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# Measurement of urinary oestrogens and 20α-dihydroprogesterone during ovarian cycles of black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses

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**Summary.** The development of a sensitive enzyme-immunoassay for 20α-dihydroprogesterone (20α-DHP) and its use in determining reproductive status in black and white rhinoceroses is reported. 20α-DHP in hydrolysed urine diluted in parallel to standards, and high-performance liquid chromatography (HPLC) confirmed the presence of 20α-DHP and the absence of pregnanediol-3α-glucuronide (PdG) in urine collected from rhinoceroses after oestrus. Conjugated oestrone was identified by HPLC as the major urinary oestrogen in the black rhineroceros and conjugated oestradiol-17β was the most abundant in the white rhinoceros. In African species, the black (*Diceros bicornis*), and northern (*Ceratotherium simum cottoni*) and southern (*Ceratotherium simum simum*) white rhinoceroses, excretion of 20α-DHP and oestrogen followed a cyclic pattern. Excretion of 20α-DHP was low before mating, at the time of peak oestrogen excretion, but high after oestrus. In the black rhinoceros, the follicular phase was 3–4 days and the luteal phase was 18 days, suggesting a cycle of 21–22 days. The inter-oestrus interval in the northern subspecies of white rhinoceros was 25 days, which correlated well with the interval between peaks of oestradiol-17β excretion. The interval between urinary oestrogen peaks in the southern subspecies of white rhinoceros suggested a cycle length of 32 days. This paper provides the first description of the pattern of excretion of urinary oestrogens and progesterone metabolites in African rhinoceroses.

*Keywords:* ovarian cycles; enzyme-immunoassay; urine; steroid metabolites; rhinoceros

## Introduction

Rhinoceroses breed poorly in captivity and, as wild populations decline, it becomes increasingly important to improve the fertility of managed herds. Methods for determining reproductive status are urgently needed to assist the captive management of these animals, but little is known about the reproductive physiology of any rhinoceros species. With the exception of the Indian rhinoceros (*Rhinoceros unicornis*), there are no methods for endocrine monitoring of the ovarian cycle.

Measurement of urinary steroid hormone metabolites has provided a non-invasive method of assessing reproductive status in many exotic species (Lasley, 1985; Hodges, 1986). As in most ungulates investigated (Loskutoff *et al.*, 1983; Hodges & Green, 1989; Hodges, 1990), the Indian rhinoceros excretes pregnanediol-3α-glucuronide (PdG) as the major progesterone metabolite in the urine (Kasman *et al.*, 1986; Hindle *et al.*, 1988). The most abundant urinary oestrogen is conjugated

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oestrone, predominantly oestrone sulphate (Kassam & Lasley, 1986; Kasman *et al.*, 1987), and measurement of these metabolites has provided a method for monitoring the ovarian cycle and pregnancy in this species. In African rhinoceroses, the black (*Diceros bicornis*), and northern (*Ceratotherium simum cottoni*) and southern (*Ceratotherium simum simum*) subspecies of white rhinoceros, concentrations of PdG, but not of oestrogens, are high in mid to late gestation (Ramsay *et al.*, 1987; Hodges & Green, 1989). Measurement of these hormone metabolites during the oestrous cycle has been uninformative, concentrations being either low or undetectable and showing no cyclic pattern in relation to ovarian function (Ramsay *et al.*, 1987; Hodges & Hindle, 1988).

In a study of the metabolism and excretion of $^{14}$C-labelled progesterone and oestradiol-17β administered intravenously (i.v.), Hindle & Hodges (1990) reported differences between *C. simum* and *R. unicornis* and suggested that these may account for the failure in monitoring ovarian function in African rhinoceroses. The study demonstrated that conjugated 4-pregnen-20α-ol-3-one (20α-dihydroprogesterone; 20α-DHP), and not PdG, is the major urinary progesterone metabolite in the white rhinoceros. Of the urinary oestrogens, oestrone was present in greatest quantities, although both isomers of oestradiol were also identified.

The present study was carried out (i) to develop an enzyme immunoassay (EIA) for the measurement of 20α-DHP—to detect 20α-DHP immunoreactivity in the urine of white and black rhinoceroses during natural cycles; and (ii) to determine the major urinary oestrogens in white and black rhinoceroses and to describe the pattern of excretion of urinary oestrogen and 20α-DHP during the ovarian cycle in African rhinoceroses.

## Materials and Methods

### Animals and sample collection

Data on cycle profiles were derived from urine samples collected daily from adult female black rhinoceroses (*Diceros bicornis*) ($n = 2$), housed and maintained by the Zoological Society of London, UK, an adult female northern white rhinoceros (*Ceratotherium simum cottoni*) ($n = 1$) housed and maintained at Dvur Kralove Zoo, Czechoslovakia, and an adult female southern white rhinoceros (*C. simum simum*) ($n = 1$) housed and maintained at Paignton Zoo, UK. Each female was allowed access to a male of the same species during the day and housed alone overnight to facilitate sample collection. Animals were observed daily by keeper staff and occurrence of behavioural oestrus, with or without mating, was recorded. Increased frequency of urine spraying, whistling and interest in the male were taken to indicate oestrus.

Each morning, urine voided during the previous night was aspirated by syringe from the floor of the enclosure, avoiding the inclusion of extraneous material. Samples were frozen at −20°C, without preservative, and transported to the Institute of Zoology, London, in solid carbon dioxide. Samples were thawed and centrifuged at 400 *g* for 10 min immediately prior to analysis. In addition, less frequent samples (~3/week) were collected from two other female black rhinoceroses, housed and maintained at St Louis Zoo, MO, USA and Audubon Zoo, LA, USA. These females were showing regular oestrous behaviour, but daily samples could not be collected for the composition of hormone profiles. The samples, standardized around the day of mating/oestrus, were used for high performance liquid chromatography (HPLC), sequential hydrolysis and in the formation of composite profiles.

### Creatinine

Upon the first thawing, all samples were analysed for creatinine concentration by the method of Brand (1981), as modified for microtitre plate format by Hodges & Green (1989), to help compensate for variation in fluid intake and output and variable periods of collection (Erb *et al.*, 1970). The sensitivity of the assay was ~0·1 mg/ml and the intra- and interassay coefficients of variation (CV) were 8·2 and 3·9% ($n = 31$), respectively. The concentration of creatinine was ~2·0 mg/ml (range 0·9 3·2 mg/ml) in the white rhinoceros and 1·0 mg/ml (range 0·3 1·8 mg/ml in the black rhinoceros.

### Hydrolysis and extraction

***Sample preparation for assay.*** Urine samples (0·05 ml) were added to 0·15 ml sodium acetate buffer, pH 5 and hydrolysed with 50 Fishman units (FU) of β-glucuronidase aryl-sulphatase (Sigma Chemical Co., Poole, Dorset, UK;

20 000 FU/g solid) at 37°C for 24 h (Hodges *et al.*, 1979). Efficiency of hydrolysis was determined by adding, in triplicate, tracer amounts of [$^3$H]oestrone sulphate (Amersham International, Buckinghamshire, UK; sp. act. 60 Ci/mmol) and [$^3$H]PdG (P. Samarajeewa, UCL, London; sp. act. 30 Ci/mmol) to 0·05 ml urine pool before hydrolysis. Amounts of unconjugated steroid recovered were 81·0 ± 2·4 and 78·2 ± 1·5% (mean ± s.e.m., $n$ = 24), respectively. After adjusting to pH 7, samples were extracted with 2 ml freshly distilled diethyl ether (BDH, Poole, Dorset, UK). Procedural losses during extraction were monitored by the addition of tracer amounts of [$^3$H]20α-DHP (New England Nuclear, Dreieich, Germany; sp. act. 45 Ci/mmol) to each sample and individual recoveries were used to correct for losses when calculating the final hormone concentration. Mean ± s.e.m. recovery was 87·0 ± 1·3% ($n$ = 180). Ether was evaporated and samples were reconstituted in 1 ml Tris buffer (20 mmol/l; pH 7·5), containing 0·01% bovine serum albumen (BSA), before assay for 20α-DHP, oestrone and oestradiol-17β.

***Sample preparation for HPLC.*** Urine (0·5 ml) to be analysed for conjugated neutral steroids was combined with an equal volume of methanol:ethanol (1:1, v/v) and centrifuged for 5 min at 400 ***g*** (Kasman *et al.*, 1986). The supernatant was collected, [$^3$H]PdG added and the volume reduced to 0·4 ml under a stream of nitrogen at 60°C.

For the chromatographic separation of unconjugated neutral steroids, urine samples (1 ml) were adjusted to pH 5 and hydrolysed with 1000 FU β-glucuronidase aryl-sulphatase at 37°C for 24 h. Before hydrolysis, [$^3$H]PdG was added to each sample to monitor the hydrolysis procedure and to provide a reference standard for 5β-pregnanediol on the HPLC. Each sample was adjusted to pH 7 and extracted with 10 ml freshly distilled diethyl ether. [$^3$H]Progesterone (Amersham; sp. act. 85 Ci/mmol) and [$^3$H]20α-DHP were added to monitor the extraction and provide reference standards. The ether was dried and the samples were reconstituted in 0·2 ml $n$-hexane:chloroform (70:30, v/v).

For oestrogen identification, urine samples (1 ml) were hydrolysed and extracted as above, after the addition of [$^3$H]oestrone (Amersham International; sp. act. 90·7 Ci/mmol, [$^3$H]oestradiol-17α (prepared according to the method of Choi *et al.*, 1989) and [$^3$H]oestradiol-17β (Amersham International; sp. act. 81·5 Ci/mmol). Samples were reconstituted in 0·2 ml $n$-hexane:chloroform (50:50, v/v).

***Sequential hydrolysis of steroid conjugates.*** Sequential enzyme hydrolysis and solvolysis was carried out on selected urine samples from females of both species. Samples for 20α-DHP analysis were taken 7 days after oestrus ($n$ = 8), those for oestrogen analysis were collected on the day before oestrus ($n$ = 8). Undiluted urine samples (0·2 ml) were extracted with 2 ml freshly distilled diethyl ether to remove the unconjugated steroids. Samples were adjusted to pH 5 and hydrolysed with 1200 FU of a specific β-D-glucuronide glucuronaosohydrolase (Sigma G3510; activity 1 250 000 FU/g solid), which contains no sulphatase, and were re-extracted to give the glucuronide fraction. Samples were then hydrolysed with 80 FU β-glucuronidase arylsulphatase followed by re-extraction to give the sulphate fraction. Acid solvolysis was carried out according to the method of Hindle & Hodges (1990) to hydrolyse any residual conjugates. After reconstitution in 1 ml Tris buffer (20 mmol/l), steroid released by each procedure was measured.

Recoveries during ether extraction, estimated by the addition of tracer amounts of [$^3$H]20α-DHP and [$^3$H]oestrone to 0·2 ml urine pool, were 93·1 ± 1·9 and 87·2 ± 2·2% (mean ± s.e.m.; $n$ = 9), respectively. The efficiency of each hydrolysis step was determined by the addition of trace amounts of [$^3$H]oestrone sulphate and [$^3$H]PdG separately to 0·2 ml volumes of a urine pool. The recoveries of [$^3$H]oestrone and [$^3$H]pregnanediol, respectively, were: 4·4 ± 3·2 ($n$ = 9) and 78·6 ± 0·8% ($n$ = 9) for β-glucuronidase; 89·2 ± 1·3 ($n$ = 9) and 71·5 ± 1·2% ($n$ = 9) for sulphatase; and 75·6 ± 0·3 ($n$ = 9) and 66·7 ± 0·6% ($n$ = 9) for solvolysis.

## Hormone assays

***Enzymeimmunoassay for 20α-DHP.*** Immunoreactive 20α-DHP was measured using a microtitration plate EIA based on the procedure described by Meyer & Hoffmann (1987). The antiserum was raised in rabbits immunized against 4-pregnen-20α-ol-3-one carboxymethyloxime:BSA and stored at −20°C at a dilution of 1:1000 in Tris buffer. The enzyme conjugate was horseradish peroxidase (Sigma Chemical Co. P8375, Poole, Dorset, UK; type VI: HRP) conjugated to 20α-DHP using the modified mixed acid anhydride procedure of Libermann *et al.* (1959), and was stored in glycerol (1:1, v/v) at −20°C.

Microtitre plates (Nunc immuno 1) were coated with sheep antirabbit IgG (1 μg/well, purified from serum using a Protein G, Sepharose 4, fast-flow column) MAbTrap G; Pharmacia, Uppsala, Sweden). Plates were then coated with Tris buffer (20 mmol/l; 0·25 ml/well) containing 0·1% sodium azide and stored at 4°C until use.

Immediately before use, plates were emptied by inversion, rinsed three times with 0·005% Tween solution and blotted dry; 4-pregnen-20α-ol-3-one (Sigma P6288) standard was double diluted in Tris buffer over the range 1600 6·4 pg/ml (80–0·32 pg/well) and 0·05 ml of each standard dispensed into wells on the plate in duplicate. Zeros (0·05 ml buffer) and samples (0·05 ml) were also added to duplicate wells on the plate. 20α-DHP-HRP (0·1 ml; 1:30 000) was then added to each well followed by 0·01 ml antiserum (1:150 000). Plates were then covered and incubated overnight in the dark at 4°C.

After incubation, plates were emptied by inversion, washed three times with 0·005% Tween solution and blotted dry. Enzyme substrate was prepared by adding 0·025 ml hydrogen peroxide solution (30%; BDH) to 100 mg O-phenylenediamine (1,2 benzenediamine; Sigma P9029) in 28 ml substrate buffer (0·1 mol citric acid monohydrate/l; pH 5), and 0·25 ml was added to each well. The plates were covered and incubated in the dark at 4°C for 45 min. The reaction was stopped by the addition of 3 mol sulphuric acid/l (0·05 ml) to each well. Absorbance was measured at 492 nm, with a reference of 620 nm, on an automatic plate reader and the amount of 20α-DHP in each well was read from a standard curve constructed by plotting the percentage bound against the amount of standard added.

***Assay validation.*** The average sensitivity of the assay, determined at 90% binding, was 0·65 pg/well or 13 pg/ml urine. Serial dilutions of urine of black and white rhinoceroses (1:20–1:320 and 1:160–1:2560, respectively) collected 7 days after oestrus gave displacement curves parallel to that obtained with 20α-DHP standards. The accuracy of the assay was determined by adding different amounts (80–0·32 pg) of unlabelled 20α-DHP to urine pools from black and northern white rhinoceroses, which contained low concentrations of endogenous hormone and had previously been hydrolysed and extracted. The samples were assayed and mean ± s.e.m. recoveries of 20α-DHP were 100·7 ± 1·0% ($n = 8$) and 101·5 ± 1·2% ($n = 8$), respectively. Interassay precision, expressed as CV, was 7·6% ($n = 21$) for a high value quality control and 6·9% ($n = 21$) for a low value quality control, and intra-assay CV was 7·4%. Steroids showing significant cross-reactivity with the 20α-DHP antiserum, determined at 50% inhibition of binding, were 5β-pregnan-20α-ol-3-one (22·0%), 5β-pregnan-3α,20α-diol (9·3%), 5-pregnen-3β-ol-20-one (6·4%), 5β-pregnen-20α-ol-3-one (3·0%) and progesterone (2·4%). All other C-21 and C-19 steroids tested cross-reacted at <1%. The specificity of the assay for measurement of immunoreactive 20α-DHP in the urine of black and white rhinoceroses was determined by co-chromatography on HPLC. A single peak of immunoreactivity, co-eluting with [$^3$H]20α-DHP, was obtained for each of eight samples on which HPLC was performed.

***EIA for PdG.*** PdG immunoreactivity was measured in fractions collected from HPLC using the EIA described and validated for African rhinoceroses by Hodges & Green (1989); the antiserum is described as cross-reacting with 5β-pregnanediol (45·1%), 20α-DHP (12·1%), 5β-pregnanedione (4·1%), 17α-hydroxyprogesterone, 5β-pregnanetriol and oestradiol-17-glucuronide (0·1%). The enzyme label was pregnanediol-3α-glucuronide conjugated to alkaline phosphatase. The sensitivity of the assay was 25 pg/well or 10 ng/ml. Intra- and interassay CV were 9·4 and 9·1% ($n = 29$), respectively.

***EIA for total oestrogens.*** Total oestrogen immunoreactivity in HPLC fractions was measured by the EIA of Möstl *et al.* (1987), which used an antiserum that cross-reacted, determined at 50% inhibition of binding with oestrone standards, with oestradiol-17β (161%), oestradiol-17α (63%), oestriol (7·2%), equilenin (3·6%), equilin (1·3%) and oestrone sulphate (0·5%) (Möstl *et al.*, 1987). The enzyme label was 1,3,5(10)-oestratriene-3,17β-diol-17-glucuronide conjugated to alkaline phosphatase and the standard was oestrone. The sensitivity of the assay was 0·32 pg/well or 6·4 pg/ml. The intra- and interassay CV were 11·8 and 8·6% ($n = 17$), respectively.

***Radioimmunoassay for oestrogens.*** Oestradiol-17β was measured by radioimmunoassay (RIA) as described by Shaw *et al.* (1989). The assay used $^{125}$I-oestradiol-17β and an antiserum that significantly cross-reacted with oestradiol-17α (27%) and oestrone (8%). The sensitivity of the assay was 4 pg/tube or 40 pg/ml. Serial dilutions of urine of a white rhinoceros collected on the day before oestrus gave displacement curves parallel to that obtained with oestradiol-17β standards. The accuracy of the assay, determined by mean ± s.e.m. recovery of unlabelled oestradiol-17β added to hydrolysed and extracted urine pool from northern white rhinoceroses, containing low concentrations of endogenous hormone, was 102·4 ± 1·7% ($n = 8$). Intra- and interassay CV were 11·2 and 9·4% ($n = 11$), respectively.

Oestrone was measured as described by Hodges *et al.* (1983). The assay used [$^3$H]oestrone and an antiserum that cross-reacted with oestradiol-17β (0·1%), oestradiol-17α (0·06%) and oestriol (0·01%). The sensitivity of the assay was 4·5 pg/tube or 9 pg/ml. Serial dilutions of black rhinoceros urine collected on the day prior to mating gave displacement curves parallel to that obtained with oestrone standards. The accuracy of the assay, determined by mean ± s.e.m. recovery of unlabelled oestrone added to black rhinoceros urine pool, containing low concentrations of endogenous hormone, was 89·3 ± 3·1% ($n = 8$). Intra- and interassay CV were 10·7 and 9·9% ($n = 10$), respectively.

## HPLC

Immunoreactive unconjugated neutral and phenolic steroids (oestrogens) in urine samples collected on the day before oestrus (presumed follicular phase of the ovarian cycle) and 7 days after oestrus (presumed luteal phase) were separated using HPLC. The system used a Lichrosorb straight phase silica 60 column (250 × 4, 10 μm particle size; Merck 50387) and steroids were eluted with solvents.

***Progesterone metabolites.*** Unconjugated progesterone metabolites were separated using a linear solvent gradient of 0–2% methanol in *n*-hexane:chloroform, 70:30 v/v, within 30 min at a flow of 2 ml/min (Carroll *et al.*, 1990). After hydrolysis and extraction, 0·1 ml of each solvent aliquot was injected onto the column and fractions (1 ml) were collected over 30 min, dried and reconstituted in assay buffer (1 ml). Efficiency of the entire procedure (including hydrolysis and extraction) was 65·7 ± 2·1% (mean ± s.e.m., $n = 17$) based on the recovery of [$^3$H]pregnanediol. Recoveries of [$^3$H]20α-DHP and [$^3$H]progesterone, added after hydrolysis, were 71·6 ± 3·3 and 67·1 ± 2·8% ($n = 17$), respectively.

***Oestrogen metabolites.*** Oestrogens were separated using a linear solvent gradient of 0–10% methanol in *n*-hexane: chloroform, 50:50 v/v, within 22 min at a flow of 2 ml/min (Hindle & Hodges, 1990). After hydrolysis and extraction, 0·1 ml of each solvent aliquot was injected onto the column and fractions were collected over 30 min, evaporated to dryness and reconstituted in assay buffer (1 ml). Recovery of [$^3$H]oestrone was 64·3 ± 2·5% (mean ± s.e.m., $n = 16$).

***Conjugated progesterone metabolites.*** Immunoreactive, conjugated, neutral steroids in urine samples collected 7 days after oestrus (presumed luteal phase) were separated by HPLC using a μBondapak reverse-phase C-18 column (30 × 3·9, 10 μm particle size; Waters 86684). Conjugated progesterone metabolites were separated using a linear

solvent gradient of 20–100% methanol in deionized, distilled water within 40 min at a flow rate of 1 ml/min (Shideler *et al.*, 1983). Portions (0·2 ml) of prepared sample were injected onto the column and fractions (1 ml) collected over 30 min, dried and reconstituted in phosphate–azide saline gel buffer (1 ml). The overall efficiency of the procedure was 73·6 ± 3·3% (mean ± s.e.m.).

## Results

### HPLC

A single peak of 20α-DHP immunoreactivity co-eluted with [$^3$H]20α-DHP in fractions eluted from HPLC of hydrolysed urine collected from female northern white and black rhinoceroses during the luteal phase of the ovarian cycle (Fig. 1a, b). Similar profiles were obtained with all luteal-phase samples analysed ($n$ = 4 for each species). In addition to confirming the presence of 20α-DHP as an abundant urinary progesterone metabolite, the HPLC profiles support the validity of the EIA for measurement of 20α-DHP immunoreactivity in the two species.

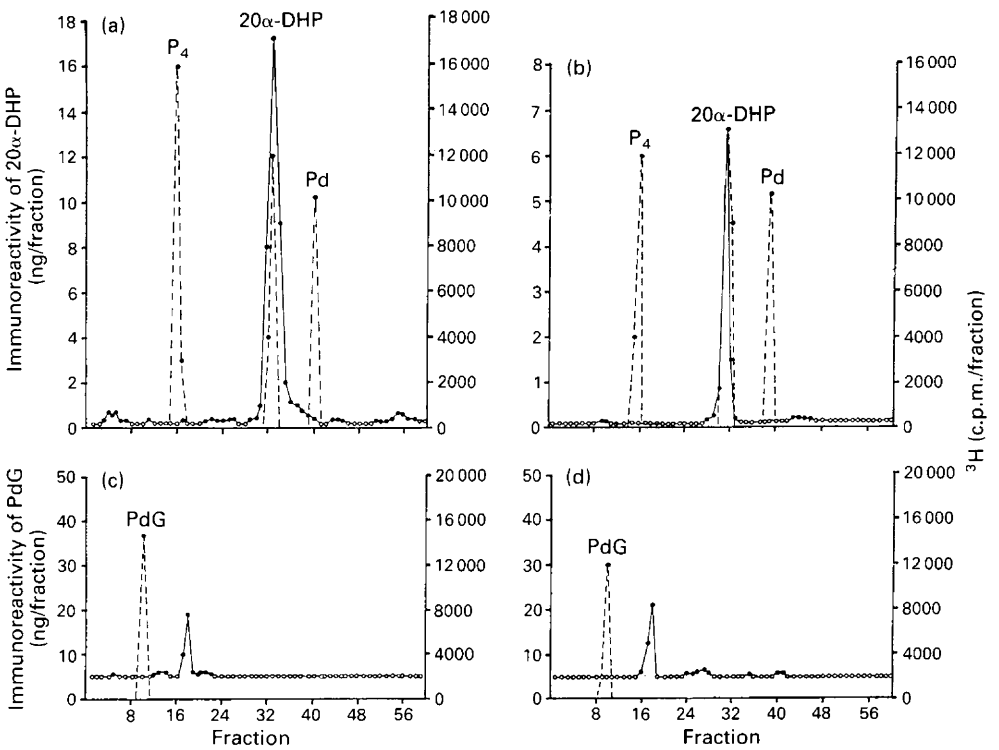


**Fig. 1.** Elution profiles from high-performance liquid chromatography of immunoreactive progesterone metabolites in urine collected from northern white (a, c) and black (b, d) rhinoceroses 7 days after oestrus. In (a) and (b), (——) elution profile of 20α-dihydroprogesterone (20α-DHP) immunoreactivity in hydrolysed urine, compared with that of $^3$H-labelled progesterone ($P_4$), 20α-DHP and pregnanediol (Pd, – – –). In (c) and (d), the elution profile of pregnanediol glucuronide (PdG) immunoreactivity (——) is shown with that of the $^3$H-labelled standard (– – –); (○) immunoreactivity below the sensitivity of the assay.

HPLC was also performed on samples collected on the day before the onset of behavioural oestrus ($n$ = 3 for each species). No 20α-DHP was detected in any of the samples.

In contrast, PdG was not detected in luteal phase samples using HPLC ($n = 6$ for each species) (Fig. 1c, d). Small amounts of immunoreactivity were measured in samples from both species but these did not co-elute with [$^3$H]PdG. The retention time of this immunoreactivity remained constant relative to that of the PdG marker.

In fractions eluted from HPLC of hydrolysed urine collected on the day before the onset of behavioural oestrus, a single peak of oestrogen immunoreactivity was consistently seen to co-elute with [$^3$H]oestradiol-17β in northern white rhinoceroses ($n = 8$), whilst in the black rhinoceros immunoreactivity was present in the fraction containing the oestrone marker (Fig. 2a, b).

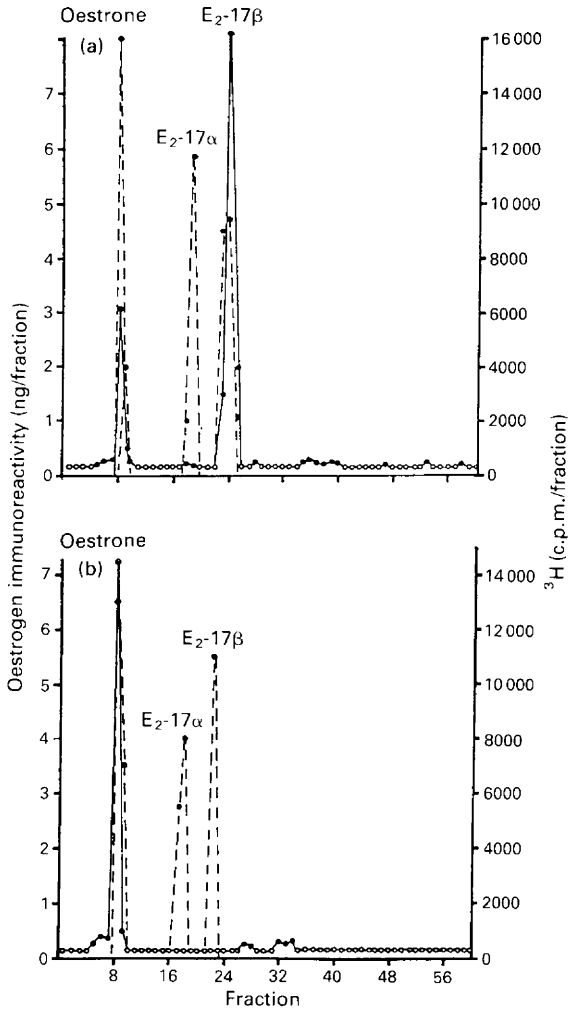


**Fig. 2.** Elution profile from high-performance liquid chromatography of $^3$H-labelled oestrogens (– – –), oestrone, oestradiol-17α ($E_2$-17α), oestradiol-17β ($E_2$-17β) and oestrogen immunoreactivity (——) in hydrolysed urine from (a) the northern white and (b) black rhinoceros on the day before oestrus; (○) immunoreactivity below the sensitivity of the assay.

## Sequential hydrolysis

The results of sequential hydrolysis of steroid conjugates in urine are shown in Table 1. Total immunoreactivity is expressed as the sum of unconjugated, glucuronide, sulphate fractions and that which was hydrolysed only after solvolysis, i.e. the residual.

**Table 1.** Conjugates (mean ± s.e.m.), expressed as percentage of the total amount of each hormone (ng/mg Cr) released at each stage of the hydrolysis and extraction procedure, contributing to the total urinary 20α-dihydroprogesterone (20α-DHP) immunoreactivity during the postoestrous period (oestrus + 7 days), and total oestrone and oestradiol-17β immunoreactivity during the oestrus period (oestrus − 1 day) of the ovarian cycle in black and northern white rhinoceroses

| | No. samples/ no. animals | Fraction | | | |
|---|---|---|---|---|---|
| | | Unconjugated | Glucuronide | Sulphate | Residual |
| Northern white rhinoceros | | | | | |
| 20α-DHP | 12/2 | 9·5 ± 1·5 | 23·6 ± 4·3 | 56·9 ± 4·5 | 13·9 ± 2·7 |
| Oestradiol-17β | 7/2 | 1·1 ± 0·4 | 80·2 ± 1·4 | 2·16 ± 0·7 | 16·1 ± 1·0 |
| Black rhinoceros | | | | | |
| 20α-DHP | 12/4 | 14·6 ± 2·2 | 49·4 ± 3·1 | 13·4 ± 2·5 | 14·9 ± 2·7 |
| Oestrone | 10/4 | 1·4 ± 0·3 | 77·8 ± 2·5 | 17·3 ± 1·2 | 3·5 ± 1·0 |

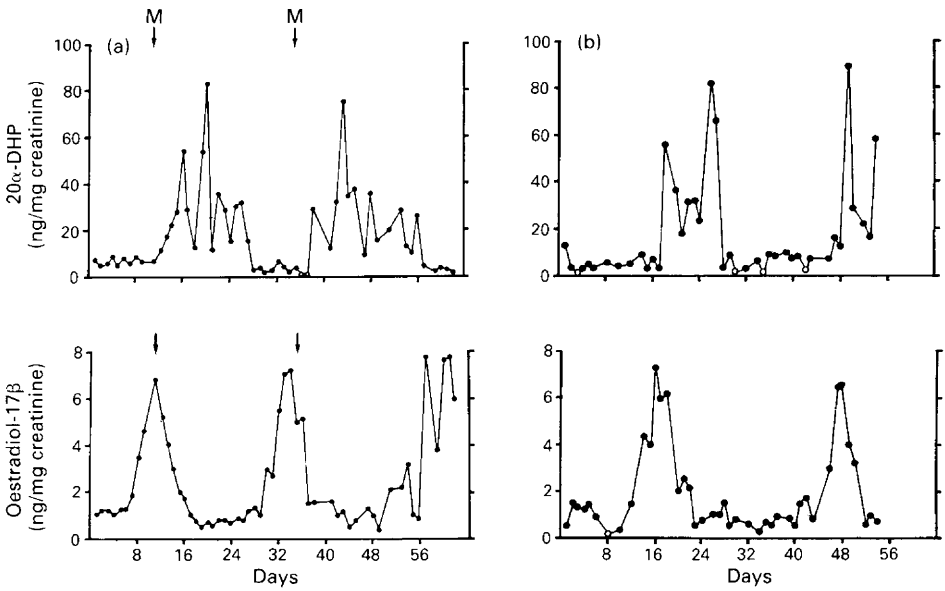


**Fig. 3.** Pattern of excretion of 20α-dihydroprogesterone (20α-DHP) and oestradiol-17β during successive oestrous cycles in a northern (a) and southern (b) white rhinoceros. Arrows indicate the time of mating (M); (○) values that were below the assay sensitivity.

The majority of urinary 20α-DHP was conjugated in both species, <15% unconjugated as indicated by immunoreactivity being measurable after ether extraction. Of the conjugated portion, cleaved by enzyme hydrolysis, glucuronides predominated in the black rhinoceros but sulphates represented a higher proportion in the northern white rhinoceros. In both species, ~15% of the total 20α-DHP immunoreactivity was measurable only after solvolysis; this represented unidentified conjugates resistant to enzymatic hydrolysis.

More than 90% of urinary oestrogen was conjugated. In the northern white rhinoceros, the majority (80%) of urinary oestradiol-17β was conjugated as a glucuronide while 2·2% was sulphated. In the black rhinoceros, >75% of the oestrone in the urine was glucuronidated. A small proportion of urinary oestrogen conjugates in both species was resistant to enzymatic hydrolysis.

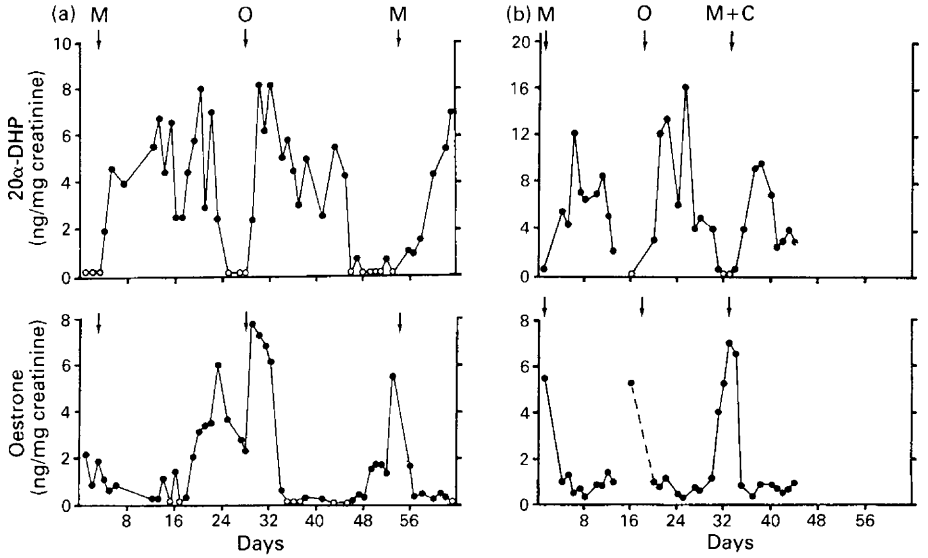


**Fig. 4.** Pattern of excretion of 20α-dihydroprogesterone (20α-DHP) and oestrone during successive oestrous cycles in two black rhinoceroses (a and b). Arrows indicate the time of observed behavioural oestrus (O) or mating (M); (○) values that were below the assay sensitivity; C, conception.

**Ovarian cycles**

Urinary 20α-DHP profiles from a northern white rhinoceros during 60 days in which mating was observed twice, showed a cyclic pattern with a marked increase immediately after presumed ovulation (Fig. 3a). In general, concentrations of 20α-DHP were low before mating, but higher and more variable after oestrus. Peak concentrations of 20α-DHP (60–80 ng/mg creatinine (Cr)) were observed between 8 and 12 days after mating, at the midluteal phase. In the southern white rhinoceros (Fig. 3b), although there were no overt signs of oestrus, the profile of 20α-DHP followed a cyclic pattern, maximum concentrations ~80 ng/mg Cr being similar to those in the northern white female. Pre-oestrus concentrations of 20α-DHP in animal (a) ranged between 1 and 9 ng/mg Cr and were < 10 ng/mg Cr during the presumed follicular phase in animal (b).

Urinary oestradiol-17β excretion was also cyclic. In the northern white rhinoceros (a) there were three distinct periods of high oestradiol-17β, maximum values occurring twice either on the day of, or on the day before behavioural oestrus; a third peak is shown (Fig. 3) although behavioural oestrus was not detected. The profile of oestradiol-17β excretion in the southern white rhinoceros (b) was also cyclic. Concentrations of oestradiol-17β fell within the same range as those of the northern white female and increased during both periods of low 20α-DHP excretion to peak values of 6–7 ng/mg Cr on the day before the initial 20α-DHP rise. The interoestrus interval in the northern white rhinoceros was 24 days, which corresponds well to cycle length as estimated from the interval between oestradiol-17β peaks. The interval between oestradiol-17β peaks in the southern white rhinoceros was 32 days.

As in the white species, two black rhinoceroses showed a cyclic pattern of 20α-DHP excretion, marked and very rapid increases occurring immediately after behavioural oestrus (Fig. 4). The profile for animal (a) indicates that concentrations of 20α-DHP before oestrous were extremely low and often below the limit of sensitivity of the assay, whilst the greatest values during the presumed luteal phase were 6–8 ng/mg Cr. Urinary oestrone concentrations also showed a cyclic pattern, highest values occurring on the day of mating. Cycle length in this animal was 25 days when estimated from either peak oestrone concentrations or interoestrous interval. Concentrations of

20α-DHP in animal (b) were generally higher, although a cyclic pattern in relation to behavioural oestrus and oestrone excretion was still evident. Cycle length could not be determined for this animal as the data presented were not from consecutive ovarian cycles.

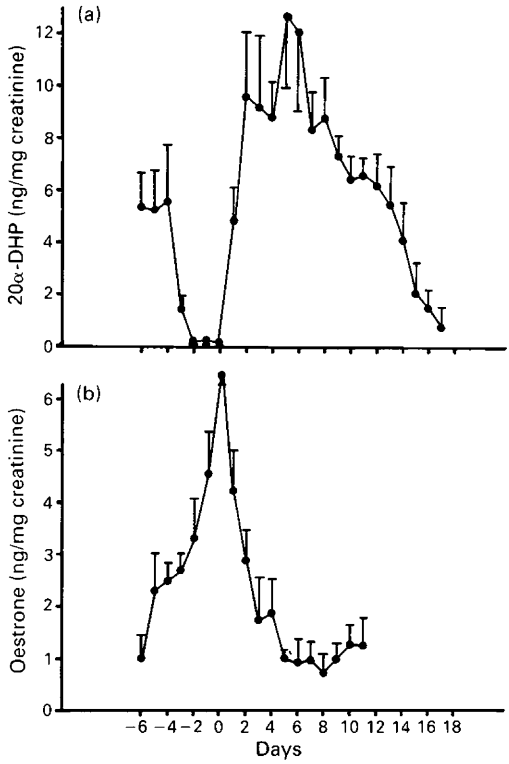


**Fig. 5.** Concentrations of (a) urinary 20α-dihydroprogesterone (20α-DHP) and (b) oestrone during nine ovarian cycles in black rhinoceroses ($n$ = 4). Individual cycles were standardized at Day 0, corresponding to the day of oestrus or mating. Each point is mean ± s.e.m. of individual values obtained for a particular day ($n$ = 6–9).

Mean ± s.e.m. urinary 20α-DHP concentrations in 144 urine samples taken from a total of nine cycles in four female black rhinoceroses, fell 3 days before oestrus and increased markedly 1 day after oestrus (Fig. 5). Using a value of 1 ng/mg Cr to distinguish between follicular and luteal phases, the composite profile indicates a follicular phase of 3–4 days and a luteal phase of ~17 days; the overall cycle length was 21–22 days. Mean oestrone rose above baseline values (1 ng/mg Cr) 5 days before oestrus to reach a maximum on the day of oestrus. Concentrations of oestrone then fell to baseline values ~5 days later.

## Discussion

This study confirms that conjugated 20α-DHP, and not PdG, is an abundant urinary progesterone metabolite in white and black rhinoceroses. The major urinary oestrogens were identified as oestradiol-17β in the white rhinoceros and oestrone in the black rhinoceros. Measurement of these metabolites by immunoassay has provided the first description of the ovarian cycle in these species.

Co-chromatography by HPLC failed to detect PdG in urine collected during the presumed luteal phase of the ovarian cycle in either black or white rhinoceroses. This observation agrees with those of Ramsay *et al.* (1987), who used similar HPLC techniques in the black rhinoceros, and of Hindle & Hodges (1990), who demonstrated that PdG was a quantitatively unimportant metabolite of exogenously administered $^{14}$C-labelled progesterone in a female southern white rhinoceros. Attempts to monitor ovarian function by measurement of PdG in black and white rhinoceroses have reported either undetectable (Ramsay *et al.*, 1987) or low and variable (Hodges & Hindle, 1988) immunoreactivity, which showed no pattern in relation to ovarian events. These observations suggest that PdG is not excreted as a physiologically significant metabolite of progesterone during the ovarian cycle in either species of African rhinoceros. In this respect, African rhinoceroses differ from the Indian species, in which PdG is the major urinary progesterone metabolite (Hindle *et al.*, 1988) and in which measurement of PdG immunoreactivity reflects corpus luteum function (Kasman *et al.*, 1986).

The urinary contaminant cross-reacting with the PdG antibody, as detected by HPLC, may account for the low PdG immunoreactivity measured in urine from black and white rhinoceroses by Hodges & Hindle (1988). The nature of the immunoreactivity was not determined in this study, although it could possibly represent 20α-DHP, which shows a 12·1% cross-reaction with the PdG antiserum, or its conjugates, the cross-reactivity of which cannot be determined as the steroid conjugates are not available.

We have recently identified 20α-DHP as a major urinary metabolite of [$^{14}$C]progesterone in the southern white rhinoceros (Hindle & Hodges, 1990). The results of the present study confirm and extend this observation by demonstrating that 20α-DHP is an abundant urinary metabolite during natural cycles in both species of African rhinoceros. The single peaks of immunoreactivity obtained on HPLC show that the measurement of 20α-DHP during the postoestrus phase is specific.

Differential hydrolysis indicated that, of the total 20α-DHP immunoreactivity, most (85%) was conjugated; glucuronides predominated in luteal phase urine from black rhinoceroses whereas sulphates were more abundant in white rhinoceroses. The profiles shown here describe concentrations of 20α-DHP after hydrolysis and thus represent a combination of unconjugated and conjugated fractions.

The time course of 20α-DHP excretion in relation to the occurrence of oestrus or mating and the pattern of oestrogen excretion suggests that the measurement of this metabolite closely reflects progesterone secretion and corpus luteum function. However, in the absence of information on the pattern of progesterone secretion in these species and the lack of direct evidence that the profiles described here represent ovulatory cycles, correlation of the timing of hormonal changes with specific ovarian events is difficult. However, in equids, circulating concentrations of 20α-DHP accurately reflect luteal function (Van Rensburg & Van Niekerk, 1968) and the measurement of urinary immunoreactive 20α-DHP by non-specific radioimmunoassay has also provided valuable information on the ovarian cycle (Kirkpatrick *et al.*, 1990). This non-specific measurement has been applied to reproductive assessment in many species, including primates (Shideler *et al.*, 1985; Monfort *et al.*, 1986), but proved insufficiently sensitive to detect low concentrations of 20α-DHP in black rhinoceros urine (M. Densmore, personal communication). The present report is the first to describe the specific measurement of 20α-DHP in urine, which in the African species of rhinoceros correlates well with behavioural oestrus and increased oestrogen excretion around the time of presumed ovulation.

The measurement of urinary oestrogens provides additional evidence that the cycles described were ovulatory. Although profiles were not always clear, changes in excretion of urinary oestrogens appeared to reflect follicular development preceding oestrus and/or ovulation. Profiles of oestradiol-17β excretion in the white rhinoceros are particularly clear and show an increase above baseline at least 2–3 days before the onset of behavioural oestrus and subsequent rise in 20α-DHP. Peak concentrations of urinary oestrogen were $>6$ times those at the time of luteal regression. Mean concentrations of urinary oestrone rose 5 days before oestrus in the black rhinoceros, when

20α-DHP declined, to reach peak concentrations on the day of oestrus. The only other data on the pattern of urinary oestrogen excretion in rhinoceroses were obtained by Kassam & Lasley (1981) and Kasman *et al.* (1986) for the Indian species, in which concentrations were generally much higher, with a more protracted increase during the pre-ovulatory period.

The results of this study suggest species differences in ovarian steroid metabolism between white and black rhinoceroses, 20α-DHP being excreted predominantly as a glucuronide in black rhinoceroses and as a sulphate in the white species. Furthermore, white rhinoceroses excreted conjugated oestradiol-17β, whilst black rhinoceroses preferentially excreted conjugated oestrone into the urine. The measurement of urinary oestradiol-17β has provided valuable information on the ovarian cycle of certain primates, e.g. marmosets (Hodges & Eastman, 1984), but is not thought to be important in ungulates (Mellin *et al.*, 1965). Conjugated oestrone is the major urinary oestrogen in the majority of ungulate species (Lasley, 1985; Lasley *et al.*, 1989) including other perrisodactyls, the horse (Hillman & Loy, 1975), tapir (Kasman *et al.*, 1985) and Indian rhinoceros (Kassam & Lasley, 1981; Kasman *et al.*, 1986). Oestrone was excreted mainly as a glucuronide in black rhinoceroses, as also described by Ramsay *et al.* (1987), and concentrations of hydrolysed urinary oestrone conjugates correlated well with reproductive events. The direct measurement of oestrone glucuronide has not provided informative data on the ovarian cycle in this species (Hodges & Hindle, 1988), perhaps due to a lack of sensitivity in the oestrone conjugate assay, or the configuration of the oestrone conjugate in black rhinoceros urine.

Cycle length in black rhinoceroses was ~25 days; although there is little information available for comparison, in behavioural studies in the wild (Hitchins & Anderson, 1983) and captivity (Dittrich, 1967; Gowda, 1967; Greed, 1967; Hallstrom, 1967; Yamamotto, 1967), cycle lengths ranging from 17 to 60 days have been reported. The cycle lengths of northern and southern white rhinoceroses were 24 and 25 days, respectively—shorter periods than the reported interoestrus interval of ~30 days observed in southern white rhinoceroses in the wild (Owen-Smith, 1973) and in captivity (Lindemann, 1984). However, in this study figures refer to the cycle length of one animal of each species. It is also impossible to determine whether these were normal ovarian cycles, as three of the four females were bred at the appropriate time, but only one mating resulted in conception.

In conclusion, we have identified the abundant progesterone and oestrogen metabolites in the urine of northern white and black rhinoceroses. The development of a sensitive enzyme immunoassay for 20α-DHP has allowed the profile of progesterone metabolite excretion during the ovarian cycle of both species to be described. The combined measurement of 20α-DHP and oestradiol-17β in white rhinoceroses and oestrone in black rhinoceroses has allowed the oestrous cycle of the African rhinoceroses to be monitored for the first time. More studies are now needed to obtain further data on steroid metabolite concentrations during the ovarian cycle and pregnancy. Determination of ovarian cyclicity will greatly aid co-ordinated breeding programmes of the African species of rhinoceros in captivity.

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