Acute intravascular hemolysis in the black rhinoceros: Erythrocyte enzymes and metabolic intermediates

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SUMMARY

Enzymes of aerobic and anaerobic glycolysis, glutathione cycling, and nucleotide metabolism were assayed on erythrocytes from 7 healthy rhinoceroses, 2 rhinoceroses during periods of intravascular hemolysis, and 1 rhinoceros without clinical signs of illness, which was the mother of 3 offspring with intravascular hemolytic syndrome. Measurements also were made of erythrocyte concentrations of glycolytic intermediates, adenine nucleotides, and glutathione. Although comparison of results for healthy and affected rhinoceroses did not identify an enzyme abnormality as a cause for the hemolytic syndrome, the data provided information regarding the metabolic characteristics of erythrocytes from healthy rhinoceroses.

In man, defective erythrocyte enzymes have been etiologically implicated in hereditary and acquired hemolytic anemia. This form of intrinsic erythrocyte abnormality may result in either chronic hemolytic anemia or in transient episodes of acute hemolysis, depending on which of several essential metabolic pathways is adversely affected. Similar syndromes have been observed in other mammalian species, eg, pyruvate kinase deficiency in dogs.[1,2] The purpose of the present study was to determine whether an enzymatic defect might be responsible for hemolytic anemia in the African black rhinoceros (*Diceros bicornis*).[3,4]

Materials and Methods

In response to a request sent to zoological veterinarians,[5] blood specimens were collected from 10 rhinoceroses: 2 rhinoceroses (rhino 1 [studbook No. 155] and rhino 2 [studbook No. 161]) during periods of acute hemolysis, 1 healthy rhinoceros (rhino 3 [studbook No. 121]) that was the mother of 3 offspring with the hemolytic syndrome, and 7 healthy rhinoceroses (rhinos 4 [studbook No. 251], 5 [studbook No. 55], 6 [studbook No. 188], 7 [studbook No. 212], and 8 to 10 [no studbook numbers]) without known histories of hemolytic disease. Venous blood containing heparin and, in 2 specimens, deproteinized with 2 volumes of 0.6*N* perchloric acid was flown under refrigeration to the University of California Los Angeles Hematology Research Laboratory for processing within 24 to 48 hours.

Enzymes of aerobic and anaerobic glycolysis, glutathione cycling, and nucleotide metabolism were assayed quantitatively, using leukocyte-free suspensions of erythrocytes that had been washed in saline solution and that had been prepared and processed according to standardized techniques.[6] Concentrations of glycolytic intermediates, adenine nucleotides, and glutathione were determined for perchloric acid extracts of whole blood or washed erythrocytes, as described by Minakami et al[7] and Beutler et al,[8] except that 2,3-diphosphoglycerate (2,3-DPG) was assayed by commercial kit.[a] Ultraviolet absorption spectra were determined for acid extracts of washed erythrocytes adjusted to pH 2 and pH 10, as described previously.[9]

Venous blood specimens from healthy people, processed in identical fashion, provided normal laboratory control values.

Results and Discussion

Erythrocyte enzyme assays—Mean (± SD) hematologic values of 7 healthy rhinoceroses were used for control values. Four of the control rhinoceroses (rhinos 4, 5, 6, and 7) were of East African origin and 3 (rhinos 8, 9, and 10) were of South African origin. Mean activities for various erythrocyte enzymes were essentially identical in both subspecies (Table 1). Affected rhinoceroses were all of East African origin.[10] Significant deviations from control values were not found for 2 rhinoceroses with hemolytic episodes, 1 of which had specimens collected and evaluated 4 and 25 days after the onset of acute hemolysis. Although the PCV decreased from 37% to 28% during this period, distinct enzymatic differences were not found between the 2 specimens.

In man, many erythrocyte enzyme defects are transmitted as autosomal recessive traits, with approximately half-normal enzymatic activities indicative of heterozygosity. Clear evidence of such heterozygosity was not found in rhino 3 (the clinically healthy mother of 3 females with previous episodes of hemolysis). A few inordinately low enzyme activities in the affected rhinoceroses were considered spurious because repeat assays did not confirm these low activities and because even lower values were

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[a] Sigma Chemical Co, St Louis, Mo.

found in some control rhinoceroses, without apparent consequence.

Although a distinct enzyme abnormality was not identified, the relative enzyme activities in rhinoceros erythrocytes deviated markedly from those observed in man. Only aldolase had the same quantitative activity in rhinoceros erythrocytes as in human erythrocytes (Fig 1). Activities of other enzymes such as hexokinase and glutathione peroxidase were an order of magnitude higher in rhinoceros erythrocytes than in man, whereas activities of numerous other enzymes were lower in rhinoceros cells. Enzymes of the Embden-Meyerhof pathway of anaerobic glycolysis, except for hexokinase and glucosephosphate isomerase, were considerably less active in rhinoceros erythrocytes than those in man. This species difference was even greater among enzymes of nucleotide metabolism, which in some instances were barely measurable (purine nucleoside phosphorylase, AMP deaminase) or were nondetectable (adenosine deaminase) in rhinoceros erythrocytes. Arnold and Faust[b] observed identical enzyme profiles in 1 affected rhinoceros and in 1 healthy rhinoceros, with similar relative activities compared with human controls.

Erythrocyte metabolites and nucleotides—Perchloric acid extracts of fresh blood were available only from rhinos 2 and 3 for complete assays of glycolytic intermediates and adenine nucleotides (Table 2). Reduced glutathione (GSH) and 2,3-DPG concentrations in washed erythrocytes from the other 8 rhinoceroses were determined by acid extraction of shipped blood.

Consistent subspecies differences in GSH and 2,3-DPG concentrations were observed. Erythrocytes from the 4 Eastern subspecies had one-third as much 2,3-DPG and twice as much GSH than did the 3 Southern subspecies. Significant differences in GSH or 2,3-DPG concentrations were not apparent between control and affected rhinoceroses.

Despite major differences in glycolytic enzyme profiles of rhinoceros and human erythrocytes, the intracellular concentrations of glycolytic intermediates were generally comparable, except that lactate concentrations were 4-fold greater in rhinoceros erythrocytes. Because erythrocytes are permeable to pyruvate and lactate, these values also reflect whole blood concentrations derived from metabolism of other tissues. Increases in glycolytic intermediates (which would have indicated metabolic blockade by a defective enzyme) were not apparent.

The most significant difference between human and rhinoceros erythrocytes was the relative dearth of adenine nucleotides in the latter. Only 2 fresh blood specimens (from rhinos 2 and 3) in perchloric acid were available

[b] Dr. H. Arnold, University of Giessen, Durchwahl, West Germany, and Dr. Richard Faust, Frankfurt Zoological Gardens, Frankfurt, West Germany: Personal communication, 1984. Assays were performed in Dr. Arnold's laboratory.

TABLE 1—Mean erythrocyte enzyme activities

	Healthy controls			Acute hemolytic syndrome			
	Eastern subspecies (n = 4)	Southern subspecies (n = 3)	Total (Mean ± SD)	Rhino 1	Rhino 2		Rhino 3 (mother of 3 affected offspring)†
				2 days*	4 days*	25 days*	
Hexokinase	5.2	6.2	5.6 ± 0.9	7.9	4.3	5.2	5.2
Glucosephosphate isomerase	22.5	28.6	25.1 ± 5.6	27.6	19.5	24.9	19.7
Phosphofructokinase	0.9	0.6	0.8 ± 0.5	0.9	1.2	1.4	0.7
Fructose-1,6-diphosphate aldolase	1.2	0.6	0.9 ± 0.4	0.8	1.1	1.1	1.0
Triosephosphate isomerase	40.5	73	54.6 ± 23.5	73	48	82	58
Glyceraldehyde-3-phosphate dehydrogenase	7.6	10.3	8.8 ± 4.1	13.4	1.5	12.4	10.6
Phosphoglycerate kinase	52.6	34.4	44.8 ± 25	49.8	39.4	50.3	25.4
Monophosphoglyceromutase	0.6	0.7	0.7 ± 0.1	0.6	0.5	1	0.5
Phosphopyruvate hydratase	0.7	0.6	0.6 ± 0.2	0.7	0.5	0.6	0.5
Pyruvate kinase 3 m*M* PEP	5.7	4	5 ± 1.3	7.3	4.6	5.9	4.3
(2 m*M* ADP) + FDP	5.8	4.5	5.3 ± 1.2	6.7	5.4	6.2	4.5
0.4 m*M* PEP	1.5	1.1	1.3 ± 0.5	3.3	0.7	1	0.8
+ FDP	4.5	3.3	4 ± 0.9	6.2	4.2	5.1	3.7
Lactate dehydrogenase	21.2	22.9	21.9 ± 2.6	27.4	29	23.7	25.5
Glucose-6-phosphate dehydrogenase	8.5	5.7	7.3 ± 1.8	13.8	8.7	10	8.9
Phosphogluconate dehydrogenase	1.2	1.1	1.1 ± 0.3	1.5	1.2	1.5	1.6
Glutathione reductase	1.3	1.3	1.3 ± 0.1	1.5	1.7	1.7	1.4
Glutathione peroxidase	55.4	32	45.4 ± 14.4	38	54	49.5	57
AMP deaminase	0.1	0.3	0.2 ± 0.1	–	0.2	0.6	0.1
Nucleoside phosphorylase	1.1	1.5	1.3 ± 1	0.6	0.4	0.3	0.9
Adenosine deaminase	0	0	0 ± 0	0	0	0	0
Adenylate kinase	8.6	9.8	9.1 ± 1	5.7	13	11.6	7.8
Ribosephosphate pyrophosphokinase	6	7.4	6.6 ± 1.2	–	11	24	6
Nucleotidase UMP	9.5	9	9.4 ± 0.8	–	12.5	–	8.6
CMP	5.3	4.1	5 ± 0.7	–	6.5	–	5.5
AMP	3.6	2.2	3.3 ± 0.9	–	5.7	–	2.6
d-TMP	5.4	3.3	5 ± 2.3	–	5.8	–	4
Glutamic oxalacetic transaminase	0.09	0.07	0.08 ± 0.04	0.4	0.04	0.06	0
Malic dehydrogenase	3	3.4	3.2 ± 1.1	5.2	3.3	3.4	3.4
Acetylcholinesterase	0	0	0 ± 0	0.1	0	0	0
Triokinase	0.5	0.5	0.5 ± 0.2	0.4	0.5	0.5	0.4

* No. of days between onset of hemolysis and obtaining blood specimen; † = mean of 2 blood specimens; PEP = phosphoenolpyruvate; FDP = fructose-1,6-diphosphate, 0.1 m*M*; UMP = uridine 5′-monophosphate; CMP = cytidine 5′-monophosphate; d-TMP = thymidine 5′-monophosphate; AMP = adenosine 5′-monophosphate; – = assay not done.

Data are expressed as micromoles of substrate converted per minute per 10^{10} erythrocytes, except for nucleotidase, which is expressed as micromoles per hour per gram of hemoglobin.

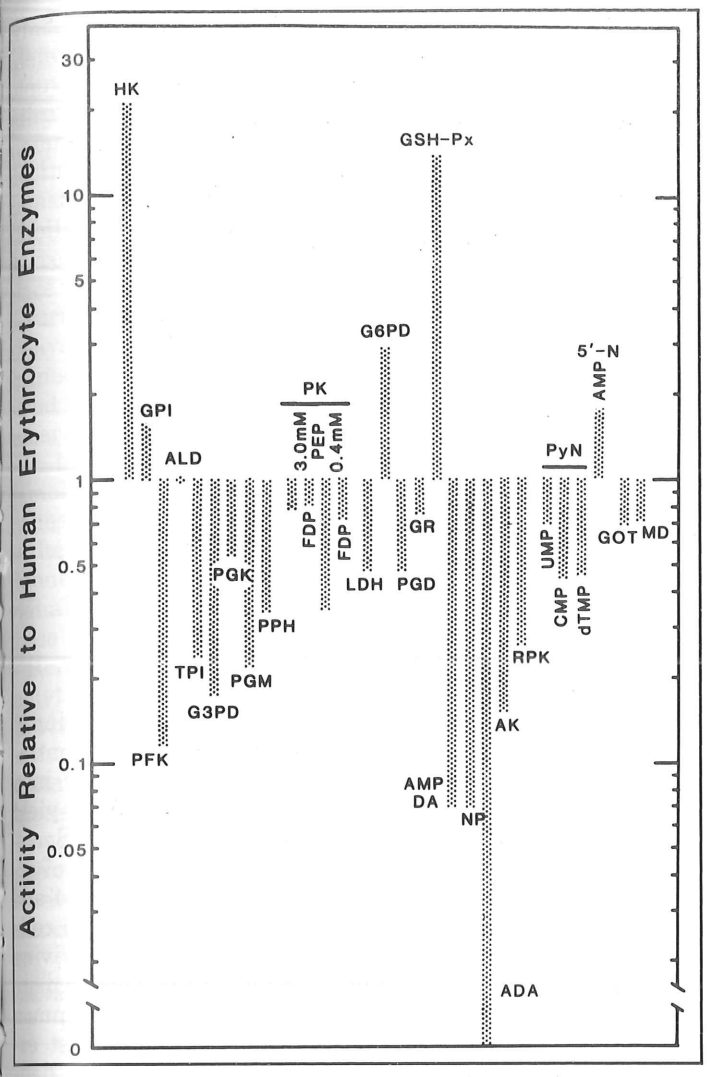

Fig 1—Enzyme activities in rhinoceros erythrocytes relative to those in normal human erythrocytes[c] (human erythrocyte values equivalent to 1). HK = hexokinase; GPI = glucosephosphate isomerase; PFK = phosphofructokinase; ALD = aldolase; TPI = triosephosphate isomerase; G3PD = glyceraldehyde-3-phosphate dehydrogenase; PGK = phosphoglycerate kinase; PGM = monophosphoglyceromutase; PPH = phosphopyruvate hydratase; PK = pyruvate kinase; PEP = phosphoenolpyruvate; FDP = fructose-1,6-diphosphate; LDH = lactate dehydrogenase; G6PD = glucose-6-phosphate dehydrogenase; PGD = phosphogluconate dehydrogenase; GR = glutathione reductase; GSH-Px = glutathione peroxidase; AMP DA = AMP deaminase; NP = nucleoside phosphorylase; ADA = adenosine deaminase; AK = adenylate kinase; RPK = ribosephosphate pyrophosphokinase; PyN = pyrimidine nucleotidase; UMP = uridine 5'-monophosphate; CMP = cytidine 5'-monophosphate; dTMP = thymidine 5'-monophosphate; 5'-N = 5'-nucleotidase; AMP = adenosine 5'-monophosphate; GOT = gluatamic oxalacetic transaminase; MD = malic dehydrogenase.

for quantitative assay[7] and these specimens contained 12% and 24%, respectively, of the total adenine nucleotide concentrations found in human controls. Differences in adenosine triphosphate (ATP) concentration were even more dramatic because adenine nucleotides in human erythrocytes are mostly (75% to 80%) in the high-energy form of ATP, with an ATP: adenosine diphosphate (ADP) ratio of > 3.5. Only 20% of the rhinoceros adenine nucleotides was in the triphosphate form, with an ATP:ADP ratio of 0.28. On an absolute scale, the mean erythrocyte ATP concentration in rhinos 2 and 3 was < 5% of that found in man. Because perchloric acid extracts of fresh blood were

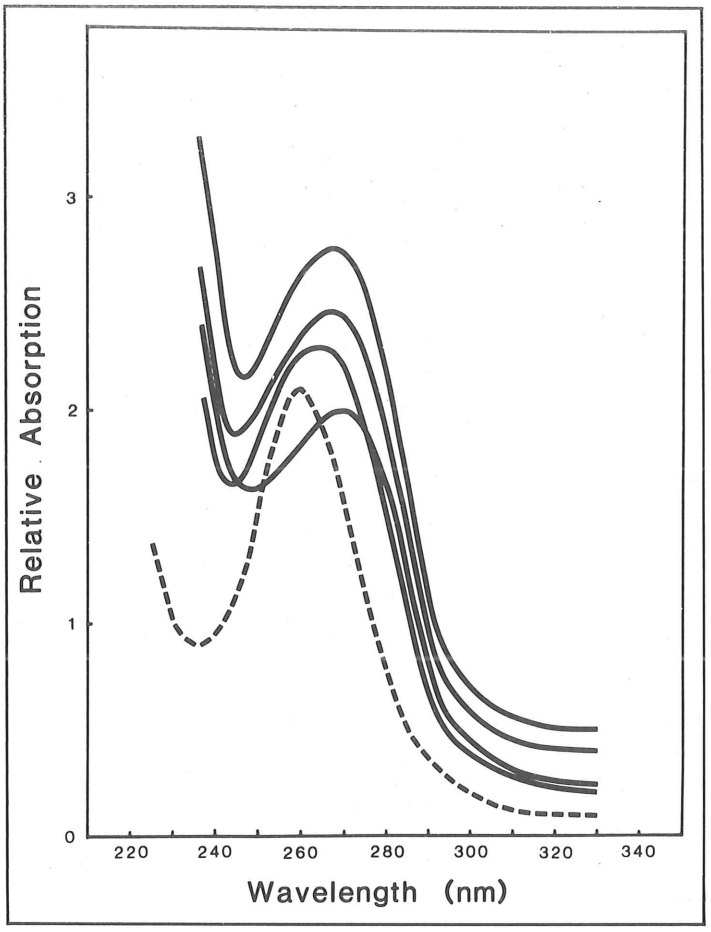

Fig 2—Ultraviolet absorption spectra (pH 2) of perchloric acid extracts of washed erythrocytes from 4 rhinoceroses (solid curves) compared with that of a representative normal human control (broken curve).

not available from control animals, it remains uncertain whether their adenine nucleotide concentrations were comparably lower.

Similar differences were observed when UV absorption spectra were determined for acid extracts of washed erythrocytes from the other 8 rhinoceroses. These spectra are dependent on pH and on the types and quantities of purine and pyrimidine compounds present. Representative spectra from 4 rhinoceros specimens were compared with a representative, normal human control specimen (Fig 2). The human control exhibits the characteristic spectrum of adenine, with maximal absorption in acid of 257 to 258 nm, because other purine or pyrimidine compounds are present only in trace amounts in normal erythrocytes. Maximum absorption (A_{max}) occurred between 266 nm and 269 nm for all but 1 rhinoceros (rhino 7, 263 to 264 nm), indicative of marked differences in intracellular nucleotide composition, compared with that of human erythrocytes. The mean A_{max} of 14 blood specimens from 8 rhinoceroses was 267 nm. Approximately 5 times the amount of rhinoceros cell extract was required to reach equivalent absorption amplitudes, compared with a given quantity of human cell extract, confirming the significantly decreased ($P < 0.001$) nucleotide concentration in rhinoceros erythrocytes. Chromatographic separation and identification of these nonadenine nucleotides were not performed in the present study.

Acute hemolytic anemia has been frequently observed

TABLE 2—Erythrocyte concentrations of glycolytic intermediates, glutathione, and adenine nucleotides

	Healthy controls		Acute hemolytic syndrome		Rhino 3 (mother of 3 affected offspring)	Human control values*
	Eastern subspecies (n = 4)	Southern subspecies (n = 3)	Rhino 1	Rhino 2		
Glucose-6-phosphate	NA	NA	NA	0.05	0.04	0.03
Fructose-6-phosphate	NA	NA	NA	0	0.01	0.02
Fructose-1,6-diphosphate	NA	NA	NA	0.02	0.02	0.01
Glyceraldehyde-3-phosphate	NA	NA	NA	0.02	0	0.02
Dihydroxyacetone phosphate	NA	NA	NA	0.07	0.03	0.02
2,3-Diphosphoglycerate	1.4	4.8	2.2	–	1.1	3.01
3-Phosphoglycerate	NA	NA	NA	0.05	0.01	0.06
2-Phosphoglycerate	NA	NA	NA	0.02	0	0.02
Phosphoenolpyruvate	NA	NA	NA	0.01	0.03	0.02
Pyruvate	NA	NA	NA	0.31	0.21	0.17
Lactate	NA	NA	NA	6.57	6.38	1.51
Reduced glutathione	1.15	0.52	2.35	1.03	1.12	0.77
ATP	NA	NA	NA	0.08	0.04	1.28
ADP	NA	NA	NA	0.29	0.14	0.35
AMP	NA	NA	NA	0.04	0.04	0.07
Total adenine nucleotides	NA	NA	NA	0.41	0.22	1.70

* Normal human control values for the University of California Los Angeles Hematology Research Laboratory.

Data are expressed as micromoles per 10^{10} RBC for all except 2,3-diphosphoglycerate (expressed as micromoles per gram of hemoglobin) and glutathione (expressed as milligrams per 10^{10} RBC).

NA = not applicable; appropriate blood specimens were not available, – = assay not done; ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate.

with high mortality (80%) in captive African black rhinoceroses in the United States and Europe.[3,4] Some clinical characteristics of this hemolytic syndrome resemble those in man and other animal species afflicted with certain intrinsic cellular defects. Such abnormalities are often heritable and may be manifested as altered structure and function of erythrocyte enzymes, membrane, or hemoglobin.

In the present study, enzyme deficiencies that might be responsible for defective cellular metabolism and premature hemolysis were not identified in comparisons of healthy vs affected rhinoceroses. The experimental design presumed that erythrocytes of the rhinoceros were metabolically similar to those of man and of other species that depend on glycolytic generation of ATP for processes crucial to normal cell viability. If this assumption were valid, a defective enzyme may have been detected by decreased activity, altered kinetics or stability, an accumulation of metabolic intermediates proximal to the ineffectively catalyzed reaction, or a decrease in the high-energy compound ATP.

Screening tests that have been useful in the study of hemolytic problems in man and a number of other species did not explain the hemolytic episodes in affected rhinoceroses. Statistically significant differences were not found between erythrocyte characteristics of affected and control rhinoceroses; however, distinct differences in erythrocyte GSH and 2,3-DPG concentrations were found between rhinoceroses of East African and South African origin.

Enzymatic or other metabolic abnormalities may exist that were not detectable in the nonhemolyzed cell subset remaining in the blood specimens. In mature anucleate erythrocytes, which are incapable of protein synthesis beyond the reticulocyte stage, enzymes cannot be replenished, and each decays with a characteristic half-life. Presumably, this gradual deterioration of the cell's metabolism eventually results in spontaneous lysis that determines the normal erythrocyte life-span for each species. Abnormalities, such as enzyme deficiencies, may accelerate this usually gradual metabolic deterioration.[11] Therefore, enzyme activities and metabolite concentrations differ considerably from one cell to another in a given specimen, and quantitative measurements yield mean values that are a function of mean cell age. Specimens obtained from rhinos 1 and 2 during periods of active hemolysis had low PCV (rhino 2 = 35% on day 4 and 28% on day 25; rhino 1 = < 5% on day 2); residual cells probably consisted of those that were capable of surviving the stress that induced premature lysis in the other more susceptible erythrocytes. This phenomenon is common among human erythroenzymopathies, perhaps best exemplified by glucose-6-phosphate dehydrogenase deficiency, in which the defect is sometimes completely masked during the immediate posthemolytic period by the preponderance of young erythrocytes, which contain more active enzymes than older cohorts. However, such masking probably does not account for the apparent lack of enzymatic defects in affected rhinoceroses because 2 specimens taken 4 and 25 days after onset of hemolysis from rhino 2 did not have significant differences in enzyme activities, and reticulocytosis was not found during the posthemolytic period. Reticulocytosis commonly occurs in people with hemolysis and is reflected by increased activities of certain age-dependent enzymes. Whether the absence of reticulocytosis was due to a lack of marrow response or to a characteristic that rhinoceroses share with horses[12] is not known.

Rhinoceros and human erythrocytes may not be sufficiently similar in their metabolic characteristics to justify direct comparisons. The enzyme activity profiles and intracellular nucleotide spectra observed with rhinoceros erythrocytes differed markedly from those of other species we have previously observed.[c] Nucleotide patterns were especially puzzling because of their low concentrations of ATP and the presence of nonadenine nucleotides. These findings may relate to diminished activities of en-

[c] D. E. Paglia and W. N. Valentine, University of California, Los Angeles: Personal observations, 1965–1986.

zymes associated with adenine nucleotide metabolism, adenosine deaminase, nucleoside phosphorylase, and adenylate kinase. If rhinoceros erythrocytes do not require ATP for vital cellular functions, as do most other mammalian species, then the importance of adenine nucleotide enzymes and those of anaerobic glycolysis is diminished. However, if ATP is important to maintain normal viability and longevity of rhinoceros erythrocytes, then the relatively low adenine nucleotide concentrations may place these cells in jeopardy of metabolic depletion if ATP is further reduced by pathologic processes. Quantitative assays of ATP, ADP, and adenosine monophosphate performed on suitably prepared perchloric extracts of fresh blood from healthy rhinoceroses may help to resolve these uncertainties.

In man, the clinical occurrence of episodic (vs chronic) hemolysis is more often associated with defects in enzymes that counter oxidative stresses, particularly those of the oxidative hexosemonophosphate shunt and glutathione cycling. These enzymes were not measured individually in the current study; therefore, investigation of these enzymes may be warranted.

Hemoglobinopathies also may present as episodic hemolytic anemia, particularly when unstable variants are involved. We have observed that Malayan tapir (*Tapirus indicus*) blood has a marked tendency for hemoglobin precipitation or crystallization, with relatively minor alterations in ionic characteristics of the hemolysate.[c] Because the tapir also is an odd-toed ungulate, rhinoceros hemoglobin might be similarly susceptible to precipitation or denaturation by exogenous toxins or biochemical changes induced in the cellular milieu. Gardner[d] recently observed a fatal episode of hemolytic anemia of unknown etiology in a South American tapir (*Tapirus terrestrus*).

Although a specific cause for severe hemolysis in the black rhinoceros was not identified, the present study provides data regarding the metabolic characteristics of healthy rhinoceros erythrocytes.

[d] Dr. Harrison Gardner, Columbus Zoological Park, Columbus, Ohio: Personal communication, 1984.

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