(d) measurement of urinary PdG appears to provide a useful method for detecting mid-late pregnancy in rhinos. Further work is needed to establish tests for early pregnancy.

3. Monitoring of estrus cycles and ovulation.
   In contrast to the Indian rhino, attempts to monitor the estrus cycle in black and white rhinos by measurement of urinary estrogen metaboiltes and pregnanediol-3x-glucuronide have so far proved unsuccessful. Other methods need to be investigated.
4. New assay methodology.
   A new, simple microtitre plate ELISA (enzyme assay) for urinary pregnanediol-3x-glucuronide has been developed and validated for all three species of rhino.

UPDATE ON DEVELOPMENT AND APPLICATION OF REPRODUCTIVE TECHNOLOGY TO RHINOS

As head of the research team of the Cincinnati Wildlife Research Federation (CWRF) which is a combined effort of the Cincinnati Zoo, Kings island Wild Animal Habitat and the University of Cincinnati College of Medicine, Dr. Betsy Dresser reported on the development and application of reproductive technologies to rhinos. At the Cincinnati Zoo , there are two breeding pairs of black rhinos and they have produced 13 offspring to date. At Kings Island, there is a group of white rhinos, and four nonpregnant cows that are being worked with now in some areas of embryo transfer technology.

It is the hope of the CWRF to eventually be able to do embryo transfer within the white and black rhino species and also at some point attempt interspecies embryo transfer between the black and white rhinos. There is a lot of talk about embryo transfer but until actual manipulation of these animals is tried, working with them is a little more difficult than is first thought. So, first there is a need to determine if catheters can be physically inserted into the cervix and uterus manipulated before superovulation by hormones can be attempted.

As has been mentioned by other investigators, there is a need to be able to determine the estrus cycle in rhinos, It will be important to know when we can artificially inseminate on when to breed these animals before embryo transfer can be attempted. And then, after that, we have to know when embryos can be recovered. Also, embryo recipients will need to be hormonally prepared in order to establish a pregnancy. At Kings Island in Ohio, in an ongoing effort to develop embryo transfer technology for white rhinos, animals were first immobilized, placed in sternal recumbancy and rectally palpated to evaluate the reproductive tract. To date, it has been determined that uterus and ovaries could be palpated. but it is often very difficult. Ultrasound equipment is now being used to aid these efforts.

Specula are being developed in order to visualize the cervix for catheter insertion. A lengthy catheter has been developed for this procedure and attempts to flush the uterus with fluids are underway. Once superovulation techniques are pursued. embryo recovery techniques will be correlated.

Another technique that the CWRF team have been trying with rhinos came out of work that is being done with domestic cattle. It involves a small radio transmitter that sends out pulses. It has been used successfully in cattle to determine internal body temperature. It is inserted into the vagina and is similar to the method used to measure internal body temperature in women when they ovulate and there is a measurable increase in body temperature. When a cow's internal body temperature increases, these pulses increase and are received through a radio receiver. Dr. David Zartman, of Ohio State University. has inserted many of these into cattle. He custom-made the transmitter for the rhinos (larger that that used in a horse). The rhino cows have been monitored for at least six months and a trend does appear to be emerging.

Dr. Terry Blasdel. research coordinator for the Houston Zoo, has organized a program to produce offspring from white rhinos at the Houston Zoo by artificial Insemination. This project involves at least eight other zoos in North America, but had not yet begun at the time of the meeting.

Session Chairman ERIC MILLER

HEMOLYTIC ANEMIA IN THE BLACK RHINO

Summary of presentation by R. Eric Miller (St. Louis Zoological Park), co-authored by Hugh Chap//n (Washington University School of Medicine), Donald E. Paglia (University of Cal/torn/a at Los Angeles) and Will/am J. Boever (St. Louis Zoological Park).

Hemolytic anemia in the black rhino *(Diceros bicornis)* is a frequent occurrence and cause of death in the captive population of this species. Twenty-eight episodes of hemolytic anemia have been identified in 21 animals in zoos in North America, Europe and Japan. Eighty percent of the affected rhinoceroses have died during their initial or a recurrent episode of the anemia.

In man and in domestic animals, hemolytic anemia may result from a variety of factors that lead to a decrease in the survival time of the red blood cells (RBC's) and their early intra- or extra-vascular destruction within the body. Intravascular destruction of the RBC's leads to the release of their hemoglobin into the serum (hemoglobinemia) and may result in its passage into the urine (hemoglobinuria). The latter results in a clear, dark red coloration in the urine that is often the first sign that a black rhinoceros is developing a hemolytic crisis.

The case that occurred in St. Louis in 1981 (studbook 183/STL 6) was typical of the majority of the cases (8). A nine-year-old nulliparious female was noted to be weak and passing red urine. She was anesthetized for further evaluation, and blood values reflected a marked anemia — a haemocrit of 14.5% (normally 45-50%) (6). in other cases this value has ranged from 4.5% to 36**%** on initial presentation. Nucleated red blood cells — cells that in the horse are indicative of intensive efforts to replace the RBC loss—— were noted Similar findings, including regenerative bone marrow, have been found in two subsequent cases. The St. Louis animal died during attempts to reverse the anesthetic, no doubt complicated by the severe anemia present. Necropsy find-

ings were unremarkable except for massive deposition of iron in the liver (3 000 ppm) and the digestive tract. Similar iron deposition had been noted in previous cases, and in one animal without any signs of hemolysis. Further evaluation is warranted to determine if this reflects a subacute or chronic stage to the peracute form of cell destruction that is the hallmark of the syndrome described here.

A common cause to link the majority of the cases of hemolytic anemia has not been identified. Leptospirosis is strongly suggested in several cases (1,4), including one recent case (Osaka 209/LAX 5). Two cases were noted in Frankfurt that temporarily responded to steroids (7). Fatal hemic parasitism has been noted in newly captured wild black rhinoceroses (9), but its relationship to hemolytic anemia is unclear (2). No evidence for similar parasitism in captive animals outside of Africa has been noted to date, and titers for *Ehrlichia* sp. and *Babesia* sp. using reagents for domestic animals have been negative. Attempts to identify the agents of equine infectious anemia, copper toxicity, equine arteritis and clostridial infection have not identified any of these as possible causes of anemia in the black rhinoceros.

In a previous survey (8), respondents reported that they had kept 98 black rhinoceroses in captivity from 1972 to 1982. Twenty-five deaths occurred in animals greater than one year of age, and 11 of these deaths were associated with hemolytic anemia. Additional animals were located, bringing the total number of episodes to 28 ln 21 individuals. No sex ratio or seasonality is apparent. The greatest difference in age at death was noted between wild-caught (average 13.6 years) and captive-bred animals (average 7.0 years). Familial groupings were evident in one vertical grouping (mother-daughter-grandaughter) at the Frankfurt Zoo and multiple siblings from pairs at St Louis (three of four) and Denver (two of three). However, these three families appear unrelated to each other and only account for eight of the 21 affected individuals. At Toronto and Memphis Zoos, two and three cases occurred at one- and ten-day time intervals, perhaps suggesting a common agent or exposure. (indeed leptospirosis was strongly suggested at Memphis). However, at the majority of institutions single deaths occurred with apparently normal black rhinoceroses in the same enclosure or nearby. Despite the pre-mortem exposure of one animal to isoniazid and two others with an inadvertent exposure to the rodenticide diaphacinon, no common environmental exposure could be found.

Further efforts to identify a cause for the syndrome were directed at finding a “common denominator”, a basic defect that could lead a number of factors, e.g. leptospirosis or a toxin exposure, to trigger a massive hemolytlc event. A”two–fold approach was chosen: (i) evaluate basic RBC parameters of stability and a possible immune basis for the anemia, and (ii) an evaluation of the function of the RBC’s via a study of their enzymes and metabolites. The former approach was designed to evaluate the stability of the black rhinoceros RBC and the apparent response of several European animals to steroids. The latter study was designed to evaluate the RBC enzymes of the black rhinoceros due to several similar hemolytic syndromes in some human populations that are due to specific enzyme defects in their RBC’s.

For the first study, specific Coomb’s reagents for the black rhinoceros were developed (3). Using black rhinoceros sera inoculated into rabbits, both anti-black rhinoceros whole sera and a more specific anti-IgG were developed. Reactions with these reagents have been negative in all presumed normal animals, and one of the two anemic black rhinoceroses studied to date. in the second individual in a hemolytic crisis, the test indicated a possible coating of the RBC’s with the C3 component of the complement system. in man, this may occur in a number of chronic conditions and does not necessarily indicate an immune, basis to the disease. The reagents continue to be available for use in any future cases of hemolytic anemia. To facilitate their use, they will most likely be disseminated to centers in North America, Europe and Africa.

Additional studies (3) also indicated an increase in osmotic fragility of the black rhinoceros RBC in saline solutions in comparison to man. The haemaglobin electrophoretic pattern of the black rhinoceros indicated two bands at a pH of 8.6, the majority (80%) of the hemoglobin migrating slightly distal to the region of the unstable human hemoglobin H. The significance of both findings remains uncertain at this time. Electrophoretic patterns of RBC membranes of affected and unaffected individuals found no discernible differences between the two.

A separate study (10) evaluated the red blood cell enzymes and metaboiltes of aerobic glycolysis, glutathione cycling, and nucleotide metabolism. Ten animals were tested’— seven of East African origin, including two during hemolytic episodes, and one who was the dam of three affected individuals; and three apparently normal animals from southern Africa. Though the values found differed markedly from human normals, no differences were noted between apparently normal and affected rhinoceroses. Values were comparable to those found in a previous study of two rhinos (5). Further tests on an anemic Individual found no differences between the time of the hemolytic crisis and the convalescent period, nor was evidence of a heterozygous carrier state evident in the dam of the affected animals.

An interesting notation to this study was the variation between the animals of the eastern and southern origin in two of the enzymes studied. The seven samples from the eastern animals had only one third of the 2,3-diphosphoglycerate activity, and twice as much reduced glutathione in their RBC’s as did the southern animals (10).

Another area of possible importance to the etiology of hemolytic anemia is the overall nutritional status of the captive black rhinoceros population. Nearly an exclusive browser in the wild, captive diets for this species often predominate in feeds more closely approximating those of a grazer. Four captive black rhinoceroses were assayed for alpha-tocopherol levels. Levels were undetectable in two, and levels 0.2 ug/ml and 0.23 ug/ml were found in two additional animals. Selenium levels were 0.122 ug/ml to 0.170 ug/ml in the four animals. Further vitamin and mineral evaluation of the captive and wild animals is planned. Assays from wild animals are needed to supply standard values for animals on natural feeds.

Suggested treatment for the syndrome at the present time remains empirical: (I) high doses of penicillin or tetracycline if the case is acute leptospirosis or other infectious agent; (ii) vitamin E and selenium supplementation due to their role in the stability of red blood cell membranes; and (iii) possible use of short-acting steroids due to the apparent response of several European cases.

In all future cases, major emphasis must be given to the re-evaluation of each of the possibilities discussed —leptospirosis equine infectious anemia, copper toxicity, clostridial infect ion, hemic parasitism, undetected infectious agent or exposure to a toxin. Wherever possible, frozen tissue and serum

should be saved and stored at -75 degrees C for future reference.

Possible future avenues of research identified at the meeting include: (I) repetition of many of the previous tests on additional black rhinoceroses and also on white and Indian rhinoceroses, (ii) further evaluation of the immunological status of these animals in addition to the continued use of the Coombs reagent, (iii) further evaluation of the stability of the black rhinoceros RBC and its hemoglobin, (iv) evaluation of the iron metabolism of this species and attempts to identify a possible chronic stage of the anemia process, and (v) an overall evaluation of the nutritional status of this species In captivity. One emphasis of the latter study should be the determination of vitamin E and selenium levels in both captive and wild populations. The importance of a multi–faceted diagnostic approach was emphasized in a species in which so little is known. in man, with a much broader data base available, the cause of less than 50% of nonspherocytic hemolytic anemia is identified.

Since the syndrome has not been reported in white and Indian rhinoceroses, results from these species may help to establish a comparative data base for the black rhinoceros. A blood collection protocol for diagnostic and genetic studies in black, white and Indian rhinoceroses has been distributed to North American and European institutions holding these species (copies are available on request from the senior author).

Finding the specific etiology for the hemolytic crisis so frequent in the captive black rhinoceros population rests on further research in the areas enumerated above and perhaps others yet to be identified. The authors welcome suggestions of additional tests and approaches to this perplexing problem in the successful maintenance of this species in captivity.

Authors' notes

(i) Collecting large volumes of blood from the black rhinoceros can be difficult if the ear vein is used as the primary venipuncture site. Animals at St. Louis have been routinely bled from a large vein that passes over the medial carpus and ante-brachium. Though it is not always visible under the thick skin, a tourniquet applied proximally on the leg allows it to be palpated and cannulated. Up to one litre of blood has been collected rapidly from this site.

(ii) Since the Cincinnati meeting, an additional three adult (14.16 and 24 years of age) black rhinoceroses have died of hemolytic anemia in North America. The deaths occurred from November 2 to December 17, 1986. Preliminary laboratory data from these cases parallels that from previous hemolytic events. Two of the cases were tested with the autoimmune reagents described in this paper, and both were negative. Further tests are pending. No common factors could be identified to link the cases.

HAEMATOLOGICAL STUDIES OF BLACK RHINOS IN ZIMBABWE

Summary of presentation by Raoul du ToIt (IUCN African Elephant and Rhino Specialist Group), co-authored by Beverley Paul (University of Zimbabwe)

Various haematological studies were carried out with blood samples from 31 black rhinos that were translocated from the Zambezi Valley, Zimbabwe, in mid-1986.

In a field laboratory, within 3 hours of the collection of each sample, the following procedures were carried out haematocrit, white blood count, red blood count measurement of haemoglobin, plasma protein, erythrocyte sedimentation rate, and osmotic fragility; preparation of slides for differential cell counts, reticulocyte counts and parasite screening Additional blood samples from each animal were transported too Harare on wet ice, where standard blood analyses were performed on a Coulter counter (within at most 48 hours, and generally within 24 hours, of collection) in Harare, additional tests were carried out to investigation haemoglobin stability: isopropanol precipitation, heat test acidified glycerol lysis time test, and staining for Heinz bodies with methyl violet. Human blood specimens stored for similar periods were used as controls. Haemoglobin electrophoresis was performed on cellulose acetate Giucose-6-phosphate-dehydrogenase was assayed using a commercial kit (Sigma), which had been supplied by St. Louis Zoo.

The findings of these investigations are to be published (Journal of Zoology, in press). Consistent results were obtained from the standard haematological tests, an measurements of haemoglobin, haematocrit, and cell count conform closely with those obtained by veterinarians at Whipanade Park, using blood from a few captive black rhinos Thus it is felt that these data constitute reliable baseline information on the haomatology of the species Reticulocytes, not generally seen in rhino blood smeared occurred in some of the samples. The osmotic fragility of the red cells was somewhat greater than that of human red cells with 50% lysis occurring at a salt concentration of about 4.9 g/l. A significant observation was that all samples showed rapid precipitation of haemoglobin when inclubated will isopropanol. Heinz bodies could be demonstrated by methyl violet staining in up to 10% of fresh red cells. Very high levels of G-6-P-D activity were found in the red cells.

These results, Indicating an inherent tendency towards collapse of haemoglobin under oxidant stress, are obvious highly relevant to the problem of intravascular haemolysis. It seems unlikely that there is any single agent responsible for triggering haemolysis episodes; these are probably the end result of a variety of oxidant stresses.

There are indications that some die-offs of black rhinos in the wild could be related to haemolytic anaemia (e.g. about 30 rhinos died in Tsavo National Park in 1960-61, due to what was tentatively described as "'nutritional anaemia"). With wild animals, it would be worth investigating if parasitaemia aggravated by Inadequate nutrition, capture stress and other debilitating factors, is associated with haemolytic anaemia-abnormal haemoglobin and red cell enzyme systems may have developed in rhino as an evolutionary response to parasitaemia (as with sickle cell anaemia and possibly G-6-P D deficiency in humans), but under extra physiological stresses the balance could tip towards excessive haemolysis The Zambezi rhinos, from which blood samples were taken. were translocated to another reserve in Zimbabwe, where at least 20% of them died some weeks after translocation. in the pathological examinations that were carried out on a couple of sick and dead rhinos in this group, an unidentified piroplasm parasite was found In blood smears to a greater extent than in biood smears taken at the time that the animals were first captured, and large amounts of haemosiderin were found in spleen and liver tissue. This indicates a possibility of the mortality being due to stress-induced parasitaemia and a degree of haemolytic anaemia (although it has also been suggested that the deaths were due to the use of the drug ivermectin, for controlling skin and gut parasites). Further