levels relatively considerably lower in the same extracts. The same steroids have also been assayed in tissue from males with gynaecomastia and with carcinoma of the breast. The endogenous steroid levels in the tumours have been related to other biochemical parameters such as "oestradiol-17β receptor" concentration.

31. Assay of hormonal steroids by gas chromatography–mass spectrometry using stable isotope internal standards

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The mass spectrometer with a multiple ion detector can be used as a very sensitive detector in the gas chromatographic estimation of hormonal steroids. The intermittent focussing in the instrument on selected ions gives high specificity to the assay. The ideal internal standards for this type of assay are hormonal steroids labelled with stable isotopes. They allow correction for any losses in the initial procedures preceding the gas chromatography. Gas chromatography/mass spectrometry with stable isotope labelled internal standards produced by deuteration of appropriate steroids has been applied to the assay of hormonal steroids in body fluids. Practical assays for testosterone and estrogens in body fluids will be compared with radioimmunoassay methods for the same compounds.

2B. Steroid radioimmunoassay—I

32. Synthesis of 11α-C_{19}-steroid–protein conjugates for new RIA antisera

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The synthesis of specific steroid antigens for use in the development of radioimmunoassay (RIA) systems has been the subject of much recent investigation by several authors. Many different sites of conjugation of the steroid to protein have been tried, including the 3, 17, 6, 7 and 11 positions. The most specific steroid derivatives yet found utilize an 11α-hydroxyl group for conjugation to protein. Only in the case of progesterone is the 11α-hydroxy compound readily available. We have succeeded in synthesizing 11α-hydroxy-androstenedione from adrenosterone which is commercially available at low cost. The simple two step method may be run in any laboratory and gives a good yield of the 11α-hydroxy compound without contamination by the 11β-hydroxy isomer. Additionally, routes will be presented to give rise to other 11α-hydroxy androgens, such as, testosterone, dihydrotestosterone and androstanediol for use in developing new RIA systems. (Supported in part by The Robert A. Welch Foundation, Q-560 and St. Luke's Episcopal Hospital).

33. Antisera specific to corticosterone

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Five rabbits were immunized with a corticosterone-3-oxime bovine serum albumin (BSA) conjugate carrying 6 steroid molecules. Antibody sensitivity and specificity were tested 10–14 weeks after first immunization. Antibody dilutions that bound 50% of ^{3}H-corticosterone were between 500 and 1000. All antisera showed similar high sensitivity as indicated by the slopes of the standard curves. Three of the 5 sera showed highest cross-reaction with cortisol (70, 43, 26%) and low cross-reaction with deoxycorticosterone (DOC) (7, 5, 5%) and progesterone (3, 4, 5%). With these sera alterations in the chemical and steric configuration at C-11 resulted in a marked decrease in cross-reaction as indicated by displacement experiments with 11-deoxycortisol (0·9, 0·8, 0·7%), cortisone (5, 0·1, 0·06%) and 11-epi-cortisol (0·03, 0·02, 0·02%). Two of the 5 sera showed highest cross-reaction with progesterone (94, 67%) and DOC (82, 88%) and low cross-reaction with cortisol (6, 8%). With these sera, changes in the chemical configuration at C-17 resulted in a decrease in cross-reactivity as indicated by displacement experiments with 17α-OH-progesterone (8,2%). Our results indicate that with corticosterone-3-oxime BSA two different antibody populations can be raised in rabbits: (a) one predominantly directed against β-C-11, the other predominantly against α-C-17. Coupling through the double bond at C-3 preventing rotation of the hapten might have favoured these findings.

34. Specific antisera for estriol-16α-glucosiduronate

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Estrogen conjugates in plasma and urine have usually been determined indirectly after hydrolysis, followed by assay of the free estrogen. Such methods often do not distinguish between individual conjugates. We have developed an antiserum to estriol-16α-glucosiduronate for use in a direct radioimmunoassay of this conjugate. Estriol-16α-glucosiduronate-bovine serum albumin was prepared by coupling the carboxylic acid group of the glucosiduronate to ε-amino groups of lyside residues in bovine serum albumin by the mixed anhydride procedure. The protein conjugate was injected intradermally into rabbits with Freund's adjuvant. At a dilution of 1:2000 the antiserum gave a useful standard curve over the range of 10 pg to 200 pg. The antiserum cross-reacted 4% with estriol-17β-glucosiduronate, < 1% with estriol-3-glucosiduronate, 2% with estradiol-17β-17-glucosiduronate, and 1% with estrone glucosiduronate. As expected, the antiserum showed significant cross reactions with certain free estrogens: 16-epiestriol (12%), estriol (22%), estrone (32%), and estradiol-17β (20%). Thus preliminary routine separation of free estrogens is necessary before radioimmunoassay.

35. Highly specific antisera for solid-phase radioimmunoassay of 11β-hydroxy-4-androstene-3,17-dione

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The contribution of the adrenal cortex to the androgenic-anabolic interrelationship in the body has not been fully explored. The secretion of androgens by the adrenal, their change in response to environmental stress, and their effect on other organs like the testes are significant features of such concerns. From what is known of the production of C_{19}-steroids by the adrenal and from the analysis of the urinary metabolites of these compounds, it is evident that they are principally 11β-hydroxy secretory products. With a conversion of over 90% of preformed 4-androstene-3,17-dione to its 11β-hydroxy derivative within the adrenal, a measurement of the plasma levels of the latter compound should serve as a rational index of the biosynthesis of

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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