Estrogen Excretory Patterns in the Indian Rhinoceros (*Rhinoceros unicornis*), Determined by Simplified Urinary Analysis

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SUMMARY

Daily urine samples obtained at random from 3 mature Indian rhinoceroses (*Rhinoceros unicornis*) were evaluated for immunologic estrogen concentration and were indexed by creatinine (Cr) concentration. Chromatographic separation and enzyme hydrolysis of the estrogenic components indicated that the major urinary estrogen in this species is immunoreactive estrone (> 95%). Cyclic variations of immunoreactive estrogens (basal values of 0.1 to 0.3 μg/mg of Cr; peak values of 5.8 to 12.5 μg/mg of Cr) were observed at an interval of 43 ± 2 days (4 cycles) which appear to reflect the apparent ovarian cycles. The method presented offers a practical approach for the monitoring of ovarian events in large, nontractable species.

Assessment of reproductive failure in exotic species is complicated by the lack of physiologic data, due to the difficulty in obtaining proper samples for evaluation. Establishing normal circulating hormone profiles and collecting blood samples from nontractable species are major problems. At a time when diminishing populations in the wild dictate efficient captive breeding programs, methods must be developed to assess and monitor reproductive events to ensure optimal reproductive success in captivity.

The purpose of the present study was to report urinary estrogen patterns in the Indian rhinoceros and to offer preliminary data on the female rhinoceros reproductive cycle.

Materials and Methods

Urine samples were collected daily from 3 adult female Indian rhinoceroses (*Rhinoceros unicornis*). Animals 1 and 2, which were considered to be cycling normally, were housed at the Los Angeles Zoo. Animal 3, which had a gastrointestinal disorder, was housed at the National Zoo in Washington, DC. Daily urine samples were collected at random between Nov 8, 1978, and Apr 6, 1979. Animal 1 was monitored over an 80-day period from Jan 17, 1979, to Apr 6, 1979, animal 2 was monitored over a 68-day period from Jan 17, 1979, to Mar 25, 1979, and animal 3 was monitored over an 89-day period from Nov 8, 1978, to Feb 4, 1979. Urine samples were collected directly in midstream while the animal was urinating or were aspirated from the trough that had been placed in the animal's enclosure. Immediately after collection, urine samples were separated into 3 equal aliquots and were frozen (−20 C) until assayed by a radioimmunoassay for immunoreactive estrogen in urine.[1]

Behavioral estrus for animals 1 and 2 was reported by the keeper staff of the Los Angeles Zoo and this appraisal was based on the keepers' experience with these animals. Clear demonstration of estrus was not reported for animal 3 during the collection interval.

Hormone Assays—Urinary creatinine (Cr) content was estimated,[2] and the value was divided into the urinary estrogen concentrations to compensate for collection errors resulting from variable fluid intake and output and from variable periods of collection.[3] Urinary values of total immunoreactive estrogen (E_T) and of immunoreactive estrone (E_1) are therefore expressed as micrograms per milligrams of Cr.

Urine (0.1 ml) was diluted with 0.4 ml of phosphate buffer (pH 5.0) before hydrolysis with 0.02 ml of β-glucuronidase-arylsulfatase[a] and was incubated at 37 C for 24 hours. Tritiated E_1 [5,000 counts per minute (cpm)] was added to the hydrolysate to monitor losses incurred during extraction with 5 ml of freshly opened anhydrous diethyl ether.[b] The ether extractants were dried under nitrogen, reconstituted in 1.0 ml of phosphate buffer (pH 7.0), and aliquoted for recovery determination and assay. An aliquot of 0.1 ml from each reconstituted sample was used for recovery evaluation.

The assay was performed by incubating 0.25 to 5 μl (ie, serial dilution of 1:4,000 to 1:200) of the reconstituted sample (made up to 0.5 ml with phosphate buffer, pH 7.0) with 10,000 cpm of [^{3}H]E_1 in 0.1 ml of phosphate buffer and an equal volume of estriol (E_3)-trisuccinyl directed antiserum.[4,c] After overnight incubation at 4 C, separation of antibody-bound steroid was achieved by the addition of 0.2 ml of charcoal-dextran (0.625% and 0.0625%, respectively, in phosphate buffer, pH 7.0) for 30 minutes at 4 C, and the supernatants were counted after centrifugation at 2,500 rpm for 10 minutes. Unknowns were compared with E_1 standards incubated in triplicate over the range 5 to 1,000 pg.

The intraassay coefficient of variation (CV) was obtained by evaluating 6 replicate urine samples in the same assay giving a value of 15.6% over the range of 12% to 15% bound. The between-assay CV was obtained by evaluating replicates of 2 individual

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[a] Boehringer, Mannheim, Germany.

[b] Mallinckrodt Inc, St Louis, Mo.

[c] Antiserum S310 No. 5, obtained from Dr. G. E. Abraham, Division of Reproductive Medicine, Harbor General Hospital, Torrance, Calif.

samples in 8 and 5 assays, respectively, giving a value of 19.2% (n = 8) and 14.6% (n = 5) over the combined range of 11% to 70% bound.

Determination of the major estrogen in rhinoceros urine was performed on 3 samples from normally cycling animals 1 and 2. Values for E_T from hydrolyzed urine samples, as indicated by the present assay system, were compared with the measurements of E_1 and estradiol (E_2) components of the same urine samples, as determined by chromatographic separation and the appropriate assay procedure. After hydrolysis of 0.5 ml of each urine sample, the hydrolysates were separated into four 0.1-ml aliquots, 2,000 cpm each of [^{3}H]E_1 and [^{3}H]E_2 were added to 2 of the hydrolysates to monitor for methodologic losses and extraction with ether, performed as before. The extractant was subjected to celite column chromatography (ethylene glycol:celite, 1:2 v/v,[5] and the appropriate E_1 and E_2 fractions were collected and assayed, using E_1 standards and tracer, and E_2 standards and tracer, respectively.

To monitor for losses incurred during extraction, 2,000 cpm of [^{3}H]E_1 was added to the remaining 2 hydrolysates (0.1 ml aliquots each). The ether extractants were blown dry with nitrogen, reconstituted in 1.0 ml of phosphate buffer (pH 7.0), and assayed for E_T.[1]

Two to 4 urine samples from each of the 3 animals were evaluated for free estrogen by enzyme hydrolysis. Aliquots (n = 2) of 0.1 ml of urine from each of the samples were diluted with 0.4 ml of phosphate buffer (pH 5.0); 0.02 ml of β-glucuronidase-arylsulfatase was added to 1 of the aliquots for enzyme hydrolysis; enzyme was not added to the other aliquot (control). Both aliquots were incubated at 37 C for 24 hours. Upon completion of the incubation, both aliquots of urine were treated and assayed for E_1 as described previously.

High Pressure Liquid Chromatography (HPLC)—Two separate urine samples (0.1 ml each) were incubated with 0.02 ml of β-glucuronidase-arylsulfatase for 24 hours at 37 C. Before extraction, 0.1 ml each of [^{3}H]E_1, [^{3}H]E_2, and [^{3}H]E_3 (2,000 cpm each) was added to each hydrolysate to monitor losses incurred during extraction. Samples were extracted with 5 ml of freshly opened anhydrous diethyl ether, blown dry with nitrogen, and reconstituted in 0.05 ml of acetonitrile.[b] Reconstituted samples (20 μl) were separated on a 3.2 × 250 mm column,[d] using acetonitrile: water (40:60 v/v) as a solvent system. Fractions were collected at 0.3-minute intervals for 12 minutes at a flow rate of 0.1 ml/minute, blown dry with nitrogen, and reconstituted to 1-ml final volume in assay buffer (phosphate-buffered saline solution, pH 7.0). Each reconstituted sample was divided into 2 aliquots (0.4 ml each). The 1st aliquot was evaluated for the recovery of [^{3}H] labels, and the 2nd aliquot was assayed for total estrogen immunoreactivity as described.[1]

Gas-Liquid Chromatography (GLC)—The urine sample (0.1 ml) was incubated with 0.02 ml of β-glucuronidase-arylsulfatase for 24 hours at 37 C. Before extraction, 0.1 ml of [^{3}H]E_1 (2,000 cpm) was added to the hydrolysate to monitor losses incurred during extraction, and the hydrolysate was extracted with 5.0 ml of freshly opened anhydrous diethyl ether. Isooctane (1.0 ml) was added to the extractant, and the resulting mixture was reduced to a volume of 0.5 ml with nitrogen. The sample was then returned to a 1.0-ml volume with isooctane and was subjected to celite column chromatography [ethylene glycol:celite (1:2 v/w)].[5] The celite fraction [3.5 ml of 15% ethyl acetate in isooctane (mobile phase)] was blown dry under nitrogen and was reconstituted in 0.01 ml of carbon disulfide.[b] This sample was subjected to flame ionization after GLC, using 200 cm × 2 mm glass columns with packing[e] at 270 C oven temperature and nitrogen carrier gas at a flow rate of 20 ml/minute, and was compared with pure crystalline E_1.[f]

[d] Licrosorb C18, Altex No. 254-69, Cole Scientific, Calabasas, Calif.
[e] Packing [3% OV 17 (80/100)], Applied Science, State College, Pa.
[f] Sigma Chemical Co, St Louis, Mo.

Results

Celite column separation was performed on four 0.1-ml aliquots of hydrolyzed urine from each of the 3 animals. Chromatographic separation and individual measurements of E_1 and E_2 in urine samples from each of these animals were performed to assess their relative contribution to the level of E_T (Table 1). Estrone represented 92% to 100% of E_T in all samples evaluated. Because these samples represent high, medium, and low E_T, these results indicated that E_1 is consistently the major estrogen catabolite in all stages of the cycles and should reflect total estrogen excretion.

Celite column cochromatography of radioactive E_1 and E_2 and immunoreactive E_1 and E_2, respectively, gave profiles indicative of single pure peaks in each fraction (Fig 1).

TABLE 1—Concentrations of E_1 and E_2 and Their Relative Contribution to E_T in Urine Samples from Three Indian Rhinoceroses

Sample	E_T	E_1	E_2
ANIMAL 1			
1	8.06	7.63	0.40
2	9.47	8.76	0.30
ANIMAL 2			
1	2.51	2.32	0.07
2	2.13	2.13	0.05
ANIMAL 3			
1	1.87	1.87	0.05
2	1.87	1.87	0.05

Data expressed as μg/mg of Cr.

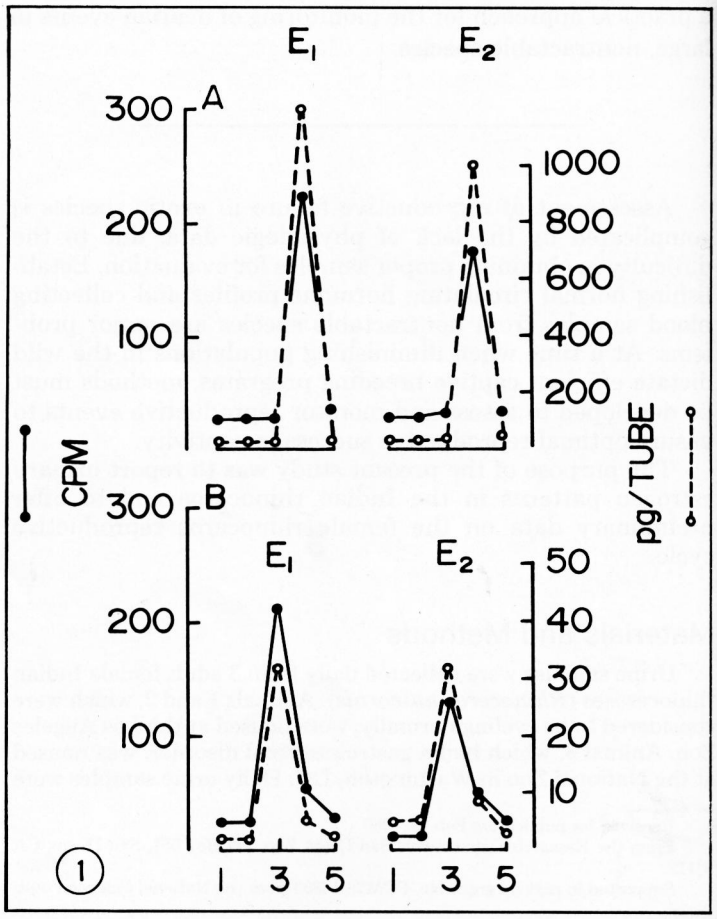

Fig 1—Celite column cochromatography of radioactive E_1 and E_2 (●—●) vs immunoreactive E_1 and E_2 (○—○) of random urine samples from 2 Indian rhinoceroses (A and B).

Furthermore, continuous elution patterns for HPLC cochromatography of tritium-labeled vs immunoreactive E_1, E_2, and E_3 from 2 urine samples demonstrated the presence of a single major immunoreactive peak corresponding to the elution pattern of tritiated E_1 (Fig 2).

The GLC evaluation of celite column E_1 fraction gave further support for the purity of E_1 in substantial quantities in this celite fraction of hydrolyzed rhinoceros urine. Taken together, the separate evaluations indicated that the major immunoreactive urine estrogen component is at least 95% E_1 (Fig 3).

The E_T in enzyme-hydrolyzed vs heat-control urine samples are compared in Table 2. In all instances, hydrolysis resulted in estrogen values 70 to 900 times higher than values in heat-control urine samples, indicating that urinary estrogens are more than 99% conjugated in this species. Thus, well over 95% of E_T in this species is represented as a form of conjugated E_1.

Profiles for the Indian rhinoceroses that exhibited overt estrus (animals 1 and 2) are shown in Figures 4 and 5. Basal values of urinary estrogens in both animals ranged from 0.1 to 0.3 μg/mg of Cr, whereas peak estrogen values, at or before the time of behavioral estrus, ranged from 5.8 to 12.5 μg/mg of Cr for animal 1 and 5.3 μg/mg of Cr on day of behavioral estrus peaking at 7.6 μg/mg of Cr on day 3 after estrus for animal 2. In the 3 complete estrous periods, urinary estrogen began to rise 8 to 11 days before behavioral estrus, reaching peak values 1 day before overt estrus in animal 1 (2 estrous cycles) to 3 days after overt estrus in animal 2 (1 estrous cycle). The periods shown in Figures 4 and 5 as representing sexual receptivity are based on observation of female behavioral patterns and do not reflect the ultimate test of receptivity, ie, male-female contact. These periods averaged 3 days (n = 5, range 2 to 5 days). The mean interval between overt behavioral estrus was 43 days (n = 4, range 41 to 45 days), whereas the mean base-line estrogen interval was 29 days (n = 2, range 27 to 31 days). Thus, estrogen excretion was accelerated for a period of 13 to 18 days (n = 2) beginning to increase approximately 10

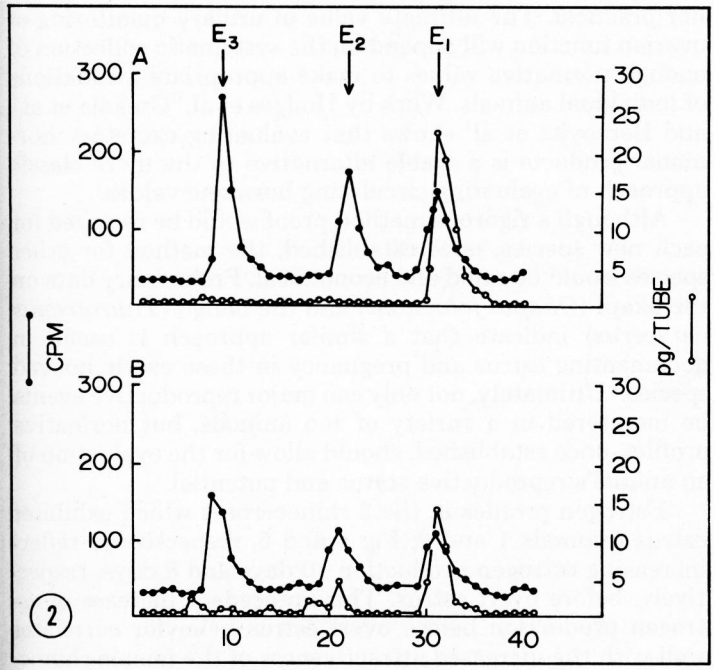

Fig 2—Continuous elution patterns of HPLC cochromatography of radioactive E_1, E_2, and E_3 (●—●) vs immunoreactive E_1, E_2, and E_3 (○—○) of separate urine samples from 2 Indian rhinoceroses (A and B). Fractions were collected at 0.3-minute intervals for 12 minutes at a flow rate of 1.0 ml/minute.

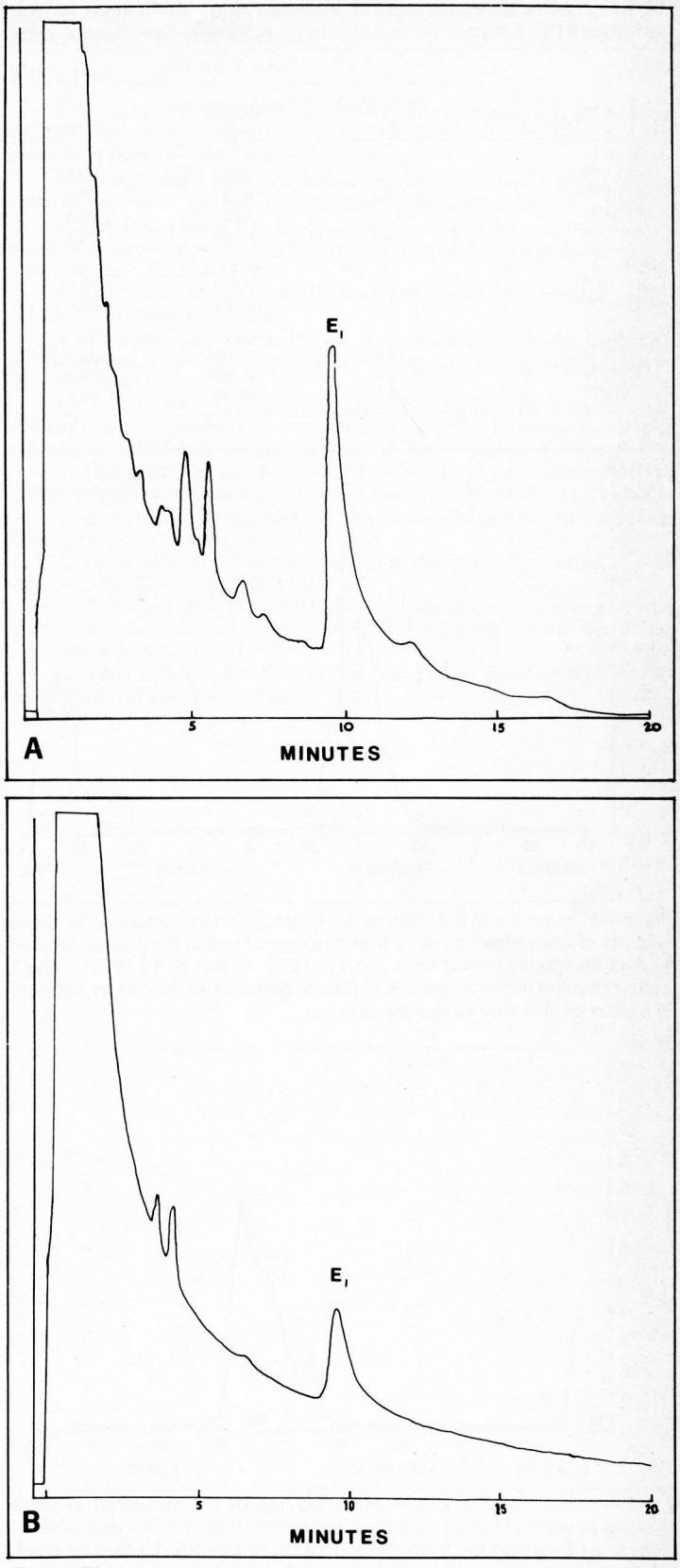

Fig 3—The GLC elution patterns of pure crystalline E_1 (A) compared with a urinary extract that had previously been subjected to celite column chromatography (B).

TABLE 2—Comparison of E_T in Hydrolyzed (Enzyme-Treated) vs Nonhydrolyzed (No Enzyme) Urine Samples from Three Indian Rhinoceroses

Date	E_T (μg/mg of Cr) Hydrolyzed urine	Nonhydrolyzed urine	% Conjugated
ANIMAL 1			
02/15/79	3.2	0.017	99.5
02/19/79	8.3	0.01	99.5
02/20/79	11.0	0.019	99.8
02/23/79	5.2	0.016	99.7
ANIMAL 2			
01/17/79	3.7	0.052	98.6
01/19/79	1.3	0.0046	99.6
ANIMAL 3			
12/15/78	4.2	0.0075	99.8
12/25/78	5.4	0.0059	99.9
01/01/79	5.0	0.025	99.5

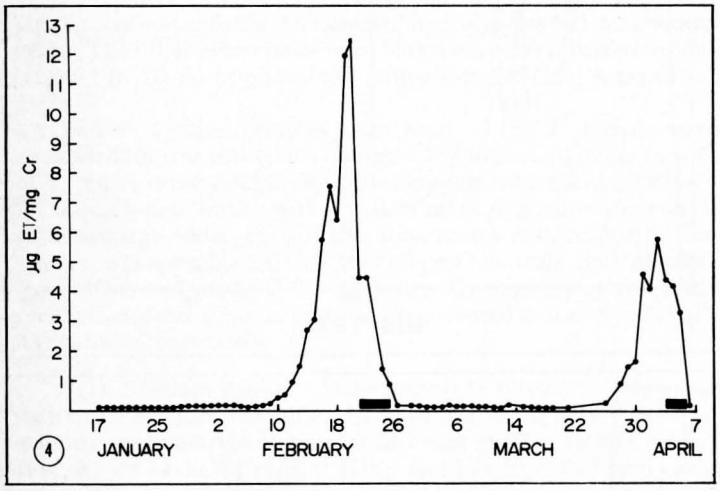

Fig 4—Immunoreactive profile of E_T (μg/mg of Cr) measured in small aliquots of urine obtained daily from the normal Indian rhinoceros (animal 1) over an 80-day period from Jan 17, 1979, to Apr 6, 1979. The black horizontal bars indicate periods of female behavior as judged by keepers to be compatible with sexual receptivity.

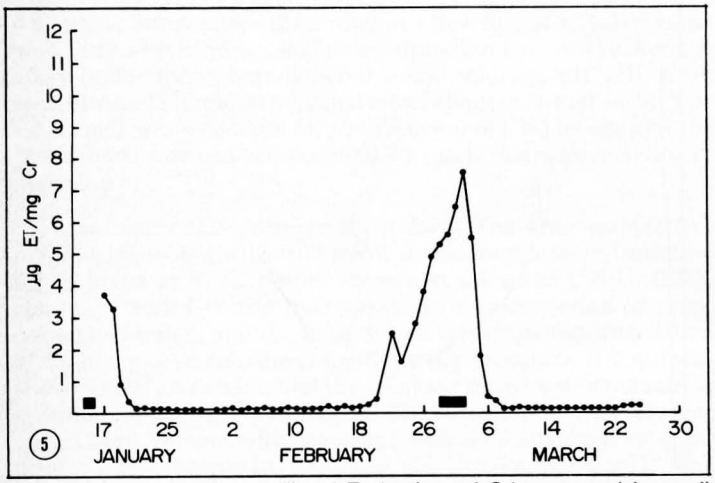

Fig 5—Immunoreactive profile of E_T (μg/mg of Cr) measured in small aliquots of urine obtained daily from the normal Indian rhinoceros (animal 2) over a 68-day period from Jan 17, 1979, to Mar 25, 1979. The black horizontal bars indicate periods of female behavior as judged by keepers to be compatible with sexual receptivity.

days before overt behavioral estrus and falling to base-line values between 3 and 8 days after estrus in all reported cases.

Figure 6 represents the urinary estrogen profile of the

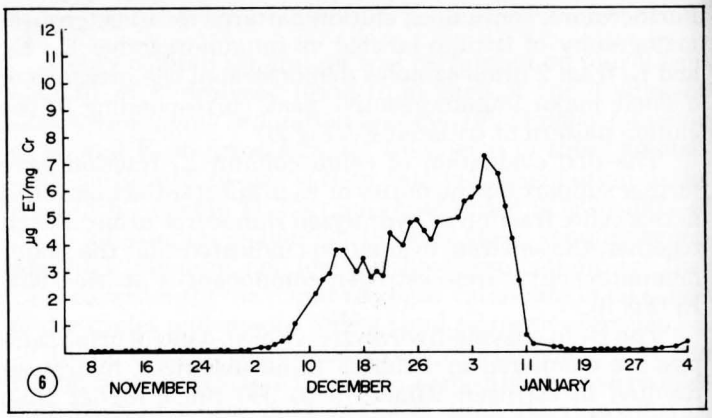

Fig 6—Immunoreactive profile of E_T (μg/mg of Cr) measured in small aliquots of daily urine from the Indian rhinoceros with chronic intestinal disorder (animal 3) over an 89-day period from Nov 8, 1978, to Feb 4, 1979.

rhinoceros that had chronic intestinal disorder (animal 3). Basal values of estrogens in this animal ranged from 0.1 to 0.2 μg/mg of Cr before increasing for 10 days, reaching values of 3.8 μg/mg of Cr. Unlike the previous 2 animals, however, estrogen values did not decrease but plateaued for 1 week, then increased to 7.3 μg/mg of Cr 15 days later. Values then decreased to previous base-line values and remained there for the duration of the study.

Discussion

The present results demonstrate the potential value of random urine collection and evaluation in the documentation of ovarian function in large, nontractable species in which the traditional method of blood collection for the evaluation of circulating hormones is neither appropriate nor practical. The ultimate value of urinary monitoring of ovarian function will depend on the systematic collection of enough normative values to make appropriate evaluations of individual animals. Work by Hodges et al,[1] Czekala et al,[6] and Bercovitz et al[7] shows that evaluating excretory hormonal products is a viable alternative to the more classic approach of evaluating circulating hormone values.

Although a rigorous method proof would be required for each new species, once established, the method for other species would be rapid and economical. Preliminary data on the okapi (*Okapia johnstoni*) and the bongo (*Taurotragus eurycerus*) indicate that a similar approach is useful in documenting estrus and pregnancy in these exotic hooved species.[8] Ultimately, not only can major reproductive events be monitored in a variety of zoo animals, but normative profiles, once established, should allow for the evaluation of an animal's reproductive status and potential.

Estrogen profiles in the 2 rhinoceroses which exhibited estrus (animals 1 and 2; Fig 4 and 5, respectively) reflect increasing estrogen production 10 days and 8 days, respectively, before overt estrus. This antecedent increase of estrogen production before overt estrus behavior correlates well with the increased attractiveness of the female rhinoceros 10 to 15 days before mating.[8] The estrogen profile of animal 3 (Fig 6) demonstrates an initial increase in estrogen

[8] Kassam AAH, Lasley BL, Research Department, San Diego Zoo, San Diego, Calif: Unpublished data, 1979.

production similar to that of animals 1 and 2. In contrast, however, there is a persistent and, in fact, increasing estrogen production after a 1-week plateau. Although ovulation itself is not necessarily indicated by the estrogen profiles alone, the constant rhythmicity of animals 1 and 2 indicated that they were, in fact, ovulating. Further documentation of ovulation will necessitate the measurements of increased progesterone production to reflect corpus luteum function.

The present estrous cycle data are consistent with previously published reports[9-11] in which an average cycle length of 45 days was reported. They also reported that sexual receptivity is limited to a 24-hour period, rather than the several days over which signs of estrus may be apparent. The estrous data obtained in the present study are not necessarily contradictory, because the female sexual receptivity was judged by the female's behavioral pattern without the direct presence of a male. It is thus possible that, although females were judged to be in estrus (by the keeper) for 3 days on the average, they may have accepted the male for a shorter period if tested with the male present.

Taken together, these data demonstrate the practicability of urinary steroid monitoring for the purpose of evaluating major reproductive events in nontractable species. The development of techniques to measure progesterone or luteinizing hormone and the accumulation of substantial base-line data should ultimately provide the essential ingredients necessary to evaluate the reproductive function in a wide variety of zoo animals.

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