Detection of the Heterozygous State in Bovine Porphyria: Analysis of Urinary Coproporphyrin Isomers[1] (34912)

WILLIAM E. MOORE,[2] BETTY D. STEPHENSON, ANNE S. ANDERSON, AND SAMUEL SCHWARTZ[3]

Departments of Veterinary Medicine and Medicine, University of Minnesota, Minneapolis, Minnesota 55455; and The Department of Infectious Disease, Kansas State University, Manhattan, Kansas 66502

Genetic studies of bovine erythropoietic porphyria in South Africa (2), Denmark (3), and the United States (4), have shown it to be inherited as a simple somatic recessive trait. Homozygous animals are recognized clinically by their photocutaneous lesions and pigmented (and red fluorescing) teeth, while chemical confirmation is based on increased porphyrin content of their blood and urine (1–5). Heterozygous animals on the other hand, have seemed entirely normal except where intended or accidental breeding trials have shown them to be heterozygous carriers of the disease. Wass and Hoyt (4) have noted the discovery of three such bulls only after their use in artificial insemination programs, where one was thought to have sired more than 100,000 calves. Despite the large numbers of such offspring and their potential economic liabilities, their study has been seriously handicapped by the presence of very few (apparently less than 1 doz) animals known to be heterozygous for this condition in the United States.

Because excessive amounts of type I isomer of uro- and coproporphyrin are excreted in this disease, preliminary studies were made (1) of the proportion of types I and III isomers in urines from 2 heterozygous animals. The relative amounts of type I coproporphyrin found were intermediate between those of 2 normal and several porphyric animals. The present studies were undertaken to extend these preliminary results and to improve the purification procedures employed.

Materials and Methods. Urines were obtained from 7 normal, 4 porphyric, and 3 heterozygous animals. All were Holstein-Friesian cattle from herds at this University. Blood samples were obtained from the same herds. We are indebted to J. J. Kaneko and to B. L. Glenn for additional urines sent from 3 of their heterozygous animals.

Most urines were single samples obtained at 7–10 a.m. Two of 3 samples from heterozygote M-38 were obtained within 1 hr after intravenous injection of a diuretic.[4] All samples were kept refrigerated or frozen until analyzed.

Urinary porphyrins were fractionated for quantitative fluorimetric analysis in HCl as described elsewhere (6). Except for the above-noted preliminary studies (1), these 1.5 *N* HCl extracts of coproporphyrin were reextracted into ethyl acetate after neutralizing with sodium acetate, washed with H_2O, and then reextracted into 0.3 *N* HCl. They were then alkalinized weakly with cold 3 *N* NaOH, frozen, and lyophilized to dryness. (Vacuum distillation to dryness from ethyl acetate; acetic acid solutions, as done previously,

[1] Supported by Research Grant No. AM 02096 from the National Institute of Arthritis and Metabolic Disease, U. S. Public Health Service. Taken in part from a dissertation (1) presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Medicine, University of Minnesota. (Approved as Scientific Journal Series Paper No. 7190, Minnesota Agricultural Experiment Station).

[2] Present address: Department of Infectious Diseases, Burt Hall, Kansas State University, Manhattan, Kansas 66502.

[3] Research Career Awardee, U. S. Public Health Service.

[4] Lasix (furosemide), Hoechst Pharmaceutical Co.

may result in appreciable loss of porphyrin). Following esterification overnight with methanol:H_2SO_4 (20:1), the porphyrin was extracted into $CHCl_3$, washed 3 times each with 0.2% NaOH, H_2O, 10% NH_4OH, and 7% NaCl, filtered, and vacuum distilled to dryness. Further purification was achieved by thin-layer chromatography on silica gel (7), using a 60:60:2.5 mixture (v/v) of $CHCl_3$, petroleum ether (bp 30–60°), and methanol for development. The fluorescing coproporphyrin zone was identified by its correspondence with spots of standard coproporphyrin applied to the same plate. It was eluted from the gel with $CHCl_3$:methanol (10:1), dried, hydrolyzed overnight in a few ml of 7.5 *N* HCl, and then lyophillized to dryness. Approximately 0.5 to 1.5 μg were dissolved in a drop of 10 to 28% NH_4OH containing 0.0007 *M* tetrasodium ethylenediaminetetraacetate (Na_4EDTA) and applied to Whatman no. 1 paper (20 cm high). Isomer separation followed the methods of Eriksen (8) and Aziz *et al.* (9), modified as follows: (a) Papers (except for approximately the top 1 in.) were presoaked in 0.0007 *M* NaEDTA and dried. (b) The solvent system contained 2,6-lutidine:H_2O (5:2), with 0.0007 *M* Na_4 EDTA in the H_2O. A beaker containing concentrated NH_4OH was included in the chromatography jar. (3) Chromatography was continued for at least 24 hr, or almost twice as long as required for the liquid front to reach the top of the paper. (Because the optimum ratio of lutidine:H_2O and duration of chromatography vary somewhat with different samples of lutidine, these factors are pretested with suitable standards). Fluorescing spots were cut out, soaked briefly in 3 ml of 28% NH_4OH containing 1 drop of 0.05 *M* Na_4 EDTA, and rinsed with several additions of H_2O to a final volume of 15 ml for fluorimetric analysis.

Under the above conditions no overlap was seen in the fluorescence of the 2 spots of coproporphyrin I and III from either urines or standard reference solutions. Absence of bluish-fluorescing spots or of significant streaking indicated that the purity of the material was superior to that employed in our earlier urine studies. In the absence of added EDTA, a dark nonfluorescing purplish spot was frequently seen slightly above the point of application. Addition of concentrated H_2SO_4 (but not HCl) to this spot yielded red-fluorescing porphyrin, suggesting that the EDTA prevents formation of a copper complex of the porphyrin.

Purification of the erythrocyte coproporphyrin did not include thin-layer chromatography of the methyl esters, but was limited to various solvent extractions described earlier (1).

Results. Results of the individual urine studies, reported as percentage of the total urinary coproporphyrin which was type I isomer, are given in Table I. The average of 65.8% distribution of this isomer found in the 6 heterozygous animals was intermediate between the 49.6 and 89.5% found in the 7 normals and 4 porphyrics, respectively. Though these differences may be relatively small, no overlap was found among the average values of individual animals in the 3 groups. There was, however, overlap with the normal values in one of the 3 samples analyzed from heterozygote M-38; this sample, obtained within 1 hr after intravenous injection of the diuretic, Lasix, showed only 40% type I isomer. Another sample from this steer obtained following injection of this diuretic showed 73% type I. Analyses of 2 or 3 different urines from normal animals M-15 and M-21 showed good agreement, as shown in footnote *b* to Table I.

A known mixture of the 2 isomers, containing 30% type I, was prepared from crystalline material. Two separate samples were carried through the extraction procedure, including thin-layer chromatography of the methyl esters. Isomer analyses of these hydrolyzed samples then showed 28 and 31% type I, respectively. (Direct chromatography of known mixtures regularly show recovery errors of less than 4%).

Concentrations of total coproporphyrin and uroporphyrin (μg/100 ml) have not differed significantly in the individual urine samples of the normal and heterozygote animals tested, though 24-hr collections have not been made.

Red cell studies showed no significant dif-

TABLE I. Distribution of Urinary Coproporphyrin Isomers (I and III) in Cattle.[a]

Fraction of Coproporphyrin as Isomer I.

Normals		Heterozygotics		Porphyrics	
No.	I (%)	No.	I (%)	No.	I (%)
M-256	50	K-1140	62	M- 84	80
M- 9	52	G- 803	67	M-102	96
M- 24	37	G- 848	64	M- 18	85
M- 21	46[b]	M- 38	67[b]	M- 95	97
M- 15	50[b]	M-2121	66		
M-364	57	M-2800	69		
M-400	55				
Av	49.6		65.8		89.5
SD	6.6		2.5		8.3

[a] "M," Univ. of Minn. herd; "K," supplied by J. J. Kaneko (Univ. of California, Davis, Cal.); "G," supplied by B. L. Glenn (Oklahoma State Univ., Stillwater, Oklahoma).

[b] Averages from 2 or 3 different urine samples: M-21, three samples: 45, 51, and 42% I; M-15, two samples: 50 and 49% I; M-38, three samples: 89, 40, and 73% I.

ference between the 4 normal and 5 heterozygous animals tested. The average for the former was 53.6% type I (range 49–67%) compared to 57.8% type I (range 49–75%) for the latter. As seen in the photographs and other details given elsewhere (1), the purity of at least some of the samples is open to question, and indicates the need for further study of this type of material.

Discussion. Few attempts have been made to chemically characterize the heterozygous state of erythropoietic porphyria. In studies limited to only a few human subjects, Heilmeyer (10) indicated that heterozygotes may have increased amounts of total uroporphyrin and of coproporphyrin I in their circulating erythrocytes. As reported elsewhere (1), the analytical method recommended by him (minus electrophoresis) yielded spectrophotometric values from uroporphyrin in our cattle which were nonspecific and meaningless. We could also find no significant differences in the distribution of coproporphyrin isomers in circulating red cells of heterozygous and normal animals, as noted herein.

Red cells or their hemolysates incubated with porphyrin precursors such as delta-aminolevulinic acid (ALA) or porphobilinogen have also been studied (1, 11). While no consistent differences were found, solution of several technical difficulties might make this approach more informative. Of special interest is the report by Levin (12) that red cell hemolysates of mature porphyric cattle have less uroporphyrinogen III cosynthetase activity than do those of normals. Since this enzyme (as well as those responsible for decarboxylation of the uroporphyrinogen I to coproporphyrinogen I) are very likely related to the relative increase in urinary coproporphyrin I observed in both homozygous and heterozygous cattle, the application of Levin's finding to heterozygous animals would be of special interest and value.

Kaneko and Mills (13) reported no significant differences in the total porphyrin content (uro-, copro-, and/or protoporphyrin) of red cells, plasma, urine, or feces of 3 heterozygotes (2 mature and 1 young) as compared to normal cattle. Of great interest, however, is their observation of diminished glutathione stability in the red cells of the heterozygous animals, without evident change in glucose-6-phosphate dehydrogenase activity. In addition to the diagnostic implications, this may have significance for the phenomenon of photohemolysis of erythrocytes from porphyric (and possibly heterozygous) animals.

Earlier studies of normal human subjects (9) showed an average of 35 ± 6% type I isomer by paper chromatographic analysis of urinary coproporphyrin in both males and females. Based on a "fluorescence quenching" technique, rabbits (14) were reported to have less than 30% type I, while dogs (14) had approximately equal amounts of the 2 isomers in urinary coproporphyrin. Significant species differences, therefore appear to be present normally. Aziz *et al.* (9), too, reported relatively less coproporphyrin I in normal children (av 19%) than in adults (9). The cattle studied here were all adults.

Breeding programs here and elsewhere are aimed at increasing the heterozygous bovine population available for study. The effect of variable such as erythropoietic activity, di-

uresis, and age can then be evaluated in a larger number of animals.

Summary. The ratios of urinary coproporphyrin isomers were found to differ significantly in normal Holstein-Friesian cattle and in those known to be heterozygous or homozygous for the porphyric trait. The average percentages of type I isomer for these 3 groups respectively, were 49.6 ± 6.6, 65.8 ± 2.5, and 89.5 ± 8.3.

1. Moore, W. E., Ph.D thesis, Univ. Minn., 1968).

2. Fourie, P. J. J., Onderstepoort, J., Vet. Sci. Anim. Ind. 7, 535 (1936).

3. Jorgensen, S. K., Brit. Vet. J. **117**, 1 (1961).

4. Wass, W. M., and Hoyt, H. H., Amer. J. Vet. Res. **26**, 654 (1965).

5. Watson, C. J., Perman, V., Spurrell, F. A., Hoyt, H. H., and Schwartz, S., Arch. Intern. Med. **103**, 436 (1959).

6. Schwartz, S., Berg, M. H., Bossenmaier, I., and Dinsmore, H., *in* "Methods of Biochemical Analysis" (D. Glick, ed.), Vol. 8, p. 221. Interscience, New York (1960).

7. Cardinal, R. A., Bossenmaier, I., Petryka, Z. J., Johnson, L., and Watson, C. J., J. Chromatogr. **38**, 100 (1968).

8. Eriksen, L., Scand. J. Clin. Lab. Invest. **10**, 319 (1958).

9. Aziz, M. A., Schwartz, S., and Watson, C. J., J. Lab. Clin. Med. **63**, 585 (1964).

10. Heilmeyer, L. "Disturbances in Heme Synthesis," p. 236. (transl. by M. Steiner) Thomas, Springfield, Ill. (1966).

11. Watson, C. J., Bossenmaier, I., and Cardinal, R., Z. klin. Chem. klin. Biochem. **7**, 119 (1969).

12. Levin, E. Y., Science **161**, 907 (1968).

13. Kaneko, J. J., and Mills, R., Amer. J. Vet. Res. **30**, 1805 (1969).

14. Schwartz, S., and Zagaria, R., *in* "Toxicology of Uranium" (A. Tannenbaum, ed.), McGraw Hill, New York (1951).

Received March 13, 1970. P.S.E.B.M., 1970, Vol. 134.