CHARACTERIZATION OF FELINE PORPHYRIA: BIOCHEMICAL FEATURES AND SELECTED ENZYME ASSAYS

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CHAPTER I

INTRODUCTION

The hereditary porphyrias are a group of relatively rare metabolic diseases involving defects at various sites in the heme biosynthesis pathway. In general, the porphyric diseases are characterized by the production of large amounts of porphyrins and/or porphyrin precursors and by clinical signs which range from photosensitivity and anemia to neurological disturbances (46). The porphyrias of man are grouped into two major categories based on the type of tissue in which the metabolic defect is expressed: those in which the defect is manifested in erythroid tissue (erythropoietic type) and those in which the defect is manifested in non-erythroid tissue (hepatic type). Subtypes within each of these categories are classified according to specific patterns of clinical signs and excretion patterns of porphyrins and porphyrin precursors (44).

Diseases of porphyrin metabolism may also be induced by certain drugs and chemicals (124). Although studies of the acquired disturbances in porphyrin metabolism have aided in the clarification and understanding of defective porphyrin metabolism, the major impetus for investigations of both normal and abnormal porphyrin metabolism has been generated through interest in the hereditary porphyrias of man.

The classification of the hereditary porphyrias of man used in this thesis follows that suggested by Goldberg (44):

I. Erythropoietic
   A. Congenital erythropoietic
   B. Erythropoietic protoporphyria
II. Hepatic Porphyria
   A. Acute intermittent porphyria
   B. Porphyria variegata (mixed) porphyria
   C. Hereditary coproporphyria
   D. Cutaneous hepatic porphyria
      1. hereditary
      2. acquired

Hereditary porphyria among domestic animal species has been reported in cattle (32), swine (68), and more recently in the domestic cat (140). The condition in cattle and swine is classified as congenital erythropoietic porphyria, although certain features of the disease in these species are dissimilar to those which characterize congenital erythropoietic porphyria in man (46). The disease in swine is inherited as an autosomal dominant trait and as an autosomal recessive trait in cattle and man. Feline porphyria was first reported in 1964 in two littermate kittens (140). The kittens were observed to have brownish discoloration of the teeth which exhibited reddish fluorescence under ultraviolet light. Limited qualitative urine analyses performed on one porphyric kitten indicated the presence of uroporphyrin, coproporphyria, and porphobilinogen. Signs of photosensitivity were absent.

A research colony of porphyric cats has been developed at Oklahoma

State University from progeny of the dam of the porphyric kittens described in the original report. Genetic studies in this colony indicate a Mendelian autosomal dominant mode of inheritance for the porphyric trait (41). The porphyric cats exhibit many features in common with those of congenital erythropoietic porphyria in other animals and man; however, certain features of feline porphyria overlap those of erythropoietic protoporphyria and the hepatic porphyrias of man (43).

The present investigation was designed to more completely characterize the metabolic defect(s) involved in feline porphyria. More specifically, the studies were designed to provide further information regarding urinary excretion patterns of porphyrins and porphyrin precursors and erythrocyte porphyrins as well as to provide data regarding activity of specific enzymes more likely to be involved in defective heme biosynthesis in the porphyric cat.

CHAPTER II

REVIEW OF SELECTED LITERATURE

Normal Heme Biosynthesis

The metalloporphyrins, chlorophyll and heme, occupy a central position in life processes. Chlorophyll performs a vital role in the photosynthetic process which is fundamental to the support of all forms of life. Heme performs a vital role as an integral, functional component of hemoglobin, myoglobin and hydroperoxidases, as well as certain of the enzymes involved in electron transport systems (46).

The enzyme mediated steps leading to the formation of heme in microorganisms, plants and higher forms of life have been elucidated; however, the exact reaction mechanisms by which certain metalloporphyrin precursors are formed remains unclear. Heme and chlorophyll are derived from protoporphyrin IX which is formed by an identical biosynthetic pathway in both plant and animal forms of life (Figure 1). Four isomer types of porphyrins are structurally possible but only types I and III are produced in biological systems but only the type III isomer can be utilized in the biosynthesis of heme.

A large body of knowledge concerning both normal and abnormal heme biosynthesis has been gained by studies of diseases of porphyrin metabolism. Fischer and co-workers (28,29,30,31), during the period of 1915-1930, resolved the structure of heme by an extensive series of studies utilizing material from a human patient with congenital

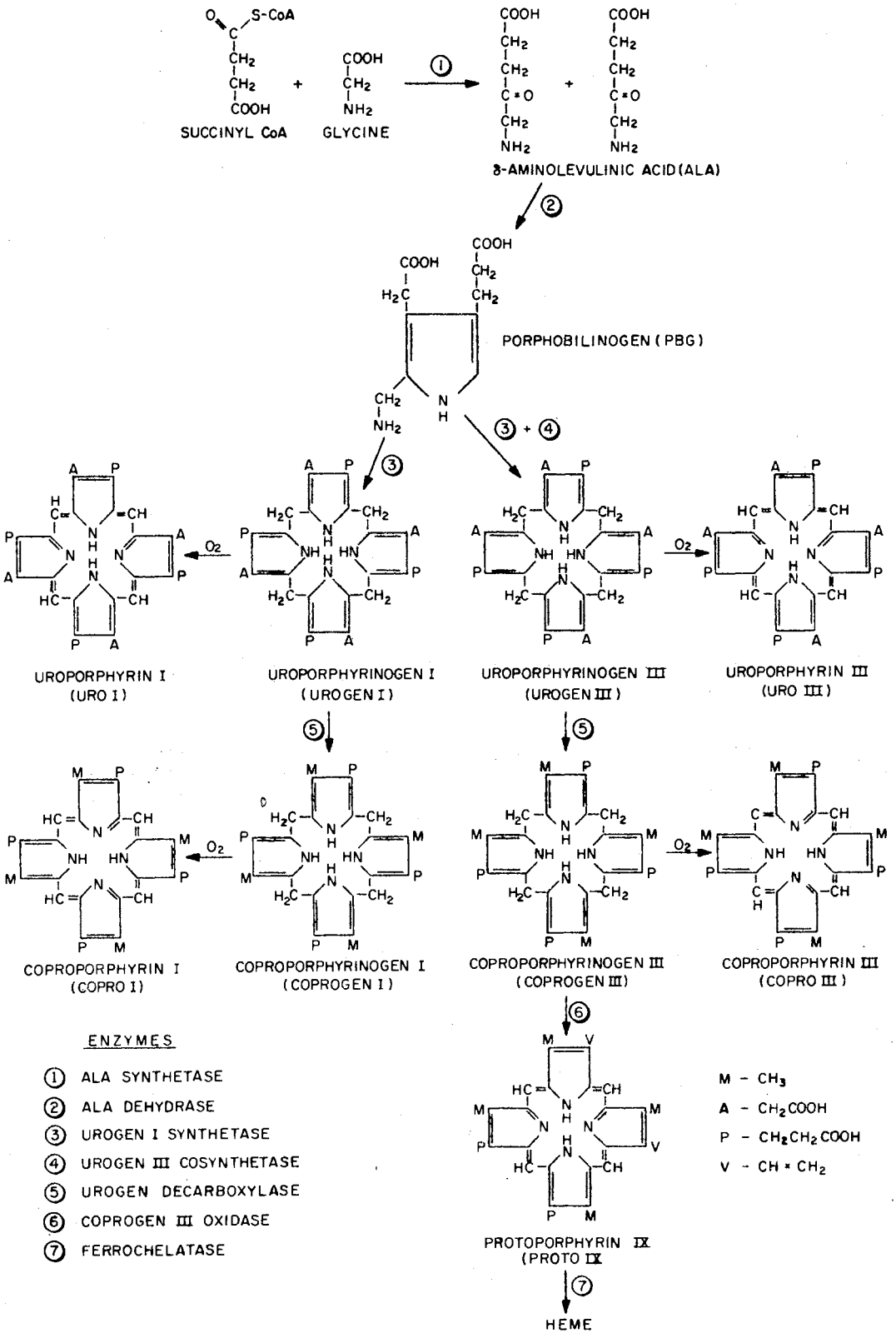

Figure 1. The Pathway of Heme Biosynthesis and Porphyrin Formation.

erythropoietic porphyria. Later investigations of defective porphyrin metabolism by numerous workers have aided in clarification of the various steps in the heme biosynthesis pathway. Studies involving chemically induced as well as hereditary porphyrias have provided information regarding possible control mechanisms regulating heme synthesis. Based on results of these studies, a postulation of feedback repression of δ-aminolevulenic acid synthetase in a manner similar to the mechanism proposed by Jacob and Monod (64) for regulation of enzyme formation in microorganism was suggested as one means of controlling heme biosynthesis (49).

Biosynthesis of δ-Aminolevulinic Acid

The initial step in heme biosynthesis is the formation of δ-aminolevulinic acid (ALA) by condensation of succinyl-CoA and glycine. Isotope labeling studies by Shemin and co-workers (110,133) demonstrated that the nitrogen atoms in protoporphyrin IX, as well as the methene bridge carbon atoms and four of the eight alpha carbons, are derived from glycine. Shemin et al. (132) further demonstrated that the remaining carbon atoms in protoporphyrin are derived from succinate. On the basis of this and other work, Shemin, Talmage, Kumar, and Kikuchi (78) concluded that succinyl CoA was the specific compound which condenses with gylcine to form ALA. Further studies using duck red cell hemolysates incubated with ^{14}C ALA and ^{14}C glycine demonstrated that ALA is an essential precursor of heme (134).

The formation of ALA from glycine and succinyl CoA is catalyzed by the enzyme, ALA synthetase, which is located in the mitochondria of avian and mammalian cells (122,37). Pyridoxal phosphate is required as a co-factor in the reaction. ALA synthesis does not occur in in vitro

systems containing the partially purified enzyme preparation without the addition of pyridoxal phosphate (78,83). Evidence that this vitamin is required *in vivo* is provided by the demonstration that pyridoxal phosphate deficient ducklings, pigs and man develop a hypochromic anemia which is corrected by administration of the vitamin (136,156,60). Additionally, Schulman and Richert (136) showed that pyridoxal phosphate deficient duck red cells synthesized heme at a reduced rate which was corrected by adding the vitamin to the system.

The conversion of glycine and succinyl CoA to ALA is considered to be the rate limiting step in the heme biosynthesis pathway (49,52). Three possible mechanisms by which heme production could be regulated at this step in the pathway are: 1) by control of the rate of enzyme degradation, 2) by negative feedback inhibition of ALA synthetase activity and 3) by control of the rate of production of the enzyme. The mechanism involved in ALA synthetase degradation has not been reported. Several investigators have suggested that heme may function as a negative feedback inhibitor of ALA synthetase. Burnham and Lascelles (12) reported that heme markedly inhibited ALA synthetase activity in preparations from the bacterium, *Rhodopseudomonas spheroides*. Evidence suggesting a similar feedback inhibition was reported to occur in rabbit reticulocytes (88); however, Granick (49) found no evidence of heme inhibition of ALA synthetase activity in mitochondrial preparations isolated from livers of guinea pigs with a chemically induced porphyria. Scholnick et al. (129) reported that heme inhibited the activity of partially purified ALA synthetase prepared from rat liver. The inhibition was blocked by a number of proteins which bind heme, including cell sap obtained from normal rat liver. These workers postulated that

the latter finding explains previous failure to demonstrate heme inhibition in crude systems.

The rate at which heme is produced also may be controlled by the cellular level of ALA synthetase. Granick (49) postulated a possible mechanism for the regulation of ALA synthetase concentrations based on studies involving the use of ALA synthetase inducing compounds. He suggests that heme binds with a protein aporepressor, thereby forming a holorepressor which inhibits ALA synthetase formation. He further postulated that the ALA synthetase inducing chemicals compete with heme for the binding site on the aporepressor, forming an inactive holorepressor, thus allowing continued synthesis of the enzyme. The half-life of ALA synthetase has been reported by Marver et al. (92) as 70 minutes in rat liver and by Granick (49) as 4-6 hours in cultured chick cells. In either instance, its half-life would be considered relatively short, thus the mechanism proposed by Granick (49) for regulation of ALA synthetase production would provide a sensitive control mechanism of heme biosynthesis.

The manner in which ALA synthetase may be involved in diseases of porphyrin metabolism has been investigated intensively in recent years. It has been established that overproduction of ALA synthetase is involved in certain of the hepatic porphyrias and, similarly, in certain of the chemically induced porphyrias (52,50,142).

Biosynthesis of Porphobilinogen

The second step in heme biosynthesis is the formation of porphobilinogen (PBG) by condensation of two molecules of ALA (127). The existence of PBG was first indicated by the finding of an Ehrlich reagent positive substance in urine from patients with acute porphyria

(148). The observation that uroporphyrin formed spontaneously in urine containing high levels of the Ehrlich positive substance suggested the possible involvement of this substance as an intermediate in porphyrin synthesis.

Westfall (33) in 1952, crystallized PBG from the urine of a patient with acute porphyria. Using these crystals, Cookson and Rimington (20) determined the structure of PBG to be 2-aminomethyl-4-2-carboxyethyl-3-carboxymethylpyrrole. Proof that PBG is a precursor of heme was demonstrated by isotope labeling studies using ^{14}C-ALA and ^{14}C-PBG as substrates in separate experiments with *in vitro* preparations of duck red cells (127). These experiments also provided proof that PBG was formed by the condensation of two molecules of ALA.

The condensation of two molecules of ALA to form PBG is catalyzed by the enzyme, ALA dehydrase (38). This enzyme is located in the soluble fraction of the cytoplasm, in contrast to the mitochondrial location of ALA synthetase (38,51). Reduced glutathione, or a similar thio-group activator, is required for ALA dehydrase activity (38). Although ALA dehydrase activity is strongly inhibited by ethylene diaminetetraacetate (EDTA), no requirement for a metal has been established (155). Lead also inhibits ALA dehydrase both *in vivo* and *in vitro* by inactivating sulfhydryl groups on the enzyme (36). As a result of this inhibition, patients with lead poisoning excrete large amounts of ALA (15).

The rate at which heme biosynthesis occurs is considered to be governed primarily by the catalytic activity and catalytic capacity of ALA synthetase. The remaining enzymes involved in heme biosynthesis are believed to have little influence on the control of heme production

rates since their catalytic capacities are normally much greater than the catalytic capacity of ALA synthetase (141). However, in vitro studies with human red cells and mouse liver have demonstrated marked inhibition of ALA dehydrase by heme, which suggests the possibility of an additional method of controlling the rate of heme biosynthesis, i.e., negative feedback inhibition of ALA dehydrase (13,19). A further means of regulating heme production at this step could occur by repression of synthesis of ALA dehydrase in a manner similar to that which has been proposed for controlling ALA synthetase production. The half-life of ALA dehydrase has been reported as 5-6 days (23). This relatively long half-life does not favor the latter mechanism as a sensitive regulator of heme synthesis.

Biosynthesis of Uroporphyrinogen III

Uroporphyrinogen III (UROgen III), the hexahydroporphyrin of uroporphyrin (URO III), is the first tetrapyrrole formed in the heme biosynthesis pathway (Figure 1). Fischer correctly hypothesized the structure of the oxidized form of UROgen and noted that four isomer types (I, II, III and IV) were possible (100). Of the different isomer types, only types I and III are known to be formed in biological material and only the type III UROgen is utilized as an intermediate in heme biosynthesis (95). Evidence suggesting that UROgen III, the reduced form of URO III, is the intermediate utilized in heme biosynthesis rather than the oxidized form, URO III, was provided by studies which showed that cellular extracts synthesized heme when UROgen III was used as a substrate but not when URO III was used (102). Mauzerall and Granick (96) provided further evidence by demonstrating that the enzyme which catalyzes the subsequent step in heme biosynthesis will

use only the reduced form of URO as a substrate.

Cellular extracts which catalyze the condensation of PBG to UROgen III have been obtained from various plants, animal tissues and bacteria (95). Two enzymes, UROgen I synthetase and UROgen III cosynthetase, both of which are located in the cytoplasm, are required for the formation of UROgen III. Normally, the type III isomer is the predominate UROgen produced and only a small amount of the unusable isomer, UROgen I, is formed. That both enzyme components are required for the formation of UROgen III was shown by Bogorad (6), who demonstrated that UROgen III cosynthetase (UROgen isomerase) is heat labile while UROgen I synthetase (PBG deaminase) is heat stable. He found that untreated plant tissue extracts formed only UROgen III and the heated extracts formed only UROgen I. He further found that UROgen I formation occurred in the heat treated preparations at rates comparable to UROgen III formation in the untreated preparations, indicating that the UROgen I synthetase enzyme was not affected by the heat treatment. These findings provided the basis for Bogorad's hypothesis that the defect in congenital erythropoietic porphyria of man and cattle involves a deficiency of UROgen III cosynthetase, thus explaining the excessive production of UROgen I, which is a prominent feature of this form of porphyria (6,8).

The exact mechanism by which both enzymes operate to condense and cyclize PBG to the usable type III isomer of UROgen, and the possible intermediate compounds which may be involved have not been clearly established. Bogorad (8) postulated that polypyrryl methane polymers of PBG are involved and, in later work (105), reported that tripyrryl methane is a likely substrate for UROgen III cosynthetase.

Although there is no direct evidence in support of a physiological

control mechanism for regulation of heme synthesis at this step, the rate of UROgen III synthesis in avian erythrocytes markedly decreases when oxygen concentration rises above or drops significantly below seven percent (26). This suggests the possibility that oxygen tension may effect an additional control mechanism for the regulation of heme production.

Biosynthesis of Coproporphyrinogen III

The reaction which forms coproporphyrinogen III (COPROgen III) involves step-wise decarboxylation of the four acetic acid groups on UROgen III. Decarboxylation is catalyzed by the cytoplasmic enzyme, UROgen decarboxylase, and appears to proceed in a random manner by the removal of one acetic acid carboxyl group at a time (146). Seven-, six-, and five-carboxyporphyrinogens are produced as UROgen undergoes progressive decarboxylation. These intermediates have been isolated in normal urine and are present in especially large amounts in urine from subjects affected with porphyria (17). Vilae and Grinstein have demonstrated that five- and six-carboxy porphyrinogens III are utilized in the formation of heme thus confirming their role as intermediates in coproporphyrinogen III formation.

Either of the type I and III isomers of UROgen can be decarboxylated by the enzyme to the corresponding isomer of COPROgen; however, the decarboxylation rate of the type III isomer is twice that of the type I (95). The reaction *in vitro* is enhanced by the presence of reduced glutathione and by anaerobic conditions (96).

It is well established that only the reduced form of URO and COPRO is utilized in heme biosynthesis. However, Bogorad (7) isolated an enzyme from spinach leaves that catalyzes formation of URO from

UROgen. The significance of this finding is unknown but the reaction does not appear to be important in mammalian tissues (124).

Biosynthesis of Protoporphyrin IX

Protoporphyrin IX (PROTO IX), a porphyrin of the type III isomer series, is the immediate precursor of heme (Figure 1). The formation of PROTO IX results from oxidation of two propionic acid groups on COPROgen III to two vinyl groups and the removal of six hydrogen atoms. This reaction is catalyzed by the mitochondrial enzyme, COPROgen oxidase, which utilizes only the III isomer of COPROgen as a substrate (123). The substrate specificity of this enzyme for the III isomer is considered to be the major reason that only the type III isomer of PROTO has been found in biological material.

The exact reaction mechanism by which COPROgen III is converted to PROTO IX has not been clearly defined. Since it has been demonstrated that β-hydroxypropionic acid deutroporphyrinogen is converted to PROTO IX by bovine liver mitochondria, Sano (121) suggested that this compound is an intermediate in the synthesis of PROTO IX from COPROgen III. Porra and Falk (107) proposed that an intermediate having one vinyl and three propionic acid groups is first formed in this reaction and then converted to PROTO IX. It is not clear whether protoporphyrinogen IX (PROTOgen IX) is produced in the reaction and spontaneously oxidizes to PROTO IX or whether PROTOgen IX is enzymatically oxidized to PROTO IX. That an enzyme may be involved in the process is suggested by the observation that the rate of oxidation is accelerated *in vitro* by the presence of liver mitochondria (123).

Biosynthesis of Heme

The synthesis of heme involves the insertion of ferrous iron into the tetrapyrrole ring of PROTO IX (66). The mitochondrial enzyme, ferrochelatase, catalyzes this reaction, although under the proper conditions, iron will react spontaneously with PROTO IX (45). A variety of porphyrins other than PROTO IX and metallic ions other than iron can be utilized as substrates by ferrochelatase. Jones and Jones (66) reported that ferrochelatase isolated from Rhodopseudomonas spheroides synthesized metalloporphyrins from such substrates as mesoporphyrin, deutroporphyrin and hematoporphyrin and incorporated various metallic ions including Co^{++}, Fe^{++}, Zn^{++} and Mn^{++} into each of these porphyrins. However, ferrochelatase cannot utilize URO, COPRO and PROTOgen as substrates in the formation of metalloporphyrins (81). For maximal in vitro activity, the enzyme requires an anaerobic atmosphere and a reducing agent, presumably to keep iron in the ferrous state (106).

The observation that relatively low concentrations of heme and other metalloporphyrins markedly inhibit ferrochelatase activity in Rhodopseudomonus spheroids suggests a regulatory mechanism for controlling the rate of heme biosynthesis (66). It has been demonstrated in this organism that ALA synthetase is subject to negative-feedback inhibition by heme (12); however, ferrochelatase appears to be even more sensitive than ALA synthetase to heme inhibition (66).

Abnormal Heme Biosynthesis

Porphyria in Man

The earliest documented case of porphyria in man was reported in 1874 (131). The patient was a 33 year old man who had suffered from cutaneous photosensitivity since infancy. Two pigments, designated as

urorubrohematin and urofuscohaematin were extracted from the patient's urine (4). The absorption spectrum of one of those pigments was found to be similar, but not identical, to that of hematoporphyrin. At autopsy, the most notable findings were reddish brown discoloration of the skeleton and enlargement of the spleen. The clinical history, chemical findings, and the pigmented skeleton indicate that the condition was the type of porphyria now designated as congenital erythropoietic porphyria.

In 1889, Stokvis (138) documented a case report of a patient who began voiding reddish urine after treatment with a newly introduced hypnotic drug, Sulphonal. During the following years additional cases were described in which treatment of patients with Sulphonal and other similar hypnotic drugs was followed by the voiding of red urine, designated as haematoporphyrinuria, and, in some instances, also by neurologic disturbances (46). However, hypnotic drugs were not involved in all cases of haematoporphyrinuria and the incidence of this condition was relatively low in patients receiving the drugs. Rankin and Pardington (46) reported the occurrence of central nervous system disturbances in conjunction with haematoporphyrinuria in two unrelated women, neither of whom had taken Sulphonal. The clinical symptoms and the pigmented urine observed both in the drug induced and spontaneous cases of haematoporphyrinuria are compatible with the features now recognized as being characteristic of certain types of hepatic porphyrias.

Photosensitivity has long been recognized as one of the major clinical features of various types of porphyria. Anderson (3) is given credit as being the first to note the relationship between the occurrence of photosensitivity and the abnormal excretion of porphyrins in

the urine. He described the skin lesions of photosensitivity in two brothers, both of whom excreted haematoporphyrin in the urine. Meyer-Betz vividly demonstrated that porphyrins could induce photosensitivity in a classic experiment in which he injected himself with 200 mg of haematoporphyrin and subsequently exposed his hands and face to sunlight. The exposed areas of skin developed urticaria and severe edema. The photosensitivity persisted for two months after the injection.

In 1911, Gunther (55) classified the diseases of porphyrin metabolism under four categories: 1) hematoporphyria acuta, 2) hematoporphyria acuta toxica, 3) hematoporphyria chronica, and 4) hematoporphyria congenita. In a later report, he provided further support for this classification scheme and suggested that hematoporphyria acuta is an inherited disease (56). Gunther also described spontaneous cases of hematoporphyria acuta and cases of hematoporphyria toxica associated with the use of Sulphonal, Trional and Veronal. The description of hematoporphyria congenita given by Gunther is cited by Garrod (35) as the first recognition that this disease is an "inborn error of metabolism".

The research of Hans Fischer and co-workers (29,28,30,31) established the structural difference between haematoporphyrin and protoheme. This led Walsenström (147) to propose a new classification for the diseases of porphyrin metabolism. Waldenström used the term porphyria in place of haematoporphyria and grouped the diseases into three categories: 1) porphyria congenita, 2) porphyria cutanea tarda, and 3) porphyria acuta. Additional knowledge gained during the past two decades has led to the subdivision of the hereditary porphyrias into the two major

categories, hepatic and erythropoietic, with subdivisions of each type (124).

Erythropoietic Porphyria.

Congenital Erythropoietic Porphyria. Congenital erythropoietic porphyria (CEP) is the rarest form of the hereditary porphyrias. The total number of recorded cases of this type of porphyria in man through 1965 was reported as 60 by Schmid (124). Although the rarity of the condition precludes detailed genetic analysis, the evidence indicates that the disease is transmitted as an autosomal recessive trait (124). Even though the condition is rare, the genetic defect is distributed among various races of widely differing genetic background including Caucasian, Japanese, Bantu, Sudanese, Aleutian Indian and Asiatic Indian (124).

The erythropoietic nature of CEP was suggested in an early study when cells from the bone marrow of a patient with the disease was examined by fluorescence microscopy and found to exhibit intense red fluorescence indicating the presence of porphyrins (9). Schmid et al. (125) later confirmed this finding in other cases of the disease and further noted that the bone marrow cells which exhibited fluorescence were primarily normoblasts although weak fluorescence was detected in non-nucleated polychromatophilic erythrocytes in marrow and circulating blood. The nuclei of fluorescing normoblasts exhibited much more intense fluorescence than the cytoplasm. Many fluorescing nuclei also contained large heme inclusion bodies which were not observed in non-fluorescing normoblasts. They estimated that 30 to 70 percent of the total population of normoblasts contained abnormal levels of porphyrins. Since both fluorescing and non-fluorescing normoblasts were observed in

all patients, it was postulated that two distinctly separate erythroid cell populations existed, i.e., a normal cell line which were non-fluorescing and a fluorescing cell line containing the defect (125,126). Similar findings of fluorescing and non-fluorescing erythroid cells were noted in marrow of cattle with CEP (120). Recent studies of bone marrow from porphyric cattle in which erythropoiesis was accelerated by bleeding have shown that a marked increase in percentage of fluorescing normoblasts occurs during the period of stimulated erythropoietic activity (120). This finding is considered as evidence against the two cell line theory. If two separate cell lines exist, it would be expected that both cell lines would be stimulated to a comparable degree.

The metabolic defect in CEP results in excessive production of URO and COPRO, which accumulate in the developing erythroid cells and plasma, are deposited in teeth and bone and are excreted in urine and feces (126,124). These porphyrins impart a pinkish to brownish pigmentation of the skeletal tissue and brownish to reddish discoloration of urine and are responsible for the characteristic bright reddish fluorescence when exposed to ultraviolet light. Additional clinical features include photosensitivity and anemia (145,46,124).

Both the types I and III isomers of URO and COPRO are produced in excessive amounts; however, the major share of these porphyrins is represented by the unusable type I isomer (62,17). The predominant porphyrin present in erythrocytes and urine is URO while COPRO constitutes the major porphyrin in feces (77,124). The porphyrin precursors, ALA and PBG, are not elevated in tissues or excreta (124,152).

Photosensitivity and anemia are common manifestations in patients with CEP. The degree of photosensitivity and anemia varies considerably

among affected individuals as well as in the same individual at different times (145,80,54). Photosensitivity results from effects of the photodynamic properties of the excess porphyrins in tissues. The exact cause and mechanism responsible for the anemia is not clear; although it is generally considered to be of hemolytic nature resulting from an intracorpuscular defect possibly related to the high concentration of porphyrins found in the erythrocytes (124). Erythrocyte life span studies made in a limited number of cases have yielded equivocal results; however, a shortened red cell survival time was observed in some of the cases studied (80,89,145).

The site of the defect in the porphyrin biosynthesis pathway and the mechanism responsible for overproduction of the type I isomer of URO and COPRO in CEP have been the subjects of considerable speculation during recent years. The demonstration by Bogorad (8) that two enzymes were involved in the conversion of PBG to UROgen III and that, in the absence of the second enzyme, only UROgen I was formed, led him to suggest that the metabolic defect in CEP was due to impaired UROgen III cosynthetase activity, or increased activity of UROgen I synthetase, or a combination of both. Watson (150) later proposed that the defect involved an overproduction of ALA synthetase, thus leading to overloading of the physiologically imbalanced dual enzyme system and favors increased production of UROgen I without impairment of UROgen III production. However, no direct evidence has been presented in support of this hypothesis.

Romeo and Levin (118) have recently reported evidence indicating that at least one of the primary defects in the metabolic pathway involves a deficiency of UROgen III cosynthetase activity. In further

studies, Romeo et al.(117) demonstrated that individuals heterozygous for the CEP trait have cosynthetase activity intermediate between that of normal and affected individuals. Although a deficiency of this enzyme would explain the major biochemical features of CEP, it remains to be proven whether or not other defective mechanisms may also be involved.

Erythropoietic Protoporphyria. Erythropoietic protoporphyria (EP) is a second type of porphyria in which a defect in porphyrin metabolism occurs in erythroid cells (57,109). This type of porphyria was first clearly defined in 1961 by Magnus et al. (91) as a distinctly separate entity. However, description in earlier case reports suggested the occurrence of EP as early as 1953 (79). Haeger-Aronsen and Krook (58) surveyed all the known cases of EP in Sweden and confirmed the previous assumption that the disease is inherited and that the gene is dominant (57). Other workers have stated that available data exclude simple autosomal dominant, x-chromosome linked, and autosomal recessive inheritance, and suggest a multifactorial inheritance or an incomplete dominant form of inheritance (159).

The primary clinical symptom of the condition is a mild to moderate photosensitivity manifested early in life, usually in the first two years (109). Affected individuals are generally less photosensitive than those affected with CEP. Anemia is not present in protoporphyria; however, there is sufficient evidence to indicate defective heme biosynthesis in erythroid cells.

The more classical cases of EP are characterized by the presence of large amounts of PROTO IX in erythrocytes, bone marrow, plasma and feces (109, 91, 58). Many of the red cells exhibit intense fluorescence due

to the high intracellular PROTO content (109,21). Concentrations of URO and COPRO are within normal limits in tissues and excreta.

Although protoporphyria is classified as an erythropoietic type, some studies have yielded results which suggest that defective porphyrin metabolism in EP patients occurs in other tissues as well as in erythroid cells. The elevated erythrocyte PROTO is indicative of an erythropoietic origin of the defect. Porter (108) demonstrated that bone marrow from two EP patients incorporated ^{59}Fe into heme at rates 10 to 20 fold greater than did normal marrow and that heme synthesis in hepatic tissue from the same patients was normal. However, other investigators have pointed out that, since erythrocyte life span is normal in EP patients, all the fecal PROTO could not be derived from circulating red cells (130). Scholnick et al. (130) recently reported the results of an experiment using a double isotope labeling procedure in an EP patient in which they interpreted the findings to indicate that excessive PROTO production occurs in hepatic tissue as well as in erythroid cells.

Hepatic Porphyrias. The hepatic porphyrias represent a much more important group of porphyric diseases than the erythropoietic types in man primarily because of their much higher incidence and generally more severe clinical manifestations. Characteristic features which are common to all of the hepatic porphyrias include: 1) autosomal dominant inheritance, 2) failure of symptoms to become apparent before a few years of age, commonly developing during middle age, 3) overproduction of type III isomer of porphyrins, and 4) excessive urinary excretion of the porphyrin precursors, ALA and PBG. Each of the separate types of hepatic porphyria, however, are characterized by differences in degree and pattern of expression of the above features as well as by additional

features not common to all types.

Acute Intermittent Porphyria. Acute intermittent porphyria (AIP) is the most common form of porphyria in man (46). The disease is found among various races but has especially high incidence among people of Scandinavian origin (14). Waldenström established the inherited nature of the disease and Gates (34), in a review of Waldenström's data, believed the transmission of the porphyric trait occurred through an irregular dominant gene. Later studies suggest that transmission of AIP occurs through a rare single autosomal gene (46). Analysis of genetic data by other workers indicates that most affected individuals are heterozygous for the dominant autosomal gene (46).

The principal clinical feature of AIP is reflected in the name designation of the disease, i.e., acute attacks followed by periods of latency. Additional clinical features include neurologic disturbance such as mental depression, neuritis, obscure abdominal pains and periods of insanity (87). The onset of symptoms in affected females commonly occurs in the third decade of life, while in males, initial symptoms more commonly occur during the fourth decade (46). It was noted in early studies of AIP that acute attacks are often precipitated by certain drugs, including the barbiturates, as well as non-drug related factors such as menstruation, alcoholism and infections (124, 87).

The primary biochemical features of AIP are elevated urinary levels of ALA and PBG which are especially high during acute attacks (141,1). During latent periods of the disease, ALA and PBG levels are variable and may be normal to elevated. Porphyrins are usually present in only small amounts in the freshly voided urine. However upon standing, any porphyrinogens present may be oxidized to porphyrins and some of the PBG

may be converted spontaneously to URO (152). Fecal COPRO and PROTO levels may be slightly elevated during acute attacks (124). Large amounts of PBG are regularly found in liver tissue from patients with AIP (126). The levels of PBG in bone marrow and spleen are normal thus, giving further indication that erythropoietic tissues are not involved.

The relationship between the biochemical findings and neurologic symptoms is intriguing but no direct evidence has been found to indicate that elevated levels of ALA and PBG produce neurologic lesions (53). The cause of the neurologic signs associated with AIP remained unexplained.

It was demonstrated by Tschudy et al. (143) and Rose et al. (119) that a high carbohydrate diet had a protective effect in rats against the porphyrin inducing capacity of porphyrinogenic drugs. This observation led to the use of high carbohydrate diets in patients affected with AIP for protection against and decreasing severity of acute attacks (153). In general, such diets have a favorable effect toward alleviation of neurologic symptoms and reduce the urinary excretion of the porphyrin precursors. The protective effects of carbohydrates has been termed the "glucose effect".

It was shown by Granick (49) that an overproduction of ALA synthetase was involved in drug-induced hepatic prophyria in experimental animals and led him to suggest that a similar metabolic disturbance may be involved in the hepatic porphyrias of man. Tschudy et al. (142) later found that patients affected with AIP showed marked increase in hepatic ALA synthetase activity. This finding provided the initial proof that an overproduction of ALA synthetase is responsible for the excessive production of ALA and PBG and prompted these workers to define

the condition as an "overproduction disease". More recently, Strand et al. (139) demonstrated marked reduction in the rate of conversion of PBG to porphyrins as well as increased ALA synthetase activity in hepatic tissue from AIP patients. On the basis of this finding, they suggest that a deficiency of UROgen I synthetase activity may be responsible for a decrease in PBG utilization and that the elevation in ALA synthetase activity results from inadequate heme synthesis in hepatic tissue so that heme repression of ALA synthetase production is decreased.

Kappas et al. (75) recently demonstrated that plasma obtained from AIP patients during acute attacks induces porphyrin synthesis in chick embryo liver cells in vitro while plasma from normal individuals and plasma from AIP patients in remission failed to show this effect. They speculated that circulating steroid hormones were possibly responsible for this effect, since it has been shown that certain steroids and steroid metabolites, as well as plasma from normal individuals taking oral contraceptives, stimulates porphyrin synthesis in the chick embryo cell system (74,113). The physiologic role that steroids may play in the control of porphyrin synthesis has not been established; however, there is mounting evidence that they do have an important effect on both normal and abnormal porphyrin metabolism (48).

Hereditary Coproporphyria. The first recognized cases of hereditary coproporphyria (HC) were reported in 1953 in a boy and both of his parents (5). These cases were recognized as being distinctly different from previously recognized cases of AIP in that a prominent feature of the disease was excretion of large amounts of COPRO III in urine and feces. Many additional cases of HC have since been described and the

chemical and clinical features of the disease have been well characterized (47).

Hereditary coproporphyria is inherited as a simple Mendelian autosomal dominant trait (47). Clinical signs of the disease are essentially the same as those in AIP, i. e., abdominal pain, mental disorders, neurological signs, vomiting and constipation (47,76). Although photosensitivity is not a usual feature of the disease, it has been described in at least one HC patient (47). The disease is similar to AIP in that it is characterized by acute attacks followed by periods of remission and also in that administration of certain drugs may precipitate acute attacks (47,76).

The principle chemical findings in affected individuals are greatly elevated fecal levels of COPRO III and large amounts of ALA and PBG in urine, especially during acute attacks. Usually, the urinary levels of URO and COPRO, the fecal PROTO and the blood porphyrin levels are normal (47). Typical porphyrin fluorescence has been demonstrated in hepatic tissue by fluorescence microscopy.

Recent studies regarding activities of enzymes involved in heme biosynthesis indicate markedly increased hepatic ALA synthetase activity, moderate increase in hepatic ALA dehydrase activity and possibly a slight increase in hepatic ferrochelatase (76). McIntyre et al. (98) have found that hepatic ALA synthetase activity was normal in a patient with HC in remission. These investigators also reported increased urinary excretion of 17-oxosteroids and increased ALA dehydrase activity in blood from two HC patients during acute attacks of the disease. Urinary levels of 17-oxosteroids and blood ALA dehydrase activity were found to be normal in HC patients during remission. The significance of

elevated urinary steroids during acute attacks is not known; however, it suggests a possible influence of steroid hormones in this disease as well as in AIP.

Porphyria Variegata. Porphyria variegata (PV) occurs most commonly among the Africaner population in South Africa; however, the condition is not limited to this group and has been reported in various other parts of the world (59,157). Genetic studies among the Africaner population indicate that the disease is inherited as an autosomal dominant trait and results from a single dose of an abnormal gene (22). The disease usually does not become apparent prior to the third decade of life (124).

Most of the clinical features of PV are similar to those of AIP and HC, including neurological manifestations and acute attacks, which may be precipitated by certain drugs followed by periods of latency (22,25). Photosensitivity is a common feature of this diasease and is present in approximately one-half of the cases of PV (124).

The chemical featuress of PV show both similarities and differences to the other hepatic porphyrias. Urinary excretion of ALA and PBG is markedly increased with URO and COPRO being only mildly elevated (124). Fecal excretion of URO, COPRO and PROTO are markedly elevated. Concentrations of the porphyrins in feces and porphyrins and porphyrin precursors in urine increase considerably during acute attacks (124). Porphyrin levels in red cells and bone marrow are normal.

Studies of the enzymes involved in the heme biosynthesis pathway in individuals affected with PV thus far have been confined to hepatic ALA synthetase. The activity of this enzyme has been shown to be increased in hepatic tissues from patients with PV (139). The rate of

utilization of PBG by hepatic tissue has been found to be normal (139), a feature differing from that in AIP.

Cutaneous Hepatic Porphyria. Cutaneous hepatic porphyria (CHP), often referred to as porphyria cutanea tarda, occurs with relative frequency among the Bantu natives of South Africa but is not limited to this ethnic group (124). A great deal of controversy exists regarding whether or not this disease is inherited or acquired. Although there are strong indications that the disease is inherited or, at least, that there is an inherited predisposition, genetic studies have not yielded evidence that clearly demonstrates an heritable nature of the disease (24). In most instances, a concurrent liver lesion, especially that produced by chronic alcoholism is present at the time the disease becomes clinically apparent (46,141). This has led to the belief by some that the disturbance in porphyrin metabolism is a secondary effect (46). Others believe that alcoholism does not cause the porphyria, but merely aggravates the pre-existing metabolic defect (116).

Onset of CHP is marked by photosensitivity and usually does not occur prior to middle age (124). The condition differs from the other hepatic porphyrias by the absence of neurological manifestations. Chemical features of CHP are increased urinary and fecal excretion of URO and COPRO without elevations in the porphyrin precursors, ALA and PBG (116,160). Fecal PROTO levels may be increased but the levels are not comparable to those found in cases of PV (116). Erythrocyte and bone marrow porphyrin levels are normal; however, plasma porphyrin levels are often elevated (46).

Zail and Joubert (160) have studied hepatic ALA synthetase activity in 12 cases of CHP. Five of these cases showed slightly elevated ALA

synthetase activity while seven showed activities within normal range. Kaufman and Marver (76) studied the activities of ALA synthetase, ALA dehydrase and ferrochelatase in a single liver biopsy specimen from a patient with CHP and determined the activity of each of these three enzymes to be normal.

Of the four types of hepatic porphyria, CHP is the only one in which hepatic ALA synthetase activity is normal. This feature is compatible with the normal ALA and PBG levels observed in the urine of patients with CHP. However, the explanation for the large amounts of porphyrins excreted by these patients remains to be determined. Rimington et al. (61) has postulated that the excessive porphyrin formation results from an increased rate of oxidation of the porphyrinogens to porphyrins by the liver. A number of cases of CHP have been reported in which no porphyrinogens were found in excreta, as well as cases in which both porphyrinogens and porphyrins were found (63,72). These contrasting findings suggest the possibility that more than one mechanism may be involved.

Porphyria in Animals

Hereditary porphyrias are relatively rare among domestic animals and have thus far been reported only in the bovine, porcine and feline species. The type of porphyria in each of these species is of the erythropoietic type and most closely corresponds to congenital erythropoietic porphyria of man. However, certain features of the disease in animals differ from those in man and differ between each of the animal species.

There have been no disturbances in porphyrin metabolism of an inherited nature among animal species that correspond to the hepatic types

of porphyria in man. However, chemically induced porphyria in experimental animals bears close resemblance to the disease induced by the same chemical agents in man. Since the heme biosynthesis pathway apparently is identical in all animals, there is no reason to suspect that similar genetic defects involving the pathway could not occur in animals as well as man. Although not considered to be a disease in the usual sense, physiologic peculiarities in porphyrin metabolism are characteristic of certain species including the American fox squirrel and certain rodents.

Bovine Porphyria. Porphyria is much more commonly encountered in cattle than in other domestic animals. The disease in cattle is almost identical to congenital erythropoietic porphyria in man. The first recognized cases in cattle were reported in 1936 by Fourie (32) who described the disease in a herd of Shorthorn cattle and recognized similarities to the disease in man. However, reports in the literature as early as 1910 recorded the observation of chocolate colored bones in cattle carcasses (32). Additional observations of abnormally pigmented cattle bones were published in the following years (32), but it was not until Fourie's early observations that the disease was described in living animals. During subsequent years, bovine porphyria has been reported in various breeds of cattle in various parts of the world (2,103, 69,112,111). Through an extensive series of studies, Fourie (32,33), in collaboration with Rimington (115,114), characterized the principal clinical, clinical, genetic and chemical features of the bovine disease.

Bovine porphyria, like its counterpart in man, is inherited as a simple Mendelian recessive trait (33). The genetic trait apparently is relatively common among the Holstein-Friesian breed in the United States as a result of widespread use of three bulls heterozygous for

the trait (149). The total number of offspring sired by these bulls is unknown but is estimated to be over 100,000. Fifty percent of the offspring would be carriers of the defective gene.

Like its counterpart in man, bovine porphyria is characterized clinically by photosensitivity, anemia, pigmentation of teeth and bone and the passage of red urine due to the presence of excessive porphyrins (32,149). Similarly, most of the chemical features that characterize bovine porphyria are identical to those of CEP in man (151,2,67). However, bovine porphyria differs from its counterpart in man in that PROTO IX is markedly elevated in red cells and in bone marrow (151,16). The significance of the elevated erythrocyte PROTO levels has not been clearly established. It could be indicative of a difference in the defective mechanisms of porphyrin metabolism in the disease of man and cattle; however, Goldberg and Rimington (46) observed that normal bovine red cell hemolysates are capable of synthesizing greater amounts of PROTO than hemolysates of normal human red cells. These authors suggest that the species difference in erythrocyte PROTO levels arises because cattle red cells retain functional mitochondrial elements for a longer period during maturation than do human red cells. Smith and Kaneko (137) suggested that the maturation rate of immature bovine red cells taken from porphyric cattle is much slower than the maturation rate of reticulocytes from phenylhydrazine-induced anemic cows.

Levin (84) has demonstrated a deficiency in UROgen III cosynthetase activity of red cells from porphyric cattle identical to that demonstrated in man (118). Although a deficiency of this enzyme could explain most or all of the chemical abnormalities observed in this type of porphyria in cattle and man, definitive information regarding activity of

other enzymes in the heme biosynthesis pathway and their possible involvement in defective porphyrin metabolism have not thus far been reported in either species.

The economic significance of the widespread distribution of the porphyric trait among cattle of the Holstein-Friesian breed has led to investigation directed toward detecting methods of identifying the heterozygous carriers of the porphyric trait. Various approaches to this problem have been used by different investigators. Kaneko and Mills (73) demonstrated that glutathione stability is greatly reduced in erythrocytes obtained from adult carrier cattle as well as adult porphyrics; however, glutathione stability was found to be normal in young porphyric cattle. Moore et al. (101) found that carrier cattle generally showed significantly larger ratios of COPRO I to COPRO III in the urine. Levin (117) has shown that adult heterozygous cattle, as well as man, have a reduced Urogen III cosynthetase activity level in red cells intermediate between those in porphyric and normal individuals. Although each of the tests show statistically significant differences between the normal and heterozygous individuals, technical difficulties in conducting the tests as well as the degree of inaccuracy in detecting all heterozygote individuals makes these tests inappropriate for routine use.

Porcine Porphyria. The first live cases of porcine porphyria were reported in New Zealand in 1944 (18); however, as in cattle, discoloration of bones in swine at abattoirs had been noted previously (70). Further cases of porcine porphyria were later reported in Danish swine in 1956 (68). Fairly extensive studies by Jorgensen and With (68,69, 158,70) were made in the Danish swine concerning the clinical, chemical

and genetic characteristics of porcine porphyria. Their studies revealed both similarities and differences between porcine porphyria and the congenital erythropoietic porphyria of man and cattle. The disease in swine was found to be inherited as an autosomal dominant trait, being completely different in this respect from the similar disease of man and cattle (68). A further difference of the disease in swine is the absence of photosensitivity (68). Porcine porphyria is similar to CEP in man and cattle with respect to porphyrin pigmentation of bone and teeth, urinary excretion of relatively large amounts of URO and COPRO, increased amounts of porphyrins in blood and other tissues, and a predominance of the type I isomer of porphyrins in urine and tissues (68,158). The indications from these investigations are that porcine porphyria is erythropoietic in type being most comparable to CEP in man and cattle; however, certain features such as fluorescence of red cells, levels of porphyrins in bone marrow and enzyme studies have not been determined in porphyric swine. The experimental herd of Danish swine died out before further studies could be conducted and other porphyric swine have not since become available for experimental study.

Feline Porphyria. Porphyria in the domestic cat (Felis catus) was first reported in 1964 (140). The original report described brownish pigmentation of the teeth and discolored urine which contained excessive amounts of URO, COPRO and PBG. Only one other case of feline porphyria in cats unrelated to those in the original report has been recorded (40). However, further studies of feline porphyria have been continued in the colony of porphyric cats established from the dam of kittens involved in the first reported case.

The inheritance pattern of feline porphyria as in swine has been established to be autosomal dominant (41). Clinical features of the feline disease, in addition to pigmentation of teeth and bone and excessive excretion of urinary porphyrins, include absence of photosensitivity and a mild to moderate anemia in the more severely affected individuals (39,43). The chemical findings in feline porphyria are similar to those in CEP of man, cattle and swine in that the type I isomer of URO and COPRO predominates in urine, feces, bone and various soft tissues (39). Feline porphyria differs from CEP in the other species in that excessive quantities of PBG are excreted in the urine and differs from CEP in man in that erythrocyte PROTO levels are markedly increased (39).

Additional biochemical features of feline porphyria are the subject of the investigations being reported in the present thesis.

"Physiological" Porphyria

The American fox squirrel (Sciurus niger) appears to have a "physiological" porphyria which mimics many of the features of congenital erythropoietic porphyria in other animal species and man (46). The condition is a species peculiarity existing in all individuals of the species and does not appear to affect the health of the animals. Turner (144) in 1937, described the condition in the fox squirrel and characterized the primary features which include brownish pigmentation of bone and teeth and porphyrinuria. He identified the skeletal chromophore as URO I. Rimington (46) demonstrated the characteristic pinkish pigmentation of bones and teeth and confirmed Turner's earlier finding that the porphyrin was primarily URO. Recently, Levin and Flyger (85) have shown that UROgen III cosynthetase activity in red cell hemolysates

and in other tissues from fox squirrels is much less than in similar preparations from grey squirrels (Sciurus carolinensis). Low activity of this enzyme explains the production of large amounts of URO I by the fox squirrel and indicates that the species peculiarity of porphyrin metabolism in the fox squirrel is analogous to the inherited defect which is responsible for CEP in man and cattle.

Certain other rodents, particularly the white rat, exhibit the peculiar physiologic feature of formation of large amounts of PROTO in the glands of Harder which are associated with the lachrymal apparatus (46). Strand et al. (139) have shown that ALA synthetase activity in this tissue is extremely high, ranging from 14,005 to 22,250 picomoles ALA/mg protein/hour as compared to ALA synthetase activity in hepatic tissue of 2.6 picomoles ALA/mg protein/hour. Such levels are much higher than those found in hepatic tissue from human patients with acute intermittent porphyria. The physiologic significance of the large amounts of PROTO in the glands of Harder of the rat is not known.

Also of interest is the presence of large amounts of the copper complex, turacin, deposited in the feathers of the touraco bird (Musophagidae), which is responsible for the deep red pigmentation in their flight feathers (124). There seems to be nothing otherwise unusual about the porphyrin metabolism of these birds.

CHAPTER III

MATERIAL AND METHODS

The investigation was conducted in two phases. Phase I consisted of characterization of the porphyrins and porphyrin precursors excreted in urine and porphyrins present in erythrocytes of porphyric and nonporphyric cats. Phase II consisted of enzyme studies in tissues obtained from affected and nonaffected cats. The specific enzymes and the tissues in which their catalytic activity was determined included: ALA dehydrase in erythrocytes, liver, and bone marrow; ALA synthetase in liver and bone marrow; and ferrochelatase in bone marrow. The assays for ALA dehydrase and ALA synthetase activities in liver and in blood were conducted in separate groups of cats for each tissue type assayed for each enzyme. All three enzymes were assayed in the same marrow specimen from each individual animal in which marrow assays were conducted.

Experimental Animals

A total of 35 male and female porphyric cats, ranging in age from six months to eight years were used in the various studies included in the two phases of these investigations. Fifty-one healthy male and female nonporphyric cats, aged six months to seven years were used as controls. Prior breeding trials indicated that the majority of the porphyric animals used were heterozygous for the porphyric trait. The

severity of the porphyric condition was estimated by an arbitrary grading system based on visual observation of the relative degree of porphyrin pigmentation in the teeth at two to three months of age. Grade 1 designates animals with the mildest degree of teeth pigmentation. Grades 2, 3, and 4 represent increasing degrees of severity respectively.

The nonporphyric animals used in the blood and urinary porphyrin studies were maintained in the porphyric cat colony for six months or longer prior to and during the studies. Nonporphyric cats used in the enzyme studies were obtained from an animal shelter and from a colony maintained for viral research. Since many of the porphyric cats were mildly to moderately anemic, three of the nonporphyric cats used in the marrow studies were intentionally made anemic by repeated bleedings and two nonporphyric cats were used that had an anemia of unknown cause. The hemogram for each animal used in the enzyme studies at the time of utilization is given in Appendix Table XLII.

Porphyrin and Porphyrin Precursor Analysis

Porphyrins and Porphyrin Precursors in Urine and Porphyrins in Erythrocytes

Individual urine specimens for the 24-hour determination of urinary porphyrins and porphyrin precursors were collected from each of five porphyric cats and five nonporphyric cats over a four day period. The urine was collected in 0.5 gm of sodium bicarbonate by means of a stainless steel metabolism cage. The specimens were analyzed immediately following the 24-hour collection period.

Fifteen ml of blood for blood porphyrin determinations was collected in ammonium and potassium oxalate anticoagulant (156). A hemogram was performed on each sample before assay for porphyrins.

URO, COPRO, and PROTO were isolated from urine and red cells by standard differential solvent extraction methods (104). A Model III Turner fluorometer was used to determine concentration of porphyrins in the final extracts. ALA and PBG were isolated from urine by ion exchange chromatography and measured as described by Mauzerall and Granick (97).

Isomer Analysis of Coproporphyrin

The ratio of I and III isomers of COPRO from the urine were determined in one porphyric cat and from red cells of one porphyric cat using a modification of the thin layer chromatography system reported by Jensen (65). The modification included use of a different proportion of 2, 6-lutidine and water (30: 9.75) and the placing of a paper wick in the beaker of 30 percent NH_4OH to aid in saturation of the atmosphere in the developing chamber. Preparation of thin layer plates and spotting of the samples was done as described by Jensen. Thin layer plates were developed in the dark until the solvent front reached the top of the plate then removed from the developing chamber and dried. Fluorescent spots corresponding to the location of I and III COPRO isomer standards were outlined under ultraviolet light. The silica gel in each area was scraped into separate 15 ml conical centrifuge tubes containing 1.5 ml of 1.5 N HCl. After mixing by vortex for one minute, each suspension was set in the dark for 0.5 hour then centrifuged at 2,000 x g for 10 minutes. The relative amount of each isomer was determined by measuring the intensity of fluorescence in the supernant using a Turner fluorometer equipped with a high sensitivity attachment.

Enzyme Assays

A total of 26 porphyric and 43 nonporphyric cats were used in the enzyme studies. A hemogram was performed on each cat before tissues were taken for enzyme analysis. Nonporphyric cats were not used as normal controls if any of the hemogram values were outside the normal range. Both porphyric and nonporphyric cats were killed by an intrathoracic injection of 400 mg of sodium pentobarbital. Liver, bone marrow, and blood were removed immediately after the animal was anesthetized. The tissues were held at 4° C during preparation for analysis.

Fixed-time incubation periods were used in determining the activity of the various enzymes. The rate of reaction for assay of each enzyme was linear with respect to the concentration of enzyme preparation and to the length of time the incubation period (Figure 2).

ALA Synthetase Activity in Liver

ALA synthetase activity was assayed in liver of three porphyric and eight nonporphyric cats by the method of Marver et al. (93). Five grams of liver were homogenized in a glass Ten Broeck homogenizer in three volumes of 0.9 percent sodium chloride solution which contained 0.5 mM EDTA and 10 mM tris buffer pH 7.4. The incubation mixture contained 1.0 ml of homogenate, 200 micromoles of glycine, 20 micromoles of EDTA, 150 micromoles of Tris-HCl buffer pH 7.2 and 0.4 micromoles of pyridoxal phosphate giving a total volume of 2 ml. Incubations were carried out in 25 ml erlenmeyer flasks. Six flasks in duplicate were used to insure sufficient material for chromatography. The flasks were shaken in air for 1 hour at 37° C and the reaction was terminated by the addition of 0.5 ml of 25 percent trichloracetic acid (TCA) to each

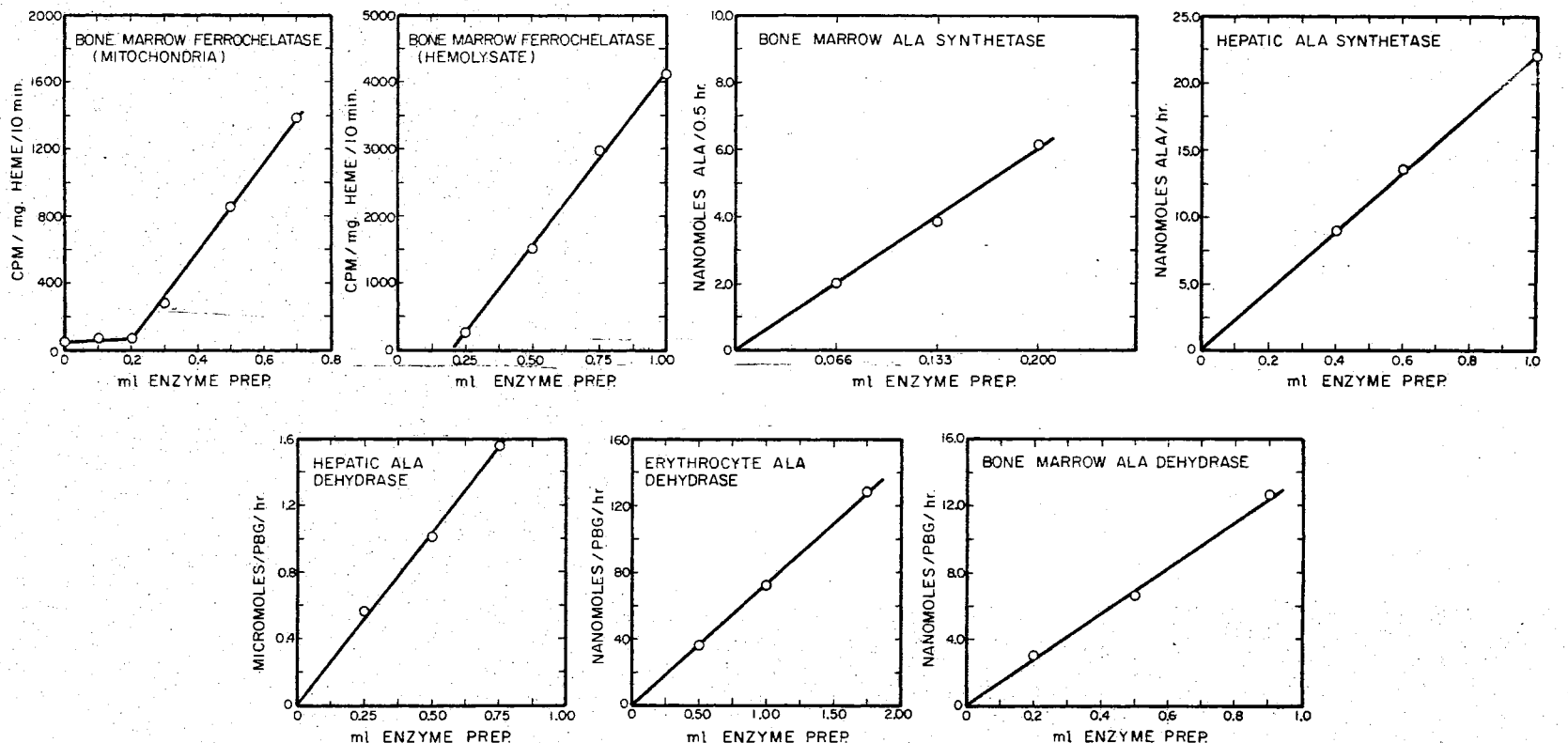

Figure 2. Enzyme Concentration versus Enzymatic Activity.

flask. The contents of the six flasks were combined and the precipitate removed by centrifugation.

Nine ml of supernant were transferred to a 50 ml volumetric flask, and the aminoketones were converted to pyrroles by the addition of 9 ml of 1M sodium acetate buffer pH 4.6 and 0.225 ml of acetylacetone. The solution was heated in a boiling water bath for 15 minutes, cooled and placed on a 1 x 7 cm Dowex-1 acetate column to separate ALA pyrrole from aminoacetone pyrrole. ALA pyrrole was eluted from the Dowex column and quantitated as outlined by Marver et al. (94).

The percent recovery of 50 nanomoles of ALA pyrrole (lower limit of detectability) added to the ion exchange column was 75± 5 percent. In thin layer and paper chromatography systems, the Rf of the standard ALA pyrrole was identical to the Rf of the Ehrlich reacting substance from the Dowex column (Table I). Evidence of PBG formation was not detectable in the assay system by the Ehrlich reagent. The activity of ALA synthetase was expressed as micromoles of ALA produced/gram liver/hour.

ALA Synthetase in Bone Marrow

ALA synthetase activity was assayed in bone marrow from 12 porphyric, 14 normal nonporphyric and five anemic nonporphyric cats. The enzyme activity in bone marrow cell mitochondria was determined by a modification of the method described by Bottomley and Smithee (11). The femora, tibiae, and humeri were taken from cats anesthetized with sodium pentobarbital. Each bone was split open and the marrow placed in 0.25 M sucrose containing 0.05 M phosphate buffer pH 7.4. A crude suspension of marrow was obtained by gently aspirating the marrow five times through the hub of a 10 ml syringe. A bone marrow smear was made

TABLE I

CHROMATOGRAPHIC RESULTS OF EHRLICH POSITIVE SUBSTANCE ASSAYED AS ALA PYRROLE IN ASSAY OF HEPATIC ALA SYNTHETASE

*Chromatographic System	Rf of Unknown Ehrlich Positive Substance	Rf of Standard ALA Pyrrole
A	0.90	0.90
B	0.78	0.78

*Paper chromatographic systems as described by Mauzerall and Granick (97).

A: Upper phase of Butanol - Acetic Acid - H_2O (4:1:5) solution.

B: Upper phase of Butanol - 1.5M aqueous ammonia (1:1) solution.

from the suspension at this time to determine the percent of erythroid cells. The marrow suspension was then further aspirated until all clumps were broken. The suspension was strained through four layers of cheesecloth and brought to a final volume of 15 ml with the 0.05 M phosphate buffered 0.25 M sucrose solution. The number of nucleated cells in this suspension was determined in a leukocyte counting chamber. The suspension was centrifuged at 750 x g for 15 minutes and, after removal of the supernant, the cells were resuspended in 10 ml of the phosphate buffered 0.25 M sucrose solution. A hemolysate was prepared by sonificating the cells for 21 seconds using a Model W 140 Sonifier Cell Disruptor set at position 8. Mitochondria were isolated by the method of Schneider (128) modified by using 0.25 M sucrose-0.05 M phosphate solution at pH 7.4 in place of 0.25 M sucrose. Mitochondrial protein concentration was determined by the method of Lowry et al. (90).

ALA synthetase activity was measured in the mitochondria by the microassay method reported by Bottomley and Smithee (11). ALA concentration was determined colorimetrically as the ALA pyrrole. Aminoacetone which will interfere with accurate measurement of the ALA pyrrole could not be detected in the assay by the solvent extraction technique employed by Granick (49).

Sonification of the bone marrow cells was the only method tried which would produce complete lysis of all cells as determined by microscopic examination of smears made from the sonificated preparation. Homogenization using a Ten Broeck homogenizer or treatment by rapid freeze-thawing did not adequately disrupt all the cells. The length of time and intensity at which the cells were sonificated was critical

(Table II). Sonification at position 8 for periods greater or less than 21 seconds resulted in a reduction in mitochondrial enzyme activity.

ALA synthetase activity was expressed both as picomoles of ALA produced/10^6 erythroid cells/30 minutes and as picomoles ALA/10^6 nucleated cells/30 minutes.

ALA Dehydrase in Liver

The activity of hepatic ALA dehydrase was determined in five porphyric and six nonporphyric cats. Two grams of liver (wet weight) were homogenized in a Ten Broeck homogenizer in 4 ml of cold 0.9 percent saline and strained through four layers of cheesecloth. ALA dehydrase activity was assayed by measuring the amount of PBG produced in a 0.25 ml aliquot of the homogenate incubated under the conditions described by Gibson et al. (38). The activity of the enzyme was stopped by adding 10 ml of a TCA-$HgCl_2$ mixture (80 ml of a 5 percent TCA plus 20 ml of 0.1M $HgCl_2$) and the precipitate removed by centrifugation. PBG was estimated by adding 1 ml of a 4N modified[1] Ehrlich's reagent to 1 ml of the TCA supernate and determining the optical density at 553 mu after three minutes. Concentration of porphobilinogen was calculated using the extinction coefficient given by Granick and Mauzerall (51) and the enzyme activity expressed as micromoles of PBG produced/gram of liver/hour. Porphyrin formation, as determined by solvent extraction procedures, did not occur in the assay under the incubation conditions used.

1. 4N modified Ehrlich's reagent was prepared daily by dissolving 0.25 gm of paradimethylaminobenzaldehyde in 5 ml of glacial acetic acid and adding 4 ml of 70 percent perchloric acid and 2.5 ml water.

TABLE II

EFFECT OF SONIFICATION ON MARROW ALA SYNTHETASE ACTIVITY

*DURATION OF SONIFICATION (Seconds)	RELATIVE MITOCHONDRIAL ENZYMATIC ACTIVITY (Percent)
15	78.0
18	82.0
21	100.0
24	86.0
27	78.0
30	75.0

*Identical aliquots of a marrow cell suspension were subjected to sonification at the intensity described in the method of assaying marrow ALA synthetase activity.

ALA Dehydrase in Erythrocytes

The red cell ALA dehydrase activity was assayed in six nonporphyric and six porphyric cats. A 10 ml sample of blood was collected in 0.66 ml of a 5 percent heparin solution. Erythrocytes were collected by centrifugation at 1500 x g for 10 minutes, washed once and reconstituted to their original volume with cold 0.9 percent saline. Complete lysis of the cells was accomplished by freeze-thawing twice in an acetone-dry ice bath.

ALA dehydrase activity was determined by measuring the amount of PBG produced in an incubation mixture composed of 1.75 ml of hemolysate, 0.25 ml of a 0.119 M solution of glutathione and 1 ml of a M/15 phosphate buffer pH 7.2 placed together in a Thunberg tube. The reaction was started by the addition of 0.2 ml of 0.1M solution of ALA from the side-arm of the Thunberg tube. Incubation and quantitation of PBG followed the liver assay method except that the reaction mixture was incubated at 37° C. Enzyme activity was expressed as nanomoles of PBG/ml of packed red cells/hour.

ALA Dehydrase in Bone Marrow

The catalytic activity of ALA dehydrase in bone marrow was assayed in seven porphyric, seven nonporphyric, and three anemic nonporphyric cats. ALA dehydrase activity was determined in marrow hemolysate prepared as described for the ALA synthetase marrow procedure. Nine-tenths ml of hemolysate was added to 0.11 ml of 0.2 M glutathione solution pH 7.2 and 0.11 ml of 0.2 M phosphate buffer pH 7.2 in a Thunberg tube under nitrogen and incubated for one hour at 37° C for activation of the enzyme. At this time the reaction was started by adding 0.066 ml of 0.1 M ALA pH 7.2 from the side arm of the Thunberg tube and incubation

was continued for an additional hour. It was found that vigorous mixing of the ALA with the incubation media resulted in false low enzymatic activity. This was avoided by gentle addition and mixing of the substrate to the hemolysate. Enzyme activity was stopped by the addition of 1 ml of freshly prepared 0.15 M $HgCl_2$ in 7.5 percent TCA. The precipitate was removed by centrifugation at 5000 x g. One ml of the supernatant was added to 1 ml of 2 N modified Ehrlich's reagent. If a precipitate formed in the Ehrlich-TCA mixture, it was removed by centrifugation and the optical density was determined at 553 mu 15 minutes after addition of Ehrlich's reagent. PBG concentration was determined from a standard curve based on known concentrations of the monopyrrole. ALA dehydrase activity was expressed both as picomoles of PBG produced/10^6 total nucleated cells/hour.

A small amount of URO was detected in the TCA supernatant by spectrophotometric examination at 406 mu. The amount of URO produced during the incubation was approximately 12 percent of the PBG produced.

Ferrochelatase in Bone Marrow

Ferrochelatase activity was measured in hemolysates of bone marrow prepared from 18 animals and in mitochondria isolated from bone marrow cells of 17 animals. Both methods were based on procedures reported by Bottomley (10).

Ferrochelatase in Bone Marrow Hemolysate. Ferrochelatase activity was assayed by the hemolysate method in five porphyric, 10 normal nonporphyric and three anemic nonporphyric cats. The hemolysate was prepared as discussed in the method for measuring bone marrow ALA synthetase activity. The incubation mixture for determination of ferrochelatase activity was prepared by adding 1.0 ml of hemolysate to a Thunberg

tube which contained 0.25 ml of 4.3×10^{-4} M ferrous-59 citrate[1] (specific activity of 1 microcurie/3 micrograms of Fe^{++}). After preincubation under nitrogen for two minutes at 37° C, 0.25 ml of 1.42×10^{-4} M protoporphyrin IX was added to the incubation mixture from the side-arm and the incubation continued for 10 minutes. The reaction was stopped by adding 20 ml of a solution composed of one part 2 percent strontium chloride hexahydrate in glacial acetic acid and three parts acetone. Carrier heme was provided by the addition of 2 ml of hemoglobin solution (10 ml of packed red cells plus 5 ml of water) to the mixture. Heme was crystallized by the method of Labbe and Nishida (82). The crystals were dried, weighed and solubilized in 0.5 ml of pyridine, after which the specific radioactivity was determined using a Model 810 Baird Atomic Scintillation detector equipped with a Baird Atomic Model 132 Multiscaler II and Model 960R Timer. The counting efficiency of the instrument for ^{59}Fe was 28 percent. Hemoglobin concentration was determined in the hemolysate and in the carrier hemoglobin solution by the cyanomethemoglobin method (156).

PROTO IX was prepared from PROTO IX dimethyl ester. The ester was recrystallized in chloroform-methanol solution, dried, then hydrolyzed to free PROTO with 7.5 N HCl. PROTO IX was precipitated as the sodium salt by slowly adding 7.5 N NaOH until the acid solution was neutralized. The precipitate was collected by centrifugation, washed twice with 1 percent acetic acid, and twice with deionized water then stored in a dessicator. A solution of protoporphyrin IX was freshly prepared for each assay as described by Bottomley (10). The labelled iron solution was

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1. Ferrous-59 citrate (specific activity of 5 to 200 ucuries/ug Fe^{++}) was purchased from Abbott Radio-Pharmaceuticals.

prepared by diluting ferrous-59 citrate with ferrous ammonium sulfate until a specific activity of 1 microcurie/3 micrograms of Fe^{++} was obtained. The ferrous ammonium sulfate was freshly made for each assay in .25 M sucrose in 0.05 M phosphate pH 7.4.

The assay for ferrochelatase did not detect enzyme activity in 0.1 ml or less of hemolysate (approximately 2.0 mg of protein). However, the velocity of the reaction was proportional to the concentration of enzyme when amounts of hemolysate in excess of 0.1 ml were used (Figure 2). Enzymatic activity was determined from the slope of a line obtained by graphing the velocity of four different enzyme dilutions, usually 0.3, 0.5, 0.7, 0.9 ml of hemolysate. Ferrochelatase activity was expressed both as picomoles of heme produced/10^6 erythroid cells/10 minutes and picomoles of heme produced/10^6 nucleated cells/10 minutes.

PROTO concentrations above 0.71×10^{-4}M in the incubation mixture markedly inhibited ferrochelatase activity (Figure 3).

Ferrochelatase in Bone Marrow Mitochondria. Ferrochelatase activity was determined by the mitochondrial method in seven porphyric, eight normal nonporphyric, and two nonporphyric anemic cats. Mitochondria were prepared in the same manner as described in the marrow ALA synthetase method. Ferrochelatase was solubilized by the addition of 0.2 ml of 11 percent sodium cholate to 2.0 ml of mitochondrial suspension (10 mg protein/ml) and the mixture slowly stirred with a magnetic mixer for 30 minutes at 4°C.

The incubation mixture was prepared by adding 0.9 ml of the solubilized mitochondrial solution to a Thunberg tube which contained 0.1 ml of 2.6×10^{-2}M L-ascorbic acid pH 7.4 and 0.25 ml of 4.3×10^{-4}M ferrous ^{59}Fe (specific activity of 1 microcurie/3 micrograms of Fe^{++}).

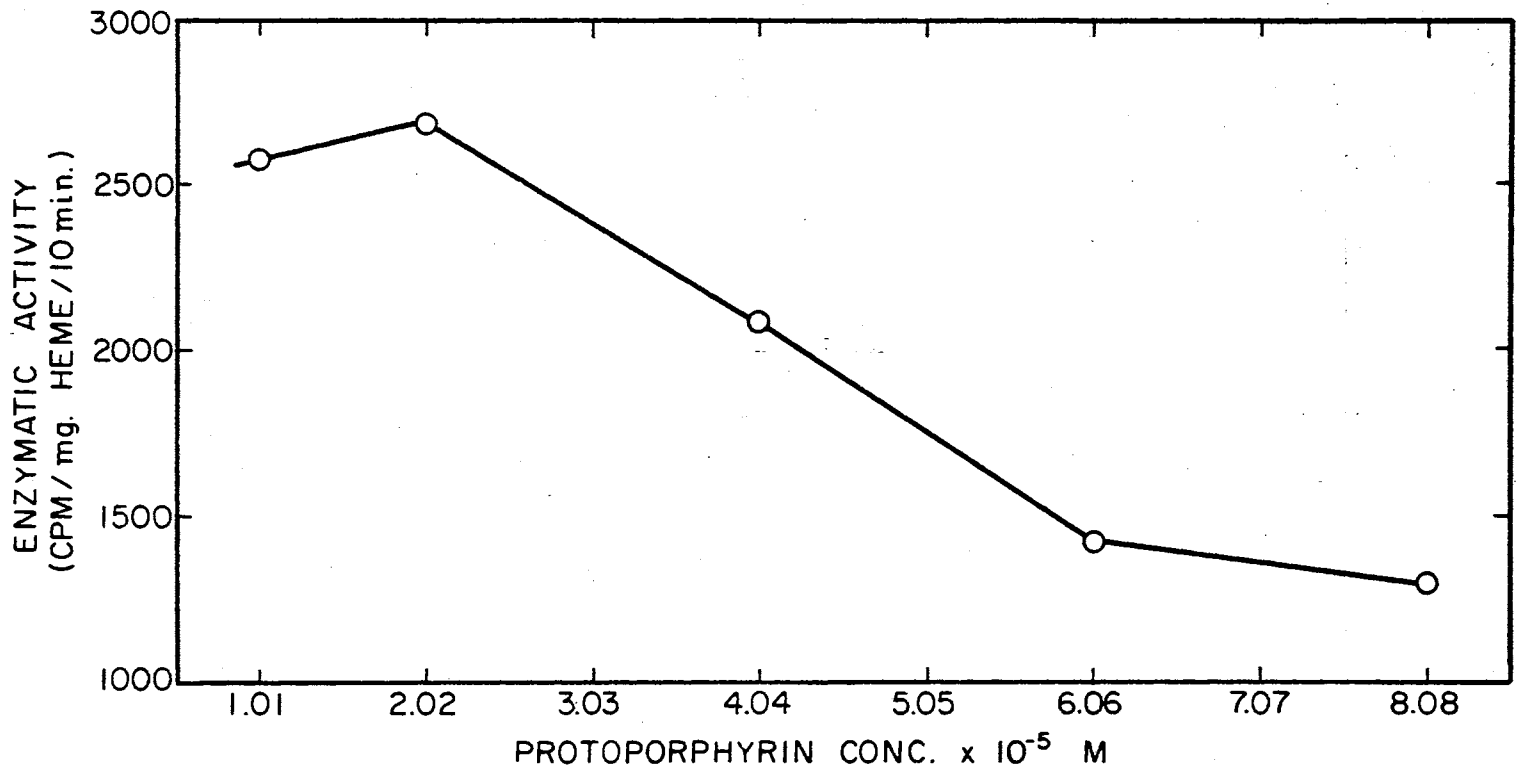

Figure 3. Hemolysate Ferrochelatase Activity versus Protoporphyrin IX Concentration.

The Thunberg tube was evacuated, flushed with nitrogen and preincubated as described in the hemolysate method. After preincubation, the reaction was started by adding 0.25 ml of 3.60×10^{-4} M protoporphyrin from the side arm. The subsequent procedure was the same as that for the hemolysate assay.

Ascorbic acid at the concentration used in the assay and treatment of the mitochondria with sodium cholate was required for maximal ferrochelatase activity (106,71). PROTO concentrations at levels which inhibited ferrochelatase in the hemolysate preparations did not affect the activity of the enzyme in the mitochondrial assay procedure. However, the mitochondrial assay was similar to the hemolysate method in that the graph of velocity versus mitochondrial suspension did not go through the intercept (Figure 2). Measurement of ferrochelatase activity was accomplished by the same procedure as in the hemolysate method. The enzyme activity was expressed both as picomoles of heme/10^6 erythroid cells/10 minutes and picomoles of heme/10^6 total nucleated cells/10 minutes.

CHAPTER IV

RESULTS

Porphyrins and Porphyrin Precursors

Urinary Porphyrins and Porphyrin Precursors

Average daily concentrations of porphyrins and porphyrin precursors and their ranges in urine collected over a four-day period for each of five normal and five porphyric cats are graphically illustrated in Figure 4. Daily levels for each animal are given in the Appendix Table XIV.

No significant difference in the concentration of urinary ALA was noted between normal and porphyric cats. The amount of ALA excreted by individuals in both groups of cats varied greatly from day to day. During the four day collection period, the widest variation in daily urinary ALA levels in the same individual of the nonporphyric group ranged from a low of 108 to a high of 1,727 micrograms/24 hours, while in the porphyric group, the greatest range in an individual animal was from 77 to 480 micrograms/24 hours. The range of ALA excretion values among the five nonporphyric cats was 22 to 1741 micrograms/24 hours with a range of the mean for each animal during the four-day period of 103 to 961 micrograms and an overall mean for the five cats of 338 micrograms/24 hours. The range in daily excretion values of ALA among the five porphyric cats was 77 to 535 micrograms/24 hours with a range of the mean for each individual of 200 to 432 micrograms and

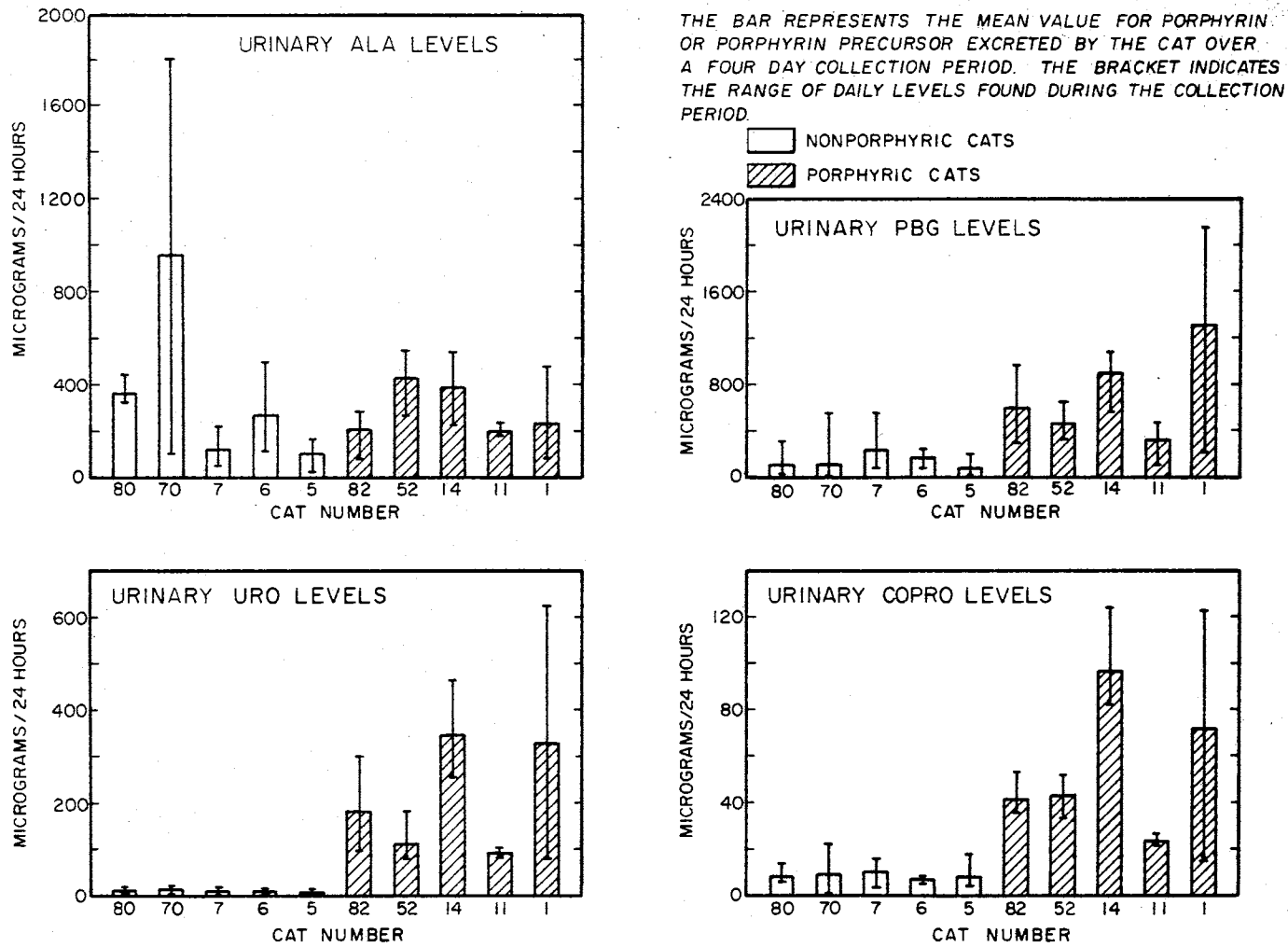

Figure 4. Urinary Levels of Porphyrins and Porphyrin Precursors for Five Normal and Five Porphyric Cats.

an overall mean for the five cats of 338 micrograms.

In contrast to ALA excretion, the urinary excretion of PBG was markedly increased in the porphyric cats. The overall mean value for the five porphyric animals during the five-day period was 729 micrograms/24 hours compared to a mean of 162 micrograms/24 hours for the five nonporphyric cats. The daily excretion of PBG in the porphyric cats ranged from 102 to 2128 micrograms/24 hours and the range in nonaffected cats was 0 to 535 micrograms/24 hours. The excretion levels of PBG fluctuated widely among the individual animals of both the porphyric and nonporphyric group over the four day collection period.

The urinary excretion of URO and COPRO was much higher in the porphyric than in the nonporphyric cats with URO being much higher than COPRO levels in the porphyric group. The average 24-hour excretion levels of URO and COPRO for the five porphyric cats during the four-day collection period were 213 and 55 micrograms, respectively, with a range of 80 to 626 micrograms of URO and 15 to 124 micrograms of COPRO. Corresponding 24-hour excretion values for nonporphyric cats were 8.2 micrograms of URO and 8.4 micrograms of COPRO, with a range of one to 22 micrograms for each porphyrin.

There was a direct correlation between the urinary levels of porphyrins and PBG in the porphyric cats (Figure 4). The two porphyric cats with the highest urinary excretion levels of porphyrins also excreted the highest amounts of PBG. Similarly, the porphyric cat with the lowest level of PBG also excreted the least amount of porphyrins. The highest urinary porphyrin levels were roughly four fold greater than the smallest amount while the highest PBG levels were approximately three fold that of the smallest PBG level. Much of the daily

variation in levels of porphyrins and porphyrin precursors may have resulted in part from the day to day variation in urine volume during the four day collection period.

Red Cell Porphyrins

Concentrations of URO, COPRO and PROTO were determined in erythrocytes from peripheral blood from four porphyric and three nonporphyric cats. The values for each of these porphyrins in each animal are given in Table III. Red cell PROTO levels were much higher than either the COPRO or URO levels in both the nonporphyric and porphyric cat groups; however, PROTO and COPRO in porphyric erythrocytes were greatly elevated above the levels found in nonporphyric red cells.

The range of values in the nonporphyric animals was 4 to 30 micrograms/100 ml red cells for PROTO and 0 to 1 micrograms for COPRO. The range of values in the porphyric animals was 152 to 349 micrograms/100 ml of red cells for PROTO and 13 to 59 micrograms/100 ml of red cells for COPRO with means of 249 and 28 micrograms respectively.

A moderate increase in URO concentration was found in erythrocytes from the porphyric cats. The mean value for URO in porphyric erythrocytes was 6.5 micrograms/100 ml red cells compared to the mean of 2.7 micrograms/100 ml of red cells in the nonporphyric animals.

The distribution of types I and III isomers of COPRO was determined in the erythrocytes from one grade 1 porphyric cat and in the urine from one grade 4 porphyric cat. The type I COPRO isomer predominated in both the red cells and urine of these individuals. The I isomer represented 93 percent of the urinary COPRO and 66 percent of the erythrocyte COPRO. Isomer distribution was not determined in erythrocytes and urine from nonporphyric cats because of the very low COPRO concentrations.

TABLE III

ERYTHROCYTE PORPHYRINS

CATS	COPRO	URO	PROTO
		µg/100 ml RBC	
Porphyric			
53 (grade 2)	21.5	0.0	330.0
353 (grade 2)	12.9	7.0	349.0
63 (grade 2)	18.4	4.0	152.0
71 (grade 1)	59.0	11.0	165.0
MEAN	28.0	6.5	249.0
Nonporphyric			
45	0.0	3.6	4.3
354	1.1	3.0	30.0
223	1.0	1.6	14.6
MEAN	0.7	2.7	14.6

Enzyme Studies

ALA Synthetase Activity in Liver and Bone Marrow

Table V summarizes the hepatic ALA synthetase activity obtained from three porphyric and eight nonporphyric cats. Individual values for ALA synthetase activity are given in Appendix Table XVI. No significant difference was noted between the two groups ($p>0.10$) although the enzyme activity for one porphyric cat was low in comparison to activities in the normal cats. The range of hepatic ALA synthetase activities in the nonporphyric cats was 4.3 to 12.5 nanomoles of ALA produced/hour/gm of liver and the range in the porphyric cats was 2.0 to 9.8 nanomoles of ALA/hour/gm of liver.

Bone marrow ALA synthetase activity was determined in 14 nonporphyric and 12 porphyric cats. Activity of the enzyme was expressed both as picomoles of ALA/10^6 nucleated cells/30 minutes and picomoles/10^6 erythroid cells/30 minutes. Mean ALA synthetase activity in marrow from porphyric cats by both methods of calculation was higher than that in marrow from porphyric cats (Table VII). Enzyme activity for nonporphyric cats ranged from 15 to 73 picomoles of ALA produced/30 minutes/10^6 erythroid cells with a mean value of 39 picomoles. In comparison, the range found in porphyric cats was 24 to 173 picomoles/30 minutes/10^6 erythroid cells with a mean value of 73.4 picomoles.

No correlation was found between ALA synthetase activity and severity of the porphyric condition. The markedly porphyric grade 3 cats had ALA synthetase activities similar to the mildly porphyric grade 1 cats (Appendix Table V). No significant difference in the levels of bone marrow ALA synthetase activity was noted between nonporphyric and porphyric cats when enzyme activity was expressed on the

TABLE IV

LIVER ALA DEHYDRASE ACTIVITY

GROUP	μmoles PBG/gm/hr.
NORMAL (6)¶	2.10† 1.7-2.3§ S.E. = 0.085*
PORPHYRIC (5)	2.30 2.2-2.5 S.E. = 0.075 p <.20

¶Number of animals in group.
*Standard error of mean.
†Mean.
§Range.

TABLE V

LIVER ALA SYNTHETASE ACTIVITY

GROUP	nmoles ALA/gm/hr.
NORMAL (8)¶	8.4† 4.3-12.5§ S.E. = 1.03*
PORPHYRIC (3)	5.5 2.0-9.8 S.E. = 2.29

TABLE VI

BONE MARROW ALA DEHYDRASE ACTIVITY

GROUP	pmoles PBG/hour	
	10^6 Erythroid Cells	10^6 Nucleated Cells
NORMAL (7)¶	1083† 790-1544§ S.E. = 105.5*	195.6 97-309 S.E. = 27.4
PORPHYRIC (7)	1953 1098-4414 S.E. = 439.8	332.1 245-442 S.E. = 25.5
	$p<0.20$	$p<0.005$

¶Number of animals in group.
*Standard error of mean.
†Mean.
§Range.

TABLE VII

BONE MARROW ALA SYNTHETASE ACTIVITY

GROUP	pmoles ALA/30 minutes	
	10^6 Erythroid Cells	10^6 Nucleated Cells
NORMAL (14)¶	39† 15-77§ S.E. = 5.51*	6.7 1.8-13.0 S.E. = 0.93
PORPHYRIC (12)	73 24-173 S.E. = 13.14	13.7 6.2-20.8 S.E. = 25.5
	$p<0.025$	$p<0.005$

basis of mitochondrial protein (Appendix Table XVII). ALA synthetase activity was also determined in bone marrow of five anemic nonporphyric cats. The values obtained in this group are given in Table VIII. The activity of ALA synthetase when calculated on the basis of 10^6 nucleated cells was considerably elevated above values found in porphyric as well as the nonanemic nonporphyric cats; however, the enzymatic activity calculated on the basis of 10^6 erythroid cells was near the mean of porphyric marrow ALA synthetase activity. ALA synthetase activities per mg of mitochondrial protein in the anemic nonporphyric cats were similar to those in the porphyric and normal groups.

ALA Dehydrase Activity in Liver, Red Cells and Bone Marrow

Hepatic ALA dehydrase activity was determined in five porphyric and six nonporphyric cats. The results of these determinations are summarized in Table IV and the complete data is given in Appendix Table XV. No significant difference in hepatic ALA dehydrase activity was found between the two groups ($P>0.10$).

ALA dehydrase activity was determined in the red cells of six porphyric and six nonporphyric cats. The enzymatic activity in porphyric erythrocytes ranged from 225 to 640 nanomoles of PBG/ml red cells/hour with a mean value of 374 nanomoles. The activity in nonporphyric erythrocytes ranged from 61 to 201 nanomoles of PBG/ml red cells/hour with a mean value of 145 nanomoles. The difference in red cell ALA dehydrase activity between the two groups was significant at $p<0.01$ level. The highest ALA dehydrase levels found in erythrocytes occurred in those from the porphyric cat which had the highest reticulocyte count (4.8 percent reticulocytes); however, there was no direct correlation between erythrocyte enzyme levels in cats with lower but slightly elevated

TABLE VIII

BONE MARROW ENZYME ACTIVITY OF NONPORPHYRIC ANEMIC CATS

ENZYME	10^6 Erythroid Cells	10^6 Nucleated Cells
ALA Synthetase *(5)	¶71 ± pmoles ALA/30 min. †51-92	19 ± pmoles ALA/30 min. 7-27
ALA Dehydrase (3)	1519 ± pmoles PBG/hr. †740-2682	357 ± pmoles PBG/hr. 104-536
Ferrochelatase		
Hemolysate Method (3)	66 ± pmoles Heme/10 min. 59-74	21 ± pmoles Heme/10 min. 19-23
Mitochondrial Method (2)	22-52	3-10

*Numbers of cats in the enzyme study.
¶Mean.
†Range.

reticulocyte values (Table IX).

Bone marrow ALA dehydrase activity was determined in seven porphyric and seven nonanemic nonporphyric cats in which marrow ALA synthetase and ferrochelatase determinations were also made. The mean ALA dehydrase activity per 10^6 erythroid cells for porphyric cats was 1.8 fold greater than that for nonporphyric cats. A range of 1098 to 4414 picomoles of PBG/10^6 erythroid cells/hour was found in porphyric cats compared to a range of 882 to 1544 picomoles of PBG/10^6 erythroid cells/hour for nonporphyric cats. However, since only one of the porphyric cats had marrow dehydrase levels markedly higher than those from the nonporphyric group, the difference between the two groups was not statistically significant ($P>0.10$).

In addition to the seven nonanemic nonporphyric cats in the preceding group, marrow ALA dehydrase activity was also determined in three anemic nonporphyric cats used in the other marrow enzyme studies. In these anemic animals, ALA dehydrase activity, when calculated on the basis of 10^6 nucleated cells, was markedly elevated, but when the calculations were based on 10^6 erythroid cells, the enzymatic activity was comparable to normal and porphyric values (Table VIII).

Ferrochelatase Activity in Bone Marrow

A total of 18 nonanemic nonporphyric, five anemic nonporphyric, and 12 porphyric cats were used in the studies of bone marrow ferrochelatase. Many of these animals were also represented among those used in marrow ALA synthetase and dehydrase studies. The hemolysate assay method was used for marrow from a total of 18 cats and the mitochondrial method on 17. Marrow from porphyric cats, as well as from nonanemic nonporphyric and anemic cats, was assayed by each method. Ferrochelatase

TABLE IX

ERYTHROCYTE ALA DEHYDRASE ACTIVITY
AND RETICULOCYTE COUNT

	nmoles PBG/ml RBC/hr.	% Reticulocytes
Normals		
6	61	1.4
8	144	Less 1.0
20	129	Less 1.0
9	127	Less 1.0
80	198	Less 1.0
7	201	Less 1.0
Mean	145	
	*S.E. = 21.1	
Porphyric		
471 (grade 2)	357	1.8
102 (grade 2)	640	4.8
21 (grade 2)	225	Less 1.0
392 (grade 3)	270	2.6
213 (grade 3)	496	Less 1.0
82 (grade 3)	258	1.8
Mean	374	
	S.E. = 66.4	

$p<0.010$
*Standard Error of Mean.

activity in marrow was similar among all three groups of cats by each of the two methods used and also similar when the activity measured by either method was expressed on the basis of 10^6 erythroid and 10^6 nucleated cells (Tables X, XI).

Values of ferrochelatase activity in marrow from porphyric cats, as determined by the mitochondrial method, ranged from 19 to 93 picomoles heme/10^6 erythroid cells/10 minutes with a mean value of 38.5. The range of ferrochelatase activities in the nonporphyric cats was 8.4 to 70.6 picomoles/10^6 erythroid cells/10 minutes with a mean value of 31.3 picomoles. Similar values were present in marrow from the anemic nonporphyric cats.

Although differences were apparent among the different grades of porphyric cats in which marrow was assayed by the hemolysate method, no significant differences were present among different grades of porphyric cats in which marrow was assayed by the mitochondrial method. Since it was noted that increased amounts of PROTO had an inhibitory effect on ferrochelatase activity in hemolysate preparations, the presence of higher levels of endogenous PROTO in the more porphyric cat marrows possibly explains the differences noted between the two assay methods.

Ratio of Bone Marrow Enzymatic Activities

A comparison of the ratios of mean activities of ALA synthetase, ALA dehydrase, and ferrochelatase in bone marrow from nonanemic nonporphyric, anemic nonporphyric, and porphyric cats are given in Table XII. ALA synthetase/ferrochelatase and ALA dehydrase/ferrochelatase ratios for the nonanemic nonporphyric cats were 1.24 and 21.4 respectively, in comparison to ratios of 1.90 and 48.1 for porphyric cats. The ratio of ALA synthetase/ferrochelatase and of ALA dehydrase/ferrochelatase in marrow from the anemic nonporphyric cats was 1.92 and

TABLE X

BONE MARROW MITOCHONDRIA FERROCHELATASE ACTIVITY

GROUP	pmoles Heme/10 minutes	
	10^6 Erythroid Cells	10^6 Nucleated Cells
NORMAL (8)	31.3† 13.3-70.6§ S.E. = 6.95*	5.63 0.9-9.8 S.E. = 1.12
PORPHYRIC (7)	38.5 19.1-93.1 S.E. = 9.58	6.77 5.3-9.3 S.E. = 0.603
	$p<0.50$	$p<0.40$

Number of animals in group.
*Standard error of mean.
†Mean.
§Range.

TABLE XI

BONE MARROW HEMOLYSATE FERROCHELATASE ACTIVITY

GROUP	pmoles Heme/10 minutes	
	10^6 Erythroid Cells	10^6 Nucleated Cells
NORMAL (10)	65.7† 13.0-164.0§ S.E. = 16.8*	10.5 1.5-28.0 S.E. = 2.47
PORPHYRIC (5)	58.2 11.5-144.0 S.E. = 24.1	11.0 3.3-21.6 S.E. = 3.6
	$p<0.50$	$p<0.50$

TABLE XII

RATIO OF ENZYME ACTIVITY IN BONE MARROW

(Based on 10^6 Erythroid Cells)

ENZYME RATIO	Nonporphyric Nonanemic	Porphyric	Nonporphyric Anemic
$\frac{\text{ALA Synthetase}}{\text{Ferrochelatase (mit.)}}$ ¶	1.24	1.90	1.92
$\frac{\text{ALA Synthetase}}{\text{Ferrochelatase (hem.)}}$*	0.59	1.20	1.08
$\frac{\text{ALA Synthetase}}{\text{ALA Dehydrase}}$	0.036	0.040	0.046
$\frac{\text{ALA Dehydrase}}{\text{Ferrochelatin (mit.)}}$	31.4	48.1	41.1
$\frac{\text{ALA Dehydrase}}{\text{Ferrochelatase (hem.)}}$	16.5	31.8	23.0

¶Mitochondrial method.
*Hemolysate method.

41.1 respectively, closely approximating those for the porphyric group. The ratio of ALA synthetase/ALA dehydrase was similar for the porphyric and anemic nonporphyric and nonanemic nonporphyric cat groups.

Hematology

The various hematologic parameters, as well as percent of marrow erythroid cells, for all cats used in the marrow enzyme studies are summarized in Appendix Table XIII. The hematologic and erythroid cell values were determined on specimens obtained at the time the animals were killed. The mean percent erythroid cells for porphyric cats was 21.6 while that for the anemic nonporphyric cats was 25.9 and was 18.4 for the nonporphyric cat group. The difference in percent of erythroid cells between the porphyric and the nonanemic nonporphyric groups was not statistically significant ($P>0.10$). Data from the anemic nonporphyric cats was not compared statistically with that of the other groups due to small numbers of animals in the group. Many of the porphyric cats used in the studies had mild anemia. The range of hemoglobin concentrations in the porphyric group was 8.7 to 15.3 gm per 100 ml of blood.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Tobias (140), in his report of the first cases of feline porphyria, mentioned the presence of excess PBG, as well as URO and COPRO, in urine obtained from one of the two porphyric cats described in the report. The reported presence of an excessive amount of PBG in the urine of this cat was significant since other features of feline porphyria described in the report more nearly corresponded to congenital erythropoietic porphyria (CEP) in man and cattle but excessive urinary excretion of PBG is not found in CEP of either of the latter species (16,151,149). Also the reported presence of urinary PBG could be questioned because the tests were of qualitative nature and it has been well established that false positive tests for PBG occur with Ehrlich's reagent unless special precautions are taken (27). The findings in the initial phase of the present investigation confirmed the presence of large amounts of PBG and URO and COPRO as characteristic features of feline porphyria. This finding showed that feline porphyria is unique among the known porphyric diseases of man and animals in certain of its features since excessive production of PBG had been previously observed only in the hepatic porphyrias of man, none of which are characterized by porphyrin pigmentation of teeth and bone (124). The latter feature has been observed only in CEP of man and animals.

The excessive production of PBG by the porphyric cat raised the

question of whether the disease in this species is merely a unique form of CEP or if the defect involved both erythropoietic and non-erythropoietic tissues. The nonerythropoietic (hepatic) porphyrias of man are characterized by urinary excretion of excessive amounts of ALA as well as PBG and, at certain times, elevated levels of fecal and urinary porphyrins which are primarily of the III isomer type (141,46, 124). Erythrocyte porphyrin levels are normal in the hepatic porphyrias of man (124). Urinary ALA levels were found to be normal in feline porphyria and the erythrocytes contained high concentrations of URO, COPRO and PROTO. In the present studies, the majority of COPRO extracted from urine and from erythrocytes of each of two separate porphyric cats was found in both instances to be predominantly of the I isomer type. Concurrent studies conducted by Glenn (39) with the same colony of porphyric cats have further shown that the primary isomer of both URO and COPRO in urine, feces and red cells is of the type I series.

An additional feature of feline porphyria which differs from hepatic porphyrias and CEP of man is the presence of large amounts of PROTO in the erythrocytes of porphyric cats. High erythrocyte PROTO levels is the primary feature of erythropoietic protoporphyria (EP) in man (91,109) but is also a prominent feature in CEP of cattle (151,16). Thus, feline porphyria has features which overlap the two types of erythropoietic porphyria of man and CEP of cattle and swine, as well as certain features of the hepatic porphyrias of man. However, since overproduction of the type I porphyrin isomers, porphyrin pigmentation of teeth and bones, and elevated erythrocyte porphyrins are features of CEP in man, cattle and swine and elevated erythrocyte PROTO levels

are a prominent feature in cattle, it would appear that the features of feline porphyria are more compatible with CEP and that excess urinary excretion of PBG in feline porphyria is a manifestation of an unique defect in the porphyric cat.

The purpose of the enzyme studies in this investigation was to determine whether or not defective heme biosynthesis in feline porphyria involved tissues other than the erythropoietic tissues and to provide more specific information regarding the site(s) of the defect(s) in the heme biosynthesis pathway responsible for porphyria in the feline.

It has been demonstrated that hepatic ALA synthetase activity is greatly increased in patients suffering from acute intermittent porphyria (AIP), porphyria variegata (PV), and hereditary coproporphyria (HC) (142,98,76,139). The activity of ALA dehydrase also tended to increase but not to the magnitude exhibited by ALA synthetase (142). Such increases were probably responsible for the elevated urinary levels of ALA and PBG found in these patients. In addition, Strand et al. (139) have demonstrated a decrease in the utilization of PBG by hepatic tissue from cases of AIP. ON the basis of this finding, they suggested that AIP is due to a partial deficiency of UROgen I synthetase. Since heme acts as a repressor for ALA synthetase production, a partial block at the UROgen I synthetase level in the heme biosynthesis pathway would result in decreased heme production thus leading to increased synthesis of ALA synthetase (49).

Kaufman and Marver (76) have suggested that HC and PV also may result from partial enzymatic deficiencies but not at the same site in the heme biosynthesis pathway.

The excessive urinary excretion of PBG by the porphyric cat

suggests the possibility that defective mechanisms in the heme biosynthesis pathway similar to those involved in hepatic porphyrias of man may be involved in feline porphyria. However, results of the enzyme assays in hepatic tissue from porphyric cats gave no indication of an increase in catalytic activity of ALA synthetase or ALA dehydrase. McIntyre et al. (98) have reported that hepatic ALA synthetase activity in HC patients was elevated only during acute attacks of the disease. It is possible that a similar situation may exist in feline porphyria and that normal values of ALA synthetase activity found in affected cats may reflect a state of remission of the disease at the time of assay. However, the activity of ALA synthetase is markedly elevated during periods of remission of AIP and in PV and further increases during acute attacks (139). Therefore, the negative findings of the enzyme studies in hepatic tissue of the porphyric cat suggest that the liver probably does not contribute significantly to the abnormal PBG and porphyrin production.

Enzyme studies concerning the erythropoietic porphyrias have been much more limited than in the hepatic porphyrias. Levin and coworkers (118,84,117) have recently shown that the activity of UROgen III cosynthetase is greatly reduced in erythrocyte hemolysates from man and cattle with CEP. Levin and Flyger (85) have also demonstrated a deficiency of this enzyme in the physiologic porphyria of the fox squirrel. In contrast, Levin and Glenn (86) found that the activity of UROgen III cosynthetase was not deficient in red cell hemolysates from porphyric cats.

Evidence that defective porphyrin synthesis occurs in erythroid tissue of the porphyric cat is provided by the presence of large

amounts of porphyrins in erythrocytes. Further indication that a metabolic defect is present in erythropoietic tissue is provided by concurrent investigations by Glenn et al. (42), who demonstrated the presence of fluorescent erythroid cells in bone marrow and peripheral blood of porphyric cats.

The excess production of PBG, type I isomers of URO and COPRO, and the presence of large amounts of PROTO in the red cells suggests several sites of possible enzyme defects in heme biosynthesis which could be involved in feline porphyria. Levin and Glenn's demonstration that UROgen III cosynthetase activity is not deficient in red cells from porphyric cats would appear to rule out a decrease in the activity of this enzyme as being involved. Other likely sites of the enzyme defect(s) are ALA synthetase, ALA dehydrase and ferrochelatase. Because of the particular biochemical features in feline porphyria, it was felt that the present investigation should include studies of all three enzymes.

ALA dehydrase activity was found to be increased in circulating red cells from porphyric cats. However, the cause and significance of the increase is difficult to assess due to the elevated reticulocyte counts in the circulating blood of the porphyric cats. It is well known that reticulocytes and young nonreticulated red cells normally have higher concentrations of various enzymes than older red cells; therefore, much of the increase in ALA dehydrase activity could be a reflection only of the increased numbers of young red cells in porphyric cat blood. Since the bone marrow is populated primarily by immature erythroid cells, it was felt that this tissue would likely provide a more standard basis for determining ALA dehydrase activity and,

because of the mitochondrial location of ALA synthetase and ferrochelatase, would allow assays for all three enzymes in the same tissue for comparative purposes. Further assays of these enzymes were therefore conducted in bone marrow.

The enzymatic activities of ALA synthetase, ALA dehydrase and ferrochelatase were calculated both on the basis of 10^6 erythroid cells and 10^6 total nucleated cells. Enzymatic activity based on 10^6 total nucleated cells takes into account the activity of the enzymes involved in non-hemoglobin heme synthesis by myeloid cells as well as enzymatic activity in erythroid cells. However, since the present studies were primarily concerned with localization of the defect in heme biosynthesis in erythropoietic tissue, it would seem that enzymatic activity expressed on the basis of erythroid cells is more appropriate, especially since it is assumed that the erythroid cells are the primary source of enzymes involved in heme biosynthesis in the bone marrow (11). Enzymatic activity based on total nucleated cells does not take into account the myeloid-erythroid ratio and therefore tends to give a higher value in marrow specimens which have significantly higher percentages of erythroid cells. This was shown to be the case with the anemic nonporphyric cats which had much higher erythroid cell percentage than either the porphyric or normal cats. When enzymatic activities for the anemic cats were expressed on the basis of total nucleated cells, the values were much higher. Expression of the data on the basis of 10^6 erythroid cells provides a more standard basis for comparison of the anemic, porphyric and normal cats.

Bone marrow ALA synthetase activity in porphyric cats was significantly elevated above the levels present in nonanemic nonporphyric

animals, but there was no apparent correlation between ALA synthetase activity and the severity of porphyric condition. There was a considerable range of values among the entire group of porphyric animals as well as among the animals of each porphyric grade group. Differences in age among the porphyric cats could be one of the factors which might explain the wide range of values in the different porphyric grade groups as well as in the nonporphyric cats. Other possible explanations for the wide variation of ALA synthetase activity among both porphyric and normal cats could be differences in levels of such physiological factors as certain of the sex steroid hormones or their metabolites and erythropoietin. It has been shown that certain steroids and their metabolites, particularly those of the 5β-H configuration, induce production of ALA synthetase in hepatic and erythroid tissues (48,74). It has also been shown that erythropoietin enhances ALA synthetase activity in rabbit cell cultures (11). ALA synthetase induction by erythropoietin is suggested by results obtained from the anemic nonporphyric cats. These cats had elevated ALA synthetase activities comparable to the mean ALA synthetase activity of the porphyric cat. ALA dehydrase activity in bone marrow from some of the porphyric cats was elevated to various degrees. The mean of the activities among the porphyric cats was 1.8 fold greater than the mean activity of the nonanemic nonporphyric cats; however, this difference was not statistically significant due to the degree of overlap among the porphyric and nonporphyric groups and the wide ranges within each group. The anemic nonporphyric cats showed elevations similar to those in the porphyric group. The level of enzyme activity in the individual animals of all three groups was generally in direct proportion to the level of ALA

synthetase activity. As was the case with ALA synthetase, there was no correlation between ALA dehydrase activity and the degree of porphyria.

Ferrochelatase activity in bone marrow hemolysates and in mitochondria isolated from bone marrow cells in general was similar for porphyric, nonanemic nonporphyric, and the anemic nonporphyric groups. However, since ALA synthetase was elevated in the porphyric cats, the ALA synthetase/ferrochelatase ratio was greater in the porphyric group. During validation of the ferrochelatase procedure, it was found that activity of the enzyme in hemolysates was inhibited by concentrations of PROTO IX greater than 7.1×10^{-5} M and it was later noted that the two most severely affected porphyric cats showed ferrochelatase activities at the lower range limit for the nonporphyric cats. The tendency of hemolysates from grade 3 porphyric cats to have low ferrochelatase activities probably was a result of the presence of large amounts of endogenous PROTO. Mitochondrial ferrochelatase activity was not affected by PROTO concentrations which were inhibitory to the enzyme in the hemolysate assay. No difference in mitochondrial ferrochelatase activity was noted among any of the cats, regardless of the severity of the porphyric condition.

Whether or not ferrochelatase is inhibited *in vivo* by PROTO which accumulates in the feline porphyric erythrocyte is not known. If excess PROTO is inhibitory to ferrochelatase *in vivo*, the excess PROTO which accumulates initially as a result of a metabolic defect would favor further PROTO accumulation because of its inhibitory effect on ferrochelatase activity. The elevated ALA synthetase activity concurrent with the normal ferrochelatase activity present in porphyric cat bone marrow may contribute to PROTO accumulation by causing

biosynthesis of more PROTO than can be effectively catalized to heme by ferrochelatase. However, the marked excess accumulation of PROTO in the red cell of the porphyric cat could occur in a manner similar to that suggested by Goldberg and Rimington (46) to explain the excess erythrocyte PROTO levels in bovine porphyria without involving a substrate overload of ferrochelatase. They noted that red cell hemolysates from nonporphyric cattle formed much more PROTO from porphyrin precursors than did hemolysates from nonporphyric humans. They concluded that mitochondria are retained in maturing bovine red cells longer than in human red cells and that this species difference accounted for the PROTO found in CEP of cattle and not in man. The feline erythrocyte may be similar in this respect to the bovine red cell and the PROTO accumulation may be only indirectly related to the metabolic defect responsible for feline porphyria. In any case, the results of this study indicated that the elevated PROTO levels in porphyric feline red cells were not caused by a ferrochelatase deficiency since in the mitochondrial ferrochelatase assay method there was no indication of a difference in enzyme activity between the porphyric and nonporphyric cats.

The only positive indication in the present investigations of a possible site of the defect in feline porphyria is the finding of increased ALA synthetase activity in bone marrow. However, a number of possibilities should be explored in considerations regarding sites in the pathway and mechanisms which may be involved in the defective porphyrin metabolism responsible for the abnormal production of porphyrins and porphyrin precursors observed in feline porphyria. The excessive production of the type I isomer of porphyrins which occurs in the

porphyric cat must be due to an imbalance between the catalytic activities of UROgen I synthetase and UROgen III cosynthetase such that the level of activity of UROgen I synthetase is much greater than that of UROgen III cosynthetase. This type of imbalance could occur if the activity of UROgen III cosynthetase is decreased without a concurrent decrease in UROgen I synthetase activity or if the activity of UROgen I synthetase is elevated in absence of an elevation in UROgen III cosynthetase. Levin and Glenn (27) found that the activity of UROgen III cosynthetase is not decreased in red cells of the porphyric cat, thus the abnormal production of the I isomer of URO and COPRO would appear to result from an increased activity of UROgen I synthetase that exceeds the catalytic capacity of UROgen III cosynthetase. However, overproduction of UROgen I synthetase, in itself, would have little effect on porphyrin metabolism, since accelerated activity at this step in heme biosynthesis is dependent upon the availability of the substrate, PBG. It would appear, therefore, that the metabolic defect responsible for the elevations of porphyrins primarily of the I isomer type centers around the mechanism(s) involved in the excessive PBG production.

PBG levels may increase as a result of either a decrease in its utilization by UROgen I synthetase, the only known enzyme that uses PBG as a substrate, or by an increase in its rate of synthesis by ALA dehydrase. Since the type I isomer of porphyrins is produced in considerable excess in feline porphyria and since Levin and Glenn have shown that UROgen III cosynthetase is not deficient, UROgen I synthetase activity cannot be decreased. Therefore the high levels of PBG must be the result of an increased rate of synthesis of PBG. ALA dehydrase,

which catalyzes the formation of PBG from two molecules of ALA, does not appear to control the rate of PBG production in porphyric and normal cats or in hepatic tissue from other animals or man unless, as suggested by two reports, heme acts as a negative feedback repressor on this enzyme (13,19). Overactivity of ALA dehydrase might occur if a structural abnormality exists in the enzyme which prevents inhibition by heme if feedback inhibition operates in the erythroid cell. However, the very high levels of ALA dehydrase in relation to the levels of ALA synthetase, as indicated by the results of this investigation, suggest that ALA synthetase activity controls the rate of PBG production by limiting the amounts of ALA available for use as a substrate by ALA dehydrase. Therefore, the elevated levels of PBG and porphyrins in feline porphyria would seem to originate by ALA synthetase overactivity.

The elevated bone marrow ALA synthetase activity in porphyric cats would explain the porphyric condition if the elevation were of sufficient magnitude to overload UROgen III cosynthetase. The mean increased levels of ALA synthetase activity in porphyric cats was twice that found in normal cats. This represents a small increase compared to increases of seven-fold or more in ALA synthetase activity of liver from patients with hepatic porphyria. However, the highest activity observed in an individual porphyric cat was almost five times the nonporphyric mean and almost three times the highest nonporphyric value. The observation that the mean ALA synthetase activity level found in the anemic nonporphyric cats approximated the mean level of the porphyric cats might suggest that the increased enzyme activity in porphyric cats was due to bone marrow stimulation

by erythropoietin; however, there was no direct correlation between the activity of ALA synthetase and the degree of anemia in the porphyric cats. In fact, the porphyric cat with the greatest bone marrow activity of ALA synthetase was not anemic. The results of the ALA synthetase studies in these investigations suggest that overactivity of this enzyme may be responsible for the biochemical abnormalities observed in feline porphyria; but, the small numbers of animals involved in these studies and the relatively low magnitude of increase do not provide conclusive evidence for overactivity of this enzyme as being responsible for the porphyric condition in the cat.

The assay techniques used in these investigations for measurement of bone marrow enzyme activity have been accepted by other workers but the heterogeneous population of cell types in the marrow may have contributed to experimental error in that differences of low magnitude between the experimental groups are masked. This, coupled with the apparent mildness of the porphyric condition in affected cats, may explain why more pronounced differences in ALA synthetase activity were not found between porphyric and nonporphyric animals. If satisfactory methods could be devised for obtaining a more homogeneous population of erythroid cells, especially cells of a similar age group, the assay procedures would provide a more standard basis for comparing data between porphyric and nonporphyric animals and might more clearly resolve differences that may be present.

It is possible that the porphyric condition in the cats is secondary to a genetically determined metabolic abnormality outside the heme synthesis pathway, i.e., steroid influence on porphyrin metabolism (75,98). Also, ALA has been shown to participate in the biosynthesis

of compounds other than those involved in heme biosynthesis (135,124). A metabolic defect causing a decreased utilization of ALA by such pathways would channel more ALA into heme biosynthesis, thereby accelerating the formation of PBG and porphyrins if substantial amounts of ALA are normally used in pathways other than heme biosynthesis.

The results of the various studies reported in this thesis have failed to define the site or the specific nature of the metabolic defect responsible for feline porphyria; however, considerable information has been obtained which provides a basis for further investigation of the problem.

CHAPTER VI

SUMMARY

The investigations were designed to more completely characterize the metabolic defect(s) involved in feline porphyria. The studies were divided into two phases. Phase I consisted of quantitation and characterization of urinary porphyrins and porphyrin precursors and of erythrocyte porphyrins in feline porphyria. Phase II consisted of measuring the comparative catalytic activities of three enzymes, ALA synthetase, ALA dehydrase and ferrochelatase, involved in the heme biosynthesis pathway in various tissues including liver, blood and bone marrow from porphyric and nonporphyric cats.

Results of the porphyrin and porphyrin precursor studies in porphyric cats showed: 1) markedly elevated urinary levels of uroporphyrin, coproporphyrin and porphobilinogen, 2) normal urinary levels of δ-aminolevulinic acid, 3) markedly elevated levels of protoporphyrin and coproporphyrin and moderate elevations of uroporphyrin in erythrocytes, and 4) a predominance of the type I isomer of coproporphyrin in urine and erythrocytes. These biochemical findings indicate that feline porphyria is an unique type of porphyria having features both of the erythropoietic and hepatic porphyrias of man. Similarities to erythropoietic porphyria include elevations in erythrocyte prophyrins and the predominance of the type I porphyrin isomer; however, elevations of urinary porphobilinogen have been previously observed only

in hepatic porphyrias.

Results of the enzyme studies in porphyric cats showed: 1) normal hepatic ALA synthetase and ALA dehydrase activities, 2) elevated erythrocyte ALA dehydrase activity, 3) normal bone marrow ferrochelatase activity, 4) slightly increased marrow ALA dehydrase activity, and 5) elevated marrow ALA synthetase activity. Mean activities of the three enzymes in marrow from five anemic nonporphyric cats were similar to the mean enzyme activities found in the porphyric group; however, marrow ALA synthetase was much higher in some porphyric cats than the highest values observed in the anemic group.

The normal levels of ALA synthetase and ALA dehydrase in the hepatic tissue of porphyric cats suggest that the liver is not involved in excessive porphyrin and porphyrin precursor production. Elevation of erythrocyte ALA dehydrase activity was interpreted as a reflection of the reticulocytosis caused by the mild anemia in several of the porphyric cats. Results of the bone marrow enzyme studies suggest that ALA dehydrase and ferrochelatase are not sites of defective heme biosynthesis in feline porphyria.

The elevated ALA synthetase activity in bone marrow of porphyric cats provides an explanation for the excessive production of porphobilinogen and porphyrins; however, anemic nonporphyric cats also had similar increases in bone marrow activity. Since many of the porphyric cats were mildly anemic, the importance of elevated marrow ALA synthetase activity in the pathogenesis of feline porphyria remains to be clarified.

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APPENDIX

TABLE XIII

HEMATOLOGIC DATA OF ANIMALS USED IN ENZYME STUDIES OF BLOOD AND BONE MARROW

CAT	Hb (gm%)	PCV	% Retic	% Erythroid
Porphyric				
82 (grade 3)	11.4	35.5	1.8	--
21 (grade 2)	12.6	38.0	Less 1.0	--
213 (grade 3)	9.1	29.0	Less 1.0	--
392 (grade 3)	12.0	39.0	2.6	--
102 (grade 2)	8.7	27.0	4.8	--
471 (grade 2)	10.8	33.5	1.8	--
744 (grade 2)	9.3	27.5	2.0	28
392 (grade 2)	12.1	35.5	1.4	26
743 (grade 2)	11.9	34.5	1.6	10
142 (grade 1	11.5	36.5	1.2	27
141 (grade 1)	11.9	34.0	1.4	20
14 (grade 3)	9.8	30.5	1.0	23
761 (grade 3)	11.6	35.0	1.0	13
213 (grade 3)	9.4	27.5	---	26
763 (grade 3)	9.5	29.5	3.0	29
65 (grade 1)	11.5	34.0	---	23
713 (grade 2)	15.3	45.0	Less 1.0	15
102 (grade 2)	9.5	27.5	1.6	20
Nonanemic Nonporphyric				
6	14.1	44.0	1.4	--
8	11.5	35.0	Less 1.0	--
20	10.9	34.0	Less 1.0	--
9	14.4	46.0	Less 1.0	--
80	10.6	37.0	Less 1.0	--
7	12.3	37.0	Less 1.0	--
J-9	13.0	38.5	---	14
J-1	11.9	35.0	---	22
J-3	11.4	32.5	---	11
J-4	12.4	37.5	---	17
J-5	10.1	31.5	---	12
J-8	10.6	31.0	---	29
J-10	12.5	36.5	---	21
J-11	13.3	38.0	---	10
J-12	12.5	41.0	---	17
J-13	12.2	35.5	---	17
M-4	12.0	37.0	---	23
M-5	13.6	41.0	---	30
M-11	12.9	38.0	---	27
M-12	11.7	34.0	---	23
M-3	11.0	32.0	---	10

TABLE XIII (continued)

CAT	Hb (gm%)	PCV	% Retic	% Erythroid
Nonanemic Nonporphyric				
M-6	13.4	40.0	---	12
M-7	10.6	28.5	---	20
M-10	16.2	45.5	---	14
M-1	11.0	34.5	---	11
M-15	15.9	46.5	---	23
Anemic Nonporphyric				
M-2	9.1	31.0	---	14
M-8	9.9	27.5	---	20
K-1	9.3	27.5	Less 1.0	27
K-2	10.5	32.0	Less 1.0	39
K-3	9.1	25.0	Less 1.0	29

TABLE XIV

DAILY URINARY LEVELS OF PORPHYRINS AND PORPHYRIN PRECURSORS OVER A FOUR DAY PERIOD

CAT	micrograms/24 hour				
Porphyric	ALA	PBG	URO	COPRO	ml Urine
52 (grade 3)	331	514	178	43	67
	217	353	82	34	53
	540	496	109	44	105
	535	666	81	52	42
82 (grade 3)	289	710	302	40	85
	86	328	94	35	34
	225	864	178	53	85
	202	508	156	36	50
1 (grade 4)	480	2128	626	121	88
	275	1189	270	80	47
	233	1732	345	72	41
	77	226	80	15	11
14 (grade 3)	481	570	257	83	90
	476	1067	363	98	75
	242	878	298	82	48
	360	1066	469	124	80
11 (grade 2)	190	458	105	25	78
	210	389	90	25	69
	232	102	88	21	75
	169	382	90	21	21

TABLE XIV (continued)

CAT	micrograms/24 hour				
Nonporphyric	ALA	PBG	URO	COPRO	ml Urine
5	170	209	9	5	31
	128	52	7	6	23
	22	7	2	18	8
	91	41	1	4	18
80	36	0	3	3	178
	441	318	9	14	96
	340	76	9	8	20
	316	35	9	6	77
70	1417	353	23	23	130
	806	0	8	6	58
	108	0	3	1	23
	1741	505	12	7	66
6	385	249	12	8	110
	170	169	6	5	50
	360	181	7	9	100
	160	80	4	5	44
7	71	237	7	6	35
	178	531	14	15	69
	50	69	3	4	16
	211	131	14	16	65

TABLE XV

HEPATIC ALA DEHYDRASE ACTIVITY

CATS	μmoles PBG/gm/hour
Porphyric	
522 (grade 1)	2.53
523 (grade 1)	2.16
521 (grade 2)	2.19
182 (grade 1)	2.43
472 (grade 2)	2.19
Nonporphyric	
A-1	1.73
A-2	2.10
A-3	2.21
A-4	2.04
A-5	2.29
A-6	2.27

TABLE XVI

HEPATIC ALA SYNTHETASE ACTIVITY

CAT	Activity (nmoles ALA/gm/hour)
Porphyric	
101 (grade 2)	4.7
653 (grade 1)	9.8
21 (grade 2)	2.0
Nonporphyric	
F-4	4.3
G-1	6.3
G-2	12.1
G-3	7.5
G-4	8.4
G-5	10.0
G-6	6.2
G-8	12.5

TABLE XVII

BONE MARROW ALA SYNTHETASE ACTIVITY

CAT	pmoles ALA/30 min.		
	10^6 Erythroid Cells	10^6 Nucleated Cells	Mg. Protein
Porphyric			
65 (grade 1)	32	7.4	893
141 (grade 1)	82	16.4	--
142 (grade 1)	66	17.8	--
713 (grade 2)	139	20.8	1762
743 (grade 2)	173	17.3	1561
342 (grade 2)	80	20.8	--
744 (grade 2)	34	9.5	940
102 (grade 2)	56	11.2	1347
761 (grade 3)	103	13.4	--
14 (grade 3)	47	10.8	783
763 (grade 3)	45	13.0	1425
213 (grade 3)	24	6.2	917
Nonporphyric			
M-1	19	2.1	--
M-4	23	5.3	--
M-5	28	8.4	--
M-6	74	8.9	--
M-12	45	10.4	--
J-1	32	7.0	601
J-4	22	3.7	649
J-5	15	1.8	--
J-9	49	6.9	945
J-10	15	3.2	576
J-11	39	3.9	--
J-12	56	9.5	1537
J-13	77	13.1	2183
M-7	52	10.0	--

TABLE XVIII

BONE MARROW ALA DEHYDRASE ACTIVITY

CAT	pmoles PBG/hour	
	10^6 Erythroid Cells	10^6 Nucleated Cells
Porphyric		
141 (grade 1)	1723	344.6
142 (grade 1)	1273	343.7
342 (grade 2)	1260	327.6
743 (grade 2)	4414	441.4
744 (grade 2)	1317	368.8
14 (grade 3)	1098	252.5
761 (grade 3)	1886	245.2
Nonanemic Nonporphyric		
M-3	969	96.9
M-6	1381	165.7
M-7	1544	308.8
M-10	918	128.5
M-11	790	213.3
M-12	882	202.9
M-15	1094	251.6
Anemic Nonporphyric Cats		
M-2	740	103.6
M-8	2682	536.4
M-13	1136	431.6

TABLE XIX

BONE MARROW MITOCHONDRIA FERROCHELATASE ACTIVITY

CAT	pmoles Heme/10 minutes	
	10^6 Erythroid Cells	10^6 Nucleated Cells
Porphyric		
141 (grade 1)	37.2	7.4
142 (grade 1)	30.8	8.3
342 (grade 2)	24.2	6.3
743 (grade 2)	93.1	9.3
744 (grade 2)	19.1	5.3
14 (grade 3)	23.5	5.4
761 (grade 3)	41.5	5.4
Nonanemic Nonporphyric		
M-1	8.4	0.9
M-4	31.7	7.3
M-5	32.5	9.8
M-12	18.4	4.2
M-11	32.0	8.6
M-10	13.3	1.9
M-6	43.3	5.2
M-3	70.6	7.1
Anemic Nonporphyric Cats		
M-2	22.3	3.1
M-8	52.3	10.4

TABLE XX

BONE MARROW HEMOLYSATE FERROCHELATASE ACTIVITY

CAT	pmoles Heme/10 minutes	
	10^6 Erythroid Cells	10^6 Nucleated Cells
Porphyric		
65 (grade 1)	72.6	16.7
713 (grade 2)	144.0	21.6
102 (grade 2)	44.9	9.0
213 (grade 3)	16.5	4.3
763 (grade 3)	11.5	3.3
Nonanemic Nonporphyric		
J-1	53.4	11.7
J-3	13.4	1.5
J-4	28.6	4.7
J-5	20.6	2.5
J-8	40.7	11.8
J-9	133.2	17.3
J-10	31.4	6.6
J-11	117.2	11.7
J-12	53.6	9.1
J-13	164.6	28.0
Anemic Nonporphyric		
K-1	74.3	20.1
K-2	59.0	23.0
K-3	65.8	19.1

VITA

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