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RADIOIMMUNOASSAY OF 17β-HYDROXY-5α-ANDROSTAN-3-ONE, 4-ANDROSTENE-3,17-DIONE, DEHYDROEPIANDROSTERONE, 17-HYDROXYPROGESTERONE AND PROGESTERONE AND ITS APPLICATION TO HUMAN MALE PLASMA

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SUMMARY

RIA methods for the measurement of 5α-dihydrotestosterone (DHT), androstenedione (Δ4), dehydroepiandrosterone (DHEA), 17-hydroxyprogesterone (17-OHP) and progesterone (P) have been developed and applied to the study of factors determining the levels of these steroids in males.

It has been shown that DHT, 17-OHP and DHEA levels are lower in elderly males than in young (<50 years) subjects, Δ4 and P levels remaining constant. All steroids, with the exception of DHT, show significant nyctohemeral variations: upon ACTH stimulation T levels decrease, whereas Δ4 and DHEA, 17-OHP and P increase significantly. Dexamethasone has the reverse effect, although T levels do not decrease. HCG stimulation results in a significant increase of T, DHT, 17-OHP and Δ4 levels, whereas DHEA and P levels are hardly influenced. In a small group of orchidectomized males, all steroids studied, with the exception of P were significantly lower than in normal males of similar age.

It is concluded that in males T, DHT and 17-OHP have an almost exclusive testicular origin; Δ4 has a mixed testicular and adrenal origin, whereas DHEA has a predominant and P an exclusive adrenal origin in males.

INTRODUCTION

The presence in human plasma of 17β-hydroxy-5α-androstan-3-one or 5α-dihydrotestosterone (DHT), 4-androstene-3,17-dione (Δ4), dehydroepiandrosterone (DHEA), 17-hydroxyprogesterone (17-OHP) and progesterone (P) is well documented. However, data concerning physiological factors influencing these plasma levels in adult males as well as concerning their origin are scarce. It was the aim of this study to determine normal plasma levels of these steroids in a group of healthy males, to investigate the existence and the importance of a nyctohemeral rhythm, to evaluate the influence of age, and to determine the adrenal, respectively gonadal contribution to these levels by studying the influence of ACTH respectively HCG stimulation as well as of dexamethasone suppression. Moreover levels were studied in a small group of gonadectomized males.

EXPERIMENTAL

Subjects studied

All subjects studied were in good health, except for the 8 gonadectomized males (age 60–72 years) who suffered from prostatic cancer; in the latter the plasma samples were taken prior to any hormonal therapy, 10–14 days after gonadectomy, when the patients were in good general condition, without clinical evidence of metastasis. For studies of circadian rhythms, as well as for stimulation and suppression tests, all subjects were hospitalized and plasma was taken not prior to 72 h after hospitalization.

Circadian rhythm was studied by taking plasma samples in the recumbent subject every 4 h for 24 h starting at 8 a.m.; the 4 a.m. sample was however omitted. Values represented on the figures are expressed as per cent of the mean of the 8 a.m., the 12 a.m. and the 8 p.m. value.

The ACTH stimulation test was started in the fasting patient between 8 and 10 a.m.; 0·25 mg tetracosactide (Synacthen) were injected i.v. and blood samples were taken at −5, 10, 30, 60 and 120 min.

Dexamethasone suppression tests were performed by giving 1 mg orally every 8 h for 72 h, blood samples being taken at 8 a.m., 48 and 72 h respectively after starting treatment; values reported are the mean of both these measurements.

HCG tests were performed by administering for 3 consecutive days at 8 p.m. 1500 I.U. of Pregnyl^{R}, plasma samples being taken at 8 a.m. the next day. Basal levels were obtained at 8 a.m. both on the day before and the first day of treatment; reported "basal levels" are the mean of both these values. For evaluation of stimulation, and suppression tests, values are expressed as per cent of the mean basal value.

Statistical evaluation was performed using Student's *t* test.

Materials

Radioactive steroids used. [1,2-^{3}H]-testosterone (TRK 340) 46·7 Ci/mmol; [7-^{3}H]-17-hydroxy-progesterone 10·2 Ci/mmol (TRK 315); [7-^{3}H]-dehydroepiandrosterone 22 Ci/mmol (TRK 163); [1,2-^{3}H]-17β-hydroxy-5α-androstan-3-one, 44 Ci/mmol (TRK 395); [1,2-^{3}H]-progesterone 53 Ci/mmol (TRK 341), all from Radiochemical Centre, Amersham; [7-^{3}H]-4-androstene-3,17-dione 45 Ci/mmol (NET 181).

Chemicals. Petroleum ether b.p. 30–45°C, Fluka No. 77·400; Benzene p.a. Merck No. 1783; Methanol p.a. Merck No. 6009; Ethylether p.a. Merck No. 921, purified by passing through an Al_2O_3 (Woelm–Basic) column. Thin layer plates with Silicagel GF 254 Merck No. 5715; Whatman paper No. 40, purified by Soxhlet extraction with methanol for 48 h; Isatine (2,3 indolinedione) Merck No. 4734; LH-20 Sephadex Pharmacia, Uppsala, Sweden; Dextran T 70 Pharmacia, Uppsala, Sweden; Charcoal p.a. Merck No. 2186. Dextran-coated charcoal: equal volumes of phosphate buffer containing respectively 1·25 g/100 ml charcoal and 0·125 g/100 ml Dextran T-70 are mixed, and further diluted 1:4 (v/v) with buffer.

Phosphate buffer is prepared by mixing 305 ml 0·2 M Na_2HPO_4-12 H_2O (Merck 6579), and 195 ml 0·2 M $NaH_2PO_4H_2O$ (Merck 6346). After addition of 9 g NaCl (Merck 6404), 1 g NaN_3 (Merck 6688) and 1 g gelatine (Merck 4070), the solution is made up to 1000 ml with aqueous bidest. The buffer is stored at 4°C. Steroids used in this study were purchased from Ikapharm (Ramat Gan Israël).

Combined determination of testosterone (T), 5α-dihydrotestosterone (DHT) and androstenedione (Δ4)

These three steroids are determined on the same plasma sample. To 0·5 ml of male plasma are added 2000 d.p.m. of respectively, [1,2-^{3}H]-testosterone, [1,2-^{3}H]-dihydrotestosterone and [7-^{3}H]-androstenedione.

The plasma is extracted with 4 ml of ether, and after freezing the plasma, the supernatant is decanted and evaporated to dryness. The extract is submitted to t.l.c. on Silicagel HF 254, in the system chloroform–ethylacetate 85:15 (v/v) with reference steroids in the side lanes; appropriate zones are eluted separately with benzene–methanol 85:15 (v/v).

R.I.A. of testosterone

R.I.A. was performed as described previously [1] using an antibody raised in rabbits against testosterone-3 CMO-BSA; Dextran-coated charcoal was used for separation of bound from free steroids. The standard curve was constructed by computer, using the "smoothing by spline function" technique described by Reinsch [2].

R.I.A. of 5α-dihydrotestosterone (DHT)

R.I.A. was performed as described for T, the eluate being however dissolved in 0·35 ml of buffer, of which 100 μl are used for recovery determination and 200 μl for R.I.A. proper. The A.B. was the same as for T determination.

Specificity can be deduced from the specificity of the antibody and separation of cross reacting steroids by t.l.c. (Table 1). For the sake of brevity none of the C-21-steroids are mentioned on Table 1 as none showed a cross reactivity of more than 0·01%. 17β-hydroxy-5β-androstan-one is poorly separated from DHT, but in view of both its low cross reactivity with the AB. and its admittedly low concentration in plasma, it is unlikely to be a significant contaminant. T on the other hand appears to be well separated from DHT. In order to verify this separation, 2 ng of T were added to 0·5 ml of 10 different plasma samples: this did not affect the DHT concentration, confirming the non-interference of T in the assay. Moreover, the DHT concentration paralleled the volume of the plasma extract assayed.

The water blank is less than 10 pg, and values observed in ovariectomized women treated with dexamethasone are below the sensitivity of the method.

The coefficient of variation (interassay) is 15·4% (n = 12) at a concentration of 53·6 ng/100 ml; the sensitivity is 6 ng/100 ml.

Recovery of 0·2 ng/ml was 108 $\pm$ 13 (S.D.)% (n = 18).

Table 1. Specificity of T and DHT determination

	Relative cross reactivity with AB No. 23573/5 testosterone 3 CMO-BSA at 50% binding	Relative mobility of cross reacting steroids on t.l.c. (chloroform-ethylacetate 85:15 v/v)
Testosterone (T)	100	1·00
4-androstene-3β,17β-diol	9	0·80
17β-hydroxy-5α-androstan-3-one (DHT)	66	1·20
17β-hydroxy-5β-androstan-3-one	12	1·11
5α-androstane-3α,17β-diol	40	0·54
5α-androstane-3β,17β-diol	23	0·80
5β-androstane-3α,17β-diol	21	0·85
4-androstene-3,17-dione	0·5	1·36
Dehydroepiandrosterone	0·5	1·42
5-androstene-3β,17β-diol	0·5	0·86
17α-hydroxy-4-androsten-3-one (epi T)	0·5	1·02

Table 2. Specificity of 4-androstene-3,17-dione determination

	Specificity AB (androstenedione-6 CMO-BSA): Relative cross reactivity of steroids at 50% binding	Relative mobility of cross reactive steroids on t.l.c. (chloroform-ethylacetate 85:15 v/v)
4-androstene-3,17-dione	100	1·36
5α-androstane-3,17-dione	85	1·46
5β-androstane-3,17-dione	<0·1	1·42
Testosterone	2	1·00
17α-hydroxy-4-androsten-3-one (epitestosterone)	0·45	1·02
Dehydroepiandrosterone	0·2	1·42
4-androstene-3,11,17-trione	0·4	1·16
3α-hydroxy-5α-androstan-17-one (epiandrosterone)	0·7	1·23
3β-hydroxy-5α-androstan-17-one (androsterone)	0·7	1·23
3α-hydroxy-5β-androstan-one (etiocholandolone)	0·2	1·06
17β-hydroxy-5α-androstan-3-one (DHT)	0·4	1·20
5β-androstan-3α,17β-diol	<0·1	0·85
5α-androstan-3β,17β-diol	0·3	0·80

R.I.A. of androstenedione (Δ4)

For male plasma 1/5th of the appropriate eluate was used in the R.I.A. assay, whereas in females only 1/15th was used. The antibody was obtained by immunizing rabbits with androstenedione-6-C.M.O.-B.S.A.; it was used at a dilution of 1:5000. Standards were prepared in triplicate, by evaporation of respectively 5, 10, 20, 50, 100, 200 and 400 pg of Δ4.

Specificity of the AB is shown in Table 2; of the naturally occurring steroids 5α-androstane-3,17-dione and testosterone are the main cross reacting steroids. The t.l.c. system used, however, completely separates T from Δ4 whereas the occurrence of 5α-androstane-3,17-dione in human plasma has never been reported. Moreover, when a paper chromatography (Bush A2) system, which separates 5α-androstane-3,17-dione completely from Δ4 (Table 3) was substituted for the t.l.c., Δ4 values were similar.

The water blank of the method is 7 ± 4 pg. In ovariectomized women treated with dexamethasone (3 mg/day), the Δ4 concentration did not differ significantly from the blank ($n = 5$).

The coefficient of variation (interassay, $n = 11$) is 7·1% at a concentration of 112 ng/100 ml.

The sensitivity is ±20 ng/100 ml.

Recovery of 400 pg/ml plasma was $108 \pm 12\%$ ($n = 12$).

Table 3. Specificity of DHEA determination

	Cross reactivity with DHEA-AB 4B(DHEA-17 CMO-BSA) at 50% binding	Relative mobility in comparison to DHEA (Bush A_2-Whatman No. 40)
Dehydroepiandrosterone (DHEA)	100	100
5-androstene-3β,17β-diol	13·7	30
3β-hydroxy-5-pregnen-20-one	13·6	155
3β,17-dihydroxy-5-pregnen-20-one	1·6	<10
4-androstene-3,17-dione	0·6	142
Testosterone	<0·04	60
Progesterone	<0·04	>200
17-hydroxyprogesterone	<0·04	<10
17β-hydroxy-5α-androstan-3-one (DHT)	0·5	125
3β-hydroxy-5α-androstan-17-one (androsterone)	<0·04	134
3α-hydroxy-5α-androstan-17-one (epiandrosterone)	3·6	118
3α-hydroxy-5β-androstan-17-one (etiocholanolone)	0·04	126
5α-androstane-3,17-dione	0·5	>200
5β-androstane-3,17-dione	<0·01	>200
5α-androstane-3β,17β-diol	1·2	<10
5β-androstane-3β,17β-diol	<0·04	<10
5β-androstane-3α,17β-diol	<0·04	<10

R.I.A. of DHEA

2000 d.p.m. of [7-^{3}H]-DHEA (± 10 pg) are added to 0·2 ml plasma and, after equilibration, extraction is performed with 4 ml of ether. The extract is purified by paper chromatography on Whatman No. 40 paper in the Bush A2 system. The zone corresponding to DHEA is eluted with 4 ml of methanol.

After evaporation, the residue is dissolved in 500 μl of buffer; 100 μl are taken for recovery and 100 μl for R.I.A. Standards covering the range between 0 and 400 pg of DHEA are prepared in triplicate

To standards and unknowns are added 100 μl of rabbit DHEA-17-CMO-BSA antibody diluted 1:2000 and containing 20.000 d.p.m. [7-^{3}H]-DHEA and, after standing overnight at 4°C, 1 ml of dextran-coated charcoal suspension is added; the bound fraction is separated and counted as usual.

The specificity of the method can be deduced from data in Table 3; the steroids cross reacting with the AB, are well separated by chromatography of the extract, with the exception of epiandrosterone which however occurs in plasma mainly as a conjugate, the free concentration being probably extremely low. Addition of 1 ng of epiandrosterone to 1 ml of plasma did not influence the results.

Moreover results obtained using a rabbit DHEA-7-CMO-BSA antibody, yielded results that were not significantly different from those obtained with the DHEA-17-CMO-BSA antibody.

A final indication of the specificity was the constancy of values found when increasing volumes of plasma were processed. As far as the precision is concerned, the coefficient of variation was 9·2% at a concentration of 596 ng/100 ml (n = 12).

The water blank of the method varies between 8 and 24 pg; the sensitivity of the method is 20 ng/100 ml.

Recovery of 5 ng added to 1 ml of plasma was 96·4 ± 10·6% (n = 10).

Progesterone and 17-hydroxy-progesterone

P and 17-OHP were determined on the same plasma sample by a R.I.A. technique, using a rabbit anti 11-oxo-progesterone-11-CMO-BSA and anti 17-hydroxy-progesterone-3-CMO-BSA antibody respectively. After addition of 2000 d.p.m. of [7-^{3}H]-17-hydroxy-progesterone as an internal standard to 2 ml of plasma and 30 min equilibrium at 4°C, the plasma is extracted with 10 ml of petroleum ether. No internal standard is used for P, the efficiency of extraction being rather constant (81 ± 2·4%); P values are not corrected for recovery.

R.I.A. of P

After freezing the plasma, the P containing petroleum ether is decanted and evaporated to dryness. The residue is dissolved in 200 μl of phosphate buffer, and after standing at room temperature for at least 30 min, according to the expected value, 25, 50 or 100 μl are taken for R.I.A.; this volume is adjusted to 100 μl with buffer. Standards are prepared in triplicate by evaporating respectively 10, 50, 100, 200, 400, 800, 1000, 1500 and 2000 pg of P. 200 μl of a 1:8000 diluted antiserum, containing 20.000 d.p.m. of [^{3}H]-progesterone, are added to standards and unknowns and, after standing overnight, bound from free fraction is separated by Dextran coated charcoal.

A standard curve is constructed by computer, as usual [2]. The specificity of the method can be inferred from data in Table 4. Taking into account their concentration in plasma, structurally related steroids do not cross react to any significant extent, except for 11-deoxycorticosterone, which however is not appreciably extracted by petroleum ether. The water blank is less than 20 pg, and plasma values of ovariectomized women treated with dexamethasone are indistinguishable from this blank.

The specificity of the method is further evidenced by the constancy of the results when increasing volumes of the plasma are extracted.

The precision of the method was evaluated from the coefficient of variation of two pools measured 10 times; the latter was 5·9% at a mean concentration of 21 ng/100 ml and 3·3% at a concentraion of 190 ng/100 ml.

The sensitivity of the assay is 0·05 ng/ml.

Recovery of 1 ng of P added to 1 ml of plasma was 0·97 ± 0·04 (S.D.) ng (n = 5), using a plasma with an initial P concentration of 0·2 ng/ml and 0·79 ± 0·09 (S.D.) ng (n = 6) using a plasma with an initial concentration of 1·9 ng/ml, no correction for incomplete extraction being applied.

Table 4. Specificity of the progesterone assay

Steroids	Cross reactivity with 11-OXO-progesterone AB on 50% binding (progesterone = 100)	Extraction by P-ether (%)
11-Deoxycorticosterone	25·7	1
Corticosterone	0·14	0·4
11-Deoxycortisol	0·04	4
20α-Dihydroprogesterone	0·55	59
17-Hydroxyprogesterone	1	14·5
Testosterone	<0·1	16
Pregnenolone	2·1	69
17-OH Pregnenolone	<0·04	20

Table 5. Specifity of the 17-OH-progesterone assay

	Cross reactivity with 17-OHP-AB at 50% binding (17-OHP = 100)	Fraction remaining in plasma after extraction by P-ether (%)	Elution in 17-OHP containing fraction from LH 20 column (% of total tracer brought on column)
17-OH-progesterone	100	85	100
17-OH-pregnenolone	16·4	80	25
Progesterone	3·7	20	85
Pregnenolone	0·75	30	86
11-Deoxycortisol	1·4	96	2
Deoxycorticosterone	0·07	99	3
20α-Dihydroprogesterone	0·5	40	25
Corticosterone	0·001	100	5
Testosterone	0·003	85	66

R.I.A. of 17-OHP

The plasma fraction remaining after extraction with P-ether (for P), is reextracted with 4 ml of diethyl-ether.

After evaporation to dryness, the residue is taken up in a few drops of benzene–methanol 85/15 (v/v), and brought onto a LH-20 microcolumn, as previously described for estradiol [3]. Elution is performed with the same solvent, 17-OHP being collected in the 1·5–3 ml eluate fraction. If desired the next 1·5 ml fraction may be collected for simultaneous determination of estradiol.

After evaporation, the residue is dissolved in 400 μl of buffer, of which 100 μl are taken for recovery, 100 μl being used for R.I.A.

Triplicate standards, covering the range between 0·01 and 2 ng are prepared.

200 μl of antiserum (1:2000 dilution) containing 20,000 d.p.m. of [7-^{3}H]-17-OHP are added to standards and unknowns and after incubation overnight at 4°C, separation of the bound from the unbound fraction is performed using 0·5 ml of dextran-coated charcoal suspension.

The supernatant is counted as usual and a standard curve is constructed as described for progesterone.

The specificity of the method can be inferred from data in Table 5.

The antibody reacts moderately with 3β,17-dihydroxy-pregn-5-ene-17-one (17-hydroxypregnenolone) and weakly with progesterone and 11-deoxycortisol; there is no cross reactivity with either corticosterone or deoxycorticosterone. The only steroid interfering in the method to a measurable degree (±2·5–3%) is 17-hydroxypregnenolone. As the concentration of the latter is of the same order of magnitude as of 17-OHP, [4, 5], the interference may be considered negligible.

The intra-assay coefficient of variation was 6·2% (n = 10) and 7·6% in two pools with a mean concentration of 0.87 and 0.84 ng/ml respectively; the interassay coefficient of variation was 8·3% (n = 22).

The sensitivity of the method is 20 pg per sample or 8 ng/100 ml.

The accuracy was evaluated from the recovery of 1 ng of 17-OHP added to 1 ml aliquots of three pools containing 0·86, 0·84 and 0·90 ng/ml respectively: recovery was 0·81 ± 0·08 (SEM, n = 5), 0·83 ± 0·10 (n = 7) and 0·95 ± 0·10 ng (n = 7).

RESULTS

Basal values

Basal values determined between 8 and 10 a.m. in young (< 50 years) and elderly (> 50 years) males respectively, and the statistical significance of the eventual differences are given in Table 6.

Nyctohemeral variations

As shown on Fig. 1, all steroids studied with the exception of DHT, show significant ($P < 0·01$) nyctohemeral variations, with highest values observed either at 8 a.m. (DHEA, P, 17-OHP) or at noon (T, Δ4) and a nadir at 8 p.m., and with a mean amplitude of ±40%; the latter is however only 24 ± 8 (SE)% for T.

The circadian rhythms in both young and elderly males were synchronous, and, although the groups are too small for separate statistical analysis, the amplitude of the variations appears to be rather similar.

ACTH test

After i.v. injection of 0·25 mg of tetracosactide (Cortrosyn^R) in males (n = 10), mean T level shows a significant ($P < 0·01$) decrease of 19·4 ± 5·7 (SEM)% at 60 min (Fig. 2); DHT levels did not show any significant variation within 120 min after ACTH injection, whereas mean Δ4, DHEA and 17-OHP levels increased within 30 min to about 200% and within 120 min to about 300% of basal levels. The most important increase, however, was observed in P levels, which, within 30′ after ACTH, averaged ±700% of basal levels and remained at this value for at least 120 min; the increase was never less than 250%. No difference was observed between young and elderly males.

Dexamethasone suppression

Dexamethasone suppression, performed as described in 10 normal males, was without any signifi-

Table 6. Basal values of plasma steroid levels in males

	ng/100 ml mean ± SEM		Stat. significance (P) of difference between groups
	< 50 years	> 50 years	
DHT*	66·6 ± 3·7 (n = 47) (26 – 126)†	51·9 ± 5·3 (n = 37) (12 – 112)	0·05
T	579 ± 39 (n = 47) (266 – 1062)	453 ± 32 (n = 37) (62 – 870)	0·05
T/DHT	10·5 ± 0·7 (5·3 – 16·5)	9·3 ± 1·7 (5·3 – 21·4)	n.s.
Δ4	109·6 + 4·5 (n = 41) (48 – 233)	125·8 ± 7·5 (n = 38) (41 – 232)	n.s.
DHEA	470 ± 58 (n = 24) (83 – 1039)	175 ± 18 (n = 25) (26 – 439)	0·001
17-OHP	112 ± 9 (n = 35) (37 – 185)	81·7 ± 10·6 (n = 19) (34 – 168)	0·05
P	18·1 ± 1·9 (n = 20) (8 – 37)	19·5 ± 2·3 (n = 15) (8 – 39)	n.s.

* For symbols see text.
† Extreme values.

cant influence on T levels, whereas only a slight, statistically non significant, decrease in DHT and 17-OHP levels was observed; Δ4 and DHEA levels on the other hand decreased to about half the basal values ($P < 0·01$), whereas P levels, being decreased in all cases, were at or below the lower detection limit of the method (Table 7).

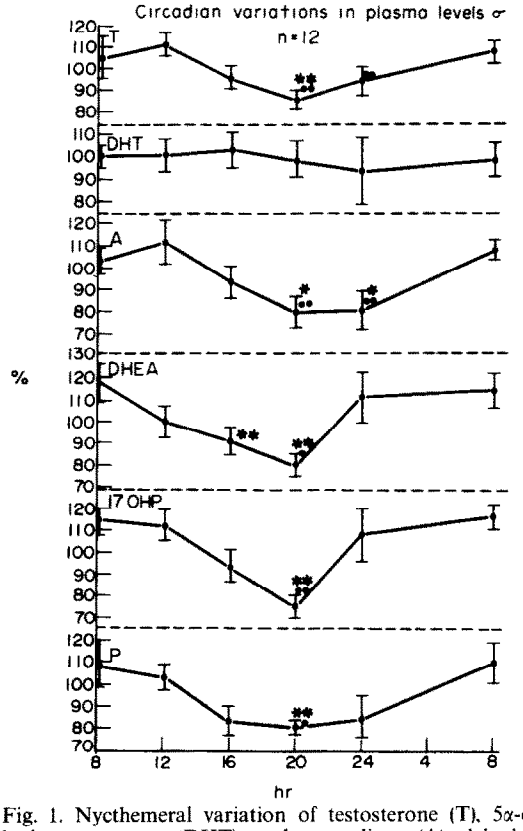

Fig. 1. Nycthemeral variation of testosterone (T), 5α-dihydrotestosterone (DHT), androstenedione (A), dehydroepiandrosterone (DHEA), 17-hydroxyprogesterone (17-OHP) and progesterone (P) in 12 males age 20–75 years. ×, × ×: Difference with 8 a.m. value significant at $P < 0·05$ (×) or $< 0·01$ (× ×). ●, ●●: Difference with 12 noon value significant at $P < 0·05$ (●) or $< 0·01$ (●●).

HCG stimulation

An HCG test was performed in 12 normal males: T, DHT, Δ4 and 17-OHP increased significantly in each individual case. P and DHEA levels on the other hand did not change significantly (Table 7).

Orchidectomy

In 8 orchidectomized males (age 60–72 years), the mean plasma T level was 23·3 ± 3·7 (SEM) ng/100 ml, DHT 14 ± 3 ng/100 ml, Δ4 79 ± 9 ng/100 ml, DHEA 84 ± 22 ng/100 ml, 17-OHP 33 ± 7 ng/100 ml and

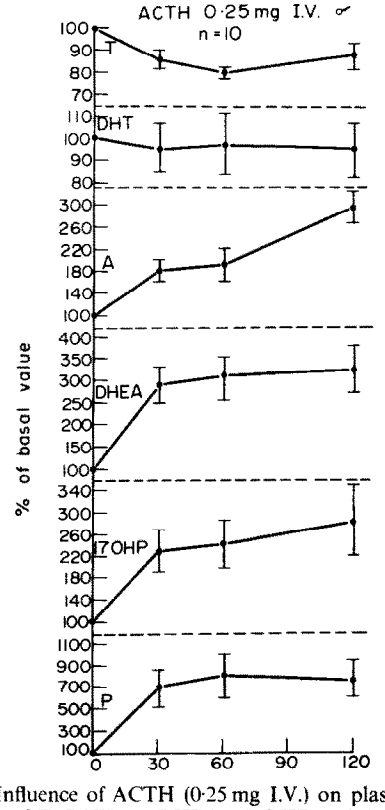

Fig. 2. Influence of ACTH (0·25 mg I.V.) on plasma level of T, DHT, A, DHEA, 17-OHP and P.

Table 7. Dexamethasone suppression ($n = 10$) and HCG stimulation ($n = 12$) tests. Plasma steroid levels in ng/100 ml and in % of basal value

T		DHT		Δ4		DHEA		17-OHP		P	
Basal	DX	Basal	DX	Basal	DX	Basal	DX	Basal	DX	Basal	DX
655 ± 79	620 ± 90 97 ± 9% n.s.	61 ± 11	58 ± 9 87 ± 8% n.s.	122 ± 11	58 ± 9 51 ± 5% $P < 0.001$	406 ± 68	158 ± 30 42 ± 8% $P < 0.001$	116 ± 13	106 ± 16 85 ± 9% n.s.	18 ± 1	10 — $P < 0.01$
Basal	HCG	Basal	HCG	Basal	HCG	Basal	HCG	Basal	HCG	Basal	HCG
574 ± 76	1210 ± 158 211 ± 18% $P < 0.001$	61 ± 11	104 ± 16 181 ± 20% $P < 0.001$	124 ± 17	161 ± 25 152 ± 17% $P < 0.01$	235 ± 75	285 ± 68 120 ± 20% n.s.	118 ± 17	280 ± 48 299 ± 94 $P < 0.001$	26 ± 3	28 ± 3 106 ± 11% n.s.

finally P 17·0 ± 4·3 ng/100 ml. Except for progesterone, all these values are significantly ($P < 0.01$) lower than in intact elderly males.

DISCUSSION

Influence of age on basal plasma levels

It appears from this study that in analogy with testosterone, DHT, DHEA and 17 OHP levels decrease significantly with age, whereas Δ4 and P levels do not show any significant decrease in aging males. DHT levels observed in this study are in agreement with most values reported in the literature [4–9], although Rosenfield and Boyar [10] as well as Coyotupa *et al.* [11] reported significantly higher levels. A decrease of DHT levels in elderly males as reported in this study, although expected, has to our knowledge not been reported previously.

The important decrease of DHEA levels from a mean value of 470 ± 58 (S.E.) ng/100 ml in young males, to a mean value of 175 ± 18 (S.E.) ng/100 ml in males over 50 years is rather unexpected, as DHEA appears to have essentially an adrenal origin. A similar decrease with age in DHEA-S has been reported by Sekihara [12]. As cortisol secretion does not decrease significantly with age, these observations suggest a change in steroid biosynthesis with age, as was already observed at puberty when the secretion of DHEA-S increases significantly [13]. The DHEA values observed in younger males are comparable to those reported in the literature [14–21], except for Abraham [22] who reports a mean basal value of 182 ± 89 ng/100 ml (range 20–380 ng/100 ml).

The moderately significant decrease of 17-OHP levels with age on the other hand, although not described previously is not unexpected in view of the essentially testicular origin of this steroid. The levels found in normal young males (110 ± 9 ng/100 ml) are similar to values reported by other authors [23–25]. The same applies to Δ4 levels [26–28]; mean P levels reported in this study on the other hand are significantly lower than those reported by most authors using competitive protein binding methods [29–31], but comparable to values reported by Furuyama [32], Youssefnejadian *et al.* [33] and Abraham *et al.* [34] using R.I.A.

Circadian variations

All steroids studied with the exception of DHT show significant circadian variations with maximal levels between 8 a.m. and 12 noon, whereas the nadir is between 8 p.m. and midnight (Fig. 1). Similar nyctohemeral variations as observed in this study have been reported previously for DHEA (15, 17, 19, 21) and for Δ4 [35], whereas the amplitude for 17 OHP is rather less important than the amplitude (60%) reported by Strott *et al.* [23]. In the view of the largely testicular origin of basal 17-OHP levels in males (see below) a more moderate amplitude as observed in this study seems more acceptable.

Like cortisol, it has been shown that for all these steroids, secretion occurs episodically [31, 36–38]. If the pulses occur at random throughout the nyctohemer, one could expect that these pulses might mask the circadian variations. However, the circadian variations appear to be at least partially the consequence of variations in frequency and importance of the secretory pulses over the nyctohemer [37]. On the other hand it is evident that, due to the existence of these secretory pulses, the circadian rhythm may be missed when studied by sampling plasma every 4–6 hr in an occasional subject; therefore circadian rhythms may only become evident when studying a larger group.

The absence of circadian variation in DHT levels is probably related to the complex origin of DHT, which is partly secreted as such [39] and partly originates from peripheral metabolism of precursors, among which testosterone accounts for 50% of DHT [40]; the longer half-life of DHT [10] might be another factor.

Influence of ACTH stimulation and dexamethasone suppression on plasma androgens

This study confirms the decrease in T levels after short term ACTH stimulation, previously observed by ourselves and others [41–46] after long term ACTH stimulation. Some authors [47, 48] did not observe this decrease in T levels, whereas Smals *et al.* [48] suggested the apparent decrease to be attributable to the spontaneous circadian rhythm. This possibility is ruled out by our results, as this decrease was already observed within 1 h of ACTH administration at a time when T levels do not decrease spontaneously.

It is remarkable that the decrease in T levels after short term ACTH stimulation, was not reflected in a decrease in DHT levels; this is possibly the consequence of the increased Δ4 secretion by the adrenal cortex, compensating the effect of the decrease in T levels. A moderate decrease in DHT levels after long term ACTH stimulation has been reported [45].

The important increase in Δ4 levels after ACTH stimulation observed in our study contrasts with the results reported by Lee *et al.* [49], who did not observe any change in Δ4 levels after ACTH, although they observed a suppression by dexamethasone from a mean level of 183 ± 14 ng to 82 ± 15 ng/100 ml ($P < 0.05$). Rosenfield *et al.* [50], however, in prepubertal children, reported a very important increase in Δ4 levels after ACTH. In the view of the important adrenal contribution to Δ4 levels, an absence of increase of Δ4 levels upon ACTH stimulation would be highly surprising. The important increase in 17-OHP levels observed after ACTH stimulation, indicates that at least under stress conditions, the adrenal cortex may contribute to a considerable extent to plasma 17-OHP. This is in accordance with the view of West *et al.* [31] that the adrenal cortex might secrete 17-OHP whenever cortisol secretion is high.

Finally the very important increase in P levels by ACTH stimulation, as well as the disappearance of

P levels after dexamethasone, point to the adrenal cortex as the source of plasma P in males.

Dexamethasone administered for 72 h did not have any significant influence on either T or DHT levels but decreased Δ4 to 50% ± 5% and DHEA levels to 42 ± 8% of basal values; 17-OHP levels decreased hardly whereas P levels decreased to levels at or below the detection limit of the method in all subjects.

These results suggest negligible adrenal contribution to basal T, DHT and 17-OHP levels.

Influence of HCG or gonadectomy on androgen levels and origin of androgens in male plasma

HCG stimulation caused significant increase in T, DHT, 17-OHP and Δ4 levels, DHEA and P levels remaining unchanged. After orchidectomy, however, DHT and 17-OHP levels are about 25% of levels in intact males, whereas Δ4 and DHEA levels are moderately but significantly decreased, P levels finally being similar to those observed in intact males.

It is evident from these results that under basal conditions both DHT and 17-OHP have an almost exclusive testicular origin. It is remarkable that after orchidectomy the T/DHT ratio is higher than in normal males and very close to the ratio observed in normal women. This might suggest that Δ4, the main precursor of androgens after orchidectomy as well as in women, yields relatively more DHT than does testosterone secreted into the general circulation.

Abraham *et al.* [34] observed similar 17-OHP levels in orchidectomized males to those reported in this study, whereas Lee *et al.* [49] observed a decrease of 17-OHP levels to about half of the pre-treatment value after gonadotropin suppression. Strott *et al.* [23] on the basis of a 90% decrease of 17-OHP levels after administration of fluoxymesterone and an absence of decrease after dexamethasone, consider 90% of 17-OHP plasma levels to have a testicular origin.

As far as the origin of Δ4 plasma levels is concerned, our results confirm the view of Longcope *et al.* [51] that in males the steroid originates in about equal parts from the testes, either by conversion of T (1/3 of total Δ4) [52] or by direct secretion [53, 54].

In accordance with most authors our results suggest that under basal conditions DHEA has mainly an adrenal origin, although the testes contribute, albeit modestly, to the plasma levels. Indeed, in accordance with Kirschner [55] but in distinction with results obtained by Nieschlag *et al.* [20] as well as by Saez and Bertrand [13], we observed no significant increase in plasma DHEA levels after HCG stimulation. In gonadectomized males however, we found a mean DHEA level significantly lower than the mean value observed in normal males of similar age, pointing to a testicular contribution to plasma levels. This was shown more directly by Hudson *et al.* [53] who observed spermatic vein blood DHEA concentration to be about 1/10th of testosterone concentration. Progesterone levels in males appear to have an almost exclusive adrenal origin: indeed levels are unaffected by either orchidectomy or HCG stimulation, whereas they increase 700% by ACTH stimulation and become almost undetectable after dexamethasone suppression. This conflicts with the views of West *et al.* [31] who, on the basis of diurnal variations of P synchronous with T variations observed in one single male, suggest P to have a testicular origin. Wasserman & Eik-Nes [56] observed, on the other hand, that progesterone is the only steroid present in the testis of the dog, which is apparently not secreted by this tissue.

In conclusion, this study indicates that plasma levels of T, DHT, DHEA and 17-OHP decrease significantly in old age. With the exception of DHEA all these steroids have an almost exclusively testicular origin and in the view of the now generally accepted decrease in Leydig cell function with age, this decrease is not unexpected. More surprising is the decrease in DHEA levels, other adrenal cortical steroid levels (Δ4, P, cortisol) remaining unchanged; this suggests a shift in adrenal cortical secretion with age.

All steroid levels studied, with the exception of DHT, show important nyctohemeral variations, the amplitude being greatest with steroids having a predominant adrenocortical origin (DHEA, Δ4, P). Finally our results indicate that in males P has an almost exclusive adrenal cortical origin, whereas basal 17-OHP levels have an almost exclusive testicular origin.

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