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A PREOVULATORY RISE OF DEHYDROEPIANDROSTERONE IN THE MARE MEASURED BY RADIOIMMUNOASSAY

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SUMMARY

3β-Hydroxy-5-androsten-19-al-17-one 19-(O-carboxymethyl) oxime was synthesised and conjugated to BSA. Antisera were raised against this antigen and characterised; the most specific of these was then used to measure dehydroepiandrosterone in the peripheral serum of the non-pregnant mare. Maximum concentrations of dehydroepiandrosterone were observed immediately before ovulation.

INTRODUCTION

Previous investigations [1, 2] have shown that there are maximum concentrations of androstenedione in the mare immediately prior to ovulation. This prompted an investigation of other metabolically related androgens, in this case dehydroepiandrosterone (DHA). Although the 5-ene-metabolic pathway is of only minor significance in the Graafian follicle of the mare [3], measureable quantities of dehydroepiandrosterone have been extracted from follicular fluid [4].

The first requirement for this study was a DHA radioimmunoassay. Antisera for this steroid have been raised previously, using antigens in which the steroid was linked through one of the native functional groups [5, 6], and this led, in each case, to low specificity around the point of attachment. It was reasoned that it would be advantageous to conjugate elsewhere on the steroid nucleus and the 19-position was selected, as models indicated that this would leave both the A. and D. rings accessable for recognition.

Accordingly, the purpose of this work was to prepare such an antigen, characterise antisera raised against it, and incorporate the antisera into the investigation of ovulation in the mare.

MATERIALS

Dehydroepiandrosterone-7-^{3}H (S.A. 16.6 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. 4-Oestrene-3,17-dione was obtained from the M.R.C. Reference Collection, 5-androstene-3β,19-dihydroxy-17-one was purchased from Aldrich and all other steroids were purchased from Steraloids Inc. General reagents were of Analar grade and obtained from B.D.H. and all solvents were redistilled before use. Freund's complete adjuvant was obtained from Difco Laboratories. Phosphate buffered saline with thiomersal (PBSM) consisted of 0.1 M-phosphate buffer (pH 7) containing sodium chloride (0.15 M) and thiomersal (0.01% w/v) and Knox gel (0.1% w/v) and was used for all dilutions. Charcoal (0.25 g; Norit A obtained from Sigma) was suspended in PBSM (100 ml) containing Dextran T-40 (2.5 mg; Pharmacia). Radioactivity was measured by liquid scintillation counting in toluene (5 ml) containing 2,5-diphenyloxazole (PPO, 4 g/l) and 1,4-bis [2(5-phenyloxazolyl)] benzene (POPOP, 0.1 g/l) using a Packard Tri-carb liquid scintillation spectrometer Model 2450.

Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. I.R. spectra were determined from mulls in Nujol on a Unicam SP 200 spectrophotometer. Ultraviolet spectra were determined for solutions in methanol on a Unicam SP 800 spectrophotometer. Nuclear magnetic resonance proton spectra were recorded on a Varian HA 100 spectrometer from solutions in deuterated dimethylsulphoxide. Mass spectra were recorded on a A.E.I. MS 12 spectrometer and only the molecular ion (M^+) is quoted.

Synthesis of antigen

17,17-Ethylenedioxy-3β,19-dihydroxy-5-androstene (**2**). A solution of 3β,19-dihydroxy-5-androsten-17-one (2.0 g) and p-toluenesulphonic acid monohydrate (0.20 g) in anhydrous benzene (200 ml) and freshly distilled ethylene glycol (5 ml) was refluxed in a Dean–Stark apparatus for 24 h. The reaction mixture was poured onto cold saturated aqueous sodium bicarbonate and extracted with ethyl acetate (50 ml × 2), dried (Na_2SO_4) and concentrated to an oil which crystallised from ethyl acetate–hexane to give 17,17-ethylenedioxy-3β,19-dihydroxy-5-androstene as white needles (1.43 g) m.p. 174–175°. ν_{max}; 3500 cm.$^{-1}$ (hydroxyl) δ; 0.78 (3H, s, 18-CH_3). 3.22 (1H, m, 3α-H) 3.46 (2H, AB system, J = 12, 19-CH_2) 3.63 (4H, s, OCH_2 CH_2O), and 5.37 (1H, m, 6-H) Mass spectrum; M^+ 348 ($C_{21}H_{22}O_4$ requires M^+ 348).

17,17-Ethylenedioxy-3β-hydroxy-5-androsten-19-al (**3**). To a well stirred solution of the above ketal (0.348 g) in anhydrous pyridine (8 ml) under nitrogen was added chromium trioxide-pyridine complex (0.60 g) [9]. After twenty minutes the reaction mixture was poured onto cold ethyl acetate (100 ml) and 5% aq. acetic acid (50 ml). After removal of the organic phase the aqueous layer was extracted with ethyl acetate (100 ml × 3). The organic solutions were washed with 5% aq. acetic acid (50 ml × 2), saturated aqueous sodium bicarbonate (50 ml × 4) saturated brine (50 ml × 2), dried (Na_2SO_4) and concentrated. Chromatography of the residue over silica (35 g; 100–200 mesh) eluting with ethyl acetate–hexane (1:4 v/v) afforded 17,17-ethylenedioxy-3β-hydroxy-5-androsten-19-al as white needles (0.220 g) m.p. 163–165°. $\nu_{max.}$ 3450 (hydroxyl) and 1710 $cm.^{-1}$ (formyl) δ: 0.71 (3H, s, 18-CH_3), 3.23 (1H, m, 3α-H), 3.63 (4H, s, OCH_2CH_2O), 5.71 (1H, m, 6-H) and 10.32 (1H, s, CHO). Mass spectrum: M^+ 346 ($C_{21}H_{30}O_4$ requires 346).

17,17-Ethylenedioxy-3β-hydroxy-5-androsten-19-al 19-(O-carboxymethyl)oxime (**4**). A solution of the above aldehyde (0.30 g) and carboxymethoxylamine hemihydrochloride (0.30 g) in anhydrous pyridine (15 ml) was stirred at 20° overnight. Removal of the solvent left an oily residue which was dissolved in a mixture of ethyl acetate (50 ml) and saturated brine (50 ml). The organic phase was separated, dried (Na_2SO_4) and concentrated to give 17,17-ethylenedioxy-3β-hydroxy-5-androsten-19-al 19-(O-carboxymethyl) oxime which was recrystallised from ethyl acetate as white prisms (0.320 g) m.p. 196–199°. ν_{max}: 3450 (hydroxyl) and 1725 cm^{-1} (carboxyl) δ; 0.71 (3H, s, 18-CH_3), 3.28 (1H, m, 3α-H), 3.74 (4H, s, OCH_2CH_2O) 4.42 (2H, s, OCH_2CO), 5.49 (1H, m, 6-H) and 7.32 (1H, s, 19-H). Mass spectrum M^+ 419 ($C_{23}H_{33}NO_6$ requires M^+ 419).

3β-Hydroxy-5-androsten-19-al-17-one 19-(O-carboxymethyl)oxime (**5**). To a stirred solution of the above ketal (0.120 g) in acetone (5 ml) was added a solution of *p*-toluenesulphonic acid monohydrate (0.004 g) in water (1 ml). After two days the acetone was removed and the residue taken up in ethyl acetate (20 ml), washed with saturated brine (10 ml), dried (Na_2SO_4) and concentrated. Crystallisation of the residue from ethyl acetate afforded 3β-hydroxy-5-androsten-19-al-17-one 19-(O-carboxymethyl)oxime as white prisms (0.103 g) m.p. 166–168°. ν_{max}; 3420 (hydroxyl) and 1720 $cm.^{-1}$ (broad; carboxyl and ketone) λ_{max}; 220 (ε 2800) δ; 0.75 (3H, s, 18-CH_3), 3.28 (1H, m, 3α-H), 4.44 (2H, s, OCH_2CO), 5.49 (1H, m, 6-H) and 7.38 (1H, s, 19-H). Mass spectrum: M^+ 375 ($C_{21}H_{25}NO_5$ requires 375).

The derivatised steroid was coupled to BSA as previously described [7]. The steroid:protein ratio of the conjugate was determined by U.V. spectroscopy to be 29.

Raising and testing of antibodies. Four male New Zealand White rabbits (2.5 kg) were immunised with the antigen using a schedule described previously [8]. Blood was withdrawn from the marginal ear vein after the second booster injection and characterised as previously described [8]. The most specific of the antisera was used at a dilution of 1:2000.

Radioimmunoassay. 100 μl serum samples in duplicate were pipetted into stoppered tubes and extracted with 2 ml of diethyl ether. The tubes were placed in an acetone-solid carbon dioxide mixture and the ether was decanted into 12 × 75 mm glass tubes.

A stock ethanolic solution of DHA (1pg/μl) was used to prepare a standard curve ranging from 10–400 pg. Solvents were evaporated under nitrogen in a water-bath at 37°C.

Equal vol. of antibody (1:2000 dilution) and hot DHA (diluted to 3000 c.p.m./100 μl in PBSM) were mixed and 200 μl was added to each tube. The tubes were vortexed and allowed to equilibrate overnight at 4°C.

The final stages of the assay were carried out at 4°C. Half a millilitre of charcoal suspension was added to each tube. The tubes were vortexed and allowed to stand for 10 min. They were then centrifuged for 10 min at 2500 *g* and the supernatants were decanted into scintillation vials.

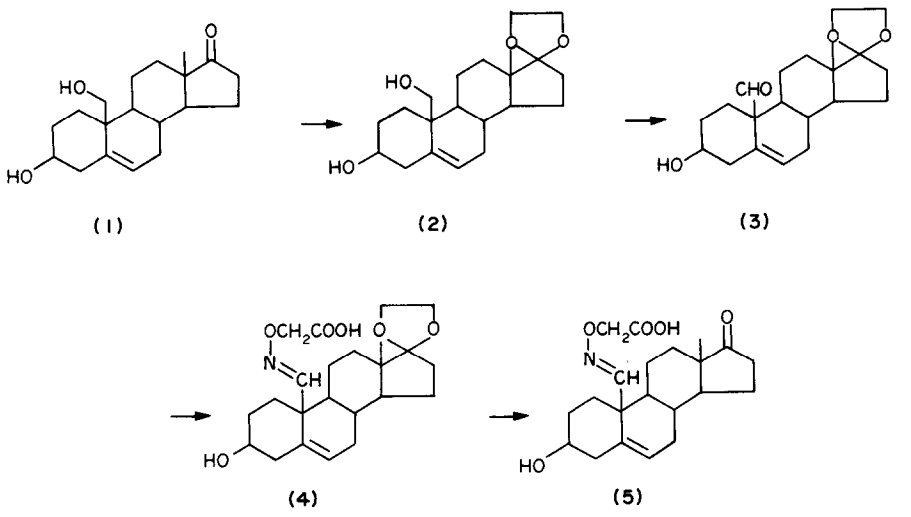
Experimental animals. From the beginning of January, eleven Dartmoor pony mares were examined regularly by rectal palpation for signs of ovarian activity. Mares showing such activity were bled daily from the jugular vein. Oestrus was determined by daily teasing with a pony stallion. Ovulation was then detected by rectal palpation and confirmed by progesterone analysis. The first six mares to ovulate (Nos. 2, 4, 5, 9, 11 and 12) were monitored throughout the season and daily blood samples were taken over an entire mid-season cycle.

One animal (a non-parous Welsh Mountain mare) was selected for a dexamethasone suppression experiment. A 6 mg dose of dexamethasone (Dexadreson, Intervet), was administered each day throughout the oestrous period. Blood samples were obtained as described above during this and the subsequent normal oestrous cycle.

RESULTS

The required 19-linked derivative [5] of DHA was synthesised from 19-hydroxydehydroepiandrosterone (**1**), as outlined in scheme 1. Three of the rabbits injected with the antigen produced usable antisera and the assay characteristics of the most specific of these are presented in Table 1 and the cross-reactions are shown in Table 2. The standard curve was found to be linear between the 10 pg point and the 400 pg point. The least detectable quantity of DHA was 5 pg ($P < 0.01$) but as this was not on the linear part of the standard curve 10 pg was considered to be the minimum quantity which could be measured reliably.

Daily serum concentrations of DHA over the first ovulatory period of the season in mares Nos. 2, 4, 5,



9, 11 and 12 and in a complete mid-season cycle (mares Nos. 2, 4, 5, 9, 11 and 12 are shown in Fig. 1).

Maximum DHA concentrations were observed prior to ovulation but were already in decline when ovulation occurred. This pattern was observed in eleven out of twelve oestrous cycles examined. Peak values before ovulation ranged from 300 pg/ml to 720 pg/ml. In the twelfth cycle there was no significant rise in DHA concentration prior to ovulation.

Elevated DHA serum values were not found to be synonymous with oestrous behaviour. One mare (No. 9), showed oestrous behaviour intermittently over a period of seven weeks before ovulating at the beginning of the season. The serum samples obtained throughout this anovulatory oestrous period had consistently low values of DHA (90–120 pg/ml) until six days before ovulation. The same mare ovulated at mid-season without exhibiting oestrus; at the end of that oestrous cycle she showed normal oestrus and ovulated on the last day of oestrus. DHA concentrations in both the silent and normal oestrus were very similar (300 pg/ml and 320 pg/ml). The pony that received daily dexamethasone treatment (Fig. 2b.) was found to have low DHA concentrations over the oestrous period, but oestrous length and ovulation were normal. Samples taken over the next oestrus (without suppression) demonstrated a peak of DHA (700 pg/ml) before ovulation.

Table 1.

DHA (pg) added to plasma	Accuracy DHA (pg) measured	increment (pg)
0	19.3 ± 1.8	—
20	43.8 ± 1.8	24.5
50	73.0 ± 7.8	53.7
100	111.5 ± 8.6	92.2
200	226.3 ± 11.4	207.0

DHA (pg) in plasma pool	Precision Intra-assay Variation (%) $n = 6$	Inter-assay Variation (%) $n = 10$
10	3.78	6.26
200	6.53	7.36

Specificity

The ratio of the results with and without chromatography was 94.65% ± 0.73 ($n = 4$). Range 93.9–95.64%.

DISCUSSION

The cross reactions of the best antiserum obtained using this antigen are presented in Table 2. The antiserum was highly specific for the D ring but showed reduced specificity for the A/B ring junction; the high cross reaction of epiandrosterone is particularly noteworthy. Since the completion of this work more specific antisera for DHA have been raised against an antigen in which the steroid was linked through the seven position [10]. It is interesting to note that the latter antiserum could discriminate between dehydroepiandrosterone and epiandrosterone, even though

Table 2. Cross-reactions [21] of various steroids with anti-DHA-19-antiserum.

Steroid	Cross-reaction (%)
Dehydroepiandrosterone	100
5α-Androstan-3α,17β-diol	<0.1
5α-Androstan-3,17-dione	9.1
5α-Androstan-3α-ol-17-one	6.0
5α-Androstan-3β-ol-17-one	99
5α-Androstan-17β-ol-3-one	<0.1
4-Androstene-3,17-dione	2.5
4-Androsten-17α-ol-3-one	<0.1
4-Androsten-17β-ol-3-one	<0.1
4-Androstene-3,11,17-trione	<0.1
5-Androstene-3β,17β-diol	<0.1
Cholesterol	<0.1
Cortisol	<0.1
Oestrone	0.68
Oestradiol	<0.1
4-Oestrene-3,17-dione	<0.1
Pregnenolone	<0.1
Progesterone	<0.1

the point of attachment is adjacent to the C5–C6 double bond.

The anti-DHA-19-BSA antiserum was considered to be adequate for the present work since there is no evidence to suggest the presence of epiandrosterone, androsterone or androstanedione in high concentrations in the mare, while androstenedione has been found to be present in similar quantities to DHA [1, 2] which would introduce an error of no more than 2.5%. As further confirmation of the specificity of this assay, preovulatory blood samples were measured with and without chromatography [11] and this showed an over-estimation of only 5%, without a chromatography step.

A preovulatory peak of DHA in the oestrous cycle has not been previously reported in the mare. Early investigations in the mare have been limited to the measurement of concentrations of this steroid in the follicular fluid. Short [4] found low concentrations of DHA (8 μg/l) in the Graafian follicular fluid, but later work [12] revealed high concentrations of the 4-ene-steroids, androstenedione (294 μg/l) and 17α-hydroxyprogesterone (272 μg/l). Furthermore, Younglai and

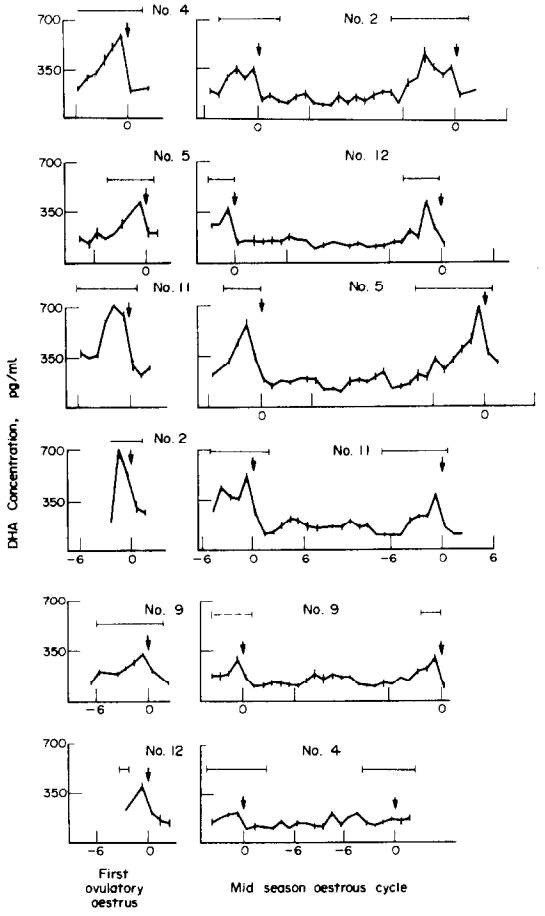


Fig. 1. DHA peripheral serum concentration during the first ovulatory oestrus and mid-season cycle of the mare. (|—| beginning, duration and end of oestrus. ------ silent oestrus; —| duration and end of oestrus; day 0 = day of ovulation; ↓ indicates time of ovulation. Vertical bars indicate assay duplicates.

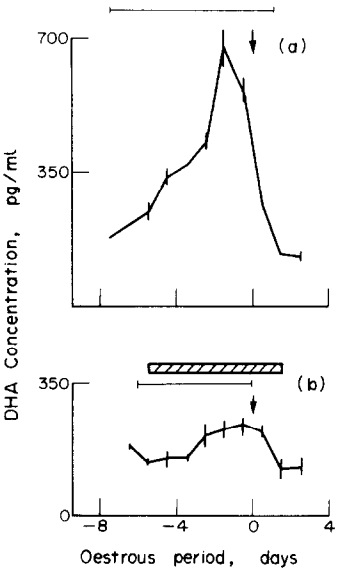


Fig. 2. DHA peripheral serum concentration during (a) normal oestrus; (b) oestrus exhibited during dexamethasone treatment (6 mg daily). (▨—period of dexamethasone treatment; |—| oestrus; day 0 = day of ovulation; ↓ indicates time of ovulation. Vertical bars indicate assay duplicates.

Short have since found no evidence of 17α-hydroxypregnenolone or DHA formation following injection of labelled pregnenolone into the follicle. They concluded that steroid synthesis occurs almost exclusively by the 4-en-3-ketone route, in the follicular fluid of the mare [3]. Thus, the major part of DHA found in peripheral blood would seem to be of extra-ovarian origin. The most probable site of synthesis is the adrenal cortex. No direct evidence is available to support this suggestion although DHA synthesis in the human adrenal cortex is well documented [13–15]. The striking depression of DHA concentrations in peripheral serum found in the dexamethasone treated mare supports the possibility that this was of adrenal origin. However, this single experiment would need to be repeated to substantiate this conclusion.

We are unable to ascribe a rôle to this preovulatory peak of DHA in the mare. As already described, low concentrations of DHA were found in mares exhibiting anovulatory oestrus. Conversely, one mare (No. 9) had a silent oestrus, followed at the beginning of the next cycle by a normal oestrus although pre-ovulatory DHA concentrations in both cycles were very similar (300 pg/ml and 320 pg/ml). From these observations, no overt connection between DHA concentrations and oestrous behaviour in the mare is apparent.

Of the twelve normal oestrous cycles examined, maximum concentrations of DHA preceded ovulation in eleven cases. In both the twelfth cycle (pony No. 4, mid-season cycle) and in the dexamethasone treated mare (Fig. 2b) ovulation occurred in spite of low concentrations of DHA in peripheral serum. Thus, it would appear that high concentrations of DHA are not necessarily involved with ovulation in the mare.

Data clarifying the adrenal–ovarian relationship in other species are scant. No distinct pattern of DHA secretion is observed during the menstrual cycle in women [16] and furthermore, in other large domestic animals no common pattern of behavioural or hormonal response is elicited by adrenal stimulation or suppression [17–20].

Also, the stimulus which provokes this increase in DHA concentration is not known. ACTH values have not been determined throughout the oestrous cycle of the mare, so this effect may not be confidently attributed to ACTH. It is possible that an ovarian steroid may facilitate the stimulus upon the adrenal cortex as concentrations of oestradiol-17β, androstenedione and testosterone are elevated at oestrus, whilst progesterone concentrations are very low [2].

It has been demonstrated that a DHA peak generally precedes ovulation in the mare and this may well originate from the adrenals. This DHA peak is provoked by an unknown factor and so far its physiological significance remains obscure.

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