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STEROIDOGENIC POTENTIAL OF THE OVARY, PLACENTA AND BLOOD OF THE ROCK HYRAX *PROCAVIA CAPENSIS*

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STEROIDOGENIC POTENTIAL OF THE OVARY, PLACENTA AND BLOOD OF THE ROCK HYRAX (*PROCAVIA CAPENSIS*)

by

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STEROIDOGENIC POTENTIAL OF THE OVARY, PLACENTA AND BLOOD OF THE ROCK HYRAX (*PROCAVIA CAPENSIS*)

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ABSTRACT

Circulating progesterone, 5α-dihydroprogesterone and oestradiol-17β concentrations, and the biosynthetic potential of blood, ovaries and placenta incubated with pregnenolone and progesterone in the absence and presence of β-NADPH, enzyme inhibitors and at various incubation times, was determined for pregnant and non-pregnant hyraxes. Plasma concentrations of 5α-

dihydroprogesterone were higher than those of progesterone and appear to be metabolically derived from progesterone in the blood of female hyraxes. Plasma concentrations of oestradiol-17β were low. White blood cells metabolised pregnenolone and progesterone, and whole blood, red blood cells and a mixture of red and white blood cells metabolised only progesterone. Plasma had no biosynthetic potential. All conversions in the blood resulted in the production of compounds A and B, partially identified as 5α- and 5β-reduced metabolites of progesterone. Progesterone metabolism was highest in pregnant animals and the formation of conversion products was lowest during late-pregnancy. White blood cells displayed the highest steroidogenic activity, which may be inhibited or negligible in blood. Whole blood had the lowest biosynthetic potential and this may be due to the presence of enzyme inhibitors or progesterone binding proteins in the plasma. Luteal, ovarian residual and placental tissues metabolised both pregnenolone and progesterone. Compound E, formed by luteal tissue, has been identified as 17α-hydroxyprogesterone and compound F, formed by ovarian residual tissue, has been partially identified as 17α-hydroxyprogesterone. Compound H was formed by placental tissue and has been partially identified as 20α-dihydroprogesterone. Luteal tissue may be important for the production and secretion of progesterone into the circulation. Pregnenolone metabolism increased in the presence of β-NADPH as well as with a longer incubation time, and was inhibited by NaF-HgCl.

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Reproductive biology of the rock hyrax

The rock hyrax (*Procavia capensis*; Pallas 1766) is found mainly in hot, arid regions and occurs throughout most of the southern African subregion (Skinner & Smithers 1990). It is a gregarious, diurnal inhabitant of rocky habitats and depends on crevices for shelter (Skinner & Smithers 1990). Adults weigh between 1.5 and 5 kg and are generalist browsers and grazers (Eley 1994). The breeding unit is a polygynous group with a dominant, territorial male, several adult females, and sub-adults and juveniles of both sexes (Fourie & Perrin 1987). Males and females become sexually mature at 16 to 17 months of age, with some animals reaching puberty when 4 to 5 months old (Millar 1971). The mean oestrous cycle length of the hyrax is 13.4 ± 1.31 days (± SEM; Gombe 1983).

In the rock hyrax, the proximate cue regulating male sexual activity is photoperiod and the mating season is reached later in the year with decreasing latitude (Millar 1971; Millar & Glover 1973). It was suggested that a critical rate of decrease in photoperiod determines the mating season (Millar & Glover 1973). A number of environmental factors may act together as ultimate cues which determine the onset of the mating season (Millar & Glover 1973). Environmental regulation of sexual activity contributes to a birth peak and in South Africa parturition can occur from September in the higher latitudes up to March in the lower latitudes (Millar 1971). Pregnancy lasts 230 days (Millar 1971). Transuterine migration of ova occurs so that foetuses are more equally distributed between uterine horns (Millar 1971). Litter size varies from 1 to 5 and seems to be affected by the predominant environmental conditions (Millar 1971). Mean litter size varies from 1.5 to 3.5 and older females have larger litters than young and very old females (Millar 1971). Foetuses of larger litters tend to weigh less than those of smaller litters (Millar 1971). The young are precocious from the day of birth, begin suckling soon after birth and are weaned from one to five months of age (Millar 1971). Lactational anoestrus does not appear to occur (Millar 1971).

In certain areas of the southern African subregion, rock hyrax numbers occasionally have increased to epidemic levels (Kolbe 1983). Both neonatal mortality and incidence of infertility in old females is low, resulting in hyraxes having a high reproductive potential (Millar 1971). Favourable environments, such as farm land, result in a greater incidence of large litters which leads to local population outbreaks (Millar 1971). There may therefore be a need to artificially reduce or regulate population growth, especially in inner city parklands in the Gauteng province.

This could be achieved by manipulating the reproductive output, possibly through the use of antifertility agents, which act on uterine tissue and may block the binding of hormones maintaining pregnancy to the receptor. In the rock hyrax the period of sexual activity (i.e. mating) is short (Millar 1971) and both ovulation and fertilization occurred between mid-March and mid-April in the central Orange Free State (Van der Merwe & Skinner 1982). Therefore, to interfere with fertilisation and implantation, a female need only be treated once a year prior to or after mating. To reduce population growth, females that have just reached puberty may be treated with an anti-fertility agent, thereby increasing their age of first reproduction. On the other hand, any sexually active female may be treated with the aim of increasing the interval between breeding periods. Such manipulation, however, depends on detailed information of the endocrine correlates of the reproductive cycle.

The elephant and the hyrax are serologically closely related (Weitz 1953; Buettner-Janusch, Buettner-Janusch & Sale 1964), whilst a number of studies including mtDNA sequencing has placed the Hyracoidea as a sistergroup to that of the African elephant (*Loxodonta africana*; De Jong, Zweers & Goodman 1981; Kleinschmidt, Czelusniak, Goodman & Braunitzer 1986; Prinsloo 1993; Lavergne, Douzery, Stichler, Catzeflis & Springer 1996; Stanhope, Smith, Waddell, Porter, Shivij & Goodman 1996). Since the hyrax is phylogenetically related to the African elephant, this study may contribute to the understanding of endocrine support of pregnancy in the elephant. In the African elephant, the possibility exists of controlling population numbers with the use of a contraceptive as an alternative to culling

(Greyling 1997). Therefore, the hyrax may serve as a useful model on which to develop possible techniques for the artificial control of reproduction in the elephant.

Progestins and the maintenance of pregnancy

Cholesterol is the precursor for steroid hormone biosynthesis and is converted to pregnenolone (3β-hydroxy-5-pregnen-20-one), which in turn is converted to progesterone (pregn-4-ene-3,20-dione; Cheesman 1982; Fig. 1.). Progesterone is the primary active progestin and is secreted in large amounts during pregnancy in most mammals (Cheesman 1982). Its functions include the establishment of the placenta, inhibition of myometrial activity and the maintenance of the developing conceptus (Heap & Illingworth 1977; Cheesman 1982). Progesterone may be converted to a number of metabolites (Fig. 1.), including those with 5α-configurations, which may maintain progestational activity (Heap & Illingworth 1977). Steroids are enzymatically metabolised to active or inactive metabolites (Chatterton 1982). The metabolism of steroids occurs via a number of pathways, including hydroxylation, hydrogenation, conjugation and oxidation (Heap & Illingworth 1977). In some tissues these active and inactive metabolites may occur in redox pairs and are interconvertible (e.g. progesterone interconverts with 20α-hydroxyprogesterone), depending on the availability of dehydrogenases and reduced or oxidised co-factors (Chatterton 1982).

Most steroids become inactive through the metabolic conversion to steroid derivatives, or due to conjugation with glucuronic acid or sulphate. Conjugated steroids may be excreted in the bile and urine, although dehydroepiandrosterone sulphate (synthesised by the foetus) may be converted by steroid sulphatases to regenerate active steroids (Johnson & Everitt 1988). Renal clearance of steroids also occurs where the kidneys metabolise steroids to water-soluble conjugates (Baird 1973). Therefore, the metabolism of progesterone, and possibly other progestins, may decrease circulating hormone

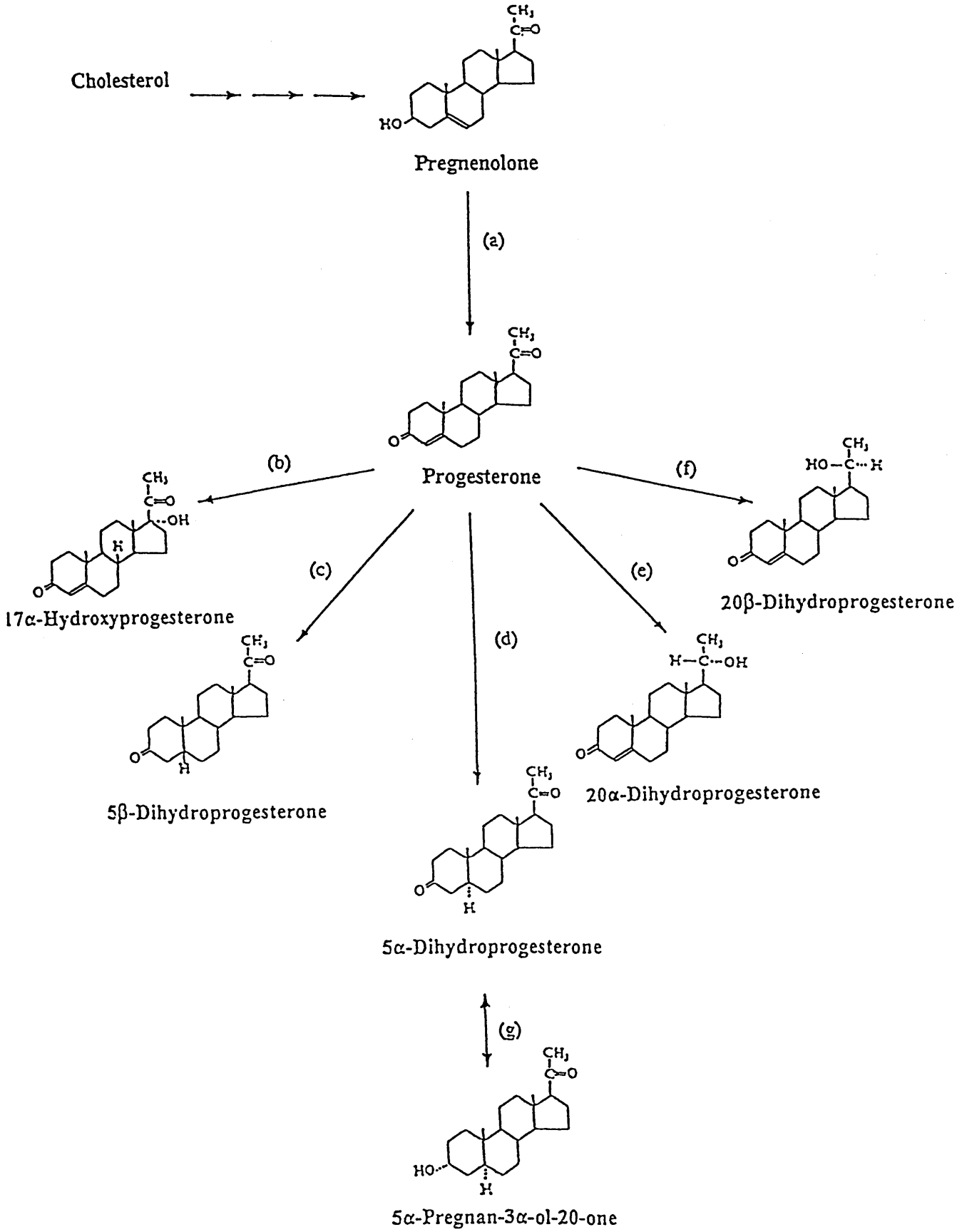


Fig. 1. The genèral steroidogenic pathway starting from cholesterol. The enzymes responsible for the conversions are (a) the 3β-hydroxysteroid dehydrogenase and isomerase complex, (b) 17α-hydroxylase, (c) 5β-reductase, (d) 5α-reductase, (e) 20α-hydroxysteroid dehydrogenase, (f) 20β-hydroxysteroid dehydrogenase and (g) 3α-hydroxysteroid dehydrogenase.

concentrations which may be present in excess amounts and the metabolites are then excreted (Johnson & Everitt 1988; Makawiti, Osaso & Gombe 1991).

Steroid precursors from the ovaries can be converted to active steroids in the placenta, and the converse can also occur (Strauss III, Martinez & Kiriakidou 1996). The syncytiotrophoblast is in direct contact with maternal blood and can take up steroid precursors from the maternal plasma and secrete hormones into the maternal compartment (Cheesman 1982; Strauss III *et al.* 1996). Hormones from the placenta can increase steroidogenesis or maintain progesterone secretion by luteal tissue (Gibori & Miller 1982; Gadsby & Keyes 1984). Ovaries synthesise androgens which enter the maternal circulation and are metabolised by the placenta to oestrogens (Cheesman 1982), and the production of progesterone by the placenta may be influenced by endogenous steroids during pregnancy (Grimshaw, Mitchell & Challis 1983). Therefore, a dynamic interaction of steroids between the placenta and ovary exists (Strauss III *et al.* 1996).

In most mammals, pregnancy is maintained through the influence of progesterone on the uterine endometrium. However, the possibility remains that a metabolite of progesterone may have greater progestational activity. For instance, in the African elephant circulating 5α-reduced metabolites are present at relatively high concentrations and are more closely related to corpus luteum function than progesterone (Hodges, Heistermann, Beard & Van Aarde 1997). In the blood of the rock hyrax, progesterone is metabolised to 5α-dihydroprogesterone (5α-pregnane-3,20-dione) and 5β-dihydroprogesterone (5β-pregnane-3,20-dione; Heap, Gombe & Sale 1975; Makawiti *et al.* 1991), suggesting that these metabolites may play an important role in maintaining pregnancy. Although 5α-dihydroprogesterone has no progestational activity (Zarrow, Neher, Lazowasem & Salhanick 1957; Sanyal & Villee 1973; Heap & Flint 1979), there is evidence to suggest that 5α-dihydroprogesterone can compete with progesterone for binding to uterine progesterone receptors (Heap & Flint 1979), that concentrations change with stage of pregnancy in the horse (Seamans, Harms, Atkins & Fleeger

1979; Chavatte & Tait 1994) and it may be important for the maintenance of pregnancy (Schutzer & Holtan 1996). Chavatte, Rossdale & Tait (1995) also suggested that Δ^5-pregnenes and 5α-pregnanes could play a role in the onset of parturition. In the African elephant, the 5α-reduced metabolites of progesterone had a higher relative binding affinity for the progesterone uterine receptor than progesterone (Greyling, Van Aarde & Potgieter 1997) and uterine progesterone receptor concentrations are down-regulated by 5α-dihydroprogesterone as pregnancy progresses, implying that 5α-dihydroprogesterone may be of biological importance (Greyling, Ford, Potgieter & Van Aarde 1998). Therefore, research on the biosynthetic potential of blood and selective tissues of the rock hyrax should contribute to understanding the endocrine support of pregnancy in this species and possibly the African elephant.

Progesterone concentrations are high in the corpora lutea of the rock hyrax but low in plasma (Gombe, Heap & Sale 1976; Van Aarde & Anderson 1989). Therefore, it has been suggested that the maintenance of pregnancy could be facilitated by a sensitive receptor mechanism in the target tissues (Heap *et al.* 1975). Alternatively, since erythrocytes metabolize progesterone *in vitro* (Makawiti *et al.* 1991) and target tissues are able to selectively remove progesterone metabolites from the blood (Wichmann 1967), it is feasible to postulate that a metabolite of progesterone may support pregnancy (Heap *et al.* 1975). However, since ovariectomy in hyraxes leads to abortion within a short period of time (Gombe, Oduor-Okelo & Amoroso 1977, in Oduor-Okelo, Musewe & Gombe 1983), hormonal support of pregnancy may be derived from the ovarian tissues in conjunction with metabolism in the blood, placenta or uterus. The ovary may supply progesterone, which in turn is metabolised to substances that support pregnancy. On the other hand, hormones which may be circulating in the blood of the rock hyrax may reach the luteal tissue and be metabolised by this tissue to hormones which support pregnancy.

Previous studies on the metabolism of progesterone by rock hyrax blood concentrated on whole blood, plasma and a mixture of red and white blood cells (Heap *et al.* 1975; Makawiti *et al.* 1991). No metabolic studies were carried out on the white blood cells and pure red blood cells. Studies on the

biosynthetic potential of the white blood cells of the hyrax may be of importance since it was reported that human white blood cells are able to transform progesterone to other progestins, such as 5α-dihydroprogesterone (Scully, Ferguson, Sirrett & Grant 1982). The purpose of this metabolic conversion of progesterone in blood is still unclear. White blood cells may be an important steroidogenic component of blood and their potential to metabolise progesterone may be of significance for the maintenance of pregnancy. Alternatively, white blood cells may be a target for progesterone or progesterone metabolites in order to inhibit the cytotoxic activity of these cells towards the foetus (Scully *et al.* 1982), thus compromising the immunocompetency of these cells.

The blood of a number of species can metabolise progesterone to other progestins, for example cow foetuses, lambs and kids (Nancarrow & Seamark 1968) as well as sheep foetuses (Nancarrow & Seamark 1967) and humans, dogs, rabbits and sheep (Van der Molen & Groen 1968). Red blood cells can also metabolise progesterone to other progestins in cow foetuses and kids (Heap *et al.* 1975) as well as in humans, dogs, rabbits and sheep (Van der Molen & Groen 1968). Van der Molen & Groen (1968) showed that red blood cells of the human had a greater capacity to convert progesterone than plasma or whole blood and progesterone was more rapidly metabolised by red blood cells than by whole blood. However, the function of the steroidogenic activity of whole blood and the red blood cells is unclear. Progesterone metabolism in the blood may play an important role in the protection of maternal and foetal tissues from the adverse effects of high hormone concentrations (Heap *et al.* 1975). Therefore, it is of biological significance to determine the steroid biosynthetic potential of whole blood and its components since steroids are transported to target tissues via the blood. Any effect that blood may have on a circulating hormone should have an effect on target tissues and consequently on pregnancy.

A number of enzymes are involved in the metabolism of pregnenolone to progesterone and several other important metabolites. The enzyme complex 3β-hydroxysteroid dehydrogenase and isomerase as

well as 5α-reductases are responsible for the metabolism of pregnenolone and progesterone to its various metabolites (Cheesman 1982). Inhibition of any of these enzymes will stop the biosynthesis of several steroid hormones (Heap *et al.* 1975; Grimshaw *et al.* 1983; Vahdat, Seguin, Whitmore & Johnston 1984; Inns & Cecchini 1989). By using enzyme inhibitors it is possible to indicate whether any of the metabolic conversions, occurring in the blood and selective tissues of the rock hyrax, are dependent on any of these enzymes.

The steroid biosynthesis of steroid hormones and their metabolites occurs in various tissues and is affected by the differentiation of tissues during follicular development, ovulation, and corpus luteum formation and regression (Cheesman 1982). The pattern of steroid secretion by endocrine glands is determined by the relative proportion of the various cell types, the anatomical organisation of the gland, the blood supply, the concentration of co-factors and precursors present in the gland, as well as the presence of appropriate tropic stimuli (Cheesman 1982). Therefore, it is reasonable to assume that steroid biosynthesis within target tissues may change as a function of the reproductive status of an individual. Studies on the biosynthetic potential of certain steroid target tissues of the hyrax may give an indication of the pattern of steroid formation and metabolism throughout its pregnancy. Examining the biosynthetic potential of the blood and reproductive tissues of the hyrax, as well as circulating steroid concentrations, should facilitate an understanding of the endocrinology of reproduction in this species.

Aims of the study

The purpose of the present study is to contribute to our understanding of the endocrinology of pregnancy in the female rock hyrax as part of a research programme directed at investigating the artificial control of reproduction in the hyrax and African elephant. In order to quantitatively describe the endocrine correlates of pregnancy in the rock hyrax, the biosynthetic potential of whole blood, plasma, red blood cells and white blood cells, luteal tissue, ovarian residual tissue and placental tissue, incubated with the

progestin precursors pregnenolone and progesterone, will be investigated. The objectives of the present study are:

- to determine progesterone, 5α-dihydroprogesterone and oestradiol-17β concentrations in plasma throughout pregnancy,
- to investigate the *in vitro* metabolism of pregnenolone and progesterone in whole blood, plasma, red blood cells, white blood cells, luteal tissue, ovarian residual tissue and placental tissue, throughout pregnancy,
- to describe temporal changes in the metabolites formed from pregnenolone and progesterone during *in vitro* manipulations, and
- to determine the influence of incubation time and the presence of the co-factor β-NADPH and of enzyme inhibitors (oestrone and sodium fluoride with mercury chloride) on the metabolism of pregnenolone.

CHAPTER 2

MATERIALS AND METHODS

Reagents used

Ketamine hydrochloride (100 mg/ml) was purchased from Kyron Labs (Pretoria, South Africa), xylazine hydrochloride (100 mg/ml) from Bayer (Isando, South Africa) and eutha-naze solution from Centaur Laboratories (Bryanston, South Africa). Heparin (5000 iu/ml) was bought from Novo Nordisk, Johannesburg, South Africa.

The radioligand [7-^{3}H]pregnenolone (specific activity 25 Ci/mmol; purified prior to use - see Appendix 1) was purchased from New England Nuclear, Massachusetts, USA. The radioligands [1,2,6,7-^{3}H]progesterone (specific activity 94 Ci/mmol; radiochemical purity 97.7%) and [2,4,6,7-^{3}H]oestradiol-17β (specific activity 75 Ci/mmol; radiochemical purity 97.9%) were bought from Amersham International, Buckinghamshire, UK.

The co-factor β-NADPH and all unlabelled steroids, namely pregnenolone (5-pregnen-3β-ol-20-one), progesterone (4-pregnene-3,20-dione), 5α-dihydroprogesterone (5α-pregnane-3,20-dione), 5β-dihydroprogesterone (5β-pregnane-3,20-dione), 5α-pregnan-3α-ol-20-one, 20α-dihydroprogesterone (4-pregnen-20α-ol-3-one), 20β-dihydroprogesterone (4-pregnen-20β-ol-3-one), 17α-hydroxyprogesterone (4-pregnen-17α-ol-3,20-dione), 11α-hydroxyprogesterone (4-pregnen-11α-ol-3,20-dione), oestradiol-17β and oestrone (1,3,5(10)-estratrien-3-ol-17-one), were purchased from Sigma, St. Louis, USA.

Hank's balanced salt solution (without antibiotics, filter sterilized) and Eagles minimum essential medium (with Earle's salts, with L-glutamine, without antibiotics, 25 mM HEPES, 0.2% sodium bicarbonate, pH ~ 7.4, filter sterilized) were provided by Highveld Biological (Kelvin, South Africa). Ficoll-paque was purchased from Pharmacia Biotech (Upsala, Sweden), Tween-20 from Sigma (St.

Louis, USA) and anti-rat IgG alkaline phosphatase conjugate from Sigma Immuno Chemicals (St. Louis, USA).

Triton X-100 was purchased from BDH Chemicals (Poole, UK). Absolute ethanol was purchased from univAR (Saarchem-holpro analytic, Krugersdorp, South Africa), acetonitrile from Rathburn (Chemicals limited, Walkburn, Scotland; HPLC grade S), dichloromethane from Analar (BDH Chemicals, Poole, UK), methanol from Romil (methanol 205, super purity solvent; Cambridge, UK), chloroform from Sky Chem (AR, Randhart, South Africa), n-hexane from Merck (Darmstadt, Germany), ethyl acetate from Robert Lundie (AR, Westgate, South Africa) and sulphuric acid from Glassworld (98% AR, Newclare, South Africa).

Diethyl ether was purchased from uniLAB Saarchem-holpro analytic (Muldersdrift, South Africa), except that used during the oestradiol-17β radioimmunoassay which was bought from Merck (Analysed Reagent, Darmstadt, Germany), and that used during the 5α-dihydroprogesterone amplified enzyme-linked immunoassay which was purchased from Ractapur (Prolabo, Fontenay S/Bois). Petroleum ether (AR, distillation range 40 - 60°C) was provided by Robert Lundie Chemicals (Westgate, South Africa).

Phosphate buffered saline (PBS; pH ~ 6.9) contained 0.1% weight/volume (w/v) gelatine powder (uniLAB, Saarchem, Krugersdorp, South Africa), 0.1% (w/v) sodium azide (NaN_3; uniLAB, Saarchem, Krugersdorp, South Africa), 2.25% (w/v) di-sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_412H_2O$. AR; univAR, Saarchem, Krugersdorp, South Africa), 0.61% (w/v) sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_42H_2O$. AR; univAR, Saarchem, Krugersdorp, South Africa) and 0.9% (w/v) sodium chloride CP (NaCl, Associated Chemical Enterprises c.c., South Africa).

Dextran-coated charcoal solutions were prepared every three weeks and consisted of 0.156% (w/v) activated charcoal (Merck, Darmstadt, Germany) and 0.0156% (w/v) dextran T70 (Pharmacia LKB, Biotechnology AB, Upsala, Sweden) in PBS, and was stored at 4°C for 24 h prior to use.

Animals

Adult female hyraxes (*Procavia capensis*) were collected from Sandton City Parks (Sandton, Gauteng, South Africa) and were also donated by the Johannesburg Zoo (Johannesburg, Gauteng, South Africa) between August 1995 and June 1996. The animals were transported to the University of Pretoria, weighed and anaesthetized using a 1:1 mixture of ketamine hydrochloride and xylazine hydrochloride, which was injected intramuscularly (1.25 ml/kg).

Blood and tissue collection

Blood was collected through cardiac puncture with a hypodermic syringe from sedated hyraxes and placed into glass tubes (10 ml) containing 500 µl heparin (600 iu/ml), on ice. Red and white blood cells were counted using a haemocytometer (Spencer Bright-line, Improved Neubauer, 1/10 mm deep). Red blood cells were counted after diluting an aliquot of blood with Dacie's fluid (99 ml 3% trisodium citrate + 1 ml formalin), while white blood cells were counted after diluting an aliquot of blood with 2% aqueous acetic acid tinted with gentian violet (Simmons 1980).

After blood collection, the animal was killed with 2 ml/kg eutha-naze solution which was injected into the heart. The reproductive tract was dissected out and cleansed in ice-cold saline solution (0.9% weight/volume NaCl; Van Aarde & Anderson 1989). Each ovary was weighed and corpora lutea were then dissected out, weighed and kept on ice. The ovarian residual and placental tissues were also weighed. For pregnant females the foetuses were weighed and these weights were used to determine the stage of pregnancy (t in days; foetal age) using the equation $t = (\sqrt[3]{w} / 0.047) + 41$, where w = total foetal weight in grams (Millar 1971). Foetal age was used to distinguish between early- (< 78 days), mid- (78 to 154 days) and late-pregnant (> 154 days) animals.

Blood and tissue incubations

Whole blood from all hyraxes (two non-pregnant and one early-, four mid- and three late-pregnant) was incubated within 20 min of collection by placing 900 µl duplicate aliquots in glass tubes (10 ml) containing 50 µl [7-^{3}H]pregnenolone (~ 120 000 dpm/50 µl PBS) and 50 µl of the co-factor β-NADPH (2.4 mM in PBS). Blood samples from four hyraxes (non-, early-, mid- and late-pregnant) were incubated with 50 µl [1,2,6,7-^{3}H]progesterone (~ 120 000 dpm/50 µl PBS) and 50 µl of the co-factor β-NADPH (2.4 mM in PBS). Whole blood from two hyraxes (one mid- and one late-pregnant) was also incubated with 50 µl of a mixture of both [7-^{3}H]pregnenolone and [1,2,6,7-^{3}H]progesterone (~ 60 000 dpm each in 50 µl PBS), with the addition of 50 µl of the co-factor β-NADPH (2.4 mM in PBS). Incubations took place in a shaking waterbath (37°C) for 3 h. The remaining whole blood was centrifuged (4°C, 1200 rpm, 15 min), the plasma removed and 900 µl of the plasma was incubated as described for the whole blood.

Following centrifugation and the removal of plasma, the red and white blood cells were separated on a ficoll-paque gradient by diluting the pellet in Hank's balanced salt solution (1:1) and placing 5 ml thereof onto 4 ml ficoll-paque in a falcon tube (Böyum 1968). These tubes were centrifuged (20°C, 1900 rpm, 20 min). The layer of white blood cells was removed and transferred into a clean falcon tube, and the ficoll and Hank's solutions were removed from the red blood cell pellet. The red and white blood cells were then washed by suspension in 3 ml PBS, centrifuging (20°C, 1400 rpm, 7 min) and removing the supernatant. The washing step was repeated by suspending the cells in 2 ml PBS, followed by centrifuging (20°C, 1200 rpm, 5 min). The supernatant was removed and the cells were suspended in 1 ml PBS. To the white blood cell suspension, 9 ml ice-cold 0.87% (weight/volume) NH_4Cl was added at 4°C to lyse any remaining red blood cells. After standing for 5 min at 4°C, the mixture was centrifuged (20°C, 1200 rpm, 10 min), the supernatant removed and the packed cells suspended in 1 ml PBS.

The isolated red and white blood cell suspensions were made up to 6 ml with Eagle's minimum essential medium and centrifuged (20°C, 1200 rpm, 7 min). All but 1 ml of the supernatant was removed and the cells were resuspended in this.

A mixture of red and white blood cells (5 ml; the pellet remaining after the whole blood has been centrifuged) from seven hyraxes (two non-pregnant and one early-, one mid- and three late-pregnant) was washed with PBS and suspended in 1 ml of Eagle's medium as described for the separated red and white blood cells. Aliquots (900 µl) of the red blood cell and white blood cell suspensions, and of the red and white blood cell mixture, were incubated in duplicate as described for the whole blood and plasma samples.

Luteal tissue (~ 20 mg), ovarian residual tissue (~ 20 mg) and placental tissue (~ 200 mg) was incubated in duplicate (except for luteal tissue since not enough tissue was available) as described for the blood incubations, with the exception that 900 µl Eagle's medium was added to the tubes to equal the volume of the blood incubations. Controls consisting of only the incubation medium (i.e. no blood, cells or tissues) were included in all incubations.

In order to determine the influence of incubation time on the metabolism of pregnenolone, whole blood, plasma, the isolated red and white blood cells and placental tissue of a mid-pregnant hyrax was incubated with 50 µl [7-^{3}H]pregnenolone (~ 120 000 dpm/50 µl PBS) and 50 µl of the co-factor β-NADPH (2.4 mM in PBS). The incubations continued for one, two and three hours. Incubations in the absence or presence of β-NADPH were carried out to determine the influence thereof on the metabolism of pregnenolone. In this case, blood and tissue samples (except luteal tissue) were incubated with 50 µl [7-^{3}H]pregnenolone (~ 120 000 dpm/50 µl PBS) and 50 µl PBS (i.e. β-NADPH absent) or 50 µl of β-NADPH (2.4 mM in PBS; i.e. β-NADPH present).

Incubations in the presence of enzyme inhibitors were carried out to determine their influence on the metabolism of pregnenolone and to establish if the metabolism results from enzyme activity. Blood and

tissue samples (except for luteal tissue) were incubated with 50 µl [7-^{3}H]pregnenolone (~ 120 000 dpm/50 µl PBS) and 50 µl oestrone (20 µM in PBS), which inhibits the metabolism of pregnenolone to progesterone (Grimshaw *et al.* 1983), or 50 µl of a solution of sodium fluoride and mercury chloride (1:1, 200 mM in PBS; NaF-HgCl; Heap *et al.* 1975; Vahdat *et al.* 1984; Inns & Cecchini 1989).

To test the effect of white blood cell number on the metabolism of pregnenolone, serial dilutions (1:0, 1:1 and 1:3) of a white blood cell stock from two non-pregnant and one early-pregnant hyrax were prepared. Aliquots (900 µl) of each dilution were incubated as described for the whole blood incubations. The number of white blood cells in the stock was counted as was described earlier when counting the number of white blood cells in the blood (p. 12).

Whole blood from the non-pregnant and early-pregnant hyraxes (two aliquots of 2 ml) was incubated with 100 µl progesterone (3.82 µM in PBS) and 100 µl β-NADPH (2.4 mM in PBS) so that any unknown compounds formed during the incubations could be identified by gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry.

Extraction of incubated samples

After incubation of the blood and its components, 200 µl of 10% triton X-100 was added to each of the blood mixtures, which then were vortexed for 2 sec to ensure that all the blood cells were lysed, thereby facilitating the extraction of substances from within the cells. Ice-cold absolute ethanol was added to each tube. These mixtures were vortexed for 10 min on a vortex mixer (IKA-Vibrax-VXR, Janke & Kunkel, Staufen, Germany), centrifuged (4°C, 2000 rpm, 10 min), the supernatants transferred to clean glass tubes (10 ml) and stored at –20°C.

After incubation of the tissues, 2 ml ice-cold absolute ethanol was added to the incubation mixtures and in the case of the control incubations 4 ml ice-cold absolute ethanol was added. These mixtures were vortexed for 10 min and then stored at –20°C. The incubated tissue samples were homogenised with a

homogeniser (Ultra-Turrax, TP 18/10, Janke & Kunkel, Staufen, Germany), after which the homogenising probe was rinsed with 2 ml absolute ethanol, which was added to the homogenised tissue sample. The tubes were vortexed for 10 min, centrifuged (4°C, 2000 rpm, 10 min), the supernatants transferred to clean glass tubes (10 ml) and stored at –20°C.

After removing the supernatants, the remaining blood and tissue incubated samples were re-extracted, but this time with 2 ml diethyl ether (peroxide free; see Appendix 2) by vortexing for 10 min, centrifuging (4°C, 2000 rpm, 10 min), freezing at –20°C for 1 h and then at –70°C for 5 min. The supernatants were decanted into the tubes containing the first extracts. All supernatants were prepared for high pressure liquid chromatography by first evaporating them at 37°C under a stream of nitrogen (N_2). These dried extracts were reconstituted in 1 ml absolute ethanol, vortexed for 20 sec, centrifuged (4°C, 2000 rpm, 7 min) and stored at 4°C until HPLC analysis. To determine the recovery of radioactivity during the extraction process, 50 µl of each extract was pipetted into a scintillation vial (6 ml Pony Vial, Chemicals and Supplies, Packard, Groningen, The Netherlands). Scintillation cocktail (4 ml; Ultima Gold XR, Packard, Downersgrove, USA) was added and radioactivity was counted with a scintillation counter (Liquid Scintillation Analyzer, Tri-carb 1500, Packard). Pre-high pressure liquid chromatography recovery was determined as the percentage of radioactivity extracted during the extraction process, and the extraction process was repeated if recovery was < 60%. The pre-high pressure liquid chromatography recovery of extracted samples was 84.4 ± 11.6% (mean ± SD; n = 270; Table 1). Subsequent calculations of the relative proportions of compounds (expressed as percentages) that eluted off the HPLC column included these recovery values to correct for extraction losses.

The incubations of whole blood with progesterone (unlabelled) were terminated by the addition of 4 ml diethyl ether. Diethyl ether was used instead of absolute ethanol because diethyl ether extracted fewer impurities, thus facilitating the detection of any conversion products during mass spectrometry. The samples were vortexed for 10 min, centrifuged (4°C, 2000 rpm, 10 min) and frozen at –70°C for 1 h. The

Table 1. Pre-HPLC recovery of radioactivity (mean ± SD) after extraction of control, blood and tissue samples incubated with [^{3}H]pregnenolone and [^{3}H]progesterone, as well as post-HPLC recovery of radioactivity (mean ± SD) following HPLC, expressed as a percentage of the total amount of radioactivity injected onto the HPLC column.

Incubation sample	Sample size	Pre-HPLC recovery (%)	Post-HPLC recovery (%)
Control	20	80.0 ± 10.9	88.9 ± 13.7
Whole blood	38	82.0 ± 11.2	58.0 ± 22.9
Plasma	38	90.7 ± 7.8	54.7 ± 23.3
Red blood cells	38	82.3 ± 11.9	86.0 ± 10.2
White blood cells	47	87.9 ± 11.0	87.8 ± 10.3
Red and white blood cells	21	82.7 ± 11.4	89.0 ± 9.6
Luteal tissue	13	85.8 ± 12.3	81.7 ± 12.4
Ovarian residual tissue	31	85.2 ± 11.7	84.8 ± 9.6
Placental tissue	24	78.2 ± 12.4	82.8 ± 12.0

organic phases were decanted into clean glass tubes (12 x 75 mm) and evaporated (37°C, N_2). The dried extract residues to be injected onto the gas chromatography-mass spectrometry system were reconstituted in 20 µl dichloromethane and those to be injected onto the liquid chromatography-mass spectrometry system were reconstituted in 200 µl absolute ethanol. The extracts were vortexed for 20 sec prior to injection.

Chromatography of extracts from incubated samples

High pressure liquid chromatography (HPLC)

Incubation extracts (100 µl) were centrifuged (Beckman microfuge B) and 80 µl of the supernatants were injected onto a HPLC column (Phenomenex Lichrosorb 5 RP-18; 150 x 4.60 mm; Cheshire, UK) protected by a precolumn with an insert (guard-pak insert; µ-Bondapak C18; Waters, Division of Millipore, Milford, USA). The HPLC system (Waters, Milford, USA) consisted of a 590 programmable solvent delivery module, an automated gradient controller and a 712 WISP autoinjector. The mobile phase consisted of 55% acetonitrile and the flow rate was 1 ml/min. Fractions were collected directly into scintillation vials on a fraction collector (2112 Redirac fraction collector, LKB, Bromma) at 0.5 min intervals over 30 min, with a total of 60 fractions per run. Scintillation cocktail (4 ml) was subsequently added to each vial and radioactivity was counted with a scintillation counter. Recovery of the amount of radioactivity injected onto the HPLC column (post-HPLC recovery) was 77.8 ± 20.3% (mean ± SD; n = 270; Table 1). Calculations of the relative proportions of compounds (expressed as percentages) that eluted off the HPLC column included these recovery values to correct for losses associated with HPLC.

Chromatography for the confirmation of pregnenolone and progesterone as well as for the identification of conversion products

High pressure liquid chromatography

HPLC was used to partially identify the unknown compounds, compound A (fractions 40 to 45), compound B (fractions 45 to 50), compound C (fractions 6 to 8), compound D (fractions 10 to 12), compound E (fractions 13 to 17), compound F (fractions 12 to 16), compound G (fractions 7 to 9) and compound H (fractions 24 to 27). The same HPLC system described above was used. Progesterone, 5α-dihydroprogesterone, 5β-dihydroprogesterone, 5α-pregnan-3α-ol-20-one, 20α-dihydroprogesterone, 20β-dihydroprogesterone, 17α-hydroxyprogesterone and 11α-hydroxyprogesterone standards (~ 1 mM in ethanol) were prepared. Each standard was injected (80 µl) onto the HPLC system. A UV detector (254 nm, Programmable multiwavelength detector, M490; Waters, Division of Millipore, Milford, USA) was attached to the HPLC system to determine the retention times of the unlabelled standards. The progesterone sample was spiked with [1,2,6,7-^{3}H]progesterone (~ 2 000 dpm) and HPLC fractions of this sample were collected on a fraction collector into scintillation vials. Radioactivity was counted as previously described, to determine the difference in retention times between fraction collection and UV detection for progesterone. An aliquot (80 µl) of an extract of a white blood cell sample and of ovarian tissue incubated with [1,2,6,7-^{3}H]progesterone and β-NADPH, and an aliquot of an extract of a placental tissue sample incubated with [7-^{3}H]pregnenolone and β-NADPH, was injected onto the HPLC system and fractions were collected into scintillation vials. Radioactivity was counted, as described earlier, to determine the retention times of compounds A, B, C, D, E, F, G and H.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was used to confirm the presence of pregnenolone and progesterone in the controls and to identify some of the conversion products. A mass spectrometer coupled to a gas chromatograph (GCQ GC, Finnigan Mat, USA) was used with splitless injection. The GC column (DB-5MS, 30 m fused silica capillary column, inner diameter of 0.25 mm, stationary phase of (5%-Phenyl)Methylpolysiloxane; J & W Scientific, Folsom, CA, USA) was operated at 40 psi helium. A temperature programme set at 240°C for the first 5 min, followed by a 5°C/min increase in temperature from 240°C to 300°C and then 5 min at 300°C was used with an injector temperature of 240°C and a transfer line temperature of 270°C. Electron impact positive ionization was used at a multiplier setting of 1375 volts. The linear velocity of the carrier gas was 40 cm/sec. The GCQ Data System and the search library NIST was used for the identification of the compounds (Finnigan Mat, USA).

Pregnenolone, progesterone, 5α-dihydroprogesterone and 17α-hydroxyprogesterone standards (~ 3.17 mM in ethanol) were prepared and 100 µl of each injected onto the HPLC system. The eluents of fractions corresponding to these standards (pregnenolone, fractions 30 to 36; progesterone, fractions 25 to 32; 5α-dihydroprogesterone, fractions 36 to 42 and 17α-hydroxyprogesterone, fractions 10 to 16) were collected into glass tubes (10 ml) and extracted with 4 ml diethyl ether. These mixtures were vortexed for 10 min, frozen at −70°C for 1 h and the organic phases of fractions relating to a compound were pooled into the same glass tube (12 x 75 mm). The organic phases were evaporated (37°C, N_2). Dichloromethane (1 ml) was added to each dried extract, vortexed for 30 sec and 1 µl was injected onto the GC-MS system. For the identification of the two unknown compounds, compound A and compound B, 1 µl of the extracts of whole blood incubated with progesterone (unlabelled), which were reconstituted in dichloromethane as described earlier (p. 15), were injected onto the GC-MS system.

Liquid chromatography-mass spectrometry (LC-MS)

As an alternative method to confirm the presence of pregnenolone and progesterone in controls, as well as to identify some of the conversion products, LC-MS was used. The HPLC system (Waters, Milford, USA) used consisted of a 2690 pump and autoinjector, a 996 photodiode array detector (PDA) and a reverse phase column (Nova-pak C_{18}, 2 x 150 mm). The system operated at 25°C on an isocratic gradient. The eluent comprised 70% methanol and the flow rate was 0.3 ml/min. The PDA wavelength was set from 220 to 350 nm with a spectral resolution of 1.2 nm. The HPLC system was coupled to a mass spectrometer (Thermabeam™ mass detector, Waters, Milford, USA) with a source temperature of 210°C, a nebulizer temperature of 90°C, an expansion temperature of 80°C and with the scan set from 120 to 320 m/z. The Millennium (version 2.21, Waters, Milford, USA) data system and the search library WILEY (Palisade Corporation, sixth edition, 1996, New York, USA) was used for the identification of the unknown compounds.

Pregnenolone, progesterone, 5α-dihydroprogesterone, 5α-pregnan-3α-ol-20-one and 17α-hydroxyprogesterone standards (~ 3.17 mM in ethanol) were prepared and 100 µl of each injected onto the LC-MS system. To identify compound A and compound B, 100 µl of the extracts of the incubations of whole blood with unlabelled progesterone, which were reconstituted in ethanol as described earlier (p. 15), were injected onto the LC-MS system.

Thin layer chromatography (TLC)

HPLC fractions corresponding to compounds A and B for isolated red and white blood cell extracts, and those corresponding to compound E for luteal extracts were extracted twice by adding 4 ml diethyl ether as described earlier. The organic phases corresponding to each of the compounds were pooled. The dried residues were reconstituted in ethanol (100 µl) and 10 µl was applied on TLC plates (Kieselgel 60 F_{254}, 5 x 20 cm, 0.25 mm thick; Merck, Darmstadt, Germany). Standards (10 µl

each; 1 mM) of 5α-dihydroprogesterone, 5β-dihydroprogesterone, 5α-pregnan-3α-ol-20-one and 17α-hydroxyprogesterone (Sigma, St. Louis, USA) were applied to the plates as references. Standards and compounds A and B were analysed under two different mobile phase conditions, where mobile phase one consisted of chloroform:ethyl acetate (9:1) and mobile phase two of hexane:ethyl acetate (5:2). Standards and compound E were analysed under mobile phase one and mobile phase three which consisted of chloroform:absolute ethanol (9:1).

Standards were first identified by UV detection (Spectroline, TC-312A, Transilluminator 312 nm ultraviolet, New York, USA) and then by spraying the plate with 70% (v/v) H_2SO_4 and drying at 110°C for 5 min. The silica containing the sample steroids was scraped from the plate at 0.5 cm fractions along the direction of the solvent front and the radioactivity of each fraction was counted.

Hormone assays

Progesterone

The radioimmunoassay method used was similar to that described by Van Aarde (1985). Duplicate plasma aliquots of 100 µl and 200 µl were extracted with 4 ml of petroleum ether in glass extraction tubes and vortexed for 10 min. After freezing at −40°C for 1 h, the organic phases were decanted into glass assay tubes and evaporated (37°C, N_2). Duplicate aliquots (100 µl) of ether blanks and quality controls (125 pg, 250 pg and 500 pg progesterone/100 µl PBS) were included in the extraction and assay process.

Triplicate 100 µl and 200 µl aliquots of plasma (in 10 ml glass tubes) were used to determine the recovery of progesterone during extraction. [1,2,6,7-^{3}H]Progesterone (100 µl; ~20 000 dpm/100 µl PBS) was added to each tube, vortexed for 2 sec and then incubated for 10 min in a waterbath at 37°C. These samples were extracted as previously described. The organic phases were decanted into scintillation

vials and evaporated (37°C, N_2). Scintillation cocktail (4 ml) was added and radioactivity was counted with a scintillation counter.

The plasma, ether blank and quality control extracts were reconstituted in 100 µl PBS. A series of standards (7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000 and 2000 pg progesterone/100 µl PBS) and a buffer blank was included in each assay in triplicate. Antiserum (1522; supplied by Prof. R.P. Millar, Department of Chemical Pathology, University of Cape Town, Cape Town, South Africa), raised in a rabbit against progesterone-6-(O-Carboxymethyl)oxime bovine serum albumin, was used at a dilution of ~ 1:5 000 in PBS. Antiserum (100 µl) was added to the reconstituted plasma, ether blanks and quality control extracts, as well as to the standards, and the final dilution of antibody was ~ 1:56 000. The tubes were vortexed for 30 sec on a multi-tube vortexer (Model 2601, Scientific Manufacturing Industries, USA) and left at room temperature for 10 min. 100 µl [1,2,6,7-^{3}H]progesterone (~20 000 dpm/100 µl PBS) was added, the tubes were vortexed for 30 sec and placed at 4°C overnight.

Cold dextran-coated charcoal (750 µl) was added to each tube at 4°C to separate progesterone bound to the antibody from free progesterone. Tubes were vortexed for 20 sec, incubated for 15 min at 4°C and centrifuged (4°C, 2500 rpm, 15 min). Supernatants were decanted into scintillation vials, 4 ml scintillation cocktail was added and radioactivity was counted. A computer package supplied by Packard (SECURIA™ PLUS RIA/QC Software Package and PC-Data Acquisition and Analysis System, United Technologies, Packard, Illinois, USA) was used to calculate hormone concentrations.

To determine parallelism, triplicate aliquots of four volumes of plasma (25 µl, 50 µl and 100 µl each made up to a volume of 200 µl with PBS, and 200 µl plasma) were assayed as described for the plasma samples. For further validation, plasma was stripped of progesterone by adding 2 ml dextran-coated charcoal to 2 ml plasma and vortexing for 20 sec. The mixture was left for 30 min at room temperature and then centrifuged (4°C, 3000 rpm, 30 min). The supernatant (stripped plasma) was

removed and the stripping process repeated. To triplicate aliquots of three volumes (100 µl, 150 µl and 175 µl) of stripped plasma and of PBS, 25 µl, 50 µl and 100 µl aliquots of progesterone (500 ng progesterone/100 µl in PBS) were added, respectively, and were assayed as for the plasma samples. To another set of triplicate aliquots of the same three volumes of stripped plasma, 25 µl, 50 µl and 100 µl PBS were added and assayed in the same way.

Oestradiol-17β

The radioimmunoassay method similar to that described by Van Aarde (1985) was used to determine plasma concentrations of oestradiol-17β. Duplicate plasma aliquots (250 µl and 500 µl) were extracted with 3 ml and 5 ml diethyl ether, respectively, in glass extraction tubes and extracted as described for the progesterone radioimmunoassay. Duplicate aliquots (100 µl each) of ether blanks and quality controls (62.5 pg and 125 pg oestradiol-17β/100 µl PBS) were included in the extraction and assay process.

Triplicate 250 µl and 500 µl aliquots of a plasma sample were pipetted in glass extraction tubes and used to determine the recovery of oestradiol-17β during extraction. [2,4,6,7-^{3}H]Oestradiol-17β (100 µl; ~20 000 dpm/100 µl PBS) was added to each tube and the tubes were processed in the same way as those of the progesterone radioimmunoassay.

Plasma, ether blank and quality control extract residues were reconstituted in 100 µl PBS. A series of standards (7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg oestradiol-17β/100 µl PBS) and a buffer blank was included in each assay in triplicate. Antiserum (E29BI; supplied by Prof. R.P. Millar, Department of Chemical Pathology, University of Cape Town, Cape Town, South Africa) was raised in a rabbit against a conjugate of oestradiol-6-(O-Carboxymethyl)oxime:BSA and 100 µl aliquots were added to the reconstituted extracts and the standards at a working dilution of 1:40 000 in PBS (final antibody dilution was 1:420 000). The mixture in each tube was vortexed for 1 min,

after which 100 µl [2,4,6,7-^{3}H]oestradiol-17β (~20 000 dpm/100 µl PBS) was added to each tube and vortexed for 1 min. This was followed by incubation in a waterbath (37°C) for 1 h, then at room temperature for 15 min and finally at 4°C for 1 h. Dextran-coated charcoal (750 µl) was added to each tube at 4°C to separate oestradiol-17β bound to the antibody from free oestradiol-17β. The tubes were vortexed for 20 sec, incubated for 12 min at 4°C and centrifuged (4°C, 2500 rpm, 15 min). Supernatants were decanted into scintillation vials, radioactivity counted and hormone concentrations determined as described for the progesterone radioimmunoassay.

To determine parallelism to the oestradiol-17β standard curve, 2.8 ml plasma was spiked with 400 µl oestradiol-17β (2000 pg/100 µl) to give a concentration of 250 pg oestradiol-17β/100 µl spiked plasma. Triplicate aliquots of 6.25 µl, 12.5 µl, 25 µl and 50 µl of the spiked plasma were prepared and the first three volumes of spiked plasma were made up to 50 µl with PBS. These four volumes of spiked plasma served to further validate the assay in determining the recovery of a known amount of oestradiol-17β. The four volumes of plasma were assayed as described for the plasma samples.

5α-Dihydroprogesterone

The amplified enzyme-linked immunoassay method described by Hamon, Clarke, Houghton, Fowden, Silver, Rossdale, Ousey & Heap (1991) was used to determine 5α-dihydroprogesterone concentration in plasma samples. A series of 5α-dihydroprogesterone standards (0, 0.5, 1, 2.5, 5, 10, 20 and 30 ng/ml) was prepared in 10% hyrax plasma to account for the effects of substances present in the plasma, since unextracted plasma samples were used in the assay. Duplicate aliquots (50 µl) of plasma were extracted with diethyl ether as described for the oestradiol-17β radioimmunoassay. The wells of an assay plate (Nunc-Immuno Plate, Nunc, Denmark) were coated with 100 µl 11α-

hydroxy-dihydroprogesterone conjugated to bovine serum albumin (0.5 μg/ml; working dilution of 1:2 000 in PBS, pH 7.0 - 7.5) and placed at 4°C overnight.

The plates were washed four times with 0.05% Tween-20, after which 50 μl of standard and plasma extracts were added. The antibody (monoclonal antibody raised in a rat) was used at a dilution of 1:400 in PBS and 50 μl was added to each well. The plates were incubated at 4°C for 1 to 2 h. Thereafter, each well was washed with 100 μl of anti-rat IgG alkaline phosphatase conjugate, used at a dilution of 1:10 000, and the plates incubated for 20 min at 4°C. The plates were washed once more with the conjugate. NADPH was used as a substrate for the alkaline phosphatases and 100 μl was added to each well. After incubation for 10 min at room temperature, 100 μl of a combination of diaphorase and alcohol dehydrogenase was added to each well for amplification. Colour development was terminated after ~ 7 min by adding 100 μl of H_2SO_4 (0.3 M) to each well. Optical density was measured at 490 nm in a V_{max} kinetic microplate reader (Molecular Devices, Novo BioLabs, Cambridge, UK) against an assay buffer blank (software used was from Novoclone, Microplate Data, Version 14-06-008).

Statistical analyses

Blood and tissue incubations

Least squares linear regression was used to determine the relationship between the number of white blood cells and the percentage of [^{3}H]pregnenolone metabolised during incubations with serially diluted white blood cells and the significance of the regression slopes were tested using *t*-tests (Fowler & Cohen 1990). Peaks were detected (see Appendix 3) and then quantified by summing the dpm counts of fractions corresponding to a peak and determining the relative percentage of each peak in a sample. The ranges of metabolism of [^{3}H]pregnenolone and [^{3}H]progesterone during incubations of controls, whole blood, plasma, red and white blood cells, luteal tissue, ovarian residual tissue and placental tissue are

expressed as the mean followed by one standard deviation of the mean (SD). The Kruskal-Wallis *H* and Mann-Whitney *U* tests were used to determine the statistical significance of differences in red and white blood cell counts, as well as in the amount of [^{3}H]pregnenolone metabolised or metabolites formed by white blood cells incubated for 3 hours with [^{3}H]pregnenolone and β-NADPH, between the various reproductive stages. Mean values are followed by one standard error of the mean (SEM). Least squares linear regression analysis was used to determine the relationship between the amount of [^{3}H]pregnenolone metabolised or metabolites formed and incubation time on log-transformed data. The significance of the regression slopes were tested using *t*-tests (Fowler & Cohen 1990). Correlations between the amount of [^{3}H]progesterone metabolised and the amount of compound A and compound B formed, during whole blood, red and white blood cell incubations with [^{3}H]progesterone and β-NADPH, were determined by means of Pearson product-moment correlations (*r*; Sokal & Rohlf 1995). Significance is taken at the 95% level.

Hormone assays

Least squares linear regression and the *t*-test was used to determine parallelism of serial volumes of plasma to the standard curve in the validation of the progesterone and oestradiol-17β radioimmunoassays and the 5α-dihydroprogesterone amplified enzyme-linked immunoassay (Fowler & Cohen 1990). Least squares linear regression was used to determine the relationship between observed and expected hormone concentrations of spiked plasma for the radioimmunoassay validations and the significance of the regression slopes were tested against a slope of 1 using a *t*-test (Fowler & Cohen 1990). The Kruskal-Wallis *H* and Mann-Whitney *U* tests were used to determine differences between the different volumes of plasma assayed for progesterone and for oestradiol-17β, as well as to determine differences in progesterone, 5α-dihydroprogesterone and oestradiol-17β concentrations between reproductive stages. The correlation between progesterone and 5α-

dihydroprogesterone circulating concentrations was determined by the Pearson product-moment correlation (*r*; Sokal & Rohlf 1995). All mean values are followed by one SEM and significance is taken at 95%.

Validations of incubations

The metabolism of [^{3}H]pregnenolone and [^{3}H]progesterone during control incubations

Control incubations with [^{3}H]pregnenolone, and both [^{3}H]pregnenolone and [^{3}H]progesterone, resulted in no metabolism of pregnenolone or progesterone. Control incubations with [^{3}H]progesterone resulted in 0 to 10% metabolism of progesterone and subsequent calculations of the relative proportions of compounds formed from progesterone were corrected for this.

The metabolism of pregnenolone during white blood cell serial dilution incubations

The amount of [^{3}H]pregnenolone metabolised by the white blood cells of two non-pregnant and an early-pregnant hyrax was not affected significantly (t_1 = 10.80, 4.81 and 4.73 respectively, $p > 0.05$, $n = 3$; Fig. 2) by the number of white blood cells[1]. This may be a result of insufficiently serially diluting the white blood cell stock.

Validations of chromatography

The pregnenolone, progesterone, 5α-dihydroprogesterone and 17α-hydroxyprogesterone standards which were injected onto the GC-MS system, and these standards (including 5α-pregnan-3α-ol-20-one) injected onto the LC-MS system, eluted separately from each other and were positively identified. The retention times for these standards are presented in Table 2.

[1] The regression lines were not used for extrapolation purposes, but for descriptive purposes only. For extrapolation, the curve described by the equation $y = ax^b$ would be more suitable.

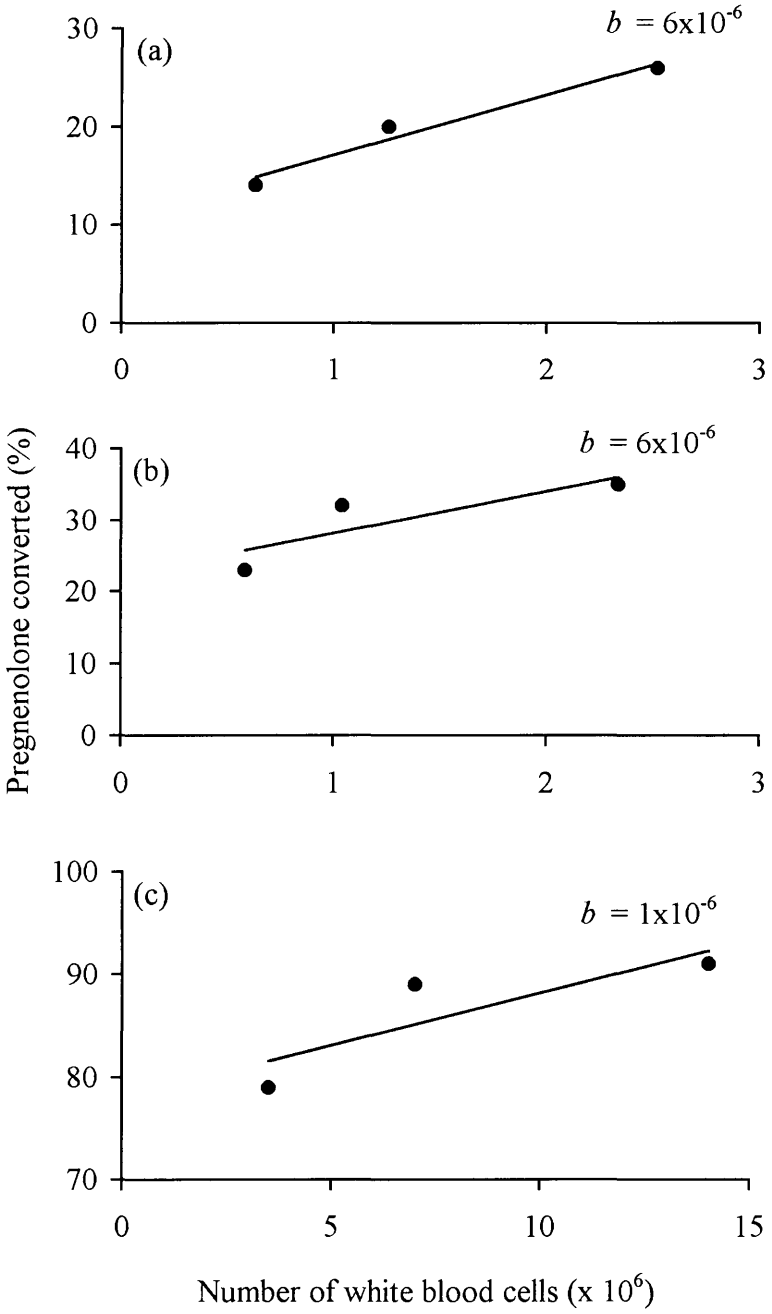


Fig. 2. The relationship between the number of white blood cells and the amount of [^{3}H]pregnenolone metabolised by (a and b) two non-pregnant hyraxes and (c) an early-pregnant hyrax. Regression lines were fitted using least squares linear regression, with *b* representing the slope of the lines.

Table 2. Retention times for pregnenolone, progesterone, 5α-dihydroprogesterone and 17α-hydroxyprogesterone standards injected onto the GC-MS and LC-MS systems and for 5α-pregnan-3α-ol-20-one injected onto the LC-MS system.

	Retention time (min)	
Compound	GC-MS	LC-MS
Pregnenolone	14.06	9.607
Progesterone	15.01	6.776
5α-Dihydroprogesterone	14.28	11.103
5α-Pregnan-3α-ol-20-one	-	13.454
17α-Hydroxyprogesterone	16.07	3.542

Validations of hormone assays

Progesterone

The cross-reactivity of the antibody for progesterone (determined by the supplier, Prof. R.P. Millar, Department of Chemical Pathology, University of Cape Town, South Africa) was as follows: progesterone 100%; 11β-hydroxyprogesterone 48.3%; 11α-hydroxyprogesterone 26.2%; 5-pregnane-3,20-dione 25.1%; pregnenolone 5.2%; 17β-hydroxyprogesterone 2.6%; 11-deoxycorticosterone 1.9%; 11-deoxycortisol 1.7%; 3-hydroxy-5-pregnane-20-one 0.4%; 20-hydroxy-4-pregnane-3-one 0.3%; cortisol < 0.1%; testosterone, Δ-4-androstenedione, oestradiol-17β and oestrone < 0.001%.

Sensitivity of the assays ranged from 0.009 to 0.039 ng progesterone/ml (n = 3). Buffer blanks contained 0.009 to 0.039 ng progesterone/ml (n = 3). Non-specific binding was 5.3% (n = 3) and specific binding of the antiserum was 34.1% (n = 3).

The intra-assay coefficients of variation were 3.2%, 3.7% and 5.3% (n = 3) for 1.25 ng, 2.50 ng and 5.00 ng progesterone/ml respectively. The inter-assay coefficients of variation were 16.4%, 25.7% and 21.4% (n = 3) for 1.25 ng, 2.50 ng and 5.00 ng progesterone/ml, respectively. Recovery estimates for the 100 µl and 200 µl plasma samples were 85% and 81%, respectively. The progesterone concentrations in the 100 µl plasma aliquots were similar to those in the 200 µl aliquots (H_1 = 0.16, p > 0.05, n = 18).

Serially diluted samples of pooled plasma from non-pregnant and pregnant females produced a displacement curve parallel to the standard curve (t_5 = 1.55, p > 0.05; Fig. 3a). Spiking plasma with 0.125 ng, 0.250 ng and 0.500 ng progesterone resulted in 116.8% (0.146 ± 0.017 ng; mean ± SEM), 122.8% (0.307 ± 0.026 ng) and 110.6% (0.553 ± 0.540 ng) recovery, respectively. The amounts recovered did not differ significantly to the amounts added (t_2 = 2.01, b = 1.07, p > 0.05, n = 3; Fig. 3b).

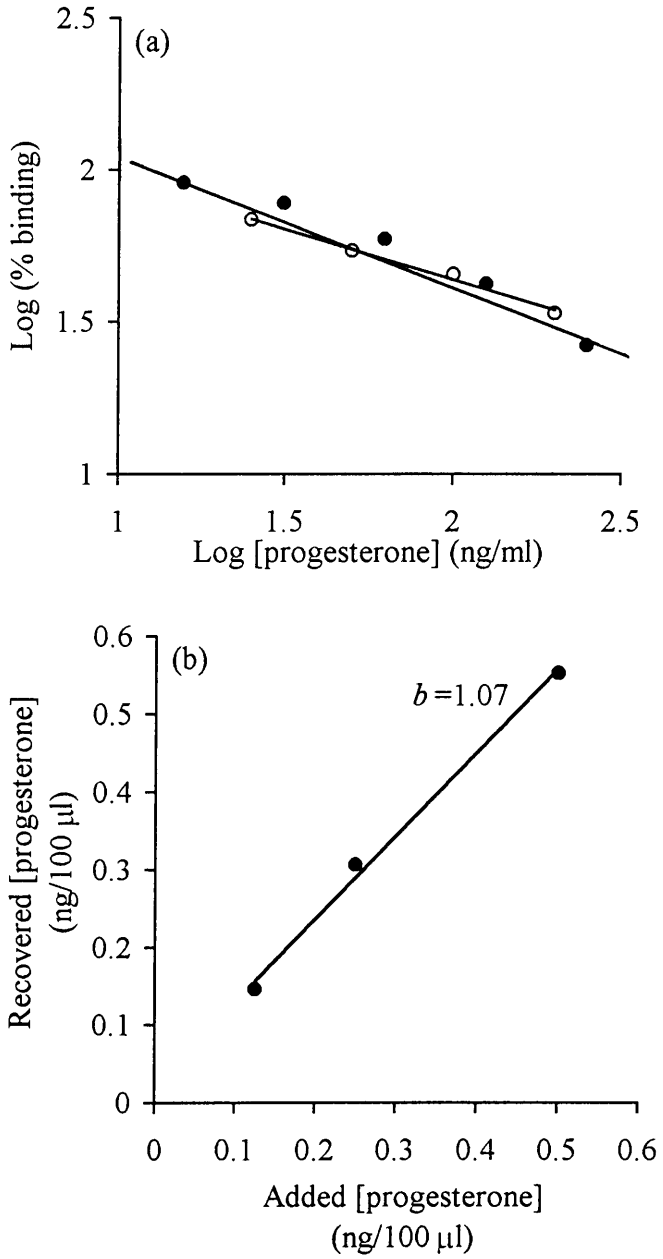


Fig. 3. (a) The displacement curve of progesterone concentrations of serially diluted hyrax plasma (o , b = -0.3) exhibiting parallelism ($t_5 = 1.55, p > 0.05$) to the progesterone standards (● , b = -0.3). (b) The relationship between added and recovered progesterone concentrations of spiked plasma. The lines were fitted using least squares linear regression and the slope of each line is represented by b.

Oestradiol-17β

The cross-reactivity of the antibody against oestradiol-17β (as determined by the supplier, Prof. R.P. Millar, Department of Chemical Pathology, University of Cape Town, South Africa) was as follows: oestradiol-17β 100%; oestrone 0.01%; cortisol 0.005%; deoxycorticosterone 0.002%; pregnanediol and corticosterone 0.001%; 17α-hydroxypregnenolone, androstenedione, progesterone and testosterone < 0.001%

Sensitivity of the assays ranged from 18.4 to 24.9 pg oestradiol-17β/ml (n = 3). Buffer blanks ranged from 18.2 to 24.5 pg oestradiol-17β/ml (n = 3). Non-specific binding was 4.3% (n = 3) and specific binding of the antiserum was 34.5% (n = 3).

Recovery estimates for the 250 µl and 500 µl plasma samples were 88% and 87%, respectively. The oestradiol-17β concentrations in the 250 µl and 500 µl plasma aliquots were similar to each other (H_1 = 0.96, p > 0.05, n = 24). The intra-assay coefficients of variation were 1.3% and 3.1% (n = 2) for 0.625 ng and 1.250 ng oestradiol-17β/ml, respectively. The inter-assay coefficient of variation was 8.8% and 9.1% (n = 2) for 0.625 ng and 1.250 ng oestradiol-17β/ml, respectively.

Serially diluted samples of pooled plasma from non-pregnant and pregnant females displayed a curve parallel to the standard curve (t_5 = 0.52, p > 0.05; Fig. 4a). Plasma spiked with 15.625 pg, 31.25 pg, 62.50 pg and 125 pg oestradiol-17β resulted in 139.6% (22.10 ± 6.29 pg; mean ± SEM), 112.0% (35.29 ± 7.08 pg), 122.9% (77.12 ± 8.72 pg) and 109.9% (137.86 ± 12.70 pg) recovery, respectively. The amounts recovered were not significantly different to the amounts added (t_4 = 0.02, b = 1.00, p > 0.05, n = 4; Fig. 4b).

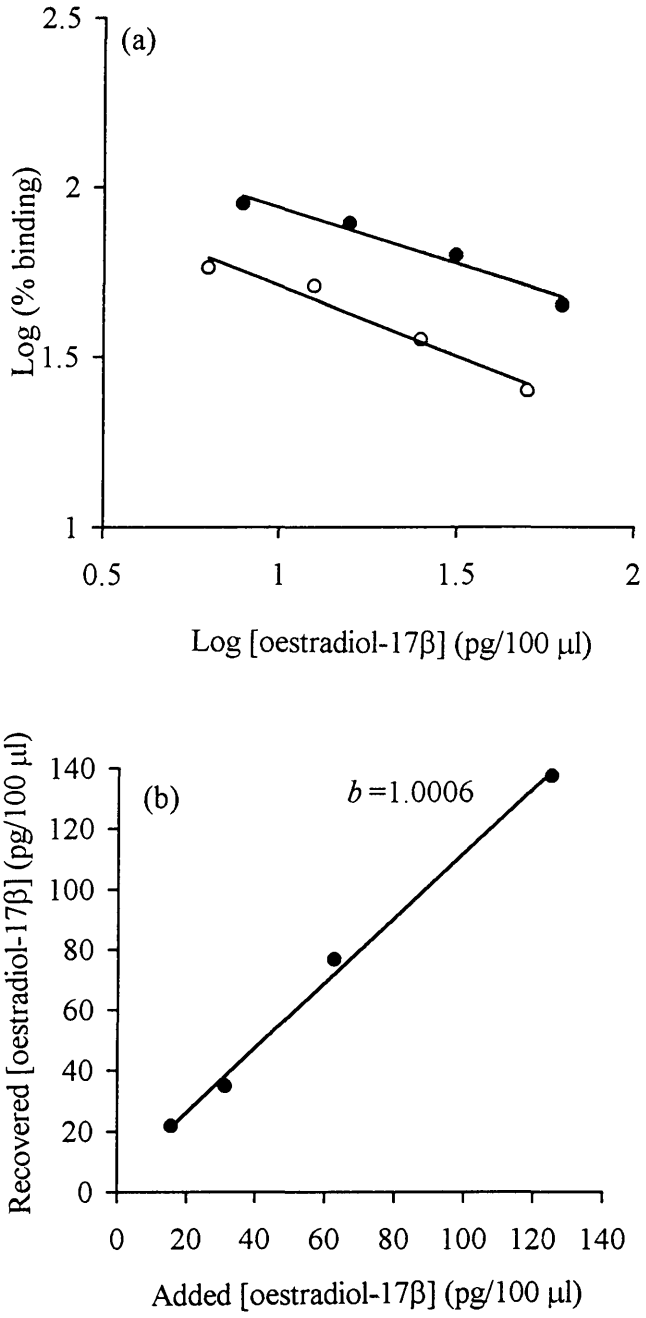


Fig. 4. (a) The displacement curve of oestradiol-17βconcentrations of serially diluted hyrax plasma (o , b = -0.4) exhibiting parallelism ($t_5 = 0.52, p > 0.05$) to the oestradiol-17βstandards (● , b = -0.4). (b) The relationship between added and recovered oestradiol-17β concentrations of spiked plasma. The regression lines were fitted using least squares linear regression with b representing the slope of each line.

5α-Dihydroprogesterone

The cross-reactivity of the antibody (as described by Hamon *et al.* (1991)) was as follows: 5α-dihydroprogesterone 100%; progesterone 56.0%; 5α-pregnane-3β-hydroxy-20-one 21.0%; 5β-dihydroprogesterone 17.0%; pregnenolone 5.0%; 20α-dihydroprogesterone 1.2%; 20β-dihydroprogesterone, 5α-pregnane-20α-hydroxy-3-one, 5α-pregnane-3β,20α-diol, Δ^5-pregnene-3β,20α-diol, Δ^5-pregnene-3β,20β-diol, equilin and equilenin < 0.01%.

Serially diluted samples of pooled plasma from non-pregnant and pregnant females displayed a curve parallel to the standard curve (t_7 = 1.09, $p > 0.05$; Fig. 5). Spiking plasma with 5 ng 5α-dihydroprogesterone resulted in 76 ± 4% (mean ± SEM, n = 6) recovery. The intra- and inter-assay coefficients of variation were 3.5% (n = 4) and 13.0% (n = 3), respectively. The limit of detection was 0.09 ng/ml.

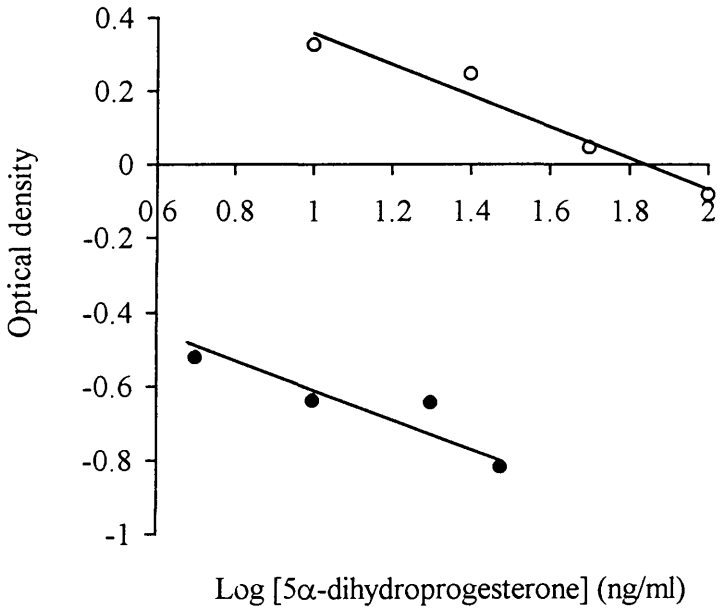


Fig. 5. The displacement curve of 5α-dihydroprogesterone concentrations of serially diluted hyrax plasma (○, b = -0.4) exhibiting parallelism (t_7 = 1.09, p > 0.05) to the 5α-dihydroprogesterone standards (●, b = -0.4). Both lines were fitted using least squares linear regression.

CHAPTER 3

CIRCULATING PLASMA CONCENTRATIONS OF PROGESTERONE, 5α-DIHYDROPROGESTERONE AND OESTRADIOL-17β

Introduction

Progesterone is the hormone that maintains pregnancy in most mammals. Plasma progesterone concentrations in pregnant hyraxes are low, falling within the range of < 0.1 to 4.6 ng/ml (Heap *et al.* 1975; Gombe *et al.* 1976; Van Aarde & Anderson 1989). Circulating progesterone concentrations in pregnant African elephant females (phylogenetically related to the hyrax) are also low (Ford, Van Aarde & Allen 1997; Hodges *et al.* 1997) and corpus luteum function seems to be reflected by 5α-dihydroprogesterone and 5α-pregnan-3α-ol-20-one which circulate at concentrations 20- and 13-fold higher, respectively, than those of progesterone (Hodges *et al.* 1997). The concentration of 5α-dihydroprogesterone in the circulation of the hyrax is unknown. Therefore, the possibility exists that a progestin other than progesterone may be circulating at higher concentrations than progesterone in the hyrax and may be responsible for the maintenance of pregnancy.

Ford *et al.* (1997) have suggested that 5α-dihydroprogesterone may be biologically important during gestation of the African elephant. This is supported by Greyling *et al.* (1997) who showed that 5α-dihydroprogesterone had a relatively high binding affinity for the uterine progesterone receptor, although it did not have a stronger binding affinity than progesterone. 5α-Dihydroprogesterone also appears to be responsible for the down-regulation of the uterine receptors as pregnancy progresses (Greyling *et al.* 1998). In the mare, 5α-dihydroprogesterone concentrations appear to be related to the stage of pregnancy (Seamans *et al.* 1979; Chavatte & Tait 1994) and may be necessary for the maintenance of pregnancy (Schutzer & Holtan 1996). 5α-Dihydroprogesterone may be an important circulating progestin in the pregnant hyrax.

In most mammals, oestrogen secretion increases as pregnancy progresses (Bedford, Challis, Harrison & Heap 1972; Anderson, Webb & Turnbull 1981), and oestradiol and oestrone concentrations increase as the placenta increases in size and weight (Cheesman 1982). Oestradiol concentrations in the plasma of the hyrax are unknown, and if oestradiol is produced by the placenta, concentrations should increase as pregnancy progresses. Oestrogens induce the synthesis of progesterone receptors in uterine tissue, thereby increasing the uptake of progesterone by the uterus (Heap & Flint 1979; Jensen 1979). Oestradiol is also required to maintain progesterone secretion by the corpus luteum of the rabbit (Gibori & Miller 1982) and therefore may be an important part of the mechanism which maintains pregnancy in the hyrax. However, abortion in sheep was associated with an increase in oestradiol-17β concentrations (Taylor 1987) and oestrogens stimulate myometrial activity in late pregnancy (Heap & Illingworth 1977) and thus oestradiol may rather be important for the termination of pregnancy in the hyrax.

Results

Plasma progesterone concentrations

Plasma progesterone concentrations in non-pregnant females ($n = 3$) ranged from 0.59 to 0.89 ng/ml with a mean ± SEM of 0.78 ± 0.10 ng/ml. Concentrations in pregnant females ($n = 9$) ranged from 0.42 to 5.91 ng/ml with a mean ± SEM of 1.60 ± 0.55 ng/ml. Mid-pregnant animals had significantly higher concentrations than those of non- and late-pregnant animals ($Z\ (U = 0) = -2.12, p < 0.05$) and concentrations in non- and late-pregnant animals were similar (Table 3). Progesterone concentrations in early-pregnant animals did not differ significantly from those in non-, mid- and late-pregnant animals ($p > 0.05$). Except for one early-pregnant animal which had a high plasma progesterone concentration, there was no convincing change in concentrations with an increase in foetal age (Fig. 6a).

Table 3. Plasma concentrations (mean ± SEM for the number of individual animals in parentheses) of progesterone, 5α-dihydroprogesterone and oestradiol-17β for each reproductive stage.

Reproductive status	Progesterone (ng/ml)	5α-Dihydroprogesterone (ng/ml)	Oestradiol-17β (pg/ml)
Non-pregnant	0.78 ± 0.10 (3)	6.32 (1)	30.99 and 33.98 (2)
Early-pregnant	1.69 and 5.91 (2)	1.41 (1)	56.23 (1)
Mid-pregnant	1.15 ± 0.07 (4)*	7.82 ± 0.70 (4)	34.20 ± 4.04 (3)
Late-pregnant	0.75 ± 0.16 (3)	6.81 ± 1.77 (3)	101.46 ± 18.70 (3)

*$p < 0.05$ for mid-pregnant versus both non-pregnant and late-pregnant animals

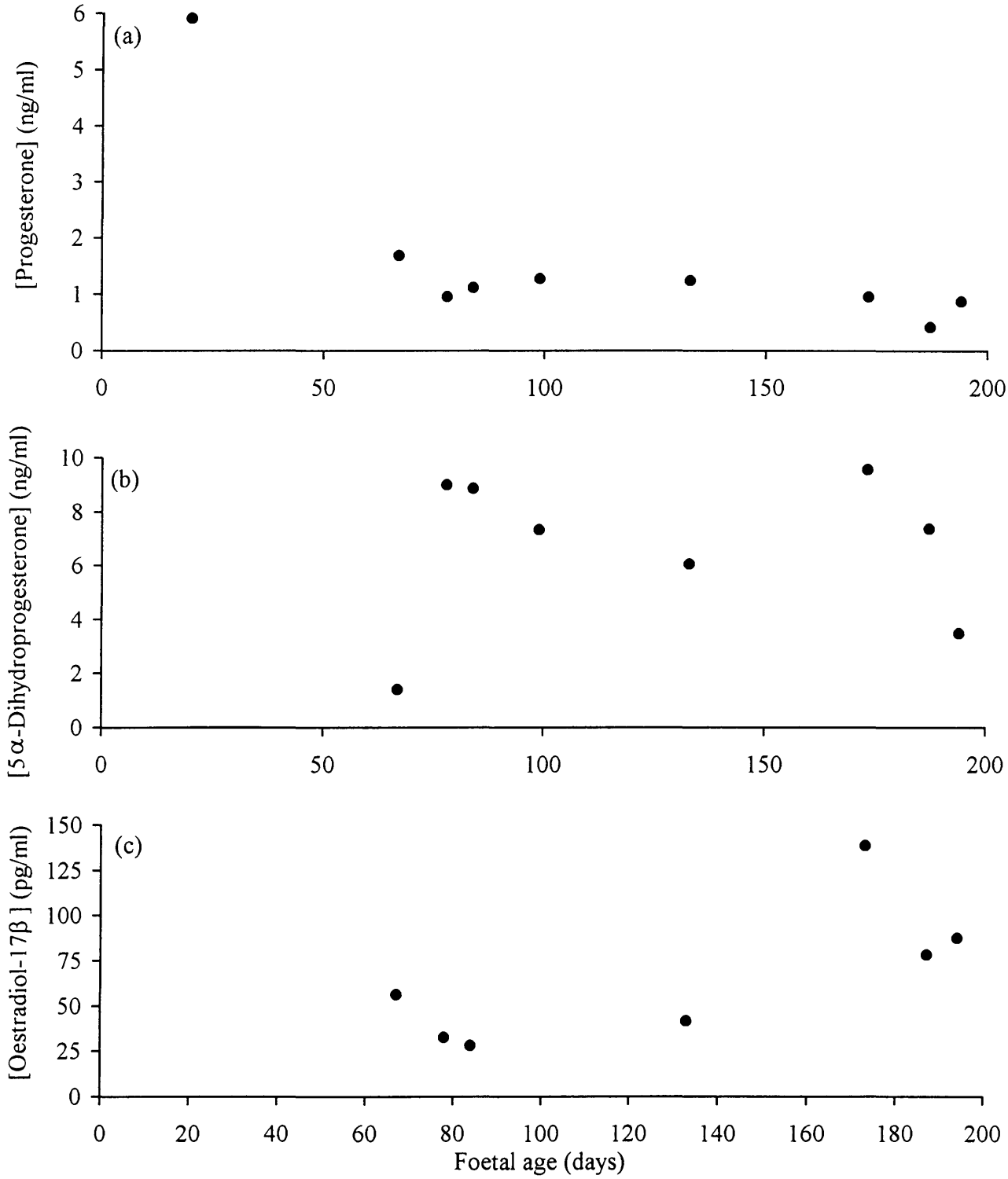


Fig. 6. The relationship between foetal age and circulating concentrations of (a) progesterone, (b) 5α-dihydroprogesterone and (c) oestradiol-17β in pregnant hyraxes.

Plasma 5α-dihydroprogesterone concentrations

The plasma concentration of 5α-dihydroprogesterone in one non-pregnant animal was 6.32 ng/ml and in pregnant animals (n = 8) ranged from 1.41 to 9.57 ng/ml, with a mean ± SEM of 6.64 ± 1.02 ng/ml. Non-, early-, mid- and late-pregnant concentrations were not significantly different to each other ($H_3 = 2.74$, $p > 0.05$, $n = 9$; Table 3). 5α-Dihydroprogesterone concentrations exhibited no trend with foetal age (Fig. 6b).

Plasma concentrations of 5α-dihydroprogesterone exceeded those of progesterone by 4 to 17 times, with a mean ± SEM ratio (5α-dihydroprogesterone:progesterone) of 7.9 ± 1.6 ($n = 9$), except for one early-pregnant animal, where 5α-dihydroprogesterone and progesterone concentrations were similar. Plasma concentrations of progesterone were not correlated to those of 5α-dihydroprogesterone ($r = -0.48$, $p > 0.05$, $n = 8$).

Individual corpus luteum weights displayed no noticeable trend with foetal age (Fig. 7a). However, it appeared that individual weights were higher during the second half of pregnancy. Total corpus luteum weight tended to increase with foetal age (Fig. 7b) and there was no indication of corpus luteum regression in weight towards the end of pregnancy. Plasma concentrations of progesterone during pregnancy exhibited no trend with total corpus luteum weight whereas those of 5α-dihydroprogesterone tended to decrease as total corpus luteum weight increased (Fig. 7c).

Plasma oestradiol-17β concentrations

Plasma concentrations of oestradiol-17β in non-pregnant animals (n = 2) ranged from 30.99 to 33.98 pg/ml, with a mean ± SEM of 32.49 ± 1.49 pg/ml, and that of one other non-pregnant animal was below the sensitivity of the assay. Concentrations in pregnant animals ($n = 7$) ranged from 28.22 to 138.49 pg/ml with a mean ± SEM of 66.17 ± 14.72 pg/ml. The plasma concentrations of oestradiol-17β for an early- and a mid-pregnant animal were below the sensitivity of the assay.

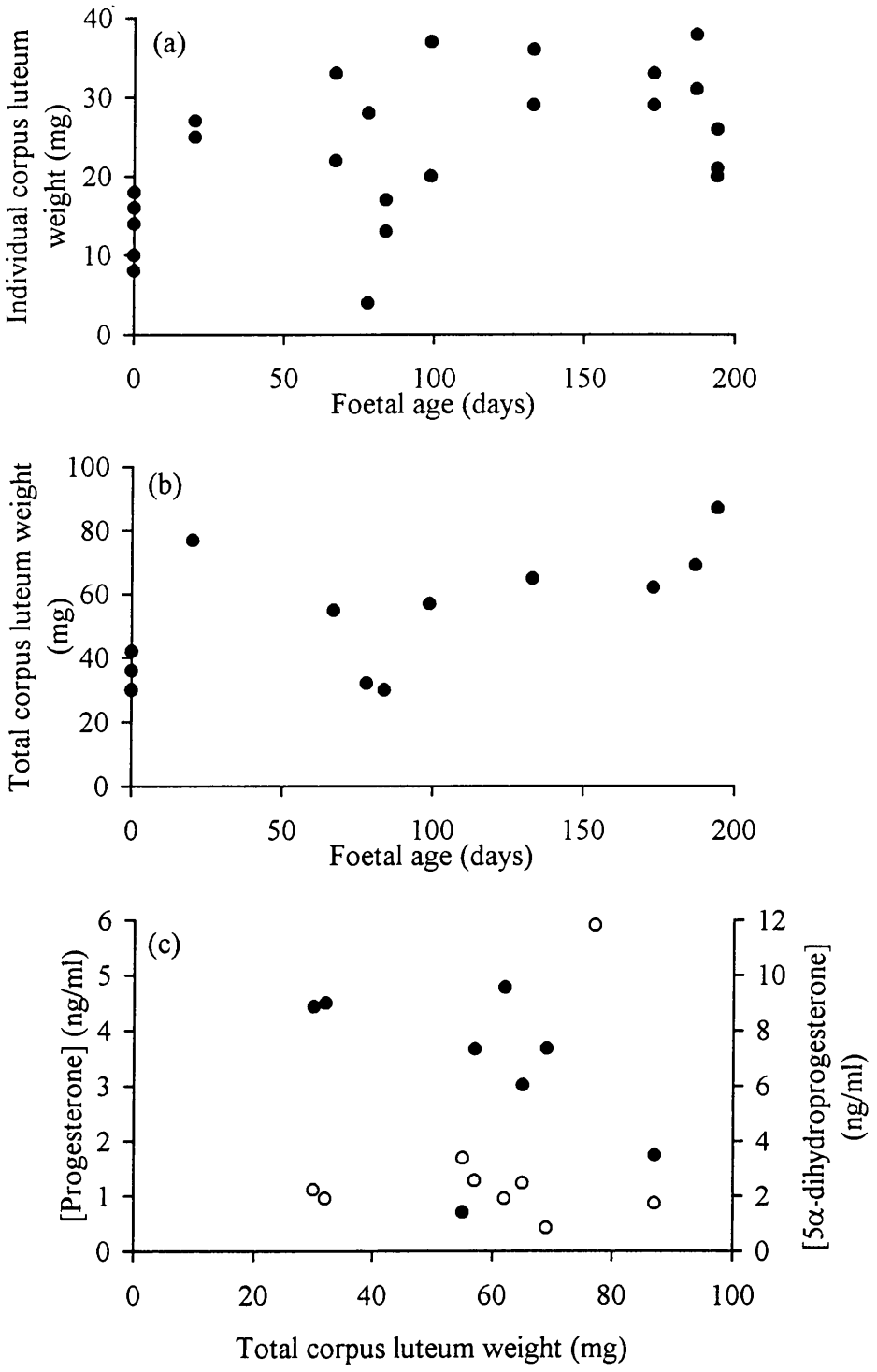


Fig. 7. The relationship between (a) individual corpus luteum weight and foetal age and (b) total corpus luteum weight and foetal age. (c) The relationship between total corpus luteum weight and circulating concentrations of progesterone (○) and 5α-dihydroprogesterone (●).

Oestradiol-17β concentrations did not differ between reproductive stages (H_3 = 6.40, p > 0.05, n = 9; Table 3), but concentrations peaked in late pregnancy (Fig. 6c). Total placental weight displayed an exponential relationship to foetal age (Fig. 8a) and oestradiol-17β plasma concentrations tended to increase with total placental weight (Fig. 8b). There was no relationship between either progesterone or 5α-dihydroprogesterone concentrations and total placental weight (Figs. 8c and d).

Discussion

Circulating 5α-dihydroprogesterone concentrations were up to 17-fold higher than those of progesterone and ranged from 1.41 to 9.57 ng/ml. Circulating progesterone concentrations ranged from 0.42 to 5.91 ng/ml, which corresponds to the findings of previous studies (Gombe *et al.* 1976; Van Aarde & Anderson 1989). Circulating progesterone concentrations in the female hyrax are lower than in most mammals. This may be due to the presence of progesterone metabolising enzymes in hyrax blood (Heap *et al.* 1975; Makawiti *et al.* 1991) and the possibility exists that a metabolite of progesterone supports pregnancy (Heap *et al.* 1975). On the other hand, a sensitive progesterone receptor mechanism in target tissues may be present (Heap *et al.* 1975) and therefore such low concentrations of progesterone are sufficient to maintain pregnancy.

The relatively high concentrations of circulating 5α-dihydroprogesterone suggest that this progestin may be of biological importance during pregnancy in the rock hyrax. However, circulating 5α-dihydroprogesterone concentrations did not appear to correlate with reproductive status or foetal age, which may be a result of individual differences in the production of this steroid. Hodges *et al.* (1997) provide evidence that 5α-dihydroprogesterone plasma concentrations in the African elephant reflect corpus luteum function. However, corpus luteum weight tended to be inversely related to 5α-dihydroprogesterone plasma concentrations suggesting that 5α-dihydroprogesterone circulating concentrations may be affected by a factor other than luteal activity.

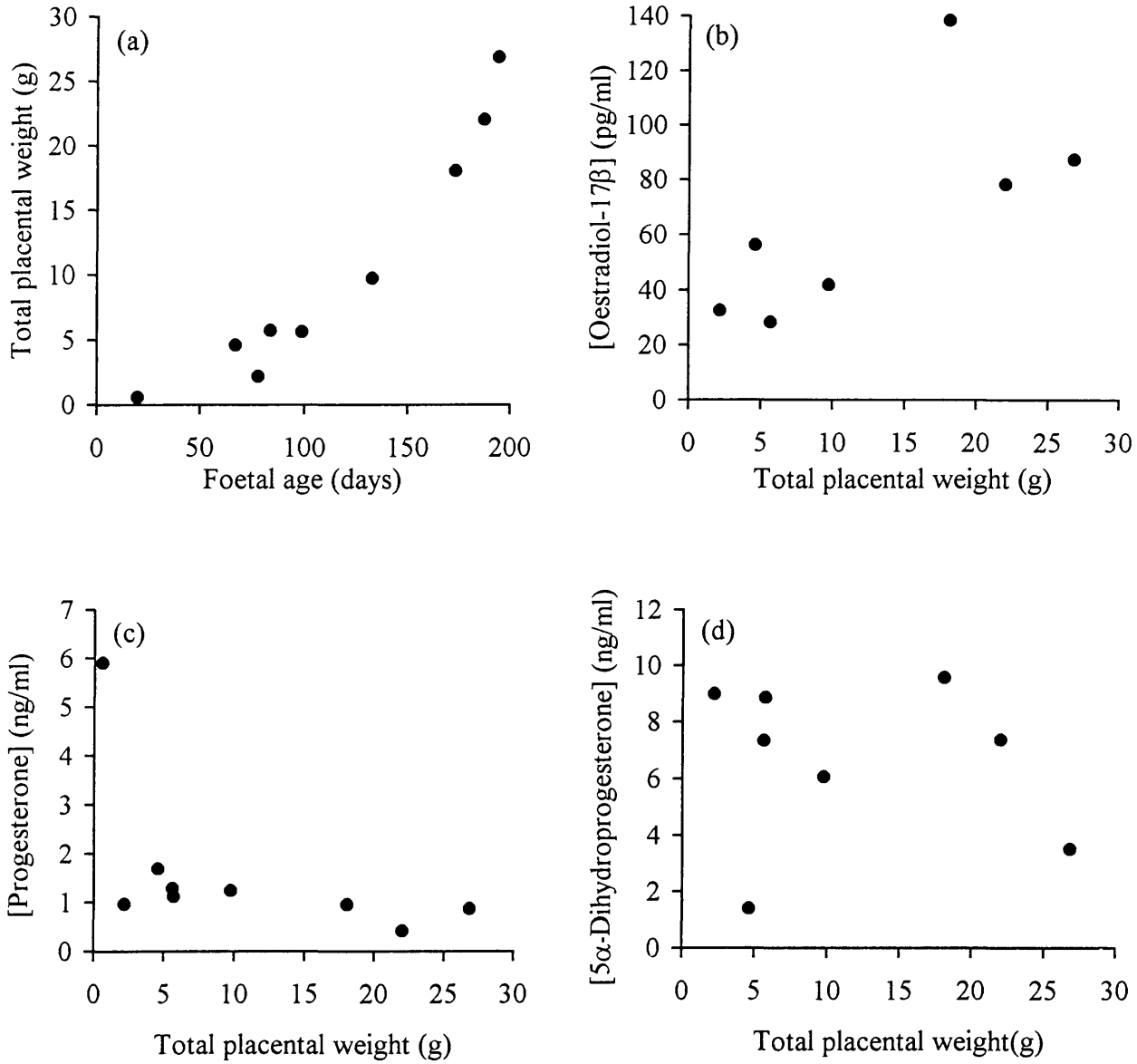


Fig. 8. (a) The relationship between total placental weight and foetal age. The relationship between total placental weight and circulating concentrations of (b) oestradiol-17β , (c) progesterone and (d) 5α-dihydroprogesterone.

5α-Dihydroprogesterone can compete with progesterone for binding to uterine progesterone receptors (Heap & Flint 1979). Greyling *et al.* (1997) reported that 5α-dihydroprogesterone has a high relative binding affinity for the progesterone receptor and also suggested (Greyling *et al.* 1998) that 5α-dihydroprogesterone down-regulates progesterone receptors in the uterine tissue of the African elephant. 5α-Dihydroprogesterone may have similar functions in the rock hyrax. Similar investigations on uterine tissue of the hyrax should be carried out so as to determine the biological significance of 5α-dihydroprogesterone in this species.

In contrast to the high circulating 5α-dihydroprogesterone concentrations, those of progesterone were lower but also showed no trend with an increase in foetal age. In some species circulating progesterone levels decrease prior to parturition (Short 1958, 1959; Tam 1973; Atkins, Harms, Sorensen & Fleeger 1976; Van der Merwe & Van Aarde 1989) whereas in other species a decrease in progesterone concentrations does not occur before the onset of parturition (Short & Moore 1959; Challis, Heap & Illingworth 1971; Lemon 1972; Milewich, Gomez-Sanchez, Madden & MacDonald 1975; Seamans *et al.* 1979; Buchanan & Younglai 1986). During studies on sheep it has also been shown that a fall in circulating progesterone concentrations before the onset of parturition may not be a prerequisite for parturition (Liggins, Grieves, Kendall & Knox 1972). Short (1959) proposed that, in the mare, progesterone was undetectable in plasma during the second half of gestation due to the local action of placental progesterone on the uterus without entering the maternal circulation. However, this does not explain the low levels of plasma progesterone in the hyrax since the placenta does not contain significant amounts of progesterone (Heap *et al.* 1975). The regulation of parturition is complex and varies in different species (Bedford *et al.* 1972) and a number of factors may be involved in the onset of parturition in the hyrax.

Plasma concentrations of progesterone and 5α-dihydroprogesterone did not correlate with each other. However, the concentration of a hormone in blood depends on its biological half-life and

distribution within the body (Heap & Illingworth 1977), and changes in reductase levels and relative metabolic clearance rates of progesterone and 5α-dihydroprogesterone may influence the plasma concentrations of these two steroids (Johnson & Everitt 1988). Therefore, a correlation between progesterone and 5α-dihydroprogesterone may not be observed. Also, progesterone is metabolized to a number of metabolites by the blood of the rock hyrax, including 5α-dihydroprogesterone (Heap *et al.* 1975). If 5α-dihydroprogesterone metabolism also occurs in the blood, then a relationship between the circulating concentrations of progesterone and 5α-dihydroprogesterone may never be observed, even though 5α-dihydroprogesterone may be a metabolite of progesterone.

Oestradiol-17β plasma concentrations in pregnant animals were low (28.22 to 138.49 pg/ml) and peaked in late-pregnant animals. These results are in agreement with measurements of unconjugated oestrogens in the plasma of the hyrax (Heap *et al.* 1975). Concentrations during non-, early- and mid-pregnancy were similar to each other, and were up to threefold higher during late pregnancy, suggesting that oestradiol-17β concentrations may be biologically important during late pregnancy. The formation of oestrogens in some species is of placental origin (Amoroso & Perry 1977; Anderson *et al.* 1981), and in sheep progesterone is metabolised to oestrogens through the activation of placental steroidogenic enzymes before labour (Anderson *et al.* 1981). A surge in foetal cortisol may also affect placental steroid metabolism by decreasing progesterone secretion and increasing oestrogen production (Anderson *et al.* 1981). The increase in oestradiol-17β plasma concentrations in the hyrax during late pregnancy may be a result of its synthesis by the placental tissue, the weight of which increases with pregnancy. A rise in biologically active oestrogens prior to parturition could trigger parturition by either converting the uterine environment from one dominated by progesterone to one dominated by oestrogens, thereby overcoming the progesterone block, or by directly stimulating the contractility of the myometrium (Bedford *et al.* 1972). Moreover, this oestrogen surge may be important in increasing the sensitivity of the uterus to

oxytocin and in stimulating the placenta to synthesise and release prostaglandin $F_{2\alpha}$, which may enhance uterine activity (Thorburn, Nicol, Bassett, Schutt & Cox 1972). Therefore, oestradiol-17β may be partly responsible for the onset of parturition.

Conclusion

5α-Dihydroprogesterone circulated at concentrations higher than those of progesterone and may be the hormone responsible for the maintenance of pregnancy in the rock hyrax, even though concentrations were not significantly different between reproductive stages. Progesterone plasma concentrations were up to 17-fold lower than those of 5α-dihydroprogesterone and did not change with an increase in foetal age. The metabolism of progesterone by hyrax blood may explain the low concentrations of progesterone in plasma and leaves open the possibility that a metabolite of progesterone may have a significant role during pregnancy. Oestradiol-17β concentrations peaked in late-pregnant hyraxes suggesting that oestradiol-17β may be biologically important during late pregnancy.

CHAPTER 4

THE METABOLISM OF PREGNENOLONE AND PROGESTERONE BY THE BLOOD OF FEMALE ROCK HYRAXES

Introduction

The metabolism of progesterone to other progestins by whole blood and red blood cells has been recorded in a number of species (Nancarrow & Seamark 1967, 1968; Van der Molen & Groen 1968; Heap *et al.* 1975). However, the endocrinological significance of these metabolic conversions in blood *in vivo* could not be established. Progesterone is metabolised to a variety of metabolites, including 5α-dihydroprogesterone and 5β-dihydroprogesterone, by the blood of the rock hyrax and this has been ascribed to erythrocyte metabolism (Heap *et al.* 1975; Makawiti *et al.* 1991). However, in the human, mononuclear and polymorphonuclear leukocytes metabolize progesterone to 20α-dihydroprogesterone, 5α-dihydroprogesterone and a pregnanolone-like metabolite (Scully *et al.* 1982). The functional significance of these metabolic conversions is not clear. Scully *et al.* (1982) suggested that the white blood cells may not only metabolize progesterone but can also be targets for progesterone so that their immunocompetency is compromised.

Progesterone concentrations are relatively high in corpora lutea of the rock hyrax but low in plasma (Heap *et al.* 1975; see Chapters 1 and 3). It has been suggested that the maintenance of pregnancy in this species is facilitated by a receptor mechanism in the target tissues which is sensitive to lower concentrations of progesterone (Heap *et al.* 1975). However, in this species, erythrocytes are able to metabolize progesterone *in vitro* (Makawiti *et al.* 1991). It is therefore possible that a metabolite associated with this metabolic conversion may support pregnancy (Heap *et al.* 1975), since target tissues are also capable of selectively removing progesterone metabolites from the blood (Wichmann 1967).

Alternatively, metabolism by the blood may very well contribute to the protection of maternal and foetal tissues from adverse effects of high steroid hormone concentrations (Heap *et al.* 1975).

The present study aims at quantifying the steroidogenic potential of hyrax blood by investigating the potential of whole blood, plasma, the isolated red and white blood cells and the mixture of red and white blood cells to metabolise pregnenolone and progesterone to metabolites *in vitro*. The effect of reproductive status on the metabolism of pregnenolone and progesterone will be determined. The influence of time, and of the presence of a co-factor (β-NADPH) and of enzyme inhibitors (oestrone as well as NaF-HgCl), is also investigated.

Results

The metabolism of [^{3}H]pregnenolone by the various blood components during incubations

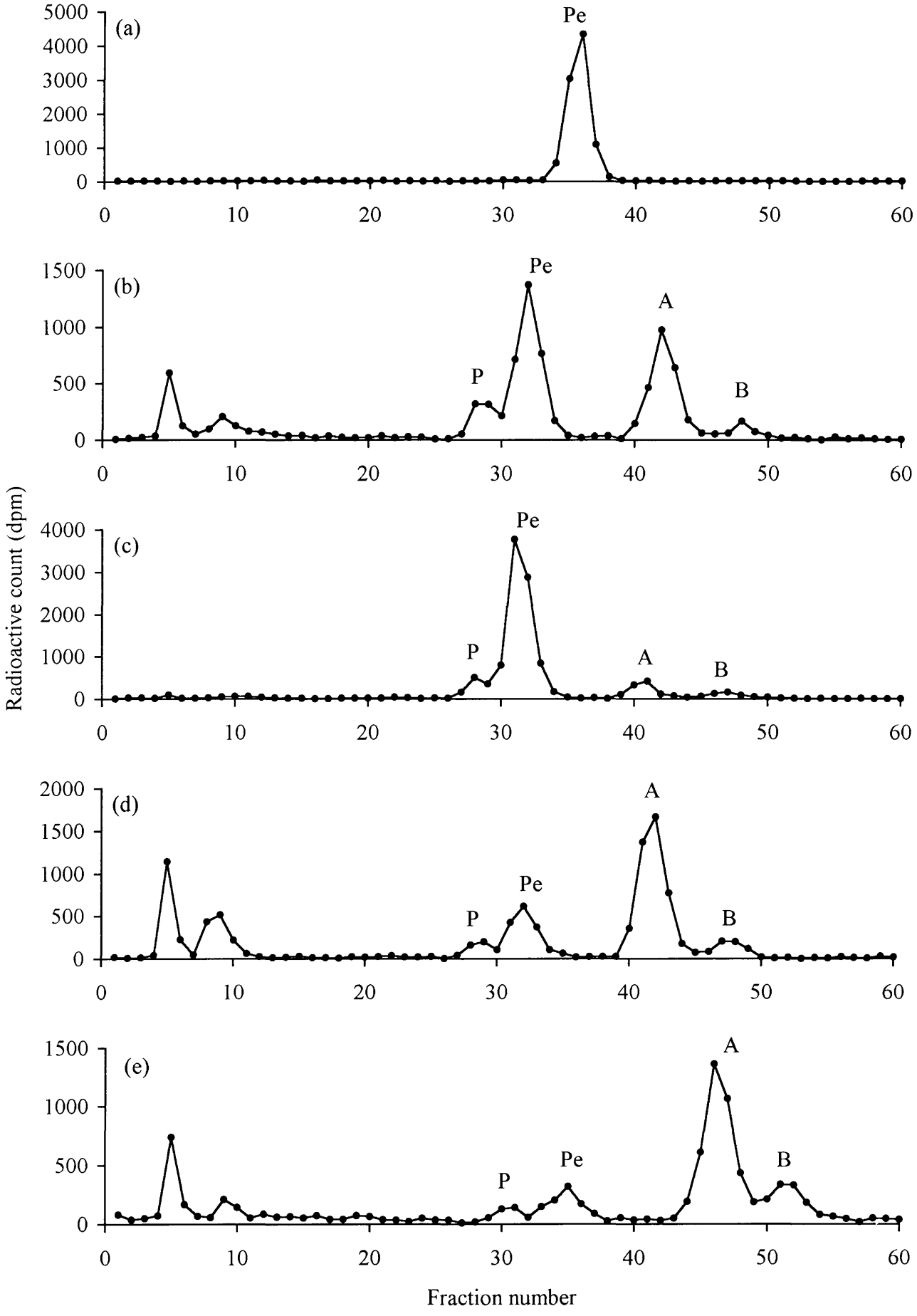
Incubations of whole blood, plasma, the isolated red blood cells and the mixture of red and white blood cells with [^{3}H]pregnenolone in the absence and presence of enzyme inhibitors, as well as in the absence and presence of the co-factor β-NADPH, resulted in very low levels of pregnenolone metabolism (Table 4). However, [^{3}H]pregnenolone appeared to be highly specifically metabolised by the isolated white blood cells to progesterone, compound A (fractions 40 to 45) and compound B (fractions 45 to 50; Fig. 9). Both compounds A and B were less polar than pregnenolone and progesterone.

The influence of incubation time, the co-factor β-NADPH and the enzyme inhibitors oestrone and NaF-HgCl on the metabolism of pregnenolone by the white blood cells is summarised in Table 5. The amount of pregnenolone metabolised was affected by incubation time and was higher after three hours of incubation when compared to the amount metabolised after one and two hours, although the increase was not linearly significant ($t_1 = 8.91$, $p > 0.05$, $n = 3$). The formation of progesterone decreased with incubation time, while the formation of both compounds A and B increased with

Table 4. The percentage metabolism of [^{3}H]pregnenolone and [^{3}H]progesterone (mean ± SD and numbers in parentheses indicate the number of animals investigated) during three hour incubations of controls, whole blood, plasma, red and white blood cells in the presence of β-NADPH.

	Amount converted (%)	
Incubation sample	Pregnenolone	Progesterone (4)
Control	0 (10)	5.3 ± 4.1
Whole blood	2.2 ± 3.5 (10)	36.4 ± 14.3
Plasma	1.8 ± 2.9 (10)	0.5 ± 0.6
Isolated red blood cells*	0.8 ± 0.9 (10)	75.8 ± 21.7
Isolated white blood cells*	55.9 ± 25.8 (9)	85.8 ± 26.8
Red and white blood cell mixture	0.4 ± 0.8 (7)	77.8 ± 26.7

* The amount of red and white blood cells incubated represent the number of the respective cells in 5 ml whole blood. The proportion of each cell type in whole blood was maintained during the incubations. Therefore, the percentage metabolism of pregnenolone and progesterone by red and white blood cells, as given in the table, represents the capacity of red and white blood cells to metabolise pregnenolone and progesterone *in vivo*. White blood cells have a greater capacity to metabolise pregnenolone and progesterone. Also, per million cells, white blood cells metabolised 6 to 19% progesterone, whereas red blood cells metabolised 0.01 to 0.02% progesterone.



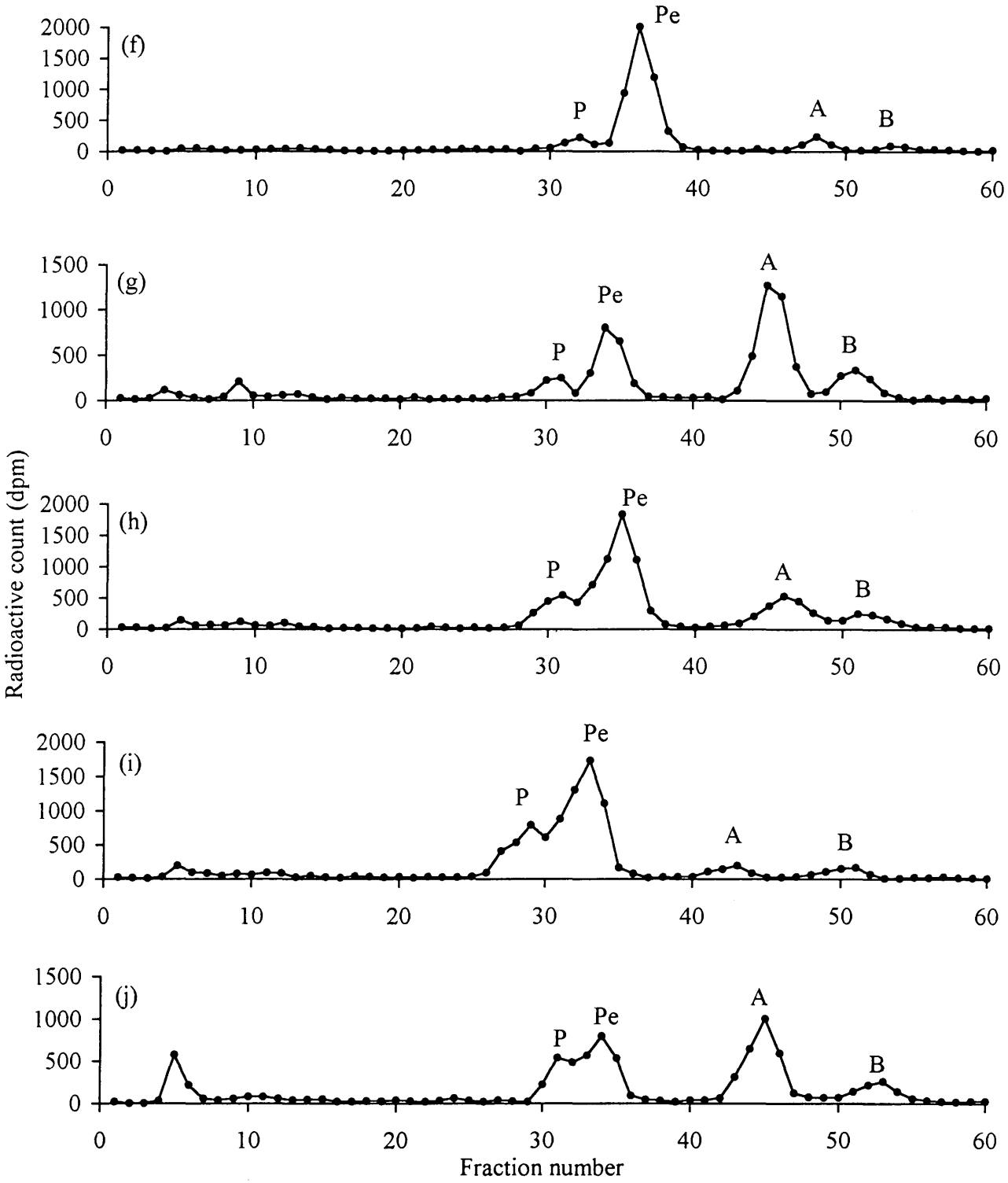


Fig. 9. HPLC elution profiles of radioactivity after incubation of (a) a control (i.e. no white blood cells), and of white blood cells from (b and c) non-, (d) early-, (e, f, and g) mid- and (h, i and j) late-pregnant hyraxes for three hours with [^{3}H]pregnenolone and β-NADPH. Peaks corresponding to pregnenolone (Pe), progesterone (P), compound A and compound B are labelled accordingly.

Table 5. The influence of time and incubation mixture on the percentage of pregnenolone metabolised and the percentage of progesterone, compounds A, B and other compounds formed by the white blood cells of a mid-pregnant hyrax.

	Amount converted/formed (%)					
	In the presence of β-NADPH			In the absence of β-NADPH (3 hours)		
Compound	1 hour	2 hour	3 hour	PBS present	Oestrone present	Sodium fluoride and mercury chloride present
Pregnenolone	39	79	89	52	16	0
Progesterone	9	8	4	13	0	0
Compound A	18	42	49	21	3	0
Compound B	7	16	16	12	0	0
Other compounds	6	13	20	6	13	0

time, suggesting that progesterone is an intermediate product. More pregnenolone was metabolised in the presence of β-NADPH than in its absence. Less progesterone was formed in the presence of β-NADPH than in its absence, but this may be the consequence of an increased metabolism to compounds A and B when β-NADPH was present. The metabolism of pregnenolone was totally inhibited by the enzyme inhibitor NaF-HgCl, and was reduced by the addition of oestrone to the incubation mixture.

The amount of pregnenolone metabolised by the white blood cells ranged from 18.0 to 89.0% (n = 9). The white blood cells of non-pregnant animals (n = 2) metabolised 22.5 and 56.5% pregnenolone and those of pregnant animals (n = 7) metabolised 60.6 ± 9.8% (mean ± SEM) pregnenolone (Table 6; see Table 20 in Appendix 4). The principal metabolite of pregnenolone was compound A. The metabolic conversion of pregnenolone was not affected by reproductive status (H_3 = 2.16, $p > 0.05$) and the formation of progesterone, compound A and B did not follow a specific pattern with stage of pregnancy (H_3 = 7.40, 2.16 and 2.42 respectively, $p > 0.05$).

The metabolism of [^{3}H]progesterone by the various blood components during incubations

Incubations of whole blood, the isolated red and white blood cells and the mixture of red and white blood cells with [^{3}H]progesterone in the presence of the co-factor β-NADPH[2] resulted in the metabolism of progesterone (Table 4) to compound A and compound B (Figs. 10, 11, 12 and 13,

[2] β-NADPH increased pregnenolone and progesterone metabolism by the white blood cells as demonstrated by incubations of white blood cells in the presence and absence of the co-factor (see Table 5). This observation validated the use of β-NADPH for all incubations. The same is true for incubations of ovarian residual and placental tissue (see Table 13). Makawiti *et al.* (1991) found that the addition of β-NADPH to the incubating medium increased progesterone metabolism by washed red blood cells, although no explanation was given for this observation. However, the results support the use of β-NADPH in the present study. The mechanism by which β-NADPH enhances pregnenolone and progesterone metabolism in the hyrax is not known and was not within the scope of this study. Although NADPH cannot enter cells, a possible explanation for the observed results was given by. Dr. SH Bissbort (*pers. comm.*, Department of Chemical Pathology, Faculty of Medicine, University of Pretoria, Pretoria, South Africa). He suggested that some of the cells used in the incubations may have been lysed, thereby releasing glutathione (GSH) and GSSG into the incubation medium. GSH binds to hormones to form conjugates so that hormones become more soluble for metabolism. GSSG is converted to GSH by NADPH and so in the presence of NADPH more GSH is formed, resulting in the formation of more hormone conjugates, thus increasing the metabolism of these hormones.

Table 6. The influence of reproductive status on the metabolism of pregnenolone and the formation of progesterone, compounds A, B and other compounds (mean ± SEM for the number of animals investigated in parentheses) after three hour incubations of white blood cells with [^{3}H]pregnenolone and β-NADPH.

		Metabolites formed (%)			
Reproductive status	Pregnenolone converted (%)	Progesterone	Compound A	Compound B	Other compounds
Non pregnant (2)	22.5 and 56.5	10.0 and 9.5	9.0 and 28.5	3.0 and 3.0	0.5 and 15.5
Early pregnant (1)	83.5	3.5	47.5	6.0	26.5
Mid pregnant (3)	60.7 ± 21.7	6.5 ± 1.3	34.2 ± 12.9	10.7 ± 4.6	9.3 ± 5.7
Late pregnant (3)	52.8 ± 9.3	15.8 ± 2.2	21.5 ± 6.4	7.3 ± 0.9	8.2 ± 2.5

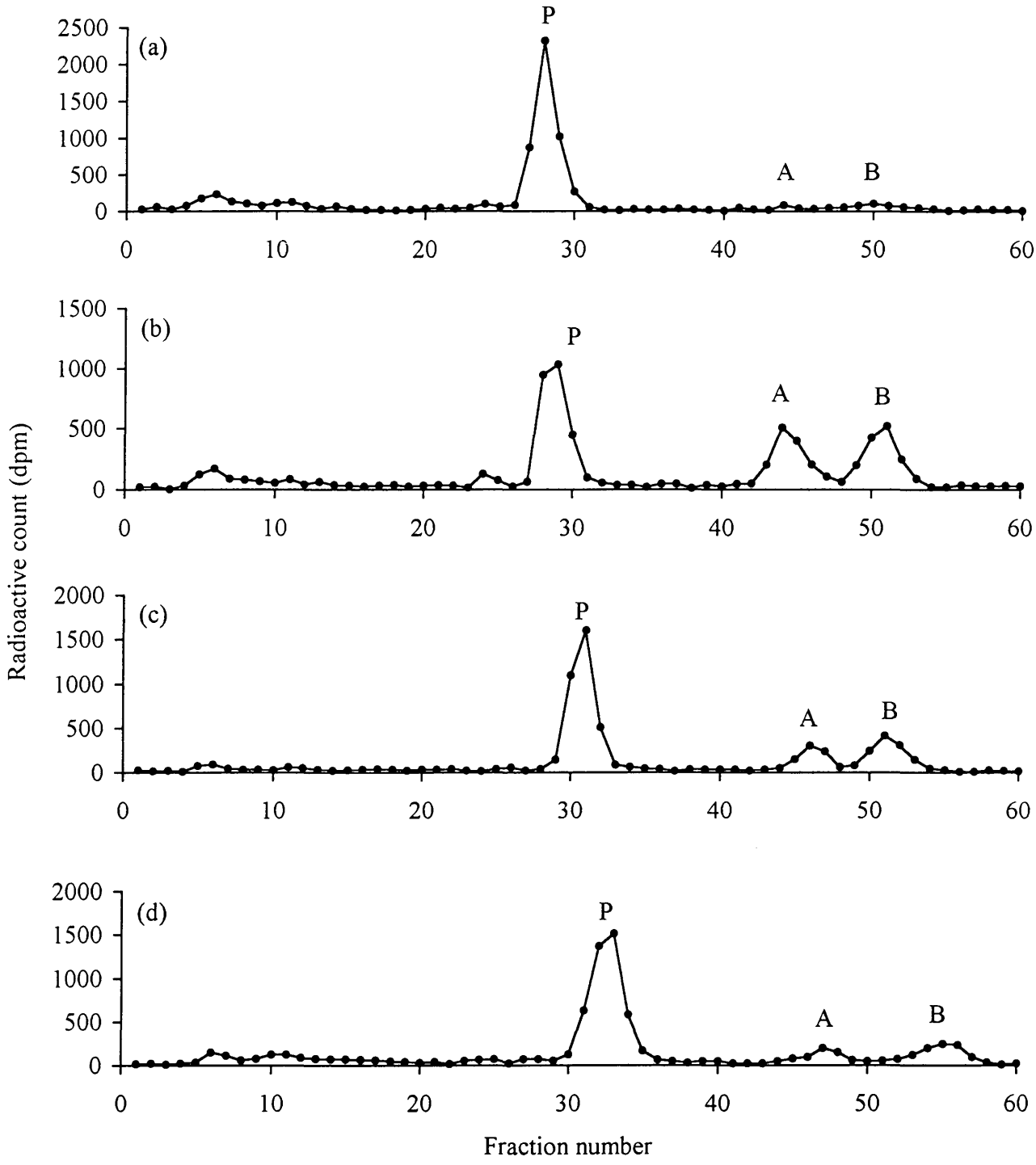


Fig. 10. HPLC profiles of radioactivity after incubation of whole blood of a (a) non-, (b) early-, (c) mid- and (d) late-pregnant hyrax, for three hours with [^{3}H]progesterone and β-NADPH. Progesterone (P), compounds A and B are labelled accordingly.

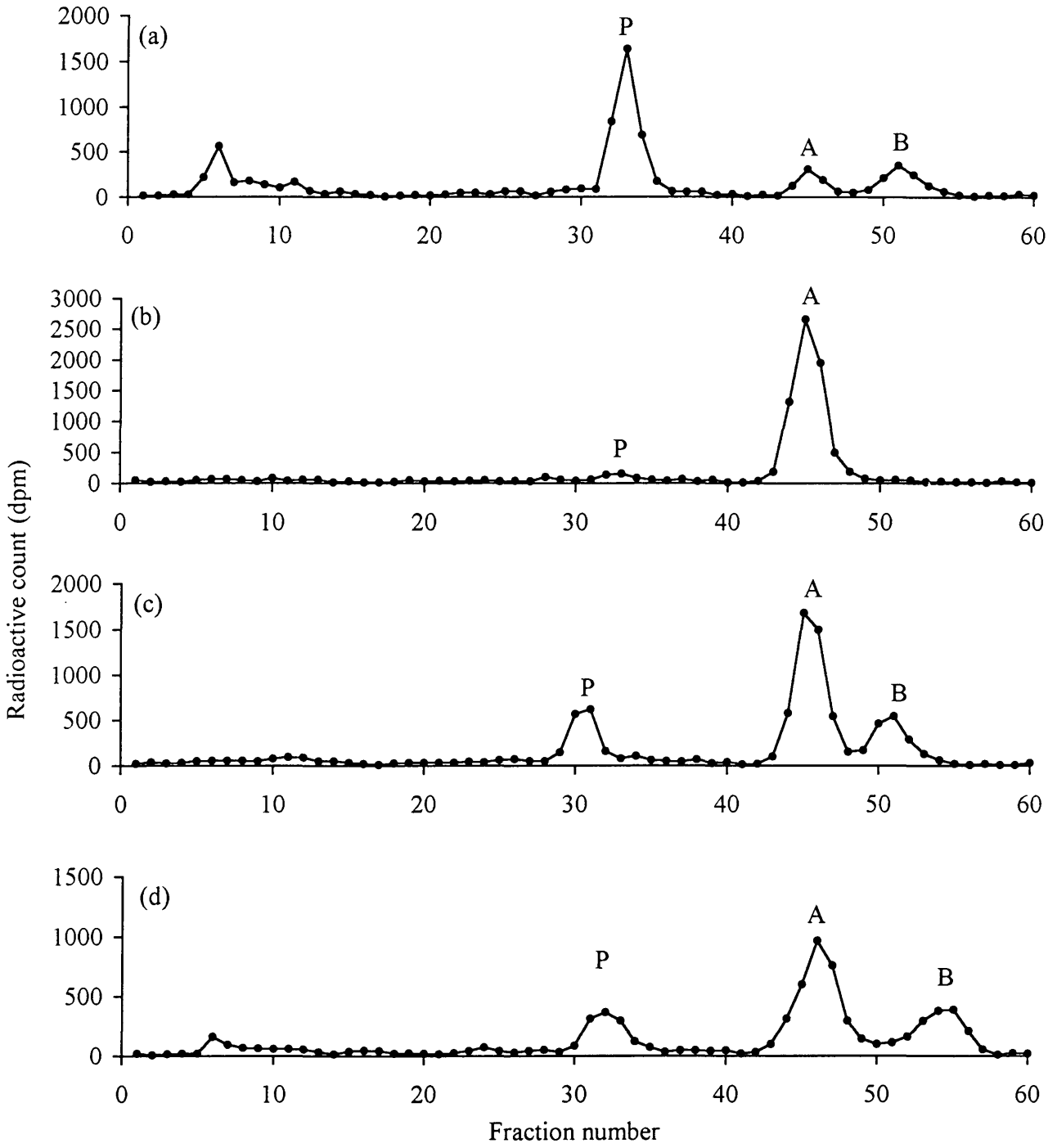


Fig. 11. HPLC radioactive profiles after three hour red blood cell incubations of a (a) non-, (b) early-, (c) mid- and (d) late-pregnant hyrax, with [^{3}H]progesterone and β-NADPH. Progesterone (P), compounds A and B are labelled accordingly.

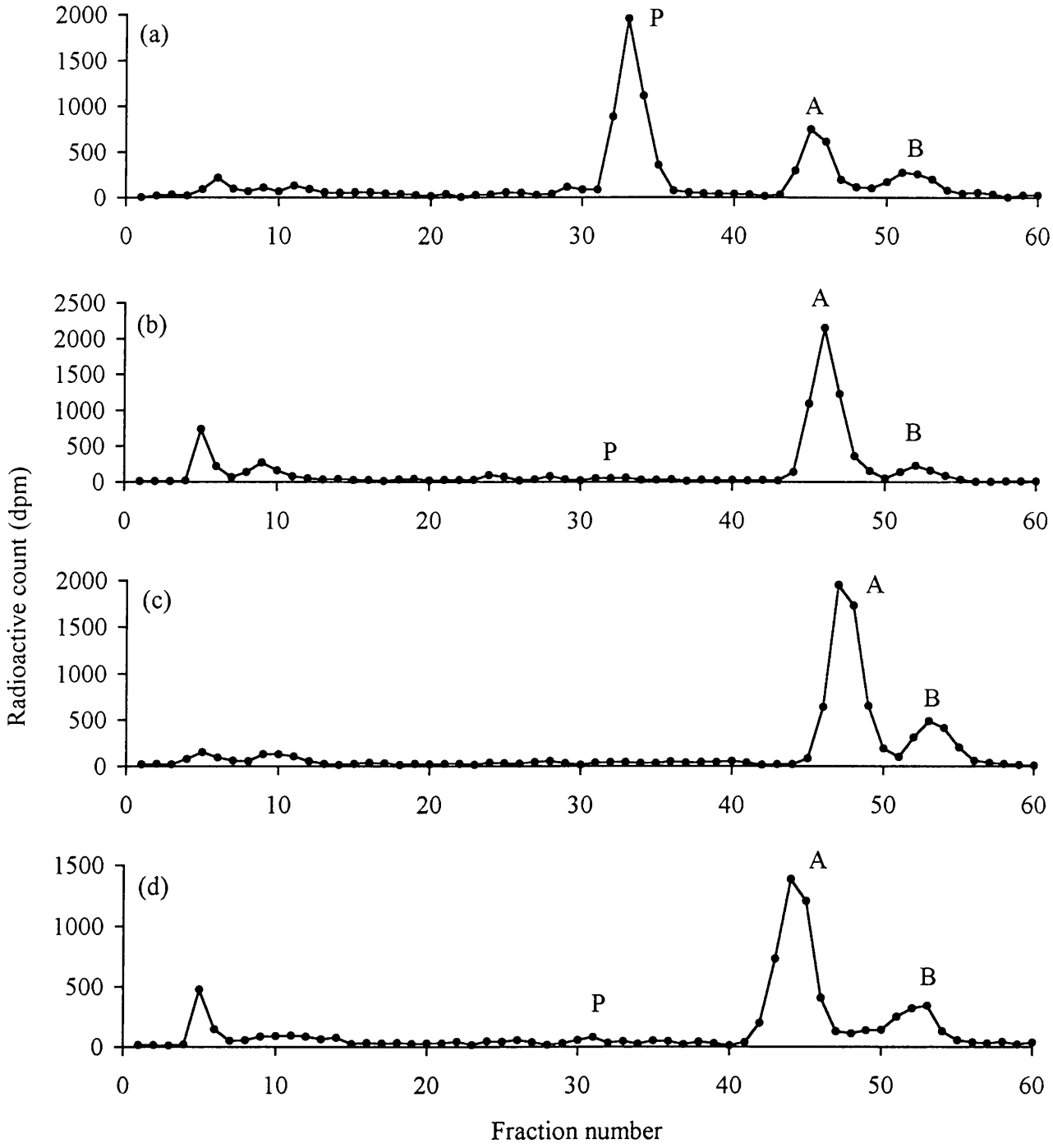


Fig. 12. HPLC radioactive profiles after three hour incubations of white blood cells of a (a) non-, (b) early-, (c) mid- and (d) late-pregnant hyrax, with [^{3}H]progesterone and β-NADPH. Progesterone (P), compounds A and B are labelled accordingly.

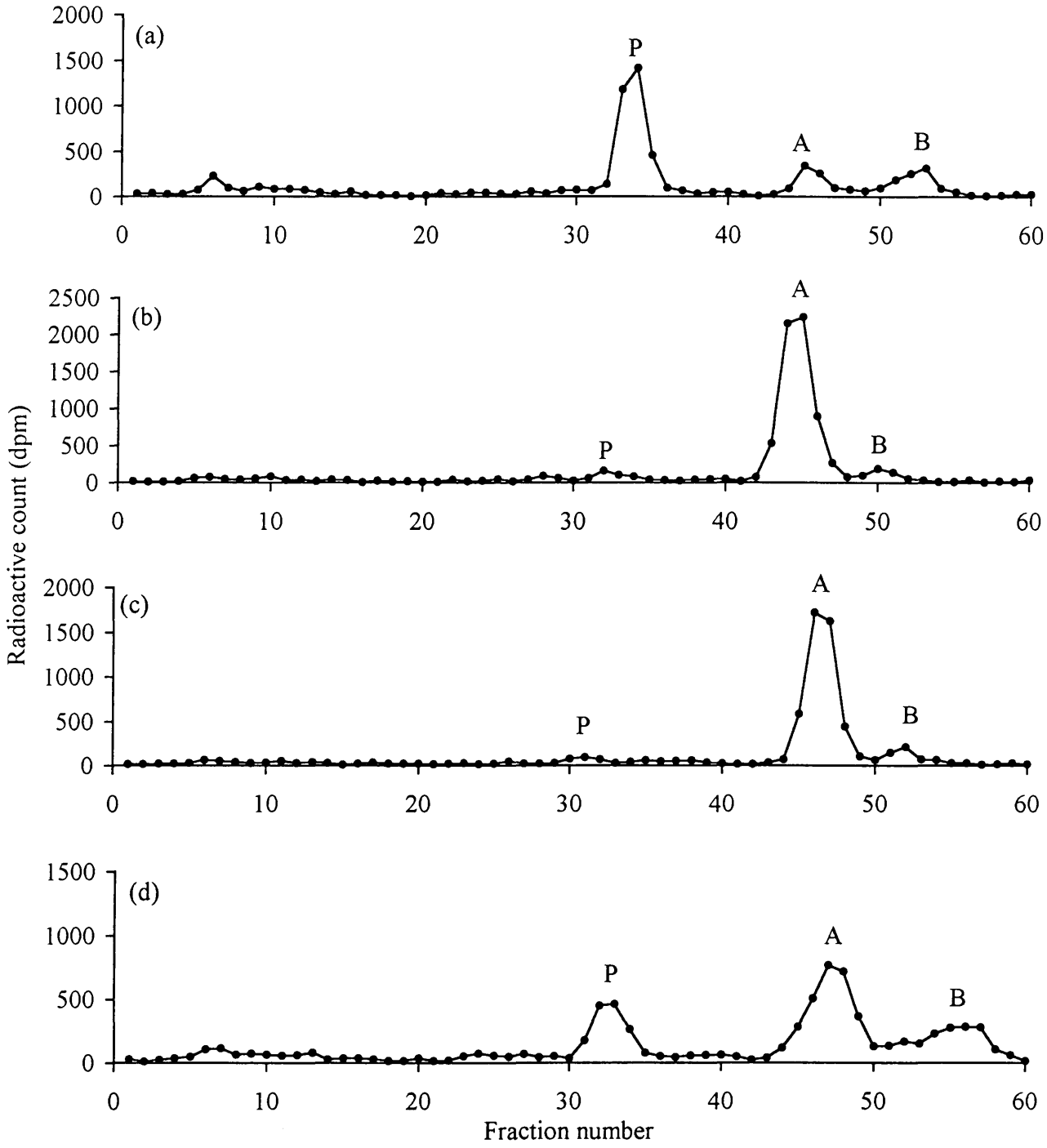


Fig. 13. HPLC profiles of radioactivity after three hour incubations of a red and white blood cell mixture of a (a) non-, (b) early-, (c) mid- and (d) late-pregnant hyrax, with [^{3}H]progesterone and with β-NADPH. Progesterone (P), compounds A and B are labelled accordingly.

respectively)[3]. Plasma alone did not metabolise progesterone. The mean percentage metabolism of progesterone in pregnant animals (n = 3) was twice as high than the amount of progesterone metabolised in the non-pregnant animal (Table 7). During pregnancy, the amount of progesterone metabolised by whole blood, the isolated red blood cells and the mixture of red and white blood cells was lowest for the late-pregnant animals. The isolated red and white blood cells metabolised similar amounts of progesterone during the non-pregnant stage. However, during pregnancy the isolated white blood cells metabolised all of the progesterone, whereas the isolated red blood cells metabolised about 86% progesterone. The red and white blood cell mixture metabolised 4% more progesterone than the isolated red blood cells during pregnancy, and whole blood metabolised the least amount of progesterone.

The amount of progesterone metabolised was positively correlated to the amount of compound A formed, but not to the amount of compound B formed (Table 8). Compound A appeared to be the main metabolite produced by the isolated red and white blood cells as well as the mixture of red and white blood cells, whereas whole blood formed similar amounts of compounds A and B. Whole blood, the isolated red blood cells and the mixture of red and white blood cells of pregnant animals formed more of compound A than those of the non-pregnant animal, and the production of this compound was lowest during late pregnancy. White blood cells formed more of compound A in pregnant animals than in the non-pregnant animal and the amount formed was similar throughout pregnancy. Whole blood formed more of compound B during pregnancy than during non-pregnancy, and amounts formed were lowest during late pregnancy. The production of compound B by the isolated red and white blood cells and the mixture of red and white blood cells was similar for non-pregnant and pregnant animals .

[3] Compound B in Figs. 10d, 11d and 13d eluted later usual (fractions 45 to 50) due to problems experienced with the HPLC system (leaks, unstable pressure and column degeneration) at the time when these samples were injected onto the HPLC column. Pregnenolone and progesterone standards which were analysed on the same day as the samples also eluted later than usual (0.5 to 2 min later). The broad peak relating to compound B may be a result of a drop in pressure.

Table 7. The influence of reproductive status on the metabolism of progesterone and the amount of compounds A, B and other compounds formed, for a animal in each reproductive stage, during three hour incubations with [^{3}H]progesterone and β-NADPH.

			Metabolites formed (%)		
Incubation substrate	Reproductive status	Progesterone converted (%)	Compound A	Compound B	Other compounds
Whole blood	Non pregnant	21.5	1.0	5.5	15.0
	Early pregnant	55.5	23.5	23.0	9.0
	Mid pregnant	37.0	13.0	21.0	3.0
	Late pregnant	31.5	8.5	13.5	9.5
	Pregnant*	41.3 ± 7.3	15.0 ± 4.4	19.2 ± 2.9	7.0 ± 2.1
Red blood cells	Non pregnant	45.0	9.5	15.5	20.0
	Early pregnant	96.0	94.5	0	1.5
	Mid pregnant	81.0	55.0	21.0	5.0
	Late pregnant	81.0	50.5	24.0	6.5
	Pregnant*	86.0 ± 5.0	66.7 ± 14.0	15.0 ± 7.6	3.3 ± 1.5
White blood cells	Non pregnant	45.5	25.5	12.5	7.5
	Early pregnant	98.5	66.0	12.0	2.5
	Mid pregnant	100	71.0	20.0	9.0
	Late pregnant	99.0	62.0	18.0	19.0
	Pregnant*	99.2 ± 0.4	66.3 ± 2.6	16.7 ± 2.4	16.7 ± 3.9
Red and white blood cells	Non pregnant	39.5	12.0	16.5	11.0
	Early pregnant	95.0	88.0	7.0	0
	Mid pregnant	97.0	88.0	9.0	0
	Late pregnant	79.5	44.5	22.0	13.0
	Pregnant*	90.5 ± 5.5	73.5 ± 14.5	12.7 ± 4.7	4.3 ± 4.3

*Mean ± SEM, $n = 3$

Table 8. Correlations of the amount of progesterone metabolised to the amounts of compounds A and B produced, by whole blood, the isolated red and white blood cells and the mixture of red and white blood cells of four animals, during three hour incubations with [^{3}H]progesterone and β-NADPH.

	Correlation (r) of progesterone with	
Blood sample	Compound A	Compound B
Whole blood	1.00*	0.90
Red blood cells	0.96*	−0.35
White blood cells	0.99*	0.54
Red and white blood cell mixture	0.96*	−0.54

* $p < 0.05$

The number of red blood cells in the hyrax was 4.93 ± 0.47 x 10^9 cells/ml blood (mean ± SEM; n = 10) and the number of white blood cells was 8.93 ± 1.03 x 10^6 cells/ml blood (n = 10). The number of red and white blood cells was not affected by reproductive stage (H_3 = 2.66 and 6.27 respectively, $p > 0.05$; Table 9). However, the number of red and white blood cells in early-pregnant females was 1.5-fold and twofold higher, respectively, than in non-pregnant females, and decreased from early to late pregnancy. The number of red and white blood cells, respectively, were similar in non- and late-pregnant animals.

The metabolism of both [^{3}H]pregnenolone and [^{3}H]progesterone by the various blood components during incubations

Incubations of whole blood, the isolated red and white blood cells and the mixture of red and white blood cells with pregnenolone and progesterone resulted in the formation of both compounds A and B. Plasma did not produce these compounds.

Partial identification of compound A and compound B by HPLC

The elution positions of the unlabelled standards, namely progesterone, 5α-dihydroprogesterone, 5β-dihydroprogesterone and 5α-pregnan-3α-ol-20-one, as well as those of [^{3}H]progesterone and the labelled unknown conversion products, compound A and compound B, are presented in Table 10. The difference in retention time between [^{3}H]progesterone and unlabelled progesterone is approximately one minute, indicating that the progesterone fraction is detected by the UV detector about one minute before the progesterone fraction is collected by the fraction collector. The fractions relating to compound A and compound B, both labelled compounds, were collected at 22 min and 25 min, respectively, by the fraction collector. As a result of the difference in retention times between UV detection and fraction collection, compound A and compound B would be detected at about

Table 9. The influence of reproductive status on the number of red and white blood cells (mean ± SEM) and the number of individual animals investigated appear in parentheses.

Reproductive status	Red blood cells (x 10^9)/ml blood	White blood cells (x 10^7)/ml blood
Non-pregnant (2)	2.7 and 6.3	0.5 and 0.8
Early-pregnant (1)	6.8	1.4
Mid-pregnant (4)	5.1 ± 0.7	1.0 ± 0.1
Late-pregnant (3)	4.3 ± 0.4	0.7 ± 0.2
Pregnant (8)	5.0 ± 4.7	1.0 ± 0.1

Table 10. HPLC retention times for the unlabelled progesterone, 5α-dihydroprogesterone, 5β-dihydroprogesterone and 5α-pregnan-3α-ol-20-one standards, for [^{3}H]progesterone and for the labelled unknown conversion products, compounds A and B.

	Retention time (min)	
Compound	Unlabelled compound	Labelled compound
Progesterone	15.7	16.5
5β-Dihydroprogesterone	20.9	-
5α-Dihydroprogesterone	25.0	-
5α-Pregnan-3α-ol-20-one	23.2	-
Compound A	-	22.0
Compound B	-	25.0

21 min and 24 min, respectively, by the UV detector. The retention times of the unlabelled standards indicate that compound A may correspond to 5β-dihydroprogesterone and compound B may correspond to 5α-dihydroprogesterone or 5α-pregnan-3α-ol-20-one.

The validation of pregnenolone and progesterone as well as an attempted identification of compounds A and B by GC-MS and LC-MS

The presence of pregnenolone and progesterone was confirmed by GC-MS analysis of the pregnenolone and progesterone standards, the fractions of which were collected after HPLC and prepared for GC-MS. Compound A and compound B could not be identified by GC-MS or LC-MS. This is probably a result of operating at steroid concentrations below the detection limits of the GC-MS and LC-MS systems. Successful positive identification of these compounds should be achieved, provided that higher concentrations of the unlabelled progesterone is incubated with the blood.

The identification of compounds A and B by TLC

The R_f values, for mobile phase one and two, of the standards (5α-dihydroprogesterone, 5β-dihydroprogesterone and 5α-pregnan-3α-ol-20-one) and of the labelled unknown conversion products (compounds A and B) formed during red and white blood cell incubations are presented in Table 11. TLC of the isolated fractions in mobile phases one and two revealed that compound A had a R_f value similar to that of 5α-pregnan-3α-ol-20-one, while compound B had a R_f value similar to that of 5α-dihydroprogesterone.

Discussion

The aim of this part of the present study was to determine the steroidogenic potential of whole blood, plasma, red and white blood cells of the rock hyrax during incubation studies with pregnenolone and

Table 11. The R_f values of the unlabelled standards (5α-dihydroprogesterone, 5β-dihydroprogesterone and 5α-pregnan-3α-ol-20-one) and of the labelled unknown conversion products formed during white and red blood cell incubations (compounds A and B), after TLC under mobile phases one and two.

		R_f value	
Compound	Incubation sample	Mobile phase one	Mobile phase two
5α-Dihydroprogesterone	-	0.85	0.54
5β-Dihydroprogesterone	-	0.81	0.46
5α-Pregnan-3α-ol-20-one	-	0.53	0.35
Compound A	White blood cells	0.46	0.35
	Red blood cells	0.46	0.35
Compound B	White blood cells	0.92	0.58
	Red blood cells	0.92	0.58

progesterone. The influence of reproductive status on the steroidogenic potential of these blood components was determined, as well as the effects of enzyme inhibitors, the co-factor β-NADPH and incubation time on the metabolism of pregnenolone. The results show that white blood cells have the ability to metabolise both pregnenolone and progesterone, whereas whole blood, red blood cells and a mixture of red and white blood cells appear to be more selective and can metabolise only progesterone. Plasma alone has no steroidogenic potential. The principal compounds biosynthesised during these metabolic conversions are compound A and compound B, identified as 5α-pregnan-3α-ol-20-one and 5α-dihydroprogesterone, respectively. Heap *et al.* (1975) and Makawiti *et al.* (1991) found that progesterone was metabolised to 5α- and 5β-dihydroprogesterone in the blood of the hyrax (Heap *et al.* 1975; Makawiti *et al.* 1991). However, the present study found no evidence that 5β-dihydroprogesterone is a metabolite of progesterone in hyrax blood. White and red blood cells mainly produced 5α-pregnan-3α-ol-20-one and whole blood produced similar amounts of compounds 5α-pregnan-3α-ol-20-one and 5α-dihydroprogesterone. The amount of 5α-pregnan-3α-ol-20-one formed was correlated to the amount of progesterone metabolised, suggesting that 5α-pregnan-3α-ol-20-one is a direct metabolite of progesterone. 5α-Dihydroprogesterone is possibly a metabolite of 5α-pregnan-3α-ol-20-one.

The present study shows that white blood cells have the greatest steroidogenic potential, and have a greater capacity than red blood cells to metabolise progesterone. The rate of metabolism of pregnenolone by white blood cells appeared to be lower than that of progesterone, indicating that the metabolism of pregnenolone to progesterone may be a limiting factor in the formation of progestin metabolites by the white blood cells. Heap *et al.* (1975) inferred that progesterone metabolism in hyrax blood could be attributed to red blood cells. However, Makawiti *et al.* (1991) reported that whole blood, red blood cells and plasma are all able to metabolize progesterone. According to the present study, conclusions in these earlier studies that attributed the metabolism of progesterone by hyrax blood

to the metabolic action of red blood cells should be amended to include white blood cells as well. However, whole blood and the mixture of red and white blood cells did not metabolize pregnenolone. This may be due to the relative higher presence of red blood cells (on average 500-fold higher) than of white blood cells in hyrax blood, or to the inhibition of the metabolic activity of the white blood cells by some other component of blood. It is therefore possible that the steroidogenic activity of white blood cells in hyrax blood is negligible *in vivo*.

It has been reported that progesterone binds to white blood cells in order to inhibit their cytotoxic activity *in vitro* (Scully *et al.* 1982). In normal human pregnancy, progesterone acts as an immunosuppressant (Scully *et al.* 1982; Szekeres-Bartho, Csernus, Hadnagy & Pacsa 1983; Szekeres-Bartho, Hadnagy & Pacsa 1985; Chiu, Nishimura, Ishii, Nieda, Maeshima, Takedani, Shibata, Tadokoro & Juji 1996) as does progesterone, 5α-dihydroprogesterone and 20α-dihydroprogesterone in sheep and goats (Staples, Binns & Heap 1983). It has also been found that the number of lymphocytes containing progesterone receptors in human peripheral blood increases as pregnancy progresses (Szekeres-Bartho, Reznikoff-Etievant, Varga, Pichon, Varga & Chaouat 1989). In the hyrax, the formation of progesterone from pregnenolone increased with pregnancy. This may indicate that as pregnancy progresses, the role of white blood cells changes from metabolising progesterone to binding progesterone, in order to reduce their immune response towards the developing foetus. However, it has been stated that the concentrations of steroids in pregnant animals do not affect the immunological status of the mother (Skinnider & Laxdal 1981) and that peripheral levels of progesterone are unable to suppress immunity (Clemens, Siiteri & Stites 1979; Van Vlasselaer, Goebels & Vandeputte 1986). Although it would appear as if the function of white blood cells in peripheral hyrax blood may not be related to a suppression of cell mediated immunity in pregnant females, further speculation is only possible once this has been investigated.

Since progesterone concentrations in hyrax plasma are normally low (Heap *et al.* 1975), the possibility exists that 5α-pregnan-3α-ol-20-one and/or 5α-dihydroprogesterone, rather than progesterone, can support pregnancy in this mammal. The metabolism of pregnenolone and progesterone by white blood cells was much higher in pregnant animals than in non-pregnant animals, suggesting that the biological activity of white blood cells is related to pregnancy, although this increased metabolic potential during pregnancy may be a result of higher white blood cell numbers when compared to those of non-pregnant animals. However, during pregnancy the amount of pregnenolone metabolised, as well as the production of 5α-pregnan-3α-ol-20-one and 5α-dihydroprogesterone, by the white blood cells was not affected by reproductive status. Consequently, the biosynthetic potential of white blood cells appears to be unassociated with reproductive status, but may be important in protecting the maternal and foetal tissues from the effects of high levels of progesterone (Makawiti *et al.* 1991).

The metabolism of progesterone by whole blood, red blood cells and a mixture of red and white blood cells was higher in pregnant animals than in non-pregnant animals, and during pregnancy the amounts formed were lowest in late-pregnant animals. This may be related to the number of red and white blood cells, which are also higher in pregnant than in non-pregnant animals, and also decrease from early- to late-pregnancy. From earlier studies (Makawiti *et al.* 1991), progesterone metabolism by the blood of pregnant hyraxes was higher than by the blood of non-pregnant animals, corroborating the findings of the present study. The formation of 5α-pregnan-3α-ol-20-one was highest in pregnant animals, and during pregnancy the lowest amounts were formed during late pregnancy. Whole blood formed more of 5α-dihydroprogesterone during pregnancy than during non-pregnancy, and amounts formed during pregnancy were lowest in late-pregnant animals.

Studies on the African elephant (Heistermann, Beard, Van Aarde & Hodges 1994) and the horse (Short 1959; Seamans *et al.* 1979; Chavatte & Tait 1994; Schutzer & Holtan 1996) demonstrated that

5α-dihydroprogesterone, rather than progesterone, is related to reproductive status and may be responsible for the maintenance of pregnancy. Both 5α-dihydroprogesterone and 5α-pregnan-3α-ol-20-one reflect corpus luteum function in the African elephant (Hodges *et al.* 1997) and both progestins had a relatively high binding affinity for the uterine progesterone receptor (Greyling *et al.* 1997), suggesting that these compounds may be biologically important during pregnancy. 5α-Dihydroprogesterone may display progestational activity in the African elephant (Greyling *et al.* 1997) and seems to be responsible for the down-regulation of uterine progesterone receptor concentrations as pregnancy progresses (Greyling *et al.* 1998). 5α-Dihydroprogesterone can also support placental growth (Sanyal & Villee 1973; Chatterton 1982). However, in the pregnant mare, 5α-pregnanes inhibit 3β-hydroxysteroid dehydrogenase activity which may lead to decreased activity in the term placenta prior to the onset of parturition, although results for 5α-dihydroprogesterone were inconsistent (Chavatte *et al.* 1995). In some cases 5α-dihydroprogesterone has no progestational activity (Zarrow *et al.* 1957; Sanyal & Villee 1973). There are a number of biological functions attributed to 5α-reduced metabolites of progesterone. Both 5α-pregnan-3α-ol-20-one and 5α-dihydroprogesterone may be biologically important during pregnancy of the rock hyrax and the trends shown may be either related to the maintenance of pregnancy or to the initiation of parturition. The biological significance of 5α-dihydroprogesterone and 5α-pregnan-3α-ol-20-one in the hyrax should be investigated by means of competitive ligand binding studies on the uterine progesterone receptors.

Whole blood metabolised less progesterone than the red and white blood cell mixture. It may be possible that enzyme inhibitors are present in hyrax plasma, capable of interfering with pregnenolone and progesterone metabolism. Enzyme inhibitors may be present in human plasma (Van der Molen & Groen 1968). However, it was also suggested that if red blood cells are capable of metabolising progesterone, then in whole blood competition might arise between the binding of steroids by plasma proteins and by red blood cells in whole blood (Van der Molen & Groen 1968). This competitive situation

may explain the low rate of steroid metabolism by whole blood compared to the high rate of steroid metabolism by a red and white blood cell mixture where plasma is absent.

Steroid binding plasma proteins are involved in the transportation of steroids, the protection of steroids against enzymatic activity and the regulation of the amount of free steroids circulating in the blood (Wagner 1978; Cheesman 1982). However, progesterone binding globulin, which serves as a progesterone-conserving mechanism, does not appear to be present in hyrax plasma (Gombe *et al.* 1976), although a preliminary investigation indicated the presence of a binding protein in hyrax plasma which binds progesterone (*pers. obs.*). Progesterone-binding globulin reduces the rate of clearance of progesterone from the blood in Hystricomorpha, which exhibit an exceptionally long gestation period relative to mature weight (Heap & Illingworth 1974). Similar to the Hystricomorpha, hyraxes also display a long gestation period relative to their mature weight. However, Gombe *et al.* (1976) could not confirm the existence of a progesterone-binding globulin in hyrax plasma since approximately 80% progesterone was metabolized by both whole blood and red blood cells. In contrast, the present study indicates that progesterone binding plasma proteins may occur in the blood of the hyrax, since whole blood metabolised up to 56% progesterone whereas red blood cells metabolised up to 96% progesterone. Progesterone in whole blood may be protected from being metabolised by red and white blood cells in whole blood because of its association with progesterone plasma binding proteins. The presence of progesterone binding plasma proteins could result in the transport of progesterone to target tissues, like the corpus luteum and placenta, where progesterone is metabolized.

White blood cell incubations with the enzyme inhibitors oestrone and NaF-HgCl confirmed that enzymes are responsible for the metabolism of pregnenolone to the conversion products, since the amount of pregnenolone metabolised in the presence of the inhibitors was negligible, compared to the amount metabolised in their absence. These findings support the suggestion that blood

steroidogenesis depends on enzymes (Heap *et al.* 1975; Makawiti *et al.* 1991) and is not merely spontaneous. during white blood cell incubations increased pregnenolone metabolism. Nevertheless, its presence was not necessary for steroid biosynthesis and the same compounds were biosynthesised whether or not the co-factor was present This is probably because white blood cells contain β-NADPH of their own.

The metabolism of pregnenolone by whole blood, plasma and red blood cells was not affected by the presence of the co-factor. These observations suggest that the enzyme complex necessary for the metabolism of pregnenolone to progesterone, possibly 3β-hydroxysteroid dehydrogenase and isomerase (Cheesman 1982; Gill 1987), is not present in plasma or red blood cells. Incubation time influenced the amount of pregnenolone metabolised and the amount of metabolites (compounds A and B) formed increased as incubation time increased.

Conclusion

White blood cells metabolically converted both pregnenolone and progesterone, whereas whole blood, red blood cells and a mixture of red and white blood cells metabolised only progesterone. Plasma had no biosynthetic potential. The main metabolites of progesterone are 5α-pregnan-3α-ol-20-one and 5α-dihydroprogesterone which are known to be biologically important during pregnancy of some mammals. Enzymes are essential for steroid biosynthesis since enzyme inhibitors decreased the metabolism of pregnenolone by white blood cells. Steroid biosynthesis increased as incubation time increased and was enhanced by the presence of β-NADPH. The metabolism of pregnenolone by white blood cells, and of progesterone by blood and its components, was higher in pregnant animals and thus the biosynthetic activity of blood may be related to pregnancy. However, the biosynthetic potential of white blood cells was not affected by pregnancy stage and these cells may rather be important in reducing the circulating progesterone levels during pregnancy. On the other hand, white

blood cells may not affect levels of circulating progesterone because there are relatively few of them in the blood.

The metabolism of progesterone by whole blood may reflect the *in vivo* situation more than the metabolism of progesterone by red and white blood cells, and the metabolites of progesterone may be responsible for the maintenance of pregnancy or for the onset of parturition. The plasma may contain enzyme inhibitors, reducing the biosynthetic activity of red and white blood cells in whole blood. On the other hand, progesterone plasma binding proteins may be present in whole blood. Studies to determine the binding affinities of 5α-pregnan-3α-ol-20-one and 5α-dihydroprogesterone to the uterine progesterone receptors in the hyrax are also necessary.

CHAPTER 5

THE METABOLISM OF PREGNENOLONE AND PROGESTERONE BY THE OVARY AND PLACENTA

Introduction

Steroid biosynthesis in luteal, ovarian residual and placental tissues changes dynamically with the reproductive status of an organism (Cheesman 1982). Steroid production and secretion is also determined by the concentration of co-factors and precursors present in the tissue, as well as by the blood supply (Cheesman 1982). Corpora lutea of pregnant hyraxes produce an appreciable amount of progesterone (from 0.61 to 24.2 ng/mg; Gombe *et al.* 1976; Van Aarde & Anderson 1989) and its biological activity may be significant during pregnancy. However, in the related African elephant, progesterone levels in corpora lutea are low and the major luteal progestins appear to be 5α-dihydroprogesterone and 5α-pregnan-3α-ol-20-one (Heistermann *et al.* 1994). The possibility therefore exists that a metabolite of progesterone may be an important luteal product in the rock hyrax. In other species, corpora lutea also secrete pregnenolone, progesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, androstenedione, oestradiol and oestrone (Cheesman 1982; Johnson & Everitt 1988). The secretory activity of the corpus luteum usually decreases or is absent during late pregnancy (Shemesh 1990; Silver 1994) and in human pregnancy, placental progesterone becomes dominant as the placenta develops (Diczfalusy 1974).

Progesterone formation in hyrax ovarian residual tissue is low (Heap *et al.* 1975; Van Aarde & Anderson 1989) and it would seem that ovarian residual tissue plays a minor role in the maintenance of pregnancy. However, in some species progesterone formation by ovarian tissue is high (Tam 1974; Johnson & Everitt 1988). Ovarian interstitial tissue contains steroidogenic cells (Johnson & Everitt 1988) and ovaries are able to biosynthesise progesterone, 17α-hydroxypregnenolone, 17α-

hydroxyprogesterone, pregnanedione and androstenedione (Nakamura, Tanabe & Katukawa 1974). It has been suggested that 3α-hydroxy-4-pregnen-20-one may be the main steroid produced by ovarian tissue and that actions associated with progesterone may actually be ascribed to actions of this metabolite of progesterone (Wiebe, De Gannes & Dallaire 1994). 3β-Hydroxysteroid dehydrogenase activity is found in corpora lutea (Lemon & Mauléon 1982; Ravindra, Bhatia & Mead 1984; Bernal 1989) and in ovaries (Galil & Deane 1966; Sasano, Mori, Sasano, Nagura & Mason 1990), which indicates that these tissues have the ability to metabolise pregnenolone to progesterone.

The placenta is a highly vascularized tissue and allows rapid and efficient transfer of materials between the mother and foetus (Cheesman 1982). 3β-Hydroxysteroid dehydrogenase isomerase activity is found in the placenta of some species (Davies, Davenport, Norris & Rennie 1966; Townsend & Ryan 1970; Tam 1974; Marcal, Chew, Salomon & Sherman 1975; Legrand 1977; Marchut 1980; Flint, Burton & Heap 1983; Das, Khan-Dawood & Dawood 1985; Dupont, Luu-The, Labrie & Pelletier 1990; Knight & Kukoly 1990), but the activity of this enzyme complex appears to be absent in some species (Davies *et al.* 1966; Galil & Deane 1966; Matsumoto, Yamane, Endo, Kotoh & Okano 1969). In the rock hyrax, the placenta apparently is not an important source of progesterone (Heap *et al.* 1975; Van Aarde & Anderson 1989). The possibility therefore remains that a hormone other than progesterone may be an important placental biosynthetic product.

To determine if luteal, ovarian residual and placental tissue of the hyrax contained steroidogenic activity, these tissues were incubated with radiolabelled progestin precursors, pregnenolone and progesterone. The influence of incubation time, as well as of the presence of the enzyme inhibitors oestrone and NaF-HgCl and of the co-factor β-NADPH, is also determined.

Results

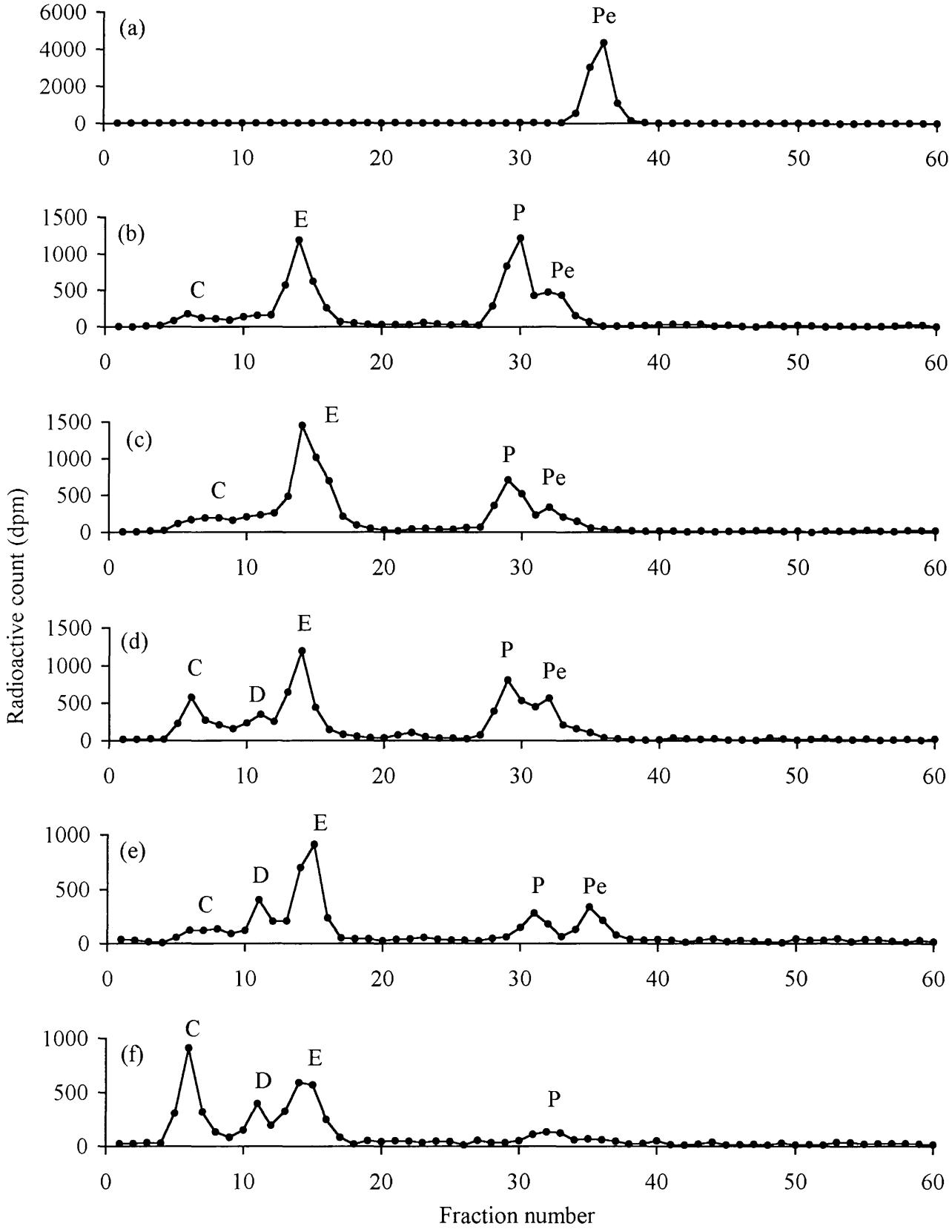
The metabolism of [^{3}H]pregnenolone by the various tissues during incubations

Incubations of luteal tissue, ovarian residual tissue and placental tissue with [^{3}H]pregnenolone and β-NADPH resulted in the metabolism of pregnenolone (Table 12). The principal metabolites of pregnenolone during luteal tissue incubations were progesterone, compound C (fractions six to eight), compound D (fractions 10 to 12) and compound E (fractions 13 to 17; Fig. 14). Ovarian residual tissue metabolised pregnenolone to progesterone and compound F (fractions 12 to 16; Fig. 15). Placental tissue metabolised pregnenolone mainly to progesterone, compound G (fractions seven to nine) and compound H (fractions 24 to 27; Fig. 16). The compounds formed by luteal, ovarian residual and placental tissues eluted before pregnenolone and progesterone and are therefore more polar than pregnenolone and progesterone.

The effect of incubation time during placental tissues incubations, and of the presence of β-NADPH and enzyme inhibitors in the incubation mixture during ovarian residual and placental tissue incubations, is presented in Table 13. The amount of pregnenolone metabolised by the placenta increased significantly with incubation time ($t_1 = 105.38$, $p < 0.05$, $n = 3$). The production of progesterone increased significantly ($t_1 = 36.66$, $p < 0.05$), and that of compound H decreased significantly ($t_1 = -38.19$, $p < 0.05$), with time. In the presence of β-NADPH, the metabolism of pregnenolone by both tissue types was twofold higher than in the absence of β-NADPH. For ovarian residual tissue, the amount of compound F formed was twofold higher in the presence of β-NADPH than in its absence. The formation of compound H by placental tissue was slightly higher, and that of progesterone threefold higher, in the presence of β-NADPH than in its absence. The metabolism of pregnenolone by ovarian residual and placental tissues was inhibited if the enzyme inhibitors NaF-HgCl were added to the incubate. However, with the addition of oestrone, the amount of pregnenolone metabolically converted by ovarian residual tissue was the same, and by placental

Table 12. The percentage metabolism of [^{3}H]pregnenolone and [^{3}H]progesterone (mean ± SD for the number of individual animals investigated which appear in parentheses) by controls, luteal tissue, ovarian residual tissue and placental tissue incubated for three hours with β-NADPH.

	Amount converted (%)	
Incubation sample	Pregnenolone	Progesterone
Control	0 (10)	5.3 ± 4.1 (4)
Luteal tissue	89.7 ± 12.6 (10)	46.7 ± 31.8 (3)
Ovarian residual tissue	73.8 ± 16.5 (10)	62.0 ± 16.3 (4)
Placental tissue	75.7 ± 4.3 (7)	49.0 and 52.0 (2)



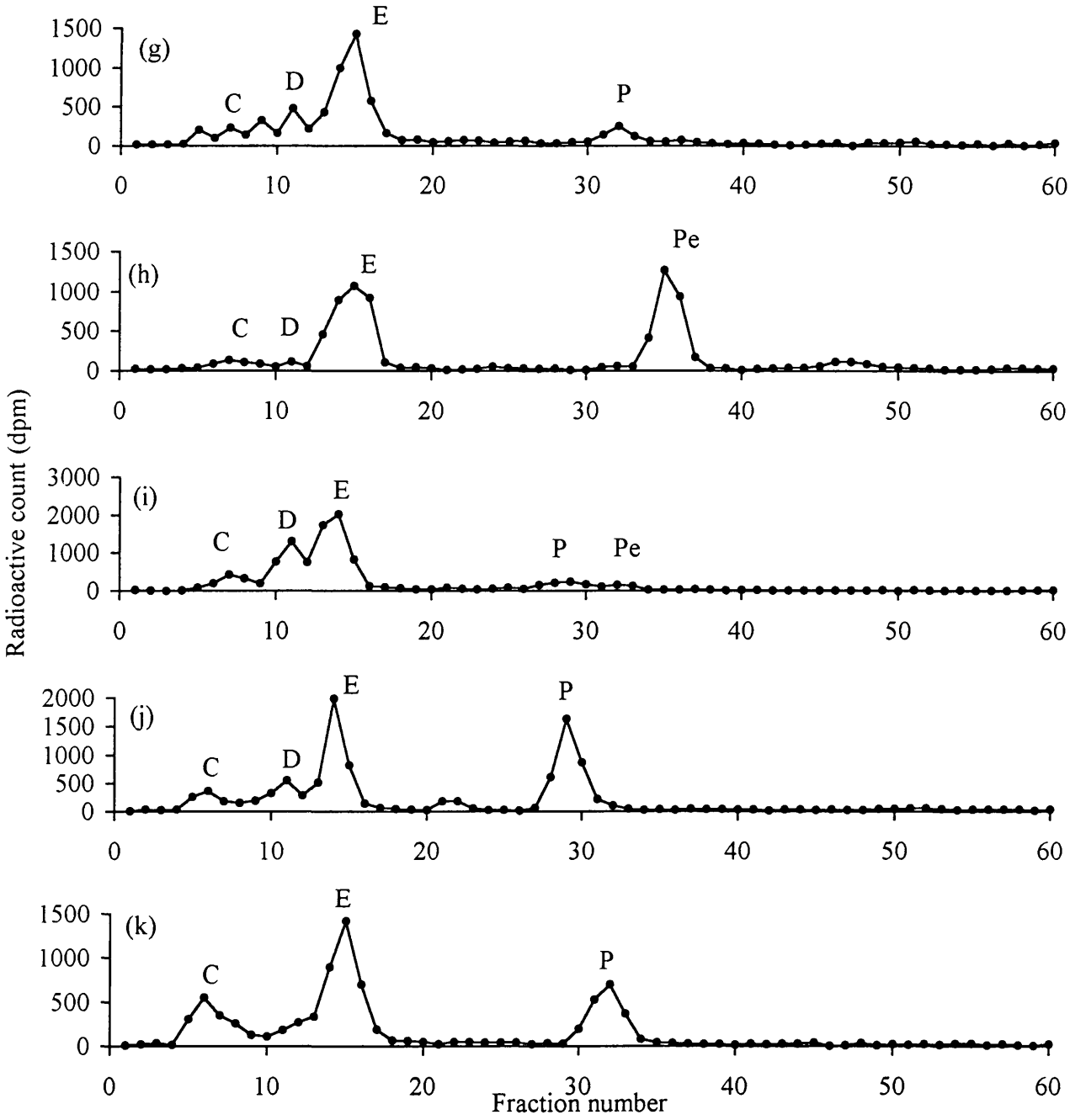
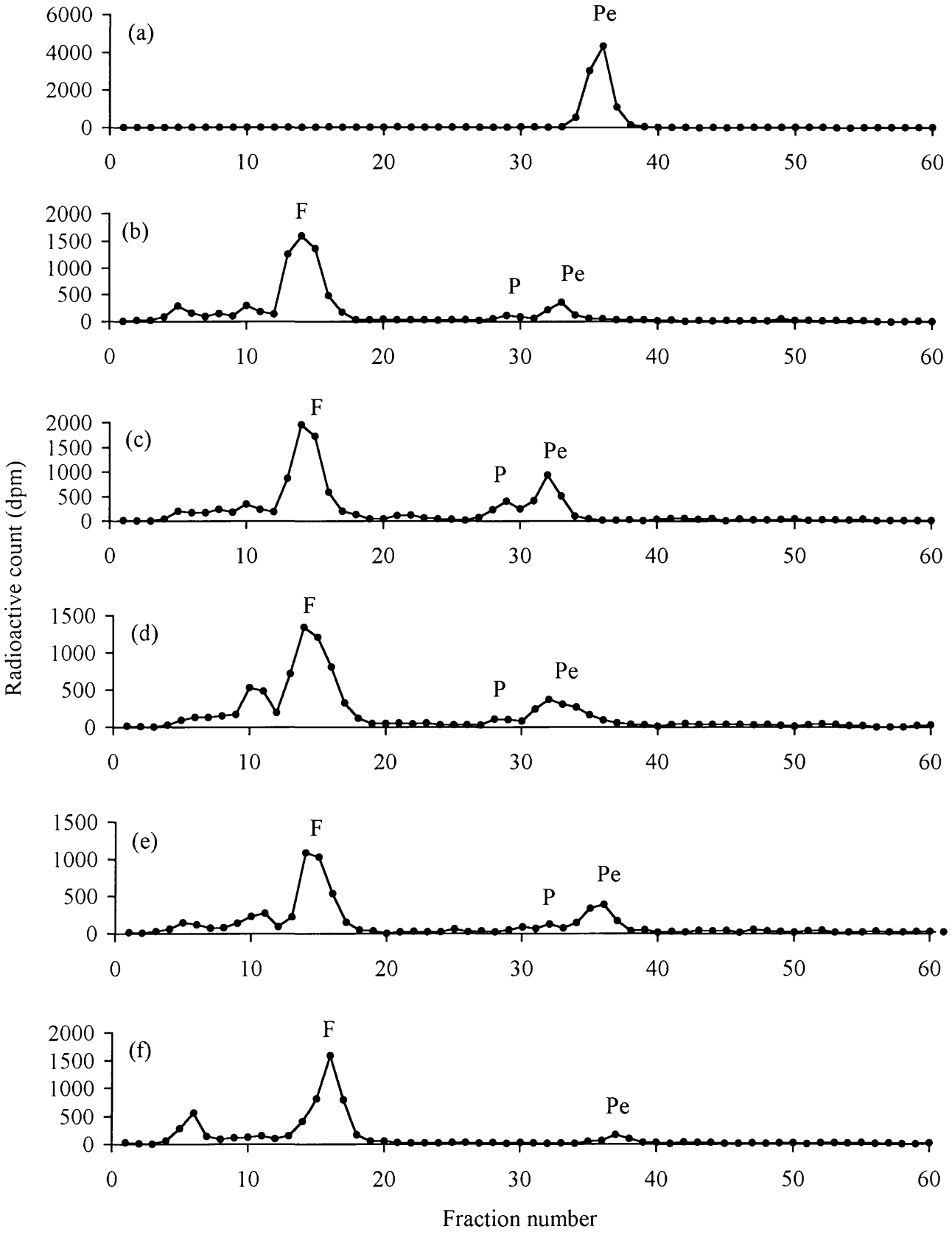


Fig. 14. HPLC radioactive elution profiles after three hour incubations with [^{3}H]pregnenolone and with β-NADPH for (a) a control (i.e. no luteal tissue), and for luteal tissue of (b and c) non-, (d) early-, (e, f, g and h) mid- and (i, j and k) late-pregnant hyraxes. Peaks corresponding to pregnenolone (Pe), progesterone (P), compound C, compound D and compound E are labelled accordingly.



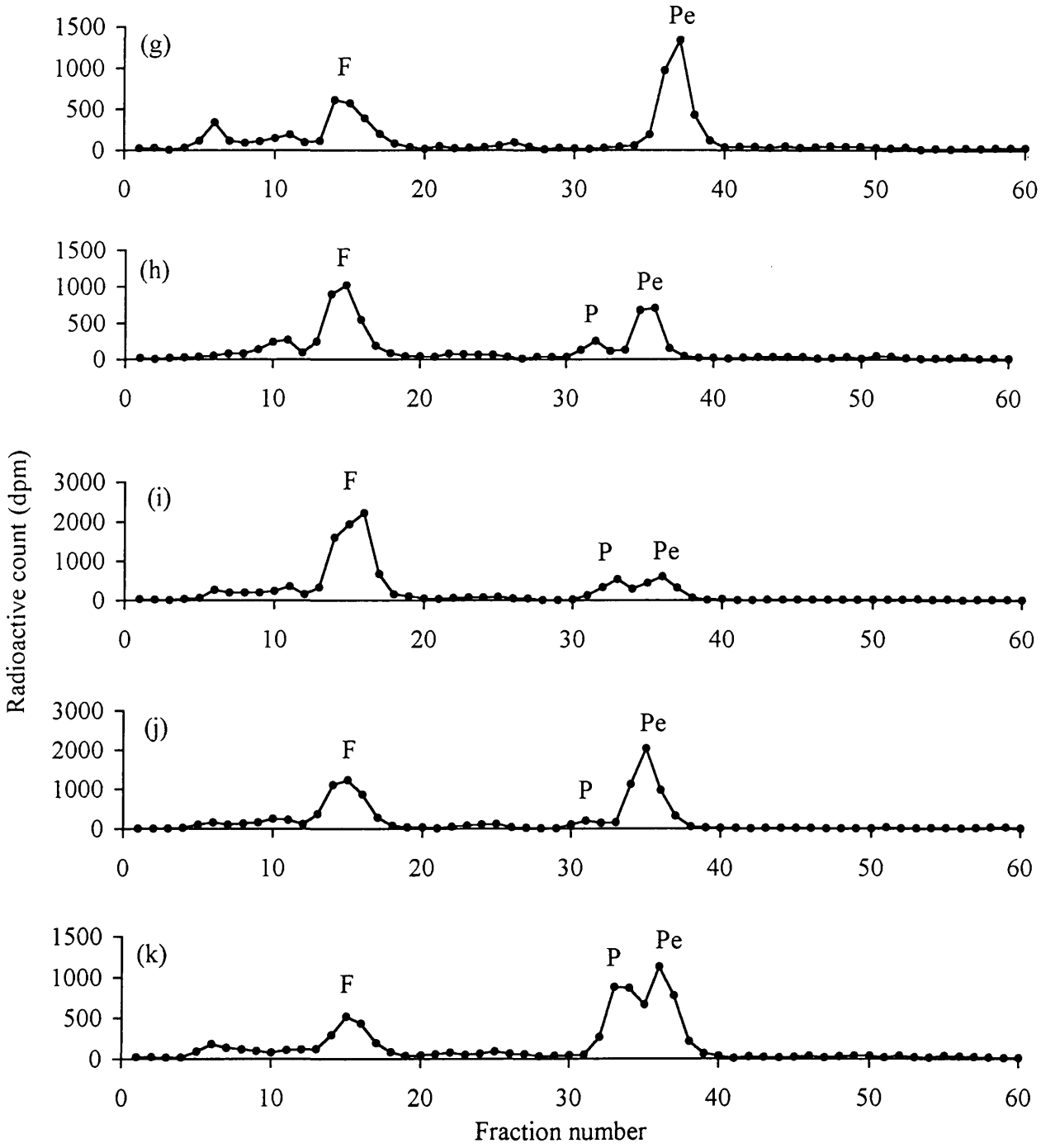
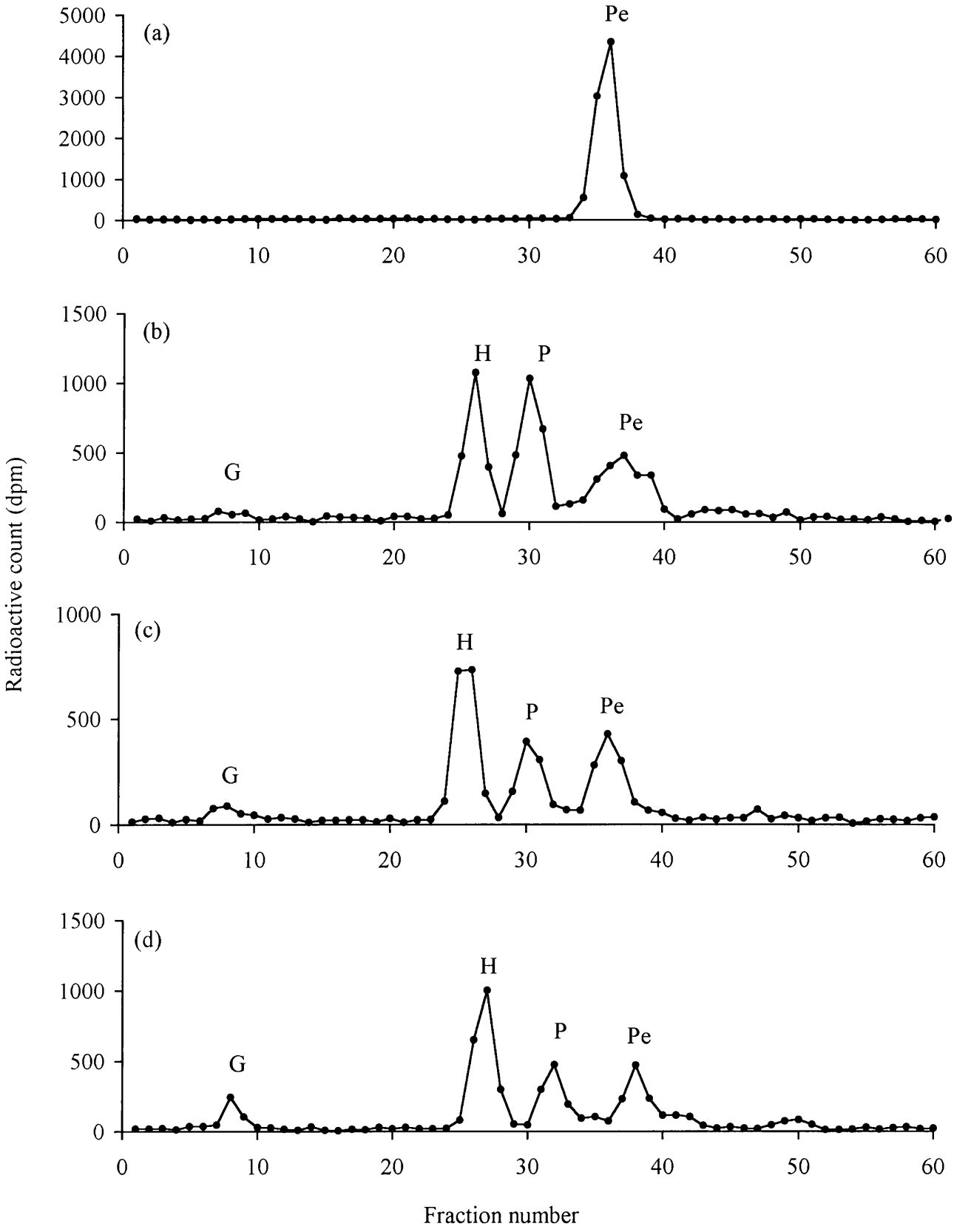


Fig. 15. HPLC elution profiles of radioactivity after three hour incubations of (a) a control (i.e. no ovarian residual tissue), and of ovarian residual tissue from (b and c) non-, (d) early-, (e, f, g and h) mid- and (i, j and k) late-pregnant hyraxes, with [^{3}H]pregnenolone and β-NADPH. Peaks corresponding to pregnenolone (Pe), progesterone (P) and compound F are labelled accordingly.



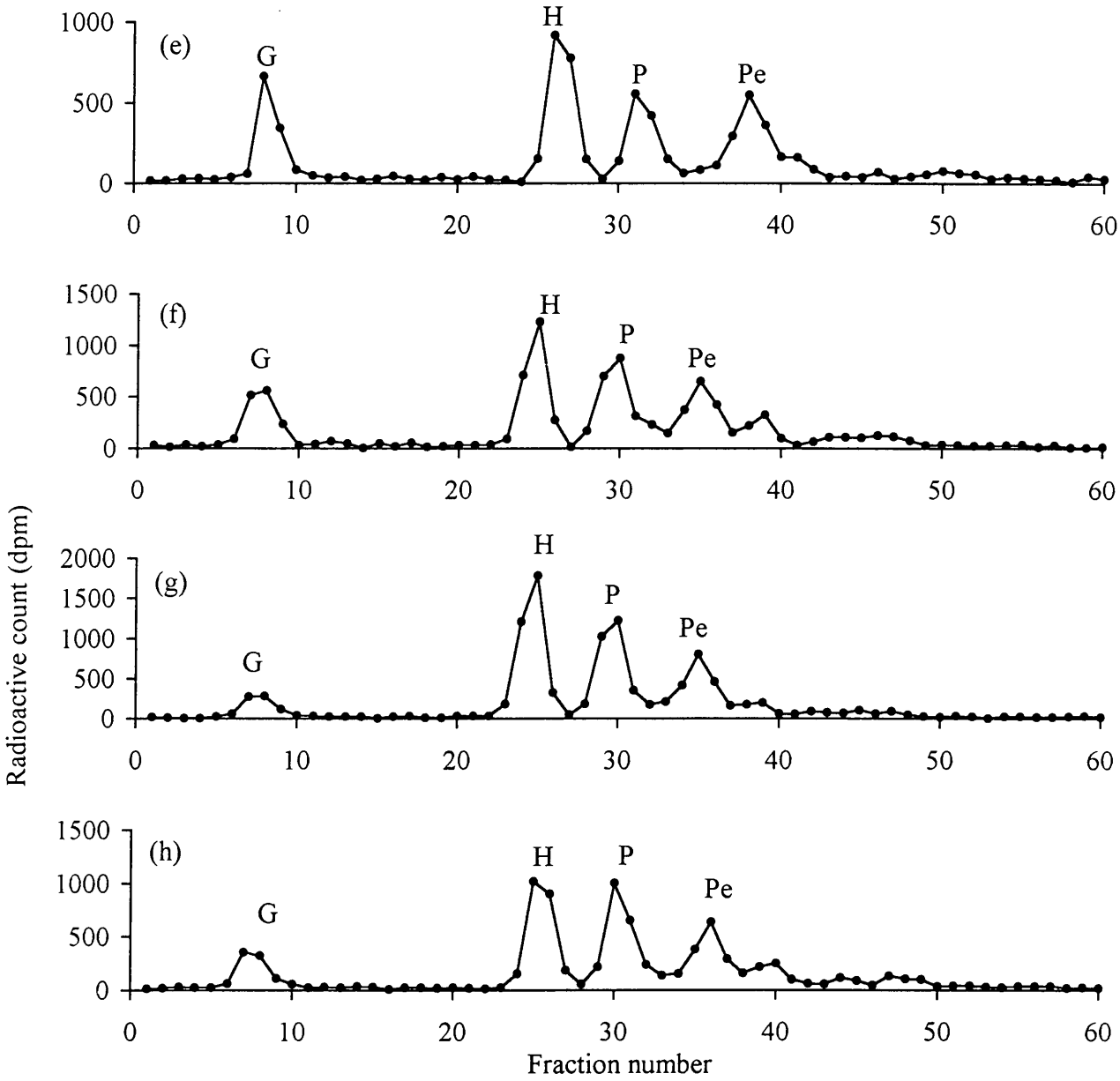


Fig. 16. HPLC elution profiles of radioactivity after three hour incubations of (a) a control (i.e. no placental tissue), and of placental tissue from (b, c, d and e) mid- and (f, g and h) late-pregnant hyraxes, with [^{3}H]pregnenolone and β-NADPH. Peaks corresponding to pregnenolone (Pe), progesterone (P), compound G and compound H are labelled accordingly.

Table 13. The influence of the incubation mixture on the metabolism of pregnenolone and the production of compound F and other compounds by the ovarian residual tissue, and the influence of incubation mixture and time on the metabolism of pregnenolone and the production of progesterone, compounds G, H and other compounds by the placental tissue of a mid-pregnant hyrax.

		Amount converted/formed (%)					
		In the presence of β-NADPH			In the absence of β-NADPH (3 hours)		
Tissue	Compound	1 hour	2 hour	3 hour	PBS present	Oestrone present	Sodium fluoride and mercury chloride present
Ovarian residual tissue	Pregnenolone	-	-	81	35	35	2
	Compound F	-	-	56	24	27	0
	Other compounds	-	-	18	0	4	2
Placental tissue	Pregnenolone	63	69	75	36	41	5
	Progesterone	0	18	32	10	17	0
	Compound G	11	10	0	5	4	5
	Compound H	53	42	28	22	20	0
	Other compounds	0	0	14	0	0	0

tissue was 5% higher than the amount metabolised in the absence of oestrone. The amount of progesterone formed by placental tissue was higher when oestrone was present.

Ovarian residual tissue metabolised 44 to 94% pregnenolone (n = 10). The amount metabolised during non-pregnancy was 63 and 90% (n = 2; Table 14) and during pregnancy (n = 8) was 73.1 ± 6.1% (mean ± SEM). Reproductive status did not affect the amount of pregnenolone that was metabolised (H_3 = 0.53, $p > 0.05$). Compound F was the principal metabolite, and the amount formed was not affected by reproductive stage (H_3 = 1.09, $p > 0.05$). The amount of progesterone formed was low and was not affected by reproductive stage (H_3 = 4.6, $p > 0.05$).

The metabolism of pregnenolone by luteal tissue ranged from 61.0 to 100% (n = 10). Luteal tissue of non-pregnant animals (n = 2) metabolised 81 and 89% and that of pregnant animals (n = 8) metabolised 90.9 ± 4.9% (mean ± SEM; Table 15[4]). The amount of pregnenolone metabolised was not affected by reproductive stage (H_3 = 3.29, $p > 0.05$), although pregnenolone was totally metabolised during late pregnancy. Compound E was the principal metabolite and amounts formed were similar between reproductive stages (H_3 = 2.74, $p > 0.05$). Relatively high amounts of progesterone were formed from pregnenolone, but amounts formed were not affected by reproductive stage (H_3 = 4.17, $p > 0.05$). Low amounts of compounds C and D were formed and these too were unaffected by stage of pregnancy (H_3 = 2.82 and 2.27 respectively, $p > 0.05$).

Placental tissue metabolised 70.5 to 80.5% (n = 7) pregnenolone and tissue of late-pregnant animals metabolised significantly more pregnenolone than that of mid-pregnant animals (Z (U = 0) = −2.14, $p < 0.05$; Table 16). The amounts of progesterone, compounds G and H formed did not differ between reproductive stages (Z (U = 3.0, 5.0 and 3.5, respectively) = −1.11, −0.35 and −0.89,

[4] Gombe (1983) found that hyraxes had a mean oestrous cycle length of 13.4 ± 1.31 days and cycled in captivity. Therefore it is possible for non-pregnant hyraxes to have corpora lutea. O'Donoghue (1963) observed in a *Dendrohyrax* sp. that corpora lutea formed after ovulation and if pregnancy did not follow the corpora lutea regressed. Van der Merwe & Skinner (1982) also suggested that corpora lutea in non-pregnant animals may be due to unfertilised ova. However, the possibility remains that, in this study, animals classified as non-pregnant may have been at the initial stage of pregnancy. Thus, the term non-pregnant used in this study refers to all females where no embryos or foetuses could be found during macroscopic observations.

Table 14. The influence of reproductive status on the metabolism of pregnenolone by ovarian residual tissue, as well as the production of progesterone, compound F and other compounds (mean ± SEM for the number of individual animals investigated in parentheses) after three hour incubations with [^{3}H]pregnenolone and β-NADPH.

Reproductive status	Pregnenolone converted (%)	Metabolites formed (%)		
		Progesterone	Compound F	Other compounds
Non pregnant (2)	63.0 and 90.0	6.0 and 3.0	42.0 and 67.0	15.0 and 20.0
Early pregnant (1)	86.0	18.5	47.0	20.5
Mid pregnant (4)	72.0 ± 10.6	2.5 ± 1.5	49.5 ± 8.3	20.0 ± 2.7
Late pregnant (3)	70.3 ± 9.3	12.8 ± 5.9	41.7 ± 9.4	15.8 ± 1.8

Table 15. The influence of reproductive status on the metabolism of pregnenolone and the formation of progesterone, compounds C, D, E and other compounds (mean ± SEM for the number of animals investigated in parentheses) by luteal tissue after three hour incubations with [^{3}H]pregnenolone and β-NADPH.

		Metabolites formed (%)				
Reproductive status	Pregnenolone converted (%)	Progesterone	Compound C	Compound D	Compound E	Other compounds
Non pregnant (2)	81.0 and 89.0	35.0 and 22.0	0 and 9.0	0 and 9.0	40.0 and 49.0	6.0 and 0
Early pregnant (1)	84.0	25.5	15.0	10.0	31.0	2.5
Mid pregnant (4)	86.5 ± 9.2	7.5 ± 2.7	11.8 ± 8.2	11.3 ± 3.2	48.3 ± 3.6	7.6 ± 3.1
Late pregnant (3)	99.0 ± 1.0	22.0 ± 8.2	13.3 ± 3.8	13.7 ± 6.8	48.0 ± 5.1	2.0 ± 1.0

Table 16. The influence of mid and late pregnancy on the metabolism of pregnenolone and the production of progesterone, compounds G, H and other compounds (mean ± SEM for the number of individual animals investigated which appear in parentheses) by placental tissue after three hour incubations with [^{3}H]pregnenolone and β-NADPH.

		Metabolites formed (%)			
Reproductive status	Pregnenolone converted (%)	Progesterone	Compound G	Compound H	Other compounds
Mid pregnant (4)	72.5 ± 0.9	23.0 ± 3.0	8.8 ± 3.7	34.6 ± 2.6	6.1 ± 2.8
Late pregnant (3)	80.0 ± 0.5	26.0 ± 0.5	10.2 ± 1.9	30.0 ± 1.8	13.8 ± 1.0

respectively, $p > 0.05$). However, the amount of "other" compounds formed during late pregnancy was twofold higher than the amount formed during mid pregnancy.

The metabolism of [^{3}H]progesterone by the various tissues during incubations

Progesterone was metabolically converted by luteal tissue, ovarian residual tissue and placental tissue during three hour incubations with [^{3}H]progesterone and β-NADPH (Table 12). Luteal tissue metabolised progesterone to compounds C, D and E (Table 17; Fig. 17). Compound E was once again the principal metabolite. The amount of progesterone metabolised was six-fold higher during early pregnancy than during late pregnancy. High amounts of compound E were formed by luteal tissue of the non- and early-pregnant animals and none was formed in the late-pregnant stage. Relatively low amounts of compounds C and D were formed.

Ovarian residual tissue metabolised progesterone to compound F (Table 18; Fig. 18). The amount of progesterone metabolised and the amount of compound F formed was higher in pregnant animals than in the non-pregnant animal. Progesterone metabolism was similar during non and late pregnancy. Ovarian residual tissue of early- and mid-pregnant animals metabolised similar amounts of progesterone and the amount metabolised was up to 30% higher than during non and late pregnancy. The formation of compound F followed the same trends as those exhibited by the amount of progesterone metabolised.

The placenta metabolised progesterone to compound G (Table 19; Fig. 19). Compound H was not formed at all. The amount of progesterone metabolised by placental tissue was similar in the mid- and late-pregnant animals. The amount of compound G formed was lower, and that of "other" compounds formed was higher, during late pregnancy than during mid pregnancy.

Table 17. The influence of reproductive stage (one animal in each stage) on the metabolism of progesterone and the formation of compounds C, D, E and other compounds after three hour incubations of luteal tissue with [^{3}H]progesterone and β-NADPH.

Reproductive status	Progesterone converted (%)	Metabolites formed (%)			
		Compound C	Compound D	Compound E	Other compounds
Non pregnant	65.0	8.0	12.0	45.0	0
Early pregnant	65.0	7.0	14.0	44.0	0
Late pregnant	10.0	5.5	4.5	0	0

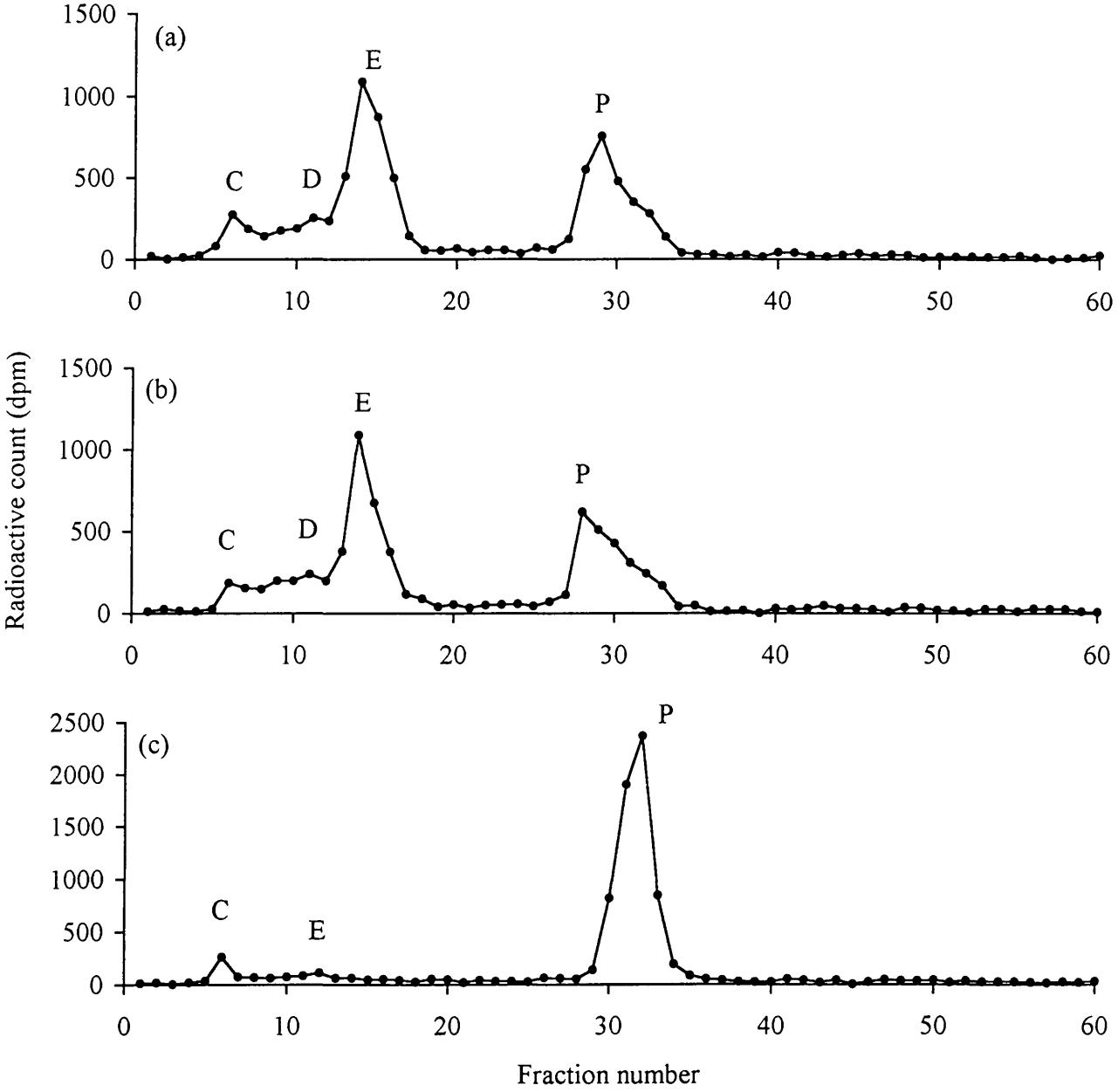


Fig. 17. HPLC radioactive profiles after three hour incubations of luteal tissue of a (a) non-, (b) early- and (c) late-pregnant hyrax, with [^{3}H]progesterone and β-NADPH. Progesterone (P), compounds C, D and E are labelled accordingly.

Table 18. The influence of reproductive status on the metabolism of progesterone and the production of compound F and other compounds by ovarian residual tissue (one animal in each reproductive stage) after three hour incubations with [^{3}H]progesterone and β-NADPH.

		Metabolites formed (%)	
Reproductive status	Progesterone converted (%)	Compound F	Other compounds
Non pregnant	50.5	32.5	18.0
Early pregnant	76.0	54.0	22.0
Mid pregnant	76.0	50.0	26.0
Late pregnant	45.5	21.5	24.0
Pregnant*	65.8 ± 10.2	41.8 ± 10.2	24.0 ± 1.2

*Mean ± SEM, $n = 3$

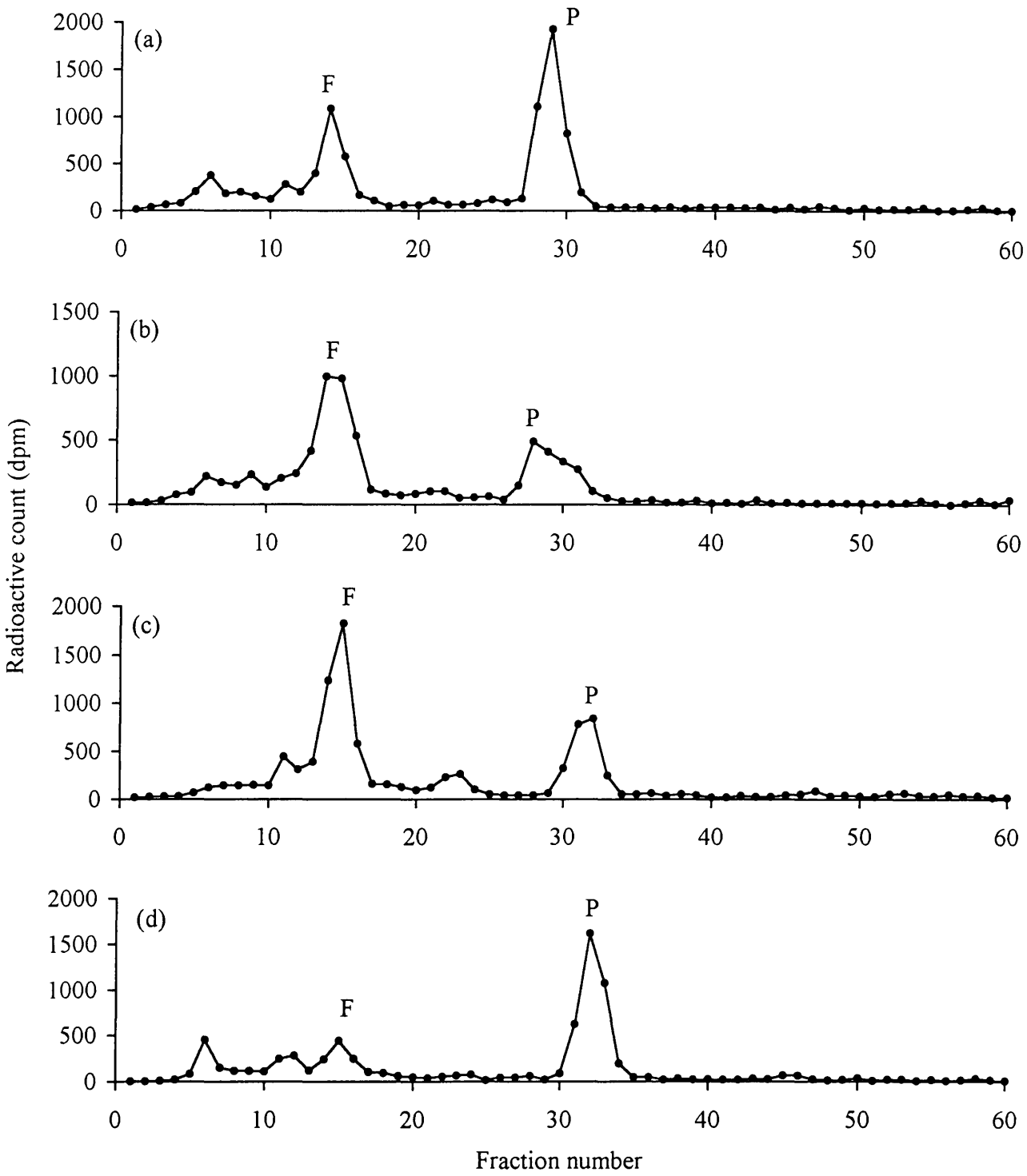


Fig. 18. HPLC profiles of radioactivity after three hour incubations of ovarian residual tissue of a (a) non-, (b) early-, (c) mid- and (d) late-pregnant hyrax, with [^{3}H]progesterone and β-NADPH. Progesterone (P) and compound F are labelled accordingly.

Table 19. The metabolism of progesterone and the production of compound G and other compounds by placental tissue of a mid- and late-pregnant animal after incubation for three hours with [^{3}H]progesterone and β-NADPH.

		Metabolites formed (%)	
Reproductive status	Progesterone converted (%)	Compound G	Other compounds
Mid pregnant	49.0	16.0	33.0
Late pregnant	52.0	11.5	40.5

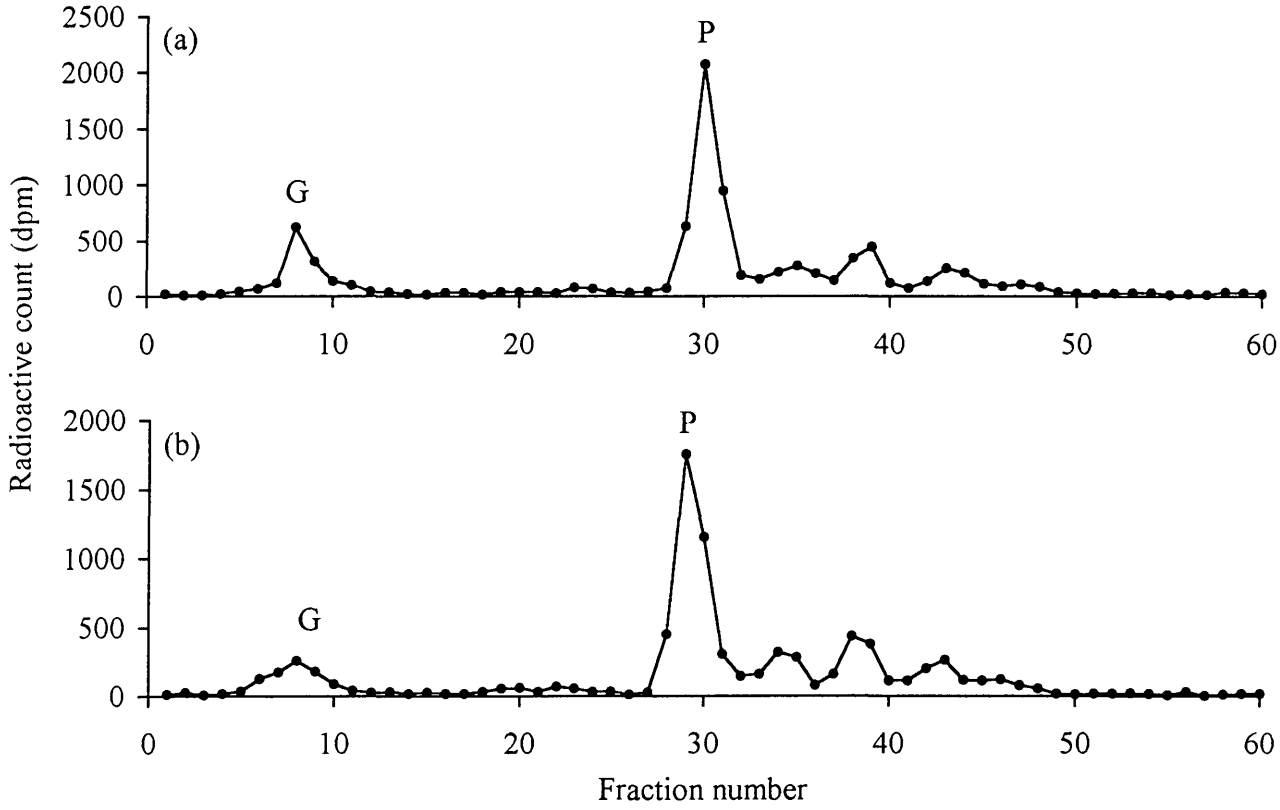


Fig. 19. HPLC radioactive profiles after incubation of placental tissue of a (a) mid- and (b) late-pregnant hyrax, for three hours with [^{3}H]progesterone and β-NADPH. Progesterone (P) and compound G are labelled accordingly.

The metabolism of both [^{3}H]pregnenolone and [^{3}H]progesterone by the various tissues during incubations

Ovarian residual tissue metabolised pregnenolone and progesterone mainly to compound F. Placental tissue metabolised both pregnenolone and progesterone, producing compounds G and H. Luteal tissue was not incubated with both pregnenolone and progesterone since there was insufficient tissue available.

Partial identification of compounds C, D, E, F, G and H by HPLC

The elution positions of the unlabelled standards, namely progesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone, 17α-hydroxyprogesterone and 11α-hydroxyprogesterone, as well as those of [^{3}H]progesterone, [^{3}H]oestradiol-17β and the labelled unknown conversion products, compounds C, D, E, F, G and H, are presented in Table 20. The difference in retention time between [^{3}H]progesterone and unlabelled progesterone is approximately 30 seconds. This indicates that the progesterone fraction is collected by the fraction collector about 30 seconds after the progesterone fraction is detected by the UV detector. Therefore, the labelled conversion products of ovarian and placental tissue would be detected about 30 seconds earlier by the UV detector than by fraction collection. Compound D may correspond to 11α-hydroxyprogesterone or oestradiol-17β, compounds E and F may correspond to 17α-hydroxyprogesterone and compound H may correspond to 20α-dihydroprogesterone. Compounds C and G did not correspond to any of the standards.

The identification of compound E by TLC

The R_f values, for mobile phase one and three, of 17α-hydroxyprogesterone were 0.25 and 0.92 cm, respectively, and for compound E were 0.23 and 0.90 cm, respectively. Compound E had an identical R_f value to 17α-hydroxyprogesterone, as revealed by TLC in mobile phases one and three.

Table 20. HPLC retention times for the unlabelled progesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone, 11α-hydroxyprogesterone and 17α-hydroxyprogesterone, for [^{3}H]progesterone, [^{3}H]oestradiol-17β and for the labelled unknown conversion products, compounds C, D, E, F, G and H.

	Retention time (min)	
Compound	Unlabelled compound	Labelled compound
Progesterone	14.5	15.0
Oestradiol-17β	-	5.5
20α-Dihydroprogesterone	10.3	-
20β-Dihydroprogesterone	13.9	-
17α-Hydroxyprogesterone	6.5	-
11α-Hydroxyprogesterone	4.2	-
Compound C	-	3.0
Compound D	-	5.0
Compound E	-	6.5
Compound F	-	6.5
Compound G	-	3.5
Compound H	-	11.5

Discussion

The objective of this part of the present study was to determine the steroidogenic potential of luteal tissue, ovarian residual tissue and placental tissue following incubation with [^{3}H]pregnenolone and [^{3}H]progesterone in the presence of the co-factor β-NADPH. The influence of the co-factor and the enzyme inhibitors was determined during incubations with ovarian residual and placental tissues and the influence of incubation time was determined during placental tissue incubations. The results revealed that luteal, ovarian residual and placental tissues have the ability to metabolise both pregnenolone and progesterone. These tissues appeared to have a higher rate of metabolism for pregnenolone than progesterone.

Metabolic conversions occurring in ovarian residual and placental tissues result from the activity of enzymes, as demonstrated by the inhibition of the metabolism of pregnenolone when the inhibitors NaF-HgCl were added to the incubate. Oestrone did not inhibit the metabolism of pregnenolone to progesterone and other metabolites. In the pig, progesterone of luteal origin is metabolized to oestrogens by the placenta and the production of oestrone is enhanced if pregnenolone is added to the incubate (Knight & Kukoly 1990). Therefore, if the placenta of the hyrax is a source of oestrone, then adding oestrone to the incubate should not inhibit steroid biosynthesis by the placenta.

The co-factor β-NADPH had a definite impact on the metabolism of pregnenolone by ovarian residual and placental tissues, and the fact that the metabolism increased in its presence also indicates that enzyme systems, which require co-factors, are involved in these metabolic conversions. However, the co-factor did not appear to be necessary for these metabolic conversions to occur. The duration of the incubation affected the metabolism of pregnenolone by placental tissue. More pregnenolone was metabolised, and more progesterone was formed, the longer the incubation.

Luteal tissue metabolised pregnenolone and progesterone to three compounds (C, D and 17α-hydroxyprogesterone) more polar than progesterone, the most important being 17α-hydroxyprogesterone. Compound D has been partially identified as 11α-hydroxyprogesterone or oestradiol-17β. The metabolism of pregnenolone to progesterone suggests the presence of the 3β-hydroxysteroid dehydrogenase and isomerase enzyme complex in hyrax luteal tissue, also present in corpora lutea of a number of mammals such as the pig (Lemon & Mauléon 1982; Bernal 1989), western spotted skunk (*Spilogale putorius latifrons*; Ravindra *et al.* 1984), rat, rabbit, guinea pig, cow and human (Davies *et al.* 1966). This observation is supported by the fact that corpora lutea of pregnant hyraxes can produce an appreciable amount of progesterone (Gombe *et al.* 1976; Van Aarde & Anderson 1989). In the African elephant, progesterone levels in corpora lutea are low and the major luteal progestins are 5α-dihydroprogesterone and 5α-pregnan-3α-ol-20-one (Heistermann *et al.* 1994). The corpus luteum of the human also secretes significant amounts of 5α-dihydroprogesterone (Bäckström, Andersson, Baird & Selstam 1986), but in the Western spotted skunk none of this steroid is formed (Ravindra *et al.* 1984). Both 5α-dihydroprogesterone and 5α-pregnan-3α-ol-20-one are less polar than pregnenolone and progesterone and no evidence of their presence in extracts from hyrax luteal tissue could be found. Therefore, 5α-dihydroprogesterone and 5α-pregnan-3α-ol-20-one do not appear to be secreted by the corpora lutea of the rock hyrax unlike the elephant. The biosynthetic pathway in the corpus luteum of the hyrax follows the metabolism of pregnenolone to progesterone, and of progesterone to compounds C, D and 17α-hydroxyprogesterone.

Ovarian residual tissue metabolised pregnenolone to progesterone and progesterone to compound F. Since pregnenolone was metabolically converted to progesterone, the activity of 3β-hydroxysteroid-dehydrogenase isomerase is present. Progesterone concentrations in hyrax ovarian residual tissue are apparently very low (Heap *et al.* 1975; Van Aarde & Anderson 1989) and this is supported by the fact that in this study little progesterone was formed from pregnenolone and that

progesterone metabolism occurs to a great extent in this tissue. Compound F is more polar than progesterone and has been partially identified as 17α-hydroxyprogesterone and does not correspond to 20α-dihydroprogesterone. Rabbit ovarian residual tissue produced 20α-dihydroprogesterone (Johnson & Everitt 1988). Wiebe *et al.* (1994) suggested that the rat ovary produces 3α-hydroxy-4-pregnen-20-one as a main steroid and that the 4-pregnene cycle in the ovary, which involves progesterone, 3α-hydroxy-4-pregnen-20-one, 20α-dihydroprogesterone and 4-pregnene-3α,20α-diol, may aid in the regulation of concentrations of biologically active steroids like progesterone and 3α-hydroxy-4-pregnen-20-one (Wiebe *et al.* 1994). Positive identification of compound F is necessary to allow further speculation on the biosynthesis of steroids by the ovarian residual tissue of the rock hyrax.

Luteal tissue had a higher biosynthetic potential than ovarian residual tissue, but for both tissues the metabolism of pregnenolone was not affected by reproductive stage. However, progesterone metabolism by luteal tissue and ovarian residual tissue appeared to be lowest during late pregnancy. The biosynthetic activity of these tissues may be related to pregnancy, although corpus luteum regression in the later stage of pregnancy does not appear to occur in the hyrax (Chapter 3). The low level of progesterone metabolism, and thus formation of progesterone metabolites, during late-pregnancy by luteal and ovarian residual tissue may be complemented by an increase in the steroidogenic potential of some other organ, for example the placenta, which takes over the maintenance of pregnancy in the sheep (Flint *et al.* 1983) and cow (Shemesh 1990). However, bilateral ovariectomy in pregnant hyraxes resulted in abortion (Gombe *et al.* 1977, in Oduor-Okelo *et al.* 1983). Steroid production and metabolism during pregnancy is characterised by the complementary functions of the foetal, placental and maternal components and removal of one of these compartments may significantly alter the function of the others (Cheesman 1982). It is therefore possible that both ovarian and placental tissues of the hyrax are contributing to the maintenance of pregnancy and the removal of either of these tissues leads to foetal loss.

The steroidogenic pathway in placental tissue follows the metabolism of pregnenolone to compound H and the metabolism of pregnenolone to progesterone. Progesterone is subsequently metabolised to compound G. Compounds G and H are less polar than progesterone. In the rock hyrax the placenta is not an important source of progesterone (Van Aarde & Anderson 1989) and placental progesterone concentrations are low (Heap *et al.* 1975). Therefore, a metabolite of progesterone, namely compound G or H, may rather be an important biosynthetic product of the placenta. Compound H has been partially identified as 20α-dihydroprogesterone. 20α-Dihydroprogesterone inhibits the metabolism of pregnenolone to progesterone in the placenta of the cow, goat and rhesus monkey (Wiener 1976). It is worthwhile to note that if 20α-dihydroprogesterone is an important biosynthetic product of the hyrax placenta, it may be involved in the control and/or maintenance of progesterone production and metabolism during pregnancy. Compound G which is formed by the placenta may correspond to compound C which is formed by the corpus luteum, since these compounds have a similar retention time. Although very low amounts of these compounds were formed, the placenta had an 8-fold (during early pregnancy) up to a 319-fold (during late-pregnancy) greater capacity to metabolise progesterone than the corpus luteum. Therefore, the possibility exists that the placenta may take over the steroidogenic activity of the corpus luteum as pregnancy progresses.

The placenta metabolised significantly more pregnenolone during late pregnancy than during mid pregnancy, although the amount of metabolites formed were similar for mid and late pregnant animals. The increased metabolic conversion of pregnenolone during late pregnancy seems to result in an increase in the formation of "other" compounds. From placental incubations with progesterone it was observed that the amount of compound G formed was lowest, and the amount of "other" compounds formed was highest, during late pregnancy. This seems to suggest that as pregnancy progresses the metabolism of compound G to its metabolites (i.e. "other " compounds) increases or, on the other hand, less progesterone is metabolised to compound G and more is metabolised to

"other" compounds. The increase in steroidogenic potential during pregnancy by the placenta may be related to the excretion of metabolites in the urine (Ainsworth & Ryan 1969), possibly represented by the "other" compounds formed by the placenta. However, since the metabolism of pregnenolone and progesterone differs between ovarian and placental tissue, the steroidogenic activity of the placenta may have a complementary function to the ovary in the maintenance or termination of pregnancy.

Conclusion

The present study indicates that luteal, ovarian residual and placental tissues of the rock hyrax exhibit steroidogenic activity, and that the metabolic rate of pregnenolone was higher than that of progesterone. Enzymes are responsible for the metabolism of pregnenolone by ovarian and placental tissues. However, oestrone did not inhibit pregnenolone metabolism in these tissues. The presence of β-NADPH increased pregnenolone metabolism but was not a prerequisite for steroid biosynthesis. More pregnenolone was metabolised by the placenta the longer the incubation. Incubations with progesterone showed that the steroidogenic activities of these tissues is highest during pregnancy, indicating that these tissues may be important in the production of steroids necessary for the maintenance of pregnancy or for the initiation of parturition. Ovarian and placental tissues could have complementary roles during pregnancy since the metabolism of pregnenolone and progesterone differed between these tissues. 17α-Hydroxyprogesterone is produced by luteal tissue. The remaining compounds formed by the ovarian and placental tissues have been partially identified. However, their positive identification is essential to understand the contribution of each tissue to the maintenance of pregnancy. *In vivo* studies concentrating on the effect of these compounds on pregnancy will increase the understanding of the biological role of steroidogenesis by these tissues.

CHAPTER 6

SYNTHESIS

The manipulation of reproductive output is necessary in cases where the population growth rate of a pest species needs to be lowered or that of an endangered species increased. In order to do this, an understanding of the endocrinology of reproduction of the target species is required. The rock hyrax is a pest species in some areas where it occurs (Kolbe 1983) and reducing reproduction output by using contraceptives may be an option in controlling their numbers, although logistically such control may be difficult or not feasible. However, not much is known about the reproductive endocrinology of the hyrax and the aim of this investigation was to increase our knowledge of the reproductive endocrinology of this species.

The steroidogenic potential of blood and the luteal, ovarian residual and placental tissues of the hyrax were investigated. It was demonstrated that whole blood and the separate components of blood differ from the tissues in their ability to metabolise the progestin precursors, pregnenolone and progesterone. Steroids are transported from their biosynthetic source via the circulation to the target tissues (e.g. luteal or placental tissues). Any metabolic changes of these steroids in the blood will affect the concentrations and types of steroids that will reach the various target tissues. Since target tissues metabolise steroids, the conversion products may have an ultimate effect on pregnancy, whether they will aid in the maintenance of pregnancy or the initiation of parturition. The white blood cells have a greater steroidogenic potential than any other component of blood, but it appears as if their biosynthetic activity is negligible in blood. Progesterone receptors are known to be present in white blood cells and the binding of progesterone to these receptors suppresses immunity, which is essential for normal human pregnancy (Chiu *et al.* 1996). If the biosynthetic activity of white blood cells is unimportant in whole blood, then the main function of white blood cells may rather be to bind progesterone. Further investigation into the binding of progesterone to the putative receptors during

the development of pregnancy would be necessary to clearly define the actual role of progesterone levels in the circulation of the hyrax, as well as the biological importance of white blood cells.

In most mammals progesterone is the pregnancy hormone, which circulates at relatively high concentrations. However, the hyrax is different to most other mammals in this respect. Circulating progesterone concentrations are very low, while those of 5α-dihydroprogesterone greatly exceed those of progesterone. Whole blood appears to metabolise progesterone to two compounds, namely 5α-dihydroprogesterone and 5α-pregnan-3α-ol-20-one. If this is the case, then the metabolism of progesterone to 5α-dihydroprogesterone by the whole blood, attributed mainly to the metabolic activity of red blood cells, since red and white blood cells together did not metabolise pregnenolone, may explain the low circulating concentrations of progesterone and the high circulating concentrations of 5α-dihydroprogesterone. The fact that the amount of progesterone metabolised by whole blood and its components did not correlate to the amount of 5α-dihydroprogesterone formed, and that progesterone and 5α-dihydroprogesterone concentrations in the plasma weren't correlated to each other, supports the view that 5α-dihydroprogesterone is not a direct metabolite of progesterone but may rather be a metabolite of 5α-pregnan-3α-ol-20-one.

In the African elephant, the concentrations of circulating 5α-reduced progestins reflected corpus luteum function more closely than progesterone (Hodges *et al.* 1997). The corpus luteum of the hyrax does not produce, and thus secrete, the same compounds that are produced by the metabolism of pregnenolone by blood, except for progesterone. The high levels of circulating 5α-dihydroprogesterone do not reflect corpus luteum, ovarian or placental function since these tissues did not produce compounds exhibiting the chromatographic behaviour of 5α-dihydroprogesterone following HPLC fractionation of tissue extracts. Therefore, the origin of circulating 5α-dihydroprogesterone seems to be from the metabolism of progesterone by the blood. Steroids that are normally present in high concentrations during normal pregnancy may have an inhibitory or

stimulatory effect on the production of progesterone by the placenta (Grimshaw *et al.* 1983). Grimshaw *et al.* (1983) reported that 5α-dihydroprogesterone stimulated the human placenta to form progesterone. The present study indicated that the placenta is capable of producing progesterone and thus the high concentrations of circulating 5α-dihydroprogesterone may stimulate progesterone synthesis by the placenta.

The luteal tissue of the hyrax contains significant concentrations of progesterone (Gombe *et al.* 1976; Van Aarde & Anderson 1989) and the ability of luteal tissue to metabolise pregnenolone to progesterone suggests that luteal tissue is able to synthesise progesterone *de novo*. Since hormonal support of pregnancy in the hyrax seems to be derived from the ovarian tissues (Gombe *et al.* 1977, in Oduor-Okelo *et al.* 1983), either progesterone or the metabolites of progesterone produced by luteal tissue may maintain pregnancy. However, the placenta may also be contributing to the maintenance of pregnancy. Since the metabolites of pregnenolone and progesterone formed by the ovarian and placental tissues differ, these tissues may have complementary functions in the maintenance of pregnancy and/or onset of parturition (Cheesman 1982). The metabolism of progesterone by the placenta may also be related to the removal of excess amounts of progesterone in the blood which may be detrimental to the foetus.

The steroidogenic pathway in the pregnant female hyrax may follow the metabolism of pregnenolone to progesterone by corpora lutea, after which the progesterone enters the circulation where it is metabolised to 5α-reduced metabolites. These metabolites may then be transported to the uterus where they bind to the progesterone receptors, resulting in the maintenance of pregnancy. Progesterone may be transported to the placenta where it is metabolised to compounds which may be necessary for the maintenance and termination of pregnancy or which may be excreted in the urine. The African elephant is phylogenetically related to the hyrax (De Jong *et al.* 1981; Kleinschmidt *et al.* 1986; Prinsloo 1993; Lavergne *et al.* 1996; Stanhope *et al.* 1996) and therefore it is possible that

the hyrax may be used as a model for developing a contraceptive in the elephant. However, these two species seem to differ greatly in their reproductive endocrinology. The white blood cells of the elephant have no steroidogenic potential and whole blood metabolically converts progesterone to metabolites that are not formed by hyrax blood (Ford 1997). The steroidogenic activity of the placenta (Ford 1997) and of corpora lutea (Heistermann *et al.* 1994; Ford 1997) of the elephant also differ from that of the hyrax. Therefore, interspecific differences exist in the ability of target tissues to transform and synthesise steroids. However, both the elephant and the hyrax have high circulating levels of 5α-dihydroprogesterone and low circulating levels of progesterone. Also, hyrax blood metabolises progesterone to the two 5α-reduced metabolites which are also formed by the luteal tissue of the elephant. Therefore, it is possible that in both species the steroid(s) responsible for the maintenance of pregnancy are similar. The hyrax may be used as a model for the development of a contraceptive which can then be applied to the elephant.

The results of this study suggest a need for further investigations, which are necessary to understand the reproductive endocrinology of the rock hyrax, as well as for the development of a contraceptive for this species. The following investigations are suggested:

- the positive identification of the principal conversion products formed by luteal, ovarian residual and placental tissues during incubations with pregnenolone and progesterone,
- the determination of progesterone binding to receptors on white blood cells throughout pregnancy,
- the investigation into the existence of progesterone binding plasma proteins,
- competitive ligand binding studies of the uterine progesterone receptors, and
- the administration of successful competitors to the animal *in vivo* in order to determine their contraceptive abilities as well as any detrimental effects they may have on the animal's health and behaviour.

SUMMARY

The objectives of this study were to determine the circulating concentrations of progesterone, 5α-dihydroprogesterone and oestradiol-17β. The *in vitro* progestin biosynthetic potential of whole blood, plasma, red and white blood cells, ovarian tissue and the placenta of pregnant and non-pregnant rock hyraxes was also determined. The temporal changes in the percentage of compounds formed from the metabolism of pregnenolone and progesterone was described and the role of red blood cells, white blood cells and plasma in such metabolic conversions was investigated. The influence of the co-factor β-NADPH and of the enzyme inhibitors oestrone and NaF-HgCl on the metabolism of pregnenolone by blood, ovarian residual and placental tissue was determined. The influence of incubation time on the amount of pregnenolone metabolised by blood and the placenta was also determined.

5α-Dihydroprogesterone plasma concentrations ranged from 1.41 to 9.57 ng/ml and were up to 17-fold higher than those of progesterone. 5α-Dihydroprogesterone plasma concentrations were not affected by foetal age. Progesterone plasma concentrations ranged from 0.42 to 5.91 ng/ml and concentrations did not change with an increase in foetal age. Progesterone and 5α-dihydroprogesterone concentrations were not correlated to each other, which may be a result of the metabolism of these steroids by the blood. Oestradiol-17β concentrations ranged from 28.22 to 138.49 pg/ml and concentrations peaked during late pregnancy. Oestradiol-17β may be biologically important prior to parturition.

The presence of β-NADPH in the incubate increased pregnenolone metabolism by white blood cells, ovarian residual tissue and the placenta, but did not affect the type of compounds formed. The presence of the inhibitors NaF-HgCl totally inhibited pregnenolone metabolism by the white blood cells, the ovarian residual tissue and the placenta. These results indicate that the metabolism of

pregnenolone and progesterone is dependent on enzymes. Oestrone reduced pregnenolone metabolism by the white blood cells, whereas it did not affect pregnenolone metabolism by ovarian residual tissue and increased pregnenolone metabolism by placental tissue. More pregnenolone was metabolised by the white blood cells and the placenta the longer the incubation period.

White blood cells metabolised both pregnenolone and progesterone, whereas whole blood, red blood cells and a mixture of red and white blood cells metabolised only progesterone. Plasma displayed no biosynthetic activity. Compound A and compound B, identified as 5α-pregnan-3α-ol-20-one and 5α-dihydroprogesterone, respectively, were formed from all metabolic conversions. The amount of progesterone metabolised was positively correlated to the amount of 5α-pregnan-3α-ol-20-one formed, but was not correlated to the amount of 5α-dihydroprogesterone formed, suggesting that 5α-pregnan-3α-ol-20-one is a direct metabolite of progesterone. White blood cells had the highest steroidogenic potential and the metabolism of pregnenolone to progesterone appears to be the limiting factor in the formation of progesterone metabolites. The biosynthetic activity of white blood cells is unassociated to pregnancy stage and may be negligible or inhibited in whole blood. The possibility exists that as pregnancy progresses the biological activity of white blood cells changes from metabolising progesterone to binding progesterone. However, it remains open to investigation whether or not the main role of white blood cells is to bind progesterone so as to compromise their immune response to the foetus.

The metabolism of progesterone by whole blood, red blood cells and a mixture of red and white blood cells was higher in pregnant than in non-pregnant animals. The formation of 5α-pregnan-3α-ol-20-one by whole blood, red blood cells and a mixture of red and white blood cells during pregnancy was lowest in late-pregnant animals, as well as the formation of 5α-dihydroprogesterone by whole blood. 5α-Pregnan-3α-ol-20-one and 5α-dihydroprogesterone may be important for the maintenance of pregnancy or initiation of parturition in the rock hyrax. The

biological significance of these progesterone metabolites during pregnancy of the hyrax may be understood once they are positively identified and once competitive ligand binding studies on uterine progesterone receptors are carried out. Whole blood exhibited the lowest biosynthetic potential and this may be due to the presence of enzyme inhibitors in the plasma or to the presence of progesterone binding plasma proteins.

Luteal, ovarian residual and placental tissues metabolised both pregnenolone and progesterone. Luteal tissue can form progesterone *de novo* and progesterone is metabolised to three relatively polar compounds (compounds C, D and E). Ovarian residual tissue formed little progesterone and mainly formed one polar compound, compound F, which appears to correspond to compound E. Compounds E has been identified as 17α-hydroxyproegsterone, compound F has been partially identified as 17α-hydroxyprogesterone and compound D as either 11α-hydroxyprogesterone or oestradiol-17β. The amount of pregnenolone metabolised by both tissues was unaffected by reproductive stage, although progesterone metabolism was lowest during late pregnancy. The placenta metabolised pregnenolone to progesterone and to two relatively polar compounds, compounds G and H, whereas compound H was not formed during incubations with progesterone. Compound H has been partially identified as 20α-dihydroprogesterone. The placenta does not appear to take over the biosynthetic role of the corpus luteum, although it may have a complementary function to the ovarian tissues in the maintenance and termination of pregnancy. The placenta of late pregnant animals metabolised significantly more pregnenolone than mid-pregnant animals, which resulted in a greater production of "other" compounds. Therefore, the placenta may be important in metabolising progesterone to metabolites which are excreted in the urine. The positive identification of compounds C to H will clarify the significance of steroidogenesis by the ovary and placenta during pregnancy.

The high concentrations of circulating 5α-dihydroprogesterone produced in the female hyrax do not relate to biosynthesis in the corpus luteum, the ovary or the placenta, but are rather a result of the metabolism of progesterone by blood. The low concentrations of progesterone in plasma are possibly a result of the metabolism of progesterone by blood. The results of this study suggest that progesterone in the pregnant female hyrax is produced by the corpora lutea. This progesterone is then secreted into the circulation where it is metabolised to its 5α-reduced metabolites. These metabolites may display progestational activity and bind to the uterine progesterone receptors for the maintenance of pregnancy.

Although differences exist between the reproductive endocrinology of the rock hyrax and the African elephant, both species are characterised by high plasma concentrations of 5α-dihydroprogesterone and low plasma concentrations of progesterone. The binding of these steroids to the uterine progesterone receptor may be similar and important for the maintenance of pregnancy in both species, and therefore the hyrax may be used as a model for the development of the contraceptive technique. The results of this study indicate that further investigations are necessary to fully understand the reproductive endocrinology of the hyrax.

SAMEVATTING

Die doelstellings van hierdie studie was om plasma konsentrasies van progesteroon, 5α-dihidroprogesteroon en estradiol-17β, en die *in vitro* potensiaal van heel bloed, plasma, rooi- en witbloedselle, ovariale weefsel en plasenta van dragtige en nie-dragtige dassies om hormone te produseer, te bepaal. Die veranderings in die vorming van verbindings vanaf pregnenoloon en progesteroon met dragtigheid is beskryf. Die funksie van rooibloedselle, witbloedselle en plasma in hierdie omskakeling is ondersoek. Die invloed van die kofaktor β-NADPH en die ensieminhibeerders estroon en NaF-HgCl op die omskakeling van pregnenoloon deur bloed, ovariale weefsel en plasenta is bepaal. Die invloed van die inkubasietyd op die omskakeling van pregnenoloon deur bloed en plasenta is ook bepaal.

Die konsentrasie van 5α-dihidroprogesteroon in die plasma het gewissel van 1.41 tot 9.57 ng/ml en was tot 17 keer hoër as dié van progesteroon. 5α-Dihidroprogesteroon konsentrasies in die plasma het nie met fetale ouderdom verander nie. Die konsentrasie van progesteroon in die plasma het gewissel van 0.42 en 5.91 ng/ml en konsentrasies het nie met fetale ouderdom verander nie. Progesteroon en 5α-dihidroprogesteroon konsentrasies het nie met mekaar gekorreleer nie en dit mag die gevolg van die metabolisme van hierdie steroïede deur die bloed wees. Die konsentrasies van estradiol-17β het gewissel tussen 28.22 en 138.49 pg/ml en die hoogste konsentrasies was gedurende laat-dragtigheid bereik. Estradiol-17β mag biologies belangrik wees voor die geboorteproses.

Die teenwoordigheid van β-NADPH het die omskakeling van pregnenoloon deur witbloedselle, ovariale weefsel en die plasenta verhoog, maar dit het nie die tipe verbindings wat gevorm is verander nie. Die teenwoordigheid van die inhibeerders NaF-HgCl het die omskakeling van pregnenoloon deur witbloedselle, ovariale weefsel en die plasenta heeltemal geinhibeer. Hierdie

resultate bevestig dat die metabolisme van pregnenoloon en progesteroon afhanklik is van ensieme. Estroon het die omskakeling van pregnenoloon deur witbloedselle verlaag, maar nie die omskakeling deur die ovariale weefsel beïnvloed nie terwyl dit die omskakeling van pregnenoloon in plasentale weefsel laat toeneem het. Hoe langer die inkubasietydperk was, hoe meer pregnenoloon is omgeskakel deur die witbloedselle en plasenta.

Beide pregnenoloon en progesteroon kan metabolies omgeskakel word deur witbloedselle terwyl slegs progesteroon deur heel bloed, rooibloedselle sowel as 'n mengsel van rooi- en witbloedselle omgeskakel kan word. Plasma het blykbaar nie die vermoë om pregnenoloon of progesteroon om te skakel nie. Verbinding A en verbinding B, onderskeidelik geïdentifiseer as 5α-pregnan-3α-ol-20-oon en 5α-dihidroprogesteroon, is die hoofprodukte van pregnenoloon- en progesteroonmetabolisme in bloed. Die hoeveelheid progesteroon wat omgeskakel is, het positief gekorreleer met die hoeveelheid van 5α-pregnan-3α-ol-20-oon wat gevorm is, maar nie met die hoeveelheid van 5α-dihidroprogesteroon wat gevorm is nie. Dit stel voor dat 5α-pregnan-3α-ol-20-oon'n direkte metaboliet van progesteroon is. Witbloedselle het die hoogste potensiaal vertoon om pregnenoloon en progesteroon te metaboliseer, en dit wil voorkom asof die omskakeling van pregnenoloon na progesteroon die beperkende faktor is in die vorming van progesteroon metaboliete. Die biosintetiese aktiwiteit van witbloedselle is nie deur die stadium van dragtigheid beïnvloed nie en mag onbeduidend of geinhibeerd wees in heel bloed. Die moontlikheid bestaan dat die biologiese aktiwiteit van witbloedselle met verloop van dragtigheid verander van die metabolisme van progesteroon tot progesteroonbinding. Verdere ondersoeke sal bepaal of dit die hoofrol van witbloedselle is om progesteroon te bind om sodoende hulle immunologiese aktiwiteit teen die fetus te inhibeer.

Die omskakeling van progesteroon deur heel bloed, rooibloedselle en 'n mengsel van rooi- en witbloedselle was hoër in dragtige diere as in nie-dragtige diere. Die vorming van 5α-pregnan-3α-ol-

20-oon deur heel bloed, rooibloedselle en 'n mengsel van rooi- en witbloedselle gedurende dragtigheid was die laagste in laat-dragtige diere, sowel as die vorming van 5α-dihidroprogesteroon in heel bloed. 5α-Pregnan-3α-ol-20-oon en 5α-dihidroprogesteroon mag belangrik wees vir die onderhoud van dragtigheid of vir die aanvang van die geboorte proses. Die biologiese betekenis van hierdie metaboliete van progesteroon gedurende dragtigheid in die dassie mag beter begryp kan word wanneer hulle positief geïdentifiseer is en wanneer kompeterende ligandbindingstudies op uterusprogesteroonreseptore uitgevoer is. Heel bloed het die laagste biosintetiese potensiaal vertoon en dit mag aan ensieminhibeerders in die plasma of aan plasma proteïne wat progesteroon bind toegeskryf word.

Luteale en ovariale weefsels en die plasenta het beide pregnenoloon en progesteroon gemetaboliseer. Luteale weefsel kan progesteroon *de novo* produseer, terwyl progesteroon omgeskakel word na drie relatiewe polêre verbindings (verbindings C, D en E). Ovariale weefsel het min progesteroon gevorm en het een polêre verbinding gevorm, verbinding F. Daar is aanduidings dat verbinding F identies aan verbinding E kan wees. Verbinding E is geïdentifiseer as 17α-hidroksiprogesteroon, verbinding F is voorlopig geïdentifiseer as 17α-hidroksiprogesteroon, en verbinding D as 11α-hidroksiprogesteroon of estradiol-17β. Die hoeveelheid pregnenoloon wat omgeskakel is deur beide weefsels is nie deur die voorplantingsstatus beïnvloed nie, alhoewel die hoeveelheid progesteroon wat omgeskakel is die laagste was gedurende laat-dragtigheid. Die plasenta het pregnenoloon na progesteroon omgeskakel en ook twee relatiewe polêre verbindings (G en H) gevorm. Verbinding H is egter nie gevorm toe dié weefsel met progesteroon geïnkubeer is nie. Verbinding H is voorlopig geïdentifiseer as 20α-dihidroprogesteroon. Dit wil voorkom asof die plasenta nie die biosintetiese rol van die corpora lutea oorneem nie, alhoewel dit 'n aanvullende funksie met die ovariale weefsel gedurende dragtigheid mag hê. Die plasenta van laat-dragtige diere het betekenisvol meer pregnenoloon omgeskakel as mid-dragtige diere, terwyl ook meer "ander"

verbindings gevorm is. Die plasenta mag derhalwe belangrik wees om progesteroon om te skakel na metaboliete wat in die urine uitgeskei word. Die uiteindelike positiewe identifikasie van verbindings C tot H sal die rol en meganisme van steroïedvorming deur die ovariale weefsel en plasenta, gedurende dragtigheid, opklaar.

Dit wil voorkom asof die hoë konsentrasies van sirkulerende 5α-dihidroprogesteroon nie deur metaboliese prosesse in die corpus luteum, ovaria of plasenta geproduseer word nie, maar wel die gevolg is van die metabolisme van progesteroon in die bloed. Die laë konsentrasies van sirkulerende progesteroon kan die gevolg wees van die metabolisme van progesteroon deur bloed. Die resultate van hierdie studie dui daarop dat progesteroon in dragtige dassie wyfies deur luteale weefsel geproduseer word. Dié progesteroon kan gevolglik in die sirkulasie omgeskakel word na die 5α-gereduseerde metaboliete. Dit is moontlik dat dié metaboliete aan die uterusprogesteroonreseptore bind as een van die stappe in die onderhoud van dragtigheid.

Alhoewel daar verskille tussen die dassie en Afrika olifant se voortplantings endokrinologie bestaan, is beide spesies gekenmerk deur hoë plasma konsentrasies van 5α-dihidroprogesteroon en laë plasma konsentrasies van progesteroon. Die binding van dié steroïede aan die progesteroonreseptore van die uterus mag dieselfde wees in beide spesies, asook belangrik wees vir die onderhoud van dragtigheid in beide spesies. Die dassie mag as 'n model gebruik word in die ontwikkeling van die tegniek om 'n voorbehoedmiddels te ontwikkel. Die resultate van hierdie studie dui egter ook daarop dat verdere studies nodig is om die voortplantings endokrinologie van die dassie beter te verstaan.

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APPENDICES

APPENDIX 1

The purification of contaminated [7-^{3}H]pregnenolone

The [7-^{3}H]pregnenolone stock (New England Nuclear, Boston, Massachusetts, USA) was contaminated with [^{3}H]progesterone (13%). The pregnenolone thus had to be purified prior to incubation. Two [7-^{3}H]pregnenolone stocks were combined (~ 150 µCi in total) and evaporated (37°C, N_2). The dried extract was reconstituted in 100 µl absolute ethanol and 80 µl was injected onto the HPLC system described earlier (Chapter 2, p. 17).

The eluents of fractions corresponding to pregnenolone were pooled in a glass tube (10 ml). The tubes which contained these eluents were rinsed with 1 ml dichloromethane, which was added to the glass tube containing the pooled eluents. The tube was vortexed and the top aqueous phase was removed. Distilled water (5 ml) was added to remove any remaining acetonitrile, vortexed thoroughly and the aqueous phase was removed once again. Magnesium sulphate (~ 500 mg; uniVAR, Saarchem, Muldersdrift, South Africa) was added to remove any remaining water. The mixture was centrifuged (4°C, 2000 rpm, 10 min), the supernatant was transferred to a clean glass tube and evaporated (37°C, N_2).

The dried extract was reconstituted in 1 ml absolute ethanol, 5 µl was pipetted into a clean glass tube and evaporated (37°C, N_2). 55% acetonitrile (100 µl) was added to the tube, which was then vortexed for 20 sec, and 10 µl was injected onto the HPLC column. Fractions were collected into scintillation vials and radioactivity counted to determine the purity of pregnenolone. At this stage, it was determined that the pregnenolone was 100% pure.

APPENDIX 2

Cleaning the diethyl ether

The purity of diethyl ether was tested using the following methods described by Vogel (1989).

To test for the presence of peroxides

Potassium iodide (1 ml, 10%; KI), HCl:distilled water (1:5, 0.5 ml), three to five drops of starch solution and diethyl ether (0.5 ml) were placed together in a glass tube (12 x 75 mm). After 1 min, the colour of the solution was checked. A clear or light purple colour indicated the absence of peroxides whereas a blue or blue-black colour indicated the presence of peroxides.

Removing peroxides

H_2SO_4 (6 ml) was added to 110 ml distilled water, and 60 g iron (II) sulphate was dissolved in this mixture. 10 to 20 ml of this mixture was added to one litre of diethyl ether in a separating funnel and mixed thoroughly. The aqueous phase was removed and the diethyl ether was tested once again for the presence of peroxides. The cleaning procedure was repeated until the peroxide test was negative. Once peroxide-free, the diethyl ether was distilled at ~ 30°C, collected into a darkened container and stored with nitrogen gas.

APPENDIX 3

Peak detection

Definition of a peak

To determine a peak the mean and standard deviation of the background dpm value for 500 µl 55 % acetonitrile were calculated. A fraction was then considered a peak when (a) its dpm value was greater than that of the fraction before and after it and (b) when its dpm value was greater than the mean plus six standard deviations (see below) of the background dpm value for 55% acetonitrile.

Determining which multiple of the standard deviation to use

Six standard deviations was decided as the optimal number. To determine this, one to seven standard deviations of the background dpm count for 500 µl 55% acetonitrile were calculated. The dpm counts of the 60 fractions for each of the control incubations (with [7-^{3}H]pregnenolone, [1,2,6,7-^{3}H]progesterone, and with both radiolabelled compounds) were submitted to the peak detection criteria (see definition of a peak above), but one, two, three, four, five, six and seven standard deviations were included in part (b) of the definition. The detection of peaks, using the different multiples of the standard deviation, is illustrated for a [7-^{3}H]pregnenolone control sample (Fig. 20). Six standard deviations gave the optimal peak detectability since using seven standard deviations would sometimes result in the exclusion of some obvious peaks.

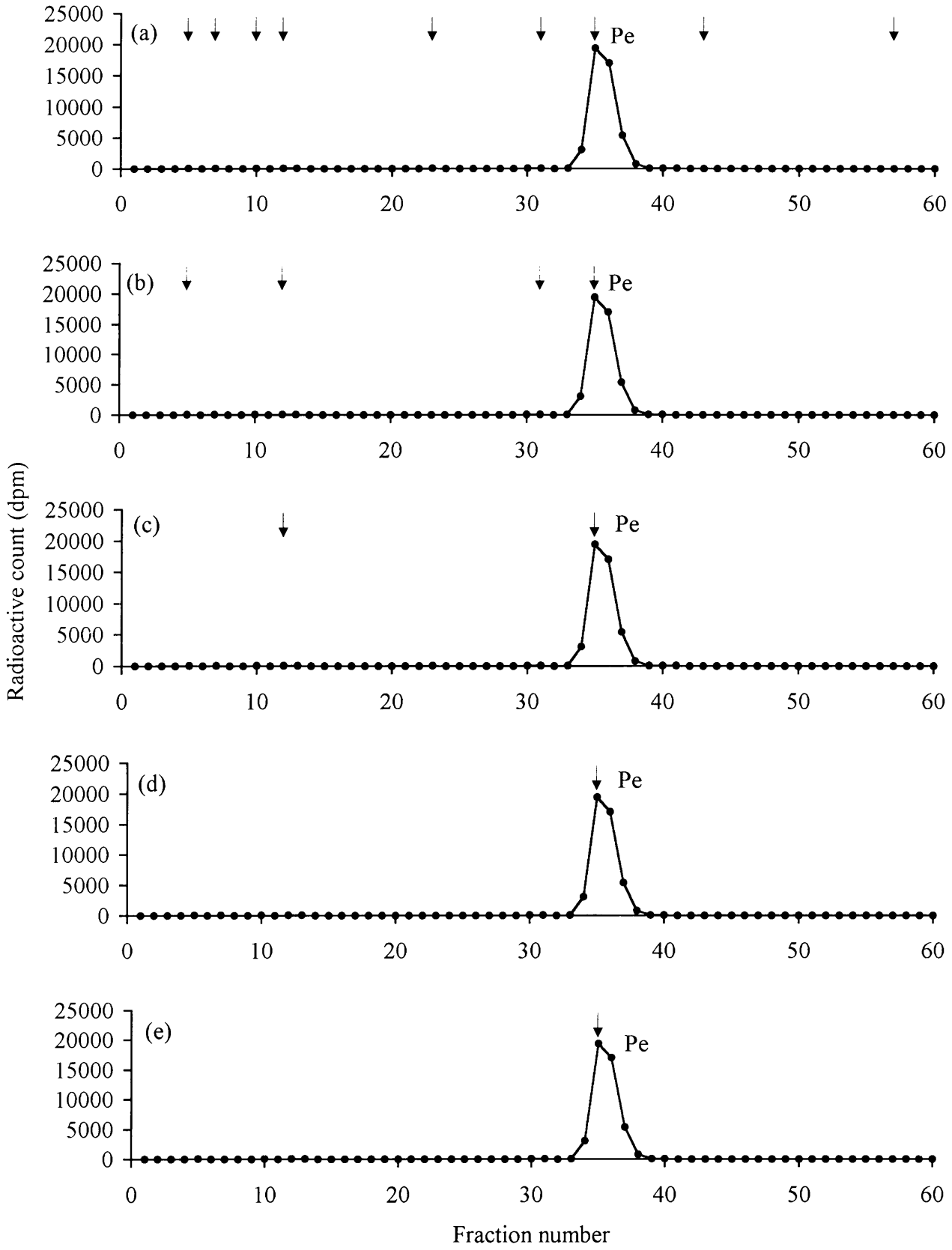

Fig. 20. HPLC radioactive profiles of a [^{3}H]pregnenolone (Pe) control incubation with peaks detected using (a) one, (b) three, (c) five, (d) six and (e) seven standard deviations. The arrows indicate the detected peaks.

APPENDIX 4

Table 21. The influence of reproductive status on the percentage metabolism of pregnenolone and percentage formation of progesterone, compounds A, B and other compounds by white blood cells after three hour incubations with [^{3}H]pregnenolone and β-NADPH.

Reproductive status	Pregnenolone converted (%)	Metabolites formed (%)			
		Progesterone	Compound A	Compound B	Other compounds
Non pregnant	56.5	9.5	28.5	3.0	15.5
Non pregnant	22.5	10.0	9.0	3.0	0.5
Early pregnant	83.5	3.5	47.5	6.0	26.5
Mid pregnant	89.0	4.0	49.0	16.0	20.0
Mid pregnant	18.0	7.5	8.5	1.5	0.5
Mid pregnant	75.0	8.0	45.0	14.5	7.5
Late pregnant	40.5	12.5	17.5	6.0	4.5
Late pregnant	47.0	20.0	13.0	7.0	7.0
Late pregnant	71.0	15.0	34.0	9.0	13.0