4-androstene-3,17-dione by the adrenal. We have therefore developed a method and obtained a highly specific antiserum for use in measuring 11β-hydroxy-4-androstene-3,17-dione by a solid-phase radioimmunoassay procedure. This antiserum was generated in rabbits using the hapten, 6β,11β-dihydroxy-4-androstene-3, 17-dione 6-hemisuccinate coupled to bovine serum albumin. The antiserum showed a titer of 68% binding of 50 pg of 11β-hydroxy-4-androstene-3,17-dione-[1,2,6,7-^{3}H] at a dilution of 1:12500 in the assay. Among the numerous steroids tested for cross-reactivity, only 4-androstene-3,17-dione and 5α-androstene-3, 17-dione showed 5% and 2% cross-reactivity respectively. All other structurally related steroids, including C_{21} compounds, showed no detectable cross-reaction. The linearity of the Scatchard plot indicated that the antibody was essentially homogeneous with respect to its binding of 11β-hydroxy-4-androstene-3,17-dione, with a K_a of $9{\cdot}7 \times 10^8$. The Rivanol-treated antiserum was coupled to Enzacryl AA, a synthetic polymer and the complex so obtained showed 50% binding with the labelled antigen. Determination of cross-reactivities employing this complex proved it retained its high specificity and can readily be adopted for a simple solid-phase RIA.

36. Preparation of antigenic conjugates of 3-oxosteroids by coupling to a macromolecule through position—1

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A general method for rendering 3-oxosteroids antigenic by coupling to a macromolecule through position 1 has been developed as part of a program aimed at generating antibodies able to discriminate between closely related steroid hormones and their metabolites. Nucleophilic attack on the 1,2-dehydro derivatives of ring A saturated 3-oxo or Δ^4-3-oxosteroids by ambidendate reagents gave the corresponding 1α-thioether alkanoic acids. Thus addition products with β-mercaptopropionic acid were obtained from 5α-dihydrotestosterone, testosterone, progesterone and androstenedione. These were covalently attached to either thyroglobulin or bovine serum albumin (BSA). Immunization of rabbits with testosterone-1α-carboxyethyl-thioether-thyroglobulin gave rise to antisera of high affinity to testosterone that showed minimal cross-reaction with 5α-dihydrotestosterone (3%), androstenedione (< 0·1%) and with a variety of 17-oxo-androstane compounds (< 0·1%). Conversely, immunization with 5α-dihydrotestosterone-1α-carboxyethyl-thioether-BSA yielded an antiserum with high affinity for 5α-dihydrotestosterone but little cross-reaction for testosterone (10–15%) and androstenedione (< 0·5%).

37. Some studies of the specificity of antisera to C_{18} and C_{19} steroid–BSA conjugates

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Antisera for use in radioimmunoassay techniques have been developed for five C_{19} and three C_{18} steroids involving exposure of alternative regions of the steroid moiety as immunogenic determinants. The ability of related steroids and other compounds to interfere with steroid-antibody binding has been investigated. Antigens prepared for this study were:—17β-hydroxy-5α-androstan-3-(O-carboxymethyl) oxime–BSA 4-androstene-3,17-dione-11α-hemisuccinate–BSA 4-androstene-3,17-dione-6β-hemisuccinate–BSA 17β-hydroxy-4-androsten-3-one, 11α-hemisuccinate–BSA 3β-hydroxy-5-androsten-17(O-carboxymethyl) oxime–BSA 1,3,5(10)-estratrien-3,16α,17β-triol-6(O-carboxymethyl) oxime–BSA 1,3,5(10)-estratrien-3,17β-diol-6(O-carboxy-methyl)oxime–BSA 1,3,5(10)-estratrien-3-ol, 17-one-6(O-carboxymethyl)oxime–BSA.

38. A high affinity testosterone antibody gives lower female testosterone values

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Antisera to testosterone reported, thus far, have an affinity of $1{-}2 \times 10^9$ L/M. We have generated an antibody to testosterone-3 conjugate which has an affinity of 2×10^{10} L/M using a conjugate containing 38 residues per albumin. This serum is of higher specificity as well (DHT = 25%, 4-Adione = 0·3%, 4-Adiol = 3·2%, F = 0·001%, Prog = 0·06%). Assay of hexane extracts directly or after alumina column chromatography gave the mean ± SD (Range) 28·4 ± 14·5 (5·6–56·2)ng% and 14·6 ± 9·0 (3·1–33·9)ng%, respectively, for 19 normal young women and 401·4 ± 174·7 (192–808)ng% and 378·1 ± 167·9 (181–795)ng%, respectively, for 14 normal men. A comparison of 10^9 L/M antisera with 10^{10} L/M antisera after alumina chromatography gave 33·6 ± 19·5 ng% vs 15·8 ± 6·5 ng% for 6 normal women. Values by the 10^9 antiserum compare with other methods. Therefore, the difference lies in the more specific antiserum rather than the methodology.

39. The use of steroid coupled bacteriophage in the steroid field

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Estradiol-17β and progesterone have been covalently conjugated to bacteriophage T4. The bacteriophages surviving the coupling process were able to form plaques on Petri dishes when mixed with their host *E. coli.* B. These "steroidated" phages could be neutralized at 0° or 37° according to pseudomolecular first order kinetics when incubated in very dilute anti-steroid sera. Preincubation with increasing amounts of free steroids resulted in a decrease of the neutralization which allowed us to construct standard curves for these two steroids and the related compounds. The average equilibrium constants of the antisera against free steroids could be directly calculated from the standard curves. We compared this "viroimmunoassay (VIA) with radioimmunoassay (RIA) for sensitivity in the antisera titer and amount of steroid detected. VIA could detect antisera concentrations 100 times lower than RIA. In addition it could quantify with good reliability 1–2 pg of estradiol or progesterone (RIA could only detect 5–10 pg). Lastly the average equilibrium constants of the antisera against the immunogenic steroids and others have been found to be the same by dialysis equilibrium and VIA. We used this VIA to assay progesterone and estradiol levels directly in diluted plasma of pregnant women. The values found in these conditions were in good agreement with those determined after extraction by RIA.

2B 2. Steroid radioimmunoassay—II

40. One column chromatography and simultaneous radioimmunoassay of testosterone and dihydrotestosterone

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A specific RIA for testosterone (T) and dihydrotestosterone (DHT) was developed using a single celite column for separation. Antibody was raised in rabbits using a conjugate of human serum albumin linked to T *via* an oxime at the three position. In an initial dilution of 1:20,000, one antiserum bound 35% of tritiated T(T^{H3}). DHT cross-reacted to the extent of 60%. There was no significant binding by any other steroid tested. With this diluted antiserum a RIA was established using T^{H3} or DHT^{H3} as the labelled hormone to measure T and DHT respectively. After incubation at room temperature for 1 h and 4 °C for 15 min separation of free from bound hormone was accomplished by dextran charcoal suspension and centrifugation. The sensitivity of each RIA was 10 pg and water blanks were consistently below this level. Recovery losses were assessed by adding a small number of counts of both labelled steroids to serum samples. The ether extract from ·25 ml serum for adult males and 1·0 for females was dried, reconstituted with isooctane, and applied to a 5 cc celite column previously washed with isooctane. The DHT fraction was eluted with 7 ml of isooctane, the column rinsed with 3 ml of isooctane and the T fraction then eluted with 5 ml of 10% ethyl acetate in isooctane. The average recovery for both T and DHT was 85%. When known amounts of T and DHT were added to sera and assayed, the mean recovery for DHT was 102% ± 3·5(S.E.M.) of the expected value, and for T 111% ± 5·5. Interassay variation for DHT was 13·8%, and for T 10·2%. Intra-assay variation was 5·7% for DHT and 4·9% for T. Values for 17 young healthy male medical students were 555 ± 131 ng%(mean ± S.D.) for T and 63·1 ± 20·2 ng% for DHT. Reproductive age female levels were 25·7 ± 11·3 ng% for T and 14·1 ± 6·5 ng% for DHT.

41. The simultaneous determination of six C_{19} steroids in human peripheral plasma using a convenient radioimmunoassay technique

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A method is described for the determination of 5α-androstane-3,17-dione(androstanedione);4-androstene-3,17-dione (androstenedione), 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone); 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone); 17β-hydroxy-4-androsten-3-one (testosterone) and 5-androstene-3β,17β-diol (androstenediol) in peripheral venous plasma using a combination of solvent extraction and thin-layer chromatography after the addition of ^3H labelled internal standards. This is followed by radioimmunoassays developed specifically to measure each of the individual steroids. The concentration of these compounds are given for healthy subjects and patients with endocrine dysfunction. The method attempts to resolve some of the problems associated with clinical assays which only measure the plasma concentration of a single C_{19} steroid.

42. Standardization of specific radioimmunoassays for plasma estrone, estradiol, progesterone and androstenedione

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The wide variety of steroid-RIA's carried out in a clinical laboratory calls for standardization of all steps involved in the procedure. Specific RIA's for estrone (E_1), estradiol (E_2), progesterone (Prog) and androstenedione (A), that require only solvent extraction of plasma and no chromatography, were developed by following identical protocols from immunization schedules to processing of RIA raw data. E_1-6-CMO:BSA, E_2-6-oxo:BSA, Prog-11-0:BSA and A-6-ol hemisuccinate were used as antigens. Antisera of different titers were generated in rabbits 6–12 weeks after the first antigen injection and after one to three booster injections of 100 μg of antigen. Working solutions of antisera were made to achieve 50% binding under standard assay conditions (300 μl reaction volume, incubation 16 h at 4 °C, charcoal separation). Further characterization of the assays included factors such as cross reactivity of antisera, sensitivity, precision and accuracy. Antisera for E_1, E_2 and Prog did not cross react significantly with other steroids at their physiological concentrations. High specificity of the A-assay was obtained with petroleum ether extraction of the plasma samples. The raw data were processed by means of a computer program based on a logit/log transformation of the dose response curve and especially designed for the Hewlett–Packard 9810. Measuring ranges for the assays were as follows: E_1: 10–500 pg, E_2: 10–500 pg, Prog: 20–2000 pg, A: 20–1000 pg. These standardized RIA's have proved to be useful in processing large numbers of samples in studies concerned with ovarian function in normal and abnormal menstrual cycles.

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43. Simultaneous determination of 6-sex-steroids on a 2 ml plasma sample

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A R.I.A. method has been developed for the determination of progesterone (P), estradiol (E_2), dehydroepiandrosterone sulphate (DS), testosterone (T), androstenedione (Δ_4) and dihydrotestosterone (DHT) in a single 2 ml plasma sample. 10 μl of plasma is used for the direct determination of DS, using DS-17HS–BSA antibody (AB). To the rest of the sample, internal ^3H-standards (1000 d.p.m.) of E_2, Δ4, T and DHT are added and the sample is extracted with petrol-ether, yielding the P fraction. The plasma is subsequently extracted with ether, and the extract fractionated through a LH-20 microcolumn, yielding the estradiol fraction and a fraction containing T, Δ4 and DHT. The latter are separated by t.l.c. on silicagel (benzene-methanol 85:15 v/v). Steroids are measured by RIA using specific antibodies.

	Normal values (ng/ml)		*C.V.* (%)	*Recovery* (%)	*Sensitivity*
	Men	Women			
DS (μg/ml)	1·8–2·9	1·0–2·9	13	102 ± 9	10 pg
Progesterone	0·1–0·22	0·2–15	18(M)–10(F)	96 ± 12	10 pg
Estradiol	0·8–2	1·0–20	6	98 ± 7	10 pg
T	2·8–8·5	0·1–0·7	7	96 ± 8	10 pg
Δ4	0·6–2·1	0·6–3·1	8	102 ± 10	10 pg
DHT	0·3–1·4	0·1–0·4	14	108 ± 11	12 pg