5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN HUMAN ERYTHROBLASTS

by

© EDWARD J FITZSIMONS

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To Alison and Ian

but not for colouring in!

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

The writing of this thesis is entirely my own work.

CONTENTS

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PUBLICATIONS AND COMMUNICATIONS TO LEARNED SOCIETIES BASED ON THE WORK IN THIS THESIS

Publications

1984 Fitzsimons E J, May A, Elder G H, Jacobs A.
Bone marrow ALA synthase activity.
British Journal of Haematology, 56, 675.

1986 Fitzsimons E J, May A, Elder G H, Jacobs A.
Measurement of 5-aminolevulinic acid synthase activity in whole and fractionated human bone marrow: effect of myeloid cell lysis by monoclonal antibody.
Analytic Biochemistry, 152 (in press)
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1986 Fitzsimons E J, May A, Barnes K, Elder G H, Jacobs A.
5-aminolevulinic acid synthase activity in human erythroblasts
British Journal of Haematology, in press

Communications

1. British Society for Haematology. London, November 1983.
   Measurement of ALA synthase activity in human bone marrow.

2. Tetrapyrrole Discussion Group. London, January 1984
   A new sensitive HPLC assay for ALA synthase activity in human bone marrow

3. The Red Cell Club. Oxford, June 1984
   ALA synthase activity in normal and PASA erythroblasts

4. The Iron Club. Rennes, France, July 1984
   Reduced ALA synthase activity in primary acquired sideroblastic anaemia and congenital dyserythropoietic anaemia

5. British Society for Haematology. Cambridge, April 1986
   Erythroblast ALA synthase activity

SUMMARY

ALA synthase, localised within the inner mitochondrial membrane, has been identified as the rate limiting enzyme of hepatic haem biosynthesis, subject to negative feedback control by haem. Liver, however, is a minor haem forming tissue as erythroblasts synthesise 85% of the total daily haem and are the cells most sensitive to haem deficiency. Within developing erythroid cells haem is required both for its specific complexing with globin and for the co-ordinate regulation of erythroblast metabolism and differentiation.

It does then seem surprising that in contrast to our understanding of hepatic haem biosynthesis and its regulation by ALA synthase, little is known about haem synthesis in erythroblasts or the factors that regulate the activities of the enzymes in the biosynthetic pathway. The principal problems that have hampered the study of erythroid haem synthesis have been the heterogeneity of cells in bone marrow samples in respect to lineage and maturity and the lack of suitably sensitive haem enzyme assays. The work detailed in this thesis has overcome these problems and has used "in vivo" human erythroblasts to examine the effect of normal and abnormal erythroid differentiation on ALA synthase activity.

A highly sensitive radiochemical assay of ALA synthase activity, capable of detecting enzyme activity in 2 x 10^5 bone marrow cells, is described. This developed assay utilised reverse phase high performance liquid chromatography to isolate [^{14}C]-ALA pyrrole from radioactive substrate and metabolites. [^{14}C]-succinate was preferred to 2-keto[^{14}C]glutarate as radioactive substrate and the enzyme assay was optimised with respect to marrow sample preparation, ALA pyrrole production, [^{14}C]-succinate, CoA, GTP, succinate thiokinase, EDTA and succinylacetone concentration to obtain maximal ALA synthase activity. The mean enzyme activity in 11 normal unfractionated bone marrow samples (893 $\pm$ 199 pmol ALA/10^6 erythroblasts/h) was approximately 50% greater than that recorded with the next most sensitive assay method.

Cytotoxic (IgM) monoclonal antibody TG-1 is specific for myeloid cells. It was used to obtain highly purified populations of marrow erythroblasts and to calculate the percentage contribution made by myeloid cells to ALA synthase activity in unfractionated bone marrow samples. In both normal and sideroblastic samples approximately 50% of the total enzyme activity was shown to be myeloid in origin. As previous studies using unfractionated human bone marrow have assumed insignificant myeloid ALA synthase activity, their results and conclusions require to be reassessed.

Age matched populations of erythroblasts were obtained by percoll equilibrium density centrifugation of the marrow erythroblasts following TG-1 lysis of myeloid cells. ALA synthase activity was

examined during congenital dyserythropoietic (CDA), thalassaemic and sideroblastic erythropoiesis. The results suggest that ALA synthase activity does not limit human erythroblast haem synthesis and that reduced ALA synthase activity is not the cause of primary acquired sideroblastic anaemia (PASA). Rather it is suggested that the reduced enzyme activity, as was found in iron loaded erythroblasts and ring sideroblasts, resulted from iron-mediated enzyme toxicity. It is further postulated that reduced ALA synthase activity as found in two cases of CDA and one of thalassaemia resulted from high transferrin saturation with consequent erythroblast iron overload, whereas in PASA enzyme inactivation and ring sideroblast formation resulted from a primary abnormality of erythroblast iron metabolism.

ABBREVIATIONS

ALA	5-aminolaevulinic acid
ATP	adenosine triphosphate
AUFS	absorbance units full scale
BFU-E	burst forming unit - erythroid
CFU	colony forming unit
-E	erythroid
-GEMM	granulocyte erythroid macrophage monocyte
-GM	granulocyte macrophage
-S	spleen
CDA	congenital dyserythropoietic anaemia
CoA	coenzyme A
cpm	counts per minute
DMAB	p-dimethylaminobenzaldehyde
DNAase	deoxyribonucleotidase
DOVA	dioxovaleric acid
dpm	disintegrations per minute
E_1	early basophilic and pro erythroblasts
E_2	intermediate polychromatic erythroblasts
E_3	late orthochromatic erythroblasts
EDTA	ethylene diamine tetracetic acid

FEL	Friend erythroleukaemia cells
GTP	guanosine triphosphate
HCR	haem controlled repressor
HPLC	high performance liquid chromatography
(RPHPLC)	reverse phase
INH	isoniazid (isonicotinic acid hydrazine)
MDS	myelodysplastic syndromes
M:E	myeloid: erythroid
MEM	minimum essential medium
ODS	octa decyl silane
PASA	primary acquired sideroblastic anaemia
PLP	pyridoxal-5-phosphate
STK	succinate thiokinase (succinyl CoA synthetase)
TCA	trichloroacetic acid
TIBC	total iron binding capacity

CHAPTER 1

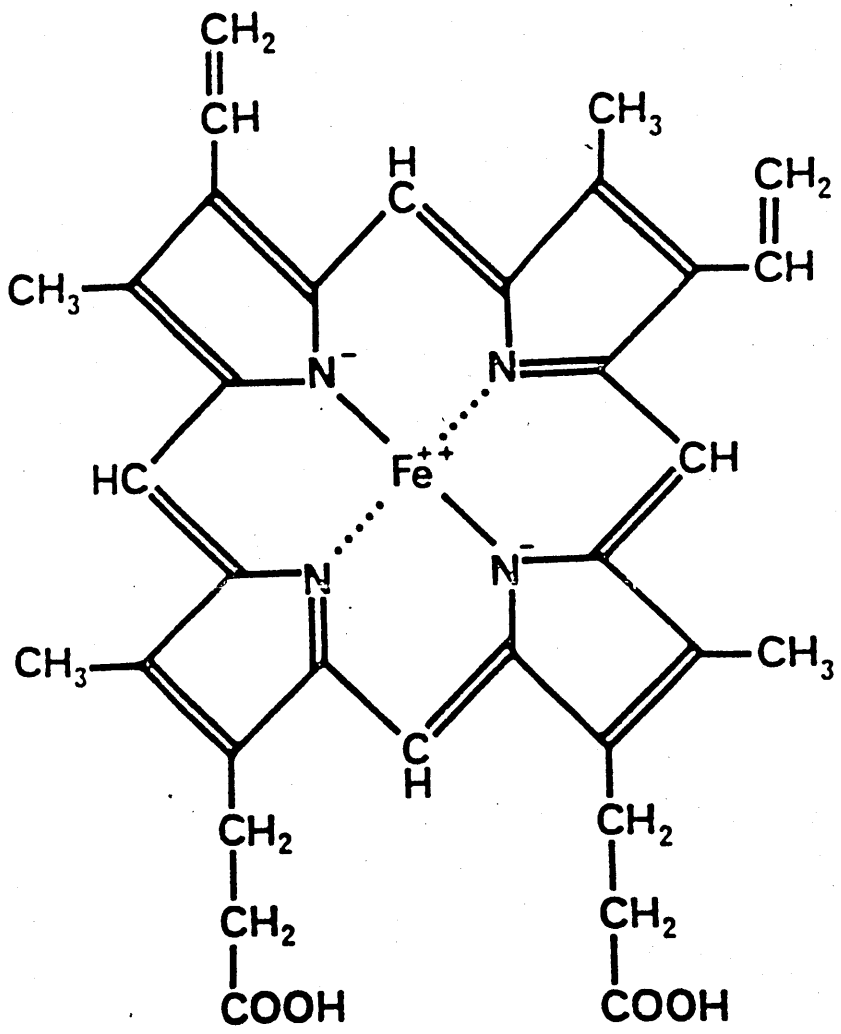
INTRODUCTION

1. INTRODUCTION

The Greek word "πορφυρου" "porphuros" meaning purple provides the stem for "porphyry" a purple rock quarried by ancient Egyptians and "porphyre" "a serpent about the bignesse of a span or more which in outward aspect was the most beautiful and well coloured purple" (Topsell, 1658). It is more frequently used in the word "porphyrin" taken from "haematoporphyrin" used by Hoppe Seyler (1871) to describe the major constituent of an iron free preparation of blood.

The structure of porphyrins may be broken down to reveal a basic tetrapyrrole arrangement. Each pyrrole represents a closed ring containing 1 nitrogen and 4 carbon atoms. The pyrroles are linked by methine bridges in either a cyclical or linear form. The vital and ubiquitous role of the cyclical tetrapyrroles is attested to by their presence in haem (Fig 1), the iron chelate of protoporphyrin IX, chlorophyll, the magnesium chelate of protoporphyrin IX and in the bacterial corrin, vitamin B_{12}, the cobalt chelate of uroporphyrinogen III derivatives.

The porphyrin structure of haem and chlorophyll was first described by Hans Fischer (1881-1945). The Nobel medal for chemistry, which shows the figure of science unveiling the goddess



The structure of heme-ferro-protoporphyrin 9.

Figure 1: The cyclical tetrapyrrole structure of haem

Isis, was awarded to Fischer in 1930 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).

Porphyrins are readily visible and emit an intense red fluorescence when exposed to long wave ultraviolet light (400 nm). This property is utilised for biological purposes and has been used to detect coproporphyrin in crocodile excrement from the Eocene age, 25 million years BC (Fikentscher, 1933) and in the search for evidence of life in samples taken from lunar rocks.

Their production in both plants and animals requires 5-aminolaevulinic acid (ALA) from which protoporphyrin IX is produced. At this point the biosynthetic pathways diverge, plants to chlorophyll, animals to haem. The pathways further differ in their production of ALA as the basic building unit. In man ALA is produced by the enzymatic coupling of succinyl CoA with glycine, whereas in plants it is produced in a three step process from L-glutamic acid through dioxovaleric acid (DOVA). Varticovski (1980) has shown that this latter pathway may not be restricted to plants, as labelled DOVA may be incorporated into ALA and haem in rats. However the balance of the paired reaction of DOVA transaminase and alanine-glyoxylate transaminase favours the reverse reaction with the formation of glycine (Moore and Disler,

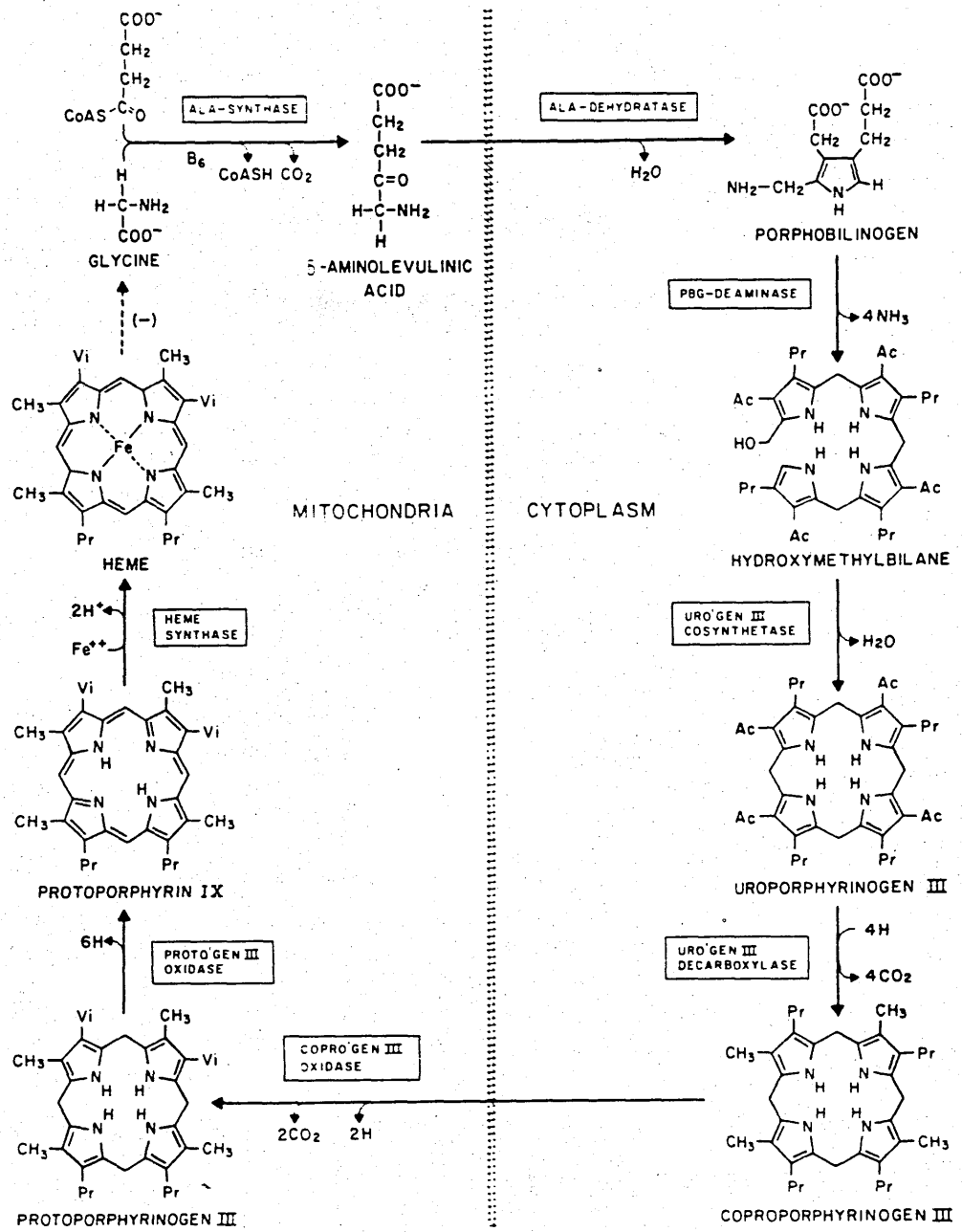
1985). If L-glutamic acid is a source of erythroid ALA in man (see Milgrom, 1985) it is likely to be a very minor one.

1.1 HAEM

Haem serves a critical metabolic function in animals as the prosthetic group of biologically important haem proteins which include haemoglobin, myoglobin, cytochromes P450 and P448, b type cytochromes, peroxidases, catalase and tryptophan pyrrolase. In addition, within developing erythroid cells haem may also be involved in the regulation of protein synthesis, iron uptake and differentiation (Chapter 5).

1.1.1 The formation of haem

The synthesis of haem occurs by an essentially unbranched pathway which consists of a series of irreversible reactions (Fig 2). The first (ALA synthase) and last three biosynthetic enzymes (coproporphyrinogen III oxidase, protoporphyrinogen III oxidase and ferrochelatase) are localised within the mitochondria with the intermediate enzymes in the cytosol. Two molecules of ALA, formed in the mitochondria, are condensed to form the colourless monopyrrole porphobilinogen (PBG). Thereafter PBG-deaminase and uroporphyrinogen III cosynthase act in concert to condense four molecules of PBG to hydroxymethylbilane from which the first porphyrin, uroporphyrinogen III is formed, with production of less than 1% of the symmetrical series I isomer. The side chains of uroporphyrinogen III are then modified by a series of



Note: B6 = pyridoxal-5'-phosphate. URO'GEN = uroporphyrinogen; COPRO'GEN = coproporphyrinogen; PROT'GEN = protoporphyrinogen. AC = acetate; Pr = propionate; Vi = vinyl.

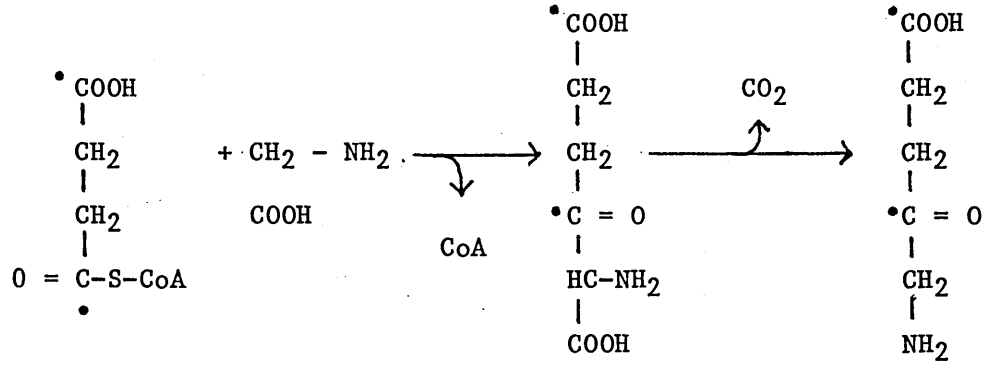
Figure 2: The pathway of haem biosynthesis

decarboxylation reactions to give protoporphyrinogen IX which is readily oxidised to protoporphyrin IX before the insertion of Fe^{2+} and haem formation.

1.1.2 ALA synthase (EC 2,3,1,37)

ALA synthase, loosely bound to the inner mitochondrial membrane, catalyses the condensation of glycine and succinyl CoA to form ALA. Glycine reacts with succinyl CoA to form the short lived enzyme bound intermediate α-amino-β-ketoadipic acid which spontaneously decarboxylates to yield ALA (Gidari and Levere, 1977). Pyridoxal 5 phosphate (PLP) is required as an essential cofactor (Shemin and Russell, 1953).

Pyridoxamine phosphate and PLP are the active coenzyme forms of pyridoxine (vitamin B6). As shown below the most common type of reaction requiring PLP is transamination, the transfer of the α-amino group of one amino acid to the carbon of an α-keto acid (Lehninger, 1972).



The carbon atoms originating from the carboxyl atoms of succinyl CoA are marked.

1.2 THE STRUCTURE OF ALA SYNTHASE

The molecular weight of isolated ALA synthase is dependent upon the nature of the animal species studied and the source of the tissue. The conditions under which the enzyme is purified are also important as buffers of low ionic strength may cause enzyme aggregation (Sassa and Kappas, 1981). The erythroid and liver iso enzymes have been shown to be structurally and immunologically different (Bishop et al, 1981; Watanabe et al, 1983). Although the enzyme is functionally active only in the presence of succinyl CoA, and hence only in the mitochondria, it is produced in the cytoplasmic polyribosomes, as a larger precursor, from which it is transported to the mitochondria in association with proteolytic processing (Yamauchi et al, 1980; Yamamoto et al, 1981). In rat liver cytosol the enzyme is thought to exist as a complex of one catalytically active and two inactive subunits of molecular weight 51,000, 79,000 and 120,000 respectively (Oshashi and Kikuchi, 1979) with which the active mitochondrial enzyme is immunochemically identical and formed as a dimer of two active subunits (Nakakuki et al, 1980). In chicken liver and bone marrow the cytosolic enzymes have molecular weights of 73,000 and 55,000 from which fragments of 8,000 and 2,000 respectively are cleaved during transport to the mitochondria (Watanabe et al, 1983).

1.3 THE ROLE OF ALA SYNTHASE IN THE REGULATION OF HEPATIC HAEM BIOSYNTHESIS

The half life of ALA synthase is very short, 34 min in foetal rat liver (Woods, 1974) and 160 min in culture chick embryo liver cells (Sassa and Granick, 1970). This is considerably shorter than the approximate 5 day half life of general mitochondrial proteins (Druyan et al, 1969) and permits the regulation of liver ALA synthase by mechanisms which control the rate of enzyme synthesis. In liver, ALA synthase is the rate limiting step in haem biosynthesis (Granick and Urata, 1963) and is subject to feedback control by "free" intracellular haem at transcriptional (Whiting, 1976) and translational (Strand et al, 1972a) levels: haem has also been shown to inhibit the translocation of cytosolic enzyme to the liver mitochondria (Yamauchi et al, 1980; Yamamoto et al, 1981). This active "free" intracellular haem pool will inevitably be small and have a rapid turnover, a necessary requisite for a controlling substance (Moore and Disler, 1985).

Within human erythroid cells there is as yet no clear picture as to what role ALA synthase plays in haem biosynthesis and what factors regulate enzyme activity. The principal reasons for this have been the lack of suitably sensitive assays of haem synthetic enzyme activity and an inability to separate bone marrow erythroid cells from each other on the basis of differentiation status and from the contaminating myeloid cells present in the marrow cavity (Ibrahim et al, 1983; Beaumont et al, 1984).

1.4 EXPERIMENTAL OBJECTIVES AND LAYOUT OF THESIS

The importance of ALA synthase activity in the regulation of hepatic haem biosynthesis and its role in the overproduction of porphyrins and/or their precursors in the different porphyrias has been defined (for review see Moore and Disler, 1985). Despite the fact that erythroblasts synthesise 85% of the total body haem produced each day (Berk et al, 1976) the importance of ALA synthase in the regulation of erythroid haem biosynthesis and its role in the development and pathogenesis of anaemias, in particular the sideroblastic anaemias, is uncertain.

It was the aim of the work discussed in this thesis to overcome the problems relating to the study of human bone marrow and erythroblast ALA synthase activity. The various chapters detail in stepwise fashion the progress made. The relevant literature and the particular problems anticipated at each step are presented in the introduction to each individual chapter.

The development of a sensitive radiochemical assay method for the measurement of ALA synthase activity in small numbers of human bone marrow cells is described in Chapter 2. The developed assay was used to measure ALA synthase activity in crude unfractionated samples of human bone marrow. The results are presented in Chapter 3 and the performance of the assay compared with published methods. Enzyme activity in unfractionated human bone marrow has been regarded, in all previous studies, as a measure of

erythroblast ALA synthase. The validity of this assumption is tested in Chapter 4 and the percentage contribution made by myeloid cells towards ALA synthase activity in unfractionated normal and sideroblastic bone marrow is reported. In Chapter 5 marrow fractionation procedures are described which have permitted the direct measurement of ALA synthase activity within age matched normal and pathological erythroblasts. The results have been used to examine the role of ALA synthase in the regulation of erythroblast haem biosynthesis and the significance of reduced ALA synthase activity in the development of primary acquired sideroblastic anaemia (PASA).

1.5 ALA SYNTHASE ACTIVITY AND SIDEROBLASTIC ANAEMIA

Iron deficiency anaemia is the most common and the clearest clinical manifestation of haem deficiency. Erythrocyte protoporphyrin levels are raised but there is as yet no information on the effects that haem deficiency have on erythroblast ALA synthase activity.

The sideroblastic anaemias are a rare group of dysplastic marrow disorders, which with respect to the congenital and primary acquired forms, have been associated with reduced bone marrow ALA synthase activity. This reduced haem synthetic enzyme activity is thought to represent the primary defect of erythroblast metabolism, responsible for the development of mitochondrial iron

loading and ineffective erythropoiesis (Aoki et al, 1974; Aoki, 1980; Ponka and Neuwrit, 1974; Bottomley, 1977, 1982). See Chapter 5.

Gruneberg (1941) applied the term "siderocyte" to the iron loaded erythrocytes of flexed-tailed mutant (f/f) foetal mice. Erythroblast iron granules were first described by Douglas and Dacie (1953). They concluded that normal erythroblasts could contain up to six small iron granules and that the number of marrow sideroblasts was related to the percentage transferrin saturation. The first description of acquired anaemia with hypochromic red cells and sideroblasts in the bone marrow (Bjorkman, 1956) was followed by the classic account by Dacie et al (1957) of refractory normoblastic anaemia with sideroblasts in the bone marrow. These seven cases undoubtedly established primary acquired sideroblastic anaemia (PASA) as a diagnostic entity. However Dacie's term "cuff" to describe the perinuclear siderotic granules, has not prevailed and has since been replaced by "ring", introduced by Bowman (1961). As used in the following text: "ring sideroblast" describes erythroblasts ($\pm$ dyserythropoietic features) with $\geqslant$ 5 large siderotic granules extending $\geqslant$ 1/3 of the nuclear circumference (see Plate 1), in which the iron granules are known to be present within mitochondria (Caroli et al, 1957); "pathological sideroblast" to describe non ring forms with $\geqslant$ 7 large iron granules in the cytoplasm.

Reduced ALA synthase activity in pyridoxine responsive congenital sideroblastic anaemia is thought to result from increased sensitivity of apo ALA synthase to an erythroblast protease specific for pyridoxal enzymes (Aoki, 1978; Aoki et al, 1979; Manabe et al, 1982). The therapeutic benefit of pyridoxine has been explained as a stabilising effect on the enzyme, through which normal sensitivity is restored (Manabe et al, 1982). In the preleukaemic form of sideroblastic anaemia, PASA, there is no therapeutic response to pyridoxine and reduced ALA synthase activity is thought to reflect reduced enzyme production.

1.6 ALA SYNTHASE ACTIVITY AND PORPHYRIA

Acute intermittent porphyria (AIP) is associated with increased ALA synthase activity in liver (Tschudy et al, 1965) and peripheral blood leucocytes (Brodie et al, 1977). This led to the initial hypothesis that AIP might represent the first genetic disease characterised by enzyme overactivity (Tschudy et al, 1965). It is now known that the human porphyrias comprise a group of inherited and acquired disorders characterised by reduced activity of specific haem enzymes as observed in liver cells, erythrocytes, leucocyte pellets, cultured fibroblasts, cultured amniotic cells and mitogen stimulated lymphocytes (for review, see Sassa and Kappas, 1980) and associated with enzyme overactivity at stages preceding the deficient enzyme. Interest in the porphyrias is considerable, however their relevance to the work described by

this Scotsman lies not with their causing transfer of the Crown from the Scottish House of Stewart to that of Hanover[+] (McAlpine and Hunter, 1966; Mcalpine et al, 1968), but with a seemingly curious paradox; while primary porphyric aberrations in haem synthetic enzymes as evidenced in many tissues, do not disturb bone marrow function, reduced ALA synthase activity in PASA is considered sufficient cause of a refractory anaemia associated with erythroid hyperplasia and mitochondrial iron loading.

[+] The Act of Settlement 1701 transferred the Crown from the Scottish House of Stewart to that of Hanover. The Act had been required to safeguard the Protestant succession, jeopardised by the failure of Queen Anne (1665-1714) to leave a Protestant heir; a failure postulated to have been caused by the "Royal Malady" porphyria.

CHAPTER 2

DEVELOPMENT OF AN ASSAY TO DETERMINE 5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN HUMAN BONE MARROW

2. DEVELOPMENT OF AN ASSAY TO DETERMINE 5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN HUMAN BONE MARROW

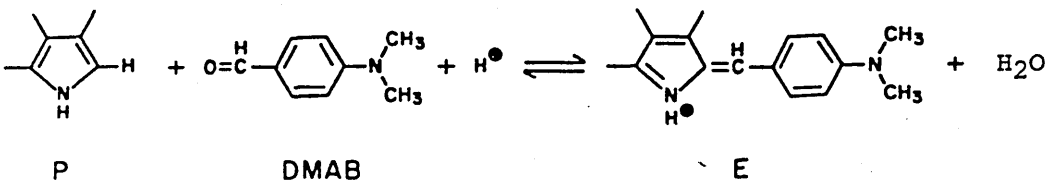
2.1 PRINCIPLE OF ENZYME ACTIVITY ASSAYS

The amount of enzyme activity in a given tissue is usually defined as the amount of product from substrate per unit time under certain specified conditions (Mahler and Cordes, 1969). Where possible enzymes are assayed in test conditions in which the pH is optimum and the substrate concentration is above the saturating level, so that the initial reaction rate is zero-order for substrate. When catalysed reactions are zero-order for substrate and cofactors the rate of the reaction is proportional to enzyme concentration alone (Lehninger, 1972).

2.2 COLORIMETRIC DETERMINATION OF ALA

The original method developed to measure ALA was a colorimetric assay first described by Mauzerall and Granick (1956) and subsequently used as the basis of colorimetric assays of tissue ALA synthase activity (see Burnham 1970). In the original method ALA was converted to a pyrrole by adding either acetylacetone or ethylacetoacetate. The resulting ALA pyrrole was reacted with modified Ehrlich reagent and quantified by spectrophotometry at 555 nm. The chemistry of pyrrole reactions with Ehrlich reagent is complex (Falk, 1964) but in relation to the reaction with ALA

pyrrole (P) a coloured salt (E) is formed by the reaction of the free α-hydrogen on the pyrrole with the dimethylaminobenzaldehyde (DMAB) in the Ehrlich reagent (Lien and Beattie, 1982).



P DMAB E

The colour is not stable and should be read at 15 min as the coloured salt (E) can react with another molecule of pyrrole (P) to form a colourless compound.

The major drawbacks of the colorimetric assay are the formation of aminoacetone pyrrole which also reacts with Ehrlich reagent and low sensitivity. Aminoacetone is formed by the condensation of glycine with acetyl CoA, catalysed by aminoacetone synthase, or by the oxidation of theonine (Lien and Beattie, 1982). The limit of detection with colorimetric assays is about 3 nmol ALA/ml (Falk 1964). As such they are not suitable for use with tissues containing low levels of ALA synthase, or with tissues such as human bone marrow available only in small quantities.

2.3 FLUORIMETRIC ASSAY OF ALA SYNTHASE

A fluorimetric assay sensitive to ALA synthase activity in the pmol/ml range has been described (Bishop et al, 1982) but has not

been used successfully to detect enzyme activity in bone marrow lysates (Bottomley and Moore, 1982).

2.4 RADIOCHEMICAL ASSAYS OF ALA SYNTHASE

ALA synthase activity in liver (Irving and Elliot, 1969), in human bone marrow (Bottomley et al, 1973), in "in vitro" cultured cell lines (Ebert and Ikawa, 1974), in "in vitro" bone marrow cultures (Ibrahim et al, 1982) and in peripheral blood leucocytes (Brodie et al, 1977) is most often detected by radiochemical assay, sensitive to the detection of 10-100 pmol ALA/ml (Irving and Elliott, 1969).

With radiochemical assays in general there are problems with the choice of radioactive substrate and the specificity of radioactive ALA isolation. In addition a problem peculiar to the use of radiochemical assays with erythroid tissues is the loss of radioactive ALA that occurs during the period of sample and substrate incubation. This is known as ALA UTILISATION (Strand et al, 1972; Bottomley et al, 1973).

2.4.1 Choice of radioactive substrate

Succinyl CoA and glycine are the direct substrates of ALA synthase and both [^{14}C]-succinyl CoA (Aoki et al, 1974) and [^{14}C]-glycine (Freshney and Paul, 1970) have been used in radiochemical assays of ALA synthase activity. There are however problems with both. The Km of ALA synthase for glycine is high 4.2 mmol/l (Freshney

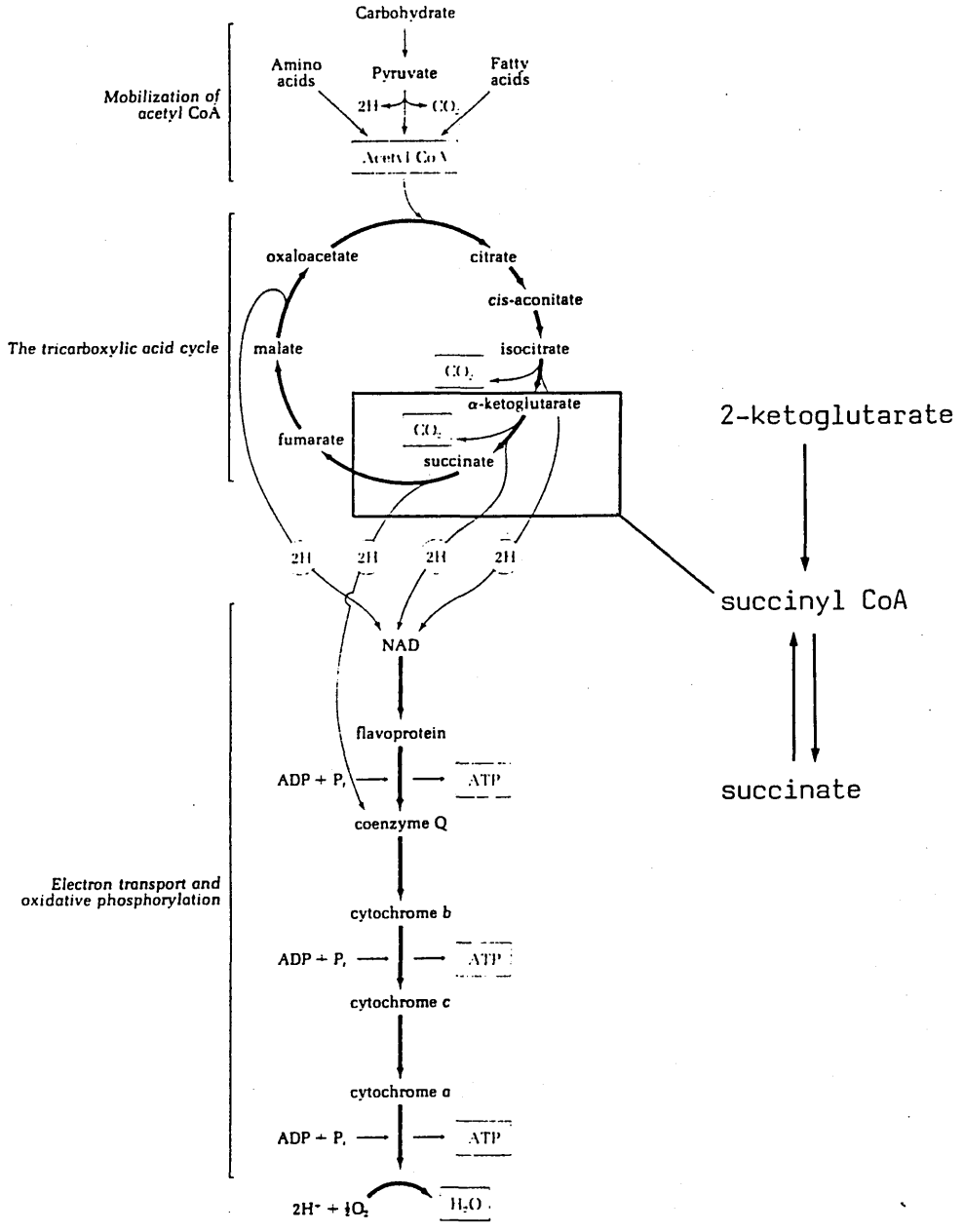
and Paul, 1970), 2.5 - 7.5 mmol/l (Nakakuki et al, 1980). As this is approximately 160 times the Km for succinyl CoA (Aoki et al, 1974) it has been thought that [^{14}C]-glycine may not be a practical choice of substrate (Bishop and Wood, 1977).

The Km for succinyl CoA is extremely low, 11 umol/l (Nakakuki et al, 1980). However, at -20°C the stability of [^{14}C]-succinyl CoA is only in the order of weeks and during incubation at 37°C for 30 min some 50% may be non enzymatically hydrolysed (Aoki et al, 1974). As [^{14}C]-succinyl CoA is not commercially available its use over a prolonged period of time would require its repeated preparation from [^{14}C]-succinic anhydride, an extremely expensive radiochemical.

These problems have led to the more frequent use of either 2-keto [^{14}C]glutarate or [^{14}C]-succinate. Neither 2-keto [^{14}C]-ketoglutarate, initially used by Ebert et al (1970) or [^{14}C]-succinate (Irving and Elliott, 1969) are direct substrates of ALA synthase. Both are intermediates of the citric acid cycle (Fig 3) and require to be metabolised to [^{14}C]-succinyl CoA.

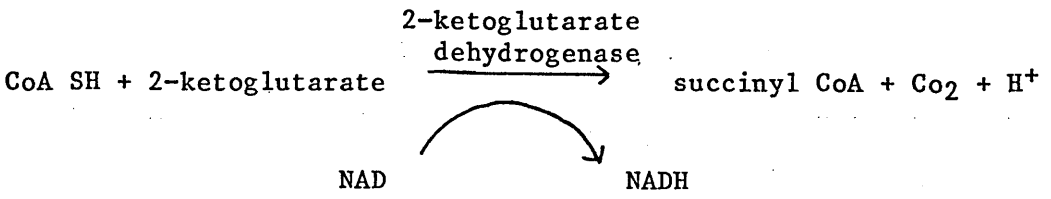
Conversion of 2-ketoglutarate to succinyl CoA:

2-ketoglutarate dehydrogenase (EC 1.2.4.2.) is an enzyme complex that catalyses the irreversible oxidative phosphorylation of 2-ketoglutarate to succinyl CoA. Thiamine pyrophosphate, lipoic acid, CoA, NAD and FAD are required as enzyme bound cofactors (Lehninger, 1972).



Note: The citric acid cycle is the central pathway for the degradation of two carbon acetyl residues derived from carbohydrate, fatty acid or amino acid metabolism. The cycle yields CO_2 and H^+ ions. The latter are led via the electron transport system to molecular oxygen.

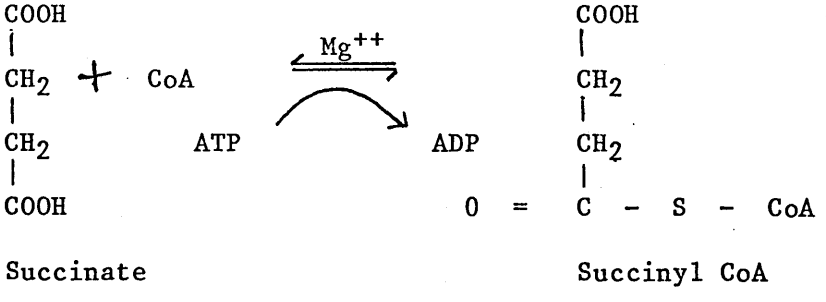
Figure 3: The citric acid cycle in relation to cell respiration



The Km of the enzyme for CoA is 10^{-7}M and for 2-ketoglutarate 1.3 x 10^{-5}M (Massey, 1960). Although both are low and 2-keto[^{14}C] glutarate is commercially available, the enzyme 2-ketoglutarate dehydrogenase is difficult to obtain. In the absence of exogenous 2-ketoglutarate dehydrogenase the assay of ALA synthase using 2-keto[^{14}C]glutarate as substrate would be subject to fluctuations in the activity of endogenous 2-ketoglutarate dehydrogenase. This would then enable reduced 2-ketoglutarate dehydrogenase activity to effect an apparent reduction in ALA synthase activity.

Conversion of succinate to succinyl CoA:

Succinate thiokinase (STK) (EC 6.2.1.5.) catalyses the reversible conversion of succinate to succinyl CoA in the presence of Mg^{++} and triphosphate.



The apparent Km of ALA synthase for succinate and CoA in an assay system containing exogenous STK and partially purified ALA synthase was 3.3×10^{-5}M (Bishop et al, 1982). [^{14}C]-succinate is used more frequently than 2-keto[^{14}C]glutarate for the measurement of ALA synthase activity. Radioactive succinate is cheap and stable. In addition GTP dependent porcine STK is readily available and so allows with ease the addition of an exogenous succinyl CoA generating system.

With each particular tissue under study however the need for provision of such a system should be determined. The assay of ALA synthase in human reticulocytes, (Strand et al, 1972) in crude liver homogenates from mice and humans but not from rats requires an exogenous succinyl CoA generating system for maximal activity (Bonkowsky and Pomeroy, 1978).

Furthermore with a view to maximising the conversion of [^{14}C]-succinate to [^{14}C]-succinyl CoA during incubation, citric acid cycle inhibitors and potassium fluoride may be added to the reaction mixture (Ebert et al, 1970; Strand et al, 1972).

Citric acid cycle inhibitors and potassium fluoride:

Sodium malonate (10mM), sodium Dl - malate (5mM) and antimycin A (2.5 ug/ml) respectively can be used to inhibit succinate dehydrogenase, trap [^{14}C]-malate and block the electron transport chain. These would effect inhibition of the citric acid cycle and might maximise [^{14}C]-succinate availability for STK (Brooker et

al, 1982). It has been shown that mitochondrial synthesis of succinyl CoA from 2-ketoglutarate may be prevented by inhibition of 2-ketoglutarate dehydrogenase with arsenite (Granick and Urata, 1963). Arsenite has been used with [^{14}C]-succinate based assays of ALA synthase to inhibit synthesis of cold succinyl CoA from endogenous 2-ketoglutarate and so prevent dilution of the [^{14}C]-succinyl CoA with cold succinyl CoA (Ebert et al, 1970). In practice however arsenite is infrequently used as it also inhibits ALA synthase activity (Irving and Elliott, 1969; Ebert et al, 1970) and presents a hazard from its use in the chemical laboratory (Muir, 1977).

The addition of potassium fluoride (100 mM) to the reaction mixture has been found to produce a twofold increase in measurable ALA synthase activity in liver homogenates (Yoda et al, 1975). Fluoride is thought to inhibit ATPases and GTPases which might limit the effect of triphosphate dependent STK and so restrict succinyl CoA generation.

In principle inhibitors are used to maximise [^{14}C]-succinate utilisation by STK and promote this route as the only source of succinyl CoA in the assay system. It is however, thought that the use of an exogenous succinyl CoA generating system may make the addition of these inhibitors unnecessary (Bishop et al, 1982).

In view of the instability and the expense involved in the preparation of [^{14}C]-succinyl CoA and the high Km value of ALA synthase for [^{14}C]-glycine, neither radiochemical was used. Both [^{14}C]-succinate and 2-keto[^{14}C]glutarate were chosen for this study to examine their suitability for measuring bone marrow ALA synthase activity.

2.5 SPECIFIC ISOLATION OF ALA

The greatest source of error in the radiochemical measurement of ALA synthase activity lies in the inability of methods to specifically isolate [^{14}C]-ALA from other radioactive substances in the enzyme reaction mixture (Patton and Beattie, 1973). The first radiochemical assay developed combined single column ion exchange chromatography with solvent extraction of ALA pyrrole by ethylacetate as a means of isolating radioactive ALA (Irving and Elliott, 1969). The method of separation was modified and made more simple by Ebert et al (1970) and so a rapid and sensitive radiochemical assay became available for use with tissues such as human bone marrow only available in small quantities. The introduction of a sequential multicolumn ion exchange procedure improved specificity (Strand et al, 1972) but replaced simplicity with a technique that was tedious and liable to produce significant variability in ALA recoveries from duplicate samples (Schacter et al, 1976). With partially purified preparations of enzyme the number of ion exchange fractionations may be reduced (Aoki et al, 1974). However, incomplete separation of [^{14}C]-ALA from other radioactive compounds has remained a problem with

assays using mitochondria or crude liver homogenates (Bishop and Wood, 1977; Managa et al, 1978; Wolfson et al, 1980). A modified Beckman aminoacid analyser has been used to improve separation (Bishop and Wood, 1977) and "clean up" procedures have been developed to rid commercial radioactive succinate of impurities that might contaminate isolated [^{14}C]-ALA (Condie et al, 1976; Bishop and Wood, 1977; Brooker et al, 1982). Sodium dodecyl sulphate used in place of trichloroacetic acid to stop the reaction eliminated contaminants that separated with ALA during ion exchange chromatography but reduced enzyme activity and produced variable recovery of the internal standard (Scotto et al, 1983). With assays using [^{14}C]-glycine (Freshney and Paul, 1971) high voltage paper electrophoresis can be used to separate [^{14}C]-ALA from [^{14}C]-glycine (Rutherford et al, 1979). The application of reverse phase (RP) HPLC to the assay of ALA synthase was first described by Tikerpae et al (1981). As RPHPLC was seen to offer a simple and rapid means of isolating [^{14}C]-ALA and also allow for the incorporation of internal standard in each sample it was decided to investigate RPHPLC as a means of achieving single column separation of [^{14}C]-ALA without need for ether extraction or freeze drying (Tikerpae et al, 1981).

2.5.1 Principles of HPLC

Liquid chromatography is a widely used chemical separation method based on interactions between solutes, liquid mobile phase and solid stationary phase. Separation takes place in a column containing the chromatographic material (ie the stationary phase)

in the form of small particles. The liquid solvent (ie the mobile phase) is pumped through the particle bed. This arrangement allows sample components dissolved in the mobile phase to interact in a reversible manner with the stationary phase. Separation is achieved on the basis of the relative affinities of the different sample components for the stationary and mobile phases. Components with a strong affinity for the stationary phase will elute later than those with a greater affinity for the mobile phase.

HPLC differs from classical chromatography in that the system operates under high pressure and has been optimised to increase speed, resolution and sensitivity. The set up for HPLC requires that a delivery system is connected to the chromatography column in order to feed it with fresh mobile phase. The system consists of a solvent reservoir and a pump capable of pumping under high pressure (6000 psi) and producing an even flow of mobile phase through the column without pulsation. The sample to be chromatographed is applied as a precise volume via the sample injector which is positioned just prior to the column. After passing through the column the separated sample components are monitored by a detector and collected by a fraction collector (LKB 1982).

The detector

ALA pyrrole formed by the condensation of ALA with ethylacetoacetate may be detected by UV spectrophotometry at

252 nm (Tikerpae et al, 1981). UV absorption is a molecular property and thus each compound will have its own specific absorbance. UV detectors are sensitive to the nanogram level of detection.

2.5.2 Principles of reverse phase HPLC

RPHPLC utilises differences in the solubility properties of compounds in a sample to achieve separation. Substances that show little solubility in water but which are readily soluble in organic solvents are called hydrophobic; organic solvents themselves are hydrophobic. On the other hand hydrophilic substances are readily soluble in water but not in organic solvents. In RPHPLC the mobile phase is always more hydrophilic than the stationary phase and so hydrophilic compounds will always move faster than hydrophobic ones.

The ability of the stationary phase to effect separation may be influenced in three different ways.

1. Alter the hydrophobicity of the stationary phase.
2. Alter the hydrophilic properties of the mobile phase. This may be done by altering the amount of organic solvent in the mobile phase.
3. Alter the hydrophobicity of the compounds in the sample to be separated. This may be done by:
    1. altering the pH, or
    2. adding an ion-pairing agent to the mobile phase.

Types of stationary phase:

In RPHPLC silica particles covered with chemically bonded hydrocarbon chains represent the hydrophobic stationary phase. The retention of hydrophobic compounds is related to the length of the hydrocarbon sidechains. A bonded sidechain with 18 carbon atoms (a C18 layer, ODS) is more hydrophobic than a C8 layer which in turn is more so than a C2 layer. RPHPLC columns are available with any one of these three packings. The C18 packing provides maximum resolving power for hydrophobic compounds.

Hydrophobicity of the sample:

Ionic compounds show low hydrophobicity because of the hydrophilic character of charged groups. These charges may be neutralised by the use of buffers or by the addition of ion-pairing agents which form electrostatic complexes with charged components in the sample. The ion-pairing agent is bulky and the ion-ion bond formed will be shielded by the rest of the molecule so rendering the complex considerably more hydrophobic than the separate parts.

2.6 INCUBATION MEDIUM FOR ALA SYNTHASE ACTIVITY

The composition of the ideal incubation medium used in an assay of enzyme activity is such as to allow maximum product synthesis.

The pH profile for ALA synthase activity has shown a broad optima around 7.4 (Strand et al, 1972), 7.5 (Bishop et al, 1982) and 7.6 for highly purified enzyme (Nakakuki et al, 1980). Enzyme

activity is almost universally measured between pH 7.2 - 7.6 and pH of 7.4 was chosen for use in this work without further experimentation. The acetate buffering system already successfully used for ALA pyrrole isolation with RPHPLC was also adopted (Tikerpae et al, 1981).

In principle the reaction mixture should contain all reagents required for the conversion of [^{14}C]-substrate to [^{14}C]-ALA. Ideally these reagents should also be present at saturating levels. The [^{14}C]-ALA formed during the reaction should be preserved and its further metabolism by ALA dehydratase inhibited. The need for individual constituents in the reaction mixture will be discussed.

2.6.1 Sucrose

Sucrose is commonly present in the incubation medium (Rutherford et al, 1979; Wolfson et al, 1980; Tikerpae et al, 1981) as it is thought that sucrose may provide osmotic stability for ALA synthase during tissue homogenisation.

2.6.2 Glycine

Glycine is the immediate substrate for ALA synthase. It has been shown that the enzyme in bone marrow is saturated with 25 mM glycine (Bottomley et al, 1973). A concentration of 50 mM glycine, as was used in previous studies (Irving and Elliott, 1969; Tikerpae et al, 1981; Ibrahim et al, 1982) was chosen for this work without further experimentation.

2.6.3 Pyridoxal phosphate

Although PLP is an essential cofactor for ALA synthase, exogenous PLP may not be necessary for the detection of maximum enzyme activity in normal bone marrow (Bottomley et al, 1973). In situations in which it has been required for maximum enzyme activity 0.1 mM PLP has been shown to be saturating (Irving and Elliott, 1969; Bishop et al, 1982). For this study 0.4 mM PLP was used.

2.6.4 2-keto[^{14}C]glutarate

2-keto[^{14}C]glutarate has been used for the detection of ALA synthase activity in liver (Ebert et al, 1970) and in bone marrow (Bottomley et al, 1973; Konopka and Hoffbrand, 1979; Tikerpae et al, 1981). The saturating concentration of 2-ketoglutarate for the detection of ALA synthase activity in both tissues has been shown to be 0.17 mM and was used at this concentration in those samples to which 2-keto[^{14}C]glutarate was added as substrate.

2.6.5. Mg^{++}

STK requires Mg^{++} as an essential cofactor (Ramaley et al, 1967). With the use of [^{14}C]-succinate as substrate $MgCl_2$ was present in the incubation medium at 20 mM while a concentration of 5 mM $MgCl_2$ was used with 2-keto[^{14}C]glutarate.

The concentration of the other reagents in the incubation medium was individually optimised for maximum ALA synthase activity. Their inclusion in the medium will be discussed here, but the

experimental work carried out to determine their optimum concentration will be reported in Experimental and Results.

2.6.6 EDTA

EDTA was added to inhibit ALA dehydratase and so prevent the condensation of ALA formed during incubation to the monopyrrole, porphobilinogen. EDTA inhibits enzyme activity by chelating Zn^{++} (Bevan et al, 1980). Failure to inhibit ALA dehydratase leads to loss of ALA via ALA utilisation in assays of both liver and bone marrow ALA synthase. ALA utilisation in liver is abolished with 1 mM EDTA (Ebert et al, 1970; Strand et al, 1972) but persists despite EDTA with the assay of erythroid ALA synthase (Strand et al, 1972; Bottomley et al, 1973).

There are other known potent inhibitors of ALA dehydratase such as sodium laevulinate, Pb^{++} and succinylacetone. Only sodium laevulinate has been used in the assay of ALA synthase activity and only then in intact cultured liver cells (Sinclair and Granick, 1977). Succinylacetone is a profound and specific inhibitor of hepatic and erythroid ALA dehydratase (Tschudy et al, 1981; Sassa and Kappas, 1983). The effect of EDTA and succinylacetone on ALA utilisation and ALA synthase activity is reported in Experimental and Results.

2.6.7 CoA

CoA may be added to the incubation medium for assays that use either [^{14}C]-succinate or 2-keto[^{14}C]glutarate as it is required

by both for the formation of [^{14}C]-succinyl CoA. The amount of exogenous CoA required for maximum ALA synthase activity is dependent upon the nature of the tissue under study. With crude liver homogenate exogenous CoA is not required for maximal ALA synthase activity (Ebert et al, 1970). For those experiments in which 2-keto[^{14}C]glutaric acid was used as substrate, CoA was added to a final saturating concentration of 0.25 mM (Tikerpae et al, 1981).

2.6.8 [^{14}C]-succinate

The use of [^{14}C]-succinate as substrate requires either that adequate amounts of [^{14}C]-succinyl CoA are generated by the tissue itself or that an exogenous succinyl CoA generating system be provided. Commercial STK is GTP dependent. The optimum combined concentrations of [^{14}C]-succinate, CoA, GTP and STK for maximal ALA synthase activity is reported in Experimental and Results.

2.7 TISSUE PREPARATION

2.7.1 Mitochondria or crude tissue homogenate

ALA synthase activity may be measured in mitochondrial fractions, in partially purified preparations of the enzyme or in crude tissue homogenate. Although it is desirable to detect functional ie mitochondrial ALA synthase, the loss of mitochondria during preparation has led to the suggestion that only crude tissue homogenate should be used when small amounts of tissue are

available (De Matteis and Hollands, 1982). In view of the small size of marrow samples crude tissue homogenate was used for all experiments.

2.7.2 Tissue homogenisation

Ultrasonic disintegration is used by most workers as a means of tissue preparation. It has been claimed that membrane solubilisation with deoxycholate in addition to sonication can increase detectable enzyme activity (Aoki et al, 1974). Both methods of preparation were tested and the results are reported.

The aim of this work was to develop a simple single column RPHPLC method for specifically isolating ALA pyrrole, without solvent extraction, for use in the assay of ALA synthase activity. With respect to the assay itself it was aimed to identify either 2-keto[^{14}C]glutarate or [^{14}C]-succinate as the more suitable radiochemical and determine the optimum conditions under which enzyme activity might be assayed.

It was at the outset decided that the reaction would take place in a final volume of 0.5 ml in plastic eppindorfs at 37°C in a shaking water bath set to 60 rpm.

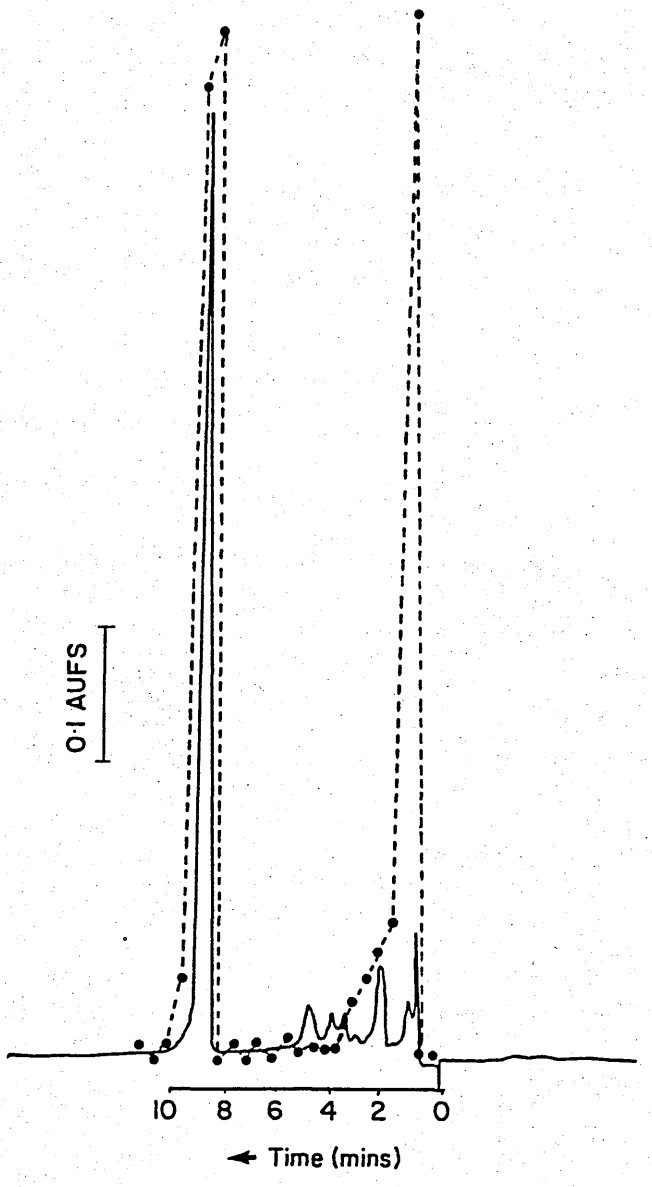
2.8 CHROMATOGRAPHIC PROPERTIES OF ALA PYRROLE

ALA pyrrole was prepared by the method of Mauzerall and Granick (1956) and was identified as 2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole by nmr spectroscopy. The chromatographic properties of the pyrrole dissolved in 50% (v/v) aqueous methanol were examined using a C18 RPHPLC column. The percentage concentration of aqueous methanol in the mobile phase was altered so as to determine the most useful concentration for routine use. Eluted pyrrole was detected by a UV spectrophotometer set to 252 nm and 1.0 AUFS, collected in 30 sec fractions on the fraction collector, and was identified by its reaction with modified Ehrlich reagent.

With 35% (v/v) aqueous methanol and 0.005 M heptane sulphonic acid (ion-pairing agent) as mobile phase, flow rate 1.5 ml/min, ALA pyrrole eluted over a 90 sec period between 8 and 9 min as a single sharp peak (Fig 4). The peak height was directly related to the volume of pyrrole solution injected.

Both 2-keto[^{14}C]-glutarate and [^{14}C]-succinate eluted at the solvent front. Injection of 25,000 cpm of either [^{14}C]-succinate or 2-keto[^{14}C]-glutarate with the prepared pyrrole showed that the cpm eluted completely within 2 minutes, with no radioactive contamination of the cold ALA pyrrole peak. Injection of 250,000 cpm of either radiochemical, still considerably less than the cpm required for an individual assay, produced significant radioactive contamination (300-400 cpm) in the cold ALA pyrrole peak (background 15-20 cpm). This indicated that RPHPLC would not by itself achieve sufficient separation of ALA pyrrole from the large number of cpm in the radiochemical substrate required to detect ALA synthase activity.

With aqueous methanol 10% (v/v) as mobile phase for RPHPLC, ALA pyrrole did not elute within 30 min. This prolonged retention of ALA pyrrole on a C18 column at low methanol concentration in the mobile phase indicated that disposable C18 cartridges might achieve partial ALA pyrrole isolation prior to complete and specific isolation by RPHPLC. There are various C18 cartridges available, however the product of Waters Associates (Sep-pak cartridge) was chosen for use.



Note: Aqueous methanol (35% v/v) containing 5 mM heptane sulphonic acid was used as mobile phase, flow rate 1.5 ml/min. Spectrophotometric monitoring (———) at 252 nm detects ALA pyrrole elution at 9 min. Radioactivity (-----) eluting at the solvent front represents [^{14}C]-substrate clear of [^{14}C]-ALA pyrrole. AUFS = absorbance units full scale.

Figure 4: The isolation of ALA pyrrole using RPHPLC on an ultrasphere C18 ODS column (4.6 x 150 mm)

2.8.1 Trial of Sep-pak cartridge

ALA added to the incubation medium of Tikerpae et al (1981) was converted to ALA pyrrole by reaction with ethylacetoacetate.

0.05 ml of ALA solution (5 mM) was added to 0.45 ml of incubation medium. Trichloroacetic acid (TCA) (10%) 0.25 ml was added and the pH brought to 5.3 by the addition of 0.5 ml sodium acetate (1 M). Ethylacetoacetate 0.05 ml was added and the mixture heated to 100°C for 20 min in a loosely capped (capped with a marble) glass boiling tube. The tube was cooled rapidly in ice and water to 5 ml was added.

A C18 Sep-pak cartridge was activated by the successive injection of acetonitrile 10 ml, water 5 ml, methanol 5 ml and water 10 ml. The ALA pyrrole solution was then injected through the cartridge at the rate of 5 ml/min (Fig 5). The ALA pyrrole solution and the Sep-pak eluant were reacted with modified Ehrlich reagent. ALA pyrrole was completely retained by the Sep-pak. Elution and 100% recovery of the pyrrole was obtained by injecting 3.0 ml of methanol through the cartridge. Each cartridge could be used four times without deterioration and ALA pyrrole was shown to remain stable during the evaporation of the methanol under N_2 at 35°C.

5×10^6 cpm of either [^{14}C]-succinate or 2-keto[^{14}C]-glutarate were added to the ALA pyrrole solution. Less than 20,000 cpm were retained on the Sep-pak. This indicated the potential use of the Sep-pak cartridge as a means of partially isolating ALA pyrrole prior to RPHPLC.

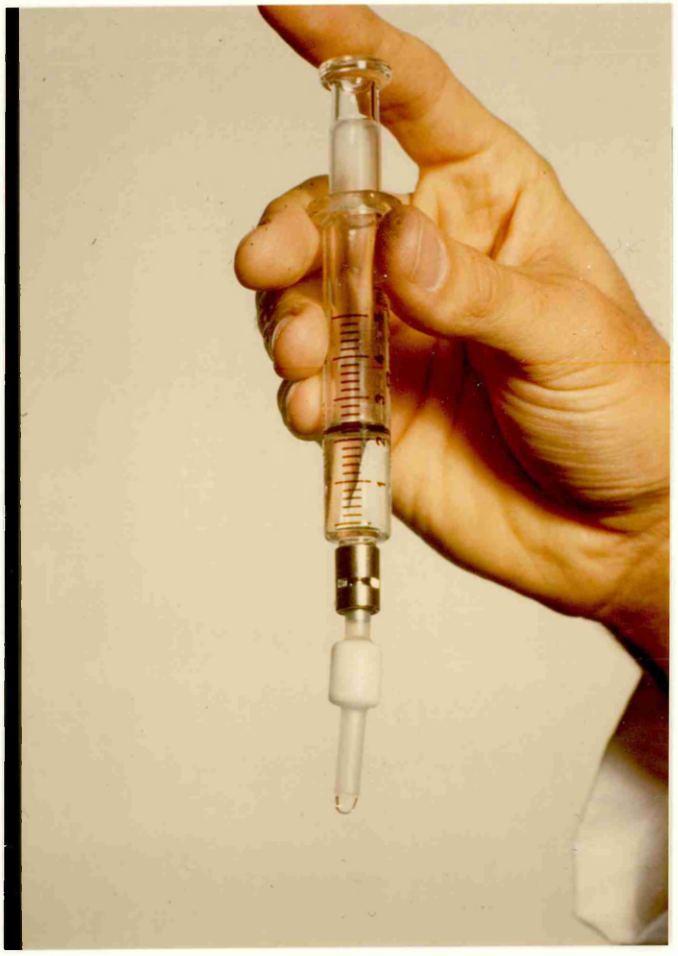


Figure 5: The use of Sep-pak cartridge for partial isolation of [^{14}C]-ALA pyrrole from [^{14}C]-substrate

Drying the Sep-pak:

For use within an assay system a technique was required which would dry the Sep-pak completely before the elution of ALA pyrrole in methanol. Failure to achieve complete drying of the Sep-pak was shown to prolong the stage of methanol evaporation.

It was shown that ALA pyrrole bound to the C18 Sep-pak was stable during drying of the Sep-pak under nitrogen blown through the cartridge or under air drawn through the cartridge by attachment to a benchside vacuum pump. Both methods produced a dry Sep-pak within 5 min and for convenience the vacuum method was adopted for regular use.

2.9 CONVERSION OF ALA TO ALA PYRROLE

ALA pyrroles may be produced from acetylacetone, succinylacetone, methylacetoacetate or ethylacetoacetate. Tikerpae et al (1981) reported that the pyrrole prepared from ethylacetoacetate had the most suitable characteristics for rapid successive applications to the RPHPLC column. The RPHPLC properties of the ALA pyrroles prepared from ethylacetoacetate and acetylacetone were examined under identical conditions.

Either ethylacetoacetate (0.05 ml) or acetylacetone (0.05 ml) was added to incubation medium (0.5 ml) (Tikerpae et al, 1981), containing 5 mM ALA (0.05 ml), 10% TCA (0.25 ml) and 1 M sodium acetate (0.5 ml). ALA pyrrole was prepared for RPHPLC as described (Section 2.8.1). Methanol from the sep-pak was evaporated and ALA pyrrole was resuspended in 0.1 ml methanol 50% (^{v}/v) from which 0.05 ml was taken for HPLC.

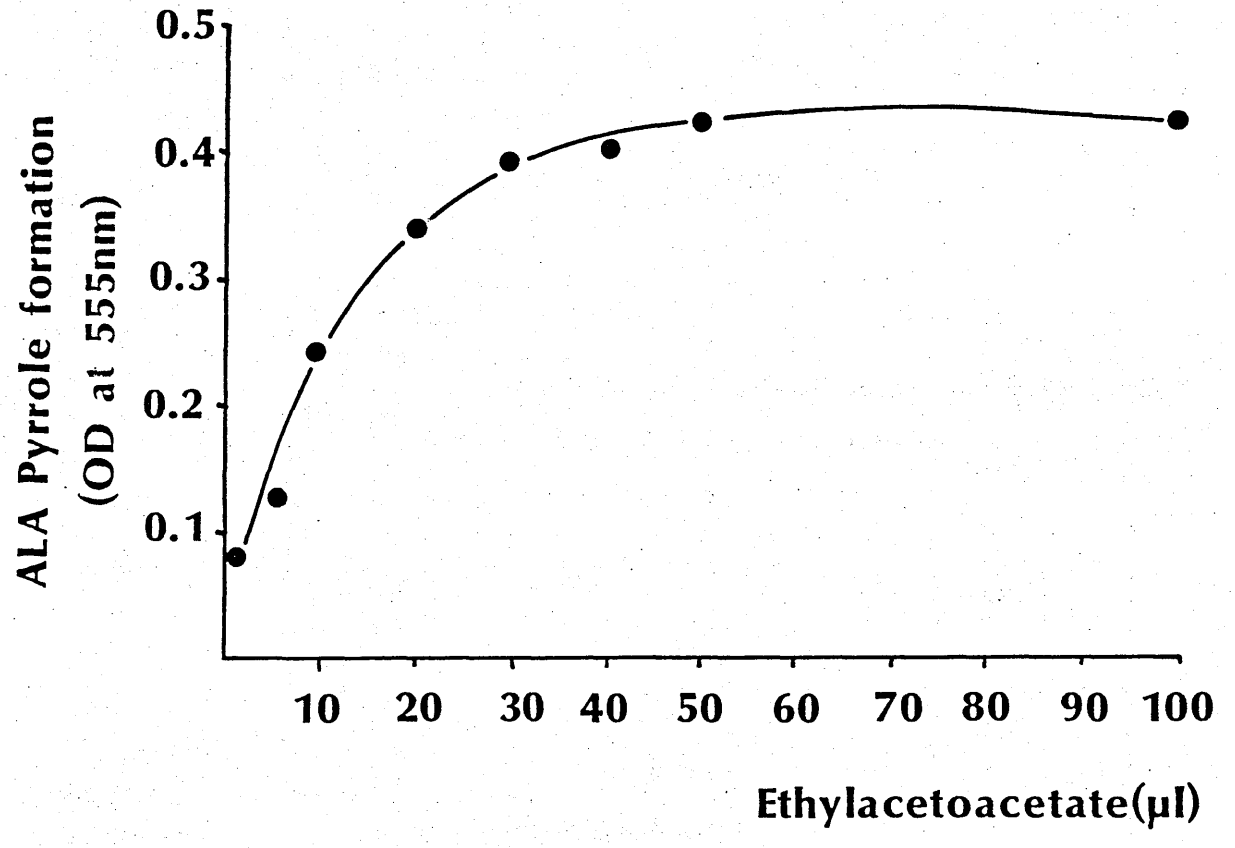
There was no loss of pyrrole at the Sep-pak stage and both pyrroles eluted at approximately the same time from the column. However, acetylacetone pyrrole peak height was well off the scale set to record eluted ethylacetoacetate pyrrole (252 nm, 1.0 AUFS). In view of this effect it was decided to continue further work with ethylacetoacetate pyrrole.

2.9.1 Formation of ethylacetoacetate pyrrole

The effect of ethylacetoacetate on ALA pyrrole production was examined.

5 mM ALA solution (0.05 ml) was added to glass boiling tubes containing incubation medium (0.5 ml) (Tikerpae et al, 1981), 10% TCA (0.25 ml), 1M sodium acetate (0.5 ml) at a final pH 5.3. Both the effect of ethylacetoacetate and time of heating to 100°C on ALA pyrrole production were assessed. Each tube was immersed in boiling water, rapidly cooled in ice then diluted with water (5.0 ml). Thereafter each sample was diluted a further 1 in 4 with water and reacted with equal volumes of modified Ehrlich reagent. The OD was read at 555 nm after standing for 15 min.

The amount of pyrrole formed after boiling for 10 min with 0.05 ml ethylacetoacetone was 82% of that formed at 20 min. With 0.1 ml, pyrrole formation at 10 min was the same as that at 20 min. The effect of altering the volume of ethylacetoacetate with boiling for 20 min was examined (Fig 6).



Note: ALA pyrrole was formed as described in text. Pyrrole production (20 min at 100°C) was measured by reaction with modified Ehrlich reagent. Each point represents the mean of two observations.

Figure 6: The effect of ethylacetoacetate on ALA pyrrole production

Maximum pyrrole formation occurred with the addition of $\geq$ 0.05 ml ethylacetoacetate which was adopted with a 20 min boiling period for routine use.

The effect of pH on ALA pyrrole formation was checked by altering the concentration of sodium acetate from 1.0 M to 2.0 M. It was found to be independent of pH over the range 5.3 to 6.5.

The extinction coefficient for ethylacetoacetate pyrrole is 7.2×10^4 molar (Mauzerall and Granick, 1956). Use of the formula

$$\text{Concentration (M)} = \frac{\text{OD}}{\text{Extinction Coefficient (M)}}$$

confirmed that under the conditions described ALA conversion to ALA pyrrole was 100%.

2.10 THE FINAL STEPS ; FROM REACTION TERMINATION TO RPHPLC

These experiments established a method for reaction termination, ALA conversion to ALA pyrrole and pyrrole isolation by RPHPLC. The method may be summarised as follows:

1. Terminate the ALA synthase reaction (final volume 0.5 ml) by the addition of 10% TCA (0.25 ml).
2. Add internal standard (0.05 ml 4 mM cold ALA) (section 2.11) and adjust the pH to 5.3 by the addition of 1 M sodium acetate (0.5 ml).
3. Spin down precipitated protein at 12,000 g for 2 min in an eppindorf centrifuge and transfer the supernatant to a 10 ml capacity glass boiling tube.

4. Add ethylacetoacetate (0.05 ml), loosely cap the boiling tube and heat to 100°C for 20 min.

5. Cool in ice, add water to 5 ml and inject the pyrrole solution through an activated C18 Sep-pak cartridge.

6. Dry the Sep-pak by attachment to a benchside vacuum and elute the pyrrole in methanol (3 ml).

7. Evaporate to dryness under N_2 in a heating block at 35°C and resuspend the pyrrole in 50% (v/v) aqueous methanol (0.1 ml). Thereafter 0.05 ml may be taken for RPHPLC. Collect the column eluate in 30s fractions (0.75 ml), add scintillant (8.0 ml) and count B emissions on a liquid scintillation counter.

2.11 ALA RECOVERY

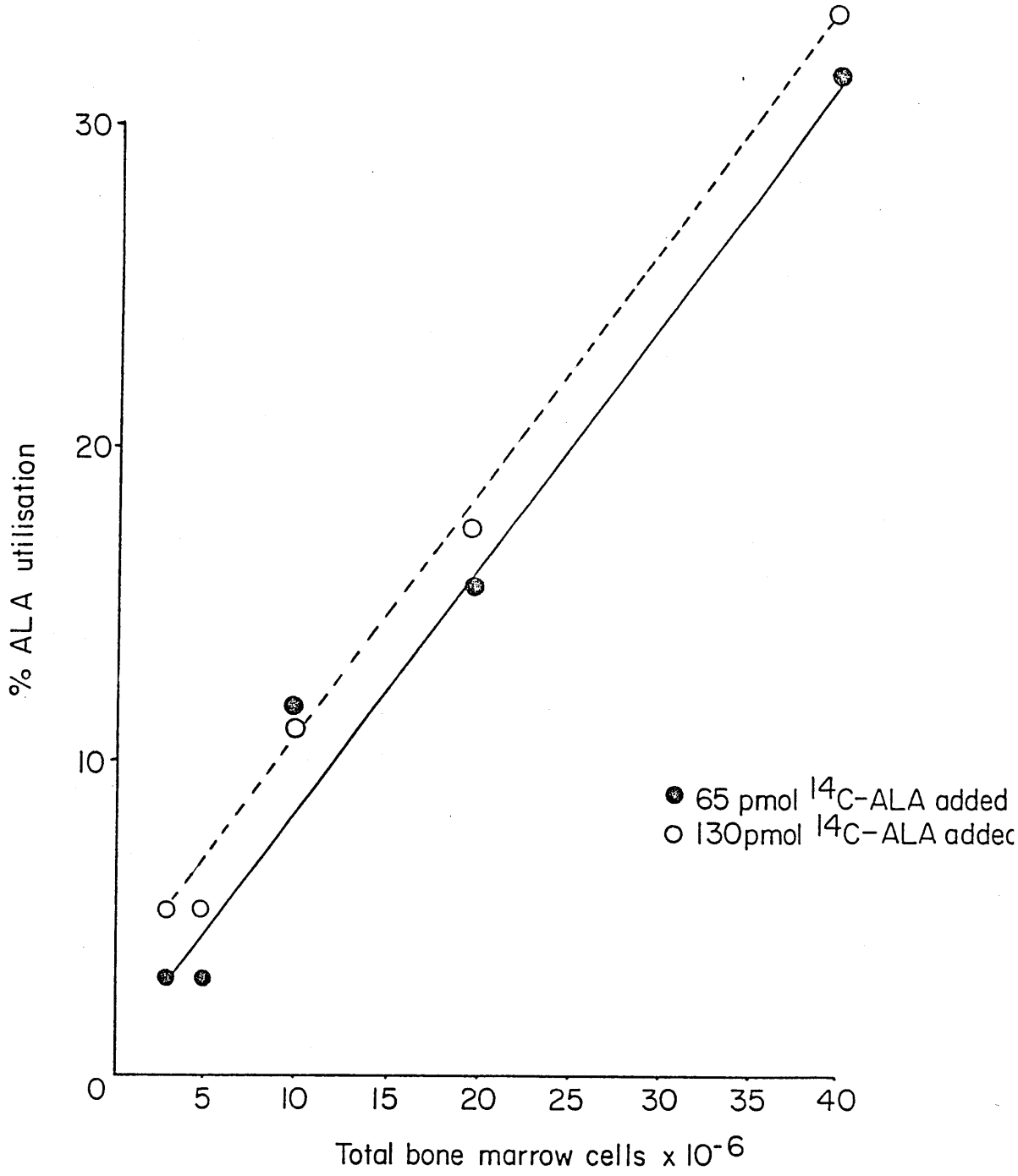
The use of RPHPLC permits the addition of large amounts of cold ALA (internal standard) at the end of incubation to each individual sample to assess ALA recovery. The ALA pyrrole peak height recorded with 0.05 ml 4 mM ALA (200 nmol), was approximately 80% of the maximum recorder deflection (spectrophotometer set to 252 nm and 1.0 AUFS). As ALA synthase activity in human bone marrow is in the pmol per hr range the [^{14}C]-ALA synthesised during incubation (pmol) would not contribute significantly to the recorded pyrrole peak height, which would reflect the conversion of cold ALA to ALA pyrrole. 200 nmol cold ALA was chosen as internal standard for routine use.

The percentage recovery of internal standard was calculated by adding [^{14}C]-ALA (0.025 uCi, 0.6 nmol) to eppindorfs containing incubation medium without either bone marrow cells or radioactive substrate. These eppindorfs were treated in an identical fashion to those containing test marrow and the ALA pyrrole from these recovery samples was taken for RPHPLC. From the recorded cpm in the 0.025 uCi [^{14}C] ALA, recovery was calculated as the percentage cpm recovered in the ALA pyrrole peak. The graph relating percentage recovery to pyrrole peak height was linear and the peak height corresponding to 100% recovery was read off.

2.12 ALA UTILISATION

Loss of [^{14}C]-ALA resulting from its metabolism during the assay of ALA synthase is called ALA utilisation. The effect of cell number on ALA utilisation was assessed.

Homogenised bone marrow cells 2-40 x 10^6 per 0.5 ml were prepared in incubation medium containing EDTA 10 mM (Tikerpae et al, 1981). Each concentration of bone marrow cells was prepared in quadruple. [^{14}C]-ALA 0.0025 uCi (65 pmol) was added to one pair and 0.005 uCi (130 pmol) to the other. From these pairs one eppindorf was incubated for zero time and the other for 60 min at 37°C. The percentage ALA utilisation for each sample was calculated from the cpm in ALA pyrrole at zero time and 60 min, corrected to 100% recovery of internal standard.



Note: ALA utilisation was assessed as described in text using both 65 pmol and 130 pmol [^{14}C]-ALA.

Figure 7: The effect of bone marrow homogenate cell number on ALA utilisation

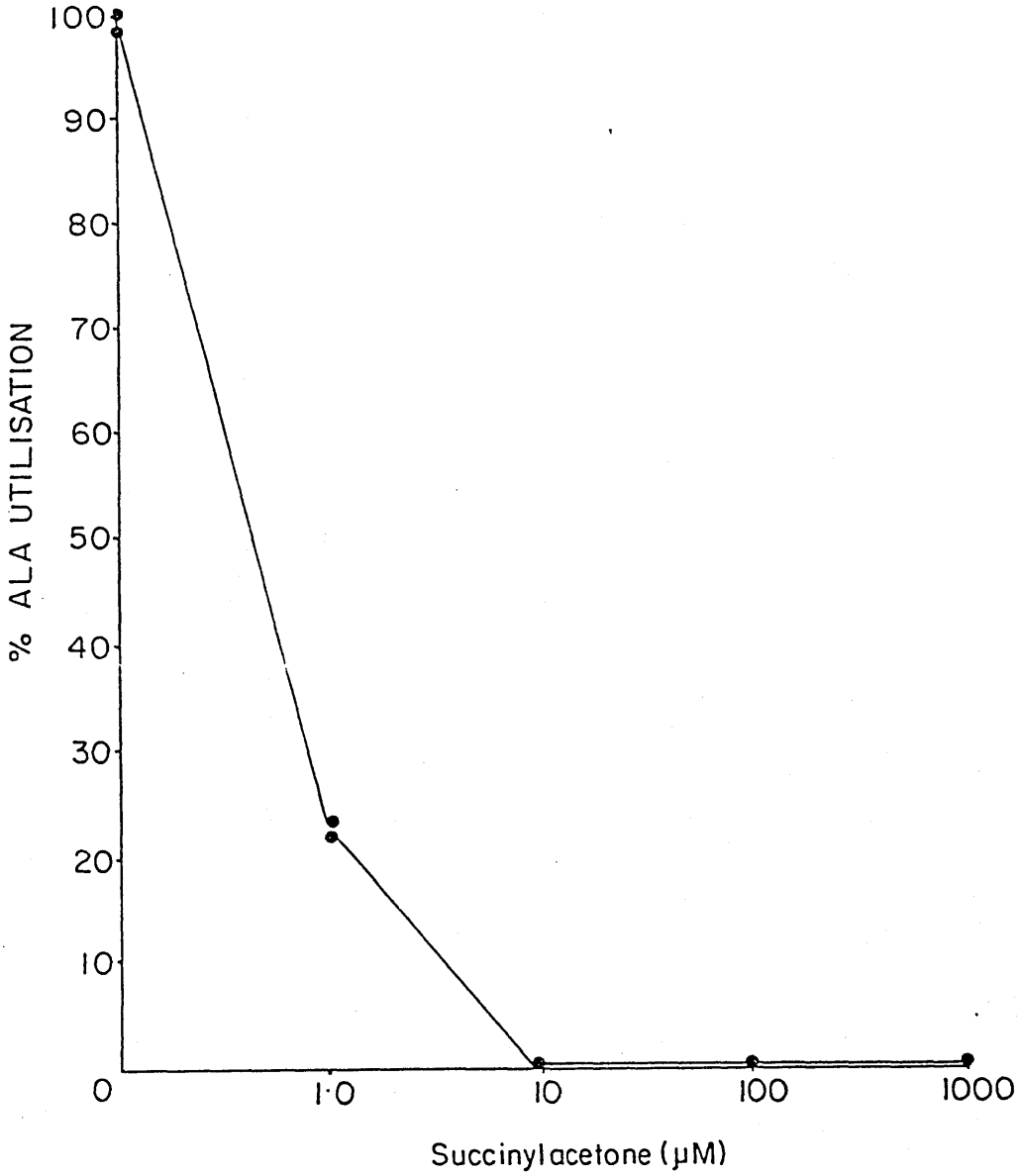
The results show (Fig 7) that ALA utilisation was linear with cell number and similar with both 65 and 130 pmol of [^{14}C]-ALA.

A linear loss of [^{14}C]-ALA during incubation was also found by Ebert et al (1970) using increasing concentrations of liver homogenate in medium without EDTA. EDTA has been shown to inhibit ALA utilisation in liver but not in erythroid tissue (Strand et al, 1972; Bottomley et al, 1973). Further assessment of ALA utilisation was carried out using 130 pmol [^{14}C]-ALA.

2.12.1 The effect of succinylacetone on ALA utilisation

The effect of succinylacetone on ALA utilisation was assessed by adding 0.005 uCi (130 pmol) [^{14}C]-ALA to 50 x 10^6 homogenised bone marrow cells in 0.5 ml incubation medium (Tikerpae et al, 1981) containing succinylacetone but no EDTA. The effect was compared with the ALA utilisation that occurred with incubation medium containing EDTA 10 mM but no succinylacetone. The marrow homogenate concentration used was high (50 x 10^6 bone marrow cells/0.5 ml) to ensure significant ALA utilisation in the absence of succinylacetone.

With incubation medium containing EDTA (10 mM) 70% utilisation occurred at 60 min. In the absence of EDTA ALA utilisation was completely inhibited by succinylacetone $\geqslant$ 0.01 mM (Fig 8).



Note: ALA utilisation was assessed by adding 130 pmol [^{14}C]-ALA to bone marrow homogenate (50 x 10^6 cells) in 0.5 ml incubation medium (see text). Each point represents the mean of two observations.

Figure 8: The effect of succinylacetone on ALA utilisation

2.13 CALCULATION OF RESULTS

With each assay ALA recovery samples were run in parallel so that the peak height corresponding to 100% recovery of the internal standard might be calculated.

2.13.1 Control tubes

The cpm in the ALA pyrrole region resulting from contaminating 2-keto[^{14}C]glutarate, [^{14}C]-succinate or radioactive metabolites were determined for each batch of samples assayed. Control tubes contained radioactive substrate incubated with bone marrow either for zero time and treated as described, or for 60 min at 37°C without the subsequent addition of ethylacetoacetate.

2.13.2 Enzyme activity calculation

For each individual sample assayed, the cpm in the ALA pyrrole region was corrected for background and for 100% recovery. For those few samples in which ALA synthase activity was measured in the absence of succinylacetone, further correction for ALA utilisation was required.

The ALA synthase activity in each eppindorf was calculated from the formula

$$\text{pmol ALA/eppindorf/h} = \frac{\text{Corrected cpm in ALA pyrrole}}{\text{cpm/pmol [}^{14}\text{C]-substrate}}$$

Enzyme activity was then related either to the total cell count or to the erythroblast content of each sample.

2.14 PREPARATION OF TISSUES

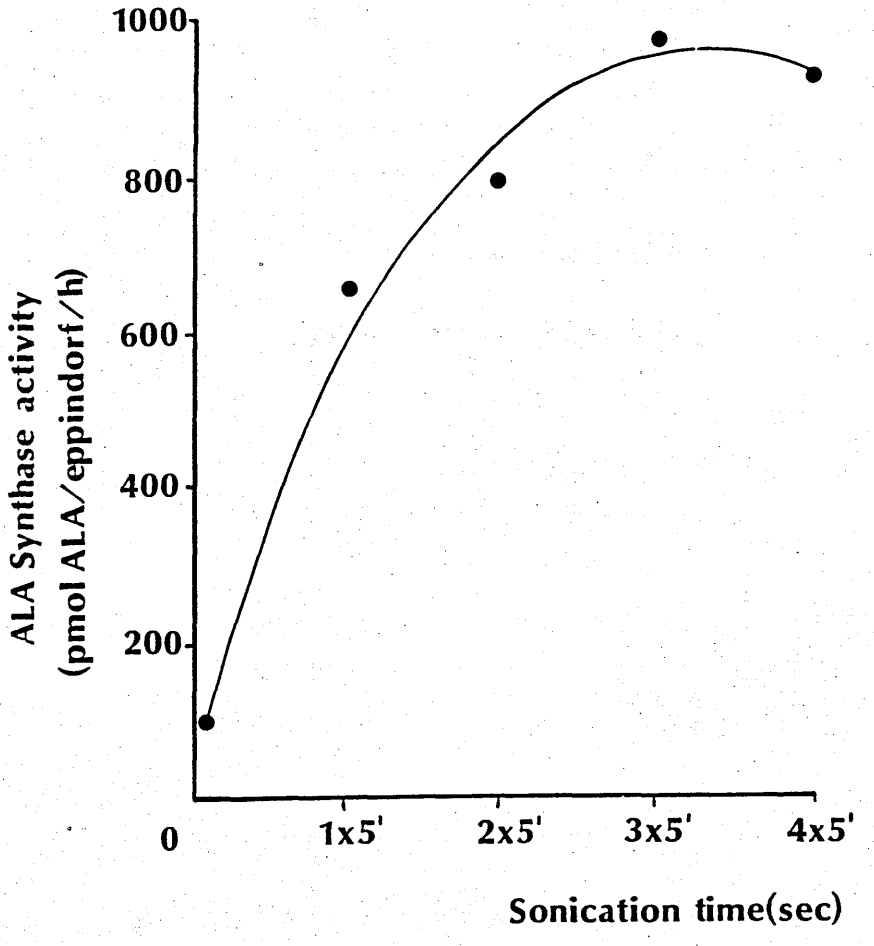
2.14.1 Sonication procedure

The optimum sonication procedure for the recovery of ALA synthase activity was determined using 2-keto[^{14}C]-glutarate (0.17 mM) with incubation medium optimised for use with this substrate (Tikerpae et al, 1981). Sonication was carried out in the small plastic tubes used by the Kemtek 3000 autoanalyser for radioimmunoassay analysis in many departments of Medical Biochemistry in this country.

120 x 10^6 bone marrow cells were obtained at sternotomy. The cells were washed and pellets of 20 x 10^6 cells were prepared in six individual Kemtek tubes. Incubation medium (1.5 ml) was added to each tube. The tubes were placed in ice and the cells were distributed using a MSE 150 KW ultrasonic disintegrator fitted with a 1/8" titanium probe. Amplitude was set to 15 microns in air which dampened to 12 microns in incubation medium.

The reaction was terminated at 60 min and the samples treated as described. ALA synthase activity per Eppindorf was calculated.

The optimum sonication procedure was 3 x 5" pulses (Fig 9). The activity recovered with one 15 sec pulse of sonication (not shown) was 30% less than that found after 3 separate pulses of 5 sec. The latter procedure was chosen for routine use.



Note: ALA synthase activity was assessed using 2-keto[^{14}C]glutarate. Each point represents the mean of two observations.

Figure 9: The effect of ultrasonication on ALA synthase activity in human bone marrow

2.14.2 The effect of deoxycholate

Aoki et al (1974) reported increased ALA synthase activity when bone marrow was prepared using a combination of sonication and membrane solubilisation with deoxycholate. Deoxycholate 0.5% was added to incubation medium prior to sonication and enzyme activity was determined using both 2-keto[^{14}C]-glutarate (0.17 mM) and [^{14}C]-succinate (2.0 mM). The incubation medium for [^{14}C]-succinate contained an exogenous succinyl CoA generating system ($MgCl_2$ 20 mM, GTP 2.5 mM, STK 0.5 U, final pH 7.4 at 37°C).

The addition of 0.5% deoxycholate to the incubation medium abolished ALA synthase activity with both succinate and 2-ketoglutarate. This was not due to a pH effect which remained unchanged at 7.4 with deoxycholate. No further experiments with membrane solubilisation were attempted and the method of sample preparation with sonication alone adopted for future use was as follows.

1. Prepare the marrow cell pellet in Kemtek tube.
2. Remove supernatant and add 1.5 ml incubation medium.
3. Pack tube in ice.
4. Disrupt cells using MSE 150 KW ultrasonic disintegrator. Set amplitude to 15 microns in air and apply 3 x 5 sec pulses.

2.15 CHOICE OF [^{14}C]-SUBSTRATE

2.15.1 Use of 2-ketoglutarate dehydrogenase

2-ketoglutarate dehydrogenase was obtained from Sigma Chemical Company and was tested for its suitability for use in the assay of ALA synthase using 2-keto[^{14}C]glutarate. Incubation medium (Tikerpae et al, 1981) containing NAD (1 mM) was used to allow conversion of 2-ketoglutarate to succinyl CoA (see section 2.4.1).

To examine this commercial enzyme preparation for contaminating ALA synthase activity, enzyme solution (0.5 U) was incubated with 2-keto[^{14}C]glutarate (0.17 mM, specific activity 6 Ci/mol) in NAD supplemented incubation medium. A control eppindorf without 2-ketoglutarate dehydrogenase was run in parallel.

The cpm in the ALA pyrrole fractions from the control sample was 20 cpm. In the test sample however the total cpm in the ALA pyrrole region was 2,200 cpm. This suggested that the enzyme preparation contained ALA synthase activity. As a result no further experimentation with 2-ketoglutarate dehydrogenase was attempted.

2.15.2 [^{14}C]-succinate V's 2-keto[^{14}C]glutarate

[^{14}C]-succinate and 2-keto[^{14}C]glutarate were examined to determine the more suitable substrate for use in the assay of bone marrow ALA synthase.

Optimum conditions for the measurement of enzyme activity with 2-keto[^{14}C]glutarate were used. Incubation medium was that described by Tikerpae et al (1981) with a final concentration of 0.17 mM 2-keto[^{14}c]glutarate (specific activity 6 Ci/mol). Incubation medium for [^{14}C]-succinate (specific activity 3.0 Ci/mol), at a final concentration 2 mM, was supplemented with an exogenous succinyl CoA generating system containing $MgCl_2$ 20 mM, GTP 2.5 mM and STK 0.5 U.

ALA synthase activity detected with 2-keto[^{14}C]glutarate was 200 pmol ALA/10^6 erythroblasts/h and with [^{14}C]-succinate was 440 pmol ALA/10^6 erythroblasts/h. Control counts were 3 x above background with [^{14}C]-succinate and 2 x above background with 2-keto[^{14}C]glutarate. This reflected the fivefold greater cpm added as [^{14}C]-succinate than as 2-keto[^{14}C]glutarate.

These results indicate that under conditions optimised for 2-keto[^{14}C]glutarate, the use of [^{14}C]-succinate with minor modifications to the incubation medium had doubled the sensitivity of the assay. Similar results have been reported with the study of hepatic ALA synthase (Ebert et al, 1970). In view of these findings and the relative ease with which commercial high purity STK may be obtained, [^{14}C]-succinate was used for all future experiments.

2.15.3 Use of STK

There are two available commercial preparations of STK. Both are GTP dependent, require Mg^{++} as cofactor and are prepared from porcine heart. The preparation from Sigma is available as a lyophilised powder whereas that from Boehringer Mannheim is an enzyme suspension in saturated ammonium sulphate.

Under identical conditions, in incubation medium containing $MgCl_2$ 20 mM and GTP 2.5 mM, 0.5 U of STK in ammonium sulphate was shown to reduce ALA synthase activity to 70% of that found with lyophilised STK resuspended in incubation medium. It was felt likely that this reduction in enzyme activity resulted from the addition of saturated ammonium sulphate.

STK 0.5 U (Sigma) was added to incubation medium (0.5 ml) containing $MgCl_2$ 20 mM, GTP 2.5 mM and [^{14}C]-succinate 2 mM (specific activity 3 Ci/mol). The mixture was incubated for 60 min at 37°C. The presence of STK in the incubation medium did not increase the cpm in the ALA pyrrole beyond the background cpm of a similar sample incubated without STK. This indicated that STK (Sigma) was free of ALA synthase activity and this preparation was used for all further experiments.

2.16 OPTIMUM CONDITIONS FOR THE MEASUREMENT OF BONE MARROW ALA SYNTHASE ACTIVITY USING [^{14}C]-SUCCINATE

The optimum concentration of reagents in the incubation medium was determined for maximum ALA synthase activity with [^{14}C]-succinate

as substrate. In particular it was decided (i) to examine the need for exogenous STK; (ii) to optimise the concentration of EDTA and succinylacetone such that ALA utilisation might be inhibited without loss of ALA synthase activity; (iii) to determine the optimum concentrations of [^{14}C]-succinate, CoA and GTP; and (iv) to assess the need for citric acid cycle inhibitors in the medium. As the conversion of succinate to succinyl CoA requires free Mg^{++} $MgCl_2$ (20 mM) was added to the medium.

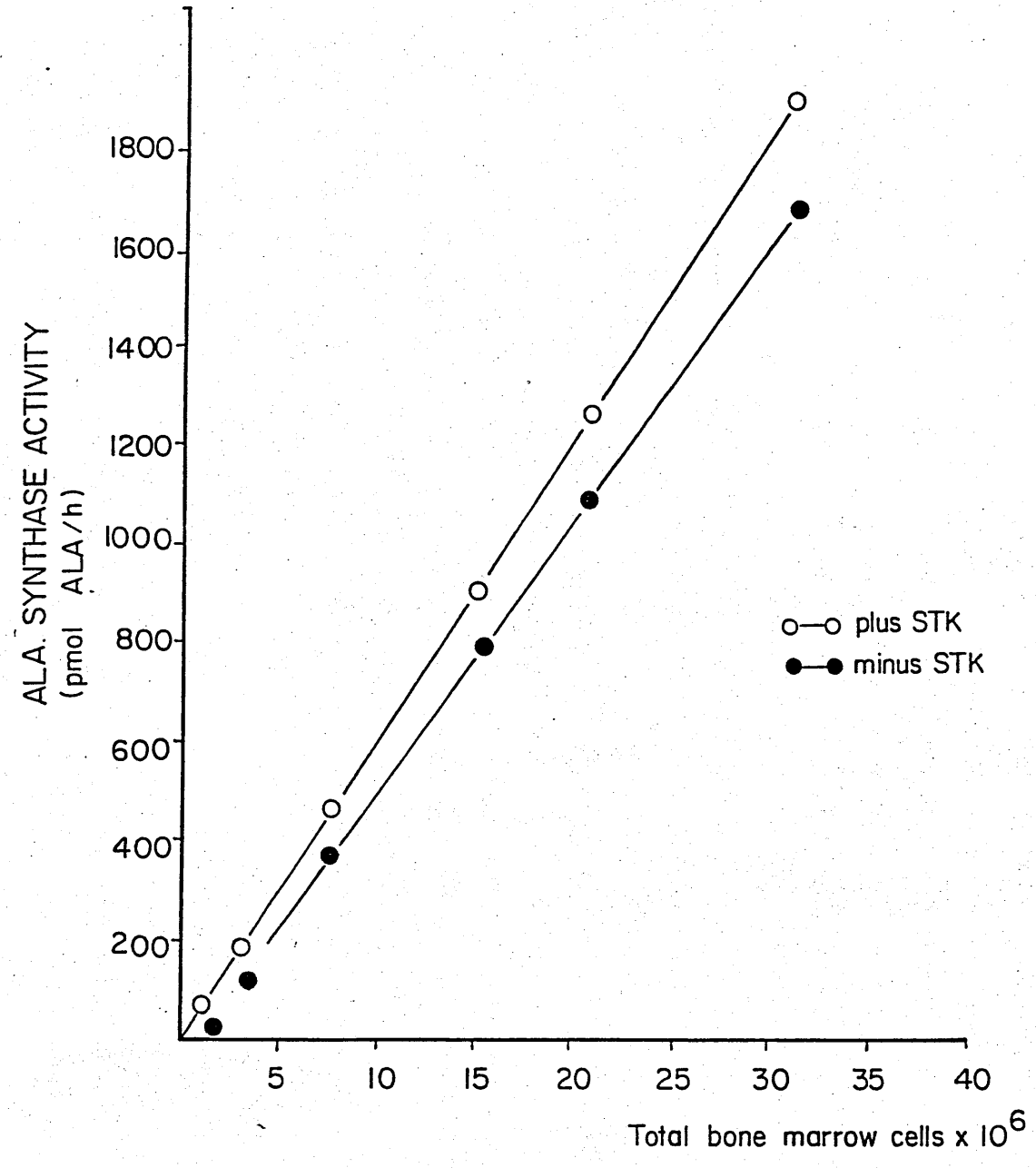
2.16.1 Need for exogenous STK

The addition of STK 0.5 U to bone marrow homogenate in incubation medium containing $MgCl_2$ 20 mM, CoA 1.35 mM and GTP 5.0 mM produced a 10% increase in ALA synthase activity (Figure 10).

2.16.2 Optimum concentrations of CoA, GTP, STK

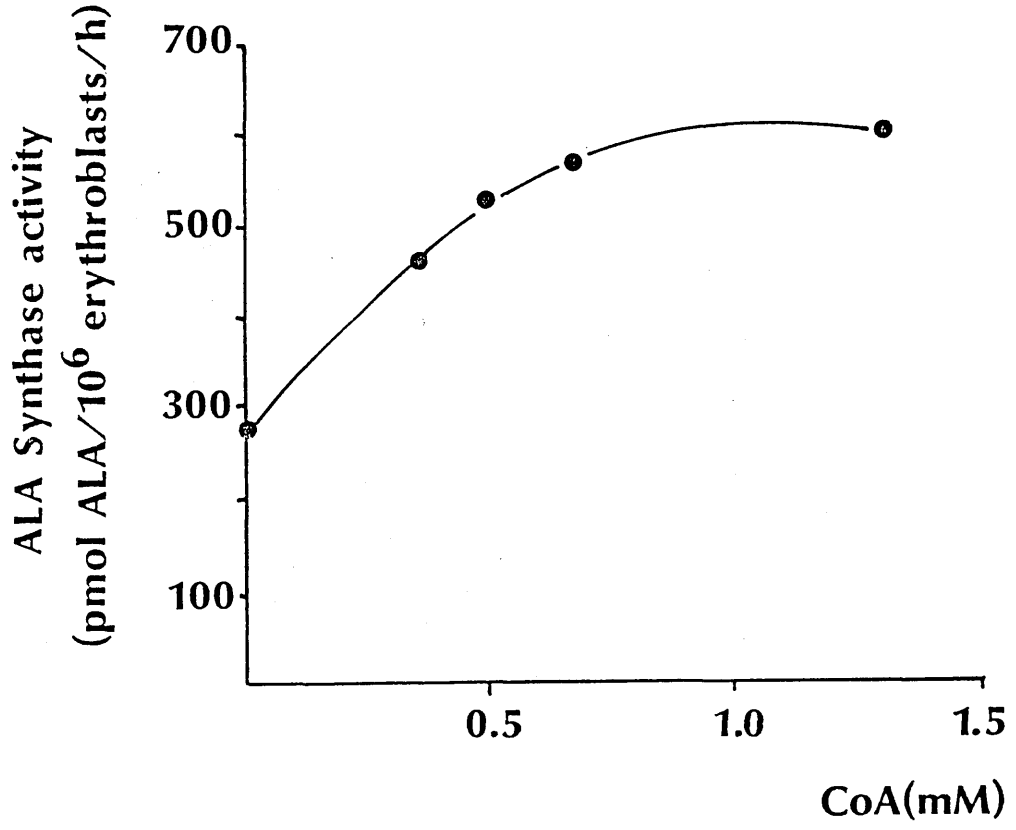
The concentrations of CoA and GTP were altered with the pH maintained at 7.4 at 37°C. Marrow homogenate was incubated in medium containing sucrose 250 mM, glycine 50 mM, GTP 2.5 mM, $MgCl_2$ 20 mM, EDTA 10 mM, PLP 0.4 mM, Tris 40 mM buffered to pH 7.4 at 37°C with KH_2PO_4 50 mM and STK 0.5 U in a final volume of 0.5 mol. The concentration of added CoA ranged from 0-1.35 mM with pH maintained at 7.4 at 37°C. ALA utilisation was calculated as described (section 2.12).

Maximum ALA synthase activity was found with CoA 1.35 mM (Fig 11).



Note: Assay conditions are described in the text.

Figure 10: The effect of STK and unfractionated bone marrow cell number on ALA synthase activity



Note: Each point represents the mean of two observations. Incubation medium is described in the text.

Figure 11: The effect of CoA concentration on ALA synthase activity

Three separate GTP concentrations were tested to determine the optimum GTP concentration in the incubation medium containing CoA 1.35 mM.

Maximum ALA synthase activity was found with GTP 5 mM (Fig 12).

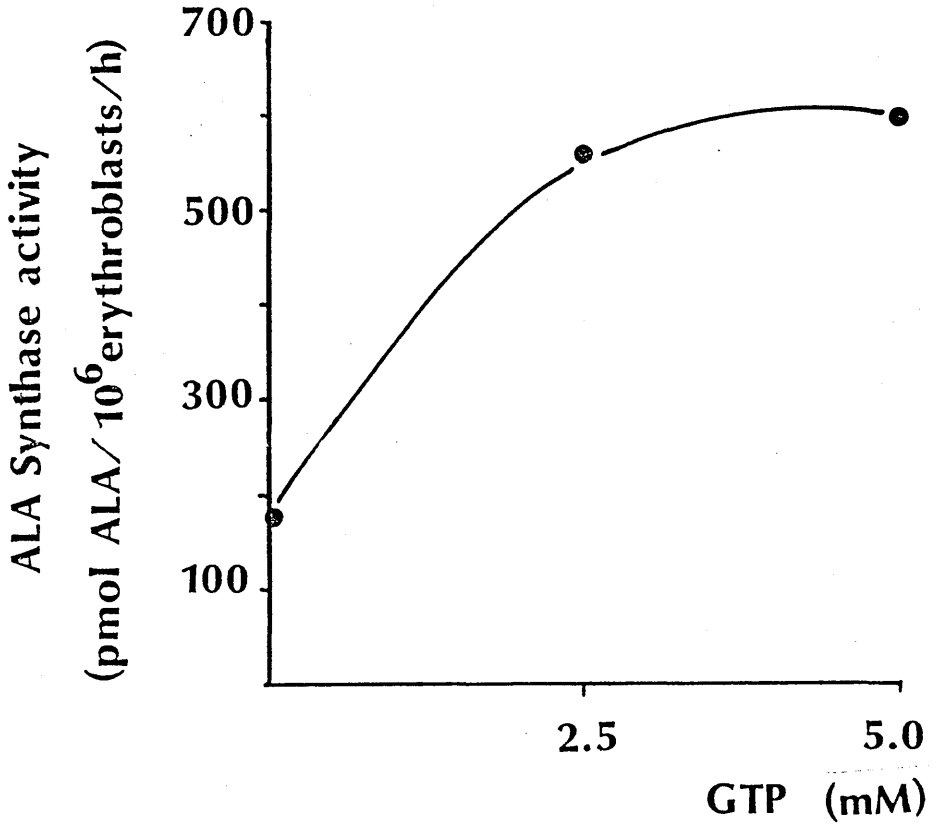
In the final group of experiments the combined optima for GTP, CoA and STK were determined. CoA, GTP and STK concentrations were altered with the pH maintained at 7.4 at 37°C. ALA synthase activity was determined in eppindorfs each containing approximately 15 x 10^6 bone marrow cells (Table 1).

The optimum combination of reagents for the detection of ALA synthase activity in human bone marrow was GTP 5.0 mM CoA 1.35 mM and STK 0.5 U in a final vol of 0.5 ml.

2.16.3 The effect of EDTA and succinylacetone on ALA synthase activity

Succinylacetone was shown to completely inhibit ALA utilisation (section 2.12.1). The effect of both EDTA and succinylacetone on ALA synthase activity was determined with a view to abolishing ALA utilisation without loss of ALA synthase activity.

Incubation medium contained glycine 50 mM, $MgCl_2$ 20 mM, sucrose 250 mM, PLP 0.4 mM, CoA 1.35 mM, GTP 5 mM, Tris 40 mM buffered with KH_2PO_4 50 mM to pH 7.4 at 37°C and 0.5 U STK in a final volume of 0.5 ml. The effect of EDTA 10 mM and succinylacetone



Note: Each point represents the mean of two observations. Incubation medium is described in the text.

Figure 12: The effect of GTP concentration on ALA synthase activity

SAMPLE	CoA (mM)	GTP (mM)	STK (U/0.5 ml)	ALA SYNTHASE (pmol ALA/Eppindorf/h
1	0.65	2.5	0.5	3078
2	0.65	2.5	1.0	3098
3	0.65	5.0	0.5	3647
4	0.65	5.0	2.0	3507
5	1.35	2.5	1.0	3589
6	1.35	2.5	1.0	3642
7	1.35	5.0	0.5	4073
8	1.35	5.0	1.0	3933

NOTE: Incubation conditions are described in the text.

TABLE 1: The effect of CoA, GTP and STK on ALA synthase activity

0.1 mM was assessed with three marrow samples, two were taken from normal subjects and one from a patient with chronic lead poisoning (free erythrocyte protoporphyrin 360 ug/100 ml packed cells, normal < 90, whole blood lead 45 ug/100 ml, normal < 40). From each bone marrow sample three identical cell pellets were prepared and assayed for ALA synthase activity in the presence of

a) 0.1 mM Succinylacetone, zero EDTA

b) 0.1 mM Succinylacetone, 10 mM EDTA

c) Zero Succinylacetone, 10 mM EDTA

In the absence of succinylacetone ALA utilisation was calculated as described (section 2.12).

The results are shown in Figure 13. Neither EDTA 10 mM nor succinylacetone 0.1 mM, separately or together, were shown to inhibit ALA synthase activity and both were chosen for routine use.

2.16.4 Optimum succinate concentration

The optimum concentration of [^{14}C]-succinate for the measurement of bone marrow ALA synthase activity was determined.

Normal marrow homogenate was incubated with different concentrations of [^{14}C]-succinate under identical conditions. The incubation medium contained glycine 50 mM, sucrose 250 mM, $MgCl_2$ 20 mM, EDTA 10 mM PLP 0.4 mM, CoA 1.35 mM, GTP 5 mM, succinylacetone 0.1 mM, Tris 40 mM buffered to pH 7.4 at 37°C with KH_2PO_4 50 mM and STK 0.5 U in a final volume of 0.5 ml.

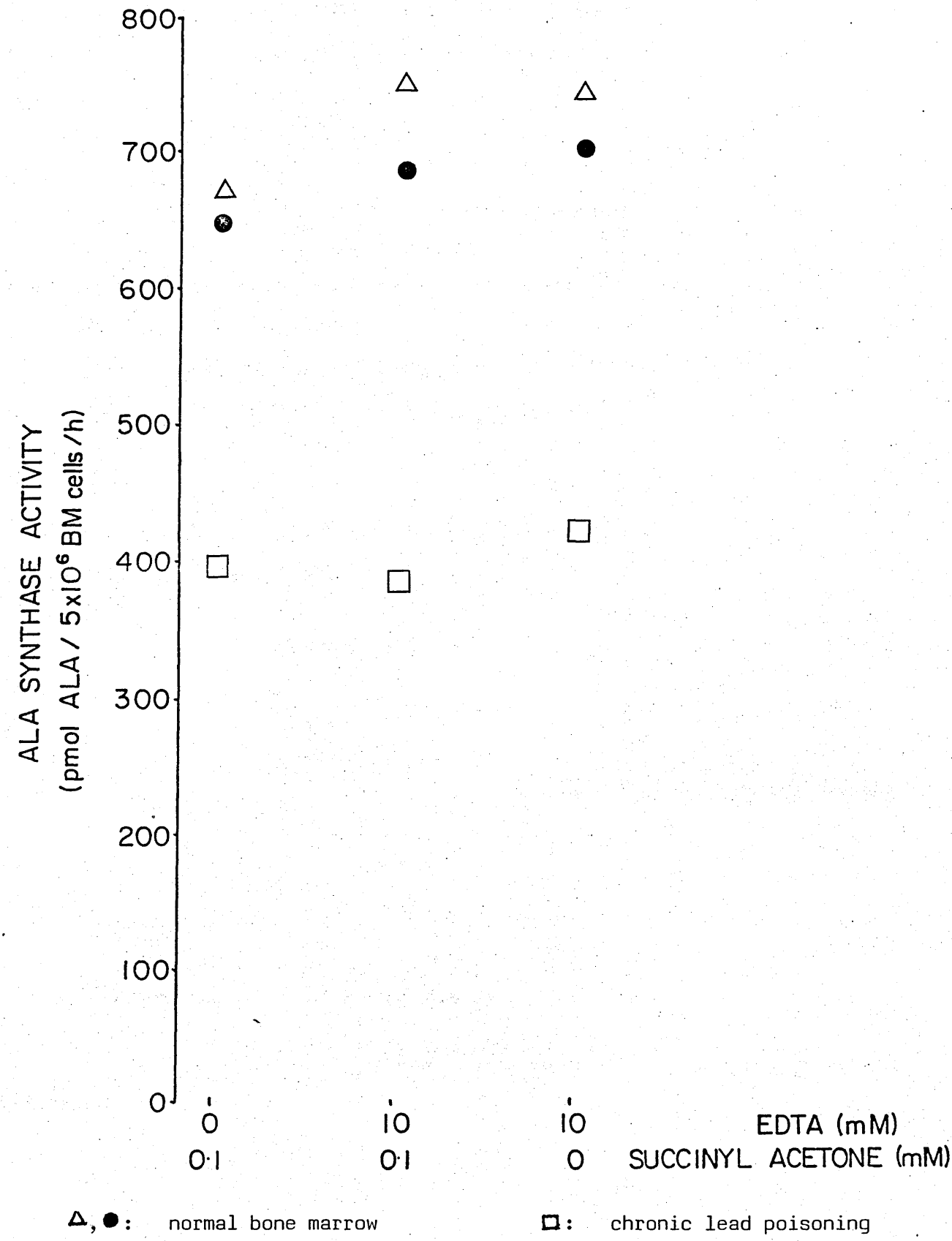


Figure 13: The effect of succinylacetone and EDTA on ALA synthase activity

The saturating level of succinate for maximal ALA synthase activity was 2 mM (Fig 14) and this concentration was chosen for routine use.

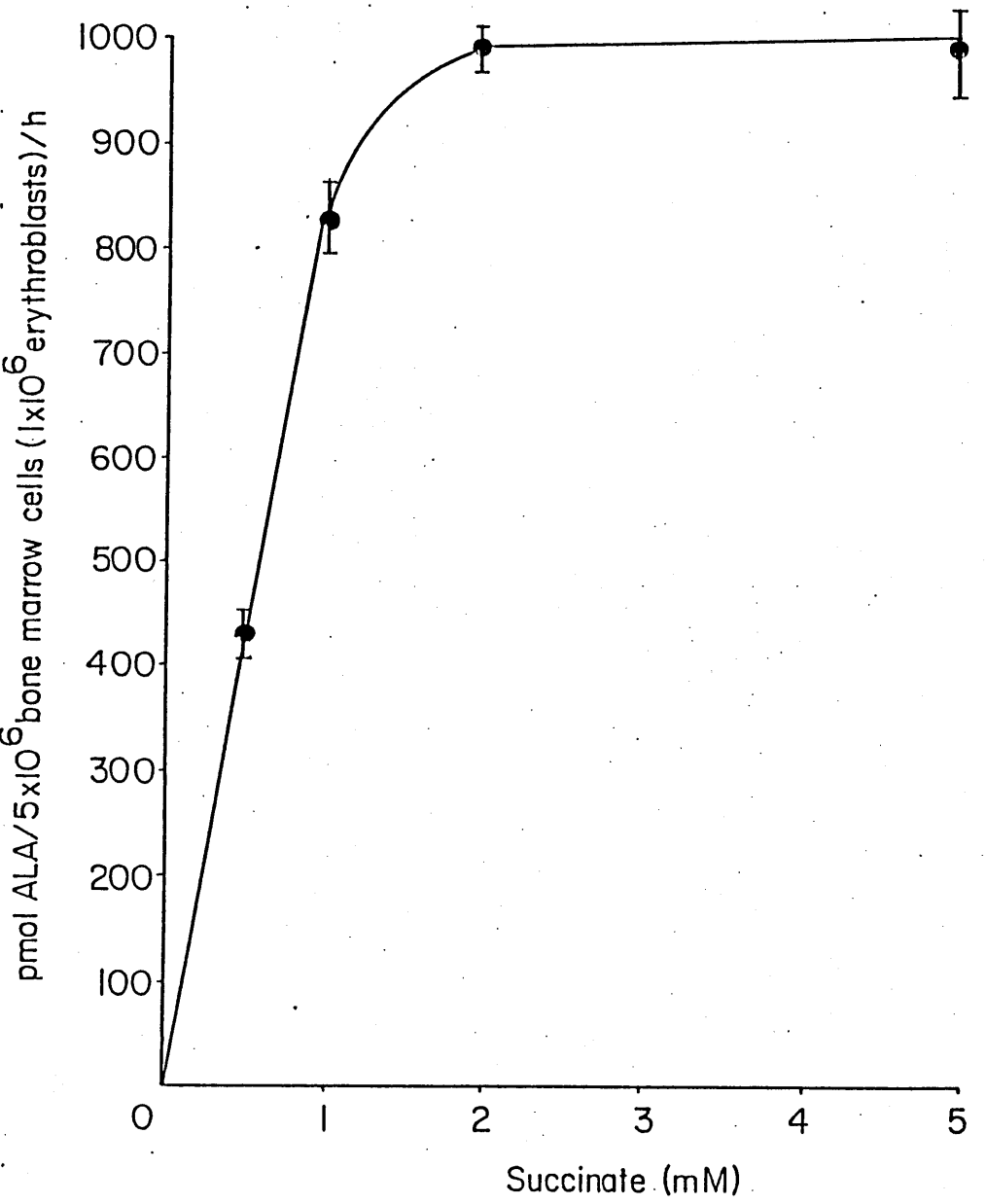
2.16.5 Effect of cell number and incubation time on ALA synthase activity

The effect of cell number in the bone marrow homogenate was determined. Enzyme activity was measured under the conditions described (section 2.16.4). Succinylacetone was omitted and ALA utilisation was calculated (see section 2.12).

The results show (Fig 10) that ALA synthase activity expressed as pmol ALA formed per eppindorf at 60 min was linear with homogenate concentration from 0.2-36 x 10^6 bone marrow cells per 0.5 ml. Each point on Figure 10 represents a single observation as duplicate eppindorfs were used to demonstrate the increase in enzyme activity apparent with the addition of STK (0.5 U).

Enzyme activity was also shown to be linear with time and the activity present at 60 min was twice that present at 30 min.

Under the conditions described ALA synthase activity was shown to be linear both with time and homogenate concentration. Such an effect is likely to indicate measurement of ALA synthase activity under optimal conditions (Patton and Beattie, 1973).



Note: Incubation was carried out for 60 min at 37°C in the presence of glycine (50 mM), sucrose (250 mM), EDTA (10 mM), PLP (0.4 mM), $MgCl_2$ (20 mM), GTP (5 mM), CoA (1.35 mM), succinylacetone (0.1 mM) and STK (0.5 U) in a final volume of 0.5 ml. Each point represents the mean of two observations.

Figure 14: The effect of [^{14}C] succinate concentration on ALA synthase activity

2.16.6 The effect of citric acid cycle inhibitors on ALA synthase activity

The effect of sodium malonate (10 mM) and sodium Dl-malate (5 mM) on ALA synthase activity in normal bone marrow was examined.

The addition of these inhibitors did not alter the pH of the incubation medium but did not increase ALA synthase activity. Added separately they reduced enzyme activity by 40% with a 60% reduction when added together. The results are similar to those found by Ebert et al (1970) with the study of liver and spleen enzyme.

2.16.7 Reproducibility

The standard error of twelve duplicate estimates was derived from the residual error in a one-way analysis of variance from the natural logarithm of the individual estimates. This type of analysis was used by Cotes and Brozovic (1982) and may be applied to look at the intrinsic variability of a measurement after that due to inter-subject variation has been "removed" statistically. It was felt that as the enzyme activity in normal marrow covered a wide normal range such an analysis would be more valuable than the coefficient of variation. It was shown that within 95% confidence limits the variation between duplicate samples was $< 5\%$.

2.17 SUMMARY

2.17.1 Optimum conditions for the assay of bone marrow ALA synthase activity

Sample preparation:

Wash bone marrow cells x 3 in cold culture medium (centrifugation 1500 g_{av} and 4°C). Resuspend the cell pellet in fresh incubation medium (1.5 ml) and disrupt the cells using a MSE 150 KW ultrasonic disintegrator fitted with 1/8" titanium probe. Maintain the sample temperature at 4°C during sonication with ice packing, set the amplitude to 15 microns in air and apply 3 x 5 sec pulses of sonication.

Preparation of incubation medium:

Prepare incubation medium in part and store in batches at -20°C until use. Prepare tetrasodium EDTA (10 mM) sucrose (250 mM) $MgCl_2$ (20 mM), glycine (50 mM) and Tris (40 mM) in a final volume of 1 litre (pH 8.0 at 37°C). Divide this solution and add KH_2PO_4 (50 mM) to 500 ml. Add aliquots of this acid solution to the original 500 ml until the pH is 7.54 at 37°C. (This pH may be achieved by the addition of 370 ml of KH_2PO_4 buffered medium). Store this preparation in 20 ml aliquots at -20°C until required.

Prepare 20 mM [^{14}C]-succinate (specific activity 3 Ci/mol) in this medium and store in aliquots of 210 ul at -20°C.

The assay:

On the day of the assay thaw an aliquot of incubation medium and add CoA (1.5 mM), GTP (5.6 mM), PLP (0.45 mM) and succinylacetone

(0.11 mM). Resuspend the marrow cell pellet (1.5 ml) and lyophilised STK (0.5 U/25 µl (Sigma) in this fresh medium.

Add 50 µl [^{14}C]-succinate (20 mM), 425 µl of marrow homogenate and 25 ul STK solution to each eppindorf. Incubate at 37°C for 60 min and assay each sample in duplicate. The final medium (0.5 ml) contains glycine 50 mM, Sucrose 250 mM, $MgCl_2$ 20 mM, EDTA 10 mM, PLP 0.4 mM, STK 0.5 U, GTP 5 mM, CoA 1.35, succinylacetone 0.1 mM, Tris 40 mM and KH_2PO_4 20 mM, pH 7.4 at 37°C.

2.18 APPENDIX TO EXPERIMENTAL AND RESULTS

2.18.1 Storage of bone marrow samples

Bone marrow samples were either assayed fresh for ALA synthase activity or after storage as cell pellets at -70°C. Under these conditions ALA synthase activity was stable for at least six months.

2.18.2 Purity of commercial [^{14}C]-ALA

At the completion of this work a method was devised to check the purity of commercial [^{14}C]-ALA.

[^{14}C]-ALA stored at -20°C for a total of two years (a further year after the completion of the experimental work) was examined. Approximately 1.2 nmol [^{14}C]-ALA was added to 0.5 ml incubation medium containing 200 nmol cold ALA (identical to that used as internal standard). ALA pyrrole was formed as described and the volume made to 5.0 ml with water. From this solution 0.5 ml was

diluted 1 in 5 with water and reacted with an equal volume of modified Ehrlich reagent. The OD at 555 nm was 0.32.

From the formula

$$\text{Conc(M)} = \frac{\text{OD}}{\text{Extinction coefficient (M)}}$$

it was determined that there had been 100% conversion of cold ALA to ALA pyrrole. 1.0 ml of this solution was taken for scintillation counting and the remainder injected through an activated C18 Sep-pak cartridge. No ALA pyrrole was detected by reaction with Ehrlich's reagent in the Sep-pak eluate thereby indicating that all ALA pyrrole had been retained by the Sep-pak. 1.0 ml of eluate was taken for scintillation counting.

Despite the absence of detectable ALA pyrrole in the eluate, 20% of the original cpm were recovered in this fraction. These cpm did not represent unconverted [^{14}C]-ALA. 1.0 ml of this fraction was reheated to 100°C for 20 min with 0.1 ml ethylacetoacetate. On cooling, no ALA pyrrole was detected with Ehrlich reagent.

ALA pyrrole was eluted from the Sep-pak with methanol (3.5 ml). An aliquot of methanol was diluted 1 in 5 with water and reacted with 1.0 ml of modified Ehrlich reagent. The OD was 0.32 at 555 nm and this indicated complete recovery of cold ALA pyrrole. 1.0 ml of methanol was taken for scintillation counting; the remainder was evaporated to dryness and resuspended in 0.1 ml 50% (v/v) aqueous methanol from which 0.05 ml was taken for RPHPLC. The cpm were determined in 30 sec fractions of HPLC column eluate.

The remaining 80% of the original cpm were recovered from the Sep-pak in the methanol and were shown by RPHPLC to represent [^{14}C]-ALA pyrrole.

These results indicate that after two years' storage the [^{14}C]-ALA was only some 80% pure. This was simply shown by demonstrating 20% of the original cpm in the Sep-pak eluate, which did not contain either ALA or ALA pyrrole. RPHPLC confirmed that the cpm retained by Sep-pak were as [^{14}C]-ALA pyrrole.

The producers of the [^{14}C]-ALA (Amersham International) were informed. Some two months after the purchase of the radiochemical their own re-testing had identified the batch to be 93.7% pure. The particular batch was withdrawn. Further deterioration to 80% within the next two years is more than would be expected from the accompanying information on [^{14}C]-ALA from Amersham. The company now feels that their prediction of 4% deterioration per annum is not realistic and will amend their product information accordingly.

DISCUSSION

There is no doubt but that the most significant feature of the developed assay method is the simple and specific technique described to isolate [^{14}C]-ALA pyrrole. The introduction of a sample preparation step using a Sep-pak cartridge obviated the need for ether extraction and freeze drying prior to RPHPLC (Tikerpae et al, 1981) and circumvented the problems relating to the specific isolation of [^{14}C]-ALA (Bishop and Wood, 1977; Minaga et al, 1978; Wolfson et al, 1980; Scotto et al, 1983). The Sep-pak facilitated the isolation of pure ALA pyrrole by RPHPLC, for which an ion-pairing agent and a C18 column were used to provide maximum resolving power for the hydrophobic ALA pyrrole. Low background counts in the controls samples indicated that the combination of Sep-pak and RPHPLC achieved separation of ALA pyrrole from radioactive succinate, contaminants in commercial [^{14}C]-succinate and metabolites. This separation procedure allowed the addition of internal standard to each individual sample and eliminated the problem of variable ALA recovery associated with multicolumn ion exchange fractionations (Schacter et al, 1976). TCA was added to stop the reaction, centrifugation removed precipitated protein and sodium acetate was added to adjust the pH to 5.3. ALA was converted to ALA pyrrole which was bound to a C18 Sep-pak cartridge prior to RPHPLC.

Utilisation of ALA formed during incubation in the presence of EDTA does not occur with liver (Ebert et al, 1970; Strand et al, 1972) but is a recognised problem with the assay of erythroid ALA synthase (Strand et al, 1972; Bottomley et al, 1973). This observation was confirmed and utilisation was shown to be linear with bone marrow cell number up to 40 x 10^6/0.5 ml (Fig 7). There was no evidence to support the report of disproportionate ALA utilisation at high marrow homogenate concentration (Bottomley et al, 1973) or of inhibition of ALA synthase activity by 10 mM EDTA (Strand et al, 1972).

Succinylacetone 10^{-5} M was shown to inhibit ALA utilisation (Fig 8) and was used at 10^{-4} M in the incubation medium to inhibit utilisation without loss of ALA synthase activity (Fig 13). This effect suggests that ALA utilisation during the assay of erythroid ALA synthase results from incomplete inhibition of ALA dehydratase by EDTA and not from alternative metabolism which might lead to the transamination of ALA and formation of $\gamma\delta$ dioxovaleric acid (Tait, 1969) or from non enzymatic degradation of ALA. The presence of succinylacetone during incubation removed the need to measure and correct for ALA utilisation and so reduced (halved) the number of samples required in each assay and improved precision.

[^{14}C]-succinate was shown to be more sensitive to bone marrow ALA synthase than 2-keto[^{14}C]glutarate; an observation already

reported with liver homogenate (Ebert et al, 1970). The method described for measurement of bone marrow ALA synthase was optimised with respect to sonication procedure ALA pyrrole production, succinate, CoA, GTP, STK, EDTA and succinylacetone concentrations to obtain maximum ALA synthase activity. The reaction was shown to be linear with time over 60 min and with cell number up to 36 x 10^6 bone marrow cells per 0.5 ml. It was capable of detecting enzyme activity in as little as 2 x 10^5 bone marrow cells (5 x 10^4 erythroblasts) (Fig 10) and was shown to be highly reproducible. The optimum conditions established for the measurement of bone marrow ALA synthase differ from those which are required for maximum liver enzyme activity (Irving and Elliott, 1969; Ebert et al, 1970; Strand et al, 1972). This would be expected as it has been recognised that problems may arise when an ALA synthase assay developed with one tissue is applied without modification to detect enzyme activity in a different tissue (Condie and Tephly, 1978). Optimum conditions for the measurement of liver ALA synthase have been shown to be suboptimal for adrenal or cardiac enzyme. Furthermore chromatographic separation techniques also require to be ascertained and modified for use with different tissues (Briggs et al, 1976; Condie et al, 1976).

The addition of citric acid cycle inhibitors sodium malonate (10 mM) and sodium malate (5 mM) reduced ALA synthase activity. This was unexpected but has been reported with the assay of the liver enzyme (Ebert et al, 1970). There would seem to be two

possible explanations. Either malonate and malate inhibited ALA synthase or they effected a dilution of the [^{14}C]-succinyl CoA pool with cold succinyl CoA. The effect of arsenite on marrow ALA synthase activity was not tested in view of the toxicity hazard (Muir, 1977) and the reported 41% inhibition of ALA synthase activity by 5 mM arsenite (Ebert et al, 1970). Inhibition of 2-ketoglutarate dehydrogenase without loss of significant ALA synthase activity would be desirable, as it would indicate whether the reduction in ALA synthase activity in the presence of malate and malonate had resulted from increased synthesis of cold succinyl CoA or from inhibition of ALA synthase. Although Ebert et al (1970) felt that the former hypothesis was not likely, future experiments with citric acid cycle inhibitors may explain these observations.

The determination of ALA synthase activity in radiochemical assays is dependent upon the purity of commercial [^{14}C]-ALA used to determine ALA recovery. In this assay it was used to calculate the peak height equivalent to 100% recovery. Problems with the purity of commercial [^{14}C]-ALA are recognised by other workers and by the manufacturers. It would seem as if the purity of the [^{14}C]-ALA used for the work reported here was about 90%. This would make no material difference to the results recorded. The description of a method to determine the purity of commercial [^{14}C]-ALA, ensures that the manufactuers claims of purity and stability may now be examined both rapidly and simply before they are accepted.

CHAPTER 3

5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN UNFRACTIONATED HUMAN BONE MARROW

3. 5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN UNFRACTIONATED HUMAN BONE MARROW

From microscopic examination it is evident that bone marrow is a dynamic continuously developing tissue, composed of cells from different lineages, at different stages of differentiation. The major cell lines are myeloid and erythroid 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 which in normal marrow 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, adult marrow is readily accessible and may be aspirated from the anterior/posterior iliac crests or from the sternum in relatively small volumes. Large volumes are more difficult to obtain as aspiration of more than 0.3 ml leads to dilution of the marrow sample with peripheral blood (Dacie and Lewis, 1984).

Bone marrow is the major iron utilising and haem producing tissue in the body (Berk et al, 1976) from which hypochromic microcytic red cells originate in response to iron and so haem deficient erythropoiesis. All mammalian cells apart from mature red blood cells synthesise haem. The myeloid cells in bone marrow are no exception and are rich in haem containing enzymes, peroxidase,

catalase and mitochondrial cytochromes. Peroxidase is most abundant and represents some 5% of the total dry weight of the polymorphonuclear leucocyte (Suzuki et al, 1983). It is then clear that although erythroid cells are the major haem forming cells and manifest the effects of haem deficiency most clearly, they are not the only haem producing cells in the marrow.

Visual evidence of haem synthesis in bone marrow is most apparent from the development of haemoglobin in erythroid cells. During erythropoiesis there is a parallel progression of haemoglobinisation and differentiation. Haemoglobin is not evident in the early basophilic erythroblasts, its presence may be detected in the intermediate polychromatic erythroblasts, while in the late orthochromatic forms it packs the cytoplasm as if to squeeze the small pyknotic nucleus from the cell. There is considerable interest as to what factors regulate the synthesis of erythroid haem and what factors are regulated by synthesised haem. It is thought that free haem may have a central role in the regulation of protein and globin synthesis, in other aspects of cell metabolism such as iron uptake and also in the control of the differentiation process itself (see Chapter 5). Although the role of ALA synthase as the rate limiting enzyme of hepatic haem biosynthesis has been identified (Granick and Urata, 1963) its place in the regulation of human erythroid haem synthesis is less certain (see Chapter 5). The level of haem synthetic activity within erythroid cells may be affected by two separate factors:

firstly the level of cell differentiation and secondly the processes adapted for short term regulation and fine adjustment of haem synthesis in cells actively producing haem (Elder, 1981). The short term regulation processes may themselves however also be affected by the level of differentiation: the presence of haem in early erythroblasts may serve to induce ALA synthase activity (Sassa, 1976; Elder, 1981; Ibrahim et al, 1983) while in reticulocytes haem has been shown to inhibit ALA synthase (Aoki et al, 1971; Ibrahim et al, 1978).

Although differentiation is likely to be the more important factor (Tait, 1978; Elder, 1981) a clear understanding of human erythroid haem biosynthesis has not yet emerged, as the heterogeneity of cells in bone marrow samples has restricted access to age matched human erythroblasts. As a result "in vivo" human erythroblasts have not been used to study the control of erythroid haem synthesis and ALA synthase activity. Investigations have been carried out in alternative erythroid systems: embryonic chick blastoderm (Levere and Granick, 1965), foetal hepatic erythroblasts (Freshney and Paul, 1971), mouse Friend erythroleukaemia cells (Sassa, 1976; Beaumont et al, 1984), "in vitro" mammalian bone marrow cultures (Ibrahim et al, 1982) and the human erythroleukaemia cell line K562 (Hoffman et al, 1980; Bottomley et al, 1985).

3.1 UNFRACTIONATED BONE MARROW ALA SYNTHASE

As the techniques for human bone marrow fractionation and cellular isolation have only recently been described (Ali et al, 1983) the study of human bone marrow ALA synthase activity has so far been confined to the detection of enzyme activity in unfractionated marrow in normal and pathological states. Enzyme activity in these bone marrow samples has been thought to reflect only that present in the marrow erythroblasts. This untested belief has been used in all previous studies. The marrow enzyme activity has been related to the sample erythroblast content and the activities found in different samples have been compared, irrespective of differences in their M:E ratios.

In certain pathological conditions the erythroid population is left shifted ie, more immature, than that found in normal marrow. Despite evidence from the alternative erythroid systems indicating that erythroblast ALA synthase activity may be dependent on differentiation status, this has not been discussed in previous studies of unfractionated marrow ALA synthase activity (Bottomley et al, 1973; Aoki et al, 1974; Konopka and Hoffbrand, 1979; Manabe et al, 1982) and comparisons have been made between marrows without regard for the state of erythroblast development.

The measurement of ALA synthase activity in crude unfractionated marrow has not provided any useful information on the regulation of erythroid haem biosynthesis or the effect of erythroid iron and

haem deficiency on ALA synthase activity (Aoki et al, 1974). It has however identified conditions in which there appears to be a reduction in bone marrow, and so by inference erythroid, ALA synthase. Reduced enzyme activity has been found in both congenital sideroblastic anaemia and PASA (Bottomley et al, 1973; Aoki et al, 1974; Konopka and Hoffbrand, 1979; Bottomley, 1980) and is now regarded as the primary metabolic defect in PASA and the likely cause of the ring sideroblastic defect (Aoki et al, 1974; Ponka and Neuwirt, 1974; Bottomley, 1977, 1980, 1982; Aoki, 1980).

3.2 AIM OF THIS STUDY

The purpose of the work described here was:

(1) to compare the developed assay of ALA synthase activity (Chapter 2) with published methods in normal and sideroblastic marrows;

(2) to examine the effect of M:E ratio on ALA synthase activity in normal marrows; and

(3) to assess the effect of erythroblast maturity on unfractionated marrow ALA synthase activity in sideroblastic and dyserythropoietic states.

3.2.1 Comparison of the developed assay with established assays

ALA synthase activity in bone marrow has been shown to be dependent on the choice of radiochemical substrate, the method of sample preparation (Aoki et al, 1974) and the concentration of

reagents in the sample incubation. It was aimed to compare results obtained with the method described in Chapter 2 with those of Aoki et al (1974), who using [^{14}C]-succinyl CoA detected more enzyme activity than had been found with either 2-keto[^{14}C]glutarate (Bottomley et al, 1973; Tikerpae et al, 1981) or [^{14}C]-succinate (Fraser and Schacter, 1980).

3.2.2 The effect of M:E ratio on ALA synthase activity in normal bone marrow

Myeloid cells, despite their content of haem enzymes have not been thought to contribute significantly to the ALA synthase activity found in unfractionated human bone marrow. If this is true a relationship between enzyme activity (per 10^6 erythroblasts) and M:E ratio would not be expected. The effect of M:E ratio on ALA synthase activity was examined in 11 normal marrow samples.

3.2.3 The effect of erythroblast maturity

ALA synthase activity in marrow samples with a similar (but non ring sideroblastic) erythroid population was compared with that found in PASA.

In PASA the marrow appearances often reveal erythroid hyperplasia with a reduced M:E ratio. The erythroid precursors may show dyserythropoietic features and have a high degree of ineffective iron turnover and ineffective erythropoiesis (Singh et al, 1970). Intramedullary erythroblast lysis leads to a relative increase in

the number of immature erythroblasts in the bone marrow. By comparison erythropoiesis in normal bone marrow is more effective and the four cell divisions between proerythroblast and late erythroblast ensure that the majority of erythroblasts are at a late stage of development (Harris and Kellermeyer, 1970).

Bone marrow ALA synthase activity in PASA was compared with that found in untreated megaloblastic anaemia, thalassaemia intermedia and congenital dyserythropoietic anaemia (CDA). Although heterogenous in their pathogenesis these disorders have in common with PASA a similar left shifted ineffective erythroid population and a reduced M:E ratio. They differed from PASA in that they were thought to be free of haem synthetic defects and were shown to lack the many visible ring sideroblasts seen with light microscopy in PASA.

MATERIALS AND METHODS

Bone marrow samples were taken from 11 normal volunteers and 11 anaemic patients. The M:E ratios on the marrows studied were calculated from a 400 cell differential count carried out on marrow cell cytospin preparations.

3.3 NORMAL SUBJECTS

Haematological information on the 11 normal volunteers is given in Table 2. Eight subjects were having minor ENT surgery but agreed to donate marrow, which was taken after the induction of general anaesthesia. Subjects 4, 5 and 6 (see Table 2) were haematologically normal but each had a thoracotomy with rib resection. Marrow was expressed from the rib immediately after resection. Patient 4 had a trans-thoracic hiatus hernia repair. Patient 5 had an opacity on chest x-ray which was found to be an arteriovenous fistula. Patient 6 had lung carcinoma confined to the thorax with no evidence of mestastatic spread. Bone marrow morphology was normal in all 11 patients.

3.4 THE ANAEMIC PATIENTS

Table 3 presents haematological information on the 11 anaemic patients studied.

3.4.1 Sideroblastic anaemia

Three patients with established PASA were studied. They were not transfusion dependent but showed ring sideroblastic change in more than 60% of their marrow erythroblasts. Blast cells were not evident and two of the patients remain stable, at 18 months after study. One patient died from a ruptured aortic aneurysm. Patient 4 had congenital non pyridoxine response sideroblastic anaemia. He was an infant of six months who had an affected brother aged 5 years.

		Hb (g/dl)	Serum ferritin (ug/l)	Transferrin saturation (%)	M:E Ratio	ALA synthase (pmol ALA/10^6 erythroblasts/h)
1	M	14.8	234	25	3:1	762
2	F	13.2	175	15	3.5:1	890
3	M	15.4	61	30	4:1	630
4	M	14.3	120	20	4:1	706
5	M	16.8	101	55	4:1	745
6	F	13.6	16	21	4:1	775
7	F	13.0	91	18	5:1	835
8	M	15.9	234	25	8:1	821
9	M	16.2	81	30	8:1	1282
10	F	13.6	29	28	10:1	1200
11	F	12.9	12	24	10:1	1115

TABLE 2: The normal volunteers

		Hb (g/dl)	Serum ferritin (ug/l)	Transferrin saturation (%)	M:E Ratio	ALA synthase (pmol ALA/10^6 erythroblasts/h)
1	M	9.1	307	50	1:1	320
2	M	8.1	219	50	2:1	370
3	M	8.7	1283	70	1:1	518
4	M	7.0	5000	100	4:1	400
5	F	7.4	169	100	1:1	922
6	F	6.7	92	50	1:1	1290
7	F	6.2	117	60	2:3	850
8	M	8.4	200	50	1:2	700
9	F	7.8	2490	100	1:2	280
10	M	10.1	572	100	3:2	450
11	M	10.0	758	70	1:2	250

NOTE: Patients 1-3 PASA; Patient 4 congenital sideroblastic anaemia; Patients 5-7 megaloblastic anaemia; Patients 8 and 9 thalassaemia intermedia; Patients 10 and 11 CDA.

TABLE 3: The anaemic patients

3.4.2 Megaloblastic anaemia

Three patients with megaloblastic anaemia were studied. Two patients (5 and 6) had pernicious anaemia, diagnosed on the basis of reduced serum vitamin B_{12} levels, correction of an abnormal Schilling test by intrinsic factor and the presence of antibodies in serum to intrinsic factor. The third patient had simple dietary folate deficiency with no history of alcohol ingestion. Bone marrow reticuloendothelial iron stores were plentiful and serum ferritin concentrations were normal in the three patients. Ring sideroblasts were not seen and all three patients responded with a brisk reticulocytosis (> 10% on day 5) to haematinic replacement.

3.4.3 Thalassaemia intermedia

Two patients with thalassaemia intermedia were studied. One patient (8), a Vietnamese boat person had HbH disease and was a compound heterozygote for α^{o}thalassaemia trait and Hb constant spring. The other patient (9) was a Greek immigrant with β thalassaemia intermedia and a considerable degree of non ring sideroblastic marrow iron overload which had resulted largely from a transfusion programme required for some years prior to splenectomy in 1978.

Examination under light microscopy demonstrated considerable reticuloendothelial iron overload in patient 9, in whom 20%

pathological and 5% ring sideroblasts were noted. Patient 8 had 10% pathological and 2% ring sideroblasts.

3.4.4 CDA

Two patients with CDA were studied. One patient (10) had features of CDA type II; however, the acidified serum lysis test was negative with the serum of five normal subjects. Approximately 10% of his marrow erythroblasts were either bi- or tri-nucleate and there was gross punctate basophilia. As with other cases of CDA (Weatherall et al, 1973) there was an imbalance of α : β globin chain synthesis ratio, 1.96 (normal range 0.97 - 1.19). Ferrokinetic studies indicated a seven fold increase in marrow iron turnover with 85% ineffective erythropoiesis. Hb electrophoresis was normal, HbF 2.2%. The second patient (11) had an atypical form of CDA with features similar to those described by Hruby et al (1973). The marrow showed erythroid hyperplasia with gross dyserythropoiesis, punctate basophilia and 2% binucleate forms. The acidified serum lysis test was negative and HbF was elevated at 17.6%. (Hb A_2 3.6%). There was minimal hypochromia (MCV 80 fl, MCH 26.5 pg), ferrokinetic evidence of 90% ineffective erythropoiesis and α :non α globin chain synthesis ratio of 2:1.

Both patients with CDA showed considerable reticuloendothelial marrow iron overload on light microscopy with 20% pathological and

8% ring sideroblasts in patient 10 and 5% pathological and 2% ring sideroblasts in patient 11.

3.5 ALA SYNTHASE ACTIVITY

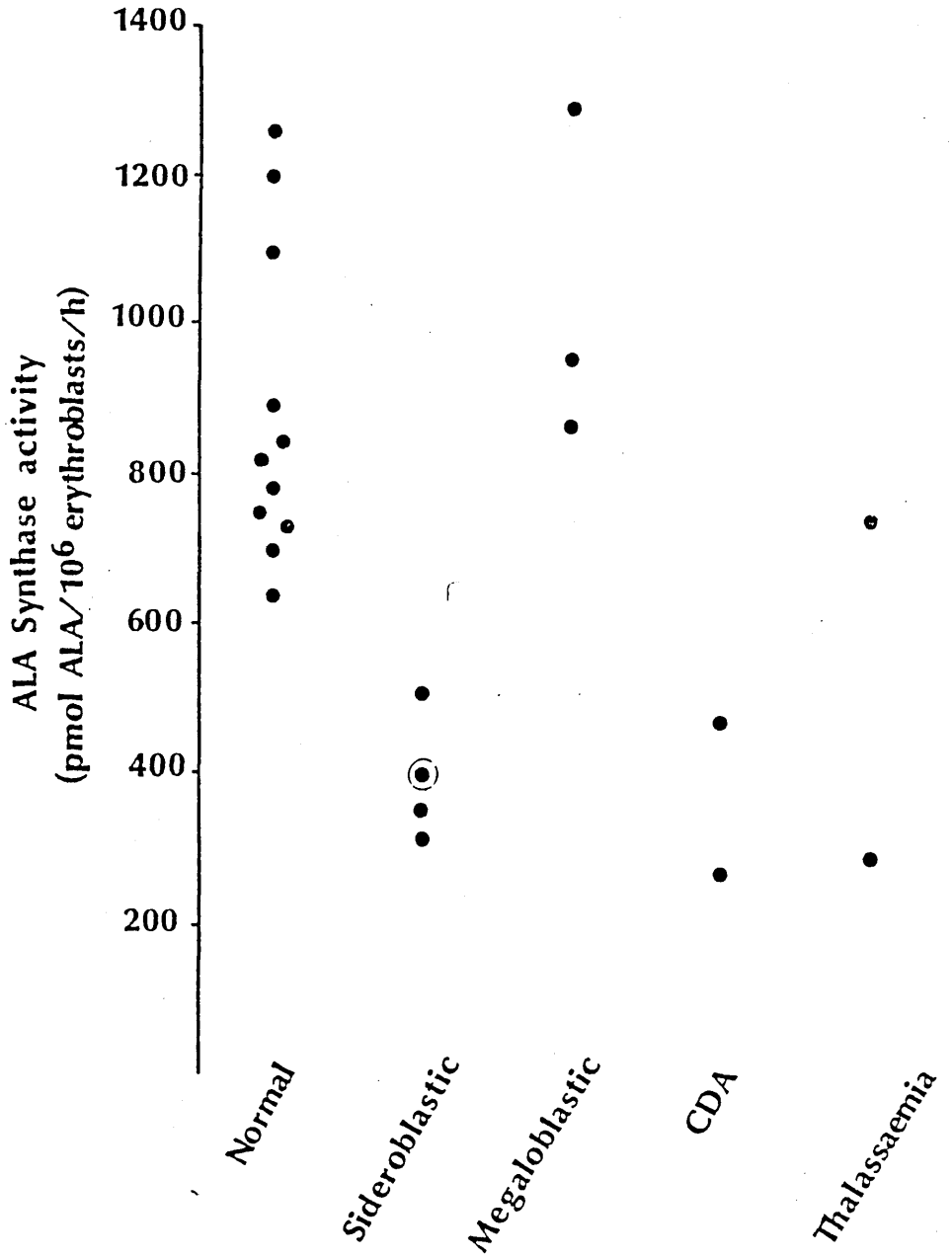
ALA synthase activity was determined as described and was related to the sample erythroblast content to allow comparison with previous studies. For the 11 normal marrows enzyme activity was also related to the M:E ratio.

RESULTS

The results of ALA synthase activity in the normal and pathological marrow samples studied are shown in Tables 2 and 3 and Figure 15.

3.6 ALA SYNTHASE ACTIVITY IN NORMAL UNFRACTIONATED HUMAN BONE MARROW

The mean ALA synthase activity in 11 normal marrow samples was 893 pmol ALA/10^6 erythroblasts/h (range 630-1282, S.D. $\pm$ 199). Enzyme activity was related to the M:E ratio and was significantly higher in those samples with a ratio of more than 4:1 ($p = 0.03$) (Table 4).



◉ congenital sideroblastic anaemia

Note: ALA synthase activity was related to the erythroblast content of the marrow samples.

Figure 15: ALA synthase activity in unfractionated human bone marrow

Number of samples	M:E ratio	Mean ALA synthase activity (± 1SD) (pmol ALA/10^6 erythroblasts/h)
6	$\leq$ 4:1	751 (± 86)
5	> 4:1	1051 (± 212)

TABLE 4: The effect of M:E ratio on ALA synthase activity in normal human bone marrow

3.7 ALA SYNTHASE ACTIVITY IN ABNORMAL UNFRACTIONATED HUMAN BONE MARROW

ALA synthase activity was reduced in the four patients with sideroblastic anaemia. In the three patients with megaloblastic anaemia and in one patient with thalassaemia (8), enzyme activity was found to be in the normal range. The other patient with thalassaemia (9) and both cases of CDA had low levels of ALA synthase activity.

DISCUSSION

Mean ALA synthase activity in 11 normal marrow samples was 893 pmol ALA/10^6 erythroblasts/h (range 630 - 1282). This activity is approximately 50% greater than that found by Aoki et al (1974) and at least twice the mean activities reported by other investigators (Takaku and Nakao, 1971; Bottomley et al, 1973; Konopka and Hoffbrand, 1979; Fraser and Schacter, 1980; Tikerpae et al, 1981). The method has been shown to be highly sensitive, capable of detecting ALA synthase activity in 0.2 x 10^6 bone marrow cells (section 2.16.5). The high sensitivity has resulted from the determination of optimum sample preparation and reagent concentration. This has allowed the detection of higher enzyme activity than has been previously reported in normal marrow samples. The performance of the assay may also indicate that RPHPLC is superior to the alternative methods available for ALA isolation.

In the normal marrow samples there was a positive relationship between ALA synthase activity and the M:E ratio. Similarly a positive relationship for coproporphyrinogen oxidase activity and M:E ratio in human bone marrow was shown by Elder and Evans (1975). These results suggest that myeloid cells may contain ALA synthase and coproporphyrinogen oxidase and so may contribute to the enzyme activities found in unfractionated marrow which should no longer be regarded only as a measure of erythroblast enzyme activity.

ALA synthase activity was shown to be reduced in the sideroblastic marrow samples, a finding in keeping with numerous previous studies (see Bottomley et al, 1973; Aoki et al, 1974; Konopka and Hoffbrand, 1979). In the three cases of megaloblastic anaemia, mean enzyme activity per 10^6 erythroblasts/h (1020 pmol) was more than twice the mean activity found in the three cases of PASA (402 pmol). It is clear that despite their similar level of erythroblast maturity and M:E ratio, ALA synthase activity is very much less in PASA than megaloblastic marrow ($p < 0.5$). Unless myeloid ALA synthase activity is sufficiently elevated in megaloblastic anaemia to account for this difference it may be inferred that megaloblasts have more ALA synthase activity than sideroblasts. It has been shown that normoblasts and megaloblasts of comparable maturity have similar levels of porphyrin synthesis (Dresel and Falk, 1954), similar levels of iron uptake (Lajtha and Suit, 1955) and similar levels of RNA synthesis (Hoffbrand and Wickramasinghe, 1982). If similar levels of ALA synthase activity are assumed, then both normoblasts, as well as megaloblasts would have more ALA synthase activity than sideroblasts. Erythroblast ALA synthase activity is the subject of Chapter 5.

One patient with thalassaemia intermedia had a level of marrow ALA synthase activity within the normal range despite gross erythroid hyperplasia and reversal in the M:E ratio (and presumably low myeloid ALA synthase). In thalassaemia patient (9) with some degree of transfusional iron overload and in both cases of CDA

reduced levels of unfractionated marrow ALA synthase activity were found. This is the first report of reduced marrow enzyme activity in the absence of prominent ring sideroblastic change and will be discussed in relation to erythroblast ALA synthase activity in Chapter 5.

CHAPTER 4

THE CALCULATED PERCENTAGE MYELOID-DERIVED 5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN NORMAL AND SIDEROBLASTIC BONE MARROW

4. THE CALCULATED PERCENTAGE MYELOID-DERIVED 5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN NORMAL AND SIDEROBLASTIC BONE MARROW

The relationship demonstrated between M:E ratio and ALA synthase activity in unfractionated normal human bone marrow (Section 3.6), suggested that myeloid cells may make a significant contribution to the unfractionated marrow ALA synthase activity.

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 has been shown to require incubation of the marrow cells with the monoclonal antibody for 30 min at 37°C.

The aim of the following series of experiments was

(1) to determine the effect of incubation at 37°C on ALA synthase activity in unfractionated human bone marrow;

(2) to use TG-1 to lyse the myeloid cells in normal and PASA bone marrow samples and so calculate the percentage of the unfractionated bone marrow ALA synthase activity that is present in myeloid cells;

(3) to examine the reduced M:E ratio in PASA as a possible cause of reduced ALA synthase activity in unfractionated PASA bone marrow.

MATERIALS AND METHODS

4.1 THE MARROW SAMPLES

Normal bone marrow was expressed from ribs resected at thoracotomy. Rib resections were performed on four patients with lung carcinoma confined to the thorax (patient 6, Section 3.3 and three others), on one patient with a hiatus hernia (patient 4, Section 3.3) and on one patient with an intrathoracic arteriovenous fistula (patient 5, Section 3.3). All the patients were haematologically normal with respect to their peripheral blood counts and bone marrow morphology. Marrow was obtained from the three patients with PASA described in Section 3.4.1.

4.2 MYELOID CELL LYSIS

TG-1 was obtained as hybridoma supernatant from Dr P Beverley, MRC Tumour Immunology Group, University College Hospital, London. The supernatant will simply be referred to as TG-1. The lysis technique was a modification of that described by Ali et al (1983). Bone marrow cells (50×10^6) and TG-1 (2.5 ml) were mixed and placed on ice for 30 min. Fresh autologous plasma (2.5 ml) was added as a source of complement with deoxyribonucleotidase (DNA ase) (DN-25, Sigma) to a final concentration of 1 mg/ml. The sample was incubated with mixing for 30 min at 37°C. The DNA ase digested the DNA liberated from lysed cells and so prevented gel formation and clumping of the viable cells. The cells were washed three times in cold culture medium to remove myeloid cell debris; cell counts, cytospin preparations and cell differential counts were made.

4.3 THE EFFECT OF INCUBATION AT 37°C ON ALA SYNTHASE ACTIVITY

ALA synthase activity was measured in three normal and three PASA bone marrow samples before and after incubation of the intact unfractionated marrow cells at 37°C for 30 min in fresh autologous plasma. Enzyme activity following incubation was expressed as a percentage of the activity in the non incubated sample.

Enzyme activity was shown to remain stable in marrow samples placed on ice for 6 hours but fell in both normal and PASA bone marrow with incubation at 37°C (Table 5).

The addition of reagents required to achieve myeloid cell lysis during incubation did not further reduce ALA-synthase activity. Normal bone marrow was incubated in:

(1) fresh autologous plasma ± DNA ase (1 mg/ml);

(2) culture medium (see Appendix 1)

(3) TG-1 and heat inactivated (ie complement inactivated) autologous serum.

As myeloid cells have been shown to contain a protease that inactivates ALA synthase (Aoki et al, 1978) the effect of myeloid cell enzymes (liberated during TG-1 lysis) on bone marrow ALA synthase activity was examined.

Marrow	Incubation time (Min)	ALA synthase activity (%)
Normal	30	61
Normal	30	60
Normal	30	55
PASA	30	70
PASA	30	74
PASA	30	66

NOTE: Marrow samples were incubated in fresh autologous plasma. ALA synthase activity was expressed as a percentage of the non incubated unfractionated marrow enzyme activity

TABLE 5: The effect of incubation at 37°C on ALA synthase activity in unfractionated bone marrow

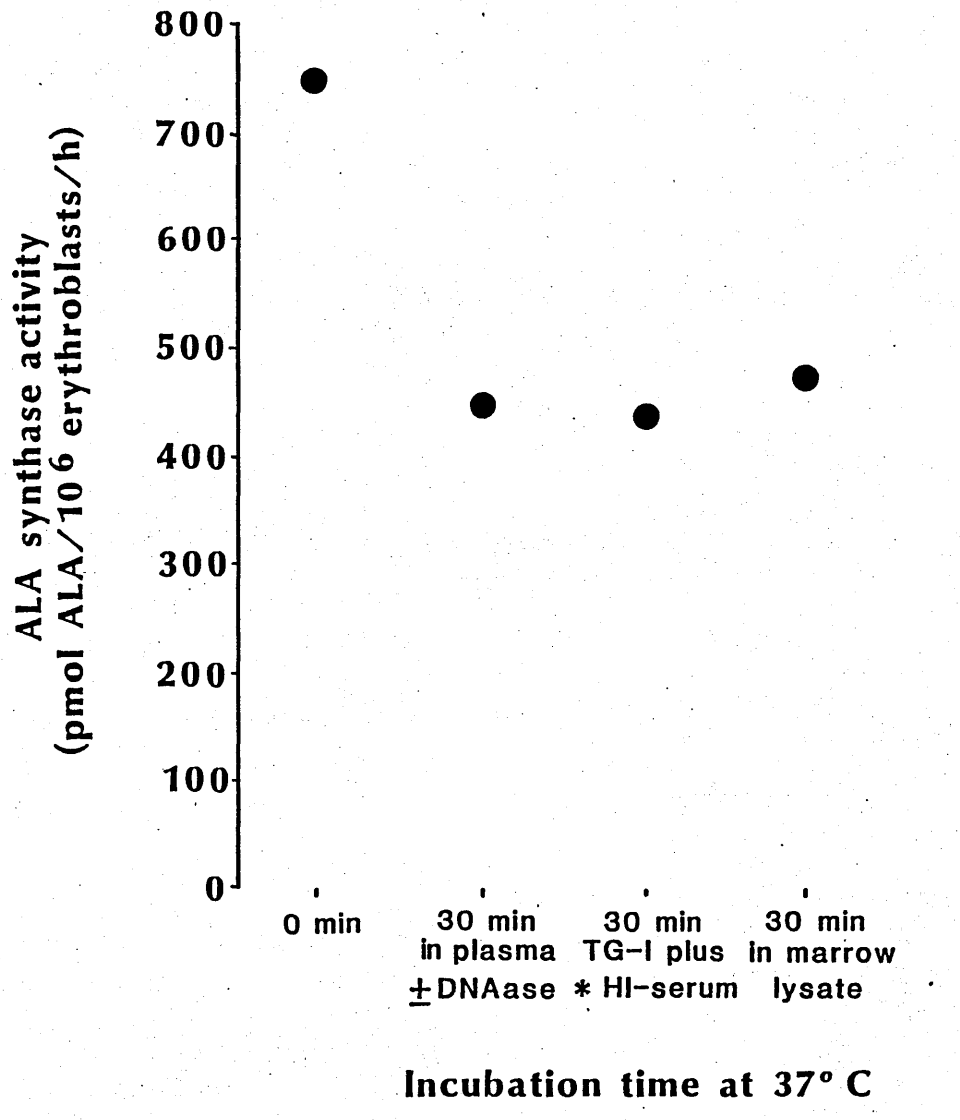
(4) marrow cells were incubated in a lysate prepared from ultrasonicated unfractionated marrow in a 1:1 mixture of buffered culture medium and autologous plasma. Lysate was prepared to expose the non lysed cells to a concentration of myeloid cell enzymes threefold greater than that reached during TG-1 lysis.

The fall in ALA synthase activity was shown to be unaffected by the bone marrow lysate or by the reagents required to achieve myeloid cell lysis (Fig 16).

4.4 THE FALL IN ALA SYNTHASE ACTIVITY DURING INCUBATION AT 37°C IN ERYTHROID AND MYELOID BONE MARROW CELLS

Any disporportionate rate of fall in ALA synthase activity within either erythroid or myeloid bone marrow cells was excluded by examining the effect of incubation at 37°C on enzyme activity in unfractionated and myeloid depleted marrow samples.

ALA synthase activity in normal unfractionated marrow was determined after incubation at 37°C in fresh autologous plasma for zero 30, 50, 70 and 90 min. Myeloid cells were lysed using TG-1 with a 30 minute period of incubation at 37°C. ALA synthase activity was measured in this erythroid preparation and after a further 20, 40 and 60 min incubation at 37°C.



Note: Each point represents the mean of two observations.

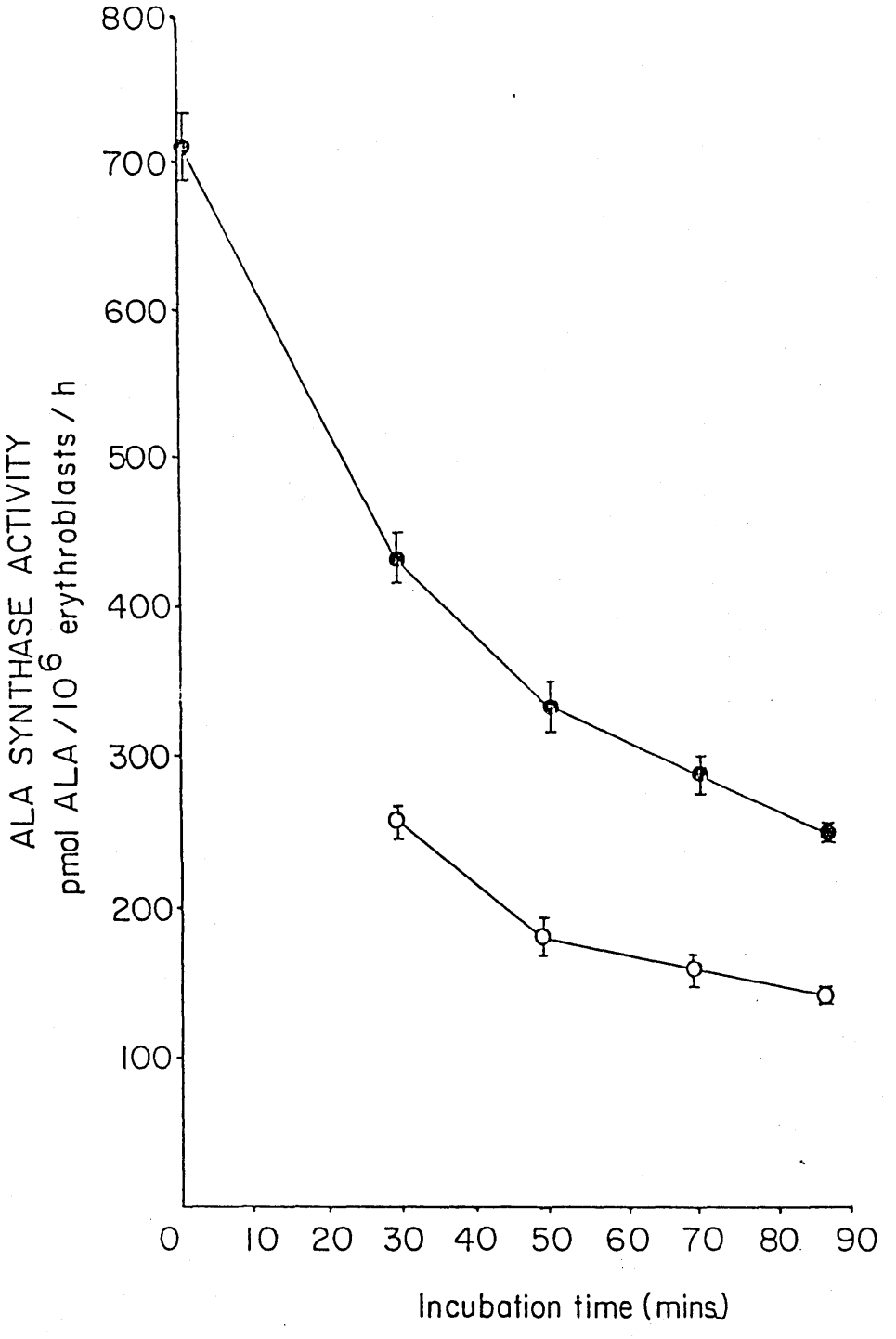
Figure 16: The effect of TG-1 and other reagents for myeloid cell lysis on ALA synthase activity in unfractionated human bone marrow

TG-1 mediated lysis of myeloid cells increased the percentage erythroid content of the bone marrow sample from 19 to 68%. The fall in ALA synthase activity both in this erythroblast preparation and in the unfractionated marrow is shown in Figure 17. As the rate of fall in activity in both samples was parallel it was concluded that the loss had occurred from both myeloid and erythroid cells, without disporportionate loss from either.

4.5 THE CALCULATED MYELOID-DERIVED ALA SYNTHASE ACTIVITY

The calculated percentage contribution of myeloid cells to the ALA synthase activity in unfractionated bone marrow was determined for four normal and three PASA marrow samples.

Myeloid cells were lysed by the addition of TG-1 and fresh autologous plasma. The effect of TG-1 lysis was to increase the mean percentage erythroid content from 17% to 64% for the normal samples and from 43% to 80% for the sideroblastic samples (residual myeloid cells < 2%). The remaining non erythroid cells were predominantly lymphocytes and monocytes. ALA synthase activity in each myeloid-free sample was expressed as a percentage of the activity found in the unfractionated sample, after an identical period of incubation in autologous plasma without TG-1: the difference was calculated and expressed as the percentage myeloid-derived ALA synthase activity.



Note: Each point represents the mean of two observations.

Figure 17: The effect of incubation at 37°C on ALA synthase activity in unfractionated (●——●) and myeloid depleted (o——o) human bone marrow

The results are shown in Table 6. In the normal and sideroblastic marrows studied the mean percentage myeloid-derived ALA synthase activity was 49% and 48% respectively of the incubated but unfractionated marrow enzyme activity. As the mean M:E ratio in the four normal marrows was 4:1, the calculated 49% contribution from myeloid cells towards the enzyme activity in unfractionated marrow indicates that in these normal marrows individual erythroid cells were in general a fourfold richer source of enzyme than individual myeloid cells.

4.6 ALA SYNTHASE ACTIVITY IN THE CELL FREE SUPERNATANT AFTER TG-1 LYSIS

As myeloid cells were shown to contain significant ALA synthase activity it was hoped to demonstrate enzyme activity in the cell free supernatant after TG-1 lysis of bone marrow myeloid cells. As the supernatant contains plasma (complement), incubation medium for the measurement of supernatant ALA synthase activity was modified to accommodate the presence of plasma.

ALA synthase activity was determined in normal bone marrow as described and in assay incubation medium containing plasma. This modified incubation medium contained glycine 50 mM, CoA 1.35 mM GTP 5 mM, [^{14}C]-succinate 2 mM, STK 0.5 U, PLP 0.4 mM, sucrose 125 mM, EDTA 5 mM, $MgCl_2$ 10 mM, Tris 25 mM buffered with KH_2PO_4 12 mM and plasma 0.125 ml in a final volume of 0.5 ml. The pH was 7.4 at 37^{o}C. ALA synthase activity was also measured in intact

	MARROW	M:E ratio	Percentage ALA synthase activity TG-1 treated : myeloid cells		
1	Normal	3:1	61	:	39
2	Normal	4.5:1	53	:	47
3	Normal	5:1	38	:	62
4	Normal	4:1	53	:	47
	Mean	4:1	51	:	49
5	PASA	1:1	50	:	50
6	PASA	2:1	46	:	54
7	PASA	1:1	60	:	40
	Mean	1.3:1	52	:	48

NOTE: ALA synthase activity was measured in unfractionated marrow incubated at 37°C for 30 min and in fractionated marrow following TG-1 lysis of myeloid cells. Enzyme activity following TG-1 lysis was expressed as a percentage of the activity found in the incubated unfractionated marrow sample.

TABLE 6: The myeloid-derived ALA synthase activity in normal and PASA bone marrow

and freeze thawed bone marrow cells, warmed to 37°C for 30 min, before enzyme assay in modified incubation medium.

The results are shown in Table 7. In the presence of the major assay reagents at normal concentration (ie glycine, CoA, GTP, STK, PLP and [^{14}C]-succinate) enzyme activity was reduced fourfold by the addition of plasma and sixfold by prior incubation of intact cells at 37°C for 30 min. ALA synthase activity was not detected in freeze thawed bone marrow warmed to 37°C for 30 min using modified incubation medium.

This inhibitory effect of plasma in the incubation mixture prevented the measurement of ALA synthase activity in the cell free supernatant after TG-1 lysis of myeloid cells. The large fluid volume required to produce cell lysis without clumping and DNA gel formation (5 ml/50 x 10^6 bone marrow cells) considerably diluted the liberated enzyme, the activity of which was further lessened by the presence of plasma in the assay incubate.

The volume of fluid required for myeloid cell lysis was reduced. TG-1 (2.5 ml) was mixed with 50 x 10^6 bone marrow cells for 30 min on ice. The TG-1 was removed from the cell pellet prior to the addition of plasma (2 ml) containing DNA ase (1 mg/ml). Cell lysis occurred with incubation at 37°C for 30 min, but the DNA liberated from the lysed cells caused gel formation and clumping of the viable cells. This was not prevented by increasing the concentration of DNA ase to 2 mg/ml. No further attempts to measure ALA synthase activity in the myeloid cell lysate were made.

Tissue preparation	Incubation medium	ALA synthase activity (pmol ALA/10^6 erythroblast/h)
Fresh bone marrow cells plus ultrasonication	Standard	780
Fresh bone marrow cells plus ultrasonication	+ plasma	210
Intact bone marrow cells incubated at 37°C for 30 min in autologous plasma plus ultrasonication	+ plasma	135
Freeze thaw lysed bone marrow cells incubated at 37°C for 30 min in autologous plasma plus ultrasonication	+ plasma	ND

TABLE 7: The effect of adding plasma to the assay incubation mixture on ALA synthase activity

DISCUSSION

These experiments have established that ALA synthase activity in unfractionated human bone marrow is not a reliable index of erythroblast enzyme activity. Myeloid cells in four normal and three PASA bone marrow samples were shown to contribute almost 50% of the activity found in the unfractionated bone marrows.

All previous studies of bone marrow ALA synthase activity have assumed that myeloid cells make an insignificant contribution towards the unfractionated marrow enzyme activity and have expressed results solely in terms of the sample erythroblast content (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 developed from the failure of Walters et al (1967) to detect ALA synthase activity in normal and leukaemic leucocytes and no doubt is supported by the visual evidence that identifies erythroblasts as 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 and the assumption of insignificant myeloid ALA synthase activity appears untenable in view of:

(1) evidence that immature, leukaemic myeloid cells can synthesise haem in significant quantities (Handler and Handler, 1972);

(2) the presence of haem containing enzymes, particularly myeloperoxidase, to more than 5% of the dry weight of polymorphonuclear leucocytes (Suzuki et al, 1983);

(3) the presence of ALA synthase activity in peripheral blood leucocyte pellets (Brodie et al, 1977).

This work indicates that ALA synthase is present in bone marrow myeloid cells and that haem synthesised in myeloid cells may contribute to the "early labelled" bile pigment pool which represents some 25% of bilirubin, but is derived from sources other than haemoglobin catabolism (Berk et al, 1976).

A protease that inactivates the apo-form of certain pyridoxal enzymes has been detected in mature and immature granulocytes at levels similar to those found in erythroblasts. It has been proposed that this protease is a regulator of erythroblast ALA synthase (Aoki, 1978). The presence of ALA synthase activity in bone marrow myeloid cells may now suggest a similar function for the protease in these cells.

Differences in the M:E ratio and myeloid-derived ALA synthase activity were not shown to cause the reduced ALA synthase activity found in unfractionated PASA bone marrow samples. Myeloid cell lysis produced a similar percentage reduction in enzyme activity in normal and PASA marrow and demonstrated low enzyme activity in the residual sideroblasts. If insignificant myeloid ALA synthase activity had been found in PASA then normal erythroid activity plus reduced myeloid activity might have combined to produce reduced whole marrow ALA synthase activity.

In the three PASA cases studied myeloid cell ALA synthase activity was not reduced. From the mean unfractionated marrow enzyme activity of 400 pmol ALA/h in PASA (Section 3.7), the mean myeloid-derived activity (approximately 50% of the total) may be calculated to be 200 pmol/h. Correction for a mean M:E ratio of 1.3:1 in the three PASA bone marrow samples provides a calculated myeloid-derived ALA synthase activity in PASA of approximately 150 pmol ALA/10^6 bone marrow myeloid cells/h. Examination of the M:E ratio and ALA synthase activity in 11 normal unfractionated bone marrow samples (Section 3.6) indicates that in all cases it would be unlikely and in most cases impossible, for the myeloid cell enzyme activity to exceed this value. As only three cases of PASA were studied these results should be considered to be of a preliminary nature. If substantiated however they would identify reduced mitochondrial ALA synthase activity in PASA to be evident only in the erythroid fraction of bone marrow, which in turn is the only cell to show mitochondrial iron loading (Larizza and Orlandi, 1964). This would contrast with the study of Aoki (1980) who demonstrated reduced activity of other myeloid mitochondrial enzymes, cytochrome oxidase, serine protease and oligomycin-sensitive ATP ase in peripheral blood polymorphs in PASA. However unlike the three cases studied here, in which peripheral white cell counts and differentials were normal, the majority of cases studied by Aoki (1980) had low circulating white cell counts.

ALA synthase activity was shown to fall during incubation of bone marrow cells at 37°C for 30 min. This period of incubation was

required to allow TG-1 mediated lysis of myeloid cells. The fall in activity was not related to the presence of reagents required for myeloid cell lysis nor was it prevented by the presence of glycine in the culture medium. The activity fell both in PASA and normal bone marrow samples and was shown to occur without a disproportionate loss in either myeloid or erythroid cells. The cause of this fall in activity is not clear and may warrant further examination in future studies. In particular the effect of proteolytic inhibitors should be determined. As the protease for ALA synthase (Aoki, 1978) is thought to be a serine protease, the effect of the serine protease inhibitor diisopropyl fluorophosphate should be examined. The effects of pyridoxine, pyridoxal phosphate and other pyridoxine metabolites require to be investigated as it has been found that pyridoxal phosphate protects against an irreversible temperature dependent inactivation of hepatic ALA synthase activity in mitochondrial matrix fraction (Beattie et al, 1985). The pattern of inactivation in the mitochondrial matrix was similar to that shown in Figure 17 with approximately 50% inactivation after 30 min at 37°C. This temperature dependent loss of activity stresses the importance of maintaining the bone marrow sample at 4°C during procedures such as centrifugation and ultrasonication which might cause an increase in sample temperature with consequent inactivation of ALA synthase.

The results reported in this chapter indicate that in the study of haem biosynthetic enzymes in bone marrow the contribution of

myeloid and erythroid cells should be clearly and separately defined using suitably sensitive enzyme assays. Even when this is done it is important to recognise the likely relationship between enzyme activity and cell age. In relation to ALA synthase activity this would apply both to myeloid and erythroid cells. Polymorphonuclear leucocytes in blood are terminally differentiated granulocytes, in transit to the tissues. They are chiefly concerned with bacterial phagocytosis and are equipped with very few mitochondria and a limited capacity for protein synthesis (Cawley and Hayhoe, 1973). In contrast immature granulocytes are rich in mitochondria and are actively engaged in protein synthesis. It is then presumably the immature bone marrow myeloid cells that provide most of the myeloid-derived ALA synthase activity. The effect of myeloid differentiation on ALA synthase activity would be difficult to study using "in vivo" bone marrow myeloid cells as no method has been described for isolating and age matching developing myeloid cells in human bone marrow. However, the study of acute myeloid leukaemia cell lines (HL-60, KG-1) has identified maximal synthesis of haem containing myeloperoxidase in promyelocytes with negligible synthesis in mature myeloid cells (Koeffler et al, 1985). The same cell lines may provide information on ALA synthase activity during myeloid cell differentiation.

The relationship between ALA synthase activity and human erythroblast differentiation in normal and pathological erythroblasts has never been examined before and is the subject of Chapter 5.

This discussion has provided possible ideas for future studies. It has also indicated the need to reassess the value of measuring ALA synthase activity in unfractionated bone marrow and the conclusions drawn from previous studies which have used this as a measure of erythroblast enzyme activity. It would seem as if unfractionated bone marrow ALA synthase activity is at best a crude indicator of erythroblast activity, insensitive to the contribution made by myeloid cells and to the differences in enzyme activity that may occur in relation to cell differentiation.

CHAPTER 5

5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN HUMAN ERYTHROBLASTS

5. 5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN HUMAN ERYTHROBLASTS

The aim of the work described in this chapter was to provide a direct measure of ALA synthase activity in human erythroblasts and examine the effect of differentiation on enzyme activity in normal and pathological states.

Within mature red cells the specific complexing of haem with globin provides the cell with its unique oxygen carrying properties. In the marrow however there is evidence that haem synthesis proceeds globin synthesis (Glass et al, 1975a, Forget et al, 1976) and that haem synthesised rapidly at the outset of differentiation may be required not for haemoglobin production but for the co-ordinate regulation of erythroblast metabolism and differentiation (Glass et al, 1975a). The activities of the various haem biosynthetic enzymes during human erythroblast differentiation require to be defined as the extent to which intracellular free haem may be involved in the control of globin synthesis, iron metabolism, haem metabolism and differentiation will depend on the ability of erythroid cells to synthesise haem during their development.

5.1 THE ROLE OF HAEM IN GLOBIN PRODUCTION IN ERYTHROID CELLS

In reticulocytes haem has been shown to exert translational control on globin synthesis through a haem controlled repressor

(HCR) of globin m RNA translation (Karibian and London, 1965; Gross, 1980).

Within developing nucleated erythroid cells however globin gene expression appears to be regulated mainly at the transcriptional level (Harrison, 1984). In the FEL cell system no HCR activity was found in a haem deficient mutant (Rutherford and Weatherall, 1979) and it has been suggested that haem may increase globin m RNA either by acting directly on the globin genes or by increasing the proportion of cells that accumulate globin messenger (Ross and Saunter, 1976; Rutherford and Weatherall, 1979).

5.2 THE ROLE OF HAEM IN REGULATING IRON UPTAKE BY ERYTHROID CELLS

Reticulocyte iron uptake is inhibited by haem (Ponka and Neuwirt, 1969) and 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 (Tschudy et al, 1981; Sassa and Kappas, 1983). It has been proposed that an intracellular free haem pool (Neuwirt et al, 1972) may regulate erythroid iron uptake by controlling the release of iron from transferrin (Ponka et al, 1974) and that haem may also alter the number of transferrin receptors on developing erythroid cells (Pelicci et al, 1982). The presence of hypochromic cells in the blood in PASA and reduced levels of ALA

synthase activity in PASA bone marrow samples have led to the hypothesis that a primary deficiency of haem synthesis in PASA allows increased erythroblast iron uptake and mitochondrial iron loading (Ponka and Neuwirt, 1974).

Not all studies support this hypothesis or the belief that haem regulates erythroblast iron uptake. Abnormal localisation of intracellular iron and reduced erythroblast iron incorporation into haem have been documented in PASA, but found to occur without any overall increase in the iron uptake of individual cells (May et al, 1982a). INH has not been found to increase human erythroblast iron uptake (May et al, 1982b) and may itself interfere with iron uptake studies, as the hydrazone formed between pyridoxal and INH is a powerful iron chelator (Ponka et al, 1979). Increased reticulocyte iron uptake in the presence of succinylacetone is not associated with chelator formation but does not in itself provide sufficient evidence to indicate that similar results might be expected with erythroblasts.

5.3 EFFECT OF HAEM ON ERYTHROID DIFFERENTIATION

In addition to there being evidence supporting haem as a major coordinator of erythroblast metabolism, it has also been suggested that haem may regulate erythroblast differentiation. Two major factors have been established in the control of erythropoiesis. Erythropoietin is primarily effective at the terminal stages of differentiation, whereas burst promoting activity (BPA) appears to

enhance the more primitive erythroid precursors (Lipton and Nathan, 1983). Of the other factors T-cell macrophage interaction may sensitise erythroid progenitors to erythropoietin (Kurland et al, 1980; Lipton et al, 1980), T suppressor cells may exercise negative regulation (Lipton and Nathan, 1983), thyroxine and growth hormone are thought to interact with erythropoietin and 5β-H steroid metabolites appear to directly affect erythroid precursors by an erythropoietin independent mechanism (Necheles and Rai, 1969; Gordon et al, 1970; Urabe et al, 1979). In FEL cells DMSO can stimulate differentiation and may do so before the cells have developed the ability to synthesise haem (Friend et al, 1971; Sassa, 1976). This suggests that haem synthesis is stimulated after differentiation has been triggered. However as the growth and differentiation of FEL cells is completely inhibited by succinylacetone (Tschudy et al, 1980) it would seem as if differentiation and haem synthesis in FEL cells are inextricably linked.

Evidence in support of haem as a regulator of erythroid differentiation has come from increased erythroid colony growth following the "in vivo" injection of haemin to mice (Monette et al, 1984) and the "in vitro" addition of haemin to murine (Monette and Holden, 1982; Ibrahim et al, 1982) and human (Lu and Broxmeyer, 1983) bone marrow cultures. The enhancement of murine BFU-E following the "in vivo" injection of haemin was compared with its failure to stimulate CFU-S (pluripotent cells), (Monette et al, 1984) and the enhancement of human BFU-E and CFU-GEMM, by

"in vitro" addition of haemin was compared with its failure to stimulate CFU-GM (Lu and Broxmeyer, 1983). These results argue strongly in favour of a specificity of haemin action localised to the most primitive erythroid progenitors.

5.4 THE ROLE OF HAEM IN THE REGULATION OF ERYTHROID HAEM BIOSYNTHESIS

It is well established that ALA synthase is rate limiting for hepatic haem biosynthesis and subject to negative feedback control by haem (Granick and Urata, 1963). The role of the enzyme in erythroid haem biosynthesis is however unclear. With reticulocytes it has been demonstrated that haem inhibits haem synthesis (Karibian and London, 1965; Neuwirt et al, 1974) and ALA synthase activity, in intact cells, in isolated mitochondria (Ibrahim et al, 1978) and in purified enzyme suspensions (Aoki et al, 1971), but does so however at concentrations 100 fold greater than that required to inhibit liver ALA synthase (Ibrahim et al, 1978). The hypothesis relating iron uptake and haem synthesis has further been extended to suggest that a reciprocal arrangement exists whereby haem may regulate iron uptake but that reduced reticulocyte iron uptake may in turn also reduce haem synthesis (Neuwirt et al, 1974). However as the inhibition of haem synthesis by the addition of haem to intact reticulocytes can be demonstrated in the absence of transferrin iron (Ibrahim et al, 1978) it would seem as if this hypothesis is unproven and perhaps unlikely.

With erythroblasts the effect of haem on haem synthesis has not been established and has been limited by the inability to obtain suitable preparations of differentiating mammalian erythroid cells. It would seem likely however as if two factors are involved in regulating erythroblast haem biosynthesis; firstly, the level of erythroid differentiation and secondly, short term regulators that operate to finely tune haem synthesis in cells actively synthesising haem (Elder, 1981). It is always difficult with erythroid cells to analyse these factors separately and no previous studies have been able to clearly dissociate the primary effect that agents such as erythropoietin (Nakao et al, 1968; Bottomley and Smithee, 1969) and 5β-H steroid metabolites (Gordon et al, 1970; Urabe et al, 1979) have on haem synthesis and ALA synthase, as distinct from effects that occur secondary to their promoting erythroid differentiation (Urabe et al, 1979).

5.4.1 The effect of erythroblast differentiation on haem synthesis

In the absence of access to purified human erythroblasts, the relationship between erythroid differentiation and haem synthesis has been studied in different erythroid systems with different results. In chick blastoderm the development of ALA synthase activity was identified as the final stage in the initiation of haemoglobin synthesis during erythroid differentiation (Levere and Granick, 1965). However in foetal mouse liver (Freshney and Paul, 1971), FEL cells (Ebert and Ikawa, 1974; Sassa, 1976) and murine bone marrow cultures (Ibrahim et al, 1982) ALA synthase was shown

not to be limiting for haemoglobin production, as enzyme activity was evident and maximal prior to haemoglobin formation. The demonstration of sequential development of the enzymes of haem synthesis in foetal mouse liver (Freshney and Paul, 1971) and FEL cells (Sassa, 1976; Rutherford et al, 1979) led to the hypothesis that haem synthesis in developing erythroid cells was limited by the late induction of ferrochelatase activity. However, in contrast, Beaumont et al (1984) demonstrated synchronous rather than sequential, development of ALA synthase and ferrochelatase activity in FEL cells and suggested that haemoglobin formation may be limited by the availability of ferrous iron to protoporphyrin rather than the late appearance of ferrochelatase activity.

5.4.2 The effect of intracellular regulators of erythroblast haem synthesis

Regulators of erythroid haem biosynthesis have also been studied in many different erythroid systems. Haem has been shown to inhibit the synthesis of ALA synthase in cultured embryonic chick liver cells (Sinclair and Granick, 1975) but not in foetal rat liver cells (Woods, 1974). In foetal mouse liver erythroblasts, haem inhibits haem synthesis at several sites, with a greater effect on ALA dehydratase than on ALA synthase (Malik et al, 1979). The addition of haem to FEL cells promotes differentiation (Ross and Sautner, 1976) and has been shown by direct measurement to induce ALA dehydratase and URO-synthase activity and by indirect measurement ALA synthase and ferrochelatase (Granick and Sassa, 1978). In view of its inhibitory effect on reticulocyte

ALA synthase (Aoki et al, 1971), it has been suggested that haem may have contradictory effects on two types of erythroid cell; in undifferentiated erythroid cells haem may induce ALA synthase (Granick and Sassa, 1978; Sassa and Kappas, 1980; Elder, 1981; Ibrahim et al, 1983) whereas in differentiated cells it may inhibit enzyme activity. Direct measurement of ALA synthase activity in DMSO stimulated FEL cells has however shown that a reduction in haem synthesis effected by succinylacetone produced a sixfold increase in ALA synthase activity after four days in culture and that this effect was reversed by the addition of haem (Beaumont et al, 1984). This then suggests that haem exerts negative, rather than positive, control over the induction of ALA synthase activity in early erythroid precursors and that haem present in the nucleus of FEL cells at day 3 of culture with DMSO (Lo et al, 1981) may be there to limit activation of the ALA synthase gene.

It has been suggested that ALA synthase activity in developing erythroblasts may be regulated by the activity of a protease specific for pyridoxal enzymes (Aoki, 1978, 1980) and that low levels of enzyme activity in sideroblastic anaemia may be effected either by reduced enzyme production or by increased sensitivity of the apo enzyme to the protease (Aoki, 1980, Manabe et al, 1982).

These results indicate that the mechanisms regulating erythroblast haem biosynthesis and ALA synthase activity are tissue specific, species specific and differentiation status dependent. The

relative importance of short term regulators in controlling erythroblast haem synthesis is uncertain. Haem proteins in liver cells turn over rapidly and so require the described system (Chapter 1) by which haem controls short term fluctuations in the rate of haem synthesis by its effect on ALA synthase activity. In contrast the long half life of haemoglobin has been argued to mitigate against the need for a similar system in erythroblasts, in which the level of differentiation has been proposed as the major determinant of ALA synthase activity and haem synthesis (Tait, 1978; Elder, 1981).

The study of haem synthesis during human erythroblast differentiation demands attention. As has been discussed erythroblast haem synthesis may not merely represent a process designed to provide haem for circulating red cells, but may also represent a process of central importance to erythroblast metabolism and normoblastic differentiation. The work in this chapter describes the effect of normal and pathological erythroblast differentiation on ALA synthase activity. Measurement of enzyme activity in age matched erythroblasts has detected reduced ALA synthase activity in sideroblastic, CDA and thalassemic erythroblasts. These results have been used to examine the proposed role of reduced ALA synthase activity in the development of mitochondrial iron loading and in the pathogenesis of PASA.

5.5 THE PATIENTS STUDIED

Bone marrow was obtained by posterior iliac crest aspiration from three patients with normoblastic erythroid hyperplasia, three patients with PASA, two patients with thalassaemia intermedia and two patients with CDA. Haematological information on the ten patients is shown in Table 8. All patients had erythroid hyperplasia and have already been described (Section 3.4).

Electron microscopy was performed to examine the ultrastructural appearance of the CDA erythroblasts to determine the degree and nature of any intracellular iron accumulation.

5.5.1 Patients with normoblastic erythroid hyperplasia

It had been hoped that marrow would be obtained from patients with brisk peripheral red cell haemolysis. However no such patients were available at the time of study. Marrow was aspirated on the fifth day of haematinic replacement from patients with severe megaloblastic anaemia. In the three cases (initially described in Section 3.4) the marrow at day 5 showed normoblastic erythroid hyperplasia. The patients had been treated with hydroxocobalamin 1000 ug im days 1-3, ferrous sulphate 200 mg tds days 1-5 and folic acid 5 mg tds days 1-5. Ferrous sulphate was given to prevent the fall in serum iron to the iron deficient levels associated with the introduction of appropriate replacement

Patient	Marrow appearance	M:E ratio	Age yrs	Hb g/dl	Transferrin saturation %	Serum ferritin (ug/l)	Unfractionated bone marrow ALA synthase activity (pmol ALA/10^6 erythroblasts/h normal range 600 - 1200)
1	Normoblastic	3:2	67	8.8	47	64	710
2	Normoblastic	1:1	72	7.1	46	109	650
3	Normoblastic	1:1	74	6.9	33	85	600
4	Sideroblastic	1:1	80	9.1	50	307	320
5	Sideroblastic	2:1	66	8.1	50	219	370
6	Sideroblastic	1:1	55	8.7	70	1283	518
7	THAL	1:2	20	8.4	50	200	700
8	THAL	1:2	51	7.8	100	2490	280
9	CDA	3:2	36	10.1	100	572	450
10	CDA	1:2	22	10.0	70	758	250

TABLE 8: Haematological information on the ten patients studied

therapy in megaloblastic anaemia and which is caused by the early change from ineffective megaloblastic to effective normoblastic erythropoiesis (Chanarin, 1979).

It was noted that ALA synthase activity in unfractionated megaloblastic marrow (see Table 3) was higher than that found in unfractionated normoblastic marrow (Table 8) aspirated from the same patients after five days of haematinic replacement.

5.6 ERYTHROBLAST FRACTIONATION

The bone marrow myeloid cells were lysed by TG-1 plus complement. The mean percentage erythroid cells in the 10 marrows after TG-1 lysis was 86% (range 75-96). Percoll equilibrium density centrifugation (Ali et al, 1982, 1983; May et al, 1982) was used to fractionate the erythroblasts at different stages of development. The cells were removed from four different density levels from each percoll density gradient.

5.6.1 Percoll gradients

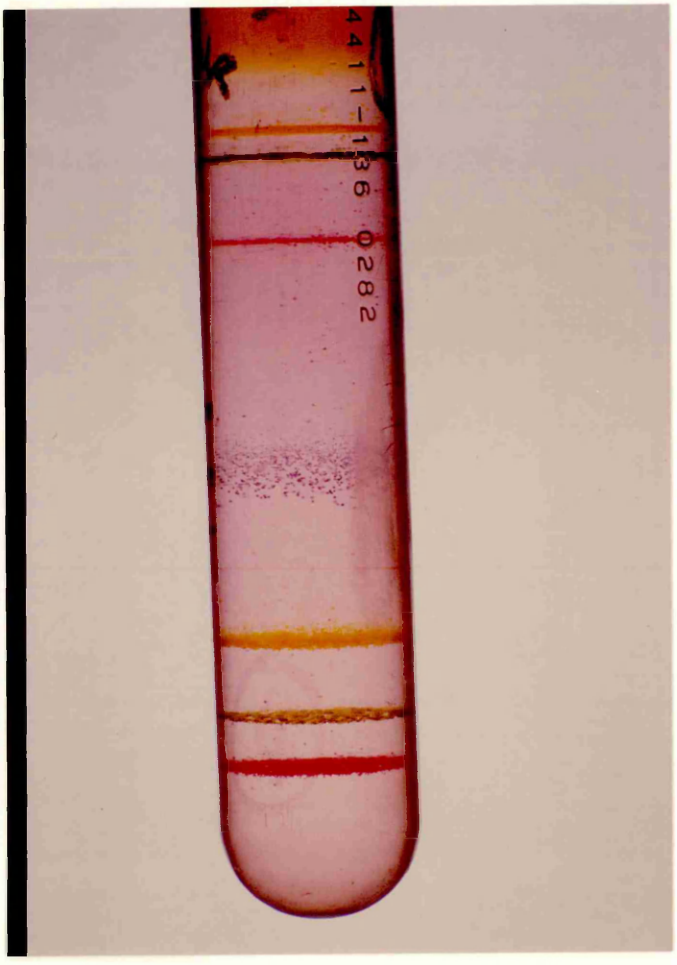
54 ml percoll was made isotonic by the addition of 6 ml 8 g/dl sodium chloride and was prepared to a specific density of 1.083 g/l by the further addition of 32 ml culture medium (Appendix 1). The density gradients were prepared in 10 ml capacity capped polycarbonate centrifugation tubes, spun with the brake off in an MSE 25 angle-head rotor for 45 min at 20,000 g and 4°C.

Bone marrow erythroblasts were washed three times in cold culture medium after TG-1 myeloid cell lysis and were suspended in heat inactivated filtered foetal calf serum to a concentration of 50×10^6/ml. 1 ml of this erythroblast suspension was carefully layered onto each preformed density gradient maintained at 4°C. Gradients were then spun in a refrigerated centrifuge at 4°C for 15 min at 1400 g. Erythroblasts were removed from four preset density fractions, identified by percoll density marker beads added to a blank percoll gradient before centrifugation at 1400 g (Fig 18). Erythroblasts were washed three times in cold culture medium at 4°C. Cell counts and cytospin preparations were made from each percoll fraction. Erythroblast pellets in capped sonication tubes were snap frozen with CO_2 ice and stored at -70°C. On the day of assay, incubation medium was freshly prepared and 1.5 ml added to the cell pellet. Sonication and measurement of ALA synthase activity was carried out as described (Chapter 2) with all samples being assayed in duplicate.

5.6.2 The erythroblasts in percoll fractions

Cytospin preparations of the erythroblasts in the four percoll fractions were examined by light microscopy. Erythroblast maturity in the percoll fractions was assessed by differential 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).

Marker beads indicate density levels after centrifugation at 1400 g_{av} for 15 min.



Note:

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

Figure 18: A percoll density gradient

Separation on the basis of density was found to provide successful separation of erythroblasts according to their state of differentiation. From microscopic examination of cells at different depths on a spun gradient, four percoll density fractions were selected as providing the most satisfactory separation of E_1 cells from E_2 cells from E_3 cells. Each fraction corresponded to the following density steps (g/ml):

fraction 1:	1.062 - 1.070
fraction 2:	1.070 - 1.080
fraction 3:	1.080 - 1.100
fraction 4:	1.100 - 1.118

As has been shown with velocity centrifugation (Denton and Arnstein, 1973) the most effective erythroblast separation was obtained from marrows that showed pronounced erythroid hyperplasia. Normal marrow lacking erythroid hyperplasia was found to contain few pro and early basophilic erythroblasts, such that the percoll fractions containing these immature erythroid cells were also found to contain large numbers of contaminating lymphocytes and monocytes.

Within the equivalent fractions from the different marrows examined, the erythroblasts were found to be at a comparable level of maturity so permitting comparison of ALA synthase activity between age matched erythroblasts from normal and pathological marrow samples. The mean erythroblast differential counts in the percoll fractions from the three normoblastic, three PASA, two CDA and two thalassaemia marrows were similar and are shown in Table 9. Pro and early basophilic (E_1) erythroblasts were most

Sample	E_1 %	E_2 %	E_3 %
NORMOBLASTIC			
Percoll 1	41	42	17
Percoll 2	19	69	12
Percoll 3	2	65	33
Percoll 4		26	74
SIDEROBLASTIC			
Percoll 1	35	50	15
Percoll 2	24	56	20
Percoll 3	8	68	24
Percoll 4		39	61
CDA			
Percoll 1	45	37	18
Percoll 2	27	48	25
Percoll 3	4	55	41
percoll 4		39	61
THALASSAEMIA			
Percoll 1	40	40	20
Percoll 2	27	48	25
Percoll 3	4	55	41
Percoll 4		39	61

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

TABLE 9: Mean erythroblast differential counts from the percoll gradients

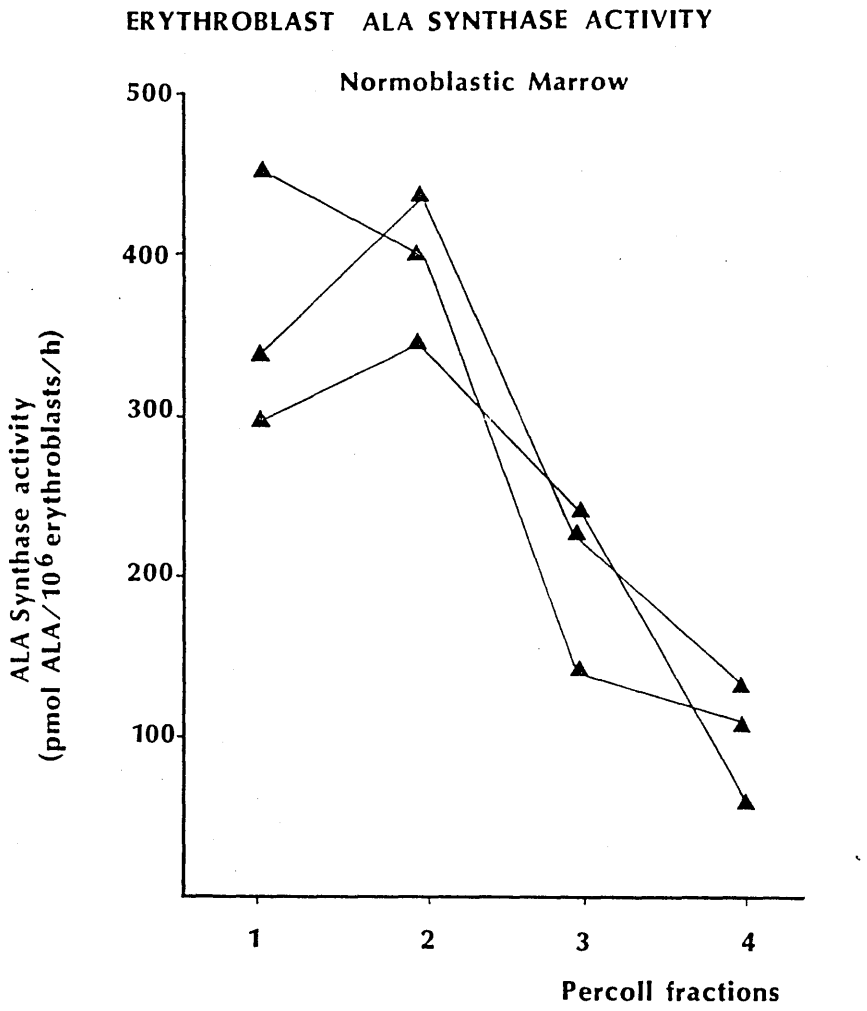
prominent in fraction 1. These immature cells were present to a lesser extent in fraction 2, but were outnumbered by intermediate (E_2) polychromatic erythroblasts. Percoll fraction 3 contained more intermediate than late (E_3) erythroblasts with the reverse situation being the case in fraction 4. (see photographic plates)

5.7 ALA SYNTHASE ACTIVITY DURING NORMOBLASTIC ERYTHROPOIESIS

Maximum ALA synthase activity was found in the erythroblasts of percoll fractions 1 and 2. The activity in these fractions was approximately four times greater than that found in the late erythroblasts of percoll fraction 4 (Fig 19).

This result indicates that the induction of ALA synthase activity occurs early during normoblastic erythroid differentiation. ALA synthase activity was found to be high in pro and early erythroblasts (percoll fraction 1) and to possibly slightly increase as these cells differentiate towards intermediate erythroblasts (percoll fraction 2). Further differentiation towards the intermediate and late erythroblasts of percoll fractions 3 and 4 was associated with a sharp fall in ALA synthase activity.

Bone marrow reticulocytes were present in large numbers in percoll fraction 4 and outnumbered E_3 cells by a factor of > 10:1. Low levels of ALA synthase activity in this fraction relative to the activity found in reticulocyte free fractions 1 and 2, indicates



Note: Each point represents the mean of two observations.

Figure 19:

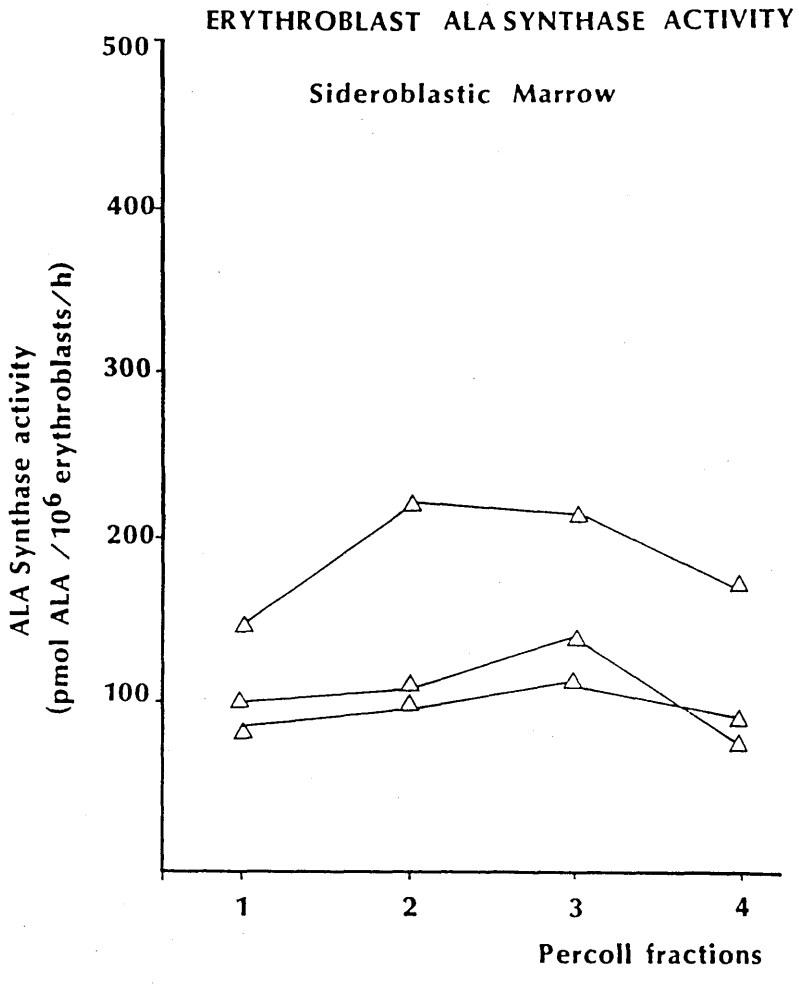
that the activity present in reticulocytes is extremely low, perhaps several hundred times lower than that of E_1 cells. The magnitude of this reduction would appear to indicate the considerable risk involved in extrapolating results from the study of haem synthesis in reticulocytes to that in erythroblasts.

It is clear from morphological examination that considerable ALA synthase activity is present in the cells of fraction 1 before the appearance of significant amounts of cytoplasmic haemoglobin.

Bone marrow red cells were recovered in large numbers together with the late erythroblasts in percoll fraction 4. As ALA synthase activity was low in this fraction, enzyme activity was measured by mixing cells from fractions 1 and 4, to exclude any inhibition of ALA synthase activity by homogenised red cells present during assay incubation. No evidence of inhibition was found and this indicated that the low levels of enzyme activity in percoll fraction 4 were a true measure of low ALA synthase activity in late erythroblasts.

5.8 ALA SYNTHASE ACTIVITY DURING SIDEROBLASTIC ERYTHROPOIESIS

ALA synthase activity during sideroblastic erythropoiesis did not show the same pattern of activity evident during normoblastic cell development (Fig 20). Rapid induction of ALA synthase activity was not seen in the early erythroblasts in PASA and mean enzyme activity was three-fourfold lower in percoll fractions 1 and 2 in sideroblastic than in normoblastic marrows ($p < 0.5$). ALA



Note: Each point represents the mean of two observations.

Figure 20:

synthase activity in the more mature sideroblasts of percoll fractions 3 and 4 was similar to that in normoblasts of equivalent maturity.

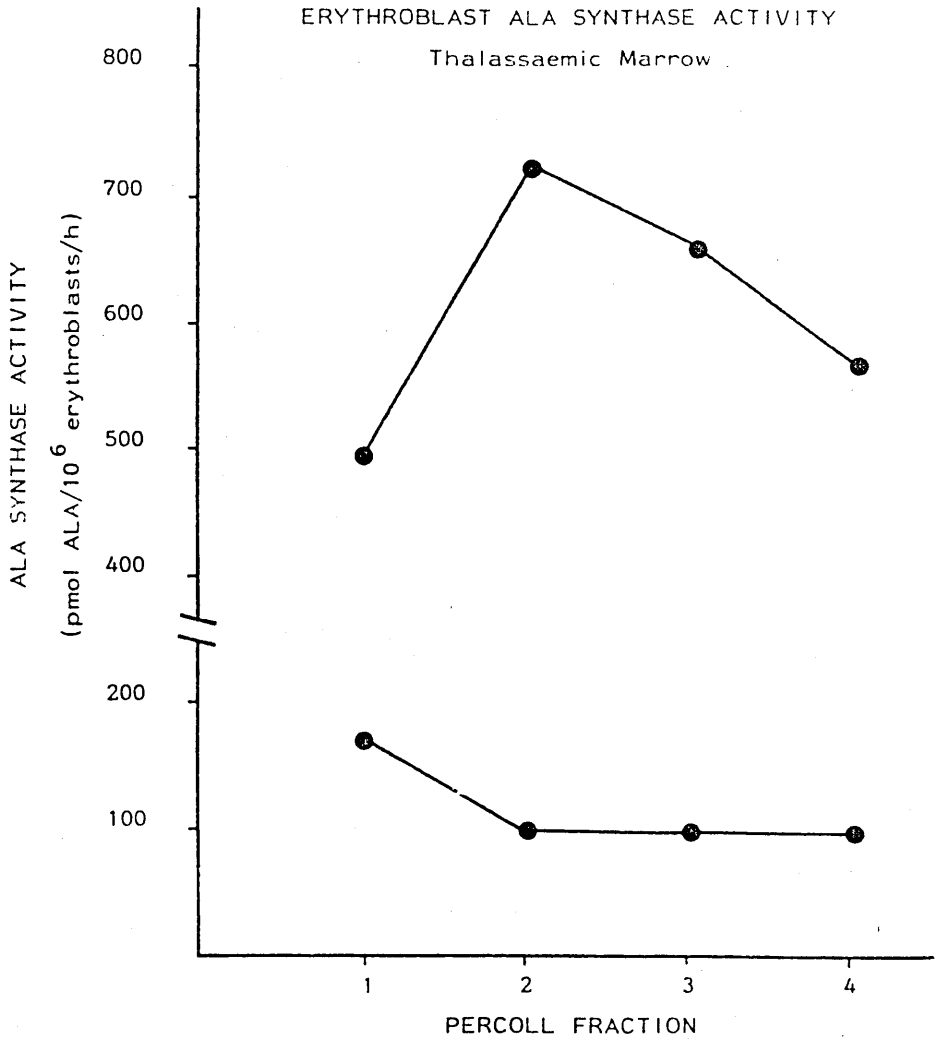
5.8.1 Intracellular iron accumulation during sideroblastic erythropoiesis

Cytochemical staining for iron was performed on cells in the percoll fractions obtained from the sideroblastic marrow samples. Pathological iron loading was seen at all stages of erythroid development. This was most apparent in the erythroblasts of percoll fractions 3 and 4. E_2 and E_3 cells showed the characteristic ring sideroblast defect, indicative of mitochondrial iron loading. E_1 cells showed iron deposits which were smaller and less numerous than those seen in the more mature erythroblasts but which appeared either to be in the cytoplasm or on/in the cell nucleus.

5.9 ERYTHROBLAST ALA SYNTHASE ACTIVITY IN THALASSAEMIA AND CDA

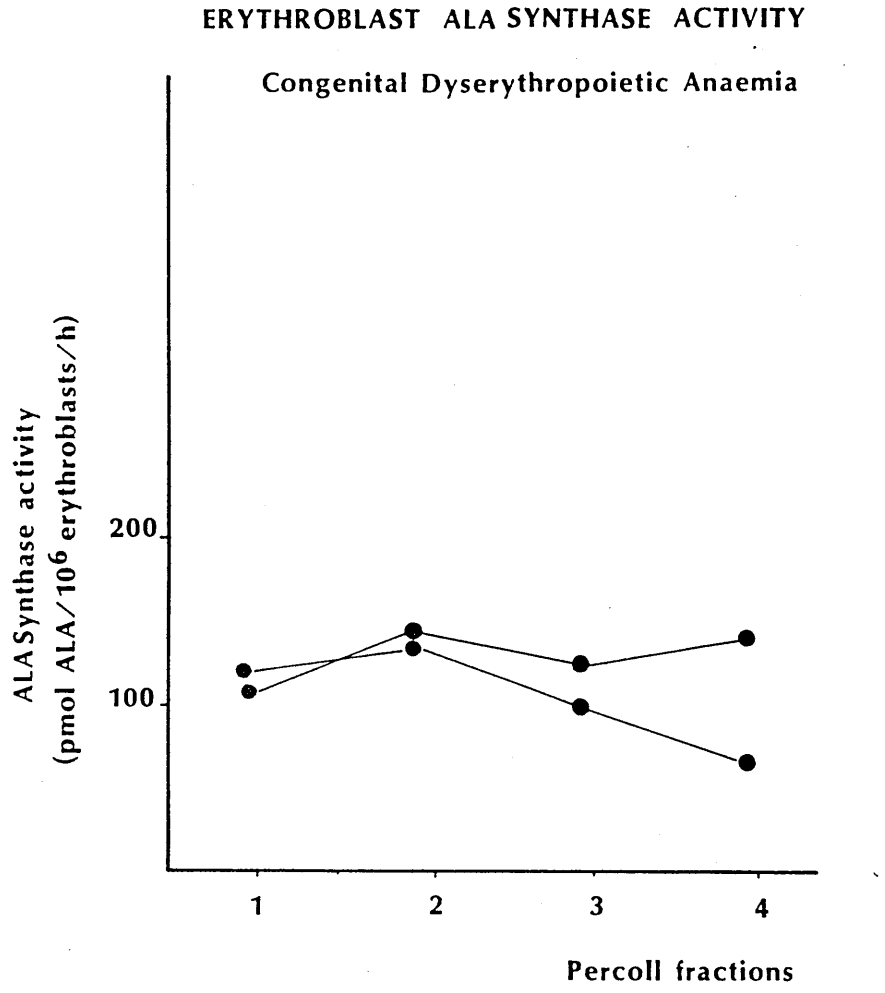
The erythroblasts from patient 7 with thalassaemia intermedia contained extremely high levels of ALA synthase activity (Fig 21). Activity was maximal in percoll fraction 2 and remained high during differentiation and in the late erythroblasts (E_3) of percoll fraction 4.

ALA synthase activity during erythroid differentiation in the other patient with thalassaemia intermedia (patient 8) and in both patients with CDA (Fig 22) was similar to that found in PASA.



Note: Each point represents the mean of two observations.

Figure 21:



Note: Each point represents the mean of two observations.

Figure 22:

Enzyme activity in percoll fractions 1 and 2 did not show the high activity evident in normoblastic cells of equivalent maturity ($p < 0.5$) but was found to be similar in fractions 3 and 4 to that of normoblastic fractions 3 and 4.

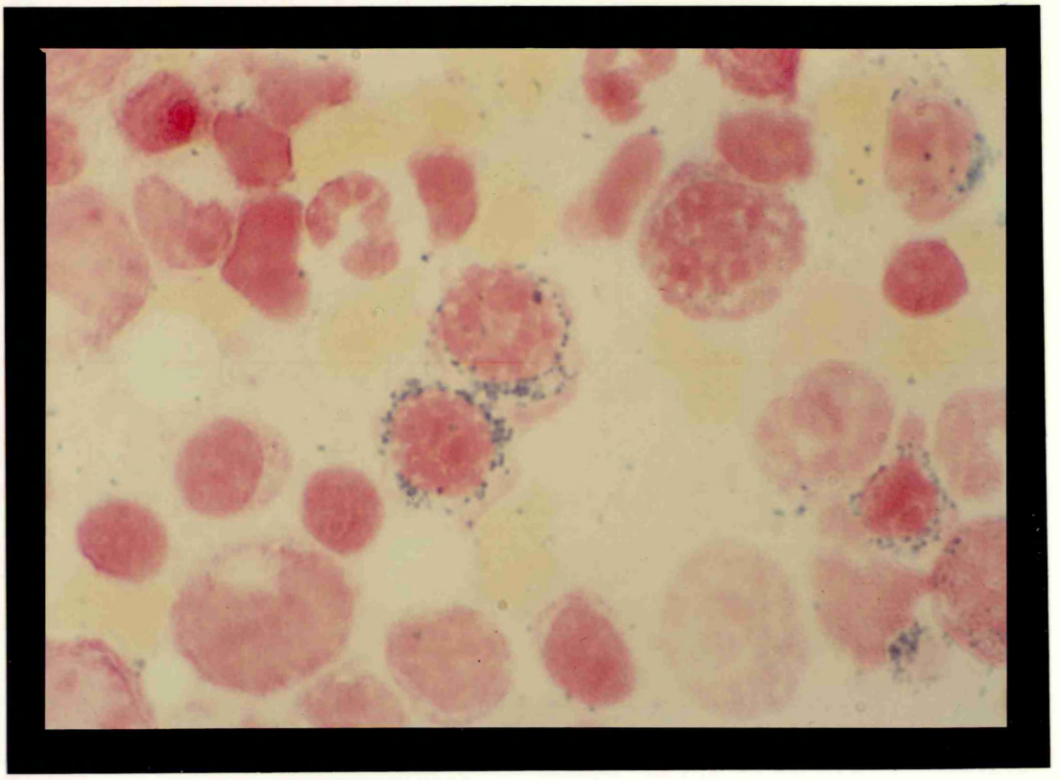
5.9.1 Erythroblast iron loading in CDA and thalassaemia

As reported in Sections 3.4.3/4 light microscopy identified 8% and 2% ring sideroblasts with 20% and 5% pathological sideroblasts in CDA patients 9 and 10 and 2% and 5% ring sideroblasts with 10% and 20% pathological sideroblasts in thalassaemia patients 7 and 8.

Electron microscopy identified mitochondrial iron loading in 47% and 31% respectively of the erythroblasts of CDA patients 9 and 10.

PHOTOGRAPHIC PLATES

Plate 1



Ring sideroblasts (x 1000)

PERCOLL EQUILIBRIUM DENSITY CENTRIFUGATION OF HUMAN BONE MARROW ERYTHROBLASTS

Erythroblast differentials

Differential counting of erythroblasts is a subjective procedure liable to interobserver variation. The differential counts on the cells in the 4 percoll fractions (reported in table 10) were made on 400 cells by two independent observers who scored the stage of erythroid development by the following criteria for cell recognition.

Proerythroblasts: large immature cells with chromatin condensation (and hence erythroid origin).

Early erythroblasts: large immature cells with basophilic cytoplasm and erythroid nuclei.

Intermediate erythroblasts: smaller cells with smaller non pyknotic nuclei and polychromatic cytoplasm.

Late erythroblasts: small well haemoglobinised cells with a small pyknotic nucleus.

Within each of the three defined erythroblast groups (E_1, E_2, E_3) different stages of differentiation were seen. This was most apparent with E_2 cells. From the erythroblast appearances as shown in Plates 5-15 it may be seen that the intermediate erythroblasts of percoll fraction 2 were in general less mature than the intermediate erythroblasts of percoll fraction 3.

PERCOLL FRACTIONS

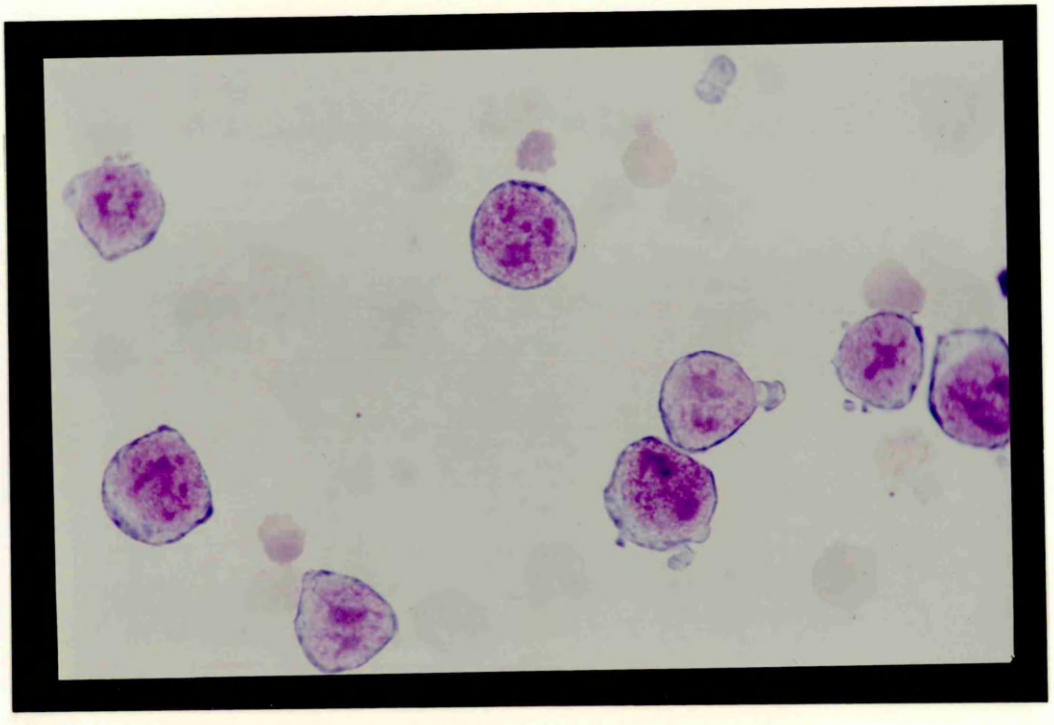
Fraction 1 (plates 2-4): proerythroblasts and early erythroblasts were prominent in this fraction which also contained equal numbers of intermediate erythroblasts.

Fraction 2 (plates 5-8): intermediate erythroblasts represented the majority of cells present. E_1 cells at 20-30% were less prominent than in percoll fraction 1.

Fraction 3 (plates 9-14): intermediate erythroblasts were again most numerous. Early erythroblasts were seen infrequently, with well haemoglobinised late erythroblasts at 30-40%.

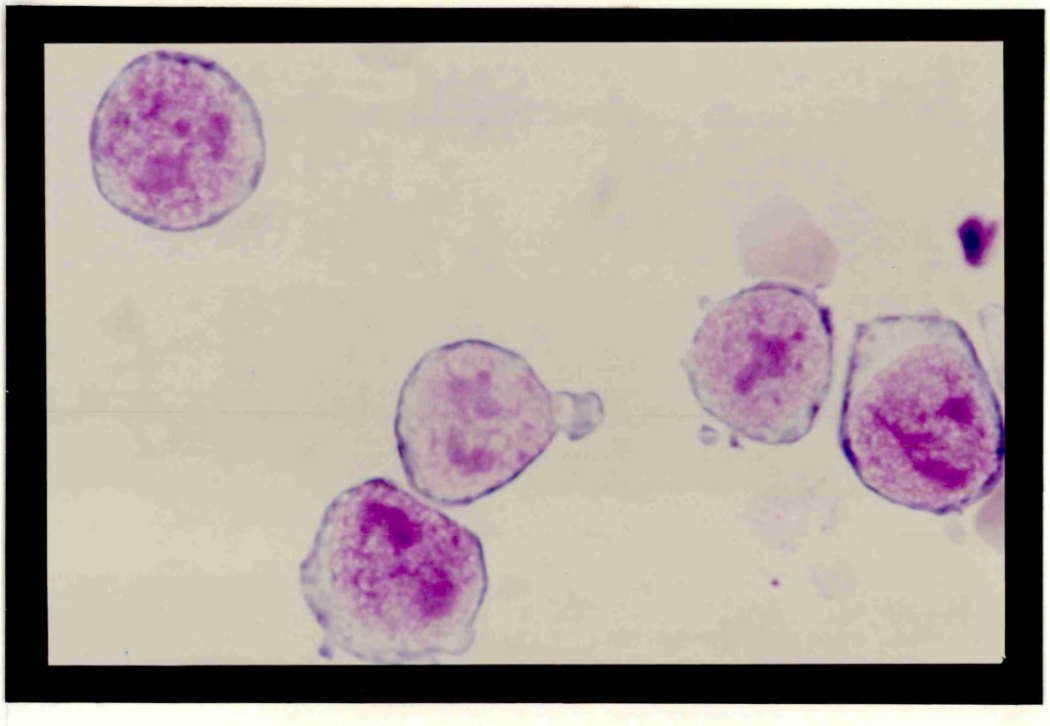
Fraction 4 (plates 15-18): this fraction contained predominantly mature red blood cells. Erythroblasts that were present were for the most part at the late stage of development.

Plate 2



Proerythroblasts (x 1000)

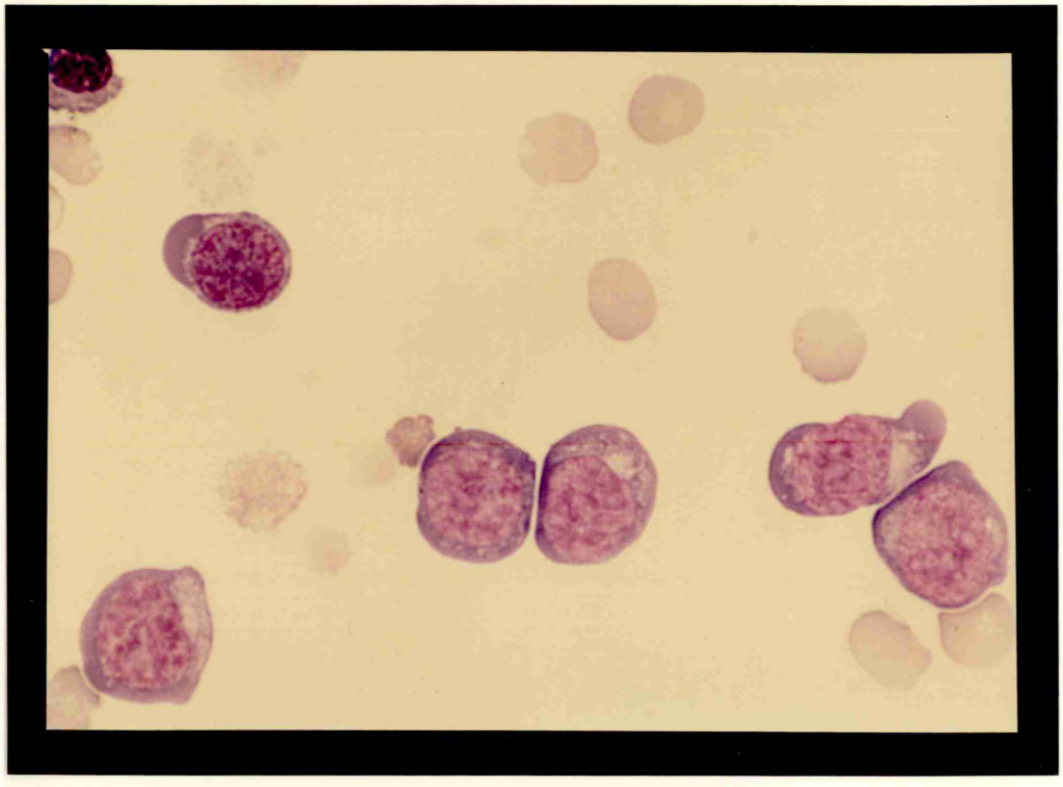
Plate 3



Proerythroblasts (x 1200)

Percoll fraction 1

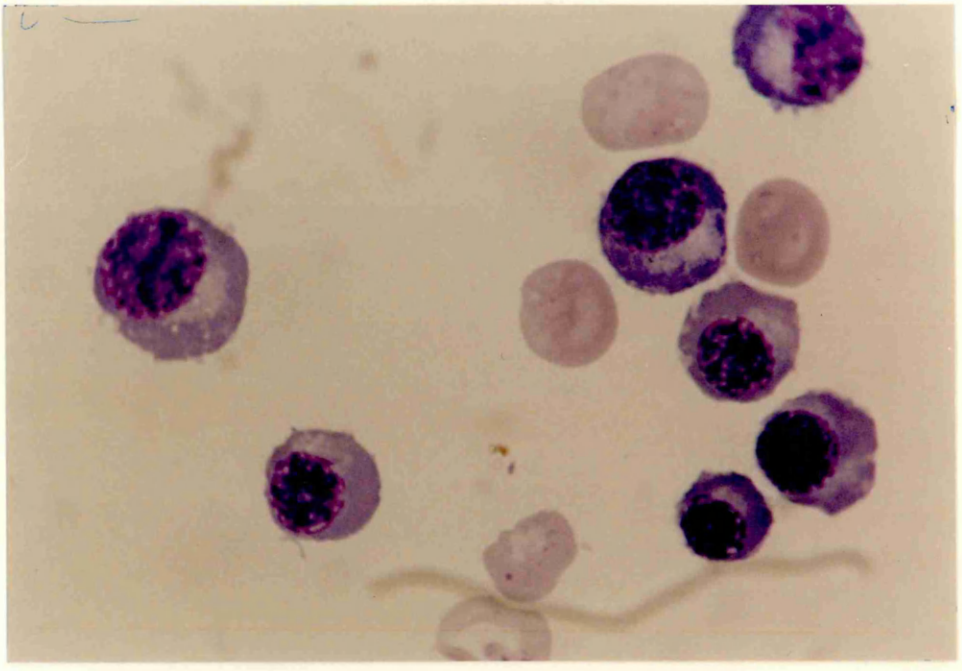
Plate 4



Early erythroblasts (x 1000)

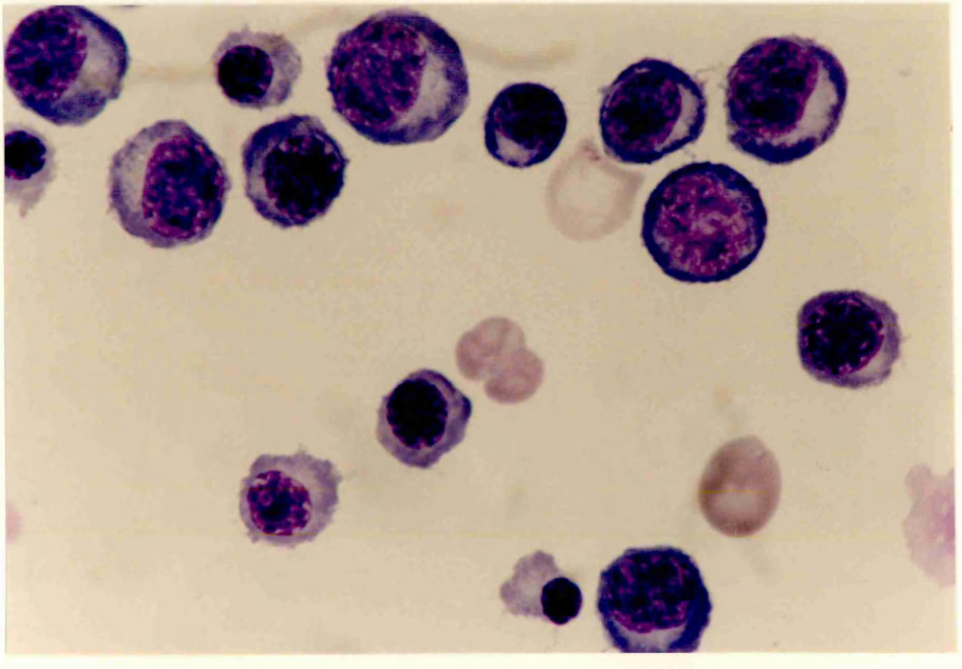
Percoll fraction 2

Plate 5



Intermediate erythroblasts (x 1000)

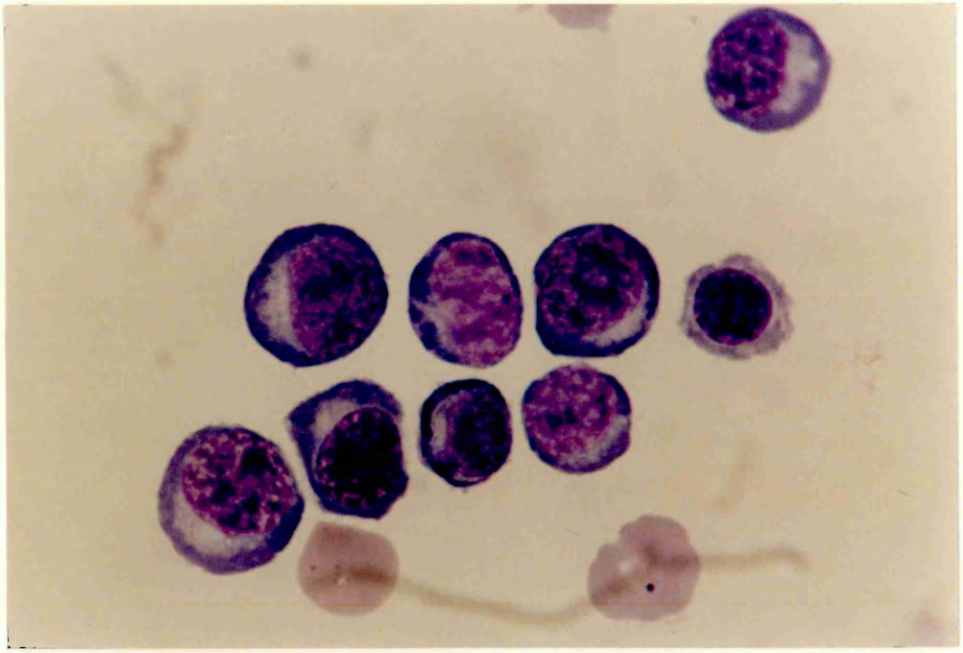
Plate 6



Early and intermediate erythroblasts (x 1000)
(late erythroblast at 6 o'clock)

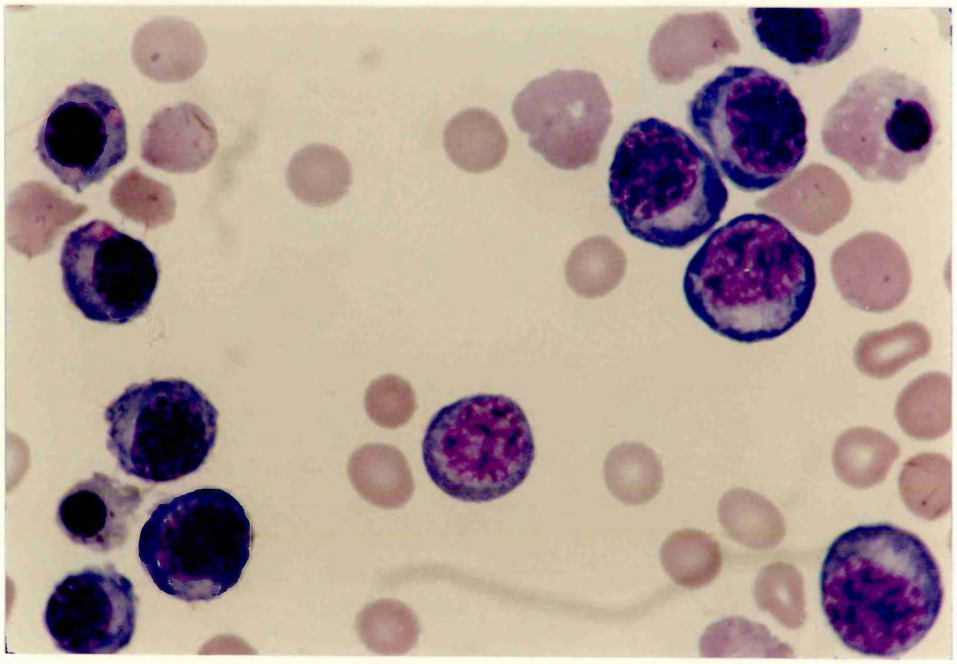
Percoll fraction 2

Plate 7



Early and intermediate erythroblasts (x 1000)

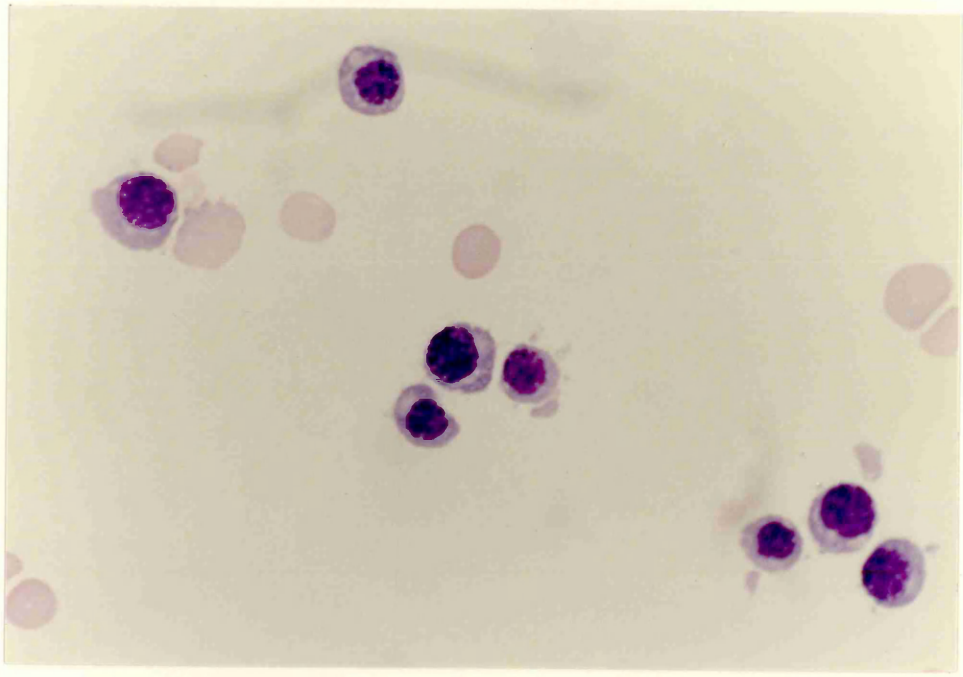
Plate 8



Early and intermediate erythroblasts (x 1000)
(late erythroblasts at 2 o'clock and 8 o'clock)

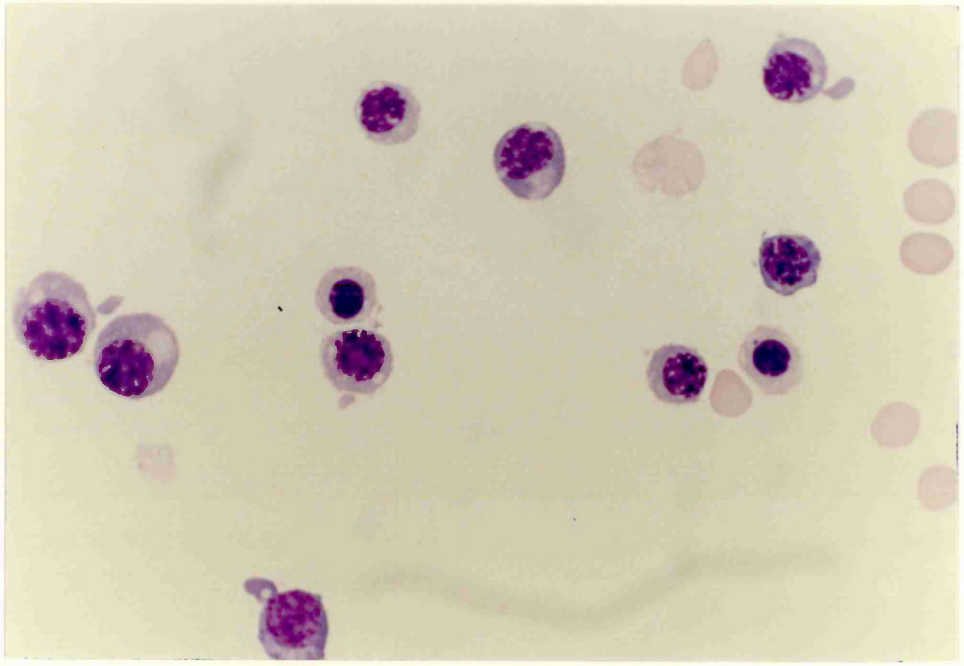
Percoll fraction 3

Plate 9



Intermediate erythroblasts x 600

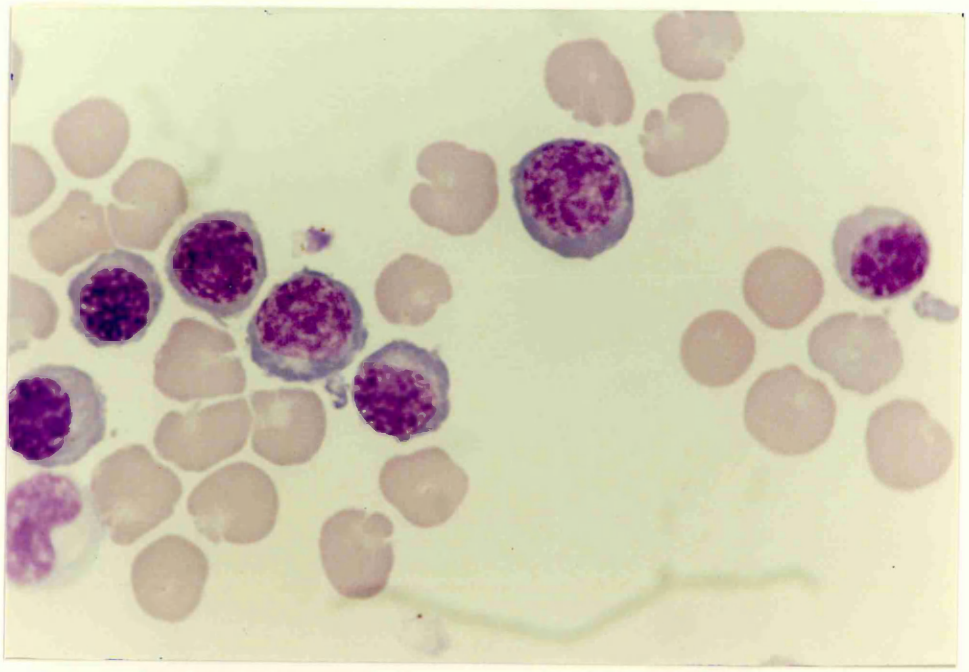
Plate 10



Intermediate erythroblasts x 600
(two late erythroblasts present)

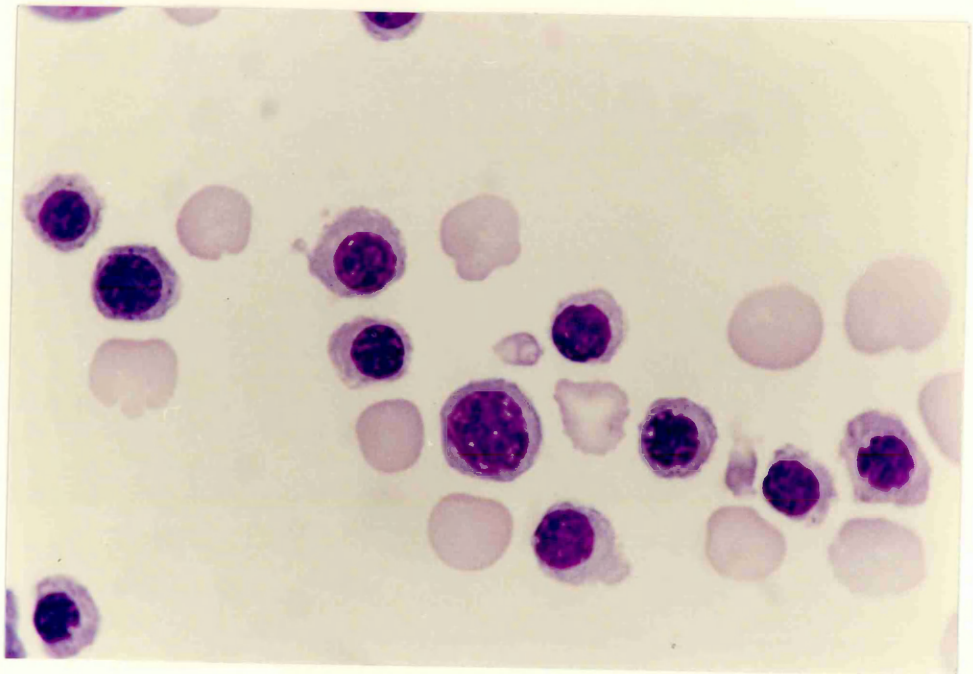
Percoll fraction 3

Plate 11



Intermediate erythroblasts x 1000

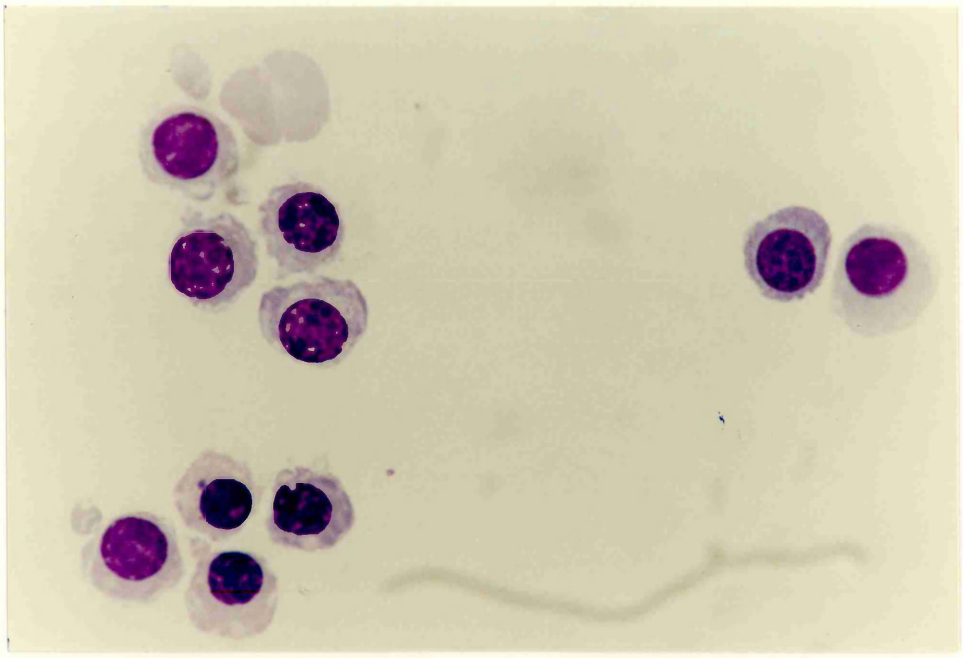
Plate 12



Intermediate and late erythroblasts (x 1000)

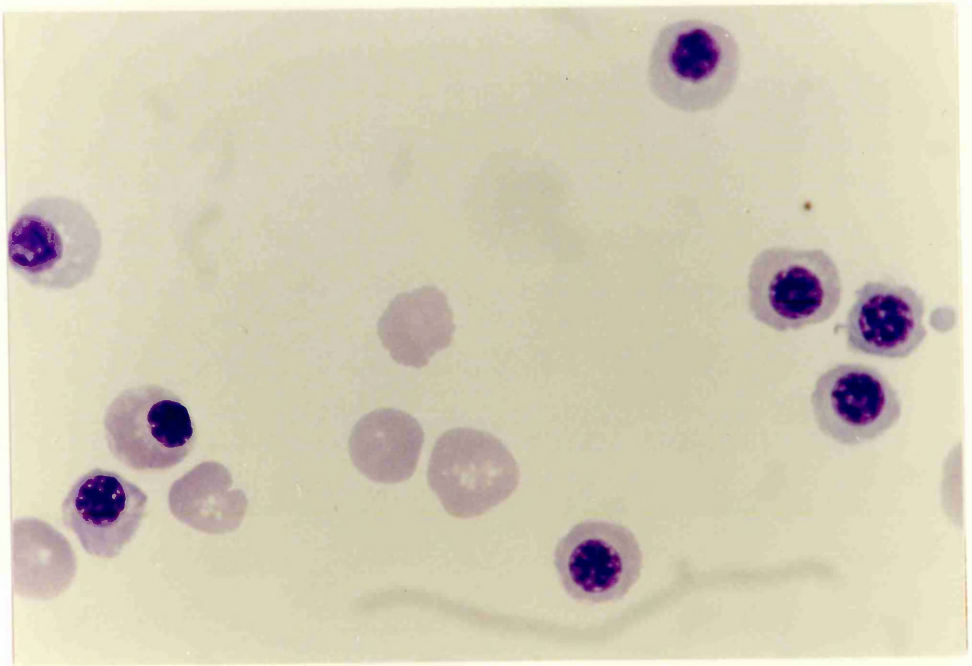
Percoll fraction 3

Plate 13



Intermediate and late erythroblasts (x 1000)

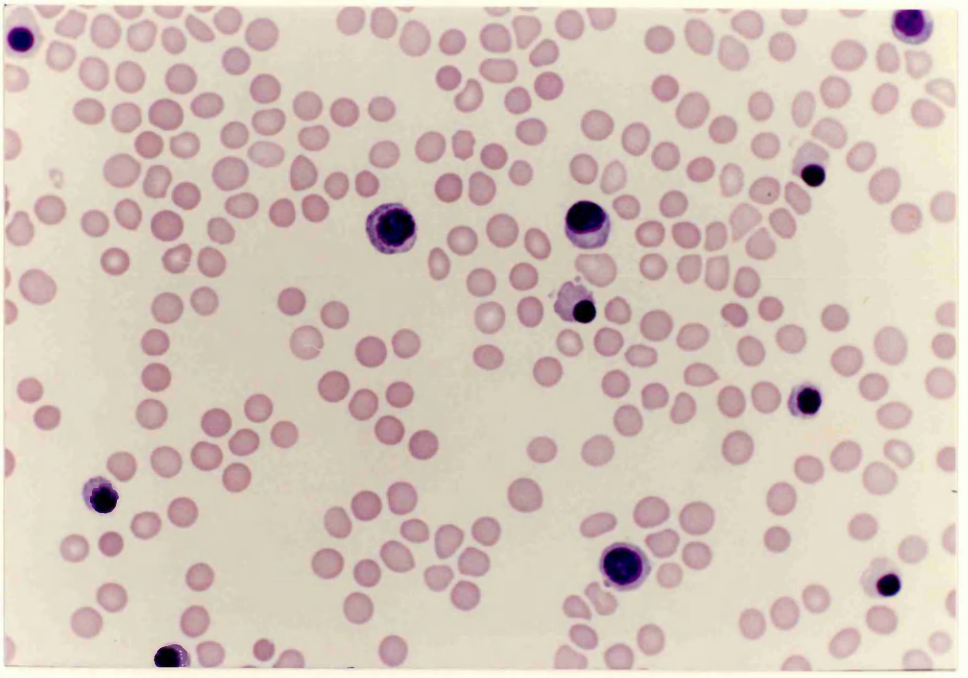
Plate 14



Intermediate and late erythroblasts (x 1000)

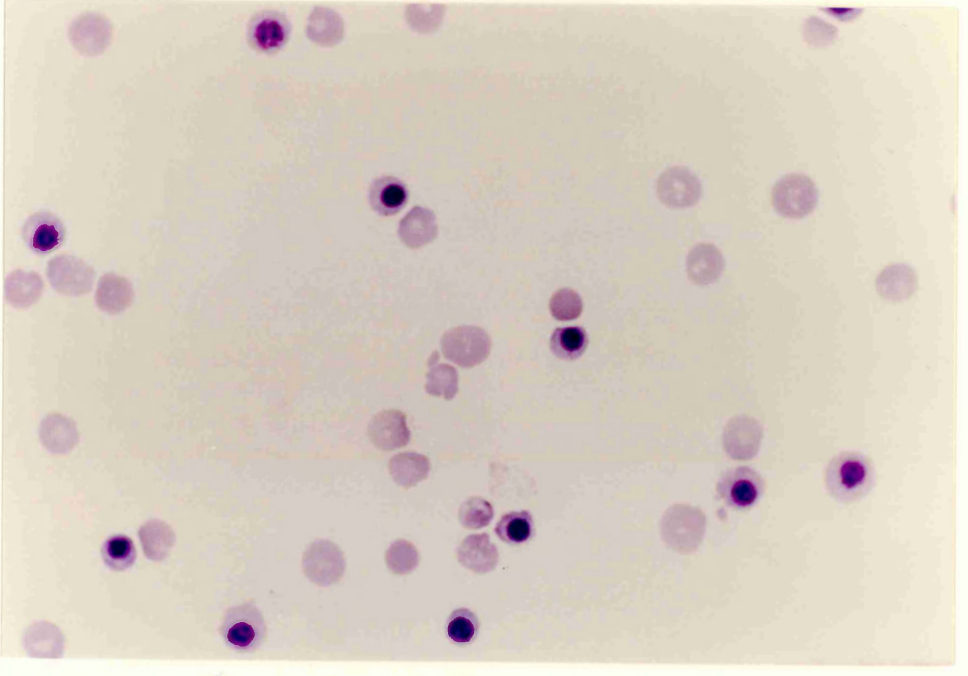
Percoll fraction 4

Plate 15



late and intermediate erythroblasts (x 450)
with large numbers of RBCs

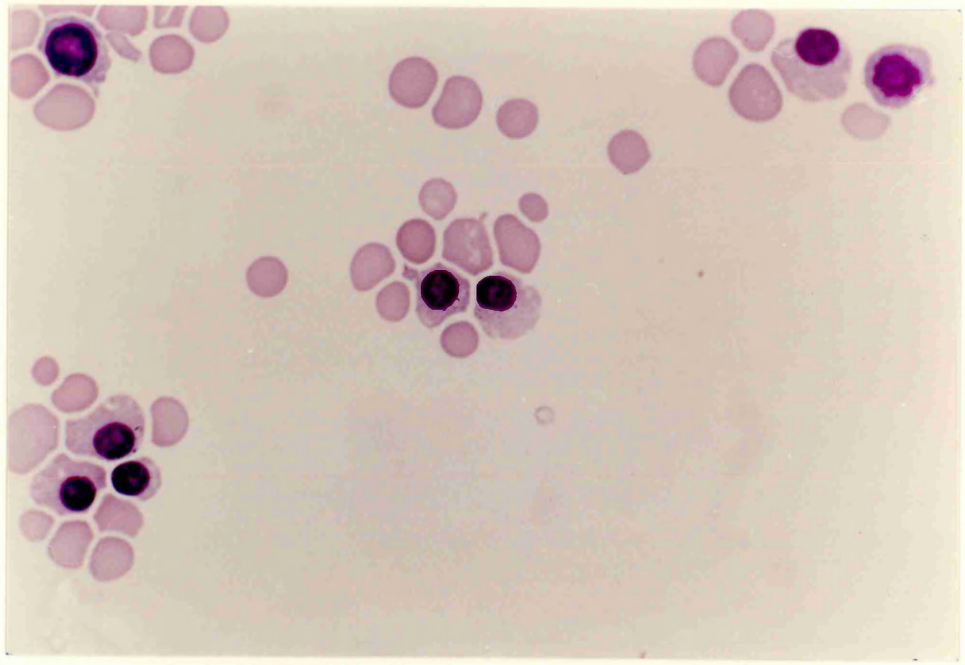
Plate 16



late and intermediate erythroblasts (x 450)
with large numbers of RBCs

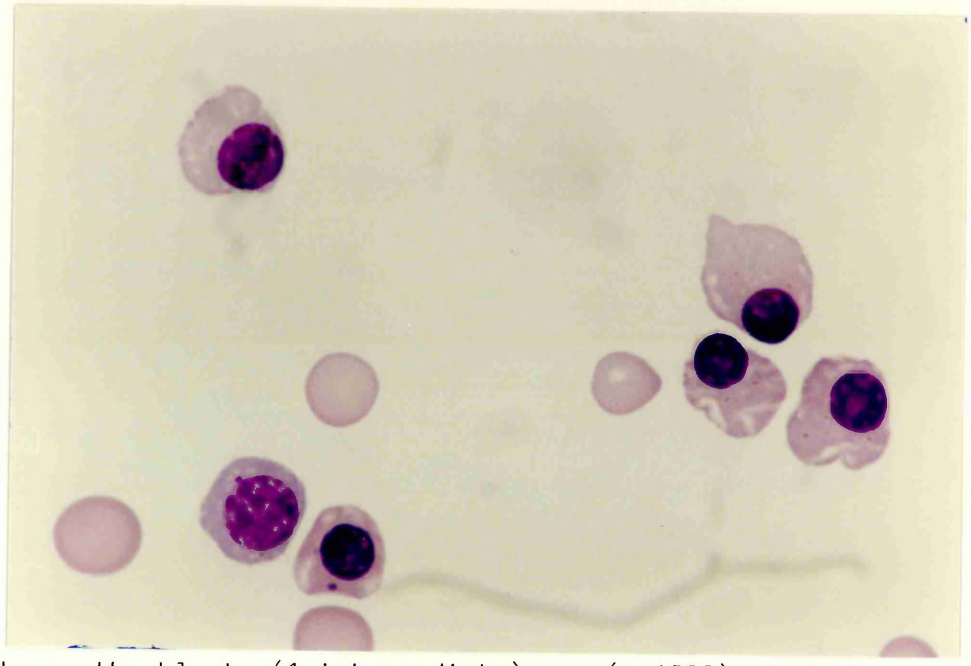
Percoll fraction 4

Plate 17



late erythroblasts (plus 2 intermediate) (x 600)

Plate 18



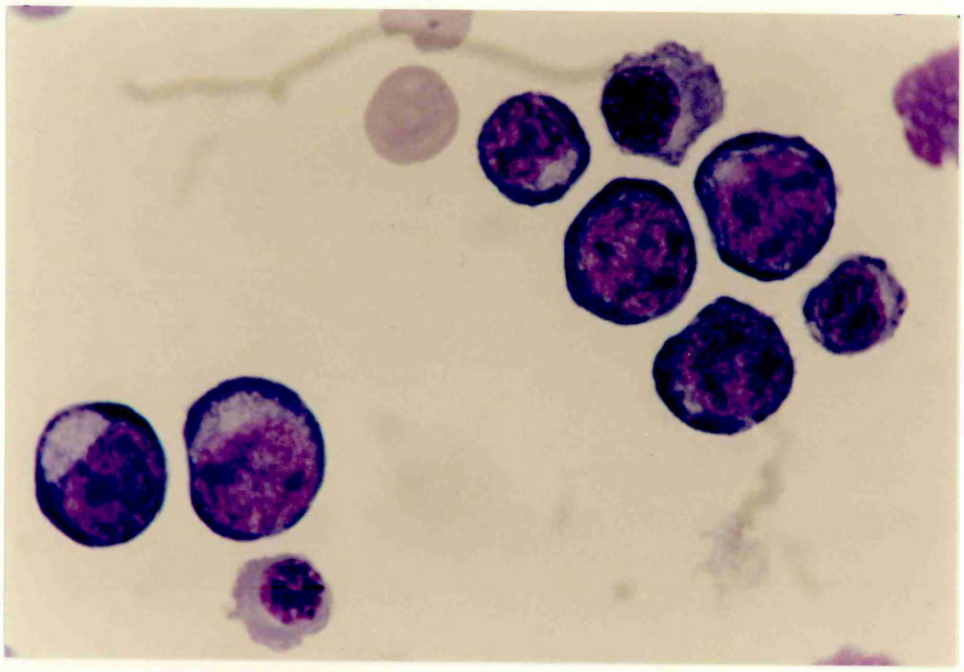
late erythroblasts (1 intermediate) (x 1000)

Percoll fractions 1 - 4

PASA erythroblasts (patient 6; table 8)

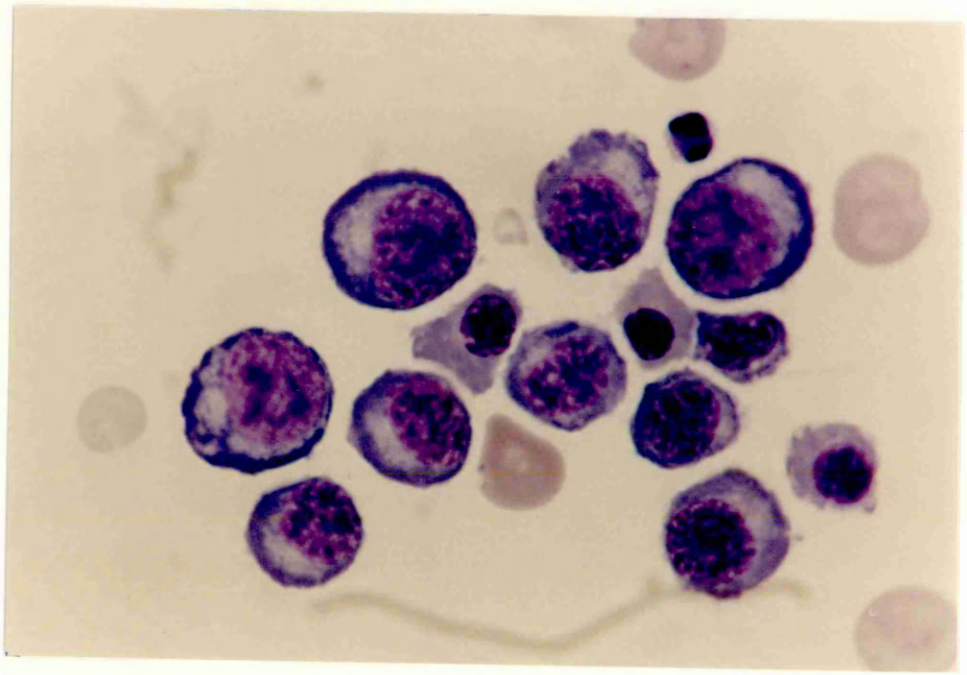
Percoll fractions 1 and 2

Plate 19



Percoll fraction 1 (x 1000)

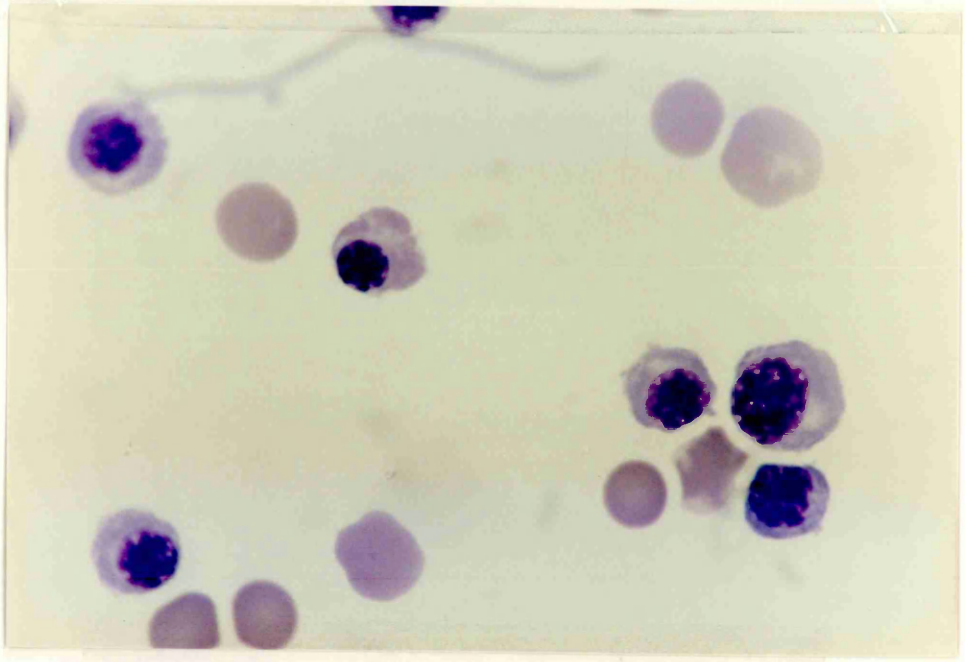
Plate 20



Percoll fraction 2 (x 1000)

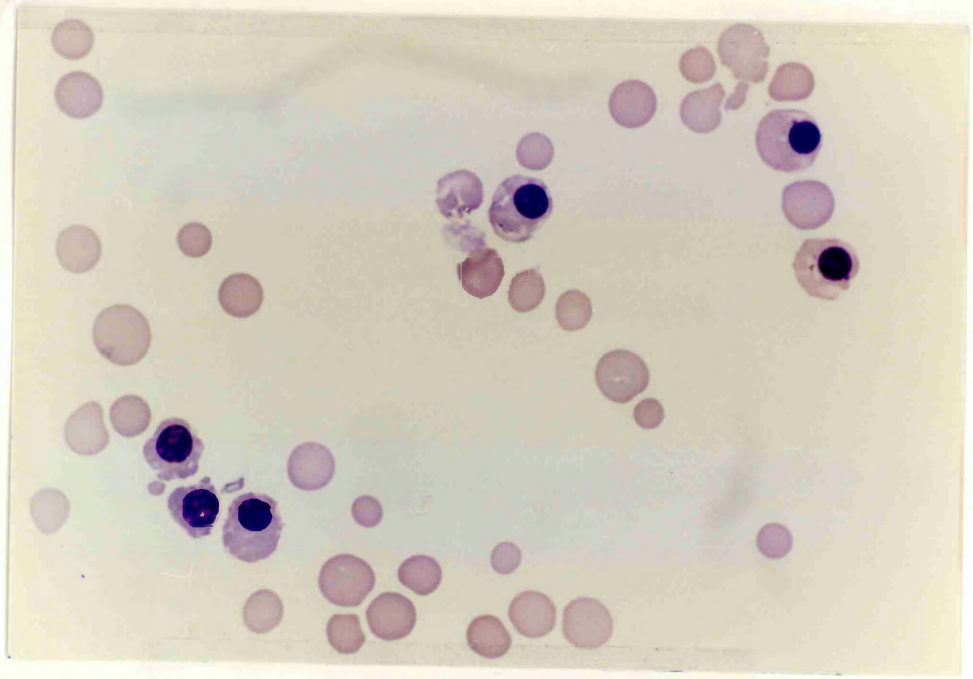
Percoll fractions 3 and 4

Plate 21



Percoll fraction 3 x 1000

Plate 22



Percoll fraction 4 (x 600)

DISCUSSION

This work has provided three points of considerable interest. Firstly, it has shown that haem synthesis and in particular ALA synthase may be examined in differentiating "in vivo" human erythroblasts so that information on human erythroid haem synthesis need no longer be derived by inference from the study of foetal mammalian erythroid systems or "in vitro" erythroid cell culture systems. Secondly, it has clearly and directly confirmed the suspicions arising from reported studies using unfractionated human bone marrow, that erythroblast ALA synthase activity is reduced in PASA. Lastly, this work has for the first time directly measured human erythroblast ALA synthase activity and has described conditions in which erythroblast ALA synthase activity was found to be reduced to levels similar to those found in PASA, but in which ring sideroblasts were not prominent on light microscopy.

Monoclonal antibody TG-1 and percoll equilibrium density centrifugation were combined to achieve physical separation of erythroid cells at different stages of development from one another and from the non erythroid cells also present in the original marrow samples. The development of such a system is an essential prerequisite to any advance in understanding the complex biochemical and molecular events of human erythroid differentiation.

Various methods have been used previously to fractionate developing erythroblasts. Bone marrow erythroblasts may be synchronised in animals by injections of actinomycin D and harvested at intervals thereafter (Konijn et al, 1979). Physical separation techniques of velocity (Denton and Arnstein, 1973) and density (Borsook et al, 1969) centrifugation have been used successfully to fractionate rabbit and mouse (Glass et al, 1975b) erythroblasts, but require either treatment of the animals with phenylhydrazine, splenectomy or lengthy and repeated centrifugations at room temperature. Human erythroblast fractionation using TG-1 and percoll centrifugation was first described by Ali et al (1983). It was modified by selecting slightly different percoll fractions from those initially used (Ali et al, 1983) and was adopted for use. The advantage of using this method was that the centrifugation step was short and refrigerated and that high percentage cell recoveries (Ali et al, 1983) permitted its use with small cell numbers.

The density of developing erythroblasts is determined mainly by the nuclear size and haemoglobin concentration (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 produced reproducible erythroblast fractionation and have provided clear information on the effects of normal and abnormal differentiation on ALA synthase activity. It is of course possible that improved fractionation could be obtained by

increasing the number of percoll fractions sampled or perhaps by combining myeloid cells lysis with refrigerated velocity centrifugation. Such steps would be those most immediately available for assessment if future experiments, perhaps regarding the molecular analysis of human erythropoiesis, demanded more complete erythroblast fractionation.

Normoblastic erythroid differentiation was associated with the early induction of high ALA synthase activity in pro and basophilic erythroblasts which fell rapidly with differentiation towards late erythroblasts. As haemoglobin cannot be demonstrated in proerythroblasts (Undritz, 1973); as its production only starts after the basophilic erythroblast (Hammarsten et al, 1953; Forget et al, 1976); and as the rapid phase of haemoglobin production occurs only after the polychromatic phase of erythroblast differentiation (Denton et al, 1975) it would appear as if ALA synthase activity does not limit human erythroblast haemoglobin production. This suggests either that erythroblast haem synthesis precedes globin synthesis (Forget et al, 1976) and/or that, as with FEL cells (Sassa, 1976) and mouse foetal hepatic erythroblasts (Freshney and Paul, 1971) and in contrast to liver cells (Granick and Urata, 1963), ALA synthase activity does not limit human erythroblast haem synthesis. Although haem and globin synthesis may indeed occur asynchronously they are sufficiently closely matched to be considered as simultaneous (Paul, 1978). This would suggest that the latter proposal is more likely.

Results from studies with FEL cells are contradictory as to whether haem synthesis is limited by the sequential development of haem enzyme activity and late induction of ferrochelatase activity (Sassa, 1976) or by the availability of ferrous iron to protoporphyrin (Beaumont et al, 1984). The direction for future experiments is to determine the temporal relationship between ALA synthase activity, ferrochelatase activity and haem synthesis in differentiating human erythroblasts.

The present work has directly identified reduced ALA synthase activity in immature PASA erythroblasts and supports the hypothesis originally developed from indirect evidence that haem synthesis is deficient in PASA. The presence of microcytes in the blood film in PASA may be seen as evidence of defective haemoglobin synthesis. However, it was the presence of free α β globin dimers in PASA reticulocytes together with the ability of exogenous haem to greatly stimulate PASA reticulocyte globin synthesis (280% increase) and restore reduced $\alpha:\beta$ globin synthetic ratios towards unity (White et al, 1971; White and Ali, 1973) that provided the first major experimental evidence in favour of reduced haem synthesis in PASA. This has since been supported by the reports of reduced ALA synthase activity in unfractionated PASA bone marrow, the association between sideroblast development and inhibition of haem synthesis with agents such as lead and INH (see Bottomley, 1979, 1982), the therapeutic benefit of pyridoxine in some cases of congenital sideroblastic anaemia and the relationship between PASA and Hb H disease (Yoo et al, 1980).

In contrast however, Peters et al (1983) found normal α:β globin synthetic ratios in PASA. Their failure to detect haem induced stimulation of PASA reticulocyte globin synthesis beyond that observed in normal reticulocytes has cast doubt on the earlier work (White et al, 1971) and its interpretation. Further doubts arose from the demonstration that mitochondrial iron loading and siderocyte formation developed in response to defective globin rather than defective haem synthesis in flexed-tailed mutant-(ff) mice (Chui et al, 1977) and from the inability of May et al (1982a) to clearly demonstrate deficient haem synthesis in PASA erythroblasts compared with age matched normal controls. The marked reduction in ALA synthase activity now demonstrated in the immature erythroblasts in PASA settles those doubts and identifies impaired haem synthesis as a feature of PASA.

Cytochemical staining for iron revealed erythroblast iron loading in all PASA percoll fractions, but more so in E_3 than E_1 cells. This does not resolve the problem as to whether reduced ALA synthase activity is the primary abnormality in PASA that produces mitochondrial iron loading (Ponka and Neuwirt, 1974; Bottomley, 1982) or whether it occurs secondary to the toxic effect of ferric/ferrous iron on mitochondria (Hunter et al, 1963) and on ALA synthase itself (Morrow et al, 1969). In contrast to the more popular belief that PASA results from a primary defect in ALA synthase activity, electron microscope autoradiography has identified mitochondrial iron loading in PASA as the likely cause, not the result of reduced protein and RNA synthesis in

sideroblasts (Wickramasinghe and Hughes, 1978). Together with the study of iron localisation in PASA erythroblasts this has led to the development of an alternative hypothesis in which a primary defect of iron metabolism is favoured as the cause of PASA (May et al, 1982a).

Iron excess certainly has adverse effects on the anaemia in PASA. Phlebotomy with iron reduction will improve the Hb concentration (Weintraub et al, 1966; Hoffbrand et al, 1974). However the extent to which generalised tissue iron overload contributes to the anaemia in PASA is unclear as the severity of the anaemia does not relate to the degree of tissue iron overload (Bottomley, 1982). Perhaps however the degree of mitochondrial iron overload is the more relevant factor.

There is no evidence from this work to support the hypothesis that ring sideroblasts develop as a result of reduced ALA synthase activity (Ponka and Neuwrit, 1974; Bottomley, 1982). The demonstration of reduced erythroblast ALA synthase in three cases of non sideroblastic anaemia (two CDA; one thalassaemia) is the first indication of reduced enzyme activity in conditions in which ring sideroblasts are not prominent on light microscopy. It is clear that reduced ALA synthase activity 'per se' is not the obligatory cause of the readily visible ring sideroblast defect in PASA.

The cause of excessive ALA synthase activity in thalassaemic patient 10, in whom iron overload was not present, is unclear. As impaired globin synthesis may be associated with an increase in the intracellular free haem pool (Ponka et al, 1973) it is possible that increased enzyme activity has resulted as proposed, by the action of free haem in stimulating ALA synthase activity in immature erythroid cells (Granick and Sassa, 1978; Sassa, 1980; Elder, 1981; Ibrahim et al, 1983).

CDA is a heterogeneous group of congenital anaemias which in all instances are associated with highly ineffective erythropoiesis and gross defects of DNA synthesis and nuclear morphology. Their pathogenesis is unclear. It is unlikely that one defect common to all cases could account for the three typical groups and the many atypical variants that have been recognised (Boogaerts and Verwilghen, 1982). These disorders have been associated with disturbed globin chain synthesis (Weatherall et al, 1973), secondary haemochromatosis (Cazzola et al, 1983) and in the HEMPAS type in particular with an increased susceptibility to lysis by normal sera in an acid media. Multiple red cell enzyme abnormalities have been described in both the congenital and the acquired myelodyoplastic syndromes (Valentine et al, 1972, 1973). This is the first report of reduced erythroblast ALA synthase activity in CDA. It is however unlikely that deficient haem synthesis is a major factor in the pathogenesis of CDA, as the absence of significant hypochromia indicates adequate rather than impaired haemoglobin formation.

The high levels of erythroblast ALA synthase activity in thalassaemic patient 7 contrast with the reduced levels in patient 8. Although these thalassaemia syndromes had arisen in response to synthetic defects affecting different globin chains it would seem unlikely that reduced α chain production might stimulate ALA synthase, whereas reduced β chain production would inhibit ALA synthase. The most notable difference between the two thalassaemic patients was in their iron loading status. Patient 7 had never received blood transfusions and was a vegetarian with normal serum ferritin concentration and transferrin saturation. On the other hand, multiple blood transfusions had contributed to the considerable iron loading of patient 8 in whom erythroblast ALA synthase activity was reduced. It is likely that electron microscope examination of the erythroblasts from this patient, as with other transfused thalassaemics (Bannerman, 1964; Polliak and Rachmilewitz, 1973), would have uncovered considerable mitochondrial iron loading not visible on routine light microscopy. The more generalised toxic effects of iron overload in transfused thalassaemics are already well recognised and provide the stimulus behind iron chelation programmes. With particular respect to erythropoiesis, there is also evidence that iron toxicity in the thalassaemia marrow may further embarrass its already highly ineffective performance. Ferrokinetic studies have identified both the predicted immediate post transfusion suppression of erythropoiesis in thalassaemia major, as well as a long term depression resulting from repeated transfusion (Cavill et al, 1978). It was postulated that this effect had resulted

from transfusional iron overload, which was already known to lead to mitochondrial iron loading in thalassaemia (Bannerman, 1964; Polliak and Rachmilewitz, 1973). This belief is supported by the report of reduced bone marrow ALA dehydratase and ferrochelatase activity in patients with thalassaemia and by the positive correlation between this reduction and the degree of iron loading as assessed by the number of blood transfusions per patient (Steiner et al, 1964).

Mitochondrial iron loading is then known to occur in transfusion dependent thalassaemia and was demonstrated at the ultrastructural level in both cases of CDA who, in common with thalassaemia patient 8, also had tissue iron overload and high transferrin saturation. In the three patients with PASA mitochondrial iron loading was readily visible on light microscopy but had developed in two of these cases in the absence of tissue iron overload or increased transferrin saturation. In these three PASA patients there was no evidence of reduced ALA synthase activity in the bone marrow myeloid cells (Section 4.5), which although probably the product of the same sideroblastic marrow clone (Prchal et al, 1978) do not show mitochondrial iron loading (Larizza and Orlandi, 1964). Erythroblast mitochondrial iron loading appears to be the only common factor shared by the six patients with reduced erythroblast ALA synthase activity.

Therefore it is postulated that in all six cases reduced enzyme activity was due to iron-mediated inhibition of ALA synthase

activity and abnormal erythroblast iron metabolism. In CDA and transfused thalassaemia it is likely that reduced enzyme activity developed as a result of erythroblast iron overload occurring secondary to tissue iron overload and high transferrin saturation. In contrast, the abnormalities in PASA developed even in the absence of tissue iron overload and high transferrin saturation. It is then postulated that reduced ALA synthase activity in PASA reflects mitochondrial iron toxicity which has resulted from a primary abnormality of erythroblast iron metabolism.

It is further possible to postulate the way in which iron inhibits ALA synthase activity. The major deficiency of ALA synthase in PASA was in the early erythroblasts, whereas maximum visible iron deposits were seen in the late erythroblasts, in which enzyme activity was not significantly lower than in the normoblastic late erythroblasts. This would suggest that reduced enzyme activity does not result from gross visible mitochondrial iron deposits, but presumably from a metabolically active "iron pool", which although not visible to the eye or electron microscopy, may be toxic and promote free radical damage. As maximum inhibition of ALA synthase by an invisible iron pool might reasonably be expected to correlate with maximal iron uptake, this hypothesis is supported by the findings of Hodgetts et al (1986), who using TG-1 and percoll marrow fractionation as described, identified maximum iron uptake in the early erythroblasts of percoll fractions 1 and 2.

SUMMARY TO CHAPTER 5

ALA synthase activity has been measured in the progressive stages of human erythroblast differentiation. Enzyme activity was fourfold higher in pro and early basophilic erythroblasts than in late orthochromatic forms. Markedly reduced erythroblast ALA synthase activity was found in six patients with three dissimilar conditions: three PASA, 2 CDA and one case of iron loaded thalassaemia. Reduced ALA synthase activity was not found in one thalassaemia patient without iron loading.

Erythroblast mitochondrial iron loading is known to occur following frequent transfusions in thalassaemia. It was demonstrated by light microscopy in PASA and by electron microscopy in CDA and was identified as the one relevant factor common to all six patients.

Therefore it is postulated that reduced erythroblast ALA synthase activity results from abnormal erythroblast iron metabolism, which may occur secondary to tissue iron overload and high transferrin saturation in CDA and transfused thalassaemia, but as a primary abnormality of erythroblast iron metabolism in PASA.

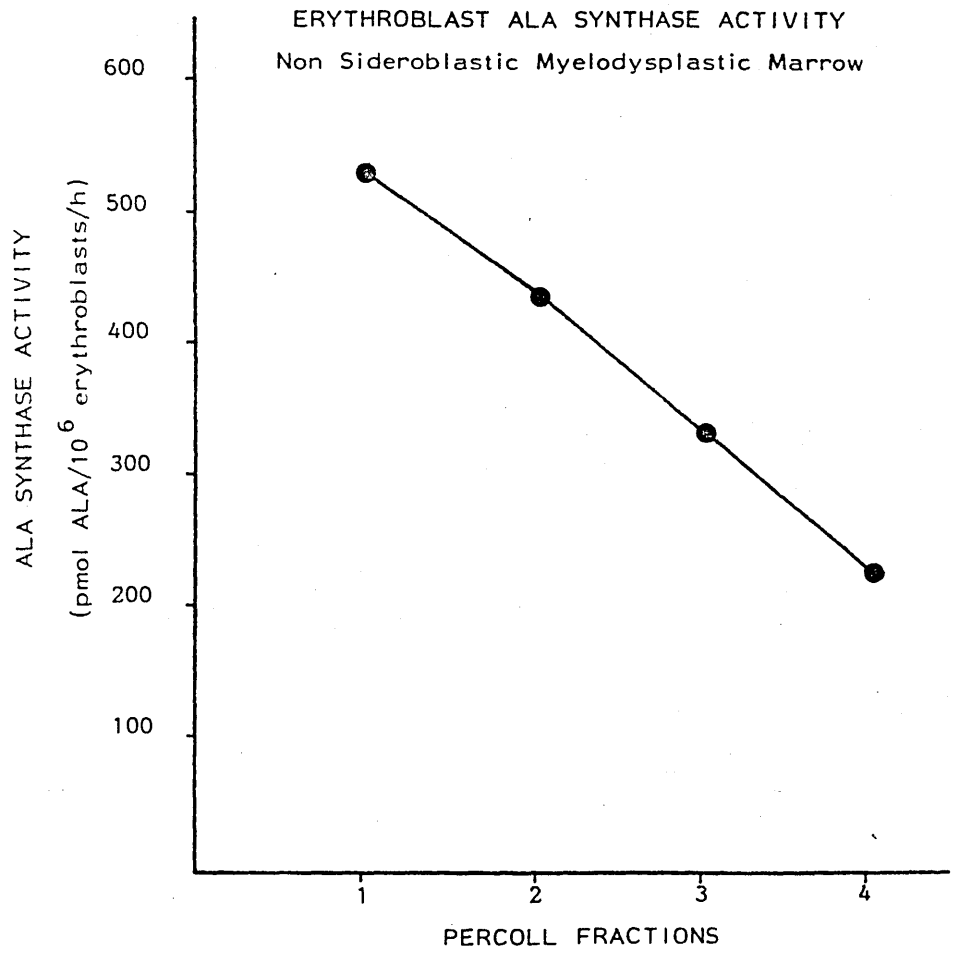
CHAPTER 6

CONCLUSIONS

6. CONCLUSIONS

The myelodysplastic syndromes (MDS) represent a clonal abnormality of the haemopoietic stem cell with a high probability of eventual acute leukaemic transformation. Although PASA represents a distinct entity in the FAB classification of MDS proposed by Bennett et al (1982) there is remarkable similarity between the different MDS groups with respect to clonal assays and ferrokinetic parameters (May et al, 1985). Indeed a diagnosis of "sideroblastic anaemia" as an anaemia with more than 15% ring sideroblasts has to be seen in the context of a wide range of sideroblast counts in patients with MDS from 1-86% (May et al, 1982a; Juneja et al, 1983; May et al, 1985). It is not known if similarities in MDS extend to a reduction in erythroblast ALA synthase activity as seen in PASA.

One patient with a non sideroblastic MDS, without tissue iron overload, has been studied. The patient was a 26 year old male with unexplained acquired pancytopenia (Hb 9.1 g/dl WC 3.0 x 10^9/l platelets 100 x 10^9/l) and ferrokinetic evidence of 90% ineffective erythropoiesis. There was no past history of anaemia. Serum ferritin was 26 ug/l and transferrin saturation 25%. Erythroblast ALA synthase activity was normal (Fig 23). This might then indicate that reduced ALA synthase activity is not a feature common to all MDS and is confined to such cases that are associated with prominent mitochondrial iron loading.



Note: Each point represents the mean of two observations.

Figure 23

The work reported in this thesis is the first of its kind as no previous studies have examined haem biosynthesis in age matched "in vivo" human erythroblasts. It has also shown that ALA synthase activity as measured in unfractionated human bone marrow reflects activity present in both myeloid and erythroid cells and is insensitive to the changes in erythroblast ALA synthase that occur during differentiation. There is then the need to reappraise all previous studies made on unfractionated bone marrow, which without exception, have reported their results as a measure of erythroblast ALA synthase activity. Further studies of haem biosynthesis in human bone marrow are required but should in future clearly and separately define the separate contributions made by myeloid and erythroid cells using suitably sensitive enzyme assay techniques.

The nature of the work has limited the number of patients studied and a first priority for further experiments would be to extend the number of observations already made. At present however there seems to be sufficient evidence to dispel the theory that reduced erythroblast ALA synthase activity is the primary abnormality in PASA (Aoki et al, 1974; Ponka and Neuwirt, 1974; Bottomley, 1977, 1982). Reduced erythroblast ALA synthase activity was found in CDA and thalassaemia, in addition to PASA, thereby indicating that reduced enzyme activity does not "per se" cause readily visible mitochondrial iron loading. Furthermore the failure to detect reduced myeloid cell ALA synthase activity in PASA bone marrow (Chapter 4) lessens the likelihood of there being a generalised primary defect of mitochondrial function as well as ALA synthase activity in PASA as proposed by Aoki (1980).

The most significant contribution made by this work has been its demonstration that we now have the ability to unveil the secrets of human erythroblast haem biosynthesis (see Chapter 1) and need no longer rely on the study of foetal erythropoiesis or "in vitro" erythroid cell cultures for information on haem synthesis, which by inference may also apply to the "in vivo" human situation. By combining a suitably sensitive assay of ALA synthase activity with bone marrow fractionation, enzyme activity was directly measured in differentiating "in vivo" erythroblasts, recovered at particular stages of development in percoll fractions containing age matched erythroblasts. The natural progression from this initial step would be to examine the activities of the other haem enzymes and establish the complete synthetic sequence from appearance to maximal activity for each individual enzyme during differentiation. Human erythroblasts and in particular early and pro erythroblasts, may now also take the place of rabbit reticulocytes and FEL cells to study the primary effects of INH and succinylacetone on erythroblast haem synthesis and their secondary effects on iron and protein metabolism and erythroblast differentiation. Lastly, as bone marrow fractionation is the only means by which pathological age matched erythroblasts may be obtained, haem synthetic enzyme activity may now be examined in secondary anaemias, iron deficiency/overload and myelodysplastic states.

The final section in Chapter 1 discussed the curious paradox apparent in the suggestion that while reduced activity of a haem

biosynthetic enzyme may cause PASA, anaemia is an uncommon manifestation of porphyria. This paradox is resolved by the proposal that reduced ALA synthase activity in the different erythroblasts studied resulted from iron toxicity mediated within the mitochondria and that reduced ALA synthase activity is not the cause of ring sideroblast development. It is likely that reduced haem synthesis contributes significantly to the anaemia in PASA but more so as a consequence of disturbed erythroblast iron metabolism than as a result of reduced ALA synthase activity.

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APPENDIX 1

GENERAL METHODS

Appendix 1. GENERAL METHODS

1. ROUTINE HAEMATOLOGICAL PARAMETERS

The measurement of haemoglobin concentration and red cell indices was performed on a Coulter Counter Model S. Serum iron, TIBC was determined by the standard colorimetric methods serum ferritin concentration by immunoradiometric assay and serum Vit B_{12} and folate levels by radioassay (Becton Dickinson).

2. CULTURE MEDIUM

Culture medium contained Eagles MEM, buffered with 20 mM Hepes to pH 7.4 at 37°C, with glycine 1 mM. 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) 1 M pH 7.3, 4 ml 1 N NaOH and 3 ml glycine 20 mg/ml were added.

3. 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.

4. BONE MARROW CELL COUNTS AND CYTOSPIN PREPARATIONS

Cell counts were made using a Coulter Counter Model ZF fitted with a channeliser attachment.

Bone marrow cells were washed x 3 in cold culture medium. Heat inactivated foetal calf serum, filtered through 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 clean cytospin holders in a Shandon Southern cytocentrifuge. Centrifugation was at 600 rpm for 10 minutes. Cell morphology was optimally preserved at 5×10^4 nucleated cells per slide.

5. BONE MARROW SAMPLES

The study was approved by the South Glamorgan Joint Ethics Committee. All patients gave their fully informed written consent.

For the development of the ALA synthase assay, bone marrow was obtained from ribs resected at thoracatomy or by curetting the cut surface of the sternum following sternotomy for cardiothoracic surgical procedures. In all cases the peripheral blood counts and bone marrow morphology was normal.

6. STAINING PROCEDURES

Morphological examination of marrow cells was made on cytospin preparation stained with Jenner Geimsa stain. Cytochemical staining for iron was made by Perls stain.

7. REFRIGERATED CENTRIFUGES

Two refrigerated centrifuges were used throughout, an MSE 2L and an MSE, Mistral 3000 refrigerated bench centrifuge.

8. ALA SYNTHASE ASSAY

(a) Incubation medium

Incubation medium for use with 2-keto [^{14}C]glutarate was that as described by Tikerpae et al (1981) and contained glycine 50 mM, sucrose 250 mM, $MgCl_2$ 5 mM, EDTA 10 mM, PLP 0.4 mM CoA 0.25 mM, Tris 40 mM buffered to pH 7.4 with KH_2 PO_4 50 mM.

All incubation media were prepared to pH 7.4 at 37^{o}C as determined by the blood gas analyser in the Department of Medical Biochemistry.

(b) Radiochemicals

Radiochemicals were purchased from Amersham International plc.

2-keto 5-[^{14}C]glutaric acid, sodium salt, was aliquoted, freeze dried and stored under nitrogen at -20°C. It was prepared to 1.7 mM (specific activity 6 Ci/mol) in incubation medium on the day of assay and used in the reaction at a final concentration of 0.17 mM.

2,3-[^{14}C]succinic acid was stored at -20°C in 210 ul aliquots of 20 mM succinate (specific activity 3.0 Ci/mol) in the basic incubation medium of Tikerpae et al (1981) to which PLP, CoA, succinylacetone, STK and GTP were added on the day of assay.

5-amino 4-[^{14}C] laevulinic acid hydrochloride was stored at -20°C in 50 ul aliquots of 0.25 mM ALA (0.5 uCi/50 ul, specific activity 40 Ci/m mol).

9. MODIFIED EHRLICHS REAGENT

Modified Ehrlichs reagent was prepared fresh daily by dissolving 1 g 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).

10. METHANOL EVAPORATION

ALA pyrrole, eluted from the Sep-pak cartridge (Waters associates), was evaporated to dryness under nitrogen in a Techne Dri Block-3 sample concentrator at 35°C.

11. RPHPLC

The HPLC system consisted of a dual piston Waters M 6000 A pump, set at flow rate 1.5 ml/min with a Rheodyne 7125 sample injector fitted with a 50 ul loop.

Mobile phase contained 35% (V/v) aqueous methanol with 0.005 M heptane sulphonic acid (PIC B-7, Waters Associates) as ion pairing agent. Mobile phase was filtered (micropore 0.45 mm filter) before use.

Two reverse phase HPLC columns (4.6 x 150 mm) were used. One was an Ultrasphere C18 ODS ion pairing column; the other an Apex C18 ODS column.

An LDC Spectro Monitor 111 was used at 252 nm and 1.0 AUFS to detect eluted ALA pyrrole. Column fractions were collected in scintillation vials at 30 sec intervals.

12. SCINTILLATION FLUID

Two interchangeable scintillation fluids were used throughout. The initial scintillant used was prepared from toluene (1 l), Triton (500 ml), PPO (4 g) and POPOP (0.1 g). This solution was left to stir overnight and maintained in the dark for 1 hour before use. In later experiments Liquiscint (National Diagnotics) was used.

13. SCINTILLATION COUNTING

All samples for counting contained 0.75 ml of the mobile phase used for HPLC. This was equivalent to the volume collected in 30 second fractions from HPLC and ensured a similar level of quenching in the radioactive standards and HPLC samples. The ratio of mobile phase to scintillant was 1:10.

A Rack Beta scintillation counter was used. Quenching was measured using the channels ratio method. This was uniform indicating that counting efficiency was the same for all tubes. As such it was not necessary to convert cpm to dpm.

14. STATISTICS

Unless otherwise stated statistical analysis of results was carried out using the students t test.