Radiometric assessment of hexose monophosphate shunt capacity in erythrocytes of rhinoceroses

Donald E. Paglia, MD; Brandon Weber, PhD; Ingrid Baumgarten, MSc; Eric H. Harley, MD, PhD

Objectives—To measure metabolic rates of the hexose monophosphate shunt (HMPS) in erythrocytes of rhinoceroses, and to test the hypothesis that low concentrations of endogenous ATP in erythrocytes impair HMPS capacity, thereby increasing susceptibility to oxidant-induced hemolysis.

Animals—13 black and 3 white rhinoceroses, free-ranging in several regions of southern Africa, and 1 Sumatran rhinoceros in US captivity.

Procedure—HMPS fluxes were measured in rhinoceros erythrocytes with carbon-labeled glucose in the presence and absence of known HMPS activators.

Results—Compared with values for human erythrocytes, mean basal state HMPS fluxes were appreciably lower (22 to 46%) in all 3 rhinoceros species studied. Shunt activators increased HMPS rates approximately 5-fold over basal rates in rhinoceros erythrocytes, compared with increases in humans of 10-fold with ascorbate and 15-fold with methylene blue. Stimulated HMPS rates in human erythrocytes were quantitatively 5- to 10-times greater than those observed in rhinoceros erythrocytes. Overall HMPS catabolic rates were completely independent of intracellular ATP concentrations.

Conclusions and Clinical Relevance—HMPS glycolytic and recycling rates and responses to activators are inherently low in erythrocytes from 3 species of rhinoceros, likely contributing to (but not solely responsible for) the high susceptibility of black rhinoceroses to oxidant-induced hemolysis. Slow erythrocyte HMPS capacities were independent of intracellular ATP concentrations, invalidating a current hypothesis regarding the pathogenesis of hemolytic anemia in captive black rhinoceroses. Limitations in HMPS capacities emphasize the importance of protecting rhinoceroses from exposure to drugs, chemicals, toxins, foodstuffs, and other conditions known to increase production of oxidizing metabolites, reactive oxygen species, and free radicals.(*Am J Vet Res* 2001;62:1113–1117)

Acute hemolytic anemia, occurring as an isolated event or as a secondary complication of other disorders, is associated with approximately 40% of deaths among captive black rhinoceroses (*Diceros bicornis*).[1] The clinical characteristics of this hemolytic syndrome are virtually identical to those of glucose-6-phosphate dehydrogenase (G-6-PD) deficiency in humans, even though that enzyme has not been demonstrably defective in any rhinoceros.[2,3] Other common causes of hemolysis, such as abnormal hemoglobins or autoimmune phenomena, have not been identified.[4,5] Results of our initial metabolic studies indicate that most enzymes in erythrocytes of black rhinoceroses were markedly lower in activities, compared with those of humans and other mammals, but there were no apparent differences between unaffected rhinoceroses and those actively hemolyzing or fully recovered from acute hemolytic episodes, nor in their relatives. Thus, no specific enzyme deficiency could be indicted as a likely cause of these hemolytic episodes.[2,3]

One of the most unusual metabolic features we observed was the presence of only 2 to 5% of the intracellular ATP concentrations normally found in most mammalian erythrocytes,[2,3] concentrations traditionally thought to be incompatible with maintenance of cation gradients that are essential to normal erythrocyte survival in most species, including *Rhinocerotidae*. These observations led to a working hypothesis that decreased availability of intracellular ATP in rhinoceros erythrocytes may impair their capacity to respond to oxidant stress by limiting production of substrate for G-6-PD, glucose-6-phosphate (G-6-P), thereby producing a metabolic restriction equivalent to G-6-PD deficiency.[3,6,7]

The purpose of the study presented here was to measure hexose monophosphate shunt (HMPS) flux rates in rhinoceros erythrocytes that had been preprimed in vitro with variable amounts of intracellular ATP. Results indicate that glucose catabolism through the HMPS is independent of erythrocyte ATP concentration in the physiological range, invalidating the original hypothesis and leaving unresolved the primary cause underlying hemolysis in black rhinoceroses. These data also provide confirmation and more precise quantification of our previous observations[3,7,8] that resting and stimulated HMPS fluxes in black and white (*Ceratotherium simum*) rhinoceroses (and now including 1 Sumatran rhinoceros, *Dicerorhinus sumatrensis*) were substantially decreased, compared with those in humans and other mammals.

Materials and Methods

Veterinarians and the authors working with game-capture teams of the Republic of South Africa, KwaZulu/Natal, and Zimbabwe obtained blood samples from free-ranging

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From the UCLA/VA Hematology Research Laboratory, Department of Pathology and Laboratory Medicine, UCLA School of Medicine, Los Angeles CA 90095-1732 (Paglia); and the Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, Republic of South Africa (Weber, Baumgarten, Harley).
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black and white rhinoceroses. One blood sample came from a captive Sumatran rhinoceros at the San Diego Zoo. In each instance, rhinoceroses were being immobilized for diverse reasons (such as examination, treatment, dehorning, radiocollaring, or relocation), and venipuncture was performed as an incidental procedure.

Whole blood samples from veins in the ears or forelimbs were drawn directly into heparinized containers, refrigerated, and transported to laboratories at the University of Cape Town or the University of California Los Angeles Hematology Research Laboratory for analyses beginning as soon as 24 to 48 hours and up to 2 to 3 weeks from time of withdrawal. Field research team members volunteered samples of human blood that were collected simultaneously from antecubital veins and transported with the rhinoceros blood samples for comparative purposes. Approximately half of the blood samples from humans and rhinoceroses obtained in the field were also flash frozen and stored in liquid nitrogen.[8] In these instances, analyses as long as 1.5 months later produced results that were comparable (± 10%) to those obtained with fresh and refrigerated blood samples.

Erythrocytes were isolated according to procedures specified by the International Committee for Standardization in Hematology[9] and suspended in isotonic PBS solution, pH 7.4, or Hank's balanced salt solution for various studies. Intracellular ATP concentrations were augmented by overnight (approx 18 hours) incubation of washed erythrocytes in Hank's balanced salt solution or PBS solution containing 10 mM glucose and 10 mM adenosine at 37 C, a procedure previously shown to increase ATP and total adenine nucleotides to concentrations equal to or greater than those found in most mammalian erythrocytes.[3] After incubation, cells were washed 3 times in isotonic saline (0.9% NaCl) solution, adjusted to approximately 10 g of Hb/dl, and aliquots were deproteinized with perchloric acid and neutralized with KOH for assays of adenine nucleotides and their products by HPLC[10] or by enzymatic techniques.[11]

Baseline metabolic flux rates through the HMPS were measured by incubation of cells at 37 C in PBS or Hank's balanced salt solution containing approximately 2 µCi of glucose tagged at the 1 position with carbon 14 (ie, [1-^{14}C]glucose) or glucose tagged at the 2 position with carbon 14 (ie, [2-^{14}C]glucose) to measure recycling of fructose-6-phosphate (F-6-P) back through the shunt. Standard HMPS stimulants, methylene blue and neutralized ascorbic acid,[b] were added at various concentrations to identical parallel systems. Reactions were stopped after various incubation periods by injection of equal volumes of 1.2 N perchloric acid. Carbon dioxide produced by catabolism of G-6-P to pentose phosphate was trapped by filter papers saturated with methyl-benzethonium chloride[c] and counted in a liquid scintillation analyzer.[d] The HMPS flux rates were calculated on the basis of nmol of $^{14}CO_2$ evolved/h/10^6 erythrocytes.

Statistical analysis—Differences in HMPS metabolic and recycling rates among groups were analyzed for significance by the Mann-Whitney 2-tailed nonparametric test. A P value of ≤ 0.05 was used to establish significance.

Results

HMPS capacity—HMPS rates, assayed under identical conditions, in intact erythrocytes from 1 Sumatran, 3 white, and 13 black rhinoceroses were compared with mean values obtained in erythrocytes from 13 humans (Table 1). Baseline rates of glucose catabolized directly through the HMPS (measured by 1-$^{14}CO_2$ evolution) were appreciably lower in erythrocytes from all 3 rhinoceros species relative to humans, with mean values varying from a fourth to less than a half of the mean HMPS flux in resting human erythrocytes. In comparison with HMPS capacity in human erythrocytes, erythrocytes from black rhinoceroses differed significantly ($P < 0.001$), as did erythrocytes from white rhinoceroses ($P = 0.011$) and the single Sumatran. The importance of interspecies variations in this value remains uncertain because of limited numbers of white and Sumatran rhinoceroses available for study, but comparisons between basal HMPS rates in erythrocytes of black and white rhinoceroses were significantly different ($P = 0.05$).

Ascorbate and methylene blue were capable of stimulating glycolysis through the HMPS pathway (Table 1), but rhinoceros erythrocytes were considerably less responsive to these activators than were human erythrocytes. In all 3 rhinoceros species, 10 mM ascorbate or 6.7 µM methylene blue increased HMPS activity approximately 5-fold over baseline rates, whereas human erythrocytes typically responded with 10- to 15-fold increases under comparable conditions. On a relativc then, responses to these HMPS stimulants were 2- to 3-times greater in human erythrocytes than in any of thc *Rhinocerotidae*. More importantly, stimulated HMPS fluxes were quantitatively lower in rhinoceros erythrocytes by a factor of 5 to 10, compared with human erythrocytes. Again, these differences compared with humans were significant for black rhinoceroses ($P < 0.001$) and for white rhinoceroses (P =

Table 1—Glucose catabolism though the hexose monophosphate shunt in rhinoceros erythrocytes

Species	HMPS metabolic rate* (nmol of 1-$^{14}CO_2$/h/10^8 RBC)			HMPS recycling rate* (nmol of 2-$^{14}CO_2$/h/10^8 RBC)		
	Control	Ascorbate (10 mM)	Methylene blue (6.7 µM)	Control	Ascorbate (10 mM)	Methylene blue (6.7 µM)
Black rhinoceros (n = 13)	31 (< 0.001)	137 (< 0.001)	119 (< 0.001)	5.1 (0.002)	137 (0.005)	110 (0.002)
White rhinoceros (n = 3)	15 (0.011)	75 (0.011)	77 (0.011)	2.2 (0.004)	11 (0.004)	37 (0.004)
Sumatran rhinoceros (n = 1)	19	72	119	4	36	60
Humans†	68 [4.4]	680 [59]	1027 [96]	28 [7]	275 [36]	787 [107]

*Values are means of duplicate assays of single samples from each subject. †n = 13 for 1-$^{14}CO_2$, n = 5 for 2-$^{14}CO_2$.
Parentheses enclose P values derived from Mann-Whitney 2-tailed nonparametric tests comparing each rhinoceros group with human controls. Brackets enclose SEM.
HMPS = Hexose monophosphate shunt.

0.011). The HMPS fluxes did not differ significantly between black and white rhinoceroses under ascorbate or methylene blue stimulation ($P = 0.080$ and 0.687, respectively).

The recycling rates of glucose catabolites back through the HMPS pathway were measured by 2-$^{14}CO_2$ evolution (Table 1). The capacity to recycle was clearly demonstrable in all erythrocytes, particularly in erythrocytes of black rhinoceroses, but again absolute flux rates were significantly ($P = 0.002$ to 0.004) lower in rhinoceros erythrocytes (again by a factor of 5 to >10) compared with human erythrocytes. Recycling rates under influence of HMPS stimulants appeared quite variable among erythrocytes of the species of rhinoceros tested. Of these, erythrocytes of black rhinoceroses had the highest activities under basal and stimulated conditions, but erythrocytes of all 3 rhinoceros species had appreciably lower HMPS capacities, compared with human erythrocytes on quantitative and relative scales. Recycling rates under shunt stimulation differed significantly ($P = 0.01$ to 0.05) between erythrocytes of black and white rhinoceroses. A notable difference between erythrocytes of black rhinoceroses and the other species, including humans, was their relatively greater response to ascorbate stimulation compared with activation by methylene blue.

Effect of increased intracellular ATP on HMPS capacity—Incubation of rhinoceros erythrocytes with adenosine in a high phosphate medium, as described, resulted in linear increases in intracellular ATP concentrations from basal concentrations of < 2.0 μ*M* to > 75 μ*M*. In 12 experiments with erythrocytes from 3 black rhinoceroses and 6 determinations with erythro-

Table 2—Effect of intracellular ATP concentration on HMPS rates in erythrocytes of black rhinoceroses

Incubation conditions	Mean erythrocyte ATP (m*M*)	HMPS metabolic rate (nmol of 1-$^{14}CO_2$/h/10^8 RBC)		
		No HMPS activator	Ascorbate	Methylene blue
4 C	1.7	16	93	120
37 C + P_i	3.8	29	139	89
37 C + P_i + adenosine	78.2	21	130	107

Values are means of duplicate assays on a single sample from a typical black rhinoceros.
P_i = 0.12*M* inorganic phosphate.
See Table 1 for key.

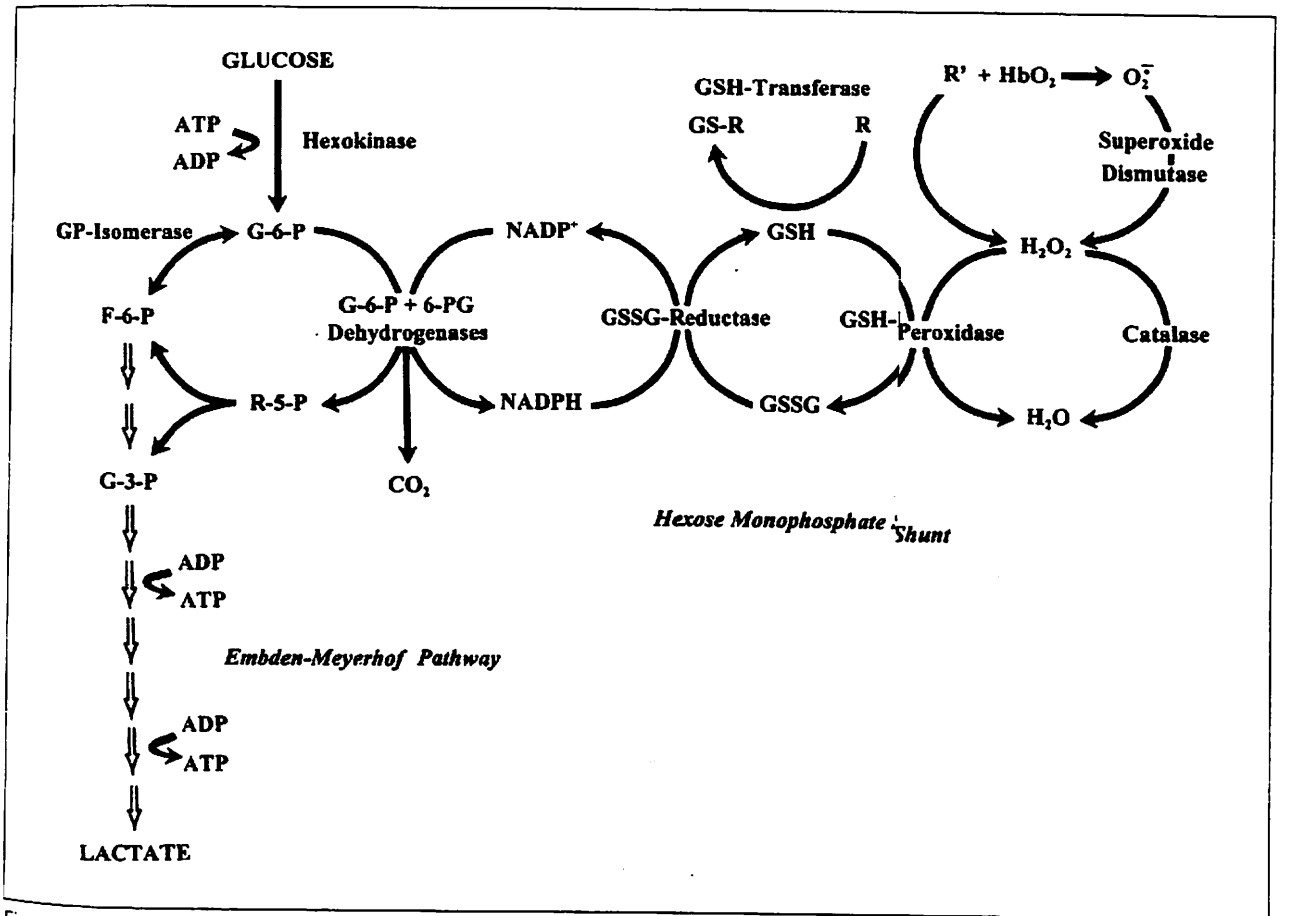

Figure 1—Anaerobic (Embden-Meyerhof) and oxidative (hexose monophosphate shunt) pathways of glycolysis in mammalian erythrocytes. Under physiologic conditions, most glucose (approx 90%) is catabolized anaerobically for maximum generation of ATP. Because of its redox potential, the tripeptide glutathione (GSH) protects cellular components from oxidation by its preferential conversion to a dimer (GSSG) by oxidants such as superoxide anion and peroxides. Glutathione reductase catalyzes conversion of GSSG back to GSH with the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) as an obligate cofactor. Increased ratios of $NADP^+$/NADPH divert the flow of G-6-P from the EMP into the HMPS, increasing oxidative glycolytic rates from approximately 5 to 10% to ≥ 95% under oxidant challenge.

cytes from 3 white rhinoceroses, no significant differences in HMPS activities or recycling rates occurred as a consequence of augmented intracellular ATP. There was minimal change in ATP concentration in erythrocytes of black rhinoceroses incubated 18 hours in high-phosphate medium alone, but the addition of 10 mM adenosine resulted in markedly increased intracellular ATP (**Table 2**). The HMPS rates were unaffected by increased ATP concentrations under basal conditions or following challenge with ascorbate or methylene blue. These findings indicate that the physiologically low intracellular concentration of ATP characteristic of rhinoceros erythrocytes is not a limiting factor in oxidative glycolytic rates through the HMPS.

Discussion

An acute hemolytic disorder has occurred with ominous frequency among African black rhinoceroses in captivity,[1] but its precise cause remains obscure. Clinically and hematologically, this syndrome is virtually identical to that observed in humans with molecular defects involving G-6-PD and other enzymes of the HMPS pathway of oxidative glycolysis (**Fig 1**).[3] As the most common enzyme deficiency disorder among humans, G-6-PD deficiency is classically manifested by acute episodes of intravascular hemolysis occurring in apparently healthy subjects after exposure to various drugs, chemicals, and toxic compounds, as well as certain foodstuffs such as fava beans. Some clinical conditions (eg, pregnancy, infection, and inflammatory disease) may also be associated with hemolysis in G-6-PD deficient subjects, all of these having in common the increased production of oxidizing catabolites or free radicals.

The HMPS (also known as the pentose phosphate pathway) is primarily responsible for protecting erythrocytes from oxidative injury. Inadequate neutralization of ambient oxidants because of defective function of this pathway results in damage to essential cellular components, such as membrane phospholipids, enzyme proteins, and hemoglobin itself. Under oxidant challenge, cells with impaired HMPS capacity exhibit declining reserves of GSH, hemoglobin denaturation with Heinz-body formation, and intravascular hemolysis with its consequent indirect hyperbilirubinemia, decrease in serum haptoglobin concentration, and hemoglobinuria with or without hemosiderinuria. All of these clinical and hematologic findings occur in the acute hemolytic syndrome of black rhinoceroses.

We previously observed that rhinoceros erythrocytes have abundant G-6-PD activity yet remain highly susceptible to oxidant stress in vitro, exhibiting GSH instability, methemoglobin production, and Heinz-body formation comparable in degree with that generally associated with human G-6-PD deficiency.[2,3,7] Glutathione peroxidase, another important enzyme of the HMPS pathway, is actually much higher in activity compared with human erythrocytes, whereas others, such as 6-phosphogluconate dehydrogenase, glutathione reductase, and glutathione S-transferase, are significantly lower.[2,3,7] Because optimal activities of individual enzymes measured in vitro provide little information about the physiologic function of an entire metabolic pathway, we sought to detect possible impairment in antioxidant capacity by quantitative radiometric measurements of glucose degradation via the HMPS, a far more sensitive and reproducible analytic technique than the nonisotopic incubation procedures previously applied.

Hexokinase catalyzes phosphorylation of glucose to G-6-P, an intermediate that can generate ATP by further anaerobic degradation or can serve as a substrate for G-6-PD (Fig 1). When G-6-P enters the HMPS, it is oxidized at the carbon-1 position to produce CO_2 and a phosphorylated pentose. The evolution of 1-$^{14}CO_2$ from glucose labeled at the carbon-1 position thus provides a direct measure of the rate of glucose catabolism via the HMPS pathway.

Because the first carbon of G-6-P is lost as CO_2 during its conversion to pentose, glucose labeled in the carbon-2 position evolves into pentose phosphate and eventually into fructose-6-phosphate (F-6-P) with a carbon-1 label via reactions of the distal HMPS pathway (not detailed in Fig 1). Isomerization of F-6-P to G-6-P can then occur, providing a substrate labeled again at carbon-1 to measure recycling of catabolites back through the HMPS.

These radiometric techniques allowed quantitative verification of our previous observations that HMPS capacities in rhinoceros erythrocytes were qualitatively decreased, compared with other mammalian species.[3,7] Basal flux rates in erythrocytes of most black rhinoceroses tested were approximately a quarter to a third those of human erythrocytes under comparable conditions. In the presence of shunt activators (neutralized ascorbic acid and methylene blue), HMPS activities were also relatively low, and maximum acceleration of oxidative glycolysis in erythrocytes of black rhinoceroses was quantitatively only about a tenth of that achievable by human erythrocytes in response to oxidant challenge. Although the numbers of blood samples available from white and Sumatran rhinoceroses were limited, statistical comparison indicated that relatively low HMPS flux rates reflected a metabolic limitation shared by all 3 species.

Additionally, these quantitative techniques allowed us to test an important working hypothesis regarding the pathogenesis of hemolytic episodes in captive black rhinoceroses, namely, that minimal reserves of ATP may be rate limiting by impairing (ATP-dependent) hexokinase-catalyzed generation of G-6-P, the obligate substrate for initiating accelerated HMPS activity in response to sudden surges in ambient oxidants. We had previously observed that very low concentrations of ATP in erythrocytes, characteristic of at least 4 species of rhinoceroses, could be increased in vitro by incubation with adenine or adenosine and various carbon sources in a high-phosphate medium.[3] Using this technique, we enhanced the ATP concentration of erythrocytes from black and white rhinoceroses and measured their capacities to catabolize and recycle glucose through the HMPS. In all instances, HMPS fluxes were independent of intracellular ATP concentration, invalidating that long-standing hypothesis.

In the absence of clear interspecies differences, it seems unlikely that limited HMPS capacity alone could

account for hemolytic tendencies that seem to be manifested only in black rhinoceroses. It remains entirely possible, however, that lower HMPS rates could compound the problems presented by a relative deficiency in catalase activity, which is far lower in erythrocytes from black rhinoceroses than in any of the other 3 species tested.[3,6,7]

Additionally, even though results of our studies indicate that low concentrations of ATP in erythrocytes are not responsible per se for slower HMPS flux rates in rhinoceros erythrocytes, it still seems likely that inadequate ATP may be the ultimate initiator of the lytic event. By stimulating HMPS cycling and recycling, sudden surges in ambient oxidants cause diversion of glucose catabolism away from the Embden-Meyerhof pathway and maximum generation of ATP. Under prolonged oxidant challenge, inherently low concentrations of endogenous ATP in rhinoceros erythrocytes would be diminished even further by ATPase activity necessary for cation pumping. In the absence of adequate ATP replenishment by anaerobic glycolysis (because of substrate diversion into the HMPS), progressive depletion of the ATP pool by ATPase activity must eventually reach concentrations incompatible with maintenance of transmembrane cation gradients, resulting in sodium and water influx and hemolysis. It therefore remains important to ensure that ATP concentrations in erythrocytes are maintained as high as possible in black rhinoceroses by dietary phosphate supplementation and by avoiding conditions that result in hypophosphatemia.[e]

[a]Weber B, Baumgarten I, Paglia DE, et al. Rhinoceros and man — comparative red cell metabolism (*abstr*). S Afr Soc Pathol 1996;CB24.

[b]Sigma Aldrich Ltd, Johannesburg, South Africa.

[c]Hyamine, Sigma Chemical Co, St Louis, Mo.

[d]Packard 1600 TR liquid scintillation analyzer, Packard Instrument Co, Meriden, Conn.

[e]Paglia DE. Rationale for phosphate supplementation in prevention and therapy of hemolytic anemia in the African black rhinoceros (*Diceros bicornis*), in *Proceedings*. Symp Zool Soc S Afr Contemp Zool S Afr, 1994;107.

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