Giampietro, Philip Francis

BIOCHEMICAL AND SOMATIC CELL GENETIC STUDIES OF HUMAN HEME BIOSYNTHETIC ENZYMES

City University of New York PH.D. 1983

BIOCHEMICAL AND SOMATIC CELL GENETIC STUDIES OF HUMAN HEME BIOSYNTHETIC ENZYMES

by

Philip Francis Giampietro

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1983

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

12/30/82
date

Robert J. Desnick
Chairman of Examining Committee

12/30/82
date

Executive Officer

David F. Bishop
Gregory A. Grabowski
Rochelle Hirschhorn

Supervisory Committee

The City University of New York

ABSTRACT

BIOCHEMICAL AND SOMATIC CELL GENETIC STUDIES OF
HUMAN HEME BIOSNYTHETIC ENZYMES

by

Philip Francis Giampietro

Advisor: Professor R.J. Desnick

The regional gene assignment for human porphobilinogen deaminase (PBG-deaminase; E.C.4.3.18) on chromosome 11 has been determined using somatic cell hybridization, immunologic, electrophoretic, and cytogenetic techniques. Dimethyl sulfoxide (DMSO) -induced erythroid differentiation of hybrid clones derived from the fusion of tetraploid Friend murine erythroleukemia (2S MEL) cells deficient in thymidine kinase and human Lesch-Nyhan fibroblasts (HLN) deficient in hypoxanthine phosphoribosyltransferase were examined for the expression of human PBG-deaminase, esterase A_4 and lactate dehydrogenase A. Examination of five primary and 10 secondary, tertiary or quaternary clones permitted the regional assignment of human PBG-deaminase to the long arm of chromosome 11. The analysis of two 2S MEL ($HPRT^-$)-human fibroblast (HX/11) hybrids, each containing the human X chromosome-autosome translocation (der 11), t(X;11)(q25-26; q23) as the only human chromosome permitted finer localization of the gene for human PBG-deaminase to the region 11q23 → 11qter.

Twelve RAG cell-human fibroblast cell hybrids were examined by electrophoretic and immunologic methods for the presence of human δ-aminolevulinic acid dehydratase (ALA-dehydratase). Using these methods it was not possible to detect human ALA-dehydratase, although the mouse

enzyme was electrophoretically and immunologically detectable. Possible reasons for the lack of expression of human ALA-dehydratase are discussed.

Further studies concerning ALA-dehydratase included the development of a sensitive fluorometric assay for determining enzymatic activity in human fibroblasts and amniocytes. Naturally occurring ALA-dehydratase polymorphisms were further characterized with respect to electrophoretic mobility, binding to DEAE-cellulose, K_m and thermostability properties. ALA-dehydratase protein levels were determined in erythrocyte lysates from normal and ALA-dehydratase deficient individuals by rocket immunoelectrophoresis. Erythrocyte lysates obtained from heterozygotes for ALA-dehydratase deficiency were found to contain normal levels of ALA-dehydratase immunoreactive protein.

FORWARD

Portions of this thesis have been presented in the following publications:

Wang, A.-L., Arrendondo-Vega, R.X., Giampietro, P.F., Smith, M., Anderson, W.F. and Desnick, R.J. Regional gene assignments for human porphobilinogen deaminase and esterase A_4 on chromosome 11. Proc. Natl. Acad. Sci. 78:5734-5738, 1981.

Giampietro, P.F. and Desnick, R.J. ALA-dehydratase: A sensitive, coupled enzyme assay. In: Assays of the Heme Biosynthetic Enzymes, Bishop, D.F. and Desnick, R.J., Eds. Enzyme 28:144-145, 1982.

Giampietro, P.F. and Desnick, R.J. Determination of δ-aminolevulinate dehydratase by a specific, fluorometric coupled-enzyme assay. Anal. Biochem. (in review).

ACKNOWLEDGEMENTS

I would like to express my gratitude to those people whose efforts greatly facilitated these studies:

Professor Robert J. Desnick, my thesis advisor was influential in several ways. His experience and scientific guidance contributed largely to my scientific development. I am also very grateful for his generosity in providing the resources to undertake my studies.

Scientific advice generously provided by Drs. Moyra Smith and David Bishop was instrumental in the completion of these studies.

Dr. Peter Anderson's prior experience in heme biosynthetic enzymes contributed largely to the initiation of these studies. I am grateful for his provision of the PBG and immune sera.

Dr. Paco Arredondo-Vega was instrumental in the characterization of the hybrids used for these studies.

I greatly appreciate the technical advice and help which was given to me by the following people: Mr. Raman Reddy, Ms. Gundula LaBadie, Dr. Ken Astrin, Dr. Gary Vorsanger, Dr. Norberto Guzman, Ms. Ludmilla Ostaciewicz and Ms. Diane Anderson.

The expert typing done by Ms. Mary Ann Dent is much appreciated.

The following people provided cells which were essential for these studies:

Dr. W. F. Anderson provided the 2S MEL-human Lesch-Nyhan fibroblast hybrids. Dr. T.D. Bird provided the ALA-dehydratase deficient erythrocyte lysates. Dr. M. Smith provided RAG-human fibroblast hybrids.

Finally, I wish to acknowledge the moral support and encouragement provided by my family members.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AIA	α-allylisopropylacetamide
ALA	δ-aminolevulinic acid
ALAD	δ-aminolevulinate dehydratase
AMP	adenosine 5'-monophosphate
BSA	bovine serum albumin
cm	centimeter
CoA	coenzyme A
DDC	1,4-dihydro-3,5,dicarbethoxy collidine
DMAB	p-dimethylaminobenzaldehyde
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ESA_4	esterase A_4
ESD	esterase D
G6PD	glucose-6-phosphate dehydrogenase
h	hour
HLN	human Lesch-Nyhan fibroblasts
$HOCH_2$bilane	hydroxymethylbilane
HPRT	hypoxanthine phosphoribosyl transferase
Ig	immunoglobulin
LDHA	lactate dehydrogenase A
M	molar
mA	milliampere
mol	mole
mg	milligram
ml	milliliter

n	nano
PBG	porphobilinogen
PCA	perchloric acid
PEPS	peptidase S
PGM2	phosphoglucomutase 2
p	pico
SH	sulfhydryl
2S MEL	tetraploid Friend erythroleukemia cells
TCA	trichloroacetic acid
U	unit
URO	uroporphyrin
UROCoS	uroporphyrinogen III cosynthetase
w	weight
v	volume
V	voltage

BACKGROUND

I. Heme Biosynthetic Regulation and the Porphyrias:

The heme biosynthetic pathway represents a series of coordinated enzymatic reactions which are essential to the maintainance of life. This pathway is diagrammed in Figure 1. Each of the enzymes will be discussed in the following sections. Current interest in the pathway has focused on the genetic and metabolic regulation of heme biosynthesis. Several seminal studies have indicated that the heme biosynthetic pathway is under tight regulatory control. In liver, heme synthesis is regulated by δ-aminolevulinic acid (ALA) synthetase. Heme has been shown to control the level of ALA-synthetase by decreasing the transcription of messenger RNA (1) and by inhibiting the translocation of newly synthesized protein from the cytoplasm to the mitochondria (2-4). In Friend erythroleukemia cells, hemin has been shown to increase hemoglobin synthesis as well as increase the activities of ALA-dehydratase and PBG-deaminase and increase the incorporation of [^{14}C] ALA and $^{59}Fe^{2+}$ into heme. These and other studies suggest that ferrochelatase, the final enzyme in the heme pathway, may be the rate limiting step in Friend erythroleukemia cells (5,6).

The porphyrias represent inherited deficiencies of specific enzymes in the heme pathway (7-9). Figure 2 shows the heme pathway with a porphyria corresponding to each enzyme in the pathway, except ALA-synthetase. ALA-synthetase activity is elevated in the hepatic porphyrias, acute intermittent porphyria (AIP), variegate porphyria, hereditary coproporphyria and ALA-dehydratase deficiency. The individual enzymatic defect diminishes the supply of regulatory heme, increasing the activity

Figure 1. Diagrammatic representation of the enzymatic reactions occurring in the heme biosynthetic pathway.

FIGURE 1

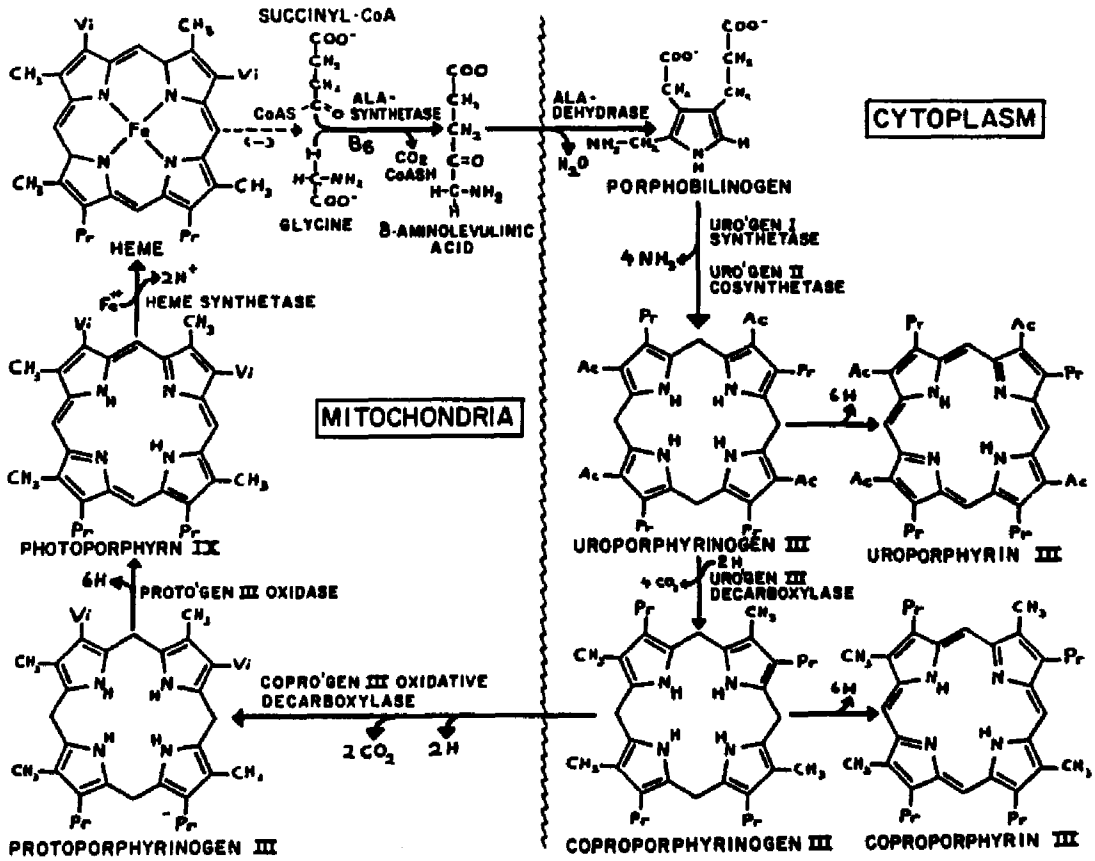
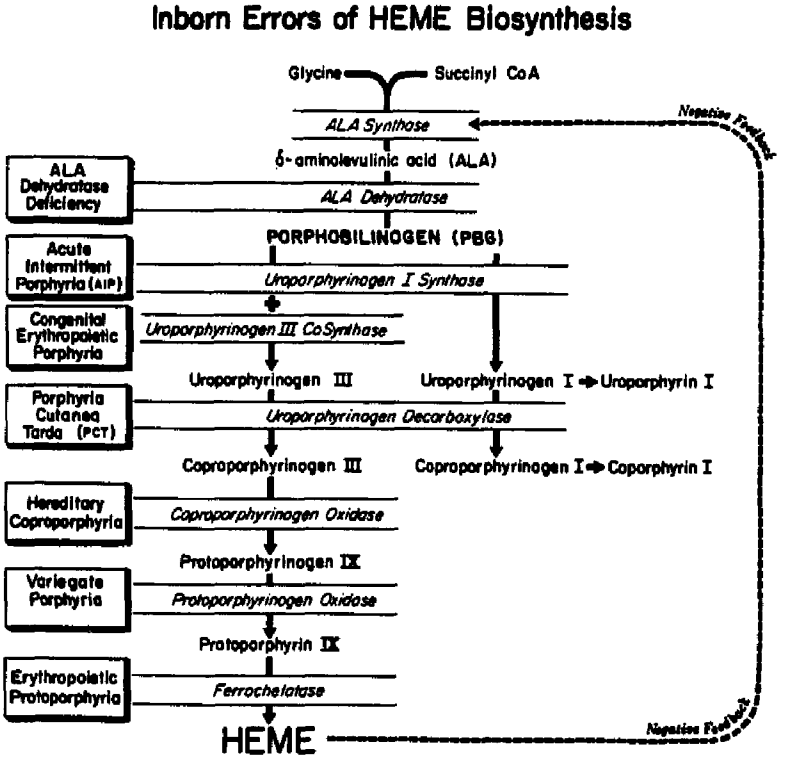

SUCCINYL-CoA
ALA-SYNTHETASE
B6
CoAS
CO2
CoASH
GLYCINE
δ-AMINOLEVULINIC ACID
ALA-DEHYDRASE
PORPHOBILINOGEN
CYTOPLASM
URO'GEN I SYNTHETASE
URO'GEN II COSYNTHETASE
UROPORPHYRINOGEN III
UROPORPHYRIN III
HEME
HEME SYNTHETASE
MITOCHONDRIA
PHOTOPORPHYRN IX
PROTO'GEN III OXIDASE
COPRO'GEN III OXIDATIVE DECARBOXYLASE
PROTOPORPHYRINOGEN III
COPROPORPHYRINOGEN III
COPROPORPHYRIN III

Figure 2. Inherited enzymatic defects in heme biosynthetic enzymes which result in porphyria.

Uroporphyrinogen I Synthase = Porphobilinogen Deaminase

FIGURE 2


Inborn Errors of HEME Biosynthesis
Glycine
Succinyl CoA
ALA Synthase
Negative Feedback
δ-aminolevulinic acid (ALA)
ALA Dehydratase Deficiency
ALA Dehydratase
PORPHOBILINOGEN (PBG)
Acute Intermittent Porphyria (AIP)
Uroporphyrinogen I Synthase
Congenital Erythropoietic Porphyria
Uroporphyrinogen III CoSynthase
Uroporphyrinogen III
Uroporphyrinogen I
Uroporphyrin I
Porphyria Cutanea Tarda (PCT)
Uroporphyrinogen Decarboxylase
Coproporphyrinogen III
Coproporphyrinogen I
Coporphyrin I
Hereditary Coproporphyria
Coproporphyrinogen Oxidase
Protoporphyrinogen IX
Variegate Porphyria
Protoporphyrinogen Oxidase
Protoporphyrin IX
Erythropoietic Protoporphyria
Ferrochelatase
HEME
Negative Feedback

of hepatic ALA-synthetase. In each of the hepatic porphyrias there is an autosomal dominant inheritance of the particular enzymatic deficiency, with the exception of ALA-dehydratase deficiency which is inherited as an autosomal recessive trait. They are also characterized by intermittent attacks of abdominal pain, autonomic dysfunction, neuropsychiatric symptoms and paresis. Porphyria cutanea tarda differs from the other hepatic porphyrias in that hepatic ALA-synthetase activity is not increased. The major symptom of the disease is cutaneous photosensitivity. Neurological dysfunction does not occur. In the erythropoietic porphyrias, congential erythropoietic porphyria and erythropoietic photoporphyria, increased erythrocyte porphyrins are responsible for the photosensitivity.

II. Heme Biosynthetic Enzymes:

A. δ-Aminolevulinic Acid Synthetase:

ALA-synthetase (E.C.2.3.1.37) catalyzes the formation of ALA from succinyl CoA and glycine in animal mitochondria (10,11). The enzyme purified from rat liver was shown to contain a sulfhydryl group (-SH) at the active site (12). The -SH group forms a thiohemiacetal with pyridoxal-5'-PO_4, the enzyme cofactor. It has been proposed that the bound pyridoxal-5'-PO_4 then forms a Schiff's base with the amino group of glycine, followed by the subsequent decarboxylation of the glycyl carboxyl group and addition of succinyl CoA (12).

Currently there are three means of detecting ALA-synthetase activity. The oldest and least sensitive procedure measures ALA by condensation with 2,4-pentanedione to form a pyrrole, followed by colorimetric determination with Erlich's reagent (13). This method is not specific

for ALA, since another compound, aminoacetone, formed by the condensation of glycine and acetyl CoA can be detected (14,15). Radiochemical methods based on [^{14}C]succinate incorporation into ALA have also been implemented (16-18), but these require multi-column steps in order to remove contaminants. A new assay for ALA-synthetase activity has been developed by Bishop et al. (19). This procedure involves the conversion of ALA to uroporphyrin I (URO I) in the presence of excess ALA-dehydratase and PBG-deaminase. This method is 10 times more sensitive than the colorimetric assay and comparable in sensitivity to the radiochemical procedure for determining ALA-synthetase. Moreover, this procedure is far more rapid that the radiochemical procedure.

ALA-synthetase is the rate limiting step for hepatic heme biosynthesis. Several lines of evidence support this. The half-life of the enzyme in mammalian liver is very short (60-70 mins) (20), as opposed to other mitochondrial enzymes which have half-lives of 3 to 5 days. The activity of this enzyme is low compared to other enzymes in the same pathway (21). ALA-synthetase is an inducible enzyme. Studies using guinea pig (22), rat liver (23-25) and cultured chick embryonic liver cells (11) have shown that certain drugs which cause increased porphyrin production in the liver accomplish this by increasing the level of ALA-synthetase. Using cultured chick embryonic liver cells, Whiting and Granick (26) demonstrated that the increases in ALA-synthetase activity following treatment with 2-allylisopropylacetamide (AIA) or 1,4-dihydro-3,5,dicarbethoxycollidine (DDC) could be accounted for by an increase in newly synthesized enzyme protein. Although Brooker et al. (27) have provided evidence for an increased level of ALA-synthetase specific mRNA following induction with AIA and DDC, the means by which this increase

occurs is different for each of these compounds. AIA is thought to induce ALA-synthetase by enhancing the destruction of heme by hepatic cytochrome P-450, thus depleting the supply of "free heme" (28). DDC has been shown to inhibit the activity of ferochelatase (29,30), resulting in a decreased amount of heme formed from protoporphyrin. A summary of the types of drugs which are capable of inducing ALA-synthetase as well as inducing attacks of porphyria is presented in Table 1.

Table 1

Some Drugs That May Induce Attacks of Prophyria (8)

Diphenylhydantoin	Ergot preparations
Barbiturates	Sulfonamides
Methyprylone	Dapsone
Meprobamate	Griseofulvin
Amidopyrene	Sulfonylureas
Glutethimide	Estrogens
Mesantoin	

ALA-synthetase is synthesized on cytoplasmic ribosomes and subsequently transported into the mitochondria. Granick (11) provided evidence for this by demonstrating that cycloheximide, an inhibitor of cytosolic protein synthesis could prevent ALA-synthetase induction, but chloramphenicol, an inhibitor of mitochondrial protein synthesis had no effect on ALA-synthetase induction. More direct studies done by using *in vitro* protein translation systems for both chick liver embryo (31) and rat liver messenger RNA (32) have shown that the cytosolic form of the enzme has a higher molecular weight than the mitochondrial form of the enzyme suggesting that ALA-synthetase is processed during its translocation from the cytoplasm to the mitochondria.

Certain developmental aspects regarding ALA-synthetase are noteworthy. Using inbred strains of mice Hutton and Gross (33,34) observed differences between various strains with respect to the basal level of hepatic ALA-synthetase activity and the ALA-synthetase activity following induction with AIA or DDC. Therefore genetic factors are responsible for mediating the levels of hepatic ALA-synthetase activity.

Woods and Dixon (35) observed that hepatic fetal guinea pig ALA-synthetase is uninducible *in vivo* by DDC, although the adult hepatic enzyme is. Bishop *et al.* (36) showed that ALA-synthetase found in erythroid tissues such as fetal liver and adult bone marrow differed from ALA-synthetase present in nonerythroid adult liver with respect to binding to AMP or CoA-agarose, K_m for glycine and inhibition by sodium chloride (NaCl). Fetal liver ALA-synthetase activity was at its peak between the 38^{th} through the 45^{th} day of gestation, and decreased substantially afterwards. The erythropoietic activity of fetal liver paralleled the ALA-synthetase activity. These data supported the existence of erythroid and non-erythroid forms of ALA-synthetase with different regulatory mechanisms.

B. ALA-Dehydratase:

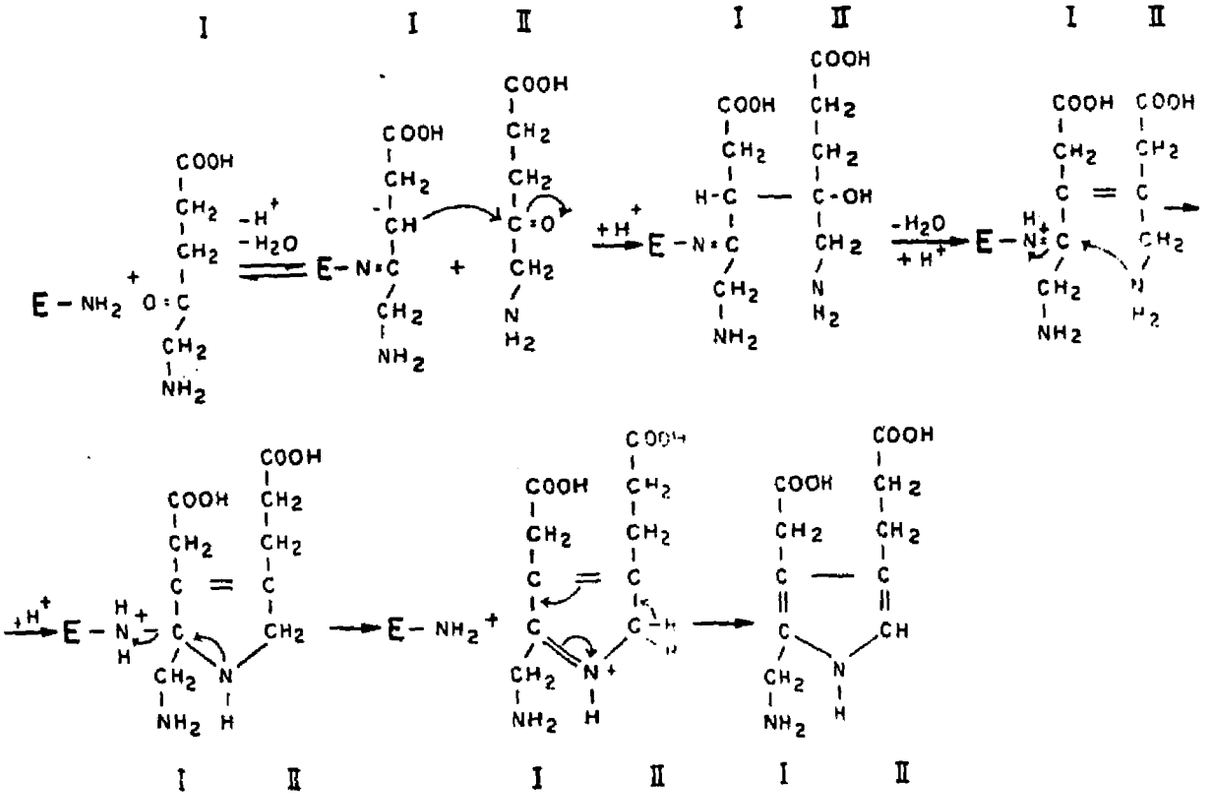
ALA-dehydratase (E.C.4.2.1.24) catalyzes the second reaction in the heme biosynthetic pathway in animals (37), the condensation of two moles of ALA to produce the monopyrrole, porphobilinogen (PBG). Elegant studies by Nandi and Shemin (38) using ALA-dehydratase obtained from *Rhodopseudomonas spheriodes* have shown that ALA forms a Schiff's base with ALA-dehydratase. Based on this observation a reaction mechanism (Fig. 3) has been proposed in which the first molecule of ALA forms

a Schiff's base with ALA-dehydratase which gives rise to a stabilized carbananion, which makes a nucleophilic attack on the second molecule of ALA. The resulting aldol loses water, and the free amino group of the second ALA molecule displaces the amino group of the enzyme by transamination, thus forming PBG. According to this mechanism, the ALA which gives rise to the acetic side of the product binds initially to the enzyme through a Schiff's base linkage. Jordan and Seehra have described a reaction mechanism by performing [^{14}C] labelling studies on purified bovine liver enzyme. These studies demonstrated that the ALA which first binds to the enzyme, and is responsible for forming the Schiff's base linkage, is the one which gives rise to the propionic acid side chain of PBG. Independent studies using ALA-dehydratase from both Rhodopseudomenas spheriodes (38) and bovine liver (39) have also shown that four of the eight R. spheriodes subunits react with the substrate to form two Schiff's bases, while eight functional catalytic sites were noted for the bovine liver octamer.

Most assays for ALA-dehydratase activity have been based on the Watson-Schwartz reaction (40), namely the condensation of PBG with Ehrlich's reagent, p-dimethylaminobenzaldehyde (DMAB), in acid solution to form a red compound (p-N,N-dimethylpyrryltoluidine). Mauzerall and Granick (41) modified this procedure by using Erlich's reagent with 2N perchloric acid (PCA) to increase the stability and intensity of the colored product. However, even with the more sensitive semi-micro colorimetric method (42), it is not possible to accurately measure ALA-dehydratase activity in cultured cells which have low specific activities or in small amounts of tissue homogenates.

Figure 3. Outline of the mechanism of PBG synthesis from two molecules of ALA as catalyzed by ALA-dehydratase from Rhodopseudumonas spheriodes (38).

FIGURE 3



ALA-dehydratase has been purified to homogeneity from murine (43-45) and bovine liver (46-48), as well as guinea pig erythrocytes (49). Anderson and Desnick have purified human erythrocyte ALA-dehydratase to homogeneity by using a series of hydrophobic and chromatographic techniques (50). Both the purified human erythrocytic and bovine hepatic enzymes were shown to be homo-octamers with subunit molecular weights of 31,000 and 35,500, respectively (50,51-53). In addition, the human and bovine enzymes contained pyridoxal-5'-phosphate and required Zn^{2+} for optimal activity (54,55).

Thiol groups are essential for ALA-dehydratase activity (53). Indeed, ALA-dehydratase is one of the first enzymes affected in lead toxicity (56-58). The reversal of ALA-dehydratase inhibition by the addition of thiol reagents indicates that lead inactivates essential thiol groups necessary for the conversion of ALA to PBG. Succinylacetone has been found to be a potent inhibitor of ALA-dehydratase (59). This compound accumulates in patients affected with hereditary tyrosinemia, and is responsible for causing a severe deficiency of liver and erythrocyte ALA-dehydratase. As a result of ALA-dehydratase inhibition, these patients excrete large quantities of ALA in their urine. Succinylacetone has also been shown to inhibit growth of murine erythroleukemia cells (60). The mechanism for this occurrence involves an inhibition of ALA-dehydratase which results in a lowered intracellular heme concentration. Low intracellular heme concentration impairs both cellular respiration and protein synthesis. Studies by Tschudy et al. have demonstrated that the inhibition of ALA-dehydratase by succinylacetone is irreversible and that succinylacetone binds at a site on the enzyme which normally forms a Schiff's base between the ALA and a lysine residue at the active site

of the enzyme (61). Recently a specific inhibitor to ALA-dehydratase in rat bone marrow cells has been reported (62). This inhibitor was sensitive to both heat and trypsin and had a molecular weight of 28,000. It inhibited the activity of ALA-dehydratase noncompetitively.

Using Sepharose-immobilized bovine ALA-dehydratase, Gurne et al. (63) demonstrated that four subunits per octamer can be removed by treatment with 4M urea leaving a catalytically active, immobilized tetrameric protein. The immobilized tetramer could be reassembled into an octameric structure with the same initial enzymatic activity by exposing the residual bound protein to a soluble pure enzyme preparation or to a crude liver extract in the presence of urea. Battle and co-workers (64), using Sepharose-immobilized bovine ALA-dehydratase, demonstrated its dissociation into catalytically active tetramers and dimers in the presence of 3M and 6M urea, respectively.

Battistuzzi and co-workers (65,66) have recently described a polymorphism for ALA-dehydratase occurring in the Italian population. Three distinct regions of ALA-dehydratase activity were observed when hemolysates obtained from 960 individuals were subjected to starch gel electrophoresis. Three forms of ALA-dehydratase were detected: a fast activity band (more electronegative); a slow band, and an intermediate band of activity. The majority of individuals (80%) had the slow anodal band of ALA-dehydratase activity referred to as the ALA-dehydratase 1-1 phenotype. The remainder of the individuals either had an intermediate band of ALA-dehydratase activity (18%) or a fast activity band (2%). These phenotypes were referred to as the ALA-dehydratase 2-1 and 2-2 phenotypes, respectively. Individuals with different ALA-dehydratase phenotypes had normal levels of ALA-dehydratase activity. The pheno-

types observed were due to the occurrence of two codominant alleles at the autosomal locus, ALA-dehydratase. Hence, the ALA-dehydratase 1-1 and ALA-dehydratase 2-2 phenotypes or isozymes are due to homozygosity for the ALA-dehydratase^1 and ALA-dehydratase^2 alleles, respectively, while the ALA-dehydratase 2-1 isozyme represents the heterozygous combination of the ALA-dehydratase^1 and ALA-dehydratase^2 alleles.

Bird et al. first described a family with an inherited deficiency of erythrocyte ALA-dehydratase (67). Individuals with this deficiency had from 22%-41% of normal mean erythrocytic ALA-dehydratase activity, and had no symptoms of porphyria. Following this observation, Doss et al. described two patients with 1% of normal mean erythrocytic ALA-dehydratase activity (68,69). The affected individuals had been excreting large quantities of ALA, PBG, coproporphyrin and protoporphyrin in their urine for several years. Both patients had acute hepatic porphyria and paralysis. Relatives of affected individuals had about 50% of normal mean erythrocytic ALA-dehydratase activity and displayed no clinical symptoms. The latter individuals are analogous to those which Bird described and presumably represent heterozygotes possessing a normal and mutant allele for ALA-dehydratase. The affected individuals described by Doss et al. represent individuals homozygous for the mutant ALA-dehydratase allele.

ALA-dehydratase activity in mice is under genetic control. Russell and Coleman (70) assayed liver extracts for ALA-dehydratase activity and found strains with high and low levels of ALA-dehydratase activity. The F_1 progeny obtained after crossing a high activity strain with a low activity strain had intermediate ALA-dehydratase activity. On the basis of these and other genetic studies it was concluded that levels of ALA-

dehydratase activity are under the control of a single genetic locus. Lv^a was designated as the allele associated with high enzymatic activity and Lv^b was designated as the allele associated with low enzymatic activity. Hence, the intermediate ALA-dehydratase levels observed in the F_1 progeny resulted from the Lv^aLv^b genotype. The Lv locus has been mapped to mouse chromosome 4 and is presumably the structural gene for this enzyme (71). Studies of ALA-dehydratase from Lv^a and Lv^b homozygous mice did not reveal different physical or chemical properties including heat stability, sedimentation coefficient, electrophoretic mobility and K_m (44-46). Combined isotopic labelling and immunochemical studies have shown that Lv^a mice had higher rates of ALA-dehydratase synthesis than Lv^b mice, and that the rate of ALA-dehydratase degradation in the two different strains was identical (45).

ALA-dehydratase activity in Lv^a and Lv^b mice had characteristic changes in developing liver (72). The specific activity was high in fetal liver, decreased prior to birth and increased to adult levels during the first three weeks following birth. The fetal enzyme was different from the adult enzyme in the following respects. The fetal enzyme was more heat labile and more stable to trypsin digestion than the adult enzyme (45). The fetal enzyme was also twice as catalytically active as the adult enzyme for a given amount of enzyme protein (45). Attempts to determine differences in the primary structure of the fetal and adult forms of ALA-dehydratase have proven unsuccessful (73). Radioactive labelling studies have demonstrated that the fetal and adult enzymes bind the same number of substrate molecules, namely six molecules of ALA per 250,000 molecular weight homo-hexamer. Tryptic digests of immunoprecipitated ALA-dehydratase from fetal and adult mice

resulted in identical profiles. However, this method is not sensitive enough to detect subtle differences in primary amino acid sequence. Further studies are required to explain the reasons for the greater catalytic efficiency of the fetal enzyme.

ALA-dehydratase activity in humans is also under genetic control (74). In normal erythrocytes, there is a four-fold variation in the levels of ALA-dehydratase activity.

ALA-dehydratase activity is markedly increased in mice and humans with hemolytic anemias (72,75). Moreover, when human erythrocytes were fractionated by specific gravity, the ALA-dehydratase activities were higher in the more buoyant, i.e., younger cells (75). In hemolytic anemias such as sickle-cell anemia there are higher proportions of younger erythrocytes. Hence, the increased erythrocyte ALA-dehydratase activity in these cases is reflective of the higher proportion of young erythrocytes.

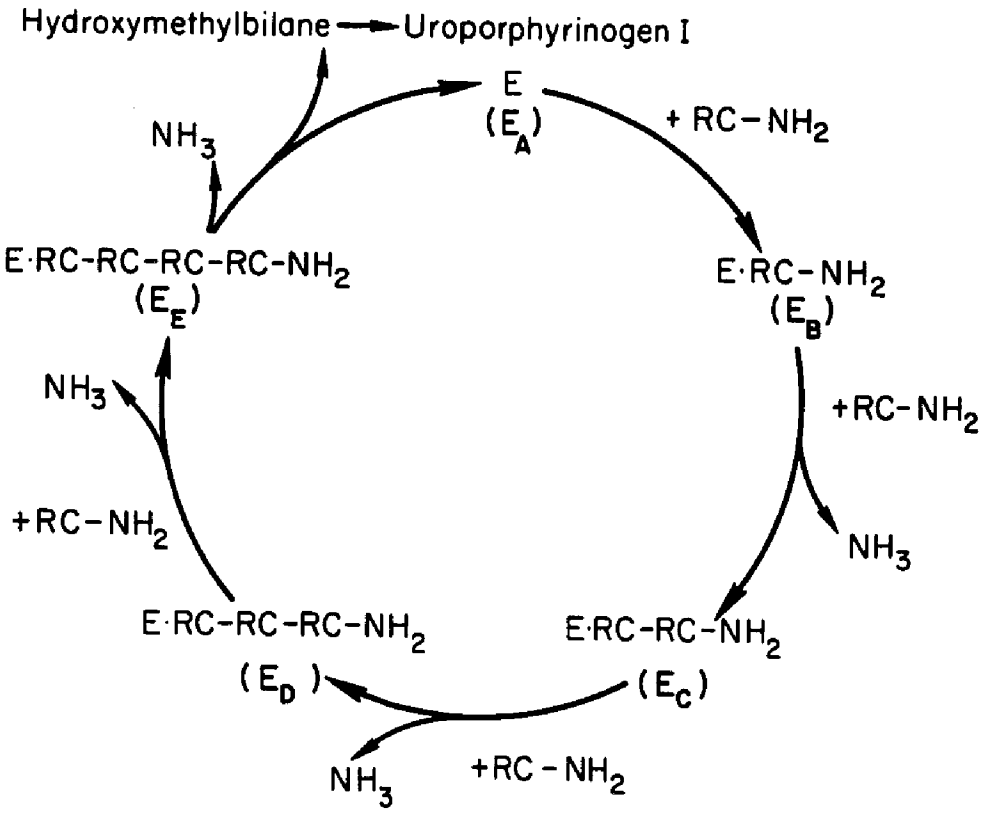
C. PBG-Deaminase:

PBG-Deaminase (E.C.4.3.1.8) is the third enzyme in the heme biosynthetic pathway (76). The enzyme sequentially deaminates four moles of the monopyrrole, porphobilinogen, to form the linear tetrapyrrole hydroxymethylbilane ($HOCH_2$bilane) which is then nonenzymatically cyclized to uroporphyrinogen I (77). In the presence of uroporphyrinogen III cosynthase (UROCoS), $HOCH_2$bilane is rapidly converted to the uroporphyrinogen III isomer, the precursor of heme, cobalamins and cytochromes (78,79). This reaction scheme is shown in Figure 3.

PBG-deaminase has been purified to homogenetiy from R. spheriodes (80,81) and spinach (82). The bacterial and plant enzymes are monomers

Figure 4. Current concept of tetrapyrrole biosynthesis. PBG-deaminase (E) catalyzes the sequential head to tail condensation of four molecules of PBG ($RC-NH_2$) to form the linear tetrapyrrole, $HOCH_2$bilane. E_A represents the native enzyme, and E_B, E_C, E_D and E_E the mono-, di- tri-, and tetrapyrrole-enzyme intermediates, respectively (80).

FIGURE 4


Hydroxymethylbilane
Uroporphyrinogen I
E
(E_A)
+ RC–NH2
E·RC–NH2
(E_B)
+RC–NH2
NH3
E·RC–RC–NH2
(E_C)
+RC – NH2
NH3
E·RC–RC–RC–NH2
(E_D)
+RC–NH2
NH3
E·RC–RC–RC–RC–NH2
(E_E)
NH3

with molecular weights of approximately 40,000. A 330-fold purification was obtained following purification of PBG-deaminase from bovine liver (83). Anderson and Desnick purified PBG-deaminase from human erythrocytes to homogeneity by a series of conventional and hydrophobic chromatographic techniques (84). Human PBG-deaminase is a monomer with a molecular weight of 37,000. DEAE-cellulose chromatography of the purified enzyme resolved the activity into five forms, designated A, B, C, D and E. Interconversion studies and experiments with [^{3}H]PBG demonstrated that the five forms were stable enzyme-substrate intermediates in the sequential condensation of four PBG molecules into the tetrapyrrole, uroporphyrinogen I. Independently, Battersby and co-workers demonstrated by using [^{13}C]-nuclear magnetic resonance studies (77) that PBG-deaminase was responsible for the stepwise, head to tail assembly of four PBG molecules to form $HOCH_2$bilane, which was nonenzymatically cyclized to form uroporphyrinogen I isomer, or converted into uroporphyrinogen III, in the presence of UROCoS.

PBG-deaminase activity can be determined by a sensitive fluorometric assay (85). $HOCH_2$bilane formed from PBG in the presence of PBG-deaminase is nonenzymatically converted to uroporphyrinogen I, which is oxidized to uroporphyrin I (URO I) in the presence of an acidic environment and ultraviolet light.

The purification of large quantities of ALA-dehydratase has made the production of milligram quantities of PBG feasible. According to the procedure developed by Anderson and Desnick (50), purified human ALA-dehydratase was immobilized on a phenyl-Sepharose CL-4B column. When ALA was pumped through the column PBG was produced. PBG was then separated from unreacted ALA by passing the Sepharose eluate through a

Dowex 1-X8 anion exchange resin.

The half-normal activity of PBG-deaminase is the primary enzymatic defect in acute intermittent porphyria (AIP) (86). During acute attacks of this dominantly inherited disease, hepatic ALA-synthase is induced; ALA and PBG accumulate because of the deficient PBG-deaminase activity, and inadequate amounts of heme are produced to retain the negative feedback control on ALA-synthase in the liver (9). The enzymatic deficiency has been demonstrated in erythrocytes (85,87-89), liver (86), cultured skin fibroblasts (90,91) and amniotic cells (91). PBG-deaminase from AIP heterozygotes has been shown to be identical to normal PBG-deaminase with respect to electrophoretic mobility (87), K_m value (87), and heat denaturation profile (85). Furthermore, mitogen-stimulated lymphocytes from AIP heterozygotes induced only 50% of the activity detected in mitogen-treated lymphocytes from normal individuals (92). Although the above are consistent with a structural gene mutation in AIP, the demonstration of cross-reacting immunologic material (CRIM) in AIP erythrocyte lysates by Anderson and Desnick (93) provides the first molecular evidence for a structural gene mutation as the primary defect in AIP. It is noteworthy that the erythrocyte assay for PBG-deaminase is not the sole criterion for determining AIP status. Mustajoki (94) reported a Finnish kindred in which all affected individuals had normal erythrocyte PBG-deaminase levels. The diagnosis of AIP was made in these members because of the urinary excretion of large amounts of ALA and PBG.

The structural gene for human PBG-deaminase has been localized to the narrow chromosomal region 11q23 → 11qter using hybrids derived from human fibroblasts and mouse RAG, LM/TK or A9 cells (95,96) and hybrids derived from human Lesch-Nyhan fibroblasts (HLN) and tetraploid Friend

murine erythroleukemia (2S MEL) cells (97). Isoelectric focusing and/or rocket immunoelectrophoresis was used to distinguish between the mouse and human PBG-deaminase. The details of these studies are the subject of the dissertation.

Using isoelectric focusing Meisler and Carter (98) have detected structural variants of PBG-deaminase in both the murine and human population. The enzymatic activity of murine and human erythrocytic PBG-deaminase has been demonstrated to be elevated in younger erythrocytes (72,75). As a consequence of this, the PBG-deaminase activity is increased in cases of hemolytic anemia.

D. Uroporphyrinogen III-CoSynthetase:

Bogorad (99) described an enzyme system consisting of PBG-deaminase and a heat labile fraction referred to as uroporphyrinogen isomerase which was capable of forming uroporphyrinogen III from PBG. As mentioned previously, Battersby et al. (77) and Burton et al. (100) showed that PBG-deaminase catalyzes the formation of a short-lived ($t_{1/2}$ = 4 min at 37°C, pH 5.2) enzyme free intermediate, $HOCH_2$bilane. In the absence of UROCoS, $HOCH_2$bilane nonenzymatically cyclizes to form uroporphyrinogen I. UROCoS is capable of converting $HOCH_2$bilane to uroporphyrinogen III, independent of PBG-deaminase in vitro but this does not exclude the possibility that PBG-deaminase and UROCoS may function as a complex in vivo to form uroporphyrinogen III.

UROCoS can be assayed by determining the ratio of uroporphyrin III to URO I formed, since only the latter is formed in the absence of UROCoS. Separation of the isomers has been achieved by esterification of the uroporphyrins, followed by decarboxylation to coproporphyrin iso-

mers, which are then separated and quantified as methyl esters by thin layer chromatography or high pressure liquid chromatography (101,102).

Jordan and co-workers (103) have developed a rapid fluorometric assay for UROCoS. This assay procedure is performed by incubation of the sample with UROS and PBG; the reaction is terminated by freezing the mixture in a dry ice-acetone slurry. With subsequent addition of benzoquinone, the uroporphyrinogen formed is oxidized to URO. The URO may be detected fluorometrically. This assay takes advantage of the fact that UROCoS catalyzes the formation of uroporphyrinogen III at a markedly faster rate than $HOCH_2$bilane cyclizes to form uroporphyrinogen I. The relative rapidity of this assay compared to previously reported methods should facilitate the purification of UROCoS from human erythrocytes.

Frydman and Feinstein (104) partially purified UROCoS from outdated human erythrocytes using DEAE-cellulose chromatography followed by ammonium sulfate fractionation. Although complete separation of PBG-deaminase and UROCoS was not achieved by the DEAE-cellulose chromatographic step, they did separate a small amount of UROCoS activity. The UROCoS was further purified by precipitation with 0-80% ammonium sulfate. The partially purified UROCoS was found to be irreversibly inhibited by -SH reagents, such as N-ethylmaleimide. Furthermore, they were able to demonstrate an association between PBG-deaminase and UROCoS by immobilizing the former on Sepharose, followed by filtration of UROCoS through the column. When PBG was passed through the column, uroporphyrinogen III was formed, demonstrating that PBG-deaminase and UROCoS associate in the absence of substrate to generate a uroporphyrinogen III forming system. In support of this concept, Higuchi and Bogorad (105) provided evidence for an association between purified

wheat germ PBG-deaminase and UROCoS by demonstrating that PBG-deaminase sediments more rapidly in the presence of UROCoS, than in the absence of UROCoS, in a sucrose density gradient.

Congential erythropoietic protoporphyria is an autosomal recessive disease due to deficient UROCoS activity (106-108). The clinical symptoms, which include photosensitivity, erythrodontia, hemolytic anemia and porphyrinuria, can be explained by the systemic deposition of uroporphyrin I and coproporphyrin I, which are the spontaneous oxidation products of uroporphyrinogen I and its decarboxylated derivative, coproporphyrinogen I.

E. Uroporphyrinogen Decarboxylase:

Uroporphyrinogen decarboxylase (URO-decarboxylase; E.C.4.1.1.37) is a cytosolic enzyme which converts uroporphyrinogen III to coproporphyrinogen III. This reaction occurs by a series of stepwise decarboxylations such that the intermediates are hepta-, hexa-, and pentacarboxylic porphyrins (109). Kardish and Woods (110) have developed a novel assay procedure for this enzyme. The substrate, uroporphyrinogen, was produced by incubation of PBG with PBG-deaminase in the assay tube under anaerobic conditions. The enzyme source was then added to the tube and the amount of coproporphyrin produced was quantitated spectrophotometrically. The enzyme activity was found to be inhibited by -SH reagents.

Porphyria cutanea tarda, the most prevalent form of porphyria is a syndrome in which cutaneous lesions are caused by the deposition of porphyrins in the skin in combination with exposure to sunlight (111). The familial form of porphyria cutanea tarda is characterized by an

autosomal dominant mode of transmission of reduced URO-decarboxylase activity (112-114). This deficiency has been found in erythrocytes and hepatocytes. Sporadic porphyria cutanea tarda is associated with a decreased hepatic URO-decarboxylase activity and normal erythrocyte URO-decarboxylase activity and is often found in alcoholics (115-117).

F. Coproporphyrinogen Oxidase:

Coproporphyrinogen oxidase (E.C.1.3.3.3) is a mitochondrial enzyme which converts coproporphyrinogen III into protoporphyrinogen IX. The enzyme has been purified 3200-fold from bovine liver (118). The enzyme had a molecular weight of 71,600 and showed a two to five-fold stimulation in the presence of mitochondrial phospholipids and commercially available phospholipids. Tyrosine has been shown to be involved in the active site of the enzyme (119), presumably in the stepwise formation of a β-hydroxypropionate porphyrinogen intermediate from the propionate side chains at positions 2 (of the upper left coproporphyrinogen III ring, Fig. 2) and 4 (of the upper right ring). The β-hydroxypropionate group on the 2 position is oxidized to a vinyl group, followed by conversion of the propionate group in the 4 position to a β-hydroxypropionate group with subsequent oxidation to a vinyl group. Kardish and Woods (120) have developed a coupled enzymatic assay procedure for coproporphyrinogen oxidase in which coproporphyrinogen III was synthesized from PBG by the sequential addition of PBG-deaminase and UROCoS followed by the addition of URO-decarboxylase. The sample to be assayed for coproporphyrinogen oxidase activity was then added and the reaction was allowed to proceed in the presence of oxygen and glutathione, without light. The coproporphyrinogen oxidase activity was then

expressed as a function of the coproporphyrinogen utilized.

Hereditary coproporphyria is an autosomal dominant disorder due to half normal levels of coproporphyrinogen oxidase (121). This deficiency has been detected in fibroblasts and leukocytes. Attacks of porphyria which are indistinguishable from those in AIP occur, precipitated mainly by the intake of barbituates and other drugs (9). The disorder is characterized biochemically by the excretion of large ammounts of coproporphyrin in the feces, and during acute attacks PBG is excreted in the urine.

G. Protoporphyrinogen Oxidase:

The oxidation of protoporphyrinogen IX to protoporphyrin IX is carried out by the mitochondrial enzyme, protoporphyrinogen oxidase. The enzyme, purified from rat liver mitochondria (122), had a molecular weight of 35,000 and was heat labile. Low concentrations of thiol reducing agents increased enzymatic activity. Brenner and Bloomer (123) have developed a fluorometric assay procedure for this enzyme. The substrate, protoporphyrinogen, was prepared by reduction of protoporphyrin with sodium amalgam. The sample to be assayed was added to the substrate and incubated at 37°C in the dark under reducing conditions. Nonenzymatically formed protoporphyrin was taken into account by heating an identical amount of sample at 75°C for 15 min prior to assay. Protoporphyrin could be measured in both samples fluorometrically and the enzymatically generated protoporphyrin represented the difference between the untreated sample and the heat treated sample. This assay procedure has been used for rat liver mitochondria and cultured skin fibroblasts.

Half normal activity of protoporphyrinogen oxidase is the enzymatic defect in variegate porphyria (124). Affected individuals may have both neuropsychiatric symptoms and skin lesions (125). A family study done by Deybach et al. (126) demonstrated the autosomal dominant inheritance of this enzyme deficiency. Variegate porphyria has a high prevalence (1 in 300) in the Afrikaner population of South Africa (127). This disease is also an example of the "founder effect". Dean and co-workers (127) demonstrated that 10,000 white South Africans alive at the time of their study inherited the gene for variegate porphyria from a common ancestor.

H. Ferrochelatase:

Ferrochelatase (protoheme ferrolyase, E.C.4.99.1.1) catalyzes the final step in heme biosynthesis, namely the insertion of Fe^{2+} into the protoporphyrin ring. Ferrochelatase activity is determined by measuring $^{59}Fe^{2+}$ incorporation into protoporphyrin or deuteroporphyrin (128). The enzyme has been purified 628-fold from rat liver mitochondria (129). The enzyme bound avidly to blue Sepharose and was eluted with high concentrations of sodium cholate with 1.5 M NaCl. This was accounted for by the high percentage of hydrophobic amino acids found following amino acid analysis. The purified enzyme was markedly stimulated by the addition of exogenous fatty acids.

Erythropoietic protoporphyria is characterized clinically by photosensitivity and biochemically by marked increases of protoporphyrin IX in erythroid cells, plasma and feces (9). A 50 percent decrease in the amount of this enzyme has been observed in bone marrow (130), nucleated peripheral blood cells (131), liver (132), cultured skin fibroblasts

(132) and mitogen stimulated lymphocytes (133) from patients with erythropoietic protoporphyria. This deficiency is inherited as an autosomal dominant trait. Bovine protoporphyria, a disease in cattle with the same clinical manifestations as erythropoietic protoporphyria is due to the deficiency of ferrochelatase which is transmitted as an autosomal recessive trait (134).

Ferrochelatase has been implicated as the rate limiting enzyme for heme biosynthesis in Friend erythroleukemia cells (135). The enzyme was not detected in uninduced cells, and in the presence of inducing compounds the induction of heme synthesis was observed to follow the same time course as the induction of ferrochelatase activity.

III. Somatic Cell Hybridization and 2S MEL-HLN Hybrids:

Somatic cell hybridization has been widely used for the localization of genes to specific chromosomes (136). In human-rodent hybrids, random human chromosomal loss occurs, generating hybrids with different human chromosomal constitutions. A gene may be localized to a particular chromosome by correlation of the presence or absence of a given human gene product in a hybrid clone with the presence or absence of a specific human chromosome. Furthermore, it is also possible to localize a given human gene to a particular region of a human chromosome by assessing the presence or absence of the human gene product in a hybrid line with different structural rearrangements of the human chromosome (e.g., a deletion or translocation of the specified chromosome).

This procedure was used to determine the chromosomal localization of the gene for human PBG-deaminase. Because the specific activity of PBG-deaminase in human fibroblasts was about one-fifth that of human

erythrocytes (93), the expression of PBG-deaminase in tetraploid Friend murine erythroleukemia (2S MEL)-human Lesch-Nyhan fibroblast (HLN) hybrid lines was examined. These hybrids previously were shown to selectively activate the synthesis of human β-globin but not δ-globin mRNA after DMSO induction of hemoglobin production (138). Tetraploid MEL-human fibroblast hybrids were chosen for these studies because the efficiency of hemoglobin inducibility was greatly increased in 2S MEL-human hybrids compared to that in 1S hybrids (138). Furthermore, the induced 2S MEL-human fibroblast hybrids expressed significantly greater amounts of PBG-deaminase than did uninduced 2S MEL cells or human fibroblasts.

IV. Objectives:

It was the purpose of these studies to examine biochemical and genetic properties of the human heme biosynthetic enzymes, ALA-dehydratase and PBG-deaminase. Previous work in this laboratory with respect to these enzymes dealt with the development of procedures for their purification (50,84). The production of highly purified ALA-dehydratase and PBG-deaminase enabled anti-sera to be raised against these proteins which facilitated the following genetic and biochemical studies:

a) Studies of the expression of human PBG-deaminase in 2S MEL-human Lesch-Nyhan fibroblast hybrids and determination of the chromosomal localization of the human gene for PBG-deaminase.

b) Studies of the expression of ALA-dehydratase in RAG-human fibroblast hybrids.

c) Development of a sensitive enzyme assay for ALA-dehydratase in order to facilitate the determination of enzymatic activity in tissues with low levels of activity or when limiting amounts of sample are available.

d) Studies of ALA-dehydratase electrophoretic variants and of ALA-dehydratase deficiency.

MATERIALS

The materials used for these studies were obtained from the following sources.

Item	Company	Location
Amicon Ultrafilters	Amicon Corp.	Lexington, MA
Hollow fiber cartridges	" "	" "
Acrylamide	Bio Rad	Bethesda, MD
Bis acrylamide	" "	" "
Sonifier 200	Branson Sonic Power	Danbury, CT
High purity acetonitrile	Burdick & Jackson Labs	Muskegan, MO
Peroxidase conjugated goat anti-rabbit IgG	Cappel Laboratories	Cochranville, PA
Starch (hydrolyzed)	Connaught Laboratories	Ontario, Canada
Polaroid Type 58 Film	Eastman Kodak Co.	Rochester, NY
DMAB	" " "	" "
TCA	Fisher Scientific Co.	Springfield, NJ
PCA	" " "	" "
Glacial Acetic Acid	" " "	" "
Sodium phosphate	" " "	" "
Tris base	" " "	" "
High resolution Tris/barbital buffer	Gelman Instrument Co.	Ann Arbor, MI
RPMI 1640 media	GIBCO	Grand Island, NY
Fetal bovine serum	"	" " "
Penicillin	"	" " "
Streptomycin	"	" " "
Dulbecco's phosphate buffered saline	"	" " "
Cellogel	Kalex Scientific	Manhasset, NY
Ampholytes (3-10)	LKB	Hicksville, NY

Item	Company	Location
Agarose IEF	Pharmacia	Piscataway, NJ
Gelbond film	"	"
Octyl-Sepharose CC-4B	"	"
Phenyl-Sepharose CC-4B	"	"
Sephadex-G100	"	"
Ampholytes (4-6.5)	"	"
Fluorescamine	Pierce Chemical Co.	Rockford, IL
Female New Zeland rabbits	Pocono Rabbit Farms	Canadensis, PA
Uroporphyrin Standards	Porphyrin Products	Logan, UT
DEAE-cellulose (DE-52)	H. Reeve Angel & Co.	Clifton, NJ
Enzyme grade ammonium sulfate	Schwartz/Mann	Orangeburg, NY
δ-aminolevulinic acid hydrochloride	Sigma	St. Louis, MO
DL-dithiothreiotol	"	"
DL-dithioerythritol	"	"
Dimethyl Sulfoxide	"	"
Triton X-100	"	"
Benzidine dihydrochloride	"	"

All other chemicals were of the highest grade commercially available.

2S MEL-HLN fibroblast hybrids were obtained from Dr. W.F. Anderson at the National Institutes of Health, Bethesda, Maryland. The 2S MEL-HX/11 hybrids used for the PBG-deaminase regional mapping were obtained from L. Sanders-Haigh at the National Institutes of Health, Bethesda, Maryland. Cultured rat hepatocyte lysates were obtained from Dr. Philip S. Guzelian at the Medical College of Virginia.

The Ascaris cuticle collegen affinity column was a gift from Dr. Norberto A. Guzman, Department of Orthopaedics, Mt. Sinai School of Medicine, New York.

ALA-dehydratase deficient erythrocytes were provided by Dr. T.D. Bird at the University of Washington School of Medicine, Seattle. Sprague-Dawley rat livers were a gift from Dr. Diana Beattie, Mt. Sinai School of Medicine, New York. Outdated human erythrocytes were obtained from the Greater New York Blood Center, New York.

METHODS

I. Assay Procedures:

A. Standard ALA-Dehydratase Colorimetric Assay:

The standard colorimetric assay was based on the methods of Doyle and Schimke (44) and Anderson and Desnick (50). Enzyme was diluted with 2.0 mg/ml of bovine serum albumin (BSA)in 0.25 M sodium phosphate buffer, pH 6.8, and was preincubated with 5.0 mM DTT for 15 min at 37°C prior to incubation with substrate. The reaction mixture consisted of the following in a total volume of 0.5 ml: 0.25 ml of 5.0 mM ALA in distilled water (pH 3.0); 0.1 ml of 0.25 M sodium phosphate buffer, pH 6.8; 0.05 ml of 20 mM DTT (freshly prepared) and of DTT-preincubated ALA-dehydratase. After 30 min at 37°C, the reaction was terminated with 0.5 ml of 10% TCA, containing 0.1 M $HgCl_2$. After centrifugation at 800 g for 10 min, 0.5 ml of the supernatant was mixed with an equal volume of freshly prepared 2 N modified Ehrlich's reagent [21 parts of 2.38% (w/v) DMAB in glacial acetic acid plus 4 parts of 70% PCA (41)]. The absorbance was measured at 555 nm after 15 min at room temperature in a Gilford Model 2000 spectrophotometer. The amount of PBG detected was calculated from a molar absorptivity of 5.8×10^4 M^{-1} cm^{-1} determined under the conditions of the assay for pure PBG acetate. One unit (U) of ALA-dehydratase activity was that amount of enzyme which catalyzed the formation of 1 nmol of PBG per h at 37°C.

B. Semi-Micro Colorimetric Assay for ALA-Dehydratase:

The semi-micro colorimetric assay for ALA-dehydratase was performed according to the method of Sasa et al. (42).

C. ALA-Dehydratase Coupled-Enzyme Assay (19):

Enzyme sources were preincubated at 37°C for 15 min in the presence of 5 mM DTT and 0.25 M sodium phosphate buffer, pH 6.7, in a total volume of 0.55 ml. Then, the tubes were placed in an ice bath and 3.8 units of purified PBG-deaminase and 0.3 ml of 5 mM ALA were added to obtain a final volume of 0.9 ml. The reaction was initiated by transfer of the sample to a 37°C water bath. After 90 min the reaction was terminated by the addition of 0.1 ml of 50% TCA and was then centrifuged at 800 g for 10 min. The supernatant was removed, photo-oxidized to URO I and then quantitated as described above in the PBG-deaminase assay. The pmol of URO I produced were multiplied by 4 to convert to pmol of PBG formed and then divided by 0.47 to correct for the 47% conversion of PBG to URO I* at equilibrium by the enzyme couple (see Results). One unit (U) of ALA-dehydratase activity was that amount of enzyme required to form 1 nmol of PBG per h at 37°C.

D. PBG-Deaminase Assay:

PBG-deaminase activity was determined by the method of Anderson and Desnick (84). The standard reaction mixture contained 0.05 ml of appropriately diluted sample, 0.5 ml of 0.1 M Tris-HCl buffer, pH 8.1, and 0.2 ml of 0.5 mM PBG. After incubation in the dark at 37°C for 30 min, the reaction was terminated by the addition of 0.25 ml of 50% TCA (w/v) and, if necessary, centrifuged at 800 g for 10 min. The supernatant was transferred to a 10 x 75 mm test tube and the uroporphyrinogen I in the supernatant was oxidized to URO I by exposure to long wave UV light for 15 min (Model XX15, Ultraviolet Products, Inc., San Gabriel, CA). The URO I was quantitated in a Turner Model 111 fluo-

rometer (standard photomultiplier tube) using Corning 7-51/Wratten 2C primary filters (405 mm) and a Wratten 25 secondary filter (540 mm). Disposable culture tubes (10 x 75 mm) were used as cuvettes with a Turner adapting sleeve for 1.0 ml samples. One U of PBG-deaminase was that amount of enzyme required to form 1 nmol of URO I per h at 37°C.

E. URO I Standard Curve:

URO I was dissolved in 1.0 N HCl to a concentration of 0.5 µg/ml. The URO I concentration also was determined by its absorbance at 406 nm using a molar absorptivity of 5.05×10^5 M^{-1} cm^{-1} as previously described (139). Known quantities of URO I were added to the ALA-dehydratase reaction mixture (devoid of ALA-dehydratase) and 0.1 ml of TCA was added to achieve a final concentration of 5.0%. Standard curves obtained under these conditions were linear for up to 100 pmol of URO I/ml solution in the cuvette, after which fluorescence quenching became significant as previously reported (140). The lower limit of detectability was about 1.5 pmol/ml.

F. Protein Determination:

Protein concentration was determined by the fluorescamine procedure (141) for purified human ALA-dehydratase and PBG-deaminase, and by the method of Lowry et al. (142) for tissue homogenates and cell lysates.

II. Enzyme Purification:

ALA-dehydratase and PBG-deaminase were purified from outdated human erythrocytes by the methods of Anderson and Desnick (50,84). Both en-

zymes were purified through the Sephadex G-200 and G-100 steps, respectively and stored at -20°C until further use. For immune serum production post-Sephadex G-200 ALA-dehydratase and post-DEAE-cellulose PBG-deaminase preparations were used. Post-Sephadex G-200 ALA-dehydratase was used to optimize the coupled enzyme assay.

III. Production of PBG:

PBG acetate was synthesized according to the method of Anderson and Desnick (50).

IV. Immune Sera:

A. Production of Rabbit Anti-Human PBG-Deaminase and ALA-Dehydratase Antibodies:

New Zealand rabbits were injected intradermally and intramuscularly with 150 µg of homogeneous human PBG-deaminase or ALA-dehydratase in a 1:1 suspension of Freund's complete adjuvant. Booster injections of 150 µg, 100 µg, and 75 µg were given at 1 month intervals. The purified antibody preparations were shown to be specific for PBG-deaminase or ALA-dehydratase by the presence of a single arc of identity following Ouchterlony double immunodiffusion.

B. Purification of Rabbit Anti-Human PBG-Deaminase and ALA-Dehydratase Antibodies:

IgG in pooled rabbit anti-sera was partially purified by the method of Harboe and Ingeld (143). The antibody was precipitated by the addition of 4 v of neutralized saturated ammonium sulfate to 6 v of anti-sera. After stirring 2 hr at 4°C, the solution was centrifuged at

2,500 x g for 20 min and then washed twice with 1.5 M ammonium sulfate. Hemoglobin and albumin were in the supernatant and over 90% of the IgG was recovered in the pellet. The pellet was resuspended in distilled water and extensively dialyzed against 5 mM potassium phosphate, pH 7.4, containing 0.9% NaCl. The antibodies were aliquoted into 1.5 ml samples and stored at -20°C.

C. Removal of Cross-reacting Mouse Antibodies from Rabbit Anti-Human ALA-Dehydratase Immune Sera:

In order to specifically detect human ALA-dehydratase immunologically in the mouse-human somatic cell hybrids used for these studies, it was necessary to remove the cross-reacting mouse antibodies from the rabbit anti-human ALA-dehydratase immune sera. This was accomplished by applying the immune sera to an Ascaris cuticle collagen affinity column. This column has been used to obtain homogeneous prolyl 4-hydroxylase from tissues which contain low concentrations of this enzyme and as a means of obtaining IgG from placenta (144). Prior to application, the sample was dialyzed against 0.01 M Tris-HCl buffer, pH 7.3, containing 0.1 M NaCl. The column was washed extensively with the above buffer, prior to and after application of the sample. The IgG was eluted by the addition of 5 M NaCl. The sample was then extensively dialyzed against deionized water and centrifuged at 20,000 x g for 15 min to remove insoluble material. The supernatant, which contained rabbit anti-human ALA-dehydratase IgG, did not cross-react with mouse ALA-dehydratase on Ochterlony double immunodiffusion or after rocket immunoelectrophoresis and was stored at -20°C until further use.

V. Electrophoretic Methods:

A. Isoelectric Focusing of PBG-Deaminase:

Isoelectric focusing was used to detect human PBG-deaminase in the mouse-human somatic cell hybrids used for these studies (see below). Polyacrylamide slab gels with a pH gradient of 3.5-9.5 were prepared with LKB Ampholines as described by the manufacturer (145). Gels were prefocused lengthwise for 1 h at 800 V (constant voltage) at 4°C. The anode contained 1 M H_3PO_4 and the cathode contaned 1 M NaOH. Aliquots of hybrid lysates (200-300 µl) were pipetted onto Whatman no. 17 paper strips which were placed 1 cm from the cathode. Gels were focused for 6 h at 800 V (constant voltage). Upon completion of the run, 0.5 x 0.5 cm strips were cut along the length of the gel. These strips were then soaked in distilled H_2O and the pH of the leached ampholytes was determined. The gels were then stained for PBG-deaminase activity by overlaying the gel with 3.0 ml of 0.3 mM PBG and incubating the gel at 50°C for 60 min in a moist chamber. Following staining the gel was then exposed to ultraviolet light for 10 min to oxidize the uroporphyrinogen to uroporphyrin, and the red fluorescent bands of PBG-deaminase activity were rapidly photographed under UV light with Polaroid type 58 film with a Wratten no. 4 filter (Eastman Kodak Co., Rochester, NY). Isoelectric focusing of mixtures, each containing varying proportions of mouse and human PBG-deaminase, revealed that this method was sensitive enough to detect human PBG-deaminase when less than 5% of the total activity was human.

B. Agarose and Starch Gel Electrophoresis of ALA-Dehydratase:

Agarose gel electrophoresis was used to detect human ALA-

dehydratase in the mouse-human somatic cell hybrids used for these studies (see below). The bridge buffer was 0.25 M Tris, 0.07 M citric acid, pH 7.6. The gel buffer was a 1 in 15 dilution of the bridge buffer. The gel was made with 1% agarose (Bio-Rad). Samples were applied to slots in the gel 3-4 cm from the cathodal end. Electrophoresis was carried out at 100 V (constant voltage) for 18 h at 4°C. Gels were stained by overlaying the surface of the gel with a solution containing 3.0 ml of 2.0 mM ALA, 0.1 ml of 100 mM DTT, 30.0 units of post-Sephadex G-100 PBG-deaminase and 0.6 ml of 0.25 M sodium phosphate buffer, pH 6.8. The solution was incubated at 37°C in a moist chamber for a minimum of 1 h. Following staining, the gel was then exposed to ultraviolet light for 10 min to oxidize the uroporphyrinogen to uroporphyrin, and the red fluorescent bands of PBG-deaminase activity were photographed as described above.

For studies of ALA-dehydratase polymorphisms in human erythrocytes, the starch gel electrophoresis and Ehrlich's staining procedures of Battistuzzi et al. (65) were used.

C. Rocket Immunoelectrophoresis of Human PBG-Deaminase:

The rocket immunoelectrophoretic system for the detection of human PBG-deaminase in mouse-human somatic cell hybrids was developed as a modification of standard methods (146,147). A 1% agarose solution was prepared in 0.06 M Tris-barbital buffer, pH 8.8, and 9.0 ml was poured into a 5 x 7.5 cm plate in the center of which a 0.4 x 2.5 x 7 cm double thickness glass microscope slide had been placed. After the agarose had gelled, the glass slide was removed and 4.0 ml of a 1% agarose solution containing 7.5 µl of rabbit anti-human PBG-deaminase was poured in the

center area.

For routine electrophoresis of hybrid lysates, 10 2 mm wells were cut into the lower portion of the antibody-containing gel and 10 µl samples were added to each well. Cotton wicks were used to make contact between the gel and the electrophoresis buffer. The plate was electrophoresed for 4 h at a constant current of 30 mA. Following electrophoresis, the gel was thoroughly washed in 0.9% NaCl for 10-12 h with several changes and then overlayed with 300 µl of goat anti-rabbit peroxidase-conjugated IgG that had been previously diluted 1:1 with 0.9% NaCl. The plate was incubated at room temperature in a moist chamber to prevent drying. The gel was again washed with 0.9% NaCl for at least 8 h and then stained for peroxidase. The staining solution was 25 mg diaminobenzidine tetrahydrochloride dissolved in 50 ml of 0.1 M Tris-HCl buffer, pH 7.6, containing 0.15 ml of 3% hydrogen peroxide. After the gel and staining solution were allowed to react for 20 min at room temperature, the gel was photographed and stored in 0.9% NaCl. The peroxidase reaction mixture stained the gel brown and the rockets appeared white against the brown background.

D. Rocket Immunoelectrophoresis of Human ALA-Dehydratase:

Rocket immunoelectrophoresis of human ALA-dehydratase was performed as described above, with the following exceptions. For studies involving ALA-dehydratase deficiency in human erythrocytes, 15 µl of the ammonium sulfate purified rabbit anti-human ALA-dehydratase immune sera was used. For ALA-dehydratase mapping studies, 30 µl of the post Ascaris cuticle collagen high speed supernatant rabbit anti-human ALA-dehydratase sera was used.

E. Crossed Immunoelectrophoresis of ALA-Dehydratase in Human Erythrocyte Lysates:

Agarose isoelectric focusing in one dimension followed by rocket immunoelectrophoresis in the second dimension was used to detect electrophoretic differences among the 1-1, 2-1 and 2-2 ALA-dehydratase phenotypes. Isoelectric focusing was performed according to the manufacturer's (Pharmacia) specifications. Gels were prepared by heating a mixture of 0.33 g Agarose IEF, 4.0 g sorbitol, and 30 ml distilled water in a boiling water bath. After cooling to 75°C, 2.1 ml of pH 4 to 6.5 Pharmalyte ampholines were added. The mixture was then poured into a horizontal casting frame (11.4 x 22.5 cm with Gelbond film backing) that had been preheated to about 60°C using a portable hairdryer. Gels were either allowed to harden at least 1 h at 4°C or stored overnight at 4°C in a moist chamber. Erythrocyte lysates (50-100 µl) were pipetted onto Whatman number 17 paper strips which were placed 1 cm from the cathode. The cathode contained 1 M NaOH and the anode, 0.05 M H_2SO_4. Focusing was carried out at 4°C for 3 h at 7 W (constant power). Following completion of the run, each lane was removed from the gel and subjected to rocket immunoelectrophoresis, as previously described above, with the exception that a larger gel (5.0 x 7.5 cm) containing 8 ml of 1% agarose and 30 µl of anti-serum was used.

F. Marker Enzyme Analysis in Somatic Cell Hybrids:

Marker enzymes for specific human chromosomes were determined by established electrophoretic methods (148-152).

VI. Cells, Cell Culture and Tissue Preparations:

Two different sets of hybrid cells were used for the PBG-deaminase mapping studies. The first was derived from the fusion of thymidine kinase-deficient 2S MEL cells with HLN, as previously described (137,138). These hybrid lines were positive for human LDHA and produced human β-globin mRNA (138). Five primary 2S MEL-HLN hybrid clones were initially examined; three of these strongly expressed human PBG-deaminase and were selected for subcloning (Table 3, see "Results" Section I.C.). Hybrids were subcloned by plating single cells into individual wells of a 96-well microtiter plate (Costar, Cambridge, MA). Of the four secondary clones generated, two derived from XX-8, [one positive (H21) and one negative (A31) for human PBG-deaminase] were chosen for further subcloning. Three cytogenetically homogeneous quaternary (H21-H7-2, -10, and -21) and two homogeneous tertiary clones (A31-18 and A31-23) were informative. The hybrid clones and the parental 2S MEL and HLN cells were cultured by standard techniques in RPMI 1640 media supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, and 100 meg/ml of streptomycin and 100 U/ml of penicillin per ml media. For subcloning, the medium was supplemented with 20% fetal calf serum.

The second set of hybrid clones (2S MEL-HX/11) were derived (L. Sanders-Haigh) from the fusion of hypoxanthine phosphoribosyltransferase-deficient ($HPRT^-$) 2S MEL cell line with human fibroblasts (153,154) carrying an X/11 chromosome translocation [46,X,t(X;11)(q25-26;q23)] (Genetic Cell Repository, GM 3552). Two hybrid clones (HX/11-2 and HX/11-3) from two independent fusions were grown in Iscove's minimal essential media (GIBCO) supplemented with 10% fetal calf serum, 4 mM L-glutamine, 0.8 mM glycine, 0.1 mM hypoxanthine, 0.1 μM aminopterin,

and 16 μM thymidine.

Erythroid differentiation was induced in 2S MEL cells and 2S MEL-human fibroblast hybrids with 2% (v/v) DMSO (137,138). Induction conditions for the 2S MEL-HX/11 hybrid cells were identical except that Iscove's minimal essential media without hypoxanthine, aminopterin and thymidine was used. Prior to harvesting, cell aliquots were taken for determination of cell viability, number and benzidine staining (137).

For ALA-dehydratase mapping studies, the parental cell lines used for somatic cell hybridization were the mouse RAG cell line (155) and human fibroblast cells derived from either fetal lung, fetal liver or fetal kidney.

Human fibroblasts, aminocytes and mouse RAG cells were cultured by standard techniques in RPMI 1640 media supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 meg/ml of streptomycin and 100 U/ml of penicillin.

Induced 2S MEL-HLN fibroblast hybrids were used for PBG-deaminase mapping studies. Cells with greater than 95% viability and 45-80% benzidine positivity were harvested by centrifugation at 700 x g for 20 min at 4°C in a Sorvall GS-3 rotor. For enzyme studies, the cell pellet was washed three times in Dulbecco's phosphate-buffered saline (GIBCO). Cells (5×10^8) for PBG-deaminase analysis were resuspended in 1.0 ml of 1.0 mM potassium phosphate buffer containing 1 mM $MgCl_2$, 1 mM DTT, and 0.05% Triton X-100. For PBG-deaminase activity assay and rocket immunoelectrophoresis, cells were lysed by three, 10-sec pulses (output of 7) using a Branson Sonifier 200. The lysate was then centrifuged at 35,000 x g for 20 min in a Sorvall SS-34 rotor; the supernatant was collected and stored at -20°C. The mean PBG-deaminase specific activi-

ties (eight experiments) in induced and uninduced hybrid cells were 1.0 and 3.7 U/mg protein, respectively. For PBG-deaminase isoelectric focusing, cell lysates were treated as above and then freeze-thawed twice in a dry ice/acetone slurry. The lysates were then heated at 60°C for 30 min and subsequently centrifuged at 35,000 x g for 10 min in a Sorvall SS-34 rotor. The supernatant was collected and immediately subjected to isoelectric focusing as described below. For enzyme marker analyses, the washed cells were resuspended in distilled water (5×10^8 cells per ml), lysed by sonication and centrifuged as described above.

For ALA-dehydratase mapping studies and enzymatic assay determinations, RAG cells, RAG-human fibroblast hybrids, human fibroblasts, Friend cells and human amniocytes were harvested by centrifugation at 800 x g for 10 min and washed twice with 0.9% NaCl. The cells were lysed by the addition of an equal volume of 7.0 mM potassium phosphate, pH 6.8, containing 1 mM DTT followed by three cycles of freezing (dry ice/acetone bath) and thawing (37°C). The lysates were centrifuged at 35,000 g for 20 min and the supernatants were assayed immediately or stored at -20°C.

Rat livers were obtained from male Sprague-Dawley rats weighing 125-150 gm. Livers were homogenized in three v of 0.1 M sodium phosphate buffer, pH 6.8, for the standard colorimetric assay and the coupled enzyme assay and 0.1 M sodium phosphate buffer, pH 7.4, for the semi-micro colorimetric assay. Homogenates were centrifuged at 17,000 x g for 30 min and the supernatant was removed and stored at -20°C until use.

Human blood was centrifuged at 800 x g for 10 min, the plasma and leukocytes removed, and the erythrocytes were washed three times with

0.9% NaCl. For routine assays the erythrocytes were lysed by the addition of two v of 1.0 mM potassium phosphate, pH 7.6, containing 1 mM DTT, 1 mM $MgCl_2$, and 0.05% Triton X-100. For starch gel electrophoresis, the erythrocytes were lysed by the addition of one v of the above buffer. The lysate was centrifuged at 35,000 x g for 20 min, and the supernatant was removed and stored at -20°C until use.

VII. Cytogenetic Analyses:

For cytogenetic analyses of 2S MEL-HLN hybrids and RAG-human fibroblast hybrids, metaphase spreads were prepared by standard techniques (156) and the chromosomes were banded to distinguish mouse from human chromosomes with the Giemsa 11 technique (157) and then destained and banded with quinacrine hydrochloride (158) or trypsin/Giemsa (156) techniques. Hybrid clones were subcloned until judged homogeneous by the examination of at least 30 metaphase spreads. It was notable that chromosomal breakage and rearrangement was observed in 2S MEL-HLN hybrids. Human PBG-deaminase assays, marker-enzyme electrophoresis, and cytogenetic analyses were performed on cell hybrids harvested from the same passage.

RESULTS

The high degree of chromosomal instability characteristic of the 2S MEL-HLN hybrids used required metaphase spreads, marker enzyme analysis and human PBG-deaminase screening to be performed on the same population of cells. The procedure implemented was as follows: uninduced hybrid cells in early logarithmic phase were harvested for chromosomal analysis prior to DMSO induction. After five days, marker enzyme analysis and human PBG-deaminase screening were performed on the induced cells from the same population. This procedure ensured valid correlation of the marker, chromosomal and human PBG-deaminase data.

I. Chromosomal Localization of Human PBG-Deaminase:

A. Rocket Immunoelectrophoresis of Human PBG-Deaminase in Hybrid Cell Lysates:

Expression of human PBG-deaminase in parental and hybrid lines was detected by rocket immunoelectrophoresis using monospecific rabbit anti-human PBG-deaminase IgG. Figure 5 shows a representative rocket immunoelectrophoretic gel demonstrating rockets for a human erythrocyte lysate and hybrid subclones, XII-4-20 and XX-8-H21-H7-10, the latter of which contained only the human chromosomal rearrangement, t(4;11). Note that rockets were not observed for lysates of 2S MEL cells or hybrid clone HX/11-2.

B. Isoelectric Focusing of Human PBG-Deaminase in Hybrid Cell Lysates:

Human PBG-deaminase expression in hybrid cells was also demon-

Figure 5. Rocket immunoelectrophoresis of human PBG-deaminase in cell lysates. Lanes 1 and 6, human erythrocytes; lane 2, 2S MEL cells; lanes 3 and 4, 2S MEL-HLN subclones, XX8-H21-H7-H10 and XII-4-20; lane 5, 2S MEL-H11/X-2 hybrid cells.

FIGURE 5

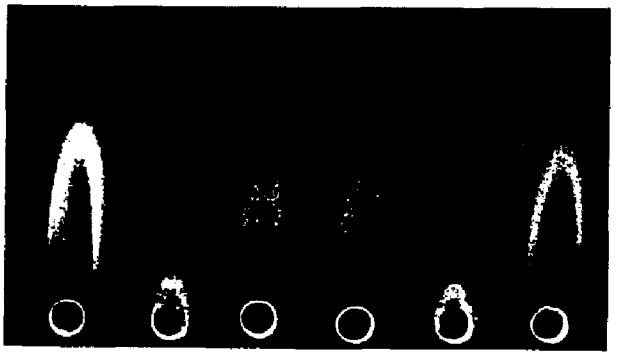
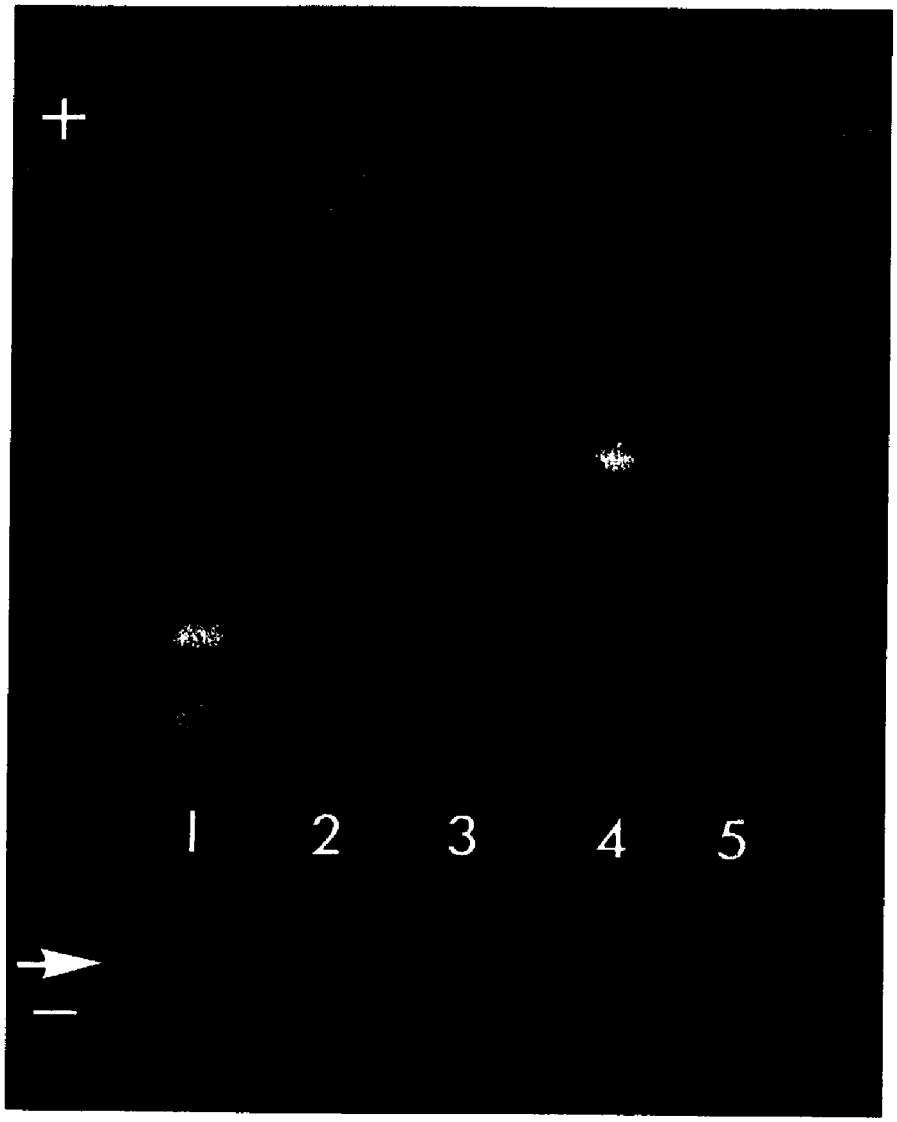


Figure 6. Isoelectric focusing of human and murine PBG-deaminase in cell lysates. Lane 1, human erythrocytes; lane 2 and 3, 2S MEL-HLN XX-8 hybrid subclones, H21-H7-10 and A31-18, respectively; lane 4, 2S MEL-HX/11-3 hybrid; and lane 5, 2S MEL cells. Note that human PBG-deaminase was detected only in human erythrocytes and in clone H21-H7-10. See text for details.

FIGURE 6



strated by isoelectric focusing as shown in Figure 6. The five PBG-deaminase intermediates, designated A-E (84), from human erythrocytes focused at pI values of about 6.6, 6.3, 6.1, 5.9 and 5.8, respectively. The PBG-deaminase intermediates from 2S MEL cells focused at pI values of 5.8, 5.7, 5.6, 5.5 and 5.4. Typically, two major murine activity bands were observed at pI values of 5.8 and 5.3 in all hybrid clones. Hybrids were scored positive for human PBG-deaminase when at least one human activity band, typically at pI 6.6, was observed in addition to those corresponding to the murine PBG-deaminase intermediates.

C. Segregation of PBG-Deaminase, ESA4, and LDHA in Hybrid Cells:

Table 3 shows the human chromosomal constitution of the 2S MEL-HLN and 2S MEL-HX/11 hybrid clones used for these analyses. Table 4 indicates the expression of human PBG-deaminase, ESA4, LDHA, peptidase-S (PEPS), phosphoglucomutase 2 (PGM2), esterase D (ESD), and glucose-6-phosphate dehydrogenase (G6PD). From the original 2S MEL-HLN hybrid line, XX-8, two cytogenetically homogeneous tertiary (A31-18 and A31-23) and three homogeneous quaternary clones (H21-H7-2, -10, and -21) provided pertinent information. Note that the tertiary subclone, H21-H7, contained only two chromosomes which were classified as human by Giemsa 11 staining. These chromosomes were identified as 9q and t(4;11) by quinacrine banding. The quaternary clone, H21-H7-10, which expressed human PBG-deaminase (Table 4), contained only the single human chromosome derivative, t(4;11),(4pter → 4q21;11q13 → 11qter), shown in Figures 7 and 8. The human PBG-deaminase gene was localized to the region 11q since the primary clone, XIX-25, contained an intact chromosome 4, but was negative for PBG-deaminase. Furthermore, the primary

clone, XII-4, and its subclone, XII-4-20, contained a mouse/human translocation (Fig. 7) in which the human derivative was 11q11 → 11qter and were positive for human PBG-deaminase. Assignment of PBG-deaminase to human chromosome 11p was further excluded by findings in the secondary and tertiary subclones of XX-8, A31 and A31-23, which were positive for LDHA and for the short arm of 11 (Fig. 7), but were negative for PBG-deaminase. In addition, the primary clone, XIX-10, contained a human-human translocation t(11;13)(11p12 → 11qter; 13q14 → 13qter) as shown in Figure 7. This clone was positive for human PBG-deaminase, ESA4, and ESD, the latter gene having been localized on 13q (159).

Examination of the 2S MEL-HX/11 hybrids provided finer localization of the gene for human PBG-deaminase. Hybrid 2S MEL-HX/11-2 contained human-human X/11 translocation, (der11),t(X;11)(q25-26;q23), as the only human chromosome. In 2S MEL-HX/11-3, the human 11p was deleted and the human X/11 derivative was translocated onto a large murine metacentric chromosome (Fig. 7 and 8). Since both of the X/11 translocations in these hybrids were deficient for the region 11q23 → 11qter and since neither hybrid expressed human PBG-deaminase, the gene for this enzyme was localized to this region.

Analogously, the structural gene for human ESA4 was found to segregate concordantly with the human PBG-deaminase gene in all the 2S MEL-human fibroblast hybrids (Table 4). Thus, the gene for human ESA4 also can be assigned to the region 11q23 → 11qter.

These hybrid clones also provided information for the finer regional localization of the genes for human PEPS and PGM2 on chromosome 4. H21-H7 quaternary subclones which contained the human-human translocation t(4;11) expressed human PEPS, thus tentatively localizing

the structural gene to the region 4pter → 4q21. The segregation of human PGM2 in A31 and H21-H7 subclones also was informative. Subclones of A31 and H21-H7 segregated for human PGM2 (Table 4). All tertiary and quaternary clones derived from H21-H7 were negative for human PGM2, suggesting that this locus was excluded from the region 4pter → 4q21 (proximal portion). However, all A31 subclones contained 4q21 (distal portion) → 4qter and expressed human PGM2. The regional gene assignments for PBG-deaminase and ESA4 and the tentative assignments for PEPS and PGM2 are diagrammatically represented in Figure 8.

Table 3

Human Chromosomal Constitution of 2S MEL-Human Fibroblast Hybrids

Hybrid clones	Human chromosomes	Human-human translocations
2S MEL-HLN:		
Primary:		
XIX-25	3,4,6,8,12,15,19	-
XVIII-5	10,14	-
XIX-10	4,6,14,19	11;13q*
XII-4	10,11,14	11q/M
XX-8	4,6,8,9,11,12,13, 14,15,18,21,22	4p11q;11p4q
Secondary:		
XIX-10-A1	4,14,9	-
XII-4-20	14	11q/M
XX-8-H21	4,9,13,15,21	4p11q
XX-8-A31	4,9q,15,21	11p4q
Tertiary:		
XX-8-A31-18	15,21	11p4q
XX-8-A31-23	4,9q,15,21	11p4q
XX-8-H21-H7	9q	4p11q
Quaternary:		
XX-8-H21-H7-2	9q	-
XX-8-H21-H7-10	-	4p11q
XX-8-H21-H7-21	9q	-

Table 3 (Continued)

Hybrid clones	Human chromosomes	Human-human translocations
2S MEL-HX/11:		
Primary:		
HX/11-2	-	X/11**
HX/11-3	-	X/11/M++

* The human chromosomal constitution of 2S MEL-HLN primary hybrids was heterogeneous (50 metaphase spreads examined). Human-human and human-mouse rearrangements were also present, but were difficult to identify cytogenetically. Therefore, in addition to cytogenetic analyses, the presence of the human chromosomes in all hybrid lines was based on marker enzyme data (151-155). PGM2 (chromosome 4) ME1 and SOD2 (6), GSR (8), ACO1, AK1 and AK3 (9), LDHA and ESA4 (11), LDHB and PEPB (12), ESD (13), NP (14), MPI (15), PEPA (18), SOD1 (21) and ACO2 (22). Identification of the human chromosomes present in all secondary, tertiary and quaternary clones was based on enzyme marker and cytogenetic analyses which corresponded in every case.

* 11:13q = t(11:13)(11p12 → 11qter;13q14 → 13qter)

11q/M = (der11)t(11:M)(11q11 → 11qter;murine)

4p11q = t(4:11),(4pter → 4q21;11q13 → 11qter)

11p4q = t(11:4)(11pter → 11q13:4q21 → 4qter)

** X/11 = (der11),t(X;11)(q25 or 26;q23)

++ X/11/M++ = (der11),t(X;11)(q25 or 26;q23;murine)

Table 4

Segregation of Human PBG-Deaminase and Enzyme Markers for Human Chromosomes 4, 11, 13 and X in 2S MEL-Human Hybrids

Clone	Human PBG-deaminase		Human chromosome					
			11		4		13	X
	RIE*	IEF*	LDHA	ESA4	PEPS	PGM2	ESD	G6PD
2S MEL-HLN:								
Primary:								
XIX-25	-	-	-	-	+	+	-	-
XVIII-5	-	-	-	-	-	-	-	-
XIX-10	+	+	+	+	+	+	+	-
XII-4	+	+	-	+	-	-	-	-
XX-8	+	+	+	+	+	+	+	-
Secondary:								
XIX-10-A1	-	-	-	-	+	+	-	-
XII-4-20	+	+	-	+	-	-	-	-
XX-8-H21	+	+	-	+	+	+	+	-
XX-8-A31	-	-	+	-	+	+	-	-
Tertiary:								
XX-8-A31-18	-	-	+	-	-	+	-	-
XX-8-A31-23	-	-	+	-	+	+	-	-
XX-8-H21-H7	+	+	-	+	+	-	-	-
Quaternary:								
XX-8-H21-H7-2	-	-	-	-	-	-	-	-
XX-8-H21-H7-10	+	+	-	+	+	-	-	-
XX-8-H21-H7-21	-	-	-	-	-	-	-	-
2S MEL-HX/11:								
Primary:								
HX/11-2	-	-	+	-	-	-	-	+
HX/11-3	-	-	-	-	-	-	-	+

* Human PBG-deaminase was specifically detected by rocket immunoelectrophoresis (RIE) and isoelectric focusing (IEF). See text for experimental details.

+ 2S MEL-HX/11 hybrids were cultured in HAT media to insure that the X-autosomal translocation was retained.

Figure 7. Partial karyotypes of the human chromosomes in the 2S MEL-human fibroblast hybrids. A, B and C) The translocation is shown between normal human chromosomes 4 (left) and 11 (right) as observed by quinacrine staining. A) The rearrangement in hybrid XX-8-H21-H7-10 was t(4;11),(4pter → 4q21;11q13 → 11qter), B) in hybrid XX-8-A31-18 was t(4;11),(4q21 → 4qter;11pter → 11q13), and C) in hybrid HX/11-2 was t(11;X),(11pter → 11q23;Xq25 or 26 → Xqter). D) In HX/11-3 a mouse-human translocation occurred at 11q11 as shown by Giemsa 11 staining. E) In XII-4-20, a mouse-human translocation occurred at 11q11, as shown by Giemsa 11 staining (left) and trypsin-Giemsa staining (center) compared to an intact human chromosome 11 (right). F) In XIX-10, the rearrangement t(11;13),(11p12 → 11qter;13q14 → 13qter) was observed, by Giemsa 11 (left) and trypsin-Giemsa (right center) staining. Trypsin-Giemsa stained intact human chromosome 11 (left center) and 13 (right) are shown for comparison.

FIGURE 7

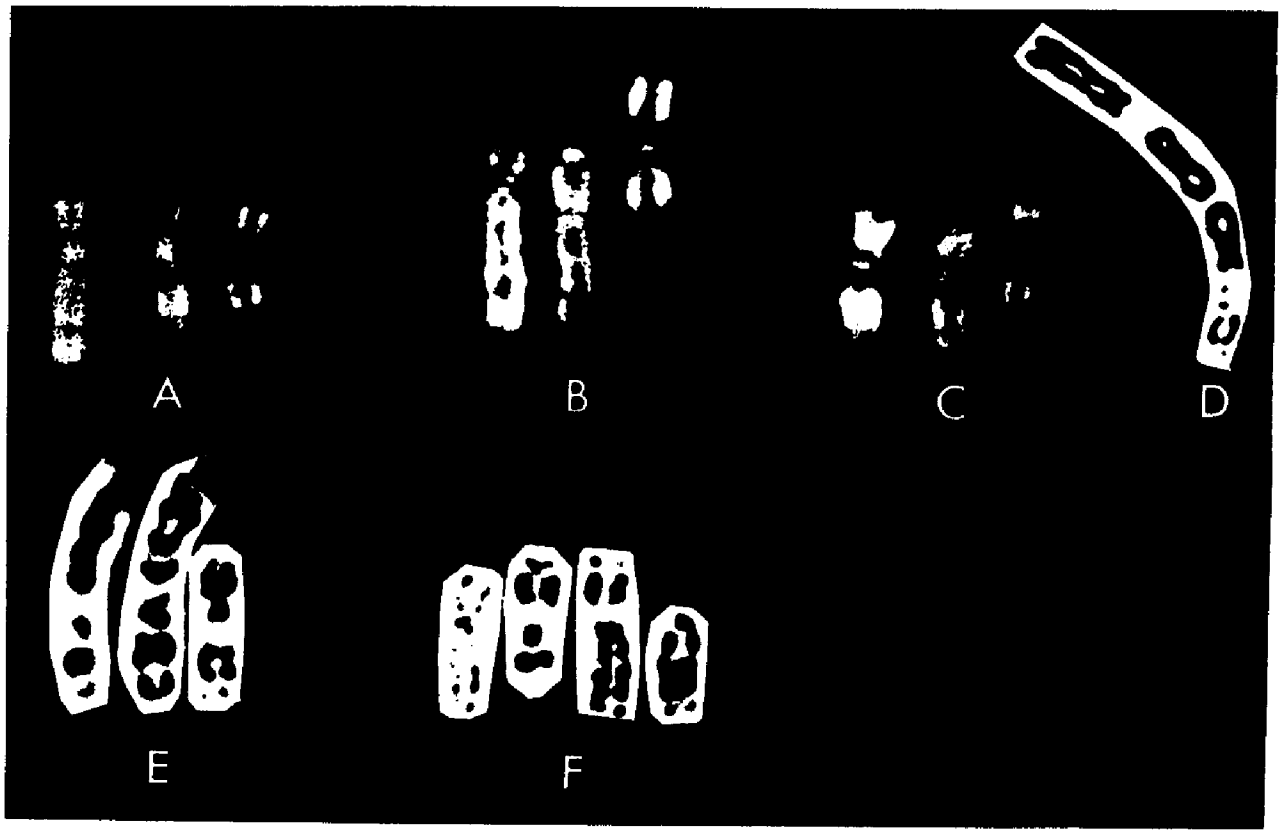
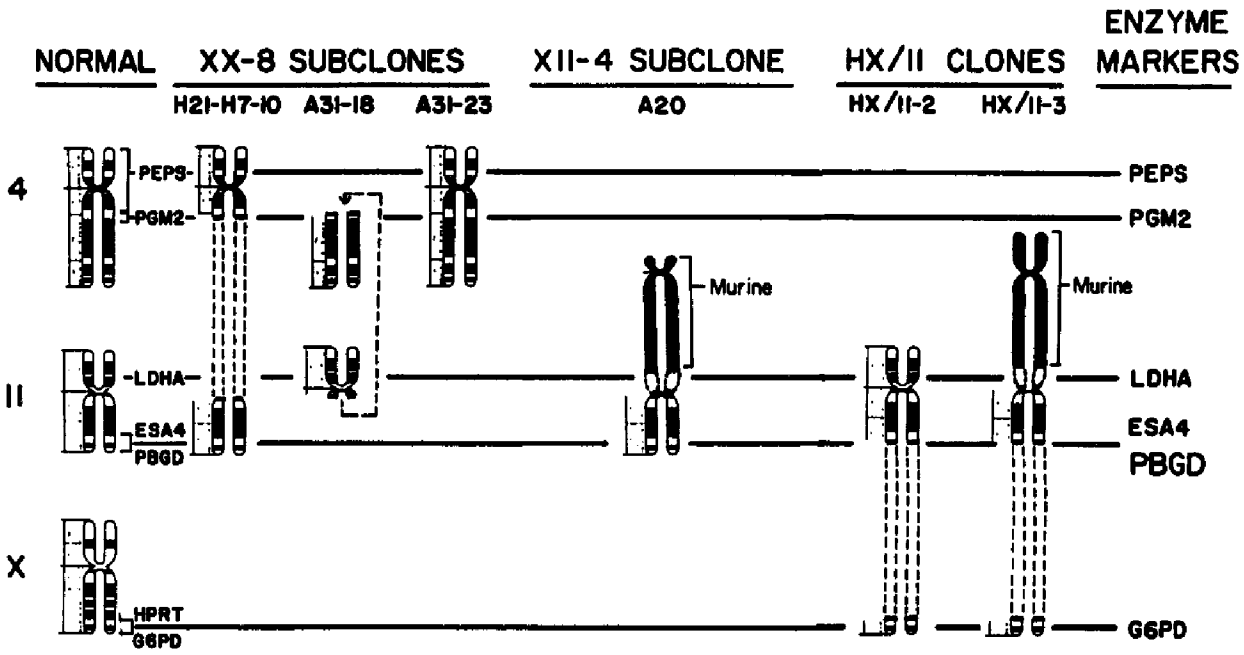


Figure 8. Diagramatic representation of the regional gene assignments for human PBG-deaminase, ESA4, PEPS and PGM2 on chromosomes 4 and 11. Chromosomes 4, 11 and X and the pertinent translocations in the 2S MEL-human fibroblast hybrid clones are shown, as observed with the Giemsa staining technique. PBG-deaminase and ESA4 were localized to the region 11q23 → 11qter and 11q22 → 11q23, respectively. PEPS and PGM2 were assigned to 4pter → 4q12 and 4q21, respectively. See text for details.

FIGURE 8


NORMAL
XX-8 SUBCLONES
H21-H7-10
A31-18
A31-23
XII-4 SUBCLONE
A20
HX/11 CLONES
HX/11-2
HX/11-3
ENZYME MARKERS
4
II
X
PEPS
PGM2
LDHA
ESA4
PBGD
HPRT
G6PD
Murine
Murine
PEPS
PGM2
LDHA
ESA4
PBGD
G6PD

D. Determination of the of Percentage of Human PBG-Deaminase Activity in Clone H21-H7-10:

When 0.7 units of human erythrocyte lysate and a lysate obtained from clone H21-H7-10 were subjected to rocket immunoelectrophoresis, the height of the rocket formed by the erythrocyte lysate was markedly greater than the height of the rocket formed by the H21-H7-10 lysate. This is to be expected since the H21-H7-10 lysate contains a mixture of both mouse and human PBG-deaminase activities, of which only the human activity is detectable. Almost equal rocket heights between the erythrocyte and H21-H710 lysate were obtained when the erythrocyte lysate was diluted 4 to 5-fold, indicating that 20-25% of the total PBG-deaminase activity in clone H21-H7-10 was human.

E. Comparison of the Levels of Human PBG-Deaminase in Uninduced and Induced H21-H7-10 Lysates:

Equal activities from uninduced and induced H21-H7-10 lysates were subjected to rocket immunoelectrophoresis. The rockets formed by both lysates were of equal height, indicating that the percentage of human PBG-deaminase activity in clone H21-H7-10 was 20 to 25% before as well as after DMSO treatment. These findings suggest that both the human and the mouse PBG-deaminase enzyme proteins were increased following DMSO induction.

II. Studies of the Expression of Human ALA-Dehydratase in RAG-Human Fibroblast Hybrid Clones:

A. Comparison of RAG and Human Fibroblast ALA-Dehydratase Activities:

ALA-dehydratase activity was determined in RAG cells and human fibroblast cells by the standard colorimetric assay. Although 10^7 RAG cells gave measurable absorbance readings, no measurable absorbance readings were obtained with 10^6 human fibroblast cells (See Table 9). Furthermore, when the semi-micro colorimetric assay was performed on human fibroblast lysates, no measurable activity was observed. Since it was not possible to detect ALA-dehydratase present in human fibroblasts by the currently available methodologies, and since large numbers of RAG cells were required for a single assay determination, it was necessary to develop a more sensitive assay for ALA-dehydratase activity. Hence, a sensitive fluorometric coupled enzyme assay procedure was developed for the determination of ALA-dehydratase activity in tissues with low specific activities. Characterization of the assay is discussed fully in Section III below. Using this assay procedure, ALA-dehydratase activity in human fibroblasts could be readily detected. As shown in Table 9 there was approximately 10 times more ALA-dehydratase activity in RAG cells as compared with human fibroblasts.

B. Agarose Gel Electrophoresis of Human and RAG ALA-Dehydratase:

Figure 9 shows the electrophoretic separation of human and RAG ALA-dehydratase, the RAG being more anodal than the human. A battery of RAG-human fibroblast hybrid clones were tested for the presence of human ALA-dehydratase. Together these clones possessed all 22 autosomes and the X and Y chromosome. The chromosomal constitution of each clone is given in Table 5. In each clone studied, all of the visable activity had the same mobility as the RAG enzyme indicating that human ALA-dehydratase was either not expressed in these clones or that the elec-

Figure 9. Agarose gel electrophoresis of human and RAG ALA-dehydratase. Lane 1, human fibroblasts; lane 2, RAG cells; lanes 3-5, RAG-human fibroblast hybrids, $RF_3KQ_3O_1$, $RF_3KQ_3U_1$ and RAG ANG F_2.

FIGURE 9

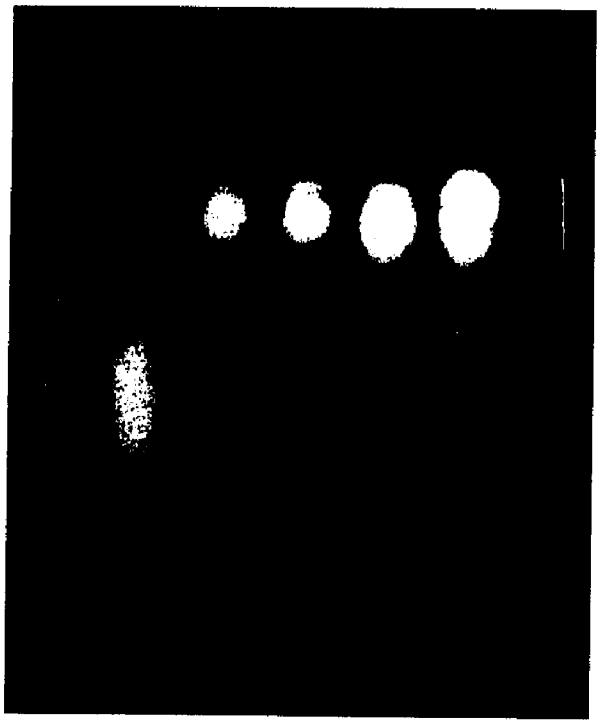


Table 5

Chromosomal Constitution of RAG-Human Fibroblast Hybrids Examined for Human ALA-Dehydratase Activity

Clone	Chromosome Number																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
RODYLA			+		+	+								+		+					+			
$RF_3KQ_3O_1$	+													+			+		+		+	+		
RAG 6323-16A			+		+			+		+		+	+		+		+	+			+	+		
R/RPM1 G21	+		+																			+	+	+
RAG ANG F_2		+	+	+		+	+	+		+	+		+			+		+				+		
$RF_3KQ_3U_1$	+									+				+	+		+				+	+		
R/RPM1 G211									+															
RAG 6323-13A	+		+	+		+		+		+		+	+		+			+			+			
RAG 6323-6B			+		+	+	+			+														
RAG ANG B	+	+	+	+	+	+	+	+		+	+	+		+										
RAG ANG G											+	+												
$RAG\ 6323\text{-}16A_1$	+	+	+	+	+	+	+	+		+		+		+	+			+		+	+			

+ Denotes chromosome present in a particular hybrid clone by marker enzyme analysis and/or cytogenetic analysis in which the particular chromosome was present in at least 50% of the metaphase spreads examined.

trophoretic procedure used was not sensitive enough to detect the small quantities of human ALA-dehydratase which might be present. Hence further studies were undertaken to detect human ALA-dehydratase activity in the hybrids used for these studies.

C. Thermostability Studies of Mouse and Human ALA-Dehydratase:

Human and mouse erythrocyte lysates were preincubated for 15, 30, 60 and 120 min at 50°C, 55°C and 60°C and then assayed for ALA-dehydratase by the standard colorimetric assay. As shown in Table 6, at all 3 temperatures the mouse enzyme was significantly more thermostable than the human enzyme. However, these differences were not great enough to accurately discriminate a positive clone from a negative clone. Therefore, this procedure was not suitable for screening for the presence of human ALA-dehydratase.

D. Rocket Immunoelectrophoresis of ALA-Dehydratase Activity Present in Hybrid Lysates:

Rocket immunoelectrophoresis was used as a means of detecting human ALA-dehydratase present in the hybrid lysates used for these studies. Preliminary studies using crude lysates obtained from RAG x human fibroblast hybrids demonstrated the need for purification of these lysates prior to rocket immunoelectrophoresis, since large streaks, indicative of high sample protein concentrations, were visable on the gel following staining. ALA-dehydratase from 3×10^8 cells was then purified by DEAE-cellulose, ammonium sulfate, octyl and phenyl Sepharose chromatography. Hybrid clones RAG ANG F_2 and RAG 6323-13A were chosen for these studies since they contained the majority of human chromo-

somes. Since preliminary studies demonstrated that approximately 10 units of human enzymatic activity were required to obtain visable rockets, at least 300 units of hybrid enzymatic activity were applied to the gel in order to compensate for dilution of human ALA-dehydratase activity by the mouse ALA-dehydratase activity present in the lysate. Under these conditions no rockets were obtained with either of these two lysates, indicating that human ALA-dehydratase could not be detected by the screening procedures used.

Table 6

Thermostability of Mouse vs. Human ALA-Dehydratase in Erythrocyte Lysates at 50°C, 55°C, and 60°C

		% Initial Activity	
Time (min)		Mouse	Human
50°C:	15	55	30
	30	50	20
	60	50	10
	120	50	10
55°C:	15	70	30
	30	45	30
	60	45	25
	90	35	25
60°C:	15	44	20
	60	40	10
	120	35	10

Aliquots of a mouse erythrocyte lysate and a human erythrocyte lysate were incubated at 50°C, 55°C and 60°C. At various time intervals, samples were removed and assayed for ALA-dehydratase activity by the standard colorimetric assay procedure.

III. ALA-Dehydratase Fluorometric Coupled Enzyme Assay:

In order to facilitate the above studies on the expression of human ALA-dehydratase in RAG-human fibroblast hybrids, and for the studies concerning the levels of ALA-dehydratase protein in ALA-dehydratase deficiency (Section IV.), a more sensitive ALA-dehydratase assay was developed. This assay procedure is based on the enzymatic conversion of ALA to uroporphyrinogen I (Fig. 10).

A. Optimization of the Enzyme Couple:

To optimize the assay sensitivity, the amount of PBG-deaminase required to give maximal conversion of PBG to uroporphyrinogen I was determined. As shown in Figure 11, increasing amounts of PBG-deaminase (ranging from 0.12 to 9.4 U) were added to 0.68 U of ALA-dehydratase. The amount of URO I formed was maximal when 3.8 U of PBG-deaminase were added. Increasing amounts of PBG-deaminase resulted in less URO I formed, presumably due to the formation of stable PBG-deaminase-substrate intermediates during the conversion of the monopyrrole PBG, to the linear tetrapyrrole, $HOCH_2$bilane (Fig. 10; 78,84). Hence, 3.8 U of PBG-deaminase were used in the standard coupled assay; this amount of PBG-deaminase was optimal for the determination of 0.085 to 0.680 U/ml of ALA-dehydratase activity.

B. Determination of the Percent Conversion of PBG to Uroporphyrinogen I:

Known amounts of PBG (ranging from 50 to 630 pmol) were added to 3.8 U of PBG-deaminase, and the reaction mixture was incubated for 90 min at 37°C. Approximately 11.8 pmol of URO I were detected for each

Figure 10. Reaction scheme for the determination of ALA-dehydratase activity by the enzymatic conversion of PBG to $HOCH_2$bilane and the fluorometric quantitation of the final product, URO I.

FIGURE 10

Fluorometric Determination of ALA-Dehydratase Activity by the Coupled Enzyme Assay

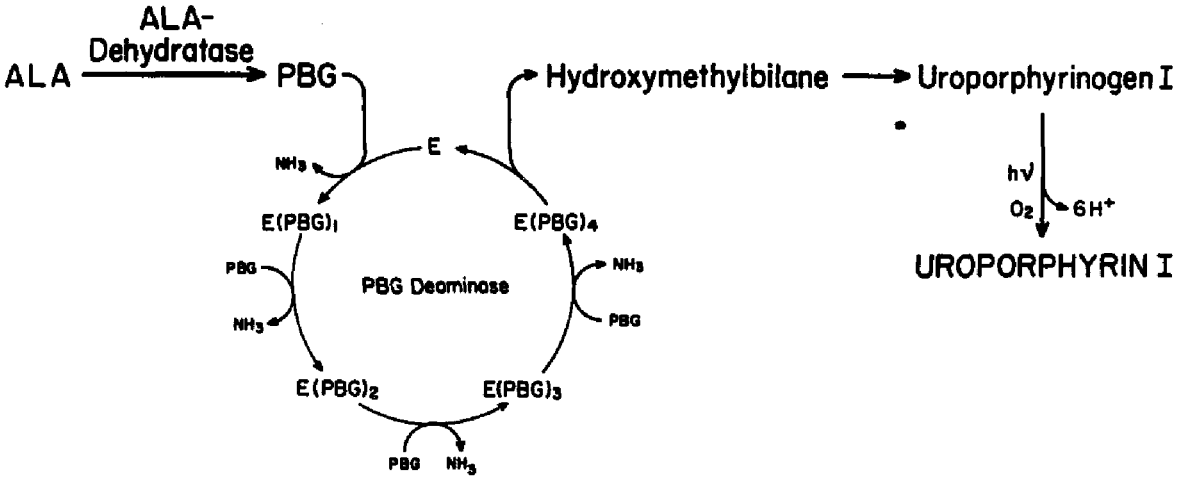
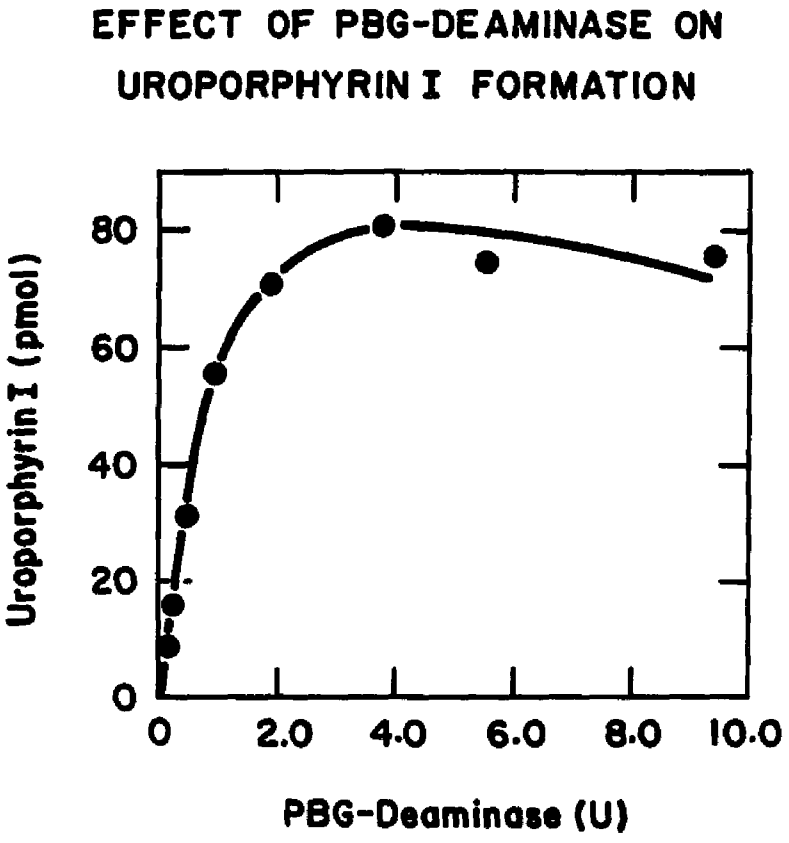


Figure 11. Effect of PBG-deaminase on URO I formation in the ALA-dehydratase coupled-enzyme assay. The conditions for the assay were as described in the "Methods" section. The amount of ALA-dehydratase was 0.68 U in each assay while the PBG-deaminase varied from 0.12 to 9.4 U.

FIGURE 11


EFFECT OF PBG-DEAMINASE ON
UROPORPHYRIN I FORMATION
Uroporphyrin I (pmol)
80
60
40
20
0
0
2.0
4.0
6.0
8.0
10.0
PBG-Deaminase (U)

100 pmol of PBG over the range of 100 to 630 pmol of added PBG (Fig.12). This corresponded to a 47% conversion of PBG to uroporphyrinogen I, assuming a mole for mole photo-oxidative conversion of uroporphyrinogen I to URO I. Therefore, the amount of URO I detected in the coupled assay was multiplied by 4 (4 moles of PBG per mole of URO I) and divided by 0.47 to obtain the total PBG produced.

C. Effect of Protein Concentration and Time:

Using purified ALA-dehydratase, the coupled-enzyme assay was linear with protein concentration over the range of 5 to 100 pmol of URO I formed (Fig. 13). The addition of 0.25 to 2.5 mg of BSA to the reaction mixture had little, if any, effect on URO I formation. However, when 5.0 or 10.0 mg of BSA was added, the URO I formation was inhibited about 10% and 20%, respectively.

Figure 14 shows the effect of time on the linearity of the coupled-enzyme assay. Note that after an initial lag of 20 min, the ALA-dehydratase assay was linear for up to 90 min. In addition, the amount of PBG present in the coupled assay at various time points were determined. After incubation for 30, 60 and 90 min, the ALA-dehydratase activities determined by the coupled assay were about 60, 90 and 100%, respectively, of the activity detected by the standard colorimetric assay. Therefore, a 90 min incubation period was used for the coupled assay.

D. Effect of pH and Substrate Concentration:

Figure 15 shows the effect of pH versus activity using sodium phosphate, succinate, Pipes and Hepes buffers. Maximum URO I formation was observed with sodium phosphate buffer, pH 6.7. The K_m for ALA in the coupled assay, determined by the Lineweaver-Burk plot, was 0.124 mM.

Figure 12. Relationship of PBG concentraton to URO I production in the coupled-enzyme assay. Varying amounts of PBG were incubated with 3.8 U of PBG-deaminase and assayed for 60 min at 37°C. See text for details.

FIGURE 12

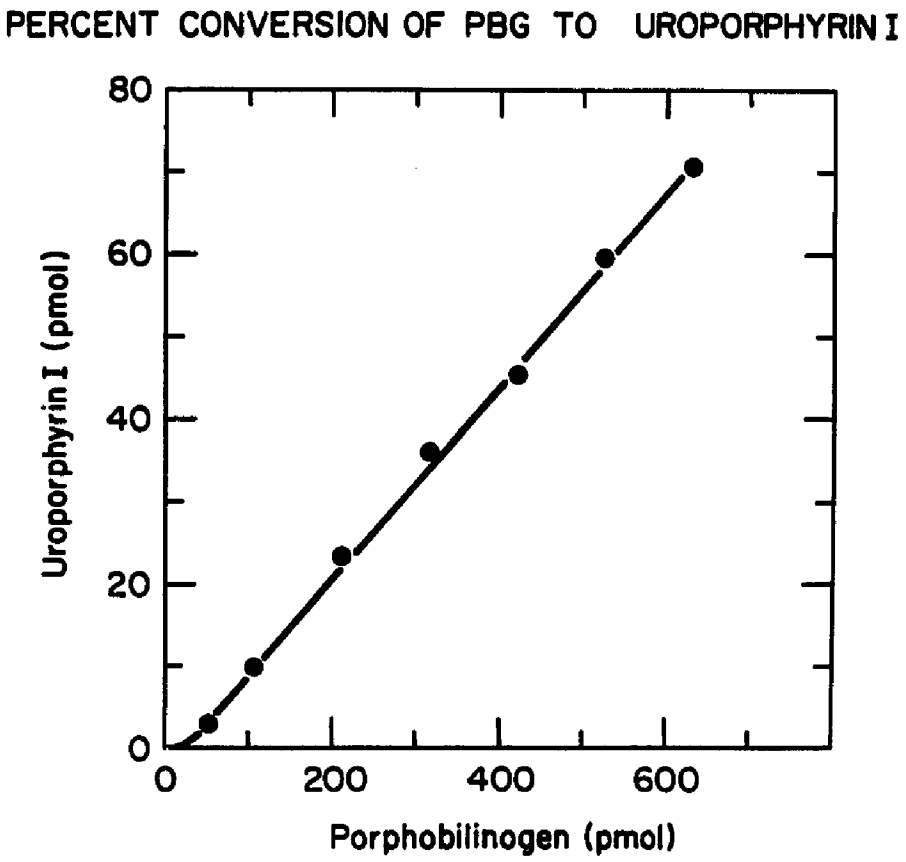

PERCENT CONVERSION OF PBG TO UROPORPHYRIN I
Uroporphyrin I (pmol)
80
60
40
20
0
0
200
400
600
Porphobilinogen (pmol)

Figure 13. Linearity of ALA-dehydratase activity with enzyme concentration for the purified enzyme (specific activity = 10.3 μmol/h/mg).

FIGURE 13

EFFECT OF ENZYME CONCENTRATION
ON ALA-DEHYDRATASE ACTIVITY

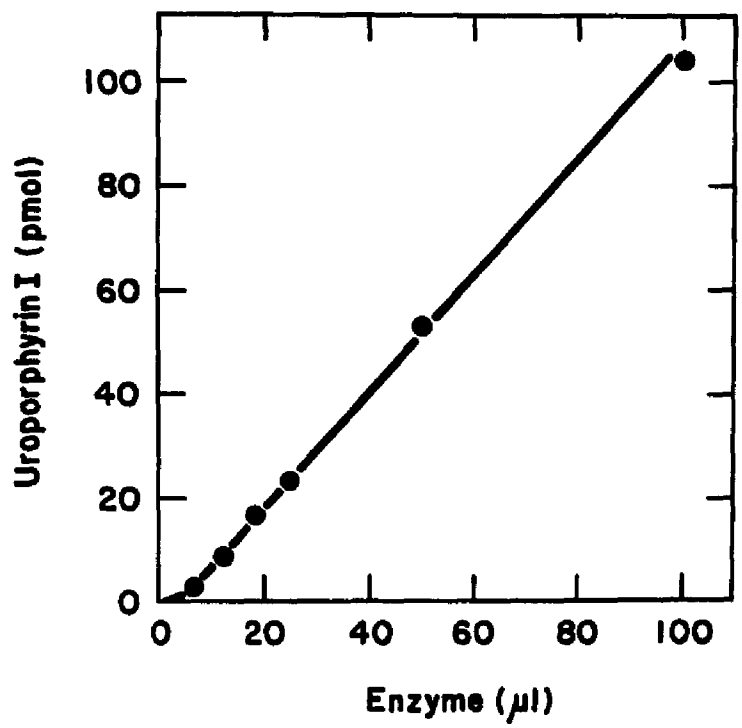


Figure 14. Linearity of ALA-dehydratase activity with time for purified enzyme. ALA-dehydratase (0.36 U/assay) was assayed for various times under the conditions described in "Methods". The indicated values represent the mean of duplicate determinations and are expressed as pmol URO/assay.

FIGURE 14

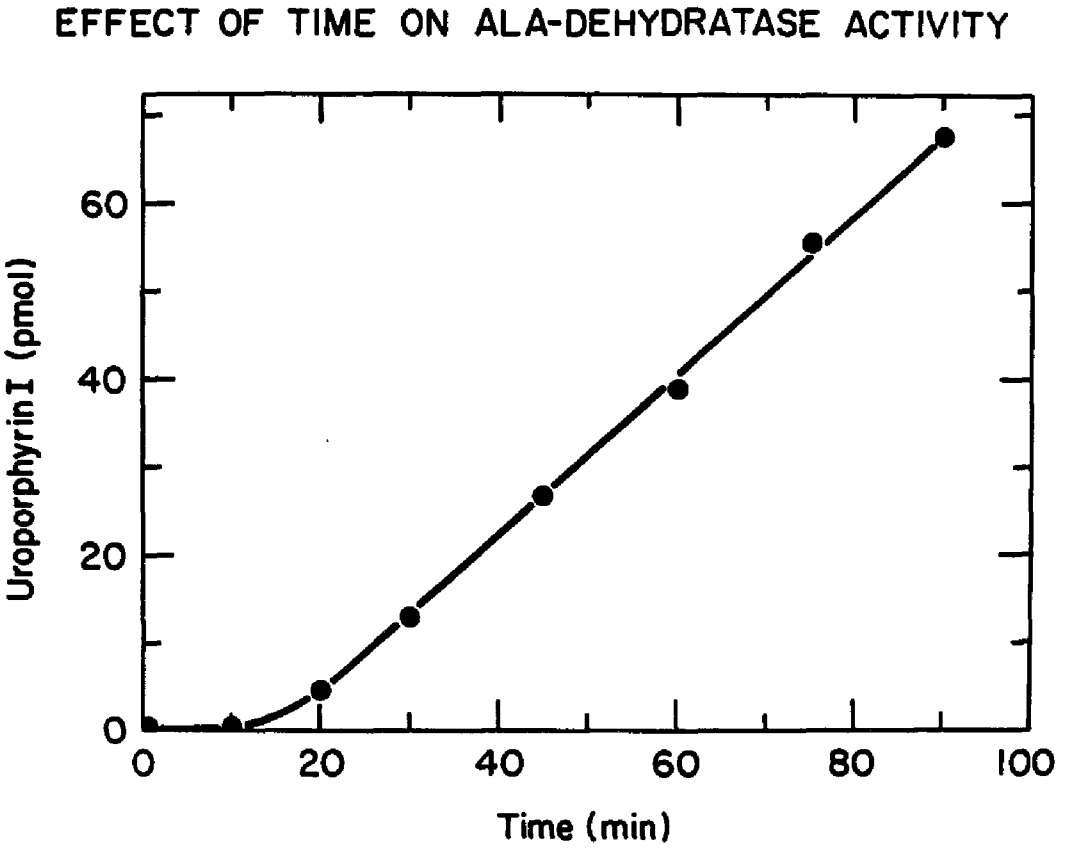
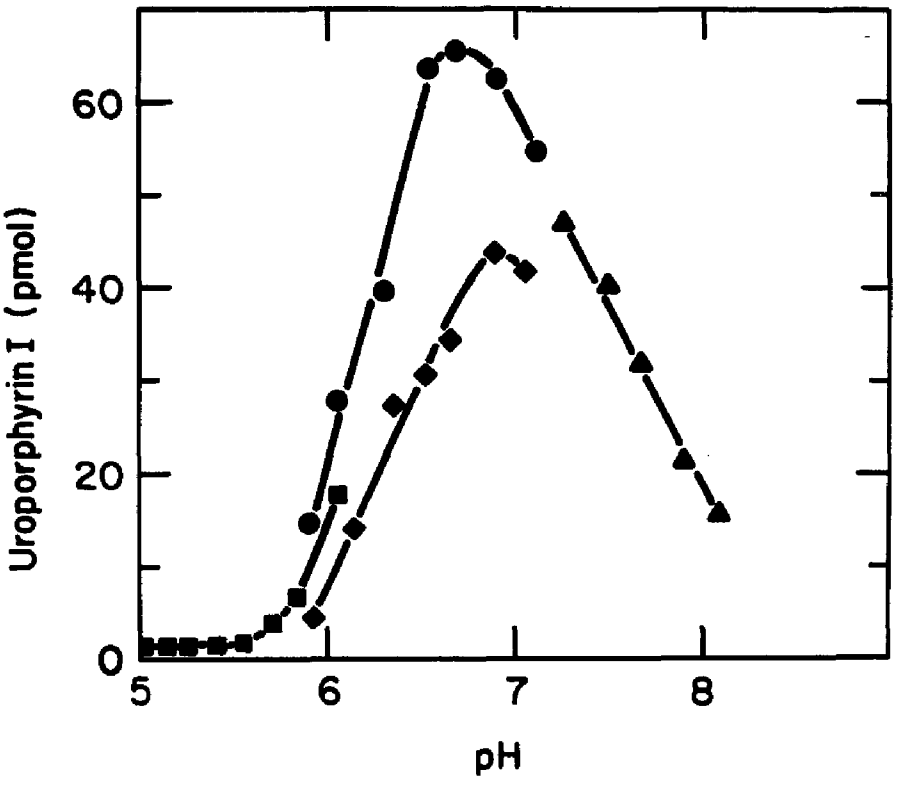

EFFECT OF TIME ON ALA-DEHYDRATASE ACTIVITY
Uroporphyrin I (pmol)
0
20
40
60
0
20
40
60
80
100
Time (min)

Figure 15. Effect of pH on ALA-dehydratase activity in the coupled-enzyme assay. Purified human ALA-dehydratase (0.55 U/assay) was used. The assay was performed as described in "Methods". The pH range was from 5.0 to 8.2 using 0.12 M sodium succinate (■), sodium Pipes (◆), sodium phosphate (●) or sodium Hepes (▲) buffer. The pH was measured at 23°C in the final assay mixture.

FIGURE 15

EFFECT OF pH ON ALA-DEHYDRATASE ACTIVITY



E. Comparison with the Colorimetric Assays:

A human erythrocyte lysate was used as enzyme source to compare the sensitivity of the fluorometric coupled-enzyme assay with the standard (44,50) and semi-micro (42) colorimetric methods. Decreasing volumes of an erythrocyte lysate were assayed in triplicate by each method. Table 7 shows the mean enzyme activity and the coefficient of variance for the triplicate determinations of each lysate volume. In general, the semi-micro colorimetric method gave slightly higher activities than the standard colorimetric or fluorometric methods for the same volume of erythrocyte lysate assayed (e.g., when 10.0 µl of lysate was assayed, the mean activities were 2.28, 2.86 and 2.11 nmol/h, for the standard colorimetric, semi-micro colorimetric and fluorometric procedures, respectively). As shown in Figure 16, the standard colorimetric assay was linear up to about 5 µl of lysate, while the semi-micro colorimetric method was linear up to 2 µl of lysate (Fig. 16 B). Not only did the range of the triplicate determinations for the semi-micro assay increase markedly, but the assay lost linearity when 1.0 µl or less lysate was assayed. This lack of sensitivity and reproducibility also was reflected by the increasing coefficients of variance for the semi-micro determinations when 1.0 µl or less lysate was assayed (Table 7). In contrast, the fluorometric assay was linear over the entire range assayed, from from 0.06 µl to 10 µl of lysate (Fig. 16 B). In addition, the sensitivity of the fluorometric coupled-enzyme assay could be increased by at least a factor of four by increasing the volume of the enzyme source in the assay. Furthermore, increased sensitivity could be obtained by the use of a Perkin-Elmer Model 204-A fluorometer.

Table 8 compares the reproducibility of the three methods when the

ALA-dehydratase activity was determined in six replicate aliquots of the same human erythrocyte lysate. The standard errors of the mean for the standard colorimetric, semi-micro colorimetric and fluorometric methods were 0.25, 0.41 and 0.028, respectively, indicating that the fluorometric assay was markedly more reproducible than either colorimetric procedure.

F. ALA-Dehydratase Activity in Selected Sources:

Table 9 compares the ALA-dehydratase activities in various tissue and cell sources when determined by the standard colorimetric, semi-micro colorimetric and fluorometric assays. Comparable results were obtained using all three methods for human erythrocytes, rat liver, Friend erythroleukemia cells and mouse RAG cells. However, the fluorometric assay proved more sensitive than either colorimetric method, permitting the measurement of ALA-dehydratase activity in cultured human skin fibroblasts and amniotic cells. When 1×10^5 cultured fibroblasts or 1×10^6 cultured amniotic cells were lysed in an equal volume of buffer, no activity was detected in the supernatants when assayed by either colorimetric method. In contrast, reliable ALA-dehydratase activities were obtained by the fluorometric assay in human fibroblast lysates from 3×10^5 to 1×10^6 cells (90-225 µg cell protein) and in cultured amniotic cells, provided the lysate was prepared from at least 6×10^5 cells (greater than 90 µg cell protein).

Figure 16. Comparison of the sensitivity standard colorimetric (A), semi-micro colorimetric (B) and fluorometric coupled enzyme (C) assays for ALA-dehydratase.

FIGURE 16

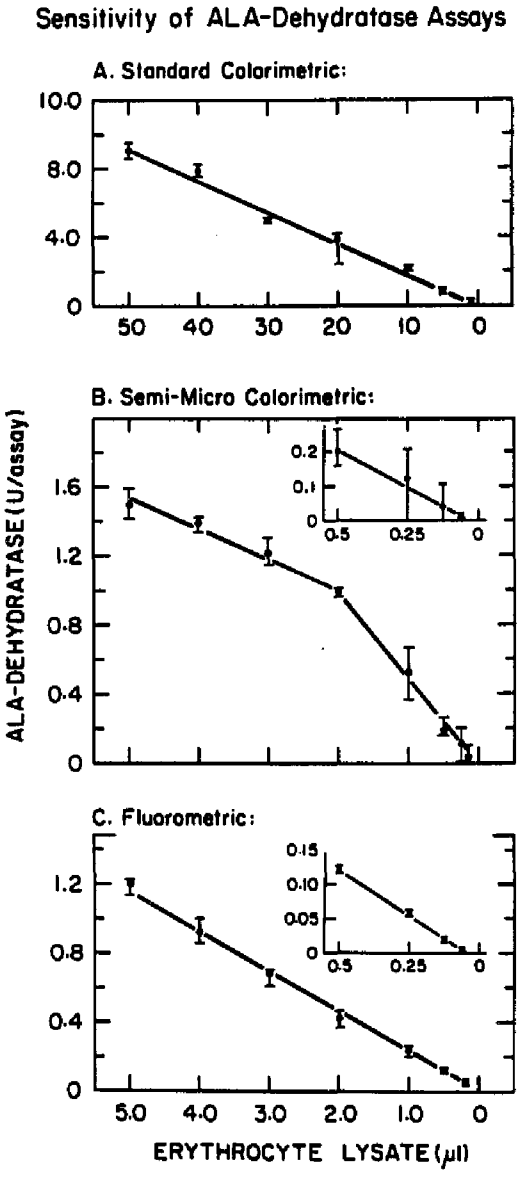

Sensitivity of ALA-Dehydratase Assays
A. Standard Colorimetric:
10.0
8.0
4.0
0
50 40 30 20 10 0
B. Semi-Micro Colorimetric:
ALA-DEHYDRATASE (U/assay)
1.6
1.2
0.8
0.4
0
0.2
0.1
0
0.5 0.25 0
C. Fluorometric:
1.2
0.8
0.4
0
0.15
0.10
0.05
0
0.5 0.25 0
5.0 4.0 3.0 2.0 1.0 0
ERYTHROCYTE LYSATE (µl)

Table 7

Comparison of the Sensitivity of the Colorimetric and Fluorometric Assays[a]

Erythrocyte Lysate/Assay (µl)	Standard Colorimetric Assay		Semi-Micro Colorimetric Assay		Fluorometric Coupled Enzyme Assay	
	Activity (U)	Coefficient[b] of Variance	Activity (U)	Coefficient[b] of Variance	Activity (U)	Coefficient[b] of Variance
50.0	8.99	0.046				
40.0	7.86	0.034				
30.0	5.90	0.048				
20.0	3.95	0.14				
10.0	2.28	0.023	2.86	0.011	2.11[c]	0.07
5.0	0.86	0.14	1.49	0.032	1.20[c]	0.03
4.0			1.39	0.030	0.92[c]	0.07
3.0			1.21	0.052	0.64	0.06
2.0			0.98	0.030	0.41	0.092
1.0	0.21	0.71	0.52	0.14	0.24	0.083
0.5	ND[d]		0.20	0.25	0.120	0.023
0.25	ND		0.12	0.73	0.057	0.072
0.12	ND		0.036	1.40	0.019	0.11
0.06	ND		0.018	1.40	0.004	0.35

[a] ALA-dehydratase activity was determined in decreasing dilutions of the same human erythrocyte lysate by the colorimetric and fluorometric assays; each observed value represents the mean of triplicate determinations.

[b] The coefficient of variance was calculated for the triplicate determinations of each value.

[c] The enzyme source was diluted with buffer in order to remain within the linearity range of 10 to 100 pmol uroporphyrinogen I produced (see Fig. 12).

[d] No detectable activity.

Table 8

Reproducibility of the Colorimetric and Fluorometric Assays for ALA-Dehydratase Activity in Human Erythrocytes[a]

	Enzymatic Activity (U/mg lysate protein)		
	Standard Colorimetric Assay	Semi-Micro Colorimetric Assay	Fluorometric Coupled Enzyme Assay
Mean (n=6)	2.84	2.71	3.17
Range	2.01-3.73	1.39-4.16	3.00-3.49
S.E.M.	0.25	0.41	0.028

[a] ALA-dehydratase activity was determined in replicate aliquots of the same human erythrocyte lysate by each of the assay procedures as described in "Methods." The lysate was diluted 1:5 for the standard and semi-micro colorimetric assays and 1:25 for the fluorometric coupled enzyme assay. For comparison, enzymatic activity was expressed as U/mg protein in the undiluted erythrocyte lysate. The standard error of the mean (S.E.M.) was calculated for six replicate determinations by each procedure.

Table 9

Levels of ALA-Dehydratase Activity in Various Sources Determined by Colorimetric and Fluorometric Assays

Enzyme Source	Enzymatic Activity (U/mg protein)		
	Standard Colorimetric Assay	Semi-Micro Colorimetric Assay	Fluorometric Coupled Enzyme Assay
Human Erythrocytes			
Mean ± 1 S.D.	4.69 ± 1.65	5.34 ± 2.62	4.80 ± 1.71
Range (n = 12)	2.87 - 9.28	4.52 - 13.4	3.13 - 9.56
Rat Liver[a]			
Mean ± 1 S.D.	7.20 ± 2.40	8.04 ± 1.70	7.40 ± 2.09
Range (n = 3)	4.10 - 9.98	5.65 - 9.53	4.56 - 9.53
Friend Erythroleukemia Cells (line 586)[b]			
Uninduced	20.6	22.2	19.3
DMSO-Induced	92.0	108	92.2
Mouse RAG Cells	13.7	14.5	11.5
Cultured Human Skin Fibroblasts			
Mean ± 1 S.D.	ND[c]	ND[c]	1.10 ± 0.62
Range (n = 6)			0.63 - 1.97
Cultured Human Amniotic Cells			
Mean ± 1 S.D.	ND[d]	ND[d]	0.488 ± 0.132
Range (n = 3)			0.340 - 0.661

[a] Sprague-Dawley Rats (125-150 gm) were fed *ad lib*.

[b] Values are the means of triplicate assays before and after induction with 2% DMSO.

[c] No detectable activity for 10^6 and 10^5 cells lysed 1:1 (v/v) with buffer for the standard and semi-micro colorimetric assays, respectively.

[d] No detectable activity for 0.6×10^6 cells lysed 1:1 (v/v) with buffer for both colorimetric assays.

IV. ALA-Dehydratase Variants:

A. Starch Gel Electrophoretic Separation of the 1-1, 1-2 and 2-2 ALA-Dehydratase Phenotypes and Determination of Their Frequency in Jewish and Non-Jewish Populations:

Figure 17 shows the electrophoretic separation of the 1-1, 1-2 and 2-2 ALA-dehydratase electrophoretic variants. The least anodal or 1-1 phenotype was found to be most common in the general population. Table 10 displays the frequencies of each of the three phenotypes in the Ashkenazi Jewish and non-Jewish population from a sample size of 120 individuals. The frequencies of each phenotype are in agreement with those reported by Petrucci et al. for an Italian population (66). It is also notable that the frequency of each phenotype was essentially the same in the Jewish and non-Jewish populations. Further studies were undertaken to characterize the differences between the three different phenotypes.

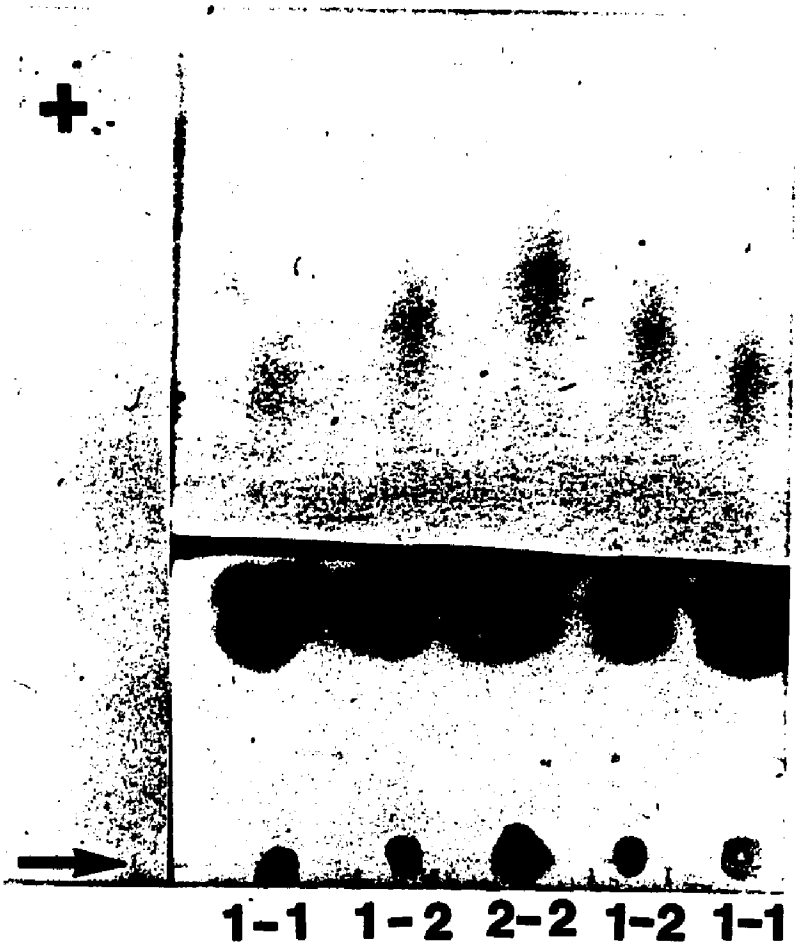
Table 10

Distribution of Human ALA-Dehydratase Phenotypes in Various Populations

Sample	N	Phenotype Frequency 1-1	2-1	2-2	Genotype 1 Frequency
Italian:					
(Petrucci et al., 1982)	762	0.81	0.17	0.02	0.90
Mount Sinai (NYC):					
Total Population	120	0.74	0.24	0.02	0.83
Ashkenazi Jewish	73	0.70	0.26	0.04	0.84
Caucasian, Non-Jewish	37	0.81	0.19	-	0.90
Other	10	0.80	0.20	-	0.89

Figure 17. Starch gel electrophoretic profile demonstrating the differences in mobility between 1-1, 1-2 and 2-2 ALA-dehydratase phenotypes.

FIGURE 17



B. Crossed Immunoelectrophoresis of 1-1, 1-2 and 2-2 ALA-Dehydratase Isozymes:

Aliquots of erythrocyte lysates from 1-1, 1-2 and 2-2 individuals were subjected to crossed immunoelectrophoresis. Figure 18 shows the profile observed for each type following staining with Coomassie Blue. It is noteworthy that the peaks for each phenotype lie in slightly different positions; the 1-2 peak having a position intermediate to those of the 1-1 and 2-2 ALA-dehydratase phenotypes.

C. DEAE-Cellulose chromatogrpahy of the 1-1 and 2-2 ALA-Dehydratase Isozymes:

Human erythrocyte lysates obtained from individuals with a 1-1 and 2-2 isozymes were subjected to DEAE-cellulose chromatography. ALA-dehydratase activity was eluted from the column by application of a 0.1 to 0.3 M NaCl gradient. Under these conditions both types were readily separated. As shown in Table 11 the 1-1 peak occurred at fraction 60 which corresponded to 0.18 M NaCl. The 2-2 peak occurred at fraction 100 which corresponded to 0.20 M NaCl. The fractions containing enzymatic activity were then concentrated and further purified by ammonium sulfate fractionation, octyl and phenyl Sepharose chromatography. K_m and thermostability studies were then performed on the purified lysates.

Table 11

Physical and Kinetic Properties of the 1-1 and 2-2 ALA-Dehydratase Isozymes from Human Erythrocytes

	1-1	2-2
Starch gel electrophoretic mobility	cathodal	anodal
NaCl concentration of peak from DEAE-cellulose chromatography	0.18 M	0.20 M
K_m (post-phenyl Sepharose)	0.76 mM	0.76 mM
Thermostability at 50°C ($t_{1/2}$)	>240 min	>240 min
Thermostability at 70°C ($t_{1/2}$)	∿15 min	∿30 min

D. K_m Studies of 1-1 and 2-2 ALA-Dehydratase Isozymes:

The K_m values for the 1-1 and 2-2 ALA-dehydratase isozymes were determined by the fluorometric coupled enzyme assay. The final concentration of ALA in the assay varied from 1.65 mM to 2.77 μM (data not shown). As indicated in Figure 19, both the 1-1 and 2-2 ALA-dehydratase isozymes had identical x-intercepts in a Lineweaver-Burke plot, indicating that the K_m values for both isozymes were essentially the same.

E. Thermostability of the 1-1 and 2-2 ALA-Dehydratase Isozymes:

Aliquots of the partially purified 1-1 and 2-2 ALA-dehydratase isozymes were preincubated at either 50°C or 70°C for 30 min, 1 h, 2 h and 4 h intervals and ALA-dehydratase activity was then determined by the fluorometric assay. The 1-1 and 2-2 enzymes behaved identically at 50°C. However, as shown in Figure 20 the 1-1 enzyme was considerably less thermostable at 70°C than the 2-2 enzyme. The 2-2 enzyme had a $t_{1/2}$ of 30 min and the 1-1 enzyme had a $t_{1/2}$ of 15 min at 70°C.

Figure 18. Crossed immunoelectrophoresis of 1-1, 1-2 and 2-2 ALA-dehydratase phenotypes.

FIGURE 18

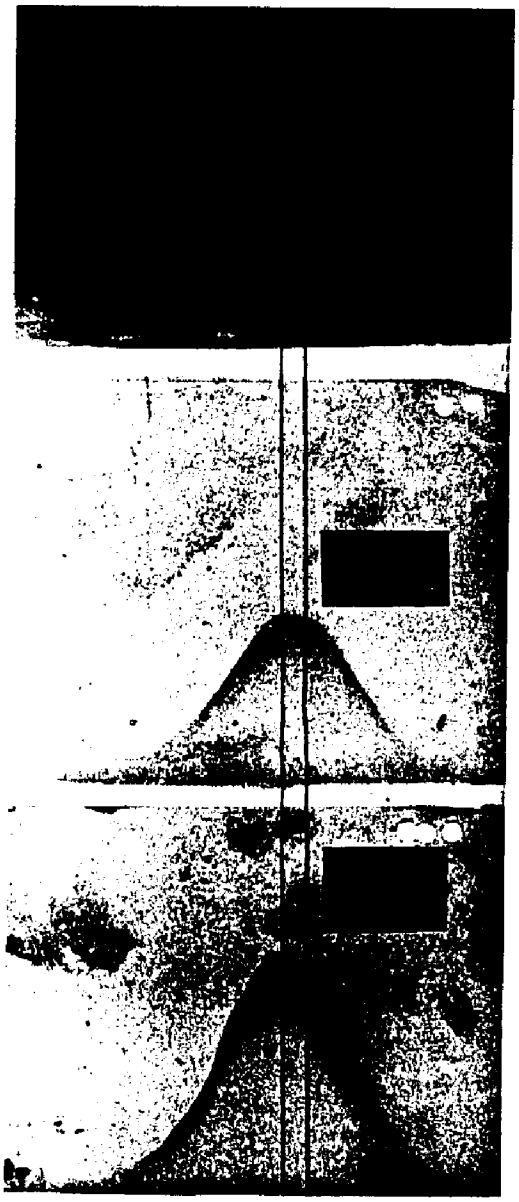
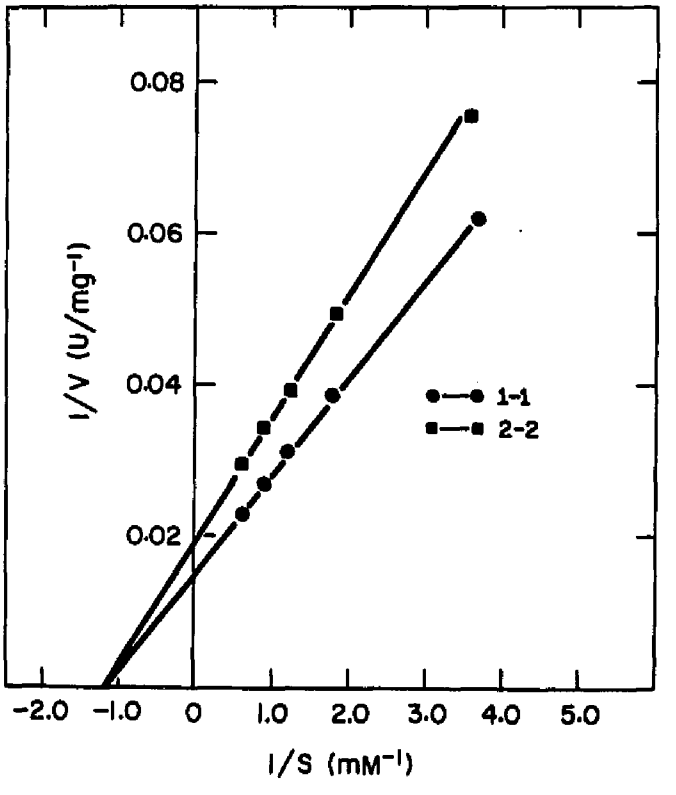


Figure 19. Lineweaver-Burke plot of partially purified ALA-dehydratase in erythrocytes obtained from phenotype 1-1, 1-2 and 2-2 individuals.

FIGURE 19

Lineweaver-Burk Plot of Partially Purified ALA-Dehydratase
Phenotypes 1-1 and 2-2 from Human Erythrocytes



F. ALA-Dehydratase Deficiency:

Bird et al. (67) reported a family with an inherited deficiency of erythrocyte ALA-dehydratase activity. Individuals with this deficiency had about 30% of normal mean erythrocyte ALA-dehydratase activity. When equal activities from a normal and ALA-dehydratase deficient erythrocyte lysate were subjected to rocket immunoelectrophoresis the rocket formed by the ALA-dehydratase deficient lysate was 3 times higher than that formed by the normal lysate (Fig. 21). These results indicated that the total amount of ALA-dehydratase protein was essentially the same in both the normal and ALA-dehydratase deficient lysates, since the amount of CRIM in the ALA-dehydratase deficient lysate corresponded to the difference in activity between the normal and mutant lysate.

Figure 20. Thermostability of 1-1 and 2-2 ALA-dehydratase isozymes at 70°C. Aliquots of erythrocyte lysates were heated at 70°C for 30 min, 1 h, 2 h and 4 h intervals. ALA-dehydratase activity was then determined by the fluorometic assay procedure.

FIGURE 20

Thermal Inactivation (70°C) of Partially Purified ALA-Dehydratase Phenotypes 1-1 and 2-2 from Human Erythrocytes

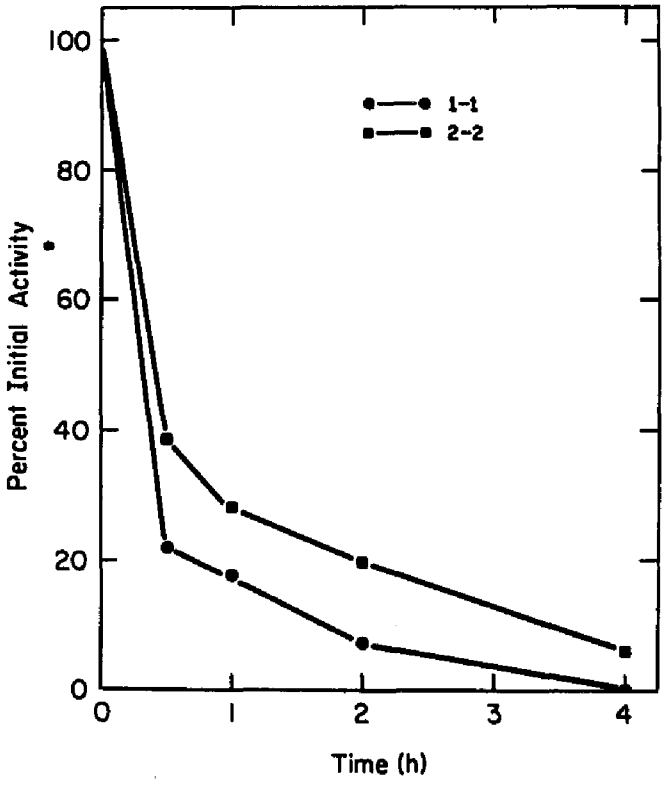


Figure 21. Rocket immunoelectrophoresis of normal and ALA-dehydratase deficient erythrocyte lysates. Normal and ALA-dehydratase deficient erythrocyte lysates were diluted to 0.014 U/ml and subjected to rocket immunoelectrophoresis according to the procedure described in the "Methods" section.

FIGURE 21

M N M N

DISCUSSION

I. Chromosomal Localization of the Gene for Human PBG-Deaminase:

Because knowledge of the genetic organization of the structural genes for each of the heme biosynthetic enzymes might provide insight into the regulation of this pathway in man, efforts were directed to determine the chromosomal localization of the human gene loci for these enzymes. While the studies reported here were in progress, Meisler et al. assigned the gene for human PBG-deaminase to chromosome 11, using hybrids derived from human fibroblasts and mouse RAG, LM/TK, or A9 cells and using isoelectric focusing for detection of human PBG-deaminase (98). The results described here, using different somatic cell hybrids, and rocket immunoelectrophoresis in addition to isoelectric focusing, confirm the assignment of the structural gene for PBG-deaminase to chromosome 11 and further localize the gene to the region q23 → qter.

Further support for this regional assignment has been based on gene dosage studies. de Verneuil et al. have supported the localization of the structural gene for human PBG-deaminase to 11q23 → 11qter by demonstrating a 1.5-fold increase in PBG-deaminase activity in erythrocyte lysates obtained from three children with a partial trisomy of the region 11q23 → 11qter (117).

It was notable that human ESA4 cosegregated with human PBG-deaminase in all hybrid clones (Table 4). The finding that HX/11 subclones were negative for ESA4 implies that the locus had been deleted from this translocation. Although the breakpoint of the X/11 translocation has been interpreted to be 11q23 (153,154), it is not possible to exclude the distal portion of band q22 as the breakpoint. Thus, these findings

and those of previous reports (160-162) imply the localization of ESA4 to the region 11q22 → 11q23.

The 2S MEL-HLN XX-8 subclones also provided data for the finer regional assignments of the genes for PEPS and PGM2 to the regions 4pter → 4q21 and 4q21 → 4qter, respectively (Fig. 8). Consistent with our findings, the smallest region of overlap for human PEPS, based on previous studies (163-165), was 4pter → 4q12. Based on several studies (163-166), the smallest region of overlap for human PGM2 was 4p14 → 4q21 (167). Bocian et al. (165) have assigned the gene to the region 4pter → 4q12. Our data are discrepant with those of Bocian et al. because homogeneous subclone A31-18 was positive for human PGM2 and did not contain the region of their assignment. Based on our findings and those of the other studies, it is likely that human PGM2 is in the region 4q21; further studies of families with 4q aberrations are required to confirm this assignment.

II. Expression of PBG-Deaminase and Human ALA-Dehydratase in RAG-Human Fibroblast Somatic Cell Hybrids:

A. Human PBG-Deaminase:

Quantitation of the percentage of human PBG-deaminase in hybrid H21-H7-10 by rocket immunoelectrophoresis demonstrated that the percentage of human PBG-deaminase activity was about 20-25% of the total enzymatic activity prior to and following DMSO treatment. Since the total enzymatic activity increased by four-fold following induction and the portion of human PBG-deaminase was not increased, it is possible that the factors which mediate induction of the murine PBG-deaminase also were capable of causing induction of the human isozyme. Sassa

(168) has shown that treatment of Friend virus-transformed murine erythroleukemia (1S MEL) cells with DMSO resulted in erythroid differentiation. These cells showed sequential increases in the activities of δ-aminolevulinic acid synthetase (ALAS), ALA-dehydratase, PBG-deaminase and ferrochelatase as well as increased heme concentration after 1, 1.5, 2, 4 and 4 days, respectively. This increase in the activities of the heme biosynthetic enzymes corresponded to the order in which these enzymes act in the heme biosynthetic pathway and is referred to as a sequential induction. It is possible that the human PBG-deaminase present in the 2S MEL-HLN hybrids was also induced along with the mouse PBG-deaminase, by such a mechanism, whatever its nature.

B. Human ALA-Dehydratase:

Twelve RAG-human fibroblast somatic cell hybrids were screened by agarose gel electrophoresis and/or rocket immunoelectrophoresis for the presence of human ALA-dehydratase. Using these methods it was not possible to detect human ALA-dehydratase in any of the hybrids examined.

Based on the observations that human PBG-deaminase was detectable in RAG-human fibroblast hybrids and that human PBG-deaminase accounted for 20-25% of the total PBG-deaminase activity in 2S MEL-human fibroblast hybrids it was not clear why the structural gene for human ALA-dehydratase was not expressed in these hybrids. A possible explanation may be that the gene is expressed but for some reason the human ALA-dehydratase protein cannot be detected by the screening procedure used. Mouse and human ALA-dehydratase are multimeric enzymes with the mouse enzyme being a homohexamer and the human enzyme being a homooctamer (50,73). It has also been demonstrated that depending upon the species,

all (38) or half (39) of the ALA-dehydratase subunit molecules form Schiff's bases with ALA. Finally, it is not firmly established whether ALA-dehydratase is active as a tetramer and/or a dimer, in addition to being active as an octamer (63,64). Since a hybrid expressing human ALA-dehydratase would also be expressing mouse ALA-dehydratase and both enzymes are multimeric, it is reasonable to assume that there could be association of the human and mouse ALA-dehydratase molecules to form a heteromultimer. However, there may be enough structural dissimilarity between the mouse and human ALA-dehydratase to prevent physical association between the two molecules. Even if the different molecules were able to associate with one another, there may be differences in the mechanisms of human and mouse ALA formation, such that the resultant protein is catalytically inactive. Hence, the human ALA-dehydratase protein may be synthesized, but not be capable of associating with the mouse ALA-dehydratase protein to form a catalytically active molecule. Finally, it is possible that once the human and mouse subunits associate to form a heteromultimer, that the human subunits are not capable of being detected by the immune sera. The interaction of mouse ALA-dehydratase subunits with human subunits may mask various domains on the human subunit which are necessary for antibody recognition.

Another possibility for the failure of detecting human ALA-dehydratase is that the ALA-dehydratase is expressed at levels below the limits of detectability of the assay used. The electrophoretic procedure was sensitive enough to detect human ALA-dehydratase when at least 20-25% of the total activity was human and the rocket immunoelectrophoretic procedure was sensitive enough to detect human ALA-dehydratase when at least 3% of the total activity was human. Therefore, if the percentage of

human ALA-dehydratase activity in a particular clone was less that 3%, it would be considered negative by these criterion.

It also is possible that the structural gene for human ALA-dehydratase was suppressed either specifically or nonspecifically after fusion. Bravo et al. (169) using two dimensional electrophoresis have demonstrated that immediately after the fusion of Chinese hamster lung fibroblasts with mouse embryo fibroblasts, all of the Chinese hamster proteins were detected, but only two thirds of the mouse embryo fibroblast proteins were expressed. Analogously, it is possible that fusion of mouse with human cells resulted in a similar loss of expression of human proteins. Finally, it is possible that the human chromosome on which the enzyme is located was lost when the hybrid lines were expanded to provide sufficient amounts of enzyme for detection. Any of these mechanisms could account for the failure to detect the human enzyme.

III. ALA-Dehydratase Coupled Enzyme Assay:

The specific, sensitive and reliable determination of picomole quantities of ALA-dehydratase has been accomplished by the enzymatic conversion of PBG to uroporphyrinogen I and the fluorometric detection of the oxidized URO I (Fig. 10). The ability to obtain large quantities of homogeneous PBG-deaminase from human erythrocytes (84) has made the coupled-enzyme assay practical. This new assay for ALA-dehydratase represents, in principle and practice, a modification of the coupled-enzyme assay for ALA-synthetase previously developed in this laboratory (19).

The following variables were sequentially optimized to maximize the sensitivity of the fluorometric coupled-enzyme assay. First, the amount

of PBG-deaminase required to maximally convert PBG to URO I (up to 80 pmol URO I formed) was determined (Fig. 11). This amount (3.8 U) was similar to that (3.6 U) used in the ALA-synthetase coupled-enzyme assay (19). In both fluorometric assays, the addition of increasing amounts of PBG-deaminase to the assay mixture caused the inhibition of URO I formation. This can be explained by the fact that PBG-deaminase catalyzes the conversion of PBG to the linear tetrapyrrole, $HOCH_2$bilane, by the sequential formation of stable mono-, di-, tri-, and tetra-pyrrole intermediates (77,84). After the individual enzyme-substrate intermediates have been saturated with PBG, any additional PBG formed (by ALA-dehydratase) is linearly converted to URO I. Conversely, if the amount of PBG-deaminase is in excess, it would bind a portion of the PBG formed (by ALA-dehydratase) as enzyme-substrate intermediates (in order to first saturate any free enzyme) rather than convert it to URO I. The requirement of 3.8 U of PBG-deaminase per assay was practical, considering that 1000 ml of packed, outdated human erythrocytes provided approximately 3000 U of PBG-deaminase activity which was sufficient for 750 coupled-enzyme assays.

Second, PBG conversion to URO I had to be established for the amount of PBG-deaminase in the standard reaction mixture. The 47% value for the conversion of PBG to URO I is lower than the value of 62% observed for the ALA-synthase coupled-enzyme assay (19). The 62% conversion value was obtained when PBG was incubated with PBG-deaminase in 0.1 M Hepes buffer, pH 7.4, whereas the 47% conversion value was obtained in 0.25 M sodium phosphate buffer at pH 6.8. Since the pH optimum of erythrocyte PBG-deaminase is 8.2 (84), a decreased conversion of PBG to URO I would be expected at the lower pH. Thus, the final ALA-

dehydratase activity was calculated by multiplying the number of pmol of URO I formed by 4 to convert to pmol of PBG formed and then dividing by 0.47 to correct for the 47% conversion of PBG to URO I.

Third, the linearity of the ALA-dehydratase coupled-enzyme assay was determined. After an initial lag period (Fig. 13), the assay was linear up to 100 pmol of URO I. This lag also was observed in the ALA-synthetase coupled-enzyme assay (19) and reflected the amount of PBG formed which is required to saturate the PBG-deaminase. The lag also was observed when only PBG was incubated with PBG-deaminase (Fig. 12). It is important to note that when determining ALA-dehydratase activity, any reading within this lag period would result in an under-estimation of enzymatic activity. The ALA-dehydratase coupled-enzyme assay also was linear with respect to time, after an initial lag of 20 min (Fig. 14). Incubation for 90 min resulted in accurate determinations of ALA-dehydratase activity, permitting at least 20 determinations in 3 h by a single technician.

The pH optimum for purified human ALA-dehydratase determined by the fluorometric assay was 6.7, a value somewhat higher than the pH optimum of 6.3 reported by Anderson and Desnick (50). This difference was presumably attributable to the PBG-deaminase in the fluorometric assay which had 2 to 3 times more activity at pH 6.7 than at pH 6.3 (87). ALA-dehydratase activity was maximal in sodium phosphate buffer, consistent with previous observations (50).

The chief advantage of the fluorometric assay is its increased sensitivity. As shown in Figure 16, the fluorometric assay was linear at activity levels which were undetectable by the standard colorimetric method and which were no longer in the linear range of detectability for

the semi-micro colorimetric procedure. Thus, the fluorometric coupled-enzyme assay should be useful, particularly for the reliable determination of low levels of ALA-dehydratase activity in cultured cells or in crude tissue homogenates.

IV. ALA-Dehydratase Variants:

The studies reported here confirm and further extend those of Battistuzzi et al. and Petrucci et al. (65,66). The observed frequency distribution of the 1-1, 1-2 and 2-2 ALA-dehydratase phenotypes is similar to those previously reported. The frequency of the most common allele, $ALAD^1$ was 0.83. Moreover, there were no significant differences in the $ALAD^1$ frequency among the Jewish and non-Jewish populations. Petrucci et al. (66) observed similar frequencies for the $ALAD^1$ allele in individuals from the 8 different cities in Italy which he studied. The observed differences in peak positions after crossed immunoelectrophoresis further confirms the charge differences among the various ALA-dehydratase phenotypes. However, the DEAE-cellulose chromatographic profiles demonstrated the most striking difference in charge among the 1-1 and 2-2 electrophoretic phenotypes. No differences were observed in the K_m of the 1-1 and 2-2 ALA-dehydratase isozymes, although the ALA-dehydratase 2-2 isozyme displayed a slightly greater thermostability at 70°C than the 1-1 ALA-dehydratase isozyme.

Meisler and Carter (98) have screened 951 individuals, representing five human ethnic groups, for PBG-deaminase variation by isoelectric focusing. Only four individuals (less than 0.5% of the population studied) were found to have a variant PBG-deaminase isoelectric focusing pattern. This stands in contrast to the high degree of electrophoretic

variation observed for ALA-dehydratase (20% of the population studied possessing either the ALA-dehydratase 2-2 or 1-2 isozyme). These data suggest that PBG-deaminase is a highly conserved enzyme among the human population, whereas ALA-dehydratase is less conserved among the human population.

Further insight has been obtained with respect to the molecular nature of the enzymatic defect in ALA-dehydratase deficiency. When equal activities of ALA-dehydratase obtained from a normal and an ALA-dehydratase deficient lysate were subjected to rocket immunoelectrophoresis, the ALA-dehydratase deficient rocket was 2 to 3 times higher than the rocket formed by normal ALA-dehydratase. These studies indicated that there was 2 to 3 times the amount of enzyme protein in the ALA-dehydratase deficient lysate as compared to the normal lysate and that the mutation produced CRIM. Therefore, the total amount of ALA-dehydratase protein (enzymatically active protein and non-enzymatically immunologically reactive protein) in the ALA-dehydratase deficient lysate was equal to the total amount of ALA-dehydratase protein present in a normal erythrocyte lysate. This rules out the possibility that the mutation responsible for ALA-dehydratase deficiency affects the regulation of ALA-dehydratase synthesis, as proposed by Bird et al. (67) and identifies the mutation as a structural gene defect.

SUMMARY AND DIRECTION FOR FUTURE WORK

The chromosomal localization of the gene for human PBG-deaminase represents the first assignment to a specific human chromosome for an enzyme in the heme biosynthetic pathway. Future understanding of the regulation of this pathway could come from a knowledge of the linkage relationships among human heme biosynthetic enzymes. With respect to linkage relationships among heme biosynthetic organisms in other organisms it has been shown in Escherichia coli that the gene for PBG-deaminase is tightly linked to the UROCoS gene; whereas, the ALA-dehydratase gene is distant from these two genes (170). Recently, Antonucci et al. have mapped the structural gene for PBG-deaminase to chromosome 9 in mice (171). Since the Lv locus, encoding the structural gene for mouse ALA-dehydratase has been mapped to mouse chromosome 4 (71), this demonstrates that the ALA-dehydratase locus and the PBG-deaminase locus are not linked in the mouse. Therefore, the regulation of heme biosynthesis in these organisms does not involve close linkage of the heme pathway enzymes. Although it was not possible to detect human ALA-dehydratase in any of the hybrid clones examined, it is anticipated that future studies could provide this information. These studies could incorporate the use of different types of anti-sera, in order to ensure recognition of the human enzyme in the hybrid clone, and/or different mouse/human somatic cell hybrids than the ones used for these studies. The rapid rate of human chromosomal loss and high frequency of chromosomal rearrangements made the use of the 2S MEL-HLN fibroblast hybrids impractical, although they had high levels of total ALA-dehydratase activity. The recent observation of genetic polymorphisms

for human ALA-dehydratase makes it possible to do family studies in order to determine whether or not ALA-dehydratase is linked to other polymorphic proteins.

Coupled enzymatic assays for heme biosynthetic enzymatic activity measurements have been devised previously for ALA-synthetase (19), UROCoS (103) URO-decarboxylase (110) and coproporphyrinogen oxidase (120). The development of a sensitive fluorometric assay procedure for ALA-dehydratase permits accurate measurement of the levels of ALA-dehydratase present in human fibroblasts and cultured amniotic fluid cells. Prior to this it was not possible to measure enzymatic activity in these tissues without using large quantities of cells.

The studies concerning ALA-dehydratase electrophoretic phenotypes confirmed and extended those previously reported by Battistuzzi et al. (65,66). Efforts in this laboratory are now being directed towards further characterization of this enzyme polymorphism by the isolation of monoclonal antibodies capable of distinguishing between the 1-1, 1-2 and 2-2 ALA-dehydratase isozymes.

The molecular nature of the enzymatic defect in ALA-dehydratase deficiency has been demonstrated to be a structural gene mutation resulting in a non-catalytic but immunologically reactive protein. It would be of interest to discern whether the homozygous ALA-dehydratase deficient variants reported by Doss (68,69) represent the same mutation described by Bird (67). One means of determining this could be to measure the amount of ALA-dehydratase protein present in homozygous ALA-dehydratase deficent erythrocyte lysates, and compare it to the level of ALA-dehydratase protein in an ALA-dehydratase deficient heterozygote and a normal erythrocyte lysate. If the amounts of ALA-dehydratase protein

are the same in all three cases, this would provide evidence for a single mutation which reduces ALA-dehydratase activity in the heterozygote state and results in a hepatic porphyria syndrome in the homozygous state.

BIBLIOGRAPHY

1. Whiting, M. and Granick, S., J. Biol. Chem. 251:1347, 1976.

2. Hayashi, N., Kurashima, Y. and Kikuchi, G., Arch. Biochem. Biophys. 148:10, 1972.

3. Brooker, J., May, B. and Elliot, W., Eur. J. Biochem. 106:17, 1980.

4. Yamauchi, K., Hayashi, N. and Kikuchi, G., J. Biol. Chem. 255:1746, 1980.

5. Granick, J.L. and Sassa, S., J. Biol. Chem. 253:5402, 1978.

6. Rutherford, T., Thompson, G.G. and Moore, M.R., Proc. Natl. Acad. Sci. USA 76:833, 1979.

7. Strand, L.J. and Marver, H., Clin. Res. 18:345, 1970.

8. Bickers, D.R., J. Inv. Dermatol. 77:107, 1981.

9. Meyer, U.A. and Schmid, R., in The Metabolic Basis of Inherited Disease, J.B. Stanbury, J.B. Wyngaarden, D.S. Fredrickson, eds., McGraw-Hill, New York, p. 1166, 1978.

10. Granick, S. and Urata, G., J. Biol. Chem. 238:821, 1963.

11. Granick, S., J. Biol. Chem. 241:1359, 1966.

12. Scholnick, P.C., Hammaker, L.E. and Marver, H.S., J. Biol. Chem. 247:4132, 1972.

13. Mauzerall, D. and Granick, S., J. Biol. Chem. 219:435, 1956.

14. Urata, G. and Granick, S., J. Biol. Chem. 238:811, 1963.

15. Marver, H.S., Tschudy, D.P., Perloth, M.G., Collins, A. and Hunter, G., Jr., Anal. Biochem. 14:53, 1966.

16. Irving, E.A. and Elliot, W.H., J. Biol. Chem. 244:60, 1969.

17. Ebert, P.S., Tschudy, D.P., Choudry, J.M. and Chirigos, M.A., Biochim. Biophys. Acta 208:236, 1970.

18. Strand, L.J., Swanson, A.L., Manning, J., Branch, S. and Marver, H.S., Anal. Biochem. 47:457, 1972.

19. Bishop, D.F., Devy, K.R., McBride, L. and Desnick, R.J., Anal. Biochem. 113:68, 1981.

20. DeMatteis, F., Pharmacol. Rev. 19:523, 1967.

21. Granick, S., Sassa, S., in Metabolic Regulation, H.J. Vogel, ed., Academic Press, New York, p. 77, 1971.

22. Marver, H.S., Collins, A., Tschudy, D.P. and Recheigl, M., Jr., J. Biol. Chem. 241:9323, 1966.

23. Hayashi, N., Yoda, B. and Kikuchi, G., Arch. Biochem. Biophys. 131:83, 1969.

24. Scholnick, P.C., Hammaker, L.E. and Marver, H.S., J. Biol. Chem. 247:4126, 1972.

25. Beattie, D.S. and Stuchell, R., Arch. Biochem. Biophys. 134:291, 1970.

26. Whiting, M.J. and Granick, S., J. Biol, Chem. 251:1347, 1976.

27. Brooker, J.D., May, B.K. and Elliott, W.H., Eur. J. Biochem. 106:17, 1980.

28. Lim, L-K., Srivastava, G., Brooker, J.D., May, B.K. and Elliot, W.H., Biochem. J. 190:519, 1980.

29. Tephly, T.R., Gibbs, A.H. and DeMatteis, F., Biochem. J. 180:241, 1979.

30. DeMatteis, F., Gibbs, A.H. and Tephly, T.R., Biochem. J. 188:145, 1980.

31. Whiting, M.J., Biochem. J. 158:391, 1976.

32. Yamauchi, K., Hayashi, N. and Kikuchi, G., FEBS Lett. 115:15, 1980.

33. Hutton, J.J. and Gross, S.R., Arch. Biochem. Biophys. 141:284, 1970.

34. Gross, S.R. and Hutton, J.J., J. Biol. Chem. 246:606, 1971.

35. Woods, J.S. and Dixon, R.L., Biochem. Pharmac. 21:1735, 1972.

36. Bishop, D.F., Kitchen, H. and Wood, W., Arch. Biochem. Biophys. 206:380, 1981.

37. Granick, S. and Mauzerall, D., J. Biol. Chem. 232:1119, 1958.

38. Nandi, D.L. and Shemin, D., J. Biol. Chem. 243:1236, 1968.

39. Jordan, P.M. and Seehra, J.S., J. Chem. Soc. Chem. Comm. 1:240, 1980.

40. Watson, C.J. and Schwartz, S., Proc. Soc. Exp. Biol. Med. 47:393, 1941.

41. Mauzerall, D. and Granick, S., J. Biol. Chem. 219:435, 1956.

42. Sassa, S., Granick, S., Bickers, S., Levere, R.D. and Kappas, A., Enzyme 16:326, 1973.

43. Coleman, D.L., J. Biol. Chem. 241:5511, 1966.

44. Doyle, D. and Schimke, R.T., J. Biol. Chem. 244:5449, 1969.

45. Doyle, D., J. Biol. Chem. 246:4965, 1971.

46. Wilson, E.L., Burger, P.E. and Dowdle, E.B., Eur. J. Biochem. 29:563, 1972.

47. Gurba, P.E., Sennett, R.E. and Kobes, R., Arch. Biochem. Biophys. 150:130, 1972.

48. Cheh, A. and Neilands, J.B., Biochem Biophys. Res. Commun. 55:1060, 1973.

49. Weissberg, J.B. and Voytek, P.E., Biochem. Biophys. Acta 364:304, 1974.

50. Anderson, P.M. and Desnick, R.J., J. Biol. Chem. 254:6924, 1979.

51. Wu, W.H., Shemin, D. and Richard, K.E., Proc. Natl. Acad. Sci. USA 71:1767, 1974.

52. Tsukamoto, I., Yoshinga, T. and Sano, S., Biochem. Biophys. Res. Commun. 67:294, 1975.

53. Shemin, D., Phil. Trans. R. Soc. Land. B. Biol. Sci. 273:109, 1976.

54. Beran, D.R., Bodlaender, P. and Shemin, D., J. Biol. Chem. 235:2030, 1980.

55. Tsukamoto, I., Yoshinaga, T. and Sano, S., Biochim. Biophys. Acta 570:167, 1979.

56. Lichtman, H.C. and Feldman, F., J. Clin. Invest. 42:830, 1963.

57. Collier, H.B., Clin. Biochem. 4:222, 1971.

58. Granick, J.L., Sassa, S., Granick, S., Levere, R.D. and Kappas, A., Biochem. Med. 8:149, 1973.

59. Lindblad, B., Lindstedt, S. and Stern, G., Proc. Natl. Acad. Sci. USA 74:4641, 1971.

60. Ebert, P.S., Hess, R.A., Frykholm, B.C. and Tschudy, D.P., Biochem. Biophys. Res. Commun. 88:1382, 1979.

61. Tschudy, D.P., Hess, R.A. and Frykholm, B.C., J. Biol. Chem. 256:9915, 1981.

62. Kondo, M., Kajimoto, M., Kimura, H., Suzuki, T., Sasaki, A., Niwa, M. and Urata, G., Arch. Biochem. Biophys. 208:189, 1981.

63. Gurne, D., Chen, J. and Shemin, D., J. Biol. Chem. 74:1383, 1977.

64. Battle, A.M.C., Stella, A.M., Ferramola, A.M., Supena, Y., Xifra, E.A.W. and Sancovich, H.A., Int. J. Biochem. 9:401, 1978.

65. Battistuzzi, G., Petrucci, R., Silvangi, L., Urbani, F.R. and Caiola, S., Ann. Hum. Genet. 45:223, 1981.

66. Petrucci, R., Leonardi, A. and Battistuzzi, G., Hum. Genet. 60:289, 1982.

67. Bird, R.D., Hamernyik, D., Nutter, J.Y. and Labbe, R.F., Am. J. Hum. Genet. 31:662, 1979.

68. Doss, M., von Tiepermann, R., Schneider, J. and Schmidt, H., Klin. Wochenschr. 57:1123, 1979.

69. Doss, M., Schneider, J., von Tiepermann, R. and Brandt, A., Clin. Biochem. 15:52, 1982.

70. Russell, R.L. and Coleman, D.L., Genetics 48:1033, 1963.

71. Hutton, J.J. and Coleman, D.L., Biochem. Genet. 3:517, 1969.

72. Sassa, S. and Bernstein, S., Proc. Natl. Acad. Sci. USA 74:1181, 1977.

73. Doyle, D., J. Biol. Chem. 246:4965, 1971.

74. Sassa, S., Granick, S., Bickers, D.R., Levere, R.D. and Kappas, A., Enzyme 16:326, 1973.

75. Anderson, K.E., Sassa, S., Peterson, C. and Kappas, A., Amer. J. Med. 63:359, 1977.

76. Bogorad, L., J. Biol. Chem. 233:501, 1958.

77. Battersby, A., Fookes, C., Matcham, G. and McDonald, E., Nature (London) 285:17, 1980.

78. Battersby, A., Fookes, J., McDonald, E. and Meegan, M., J.C.S. Chem. Commun. 5:185, 1978.

79. Jordan, P. and Berry, A., FEBS Lett. 112:86, 1980.

80. Jordan, P.M. and Shemin, D., J. Biol. Chem. 248:1019, 1973.

81. Davies, R.C. and Neuberger, A., Biochem. J. 133:471, 1973.

82. Higuchi, M. and Bogorad, L., Ann. N.Y. Acad. Sci. 294:401, 1975.

83. Sancovich, H.A., Battle, A.M.C. and Grinstein, M., Biochim. Biophys. Acta 158:496, 1969.

84. Anderson, P.M. and Desnick, R.J., J. Biol. Chem. 255:1993, 1980.

85. Sassa, S., Granick, S., Bickers, D.R., Bradlow, H.L. and Kappas, A., Proc. Natl. Acad. Sci. USA 71:732, 1975.

86. Strand, L., Felsher, B., Redeker, A. and Marver, H., Proc. Natl. Acad. Sci. USA 67:1315, 1970.

87. Strand, L., Meyer, U., Felsher, B., Redeker, A. and Marver, H., J. Clin. Invest. 51:2530, 1972.

88. Meyer, U.A., Strand, L., Doss, M., Rees, C.A. and Marver, H.S., N. Engl. J. Med. 286:1277, 1972.

89. Magnussen, C.R., Levine, J.B., Doherty, J.M., Cheeseman, J.O. and Tschudy, D.P., Blood 44:857, 1974.

90. Bonkowski, H.L., Tschudy, D.P., Weinbach, E.C., Ebert, P.S. and Doherty, J.M., J. Lab. Clin. Med. 85:93, 1975.

91. Sassa, S., Solish, G., Levere, R.D. and Kappas, A., J. Exp. Med. 142:722, 1975.

92. Sassa, S., Zalar, G.L. and Kappas, A., J. Clin. Invest. 61:499, 1978.

93. Anderson, P.M., Reddy, R.M., Anderson, K.E. and Desnick, R.J., J. Clin. Invest. 68:1, 1981.

94. Mustajoki, P., Ann. Int. Med. 95:162, 1981.

95. Meisler, M., Wanner, L., Eddy, R. and Shows, T., Biochem. Biophys. Res. Commun. 95:170, 1980.

96. Meisler, M.H., Wanner, L., Kao, F.T. and Jones, C., Cytogenet. Cell Genet. 31:124, 1981.

97. Wang, A.L., Arredondo-Vega, F.X., Giampietro, P.F., Smith, M., Anderson, W.F. and Desnick, R.J., Proc. Natl. Acad. Sci. USA 78:5734, 1981.

98. Meisler, M.H. and Carter, M.L.C., Proc. Natl. Acad. Sci. USA 77:2848, 1980.

99. Bogorad, L., J. Biol. Chem. 233:510, 1958.

100. Burton, G., Fagerness, P.E., Hosozawa, S., Jordon, P.M. and Scott, A.I., J. Chem. Soc. Chem. Commun. 1:202, 1979.

101. Chu, T.C. and Chu, E.J., J. Biol. Chem. 227:505, 1957.

102. Battersby, A.R., Buckly, D.G., Hodgson, G.L., Markwell, R.S. and McDonald, E., in High Pressure Liquid Chromatography in Clinical Chemistry, P.F. Nixon, et al., eds., Academic Press, New York, p. 63, 1976.

103. Jordan, P.M., Nordlov, H., Burton, G. and Scott, A.I., FEBS Lett. 115:269, 1980.

104. Frydman, R.B.and Feinstein, G., Biochim. Biophys. Acta 350:358, 1974.

105. Higuchi, M. and Bogorad, L., Ann. N.Y. Acad. Sci. 244:401, 1975.

106. Romeo, G., Glenn, B.L. and Levin, E.Y., Biochem. Genet. 4:719, 1970.

107. Miyagi, K., Petryka, Z.T., Bossenmaier, I., Cardinal, R. and Watson, C.J., Am. J. Hematol. 1:3, 1976.

108. Romeo, G., Kaback, M.M. and Levin, E.Y., Biochem. Genet. 4:659, 1970.

109. Jackson, A.H., Sancovich, H.A., Ferramola, E.N., Ganes, D.E., Matlin, S.A., Elder, G.H. and Smith, S.G., Phil. Trans. R. Soc. Lond. 273:191, 1976.

110. Kardish, R.M. and Woods, J.S., J. Appl. Biochem. 2:159, 1980.

111. Rimington, C., Magnus, I.A., Ryan, E.A. and Cripps, D.J., Q. J. Med. 36:29, 1967.

112. Kushner, J.P., Barbuto, A.J. and Lee, G.R., J. Clin. Invest. 58:1089, 1976.

113. Benedetto, A.V., Kushner, J.P. and Taylor, J.S., N. Engl. J. Med. 298:358, 1978.

114. Felsher, B.F., Carpio, N.M., Engleking, D.W. and Nunn, A.T., N. Engl. J. Med. 306:766, 1982.

115. Elder, G.H., Lee, G.B. and Tovey, J.A., N. Engl. J. Med. 299:274, 1978.

116. Felsher, B.F., Norris, M.E. and Shih, J.C., N. Engl. J. Med. 299:1095, 1978.

117. de Verneuil, H., Aitken, G. and Nordmann, Y., Hum. Genet. 44:145, 1978.

118. Yoshinaga, T. and Sano, S., J. Biol. Chem. 255:4722, 1980.

119. Yoshinaga, T. and Sano, S., J. Biol. Chem. 255:4727, 1980.

120. Kardish, R.M. and Woods, J.S., J. Appl. Biochem. 2:168, 1980.

121. Elder, G.H., Evans, J.O., Cox, T.N., Brodie, M.J., Moore, M.R., Goldberg, A. and Nicholson, P.C., Lancet 2:1217, 1976.

122. Poulson, R., J. Biol. Chem. 251:3730, 1976.

123. Brenner, D.A. and Bloomer, J.R., Clin. Chimica. Acta 100:259, 1980.

124. Brenner, D.A. and Bloomer, J.R., N. Engl. J. Med. 302:765, 1980.

125. Mustajoki, P., Ann. Int. Med. 89:238, 1978.

126. Deybach, J.Ch., de Verneuil, H. and Nordmann, Y., Hum. Genet. 58:425, 1981.

127. Dean, G., in The Porphyrias: A Story of Inheritance and Environment, 2nd edition, Pittman Medical, London, pp. 103-113, 1971.

128. Bonkowsky, H.L., Bloomer, J.R., Ebert, P.S. and Mahoney, M.J., J. Clin. Invest. 56:1139, 1975.

129. Taketani, S. and Tokunaga, R., J. Biol. Chem. 256:12748, 1981.

130. Bottomley, S.S., Tanaka, M. and Everett, M.A., J. Lab. Clin. Med. 86:126, 1975.

131. de Goeij, F.P.M., Christianase, K. and von Steveninck, I., Eur. J. Clin. Invest. 5:397, 1975.

132. Bonkowsky, H.L., Bloomer, J.R., Ebert, P.S. and Mahoney, M.J., J. Clin. Invest. 56:1139, 1975.

133. Sassa, S. Zalar, G.L., Poh-Fitzpatrick, M.B., Anderson, K.E. and Kappas, A., J. Clin. Invest. 69:809, 1982.

134. Bloomer, J.R., Morton, K.O., Reutler, R.J. and Ruth, G.R., Am. J. Hum. Genet. 34:322, 1982.

135. Rutherford, T., Thompson, G.G. and Moore, M.R., Proc. Natl. Acad. Sci. USA 76:833, 1979.

136. Shows, T.B. and Sakaguchi, A.Y., In Vitro 16:55, 1980.

137. Willing, M., Neinhuis, A. and Anderson, W., Nature 277:534, 1979.

138. Axelrod, D., Gopalkrishnan, T., Willing, M. and Anderson, W.F., Somatic Cell Genet. 4:157, 1976.

139. National Research Council Committee on Specifications and Criteria for Biochemical Compounds (1972). Specifications and Critera for Biochemical Compounds, National Academy of Sciences, Washington, D.C.

140. Schwartz, S., Berg., M.H., Bossenmaier, I. and Dinsmore, H., Meth. Biochem. Anal. 8:221, 1960.

141. Bohler, P., Stein, S. Dairman, W. and Underfriend, S., Arch. Biochem. Biophys. 155:213, 1973.

142. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem. 193:265, 1951.

143. Harboe, N. and Ingeld, A., Scand. J. Immunol. 2, Suppl. 1:161, 1973.

144. Guzman, N.A., Oronsky, A.L., Suarez, G., Meyerson, L.R., Cutroneo, K.R., Olson, B.R. and Prockop, D.J., Collagen Col. Res. 2:365, 1982.

145. Winter, A., Ek, K. and Anderson, U., LKB Application Note 250, 1977.

146. Alpert, E., Coston, R. and Perrotto, J., Lancet 1:626, 1974.

147. Ainbender, E., Brown, E., Kierney, L. and Hirschhorn, K., in Prevention of Neural Tube Defects, B.F. Crandell, M.A. Brazier, eds., Academic Press, New York, 1978.

148. Harris, H. and Hopkinson, D., in Handbook of Enzyme Electrophoresis in Human Genetics, North-Holland, Amsterdam, 1976.

149. van Someren, H., van Henegouwen, H., Los, W., Wurzer-Figurelli, E., Doppert, B., Vervloet, M. and Meera Khan, P., Humangenetik 25:189, 1974.

150. George, D. and Franke, U., Cytogenet. Cell Genet. 22:437, 1978.

151. Povey, S., Slaughter, C., Wilson, D., Gormiley, I., Buckton, K., Perry, P. and Bobrow, M., Ann. Hum. Genet. 39:413, 1976.

152. Meera Kahn, P., Wignen, L. and Pearson, P., Cytogenet. Cell Genet. 22:212, 1978.

153. Scott, A., Phillips, S. and Migeon, B., Proc. Natl. Acad. Sci. USA 76:4563, 1979.

154. Rudak, E., Mayer, M., Jacobs, P., Sprenkel, J., Po, T. and Migeon, B., Cytogenet. Cell Genet. 25:198, 1979.

155. Klebe, R.J., Cheh, T-R. and Ruddle, F.H., J. Cell Bio. 45:74, 1970.

156. Smith, M., Gold, P., Freedman, S. and Shuster, J., Ann. Hum. Genet. 39:21, 1975.

157. Friend, K., Dorman, B., Kucherlapati, R. and Ruddle, F., Exp. Cell Res. 99:31, 1976.

158. Breg, W., Stain Technol. 47:87, 1972.

159. Fergusun-Smith, M. and Westerveld, A., Cytogent. Cell Genet. 25:59, 1979.

160. Busby, N., Courval, J. and Francke, U., Cytogenet. Cell Genet. 16:105, 1976.

161. Kao, F. and Puck, T., Proc. Natl. Acad. Sci. USA 69:3273, 1972.

162. Bootsma, D. and McAlpine, P., Cytogenet. Cell Genet. 25:21, 1979.

163. McAlpine, P., Mohandas, T., Komarnicki, L., Nieweczas-Late, V., Vust, A. and Hamerton, J., Cytogenet. Cell Genet. 16:198, 1976.

164. Francke, U. and Brown, S., Cytogenet. Cell Genet. 22:401, 1978.

165. Bocian, M., Mohanadas, T., Sparkes, R., Sparkes, M. and Fundersburk, S., Cytogenet. Cell Genet. 25:138, 1979.

166. Sparkes, R., Mohandas, T., Sparkes, M. and Shulkin, J., Exp. Cell Res. 111:492, 1978.

167. Bootsma, D. and Ruddle, F., Cytogenet. Cell Genet. 22:74, 1978.

168. Sassa, S., J. Exp. Med. 143:305, 1976.

169. Bravo, R., Schafer, R., Willecke, K., MacDonald-Bravo, H., Fey, S.J. and Celis, J.E., Proc. Natl. Acad. Sci. USA 79:2281, 1982.

170. Bachmann, B. and Low, K., Microbiol. Rev. 44:1, 1980.

171. Antonucci, T.K., Chapman, V.C. and Meisler, M.H., Biochem. Genet. 20:703, 1982.