HEALTH & HUSBANDRY REPORTS

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for

RHINOCEROS

1997

PATHOPHYSIOLOGIC BASIS OF DISEASES AFFECTING CAPTIVE AFRICAN BLACK RHINOCEROSES

PROGRESS REPORT: JANUARY 1996 TO JULY 1997 INTERNATIONAL RHINO FOUNDATION GRANT R-96-1

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In the first 18 months of this grant period, the UCLA Hematology Research Laboratory has evaluated 62 separate blood specimens from a total of 25 rhinoceroses quartered in U.S. institutions. All were black rhinoceroses *(Diceros bicornis)* except for one white *(Ceratotherium simum)* and four Indian *(Rhinoceros unicornis)*. Results of these assays have added substantially to our expanding base of red cell metabolite data that now includes four of the five extant species of rhinoceros (only the Javan is not represented). These metabolic profiles provide more precise definition of the similarities and differences in red cell enzyme activities and metabolite concentrations and, in turn, now allow reliable distinctions to be made among these four species as well as between the eastern *(D.b.michaeli)* and southern *(D.b.minor)* subspecies of black rhinoceroses. As an example, the red cells of all four species studied have been confirmed to possess uniformly low amounts of the essential high-energy phosphate ATP, suggesting that this extremely unusual finding, (matched only by the monotreme, *Echidna*), cannot be etiologically related to the several serious disorders that appear to affect black rhino almost exclusively.

As a consequence of these observations and our in vitro studies of ATP-primed erythrocytes, the previous working hypothesis that focused on ATP deficiency has now been disproven, and emphasis has shifted to other aspects of anti-oxidant metabolism.

Results of theses studies have contributed to the **first objective,** ***(to determine the nature, extent and significance of unusual metabolic features of rhinoceros red cells)***, by identifying several other areas of red cell metabolism that *do* appear to be unique to the black rhinoceros. These metabolic features have become the focus of current investigations to evaluate their potential etiologic roles in hemolytic anemia or any of the other diseases of high morbidity and mortality affecting captive black rhinoceroses. These data will be augmented in September with the scheduled acquisition of specimens from the three surviving *Dicerorhinus sumatrensis* and from additional white and Indian greater one-horned rhinos scheduled for the following months.

In pursuit of the **second objective,** ***(to establish the basis for susceptibility to oxidant stress),*** we have previously developed *in vitro* and *in vivo* techniques to augment rhino red cell ATP up to human levels and above, and have demonstrated that *in vitro* responses to oxidant challenges are completely independent of ATP concentration. We have also determined that black rhinoceroses uniformly possess only 2-4% of the catalase activity found in human red cells, whereas the three other rhinoceros species so far evaluated have intermediate activities of this crucial enzyme. It is now firmly established that human catalase provides the major mechanism for detoxification of hydrogen peroxide that is generated constantly by normal physiologic processes and produced copiously in many pathologic conditions. Recent studies by others have also shown that the pyridine nucleotide cofactor, NADPH, is essential for both the stability and catalytic effectiveness of catalase, in addition to its well-established role as the obligate cofactor for initiation of hexose monophosphate shunt activity (the metabolic pathway primarily responsible for neutralization of reactive oxidants and free radicals.) Because of these multiple pivotal roles for NADPH in anti-oxidant metabolism, we are now concentrating on developing new assays for this compound and for NADH (the obligate cofactor for methemoglobin reduction) and for their oxidized forms to determine

whether deficiencies of these inherently unstable nucleotide cofactors could be responsible for the relative catalase deficiency and sensitivity to oxidant-induced methemoglobin and Heinz-body formation exhibited by black rhino erythrocytes.

In further pursuit of the **second objective**, we have fractionated the granulocytes and lymphocytes from 27 specimens obtained from 11 black, one white, and three Indian rhinoceroses, and measured the activities of approximately 25 enzymes involved in ATP generation, oxidant detoxification, and nucleotide metabolism, and the concentrations of six essential metabolites in each fraction. Preliminary results indicate that a number of the unusual metabolic features we have found to be characteristic of rhinoceros erythrocytes are shared to varying degrees by leukocytes as well. We anticipate beginning to perform similar assays on cultured fibroblasts within the next few months, as well as on additional leukocyte fractions sufficient in number to provide statistically meaningful comparisons. Techniques to assay these enzymes and metabolites in other tissues remain to be developed.

The **third objective,** ***(to test preventative and therapeutic strategies)***, has been served by collaborative studies of six black rhinoceroses undergoing dietary or therapeutic regimens for a variety of conditions. In each instance, multiple blood specimens (as many as six to nine) were obtained to monitor metabolic changes in red cells and leukocytes as a function of time following specific therapeutic interventions. The effectiveness of oral and parenteral phosphate supplementation in raising red cell ATP concentrations continues to be documented. Although ATP deficiency *per se* does not appear to be etiologically related to hemolytic anemia or other disorders, the extremely low reserves of this crucial high-energy compound may be the proximate cause for eventual failure of the red cell membrane's cation pump (which relies on ATP for fuel), allowing influx of water and its consequent cell swelling to the ultimate point of rupture (hemolysis). Elevating red cell ATP by phosphate loading, therefore, may provide a buffer against those conditions that tend to deplete ATP stores, and no adverse effects of phosphate supplementation have yet been reported. The absence of any new cases of primary acute hemolytic anemia in the past three years is encouraging evidence that the preventive and therapeutic techniques already in place may be at least partially effective. These strategies and their metabolic bases were reviewed in a presentation to the 1996 meeting of the American Association of Zoo Veterinarians.

(N.B. In early August 1997, a new case of acute hemolytic anemia developed in a black rhinoceros at the Kansas City Zoo, and specimens are currently under study in both UCLA and Cape Town laboratories.)

We have also been collaborating with several other groups nationally to investigate a new syndrome characterized by ***non*hemolytic anemia** and hemorrhagic extravasation into soft tissues that has affected four black rhinos at separate institutions in Texas and Colorado. Clinical similarities with another Fossil Rim black rhino that we helped necropsy in 1992 led us to consider a possible vasculitis or immunologic etiology, and evidence has been accumulating for both. We observed prominent erythrophagocytosis in the necropsy slides, and have detected a possible cold agglutinin in one of the new cases and a monoclonal gammopathy by electrophoresis in another, with no strong evidence for a coagulopathy by conventional laboratory criteria. Additional sera from normal and affected animals are being studied in our laboratory, and more extensive immunologic and pathologic studies are being coordinated by Dr. Richard Montali of the National Zoo.

(N.B. In early August 1997, the sibling of one of these affected rhinos, also at the Dallas Zoo, began to develop similar chemical signs and symptoms, suggesting a possible infectious component, and additional specimens are under current study.)

While the studies to date have contributed substantially to each of our primary objectives, we do not yet know the precise cause(s) of acute hemolytic episodes or any of the other major disorders that all too commonly affect black rhinoceroses, such as mucocutaneous ulcerative disease, fungal pneumonia, primary and secondary hepatopathies, leukoencephalomalacia, and the new hemorrhagic syndrome. Additionally, some of these results have raised new questions and opened new avenues for potential investigation into the remarkably unusual metabolic characteristics of rhinoceroses in general and black rhinos in particular.

The following are among the most promising areas for continued and future investigation:

(1) After more than a dozen years of searching, the identification of the amino acid amino as "substance X" (reported in detail by Co-PI Eric Harley in his portion of this progress report) represents a major contribution and potential breakthrough toward understanding the perplexing nature of rhinoceros red cell metabolism. The presence of unprecedented amounts of this compound in rhino is a startling as the virtual absence of ATP. Potentially tyrosine could have a positive role in anti-oxidant metabolism, or a negative role as a highly insoluble amino acid capable of crystallizing and inducing hemolysis itself. The need for further investigation is crucial.

(2) Given the current understanding of the complex relationship between human catalase activity and NADPH, and the multiple roles the latter plays in anti-oxidant metabolism (including incidentally, serving as an electron transfer co-factor in tyrosine catabolism), development of assays for this unstable nucleotide and its companion NADH, will have a high priority in future studies This is particularly important in view of our previous demonstration that **methylene blue** is a most powerful *in vitro* stimulant of hexose monophosphate shunt activity. Since that redox dye links electron transfer with NADPH, it **could theoretically serve as an effective therapeutic agent for treating oxidant-induced hemolysis** as it is in humans and other animals for a number of related conditions.

(3) Observation continues to accumulate that suggest a re-investigation of rhinoceros hemoglobin is warranted. Most of these relate to its apparent susceptibility to oxidative conversion to methemogloblin (potentially reversible by methemoglobin reductase), or irreversible de-naturation into Heinz bodies. We have observed microscopically that oxidants can induce cell-rupturing crystalloids that could represent hemoglobin tactoids similar to those occurring in sickle-cell disease. That they might also represent tyrosine crystals makes further investigation of this phenomenon even more important.

(4) Development of techniques to accurately assay catalase and other enzymes in tissue preparations will require far more time than originally anticipated. Collaborative studies are planned with colleagues who already have cell cultures available, but many of the questions about a potential common denominator among all the clinically disparate disorders of black rhino cannot be answered until we develop the capacity to analyze fresh and frozen preserved tissues as well. Culture techniques for endothelial cells also need to be developed, for example, before we can determine whether the new hemorrhagic syndrome might be related to metabolic impairment at the vascular level.

In view of the questions remaining unanswered and the emerging areas for future investigation, we can envision pursuing these objectives well beyond the three years outline in our initial proposal, of which IRF has generously funded the first two years. We should be able to continue this research at the current level of support. **Therefore, we respectfully request that IRF consider continuing support of our research at approximately the same level for another 1-4 years. (Ed. Note: Current Level ~ $ 50,000/yr.)**

PATHOPHYSIOLOGIC BASIS OF DISEASES AFFECTING CAPTIVE BLACK RHINOCEROSES

PROGRESS REPORT - U. Cape Town Lab. Aug. 1997

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Captive Black Rhinoceros (*Diceros bicornis*) display high mortality from a haemolytic anaemia of unknown origin, together with disorders such as mucocutaneous ulceration with a possible common aetiology.

Two laboratories- the UCLA Hematology Research Laboratory and the University of Cape Town (UCT) Dept of Chemical Pathology, have combined their expertise and resources to provide a co-ordinated attack on the problem, with the former conducting primarily enzymological and the later primarily metabolic approaches to determine the pathophysiological basis for the anaemia Results from the UCT laboratory will be discussed below in the context of the original three objectives :

Objective 1) ***Determination of the nature, extent and significance of the uniquely divergent patterns of red cell metabolism characteristic of rhinoceroses.***

Comparative studies in other mammals are useful in placing the findings in black rhino red cells in context. Red cell nucleotide profiles were therefore compared in a range of species representing five mammalian orders, including rhinos, humans, cats, horses, cattle, and rabbits. ATP levels in black rhino red cells are known to be only 1/20 to 1/50 those of human red cells. We have examined ATP levels in white rhino and Sumatran rhino and shown that these are equally low. In horses, which are also in the same order, Perissodactyla, as rhinos, ATP levels are more like those in man, so very low ATP levels seem to be a characteristic of the family Rhinocerotidae. Interestingly each species showed unique features on their nucleotide profiles, horses had more CTP than other species, cattle had large quantities of a species confirmed as urate riboside, and rabbits had two to three times the amount of ATP as man or any of the other species. This unexpected and marked variation in red cell nucleotide profiles between species we suspect may be a consequence of adaptive changes to intracellular parasites. The very low ATP in rhinos, for example, may protect against protozoal parasites such as Plasmodium which need red cell nucleotides for their own DNA synthesis.

From the earliest stages of this work we were aware that both rhinos and cattle exhibit a major amount of a u/v absorbing fast-eluting material (substance X) on HPLC columns with A280/260 ratios similar to that of cytidine compounds. However in cattle it soon became clear that this species in fact represented urate riboside, which seems to be a species uniquely found in cattle red cells. We undertook radio-labelling studies in rhino red cells using either ^{14}C-uridine or ^{14}C-cytidine and found that both of these substrates were poorly incorporated and did not accumulate in the unknown substance X, implying that despite its spectral properties it was not a cytidine compound. Neither was it urate riboside as in cattle. An exhaustive search was therefore made for naturally occurring u/v absorbing compounds with the chromatographic properties of substance X and to our great surprise we found that the amino acid tyrosine corresponded in every respect to substance X. Its

elution properties on a number of different chromatographic systems, its u/v spectrum, and its reaction properties with a number of substances such as ninhydrin and hypochlorous acid confirmed that this was indeed the identity of substance X. Not only that, but quantitatively it was present in concentrations in the rhino red cell as high as 1 m molar, about 50 times the level found in human red cells (or in any other cells).

The presence of such high levels of tyrosine in rhino red cells therefore represents another unique feature of the rhinoceros and this finding has no precedent. No other mammalian cell, nucleated or otherwise, has such high levels of any aromatic amino acid. Examination of serum and nucleated cells (fibroblast cell cultures)showed normal (by human standards) levels of tyrosine. There is no known mechanism for concentrating tyrosine intracellularly in this way and it is highly unlikely that such a mechanism would have evolved without it having some selective advantage for the animal.

Possible functions for this red cell tyrosine are:
1) a buffer or osmotic effect, although the levels are probably not high enough for this;
2) a free radical trap;
3) an anti-parasite effect;
4) a transport function.

However, reference to the literature revealed some interesting observations with respect to tyrosine and free radical metabolism which may be highly relevant in the context of this investigation, and suggest possible functions under category 2 above for such high levels of tyrosine:

Activated macrophages secrete myeloperoxidase which can produce hydrogen peroxide, H_2O_2 , and from this the powerful oxidant hypochlorous acid, HOCl, (J.Biol.Chem 259 (1984) 10404-10413). Natural moderators of the toxic effect of HOCl are substances such as taurine, which reacts to form taurine chloramine, and it has been observed that the latter is transported into (human) red cells by the anion-transport system, there to be reduced by glutathione (GSH) back to taurine. Taurine can not escape from the red cell, so can accumulate to very high levels. It has been proposed (J. Biol. Chem. 260 (1984) 3321-3329) therefore that detoxification of taurine chloramine is an important function of red cells. In situations of high leucocyte to red cell ratios, as in inflammatory exudates, this activity could however exceed the detoxifying capability of the red cell and result in haemolysis.

The relevance of these observations to the present problem is the possibility that tyrosine in rhinos plays an analogous role to taurine in humans. It has already been observed that about 2% of the HOCl produced by neutrophils is accounted for by the formation of chlorotyrosine, albeit in protein bound form (FEBS Letters 379 (1996) 103-106). This, in an analogous way to taurine chloramine, could enter the red cell via the anion transporter and be reduced by GSH back to tyrosine, which might then be unable to escape. It may also be relevant that high levels of serum and erythrocyte tyrosine are found in a human inherited metabolic disease -tyrosinaemia type II- and can cause haemolysis! Reactive nitrogen oxide species such as peroxynitrite are also produced in pathological conditions such as inflammation and have been shown to form 3-nitrotyrosine upon reaction with free or protein-bound tyrosine(PNAS 94 (1997) 3211-3216), so chlorination of tyrosine is not the only reaction to consider..

Objective 2) Determination of the extent and precise cause(s) of susceptibility to oxidative stress exhibited by rhinoceros red cells.

The remarkably low levels of ATP in rhino red cells raises a number of questions:

- Is this only a feature of the erythrocyte ?

  We have measured ATP levels in rhino fibroblasts and found them to be the same as in other mammalian nucleated cells, so this feature seems specific to the red cell .

- Does the low level limit response to oxidative stress ?

  The results of the experiments to be described below suggest not, or at least not to a major degree.

- How does the red cell survive with such low levels of such an essential metabolite?

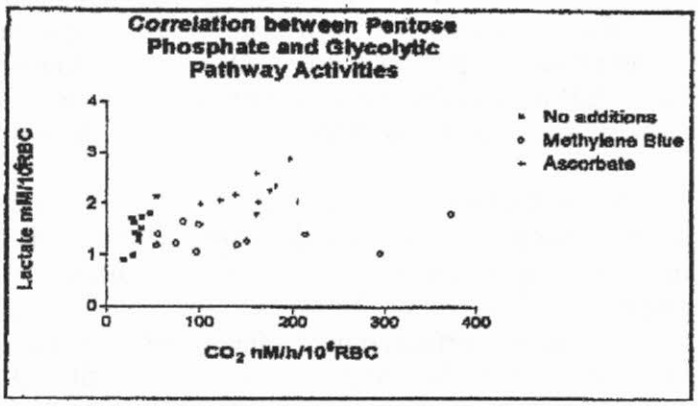
  The most likely answer is that there is no reason why a small pool of ATP turning over fast should not be as effective in driving ATP-utilizing reactions as a large pool turning over slowly. It even begs the question as to why nucleated cells need such relatively high ATP levels, with rhino red cells in healthy animals setting this unusual precedent. The answer could be that ATP is a substrate for low Km reactions, which by definition run at maximum velocity at low ATP levels, but when used for regulatory functions ATP is required at much higher concentrations. In the simple metabolic context of the red cells there are probably no such regulatory functions required. Studies are in progress to compare the turnover rate of ATP in rhino and other mammalian cells.

- Can ATP levels be boosted artificially to human levels?

  The answer is they can - incubation overnight with adenine and high phosphate concentrations resulted in ATP levels accumulating to levels equivalent to those in humans, and this result is being applied to treatment in vivo with oral phosphate, the results of which are being monitored in the UCLA lab. Studies will next be performed to measure how long elevated ATP levels can be maintained.

Measurements of $^{14}CO_2$ production after incubating red cells with ^{14}C-glucose provides a measure of hexose-monophosphate shunt (HMP) activity. Baseline levels in cells from humans, 13 opportunistically sampled wild black rhinoceroses (minor subspecies), 3 wild southern white rhinos and a captive Sumatran rhino showed that all the rhinos had activities about one third those in human red cells. Stimulation of HMP activity using methylene blue (which directly oxidizes NADPH -the first product of the HMP pathway) showed about a 12 fold stimulation in humans, compared with about 6-fold in rhinos. Stimulation with ascorbate (Vitamin C), which acts more indirectly by forming H_2O_2 in the red cell and by oxidizing glutathione (GSH, which is then itself reduced by NAPD) showed a somewhat different effect: human cells were stimulated six-fold and black rhino cells by a similar amount. Results from three white rhinos showed a lesser degree of stimulation, which needs further samples for confirmation, but if true could represent a beneficial effects of catalase: white rhinos remove H_2O_2 with catalase, but catalase deficient black rhinos need to use glutathione peroxidase to remove H_2O_2 , which requires NADPH generated from the HMP shunt.

In an extension of these studies in black rhino red cells the production of $^{14}CO_2$ was compared with production of lactate from glucose, which is a measure of flux down the glycolytic pathway and hence of ATP production. The results are illustrated below in the Figure and show graphically the stimulation of the HMP shunt by methylene blue and by ascorbate, but also show that whereas methylene blue has no effect on the glycolytic pathway, ascorbate produces a significant stimulation for comparable HMP shunt activity, and the requirement for additional ATP synthesis.



This experiment was repeated with 2-^{14}C-glucose which measures cycling of glucose through the HMP shunt and the results were similar to the above, but with enhanced MB and ascorbate effects, since the baseline $^{14}CO_2$ production was close to zero. These experiments have still to be repeated on white rhinos, which is somewhat problematical since it has so far been difficult to sample an appropriate number to give a comparable scattergram to that in the above figure.

Although differential susceptibility to oxidative stress has been seen in these experiments at a between species level, there has not been a clear-cut and repeatable differential within black rhino individuals, although the scatter in the above figure is quite marked. In contrast Paglia's studies indicate variability in, particularly, adenosine kinase and glutathione peroxides levels which might compound certain individuals susceptibility to oxidant-induced haemolysis. As a complement to these studies we have started to screen our population of rhino cell cultures for variability at microsatellite loci to give an indication of both the genetic diversity of black rhinos as a species, as well as the amount of differentiation between subspecies. For this survey we have the advantage of a large data base in the form of cell cultures accumulated over the last few years from 45 *Diceros bicornis minor*, 15 *D.b. michaeli*, 19 *D.b. bicornis*, 1 *D.b.longipes* (from Cameroon), *1 D.b.chobiensis* (from Angola), 28 *Ceratotherium simum simum* and 6 *C.s. cottoni* (from Garamba).

Objective 3) Generation of strategies for prevention and/or therapy of hemolytic crises and other diseases.

The increase in our basic knowledge of rhino red cell metabolism resulting from these studies is beginning to show new ways for generation of such strategies which would not have been previously apparent.

The haemolytic anaemia in captivity, and perhaps also the mucocutaneous ulcerative disorder, is looking increasingly likely to have its causation in an inability to cope with free radical damage. Evidence for this are the presence of Heinz bodies, the sluggish HMP shunt, the very low catalase, and the presence of unusual substances (tyrosine) which are known to play a role in free radical metabolism in other contexts. The precipitating event which results in the acute haemolysis is still not known but is likely to be dietary - either something essential which is missing, or something toxic specifically to the black rhinos unique red cell metabolism.

The ability to boost ATP to "normal" (for most mammals) levels is relevant only if higher levels are necessary to offset free radical attack, and there is little direct evidence to suggest that this is the case.

More significant may be to understand fully the role played by the extraordinarily high levels of tyrosine which we have recently found in rhino red cells. It seems likely that it represents a novel mechanism for coping with free radical attack, and therefore current and future experiments are being designed to test the possibility that rhinos have evolved a unique method of trapping free radicals based on reactions with free tyrosine. These experiments will require quantitative studies on the effect of free radicals and oxidizing agents, such as H_2O_2 and hypochlorous acid, on the levels of tyrosine, taurine, ATP and GSH in red cells of black and white rhinos as well as those of humans and other mammals. This will be the emphasis of work for the remainder of this year and the next.

In addition, these new insights will enable us to investigate red cells from rhinos undergoing haemolytic crisis (or suffering from mucocutaneous ulceration) in totally novel ways: we will be looking especially for qualitative and quantitative changes in red cell and serum tyrosine and taurine levels, which will not only help to confirm our suspicions as to the involvement of these metabolites in the haemolytic process, but will be the starting points for therapeutic strategies based on modifying how these metabolites may be utilized, replenished, enhanced, or substituted.

SUMMARY

To a large extent the first two of our objectives have been met, but as we find out more and more about the highly unusual metabolism of the rhino red cell, the more we are finding new questions to answer. These are relevant not only to the main purpose of this research, which is to apply the findings in novel treatment or prevention strategies, but by opening a Pandora's box of novel metabolic processes, are relevant to mammalian physiology generally, and suggest new insights and understanding especially of the mechanisms used by cells to protect against free radical and oxidant injury.

Totally new, but clearly relevant findings, such as the 50-fold elevation in red cell tyrosine, are requiring shifts of emphasis and novel experimental approaches. This will not enable the complete story of the mechanism of protection against oxidant damage and consequent therapeutic opportunities, to be completed this year. However, there is a clearly defined set of objectives relating to elucidating the role of red cell tyrosine and this should be achievable in one more year's work (taking us until the end of 1998). We would therefore like to build on what we consider are very exciting findings for both rhinos in particular, and mammalian metabolism in general, by requesting support at a similar level for one more additional year.

Personnel at UCT:
Eric H. Harley, M.D., Ph.D.,
Colleen O'Ryan Ph.D.(genetic aspects),
Brandon Weber, B.Sc.(metabolic aspects),
Ingrid Baumgarten, M.Sc.Med.Tech.,(cell culture).

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BLACK RHINOCEROS (*Diceros bicornis*) VETERINARY AND NUTRITION RESEARCH UPDATE

1997

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Black Rhinoceros Species Survival Plan
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Perhaps the "biggest" news in the past year is the fact that the veterinary and nutrition portions of a revised protocol for rhinoceros blood and tissue sample collection are near completion and will be distributed in the Fall of this year to all North American holding institutions.

Perhaps the most significant medical event for black rhinoceroses in the past year has been the occurrence of a clinical syndrome characterized by limb swelling and skin pathology. Histologically, the syndrome is characterized by a vasculitis. To date, 5 animals have been affected (one has had a recurrence) at the Dallas, Denver and Fort Worth Zoos, and at the Fossil Rim Wildlife Center. A more detailed description of the lesions appears below under the report of Dr. Richard Montali.

Also in 1997, Dr. David Kenny of the Denver Zoo, distributed a questionnaire on the occurrence of salmonellosis in rhinoceroses held in zoological parks. He had an 81% response rate. At the present time, he is compiling the data and he will distribute the resultant data to rhinoceros holding institutions.

In June 1997, a 22 year old female black rhinoceros at the St. Louis Zoo developed a nasal ulcer that was markedly hemorrhagic in nature. When her hematocrit dropped to 24%, attempts were made to cauterize the lesion with a CO_2 laser. That therapy was unsuccessful and the bleeding reoccurred. One week later, Dr. Randy Junge suggested cryosurgery of the lesion using liquid nitrogen. That therapy produced an immediate effect, and within 2 weeks, only a small scar remained. Thus, cryosurgery may offer another alternative for the treatment of severe mucocutaneous ulcers that are leading to blood loss.

Please remember that due to several allergic-like reactions that have occurred in the past several years, the vaccination recommendations for the 5-way leptospiral vaccine (Leptoferm-5, Norden Co.) have been amended to be done annually (no bi-annually as previously suggested). However, due to the evidence linking leptospirosis with approximately 50%+ of the acute cases of hemolytic anemia, we continue to recommend leptospiral vaccination.

Dental tartar continues to be a serious problem in adult black rhinoceroses, and all animals should have a thorough dental examination while anesthetized.

Please read to the end of this report, as the last entry (#10) is Dr. Dierenfeld's comprehensive report on a variety of ongoing nutritional projects. Finally, thank you to Dr. Evan Blumer, Research Coordinator for the Rhinoceros TAG for his assistance and support, and once again, to all who submitted samples and participated in the rhinoceros research projects in the past year.

1. Project: Comparative cell metabolism

Researchers: Drs. Don Paglia and Stephen W. Renner, UCLA School of Medicine, Los Angeles, CA 90095-1732, and Dr. Eric Harley, University of Capetown, Medical School Observatory 7925, Capetown, Republic of South Africa.

Assays of critical cell metabolites and enzymes involved in ATP generation, oxidant detoxification and nucleotide metabolism have been performed on 4 of the 5 extant species of rhinoceroses. These metabolic patterns now allow reliable distinctions to be made among these species and between the eastern and southern subspecies of black rhinoceroses (*D.b. michaeli* and *minor*). All species have been confirmed to possess uniformly low amounts of red cell ATP, suggesting that this extremely unusual finding is *not* directly related etiologically to hemolytic anemia or other disorders of black rhinoceroses, as had been previously postulated. Nonetheless, the extremely low reserves of this crucial high energy compound may be the proximate cause for the eventual failure of the red cell membrane's (ATP-fueled) cation pump, with consequent influx of water to the point of lysis. The effectiveness of oral and parenteral phosphate supplementation in raising red cell ATP concentrations continues to be documented. Studies of fractionated leukocytes indicate that other cells and tissues may also share some of the unusual metabolic features characteristic of rhinoceros erythrocytes.

At this point, the principal metabolic difference observed is in the relative catalase activity, black rhinoceroses uniformaly possess only 2-4% of the activities found in human red cells, whereas the three other rhinoceros species evaluated so far have activities intermediate between the two.

The role of the pyridine cofactor NADPH inactivating and stabilizing catalase is now under study, as is its role in regulation the metabolic influx of the pathway responsible for neutralization of reactive oxidants and free radicals and its role in the catabolism of tyrosine. The latter compound has been detected in high concentrations in rhinoceros erythrocytes by collaborative studies in Prof. Harley's laboratory, a study supported by a grant from the International Rhinoceros Foundation. If confirmed, this highly unusual finding could have major significance in understanding normal, as well as pathological changes, in rhinoceros red cell metabolism.

We have continued to collaborate with several groups nationally to investigate a new syndrome characterized by *non*hemoltyic anemia and hemorrhagic extravasation into soft tissues that has now affected at least 5 black rhinoceroses at separate institutions in Texas and Colorado (See report below by Dr. Richard Montali). Evidence has been accumulating for an immune-complex-mediated vasculitis, rather than a coagulopathy, and we have detected a possible cold agglutinin in one of the new cases, a monoclonal gammopathy by electrophoresis in two others, and erythrophagocytosis in an earlier case with similar clinical features. Additional sera from normal and affected animals are under study and a more extensive immunological and pathologic studies are being conducted and coordinated by Dr. Montali.

2. Project: Pathological review of black rhinoceros tissues

Researcher: Dr. Richard Montali, National Zoological Park, Washington, DC 20008

Further work on the "anemia/hemorrhagic diathesis syndrome reported in 3 black rhinoceroses last year (at Denver, Fort Worth and Fossil Rim) has indicated that it is not a true coagulopathy as there are no abnormalities in the clotting factors. Additionally, a definitive relationship with equine herpes virus (EHV) infection has not been established. A rising, but low EHV titer in the Denver animal was probably related to an incidental exposure during an outbreak in the equid collection. The other two black rhinos affected in 1996 showed no EHV titers nor was there any evidence of exposure to any other infectious agents including those that are often associated with vasculopathies like EIA, equine arteritis, blue tongue, EHD, and others. Over the course of several months, all three black rhinos recovered from the syndrome. A new case occurred at the Dallas Zoo in

a male black rhinoceros who presented in February 1997 with progressive limb and ventral body swellings from subcutaneous hemorrhage and edema, gingival ulcers, coronary band lesions, and anemia. Over a 5 month course, there was one relapse and eventual remission. During this time the animal appeared to be responsive to corticosteroids and supportive treatment. Periodic blood samples and biopsies of the skin/subcutis and oral/hoof lesions were taken for hematological and serological screening as well as for culture, histopathology, and numerous tests for possible immune-mediated diseases. Because the syndrome has some features of purpura hemorrhagica of horses (which is a post-infection immune-mediated vasculopathy usually associated with streptococcal infections), a search for immune complexes is being conducted by Dr. John Timoney at the University of Kentucky. Other immunological testing (for cold agglutinins, gammopathies, etc.) has been conducted by Dr. Don Paglia at UCLA as well as the Texas Veterinary Medical Diagnostic Laboratory and the University of Miami. To look for additional autoimmune changes, Dr. Montali has also initiated immunohistochemistry studies on biopsy material with Dr. Mark Mense at the Walter Reed Army Institute of Research. All of these studies are ongoing.

3. Project: Evaluation of the mucocutaneous ulcerative syndrome

Researcher: Dr. Linda Munson, School of Veterinary Medicine, Davis, CA 95616

A report of this syndrome has been submitted to and accepted by *Veterinary Pathology*.

4. Project: Routine hematological and clinical chemistry assays

Researcher: Dr. Steve Stockham, Colege of Veterinary Medicine, University of Missouri, Columbia, MO 65211

Samples are still being accepted and analyzed at no charge to the submitter. The previous data is in the process of being compiled and evaluated for publication.

5. Project: Measurement of cytokines (gamma interferon) in rhinoceroses as a diagnostic aid for tuberculosis.

Researchers: Drs. Mitch Bush and Janine Brown, Conservation and Research Center, Front Royale, VA 22630, and Dr. Terry Phillips, George Washington Medical School, Washington, DC 20037.

Gamma interferon has proven useful as a pre-mortem diagnostic

aid for identifying tuberculosis in cattle, deer and African buffalo. Since interferon is relatively species specific, it would be necessary to make an anti-rhinoceros gamma interferon and validate it before it could be used in the classical tests. Recently, we have been able to measure rhinoceros gamma interferon directly by using capillary electrophoresis. This procedure requires further validation, but promises to be a significant aid in understanding tuberculosis in rhinoceroses. To participate in this study, interested parties should mail 10-20ml heparinized blood via overnight express and at room temperature to: Dr. Terry Phillips, Immunohistochemistry Lab, 413 Ross Hall, 2300 Eye Street NW, Washington, DC 20037, 202-944-8858.

6. Project: Evaluation of ante mortem tests for bovine tuberculosis

Researchers: Dr. Mitchell Bush, Conservation and Research Center, Front Royale, VA and Dr. J.P. Raath, South African National Parks, Skukuza, RSA.

Diagnosis of tuberculosis in rhinoceroses remains confounded by the lack of a standardized, validated testing regimen for this species. This project proposes vaccinating a black rhinoceros scheduled for euthanasia (but which could be humanely delayed for 4-8 weeks) with BCG, and then monitor the animal with a variety of tests. Please contact Dr. Bush if such a rhinoceros becomes available at your institution.

7. Project: Evaluation of stress in rhinoceroses

Researcher: Dr. Mitchell Bush, National Zoological Park, Washington, DC

This project is designed to evaluate stress levels of captive, wild, and translocated rhinoceroses. By conducting an ACTH test with subsequent monitoring of cortisol levels in blood, urine, and feces, this project is designed to obtain parameters for evaluation of stress levels. To participate, please contact Dr. Bush.

8. Project: Blood gas monitoring of anesthetized rhinoceroses.

Researchers: Dr. Mitchell Bush, Conservation and Research Center, Front Royale, VA and Dr. J.P. Raath, South African National Parks, Skukuza, RSA.

If a rhinoceros requires anesthesia, this project requests that the resident veterinarian(s) consider oxygen insufflation of the trachea during anesthesia. The researchers would be interested in participating in the procedure and concurrently measuring blood gases and monitoring by pulse oximetry and indirect blood pressure.

9. Project: Basic evaluation of the immunological status of black rhinoceroses.

Researcher: Dr. Raymond Slavin, School of Medicine, St. Louis University, St. Louis, MO 63104.

Due to the incidence of fungal pneumonia and other diseases in the black rhinoceros that might suggest an immuno-compromised state, Dr. Slavin is proposing a project that would evaluate the following: *in vitro* lymphocyte proliferative response testing using a variety of specific and non-specific antigens, and evaluating aspergillus antibodies in affected and unaffected black rhinoceroses. A funding proposal has been submitted to the AZA's Conservation Endowment Fund (CEF).

10. Project: Baseline physiological data for assessment of nutrient status in rhinoceros: a comparative approach.

Researcher: Dr. Ellen Dierenfeld, Wildlife Conservation Society, Bronx, NY 10460.

1) Fatty acids: Adipose tissue samples (6 from black rhinos, 1 greater one-horned, 1 Sumatran) received by Dr. Craig Frank, Fordham University, have been evaluated via gas chromatography. Typically, animals feeding on a diet with appropriate levels of linoleic acid (C18:2) have depot fats containing greater than 5% linoleic acid. Five of eight samples analyzed contained linoleic acid concentrations below that threshold, indicating possible linoleic acid deficient diets. Only two of the rhino adipose tissue samples contained detectable linolenic acid (C18:3) concentrations, another possibly essential fatty acid found in natural browse. This data would indicate that fatty acid nutrition of rhinoceroses needs to be evaluated in much greater detail, and that diets should likely be supplemented to provide adequate levels of both of these essential fatty acids.

2) Mineral status: Mineral concentrations in livers from the frozen tissue bank have been assayed in 26 individual black rhinos, 6 white rhinos, 5 greater one-horned rhinos and 3 Sumatran rhinos at the laboratory of Dr. Morrie Craig at Oregon State University. A further set of

tissues (n=3) from captive animals remains to be evaluated, and in the coming year, comparative tissues from free-ranging. Data are being summarized and compared with normal ranges for domestic equids. Of note, Zn, Cu and Mo interactions were apparent in young animals (all < 10 days of age at death); Ca, Fe and Se concentrations were excessive in a high proportion of samples, and heavy metal accumulations were associated with specific institutions. Excesses of Fe, specifically, may be associated with oxidative damage and hemosiderosis reported in rhinos., and should be investigated in detail at specific institutions and/or with individual animals. Se is potentially toxic, and it is often administered with vitamin E, therefore supplementation regimens at individual facilities need to be reviewed and based in animal results. The two institutions with potentially toxic heavy metal concentrations were contacted, and in cooperation with their animal health staffs, more detailed assessment is underway. Mineral imbalances may underlay some of the disease problems identified in captive rhino populations, therefore, this baseline information may provide a valuable assessment tool. Normal mineral concentrations in rhinoceros plasma is also being characterized so that they can be used as pre-mortem indicators.

3) Fat soluble vitamins: Alpha-tocopherol, retinol, and carotenoid concentrations in both liver and tissue samples collected from all rhino species continue to be quantified at the Wildlife Conservation Society's Nutrition Laboratory, in association with Dr. Ed Norkus at Our Lady of Mercy Medical Center. Low circulating concentrations of both vitamin E (alpha-tocopherol) and vitamin A (retinol) appears normal in rhinoceros species and excessive supplementation of either nutrient is not recommended.

4) Tocopherol transfer protein: Following mineral evaluations, liver tissues were shipped from Oregon State University to Dr. Margaret Traber at the University of California-Berkeley for characterization of tocopherol transfer protein. This protein is responsible for distribution of tocopherol (vitamin E activity) into plasma lipoproteins for active transport in the body. Understanding these basic mechanisms of transport and delivery are essential for defining vitamin E metabolism and ultimately, its requirements in rhinoceros species.

5) Development of a pelleted diet: A pelleted diet containing nutrient specifications based on natural browses consumed by black rhinoceroses has been manufactured by Nutrena Feeds, Inc., with the support of

the White Oak Conservation Center. It is currently in palatability, digestibility, and efficacy trials in several facilities in the United States.

6) Investigation into skewed natal sex ratios in captive rhinoceroses in North America: A report was prepared for the International Rhinoceros Foundation (IRF) by Shirley Atkinson that summarizes current literature and data on factors that may be influencing sex ratios of black and greater one-horned rhinoceros populations in North America. While no discernible dietary patterns were identified, it was recommended that the role of nutrition should not be dismissed as an underlying factor for future investigation. Nutritional information provided by many North American institutions was not detailed enough to allow meaningful comparisons, but should be a priority. Detailed diet and nutritional information from European and Japanese facilities should also be included in future investigations.

7) Sumatran rhino intake and digestion: Intake, digestion and composition of browses offered to 3 Sumatran rhinoceroses are being investigated at the Cincinnati Zoo. This study is coordinated by Dr. Robert Wildman from the University of Delaware and Steve Romo (Cincinnati). The Los Angeles Zoo, the International Rhinoceros Foundation, and the Cincinnati Zoo are providing financial support for the project.

8) Fatty acids: Jaqualine Wright, a graduate student at Cornell University, has investigated fatty acid composition of fresh browses eaten by black rhinoceroses in Zimbabwe, and compared those with browses and diets fed to rhinoceroses in North American zoos and from El Coyote Ranch in Texas. n-6 linoleic acid, and n-3 linoleic acid have been detected in all samples, however, samples from Zimbabwe appear to contain significantly higher levels of n-3 linoleic acid than zoo samples. In addition, the Zimbabwean browses contain low levels of n-6 linoleic acid which was not found in zoo samples. Date ware being correlated with health aspects of individual rhinoceroses at various facilities - this thesis should be completed in 1997.

BLACK RHINOCEROS DEATHS IN NORTH AMERICA 1996-1997
(July 1996 - July 1997)

Diceros bicornis michaeli

STUDBK.#	SEX	DOB	DOD	CAUSE OF DEATH
192/ San Diego WAP	F	2MAY72	25APR97	Cecal and colonic volvulus, gravel impaction
328/ Denver	F	15NOV82	11SEP96	Undetermined, possible mineral (Cu?) imbalance
538/ Caldwell Zoo	F	20APR96	21OCT96	Self-inflicted head trauma after shipment
205/ Miami	F	9JUL69	14AUG97	Pulmonary lesions, aspergillosis suspect

LITERATURE CITED AND RECENT PUBLICATIONS

Atkinson, SJ: Maintenance of captive black rhinoceros (*Diceros bicornis*) on indigenous browse in Zimbabwe: energetics, nutrition and implications for conservation. MSc. Thesis, University of Zimbabwe, 1995.

Atkinson, SJ: Possible determinants of skewed natal sex ratios in captive black (*Diceros bicornis*) and Indian (*Rhinoceros unicornis*) rhinoceros in North America. Unpublished report, International Rhinoceros Foundation, Cumberland, Ohio, 50 pages, 1997.

Atkinson, SJ: Maintenance of captive black rhinoceros (*Diceros bicornis*) on indigenous browse in Zimbabwe: energetics and nutrition. Proc. AZA Nutrition Advisory Group, 1995.

Graffam, W, ES Dierenfeld, G Patillo, L Bass: Evaluation of 6 species of native Texas browses as suitable forage substitutes for black rhinoceros (*Diceros bicornis*). Proc. AZA Nutrition Advisory Group Conference, 1997.

BASELINE PHYSIOLOGICAL DATA FOR ASSESSMENT OF NUTRIENT STATUS IN RHINOCEROS: A COMPARATIVE APPROACH

International Rhino Foundation: Interim Report (August, 1997)
Ellen S. Dierenfeld, Wildlife Conservation Society

SUMMARY

Inadequate nutrition may underlie some of the health and reproductive management problems documented for rhinoceros in captivity, yet a dearth of information on adequate nutrient concentrations in either diets or animal tissues exists. This study was designed to evaluate mineral, fatty acid, fat-soluble vitamin, and carotenoid concentrations in biological tissues (blood, liver, and adipose) harvested from rhinoceros at necropsy in North American facilities, for comparison with similar samples from free-ranging rhinos and those available from European zoo populations, to meet specific objectives:

1) to establish intra-and interspecies differences in biochemical profiles for use in assessing nutrient status of rhinoceros populations in comparison with the horse as a domestic animal model and

2) to provide quantitative data to assist in health assessment of rhinoceros, and support the development of dietary guidelines for these species.

Even with no detailed dietary analysis as part of this study, physiological and long-term indicators of imbalanced mineral status and fatty acids were identified in rhinoceros (primarily black rhinos) from North American zoological facilities. These baseline data with captive rhinos must be supplemented with biological samples from other populations - most especially free-ranging rhinos and captive populations from other continents - to best interpret the results. We currently have no detailed dietary assessments from which these tissue concentrations originated, due to the retrospective and opportunistic nature of the sampling regimen. Future nutritional priorities should include in-depth dietary evaluation in rhinos, with a focus on both mineral and fatty acid components. In addition, more data on naturally-occurring levels of these nutrients in forages consumed by, particularly, the black rhinoceros, is needed.

Due to the collaborative nature of the analytical components of this project (i.e. spread over several laboratories), analysis of samples has not been as timely as originally envisioned. Additionally, efforts will be redoubled to obtain available samples from both free-ranging and European populations of rhinoceros for comparison, and an extension of 6 months is requested to complete the project as outlined. Expenditures to date, and future projections, are within the original approved budget.

SAMPLE ACQUISITION

No animal manipulations were necessary for this study. Tissues collected at necropsy and stored frozen as part of the AZA (American Zoo and Aquarium Association) Rhinoceros Taxon Advisory Group (TAG) were utilized. From an estimated original sample size of 35 individuals, we obtained livers from 29 black rhinos, 6 white rhinos, 5 one-horned rhinos, and 3 Sumatran rhinos (n=43). A request for tissues from the European zoo community was issued, with no response to date. Similarly, a protocol for tissue sampling was developed and distributed to field researchers with potential access to fresh tissues on an opportunistic basis; biological tissues apart from blood are not expected from non-AZA facilities at this time.

We anticipated frozen plasma samples from an estimated 60 individual zoo rhinos, and 100 free-ranging rhinos from 4 African locations. To date, we have received no free-ranging rhino plasma samples for comparative purposes; obtaining and analyzing this latter set of samples remains a focus for the coming 6 months.

Additionally, we were able to utilize 8 frozen adipose tissue samples (6 from black rhinos, 1 greater one-horned, 1 Sumatran) for assessment of fatty acids.

PRELIMINARY RESULTS

MINERAL STATUS:

Minerals are being quantified through the laboratory of Dr. Morrie Craig, Oregon State University. Unfortunately, we have received no comparative tissues from free-ranging rhinoceros; all zoo data are being summarized against ranges considered normal for domestic equids (Table 1). Of note,

Zn, Cu and Mo interactions were apparent in young animals (all < 10 days of age at death), which may indicate problems with maternal nutrition during pregnancy.
Ca, Fe, and Se concentrations were excessive in a high proportion of samples. High Ca may be associated with the hypophosphatemia reported in many captive rhinos, and underlies the dietary recommendation not to feed alfalfa as an exclusive forage. Excesses of dietary Fe, specifically, may be associated with oxidative damage and hemosiderosis reported in rhinos, and should be investigated in detail with specific facilities and/or individual animals. Se is potentially toxic, and is often administered with vitamin E without prior regard to selenium status. Supplementation regimes at individual facilities needs to be reviewed in detail based on animal results.
Two institutions with potentially toxic heavy metal concentrations in rhinoceros liver tissues (As, Cd, Pb) were contacted, and more detailed dietary and habitat assessment is under way in association with animal health staff at those facilities.

Mineral concentrations in rhinoceros plasma samples are found in Table 2, in comparison with domestic equid normal ranges.

Implications:

Mineral imbalances (some excesses, some deficiencies) are indicated from the available data; both diets and utilization of minerals should be investigated in greater detail in captive rhinoceros. No published study to date evaluates mineral intake or output of rhinoceros, and few data on mineral composition of native forages or mineral licks are available.

FAT-SOLUBLE NUTRIENT STATUS:

Alpha-tocopherol, retinol, and carotenoid concentrations in both liver and plasma samples collected from all rhino species continue to be quantified through the Nutrition Laboratory at the Wildlife Conservation Society, in association with Dr. Ed Norkus, Our Lady of Mercy Medical Center, Bronx, NY (Table 3). Carotenoids have not yet been quantified, as mineral nutrition was felt to be a higher priority, but will be completed within the coming 6 months. Low circulating concentrations of both vitamin E (a-tocopherol) and vitamin A (retinol) appear normal in rhinoceros species, and excessive dietary supplementation of either nutrient is not recommended.

Implications:

Suspected vitamin E deficiency in rhinoceros species appears to be unsubstantiated, and current circulating concentrations of this nutrient do not warrant excessive supplementation of diets. Vitamin E/Se-mixed supplements should not be administered without prior assessment of status for both nutrients.

FATTY ACID STATUS:

Available adipose tissue samples (n=8) have been evaluated by Dr. Craig Frank, Fordham University, via gas chromatography. While the original proposal stated that blood samples would be evaluated for fatty acid components, Dr. Frank advised that only red cell membranes (in blood) would provide useful assessment of fatty acid status. Since plasma, not cell membranes, comprise the available samples from the tissue bank, this component of the original design cannot be realized. Rhinoceros depot fat analysis is detailed in Table 4. Typically, animals feeding on diets with appropriate levels of linoleic acid (C18:2) have depot fats containing > 5% linoleic acid.
Five of eight samples analyzed contained linoleic acid concentrations below that threshold, indicating possible linoleic acid-deficient diets.
Further, only two of the rhino adipose tissue samples contained detectable linolenic acid (C18:3) concentrations, another possibly essential fatty acid found in natural browses. In the single literature report, linolenic acid is found at higher dietary concentration in black rhino browses than linoleic acid.

Implications:

These data would indicate that fatty acid nutrition of rhinoceros needs to be evaluated in much greater detail, and that captive diets should be analyzed in detail (and likely be supplemented) to ensure adequate levels of both these essential fatty acids. Only a single report of fatty acid composition in native rhinoceros browse is currently available, and little information on this nutrient group exists in the domestic animal literature. Fatty acid nutrition is intimately tied with general health, immune function, and reproduction in other species.

INTAKE AND DIGESTION IN SUMATRAN RHINOCEROS

An opportunity to monitor diets along with physiological parameters in the entire living North American population of Sumatran rhinos was realized, and supported with funding from the overall grant budget. Trials were conducted by Dr. Rob Wildman, University of Delaware, with assistance from the zoo staff, especially Steve Romo. A protocol was developed and implemented; intake and digestion in Sumatran rhinos housed at the Cincinnati Zoo was measured over a one-month period. Browses (n=10), hay, pellets, and salt blocks consumed by three individuals were quantified in triplicate trials; feed and fecal samples are currently under laboratory analysis. Body mass, and plasma samples were obtained simultaneously from animals to assist in nutritional evaluation of diets; a project report should be complete by Jan 98.

Implications:

While other intake and digestion trials have been conducted with animals in Indonesia fed natural browses, this study represents the only detailed trial conducted with North American animals and will provide direct comparisons of diet utilization compared with native forages. Additionally, simultaneous blood sampling along with the intake study will provide a direct link for interpreting circulating nutrient concentrations.

FUTURE DIRECTIONS:

DIET EVALUATION - US FACILITIES

Even though simultaneous diet evaluation was not conducted in association with baseline tissue analyses in this study, mineral and fatty acid imbalances were identified in specific individuals. Detailed diet evaluation (intake, analysis, digestion, utilization) is warranted and strongly encouraged, particularly for North American facilities with extreme values (which can be identified from this study). Each dietary component will cost approximately $150 to $200 per full assay (minerals, fiber, protein, fatty acids, amino acids, fat-soluble vitamins), with a minimum of 5 diet components (i.e. hay, 2 browses, pellet, produce). Blood evaluation for mineral and fat-soluble components would be approximately $50 per individual assessed. Six to ten institutions and/or individuals should be evaluated at a cost ranging from **$6000 to $10000** for analysis alone.

INTERCONTINENTAL DIETARY COMPARISON

Because rhinoceros health problems appear to be focused in North American facilities, detailed diet assessments across continents (Europe, Japan) may provide even further insight into appropriate dietary management for these animals. Thus dietary evaluation as described above, 2 selected institutions per continent, would provide excellent comparative data at an estimated cost of $6000 to $10000 for analysis, with an additional $2500 for a research assistant totaling **$8500 to $12,500.**

MINERAL COMPOSITION OF NATIVE BROWSES

Detailed evaluation of mineral constituents in native rhinoceros browses is limited in the literature. Minerals analysis would cost approximately $25/sample. A minimum of 30 to 50 rhino browses (possibly as many as 100 to 200 -- to correspond with natural diet diversity) should be evaluated: **$5000.**

Fatty Acids in Native and Substitute Browses
Further investigation of fatty acid constituents in native and substitute browses consumed by captive rhinos @ $50 to 100/sample X 30 to 50 browses: **$3000 to $5000.**

TOTAL ESTIMATED BUDGET FOR DETAILED ANALYSIS OF RHINO DIETS **$32,500**

(The most comprehensive study may be to fund a Master's thesis
or post-doctoral project to encompass these various aspects)

IRF GRANT R-96-2
BUDGET EXPENDITURES
DATE: AUGUST 1997

Oregon State University - partial payment for mineral analyses	$10,000.00
Oregon State University - partial payment for mineral analyses	6,000.00
Transportation Expenses - Sumatran Rhino trial	332.50
Rhino Husbandry Manuals to research collaborators	60.00
TOTAL EXPENDED TO DATE	**$16,392.50**

OUTSTANDING ANALYTICAL EXPENSES

Oregon State University - partial payment (mineral analyses & Medical Technologist)	$ 9,163.00
Sumatran Rhino feed sample analyses (13 samples X $150)	1,950.00
Sumatran Rhino fecal samples (9 samples X $100)	900.00
Carotenoids & vitamin analysis (40 livers @ $80)	3,200.00
50 plasma samples @ $40	2,000.00
Fordham University - fatty acids (6 adipose tissues @ $100)	600.00
Texas A & M University - fatty acids (50 plasma @ $100)	5,000.00
TOTAL ANALYTICAL COSTS BUDGETED NOT YET INVOICED	$22,813.00
Shipments, Office Supplies, Publication Costs	$ 2,536.50
AVAILABLE FORCONTINUED RESEARCH	$ 5,000

Note: All grant money $ 46,742 will have been transferred by 1 October 1997.

Note: non-U.S. samples have not been obtained yet; adipose tissue, but not liver, was utilized for fatty acids analysis (with only 6 samples available); total sample numbers estimated will likely be reduced from original budget estimate.

Table 1.
Preliminary Results
Mineral Concentrations in Rhinoceros Liver Tissues (Mean ±SD, μg/g), August 1997.

Element	White (n=5)	Sumatran (n=2)	Black (n=15)	Indian (n=4)	Horse
As (μg/kg)	31.4±17.1	36, bdl	most bdl one 630	most bdl	<400
Al	5.0 ±0.0	6.5±0.7	5.9±1.2	6.3±1.3	<5
Ca	67.0±12.1	69.0±8.5	120.0±39.0	84.±9.9	40-60
Cd	1.8±0.6	5.1±3.7	2.9±2.4	0.6±0.5	0.1-5.0
Co	0.4±0.04	0.9±0.5	0.7±0.4	0.3±0.2	<1.0
Cu	115.3±72.1	5.0±0.0	77.5±181.1	125.5±140.9	4-7.5
Cr	bdl	bdl	bdl	bdl	0.4-3.8
Fe	670±269.1	4960±6279	4075±4880	485±157.0	100-300
Mg	195±15	230±28.3	202.7±29.9	160±27.1	130-200
Mn	bdl	bdl	4 animals	bdl	1.0-6.0
Mo	0.9±0.3	2.6±1.9	4.5±5.7	0.5±0.6	0.5-6.0
Ni	bdl	bdl	3 animals	bdl	0.04-0.12
P	3000±340	3550±353	2980±643	2475±640	2600-4000
Pb	bdl	1 animal	5 animals	bdl	0.08-1.4
S	2350±189	2900±0	3007±500	2767±378	
Se	1844±1597	423±29.7	2287±2004	414±125	300-1000
Sn	bdl	bdl	bdl	bdl	
V	bdl	bdl	bdl	bdl	
Zn	102.5±21.8	58.5±0.7	97.7±42.8	73.5±45.7	40-125

bdl = below detection limit

Table 2.
Preliminary Results
Mineral Concentrations in Rhinoceros Plasma (Mean ±SD, μg/ml), August 1997.

Element	White (n=1)	Sumatran (n=1)	Black (n=11)	Indian (n=1)	Horse
Ca	150	117	124±19	120	100-130
Cu	3	3	3±1	4	0.85-2.0
Fe	1	2	3±1	1	8-25
Mg	23	13	21±7	21	18-35
P	32	16	37±9	30	27-50
Se	391	173	227±37	183	140-250
Zn	1.7	0.9	1.5±0.9	4	0.6-1.7

Table 3.
Preliminary Results
Retinol and Tocopherol Concentrations in Rhinoceros Plasma (Mean ±SD, μg/ml), August 1997.

Element	White (n=1)	Sumatran (n=1)	Black (n=11)	Indian (n=1)	Horse
Retinol	0.08	0.06	0.08±0.03	0.07	.
a-tocopherol	1.90	1.33	0.72±0.63	1.28	>2.0

Table 4.
Preliminary Results -
Fatty Acid Concentrations in Rhinoceros Adipose Tissue (as % Total Fatty Acids), August 1997.

Fatty Acid	Sumatran (n=1)	Indian (n=1)	Black (n=6)					
14:0	1.1		1.9	3.9	0.4	1.7	0.0	1.2
16:0	32.2	29.7	35.5	25.6	21.1	25.6	31.9	29.8
16:1	6.9		4.3	8.0	2.9	3.8	4.6	3.8
18:0	3.6	8.9	5.2	4.1	7.3	8.1	8.8	7.4
18:1	54.7	61.4	35.0	47.5	58.4	35.9	42.0	45.0
18:2 Linoleic	1.6		15.1	2.1	3.9	21.3	5.1	10.2
18:3 Linolenic						1.0		1.8
20:1					2.6			0.8
Unkn#1				1.5		2.7	4.3	
Unkn#2							3.3	

Black rhino diets for Kanazawa Zoo, Yokohama:

The zoo currently houses 1.2 rhinos, a breeding pair and offspring. The calf is approximately three years old and the female is pregnant again. During the day all three animals are exhibited together. At night the male is separated out to his own stall.

Browse

Browse is fed out year round and considered an important component to the animals diet. The male receives 7 kilos of leaves daily, the female and calf 9 kilos.
The following species of browse are offered:

Lithocarpus edulis - this species is offered the most, as it is available year round. It is also the rhinos' favorite.

Oak - they were unable to tell me the species

Myrica - this should be the genus name, again unable to tell me the species

Hay

Timothy hay is offered 2x daily to the female and calf. The male will not eat the timothy, so he is fed alfalfa.

Grain

Two types of pelleted feed are offered in a 50/50 mix, 1x daily. Both are milled in Japan. The first is a low protein grass-eater diet (called C-12):

Protein	12.0%
Crude fat	2.5%
Crude fiber	10.0%
Crude ash	9.0%
TDN	65.0%
DCP	9.0%

The order is made for racehorses, with much higher protein:

Protein	32.2%
Crude fat	2.4%
Crude fiber	9.0%
Crude ash	7.8%
TDN	28.8%

Supplements:

Vitamin D, calcium and salt

Alfalfa cubes and produce:

7 kilos of alfalfa cubes are offered to the male, 9 kilos to the female and calf. Produce, consisting of apple, carrot & sweet potato are also offered daily. The alfalfa and produce are not considered important to their diets, fed more as a treat.

WILDLIFE CONSERVATION SOCIETY
FOUNDED IN 1895 AS THE NEW YORK ZOOLOGICAL SOCIETY

23 October 1997

Thought you might enjoy a copy of the paper (presented as a poster) from the recent AZA Nutrition Advisory Group meeting held in Fort Worth, TX. It was very well received.

Thanks for your input to the success of this project!

Sincerely,

Ellen S. Dierenfeld & Wendy Graffam

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Evaluation of Eight Species of Native Texas Browses as Suitable Forage Substitutes for Black Rhinoceros (*Diceros bicornis*)

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[2] El Coyote Ranch, Fort Worth, TX

ABSTRACT

Duplication of natural foodstuffs for animals in captivity can be a difficult task. While it may not be possible to provide food sources normally available in an animal's natural habitat, a prospective goal might be to provide similar nutrients from locally available foods. Eight species of native Texas browse were studied for adequacy as a source of browse for black rhinoceros (*Diceros bicornis*) in captivity. Samples of browse were analyzed for moisture, crude and bound protein, neutral (NDF) and acid detergent fiber (ADF), lignin, vitamin E, ash, macrominerals and selected microminerals. Texas browse samples (whole plants) contained 42.5%-77.8% water, 7.5-24.8% protein, 0.81-2.43% bound protein, 30.1-61.6% NDF, 16.2-42.7% ADF, 5.8-22.5% lignin, 4.8-21.3% ash and 94.1-509.0 IU/kg vitamin E (all on a dry matter basis except water). Leaves contained significantly ($P<0.05$) higher protein and vitamin E than twig portions of the same plants. Twigs contained significantly higher NDF, ADF and lignin than leaves. Twig and leaf fractions did not differ in water or ash concentrations. Texas browses were compared to previously published values for black rhino browses from Zimbabwe and found to have similar concentration of nutrients. Overall, available Texas browses appear to be nutritionally adequate substitutes for the plants that black rhinos consume in nature, at least for the constituents evaluated.

Key words: Browse, rhino, fiber, minerals, vitamin E

INTRODUCTION

The black rhinoceros (*Diceros bicornis*), an extremely endangered browser, consumes a wide variety (often >100 species) of herbs, succulents, and woody plants throughout the year [Goddard, 1970; Mukinya, 1977; Hall-Martin et al., 1982; Oloo et al., 1994]. Grasses are not generally consumed except when accidentally taken with other browse items [Goddard, 1970; Mukinya, 1977]. Most captive rhinos are housed in settings where natural browse acquisition is limited, thus are generally sustained on a diet consisting of hay (grass, alfalfa or mixed) with herbivore pellets, produce and occasional browse [AAZK, 1988]. The AZA Rhino Taxonomic Advisory Group (TAG) dietary recommendations [Dierenfeld, 1996] for browsing black rhinos are to feed mixed grass:legumes hays and/or a mixture of legume hay and less digestible browse (rather than straight legume hay) as the forage source(s), with water and salt blocks available at all times.

Proceedings of the Second Conference of the Nutrition Advisory Group American Zoo and Aquarium Association on Zoo and Wildlife Nutrition. 1997.
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protein, 30.1-61.6% NDF, 16.2-42.7% ADF, 5.8-22.5% lignin, 4.8-21.3% ash and 94.1-509.0 IU/kg vitamin E (all on a dry matter (DM) basis except water). On average, leaves contained significantly ($P<0.05$) higher protein and vitamin E concentrations than twig portions of the same plants. Twigs contained significantly higher NDF, ADF and lignin values than leaves. Twig and leaf fractions did not differ in water or ash content.

Mineral analyses of are included in Table 2. Plants (whole) contained an average of 2.4% Ca, 1.5% K, 0.45% Mg, 0.14% Na, 0.11% P, 7.3 IU/kg Cu, 122.5 IU/kg Fe, 34.6 IU/kg Mn, 27.4 IU/kg Zn. Ranges for native browses collected in the Zambezi Valley, Zimbabwe, are also included in Table 2 for comparison.

DISCUSSION

Using horse and pony NRC [1989] dietary nutrient recommendations as a guide, protein requirements for maintenance of mature rhinos should be met with diets containing 8% CP (DM basis). Other physiological states (growth, pregnancy or lactation) would require higher-protein diets ranging from 10 to 15%. Leaves of the seven species analyzed contained considerably higher crude protein levels (14.3 to 43.7%, average = 22.7%), with <3.0% as chemically bound protein for any single species. Twigs contained less protein ($9.9 \pm 3.8\%$; mean $\pm$ SD), but were generally similar in CP content to many grass hays by comparison (6.4-12.9 % CP) [NRC, 1989]. Bound protein fractions were considerably higher (as a percentage of total CP assayed) in twig fractions versus leaves (7% bound in leaves compared with 25% in twigs); as much as 67% of protein measured in mesquite browse twigs was chemically bound, thus presumably unavailable from the diet.

Whole browses (leaves plus twigs) averaged approximately 15% available CP (CP less bound protein) in this study (range 9. 0 to 22.4%), comparable to values reported from other studies (4 to 22% of DM) [see summary in Dierenfeld et al., 1995]. Although browses can be high in CP, particularly leaf fractions, diets consumed by black rhinoceros in nature appear, in general, to contain a protein concentration similar to that of equid dietary recommendations [NRC, 1989].

Texas browses were rather fibrous and highly lignified, with leaves containing significantly lower levels of all fiber fractions evaluated than twigs. Despite these differences in total fiber content, the degree of lignification (lignin/NDF; approximately 30%) did not differ between leaves and the woodier twig samples. Because lignin constitutes a theoretically indigestible fiber fraction, cell wall lignification can be an indicator of the degree of fiber digestibility. From these data, both leaves and twigs may have limited digestibility. As with browses from Zimbabwe [Dierenfeld et al., 1995], the total fiber in these Texas samples was higher overall, and more highly lignified, than forages (hays) commonly fed to black rhinos in captivity. Despite the lower fiber and higher protein content of leaves in browses, leaves are not necessarily preferentially consumed by rhinos, with twigs up to approximately 3 cm in diameter completely consumed. Both total amount and type of dietary fiber may have important health consequences, as diets which are too digestible have been implicated as a possible cause underlying gastrointestinal problems in captive rhinos [Dierenfeld, 1996].

ADF, 14% lignin, 308 IU/kg vitamin E and adequate minerals compared with equine recommendations.

Depending upon habitat and season, black rhinos consume a wide variety of plants in nature. It is impossible to duplicate this type of diversity in most captive management situations, but nutrients contained within those dietary ingredients can be reproduced. The browses analyzed in this study contained concentrations of protein, fiber, vitamin E, and some macrominerals similar to those in plants which black rhinos consume in their native environments; however, other nutrients (Na, some microminerals) were limiting in these single samples, and must be supplied through other dietary ingredients. Nonetheless, significant nutritional contributions from available browses, provided as a staple ingredient in diets of browsing species, should not be discounted. Although not quite half of DM intake, browse contributed >55% of the protein and fiber, and about 25% of total vitamin E to the diet, accentuating the absolute need for more detailed investigations of the nutrient composition of browses in managed feeding programs.

CONCLUSIONS

1. Texas browses contained protein, vitamin E, calcium, potassium and magnesium concentrations which would meet dietary recommendations for domestic equids, and may be nutritionally adequate for the browsing black rhinoceros.
2. However, these same browses contained low levels of phosphorus, sodium, copper, manganese and zinc when compared to domestic equid requirements, and may be unsuitable as sources of these nutrients for black rhinos.
3. Rhinoceros diets should be evaluated based on, and balanced in relation to, composition of browses and other dietary components.

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Table 2. Mineral composition of whole native Texas browses fed to black rhinoceros at El Coyote Ranch (dry matter basis).

Plant	L:T ratio	Ca	K	Mg	Na	P	Cu	Fe	Mn	Zn
		< ---------- % ---------- >					< ------- IU/kg ------- >			
Acacia farnesiana	47:53	1.40	0.81	0.22	0.07	0.12	3.8	145	20.0	12.0
Acacia roemeriana	50:50	-	-	-	-	-	-	-	-	-
Acacia spp	48:52	0.79	1.71	0.2	0.01	0.15	16.1	100	33.5	18.6
Cassia fasciculata	73:27	-	-	-	-	-	-	-	-	-
Celtis pallida	72:28	4.15	2.04	0.66	0.03	0.13	6.1	104	38.2	13.1
Condalia obovata	61:39	1.40	1.50	0.47	0.04	0.05	3.2	100	21.7	5.1
Opuntia engelmannii		6.13	1.95	0.88	0.04	0.08	3.2	141	54.7	96.3
Prosopis juliflora	91:9	0.61	0.90	0.29	0.65	0.13	11.6	145	39.4	19.2
Average		2.41	1.49	0.45	0.14	0.11	7.3	122.5	34.6	27.4
SD		2.23	0.52	0.27	0.25	0.04	5.4	23.3	12.8	34.2
SSP recommendations[a]										
Growing		0.6	0.3	0.1	-	0.3	-	-	-	-
Mature		0.3	0.3	0.1	0.1	0.2	10	50	40	40
Pregnant/lactating		0.4	0.4	0.1	-	0.3	-	-	-	-
Zambezi native browse samples[b]		0.55-4.27	0.28-1.77	0.12-0.65	0.001-0.094	0.06-0.19	3.0-12.2	29.0-215	10.8-269	2.5-67.4

[a] Modified from NRC [1989] for horses and ponies
[b] From Dierenfeld et al. [1995]