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Identification of a series of $C_{21}O_2$ pregnanes from fecal extracts of a pregnant black rhinoceros (*Diceros bicornis minor*)

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Abstract

Fecal extracts from a pregnant black rhinoceros, *Diceros bicornis*, were analyzed by radioimmunoassay, HPLC, and by GC-mass spectrometry. From 40 g of dried feces a total of 33 pregnanes in the $C_{21}O_2$ series, including a number of novel 17α epimers were identified. No progesterone was recovered. The principal progesterone metabolite by mass was 5α-pregnan-3β,20α-diol (44.5%), which did not cross react with the antibody used in our RIA. The antibody recognized progesterone and pregnanes with 20-one configuration, which when combined made up less than 15% of the total $C_{21}O_2$ steroid mass. Of the 33 pregnanes in the $C_{21}O_2$ series identified, 81%, by mass, were in the 5α-configuration. These results are compared with studies in other rhinoceros species (Asian and Sumatran) in which pregnanes in the 5β-configuration are the major fecal metabolites, and the white rhinoceros in which pregnanes in the 5α-configuration are the dominant form.

Keywords: Pregnanes; Pregnancy; Fecal metabolites; Rhinoceros

1. Introduction

The use of fecal extracts to monitor reproductive steroids in a variety of wild and domestic species has gained popularity in recent years [1], and is the method of choice to study reproductive events in animals in which it is difficult if not impossible to collect blood or urine samples. Rhinoceros, in particular, are extremely difficult to bleed on a regular basis, and thus fecal steroid analysis is about the only method available to study reproduction in these animals. A number of recent studies have reported successful monitoring of estrus cycles and pregnancy by measuring fecal progesterone metabolites in black rhinoceros, white rhinoceros, and Indian rhinoceros using immunoassays [2–7]. The identity of the metabolites, however, is still a matter of conjecture. Schwarzenberger et al. [5] tentatively identified 5α-pregnane-3,20-dione, 5α-pregnane-3α-ol-20-one and 5α-pregnane-3β-ol-20-one as the principal fecal pregnanes in black rhinoceros, based on cross-reactivity against progesterone antibodies that recognized 5α and 5β-reduced pregnanes, and from elution profiles following HPLC. Hindle and Hodges [8] injected ^{14}C-labeled estradiol 17β and progesterone into a southern white rhinoceros and measured urinary and fecal metabolites. A small amount of labeled progesterone, but curiously, no progesterone metabolites were recovered from ethanolic extracts of the feces. In a similar experiment, however, in which a Sumatran rhinoceros was injected with ^{3}H-labeled estradiol-17β and ^{14}C-labeled progesterone, numerous progesterone metabolites were recovered in ether extracts of feces [9]. The three most abundant pregnanes were, 5β-pregnane-3α,20α-diol, 5β-pregnane-3α-ol-20-one and a second pregnanediol for which an exact structure was not determined.

In a preliminary report, we presented data on the structural identity of several pregnanes isolated from the feces of a black rhinoceros [10]. In this paper we present data on the quantification and structural identity of a large number of pregnanes in the $C_{21}O_2$ series isolated from the feces of a pregnant black rhinoceros.

2. Materials and methods

2.1. Animal and sample collection

Fecal samples were collected from a pregnant black rhinoceros maintained at the Dallas Zoo. This animal was

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caught wild in Mana Pools in the Zambezi valley of Zimbabwe and was estimated to have been born in 1974. She had one female offspring in 1990 and one male in 1994 before she died on March 27, 1995. In Dallas she was maintained in an indoor/outdoor enclosure, given free access to water, and was fed ad libitum on a standard zoo diet. Fecal samples were collected from the barn floor and placed into labeled plastic bags and stored at −20°C before being shipped to San Diego for analysis. Samples used for metabolite identification were pooled from the last two months of pregnancy when concentrations of immunoassayable progesterone metabolites exceed 50 μg/g dry fecal mass (see results).

2.2. Chemicals

Steroids in the pregnane series were obtained from Steraloids Inc., Newport, Rhode Island. Organic solvents were obtained from Mallinckrodt, Paris, KY.

2.3. Extraction of fecal samples

To reduce inter-sample variability in water content, fecal samples were placed into labeled small plastic cups with lids (Starstedt Inc., Newton, NC) and lyophilized for 72 h in a Flexi-Dry microprocessor manifold lyophilizer (FTS System, Inc., Stone Ridge, NY). Following lyophilization, the samples were sifted through a 2 × 2 mm wire mesh to remove larger pieces of vegetation.

For radioimmunoassay (RIA), 0.2 g of the dried feces was extracted with 5 ml of anhydrous diethyl ether (see Patton et al. [3] for details). For structural identity of the metabolites, extracts from a series of 0.2 g samples from the last two months of pregnancy were combined, such that a total of 40 g of the dried feces was available for analysis. Each 0.2 g sample was placed into a borosilicate culture tube (16 × 150 mm; 0.2 g/tube), solubilized in distilled water (2 ml) and vortexed (30 s). Five ml of anhydrous diethyl ether was added and the tube agitated on a mechanical shaker for 2 min. The mixture was then flash frozen in a methanol:dry ice bath. The supernatant was poured into a beaker and allowed to evaporate at 37°C. The combined ether extracts were dried and resolubilized in ethyl acetate: hexane (1:1).

2.4. Chromatography

The solubilized extract was subjected to flash chromatography using a 30 × 5 cm column (Ace Glass Inc., Vineland, N.J) packed to 21 cm with silica gel, 40 μm flash chrom pack (J.T. Baker, Phillipsburg, NJ). Five ml fractions were collected using ethyl acetate:hexane 50:50 for the first 250 ml, followed by the same solvent pair at 60:40 v/v until 160 fractions were collected. No attempt was made to identify or quantitate the later, more polar fractions which, we assumed would contain the $C_{21}O_3$ and $C_{21}O_4$ series of pregnanes. Each fraction was dried and reconstituted in 10 ml phosphate-buffered saline (PBS), pH 7.0 with 1% gelatin (PBS). Ten μl of the reconstituted sample was diluted 1:50 in PBS and analyzed for progestin immunoreactivity by RIA according to Patton et al. [3].

2.5. Gas-liquid chromatography-mass spectrometry (GLC-MS)

Each 5 ml fraction was then dried, mixed with internal standard, and progesterone metabolites analyzed by gas-liquid chromatography-mass spectrometry (GLC-MS) as methyl esters, methyl ester acetates, or methyl ester trimethylsilyl derivatives. The instrument was a Hewlett-Packard 5890 Gas Chromatograph-5970-MSD, controlled by HP/UX Chem Station software. The column was a Supelco 30 m 0.25 ID SPB-35 (35% phenyl methyl silicone) operated at 275°C (isothermal). A splitless injection was used with an injection temperature of 290°C. Helium was used as the carrier gas with a 6 psi column head pressure. Relative retention times and fragmentation spectra of peaks obtained by GLC-MS were compared with those of known standards for identification [11].

Pregnane standards for GC-MS with the 17α-side chain configuration were unavailable commercially and thus were synthesized in the laboratory according to Tökés et al. [12]. One mg of each of the 3α- and 3β-hydroxy epimers in the 5α- and 5β-pregnane series was dissolved in 110 μl ethanol and 100 μl 4N sodium hydroxide and heated over an oil bath at 80°C for 3.5 h. The reaction mixture was neutralized with HCl and the steroid mixture extracted with ethyl acetate and dried. The four reaction mixtures were derivatized and identified by GS-MS without further purification. Each of the four samples was found to contain a pair of 17α- and 17β-side chain isomers. For example, the product of the first set was a mixture of 3α-hydroxy-20-oxo (5α, 17α) pregnane and 3α-hydroxy-20-oxo (5α, 17β) pregnane. The reaction yields for the 17α-isomer averaged 41%. A portion of each of these four product mixtures was then dissolved in 1 ml methanol, and 1 mg sodium borohydride was added to the solution, which was then incubated for 1.5 h at room temperature. The methanolic solution was evaporated to a minimum volume, water was added to quench the reaction, and the steroid mixture was extracted with ethyl acetate. The resulting mixtures were derivatized and identified by GC-MS without further purification. Each contained the set of four dihydroxy steroids, two (20α- and 20β) with a 17α-side chain and two (20α- and 20β) with the 17β-side chain. Examples of two such conversions are shown in Fig. 1.

An internal standard (1-methyl-17β-estradiol) was added to each fraction prior to GC-MS analysis. The mass/charge responses of individual steroids in the sample were integrated and a value obtained relative to the internal standard. The amount of an individual steroid in each fraction was

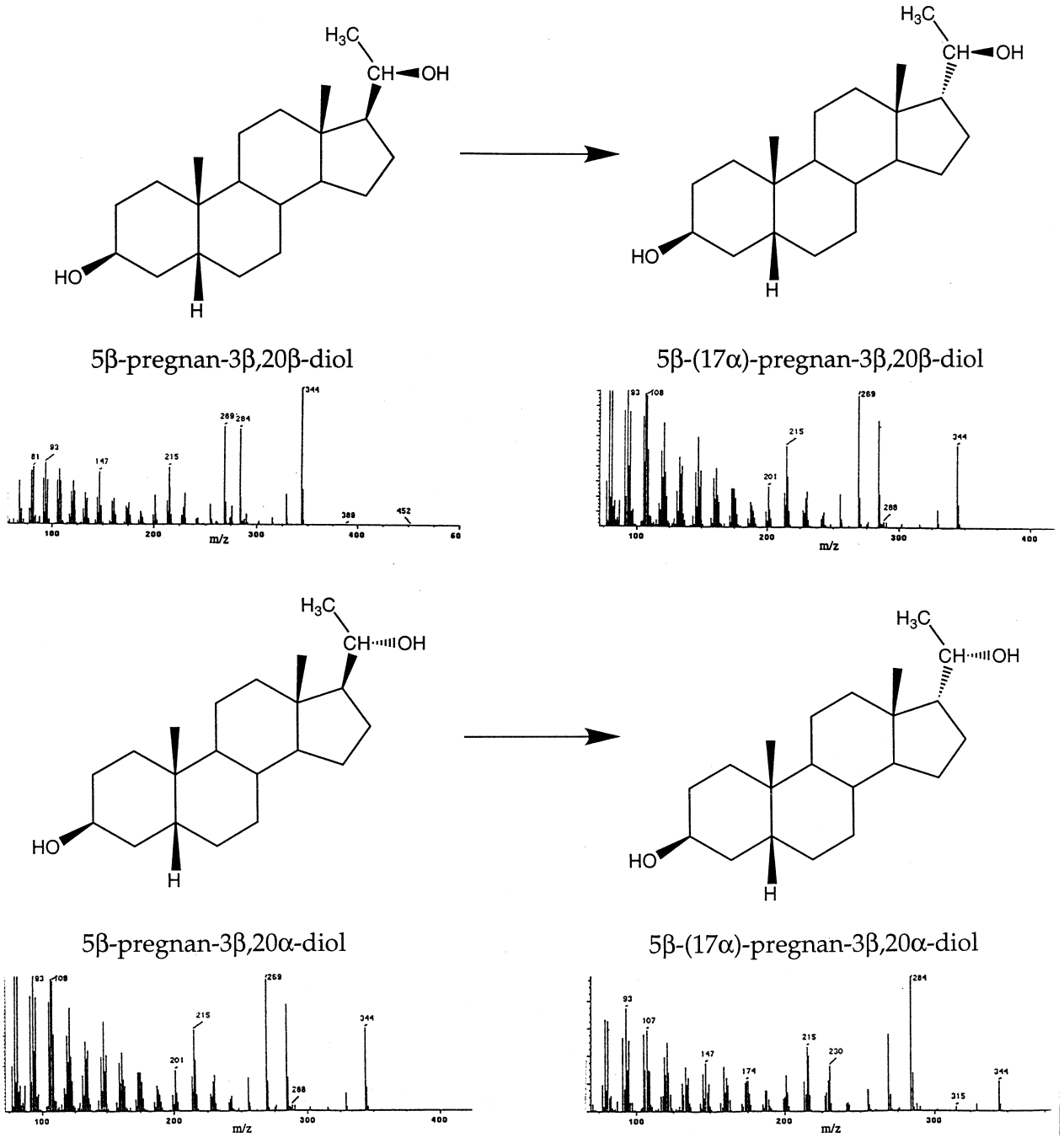

Fig. 1. Conversion of 5β-pregnan-3β,20β-diol to 5β,17α-pregnan-3β,20β-diol and conversion of 5β-pregnan-3β,20α-diol to 5β,17α-pregnan-3β,20α-diol. The mass spectra are presented below each steroid.

then summed over all the fractions in which it appeared to obtain a total overall value.

2.6. Cross reactivity

Pregnanes identified by GLC/MS were tested against the antibody used in the RIA. The antibody used in the RIA was raised against progesterone, but did cross react significantly with pregnanes showing a 20-one configuration. A detailed description of the assay and its validation is given in Ref. [3]. Each pregnane was serially diluted from 10 μg to 10 pg/assay tube and tested for linearity against the standard curve.

3. Results

Total immunoreactive fecal pregnanes for an entire pregnancy in a black rhinoceros are presented in Fig. 2. As shown in Fig. 2, fecal pregnane values begin a steady

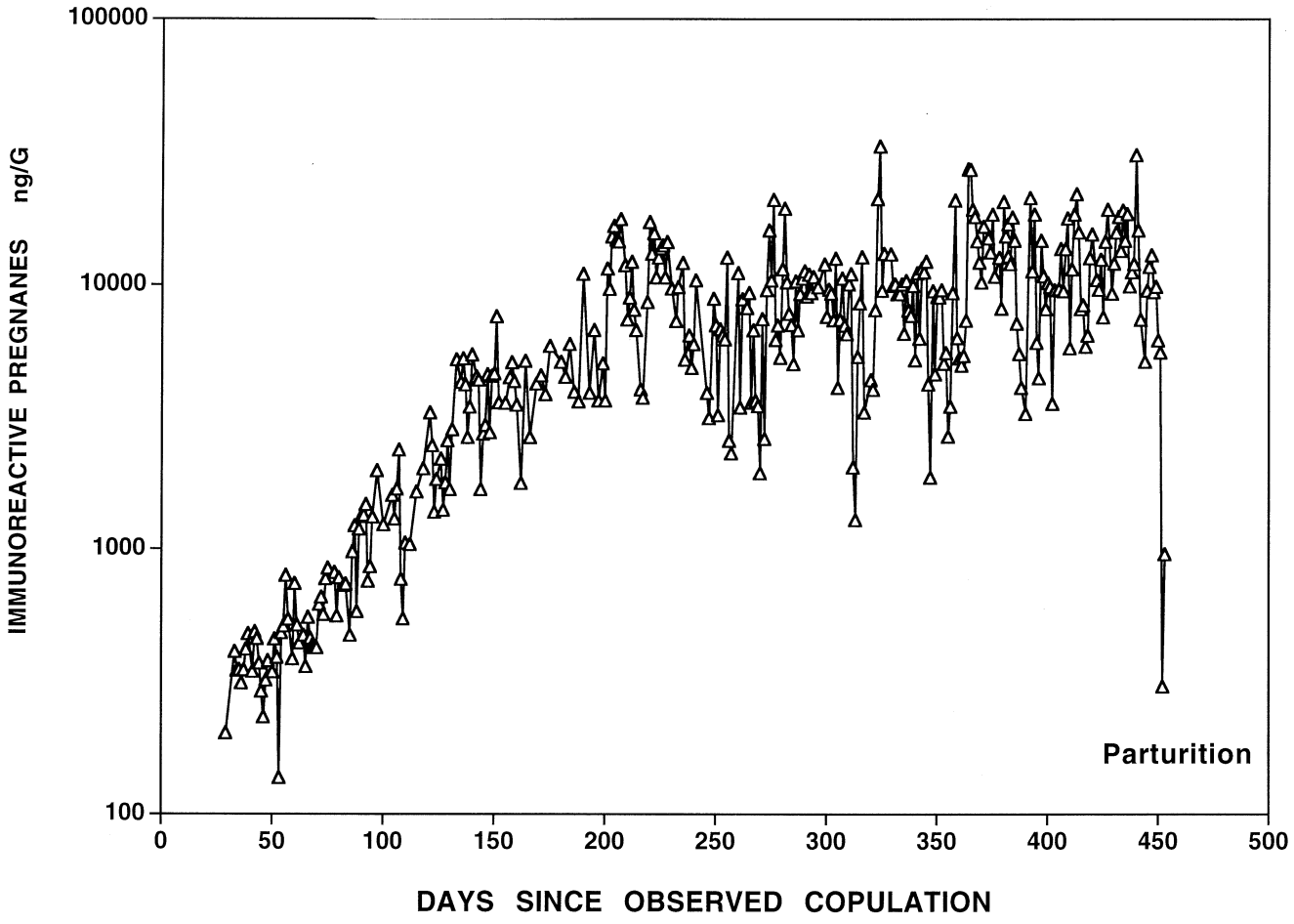

Fig. 2. Fecal pregnanes, as measured by immunoassay, during an entire pregnancy of a black rhinoceros. Note that the Y-axis is in the log scale.

rise, starting between days 90 and 100 and continue to rise during pregnancy. During the latter third of pregnancy the concentration may exceed 80 000 ng/g feces. After parturition pregnanes drop to below 300 ng/g feces (Fig. 2).

The elution pattern of immunoreactive and non-immunoreactive pregnanes, as detected by GC-MS, following flash chromatography of the combined extract of 40 g of feces, is presented in Fig. 3. The relative amount of steroid present (as a percent of total) in any given fraction is indicated by the size of the individual peaks. The shallowness of certain peaks does not suggest that they were difficult to observe, but that relative to other peaks in the figure they were proportionately low in concentration. The GC-MS was able to identify unambiguously all of the steroids listed in Table 1. The very large differences in the concentration of the different pregnanes result in some relatively 'flat' peaks. Nevertheless these small peaks contained sufficient steroid for analysis. The peaks are displayed in approximate order of elution and the size of each peak proportional to its relative mass. By 'relative mass' as in Table 1, we mean that each pregnane is reported as a percent of the total mass of the $C_{21}O_2$ series. The major immunoreactive peak corresponds to 5α-pregnan-3β-ol-20-one, whereas the principal metabolite by mass is 5α-pregnen-3β,20α-diol, which shows little cross reactivity with the antibody (Fig. 3, Table 1). The antibody also recognizes 5α-pregnan-3,20-dione and 5α-pregnan-3α-ol-20-one which together account for less than 15% of the total pool of fecal metabolites.

In Table 1, a complete list of the C_{21}-dihydroxy pregnanes identified, and their cross-reactivity in the radioimmunoassay is presented. All steroids exhibiting a cross-reactivity greater than 0.01% showed parallelism to the standard curve. For example, 5β-pregnan-3β,20α-diol (number 22 in Table 1) gave a value of only 0.42% in the RIA, but in a simple regression test for parallelism gave an R^2 value of 0.985 ($P = 0.007$), and 5α-pregnan-3β-ol-20-one (number 11 in Table 1) gave a value of 97% in the RIA and an R^2 value of 0.973 ($P = 0.0003$).

Several of the 17α-epimers of 5α- and 5β-pregnane, which have not been previously reported, were present in significant amounts. The combined mass of the 17α-epimers accounted for approximately 19% of the total.

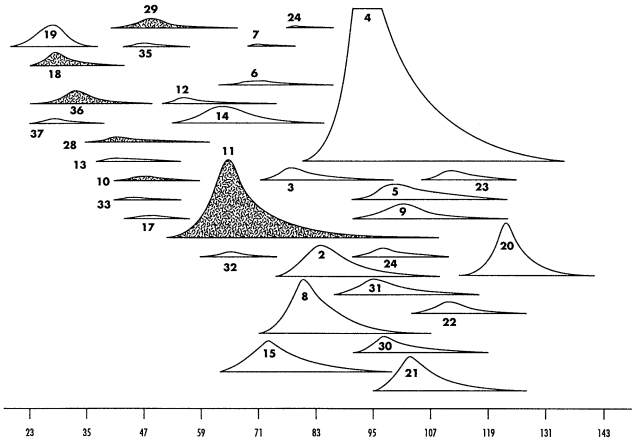

Fig. 3. Elution profile of $C_{21}O_2$ pregnanes following flash chromatography. The peaks are displaced in approximate order of elution and the size of the peak is proportional to their relative mass as determined by GC-MS. The number under each peak refers to the number in Table 1. Shaded peaks represent those pregnanes detected in the radioimmunoassay. The numbers on the X-axis represent individual 5 ml fractions.

4. Discussion

Pregnancy in the black rhinoceros lasts between 442 and 469 days [2] during which time fecal pregnane concentrations, as measured by immunoassay, can exceed 50 μg/g dry wt. (see Fig. 1). In non-pregnant cycling animals, fecal pregnane concentrations, as measured by RIA, typically range from 100 to 1500 ng/g feces, with considerable individual variation. Our results for duration of pregnancy in a captive black rhinoceros are in the same range as those reported by Garnier et al. [2] for pregnancy in wild black rhinoceros, and to those of Schwarzenburger et al. [5] for captive black rhinoceros. These high concentrations of pregnanes, especially in the last two months of pregnancy, made it possible for us to isolate and identify a large number of progesterone metabolites from a relatively small mass of material. We identified a total of 33 pregnanes in the $C_{21}O_2$ series, but did not attempt structural identity of the $C_{21}O_3$ series. A large number of pregnane triols have been identified in the feces of pregnant humans, the combined mass of which accounted for about 20% of the total steroids excreted in 24 h [13]. As far as we are aware, no pregnane triols have been identified in fecal extracts of any wild mammalian species.

We were unable to detect progesterone in the feces of the black rhinoceros. In the study by Heistermann et al. [9] in which ^{14}C-progesterone was injected into a female Sumatran rhinoceros, no radiolabeled progesterone was recovered in the feces, but more than 99% of injected radioactivity was accounted for in the fecal fraction and only trace amounts in the urine. The principal route of excretion of progesterone metabolites in most mammals studied is via the feces [14]. Hindle and Hodges [8] however, did report recovering a small amount of ^{14}C-progesterone in the feces of a female white rhinoceros that had been similarly injected. In general, however, it appears that very little free or unmetabolized progesterone is excreted in the feces of mammals. In the few studies in which attempts have been made to identify the fecal progestins in exotic mammals, African wild dog [15], baboon [16], African elephant [17], free progesterone was not recovered.

In rats and humans, and presumably other mammalian species, the passage of ovarian venous blood through the liver reduces the double bond between carbons 4 and 5 in the progesterone molecule to 5α (allopregnane) or 5β (pregnane) compounds that are then secreted into the bile [18–20,22]. In the gut the steroids are further modified. Hydroxylases for all positions in the progesterone molecule are known to occur in vertebrates except for C-4, 5, 8, 9, 10, 12 and 13. Micro-organisms have been identified, however, that can hydroxylate steroids not only in the same position as vertebrates, but also at C-8, 9, 10 and 12 [18–20].

We are not aware of any steroids in the pregnane series known from grasses that are the principal food of captive rhinoceros, but this does not rule out the possibility that some of the fecal steroids we identified came from dietary sources. A number of observations argue against this likelihood: All of the 17α-epimers identified were present in the same proportion as their 17β- precursors, indicating that the conversions occurred in the animal. The huge increase in fecal pregnanes during pregnancy, from values in the 100 ng/g range to concentrations in excess of 50 000 ng/g could not be the result of increased dietary intake, and, finally, pregnanes are virtually undetectable in the feces of male black rhinoceros (M.L. Patton, unpublished observations). All of these observations would suggest that the pregnanes we identified in feces from pregnant rhinoceros came from endogenous progesterone.

The 17α-epimers of pregnane are formed by the action of the microbial flora in the digestive tract using 16-hydroxylated-17β precursors [20,21]. Our results are first to document pregnane 17α-epimers in a herbivore. Little is known of the microflora in the gut of the rhinoceros, but it is evident that they are able to metabolize the 17β-pregnanes to their 17α-epimers. We identified a total of 14 pregnanes with the 17α-configuration (Table 1) only two of which have been previously reported [13]. A significant proportion of the fecal pregnanes were in the 17α configuration. Approximately 19% of the total by mass of the steroids we identified were 17α-epimers. The relative amount of each epimer was matched by the relative amount of its precursor. For example, 5α-pregnane-3β,20α-diol, which made up more than 44% of the total, was the precursor of the most abundant epimer, 5α(17α)-pregnane-3β,20α-diol, which accounted for 6.4% of the total.

Interestingly, the most abundant pregnane identified in our study, 5α-pregnane-3β,20α-diol, is barely detected in the RIA. The monoclonal antibody we used in our assay detects only progesterone and pregnanes with the 20-one configuration (see Table 1). Schwarzenberger et al. [4] reported 5α-pregnane-3,20-dione, 5α-pregnane-3α-ol-20-one

Table 1
Pregnanes in Rhinoceros feces

	Pregnane	Percent*	RIA cross-reactivity
1.	4-pregnen-3,20-dione	–	100
2.	5α-pregnan-3α,20α-diol	3.74	0.20
3.	5α-pregnan-3α,20β-diol	1.15	<0.03
4.	5α-pregnan-3β,20α-diol	44.46	<0.10
5.	5α-pregnan-3β,20β-diol	2.24	<0.001
6.	5α(17α)-pregnan-3α,20α-diol	0.23	N/A
7.	5α(17α)-pregnan-3α,20β-diol	0.03	N/A
8.	5α(17α)-pregnan-3β,20α-diol	6.40	N/A
9.	5α(17α)-pregnan-3β,20β-diol	1.74	N/A
10.	5α-pregnan-3α-ol-20-one	0.40	35.00
11.	5α-pregnan-3β-ol-20-one	10.69	97.00
12.	5α-pregnan-20α-ol-3-one	0.59	<0.10
13.	5α-pregnan-20β-ol-3-one	0.18	<0.04
14.	5α(17α)-pregnan-3α-ol-20-one	2.31	N/A
15.	5α(17α)-pregnan-3β-ol-20-one	4.36	N/A
16.	5α(17α)-pregnan-20α-ol-3-one	nd**	N/A
17.	5α(17α)-pregnan-20β-ol-3-one	0.16	N/A
18.	5α-pregnan-3,20-dione	0.99	90.00
19.	5α(17α)-pregnan-3,20-dione	1.92	N/A
20.	5β-pregnan-3α,20α-diol	4.57	<0.001
21.	5β-pregnan-3α,20β-diol	3.79	<0.001
22.	5β-pregnan-3β,20α-diol	1.17	0.42**
23.	5β-pregnan-3β,20β-diol	0.76	<0.001
24.	5β(17α)-pregnan-3α,20α-diol	0.73	N/A
25.	5β(17α)-pregnan-3α,20β-diol	0.03	N/A
26.	5β(17α)-pregnan-3β,20α-diol	nd	N/A
27.	5β(17α)-pregnan-3β,20β-diol	nd	N/A
28.	5β-pregnan-3α-ol-20-one	0.54	7.40
29.	5β-pregnan-3β-ol-20-one	0.74	12.00
30.	5β-pregnan-20α-ol-3-one	1.67	<0.01
31.	5β-pregnan-20β-ol-3-one	2.02	<0.10
32.	5β(17α)-pregnan-3α-ol-20-one	0.39	N/A
33.	5β(17α)-pregnan-3β-ol-20-one	0.09	N/A
34.	5β(17α)-pregnan-20α-ol-3-one	nd	N/A
35.	5β(17α)-pregnan-20β-ol-3-one	0.22	N/A
36.	5β-pregnan-3,20-dione	1.38	4.50
37.	5β(17α)-pregnan-3,20-dione	0.31	N/A

* Relative amount as a percent of total detected following chromatography: see Fig. 2.
** The signal for this epimer could not be clearly distinguished from that of the much larger peak 11.
N/A: not available; nd: not detected by the antibody in the RIA.

and 5α-pregnane-3β-ol-20-one as the most abundant progesterone metabolites in the feces of black rhinoceros. These three metabolites are also the principal three recognized by our antibody, but together comprise less than 15% of the total mass of pregnanes in the $C_{21}O_2$ series. Our figure of 50 μg/g dry wt. fecal pregnanes in the pregnant black rhinoceros is clearly an underestimate of total steroid mass. The pregnanediols, which are not recognized by the antibodies Swarzenberger [5] or we used, account for more than 50% of the fecal progesterone metabolites in the $C_{21}O_2$ series. In the Indian rhinoceros however, Schwarzenberger et al. [7] used an antibody that detected 5β-pregnane-3α,20α-diol as the principal immunoreactive fecal metabolite during pregnancy.

Based on analysis of biliary secretion in human and rat, the liver rapidly reduces progesterone to mostly 5β-pregnanes and a lesser amount of 5α-pregnanes [13,21]. The liver of mammals contains both 5α- and 5β-reductases, but it is not clear what determines the reduction of progesterone to one isomer over the other. In the African elephant, the principal fecal progesterone metabolites were in the 5α-configuration [17]. In the African wild dog, both 5α and 5β fecal metabolites were recovered, but it was not clear which were the most abundant [15]. Similarly, in the baboon both 5α and 5β fecal pregnanes were recovered [16]. The most abundant fecal pregnane found in human pregnancy, 5β-pregnane-3α,20α-diol [13] was also the most abundant pregnane identified in the feces of a non-pregnant Sumatran rhinoceros [9] and in the Indian rhinoceros [7]. In the pregnant black rhinoceros on the other hand, we found the most abundant pregnane to be 5α-pregnane-3β,20α-diol (Table 1). Schwarzenberger et al. [6] reported 5α-pregnanes as the most abundant progestins in white rhinoceros feces. Thus the two Asian species of rhinoceros (Indian, *Rhinoc-*

eros unicornis; Sumatran, *Dicerorhinus sumatrensis*) show a preponderance of 5β–pregnanes, whereas in the two African species, (white rhinoceros, *Ceratotherium simum;* black rhinoceros, *Diceros bicornis*), most of the fecal pregnanes are in the 5α-configuration. In the black rhinoceros, 81% of the pregnanes by mass were in the 5α-configuration (Table 1).

Evolutionary relationships within the family Rhinocerotidae are controversial. It has been argued that the two-horned species (two African plus the Sumatran) form one group separate from the Indian one-horned rhinoceros. Another theory posits the two African and two Asian species as separate lineages (see Xu and Arnason [23] for discussion and references). The results of our study and those from different rhinoceros species are interesting in that they lend support to the argument that the two Asian rhinoceroses and the two African species evolved separately.

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