Reproductive Assessment in Female African Rhinoceroses by Urinary Steroid Analysis

by

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ABSTRACT OF THESIS

Measurement of urinary pregnanediol glucuronide (PdG) and oestrone conjugates provides a reliable method of monitoring ovarian function and pregnancy in the Indian rhinoceros, but application is limited in the African species. The possibility of species differences in the metabolism and excretion of ovarian steroids was investigated by a metabolism study involving the i.v. injection of 100μCi each of ^{14}C-labelled progesterone and oestradiol-17β into an adult female white rhinoceros. Of the radiolabel injected, significant amounts were recovered in both urine (25%) and faeces (36%) collected over the following 4 days. Almost all (92%) of the label recovered in urine was accounted for in the Day 2 sample, of which 41% steroids were present in the conjugated form. 20α-Dihydroprogesterone(20α-DHP) was the only conjugated progesterone metabolite in urine with which radioactivity was associated and no radioactivity could be found to co-elute with pregnanediol after high performance liquid chromatography (HPLC) separation of conjugated neutral steroids. Whereas progesterone metabolites predominated in urine, isomers of oestradiol were the most abundant steroids in faecal extracts.

The metabolism and excretion of endogenous ovarian steroids in African rhinoceroses was then examined by gas chromatography/mass spectrometry (GC/MS) and HPLC in conjunction with differential hydrolysis techniques. GC/MS identified pregnanediol as the major progesterone metabolite in the Indian rhinoceros, but neither PdG nor 20α-DHP was detected in urine from African rhinoceroses during any stage of the reproductive cycle. However, HPLC confirmed the presence of conjugated 20α-DHP and the absence of PdG in post-oestrus urine. Oestrone-glucuronide was the major urinary oestrogen in the black, whilst oestradiol-17β-glucuronide predominated in the white, rhinoceros.

A sensitive enzymeimmunoassay was developed for the measurement of 20α-DHP. This was initially validated by measurement of 20α-DHP in hydrolysed rhinoceros urine, which showed parallel dilution-response curves with the standards, and subsequently by co-chromatography on HPLC. In hydrolysed urine from the black rhinoceros (9 cycles, 4 animals) levels of 20α-DHP and oestrone, indexed to creatinine (Cr), followed a cyclic pattern which correlated well with behavioural events. Levels of 20α-DHP were low for 3 days prior to mating during which oestrone levels rose to reach a peak. This was followed by a rapid rise in 20α-DHP to reach maximum levels (7·64±3·82 ng/mgCr) within 2 days. The pattern of 20α-DHP excretion in the white rhinoceros (6 cycles, 3 animals) differed in that the presumed follicular phase was longer than in the black species (12-17 d), and maximum levels of 20α-DHP (80-90 ng/mgCr) were not reached until approximately 7 days after peak oestradiol-17β excretion at the time of oestrus . A mean cycle length of 22 days was recorded for the black rhinoceros whilst cycle lengths of 26 and 32 days were obtained for the northern and southern white respectively.

In contrast to the ovarian cycle, PdG was present in late pregnancy urine from African rhinoceroses. Elevated levels were recorded during early pregnancy in the black rhinoceros, whilst concentrations did not increase for 5-6 months in the white species. Urinary oestrogen levels also rose throughout gestation. A gestation period of approximately 16 months was recorded for both species.

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ABBREVIATIONS

Abbreviations used throughout this thesis, in alphabetical order.

A; 5α-androstane-3α, 17α-diol

androstenedione; 4-androstene-3,17 dione

avs; "Analar" volumetric solution

B; blank

BSA; bovine serum albumen

C; cholesterol butyrate

C of V; coefficient of variation

CMO; carboxymethyl oxime

cortisol; 11β, 17α,21-trihydroxy-4-pregnene-3,20-dione

Cr; creatinine

dehydroepiandrosterone; 3-hydroxy-5-androsten-17-one

20α-DHP; 20α-dihydroprogesterone; 20α-hydroxy-4-pregnen-3-one

E_o; phenolic steroids

E_1; oestrone; 3-hydroxy-1,3,5{10}-oestratrien-17-one

E_2-17α/β; oestradiol-17α/β; 1,3,5{10}-oestratriene-3,17α/β-diol

E_3; oestriol; 1,3,5{10}-oestratriene-3,16α,17β-triol

EIA; enzyme immunoassay

E_1G; oestrone-3-glucuronide

equol; 7-hydroxy-3-{4'-hydrophenyl}chroman

ES; oestrone-3-sulphate

FSH; follicle stimulating hormone

GC/MS; gas chromatography/mass spectrometry

GFR; glomerular filtration rate

GnRH; gonadotrophin releasing hormone

hCG; human chorionic gonadotrophin

HCl; hydrochloric acid

11α-hydroxyandrosterone; 3α,11α-dihydroxy-5α-androstan-17-one

17α-hydroxypregnenolone; 3β,17α-dihydroxy-5-pregnen-20-one

16α-hydroxyprogesterone; 16α-hydroxy-4-pregnene-3,20-dione

HMDS; hexamethyldisilazane

HPLC; high performance liquid chromatography

HRP; horse radish peroxidase

17α-hydroxyprogesterone; 17α-hydroxy-4-pregnene-3,20-dione

IgG; immuno gamma globulin

LH; luteinizing hormone

LSB; low salt buffer

M+; molecular ion

MO-TMS; methyloxime-trimethylsilyl

m/z; mass to charge ratio

NSB; non-specific binding

O-1 and O-3; oestrus-1 and oestrus-3 (follicular)

O+7; oestrus + 7 (luteal)

OHSD; hydroxysteroid dehydrogenase

O.D.; optical density

P_4; progesterone; 4-pregnene-3,20-dione

P_o; neutral steroids

PBS; phosphate buffered saline

Pd; pregnanediol; 5β-pregnane-3α,20α-diol

PdG; pregnanediol-3α-glucuronide

pregnanetriol; 5α/β-pregnane-3α/β,17α/β,20α/β-triol

pregnenolone; 3α/β-hydroxy-5-pregnen-20-one

QC; quality control

RIA; radioimmunoassay

S; stigmasterol

SIC; selected ion current chromatogram

SpA; specific activity

TB; total bound

TBS; tricine buffered saline

TC; total counts

testosterone; 17β-hydroxy-4-androst n-3-one

TIC; total ion current chromatogram

TLC; thin layer chromatography

vol; volumes

Z; zero

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PUBLICATIONS ARISING FROM THIS THESIS.

HINDLE, J.E. (1989). Aspects of the reproductive endocrinology of rhinoceroses. In Proc. Soc. Study Anim. Breed., pp. 78-83. Ed. R.T.Pepper. Upjohn Ltd., Crawley.

HINDLE, J.E., COULSON, W.F., HONOUR, J.W. & HODGES, J.K. (1988). Comparative aspects of progesterone metabolism in the rhinoceros. J.Reprod. Fert. Abstr. Ser. 1, 77.

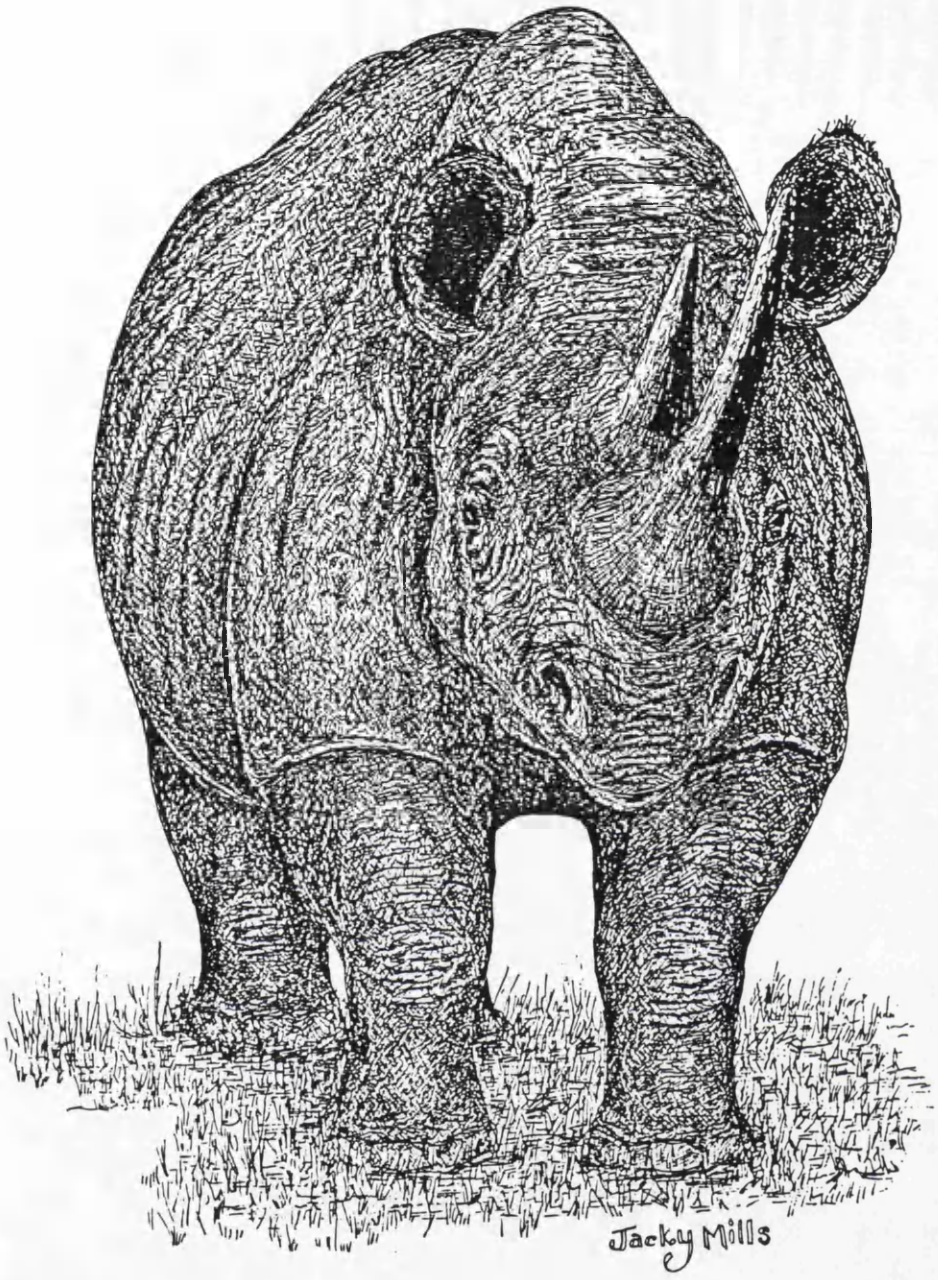
HINDLE, J.E., & HODGES, J.K. (1989). Metabolism of oestradiol and progesterone in the white rhinoceros (*Ceratotherium simum*). J.Reprod. Fert. Abstr. Ser. 3, 101.

HINDLE, J.E. & HODGES, J.K. (1990). Metabolism of oestradiol-17β and progesterone in the white rhinoceros (Ceratotherium simum simum). J.Reprod. Fert. 90, 571-580.

HINDLE, J.E., MÖSTL, E. & HODGES, J.K. (1990). Measurement of urinary 20α-dihydroprogesterone in the African species of rhinoceros. J. Reprod. Fert. Abstr. Ser. 5, 49.

HINDLE, J.E., MÖSTL, E. & HODGES, J.K. (1991). Measurement of urinary oestrogens and 20α-DHP during the ovarian cycle of the black (*Diceros bicornis*) and the white (*Ceratotherium simum*) rhinoceros. (submitted).

HODGES, J.K. & HINDLE, J.E. (1988). Comparative aspects of urinary hormone excretion in rhinoceroses: implications for reproductive assessment. In Proc. 5th World Conf. on Breeding Endangered species in captivity, pp. 83-91. Eds. B.L.Dresser, R.W.Reece and E.J.Maruska. Cincinnati zoo, Ohio.


Jacky Mills

CHAPTER 1.
INTRODUCTION

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1.1 RHINOCEROS BIOLOGY

1.1.1 Taxonomy

There are five extant species in the family *Rhinocerotidae* which together with the *Equidae* and *Tapiridae* constitute the order *Perissodactyla* or odd-toed ungulates. Rhinoceroses are often grouped with tapirs in the sub-order *Ceratomorpha* to distinguish these species, synonomous prior to the late Eocene, from the equids (*Hippomorpha*).

Three species of rhinoceros inhabit the tropical regions of Asia, namely the Indian (*Rhinoceros unicornis*) (Linnaeus, 1758), Sumatran (*Dicerorhinus sumatrensis*) (Fischer, 1814) and Javan (*Rhinoceros sondaiacus*) (Desmarest, 1822) rhinoceroses. The remaining species, the black (*Diceros bicornis*) (Linnaeus, 1758) and the white (*Ceratotherium simum*) (Burchell, 1817) rhinoceroses, inhabit the African plains.

The Asian and African rhinoceroses are thought to have evolved independently. The Sumatran rhinoceros is the only living member of the *Dicerorhinae* (Gloger, 1841), whilst Indian and Javan rhinoceroses both belong to the *Rhinocerinae*. The African species evolved from the same stock until divergence in the Pliocene led to the formation of the black and white species (Macdonald, 1985). There is some doubt whether the black and white rhinoceroses are dissimilar enough to warrant two different genera, indeed in the past the two were located over a similar range but differed in their ecological requirements. However, it is now generally accepted that they are two different genera with sub-species within each genera. There are two recognised sub-species of white rhinoceros, the northern (*Ceratotherium simum cottoni*) (Lydekker, 1908) and the

southern (*Ceratotherium simum simium*) (Burchell, 1817) races, which are not dissimilar in appearance but are now geographically separated. There is also geographic variation with the black rhinoceros population. Zukowsky (1964) suggested the existance of 17 sub-species, reinterpreted as 7 main subspecies *Diceros bicornis bicornis, D. b. chobiensis, D. b. minor, D. b. michaeli, D. b. ladoensis, D. b. longipes, D. b. brucii* (Groves, 1967a).

1.1.2 Distribution and status in the wild

The Indian rhinoceros once ranged from the Indus valley in the east, across the Ganges to Assam in the west (Laurie, 1978). In the 1900s the Indian rhinoceros was on the verge of extinction, but has recovered dramatically with conservation help (Martin, 1985; Martin, Martin and Vigne, 1987). Around 1,800 individuals now remain in Assam, Nepal and Bengal (Penny, 1987) with approximately 84% of the population in Kaziranga and Chitwan national parks (Sale and Singh, 1987). The Javan rhinoceros lived alongside the Indian species on the mainland, and in Burma, Malaya, Thailand and most of Indo-China (Prater, 1971). Today its range is limited to the Ujung Kulon reserve in western Java (Laurie, 1978; Schenkel and Schenkel-Hulliger, 1977) where it is thought that there are 50 animals (Nardelli, 1987), although there is evidence that a few individuals may persist in Thailand, Laos and Cambodia (Rookmaker, 1980). Recently, reports of sightings of the Javan rhinoceros in Vietnam (Schaller, Dang, Thuy, and Son, 1990) suggest that a population of 10-15 animals may have been rediscovered in the Nam Cat Tien reserve (Dang, 1986; 1988; Thai, 1987).

Until the turn of the century, the Sumatran rhinoceros had a very wide distribution throughout continental south-east Asia, Sumatra and Malaya (Groves, 1967b; Groves and Kurt, 1972). The species is now located in two protected reserves in Indonesia, Kerinci and Gunung Leuser, each carrying a population of around 300 animals (Penny, 1987).

The past and present distribution of wild black rhinoceroses is shown in Figs. 1.1a and b. The black rhinoceros was once found throughout East Africa, from the cape in the south to Sudan in the north, with Nigeria being the western limit of its range (Groves, 1967a; Roth, 1967; Hillman, 1982). However, relentless poaching during the 1960s and 1970s caused the population to fall from 100,000 in 1961 to around 14,700 individuals in 1980 (Western and Vigne, 1986). Between 1981 and 1987 the numbers further declined by 70% to an estimated total population size of 3,800 (Cumming, Du Toit and Stuart, 1987). The downward trend in numbers continues, the most recent estimate being 3,000 (Emslie and Adcock, 1990). The decline in numbers has been accompanied by fragmentation into small populations, the largest of which comprises 400 animals, and between which there is no exchange of genetic material. Some sub-species of black rhinoceros may have already been lost, and the continental population has now been divided into four regional units which contain viable populations, south-western desert (*Diceros b. bicornis*), south-central Africa (*Diceros b. minor*), East Africa (*Diceros b. michaeli*) and those animals surviving north of the rainforest belt (Cumming, Du Toit and Stuart, 1990).

The past and present distribution of the white rhinoceros is shown in Fig. 1.2 a and b. The southern white rhinoceros once ranged over most of Southern Africa from Angola to Zululand (Player and Feeley, 1960; Owen-Smith, 1973). However, at the turn of the century, its numbers had fallen to only 200 individuals in a small area of Natal (Turner, 1982) and Uganda (Pittman, 1931). The population has since recovered and, in contrast to the black rhinoceros, has continued to show a clear upward trend and is now spread throughout much of the South African subregion. Between 1981 and 1987 the total African population of Southern white rhinoceros rose from 2,861 to 4,560 (Cumming *et al.*, 1987), an increase of 8% per annum.

The northern white subspecies of rhinoceros has been less fortunate. In the 1900s, the northern white rhinoceros occurred

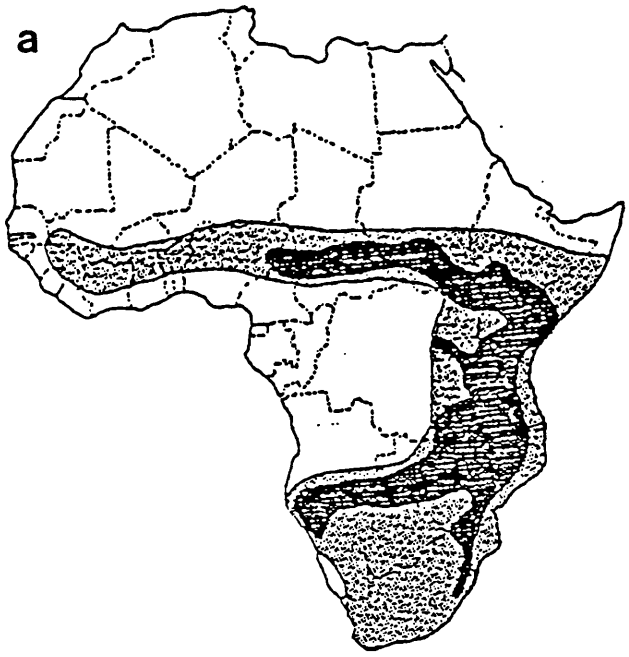
Figure 1.1a. Former distribution of the black rhinoceros. Light shading indicates the probable distribution around 1700; dark shading indicates distribution in 1900 (adapted from Cumming *et al.*, 1987).

Figure 1.1b. Distribution of the black rhinoceros in 1987 (Cumming *et al.*, 1987).

Figure 1.2a. Probable distribution of the white rhinoceros around 1800 (Cumming *et al.*, 1990).

Figure 1.2b. Distribution of the white rhinoceros in 1987 (Cumming *et al.*, 1990).

a



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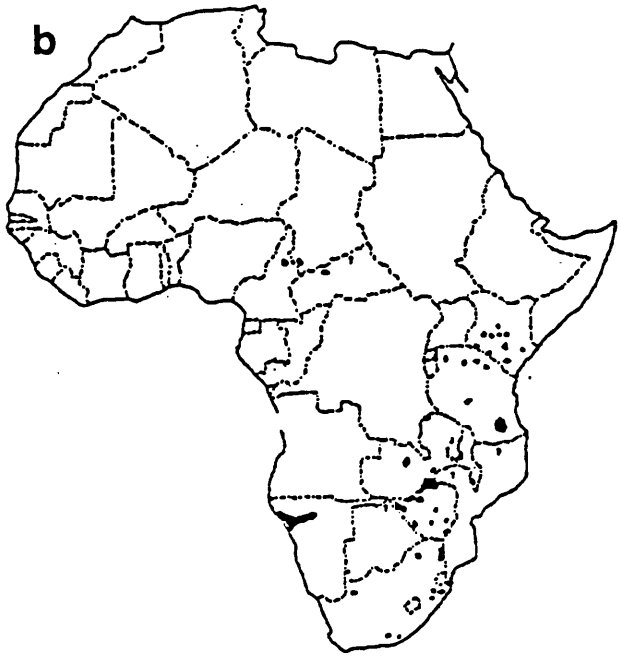

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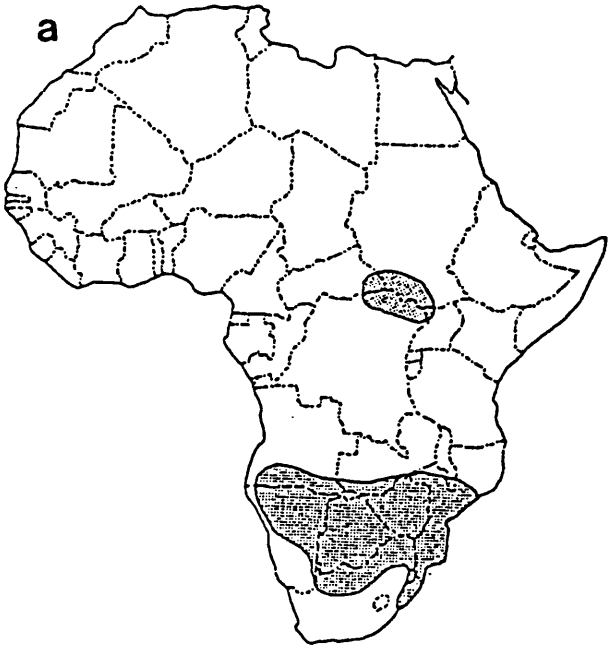

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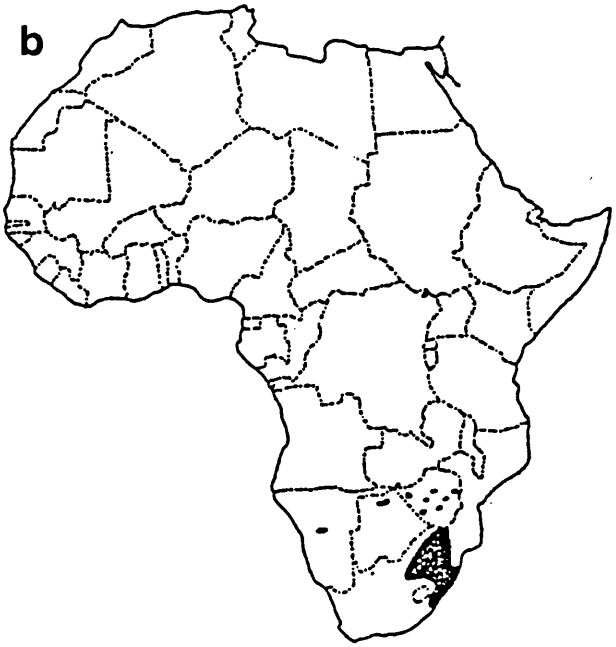


from Southern Chad, west to Sudan and south to Uganda. However, numbers declined rapidly and, in 1980, 821 individuals were known to survive in the Central African Republic, Sudan, Uganda and Zaire (Sidney, 1985). Within four years the population had crashed to 17 animals, all located in Garamba national park in Zaire (Smith, 1986). The decline was subsequently arrested and the population increased to 22 animals in 1986 (Cumming *et al.*, 1987).

The rapid decline in numbers of wild rhinoceroses has made these key species of great interest in the field of conservation. Although the prevention of poaching and exportation of rhinoceros products has been the priority for species protection in the past, it is now recognised that there is an urgent need for carefully planned genetic and reproductive management programmes for the existing populations, if long term conservation of the rhinoceros is to be successful.

1.1.3 Status in captivity

Little information is available on the breeding of Asian rhinoceroses in captivity. Certainly, the Javan and Sumatran species are rarely seen in zoological collections, with the only births being recorded in the 19th century (Bartlett, 1873). The Indian rhinoceros has bred fairly regularly in captivity since a birth in Basle zoo in 1975 (Lang, 1975). However, the 80 animals in captivity (Olney and Ellis, 1989) cannot safeguard against the declining numbers in the wild.

The black rhinoceros has been exhibited in zoos for over 100 years but, with most animals housed as single specimens (Hindle, 1947), the first birth was not recorded until 1958 (Banks, 1986). In 1987 the world captive population of black rhinoceros numbered only 128 animals (Olney and Ellis, 1989) and, despite attempts to relocate animals to stimulate breeding (Shapcott, 1986), was recently estimated to be decreasing by approximately 7% per annum

(Lindemann, 1984). The southern white rhinoceros has bred more successfully in zoological collections (Rawlins, 1979), and the total captive population now numbers 371 (Olney and Ellis, 1989). The number of southern white rhinoceros in captivity is a dramatic contrast to the 13 northern white rhinoceroses in zoological collections. Until recently, 11 of these animals were housed in one location, at Dvůr Králové Zoo in Czekoslovakia. Out of these animals a single female, Nasima, has bred. Two female and a male Northern white rhinoceroses have now been moved to San Diego Wild Animal Park to join a lone male, where it is hoped that further breeding may occur.

Although the numbers of southern white rhinoceroses in captivity has continued to increase steadily, the populations of northern white and black rhinoceroses are declining. There are several factors which may contribute to the decline in number of African rhinoceroses in captivity. A high still birth rate and general, premature mortality has added to the degeneration of the population. The situation is particularly serious in the black rhinoceros. Through probable deficiencies in nutrition (Dierenfeld, Du Toit and Miller, 1988; Ghebremeskel, Williams, Lewis and Du Toit, 1988), perhaps due to mismanagement, haemolytic anaemia had afflicated around 25% of the captive population causing death in over 15 animals (Miller and Boever, 1982). Inappropriate management may also account for social deficiencies leading to reproductive failure within the population. Undoubtedly one of the main contributory factors to the poor breeding record in captivity is the lack of knowledge of the reproductive biology of the rhinoceros and the factors required for reproductive success. The importance of obtaining such information is illustrated in the species survival plan for African rhinoceroses which is produced by the Captive Breeding Specialist Group (part of the American Association of Zoo Parks and Aquariums) (Cumming *et al.*, 1989). This plan ascribes priority status to reproductive studies on the animals which remain in captivity in order to gain an insight into the biology and physiology of the species.

1.2 REPRODUCTIVE BIOLOGY OF AFRICAN RHINOCEROSES

1.2.1 Reproductive behaviour

Very little is known about the reproductive behaviour of African rhinoceroses, and the information available comes largely from studies carried out in the wild. However, it is clear from these studies that there are differences in the pattern of social behaviour of the white and the black species.

White rhinoceros bulls of reproductive age are strictly territorial (Owen-Smith, 1971). Groups may occur within each territory, each group consisting of females and sub-adults with which the male is associated (Ripley, 1958; Guggisberg, 1966). As a consequence, breeding of the white rhinoceros in captivity is most successful when the animals are kept in herds, although this species is often exhibited in pairs for ease of management.

An indepth study of the white rhinoceros herd at Whipsnade Wild Animal Park (O'Connor, 1982; O'Connor, 1986) demonstrated that the establishment of dominance by the bull provides stimulation for reproduction within the herd, and studies in the wild have confirmed this observation (Owen-Smith, 1975; Eriksen, 1977). Studbook keepers Klös and Frese (1978) further suggested that several females must be present to ensure breeding success in captivity, and similar conclusions were drawn from a survey by Rawlins (1979) which demonstrated that zoos with more than 3 animals were the most successful in breeding white rhinoceroses. However, the relative extent to which behavioural and physiological factors contribute to the failure of reproduction in captivity is difficult to assess as no method of monitoring reproductive function is available.

The black rhinoceros is thought to be less strictly territorial (Schenkel and Schenkel-Hülliger, 1969), and males and females are rarely seen together except when the female is in oestrus (Goddard, 1967). Limited information is available on the

reproductive behaviour of this species in captivity as few females have bred. Black rhinoceroses are often exhibited in pairs which has been shown to adversely affect mutual sexual interest (Klös and Frädrich, 1970), although the significance of this management policy over reproductive dysfunction due to physiological problems is unknown.

1.2.2 Sexual maturity

Accurate determination of the age of animals in the wild is a difficult process and most studies have had to rely on estimation of body length (Guérin, 1980) and horn size (Roth and Child, 1968). However, it has been estimated that, although the animal may be capable of reproducing at an earlier age, white rhinoceros bulls will not rise to the position of dominant males until the age of 10-12 years. However, female white rhinoceroses will reproduce as soon as sexual maturity is attained at 5-6 years of age (Heppes, 1958; Owen-Smith, 1971). More accurate figures for the age of sexual maturity, obtained from a census of animals in captivity (Lindemann, 1982), correspond well with results obtained in the wild.

Black rhinoceros bulls become capable of siring young at around 6 years of age, both in the wild (Ritchie, 1963; Schenkel and Schenkel-Hülliger, 1969; Mentis, 1972; Reuther, 1972; Hall-Martin, 1986) and in captivity (Fraust, 1958; Dittrich, 1967; Yamamotto, 1967). Data from the rhinoceros population in Tsavo East (Schenkel and Schenkel-Hülliger, 1969) suggests that females reach sexual maturity at 3½-4 years. Although a similar observation was made by Jones (1979), more recent and representative evidence suggests that black rhinoceros cows in zoological collections will not breed until aged 5-7 years (Lindemann, 1982; Lindemann, 1984).

1.2.3 Oestrous cycle

The length of the oestrous cycle of the white rhinoceros has been estimated to be 30 days, based on the intervals between matings observed in the wild (Owen-Smith, 1971). The 30 day cycle length has not been satisfactorily confirmed in captivity, and a range of inter-oestrus intervals of 27-265 days has been reported in a single study which collated data on 29 females provided by zoos around the world (Lindemann, 1982). However, the same study suggested that behavioural oestrus in the white rhinoceros is very short and that females may suspend overt signs of oestrus for long periods of time, both of which may account for the wide range of cycle lengths reported from behavioural observations. Without hormonal data, which are currently unavailable for this species, accurate determination of the length of the ovarian cycle of the white rhinoceros is difficult.

A single study has estimated the length of the oestrous cycle of the black rhinoceros in the wild to be 35 days (Hitchins and Anderson, 1983). Female black rhinoceroses in oestrus urinate frequently, leaving a white streaky deposit around and beneath the vulva (Goddard, 1967); together with a swollen vulva, and the close attentions of males, these are the signs of oestrus in this species (Mukinya, 1973). However, the observation of subtle changes in behaviour associated with oestrus is difficult in the wild where there is restricted view of animals (Brett, Hodges and Wanjohi, 1988). In captivity, where close behavioural observation is possible, inter-oestrus intervals of between 17 and 60 days have been reported (Dittrich, 1967; Gowda, 1967; Greed, 1967; Hallstrom, 1967; Yamamotto, 1967). Once again, such variation demonstrates the unreliability of estimating cycle length by behavioural observation and illustrates the requirement of hormonal data. Ramsay, Kasman and Lasley (1987) were unable to monitor the ovarian cycle of the black rhinoceros by endocrine methods, and could therefore not offer any further information on the length of the oestrous cycle in this species. Bamberg and Schwarzenberger

(1990) have suggested a cycle length of 25 days in the black rhinoceros by estimating the time interval between nadirs of faecal progestagen excretion, although the data is preliminary and the methods used have yet to be validated. No other published information is available.

1.2.4 Gestation period

Both black and white rhinoceroses normally give birth to a single calf. The gestation period in the white rhinoceros has been estimated to be 15-18 months in both the wild (Owen-Smith, 1971) and in captivity (Rawlins, 1979; Lindemann, 1982; Hodges and Green, 1989). A shorter length of 13-15 months has been reported for the black rhinoceros both in the wild (Goddard, 1967; Joubert and Eloff, 1971; Hall-Martin and Penzhorn, 1977) and in captivity (Dittrich, 1967; Goddard, 1967; Gowda, 1967; Hays, 1967; Yamamotto, 1967; Roomaker, 1973; Ramsay *et al.*, 1987; Wanjohi, 1989).

For both species, most estimates of gestation length are based on calculating the interval between the last observed mating and parturition. However, determining the mating that led to conception is often difficult. The only reliable data have been provided by Wanjohi (1989) who determined the gestation length of a single black rhinoceros to be 494 days by monitoring endocrine changes during pregnancy.

In summary, current knowledge of the reproductive biology of the African species of rhinoceros is derived mainly from behavioural studies in the wild where reliable observations are difficult to obtain. Information on reproductive physiology is limited to a couple of studies on the black rhinoceros where some useful data have been provided on the length of gestation, but no information is available regarding the ovarian cycle. There are no published data on the endocrinology of the ovarian cycle or gestation length in the white rhinoceros.

There are a number of reasons for this lack of physiological information. The main reason is that there have been limited opportunities to study "normal" reproductive physiology within the captive rhinoceros population due to the poor reproductive success and difficulties presented by management policies. Another important point is the physical difficulty of working with the animals due to their size and intractable nature. This is a problem experienced by many scientists who work with wild animals, and thus methods have been devised to overcome this obstacle. The requirements for assessing reproductive status in exotic species, and methods currently available with respect to the female, are considered in the next section.

1.3 METHODS FOR ASSESSING FEMALE REPRODUCTIVE STATUS

There are a variety of methods available for assessing reproductive status in female mammals, as shown in Fig. 1.3. For use in the monitoring of reproductive function in exotic animals, such methods need to be accurate, reliable and sufficiently versatile to allow application to different species. With the ultimate aim of performing assessments in zoos and in the wild, methods should be practical, economical, simple and require the minimum amount of sophisticated laboratory equipment. Few methods satisfy all the requirements listed but invasive methods, which require capture and restraint or sedation of the animal, are not suitable for use in the case of many exotic species. Non-invasive methods may therefore provide the only possible alternative for assessing reproductive status in these animals.

1.3.1 Non-endocrine methods of assessing reproductive status

The observation of changes in vaginal contents in response to cyclic fluctuations in circulating hormones, is a potentially useful method for determining reproductive status and predicting the time of ovulation. However, there are difficulties in obtaining

Figure 1.3 Methods for assessing reproductive status in female exotic mammals. Adapted from Hodges (1990).

Non-endocrine	Endocrine
Laparotomy	Blood sample
Laparoscopy	Saliva sample
Ultrasonography	Urine sample*
Behavioural observation*	Faecal sample*
Vaginal cytology	Milk sample

*Methods requiring no contact with the animal

informative measurements across a diverse range of species (D'Souza, 1978) and, in practice, the usefulness of such methods is limited by the need to restrain or sedate the animal for daily collection of data.

Real-time ultrasonography has been applied to investigate ovarian dynamics and pregnancy in primates (Morgan, Hutz, Kraus and Cormie, 1985; Tarantal and Hendrickx, 1988) and other exotic mammals (Wilson and Du Boulay, 1988). Although ultrasound has the advantage that organs may be visualised and a rapid diagnosis may be obtained, use of this technique requires direct contact with the animal which involves sedation in many cases. Use in the rhinoceros is further limited as this is a large animal in which the internal organs and situated deep within the body; rectal probing would be necessary in this species which would require immobilisation possibly causing stress and damage to the spinal cord whilst the animal is recumbent (King, 1969). Similar restictions limit the use of rectal palpation (Mahoney, 1970; Studer, 1975) in the rhinoceros species.

The surgical procedures of laparoscopy and laparotomy allow direct observation of the ovaries and thus enable the precise time of ovulation to be determined for successful artificial insemination. Such techniques are also invaluable in detecting anatomical abnormalities which cause reproductive dysfunction, and can therefore be useful diagnostic tools. Despite the obvious advantages, laparoscopy and laparotomy are invasive procedures, requiring surgery, expensive equipment and skilled labour. The need for regular sedation or anaesthesia also limits the application of these methods for assessing reproductive status in large exotic species. It has also been shown that the surgical stress involved with laparoscopy and laparotomy may in itself be sufficient to inhibit or alter the pattern of reproduction in many wild animals (Bosu, Johansson and Gemzell, 1973). However, in some exotic species, eg. carnivores (Wildt and Seager, 1979; Bush, Seager and Wildt, 1980; Wildt, 1980; Bonney, Moore and Jones, 1981), non-

invasive, endocrine methods for assessing reproductive status have been unsuccessful and laparoscopy and laparotomy continue to provide valuable information to reproductive physiologists.

With intractable and dangerous animals such as rhinoceroses, it is hardly suprising that the majority of reports on reproductive assessment have relied upon behavioural observation. Although such observations allow the detection of reproductive events they are not, on their own, sufficiently accurate to determine the exact timing of ovulation and to diagnose reproductive dysfunction (Hodges, 1986). In order to utilise artificial breeding techniques to aid captive management of exotic species, behavioural observations must be combined with a sound knowledge of the animal's reproductive endocrinology and physiology (Hodges and Hearn, 1983).

1.3.2 Endocrine methods for assessing reproductive status

The female reproductive cycle is controlled by circulating gonadotrophins (luteinizing hormone, LH, and follicle stimulating hormone, FSH) which stimulate oocytes within the ovary to mature, ovulate and luteinize, producing the ovarian steroids, oestrogens and progestagens, which in turn regulate gonadotrophin release. Measurement of changes in concentration of these reproductive hormones in the peripheral circulation provides an accurate indication of ovarian function.

New and highly sensitive immunoassays to measure hormones in the circulatory system have enabled the endocrinology of the ovarian cycle and pregnancy of women, laboratory animals and most domestic species to be characterised in detail. Such knowledge has allowed artificial breeding techniques to be employed to manipulate reproduction in the human (Collins, 1983) and increase productivity of many farm animals (Gordon, 1983). However, the hormonal control of reproduction varies significantly between species, and detailed investigation into the reproductive endocrinology of each new animal studied is necessary.

In order to obtain reliable information on the fluctuations in circulating hormones, frequent blood samples must be collected. Whereas regular blood sampling is possible in some small, exotic mammals which may be easily restrained or trained for laboratory purposes , eg. *Callitrichids* (Chambers and Hearn, 1979; Hearn, 1983), sequential samples are seldom available from an intractable species such as the rhinoceros. The degree of restraint, and even sedation, that may be required to obtain blood samples from large, exotic species is sufficient to prohibit the use of this method of reproductive monitoring in most zoo animals. One exception is the elephant, a species which may be trained to accept blood sampling without stress (Brannian, Griffin, Papkoff and Terranova, 1988; Plotka, Seal, Zarembka, Simmons, Teare, Phillips, Hinshaw and Woods, 1988). In the African and Asian elephants, in addition to monitoring the ovarian function by changes in circulating levels of progesterone, the measurement of prolactin in the blood is a reliable way of detecting pregnancy (Hodges, Henderson and McNeilly, 1983; McNeilly, Martin, Hodges and Smuts, 1983) and, as frequent sampling is not required, the information obtained justifies the use of the procedure.

It is clearly not possible to collect regular blood samples from rhinoceroses although detailed knowledge of the reproductive endocrinology is required to improve breeding preformance in captivity. Endocrine methods of assessing reproductive status, accompanied by non-invasive sample collection therefore offer the only feasible approach for long term studies on animals in zoological collections. The measurement of ovarian steroids in body fluids other than plasma potentially provides a reliable method for monitoring ovarian function providing levels of the hormone, or its metabolite, accurately reflect the levels in the peripheral circulation.

Measurement of the progesterone content of milk (Sauer, Foulkes, Worsfold and Morris, 1986) has been useful in determining the reproductive status of animals in which lactation is concurrent

with ovarian cycles and pregnancy, eg. the cow (Hoffman and Hamburger, 1973), goat (Murray and Newstead, 1988) and horse (Gunther, Fowley, Gaverick and Plotka, 1980). Salivary progesterone levels have also been shown to correlate well with circulating concentrations during ovarian cycles in the human (Finn, Gosling, Tallon, Madden, Meechanm and Fottrell, 1988; Lenton, Gelsthorp and Harper, 1988) and cow (Gao, Short and Fletcher, 1988), and may also be useful in detecting pregnancy in the black rhinoceros (N.M.Czekala, personal communication). Obscure non-invasive methods of determining reproductive status, such as hair progesterone analysis (Liu, Chen, Guo, Song and Zhang, 1988), have been proposed but have yet to be validated.

The analysis of hormone metabolites in urine and faeces has provided a basis for the characterization of the ovarian cycle and pregnancy in many exotic species (reviewed by Loskutoff, Ott and Lasley, 1983; Lasley, 1985; Hodges, 1986; 1990). The major advantage of hormonal assessment of reproductive status by measurement of excreted hormones is that sample collection avoids direct contact with the animal and such procedures are therefore possible in species which are difficult to handle or easily stressed. However, as corresponding blood samples are usually unavailable, it is not possible to correlate results obtained with plasma hormone profiles, and behavioural observations must be used to determine the time of reproductive events. The clearance rate of hormones from the body results in a certain delay which makes the exact time of ovulation difficult to determine. Furthermore, this delay results in an integrated picture rather than a description of short-term changes and does not enable pulsatile release of hormones to be detected.

Equally important is the identification of the hormone metabolites in urine and faeces which will, when measured by immunoassay, most accurately reflect ovarian function. This is of particular importance when considering the measurement of excreted, ovarian steroid hormone metabolites. Whilst circulating ovarian

steroids do not differ significantly between taxa, species differences in the metabolism and route of excretion of progesterone and oestradiol-17β have been demonstrated. It is therefore very important to identify the oestrogen and progesterone metabolites that are excreted into the urine and faeces of each new species studied. The biosynthesis of reproductive steroids and the possible end products produced by *in vivo* steroid metabolism are discussed in the following section.

1.4 BIOSYNTHESIS AND METABOLISM OF REPRODUCTIVE HORMONES

Both circulating gonadotrophins and ovarian steroid hormones are metabolised in the body prior to excretion via the urine or faeces. Although the measurement of urinary gonadotrophins has provided valuable information on the endocrinology of the ovarian cycle in a number of species there are specific problems associated with their determination (see next section). Such problems limit the use of urinary gonadotrophin analysis and, although gonadotrophin measurement can enhance results obtained by steroid analysis, the measurement of steroid metabolites is a more reliable and feasible method of assessing reproductive status. For this reason the emphasis of this section, and indeed this thesis, is on the metabolism and excretion of steroid hormones.

1.4.1 Glycoprotein hormones

The glycoprotein hormones include the pituitary hormones, LH, FSH and thyroid-stimulating hormone, and the placental hormones, human and equine chorionic gonadotrophins. All of these hormones are composed of a protein core with branched carbohydrate side chains usually terminating with sialic acid. The glycoprotein hormones share a common quarternary structure, each hormone possessing two non-identical α and β subunits with hydrophobic binding between the subunits (Aloj, Edeloch, Ingham, Morgan, Canfield and Ross, 1973). The α-subunits of all the homologous

glycoprotein hormones are nearly identical, whilst the β-subunits confer specificity on each of these hormones (Pierce, 1971).

Glycoprotein hormones are metabolised in the liver, where the protein component of the hormone is denatured. However, although the structure of the molecule changes dramatically on metabolism, the functional site remains intact. The bioactive hormone metabolite is then excreted via the kidney, into the urine.

1.4.1.1 Pituitary gonadotrophins

The biosynthesis of gonadotrophins, LH and FSH, in the pituitary is under the control of gonadotrophin releasing hormone (GnRH), produced in the hypothalamus and discharged in a pulsatle manner via the portal network to reach the pituitary (Clarke and Cummins, 1982).

Extraction studies of normal pituitary tissue have revealed the presence of an excess concentration of the α-subunit of LH and FSH (Prentice and Ryan, 1975; Kaplan, Grumbach and Aubert, 1976), but smaller and perhaps more significant amounts of free β-subunit of either gonadotrophin (Dubois and Dubois, 1974; Hagen and McNeilly, 1976). These observations suggest that control of synthesis of gonadotrophins within the pituitary resides in the control of synthesis of their unique β-subunit.

1.4.1.2 Chorionic gonadotrophins

Human chorionic gonadotrophin (hCG) is the best known placental gonadotrophin, secreted by the syncitiotrophoblast cells of the placenta. The structure of hCG is very similar to that of human LH, consisting of two non-identical subunits. The βsubunit of hCG is unique among the gonadotrophins with an additional 30 amino acids at its COOH-terminus (Birken and Canfield, 1978). Human placental tissue contains native hCG as well as large quantities of free α-subunit, but insignificant quantities of hCGβ (Vaitukaitis, 1974).

1.4.2 Reproductive steroid hormones

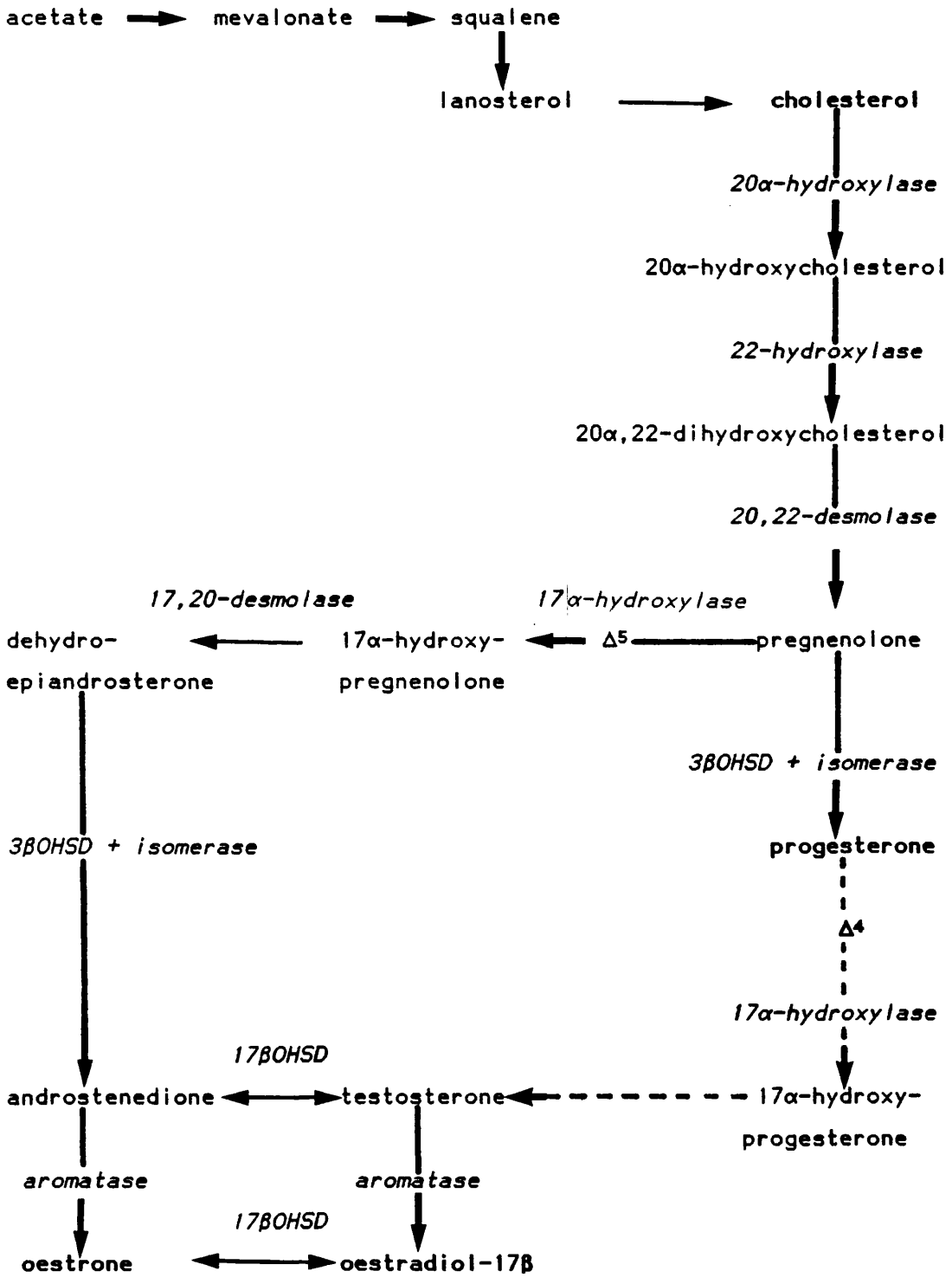
The steroid hormones comprise a large group of molecules all derived from a common steroid precursor, cholesterol (Fig. 1.4), which is synthesised from acetate in many tissues of the body. Removal of the hydroxyl group at the C-3 position of cholesterol, and reduction of the double bond between C-5 and C-6 converts cholesterol into the fully saturated compound, cholestane, which is the parent compound of all C_{27} steroids. Fission occurs between C-20 and C-22 and leads to the formation of a large group of compounds based on the parent pregnane (C_{21}). Bond fracture between C-17 and C-20 leads to the formation of androstane (C_{19}), and further fission between C-10 and C-19 produces compounds of the oestrane (C_{18}) series, a structure found in all mammalian oestrogens (Kellie, 1984).

Interconversion from one class of steroids to another is undertaken by a series of enzymes arranged together to form a "biosynthetic unit" for the synthesis of each steroid (Johnson and Everitt, 1988). In the female, steroid biosynthesis occurs in a wide range of tissues within the body including the adrenal cortex (Macdonald, Grodin and Siiteri, 1971), thyroid (Lyne and Gower, 1977) and brain (Benagiano, Mancuso, Mancuso, Wiqvist and Diczfalusy, 1968). However, the largest contribution to the total steroid output is made by the ovaries in the non-pregnant mammal (De Jongh, Baird and Van der Molen, 1974) and, in the pregnant mammal, the ovaries and/or the foeto-placental unit (Diczfalusy, 1964).

1.4.2.1 Biosynthesis of steroids by the ovary

The general biosynthetic pathway of ovarian steroids is shown in Fig. 1.4. The biosynthesis of steroids by the ovary is profoundly influenced by the constantly changing population of cells in the process of follicular development, ovulation, corpus luteum formation and regression. Pioneering experiments by Popjak and Tietz (1954) suggested that granulosa and theca cells

Figure 1.4. Biosynthetic pathway of steroids in the ovary. The dotted line represents the Δ^4 pathway; OHSD= hydroxy-steroid dehydrogenase.



surrounding ova in hens were active in the synthesis of cholesterol from acetate, as confirmed in the ovary of many species including the dog (Rabinowitz and Dowben, 1955), horse (Ryan and Short, 1966), cow (Hellig and Savard, 1966) and human (Ryan and Smith, 1961). Intermediates in this synthesis have been identified as mevanolate, squalene and lanosterol (Popjack, 1954; Hellig and Savard, 1966).

In ovarian tissue, the side chain of cholesterol is removed by a series of three enzymes, 20α-hydroxylase, 22-hydroxylase and 20,22 desmolase, to form pregnenolone (Lynn, Staple and Gurin, 1955; Ryan and Smith, 1965), via the intermediates of 22-hydrocholesterol and 20,22-dihydroxycholesterol. Hall and Koritz (1964) provided evidence for the presence of this series of enzymes in the mitochondria of bovine corpus luteum cells, and the role of mitochondria as the sub-cellular site of cleavage of the cholesterol side chain was subsequently confirmed in the rat ovary (Sulimovici and Boyd, 1967).

Growing antral and preovulatory follicles within the ovary secrete steroids including progestins, androgens and oestrogens. In follicular tissue C-17,20 lyase (17α-hydroxylase) enzymes may convert pregnenolone to 17α-hydroxypregnenolone (Gower, 1984) by what has become to be known as the Δ^5 pathway. Alternatively, 3β-ol-dehydrogenase and isomerase enzymes convert pregnenolone to progesterone which is in turn converted to 17α-hydroxyprogesterone by 17α-hydroxylase (the Δ^4 pathway). Both 17α-hydroxypregnenolone and 17α-hydroxyprogesterone are precursors for the androgens and oestrogens produced during the follicular phase of the reproductive cycle.

Biosynthesis of oestrogens by the preovulatory follicle requires the co-ordinated activity of both granulosa and theca cell types in response to LH and FSH stimulation (Hillier, 1985). Androgens are produced by the ovary throughout the phase of antral growth in many species (Moore, 1977; McNatty, 1982). The major site of androgen biosynthesis is the theca interna, where

steroidogenesis is under the control of LH (England, Webb and Dahmer, 1981). LH acts by receptor mediation, via intracellular cyclic AMP (Tsang, Armstrong and Whitfield, 1980), and stimulates the side chain cleavage of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone by 17,20 desmolase enzymes (Savard, 1968) to form dehydroepiandrosterone and androstenedione respectively.

Granulosa cells are unable to *de novo* synthesise androgens but, as these cells mature in response to FSH, they acquire the ability to aromatise androgens to oestrogens (Fortune and Armstrong, 1978; Dorrington and Armstrong, 1979). Aromatisation occurs in the microsomal fraction of granulosa cells and involves the oxidation and elimination of C-19 to form an unstable intermediate (Axelrod and Goldzieher, 1962; Ryan and Smith, 1965) which then rearranges to give the aromatic A ring. Androstenedione has been shown to be the substrate most effectively aromatised to form oestradiol-17β (Ryan and Short, 1965), the major follicular and circulating oestrogen. The newly synthesised oestradiol-17β binds to receptors in the granulosa tissue and promotes cell proliferation (Goldenberg, Vaitukaitis and Ross, 1972) and increased sensitivity to FSH which then results in further oestrogen production (Richards and Midgley, 1976; Carson, Findlay, Burger and Trounson, 1979). In the preovulatory follicle, oestrogen biosynthesis is thought to be under the dual control of FSH and LH, and long-loop feedback on pituitary gonadotrophin release is responsible for co-ordination of biosynthesis (Bolt, Kelly and Hawk, 1971; Diekman and Malvern, 1973). Oestradiol-17β is released into the circulation in greatest amounts as the follicle completes its late preovulatory stage of development. The direct or indirect measurement of increasing concentrations of oestradiol-17β in the blood is thus a good indicator of follicular development in the majority of mammalian species. The rising peripheral oestradiol levels eventually triggers the surge of LH from the anterior pituitary, which results in ovulation (Hoff, Quigley and Yen, 1983).

Granulosa cells may also secrete progesterone prior to the preovulatory LH surge (Channing, Thanki, Lindsay and Ledwitz-Rigby, 1978) although, *in vivo*, the high levels of oestradiol-17β in folliclular fluid may help to supress progesterone synthesis in these cells by inhibition of 5-ene-3β-hydroxysteroid dehydrogenase activity (Williams, Roth, Marsh and LeMaire, 1979). However, gonadotrophic stimulation of granulosa cell maturation in the preovulatory follicle results in increased cholesterol metabolism and stimulation of progesterone biosynthetic processes (Henderson, Gorban and Boyd, 1981 and Dorrington and Armstrong, 1979 for review). The granulosa cell is denied access to circulating cholesterol until follicle rupture at ovulation, and cholesterol derived from the uptake and metabolism of low-density lipoprotein is thought to be an important precursor for steroid synthesis at this time (Gwynne and Strauss, 1982).

After ovulation, granulosa lutein cells are the major source of progesterone (Hay and Moore, 1978) due to increased activity of Δ^5-3β-ol dehydrogenases and Δ^5-isomerases (often classed as one enzyme 5-ene-3β-hydroxysteroid dehydrogenase-isomerase) (Cheatum and Warren, 1966). These enzymes are located predominantly in the microsomes (Davenport and Mallette, 1966), converting pregnenolone to progesterone. Thus the measurement of progesterone during the post-ovulatory period indicates the functional lifespan of the corpus luteum, ie. the length of the luteal phase of the ovarian cycle. The time period over which progesterone production increases, in relation to ovulation, varies between species.

In most species, the increased production of progesterone inhibits C-17,20 lyase activity preventing the formation of 17α-hydroxypregnenolone (Beyer and Samuels, 1956) and further synthesis of oestrogens within the luteinized cells. High circulating progesterone levels also prevent the release of LH and FSH from the pituitary. However, the human corpus luteum also secretes as much, if not more oestradiol than the preovulatory follicle does during the follicular phase of the cycle (Baird, 1977). Due to the

combined feedback action of progesterone and oestradiol on the hypothalamic-pituitary axis, FSH and LH levels during the human luteal phase are sufficiently supressed to prevent follicular development to the preovulatory stage. When the feedback effect is withdrawn, gonadotrophin dependant pre-ovulatory follicular development can proceed (Clark, Dierschke, Meller and Wolf, 1979). In species in which oestrogen feedback from the corpus luteum is minimal (eg. the sheep), gonadotrophin levels during the luteal phase remain adequate to sustain follicular maturation (McNatty, 1982; Baird, 1983) and the subsequent follicular phase is shorter.

1.4.2.3 Steroidogenesis during pregnancy

Progesterone and oestrogens are also the principal reproductive steroids secreted in pregnancy, the biosynthetic pathways being essentially the same as described for ovarian production. However, there are species differences in the steroidogenic potential of the tissues involved in pregnancy, ie. the extent to which the ovary and feto-placental unit are involved in the production of steroid hormones. In certain mammals the placenta becomes the major or sole source of progesterone, eg. the horse, sheep, human and non-human primates (Heap, Perry and Challis, 1973); in others the placenta produces little or no progesterone,eg. the cow, goat, rabbit and sow (Heap *et. al.*, 1973). For the species that fall into the latter category, luteal function in the maternal ovaries is essential throughout gestation as the corpus luteum continues to be the major source of progesterone.

In those species in which the placenta is responsible for the steroid production during pregnancy, the perfusion of the placenta with ^{3}H-labelled steroids *in vitro* have shown that cholesterol from the maternal circulation may be used to synthesise pregnenolone (Jaffe and Peterson, 1966). Pregnenolone is rapidly converted to progesterone by Δ^{5}-3β-hydroxysteroid dehydrogenase-isomerase enzymes in the trophoblast layer of the human (Ferguson and Christie, 1967)

and equine placenta (Ainsworth and Ryan, 1969; Flood and Marrable, 1975).

Progesterone passes into the maternal circulation and is metabolised by the endometrium to 20α-dihydroprogesterone in humans (Billiar, Rahman and Little, 1978; Palmer, Blair, Eriksson and Diczfalusy, 1966), rats (Flint and Armstrong, 1973; Wichmann, 1967) and sheep (Paterson, Harrison, Sheldrick and Heap, 1983), or by the liver to form pregnanediol (Short, 1959) which is excreted via the urine. Progesterone reaching the foetal liver is converted to 20α-dihydroprogesterone and pregnanediol (Greig and Macnaughton, 1967); the 20α-dihydroprogesterone can be converted back to progesterone by the placenta (Barnes, Nathanielsz, Rossdale, Comline and Silver, 1975; Maeyama and Ikemoto, 1967) thus providing a recycling system.

This system is of particular importance as the foetal adrenal glands and gonads possess little or no Δ^5-3β-hydroxysteroid dehydrogenase activity and are therefore unable to produce progesterone from pregnenolone (Hay and Allen, 1975; Flood and Marrable, 1975). The maternal-placental unit is responsible for the secretion of large amounts of both progesterone and 20α-dihydroprogesterone into the maternal circulation of many species (Solomon, Bird, Ling, Iwamiya and Young, 1967), including the horse (Seren, Tamanini, Gaiani and Bono, 1981), cow (Schnider, 1989) and some non-human primates (Waddell, Pepe and Albracht, 1988). Inter-conversion has a major impact on total progestational activity (Waddell and Bruce, 1989) and measurement of these steroids in maternal blood is regarded as a good indicator of normal pregnancy and foetal viability.

As pregancy progresses, changes in foetal steroidogenesis occur. However, there are also species differences in the role of the foetus, as the enlargement of the foetal adrenal glands is responsible for increased steroidogenesis in the human (Crowder, 1967), whilst foetal gonads are of greater importance in the mare

(González-Angulo, Hernandez-Jóuregui and Martinez-Zedilo, 1975; Hay and Allen, 1975).

In the human and non-human primate (Challis, Davies, Benirschke, Hendrickx and Ryan, 1974), the main role of the foetus is with regard to oestrogen and not progesterone production. In the foetal adrenals, 17α-hydroxylase,C-17,20-lyase enzymes convert pregnenolone to androgens such as dehydroepiandrosterone (Villee, 1969a; b) which may be aromatised by the placenta to oestrone and oestradiol-17β. Alternatively, 16α-hydroxylation of the steroids in the foetal liver, and aromatisation in the placenta forms oestriol (Reynolds, Mancuso, Wiqvist and Diczfaluzy, 1968). Oestrone and oestradiol-17β produced by the placenta may also be metabolised in this way to form oestriol.

In the mare, different methods of oestrogen synthesis have been suggested as concentrations of different oestrogens change independently throughout gestation (Cox, 1975; Savard, 1961). Although it is possible that the production of oestrogens by the equine foeto-placental unit is essentially similar to that of the human, the formation of the pregnancy specific unsaturated B ring steroids (equilin and equilenin) has posed problems for investigators. Stárka and Breuer (1966) proposed that dehydroepiandrosterone is the precursor of both oestrone and equilin, 7α-hydroxylation in the foetal liver producing the obligatory intermediate for equilin synthesis. However, other workers (Bhavnani and Woolever, 1978) proposed a pathway of synthesis for the B ring unsaturated steroids involving a break in the chain of synthesis of cholesterol from acetate.

1.4.3 Metabolism and excretion of reproductive steroids

Steroid hormones are secreted into the peripheral circulation, where they are nearly all bound to plasma proteins (Wiest and Kidwell, 1969), before reaching the target tissues. Steroids are metabolised by many tissues including the gut and skin, but the

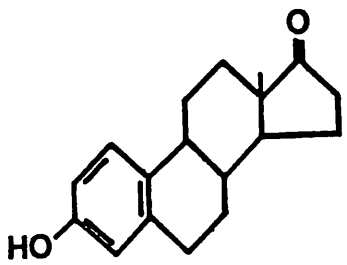
major site of metabolism is the liver. The rate at which a steroid is removed from the body is reflected in its metabolic clearance rate (Bedford, Harrison and Heap, 1972). The metabolic clearance rate for the majority of steroids is rapid and, although some removal of steroid from the peripheral circulation is affected by receptor binding at the target tissues, the bulk of steroids which enter the blood stream are removed by inactivation and excretion from the body.

The structural modifications of the steroid hormones in the peripheral tissues, and ultimately in the liver, are generally regarded as prerequisites for their biological inactivation. Steroids are hydrophobic substances and the catabolic reactions both inactivate the physiologically active steroid hormones and render the molecules hydrophilic. Peripheral metabolism involves mainly oxidation and reduction of steroid hormones. To make the catabolic products of secreted hormones even more water-soluble, the majority are conjugated as glucuronides or sulphates, before being excreted into the bile or urine.

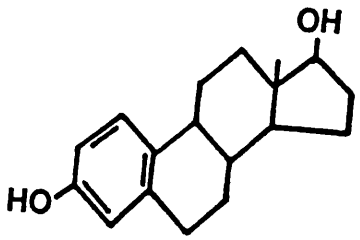
The biosynthesis of oestrogens by the ovary and foeto-placental unit results in the formation of oestrone, oestradiol-17β and oestriol (see Fig. 1.5 for structures). These oestrogens are close to the terminal end of the biosynthetic pathway and are thus excreted without major modification in peripheral tissues. Whereas this general principle applies to most mammals, intensive studies in the cow have proposed further interconversion of oestrogens in peripheral tissues which may be applied to other species in the future. Most significantly, bovine red blood cells are capable of converting oestradiol-17β to the stereoisomer, oestradiol-17α *in vitro* (Choi, Möstl and Bamberg, 1989) and *in vivo* (Dobson and Dean, 1974). In addition, the interconversion of oestradiol-17β, oestradiol-17α and oestrone has been demonstrated in the peripheral tissues of the cow (Axelrod and Werthessen, 1960) and other species eg. the dog (Siegel, Dorfman, Brodey and Friedman, 1962). The

Figure 1.5. Structure of the major mammalian oestrogens.

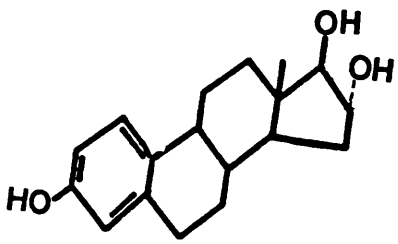
oestrone



oestradiol



oestriol



conversion of small amounts of circulating oestrogens to the isomers of 16-hydroxy oestrone and 3-methoxy oestrone has also been suggested in the human (Adlercreutz, Martin, Pulkkinene, Denckar, Rimer, Sjoberg and Tekanen, 1976).

In contrast, the catabolic products of progesterone may be formed in a number of ways and the major transformations are shown in Fig. 1.6 (see Fig. 1.7 for compound structures).

Firstly, reduction at C-20, by means of 20α- and 20β-hydroxysteroid dehydrogenase, results in the formation of perhaps the most important biologically active progesterone metabolites, the dihydroprogesterones, of which 20α-dihydroprogesterone (20α-DHP) is most common. It is interesting to speculate which organs convert progesterone to 20α-DHP. Studies in the rhesus monkey, have reported that a small proportion of this conversion can be accounted for by metabolism in the arm, head, renal and uterine tissues *in vivo* (Billiar, Takaoka, Reddy, Hess, Longscope and Little, 1981) and in the liver (Shirley and Cooke, 1968), kidney (Chatterton, Chatterton and Hellman, 1969a) and mammary gland (Chatterton, Chatterton and Hellman, 1969b) *in vitro*. The erythrocytes of the hyrax also have this potential (Heap, Gombe and Sale, 1975). However, extensive incubation studies have concluded that the ovary is the major site for the conversion of progesterone to 20α-DHP in many species.

The soluble fraction of ovarian homogenates of rats (Wiest, 1959) and rabbits (Davenport and Mallette, 1966) contains 20α-hydroxysteroid dehydrogenase activity. The enzyme is active in both luteal (Naito, Takahashi and Homma, 1986; Ingamells and Peddie, 1988) and follicular tissue (Takahashi, Duleba, Yuen and Moon, 1984) from the rat, and functions during pregnancy in this species when the ovary may take up circulating progesterone and convert it to 20α-dihydroprogesterone (Bruce, Swann and Waddell, 1983). Conversion occurs in response to gonadotrophic stimulation (Wiest, Kidwell and Kirschbaum, 1963) and is regulated by progesterone concentrations within the ovary (Uilenbroek, 1988). 20α-DHP has

Figure 1.6. Possible pathways in the metabolism of progesterone.

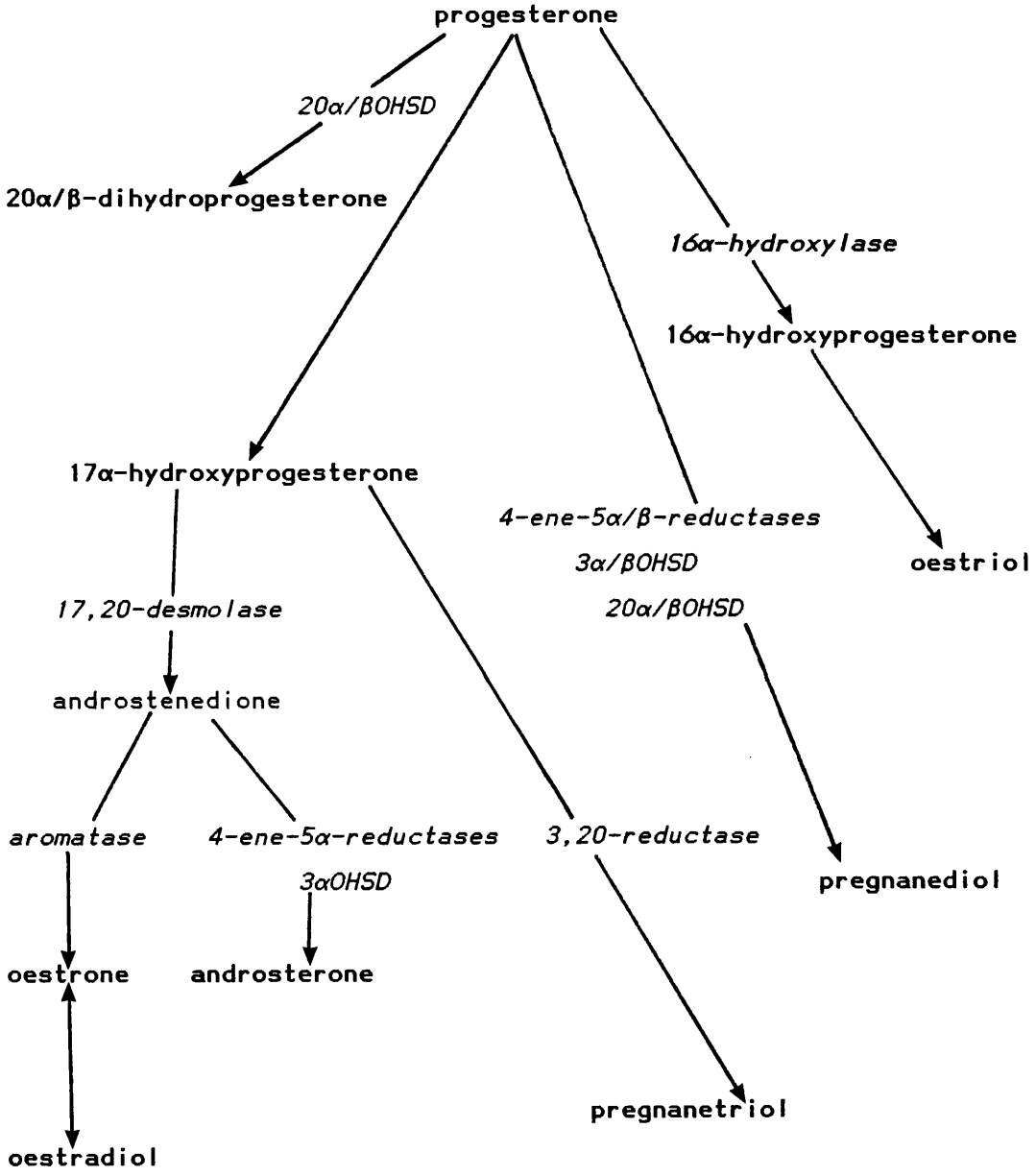
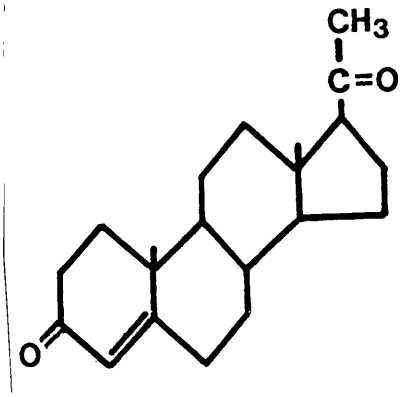
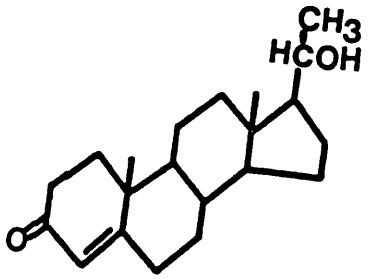


Figure 1.7. Structure of the major metabolites of progesterone.

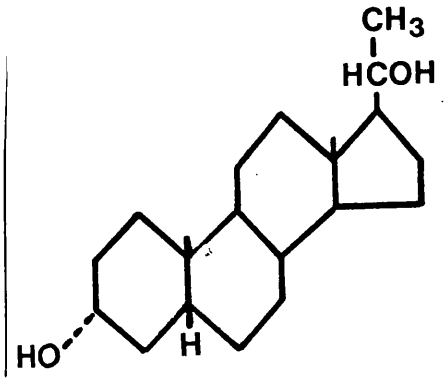
progesterone



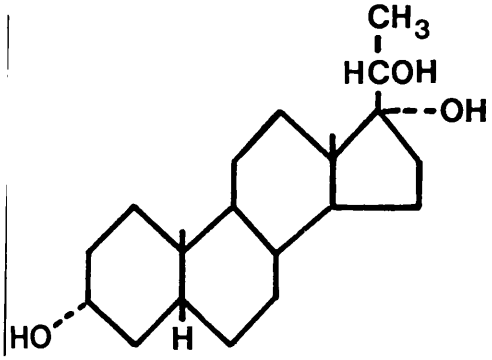
20-dihydroprogesterone



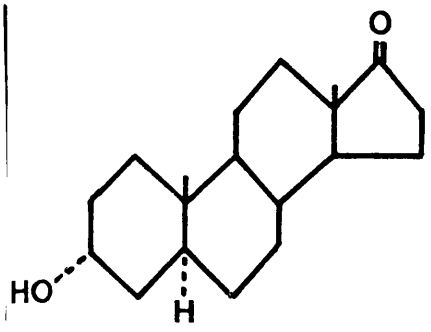
pregnanediol



pregnanetriol



androsterone



been shown to be an important ovarian progestagen, not only in the rat, but also in the mouse (Loutfi, Peron and Dorfman, 1962), human (Billiar, Lin and Little, 1973), horse (van Rensburg and van Niekerk, 1968) and quail (Ongabesan and Peddie, 1988). However, species differences do exist as both the cow (Savard, Marsh and Rice, 1965) and the trout (Canario, Scott and Flint, 1989) ovary produce the stereo-isomer 20β-dihydroprogesterone.

In some cases C-20 reduced progesterone metabolites may themselves be the end products of progesterone catabolism, or may be further reduced in the A ring by means of 4-ene-5α- and 5β-reductases to form 5α- and 5β-pregnane-3,20-diones. Reduction at C-3 by 3α- and 3β-hydroxysteroid dehydrogenases results in the formation of some isomeric pregnanolones. A final reduction at C-3, C-20 and in the A ring leads to the formation of the pregnanediols, most importantly 5β-pregnane-3α,20α-diol, which have been shown to be quantitatively the most important end products of progesterone metabolism in most species (Gower, 1984).

Although the formation of pregnanediols is the major route of metabolism of progesterone in the majority of species, other minor catabolic pathways have been demonstrated in a variety of mammals. In the ovarian tissue of the bovine (Solomon, Vande Wiele and Liberman, 1956) and human (Miron, Hay, Johnston, McKenna and McBain, 1988), progesterone may be hydroxylated in the 17α-position to form 17α-hydroxyprogesterone. Further tetrahydro-reduction may also occur which produces the C_{19}-17-oxosteroids, androsterone and acetiocholanolone. Alternatively, reduction in the A ring of 17α-hydroxyprogesterone, at C-3 and C-20 produces a number of pregnanetriols, the most important being 5β-pregnane-3α,17,20α-triol (Burger and Summerville, 1963; Samarajeewa and Kellie, 1985).

The human ovary can also hydroxylate progesterone in the 16α-position (Warren and Salhanick, 1961) within the corpus luteum (Huang, 1967). It is thought that a similar hydroxylation occurs in

the sow ovary to form the intermediate for oestriol biosynthesis (Kadis, 1964).

Despite the differences in site and pathway of catabolism of ovarian steroids, metabolites are ultimately formed in the hepatocytes of the liver. In these cells, the majority of steroids are conjugated with either glucuronic acid or a sulphate molecule to render the steroid metabolite more water soluble to aid excretion. The stereochemistry of the A ring appears to direct the type of conjugation formed in the liver. Neutral steroids with 3α-hydroxy-5β-pregnane structure are preferentially conjugated with glucuronic acid, whereas oestrogens may be conjugated as either glucuronides or sulphates. The biosynthesis of steroid conjugates involves three consecutive stages, resulting in the transference of the conjugate residue from a nucleotide source to the steroid acceptor (Dutton, 1966).

From the hepatocytes, some metabolites return into the circulation before renal excretion whilst others cross the membrane of the hepatic canaliculi to be excreted in the bile. Except for the polyhydroxylated compounds, the majority of the various steroid metabolites are excreted into the urine either as the free steroids or as conjugates. There is some preference for the excretion of steroid glucuronides into the urine. The clearance rate of glucuronides approximates to the glomerular filtration rate (GFR), whereas renal clearance of steroid sulphates is only 10% of the GFR.

Faecal excretion of steroid hormone metabolites is less clearly defined, and early investigations of the biliary excretion of steroids were hindered by complex experimental processes, eg. cannulation of the bile duct. However, the use of exogenously administered, radiolabelled hormones suggested an enterohepatic circulation of steroids (Howard, Robinson, Schmidt, McCord and Preedy, 1969). Despite the high concentrations of steroids in the bile, relatively low levels were recovered in the faeces. Such studies also concluded that urine is the major route of excretion

for steroid elimination in the range of species investigated, with only small amounts of biliary steroids remaining in the intestine to be excreted in faeces.

There appears to be a preference for excretion of double conjugates such as oestriol-3-sulphate-16-glucuronide (Støa and Skulstad, 1972) and the N-acetylglucosaminides and sulphate-N-acetylglucosamines of phenolic steroids (Jirku and Evitz, 1969) into the bile. Progesterone metabolites are also present in bile and preferential excretion of mono- and di-sulphate conjugates of pregnanediol, 5α-pregnandione and pregnanolone has been suggested (Laatikainen, 1970).

Despite the high proportion of steroid conjugates present in bile, the steroids present in faeces are generally unconjugated and exhibit a higher degree of saturation. Biliary steroid conjugates are hydrolysed in the intestine where the steroid moiety is subjected to hydrolase, dehydroxylase, reductase and epimerase activity of the intestinal bacteria (Jänne, Laatikainen and Vihko, 1971). The caecum is probably the major site for steroid transformation (Kelly, de Leon, Winter and Bokkenheuser, 1977). Steroids may be reabsorbed in the gut to undergo further metabolism in the intestinal wall (Hartiala, 1973) or in the liver, and products may again be excreted in the bile or in urine.

Of the steroid hormones excreted in faeces, unconjugated oestrogens predominate in the majority of species, especially during pregnancy (Honour, 1984). The major oestrogens, ie. oestrone, oestradiol-17α and -17β and oestriol have been identified in faeces (Adlercreutz and Järvenpää, 1982), along with the less common metabolites, 16α- and 16β-hydroxyoestrone and 3-methoxy oestrone (Adlercreutz and Martin, 1976). Progesterone metabolites have been identified in faeces, in lesser proportions, mainly as free steroids although mono-sulphates are common (Eriksson and Gustafsson, 1971).

1.5 MEASUREMENT OF EXCRETED STEROID HORMONE METABOLITES

The evaluation of excreted hormones and their metabolites is the most practical approach to monitoring ovarian function in many species (Hodges, 1986; 1990). Urine provides a medium for the accurate, detection of fluctuations in circulating steroid and gonadotrophic hormones. Although the metabolism of gonadotrophins chemically alters the structure of the hormone, the bioactive site remains functional and is excreted into the urine. Furthermore, the functional site is not species specific. Therefore, whereas the validation of immunoassays developed for the measurement of FSH and LH in serum is often difficult for the metabolised glycoprotein hormones in urine, biological assays may be applied for the measurement of "bioactive" gonadotrophins in urine.

One example of such a biological assay was developed by Dufau, Mendelson and Catt (1974) specifically for the measurement of LH. This *in vitro* bioassay quantified the testosterone production by dispersed Leydig cells in response to applied LH in plasma, and has now been adapted to measure urinary LH in a wide range of primates (Abbott, Hodges and George, 1988; Harlow, Hearn and Hodges, 1984; Hodges, Czekala and Lasley, 1979), but has yet to be validated for ungulate species. Urinary FSH has also been measured by an *in vitro* granulosa cell aromatase bioassay (Dahl, Czekala and Hseuh, 1987a; Dahl, Jia and Hsueh, 1989) in a wide range of species including great apes (Dahl, Czekala, Lim and Hsueh, 1987), ungulates (Dahl, Czekala and Hsueh, 1987b), the giant panda (Monfort, Dahl, Czekala, Stevens, Bush and Wildt, 1989) and killer whale (Walker, Cornell, Dahl, Czekala, Dargen, Joseph, Hseuh and Lasley, 1988).

McFarlane, Cabrera, Coulson and Papkoff (1990) have shown that rhinoceros pituitary hormones may be detected by radioimmunoassay, and it has been suggested that it may be possible to measure bioactive LH in rhinoceros urine (N.M.Czekala, personal communication). However, biological assays are labour intensive and subject to wide biological variation as they deal with living

tissue. Although such methods can supply useful information in the investigation of the hormonal control of the oestrous cycle in exotic species, it is unlikely that they will be used for routine monitoring. Application may be limited to the detection of pregnancy in species where placental or chorionic gonadotrophins play an important role in gestation, eg. primates (Findlay, 1980) and equine species (Roser and Lofstedt, 1989). The development of monoclonal antibodies to detect gonadotrophins from diverse mammalian species may increase the viability of the measurement of urinary gonadotrophins (Matteri, Roser, Baldwin, Lipovetsky and Papkoff, 1987), but for the present, urinary steroid analysis provides a more simple and cost-effective method of monitoring reproductive function in the majority of exotic species.

1.5.1 General principles of steroid hormone analysis

Perhaps the most important practical consideration in monitoring excreted hormone metabolites, is the route of excretion and molecular structure of the catabolic products. Although circulating hormones maintain the same structure throughout taxa, their catabolic products may differ considerably in each species. As demonstrated in the previous section, progesterone has an array of possible metabolic products, and different molecular forms may be found even in closely related species. For example, within the subfamily of *Cercopithecinae*, androsterone has been identified as the major progesterone metabolite in the urine of the pig-tailed macaque (Jeffery, 1966) and the rhesus macaque (Liskowski and Wolf, 1972), whilst the measurement of 20α-DHP immunoreactivity is necessary for the characterization of the luteal phase of the ovarian cycle in the cynomolgus macaque (Monfort, Jayaraman, Shideler, Lasley and Hendrickx, 1986) and the lion-tailed macaque (Shildeler, Mitchell, Lindburg and Lasley, 1985) while PdG is probably the most useful progesterone metabolite for the vervet monkey (Andelman, Else, Hearn and Hodges, 1985). Oestrogens, however, are at the end of the metabolic pathway and the

number of catabolic products are therefore fewer and less variable between species.

Knowledge of the metabolism and excretion of ovarian steroids has been obtained by investigating the fate of exogenously administered radiolabelled steroids in several species including primates, eg. the baboon (Goldzieher and Axelrod, 1969), carnivores, eg. the cat (Shille, Wing, Lasley and Banks, 1984), domestic and exotic ungulates, eg. the cow (Ivie, Christopher, Munger and Coppock, 1986) and okapi (Loskutoff, Kasman, Raphael, Ott-Joslin and Lasley, 1987). Other, more advanced techniques such as gas-liquid chromatography (Tomosova, Gregorova and Horky, 1981), high performance thin layer chromatography (Heger and Neubert, 1987), nuclear magnetic resonance and mass spectrometry (Heger, Hoyer and Neubert, 1988) have also been applied to identify excreted steroid metabolites. Although these procedures have the advantage over radiometabolism studies of requiring no contact with the animal, the steroid from which the metabolites originated cannot be directly determined.

Having identified the major excreted steroid metabolites, purification techniques are required to assess the relative abundance of individual metabolites and determine the most informative measurement for monitoring reproductive function. For example, high performance liquid chromatography (HPLC) is a powerful technique which provides an efficient system for steroid separation and useful method for assay validation (Lasley, Monfort, Hodges and Czekala, 1981; Lin and Heftmann, 1981). The use of solvent extraction procedures enables the proportion of steroids excreted in the unconjugated and conjugated forms to be determined. Sequential enzyme hydrolysis may then be used to identify the relative amounts of each conjugate present (Hodges and Eastman, 1984). Such purification techniques are essential for the validation of hormone measurement in each new species studied, for accurate and reliable results to be obtained.

Care must be taken when substituting urinary and faecal measurements in place of circulating hormone concentrations. Studies in the human have suggested that 24 hour collection of urine provides the closest correlation with circulating hormone levels (Collins, Collins, Kilpatrick, Manning, Pike and Tyler, 1979). However, prolonged periods of isolation are necessary and this is undesirable with many animals, especially in zoological collections. Overnight or opportunistic sample collection is more acceptable although this may result in daily variation in the water content of the samples. Thus the water content and variable time of collection of the sample makes the quality unpredictable and compensation must be made by indexing the hormone concentration to a marker excreted in constant daily amounts.

1.5.2 Faecal analysis

As a result of radiolabel metabolism studies, it has become obvious that significant amounts of steroid metabolites are excreted into the faeces in a wide range of species. For many mammalian species, faeces may be the most important route of steroid excretion and analysis of faecal steroids may provide the only non-invasive method of monitoring ovarian function, eg. exotic felidae (Shille *et al.*, 1985; Hodges, 1990).

Early studies on human faeces (Adlercreutz *et al.*, 1976) indicated the importance of the faecal route in the excretion of oestrogens. Adlercreutz and Järvenpää (1982) developed methods for the quantification of faecal steroids, and reported the use of faecal oestrogen analysis to determine reproductive status in women. In the last 3-5 years there has been a growing interest in the application of faecal steroids analysis to monitor reproductive events in domestic and exotic mammals.

The collection of faeces from domestic and captive exotic species is a simple procedure requiring no contact with the animal, and the feasibility of sequential sample collection from free-

ranging animals has also been demonstrated (Wasser, Risler and Steiner, 1988). However, the distribution of particulate matter and steroids in faeces is somewhat uneven and complicated by changes in faecal water content and retention time in the gut. These parameters are further affected in the wild situation by wide variations in dietary fiber intake (Martin, Peltonen, Laatikainen, Pulkkinen and Adlercreutz, 1975; Goldin, Adlercreutz, Dwyer, Swenson, Warram and Gorbach, 1981 for examples). The administration of indigestible markers (see Grace and Body, 1981; Gosden and Mosely, 1984; Mayes, Lamb and Colgrove, 1986) and use of lyophilization procedures enable corrections to be made for variable sample quality. However the cost, coupled with the complex extraction techniques required for faecal analysis, may inhibit the use of such methods in situations where other monitoring techniques may be employed.

Despite the problems associated with faecal steroid analysis, levels of oestrogens and progestagens in faeces have been shown to correlate well with serum levels during ovarian cycles in some non-human primates in captivity (Risler, Wasser and Sackett, 1987; Ziegler, Sholl, Scheffler, Haggerty and Lasley, 1989). Furthermore, a pilot study on faecal steroid excretion by wild yellow baboons in Tanzania (Wasser *et al.*, 1988) reported levels of oestrogens and progesterone consistent with data obtained in captivity (Townsley, 1974; Albrecht and Townsley, 1976). Limited data suggest that faecal oestrogen analysis may also be used for diagnosing pregnancy in endangered primate species in the future (Bamberg, King, Von Hegel, Patzl and Möstl, 1988; Wasser *et al.*, 1988).

Faecal excretion of ovarian steroids in domestic ungulates has been well documented, and techniques have been applied to closely related exotic species. The excretion of progesterone into the faeces follows a cyclical pattern which correlates well with the time of oestrus in the cow and the muskox (Desauliners, Goff, Bettridge, Rowell and Flood, 1989). In addition, elevated levels of 20α-gestagens have been measured in the faeces of domestic and

exotic equids following oestrus (Schwarzenberger, Möstl, Bamberg and Von Hegel, 1988). Bamberg and Schwarzenberger (1990) have recently shown an increase in faecal 20α-gestagens during the presumed luteal phase of the cycle in a black rhinoceros, although the procedure has yet to be fully validated.

Faecal oestrogen analysis has also been used to diagnose pregnancy in many ungulate species. The faecal oestrogen most accurately reflecting the presence of a conceptus in cattle is oestradiol-17α (Möstl, Choi, Wurm, Ismail and Bamberg, 1984), whilst a significant increase in the excretion of oestradiol-17β and oestrone occurs after 120 days of gestation in the mare (Bamberg, Choi, Möstl, Wurm, Lorin and Arbeiter, 1984; Bamberg, Möstl, Wurm and Choi, 1986a). In the sow, the excretion of oestrogens into the faeces during pregnancy reflects serum levels so clearly, that a peak of oestradiol-17β in the peripheral blood between days 20 and 36 of gestation (Robertson and King, 1979) may be detected in the faeces (Bamberg, Choi and Hois, 1986b; Choi, Kiesenhofer, Gantner, Hois and Bamberg, 1987).

Safar-Hermann, Ismail, Choi, Möstl and Bamberg (1987) investigated the use of faecal oestrogen analysis for pregnancy diagnosis in exotic herbivorous species. Pregnancy was revealed in four species of exotic ungulate (red buffalo, yak, Grevy's zebra and nubian ibex), with levels ten times higher than in the non-pregnant female or male animals. However, such evaluations can only be used after the first trimester of pregnancy and are not applicable in all species, eg. the hippopotamus, where levels of faecal oestrogens were not significantly different between pregnant and non-pregnant animals.

1.5.3 Urinary analysis

For many years, quantification of steroid hormone metabolites in urine has been regarded as a valuable diagnostic tool, and crude chemical methods were developed for this purpose (Brown, 1955;

Stern, 1957; Pickett, Kyriakides, Stern and Sommerville, 1959). With the advent of sensitive radioimmunoassays for circulating hormones, it became more common to measure steroids in serum which gave a more accurate reflection of reproductive function than the determination of urinary hormone metabolites. However, it is now recognised that, although urinary steroid metabolite analysis will never fully replace the direct measurement of circulating hormones, there are several advantages to this procedure. In most cases, urinary hormone analysis provides the most feasible and reliable approach to long-term studies of reproductive function in exotic species either in captivity or in the wild.

Like faecal sampling, the collection of urine is non-invasive requiring no contact with the animal. Although the collection of faeces is often simpler than the collection of urine, due to the nature of the sample, there are examples of the application of urinary steroid analysis for assessing reproductive function in exotic species in their natural habitat (Poole, Kasman, Ramsay and Lasley, 1984; Andelman *et al.*, 1985; Brett *et al.*, 1988; Kirkpatrick, Kasman, Lasley and Turner, 1988; Chaudhuri and Ginsberg, 1990).

Opportunistic collection often results in a single urine sample of unpredictable quality. However, unlike in faeces, a natural marker may be used to make adjustments to compensate for variations in fluid intake and output, and for variable periods of collection. The marker, creatinine, is formed as a by-product of amino-acid metbolism in the body, and is excreted in relatively constant amounts per day (Paterson, 1967; Pierro and Johnson, 1970). Creatinine may be measured in the urine by a simple colorimetric reaction (Taussky, 1954; Brand, 1981) to provide an index for hormone concentrations which correlate well with 24 hour excretion rates (Metcalf and Hunt, 1976).

Many of the advances in the field of urinary steroid analysis have stemmed from studies on primates. Initially, the application

of this method was based on the non-specific measurement of total immunoreactive oestrogens. As the majority of oestrogens present in the urine are conjugated to a glucuronide or sulphate molecule, this procedure required hydrolysis of the conjugates (by enzymes or chemical means, ie. solvolysis) and solvent extraction of urine prior to assay. Total urinary oestrogen analysis was successfully used to monitor ovarian function in many old and new world monkeys and Great apes (Hodges, *et al.*, 1979; Lasley *et al.*, 1981; Lasley, 1985). Although this measurement has been found to reflect general oestrogen excretion in many primates, it does not reflect the differences in oestrogenic components between samples and between species.

The measurement of individual oestrogen metabolites, identified by a combination of HPLC and the total oestrogen assay, may provide more specific information on ovarian events. This procedure, used in conjunction with sequential hydrolysis, has been used to identify conjugated oestrone as the major urinary oestrogen metabolite during the ovarian cycle of most primate species (Hodges *et al.*, 1979), although species differences in oestrogen metabolism have been demonstrated. For example, oestradiol-17β may be excreted into the urine as in the Goeldi's monkey (Christen, Dobeli, Kempken, Zachmann and Martin, 1989), pied-barefaced tamarin (Heistermann, Pröve, Wolters and Mika, 1987) and common marmoset (Eastman, Makawiti, Collins and Hodges, 1984), or circulating oestradiol-17β may be metabolised to its epimer, oestradiol-17α as in the ruffed lemur (Shideler, Czekala, Benirschke and Lasley, 1983).

One area in which urinary hormone measurement has been greatly simplified is in the development of direct, non-extraction assays for steroid conjugates (eg. Samarajeewa, Coolie and Kellie, 1979). Direct immunoassay of steroid conjugates avoids both the hydrolysis and subsequent extraction and purification procedures which are expensive and laborious. Therefore direct assays offer a much more practical and simple approach to routine urinary analysis than was previously available. Furthermore, by avoiding the hydrolysis step

a more representative and informative profile can sometimes be obtained, as hydrolysis has been shown to be inefficient in cleaving certain conjugates (Shideler *et al.*, 1983; Eastman *et al.*, 1984). However, when monitoring reproductive function by direct assay, it is particularly important to determine the nature and relative abundance of individual conjugates in the urine for each new species studied.

Conjugated oestrone has been identified as the major oestrogen in the urine of most species of primates (Lasley *et al.*, 1981) and ungulates (Loskutoff *et al.*, 1983). Conjugated oestrone is commonly excreted in two forms, oestrone-3-sulphate or oestrone -3-glucuronide. An assay utilising an antiserum that cross reacts with both conjugates has provided valuable information on the timing of ovulation, implantation and early pregnancy in many species of primate, eg. the baboon (Hodges, Tarara, Hearn and Else, 1986), gorilla (Czekala, Mitchell and Lasley, 1986) and mangaby (Calle, Chaudhuri and Bowen, 1990), and has also been applied to assess reproductive function in a variety of exotic ungulates, eg. the tapir (Kasman, McCowan and Lasley, 1985) and zebra (Czekala, Kasman, Allen and Lasley, 1990).

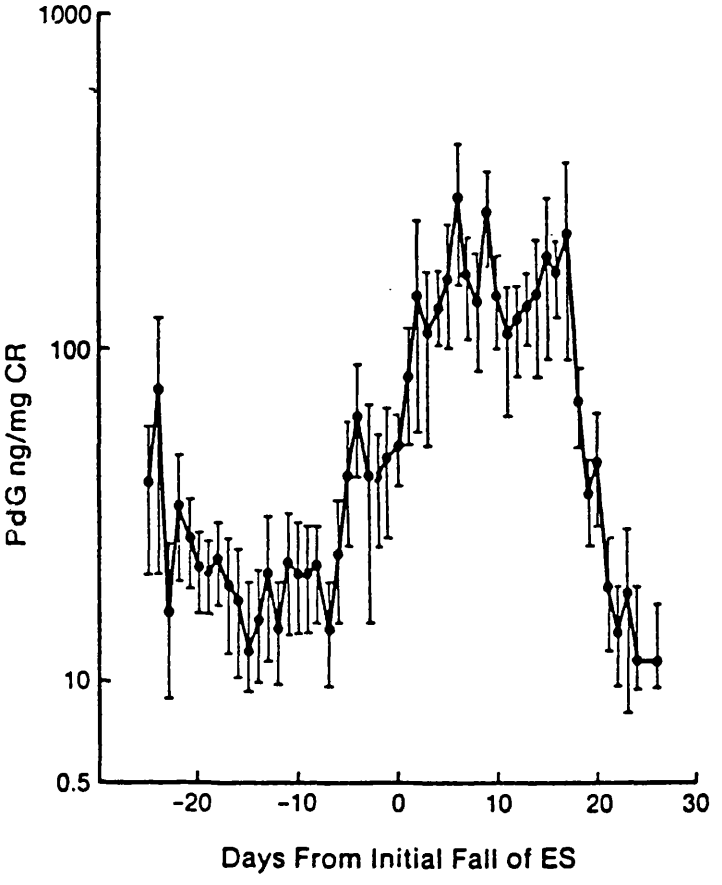
Patterns of oestrone conjugate excretion alone are insufficient to provide reliable information on ovarian function and data are often combined with measurements of urinary progesterone metabolites, mainly in the form of pregnanediol-3-glucuronide (PdG). Urinary PdG has been used to monitor reproductive events in a diverse range of species including the gorilla (Mitchell, Presley, Czekala and Lasley, 1982), vervet monkey (Andelman *et al.*, 1985), giant panda (Hodges, Bevan, Celma, Hearn, Jones, Kleiman, Knight and Moore, 1984; Chaudhuri, Kleiman, Wildt, Bush, Frank and Thau, 1988), blackbuck (Holt, Moore, North, Hartman and Hodges, 1988), suni (Loskutoff, Raphael, Nemec, Wolfe, Howard and Kraemer, 1990), eld's deer (Monfort, Wemmer, Kepler, Bush, Brown and Wildt, 1990), okapi and giraffe (Loskutoff, Ott-Joslin and Lasley, 1982; Loskutoff, Walker, Ott-Joslin, Raphael and Lasley, 1986). However,

other C_{21} steroids such as progesterone and 20α-DHP have been shown to accurately reflect luteal function in other species, eg. Goeldi's monkey (Carroll, Abbott, George, Hindle and Martin, 1990) and horse (Kirkpatrick, Lasley and Shideler, 1990) respectively.

Within the rhinoceros species the application of the well established assays for PdG and oestrone conjugates has met with differing degrees of success. The measurement of urinary PdG and oestrone conjugates, in the form of oestrone sulphate, has provided valuable information on the ovarian cycle and pregnancy in the Indian rhinoceros (Kassam and Lasley, 1981; Czekala, Gallusser, Meier and Lasley, 1986; Kasman, Ramsay and Lasley, 1986; Hodges and Green, 1989). The profile of conjugated oestrone and PdG excretion during the oestrous cycle of the Indian rhinoceros is shown in Fig. 1.8. The data are derived from a study by Kasman *et al.* (1986) and clearly show a marked increase in conjugated oetrone, in this case oestrone sulphate, to reach peak levels of 100 µg/mgCr at the presumed time of ovulation. Elevated levels of PdG (greater than 100 ng/mgCr) followed the peak of conjugated oestrone, characterizing the luteal or post-ovulatory period. However, Ramsay *et al.* (1987) reported undetectable levels of PdG in sequential urine samples collected from female black rhinoceroses during oestrous cycles. Oestrone conjugates were detectable in the urine, but the pattern of excretion showed no correlation with reproductive events. There are no reports of similar studies in the white rhinoceros.

In contrast to the situation during the ovarian cycle, Ramsay *et al.* (1987) reported the measurement of PdG immunoreactivity in urine collected after mid-gestation in the black rhinoceros. Elevated levels of PdG immunoreactivity during mid-to late gestation in this species were later confirmed by Hodges and Green (1990), who also provided data to show a similar pattern of PdG excretion during the last trimester of pregnancy in a white rhinoceros. In both the Indian and African species, a precipitous fall in the levels of urinary immunoreactive PdG were seen at the time of parturition or

Figure 1.8. Profile of conjugated oestrone and immunoreactive PdG excretion during the oestrous cycle in the Indian rhinoceros. All values (mean±sem, taken from two successive cycles in five individuals) are aligned to the day of the fall in conjugated oestrone. Data adapted from Kasman *et al.* (1986).
Urinary pregnanediol-3α-glucuronide (PdG) and oestrone sulphate (ES) concentrations are indexed to creatinine (Cr).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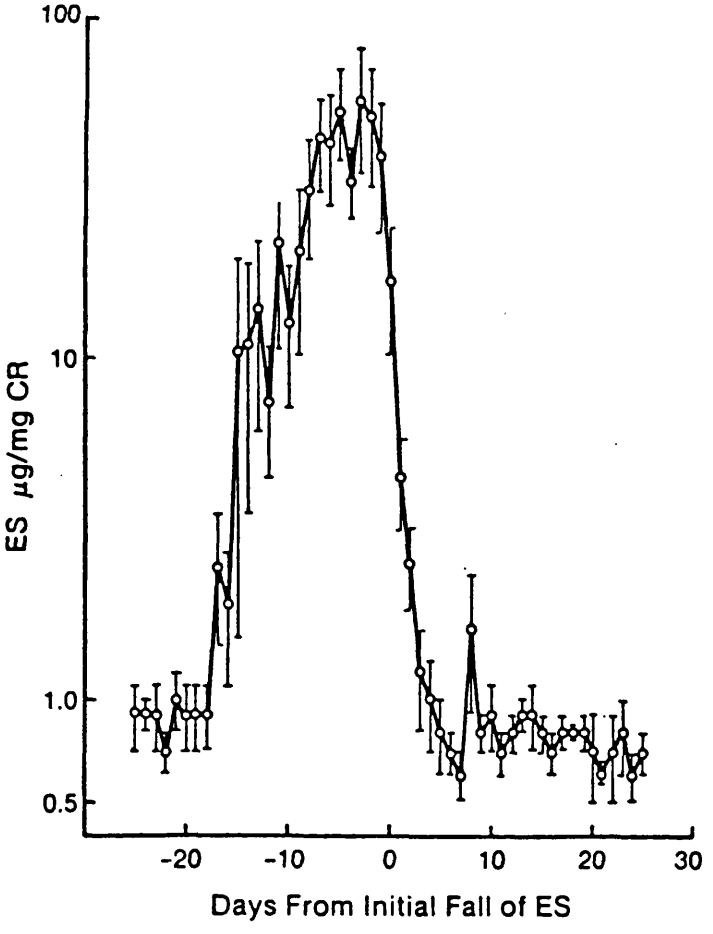
100
10
0.5
PdG ng/mg CR
-20
-10
0
10
20
30
Days From Initial Fall of ES


100
10
1.0
0.5
ES µg/mg CR
-20
-10
0
10
20
30
Days From Initial Fall of ES

abortion. Although there was much variation between the levels of PdG in the urine of different individuals at the same stage of gestation (Ramsay *et al.*, 1987), elevated levels of PdG immunoreactivity above those measured during the ovarian cycle would appear to indicate the presence of a conceptus in all three species of rhinoceros. To date this is the only useful data available on the reproductive endocrinology of the African species of rhinoceros.

1.5.4 Assay methods

The quantitative determination of steroids is difficult as their structures are closely similar, containing few functional groups, and they are generally present in low concentrations in body fluids. However, in the 1960s, the development of sensitive radioimmunoassay methods made possible the direct quantification of low levels of hormone in the circulation to monitor specific physiological processes. Such assays may also be applied to the quantitation of steroids in the urine and faeces. Radioimmunoassay techniques are relatively simple, robust and specific, and the principle of radioimmunoassay is straightforward (Hunter and Corrie, 1983). The assay is based on the competition between radioactively labelled and unlabelled hormone for a fixed, but limiting, number of binding sites on antibody molecules. Radioactively labelled hormone is added in excess to all assay tubes and, as the concentration of unlabelled hormone increases, more label will be displaced from the bound fraction. Using standards of known concentration, the binding of label at each point may be determined and a calibration curve (standard curve) constructed. Hormone concentrations from biological test samples are obtained from the curve by interpolation.

The simplification of hormone assay techniques has become of great importance to minimise costs and facilitate wider practical application. This is especially important when dealing with exotic species if the assays are to be used for routine monitoring of reproductive events in zoological collections, where expertise and

time for complicated extraction and purification procedures is limited. Furthermore, if the assays are to be applied to reproductive assessment in the field, they must be as simple and robust as possible.

The development of direct, non-extraction assays for urinary steroid conjugates is one area in which hormone measurement has been greatly simplified. Direct immunoassay avoids the enzyme hydrolysis of steroid conjugates and the following extraction and purification procedures, all of which are inefficient, laborious and expensive. Direct assays provide a practical and simple approach to routine urinary analysis, and can provide greater resolution in measurement and generate a more informative hormone profile as demonstrated in the lion-tailed macaque (Shideler, Czekala, Kasman, Lindburg and Lasley, 1983).

Although the application of radioimmunoassays has provided the basis for the current concepts in reproductive endocrinology, the use of radioisotopes introduces a number of disadvantages. Firstly, there is the quoted hazard to health. Radioimmunoassays are also expensive to perform, requiring sophisticated end-point analysis and utilizing radioisotopes with a relatively short shelf-life. Thirdly, radioimmunoassay procedures are laborious to perform and relatively long counting times are required. Finally, radioisotopes tend to complicate attempts to automate immunoassay.

The development of enzyme immunoassays (EIAs) for reproductive steroids offers an attractive alternative to conventional assays using radioisotopes. EIA systems operate on the same principle as radioimmunoassay, although the label in the assay is an enzyme label rather than a radiolabelled hormone. Once again, labelled and unlabelled hormone compete for a limited number of binding sites on antibody molecules. As the concentration of unlabelled hormone increases, more enzyme-labelled hormone will be displaced from the bound fraction. Once an equilibrium has been reached, the amount of bound enzyme label is determined by the

addition of enzyme substrate resulting in a measurable end point, usually a colour reaction. Such assay methods were initially developed for use in the human (eg. Joyce, Wilson, Read and Riad-Fahmy, 1978), and offer several advantages over radioimmunoassay. Firstly, EIAs are safe to perform in non-licensed laboratories. The procedures are relatively simple and the assays sufficiently robust to be performed in the field and in small, non-skilled establishments. Furthermore, the cost of EIAs is lower than that of radioimmunoassays, although the efficiency and sensitivity are often enhanced. Expensive antibodies may often be used at much higher dilutions than in radioimmunoassays and the end-point is easy to determine without the need for sophisticated equipment. The end-point is also formed over a relatively short period of time allowing rapid processing of results.

The development of rapid and sensitive solid phase assays using microtitre plates (eg. Sauer *et al.*, 1986) has further simplified the assay procedure to give results that compare favourably with those obtained by RIA. Such assays are now available to measure a variety of unconjugated steroids in urine and faeces, including oestradiol-17β (Maurel, Labrousse, Terqui and Avrameas, 1987) and oestradiol-17α (Möstl *et al*, 1984) in urine and faeces.

Recently, simplification of assay techniques by a combination of the direct measurement of urinary steroid conjugates by EIA on microtitre plates has produced sensitive and economical assays for oestrone conjugates* (Czekala, *et al.*, 1986) and PdG (Hodges and Green, 1989; Mitsuma, Yoshimura, Kambegawa, Okinaga and Arai, 1989) which have been applied to monitor ovarian function and pregnancy in a wide range of primates and exotic ungulate species.

*
(including both oestrone sulphate and oestrone glucuronide)

1.6 AIMS

This study was carried out to investigate the metabolism and excretion of ovarian steroids in African rhinoceroses, with a view to advancing our understanding of the reproductive physiology of these species and developing methods of assessing reproductive status in the white and the black rhinoceros.

The initial aim of this study was to investigate the metabolic fate of exognously administered radiolabelled oestradiol-17β and progesterone in a white rhinoceros, with the specific objective of identifying the major excreted metabolites of ovarian steroids and to determine the relative abundance of these metabolites in urine and faeces.

Secondly, the findings of the metbolism study were to be confirmed and extended by identifying and determining the form of the urinary metabolites of endogenous oestradiol-17β and progesterone, excreted during the ovarian cycle and pregnancy in both the black and white species of African rhinoceros.

The final aim was to establish sensitive immunoassays for the measurement of the major ovarian steroid metabolites in urine. By describing the pattern of excretion of reproductive steroid hormone metabolites, methods of detecting ovulation and pregnancy may be developed. The availability of non-invasive methods of assessing reproductive status will then provide an aid to management and breeding of these species in captivity and possibly in the wild.

CHAPTER 2.
GENERAL METHODS.

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GENERAL METHODS.

2.1 ANIMALS AND SAMPLE COLLECTION

The studies reported in this thesis were carried out on a juvenile female black rhinoceros (*Diceros bicornos*) housed and maintained by the Zoological Society of London (Regent's Park), seven adult, female black rhinoceroses (*Diceros bicornis*) housed and maintained by the Zoological Society of London, UK (at London zoo and Whipsnade Wild Animal Park), St. Louis Zoological Park, USA, Dvur Kralove Zoo, Czechoslovakia and Audubon Zoo, USA; two adult, female northern white rhinoceroses (*Ceratotherium simum cottoni*) housed and maintained at Dvur Kralove Zoo; two adult, female southern white rhinoceroses (*Ceratotherium simum simum*) housed and maintained by Edinburgh Zoo, UK and Paignton Zoo, UK and an adult female Indian rhinoceros (*Rhinoceros unicornis*) housed and maintained by the Zoological Society of London at Whipsnade Wild Animal Park. In the presentation of results in chapters 4 and 5, animals are described by number. Table 1.1 identifies these animals.

Except during pregnancy, each female was allowed access to a male of the same species during the day. Black, Indian and southern white rhinoceroses were kept in pairs, whilst the nothern white rhinoceroses were exhibited in a herd consisting of 4 females with a male. Signs of mating or oestrous behaviour (characterized by increased frequency of urine spraying, whistling, aggression or interest in the male) were recorded by the animal keepers. In most cases, females under investigation were housed alone overnight to facilitate sample collection. First morning urine or urine voided overnight was collected each day from the floor of the enclosure using a plastic syringe, or from plastic recepticles placed below the external outlets of the enclosure drains. Care was taken to minimise the possibility of contamination with faeces, water or

Table 2.1. Table to identify Indian, white and black rhinoceroses (referred to as numbers throughout this thesis), and the place of origin of urine samples analysed.

species	animal number	name	origin
Indian	1	Roopa	Whipsnade
northern white	2	Nesari	Dvur Kralove
	3	Nasima	Dvur kralove
southern white	4	Umfolozi	Edinburgh
	5	Gracie	Paignton
black	6	June	London
	7	Olive	St. Louis
	8	Jessie	Audubon
	9	Mama kidogo	Whipsnade
	10	Sali	Dvur Kralove
	11	Jimmi	Dvur Kralove
	12	Betsy	St. Louis
	13	Rosie	London

bedding. Following collection, urine was transferred to plastic containers and placed in a freezer at -20°C. Samples were generally stored in 5 ml volumes. In some cases, however, smaller quantities of urine were collected as and when possible throughout the day, and at irregular intervals. Analysis of these samples was limited to additional validation of the procedures used, and was not used for compiling individual profiles. Samples were frozen without preservatives at -20°C and transported in dry ice (if applicable) to the Institute of Zoology without thawing. Immediately prior to analysis, urine samples were allowed to thaw by standing at room temperature, and were then centrifuged at 400 g for 10 min to precipitate particulate matter.

Sample collection from an adult female southern white rhinoceros, housed and maintained at Whipsnade Wild Animal Park, during the course of a radiometabolism study followed a separate protocol which is described in detail in chapter 3.

2.2 CREATININE DETERMINATION

Reagents used throughout this thesis were Analar-grade from BDH (Poole, Dorset) unless otherwise stated.

Immediately upon the first thawing, urine samples were analysed for creatinine concentration according to the method of Brand (1981), as adapted by Hodges and Green (1989) for microtitre plate format. The measurement of creatinine helps to compensate for variation in fluid intake and output and variable periods of collection (Erb, Tillson, Hodgen and Plotka, 1970), and all results are reported as mass of hormone/mg creatinine (ng/mgCr) as previously described by several workers (eg. Shideler and Lasley, 1982; Hodges and Eastman, 1984). However, a comparison of hormone excretion over a 24 hour period with hormone concentrations indexed to creatinine has not been made for any species of rhinoceros. Creatinine excretion varies with muscle mass and level of activity. The concentration of creatinine in rhinoceros urine was thus variable between both individuals and species, approximately 2·0 mg/ml (range 0·9-3·2 mg/ml) in the white and 1·0 mg/ml (range 0·3-1·8 mg/ml) in the black rhinoceros. Fluctuations within individuals were ±0·5 mgCr/ml in the white and ±0·2 mgCr/ml in the black rhinoceros.

2.2.1 Assay materials and reagents

Creatinine standards were prepared over a range of 0·5-3·0 mg/ml by diluting a stock solution of 3·0 mg/ml in distilled water. Low and high quality controls (QCs) were prepared by diluting human urine, and stored in aliquots of 0·15 ml at -20°C.

Saturated picric acid was prepared in advance by dissolving 30 g picric acid slurry in approximately 1 litre distilled water, on a stirrer hotplate at 80°C. Whilst still hot, the acid solution was poured into a stoppered bottle and allowed to cool and crystalise. Alkaline triton X-100 was prepared by combining triton X-100 (4·2 ml), 1 N sodium hydroxide solution (12·5 ml; avs) and 66·0 ml distilled water. Saturated picric acid, alkaline triton X-100 and deionised distilled water were mixed in the ratio 1:1:10 (v/v) in order to provide the picrate reagent for the assay.

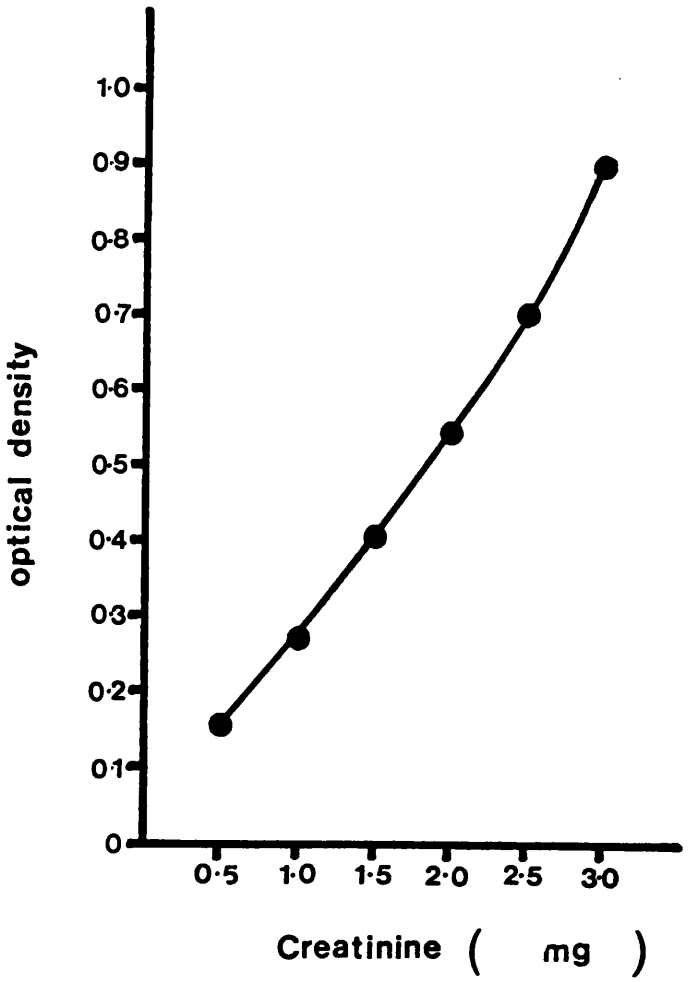
2.2.2 Assay method

Creatinine standards (0·5-3·0 mg/ml), QCs and samples were added to duplicate wells on a microtitre plate (Immuno 1; Nunc, Denmark) in 5 µl volumes. Zero wells, containing 5 µl distilled water, were also prepared in duplicate. Picrate reagent (0·3 ml) was then added to each well. The plate was incubated in the dark at room temperature for 1·5-2 h, after which the optical density was read at 490 nm using an automatic micro-plate reader (Dynatech MR 700). The amount of creatinine/ml urine was calculated by comparison with a standard curve constructed from optical density and creatinine concentration.

2.2.3 Assay evaluation

A typical standard curve for the creatinine assay is shown in Fig. 2.1. The sensitivity of the assay was approximately 0.1 mg creatinine/ml, based on the smallest change in optical density which could be readily detected, and samples containing less than 0·1 mg

Figure 2.1. Typical creatinine assay standard curve over the range of standards 0-3 mg. The curve is plotted as optical density against the creatinine concentration.



creatinine were taken to be too dilute for analysis, and were discarded. The intra-assay precision was determimed by the repeated measurement of the same urine pool in 1 assay, ie. on 1 plate, and was found to be 8·2% (n=30) expressed as a coefficient of variation (C of V). Inter-assay C of V, determined by measuring the same samples (QCs) in a total of 31 assays, was 3·9% for a high value QC and 5·7% for a low value QC.

2.3 SAMPLE PREPARATION

2.3.1 Enzyme hydrolysis

Prior to assay for oestrone, oestradiol-17β and 20α-DHP, urine samples (0·05 ml) were hydrolysed according to the method of Hodges *et al.*, (1979), and ether extracted as described below.

Urine samples were diluted with sodium acetate "hydrolysis" buffer (1:2; Appendix) to give a pH 5 solution, and hydrolysed with 50 FU β-glucuronidase-aryl sulphatase (Sigma, activity 20,000 FU/g solid; 50 FU/ 0·05 ml hydrolysis buffer) at 37°C for 24 h.

2.3.2 Ether extraction

After hydrolysis, urine samples were adjusted to pH 7 with 3 M sodium hydroxide solution and tracer amounts (2,000 cpm) of radiolabel were added to monitor the efficiency of the extraction procedure. Unconjugated steroids were extracted by vigorously mixing with 2 ml (10 vol) diethyl ether, distilled at 36°C immediately prior to use. Samples were mixed for 15 min on a multi-vortex mixer (multitube model 2601) and allowed to stand for 5 min to allow separation of the aqueous and ether phases. The aqueous phase was snap-frozen by standing the tubes in a mixture of acetone and liquid nitrogen. The ether was decanted into clean test tubes and evaporated to dryness under nitrogen in a dri-block (DB-3, Techne) at a temperature of 30°C. Steroids, formerly present in the urine in the unconjugated form or as a conjugate hydrolysed by

enzyme action, were reconstituted in 1 ml appropriate buffer for assay.

2.3.3 Hot ethanol extraction

Hot ethanol extraction was used to remove steroids from the solid vegetable matter of faeces. Faecal samples were weighed, to determine the wet mass, and thoroughly mixed to break up the solid matter. Faeces was placed in a large glass beaker with 10 vol. absolute ethanol. The ethanol was heated on a stirrer hotplate, whilst stirring constantly, until boiling point was reached and then allowed to boil vigorously for 10 min. The vegetable matter was strained and the residue squeezed before re-extraction by boiling for 20 min with 5 vol. fresh absolute ethanol. The vegetable matter was strained and the ethanol extracts combined in a 5 l refluxing vessel. The extracts were refluxed for 2 h at 70°C, filtered under pressure and concentrated on a Bucchi rotary evaporator (Rotorvapor-R). The steroids, formerly present in the faeces in both the conjugated and unconjugated forms were reconstituted in absolute ethanol.

2.3.4 Phenolic extraction of steroid extracts from the metabolism study

Neutral and phenolic steroids were separated by phenolic extraction according to the method of Brown (1955). Steroids were dissolved in 10 ml petroleum ether (fraction 40-60°C) and transferred to a separating funnel. Phenolic steroids were removed by shaking vigorously with 2N sodium hydroxide (2 ml) for 2 min. The contents of the funnel were allowed to settle for 5 min and the sodium hydroxide was removed. This procedure was repeated 3 times and the sodium hydroxide (6 ml) was pooled. The ether was washed by shaking with 2 ml distilled water. The water was removed and added to the sodium hydroxide to make the aqueous fraction. The ether was transferred to a round bottomed flask and evaporated to dryness on a rotary evaporator. Steroids were re-dissolved in ethanol to give the first neutral steroid fraction.

The aqueous phase was returned to the separating funnel and further extracted with petroleum ether (50 ml). The ether was removed, evaporated to dryness and steroids re-dissolved in ethanol to give the second neutral steroid fraction.

The aqueous phase, containing phenolic steroids, was adjusted to pH 7 with dilute hydrochloric acid and returned to the separating funnel. Phenolic steroids were extracted 3 times, by shaking with 50 ml freshly distilled diethyl ether. Ether extracts were removed, pooled and evaporated to dryness. Steroids were redissolved in ethanol to give the phenolic steroid fraction.

2.3.5 Sequential enzyme hydrolysis for identification of steroid conjugates

Urine samples were subjected to sequential hydrolysis according to the method of Eastman *et al.* (1984).

Undiluted urine samples (0·2 ml) were placed in a large, glass test tube and extracted with 2 ml diethyl ether. Steroids, formerly present in the urine in the unconjugated form, were reconstituted in 1 ml buffer. Procedural losses during the extraction were estimated in triplicate by the addition of tracer amounts of [^{3}H]20α-dihydroprogesterone ([^{3}H]20α-DHP; hydroxypregn-4-ene-3-one, 20α-[1,2-^{3}H(N)]-; New England Nuclear, Dreiech, W.Germany; specific activity 45 Ci/mmol) to 0·2 ml rhinoceros urine pool, and recoveries were 93·1±1·9% (mean±s.e.m., n=9).

The aqueous phase, containing conjugated steroids, was adjusted to pH 5 by the addition of hydrolysis buffer (1:2). Samples were then hydrolysed with 1,200 FU of a specific β-D-glucuronide glucuronaosohydrolase (Sigma G3510; activity 1250,000 FU/g solid; 1,200 FU/0·1 ml hydrolysis buffer), containing no sulphatase activity, at 37°C for 24 h. Samples were adjusted to pH 7 with 3 M sodium hydroxide solution and extracted with 4 ml diethyl ether. The steroids, formerly present in the urine as conjugates of glucuronic acid, were reconstituted in 1 ml buffer. The

efficiency of the β-glucuronidase hydrolysis procedure was determined by hydrolysing, in triplicate, tracer amounts of [^{3}H]pregnanediol glucuronide ([^{3}H]PdG: Dr.P.Samarajeewa, Dept. Biochem., UCL; specific activity 30 Ci/mmol) to pregnanediol in a 0·2 ml urine pool, and recoveries obtained were 78·6±0·8% (mean±s.e.m., n=9). The specificity of the enzyme was monitored by the addition of tracer amounts of [^{3}H]oestrone sulphate (Amersham International, Bucks.; specific activity 60 Ci/mmol) to 0·2 ml urine pools, in triplicate, prior to hydrolysis. Recoveries of radiolabel (ie. [^{3}H]oestrone) were 4·4±3·2% (mean±s.e.m., n=9) confirming the specificity of β-glucuronidase in cleaving glucuronide conjugates (Eastman *et al.*, 1984).

The aqueous phase, containing conjugated steroids which remained unhydrolysed by β-glucuronidase activity, was adjusted to pH 5 by the addition of hydrolysis buffer (1:2) and hydrolysed at 37°C for 24 h with 400 FU β-glucuronidase-aryl-sulfatase in 0·1 ml hydrolysis buffer. Samples were then adjusted to pH 7 with 3 M sodium hydroxide solution and extracted with 6 ml freshly distilled diethyl ether. The steroids, formerly present in the urine as steroid sulphates, were reconstituted in 1 ml buffer. The efficiency of the sulfatase hydrolysis procedure was determined by hydrolysing, in triplicate, tracer amounts of [^{3}H]PdG to pregnanediol and [^{3}H]oestrone sulphate to oestrone in 0·2 ml urine pools, and recoveries obtained were 71·5±1·2% and 89·2±1·3% (mean±s.e.m, n=9) respectively.

2.3.6 Acid solvolysis

Acid solvolysis was carried out to hydrolyse steroid conjugates that may have been present in the urine, but were not hydrolysed by enzymatic procedures. Solvolysis is a stringent procedure which has been reported to liberate most ovarian steroid conjugates (Carroll *et al.*, 1990). Solvolysis was carried out according to the origional method of Jacobsohn and Lieberman (1962) as modified by Eastman *et al.* (1984).

After enzyme hydrolysis (see above) the residual aqueous phase was transferred to a small, glass bottle and diluted with an equal volume (0·6 ml) phosphate buffered saline (PBS; Appendix). Samples were then vortexed for 15 min with saturated sodium chloride solution (0·6 ml), 2·5 M sulphuric acid (0·3 ml) and 15 ml ethyl acetate and incubated overnight at 37°C. The aqueous phase was snap-frozen and the ethyl acetate decanted. The samples were again vortexed for 15 min with fresh ethyl acetate (15 ml), snap-frozen, and the ethyl acetate extracts combined. Ethyl acetate extracts were washed with distilled water (1 ml) prior to evaporating to dryness under nitrogen at 60°C. The dried extracts were reconstituted in 1 ml buffer. The solvolysis procedure was monitored by the addition of tracer amounts of [^{3}H]PdG and [^{3}H]oestrone sulphate to urine pools (0·6 ml), and recoveries were 66·7±0·6% and 75·6±0·3% (mean±s.e.m., n=9) respectively.

2.3.7 Derivatization for confirmation of steroid identity

Steroids were derivatized by selective reduction of the 3-oxo groups (Bush, 1961). Steroids were dissolved in methanol (9 ml) and dry nitrogen was bubbled through. A mixture of 2·5 M sodium hydroxide (0·1 ml), methanol (3 ml) and 0·18 M sodium borohydride in pyridine (2·8 ml) was added and the solution agitated with nitrogen for 10 min. After adjusting the solution to pH 7 with hydrochloric acid, steroids were extracted with 200 ml diethyl ether. The ether was washed with dilute sodium hydroxide solution followed by distilled water, and dried over anhydrous sodium sulphate for 12 h before evaporating to dryness.

2.4 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC), using plastic plates pre-coated with silica (Kodak, Hemel Hempstead, UK), was used to separate steroid metabolites to allow preliminary identification prior to high performance liquid chromatography. The plate was divided into 0·5 cm portions by horizontal lines of pencil. The

plate was further divided into vertical portions (approximately 2·5 cm in width), each labelled for standard or unknown.

Unknown steroids, in diethyl ether, were applied to the plate with a fine Pasteur pipette under a gentle stream of warm air to evaporate the solvent and give a discrete area of sample at the origin. Unlabelled reference standards, also dissolved in diethyl ether, were applied alongside the unknown material in a similar manner. Standards of progesterone and oestradiol-17β and the predicted end products of metabolism, pregnanediol, 17α-hydroxyprogesterone, 20α-DHP and oestriol, oestradiol-17α and oestrone respectively, were chosen so as to give a ready reference for the chromatographic mobility and identity of the unknown compounds.

Neutral steroids (progesterone metabolites) were separated in toluene:ethyl acetate (60:40, v/v) and phenolic steroids (oestrogens) were separated in cyclohexane:ethyl acetate (65:35, v/v). Chromatography tanks were prepared by the addition of 100 ml solvent, and allowed to equilibrate for one hour prior to use. Plates were placed in the respective tanks and allowed to develop at room temperature until the solvent front had migrated to within 2 cm of the top of the plate. At the completion of the run, plates were removed, the position of the solvent front was noted, and the plates were dried in air.

The vertical portion of plate containing unknown steroids was removed and cut into 0·5 cm horizontal strips. The mobility of the reference steroids was determined by spraying the plate with sulphuric acid, and developing in an oven at 80°C.

2.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) was used to separate unconjugated and conjugated progesterone metabolites, and unconjugated oestrogens in rhinoceros urine.

For HPLC a chromatographic system consisting of two single piston pumps (510 and 501; Waters, Milford, MA), a 1 ml injection loop and an automated gradient controller was used. For the separation of conjugated steroids the system used a μBondapak reverse phase C_{18} column (30x3·9, 10 μm particle size; Waters 86684), and for unconjugated steroids the system was fitted to a Lichrosorb straight phase silica 60 column (250x4 mm, 10 μm particle size; Merck, Sardsedt, Germany, 50387) and a solvent gradient system used to separate the urinary steroids. All solvents used in HPLC were Hipersolve (HPLC grade) from BDH.

2.5.1 Separation of conjugated progesterone metabolites

Conjugated progesterone metabolites were separated using a linear solvent gradient of 20-100% methanol in deionised, distilled water within 40 min at a flow rate of 1 ml/min, as described by Shideler *et al.* (1983). A blank run, with an injection of 0.2 ml methanol, was carried out prior to each series of sample runs to equilibrate the column.

Sample preparation was according to the method of Kasman *et al.* (1986). Urine samples (0·5 ml) were combined with an equal volume of methanol:ethanol (1:1, v/v) and centrifuged for 5 min at 400 g to precipitate particulate matter. The supernatant was removed and transfered to a glass test tube. [^{3}H]PdG (20,000cpm in 0·05 ml absolute ethanol) was added to the tube, and the volume of liquid therein reduced to 0·4 ml by evaporation under nitrogen at a temperature of 60°C. A sample volume of 0·2 ml was injected onto the column and fractions (1 ml) were collected by means of an automated fraction collector (Frac-100; Pharmacia, Uppsala, Sweden), into 1·5 ml polypropylene micro-centrifuge test tubes (Scotlab, Midlothian).

Fractions were evaporated under negative pressure in a vortex evaporator and reconstituted overnight in phosphate-azide-saline (PAS) gel buffer (1 ml; Appendix). Part (0·1 ml) of each fraction

was placed in a scintillation vial (Pony vials; Packard, Pangbourne, Berks.) with 5 ml scintillation fluid (Picofluor-15; Packard) and radioactivity counted on a Tricarb liquid scintillation analyzer (Packard 1900CA; efficiency 30%; $^{3}HE_{max}$=18·6 keV), to determine the retention time and recovery of the added radiolabel. The efficiency of the procedure (mean±s.e.m., n=23) was 69·5±3·8%

2.5.2 Separation of unconjugated progestagens

In order to separate unconjugated progesterone metabolites, a linear solvent gradient of 0-2% methanol in n-hexane : chloroform, 70:30 v/v, within 24 min at a flow rate of 2 ml/min was used (E.Möstl, personal communication). A blank run, after injecting 0·1 ml chromatography solvent, was carried out prior to each series of sample runs to equilibrate the column, and the concentration of methanol was increased to 6% over 10 min at the end of each run to ensure elution of all substances from the column.

Urine samples (1 ml) were placed in a small, glass bottle and adjusted to pH 5, by the addition of 1·5 ml hydrolysis buffer. [^{3}H]PdG (20,000 cpm in 0·05 ml hydrolysis buffer) was added to each sample to monitor the efficiency of the hydrolysis procedure and provide a reference standard for pregnanediol on the HPLC. Each sample was hydrolysed with 1,000 FU β-glucuronidase-aryl-sulphatase (1,000 FU/0·05 ml hydrolysis buffer) containing both glucuronidase and sulphase activity, at 37°C for 24 h. Samples were then adjusted to pH 7 by the addition of 3 M sodium hydroxide solution and [^{3}H]20α-DHP and [^{3}H]progesterone (Amersham International, Bucks.; specific activity 85 Ci/mmol) added (20,000 cpm each in 0·05 ml PAS gel buffer). Each sample was extracted with 20 ml freshly distilled diethyl ether. Samples were allowed to stand for 5 min to allow the ether and aqueous phases to separate, after which the aqueous phase was snap-frozen. The ether was decanted into clean test tubes, in a series of small volumes, and evaporated to drynessunder nitrogen at 30°C. Samples were reconstituted in 0·2 ml n-hexane : chloroform (70:30, v/v), and 0·1 ml injected onto the column. Fractions (1

ml) were collected over a period of 30 min, evaporated to dryness under pressure in a vortex evaporator and reconstituted overnight in 1 ml tris buffer (Appendix). The recovery of [^{3}H]PdG (65·7±2·1%; mean±s.e.m., n=29) indicated the efficiency of the procedure including the initial hydrolysis. The recoveries of [^{3}H]20α-DHP and [^{3}H]progesterone, added after hydrolysis, were 71·6 ± 3·3% and 67·1±2.8% (mean±s.e.m; n=29)

2.5.3 Separation of unconjugated oestrogens

Unconjugated 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 rate of 2 ml/min (E.Möstl, personal communication). A blank run, injecting 0·1 ml chromatography solvent, was carried out prior to each series of sample runs to equilibrate the column, and the concentration of methanol was increased to 20% over a period of 15 min at the end of each run to ensure the elution of all substances from the column.

Urine samples (1 ml) were adjusted to pH 5, by the addition of 1·5 ml hydrolysis buffer, and hydrolysed with 1,000 FU β-glucuronidase-aryl-sulphatase (1,000 FU in 0·05 ml hydrolysis buffer) at 37°C for 24 h. Samples were adjusted to pH 7 and [^{3}H]oestrone (Amersham International, Bucks., specific activity 90·7 Ci/mmol), [^{3}H]oestradiol-17β (Amersham International, Bucks.; specific activity 81·5 Ci/mmol) and [^{3}H]oestradiol-17α (donated by E.Möstl, Institut für Biochemie, Vienna; 36 µCi in 2 ml prepared according to the method of Choi *et al.*, 1989) added (20,000 cpm each in 0·05 ml PAS gel buffer). Each sample was extracted with 20 ml freshly distilled diethyl ether. Samples were allowed to stand for 5 min, then the aqueous phase was snap-frozen. The ether was decanted and evaporated to dryness, in very small volumes, under nitrogen at 30°C. Samples were reconstituted in 0·2 ml n-hexane : chloroform (50:50, v/v), and 0·1 ml injected onto the column. Fractions (1 ml) were collected over a period of 30 min, evaporated to dryness in a vortex evaporator and reconstituted overnight in

assay buffer (1 ml). The recovery of [^{3}H]oestrone was 64·3±2.6% (mean±s.e.m., n=16).

2.6 GAS CHROMATOGRAPHY/MASS SPECTROMETRY

2.6.1 Sample preparation

A Sep-pak C18 chromatography cartridge (Waters) was attached to a 5 ml syringe and primed for sample extraction by the application of 5 ml absolute ethanol followed by 5 ml deionised distilled water. The cartridge was then removed and attached to a 100 ml syringe containing the urine sample (20 ml) for GC/MS analysis. The urine was loaded onto the cartridge and the syringe washed through with water (5 ml). The sample was eluted with 5 ml absolute ethanol into a round bottomed tube. The ethanol was evaporated to dryness on a rotary evaporator and the sample redissolved in 10 ml hydrolysis buffer (pH 5) and sonicated to aid the reconstitution process.

Samples were incubated with β-glucuronidase-aryl sulphatase (10,000 FU/in 0·1 ml hydrolysis buffer) at 55°C for 3 h in order to hydrolyse steroid conjugates present in the sample. The samples were applied to Sep-pak cartridges, primed as before, and the tubes were washed with water which was also loaded onto the cartridge. The sample was eluted with absolute ethanol (5 ml) into a large, round bottomed flask. The flask was partially immersed in a water bath (60°C) and the ethanol evaporated to dryness on a rotary evaporator. The sample was redissolved in absolute ethanol (2 ml) and sonicated.

2.6.2 Sephadex LH20 chromatography

Steroids were fractionated on a column of Sephadex LH20 according to the method of Shackleton, Honour, Dillon and Milla, 1976). Sephadex LH20 (6·1 g; Pharmacia) was dissolved in 30 ml chromatography solvent (freshly distilled cyclohexane : absolute

ethanol, 4:1, v/v) and allowed to swell at room temperature for 10 min. The Sephadex LH20 was loaded into a 0·5 m glass column (2 cm diameter) to form the stationary phase for chromatography. Any residual chromatography solvent was allowed to elute from the column until a dry surface was obtained.

A volume of the hydrolysed sample extract (1 ml) was placed in a test tube and the ethanol evaporated under nitrogen at 60°C. The sample was redissolved in absolute ethanol (0·4 ml), and 1·6 ml freshly distilled cylohexane was added in a dropwise manner, whilst mixing the contents of the tube in a sonicating waterbath. The sample was then loaded slowly onto the dry surface of the Sephadex LH20 column by means of a Pasteur pipette. Once the sample had been absorbed into the stationary phase, 8 ml chromatography solvent was applied. The column was run to dryness and the eluent discarded. Four fractions were collected into round bottomed flasks by eluting compounds from the column with 43 ml, 65 ml, 40 ml and 90 ml volumes of cyclohexane:ethanol (4:1, v/v) sequentially. The solvent was evaporated from each fraction on a rotary evaporator, and fractions reconstituted in absolute ethanol (2 ml).

2.6.3 Derivatization for GC/MS

Methyloxime-trimethylsilyl ether (MO-TMS) steroid derivatives were prepared in a 2 step reaction (Prost, Bournot and Maume, 1975). Firstly, a volume of each reconstituted fraction (1 ml) was placed in a small test tube. Internal standards, 0·05 ml absolute ethanol containing 5 ng each of 5α-androstane-3α,17α-diol, stigmasterol and cholesterol butyrate (Sigma Chemical Co.), were added to the tube and the solvent evaporated to dryness under nitrogen at 60°C. The residue was oximised by incubating at 60°C for 1 h with a solution of methoxyamine hydrochloride in pyridine (2%, w/v; 0·2 ml). Secondly, trimethylsilylimidazole (0·1 ml) was added and the mixture incubated at 100°C overnight for silylation to occur.

Solvent was evaporated from the sample under nitrogen at 60°C and the residue redissolved in 1ml lipidex solvent (freshly distilled cyclohexane : pyridine : HMDS, 98:1:1 v/v) and sonicated. Lipidex-5000 (Pharmacia; stored in methanol) was placed in a filter funnel over a conical flask and a vacuum applied. The powder was washed three times with freshly distilled cyclohexane followed by two washes with lipidex solvent. A small pad of glass wool was placed in a pasteur pipette and 2 ml lipidex added. The solvent was eluted and the column washed with lipidex solvent (0·5 ml). The reconstituted sample was loaded onto the column, the tube rinsed with lipidex solvent (0·5 ml) which was also applied to the column. Excess reagents were removed by chromatography (Axelson and Sjövall, 1974) and the derivatised compounds were eluted with lipidex solvent (1 ml) into a test tube under nitrogen pressure to force through all the liquid. The solvent was evaporated under nitrogen at 60°C and the sample reconstituted in freshly distilled cyclohexane (0·5 ml) and stored in an air-tight vial.

2.6.4 Gas chromatography/mass spectrometry (GC/MS)

Steroid derivatives were analysed on a Packard 437A gas chromatograph equipped with flame ionization detectors. Derivatised samples (2 µl) were applied to the column via a solid injection system. Helium was used as the carrier gas and the flow rate through the column was 1-2 ml/min. Gas chromatography was carried out using temperature programming conditions of 260-290°C with increments of 3°C/min.

Repetitive magnetic scanning over the mass range 98-800 atomic mass units (amu) was performed using a Hewlett Packard model 5890 (GC) and 5970 series mass spectrometer detector (MSD) . For GC/MS the temperature of the separator and transfer line and ion source was 250°C, the ionisation current and voltage were 300 mA and 70 eV respectively.

Steroid identification was based on gas chromatographic retention time of the steroid derivative and the ionic spectrum produced by mass spectrometry. Steroids were quantified by relating the peak height on the gas chromatograph to that of a line drawn between the peak heights of the internal standards.

2.7 RECRYSTALLIZATION

Recrystallization was carried out according to the method of Axelrod, Matthijssen, Goldzieher and Pulliam (1965), as modified by Pashen (1980).

Authentic steroid (20-30 mg) was dissolved in absolute ethanol containing ^{3}H-labelled steroid and ^{14}C-labelled unknown steroid, and the solution was evaporated to dryness. Chloroform (0·5 ml) was added to redissolve the steroids and 0·05 ml of this solution was dispensed onto a pre-weighed planchette and place in a dust-free covered box. The remaining chloroform was evaporated to dryness and redissolved in warm acetone which was gradually reduced in volume by evaporation, while simultaneously n-hexane was added in a drop-wise manner to decrease the solubility of the steroid. As soon as crystals began to form, the solution was transferred to 4°C where the crystals were allowed to grow for 24 h. After a short centrifugation at 400 g, the supernatant was decanted and the crystals redissolved in 0·5 ml chloroform and the process repeated. Four recrystallizations were performed for each unknown steroid.

The chloroform was allowed to evaporate spontaneously from the steroid solutions in the pre-weighed planchettes leaving behind a small mass of solid hormone, the weight of which was calculated by subtracting the weight of the empty planchette from the weight of the planchette plus hormone residue. Following weighing, each planchette was transferred to a separate scintillation vial and 0·5 ml methanol was added to redissolve the steroids. Scintillation fluid (5 ml) was added and the ^{3}H and ^{14}C-radioactivity counted for 10 min. The specific activity (SpA) and $^{3}H/^{14}C$ ratio was

calculated. The SpA of the steroid crystallized at each step was determined from the mass of material in the planchette and the ^{14}C-radioactivity associated with it as follows:

$$SpA = \frac{\text{c.p.m.}}{2.2 \times 10^6 \times \text{mass of crystals (g)}}$$

2.8 RADIOIMMUNOASSAY FOR OESTRONE

Oestrone was measured in hydrolysed urine samples according to the method of Hodges, Brand, Henderson and Kelly (1983a).

2.8.1 Assay materials and reagents

For radioimmunoassay of oestrone, the assay buffer was WHO buffer (pH 7·2; Appendix). The anti-oestrone serum used in the assay was prepared in a rabbit immunized against oestrone-6-carboxymethyloxime conjugated to bovine serum albumin and was supplied by Steranti Research Ltd., St. Albans, UK. The cross reactivities of the antiserum, determined by the supplier, were oestradiol-17β (0·1%), oestradiol-17α (0·06%), oestriol (0·01%) and <0·02% with other C_{19} and C_{21} steroids tested. The antibody was supplied freeze dried and was reconstituted in buffer and 25 µl aliquots stored at -20°C until used in the assay.

Tritiated oestrone ([^{3}H]E_1; [2,4,6,7-^{3}H]oestrone) was used as the label in the assay. The original supply was diluted to 50 µCi/ml in toluene : ethanol (9:1) and stored at -20°C. For assay, radiolabel was dried down under a stream of nitrogen and reconstituted in buffer to give a solution of 10,000 cpm/ 0·1 ml.

Oestrone standards (3-hydroxy-1,3,5{10}-oestratrien-17-one; MW=270·4; Sigma Chemical Co. E9750) were double diluted over the range 400-6·25 pg/0·5 ml (800-12·5 pg/ml) after the initial dilution of a stock solution of 20 ng/ml buffer. A QC was prepared by

diluting urine from a pregnant, northern white rhinoceros, which had previously been hydrolysed and extracted, and was stored at -20°C.

Free and antibody-bound oestrone were separated using a dextran activated charcoal suspension containing 0·625% activated charcoal (previously washed in distilled water and dried in a heated cabinet) and 0·0625% dextran T-70 (Pharmacia) in buffer, ie. 1·25 g charcoal and 0·125 g dextran T-70 in 200 ml buffer.

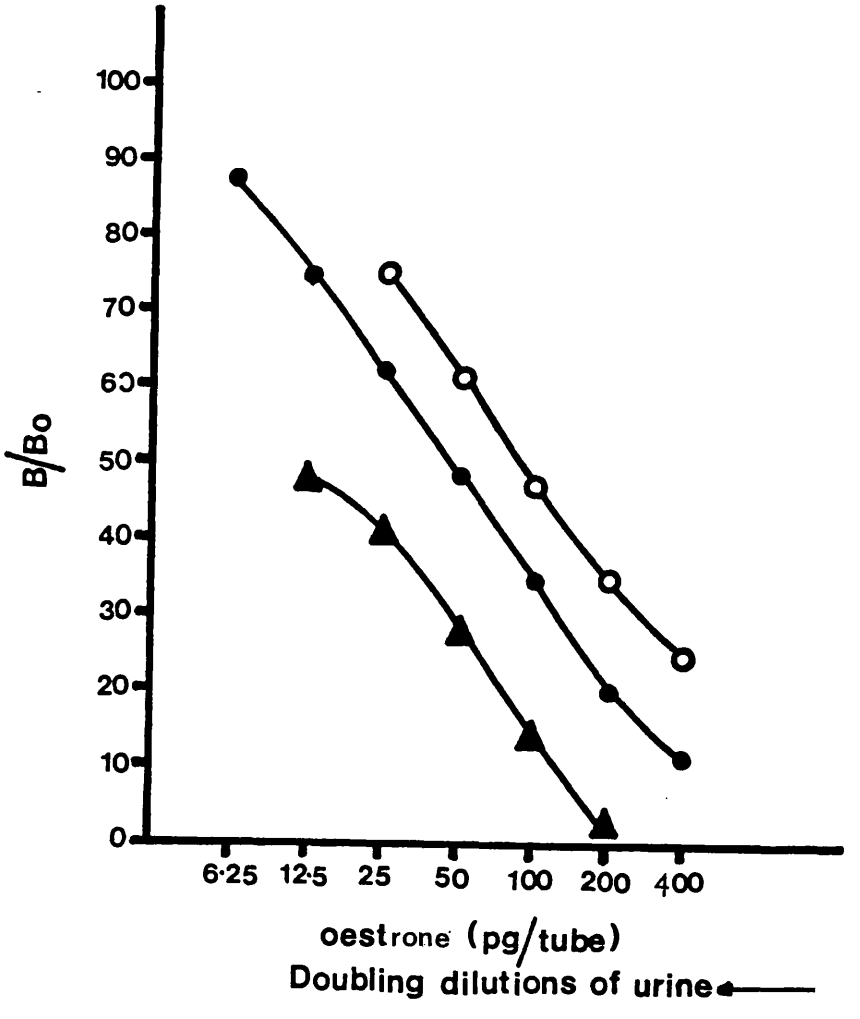
2.8.2 Sample preparation

Urine samples (0·05 ml) were hydrolysed with β-glucuronidase-aryl-sulphatase and extracted with diethyl ether, prior to assay for oestrone (see section 2.3). Procedural losses during the extraction were monitored by the addition of tracer amounts of [^{3}H]E_1 to each sample prior to extraction. Recoveries were 86·2±2·0% (mean±s.e.m, n=378), and individual recoveries were used to correct for procedural losses when calculating the final concentration of oestrone in urine. Hydrolysis efficiencies were determined in triplicate for each assay by adding tracer amounts of [^{3}H]oestrone sulphate and [^{3}H]oestrone glucuronide (Dr. P.Samarajeewa, Dept. Biochem., UCL, London; specific activity 53 Ci/mmol) respectively to 0·05 ml urine pool, hydrolysed and extracted as above. Recoveries were 79·2±3·0% and 80·2±0·9% (mean±s.e.m., n=16) respectively.

2.8.3 Assay protocol

For the assay, standards were prepared in triplicate over the range of 400-6·25 pg/0·5 ml, along with total count (TC) and non-specific binding (NSB) tubes. Total binding (TB) and QC tubes (0·5 ml) were included in duplicate. Radiolabel (10,000 cpm in 0·1 ml) was added to each tube; antiserum (0·1 ml; working dilution of 1:400) was added to all tubes except TC and NSB. The contents of each tube were mixed thoroughly, tubes were covered and incubated overnight at 4°C.

Figure 2.2. Typical oestrone assay standard curve with standards over the range 6·25–400 pg. The curve is plotted as % bound against the log of the mass of oestrone. The standard dose response curve for the oestrone RIA (●—●) is compared with serial dilutions of urine from the black rhinoceros during the pre-oestrus period (○—○) and the white rhinoceros during pregnancy (▲—▲).



The final assay layout was as follows:

TC	0·1ml [^{3}H]E_1 + 0·8ml buffer
NSB	0·1ml [^{3}H]E_1 + 0·6ml buffer
TB	0·1ml [^{3}H]E_1 + 0·5ml buffer + 0·1ml antiserum
STANDARDS	0·1ml [^{3}H]E_1 + 0·5ml (6·25-400pg)E_1 + 0·1ml antiserum
QCs and SAMPLE	0·1ml [^{3}H]E_1 + 0·5ml QC or sample + 0·1ml antiserum

After incubation, ice cold dextran activated charcoal suspension (0·2 ml) was added to all tubes except the TC. The tubes were vortex mixed and incubated for 15 min at 4°C prior to centrifuging at 2400 rpm (500 g) for 10 min in a cool spin (Fisons Scientific Apparatus Ltd., Loughborough) centrifuge (4°C). The supernatants were decanted into scintillation vials containing 2 ml scintillation fluid, mixed and allowed to equilibrate for 1 h before radioactivity was counted for 3 min.

2.8.4 Assay evaluation

A typical standard curve, obtained by plotting % bound (B/B_0) against mass (pg) oestrone standard on a logarithmic scale, is shown in Fig. 2.2. Values of oestrone in samples were calculated from the standard curve, initially as pg/tube, and then transformed to ng/mgCr. Serial dilutions of hydrolysed urine from female black rhinoceroses on the day prior to oestrus and northern white rhinoceroses during pregnancy gave displacement curves parallel to that obtained with oestrone standards (Fig. 2.2).

The sensitivity of the assay, determined at 90% binding was 4·5 pg/tube or 9·0 ng/ml. The accuracy of the assay was determined by adding varying amounts of unlabelled oestrone (6·25-400pg) to 0·5 ml volumes of hydrolysed and extracted black and white rhinoceros urine pools, containing low levels of endogenous hormone. The samples were assayed and means±s.e.m recoveries of added oestrone were 89·2±3·1% and 97·8±1·6% respectively. Intra-assay precision was determined by repeated measurement of a sample prepared from pooled black rhinoceros urine which had previously been hydrolysed and

extracted. The intra-assay C of V was found to be 11·2% (n=45). Inter-assay precision was calculated by determining the C of V of measurement of a QC (which gave a value of approximately 50% bound) in each assay and was found to be 9·9% (n=10).

2.9 RADIOIMMUNOASSAY FOR PROGESTERONE

Urinary progesterone was measured by radioimmunoassay without chromatography, as described by Hodges, Eastman and Jenkins (1983b).

2.9.1 Assay reagents

For the assay of progesterone, the assay buffer was low salt buffer (LSB; pH 7·0; Appendix). The anti-progesterone serum was obtained from the World Health Organisation (WHO CHWIB), stored at -20°C in 80 µl aliquots of 1:100 dilution. The antibody was raised in rabbits against progesterone-carboxymethyloxime-BSA, and the cross reactivity with other steroids were described by Hodges *et al.* (1983b) as follows: 17α-hydroxyprogesterone (3·0%), testosterone (0·1%) and cortisol (0·01%).

Tritiated progesterone ([^{3}H]P$_4$, {1,2,6,7-^{3}H}-progesterone) was used as the label in the assay. The original supply was diluted to 50 µCi/ml in toluene:ethanol (9:1) and stored at -20°C. For assay, an aliquot was dried down under a stream of nitrogen and reconstituted in buffer to give a solution of 10,000 cpm/ 0·1 ml.

Progesterone standards (4-pregnene-3,20-dione; MW=314·5; Sigma Chemical Co. P0130) were double diluted over a range of 400-6·25 pg/0·5 ml (800-12·5 pg/ml) after the initial dilution of a stock solution of 4 ng/ml buffer. A QC was prepared from extracted, marmoset plasma and stored at -20°C.

Free and antibody-bound progesterone were separated using a dextran activated charcoal suspension containing 0·625% activated

charcoal and 0·0625% dextran T-70 in buffer, ie. 1·25 g charcoal and 0·125 g dextran T-70 in 200 ml buffer.

2.9.2 Sample preparation

Urine samples (0·05 ml) were extracted with 0·5 ml freshly distilled petroleum ether (40-60°C) prior to assay for progesterone. Procedural losses during the extraction were monitored by the addition of tracer amounts of [^{3}H]P_4 to each sample prior to extraction. Recoveries were 89·1±2.6% (mean±s.e.m, n=126), and individual recoveries were used to correct for procedural losses when calculating the final concentration of progesterone in urine.

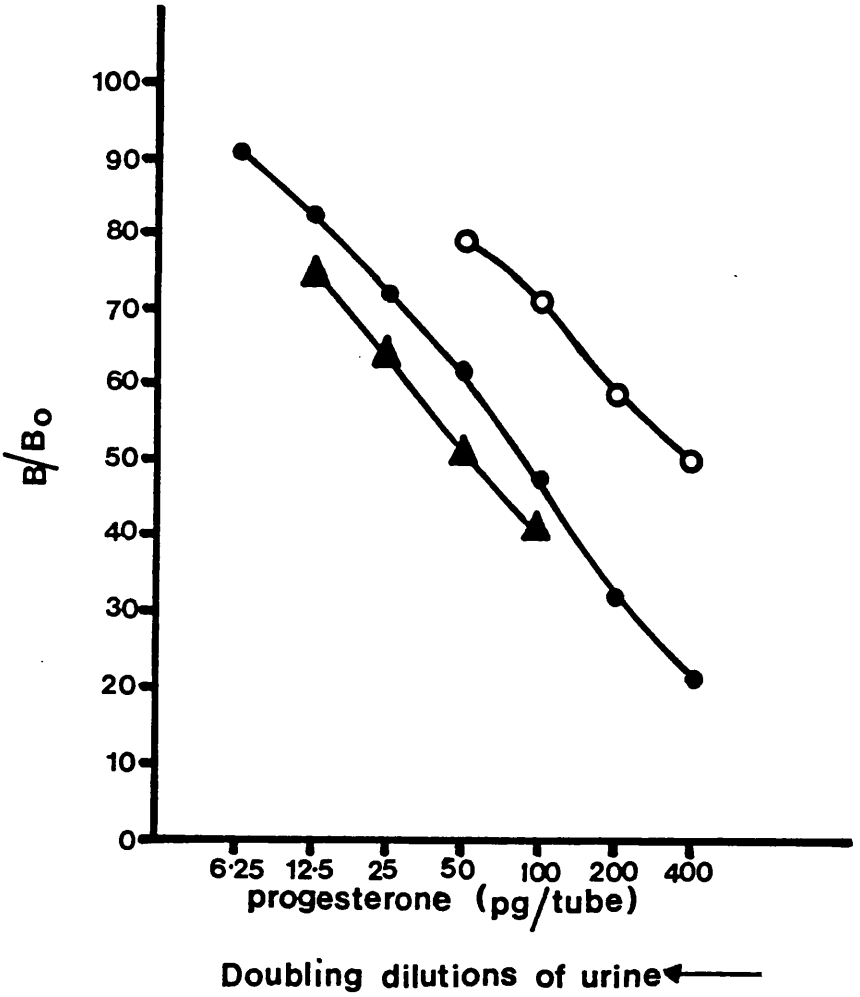
2.9.3 Assay protocol

Standards were prepared in triplicate over the range of 400-6·25 pg/0·5 ml, along with total count (TC) and non-specific binding (NSB) tubes. Total binding (TB) and QC tubes (0·5 ml) were included in duplicate. Radiolabel (10,000 cpm in 0·1 ml) was added to each tube followed by antiserum (0·1 ml; working dilution of 1:12,500), which was omitted from the TC and NSB tubes. The contents of each tube were mixed throughly, and the tubes were covered and incubated overnight at 4°C. The final assay layout was as follows:

TC	0·1ml [^{3}H]P_4 + 0·8ml buffer
NSB	0·1ml [^{3}H]P_4 + 0·6ml buffer
TB	0·1ml [^{3}H]P_4 + 0·5ml buffer + 0·1ml antiserum
STANDARDS	0·1ml [^{3}H]P_4 + 0·5ml (6·25-400pg)P_4 + 0·1ml antiserum
QCs and SAMPLE	0·1ml [^{3}H]P_4 + 0·5ml QC or sample + 0·1ml antiserum

After incubation, ice cold dextran activated charcoal suspension (0·2 ml) was added to all tubes except the TC. The tubes were vortex mixed and incubated for 15 min at 4°C prior to centrifuging at 2400 rpm (500 g) for 10 min at 4°C. The supernatants were decanted into scintillation vials containing 4 ml

Figure 2.3. Typical progesterone assay standard curve with standards over the range 6·25–400 pg. The curve is plotted as % bound against the log of the mass of progesterone. The standard dose response curve for the progesterone RIA (●—●) is compared with serial dilutions of urine from the black (o—o) and the white rhinoceros (▲—▲) during the post-oestrus period.



scintillation fluid, mixed and allowed to equilibrate for 1h before radioactivity was counted for 3 min.

2.9.4 Assay evaluation

A typical standard curve, obtained by plotting B/B_0 against mass (pg) progesterone standard on a logarithmic scale, is shown in Fig. 2.3. Values of progesterone in samples were calculated from the standard curve, initially as pg/tube, and then transformed to ng/mgCr. Serial dilutions of urine collected from female black and northern white rhinoceroses during the post-oestrus period gave displacement curves parallel to that obtained with progesterone standards (Fig. 2.3).

The sensitivity of the assay, determined at 90% binding was 7·0pg/tube or 14 pg/ml. The accuracy of the assay was determined by adding varying amounts of unlabelled progesterone (6·25-400pg) to 0·5 ml volumes of extracted black and white rhinoceros urine pools, containing low levels of endogenous hormone. The samples were assayed and means±s.e.m recoveries of added progesterone were 102·8±1·6% and 82.8±3.7% respectively. Intra-assay precision was determined by repeated measurement of a sample prepared from pooled black rhinoceros urine which had previously been extracted. The intra-assay C of V was found to be 7·2% (n=45). Inter-assay precision was calculated by determining the C of V of measurement of a QC (which gave a value of approximately 60% bound) in each assay and was found to be 11·4% (n=5).

2.10 RADIOIMMUNOASSAY FOR OESTRONE CONJUGATES

Conjugated oestrone was measured using the direct assay of Hodges and Eastman (1984) and Hodges *et al.* (1984). Following results obtained by sequential hydrolysis of oestrone conjugates in this study, and results obtained by Ramsay *et al.* (1987), oestrone-3-glucuronide standards and tracer were used to measure oestrone conjugates in rhinoceros urine.

2.10.1 Assay reagents

For the assay of oestrone conjugates, the assay buffer was tricine buffered saline (TBS; pH 7·0; Appendix). The antiserum used in this assay was raised in a rabbit against oestrone-3-glucuronide conjugated to bovine serum albumen (Dr. P.Samarajeewa, Dept. Biochem., UCL, London), and was stored at -20°C in 50µl aliquots at a dilution 1:75. Using oestrone-3-glucuronide as a standard, the major reacting steroids were described by Hodges and Eastman (1984) as oestrone (126%), oestrone-3-sulphate (100%), oestrone-2-glucuronide (84%) and oestrone-17-sulphate (5·4%).

Tritiated oestrone-3-glucuronide ([^{3}H]E_1G) was diluted to 50 µCi/ml in toluene:ethanol (9:1) and stored at -20°C. An aliquot was dried down under nitrogen and reconstituted in an appropriate amount of buffer, to give a solution containing 10,000 cpm/0·1 ml.

Oestrone-3-glucuronide standards (estrone β-D-glucuronide; MW=446·5; Sigma Chemical Co. E1752) were double diluted over a range of 2-0·0313 ng/0·1 ml (20-0·313 ng/ml) after the initial dilution of a stock solution of 1 µg/ml buffer. A QC was prepared from diluted, human urine and stored in 0·1 ml aliquots at -20°C.

Free and antibody-bound conjugated oestrone were separated using a dextran activated charcoal suspension containing 1% activated charcoal and 0·1% dextran T-70 in buffer, ie. 2·0 g charcoal and 0·2 g dextran T-70 in 200 ml buffer.

2.10.2 Assay protocol

Standards were prepared in triplicate over the range of 2·0-0·0313 ng/0·1 ml, along with total count (TC) and non-specific binding (NSB) tubes. Total binding (TB) and QC tubes (0·1 ml) were included in duplicate. Radiolabel (10,000 cpm in 0·1 ml) was added to each tube; antiserum (0·1 ml; working dilution of 1:15,000) was added to all tubes except TC and NSB tubes. The contents of each

tube was mixed throughly, and the tubes covered and incubated overnight at 4°C. The final assay layout was as follows:

TC	0·1ml [^{3}H]E_1G + 0·7ml buffer
NSB	0·1ml [^{3}H]E_1G + 0·2ml buffer
TB	0·1ml [^{3}H]E_1G + 0·1ml buffer + 0·1ml antiserum
STANDARDS	0·1ml [^{3}H]E_1G + 0·1ml (0·0313-2·0ng)E_1G + 0·1ml antiserum
QCs and SAMPLES	0·1ml [^{3}H]E_1G + 0·1ml QC or sample + 0·1ml antiserum

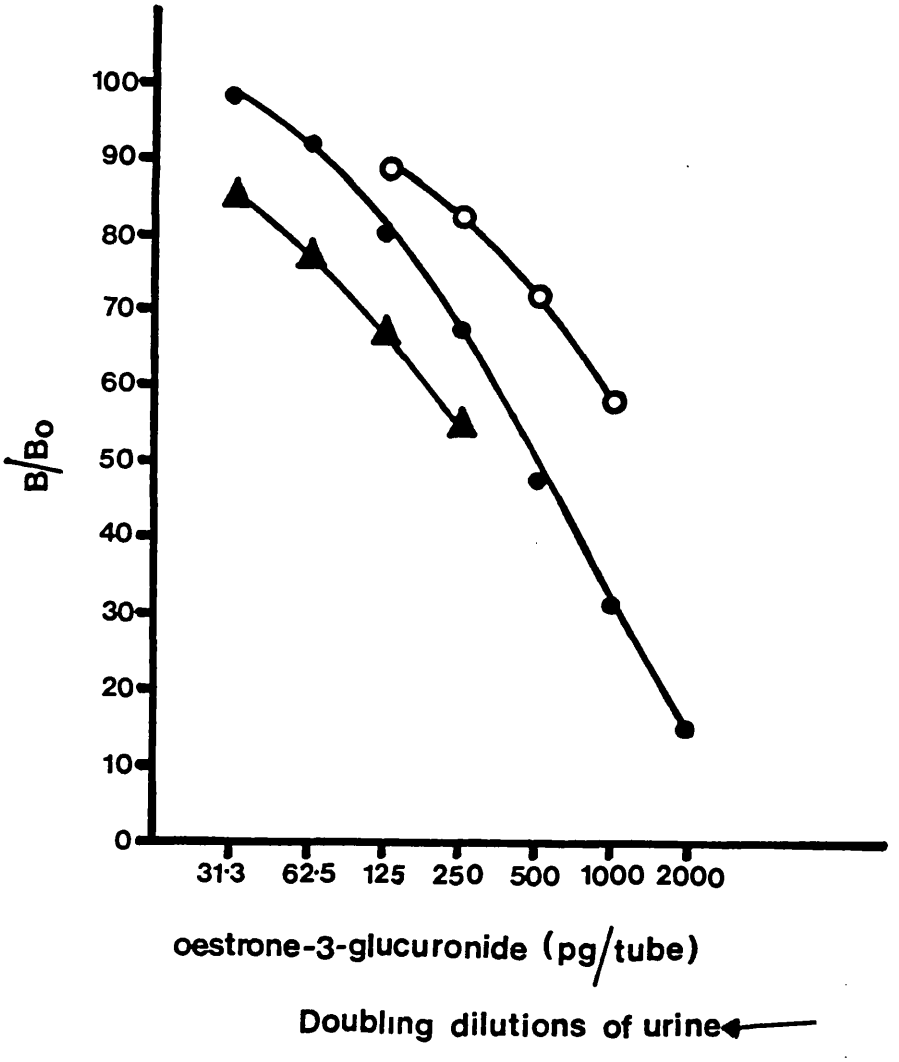
After incubation, ice cold dextran activated charcoal suspension (0·2 ml) was added to all tubes except the TC. The tubes were vortex mixed and incubated for 15 min at 4°C prior to centrifuging at 2400 rpm (500 g) for 10 min at 4°C. The supernatants were decanted into scintillation vials containing 4ml scintillation fluid, mixed and allowed to equilibrate for 1h before radioactivity was counted for 3 min.

2.10.3 Assay evaluation

A typical standard curve, obtained by plotting B/B_o against mass (pg) oestrone glucuronide standard on a logarithmic scale, is shown in Fig. 2.4. Values of conjugated oestrone in samples were calculated from the standard curve initially as pg/tube, and then transformed to ng/mgCr. Serial dilutions of urine collected on the day prior to oestrus from female black and northern white rhinoceroses gave displacement curves parallel to that obtained with with oestrone glucuronide standards to assume the assay fully validated for the measurement of oestrone glucuronide in rhinoceros urine (Fig. 2.4).

The sensitivity of the assay, determined at 90% binding was 70 pg/tube or 0·7 ng/ml. The accuracy of the assay was determined by adding varying amounts of unlabelled oestrone glucuronide (0·313-20 ng) to 0·1 ml undiluted black and white rhinoceros urine pools, containing low levels of endogenous hormone. The samples were

Figure 2.4. Typical oestrone conjugate assay standard curve with oestrone glucuronide standards over the range 6·25–400 pg. The curve is plotted as % bound against the log of the mass of oestrone glucuronide. The standard dose response curve for the oestrone conjugate RIA (●—●) is compared with serial dilutions of urine from the black (o—o) and the white rhinoceros (▲—▲) on the day prior to oestrus.



assayed and mean±s.e.m recoveries of added oestrone glucuronide were 98.4±1.1% and 92.8±2.7% respectively. Intra-assay precision was determined by repeated measurement of a sample prepared from pooled black rhinoceros urine. The intra-assay C of V was found to be 10·6% (n=45). Inter-assay precision was calculated by determining the C of V of measurement of a QC (which gave a value of approximately 55% bound) in each assay and was found to be 13·2% (n=5).

2.11 RADIOIMMUNOASSAY FOR OESTRADIOL-17β

Oestradiol-17β in hydrolysed urine samples was measured by iodinated radioimmunoassay according to the method described by Shaw, Hillier and Hodges (1989).

2.11.1 Assay reagents

For the assay of oestradiol-17β, the assay buffer was WHO buffer. The anti-oestradiol-17β serum used in this assay was raised in a rabbit against oestradiol-11β-succinyl BSA (Medical Research Council, Edinburgh, Scotland), and was stored at -20°Cin 100 µl aliquots at a dilution of 1:100. Shaw *et al.* (1989) reported oestrone to be the only steroid which cross reacts significantly (8%) with the antiserum, all other steroids tested cross reacted less than 0·1% by mass. For this study the cross reaction of oestradiol-17α with the oestradiol-17β antiserum was determined as 27%. The second antibody was donkey anti-rabbit gamma globulin (donated by Prof. G.R.Foxcroft, Uni. Nottingham, School of Agriculture, Loughborough) which was added in combination with normal rabbit serum (Regal Rabbits, Great Bookham, Surrey).

Iodinated oestradiol-17β ($[^{125}I]E_2$; 17β-oestradiol-11α-tyrosine methyl ester-^{125}I; MRC Edinburgh) prepared from 17β oestradiol-11α-succinyl-tyrosine methyl ester according to the method of Greenwood , Hunter and Glover (1963), was the label in the assay. The origiǿnal supply was stored at 4°C and an aliquot

was dried down under nitrogen and reconstituted in buffer to give a solution containing 10,000 cpm/ 0·1 ml.

Oestradiol-17β standards (MW=272·4; Sigma Chemical Co. E8875) were diluted over the range of 400-3·15 pg/0·1 ml (4-0·0315 ng/ml) from a stock solution of 4 ng/ml buffer. A QC was prepared from hydrolysed and extracted urine, collected from a northern white rhinoceros on the day of oestrus, and stored in 0·1 ml aliquots at -20°C.

Free and antibody-bound steroid were separated using a mixture of 0·9% saline and 0·2% triton X-100 in buffer.

2.11.2 Sample preparation

Urine samples (0·05 ml) were hydrolysed with β-glucuronidase-aryl-sulphatase and extracted with diethyl ether prior to assay for oestradiol-17β. Procedural losses during the extraction were monitored by the addition of tracer amounts of [^{3}H]oestradiol-17β to each sample prior to extraction. Recoveries were 90·6±1·3% (mean±s.e.m, n=136), and individual recoveries were used to correct for procedural losses when calculating the final concentration of oestradiol-17β in urine. Hydrolysis efficiencies were determined in triplicate by adding tracer amounts of [^{3}H]oestrone sulphate and [^{3}H]E_1G to 0·05 ml rhinoceros urine pool, hydrolysed and extracted as above. Recoveries were 76·2±3·3% and 79·3±1·2% (mean±s.e.m., n=16) respectively.

2.11.3 Assay protocol

Standards were prepared in triplicate over the range of 400-3·15 pg/0·1 ml, along with total count (TC) and non-specific binding (NSB) tubes. Total binding (TB) and QC tubes (0·1 ml) were included in duplicate. Radiolabel (10,000 cpm in 0·1 ml) was added to each tube; antiserum (0·1 ml; working dilution of 1:10,000) was added to all tubes except TC and NSB. The contents of each tube were mixed

thoroughly, and the tubes were covered and incubated at room temperature for 3 h. The final assay layout was as follows:

TC	0·1ml [^{125}I]E_2
NSB	0·1ml [^{125}I]E_2 + 0·2ml buffer
TB	0·1ml [^{125}I]E_2 + 0·1ml buffer + 0·1ml antiserum
STANDARDS	0·1ml [^{125}I]E_2 + 0·1ml (3·15-400pg)E_2 + 0·1ml antiserum
QCs and SAMPLES	0·1ml [^{125}I]E_2 + 0·1ml QC or sample + 0·1ml antiserum

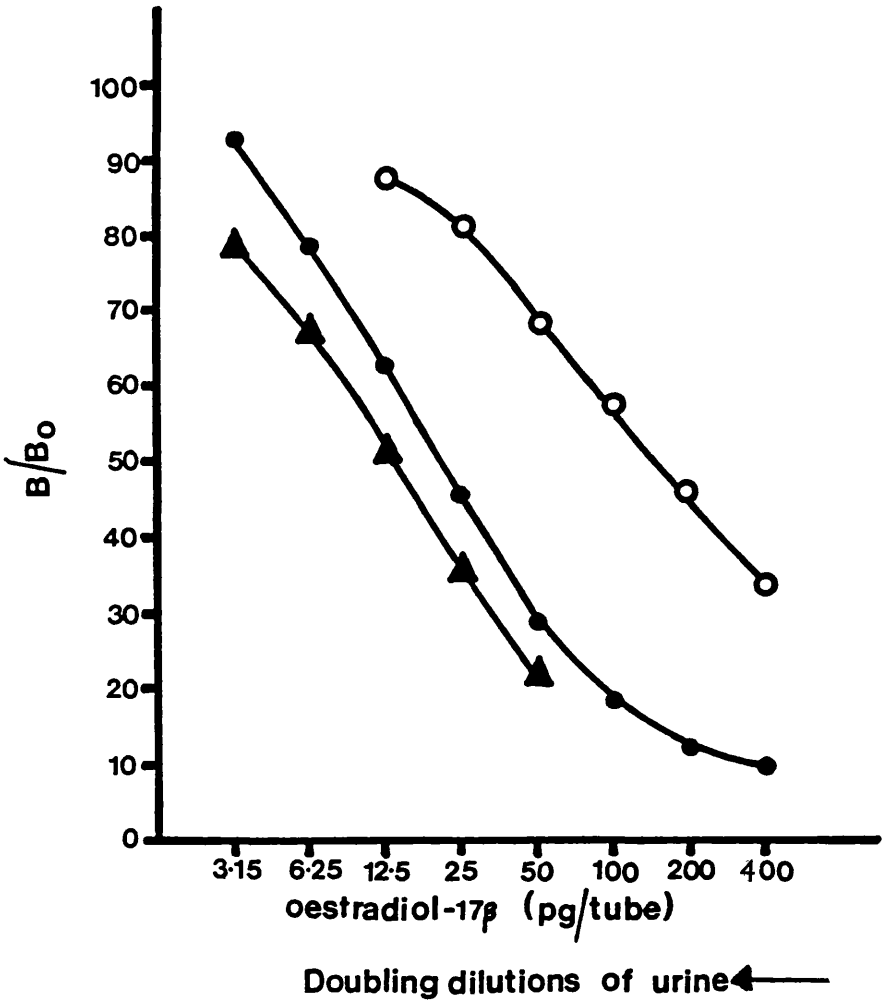
After incubation, normal rabbit serum (0·1 ml, 1:8,000) and donkey anti-rabbit gamma globulin (0·1 ml, 1:40) was added to all tubes except the TC. The tubes were vortex mixed and incubated overnight at 4°C. Separation was achieved by the addition of 1·0 ml saline and triton X-100 mixture prior to centrifuging at 2400 rpm (500 g) for 30 min at 4°C. The supernatants were removed by aspiration and radioactivity in the precipitate counted on a Cobra-QC auto-gamma counting system (Packard 5005).

2.11.3 Assay evaluation

A typical standard curve, obtained by plotting B/B_o against mass (pg) oestradiol-17β on a logarithmic scale, is shown in Fig. 2.5. Values of oestradiol-17β in samples were calculated from the standard curve, initially as pg/tube, and then transformed to ng/mgCr. Serial dilutions of hydrolysed and extracted urine collected on the day prior to oestrus from female northern white rhinoceroses gave displacement curves parallel to that obtained with the oestradiol-17β standards (Fig. 2.5).

The sensitivity of the assay, determined at 90% binding, was 3·8pg/tube or 38 pg/ml. The accuracy of the assay was determined by adding varying amounts of unlabelled oestradiol-17β (3·15-400pg) to 0·1 ml volumes of hydrolysed and extracted white rhinoceros urine pool, containing low levels of endogenous hormone. The samples were assayed and mean±s.e.m recoveries of added oestradiol-17β were

Figure 2.5. Typical oestradiol-17β assay standard curve with standards over the range 3·15–400 pg. The curve is plotted as % bound against the log of the mass of oestradiol-17β. The standard dose response curve for the oestradiol-17β RIA (●—●) is compared with serial dilutions of urine from the white rhinoceroses (n=2) on the day prior to oestrus.



102·4±1·7%. Intra-assay precision was determined by repeated measurement of a sample prepared from pooled white rhinoceros urine which had previously been hydrolysed and extracted. The intra-assay C of V was found to be 9·0% (n=45). Inter-assay precision was calculated by determining the C of V of measurement of a QC (which gave a value of approximately 50% bound) in each assay and was found to be 10·9% (n=11).

2.12 ENZYME-IMMUNOASSAY FOR PREGNANEDIOL-3α-GLUCURONIDE

Urinary PdG was measured by the direct EIA system described by Hodges and Green (1989).

2.12.1 Assay materials and reagents

For the assay of PdG, the assay buffer was PAS gel buffer. The anti-PdG serum was raised in a rabbit against pregnanediol-3α-glucuronide conjugated to BSA. The IgG fraction was obtained by purification on a protein A column (Pharmacia) followed by dialysis and concentration using ethylene glycol. The antiserum is described by Hodges and Green (1989) as showing significant cross reaction with 5β-pregnanediol (45·1%), 20α-dihydroprogesterone (12·1%), 5β-pregnanedione (4·1%), 17α-hydroxyprogesterone, 5β-pregnanetriol and oestradiol-17-glucuronide (0·1%) in this system.

The enzyme label for the assay was pregnendiol-3α-glucuronide conjugated to alkaline phosphatase (EC 3.1.3.1; type VIII-T; Sigma Chemical Co.) at a steroid : enzyme molar ratio of 10:1, by the active ester technique of Sauer *et al.* (1986). The enzyme label was dialysed against 0·1 M PBS and stored at 4°C in PBS containing 0·1% gelatin and sodium azide. Enzyme substrate was p-nitrophenyl phosphate disodium (0·13 g; Sigma 104) dissolved in 24 ml diethanolamine buffer (Appendix).

PdG standards (5β-pregnan-17α, 20α-diol-3α-glucuronide, MW=495·6; Sigma P3635) were double diluted over a range of 1600-

12·5 pg/0·1 ml (16-0·125 ng/ml) from a stock solution of 16 ng/ml buffer. A QC was prepared by diluting urine collected from an Indian rhinoceros during late pregnancy and stored at -20°C in 0·1 ml aliquots.

2.12.2 Coating of microtitre plates

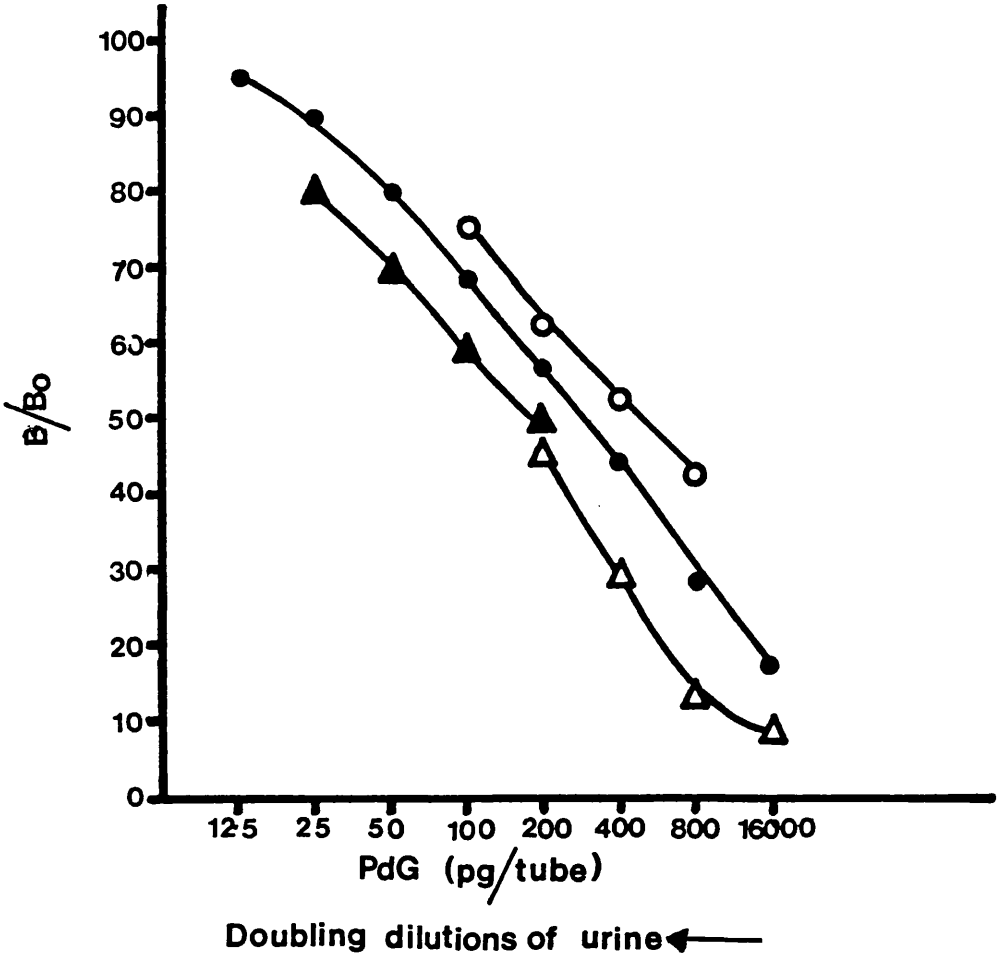
Wells on a microtire plate were coated with 0·2 ml PdG IgG (1:10,000) in sodium acetate buffer. Plates were incubated in a humid chamber overnight at 4°C. The plates were then emptied by inversion and each well filled with 0·4 ml PAS gel buffer, covered with polythene film and stored at 4°C, under which conditions they will remain stable for at least 6 months .

2.12.3 Assay protocol

PdG standards, diluted over the range 1600-12·5 pg/0·1 ml, and 0·1 ml PAS gel buffer (zeros), QCs and samples were pipetted into clean, glass test tubes. Enzyme conjugate (0·4 ml; 1:10,000) was added to each tube and the contents mixed throughly. Zeros (Z), standards (S1-S8), QCs and samples (UK) (0·2 ml each) were pipetted into duplicate wells in columns across the microtitre plate. Two wells remained empty to represent blanks (B). The plate was covered with polythene film and incubated at room temperature for 3 h in a humid chamber. The final assay layout on the microtitre plate is shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	S7	S7								
B	Z	Z	S8	S8								
C	S1	S1	QC	QC								
D	S2	S2	UK1	UK1								
E	S3	S3	UK2	UK2								
F	S4	S4	UK3	UK3								
G	S5	S5	UK4	UK4								
H	S6	S6	UK5	UK5	etc.							

Figure 2.6. Typical PdG assay standard curve with standards over the range 12·5-1600 pg. The curve is plotted as % bound against the log of the mass of PdG. The standard dose response curve for the PdG EIA (●—●) is compared with serial dilutions of urine from the black (o—o), white (▲—▲) and Indian (△—△) rhinoceroses during mid to late gestation.



After incubation, the plate was emptied by inversion, to separate the antibody-bound and unbound PdG, and washed four times with PAS gel buffer. Enzyme substrate (0·2 ml) was added to each well on the plate, and incubated at room temperature for 30 min. The reaction was terminated with 3 M sodium hydroxide solution (0·05 ml/ well), and absorbance was measured at 410 nm using a microplate reader. After subtracting blank values, the system transformed readings of optical density (O.D.) into ng/ml urine from a standard curve plotted from O.D. against concentration PdG. For each assay, a standard curve was constructed by hand, by plotting B/B_o against mass (pg) PdG, to determine the sensitivity of the assay at 90% binding.

2.12.4 Assay evaluation

A typical standard curve, obtained by plotting B/B_o against mass (pg) PdG on a logarithmic scale, is shown in Fig. 2.6. Serial dilutions of urine collected during late pregnancy in Indian, black and white rhinoceroses gave displacement curves parallel to that obtained with PdG standards (Fig. 2.6). The identification of a single peak of immunoreactivity in urine, collected from mid-late pregnant Indian, black and white rhinoceroses, co-eluting with the PdG standard on HPLC further validated this assay (see chapter 4).

The sensitivity of the assay, determined at 90% binding was 25 pg/tube or 10 ng/ml. The accuracy of the assay was determined by adding varying amounts of unlabelled PdG (1600-12·5 pg) to 0·1 ml volumes of black and white rhinoceros urine pools, containing low levels of endogenous hormone. The samples were assayed and means±s.e.m recoveries of added PdG were 101·5±1·3% and 107·8±3·2% respectively. Intra-assay precision was determined by repeated measurement of a sample prepared from pooled Indian rhinoceros urine on one plate. The intra-assay C of V was found to be 9·4% (n=30). Inter-assay precision was calculated by determining the C of V of measurement of a QC (which gave a value of approximately 45% bound) in each assay and was found to be 9·1% (n=29).

2.13 ENZYME-IMMUNOASSAY FOR 20α-DIHYDROPROGESTERONE

In order to measure 20α-DHP in this study, a heterologous, microtitre plate, EIA was developed, based on the procedure described by Meyer and Hoffmann (1987). The assay utilised a 20α-DHP antiserum and an enzyme label of 20α-DHP conjugated to horse radish peroxidase.

2.13.1 Sample preparation

Urine samples (0·05 ml) were hydrolysed with β-glucuronidase-aryl-sulphatase and extracted with diethyl ether, prior to assay for 20α-DHP. Procedural losses during the extraction were monitored by the addition of tracer amounts of [^{3}H]20α-DHP to each sample prior to extraction. Recoveries were 87·0±1·3% (mean±s.e.m, n=180), and individual recoveries were used to correct for procedural losses when calculating the final concentration of 20α-DHP in urine. Hydrolysis efficiencies were determined in triplicate by adding tracer amounts of [^{3}H]oestrone sulphate and [^{3}H]PdG respectively to 0·05 ml urine pool, hydrolysed and extracted as above. Recoveries were 81·0±2·4% and 78·2±1.5% (mean±s.e.m., n=24) respectively.

2.13.2 Assay reagents

For the assay of 20α-DHP, the assay buffer was tris buffer.

2.13.2.1 Preparation of coating antibody

The coating antibody was a non-specific sheep anti-rabbit gamma globulin (IgG), purified from sheep plasma using a Protein G sepharose 4 fast flow column (MAbTrap G; Pharmacia, Uppsala, Sweden) according to the method of Nilsson, Myhre, Kronvall and Sjögren (1982). Protein G binds only IgG and its subclasses, and is therefore effective in separating out IgA, IgM, IgD and albumin which may bind to other affinity gels. Plasma (2 ml) was centrifuged for 10 min and filtered through a 0.22 μm filter

(Waters). The filtrate was diluted with 2 ml binding buffer (0·2M sodium phosphate, pH 7·0) and applied to the column, previously equilibrated with binding buffer (30 ml). The sample was allowed to absorb into the gel, and unbound proteins were eluted with a further 30 ml binding buffer. The bound IgG was eluted from the column with elution buffer (15 ml; 1·0 M glycine-HCL, pH2·7), and the antibody fraction was collected into prepared test-tubes. The concentration of IgG in each fraction was measured by absorbance at 280 nm (Pye unicam SP-550 UV/Vis spectrophotometer; Philips). The fractions containing the highest concentrations of IgG were pooled, the final concentration recorded and aliquots stored at -20°C.

2.13.2.2 Preparation of 20α-DHP antibody

The antibody was donated by Dr. M.J.Peddie (Dept. Phys. and Pharm., Univ. Southampton). New Zealand white rabbits were injected intra-dermally with 250 µg of 20α-DHP conjugated at C3 to bovine serum albumen (BSA) through carboxymethyl oxime (CMO) (4-pregnen-20α-ol-3-one CMO:BSA; Steraloids Inc., Wilton, USA; Ref. Q3606; 28-30 moles steroid/mole BSA) in a 50:50 emulsion of 0·9% saline : complete Freund's adjuvant (Difco). Second and third booster, injections (100 µg each) were given at 6 and 8 weeks after the primary injection. The rabbits were bled from the ear vein at 10 weeks, and then re-immunized for subsequent bleeds. The blood was centrifuged, serum aliquoted into individual plastic vials and stored at
-20°C.

2.13.2.3 Preparation of enzyme conjugate

The enzyme label was horseradish peroxidase (HRP) conjugated to 20α-DHP using the modified mixed acid anhydride procedure of Liebermann, Erlanger, Beiser and Agate (1959).

HRP (2·5 mg; Type VI; Sigma P8375) was dissolved in 0·4 ml distilled water and cooled on ice to 0°C. Dimethylformamide

(0·375ml; Sigma) was added slowly to the enzyme solution whilst gently shaking by hand. Meanwhile, 20α-DHP (0·25 mg) was dissolved in 0·5 ml dimethylformamide in a small conical flask which was placed in a beaker surrounded by salt-ice. Methylmorphine (6·25 µl; Sigma) was added and the solution stirred on a magnetic stirrer at 4°C. Isobutyl-chloroformate (6·25 µl; Sigma) was then added and the solution stirred for 3 min before the combination of enzyme and hormone preparations. The solution was immediately adjusted to pH8·0 by the addition of 3 M sodium hydroxide solution, and stirred for 1 h at -15°C, adjusting the pH regularly during this period. The solution was stirred for a further 2 hours at 0°C prior to the addition of 10 mg sodium hydrogen carbonate and loading into dialysis tubing. The conjugate solution was dialysed for 48 h at 4°C against PBS (2·5 l; without azide), changing the buffer every 2-3 hours when possible. After dialysis, the solution was loaded onto a G25 chromatography column (Pharmacia) and the enzyme conjugate eluted with 200 ml dialysis buffer. Fractions (approximately 1 ml) were collected by hand and 5 µl of fractions 10-25 tested with enzyme substrate (0·25 ml; see below). Samples giving a strong and rapid colour reaction were pooled, diluted with glycerol (1:1) and stored in aliquots at -20°C.

Enzyme substrate was O-phenylenediamine (100 mg; 1,2 benzenediamine; Sigma P9029) dissolved in citric acid buffer (28 ml; Appendix), to which 0·025 ml of 30% hydrogen peroxide solution (BDH) was added immediately prior to use.

2.13.2.4 Preparation of standards and QCs

A stock solution of 20α-DHP standard (4-pregnen-20α-ol-3-one, MW= 316·5; Sigma P6288) was prepared at a concentration of 1 mg/ml absolute ethanol, and stored at -20°C. A second, less concentrated stock solution of 1 µg/ml buffer was prepared from the initial stock and stored at -20°C in aliquots of 16 µl. For assay, 10 ml buffer was added to this aliquot to make a solution of 1·6 ng/ml buffer

from which the assay standards were double diluted over the range 80-0·32 pg/0·05 ml (1600-6·4 pg/ml).

QCs were prepared from urine pooled from the post-oestrous phase of the cycle in the white rhinoceros. Urine samples were pooled prior to hydrolysis and extraction, the concentration of 20α-DHP was determined and the urine was divided into two portions, one of which was diluted to contain levels of 20α-DHP which gave 75% displacement (QCL), and the other to give 35% displacement (QCH).

2.13.3 Coating of microtitre plates

For the coating of each microtite plate, 0.1 mg sheep anti-rabbit IgG were diluted with 25 ml carbonate "coating" buffer (Appendix) and 0·25 ml pipetted into each well. The plates were incubated overnight at room temperature after which they were emptied by inversion and each well post-coated with 0·25 ml tris "storage" buffer (Appendix) containing 0.1% sodium azide under which conditions the plate will remain stable in excess of 6 months at 4°C. The plates were sealed with plastic film and stored in airtight polythene boxes.

2.13.4 Assay protocol

Immediately prior to use, pre-coated plates were emptied by inversion and rinsed 3 times with 0.005% Tween (Sigma) solution, and blotted dry. 20α-DHP standards (S1-S9), diluted over the range 0·32-80 pg/0·05 ml, 0·05 ml buffer (zeros; Z), QCs and samples (UK) were pipetted into duplicate wells in rows down the microtitre plate. Enzyme conjugate (0·1 ml), at a working dilution of 1:30,000, was added to each well using a multi-channel pipette. The titre of the anitserum, at an enzyme conjugate dilution of 1:30,000, was determined as 1:150,000, and 0·1 ml antiserum was added to each well at this working dilution. Duplicate blank wells (without antiserum) were included to monitor non-specific binding (NSB) of enzyme conjugate to the sheep anti-rabbit IgG. Plates were covered

with plastic film and incubated overnight at 4°C in the dark. The final layout on the microtitre plate is shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NSB	S3	S7	QCL								
B	NSB	S3	S7	QCL								
C	Z	S4	S8	UK1								
D	Z	S4	S8	UK1								
E	S1	S5	S9	UK2								
F	S1	S5	S9	UK2								
G	S2	S6	QCH	UK3								
H	S2	S6	QCH	UK3	etc.							

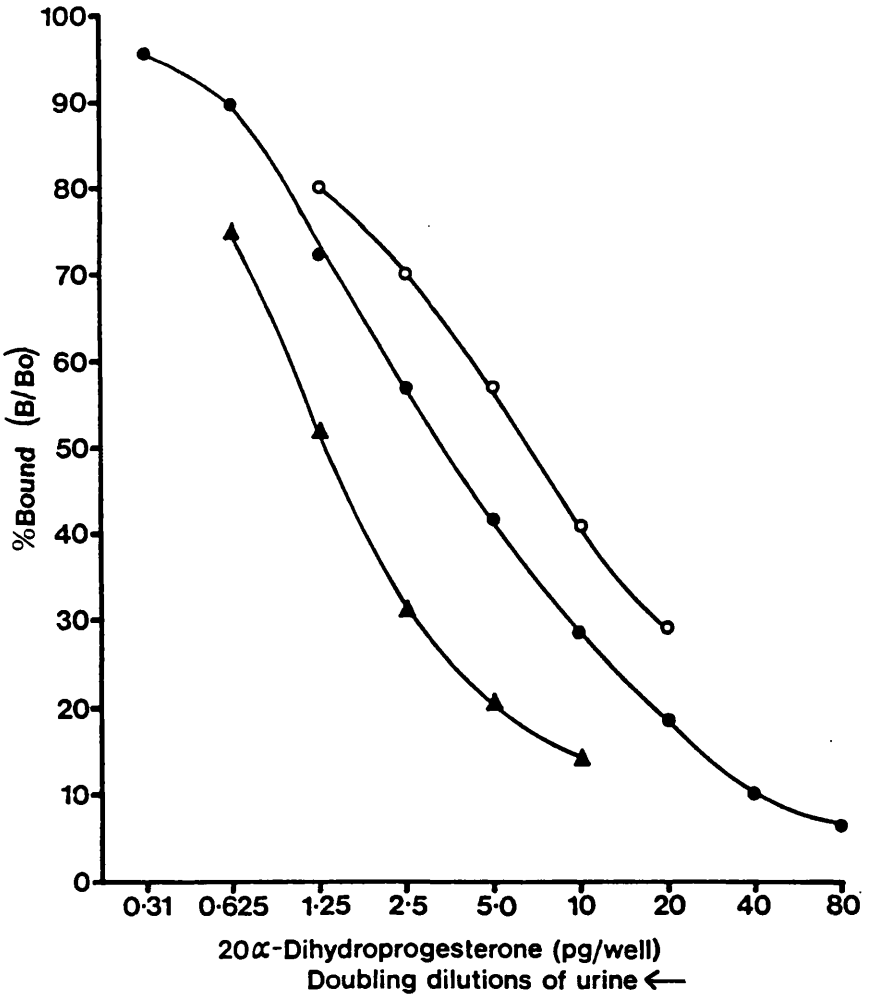
The plates were emptied by inversion, to remove the unbound reagents, and washed 3 times with 0·005% Tween solution and blotted dry. Enzyme substrate was added to each well (0·25 ml), and the plates were covered with plastic film and incubated in the dark at 4°C for 45 min. The reaction was stopped by the addition of 3 M sulphuric acid (0·05 ml) to each well. Absorbance was measured at 494 nm, with a reference of 620 nm, on an micro-plate reader.

2.13.5 Assay validation

A typical standard curve, obtained by plotting % bound B/B_0 against mass (pg) 20α-DHP on a logarithmic scale, is shown in Fig. 2.7. Values of 20α-DHP in samples were calculated from the standard curve, initially as pg/well, and transformed to ng/mgCr. Serial dilutions of hydrolysed and extracted urine from both black and northern white rhinoceros during the post-oestrus phase of the ovarian cycle gave displacement curves parallel to that obtained with the 20α-DHP standards (Fig. 2.7).

The average of the assay, determined at 90% binding, was 0.65 pg/well or 13 pg/ml. The accuracy of the assay was determined by adding varying amounts of unlabelled 20α-DHP (0·32-80 pg) to 0·05 ml

Figure 2.7. Typical 20α-DHP assay standard curve with standards over the range 0·31-80 pg. The curve is plotted as % bound against the log of the mass of 20α-DHP. The standard dose response curve for the 20α-DHP EIA (●—●) is compared with serial dilutions of urine from the black (o—o) and the white rhinoceros (▲—▲) during the post-oestrus period.



of hydrolysed and extracted urine pool from black and northern white rhinoceroses containing low levels of endogenous hormone. The samples were assayed and the mean±s.e.m. recoveries of 20α-DHP were 100·7±0·9% (n=8) and 101·5±1·2% (n=8) respectively. Intra-assay precision was determined by repeated measurement of a sample prepared from a pool of white rhinoceros urine which had been hydrolysed and extracted, on a single plate. The intra-assay C of V was found to be 7.4% (n=30). Inter-assay precision was calculated by determining the C of V of measurement of the same QCs in each assay, and was found to be 7·6% for a high value quality control and 6·9% for a low value quality control (n=21).

Cross reactions of many steroids with the 20α-DHP antiserum in the enzyme immunoassay system were determined. Serial dilutions of steroids (ng quantities) were assayed and the cross reactivity determined at the level of 50% inhibition of binding. The specificity of the antiserum is shown in Table 2·2. The major cross reacting substances were 20α-hydroxy-5β-pregnan-3-one(22%), 5β-pregnane-3α,20α-diol (9·3%), 5-pregnen-3β,20α-diol (6·4%), 20α-hydroxy-5-pregnen-3-one (3%) and progesterone (2·4%). All other steroids tested cross reacted less than 1% by mass.

2.14 ENZYME-IMMUNOASSAY FOR TOTAL OESTROGENS

The qualitative enzyme-immunoassay for total oestrogens was performed according to the method of Möstl, Meyer, Bamberg and Von Hegel (1987).

2.14.1 Assay materials and reagents

The assay buffer was tris buffer. The antiserum used in this assay was raised in rabbits and was donated by Dr. H.H.D.Meyer (Institüt für Physiology, Uni. Münich, W.Germany). The antibody was stored in 0·2 ml aliquots at 1:1,000 dilution at -20°C. Using oestrone as a reference, the major cross reacting steroids were oestradiol-17β (161%), oestradiol-17α (63%), oestriol (7·2%),

Table 2.2. Specificity of the 20α-DHP antiserum as determined by enzyme immunoassay. Figures for the cross reactivity were determined at 50% inhibition of binding.

steroid	cross reaction %
20α-hydroxy-4-pregnen-3-one	100·0
20α-hydroxy-5β-pregnan-3-one	22·0
5β-pregnane-3α,20α-diol	9·3
5-pregnene-3β,20α-diol	6·4
20α-hydroxy-5-pregnen-3-one	3·0
4-pregnene-3,20-dione	2·4
5α-pregnane-3α,20α-diol	0·9
20α-hydroxy-5α-pregnan-3-one	0·8
17α-hydroxy-4-pregnene-3,20-dione	0·5
5β-pregnan-3β,20α-diol	0·5
5α-pregnan-3α,20β-diol	0·3
3β-hydroxy-5-pregnen-20-one	0·2
1,3,5 (10) estratriene-3,17α-diol	<0·1
20α-hydroxy-4-pregnen-3-one	<0·1
17α,20α-dihydroxy-4-pregnen-3-one	<0·1
17α,20β-dihydroxy-4-pregnen-3-one	<0·1
11β,17α,21-trihydroxy-4-pregnen-3,20-dione	<0·01
5β-pregnan-3α,20β-diol	<0·01
5α-pregnan-3,20-dione	<0·01

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 by the method of Meyer and Hoffmann (1987). The label was stored in 0·12 ml aliquots at a dilution of 1:4 at -20°C. Enzyme substrate was p-nitrophenyl phosphate disodium (0·16 g) dissolved in 28 ml diethanolamine buffer.

Oestrone standards were double diluted over a range of 40-0·31 pg/0·05 ml buffer (800-6·2 pg/ml) from a stock solution of 0·8 ng/ml. A QC was prepared by diluting oestrone standard to give a concentration equivalent to 50% binding in the assay.

Wells on a microtitre plate were coated with sheep anti-rabbit IgG as for 20α-DHP assay.

2.14.2 Assay protocol

Immediately prior to use, pre-coated plates were emptied by inversion and rinsed 3 times with 0.005% Tween solution, and blotted dry. Oestrone standards (S1-S8), diluted over the range 0·32-40 pg/0·05 ml, 0·05 ml buffer (zeros; Z), QCs and samples (UK) were pipetted into duplicate wells in rows down the microtitre plate. Enzyme conjugate (0·1 ml), at a dilution of 1:400, was added to each well using a multi-channel pipette followed by antiserum at a working dilution of 1:70,000. Duplicate blank wells (without antiserum) were included to monitor non-specific binding (NSB) of enzyme conjugate to the sheep anti-rabbit IgG. Plates were covered with plastic film and incubated overnight at 4°C in the dark. The final layout on the microtitre plate is shown overleaf.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NSB	S3	S7	UK2								
B	NSB	S3	S7	UK2								
C	Z	S4	S8	UK3								
D	Z	S4	S8	UK3								
E	S1	S5	QC	UK4								
F	S1	S5	QC	UK4								
G	S2	S6	UK1	UK5								
H	S2	S6	UK1	UK5	etc.							

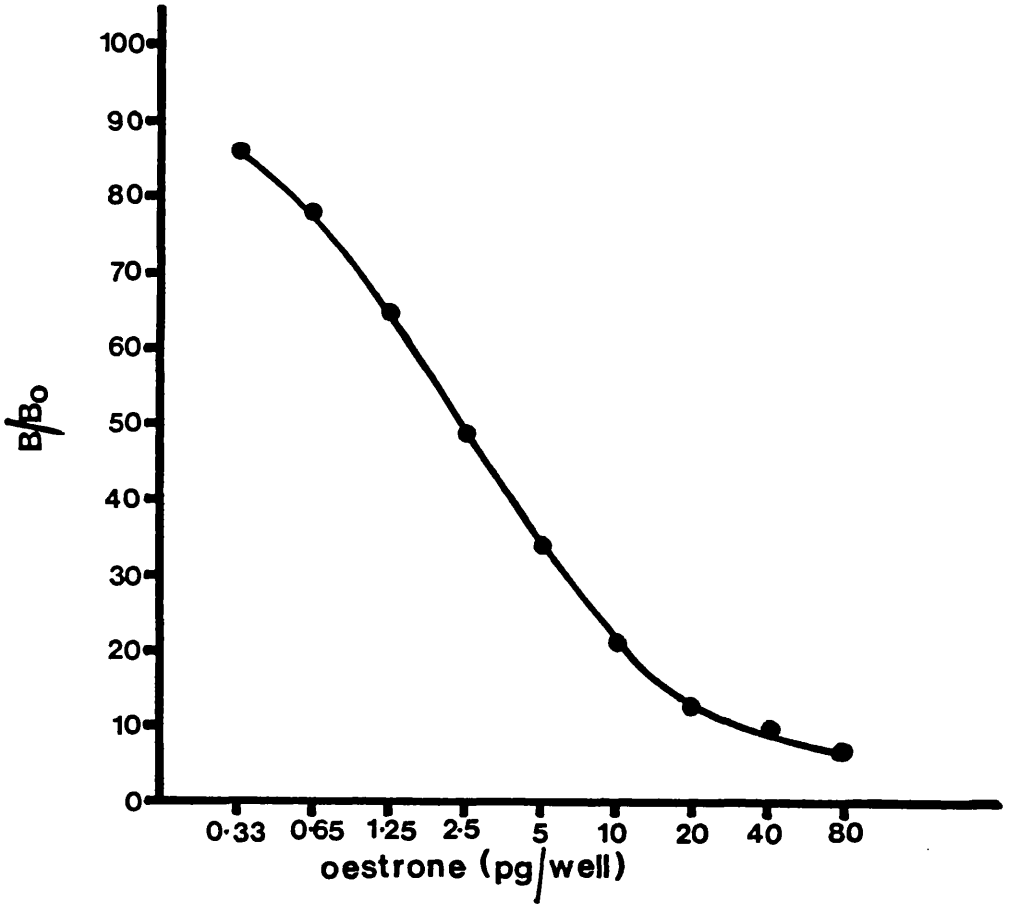
The plates were emptied by inversion, to remove the unbound reagents, and washed 3 times with 0·005% Tween solution and blotted dry. Enzyme substrate (0·25 ml) was added to each well, the plates were covered with plastic film and incubated at 37°C for 3 h, after which absorbance was measured at 410 nm, with a reference of 620 nm, on an micro-plate reader.

2.14.3 Assay evaluation

A typical standard curve, obtained by plotting B/B_0 against mass (pg) oestrone on a logarithmic scale, is shown in Fig. 2.8. Semi-quantitative values of oestrogen metabolites were calculated from the standard curve, initially as pg/well, and transformed to ng/ml.

The sensitivity of the assay, determined at 90% binding was 0·3 pg/tube or 6 pg/ml. Intra-assay precision was determined by repeated measurement of a sample prepared from a pool of northern white rhinoceros urine, which had been hydrolysed and extracted, on one plate. Intra-assay C of V was found to be 11.8% (n=30). Inter-assay precision was calculated by determining the C of V of measurement of a QC (which gave a value of approximately 45% bound) in each assay and was found to be 8.6% (n=17).

Figure 2.8. Typical total oestrogen assay standard curve with oestrone standards over the range 0·33–80 pg. The curve is plotted as % bound against the log of the mass of oestrone.



CHAPTER 3

METABOLISM OF OESTRADIOL AND PROGESTERONE IN A WHITE RHINOCEROS

CHAPTER 3

METABOLISM OF OESTRADIOL AND PROGESTERONE IN A WHITE RHINOCEROS

3.1 INTRODUCTION

The ovarian cycle of the Indian rhinoceros has been described using urinary hormone analysis. Studies in the Indian species have indicated that PdG is the major urinary metabolite of progesterone, whilst circulating oestradiol-17β is excreted predominately as oestrone sulphate (Kassam and Lasley, 1981; Kasman *et al.*, 1986). Although the measurement of PdG immunoreactivity is informative in the determination of mid-late pregnancy in both the black and the white rhinoceros (Hodges and Green, 1989), attempts to describe ovarian function using the same measurements in the African species of rhinoceros have been unsuccessful (Ramsay *et al.*, 1987; J.E.Hindle and J.K.Hodges, unpublished observations). Thus no method exists for monitoring the ovarian cycle in the black and the white rhinoceros and the primary metabolites of oestradiol and progesterone are not known for these species.

There are a number of possible explanations for the lack of success in monitoring ovarian function by urinary steroid hormone analysis in the African species of rhinoceros.

1. The African species may favour the biliary route of excretion, with a much larger proportion of steroid hormone metabolites being voided via the faeces, rather than into the urine.
2. The African species may excrete steroid homone metabolites into the urine, and these metabolites may be the same as in the Indian rhinoceros (ie. PdG and oestrone conjugates), but at levels below the sensitivity of existing assays.
3. Qualitative differences may exist between species in the metabolism of ovarian steroid hormones. Thus, the principal metabolites of progesterone and oestradiol excreted by the African

rhinoceroses may be different from those previously measured in the Indian rhinoceros.

The aim of this experiment was to examine these possibilities by carrying out a radiometabolism study to investigate the metabolic fate of exogenously administered ^{14}C-labelled oestradiol-17β and progesterone in a white rhinoceros. Specific objectives were to identify the major metabolites of oestradiol-17β and progesterone and to determine the relative abundance of these metabolites in urine and faeces. It was hoped that these results would identify the hormone metabolites whose measurement would most accurately reflect ovarian function in the African rhinoceroses, to provide methods of monitoring oestrous cycles and pregnancy in these species.

3.2 MATERIALS AND METHODS

3.2.1 Animal and housing

For this study, the experimental animal was an adult female southern white rhinoceros (*Ceratotherium simum simum*). The animal was estimated to be 15 years of age when captured in the Umfolozi Game Reserve, South Africa in 1976, and had been housed in the white rhinoceros exhibit at Whipsnade Wild Animal Park, Zoological Society of London, for 12 years prior to the experiment. It was thus estimated to be 27 years old at the time of the study. The animal was chosen for the experiment as it was to be euthanised, its last two calves being still born with congenital disorders. No sign of oestrus had been observed since the birth of the second still born calf 8 months previously.

The rhinoceros was housed alone, without bedding, in a stable with a concrete floor and a single outlet drain in the centre of the enclosure. The animal was allowed unlimited access to water and hay throughout the experimental period.

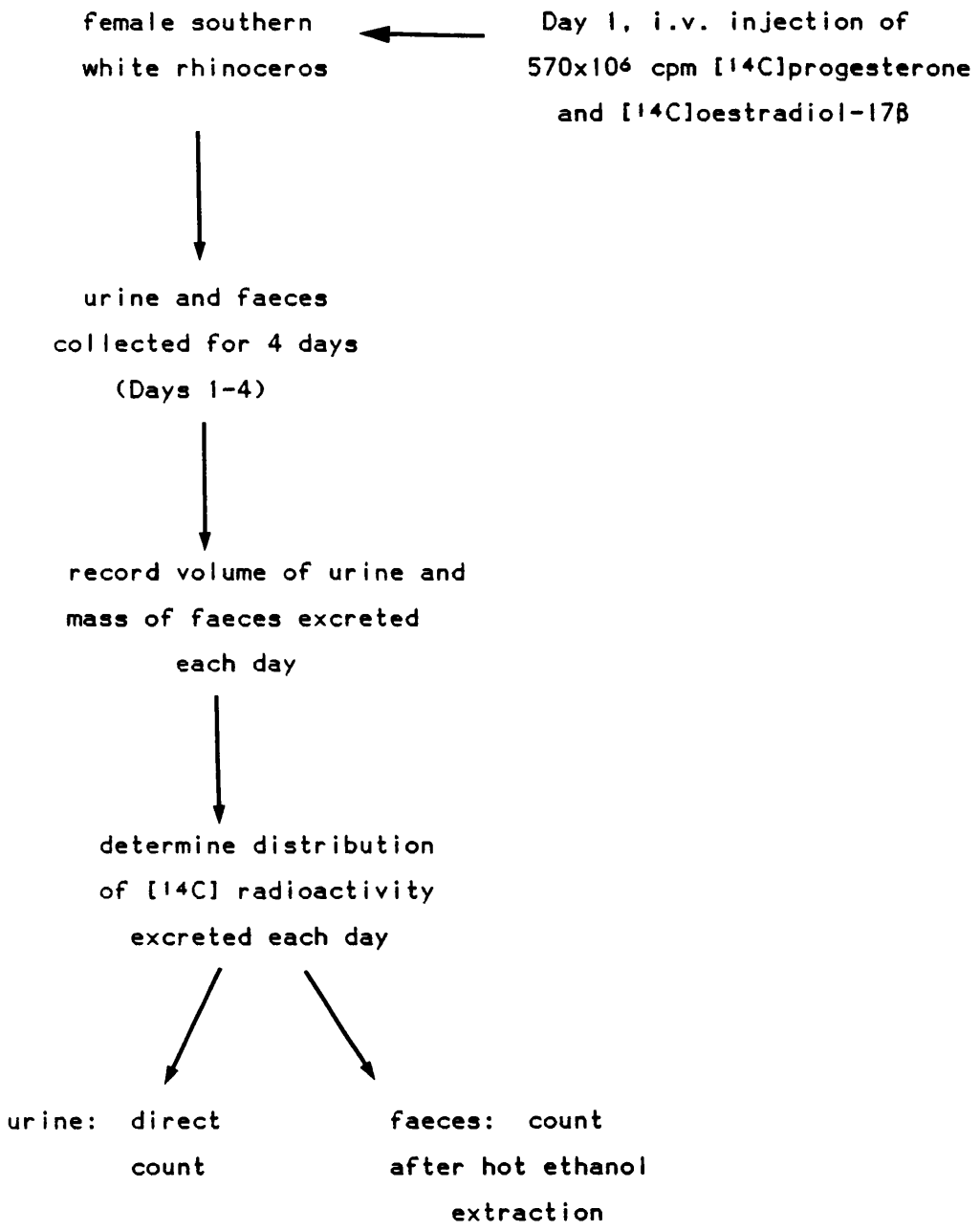
3.2.2 Preparation and injection of radiolabel

* [4-^{14}C]oestradiol-17β (sp. act. 56 mCi/mmol) and [4-^{14}C]progesterone (sp. act. 56 mCi/mmol) were obtained commercially (Amersham International plc.). The radiochemical purity of each steroid was determined by the manufacturers by HPLC, and was quoted as 98·0% for [4-^{14}C]oestradiol-17β and 95·4% for [4-^{14}C]progesterone. Two 1·0 ml aliquots of toluene:ethanol (1:1, v/v), containing 50 µCi [4-^{14}C]progesterone each, were evaporated to dryness under nitrogen. Each aliquot of radiolabel was redissolved overnight in 0·5 ml absolute ethanol, and the two aliquots were combined to give 1·5 ml ethanol including a rinse. The radioactivity in a small portion (10 µl) was counted to gain an estimate of the radioactivity present. Similarly, two 2·5 ml portions of toluene:ethanol (1:1, v/v), containing 50 µCi [4-^{14}C]oestradiol-17β each, were evaporated to dryness and the radiolabel redissolved in two 1·0 ml volumes of absolute ethanol overnight. The aliquots were combined to give 2·5 ml including a rinse. The radioactivity in a small portion (10 µl) was counted. The two volumes of ethanol were combined to give 4·5 ml which included a rinse and contained a total of 570·13x10^6 cpm. The ethanol was evaporated under nitrogen and the radiolabelled steroids redissolved in 1·0 ml absolute ethanol, and stored at -20°C.

Unlabelled steroids were also prepared for injection with the radiolabel, to act as carriers. Crystals of oestradiol-17β (1 mg) were dissolved in 0·5 ml absolute ethanol and 30% (w/v) propylene glycol in water (30% PG; 0·5 ml) was added. A 10 mg mass of progesterone was also dissolved in 0·5 ml absolute ethanol to which 0·5 ml 30% PG was added. The steroids were combined with a further 3·0 ml of 30% PG, to give a volume of 4·0 ml which was added to the 1·0 ml ethanol containing the radiolabelled steroids. Prior to injection the volume was increased to 10·0 ml, by the addition of 5·0 ml 30% PG, and the solution was filtered through a 0.22 µm (Millipore) filter unit.

* The high levels of ^{3}H-labelled steroid required for this experiment prevented the injection of two different radiolabels.

Figure 3.1. Protocol for the i.v. injection of radiolabelled oestradiol-17β and progesterone in an adult female southern white rhinoceros, and the time course of sample collection.



At 10·23 h on the morning of Day 1 , the animal was anaesthetized with 1.25 ml etorphine hydrochloride (Immobilon, C-vet, Bury St. Edmunds, Suffolk, UK) and monitored whilst under the anaesthetic. At 11·10 h the radiolabel solution was injected into an ear vein. After 10 min the animal was revived with 2 ml diprenorphine hydrochloride (Revivon; C-vet) and was standing within 5 min.

3.2.3 Collection and storage of samples

The protocol for the experiment is shown in Fig. 3.1. All urine and faeces excreted were collected separately each day for 4 days (Days 1-4) following the injection of radiolabel on Day 1. Urine was collected in a receptacle beneath the external outlet of the enclosure's single drain. The urine was mixed thoroughly by pouring between buckets, and the total volume excreted on each day of the experiment was determined, by measuring the volume collected in a 1 litre measuring cylinder. Portions (1 litre) were stored in plastic bottles with sodium azide (final concentration 0·1%, w/v), at 4°C until the end of the experiment. The urine was then frozen and stored at -20°C.

To prevent contamination with urine, faeces were removed from the enclosure within 2 min of voiding. Faeces was mixed with a garden fork and kneaded manually between strong, plastic bags. The total weight of faeces excreted during each day was determined by weighing bags on a 10 kg hook scale. Portions (1 kg) were frozen immediately and stored at -20°C.

3.2.4 Distribution of radioactivity

When counting radioactivity in undiluted urine or after reconstitution in ethanol, the counting efficiency was low due to a quenching effect. Therefore, when counting radioactivity throughout the study, quench corrections were made by internal standardization with [^{14}C]toluene. The liquid scintillation counter

provided a value for the degree of quench in each sample (s), and the efficiency (%) of the counting procedure for this sample was determined by comparison to a quench curve constructed from ^{14}C-quenched standards (G & G Chemical Services Ltd., Ascot, UK) as shown in Fig. 3.2. The average efficiency of counting, after quench correction, was 80·5%.

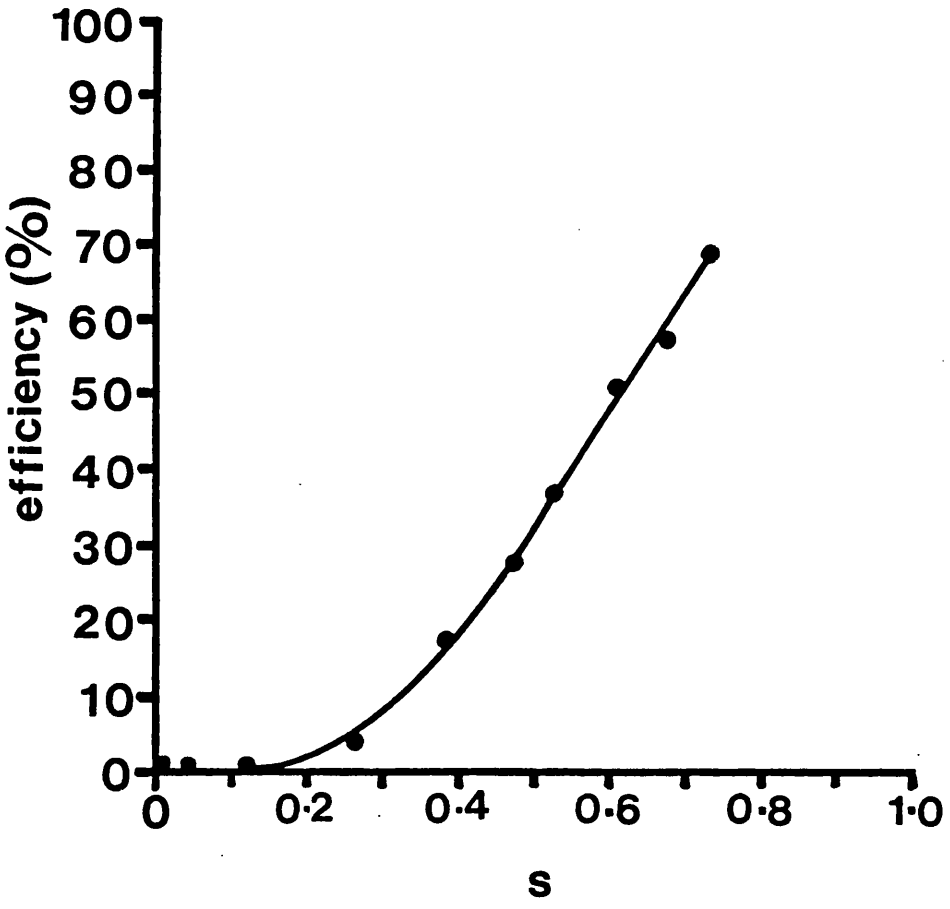
3.2.4.1 Urine

Three volumes of urine (1, 0·5 and 0·25 ml) collected on each day of the experiment, were placed in separate scintillation vials in duplicate, with 10ml scintillation fluid (Scintran; BDH, Poole, UK). Radioactivity was counted for 10 min in a Beckman LS200 liquid scintillation counter. The amount of radioactivity in 1·0 ml of urine collected on each day was calculated, and the radioactivity recovered per day was determined by multiplying the amount of radioactivity in 1·0 ml urine by the total volume collected on each of the 4 days.

3.2.4.2 Faeces

Radioactivity was extracted from 250 g (wet weight) aliquots of faeces, collected on each day of the experiment, by hot ethanol extraction (see section 2.3.3 for method). Each aliquot of faeces displaced a volume of approximately 50 ml, so 500 ml (10 vol) and 250 ml (5 vol) ethanol were used for the first and second extractions respectively. The ethanol extracts were then refluxed for 2 h at 70°C. Purified extracts were concentrated on a Buchi rotary evaporator (Rotavapor-R) and the residue, containing the radiolabelled steroids, reconstituted in absolute ethanol (200 ml). An aliquot (1 ml) of each extract was evaporated to dryness and the steroids reconstituted overnight in 10 ml PBS. Portions (1, 0·5 and 0·25 ml) of each reconstituted extract were added to scintillation vials in triplicate, with 10 ml scintillation fluid. The radioactivity in each portion was determined by counting for 10 min.

Figure 3.2 Quench curve constructed from [^{14}C] quenched standards and internal standardization with [^{14}C]toluene (s) on a Beckmann LS2000 liquid scintillation counter. When counting radioactivity in urine and faecal extracts, an s (quench) value was obtained for each sample and the efficiency of counting determined. All levels of radioactivity in samples were then corrected for quench.



From each faecal sample, approximately 83% of the total radioactivity recovered by extraction was present in the initial extract, with 17% in the second. It was therefore concluded that the majority of extractable radiolabel had been removed from the faeces and the residual vegetable matter was not analysed. The efficiency of the extraction procedure was not monitored by the addition of a known activity of [^{3}H] radiolabel. Such recoveries would be artificially high as the label would not be incorporated into the vegetable matter, as is the case with steroids excreted via the bile into the faeces.

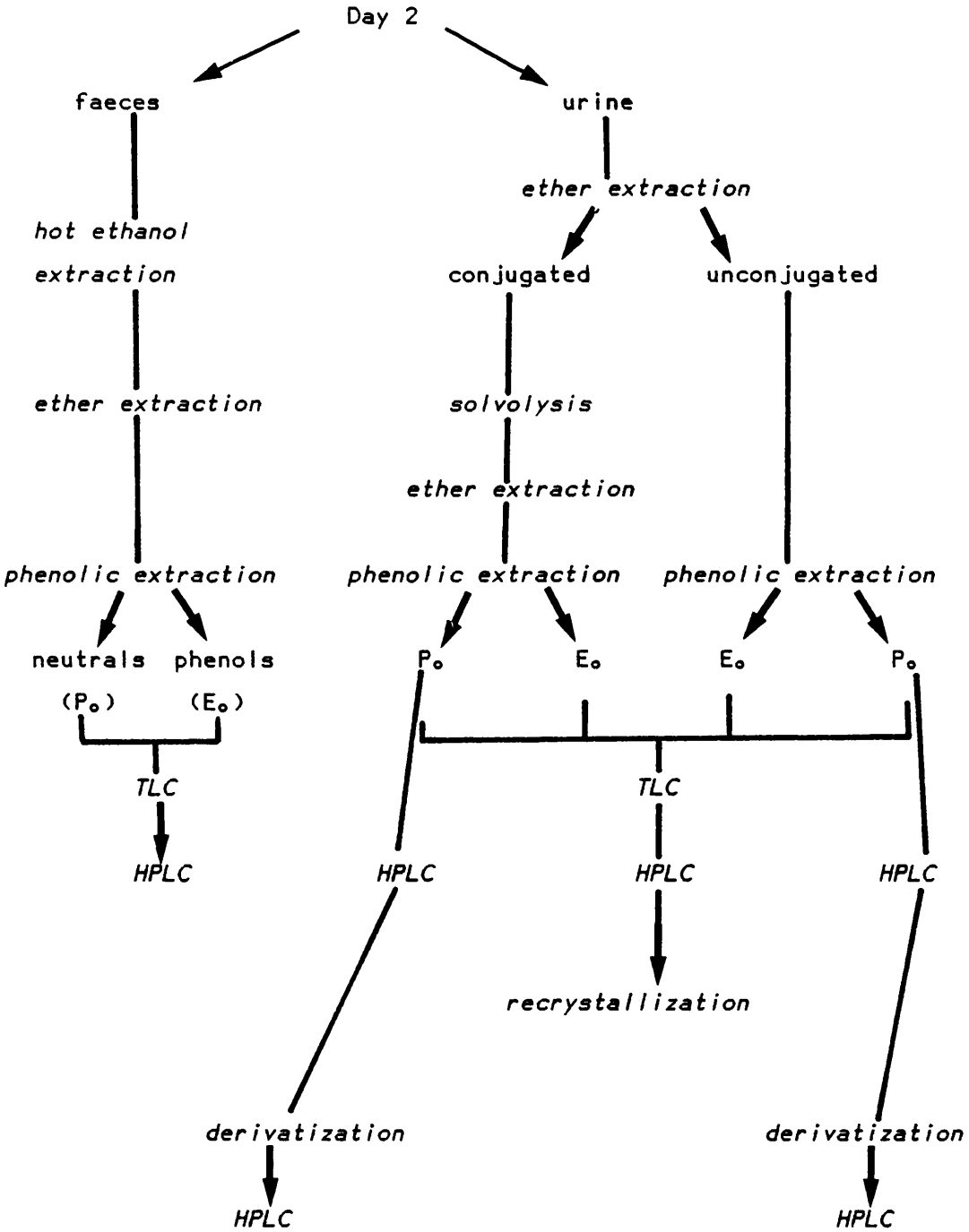
3.2.5 Separation of conjugated and unconjugated steroids

After determining the distribution of radioactivity in urine and faeces collected on Days 1-4 of the study, it was found that the majority of injected radiolabel was recovered in the urine and faeces excreted on Day 2 (see Table. 3.7). All subsequent analysis was carried out on the samples collected on this day. The scheme for the separation and identification of oestradiol-17β and progesterone metabolites in Day 2 urine and faeces is shown in Figure 3.3.

3.2.5.1 Urine

Unconjugated steroids were extracted from duplicate 50 ml portions of urine with 300 ml freshly distilled diethyl ether by shaking vigourously by hand in a separating funnel. The contents were left to separate for 10 min and the aqueous fraction (containing the conjugated steroids) was removed. The ether was evaporated to dryness on a rotary evaporater and the residue redissolved in absolute ethanol (50 ml). The radioactivity in duplicate 1·0 ml portions of aqueous (conjugated steroids) and solvent (unconjugated steroids) phases was determined by counting for 10 min with 10 ml scintillation fluid . Procedural losses were monitored in separate extractions by the addition of trace amounts of [^{3}H]progesterone to 50 ml rhinoceros urine pool (containing no

Figure 3.3. Scheme for the separation and identification of oestrogen and progesterone metabolites in urine and faeces. The analysis was carried out on material collected 2 days after i.v. injection of 570×10^6 cpm ^{14}C-labelled oestradiol-17β and progesterone.



endogenous radioactivity), in which recoveries of 82·9±4·6% (mean±s.e.m, n=7) were obtained. The procedure was repeated after the addition of [^{3}H]oestrone sulphate (Amersham International, Bucks., UK) and [^{3}H]pregnanediol glucuronide (Dr P.Samarajeewa, University College, London) to separate 50 ml portions of rhinoceros urine pool (containing no endogenous radioactivity). Less than 3% of the steroid conjugates added were extracted into the solvent by the extraction procedure. The unconjugated fraction was retained.

Having separated conjugated and unconjugated radiolabelled steroids in the urine, acid solvolysis was used to hydrolyse the steroid conjugates present (as described in section 2.3.6). Volumes of the aqueous fraction (50 ml), containing conjugates remaining after ether extraction as above, were placed in a large conical flask and solvolysed for 24 h at 37°C. The contents of the flask were transferred to a separating funnel, and the solvent removed. The remaining aqueous phase was re-extracted and solvent fractions were pooled, washed with water and evaporated to dryness on a rotary evaporator. The residue was re-dissolved in absolute ethanol (50 ml). Duplicate 1 ml portions of the solvent and aqueous residue were added to a scintillation vial with 10 ml scintillation fluid, and the radioactivity counted.

Residual radioactivity in the aqueous phase (5.2 ± 2.4%, n=7) was not analysed but could possibly represent 17-sulphated metabolites, unhydrolysed by solvolysis. The efficiency of the hydrolysis for glucuronides, monitored by repeating the procedure with phosphate buffer (50 ml) containing trace amounts of [^{3}H]pregnanediol glucuronide was 91·9±3·7% (n=7). The hydrolysed conjugate fraction was retained.

3.2.5.2 Faeces

Ethanolic extracts of faeces were evaporated to dryness and the residue reconstituted in 10 ml phosphate buffered saline and extracted with 100 ml freshly distilled diethyl ether. The ether

was evaporated to dryness on a rotary evaporator and the residue redissolved in 50 ml ethanol. Duplicate 1 ml portions were added to scintillation vials with 10 ml scintillation fluid and the radioactivity counted. Since 93% of the radioactivity present in the original faecal extract was now present in the ether extract (associated with unconjugated steroids), further analysis was limited to this fraction.

3.2.6 Separation of phenolic and neutral steroids

3.2.6.1 Urine

Phenolic and neutral compounds in the hydrolysed conjugated and unconjugated fractions were separated by phenolic extraction (see section 2.3.4 for method). Ethanolic extracts containing unconjugated and hydrolysed conjugated steroids in urine and unconjugated steroids in faeces were dried and redissolved in 10 ml petroleum ether prior to the separation procedure. Neutral and phenolic fractions were evaporated to dryness on a rotary evaporator, and the radiolabelled steroids re-dissolved in absolute ethanol (10 ml). Duplicate portions (1 ml) of each fraction and the aqueous residue were added to scintillation vials with 10 ml scintillation fluid and radioactivity counted. The entire procedure was repeated on 7 separate occasions. Phenolic and neutral extracts (in ethanol) were evaporated to dryness, the residue reconstituted in 250 ml diethyl ether and dried over excess, anhydrous sodium sulphate for 12 h.

The specificity of the phenolic extraction procedure used was monitored by the addition of trace amounts of radiolabelled progesterone and oestradiol-17β to separate 10 ml volumes of petroleum ether. Typical proportions of recovered [^{3}H]progesterone (93·9% of total added) in each of the neutral and phenolic extracts were 95·7% and 3·2% respectively, with less than 2% of the radiolabel remaining unextracted in the residual portion. Of the [^{3}H]oestradiol-17β recovered (91·0% of the total added) 14·8% was

extracted into the neutral portion whilst 84·0% was present in the phenolic extract with less than 2% in the residual portion.

After 12 h, the solid sodium sulphate was removed from the ether by fitration, and all separate neutral and phenolic extracts were combined. The diethyl ether was evaporated to dryness on a rotary evaporator, and the steroids were redissolved in 50 ml absolute ethanol. A portion (20 ml) of the ethanol was taken for TLC and the remainer (30 ml) was taken for HPLC.

3.2.6.2 Faeces

Ethanolic extracts were evaporated to dryness and the residue reconstituted in 10 ml petroleum ether before phenolic extraction. Duplicate portions (1 ml) of neutral and phenolic extracts, and aqueous residue, were added to scintillation vials with 10 ml scintillation fluid and the radioactivity counted for 10 min. Ethanolic extracts were evaporated, the residue reconstituted in 250 ml diethyl ether and dried over excess, anhydrous sodium sulphate for 12 h. The solid matter was removed from the ether by filtration, and all neutral and phenolic extracts were combined. The diethyl ethyer was evaporated to dryness on a rotary evaporator, and the steroids redissolved in 50 ml absolute ethanol. A portion (20 ml) of the ethanol was taken for TLC and the remainder (30 ml) was taken for HPLC.

3.2.7 Chromatographic separation and identification of steroids

3.2.7.1 Thin layer chromatography.

Thin layer chromatography (TLC) was used for the preliminary separation of radiolabelled urinary steroid metabolites, to indicate which standards would be most informative to run on HPLC. Neutral and phenolic extracts from urine and faeces were evaporated to dryness on a rotary evaporator and steroids were redissolved in 1·5 ml freshly distilled diethyl ether.

TLC plates were prepared in duplicate for neutral and phenolic steroids separately by spotting extracts (0·5 ml) and standards (1 µg in 0·5 ml diethyl ether) on the origin of the plate. Neutral steroids were separated with a solvent of toluene:ethyl acetate (60:40, v/v), and phenolic steroids were separated with a solvent of cyclohexane:ethyl acetate (65:35, v/v) as described in section 2.4. Plates were divided into two portions, one containing separated radiolabelled steroids and one containing standards. Standards were developed and the position and Rf value of each steroid recorded. Strips (0·5 cm in height) of plate containing radiolabelled steroids were placed in scintillation vials with 10 ml scintillation fluid. Vials were left to stand for 3 h to equilibrate before the radioactivity in each was counted for 10 min to determine the position and Rf value of radiolabelled steroids.

3.2.7.2 High performance liquid chromatography.

Separations were achieved using a Lichrosorb silica 60 column as described in section 2.5. For faecal steroids 2 HPLC runs were completed for both neutral and phenolic steroids (according to the methods in sections 2.5.2 and 2.5.3 respectively). For the analysis of urine, 4 HPLC runs were carried out on both the conjugated and unconjugated phenolic steroid fractions and for each of the neutral steroid fractions (unconjugated and conjugated).

Neutral and phenolic extracts from urine and faeces were evaporated to dryness on a rotary evaporator. Neutral steroids were redissolved in 4·0 ml of a mixture of chloroform and *n*-hexane (70:30, v/v) and a further 0·5 ml solvent, containing approximately 60,000 cpm each of [^{3}H]progesterone, [^{3}H]20α-DHP and [^{3}H]pregnanediol was added. Aliquots of 1 ml (n=4) were injected onto the HPLC. Phenolic steroids were redissolved in 4·0 ml of a mixture of chloroform and *n*-hexane (50:50, v/v), and a further 0·5 ml solvent, contining approximately 60,000 cpm each of [^{3}H]oestrone, [^{3}H]oestradiol-17β and [^{3}H]oestradiol-17α was added. Aliquots of 1 ml (n=4) were injected onto the HPLC.

Fractions (1 ml) were collected over 30 min, the solvent evaporated to dryness in a vacuum centrifuge. Residues were redissolved in 1 ml ethanol and duplicate portions (0·1 ml) of each fraction were added to a scintillation vial with 3 ml scintillation fluid and ^{3}H and ^{14}C radioactivity counted in each on a tricarb liquid scintillation analyser (Packard). Using a spectrum analyser, calibrated in keV, the region 0-18.6 keV (^{3}H E_{max}=18.6 keV) was used to measure ^{3}H radioactivity and the region 0-156 keV (^{14}C E_{max}=156 keV) used to measure ^{14}C radioactivity. By considering the composite spectrum obtained, corrections were made for ^{14}C to ^{3}H crossover by the scintillation analyser which also provided a $^{3}H/^{14}C$ ratio value for each fraction. The efficiency of counting ^{14}C-label was 85%, whilst the efficiency of counting ^{3}H was 54%.

HPLC elution fractions containing ^{14}C-labelled neutral steroids in urine, co-eluting with ^{3}H-labelled standards, were combined and divided into two portions. Steroids in one portion underwent derivatization, whilst those in the other portion were recrystallized. ^{14}C-labelled phenolic steroids were combined and underwent recrystallization only. Due to insufficient radiolabel, ^{14}C-labelled steroids in faeces were not analysed further.

3.2.7.3 Derivatization.

^{14}C-labelled neutral steroids in HPLC fractions identified by coelution with ^{3}H standards were derivatized by selective reduction of the 3-oxo groups (n=2). This method of derivatization was chosen as it produces good yields of related 3-hydroxysteroids, with the equitorial isomer being formed almost exclusively. The ketone groups at positions 11, 12, 17 and 20 remain unchanged which prevents the formation of many different compounds which would complicate the HPLC profile obtained. HPLC fractions were evaporated to dryness in a vacuum centrifuge and the steroids redissolved in 9 ml methanol prior to derivatisation (see section 2.3.7).

After the derivatization procedure, steroids were reconstituted in initial chromatography solvent (2·25 ml) and two portions (1·0 ml) analysed by HPLC. Fractions (1 ml) were collected and the solvent evaporated to dryness on a vacuum centrifuge. Steroids were redissolved in 1 ml phosphate buffer, and duplicate portions (0·1 ml) added to a scintillation vial with 3 ml scintillation fluid. Fractions were counted for ^{3}H and ^{14}C radioactivity.

3.2.7.4 Recrystallization

The identity of ^{14}C-labelled steroid metabolites co-eluting with ^{3}H-steroids on HPLC was determined by recrystallization to constant specific activity and $^{3}H/^{14}C$ ratio as described in section 2.7.

3.3 RESULTS

3.3.1 Distribution of radiolabelled steroids

The volumes of urine and mass of faeces excreted on each of the 4 days of the experiment are shown in Table 3.1. Very little urine and no faeces were excreted on Day 1, the day of the radiolabel injection, presumably whilst the animal recovered from the sedation procedure. Over the following 3 days, the volume of urine excreted ranged from 8·7-21·4 litres and the mass of faeces ranged from 25·5-47·7 Kg.

By directly counting aliquots of urine, the radioactivity in 0·25, 0·5 and 1 ml volumes of urine excreted on each day of the experimental period was determined and an average value for the cpm in 1 ml of each sample was obtained (Table 3.2). The small quantity of urine excreted on Day 1 of the experiment contained very low levels of radioactivity (117·8 cpm/ml). The average radioactivity

Table 3.1. Volume of urine and mass of faeces excreted on each day of the experiment, following the i.v. injection of ^{14}C-labelled oestradiol-17β and progesterone on Day 1.

day	volume urine excreted (l)	weight faeces excreted (kg)
1	0·03	-
2	20·0	26·5
3	8·7	30·9
4	21·4	47·7

Table 3.2. Radioactivity in duplicate volumes (1, 0·5 and 0·25 ml) of urine collected on each day of the experiment, from which the average cpm/ml was calculated.

	volume urine (ml)			
	0·25	0·5	1·0	average cpm/ml
Day 1	13	56	189	
	16	46	198	
Av/ml	58	102	193·5	117·8
Day 2	1586	2881	5317	
	1582	2921	5348	
Av/ml	6336	5802	5332·5	5823·5
Day 3	114	217	387	
	109	225	408	
Av/ml	446	442	397·5	428·5
Day 4	79	153	285	
	75	149	268	
Av/ml	308	302	276·5	295·5

in 1 ml urine excreted on Day 2 was relatively constant when calculated from each volume counted, and resulted in a mean value of approximately 5000 cpm/ml. Lesser amounts of radioactivity, 428·5 and 295·5 cpm/ml, were excreted on Days 3 and 4 respectively.

The total radioactivity excreted into the urine each day was calculated and is shown in Table 3.3. The greatest amount of radioactivity ($1·17x10^8$ cpm) was excreted into the urine on Day 2 with relatively small numbers of counts ($3·73x10^6$ and $6·32x10^6$) being excreted on Days 3 and 4 of the experiment.

The radioactivity in both the first and second ethanol extracts of faeces was determined , and the results are shown in Table 3.4. The values for the two ethanol extracts obtained from faeces collected on each day were then combined and an average figure for the cpm/ml of each extract (a total of 200 ml) was then calculated. No faeces, and thus no radioactivity was excreted on Day 1 of the experiment. Again the greatest amount of radioactivity was found in the extract of the Day 2 faecal sample (4201·2 cpm/ml) with smaller but significant amounts of radioactivity being excreted into the faeces on Days 3 and 4 (2685·7 and 730·9 cpm/ml respectively).

Table 3.5 shows the total radioactivity in each 250 g faecal extract. The total radioactivity excreted via the faeces per day was then calculated. The majority of radioactivity ($9·3x10^7$ cpm) was excreted on Day 2 of the experiment, although, in contrast to the situation in the urine, significant amounts of radioactivity ($6·64x10^7$ and $2·79x10^7$ cpm) were also excreted on the final 2 days of the experiment.

Table 3.6 shows the radioactivity (expressed both as a percentage of the total recovered and total administered) in the urine and faeces on each of the 4 days. From this table it can be seen that over 65% of the recovered label was present in urine and faeces excreted on Day 2 of the experiment. Whilst a slightly

Table 3.3. Total radioactivity (cpm) excreted into the urine by a female southern white rhinoceros on each of the 4 days of the experiment.

day	average cpm/ml urine	volume urine excreted (l)	total cpm excreted per day
1	114·5	0·03	$3·43 \times 10^3$
2	5823·5	20·0	$1·17 \times 10^8$
3	428·5	8·7	$3·73 \times 10^6$
4	295·5	21·4	$6·32 \times 10^6$

Table 3.4. Radioactivity in four volumes (1, 0·5 and 0·25 ml) of 2 ethanol extracts of faeces collected on each day of the experiment, from which the average cpm/ml extract was calculated.

day	cpm/ vol (ml) diluted ethanol extract of faeces			
	0·25	0·5	1·0	average cpm/ml (equivalent to cpm/ 0·1 ml extract)
1	-	-	-	-
2	80	173	366	
first	81	159	316	
extraction	72	154	419	
	91	203	364	
Av/ml	324	344·5	366	344·8
second	18	27	138	
extraction	15	36	68	
	21	30	75	
	23	34	51	
Av/ml	77	63·5	83	74·52
3	36	119	242	
first	37	111	243	
extraction	29	114	216	
	48	109	213	
Av/ml	150	226·5	228·5	201·67
second	27	26	39	
extraction	49	22	31	
	11	22	46	
	27	21	49	
Av/ml	114	45·5	41.25	66·9
4	9	21	76	
first	13	22	92	
extraction	17	26	41	
	14	25	87	
Av/ml	53	47	74	50·09
second	5	8	12	
extraction	4	9	11	
	5	10	11	
	3	7	11	
Av/ml	17	17	11	15·0

Table 3.5. Total radioactivity (cpm) excreted into the faeces by a female southern white rhinoceros on each of the 4 days of the experiment.

day	average cpm/ml extract	cpm/200 ml extract (250 g)	cpm/Kg	mass excreted per day (kg)	total excreted cpm/day
1	-	-	-	-	-
2	4201·2	$8{\cdot}40\times10^{5}$	$3{\cdot}36\times10^{6}$	26·5	$8{\cdot}90\times10^{7}$
3	2685·7	$5{\cdot}37\times10^{5}$	$2{\cdot}15\times10^{6}$	30·9	$6{\cdot}64\times10^{7}$
4	730·9	$1{\cdot}46\times10^{5}$	$5{\cdot}85\times10^{5}$	47·7	$2{\cdot}79\times10^{7}$

Table 3.6. ^{14}C-Radioactivity excreted into the urine and faeces expressed as a percentage of the total ^{14}C-radiolabel recovered in urine and faeces combined.

	urine			faeces		
day	cpm/day	% total recovered	% total injected	cpm/day	%total recovered	% total injected
1	$3{\cdot}43\times10^{3}$	$1{\cdot}1\times10^{-7}$	$6{\cdot}0\times10^{-6}$	-	-	-
2	$1{\cdot}17\times10^{8}$	38·0	23·0	$8{\cdot}90\times10^{7}$	28·1	17·0
3	$3{\cdot}73\times10^{6}$	1·2	0·7	$6{\cdot}64\times10^{7}$	21·6	13·1
4	$6{\cdot}32\times10^{6}$	2·0	1·2	$2{\cdot}79\times10^{7}$	9·1	5·5
total		41·2	24·9		58·8	35·6

higher proportion of the recovered label was present in faeces, both urinary (24·9%) and faecal (35·6%) routes of excretion were seen to be important in the overall recovery of 61% of the administered radiolabel over the 4 day experimental period. Over 40% of the injected radiolabel was excreted in the urine and faeces on Day 2, and it was these samples that were further analysed.

A typical separation of counts into the solvent (unconjugated) and aqueous (conjugated) fractions after ether extraction of Day 2 urine (50 ml) is shown in Table 3.7a. The counts presented are corrected for procedural losses (see section 3.2.5.1 for method of determination) and for quench during counting. Of the $2{\cdot}0\times10^5$ cpm present in the 50 ml of urine, 54·1% ($1{\cdot}573\times10^5$ cpm) was present in the ether extract, ie. associated with unconjugated steroids, whilst 39·0% ($1{\cdot}134\times10^5$) remained in the aqueous phase, ie. associated with conjugated steroids. An overall recovery of 93·1% of the total counts (TC) initially present in the urine was obtained. The proportions of radioactivity associated with conjugated and unconjugated steroids, after 7 such ether extractions of Day 2 urine, are shown in Table 3.7b. Mean values for the percentage of radioactivity in each fraction were similar to those obtained in the example described above. Consistently higher amounts of radioactivity were detected in the ether phase (51·4%) than in the aqueous fraction (41·2%) in all extractions. However, after ether extraction of faecal extracts (n=2, not shown), 92.4% of steroids were found to be present in the ether, ie. in the unconjugated form.

Both the unconjugated and conjugated steroids in urine underwent phenolic extraction on 7 separate occasions, and the proportions of radioactivity associated with neutral and phenolic steroids are shown in Table 3.8b. The actual separation of counts in phenolic extraction 1 is shown above in Table 3.8a. From a total of 141,061 cpm in the 10 ml of reconstituted ether extract (containing unconjugated steroids) 97,614 cpm (69·2%) were present in

Table 3.7a. ^{14}C-Radioactivity (cpm) in a portion of Day 2 urine (50 ml), and in the ether (unconjugated) and aqueous (conjugated) phases after a representative ether extraction.

	cpm/ml	cpm/50ml	% total counts
total counts	5814·3	$2{\cdot}907 \times 10^5$	
ether phase	3146·5	$1{\cdot}573 \times 10^5$	54·1
aqueous phase	2267.6	$1{\cdot}134 \times 10^5$	39·0
total recovery of radiolabel			93·1

Table 3.7b. Mean±sem percentage of ^{14}C-radioactivity in the ether extract and aqueous phase after 7 separate ether extractions of Day 2 urine, to separate unconjugated and conjugated steroids.

extraction	% radiolabel	
	ether extract (unconjugated)	aqueous phase (conjugated)
1	54·1	39·0
2	60·8	38·8
3	49·9	40·6
4	54·0	38·1
5	49·0	43·2
6	51·1	45·0
7	44·0	41·1
mean±s.e.m.	51·4±2·10	41·2±0·98
coefficient of variation	10·8	6·2

Table 3.8a. ^{14}C-Radioactivity (cpm) associated with neutral (progesterone metabolites) and phenolic (oestrogens) steroids in unconjugated and hydrolysed conjugated fractions separated from 50 ml urine, after a representative phenolic extraction.

	unconjugated			conjugated		
	cpm/ml extract	cpm /10ml	% total counts	cpm/ml extract	cpm /10ml	%total counts
total counts	14106·1	141061		11012·9	110129	
neutrals	9761·4	97614	69·2	4515·3	45153	41·0
phenols	4076·7	40767	28·9	4812·6	48126	43·7
%total recovery of label			98·1			84·7

Table 3.8b. Mean±sem percentage of ^{14}C-radioactivity in the neutral and phenolic phases after 7 separate phenolic extractions of Day 2 urine, to separate progesterone metabolites and oestrogens.

phenolic extraction	unconjugated		conjugated	
	neutrals	phenols	neutrals	phenols
1	69·2	28·9	41·0	43·7
2	72·3	21·6	50·5	44·2
3	68·0	30·1	48·5	41·2
4	64·0	29·4	50·0	38·1
5	69·1	29·3	52·1	40·3
6	71·5	24·0	52·2	46·1
7	73·0	26·0	52·4	36·3
mean±s.e.m.	69·59±1·08	27·04±1·14	49·53±1·41	41·40±1·23
C of V (%)	4·1	11·1	7·5	7·8

the neutral portion whilst 40,767 cpm (28·9%) were extracted by alkali into the phenolic portion. Similarly, of the radioactivity (110,129cpm) associated with conjugated steroids, 45,153 cpm (41·0%) were present in the neutral portion whilst 48,126 cpm (43·7%) were associated with phenolic steroids In both cases less than 15 % of the total radiolabel present remainined in the aqueous (ie. dilute sodium hydroxide solution) and was not available for further analysis. These results are typical for the phenolic extractions performed which overall demonstrated that, in the unconjugated portion a larger proportion of radioactivity was associated with neutral (69·6%) than phenolic steroids (27·0%), whereas similar proportions of neutral and phenolic steroids were seen as conjugates (49·5% and 41·4% respectively). Overall, a higher percentage of radioactivity in the urine was present as progesterone metabolites (63·5%; 37·1% unconjugated and 26·4% conjugated) than as oestrogens (36·5%; 14·4% unconjugated and 22·1% conjugated). Whereas progesterone metabolites were predominant in the urine, repeated phenolic extraction (n=4) demonstrated that the majority of the radioactivity in faeces (71·0%) was associated with oestrogens (see Table 3.9).

3.3.2 Identification of radiolabelled steroids

3.3.2.1 Urine

The position of radioactivity on the TLC plate, in relation to the mobility of the reference standards for neutral and phenolic steroids in urine is shown in Figures 3.4a and b respectively. The data presented represents that obtained from 1 of 3 similar TLC runs for each group of steroids.

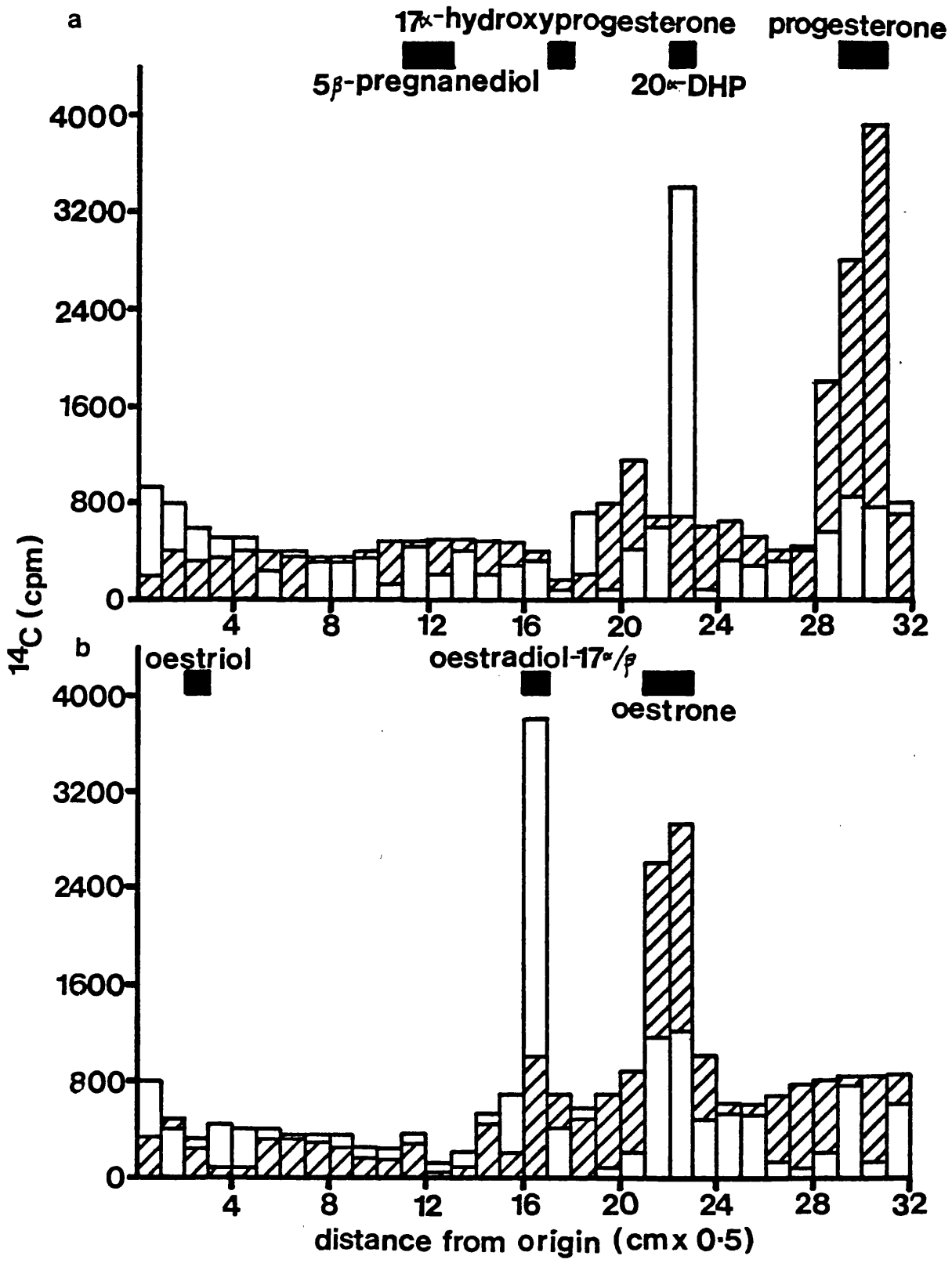
For neutral steroids, in the unconjugated fraction a single peak of radioactivity was seen with the same Rf value as the progesterone standard. Similarly, in the conjugated fraction, a single radioactive peak was seen with an Rf value similar to that of

Table 3.9. Mean±sem percentage of ^{14}C-radioactivity in the neutral and phenolic phases after 4 separate phenolic extractions of Day 2 faeces, to separate progesterone metabolites and oestrogens.

phenolic extraction	unconjugated	
	neutrals	phenols
1	21·1	72·6
2	24·7	71·2
3	26·0	69·9
4	20·8	70·3
mean±s.e.m	23·15±1·12	71·00±0·52
C of V	9·7	1·4

Figure 3.4a. Separation of [^{14}C] radioactivity associated with neutral steroids in Day 2 urine on TLC plates. Hatched boxes represent the migration of unconjugated radioactivity on the plate, whilst open boxes indicate the level of radioactivity associated with conjugated (hydrolysed) neutral steroids. The position of the unlabelled standards on the TLC plate, 5β-pregnanediol, 17α-hydroxyprogesterone, 20α-DHP and progesterone, are shown above (closed boxes).

Figure 3.4b. Separation of [^{14}C] radioactivity associated with phenolic steroids in Day 2 urine on TLC plates. Hatched boxes represent the migration of unconjugated radioactivity on the plate, whilst open boxes indicate the level of radioactivity associated with conjugated (hydrolysed) phenolic steroids. The position of the unlabelled standards on the TLC plate, oestriol, oestradiol-17α/β and oestrone, are shown above (closed boxes).


a
17α-hydroxyprogesterone
progesterone
5β-pregnanediol
20α-DHP
4000
3200
2400
1600
800
0
14C (cpm)
4
8
12
16
20
24
28
32
b
oestriol
oestradiol-17α/β
oestrone
4000
3200
2400
1600
800
0
4
8
12
16
20
24
28
32
distance from origin (cm x 0·5)

20α-DHP. Only very low, background levels of radioactivity in the conjugated fraction were seen to correspond with the pregnanediol standard (11-13 cm).

Of the phenolic steroids, a single peak of radioactivity was seen in the unconjugated fraction with the same Rf value as that of oestrone. In the unconjugated fraction a similar radioactive area was seen with the same Rf value as the 17α and 17β-isomers of oestradiol, indistinguishable in the TLC system used.

A typical HPLC elution profile of unconjugated, neutral steroids in urine, in relation to that of ^{3}H-labelled P_4, is shown in Fig 3.5 a. Similar separations were obtained in four HPLC runs of this fraction. A single peak of ^{14}C-label was identified in the unconjugated fraction with a retention time of 10 min. This peak of ^{14}C-label co-eluted with the [^{3}H]progesterone standard in the ratio 1·12 ($^{3}H/^{14}C$). The fractions containing both ^{14}C and ^{3}H-radioactivity (ie. fractions 19 and 20) from 2 of the 4 HPLC runs were taken for recrystallization whilst the fractions from the two remaining runs underwent derivatization. Derivatized radiolabels were once again separated on HPLC to give an elution profile as shown in Fig 3.5b. A single peak of both ^{14}C and ^{3}H-radioactivity was seen in fraction 28. Peaks of ^{3}H- and ^{14}C-radioactivity not only had the same retention time but continued to elute in similar proportions (1·25) to those obtained prior to derivatization suggesting that they are infact the same compound.

Similarly, 4 HPLC runs of conjugated, neutral steroids in urine gave similar elution profiles, as illustrated in Fig. 3.6a. HPLC identified a single peak of ^{14}C-label in fraction 32 which co-eluted with [^{3}H]20α-DHP in the ratio 1·81 ($^{3}H/^{14}C$). No ^{14}C-label was seen in fraction 38, that fraction containing the [^{3}H] pregnanediol marker. Fractions 32 (n=2) were taken for recrystallization, whilst the same fractions from the 2 remaining HPLC runs were derivatized. HPLC was repeated and the elution profile of ^{3}H- and ^{14}C-radioactivity in the conjugated neutral

Figure 3.5. HPLC elution profiles of unconjugated ^{14}C-labelled neutral steroids (progesterone metabolites) in Day 2 urine (——————) and [^{3}H]progesterone (- - - -) before (a) and after (b) derivatization..

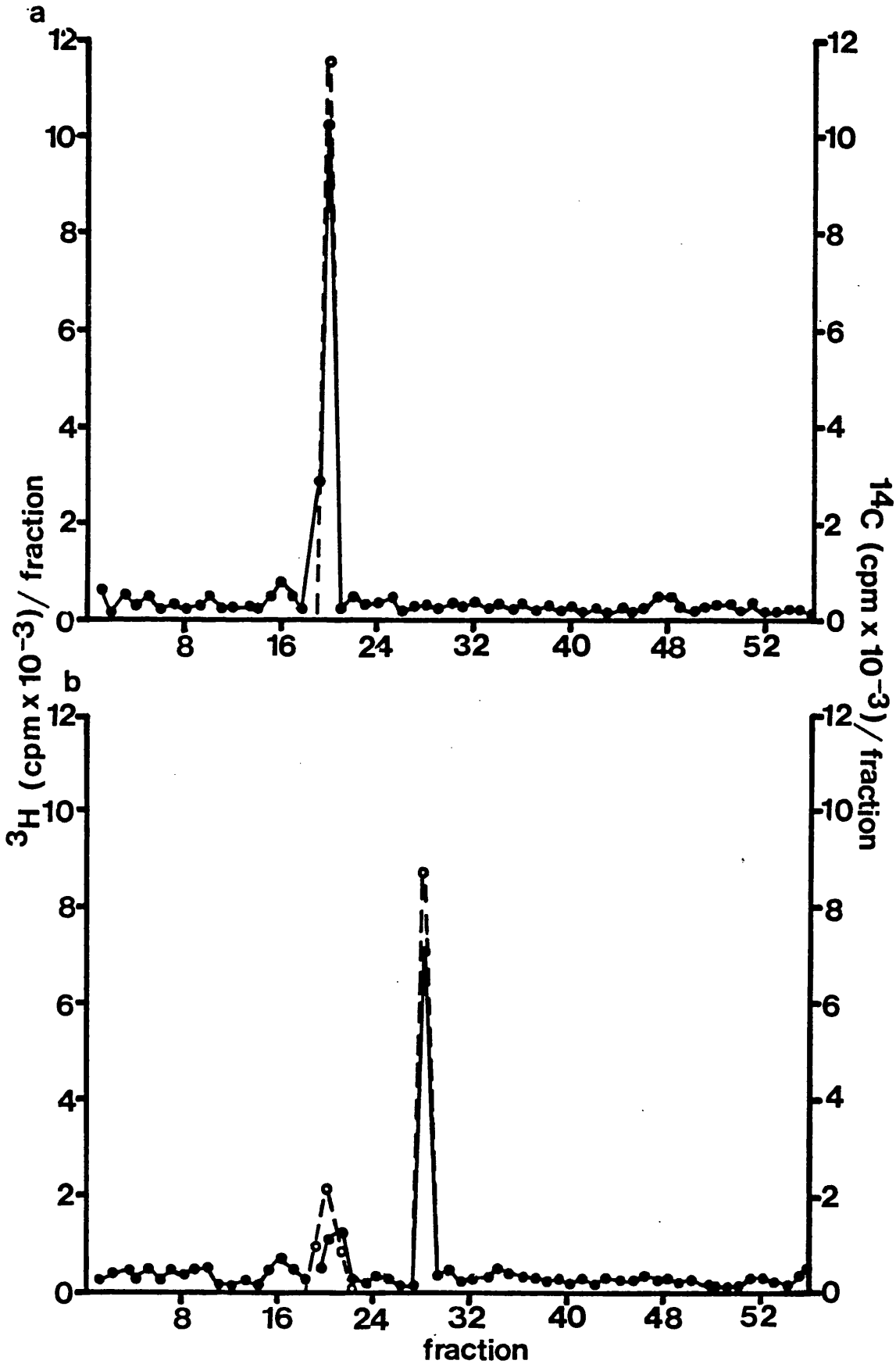
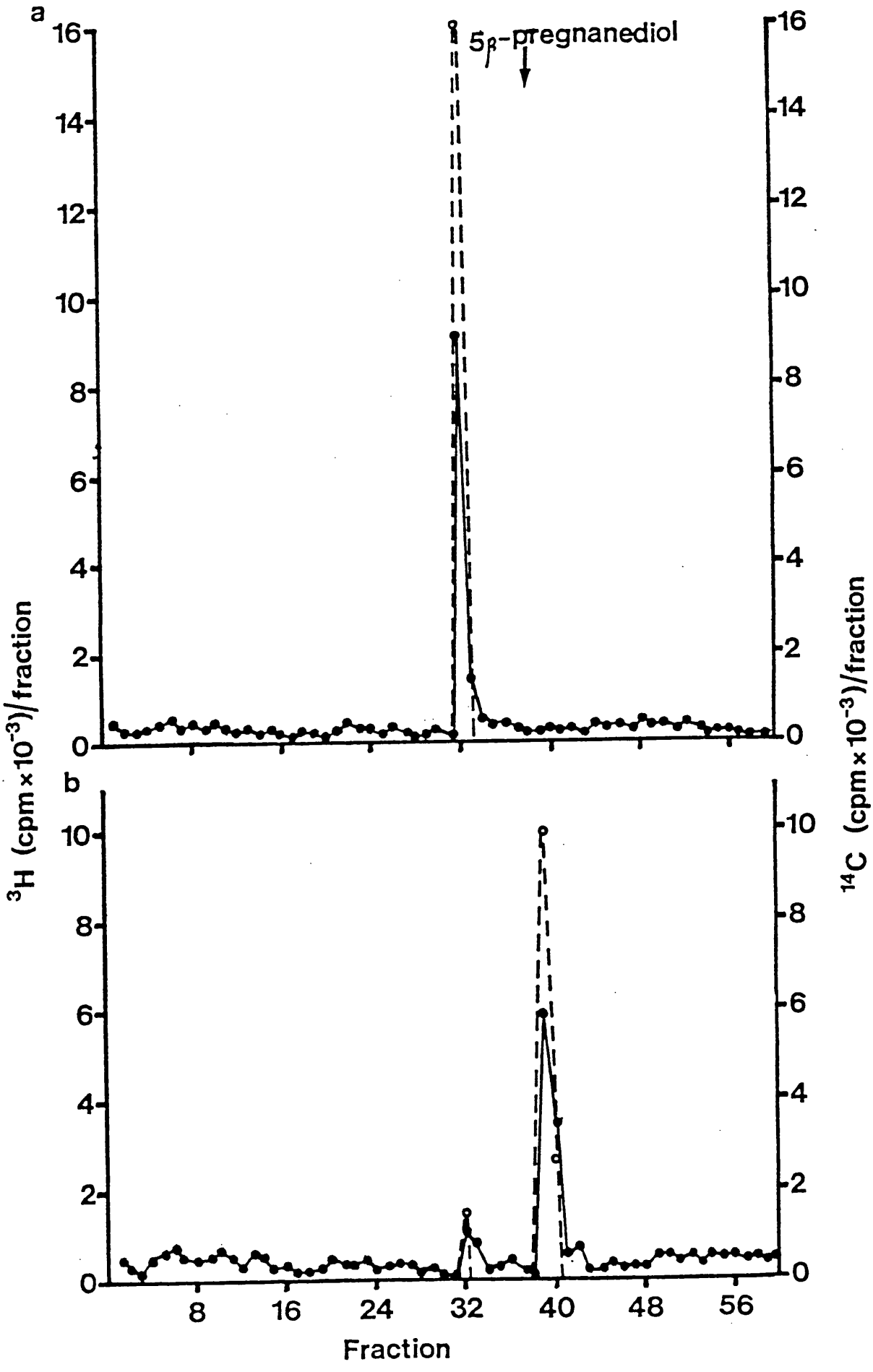

a
b
12
10
8
6
4
2
0
8
16
24
32
40
48
52
^{3}H (cpm x 10^{-3})/ fraction
^{14}C (cpm x 10^{-3})/ fraction
fraction

Figure 3.6. HPLC elution profiles of conjugated ^{14}C-labelled neutral steroids (progesterone metabolites) in Day 2 urine (——————) that had previously undergone solvolysis and [^{3}H]20α-DHP (- - - -) before (a) and after (b) derivatization. The elution position of 5β-pregnanediol on this HPLC system is indicated above.


a
5β-pregnanediol
b
^{3}H (cpm×10^{-3})/fraction
^{14}C (cpm×10^{-3})/fraction
Fraction
0
2
4
6
8
10
12
14
16
8
16
24
32
40
48
56

fraction after derivatization is shown in Fig. 3.6b. A single peak of ^{14}C and ^{3}H radioactivity was seen in fractions 39 and 40. Radioactivity continued to elute in the same position, and in similar proportions of $^{3}H/^{14}C$ radiolabel (1·67), suggesting that all the ^{14}C-radioactivity in the conjugated neutral portion of urine was 20α-DHP.

The HPLC elution profiles of unconjugated and conjugated, phenolic steroids in the urine are shown in Fig. 3.7 a and b. The elution profiles described are representative of the results obtained from 4 separate runs. Two peaks of equal amounts of ^{14}C-radioactivity were seen in the unconjugated portion in fractions 9 and 23, those also containing [^{3}H]oestrone and [^{3}H]oestradiol-17β respectively. (Fig. 3.7 a). In the conjugated portion, ^{14}C-label was also seen to elute in 2 peaks (fractions 9 and 18) which co-eluted with [^{3}H]oestrone and [^{3}H]oestradiol-17α (Fig. 3.7 b). Fractions contining co-eluting ^{14}C and ^{3}H radioactivity were taken for recrystallization.

Data obtained for the mass and radioactivity content of crystals formed over 4 successive recrystallizations of HPLC fractions with pure, unlabelled steroids are shown in Table 3.10. Fractions containing radiolabel after HPLC of unconjugated neutral steroids were recrystallized with unlabelled progesterone to a constant specific activity (0·36-0·34 μCi/g) and $^{3}H/^{14}C$ (1·15-1·14) which provides further evidence that the ^{14}C-label in this portion of the urine was progesterone. Similar results were obtained for the recrystallization of HPLC fractions containing radiolabel present in the conjugated neutral portion of urine. The recrystallization of radiolabel with authentic 20α-hydroxy-4-pregnen-3-one (20α-DHP) to constant specific activity (initially 2·33 μCi/g and finally 2·11 μCi/g with a c of v of 3·9 throughout the 4 recystallizations) and $^{3}H/^{14}C$ (1·80-1·71) supported the results obtained by derivatization that all the conjugated neutral radioactivity in white rhinoceros urine was in the form of 20α-DHP.

Figure 3.7. HPLC elution profiles of unconjugated (a) and conjugated (b) ^{14}C-labelled phenolic steroids (oestrogens) in Day 2 urine (——) and ^{3}H-labelled standards, oestrone, oestradiol-17α ($E_2$17α) and oestradiol-17β ($E_2$17β) (- - - -).

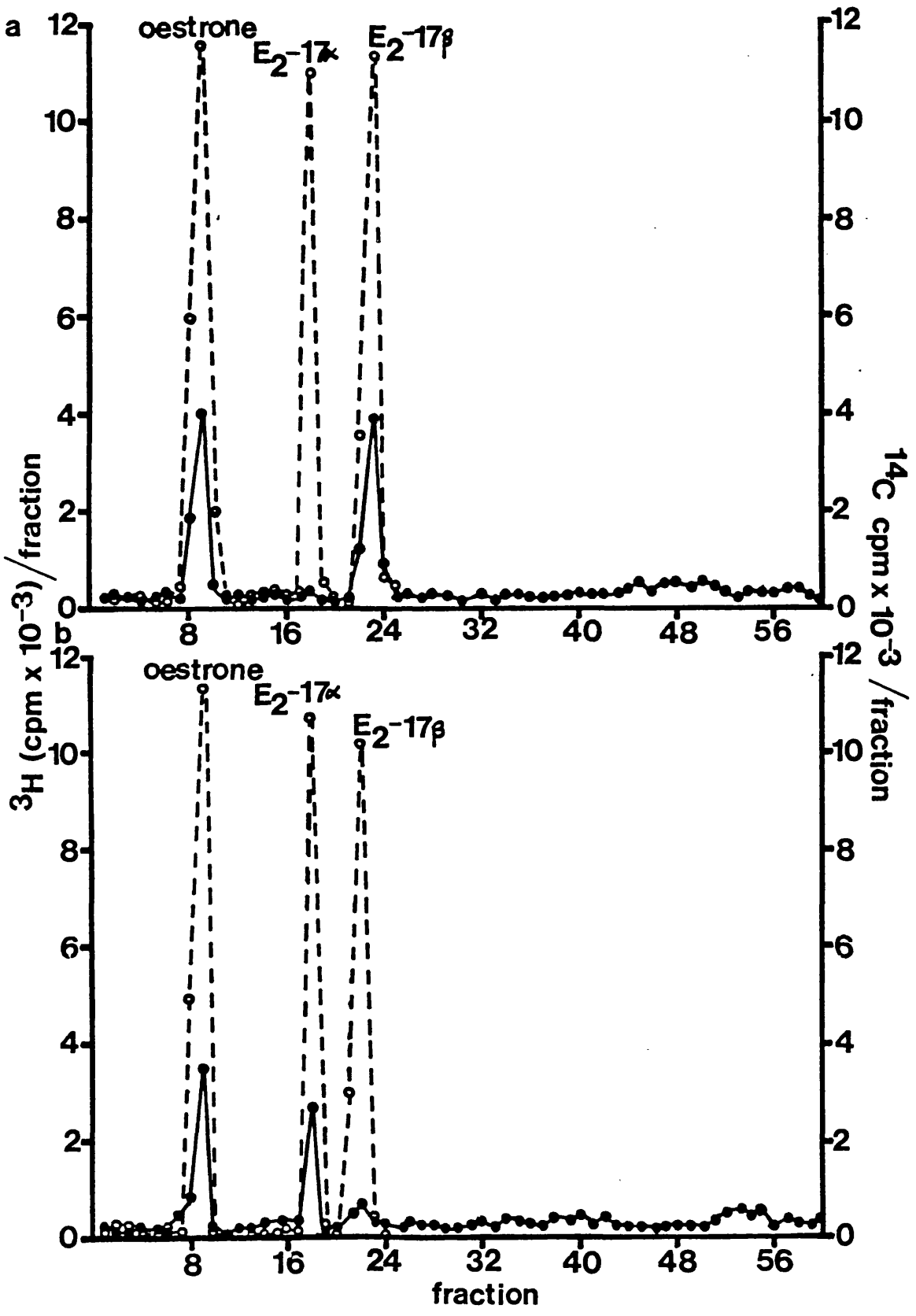

a
b
oestrone
E2-17α
E2-17β
3H (cpm x 10-3)/fraction
14C cpm x 10-3 /fraction
fraction
0
2
4
6
8
10
12
8
16
24
32
40
48
56

Table 3.10. Specific activity and $^{3}H/^{14}C$ and the coefficient of variation between 4 successive recrystallizations of ^{14}C- and ^{3}H-labelled steroids co-eluting on HPLC with unlabelled steroid.

steroid	crystalln number	mass crystals (g)	^{3}H (cpm)	^{14}C (cpm)	specific activity (μCi/g)	$^{3}H/^{14}C$
progesterone	initial	0·0061	5543	4820	0·36	1·15
	1	0·0062	4771	4260	0·31	1·12
	2	0·0062	5113	4525	0·33	1·13
	3	0·0059	4287	3862	0·30	1·11
	4	0·0029	2506	2197	0·34	1·14
C of V (%)					6·5	1·2
20α-DHP	initial	0·0010	9227	5126	2·33	1·80
	1	0·0007	5729	3292	2·17	1·74
	2	0·0009	7397	4326	2·14	1·71
	3	0·0006	4972	2925	2·09	1·70
	4	0·0009	7285	4260	2·11	1·71
C of V (%)					3·9	2·1
oestrone (unconj.)	initial	0·0006	2299	1277	0·97	1·80
	1	0·0007	2262	1396	0·92	1·62
	2	0·0006	1766	1104	0·89	1·60
	3	0·0006	2077	1290	0·91	1·61
	4	0·0007	2240	1427	0·89	1·57
C of V (%)					3·2	5·0
oestradiol -17β	initial	0·0006	1298	827	0·65	1·57
	1	0·0013	2208	1462	0·52	1·51
	2	0·0008	1389	926	0·51	1·50
	3	0·0008	1526	991	0·57	1·54
	4	0·0010	1549	1046	0·50	1·48
C of V (%)					10·1	2·1
oestrone (conjugated)	initial	0·0017	4064	1926	0·51	2·11
	1	0·0016	4390	2042	0·57	2·15
	2	0·0017	3952	1873	0·50	2·11
	3	0·0008	1889	926	0·48	2·04
	4	0·0014	2820	1410	0·46	2·00
C of V (%)					7·4	2·6
oestradiol -17α	initial	0·0004	2779	989	1·12	2·81
	1	0·0004	2365	863	1·04	2·74
	2	0·0003	1670	621	0·92	2·69
	3	0·0005	2497	946	0·89	2·64
	4		crystals lost			
C of V (%)					9·3	2·3

For the phenolic steroids, in the unconjugated portion HPLC fraction 9 containing radiolabel were recrystallized to constant specific activity (0·97-0·89 μCi/g) and $^{3}H/^{14}C$ (1·80-1·57) with pure oestrone whilst those in fraction 23 were recrystallized with oestradiol-17β. Of the conjugated oestrogens, oestrone was also identified in this fraction by recrystallizing radiolabel in fractions 9 with authenic standard to constant specific activity (C of V=7·4) and $^{3}H/^{14}C$. The radiolabel in fraction 18 was recrystallized with oestradiol-17α although the crystals of the fourth procedure were lost.

3.3.2.2 Faeces

The position of radioactivity on the TLC plate, in relation to the mobility of the reference standards for neutral steroids in faeces is shown in Fig. 3.8a. No significant elevation of the level of radioactivity above background was seen using TLC. However, a small, single peak of ^{14}C-radioactivity was identified by HPLC (Fig 3.9a) which co-eluted with [^{3}H]progesterone.

The position of radioactivity on the TLC plate, in relation to the mobility of the reference standards for phenolic steroids in faeces is shown in Fig. 3.8b. One area of radioactivity was seen with the same Rf value as oestradiol-17α/β. The only detectable peaks of ^{14}C-radioactivity in faecal extracts co-eluted with [^{3}H]oestradiol-17α and [^{3}H]oestradiol-17β in the phenolic fraction on HPLC (Fig. 3.9b).

3.4 Discussion

This study has identified the major metabolites of i.v. injected radiolabelled oestradiol-17β and progesterone in urine and faeces collected from a female southern white rhinoceros.

Figure 3.8a. Separation of [^{14}C] radioactivity associated with unconjugated neutral steroids in Day 2 faeces on TLC plates. The position of the unlabelled standards on the TLC plate, 5β-pregnanediol, 17α-hydroxyprogesterone, 20α-DHP and progesterone, are shown above (closed boxes).

Figure 3.8b. Separation of [^{14}C] radioactivity associated with unconjugated phenolic steroids in Day 2 faeces on TLC plates. The position of the unlabelled standards on the TLC plate, oestriol, oestradiol 17α/β and oestrone, are shown above (closed boxes).

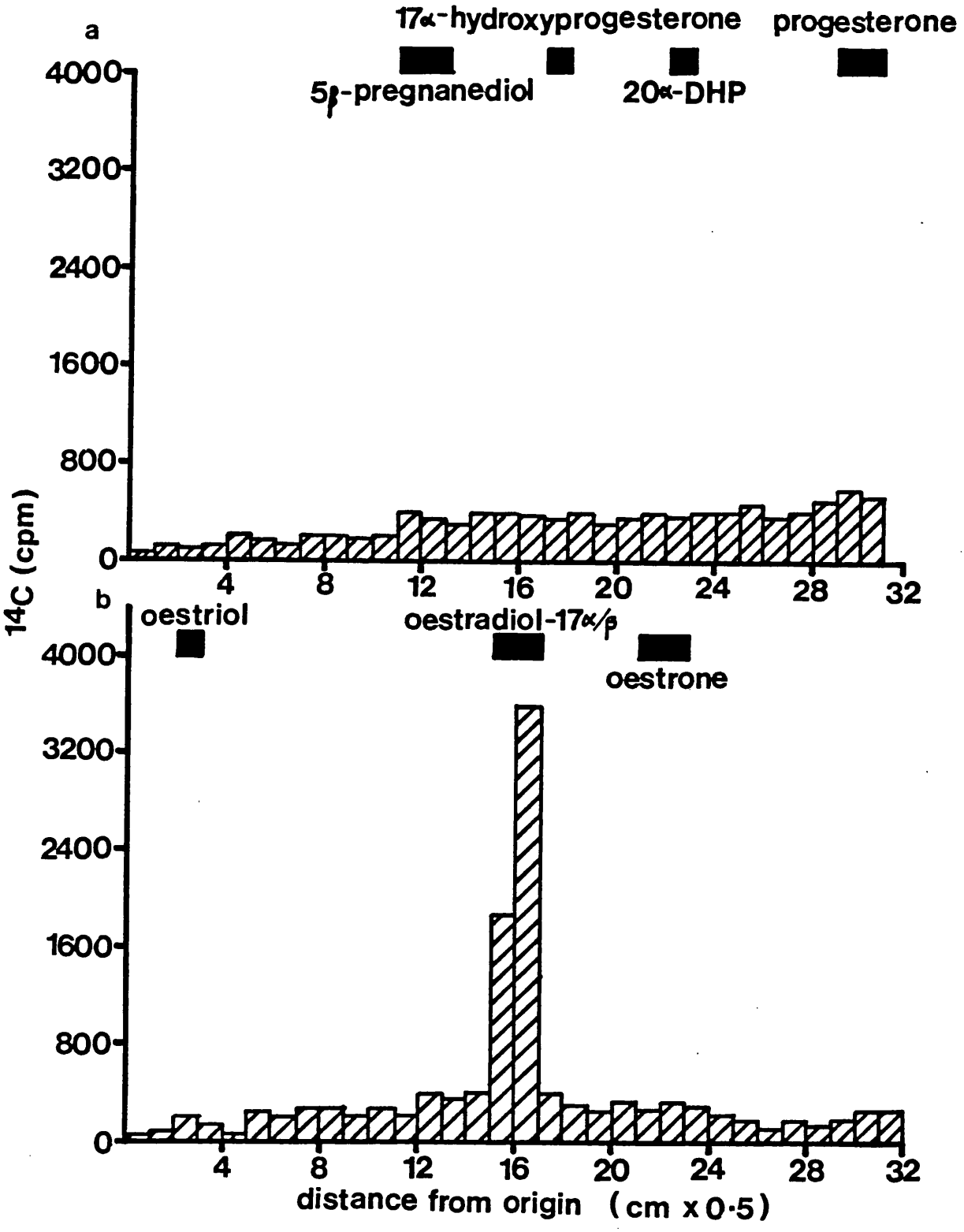
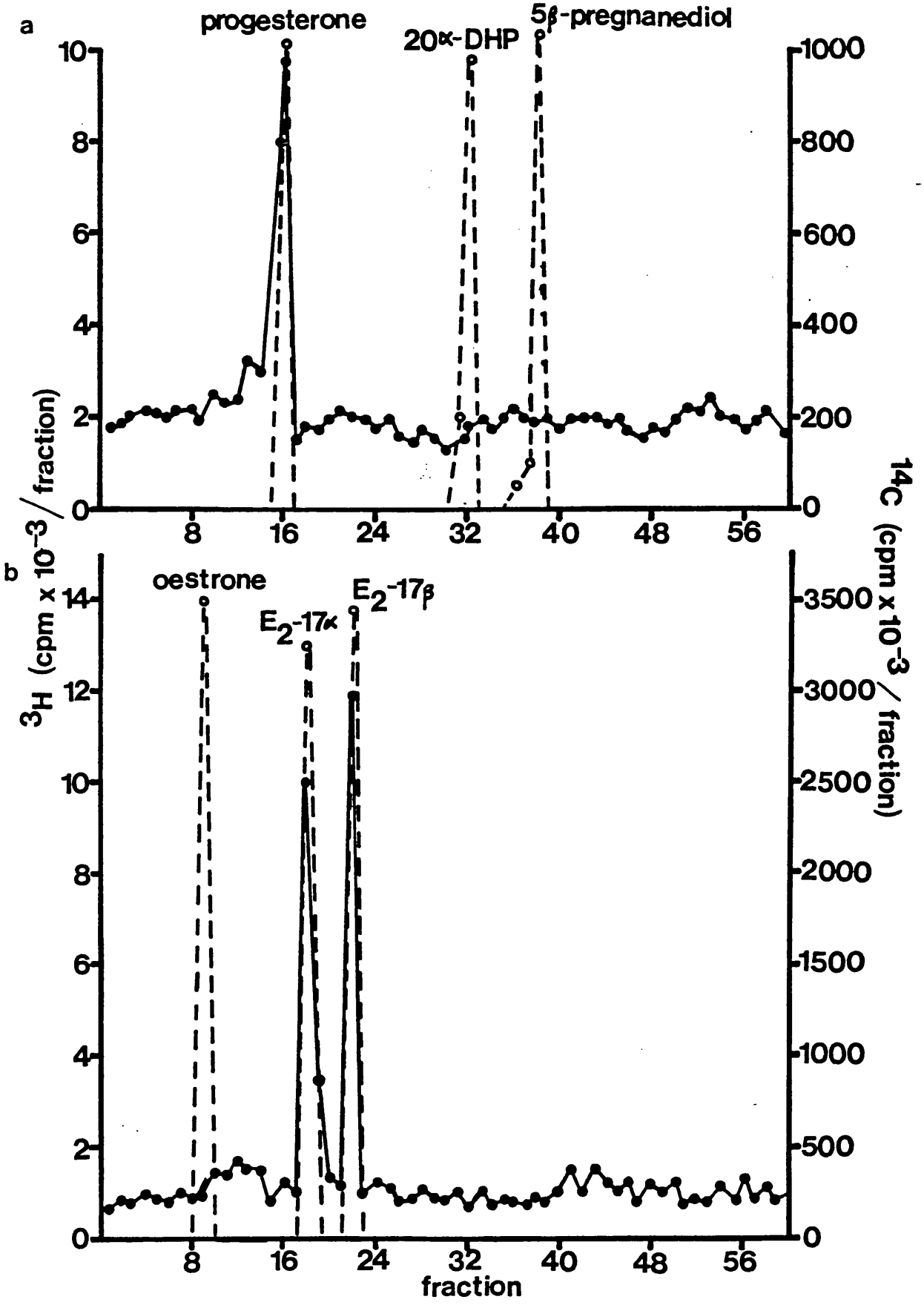

a
17α-hydroxyprogesterone
progesterone
5β-pregnanediol
20α-DHP
4000
3200
2400
1600
800
0
14C (cpm)
4
8
12
16
20
24
28
32
b
oestriol
oestradiol-17α/β
oestrone
4000
3200
2400
1600
800
0
4
8
12
16
20
24
28
32
distance from origin (cm x 0·5)

Figure 3.9a. HPLC elution profile of unconjugated ^{14}C-labelled neutral steroids in Day 2 faeces (——————) and ^{3}H-labelled standards, progesterone, 20α-DHP and 5β-pregnanediol (- - - -).

Figure 3.9b. HPLC elution profile of unconjugated ^{14}C-labelled phenolic steroids in Day 2 faeces (——) and ^{3}H-labelled standards, oestrone, oestradiol-17α and oestradiol-17β (- - - -).


a
progesterone
20α-DHP
5β-pregnanediol
^{3}H (cpm x 10^{-3} / fraction)
^{14}C (cpm x 10^{-3} / fraction)
b
oestrone
E_2-17α
E_2-17β
fraction

As with any study in which the fate of exgenously administered steroids is investigated, the results obtained in this experiment must be treated with some degree of caution. For example, it is not known whether the findings presented here represent the normal pattern of metabolism and excretion of steroids in the southern white rhinoceros. It is possible the the levels of unlabelled "carrier" steroids (1 and 10 mg of oestradiol-17β and progesterone respectively), administered in the same preparation as the radiolabel, were above the physiological circulating levels in this species and thus altered the natural metabolism of ovarian steroids *in vivo*.

The results presented here also apply to a single female of unknown reproductive status and therefore the results may not be representative of all animals of the species. As very little is known about the reproductive physiology of the white rhinoceros it is impossible to say whether this animal, at the age of 27 years, would have had functional ovaries and whether such functional ovaries would alter the metabolism of administered radiolabelled steroids is unclear. However, similar studies have provided valuable information on the reproductive physiology of exotic ungulates by investigating steroid metabolism and excretion in a single animal, eg. the okapi (Loskutoff *et al.*, 1987) and the elephant (Czekala, Roocroft and Bates, 1989). Other studies in the cat (Shille *et al.*, 1984) and slow loris (Perez, Czekala, Weisenseel and Lasley, 1988) have shown that the reproductive status of the animal at the time of injection has no effect on the excreted steroid metabolites,even after the induction of ovulation with artificial gonadotrophins. Therefore, considering the limitations of the experiment, it is important to confirm the results of this metabolism study by further investigating the metabolism and excretion of endogenous ovarian steroids during natural cycles.

In addition, certain methodological problems were encountered in this study, and these were exacerbated by the large size of the species being investigated. It can be seen from Table 3.1 that

large quantities of urine and faeces (up to 21·4 litres and 47·7 Kg respectively) were excreted by this animal. The measurement of the daily output of urine and faecal matter illustrates a problem encountered with all large species, that small concentrations of steroids are diluted in large quantities of excreta and are thus present in very low levels when small volumes are assayed by immunological techniques. The analysis of such large volumes of urine and faeces also resulted in lower procedural efficiencies than would be obtained in the analysis of smaller volumes. Large errors were also encountered in the efficiency of counting radioactivity in strongly coloured urine and solvent extracts. Originally, the efficiency of counting was approximately 17·5%, but accuracy was improved by the quench correction procedures illustrated in section 3.2.4 although these corrections also incorporate a degree of error.

Additional identification procedures, ie. derivatization, were used to positively identify progesterone metabolites in the urine as the range of metabolites that could have been formed was extensive. In order to identify species differences in progesterone metabolism, and to develop immunological methods for the measurement of a new urinary progestagen, it was necessary to have further proof that 20α-DHP was the major metabolite of progesterone in the white rhinoceros. However, recrystallization alone was used to identify urinary oestrogens since the endproducts of oestradiol-17β metabolism are less variable between species than those of progesterone. There was insufficient radioactivity for derivatization and the results of the oestrogen identification are thus less reliable. Furthermore, there was insufficient radioactivity in the faecal extracts to take the identification procedures further than co-elution on HPLC.

The simultaneous injection of ^{14}C-labelled progesterone and oestradiol-17β also caused some uncertainty in the interpretation of the results of this study, since a small amount of ^{14}C-labelled oestrogens could have been formed by the metabolism of injected progesterone and not from [^{14}C]oestradiol-17β. Given the difficulty

of performing such a study on an endangered species in a zoological collection, it was not possible to repeat the study by investigating the metabolism of oestradiol-17β and progesterone separately in two female rhinoceroses. The injection of two different radiolabels, eg. [^{14}C] and [^{3}H], would have eliminated the possible complication of the results by the formation of oestrogens from the metabolism of progesterone. However, the risk of radioactive contamination in an area of public access from using high levels of [^{3}H]-radiolabel necessary for this experiment was too great and a compromise had to be made. Despite the problems encountered in the design and execution of this study, useful data have been obtained on the metabolism and excretion of ovarian steroids in the white rhinoceros which will be valuable in future studies on the reproductive physiology of this species.

During the sample collection period a total of 61% of the injected label was recovered in excreted urine and faeces, an amount comparable to that recovered in metabolism studies in the cow (Mellin and Erb, 1966; Estergreen, Lin, Martin, Moss, Branden, Luedecke and Shimoda, 1977), sheep (Stupnicki, McCracken and Williams, 1969; Miyazaki, Peric-Golia, Slaunthwhite and Sandberg, 1972), sow (Schomberg, Jones, Featherstone and Erb, 1966) and some primate species (Jeffery, 1966; Goldzieher and Axelrod, 1969; Plant, James and Michael, 1969). Since radioactivity was still present in excreted material (especially faeces) collected at the end of the study (Day 4), a longer period of sample collection would have resulted in a higher overall recovery of label and probably a higher proportion of radioactivity present in faeces. Nevertheless, 39% of the radioactivity recovered was excreted in the urine indicating that, whilst faecal excretion may predominate, significant amounts of gonadal steroids are excreted into the urine in this species.

Radioactivity in faeces was associated almost exclusively (93%) with steroids in the unconjugated form. The possibility exists that 3-sulphoconjugated products present in faeces were

hydrolysed during the ethanol extraction procedure, and were therefore not detected. Whilst biliary steroids can exist in the conjugated form (Støa & Skulstad, 1972), it has been demonstrated from studies in the cow (Bamberg *et al.*,1986a), sheep (Choi *et al.*,1987) and horse (Bamberg *et al.*, 1984) that steroids are excreted into the faeces predominantly in the unconjugated form, probably after bacterial hydrolysis in the gut (Miyazaki *et al.*,1972).

Over 50% of the radioactivity in urine was also associated with steroids in the unconjugated form, in contrast to the findings of radiometabolism studies in other ungulates, e.g. the cow (Ivie *et al.*, 1986) and pig (Schomberg *et al.*, 1966), in which less than 20% of excreted radiolabel was unconjugated. Furthermore, Kassam & Lasley (1981) demonstrated, using sequential hydrolysis, that more than 95% of urinary oestrogens were present as conjugates in the Indian rhinoceros, with a comparable figure being reported for the black rhinoceros (Ramsay *et al.*, 1987). There are several factors which may have influenced the concentration of unconjugated steroids in the urine. Firstly, it is possible that, in the present experiment, conjugated steroids were hydrolysed during storage at -20°C despite the addition of a preservative. However, this process does not appear to have led to generation of unconjugated 20α-DHP in the urine. Alternatively, it is possible that the large dose of unlabelled steroids administered affected the pattern of metabolism, and the fact that the major unconjugated steroids in urine were unmetabolised progesterone and oestradiol-17β would agree with this interpretation. However, it is possible that these results are accurate and that a higher proportion of steroids are present in the unconjugated form in the white rhinoceros than in the Indian species.

In contrast to the small proportion of radioactivity associated with neutral steroids in the faeces, progestagens predominated in the urine, a distribution similar to that observed in other ungulates including the sheep (Stupnicki *et al.*, 1969).

The only unconjugated neutral steroid that was present in the urine was unmetabolised progesterone. HPLC of the conjugated neutral steroids in urine revealed a single peak of radioactivity which was identified as 20α-DHP, and accounted for more than 90% of the radiolabel in the conjugated fraction. Significantly, in this fraction there was no peak of radioactivity that co-eluted from HPLC with the pregnanediol marker, indicating that conjugated pregnanediol was not a urinary metabolite of the progesterone administered. This finding is consistent with observations on the excretion of progesterone metabolites during natural cycles in the African species of rhinoceros. Ramsay *et al.* (1987) used HPLC analysis of urine from non-pregnant black rhinoceroses to demonstrate that PdG immunoreactivity did not co-elute with [^{3}H]PdG in this species, and was not detectable at any stage of the ovarian cycle. However, the absence of conjugated pregnanediol in the urine of the white rhinoceros is in marked contrast to observations made on the Indian species in which PdG has been identified as the major progesterone metabolite during the luteal phase of the ovarian cycle (Kasman *et al.*, 1986) and during pregnancy (Hodges & Green, 1989).

In addition to confirming the absence of pregnanediol glucuronide, the present results have identified conjugated 20α-DHP as the major urinary metabolite of administered progesterone. The pattern of excretion of 20α-DHP has not been described during natural cycles in the white or the black rhinoceroses, although its measurement in urine by non-specific immunoassay has been useful in monitoring the ovarian cycle and pregnancy in some species, eg. the lion-tailed macaque (Shideler *et al.*, 1985) and killer whale (Walker *et al.*, 1988). However, the significance of these measurements in relation to ovarian progesterone secretion is unknown. In the rat (Naito *et al.*, 1986) and some avian species (Llewellyn, 1981), the presence of 20-hydroxylase enzymes in thecal tissue has been demonstrated and these enzymes convert progesterone to isomers of 20-dihydroprogesterone which are secreted into the circulation. Although such detailed studies have not been carried out on any ungulate species, serum concentrations of 20α-DHP have been shown to

accurately reflect corpus luteum function in the horse (van Rensburg & van Niekerk, 1968; Seren *et al.*, 1981) which, along with the rhinoceros is a species of *Perrisodactyl*. Studies by Bamberg and Schwarzenberger (1990) indicated that a large proportion of the circulating 20α-DHP in the horse is excreted via the faeces, rather than into the urine, and have extended this finding to exotic equids including Przewalski's horse. However, recent results of Kirkpatrick *et al.* (1990) have indicated that the non-specific measurement of urinary 20α-DHP correlates well with fluctuations in plasma progesterone in equidae. As yet, no other published data are available on the levels of urinary 20α-DHP in any ungulate species, but the present results suggest that its measurement may reflect circulating progesterone levels and therefore corpus luteum function in the white rhinoceros to enable non-invasive monitoring of the ovarian cycle in this species.

In contrast to the distribution of neutral steroids the amounts of radioactive phenolic steroids excreted into the urine and faeces were similar. Like other *Perissodactyls*, including the tapir (Kasman *et al.*, 1985), horse (Raeside & Liptrap, 1975) and Indian rhinoceros (Kassam & Lasley, 1981; Kasman *et al.*, 1986), the white rhinoceros excretes conjugated oestrone as an abundant oestrogen metabolite in urine. Furthermore, in the Indian rhinoceros, the measurement of urinary oestrone sulphate during the ovarian cycle provides a good indication of follicular development with maximum levels being attained at the time of oestrus or mating. Sequential hydrolysis studies by Ramsay *et al* . (1987) have shown that the black rhinoceros also excretes conjugated oestrone, mainly oestrone glucuronide, into the urine although its measurement was not informative in the monitoring of reproductive function in this species. However, as in the present study, it was not possible to determine the position and configuration of the conjugation.

The 17α- and 17β-isomers of oestradiol have also been shown to be important oestrogens in the serum and urine of many species. Unmetabolised oestradiol was identified as the major urinary

oestrogen in the Okapi by means of a radiometabolism study (Loskutoff *et al.*, 1987), although the isomer of oestradiol was not definitively identified. Similarly, Ramsay *et al.* (1987) suggested oestradiol-17β to be an important urinary oestrogen in the black rhinoceros by sequential hydrolysis and radioimmunoassay. In this study, whereas unconjugated oestradiol-17β was present in the urine, oestradiol-17α was also an abundant conjugated metabolite of the injected oestradiol-17β. Formation of a 17α-epimer from [^{3}H]oestradiol-17β has been demonstrated in the dog (Siegel *et al.*, 1962), and measurement of oestradiol-17α by immunoassay has been shown to reflect ovarian function accurately in the cow (Dobson & Dean, 1974) and ruffed lemur (Shideler *et al.*, 1983), and may be informative in the white rhinoceros.

Over 70% of radioactivity excreted into the faeces in this study was exclusively associated with oestrogenic steroids, and it is possible that this species can preferentially excrete oestrogens into the faeces as is the case in the bovine (Mellin and Erb, 1966). Steroid excretion in the African species of rhinoceros thus appears more like a ruminant than other species of Perrisodactyls, in which the majority of oestrogens are excreted via the urine. Only very small amounts of progesterone were excreted into the faeces, in marked contrast to the high levels of 20α-gestagens which have been measured in the faeces of the horse and other exotic equids (Schwarzenberger *et al.*, 1988) and the black rhinoceros (Bamberg and Schwarzenberger, 1990). However, the measurement of faecal oestrogens either collectively as total oestrogens (Möstl *et al.*, 1987) or individually as oestradiol-17α (Möstl *et al.*, 1984) may be useful in determining reproductive status in the white rhinoceros. Such methods have been used for pregnancy diagnosis in a variety of species, including exotic ungulates (Safar-Hermann *et al.*, 1987), and may also be applicable to the detection of pregnancy in the white rhinoceros.

In conclusion, it has been shown from this study that steroid metabolites are excreted into both the urine and the faeces of the

white rhinoceros. Species differences in steroid hormone metabolism and excretion have also been demonstrated between the Indian and the white rhinoceros. It is not possible to determine whether the results obtained in this study may also be applied to the black rhinoceros as, although the black and the white rhinoceroses inhabit the same areas of Africa, they are in fact separate species with different social and ecological requirements. However, from the limited data that is currently available on the metabolism and excretion of ovarian steroids in the black rhinoceros, the species would appear to be alike in this respect.

Clearly the results obtained from this study require confirmation by analysis of urine collected during natural cycles, but the identification of the major metabolites of injected oestradiol-17β and progesterone as described here, increases the knowledge available on the metabolism and excretion of ovarian steroids in the African species of rhinoceros. This knowledge may now form the basis for the development of improved hormone assays for assessing reproductive status and monitoring of the ovarian cycle in the African rhinoceroses.

CHAPTER 4

EXCRETION OF OESTROGEN AND PROGSTERONE METABOLITES DURING THE OVARIAN CYCLE AND PREGNANCY IN WHITE AND BLACK RHINOCEROSES

CHAPTER 4

EXCRETION OF OESTROGEN AND PROGSTERONE METABOLITES DURING THE OVARIAN CYCLE AND PREGNANCY IN WHITE AND BLACK RHINOCEROSES

4.1 INTRODUCTION

The results of the previous chapter provided information on the metabolic fate of exogenously administered, radiolabelled oestradiol-17β and progesterone in a white rhinoceros. The results indicated that steroids were excreted into the urine and that 20α-DHP, and not PdG, was the major urinary progesterone metabolite whilst ^{14}C-labelled oestradiol-17β was excreted as oestrone, oestradiol-17α and in the unmetabolised form.

The results, however, are based on the metabolism of injected material in a single animal. The objectives of the study described in this chapter, were to confirm the findings of the metabolism study by determining the urinary metabolites of endogenous oestradiol-17β and progesterone during the ovarian cycle and pregnancy in several individuals, and to extend the observations to both species of African rhinoceros.

The metabolism of endogenous ovarian steroids was studied by three different, but related, approaches. Urine samples were collected from female Indian, black and white rhinoceroses of known reproductive status and analysed by gas chromatography/mass spectrometry (GC/MS) in order to identify the major steroid metabolites. Secondly, the steroid components of urine from female rhinoceroses during the ovarian cycle and pregnancy were examined by co-chromatography on HPLC, to determine the major immunoreactive metabolites of ovarian steroids. Thirdly, the excretion of free and conjugated steroids and the relative proportions of different conjugated fractions was determined by sequential hydrolysis.

4.2 MATERIALS AND METHODS

4.2.1 GC/MS

Urine samples collected from female Indian, black and white rhinoceroses at various stages of the reproductive cycle were analysed by GC/MS. Because of the large sample volume (20 ml) required for this procedure, 2 out of the 5 samples analysed consisted of urine pooled from 4-5 separate collections over a short period of time. All urine samples, pooled or from a single collection, had similar creatinine contents (between 0·5 and 1·0 mg/ml), and were thus presumed to be of similar water content and dilution.

A large sample (20 ml) was collected from a single Indian rhinoceros and a single southern white rhinoceros during late pregnancy (2 months prior to birth). A second sample from the same southern white rhinoceros during the late stages of a preceeding pregnancy was formed by pooling urine collected over 4 weeks (6-10 weeks prior to parturition). A similar procedure was undertaken to acquire large urine samples from 2 black rhinoceroses during late pregnancy.

Urine samples were also collected during the ovarian cycle in one black and one northern white rhinoceros. One pooled sample was collected 1-3 days prior to mating, ie. during the presumed follicular phase of the ovarian cycle. A post-oestrus or luteal phase sample was pooled from urine collected between 5 and 9 days after mating.

A summary of the samples taken for GC/MS can be seen in Table 4.2. Urine samples (20 ml) were applied to a primed Sep-pak cartridge and prepared for GC/MS analysis by the procedure described in section 2·6.

4.2.2 HPLC

Table 4.1 shows the source of urine samples taken for HPLC analysis. All samples chosen for HPLC analysis had creatinine contents between 0·5 and 1·5 mg/ml.

To ensure that comparisons could be made between the results from different animals, certain criteria were used in the choice of urine samples for HPLC analysis. For identification of steroids excreted during pregnancy, urine samples collected during the early and late stages of gestation were analysed. All pregnancies from which urine samples were collected led to the birth of a live calf. Early pregnancy samples were those collected between 2 and 3 months following the observed mating that is thought to have led to conception. Although this may not be accurate in all cases, samples were back dated from the time of parturition to ensure that all animals were in the early stages of pregnancy. Late pregnancy urine samples were taken 2-3 months prior to parturition.

Urine samples from the presumed luteal phase were collected 7 days after oestrus and/or mating (oestrus+7; O+7). Samples were also collected on the third and first day prior to oestrus and/or mating (oestrus-1 and oestrus-3; O-1 and O-3) ie. during the presumed follicular phase of the ovarian cycle.

4.2.2.1 HPLC separation of conjugated progesterone metabolites

Samples from early and late pregnancy in one Indian rhinoceros were analysed. The late pregnancy sample was the same as previously analysed by GC/MS. One luteal phase sample was also analysed.

Of the African species, 2 urine samples were analysed from early pregnancy in each of one black and one northern white rhinoceros. In the black rhinoceros, late gestation samples were collected from 4 separate pregnancies in 4 females. One sample from

Table 4.1. Urine samples used for HPLC separation of conjugated (CP) and unconjugated (UCP) progesterone metabolites and unconjugated oestrogens (UCE). The samples which underwent both HPLC analysis and sequential enzyme hydrolysis (SEH) are also indicated. Subscript numbers denote samples from separate pregnancies/ovarian cycles from the same animal

species	animal	reproductive status	HPLC CP	HPLC UCP	HPLC UCE	SEH
Indian	1	0+7	✓	-	-	-
		early pregnant	✓	-	-	-
		late pregnant	✓	-	-	-
northern white	2	follicular (0-3)	-	-	✓	-
		follicular ($0-1^1$)	-	✓	✓	✓
		$0-1^2$	-	✓	✓	✓
		luteal ($0+7^1$)	✓	✓	-	✓
		$0+7^2$	✓	✓	-	✓
	3	0-3	-	-	✓	-
		$0-1^1$	-	✓	✓	✓
		$0-1^2$	-	✓	✓	✓
		$0+7^1$	✓	✓	-	✓
		$0+7^2$	✓	✓	-	✓
		early pregnant	✓	✓	✓	-
		early pregnant	✓	✓	✓	-
		late pregnant	✓	✓	✓	-
southern white	4	late pregnant^1	✓	✓	✓	-
		late pregnant^2	✓	✓	✓	-
black	6	0-3	-	-	✓	-
		0-1	-	✓	✓	✓
		0+7	✓	✓	-	✓
	7	0-3	-	-	✓	-
		0-1	-	✓	✓	✓
		0+7	✓	✓	-	✓
	8	0-3	-	-	✓	-
		0-1	-	✓	✓	✓
		0+7	✓	✓	-	✓
	9	0-1	-	✓	✓	✓
		0+7	✓	✓	-	✓
		early pregnant	✓	✓	✓	-
		early pregnant	✓	✓	✓	-
		late pregnant	✓	✓	✓	-
	10	late pregnant	✓	✓	✓	-
	11	late pregnant	✓	✓	✓	-
	12	late pregnant	✓	✓	✓	-

late pregnancy in a northern white rhinoceros and two samples from separate pregnancies in a southern white rhinoceros were analysed.

Urine samples (n=4) were analysed from the luteal phase of the cycle for each of the black and the northern white rhinoceros. Samples were collected from 4 separate black rhinoceroses, and during 2 separate luteal phases of 2 females of the northern white species.

HPLC was performed as described in section 2.5.1. After reconstitution of HPLC fractions in PAS gel buffer, part (0·1 ml) each fraction was placed in a scintillation vial with 5 ml scintillation fluid, and the radioactivity counted to determine the retention time of [^{3}H]PdG. A further 0·1 ml of each fraction was diluted (1:10 for luteal samples in all species and for pregnancy samples in the African rhinoceroses, and 1:20 for pregnancy samples in the Indian species) and 0·1ml taken to assay for PdG as described in section 2.12. The level of PdG immunoreactivity in each HPLC fraction (1 ml) was calculated.

4.2.2.2 HPLC separation of unconjugated progesterone metabolites

Hydrolysed progesterone metabolites in urine samples from black and white rhinoceroses at various stages of pregnancy and the ovarian cycle were separated by HPLC. In order to determine the presence of immunoreactive 20α-DHP in fractions collected from HPLC of hydrolysed rhinoceros urine, a highly sensitive EIA was developed (see section 2.13) as no specific method for the measurement of 20α-DHP was available.

HPLC was performed on the same samples as in section 4.2.2.1. In addition, follicular phase samples (0-1) from 4 cycles in 4 separate female black rhinoceroses, and 4 samples from 2 separate follicular phases in 2 northern white females were also analysed.

HPLC was performed as described in section 2.5.2. After reconstitution of HPLC fractions in assay buffer, the radioactivity in part (0·1 ml) of each fraction was counted to determine the retention time of [^{3}H]progesterone, [^{3}H]20α-DHP and [^{3}H]pregnanediol. A portion of each fraction (0·1 ml) was further diluted (1:50 for pregnancy and luteal phase samples, and 1:5 for follicular phase samples in the northern white rhinoceros; 1:5 and 1:2 respectively for the black rhinoceros) and duplicate aliquots of 0·05 ml were taken to assay for 20α-DHP as described in section 2.13. The level of 20α-DHP immunoreactivity in each HPLC fraction (1 ml) was calculated.

4.2.2.3 HPLC separation of unconjugated oestrogens

Urine samples from early and late pregnancy were the same as those analysed for progesterone metabolites (see above). HPLC was also performed on urine samples from the 2 stages of the follicular phase of the ovarian cycle (0-1 and 0-3). Follicular samples (n=3 at each stage) from 3 cycles in 3 separate female black rhinoceroses were analysed. In the northern white rhinoceros, samples (n=4) were collected from 2 separate follicular phases of 2 females.

Urine samples (1 ml) underwent HPLC as described in section 2.5.3. After reconstitution of HPLC fractions in assay buffer, the radioactivity in part (0·1 ml) of each fraction was counted to determined the retention time of [^{3}H]oestrone, [^{3}H]oestradiol-17α and [^{3}H]oestradiol-17β. A portion of each fraction (0·1 ml) was further diluted (1:50 for pregnancy samples and 1:25 for follicular phase samples in both species) and duplicate aliquots of 0·05 ml were taken to assay for total oestrogens as described in section 2.14. The level of oestrogen immunoreactivity, relative to oestrone standards, in each HPLC fraction (1 ml) was calculated.

4.2.3 Sequential enzyme hydrolysis and solvolysis

Sequential enzyme hydrolysis was performed on urine samples from black and white rhinoceroses as indicated in Table 4.1. Luteal samples (0+7) were collected from 5 separate ovarian cycles in 2 northern white females, and from 5 cycles in 4 black rhinoceroses. Follicular samples (0-1) were also analysed from each of these cycles.

Sequential enzyme hydrolysis of undiluted urine samples (0·2 ml) was carried out as described in section 2.3.4. Once the conjugates (hydrolysed by enzymatic procedures) had been removed from the urine by separate ether extractions, acid solvolysis (method as in section 2.3.5) was used to hydrolyse any conjugates remaining in the urine which were resistant to the action of enzymes.

Steroids removed by ether extraction (present in urine in the unconjugated form) and those liberated by β-glucuronidase (previously conjugated as glucuronides), sulphatase (previously conjugated as sulphates) and acid solvolysis (previously present as conjugates which could withstand enzyme hydrolysis procedures) were reconstituted in assay buffer (1 ml). Duplicate portions of each extract (0·05 ml) obtained by sequential hydrolysis and solvolysis of samples collected during the luteal phase of the ovarian cycle were assayed for 20α-DHP immunoreactivity (see section 2.13). Duplicate portions (0·1 ml) of each extract from samples collected from the follicular phase of the ovarian cycle in the black rhinoceros were assayed for oestrone immunoreactivity as described in section 2.6. Similarly, duplicate portions (0·1 ml) of each extract from samples collected during the follicular phase in the northern white rhinoceros were assayed for oestradiol-17β immunoreactivity as described in section 2.11.

A value for total immunoreactivity was obtained by the summation of immunoreactivity in unconjugated, glucuronide, sulphate

and residual (hydrolysed by solvolysis) fractions. The amount measured in each fraction was then expressed as a percentage of the total value.

4.3 RESULTS

4.3.1 GC/MS

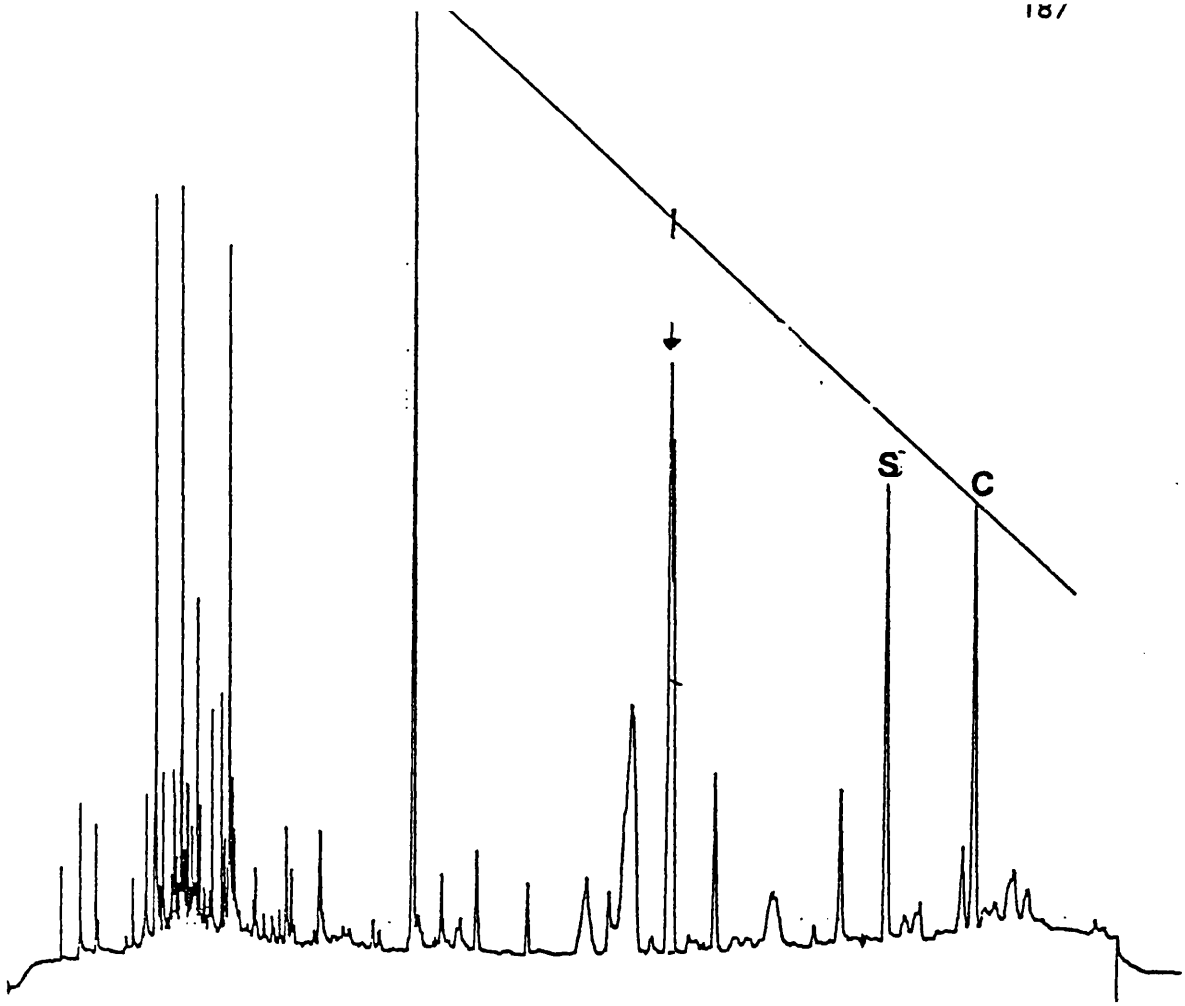
The gas chromatogram, total ion current chromatogram (TIC) and the selected ion current chromatogram (SIC) of the O-methyloxime-trimethylsilyl ether of 20α-hydroxy-4-pregnen-3-one formed by GC/MS are shown in Fig 4.1 (a, b and c respectively), to illustrate the method of identification and quantification of steroids by this procedure. The retention time of the derivatized 20α-DHP standard on the GC is shown on the gas chromatogram in relation to the internal standards 5α-androstane-3α,17α-diol, stigmasterol and cholesterol butyrate (A, S and C respectively). From the GC the quantity of steroid injected was determined as 4·1 ng by comparison to the 5 ng of internal standard added prior to derivatization. Considering that an accurate mass of 10·00 µg 20α-DHP was derivatized, and that 1/1000 of the derivatized product was injected onto the machine, the recovery of 20α-DHP (41·0%) was low. The SIC of steroidal peaks on the TIC were used to identify steroids. The ion of the highest mass in the spectrum is the molecular ion (M^+), in this case at mass/charge (m/z) 417. All other ions are formed from the fragmentation of the molecular ion. The ion at m/z 153 provides important information on the structure of the compound, which includes a pregnane group. The strong ion at m/z 117, indicates the presence of a methyl ketone group. By compiling a library of the mass spectra of pure standards, derivatized steroids in urine samples may be identified.

The TIC of the progestagen fraction, separated by Sephadex LH-20 chromatography of hydrolysed urine collected from a pregnant Indian rhinoceros is shown in Fig. 4.2a. Four peaks on the TIC

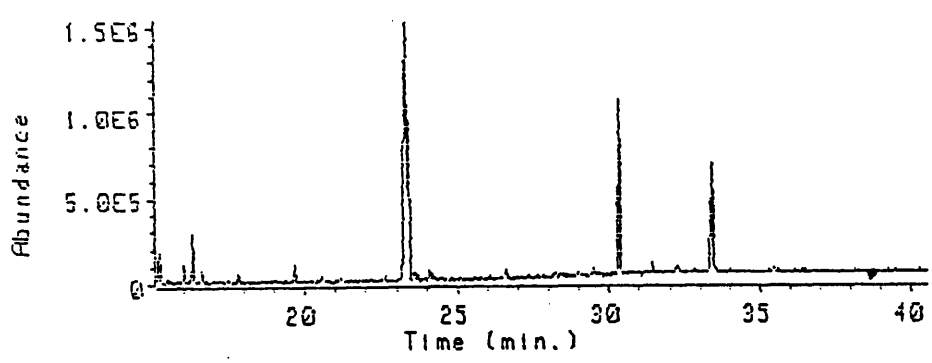
Figure 4.1a. Gas chromatogram of the O-methyloxime-trimethylsilyl ether of 4-pregnen-20α-ol-3-one (20α-DHP). A, S and C represent the elution positions of the internal standards (5α-androstane-3α, 17α-diol, stigmasterol and cholesterol butyrate respectively). The quantity of 20α-DHP was calculated by relating its peak height to that of a line drawn between the peak heights of the internal standards. An arrow indicates the 20α-DHP peak.

Figure 4.1b. Total ion current chromatogram of a derivatised sample of pure 20α-DHP standard produced by mass spectrometry.

Figure 4.1c. Selected ion current chromatogram of the O-methyloxime-trimethylsilyl ether of 20α-hydroxy-4-pregnen-3-one


S
C

b


1.5E6
1.0E6
5.0E5
0
Abundance
20
25
30
35
40
Time (min.)

c

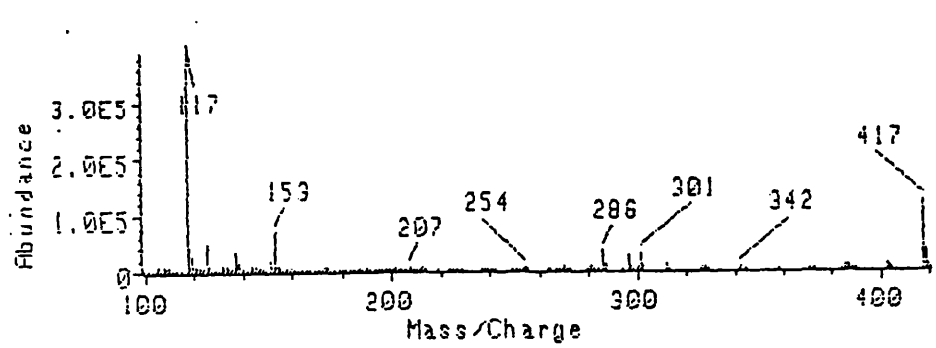
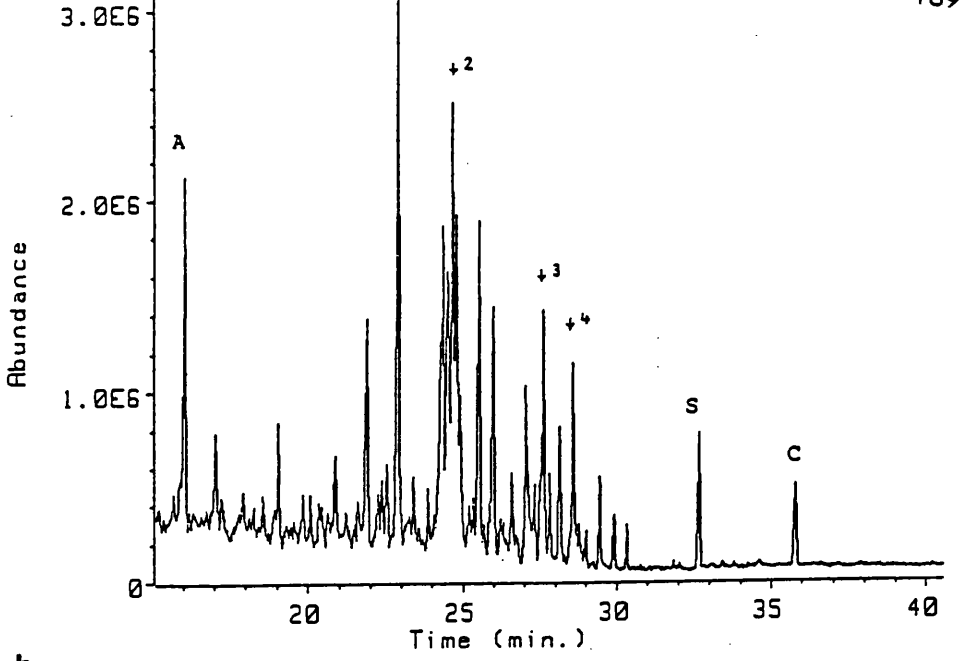
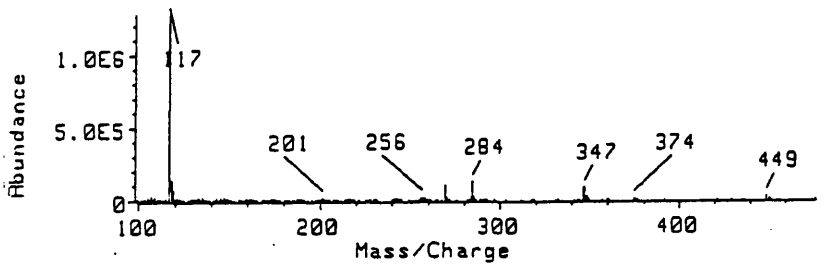

3.0E5
2.0E5
1.0E5
0
Abundance
117
153
207
254
286
301
342
417
100
200
300
400
Mass/Charge

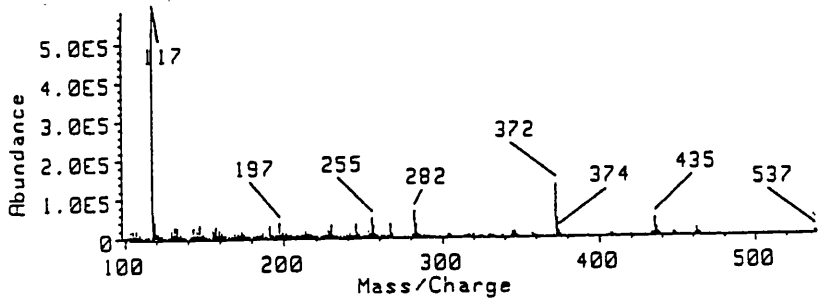
Figure 4.2. Total ion current chromatogram (a) representing the separation of neutral steroid derivatives in urine collected during the third trimester of pregnancy in an Indian rhinoceros. The selected ion current chromatograms for peaks 1-4 (indicated by the arrows on the TIC) are shown in Figures 4.2b,c,d and e. Peak 1 is 5β-pregnanediol, peak 2 is pregnanetriol, peak 3 is 5β-pregn-3α, 17, 20β, 21 tetrol and peak 4 is 17α-hydroxypregnanolone.


3.0E6
2.0E6
1.0E6
0
Abundance
A
+2
+3
+4
S
C
20
25
30
35
40
Time (min.)

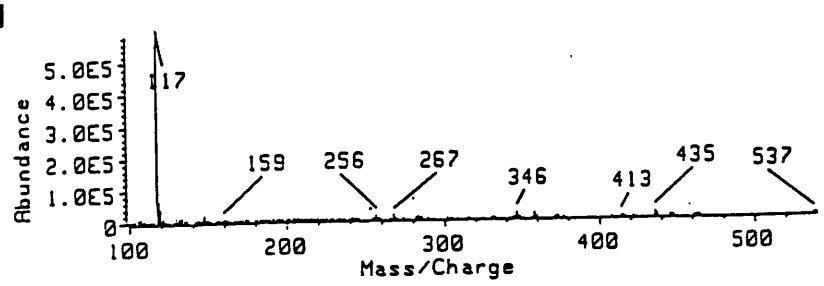
b


1.0E6
5.0E5
0
Abundance
117
201
256
284
347
374
449
100
200
300
400
Mass/Charge

c


5.0E5
4.0E5
3.0E5
2.0E5
1.0E5
0
Abundance
117
197
255
282
372
374
435
537
100
200
300
400
500
Mass/Charge

d


5.0E5
4.0E5
3.0E5
2.0E5
1.0E5
0
Abundance
117
159
256
267
346
413
435
537
100
200
300
400
500
Mass/Charge

e

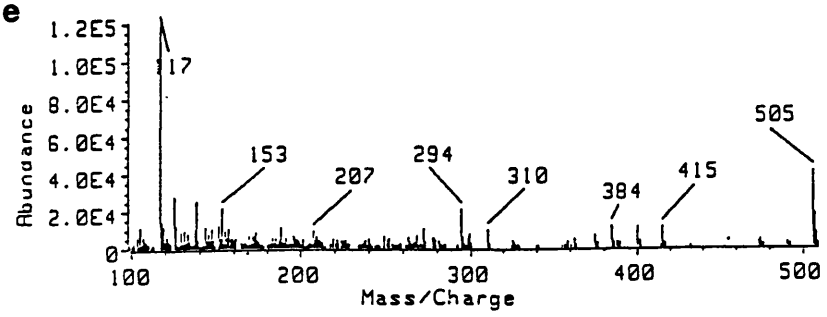
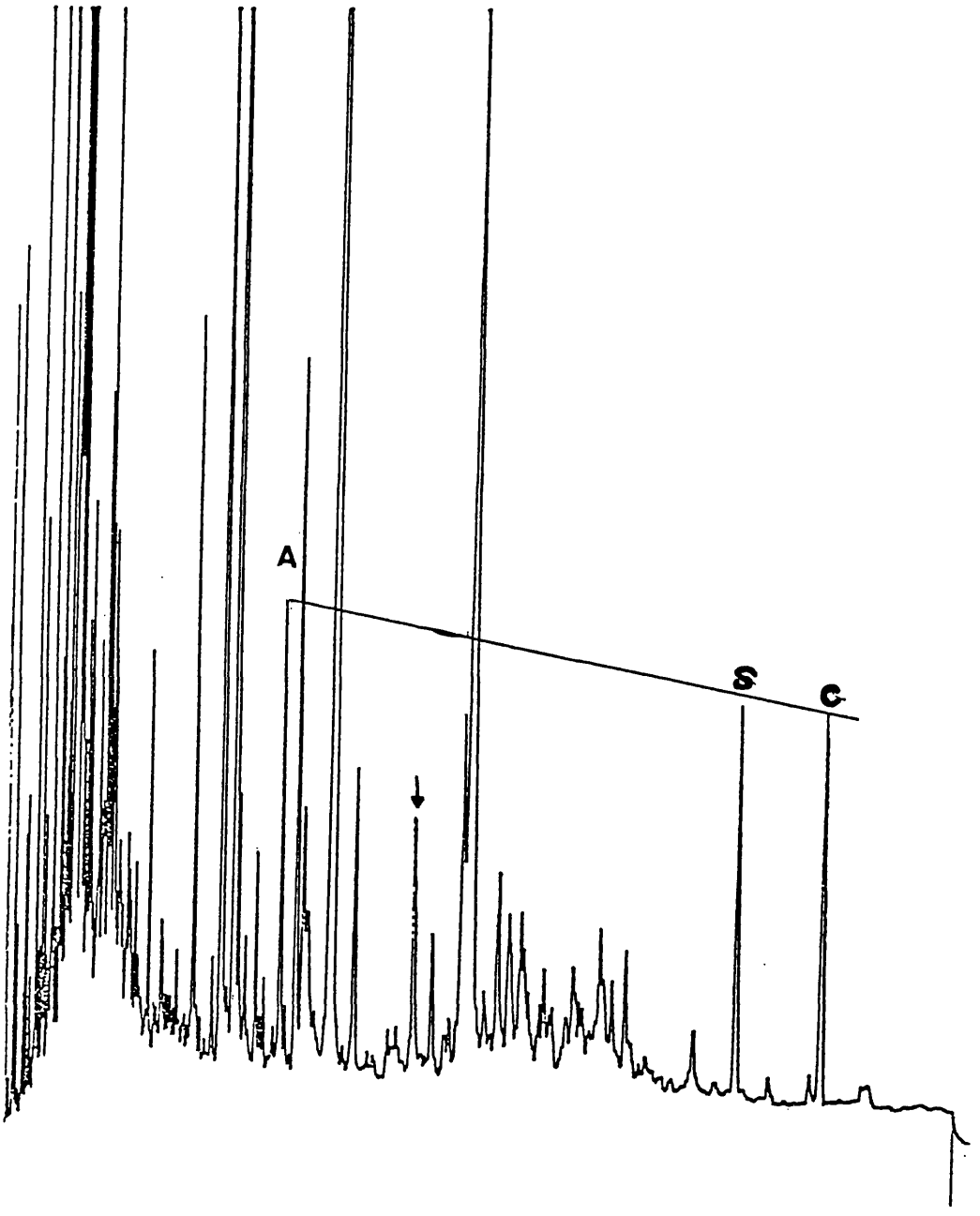

1.2E5
1.0E5
8.0E4
6.0E4
4.0E4
2.0E4
0
Abundance
117
153
207
294
310
384
415
505
100
200
300
400
500
Mass/Charge

Figure 4.3. Gas chromatogram of the O-methyloxime-trimethylsilyl ethers of neutral steroids in pregnant Indian rhinoceros urine. The position of the internal standards are indicated by A,S and C. The peak indicated by the arrow represents 5β-pregnanediol, the most abundant progesterone metabolite in this urine sample.

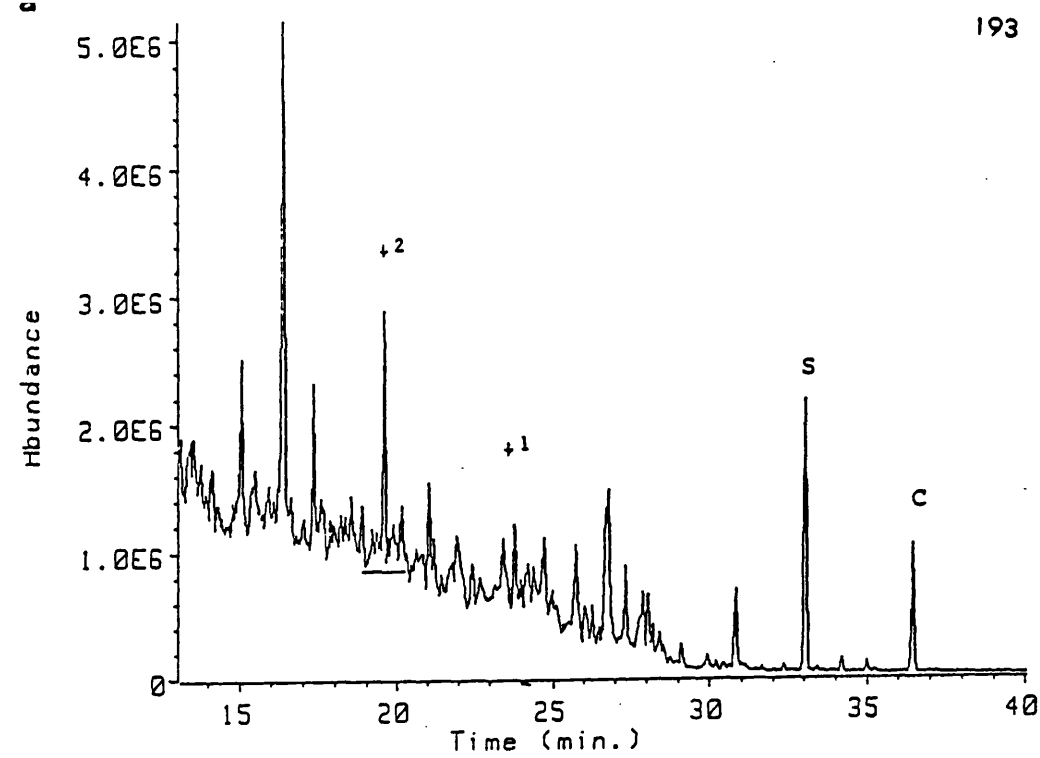


were seen to have characteristic steroidal structures and could be identified from the ionic spectra and relative abundance of the molecular ion as O-methyloxime-trimethylsilyl ethers of progestagen metabolites. The most abundant derivatised progesterone metabolite was identified as 5β-pregnanediol (M^+, m/z=449; Fig. 4.2 b) which had a retention time of 22·922 min and the same ionic spectrum as determined by mass spectrometry of the pure standard. From the GC (Fig. 4.3) of this sample, the amount of 5β-pregnanediol was 208 ng/ml.

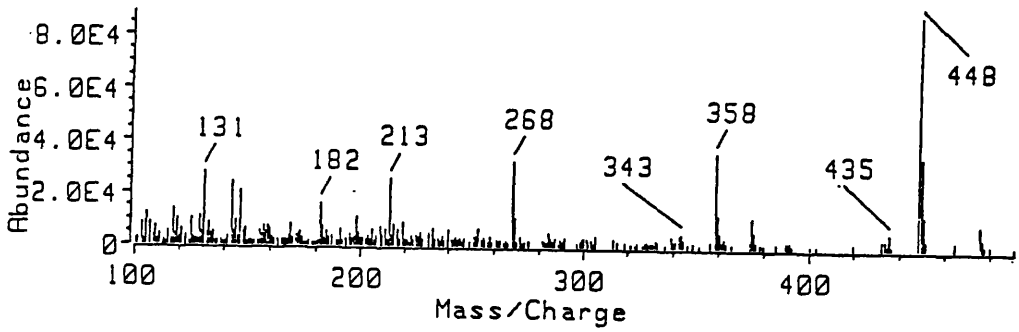
The other neutral steroids identified were less abundant in relation to pregnanediol, but remained significant progesterone metabolites in the urine. The SIC of peak 2 (Fig. 4.2c) was used to identify this metabolite as as a 5β-pregnanetriol (M^+, m/z=537; Quilliam and Westmore, 1980). Peak 3 (Fig. 4.2d) was identified as 5β-pregn-3α, 17, 20β, 21 tetrol with a retention time of 27·604 min and peak 4 (Fig. 4.2e) represented 17α-hydroxypregnanolone with a retention time of 28·587 min (M^+, m/z=415).

The TIC of neutral steroids in the urine of a southern white rhinoceros collected during late pregnancy is shown in Fig. 4.4a. The higher baseline values for the abundance of urinary compounds indicates that this sample contained more components than that of the Indian rhinoceros. Despite the fact that a concentration of 105 ng PdG/ml had been measured in this urine sample by EIA, none of the peaks seen on the TIC had an ionic spectrum corresponding to the O-methyloxime-trimethylsilyl derivative of 5β-pregnanediol. Indeed, the most abundant steroid in the urine (peak 2) was identified as androsterone from the ionic spectrum, shown in Fig. 4.4 b, with a retention time of 19·574 min and a M^+, m/z=376. From the GC (not shown) the estimated concentration of androsterone was 135 ng/ml. The only other steroid in the neutral fraction of the urine was 11β-hydroxyandrosterone with a retention time of 23·797 min (peak 1) and an ionic spectrum shown in Fig. 4.4c. No steroids were detected in the second pregnancy urine sample from this animal.

Figure 4.4. Total ion current chromatogram (a) representing the separation of neutral steroid derivatives in urine collected during late pregnancy in a southern white rhinoceros. The selected ion current chromatograms for peaks 1 and 2 are shown in Figures 4.4b and c. Peak 1 is 11β-hydroxyandrosterone and peak 2 is androsterone.


5.0E6
4.0E6
3.0E6
2.0E6
1.0E6
0
Abundance
+2
+1
S
C
15
20
25
30
35
40
Time (min.)

b


8.0E4
6.0E4
4.0E4
2.0E4
0
Abundance
131
182
213
268
343
358
435
448
100
200
300
400
Mass/Charge

c

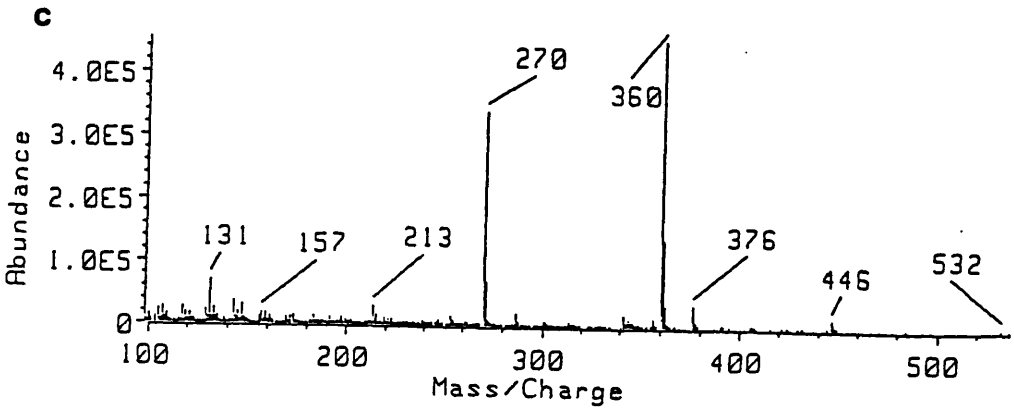
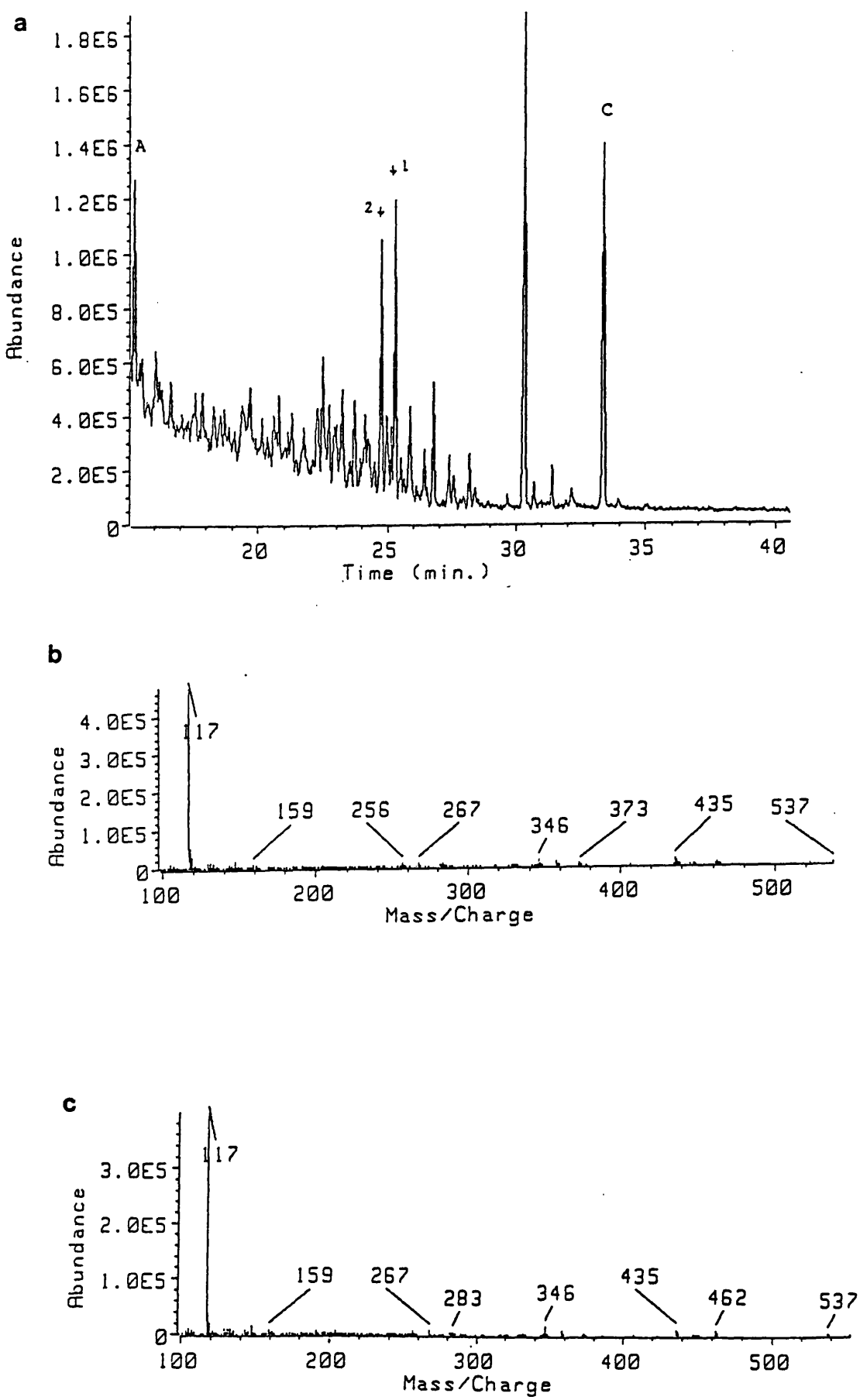

4.0E5
3.0E5
2.0E5
1.0E5
0
Abundance
131
157
213
270
360
376
446
532
100
200
300
400
500
Mass/Charge

Figure 4.5. Total ion current chromatogram (a) representing the separation of neutral steroid derivatives in urine collected during late pregnancy in a black rhinoceros. The selected ion current chromatograms for peaks 1 and 2 are shown in Figures 4.5b and c. Peak 1 is 5β-pregn-3α, 17, 20β, 21 tetrol, and peak 2 is another pregnanetetrol which could not be identified from the library of steroids available.


a
Abundance
1.8E6
1.6E6
1.4E6
1.2E6
1.0E6
8.0E5
6.0E5
4.0E5
2.0E5
0
A
1
2
C
20
25
30
35
40
Time (min.)
b
Abundance
4.0E5
3.0E5
2.0E5
1.0E5
0
117
159
256
267
346
373
435
537
100
200
300
400
500
Mass/Charge
c
Abundance
3.0E5
2.0E5
1.0E5
0
117
159
267
283
346
435
462
537
100
200
300
400
500
Mass/Charge

PdG could not be detected in urine collected from black rhinoceroses during late pregnancy. Furthermore, none of the steroids identified in samples from the white species at the same stage of gestation were present in the black rhinoceros urine. In both samples the only steroid peaks (Fig. 4.5a) represented O-methyloxime-trimethylsilyl derivatives of two isomers of pregnane tetrol (ionic spectra shown in Figs. 4.5 b and c), one of which was positively identified from the analysis of pure standards, as 5β-pregn-3α,17,20β,21-tetrol. The total concentration of pregnanetetrol excreted by the pregnant rhinoceros, as determined from the GC, as 66 ng/ml. A similar ionic spectrum was obtained for the only steroidal peak detected by GC/MS analysis of urine from a black rhinoceros in the presumed luteal phase of the ovarian cycle (Fig. 4.6). Although the ionic spectrum obtained for this steroid (Fig. 4.6b) was not identical to that in Fig. 4.5b , the M^+ (m/z=537) was present, and the steroid had the same retention time with respect to the internal standards as 5β-pregn 3α, 17, 20β, 21 tetrol. The concentration of pregnanetetrol in this luteal sample was 38 ng/ml. This was the only urine sample from the ovarian cycle of either species of African rhinoceros to contain any peaks that were identifiable as steroids.

The results of the GC/MS analysis are summarised in Table 4.2. The most abundant urinary steroid during pregnancy in the Indian rhinoceros was identified as 5β-pregnanediol. In the African species, no pregnanediol was detected in the urine from either pregnancy or the ovarian cycle; of those steroids that were detected, androsterone was the major urinary steroid during pregnancy in the white rhinoceros whilst pregnanetetrol was the only steroid identified in the black species.

Identification of oestrogens using GC/MS was not possible due to very high concentrations of phyto-oestrogens (eg. equol), lignins and other dietary compounds which were present in the phenolic fractions eluted from Sephadex LH-20. The dilution of samples to reduce the quantity of contaminents to suitable levels to prevent

Figure 4.6a. Total ion current chromatogram representing the separation of neutral steroid derivatives in urine collected during the luteal phase of the ovarian cycle in a black rhinoceros.

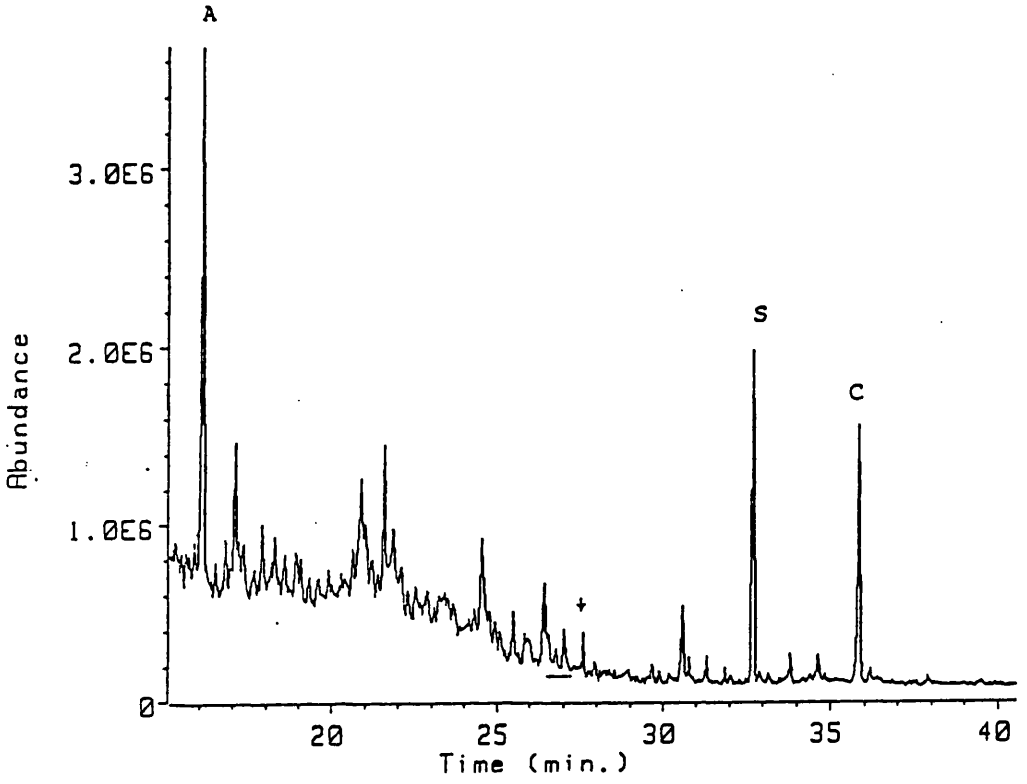


Figure 4.6b. Selected ion current chromatograms of peak 1, representing the O-methyloxime-trimethysilyl ether of 5β-pregn 3α, 17, 20β, 21 tetrol.

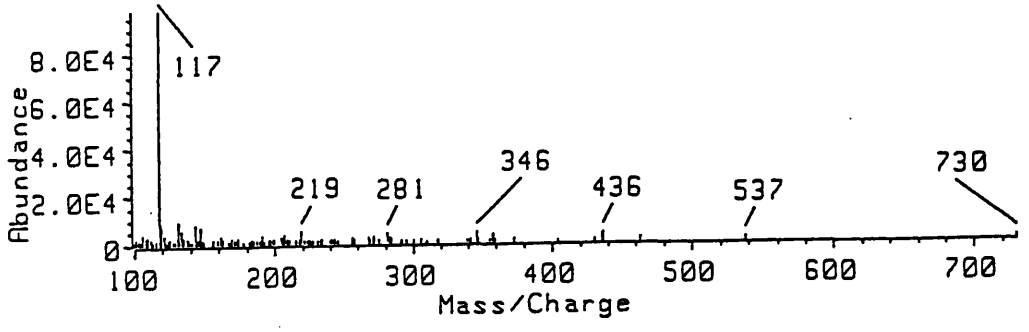


Table 4.2. Summary of the results obtained by GC/MS identification of steroid metabolites in rhinoceros urine. The steroids present in each sample represent the only detectable peaks of a steroidal nature in the sample, and are listed in order of relative abundance.

species	animal	reproductive status	steroids identified
Indian	1	pregnant	5β-pregnanediol 5β-pregnane 3α,17α,20α-triol 5β-pregn-3α,17,20β,21-tetrol 17α-hydroxypregnanolone
southern white	4	pregnant[1] pregnant[2]	no steroids detected androsterone 11β-hydroxyandrosterone
northern white	3	follicular luteal	no steroids detected no steroids detected
black	9	pregnant follicular luteal	pregnanetetrols no steroids detected 5β-pregn-3α,17,20β,21-tetrol
	10	pregnant	pregnanetetrols

damage to the machine would have prevented the detection of estimated concentrations of oestrogens.

4.3.2 HPLC analysis of urinary steroids during the ovarian cycle

4.3.2.1 Progesterone metabolites

Elution profiles of immunoreactive progesterone metabolites in urine collected from Indian and African rhinoceroses during the ovarian cycle are shown in Figs. 4.7-4.11.

The HPLC elution profile of PdG immunoreactivity in the urine of an Indian rhinoceros during the presumed luteal phase of the ovarian cycle is shown in Fig. 4.7. The position of the [^{3}H]PdG standard is shown by the dotted line, indicating a retention time of 4·5 min for PdG. Low levels of immunoreactivity were seen in many of the HPLC fractions, but a single prominent peak of immunoreactivity was present in fraction 9, which corresponded with the [^{3}H]PdG standard. The co-elution of PdG immunoreactivity with the tritiated PdG marker gave a good indication that PdG was present in the urine of the Indian rhinoceros during the luteal phase of the cycle.

Representative elution profiles of PdG immunoreactivity in urine collected from northern white and black rhinoceroses during the presumed luteal phase of the cycle are shown in Fig. 4.8 (a and b respectively). In contrast to the sample from the Indian rhinoceros, no immunoreactivity co-eluted with the [^{3}H]PdG marker, ie. in fractions 9 and 10. The absence of immunoreactivity in the fractions containing the tritiated PdG standard suggested that no immunoreactive PdG was present in these urine samples, ie. PdG was not present in urine collected during the luteal phase of the ovarian cycle of the white or the black rhinoceros. However, HPLC indicated the presence of a substance in the urine of the African rhinoceroses during the luteal phase of the cycle that cross reacts with the PdG antiserum. The substance is indicated by relatively

Figure 4.7. Elution profile showing the levels of PdG immunoreactivity in 60 fractions collected over a period of 30 min during the HPLC of urine collected from an Indian rhinoceros during the presumed luteal phase of the ovarian cycle. The elution profile of [^{3}H]PdG is indicated by the dotted line.

In all the following profiles, open circles denote concentrations below the assay sensitivity.

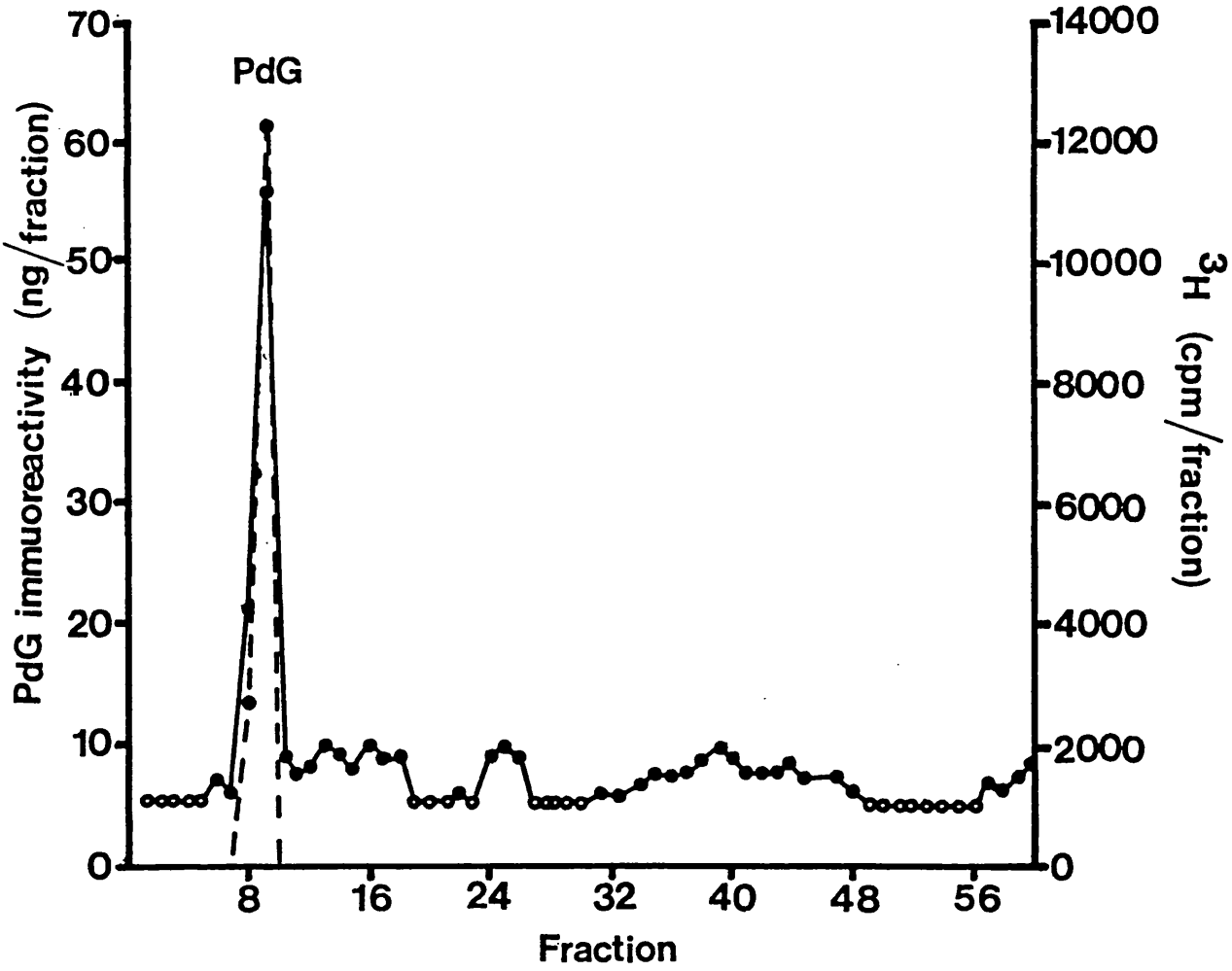
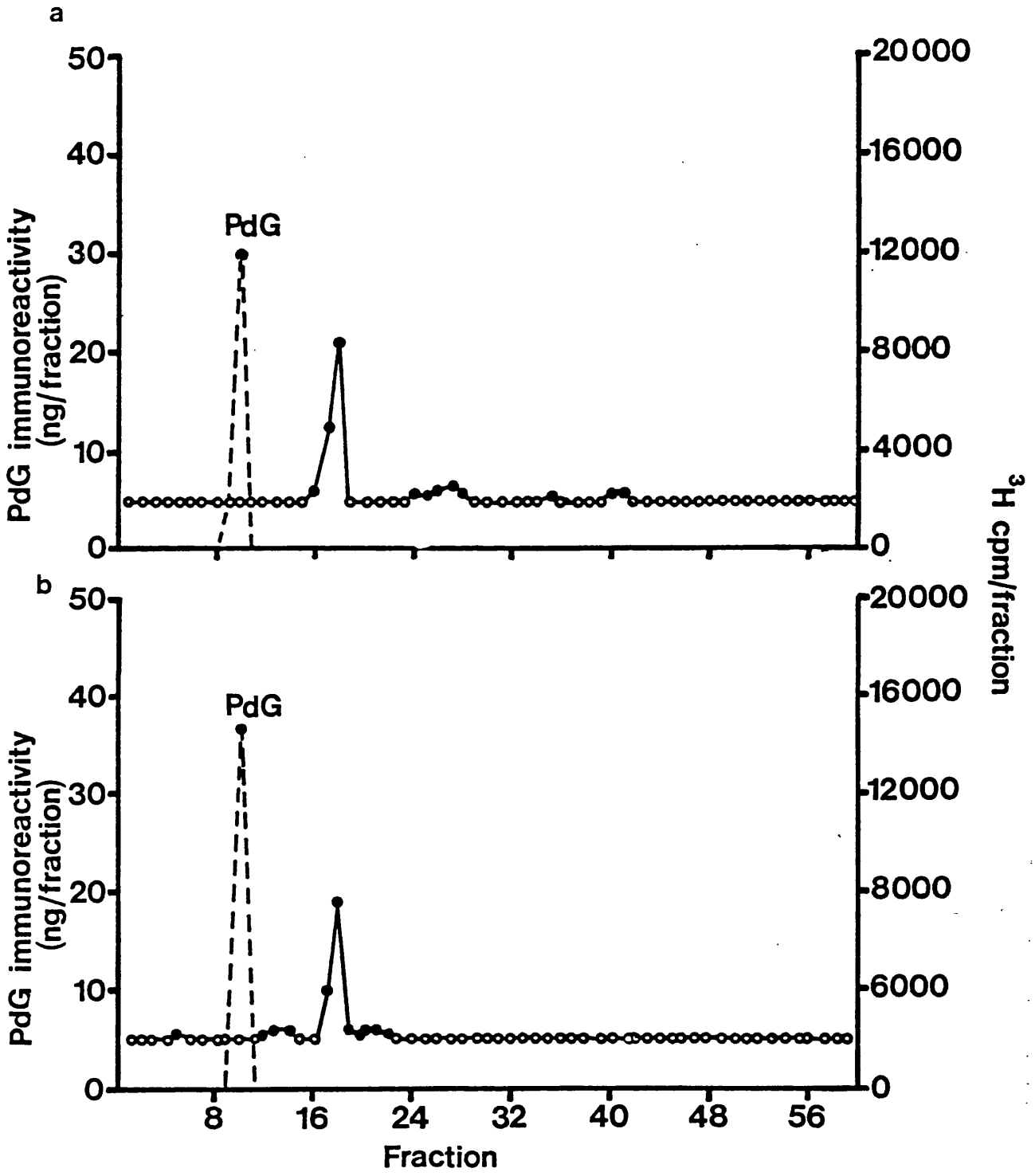


Figure 4.8a and b. Representative HPLC elution profiles of PdG immunoreactivity in urine collected from northern white and black rhinoceroses (a and b respectively) during the presumed luteal phase of the ovarian cycle (O+7).


a
b
PdG
PdG
PdG immunoreactivity (ng/fraction)
PdG immunoreactivity (ng/fraction)
^{3}H cpm/fraction
Fraction
0
10
20
30
40
50
0
4000
8000
12000
16000
20000
0
6000
8000
12000
16000
20000
8
16
24
32
40
48
56

high levels of PdG immunoreactivity with a longer retention time on HPLC than [^{3}H]PdG, ie. in fractions 16-18. Furthermore, the cross-reacting substance was present in all luteal phase urine from both species (n=8), and continued to elute with the same retention time with respect to [^{3}H]PdG in all samples analysed. The identity of this cross reacting substance was not determined in this study.

The elution profiles of 20α-DHP immunoreactivity in urine collected from a northern white rhinoceros during the presumed follicular (a) and luteal (b) phase of the ovarian cycle are shown in Fig. 4.9. The elution positions of [^{3}H]labelled progesterone (a), 20α-DHP (b) and pregnanediol (c) are shown by the dotted lines. No 20α-DHP immunoreactivity could be detected in any fraction resulting from the HPLC analysis of urine samples from 2 separate follicular phases. However, in both urine samples from the post-oestrus period, a single peak of 20α-DHP immunoreactivity was detected in the HPLC fractions containing the [^{3}H]20α-DHP marker (fractions 31 and 32), indicating the presence of 20α-DHP in the urine of the northern white rhinoceros during the luteal phase of the ovarian cycle.

Elution profiles of 20α-DHP immunoreactivity in fractions collected from HPLC of urine from the ovarian cycle of a second northern white female are shown in Fig. 4.10. Analysis of luteal phase urine revealed a single peak of 20α-DHP immunoreactivity that co-eluted with the radioactive 20α-DHP marker (4.10b). However, HPLC analysis of urine collected during the pre-oestrus period resulted in a different pattern of elution of 20α-DHP immunoreactivity between this animal and the female previously investigated. Although neither of the presumed follicular samples contained 20α-DHP immunoreactivity which co-eluted with [^{3}H]20α-DHP, a separate peak of immunoreactivity was seen in each of the samples analysed, which consistently eluted 4 fractions later than the 20α-DHP marker. This immunoreactivity indicated the presence of a substance in the urine of this animal that cross reacted with the 20α-DHP antibody. However, it is was not possible to say whether

Figure 4.9a and b. HPLC elution profiles of 20α-DHP immunoreactivity in hydrolysed urine collected from a northern white rhinoceros during the presumed follicular and luteal phases of the ovarian cycle (a and b respectively). The elution profile of ^{3}H-labelled progesterone (a), 20α-DHP (b) and pregnanediol (c) is indicated by the dotted line.

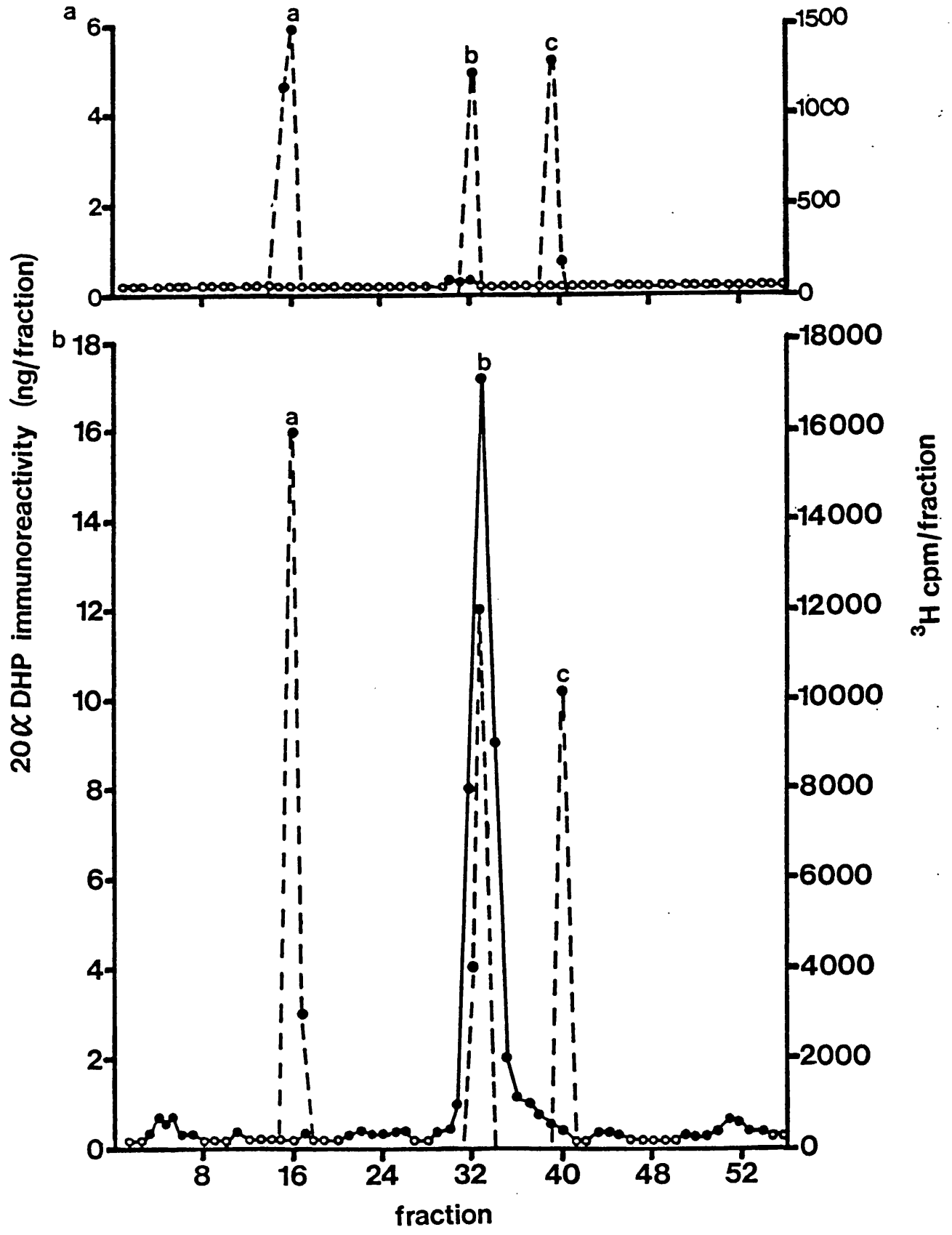

a
b
c
20α DHP immunoreactivity (ng/fraction)
^{3}H cpm/fraction
fraction
0
2
4
6
8
10
12
14
16
18
500
1000
1500
2000
4000
6000
8000
10000
12000
14000
16000
18000
8
16
24
32
40
48
52

Figure 4.10 a and b. HPLC elution profiles of 20α-DHP immunoreactivity in hydrolysed urine collected from a second northern white rhinoceros (animal 3) during the follicular and luteal phase of the ovarian cycle (a and b respectively).

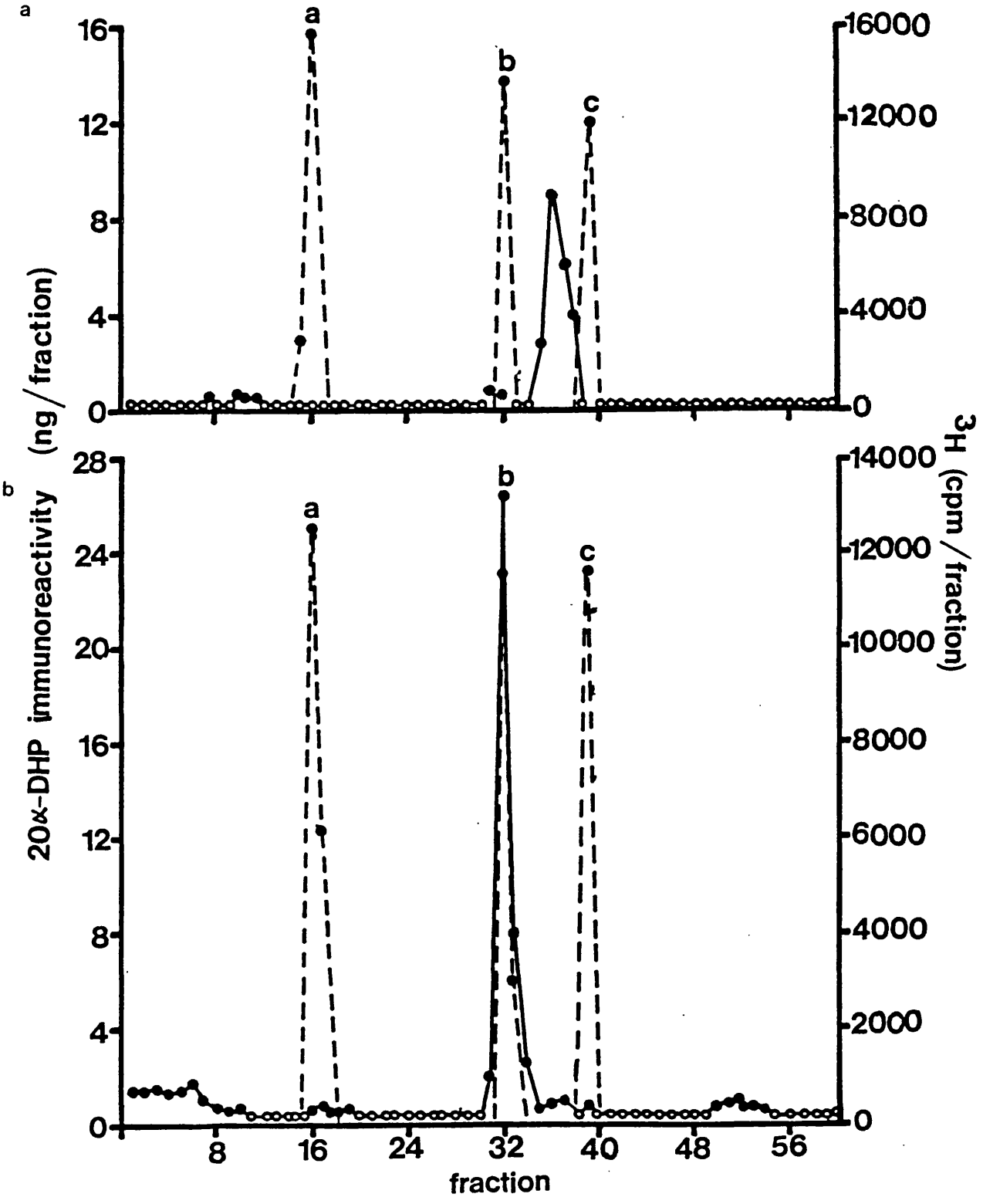
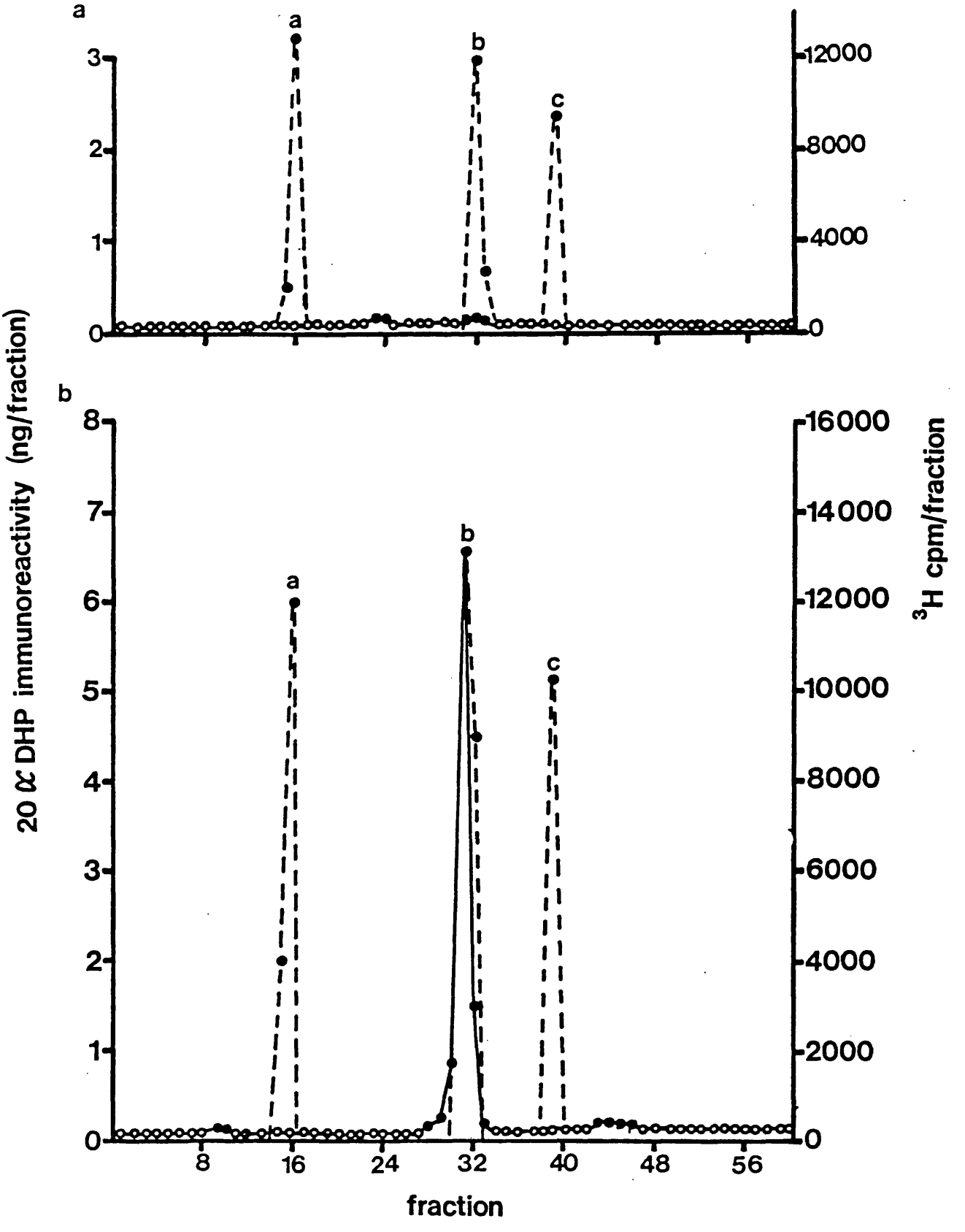

a
b
a
b
c
16
12
8
4
0
16000
12000
8000
4000
0
28
24
20
16
12
8
4
0
14000
12000
10000
8000
6000
4000
2000
0
20α-DHP immunoreactivity (ng/fraction)
^{3}H (cpm/fraction)
8
16
24
32
40
48
56
fraction

Figure 4.11 a and b. Representative HPLC elution profiles of 20α-DHP immunoreactivity in hydrolysed urine collected from black rhinoceroses during the follicular and luteal phase of the ovarian cycle (a and b respectively).


a
a
b
c
3
2
1
0
12000
8000
4000
0
b
8
7
6
5
4
3
2
1
0
a
b
c
16000
14000
12000
10000
8000
6000
4000
2000
0
8
16
24
32
40
48
56
fraction
20 α DHP immunoreactivity (ng/fraction)
^{3}H cpm/fraction

this cross-reacting substance was present only in the follicular phase samples, as greater dilution of luteal samples prior to assay may have prevented the detection of this immunoreactivity.

Results of equivalent HPLC analysis of samples from black rhinoceroses are shown in Fig. 4.11. HPLC of follicular phase samples (O-1; 4 samples from 4 animals) detected no immunoreactivity in any of the samples. A single peak of immunoreactivity co-eluting with [^{3}H]20α-DHP (ie. in fraction 31) was detected in all luteal samples analysed (n=4), indicating the presence of 20α-DHP in the urine of the black rhinoceros during the post-oestrus period.

The results for the HPLC analysis of urinary progesterone metabolites are summarised in Table 4.3. The levels of PdG immunoreacitivity determined during the luteal phase of the cycle in the Indian rhinoceros represents a direct measurement of PdG. However, PdG immunoreactivity detectable in the urine of the African species of rhinoceros at this time is due to the presence of a compound that cross-reacts with the PdG antiserum and is not a direct measurement of PdG. In all urine samples collected from African rhinoceroses, 20α-DHP was present during the luteal phase and absent during the pre-oestrus period. The exception was one female northern white rhinoceros in which 20α-DHP immunoreactivity was detectable in the follicular phase of the cycle although this immunoreactivity was not due to the presence of 20α-DHP but a cross-reacting substance.

4.3.2.2 Oestrogens

The elution profiles of oestrogen immunoreactivity in urine from African rhinoceroses during the ovarian cycle are shown in Figs. 4.12 and 4.13.

Profiles of immunoreactive oestrogens in fractions eluted from HPLC of hydrolysed urine samples collected from a female northern

Table 4.3. Summary of the results obtained by HPLC analysis of urine samples collected from female rhinoceroses at various stages of the reproductive cycle. A ✓ indicates the presence of immunoreactivity co-eluting with the respective ^{3}H-labelled standard (*), an X indicates that no immunoreactivity co-eluted with the tritiated standard. Samples that were not analysed are identified (-). Immunoreactivity in fractions other than those containing the radiolabel is noted, and the retention time with respect to the radiolabel recorded.

species	animal	stage cycle	PdG immunoreactivity		20α-DHP immunoreactivity	
			PdG*	other (ret. time wrt PdG)	20α-DHP*	other (ret. time wrt 20α)
Indian	1	luteal	✓	-	-	-
black	6	follicular	-	-	X	-
		luteal	X	7&8	✓	-
"	7	follicular	-	-	X	-
		luteal	X	7	✓	-
"	8	follicular	-	-	X	-
		luteal	X	6-8	✓	-
"	9	follicular	-	-	X	-
		luteal	X	7&8	✓	-
northern white	2	follicular	-	-	X	-
		follicular	-	-	X	-
		luteal	X	6&7	✓	-
		luteal	X	6-8	✓	-
"	3	follicular	-	-	X	4
		follicular	-	-	X	4
		luteal	X	7	✓	-
		luteal	X	6-8	✓	-

white rhinoceros 3 days and 1 day prior to mating are shown in Fig. 4.12. The elution positions of [^{3}H]labelled oestrone (a), oestradiol-17α (b) and oestradiol-17β (c) are shown by the dotted line. In the first profile (O-3) no oestrogen immunoreactivity was seen in the fraction containing the oestradiol-17α standard (fraction 18), but immunoreactivity co-eluted with both the [^{3}H]oestrone and [^{3}H]oestradiol-17β markers. Although the cross-reactivity of the antiserum with these oestrogens differs in the EIA system used (100% with oestrone versus 161% with oestradiol-17β), it is possible to estimate the relative proportions of each steroid and identify oestradiol-17β as the most abundant oestrogen in urine collected 3 days prior to oestrus. Analysis of urine collected on the day prior to oestrus (Fig. 4.12 b) also showed oestrogen immunoreactivity in HPLC fractions containing radiolabelled oestrone and oestradiol-17β, with no immunoreactivity co-eluting with [^{3}H]oestradiol-17α. The constant creatinine content, indicating consistent water content of the samples, allowed comparison of oestrogen levels between the samples. Oestradiol-17β concentrations increased over the 3 days prior to oestrus, whilst oestrone levels remained constant. A similar pattern of excretion of oestrogens over the 3 days prior to oestrus was seen for the second northern white female under investigation.

An example of the elution profiles obtained after HPLC separation of urinary oestrogens in follicular phase urine from black rhinoceroses (6 samples from 3 females) is shown in Fig. 4.13. Oestrogen immunoreactivity was seen to co-elute consistently with [^{3}H]oestrone, with higher levels being observed in the samples collected on the day prior to the onset of behavioural oestrus than in those collected 3 days prior to oestrus. No oestrogen immunoreactivity was detectable in the fractions containing [^{3}H]oestradiol-17β in any of the samples tested. Small amounts of immunoreactivity were measurable in the position of the oestradiol-17α marker in urine collected 3 days prior to oestrus in 2 out of the 3 females.

Figure 4.12 a and b. Representative HPLC elution profiles of oestrogen immunoreactivity in hydrolysed urine collected from northern white rhinoceroses 3 days prior to mating (a) and the day prior to mating (b). The level of ^{3}H-radioactivity in each fraction is indicated by the dotted line. The peak of radioactivity with the shortest retention time represents [^{3}H]oestrone whilst the second and third peaks represent [^{3}H]oestradiol-17α ($E_2$17α) and [^{3}H]oestradiol-17β ($E_2$17β) respectively.

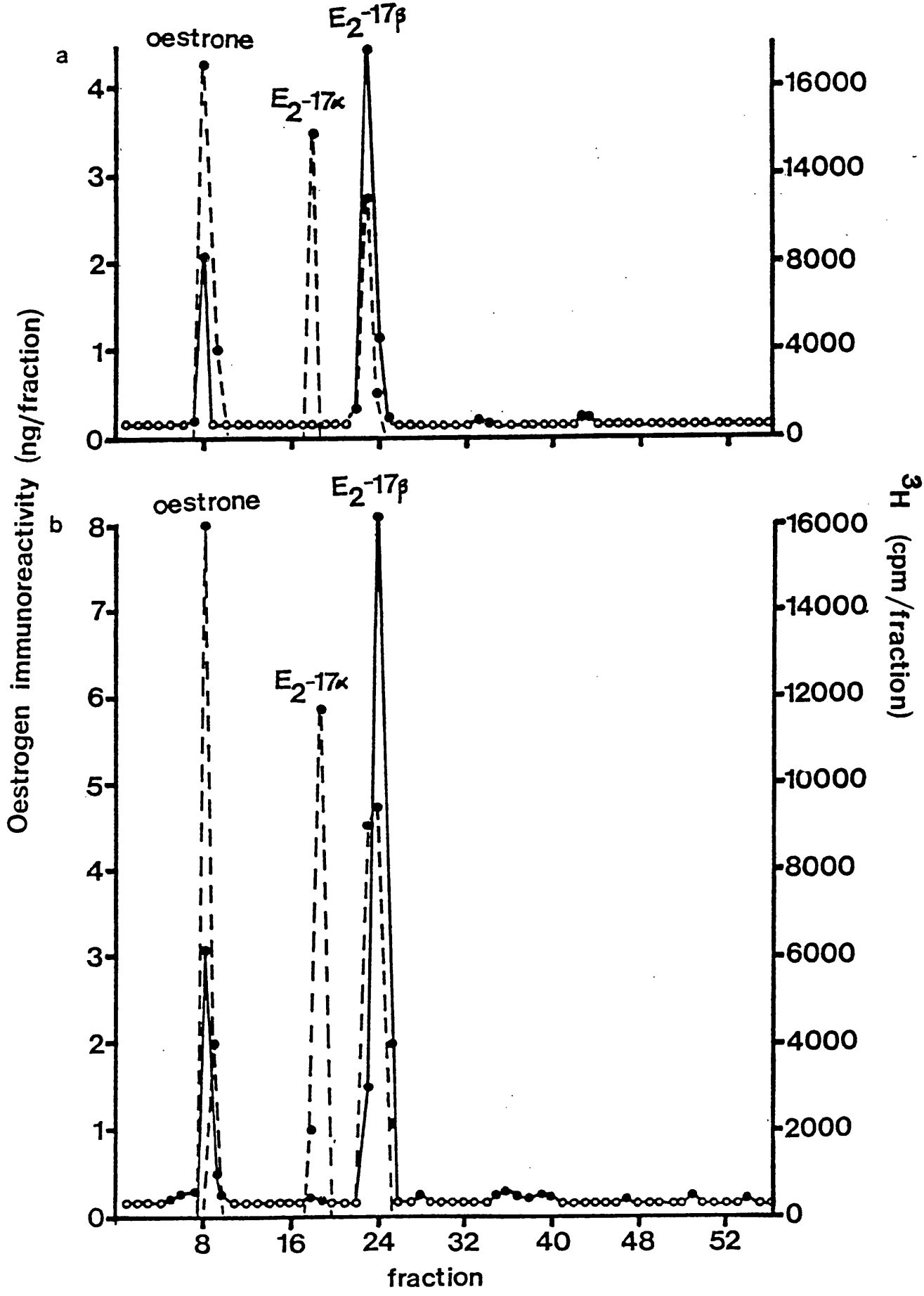
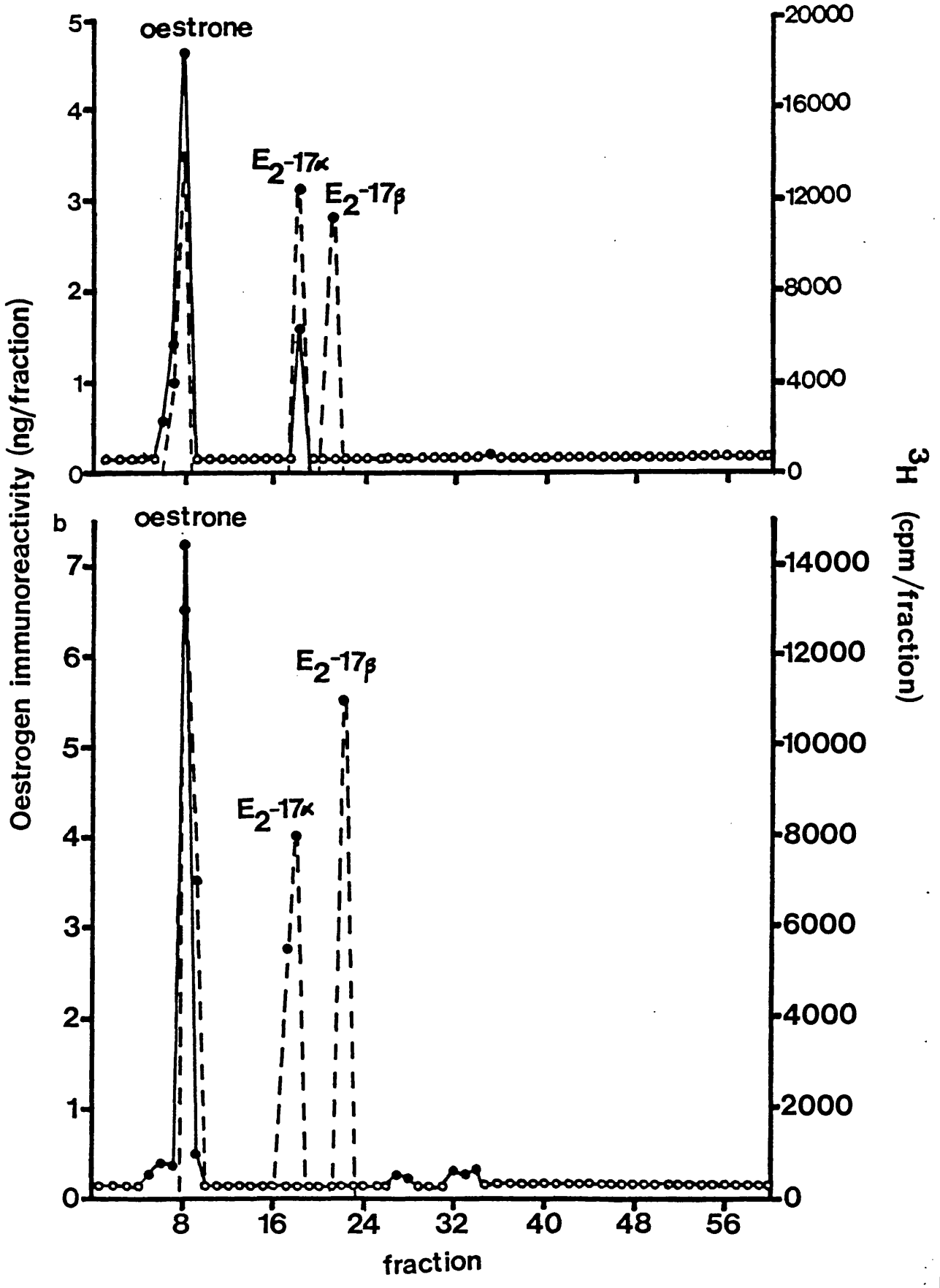

a
oestrone
E_2-17β
E_2-17α
b
oestrone
E_2-17β
E_2-17α
Oestrogen immunoreactivity (ng/fraction)
^{3}H (cpm/fraction)
fraction
0
1
2
3
4
5
6
7
8
0
2000
4000
6000
8000
10000
12000
14000
16000
8
16
24
32
40
48
52

Figure 4.13 a and b. Representative HPLC elution profiles of total oestrogen immunoreactivity in hydrolysed urine collected from black rhinoceroses 3 days prior to mating (a) and the day prior to mating (b).


a
oestrone
E_2-17α
E_2-17β
b
oestrone
E_2-17β
E_2-17α
Oestrogen immunoreactivity (ng/fraction)
^3H (cpm/fraction)
fraction
0
1
2
3
4
5
6
7
8
16
24
32
40
48
56
0
4000
8000
12000
16000
20000
2000
6000
10000
14000

The results for the HPLC analysis of urinary oestrogens are summarised in Table 4.4. The figures shown are the concentrations of each oestrogen (ng/ml) which have been corrected for the % cross reaction with the total oestrogen antiserum relative to oestrone (100%).

4.3.3 HPLC analysis of urinary steroids during pregnancy

4.3.3.1 Urinary progesterone metabolites during pregnancy

Elution profiles of urinary progesterone metabolites during pregnancy in Indian and African rhinoceroses are shown in Figs. 4.14-4.18.

The elution profiles of PdG immunoreactivity in urine excreted by an Indian rhinoceros during early and late pregnancy are shown in Fig.4.14 (a and b respectively, n=1). In the sample collected during the early stages of gestation, a single peak of immunoreactivity was seen in the same fraction as the [^{3}H]PdG marker. In contrast, the sample from late pregnancy contained many substances that cross-reacted with the PdG antiserum resulting in measurable amounts of immunoreactivity in almost all HPLC fractions. However, the largest peak of immunoreactivity continued to co-elute with the PdG standard, ie. in fraction 10.

Fig. 4.15 shows the elution profile of PdG immunoreactivity (a and c) and 20α-DHP immunoreactivity (b and d) in urine from early and late pregnancy in a northern white rhinoceros. PdG immunoreactivity was not measurable in early pregnancy urine but a single peak of immunoreactivity was seen to co-elute with the [^{3}H]20α-DHP marker after HPLC of this sample. In contrast to the situation during early pregnancy, all samples collected during late gestation contained large amounts of immunoreactivity which were almost exclusively associated with the PdG standard. Some 20α-DHP was detected in late pregnancy urine, although levels were

Table 4.4. Levels of oestrogen immunoreactivity co-eluting with ^{3}H-labelled standards, corrected for differing cross-reactivities with the total oestrogen antiserum with oestrone as a standard (ie. oestradiol-17α 63%, oestradiol-17β 161%). Results are expressed as ng/ fraction (1 ml) collected after HPLC of urine from days 3 and 1 prior to oestrus in northern white and black rhinoceroses. A (-) indicates that no immunoreactivity was seen to co-elute with the ^{3}H-labelled standard.

species	animal	time wrt oestrus	oestrogen immunoreactivity (ng/ml)*		
			oestrone	oestradiol-17α	oestradiol-17β
northern white	2	0-3	2·13	-	7·45
		0-1	3·02	-	13·04
	3	0-3	1·77	-	9·43
		0-1	1·98	-	19·28
black	6	0-3	4·65	2·50	-
		0-1	7·90	-	-
	7	0-3	14·01	3·42	-
		0-1	16·54	-	-
	9	0-3	2·97	-	-
		0-1	3·22	-	-

* corrected for differing cross reactivity of the antiserum for these steroids.

Figure 4.14 a and b. Elution profiles of PdG immunoreactivity in urine collected from an Indian rhinoceros during early (a) and late (b) pregnancy.

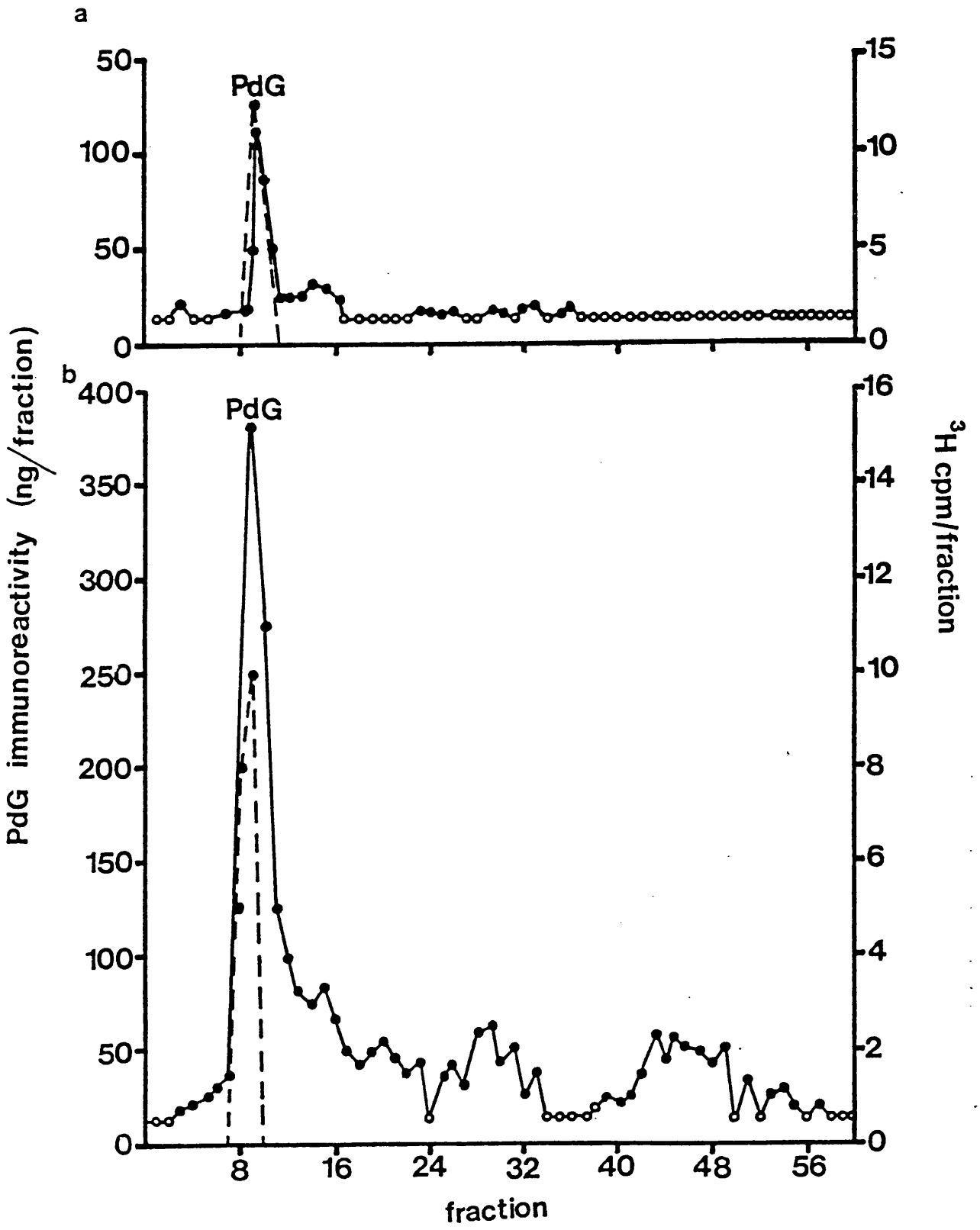
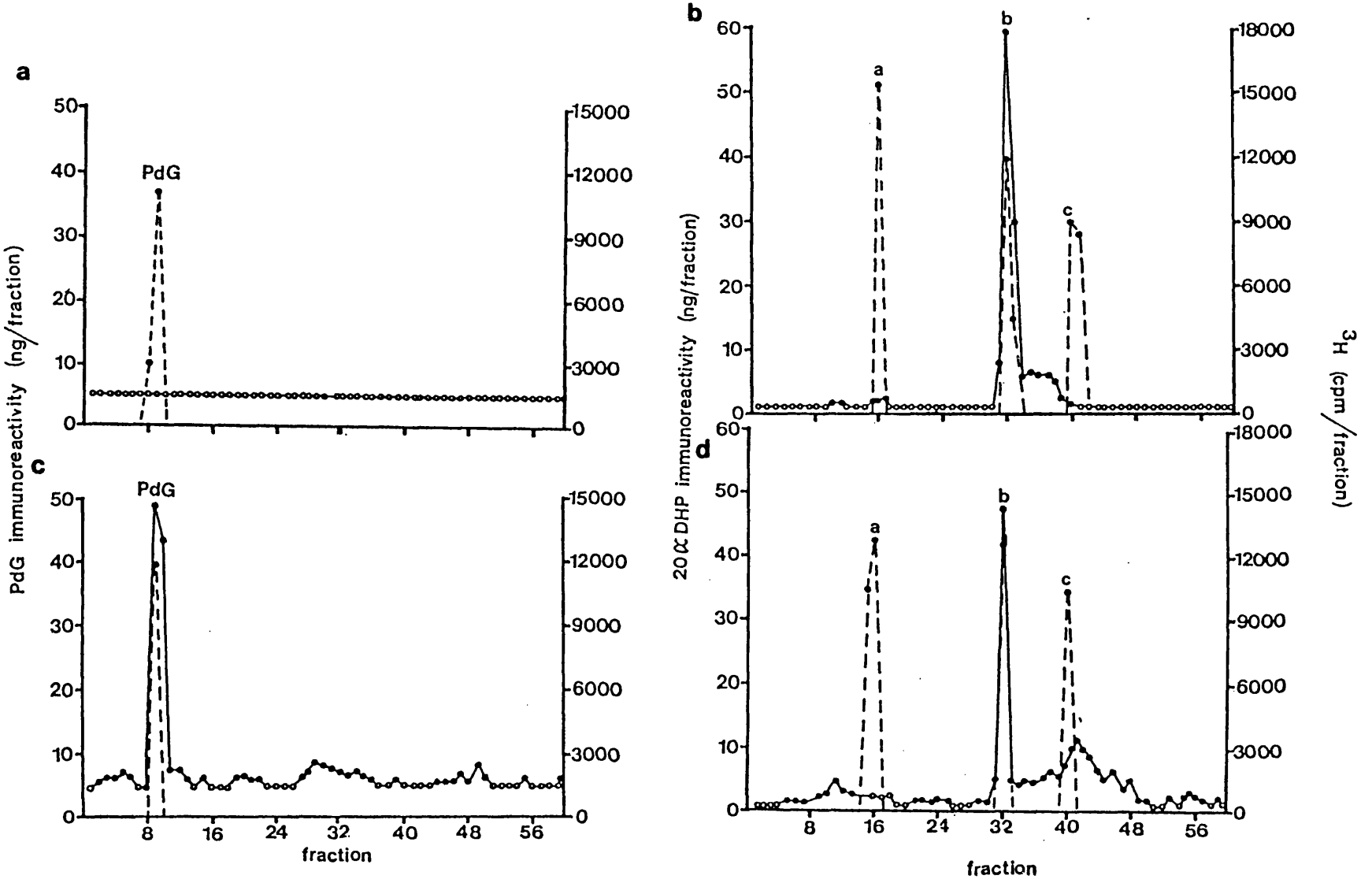

a
b
PdG
PdG
PdG immunoreactivity (ng/fraction)
^{3}H cpm/fraction
fraction
0
50
100
50
0
5
10
15
0
50
100
150
200
250
300
350
400
0
2
4
6
8
10
12
14
16
8
16
24
32
40
48
56

Figure 4.15. Elution profiles of PdG immunoreactivity (a and c) and 20α-DHP immunoreactivity (b and d) in urine collected from white rhinoceroses during early (a and b) and late (c and d) pregnancy. Peaks a, b and c represent progestesterone, 20α-DHP and pregnanediol respectively.


a
b
c
d
PdG
PdG immunoreactivity (ng/fraction)
20α DHP immunoreactivity (ng/fraction)
^{3}H (cpm/fraction)
fraction
0
10
20
30
40
50
60
3000
6000
9000
12000
15000
18000
8
16
24
32
40
48
56

consistently lower than those detected during the early stages of gestation.

Results for the analysis of urine from pregnant black rhinoceroses are shown in Fig. 4.16. No 20α-DHP immunoreactivity was measurable in any HPLC fraction after analysis of samples from early pregnancy. However, low levels of PdG immunoreactivity were detected (Fig. 4.16 a) which co-eluted exclusively with the PdG marker, indicating that PdG is excreted during early pregnancy in the black rhinoceros. In late pregnancy urine samples (n=4, from 4 animals), HPLC elution profiles from all animals showed large amounts of PdG immunoreactivity in a single peak which corresponded to the PdG marker. Low levels of 20α-DHP were also measured in each sample during late gestation.

4.3.3.2 Urinary oestrogens during pregnancy

Representative HPLC elution profiles of oestrogen immunoreactivity in urine collected during early and late pregnancy from white and black rhinoceroses have been combined in Fig. 4.17.

Whilst the largest peak of oestrogen immunoreactivity co-eluted with the oestradiol-17β marker in early pregnancy urine collected from a northern white rhinoceros, the major oestrogen excreted into the urine by the black rhinoceros at this time was oestrone. As during the follicular phase of the ovarian cycle, oestrone was the only oestrogen measurable in the urine of the black rhinoceros, although small amounts of oestrone accompanied oestradiol-17β in the northern white rhinoceros.

During late pregnancy in white rhinoceroses, no oestrogen immunoreactivity was seen in the position of [^{3}H]oestradiol-17β, but a single peak co-eluted with [^{3}H]oestrone in all animals tested. In the black rhinoceros, oestrone continued to be the major oestrogen in the urine during late gestation. However, a second peak of immunoreactivity was measured in fractions 32 and 33 which

Figure 4.16. Elution profiles of PdG immunoreactivity(a and c) and 20α-DHP immunoreactivity (b and d) in urine collected from black rhinoceroses during early (a and b) and late (c and d) pregnancy. Peaks a, b and c represent progesterone, 20α-DHP and pregnanediol respectively.

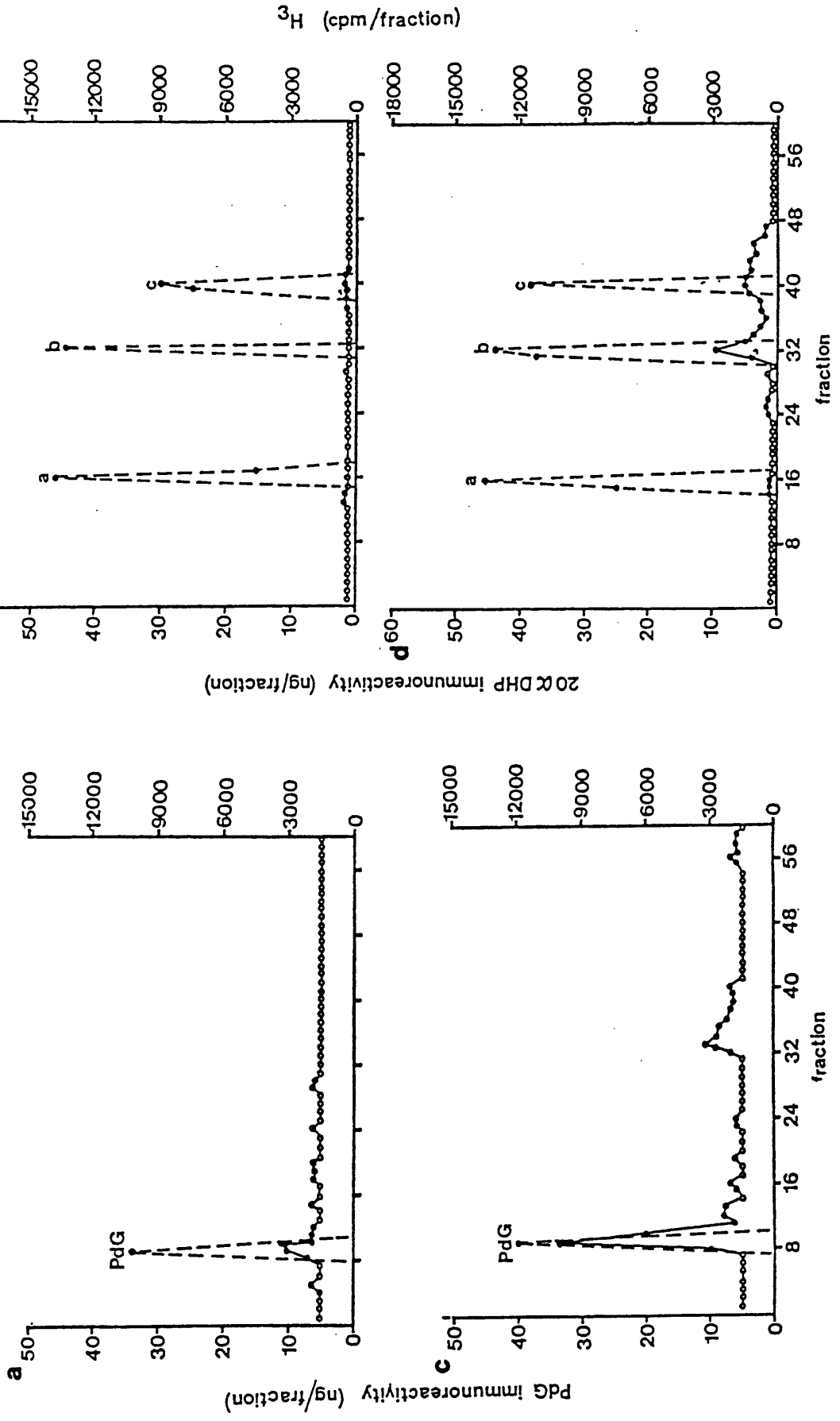
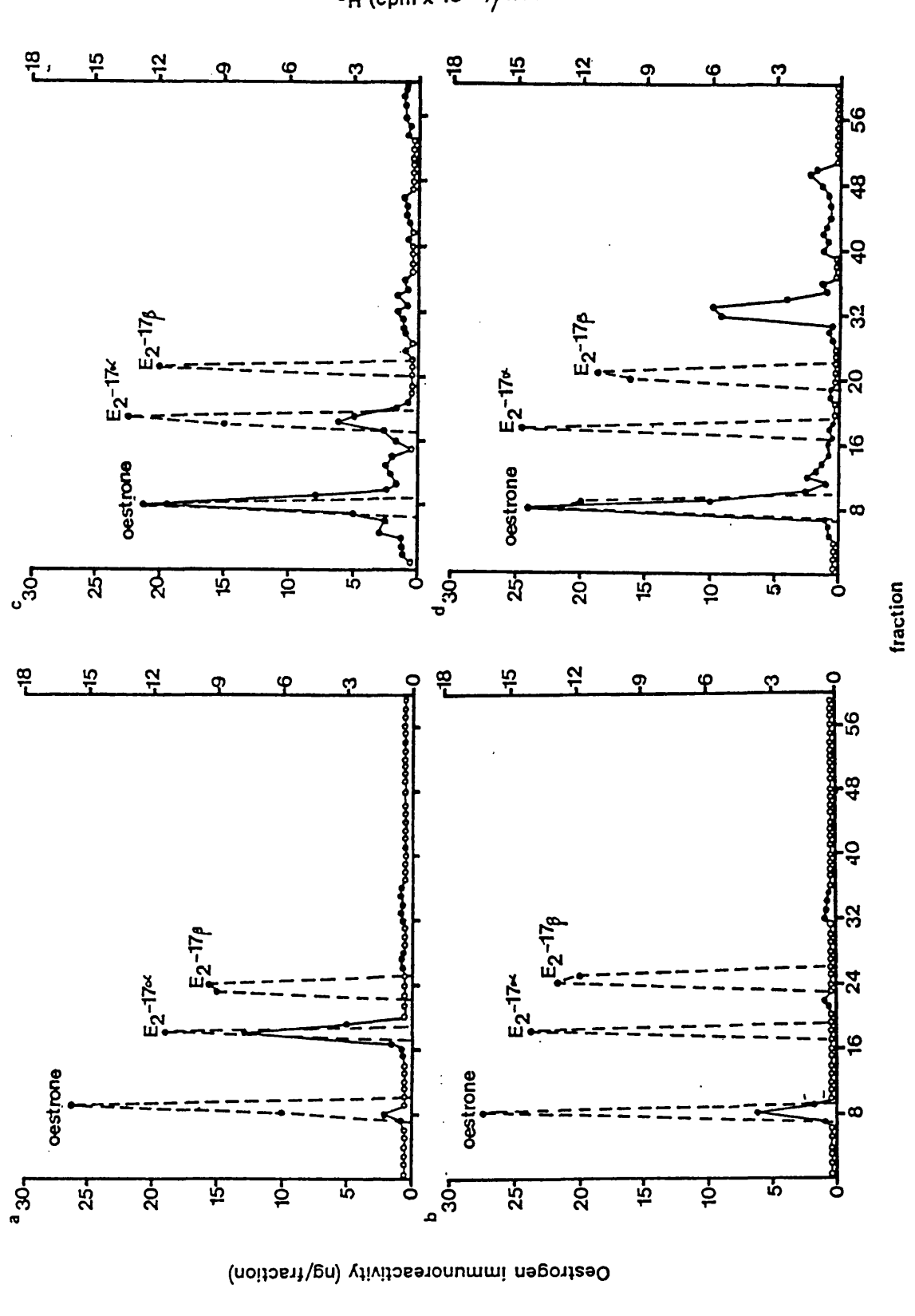

a
b
c
d
PdG
PdG immunoreactivity (ng/fraction)
20α DHP immunoreactivity (ng/fraction)
^3H (cpm/fraction)
fraction
fraction

Figure 4.17. Elution profiles of total oestrogen immunoreactivity in urine collected during early (a and c) and late (b and d) pregnancy in white (a and b) and black (c and d) rhinoceroses.


^3H (cpm x 10^{-3})/fraction
Oestrogen immunoreactivity (ng/fraction)
fraction
oestrone
E_2-17α
E_2-17β
a
b
c
d

was later identified as the elution position of oestriol (E.Möstl, personal communication).

The results of HPLC analysis of oestrogens and progesterone metabolites in urine collected during the early and late stages of pregnancy in both species of rhinoceros are summarised in Table 4.5.

4.3.3 Sequential hydrolysis and solvolysis

4.3.3.1 Sequential hydrolysis and solvolysis of 20α-DHP conjugates

Results of sequential hydrolysis of 20α-DHP conjugates in urine samples collected during the presumed luteal phase of the ovarian cycle in northern white and black rhinoceroses are shown in Tables 4.6 and 4.7. Table 4.6 shows the concentration of 20α-DHP immunoreactivity (ng/ml) present (a) in the unconjugated form, (b) as steroids conjugated to glucuronides or sulphates and (c) as conjugates which had remained unhydrolysed by enzyme action but were cleaved by solvolysis (residual). In Table 4.7, the results are expressed as a % of the total amount of 20α-DHP immunoreactivity (ng/ml) measurable in each sample (ie. total= a+b+c). Also shown are the mean±s.e.m. % of the total 20α-DHP concentration present for each conjugate.

In both the northern white and black rhinoceroses, the majority of the urinary 20α-DHP was present in the conjugated form. Less than 14% was unconjugated as indicated by the immunoreactivity measurable after ether extraction. Of the conjugates cleaved by enzyme hydrolysis over 53% were found to be sulphates in the northern white rhinoceros, with the percentage of glucuronide conjugates ranging from as little as 7·4% to 31·12%. In the black rhinoceros, only 15·46% of the total 20α-DHP released by hydrolysis and solvolysis was present as a sulphate. In this species, glucuronides predominated and accounted for over 50% of the total 20α-DHP immunoreactivity. In both species less than 22% of the total immunoreactivity was in the residual portion, ie. measurable

Table 4.5. Summary of the results of HPLC separation of progesterone metabolites and oestrogens in urine collected from Indian, white and black rhinoceroses at various stages of pregnancy.

species	animal	stage of gestation	pregnancy	PdG	20α-DHP	E_1	E_2-17α	E_2-17β	E_3
Indian	1	early	1	✓	-	-	-	-	-
		late	1	✓	-	-	-	-	-
northern white	3	early	1	X	✓	✓	X	✓	X
		early	1	X	✓	✓	X	✓	X
		late	1	✓	✓	✓	X	✓	X
southern white	4	late	1	✓	✓	✓	X	✓	X
		late	2	✓	✓	✓	X	✓	X
black	9	early	1	✓	X	✓	X	X	X
		early	1	✓	X	✓	X	X	X
		late	1	✓	✓	✓	X	X	✓
	10	late	1	✓	✓	✓	X	X	✓
	11	late	1	✓	✓	✓	X	X	✓
	12	late	1	✓	✓	✓	X	X	X

Table 4.6. Concentrations of 20α-DHP immunoreactivity in ng/ml urine from the luteal phase of black and northern white rhinoceroses, measurable after ether extraction (in the unconjugated form, U), and released after sequential hydrolysis with glucuronidase (conjugated as a glucuronide, G), sulphatase (S) and solvolysis (R).

20α-DHP immunoreactivity (ng/ml)									
northern white rhinoceros[1]					black rhinoceros[2]				
U	G	S	R	total	U	G	S	R	total
2·93	10·67	16·82	3·87	34·29	0·95	5·13	1·63	2·15	9·86
2·24	8·00	16·82	2·96	30·02	1·40	5·47	1·96	5·13	13·96
3·17	6·05	10·09	1·34	20·67	0·80	4·86	0·66	1·81	8·13
2·33	1·39	12·69	2·33	18·74	3·26	7·41	2·37	1·07	14·10
2·36	8·73	18·14	9·30	38·53	4·12	13·75	6·00	4·62	27·50

[1] 5 samples from 2 animals

[2] 5 samples from 4 animals

Table 4.7. Proportion of the total 20α-DHP immunoreactivity (total=unconjugated + glucuronide + sulphate + residual) measurable after each sequential hydrolysis procedure in urine from the northern white (a) and black rhinoceroses (b) during the luteal phase of the ovarian cycle.

a

	% total 20α-DHP immunoreactivity			
	unconjugated	glucuronide	sulphate	residual
	8·50	31·12	49·05	11·29
northern	7·46	26·65	56·03	9·86
white	15·34	29·27	48·81	6·48
rhinoceros	12·43	7·42	67·72	12·43
	6·12	22·65	47·08	24·14
mean ±sem	9·97±1·53	23·42±3·80	53·74±3·41	12·84±2·68

b

	% total 20α-DHP immunoreactivity			
	unconjugated	glucuronide	sulphate	residual
	9·62	52·02	16·53	21·80
black	10·03	39·18	14·04	36·74
rhinoceros	9·84	59·77	8·11	22·26
	23·12	52·55	16·80	7·59
	14·98	50·00	21·82	16·80
mean ±sem	13·52±2·33	50·70±2·97	15·46±1·99	21·04±4·23

after solvolysis, representing those conjugates of unknown identity which are resistant to enzyme hydrolysis.

4.3.3.2 Sequential hydrolysis and solvolysis of oestrogen conjugates

Immunoreactive oestradiol-17β (expressed as ng/ml and % total oestradiol-17β immunoreactivity) cleaved from conjugates by sequential hydrolysis and solvolysis of urine collected from northern white rhinoceroses on the day prior to oestrus, are shown in Table 4.8.

A small proportion of oestradiol-17β immunoreactivity (less than 2%) was measurable after ether extraction (ie. present in the unconjugated form). Whilst oestradiol-17β immunoreactivity liberated by sulphatase enzymes represented only 2·16% of the total immunoreactivity present, specific glucuronidase enzymes liberated over 80% of the total immunoreactivity, indicating that the majority of oestradiol-17β excreted into the urine by the northern white rhinoceros is in the form of a glucuronide. However, approximately 16% of the oestradiol-17β immunoreactivity remained unhydrolysed by enyzme hydrolysis alone and was only measurable after acid solvolysis.

Table 4.9a shows the amounts of oestrone immunoreactivity (ng/ml) measurable by immunoassay after sequential hydrolysis and solvolysis of urine collected from black rhinoceroses during the follicular phase of the ovarian cycle. These results are also expressed as a percentage of the total oestrone immunoreactivity recovered in Table 4.9b.

Very little (1·42%) oestrone immunoreactivity was present in the unconjugated form, whilst over 97% of the oestrone measurable was present in the urine as conjugates. Of these conjugates,

Table 4.8a. Concentrations of oestradiol-17β immunoreactivity in ng/ml urine from the day prior to mating in northern white rhinoceroses[1], measurable after ether extraction (in the unconjugated form), and released after sequential hydrolysis with glucuronidase (conjugated as a glucuronide), sulphatase and solvolysis (residual).

Oestradiol-17β immunoreactivity (ng/ml)				
unconjugated	glucuronide	sulphate	residual	total
0·23	16·14	0·24	2·97	19·58
0·13	12·26	0·61	2·76	15·76
0·25	8·58	0·42	2·09	11·34
0·18	8·29	0·19	1·21	9·87
0·09	8·35	0·08	1·75	10·28

Table 4.8b. Proportion of the total oestradiol-17β immunoreactivity (total=unconjugated + glucuronide + sulphate + residual) measurable after each sequential hydrolysis procedure in urine from the northern white rhinoceros on the day prior to mating.

% total oestradiol-17β immunoreactivity			
unconjugated	glucuronide	sulphate	residual
1·16	82·43	1·23	15·17
0·08	77·79	3·87	17·51
2·20	75·66	3·70	18·43
1·82	83·99	1·92	12·26
0·09	81·23	0·08	17·02
mean ±sem 1·07±0·39	80·22±1·37	2·16±0·65	16·08±0·98

1 5 samples from 2 animals

Table 4.9a. Concentrations of oestrone immunoreactivity in ng/ml urine from the day prior to mating in black rhinoceroses[1], measurable after ether extraction (in the unconjugated form), and released after sequential hydrolysis with glucuronidase (conjugated as a glucuronide), sulphatase and solvolysis (residual).

oestrone immunoreactivity (ng/ml)				
unconjugated	glucuronide	sulphate	residual	total
0·09	7·08	1·25	0·15	8·57
0·16	4·52	1·27	0·34	6·29
0·07	5·91	1·07	0·10	7·15
0·08	7·85	1·50	0·18	9·61
0·12	4·83	1·46	0·49	6·90

Table 4.9b. Proportion of the total oestrone immunoreactivity (total=unconjugated + glucuronide + sulphate + residual) measurable after each sequential hydrolysis procedure in urine from the black rhinoceros on the day prior to mating.

% total oestrone immunoreactivity			
unconjugated	glucuronide	sulphate	residual
1·02	82·65	14·63	1·70
2·51	71·92	20·21	5·36
0·91	82·72	14·97	1·41
0·86	81·63	15·63	1·88
1·79	69·92	21·16	7·16
mean ±sem 1·42±0·29	77·77±2·52	17·32±1·24	3·50±1·04

1 5 samples from 2 animals

oestrone glucuronide predominated in the urine, representing 78% of the total immunoreactivity liberated by hydrolysis and solvolysis procedures. However, significant amounts of oestrone immunoreactivity remained unhydrolysed by glucuronidase alone, with 17% present as sulphate conjugates and 3·5% remaining unhydrolysed by enzymatic procedures but liberated by solvolysis.

4.4 DISCUSSION

The aim of this study was to investigate the metabolism and excretion of endogenous oestradiol-17β and progesterone during the ovarian cycle and pregnancy in the black and white rhinoceroses. Three approaches were used to achieve this end.

Firstly, GC/MS was used to identify the steroid components of urine. Mass spectrometry has been widely used to elucidate the molecular structure of biological compounds and to study metabolic pathways (Wilson and Goulding, 1986). The coupling of a gas liquid chromatograph simplifies the interpretation of the structure of any urinary compound by ensuring that it is in a relatively pure state. Such GC/MS analysis has been useful for the definitive identification of steroids in the urine of humans (Shackleton and Honour, 1977) and other mammals, eg. the horse (Houghton, Copsey, Dumasia, Haywood, Moss and Teale, 1984; Marshall, Gower, Houghton, Dumasia, Patel, Byrne and Mortishire-Smith, 1990).

The GC/MS procedure was initially tested by the application of pure 20α-hydroxy-4-pregnen-3-one standard, which also provided a mass spectrum of the authentic steroid for reference. Urine from late pregnancy in an Indian rhinoceros was used as a biological test as previous studies (Kasman *et al.*, 1986; Hodges and Green, 1989) had provided a good indication that PdG was an abundant urinary progesterone metabolite at this time. GC/MS analysis of this urine confirmed that pregnanediol was the most

abundant progesterone mtabolite during pregnancy in the Indian rhinoceros, and also revealed the presence of other, less abundant C_{21} steroids. Amongst these metabolites were 17α-hydroxypregnanolone and a pregnanetriol, both of which are urinary metabolites of 17α-hydroxyprogesterone (Samarajeewa and Kellie, 1985). 17α-Hydroxyprogesterone has been shown to be an important progesterone metabolite during pregnancy in other mammalian species including the human (Harkness and Love, 1966) and the goat (Flint, Kingston, Robinson and Thorburn, 1978).

In the black and white rhinoceros, urine samples from late pregnancy were initially analysed by GC/MS as the presence of PdG had already been indicated by HPLC (Ramsay *et al.* 1987) and the measurement of elevated levels of PdG immunoreactivity during the third trimester of gestation (Hodges and Green, 1989). Furthermore, urine samples from late pregnancy were presumed to contain higher levels of progesterone metabolites than samples voided during non-pregnancy when the reproductive status of the animal could not be accurately determined.

Pregnanediol was not detected in late pregnancy urine of either the white or the black rhinoceros by GC/MS. Furthermore, there was no evidence of the presence of 20α-DHP in the urine at this time. Androsterone was identified as the major neutral steroid in the urine of the southern white rhinoceros, whilst a pregnanetetrol was the major steroid metabolite in the black rhinoceros. Androsterone has been shown to be an important metabolite of exogenously administered [^{14}C]progesterone in the female pig-tail macaque (Jeffery, 1966) and may be an important C_{19}-17-oxosteroid during pregnancy in the white rhinoceros. However, this steroid was only identified in one out of the two samples analysed from two pregnancies in a single individual and the significance of this finding is not clear.

Pregnanetetrol was identified as the only steroid in the urine of 2 pregnant black rhinoceroses. However, there is no literature

available on the urinary excretion of pregnanetetrol in any other mammalian species. Indeed, multi-hydroxylated C_{19} steroids are generally thought to be formed by the metabolism of corticosteroids, eg. 3α,5α- and 3β,5β-tetrahydro derivatives of aldosterone which are found in human urine (Kelly and Lieberman, 1964), and the pregnanetetrol excreted by this species may simply be a metabolite of a range of corticoids produced by the maternal and/or foetal adrenals during late pregnancy. Furthermore, the identification of the same pregnanetetrol in urine collected from a female black rhinoceros during the ovarian cycle indicates that this steroid is not pregnancy specific, and thus the relevance of its measurement for pregnancy diagnosis may be limited.

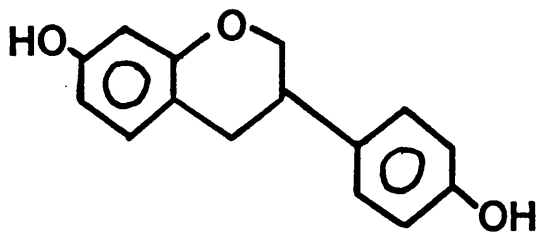
Thus GC/MS failed to confirm the presence of PdG or 20α-DHP during pregnancy in the African species of rhinoceros but indicated that other steroids, eg. androsterone in the white and pregnanetetrol in the black rhinoceros, are detectable. These findings are surprising in view of the HPLC results obtained in this study, and question the use of GC/MS for steroid identification in animals in which relatively low levels of hormones are excreted into the urine. A similar study using GC/MS to identify the major urinary progesterone metabolite in the baboon, reported that 3α,6β,11β,17 21-pentahydroxy-5β-pregnan-20-one (6β-hydroxy-THF) was the only steroid detectable by this method (Setchell, Gontscharow, Axelson and Sjövall, 1976). However, pregnanediol had previously been identified as the major metabolite of [^{14}C]progesterone in this species (Goldzieher and Axelrod, 1969) and the measurement of PdG had been informative in characterising the ovarian cycle and pregnancy in the female baboon (Hodges *et al.*, 1986).

Even though large volumes of urine are generally prepared for GC/MS analysis, only a fraction (1/1000) of the original sample is injected onto the GC/MS. Procedural losses during the preparation of samples for GC/MS by the methods used in this study are high, and it was indicated that a 40% recovery of steroid may be expected after derivatization procedures. Future studies may be improved by

the extraction and purification of steroids on Amberlite XAD-2 columns which have been shown to give optimal recoveries of steroids whilst retaining any non-polar matter (Axelson and Sjövall, 1974). In addition to the high procedural losses, repetitive magnetic scanning used in this and other such experiments has a detection limit of 5-500 ng (Hewlett Packard, personal communication). The urine samples from pregnant black and white rhinoceroses that underwent GC/MS were known to have a PdG concentration of between 100 and 200 ng/ml , as determined by EIA. After procedural losses and the application of a small fraction of the sample, levels of steroid may have been below the detection limit of the machine. Repeated analysis with repetitive acceleration voltage scanning (Brooks,Middleditch and Harvey, 1971) would increase the sensitivity of the machine to detect 10 pg and may enable low levels of ovarian steroid metabolites to be identified. Whatever the reason for the failure to detect PdG in late pregnancy urine of African rhinoceroses, samples from non-pregnant animals (presumed to contain even lower levels of steroid) were not extensively analysed.

It is not possible to comment on the use of GC/MS for the identification of urinary oestrogens. The initial injection of phenolic extracts identified the non-steroidal oestrogen, equol (7-hydroxy-3-{4'-hydrophenyl}chroman) as the major phenolic constituent of rhinoceros urine (see Figure 4.18 for structure).

Figure 4.18. The chemical structure of equol (7-hydroxy-3-{4'-hydrophenyl}chroman).



Equol was present in rhinoceros urine in very large quantities which prevented the identification of any other oestrogen and also prevented injection of further phenolic extracts onto the machine. Over the past 10 years there has been much interest in the levels of dietary oestrogens and their possible physiological significance. High levels of equol have been reported in the urine of many species, including the cow (Dewar, Glower, Elsworth and Sandler, 1986), sheep (Cox and Braden, 1974) and primates (Adlercreutz, Musey, Fotsis, Bannwart, Wähälä, Mäkelä, Brunon and Haste, 1986). This poses a concern, as equol is the molecule responsible for clover disease in sheep which leads to female infertility and foetal mortality resulting in abortion (Moule, Braden and Lamond, 1963). However, large quantities of equol in the female rhinoceros would appear to have no affect on fertility. Urine from 4 pregnancies was analysed by GC/MS, and all animals were shown to excrete considerable amounts of equol. However, only one of these pregnancies resulted in abortion, and all females subsequently underwent fertile ovarian cycles that led to conception. It is possible that, like other species including the macaque (Thompson, Lasley, Rideout and Kasman, 1984), the rhinoceros is resistant to equol poisoning.

In addition to GC/MS analysis, the metabolism and excretion of steroids was also investigated by HPLC. The wide applicability, speed and sensitivity of HPLC have resulted in it becoming the most popular form of chromatography, especially for polar compounds such as steroids. Using reverse or normal phase HPLC, the steroid components of urine may be separated, each eluting from the column separately, in order of polarity, with a unique retention time. Immunoreactivity in each HPLC fraction may then be determined by immunoassay. By combining the injection of urine with the application of radiolabelled steroids, co-chromatography of immunoreactivity with radiolabelled standard provides a good indication of the presence of a steroid in urine. Immunoreactivity which elutes with a different retention time than the radiolabelled marker indicates the presence of a another substance in the urine

that cross-reacts with the antiserum used. HPLC may thus be used to confirm the presence of a urinary steroid and to validate immunoassays for the measurement of that steroid in a particular species. However, HPLC is limited to the identification of steroids for which there are immunoassays available. This technique can only confirm the presence or absence of steroids in biological fluids, and it is not possible to detect all steroids present or to determine their structural identity.

The results of HPLC analysis of urine from the Indian rhinoceros indicate that PdG was present and detectable by immunoassay during the luteal phase of the ovarian cycle and during pregnancy. In contrast, PdG could only be detected during pregnancy in the African species and no PdG could be measured in any sample from the non-pregnant animal. These results together with those of chapter 3 provide a clear indication that PdG is not excereted as a metabolite of progesterone during ovarian cycles in the white and the black rhinoceros. In this study it was not possible to determine whether urine samples were taken from ovulatory cycles. However, the failure to detect PdG in post-oestrus urine is consistent with the observations made by Ramsay *et al.* (1987) who used similar HPLC techniques in the black rhinoceros. In this respect it can be concluded that the African rhinoceroses differ from the Indian species in which PdG is the major urinary progesterone metabolite durine the luteal phase of the ovarian cycle.

Although PdG immunoreactivity was not seen to co-elute with [^{3}H]PdG in post-oestrus urine, a separate peak of immunoreactivity was observed in all animals. Furthermore, this immunoreactivity eluted with the same retention time with respect to the PdG marker in all samples. This peak of immunoreactivity indicated the presence of a substance in the urine of both the white and the black rhinoceros that cross-reacted with the PdG antiserum. The identity and origin of this immunoreactive substance was not determined in this study, although it was a more polar substance than PdG as

indicated by its longer retention time on reverse phase HPLC. Suggestions as to its identity may be made by considering the specificity of the PdG antiserum used in this study and that of Ramsay *et al.* (1987) who failed to detect this immunoreactivity by HPLC analysis of post-oestrus urine in the black rhinoceros. The antiserum of Ramsay *et al.* (1987) is more specific for PdG than that used in this study, the specificity of which has been determined by Hodges and Green (1989) who found that 20α-DHP cross reacted significantly (12·1% by mass) with the antiserum. The immunoreactive substance detected by HPLC could possibly be 20α-DHP, but is more likely to represent the conjugated form of this steroid (20α-DHP glucuronide or 20α-DHP sulphate) which has been shown by sequential hydrolysis to predominate in the urine of white and black rhinoceroses. However, it is not possible to determine the cross reaction of conjugated 20α-DHP with the PdG antiserum, or the elution pattern on HPLC, as 20α-DHP conjugates are not commercially available. Furthermore, it is not possible to deduce whether this substance is of ovarian origin as urine samples from the follicular phase were not analysed.

Combined HPLC and EIA techniques detected 20α-DHP immunoreactivity that co-eluted with [^{3}H]20α-DHP in all luteal phase urine samples from both species of African rhinoceros. These results confim the findings of chapter 3 for the white rhinoceros and extend the observations to the black species. Although the 20α-DHP antiserum is non-specific for several C_{21} steroids, the single peaks of immunoreactivity obtained on HPLC suggest that, in most cases, the measurement of 20α-DHP in hydrolysed, post-oestrus urine samples is specific. Together these results suggest tht 20α-DHP is the major urinary progesterone metabolite in African rhinoceroses. 20α-DHP was not detectable in any urine sample from the follicular phase, therefore its presence varies according to the stage of the ovarian cycle and the measurement of 20α-DHP should be useful for reflecting progesterone secretion and therefore luteal function. The only exception was one northern white rhinoceros (animal 3), in which small amounts of an immunoreactive substance other than 20α-

DHP were detected in some samples (O-1, n=2) after HPLC, giving rise to elevated 20α-DHP immunoreactivity during the pre-oestrus period (see chapter 5).

Sequential hydrolysis indicated that, of the total 20α-DHP immunoreactivity, approximately 15% exists in the unconjugated form, suggesting that the African species of rhinoceros excrete the majority (85%) of 20α-DHP in the conjugated form. However, the results from chapter 3 suggest that all 20α-DHP is excreted into the urine in the conjugated form with no 20α-DHP present in the unconjugated fraction. In the present study the large proportion of unconjugated 20α-DHP immunoreactivity present in the urine may represent hydrolysis of the conjugate during storage and subsequent thawing, as urine is frozen without preservative. Of the 20α-DHP present in the conjugated form, glucuronides predominated in luteal phase urine from the black rhinoceros whereas sulphates were more abundant in the northern white species. However, in both species a range of conjugates were excreted including over 13% which remained uncleaved by enzymatic hydrolysis and were not represented in the HPLC elution profiles described.

It is difficult to compare the excretion of 20α-DHP by African rhinoceroses to studies on other mammalian species in which 20α-DHP is thought to be an important metabolite of progesterone during the ovarian cycle and pregnancy, eg. the lion-tailed macaque (Shideler *et al.*, 1985) and the killer whale (Walker *et al.*, 1988). In these studies, urinary 20α-DHP was not measured by a specific enzyme immunoassay as described here, but by cross reaction in a radioimmunoassay system with a non-specific antibody raised against PdG and 20α-DHP standards. The authors of such reports indicated that the data represent the amount of 20α-DHP-like immunoreactivity in urine, although HPLC revealed the presence of many immunoreactive substances and therefore the measurement is likely to be non-specific for a range of urinary progesterone metabolites. Furthermore, the results represent a total concentration of conjugated and unconjugated steroid and in no report was sequential

hydrolysis performed to establish the proportions of 20α-DHP in the conjugated and unconjugated form.

HPLC analysis of urine from late pregnancy in black and white rhinoceroses revealed the presence of PdG immunoreactivity co-chromatographing with the tritiated PdG marker in all cases. The measurement of elevated levels of PdG during mid-late gestation has been described in the black (Ramsay *et al.*, 1987; Hodges and Green, 1989) and the southern white rhinoceroses (Hodges and Green, 1989), and the results obtained in this study also indicate tht PdG is an abundant metabolite of progesterone during the late stages of pregnancy in these species.

Such accounts are limited to the measurement of PdG during mid to late pregnancy, and Ramsay *et al.* (1987) described a possible delay in PdG excretion reflecting a mid-gestational shift in progesterone metabolism in the black species. Although the HPLC results presented here for early pregnancy are limited to a single black rhinoceros, PdG immunoreactivity co-eluting with [^{3}H]PdG was identified in two seperate samples collected during this period. A possible explanation for the earlier detection of PdG during pregnancy in this study is the use of a different PdG antibody to that of Ramsay *et al.* (1987) in an EIA system of increased sensitivity (Hodges and Green, 1989). Certainly, when the PdG assay used by Ramsay *et al.* (1987), was validated for use in the giant panda (Hodges *et al.*, 1984) it was found that the assay underestimated the amount of PdG in the urine by approximately 40%, as determined by recovery of known amounts of PdG from panda urine containing low levels of endogenous hormone. The accuracy of the PdG RIA with black rhinoceros urine has not been determined, but could possibly be lower than 101·5% determined by EIA in this study. Such a reduction in accuracy, coupled with a decrease in sensitivity may have led to the failure to detect PdG in early pregnancy samples from black rhinoceroses in previous studies. The PdG EIA of Hodges and Green (1989) may therefore provide a diagnostic test for early pregnancy in the black rhinoceros, and increased efficiency may be

obtained if the immunoreactive substance present during the luteal phase of the ovarian cycle can be removed.

In contrast to the black rhinoceros, no PdG immunoreactivity was seen to co-elute with the PdG marker in urine collected during early pregnancy in a northern white rhinoceros. Even though this observation is restricted to one individual, HPLC analysis suggests that PdG is not a major urinary prgesterone metabolite during early gestation in the white rhinoceros. However, a single peak of 20α-DHP immunoreactivity in early pregnancy urine from the northern white rhinoceros was seen to co-elute with [^{3}H]20α-DHP, indicating that 20α-DHP is the most abundant progesterone metabolite during early pregnancy as well as during the ovarian cycle. In contrast, no 20α-DHP immunoreactivity was seen in urine from an early pregnant black rhinoceros.

Late pregnancy urine from white and black rhinoceroses revealed immunoreactivity that co-eluted with the 20α-DHP marker in all samples. Thus in both species of rhinoceros, PdG and 20α-DHP are excreted into the urine during the third trimester of pregnancy.

Using HPLC, the major urinary oestrogens excreted 3 days and 1 day prior to the onset of behavioural oestrus in the northern white and the black rhinoceros were identified. Co-chromatography of oestrogen immunoreactivity on HPLC indicated that oestradiol-17β was the most abundant urinary oestrogen in the northern white rhinoceros, with small, and relatively constant amounts of oestrone also being excreted around the time of oestrus. Sequential hydrolysis indicated that over 98% of the oestradiol-17β excreted was present in the urine in the conjugated form, predominantly as a glucuronide. There are inconsistencies in the results obtained for the excretion of endogenous oestrogens and those identified after i.v. injection of ^{14}C-labelled oestradiol-17β in the white rhinoceros. In the metabolism study, unmetabolised [^{14}C]oestradiol-17β was excreted into the urine, all of which was removed by ether extraction, ie. present in the unconjugated form. Oestrone, present

in both the conjugated and unconjugated form was found to be the major urinary metabolite of [^{14}C]oestradiol-17β, although HPLC indicated that oestrone accounted for only a small proportion of the total oestrogen immunoreactivity. Furthermore, no oestradiol-17α was identified by HPLC, although this was the second most abundant metabolite identified by the metabolism study.

The results of metabolism study therefore differ from those obtained in this study. Although it is not clear which result is correct, the findings of the present study on the metabolism and excretion of endogenous ovarian steroids is likely to be the most reliable as the pattern of metabolism of exogenously administered radiolablled steroids may be altered by the experimental procedure. Oestradiol has been identified in the urine of some ungulates, eg. the okapi (Loskutoff *et al.*, 1987) and primate species, eg. Goeldi's monkey (Carroll *et al.*, 1990) and may be useful in the detection of ovulation in the white rhinoceros. Furthermore, the increase in oestradiol-17β excretion between days 3 and 1 prior to oestrus indicates that the measurement of urinary oestradiol-17β may be used for monitoring follicular development in this species.

In the black rhinoceros, as in the majority of other ungulate species including the Indian rhinoceros (Kassam and Lasley, 1981), the major urinary oestrogen was identified as oestrone. Over 98% of the total oestrone immunoreactivity measurable after sequential hydrolysis was found to be present in the conjugated form, a similar percentage to that obtained by Ramsay *et al.* (1987). In their study, Ramsay *et al.* (1987) also identified oestrone glucuonide as the major oestrone conjugate with smaller (30%) amounts of the total oestrone conjugate immunoreactivity being present as a sulphate. This is in accordance with the results obtained in this study, but in contrast to the conjugation of oestrone in the majority of ungulate species which excrete oestrone sulphate (Lasley, Troedsson and Haggerty, 1989).

Co-chromatography on HPLC of pre-oestrus urine failed to detect oestradiol-17β in all samples collected from black rhinoceroses. Ramsay *et al.* (1987) report the identification of oestradiol-17β in urine using similar HPLC techniques, and further reported the measurement of comparable levels of oestrone and oestradiol-17β in urine collected from 3 female black rhinoceroses. However, these measurements were made with a total oestrogen RIA, the specificity of which is not fully known, and represent oestrogen metabolites in urine collected on the day of presumed fertile breeding. The results presented here refer to the proportion of oestrogens in urine collected on the day prior to those analysed by Ramsay *et al.* (1987). The presence of oestradiol-17α in urine collected 3 days prior to oestrus, and its absence in urine collected 2 days later indicates the rapid change in the metabolism of oestrogens in this species, and it may be possible that yet another oestrogen, eg. oestradiol-17β, may be present in the urine at the time of oestrus that is usually associated with peak levels of oestrogen excretion.

In addition to suggesting species differences in oestrogen metabolism between the white and the black rhinoceros during the pre-oestrus period, HPLC analysis of urine indicated that such differences may also exist throughout pregnancy. During early pregnancy, oestradiol-17β continued to be the major oestrogen in the urine of the northern white rhinoceros, whilst oestrone remained the only immunoreactive oestrogen in the urine of the black rhinoceros. During late gestation, oestrogen immunoreactivity co-eluted with [^{3}H]oestrone in the urine of both the white and the black rhinoceroses, indicating that oestrone is the quantitatively most important urinary oestrogen during late pregnancy in both African rhinoceroses. The absence of immunoreactivity co-eluting with the oestradiol-17β marker in late pregnancy urine from northern and southern white rhinoceroses suggests a shift in the metabolism of oestrogens during mid gestation in this species.

In conclusion, this study has confirmed the results of the metabolism study described in chapter 3, that 20α-DHP and not PdG is the most abundant progesterone metabolite in the non-pregnant, female white rhinoceros and extended this observation to the black species. The results also illustrate species differences in progesterone metabolism between the African rhinoceroses and the Indian species, in which PdG is the most abundant urinary progesterone metabolite at all stages of the reproductive cycle. Co-chromatography on HPLC suggested that, whilst 20α-DHP was present in post-oestrus urine from white and black rhinoceroses, in all but one animal no 20α-DHP immunoreactivity was seen in follicular phase samples. The measurement of urinary 20α-DHP in hydrolysed urine from African rhinoceroses may therefore provide a method of monitoring luteal function in these species. During pregnancy however, the African species of rhinoceros excrete PdG into the urine although the time at which PdG becomes an important metabolite of progesterone would appear to vary between the black and the white rhinoceroses.

Oestrogen excretion during the follicular phase of the ovarian cycle and in pregnancy also differed between the white and black rhinoceroses. Oestrone glucuronide was the most abundant oestrogen in the urine of the black rhinoceros during the ovarian cycle and pregnancy. In the white rhinoceros, oestradiol-17β (conjugated as a glucuronide) was the most abundant oestrogen during the ovarian cycle, whilst oestrone appeared to be important during late gestation. The identification of the major urinary metabolites of endogenous ovarian steroids in the African rhinoceros should enable appropriate assay methods to be developed for their measurement throughout the reproductive cycle in these species.

CHAPTER 5.

MONITORING THE REPRODUCTIVE CYCLE OF AFRICAN RHINOCEROSES BY THE MEASUREMENT OF STEROID HORMONE METABOLITES IN URINE

CHAPTER 5

MONITORING THE REPRODUCTIVE CYCLE OF AFRICAN RHINOCEROSES BY THE MEASUREMENT OF STEROID HORMONE METABOLITES IN URINE

5.1 INTRODUCTION

The results of chapters 3 and 4 indicate that conjugated 20α-DHP is the major urinary metabolite of progesterone during the ovarian cycle in both the white and the black rhinoceros. Furthermore, species differences in the metabolism of oestradiol were suggested. The white rhinoceros excretes oestradiol-17β whilst the black rhinoceros preferentially excretes oestrone into the urine around the time of oestrus.

In contrast to the situation during the ovarian cycle, the measurement of urinary PdG immunoreactivity has been informative in monitoring mid to late gestation in the African species of rhinoceros (Ramsay *et al.*, 1987; Hodges and Green, 1989), and HPLC results from Chapter 4 have confirmed the presence of PdG in the urine of the black and white rhinoceros at this time. There is, however, no data available on the pattern of excretion of steroid metabolites into the urine during the early stages of pregnancy, nor have any studies investigated in detail the excretion of oestrogens during gestation.

The aim of this study was to investigate the pattern of excretion of oestrogens and progesterone metabolites into the urine of African rhinoceroses during the ovarian cycle and pregnancy. By comparing the profile of excretion of several different steroids, the metabolites that most accurately reflect ovarian function and gestation may be identified. The overall objective of this study was to measure urinary oestrogens and progesterone metabolites with a view to monitoring ovarian function and pregnancy in African rhinoceroses.

5.2 MATERIALS AND METHODS

5.2.1 Animals and sample collection

Hormonal profiles of the ovarian cycle are derived from daily samples collected over a 60 day period from a juvenile female black rhinoceros (aged 4-6 months over the collection period; animal 13), adult female black rhinoceroses (n=4, animals 6-9), adult female northern white rhinoceroses (n=2, animals 2 and 3) and an adult female southern white rhinoceros (animal 5). Animals were observed daily by keeper staff and the occurence of mating and/or oestrous behaviour were recorded. Oestrous behaviour was characterized by swelling of the vulva, increased frequency of urine spraying, whistling and interest in the male (Ritchie, 1963; Mukinya, 1973)

Hormonal profiles during mid to late pregnancy are derived from weekly samples collected within the last 12 months prior to birth of a live calf from female black rhinoceroses (n=4, animals 9-12), a female northern white rhinoceros (animal 3), a female southern white rhinoceros (animal 4) and a female Indian rhinoceros (animal 1). Hormonal profiles for complete pregnancies were derived from weekly samples collected from a female black rhinoceros (animal 9) and a female northern white rhinoceros (animal 3) who concieved and gave birth during the course of this study.

Samples were collected according to the procedure outlined in section 2.1. Each sample was analysed for creatinine content immediately upon the first thawing (section 2.2) and hormone concentrations were indexed to creatinine throughout this study.

5.2.2 Hormone assay of conjugated steroids

Conjugated hormones were measured directly in urine after centrifugation to remove particulate matter. Hydrolysis and extraction were not carried out.

Oestrone conjugates were measured directly in urine by RIA (see section 2.10 for method and validation for use in the rhinoceros). Prior to analysis, urine from both black and white rhinoceroses was diluted 1:2.

PdG was measured in unextracted urine by EIA according to the method of Hodges and Green (1989) (see section 2.12 for method and validation). Prior to analysis, urine collected during pregnancy was diluted 1:20 for samples from the Indian rhinoceros and 1:5 for samples from the African species. Urine samples from the ovarian cycle in the white and black rhinoceroses were assayed at a dilution of 1:5 in the first instance, samples containing undetectable levels of PdG immunoreactivity were then re-assayed at a dilution of 1:2.

5.2.3 Hormone assay of unconjugated steroids

Urine samples were hydrolysed and extracted before assay for oestrone, oestradiol-17β, progesterone and 20α-DHP. Results were corrected for hydrolysis and extraction efficiencies. Data from these assays represent a summation of levels of steroids formerly present in the urine in the unconjugated form and those as conjugates which were hydrolysed by the enzymatic procedures.

Unconjugated oestrone was measured in urine after hydrolysis and extraction by the RIA of Hodges *et al.* (1983a; see section 2.8 for method and validation). Results presented therefore represent total oestrone immunoreactivity in urine (ie. unconjugated oestrone in addition to those conjugates cleaved by enzyme hydrolysis). Urine samples were assayed at a dilution of 1:40 which also accounted for the dilutions made during sample preparation. The concentration of oestrone immunoreactivity in each sample, as determined by RIA, was then corrected for hydrolysis efficiency and each individual extraction recovery (see section 2.8.1 for method).

Unconjugated oestradiol-17β was measured in urine after hydrolysis and extraction according to the method of Shaw *et al.*

(1989) (see section 2.11 for method and validation). Urine samples were assayed at a dilution of 1:20. Progesterone was measured in urine after hydrolysis and extraction according to the method of Hodges *et al.* (1983b) (see section 2.9 for method and validation for use with rhinoceros). Urine samples were assayed at a dilution of 1:30. Unconjugated 20α-DHP was measured in urine after hydrolysis and extraction as described in section 2.13. Urine samples from the white rhinoceroses were assayed at a dilution of 1:400 and samples from black rhinoceroses were assayed at a dilution of 1:40.

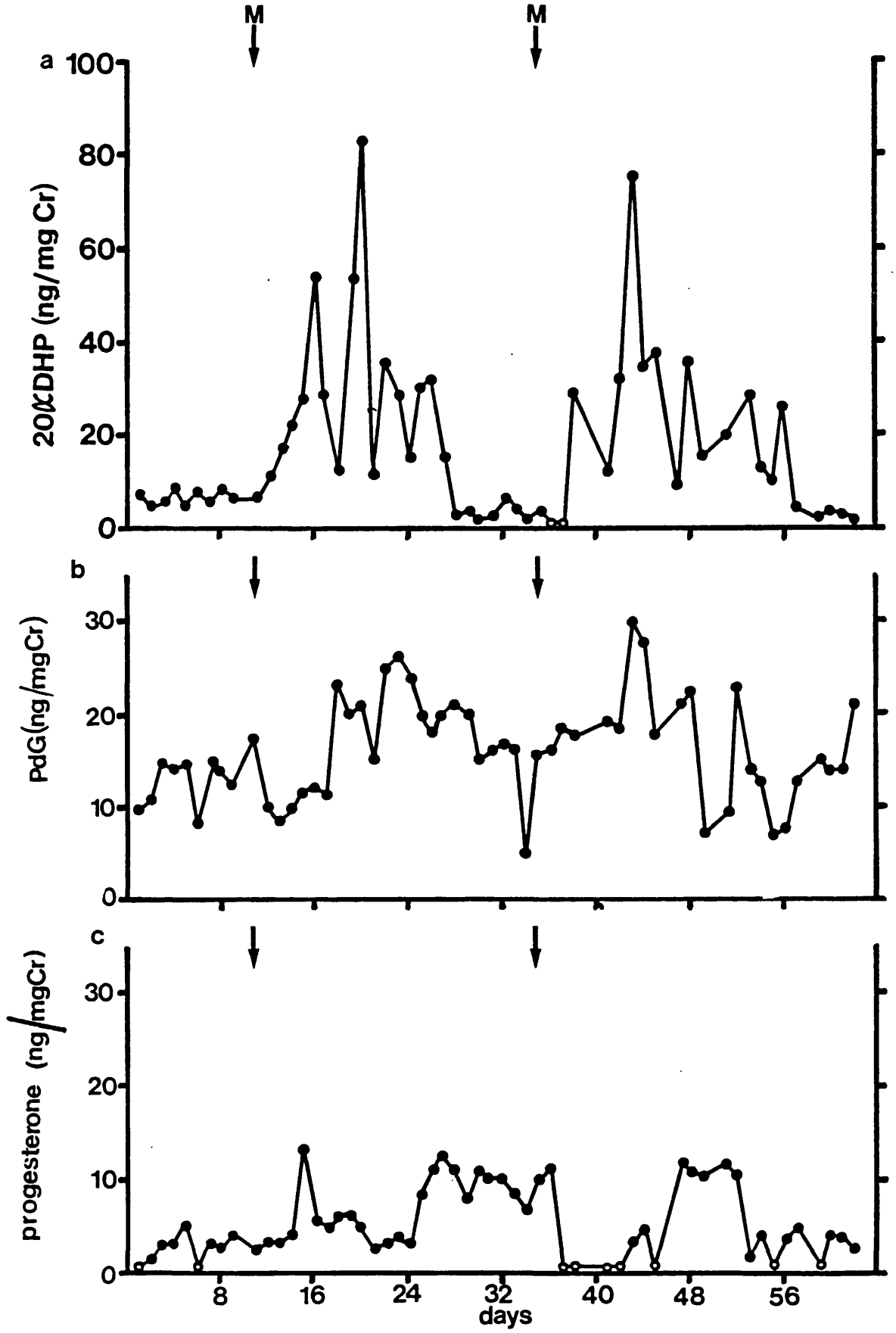
5.3 RESULTS

5.3.1 Measurement of urinary steroids during the ovarian cycle

5.3.1.1 Urinary steroid excretion during the ovarian cycle in the white rhinoceros

The pattern of excretion of 20α-DHP, PdG and progesterone throughout 2 oestrous cycles in a northern white rhinoceros (animal 2) are shown in Fig. 5.1. During the sample collection period, positive matings (M) were observed on days 11 and 35 as indicated by an arrow. Levels of urinary immunoreactive PdG (Fig. 5.1.b) were low (less than 30 ng/mgCr), but detectable throughout the sample collection period. Although some fluctuations in levels were seen, levels of PdG were elevated at the time of oestrus and there were no cyclical changes which corresponded to the time of observed mating. Therefore, changes in levels of PdG were not related to the stage of the ovarian cycle. Urinary immunoreactive progesterone levels were also low (less than 15 ng/mgCr) during the 60 day period and showed no clear cyclic pattern.

Figure 5.1. Pattern of excretion of 20α-DHP (a), PdG (b) and progesterone (c) during successive oestrous cycles in a northern white rhinoceros (animal 2). The arrows represent the time of mating (M).


M
M
a
100
80
60
40
20
0
20αDHP (ng/mg Cr)
b
30
20
10
0
PdG(ng/mgCr)
c
30
20
10
0
progesterone (ng/mgCr)
8
16
24
32
40
48
56
days

Peak levels of 20α-DHP in the urine were considerably higher than those measured by immunoassays for progesterone and PdG. There was a clear, cyclic pattern of 20α-DHP excretion in which a marked increase in 20α-DHP occurred immediately following the day of mating. Levels of 20α-DHP were either low (less than 10 ng/mgCr) or undetectable for at least 10 days prior to mating. Elevated, (greater than 10 ng/mgCr) although somewhat variable levels were measured during both post-oestrus periods, during which levels remained elevated above 10 ng/mgCr for 16 and 19 days. Maximum levels of 20α-DHP (70-80 ng/mgCr) were observed 8 and 9 days after oestrus, corresponding to the middle of the presumed luteal phase.

Behavioural data suggest that the cycles described by 20α-DHP excretion were ovulatory. Additional evidence is provided by the urinary oestrogen profile. Urinary oestrogen profiles (oestrone conjugates, oestrone and oestradiol-17β) from the same northern white female (animal 2) are shown in Figure 5·2. Levels of oestrone conjugates in the urine were consistently low and showed no elevation prior to or around the time of observed mating. Similarly, the profile of unconjugated oestrone after hydrolysis showed low levels (0·25-2 ng/mg Cr) prior to mating although elevated levels were observed during the oestrous period. Maximum values during this period were approximately 5 ng/mgCr.

In contrast, the levels of oestradiol-17β showed a cyclic pattern of excretion. In this animal there were three distinct periods of elevated oestradiol-17β, with maximum values (7-8 ng/mgCr) on two occasions occurring either the day of or the day preceeding mating. A third peak of oestradiol-17β excretion is shown although behavioural oestrus was not detected. The durations of the increases in levels of oestradiol-17β were 9 and 7 days.

The combined profiles of 20α-DHP and oestradiol-17β excretion during the sample collection period from this northern white rhinoceros are shown in Fig. 5.3. From this figure the temporal relationship between 20α-DHP and oestradiol-17β excretion can be

Figure 5.2. Pattern of excretion of oestrone conjugates (a), oestrone (b) and oestradiol-17β (c) during successive oestrous cycles in a northern white rhinoceros (animal 2). The arrows represent the time of mating (M).

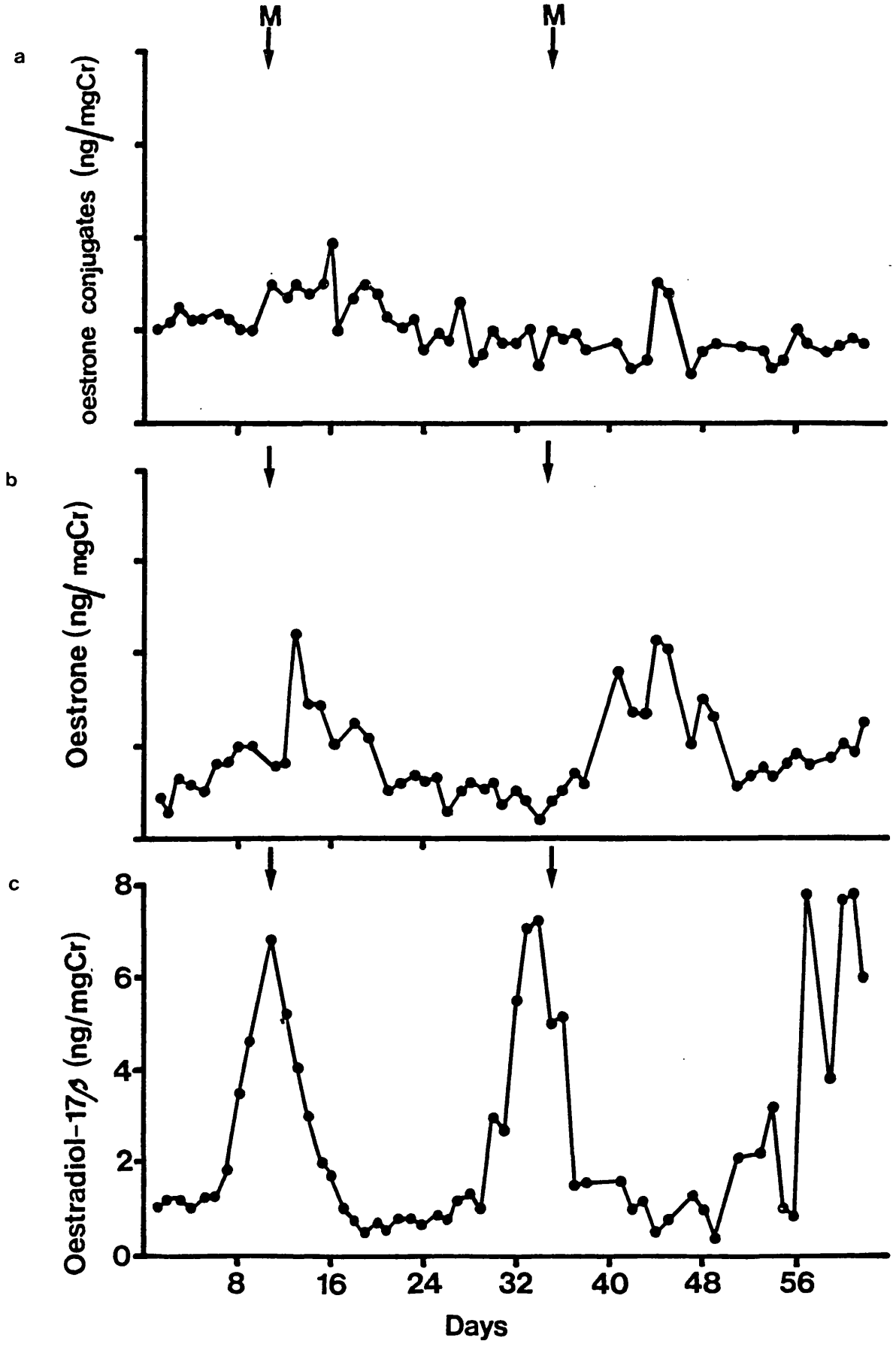
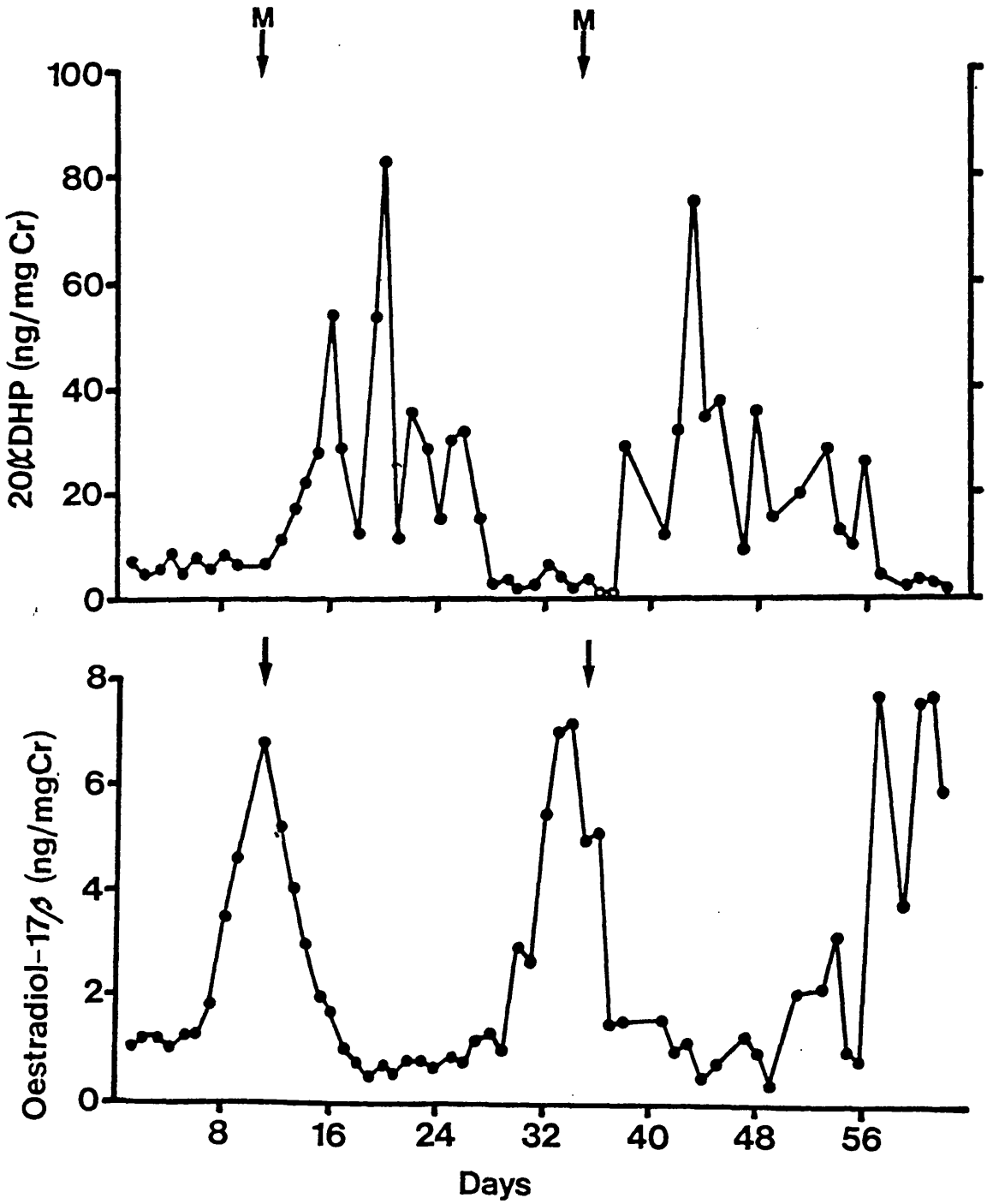

M
M
a
oestrone conjugates (ng/mgCr)
b
Oestrone (ng/ mgCr)
c
Oestradiol-17β (ng/mgCr)
0
2
4
6
8
8
16
24
32
40
48
56
Days

Figure 5.3. Pattern of excretion of 20α-DHP and oestradiol-17β during successive oestrous cycles in a northern white rhinoceros (animal 2). The profiles are compiled from Fig. 5.1a and 5.2c.


M
M
100
80
60
40
20
0
20αDHP (ng/mg Cr)
8
6
4
2
0
Oestradiol-17β (ng/mgCr)
8
16
24
32
40
48
56
Days

seen. Peaks of oestradiol-17β excretion occured during periods of low 20α-DHP excretion in all cases, ie. at the time of mating. Levels reached a nadir at the time of maximum 20α-DHP excretion. As 20α-DHP levels fell, eg. at days 27 and 56, the concentration of oestradiol-17β in the urine began to rise and thus may be interpreted as signifying the onset of the follicular phase. The time between observed matings, ie. the inter-oestrus interval, in this animal was 24 days. The cycle length as determined by oestradiol-17β excretion, ie. the time between peak levels of oestradiol-17β, was 23 days for both cycles illustrated.

The combined profiles of 20α-DHP and oestradiol-17β excretion in a second female northern white rhinoceros (animal 3) over a 60 period are shown in Fig. 5.4. Pre-oestrus levels of 20α-DHP in this animal were considerably higher and more variable (18-35 ng/mgCr) than the levels seen in animal 2 (the first northern white rhinoceros described). However, as in animal 2, there was a clear and marked increase in 20α-DHP excretion following the day of observed mating and the profile of 20α-DHP excretion followed a similar cyclic pattern. Maximum levels of 20α-DHP (75-90 ng/mgCr) were similar to those in animal 2 (60-80 ng/mgCr) and were also reached around 8-12 days after oestrus, in the middle of the luteal phase.

The pattern of urinary oestradiol-17β excretion in this animal is less clear, although levels do fall within the same range as those in animal 2 and increase during both periods of low 20α-DHP excretion. The inter-oestrus interval in this animal was 29 days. The time between oestradiol-17β peaks was 24 days.

Figure 5.5 shows the combined 20α-DHP and oestradiol-17β profiles in urine samples collected from a southern white rhinoceros (animal 5). No behavioural signs of oestrus were seen, although the hormonal profiles clearly show a cyclic pattern of excretion. Low levels of 20α-DHP (less than 10 ng/mgCr) were seen for 16 and 19 days prior to a rapid increase on days 18 and 47

Figure 5.4. Pattern of excretion of 20α-DHP and oestradiol-17β during successive oestrous cycles in a northern white rhinoceros (animal 3). The arrows indicate the time of observed mating (M).

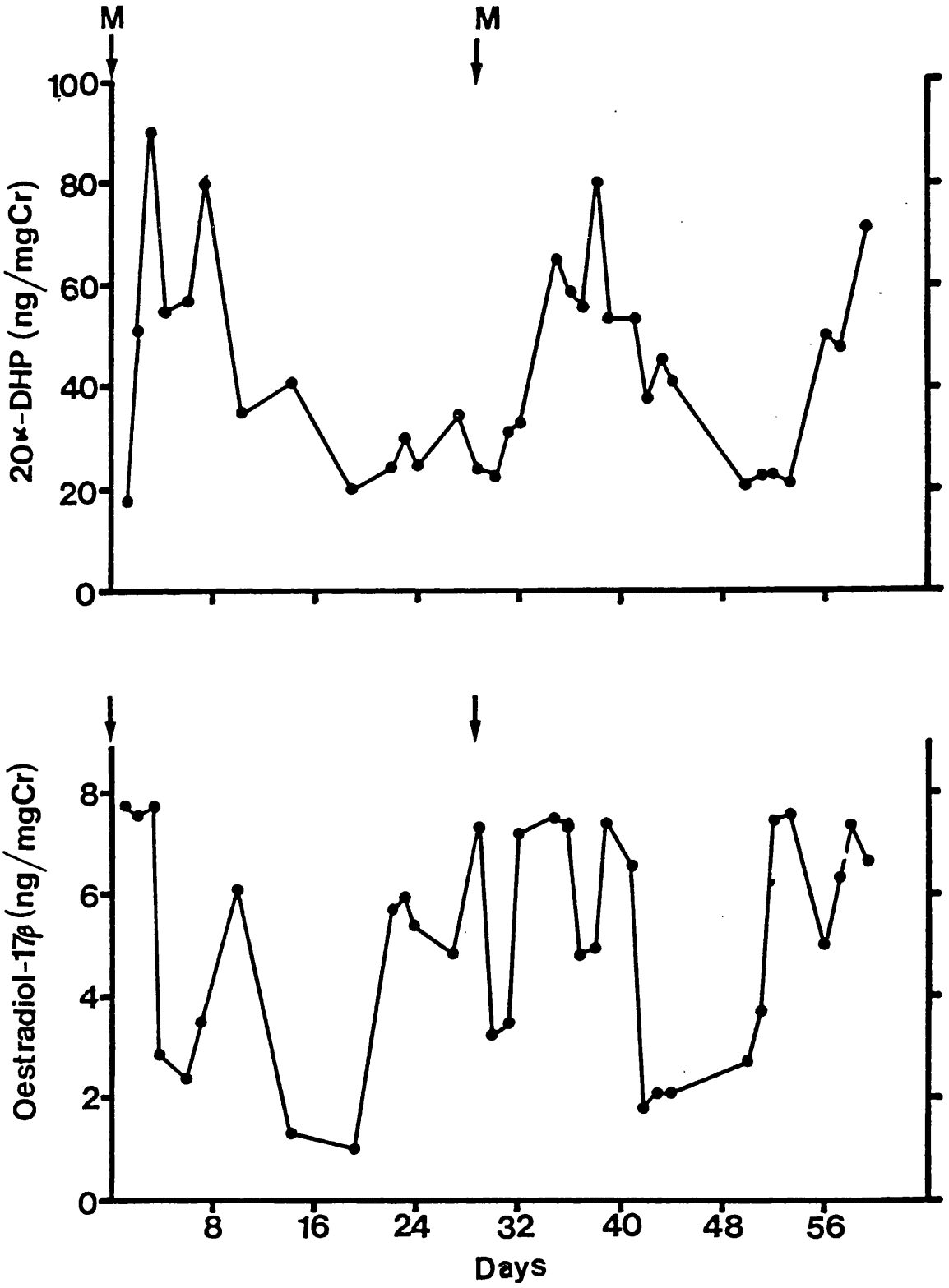
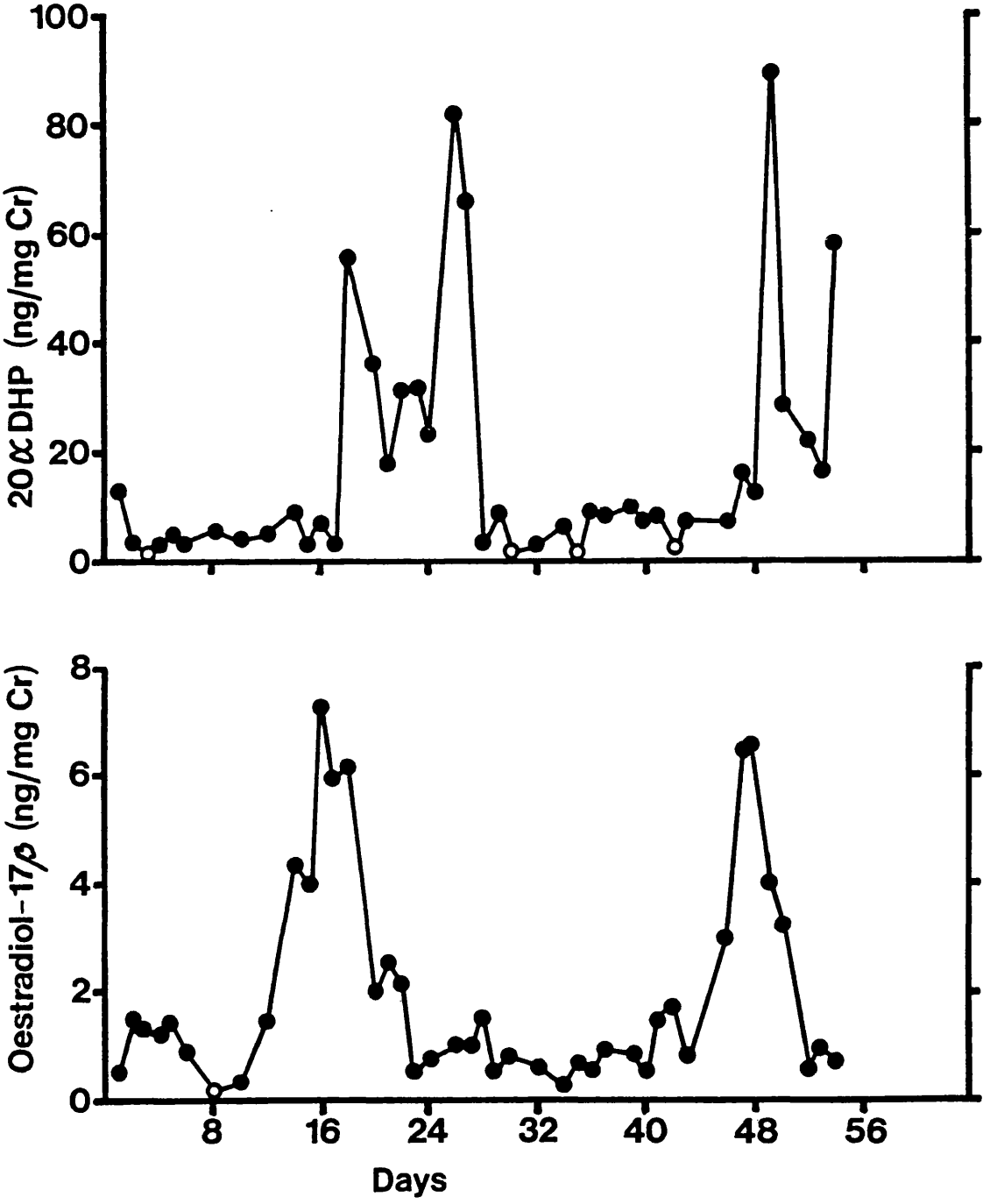

M
M
100
80
60
40
20
0
20α-DHP (ng/mgCr)
8
6
4
2
0
Oestradiol-17β (ng/mgCr)
8
16
24
32
40
48
56
Days

Figure 5.5. Pattern of excretion of 20α-DHP and oestradiol-17β during successive oestrous cycles in a southern white rhinoceros (animal 5). There was no behavioural data to accompany these hormonal profiles.


100
80
60
40
20
0
20α DHP (ng/mg Cr)
8
6
4
2
0
Oestradiol-17β (ng/mg Cr)
8
16
24
32
40
48
56
Days

respectively. Maximum levels of 20α-DHP (70-90 ng/mgCr) during the post-oestrus period, were similar to those in the northern white rhinoceroses.

The cyclic pattern of oestradiol-17β excretion in this animal further suggests that it was undergoing oestrous cycles at the time of sample collection. Although there was no outward signs of oestrus, peaks in oestradiol-17β excretion were clearly seen on days 16 and 48. Peak levels of oestradiol-17β (6-8 ng/mgCr) which were similar to those seen during oestrus in the northern white rhinoceros, also occurred at a time of low 20α-DHP excretion, and levels began to fall at the time of increasing 20α-DHP excretion. As there was no oestrus and/or mating in this animal, the inter-oestrus interval cannot be estimated. However, the distance between peaks in oestradiol-17β excretion in the southern white rhinoceros was 31 days.

Profiles shown in Figs 5.1-5.5 indicate cyclic ovarian function. Accurate description of "follicular" and "luteal" phase components of the cycle is not possible without knowledge of the time of ovulation. However, in order to attempt some partitioning between the two phases of the cycle, the following criteria have been used. The onset of the luteal phase was co-incident with the first rise in 20α-DHP levels above 10 ng/mgCr (animals 2 and 5) and 35 ng/mgCr for animal 3, after at least 8 days below this level. On this basis the mean follicular and luteal levels of 20α-DHP and peak levels of oestradiol-17β in the white rhinoceros are summarised in Table 5.1. From these data the difference between levels of urinary 20α-DHP between the follicular and luteal phase can be clearly seen in all three animals. It is not possible to calculate average levels of 20α-DHP during each period for the white rhinoceros as the results would not be meaningful because of the large difference between animal 3 and the other animals investigated. In animals 2 and 5, the average follicular phase values of 20α-DHP appear to be below 5 ng/mg Cr, whilst mean luteal

Table 5.1. Mean±sem urinary 20α-DHP (ng/mgCr) in samples collected during the presumed follicular and luteal phase of the ovarian cycle, and mean±sem peak levels of oestradiol-17β in the white rhinoceros (*n*=3).

animal	20α-DHP (ng/mgCr)				peak oestradiol-17β (ng/mgCr)
	follicular		luteal		
	mean ±sem	range	mean ±sem	range	
2[1]	4·38±0·46	0·0-9·0	29·30±3.11	11·0-83·0	7·20±0·14
3[1]	25·07±1·30	18·0-25·0	58·79±3·69	35·0-90·0	7·50±0·14
5[2]	4·60±0·56	0·0-9·0	38·87±5·86	12·0-89·0	7·00±0·14

[1] northern white rhinoceros

[2] southern white rhinoceros

Table 5.2. Mean±sem cycle length (days) in the white rhinoceros (n=3) as determined by inter-oestrus interval, 20α-DHP and peak oestradiol-17β (E_2-17β) excretion.

subspecies	animal	cycle length (days)		
		inter-oestrus interval	first rise in 20α-DHP	peak E_2-17β
northern white	2	24	25	23
				23
	3	29	33	24
southern white	5	-	31	31
mean±sem		26·5±1·8	29·7±2·0	25·2±1·7

levels were approximately 30 ng/mgCr. Peak levels of oestradiol-17β were similar (approximately 7 ng/mgCr) in all three animals.

Table 5.2 shows the mean cycle length as determined by inter-oestrus interval, 20α-DHP levels and peak oestradiol-17β excretion in the white rhinoceros. From the limited data available for this species, the mean cycle length was calculated as 27·14 days from means of cycle length as determined by inter-oestrus interval, 20α-DHP and oestradiol-17β excretion.

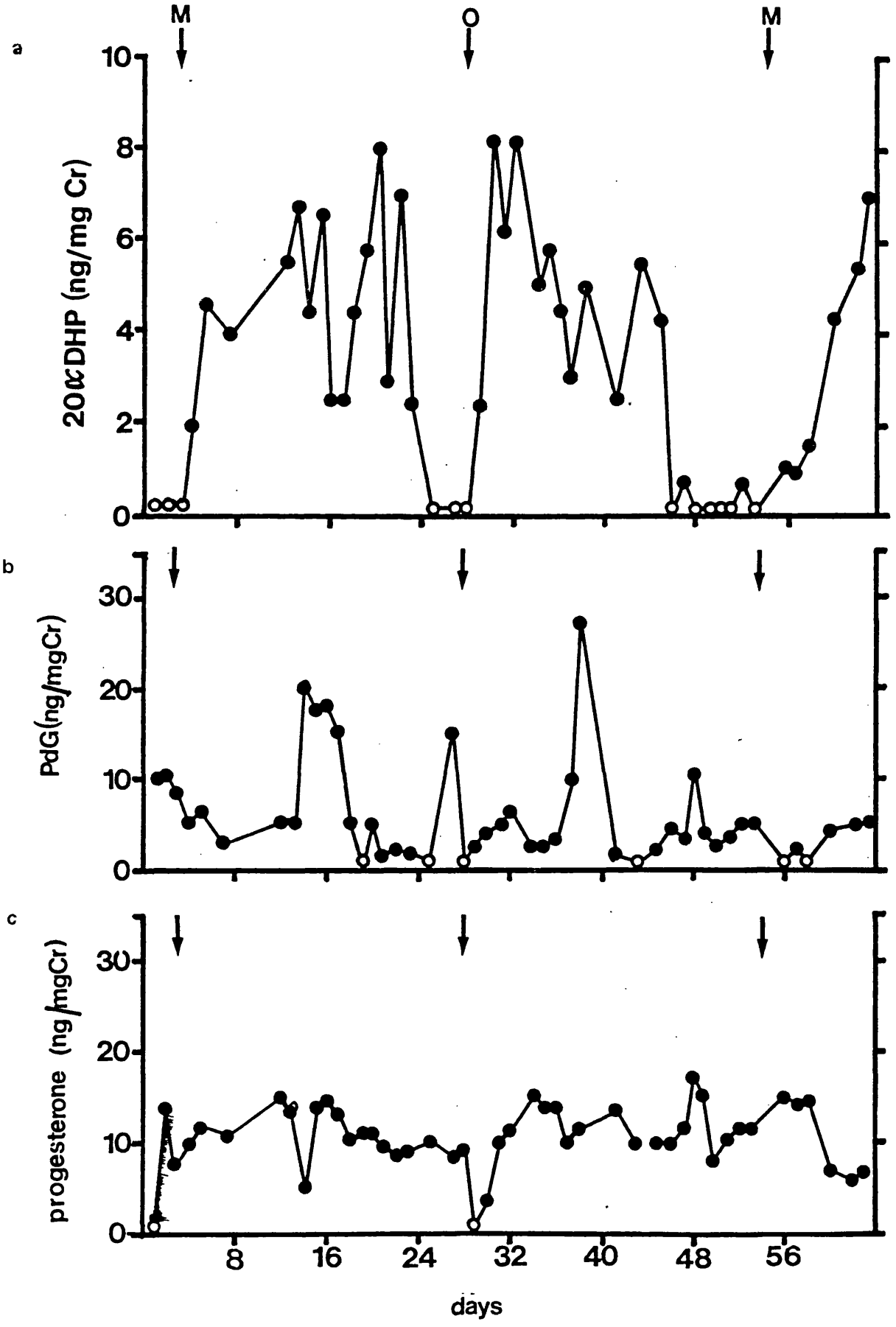
5.3.1.2 Urinary steroid excretion during the ovarian cycle in the black rhinoceros.

The pattern of 20α-DHP excretion over a 60 day period in a juvenile female black rhinoceros revealed low (less than 0·5 ng/mgCr) and mainly undetectable levels in all samples analysed. The profile of excretion is therefore not shown.

Matched profiles of 20α-DHP, PdG and progesterone excretion throughout successive oestrous cycles in an adult female black rhinoceros (animal 6) are shown in Fig. 5.6. The occurrence of two matings and a period of observed oestrous behaviour are indicated by arrows. As in the white rhinoceros, levels of urinary progesterone were low, variable and showed no changes which corresponded to the times of observed sexual activity. Levels of urinary PdG immunoreactivity were generally lower than in the white rhinoceros, with many samples below the sensitivity of the assay. Some peaks of PdG excretion were seen, eg. on days 14 and 38, but overall there was no cyclic pattern of excretion.

In contrast, the levels of urinary 20α-DHP (Fig. 5.6a) showed a cyclic pattern of excretion with marked and very rapid increases occurring immediately following oestrus and/or mating. The figure demonstrates that levels of 20α-DHP excretion during the pre-oestrus period are extremely low (less than 1 ng/mgCr) and often below the assay sensitivity. The period of low 20α-DHP excretion

Figure 5.6. Pattern of excretion of 20α-DHP (a), PdG (b) and progesterone (c) during successive oestrous cycles in a black rhinoceros (animal 6). The arrows indicate the time of observed oestrus (O) or mating (M).


a
M
O
M
10
8
6
4
2
0
20αDHP (ng/mg Cr)
b
30
20
10
0
PdG(ng/mgCr)
c
30
20
10
0
progesterone (ng/mgCr)
8
16
24
32
40
48
56
days

(less than 1 ng/mgCr) was short, in this case less than 7 days prior to mating. Maximum values of 20α-DHP during the presumed luteal phase were between 6 and 8·5 ng/mg Cr, much lower than in the white rhinoceros. Urinary 20α-DHP remained elevated for a post-oestrus period of at least 17 days.

Profiles of oestrone conjugates, oestrone and oestradiol-17β excretion over the 60 day sample collection period are shown in Fig, 5.7. Levels of oestrone conjugates were generally low (0-5 ng/mgCr) with elevations up to 20 ng/mgCr around the time of observed mating and/or oestrus. However, although increased levels of oestrone conjugates were observed at the time of mating and/or oestrus, fluctuations were not exclusively associated with these periods of sexual activity. The pattern of excretion of oestrone conjugates was not reliable enough to provide a good indication of the time of presumed ovulation in this species. However, levels of urinary oestrone after hydrolysis (Fig. 5.7.b) showed a clear cyclical pattern, with the highest values occurring on the day preceeding or the day after oestrus or mating. These peaks (5.5-7.8 ng/mgCr) were also co-incident with the time of peak levels of oestrone conjugate excretion. A third period of observed mating is indicated during the sample collection period although there is no peak of oestrone excretion to accompany this.

In contrast to the pattern of oestrone excretion, the profile of urinary oestradiol-17β showed no elevation from baseline levels (less than 2 ng/mgCr).

Figure 5.8 shows the matched profiles of 20α-DHP and oestrone excretion in this black rhinoceros (animal 6). In both cycles shown, levels of oestrone rise to peak values at the time of low levels of 20α-DHP excretion, and fall rapidly to reach a nadir at the time of maximum excretion of 20α-DHP. The inter-oestrus intervals in this animal were 25 and 26 days respectively, and the time between peaks in oestrone excretion was 24 days.

Figure 5.7. Pattern of excretion of oestrone conjugates (a), oestrone (b) and oestradiol-17β (c) during successive oestrous cycles in a black rhinoceros (animal 6). The arrows indicate the time of observed oestrus (O) or mating (M).

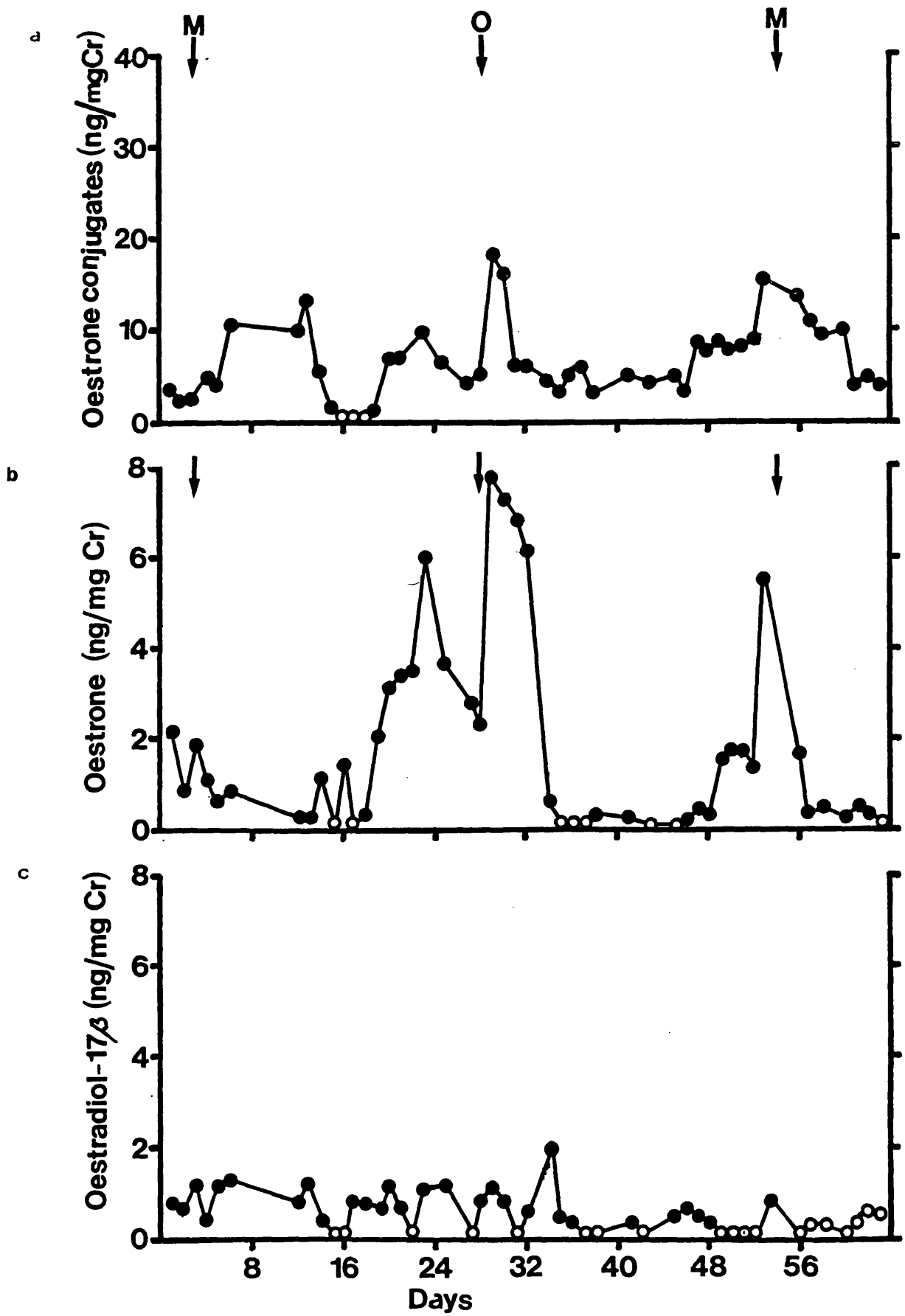

a
M
O
M
Oestrone conjugates (ng/mgCr)
40
30
20
10
0
b
Oestrone (ng/mg Cr)
8
6
4
2
0
c
Oestradiol-17β (ng/mg Cr)
8
6
4
2
0
8
16
24
32
40
48
56
Days

Figure 5.8. Pattern of excretion of 20α-DHP and oestrone during successive oestrous cycles in a black rhinoceros (animal 6). The profiles are compiled from Fig. 5.6a and 5.7b.

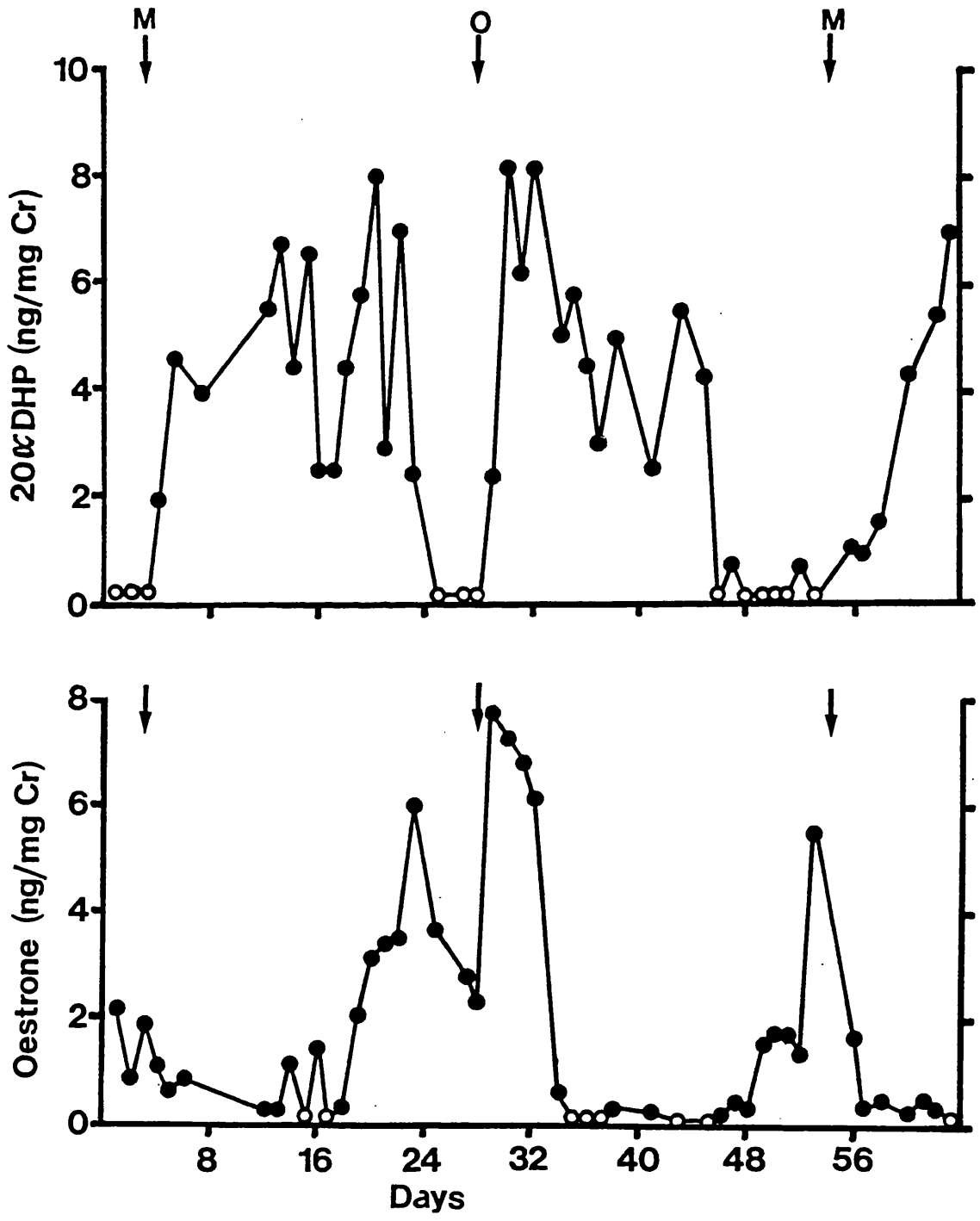

M
O
M
10
8
6
4
2
0
20αDHP (ng/mg Cr)
8
6
4
2
0
Oestrone (ng/mg Cr)
8
16
24
32
40
48
56
Days

Figure 5.9 shows the pattern of excretion of 20α-DHP and oestrone during a sample collection period in which mating was observed 3 times (animal 8). Urinary 20α-DHP excretion showed a similar cyclic pattern to that of animal 6, with low levels of 20α-DHP for at least 3 days preceeding mating. The period of sexual activity was followed by a very rapid rise in 20α-DHP reaching maximum levels within 7 days. Pre-oestrus levels of 20α-DHP were similar to those of animal 6, ie. below 2 ng/mgCr and often undetectable, although urinary concentrations of 20α-DHP were generally higher (9-15 ng/mgCr) and less variable.

The pattern of oestrone excretion in this female was clearer than that in animal 6, with peak levels of oestrone (6-8 ng/mgCr) being observed on the day of mating in both cases. Inter-oestrøus intervals for this animal were 20 and 16 days, the latter of which was also the cycle length as determined by oestrone excretion. Considering the pattern of 20α-DHP excretion, the cycle length was 17 days, considerably shorter than that observed in animal 6.

Matched urinary 20α-DHP and oestrone profiles throughout 3 cycles in another black rhinoceros (animal 9) are shown in Figure 5.10. Conception occurred following the mating on day 33. Similar results were obtained for the pattern of 20α-DHP excretion in this animal as those previously recorded. Due to infrequent sample collection, only one peak of oestrone is shown, which occurred on the day of mating from which conception resulted. The inter-oestrus interval was 15 days.

The profile of excretion of 20α-DHP and oestrone in a fourth animal (animal 8) over a 60 day period, where mating was observed on two occasions, is shown in Figure 5.11. Urinary 20α-DHP levels in this animal were generally higher (peak levels of over 20 ng/mgCr) than those observed previously, although a clear cyclic pattern in relation to mating and oestrone excretion was still evident. Peaks of oestrone excretion (5-6 ng/mgCr) were similar to those observed in other female black rhinoceroses and occurred on the

Figure 5.9. Pattern of excretion of 20α-DHP and oestrone during successive oestrous cycles in a black rhinoceros (animal 8). The arrows indicate the time of observed mating (M).

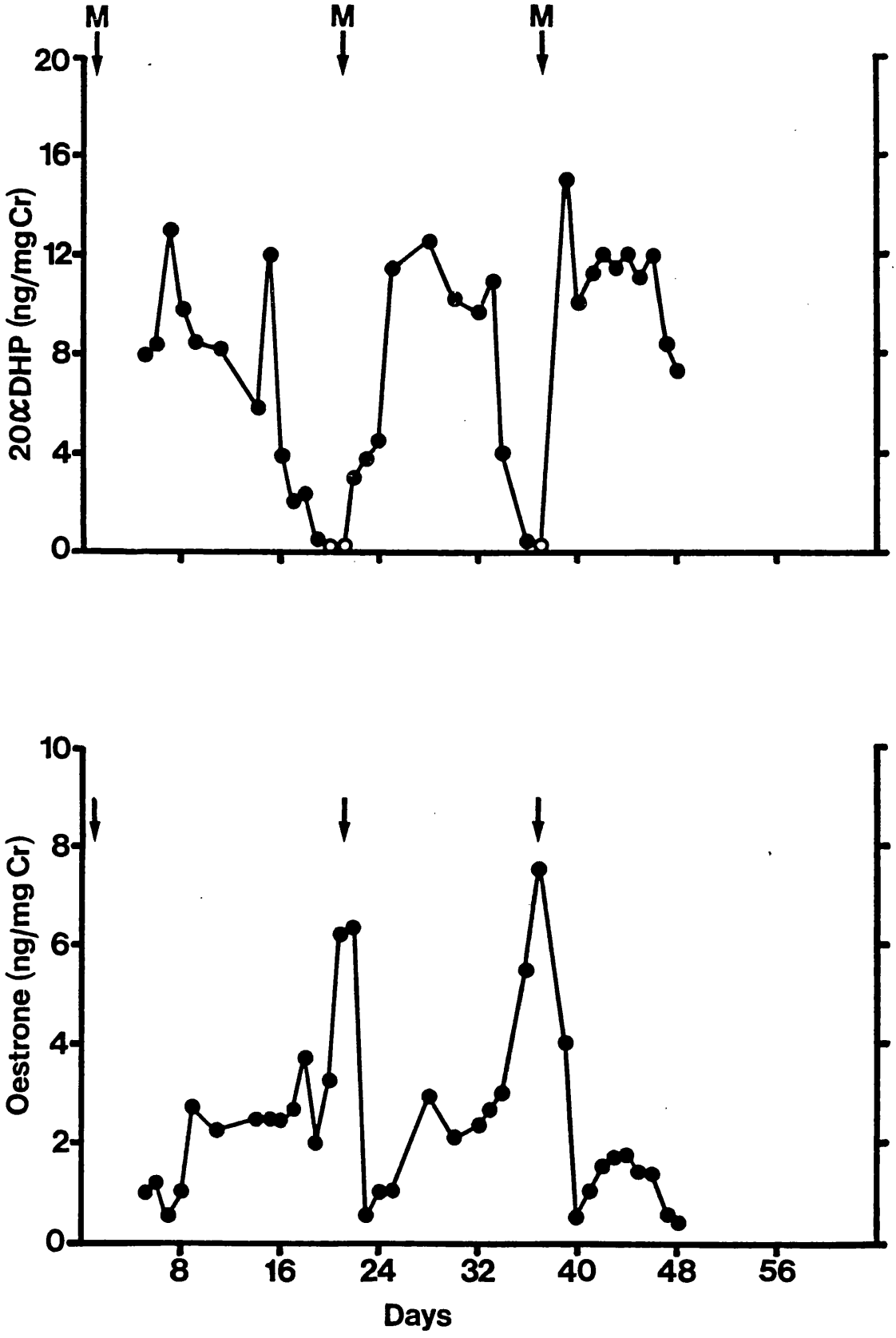

M
M
M
20
16
12
8
4
0
20αDHP (ng/mg Cr)
10
8
6
4
2
0
Oestrone (ng/mg Cr)
8
16
24
32
40
48
56
Days

Figure 5.10. Pattern of excretion of 20α-DHP and oestrone during successive oestrous cycles in a black rhinoceros (animal 9). The arrows indicate the time of observed oestrus (O) and mating (M). The period of oestrus indicated on the figure resulted in conception (M+C).

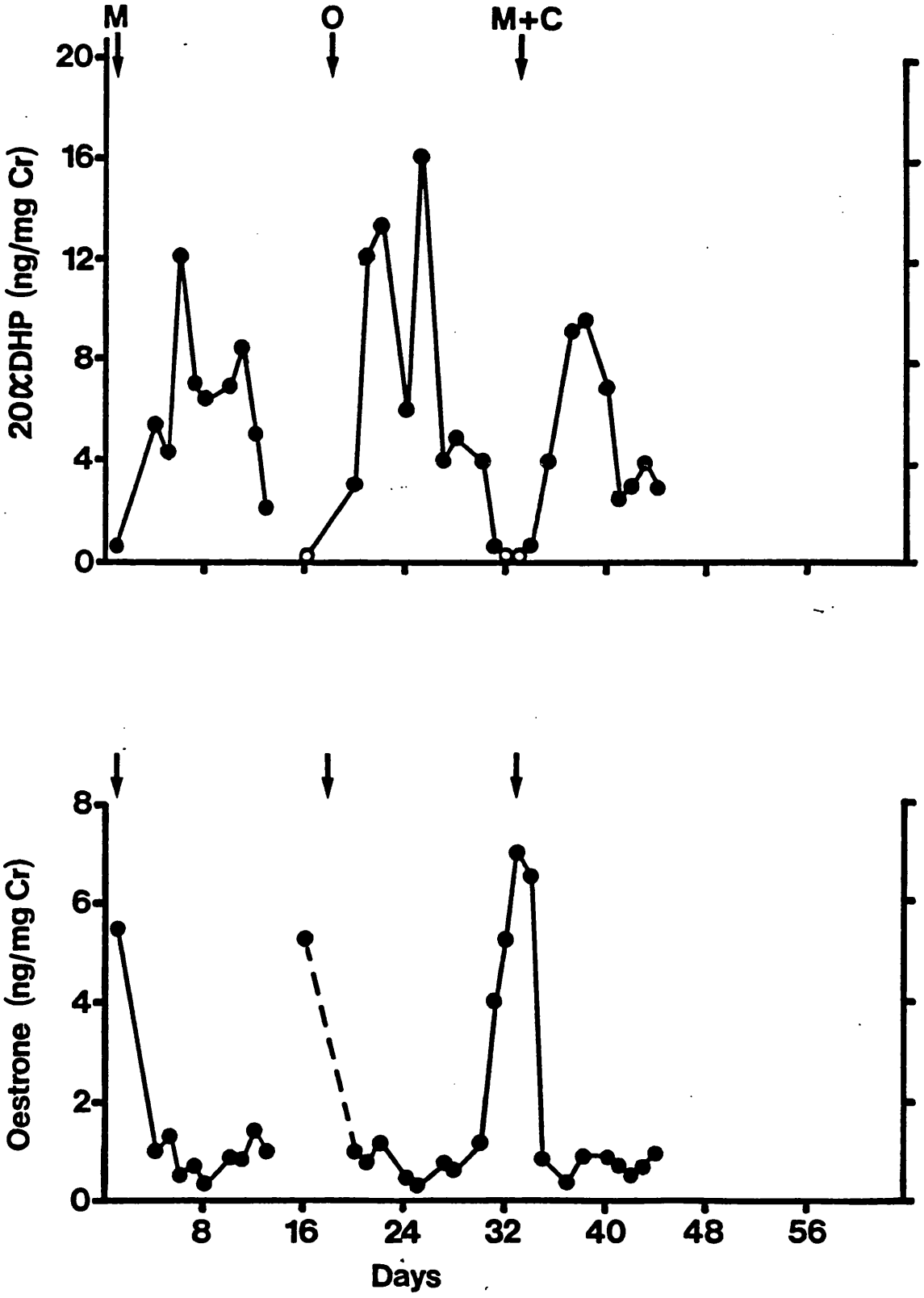
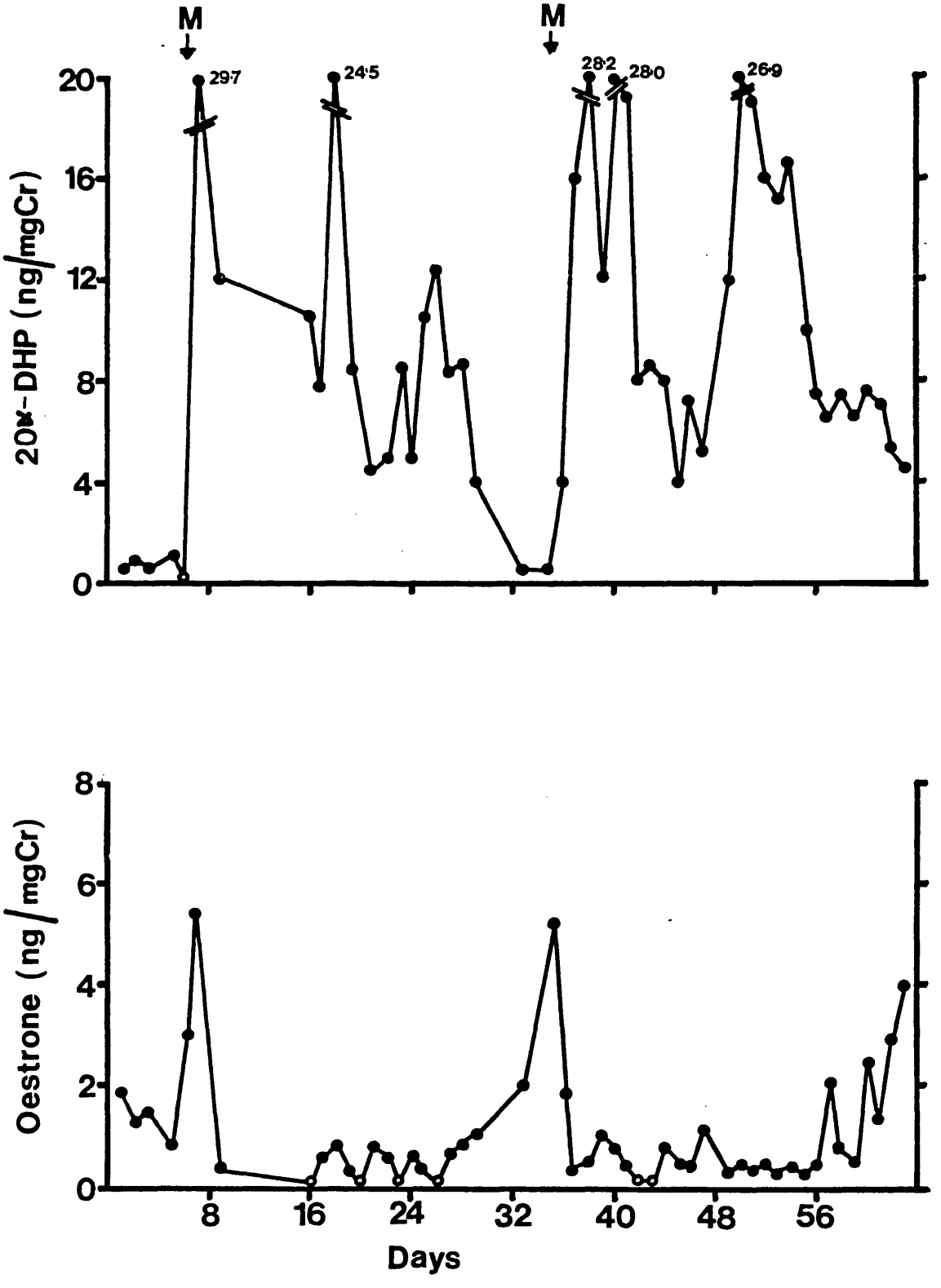

M
O
M+C
20
16
12
8
4
0
20αDHP (ng/mg Cr)
8
6
4
2
0
Oestrone (ng/mg Cr)
8
16
24
32
40
48
56
Days

Figure 5.11. Pattern of excretion of 20α-DHP and oestrone during successive oestrous cycles in a black rhinoceros (animal 7). The arrows indicate the time of observed mating (M).


M
M
29·7
24·5
28·2
28·0
26·9
20α-DHP (ng/mgCr)
0
4
8
12
16
20
Oestrone (ng/mgCr)
0
2
4
6
8
8
16
24
32
40
48
56
Days

day of mating in both cases. The inter-oestrus interval and time between peaks of oestrone excretion were a constant 28 days.

In the black rhinoceros, the criteria used to determine the onset of the luteal phase was the first rise in 20α-DHP levels above 1 ng/mgCr , after at least 3 days below this level. On this basis the mean presumed follicular and luteal levels of 20α-DHP and peak levels of oestrone in the black rhinoceros are summarised in Table 5.3. Once again the difference between levels of urinary 20α-DHP between the follicular and luteal phase can clearly be seen in all four animals. Mean follicular levels of 20α-DHP were 0·23 ng/mgCr and luteal levels were 7·64 ng/mgCr. Peak levels of oestrone were similar in all animals (mean ± sem, 6·44±0·35 ng/mgCr, c of v=10·8%). Table 5.4 shows the mean cycle length as determined by inter-oestrus interval, 20α-DHP levels and peak oestrone excretion in the black rhinoceros. The mean cycle length was calculated as 22·51 days from inter-oestrus interval, 20α-DHP and oestrone excretion.

Figure 5.12 shows the mean±sem urinary 20α-DHP and oestrone concentrations from 144 urine samples taken from a total of 9 cycles in 4 female black rhinoceroses. Individual cycles were aligned at Day 0, corresponding to the day of observed oestrus and/or mating, and combined to produce composite urinary 20α-DHP and oestrone profiles for this species. Mean levels of 20α-DHP fell 3 days before oestrus and increased markedy 1 day after oestrus. Using a value of 1 ng/mgCr to distinguish between follicular and luteal phases, the composite profile indicated a follicular phase length of 3-4 days and a luteal phase of approximately 17 days. Thus the overall cycle length was 21-22 days. Mean oestrone levels rose above baseline (1 ng/mgCr), 5 days prior to oestrus. Maximum levels were attained on the day of oestrus, and fell to basal values approximately 5 days later.

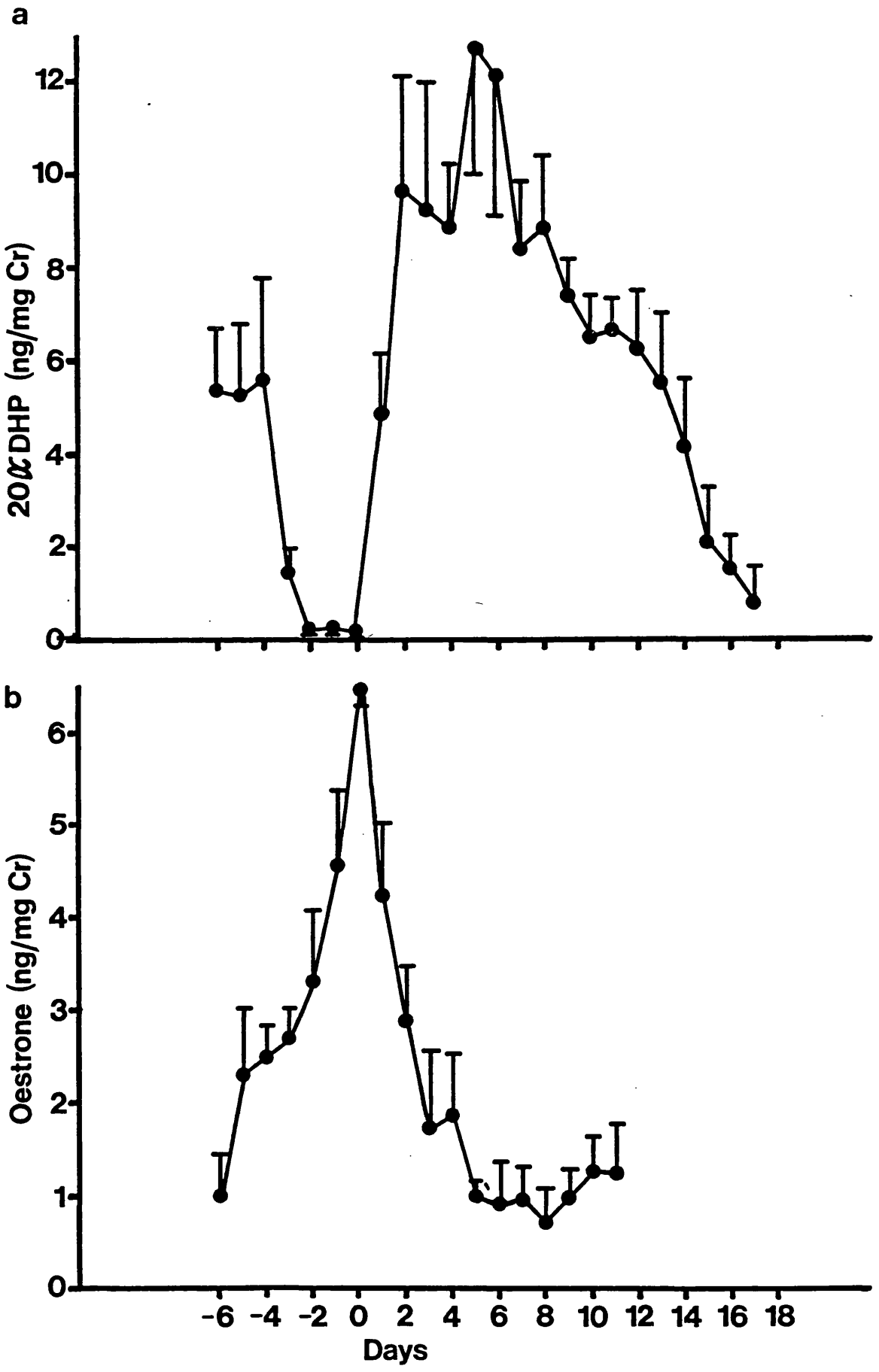
Table 5.3. Mean±sem urinary 20α-DHP (ng/mgCr) in samples collected during the presumed follicular and luteal phase of the ovarian cycles, and measn±sem peak levels of oestrone in the black rhinoceros (n=3).

animal	20α-DHP (ng/mgCr)				peak oestrone (ng/mgCr)
	follicular		luteal		
	mean ±sem	range	mean ±sem	range	
6	0·09±0·06	0-0·8	4·53±0·37	1·2-8·2	6·65±0·81
8	0·16±0·10	0-1·0	8·31±0·69	1·2-15·0	6·88±0·44
9	0·30±-0·12	0-0·6	6·66±0·72	2·0-16·2	7·00
7	0·37±0·05	0-0·5	11·07±1·09	4·0-29·7	5·25±0·11
mean±sem	0·23±0·05		7·64±3·82		6·44±0·35

Table 5.4. Mean±sem cycle length in the black rhinoceros as determined by inter-oestrus interval, first rise in 20α-DHP levels above those during the follicular phase and peak oestrone excretion.

animal	cycle length (days)		
	inter-oestrus interval	first rise in 20α-DHP	peak E_2-17β
6	25 26	24 31	24
8	20 16	17	16
9	15	15	-
7	28	29	28
mean±sem	21·67±2·04	23·2±2·83	22·67±2·88

Figure 5.12. Mean±sem urinary 20α-DHP (a) and oestrone (b) concentrations from 144 urine samples taken from a total of 9 cycles in 4 black rhinoceroses. Individual cycles were standardized at Day 0, corresponding to the day of observed behavioural oestrøus and/or mating, and combined to produce composite urinary profiles of 20α-DHP and oestrone for the ovarian cycle in the black rhinoceros. Each point is mean±sem of individual values obtained for a particular day (n=6-9).


a
20α DHP (ng/mg Cr)
0
2
4
6
8
10
12
b
Oestrone (ng/mg Cr)
0
1
2
3
4
5
6
-6 -4 -2 0 2 4 6 8 10 12 14 16 18
Days

5.3.2 Measurement of urinary steroids during pregnancy in rhinoceroses

Levels of PdG immunoreactivity during the last 6 months of pregnancy in the Indian, black and white rhinoceroses are shown in Figure 5.13. All three profiles show increased PdG immunoreactivity associated with pregnancy and a rapid fall in levels at the end of the gestation period. Although PdG immunoreactivity was elevated in all three species, the absolute values for PdG immunoreactivity varied considerably between the species. Whilst levels of PdG were consistently above 4 µg/mgCr in the Indian rhinoceros over the last 6 months of gestation, levels in the black rhinoceros and white rhinoceros were 10 fold lower, being between 0·2 and 0·6 µg/mgCr.

During the course of this study, immunoreactive PdG was measured in mid to late pregnancy urine of 4 black rhinoceroses during 6 pregnancies. Figure 5·14a shows mean±sem values from 232 urine samples over the last 11 months of pregnancy. Each point represents the mean values for samples collected during each week prior to birth. A clear elevation of PdG immunoreactivity was seen throughout the 11 months of gestation as compared with the average PdG immunoreactivity measurable following parturition. Mean PdG immunoreactivity remained above 100 ng/mgCr throughout the second and third trimester of pregnancy, but fell rapidly from 475 ng/mgCr on the day prior to birth to 10 ng/mgCr on the day after parturition. Urinary concentrations of PdG immunoreactivity then remained low.

However, Fig. 5.14b shows the urinary PdG immunoreactivity from each of 6 pregnancies in 4 black rhinoceroses, from which the composite profile was obtained and plotted in respect to the day of parturition. Although each animal excreted consistently higher amounts of PdG throughout the last 11 months of gestation than during the ovarian cycle, a wide range of values were obtained for individual animals and between urine samples collected from a single animal.

Figure 5.13. PdG immunoreactivity during the last 6 months of pregnancy in an Indian (a), black (b) and southern white (c) rhinoceros. All pregnancies were full term.

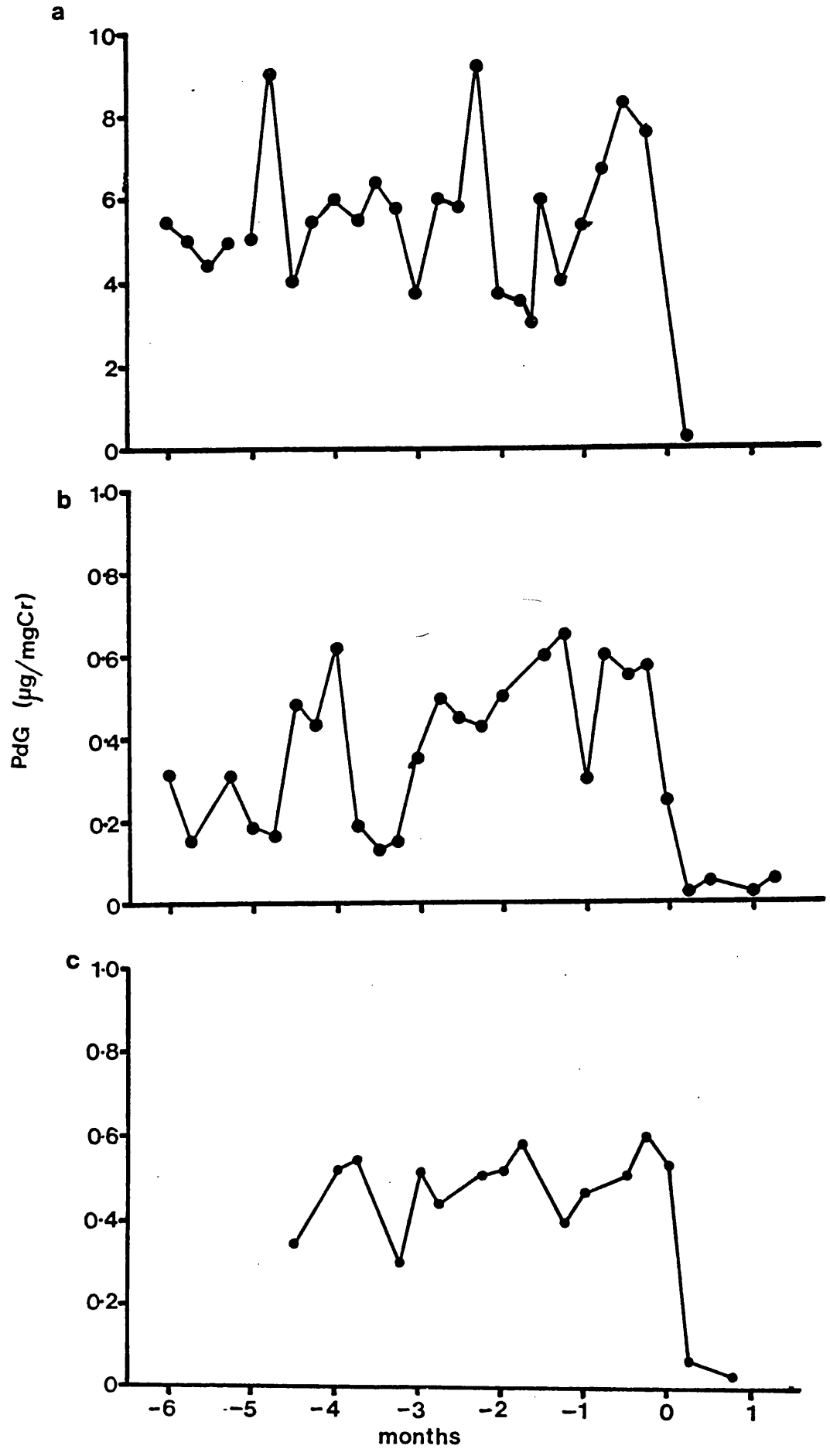
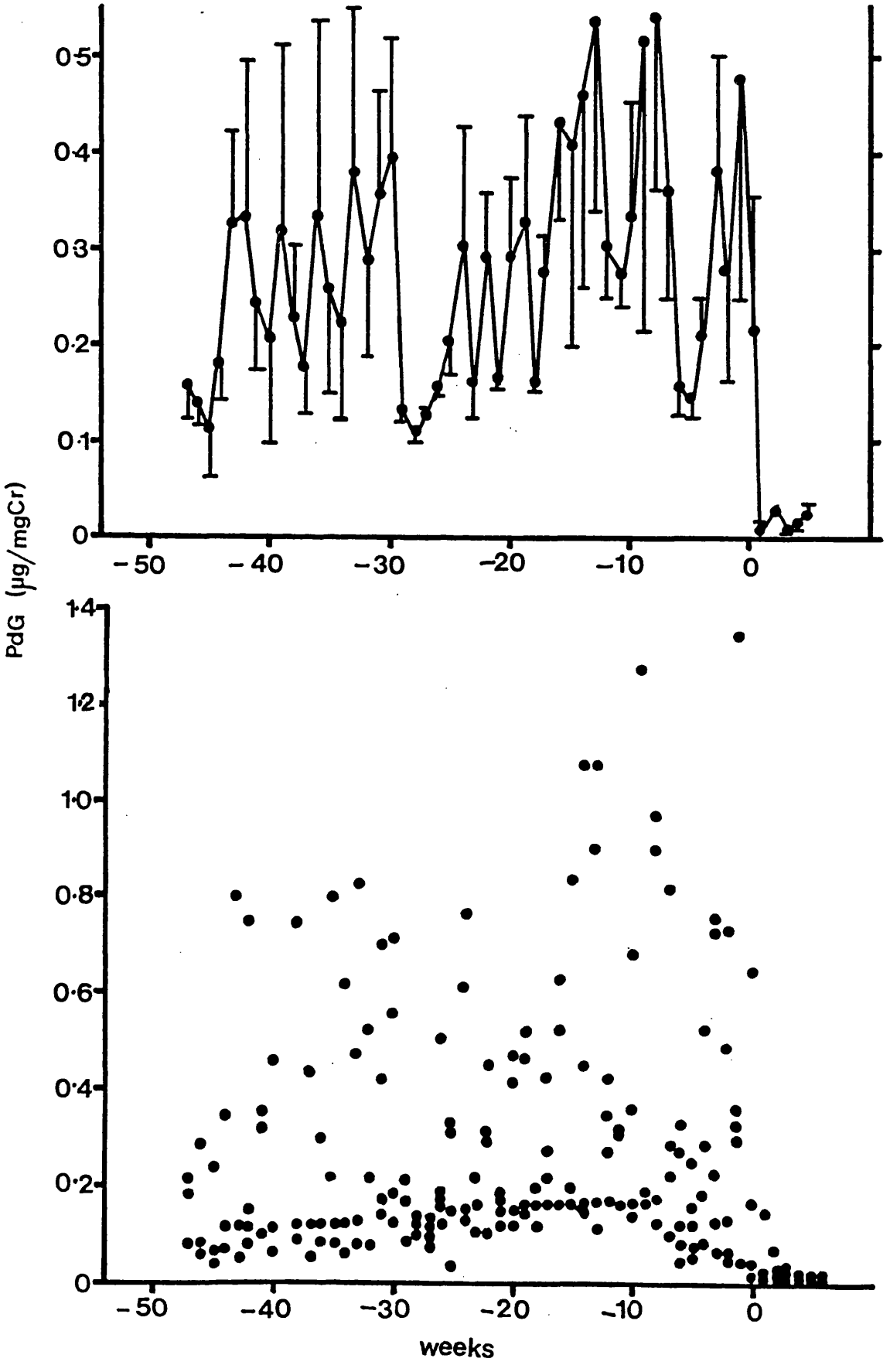

a
10
8
6
4
2
0
b
1·0
0·8
0·6
0·4
0·2
0
PdG (µg/mgCr)
c
1·0
0·8
0·6
0·4
0·2
0
-6
-5
-4
-3
-2
-1
0
1
months

Figure 5.14a. Mean±sem urinary PdG immunoreactivity from 232 urine samples taken from a total of 6 pregnacies in 4 black rhinoceroses. Individual pregnancies were standardized at Day 0, corresponding to the day of parturition, and combined to produce a composite profile of PdG excretion during the last 11 months of pregnancy in the black rhinoceros. Each point is mean±sem of individual values obtained for a particular week prior to birth (n=3-6).

Figure 5.14b. Urinary PdG immunoreactivity values from 6 pregnancies in 4 black rhinoceroses, aligned to the day of parturition (Day 0).


PdG (µg/mgCr)
0·5
0·4
0·3
0·2
0·1
0
-50
-40
-30
-20
-10
0
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
-50
-40
-30
-20
-10
0
weeks

The patterns of 20α-DHP and PdG excretion throughout a complete pregnancy in a northern white rhinoceros (animal 3) are shown in Fig.5.15. This animal was presumed to have concieved in March 1988 when the last positive mating was observed. Thus the length of gestation for this animal was determined to be 482 days (approximately 16 months). Thus 1 month after conception 20α-DHP levels were similar to peak luteal phase values (ie. 90 ng/mgCr) and then fell to below 20 ng/mgCr for a period of 3 weeks. After this period, 20α-DHP levels remained elevated although variable and rose steadily to reach levels of 0·18 µg/mgCr at mid-pregnancy. Samples were not collected during the period October 1988-April 1989 by which time levels had fallen. Levels of 20α-DHP rose once more to reach 0·17 µg/mgCr a week prior to birth, and fell to less than 20 ng/mgCr at the time of parturition. Thereafter levels remained within the follicular phase range for this animal.

Low levels of PdG immunoreactivity were measurable in the urine at 2 months after conception and increased steadily throughout gestation to reach maximal levels of 1·2 µg/mgCr 2 months prior to birth. After reaching this peak, levels of PdG immunoreactivity fell to around 0·4 µg/mgCr where they remained until parturition, when the characteristic precipitous fall in levels was seen.

The excretion of oestrogens during pregnancy was also investigated, and the profiles of oestradiol-17β and oestrone excretion are shown in Fig. 5.16. Levels of both oestrogens were low throughout pregnancy (less than 40 ng/mgCr) although some apparently pregnancy specific changes were observed. Oestradiol-17β remained the most abundant urinary oestrogen throughout the first and second trimesters of gestation, with levels consistently elevated above baseline values seen in the luteal phase of the ovarian cycle (approximately 2 ng/mgCr). Levels of urinary oestradiol-17β increased throughout gestation, although there was much variation between individual samples, to reach a maximum of 34 ng/mgCr 2 months prior to birth. Levels of oestradiol-17β then fell and remained low (around 4 ng/mgCr) until birth which was followed

Figure 5.15. Profiles of 20α-DHP (——) and PdG (- - - -) excretion throughout a complete pregnancy in a northern white rhinoceros.

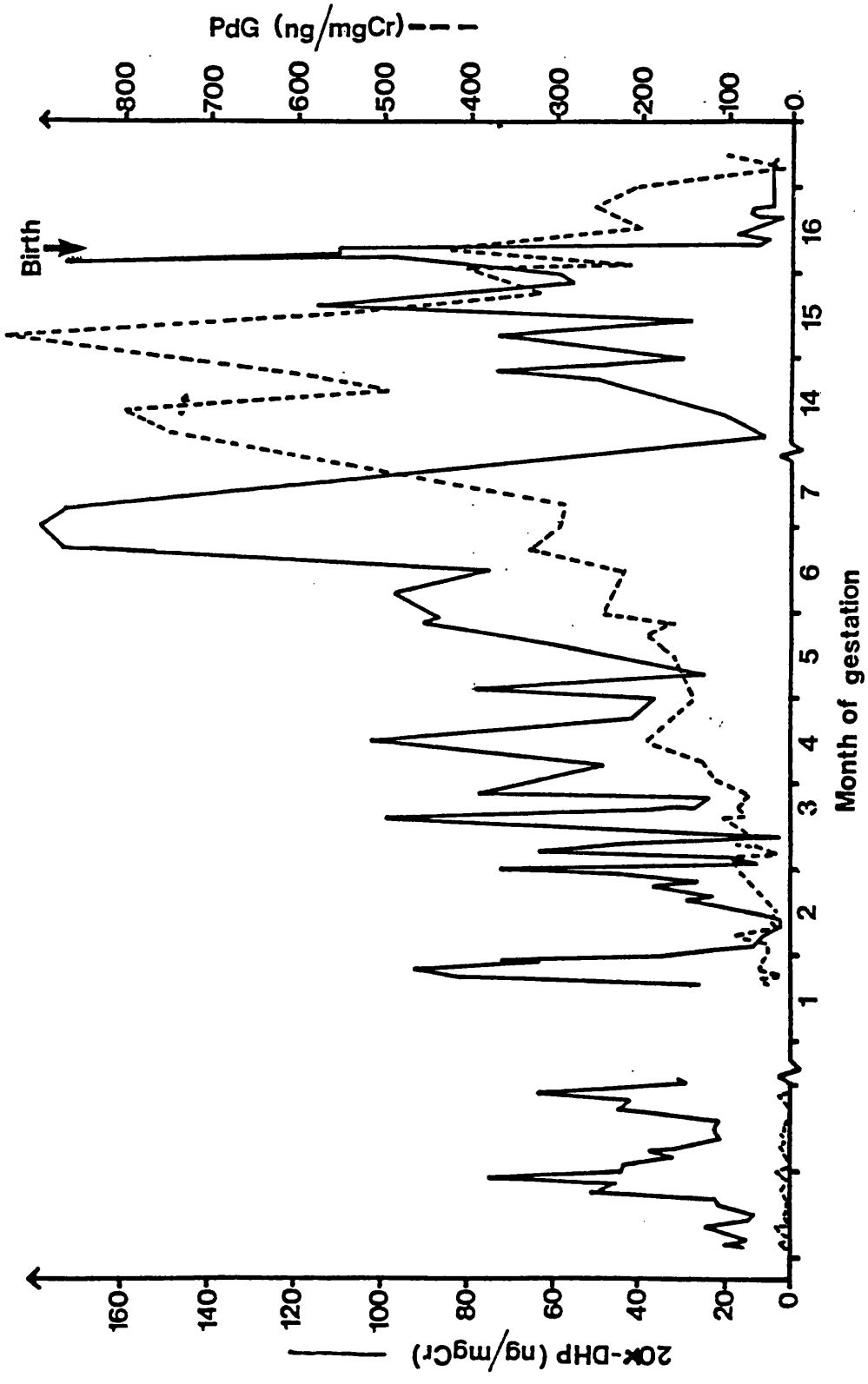
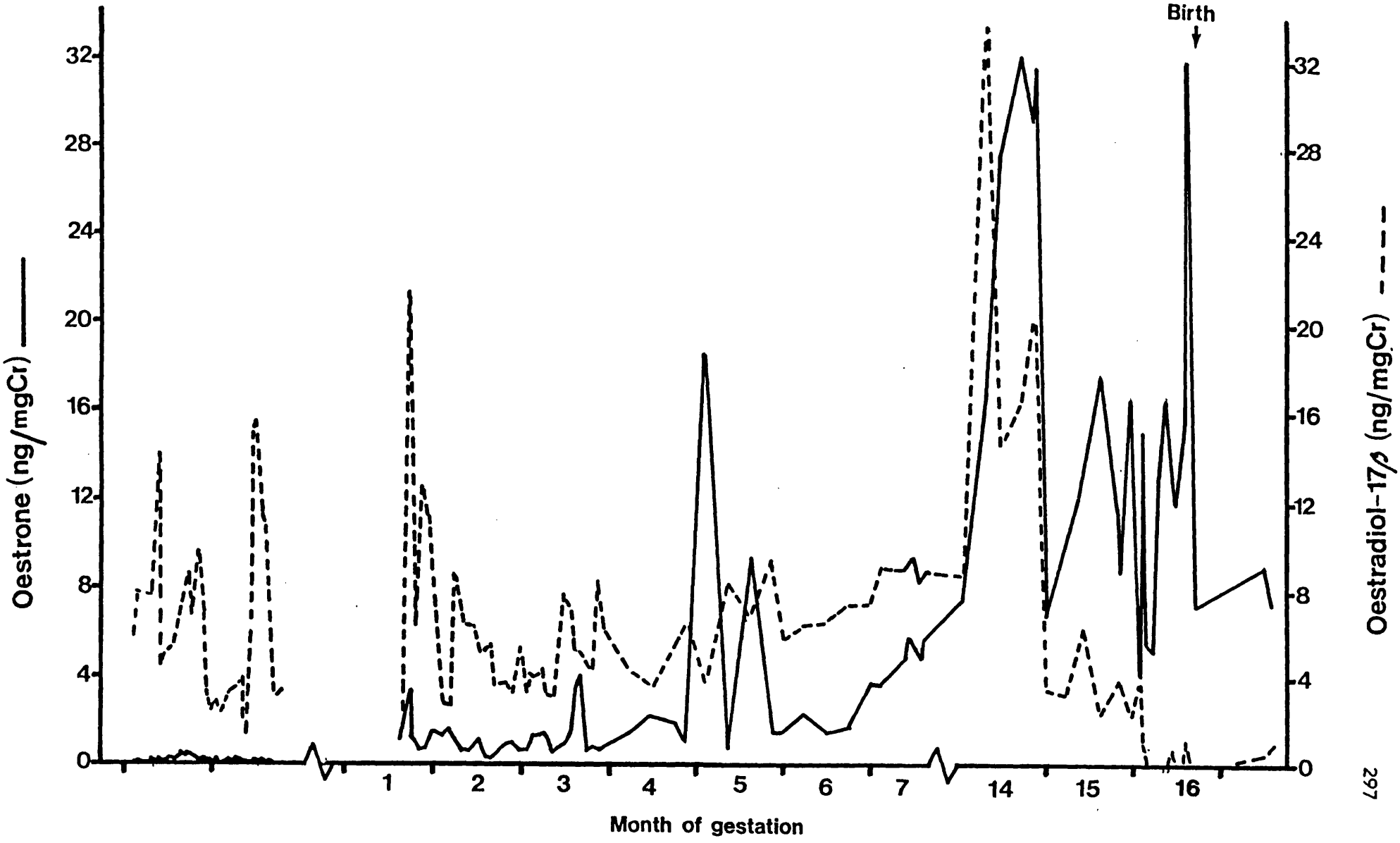

PdG (ng/mgCr)
800
700
600
500
400
300
200
100
0
Birth
16
15
14
7
6
5
4
3
2
1
Month of gestation
20α-DHP (ng/mgCr)
160
140
120
100
80
60
40
20
0

Figure 5.16. Profiles of oestradiol-17β (- - - -) and oestrone (⟵⟶) excretion throughout a complete pregnancy in a northern white rhinoceros.


Birth
Oestrone (ng/mgCr) ——
Oestradiol-17β (ng/mgCr) - - - -
0
4
8
12
16
20
24
28
32
1
2
3
4
5
6
7
14
15
16
Month of gestation

by a period of elevated oestradiol-17β which lasted for at least 2 weeks *post partum*.

Apart from two samples that contained high levels of oestrone immunoreactivity, values remained below 4 ng/mgCr for at least the first 7 months of gestation. However, a rapid increase in urinary oestrone was seen 2 months prior to birth, almost co-incident with the increased oestradiol-17β excretion, to reach levels greater than 12 ng/mgCr. Oestrone levels fell to undetectable values 6 days prior to parturition and remained so throughout the *post partum* period.

Similar profiles of progesterone metabolite and oestrogen excretion throughout pregnancy in the black rhinoceros (animal 9) are shown in Figs 5.17 and 5.18. Urine samples were collected throughout the conception cycle in this animal and urinary steroid levels during this period are shown on the graphs. The gestation length of this animal could thus be accurately determined as 494 days. In contrast to the northern white rhinoceros, levels of urinary 20α-DHP fell to below 1 ng/mgCr following conception (ie. follicular phase levels) and remained low throughout the first 10 months of gestation. Levels of urinary 20α-DHP then rose gradually to reach maximum levels of 4 ng/mgCr a month prior to birth, before falling to undetectable a week prior to parturition.

The profile of PdG excretion throughout gestation is also shown in Fig. 5.17. Within 3 months of conception, levels of immunoreactive PdG were elevated (40-60 ng/mgCr) above those seen during the ovarian cycle in this animal (maximum of 5-7 ng/mgCr). PdG immunoreactivity continued to rise substantially throughout the gestation period, reaching maximum concentrations of 0·18 µg/mgCr 2 months prior to birth. In the remaining two months, PdG levels fell gradually to be less than 5 ng/mgCr on the day of parturition.

The profile of oestrone excretion throughout pregnancy in the black rhinoceros is shown in Fig. 5.18. A peak of oestrone was

Figure 5.17. Profiles of 20α-DHP (——————) and PdG (- - - -) throughout a conception cycle and complete pregnancy in a black rhinoceros.

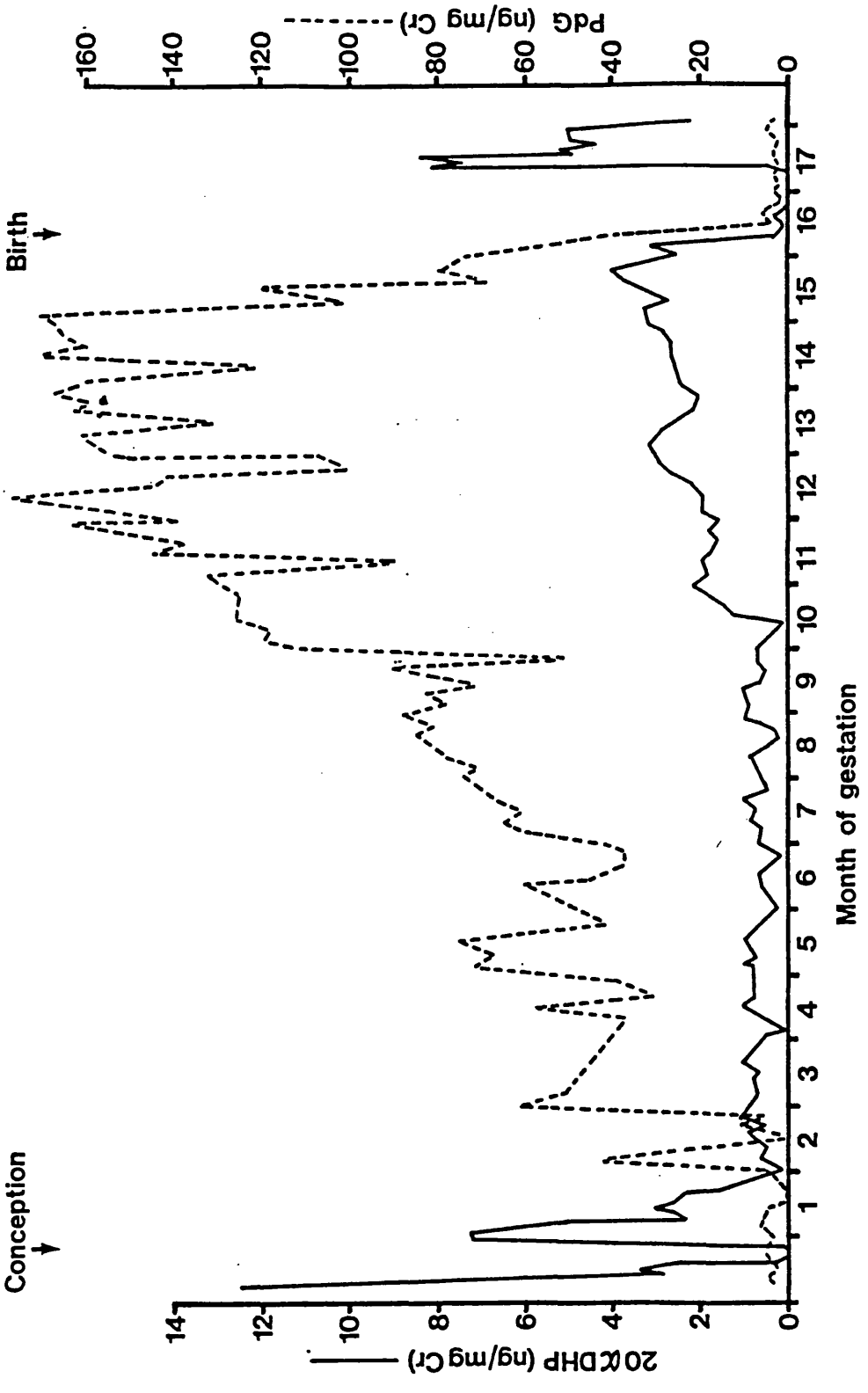
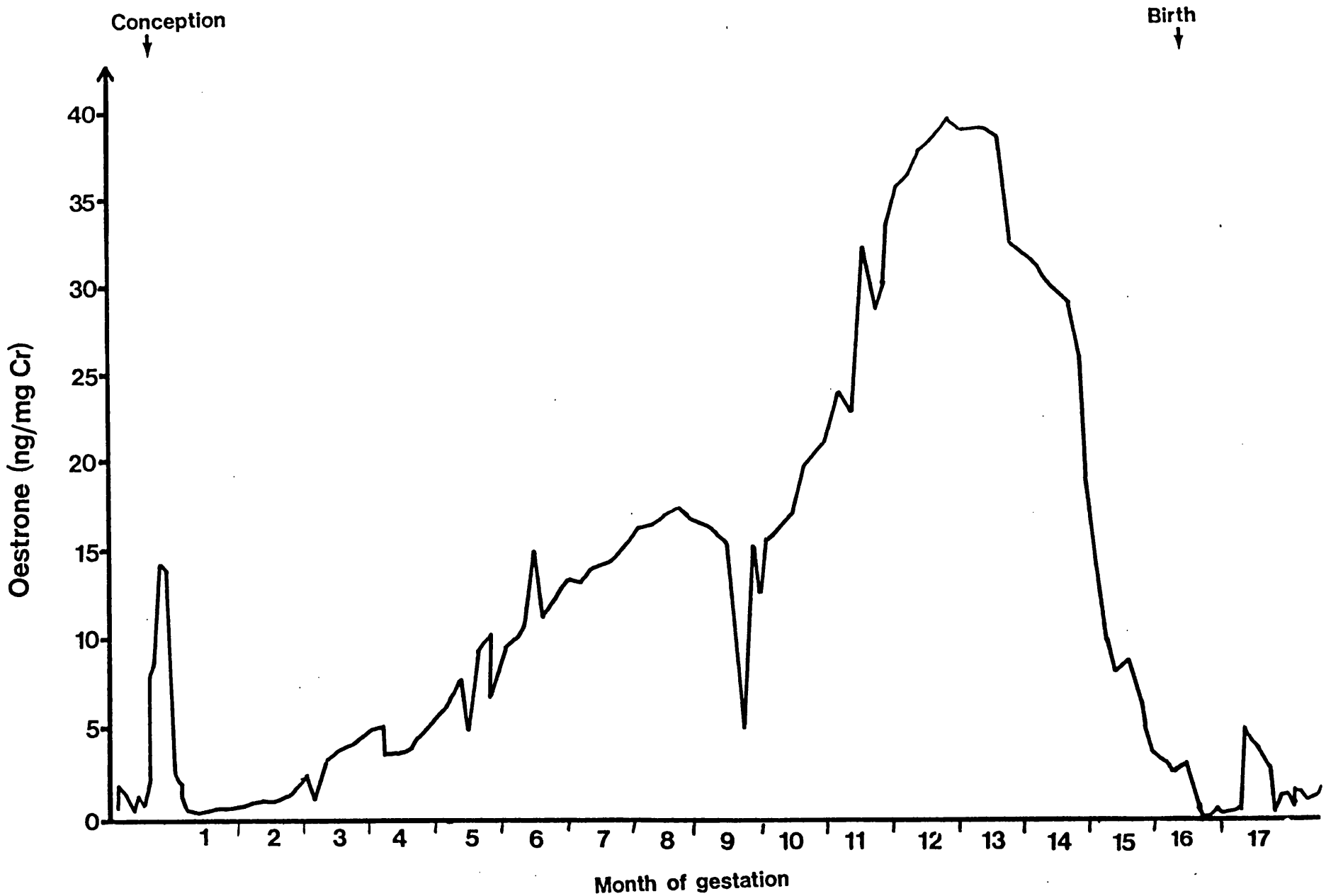

Conception
Birth
20αDHP (ng/mg Cr)
PdG (ng/mg Cr)
Month of gestation
0
2
4
6
8
10
12
14
0
20
40
60
80
100
120
140
160
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

Figure 5.18. Profile of oestrone excretion throughout a conception cycle and complete pregnancy in a black rhinoceros.


Conception
Birth
Oestrone (ng/mg Cr)
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Month of gestation

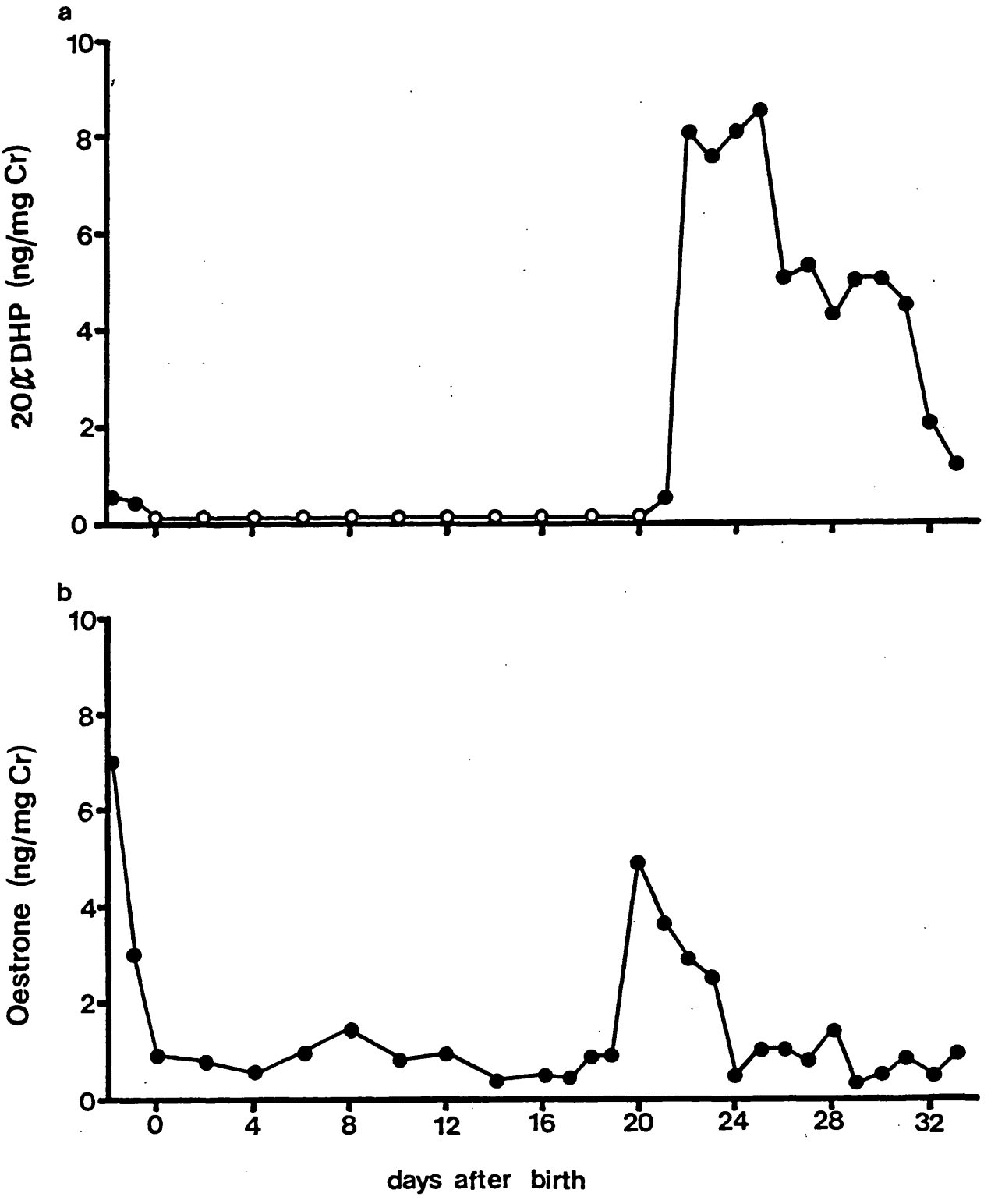
seen in the urine on the day of mating from which conception occurred. Oestrone levels then fell below 2·5 ng/mgCr where they remained for the first 2 months of gestation. Levels then rose, following a similar pattern to that of PdG excretion, and reached maximum levels of 40 ng/mgCr 4 months prior to birth. Urinary oestrone then fell gradually to become undetectable at the time of parturition. The profile of oestradiol-17β excretion is not shown as it did not vary from the pattern obtained during the ovarian cycle (see Fig. 5.7).

In both of these pregnant animals it was also interesting to investigate the excretion of ovarian steroids during the *post partum* period. Whilst all urinary hormone concentrations in the northern white rhinoceros remained low or undetectable for at least 3 months after birth, the pattern of excretion of oestrone and 20α-DHP in the black rhinoceros suggested the presence of a *post partum* oestrus in this species. The levels of 20α-DHP and oestrone in daily urine samples collected over a 33 day period after parturition are presented in Fig. 5.19, aligned to the day of birth (Day 0). Urinary 20α-DHP remained undetectable for 20 days *post partum*, when levels rose rapidly to reach maximum levels of 8-9 ng/mgCr within 4 days. After an elevated period of 20α-DHP excretion which lasted for 14 days, levels returned to below 1 ng/mgCr. Levels of urinary oestrone also remained low after parturition until an immediate increase to 5 ng/mgCr was seen on day 20 post partum, the day prior to an increase in 20α-DHP. However, no behavioural oestrus was observed although the female was not given access to a male.

5.4 DISCUSSION

This study describes the pattern of excretion of the major steroid hormone metabolites in the urine of the black and white species of rhinoceros throughout the ovarian cycle and pregnancy.

Figure 5.19. Profile of 20α-DHP (a) and oestrone (b) throughout the *post partum* period in a black rhinoceros.


a
10
8
6
4
2
0
20α DHP (ng/mg Cr)
b
10
8
6
4
2
0
Oestrone (ng/mg Cr)
0
4
8
12
16
20
24
28
32
days after birth

The results presented confirm that 20α-DHP, and not PdG, is the urinary metabolite of progesterone that most accurately reflects ovarian function during the oestrous cycle in African rhinoceroses. PdG has been informative in monitoring ovarian function in the Indian rhinoceros (Kasman *et al.*, 1986) and in many other ungulate species including the cow (Fehér, Baksai and Karaszti, 1970), pig (Schomberg, Jones, Erb and Gomes, 1966), okapi (Loskutoff *et al.*, 1982), giraffe (Loskutoff *et al.*, 1986), black buck (Holt *et al.*, 1988), Eld's deer (Monfort, Wemmer, Kepler, Bush, Brown and Wildt, 1990) and oryx (Hodges and Hearn, 1983; Loskutoff *et al.*, 1983). Contrary to the results of Ramsay *et al.* (1987), PdG immunoreactivity was detectable during the oestrous cycle of the black rhinoceros. However, levels of urinary immunoreactive PdG were low (below 30 ng/mgCr) throughout the ovarian cycle in both African species of rhinoceros and the profile of excretion showed no indication of cyclic ovarian activity.

The measurement of PdG immunoreactivity during this study could possibly be attributed to the fact that the PdG EIA is more sensitive that the RIA used by Ramsay *et al.* (1987). Alternatively, differences in specificity between the PdG antiserum used in this study (Hodges and Green, 1989) and Ramsay *et al.* (1987) may account for the measurement of PdG immunoreactivity as reported here. Our antiserum showed significant cross reactions with 5β-pregnanediol, 20α-DHP and 5β-pregnanedione but data was not available for the antiserum used by Ramsay *et al.* (1987).

HPLC analysis of urine from the luteal phase in both black and white rhinoceroses indicated that the PdG immunoreactivity measurable during the ovarian cycle was due to the presence of a substance in the urine the cross reacted with the anti-PdG serum used in the EIA (see chapter 4). The nature and origin of the cross reacting component of urine that leads to the measurement of PdG immunoreactivity in African rhinoceroses during the ovarian cycle is not known, However, the substance is unlikely to be of ovarian origin as levels were relatively constant throughout the ovarian

cycle in both species. The compound is more likely to be a urinary steroid metabolite of adrenal origin, perhaps one identified by GC/MS (eg. pregnanetetrol) in the urine of non-pregnant African rhinoceroses.

In contrast, levels of 20α-DHP in the urine showed a cyclic pattern of excretion in both species of rhinoceros. Low levels of urinary 20α-DHP were measured prior to mating, during the presumed follicular phase, with elevated levels during the post oestrus period. The exception was one female northern white rhinoceros, (animal 2), which showed high levels of 20α-DHP throughout the ovarian cycle. However, clear increases in 20α-DHP excretion were seen to follow each period of mating in this animal.It is of interest to note that this is the only animal in which immunoreactivity did not co-elute with the 20α-DHP marker on HPLC, incicating that a cross reacting substance was present in the urine during the follicular phase of the ovarian cycle (see chapter 4). The nature of this substance was not determined although it seems unlikely that its excretion is related to reproductive dysfunction as this female subsequently conceived and maintained a normal pregnancy.

Levels of urinary 20α-DHP differed between the black and white species with much higher levels (generally 10 fold higher) being measured in the urine of the white rhinoceros. There was also some variation between levels of 20α-DHP excreted during the post oestrus period in animals of the same species, which made criteria for separation of follicular and luteal phases of the cycle difficult to establish. Furthermore, there were wide daily fluctuations in hormone concentration in the same animal, as are often experienced when monitoring ovarian function by urinary hormone analysis. Loskutoff *et al*, (1990) reported daily fluctuations in levels of urinary PdG of 0·4-0·7 µg/mgCr during the luteal phase of a suni antelope, whilst Walker *et al.*, (1988) demonstrated that the excretion of 20α-DHP immunoreactivity could vary from 50-150 ng/mgCr during the luteal phase in a killer whale. These

fluctuations may reflect alterations in secretion of progesterone by the corpus luteum. However, they are more likely to be due to changes in quality of the urine sample, ie. changes in the concentration of electrolytes and possible contamination with bedding material or cleaning fluids which can affect hormone measurement in sensitive immunoassays. Although attempts were made to account for changes in the water content of urine samples by indexing hormone concentrations to the mass of creatinine excreted, a low creatinine content can lead to an artificially high hormone value.

In addition to the daily fluctuations and species differences in levels of urinary 20α-DHP, the pattern of excretion during the oestrous cycle also varied considerably between black and white rhinoceroses. In the black species, levels of urinary 20α-DHP were low for a short period (3-4 d) prior to mating and rose very rapidly after oestrus. This pattern of excretion suggests that, as in the horse (Witherspoon, 1975), corpus luteum formation rapidly follows ovulation to give an early increase in plasma progesterone. In contrast, levels of urinary 20α-DHP were low for approximately half of the cycle length in white rhinoceroses, rising slowly after oestrus to reach peak levels after 7 days. This pattern of excretion suggests that, as in the cow (Ayalon and Shemesh, 1974), luteal tissue slowly achieves functional significance in this species. Alternatively, the enterohepatic circulation of ovarian steroids in the white rhinoceros may be responsible for the time lapse between ovulation, during oestrus, and the excretion of maximum levels of 20α-DHP.

It can be concluded that 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 in the African species of rhinoceros. However, in the absence of information on the pattern of progesterone secretion in these species, together with 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. Invasive techniques to provide such evidence, eg. laparotomy and laparoscopy, are not acceptable in such non-tractable species as rhinoceroses, and so far ultrasound has not been informative. However, the oestrogen data presented in this study would suggest that follicular development was occurring, and perhaps the measurement of bioactive gonadotrophin levels in the urine would provide further evidence that these cycles were ovulatory.

Although the precise relationship between 20α-DHP and ovarian function is not clear for any species, the measurement of circulating levels of 20α-DHP accurately reflect luteal function in the horse (Van Rensburg and Van Niekerk, 1968). Furthermore, the mesurement of urinary immunoreactive 20α-DHP, by non-specific radioimmunoassay, has enabled the ovarian cycle of domestic and exotic equids to be successfully characterised for the first time by non-invasive methods (Kirkpatrick *et al.*, 1990). It is perhaps important to note that the profile of PdG excretion during the oestrous cycle of the horse has been found to show no cyclic changes corresponding to ovarian events (Kasman *et al.*, 1985), a similar situation to that in the African species of rhinoceros. In addition, the non-specific measurement of 20α-DHP has provided valuable information on the ovarian cycle in a diverse range of mammals including the giant panda (Chaudhuri *et al.*, 1988), killer whale (Walker *et al.*, 1988) and primates (Shideler *et al.*, 1985; Monfort *et al.*, 1986). The present study is however the first to describe the specific measurement of urinary 20α-DHP and its application to monitoring ovarian function in an ungulate.

The measurement of urinary oestrogens in the black and the white rhinoceros is useful in providing additional evidence that the cycles described are ovulatory, and aids the interpretation of the 20α-DHP data in relation to presumed ovarian events. Although profiles of oestrogen excretion were not always clear, they show that it is possible to measure changes in levels of urinary

oestrogens which appear to reflect the period of follicular development preceeding the time of oestrus and/or ovulation. The profiles of oestradiol-17β and oestrone in Figs 5.3, 5.5 and 5.10 are particularly clear for the northern white, southern white and black rhinoceroses respectively. Each profile shows an increase above baseline at least 2-3 days prior to the onset of behavioural oestrus, with peak levels measuring 6 fold higher than those at the time of luteal regression. It should therefore be possible to use the measurement of urinary oestrogens to monitor follicular activity and predict ovulation in both species of African rhinoceroses. The only other data on the pattern of urinary oestrogen excretion in a rhinoceros were obtained by Kassam and Lasley (1981) and Kasman *et al.* (1986) for the Indian species, in which levels are generally much higher with a larger and more protracted increase during the pre-ovulatory period.

Results of this study also confirm the species differences in oestrogen metabolism and excretion suggested by HPLC analysis of pre-oestrus urine in chapter 4. Both the northern and southern white rhinoceroses were shown to excrete oestradiol-17β and oestrone, whilst black rhinoceroses excreted primarily oestrone into the urine. The measurement of oestradiol-17β has been useful for monitoring the ovarian cycle of certain primates, eg. Goeldi's monkey (Carroll *et al.*, 1990) and the marmoset (Hodges and Eastman, 1984), but is thought to be a minor oestrogen in the urine of ungulates (Mellin, Erb and Estergreen, 1965). The pattern of excretion of oestradiol-17β would appear to accurately reflect ovarian function in at least 2 of the 3 white rhinoceroses studied here. The pattern of oestrone excretion in this species, Fig. 5.2, shows an increase during the post-oestrus period, with low levels around the time of ovulation. A post-ovulatory rise in urinary oestradiol-17β has been demonstrated in certain new world primates (Hodges, 1987) and oestrogen measurements have been used as an alternative to progesterone metabolites for monitoring luteal function in these species (Eastman *et al.*, 1984). The excretion of oestrone during the luteal phase of the ovarian cycle in the white

rhinoceros suggests that the corpus luteum produces oestrogens which may be in a different form to those produced during follicular development (ie. indicating the ovarian production of oestrone rather than oestradiol-17β). Alternatively, the metabolism of circulating oestradiol-17β may alter following ovulation resulting in the excretion of conjugated oestrone rather than oestradiol-17β.

The most abundant oestrogen in the urine of the black rhinoceros was oestrone. The measurement of conjugated oestrone has been used extensively to determine the time of ovulation in many primates (Lasley, Hodges and Czekala, 1980; Czekala *et al.*, 1986) and ungulate species (Lasley, 1985; Lasley *et al.*, 1989). Although over 90% of oestrone in black rhinoceros urine is conjugated as a glucuronide (Ramsay *et al.*, 1987 and chapter 4), the pattern of excretion of oestrone conjugates was not informative in determining the time of ovulation in this species. Figure 5.7 shows that levels of urinary oestrone conjugates were elevated around the time of observed mating although the pattern of excretion was not cyclic. In contrast, the measurement of unconjugated oestrone in hydrolysed urine gave a clear profile, indicating a cyclic pattern of excretion with peak levels of oestrone being observed either the day after or the day preceeding mating. However, peak urinary oestrone concentrations were co-incident with elevated period of oestrone conjugate excretion during which oestrone conjugate levels were 2 fold higher than those of oestrone. A possible explanation for the measurement of unconjugated oestrone being more informative in monitoring ovarian function than oestrone conjugates may be obtained by considering the nature of the assays involved. The oestrone conjugate assay is a direct RIA, which measures the mass of oestrone glucuronide in unextracted urine. The elevated levels of oestrone conjugates throughout the ovarian cycle in the black rhinoceros could be due to the presence of substances in the urine, perhaps cross-reacting with the antiserum, that interfere in the assay. This may also explain the measurement of high levels of oestrone conjugates (5-10 ng/mgCr) in the urine of the white rhinoceros when oestrone levels were less

than 2 ng/mgCr. Such substances may not cross-react with the oestrone antiserum or interfere in the oestrone assay. Alternatively, sample preparation procedures prior to assay for oestrone may remove these contaminants from the urine.

The profiles of 20α-DHP and oestrogen excretion described in this chapter represent the measurement of unconjugated steroids in addition to those conjugates which were cleaved by enzyme hydrolysis. Care must be taken in the expression of absolute hormone values, as differential hydrolysis suggests that a significant proportion of 20α-DHP and oestrogen conjugates in both species remain unhydrolysed by enzymatic procedures. The future measurement of steroid metabolites in urine following solvolysis would give a more accurate indication of the hormone concentration of urine, although it is not known whether this would improve the profile obtained.

An approximate cycle length of 22 days for the black rhinoceros and a slightly longer cycle length of 27 days for the white rhinoceros is suggested. Little information is available for the black rhinoceros, and limited behavioural observation of animals in the wild (Hitchins and Anderson, 1983) and in captivity (Dittrich, 1967; Gowda, 1967; Greed, 1967; Hallstrom, 1967; Yamamotto, 1967) have reported cycle lengths varying from 17-60 days. The cycle lengths for four captive black rhinoceroses, determined in this study by both behavioural and hormonal means, also show much individual variation. However, with the exception of one animal which had a short cycle length of 15 days, the ovarian cycle lengths obtained for the black rhinoceros fell between 17 and 30 days, a much smaller range than previously reported. This demonstrates the increased reliability of hormonal data in comparison to behavioural observation in the calculation of cycle length. However, a more accurate estimation of cycle length, as determined by 20α-DHP and oestrone excretion may have been obtained if samples were collected daily around the time of oestrus in all cases.

The only published data available on the cycle length of the white rhinoceros refers to that of the southern white sub-species, and behavioural data indicates an inter-oestrus interval of approximately 30 days both in the wild (Owen-Smith, 1973) and in captivity (Lindemann, 1982). The cycle length of the southern white rhinoceros under investigation in this study was 31 days when estimated by both 20α-DHP and oestradiol-17β excretion. It was not possible to determine the inter-oestrus interval in this animal as no overt signs of oestrus were detected. The average cycle length for the northern white rhinoceros was found to be 26 days, a slightly shorter cycle than that of the southern white rhinoceros in this study. There is no data available for the northern white rhinoceros for comparison and the lack of samples makes the accurate determination of cycle length by hormone measurement very difficult. It is therefore not possible to comment on whether the shorter cycle length of the northern white rhinoceros compared to that of the southern white represents sub-species differences or merely reflects individual variation.

In contrast to the lack of success in monitoring ovarian function, the measurement of PdG immunoreactivity was informative in indicating pregnancy in the African and Indian rhinoceroses. In this study, elevated levels of PdG immunoreactivity were measured during mid to late gestation in the black and white rhinoceroses, in accordance with data previously published by Ramsay *et al.* (1987) and Hodges and Green (1989). The lower levels of urinary PdG during mid to late pregnancy in the African rhinoceros, compared to those measured in the Indian species, substantiated the results of Hodges and Green (1989) who suggested quantitative species differences in progesterone secretion during gestation.

In chapter 4, co-chromatography on HPLC confirmed that the PdG immunoreactivity detected during mid to late pregnancy in the black and the white rhinoceros represented a real measurement of levels of PdG in the urine. In contrast, PdG immunoreactivity in urine from the ovarian cycle in these species did not co-elute with the PdG

marker on HPLC, suggesting the presence of a cross-reacting substance. Therefore, in the African species of rhinoceros, the excretion of PdG into the urine would appear to be pregnancy specific. The combined use of HPLC and the enzymeimmunoassay to obtain a specific measurement of PdG would allow mid to late pregnancy to be detected by analysis of a single urine sample from both black and white rhinoceroses. Alternatively, the EIA of Hodges and Green (1989) may be adapted to incorporate the antibody used by Ramsay *et al.* (1987) with which the substance identified in this study did not appear to cross react. However, the conversion of a RIA to an EIA can lead to changes in the specificity of an antiserum as demonstrated by Hodges and Green (1989).

The measurement of PdG immunoreactivity during the conception cycle and early pregnancy in a black rhinoceros (Fig. 5.17) showed that levels of urinary PdG immunoreactivity begin to rise during the early stages of pregnancy, 2-3 months after conception. HPLC analysis confirmed that the PdG immunoreactivity measurable at this stage of pregnancy was due to the excretion of PdG (chapter 4). The only other published data on PdG during early gestation in the black species (Ramsay *et al.*, 1987) reported that no PdG immunoreactivity was detectable at this stage of pregnancy. The use of a more sensitive PdG EIA (Hodges and Green, 1989) could account for the detection of PdG during early pregnancy in this study, although the results are from a single animal and require confirmation in more animals before any conclusion can be drawn on the stage of gestation at which PdG becomes detectable in the urine. In the black rhinoceros in this study, PdG levels were seen to increase 4 fold 3 months after conception whereas 20α-DHP excretion fell to very low levels after the luteal phase of the conception cycle and remained low (< 1ng/mgCr) until a short increase to around 4 ng/mgCr in the third trimester of pregnancy.

In contrast to the situation in the black rhinoceros, PdG immunoreactivity remained low until approximately 5-6 months of gestation in the northern white rhinoceros (Figure 5.15). As this

is the first report on early pregnancy in the white rhinoceros there are no other data for comparison. Levels of urinary immunoreactive PdG increased during mid-gestation and remained high until parturition, as demonstrated by Hodges and Green (1989) and further by the data of this study. Differences between the pattern of excretion of 20α-DHP during early pregnancy in the black and white rhinoceroses were also evident. Whilst levels of urinary 20α-DHP were low after conception in the black rhinoceros, elevated levels of 20α-DHP (at least 20-30 ng/mgCr higher than during the luteal phase) were observed 3 months post-conception in the northern white species. Levels of urinary 20α-DHP remained high until 4-8 months prior to parturition, when levels fell coincident with an increase in PdG excretion.

The data obtained in this study may afford an insight into the physiology of pregnancy in the black and white rhinoceroses, with specific regard to the relative importance of ovarian and placental progestagen secretion. The absence of PdG in urine during the ovarian cycle suggests that this is not a major metabolite of progesterone secreted by the ovary. The presence of PdG during early gestation in the black rhinoceros suggests an alternative source of progestagens for the maintenance of pregnancy in this species. It is possible that the ovary of the black rhinoceros plays very little role in steroidogenesis during pregnancy in the black rhinoceros, and as in the sheep (Heap, *et al.*, 1973) progestagens may be produced by the placenta throughout gestation. The placental production of pregnanediol has been documented in goats (Currie, 1977) and may be the primary source of this urinary progestagen in the black rhinoceros. Alternatively, the excretion of PdG in early pregnancy may be a result of a change in the metabolism of ovarian steroids during gestation, resulting in the excretion of PdG rather than 20α-DHP.

In the white rhinoceros, elevated levels of 20α-DHP throughout the first 7 months of pregnancy suggest that early pregnancy may be maintained by the ovary as in the horse (Squires, Wentworth and

Ginther, 1974) and the cow (Henricks, Dickey and Niswender, 1970). The hormonal mechanisms for the maintenance of the lifespan of the corpus luteum cannot be determined from the profiles obtained. The corpus luteum may be maintained by the production of a chorionic gonadotrophin, as in primates (Findlay, 1980) and the horse (Allen, 1970), and it may be interesting to look for bioactive gonadotrophin levels in the urine of this species post-conception. Alternatively, the sharp fall in 20α-DHP 2 months after conception, followed by a substantial increase to levels exceeding 0·1 μg/mgCr in the third and fourth months of pregnancy may indicate the formation of accessory corpora lutea. Certainly in the horse, a reduction in the secretory activity of the primary corpus luteum from around day 16 causes a decline in the production of progesterone (van Niekerk, 1976), and a secondary rise between days 35 and 45 results from the formation of accessory corpus lutea (Squires and Ginther, 1975). Assuming that the pattern of 20α-DHP excretion reflects that of progesterone secretion, this may also be the case in the white rhinoceros. Furthermore the mid-gestational rise in PdG, suggesting that pregnancy may now be maintained by progestagens from a source other than the ovary, ie. the placenta, indicates further similarities to the horse. Although PdG has not been identified as a major urinary progestagen during pregnancy in the horse (Kasman *et al.*, 1985), the placental production of progesterone has been shown to play a vital role in the maintenance of late pregnancy in this species (Short, 1959; Holtan, Squires, Lapin and Ginther, 1979), and also in the pig (Godke, 1975).

The second rise in 20α-DHP excretion during the last trimester of pregnancy in both species of rhinoceros is common in many ungulates including the horse (Barnes *et al.*, 1975) and the cow (Schnider, 1989). It has been suggested that 20α-DHP may be the principal foetal metabolite of progesterone (Moss, Estergreen, Becker and Grant, 1979), which may be reconverted to progesterone in the placenta (Ferguson and Christie, 1967). An overall increase in 20α-DHP secretion into the maternal circulation in the rhinoceros would possibly lead to the rise in excretion seen in this study.

The biological function of 20α-DHP and many other progesterone metabolites formed in late gestation, is unknown, but their gradual elevation in the circulation, and subsequently the urine, suggests that a general alteration in progesterone metabolism occurs near term.

The pattern of oestrogen excretion during pregnancy in the African rhinoceroses is similar to that in many other mammalian species, ie. a gradual increase in the total oestrogen production is seen as pregnancy progresses. In the human, the increased growth and secretory activity of the foetal adrenal glands occurs in parallel with an increase in the 16α-hydroxylating capacity of the foetal liver. Therfore, the rate of synthesis and secretion of oestrogens by the placenta rises steadily during the second and third trimester of pregnancy (Diczfalusy, 1964). Since the placental secretion of oestrogens depends basically on the action of the foetal organs, measurement of urinary oestrogens in pregnant women provides a satisfactory means of assessing foetal viability. This increase in oestrogen secretion is also seen in the African species of rhinoceros. However, this is in contrast to the Indian rhinoceros in which the levels of conjugated oestrone were not raised above those measured during the luteal phase of the ovarian cycle thoroughout gestation (Kasman *et al.*, 1986). This may suggest that another oestrogen is the most abundant urinary oestrogen during pregnancy in the Indian rhinoceros. In the black rhinoceros oestrone was the major urinary oestrogen throughout the ovarian cycle and pregnancy although a 3 fold increase in levels was observed 6 months after conception. HPLC revealed the presence of oestriol during the third trimester of pregnancy in the black rhinoceros, and this oestrogen may be more important than oestrone in the urine at this time, as is the case in the ewe (Challis, 1971).

In the northern white rhinoceros, levels of urinary oestrone did not become quantitatively more important than oestradiol-17β until approximately half way through the gestation period suggesting

that concentrations of different circulating oestrogens change independantly throughout gestation in this species, as in the horse (Savard, 1961; Cox, 1975). A peak in oestrone excretion was observed 3 months prior to parturition followed by a gradual decline in levels as seen in the black rhinoceros. However, levels of oestradiol-17β in the urine remained elevated throughout late pregnancy and into the *post partum* period. In both the black and the white rhinoceros, levels of urinary oestrone fell rapidly during the last month of pregnancy to be baseline at the time of parturition.

The gestation period determined by hormone measurement for the white and black rhinoceroses in this study was 16 months. These figures correlate well with the gestation period of the southern white rhinoceros, estimated from the date of mating to the time of birth as 15-18 months in both the wild (Owen-Smith, 1971) and captivity (Rawlins, 1979; Lindemann, 1982; Hodges and Green, 1989). The gestation period of the black rhinoceros has been reported as 13-15 months both in the wild (Goddard, 1967; Joubert and Eloff, 1971; Hall-Martin and Penzhorn, 1977) and in captivity (Dittrich, 1967; Goddard, 1967; Gowda, 1967; Yamamotto, 1967; Rookmaker, 1973; Ramsay *et al.*, 1987). However, results from this study, the first to monitor a complete pregnancy by endocrine means in either species, suggest a gestation period of 494 days for the black rhinoceros which is slightly longer than those previously reported. The black rhinoceros in this study clearly underwent no further ovarian cycles after the last observed mating. It is possible that a shorter gestation period described from behavioural data, is a result of observations of matings which have been reported during pregnancy in this species (Schenkel and Schenkel-Hulliger, 1967).

The results of this study also describe the excretion of oestrogens and progesterone metabolites during the *post-partum* period in both the black and the white rhinoceroses. In the northern white species, levels of both progesterone metabolites and

oestrogens remained basal throughout the 3 month sample collection period *post-partum*. This data suggests that, unlike the horse (Ginther, 1979) but as in the sheep (van Neikerk, 1976) and the pig (Parvizi, Elsaesser, Smidt and Ellendorff, 1976), there is no ovulatory *post-partum* oestrus in the white rhinoceros. There are no published reports on the reproductive activity of white rhinoceros after birth, although the inter-calf interval of 30 months in the wild (Owen-Smith, 1973) and 27 months in zoological collections (Klös and Frese, 1981) indicates that reproductive activity may be suspended for up to a year after parturition, perhaps due to a lactational anoestrus.

In the black rhinoceros, however, the excretion of high levels of oestrone 20 days *post-partum* indicated that ovarian activity may have recommenced. In addition, the increase in levels of urinary 20α-DHP which immediately followed this oestrone rise indicating that ovulation had occurred. It is not possible to determine whether this ovulation was silent or accompanied by oestrus as the animal was not in the presence of a male. Sexual activity has been seen 25 days after birth in a female black rhinoceros at St. Louis Zoo (C.A.Asa, personal communication) which also indicates the presence of a *post-partum* oestrus in the black rhinoceros. Furthermore, the inter-calf interval for this species may be as low as 17 months in captivity (Lindemann, 1982) indicating that ovulatory oestrous cycles resume soon after birth.

However, the two females that have been reported to have undergone a *post-partum* oestrus had rejected their calves at birth and were thus not lactating. It is not known what effect lactation may have upon these *post-partum* ovarian cycles. Previous reports from the wild have suggested that ovarian activity resumes soon after birth regardless of lactation (Goddard, 1967) in the black rhinoceros, although the interval to time of conception may be longer. This period of reduced fertility is presumably responsible for inter-calf intervals of up to 112 months that have been reported in captivity and in the wild (Lindemann 1982; Western and

Sindiyo, 1972).

It should be stressed that the results presented in this chapter refer to a limited number of black and white rhinoceroses and the results clearly require confirmation in a larger number of animals. Such confirmation is difficult to obtain as few female rhinoceroses in captivity show "normal" reproductive activity and regular urine sample collection is often difficult, especially in the white rhinoceros where the majority of captive animals are exhibited in herds. However, it is obvious that more studies are urgently needed to obtain further data in order to fully characterize the reproductive cycle of the African rhinoceroses.

In summary, the development of a novel EIA for 20α-DHP has allowed the profile of progesterone metabolite excretion during the ovarian cycle of black and white rhinoceroses to be described. The combined measurement of 20α-DHP and oestradiol-17β in the white and oestrone in the black rhinoceros has allowed the oestrous cycle of the African rhinoceroses to be monitored for the first time. The combined results for chapters 4 and 5 suggest that PdG is the major metabolite of progesterone during mid to late pregancy in both the African and Indian rhinoceroses, although the time at which the excretion of PdG indicates the presence of a conceptus varies between species. Whilst PdG is present in the urine of the black rhinoceros during early pregnancy, 20α-DHP is the major urinary metabolite of progesterone at this stage of gestation in the white rhinoceros, perhaps suggesting species differences in the source of progestagens for the maintenance of pregnancy. Oestrone is the major urinary oestrogen throughout pregnancy in the black rhinoceros. In the white species, oestradiol-17β is the most abundant oestrogen in urine until mid-gestation at which time elevated levels of oestrone are measurable in the urine. More studies are now needed to obtain further data on steroid metabolite levels during the ovarian cycle, conception, early pregnancy and the *post-partum* period. Determination of ovarian cyclicity and diagnosis of pregnancy will greatly aid co-ordinated breeding

programmes of the African species of rhinoceros in captivity and possibly in the wild.

CHAPTER 6.
GENERAL DISCUSSION

Chapter 6
General Discussion

This study was designed to examine steroid metabolism and excretion in the black and white rhinoceroses in order to advance our understanding of the reproductive physiology of these species. A second objective of the study was to develop non-invasive methods for monitoring reproductive function in African rhinoceroses.

The identification of the major urinary metabolites of progesterone and oestradiol-17β in the white and the black rhinoceros has shown species differences between the Indian and African rhinoceroses in the metabolism of ovarian steroids. The species differences in steroid metabolism were present throughout, but perhaps the most significant difference was in regard to progesterone metabolism in the non-pregnant animal. Whilst PdG was identified as the major progesterone metabolite in the urine of the Indian rhinoceros during the ovarian cycle, in white and black rhinoceroses the only detectable immunoreactive progesterone metabolite during the post-oestrus period was conjugated 20α-DHP.

There are several possible explanations for the species differences in progesterone metabolism between the Indian and African rhinoceroses. Firstly, the erythrocytes of the black and the white rhinoceros may possess the potential to convert progesterone to 20α-DHP as in the hyrax (Heap *et al.*, 1975). Secondly, the 4-ene-5α/β-reductase enzymes that convert 20α-DHP to pregnanediol in the liver prior to conjugation may be low or absent in the African species of rhinoceros. Alternatively, the liver may not be an important site of progesterone metabolism in black and white rhinoceroses and 20α-DHP may be secreted by the corpus luteum on the ovary. The latter is certainly possible in view of the fact that 20α-DHP is known to be secreted by the ovary of the horse (a close relative of the rhinoceros) and its measurement in the circulation accurately reflects luteal function in this species (Van Rensburg and Van Niekerk, 1968; Seren *et al*, 1981).

One way to examine these possibilities would be to investigate the *in vitro* metabolism of radiolabelled progesterone by blood or liver and ovarian tissue collected *post mortem*. It would also be interesting to establish whether progesterone or 20α-DHP is the major circulating progestagen during normal ovarian cycles in the African species of rhinoceros. By measuring 20α-DHP in the peripheral circulation, its secretion in relation to ovarian events may be determined. The profile of 20α-DHP secretion may provide an indication of the origin of this progestagen in African rhinoceroses. However, such experiments were not possible during the course of this study as *post mortem* tissue was not available and blood samples could not be collected from any of the captive animals under investigation.

In terms of progesterone metabolism, the African species of rhinoceros resembles the the equids (perrisodactyls), whilst the Indian rhinoceros exhibits pathways of progesterone metabolism that more closely equate to those in artiodactyl species. Certainly in all artiodactyls studied, high levels of PdG are present in the urine during the luteal phase ovarian cycle (see Loskutoff *et al.*, 1983; Lasley, 1985; Hodges, 1990 for references). However, the measurement of urinary PdG does not reflect corpus luteum function in domestic and exotic equids (Loskutoff *et al.*, 1983) or tapirs (Kasman *et al.*, 1985). Furthermore, a recent study by Kirkpatrick *et al.* (1990) has shown that the non-specific measurement of urinary 20α-DHP is useful in monitoring ovarian function in exotic equids, eg. zebras, indicating that 20α-DHP is possibly more abundant than PdG in the urine of the horse and perhaps, by inference, the tapir.

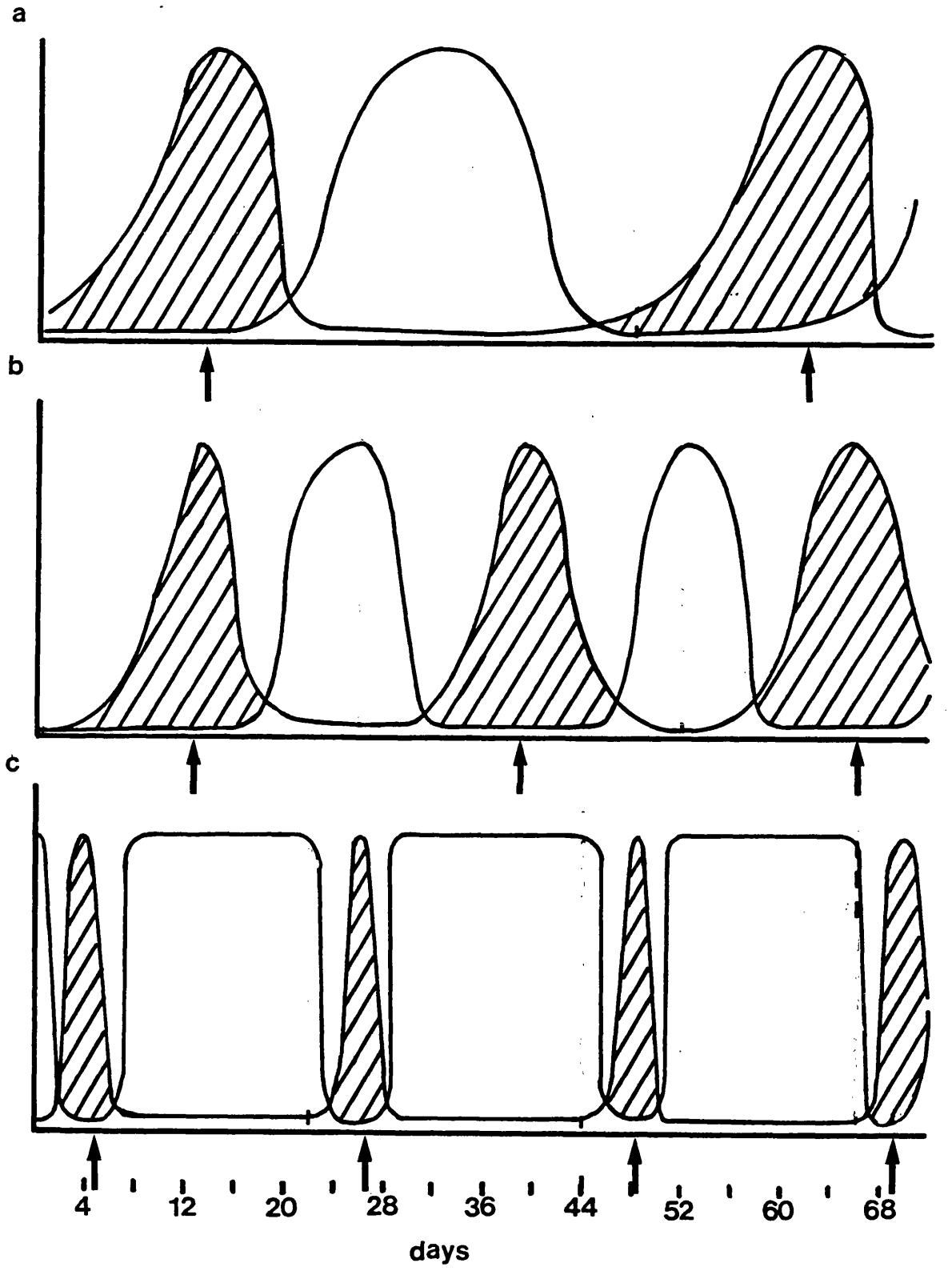
Therefore, species differences exist in the metabolism of progesterone between Indian and African rhinoceroses. In addition, differences in the metabolism and excretion of oestrogens were observed, not only between Indian and African rhinoceroses, but

also within the African species. The most abundant urinary oestrogen in the Indian rhinoceros is oestrone sulphate (Kassam and Lasley, 1981; Kasman *et al.*, 1986). The pattern of excretion and levels of oestrone sulphate found in the urine of the Indian rhinoceros are similar to those seen in equids (Kirkpatrick *et al.*, 1988) Similarly the black rhinoceros, like the majority of ungulates, excretes conjugated oestrone into the urine during the ovarian cycle, although levels were lower than those seen in the Indian rhinoceros. However, the white rhinoceros was shown to excrete conjugated oestradiol-17β as the major oestrogen.

The species differences in steroid metabolism may stem from the separate evolution of the Asian and African rhinoceroses. It is possible that, of the *Perrisodactyl* species, the Indian rhinoceros has evolved more closely with the artiodactyls, whilst the black and white rhinoceroses have evolved more closely with the path of other perrisodactyls. Furthermore, evolution may account for the species differences in oestrogen metabolism between black and white rhinoceroses. Examination of the blood proteins in black and white rhinoceroses (Osterhoff and Keep, 1970) revealed great genetic variability in the white rhinoceros whilst the black species showed no variability, suggesting that the black rhinoceros is more stabilized. *Diceros* is thought to be ancesteral to *Ceratotherium* (Hooijer and Patterson, 1972) since the split in evolution during the Pliocene. The differences in oestrogen metabolism may represent another consequence of separate evolution.

In addition to the qualitative species differences in steroid metabolism, the pattern of steroid excretion also differed. Figure 6.1 shows stylised profiles of excretion of oestrogens and progesterone metabolites during the ovarian cycle of the Indian, black and white rhinoceroses. The typical ovarian cycle, as reflected by urinary steroid measurement, representative of Indian rhinoceroses shows a long follicular phase characterised by a slow, gradual increase in oestrogen levels prior to the pre-ovulatory peak. Progesterone metabolite levels rise after ovulation and

Figure 6.1. Stylised profiles of oestrogen and progesterone metabolite excretion during the ovarian cycle of the Indian (a), white (b) and black (c) rhinoceroses. The shaded area represents the excretion of oestrogens, whilst the pattern of excretion of progesterone metabolites is denoted by the open area. The time of mating in relation to ovarian steroid metabolite excretion is indicated by an arrow.


a
b
c
4
12
20
28
36
44
52
60
68
days

remain high for a luteal period that is equivalent to half the cycle length of 43 days (Kasman *et al.*, 1986). From the limited data collected in this thesis, the profile of excretion of ovarian steroids in the white rhinoceros demonstrates a similar trend in the gradual increase in oestrogen excretion during a relatively long follicular phase preceeding ovulation, followed by a luteal period of elevated progesterone metabolite excretion. In addition to elevated levels of urinary oestradiol-17β at the time of oestrus, cyclical changes in the excretion of oestrone conjugates were seen to correlate with luteal function in the white rhinoceros (see Fig. 5.2). In this species, the excretion of oestradiol-17β may be associated with follicular development, whilst oestrogens produced by the corpus luteum are excreted in the form of oestrone conjugates.

However, the pattern of excretion of steroids during the ovarian cycle of the black rhinoceros more closely resembles that of artiodactyls, eg. the scimitar-horned oryx (Loskutoff *et al.*, 1983) and cow (Gaverick, Erb, Niswender and Callahan, 1971), and the equids, the horse (Lowe, Foote, Baldwin, Hillman and Kallfelz, 1987) and donkey (Henry, Figueiredo, Palhares and Coryn, 1987). In these species the follicular phase is compressed (approximately 4 days in the black rhinoceros) and urinary oestrogens rise sharply after luteal regression. The peak in oestrogen excretion is followed by a prolonged period of progesterone metabolite excretion, the luteal phase, that occupies approximately 80% of the cycle length.

The results presented in this thesis therefore stimulate some interesting discussion on species differences in steroid metabolism and pattern of excretion during the ovarian cycle of rhinoceroses. Firstly, whilst PdG is the most abundant immunoreactive progesterone metabolite in the urine of the Indian rhinoceros, PdG is not present during the luteal phase of the ovarian cycle in the African species. In contrast, conjugated 20α-DHP was identified as the most abundant urinary immunoreactive progestagen in both the black and white rhinoceroses. Secondly, differences in the metabolism and excretion

of oestrogens were observed during the follicular phase of the ovarian cycle between the black and the white rhinoceros. Oestrone was the major oestrogen in the urine of the black rhinoceros, whilst the measurement of oestradiol-17β provided a more informative profile in the white species.

The results of studies in this thesis also provide the first successful attempt to monitor ovarian function in the African species of rhinoceros. The development of such methods have far reaching implications in the management of rhinoceroses in captivity and in the wild. The major function of such monitoring methods is to aid the management of natural breeding in captivity. By increasing our knowledge of the excretion of reproductive steroid hormone metabolites during normal ovarian cycles, causes of reproductive dysfunction within the captive population may be determined. The administration of reproductive hormones, such as gonadotrophin releasing hormone (GnRH) and its antagonists have been used to stimulate steroid secretion and cause ovulation or luteal regression in exotic species ie. wild antelope (Illius, Haynes, Lamming, Howles and Fairall, 1983), the giant panda (Chaudhuri *et al.*, 1988) and marmoset (Hodges, Green, Cottingham, Sauer, Edwards and Lightman, 1988), and may be tested for use in rhinoceroses. Furthermore, the ability to monitor oestrous cycles in African rhinoceroses enables the effect of social behaviour and herd size on reproduction to be assessed.

In addition to the application of non-invasive monitoring of ovarian function to the management of natural breeding in captivity, methods of assessing reproductive function may be used in the development and application of reproductive technologies for assisted breeding. Measurement of ovarian steroid hormone metabolites in the urine provides a non-invasive method for determining the time of ovulation in many exotic species. Such information has assisted artificial insemination and embryo transfer in non-human primates eg. the black mangaby (Calle *et al.*, 1990) and exotic ungulates, eg. blackbuck (Holt *et al.*, 1988) and

suni (Loskutoff *et al.*, 1990). Although there have been successful attempts to apply artificial breeding techniques for exotic species without the endocrine knowledge that urinary analysis can provide (Stover, Evans and Dolensek, 1981; Dresser, Kramer, Pope, Dahlhausen and Blauser, 1982; Dresser, 1986; Summers, Shephead, Hodges, Kydd, Boyle and Allen, 1987), more reliable results would have been obtained if the reproductive status of the animal had been monitored.

If the measurement of urinary oestrogens and 20α-DHP are to be used to determine the exact time of ovulation and thus form the basis for the development of artificial breeding techniques for African rhinoceroses , it is essential to correlate changes in urinary hormone concentration with ovarian events. Such a correlation could be obtained by comparing the changes in levels of urinary steroid metabolites with the secretion of oestrogens and progestagens by the ovary into the peripheral circulation. However, to date there has been no satisfactory method of collecting regular blood samples from rhinoceroses on which to carry out such a comparison. The Captive Breeding Specialist Group has encouraged zoological collections in America to construct apparatus for restraining animals so that blood may be collected in the future (Cumming *et al.*, 1990). By employing crushes it may be possible to use real time ultrasonography to investigate ovarian dynamics and determine the exact time of ovulation with respect to the endocrine changes that may be detected in the urine.

For the female rhinoceros, there is only one account of the application of artificial breeding techniques in captivity. Godfrey, Pope, Dresser, Bavister, Andrews and Olsen (1990) attempted to superovulate a female southern white rhinoceros with a view to harvesting embryos after natural mating. However, this experiment was unsuccessful, perhaps a direct consequence of using a female of unknown reproductive status. Furthermore, without a method for monitoring endocrine changes during the superovulation procedure, the effect of hormone analogues on ovarian function could

not be determined. With respect to the male, there have been reports of the sucessful collection of semen from both Indian (Schaffer, Beehler, Jeyendran and Balke, 1990) and African rhinoceroses (Young, 1967; Platz, Seager and Bush, 1979), although studies on cryopreservation are urgently needed before artificial insemination may become practical in this species. The development of artificial insemination in captivity would enable the transfer of gametes between countries without the expense and risk of re-locating a male rhinoceros which is presently the only method used to transfer genetic information within the captive population (Shapcott, 1986).

Non-invasive methods of assessing reproductive status will not only aid the natural and artificial breeding of rhinoceroses in captivity, but may also be applied in the wild. The rapidly increasing human population, loss of habitat and illegal poaching in many of their native countries (Hillman and Martin, 1979; Martin and Vigne, 1986), has led to a rapid decline in numbers of wild African rhinoceroses. Both white and black rhinoceroses are now being moved to ranches or sanctuaries where they can be effectively protected from poachers and where the habitiat is secure. Southern white rhinoceroses have been translocated to strategic sites within South Africa (Player, 1967), although the practice is more common with the black rhinoceros in countries such as Kenya that have well established conservation programmes for the species (Lever, 1990). Kenya has 11 well protected areas containing 290 of the country's 350 black rhinoceroses, as a conservation policy with the aim of maintaining and managing healthy rhinoceros populations within them. Indeed, since 1986 the record of breeding and security has been encouraging with 59 births versus 13 deaths (R.A.Brett, personal communication). However, although the black rhinoceros population is now increasing in number in Kenya, the migration of animals and transfer of genetic material between these populations is impossible. Given that the largest black rhinoceros sanctuary, Solio, contains only 60 animals inbreeding is inevitable. In order to prevent inbreeding and infertility that may result within the

sanctuary populations, juvenile males are regularly translocated between the reserves (Henwood, 1989; Raath and Hall-Martin, 1989). However, the movement of animals is expensive and hazardous, and many translocated males have been killed by older bulls at their destination. Genetic diversity could be maintained, and the risk to animals minimised, by the transfer of gametes alone between the protected population.

The feasibility of collecting semen in the field by electroejaculation of black rhinoceroses during routine sedation has been demonstrated (Howard, Bush, Collby, de Vos and Wildt, 1983). However, studies have indicated that semen collected by electroejaculation of anaesthatised rhinoceroses in captivity contains very few viable sperm (R.Kock and W.Holt, personal communication). An alternative may be to collect epididymal sperm from deceased males. Information on the physiology of the black rhinoceros has been gained using material collected during *post-mortem* examinations in the wild (Morton and Kock, 1990) and in captivity (McFarlane,*et al.*, 1990). Ideally both sperm and oocytes should be collected *post mortem* and studies carried out to establish protocols for cryopreservation of these gametes. Once frozen, gametes may be stored until a time that *in vitro* maturation and fertilization techniques, such as those in regular use in exotic felids (Donoghue, Johnston, Seal, Armstrong, Tilson, Wolf, Petrini, Simmons, Gross and Wildt, 1990; Miller, Roelke, Goodrowe, Howard and Wildt, 1990), are established for this species.

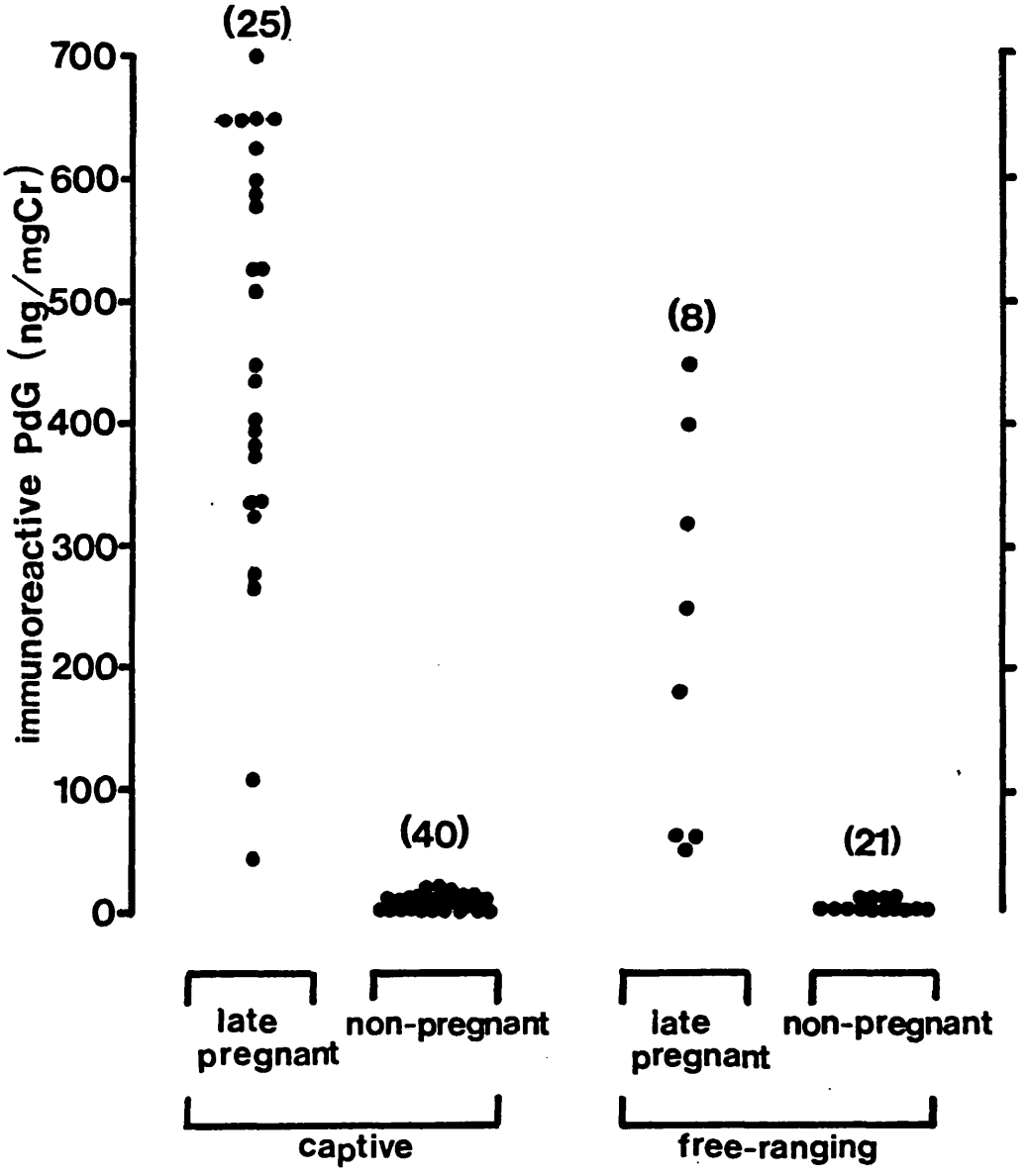
The application of artificial breeding techniques in the wild is a study for the future. Much work will have to be done on the captive population before the feasibility of such a project in the wild can be determined. The measurement of urinary steroid metabolites, however, may be immediately applied to the non-invasive assessment of reproductive function in wild rhinoceroses. Collection of urine from free-ranging animals has been demonstrated in a number of studies (Poole *et al.*, 1984; Chaudhuri and Ginsberg, 1990) and a technique of syringing urine desposited on leaves during

regular tracking of female and male rhinoceos on Ol Ari Nyiro ranch in Kenya has been documented (Brett *et al.*, 1988). The priorities for long-term propagation of rhinoceroses in the wild, with respect to the female, are easy detection of oestrus and pregnancy. Regrettably, in the majority of cases it is not possible to collect daily samples from wild rhinoceroses. This makes the use of urinary 20α-DHP analysis for monitoring ovarian function in wild animals difficult, especially in the black rhinoceros where a 4 day period of depressed 20α-DHP may be missed. Pregnancy diagnosis is however possible and preliminary data suggests that the measurement of PdG may be useful in the detection of mid-late pregnancy in free ranging females. Brett *et al.* (1988) reported that known non-pregnant females in the wild had low urinary PdG levels similar to those in captivity. During the course of this study, it was possible to collect urine samples from a free-ranging female who was known to be in the late stages of pregnancy. Figure 6.2 shows the PdG levels in this female, which were clearly elevated above those seen in non-pregnant animals. Although this data pertains to a single animal, it represents the first data on non-invasive assessment of reproductive status of a wild African rhinoceros, and demonstrates the feasibility of this work for application in the field.

The principle behind pregnancy diagnosis in African rhinoceros is the apparent shift in excretion of progesterone metabolism from 20α-DHP to PdG. Although the time at which PdG becomes the major progesterone metabolite varies between black and white rhinoceroses, the presence of elevated levels of PdG indicates the presence of a conceptus in both species. However, the measurement of PdG by immunoassay is not sufficient evidence of pregnancy as low levels of PdG immunoreactivity are measurable during the ovarian cycle in both species. HPLC results in this thesis have shown that this immunoreactivity is not PdG itself, but is due to the cross reaction of another substance with the PdG antiserum. If this substance could be efficiently removed from the urine eg. by chromatography, the measurement of PdG by EIA in a single urine

Figure 6.2. **Levels of immunoreactive PdG in urine samples from pregnant and non-pregnant black rhinoceroses in captivity and in the wild.**

Urine samples were collected from late pregnant (25 samples 9 animals) and non-pregnant (40 samples from 8 animals) black rhinoceroses in captivity, ie. in zoological collections. Samples from non-pregnant animals (21 samples from 3 animals) and a single late pregnant female (8 samples) were collected in the field, ie. from free-ranging black rhinoceroses.


(25)
700
600
500
400
300
200
100
0
immunoreactive PdG (ng/mgCr)
(40)
(8)
(21)
late pregnant
non-pregnant
late pregnant
non-pregnant
captive
free-ranging

samples would provide a diagnosis of pregnancy in African rhinoceroses.

Such random checks would be useful in detecting pregnancy from an early stage in the black rhinoceros as this study has shown that PdG is excreted soon after conception. In the white rhinoceros, the measurement of PdG would only be useful in the detection of mid-late pregnancy as PdG is not detectable in the urine until 6 months after conception. This is a similar situation to that seen in the horse (Clegg, Boda and Cole, 1954) and it would be interesting to look for a luteotrophic factor, possibly a chorionic gonadotrophin that may be measured in the urine during early pregnancy, possibly by biological assay. African species of rhinoceroses may also produce the pregnancy specific oestrogens, equilin and equilenin, as in the mare (Allen, 1970) and measurement of these compounds in urine may be useful in the detection of early gestation.

There are a number of other methods for the diagnosis of pregnancy in ungulates which could be applied to rhinoceros species. Firstly, it is possible to diagnose pregnancy in equids by the measurement of oestrone conjugates in urine, both in captivity (Evans, Kasman, Hughes, Couto and Lasley, 1984; Czekala *et al.*, 1990) and in the wild (Kirkpatrick *et al.*, 1988). An increase in secretion of oestrogens into the circulation around 120 days after conception in the mare (Hillman and Loy, 1975) is detectable in the urine and has formed the basis for simple clinical pregnancy tests such as the Cuboni test (Cox, 1971). The Cuboni test relies upon the development of a green fluorescence produced when an extract of hydrolysed urine is heated with sulphuric acid, and has been applied to other ungulates including the camel (Ghannam, Azab and Sawae, 1974). In the black rhinoceros, conjugated oestrone is present in the urine during the follicular phase of the ovarian cycle and throughout pregnancy, so such methods may have little application in this species. However, in the white rhinoceros an increase in levels of oestrone in hydrolysed urine have been shown 4 months after conception, as in the horse, so the measurement of oestrone

conjugates may be accurate in detecting pregnancy in the white rhinoceros.

Similarly, the increase in circulating oestrogens during pregnancy in the horse is reflected in the measurement of total oestrogens in the faeces (Bamberg *et al.*, 1984; Bamberg *et al.*, 1986a). In addition, the measurement of total oestrogens, using the EIA of Mostl *et al.*, (1987), has enabled the detection of pregnancy in a variety of non-human primates (Bamberg *et al.*, 1988), domestic (Choi *et al.*, 1987) and exotic ungulates (Safar-Hermann *et al.*, 1987). The results of the metabolism study in chapter 3 of this thesis indicated that the majority of exogenously administered ^{14}C-labelled oestradiol-17β was excreted via the faeces in the white rhinoceros. The oestrogens in the faeces were identified as the 17α- and 17β-isomers of oestradiol, and the combined measurement of these oestrogens may be useful in detecting pregnancy in the white rhinoceros. Alternatively, the proportion of different oestrogens in the faeces may alter after conception, and the measurement of a specific faecal oestrogen may indicate the presence of a conceptus in African rhinoceroses. As faeces is easier to collect than urine in a field situation, a pregnancy test based upon the measurement of faecal oestrogens could have wide application for free-ranging animals. Therefore, studies are needed to identify the major oestrogens in the faeces of the black and white rhinoceroses during natural ovarian cycles and pregnancy.

As a results of the work presented in this thesis, methods for monitoring ovarian function and detecting pregnancy in the black and white rhinoceros are now available based on sensitive immunoassay procedures. A natural progression would be to simplify the assays for use in zoos and in the field, to be carried out by non-skilled personnel. Wanjohi (1988) descibed the use of a portable plate reader to measure the colour change associated with the end point of the PdG EIA. Such equipment is inexpensive and sufficiently robust to be used under field conditions. There are also commercial kits for the detection of PdG and oestrone conjugates (Bahar, 1988)

kits for the detection of PdG and oestrone conjugates (Bahar, 1988) which may be useful in pregnancy detection in black and white rhinoceroses. Although direct assays for conjugated steroids may be simplified for use in the field, the novel assay for 20α-DHP could not be used in a poorly equipped laboratory because of the need for hydrolysis and extraction of the urine. It would therefore be interesting to develop a direct assay for 20α-DHP conjugates. However, the results of the sequential hydrolysis of urine from black and white rhinoceroses indicated species differences in the conjugation of 20α-DHP. The black rhinoceros was found to excrete 20α-DHP-glucuronide whilst in the white rhinoceros 20α-DHP was predominantly conjugated to a sulphate moiety . A direct assay for 20α-DHP conjugates would therfore need to be non-specific for glucuronide and sulphate conjugates to be applied to monitoring ovarian function in both species of African rhinoceroses.

In conclusion, this study has provided valuable information on the excretion of ovarian steroid hormone metabolites in the African species of rhinoceros. The methods of monitoring ovarian function, which have resulted from the investigation of steroid hormone metabolism and excretion in these species, may be applied to the management of natural breeding of African rhinoceroses in captivity and may allow the development and application of reproductive techniques for artificial (assisted) breeding. Methods for assessing reproductive status may also be applied to reproductive studies and genetic management of small populations of African rhinoceroses in the wild.

BIBLIOGRAPHY.

BIBLIOGRAPHY.

ABBOTT, D.H., HODGES, J.K. & GEORGE, L.M. (1988). Social status controls LH secretion and ovulation in female marmoset monkeys (*Callithrix jacchus*). J.Endocr. 117, 329-339.

ADLERCREUTZ, H. & JÄRVENPÄÄ, P. (1982). Assay of oestrogens is human faeces. J.Steroid Biochem. 17, 639-645.

ADLERCREUTZ, H. & MARTIN, F. (1976). Oestrogens in human pregnancy feces. Acta Endocr., Copenh. 83, 410-419.

ADLERCREUTZ, H., MARTIN, F., PULKKINENE, M., DENCKAR, H., RIMER, U., SJOBERG, M.O. & TEKANEN, M.J. (1976). Intestinal metabolism of oestrogens. J.Clin. Endocr. Metab. 43, 497-505.

ADLERCREUTZ, H., MUSEY, P.I., FOTSIS, T., BANNWART, C., WÄHÄLÄ, K., MÄKELÄ, T., BRUNON, G. & HASE, T. (1986). Identification of lignans and phytoestrogens in urine of chimpanzees. Clinica. Chimica. Acta. 158, 147-154.

AINSWORTH, L. & RYAN, K.J. (1969). Steroid hormone transformation by endocrine organs from pregnant mammals III. Biosynthesis and metabolism of progesterone by the mare placenta *in vitro*. Endocrinology 84, 91-97.

ALBRECHT, E.D. & TOWNSLEY, J.D. (1976). Serum progesterone in the pregnant baboon (*Papio papio*). Biol. Reprod. 14, 610-612.

ALLEN, W.R. (1970). Endocrinology of early pregnancy in the mare. Equine Vet. J. 2, 64.

ALOJ, S.M., EDELOCH, H., INGHAM, K.C., MORGAN, F.J., CANFIELD, R.E. & ROSS, G.T. (1973). The rates of dissociation and reassociation of the subunits of human chorionic gonadotrophin. Arch. Biochem. Biophys. 159, 497.

ANDELMAN, S.J., ELSE, J.G., HEARN, J.P. & HODGES, J.K. (1985). The non-invasive monitoring of reproductive events in wild vervet monkeys (*Cercopithecus aethiops*) using urinary pregnanediol-3α-glucuronide and its correlation with behavioural observations. J.Zool., Lond. 205, 467-477.

AXELROD, L.R. & GOLDZIEHER, J.W. (1962). Mechanism of biochemical aromatization of steroids. J.Clin. Endocr. Metab. 22, 537-542.

AXELROD, L.R., MATTHIJSSEN, C., GOLDZIEHER, J.W. & PULLIAM, J.E. (1965). Definitive identification of microquantities of radioactive steroids by recrystallization to constant specific activity. Acta. Endocr., Copenh., suppl., 99, 7-66.

AXELROD, L.R. & WERTHESSEN, N.T. (1960). The metabolism of estrone-16-C^{14} in bovine blood. Arch. Biochem. Biophys. 86, 53-55.

AXELSON, M. & SJÖVALL, J. (1974). Separation and computerized gas chromatography-mass spectrometry of unconjugated neutral steroids in plasma. J. Steroid Biochem. 5, 733.

AYALON, N. & SHEMESH, M. (1974). Pro-oestrous surge in plasma progesterone in the cow. J.Reprod. Fert. 36, 239-243.

BAHAR, I. (1988). The development of over-the -counter assays for pregnanediol-3-glucuronide and estrone-3,D-glucuronide. Prog. Clin. Biol. Res. 285, 139-151.

BAIRD, D.T. (1977). Evidence for the two-cell hypothesis of oestrogen synthesis by the sheep Graafian follicle. J.Reprod. Fert. 50, 183-185.

BAIRD, D.T. (1983). Factors regulating the growth of the pre-ovulatory follicle in the sheep and human. J. Reprod. Fert. 69, 343-352.

BAMBERG, E., CHOI, H.S. & HOIS, J. (1986b). Comparison of oestrogen concentrations in faeces and blood plasma of cyclic and pregnant sows. IAES-SM-292/13 P, 105-106.

BAMBERG, E., CHOI, H.S., MÖSTL, E., WURM, W., LORIN, D. & ARBEITER, K. (1984). Enzymatic determination of unconjugated oestrogens in faeces for pregnancy diagnosis in mares. Equine Vet. J. 16, 537-539.

BAMBERG, E., KING, G.J., VON HEGEL, G., PATZL, M. & MÖSTL, E. (1988). EIA determination of oestrogens in primate faeces. J.Reprod. Fert. Abstr. Ser. 1, 111.

BAMBERG, E., MÖSTL, E., WURM, W. & CHOI, H.S. (1986a). Confirmation of pregnancy in mares and cattle by determination of estrogens in faeces. 10th Int. Congress on Anim. Reprod. and AI II, 75.

BAMBERG, E. & SCHWARZENBERGER, F. (1990). Fecal steroid assay for monitoring of estrous cycles and pregnancy. Proc. 4th Congress of Int. Soc. for Anim. Clinical Biochem. (In press).

BANKS, M. (1986). Breeding the black rhinoceros in Great Britain. Int. Zoo News 33/5-6, 10-15.

BARNES, R.J., NATHANIELSZ, P.W., ROSSDALE, P.D., COMLINE, R.S. & SILVER, M. (1975). Plasma progestagens and oestrogens in fetus and mother in late pregnancy. J.Reprod. Fert. (Suppl.) 23, 617-623.

BARTLETT, A.D. (1873). On the birth of a Sumatran rhinoceros. Proc. Zool. Soc. Lond., pp. 104-106.

BEDFORD, C.A., HARRISON, F.A. & HEAP, R.B. (1972). The metabolic clearance rate and production rate of progesterone and the conversion of progesterone to 20α-hydroxypregn-4-en-3-one in the sheep. J. Endocr. 55, 105-118.

BENAGIANO, G., MANCUSO, S., MANCUSO, F.P., WIQVIST, N. & DICZFALUSY, E. (1968). Studies on the metabolism of C_{19} steroids in the human foeto-placental unit. 3. Dehydrogenation and reduction products formed by previable foetuses with androstenedione and testosterone. Acta. Endocr., Copenh., 57, 187-207.

BEYER, K.F. & SAMUELS, L.T. (1956). Distribution of steroid 3β-ol-dehydrogenase in cellular structures of adrenal gland. J.Biol. Chem. 219, 69-76.

BHAVNANI, B.R. & WOOLEVER, C.A. (1978). Formation of steroids by the pregnant mare VI. Metabolism of [^{14}C] farnesyl pyrophosphate and [^{3}H] dehydroepiandrosterone injected into the fetus. Endocrinology 103, 2291-2298.

BILLIAR, R.B., LIN, T.J. & LITTLE, B. (1973). Metabolic clearance rate and production rate of 20α-hydroxypregn-4-en-3-one in the menstrual cycle. Gynecol. Invest. 4, 148-154.

BILLIAR, R.B., RAHMAN, S.S & LITTLE, B. (1978). The metabolic clearance rate and uterine metabolism and retention of progesterone and 20α-hydroxypregn-4-en-3-one during the secretion of uteroglobulin in ovariectomized, steroid-treated rabbits. Endocrinology 103, 990-996.

BILLIAR, R.B., TAKAOKA, Y., REDDY, P.S., HESS, D.L., LONGSCOPE, C. & LITTLE, B. (1981). Specific tissue metabolism of progesterone *in vivo* in the anesthetized female rhesus monkey during the follicular and luteal phases of the menstrual cycle. Endocrinology 108, 1643-1648.

BIRKEN, S. & CANFIELD, R.E. (1978). Structure and immunological properties of human chorionic gonadotrophin. In: Structure and function of the gonadotrophins. (Ed. K.W.McKerns), (Plenum press, London), pp. 47-80.

BOLT, D.T., KELLY, H.E. & HAWK, H.W. (1971). Release of LH by estradiol in cycling ewes. Biol. Reprod. 4, 35-40.

BONNEY, R.C., MOORE, H.D.M. & JONES, D.M. (1981). Plasma concentrations of oestradiol-17β and progesterone and laparoscopic observations of the ovary in the puma (*Felis concolor*) during oestrus, pseuderpregnancy and pregnancy. J.Reprod. Fert. 63, 523-531.

BOSU, W.T.K., JOHANSSON, E.D.B. & GEMZELL, C. (1973). Ovarian steroid patterns in peripheral plasma during the menstrual cycle in the Rhesus monkey. Folia Primatol. 19, 218-234.

BRAND, H.M. (1981). Urinary oestrogen excretion in the female cotton-topped tamarin (*Saguinus oedipus oedipus*). J.Reprod. Fert. 62, 467-473.

BRANNIAN, J.D., GRIFFIN, F., PAPKOFF, H. & TERRANOVA, P.F. (1988). Short and long phases of progesterone secretion during the oestrous cycle of the African elephant (*Loxodonta africana*). J.Reprod. Fert. 84, 357-365.

BRETT, R.A., HODGES, J.K. & WANJOHI, E. (1988). The assessment of reproductive status and its effect in the bionomics of the black rhinoceros. Proc. Symp. Zool. Soc. Lond. 63, pp. 167-179

BROOKS, C.J.W., MIDDLEDITCH, B.S. & HARVEY, D.J. (1971). The mass spectra of some corticosteroid boronates. Org. Mass. Spectrom. 5, 1429.

BROWN, J.B. (1955). A chemical method for the determination of oestriol. oestrone and oestradiol in human urine. Biochem. J. 60, 185-193.

BRUCE, N., SWANN, R. & WADDELL, B. (1983). An *in vivo* model for studying metabolism and transport in the rat corpus luteum. Biol. Reprod. (Suppl) 28, 229.

BURCHELL, W.J. (1817). Note sur une nouvelle espèce de rhinocéros. Bulletin des sciences, par la Société philomatique 96-97.

BURGER, H.G. & SUMMERVILLE, I.F. (1963). Further evidence for an ovarian source of urinary pregnanetriol. Acta Endocr., Copenh. 43, 95-100.

BUSH, I.E. (1961). Microchemical reactions for steroids. In: The chromatography of steroids. (Pergamon press, Oxford), pp. 358-371.

BUSH, M., SEAGER, S.W.J. & WILDT, D.E. (1980). Laparoscopy in zoo mammals. In: Animal Laparoscopy (Eds. R.M.Harrison and D.E.Wildt), (Williams and Wilkins, Baltimore), pp. 31-72.

CALLE, P.P., CHAUDHURI, M.C. & BOWEN, R. (1990). Menstrual cycle characterization and artificial insemination in the black mangabey (*Cercicebus aterrimus*). Zoo Biol. 9, 11-24.

CANARIO, A.V.M., SCOTT, A.P. & FLINT, A.P.F. (1989). Radioimmunoasay investigations of 20β-hydroxylated steroids in maturing/ovulating female rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocr. 74, 77-84.

CARROLL, J.B., ABBOTT, D.H., GEORGE, L.M., HINDLE, J.E. & MARTIN, R.D. (1990). Urinary endocrine monitoring of the ovarian cycle and pregnancy in Goeldi's monkey. J.Reprod. Fert. 89, 149-161.

CARSON, R.S., FINDLAY, J.K., BURGER, H.C. & TROUNSON, A.O. (1979). Gonadotrophin receptors of the ovine ovarian follicle during follicular growth and atresia. Biol. Reprod. 21, 75-87.

CHALLIS, J.R.G. (1971). Sharp increases in free circulating oestrogens immediately before parturition in sheep. Nature (Lond.), 229, 208.

CHALLIS, J.R.G., DAVIES, I.J., BENIRSCHKE, K., HENDRICKX, A.G. & RYAN, K.J. (1974). The effects of dexamethasone om plasma steroid levels and fetal adrenal histology in the pregnant rhesus monkey. Endocrinology 95, 1300-1305.

CHAMBERS, P.L. & HEARN, J.P. (1979). Peripheral plasma levels of progesterone, oestradiol-17β, oestrone, testosterone, androstenedione and chorionic gonadotrophin during pregnancy in the marmoset monkey (*Callithrix jacchus*). J.Reprod. Fert. 56, 23-32.

CHANNING, C.P., THANKI, K., LINDSAY, A.M. & LEDWITZ-RIGBY, F. (1978). Development and hormonal regulation of gonadotrophin responsiveness in granulosa cells of the mammalian ovary. In: Receptors and hormone action. (Ed. L.Birnbaumer & B.W.O'Malley), (Academic press, New York), pp. 435-455.

CHATTERTON, R.T., CHATTERTON, A.J. & HELLMAN, L. (1969a). Metabolism of progesterone by the rabbit kidney. Endocrinology 84, 1089-1097.

CHATTERTON, R.T, CHATTERTON, A.J. & HELLMAN, L. (1969b). Metabolism of progesterone by the rabbit mammary gland. Endocrinology 85, 10-24.

CHAUDHURI, M. & GINSBERG, J.R. (1990). Urinary androgen concentrations and social status in two species of free ranging zebra (*Equus burchelli* and *Equus grevyi*). J.Reprod. Fert. 88, 127-133.

CHAUDHURI, M., KLEIMAN, D.G., WILDT, D.E., BUSH, M., FRANK, E.S. & THAU, R.B. (1988). Urinary steroid concentrations during natural and gonadotrophin-induced oestrus and pregnancy in the giant panda (*Ailuropoda melanoleuca*). J.Reprod. Fert. 84, 23-28.

CHEATUM, S.G. & WARREN, J.C. (1966). Purification and properties of 3β-hydroxysteroid dehydrogenase and Δ^5-3-ketosteroid isomerase from bovine corpora lutea. Biochem. Biophys. Acta. 122, 1-13.

CHOI, H.S., KIESENHOFER, E., GANTNER, H., HOIS, J. & BAMBERG, E. (1987). Pregnancy diagnosis in sows by estimation of oestrogens in blood, urine or faeces. Anim. Reprod. Sci. 15, 209-216.

CHRISTEN, A., DOBELI, M., KEMPKEN, B., ZACHMANN, M. & MARTIN, R.D. (1989). Urinary excretion of oestradiol-17β in the female cycle of Goeldi's monkey (*Callimico goeldii*). Folia Primatol. 52, 191-200.

CLARKE, I.J. & CUMMINS, J.T. (1982). The temporal relationship between gonadotrophin releasing hormone (GnRH) and leuteinizing hormone (LH) secretion in ovariectomized ewes. Endocrinology 111, 1737-1739.

CLARKE, J.R., DIERSCHKE, D.J., MELLER, P.A. & WOLF, R.C. (1979). Hormonal regulation of ovarian folliculogenesis in rhesus monkeys. II. Serum concentrations of oestradiol-17β and FSH associated with growth and identification of the preovulatory follicle. Biol. Reprod. 21, 497-503.

CLEGG, M.T., BODA, J.M. & COLE, H.H. (1954). The endometrial cups and allantochorionic pouches in the mare with emphasis on the source of equine gonadotrophin. Endocrinology 54, 448.

COLLINS, W.P. (1983). Biochemical approaches to ovulation prediction and detection and the location of the fertile period in females. In: Ovulation: methods for its prediction and detection. (Ed. S.L.Jeffcoate), (John Wiley and sons Ltd., N.Y.). pp. 49-66.

COLLINS, W.P., COLLINS, P.O., KILPATRICK, M.J., MANNING, P.A., PIKE, J. & TYLER, J.P.P. (1979). The concentrations of urinary oestrone-3-glucuronide, LH and pregnanediol-3α-glucuronide as indices of ovarian function. Acta Endocr., Copenh. 90, 336.

COX, J.E. (1971). Urinary tests for pregnancy in the mare. Vet. Rec. 89, 606-607.

COX, J.E. (1975). Oestrone and equilin in the plasma of the preganant mare. J.Reprod. Fert. (Suppl) 23, 463-468.

COX, R.I. & BRADEN, A.W.H. (1974). A new phyto-oestrogen metabolite in sheep. J. Reprod. Fert. 36, 492.

CROWDER, R.E. (1967). Development of adrenal glands in man with special reference to origin and ultimate location of cell types and evidence in favour of cell migration theory. Contr. Embryol. 36, 195-210.

CUMMING, D.H.M., DU TOIT, R.F. & STUART, S.N. (1987). African elephants and rhinos: status survey and action plan. IUCN/ISSC African elephant and rhino specialist group.

CUMMING, D.H.M., DU TOIT, R.F. & STUART, S.N. (1990). African elphants and rhinos: status survey and action plan. IUCN/ISSC African elephant and rhino specialist group.

CURRIE, W.B. (1977). Endocrinology of pregnancy and parturition in sheep and goats. In: Management of reproduction in sheep and goats (Uni. Wisconsin, Madison), pp. 72-78.

CZEKALA, N.M., GALLUSSER, S., MEIER, J.E. & LASLEY, B.L. (1986). The development and application of an enzyme immunoassay for urinary estrone conjugates. Zoo Biol. 5, 1-6.

CZEKALA, N.M., KASMAN, L.H., ALLEN, J.O. & LASLEY, B.L. (1990). Urinary steroid evaluations to monitor ovarian function in exotic ungulates. VI. Pregnancy detection in exotic equids. Zoo Biol. 9, 43-48.

CZEKALA, N.M., MITCHELL, W.R. & LASLEY, B.L. (1986). Direct measurement of urinary oestrone conjugates during the normal menstrual cycle of the gorilla (*Gorilla gorilla*). Am. J. Primatol. 68, 1988-1993.

CZEKALA, N.M., ROOCROFT, A. & BATES, M. (1989). Estrogen metabolism in the Asian elephant (*Elephas maximus*). Biol. Reprod. (Suppl.) 40, 214.

DAHL, K.D., CZEKALA, N.M. & HSUEH, A.J.W. (1987a). Estrogen-producing ovarian granulosa cells: use of the granulosa cell aromatase bioassay to monitor FSH levels in body fluids. Adv. Exp. Med. Biol. 219, 275-298.

DAHL, K.D., CZEKALA, N.M. & HSUEH, A.J.W. (1987b). Measurement of urinary bioactive follicle-stimulating hormone (FSH) levels during reproductive cycles in diverse mammalian species. Biol. Reprod. (Suppl.) 36, 366.

DAHL, K.D., CZEKALA, N.M., LIM, P. & HSEUH, A.J.W. (1987). Monitoring the menstrual cycle of humans and lowland gorillas based on urinary profiles of bioactive follicle-stimulating hormone and steroid metabolites. J.Clin. End. Metab. 64, 486-493.

DAHL, K.D., JIA, X.C. & HSUEH, A.J.W. (1989). Granulosa cell aromatase bioassay for FSH. Meth. Enzym. 168, 414-422.

DANG, H.H. (1986). Biology and ecology of ungulates in Vietnam. (Science and Technology Publishers, Hanoi).

DAVENPORT, G.R. & MALLETTE, L.E. (1966). Some biochemical properties of rabbit ovarian hydroxysteroid dehydrogenases. Endocrinology 78, 672-678.

DEJONGH, F.H., BAIRD, D.T. & VAN DER MOLEN, H.J. (1974).* Acta Endocr., Copenh., 77, 575-587

DESAULINERS, D.M., GOFF, A.K., BETTRIDGE, K.J., ROWELL, J.E. & FLOOD, P.F. (1989). Reproductive hormone concentrations in faeces during the oestrous cycles and pregnancy in cattle (*Bos taurus*) and muskoxen (*Ovibos moschatus*). Can. J. Zool. 67, 1148-1154.

DESMAREST, A.G. (1822). Mammalogie ou description des espèces de mammifères, seconde partie: Contenant les ordres des rongeurs, des edeentes, des pachyderms, des ruminans et des cétacés: viii. (Veuve Agasse, Paris) pp. 277-555.

DEWAR, D., GLOWER, V., ELSWORTH, J. & SANDLER, M. (1986). Equol and other compounds from bovine urine as monoamine oxidase inhibitors. J. Neural Transmission 65, 147-150.

DICZFALUSY, E. (1964). Endocrinological funtions of the human feto-placental unit. Fed. Proc. 23, 791-798.

DIEKMAN, M.A. & MALVERN, P.V. (1973). Effect of ovariectomy and estradiol on LH patterns in ewes. J. Anim. Sci. 37, 562-567.

DIERENFELD, E.S., DU TOIT, R. & MILLER, R.E. (1988). Vitamin E in captive and wild black rhinoceroses. J. Wildl. Dis. 24, 547-550.

DITTRICH, L. (1967). Breeding the black rhinoceros at Hanover zoo. Int. Zoo Yb. 7, 161-162.

*

Ovarian secretion rates of oestrogens, androgens and progesterone in normal women and in women with persistent ovarian follicles.

DOBSON, H. & DEAN, P.D.G. (1974). Radioimmunoassay of oestrone, oestradiol-17α and -17β in bovine plasma during the oestrous cycle and last stanges of pregnancy. J.Endocr. 61, 479-486.

DONOGHUE, A.M., JOHNSTON, L.A., SEAL, U.S., ARMSTRONG, D.L., TILSON, R.L., WOLF, P., PETRINI, K., SIMMONS, L.G., GROSS, T. & WILDT, D.E. (1990). In vitro fertilization and embryonic development in vitro and in vivo in the tiger (*Panthera tigris*). Biol. Reprod. 43, 733-744.

DORRINGTON, J.H. & ARMSTRONG, D.T. (1979). Effects of FSH on gonadal function. Rec. Prog. Horm. Res. 35, 301-342.

DRESSER, B.L. (1986). Embryo transfer in exotic bovids. Int. Zoo Yb. 24/25, 138-142.

DRESSER, B.L., KRAMER, L., POPE, C.E., DAHLHAUSEN, R.D. & BLAUSER, C. (1982). Superovulation of African eland (*Taurotragus oryx*) and interspecies embryo transfer to Holstein cows. Theriogen. 21, 232-240.

D'SOUZA, F. (1978). The detection of oestrus. Symp. Zool. Soc. Lond. 43, 175-193.

DUBOIS, P.M. & DUBOIS, M.P. (1974). Mise en evidence pour immunofluorescence de l'activity gonadotrope LH dans l'ante-hypophyse foetale humane. In: Endocrinologie sexualle de la periode perinatale. (Editions, Paris), pp. 37.

DUFAU, M.L., MENDELSON, C.R. & CATT, K.J. (1974). A highly sensitive *in vitro* bioassay for luteinizing hormone and chorionic gonadotrophin: testosterone production by dispersed leydig cells. J.Clin. Endocr. Metab. 39, 610-613.

DUTTON, G.J. (1966). Glucuronic acid free and combined. (Academic press, N.Y.).

EASTMAN, S-A., MAKAWITI, D.W, COLLINS, W.P. & HODGES, J.K. (1984). Pattern of excretion of urinary steroid metabolites during the ovarian cycle and pregnancy in the marmoset monkey. J.Endocr. 102, 19-26.

EMSLIE, R.H. & ADCOCK, K. (1990). Status, history and performance of black rhinoceros population in South Africa, the TBVC states and Namibia. (Unpub. man.).

ENGLAND, B.G., WEBB, R. & DAHMER, M.K. (1981). Follicular steroidogenesis and gonadotrophin binding to ovine follicles during the estrous cycle. Endocrinology 109, 881-889.

ERB, R.E., TILLSON, S.A., HODGEN, G.D. & PLOTKA, E.D. (1970). Urinary creatinine as an index compound for estimating the rate of excretion of steroids in the domestic sow. J. Anim. Sci. 30, 79-85.

ERIKSEN, E. (1977). Birth of two white rhinoceroses (*Ceratotherium simum simum*) at the Copenhagen zoo. Zool. Gert., Lpz. N.F. 47, 33-44.

ERIKSSON, H. & GUSTAFSSON, J-A. (1971). Excretion of steroid hormones in adults. Steroids in faeces from adults. Eur. J. Biochem. 18, 146-150.

ESTERGREEN, V.L., LIN, M.T., MARTIN, E.L., MOSS, G.E., BRANEN, A.L., LUEDECKE, L.O. & SHIMODA, W. (1977). Distribution of progesterone and its metabolites in cattle tissue following administration of progesterone-4-14C. J. Anim. Sci. 45, 642-651.

EVANS, K.L., KASMAN, L.H., HUGHES, J.P., COUTO, M. & LASLEY, B.L. (1984). Pregnancy diagnosis in the domestic horse through direct urinary estrone conjugate analysis. Theriogen. 22, 615-620.

FEHÉR, T., BAKSAI, E.H. & KARASZTI, J. (1970). Determination of pregnanediol in non-pregnant cow's urine by thin-layer chromatography. Endokrinologie Bd. 56 Heft 1, 55-60.

FERGUSON, M.M. & CHRISTIE, G.A. (1967). Distribution of hydroxysteroid dehydrogenase in placental and foetal membranes of various mammals. J.Endocr. 38, 291-306.

FINDLAY, J.K. (1980). Immunological diagnosis of early pregnancy. In: Immunological approaches to fertility control. (Ed. J.P.Hearn), (MTP Press Ltd., Lancaster). pp.63-81.

FINN, M.M., GOSLING, J.P., TALLON, D.F., MADDEN, A.T.S., MEEHANM, F.P. & FOTTRELL, P.F. (1988). Normal salivary progesterone levles throughout the ovarian cycle as determined by direct enzyme immunoassay. Fertil. Steril. 50, 882-887.

FISCHER, G. (1814). Zoognosia tabulis synopticis illustrata, volumen III: quadrupedum reliquorum, certorum et monotrymatum escriptionem continens: xxiv, 734. Mosquae, Nicolai Sergeidis Vsevolozsky.

FLINT, A.P.F. & ARMSTRONG, D.T. (1973). The appearance of an endometrial 20α-hydroxysteroid dehydrogenase towards the end of pregnancy in the rat. Endocrinology 82, 624-627.

FLINT, A.P.F., KINGSTON, E.J., ROBINSON, J.S. & THORBURN, G.D. (1978). Initiation of parturition in the goat: evidence for control by foetal glucocorticoid through activation of placental C_{21}-steroid 17α-hydroxylase. J. Endocr. 78, 367-378.

FLOOD, P.F. & MARRABLE, A.W. (1975). A histochemical study of steroid metabolism in the equine fetus and placenta. J.Reprod. Fert. (Suppl.) 23, 569-573.

FORTUNE, J.E. & ARMSTRONG, D.T. (1978). Androgen production by theca and granulosa cells isolated from pro-oestrus rat follicles. Endocrinology 100, 1341-1347.

FRAUST, R. (1958). Die geburt eines spizmaulnashornes (*Diceros bicornis L.*) in Frankfurt zoo. Garten (Nf) 22, 208-214.

GAO, Y., SHORT, R.V. & FLETCHER, T.P. (1988). Progesterone concentrations in plasma, saliva and milk of cows in different reproductive states. Brit. Vet. J. 144, 262-268.

GARVERICK, H.A., ERB, R.E., NISWENDER, G.D. & CALLAHAN, C.J. (1971). Reproductive steroid in the bovine. III. Changes during the estrous cycle. J.Anim. Sci. 32, 946-956.

GHANNAM, F.El-., AZAB, E.A.El-. & SAWAE, S.El-. (1974). Preliminary study on the application of Cuboni test for pregnancy diagnosis in the camel. Zuchthyg. 9, 46.

GHEBREMESKEL, K., WILLIAMS, G., LEWIS, J.C.M. & DU TOIT, R. (1988). Serum alpha-tocopherol, all-trans retinol, total lipids and cholesterol in the black rhinoceros (*Diceros bicornis*). J. Wildl. Dis. 25, 887-965.

GINTHER, O.J. (1979). Reproductive biology of the mare. (McNaughton & Gun, Ann, Arbor.)

GLOGER, C.W.L. (1841). Gemeinnütziges Hand-und Hilfsbuch der Naturgeschichte: für gebildete leser aller stände, besonders für die reifere Jugend und ihre Lehrer I: xxxiv. (Breslau, Aug. Schultz and Co.), pp. 496.

GODDARD, J. (1967). Home range, behaviour and recruitment rates of two black rhinoceros populations. E.Afr. Wildl. J. 5, 133-150.

GODFREY, R.W., POPE, C.E., DRESSER, B.L., BAVISTER, B.D., ANDREWS, J.C. & OLSEN, J.H. (1990). An attempt to superovulate a southern white rhinoceros (*Ceratotherium simum simum*). Theriogen. 33, 231.

GODKE, R.A. (1975). Maternal and fetal endocrine involvement in pre-partum pigs. Diss. Abstr. Int. B. 36B, 986-987.

GOLDENBERG, R.L., VAITUKAITIS, J.L. & ROSS, G.T. (1972). Estrogen and follicle stimulating hormone interactions on follicle growth in rats. Endocrinology 90, 1492-1498.

GOLDIN, B.R., ADLERCREUTZ, H., DWYER, J.T., SWENSON, L., WARRAM, J.H. & GORBACH, S.L. (1981). Effect of diet on excretion of oestrogens in pre- and post-menopausal women. Cancer Res. 41, 3771-3773.

GOLDZIEHER, J.W. & AXELROD, L.R. (1969). Urinary metabolites of 4-^{14}C-progesterone in the baboon (*Papio ssp.*). J.Gen. & Comp. Endocr. 13, 201-205.

GONZALEZ-ANGULO, A., HERNANDEZ-JÁUREGUI, P. & MARTINEZ-ZEDILO, G. (1975). Fine structure of the gonads of the horse and its functional implications. J.Reprod. Fert. (Suppl.) 23, 563-567.

GORDON, I. (1983). Controlled breeding in farm animals. (Pergamon press, Oxford).

GOSDEN, A.F. & MOSELY, G. (1984). Naturally occuring forage hydrocarbons as intake markers. Welsh Plant Breeding Station Ann. Report 109-110.

GOWDA, K. (1967). Breeding the black rhinoceros at Mysore zoo. Int. Zoo Yb. 7, 163-164.

GOWER, D.B. (1984). Steroid catabolism and urinary excretion. In: Biochemistry of steroid hormones. (Ed. H.L.J.Makin), (Blackwall Scientific Publications, Oxford.) Pp 349-382.

GRACE, N.D. & BODY, D.R. (1981). The possible use of long chain (C_{19}-C_{32}) fatty acids in herbage as an indigestible faecal marker. J.Agric. Sci., Camb. 97, 743-745.

GREED, G.R. (1967). Notes on breeding the black rhinoceros at Bristol zoo. Int. Zoo Yb. 2, 106.

GREENWOOD, F.C., HUNTER, W.M. & GLOVER, J. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89, 114-123.

GREIG, M. & MACNAUGHTON, M.C. (1967). Radioactive metabolites in the liver and adrenals of the human foetus after administration of [4-^{14}C] progesterone. J.Endocr. 39, 153-162.

GROVES, C.P. (1967a). Geographic variation in the black rhinoceros (*Diceros bicornis*) (L., 1758). Z. Säugetierk. 32, 267-276.

GROVES, C.P. (1967b). On the rhinoceroses of S.E.Asia. Säugertiek. Mitt. 15, 221-237.

GROVES, C.P. & KURT, F. (1972). *Dicerorhinus sumatrensis*. Mammalian species 21, 1-6.

GUÉRIN, C. (1980). Les rhinocéros (Mammalia Perrisodactyla) du miocène terminal du pleistocène supérieur en Europe occidentale: comparaison avec les espèces actuelles. Documents les lab. de géologie Lyon 79, 3-1185.

GUGGISBERG, C.A.W. (1966). S.O.S. Rhino. (Andre Deutche Ltd., London), pp. 75-100.

GUNTHER, J.D., FOWLEY, C.W., GAVERICK, H.A. & PLOTKA, E.D. (1980). Comparison of milk and blood plasma progesterone concentrations in cycling and pregnant mares. J.Anim. Sci. 51, 1131-1138.

GWYNNE, J.T. & STRAUSS, J.F. (1982). The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocr. Rev. 3, 299-329.

HAGEN, C. & McNEILLY, A.S. (1975). The gonadotrophin hormones and their subunits in human maternal and fetal circulation at delivery. Am. J. Obstet. Gynecol. 121, 926-930.

HALL, P.F. & KORITZ, S.B. (1964). Conversion of cholesterol and 20α-hydroxycholesterol to steroids by acetone powder of particles from bovine corpus luteum. Biochemistry 3, 129-134.

HALL-MARTIN, A.J. (1986). Recruitment in a small black rhinoceros population. African elephant and rhinoceros specialist newsletter 7, 6-8.

HALL-MARTIN, A.J. & PENZHORN, B.L. (1977). Behaviour and recruitment of translocated black rhinoceros, *Diceros bicornis*. Koedoe 20, 147-162.

HALLSTROM, E. (1967). Notes on breeding the black rhinoceros at Sydney zoo. Int. Zoo Yb. 7, 165.

HARKNESS, R.A. & LOVE, D.N. (1966). Studies on the estimation of urinary pregnanetriol during pregnancy and childhood. Acta Endocr., Copenh., 51, 526-534.

HARLOW, C.R., HEARN, J.P. & HODGES, J.K. (1984). Ovulation in the marmoset monkey: endocrinology, prediction and detection. J. Endocr. 103, 17-24.

HARTIALA, K. (1973). Metabolism of hormones, drugs and other substances by the gut, Physiol. Rev. 53, 496-534.

HAY, M.F. & ALLEN, W.R. (1975). An ultrastructural and histochemical study of the interstitial cells in the gonads of the fetal horse. J.Reprod. Fert. (Suppl.) 23, 557-561.

HAY, M.F. & MOORE, R.M. (1978). Changes in the Graafian follicle population during the follicular phase of the oestrous cycle. In: Control of ovulation (Eds. D.B.Crighton, N.B.Haynes, G.R.Foxcroft and G.E.Lamming), (Butterworths, London), pp. 177-196.

HAYS, H.R. (1967). Notes on breeding the black rhinoceros at Pittsburg zoo. Int. Zoo Yb. 7, 164-165.

HEAP, R.B., GOMBE, S. & SALE, J.B. (1975). Pregnancy in the hyrax and erthrocyte metabolism of progesterone. Nature (Lond.) 257, 809-811.

HEAP, R.B., PERRY, J.S. & CHALLIS, J.R.G. (1973). Hormonal maintenance of pregnancy. In: Handbook of physiology, Section 7, Endocrinology, Vol. 7, Female reproductive system, Part 2. (American Phys. Society, Washington, D.C.), pp. 217-260.

HEGER, W., HOYER, G-A & NEUBERT, D. (1988). Identification of the main gestagen metabolite in marmoset (*Callithrix jacchus*) urine by NMR and MS spectroscopy. J.Med. Primatol. 17, 19-29.

HEGER, W. & NEUBERT, D. (1987). Determination of ovulation and pregnancy in the marmoset monkey (*Callithrix jacchus*) by monitoring of urinary hydroxy-pregnanolone excretion. J.Med. Primatol. 16, 151-164.

HEISTERMANN, M., PRÖVE, E. WOLTERS, H.J. & MIKA, G. (1987). Urinary oestrogen and progesterone excretion before and during pregnancy in a pied bare-faced tamarin (*Saguinus bicolor bicolor*). J.Reprod. Fert. 80, 635-640.

HELLIG, H.R. & SAVARD, K. (1966). Sterol biosynthesis in the bovine corpus luteum *in vitro*. Biochemistry 5, 2944-2956.

HENDERSON, K.M., GORBAN, A.M.S. & BOYD, G.S. (1981). Effect of LH factors regulating ovarian cholesterol metabolism and progesterone synthesis on PMSG-primed imature rats. J. Reprod. Fert. 61, 373-380.

HENRY, M., FIGUEIREDO, A.E.F., PALHARES, M.S. & CORYN, M. (1987). Clinical and endocrine aspects of the oestrous cycle in donkeys (*Equus asinus*). J. Reprod. Fert. (Suppl.) 35, 297-303.

HENWOOD, R.R. (1989). Black rhinoceros (*Diceros bicornis*) capture, translocation and boma management by the Natal Parks Board. Koedoe 32, 43-47.

HEPPES, J.B. (1958). The white rhinoceros in Uganda. Afr. Wildlife 12, 272-280.

HILLIAR, S.G. (1985). Sex steroid metabolism and follicular development in the ovary. Oxford Rev. of Repro. Biol. 7, 168-222.

HILLMAN, K. (1982). Panafrican survey of the black rhino and status of the northern white rhino. Int. Symp. Mgmt. Rhino in Captivity (Unpub. man.).

HILLMAN, K. & MARTIN, E.B. (1979). Will poaching exterminate Kenya's rhinos? Oryx 15, 131-132.

HILLMAN, R.B. & LOY, R.G. (1975). Oestrogen excretion in mares in relation to various reproductive states. J.Reprod. Fert. (Suppl.) 23, 223-230.

HINDLE, E. (1947). Report on the additions to the Society's menagerie during June, July, August and September 1947. Proc. Zool. Soc. Lond. 117, 792.

HITCHINS, P.M. & ANDERSON, J.L. (1983). Reproduction, population characteristics and management of the black rhinoceros population in the Hluhluwe/Corridor/Umfolozi compex. Natal Park board: internal report, pp. 1-27.

HODGES, J.K. (1986). Monitoring changes in reproductive status. Int. Zoo Yb. 24/25, 126-130.

HODGES, J.K. (1990). Determining and manipulating female reproductive parameters. In: Management of wild mammals in captivity. (Ed. D.Kleiman and S.Lumpkin), (Smithsonian Institution, Washington). (In press).

HODGES, J.K., BEVAN, D.J., CELMA, M., HEARN, J.P., JONES, D.M., KLEIMAN, D.G., KNIGHT, J.A. & MOORE, H.D.M. (1984). Aspects of the reproductive endocrinology of the female giant panda (*Ailuropoda melanoleuca*) in captivity with specific reference to the detection of ovulation and pregnancy. J. Zool. (Lond.) 203, 253-268.

HODGES, J.K., BRAND, H.M., HENDERSON, C. & KELLY, R.W. (1983a). The levels of circulating and urinary oestrogens during pregnancy in the marmoset monkey (*Callithrix jacchus*). J.Reprod. Fert. 67, 73-82.

HODGES, J.K, CZEKALA, N.M. & LASLEY, B.L. (1979). Estrogen and luteinizing hormone secretion in diverse primate speices from simplified urinary analysis. J.Med. Primatol. 8, 349-364.

HODGES, J.K. & EASTMAN, S-A, K. (1984). Monitoring ovarian function in marmosets and tamarins by the measurement of urinary estrogen metabolites. Am. J. Primatol. 6, 187-197.

HODGES, J.K., EASTMAN, S-A., K. & JENKINS, N. (1983b). Sex steroids and their relationship to binding proteins in the serum of the marmoset monkey (*Callithrix jacchus*). J. Endocr. 96, 443-450.

HODGES, J.K. & GREEN, D.I. (1989). A simplified enzymeimmunoassay for urinary pregnanediol-3-glucuronide: application to reproductive assessment in exotic species. J.Zool., Lond. 219, 89-99.

HODGES, J.K., GREEN, D.I., COTTINGHAM, P.G., SAUER, M.J., EDWARDS, C. & LIGHTMAN, S.L. (1988). Induction of luteal regression in the marmoset monkey (*Callithrix jacchus*) by a gonadotrophin-releasing hormone antagonist and the effects on subsequent follicular maturation. J.Reprod. Fert. 82, 743-752.

HODGES, J.K. & HEARN, J.P. (1983). The prediction and detection of ovulation: applications to comparative medicine and conservation. In: Ovulation: methods for its prediction and detection. (Ed. S.L.Jeffcoate) (Wiley and sons Ltd., New York), pp. 103-122.

HODGES, J.K., HENDERSON, C. & McNEILLY, A.S. (1983). Circulating oestrogen concentrations during pregnancy in the African elephant (*Loxodonta africana*). J.Reprod. Fert. 67, 121-127.

HODGES, J.K., TARARA, R., HEARN, J.P. & ELSE, J.G. (1986). The detection of ovulation and early pregnancy in the baboon by direct measurement of conjugated steroids in urine. Am. J. Primatol. 10, 329-338.

HOFF, J.D., QUIGLEY, M.E. & YEN, S.S.C. (1983). Hormonal dynamics at mid-cycle: A re-evaluation. J. Clin. Endocr. Metab. 57, 792-796.

HOFFMAN, B. & HAMBURGER, R. (1973). Progesterone in milk; determination by means of radioimmunoassay relationships with corpus luteum function and milk fat concentration. Zuchthyg. 8, 154-162.

HOLT, W.V., MOORE, H.D.M., NORTH, R.D., HARTMAN, T.D. & HODGES, J.K. (1988). Hormonal and behavioural detection of oestrus in blackbuck, *Antilope cervicapra*, and successful artificial insemination with fresh and frozen semen. J.Reprod. Fert. 82, 717-725.

HOLTAN, D.W., SQUIRES, E.L., LAPIN, D.R. & GINTHER, O.J. (1979). Effect of ovariectomy on pregnancy in mares. J.Reprod. Fert. (Suppl.) 27, 457-463.

HONOUR, J.W. (1984). Biliary excretion and enterohepatic circulation. In: Biochemistry In: Biochemistry of steroid hormones. (Ed. H.L.J.Makin), (Blackwall Scientific Publications, Oxford.) Pp 382-407.

HOUGHTON, E., COPSEY, J., DUMASIA, M.C., HAYWOOD, P.E., MOSS, M.S. & TEALE, P. (1984). The identification of C_{18} neutral steroids in normal stallion urine. Biomed. Mass. Spectrom. 11, 96-99.

HOWARD, C.M., ROBINSON, H., SCHMIDT, F.H., McCORD, J.R. & PREEDY, J.R.K. (1969). Evidence for a two-pool system governing the excretion of radioactive urinary oestrone conjugates during the first 8 hours following the administration of estrone-6,7-^{3}H to male subjects, probable role of the enterohepatic circulation. J. Clin. Endocr. Metab. 29, 1618-1629.

HOWARD, J.G., BUSH, M., COLLBY, L., DE VOS, V. & WILDT, D.E. (1983). Electroejaculation techniques and semen evaluation in rhinoceroses. Proc. Conf. Am. Ass. Zoo Vet. (Tampa, Florida), pp. 74-75.

HUANG, W.Y. (1967). Conversion of progesterone to 16α-hydroxyprogesterone in normal human corpus luteum tissue. Steroids 10, 107-114.

HUNTER, W.M. & CORRIE, J.E.T. (1983). Immunoassays for clinical chemistry. (Churchill Livingstone, Edinburgh).

ILLIUS, A.W., HAYNES, N.B., LAMMING, G.E., HOWLES, C.M. & FAIRALL, N. (1983). Evaluation if LH-RH stimulation of testosterone production as an index of reproductive status in rams and its application in wild antelope. J.Reprod. Fert. 68, 107-112.

INGAMELLS, S. & PEDDIE, M.J. (1988). 20α-hydroxyprogesterone and progesterone secretion by dispersed rat luteal cells. J.Endocr. (Suppl.) 119, 141.

IVIE, G.W., CHRISTOPHER, R.J., MUNGER, C.E. & COPPOCK, C.E. (1986). Fate and residues of [4-^{14}C]estradiol-17β after intramuscular injection into Holstein steer calves. J.Anim. Sci. 62, 681-690.

JACOBSOHN, G. & LIEBERMAN, S. (1962). Studies on the chemical cleavage of urinary glucuronosides of the 17-ketosteroids. J. Biol. Chem. 237, 1469-1475.

JAFFE, R.B. & PETERSON, E.P. (1966). *In vivo* steroid biosynthesis and metabolism in the human term placenta. 2. *In situ* placental perfusion with cholesterol-7α^{3}H. Steroids 8, 695-710.

JÄNNE, O.A., LAATIKAINEN, T.J. & VIHKO, R.K. (1971). Effect of reduction of the intestinal microflora on the excretion of neutral steroids in human faeces and urine. Eu. J. Biochem. 20, 120-123.

JEFFERY, J.D'A. (1966). Metabolism of progesterone in the pig-tailed monkey (*Macaca nemestrina*). J.Endocr. 34, 387-392.

JIRKU, H. & LEVITZ, M. (1969). Biliary and urinary metabolites of estrone-6,7-^{3}Hsulphate-^{36}S in a woman. J. Clin. Endocr. Metab. 29, 615-637.

JOHNSON, M.H. & EVERITT, B.J. (1988). Essential reproduction. (Blackwell Scientific publications, Oxford), pp. 35-49.

JONES, D.M. (1979). The husbandry and veterinary care of captive rhinoceroses. Int. Zoo Yb. 19, 239-252.

JOUBERT, E. & ELOFF, F.C. (1971). Notes on the ecology and behaviour of the black rhinoceros *Diceros bicornis* Linn. 1758 in South west Africa. Madoqua 3, 5-53.

JOYCE, WILSON, READ & RIAD-FAHMY (1978). An improved EIA for progesterone in human plasma. Clin. Chem. 24, 2099-2102.

KADIS, B. (1964). Estriol biosynthesis by the sow ovary. Biochemistry 3, 2016-2019.

KAPLAN, S.L., GRUMBACH, M.M. & AUBERT, M.L. (1976). α and β glycoprotein hormone subunits (hLH, hFSH, hCG) in the serum and pituitary of the human fetus. J. Clin. Endocr. Metab. 42, 995.

KASMAN, L.H., McCOWAN, B. & LASLEY, B.L. (1985). Pregnancy detection in tapirs by direct urinary estrone sulphate analysis. Zoo Biol. 4, 301-306.

KASMAN, L.H., RAMSAY, E.C. & LASLEY, B.L. (1986). Urinary steroid evaluations to monitor ovarian function in exotic ungulates : III. Estrone sulfate and pregnanediol-3-glucuronide excretion in the Indian rhinoceros (*Rhinoceros unicornis*). Zoo Biol. 5, 355-361.

KASSAM, A.A.H. & LASLEY, B.L. (1981). Estrogen excretion patterns in the Indian rhinoceros (*Rhinoceros unicornis*), determined by simplified urinary analysis. Am. J.Vet. Res. 42, 251-255.

KELLIE, A.E. (1984). Structure and Nomenclature. In: Biochemistry of steroid hormones. (Ed. H.L.J.Makin), (Blackwall Scientific Publications, Oxford.). pp 1-19.

KELLY, W.G., DE LEON, O., WINTER, J. & BOKKENHEUSER, V.D. (1977). Exchange of hydrogen at C_{21} during dehydroxylation of deoxycorticosterones by mixed cultures of human faecal flora. J. Steroid Biochem. 2, 73.

KELLY, W.G. & LIEBERMAN, S. (1964). Aldosterone. (Ed. E.E. Baulier & P. Robel), (Blackwall scientific publishers, Oxford), pp. 215.

KING, J.M. (1969). The capture and translocation of the black rhinoceros. E.Afr. Wildl. J. 7, 115-130.

KIRKPATRICK, J.F., KASMAN, L.H., LASLEY, B.L. & TURNER, J.W. (1988). Pregnancy determination in uncaptured feral horses. J.Wildl. Mgt. 52, 305-308.

KIRKPATRICK, J.F., LASLEY, B.L. & SHIDELER, S.E. (1990). Urinary steroid evaluation to monitor ovarian function in exotic ungulates: VII. Urinary progesterone metabolites in the Equidae assessed by immunoassay. Zoo Biol. 9, 341-348.

KLÖS, H.G. & FRÄDRICH, H. (1970). Ein überbück über die in zoologischen gärten gehaltenen afrikanischen Näshörner. Zool. Gart., Lpz. N.F. 38, 227-245.

KLÖS, H.G. & FRESE, R. (1978). Population trends in African rhinoceroses living in zoos and safari parks. Int. Zoo Yb. 18, 231-234.

LAATIKAINEN, T.J. (1970). Identification of $C_{21}O_3$ and $C_{21}O_4$ steroids in human bile. Eur. J. Biochem. 14, 372.

LANG, E.M. (1975). The Indian rhino in captivity. In: Breeding endangered species in captivity. (Ed. R.D.Martin) (Academic press, London), pp. 295-302.

LASLEY, B.L. (1985). Methods of evaluating reproductive function in exotic species. Adv. Vet. Sci. and Comp. Med. 30, 209-228.

LASLEY, B.L., HODGES, J.K. & CZEKALA, N.M. (1980). Monitoring the female reproductive cycle of great apes and other primate species by determinations of oestrogen and LH in small volumes of urine. J.Reprod. Fert. 28, 121-129.

LASLEY, B.L., MONFORT, S.L., HODGES, J.K. & CZEKALA, N.M. (1981). Comparison of urinary oestrogens during pregnancy in diverse primate species. In: Foetal Endocrinology, proceedings of the 1st ORPRC symposium on Repro. Biol. (Ed. Novy and Resko), (Academic press, London).

LASLEY, B.L., TROEDSSON, M., BRAVO, W. & HAGGERTY, M.A. (1989). Estrogen conjugate measurements to monitor ovarian activity. Theriogen. 31, 127-139.

LAURIE, W.A. (1978). The ecology and behaviour of the greater one-horned rhinoceros. Unpub. PhD thesis, Uni. of Cambridge.

LAURIE, W.A. (1982). Behavioural ecology of the greater one-horned rhinoceros. J.Zool., Lond. 196, 307-361.

LENTON, E.A., GELSTHORP, C.H. & HARPER, R. (1988). Measurement of progesterone in saliva: assessment of the normal fertile range using spontaneous conception cycles. Clin. Endocr., 28, 637-646.

LEVER, C. (1990). Lake Nakuru black rhinoceros sanctuary. Oryx 24, 90-94.

LIBERMANN, S., ERLANGER, B.F., BEISER, S.M. & AGATE, F.J.Jr. (1959). Steroid-protein conjugates: their chemistry, immunochemistry and endocrinological properties. Rec. Prog. Horm. Res. 15, 165-200.

LIN, J-T. & HEFTMANN, E. (1981). High performance liquid chromatography of naturally occuring oestrogens. J. Chromatog. 212, 239-244.

LINDEMANN, H. (1982). African rhinoceroses in captivity. Unpub. MPhil. thesis, Uni. of Cambridge.

LINDEMANN, H. (1984). Demographic survey of the black rhinoceros in captivity. Int. Zoo Yb. 23, 233-255.

LINNAEUS, C. (1758). Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus dufferentiis, synonymis, locis. (Holmiae, Laurentius Salvius), 10th ed. I: iv, 824.

LISKOWSKI, L. & WOLF, R.C. (1972). Urinary excretion of progesterone metabolites in pregnant rhesus monkeys. Proc. Soc. Exp. Biol. Med. 139, 1123-1126.

LIU, X.Y., CHEN, F.J., GUO, D.Z., SONG, X.H. & ZHANG, Y.G. (1988). Early pregnancy diagnosis in dairy cows based on hair progesterone analysis. Int. J. Anim. Sci. 3, 123-127.

LLEWELLYN, C.A. (1981). Conversion of progesterone to 20β-hydroxy-4-pregnen-3-one by the pre-ovulatory follicle in the hen (*Gallus domesticus*). J. Endocr. 89, 283-288.

LOSKUTOFF, N.M., KASMAN, L.H., RAPHAEL, B.L., OTT-JOSLIN, J.E. & LASLEY, B.L. (1987). Urinary steroid evaluations to monitor ovarian function in exotic ungulates: IV. Estrogen metabolism in the okapi (*Okapia johnstoni*). Zoo Biol. 6, 213-218.

LOSKUTOFF, N.M., OTT, J.E. & LASLEY, B.L. (1983). Stratergies for assessing ovarian function in exotic species. J. Zoo Anim. Med. 14, 3-12.

LOSKUTOFF, N.M., OTT-JOSLIN, J.E. & LASLEY, B.L. (1982). Urinary steroid evaluations to monitor ovarian function in exotic ungulates: I. Pregnanediol-3-glucuronide immunoreactivity in the okapi (*Okapia johnstoni*). Zoo Biol. 1, 45-53.

LOSKUTOFF, N.M., RAPHAEL, B.L., NEMEC, L.A., WOLFE, B.A., HOWARD, J.G. & KRAEMER, D.C. (1990). Reproductive physiology of suni (*Neotragus moschatus zuluensis*). I. Female anatomical traits, manipulation of ovarian activity and non-surgical embryo recovery. J. Reprod. Fert. 88, 521-532.

LOSKUTOFF, N.M., WALKER, L., OTT-JOSLIN, J.E., RAPHAEL, B.L. & LASLEY, B.L. (1986). Urinary steroid evaluations to monitor ovarian function in exotic ungulates: II. Comparison between the giraffe (*Giradda camelopardalis*) and the okapi (*Okapi johnstoni*). Zoo Biol. 5, 331-338.

LOUTFI, G., PERON, F. & DORFMAN, R.I. (1962). Formation of 20α-hydroxy-Δ^4-pregnen-3-one and Δ^4-pregnen-3,17-dione in rodent ovaries. Endocrinology 71, 983-985.

LOWE, J.E., FOOTE, R.H., BALDWIN, B.H., HILLMAN, R.B. & KALLFELZ, F.A. (1987). Reproductive patterns in cyclic and pregnant thyroidectomized mares. J. Reprod. Fert. (Suppl.) 35, 281-288.

LYDEKKER, R.B.A. (1908). The white rhinoceros. The Field III, 319.

LYNE, C. & GOWER, D.B. (1977). Steroid metabolism in thyroid tissue: effect of compound SU10'603 (3(-7-chloro-1,2,3,4-tetrahydro-4-oxonapth-2-yl) pyridine. Biochem. Soc. Trans. 5, 1304-1307.

LYNN, W.S., STAPLE, E. & GURIN, S. (1955). Catabolism of cholesterol by *in vitro* systems. Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 14, 783-785.

MACDONALD, D. (1985). Encyclopaedia of mammals II. (Equinox, Oxford).

MACDONALD, P.C., GRODIN, J.M. & SIITERI, P.K. (1971). In: Control of gonadal steroid secretion. (Ed. D.T.Baird & J.A.Strong), (Edinburgh Uni. Press, Edinburgh), pp. 158.

MAEYAMA, M. & IKEMOTO, M. (1967). Progesterone metabolism by human fetal and placental tissues: interconversions of progesterone and 20α-hydroxypregn-4-en-3-one in feto-placental unit *in vitro*. Endocr. Jap. 14, 232-238.

MAHONEY, C.J. (1970). Study of the menstrual cycle in *Macaca irus* with special reference to the prediction of ovulation. J.Reprod. Fert. 21, 153-163.

MARSHALL, D.E., GOWER, D.B., HOUGHTON, E., DUMASIA, M.C., PATEL, B., BYRNE, T. & MORTISHIRE-SMITH, R.J. (1990). The identification of some neutral C_{18} steroids in pregnant mare urine. J. Endocr. (Suppl.) 127, 93.

MARTIN, E.B. (1985). Religion, royalty and rhino conservation in Nepal. Oryx 19, 11-16.

MARTIN, E.B., MARTIN, C.B. & VIGNE, L. (1987). Conservation crisis- the rhinoceros in India. Oryx 21, 212-218.

MARTIN, E.B. & VIGNE, L. (1986). Rhino poaching and conservation. Swara 9, 25-27.

MARTIN, F., PELTONEN, J., LAATIKAINEN, T., PULKKINEN, M. & ADLERCREUTZ, H. (1975). Excretion of progesterone metabolites and oestradiol in faeces from pregnant women during ampicillin administration. J.Steroid Biochem. 6, 1339-1346.

MATTERI, R.L., ROSER, J.F., BALDWIN, D.M., LIPOVETSKY, V. & PAPKOFF, H. (1987). Charaterisation of a monoclonal antibody which detects luteinizing hormone from diverse mammalian species. Dom. An. Endocr. 4, 157-165.

MAUREL, M-C., LABROUSSE, H., TERQUI, M. & AVRAMEAS, S. (1987). A highly sensitive microtitre plate enzyme immunoassay for oestadiol-17β. J. Imm. Methods 102, 165-172.

MAYES, R.W., LAMB, C.S.& COLGROVE, P.M. (1986). The use of dosed and herbage n-alkanes as markers for the determination of herbage intake. J.Agric. Sci., Camb. 107, 161-170.

MELLIN, T.N. & ERB, R.E. (1966). Estrogen metabolism and excretion during the bovine estrous cycle. Steroids 7, 589-606.

MELLIN, T.N., ERB, R.E. & ESTERGREEN, V.L. (1965). Quantitative estimation and identification of estrogens in bovine urine. J.Dairy Sci. 48, 895.

MENTIS, M.T. (1972). A review of some life history features of the large herbivores of Africa. Lammerrgeyer 16, 1-89.

METCALF, M.G. & HUNT, E.G. (1976). Calculation of estrogen excretion rates from urinary estrogen to creatinine ratios. Clin. Biochem. 9, 75-77.

MEYER, H.H.D & HOFFMANN, S. (1987). Development of a microtitration plate enzyme-immunoassay for the anabolic steroid trenbolone. Food Additives and Contaminants 4, 149-160.

MILLER, A.M., ROELKE, M.E., GOODROWE, K.L., HOWARD, J.G. & WILDT, D.E. (1990). Oocyte recovery, maturation and fertilization *in vitro* in the puma (*Felis concolor*). J. Reprod. Fert. 88, 249-258.

MILLER, R.E. & BOEVER, W. (1982). Fatal hemolytic anaemia in the black rhinoceros: case report and a survey. J. Am. Vet. Med. Ass. 181, 1228-1231.

MIRON, P., HAY, D.L., JOHNSTON, W.I.H., McKENNA, M. & McBAIN, J.C. (1988). Mid-cycle changes of 17-hydroxyprogesterone levels in women superovulated with clomiphene citrate and HMG for *in vitro* fertilization. Fert. Steril. 49, 848-853.

MITCHELL, W.R., PRESELY, S., CZEKALA, N.M. & LASLEY, B.L. (1982). Urinary immunoreactive estrogen and pregnanediol-3-glucuronide during the normal menstrual cycle of the female lowland gorilla (*Gorilla gorilla*). Am. J.Primatol. 2, 167-175.

MITSUMA, M., YOSHIMURA, S., KAMBEGAWA, A. OKINAGA, S. & ARAI, K. (1989). Development of a direct microplate enzyme immunoassay for the determination of pregnanediol-3-glucuronide in urine. J.Steroid Biochem. 32, 467-471.

MIYAZAKI, T., PERIC-GOLIA, L., SLAUNTHWITE, W.R. & SANDBERG, A.A. (1972). Estriol metabolism in sheep: excretion of biliary and urinary conjugates. Endocrinology 90, 516-524.

MONFORT, S.L., DAHL, K.D., CZEKALA, N.M., STEVENS, L., BUSH, M. & WILDT, D.E. (1989). Monitoring ovarian function and pregnancy in the giant panda (*Ailuropoda melanoleuca*) by evaluating urinary bioactive FSH and steroid metabolites. J.Reprod. Fert. 85, 203-212.

MONFORT, S.L., JAYARAMAN, S., SHIDELER, S.E., LASLEY, B.L. & HENDRICKX, A.G. (1986). Monitoring ovulation and implantation in the cynomologus macaque (*Macaca fasicularis*) through evaluations of urinary estrone conjugates and progesterone metabolites : a technique for the routine evaluation of reproductive parameters. J.Med. Primatol. 15, 17-26.

MONFORT, S.L., WEMMER, C., KEPLER, T.H., BUSH, M., BROWN, J.L. & WILDT, D.E. (1990). Monitoring ovarian function and pregnancy in Eld's deer (*Cervus eldi thamin*) by evaluating urinary steroid metabolite excretion. J.Reprod. Fert. 88, 271-281.

MOORE, R.M. (1977). Sites of steroid production in ovine Graafian follicles in culture. J.Endocr. 73, 143-150.

MORGAN, P.M., HUTZ, R.J., KRAUS, E.M. & CORMIE, J.A. (1985). Ultrasounic assessment of ovarian folliculogenesis in rhesus monkeys. Biol. Reprod (Suppl.) 32, 190.

MORTON, D.J. & KOCK, N. (1990). Some properties of pineal gland hydroxyindole-O-methyltransferase from black rhinoceros (*Diceros bicornis*). J. Pineal Res. 8, 35-40.

MOSS, G.E., ESTERGREEN, V.L., BECKER, S.R. & GRANT, B.D. (1979). The source of 5α-pregnanes that occur during gestation in mares. J. Reprod. Fert. (suppl.) 27, 511-519.

MÖSTL, E., CHOI, H.S., WURM, W., ISMAIL, N. & BAMBERG, E. (1984). Pregnancy diagnosis in cows and heifers by determination of oestradiol-17α in faeces. Br. Vet. J. 140, 287-291.

MÖSTL, E., MEYER, H.H.D., BAMBERG, E. & VON HEGEL, G. (1987). Oestrogen determination in faeces of mares by enzyme immunoassay on microtitre plates. Proc. Symp. Analysis of Steroids, pp. 219-224.

MOULE, G.R., BRADEN, A.W.H. & LAMOND, D.R. (1963). The significance of oestrogens in pasture plants in relation to animal production. Anim. Breed. Abstr. 31, 139-157.

MUKINYA, J.T. (1973). Density, distribution, population structure and social organization of the black rhinoceros in Masai Mara Game Reserve. E. Afr. Wildl. J. 11, 385-400.

MURRAY, R.D. & NEWSTEAD, R. (1988). Determination of steroid hormones in goat's milk and plasma as an aid to pregnancy diagnosis using an ELISA. Vet. Rec. 122, 158-161.

McFARLANE, J.R., CABRERA, C.M., COULSON, S.A. & PAPKOFF, H. (1990). Purification and assay of rhinoceros pituitary gonadotrophins, growth hormone and prolactins. Biol. Reprod. (Suppl.) 42, 333.

McNATTY, K.P. (1982). Ovarian follicular development from the onset of luteal regression in the human and sheep. The "Reinier de Graaf Lecture".

McNEILLY, A.S., MARTIN, R.D., HODGES, J.K. & SMUTS, G.L. (1983). Blood concentrations of gonadotrophins, prolactin and gonadal steroids in males and in non-pregnant and pregnant female African elephants (*Loxodonta africana*). J.Reprod. Fert. 67, 113-120.

NAITO, K., TAKAHASHI, M. & HOMMA, K. (1986). *In vitro* secretion of progestins by rat luteal cells and their 20α-hydroxysteroi dehydrogenase activity. Endocrinol. Japan 33, 43-50.

NARDELLI, F. (1987). The conservation of the Javan rhinoceros (*Rhinoceros sondaicus*). Int. Zoo News 34/3, 18-22.

NILSSON, R., MYHRE, E.B., KRONVALL, G. & SJÖGREN, H.O. (1982). Fractionation of rat IgG subclasses and screening for IgG Fc-binding to bacteria. Mol. Immunol. 19, 119-126.

O'CONNOR, S.M. (1982). The behavioural ecology of the white rhinoceros, *Ceratotherium simum simum*, at Whipsnade zoological park. Unpub. MPhil thesis, Uni. of Cambridge.

O'CONNOR, S.M. (1986). Activity cycles in the southern white rhinoceros in captivity: implications for management. Int. Zoo Yb. 24/25, 297-303.

OLNEY, P.J.S. & ELLIS, P. (1989). International zoo yearbook, (Zoological Society of London), pp. 523-524.

ONGABESAN, O.M. & PEDDIE, M.J. (1988). Steroid secretion by ovarian cells of the japanese quail (*Coturnix coturnix japonica*). Gen. Comp. Endocr. 72, 272-281.

OSTERHOFF, D.R. & KEEP, M.E. (1970). Natural variation in the blood proteins of white and black rhinoceroses. Lammergeyer II.

OWEN-SMITH, R.N. (1971). Territoriality in the white rhinoceros (*Ceratotherium simum*, Burchell). Nature (Lond.) 231, 294-296.

OWEN-SMITH, R.N. (1973). The behavioural ecology of the white rhinoceros. Unpub. PhD thesis, Uni. of Wisconsin.

OWEN-SMITH, R.N. (1975). The social ethology of the white rhinoceros *Ceratotherium simum* (Burchell, 1817). Z. Tierpsychol. 38, 337-384.

PALMER, R. BLAIR, J.A., ERIKSSON, G. & DICZFALUZY, E. (1966). Studies on metabolism of C-21 steroids in human foeto-placental unit. 3. Metabolism of progesterone and 20α and 20β-dihydroprogesterone by midterm placentas perfused *in situ*. Acta Endocr., Copenh. 53, 407-419.

PARVIZI, N., ELSAESSER, F., SMIDT, D. & ELLENDORFF, F. (1976). Plasma LH and progesterone in the adult female pig during the oestrous cycle, late pregnancy and lactation, and after ovariectomy and pentobarbitone treatment. J.Endocr. 69, 193-203.

PASHEN, R.L. (1980). Studies on the endocrinology of pregnancy in the mare. Unpub. PhD thesis, Uni. of Cambridge.

PATERSON, J.Y.F., HARRISON, F.A., SHELDRICK, E.L. & HEAP, R.B. (1983). An improved model of the distribution and metabolism of progesterone and 20α-dihydroprogesterone in sheep. J.Reprod. Fert. 68, 81-90.

PATERSON, N. (1967). Relative constancy of 24 hour urine volume and 24 hour creatinine output. Clin. Chim. Acta 18, 57-58.

PENNY, M. (1987). Rhinos: an endangered species. (Butler & Tanner, Frome). Pp 5-117.

PEREZ, L.E., CZEKALA, N.M., WEISENSEEL, K.A. & LASLEY, B.L. (1988). Excretion of radiolabelled estradiol metabolites in the slow loris (*Nycticebus coucang*). Am.J.Primatol. 16, 321-330.

PICKETT, M.T., KYRIAKIDES, E.C., STERN, M.J. & SOMMERVILLE, I.F. (1959). Urinary pregnanetriol throughout the menstrual cycle. The Lancet 2, 829-830.

PIERCE, J.G., LIAO, T.H., HOWARD, S.M., SHOME, B. & CORNELL, J.S. (1971). Studies on the structure of tyrotropins: its relationship to leuteinizing hormone. Res. Prog. Horm. Res. 27, 165.

PIERRO, A. & JOHNSON, R.E. (1970). Creatinine excretion. The Lancet 1, 784.

PITTMAN, C.R.S. (1931). A game warden among his charges. (James Nisbet, London).

PLANT, T.M., JAMES, V.H.T. & MICHAEL, R.P. (1969). Metabolism of [4-^{14}C] progesterone in the rhesus monkey (*Macaca mulatta*). J.Endocr. 43, 493-494.

PLATZ, C., SEAGER, S.W.J. & BUSH, M. (1979). Collection and analysis of semen from a black rhinoceros. J. Am. Vet. Med. Ass. 175, 1002-1004.

PLAYER, I.C. (1967). Translocation of white rhinoceros in South Africa. Oryx 9, 137-150.

PLAYER, I.C. & FEELEY, J.M. (1960). A preliminary report on the square-lipped rhinoceros, *Ceratotherium simum simum.* Lammergeyer (Natal) 1, 3-23.

PLOTKA, E.D., SEAL, U.S., ZAREMBKA, F.R., SIMMONS, L.G., TEARE, A., PHILLIPS, L.G., HINSHAW, K.C. & WOOD, D.G. (1988). Ovarian function in the elephant: luteinizng hormone and progesterone cycles in African and Asian elephants. Biol. Reprod. 38, 309-314.

POOLE, J.H., KASMAN, L.H., RAMSAY, E.C. & LASLEY, B.L. (1984). Musth and urinary testosterone concentrations in the African elephant (*Loxodonta africana*). J.Reprod. Fert. 70, 255-260.

POPJAK, G. (1954). Biosynthesis of squalene and cholesterol *in vitro* from acetate-1-C^{14}. Archs. Biochem. Biophys. 48, 102-106.

POPJAK, G. & TIETZ, A. (1954). Biosynthesis of fatty acids by slices and cell-free suspensions of mammary gland. Biochem. J. 56, 46-54.

PRATER, S.H. (1971). Book of Indian Animals. (Bombay Nat. Hist. Soc.), pp. 323.

PRENTICE, L.G. & RYAN, R.J. (1975). LH and its subunits in human pituitary, serum and urine. J. Clin. Endocr. Metab. 40, 303.

PROST, M., BOURNOT, P. & MAUME, B.F. (1975). Adrenal and liver metabolites of 18-hydroxy-11-deoxycorticosterone in the rat. Identification of reduced compounds (18-OH-TH-DOC). Steroids 25, 177.

QUILLIAM, M.A. & WESTMORE, J.B. (1980). Mass spectral studies of partial and mixed silyl derivatives of 5β-pregnance-3α, 17α, 20α-triol. Eur. J. Mass Spec. in Biochem. Med. and Env. Res. 1, 53-56.

RAATH, J.P. & HALL-MARTIN, A.J. (1989). Transport and boma management techniques for black rhinoceroses (*Diceros bicornis*) as used in the Etosha Vaalbos operation. Koedoe 32, 69-76.

RABINOWITZ, J.L. & DOWBEN, R.M. (1955). Biosynthesis of radioactive estradiol. I. Synthesis by surviving tissue slices and cell-free homogenates of dog ovary. Biochim. Biophys. Acta. 16, 96-98.

RAESIDE, J.I. & LIPTRAP R.M. (1975). Patterns of urinary oestrogen excretion in individual pregnant mares. J.Reprod. Fert. (Suppl.) 23, 469-475.

RAMSAY, E.C., KASMAN, L.H. & LASLEY, B.L. (1987). Urinary steroid evaluations to monitor ovarian function in exotic ungulates: V. Estrogen and pregnanediol-3-glucuronide excretion in the black rhinoceros. Zoo Biol. 6, 275-282.

RAWLINS, C.G.C. (1979). The breeding of white rhinos in captivity- a comparative study. Zool. Gart., Lpz. N.F. 49, 1-7.

REUTHER, R.T. (1972). Rhinoceros data. AAZPA newsletter 13, 5-6.

REYNOLDS, J.W., MANCUSO, S., WIQVIST, N. & DICZFALUSY, E. (1968). Studies on the aromatisation of neutral steroids in women. 7. Aromatisation of 3β, 17β-dihydroxy-androst-5-en-16-one by placentas perfused *in situ* in mid pregnancy. Acta. Endocr., Copenh. 58, 377-395.

RICHARDS, J.S. & MIDGLEY, A.R.Jr (1976). Protein hormone action: a key to understanding ovarian follicular and luteal cell development. Biol. Reprod. 14, 82-94.

RIPLEY, S.D. (1958). Comments on the black and squrare-lipped rhinoceros species in South Africa. Ecology 39, 172-174.

RISLER, L., WASSER, S.K. & SACKETT, G.P. (1987). Measurement of excreted steroids in *Macaca nemestrina*. Am. J. Primatol. 12, 91-100.

RITCHIE, A.T.A. (1963). The black rhinoceros (*Diceros bicornis l.*). E.Afr. Wildl. J. 1, 54-62.

ROBERTSON, H.A. & KING, G.J. (1979). Conjugated and unconjugated oestrogens in fetal and maternal fluids of the cow throughout pregnancy. J.Reprod. Fert. 55, 463-470.

ROOKMAKER, L.C. (1973). Breeding of rhinoceros in zoological gardens. AAZPA newsletter 14, 5-8.

ROOKMAKER, L.C. (1980). The distribution of the rhinoceros in eastern India, Bangladesh, China and the Indo-Chinese region. Zoologischer Anzeiger 205, 253-268.

ROSER, J.F. & LOFSTEDT, R.M. (1989). Urinary and CG patterns in the mare during pregnancy. Theriogen. 32, 607-682.

ROTH, H.H. (1967). White and black rhinoceros in Rhodesia. Oryx 4, 217-231.

ROTH, H.H. & CHILD, G. (1968). Distribution and population structure of black rhinoceros (*Diceros bicornis*) in the Lake Kariba basin. Zeitschrift fur saugetierkundle 33, 214-226.

RYAN, K.J. & SHORT, R.V. (1965). Formation of estradiol by granulosa and theca cells of the equine ovarian follicle. Endocrinology 76, 214-216.

RYAN, K.J. & SHORT, R.V. (1966). Cholestrol formation by granulosa and thecal cells of the equine follicle. Endocrinology 78, 214-216.

RYAN, K.J. & SMITH, O.W. (1961). Biogenesis of oestrogens by the human ovary. J.Biol. Chem. 236, 705-709.

RYAN, K.J. & SMITH, O.W. (1965). Biogenesis of steroid hormones by the human ovary. Rec. Prog. Horm. Res. 21, 367-403.

SAFAR-HERMANN, N., ISMAIL, M.N., CHOI, H.S., MÖSTL, E. & BAMBERG, E. (1987). Pregnancy diagnosis in zoo animals by estrogen determination in feces. Zoo Biol. 6, 189-193.

SALE, J.B. & SINGH, S. (1987). Reintroduction of greater indian rhinoceros into Dudhwa national park. Oryx 21, 81-84.

SAMARAJEEWA, P., COOLIE, W. & KELLIE, A.E. (1979). The radioimmunoassay of pregnanediol glucuronide. J. Steroid Biochem. 11, 1165-1171.

SAMARAJEEWA, P. & KELLIE, A.E. (1985). The radioimmunoassay of pregnanetriol-3α-glucuronide. J. Steroid Biochem. 22, 843-849.

SAUER, M.J., FOULKES, J.A., WORSFOLD, A. & MORRIS, B.A. (1986). The use of progesterone 11-glucuronide-alkaline phosphatase conjugate in a sensitive microtitre plate enzymeimmunoassay of progesterone in milk: application to pregnancy testing in dairy cattle. J. Reprod. Fert. 76, 375-391.

SAVARD, K. (1961). The estrogens of the pregnant mare. Endocrinology 68, 411-416.

SAVARD, K. (1968). The biogenesis of steroids in the human ovary. In: The ovary (Ed. H.C.Mack), (Springfield, Charles C.Thomas), pp. 10-26.

SAVARD, K., MARSH, J.M. & RICE, B.F. (1965). Gonadotrophins and ovarian steroidogenesis. Rec. Prog. Horm. Res. 21, 285-356.

SCHAFFER, N.E. BEEHLER, B., JEYENDRAN, R.S. & BALKE, B. (1990). Methods of semen collection in an ambulatory greater one-horned rhinoceros (*Rhinoceros unicornis*). Zoo Biol. 9, 211-221.

SCHALLER, G.B., DANG, N.X., THUY, L.D. & SON, V.T. (1990). Javan rhinoceros in Vietnam. Oryx 24, 77-80.

SCHENKEL, R. & SCHENKEL-HULLIGER, L. (1969). Ecology and behaviour of the black rhinoceros (*Diceros bicornis l.*). (Verlag Paul Parey, Hamberg).

SCHENKEL, R. & SCHENKEL-HULLIGER, L. (1977). Ujung Kulon Nature Reserve protection and management programme. In: WWF Yearbook (Ed. P.Jackson) (Morges Press) pp. 129-132.

SCHNIDER, M. (1989). Progesterone, 20α-dihydroprogesterone and cortisol in the blood of cows at parturition. Wiener tierarztl mschr. 76, 56.

SCHOMBERG, D.W., JONES, P.H., ERB, R.E. & GOMES, W.R, (1966). Metabolites of progesterone in urine compared with progesterone in ovarian venous plasma of the cycling domestic sow. J.Anim. Sci. 25, 1181-1185.

SCHOMBERG, D.W., JONES, P.H., FEATHERSTONE, W.R. AND ERB, R.E. (1966). Identification of metabolites of progesterone-4-^{14}C in domestic sow urine. Steroids 8, 277.

SCHWARZENBERGER, F., MÖSTL, E., BAMBERG, E. & VON HEGEL, G. (1988). Gestagens in mare faeces during the oestrous cycle. J.Reprod. Fert. Abstr. Ser. 2, 82.

SEREN, E., TAMANINI, C., GAIANI, R. & BONO, G. (1981). Concentrations of progesterone, 17α-hydroxyprogesterone and 20α-dihydroprogesterone in the plasma of mares during pregnancy and at parturition. J.Reprod. Fert. 53, 443-448.

SETCHELL, K.D.R., GONTSCHAROW, N.P., AXELSON, M. & SJÖVALL, J. (1976). The identification of 3,6,11,17,21-penhydroxy-5-pregnan-20-one (16β-hydroxy-THF) the major urinary steroid of the baboon (*Papio papio*). J. Steroid Biochem. 7, 801-808.

SHACKLETON, C.H.L. & HONOUR, J.W. (1977). Identification and measurement of 18-hydroxycorticosterone metabolites by gas chromatography-mass spectrometry. J. Steroid Biochem. 8, 199.

SHACKLETON, C.H.L., HONOUR, J.W., DILLON, M. & MILLA, P. (1976). Multicomponent gas chromatographic analysis of urinary steroids excreted by an infant with a defect in aldosterone biosynthesis. Acta Endocr., Copenh. 81, 762.

SHAPCOTT, P. (1986). Catching the black rhinoceros for transportation at Bristol zoo. Int. Zoo News 33/4, 6-10.

SHAW, H.J., HILLIER, S.G. & HODGES, J.K. (1989). Developmental changes in leuteinising hormone/human chorionic gonadotrophin. Steroidogenic responsiveness in marmoset granulosa cells: effects of follicle-stimulating hormone and androgens. Endocrinology 124, 1669-1677.

SHIDELER, S.E., CZEKALA, N.M., BENIRSCHKE, K. & LASLEY, B.L. (1983). Urinary estrogens during pregnancy of the ruffed lemur (*Lemur variegatus*). Biol. Reprod. 28, 963-969.

SHIDELER, S.E., CZEKALA, N.M., KASMAN, L.H., LINDBURG, D.G. & LASLEY, B.L. (1983). Monitoring ovulation and implantation in the lion-tailed macaque (*Macaca silenus*) through urinary estrone conjugate exaluations. Biol. Reprod. 29, 905-911.

SHIDELER, S.E. & LASLEY, B.L. (1982). A comparison of promate ovarian cycles. Am. J. Primatol. (Suppl.) 1, 171-180.

SHIDELER, S.E., MITCHELL, W.R., LINDBURG, D.G. & LASLEY, B.L. (1985). Monitoring luteal function in the lion-tailed macaque (*Macaca silenus*) through urinary progesterone metabolite measurements. Zoo Biol. 4, 65-73.

SHILLE, V.M., WING, A.E., LASLEY, B.L. & BANKS, J.A. (1984). Excretion of radiolabelled estradiol in the cat (*Felis* catus, *L.*): a preliminary report. Zoo Biol. 3, 201-209.

SHIRLEY, I.M. & COOKE, B.A. (1968). Ring A and side-chain reduction of [4-^{14}C] progesterone by the rat liver *in vitro*. J.Endocr. 40, 477-483.

SHORT, R.V. (1959). Progesterone in blood: III. Progesterone in the peripheral blood of pregnant women. J.Endocr. 18, 418-425.

SIDNEY, J. (1985). The past and present distribution of some African Ungulates. Trans. Zool. Soc. Lond. 30, 51-68.

SIEGEL, E.T., DORFMAN, R.I., BRODEY, R.S. & FRIEDMAN, M.F.H. (1962). Conversion of 6,7-H^3 estradiol-17β into estrone and estradiol-17α in the mature male dog. Proc. Soc. Exptl. Biol. Med. 3, 533.

SMITH, K. (1986). Northern white rhinos (*Ceratotherium simum cottoni*). (Unpub. man.)

SOLOMON, S., BIRD, C.E., LING, W., IWAMIYA, M. & YOUNG, P.C.M. (1967). Formation and metabolism of steroids in the fetus and placenta. Rec. Prog. Horm. Res. 23, 297-335.

SOLOMON, S., VANDE WIELE, R. & LIBERMAN, S. (1956). The *in vitro* synthesis of 17α-hydroxyprogesterone and 4-androstene-3,17-dione from progesterone by bovine ovarian tissue. J.Am. Chem. Soc. 78, 5453.

SQUIRES, E.L. & GINTHER, O.J. (1975). Collection techniques and progesterone concentrations of ovarian and uterine venous blood in mares. J. Anim. Sci. 38, 330-338.

SQUIRES, E.L., WENTWORTH, B.C. & GINTHER, O.J. (1974). Progesterone concentration in blood of mares during the oestrous cycle, pregnancy and after hysterectomy. J.Anim. Sci. 39, 759-767.

STÁRKA, L. & BREUER, H. (1966). Biogenese von C_{18} steroiden beim pferd. Hoffe Seyler Z Physiol. Chem. 344, 124-139.

STERN, M.J. (1957). A method for the determination of 5β-pregnane-3α,17α, 20α-triol in urine. J.Endocr. 16, 180-188.

STØA, K.F. & SKULSTAD, P.A. (1972). Biliary and urinary metabolites of intravenously and intraduodenally administered 17β-oestradiol and oestriol. Steroids Lipids Res. 3, 299-314.

STOVER, J., EVANS, J. & DOLENSEK, E.P. (1981). Interspecies embryo transfer from the Gaur to domestic Holstein. Proc. Am. Ass. Zoo Vet. 122-124.

STUDER, L. (1975). Palpation of the genital tract for prediction of oestrus in the cow. Vet. Med. Small Ani. Clin. 70, 1337-1341.

STUPNICKI, R., McCRACKEN, J.A. & WILLIAMS, K.J.H. (1969). Progesterone metabolism in the ewe. J.Endocr. 45, 67-74.

SULIMOVICI, S. & BOYD, G.S. (1967). Steroid hydroxylation and the cholesterol side-chain cleavage system in the rat ovary. Biochem. J. 103, 16-17.

SUMMERS, P.M., SHEPHEAD, A.M., HODGES, J.K., KYDD, J., BOYLE, M.S. & ALLEN, W.R. (1987). Successful transfer of the embryos of Przewalskis horses (*Equus Przewalski*) and Grant zebras (*Equus burchelli*) to domestic mares (*Equus calallus*). J. Reprod. Fert. 80, 13-20.

TAKAHASHI, H., DULEBA, A.J., YUEN, B.H. & MOON, Y.S. (1984). Steroidogenic capabilities of various sompartments of rat ovarian follicles in culture. Steroids 44, 337-348.

TARANTAL, A.F. & HENDRICKX, A.G. (1988). Use of ultrasound for early pregnancy detection in the shows and cynomolgus macaque (*Macaca muletta* and *Macaca fasicularis*). J.Med. Primatol. 17, 105-112.

TAUSSKY, H.H. (1954). A microcolorimetric determination of creatinine in urine by the Jaffe reaction. J.Biol. Chem. 208, 853-861.

THAI, V.T. (1987). The general features of oceogenic factors and vegetation types in the tropical low-land mixed dipterocarp rainforest ecosystems at Nam Cat Tien Forest Reserve. In: Nat. Workshop of future biosphere res. Nam Cat Tien (Res. Centre for ecology, Ho Chi Minh City) pp. 7-11.

THOMPSON, M.A., LASLEY, B.L., RIDEOUT, B.A. & KASMAN, L.H. (1984). Characterization of the estrogenic properties of a non-steroidal estrogen from urine of pregnant macaques. Biol. Reprod. 31, 705-713.

TOMOSOVA, Z., GREGOROVA, I. & HORKY, K. (1981). Determination of 26 urinary steroids in one urine sample by gas-liquid chromatography. Proc. Symp. Analysis Steroids, 259-263.

TOWNSLEY, J.D. (1974). Estrogen excretion of the pregnant baboon (*Papio papio*). Endocrinology 95, 1759-1762.

TSANG, B.K., ARMSTRONG, D.T. & WHITFIELD, J.F. (1980). Steroid biosynthesis by isolatedhuman ovarian follicle cells in vitro. J. Clin. Endocr. Metab. 51, 1407-1411.

TURNER, E. (1982). White rhino: a critical species. AAZPA Ann. Proc. 31-35.

UILENBROEK, J.Th.J. (1988). Maintenace of a low 20α-HSD activity in rat corpora lutea *in vitro* by the anti-progesterone RU-486. J.Reprod. Fert. Abstr. Ser. 2, 79.

VAITUKAITIS, J.L. (1974). Changing placental concentrations of hCG and its subunits during gestation. J. Clin. Endocr. Metab. 38, 755.

VAN NIEKERK, C.H. (1976). Limitations to female reproductive efficiency. Proc. Int. Congr. Sheep Breed. Muresk, 299-309.

VAN RENSBURG, S.J. & VAN NIEKERK, C.H. (1968). Ovarian function, follicular oestradiol-17β and luteal progesterone and 20α-hydroxy-preg-4-en-3-one in cycling and pregnant equines. Onderspoort J. Vet. Res. 35, 589-610.

VILLEE, D.B. (1969a). Development of endocrine functions in the human placenta and fetus (Part I). New Eng. J. Med. 281, 473-484.

VILLEE, D.B. (1969b). Development of endocrine function in the human placenta and fetus (Part II). New Eng. J. Med. 281, 533-541.

WADDELL, B.J. & BRUCE, N.W. (1989). Changes in the blood concentrations of progesterone and 20a-hydroxypregn-4-en-3-one during late pregnancy in the conscious rat: a specific role for metabolic clearance rate. Biol. Reprod. 40, 1231-1236.

WADDELL, B.J., PEPE, G.J. & ALBRACHT, E.D. (1988). Changes in the placental secretion of progesterone and 20α-hydroxypregn-4-en-3-one into the fetus between mid and late pregnancy in the baboon. Biol. Reprod. (Suppl.) 38, 64.

WALKER, L.A., CORNELL, L., DAHL, K.D., CZEKALA, N.M., DARGEN, C.M., JOSEPH, B., HSUEH, A.J. & LASLEY, B.L. (1988). Urinary concentrations of ovarian steroid hormone metabolites and bioactive follicle-stimulating hormone in killer whales (*Orcinus orchus*) during ovarian cycles and pregnancy. Biol. Reprod. 39, 1013-1020.

WANJOHI, E.W. (1989). Non-invasive methods of assessment of reproductive status of the black rhinoceros, *Diceros bicornis*, in captivity and in the wild. Unpub. MPhil. thesis, Uni. of London.

WARREN, J.C. & SALHANICK, H.A. (1961). Steroid biosynthesis in the human ovary. J.Clin. Endocr. Metab. 21, 1218-1230.

WASSER, S.K., RISLER, L. & STEINER, R.A. (1988). Excreted steroids in primate feces over the menstrual cycle and pregnancy. Biol. Reprod. 39, 862-872.

WESTERN, D. & VIGNE, L. (1986). The deteriorating status of African rhinos. Oryx 19, 215-220.

WESTERN, D. & SINDIYO, D.M. (1972). The status of the Amboseli rhino population. E.Afr. Wild. J. 10, 43-57.

WICHMANN, K. (1967). On the metabolism and sub-cellular distribution of progesterone in the myometrium of the pregnant rat. Act. Endocr., Copenh. 57, 1-98.

WIEST, W.G. (1959). Conversion of progesterone to 4-pregnen-20-ol-3-one by rat ovarian tissue *in vitro*. J.Biol. Chem. 234, 3115-3121.

WIEST, W.G. & KIDWELL, W.R. (1969). The regulation of progesterone secretion by ovarian dehydrogenases. In: The gonads (Ed. K.W.McKerns), (Appleton-Century -Crofts, N.Y.), pp. 295-325.

WIEST, W.G., KIDWELL, W.R. & KIRSCHBAUM, T.H. (1963). Induction of rat ovarian 20α-OHSD activity by gonadotrophin hormone administration. Steroids 2, 617-130.

WILDT, D.E. (1980). Laparoscopy in the dog and cat. In: Animal laparoscopy (Ed. R.M.Harrison and D.E.Wildt), (Williams and Wilkins, Baltimore), pp. 31-72.

WILDT, D.E. & SEAGER, S.W.J. (1979). Laparoscopic determination of ovarian and uterine morphology during the reproductive cycle. In: Current therapy in theriogenology (Ed. D.Harroe), (W.B.Saunders, Philadelphia).

WILLIAMS, M.T., ROTH, M.S., MARSH, J.M. & LEMAIRE, W.J. (1979). Inhibition of human chorionic gonadotrophin-induced progesterone synthesis by estradiol in human luteal cells. J. Clin. Endocr. Metab. 48, 437-440.

WILSON, K.W. & GOULDING, K.H. (1986). Principles and techniques of practical biochemistry, (Edward Arnold, London), pp. 307-311.

WILSON, O.L. & DU BOULAY, G.H. (1988). Real time ultrasonic diagnosis of pregnancy in exotic species. Symp. Zool. Soc. Lond. 60, 135-150.

WITHERSPOON, D.M. (1975). The site of ovulation in the mare. J.Reprod. Fert. (Suppl.) 23, 329-330.

YAMAMOTTO, S. (1967). Notes on breeding of black rhinoceroses at Kobe zoo. Int. Zoo Yb. 7, 163.

YOUNG, E. (1967). Semen extraction by manipulation techniques in the black rhinoceros. Int. Zoo Yb. 7, 166-167.

ZIEGLER, T.E., SHOLL, S.A., SCHEFFLER, G., HAGGERTY, M.A. & LASLEY, B.L. (1989). Excretion of estrone, estradiol and progesterone in the urine and feces of the female cotton-top tamarin (*Saguinus oedipus oedipus*). Am. J. Primatol. 17,

ZUKOWSKY, L. (1964). Die systemsatik der Gettung *Diceros* Gray, 1821. Zool. Gart., Lpz. N.F. 30, 1-178.

APPENDIX

Appendix

Assay buffers

Hydrolysis buffer (pH 5·0)

sodium acetate	27·00 g
acetic acid (99%)	12·00 g
deionised distilled water	1·00 l

Phosphate buffered saline (pH 7·0)

Na_2HPO_4	17·20 g
$NaH_2PO_4.H_2O$	12·16 g
NaCl	18·00 g
methiolate (thinerosal)	0·20 g
deionised distilled water	1·00 l

Phosphate-azide-saline (PAS) gel buffer (pH 7·0)

KH_2PO_4	4·42 g
$Na_2HPO_4.12H_2O$	24·16 g
NaCl	9·00 g
gelatin	1·00 g
NaN_3	1·00 g
deionised distilled water	1·00 l

Tris assay buffer (pH 7·5)

trishydroxy-aminomethane	2·42 g
NaCl	17·52 g
BSA (fraction V)	1·00 g
tween 80	1·00 ml
deionised distilled water	1·00 l

WHO buffer (pH 7·2)

$Na_2HPO_4.12H_2O$	146.25 g
$Na_2HPO_4.2H_2O$	15·30 g
NaCl	44·00 g
NaN_3	0·50 g
gelatin	5·00 g
deionised distilled water	1·00 l

Low salt buffer (pH 7·0)

$NaH_2PO_4.2H_2O$	30·44 g
$Na_2HPO_4.12H_2O$	109·25 g
NaN_3	0·50 g
gelatin	5·00 g
deionised distilled water	1·00 l

Tricine buffered saline (pH 7·0)

tricine	17·90 g
NaCl	9·00 g
methiolate (thimerosal)	0·10 g
gelatin	1·00 g
deionised distilled water	1·00 l

Diethanolamine buffer (pH 9·8)

diethanolamine	105·10 g
$MgCl_2$	0·10 g
NaN_3	1·00 g
deionised distilled water	1·00 l

Coating buffer (pH 9·6)

Na_2CO_3	1·59 g
$NaHCO_3$	2·93 g
deionised distilled water	1·00 l

Storage buffer (pH 7·5)

trishydroxy-aminomethane	3·16 g
NaCl	23·30 g
BSA (fraction V)	13·00 g
NaN_3	1·30 g
deionised distilled water	1·00 l

HRP Substrate buffer (pH 5·0)

citric acid	21·01 g
tween 80	1·00 ml
deionised distilled water	1·00 l