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STUDIES OF HUMAN HEME BIOSYNTHETIC ENZYMES AND ACUTE INTERMITTENT PORPHYRIA

City University of New York PH.D. 1980

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STUDIES OF HUMAN HEME BIOSYNTHETIC ENZYMES AND ACUTE INTERMITTENT PORPHYRIA

by

PETER MEADE ANDERSON

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.

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ABSTRACT

STUDIES OF HUMAN HEME BIOSYNTHETIC ENZYMES

AND ACUTE INTERMITTENT PORPHYRIA

by

Peter Meade Anderson

Advisor: Professor R.J. Desnick

Human δ-aminolevulinic acid dehydratase (ALAD, E.C.4.2.1.24) was purified from human erythrocytes more than 38,000-fold with a 69% yield. The purification procedure included DEAE-cellulose chromatography, ammonium sulfate precipitation, hydrophobic chromatography on octyl- and phenyl-Sepharose and gel filtration. The unusual hydrophobic properties of the enzyme permitted substantial purification (36-fold) using the hydrophobic supports. Purified human ALAD appeared to be homogeneous by disc gel electrophoresis and had a specific activity of 18.5 units/mg protein (one unit of ALAD activity is the amount required to produce 1 μmole of porphobilinogen (PBG) per hour). In the presence of 0.1 mM zinc, the specific activity was 23.8 units/mg protein. Zinc activated the enzyme at 0.1 and 0.02 mM concentrations but inhibited the enzyme at 1 mM concentrations. The K_m of human ALAD was 0.27 mM and the V_{max} was 21.6 units/mg. Activity of enzyme was inhibited noncompetitively by ferrous iron (K_i = 0.26 mM) and competitively by pyridoxal 5'-phosphate (K_i = 0.031 mM). Lead

inhibition (K_i = 0.0017) altered both the K_m and the V_{max} values. The enzyme had a pH optimum of 6.3 to 6.7 and an isoelectric point of 4.9. The molecular weight of the native enzyme was estimated to be 252,000 by gel filtration. The subunit molecular weight as determined by SDS polyacrylamide gel electrophoresis was approximately 31,000 indicating that human ALAD is composed of 8 apparently identical subunits.

Purified human ALAD was immobilized on Sepharose 4B and on phenyl-Sepharose CL-4B and packed into a jacketed column maintained at 37°C. When δ-aminolevulinic acid (ALA) was pumped through the enzyme reactor, gram quantities of PBG were produced. PBG was separated from unreacted ALA using Dowex 1-X8 anion exchange resin and isolated as the acetate or hydrate salt. Homogeneous human ALAD was also sucessfully employed to synthesize [^{14}C]PBG and [^{3}H]PBG. The radiolabeled pyrrole was used to monitor 1) binding of PBG to supports in an attempt to construct an affinity support for human uroporphyrinogen I synthase (UROS, E.C.4.3.1.8) and 2) the production of UROS enzyme-substrate complexes.

Human UROS was purified from erythrocytes more than 42,000-fold with a 25% yield. The purification procedure included a preparative DEAE-cellulose step followed by sequential chromatography on octyl-Sepharose, phenyl-Sepharose, Sephadex G-100 and DEAE-cellulose. The final anion exchange step resolved UROS activity into 5 forms which were designated UROS A, B, C, D and E. These forms also were separated by cellulose acetate and polyacrylamide disc gel electrophoresis

and isoelectric focusing. UROS A and B, the least charged forms, represented about 75% of total activity and appeared homogenous by SDS and analytical polyacrylamide disc gel electrophoresis at pH 6.8 and 8.2. UROS A and B had similar physical and kinetic properties including specific activities of about 2,300 units/mg protein (1 unit of UROS activity is the amount required to produce 1 nmole uroporphyrinogen per hour), pH optima of 8.2, K_m values of about 6 μM, inhibition by sulfhydryl reagents, and identical amino acid compositions which were characterized by the absence of tryptophan and methionine. The molecular weights of UROS A and B were estimated by gel filtration as 36,000 and 38,000 respectively. After SDS polyacrylamide electrophoresis under denaturing conditions, UROS A and B had identical mobilities and a molecular weight of 37,000 indicating that human UROS is a monomeric enzyme.

Radiolabeled PBG was used to demonstrate that the multiple forms of human UROS represented intermediates in the stepwise conversion of the monopyrrole to the tetrapyrrole. When homogenous UROS A and B were incubated with [^{3}H]PBG and then subjected to electrophoresis, all 5 forms of the enzyme were observed; the more anodal bands contained proportionately more radiolabel. These data are consistent with UROS A being the native enzyme with no bound PBG and the UROS B, C, D and E charge isomers corresponding to the enzyme-substrate (mono-, di-, tri-, and tetrapyrrole) intermediates.

The patterns of UROS enzyme-substrate intermediates were investigated in normal erythrocytes, liver and brain, and evi-

dence for a physiologic excess of PBG in liver and brain but not erythrocytes was obtained. When UROS enzyme-substrate patterns in erythrocytes in AIP (acute intermittent porphyria) and normal individuals were compared, two types of patterns were observed. One pattern observed in AIP (n = 7) was similar to the normal pattern but was characterized by approximately a 50% reduction in the activity of UROS A, B and C. The second pattern in AIP individuals (n = 7) was characterized by elevation of UROS C with respect to UROS A and UROS B. Patients with the latter pattern of UROS enzyme-substrate intermediates had high urinary PBG levels and/or were experiencing neurologic symptoms of their disease. Thus, the overproduction of PBG in AIP is reflected by UROS enzyme-substrate intermediates in the erythrocyte.

Finally, homogenous human UROS was used to raise anti-UROS immune sera in rabbits. Preliminary immunochemical studies indicated that several AIP individuals possess material in erythrocyte lysates that is non-catalytic but immunologically cross-reactive (CRM-positive). Thus the enzymatic deficiency of UROS in these patients is probably the result of a point mutation in the structural gene for UROS. Preliminary studies on another AIP patient did not identify CRM. The molecular pathology of this CRM-negative individual remains to be elucidated.

FORWARD

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

Anderson, P.M. and Desnick, R.J., 1978, Biosynthesis of Porphobilinogen by Immobilized Human δ-aminolevulinate Dehydratase. Federation Proceedings 37:1515.

Anderson, P.M. and Desnick, R.J., 1979, Purification and Properties of δ-aminolevulinate Dehydrase from Human Erythrocytes. Journal of Biological Chemistry 254:6924-6930.

Anderson, P.M. and Desnick, R.J., 1979, Purification and Properties of Uroporphyrinogen I Synthase from Human Erythrocytes. Identification of Stable Enzyme-Substrate Intermediates. Journal of Biological Chemistry (in review).

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LIST OF ABBREVIATIONS

allylisopropylacetamide	AIA
acute intermittent porphyria	AIP
δ-aminolevulinic acid	ALA
δ-aminolevulinic acid dehydratase	ALAD
δ-aminolevulinic acid synthetase	ALAS
immunologically cross-reactive material	CRM
3,5-dicarbethoxy-1,4 dihydrocollidine	DDC
dimethylsulfoxide	DMSO
dithioerythritol	DTE
dithiothreitol	DTT
ethylenediaminetetracetic acid	EDTA
high pressure liquid chromatography	HPLC
hour(s)	hr
low density lipoprotein	LDL
minutes	min
molecular weight	M_r
porphobilinogen	PBG
sodium dodecyl sulfate	SDS
trichloroacetic acid	TCA
thin-layer chromatography	TLC
uroporphyrinogen III cosynthase	UROCoS
uroporphyrinogen decarboxylase	UROD
uroporphyrinogen I synthase	UROS

I. BACKGROUND AND RATIONALE

A. Heme Biosynthesis:

The biosynthesis of heme was not understood until the late 1940's when the application of radiotracer technology and the work of Shemin and others led to the elucidation of the heme biosynthetic pathway (1). Although the porphyrias were recognized early in the 20th century as diseases of abnormal heme metabolism characterized by specific patterns of porphyrin excretion, the enzymatic deficiencies in these disorders were not identified until the 1970's. The current concept of mammalian heme biosynthesis is illustrated in Figure 1.

Heme biosynthesis is modulated by genetic and metabolic mechanisms in both prokaryotic and eukaryotic organisms (2,3). In animals, *de novo* δ-aminolevulinic acid synthetase (ALAS), the first and rate-limiting enzyme in the pathway, is inducible by various compounds (3) and is modulated by the ultimate product of the pathway, heme (4). Although no data are available for humans, studies in several mammalian systems have indicated that the control for the induction and negative feedback repression of ALAS synthesis occurs at both transcriptional and translational levels (5-8). In addition, another aspect of the genetic regulation of heme biosynthesis is the recent demonstration of tissue-specific isozymes for ALAS in the guinea pig (9). Hepatic and erythroid ALAS isozymes were identified and evidence for the metabolic regulation of the hepatic isozyme was found (10). Other investi-

gators have also demonstrated that four heme biosynthetic enzymes and globin mRNA are induced sequentially with time during erythropoiesis in the mouse (11,12,13). Thus although the necessity of heme during erythropoietic differentiation is obvious, the mechanisms which are responsible for the tight genetic regulation of this pathway are only beginning to be understood.

It is interesting to note that the enzymes of the heme pathway are linked in a sequential arrangement in the genome of *Staphylococcus aureus* (14), but not in *Escherichia coli* (15). At the present time none of the structural genes for the enzymes participating in heme biosynthesis have been assigned to chromosomes or linkage groups in man. With the development of somatic cell hybridization techniques (16) this should now be possible. Chromosome localization, however, must await the development of sensitive methods to discriminate between the human and rodent isozymes.

B. The Porphyrias:

Rapid advances have been made in the understanding of the molecular pathology of recessively inherited diseases which are due to functional defects of catalytic proteins (the inborn errors of metabolism) or transport proteins (the hemoglobinopathies and thalassemias). However, and in marked contrast, knowledge of the specific molecular defects in the over 1,000 known, dominantly inherited diseases of man is extremely limited (16). Since deficient activities of specific heme biosynthetic enzymes have been described in the

porphyrias, these diseases provide a unique opportunity to investigate the molecular mechanisms responsible for the autosomal dominant inheritance of human disorders (17).

The porphyrias are classified on clinical grounds into erythropoietic and hepatic types (18,19) as detailed in Table 1. The hepatic types of porphyria are particularly interesting diseases in that individuals with hepatic porphyria are healthy all or most of their lives with the exception of acute episodes. These acute episodes are characterized clinically by abdominal pain and neurologic dysfunction and may be severe enough to cause respiratory paralysis and death. During acute attacks patients have increased excretion of δ-aminolevulinic acid (ALA) and porphobilinogen (PBG). These acute attacks often are related to induction of the cytochrome P-450 system and heme biosynthesis by drugs listed in Table 2 but may be spontaneous. In many instances, therapy with hematin (20,21) has been life-saving. Thus, like many dominantly inherited genetic diseases, acute hepatic porphyrias have a large spectrum of clinical manifestations. What is exceptional in the porphyrias is that the site of disturbed metabolism is known and can be studied.

The inherited porphyrias and their respective enzyme deficiencies are illustrated in Figure 2. Acute intermittent porphyria (AIP) has been shown to result from the reduced activity of uroporphyrinogen-I-synthetase (UROS) (22-25); in porphyria cutanea tarda reduced uroporphyrinogen decarboxylase (UROD) has been found (26); in hereditary coproporphyria

reduced coproporphyrinogen oxidase activity has been recently reported (27-29); in variegate porphyria reduced protoporphyrinogen oxidase activity has been reported (30); in erythropoietic protoporphyria diminished activity of heme synthetase has been demonstrated (31). The above types of porphyria are inherited as autosomal dominant traits. The only hereditary porphyria which is inherited in an autosomal recessive manner is congenital erythropoietic porphyria. The defect in this disease may involve uroporphyrinogen-III-cosynthetase (UROCoS) (32).

Table 1

Classification of the Porphyrias

I. Erythropoietic Porphyrias

   A. Congenital erythropoietic porphyria, Gunther's disease.

   B. Erythropoietic protoporphyria.

II. Hepatic Porphyrias

   A. Acute intermittent porphyria (AIP), Swedish genetic porphyria.
      1. Manifest
      2. Latent

   B. Variegate porphyria, mixed porphyria, South African genetic porphyria, protocoproporphyria.
      1. Cutaneous manifestations only
      2. Acute manifestations only
      3. Both cutaneous and acute manifestations
      4. Latent

   C. Hereditary coproporphyria.

   D. Porphyria cutanea tarda, symptomatic porphyria.

Table 2

Drugs and Acute Porphyria (19)

A. Drugs that have been reported to precipitate acute neurological attacks:

barbiturates
sulfonamides
griseofulvin
chlordiazepoxide
meprobamate
isopropylmeprobanate
diphenylhydantoin
methsuximide
dichloralphenazone
glutethimide
amidopyrine
antipyrine
isopropylantipyrine
dipyrone
methprylon
sulfonal
trional
imipramine
ergot preparations
tolbutamide

B. Drugs which produce significant porphyria in one or more experimental systems (rats, mice or liver cell cultures, etc.), but have not been implicated in precipitating acute attacks in patients.

mephenytoin
phensuximide
chloroamphenicol
2-allyloxy-3methyl benzamide
lead
arsenic

C. Safe and probably safe drugs

morphine group
hyoscine
methadone
codeine
chloral hydrate
meperidine
penicillins
streptomycin
tetracyclines
furadantin
mandelamide
corticosteroids
rauwolfia alkaloids
guanethidine
diphenylhydramine
promethazine
chlorpromazine
trifluroperazine
perchlorperazine
meclizine
vitamin B, C, E groups
digoxin
mersalyl
atropine
prostigmine
neostigmine
tetraethylammonium bromide
propoxyphene
diazepam
lithium chloride

Figure 1. Current Concept of Mammalian Heme Biosynthesis.

In mammals the first enzyme, ALAS, catalyzes the formation of ALA at the expense of the high energy bond in succinyl CoA. In plants, however, this step is modified with dioxovaleric acid being the precursor of ALA (149). Mammalian ALAS seems to be rate-limiting in hepatic tissue and is subject to negative feedback control by heme. Under some conditions such as hepatic porphyria, UROS may become rate-limiting. Both ALAS and UROS are enzymes which are inducible with increases as much as 300 - fold having been reported (4,93).

Current Concept of HEME Biosynthesis

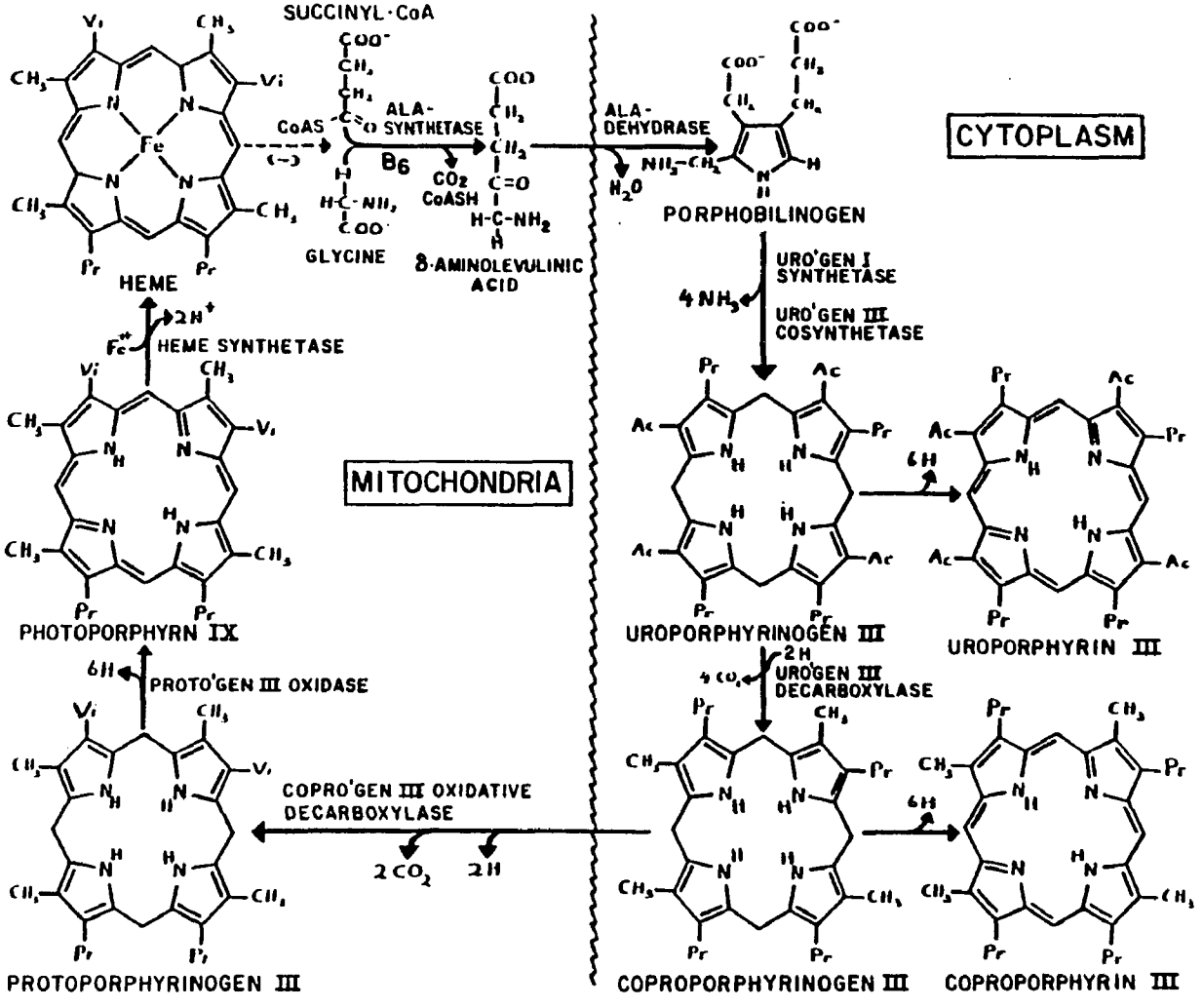
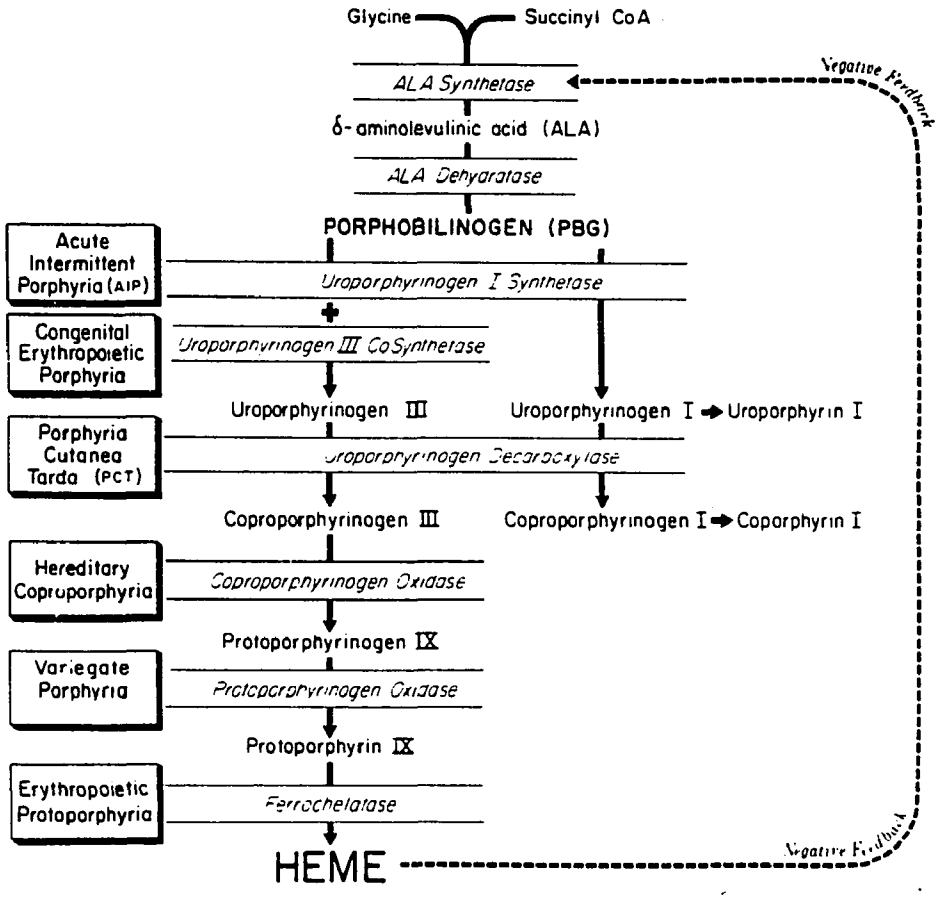


Figure 2. The Inherited Porphyrias and Their Enzymatic Defects.

Each type of inherited porphyria is associated with a specific enzymatic deficiency in the heme biosynthetic pathway. With the exception of congenital erythropoietic porphyria, the inherited porphyrias are inherited as autosomal dominant traits.

THE INHERITED PORPHYRIAS AND THEIR ENZYMATIC DEFECTS



C. Biochemical and Genetic Aspects of Heme Biosynthetic Enzymes:

Comprehensive reviews of the biochemistry, metabolism and inherited defects of heme biosynthesis in man are available (2,18,19,33-35). It is the purpose of this section to provide a selected and critical review of the enzymes in the heme biosynthetic pathway, with special emphasis on assay methods and the biochemical genetics of each enzyme in man and other species.

1. δ-Aminolevulinic Acid Synthetase (E.C.4.2.1.24):

The condensation of succinyl-CoA and glycine to form δ-aminolevulinic acid (ALA) with the release of CO_2 and CoA is catalyzed by the mitochondrial enzyme, δ-aminolevulinic acid synthetase (ALAS). The enzyme requires pyridoxal-5'-phosphate, is stabilized by dithiothreitol (DTT) and is solubilized by detergent or high salt concentrations (35,36).

In mammalian systems, the activity of ALAS is probably rate-limiting for the pathway and its synthesis is negatively controlled by the final product, heme (5, 37). These studies indicate that the regulation of ALAS is an important locus in the genetic control of heme biosynthesis. However, the molecular mechanisms responsible for this process are yet to be elucidated. An approach employing somatic cell genetic techniques would be a powerful way to investigate ALAS action but at the present time methodologic problems make this an extremely difficult proposition. One of the limiting factors

in investigations of the properties and regulation of ALAS has been the lack of a rapid, specific and sensitive assay. Until recently, ALA formation was estimated by condensation with 2,4-pentanedione followed by colorimetric determination with Ehrlich's reagent (38). Subsequent modifications improved specificity and sensitivity (39-42); however, the methods are limited in sensitivity and are prone to errors as well as interference by aminoacetone, the condensation product of glycine and acetyl-CoA. The introduction of radiochemical assays utilizing ^{14}C-succinyl-CoA has lowered the detection limit by a factor of 10 (43-46). While this assay uses multiple columns making it tedious and subject to error, it is presently the most reliable and readily available method. The single column modifications are more reproducible but have been shown to lack selectivity against contaminants generated with impure enzyme preparations (47). Clearly, a more rapid, sensitive and reliable assay for ALAS would facilitate future studies of this enzyme.

Little is known about the genetic control of ALAS in mammals, especially in humans. Although there are no known inherited defects of ALAS activity, theories on the control of ALAS had their origin in studies of the porphyrias. Many drugs including steroids and barbiturates which precipitate acute porphyric attacks have been found to induce ALAS. For example, 5-OH steroids and 3,5-dicarbethoxy - 1,4 dihydrocollidine (DDC) have been shown to induce ALAS synthesis by exerting their effects at the level of transcription while allylisopropylacetamide (AIA) acts at the level of translation (8).

Quantitative immunochemical studies have shown that the increase in ALAS activity induced by DDC or AIA actually represent an increase in the number of enzyme molecules present (6).

Immunochemical studies have shown that heme specifically blocks the induction of ALAS in chick embryo liver cell cultures (6). Some investigators have suggested that control of ALAS levels resides in the heme-dependent repression of mRNA translation (33,48). Granick proposed that a repressor substance, which is sensitive to heme concentration is responsible for the genetic control of heme biosynthesis (33). This was supported by the studies which found cyclic, complimentary oscillations in rat hepatic heme oxygenase and ALA synthetase activities following heme injections (49). Some success has also been achieved recently in the clinical remission of hepatic porphyria in man by intravenous administration of hematin (20,21).

2. δ-Aminolevulinic Acid Dehydratase (ALAD; E.C.4.2.1.24):

ALAD catalyzes the self-condensation of two moles of ALA to produce the pyrrole, porphobilinogen (PBG). Shemin and colleagues have extensively studied ALAD purified from bovine liver and the photosynthetic bacterium *Rhodopseudomonas spheriodes* (50,51). The enzyme has also been purified from murine (52-54) and guinea pig (55) sources. ALAD is a sulfhydryl enzyme as indicated by its sensitivity to thiol reagents; it is one of the first enzymes affected in lead toxicity (56).

The mammalian enzyme has a molecular weight of 250,000 to 290,000 and is composed of 6 to 8 subunits (53,56-58). The K_m for ALA is around 0.2 mM and the activity is optimal at pH 6.8 (57,59).

To determine ALAD activity a colorimetric assay is employed. This assay detects PBG formed by reacting the pyrrole with modified Ehrlich's reagent (38) to yield a colored product. The assay is capable of detecting PBG in the micromole range. However, it does not provide the sensitivity necessary to detect ALAD in many types of cultured cells. In addition, the colorimetric assay cannot be adapted to detect PBG formed on starch, agarose or cellulose acetate gels due to the very acidic conditions necessary for color formation. Therefore, a more sensitive method of staining for ALAD on these gels is desirable. This would facilitate chromosomal assignment of the structural gene(s) for human ALAD.

The genetic regulation of ALAD activity has been the subject of recent interest (53,56,60,61). The variation in the rate of hepatic synthesis of murine ALAD activity has been shown to be under the control of at least two different alleles at the levulinate (Lv) locus. Homozygous Lv^a mice synthesize over 3 times as much hepatic ALAD as homozygous Lv^b mice (60). Possible explanations of this phenomenon include gene-dosage effect (i.e., few copies of the ALAD structural gene in Lv mice) or a regulatory mutation (i.e., the Lv^b locus regulates transcription or translation of the structural gene).

A significant variation (4- to 10-fold) in the levels of enzyme activity in erythrocytes from baboons and rhesus

monkeys has been reported (56). Similarly, a 4-fold range in the levels of assayable activity in normal human erythrocytes has been observed (61). In both monozygotic and dizygotic twins, there is a much closer correlation between ALAD activities suggesting some form of genetic control of expression (61). These factors suggest that genetic factors (i.e., regulatory genes or structural alleles) are involved in the regulation of ALAD activities in primates, although immunochemical studies are required to determine whether the differences in activity are the result of qualitative or quantitative variation. Further understanding of the genetic regulation of ALAD activity in man requires the availability of homogeneous human enzyme for biochemical and immunologic characterization.

Previously, human ALAD has been partially purified 1,200-fold (specific activity 4.1 units/mg) with a 47% yield (62). However, the enzyme was only 20% pure. What is needed is a convenient method of purification using a readily available human tissue such as erythrocytes or placenta.

Recently, Bird and coworkers discovered a family with an inherited deficiency or erythrocyte ALAD activity occurring over three generations in an autosomal dominant pattern (63). None of the individuals with low ALAD activity had symptoms of porphyria nor did they excrete abnormal amounts of urinary ALA or porphyrin precursors. Furthermore, the deficiency of ALAD activity in this family was not similar to that described in patients with hereditary tyrosinemia. A total of ten individuals in this kindred were found to have the variant ALAD

with a mean erythrocyte activity of 28% of the mean for 11 other normal blood relatives. No inhibitor was demonstrated by mixing experiments using normal blood with blood or urine from a deficient individual. Added zinc in the assay system stimulated enzyme activity to an equal degree (20% mean increase) in both deficient and normal erythrocytes. K_m values, temperature and pH optima for the reaction were similar for blood with low or normal ALAD activity. Based on these findings, these investigators suggested that the mutation producing low ALAD activity may affect a regulatory rather than a structural gene, consistent with the Lv locus mutations in the mouse. However, they recognized that a mutation at a structural locus remained a strong possibility, and that purification of the enzyme was necessary to define the precise nature of the mutation. It is intriguing to speculate that persons with genetically low ALAD activity may be especially sensitive to environmental lead exposure.

3. Uroporphyrinogen-I-Synthetase (UROS; E.C.4.3.1.8.):

UROS catalyzes the conversion of 4 moles of PBG to 1 mole of uroporphyrinogen I and 4 moles of ammonia, hence it is also known as porphobilinogen deaminase. UROS has been purified to homogeniety from *Rhodopseudomonas spheriodes* (64,65) and spinach (66). The bacterial and plant enzymes are monomers with molecular weights of approximately 40,000.

Mammalian UROS, however, has been difficult to isolate. Using bovine liver as an enzyme source, a 330-fold purification was reported (67). After UROS from human erythrocytes was

partially purified 160-fold (68), the enzyme was reported to be unstable. Another preparation from human erythrocytes appeared to contain separate porphobilinogen consuming activities and uroporphyrinogen forming activities (69).

UROS activity is easily quantitated by a sensitive fluorometric assay (23). The colorless uroporphyrinogen I formed is oxidized by light and oxygen to uroporphyrin I, which exhibits the brilliant red fluorescence characteristic of porphyrins.

A major limitation to the study of UROS in health and disease has been the difficulty in obtaining adequate quantities of PBG substrate. Several organic methods for PBG synthesis have been reported. The earlier methods have yields of approximately 1% and the most recent organic method yields 8% (70,71). On a preparative scale these procedures are not feasible in terms of time-energy, equipment requirements, and cost considerations. In addition, when synthesizing radiolabeled PBG, higher yields are desirable. Alternatively, PBG has been obtained from the urine of individuals with AIP (72). Major limitations of this procedure are unavailability of such individuals and the fact that radiolabeled PBG cannot be prepared by this approach.

Enzymatic procedures are currently the most efficient methods for PBG synthesis. Preparation of PBG using heat-treated *Pseudomonas shermanii* has been reported (73). Unfortunately this method requires large volumes of cells due to product inhibition of ALAD by low concentrations (2 mM) of PBG. Furthermore, isolation of PBG from bacterial lysates

containing high concentrations of amino acids and protein is difficult. The covalent linkage of ALAD to Sepharose 4B has eliminated the drawbacks of the biosynthetic approach. However, now the time-consuming step is the purification of ALAD from large quantities of photosynthetically grown *Rhodopseudomonas spheriodes* or bovine liver (74,75). To facilitate PBG production, methods that utilize a readily available enzyme source and a convenient procedure to purify the ALAD in high yield are needed.

Recent interest in human UROS has been prompted by the finding of reduced activity in tissues of individuals with AIP. A 50% reduction in the activity of UROS has been demonstrated in erythrocytes, cultured skin fibroblasts, amniotic cells, and lymphoid cells of AIP heterozygotes (22-25). Although individuals with AIP have approximately 50% levels of enzyme activity compared to the unaffected parent or unaffected siblings, overlap of UROS activities in normal and porphyric subjects has been repeatedly observed (76).

The activity of UROS is nearly as low as ALAS and has been proposed as a secondary control mechanism in the biosynthesis of heme (77,78). After mitogen stimulation of human lymphocytes, the enzyme was induced between 10- and 30-fold and UROS induction was not accompanied by a corresponding increase in ALAS activity. Also UROS, in lymphoblasts from individuals with AIP was induced only 50% compared to the induced levels in normal cells (79). Since induced enzyme had a normal apparent K_m and the level of UROS in AIP lymphocytes did

not correlate with clinical status, it was proposed that AIP was the result of a dominantly inherited mutation regulating the rate of synthesis of normal enzyme. Other possible explanations of reduced UROS activity in AIP include: a point mutation that results in a non-catalytic enzyme due to a kinetic and/or stability defect or a complete or partial deletion of the UROS structural gene.

An anti-UROS antibody would be a very useful tool to aid in the discrimination among these possibilities. The highest purification of human UROS reported to date is only 160-fold (68). What is needed is a method that would yield sufficient amounts of homogenous human UROS to raise monospecific anti-UROS antibodies. Such antibodies would allow the investigation of the amount of cross-reacting material (CRM) in various AIP families. For example, if CRM status in AIP is the same as in normals, this evidence would support a point mutation rendering the UROS structural gene product non-catalytic due to a kinetic mutation. If only 50% levels of CRM were found, this would provide the impetus to design experiments exploring the possibility of a defective regulatory gene in AIP.

4. Uroporphyrinogen-III-CoSynthetase (UROCoS):

In the presence of UROS, UROCoS acts as a specifier protein to form the biologically important stereoisomer, uroporphyrinogen III. In the absence of UROCoS, only the uroporphyrinogen I isomer is formed. Many investigators (80-83) have attempted to determine the mechanisms by which the asymmetric uroporphyrinogen III isomer (rather than the symmetric uropor-

phyrinogen I isomer) is synthesized by the UROCoS-UROS complex (see Figure 1).

Investigation of the biochemistry of UROCoS has been extremely difficult due to 1) the lability of UROCoS and 2) cumbersome assay procedures, since no accurate and convenient method to separate the uroporphyrin I and III isomers existed until recently (83). Analysis of uroporphyrin isomers in most laboratories requires that the uroporphyrins are esterified, extracted, dried, hydrolyzed in 7.5 N HCl, then heated at 180°C for 4 hours at reduced pressure to decarboxylate the uroporphyrins to coproporphyrin isomers (84). The latter compounds are readily separated by TLC in a lutidine/water system and can be quantified after elution from the thin-layer plate (85). Recently, HPLC methods have been developed to separate uroporphyrin isomers (83). However, even this method still takes considerable time when multiple samples are analyzed.

Congenital erythropoietic porphyria is an autosomal recessive disease considered due to UROCoS deficiency. A variant has been reported with autosomal dominant inheritance (86).

5. Uroporphyrinogen Decarboxylase (UROD; E.C.4.1.1.37):

UROD catalyzes the decarboxylation of uroporphyrinogen to coproporphyrinogen by the removal of 4 moles of CO_2 from the acetyl groups of the tetrapyrrole. The most specific but time-consuming assay of UROD uses tritiated uroporphyrinogen as substrate, isolation by thin-layer chromatography and liquid

scintillation quantitation of the reaction products (87). Deficiencies of 50% of the normal UROD activities have been described in porphyria cutanea tarda (26).

6. Coproporphyrinogen Oxidase (E.C.1.3.3.3):

Coproporphyrinogen oxidase catalyzes the oxidative decarboxylation of coproporphyrinogen III to yield protoporphyrinogen III as shown in Figure 1. Like ALAS, this enzyme and subsequent enzymes in the heme pathway are found in mitochondria. The most specific assay for coproporphyrinogen oxidase measures $^{14}CO_2$ liberated from coproporphyrinogen labeled in the carboxyl positions (88).

A deficiency of coproporphyrinogen oxidase has been demonstrated in hereditary coproporphyria, an autosomal dominant disease. In addition, the enzymatic activity was almost totally deficient in a homozygous individual with this disorder (89), suggesting a structural gene mutation as the molecular basis of hereditary coproporphyria.

7. Protoporphyrinogen Oxidase:

Protoporphyrinogen oxidase catalyzes the oxidation of protoporphyrinogen III to protoporphyrin IX. Investigation of the activity of this enzyme is difficult because in the presence of oxygen, protoporphyrinogen is readily oxidized to protoporphyrin nonenzymatically. Poulson has used a differential spectrophotometric assay to measure the enzyme from a variety of sources (90,91).

Variegate porphyria has a prevalence of 1 in 300 in the Afrikaner population of South Africa (92). The disorder is

inherited as an autosomal dominant and shares many of the clinical features of AIP. Recently a 50% reduction of protoporphyrinogen oxidase activity has been demonstrated in individuals with variegate porphyria (30).

8. Heme Synthetase (E.C.4.99.1.1):

This enzyme catalyzes the insertion of Fe^{2+} into the protoporphyrin IX ring thus resulting in the synthesis of heme. Hence, the enzyme has been known as ferrochelatase or heme synthetase. Relatively little is known about its properties since the enzyme is membrane-bound and difficult to solubilize (2). The substrate for heme synthetase, protoporphyrin IX, also exhibits poor solubility. Currently, the best assay measures $^{59}Fe^{2+}$ incorporation into deuteroporphyrin (30). While erythropoietic protoporphyria is an autosomal dominant disorder and one would expect a 50% reduction in heme synthetase activity, between 75% and 90% reductions in enzyme activity are observed (30,31).

Recently the non-inducible Friend erythroleukemic cell line FW has been shown to be deficient in heme synthetase (93). These cells, however, were able to induce in the presence of hemin or butyric acid. An interesting feature of the FW cell line is the observation of extremely high levels of UROS. An observation in our laboratory (Phillip Giampetro, unpublished results) is that a human cell line (K562) also has high UROS activity.

D. Genetic Regulation of Heme Synthesis - Studies in the Mouse.

The genetic events governing regulation of the activity of enzymes in the heme biosynthetic pathway are largely obscure. In fact, no control model for any eukaryotic enzyme has been firmly established. One possible regulatory locus, the levulinate locus (Lv), has been described in inbred mice; homozygous Lv^a strains exhibit 3 to 4 times the amount of ALAD protein as Lv^b strains (53). This locus has been mapped to mouse chromosome 4 and is closely linked (or allelic) to the ALAD structural gene locus (94). In addition, a fetal isozyme of ALAD has been identified at or near the Lv locus which may also be expressed in a mouse hepatoma cell line (95). The fetal enzyme is probably erythroid and has been shown to be twice as efficient catalytically as the adult enzyme (33).

Erythroid differentiation of cultured cells presents a unique opportunity to study the genetic control of heme biosynthesis. The Friend T-3-C12 erythroleukemia cell line has been shown to produce hemoglobin within 4 days of treatment with 2% dimethylsulfoxide (DMSO) (96). ALAS activity in these cells was elevated after 28 hours and increased to 5 times the level of the control cells within 4 days (96). Furthermore, there was a sequential induction of the heme pathway enzymes in these cells (11). This induction pattern was also found during erythroid differentiation in fetal mouse liver (17).

E. Somatic Cell Hybridization and the Investigation of the Genetic Regulation of Hemoglobin Biosynthesis.

1. Chromosomal Localization of Human Genes.

The extraordinary increase in knowledge about the human gene map in recent years has been due to the development of somatic cell hybridization methodology (16,97). Application of these techniques will eventually permit an important first step in the elucidation of the genetic regulation of heme biosynthesis via the establishment of the chromosomal localization of genes responsible for heme biosynthesis.

To induce fusion of somatic cells a factor from myxoviruses such as Sendai virus or Newcastle disease virus has been used (98). In recent years polyethylene glycol also has been shown to be a potent fusogenic agent of mammalian cells (99). The immediate results of fusion are bi- or multi-nucleate cells which may contain nuclei derived from the different cell types. Following fusion, the nuclei enter mitosis in synchrony and thereafter mononuclear cells containing genetic material from both parental cell types occur. It has been demonstrated that when cells of different species are fused, random chromosomal loss from one or both parental genomes in the hybrid cells occurs during post-fusion mitoses (100). Human chromosomal loss, which occurs in human/rodent hybrids, produces hybrid cell lines that may or may not contain a particular human chromosome (16,101). Thus correlation of the karyotype of different hybrid cell lines and of the presence or absence of a particular gene (97) or production of a

gene product (101) permits the identification of the human chromosome(s) necessary for the synthesis of a protein or enzyme. An example of the use of somatic cell hybridization techniques is the identification of chromosomes 17 and 11 as the sites of human α- and β- globin genes, respectively, using human fibroblast x mouse fibroblast hybrid clones (102,103).

2. Expression of Erythroid Functions by Somatic Cell Hybrids:

Recently, several investigators have employed somatic cells and somatic cell hybrids to probe the events responsible for erythropoiesis and, in particular, hemoglobin production. Hybrid somatic cells derived from undifferentiated mouse embryonal carcinoma and Friend erythroleukemia cells were found to synthesize hemoglobin after DMSO induction (104,150). Some of these hybrid lines also were able to synthesize hemoglobin after erythropoietin induction. Fusion of a lymphoma cell line (L5178Y) with Friend erythroleukemia cells resulted in hybrids which produced globin mRNA, but no hemoglobin after induction with DMSO. Hemin, however, was found to induce synthesis of both mRNA and globin chains in these hybrids (105).

Experiments with enucleated mouse fibroblasts fused with Friend erythroleukemia cells resulted in extinction of hemoglobin inducibility (106). Induction was defective in cells even after passage for 6 months thus suggesting that the cytoplasm of non-erythroid cells may contain an inherited factor(s) which prevents erythroid differentiation. These results are consistent with the failure of Friend erythroleukemia x human fibro-

blast hybrids to produce detectable globin or globin mRNA from either species (107).

Recently, human α- and β- globin genes have been activated in human fibroblast x tetraploid Friend erythroleukemia cell hybrids (108). DMSO induction in some of the hybrids resulted in a greater percentage of benzidine positive cells than the parental line. Another interesting finding by these investigators was the observation of the selective activation of β- but not γ- globin genes in these hybrids.

A cell line of current interest is the human K562. This cell line was established from a patient with chronic myelogenous leukemia (109), a stem cell leukemia. The cells do not possess surface immunoglobins, myeloperoxidase or alkaline phosphatase. K562 cells, however, possess erythroid markers including spectrin and glycophorin A, the major sialoglycoproteins of red cells (110). To date, there is no information on the presence or absence of platelet markers in K562 cells. When K562 cells were cultivated for 4 days in the presence of 1 mM sodium butyrate, eosinophilic particles which resembled erythrocytes accumulated in the cultures. These particles were benzidine positive and contained hemoglobin as determined by radioimmunoassay (111). Recently, it has been demonstrated (112,113) that hemin is an apparent inducer of erythroid differentiation in K562 cells.

F. Molecular Pathology of Mendelian Inherited Disease: Some Biochemical and Immunologic Considerations.

Rapid advances in biochemical genetics have made it

possible to specifically describe the molecular pathology in many of the recessively inherited inborn errors of metabolism (16,118). With exception of the hemoglobinopathies (16,114-117), the inaccessability and/or small quantities of gene products in other genetic disorders have prevented detailed analysis of these abnormalities at the molecular level. Nevertheless, in many X-linked and autosomal recessive abnormalities, biochemical and immunologic investigations have characterized the nature of the specific enzymatic deficiency occurring in these diseases. Table 3 summarizes types of mutations which may result in a deficiency of an enzyme (118). Figure 3 (from R.J. Desnick *et al.*, 118) illustrates the effect on catalytic activity and presence or absence of immunologically cross-reactive material (CRM) of various mutations in the enzyme's structural gene. Recent evidence has indicated that most X-linked and autosomal recessive diseases are associated with the presence of a mutant protein which is detectable immunologically (119), but has little or no catalytical activity (118). Pathologic processes in these enzyme deficiencies result either from the accumulation of substrate and its metabolites, or the lack of a crucial product normally produced by the wild-type enzyme, or both. Heterozygotes are usually phenotypically unaffected by disease manifestations since 50% levels of enzyme apparently are adequate to prevent pathologic substrate accumulation or product deficiency.

In contrast to individuals with X-linked and autosomal recessive inherited diseases, individuals with dominantly

inherited disorders possess not only a mutant allelle but also a normal allelle. Little, if anything, is known about the molecular pathology in the over 1,000 catalogued (16) dominantly inherited disorders of man. The underlying molecular mechanisms responsible for these defects has not yet become the focus of intensive research, with several notable exceptions. The dominantly inherited deficiency of C'1 esterase inhibitor in angioneurotic edema has been the subject of investigation (120). Studies of Von Willibrand's disease (121) have demonstrated that the disorder results from a deficiency of Von Willibrand factor, a protein associated with Factor VIII activity. Another disorder, hypercholesterolemia type IIb is of note. This recessive disorder is manifested phenotypically in the heterozygote and for the purpose of discussion can be considered a dominant disorder (16). In type IIb hypercholesterolemia most heterozygous patients have defective functioning of half of their receptors for low density lipoprotein (LDL). These patients would be expected to have normal or nearly normal levels of CRM. Another group of type IIb heterozygotes have a 50% deficiency of LDL receptor protein; they would be expected to have 50% of normal CRM levels.

With the exception of the above diseases it is not known whether the mutant allelle in dominantly inherited diseases produces defective enzyme (or structural protein) or whether the disorder is due to defective regulation at or before the level of protein synthesis.

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The porphyrias represent a unique opportunity to assess whether a dominantly inherited enzyme deficiency is due to a structural gene mutation or a mutation affecting the rate of enzyme synthesis. If the deficiency of UROS in AIP was due to a point mutation rendering the mutant enzyme non-catalytic (but stable), the amount of CRM should closely correspond to that in normal individuals. If less than normal amounts of CRM occur in AIP then this could be due to the occurrence of an unstable enzyme, a deletion of the UROS structural gene as occurs for hemoglobin in α-thalassemia (116) and hereditary persistance of fetal hemoglobin (117), or a mutation affecting the rate of synthesis of normal enzyme (79). Since the enzymatic activity in ALAD deficiency and erythropoietic protoporphyria is only 25% of the respective normal levels, these dominantly inherited disorders could be due to the inheritance of a gene which codes for a "killer subunit" or a gene which alters the rate of synthesis of normal enzyme. At the present time immunochemical investigation of the molecular pathology of the dominantly inherited disorders of heme biosynthesis awaits the development of purification procedures for the human heme biosynthetic enzymes and production of antibodies to these enzymes.

TABLE 3

Types of Mutations Causing

Enzymatic Deficiencies (118)

Structural gene mutations

- Normal rate of enzyme synthesis, but:
  - Altered kinetic properties (kinetic mutations)
    - Raised K_m value
    - Lowered V_{max} value
  - Elevated rate of enzyme degradation (stability mutations)
    - Physical instability
    - Increased susceptibility to endogenous proteases
  - Other structural defects
    - Altered secondary binding sites (e.g., allosteric effectors, cofactors, etc.)
    - Altered intereactions (e.g., killer subunits)
    - Incomplete polypeptide synthesis

Posttranslational mutations

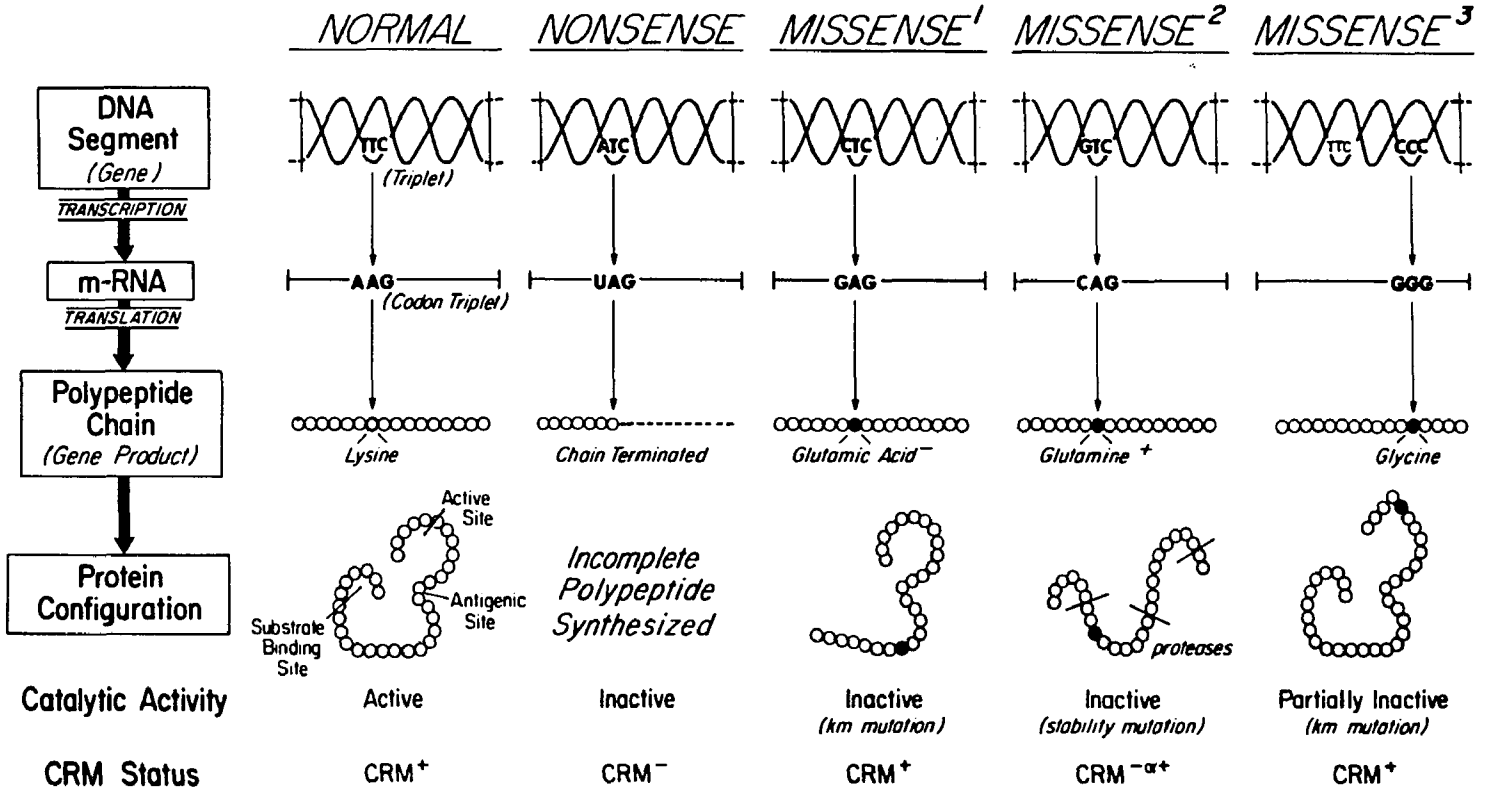
- Normal rate of enzyme synthesis and normal structural gene product, but:
  - Altered enzyme modification (e.g., glycosylation, phosphorylation, peptide cleavage or addition, etc.)
  - Altered tissue or subcellular localization

Regulatory gene mutations

- Low rate of normal enzyme synthesis

Figure 3. Possible Effects of a Point Mutation on Catalytic Activity and CRM (Immunologically Cross-Reacting Material).

The occurrance of a single base substitution in a structural gene can result in a mutant protein which may have normal or altered catalytic and/or immunologic properties.


NORMAL
NONSENSE
MISSENSE 1
MISSENSE 2
MISSENSE 3
DNA Segment (Gene)
TRANSCRIPTION
m-RNA
TRANSLATION
Polypeptide Chain (Gene Product)
Protein Configuration
TTC
(Triplet)
AAG
(Codon Triplet)
Lysine
ATC
UAG
Chain Terminated
CTC
GAG
Glutamic Acid⁻
GTC
CAG
Glutamine ⁺
TTC
CCC
GGG
Glycine
Active Site
Antigenic Site
Substrate Binding Site
Incomplete Polypeptide Synthesized
proteases
Catalytic Activity
Active
Inactive
Inactive (km mutation)
Inactive (stability mutation)
Partially Inactive (km mutation)
CRM Status
CRM⁺
CRM⁻
CRM⁺
CRM⁻ᵅ⁺
CRM⁺

II. OBJECTIVES

It was the purpose of these studies to investigate the biochemical and genetic characteristics of selected heme biosynthetic enzymes in man and the molecular pathology of AIP. In order to accomplish this research, development of the tools and methods necessary for these investigations was required. Therefore, the intention of this research was to:

1) purify and characterize human ALAD from a readily obtainable tissue source
2) synthesize gram quantities of PBG to permit study of the biochemical characteristics of human UROS
3) purify and characterize human UROS from a readily obtainable tissue source
4) investigate the molecular pathology of UROS in AIP, and
5) facilitate future studies concerning the genetic regulation of human heme biosynthesis including the chromosomal localization of enzymes responsible for this vital process.

III. MATERIALS

Sepharose 4B, octyl-Sepharose CL-4B, phenyl-Sepharose CL-4B, Sephadex G-100, and Sephadex G-200 were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Pyridoxal 5'-phosphate, δ-aminolevulinic acid hydrochloride, coproporphyrin standards, cytochrome c, aldolase, DL-dithiothreitol (DTT), DL-dithioerythritol (DTE), and low-fluorescence imidazole were purchased from Sigma Chemical Co., St. Louis, MO. Bovine serum albumin was obtained from Schwartz/Mann Research Labs, Orangeburg, NY. Catalase was purchased from Worthington Biochemical Corp., Freehold, NJ. Chymotrypsinogen was obtained from Calbiochem, La Jolla, CA. Bio-Gel A-1.5m, Bio-Rex RG 501-X8 mixed ion exchange resin, Dowex 1-X8 (acetate) ion exchange resin, and materials used for polyacrylamide gel electrophoresis were purchased from BioRad Laboratories, Richmond, CA. NCS tissue soluabilizer was obtained from Amersham, Arlington Heights, IL. Cellogel was purchased from Kalex Scientific, Manhasset, NY. Ampholytes were obtained from LKB, Hicksville, NY. Uroporphyrin standards were purchased from Porphyrin Products, Logan, Utah. Cyanogen bromide, 3-aminopropyltriethoxysilane, 1,6-diaminohexane, and 6-aminocaproic acid were obtained from Aldrich, Milwaukee, WI. [5-^{14}C]δ-aminolevulinic acid and [3,5-^{3}H]δ-aminolevulinic acid were obtained from New England Nuclear, Boston, MA. High purity acetonitrile was purchased from Burdick and Jackson Labs, Muskegon, MI. Fluorescamine, zirconium-clad porous

glass, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were obtained from Pierce Chemical Co., Rockford, IL. Amicon ultrafilters and hollow fiber cartridges were purchased from Amicon Corp., Lexington, MA. Goat anti-rabbit serum was a gift from Dr. Gregory Grabowski, Mt. Sinai School of Medicine. Female New Zealand rabbits were obtained from the Pocono Rabbit Farm, Canadensis, PA. Rabbit IgG was obtained from Miles Laboratories, Kankakee, IL. All other chemicals were of the highest purity available.

Outdated erythrocytes were obtained from the Greater New York Blood Center, New York, NY. Fresh erythrocytes were obtained from normal volunteers with informed consent. Samples of AIP erythrocytes were obtained from Dr. S. Zak, Minneapolis, MN., Dr. D.P. Tschudy and Dr. J. Lamon at the National Institutes of Health, Bethesda, MD, and Dr. B. Felsher, Long Beach Veterans Administration Hospital, Long Beach, CA. Tissues used in these studies were quickly obtained at autopsy, frozen and maintained at -70°C.

IV. METHODS

A. Standard ALAD Assay:

Crystalline ALA hydrochloride was dissolved in distilled water immediately prior to assays or stored at 4°C. This solution had a pH of about 3.2 which prevented bimolecular cyclization of the substrate. The standard enzyme assay contained 0.25 ml of 5.0 mM ALA hydrochloride in distilled water; 0.1 ml of 0.25 M sodium phosphate buffer, pH 6.8, 0.05 ml of 20 mM DTT, 0.05 ml of water or effector and 0.05 ml of appropriately diluted enzyme (0.005 to 0.05 units/assay). After incubation for 30 min at 37°C, the reaction was terminated by the addition of 0.5 ml of 0.1 M $HgCl_2$ in 10% trichloroacetic acid (TCA). The resultant precipitate was pelleted by centrifugation at 2,000 x g for 10 min and 0.5 ml of the supernatant was mixed with 0.5 ml modified Ehrlich's reagent (38). After 15 min at 23°C, the color intensity was quantitated at 555 nm in a Gilford 2000 spectrophotometer. One unit of enzymatic activity equals 1 umole of PBG formed/hr at 37°C. The effect of pH on enzyme activity was determined in 0.05 M sodium phosphate, sodium succinate, sodium PIPES and sodium Hepes buffers. For experiments investigating the effects of metals and pyridoxal 5'-phosphate on ALAD activity, the assay was modified by the addition of 0.05 ml of the effector and the use of 0.25 ml of 0.25 M PIPES buffer pH 6.8 instead of phosphate buffer. The production of nanomolar quantities of PBG were detected with this assay.

B. Coupled ALAD Assay:

UROS purified from erythrocytes was used in a 5-fold excess to produce uroporphyrin. A mixture of 50 µl sample, 50 µl UROS, 50 µl 0.25 M sodium phosphate pH 7.4, 50 µl, 100 mM DTT, and 200 µl 5 mM ALA was incubated in a 10 x 75 mm disposable test tube at 37°C for 30 min. The reaction was terminated by the addition of 50% TCA and the samples centrifuged 10 min at 2,000 x g. The tubes were then exposed to light for 20 min to oxidize from uroporphyrinogens to uroporphyrin. Fluorescence was then quantitated on a Turner model 111 fluorometer using a 405 nm primary and a 595 nm secondary filter. The production of picomole quantities of PBG was easily detectable using this assay.

C. UROS Assay:

The enzyme was also assayed by quantitation of uroporphyrin fluorescence using a Turner model 111 fluorometer equipped with a 405 nm primary filter and a 595 secondary filter. For routine assays the standard reaction mixture contained 50 µl of appropriately diluted sample, 750 µl of 0.1 M Tris-HCl, pH 8 buffer containing 0.1 M DTT, and 100 µl of 0.1 mM PBG. After mixing and incubation in the dark at 37°C for 20 min, the reaction was stopped by the addition of 100 µl of 5 N HCl and exposed to light for 20 min to oxidize porphyrinogens to porphyrins. When large amounts of hemoglobin were present the procedure was modified by the use of larger sample volumes and the addition of 100 µl 50% TCA to terminate the reaction. Precipitated protein was then pelleted by centrifugation at

2,000 g for 10 min. If samples to be assayed had protein concentrations of less than 0.5 mg/ml, bovine serum albumin (2mg/ml) was included in the assay buffer. The assay was linear from 0.005 to 0.12 nmole uroporphyrin/ml. One unit of UROS activity represents production of 1 nmole of uroporphyrin/hr at 37°C.

D. Analysis of PBG and Porphyrins:

Production and purity of PBG was determined by 1) co-migration on silica gel thin-layer plates with an authentic sample (provided by Dr. David Shemin) in an acetic acid/butanol/water (1:4:5) solvent system and 2) production of uroporphyrins after heating in acid at 100°C for 10 min.

Identification of uroporphyrin was carried out using procedures detailed by Falk (85). Uroporphyrin was identified by its slow migration on silica gel TLC plates in a 2,6 lutidine/H_2O (5:2) solvent system using an ammonia atmosphere. Alternatively, uroporphyrin could be identified by its rapid migration in an ascending aqueous lithium chloride system with an ammonia atmosphere. Methyl esters of porphyrins were prepared as described by Falk (85). Since the dioxan method (85) of identification of uroporphyrin isomers was found to be unreliable, isomer characterization was accomplished by the method of Edmonson and Schwartz (84).

E. Protein Assay:

Protein concentrations were determined by an adaptation of the fluorescamine method described by Anderson and Desnick (122); this assay was considerably less time-consuming and

more reproducible than that of Lowry *et al.* (123). Reagent grade acetonitrile was dried by the addition of 100 g of non-indicating Drierite followed by filtration through a filter paper cone containing another 100 g of Drierite. The dry acetonitrile was immediately used to prepare a solution of fluorescamine (60 mg/200 ml). If capped tightly the fluorescamine reagent was stable for several weeks. Since the fluorescamine assay detects primary amines, protein samples to be assayed were in phosphate, borate or other non-amine buffers; Tris, an amine, interferes with the assay. For assay, samples were diluted with 0.2 M sodium borate buffer, pH 9.0 to 1.0 ml in a 10 x 75 mm disposable test tube. Then 0.35 ml of the fluorescamine/acetonitrile solution was rapidly added while blending on a test tube mixer. Fluorescence was then quantitated in the same tube using a Turner model 111 fluorometer equipped with Corning 7-51 (360 nm) primary and Wratten #4 (465 nm) secondary filters and either a Wratten 10% or 1% neutral density filter. Using bovine serum albumin as a standard, the assay was linear between 0.02 and 2.5 µg/assay. The extreme sensitivity and reliability of the fluorescamine assay made it especially useful to monitor protein profiles from chromatography columns and other protein determinations with a minimum of sample loss.

F. Cellulose Acetate Electrophoresis of UROS and ALAD:

A sheet of 17 x 17 cm x 0.35 mm Cellogel was placed in 0.025 M potassium phosphate pH 8 for 2 hours at 4°C. Then the gel was blotted gently, placed into an electrophoresis

apparatus at 4°C, and pre-run 20 min, at 15 ma constant current. ALAD and UROS samples were then run 3 and 4 hr respectively. To visualize enzyme activity the gel was placed in activity staining solution (described below), gently blotted and incubated in a moist chamber which consisted of two 25 x 25 cm glass plates with 1 cm wet felt strips around the perimeter. Drying out of the gel was efficiently prevented by the chamber.

G. Analytical Polyacrylamide Gel Electrophoresis of UROS and ALAD:

Samples were electrophoresed in polyacrylamide disc gels using a constant current of 1 ma/gel in a pH 6.8, low fluorescence imidazole/sodium Hepes buffer system (122) or the pH 8.2 Tris buffer system described below. A 2 cm 2.5% polyacrylamide stacking gel and a 10 cm 7% polyacrylamide running gel were prepared as described by Cawley (124). The reservoir buffer was 0.05 M Tris/0.004 M glycine pH 8.3. Electrophoresis was carried out at 4°C using a constant current of 1 ma/gel until samples entered the running gel and 2 ma/gel until the tracking dye reached the end of the gel. Prior to staining, a copper wire was used to mark the dye front. To detect UROS activity the gel was incubated with 0.2 mM PBG in 0.1 M Tris/HCl, pH 8.1, at 37°C. Polyacrylamide disc gels required 12 min, cellulose acetate gels 40 min, and polyacrylamide slab gels 60 min of incubation. Oxidation of uroporphyrinogen to uroporphyrin was accomplished with ultraviolet light. The red fluorescence of the uroporphyrin permitted direct visualization of bands of enzyme activity.

ALAD activity was detected by sensitive coupled enzymatic stain in which the PBG produced by ALAD was converted to uroporphyrinogen using UROS partially purified from human erythrocytes. The gel was incubated at 37°C in the activity stain which consisted of 0.1 M potassium phosphate buffer, pH 7.6 containing 0.3 mM DTT, 0.5 mM ALA and 0.1 to 1 units of UROS (a > 10-fold excess of UROS). After incubation for 30 min ALAD activity was visualized in the gels by using ultraviolet light to oxidize the uroporphyrinogen to uroporphyrin.

Gels were stained for protein by Coomassie brilliant blue G-250 in 10% methanol using the method Blakesley and Boezi (125). This method required a minimal amount of destaining.

Electrophoresis in 0.1% SDS was carried out according to the method ofWeber and Osborne (126). Usually 4 to 10 µg of sample were applied to each gel.

H. Isoelectric Focusing Studies:

Isoelectric focusing of ALAD was performed essentially according to the analytical column method of Behnke *et al.* (127) with the following modifications. The density gradient consisted of 0-75% ethylene glycol instead of 5-20% sucrose. The gradient contained ALAD (50 µg), pH 3.5 to 10 ampholytes (6.09 ml), pH 5-7 ampholytes (0.22 ml), and 0.1 mM DTT. The anodal solution was 3% sulfuric acid (250 ml) and the cathodal solution was 3% ethylene diamine with 1.0 mM DTT in a volumne of 250 ml. During electrofocusing a voltage of 200 v was applied to the column for 3 hr at 4°C, then 0.2 ml fractions were collected from the column and enzyme activity was

determined. The pH was measured at 4°C.

Isoelectric focusing of UROS was performed using flat bed pH 4.5 to 8 polyacrylamide gels prepared according to the LKB isoelectric focusing instruction manual (128) with the addition of 20% ethylene glycol. A constant voltage of 800 v was applied to the gel for 16 hr with cooling. Bands of UROS activity were identified by staining with a filter paper overlay containing 0.1 mM PBG in 0.1 M Tris/HCl pH 8.1 and visualizing uroporphyrin as described earlier.

I. Molecular Weight Determinations of ALAD:

The molecular weight of the native enzyme was estimated by chromatography on Sephadex G-200 Superfine in 10 mM potassium phosphate, pH 6.8, containing 0.05 M KCl and 1 mM DTT. ALAD (200 µg) and standards (5 mg each) were run simultaneously. Molecular weight standards used for gel filtration were urease (M_r = 480,000), catalase (M_r = 250,000), muscle aldolase (M_r = 160,000), yeast alcohol dehydrogenase (M_r = 141,000), bovine serum albumin (M_r = 67,000), and cytochrome c (M_r = 12,400).

The subunit molecular weight of ALAD was estimated by SDS electrophoresis in polyacrylamide gels using the method of Weber and Osborne (126). Standards used were bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 43,000), aldolase (subunit M_r = 40,000), chymotrypsinogen (M_r = 25,700), and cytochrome c (M_r = 12,400). Destaining of gels was greatly facilitated by placing the gels in 12 ml destaining solution with 3 ml of Bio-Rex RG 501-X 8 mixed ion exchange resin and

rotating the tubes at 37°C by attachment to a roller bottle assembly for 24 hr.

J. Determination of the Molecular Weight of UROS and its Enzyme-Substrate Intermediates:

Each peak of enzyme activity after the DEAE-cellulose purification step was rechromatographed on DEAE-cellulose and enzyme from the peak fraction was used to estimate the molecular weight of the native enzyme. Molecular weight standards used were: bovine serum albumin (M_r = 64,000), ovalbumin (M_r = 44,000), chymotrypsinogen (M_r = 25,700) and cytochrome c (M_r = 12,400). Enzyme and 5 mg of each standard were applied to a column of Sephadex G-100 Superfine previously equilibrated with 10 mM potassium phosphate, pH 8, containing 0.05 M KCl. Fractions (2 ml) were collected and assayed for protein and UROS activity. The molecular weights were then estimated from the elution volumes.

Like ALAD, the molecular weight of denatured UROS (i.e., the subunit molecular weight of UROS) was estimated using the method of Weber and Osborne (126). In addition to the calibration of proteins used above, aldolase (subunit M_r = 40,000) was also employed.

K. Purification of Human ALAD:

All purification procedures were carried out at 4°C unless stated otherwise. Outdated human blood was centrifuged at 2,000 x g for 15 min and the buffy coat and plasma were discarded. The cells were washed three times with cold isotonic sodium chloride, and lysed by the addition of three

volumes of deionized water containing 3 mM DTE. Stroma and incompletely lysed cells were removed by centrifugation at 12,500 x g for 90 min. The resultant supernatant was then dialyzed against 0.007 M potassium phosphate, pH 6.80, containing 0.1 mM DTE and 0.017% sodium azide.

1. Preparative DEAE-Cellulose Chromatography:

A 10 cm x 40 cm column containing DEAE-cellulose was thoroughly equilibrated with the above dialysis buffer. It was important to use DEAE-cellulose which had been precycled in both acid and base. The pH was maintained below 6.87 (measured at 23°C) to prevent appreciable quantities of hemoglobin from binding and overloading the anion exchanger. The pH was maintained above 6.75 in order to bind UROS and elute hemoglobin in the buffer wash. The dialyzed lysate was pumped through the column at a flow rate of 8.0 ml/min and the column was washed with 3 liters of buffer. UROS was eluted batchwise using the same buffer containing 0.12 M NaCl and was concentrated for subsequent purification using an Amicon CH-4 hollow fiber concentration unit. Then ALAD was eluted from the preparative DEAE column using 0.025 M potassium phosphate buffer, pH 6.8, containing 0.1 mM DTT and 0.25 M KCl. Fractions were collected and assayed for protein and enzyme activity.

2. Ammonium Sulfate Fractionation:

Solid ammonium sulfate was added to the solution containing ALAD until the ammonium sulfate concentration reached 45% of saturation (277 g/1000 ml). The mixture was stirred

for 30 min and then centrifuged at 5,000 x g for 20 min. The precipitate containing nearly all of the ALAD activity was resuspended in 0.05 M potassium phosphate, pH 6.8, containing 0.25 M KCl and 1 mM DTT.

3. Octyl-Sepharose Chromatography:

The resuspended ALAD was passed through a 5 cm x 10 cm column of octyl-Sepharose CL-4B using the above buffer. The enzyme did not bind to the hydrophobic support and was eluted in the buffer wash. The octyl-Sepharose was regenerated after use by washing with 3 volumes each of distilled water, 1.0 M KOH, ethanol, butanol, ethanol, distilled water, and 0.05 M potassium phosphate buffer, pH 6.8.

4. Phenyl-Sepharose Chromatography:

Although ALAD did not bind to octyl-Sepharose, the enzyme bound avidly to phenyl-Sepharose, which is less hydrophobic. The post octyl-Sepharose solution containing ALAD activity was applied to a 2.6 cm x 40 cm column of phenyl-Sepharose CL-4B. The column was washed with 200 ml of 0.02 M potassium phosphate, pH 6.8, containing 0.3 mM DTT. Elution of the enzyme was accomplished using a 0 to 80% ethylene glycol gradient in 0.02 M phosphate buffer, pH 6.8. The gradient was constructed in a four-chamber apparatus with equal volumes of 0, 20, 70 and 80% ethylene glycol. The fractions containing ALAD were pooled and then concentrated to 3.0 ml in an Amicon ultrafiltration apparatus equipped with a PM 30 filter. Phenyl-Sepharose was regenerated by the method described above for octyl-Sepharose.

5. Gel Filtration:

The concentrated ALAD preparation was applied to a 2.5 cm x 100 cm column of Bio-Gel A-1.5m or Sephadex G-200 equilibrated with 0.01 M potassium phosphate, pH 6.8, containing 0.5 M KCL and 0.1 mM DTT. The major peak of enzyme activity was concentrated in an Amicon 8 MC ultrafiltration apparatus and stored at -20°C.

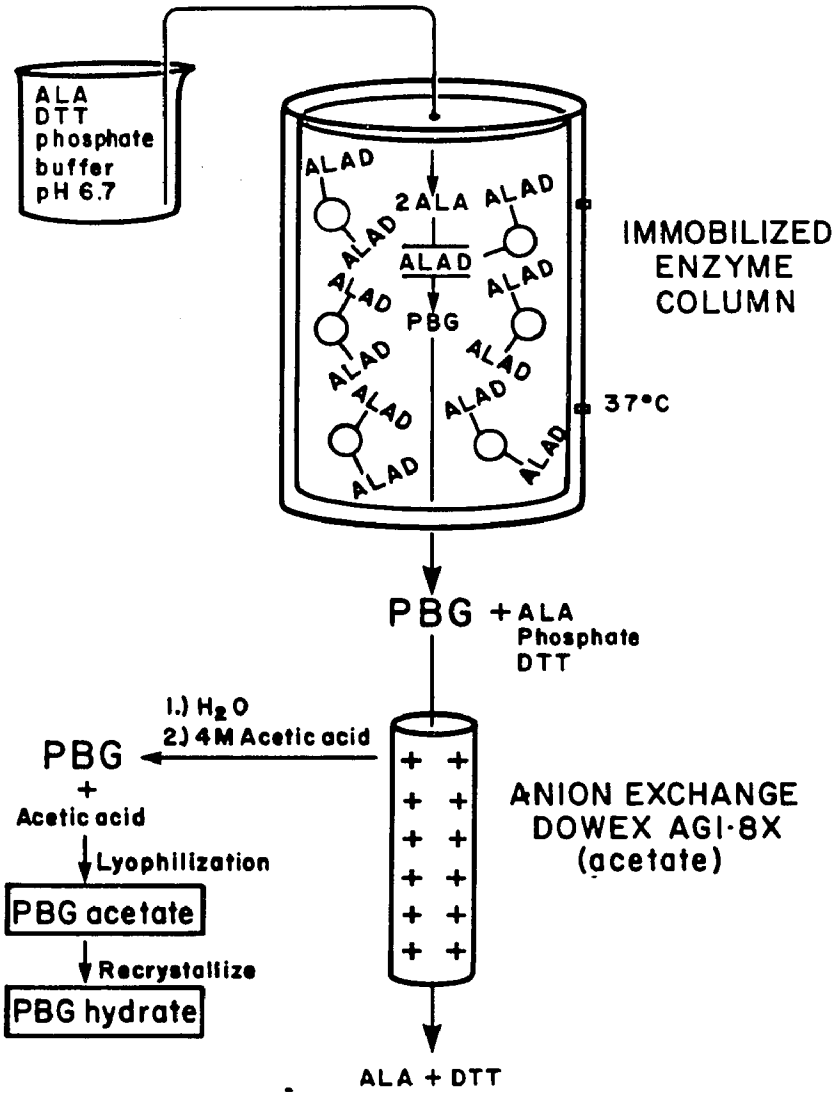
L. Synthesis of PBG:

This biosynthetic procedure is illustrated in Figure 4. Using immobilized human ALAD which was purified from human erythrocytes as described by Anderson and Desnick (122), PBG was synthesized by modification of the procedure of Gurne and Shemin (74). Using the procedure of March *et al.* (129), ALAD was covalently bound to CNBr activated Sepharose 4B (130); alternatively the enzyme could be successfully immobilized using phenyl-Sepharose. The enzyme gel was placed in a 5 cm x 10 cm column maintained at 37°C and a solution of 2 mM ALA in 20 mM potassium phosphate buffer, pH. 6.7, with 0.3 mM DTT was passed through the column. PBG produced by the enzyme reactor was collected and separated from ALA (74) by using a 2.5 x 40 cm column of Dowex 1-X8 ion exchange resin in the acetate form placed in series with the enzyme reactor column. When the effluent of the Dowex 1-X8 column became Ehrlich's positive the column was washed with cold distilled water and the PBG was eluted using 4 M acetic acid. Lypholyzation resulted in a white powder of PBG acetate. A portion of acetate salt was converted to PBG hydrate by dis-

Figure 4. Synthesis of Porphobilinogen (PBG).

Purified human ALAD was covalently immobilized on Sepharose 4B using CNBr or alternatively, immobilized on phenyl-Sepharose CL4B. The immobilized enzyme was maintained in a jacketed column at 37°C using a water recirculator. When a solution of ALA was percolated through the column, the immobilized enzyme produced PBG which was collected on an ion exchange column in series with the enzyme reactor. Unreacted ALA did not bind the Dowex column. Using 4 M acetic acid, PBG was eluted from the Dowex column; and the acetic acid and water removed by lyophilization to yield pure PBG acetate. PBG hydrate was also obtained by dissolving the acetate salt, adjusting the pH to 6 with 4 M NH_4OH, and lyophilization to remove ammonium acetate.

SYNTHESIS OF PORPHOBILINOGEN



solving in water, adjusting the pH to 6 with 4 M NH_4OH and lypholyzing to remove ammonium acetate.

M. Synthesis of Radiolabeled PBG:

A 1 ml sample consisting of 1 mc[3,5-^{3}H]ALA in 0.1 M potassium phosphate, pH 6.7, and 25 units of human ALAD were incubated at 37°C for 1 hr and then placed on ice. PBG and ALA were then separated from the ALAD by gel filtration on Sephadex G-50. The tritium labeled PBG was isolated as the acetate salt by ion-exchange chromatography on Dowex-x8 and lypholyzation as described earlier. The specific activity of the ^{3}H-PBG was 4.7 x 10^8 dpm/μg PBG.

N. Immobilization of PBG:

Carboxyhexyl-Sepharose 4B and aminohexyl-Sepharose 4B were prepared by coupling 6-aminocaproic acid or 1,6-diaminohexane to CNBr activated Sepharose 4B using the method of March *et al.* (129). Amino porous glass beads and arylamino porous glass beads were synthesized as described by Weetal and Filbert (131). To attach the carboxyl group(s) of PBG to the support the amino group of the pyrrole was first blocked using trifluoroacetic anhydride. The blocked pyrrole was attached to aminohexyl-Sepharose 4B using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide using conditions described in the Pharmacia affinity chromatography handbook. The trifluoroacetic group was removed with dilute alkali. To attach the amino group of PBG to carboxyhexyl-Sepharose 4B the support was activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, the gel was then washed with 0.01 M sodium

phosphate with 0.5 M NaCl to remove the coupling agent and PBG hydrate added to the activated gel. The coupling of PBG to a zirconium-clad arylamino porous glass support through the pyrrole nucleus was accomplished via the diazo linkage procedure described by Weetal and Filbert (131). All supports contained significant quantities of PBG as determined by 1) incorporation of radioactively labeled PBG into the support and 2) a positive Ehrlich's test which produced magenta beads.

O. Purification of Human UROS:

The initial procedure (lysis of erythrocytes and preparative DEAE chromatography) were often carried out simultaneously with ALAD purification. Since outdated human erythrocytes contained much less UROS than ALAD, the procedure was scaled up from 6 liters to 10 liters for UROS purification. Pilot studies indicated that UROS isolated from older erythrocytes did not differ from enzyme isolated from fresh erythrocytes with respect to kinetic properties, isoelectric point(s), pH optimum and molecular weight. Therefore, the starting material of large-scale purification procedures was outdated human blood. After centrifugation at 2,000 x g for 15 min and removal of the buffy coat and plasma, erythrocytes were washed with cold isotonic sodium chloride. Osmotic lysis of the 10 liters of erythrocytes was performed in liter increments by the addition of 3 volumes of cold deionized water containing 0.3 mM DTE. Stroma and incompletely lysed cells were removed by centrifugation at 12,500 x g for 90 min. The

supernatant was dialyzed against 7 mM potassium phosphate, pH 6.80, containing 0.1 mM DTE and 0.017% sodium azide.

1. Preparative DEAE-Cellulose Chromatography:

The preparative DEAE-cellulose column (10 x 60 cm) was thoroughly equilibrated with the above dialysis buffer. To prevent undue back pressure, it was important to use DEAE-cellulose which had been precycled in both acid and base. In order to elute the hemoglobin in the void volume and buffer wash, the pH was maintained below 6.87 (measured at 23°C), It was necessary to maintain the pH above 6.75 to bind UROS.

The dialyzed erythrocyte lysate was pumped through the column at a flow rate of 10 ml/min and the column was washed with 3 liters of buffer. The UROS was eluted batchwise using the same buffer containing 0.12 M NaCl and was concentrated to a volume of approximately 700 ml using an Amicon CH-4 hollow fiber concentration unit. Elution of ALAD remaining on the column was also accomplished with 0.30 M KCl as described earlier.

2. Octyl-Sepharose Chromatography:

The concentrated UROS was passed through a 5 x 20 cm column of octyl-Sepharose CL-4b in the same buffer used for elution of the preparative ion-exchange column. UROS was only slightly retarded on the hydrophobic support and was easily eluted in the buffer wash which was 20 mM potassium phosphate, pH 8.0, containing 0.25 M KCl and 0.1 mM DTE.

3. Phenyl-Sepharose Chromatography:

Although UROS failed to bind to octyl-Sepharose, the

enzyme avidly bound to phenyl-Sepharose. The eluate containing UROS from the octyl-Sepharose column was applied to a 10 x 10 cm column of phenyl-Sepharose CL-4B at a flow rate of 100 ml/hr and the column was washed with 500 ml of the above buffer. The enzyme was eluted using a linear 0 to 80% ethylene glycol gradient. The gradient was constructed in a 5-chambered (2,000 ml) apparatus with equal volumes of 0, 40, 60, 80, and 80% ethylene glycol. The last chamber was held in reserve until approximately 1,500 ml of the gradient had been pumped into the column. To achieve good yields of human UROS it was necessary when constructing the gradient to adjust the pH of the alkaline ethylene glycol from pH 9.2 to pH 8.0 using dilute phosphoric acid. Fractions containing UROS activity were pooled and concentrated in an Amicon ultrafiltration cell using a PM-10 filter.

4. Gel Filtration:

The concentrated UROS was applied to a 5 x 100 cm Sephadex G-100 column previously equilibrated with 10 mM potassium phosphate, pH 8.0, containing 0.05 M KCl and 0.1 mM DTE. Slow flow rates (0.3 ml/min) resulted in the separation of the enzyme from several visible protein bands. Fractions containing the highest specific activity were pooled, concentrated as described above and dialyzed against 10 mM potassium phosphate, pH 8.0, containing 0.1 mM DTE.

5. DEAE-Cellulose Chromatography:

The dialyzed UROS was applied to a 1.6 x 40 cm column of DEAE-cellulose which had been previously equilibrated with

dialysis buffer. A shallow, linear 0 to 0.12 M sodium chloride gradient was used to elute the enzyme. Peaks of enzyme activity were separately pooled, concentrated and stored at -20°C.

P. Amino Acid Analysis of Human ALAD and UROS:

Amino acid composition of homogenous ALAD and UROS were determined following hydrolysis of 0.5 mg of purified enzyme in 0.5 ml of 3.0 M toulene sulfonic acid containing 10 µl of 1,2 ethanedithiol and 10 µl of phenol in evacuated sealed tubes at 110°C for 24 hr. The amino acid concentrations in the hydrolysate were measured using a Beckman 119 CL amino acid analyzer.

Q. Production and Identification of UROS Enzyme-Substrate Intermediates:

A 80 µg sample of pure human UROS A and B was incubated with an excess 1 µg ^{3}H-PBG (4.7×10^{8} dpm/µg) for 10 min at 37°C, placed in an ice bath then immediately run on pH 8.2 polyacrylamide disc gels at 4°C as described earlier. Control gels consisted of enzyme or substrate only. Triplicate gels were stained for protein, sliced, and stained for UROS activity. Bands containing UROS and spaces between bands were cut out of the gels and the gel slices were dissolved with NCS tissue solubilizer. Using an organic scintillation cocktail, ^{3}H-PBG and ^{3}H-uroporphyrin were then quantitated in a liquid scintillation counter.

R. Determination of Patterns of UROS Enzyme-Substrate Intermediates in Normal Erythrocytes and Tissues and AIP Erythrocytes:

Human blood was placed into a heparinized tube, centrifuged at 2,500 rpm for 10 min and the plasma and buffy coat discarded. Erythrocytes were then washed twice with isotonic saline and 2.0 ml placed into a 40 ml plastic centrifuge tube. The red cells were lysed by the addition of 20 ml of cold distilled water and centrifuged at 35,000 g for 20 min. Frozen washed erythrocytes from heterozygotes with AIP were lysed in the same manner. The supernatant was then dialyzed against 7 mM potassium phosphate, pH 6.8, containing 0.1 mM DTE (2 x 2 liters, 6 hr). Small tissue samples were homogenized in a Ten-Broek glass homogenizer and larger tissue samples were homogenized using a polytron tissue homogenizer. For most tissues, 10% w/v tissue homogenates were prepared. Homogenates were centrifuged at 35,000 g for 30 min and the supernatants dialyzed as described above.

Dialyzed erythrocyte lysates were applied to a 0.9 x 30 cm columns of DEAE-cellulose (prepared as described in purification procedures) at a flow rate of 1.0 ml/min and 60 ml of buffer passed through the column. UROS enzyme-substrate intermediates were eluted as peaks of enzymatic activity using a linear 400 ml 0 to 0.11 M NaCl gradient. Fractions (2.0 ml) were collected and 0.5 ml were assayed 2 hr for UROS activity to quantitate enzyme-substrate patterns.

Dialyzed tissue homogenates (~100 ml) were applied to

1.6 x 40 DEAE-cellulose columns at a flow rate of 1.0 ml/min. The columns were washed with 120 ml of buffer and UROS eluted using an 800 ml 0 to 0.11 ml NaCl gradient. Peaks of UROS activity were determined by assaying 0.5 ml of each column fraction for 2 hr at 37°C.

S. Production and Quantitation of Rabbit Anti-Human UROS Antibodies:

New Zealand rabbits were injected intradermally and intramuscularly with 150 μg of homogeneous human UROS 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 titers of rabbit anti-human UROS were determined by an immunotitration assay. Antigen (150 μl; 3 μg of homogeneous UROS), 50 μl immune sera (in serial dilution, and 100 μl of rabbit IgG (0.4 mg/ml) were mixed and incubated at 37°C. After 30 min, 50 μl of goat anti-rabbit IgG was added and the mixture incubated at 37°C for 30 min. To insure quantitative precipation of the anti-UROS antibodies the samples were left at 4°C overnight. The mixtures were then centrifuged at 7,500 rpm for 15 min. The supernatant, which contained unbound enzyme, was then assayed for activity by the standard assay described above.

T. Partial Purification of Immune Serum:

IgG in pooled rabbit antisera was partially purified by the method of Harboe and Ingeld (132). The antibody was precipitated by the addition of 4 volumes of neutralized

saturated ammonium sulfate to 6 volumes 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 antibody was aliquoted into 1.5 ml samples and stored at -20°C.

U. Immunological Assays:

Erythrocytes from normal individuals and AIP patients were lysed by the addition of 3 volumes of 1 mM sodium phosphate buffer, pH. 7.6, containing 1 mM DTT, 1 mM $MgCl_2$ and 0.05% Triton X-100. The lysate was centrifuged at 30,000 x g for 20 min and the supernatant was removed and used for assays. The amount of CRM in normals and AIP patients was compared by two procedures 1) quantitation of amount of enzyme immunoprecipitated by 1:50, 1:100, 1:200 and 1:800 dilutions of antibody when an equivalent amount of enzymatic activity was present and 2) quantitation of the degree of inhibition of immunotitration when a constant amount of normal lysate was added to AIP or control samples. The immunoprecipitation procedure was essentially the same as the method described to determine the titer of the anti-sera. After immunoprecipitation, the supernatants were assayed for UROS activity by standard techniques described above.

V. RESULTS

A. Purification and Properties of ALAD from Human Erythrocytes:

1. Purification of the Enzyme:

Table 4 summarizes the results of a typical purification of ALAD from 6 liters of packed human erythrocytes. The enzyme was purified approximately 38,000-fold with nearly a 70% overall yield. The DEAE-cellulose preparative chromatographic step resulted in a 600-fold purification of the enzyme, primarily due to the removal of hemoglobin. This step also provided complete resolution of ALAD from UROS activity. Nearly complete recovery of ALAD activity occurred after the DEAE-cellulose, ammonium sulfate precipitation and octyl-Sepharose steps. Although ALAD did not bind to octyl-Sepharose, this step removed other hydrophobic proteins. In contrast, the enzyme was avidly bound to the less hydrophobic phenyl-Sepharose (Figure 5), providing an additional 6-fold purification. The interaction of the enzyme with this support appeared to be hydrophobic in nature in that complete elution was accomplished with 80% ethylene glycol or less effectively with distilled water; aromatic compounds such as benzoic acid, imidazole or tryptophan did not elute the enzyme. Gel filtration was required as a final step to remove two minor protein contaminants as well as the ethylene glycol. BioGel A-1.5m worked slightly better than Sephadex G-200 in the gel filtration step.

TABLE 4

Purification of ALAD from Human Erythrocytes

The results in the table represent typical values for purification of the enzyme from 6 liters of packed erythrocytes.

Step	Volume	Total Activity	Specific Activity	Yield	Purification
	ml	*units**	*units/mg*	*%*	*-fold*
Erythrocyte lysate	14,250	807	0.00045	100	
DEAE-cellulose	2,402	789	0.29	98	602
45% $(NH_4)_2SO_4$ ppt.	125	780	0.49	97	1,020
Octyl-Sepharose	176	741	2.97	92	6,140
Phenyl-Sepharose	3.0	702	17.6	87	36,500
Bio-Gel A-1.5m	7.6	554	18.5	69	38,200

* *One unit of ALAD activity is equivalent to 1 umole PBG produced per hour.*

2. Purity of ALAD:

The enzyme appeared to be homogeneous, yielding a single band on polyacrylamide disc gel electrophoresis at pH 6.9 and 8.1 which stained for both protein (Figure 6) and enzymatic activity.

3. Molecular Weight and Subunit Composition of Human ALAD:

The apparent molecular weight of the native enzyme was 252,000 as determined by gel filtration on Sephadex G-200 (Figure 7a). After SDS polyacrylamide gel electrophoresis, a subunit molecular weight of approximately 31,000 was obtained (Figure 7b). These results are consistent with the enzyme being a homo-octamer.

4. Isoelectric Focusing of ALAD:

A single isoelectric point of 4.9 was obtained by the analytical column method. When the enzyme was subjected to flatbed isoelectric focusing, considerable loss of activity occurred below pH 5.5, probably due to precipitation. The ethylene glycol gradient in the column method successfully circumvented this problem.

5. Amino Acid Composition:

Table 5 summarizes the results of the amino acid analysis of the homogeneous human ALAD. In most cases the number of amino acid residues per molecular weight of 31,000 closely approximated integral multiples of four.

6. Effect of pH:

Figure 8 shows the effect of pH on the formation of PBG by human ALAD. A pH optimum of 6.3 to 6.7 was observed

Figure 5. Phenyl-Sepharose Chromatography of Human ALAD.

Although the enzyme did not bind to octyl-Sepharose it was tightly bound to phenyl-Sepharose. Thus substantial purification was achieved by sequential chromatography of ALAD on octyl-Sepharose and phenyl-Sepharose. When the post octyl-Sepharose enzyme preparation was applied to phenyl-Sepharose, the majority of the protein (■-■) appeared in the buffer wash and the enzyme was eluted using a 0 to 80% ethylene glycol gradient. Units of ALAD activity (●-●) are umole PBG/hr.

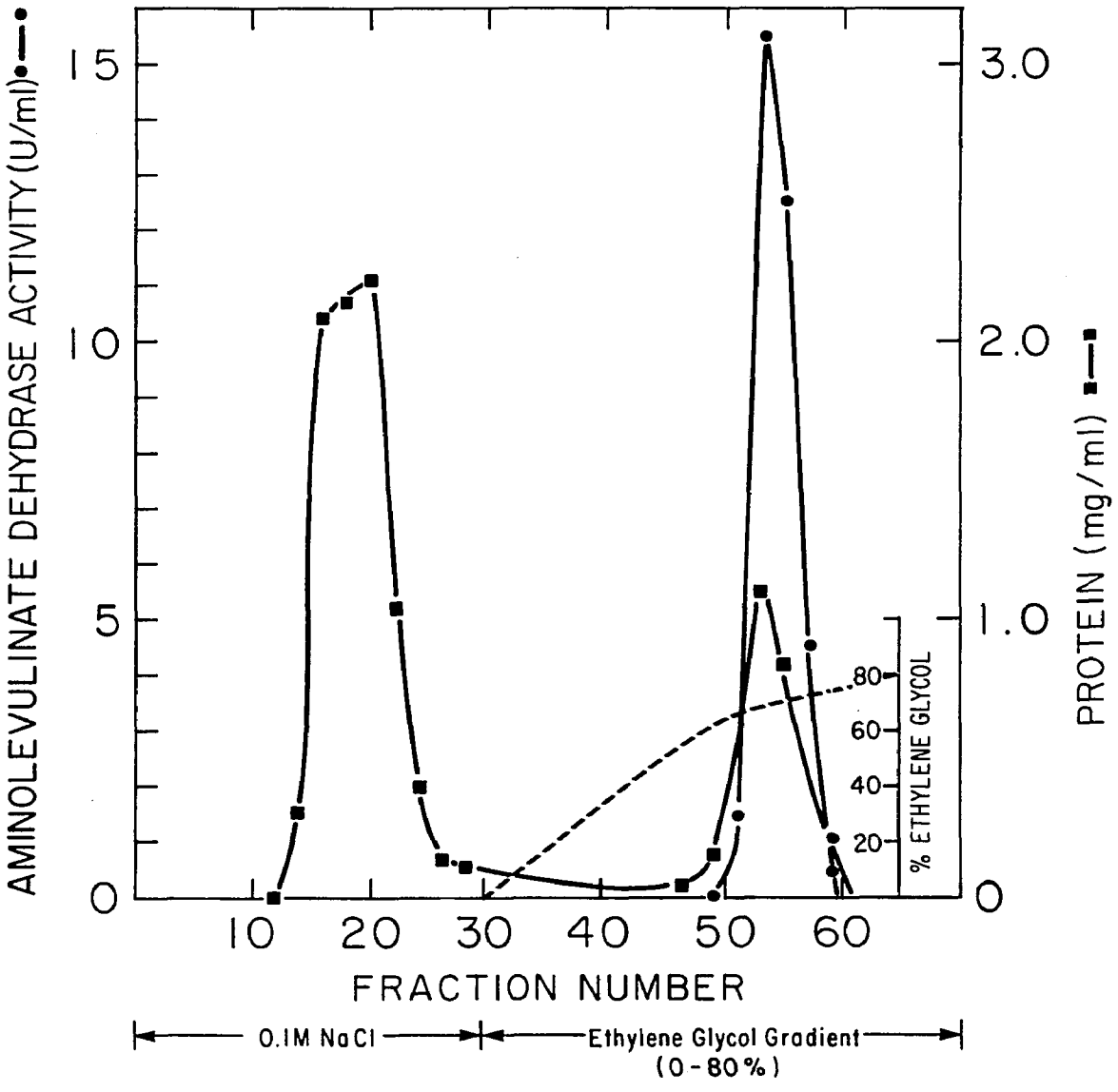

AMINOLEVULINATE DEHYDRASE ACTIVITY (U/ml)
PROTEIN (mg/ml)
% ETHYLENE GLYCOL
FRACTION NUMBER
0.1M NaCl
Ethylene Glycol Gradient
(0-80%)

Figure 6. Analytical Polyacrylamide Electrophoresis of Human ALAD.

Homogenous enzyme (15 µg) was applied to 10 cm polyacrylamide disc gels and electrophoresed at pH 6.8 (left) and 8.2 (right). In each case a single band which stained both for protein and enzymatic activity was observed.

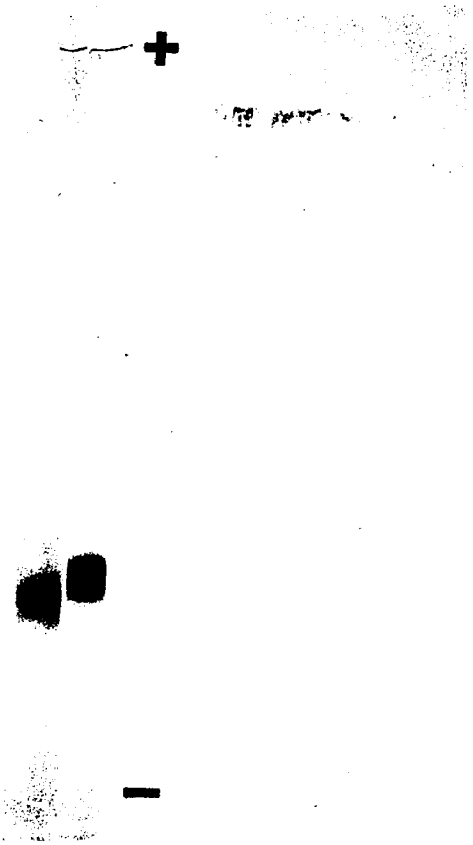


Figure 7. Molecular Weight of Human ALAD in Native(a) and Denatured(b) Forms.

To estimate the molecular weight of the native enzyme 5 mg of each protein standard and 1 mg of purified human ALAD were applied to a 2.5 x 100 cm column of Sephadex G-200. Enzymatic activity and protein were determined as described in "Methods". To estimate the subunit molecular weight of human ALAD (b), homogenous enzyme and protein standards were denatured in the presence of SDS, electrophoresed in SDS-polyacrylamide disc gels, and stained for protein as described in "Methods". Mobilities of protein standards are expressed relative to the bromophenol blue dye front. The results obtained are consistent with human ALAD being a homo-octamer with a subunit molecular weight of 31,000.

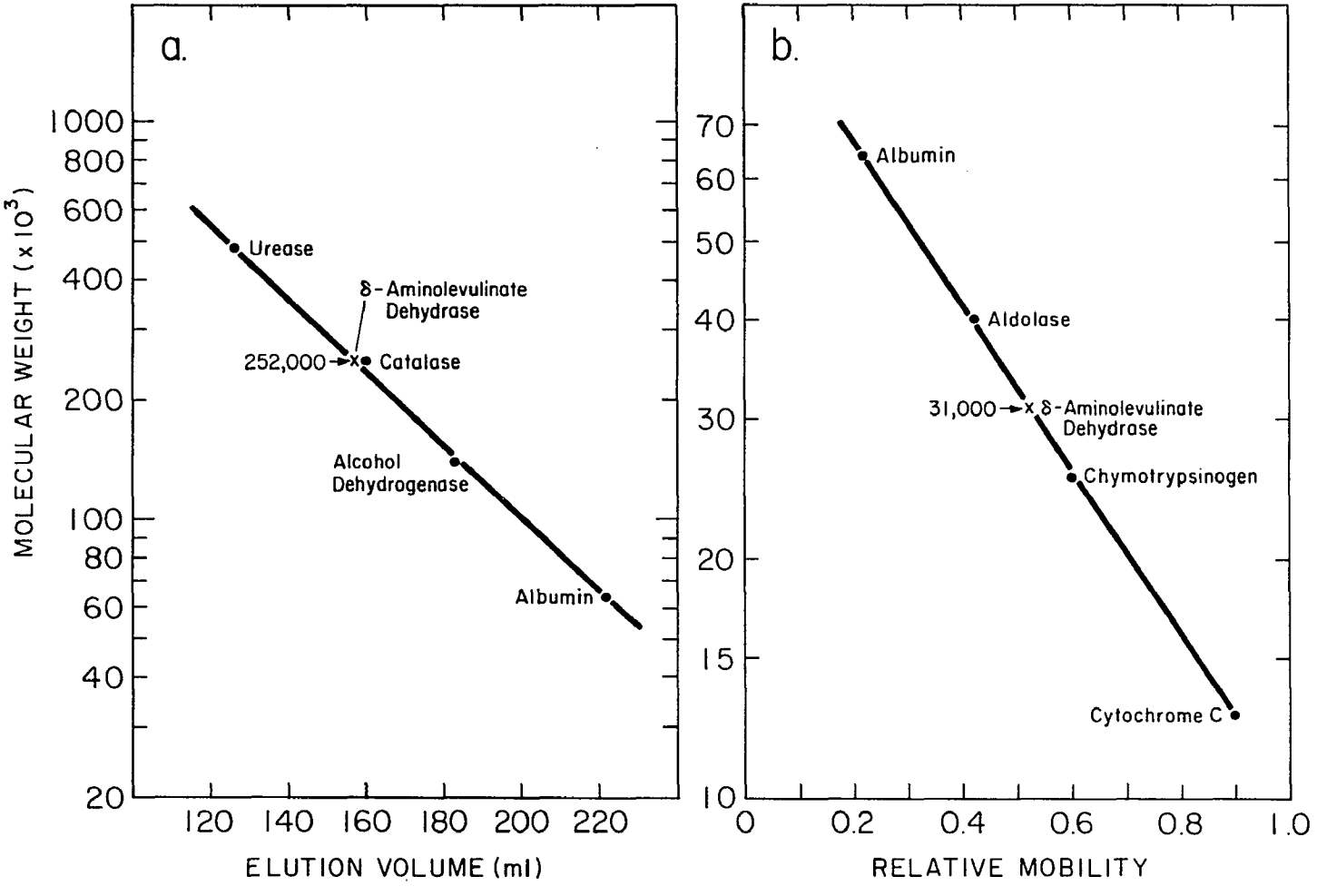

a.
MOLECULAR WEIGHT (x10³)
1000
800
600
400
200
100
80
60
40
20
Urease
δ-Aminolevulinate Dehydrase
252,000
Catalase
Alcohol Dehydrogenase
Albumin
120
140
160
180
200
220
ELUTION VOLUME (ml)
b.
70
60
50
40
30
20
15
10
Albumin
Aldolase
31,000
δ-Aminolevulinate Dehydrase
Chymotrypsinogen
Cytochrome C
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

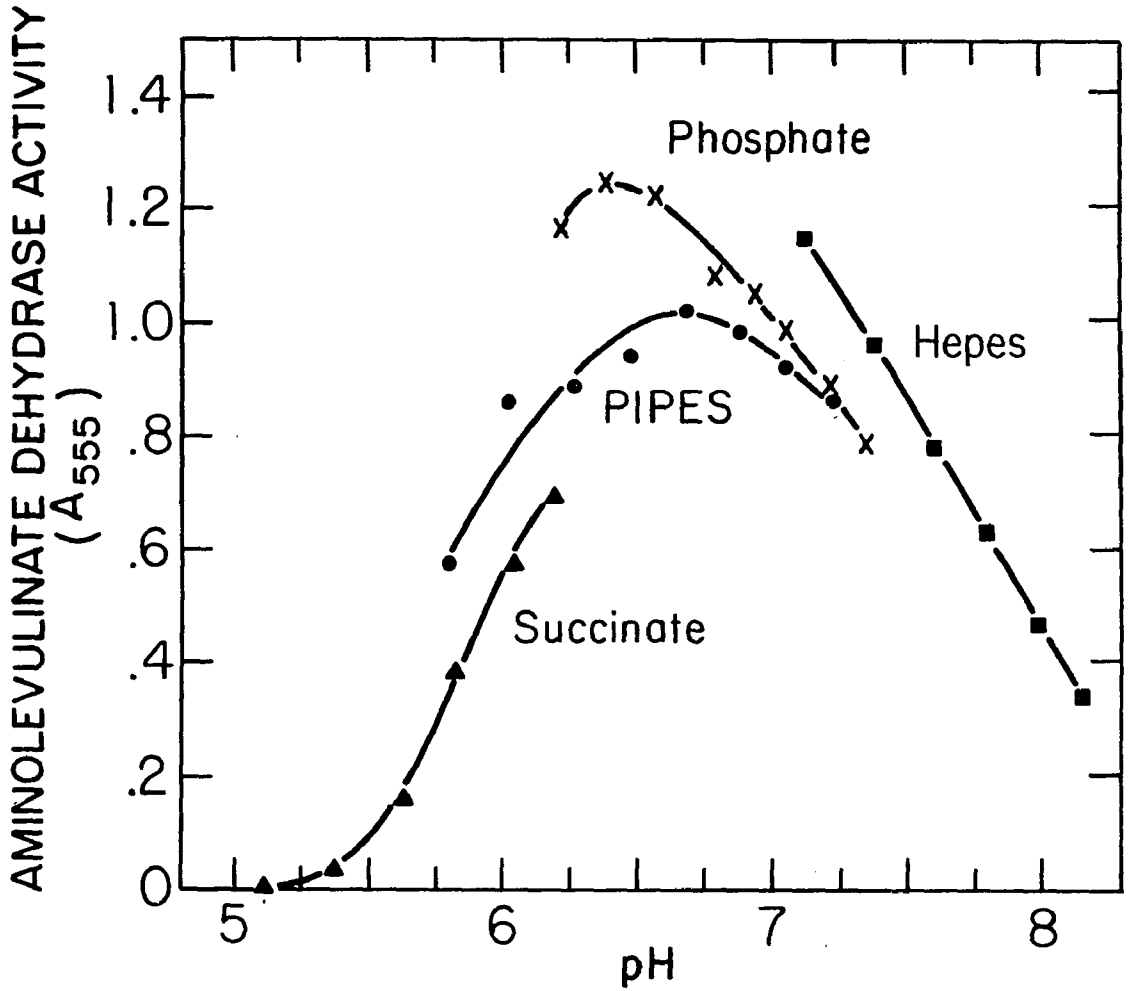
TABLE 5

Amino Acid Composition of Human Erythrocyte ALAD

Amino acid	Moles per 31,000 g of protein	Nearest integer per 31,000 g of protein
Aspartic acid	8.0	8
Threonine	10.8	11
Serine	10.8	11
Glutamic acid	28.0	28
Proline	18.4	18
Glycine	19.2	19
Alanine	37.6	38
Valine	16.0	16
Half-cystine	8.0	8
Methionine	8.0	8
Isoleucine	4.8	5
Leucine	28.8	29
Tyrosine	8.8	9
Phenylalanine	8.0	8
Lysine	8.8	9
Histidine	4.8	5
Arginine	16.8	17
Tryptophan	8.0	8

Figure 8. Effect of pH on Human ALAD Activity.

Enzyme activity was determined as described in "Methods" over a pH range from 5.2 to 8.2 using 0.05 M concentrations of sodium succinate (▲), sodium PIPES (●), sodium phosphate (x) and sodium Hepes (■).


AMINOLEVULINATE DEHYDRASE ACTIVITY
(A_{555})
1.4
1.2
1.0
.8
.6
.4
.2
0
5
6
7
8
pH
Phosphate
Hepes
PIPES
Succinate

using succinate, phosphate, Hepes and PIPES buffers. The activity of the enzyme was greatest in phosphate buffer.

7. Kinetic Analyses:

For kinetic studies, homogeneous enzyme which had been desalted over Sephadex G-50 equilibrated with 0.5 M PIPES buffer, pH 6.8, was used. The reaction was linear with time. The K_m was determined by plotting 1/v against 1/s (Figure 9); the enzyme demonstrated Michaelis-Menten kinetics and no cooperactivity was observed. The K_m was 0.27 mM and the V_{max} was 21.6 units/mg. The calculated maximal turnover number, assuming one active site per mole of enzyme, was 90 min^{-1}; however, if all subunits were catalytically active, the minimal turnover number would be 11 min^{-1}.

8. Inhibitors and Activators:

The effects of several known inhibitors of mammalian ALAD activity on the kinetics of the human enzyme were determined. Zinc inhibited the human ALAD at millimolar concentrations but activated the enzyme at 20 and 100 μM concentrations (Figure 9). The activation with zinc is consistent with an effective increase in the number of active catalytic sites. As shown in Figure 10, EDTA affected both the K_m and V_{max} of the enzymatic reaction. The addition of a stoichiometric amount of zinc effectively blocked these effects and in fact resulted in slight activation. Lead was found to be an extremely potent inhibitor affecting both the K_m and the V_{max} of the enzyme (Figure 11); a K_i of 0.0017 mM was obtained. In contrast, ferrous iron inhibited ALAD activity by lowering the velocity of the enzymatic reaction (Figure 11) indicating

Figure 9. Activation of Human ALAD by Zinc.

The sample was prepared by incubating 1 ml of the purified ALAD (5 mg/ml) with 5 mM DIT for 10 min at 37°C. Then pyridoxal 5'-phosphate (0.2 mM) and zinc acetate (25 mM) were added and the mixture was incubated an additional 5 min at 37°C. The sample was then desalted on a column (0.8 x 15 cm) of Sephadex G-50 (medium) equilibrated with 0.05 M PIPES buffer, pH 6.8. The specific activity of the resultant enzyme preparation was 16.9 units/mg. Each assay contained 3.5 µg of the enzyme. Additions were: none (●), 0.1 mM (■), or 0.02 mM (▲) zinc acetate.

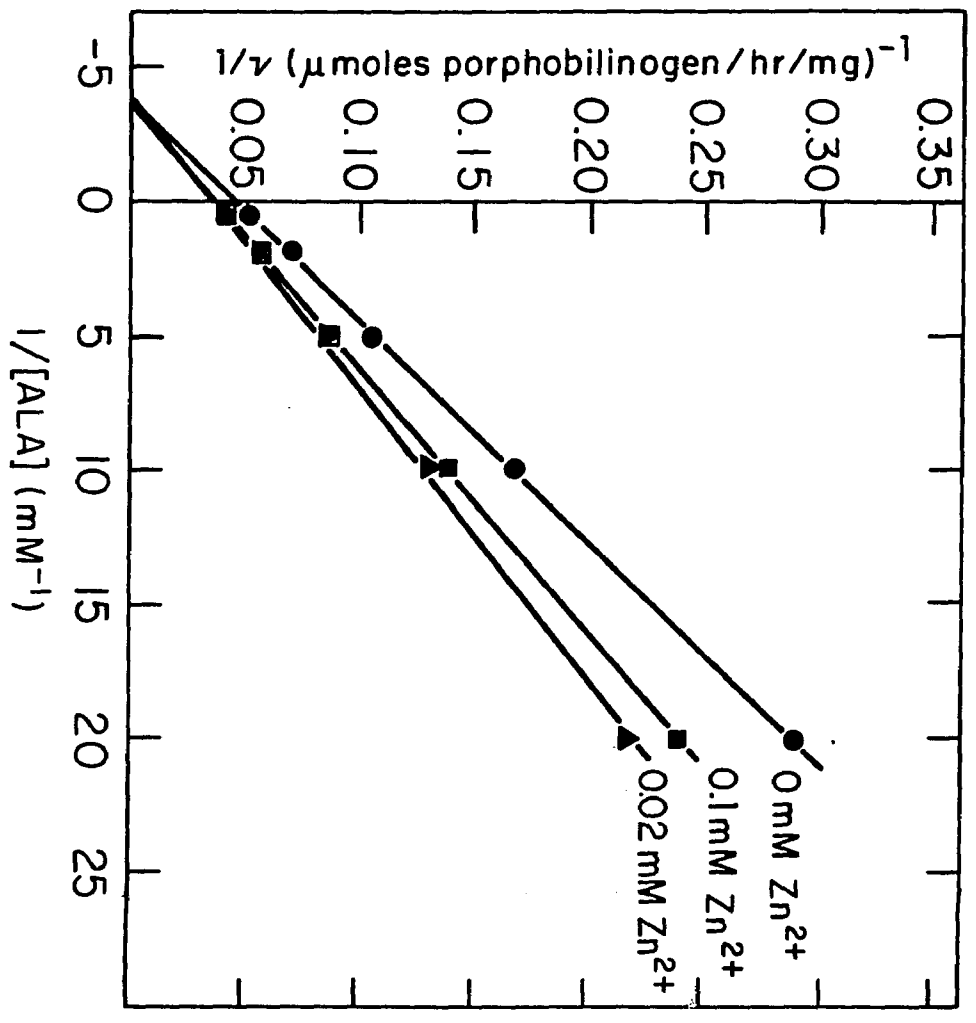

1/ν (μmoles porphobilinogen/hr/mg)$^{-1}$
0.05
0.10
0.15
0.20
0.25
0.30
0.35
-5
0
5
10
15
20
25
1/[ALA] (mM^{-1})
0 mM Zn^{2+}
0.1 mM Zn^{2+}
0.02 mM Zn^{2+}

Figure 10. Effect of EDTA on ALAD from Human Erythrocytes.

Each assay contained 3.5 µg of enzyme (16.9 units/mg) and was carried out as described in Figure 9. Concentrations of EDTA in the assay: none (●), 10 µM (■), 25 µM (▲), and 25 µM EDTA + 50 µM Zn^{2+} (◆).

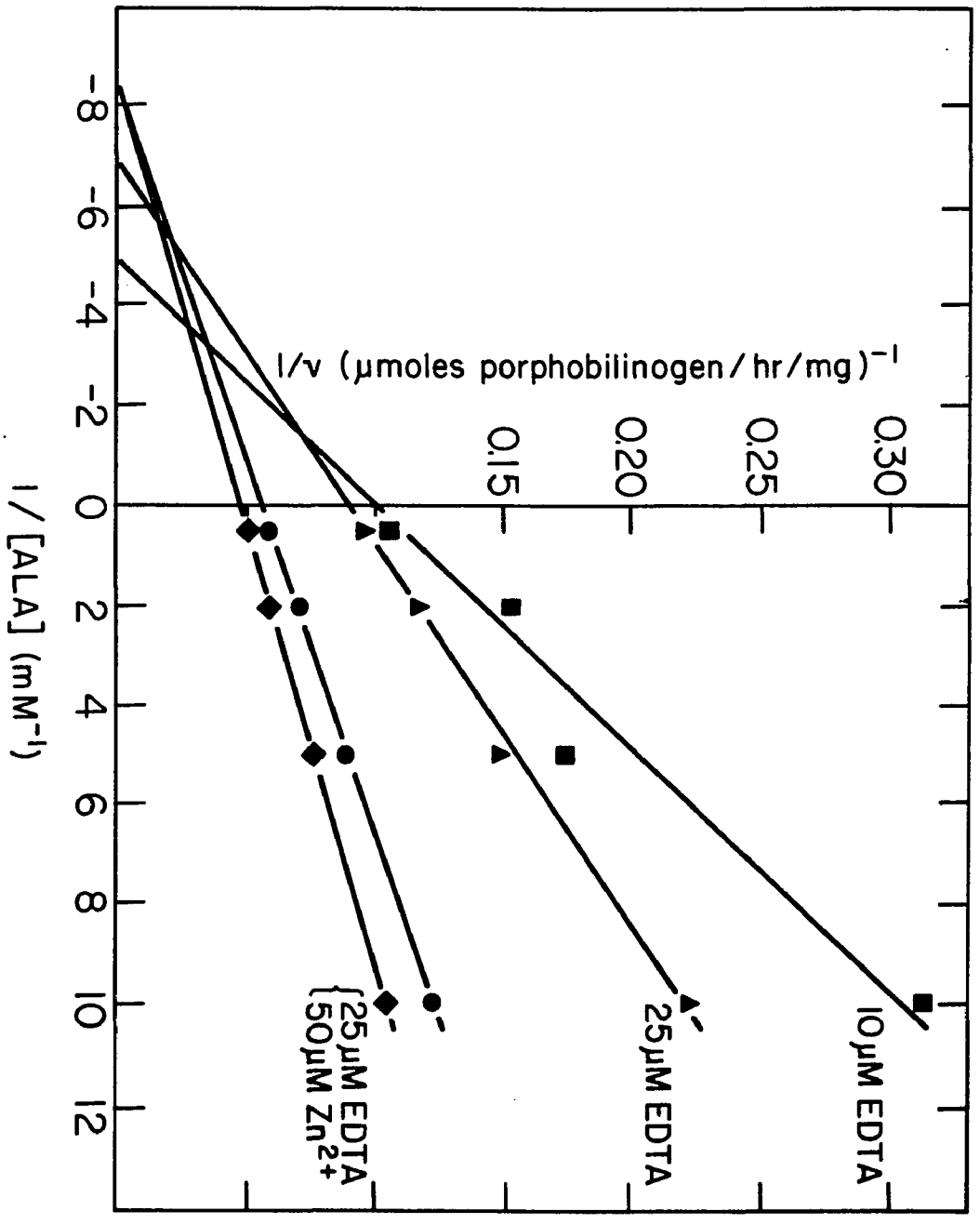

1/v (μmoles porphobilinogen / hr / mg)⁻¹
0.15
0.20
0.25
0.30
1 / [ALA] (mM⁻¹)
-8
-6
-4
-2
0
2
4
6
8
10
12
10μM EDTA
25μM EDTA
{25μM EDTA
{50μM Zn²⁺

Figure 11. Inhibition of Human ALAD by Ferrous Iron and Lead.

The assays were performed as described in Figure 9. Each assay contained 3.5 µg of ALAD (16.9 units/mg). Concentrations of effectors were: (Top) none (●), 0.01 mM (▲), and 0.1 mM ferrous ammonium sulfate (■); (Bottom) none (●), 1.0 µm (■), and 5.0 µM lead nitrate (▲).

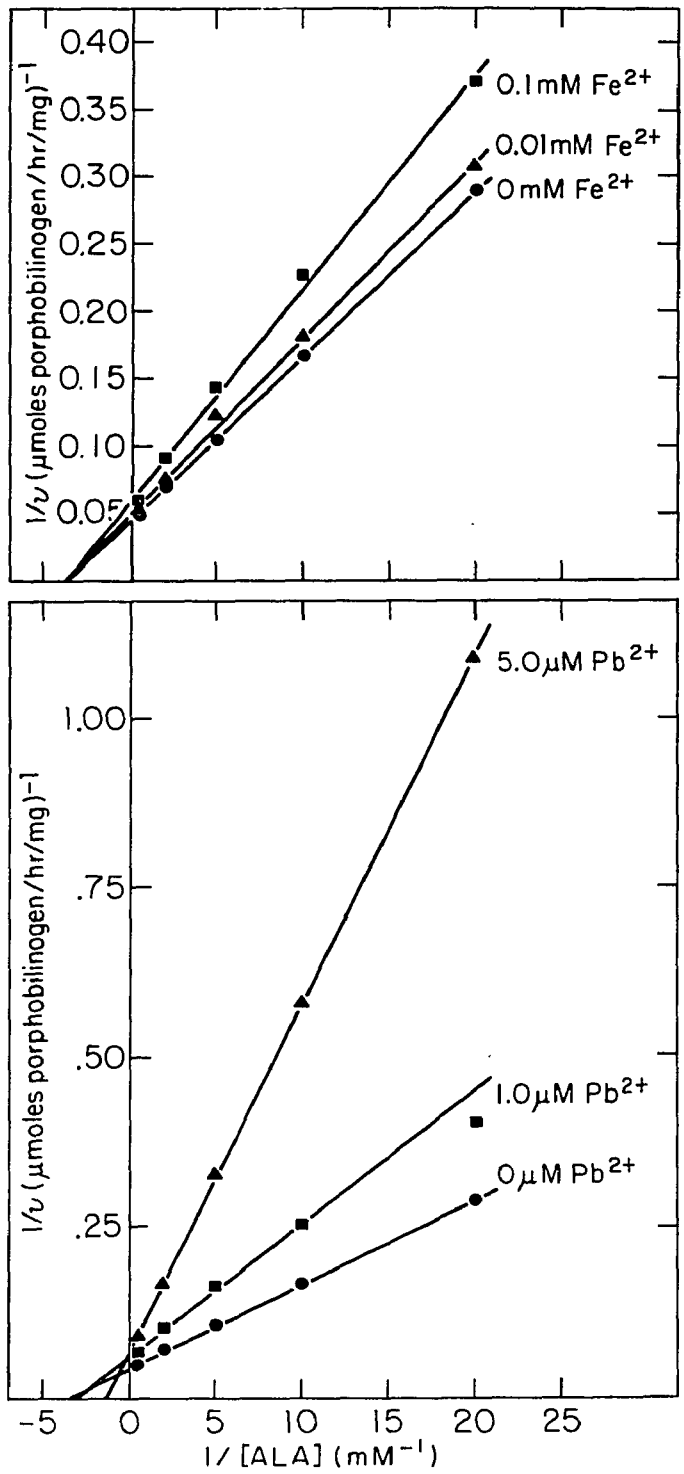
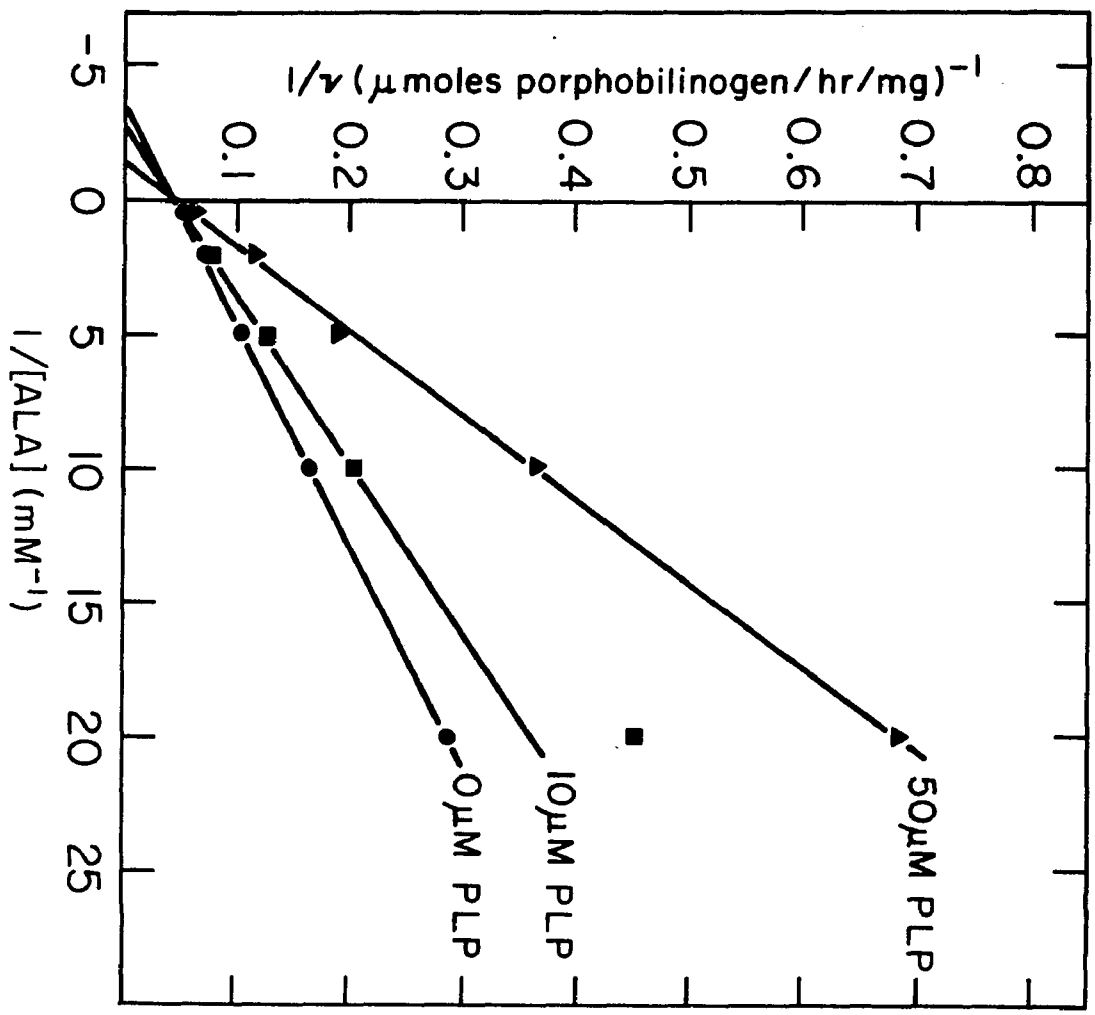

0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
1/v (μmoles porphobilinogen/hr/mg)⁻¹
0.1mM Fe2+
0.01mM Fe2+
0mM Fe2+
1.00
.75
.50
.25
1/v (μmoles porphobilinogen/hr/mg)⁻¹
5.0μM Pb2+
1.0μM Pb2+
0μM Pb2+
-5
0
5
10
15
20
25
1/ [ALA] (mM⁻¹)

Figure 12. Competitive Inhibition of Human ALAD by Pyridoxal Phosphate.

Each assay contained 3.5 µg of enzyme (16.9 units/mg) and was carried out as described in Figure 9. Concentrations of pyridoxal 5'-phosphate in the assay were: none (●), 10 µM (■), and 50 µM (▲).


1/v (μ moles porphobilinogen/hr/mg)⁻¹
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
-5
0
5
10
15
20
25
1/[ALA] (mM⁻¹)
0μM PLP
10μM PLP
50μM PLP

a non-competitive type of inhibition (K_i = 0.26 mM). Interestingly, pyridoxal 5'-phosphate inhibited the enzyme (K_i = 0.31 mM) by reduction in substrate affinity indicating competitive inhibition (Figure 12).

9. Spectral Analysis:

The homogeneous enzyme preparation appeared to have a pale yellow color. Since purified bovine ALAD has pyridoxal 5'-phosphate associated with it (133) the absorption spectrum of the human enzyme was determined. As shown in Figure 13, increased absorption was found at about 330 and 410 nm consistent with the known spectra for pyridoxal 5'-phosphate (134).

10. Thermal Stability:

Figure 14 shows the effect of heat on the purified enzyme. At 50°, 55° and 60°C, the enzyme was inactivated to 50% of initial activity at 200, 90 and 30 min, respectively. In each case a rapidly inactivated and a more stable component was observed suggesting possible subunit dissociation or conformational changes with heat.

11. Stability of Stored Enzyme:

Homogenous human ALAD was stable for 6 months when stored at high concentration (>2 mg/ml) in phosphate buffer containing 5 mM DTT, ph 6.8, at -20°C. Partially purified fractions were stored at -20°C in phosphate buffer with 5 mM DTT for 18 months with little or no loss of activity. In the absence of DTT enzyme activity was rapidly lost due to air oxidation. Addition of adequate concentrations of reducing agents restored

Figure 13. Absorbtion Spectrum of Human ALAD.

Enzyme (3.5 mg/ml) in 0.05 M sodium PIPES, pH 6.8 buffer was analyzed in a Gilford model 2000 spectrophotometer. The following absorbances were determined: $E^{1\%}_{260} = 18$; $E^{1\%}_{280} = 28$; $E^{1\%}_{325} = 3.3$; $E^{1\%}_{410} = 1.4$. The increased absorbtion at about 330 and 410 is consistent with the presence of pyridoxal phosphate in the enzyme.

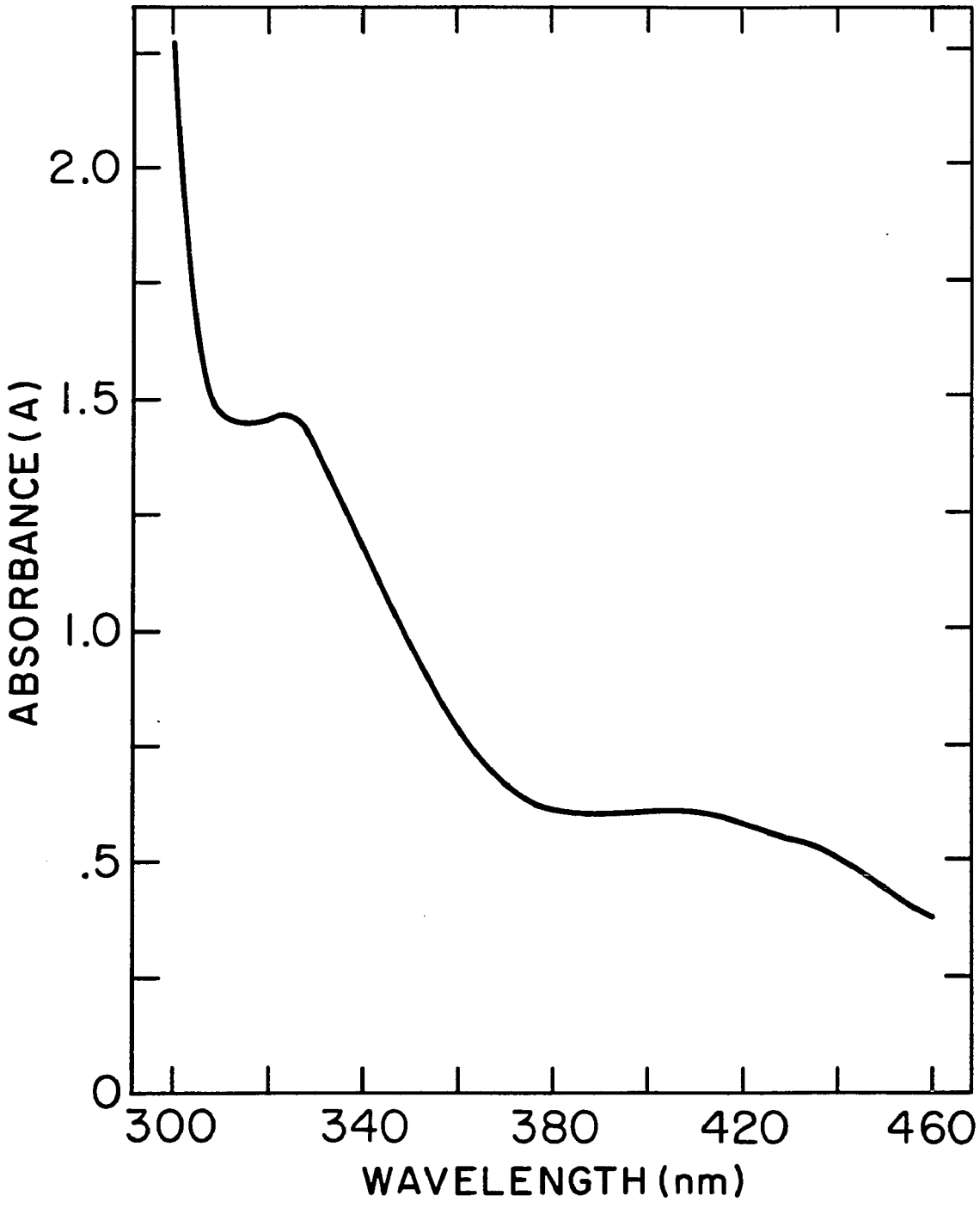
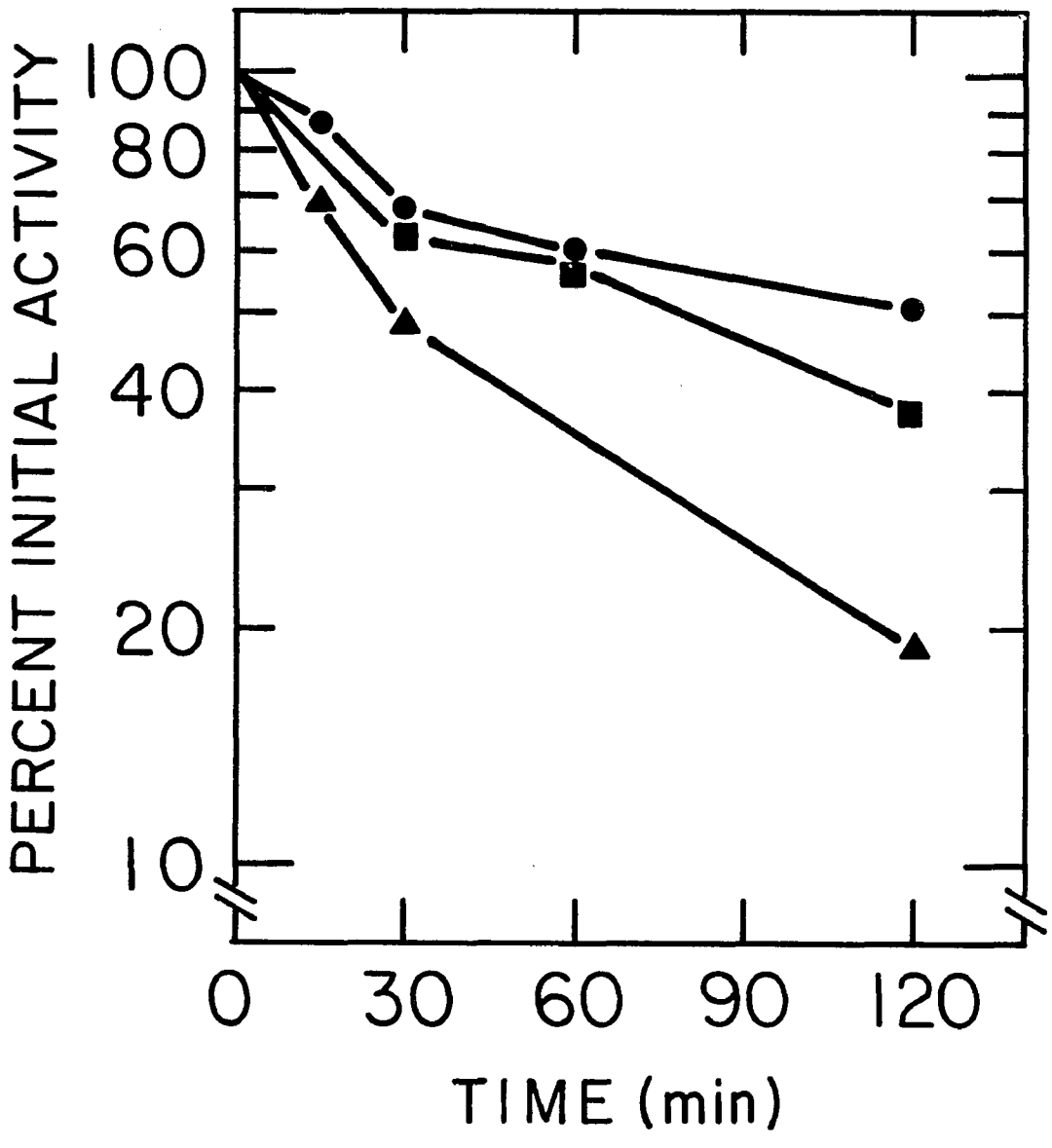

2.0
1.5
1.0
.5
0
ABSORBANCE (A)
300
340
380
420
460
WAVELENGTH (nm)

Figure 14. Thermal Stability of ALAD.

Aliquots of the purified enzyme were incubated at 50°C (●), 55°C (■), or 60°C (▲) in 25 mM phosphate buffer containing 5 mM DTT, pH 6.8. At various time intervals, samples were removed and assayed for activity as described under "Methods".


PERCENT INITIAL ACTIVITY
100
80
60
40
20
10
0
30
60
90
120
TIME (min)

activity. The conditions for increasing the stability of the enzyme in frozen supernatants of cultured cells have also been determined. ALAD in cells which had been lysed using 0.05% Triton X-100 in 5 mM sodium phosphate, pH 7.6, containing 1 mM DTT and 1 mM $MgCl_2$ was stable for at least 1 month at -20°C.

B. Synthesis of PBG:

ALAD from spinach and bovine liver was initially evaluated to see whether 1) these sources would be suitable for large scale purification and 2) enzyme from these sources possess physical properties compatible for immobilized biosynthesis of large quantities of PBG. The large amount of protein in bovine liver made this source unacceptable for purification using standard methods. However, this was before the unique hydrophobic properties of ALAD were discovered. The spinach enzyme proved to have a short half-life when incubated at 37°C or temperatures that resulted in appreciable PBG synthesis. Fortunately, the human enzyme from erythrocytes was readily purified in sufficient quantity and possessed adequate stability for synthesis of gram quantities of PBG. Initially, 4.5 gm of highly pure PBG was synthesized by means of covalently immobilized human ALAD (130). After being used for 28 days to make pyrrole, the column still possessed 30% of its initial activity.

A second synthesis was undertaken and another 4 grams of the pyrrole was synthesized using phenyl-Sepharose to immobilize the enzyme. This column, however, had a life time of

only 2 weeks. The efficiency of PBG production for both immobilized ALAD columns was nearly 100% initially and slowly decreased thereafter. The efficiency and amount of pyrrole synthesized was dependent on substrate concentration and flow rate. Since ALA cyclized (resulting in a pink adduct) when at pH 6.8 and especially when at high concentrations, 2 mM was determined to be the most suitable concentration of ALA. Flow rates greater than 2 ml/min did not permit sufficient time to elapse for efficient catalysis and over 50% of the ALA appeared in the void. Therefore flow rates between 0.7 and 1.5 ml were employed. These concentrations resulted in maximal PBG production.

Using human ALAD, ^{14}C-PBG was synthesized in a third preparation. The label was invaluable in monitoring the binding of PBG to solid supports. A subsequent preparation of ^{3}H-PBG with a specific activity of 4.7×10^{8} dpm/μg was synthesized; a total of 5 μg of the tritium labeled PBG in 0.5 ml was made. The biosynthetic approach was extremely efficient with yields approaching 95%. Although [3,5-^{3}H]ALA lost 25% of its label as $^{3}H_2O$ during the enzymatic reaction, this was readily removed by lypholyzation. Thus, the ^{3}H-PBG had more than twice as much radiolabel/molecule than the ALA starting material. This ^{3}H-PBG of high specific activity proved invaluable in the demonstration that multiple forms of UROS were enzyme-substrate intermediates.

C. Purification and Properties of UROS from Human Erythrocytes:

UROS activity was obtained from the same preparative DEAE column from which human ALAD was isolated. Conditions were determined which resulted in little or no loss of UROS activity in this preparation upon storage at -20°C and -70°C. This was in contrast to the cryolability of the human erythrocyte UROS purified by Stevens *et al.* (69). The stable UROS preparation permitted aliquots to be used in pilot studies of purification procedures.

1. Affinity Purification Studies:

The availability of PBG enabled immobilization of the pyrrole on several types of supports in an effort to use the affinity of human UROS for its substrate as a purification procedure. Attachment of PBG through its carboxyl group(s) to aminohexyl-Sepharose 4B or to aminopropyl porous glass beads and covalent linkage via the amino group of PBG to carboxyhexyl-Sepharose 4B were the initial affinity schemes investigated. Even after careful adjustment of conditions including ionic strength buffer, pH, and temperature, the enzyme did not bind to the affinity support. In contrast, a diazo linkage of the pyrrole nucleus of PBG to a zirconium-clad arylamino porous glass support resulted in 100% binding of partially purified human UROS. However, bound enzyme was not eluted with 1 mM substrate. The enzyme could be eluted using 8 M urea with recovery of 80% of activity after dialysis. Subsequently, it was determined that 80% ethylene glycol eluted UROS quantatively with little or no loss of acti-

vity. These findings suggested that the PBG arylamine support acted as an efficient hydrophobic support, not an affinity column. Therefore octyl-Sepharose and phenyl-Sepharose chromatography were investigated as possible purification steps in the purification of human UROS. These supports revealed unusual hydrophobic properties which permitted human UROS to be purified. The results of the purification of UROS are detailed below.

2. Purification of Human UROS:

Table 6 summarizes the results of a typical purification of UROS from 10 liters of outdated human erythrocytes. The enzyme was purified approximately 42,000-fold with a 25% overall yield. The initial preparative DEAE-cellulose chromatographic step resulted in separation of the enzyme from hemoglobin with a recovery of over 90%. DTE in the chromatography buffers resulted in better recovery of the enzyme during this and other steps. Concentration of the enzyme by ammonium sulfate precipitation required at least 80% saturation and resulted in lower overall yields. Therefore, a hollow fiber apparatus was used to concentrate the preparative DEAE eluate before octyl-Sepharose chromatography. Most of the protein including the enzyme was eluted in the void volume of octyl-Sepharose. After the octyl-Sepharose procedure a slight stimulation of enzyme activity was observed. Although the increase in specific activity of UROS after octyl-Sepharose chromatography was slight, removal of some hydrophobic proteins during this step enhanced subsequent purification of

TABLE 6

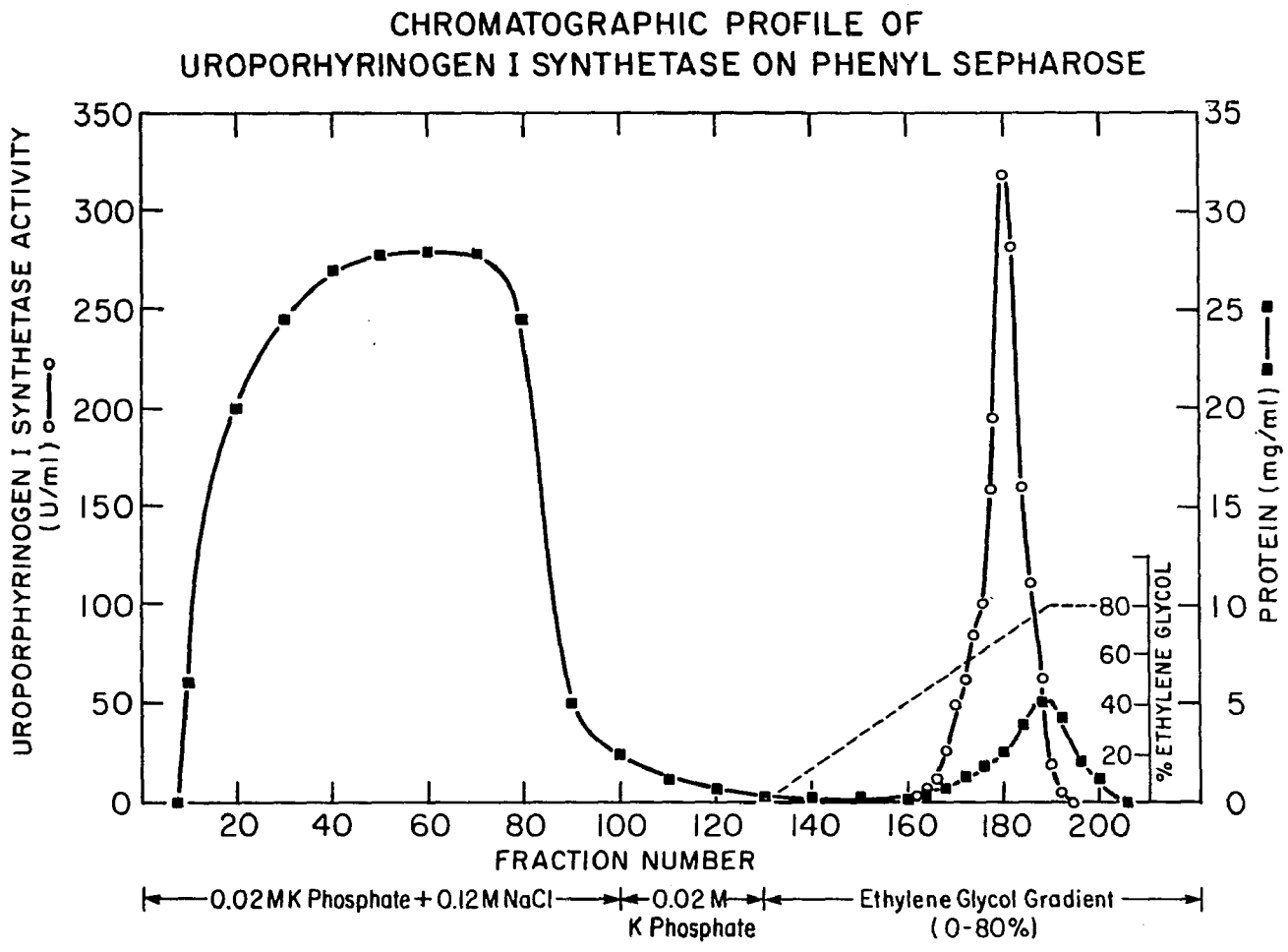
Purification of UROS from Human Erythrocytes

Step	Volume	Total Activity	Specific Activity	Yield	Purification
	ml	*units*	*units/mg*	*%*	*-fold*
Outdated Erythrocytes	10,000	108,500	0.0547	100	1
Erythrocyte Lysate	29,800	104,100	0.0733	96	1.3
DEAE-Cellulose	611	96,200	4.50	89	82.3
Octyl-Sepharose	727	99,800	5.36	92	98
Phenyl-Sepharose	15	82,900	134	76	2,440
Sephadex G-100	13.3	55,900	1,080	52	19,700
DEAE-Cellulose					
UROS A Form	5.4	13,700	2,320	12.6	42,400
UROS B Form	4.8	13,500	2,340	12.4	44,500

One unit of UROS activity is equivalent to 1 nmole uroporphyrinogen produced per hour.

Figure 15. Phenyl-Sepharose Chromatography of Human UROS.

The buffer wash from the preceeding octyl-Sepharose chromatographic step was applied to a 5 x 10 cm column of phenyl-Sepharose. The column was washed with 0.02 M potassium phosphate, pH 8.0, and eluted with ethylene glycol using a gradient constructed as described in "Methods". 0——0 human UROS activity; ■——■ protein concentration.


CHROMATOGRAPHIC PROFILE OF
UROPORHYRINOGEN I SYNTHETASE ON PHENYL SEPHAROSE
UROPORPHYRINOGEN I SYNTHETASE ACTIVITY (U/ml)
PROTEIN (mg/ml)
% ETHYLENE GLYCOL
FRACTION NUMBER
0.02M K Phosphate + 0.12M NaCl
0.02M K Phosphate
Ethylene Glycol Gradient (0-80%)

the enzyme on phenyl-Sepharose. A property of the enzyme which was key to the success of the purification was the ability of UROS to bind tightly to the less hydrophobic phenyl-Sepharose (Figure 15). The interaction of human UROS with this support permitted a 25-fold purification and appeared to be hydrophobic in nature. Although both distilled water and 80% ethylene glycol resulted in elution of the enzyme from phenyl-Sepharose, the latter resulted in better recovery of the enzyme. Aromatic compounds such as tyrosine, imidazole, tryptophan, or benzoic acid were unable to elute the enzyme.

Gel filtration (Figure 16) resulted in not only elimination of the ethylene glycol and contaminating UROCoS, but also significant purification (8-fold). The final purification step, DEAE-cellulose chromatography, resulted in the isolation of 5 peaks of human UROS (Figure 17). If only the first two peaks are considered, the yield for this step was approximately 60%. The identification of 5 peaks of enzymatic activity after ion exchange chromatography proved to be a general phenomonema and was observed not only in outdated erythrocyte lysates but also in fresh erythrocyte lysates, livers and brain (Figure 18). Although the specific activity of the enzyme in cultured lymphoid cell lines was quite low, these cells also had 5 peaks of enzyme activity after ion exchange chromatography.

3. Purity of UROS:

The enzyme derived from the first two (i.e., the UROS A and B) activity peaks from the DEAE-cellulose step appeared

Figure 16. Gel Filtration of Human UROS.

Concentrated post phenyl-Sepharose enzyme containing ethylene glycol was applied to a 5 x 100 cm column of Sephadex G-100. The enzyme was eluted as a single peak which only produced uroporphyrinogen of the type I isomer. 0——0 UROS activity; ■——■ protein.

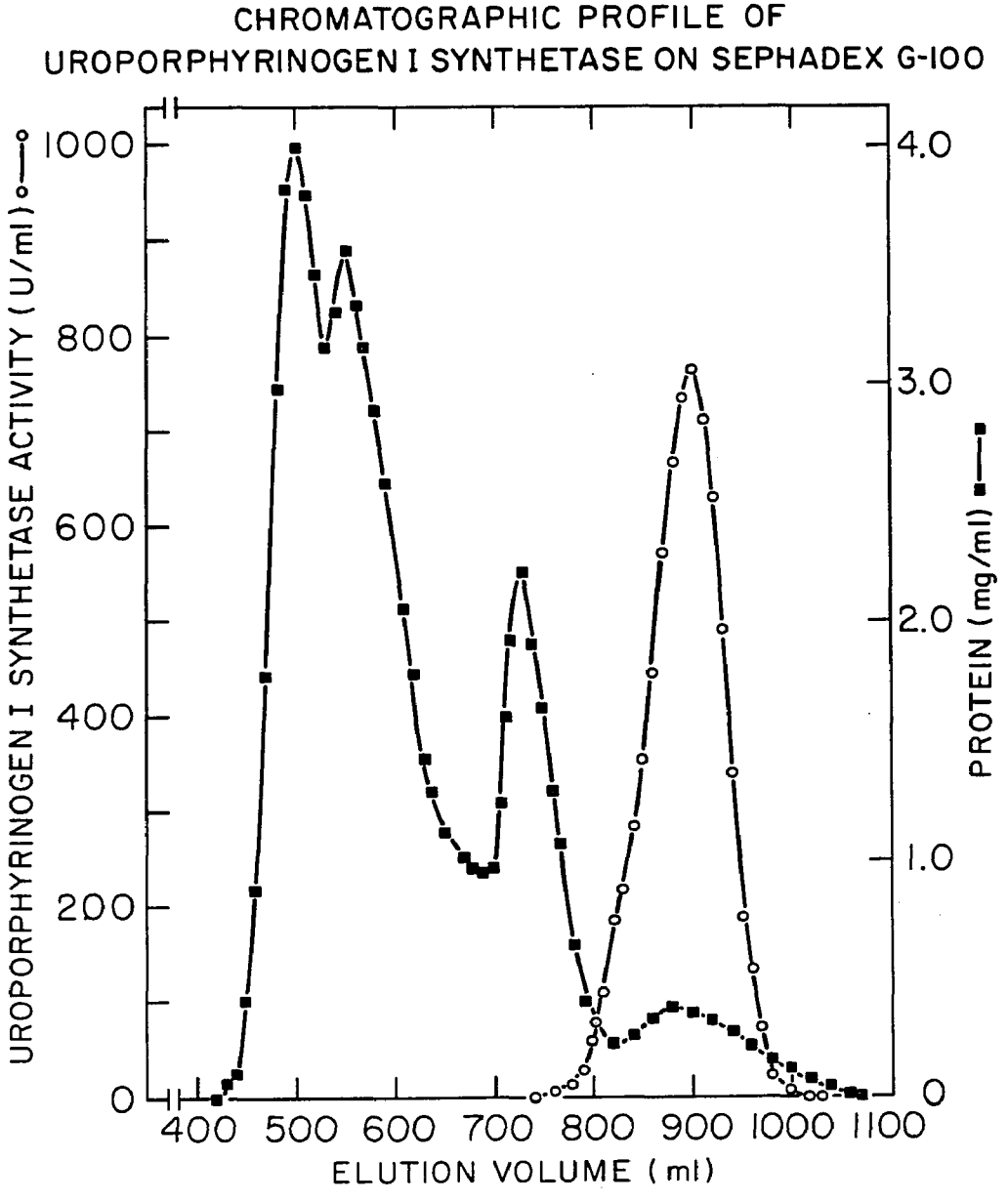

CHROMATOGRAPHIC PROFILE OF
UROPORPHYRINOGEN I SYNTHETASE ON SEPHADEX G-100
UROPORPHYRINOGEN I SYNTHETASE ACTIVITY (U/ml)
PROTEIN (mg/ml)
0
200
400
600
800
1000
1.0
2.0
3.0
4.0
400 500 600 700 800 900 1000 1100
ELUTION VOLUME (ml)

Figure 17. Chromatographic Profile of Human Erythrocyte UROS on DEAE-Cellulose.

The post Sephadex G-100 enzyme was applied and eluted from 1.6 x 40 column of DEAE-cellulose as described in "Methods." The 5 peaks of UROS activity were designated A, B, C, D and E from least to most charged. 0——0 UROS activity; ■——■ protein.

CHROMATOGRAPHIC PROFILE OF
UROPORPHYRINOGEN I SYNTHETASE ON DEAE CELLULOSE

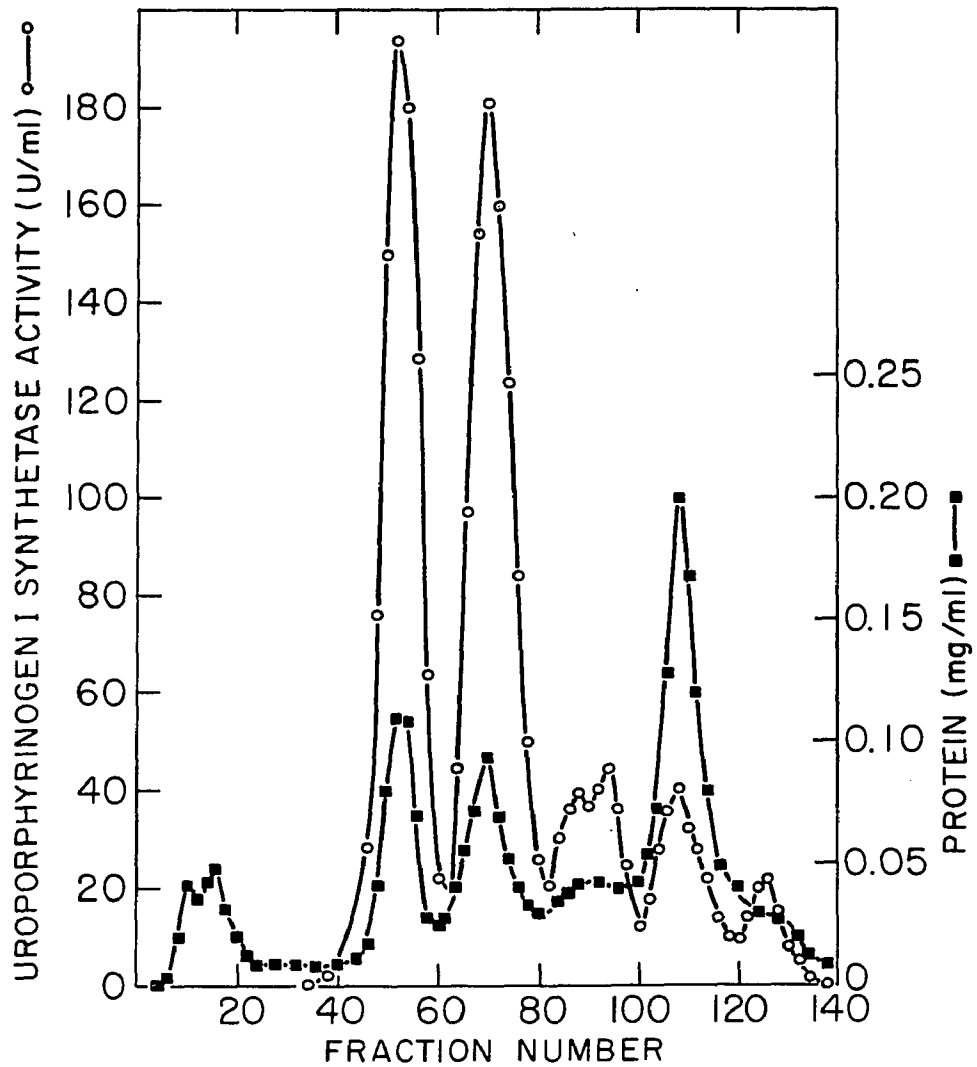


Figure 18. Multiple Forms of Human UROS in Erythrocytes, Liver and Brain.

Erythrocyte lysates and tissue homogenates were prepared as described in "Methods", dialyzed against 7 mM potassium phosphate, pH 6.8, and chromatographed on DEAE-cellulose. Five distinct peaks of enzymatic activity were observed in each tissue.

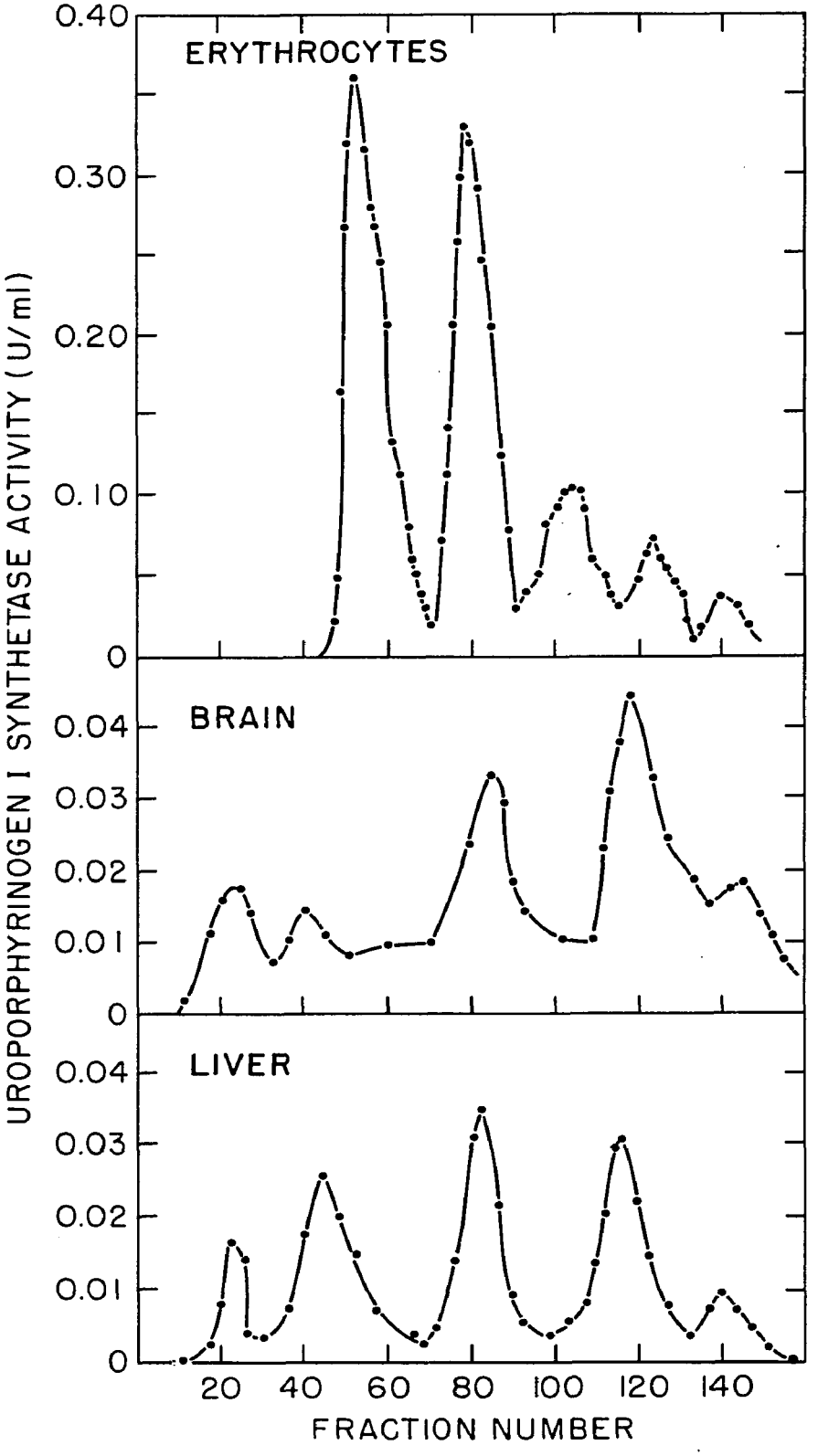
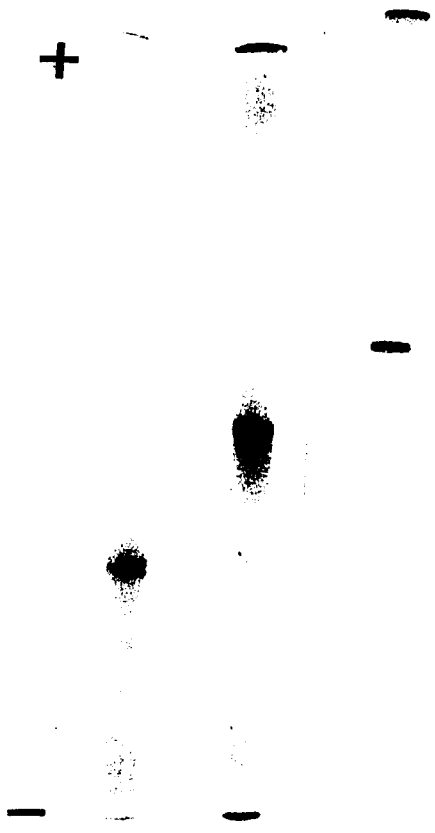

ERYTHROCYTES
BRAIN
LIVER
UROPORPHYRINOGEN I SYNTHETASE ACTIVITY (U/ml)
0.40
0.30
0.20
0.10
0
0.04
0.03
0.02
0.01
0
0.04
0.03
0.02
0.01
0
20
40
60
80
100
120
140
FRACTION NUMBER

Figure 19. Analytical Polyacrylamide Electrophoresis of Human UROS.

(Left) homogenous UROS electrophoresed at pH 6.8; (Center) enzyme electrophoresed at pH 8.2. A single band which stained for protein and activity was found in both cases.

(Right) After treatment at 100°C in the presence of SDS, UROS was electrophoresed in SDS polyacrylamide gels. When 8 µg of protein was applied to the gel a single band was observed.



to be homogeneous. Polyacrylamide disc gel electrophoresis at pH 6.8 and 8.2 yielded single bands of differing mobility which stained both for protein and activity. The enzyme from both peaks exhibited identical mobility and a single protein band after SDS electrophoresis as illustrated in Figure 19.

4. Molecular Weight and Subunit Composition:

The 5 peaks of UROS activity resolved by DEAE-cellulose chromatography were subjected to analytical gel filtration to determine the approximate molecular weights of these enzymes. As illustrated in Figure 20a, the various forms of the enzyme had molecular weights between 36,000 and 42,000 with the more highly charged forms having somewhat higher molecular weights. After SDS polyacrylamide electrophoresis (Figure 20b), a subunit molecular weight of approximately 37,000 was obtained for UROS A and UROS B, indicating that human UROS is a monomeric protein.

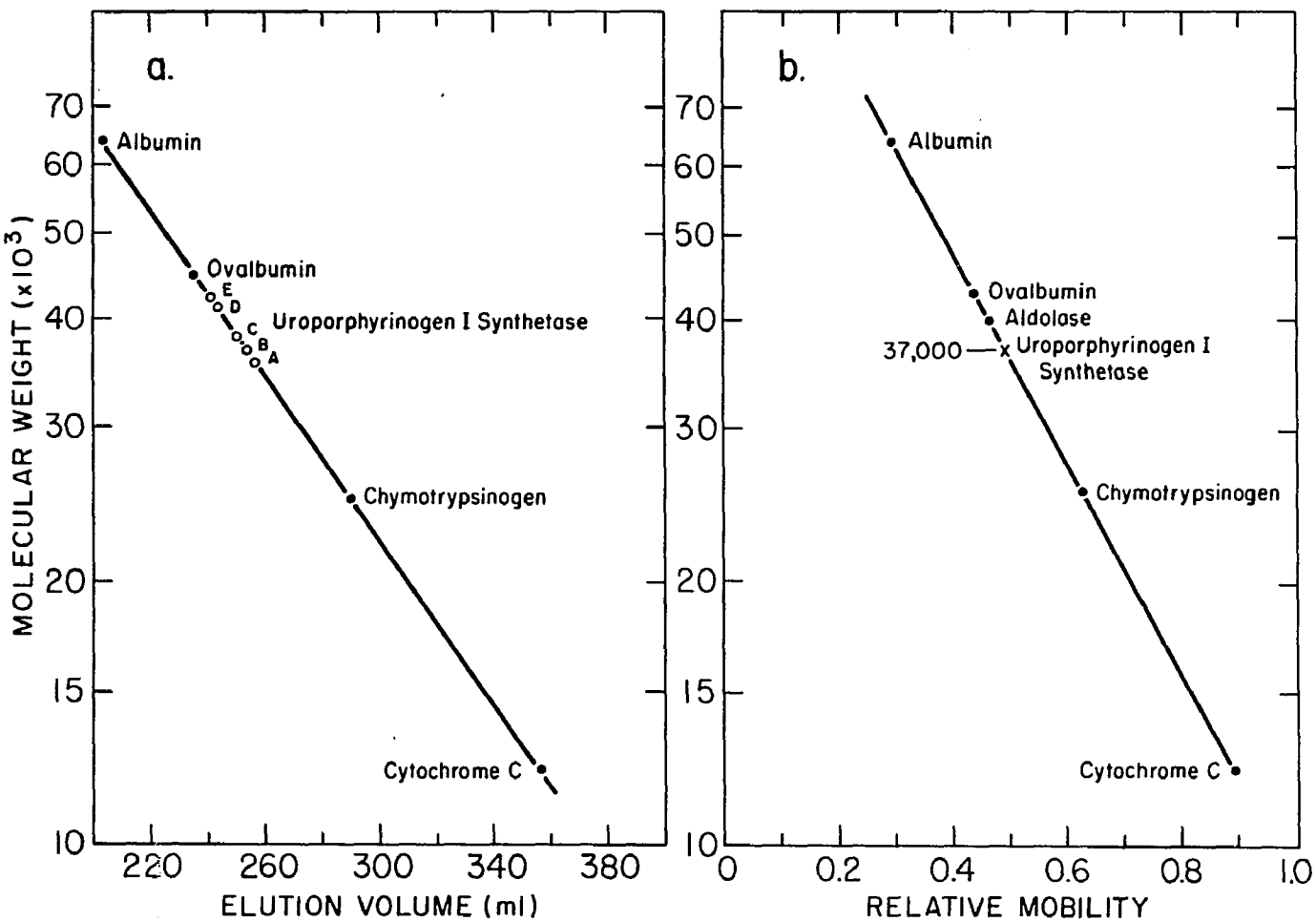
5. Occurrance of Human UROS Enzyme-Substrate Intermediates:

The intriguing finding of 5 forms of the enzyme (Figures 17 and 18) which had similar molecular weights (Figure 20) led to a series of experiments to determine their origin. As illustrated in Figure 18, the multiple forms were present not only in erythrocytes but also liver and brain. Chromatographic analysis of other tissues and human lymphoid cell lines also demonstrated the presence of 5 peaks of enzyme activity. The chromatographic profiles were related to the enzymatic activity in the tissue; erythrocytes and liver had

Figure 20. Molecular Weight of Human UROS from Erythrocytes.

A) Molecular weight of the native enzyme. UROS A, B, C, D and E were analyzed in separate runs. Enzyme (50 units) and 5 mg of each protein standard were applied to a 2.5 x 100 cm column of Sephadex G-100. UROS A, the least charged form, had the lowest molecular weight.

B) SDS polyacrylamide gel electrophoresis of human UROS. 10 µg of each standard and 10 µg of homogenous UROS A and B activities treated and electrophoresed as described in "Methods". Mobilities of protein standards electrophoresed simultaneously are expressed relative to that of the bromophenol blue dye front. The A and B activities had identical mobilities.


a.
b.
MOLECULAR WEIGHT (x10³)
70
60
50
40
30
20
15
10
Albumin
Ovalbumin
E
D
C
B
A
Uroporphyrinogen I Synthetase
Chymotrypsinogen
Cytochrome C
220
260
300
340
380
ELUTION VOLUME (ml)
Albumin
Ovalbumin
Aldolase
37,000
Uroporphyrinogen I
Synthetase
Chymotrypsinogen
Cytochrome C
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

higher UROS activity than brain (Table 7). Finally, a series of experiments demonstrated that when PBG was briefly incubated with UROS A, the least charged form of the enzyme, 5 peaks of enzyme activity were obtained after DEAE chromatography. These results suggested that the charge differences which resulted in 5 peaks of enzyme activity were due to the sequential binding of the pyrrole by human UROS to generate enzyme-substrate intermediates. This conclusion was further supported by experiments in which ^{3}H-PBG was incubated with the enzyme and the mixture run on polyacrylamide gels at 4°C. As illustrated in Figure 21, after incubation with ^{3}H-PBG with a mixture of UROS A and B, all 5 enzyme-substrate intermediates were generated. Bands stained both for protein and enzymatic activity. Increasing radiolabel was associated with bands possessing faster mobility. A band of uroporphyrin near the dye front contained eight to ten times more radiolabel than any enzyme-substrate intermediate.

6. Isoelectric Focusing Studies:

Multiple isoelectric points of 6.8, 6.6, 6.5, 6.3 and 6.2 were obtained for human UROS, consistent with the occurrence of 5 charge isomers of the enzyme.

7. Effect of pH:

Figure 22 shows the effect of pH on the activity of human UROS. The enzyme had a pH optimum of approximately 8.2. Activity was not detected below pH 6.0. Activity was similar in phosphate, Tris and barbital buffers and somewhat less in borate buffer.

TABLE 7

UROS Activity in Human Tissues

Organ	% Homogenate	UROS activity pmole/hr·ml	Specific activity pmole/hr·ml·mg
Brain	10	49.3	34.5
Heart	10	133	58.5
Lung	10	407	96.6
Spleen	10	436	56.9
Liver	10	493	73.6
Erythrocytes	10	35,000	87.5

Figure 21. Production of Enzyme-Substrate Intermediates and their Separation on Analytical Polyacrylamide Disc Gels.

A preparation of homogenous UROS A and UROS B (80 µg) consisting primarily of the A form (no bound substrate) was incubated at 37°C with 0.1 ml of ^{3}H-PBG containing 10^{7} cpm. After 10 min the mixture was chilled at 4°C and immediately subjected to analytical polyacrylamide disc gel electrophoresis at 4°C as described in "Methods". (Upper gel) enzyme incubated without PBG. (Lower gel) enzyme incubated in the presence of radiolabeled PBG which generated the B, C, D and E forms. The enzymatic activity, protein, and radioactivity in each band were determined as described in "Methods".

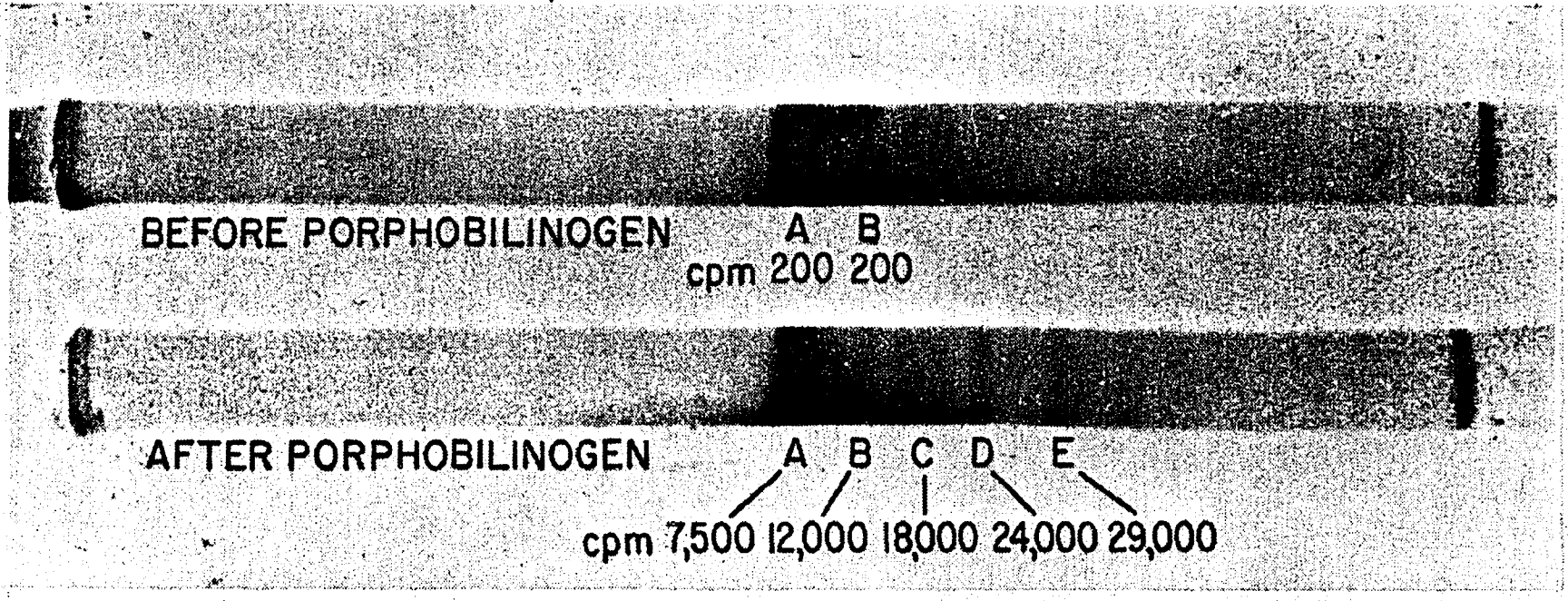
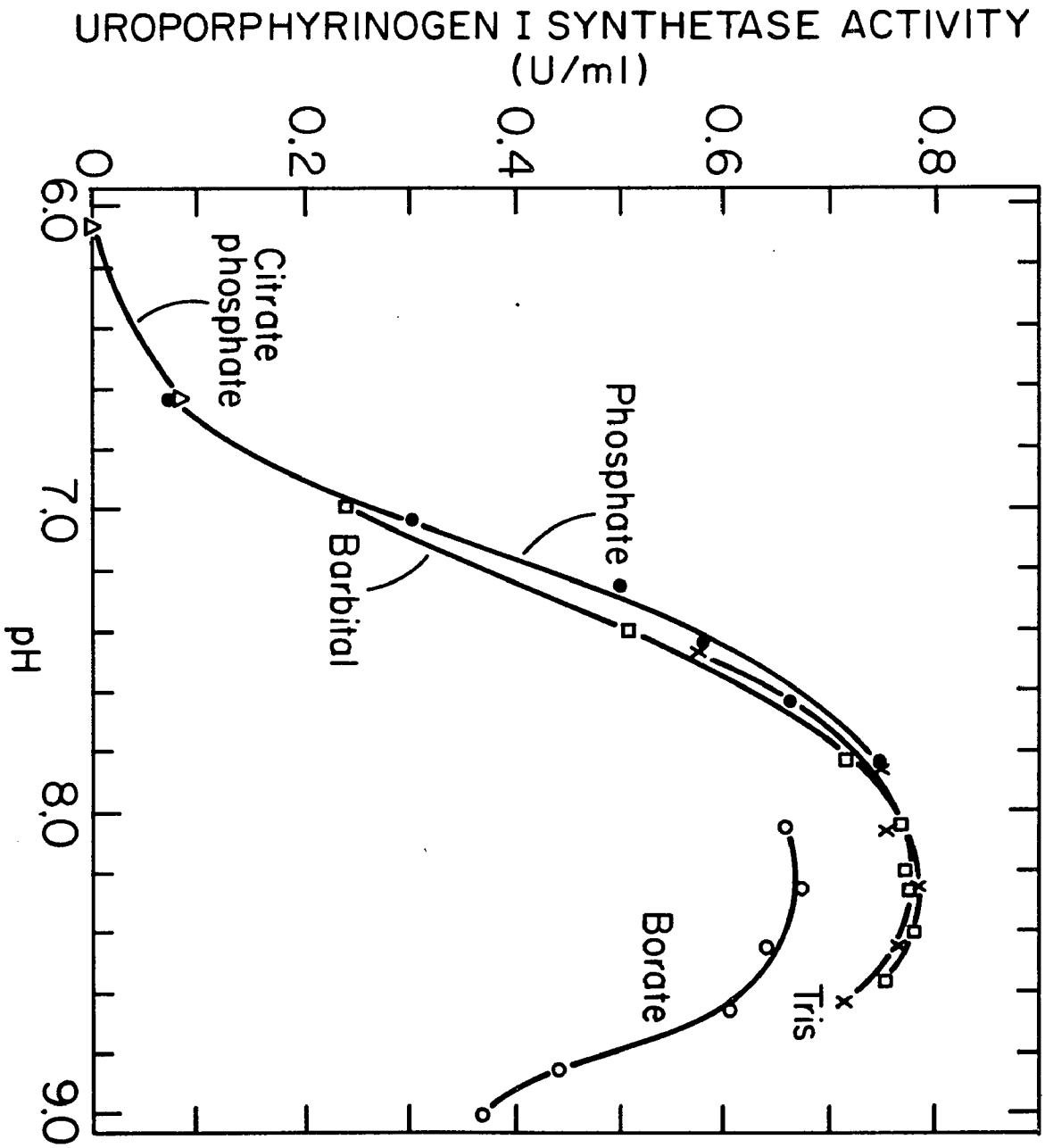

BEFORE PORPHOBILINOGEN
A B
cpm 200 200
AFTER PORPHOBILINOGEN
A B C D E
cpm 7,500 12,000 18,000 24,000 29,000

Figure 22. Effect of pH on Human UROS Activity.

Enzymatic activity was determined as described in "Methods" over the pH range from 6.0 to 9.0 using 0.1 M citrate phosphate (Δ), potassium phosphate (●), barbital (□), Tris HCl (x) or sodium borate (o) buffer. The pH measured at 23°C in the final assay mixture.


UROPORPHYRINOGEN I SYNTHETASE ACTIVITY
(U/ml)
0
0.2
0.4
0.6
0.8
6.0
7.0
8.0
9.0
pH
Citrate phosphate
Barbital
Phosphate
Borate
Tris

8. Kinetic Studies:

For kinetic studies homogenous UROS which had been dialyzed against 5 mM sodium phosphate buffer, pH 8.0, was used. The reaction was linear with time and protein. The K_m was determined to be 6 μM and the V_{max} was approximately 2,300 units/mg. The calculated turnover number, assuming one active site/mole of enzyme was 15 mole of product/min. The activity of the enzyme was found to be extremely temperature dependent with little or no activity occurring at 20°C and increasing amounts of activity thereafter. These data account for the stability of enzyme-substrate intermediates in polyacrylamide disc gels run at 4°C.

9. Inhibitors:

Results of the effects of several known inhibitors on enzyme activity are summarized in Table 8. Concentrations of inhibitors which partially inhibited activity are detailed. Agents which react with sulfhydryl groups were particularly effective inhibitors of UROS from human erythrocytes with significant inhibition occurring in the presence of micromolar concentrations of inhibitors. Iron and copper salts were effective inhibitors when employed in millimolar concentrations. Calcium and magnesium were the least effective inhibitors studied.

10. Amino Acid Composition of Human UROS:

Table 9 summarizes the results of the amino acid analysis of the homogenous A or B enzyme. The enzyme had little, if any, methionine. Despite use of protective conditions,

TABLE 8

Inhibitors of Human UROS

Inhibitor	Concentration	Inhibition
	mM	%
$HgCl_2$	0.0004	80
p-chloromercuric benzoate	0.002	74
$PbNO_3$	0.005	35
$CdCl_2$	0.1	80
n-ethyl maliemide	1.0	38
$CuSo_4$	1.0	87
$FeCl_3$	1.0	62
$FeSO_4$	1.0	58
$MgCl_2$	50.0	55
$CaCl_2$	50.0	73

TABLE 9

Amino Acid Composition of UROS from Human Erythrocytes

Amino Acid	Moles per 37,000 g of protein	Nearest integer per 37,000 g of protein
Aspartic acid	36.4	36
Threonine	18.0	18
Serine	20.7	21
Glutamic acid	40.1	40
Proline	12.0	12
Glycine	29.9	30
Alanine	22.9	23
Valine	17.0	17
Half-cystine	2.4	2
Methionine	0.1	0
Isoleucine	15.0	15
Leucine	39.1	39
Tyrosine	1.0	1
Phenylalanine	7.8	8
Lysine	16.7	17
Histidine	3.4	3
Arginine	10.2	10
Tryptophan	0.0	0

no evidence was obtained for the presence of tryptophan and only one tyrosine residue per molecular weight of 37,000 was detected. When the hydrolysate from the homogenous B enzyme was chromatographed, a red fluorescent peak with an absorption maximum in the Soret region was eluted just before cystathionine. The fluorescent material co-migrated with authentic coproporphyrin using standard thin-layer chromatographic techniques (84,95). In contrast, no such fluorescent material was observed when the UROS A hydrolysate was analyzed, thus confirming that this form of the enzyme possesses little, if any, bound substrate.

11. Thermal Stability:

Human UROS was an extremely heat stable enzyme. When the enzyme was incubated and assayed in the presence of adequate protein concentrations, (>1 mg/ml), no enzymatic activity was lost after incubation at 56°C for 120 min and greater than 90% of initial activity remained after incubation at 60°C for 120 min. Enzyme which had been heat treated for 1 hr at 60°C during purification ran as a single band in pH 6.8 disc gels, however, SDS electrophoresis revealed cleavage of the enzyme into two fragments with approximate molecular weights of 14,000 and 24,000. Analagous to the extreme heat stability of ribonuclease, the heat stability of human UROS is probably due to the absence of tryptophan.

12. Stability of Stored Enzyme:

Homogenous UROS from human erythrocytes was stable for at least 12 months when stored at high concentrations (>1 mg/

ml) in 5 mM potassium phosphate, pH 8.0, containing 0.2 mM DTE at -20°C. Lysates of cultured cells were stored in 1 mM potassium phosphate, pH 7.6, containing 0.05% Triton X-100; 1 mM DTT and 1 mM $MgCl_2$ with little or no loss of activity after 12 months.

D. UROS Enzyme-Substrate Intermediates in AIP and Normal Individuals:

Since the availability of hepatic and nervous tissue from individuals with AIP is extremely limited, investigations of the patterns of UROS enzyme-substrate intermediates in AIP was limited to erythrocytes. Analysis of normal (n = 10) and AIP (n = 12) erythrocytes revealed interesting differences in the DEAE-cellulose elution profiles of UROS enzyme-substrate intermediates. Half of the patients exhibited profiles with normal proportions of enzyme-substrate intermediates. These individuals had approximately half of the activity of UROS A, B and C when compared to normal individuals as illustrated in Figure 23. The low activities of UROS D and E in both normals and AIP patients did not permit comparison of these forms. Samples with this type of pattern occurred always in patients who were in clinical remission. These patients had urinary PBG excretion values less than 5 mg/liter.

A different pattern of enzyme-substrate intermediates was observed in 7 AIP patients as illustrated in Figure 23b. Six of these individuals with AIP were from the California sample and only one was from the NIH sample. These AIP

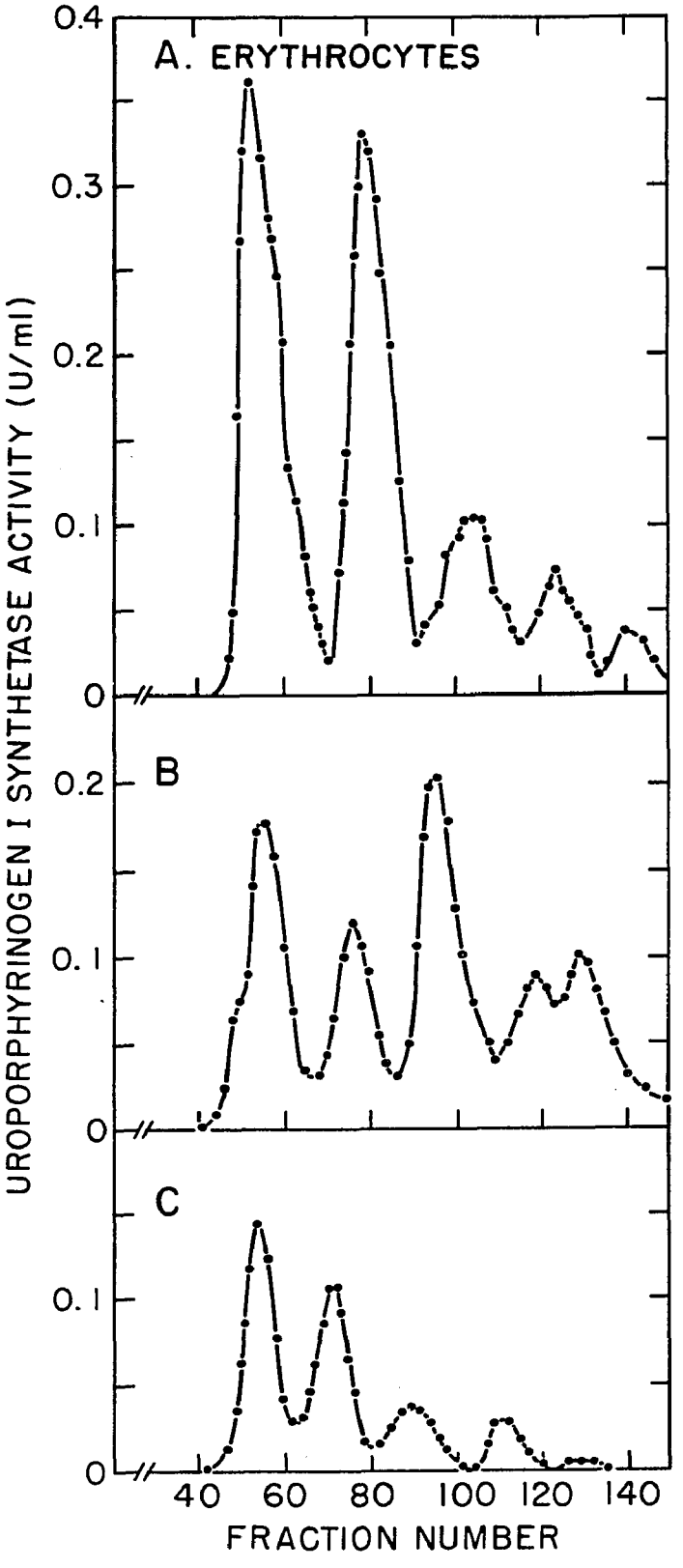
Figure 23. DEAE-Cellulose Chromatography of UROS from Erythrocytes of Normal Individuals and AIP Patients.

Red cells from normal individuals were lysed, centrifuged, dialyzed and chromatographed on DEAE-cellulose and and assayed as described in "Methods".

A) Normal profile of UROS enzyme-substrate intermediates.

B) Representative profile obtained from AIP patients during acute clinical episodes and patients in remission who had elevated levels of urinary PBG.

C) Representative profile obtained from several AIP patients in remission.


A. ERYTHROCYTES
B
C
0.4
0.3
0.2
0.1
0
0.2
0.1
0
0.1
0
40
60
80
100
120
140
FRACTION NUMBER
UROPORPHYRINOGEN I SYNTHETASE ACTIVITY (U/ml)

patients were experiencing acute clinical symptoms of the disease and/or had elevated (5 mg/liter) urinary PBG excretion when blood samples were obtained. The elevation of UROS C was characteristic of this pattern and sometimes even exceeded the UROS C activity in normal individuals. In one patient for whom samples from both remission and exacerbation periods of AIP was available, the UROS B and UROS C activities were increased during the acute attack compared to the levels in remission. These preliminary findings are interesting in that they demonstrate that although erythrocytes do not possess mitochondria or ALAS, the overproduction (or back-up) of PBG appears to be reflected in the erythrocyte.

E. Studies of CRM in AIP and Normal Individuals:

Two New Zealand rabbits were immunized against homogeneous human UROS. The titer of immune sera which resulted in 50% precipitation of purified enzyme was approximately 1:300. This immune sera was ammonium sulfate precipitated at 40% saturation and hemoglobin and albumin were successfylly removed. After dilution and dialysis the anti-UROS IgG had a titer of approximately 1:150.

Table 10 summarizes the preliminary data obtained from 2 normal and 3 AIP individuals with respect to immunoprecipitation of UROS in erythrocyte lysates. In these two individuals with AIP, inhibition of immunoprecipitation occurred, thus indicating the presence of CRM. In another AIP sample, no inhibition of immunoprecipitation occurred. These results

suggest that the anti-sera did not recognize any CRM except for the normal catalytically active enzyme present in the sample. This finding is consistent either with the absence of mutant enzyme in the AIP patient or the inability of the anti-sera to recognize CRM if it was present. Further studies are required to document these data.

TABLE 10

CRM in AIP and Normal Individuals. Preliminary Studies.

	Total UROS Activity in the lysate	UROS Activity in the Supernatant			
		Anti-UROS antibody dilution 1:100		Anti-UROS antibody dilution 1:200	
	units/ml	*units/ml*	*%*	*units/ml*	*%*
Control					
R.D.	424	44	10	154	36
P.A.	424	58	14	180	42
AIP					
J.M.	385	139	36	195	51
S.G.	480	172	36	277	58
T.L.	409	55	14	175	43

** 1 unit is equivalent to 1 pmole uroporphyrinogen per hour.*

VI. DISCUSSION

Prior to these studies, no human heme biosynthetic enzyme had been purified to homogeneity. ALAD was first purified to homogeneity because it not only was present in considerable excess compared to UROS but also was obligatory for the manufacture of the large quantities of PBG required for studies of human UROS. Like ALAD, UROS was also purified to homogeneity and its physical and kinetic properties studied.

To obtain adequate quantities of human ALAD and UROS, preparative procedures using human erythrocytes were successfully developed. The logistics of lysing, centrifuging, dialyzing and chromatographing extremely large volumes were formidable and preparative methods were developed only after many preliminary studies and several large pilot preparations. Since centrifuging the large volumes of lysate (20 liters) was the most difficult step in the preparative procedure, the utilization of a large Sorvall HS-4 centrifuge head facilitated the process and allowed the scale-up from 6 to 10 liters of packed erythrocytes (38 liters of lysate). Although the specific activity of both ALAD and UROS was quite low in the starting material, the major protein contaminant, hemoglobin, was readily removed in the initial preparative purification step through the use of DEAE-cellulose at a pH just below its isoelectric point. Fortunately at this pH both enzymes bound to the anion exchanger and could be eluted with good yields. Considerable purification, 600-

fold for ALAD and 100-fold for UROS was achieved by the preparative anion exchange procedure. In addition, ALAD and UROS were completely separated by the preparative DEAE step. This enabled ALAD to be concentrated by ammonium sulfate precipitation. UROS, on the other hand, lost activity when subjected to ammonium sulfate precipitation. Therefore, the enzyme was concentrated using hollow fiber technology. After concentration, ALAD or UROS could be frozen and stably stored at -20°C or -70°C. Thus, UROS was stockpiled while procedures were developed for the purification of ALAD and the synthesis of PBG.

In comparison to other reported methods (53-55,58), a major improvement in the purification of mammalian ALAD was made by exploitation of the enzyme's hydrophobic properties. Human ALAD, even in the presence of high concentrations of salt, did not bind to octyl-Sepharose; however, most other hydrophobic proteins were bound to this support. Interestingly, the enzyme could be bound and eluted from phenyl-Sepharose which is a less hydrophobic support. As judged by polyacrylamide gel electrophoresis, the enzyme peak from phenyl-Sepharose contained only three proteins, with ALAD accounting for approximately 85% of the protein in the sample. Thus, sequential chromatography of the enzyme on hydrophobic columns provided an extremely efficient purification procedure. The two minor protein contaminants were separated from human ALAD by gel filtration.

The final ALAD preparation has a specific activity of

18.5 units/mg. This specific activity is higher than previously reported values for homogeneous mammalian ALAD including enzyme isolated from guinea pig erythrocytes, 3.6 units/mg (58); murine liver, 9 and 12 units/mg (52-54); and bovine liver, 10.9-16.9 units/mg (57-58). Shemin and coworkers (135) recently reported that the specific activity of the enzyme from bovine liver was increased to 20-25 units/mg by the use of an affinity column of immobilized ALAD subunits. Although this strategy has not been explored for the purification of the human enzyme, the addition of 0.1 or 0.02 mM Zn^{2+} to the purified human enzyme increased the specific activity to 23.8 units/mg.

Typically, an overall yield of about 70% of human ALAD was obtained from erythrocytes. This was significantly greater than that obtained for the purified enzymes from guinea pig erythrocytes, 44% yield (58), or bovine liver, 6-31% yields (57,58,136). The amount of homogenous human ALAD isolated from 6 liters of erythrocytes was approximately 30 mg.

Native human ALAD had a molecular weight of approximately 252,000 and appeared to be composed of identical subunits of 31,000 molecular weight. These data indicated that the enzyme was homo-octamer. Although purified murine ALAD was reported to be a homo-hexamer, recent studies of bovine ALAD , including electron microscopic studies (137-139), indicated that the enzyme was a homo-octamer with a subunit molecular weight of 35,500.

Another similar property of human and bovine ALAD was the pale yellow color of the homogeneous preparations. Since pyridoxal 5'-phosphate appears to be associated with the bovine enzyme (133), spectral analysis of the human ALAD was performed. Slight increases in the absorption spectrum at 330 and 410 nm were consistent with the reported spectrum for pyridoxal 5'-phosphate (134). In addition, kinetic studies demonstrated pyridoxal 5'-phosphate to be a competitive inhibitor of ALAD. Previous studies (53,54,140) have indicated that ALA forms a Schiff's base with an ε-amino group of a lysine residue of bovine or bacterial ALAD. The spectral analysis and inhibition studies are consistent with the possibility that a small, but not necessarily stoichiometric amount, of pyridoxal 5'-phosphate is associated with the human enzyme. The possibility that pyridoxal 5'-phosphate is bound to the active site is unresolved and studies demonstrating that radiolabeled ALA and/or pyridoxal 5'-phosphate compete for the active site and are stably bound (via reduction) are necessary to support this hypothesis. Unfortunately, radiolabeled pyridoxal 5'-phosphate is not commercially available.

Purified ALAD had a pH optimum which was similar to those reported by others for the partially purified human erythroid activity (141,142). The K_m found for the human enzyme was essentially the same as those reported for purified enzyme from bovine (58) and murine sources (54), but was higher than that reported for the partially purified

ALAD from human erythrocytes (62). A possible explanation for the latter value may be the fact that their kinetic analyses were performed at pH 7.5 where cyclization of substrate during incubation results in increased amounts of Ehrlich's positive material.

The inhibitory effect of lead on the activity of ALAD is well-documented (57) and inhibition of the erythroid ALAD is used as a sensitive indicator of lead poisoning. Lead inhibited the purified human enzyme in a manner affecting both K_m and V_{max}. Similar results were obtained for the guinea pig erythroid enzyme (55). The bovine hepatic enzyme, however, has been reported to be inhibited by lead in a non-competitive manner (58). These differences may be due to either tissue-specific or species differences.

In order to purify and characterize human UROS the enzyme's substrate, PBG, had to be synthesized since its expense (50 mg is $146 from Sigma) prohibited its commercial purchase. The isolation of human ALAD permitted the biosynthetic approach to be utilized to produce gram quantities of PBG. This method had considerable advantages over organic methods in that larger quantities of extremely high quality PBG could be produced cheaply and efficiently in terms of material and time/effort requirements.

Since both [5-^{14}C] and [3,5-^{3}H]ALA were commercially available, PBG was also made using the purified ALAD. This radiolabeled pyrrole was extremely useful in monitoring binding of PBG to solid supports and UROS enzyme-substrate

complexes. The use of ALAD in conjunction with [1-^{14}C]ALA will permit the radiolabeled substrate for UROD and coproporphyrinogen oxidase to be synthesized much more easily than previously. Since porphyria cutanea tarda is the most common type of porphyria, the biosynthesis of [^{14}C]uroporphyrinogen using technology detailed in this work could have both research and clinical value.

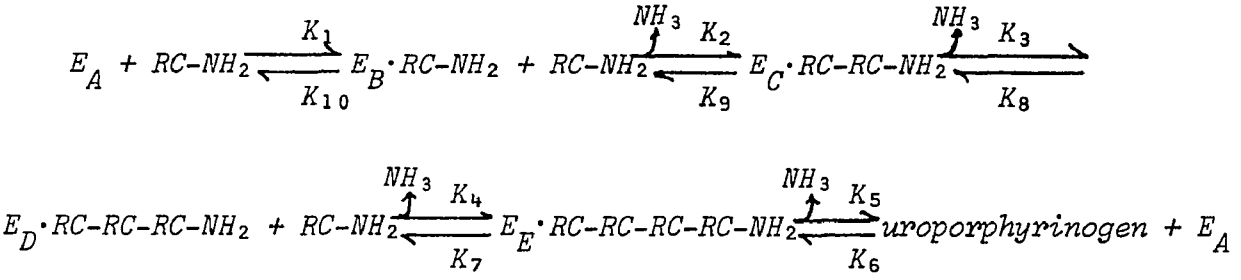
In addition to ALAD, the next enzyme in the heme biosynthetic pathway, UROS, was purified to homogeneity. This is the first time mammalian UROS has been purified to homogeneity. The human enzyme was purified from erythrocytes approximately 42,000-fold with a recovery of 25%. The physiologic low activity of the enzyme in human erythrocytes necessitated the use of preparative scale procedures in order to obtain milligram quantities of homogeneous enzyme. The initial DEAE-cellulose chromatographic step provided over 80-fold purification primarily by the removal of hemoglobin, the major protein contaminant.

Subsequent chromatography of UROS on hydrophobic supports provided a 30-fold purification. Although the enzyme did not bind to octyl-Sepharose even in the presence of 0.25 M potassium chloride, it bound tightly to phenyl-Sepharose (Figure 15). Exploitation of these hydrophobic properties resulted in an enzyme preparation with a specific activity more than 8 times greater than the highest value previously achieved for the human enzyme (68). Gel filtration not only removed ethylene glycol from the preparation but also sepa-

rated UROS from higher molecular weight proteins including any comtaminating UROCoS. The final anion exchange step resolved UROS activity into five forms, designated A through E.

The following experimental findings demonstrated that the A form was the native enzyme and that the charge isomers B, C, D and E were enzyme-substrate intermediates in the step-wise condensation of four monopyrrole substrates to the tetrapyrrole, uroporphyrinogen 1) Incubation of the A enzyme with radiolabeled PBG resulted in the generation of the B, C, D and E forms, each with a proportionately increased amount of radioactivity. Similarly, when the B enzyme was incubated with the radiolabeled substrate, all five forms were observed; the A form had minimal radioactivity, presumably due to substrate diffusion in the polyacrylamide gel or $^{3}H_2O$ in the sample. 2) Coproporphyrinogen was detected when the homogenous B enzyme hydrolysate was subjected to amino acid analysis. In contrast, no porphyrin was detected when the A enzyme hydrolysate was analyzed. This was consistent with the fact that PBG can be non-enzymatically condensed to uroporphyrin and subsequently decarboxylated to coproporphyrin by heat (84,85). 3) The A, B, C, D and E forms had similar kinetic properties and native molecular weights (Figure 20). The monomeric structure of the enzyme forms precluded subunit intereactions or aggregate formations. These findings are most consistent with the occurence of stable enzyme-substrate intermediates which may be described

by the following equilibrium equation in which $RCNH_2$ represents the substrate, PBG. The overall K_m of the reaction is 6 μM.



In support of this reaction scheme, recent studies of the mechanism of the enzymatic synthesis of uroporphyrinogen by NMR concluded that the product is formed by the stepwise head-to-tail condensation of monopyrroles to form the tetrapyrrole (83). Since milligram quantities of homogeneous stable intermediates can be isolated, the nature of enzyme substrate binding and the reaction stereochemistry can be investigated by diffraction techniques.

The studies of the patterns of UROS enzyme-substrate intermediates in tissues yielded information concerning the role of the enzyme *in vivo*. The patterns of enzyme-substrate intermediates observed in normal liver, normal brain, and AIP erythrocytes suggest that in these tissues PBG may occur in excess of the capacity of UROS to metabolize the substrate to uroporphyrinogen. This "bottleneck" in the heme pathway may be physiologic in normals. It may be speculated that when a genetic deficiency of UROS exists as in AIP, the ability of UROS in hepatic and nervous tissues to efficiently clear PBG is probably exceeded and pathologic accumulation of sub-

strate or its metabolites (143-145) may occur. This proposed process is consistent with the studies of UROS activity in various human tissues (Table 7) which demonstrated that brain had the least UROS activity of any tissue studied.

On the other hand, Schmid (146) has recently hypothesized that liver may synthesize heme for export to nervous tissue. When this vital hepatic function is not adequate, a relative deficiency of heme may occur in other tissues. Thus the encephalopathy and peripheral neuropathies in lead poisoning, AIP, and hepatic insufficiency may have the same pathologic basis—i.e., heme deficiency.

The observation that hematin administration results in remission of acute attacks in AIP (20) could be due either to feedback inhibition of ALAS to decrease the amount of PBG and/or toxic metabolites or to uptake and utilization of heme by heme-deficient nervous tissue. Analysis of AIP tissue with respect to ALA, PBG, UROS, and heme levels could enable discrimination between these possibilities. Using purified UROS A, it may be possible to develop a method to measure the small quantities of PBG present in tissue extracts. Small quantities of ALA in tissue extracts could also be measured by means of an ALAD-UROS enzymatic couple to produce readily identifiable uroporphyrinogen. The availability of homogenous, stable ALAD and UROS should permit the development of this procedure. A method to measure small quantities of free heme could employ tryptophan pyrrolase apoenzyme. This is one of the few enzymes that has

freely dissociable heme (147). In the presence of free heme, the enzyme would become active.

Finally, immunochemical studies of the enzymatic defect in AIP appeared to demonstrate CRM in several AIP patients. Further studies on a large series of AIP patients in kindreds are necessary to document this finding. However, based on this preliminary data, it is tempting to speculate that these AIP individuals may possess a protein which has some immunologically similar sites to normal catalytically active UROS. The CRM most probably represents a mutant UROS which is catalytically inactive since no differences in the kinetic properties, thermal stability or electrophoretic mobility of UROS in AIP have been documented (11,18). Thus the molecular pathology of the enzymatic defect in the AIP patients may be analogous to the heterozygote in many autosomal recessive diseases (118,119) in that normal enzyme and mutant CRM are present.

Further studies are needed, however, before these CRM positive AIP individuals can be characterized as having a point mutation in the UROS structural gene. First, the families of CRM-positive AIP patients should be studied. Siblings and parents with AIP should also have CRM. In another patient, preliminary studies revealed the absence of CRM. The family of this CRM-negative AIP patient should contain only affected individuals with the same CRM-negative type of mutation. Secondly, CRM should be investigated in AIP tissues other than erythrocytes. Cultured skin fibroblasts and cultured lymphoid lines would probably be the best tis-

sues for these studies. Thirdly, the CRM in AIP should be isolated and its amino acid sequence analyzed to determine the precise molecular defect in the UROS protein which renders it non-catalytic. It is possible that the molecular defects in the UROS protein may be different in families from various racial or ethnic backgrounds. This situation is analogous to the multiple defects found in families with glucose-6-phosphate dehydrogenase deficiency (148) and many other inborn errors of metabolism (16).

VII. CONCLUDING REMARKS

The ability to purify milligram quantities of homogenous human UROS and ALAD has been demonstrated. The physical and kinetic properties of these enzymes have been investigated. The studies of UROS in normal individuals and AIP have shed some light on the nature of the molecular pathology of this dominantly inherited disorder. Antibodies to not only human UROS, but also ALAD have been raised in rabbits so immunochemical investigations of the quantitative variation of these enzymes in man is now possible.

When stored at -20°C under appropriate conditions, purified human ALAD and UROS were stable. Thus it is likely that in the future mammalian ALAD and UROS will become commercially available as tools for the synthesis and generation of substrates for enzymes in the heme biosynthetic pathway. Furthermore, purified ALAD and UROS may be useful in the development of convenient and sensitive coupled assays for ALAS and ALAD and the biospecific detection of physiologic quantities of ALA and PBG. These procedures would enable elegant studies of the rates of enzymatic production of porphyrin precursors, porphyrins, and heme to be conducted. Such studies could have relevance in the elucidation of metabolic abnormalities occurring in AIP, other hepatic porphyrias, lead intoxication, and possibly hepatic encephalopathies. This technology also may facilitate the construction and testing of models concerning the rates and equilibrium of the enzymatic reactions of heme biosynthesis in various

tissues.

The nature of the exquisite metabolic and genetic control of differentiated cells in eukaryotes is an extremely important area of inquiry in the biomedical sciences. Future investigations concerning the genetic regulation and control of globin and heme synthesis and their intimate relationship will undoubtedly yield valuable information about the genetic control of differentiation. Several studies investigating the genetic regulation of the biosynthesis of heme are currently under way in the laboratory of Dr. R.J. Desnick; these include studies of K562 cells and the chromosomal localization of human ALAS, ALAD, and UROS using somatic cell hybridization techniques. It is hoped that these studies have provided the essential enzymatic tools for the biochemical geneticist to dissect the molecular nature of the enzymatic defect in AIP and the newly discovered genetic deficiency of ALAD. In addition, the availability of these enzymes should permit the production of monoclonal antibodies and electrophoretic methods useful for the chromosomal localization of these heme biosynthetic enzymes.

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