE GRAPH FOR DETERMINATION OF HAEM RECOVERY DURING SORBENT EXTRACTION USING C2 'BOND ELUT' COLUMNS.

^{59}Fe and haem could be successfully separated on C2 columns following organic solvent extraction such as to make the use of RPHPLC unnecessary.

^{59}Fe (2.5μCi) was applied to five activated C2 'Bond Elut' columns in 3.0ml neutral tris buffer (50mM). Both eluate and columns were then counted on a gamma counter for 1 minute each.

	average cpm
Activity applied to columns	484,293
Activity retained by columns	50,361 (± 13 871)
Activity in eluate	433,931 (± 12 886)
	(± 1 S.D.)

11.6% of the ^{59}Fe was retained by the columns. Since it was intended that a similar amount of radioactive iron should be used in the assay of ferrochelatase activity, C2 columns were suitable for the removal of up to 90% of the free $^{59}Fe^{2+}$ ions. ^{59}Fe retention did not alter in the presence of haem (100μM) and/or protoporphyrin (100μM).

The retention of ^{59}Fe ions was not reduced either by saturating the C2 column with cold $FeSO_4$ prior to the addition of ^{59}Fe or by repeated washing of the column with water or tris buffer, pH 7.0. Tris buffers in the lower pH range (pH 4.0) removed up to 50% of the retained ^{59}Fe but also allowed haem to elute from the column.

Although it was not possible to isolate haem using sorbent extraction alone, this stage was useful for the partial isolation of haem prior to RPHPLC. This significantly reduced the ^{59}Fe cpm injected into the HPLC and facilitated ^{59}Fe-

labelled haem isolation by RPHPLC. In a typical assay 500000 ^{59}Fe cpm could be reduced to 7500 cpm using organic solvent extraction followed by sorbent extraction prior to RPHPLC.

5.7.3 Summary of Sample Preparation Prior to RPHPLC

Sample preparation prior to RPHPLC may be summarised by the following steps:

Haem was extracted from the incubation medium with acidified organic solvent.

1. HCl (1.0M, 0.5ml) was added to incubation medium (1.0ml) containing haem, protoporphyrin and iron.
2. Methylethyl ketone (1.5ml) was then added to each sample and haem extraction effected as described in Section 5.6.2. As non-specific contamination of organic solvent with 15% of original ^{59}Fe cpm is known to occur (Section 5.6.4), further sample preparation to reduce this contamination is required prior to RPHPLC.
3. The samples were evaporated to dryness under N_2 at 37°C and reconstituted in 2.0ml of ammonium acetate (1M), pH 7.0 by the sequential addition of 0.2ml ammonia (5M), 1.7ml ammonium acetate (1M) and 0.1ml glacial acetic acid.

   The samples were then prepared for RPHPLC by sorbent extraction.
4. C2 'Bond-Elut' columns were activated by 2.0ml of methanol, followed by 5.0ml of ammonium acetate (1M), pH 7.0.
5. Samples were applied to the columns in 2.0ml of ammonium acetate (1M), pH 7.0 at a rate of 1ml min^{-1}.
6. The columns were washed with 2.0ml of ammonium acetate (1M), pH 7.0.

7. Haem was eluted in 3 volumes (3 x 1.0ml) of the eluting solvent, methanol : ammonium acetate (1M) (95:5), pH 6.9 at a flow rate of 1ml min^{-1}.

8. Each sample was evaporated to dryness under N_2 at 37°C in a sample concentrator.

9. The samples were reconstituted in 0.2ml of the eluting solvent and an aliquot taken for RPHPLC (0.05ml).

Losses of haem were most likely to occur at haem extraction into organic solvent (Section 5.6.3). Losses were minimal at the stage of sorbent extraction.

With conditions now established for haem isolation the optimum conditions for the enzyme reaction were determined.

5.8 DETERMINATION OF OPTIMUM ASSAY CONDITIONS

5.8.1 The optimum assay conditions for ferrochelatase activity were determined in normal whole human bone marrow.

Cells

(a) Cell Number

1 x 10^7 cells were used per assay. This was approximately equivalent to the number of cells available following bone marrow fractionation.

(b) Cell Preparation

The cells were prepared for initial work by ultrasonic disintegration in incubation medium (0.85ml per 2 x 10^7 cells) as previously described for the ALA synthase assay (Chapter 3, Section 3.8.8). 0.4ml of cell suspension were taken for assay.

(i) Incubation Medium

The reaction took place in tris buffer, final pH 7.4 in a volume of 1.0ml.

(a) Reaction Under N_2

The reaction took place under N_2 at 37°C in the dark.

(b) Presence of ascorbic acid as reducing agent

Ascorbic acid was present at a concentration of 4.25mM.

(iii) Means of assessing haem recovery

The endogenous haem peak in identical marrow samples, as determined by RPHPLC, was used as a means of assessing relative recoveries. All cpm were then corrected to the peak area of the sample with the highest recovery.

5.8.2 Optimum Iron Concentration

(i) The Radioactive : Nonradioactive Iron Ratio (Specific Activity)

The most suitable radioactive iron substrate was ferric chloride ($^{59}FeCl_3$) in hydrochloric acid (0.1M) (Amersham International) purchased in batches of 100μCi in a volume of 1.0ml. The iron content per batch of $^{59}FeCl_3$ could be calculated, and, on average -

$$1\mu Ci \approx 1nmole \approx 10\mu l.$$

Other workers have used large amounts of radioactive iron (e.g. 10μCi) in the assay of ferrochelatase activity (Bloomer and Morton, 1982). However, this was undesirable and perhaps unnecessary. The aim, therefore, was to determine the minimum amount of radioactivity per assay which could provide adequate

sensitivity.

The radioactive iron would primarily be in the form of ferric ions ($^{59}Fe^{3+}$) and so would be unable to act as substrate for ferrochelatase. To correct this, non-radioactive ferrous ions (Fe^{2+}) were added in excess such that mass action favoured the conversion of $^{59}Fe^{3+}$ ions to $^{59}Fe^{2+}$ ions. The presence of the reducing agent, ascorbic acid (in which the non-radioactive iron was dissolved), maintained the radioactive iron as $^{59}Fe^{2+}$ ions.

Experimental

Ascorbic acid was prepared fresh on the day of assay by dissolving ascorbic acid (0.25g) in distilled deionised water (10ml). The addition of 0.03ml of this solution to the final reaction mixture (0.97ml) gave a final ascorbic acid concentration of 4.25mM. Ferrous chloride ($FeCl_2$) was included in the ascorbic acid solution at various concentrations to produce final assay concentrations in the range (10 - 500μM). Radioactive iron (2μCi) was added to each assay. The enzyme reaction was initiated by the addition of 0.05ml of protoporphyrin (final concentration 100μM). The reaction was terminated at 1 hour and the haem extracted then isolated by RPHPLC.

Results

The results were expressed as the mean of duplicate samples.

[Iron] μM	^{59}Fe:Fe	Corrected average cpm in haem
500	1 : 250	86
100	1 : 50	385
50	1 : 25	983
20	1 : 10	2311
10	1 : 5	5918

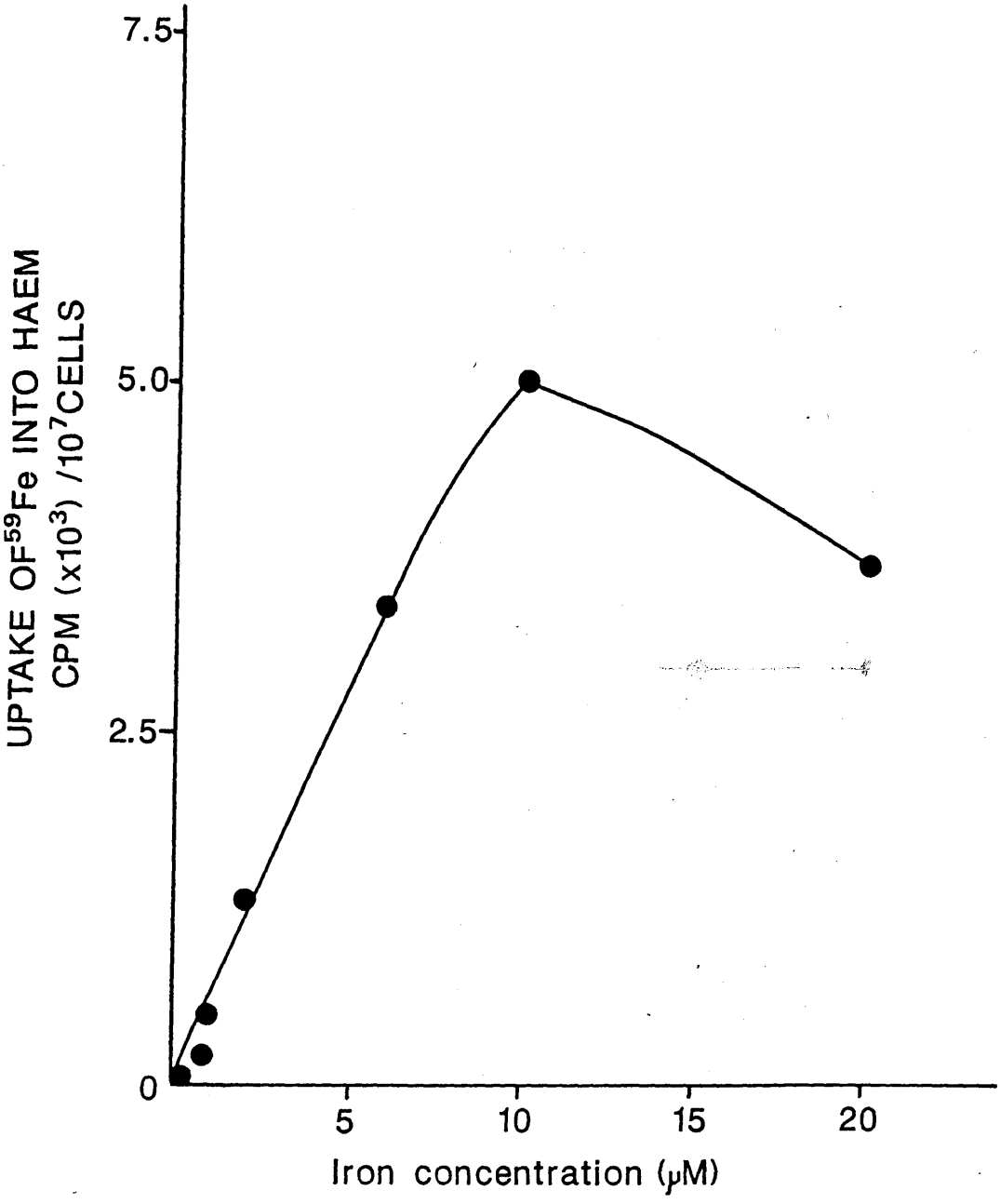
The results indicated that at iron concentrations of 20 and 10μM a ^{59}Fe:Fe ratio of 1:10 or 1:5 provides suitable sensitivity enabling ^{59}Fe-haem to be easily detected (background counts up to 100 cpm). Various concentrations of iron (specific activity 0.2) were examined in the range 0-40μM to determine the saturating concentration of iron for ferrochelatase. Corrected cpm in haem were then plotted as a function of iron concentration.

The results from three experiments, each using duplicate samples, indicated that a plateau in enzyme activity was attained at an iron concentration of between 5 and 10μM. Thereafter, the addition of iron to a concentration of 40μM may cause slight enzyme inhibition (Figures 18 and 19). This was consistently observed and it may be speculated that iron has a regulatory role in ferrochelatase function. However, any firm conclusions must await more detailed experimentation.

A concentration of 10μM iron (specific activity 0.2) was chosen for routine use i.e. in 1.0ml of incubation medium 8nmoles of non-radioactive iron and 2nmoles of radioactive iron were present, the equivalent of 2μCi of $^{59}FeCl_3$ per assay.

5.8.3 Time Course for Ferrochelatase Activity

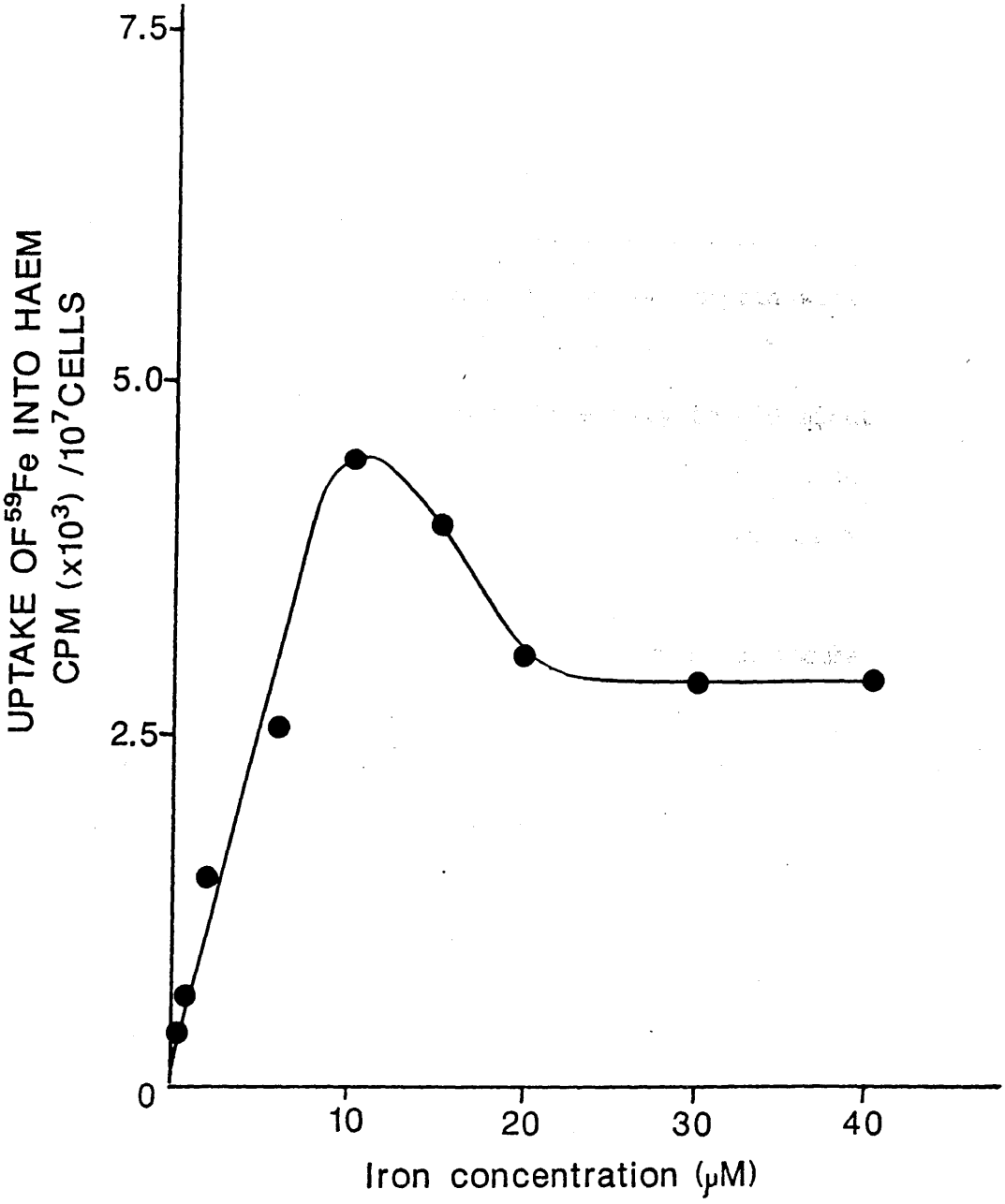
The rate of haem formation was investigated for comparison



NOTE :
Each point represents the mean of two observations.
Assay conditions are described in the text.

FIGURE 18

UPTAKE OF ^{59}Fe INTO HAEM AS A FUNCTION OF IRON CONCENTRATION.



NOTE :
Each point represents the mean of two observations.
Assay conditions are described in the text.

FIGURE 19

UPTAKE OF ^{59}Fe INTO HAEM AS A FUNCTION OF IRON CONCENTRATION.

with previous reports (Krueger et al, 1956; Nishida and Labbe, 1959; Porra and Jones, 1963; 1963; Porra et al, 1967). A plot of haem formation as a function of reaction time is shown over a period of three hours (Figure 20). The concentrations of iron (specific activity 0.2) and protoporphyrin were 10μM and 100μM respectively.

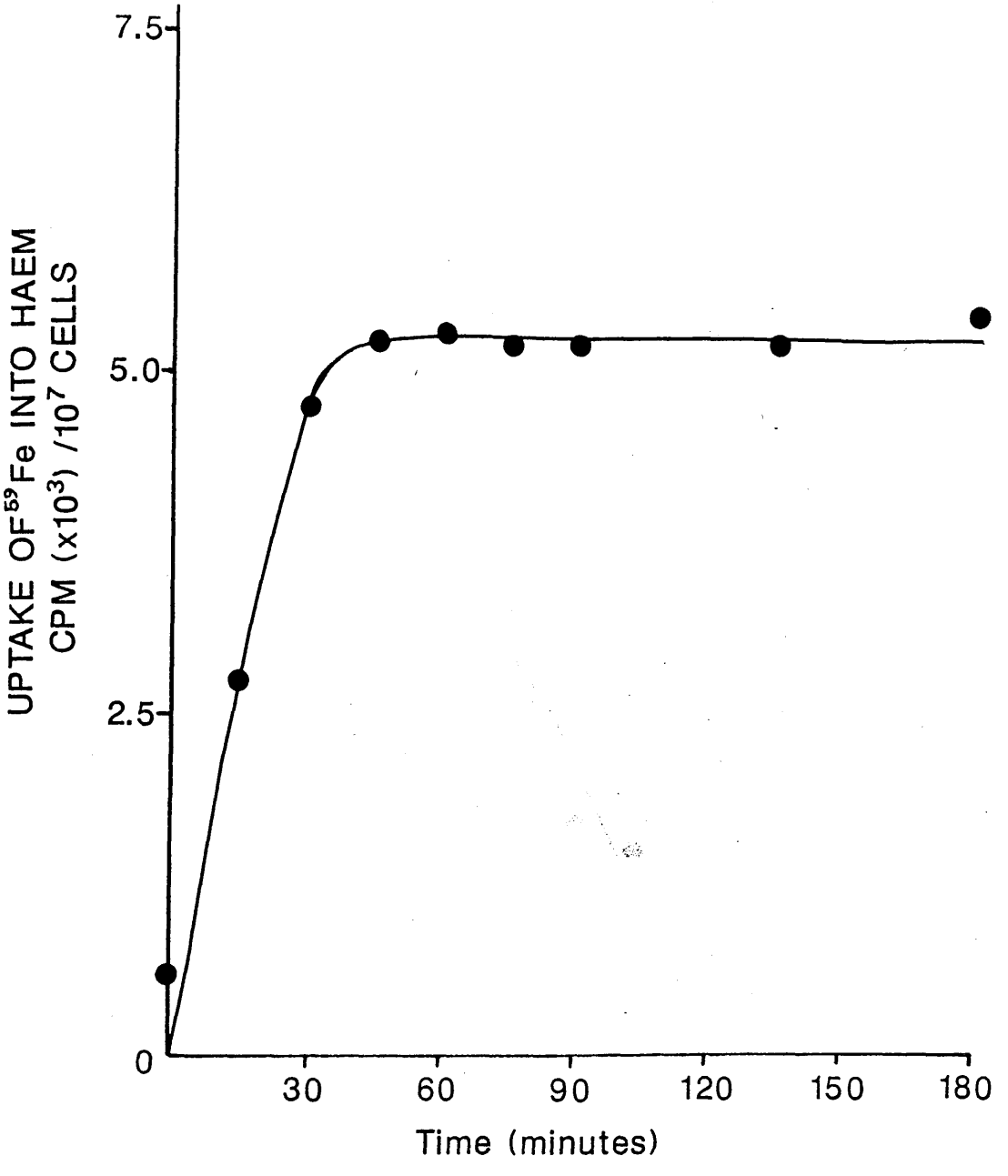
The reaction maintained linearity for 30 minutes after which a plateau was reached, presumably due to a shortage of substrate or enzyme instability. The results were verified using a second bone marrow sample.

To ensure linearity of haem formation an incubation time of 20 minutes was chosen for routine use.

5.8.4 The Effect of pH on Ferrochelatase Activity

Haem formation during a 20 minute incubation was plotted as a function of pH over the pH range 6.0 - 8.7 (Figure 21). The results clearly demonstrated optimum bone marrow ferrochelatase activity at pH 7.3 in agreement with that reported by Bottomley (1968).

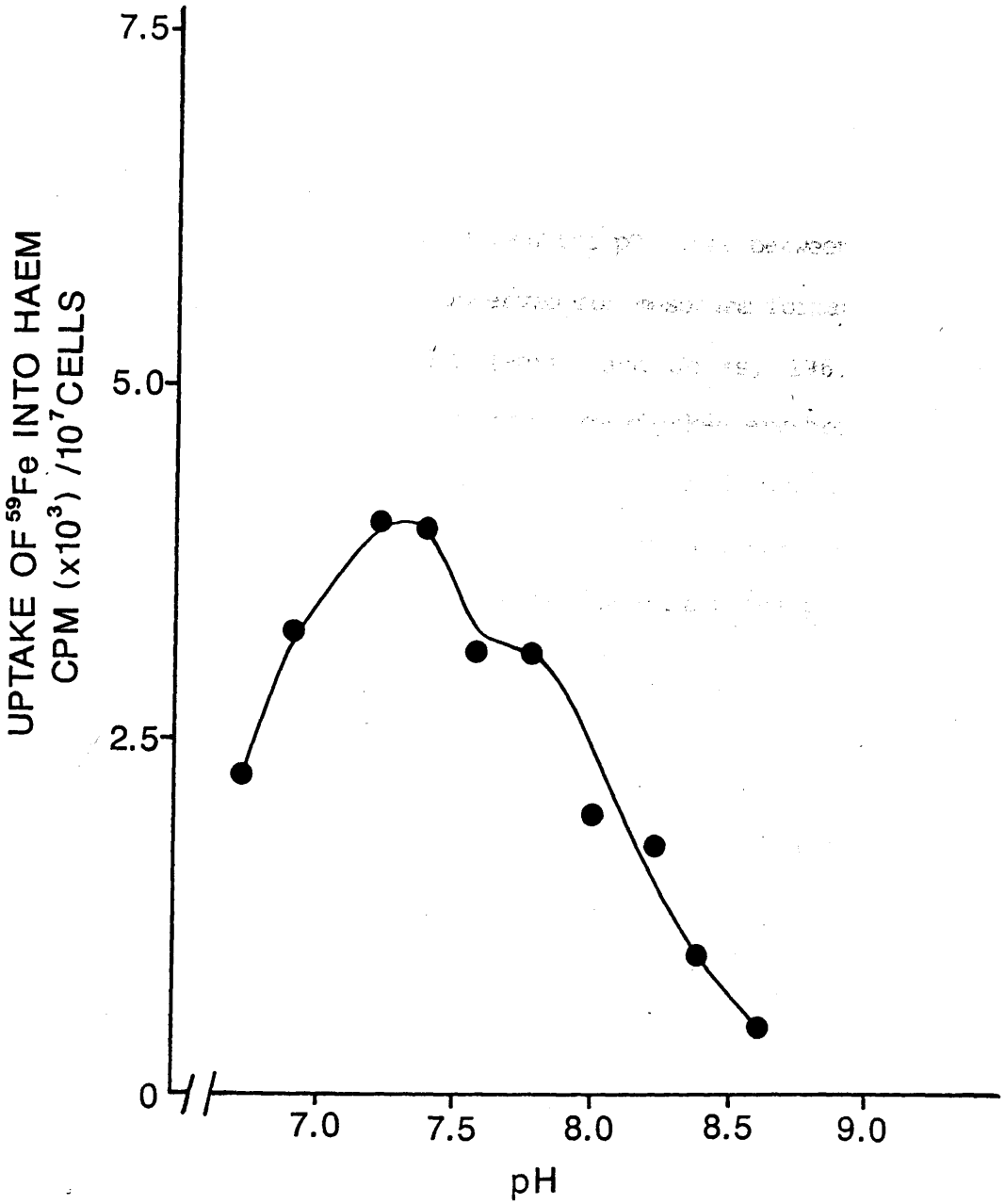
The pH optimum of 7.3 for human bone marrow ferrochelatase activity differs from that determined in other tissues. For example, a pH optimum for ferrochelatase activity of 7.9-8.0 has been reported in avian erythrocyte (Schwartz et al, 1959) and rat liver (Labbe and Hubbard, 1960; Li et al, 1988; Taketani and Tokunaga, 1981) while a value of 8.2 has been recorded for the reticulocyte enzyme in peripheral blood (Langelaan et al, 1970). However, it would seem logical that enzyme activity is optimal at physiological pH when using the



NOTE :
Each point represents the mean of two observations.
Assay conditions are described in the text.

FIGURE 20

UPTAKE OF ^{59}Fe INTO HAEM AS A FUNCTION OF TIME.



NOTE :
Each point represents the mean of two observations.
Assay conditions are described in the text.

FIGURE 21

UPTAKE OF ^{59}Fe INTO HAEM AS A FUNCTION OF pH.

natural substrates for the enzyme. Discrepancies may possibly be explained by the use of different substrates such as Zn^{2+} and mesoporphyrin (Li et al, 1988; Taketani and Tokunaga, 1981).

A shoulder was observed on the pH curve between pH 7.6 and 7.8. This has also been observed for mesohaem formation by pig liver extract at pH 9.0 (Porra and Jones, 1963) and for protohaem formation in extracts from chicken erythrocytes also at pH 9.0 (Sawada et al, 1969). Dailey and Lascelles (1974) have suggested that this biphasic pH optima curve is an artifact due to the known propensity of aqueous porphyrins to aggregate (Gallacher and Elliot, 1973).

5.8.5 Optimum Protoporphyrin Concentration

The concentration of protoporphyrin in the reaction mixture was examined over the range 0-100μM. Haem formation was plotted as a function of protoporphyrin concentration (Figure 22). A plateau in ferrochelatase activity was attained at a protoporphyrin concentration of approximately 40μM.

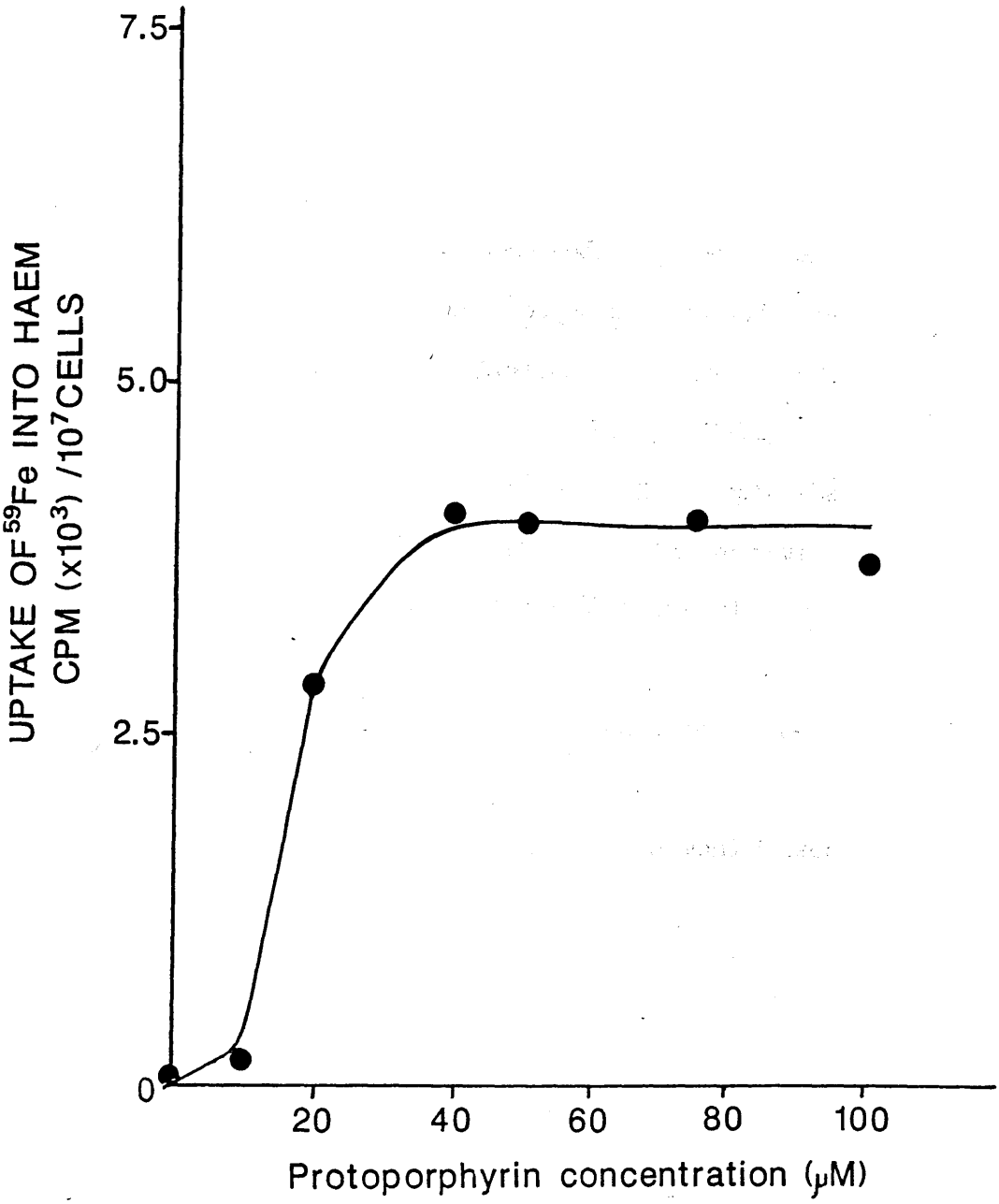
To ensure optimum ferrochelatase activity the protoporphyrin concentration for routine use was 100μM.

5.8.6 Choice of Reducing Agent

A reducing agent is essential for the assay of ferrochelatase activity -

(1) to maintain the iron substrate as Fe^{2+} as opposed to Fe^{3+};

(2) to maintain the active sulphydryl groups of the ferrochelatase molecule in the reduced form.



NOTE :
Each point represents the mean of two observations.
Assay conditions are described in the text.

FIGURE 22

UPTAKE OF ^{59}Fe INTO HAEM AS A FUNCTION OF PROTOPORPHYRIN CONCENTRATION

Several different reducing agents have been chosen for this purpose e.g. dithiothreitol (DTT), cysteine, 2-mercaptoethanol, glutathione (GSH) or ascorbic acid (Dailey and Fleming, 1986; Krueger et al, 1956; Goldberg et al, 1956; Bloomer and Morton, 1982). Some reducing agents may facilitate non-enzymatic haem formation thought to occur via the formation of an iron-sulphur complex (Tokunaga and Sano, 1972). Based on these observations, Kassner and Walchak, (1972), demonstrated that thiol reductant DTT, cysteine or 2-mercaptoethanol act to reduce Fe^{3+} ions and form a catalytically active iron-thiol complex. GSH, however, is ineffective at promoting non-enzymatic haem formation and this may be attributed to the formation of a stable iron-thiol complex which is an inactive species for haem formation.

DTT and ascorbic acid are most commonly used and were examined for use in the assay of ferrochelatase in bone marrow cells.

(i) DTT (Cleland's Reagent)

DTT was an unsatisfactory reducing agent in the assay of bone marrow ferrochelatase activity. It interfered with haem extraction into methylethyl ketone and so reduced haem recovery. Similar findings have been reported (Bloomer and Morton, 1982) and its use was therefore discontinued.

(ii) Ascorbic Acid

Ascorbic acid (Bloomer and Morton, 1982) was an effective reducing agent which facilitated the ferrochelatase enzyme reaction. A suitable concentration was determined for routine use.

(iii) Optimum Ascorbic Acid Concentration

The concentration of ascorbic acid (reagent grade, BDH) was examined over the concentration range 4.25-42.50mM, similar to that used in the measurement of rat liver ferrochelatase (Bloomer and Morton, 1982). Haem formation was plotted as a function of ascorbic acid concentration (Figure 23).

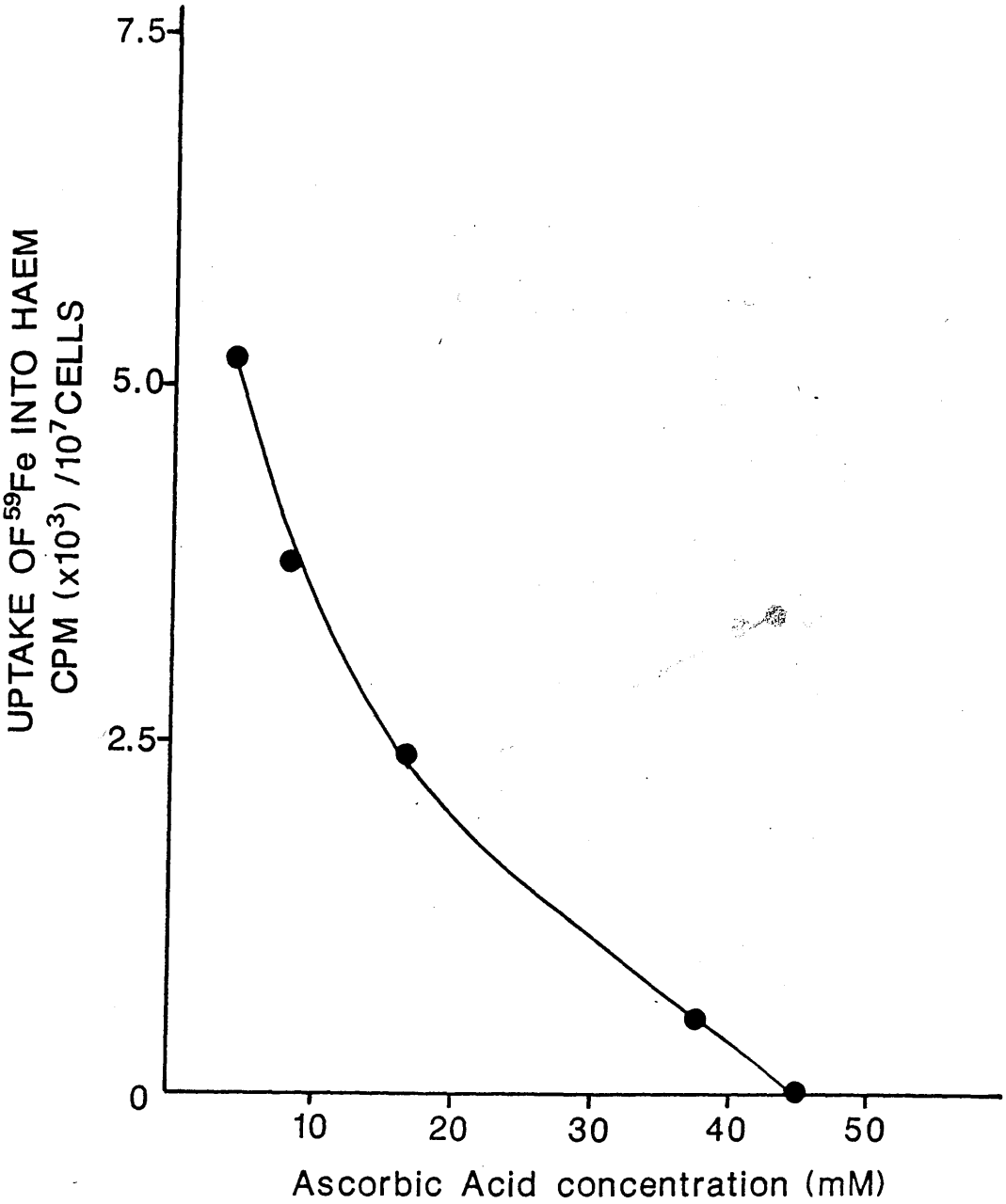
The results demonstrate a steady decline in ferrochelatase activity with increasing ascorbic acid concentration at a fixed pH of 7.4. The aerobic degradation of haems in the presence of thiols or ascorbic acid has been reported (Porra et al, 1967) but has not been observed under anaerobic conditions similar to those used in this assay.

The experiment was repeated over the concentration range 0-4.25mM and optimum ferrochelatase activity was observed at an ascorbic acid concentration of 4.25mM (Figure 24). In all subsequent experiments ascorbic acid was maintained at this concentration.

The removal of ascorbic acid from the incubation medium resulted in a 50% reduction in enzyme activity. Significant enzyme activity remained indicating that mass action favours the conversion of $^{59}Fe^{3+}$ ions to $^{59}Fe^{2+}$ ions even in the absence of a reducing agent. This activity was shown not to be due to non-enzymatic haem formation (Section 5.11).

5.8.7 **The Need for Anaerobiosis**

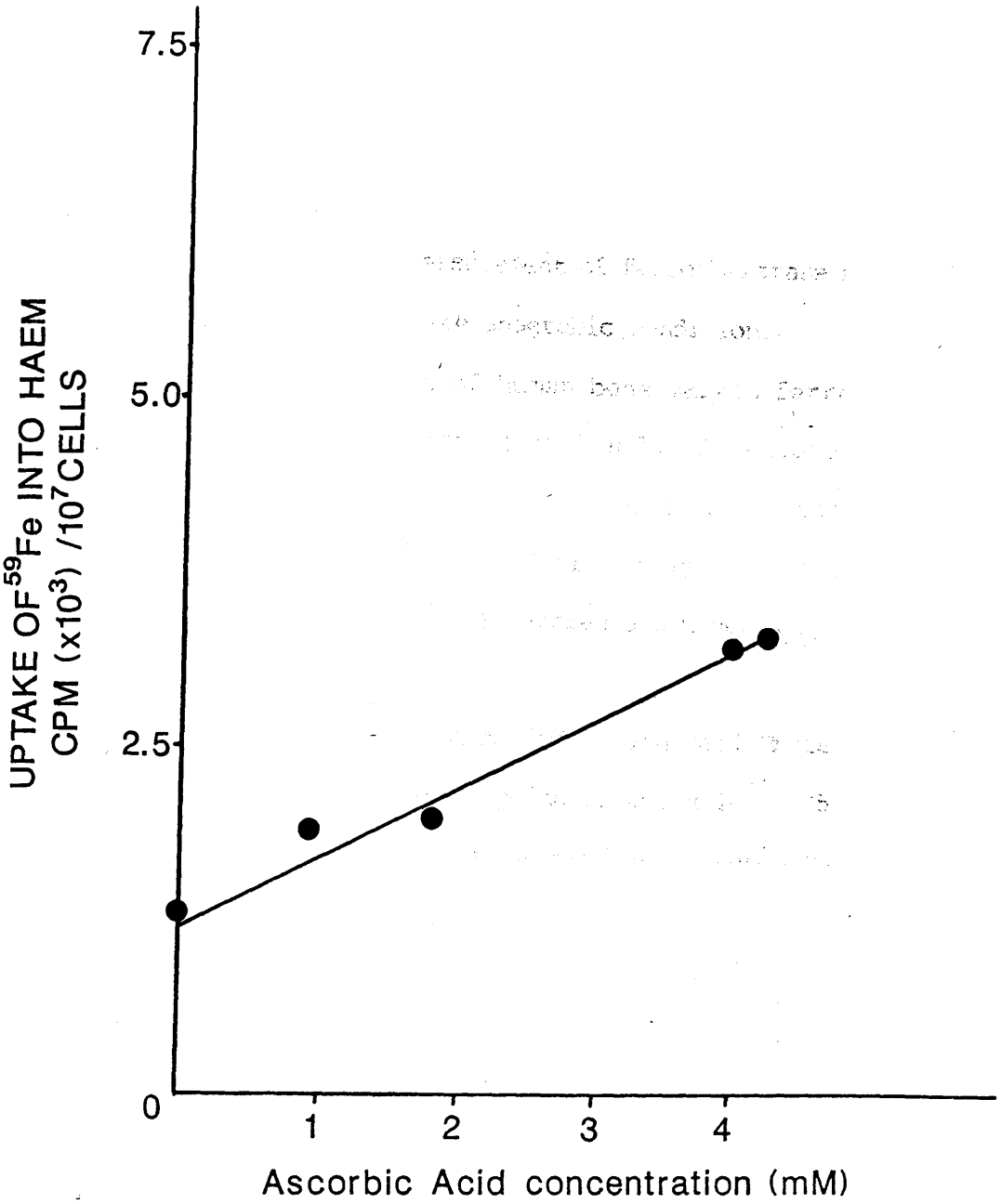
Anaerobic conditions have generally been utilised for enzyme assays of ferrochelatase. The reaction has been reported to be inhibited by oxygen (Labbe and Hubbard, 1960;



NOTE :
Each point represents the mean of two observations.
Assay conditions are described in the text.

FIGURE 23

UPTAKE OF ^{59}Fe INTO HAEM AS A FUNCTION OF ASCORBIC ACID CONCENTRATION.



NOTE :
Each point represents the mean of two observations.
Assay conditions are described in the text.

FIGURE 24

UPTAKE OF ^{59}Fe INTO HAEM AS A FUNCTION OF ASCORBIC ACID CONCENTRATION

Porra and Jones, 1963) and so is normally carried out under N_2. This provides optimum conditions for maintaining the iron substrate as Fe^{2+} and ensures that active sulphydryl groups do not undergo oxidation. It has, however, been reported (Dailey et al, 1986) that the measurement of ferrochelatase activity in FEL cells does not require anaerobic conditions.

In the measurement of human bone marrow ferrochelatase activity it was consistently found that anaerobiosis was indeed necessary as up to 50% of the enzyme activity was lost in the presence of O_2. All assays of bone marrow ferrochelatase activity were carried out under N_2.

5.8.8 Linearity of Ferrochelatase Activity with Cell Number

Various cell numbers ranging from 1×10^6 - 35×10^6 were assayed for ferrochelatase activity. Haem formation was plotted as a function of cell number (Figure 25) and the reaction was found to be linear to a cell number of 30×10^6. Similar results were also obtained from a second experiment.

Other workers have also demonstrated a linear relationship between cell number and ferrochelatase activity in human bone marrow (Bottomley, 1968) and in cultured skin fibroblasts (Bonkowsky et al, 1975). However, linearity of enzyme activity with cell number has not been demonstrated for ferrochelatase in the liver (Porra et al, 1963).

5.9 RECOVERY OF HAEM FROM HUMAN BONE MARROW: INTERFERENCE FROM ENDOGENOUS Hb

Endogenous haem is present in substantial amounts in bone marrow in the form of haemoglobin (Hb). It has been shown that

following the incubation stage of the ferrochelatase assay a minimum concentration of 0.3M HCl (0.5ml) is required to terminate the enzyme reaction, precipitate protein and to facilitate haem extraction (Section 5.6). However, the presence of HCl at this concentration also led to Hb dissociation thereby releasing large quantities of free haem. This endogenous haem resulted in poor chromatographic isolation of haem. This gross excess of endogenous haem liberated from Hb made it impossible to add cold carrier haem at reaction termination to assess haem recovery. Mitochondrial preparations are free of Hb and the assay of enzyme activity using carrier haem and mitochondrial preparations is discussed in Section 5.9.8. Several other steps were examined in the hope of overcoming endogenous haem release and so allow for the addition of cold carrier haem as internal standard to each enzyme assay.

5.9.1 **Use of an External Standard**

The inclusion of an external standard in the reaction mixture was considered. Such a compound had to satisfy two criteria i.e. to have:-

(1) an extraction efficiency into acidified methylethyl ketone which was identical to that of haem;

(2) the potential to chromatograph by RPHPLC independently from haem and iron producing a recognisable peak which could be accurately quantified.

No such compound could be identified.

5.9.2 **The Consistency of Haem Extraction**

(1) Haem (0.1ml, 100μM) in ammonium acetate (1M), pH 7.0 was

extracted from incubation medium (1.0ml) into acidified methylethyl ketone. The percentage recovery was assessed by relating the peak area of haem as determined by RPHPLC following extraction with the peak area of haem prior to extraction.

The average recovery was 75% $\pm$ 7.6% ($\bar{x} \pm$ 1.S.D., n = 10) with a coefficient of variation (C.V.) of 9.7%.

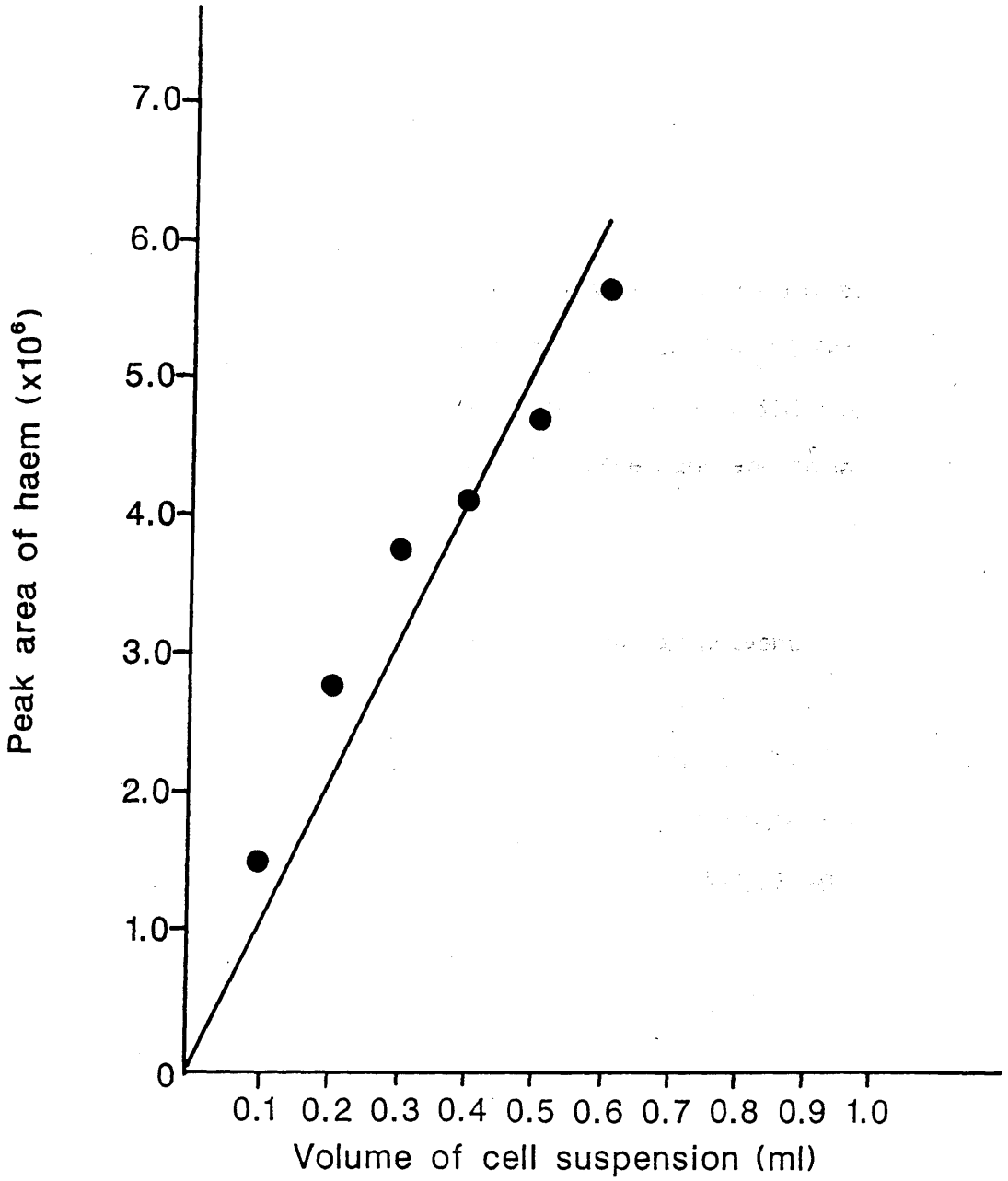
(2) Cell pellets from 0.25ml of blood were sonicated in incubation medium. The samples were extracted to investigate whether endogenous haem liberated from Hb behaved in a manner similar to carrier haem.

Actual recoveries could not be determined as haem could not be accurately quantified, but variations in recovery were observed and the C.V. found to be 8.8% (n = 10).

(3) Haem was extracted from samples of both blood and bone marrow samples suspended in incubation medium. A linear relationship was demonstrated between haem extraction and cell number (Figure 26).

The results from (1), (2) and (3) above indicated that the recovery of haem was relatively consistent at 75% with a variation of less than 10%.

Ideally, for a sensitive assay, the precision should fall within 5% variation. Several possibilities, i.e. protein precipitation by various means, were explored in an attempt to enable haem recovery to be assessed more accurately by avoiding the release of endogenous haem from Hb. All of these methods were unsuccessful.



NOTE :
Blood was used as a source of haem in this instance (see text for details).
Each point represents the mean of duplicate observations.

FIGURE 26

THE RELATIONSHIP BETWEEN THE VOLUME OF CELL SUSPENSION FROM WHICH HAEM IS EXTRACTED AND THE PEAK AREA OF HAEM AS DETERMINED BY RPHPLC.

5.9.3 Comparison of TCA and HCl for Use at Reaction Termination

A comparison was made between 0.5ml of trichloroacetic acid (TCA, 10% w/v) and 0.5ml of HCl (1.0M) for use at reaction termination.

Both HCl (1M) and TCA (10% w/v) could precipitate protein, however no haem was extracted into methylethyl ketone in the presence of TCA (10% w/v). Although TCA did not cause Hb dissociation it did precipitate free haem and so was useless for the purposes of this assay.

5.9.4 Haem Extraction in the Absence of Organic Solvent

In an attempt to prevent Hb dissociation the enzyme reaction was terminated by placing the samples in ice following incubation. This resulted in protein precipitation in the absence of acid. The supernatents were then applied to the C2 'Bond-Elut' columns, omitting the process of haem extraction into acidified organic solvent. However, this was not found to be feasible as haem recovery was extremely low. This was possibly due to precipitation of haem along with protein and/or reduced haem retention on the Bond-Elut column.

The next approach was to attempt to remove Hb from the bone marrow. This would enable the addition of a known quantity of exogenous cold carrier haem to allow haem recovery to be calculated.

Hb required to be removed quickly and efficiently, without removing, or inactivating, ferrochelatase. Several methods were assessed for their ability to extract endogenous Hb without causing haem dissociation.

5.9.5 Salt Precipitation of Protein

Salt precipitation of protein was examined as a possible means for the removal of endogenous Hb for use prior to addition of internal standard.

(i) Principles of Salt Precipitation

Proteins precipitate at low and high salt concentrations. Precipitation at high concentrations is useful; the proteins do not denature and different proteins precipitate at different concentrations. The agent most frequently used is ammonium sulphate $(NH_4)_2SO_4$.

(ii) Experimental

$(NH_4)_2SO_4$ (reagent grade, May and Baker) was added at saturating concentration to several samples containing sonicated red cells from 0.25ml of blood in incubation medium (3ml). The samples were thoroughly mixed (1 min) then spun at 1000g for 5 minutes. The supernatant was removed and the procedure repeated (x 2) to ensure maximal protein removal.

(iii) Results

Following salt fractionation, the samples remained red in colour and turned brown upon the addition of 0.5ml of HCl (1.0M) indicating the release of free haem. Extraction and RPHPLC analysis demonstrated that substantial quantities of Hb remained following salt fractionation. This method of Hb removal was therefore inefficient.

5.9.6 **Ion-Exchange Removal of Protein**

Ion-exchange was examined as a possible means for the removal of endogenous Hb.

(i) Principles of Ion-Exchange

Proteins bind to ion exchangers via electrostatic interactions. Protein binding depends on (1) the type of ion exchanger; (2) the overall charge of the protein (hence the pH of the solution); (3) the ionic strength of the solution. The solution contains ions of opposite charge to the ion-exchanger which compete with the protein for the ion-exchanger. A protein that binds at low ionic strength will be displaced at high ionic strength because of increased competition.

Conventional ion exchange chromatography takes place in a column. This is impractical for the removal of Hb during the assay of ferrochelatase activity as limited time is available and the enzyme must be maintained at $4^{o}C$ throughout.

(ii) Experimental

Sephadex beads (Pharmacia, G-75) were added to samples containing Hb then removed by centrifugation (1000g, 5min). Two types of ion exchangers were tested -

CM-sephadex	=	carboxymethyl	CH_2COO^-
DEAE-sephadex	=	diethylaminoethyl	$CH_2H_4-N^+H(C_2H_5)_2$

The beads (5g) were swollen in 20ml of tris buffer (50mM) at various pH's. (Note: isoelectric point of Hb is 6.8).

	pH of Tris Buffer (50mM)
CM-sephadex	5.5, 6.0, 6.5
DEAE-sephadex	8.0, 8.5, 9.0

1ml of beads was added to duplicate samples, one of which contained a known quantity of free haem (0.1ml of haem (100μM

in ammonium acetate 1M)). The samples were mixed and the beads removed. This step was repeated twice more.

(iii) Results

All Hb could be removed from the samples using CM-sephadex beads at pH 5.5, 6.0 and 6.5 but no free haem could be detected by RPHPLC in the samples to which free haem had been added. DEAE-sephadex beads failed to achieve complete Hb removal.

Hence, this method of endogenous Hb removal was not viable due to the additional loss of free haem.

5.9.7 Separation of Haem and Hb by Density Centrifugation

Density centrifugation was examined as a possible means for removal of endogenous Hb to facilitate the use of exogenous haem as internal standard.

(i) Principles of Density Centrifugation

Lymphocyte separation medium (L.S.M.) (Pharmacia) is an aqueous solution of ficoll (Pharmacia Fine Chemicals) and sodium metrizoate commonly used for the separation of blood components according to density. The density (1.077 $\pm$ 0.001g/ml) may be altered by the addition of a salt solution.

(ii) Experimental

(1) L.S.M. (4.0ml) preparations of various densities were added to 10ml capacity glass centrifuge tubes:

% L.S.M.	% NH_4Cl (0.15M)
100	0
95	5
90	10
85	15
80	20
75	25
70	30

(2) Sonicated blood in incubation medium (2ml), haem (5μM) in incubation medium (2ml) or haem (5μM) plus sonicated blood in incubation medium (2ml) were applied to duplicate tubes at each density.

(3) The tubes were then spun at 400g (1500rpm) for 30 minutes in a refrigerated centrifuge.

(iii) Results

In each case, with the exception of 100% L.S.M., separation of haem and Hb occurred. Haem was recovered by diluting the L.S.M. followed by centrifugation. Haem was resuspended in 2ml of ammonium acetate (1M), pH7, then passed through an activated C2 'Bond-Elut' column prior to isolation by RPHPLC.

In each case, all Hb was removed. However, the recovery of haem in those tubes to which free haem had been added was poor and very variable, reaching a maximum of 32% with L.S.M : NH_4Cl (0.15M) (80:20) ($\bar{x}$ = 21%, S.D. = 7.5, C.V. = 30, n = 5).

Density centrifugation was therefore abandoned as a means of separating carrier haem and Hb because the recovery of haem was unacceptably low and inconsistent.

5.9.8 Mitochondrial Preparation of Bone Marrow

Mitochondrial preparations would provide a Hb-free deposit for the measurement of enzyme activity to which cold carrier haem could be added to assess recovery. The use of mitochondrial preparations has often been favoured in enzyme assays and may provide higher activity than a crude tissue

preparation.

A method for preparing Hb-free mitochondria was developed. The aim was to determine the ultracentrifuge setting required to separate Hb from cell debris. A concentrated red cell pellet of peripheral blood (0.25ml) was used in each case.

(i) Experimental

(1) Six cell pellets were sonicated (3 x 5' at 15 microns) in 1ml of culture medium in a 6ml capacity centrifuge tube to release Hb.

(2) The tubes were filled with culture medium then spun in a refrigerated ultracentrifuge (Beckman, model L2-65B, rotor head 40.3) at 20,000rpm for 10 minutes.

(3) The culture medium was removed.

(4) Two pellets were resuspended in 1ml of ferrochelatase incubation medium then extracted into acidified methylethyl ketone and treated as for a ferrochelatase assay through to the RPHPLC stage.

(5) Of the remaining four pellets, all were resuspended in culture medium; two were centrifuged once more at 20,000 rpm (10 minutes) and two were centrifuged twice more at 20,000 rpm (10 minutes).

(6) These pellets were then treated as described in step (4).

No free haem was added to any of the samples such that any free haem detected by RPHPLC would indicate its release from Hb upon treatment with 0.5ml of 1.0M HCl (1M).

(ii) Results

No. of Spins on Ultracentrifuge	Area of Haem on HPLC
1	231,101
1	247,529
2	39,887
2	34,056
3	--
3	—

Complete removal of Hb was achieved by three spins at 20,000 rpm (10 minutes). Under these circumstances it would be possible to add a known quantity of exogenous haem to each sample to assess haem recovery.

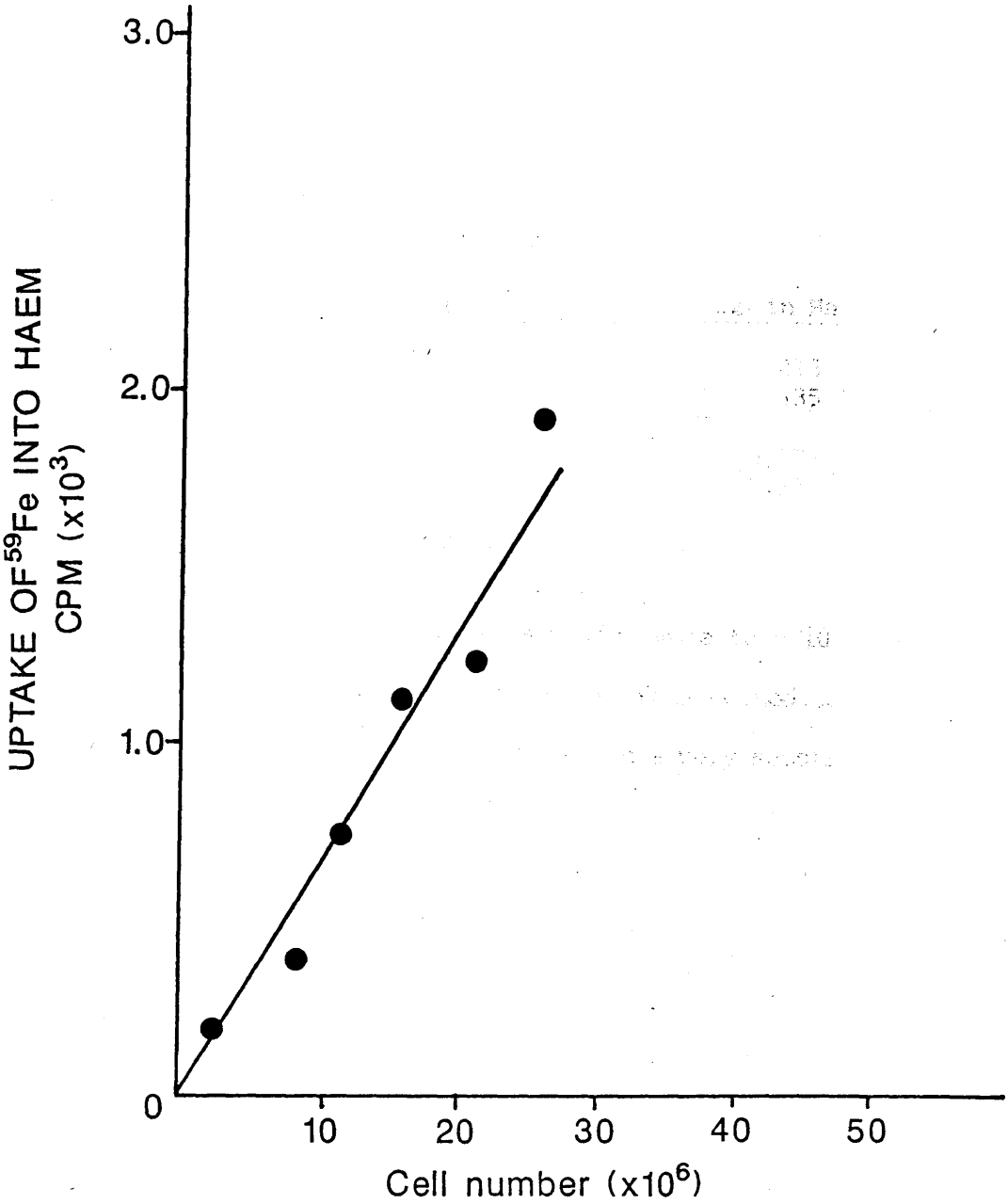
(iv) Linearity of Ferrochelatase Activity in Mitochondrial Preparations with Cell Number

Bone marrow mitochondrial preparations were made at cell numbers ranging from 5×10^6 - 30×10^6 and assayed for enzyme activity. Haem formation was plotted as a function of cell number (Figure 27) and the reaction was found to be linear to a cell number of 25×10^6. Similar results were also obtained from a second experiment.

The results were similar to those for whole untreated bone marrow (Section 5.8.8) where linearity of enzyme activity was maintained to a cell number of 30×10^6. However enzyme activity was significantly less in mitochondrial preparations. This observation was further investigated.

(v) Loss of Ferrochelatase Activity During Mitochondrial Preparation

Bone marrow from the same individual was divided into four



NOTE :
Each point represents the mean of duplicate observations. Mitochondrial preparations were made prior to the assay.
Assayconditions are described in the text.

FIGURE 27

UPTAKE OF ^{59}Fe INTO HAEM AS A FUNCTION OF CELL NUMBER.

cell pellets of 20 x 10^6 cells. Two pellets were placed on ice whilst mitochondrial preparations were made from the remaining two. The four samples were sonicated (3 x 5' at 15 microns) in incubation medium then assayed for ferrochelatase activity.

Sonicated Sample	cpm in Haem
Mitochondrial prep.	418 *
	335 *
Whole bone marrow	5,573
	5,538

(* Corrected to 100% recovery)

Whole bone marrow had an activity more than 10 times that of a mitochondrial preparation. Thus, this method for removing Hb from bone marrow samples resulted in a very substantial loss of ferrochelatase activity.

Freeze-thawing was examined as a possible means of reducing the loss of enzyme activity evident in mitochondrial preparations. Bone marrow from a second individual which had been stored at -80°C for several weeks was prepared in the same manner. The samples were however freeze-thawed (x 3) in incubation medium : glycerol (80:20 vol/vol, 0.85ml) prior to assay as an alternative to sonication.

Freeze-thawed Sample in Presence of Glycerol	cpm in Haem
Mitochondrial prep.	345 *
	401 *
Whole bone marrow	1,665
	1,580
	1,972
	1,594

(* Corrected to 100% recovery)

These results showed that for samples prepared by freeze-thawing in the presence of glycerol, the activity in whole bone marrow was 4-5 times that of mitochondria.

The preparation of mitochondria for assay resulted in such a dramatic loss of ferrochelatase activity that this was no longer regarded as a feasible means of removing endogenous haem. Centrifugation of samples at 40,000 rpm instead of 20,000 rpm did not reduce the loss of activity.

In whole bone marrow the activity detected in the second marrow sample was significantly less (69%) than that of the first sample. This discrepancy was too large to be explained by the expected natural variation in enzyme activity between normal individuals. It therefore seemed likely that this could be attributed to differences in either:-

(1) the method of sample preparation -
i.e. sonication in the absence of glycerol versus freeze-thawing in the presence of glycerol.

(2) the instability of the enzyme during storage at -80°C - the first bone marrow sample was assayed fresh while the second sample had been stored for several weeks at -80°C.

These possibilities were examined experimentally. Section 5.10 describes the investigation to find the most suitable sample preparation method prior to enzyme assay. Section 5.14 addresses the question of enzyme stability in an attempt to find the most suitable means of bone marrow storage prior to assay.

(vi) Rejection of Mitochondrial Preparation as a Useful Sample Preparation Method

No alternative remained but to assay bone marrow and

erythroblast activity directly without making a mitochondrial preparation. As a means of removing mature erythrocytes from aspirated bone marrow cells, the samples were centrifuged at 3000 rpm for 5 min. The "buffy coat" (i.e. nucleated cells) was then carefully removed leaving most mature erythrocytes in the cell pellet.

5.9.9 Chosen Method for Assessing Haem Recovery

Under circumstances whereby large quantities of endogenous haem were present, individual sample recoveries could not be exactly determined. Following these extensive investigations the decision was taken to accept the final percentage recovery as 75% (± 7.6%) (as determined in Section 5.9.2(i)).

5.10 SAMPLE PREPARATION

Since the method of sample preparation was originally optimised for the measurement of ALA synthase activity in bone marrow and may not represent the optimum procedure for measurement of ferrochelatase activity, other methods of sample preparation were examined i.e.

(1) sonication

(2) freeze-thawing

Note: manual homogenisation was not possible as the cell pellets were very small.

5.10.1 Comparison of Freeze-thawing and Sonication

Several samples (2×10^7) cells were prepared for assay either by freeze-thawing or by sonication in the presence or

absence of glycerol (20% v/v). The samples were assayed for ferrochelatase activity. The sample with the largest haem peak area as determined by HPLC was taken to represent 100% recovery. All other sample recoveries were corrected to this value.

(i) Experimental

Freshly prepared cell pellets were suspended in culture medium (1.0ml) in an ultracentrifuge tube (6ml capacity):

(a) Freeze-thawing

The tubes were packed in dry ice/methanol for 5 minutes, then placed in hot water (70°C) for 5 minutes. This process was carried out in triplicate.

(b) Sonication

Sonication was carried out using a $^1/_8$" titanium probe set to 15 microns in air dampened to 12 microns in culture medium (3 pulses of 5 seconds).

The tubes were filled with culture medium and a mitochondrial preparation made to remove endogenous Hb. The samples were assayed in duplicate for ferrochelatase activity and a known quantity of haem was added at reaction termination as internal standard.

(ii) Results

Preparation of Sample	Corrected cpm in Haem
Sonication in culture medium	415 335
Sonication in culture medium/20% glycerol	834 1,007
Freeze-thawed in culture medium	960 1,119
Freeze-thawed in culture medium/20% glycerol	1,550 1,602

The results demonstrated that:

(1) Freeze-thawed samples had ferrochelatase activities 2-3 times greater than sonicated samples.

(iii) Inclusion of Glycerol During Sample Preparation

(2) The presence of 20% glycerol (v/v) during sonication resulted in ferrochelatase activities similar to freeze-thawed samples.

(3) The presence of 20% glycerol during freeze-thawing resulted in ferrochelatase activities 50% greater than those in the absence of glycerol.

5.10.2 Optimum Procedure for Sample Preparation

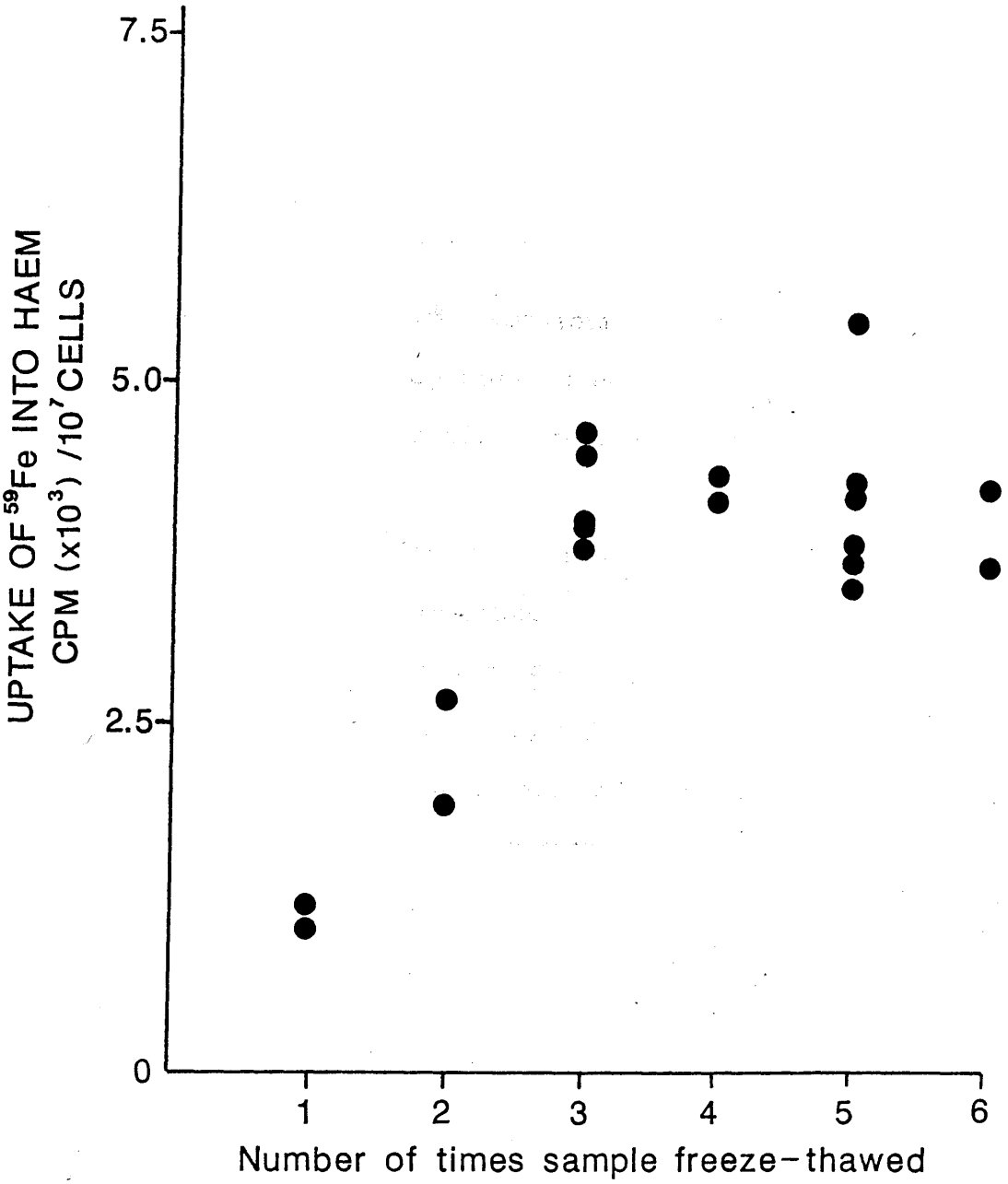
Freeze-thawing of bone marrow samples in the presence of 20% glycerol provided maximum ferrochelatase activity (Section 5.10.1). To optimise this procedure it was necessary to establish the most suitable number of times to freeze-thaw the sample.

(i) Experimental

Several fresh bone marrow samples (10^7 cells) were freeze-thawed in incubation medium in the presence of 20% glycerol various numbers of times, ranging from 1 to 6. The cells were then assayed for enzyme activity.

(ii) Results

Haem formation was plotted as a function of freeze-thaw episodes (Figure 28). The optimum number of times to freeze-thaw was three.



NOTE :
Each point represents individual observations.
Assay conditions are described in the text.

FIGURE 28

UPTAKE OF ^{59}Fe INTO HAEM AS A FUNCTION OF THE NUMBER OF TIMES THE SAMPLE WAS FREEZE-THAWED.

5.11 NON-ENZYMATIC HAEM FORMATION

Haem formation has been demonstrated in the absence of ferrochelatase (Granick and Mauzerall, 1958; Kassner and Walchak, 1973; Heikel, Lockwood and Rimington, 1958; Tokunaga and Sano, 1972).

Although non-enzymatic haem formation has characteristics which parallel those reported for enzymatic haem formation, non-enzymatic and enzymatic haem formation differ in several respects:

(1) The optimum pH of haem formation normally differs for the non-enzymatic and enzymatic reaction.

(2) Ferrochelatase is activated by GSH and ascorbate (Taketani and Tokunaga, 1981; Yoneyema et al, 1965) but the non-enzymatic reaction is not observed under such conditions. The formation of an active iron-thiol complex such as that formed with DTT, cysteine or 2-mercaptoethanol seems to be more essential for non-enzymatic synthesis (Taketani and Tokunaga, 1982; Kassner and Walchak, 1973).

(3) Non-ionic detergents decrease non-enzymatic haem formation but ferrochelatase can react with completely solubilised porphyrins in the presence of non-ionic detergents.

(4) The enzyme itself catalyzes the formation of haem in mitochondria since heat and trypsin treatment completely destroy activity (Taketani and Tokunaga, 1981; Yoneyema et al, 1962).

(i) Experimental

The reaction of iron with protoporphyrin IX was studied in aqueous solution in the absence of cellular material.

(ii) Results

Non-enzymatic haem formation did not occur supporting the view that non-enzymatic and enzymatic syntheses are independent. This lack of non-enzymatic haem formation may partly be due to the presence of ascorbate as reducing agent and the absence of exogenous fatty acids.

Routinely, control tubes were included alongside each set of enzyme assays. These contained all the requirements for enzyme assay with the exception of cells.

5.12 THE USE OF NON-IONIC DETERGENTS

A non-ionic detergent such as tween-20 or triton X-100 is frequently included in the incubation medium for ferrochelatase activity. It is believed that the presence of a detergent may:

(1) prevent porphyrin aggregation;

(2) inhibit the non-enzymatic formation of haem;

(3) release the enzyme from the mitochondrial membrane.

For example, Bloomer and Morton (1982) incubated a liver tissue preparation with tween-20 for 20-30 minutes on ice before commencing the enzyme reaction. This resulted in a more homogeneous suspension and increased the activity in the sample.

In the assay of ferrochelatase activity in bone marrow it was decided following experimentation to exclude detergent from the reaction mixture because -

(1) Triton X-100 (reagent grade, BDH) used over the concentration range 0.01%-2% markedly reduced haem recovery at sorbent extraction.

(2) Under the established conditions non-enzymatic haem formation did not occur thereby making inhibition unnecessary.

(3) Freeze-thawing in the presence of glycerol (20% v/v) was a suitable means of providing homogeneous suspension with ferrochelatase available for enzyme assay.

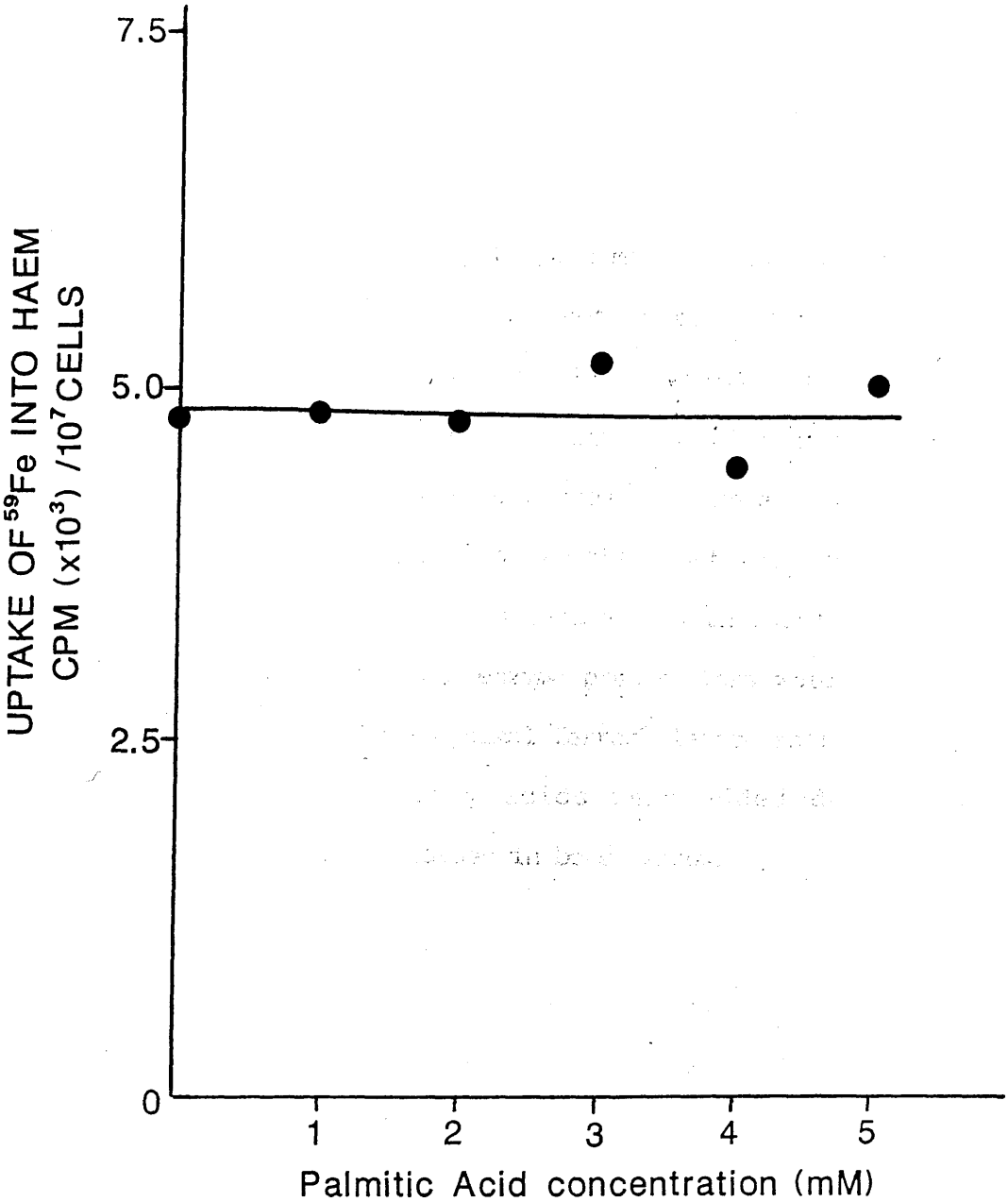
High concentrations of detergent may have inhibited ferrochelatase activity. In the presence of non-ionic detergents, the porphyrin nucleus is surrounded by the micelle and at high concentrations is unable to come into contact with the iron (Phillips, 1967).

5.13 THE USE OF FATTY ACIDS

It has been reported that lipids activate mitochondrial ferrochelatase (Sawada et al, 1969; Simpson and Poulson, 1977). This was clearly demonstrated for ferrochelatase purified from rat liver mitochondria. Fatty acids markedly increased enzyme activity while phospholipids were less effective (Taketani and Tokunaga, 1981). It has been shown that the addition of fatty acids but not neutral lipids and phospholipids causes non-enzymatic haem formation (Taketani and Tokunaga, 1982). It is generally believed that fatty acids interact with the porphyrin and aid haem formation via increasing electrostatic effects.

Experimental

Bone marrow samples were incubated with varying concentrations of palmitic acid (hexadecanoic acid, Sigma) in the range 0-5mM (Figure 29) to examine the effect of fatty acids on ferrochelatase activity. Palmitic acid was chosen because a ten-fold stimulation of rat liver ferrochelatase



NOTE :
Each point represents the mean of duplicate observations.
Assay conditions are described in the text.

FIGURE 29

UPTAKE OF ^{59}Fe INTO HAEM AS A FUNCTION OF PALMITIC ACID CONCENTRATION.

activity had been demonstrated with 2mM palmitic acid (Taketani and Tokunaga, 1981). Fatty acids of shorter chain length were less effective.

The results obtained with bone marrow, however, indicated that palmitic acid (0-5mM) did not increase ferrochelatase activity beyond that of the control. It would seem likely that the bone marrow preparation contained sufficient endogenous quantities of fatty acids to sustain enzyme activity at the optimum level. It is noteworthy that most workers who have observed an increase in enzyme activity with fatty acids have studied partially purified enzyme preparations which may lack fatty acids required for optimal ferrochelatase activity.

Therefore, no fatty acids were added during the measurement of ferrochelatase in bone marrow.

5.14 STABILITY OF FERROCHELATASE

5.14.1 Examination of Storage Methods for Human Bone Marrow

The stability of ferrochelatase was examined. Pellets of normal human bone marrow (2×10^7 cells) were stored under different conditions, then assayed for ferrochelatase activity.

(1) Two cell pellets of fresh bone marrow were assayed directly.

(2) Two cell pellets were stored for 3 days at $-80^{o}C$.

(3) Two cell pellets were stored for 3 days at $-20^{o}C$.

(4) Two cell pellets were stored overnight in 1.0ml of culture medium at $4^{o}C$.

Duplicate samples were assayed from each pellet.

Results

The results are expressed in Table 15.

		cpm in haem	average cpm in haem
(1)	Fresh bone marrow	13,720 13,796 14,000 13,976	13,873
(2)	Bone marrow stored at -80°C	6,047 7,842 7,763 7,822	7,369
(3)	Bone marrow stored at -20°C	6,677 6,622 6,096 5,692	6,272
(4)	Bone marrow stored at 4°C (overnight)	13,978 13,851 14,005 14,011	13,961

TABLE 15

EFFECTS OF STORAGE METHOD ON FERROCHELATASE ACTIVITY

These results demonstrate that bone marrow stored frozen at either -20°C or -80°C loses up to 50% ferrochelatase activity, while overnight storage at 4°C leads to no such loss. This was verified using bone marrow from a further five individuals.

In the light of these results, all bone marrow samples whether whole or fractionated (Chapter 2) were stored overnight at 4°C, then assayed for ferrochelatase activity the following day.

5.14.2 The Effect of Temperature on Ferrochelatase Activity

Enzyme stability during the period of incubation at 37°C required to achieve white cell lysis was examined.

Two samples of whole unfractionated bone marrow (2×10^7 cells) were obtained from several patients. One sample was incubated at 37°C for 30 minutes prior to enzyme assay (equivalent to the incubation period with the monoclonal antibody TG-1) (Chapter 2, Section 2.5). No loss of activity was detected in this sample relative to the sample maintained at 4°C.

Ferrochelatase was not therefore prone to the loss of activity which was observed for ALA synthase under the same conditions (Chapter 3, Section 3.10).

5.15 SUMMARY OF ASSAY METHOD FOR THE MEASUREMENT OF FERROCHELATASE ACTIVITY IN HUMAN BONE MARROW

5.15.1 Materials

The following chemicals were purchased from Sigma Chemical Company - tris HCl (reagent grade); tris base (reagent grade);

ammonium acetate (reagent grade); tetrabutylammonium hydrogen sulphate (TBAHS) (reagent grade).

Ferrous chloride ($FeCl_2$) (reagent grade); ascorbic acid (reagent grade); trichloroacetic acid (TCA) (reagent grade) and ammonium hydroxide (reagent grade) were purchased from BDH.

Protoporphyrin IX was purchased from Porphyrin Products Inc., U.S.A., $^{59}FeCl_3$ from Amersham International in 0.1M HCl, 100μCi ml^{-1}; glacial acetic acid (reagent grade) from May and Baker and methanol (HPLC grade) was purchased from Rathburn Chemicals.

5.15.2 **Methods**

Assays were carried out in glass tubes (methylethyl ketone damaged plastic) which were then discarded to avoid contamination problems since iron adheres very strongly to glassware.

Ascorbic Acid

0.25g ascorbic acid in incubation medium (10.0ml)

(1) Sample Preparation

Cells were either assayed fresh for ferrochelatase activity or after storage overnight at 4°C in culture medium (Appendix I).

Incubation medium (0.85ml of tris buffer (50mM), pH 8.3 / glycerol, 80:20 vol/vol) was added to cell pellets. The samples were then freeze-thawed (x 3) via alternate submersion in dry ice/methanol and hot water (70°C).
Two aliquots of 0.4ml were taken from each tube for assay. Linearity has been demonstrated for 1-30 x 10^6 bone marrow cells per assay.

(2) Assay Mixture

0.02ml $^{59}FeCl_3$ (~2μCi, ~2nmoles)

0.03ml $FeCl_2$ (~ 8nmoles) in ascorbic acid

Radioactive and non-radioactive iron were thoroughly mixed.

The following were then added:

0.50ml incubation medium

0.40ml sample in incubation medium.

The reaction was initiated by the addition of 0.05ml protoporphyrin (100nmoles) in incubation medium.

(3) Enzyme Incubation

The tubes were sealed under N_2, thoroughly mixed, then incubated at 37°C in the dark for 20 minutes.

(4) Reaction Termination

The reaction was terminated by the addition of 0.5ml HCl (1M).

(5) Blank

(a) As for samples, but reaction terminated at 0 time

or

(b) As for samples, but no cells added.

(6) Methylethyl Ketone Extraction of Haem

(a) Methylethyl ketone (1.5ml) was added to each sample.

(b) The samples were mixed, then centrifuged at 3,000rpm for 5 minutes to allow phase separation.

(c) The organic phase was removed.

(d) Extraction was repeated twice more, using 1.0ml of methylethyl ketone each time.

(e) The organic phases were pooled for each sample, then evaporated to dryness under N_2 at 37°C.

(7) Sample Reconstitution

Samples were reconstituted in ammonium acetate (1M), pH 7.0, (2.0ml) by the sequential addition of 0.3ml ammonium hydroxide (5M), 1.7ml ammonium acetate (1M), pH 7.0 and 0.15ml glacial acetic acid.

(8) Partial Isolation of Haem by Sorbent Extraction

(a) Activation of C2 'Bond-Elut' Columns

Columns were activated by methanol (2.0ml) followed by ammonium acetate (1M), pH 7.0 (5.0ml).

(b) Application of Samples

Reconstituted samples were applied to activated columns at a flow rate of 1.0ml min^{-1}. Columns were washed with ammonium acetate (1M), pH 7.0, (2.0ml). Haem was then eluted in 3 volumes (1.0ml) of methanol: ammonium acetate (1M) (95:5) at pH 6.9.

(c) Sample Concentration

Samples were evaporated to dryness under N_2 at 37°C. At this stage samples could be stored overnight at 4°C under N_2.

(9) Sample Reconstitution

Samples were reconstituted in 0.2ml methanol: ammonium acetate (1M), (95:5), pH 6.9. 0.05ml was then taken for RPHPLC.

(10) RPHPLC Separation of Haem, Iron and Protoporphyrin

(a) Mobile Phase

Methanol: water (83:17)

Acetic acid (10mm)

TBAHS (2mM)

pH' 6.5 (actual pH 7.0)

Flow rate: 1.0ml min^{-1}

(b) Detector

Measuring wavelength = 398nm

AUFS = 0.1

(c) Column

Apex octadecyl 15cm x 4.6 I.D., particle size 5μM.

(d) Fraction Collector

Fractions were collected at 0.5 min intervals at 3.5 - 9.5 minutes following sample injection.

(11) Calculation of Results

Results are expressed as:

$$\text{pmoles haem formed}/10^6 \text{ cells}/20 \text{ minutes} = \frac{\text{corrected cpm in haem}}{\text{cpm/pmole } ^{59}\text{Fe substrate}}$$

(12) Recoveries

Recoveries were calculated on an extraction efficiency of 75% (± 7.6%). No suitable method was found for the incorporation of an internal standard.

PATIENT STUDIES

CHAPTER 6

ALA SYNTHASE AND FERROCHELATASE ACTIVITIES DURING NORMOBLASTIC ERYTHROPOIESIS

6 ALA SYNTHASE AND FERROCHELATASE ACTIVITIES DURING NORMOBLASTIC ERYTHROPOIESIS

6.1 AIMS OF THE STUDY

The aim of the work described in this chapter was to provide a measure of ALA synthase and ferrochelatase activities in whole bone marrow and isolated human erythroblasts. The effect of differentiation on enzyme activity during normoblastic erythropoiesis was examined. Specifically, it was intended to resolve the conflict as to whether ALA synthase and ferrochelatase appear simultaneously (Beaumont et al, 1984) or sequentially (Sassa, 1976) during normal erythroid differentiation. It was hoped also to determine if either ALA synthase (Levere and Granick, 1965) or ferrochelatase (Sassa, 1976) might be rate limiting for erythroid haem biosynthesis.

The multiple roles for erythroblast haem beyond its specific complexing with globin have been discussed in Chapter 1. A clear understanding of the ability of erythroid cells to synthesise haem during their development is then an essential prerequisite to gaining an insight into the manner in which intracellular free haem may be involved in the regulation of globin synthesis, iron metabolism, haem metabolism and differentiation.

6.2 EXPERIMENTAL

6.2.1 Patients Studied

Bone marrow was obtained from 15 patients, all of whom showed normoblastic erythroid hyperplasia in the marrow. Erythroid hyperplasia was required to provide adequate erythroid cell numbers for fractionation. All patients were

shown to have normal haematinic levels (B_{12}, folate and ferritin-ferritin as a measure of iron status). In all cases transferrin saturation was >30% ensuring adequate iron availability to the erythroblasts. For complete patient details see Appendix 2.

One patient with erythroid hyperplasia taking high dose steroids (prednisolone 60mg/day) was also examined. The results from this patient are presented separately.

6.2.2 Enzyme Measurements

ALA synthase and ferrochelatase activities were first measured as described (Chapters 3 and 5 respectively) in whole unfractionated bone marrow to allow comparison with previous assays which have only measured enzyme activity in whole marrow (Bottomley et al, 1968; 1973; Aoki et al, 1974; Konopka and Hoffbrand, 1979; Manabe et al, 1982; Tanaka and Ohta, 1972; Stavem et al, 1985). ALA synthase and ferrochelatase activities were then measured in each of the four percoll fractions obtained from bone marrow (Chapter 2). Fractions 1 to 4 contained purified age-matched erythroblasts. The differentiation status of the erythroblasts in each fraction has been described (Chapter 2, Table 1). The least dense erythroblasts were recovered in fraction 1. Light microscopy and differential erythroblast counting showed that most pro and early erythroblasts (E_1 cells) were found in this fraction. Fraction 2 contained predominantly intermediate erythroblasts (E_2 cells) with less E_1 cells. Fraction 3 contained E_2 cells with some late erythroblasts (E_3 cells). Fraction 4 (i.e. the

most dense fraction) contained predominantly E_3 cells with less E_2 cells. Measurement of enzyme activity in the fractions therefore provided a measure of haem synthesis during erythropoietic development.

Fractionated samples from 10 marrows were stored in duplicate at -80°C during the developmental period of the ferrochelatase assay. All samples were assayed for ALA synthase activity. As it was shown that, unlike ALA synthase, ferrochelatase was unstable at -80°C (Chapter 5, Section 5.14) these samples were unsuitable for ferrochelatase measurement. Ferrochelatase activity was measured in the fresh non-frozen erythroblast fractions obtained from the other 5 marrow samples.

6.3 RESULTS

The results for whole bone marrow haem enzyme activity are expressed as enzyme activity per 1 x 10^6 erythroblasts per hour. The results for fractionated samples are similarly expressed per 1 x 10^6 erythroblasts per hour and are related to fraction number (and hence differentiation status).

6.3.1 ALA Synthase Activity in Whole Human Bone Marrow

ALA synthase activity was measured in whole unfractionated bone marrow (10 x 10^6 cells) from each patient to establish a normal range of enzyme activity (Table 16). This served only as a crude indicator of erythroblast enzyme activity as the contribution made by the white cell populations in the bone marrow and the effects of erythroblast differentiation status on enzyme activity were not considered (Chapter 2, Section

	ALA SYNTHASE ACTIVITY (p moles ALA/10^6 erythroblasts/hour)
WHOLE BONE MARROW	608 ± 334

	FERROCHELATASE ACTIVITY (p moles haem/10^6 erythroblasts/hour)
WHOLE BONE MARROW	164 ± 50

The results are expressed as $\bar{x} \pm 1$ S.D. where n = 15 (ALA synthase activity) and n = 4 (ferrochelatase activity).

TABLE 16

THE NORMAL RANGES OF ALA SYNTHASE AND FERROCHELATASE ACTIVITIES IN WHOLE UNFRACTIONATED HUMAN BONE MARROW

2.2). However, since other workers have measured ALA synthase activity in whole bone marrow, this enabled direct comparisons of enzyme activity to be made. The ALA synthase activity detected in whole bone marrow (608 $\pm$ 334 pmoles ALA/10^6 erythroblasts/hour, $\bar{x} \pm 1$ S.D., n = 15) was similar to that reported by Fitzsimons et al (1986).

Furthermore, measurement of ALA synthase activity in whole unfractionated bone marrow (10 x 10^6) following preincubation for 30 minutes at 37°C permitted assessment of the effect of incubation on enzyme activity. ALA synthase was prone to a temperature-dependent inactivation at 37°C due to the dissociation of the enzyme cofactor, PLP (Chapter 3, Section 3.10). This loss of activity during incubation at 37°C (30 minutes) was routinely measured. The loss of enzyme activity was found to be consistent at 45% $\pm$ 14%.

6.3.2 Ferrochelatase Activity in Whole Human Bone Marrow

Ferrochelatase activity was also measured in whole unfractionated bone marrow (20 x 10^6 cells) from four samples to establish a normal range of enzyme activity (Table 16). The activity detected (164 $\pm$ 50 pmoles haem/10^6 erythroblasts/hour, $\bar{x} \pm 1$ S.D., n = 4) was similar to that reported by Bottomley (1968) (112-300 pmoles haem/10^6 nucleated red cells/hour) and slightly higher than that reported by Tanaka and Ohta (1972) (80.6 $\pm$ 18.6 pmoles haem/10^6 erythroblasts/hour) in normal human bone marrow. Bone marrow ferrochelatase activities in much higher ranges have also been reported (Stavem et al, 1985) i.e. 4800-9420 and 16500-17100 pmoles/10^6 erythroid cells/hour

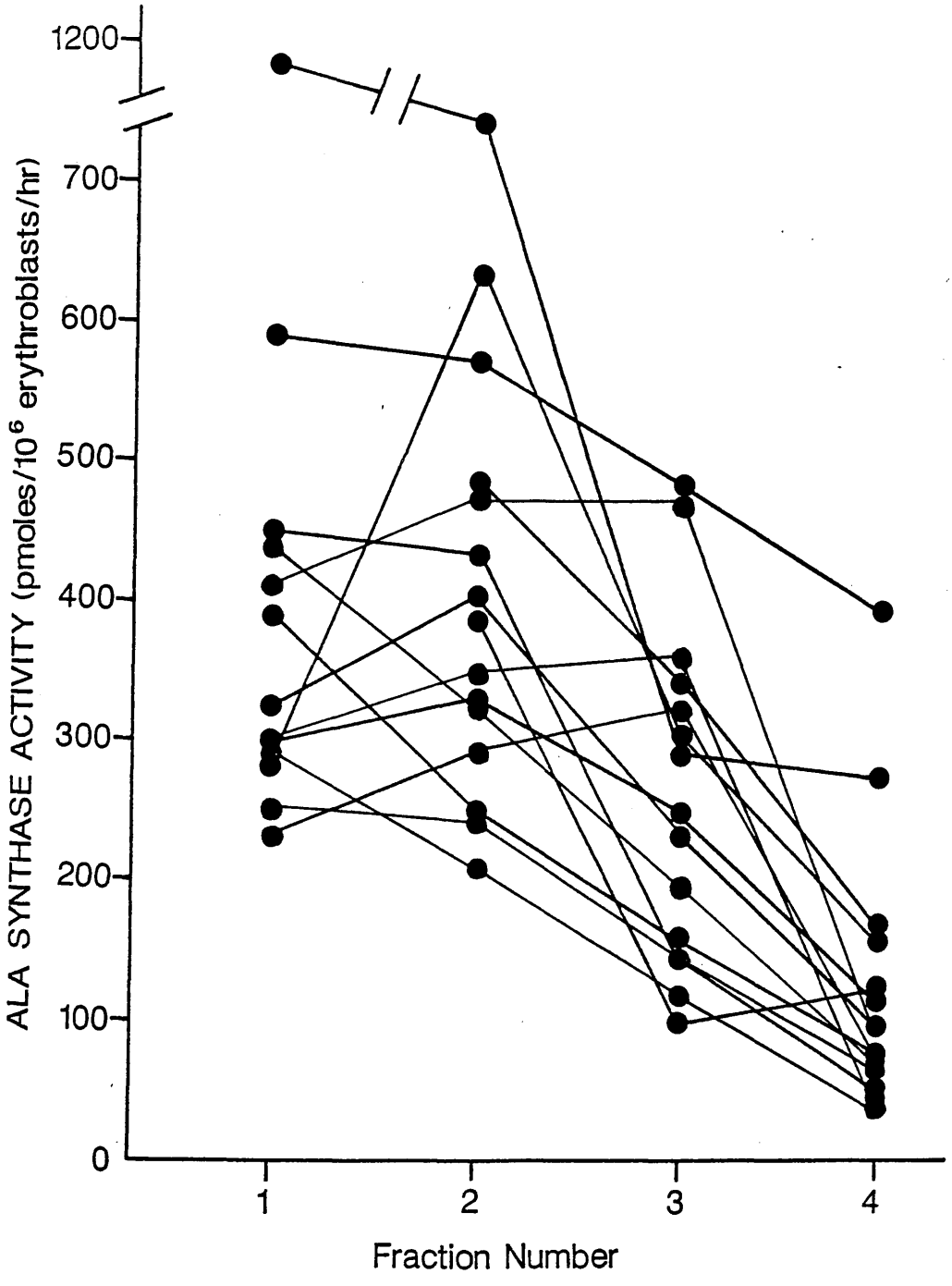
respectively. These seemingly high results were obtained using two different assay methods and may be explained by a lack of assay specificity. The first method measured the decline in fluorescence in the HCl-acid phase while the second attempted to isolate radiolabelled haem by organic solvent extraction alone. The latter method is now known to be ineffective (Chapter 5, Section 5.6.4). It is therefore unlikely that either method was a reliable measure of ferrochelatase activity.

Routine measurement of ferrochelatase activity in normal human bone marrow following preincubation for 30 minutes at 37^{o}C indicated that ferrochelatase did not show the temperature-dependent inactivation (Chapter 5, Section 5.14.2) as found with ALA synthase (Chapter 3, Section 3.10).

6.3.3 ALA Synthase Activity During Normoblastic Erythropoiesis

ALA synthase activity was maximal in the erythroblasts of percoll fractions 1 and 2 and was approximately four times that found in the late erythroblasts of percoll fraction 4 (Figure 30) ($p < 0.001$). Enzyme activity was high in pro and early erythroblasts (percoll fraction 1) and remained high as the cells differentiated towards intermediate erythroblasts (percoll fraction 2). Further differentiation towards the intermediate and late erythroblasts of percoll fractions 3 and 4 was associated with a marked decline in enzyme activity. This indicates that induction of ALA synthase activity occurs early during normoblastic erythroid differentiation and confirms the preliminary findings of Fitzsimons et al (1986).

Bone marrow reticulocytes were present in significant



NOTE :
Each point represents the mean of two observations.

FIGURE 30

ALA SYNTHASE ACTIVITY IN FRACTIONATED HUMAN ERYTHROBLASTS : NORMOBLASTIC ERYTHROPOIESIS

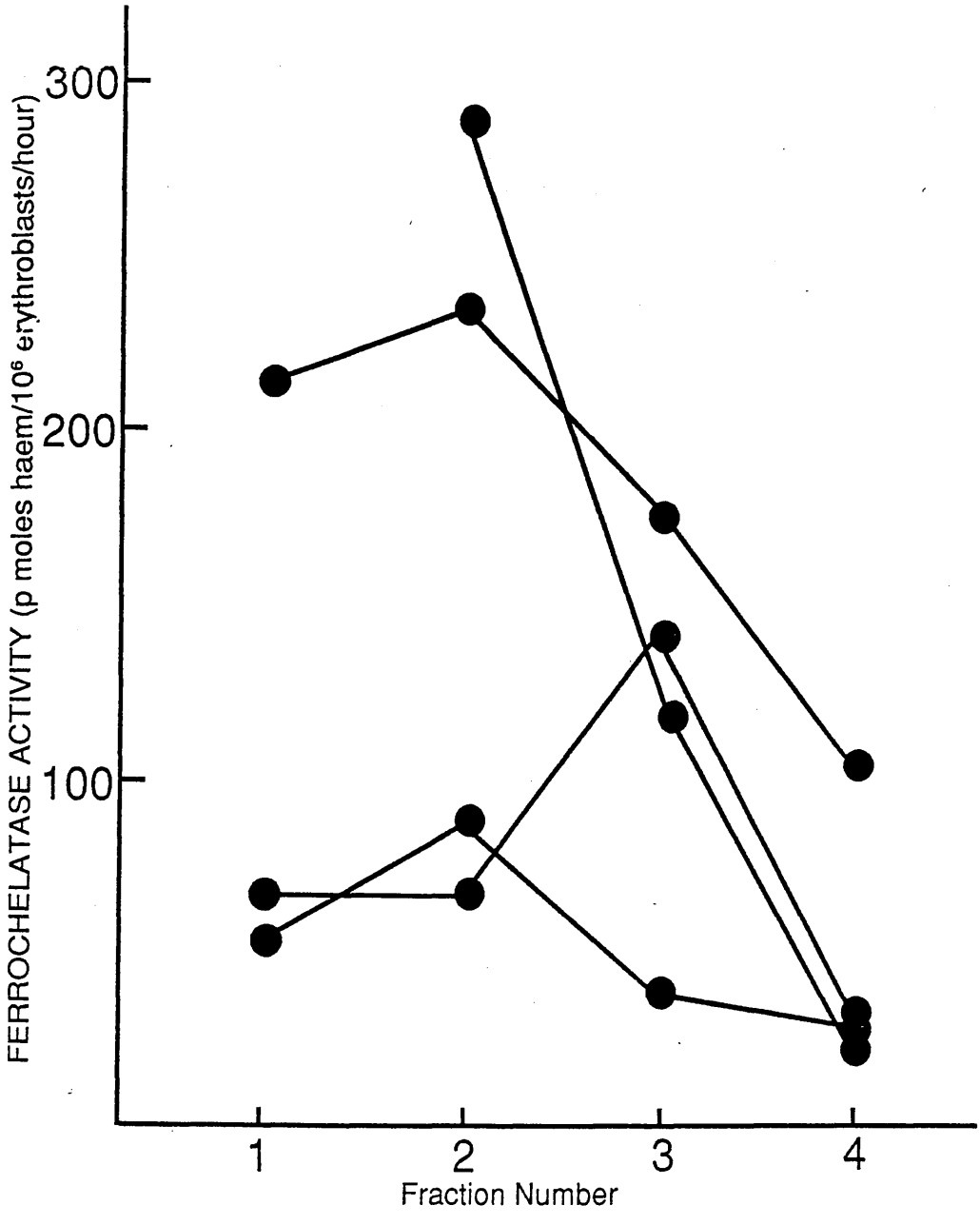
quantities in percoll fraction 4 and outnumbered E_3 cells by a ratio of > 10:1. Low levels of ALA synthase activity in this fraction relative to the activity found in reticulocyte free fractions 1 and 2, indicate that the enzyme activity present in reticulocytes may be several hundred fold lower than that of E_1 cells (Fitzsimons et al, 1986). The magnitude of this difference highlights the dangers involved in extrapolating results obtained from the study of haem synthesis in reticulocytes to that in erythroblasts.

Morphological examination clearly demonstrated that considerable ALA synthase activity was present in the cells of fraction 1 before the appearance of significant quantities of cytoplasmic Hb.

6.3.4 Ferrochelatase Activity During Normoblastic Erythropoiesis

The ferrochelatase activity observed in the different fractions in 4 patients with normoblastic erythroid hyperplasia is shown in Figure 31. In one patient insufficient erythroid cells were recovered in percoll fraction 1 to permit measurement of ferrochelatase activity. The reproducibility of the assay was impressive in every instance with enzyme activity of duplicate samples within 7.5% variation.

The recorded enzyme activity from the 4 patients showed considerable individual variation. The pattern of enzyme development in 3 of the patients showed a peak in enzyme activity in fractions (2 or 3) containing erythroid cells more mature than those found in fraction 1. In the fourth sample no enzyme activity was measured in fraction 1 because of the low



Note:
Each point represents the mean of two observations.

FIGURE 31

FERROCHELATASE ACTIVITY IN FRACTIONATED HUMAN ERYTHROBLASTS : NORMOBLASTIC ERYTHROPOIESIS

number of erythroblasts obtained from the percoll gradient. It was not therefore possible to determine which fraction contained peak ferrochelatase activity in this sample.

As it is difficult to draw conclusions from only 4 samples it was thought more worthwhile to examine enzyme activity in the individual patients in relation to their ALA synthase activity.

6.3.5 The Relationship Between ALA Synthase and Ferrochelatase Activities During Normoblastic Erythropoiesis

The relationship between ALA synthase and ferrochelatase enzyme activities was examined during normoblastic erythropoiesis in the four individuals described in the previous section.

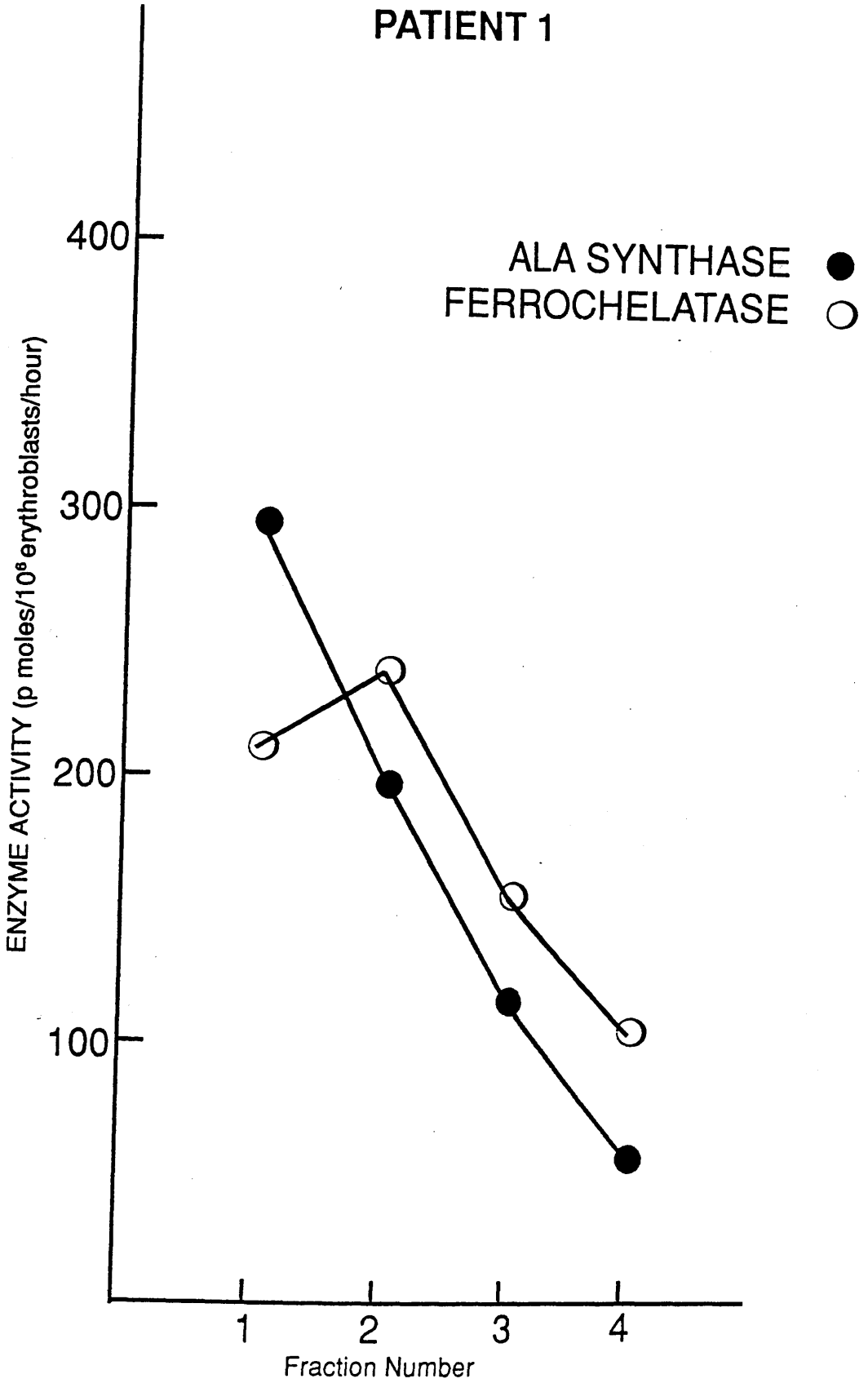
To permit comparison, consideration was given to two points. Firstly, the time scales of the enzyme incubations were considered. The ALA synthase assay incubation time was one hour whereas a 20 minute incubation period was used for ferrochelatase. However, as the ferrochelatase reaction demonstrated linearity over 20 minutes (Chapter 5, Section 5.8.3) it was possible to extrapolate the recorded enzyme activity to one hour. Secondly, it was estimated that the activity of ALA synthase would need to be approximately eight fold that of ferrochelatase as eight molecules of ALA are required to form each molecule of haem (Chapter 1, Section 1.3).

With these factors considered it was possible to demonstrate a close temporal relationship between ALA synthase and ferrochelatase activities during normoblastic

erythropoiesis (Figures 32-35). In 3 of the marrows examined, peak ferrochelatase activity was seen to develop later than peak ALA synthase activity (Figures 32-34). The results from the fourth individual (Figure 35) do not permit such a comparison as peak ferrochelatase activity cannot be judged. It is, however, clear that peak activity in this patient occurs either in the erythroblasts of fraction 2 or would have been found in the early cells of fraction 1 had sufficient cells been obtained at fractionation. It is, however, unlikely that peak activity would have been found in fraction 1 because no dpm were recovered from the few cells that were present in this fraction, thereby suggesting that their enzyme activity was low and that peak activity is indeed present in Fraction 2.

These results suggest that the development of ALA synthase and ferrochelatase activities during visually recognisable normoblastic erythropoiesis may be a sequential rather than a synchronous process (Sassa, 1976). In 3 of the 4 patients ALA synthase activity in fraction 1 and/or 2 is considerably higher than ferrochelatase activity in the same fractions. Nevertheless, the quantitative relationship between the two enzymes in most cases indicates an activity of ferrochelatase relative to ALA synthase which is several times greater than would in theory be required to cope with the estimated ALA production. It is not, therefore, possible to establish whether the delay in achieving peak ferrochelatase activity relative to ALA synthase is sufficient to support the argument that delayed development of ferrochelatase activity is limiting for haem biosynthesis during erythroid differentiation.

The fifth patient (Figure 36) had been on high dose

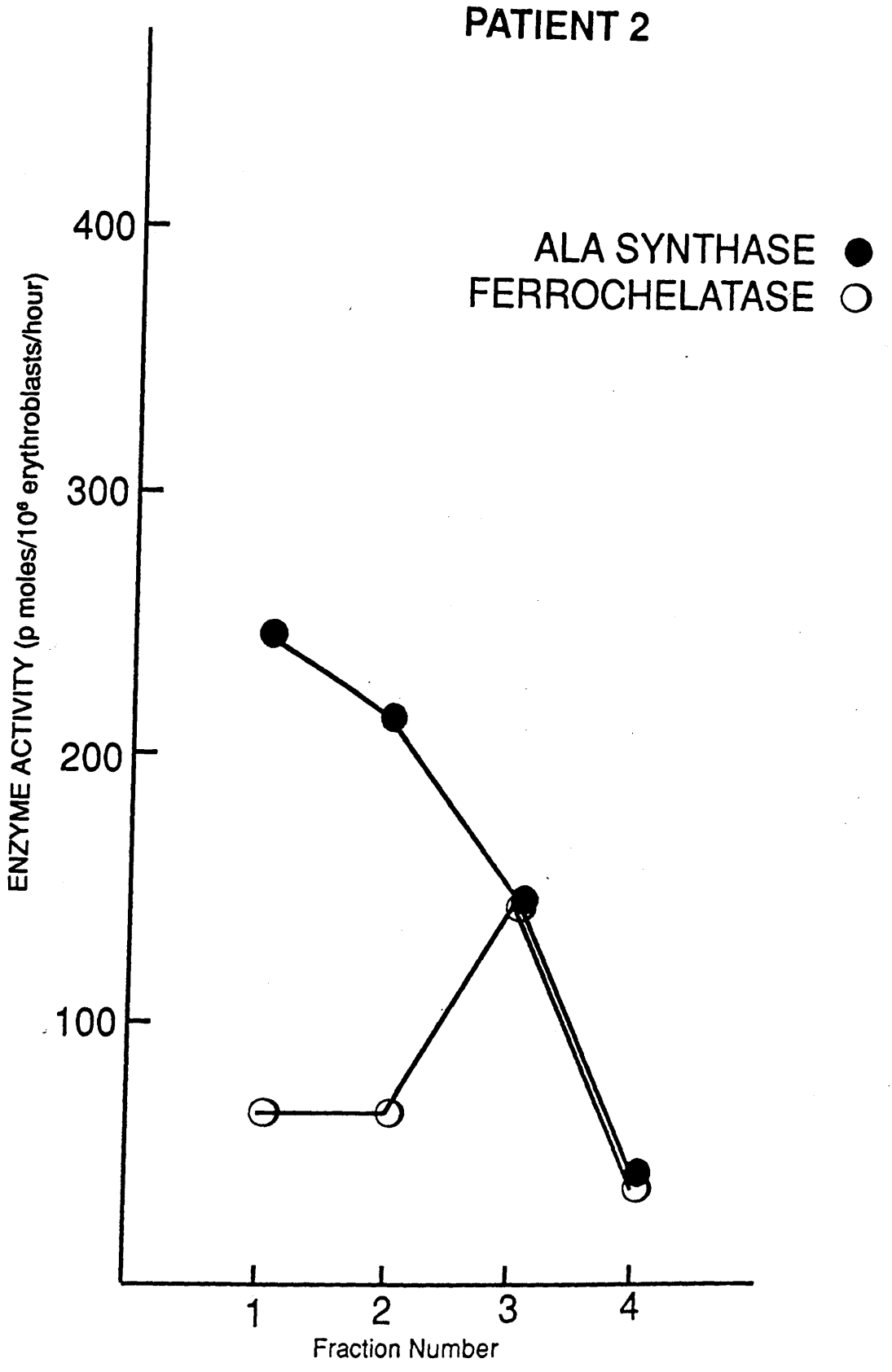


Note:

Each point represents the mean of two observations.

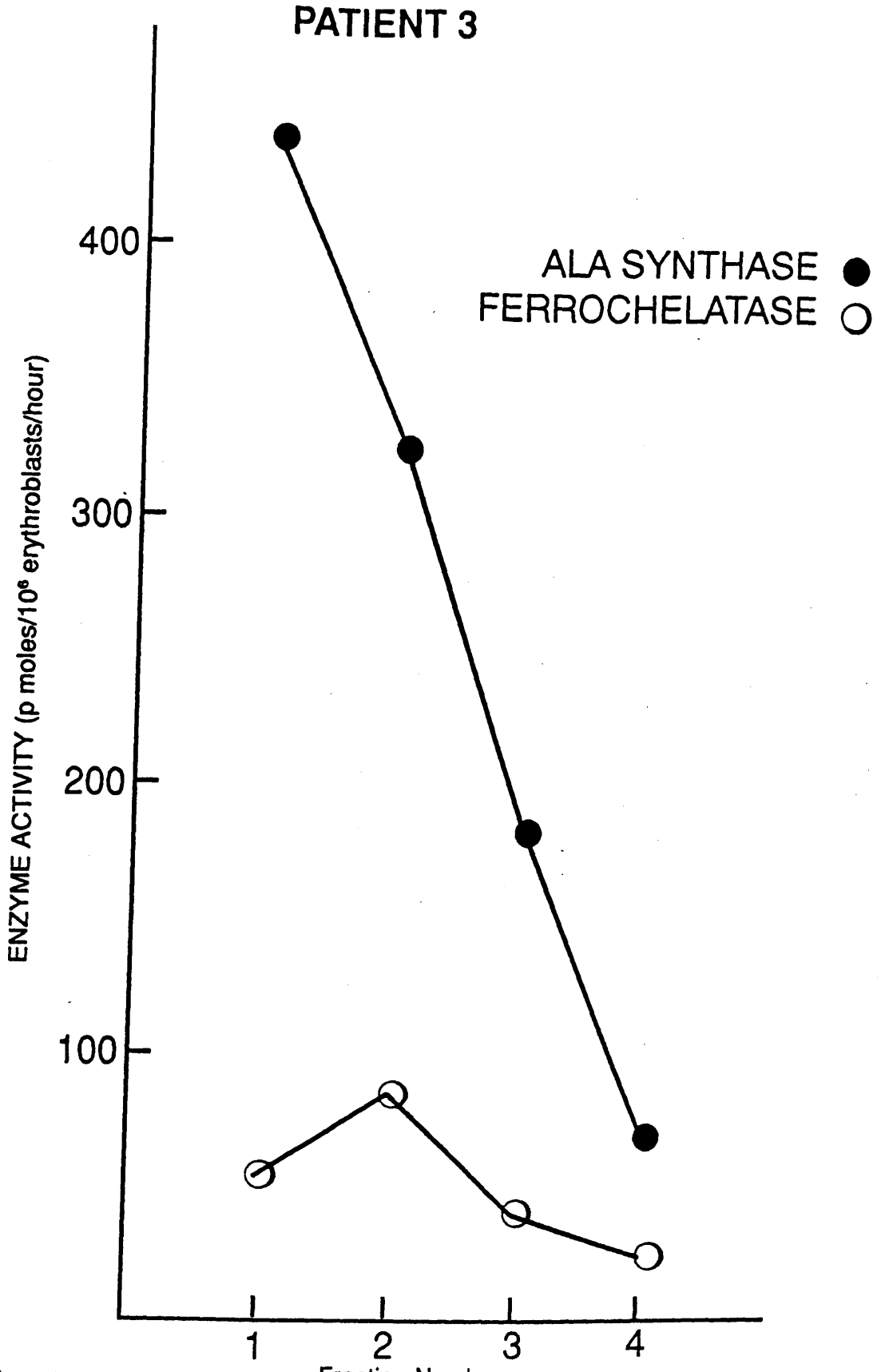
FIGURE 32

THE RELATIONSHIP BETWEEN ALA SYNTHASE AND FERROCHELATASE ACTIVITIES DURING NORMOBLASTIC ERYTHROPOIESIS



Note:
Each point represents the mean of two observations.

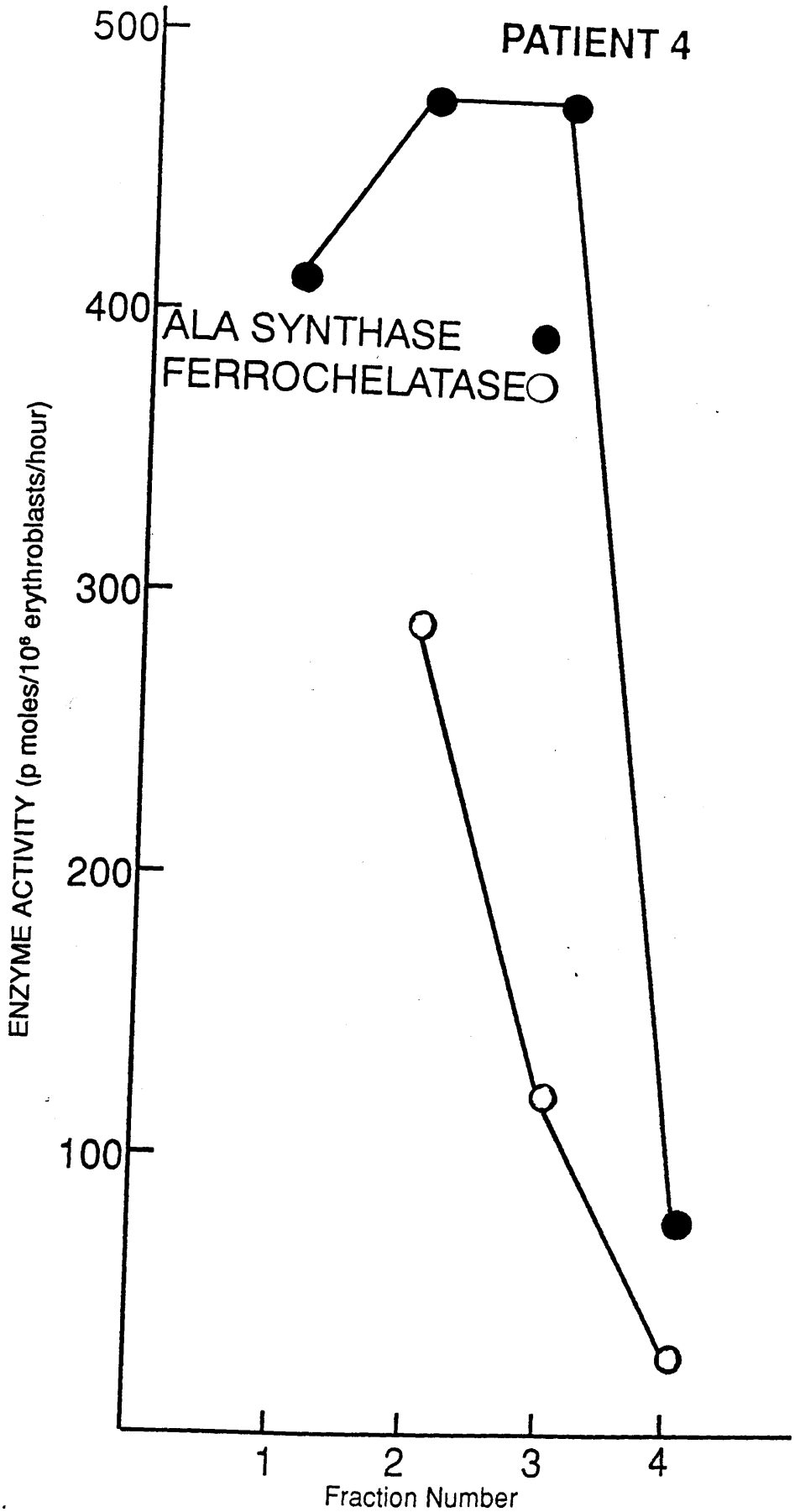
FIGURE 33
THE RELATIONSHIP BETWEEN ALA SYNTHASE AND FERROCHELATASE ACTIVITIES DURING NORMOBLASTIC ERYTHROPOIESIS



Note:
Each point represents the mean of two observations.

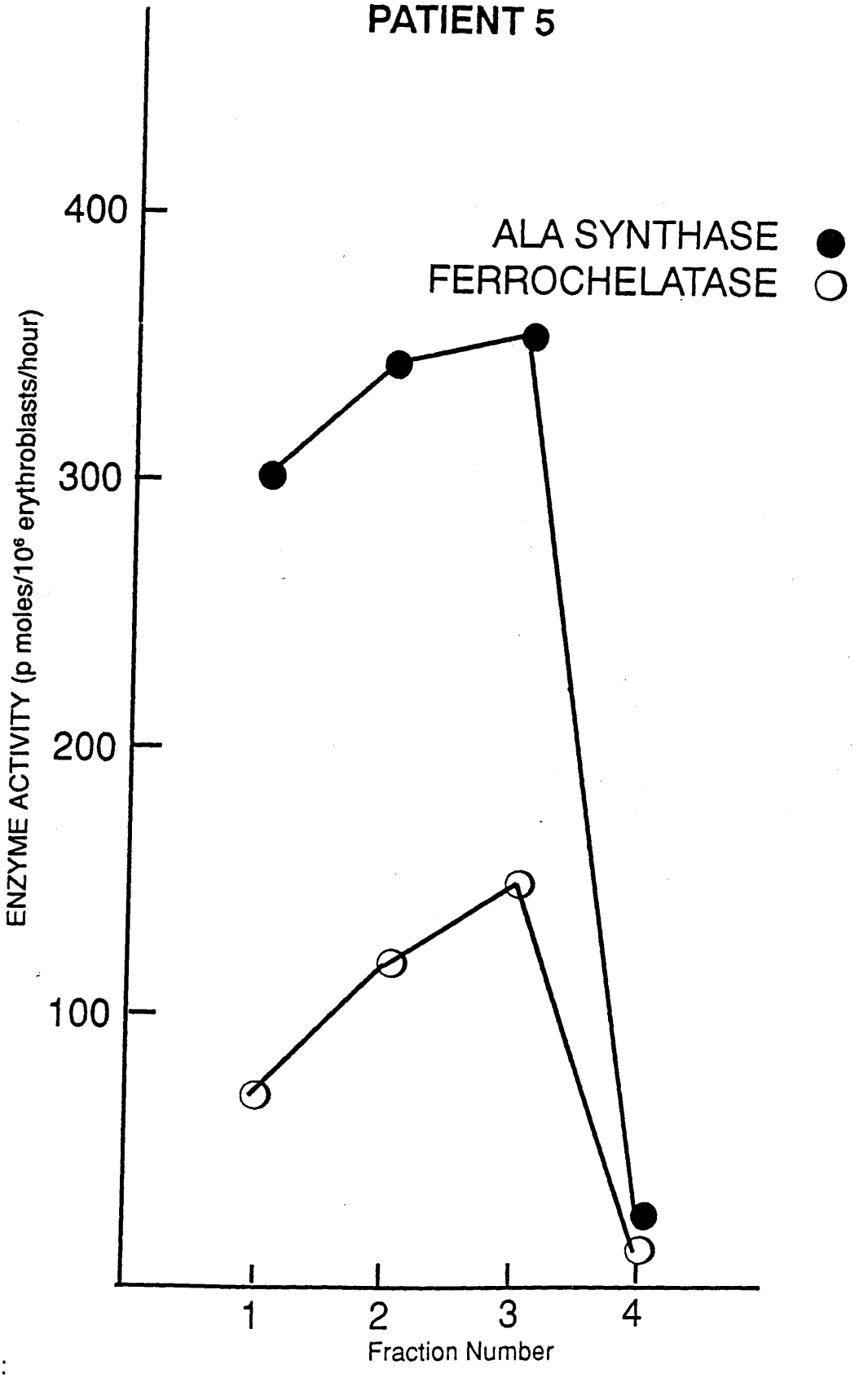
FIGURE 34

THE RELATIONSHIP BETWEEN ALA SYNTHASE AND FERROCHELATASE ACTIVITES DURING NORMOBLASTIC ERYTHROPOIESIS



Note:
Each point represents the mean of two observations.

FIGURE 35
THE RELATIONSHIP BETWEEN ALA SYNTHASE AND FERROCHELATASE ACTIVITIES DURING NORMOBLASTIC ERYTHROPOIESIS



Note:
Each point represents the mean of two observations.

FIGURE 36
THE RELATIONSHIP BETWEEN ALA SYNTHASE AND FERROCHELATASE ACTIVITIES DURING NORMOBLASTIC ERYTHROPOIESIS

steroid treatment at the time of study. This patient therefore must be examined separately due to the possible effect of steroid hormones on erythroblast haem enzyme activity (Urabe et al, 1979; Granick and Kappas, 1967; Levere and Gidari, 1974). In this case the development of enzyme activity during erythropoiesis was similar to that of the first four patients but appeared at first to be synchronous rather than sequential. The results are, however, more easily interpretated when enzyme activities are examined separately. ALA synthase activity is high in Fraction 1 and increases by only 15% in Fraction 2 and 3, before declining sharply in Fraction 4. Thus, in this patient, ALA synthase activity is initially high (Fraction 1) and does not change appreciably during early to intermediate erythroid development (Fractions 2 and 3). This implies that ALA synthase has attained peak activity early during erythroid development. Ferrochelatase activity, on the other hand, is relatively low in Fraction 1 at 15% of ALA synthase activity. However, activity increases by as much as 100% in Fractions 2 and 3, before declining sharply in Fraction 4. This implies that ferrochelatase activity does not peak until the intermediate stages of erythroid development and hence lags behind the peak in ALA synthase activity.

These findings are consistent with the results obtained for two of the four patients previously discussed (Figures 33 and 34). In terms of the fold increase in enzyme activity, ferrochelatase activity almost doubled during the intermediate stages of erythroid development (Fractions 2 and 3) in both patients. ALA synthase activity in the same patients is highest in the more immature cells (Fraction 1) and decreases

during further development. This again indicates that peak ALA synthase activity precedes peak ferrochelatase activity and hence that the development of enzyme activity is a sequential process.

The results obtained from this work enabled a normal range of ALA synthase and ferrochelatase activities to be established for fractions 1 to 4 (Table 17). This represented the normal pattern of ALA synthase and ferrochelatase activity during normoblastic erythropoiesis. All further measurements of enzyme activity in abnormal or pathological states were then related to these values.

6.3.6 Summary

ALA synthase and ferrochelatase enzyme activities were measured in highly purified age-matched human erythroblasts using improved and newly developed sensitive, specific enzyme assays. The results suggest that during normoblastic erythropoiesis, sequential development of enzyme activity may occur. The results for ALA synthase activity confirmed previous findings (Fitzsimons et al, 1986) while the results for ferrochelatase activity demonstrated for the first time the pattern of enzyme activity during normoblastic erythropoiesis. Enzyme activity for the two enzymes demonstrated a similar pattern of development in three of four cases, although a delay in peak activity for ferrochelatase relative to peak ALA synthase activity was evident in three cases. Despite this, quantitative analysis of enzyme activity was unable either to establish or refute the suggestion that ferrochelatase is limiting for haem formation (Sassa, 1976).

ALA SYNTHASE ACTIVITY
(p moles/10^6 erythroblasts/hour)

PERCOLL FRACTION	($\bar{x} \pm 1$ S.D., n = 15)
FRACTION 1	418 ± 249
FRACTION 2	406 ± 156
FRACTION 3	261 ± 123
FRACTION 4	120 ± 99

FERROCHELATASE ACTIVITY
(p moles/10^6 erythroblasts/hour)

PERCOLL FRACTION	($\bar{x} \pm 1$ S.D., n = 4)
FRACTION 1	92 ± 68
FRACTION 2	149 ± 92
FRACTION 3	123 ± 48
FRACTION 4	39 ± 33

TABLE 17

THE NORMAL RANGES OF ALA SYNTHASE AND FERROCHELATASE ACTIVITIES DURING NORMOBLASTIC ERYTHROPOIESIS

CHAPTER 7

ALA SYNTHASE ACTIVITY DURING IRON AND HAEM DEFICIENT ERYTHROPOIESIS

7 ALA SYNTHASE ACTIVITY DURING IRON AND HAEM DEFICIENT ERYTHROPOIESIS

7.1 AIMS OF THE STUDY

The aim of the work described in this chapter was to closely examine ALA synthase activity during iron and haem deficient erythropoiesis. It is well established that hepatic ALA synthase is induced in response to haem deficiency and repressed by excess haem via negative feedback inhibition. However, erythroid ALA synthase has been less well studied and the effects of haem deficiency on the haem enzymes in erythroid cells are currently poorly understood.

7.2 EXPERIMENTAL

7.2.1 Patients Studied

Bone marrow was obtained from 10 patients with iron deficient polycythaemia (IDP) (and consequent haem deficiency). More clinical details are presented in Appendix 2. Polycythaemic patients were chosen as the increased cellularity in such marrow is sufficient to provide adequate erythroblast numbers for fractionation. In each case, iron deficiency was established by serum ferritin concentrations less than 10µg/ml and MCV values less than 75fl (normal range 80 - 100 fl). No stainable iron was demonstrated in these marrows using conventional light microscopy techniques.

7.2.2 Enzyme Measurement

ALA synthase activity was measured in each of the four percoll fractions obtained from TG-1 treated bone marrow (Chapter 2). Fractions 1 to 4 contained purified age-matched

erythroblasts predominantly of pro, early, intermediate and late erythroblast status respectively. Differential counting of E_1, E_2 and E_3 cells in the different fractions confirmed erythroblast maturity per fraction equivalent to that seen with normal marrow (Chapter 2, Table 1).

7.3 RESULTS

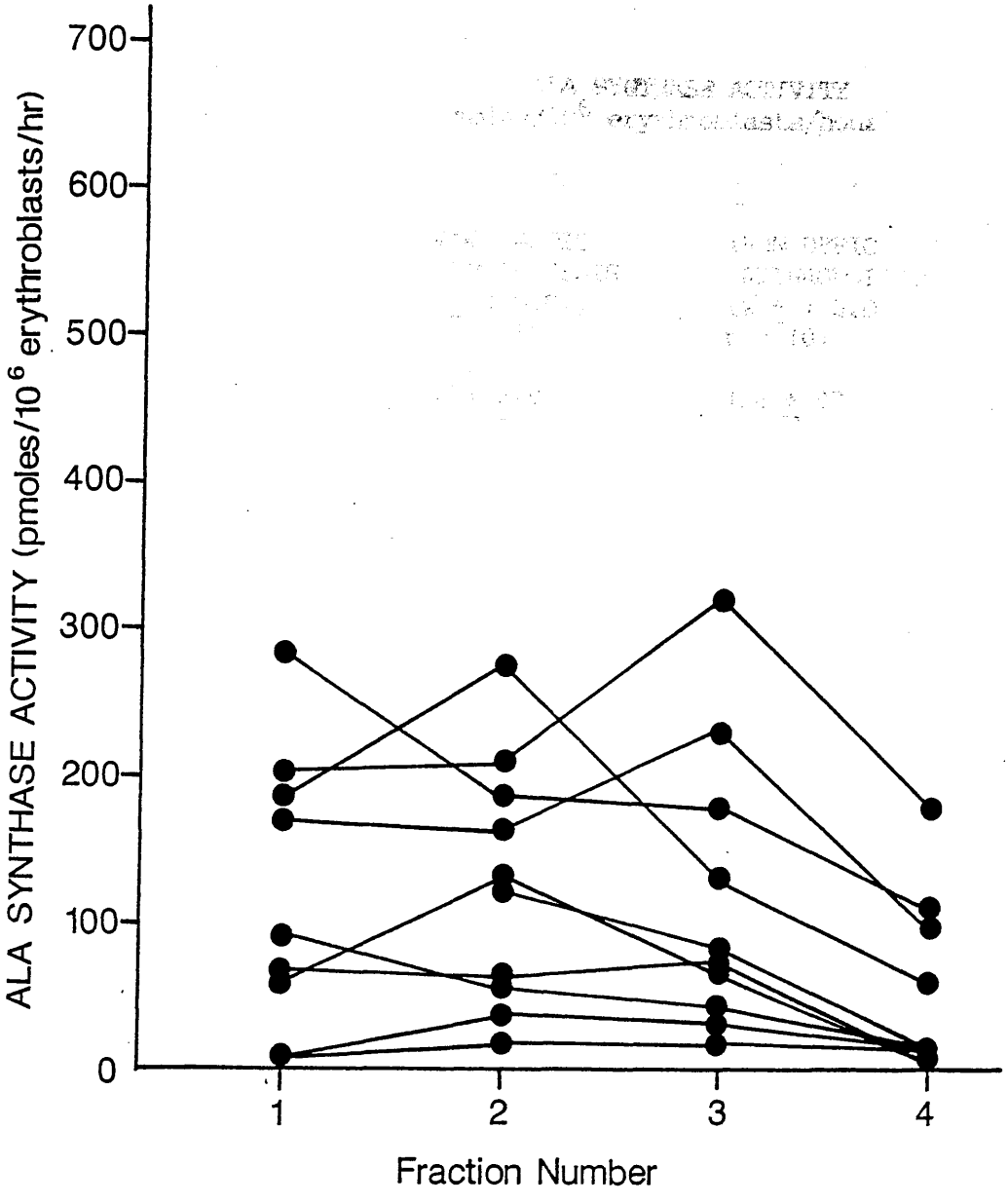
7.3.1 ALA Synthase Activity During Iron Deficient Erythropoiesis

The results expressed in Figure 37 and Table 18 indicate that ALA synthase activity in those immature erythroblasts of fractions 1 and 2 and in the more mature erythroblasts of fractions 3 and 4 was not increased by haem deficiency as might be expected for the "hepatic" enzyme. On the contrary, a significant reduction in ALA synthase activity was demonstrated during haem deficient erythropoiesis relative to normoblastic erythropoiesis (Chapter 6, Figure 30). The reduction in enzyme activity was particularly marked in fractions 1 and 2 ($p < 0.001$) but was also significant in fractions 3 and 4 ($p < 0.01$).

The results suggest that ALA synthase activity in erythroblasts, in contrast to hepatocytes, may be reduced in response to haem deficiency. This further indicates that the regulation of erythroid ALA synthase may be different from hepatic ALA synthase.

7.3.2 Discussion

Iron deficiency results in the inadequate production of haem to form Hb. There is also secondary inhibition of globin



NOTE :
Each point represents the mean of two observations.

FIGURE 37

ALA SYNTHASE ACTIVITY IN FRACTIONATED HUMAN ERYTHROBLASTS : IRON DEFICIENT ERYTHROPOIESIS

ALA SYNTHASE ACTIVITY
(p moles/10^6 erythroblasts/hour)

PERCOLL FRACTION	NORMOBLASTIC ERYTHROPOIESIS ($\bar{x} \pm 1$ S.D., n = 15)	IRON DEFICIENT ERYTHROPOIESIS ($\bar{x} \pm 1$ S.D., n = 10)	p<
FRACTION 1	418 ± 249	134 ± 90	0.000
FRACTION 2	406 ± 156	127 ± 83	0.001
FRACTION 3	261 ± 123	116 ± 99	0.010
FRACTION 4	120 ± 99	49 ± 61	0.010

TABLE 18

ALA SYNTHASE ACTIVITY IN FRACTIONATED BONE MARROW FROM PATIENTS WITH NORMOBLASTIC AND IRON DEFICIENT ERYTHROPOIESIS

synthesis (Freedman et al, 1974; 1976) and a marked accumulation of protoporphyrin in erythrocytes (Pagliardi et al, 1959; Heilmeyer and Clotten, 1962). There have been conflicting reports concerning alterations of other porphyrins and porphyrin precursors. No clear pattern has yet emerged with regard to the effect of iron deficiency on the enzymes of haem biosynthesis.

Several groups have demonstrated that ALA dehydratase activity in human erythrocytes does not differ in iron deficient patients from normal controls (Lichtman et al, 1963; Battistini et al, 1971), while others have reported increased ALA dehydratase activity in iron deficient patients (Chalevelakis et al, 1977; Campbell et al, 1978). Conversely, reduced ALA dehydratase has been shown in the whole blood, liver and bone marrow of iron deficient rats (Sharma et al, 1973). When erythrocytes from patients with iron deficiency were incubated with ALA they produced more PBG and less porphyrins than controls, suggesting reduced activity of PBG deaminase (Prato et al, 1968). In such studies, reticulocytosis may influence the recorded activities of the haem enzymes as both ALA dehydratase and PBG deaminase activity in peripheral blood may be increased by elevated reticulocyte counts (Anderson et al, 1977).

It has been shown that ALA synthase activity is not significantly different in bone marrow in iron deficient patients than in normal subjects (Aoki et al, 1974).

It has been claimed that the accumulation of protoporphyrin in iron deficiency anaemia is less than one would expect if the pathway prior to ferrochelatase continued

to operate at its normal rate (Moore and Goldberg, 1974). This would suggest that in addition to the block at the level of iron and protoporphyrin utilisation, there is a general slowing of the pathway prior to this. It has also been suggested that protoporphyrin may inhibit ferrochelatase activity in normal human bone marrow (Bottomley, 1968).

A reduction in ALA synthase activity during iron deficient erythropoiesis has been demonstrated for the first time in the work described in this chapter. Moreover, this work has identified that this reduction is most marked in the more immature erythroid cells, normally most active with regard to ALA synthase activity. This is in apparent contrast to the inducing effect of haem deficiency on ALA synthase activity in hepatic tissue. This finding provides support for there being different methods of regulation for the haem biosynthetic pathway in hepatic and erythroid tissue and may in part be explained by the recent confirmation of distinct housekeeping (Sutherland et al, 1988) and erythroid-specific (Cox et al, 1990) genes for ALA synthase. ALA synthase is well established as the rate controlling enzyme of haem biosynthesis in hepatic tissue, but its role in erythroid tissue has not been defined. It is clear from this work that the regulation of erythroid ALA synthase is different from that of hepatic ALA synthase.

The reduction in erythroid ALA synthase activity in iron deficient patients may be explained in several ways:

(1) Erythroid ALA synthase may be inhibited by the accumulation of protoporphyrin. There is, however, currently no experimental evidence to support this.

(2) Adequate free haem concentrations may be required for optimal erythroid ALA synthase activity. Haem may therefore affect erythroid ALA synthase in an opposite manner to that of the housekeeping enzyme i.e. it may induce erythroid ALA synthase activity. This is most likely to occur post-transcriptionally i.e. at translational or translocational levels or through a direct effect on enzyme activity (Chapter 3, Section 3,5).

(3) Zinc protoporphyrin has been reported to accumulate in iron deficiency (Lamola and Yamane, 1974). As ferrochelatase is required to catalyze the formation of zinc protoporphyrin this suggests that enzyme activity is not compromised and that ferrous iron supply limits haem formation (Bottomley, 1977). It is possible that zinc protoporphyrin may exert an inhibitory role, not unlike haem in hepatic tissue, at the level of erythroid ALA synthase (Vreman and Stevenson, 1987). This observation requires clarification.

(4) A low iron diet plus a single dose of desferroxamine results in reduced cytochrome P_{450}, but no increase in ALA synthase activity in rat hepatic tissue (White et al, 1978). This suggests that adequate iron may be required for normal ALA synthase activity.

This theory is supported by the recent suggestion of a possible role for iron in the regulation of erythroid ALA synthase mRNA translation (Chapter 3, Section 3.5.2) (Casey et al, 1988).

It is therefore difficult to determine whether the reduced erythroid ALA synthase activity observed during iron deficient

erythropoiesis is a direct consequence of iron deficiency or of the resultant haem deficiency. In an attempt to resolve this uncertainty a patient with adequate iron stores, yet likely to have erythroid haem deficiency as a feature of hepatoerythropoietic porphyria (HEP), was examined.

7.4 BIOCHEMICAL INVESTIGATION OF HEPATOERYTHROPOIETIC PORPHYRIA - HOMOZYGOUS PORPHYRIA CUTANEA TARDA

A unique opportunity arose to examine the effect of haem deficiency on both erythroid and "hepatic" ALA synthase activities in a patient with hepatoerythropoietic porphyria (HEP). This provided further insight into the tissue-specific regulation of ALA synthase.

HEP is a rare type of porphyria believed to represent homozygous familial porphyria cutanea tarda (PCT). It is generally accepted that HEP is characterised by decreased uroporphyrinogen (URO) decarboxylase activity (Fujita et al, 1987). However, only 15 cases have been reported in the world literature to date (Smith, 1986) and this has created difficulty with regard to achieving full clinical and biochemical characterisation of this disease. Two very similar cases, originally diagnosed and intensively investigated over several years (Gunther, 1967; Pinõl Aquadé et al, 1975) may be considered as "typical" HEP.

HEP is present from infancy and excess porphyrins (primarily uroporphyrin III) are found in urine, faeces and erythrocytes. This distinguishes HEP from the acute porphyrias, which present at puberty and have a normal erythrocyte porphyrin content. Although HEP is clinically

similar to congenital erythropoietic porphyria (CEP), it differs biochemically in that the enzyme deficiency in CEP is uroporphyrinogen III cosynthetase. Markedly depressed liver and erythrocyte URO decarboxylase activity has been demonstrated in nine of the fifteen previously reported cases of HEP. Biochemically, HEP has many similarities with familial PCT, including porphyrin excretion patterns in urine and faeces, the presence of isocoproporphyrin and isomer III being predominant over isomer I porphyrin. The activity of URO decarboxylase is depressed in cases of PCT (Chapter 1, Section 1.7.6) and is even further reduced in HEP.

7.5 EXPERIMENTAL

The study included porphyrin measurements in urine, faeces and erythrocytes; ferrokinetic studies; and haem biosynthetic enzyme activity measurements in bone marrow and liver of a 37 year old male patient with suspected HEP.

Until recently ferrokinetic studies have been confined to measurement of plasma iron turnover (PIT), calculated from the clearance of [^{59}Fe]-transferrin from plasma and the serum iron concentration. In addition, red cell iron utilisation was usually measured after 14 days. These methods did not provide any indication of the rate or characteristics of erythropoiesis (Cavill and Ricketts, 1980) but could demonstrate changes in the total plasma iron flow. Modern computer-assisted analytical methods applied to ferrokinetic data allow the distinction of erythroid from non-erythroid iron turnover and thus provide more accurate information regarding erythropoiesis (Cavill and Ricketts, 1980). Furthermore, the degree of

ineffective erythropoiesis can be assessed accurately and a precise value for red cell lifespan obtained.

This study was carried out in collaboration with the Porphyrin Research Laboratories, Department of Medicine and Therapeutics, Western Infirmary, Glasgow and the Department of Haematology, University Hospital, Cardiff.

Clinical Features

The patient was a 37 year old male. The major clinical features exhibited by the patient were: (a) lifelong severe photosensitivity; (b) hypertrichosis; (c) scleroderma-like changes; (d) anaemia (Hb = 10.9g/dl). Splenomegaly 8cm. (See photographic plate 7). No family members were available for study.

7.6 RESULTS

7.6.1 Porphyrin Measurements

Porphyrin analysis (Moore, 1983) (Table 19) showed excessive amounts of porphyrins in urine, faeces and blood. There were, in particular, significant quantities of uroporphyrin in blood and faeces. Total porphyrins in urine were increased 120-fold relative to the normal range; coproporphyrin excretion in faeces was increased 160-fold, while protoporphyrin levels in whole blood were increased 5-fold. HPLC analysis (Seubert and Seubert, 1978) revealed excessive amounts of uroporphyrinogen, together with hepta (7-COOH); hexa (6-COOH); penta (5-COOH) intermediates and coproporphyrinogen in urine. This excretion pattern would

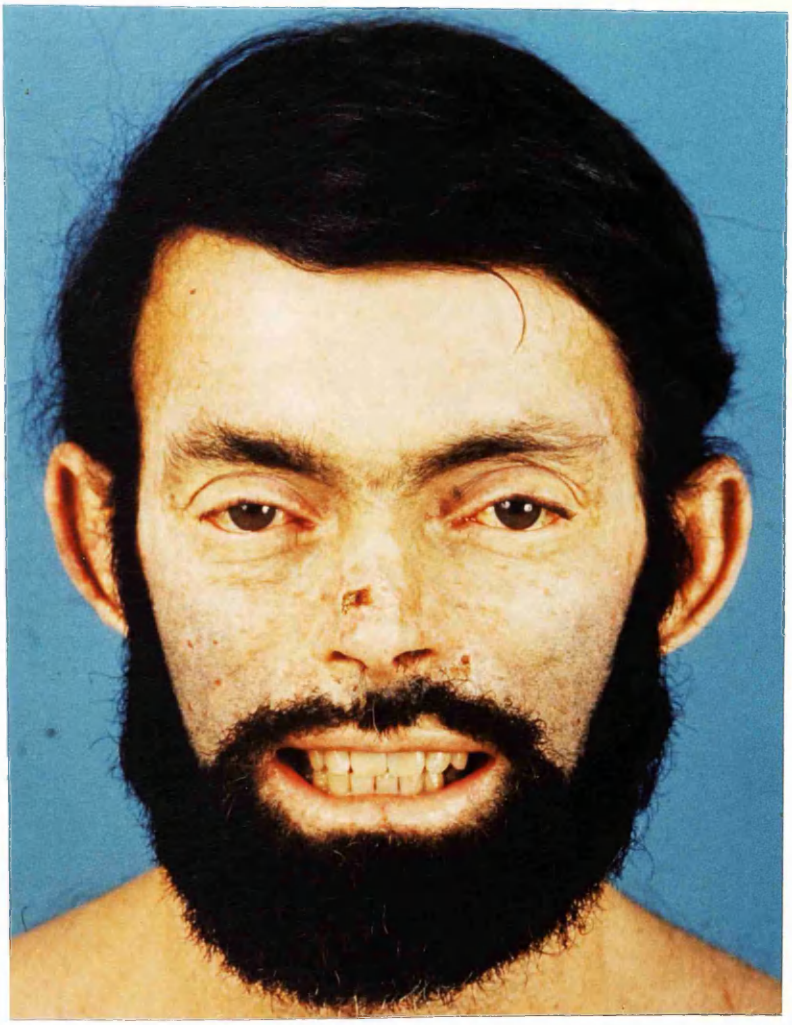


PLATE 7 (a), (b)

FACE AND HANDS OF A PATIENT WITH HEPATOERYTHROPOIETIC PORPHYRIA

	PORPHYRINS					ANALYSIS OF PORPHYRINS BY HPLC					
	ALA	PBG	COPRO	PROTO	T.P.	PROTO	COPRO	5-COOH	6-COOH	7-COOH	URO
URINE	17.6 ± 6.3	1.9 ± 2.8			36489 ± 7016	0.5	5.0	2.0	1.0	3.5	88.0
FAECES	-	-	12177 ± 4307	292 ± 104		-	96.75	-	-	0.75	2.5
BLOOD	-	-	-	4552 ± 1092		5.5	13.5	2.0	3.5	27.0	48.5
LIVER						-	-	-	-	9.0	91.0
BONE-MARROW						17.0	3.5	13.0	1.2	3.3	62.0

Abbreviations and Units: **Normal Range:**

Urine: ALA = 5-aminolaevulinate — 0 - 40μmol/24h
PBG = porphobilinogen — 0 - 16μmol/24h
T.P. = total porphyrins — 0 - 300μg/24h

Faeces: COPRO = coproporphyrin — 0 - 76nmol/g dry wt
PROTO = protoporphyrin — 0 - 200nmol/g dry wt

Blood: PROTO = protoporphyrin — 0 - 900nmol/hr/ml of red blood cells

Porphyrin results are expressed as the mean ± 1 S.D. where n = 25(urine), n = 8(faeces) and n = 13(blood)

TABLE 19
ANALYSIS OF PORPHYRINS AND PRECURSORS IN HEPATOERYTHROPOIETIC PORPHYRIA

suggest a defect in the decarboxylation catalyzed by URO decarboxylase. Coproporphyrinogen was prominent in faeces, while erythrocytes were found to contain excess protoporphyrinogen, uroporphyrinogen, coproporphyrinogen and 7-COOH, 6-COOH and 5-COOH intermediates. Liver and bone marrow both contained excess uroporphyrin.

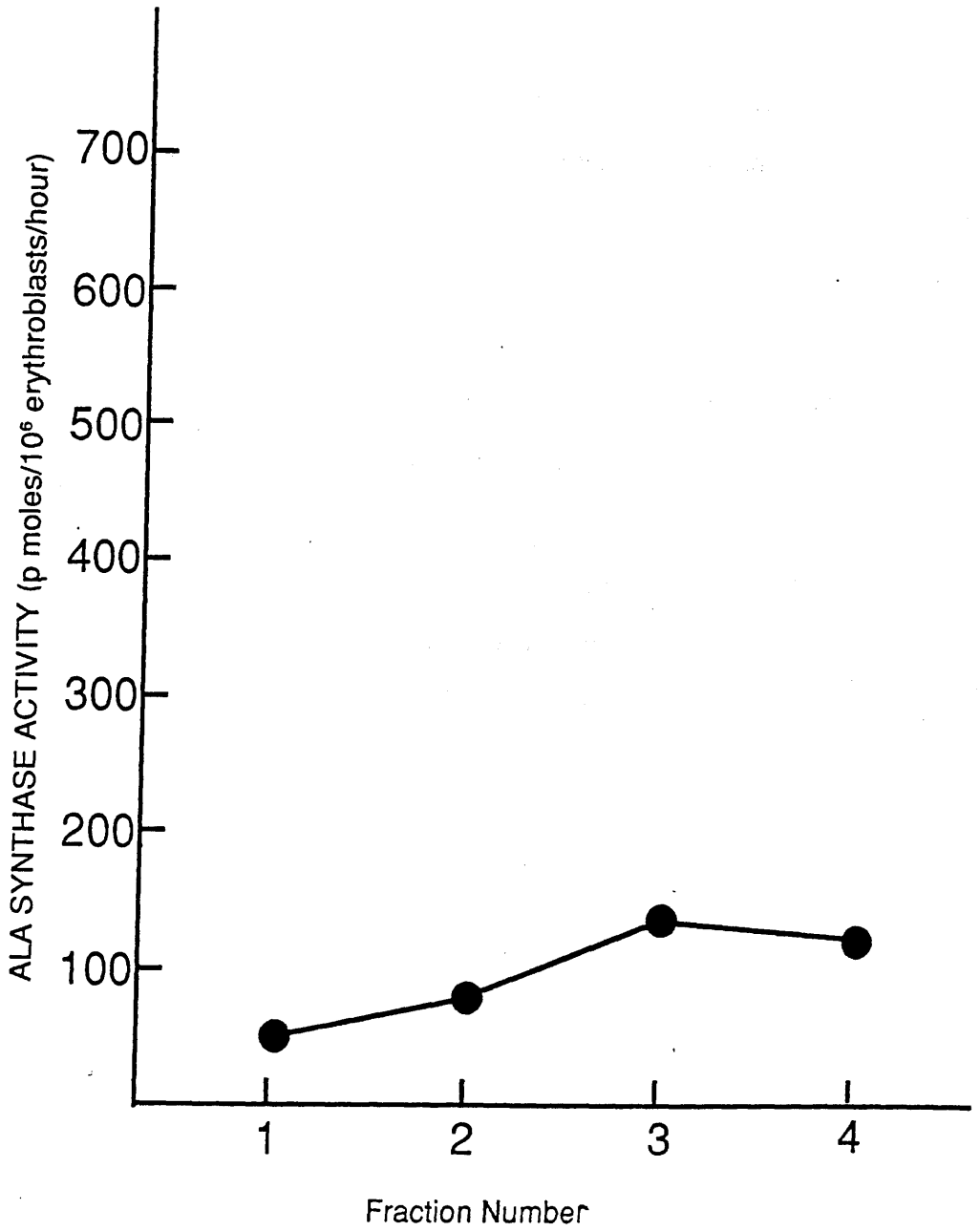
Thus, the general porphyrin profile was consistent with that observed in HEP. This conclusion was further substantiated by porphyrin isomer examination which indicated the predominance of uroporphyrin isomer III. In CEP isomer I would be expected.

7.6.2 Haem Biosynthetic Enzyme Activity Measurements

Hepatic URO decarboxylase activity was undetectable. Significantly, ALA synthase (5280nmoles ALA/h/g protein) was elevated 25-fold in liver (normal range = 116-240nmoles ALA/h/g protein) but was markedly reduced in fractionated bone marrow erythroblasts. This was particularly evident in the more immature cells of fractions 1 and 2 which normally contain highest ALA synthase activity. In these cells ALA synthase activity was reduced to approximately one sixth of normal (Figure 38).

7.6.3 Ferrokinetics

Ferrokinetic studies demonstrated highly ineffective erythropoiesis (> 75% ineffective). Red cell life span was not significantly reduced at 77 days which is towards the lower limit of normal for this ferrokinetic method. It was then clear that ineffective erythropoiesis (intramedullary



Note:
Each point represents the mean of two observations.

FIGURE 38

ALA SYNTHASE ACTIVITY IN FRACTIONATED ERYTHROBLASTS FROM A PATIENT WITH HEPATOERYTHROPOIETIC PORPHYRIA

lysis of developing erythroblasts) rather than haemolysis (reduced red cell lifespan) was the cause of the anaemia. As haem has been shown to play a major role in the regulation of normal erythropoiesis (Chapter 1, Section 1.13) it is likely that erythroblast haem deficiency in HEP is the cause of the highly ineffective erythropoiesis.

7.6.4 Conclusions

This study has identified severely depressed URO decarboxylase activity which would explain the observed porphyrin excretion profile and the clinical and biochemical characteristics associated with HEP. PCT and HEP are characterised by reduced URO decarboxylase activity. In cases of PCT enzyme activity is reduced to approximately 50% of normal, while enzyme depression in HEP is more severe due to the homozygous nature of the defect. The anaemia has been shown to result from ineffective erythropoiesis as demonstrated by ferrokinetic studies.

Perhaps most significantly this study has shown that in response to reduced haem synthesis, ALA synthase activity increased in hepatic tissue and decreased in erythroblasts. This suggests that the regulation of haem synthesis in erythroblasts is different to that recognised to operate in hepatic tissue and supports the earlier finding of reduced ALA synthase activity during iron deficient erythropoiesis (Section 7.3.1). This observation would support the view that haem deficiency rather than iron deficiency causes reduced erythroid ALA synthase activity. However, a possible contributory role

for iron in the attenuation of erythroid ALA synthase activity cannot be discounted on the basis of this observation. This latter view is supported by the suggestion of a role for iron in the regulation of erythroid ALA synthase mRNA translation (Chapter 3, Section 3.5.2) (Casey et al, 1988). In addition, the possible existence of an erythroid-specific inhibitor for ALA synthase activity cannot be overlooked, although there is currently no experimental evidence to support this theory.

7.6.5 Summary

ALA synthase activity was measured in highly purified age-matched human erythroblasts from 10 patients with iron deficient polycythaemia.

It was demonstrated for the first time that during iron deficient erythropoiesis ALA synthase activity is seriously impaired. This was particularly evident in the more immature erythroid cells, normally most active with regard to ALA synthase activity. In order to determine whether the reduction in erythroid ALA synthase activity was a direct consequence of the iron deficiency or of the resultant haem deficiency a patient with erythroid haem deficiency hepatoerythropoietic porphyria (HEP) but adequate iron stores was examined. This patient demonstrated a similar pattern of reduced ALA synthase activity suggesting that the reduction in ALA synthase activity was most likely to be a consequence of the haem deficiency. A secondary role for iron in the regulation of ALA synthase activity cannot however be eliminated on the basis of these results.

The regulation of erythroid ALA synthase activity would

therefore appear to be different from that recognised to operate in hepatic tissue whereby ALA synthase activity is induced in response to haem deficiency. This may be explained by the existence of tissue-specific isoenzymes for ALA synthase in erythroid and hepatic tissue which are encoded for by separate genes on the X chromosome and chromosome 3 respectively (Cox et al, 1990; Sutherland et al, 1988).

CHAPTER 8

ALA SYNTHASE AND FERROCHELATASE ACTIVITIES DURING SIDEROBLASTIC ERYTHROPOIESIS

8 ALA SYNTHASE AND FERROCHELATASE ACTIVITIES DURING SIDEROBLASTIC ERYTHROPOIESIS

8.1 AIMS OF THE STUDY

It was the aim of the work described in this chapter to examine ALA synthase and ferrochelatase activities during sideroblastic erythropoiesis (Chapter 1, Section 1.15.2).

Erythroid ALA synthase has been reported to be reduced in most types of sideroblastic anaemia (S.A.) (Cartwright and Deiss, 1975; Aoki et al, 1974; Bottomley, 1982) and it is generally believed that this is the primary defect which results in sideroblast formation. However, controversy surrounds this issue. Several reports have implicated abnormal intracellular iron metabolism (May et al, 1982; Wickramasinghe and Hughes, 1978) and abnormalities of other haem enzymes, particularly ferrochelatase (Ali and Sweeney, 1974; Ali and Brain, 1974; Labbe and Nielsen, 1976) as possible sites of the primary defect. A clear answer has not been forthcoming due to the hitherto inability to closely examine haem enzyme activity during erythropoietic development.

There is a broad spectrum of clinical settings in which the ring sideroblast abnormality occurs. SA may present as a congenital anaemia, usually X-linked (CSA); as an irreversible acquired erythroid defect (Primary Acquired Sideroblastic Anaemia, PASA) which may either be stable or may evolve relatively rapidly into acute leukaemia; and as a reversible phenomenon (Secondary Sideroblastic Anaemia, SSA) secondary to alcohol intoxication, copper deficiency, occasional haemolytic processes and the administration of certain drugs such as isoniazid or chloramphenicol (Bottomley, 1982). These varied

associations have provided no clue for the etiologic or pathogenic mechanisms of the erythropoietic defect(s).

8.2 EXPERIMENTAL

8.2.1 Patients Studied

Bone marrow was obtained at the time of routine bone marrow examination from 11 patients with several types of SA. Three patients had congenital SA (CSA); seven patients had primary acquired SA (PASA); and one patient had secondary SA (SSA). Further patient details are presented in Appendix 2.

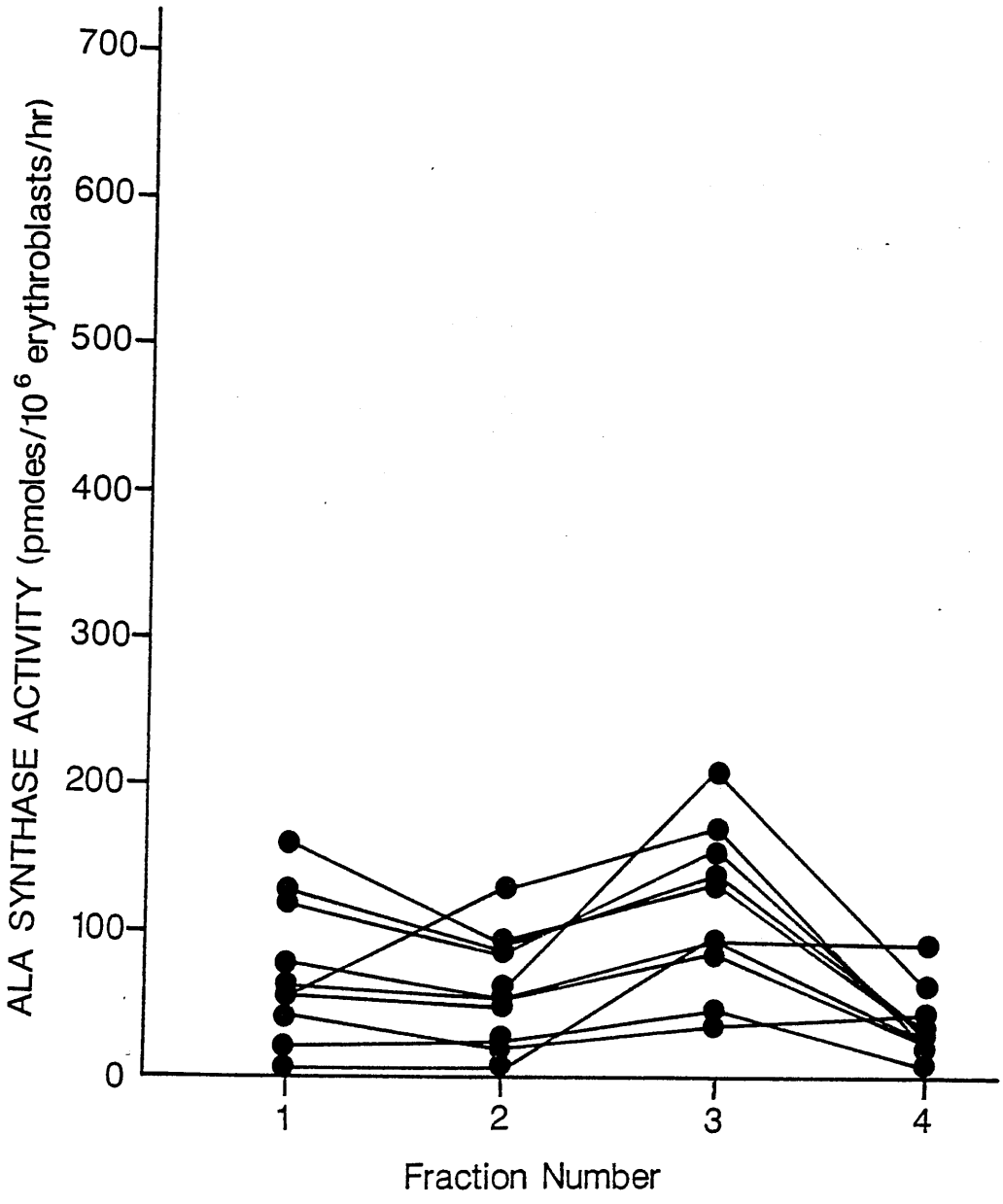
8.2.2 Enzyme Measurements

ALA synthase activity was measured in the four percoll fractions obtained from fractionated bone marrow (Chapter 2). Ferrochelatase activity was measured in six patients (1 CSA, 5 PASA). Fractions 1 to 4 contained purified age-matched erythroblasts predominantly of pro, early, intermediate and late erythroblast status respectively.

8.3 RESULTS

8.3.1 ALA Synthase Activity During Sideroblastic Erythropoiesis

The results expressed in figure 39 represent ALA synthase activity during CSA and PASA. In each case ALA synthase activity was markedly decreased throughout erythropoiesis relative to that observed during normoblastic erythropoiesis (Chapter 6, Figure 30). The reduction in enzyme activity was particularly marked in fractions 1, 2 and 4 ($p < 0.001$), but was also significant in fraction 3 ($p < 0.001$) (Table 20). It



NOTE :
Each point represents the mean of two observations.

FIGURE 39

ALA SYNTHASE ACTIVITY IN FRACTIONATED HUMAN ERYTHROBLASTS : SIDEROBLASTIC ERYTHROPOIESIS

ALA SYNTHASE ACTIVITY
(p moles/10^6 erythroblasts/hour)

PERCOLL FRACTION	NORMOBLASTIC ERYTHROPOIESIS ($\bar{x} \pm 1$ S.D., n = 15)	SIDEROBLASTIC ERYTHROPOIESIS ($\bar{x} \pm 1$ S.D., n = 10)	P <
FRACTION 1	418 ± 249	71 ± 54	0.000
FRACTION 2	406 ± 156	53 ± 30	0.001
FRACTION 3	261 ± 123	115 ± 55	0.010
FRACTION 4	120 ± 99	35 ± 29	0.001

TABLE 20

ALA SYNTHASE ACTIVITY IN FRACTIONATED BONE MARROW FROM PATIENTS WITH NORMOBLASTIC AND SIDEROBLASTIC ERYTHROPOIESIS

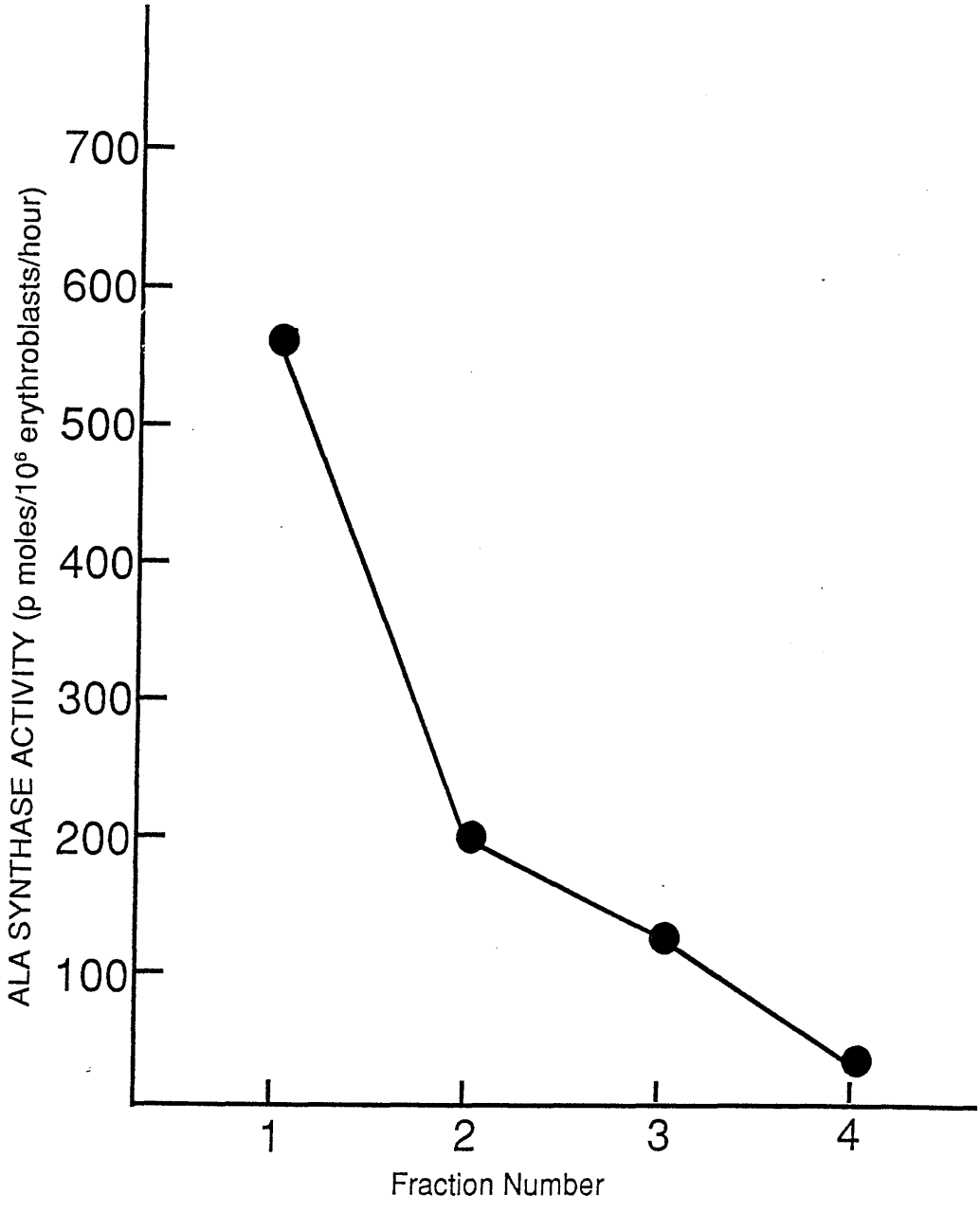
is therefore clear that ALA synthase activity is reduced throughout erythropoiesis in CSA and PASA.

In the case of SSA, however, ALA synthase activity in Fractions 1 to 4, i.e. throughout erythropoiesis, was not significantly reduced and remained within the normal range (Figure 40). Although enzyme activity in percoll Fraction 2 was perhaps a little low, abundant activity was evident in Fraction 1.

One transfusion-dependent CSA patient (male, aged 4 years) was studied over a period of 1 year with regard to Hb levels and ALA synthase activity. The patient originally presented with a severe non pyridoxine responsive anaemia, Hb of 4.6g/dl and the marrow showed evidence of dyserythropoiesis with 24% ring sideroblasts. ALA synthase activity was markedly reduced in all 4 percoll fractions (Figure 41). Family studies were not available as the patient lived in England. During the period of regular transfusion Hb was maintained between 8 and 9 g/dl. However, one year later the patient underwent spontaneous remission. Hb returned to normal/or possibly raised levels (14-15 g/dl), as did ALA synthase activity in all four percoll fractions (Fraction 41). This remission was associated with a switch to HbF production. This represents the first documented case of a patient with CSA making a full spontaneous recovery.

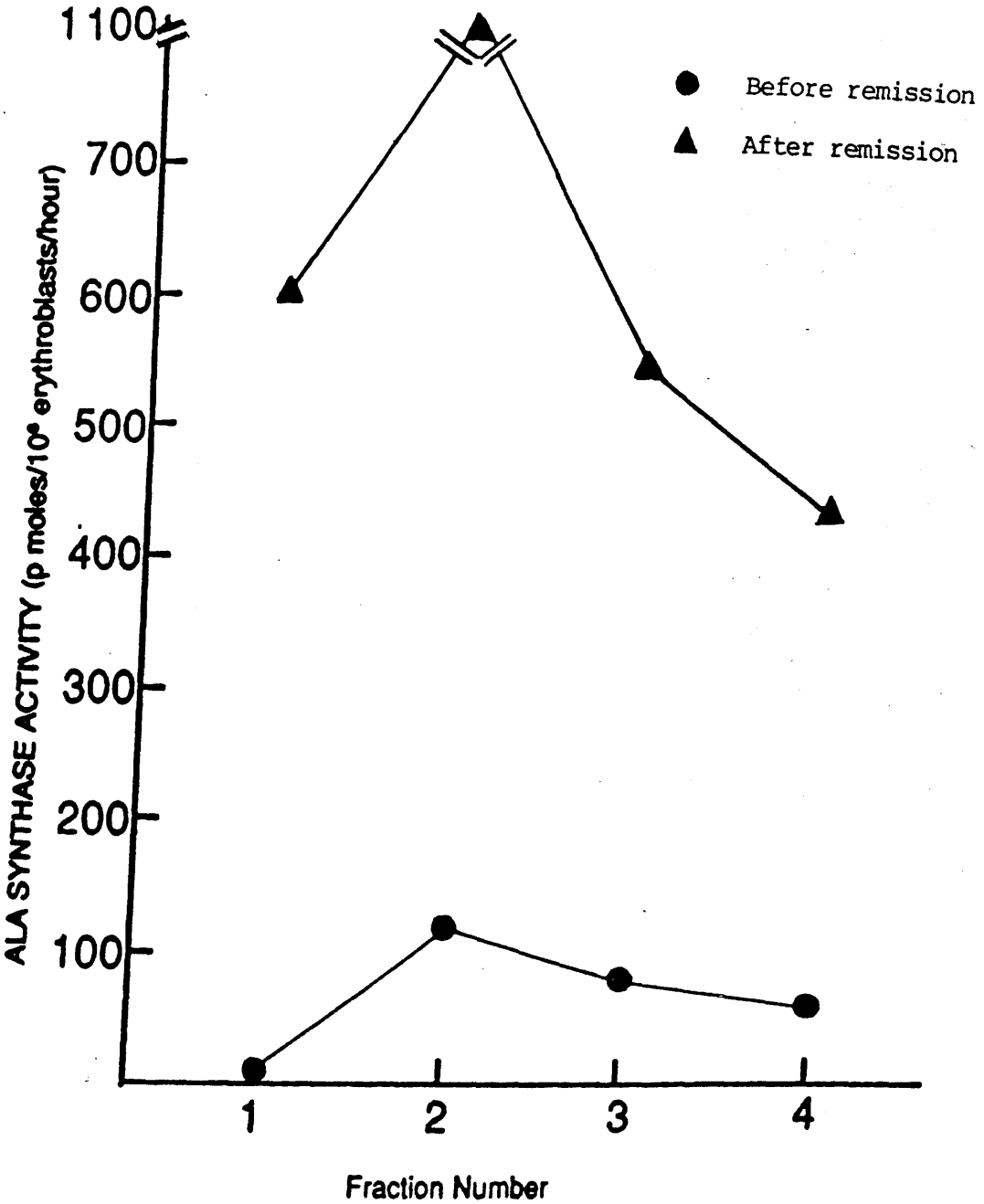
8.3.2 Ferrochelatase Activity During Sideroblastic Erythropoiesis

Fractionated sideroblastic bone marrow samples from six patients (1 CSA, 5 PASA) were assayed for ferrochelatase activity.



Note:
Each point represents the mean of two observations.

FIGURE 40
ALA SYNTHASE ACTIVITY IN FRACTIONATED HUMAN ERYTHROBLASTS : SIDEROBLASTIC ERYTHROPOIESIS (SECONDARY SIDEROBLASTIC ANAEMIA)



Note:
Each point represents the mean of two observations.

FIGURE 41

ALA SYNTHASE ACTIVITY IN FRACTIONATED HUMAN ERYTHROBLASTS FROM A PATIENT WITH CONGENITAL SIDEROBLASTIC ANAEMIA (BEFORE AND AFTER SPONTANEOUS REMISSION).

Enzyme activity in all patients was difficult to detect in fractions 1 to 4 despite the use of a highly sensitive radiochemical assay. In four cases enzyme activity was completely undetectable whereas in the remaining two cases enzyme activity could just be detected. This indicated that ferrochelatase activity was impaired throughout erythropoiesis in a similar fashion to ALA synthase activity.

8.3.3 Discussion

SA is characterised by defective haemoglobinisation of red cells, ineffective erythropoiesis and an abnormal accumulation of iron in the maturing erythroblasts distributed in the form of a perinuclear ring (Chapter 1, Section 1.15.2). Erythroblasts from individuals with either the inherited or acquired types of SA have an inability to utilise iron and synthesise haem, although iron uptake and iron transfer to mitochondria remain normal (Bottomley and Moore, 1986).

Several theories have been forwarded to explain the reduction in haem synthesis. It has been argued that virtually all cases of SA show impaired ALA synthase activity and that this is the primary defect which leads to reduced haem synthesis (Cartwright and Deiss, 1975; Aoki et al, 1974). Other workers believe the primary defect to be an abnormality of mitochondrial iron metabolism (May et al, 1982) possibly associated with impaired mitochondrial function (Wickramasinghe and Hughes, 1978). However, in cases of pyridoxine-responsive CSA only ALA synthase is thought to be impaired. CSA usually follows X-linked inheritance (Lee et al, 1968; Bottomley, 1982; McKusick, 1986) and the erythroid ALA synthase gene has

recently been located on the X chromosome (Cox et al, 1990) supporting the theory of a possible defect at the level of the erythroid ALA synthase gene. Moreover, the response of patients with CSA to pyridoxine is strong evidence in favour of decreased ALA synthase activity contributing to the anaemia. It has been argued that pyridoxine may improve haem synthesis and lessen the anaemia by preventing the temperature-dependent inactivation of ALA synthase by a controlling protease present in erythroid cells (Aoki, 1978; Aoki et al, 1979). In experimental models of pyridoxine deprivation or antagonism, mitochondrial iron-loading, reduced ALA synthase activity and hypochromic-microcytic anaemia are prominent findings (Bottomley, 1980). Most of the drugs (and in particular isoniazid) which induce SA in man and animals are thought to impair haem biosynthesis by diminishing the availability of PLP for ALA production (Haden, 1967; McCurdy and Donohoe, 1966; Roberts and Hoffbrand, 1966; Harris and MacGibbon, 1965; Tanaka and Bottomley, 1974). In acquired SA pyridoxine responsiveness is infrequently observed.

Reduced ALA synthase activity was found in the CSA and PASA patients included in this study. The low enzyme activity was mainly evident during early erythroid development (i.e. percoll Fractions 1 and 2). Mitochondrial iron deposits are, on the other hand, normally more evident in the later siderotic erythroblasts (Bottomley, 1982). In vitro studies of erythroid cell iron metabolism have not disclosed any abnormalities of iron uptake or transfer to mitochondria and merely reflect reduced incorporation of iron into haem

(Bottomley and Moore, 1986). The patient with SSA demonstrated normal levels of ALA synthase. This latter observation identified normal ALA synthase activity in ring sideroblastic erythroid cells and so casts doubt on the theory that reduced ALA synthase is the primary cause of ring sideroblast formation.

Conversely, increased bone marrow ALA synthase activity has been found in the acute reversible SA of alcoholics (Fraser and Schacter, 1980). Scrutiny of the clinical state of the alcoholic patient has led to the conclusion that chronic malnutrition and folate deficiency with megaloblastic marrow maturation are prerequisites for the occurrence of sideroblastic changes (Pierce and McGuffin, 1976). Comparison of enzyme activity in the presence and absence of pyridoxal phosphate (PLP) revealed that PLP metabolism in alcoholics may not be important in the inhibition of haem biosynthesis at the level of ALA synthase, but that rather an enzymatic block should be sought at a more distal site in the haem pathway. The biochemical pathogenesis of this disorder therefore remains unclear. A block at ferrochelatase is supported by increased red cell protoporphyrin and coproporphyrin levels in alcoholic patients with marrow ring sideroblasts (Ali and Sweeney, 1974; Moore, 1974). The observations that 'in vitro' inhibition of globin synthesis by ethanol may be corrected by the addition of haem, but not by ALA (Ali and Brain, 1974) also suggests a blockade distal to ALA synthase. A proposed association of PLP as a cofactor requirement for ferrochelatase as well as ALA synthase (Labbe and Nielsen, 1976) provides a novel solution by which the postulated PLP deficiency noted in some studies could

also explain the observations that support a distal block in haem synthesis.

Moore and co-workers have described diminished activity of erythrocyte ALA dehydratase following alcohol ingestion (Krasner et al, 1974; Moore et al, 1971) and suggest that this change may be mediated through a relative increase in reduced glutathione concentration. However, since the enzyme is dependent on zinc, an alternative mechanism may involve a concomitant zinc deficiency through excessive renal excretion in alcoholism (Sullivan, 1974). Impaired metabolism of pyridoxine has also been demonstrated in alcoholic patients. Blood concentrations of PLP are frequently diminished in alcoholics (Hines, 1975; Hines and Cowan, 1970; Lumeng and Li, 1974; Pierce and McGuffin, 1976) but do not correlate consistently with morphological evidence of SA.

Reduced ferrochelatase activity during sideroblastic erythropoiesis in the patients examined in this study supports the theory of a distal blockade at ferrochelatase and/or iron metabolism. It is then possible that reduced ferrochelatase activity may cause haem deficiency which in turn leads to reduced ALA synthase activity. The results reported in Chapter 7 suggest that reduced ALA synthase activity may occur secondary to haem deficiency. This argument is further developed in Chapter 9 in which the case for and the results of a therapeutic trial of haem therapy in sideroblastic anaemia are described.

The spontaneous remission seen in a patient with transfusion-dependent CSA does, however, cast doubt over

reduced haem synthesis being the primary defect in SA. Prior to remission, ALA synthase was markedly reduced throughout erythroid development and HbA was predominantly produced (i.e. globin chain production was of type α and β). Following remission, 95% HbF was produced (i.e. β globin chain production was replaced by δ globin chain production) and ALA synthase levels were returned to normal. Hence, this patient has apparently overcome CSA by switching from β globin chain to δ globin chain production. This suggests that in this case of CSA the primary defect may lie at globin synthesis rather than at haem synthesis. Haem deficiency may, therefore, have been secondary, resulting in reduced ALA synthase activity. This remission may indicate a therapeutic role for agents which favour HbF production (i.e. increase δ globin chain production) in CSA , e.g. hydroxyurea or 5-AZA cytidine, as have been used to promote HbF production in sickle cell anaemia.

8.3.4 Summary

ALA synthase and ferrochelatase activities were measured in highly purified age-matched human erythroblasts from patients with CSA, PASA and SSA.

One patient with SSA had ALA synthase activity within the normal range. In the remaining patients (CSA/PASA) ALA synthase and ferrochelatase activities were reduced throughout erythropoiesis, particularly in the more immature erythroid cells. These results indicate that reduced ALA synthase activity is not present in all types of S.A. and is therefore unlikely to be the primary defect responsible for ring sideroblast formation. The marked reduction in ferrochelatase

activity in the six patients examined suggests a possible role for ferrochelatase deficiency and/or abnormal iron metabolism in the pathogenesis of this disease. As previously demonstrated (Chapter 7) haem deficiency may produce secondary inhibition of ALA synthase. This indicates a possible role for haem therapy in the treatment of patients with SA. This may not only replace the haem deficiency but may also stimulate the impaired ALA synthase activity.

However, the spontaneous remission observed in a patient with transfusion-dependent CSA suggests that the primary defect in this case of CSA may lie at globin rather than haem synthesis. This patient was apparently able to correct for the defect in CSA by switching from β globin chain to δ globin chain production.

CHAPTER 9

TREATMENT OF SIDEROBLASTIC ANAEMIA WITH HAEM ARGINATE: EFFECT ON HAEM SYNTHESIS

9 TREATMENT ON SIDEROBLASTIC ANAEMIA WITH HAEM ARGINATE: EFFECT ON HAEM SYNTHESIS

9.1 AIMS OF THE STUDY

It was the aim of this study to examine the effects of haem therapy on haem synthesis and Hb concentration in individuals with various types of SA.

Most cases of SA are refractory to treatment. Haem arginate is a new stable form of haem which has been used with some success in the treatment of porphyria (Herrick et al, 1987; Mustajoki et al, 1986). It has been reported to be of potential therapeutic benefit in the treatment of various haematological disorders (Ruutu et al, 1987). The use of haem arginate in the treatment of SA is studied in detail with regard to ALA synthase and ferrochelatase activities and Hb concentration in the work described here.

9.1.1 Haem in the Treatment of Haematological Disorders

Haem was first used to treat porphyria nearly 20 years ago (Bonkowsky et al, 1971). Extensive investigations of porphyrin metabolism have shown that the administration of exogenous haem in the form of haemin or haematin results in the repression of the overproduction of porphyrin precursors in acute intermittent porphyria (AIP) and corrects the haem deficiency.

Abnormalities of enzymes in the haem biosynthetic pathway, such as ALA synthase and ferrochelatase, have also been found in SA and myelodysplastic syndromes (MDS) (Chapter 8). The rationale for haem treatment in MDS can be described as follows. A characteristic feature of SA is defective Hb synthesis morphologically evident as hypochromia despite

abundant iron in the erythroblasts. Disturbances have been demonstrated in both haem and globin synthesis (White et al, 1971). In the study of White and Ali (1973) exogenous haem normalised globin synthesis and so it was concluded that haem deficiency was the basic disturbance in Hb production. This finding has, however, been disputed (Peters et al, 1983). Abnormalities in the enzymes of haem synthesis have been demonstrated in patients with MDS (Pasanen et al, 1981).

The beneficial effects of exogenous haem on 'in vivo' and 'in vitro' haematopoiesis are well established. Haemin increases the number of erythroid burst forming units (BFU-E) in the rat bone marrow 'in vivo' and enhances the cell cycling of BFU-E (Monette et al, 1984), augments colony formation by rat BFU-E 'in vitro' (Monette and Holden, 1982) and also induces maturation of leukaemic cell lines 'in vitro' (Rutherford et al, 1979), (further details in Chapter 1, Section 1.13).

The work so far described (Chapter 7) has confirmed the proposal that the regulation of the haem biosynthetic pathway is different in hepatic and erythroid cells. It has been demonstrated that in contrast to liver, haem deficiency in erythroid cells results in decreased ALA synthase activity. It has been suggested that the converse could be true (Granick and Sassa, 1978; Elder, 1981) i.e. that the administration of exogenous haem would result not only in the correction of the haem deficiency but also in increased activity of those enzymes of haem synthesis which are repressed. Therefore, if the primary abnormality in SA is reduced haem synthesis it would seem reasonable to attempt to correct this deficiency with

exogenous haem and assess the effects of treatment on enzyme activity and haematopoiesis.

The role of haem therapy in the treatment of haematological disorders is unclear as it is not yet known to what extent exogenous haem is able to enter bone marrow cells. Furthermore, it is not clear if haem deficiency in SA is a primary abnormality or is related to abnormal intracellular iron metabolism or whether both reduced haem biosynthetic enzyme activity and iron abnormalities are secondary events in the pathogenesis.

(i) Haematin

Haematin (haemin hydroxide), is an effective form of treatment for acute porphyric attacks. It can, however, cause side-effects such as phlebitis at the site of infusion (Dhar et al, 1975). Moreover, a transitory renal failure with relatively large doses has been reported (Dhar et al, 1978). Treatment may also cause disturbances in haemostasis and even haemorrhagic complications (Glueck et al, 1983; Morris et al, 1981; Petersen and Pierach, 1984). In solution, haem has a well known tendency both to aggregate (Shack and Clark, 1947) and to degrade (Jones, 1986; Goetsch and Bissell, 1986). It has been shown that the coagulation defects seen after haematin treatment are mainly caused by degradation and metabolisation products that bind to the coagulation proteins and reversibly inhibit their function (Jones, 1986).

(ii) Haem Arginate (Normosang)

Haem arginate was developed to find a well-tolerated and more stable haem compound (Tenhunen et al, 1987).

Theoretically, a stable haem compound with a low tendency to degrade may have fewer side effects.

Haem arginate is a salt of haem and the physiological, basic amino acid arginine. The active ingredient is haemin which is utilised effectively through the same pathways as physiological haem (Tokola, 1987).

9.2 EXPERIMENTAL

9.2.1 Patients Studied

Six male patients took part in the study (age range 23-83 years); one had CSA, the remainder had PASA. Of the five patients with PASA, three were stable and non transfusion dependent, one was newly diagnosed and one was transfusion dependent - see Appendix 2.

9.2.2 Haem Arginate Infusion

Haem arginate (3mg/kg) was given as an intravenous infusion over 30 minutes on days 1-5 and thereafter twice weekly for a further five weeks (i.e. total treatment period was 6 weeks).

9.2.3 Enzyme Measurements

ALA synthase and ferrochelatase activities were measured in whole and fractionated human bone marrow prior to treatment (Day 0) and during treatment (Day 5, Week 6).

9.3 RESULTS

9.3.1 Haemoglobin Levels

Hb levels remained unchanged throughout treatment in all patients.

9.3.2 Bone Marrow Morphology

Bone marrow morphology did not change during treatment. The percentage ring sideroblasts did not alter.

9.3.3 Haem Enzyme Activity in Whole Unfractionated Bone Marrow

No results were obtained for Patient 4 on day 5 due to inadequate cell numbers in the marrow sample taken on that day.

(i) ALA Synthase Activity

Prior to haem arginate treatment, the mean recorded ALA synthase activity in whole unfractionated bone marrow was 242 $\pm$ 142 ($\pm$ 1 S.D.) (Table 21). Enzyme activity rose to a level of 393 $\pm$ 141 ($\pm$ 1 S.D.) by day 5 of treatment. However, this rise in activity was not sustained and by week six had returned to a value of 215 $\pm$ 144 ($\pm$ 1 S.D.). With regard to the individual patients, four of the five (1 CSA, 3 PASA) showed significant increases in enzyme activity on day 5 of treatment, two of which were restored into the normal range (Table 21). The increase in enzyme activity was not sustained to week 6 of treatment in any of the four patients (Table 21).

(ii) Ferrochelatase Activity

Prior to haem arginate treatment, ferrochelatase activity was undetectable in four of the six patients and well below the normal range in the remaining two patients (Table 22). At day 5, three of five patients showed a marked improvement with enzyme activity returning to near normal levels. The observed increase in enzyme activity was sustained to week 6 in only one patient. In the remaining two patients ferrochelatase activity did not improve during haem arginate treatment.

ALA SYNTHASE ACTIVITY
(p moles ALA/10^6 erythroblasts/hour)

DAY OF TREATMENT WITH HAEM ARGINATE	PATIENT 1 PASA	PATIENT 2 CSA	PATIENT 3 PASA	PATIENT 4 PASA	PATIENT 5 PASA	PATIENT 6 PASA	$\bar{x} \pm$ 1S.D.
0	472	188	248	598	211	89	242 ± 142
5	366	419	608	*	353	219	393 ± 141
42	339	231	369	448	50	88	215 ± 144

NOTE:

ALA synthase activity: normal range = 608 ± 334 ($\bar{x} \pm$ 1 S.D.)

PASA = primary acquired sideroblastic anaemia

CSA = congenital sideroblastic anaemia

* = no result available due to small sample size

TABLE 21

THE EFFECT OF HAEM ARGINATE TREATMENT ON ALA SYNTHASE ACTIVITY IN WHOLE BONE MARROW FROM PATIENTS WITH SIDEROBLASTIC ANAEMIA

FERROCHELATASE ACTIVITY
(p moles haem/10^6 erythroblasts/hour)

DAY OF TREATMENT WITH HAEM ARGINATE	PATIENT 1 PASA	PATIENT 2 CSA	PATIENT 3 PASA	PATIENT 4 PASA	PATIENT 5 PASA	PATIENT 6 PASA
0	N.D.	N.D.	42	42	N.D.	N.D.
5	72	144	84	*	N.D.	N.D.
42	81	N.D.	24	N.D.	N.D.	N.D.

NOTE:

Ferrochelatase activity: normal range = 164 $\pm$ 50 ($\bar{x} \pm 1$ S.D.)

PASA = primary acquired sideroblastic anaemia

CSA = congenital sideroblastic anaemia

N.D. = not detectable

* = no result available due to small sample size

TABLE 22

THE EFFECT OF HAEM ARGINATE TREATMENT ON FERROCHELATASE ACTIVITY IN WHOLE BONE MARROW FROM PATIENTS WITH SIDEROBLASTIC ANAEMIA

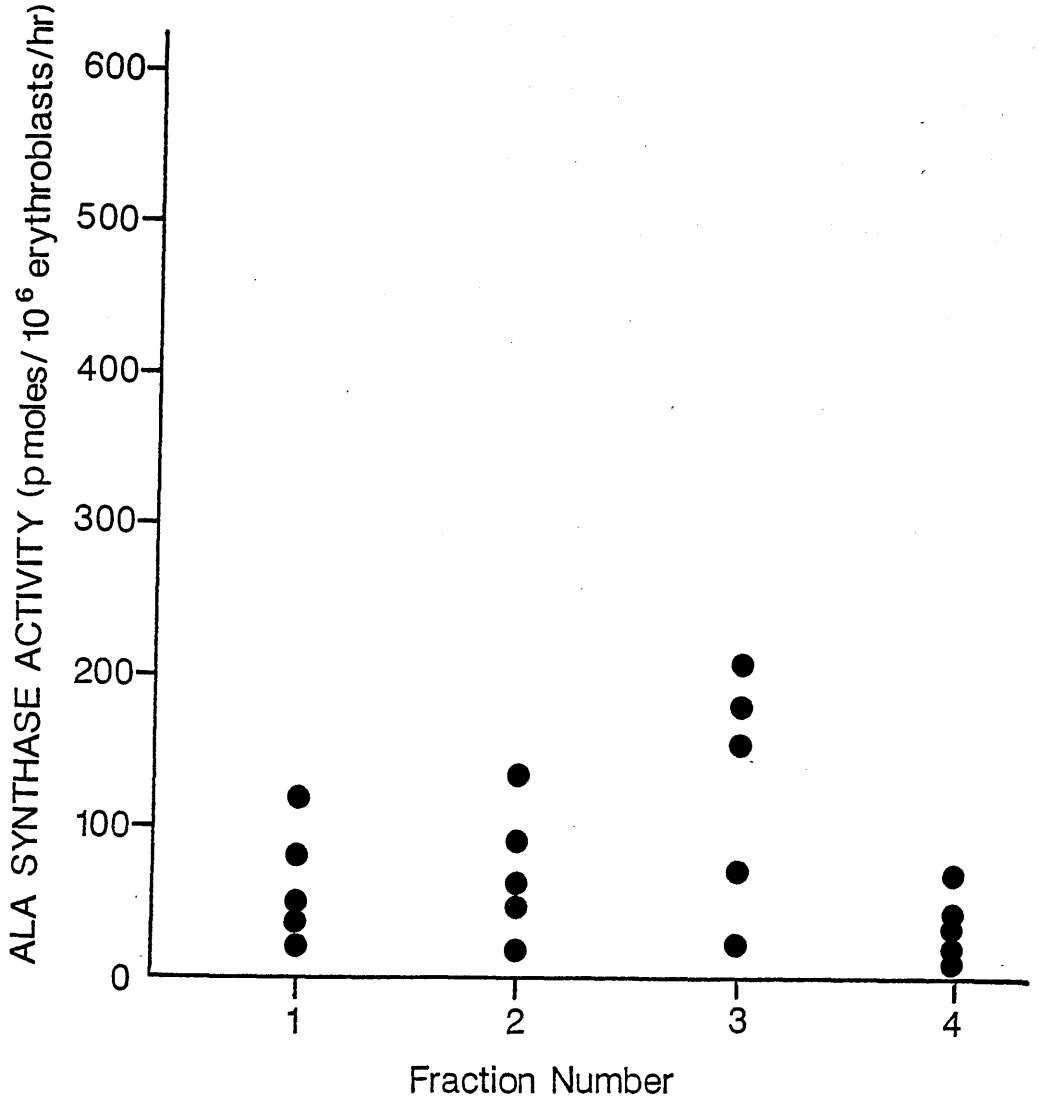
These results suggest a possible positive effect of haem arginate on haem enzyme activity in certain patients with SA. However, no firm conclusion may be drawn from this study of unfractionated marrow due to the difficulty in assessing the relative contribution of the erythroid cells to the total marrow activity (Chapter 2). Fractionation of the bone marrow samples was carried out to gain insight into the effects of haem therapy on erythroid haem synthesis during the various stages of erythroid differentiation.

9.3.4 Haem Enzyme Activity in Fractionated Bone Marrow

Prior to treatment, all patients (1 CSA, 5 PASA) demonstrated reduced erythroblast ALA synthase activity (as described in Chapter 8) in each of the four percoll fractions (Figure 42). Enzyme activity remained low throughout erythropoietic development and failed to display the characteristic peak of ALA synthase activity in fractions 1 and 2 as demonstrated during normoblastic erythropoiesis (Chapter 6, Figure 30). All reductions in enzyme activity were statistically significant (Mann Whitney U test, $p < 0.001$).

Ferrochelatase activity was also significantly reduced in all four percoll fractions such that four of the six patients had no detectable enzyme activity. The remaining two patients had low levels of ferrochelatase activity in each fraction which were significantly reduced ($p < 0.001$) relative to those found during normoblastic erythropoiesis (Chapter 6, Figure 31).

Results for five patients indicate an increase in ALA synthase activity following five days of treatment with haem



NOTE :
Each point represents the mean of two observations.

FIGURE 42

ALA SYNTHASE ACTIVITY IN FRACTIONATED ERYTHROBLASTS FROM THE SIDEROBLASTIC PATIENTS (PRIOR TO TREATMENT WITH HAEM ARGINATE)

arginate (Table 23, Figure 43). This was particularly significant ($p < 0.01$, $p < 0.05$) in the more immature cells of fractions 1 and 2 i.e. those cells normally most active with regard to ALA synthase activity. In these fractions, all patients showed increased ALA synthase activity on Day 5. This increase in activity was partially sustained until week six of treatment (Figure 44) with three of five patients maintaining ALA synthase activity in fractions 1 and 2 markedly above that of Day 0 values. Enzyme activity in the remaining two patients returned to Day 0 values.

Due to the small patient numbers in the study, the results were also examined on an individual basis. Two of the six patients demonstrated a dramatic response to haem arginate treatment with regard to haem enzyme activity. Patient One with PASA (Figure 45) and Patient Two with CSA (Figure 46) displayed a sharp rise in ALA synthase activity on day five achieving values within the normal range for percoll Fractions 1 and 2. Normal ALA synthase levels were sustained until week six in Patient 1 but fell back outwith the normal range in Patient 2 (but still remained significantly higher than those recorded on Day 0).

Ferrochelatase activity, however, was very low in all the patients. By day 5 Patients 1 and 2 again demonstrated levels of activity in the normal range (Tables 24 and 25). This increase in enzyme activity was not, however, sustained until week 6 (Tables 24 and 25).

9.4 DISCUSSION

Markedly reduced ALA synthase and ferrochelatase

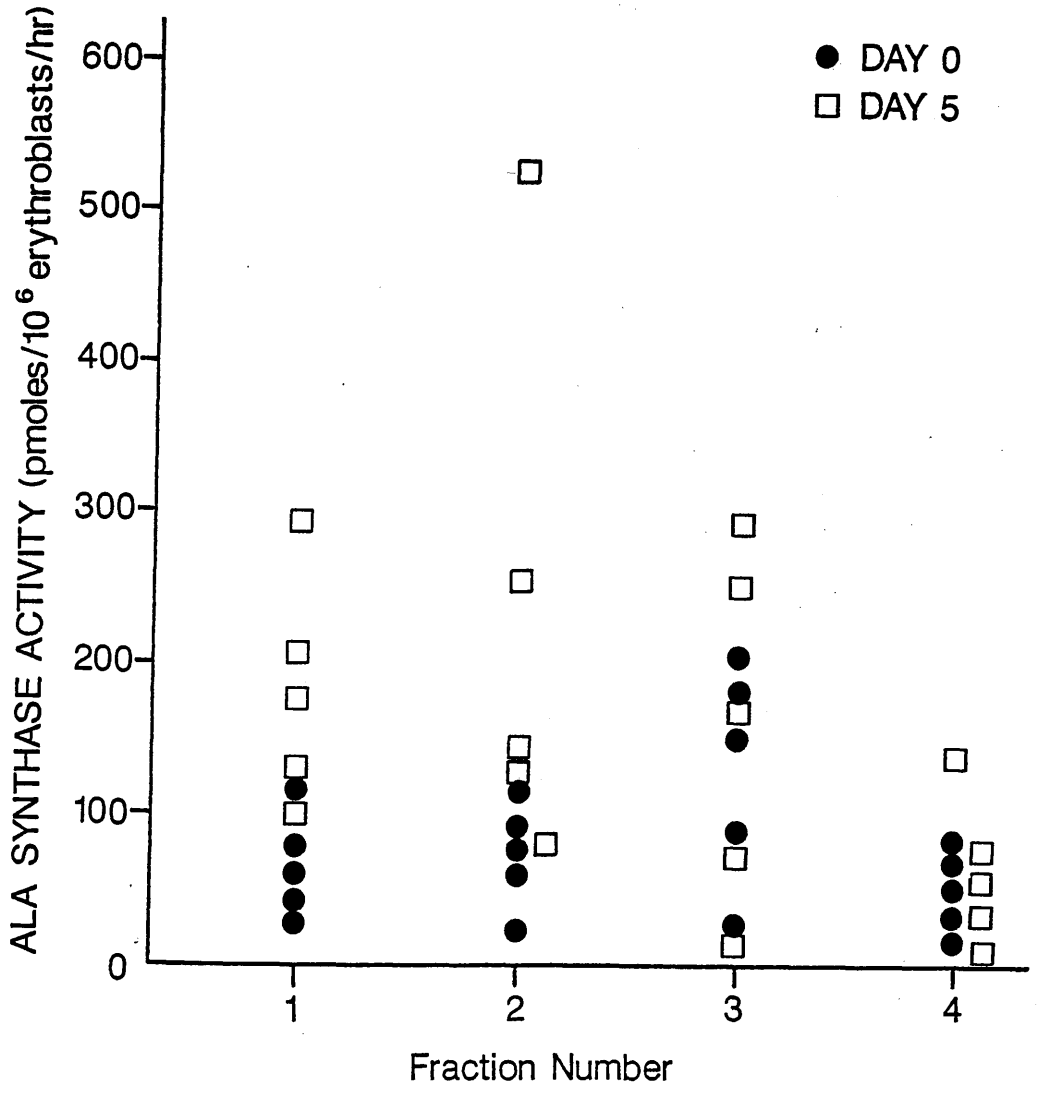
	ALA SYNTHASE ACTIVITY (pmoles/10^6erythroblasts/hour)		
DAY OF TREATMENT WITH HAEM ARGINATE	DAY 0	DAY 5	WEEK 6
FRACTION 1	70 ± 31	175 ± 127	166 ± 135
FRACTION 2	68 ± 40	218 ± 191	160 ± 116
FRACTION 3	133 ± 61	154 ± 112	105 ± 59
FRACTION 4	39 ± 17	53 ± 53	47 ± 41

NOTE:

Each point represents the mean of six duplicate observations ($\bar{x}$ ± 1S.D.)

TABLE 23

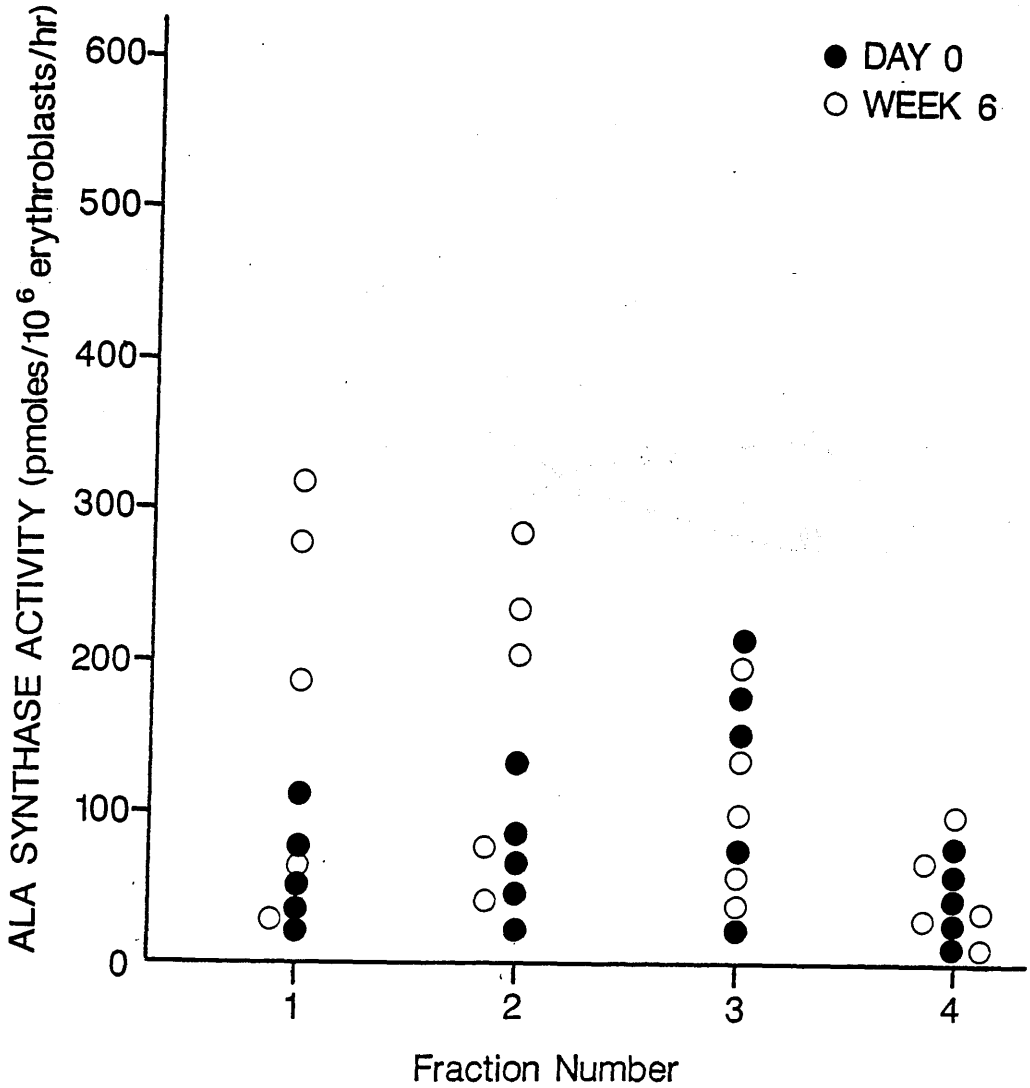
THE EFFECTS OF HAEM ARGINATE TREATMENT ON ALA SYNTHASE ACTIVITY DURING SIDEROBLASTIC ERYTHROPOIESIS



NOTE :
Each point represents the mean of two observations.

FIGURE 43

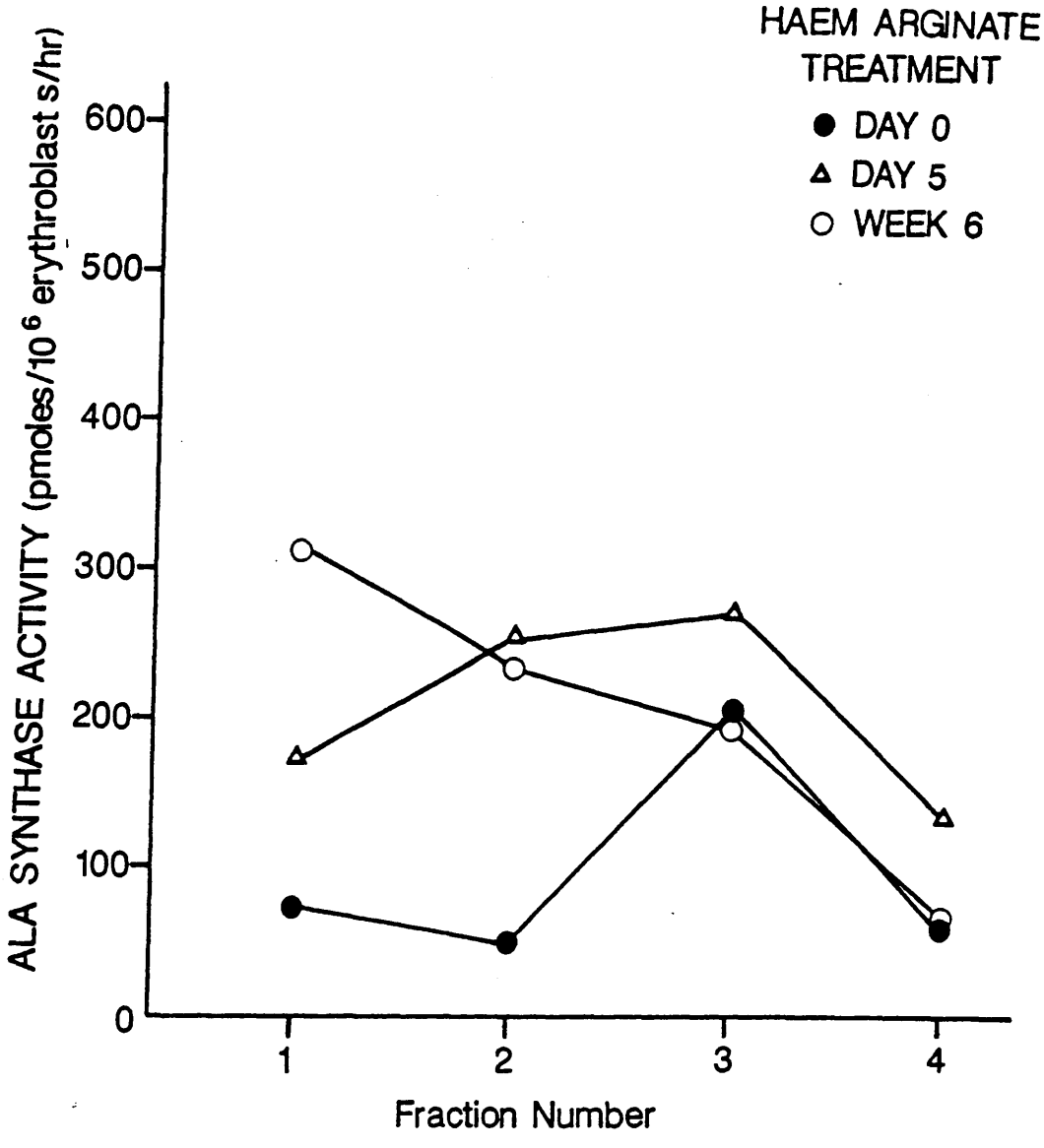
EFFECT OF HAEM ARGINATE ON ERYTHROBLAST ALA SYNTHASE ACTIVITY : DAY 5 OF TREATMENT



NOTE :
Each point represents the mean of two observations.

FIGURE 44

EFFECT OF HAEM ARGINATE ON ERYTHROBLAST ALA SYNTHASE ACTIVITY : WEEK 6 OF TREATMENT

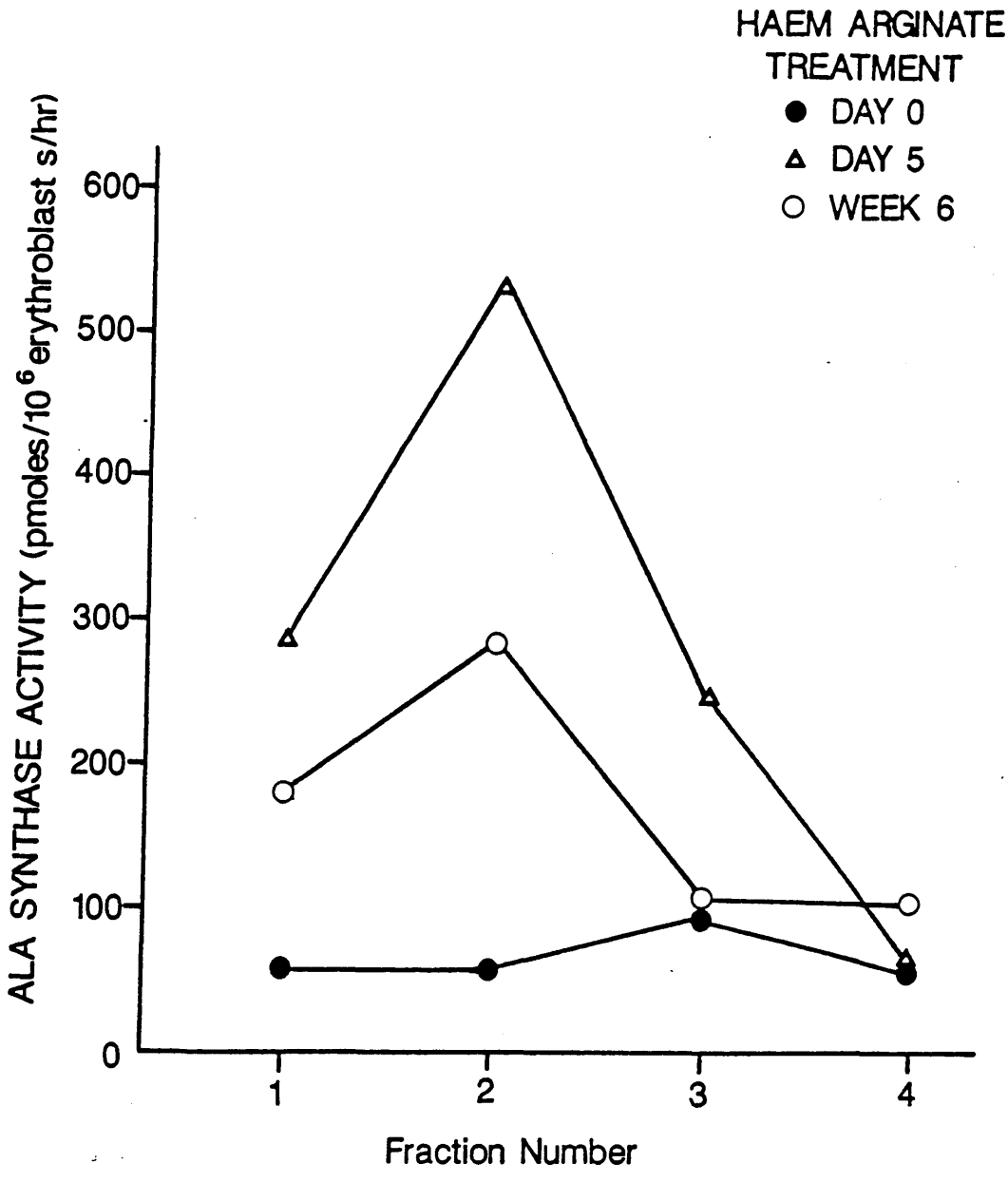


NOTE :
Each point represents the mean of two observations.

FIGURE 45

PATIENT 1 : 1°ACQUIRED SIDEROBLASTIC ANAEMIA :

EFFECT OF HAEM ARGINATE THERAPY ON ALA SYNTHASE ACTIVITY



NOTE :
Each point represents the mean of two observations.

FIGURE 46

PATIENT 2 : CONGENITAL SIDEROBLASTIC ANAEMIA :

EFFECT OF HAEM ARGINATE THERAPY ON ALA SYNTHASE ACTIVITY

FERROCHELATASE ACTIVITY
(pmoles/10^6 erythroblast s/hr)

HAEM ARGINATE TREATMENT	Fraction Number 1	2	3	4
DAY 0	N.D.	N.D.	N.D.	N.D.
DAY 5	9	36	123	6
WEEK 6	N.D.	18	21	N.D.

NOTE :
Each point represents the mean of two observations.
N.D. = not detectable

TABLE 24

PATIENT 1 : 1°ACQUIRED SIDEROBLASTIC ANAEMIA :
EFFECT OF HAEM ARGINATE THERAPY ON FERROCHELATASE ACTIVITY

FERROCHELATASE ACTIVITY
(pmoles/10^6 erythroblast s/hr)

HAEM ARGINATE TREATMENT	Fraction Number 1	2	3	4
DAY 0	N.D.	N.D.	N.D.	N.D.
DAY 5	45	72	36	18
WEEK 6	N.D.	N.D.	N.D.	N.D.

NOTE :
Each point represents the mean of two observations.

N.D. = not detectable

TABLE 25

PATIENT 2 : CONGENITAL SIDEROBLASTIC ANAEMIA :
EFFECT OF HAEM ARGINATE THERAPY ON FERROCHELATASE ACTIVITY

activities were demonstrated throughout erythropoiesis in six patients with CSA and PASA (in agreement with the results previously discussed in Chapter 8). Following haem arginate treatment, the six patients demonstrated no change in Hb levels. Therefore, haem did not correct the inefficient erythropoiesis that characterised all the cases of SA. However, despite the absence of a direct therapeutic benefit, two of the six patients demonstrated significant changes in haem enzyme activities. In response to haem therapy, both ALA synthase and ferrochelatase activities in the early percoll fractions returned to values within the normal range at day 5.

This study provides evidence that during systemic haem arginate therapy haem is able to enter developing erythroblasts and influence haem biosynthetic activity and thereby suggests a potential therapeutic role for haem arginate in the treatment of patients with abnormalities of erythroid biosynthesis.

Two patients demonstrated increased haem enzyme activity in response to treatment, one PASA the other CSA. A further four patients with PASA failed to respond to treatment. Such findings highlight the possible heterogeneous nature of the defect responsible for the pathogenesis of PASA. The normalisation of ALA synthase with haem arginate treatment in CSA without morphological or clinical improvement casts doubt over the belief that reduced ALA synthase is the primary defect in CSA. This theory has been supported by several workers (Cartwright and Deiss, 1975; Aoki et al, 1974) and has been reinforced by the recent localisation of the erythroid ALA synthase gene to the X chromosome (Cox et al, 1990) (CSA usually has an X-linked inheritance pattern, Bottomley, 1982).

However, the demonstration here of marked improvement in ALA synthase activity in response to haem arginate treatment without clinical improvement suggests that impaired ALA synthase activity is not the primary defect in this case of CSA. This is consistent with evidence that, in this patient, CSA is unlikely to be X-linked. The patient's mother could not be shown to be a carrier for CSA and five brothers were unaffected. The patient was, however, pyridoxine responsive suggesting a possible abnormality of the enzyme cofactor, PLP. ALA synthase is unstable in the absence of PLP, but in the presence of haem the resultant increase in enzyme activity may perhaps be sufficient to mask the effects of reduced enzyme stability. Ferrochelatase activity in this CSA patient also responded well to treatment, returning into the normal range on day five. This implies that ferrochelatase deficiency does not represent the primary defect in this case of CSA which may instead lie in a defect at an intermediate enzyme in the pathway or at an abnormality of intracellular iron metabolism. Hence, the nature of the defect in CSA remains uncertain. The same argument could apply to the patient with PASA whose haem enzymes responded well to haem therapy.

The non-sustained effect of haem therapy on haem enzyme activity (Patient 2) may indicate induction of haem oxygenase activity in response to the presence of exogenous haem. This observation may warrant follow up with a therapeutic trial of combined haem arginate and tin protoporphyrin, a potent inhibitor of haem oxygenase (Drummond and Kappas, 1981), as a means of sustaining the enzyme response. Tin protoporphyrin

has recently been reported to have therapeutic value in the mangement of hyperbilirubinaemia in newborn infants (Kappas et al, 1988).

Haem arginate may therefore be of therapeutic benefit in future studies. The possibility remains whereby further patients with SA may be treated with haem arginate, perhaps in conjunction with tin-protoporphyrin. However, the failure of two patients to show any clinical improvement despite normalisation of haem enzyme activity may dampen enthusiasm for this study. Nevertheless, patients with other haematological abnormalities may be potential candidates for haem arginate therapy.

As a direct consequence of this work a further study has been funded (Nuffield Foundation) to examine the effects of haem arginate treatment on haem synthesis in patients with anaemia of chronic disease such as rheumatoid arthritis. The anaemia is characterised by iron deficient red cell indices with the paradoxical association of reduced serum iron despite normal/increased reticuloendothelial iron stores (Cartwright, 1966). Macrophages have been widely considered as responsible for this reticuloendothelial iron block (Van Snick et al, 1974). However, ferrokinetic studies have established that the hypoferraemia does not restrict marrow iron supply (Dinant and De Maat, 1978; Cavill and Bentley, 1982). Hypochromic microcytic red cells provide absolute evidence of Hb deficiency. Limited studies have identified abnormalities of haem biosynthesis. Erythrocyte protoporphyrin levels are raised and unexplained (Lee, 1983) and coproporphyrinogen oxidase activity is markedly reduced (Campbell et al, 1978).

It has been postulated that a failure of ALA synthase and haem synthesis to increase in response to anaemia leads to marrow unresponsiveness (Campbell et al, 1978).

Finally, a finding of major significance from this work is the demonstration that erythroid ALA synthase has the ability to respond to haem argiante therapy in a manner which is opposite to that of "hepatic" ALA synthase i.e. the erythroid enzyme may be induced in response to exogenous haem. This is consistent with the earlier findings (Chapter 7) whereby erythroid ALA synthase was reduced in response to haem deficiency and thus represents very strong evidence in favour of the theory that the isoenzymes of ALA synthase in "hepatic" and erythroid tissue operate under strict and specific control mechanisms. Haem synthesis appears to be regulated by the size of the free haem pool in both tissues. However, in the case of the erythroid enzyme the effects of haem are linked to the differentiation status of the cells. Haem is thought to inhibit reticulocyte ALA synthase (Bruns and London, 1965; Ibrahim et al, 1978) but stimulate activity in immature erythroblasts (Granick and Sassa, 1978; Elder, 1981; Abraham and Levere, 1990). Other workers, however, have concluded from studies using FEL cells that the induction of erythroid ALA synthase gene transcription is developmentally regulated and largely unaffected by intracellular haem balance (Young and Dierks, 1990).

Due to the complicated nature of the erythropoietic process and the multiplicity of factors which are involved in its regulation (Chapter 1), it is unlikely that the regulation of erythroid haem synthesis will be fully understood for some

time yet. Further work is required to examine the contribution of other factors to the regulatory mechanisms which operate. The role of other haem enzymes and intracellular iron metabolism in regulating erythroid haem synthesis have yet to be established.

9.4.1 Summary

Six patients with SA (1 CSA, 5 PASA) were given haem arginate infusions (3mg/kg) over a 6 week period in an effort to correct the haem deficiency, stimulate erythroid haem synthesis and restore more effective erythropoiesis to the sideroblastic clone. At day 5, two of six patients (1 CSA, 1 PASA) demonstrated significant rises in ALA synthase and ferrochelatase activities in fractionated bone marrow with levels rising into the normal range. Despite the dramatic increase in enzyme activity none of the patients showed any change in Hb levels. The remaining four patients (PASA) showed no enzyme response to treatment.

These results question the nature of the primary defect in CSA. The primary defect in X-linked CSA is generally believed to lie at ALA synthase, a view reinforced by the recent localisation of the erythroid ALA synthase gene to the X chromosome. However, the dramatic response of ALA synthase to haem therapy in the patient described here was not associated with clinical benefit. Moreover, the similar response of ferrochelatase to haem therapy in this patient suggests that the primary defect is unlikely to lie at ferrochelatase.

The heterogeneous nature of PASA is highlighted by the

fact that only one of five patients responded to haem therapy (in a similar manner to the patient with CSA) while the others did not.

The increase in enzyme activity in the two patients was only partially sustained at week six and so suggested haem oxygenase induction. This further suggests a role for combined therapy of haem arginate with tin-protoporphyrin, a potent inhibitor of haem oxygenase.

Although a direct therapeutic benefit of haem arginate therapy in the treatment of SA was not apparent, the response of the haem enzymes in two of six patients indicates that haem arginate does enter erythroblasts and is able to influence enzyme activity. This may indicate a potential role for haem arginate in the treatment of other haematological disorders in the future.

Finally, this work has demonstrated that the isoenzymes of ALA synthase in hepatic and erythroid tissue operate under distinct control mechanisms. In response to exogenous haem, erythroid ALA synthase may be induced while conversely hepatic ALA synthase is inhibited under the same conditions. The increase of erythroid ALA synthase in response to exogenous haem is compatible with the earlier observation (Chapter 7) that erythroid ALA synthase activity is reduced by haem deficiency.

CHAPTER 10

CONCLUSIONS

10 CONCLUSIONS

This thesis has presented evidence supporting different regulatory mechanisms for haem biosynthesis in erythroid and hepatic tissue. This is consistent with the recent identification of tissue-specific isoenzymes for ALA synthase which are encoded for by separate genes (Sutherland et al, 1988; Cox et al, 1990).

ALA synthase is rate-limiting for hepatic haem biosynthesis and is under negative feedback control by haem. In erythroid tissue, however, ALA synthase activity appears to be decreased in response to haem deficiency. The reduced ALA synthase activity that characterises SA may be increased by haem infusion. This work has further shown that although exogenous haem increases ALA synthase and ferrochelatase activity in certain patients with SA, this improvement did not lead to increased Hb concentration. The ability of exogenous haem to influence erythroblast haem synthesis does nevertheless demonstrate that haem arginate can enter developing red cells and indicates a potential therapeutic role for haem in the treatment of haematological disorders associated with reduced haem synthesis. These observations cast doubt over the belief that reduced ALA synthase activity is the primary abnormality which results in impaired haem synthesis in SA. Reduced ALA synthase activity may, in fact, be secondary to the haem deficiency.

The primary site(s) of regulation of erythroid haem synthesis has not yet been identified. This work has demonstrated that erythroid ALA synthase is sensitive to intracellular haem levels, particularly in the most immature

erythroid cells. Ferrochelatase in sideroblasts is sensitive to haem, but the effect of haem deficiency on ferrochelatase is, as yet, unknown.

Control of the haem enzymes is likely to occur at gene level. As with ALA synthase, different isoenzymes of PBG deaminase have been identified in human liver and erythroid tissue (Grandchamp et al, 1987). They are encoded by distinct tissue-specific mRNAs but, unlike ALA synthase, are transcribed from a single gene (Raich et al, 1986). The genes for erythroid and hepatic ALA synthases have been located on chromosome X and chromosome 3 respectively (Cox et al, 1990; Sutherland et al, 1988). Separate promoters of a single gene for PBG deaminase may account for the tissue-specific and tissue-nonspecific mRNA transcripts and their respectively encoded enzymes (Chretien et al, 1988; Mignotte et al, 1990).

Gene studies will provide clearer understanding of erythroid haem synthesis, in the near future, with the cloning of all the cDNAs and genes for the haem enzymes. Cloned cDNA may be expressed in microorganisms to produce large quantities of encoded proteins. This would allow crystallisation and subsequent determination of the three-dimensional structure of each enzyme. Site-directed mutagenesis can create mutant enzymes with altered properties. Analysis of such enzymes may then allow a better understanding of enzyme function in relation to structure.

Determination of DNA sequences responsible for the transcriptional regulation of haem enzyme genes may explain genetic expression and regulation. The most challenging issue

is to understand what links erythropoietic differentiation with increased expression of the haem enzyme genes. The mammalian genes are not clustered and it is therefore likely that some common factor(s) coregulate their expression. These factors and the DNA sequences that they bind have not been identified.

APPENDICES

Appendix 1 - General Methods

Culture Medium

Culture medium contained Eagles MEM, buffered with 20mM Hepes to pH 7.4 at 37°C, with glycine 1mM. This was prepared in 500 ml aliquots; 90 ml was removed from a 500 ml bottle of sterile distilled water to which 50 ml Eagles MEM (x 10 concentrated), 10 ml Hepes (Gibco) 1M pH 7.3, 4 ml 1N NaOH and 3 ml glycine, 20 mg/ml were added.

Bone Marrow Collecting Medium

Bone marrow samples were collected into culture medium containing 25% (v/v) heat inactivated foetal calf serum (Gibco) and preservative free heparin 15 U/ml.

Bone Marrow Cell Counts

Cell counts on whole and fractionated bone marrow were made using a Coulter Counter Model ZF fitted with a channelyzer attachment.

Bone Marrow Cytospin Preparations

Bone marrow cells were washed x 3 in cold culture medium. Heat inactivated foetal calf serum, filtered through a 0.22 μm micropore filter, was added to the bone marrow cells to achieve a final concentration of 2×10^5 nucleated cells per ml. 0.25 ml was added to cytospin holders in a Shandon Southern cytocentrifuge. Centrifugation was at 600 rpm for 14 minutes. Cell morphology was optimally preserved at 5×10^4 nucleated cells per slide.

Staining Procedures

Morphological examination of marrow cells was made on

cytospin and marrow aspirate preparations stained with Jenner Geimsa stain. Cytochemical staining for iron or aspirated bone marrow material was made by Perls stain.

ALA Sythase Assay

(i) Incubation Medium

Incubation medium used was a modification of that described by Tikerpae et al (1981) and contained glycine 50 mM, sucrose 250 mM, $MgCl_2$ 2mM, EDTA 5 mM, Tris 40 mM buffered to pH 7.4 with KH_2PO_4 50 mM. This was prepared in distilled H_2O (N.B. not deionised) in a volume of 1 litre, then stored in aliquots of 20 ml at -20°C. On the day of the assay PLP 0.4 mM, CoA 1.35 mM, succinylacetone 2 mM, succinate thiokinase (STK) 1 unit per ml and GTP 5.0 mM were added.

(ii) Radiochemicals

Radiochemicals were purchased from Amersham International plc. 2-3-[^{14}C] succinic acid was stored at -20°C in 0.21 ml aliquots of 20 mM succinate (specific activity 3.0 Ci/mol) in incubation medium.

5-amino 4-[^{14}C] laevulinic acid hydrochloride was stored at -20°C in 0.05ml aliquots of 0.25 mM ALA (0.5 μCi/0.05 ml, specific activity 40 Ci/m mol).

Modified Ehrlichs Reagent

Modified Erhlichs reagent was prepared fresh daily by dissolving 1g p-DMAB in 30 ml glacial acetic acid. 8 ml 70% perchloric acid was added and the final volume made up to 50 ml with glacial acetic acid (Falk, 1964).

Statistics

Statistical analysis of results was performed using the Mann Whitney U test.

Appendix 2 - Patient Details

Ethical Approval for the studies was obtained from the Ethical Committee of Monklands District General Hospital and the Western Infirmary, Glasgow.

Normoblastic Marrow

Marrow was aspirated from 15 patients with normoblastic erythroid hyperplasia. Hyperplasic marrow is required to guarantee satisfactory erythroid fractionation on the percoll gradients.

Ten patients had Pernicious Anaemia (PA) with Hb concentrations < 8g/dl. Marrow was sampled on day 5 of treatment with B_{12} 1000µg/day, folic acid 5mg bd and ferrous sulphate 200mg tds. Marrow taken on day 0 had in all cases shown frank megaloblastic changes. These changes are corrected within 48 hours of treatment and by day 5 all appearances were hyperplastic but normoblastic. Ferrous sulphate was given to prevent the fall in serum iron concentration which follows B_{12} administration in PA. Stainable iron was demonstrated in the marrow reticuloendothelial cells in all cases. Transferrin saturation was between 25 and 54% ensuring adequate iron supply to erythroblasts. Reticulocyte counts on day 5 were between 18 and 40% reflecting conversion of ineffective megaloblastic erythropoiesis to normoblastic and effective.

Five patients had hereditary haemolytic anaemias - three patients had hereditary spherocytosis, one had pyruvate kinase deficiency and the fifth had hereditary pyropoiklocytosis. All patients had active normoblastic erythropoiesis with

reticulocyte counts greater than 15%. Haematinic levels (B_{12}, folate, ferritin) were normal in all patients.

One patient had autoimmune haemolytic anaemia. Again the marrow showed erythroid hyperplasia with normal haematinic levels.

One additional patient with autoimmune haemolytic anaemia is described in Chapter 6. This patient became unwell prior to marrow aspiration requiring treatment with prednisolone 60mg/day. This steroid treatment started 5 days prior to marrow sampling. As such, the results of ALA synthase and ferrochelatase activities in this patient are reported separately.

Iron Deficient Polycythaemia (IDP)

Ten patients were studied. Eight patients had primary polycythaemia i.e. Polycythaemia Rubra Vera. Two patients had IDP secondary to chronic obstructive airways disease and chronic hypoxia. Serum ferritin concentrations were reduced (< 10ng/ml) in all caess. Serum iron levels were low and TIBC values raised. In all cases transferrin saturation was < 10% and no stainable iron was evident in the marrow.

Sideroblastic Patients

Sideroblastic anaemia was established by the demonstration of ring sideroblasts in the bone marrow to exceed 30% of the nucleated erythroid cells.

Congenital Sideroblastic Anaemia (CSA)

Three patients with hypochromic microcytic CSA have been studied in this work. Two of the patients were children aged 3

and 4 years with transfusion dependent CSA. Family studies were not available as both children lived in England. In neither case, however, was there a positive family history. One child underwent spontaneous remission of his CSA and this remission is discussed in detail in Chapter 8.

The third patient with CSA was a 21 year old male student studied in Chapter 9. His anaemia was congenital and pyridoxine responsive. The mode of inheritance was unclear, but did not appear to be X-linked as no abnormal cells were seen in the mother (who would be an obligate carrier for an X-linked disorder) and five brothers were unaffected. At the time of study his Hb concentrations was 8.0g/dl MCV 52 fl. Without pyridoxine supplementation he was unable to maintain his Hb above 6.0g/dl and was transfusion dependent.

Secondary Sideroblastic Anaemia (SSA)

One woman (aged 24 years) with Secondary Sideroblastic Anaemia (SSA) was studied. This woman had Wilson's disease and developed sideroblastic erythropoiesis in response to treatment with TRIENTENE (a copper chelating agent). She had a mild (Hb 10.5g/dl) microcytic anaemia (MCV 72fl) with adequate iron stores (serum ferritin 70ng/ml) and abundant ring sideroblasts (> 30%) in her marrow.

Primary Acquired Sideroblastic Anaemia (PASA)

Eight patients had primary acquired sideroblastic anaemia (PASA). Seven patients were long established cases, each of more than 3 years duration. These seven cases were all significantly anaemic (Hb < 10g/dl). One patient was transfusion dependent while the other six were not. All cases

showed gross erythroid hyperplasia, dyserythropoiesis and > 80% ring sideroblasts. One patient was studied within 3 months of diagnosis. His Hb was slightly reduced (13.0g/dl) but his marrow showed abundant ring sideroblast formation (> 75%).

Cytogenetic studies were carried out on the six patients treated with haem arginate (Chapter 9). All cases showed a normal male karyotype. In two patients random loss of individual chromosomes was noted.

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