Beta-globin chain hemoglobin polymorphism and hemoglobin stability in black rhinoceroses (*Diceros bicornis*)

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SUMMARY

To evaluate the syndrome of acute intravascular hemolytic anemia in black rhinoceroses (*Diceros bicornis*), the hemoglobin of this species was evaluated by use of isopropanol- and heat-stability tests and was further characterized by electrophoretic studies. Samples were obtained from 22 apparently healthy captive North American black rhinoceroses, though 3 of the study animals had survived previous hemolytic events, and 3 others were parents of 3 offspring that had suffered hemolysis. The eastern African (*Diceros bicornis michaeli*) and the southern African subspecies (*D b minor*) were represented. Comparative samples were also obtained from 2 white (*Ceratotherium simum*) and 1 Indian (*Rhinoceros unicornis*) rhinoceroses. The hemoglobin of all 3 species appeared stable when tested by use of the heat and isopropanol methods. Thus, an unstable hemoglobin does not appear to be involved in the hemolytic crises of captive black rhinoceroses.

Black rhinoceros hemoglobin had a striking polymorphism. Thirteen of the samples from black rhinoceroses had a single hemoglobin band, based on results of alkaline electrophoresis. Nine had, in addition to this major band, a slow (more cathodic) minor band that comprised about 10% of the total hemoglobin. Further studies indicated that the major band and the slower minor band may contain globin chains analogous to human β- and δ-chains respectively; these bands have been tentatively designated B and C. Phenotypes B and BC are common, in a ratio of 4:3. A genetic mechanism is proposed that assumes β_b and β_c gene loci and that β_c-locus-expressed (β_c^+) and β_c-locus-inhibited (β_c^o) are common alleles for the β_c-locus. The polymorphism of rhinoceros hemoglobins appears to be unrelated to the acute hemolytic anemia that occurs in this species.

Received for publication Jan 13, 1989.

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Supported by the Nixon Griffis Fund for Zoological Research from the New York Zoological Society.

The authors thank Drs. William Boever and Randy Junge, and Mr. Bruce Read, St Louis Zoological Park, St Louis, Mo, Dr. Roger Brannian, Kansas City Zoological Gardens, Kansas City, Mo, Dr. William Bryant, Sedgewick County Zoological and Botanical Gardens, Wichita, Kan, Dr. Richard Cambre, Denver Zoological Gardens, Denver, Colo, Drs. Ben Gonzales and Gary Kuehn, Los Angeles Zoo, Los Angeles, Calif, Drs. Don Janssen, Michael Loomis, and Amy Shima, San Diego Zoo, San Diego, Calif, Dr. Andrea Lenhard, Milwaukee County Zoological Gardens, Milwaukee, Wis, Dr. Albert Lewandowski, Detroit Zoological Park, Royal Oak, Mich, Dr. Rita McManamon, Zoo Atlanta, Atlanta, Ga, Dr. Pat Morris, Knoxville Zoological Park, Knoxville, Tenn, Dr. Robert Wagner, Pittsburgh Zoo, Pittsburgh, Pa, and Dr. Paul Zollman, Mayo Institute Hills, Rochester, Minn, for supplying the samples used in the study, and Ms. Rose Currie, Kathleen Herron, Margaret Karst, Susan Keel, Carolyn McDonald, and Mr. Robert Sandeen, Mayo Clinic, Rochester, Minn, for performing laboratory tests.

Hemolytic anemia is the leading cause of death among captive black rhinoceroses.[1,2] In 1 survey,[1] it was observed that 70% of the affected rhinoceroses died during their initial or subsequent hemolytic episode.[1] Despite efforts to identify an underlying cause for the disease,[1-5] one that accounts for all of the cases has not been identified.

In human beings, unstable hemoglobin variants are known causes of hemolysis that may be mild or severe or chronic or paroxysmal. By analogy, the question has been raised whether hemoglobin instability might underlie the acute hemolytic anemia of black rhinoceroses. In an unpublished study of 2 black rhinoceroses,[a] 1 of whom had survived a hemolytic episode, a marked proclivity to form Heinz bodies spontaneously was described. Although in human erythrocytes, Heinz bodies are composed in part of denatured hemoglobin, the relevance of this finding in rhinoceros blood is uncertain. In the same study, results of alkaline hemoglobin electrophoresis in agarose gel revealed, for both subjects, a single band that migrated markedly anodic to human hemoglobin (Hb) A. In a subsequent study,[6] hemoglobin instability was reported in all specimens obtained from a group of black rhinoceroses and tested by the isopropanol method, and it was asserted that hemoglobin instability contributes to the acute hemolytic anemia observed in this subspecies.[6] The objective of the study reported here was to assess the possible contribution of hemoglobin instability, or of hemoglobin variants to the hemolytic anemia of black rhinoceroses.

Materials and Methods

Blood samples—In response to a blood collection protocol distributed to all rhinoceros-holding institutions in North America, blood samples for this study were collected on an opportunistic basis and preserved, using EDTA. Blood was collected from peripheral (limb or ear) veins in 25 rhinoceroses anesthetized with

[a] Faust R, Frankfurt Zoological Gardens, Frankfurt, Federal Republic of Germany: Personal communication, 1987.

etorphine hydrochloride[b] or carfentanil citrate.[c] Ten rhinoceroses also were administered xylazine hydrochloride[d] (n = 4), acepromazine maleate[e] (n = 4), or metomidine hydrochloride[f] (n = 1), as supplemental tranquilization. An additional rhinoceros (No. 360) had been administered acepromazine, haloperidol,[g] and azaperone[h] because of a hyperexcitable neurologic disturbance. Samples were packed in wet ice and mailed overnight express to the laboratory of one of the authors (VFF). All rhinoceroses were identified with their numbers from the International Studbook for African Rhinoceroses.[7]

The 22 samples received reflected 19 black rhinoceroses of eastern African origin (*Diceros bicornis michaeli*), and 3 rhinoceroses (No. 333, 334, 336) of southern African origin (*Diceros bicornis minor*). Though blood samples were not obtained from rhinoceroses during a hemolytic event, 3 rhinoceroses (No. 162, 163, and 328) constituted a family group at the Denver Zoo (Fig 1), of which each member had had at least 1 previous episode of hemolysis. In this kindred, 2 rhinoceroses (No. 124 and 125) had 3 offspring (including No. 163), and were grandparents of a fourth; each of the progeny had had a hemolytic event.

Hemoglobin evaluation—Hemolysates were prepared and hemoglobin electrophoresis was performed, using cellulose acetate membranes in tris-EDTA-borate buffer, pH 8.6, and an acid citrate agar, pH 6.2.[8-10] All samples were tested for hemoglobin instability in buffered 17% isopropanol and for thermal instability.[11-12] In addition, a few samples were examined, using globin chain electrophoresis in 8*M* urea, at alkaline and acid pH[10,12,13] or isoelectric focusing in polyacrylamide gel.

For comparison and as position markers in the electrophoresis studies, blood samples from human beings with sickle cell trait were used to provide human Hb bands A, S, and A_2. A sample of equine blood was also used to compare rhinoceros hemoglobin(s) with equine hemoglobins.

Results

Test results for hemoglobin instability, using isopropanol- and heat-precipitation methods, were generally interpreted as negative. In 4 samples of black rhinoceros blood, use of the isopropanol test produced faint turbidity, a result considered to be equivocal. In the other 18 samples from this species and in the 3 samples from white and Indian rhinoceroses, the isopropanol test result for hemoglobin stability was unequivocally negative. The heat stability test result was negative in all 25 samples examined (Table 1). None of the rhinoceroses with equivocal isopropanol hemoglobin stability test results was known to have had a hemolytic episode.

Electrophoresis at alkaline pH revealed a striking hemoglobin polymorphism. Samples from 13 black rhinoceroses had a single band that was as far anodic to human Hb A as human Hb A_2 is cathodic to human Hb A. We designated this principal black rhinoceros hemoglobin band as Hb B. Thus, these 13 samples were of hemoglobin phenotype B. Samples from 9 black rhinoceroses also had this major band B, and, in addition, a minor, more cathodal band, which we designated Hb C (Fig 2); thus, these 9 samples were of hemoglobin phenotype BC. The sample from the Indian rhinoceros had a band electrophoretically identical with Hb B, and in addition, a more anodic band, which we designated as Hb A; thus, this sample was of phenotype AB. The 2 samples from white rhinoceroses had a single hemoglobin band that was identical electrophoretically with black rhinoceros Hb C; thus, they were of phenotype C. The phenotypes of rhinoceroses tested are seen in Table 1.

In all samples with the BC pattern, Hb B comprised approximately 90% of total hemoglobin. In the samples from the Indian rhinoceros, with pattern AB, the proportion of A:B was 60:40. On the basis of acid citrate agar gel electrophoresis, all rhinoceros hemoglobins migrated

[b] M99, Lemmon Co, Sellersville, Pa.
[c] Wildnil, Wildlife Laboratories, Fort Collins, Colo.
[d] Rompun, Haver Mobay Corp, Shawnee, Kan.
[e] Acepromazine maleate injection, TechAmerica, Ellwood, Kan.
[f] Dormosedan, Farmus Group, Turku, Finland.
[g] Haldol, McNeil Pharmaceutical Co, Spring House, Pa.
[h] Stresnil, Pitman-Moore Inc, Washington Crossing, NJ.

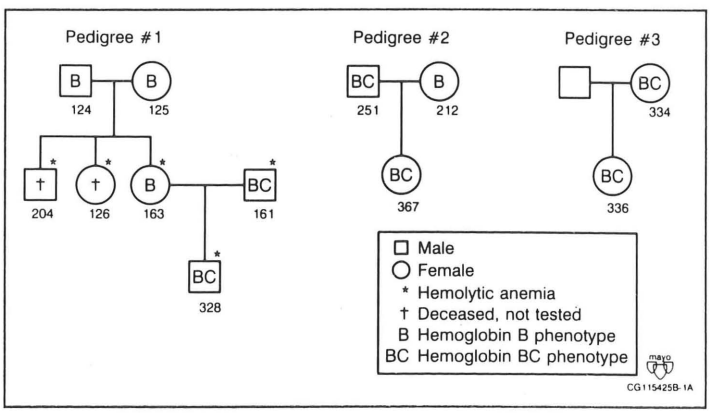

Fig 1—Available pedigree data for black rhinoceroses of this study, indicating autosomal genetic transmission of the B and BC phenotypes. Beneath each square or circle are studbook numbers for comparison with data in Tables 1 and 3. Also indicated (*) are the rhinoceroses that were known to have hemolytic anemia.

TABLE 1—Hemoglobin phenotypes and hemoglobin stability test results

Studbook No.	History of hemolysis	Hemoglobin phenotype	Stability test	
			Isopropanol	Heat
Black rhinoceroses				
360	–	B	Neg	Neg
212	–	B	Tr	Neg
332	–	B	Neg	Neg
281	–	B	Neg	Neg
163	R	B	Neg	Neg
125	–	B	Tr	Neg
188	–	B	Neg	Neg
302	–	B	Neg	Neg
38	–	B	Neg	Neg
34	–	B	Neg	Neg
192	–	B	Tr	Neg
267	–	B	Neg	Neg
124	–	B	Tr	Neg
333*	–	BC	Neg	Neg
334*	–	BC	Neg	Neg
121	–	BC	Neg	Neg
328	R	BC	Neg	Neg
9031	–	BC	Neg	Neg
367	–	BC	Neg	Neg
161	R	BC	Neg	Neg
336*	–	BC	Neg	Neg
251	–	BC	Neg	Neg
White rhinoceroses				
482	–	C	Neg	Neg
452	–	C	Neg	Neg
Indian rhinoceros				
202	–	AB	Neg	Neg

* Southern African subspecies of black rhinoceros (rhino).
– = no hemolysis; R = after recovery from acute hemolytic anemia; Neg = negative result for hemoglobin instability; Tr = faint turbidity.

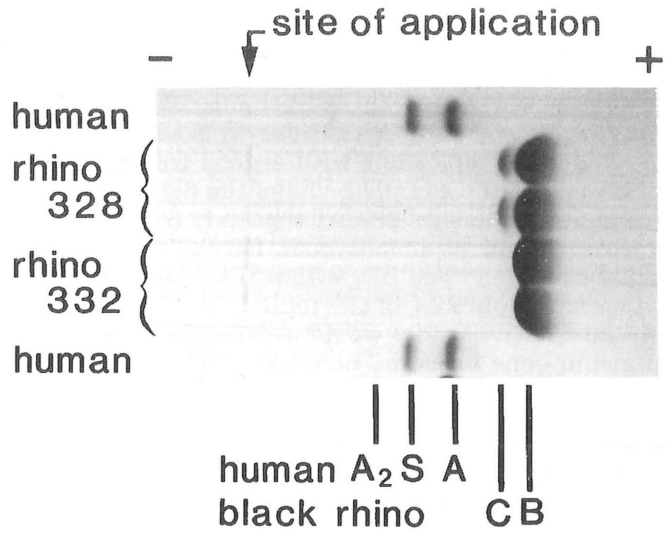

Fig 2—Alkaline electrophoresis results for 2 specimens of black rhinoceros hemoglobin, compared with human hemoglobin. One of the rhinoceros specimens has a single (B) band, and the other has a major (B) band and a minor (C) band.

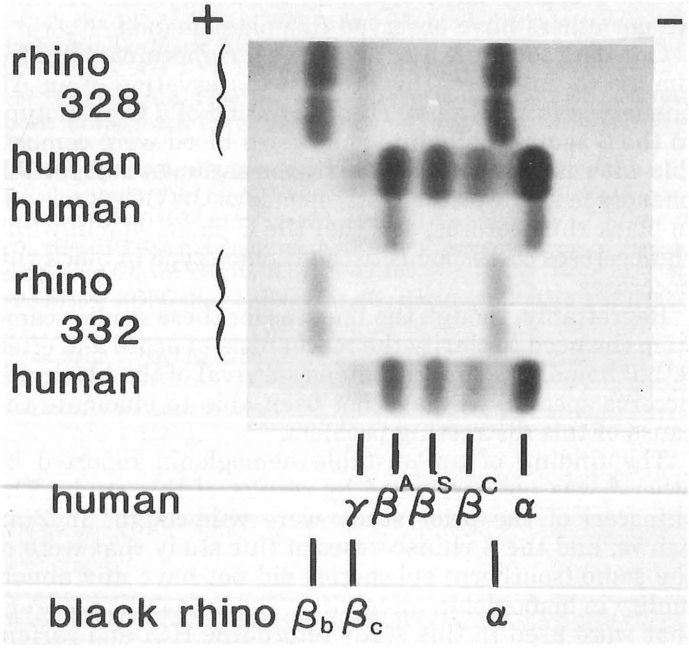

Fig 3—Globin chain electrophoresis in 8*M* urea, pH 9.0, comparing results for the same 2 black rhinoceroses seen in Figure 2, using human globin chains γ, β^A, β^S, β^C, and α. The black rhinoceros of B phenotype has a single β-globin band (β_b), the rhinoceros of BC phenotype has, in addition, a minor β_c band.

in the electrophoretic position of human Hb F. Results of isoelectric focusing were the same as those obtained, using alkaline electrophoresis at pH 8.6. Globin chain electrophoresis in 8*M* urea at pH 9.0 and pH 6.3 revealed major α- and β-chains only in rhinoceroses of phenotype B and, in addition, a minor band slightly cathodic to the major β-globin chain band in rhinoceroses of phenotype BC (Fig 3). By contrast, electrophoresis of equine globin chains in 8*M* urea disclosed a minor band (α_f) slightly anodic to the major α-globin chain band. The relative mobilities in 8*M* urea, of the various globin chains examined are seen in Table 2.

Hematologic data were available for 15 rhinoceroses (Table 3). With 3 exceptions (No. 34, 38, and 163), these data do not appear to differ appreciably from the limited previously published normal values for this species.[6,14,15] There appears to be little difference in hematologic values between rhinoceroses of B and BC phenotypes.

TABLE 2—Relative mobilities of globin chains at pH 8.6 in 8*M* urea*

Species	Chain	Mobility
Human being	β	1.00
Horse	α_s	0.00
	α_f	0.24
	β	1.27
Black rhinoceros	α	0.27
	β_b	1.51
	β_c	1.34
White rhinoceros	α	0.27
	β_c	1.21

* Relative to the distance between human α and β chains, ie, distance between the rhinoceros globin chain band and human α globin chain band ÷ distance between human α and β^A globin chain bands. Relative mobility > 1.0 indicates that the rhinoceros globin chain band is anodic to the human β^A globin chain band.

TABLE 3—Hematologic data available for rhinoceroses tested, grouped according to sex and hemoglobin phenotype

Studbook No.	Sex	Hemoglobin phenotype	Hemoglobin concentration (g/dl)	RBC count ($\times 10^{12}$/L)	MCV (fl)
332	M	B	14.4	4.48	88.4
34*	M	B	1.6	0.63	100.4
281	M	B	16.5	5.43	84.0
124	M	B	14.6	4.51	96.7
188	F	B	16.6	4.83	91.9
38	F	B	9.8	2.90	99.8
212	F	B	9.3	2.90	99.8
360	F	B	17.5	6.19	83.0
192	F	B	15.6	4.71	96.3
267	F	B	16.0	5.60	85.7
125	F	B	14.2	3.94	105.1
163	F	B	12.2	3.31	102.4
Means†	M (n = 3)		15.2	4.81	89.7
	F (n = 6)		15.9	4.99	92.5
9031	M	BC	17.3	7.08	70.0
251	M	BC	16.6	5.02	85.6
328	M	BC	15.7	4.85	93.2
161	M	BC	14.6	4.36	95.6
367	F	BC	18.0	6.39	77.6
336	F	BC	18.7	6.18	79.0
121	F	BC	16.5	4.92	94.0
Means	M (n = 4)		16.0	5.33	86.0
	F (n = 3)		17.8	5.82	83.6

* At time of fatal exsanguinating hemorrhage from nasal granuloma. † Mean values were calculated after excluding data for rhinoceroses 34, 38, and 163, that were clearly outliers. The number of rhinoceroses tested was too small for meaningful statistical analysis.

Discussion

The B/BC hemoglobin polymorphism in black rhinoceroses was consistently observed when samples were reexamined after prolonged storage, or following further tenfold dilution of the samples, or when additional samples were obtained from the same rhinoceroses after lapse of several months. Available pedigree data were consistent with autosomal transmittance of the B and BC phenotypes (Fig 1). Thus, this hemoglobin polymorphism is not likely to be artefactual. Both B and BC phenotypes

were observed in adult rhinoceroses; hence, the minor C band is not analogous to human fetal hemoglobin. Both phenotypes were observed in rhinoceroses that had had acute hemolytic anemia.[i] Thus, the hemoglobin polymorphism appears to be unrelated to the problem of acute hemolytic anemia in black rhinoceroses.

Although most of what we know of hemoglobin polymorphisms is attributable to studies of human hemoglobins, the hemoglobins of many non-primate species have also been studied, and hemoglobin polymorphisms have been recognized in horses and domestic cats, as well as in other species.[6,16-22]

Previous studies of hemoglobin in rhinoceri are limited. Using alkaline electrophoresis, Osterhoff and Keep[16] reported 2 hemoglobin bands in southern African black rhinoceroses and 1 band in white rhinoceroses.[17] Others have also described 2 hemoglobin bands on the basis of results of alkaline electrophoresis of hemoglobin solutions from black rhinoceros blood.[18] Altogether, however, only a small number of samples have been examined previously. Thus, our results may be reconciled with previous observations that may, by chance, have included only rhinoceroses of BC phenotype. It is also possible that members of the southern subspecies of black rhinoceroses that others studied may have a lower frequency of the B phenotype than that which we observed in members of the eastern subspecies that predominated in this study, because all 3 of the southern subspecies of rhinoceroses that we examined were of BC phenotype. Indeed, the B phenotype, that predominates in the eastern subspecies, may prove to be uncommon in the southern African subspecies of black rhinoceroses.

In blood samples from horses, 2 bands of hemoglobin almost always are detected, using alkaline electrophoresis.[19,21] Similar to 9 black rhinoceroses of this study, the hemoglobin bands in horses consist of a major fast band and a minor slow band. Clegg et al[20,21] have reported that this phenomenon in horses is attributable to different alleles of the α-globin genes. However, the variations in hemoglobin patterns of rhinoceroses appear to be the expression of a different mechanism.

We can explain our data by postulating major and minor β-globin gene loci, or a predominant β-like locus and a subordinate β-like locus. In this hypothesis, the major locus determines synthesis of β_b-globin chains and the minor locus determines synthesis of β_c-globin chains. Hemoglobin B contains α-chains and β_b-chains; Hb C contains α-chains and β_c-chains. We further postulate 2 prevalent alleles for the minor β (β_c)-locus. One of these permits expression of the β_c-locus (ie, is β_c^+). The other allele inhibits synthesis of β_c-globin chains (ie, is β_c°). A rhinoceros of B phenotype would be homozygous for the β_c° allele. This would be analogous to the few reported cases of homozygous δ-thalassemia of human beings. Alternatively, the β_c° allele may reflect completion of the evolution of the β_c-locus to a pseudogene.

According to the model proposed here, rhinoceroses of BC phenotype are heterozygous for the β_c^+ and β_c° alleles. This model predicts that rhinoceroses that are homozygous for the β_c^+ allele should be encountered, and they should be of BC phenotype, but with at least 20% Hb C. However, such individuals are likely to be uncommon, and it may only have been by chance that we did not observe any such instances in a survey of this sample size. Our data were consistent with a gene frequency of the postulated β_c° allele of 0.76, and of the β_c^+ allele of 0.24. Such gene frequencies predict that only 6% of black rhinoceroses would be homozygous for the β_c^+ allele and would be of phenotype BC, with a Hb C band that would be approximately 22% of the total.

An alternative model would assume that there is only 1 β-globin gene locus, ie, no δ-like β_c-globin gene locus, and that the BC phenotype reflects heterozygosity for 2 alleles of the β-locus. These 2 alleles would determine synthesis of the β_b- and β_c-globin chains. This alternative model would require the additional assumption that the β_c allele is, like the human β^E allele, responsible for reduced globin chain synthesis, ie, a kind of thalassemia allele. Our data did not exclude this alternative hypothesis. However, this model would predict that 6% of black rhinoceroses have only Hb C bands, and such individuals would probably have erythrocyte microcytosis. Neither we nor others have observed this phenomenon.

Our data for the white and Indian rhinoceroses are too limited to interpret. However, the observation of an AB phenotype in the Indian rhinoceros and of a C phenotype in the 2 samples of white rhinoceros blood were compatible with our proposed model, if one assumes that the AB phenotype in the former corresponds to the BC phenotype in black rhinoceroses, and that the C phenotype in white rhinoceroses corresponds to the B phenotype in black rhinoceroses.

Regrettably, though the impetus for these studies came from the need to clarify the mechanism of acute and often lethal hemolysis that threatens survival of the black rhinoceros species, we have not been able to elucidate the cause of this distressing problem.

The finding of an unstable hemoglobin reported by others[6] was not confirmed by results of this study. The rhinoceri of the prior study were wild-caught in Zimbabwe, and the 3 rhinoceroses of this study that were of the same (southern) subspecies did not have any abnormality in hemoglobin instability. The immobilizing drugs that were used in this study (etorphine HCl and carfentanil citrate) were the same as those used in the Zimbabwe study.[6] Therefore, differing drug treatments do not explain the divergent results of the 2 studies. Additionally, the supplemental tranquilizers used in several rhinoceroses of this study had no apparent effect on hemoglobin stability.

A caveat should be observed when interpreting results of hemoglobin studies in one species by analogy with observations in another species. Even if results of the isopropanol test or heat test for hemoglobin instability were positive for black rhinoceros hemoglobin (which we have not documented), it would not prove that rhinoceros hemoglobin is unstable in vivo or that hemoglobin instability contributes to hemolysis in rhinoceroses. Human Hb F, when present in > 10% of total hemoglobin concentration, gives positive test results for hemoglobin instability, although is not a cause of hemolysis. Human Hb E, possibly the commonest human hemoglobin variant worldwide, is invariably unstable in vitro, using the

[i] If, to the 2 rhinoceroses of BC phenotype and 1 of B phenotype that had history of acute hemolytic anemia, are added 2 rhinoceroses of the first kindred in Figure 1 that also had history of hemolytic anemia and may be presumed to have been of B phenotype (No. 204 and 126), of those with history of hemolysis, 3 were known or presumed to be of B phenotype and 2 were of BC phenotype.

isopropanol and heat tests, yet there is no hemolytic diathesis in people who are heterozygous, or even homozygous, for Hb E.[23] The converse is, of course, also true: failure of tests used for human hemoglobin to detect instability of rhinoceros hemoglobin do not prove that the latter is not unstable. It is possible that in some rhinoceroses, there may be a subtle but critical instability of hemoglobin that is not identifiable by the tests we use for human hemoglobin. However, at this time there is no clue that points to a hemoglobin variant or hemoglobin instability as the basis for the acute hemolytic crisis of black rhinoceroses. Most likely, the resolution of this problem lies elsewhere.

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