Erythropoietic Porphyria of the Fox Squirrel *Sciurus niger*

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ABSTRACT Uroporphyrin I is found in high concentration in the bones, teeth, blood, soft tissues, and urine of the fox squirrel, *Sciurus niger*. The concentration of uroporphyrin in fox squirrel spleen is much higher than in liver, kidney or bone marrow, probably because of accumulation from phagocytosed red cells. Bleeding causes a marked increase in the uroporphyrin concentration of red cells and spleen, and a 3–8-fold increase in uroporphyrin excretion. Urinary excretion of δ-aminolevulinic acid and porphobilinogen is not greater in fox squirrels than in nonporphyric gray squirrels, *Sciurus carolinensis*, used as controls. In all these characteristics, as well as in the previously demonstrated deficiency of the enzyme uroporphyrinogen III cosynthetase in red cells, the physiological porphyria of fox squirrels resembles congenital erythropoietic porphyria, a hereditary disease of man and cattle. Fox squirrels differ in showing no evidence of cutaneous photosensitivity or hemolytic anemia.

Uroporphyrinogen III cosynthetase activity is present in fox squirrel bone marrow at $\frac{1}{10}$ its concentration in gray squirrel marrow. The fox squirrel enzyme is much more unstable than the gray squirrel enzyme, which provides a possible explanation for its low activity and for the overproduction of uroporphyrin I. It is unlikely that the deficiency of cosynthetase is due to its inactivation by excessive amounts of uroporphyrinogen I synthetase, because activity of the latter enzyme is the same in blood from fox and gray squirrels.

Fox squirrel porphyria provides a convenient model for studies of pathogenesis of human congenital erythropoietic porphyria.

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INTRODUCTION

The bones of the fox squirrel, *Sciurus niger,* are red. This phenomenon is so characteristic of the species that it is used by conservation officers as a means of identifying field specimens (1). In 1937, Turner showed that the chromophore in fox squirrel bones is uroporphyrin I, and that this animal excretes large amounts of porphyrins in the urine (2). He proposed that the physiological porphyria of the fox squirrel might be a useful animal model for human metabolic disease.

An accumulation of uroporphyrin I in bones and teeth similar to that seen in the fox squirrel also occurs in human and bovine individuals with the hereditary disease congenital erythropoietic porphyria. The overproduction of uroporphyrin I in this disorder is due to a partial deficiency of the enzyme uroporphyrinogen III cosynthetase (3, 4), inherited as an autosomal recessive trait (5). Cosynthetase activity is also low in blood and tissue extracts from the fox squirrel as compared with extracts from the closely related gray squirrel, *Sciurus carolinensis* (6). This result indicates that the production of large amounts of uroporphyrin I by normal fox squirrels is due to the same partial enzymatic block as is its abnormal production by porphyric human beings and cattle.

The present investigation was undertaken to determine how closely the physiological porphyria of *Sciurus niger* mimics the pathologic erythropoietic porphyria of *Homo sapiens,* and the possible utility of sciurine porphyria as an animal model for studies of pathogenesis of the human disease.

METHODS

Sciurus niger is a large squirrel, weighing 1½–2 times as much as *S. carolinensis*. It is widely distributed across the United States, especially in regions of open woodland and along river valleys. The animal commonly occurs in a red color phase (as well as in gray and black), but the proper-

ties of the red fur pigment resemble those of human red hair pigment, and are not those of a porphyrin or metalloporphyrin.[1] Bones dissected from freshly killed fox squirrels are a bright reddish-pink color, which turns brown if the bones are boiled, or fades on exposure to light. When illuminated by a Wood's lamp, the bones of the trunk and extremities, and the entire surface of the skull, fluoresce a brillant crimson, although in very old animals the skull fluorescence may be concentrated near the sutures. The incisors, the premolars, and the third molar fluoresce brilliantly; the first and second molars are usually much less fluorescent.

Fox squirrels for these studies (450–1000 g) were live-trapped on the Delmarva peninsula or in the mountains of Western Maryland, or they were purchased from Starling Flying Squirrel Ranch (Palestine, Tex.). Gray squirrels (400–700 g) were trapped in the vicinity of Washington, D. C. Two California gray squirrels, *Sciurus griseus* (760 g), were a gift of the California Department of Fish and Game. Squirrels were housed individually or in pairs, in cages $4 \times 6 \times 12$ feet, containing wooden nesting boxes $18 \times 10 \times 10$ inches. Feed consisted of peanuts, corn, sunflower seeds, and apples. For periods of urine collection, the animals were kept in screen-bottomed rabbit metabolism cages $1 \times 1.5 \times 1.5$ feet, underlaid by a sloping pan. In the rear of each cage was an $8 \times 6 \times 6$ inch nesting box. The animals would leave the nesting box to urinate in the front of the cage, where the urine passed through a wire screen which removed feces and food debris, and trickled into a light-shielded reservoir, to be collected under oil.

When urine samples were to be analyzed for δ-aminolevulinic acid, the reservoirs contained 0.5 cm^3 of 1 N HCl; for porphobilinogen, they contained 1 cm^3 of 0.05 M potassium phosphate buffer, pH 7.9, or no additive at all; and for porphyrins, no additive. Urine samples were stored in the dark at 2–4°C for several days before being analyzed for δ-aminolevulinic acid, and in the dark at −20° for porphobilinogen or porphyrins. The urine was filtered and the pH was measured before analysis. No alkaline samples were analyzed for δ-aminolevulinic acid, nor any acid samples for porphobilinogen or porphyrins. Urinary δ-aminolevulinic acid and aminoacetone were determined by a three-column technique, modified[2] from the method of Urata and Granick (7). For chromatographic standards, δ-aminolevulinic acid was purchased from ICN Nutritional Biochemicals Div. (Cleveland, Ohio) and aminoacetone was prepared as described in Organic Syntheses (8). Porphobilinogen in urine was determined by the method of Mauzerall and Granick (9), and urinary porphyrins and porphyrinogens according to Fernandez, Henry, and Goldenberg (10). Results are expressed as nanomoles of porphyrin per milligram creatinine, in order to minimize differences in daily excretions caused by variations in the times of voiding. Individual gray squirrels usually excreted about 10 mg of creatinine per day, and fox squirrels about 20 mg. The values listed for porphyrins, porphobilinogen, and δ-aminolevulinic acid in urine, are usually averages for two to five 24 h samples from individual animals, except where sequential daily values are given.

For blood drawing, animals were anesthetized with methoxyflurane, and heparinized blood was obtained by cardiac puncture. The cells were collected by centrifugation and washed with saline. Porphyrins in these cells were extracted with acetone-HCl and diluted into ethyl acetate (11). The uroporphyrin was extracted from this mixture with 1 M acetate buffer, pH 4.6 (pH of the extract 4.6–5.2), the coproporphyrin with 0.1 M HCl (pH of the extract 0.5–1.0), and the protoporphyrin with 3 N HCl (11, 12). The pH of each uro- and coproporphyrin extract was measured; if necessary, the pH was adjusted and the extract was reequilibrated with the organic layer. The porphyrin extracts were purified (11), and analyzed spectrophotometrically (13). For the determination of porphyrins in tisues, aqueous extracts were prepared in a hand homogenizer or a Waring blender. These extracts were diluted to 5% (wt/vol), extracted with ethyl acetate-acetic acid (12), and then fractionated by a method similar to that used for blood. Porphyrin concentrations in blood were expressed per gram of hemoglobin, since the latter could be determined directly on the hemolysate analyzed, rather than per 100 ml of packed cells, as is more commonly done. Concentrations in tissue were expressed as nanomoles per milligram protein, as determined by the biuret method (14).

Uroporphyrinogen III cosynthetase activity in blood, tissue, and bone marrow extracts was assayed by measuring the isomer composition of the uroporphyrinogen formed from porphobilinogen in the presence of partially purified uroporphyrinogen I synthetase (3, 4). When the amount of cosynthetase added is appropriately limiting, the product is a mixture of uroporphyrinogens I and III, and the percentage of III is proportional to the amount of cosynthetase added (15). 1 U of cosynthetase is defined as the amount that produces 50% isomer III, in the presence of enough synthetase to catalyze the formation of 5 nmol of uroporphyrinogen in 30 min at 31° (4). Uroporphyrinogen I synthetase activity was assayed by the disappearance of porphobilinogen (16). 1 U of synthetase is defined as the amount which catalyzes the disappearance of 1 nmole of porphobilinogen per hour at the temperature of the assay.

The isomer composition of isolated uroporphyrin was determined by decarboxylating it (17) and separating the resulting coproporphyrin isomers by thin-layer chromatography in lutidine-ammonia (4, 18). The areas for isomers I and III were eluted with 0.7 M NH_4OH, diluted into 1 N HCl, and analyzed for coproporphyrin spectrophotometrically (13) or fluorometrically (19).

All other materials and methods, including the preparation of porphobilinogen and the porphyrin standards, were those previously described or cited (6, 20).

RESULTS

Nature of the porphyrin accumulated and excreted by fox squirrels. The chromophore from the bones and urine of fox squirrels was initially identified as uroporphyrin I on the basis of its absorption spectrum and melting point (2). In addition, the material behaves like uroporphyrin on chromatography in lutidine-ammonia (18). After esterification, it has the chromatographic properties of uroporphyrin methyl ester in dioxane-kerosene (21). Chromatography (18) after decarboxylation to coproporphyrin (17) showed that it consists of more than 90% isomer I.

In a similar way, porphyrin isolated from the red cells and spleen of several animals has been shown to be predominantly uroporphyrin (see below), of which 97–100% was isomer I. Urinary porphyrin isolated from six animals was about half uroporphyrin and half coproporphyrin, both ranging from 92 to 99% isomer I.

[1] Levin, E. Y. Unpublished data.

[2] Chisolm, J. J. Personal communication.

TABLE I

Hematologic Values in Squirrels (Mean ±SD). The Figures in Parentheses Indicate the Number of Animals Tested

	Gray squirrels	Fox squirrels
Hemoglobin (*g/100 ml*)	12.7±2.3 (30)	12.5±1.7 (15)
Hematocrit (%)	42.6±4.7 (15)	42.1±5.7 (10)
Mean corpuscular hemoglobin concentration (*g/100 ml*)	29.7±3.2 (15)	28.6±1.9 (10)
Reticulocytes (%)	2.7±1.3 (30)	2.3±1.9 (15)
White blood cell count (*cells/mm³*)	3,450±1,872 (15)	4,020±1,916 (10)

TABLE II

Excretion of Porphyrins and Porphyrinogens in the Urine

	Gray squirrels	Fox squirrels
	nmol/mg creatine	
Number of animals	8	9
Copro		
Range	0.14–0.45	1.8–7.7
Mean	0.25	3.7
Uro		
Range	0.02–0.11	1.11–6.1
Mean	0.07	3.6

Hematologic data on fox and gray squirrels. There is no evidence that fox squirrels commonly suffer from a hemolytic anemia like that seen in human and bovine subjects with congenital erythropoietic porphyria. Table I demonstrates that the usual hematologic indices in fox squirrels are similar to those in gray squirrels, and that the reticulocyte counts do not differ significantly. Moreover, measurement of the mean overall red cell life-span by the ^{14}CO method (22) in one fox and one gray squirrel gave values of 66.4 and 56.4 days, respectively.[3] The Wright's-stained smears of peripheral blood from both species were unremarkable. The relatively low white counts reported in Table I have been found previously to be characteristic of wild squirrels (23).

Excretion of porphyrins and porphyrinogens in urine. Healthy wild-trapped fox squirrels, which have not previously been used in any laboratory experiment, excrete urine which is usually clear and yellow. If the urine is treated with iodine, or acidified and allowed to stand for a few hours in the light, it develops a Burgundy-red color, indicating that large amounts of porphyrinogens are present. Results of analyses of total porphyrins and porphyrinogens in the urine of nine fox squirrels and eight gray squirrels are given in Table II. The average values for the excretion of copro- and uroporphyrins by the fox squirrels were, respectively, 15 and 50 times as high as the average for gray squirrels. Assuming that *Sciurus carolinensis* is a valid control animal for *S. niger*, the results show that the fox squirrel normally produces large amounts of a porphyrin which is abnormal for man and domestic animals.

Excretion of δ-aminolevulinic acid and porphobilinogen in the urine. Despite this unusually high excretion of uro- and coproporphyrin by fox squirrels, the amounts of the porphyrin precursors δ-aminolevulinic acid and porphobilinogen in the urine are not greater than the amounts in the urine of gray squirrels (Table III). In this respect, sciurine porphyria resembles human and bovine erythropoietic porphyria, and differs from human acute hepatic porphyria and feline erythropoietic porphyria (24). Table III also shows the unusually high excretion of aminoacetone, which is greater than that of δ-aminolevulinic acid in both species. The reason for this is unknown.

Porphyrin content of red blood cells. Some of the circulating red cells of fox squirrels, and nucleated red cell precursors in the bone marrow, exhibit brilliant fluorescence when illuminated with long-wave ultraviolet light (2). Analysis of the porphyrin content of red cells (Table IV) indicated that this fluorescence is due to the presence of large amounts of uroporphyrin, similar to those which are found in the red cells of patients with congenital erythropoietic porphyria (25).

TABLE III

Excretion of δ-Aminolevulinic Acid, Aminoacetone, and Porphobilinogen in the Urine (Mean ±SD). The Figures in Parentheses Indicate the Number of Animals Tested

	Gray squirrels	Fox squirrels
	nmol/mg creatine	
δ-Aminolevulinic acid	27.5±16.5 (6)	25.6±9.3 (5)
Aminoacetone	47.4±28.3 (7)	43.2±17.9 (5)
Porphobilinogen	4.3±2.5 (5)	4.0±2.6 (5)

TABLE IV

Porphyrin Content of Red Cells

	Gray squirrels	Fox squirrels
	nmol/g Hb	
Number of animals	9	6
Protoporphyrin	0.19–1.47	0.31–0.62
Coproporphyrin	0–0.022	0–0.016
Uroporphyrin	0–0.03	0.26–26.6

[3] Kindly performed by Dr. S. Landaw, Donner Laboratory, University of California, Berkeley.

The uroporphyrin content of the red cells in six fox squirrels ranged from 9 to 900 times as high as the highest amount seen in nine gray squirrels, and was as high or higher than the amount of protoporphyrin present. The amounts of coproporphyrin and protoporphyrin in the red cells were not significantly different in the two species.

Porphyrin content of tissues. The uro-, copro-, and protoporphyrin content of the liver, spleen, and kidney from four fox squirrels is shown in Table V. Although the amount of uroporphyrin in liver and kidney was very high, its concentration was in the same range as that of protoporphyrin, which suggests that much of this porphyrin may have been due to blood trapped in those organs. In the spleen, on the other hand, the concentration of uroporphyrin was far greater than that of protoporphyrin, and was 50–500 times as high as the concentration of uroporphyrin in other fox squirrel tissues. The four animals used for this study had previously been utilized in other experiments, and had been bled at least once. In four additional animals previously bled once or twice, the uroporphyrin concentration in the spleen ranged from 174 to 490 nmol/g protein, while the concentration in spleens from five animals which had not been bled previously was only 16–56 nmol/g protein. However, even the lowest of these values on spleen from an unbled animal is twice the highest value found in any other tissue of a bled animal (Table V).

In four gray squirrels, uro- and coproporphyrin concentrations were not measureable by the methods used in liver, spleen, and kidney, except for two spleens in which the uroporphyrin concentration was about 2 nmol/g protein. The protoporphyrin in extracts from tissues of these gray squirrels ranged from 1 to 4 nmol/g protein.

It is difficult to obtain enough bone marrow from squirrels to carry out accurate porphyrin analyses, and interpretation of the results is complicated by the presence of varying amounts of fatty tissue and varying levels of erythropoiesis. However, the uroporphyrin content of bone marrow from six fox squirrels ranged from 0.67 to 2.7 nmol/g protein, while it was not measureable in a pooled sample from the marrow of six gray squirrels.

TABLE V

Porphyrin Content of Fox Squirrel Tissues

Animal	Liver			Spleen			Kidney		
	Proto	Copro	Uro	Proto	Copro	Uro	Proto	Copro	Uro
	nmol/g protein			*nmol/g protein*			*nmol/g protein*		
1	10.6	1.6	6.4	2.7	7.4	294	7.1	<0.8	4.5
2	<0.8	<0.8	1.7	3.7	13.8	821	2.6	<0.8	8.7
3	3.6	<0.8	4.2	5.9	1.4	250	3.2	<0.8	5.9
4	1.0	<0.8	1.3	—	1.0	490	<0.8	<0.8	2.0

Effect of bleeding on urinary porphyrin excretion. The effect of the erythropoietic stimulus provided by bleeding on the urinary excretion of porphyrins has been measured in five fox squirrels. These animals, which were excreting 2–5 nmol of uro- and coproporphyrin per milligram of creatinine, were acutely bled of 15–30% of their blood volume (assumed to be 8% of the body weight). By the 5th to the 12th day after phlebotomy, the freshly voided urine had become pink or red. Excretion of coproporphyrin increased from two- to sixfold, and uroporphyrin by three- to eight fold. A representative experiment is shown in Fig. 1*A*, which demonstrates the usual time course of this response, and the typically greater effect of the bleeding on the uroporphyrin excretion than on the coproporphyrin excretion. Fig. 1*B* shows a similar experiment, in which a fox squirrel was bled twice in a 1 wk period, with removal of a total of 40% of the calculated blood volume. Porphyrin excretion initially rose, but by the end of a 3 wk period of recuperation, it had returned to prebleeding levels; it rose again when bleeding was resumed. These experiments demonstrate that a stimulus to erythropoiesis markedly increases the excretion of uro- and coproporphyrin I in fox squirrels. The possibility that the porphyrin-laden spleen is the source of the urinary uroporphyrin (25) was eliminated by splenectomizing two fox squirrels, and then acutely depleting them of 30% of their blood volumes. In these animals, there was a rapid and marked rise in the porphyrin excretion like that shown in Fig. 1 for nonsplenectomized animals.

Similar experiments designed to measure the effect of bleeding on urinary porphyrin excretion were carried out on five normal gray squirrels. As with the fox squirrels, sudden removal of 28–31% of the calculated blood volume led to an increase in uro- and coproporphyrin excretion over the next 13 days, but the maximal excretions were one to two orders of magnitude lower than the maxima in fox squirrels. In four of the five animals, uroporphyrin excretion remained below 0.1 nmol/mg creatinine, and coproporphyrin excretion less than 0.9 nmol/mg creatinine. The fifth animal developed maximal excretions on the 8th day after bleeding of 0.9 and 2.4 nmol/mg creatinine for uro- and coproporphyrin, respectively; the values in this last animal were as high as those seen in some unbled fox squirrels.

Effect of bleeding on red cell porphyrin content. The reticulocytosis which occurs in response to bleeding is associated in fox squirrels not only with an increase in urinary porphyrin excretion but also with a large increase in the red cell porphyrin content. After removal of 17–27% of the blood volume, the amount of uroporphyrin in the red cells of fox squirrels rose 2–25-fold, reaching levels from 10–300 times as high as those seen in gray squirrels treated in a similar way (Table VI).

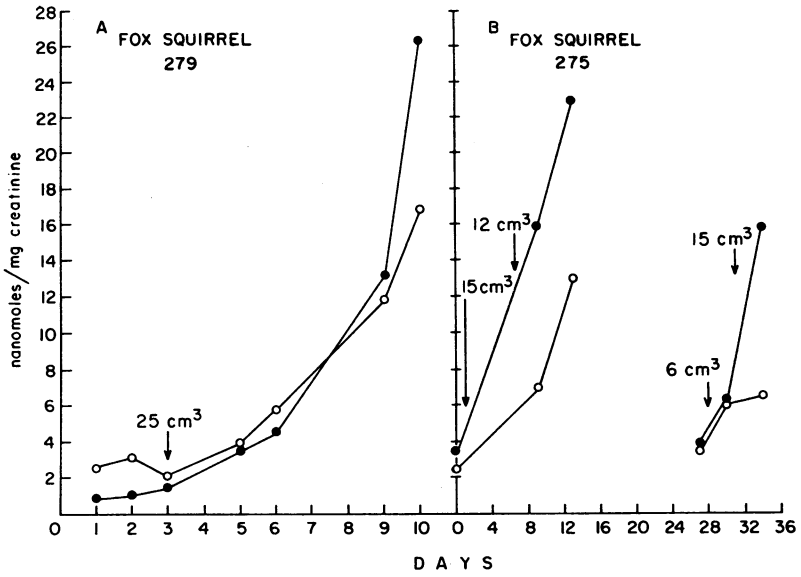

FIGURE 1 Typical experiments illustrating the effect of bleeding on the porphyrin excretion of fox squirrels. Blood was removed at the times indicated by the arrows. Open circles, coproporphyrin in the urine; solid circles, uroporphyrin in the urine.

Much smaller increases (not shown) also occurred in the coproporphyrin and protoporphyrin content of red cells in both species.

Activity of uroporphyrinogen III cosynthetase in blood. The excretion of uroporphyrin I by fox squirrels has been explained by a very low activity of the enzyme uroporphyrinogen III cosynthetase in hematopoietic tissue (6). Activity of this enzyme has now been measured in hemolysates from a total of 18 gray squirrels and 15 fox squirrels, including those 21 animals for which the data was previously reported (6). Cosynthetase activity in the gray squirrels ranged from 3,660 to 13,250 U/g hemoglobin, with a mean of 8913±2647 (SD). The activity in hemolysates from two California gray squirrels was higher, 21,000 and 25,000 U/g hemoglobin. In contrast, cosynthetase activity in the hemolysates from the 13 fox squirrels was not measurable in the amounts of hemolysate which could practicably be added to the assay mixture. The data indicated that cosynthetase activity was present in the 13 fox squirrel hemolysates at concentrations of less than 400 U/g hemoglobin, or less than 6% of the mean in the blood of gray squirrels. This

TABLE VI
Effect of Bleeding on Red Cell Uroporphyrin Content

		Before bleeding			7th day after bleeding		
Animal	Bleeding	Hb	Reticulocytes	Uroporphyrin	Hb	Reticulocytes	Uroporphyrin
	% of blood vol	*g/100 ml*	*%*	*nmol/g Hb*	*%*	*%*	*nmol/g Hb*
Gray squirrels							
1	27	11.1	3.5	0.11	9.7	11.3	0.09
2	27	12.2	1.5	0	10.7	6.0	0.07
3	27	12.6	1.0	0	10.7	8.0	0.04
4	32	12.4	2.8	0.03	11.0	12.6	0.11
Fox squirrels							
1	27	11.3	3.3	0.5	9.1	11.3	1.1
2	18	11.3	2.1	0.3	9.1	8.3	3.8
3	24	12.1	1.6	1.3	8.2	6.6	36.0
4	17	13.5	1.8	1.0	9.4	6.5	24.4

difference in activity between normal and porphyric animals is greater than the difference between normal human or bovine subjects and those with congenital erythropoietic porphyria (4, 5). Control experiments demonstrated that hemolysates from fox squirrels do not inhibit the activity of hemolysates from gray squirrels, or the cosynthetase activity of mouse spleen extracts (15).

Previous work in cattle and in man had shown that activity of uroporphyrinogen III cosynthetase is much higher in young red cells than in old ones, and that an increase in the reticulocyte count is associated with an increase in the activity of the enzyme (5). Fig. 2 demonstrates that there is a similar relationship between the cosynthetase activity and the reticulocyte count in gray squirrels. The correlation coefficient between these two variables for the previously unbled animals shown in this table was 0.69.

Effect of bleeding on the activity of uroporphyrinogen III cosynthetase. These data suggested that it might be possible to demonstrate cosynthetase activity in fox squirrel blood by increasing the proportion of immature red cells in the circulation. For this experiment, five fox squirrels and six gray squirrels were acutely depleted of 18–40% of the blood volume in order to raise the reticulocyte count, and the effect on cosynthetase activity was followed. In the gray squirrels, cosynthetase activity rose concomitantly with the rise in the reticulocyte count; the data have been included in Fig. 2. In the fox squirrel, however, cosynthetase activity was not demonstrable even when the reticulocyte count was raised to 17% by bleeding. In another experiment a gray squirrel and a fox squirrel were each bled of 5–10 cm^3 at 3-day intervals.

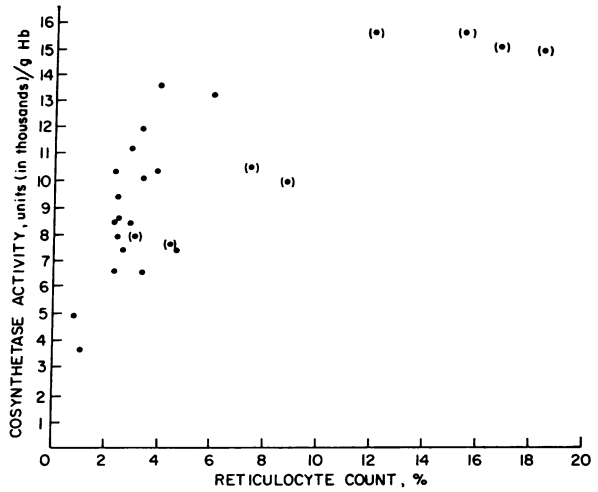

FIGURE 2 Relationship between the reticulocyte count and uroporphyrinogen III cosynthetase activity in the blood of nonporphyric gray squirrels. The points in parentheses (●) indicate animals which had been bled in order to elevate their reticulocyte counts.

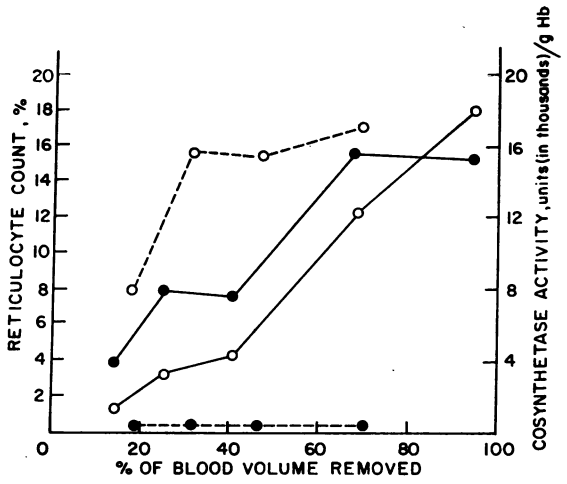

FIGURE 3 Effect of bleeding at 3-day intervals on the reticulocyte count and blood cosynthetase activity. Solid line, gray squirrel; broken line, fox squirrel. Reticulocyte counts are indicated by open circles and cosynthetase activities by solid circles.

As the hemoglobin in the gray squirrel fell from 10.4 to 8.3%, the reticulocyte count rose from about 1% to nearly 18%, and the cosynthetase activity increased in parallel, rising from 4,000 to 15,000 U/g hemoglobin (Fig. 3). The fox squirrel initially had a reticulocyte count of 8%, which rose to 17% while the hemoglobin fell from 10.1 to 9.1 g/100 ml. Despite this reticulocytosis, cosynthetase activity in the blood of this animal remained undetectable at a sensitivity level of 200 U/g hemoglobin.

These data indicate that deficiency of cosynthetase in erythropoietic tissue of the fox squirrel is even more profound, compared with the gray squirrel, than had been suspected on the basis of a comparison of the activities in blood samples with normal reticulocyte counts (6). It suggests that the low activity of cosynthetase in the erythropoietic cell series of the fox squirrel extends back at least to the reticulocyte.

Uroporphyrinogen III cosynthetase activity in bone marrow. Although uroporphyrinogen III cosynthetase activity does not reach measureable levels in reticulocyte-rich blood of fox squirrels, it is possible to demonstrate activity of the enzyme in extracts of erythropoietic tissue. To obtain marrow, anesthetized animals were asphyxiated, and the femura and humeri were rapidly removed, trimmed of muscle and sinew, and frozen in solid CO_2. The bones were split and the marrow either scraped out while still frozen, or flushed out with ice-cold 0.05 M potassium phosphate buffer, pH 7.9. The marrow was homogenized in a convenient volume of the same buffer, and then centrifuged at 15,000 *g* and 4°C for 20 min. These crude extracts (22–55 mg protein/ml) were fil-

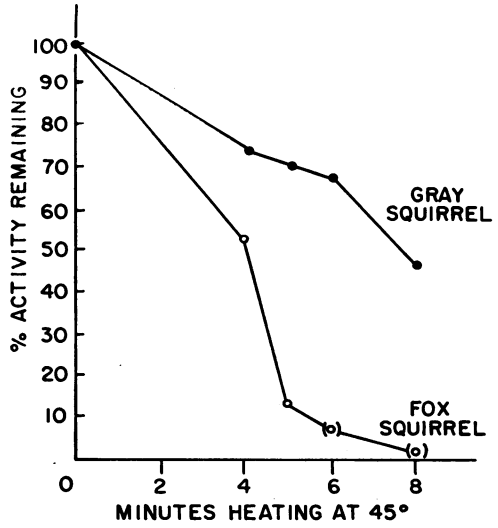

FIGURE 4 Thermolability of bone marrow cosynthetase from fox and gray squirrels. At the points in parentheses (○) no cosynthetase activity was actually detected; the values plotted represent the maximum amount which could have been present, calculated on the basis of the sensitivity of the assay.

tered to remove fat if necessary, and cosynthetase activity was assayed. In most cases, fox squirrels (but not gray squirrels) were bled of 10–15 cm^3 about 5–7 days before sacrifice in order to stimulate marrow erythropoietic activity, in an attempt to obtain higher levels of cosynthetase activity.

Extracts of marrow from three groups of one to three fox squirrels contained easily measureable amounts of uroporphyrinogen III cosynthetase, with a specific activity of 3–5 U/mg protein. In contrast, extracts of marrow pooled from three groups of four or more gray squirrels contained enzyme at a specific activity of 30–35 U/mg protein. The presence of cosynthetase in fox squirrel marrow extracts, although at levels only $\frac{1}{10}$ as high as those in gray squirrels, made it possible to compare some of the properties of the enzyme from the two species.

Instability of cosynthetase from fox squirrel marrow. The presence of a low activity of cosynthetase in fox squirrel bone marrow, and its virtual absence from peripheral blood, might be accounted for by a decreased survival of this enzyme in maturing red cells. To test the possibility that cosynthetase from fox squirrels is more labile than that from gray squirrels, marrow extracts from the two species were heated for various times, cooled, and diluted to an appropriate concentration for cosynthetase assay. For the experiment shown in Fig. 4, the heating was carried out at 45°, at a protein concentration of 5.5 mg/ml. This figure shows the much greater thermolability of the fox squirrel enzyme. After 6 min, when 70% of the gray squirrel enzyme was still intact, the fox squirrel enzyme was no longer measureable under these conditions. Data of this kind are of course not a direct indication of the destruction of the enzyme at physiological temperatures in vivo, but they do suggest that the enzyme from the porphyric squirrel has a much less stable structure than the same enzyme from the control animal. Another possibility is that the fox squirrel lacks an enzyme-stabilizing factor present in gray squirrels.

The rectal temperature of three fox squirrels was found to be about 38°, and experiments like those described above were repeated at this temperature with similar results: for periods of heating from 15 to 45 min, the enzyme from fox squirrel marrow was less stable than the one from gray squirrel marrow. Control experiments demonstrated that the stability of the gray squirrel enzyme was not decreased in the presence of fox squirrel enzyme.

Uroporphyrinogen I synthetase activity in blood. It has been proposed that the basic defect in bovine and human congenital erythropoietic porphyria is an increased activity of the enzyme uroporphyrinogen I synthetase (26). Such an increase could possibly lead to the low cosynthetase activities observed in the disease, because in vitro cosynthetase is inactivated in the presence of synthetase and porphobilinogen (15). Definitive studies of tissue levels of uroporphyrinogen I synthetase in congenital erythropoietic porphyria have not been reported, but it has been possible to measure the activity of this enzyme in fox squirrel blood, and determine whether the low levels of cosynthetase observed are as-

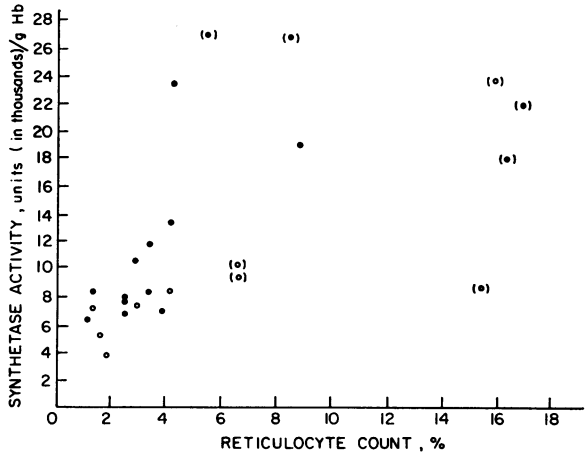

FIGURE 5 The relationship between the reticulocyte count and uroporphyrinogen I synthetase activity in blood. Solid circles, gray squirrels; open circles, fox squirrels. The points in parentheses (●) (○) indicate animals which had been bled in order to elevate their reticulocyte counts.

sociated with elevated levels of uroporphyrinogen I synthetase in this animal model.

A direct comparison of synthetase levels in blood from fox and gray squirrels is complicated by an effect of the age of the cells on the apparent synthetase activity in both species, similar to that seen with cosynthetase in gray squirrels (Fig. 2). The relationship between synthetase activity in hemolysates, and the reticulocyte count, for both fox squirrels and gray squirrels, is shown in Fig. 5. In the range from 1 to 16% reticulocytes, synthetase activity is not higher in fox squirrels than in gray squirrels, and may even be lower. These data do not support the idea that a low activity of uroporphyrinogen III cosynthetase is a secondary result of an elevated activity of uroporphyrinogen I synthetase.

DISCUSSION

Sciurine porphyria resembles congenital erythropoietic porphyria of man and cattle in many ways: staining of the teeth and bones with uroporphyrin I, accumulation of uroporphyrin I in soft tissues, excretion of increased amounts of uroporphyrin I and coproporphyrin I in the urine, fluorescence of erythroblasts and red blood cells, the effect of accelerated erythropoiesis on porphyrin concentration in red cells and urine, and the relative deficiency of uroporphyrinogen III cosynthetase. The concentration of porphyrin in spleen and blood of fox squirrels, and the amounts excreted in the urine, are in the same range as those values found in samples from porphyric human patients. For example, the uroporphyrin concentration in the spleen of a typical patient was 3400 μg/100 g of spleen (25). Assuming 150 mg protein/g of wet weight, this is about 240 nmol/g of protein, which is in the range of values reported for fox squirrels in Table V. The concentration of uroporphyrin in the spleen of the human patient was much higher than in marrow, as it was in the fox squirrels; this is probably a result of accumulation in the spleen of porphyrin released by red cell destruction. The patient's blood uroporphyrin varied from 11 to 440 μg/100 ml of red blood cells, or roughly 0.5–20 nmol/g hemoglobin, while the range in the fox squirrels studied was from 0.3 to 27 nmol/g hemoglobin (Table IV). The patient excreted 1,200–52,000 μg of uroporphyrin per day, or 60–2600 μg/kg; previously unbled squirrels (Table II) excreted an average of 72 μg/day, or 100–150 μg/kg.

In bovine and human congenital porphyria, and in the normal fox squirrel, a stimulus to erythropoiesis leads to an increase in porphyrin excretion (11), because much uroporphyrin adheres to nuclei extruded as the red cell matures (25, 27). As the proportion of young red cells in the circulation rises, the blood concentration of uroporphyrin increases (28, 29), because the half-time for uroporphyrin survival in red cells is very short (30). In fox squirrels, phlebotomy seems also to produce an increase in the concentration of uroporphyrin I in the spleen, presumably as a result of the destruction of rapidly-formed, short-lived red cells. These observations strengthen the conclusion that in sciurine porphyria, as in the human and bovine diseases, the large amount of uroporphyrin I stored and excreted originates in erythropoietic tissue.

The porphyria of fox squirrels, is, of course, a physiological condition, and it differs from the disease state in lacking demonstrable skin lesions and hemolytic anemia. Both of these phenomena are probably a response to sunlight, since improvement occurs when porphyric cattle are maintained indoors (29). Lack of photosensitivity in the fox squirrel does not seem to be due to avoidance of light; the fox squirrel is normally active during daylight hours on open, park-like range, while it is the nonporphyric gray squirrel which frequents the shaded canopy of dense forests. Possibly the fox squirrel is protected against the photodynamic effects of its biochemical peculiarity by long hair, but we have not been able to induce photosensitivity in partially shaved animals, phlebotomized to increase their porphyrinemia. The lack of apparent photosensitivity in this porphyric animal remains to be explained.

As far as is known, all members of the species *S. niger* are porphyric. It seems most likely that the condition is hereditary, although it is conceivable that it results from some dietary or behavioral idiosyncrasy. Tree squirrels probably originated from ancestral rodent stock about 25 million years ago, during the Miocene. A gene for porphyria could have appeared then, or subsequently, in a breeding isolate which later became the fox squirrel line. It could have become general by genetic drift before widespread dispersion of the species. Persistence of the porphyria under present circumstances means either that the porphyria is harmless in fox squirrels, or that it is in some way favorable to them. Proof of the genetic nature of the partial enzyme deficiency will have to wait until squirrels can be bred in the laboratory, and hybrids produced.

The partial deficiency of uroporphyrinogen III cosynthetase which accounts for fox squirrel uroporphyria also accounts for the overproduction of uroporphyrin I in bovine and human congenital erythropoietic porphyria. This enzyme, along with uroporphyrinogen I synthetase, is involved in the formation of the tetrapyrrole ring from the monopyrrole precursor, porphobilinogen. In the presence of both enzymes, the product formed is uroporphyrinogen III, the normal physiological precursor of the heme ring. When cosynthetase is present in inadequate amounts, synthetase catalyzes the formation of uroporphyrinogen I, which can be decarboxylated to coproporphyrinogen I or oxidized to uroporphyrin, but

is not an intermediate in heme biosynthesis. The mechanism of action of cosynthetase is unknown, but it is presumed to act by catalyzing the cyclization of a synthetase-formed linear polypyrrole, which in the absence of cosynthetase can cyclize spontaneously to form uroporphyrinogen I (31). Because cosynthetase is necessary for the formation of an obligatory intermediate in heme biosynthesis, a mutation which leads to a complete absence of this enzyme in erythropoietic tissue results in a nonviable organism. If the loss of enzyme is incomplete, however, the affected individual could possibly overcome the block by increasing the metabolic flux through the pathway, either by increasing the supply of precursor, by increasing the number of erythropoietic cells, or by lengthening the time for maturation of the cells, so that each mature cell ultimately contains a normal amount of the desired end product, heme. However, when the concentration of cosynthetase is low, the utilization of the intermediate formed by synthetase is inefficient, and some uroporphyrinogen I is formed. It is possible that in fox squirrels this low activity is due to the observed increased lability of the enzyme, rather than to any defect in its rate of synthesis or its catalytic activity.

Studies of cosynthetase from the peripheral blood of two human and two bovine porphyric subjects did not reveal any increase in thermolability of the enzyme, relative to normal, comparable to that seen in the fox squirrel.[1] The low activity of cosynthetase in these individuals apparently has some other explanation. It would not be surprising if the precise protein abnormality which results in a cosynthetase deficiency would be different in the human and bovine disease states than in the physiological porphyria of fox squirrels. Nevertheless, since the functional enzyme defect in the heme biosynthetic pathway is the same, the fox squirrel provides an inexpensive, widely available, easily maintained laboratory model for experimental studies of the cosynthetase deficiency of congenital erythropoietic porphyria.

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