A Solid-Phase Radioimmunoassay for Plasma Progesterone

By KAILAS K. DIGHE and WILLIAM M. HUNTER

Medical Research Council Radioimmunoassay Team, 2 Forrest Road, Edinburgh EH1 2QW, U.K.

(Received 22 February 1974)

A detailed procedure is presented for the assay of plasma progesterone. The routine assay is based on the use of antiserum which is covalently linked to microcrystalline cellulose, the double-antibody method being used as a reference separation system. This procedure gives high precision accompanied by small and acceptable losses of antiserum titre but without loss of sensitivity when compared with the double-antibody method. Ethanol is first added to the plasma (10 vol. of plasma + 1 vol. of ethanol) after which a single extraction with light petroleum yields a constant recovery $[92.4 \pm 1.2 \text{ (S.D.)}\%$ of added $[^3\text{H}]\text{progesterone}$] and obviates the need for tracer recoveries on each sample being assayed. Distortions of the response curve owing to solvent residues have been almost eliminated. The assay can measure progesterone at all stages of the menstrual cycle when volumes of $200 \mu\text{l}$ of plasma are used and this permits the detection of the periovulatory rise at its inception. Detailed specificity studies are presented for the assay end point itself and these are related to the responses to be expected in extracts of plasma. Progesterone-like activity was found in urine and a fourfold increase in excretion rates was observed between the follicular and luteal phase of the normal menstrual cycle.

Although some interest may attach to measurements of plasma progesterone in men and post-menopausal women, their main value in the human is that they can serve to indicate the presence and functional competence of the corpus luteum. Methods based on the use of the cortisol-binding protein give limited specificity when combined with a simple extraction in light petroleum or a similar non-polar solvent (Johansson, 1969), and they therefore tend to suffer some interference notably from 17-hydroxyprogesterone. Also these procedures are less sensitive and consequently require more plasma than assays based on the use of specific antisera. Further, antisera are highly stable and a single venous sample may suffice for many thousands or even millions of assays.

That the method, if based on the use of antisera raised to progesterone conjugates linked at the 11α position to a macromolecular carrier, was likely to achieve a successful assay, was clear when reports of the specificity of such antisera began to appear (Midgley & Niswender, 1970). However, the development of a viable convenient robust and reproducible assay from such general principles involves the procurement of antisera of adequate sensitivity, the selection of a suitable system for separating antibody-bound from free progesterone and the development, optimization and integration of the detailed procedures to be used for each of the assay steps. Of the reports that have so far appeared few contain sufficient detail to enable the establishment of the procedures described, and none has produced convincing evidence that would amount to a claim

for consideration as a standard procedure. We report here an assay which uses a single light petroleum extraction and a 90 min incubation with an antiserum that was raised to an 11α -hydroxyprogesterone hemisuccinate-bovine serum albumin conjugate which has been covalently linked to cellulose. The assay can measure progesterone in 0.2 ml of plasma, or less, at all concentrations encountered in menstruating women. The reliability of the extraction procedure is such that ^3H -labelled tracer recoveries need not be undertaken on each plasma sample being assayed.

Experimental

Materials

$[1,2,6,7\text{-}^3\text{H}]\text{Progesterone}$ (specific radioactivity $80\text{--}110 \text{ Ci/mmol}$) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and checked for homogeneity on a column of Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) run in heptane-chloroform-ethanol (200:200:1, by vol.) saturated with water. Butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-oxo-3,4-diazole] scintillator was from CIBA Geigy (U.K.) Ltd., Duxford, Cambridge, U.K.). The liquid-scintillation fluid was prepared by dissolving 4 g of butyl-PBD in 1 litre of toluene (AnalaR, BDH Chemicals Ltd., Poole, Dorset, U.K.).

Light petroleum (b.p. $40\text{--}60^\circ\text{C}$; BDH AnalaR or from Hopkins and Williams Ltd., Chadwell Heath, Essex, U.K.), heptane (BDH, Laboratory grade), chloroform (BDH AnalaR) and ethanol (James Burrough Ltd., 60 Montford Place, London S.E.11,

U.K.), were all redistilled before use. Sodium salts, Na_2HPO_4 and NaH_2PO_4 (BDH AnalaR), were used to prepare the phosphate buffer. Silica gel (60–120 mesh) and gelatine were BDH laboratory grade and all other chemicals were AnalaR grade.

Progesterone, 11α -hydroxyprogesterone, 17α -hydroxyprogesterone, corticosterone and cortisone were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and androst-4-ene-3,17-dione was from Organon Laboratories Ltd., Newhouse, Scotland, U.K. 11β -Hydroxyprogesterone, 11α -oxoprogesterone, 11α -deoxycorticosterone, 11α -deoxycortisol (compound S), cortisol, dehydroepiandrosterone and cholesterol were from Sigma (London) Chemical Co., Ltd., Kingston-upon-Thames, U.K. 6β -Hydroxyprogesterone, 5α -pregnane-3,20-dione, 5β -pregnane-3,20-dione were from Steraloids, 31 Radcliffe Road, Croydon, Surrey, U.K. 20α -Hydroxypregn-4-en-3-one, 20β -hydroxypregn-4-en-3-one, 3α -hydroxy- 5β -pregnan-20-one, 5α -pregnane- $3\alpha,20\alpha$ -diol, 5α -pregnane- $3\beta,20\alpha$ -diol, 5β -pregnane- $3\alpha,20\alpha$ -diol and pregnenolone (3β -hydroxypregn-5-en-20-one), were from the M.R.C. reference collection, Department of Chemistry, Westfield College, Hampstead, London N.W.3, U.K. Bovine serum albumin was Cohn fraction IV (Armour Pharmaceutical Co., Ltd., Eastbourne, Sussex, U.K.).

The assay diluent was 0.05M-phosphate buffer, pH 7.5, containing gelatine (0.1%).

Methods

Preparation of immunogens. The hemisuccinate of 11α -hydroxyprogesterone was prepared from 11α -hydroxyprogesterone and conjugated to bovine serum albumin by the method of Erlanger *et al.* (1959). As judged by u.v. spectrometry 18 mol of progesterone were conjugated/mol of bovine serum albumin. A second conjugate was similarly prepared from 6β -hydroxyprogesterone, a ratio of 30 mol of steroid/mol of bovine serum albumin being achieved.

Immunization. New Zealand white rabbits were initially given 1 mg of immunogen in 4×0.25 ml, subcutaneously over the scapuli and in the thighs, of an emulsion of 0.9% NaCl in Freund's complete adjuvant (1 vol.+1 vol.). This was followed by identical administrations generally at intervals of 5–6 weeks, the animals being bled 7–10 days after each such booster dose. Antisera were designated by an animal number (e.g. R1–R6) and sample number (1–6), e.g. R1(4).

Separation of bound and free tracer. All incubation mixtures were allowed to reach equilibrium (90–120 min at 20–22°C). The double-antibody method which was used as a reference method was based on an already selected batch of donkey anti-rabbit serum and an optimized system (Hunter, 1973), which gave a good precipitation plateau when incubated at 4°C for 16 h.

Once a given batch of primary antiserum had been selected it was used for the routine assay procedure after being covalently linked to cellulose (micro-crystalline for t.l.c.; E. Merck, A.G., Darmstadt, Germany) by the method of Cuatrecasas (1970).

Further purification of solvent. Light petroleum was purified by stirring over different samples of concentrated H_2SO_4 until the acid remained colourless after 6 h of mixing; the acid-treated solvent was washed with water, dried over anhydrous Na_2SO_4 and then passed through a preheated silica-gel column; 320 g of silica gel was used/litre of solvent and each sample of silical gel was used only once.

Distilled water. Deionized double-glass-distilled water was used.

Radioactivity counting. A Tracerlab Corumatic 200 or a Packard Tricarb liquid-scintillation spectrometer was used. The [^3H]progesterone present in the aqueous supernatant which represented the 'free' hormone from the centrifuged assay tubes was decanted into scintillation vials containing 10 ml of liquid-scintillation fluid. The vials were shaken for 2 min to extract the tracer into the organic phase and radioactivity was counted without separation of the two phases (Abraham *et al.*, 1971). In the routine assay procedure in which the incubation mixtures contained 10 pg of [$1,2,6,7\text{-}^3\text{H}$]progesterone, which yielded 6070–8200 d.p.m., a counting time of 5 min was used.

Results

Titre and sensitivity of antisera

Maximum assay sensitivities were generally obtained at 3–7 months from the primary immunization and thereafter in every case remained unchanged after further booster injections. Such antisera gave 95–100% binding of the [^3H]progesterone tracer when present at 10 pg/ml in excess of antiserum, whereas non-specific binding in the absence of antibody was 5–8%, when tested with the double-antibody system. With the same concentration of tracer, three of the six rabbits given 11α -hydroxyprogesterone-bovine serum albumin conjugate over periods of 2, 3 and 24 months respectively, gave antisera which yielded standard curves with the following essentially identical characteristics: 65–75% of a [^3H]progesterone tracer (10 pg/ml) bound to antibody in the absence of unlabelled progesterone with 25, 50 and 75% inhibition of binding being given by unlabelled progesterone at concentrations of 50, 150 and 450 pg/ml respectively. Final dilutions of antisera to achieve this were 1/30000 in all three rabbits at 3–4 months, increasing to 1/60000 during the second year of the one surviving animal. These three antisera had average affinity constants (K) of 3.15×10^9 , 3.77×10^9 and 3.92×10^9 l./M.

Fig. 1. Detailed dose-response curves for progesterone (■) and three cross-reacting steroids, 17-hydroxyprogesterone (□), androst-4-ene-3,17-dione (●) and cortisol (○)

The responses are plotted with the binding of [^3H]progesterone tracer (10 pg in 1 ml final incubation volume) in the absence of unlabelled progesterone as 100% bound [first point (●) $\pm$ s.d. in the Figure] and non-specific binding controls as 0% bound. The response curves for the cross-reacting compounds are not strictly parallel to that of progesterone itself but show diminishing slopes as the degree of cross-reactivity falls. Each point represents the mean of duplicate tubes. About 70% of the tracer was bound in the absence of unlabelled steroid.

The remaining three rabbits from this group were rejected 6 months after the primary immunization because their titres were consistently greater than 1/20000.

Of the six rabbits immunized with 6 β -hydroxyprogesterone conjugate three yielded standard curves having characteristics essentially identical with the three animals which were successful in producing antibodies to 11 α -hydroxyprogesterone-bovine serum albumin conjugates and had average affinity constants of 2.92×10^9 , 3.55×10^9 and 3.92×10^9 l/m. This was achieved at final dilutions which generally increased steadily to reach 1/30000–1/60000 at 6 months. The remaining three rabbits had similar titres but gave less sensitive standard curves.

Specificity of antisera

Response curves over the range 1 pg–10 μ g were run on 24 compounds for the antiserum finally selected for use [11 α -hydroxyprogesterone antiserum R1(4)] and most of these compounds were similarly tested by using antisera from the five other rabbits (two 11 α -hydroxyprogesterone and three 6 β -hydroxyprogesterone antisera) which yielded acceptable standard curves for progesterone. The dose-response curves of steroids which cross-react

in the assay system were not strictly parallel to those of progesterone itself. Changes in structure resulted not only in the expected lateral displacement of such curves (to the right in plots such as that used in Fig. 1) but generally also in progressively diminishing slopes as the relative cross-reactivity decreased. Fig. 1 illustrates this for three compounds, 17-hydroxyprogesterone, androst-4-ene-3,17-dione and cortisol. It follows that the degree of cross-reactivity of a given compound cannot be fully represented by a single value expressing its relative potency, since this differs throughout its response curve. For example, cortisol gave relative potencies of 0.038, 0.018 and 0.011%, whereas the values for 17-hydroxyprogesterone were 1.0, 0.83 and 0.61 respectively at 20, 50 and 80% inhibition of tracer binding in Fig. 1. The general practice of expressing relative potencies in terms of single ratios from the intercept of their dose-response curve at 50% inhibition of tracer binding is not strictly valid unless the responses are parallel, and where they are not its use implies a specificity which is better than can be achieved in practice at the upper half of the response curve but which is less than can be expected in its lower half. Even so, this practice is now widespread and Table 1 sets out the specificities of the three 11 α -hydroxyprogesterone-bovine serum albumin and the three 6 β -hydroxyprogesterone-bovine serum albumin antisera which yielded sensitive standard curves. The results were derived from several experiments, but the conditions, which have been set out in detail in the legend to Table 1, were constant and were identical with those used in the routine assay. The specificities of the three 11 α -hydroxyprogesterone antisera were remarkably similar to each other, and those of the 6 β -hydroxyprogesterone antisera, though different from the 11 α -hydroxyprogesterone group, were again remarkably similar to each other. As expected, the antisera to 11 α -hydroxyprogesterone-bovine serum albumin showed relatively little ability to detect changes in substituents at the 11 position. Divergencies from the structure of progesterone which were distal to the conjugation site were equally well seen whether they were present in the A or D ring or on the C-21 side chain. Generally, a single change on the A, B or D ring (e.g. H to OH or =O to OH) produced an approximately 100-fold loss in potency. Multiple changes appeared to be essentially additive in their effect. The overall specificity of these antisera was such that substances likely to be present in blood, and extractable with progesterone by non-polar solvents, are present at concentrations which would not be expected to interfere in the assay for progesterone.

Again as expected, the antisera to 6 β -hydroxyprogesterone-bovine serum albumin did not detect well different substituents at the 6 position and

Table 1. *Specificity studies on rabbit antisera to 11 α -hydroxyprogesterone-bovine serum albumin and 6 β -hydroxyprogesterone-bovine serum albumin conjugates*

Steroids were dissolved in the assay diluent. Tubes with a maximum concentration of 10 μ g/ml contained 1% (v/v) ethanol and these were serially diluted down to a concentration of 1 pg/ml. Incubation was for 2 h and separation was achieved by the double-antibody method. Responses were calculated at 50% inhibition of tracer binding except for cholesterol, whose maximum displacement (10% at 10 μ g/ml) was used.

Compounds	Specificities of rabbit antisera to 11 α -hydroxyprogesterone-bovine serum albumin (% cross-reaction)			Specificities of rabbit antisera to 6 β -hydroxyprogesterone-bovine serum albumin (% cross-reaction)		
	R1	R3	R6	R388	R389	R390
	4th booster	4th booster	4th booster	5th booster	5th booster	3rd booster
Progesterone	100	100	100	100	100	100
11 α -Hydroxyprogesterone	31	33	36	9.3	9.1	12.0
11 α -Hydroxyprogesterone hemisuccinate	78	69	49	—	—	—
6 β -Hydroxyprogesterone hemisuccinate	—	—	—	69.0	83.0	82.0
11 β -Hydroxyprogesterone	12	4	14	1.7	0.8	3.3
11-Oxoprogesterone	15	12	16	4.0	5.3	3.3
6 β -Hydroxyprogesterone	0.8	0.9	1.0	56.0	20.0	28.0
20 α -Hydroxypregn-4-en-3-one	1.0	1.0	2.4	1.8	0.91	1.1
20 β -Hydroxypregn-4-en-3-one	0.05	0.1	0.1	0.1	0.06	0.04
5 α -Pregnane-3,20-dione	13	7.5	17	68.0	55.0	57.0
5 β -Pregnane-3,20-dione	7	17	7	18.0	14.0	20.0
17-Hydroxyprogesterone	1.2	2.4	1.4	0.2	1.25	0.53
3 α -Hydroxy-5 β -pregnan-20-one	0.2	0.2	0.4	0.5	0.15	0.4
5 α -Pregnane-3 α -20 α -diol	0.0001	0.002	0.0003	—	—	—
5 α -Pregnane-3 β -20 α -diol	0.003	0.03	0.003	—	—	—
5 β -Pregnane-3 α -20 α -diol (pregnanediol)	0.008	0.035	0.05	0.014	0.002	0.043
3 β -Hydroxy-pregn-5-en-20-one	0.13	0.11	0.05	8.0	1.0	5.3
3 β ,17-Hydroxypregn-5-en-20-one (17-hydroxypregnenolone)	0.02	—	—	0.01	0.003	0.01
11-Deoxycorticosterone	0.9	4.0	1.9	0.7	1.43	1.54
11-Deoxycortisol (compound S)	0.15	0.07	0.16	0.47	0.45	0.53
Cortisol	0.02	0.03	0.03	0.02	0.002	0.001
Corticosterone	0.43	—	—	0.025	0.005	0.06
Cortisone	0.02	—	—	0.01	0.003	0.01
Androst-4-ene,3,17-dione (androstenedione)	0.02	0.09	0.03	0.09	0.27	0.08
3 β -Hydroxyandrost-5-en-17-one (dehydroepiandrosterone)	0.004	—	—	0.025	0.01	0.01
Cholesterol	0.0004	0.0008	0.0007	0.005	0.002	0.002
Cholic acid (3 α ,7 α ,12 α -trihydroxy- cholanilic acid]	Not detected	—	—	—	—	—

changes close to this (e.g. loss of the double bond at Δ^4 ; its presence instead at Δ^5 with or without reduction of the $-3=O$ to $-3-OH$) were less well seen by these antisera than by the 11 α -hydroxyprogesterone-bovine serum albumin antisera. Assays based on these antisera would therefore be more vulnerable to interference from pregnenolone and metabolites of progesterone which embrace such changes. Conversely, the use of a conjugate involving the 6 position has not conferred a proportionate improvement in specificity with respect to progesterone derivatives which have small changes at the

11 position. Changes in the C-21 side chain were equally well detected by both groups of antisera. The overall picture therefore leads to a clear, though not overwhelming, preference for the antisera to 11 α -hydroxyprogesterone-bovine serum albumin.

Routine assay procedure

Antiserum to 11 α -hydroxyprogesterone-bovine serum albumin no. R1(4) was chosen for the establishment of the routine assay procedure and portions

were conjugated to CNBr-activated cellulose (micro-crystalline). The ratio of antiserum to cellulose was arranged so that each assay tube would contain sufficient antiserum to give 65–75% binding of the 10pg of tracer (in an incubation volume of 1ml) when 50–100µg of solid-phase antiserum was added. Covalently coupled antisera yielded standard curves which were superimposable on those obtained from the same antiserum by using the double-antibody method. Small and acceptable losses in titre resulted, but sensitivity was undiminished (Bolton & Hunter, 1973). The tubes were incubated on a reciprocating shaker at 170 strokes/min (Baird and Tatlock, Chadwell Heath, Essex, U.K.). After 90min incubation at 20–30°C, 1ml of diluent was added to minimize non-specific adsorption and the mixture was then ready for radioactivity counting after a single final centrifugation (5min at 2000g). Fig. 2 shows the mean standard curve (±s.d.) calculated from ten consecutive routine assays. Also included in Fig. 2 is a single standard curve in which the concentrations of all reactants were as in the routine assay but the total incubation was 0.5ml instead of the customary 1.0ml.

Extraction of progesterone from plasma

A somewhat variable proportion (70–90%) of [³H]progesterone added to plasma was extracted by 10vol. of light petroleum alone and extraction was much improved and became more constant if ethanol (10vol. of plasma+1 vol. of ethanol) was first added to the plasma. Thereafter extraction by 10vol. of light petroleum for 2min on a hand or Multivortex mixer (Baird and Tatlock) which could accommodate and mix simultaneously 100 unstoppered tubes (75mm×17mm) gave the results set out in Table 2. A single extraction gave a mean of 92.4±1.0 (s.d.) for 50–200µl of plasma when 2ml of solvent was used, and extraction was quantitative if a second 10vol. of extraction was included.

For the larger plasma volume (500µl) necessarily used when progesterone was to be measured in men or postmenopausal women, extraction with 2×5vol. was little better than that given by 1×10vol. For the routine assay therefore ethanol (10vol. of plasma+1vol. of ethanol) was added to plasma. After 15min the ethanolic plasma was extracted with light petroleum, 2ml being used for plasma volumes of 200µl or less, and 5ml for 500µl. After extraction the aqueous layer was frozen by standing the rack of tubes in solid CO₂-ethanol; the organic layer was then decanted into glass tubes (75mm×17mm), evaporated to dryness and the residue assayed *in situ*.

Effect of solvent residues on assay

A series of experiments were carried out to examine whether solvent residues produced by the above procedure might distort the standard curve

Fig. 2. Radioimmunoassay standard curve for progesterone from ten consecutive assays

Mean (±s.d.) obtained in 1 ml of final incubation volume from ten consecutive routine assays; ○, mean of duplicates for a single standard curve with the routine reactant concentrations but in a final incubation volume of 0.5 ml.

Table 2. Extraction of [³H]progesterone from plasma by light petroleum

Plasma was equilibrated with [³H]progesterone for 15min at 20°C. Ethanol was then added to the plasma (10vol. of plasma+1 vol. of ethanol) and after a further 15min at 20°C the plasma was extracted with light petroleum. Four replicates were used for each group.

Vol. of plasma (µl)	Vol. of light petroleum (ml)	Extraction (%)			
		One extraction		Two extractions	
		Mean	S.D.	Mean	S.D.
50	2.0	93.12	1.24	99.22	1.5
100	2.0	93.45	1.40	99.72	2.0
200	2.0	91.52	1.54	100.3	1.5
500	5.0	91.5	0.7	99.4	0.93
Mean		92.4	1.2	99.7	1.5
500	2.5	79.6	3.5	96.8	1.1

Fig. 3. Effect of solvent residues on the dose-response curve

In each case the curve with open circles represents a standard curve in diluent. The curve with open triangles represents the standard curve from tubes in which the dried progesterone standards were redissolved in water (200 μ l) and extracted with light petroleum by the standard extraction procedure as described in the text. The first values on each curve represent ($\pm$ s.d.) the percentage [3 H]progesterone tracer (10 pg in 1 ml final incubation volume) bound in the absence of unlabelled progesterone. All other values are expressed as the means of duplicates. The four parts to the Figure show the standard curve as (a) water-extracted in impure solvent with drying of tubes at room temperature; (b) water-extracted in purified solvent with drying of tubes at room temperature; (c) water-extracted in incompletely purified solvent but with drying of tubes under air at 60°C; (d) water-extracted in purified solvent with drying of tubes under air at 60°C.

of the assay. Progesterone was added over the range 5pg–10ng in ethanol solution which was then evaporated to dryness and redissolved in water (200 μ l). Ethanol (10 vol. of water+1 vol. of ethanol) was added and the progesterone was then extracted into light petroleum (2ml), the aqueous phase was frozen and the organic phase decanted into the assay tubes. After evaporation, these tubes were assayed together with similar standards which were made up by adding the assay reagents (tracer and antiserum) directly into tubes containing the residues from the ethanolic solution of progesterone. Fig. 3 shows the two different ways in which the solvent residues could distort the dose-response curve of the standards. In each case the curve obtained for the standards in diluent only is shown by open circles, and those run in tubes containing solvent residues are shown by open triangles. Most batches of solvent yielded curves of the kind shown in Fig. 3(a). Two factors appeared to be distorting the dose-response curve. In Fig. 3(b) the light petroleum had been purified as described above, although some batches of solvent gave curves of this kind without purification. The distortion at this end of the curve were generally less severe in solvent

blanks than in water blanks. The remaining perturbation which is seen at higher concentrations of progesterone in Fig. 3(a) was always seen when the light petroleum was evaporated under a stream of N_2 or air at room temperature. When assay diluent and then [3 H]progesterone was added to tubes containing these residues only 70–85% of the tracer was removed when the aqueous solution was decanted into counting vials. This was presumably due to the presence of solvent contaminant(s) which dissolved some of the tracer and rendered it unavailable for aqueous dissolution. This effect was only slightly diminished by the solvent purification described above (Fig. 3b). However, it was completely abolished (Fig. 3c) by heating the residues to 60°C for 15 min under N_2 , or more conveniently by carrying out the entire solvent evaporation process under N_2 or air at 60°C before addition of assay diluent. When purified solvent was used and the evaporation temperature also raised, the curve almost invariably was identical with that obtained in diluent (Fig. 3d). Since distortions caused by solvent residues can, if unrecognized, give rise to both positive and negative artifacts, two standard curves were run in all assays. One standard curve was carried out without

Table 3. *Assay recoveries of non-radioactive progesterone added to pooled male plasma*

Ethanol (10 vol. of plasma + 1 vol. of ethanol) was added after equilibration of the plasma samples with dried unlabelled progesterone and extraction with light petroleum performed after another 15 min. Progesterone was assayed as described in the text. In each case four replicates were run. The mean and s.d. show their scatter in each group.

Progesterone added	Vol. of plasma (μ l)	Vol. of light petroleum (ml)	Recoveries (%)			
			One extraction		Two extractions	
			Mean	S.D.	Mean	S.D.
80pg	100	2	87.5	8.7	—	—
	200	2	89.5	8.8	—	—
	500	5	84.5	14.3	—	—
	Mean:		87.2	10.6		
160pg	50	2	100.9	7.75	99.8	7.0
	100	2	94.5	6.4	99.2	9.9
	200	2	100.0	14.25	93.9	13.4
	500	5	90.3	8.1	—	—
	Mean:		96.4	9.1	97.6	6.8
640pg	50	2	90.1	8.2	98.1	7.3
	100	2	90.2	5.6	92.1	7.5
	200	2	95.2	8.3	97.5	4.7
	500	5	91.8	2.7	—	—
	Mean:		92.4	6.2	95.9	6.5
Overall mean $\pm$ S.D.			92.2	8.6	96.8	6.6

extracting the standards, these being added, in ethanol, to the assay tubes, evaporated under N_2 and dissolved in diluent. In the second standard curve the standards were added in ethanol, evaporated to dryness, and the residues were dissolved in 200 μ l of water and then extracted with light petroleum after the addition of 20 μ l of ethanol as in the standard extraction procedure. These two standard curves have been superimposable in seven out of eleven consecutive assays carried out over 13 weeks with four different batches of solvent. The remaining four assays gave slightly displaced curves (similar to that shown in Fig. 3c) for the standards based on water blanks and these, rather than the curve for standards in diluent, were used to calculate the results of these assays.

Table 3 shows results for the recovery of unlabelled progesterone added in different amounts over the range 160 pg/ml to 12.8 ng/ml to portions from a pool of male plasma from which different volumes were extracted and assayed. By using a single extraction, recoveries over the whole range of different amounts of progesterone and of different plasma volumes of $92.2 \pm 8.6\%$ (s.d.) were obtained, and two such extractions over the plasma volume range 50–200 μ l gave $96.8 \pm 6.6\%$

(s.d.). These findings confirm the high and constant recoveries obtained with [3H]progesterone and provide further evidence that the assay is free of distortion of the plasma dose-response curve by solvent residues. The absence of such distortion was further shown by the finding (Table 4) that plasma samples containing high concentrations of endogenous progesterone gave identical concentrations when different volumes covering the entire working range of the dose-response curve were extracted and assayed. For reasons of practicality 2 ml of solvent was used even when volumes below 200 μ l of plasma were being extracted. The fact that these gave values identical with those found when 200 μ l of plasma was extracted suggested also that no important interference was produced by increasing the solvent/plasma ratio to above the minimum of 10:1 regularly used.

Specificity of assay for plasma progesterone

The specificity of the assay for measuring plasma progesterone depends on a combination of the specificity of the assay end-point and the selective nature of the extraction procedure. The extraction rates of the relevant compounds are set out in Table 5. In women the plasma concentrations of 17-hydroxy-

Table 4. Progesterone concentration from varied volumes of plasma

Apparent plasma progesterone concentration (ng/ml) was measured in samples from which varied portions were taken and each was extracted with 2ml of light petroleum. Extraction was performed after the addition of ethanol to plasma (10vol of plasma+1 vol. of ethanol).

Plasma no. Vol. of plasma extracted (ml)	Apparent plasma progesterone concentration (ng/ml)					
	1	2	3	4	5	6
0.01	14.0	26.0	5.9	17.5	29.0	
0.05	14.8	18.8	—	—	34.0	29.4
0.10	—	—	5.9	17.0	—	29.2
0.15	—	—	—	—	—	28.0
0.20	—	—	—	—	—	29.7

Table 5. Extraction of ^3H -labelled steroids from male plasma by light petroleum (1vol. of plasma+10vol. of light petroleum) with and without prior addition of ethanol to plasma

Samples of plasma (500 μl) were equilibrated with dried ^3H -labelled steroids for 1h and extraction with light petroleum was performed 15min after the addition of ethanol (10vol. of plasma+1vol. of ethanol). Four replicates were used for each steroid.

	Recovery (% $\pm$ S.D.)	
	Without ethanol	With ethanol
Progesterone	81.5 $\pm$ 9.4	92.8 $\pm$ 2.0
Cortisol	0.55 $\pm$ 0.15	0.29 $\pm$ 0.03
3 β -Hydroxypregn-5-en-3-one	84.3 $\pm$ 10.8	90.9 $\pm$ 1.3
20 α -Hydroxypregn-4-en-3-one	78.1 $\pm$ 7.0	88.0 $\pm$ 4.0
Androst-4-ene-3,17-dione	77.8 $\pm$ 6.5	90.1 $\pm$ 1.7
3 β -Hydroxyandrost-5-en-17-one	76.3 $\pm$ 7.1	89.1 $\pm$ 2.1
17-Hydroxyprogesterone	65.2 $\pm$ 7.4	78.9 $\pm$ 1.8

progesterone, 20 α -hydroxypregn-4-en-3-one, 3 β -hydroxypregn-5-en-20-one and androst-4-ene-3,17-dione can reach 2.2ng/ml (Youssefnejadian *et al.*, 1972b), 4ng/ml (Florensa & Somerville, 1973), 2ng/ml (Abraham *et al.*, 1973) and 2ng/ml (Judd & Yen, 1973) respectively, but such values are recorded only during the mid-luteal phase and their equivalents at the assay end-point (see Table 1) would amount respectively to 26, 40, 3 and 0.4pg of progesterone/ml which would be insignificant at that time. In men and postmenopausal women the maximum concentration of 20 α -hydroxypregn-4-en-3-one and 3 β -hydroxypregn-5-en-20-one is about one-tenth of those found in the female luteal phase, so that here again they would not contribute more than 5% of the progesterone as measured. Also in men plasma 17-hydroxyprogesterone concentrations show a mean of 1.23ng/ml, whereas

the highest value reported was 3.2ng/ml (Youssefnejadian *et al.*, 1972b). These would give responses equivalent to 14.8 and 38.5pg of progesterone/ml. As such they could represent a small intrusion into the assay's specificity, which should, however, be generally acceptable since the mean recorded value in men was 205pg/ml.

The more polar adrenal steroids are poorly extracted by light petroleum and as judged from the extraction of cortisol (Table 5) are likely to be even less well extracted when ethanol is first added to the plasma.

The situation with respect to cholesterol is anomalous. Samples from each of six hypercholesterolaemic men and two postmenopausal women whose plasma cholesterol concentrations ranged from 3.7 to 4.6mg/ml and three with cholesterol concentrations ranging from 0.87 to 1.0mg/ml gave progesterone values of 72–220pg/ml, which were not different from those of the normal group. Although it would be tempting to suggest that the specificity of the progesterone assay was maintained because of poor extraction of cholesterol, direct evidence from its measurement (Abell *et al.*, 1952) in the light petroleum extracts indicated that these contained almost all of the total plasma cholesterol. This suggested that the response obtained in the specificity studies represented in Table 1 and consisting of a small fall in the percentage of [^3H]progesterone bound to antibody when cholesterol was present at 10 μg /ml (which was at the limit of its solubility in the diluent solution) did not represent a true cross-reaction.

Assay of progesterone in human plasma

The plasma progesterone value found with the assay by assaying duplicate 0.5ml portions of single samples from 50 normal men was 205 $\pm$ 20.6 (S.D.). The value found for a pool of plasma from three postmenopausal women was

88pg/ml. Fig. 4 shows the mean ($\pm$ s.d.) of plasma progesterone concentrations found from daily sampling throughout a single menstrual cycle from five women, day 0 being the centre of the mid-cycle plasma luteinizing-hormone surge. From a mean follicular-phase base-line concentration of 332pg/ml a mean luteal-phase peak of 14.6 ± 3.7 (s.d.) ng/ml was found on the sixth day after the luteinizing-hormone peak. Table 6 compares these results with previously published values.

Six portions each of follicular-phase plasma (0.5ml) and of luteal-phase plasma (50 μ l) and a mixture of male and female luteal-phase plasma (0.1ml) were extracted by the routine procedure: in each case four were assayed directly and the remainder were subjected to gel filtration on Sephadex LH-20 by using the solvent system of Murphy (1971). The eluates having the elution characteristics of added non-radioactive and [3 H]progesterone gave mean values of 0.305, 14.0 and 3.0ng/ml respectively, and the unchromatographed samples gave means of 0.297, 14.0 and 2.82 ng/ml respectively.

Behaviour of unextracted plasma in the assay

It became apparent during the above studies on specificity that if only the known steroid and sterol substances present in plasma could affect the assay then assays on unextracted plasma should be acceptable. Even if all of the compounds listed in Table 1 were to be present together in the highest concentrations yet reported and their responses were to be additive, then their combined effect would be equivalent to about 220pg of progesterone/ml. An assay having a non-specific base-line of this order would be unsuitable for measuring progesterone in men and in women during the follicular phase but would nevertheless be perfectly acceptable for monitoring the presence and function of the corpus luteum. However, when the assays were conducted on unextracted plasma from combined samples from (a) normal men, (b) postmenopausal women, (c) plasma from menstruating women collected

during their luteal phase and (d) a mixture of (a) and (c) used as a quality-control pool, grossly high values were obtained (Table 7). The dose-response curves for different quantities of each pool were parallel to those of the progesterone standards. These high values were not materially altered when the plasma was diluted in water (1 vol. of plasma +3vol. of water) and heated at 100°C for 60min, centrifuged and the supernatant assayed.

Precision of assay

The intra-assay precision was assessed by calculating the coefficient of variation from the duplicate determinations on 203 plasma samples, whose values ranged from 85pg to 35ng/ml, which were assayed in the ten consecutive assays whose standard curves are represented in Fig. 2 above. By using the formula coefficient of variation =

$$\sqrt{\frac{\varepsilon \left(\frac{d}{\bar{x}} \times 100 \right)^2}{2n}}$$
 where d = difference between duplicate estimations, $\bar{x}$ = mean of duplicate estimations and n = number of duplicate estimations, a value of 9.7%

Fig. 4. Mean plasma progesterone concentration ($\pm$ s.d.) in five menstrual cycles

Day 0 is the centre of the mid-cycle plasma luteinizing-hormone surge.

Table 6. Plasma progesterone values as reported by various authors

		Plasma progesterone (pg/ml) mean $\pm$ s.d. or range			
	Whether chromatography used	Men	Postmenopausal women	Women in follicular phase	Women in luteal phase
Abraham <i>et al.</i> (1971)	Yes	233 $\pm$ 64	180 $\pm$ 63	545 $\pm$ 103	8560 $\pm$ 470
Furuhama & Nugent (1971)	Yes	266 $\pm$ 97	—	265 $\pm$ 192	8300 $\pm$ 630
Thornycroft & Stone (1972)	No	193	63–225	60–371	8900
De Villa <i>et al.</i> (1972)	No	260 $\pm$ 80	260 $\pm$ 80	500 $\pm$ 440	4300–19400
Kutas <i>et al.</i> (1972)	No	195	—	933 $\pm$ 86	12100 $\pm$ 4810
Youssefnejadian <i>et al.</i> (1972a)	Yes	230 $\pm$ 6.8	—	424 $\pm$ 187	5600 $\pm$ 2100
Present method	No	205 $\pm$ 21	88.0 $\pm$ 8.4	332 $\pm$ 187	7700 $\pm$ 1900
					7320 $\pm$ 4260

Table 7. *Comparison of progesterone-like activity in extracted and unextracted plasma*

Combined plasma samples were taken and suitable portions from each were assayed for progesterone before and after the extraction by the standard method described in the text.

	Apparent progesterone concentration (ng/ml)			
	11 α -Hydroxyprogesterone antiserum R1(4)		6 β -Hydroxyprogesterone antiserum R390(5)	
	Extracted	Unextracted	Extracted	Unextracted
Plasma pool from				
(a) Normal men	0.12	1.9	0.1	3.3
(b) Postmenopausal women	0.09	3.9	0.08	4.10
(c) Luteal phase	17.2	15.3	13.2	23.4
(d) Luteal phase diluted with normal male (used as quality control)	2.9	7.5	2.3	8.6

Fig. 5. Mean urinary excretion ($\pm$ S.D.) of progesterone-like activity in 24 h samples from three menstrual cycles

Day 0 is the centre of the mid-cycle urinary luteinizing-hormone surge.

was found. The inter-assay precision was examined by calculating the coefficient of variation of the means of triplicate determinations carried out on a quality-control pool in the ten consecutive assays. A mean value of 2.78 ng/ml was found and the coefficient of variation was 5.0%. In five of these assays the same pool was assayed a second time (again in triplicate), the two sets of tubes being placed at the beginning and end of the assays. Mean values of 2.76 and 2.98 ng/ml respectively were obtained.

Sensitivity and working range of the routine assay

Mathematical representations of the sensitivity of the radioimmunoassay systems almost inevitably assume complete specificity. In practice this assumption is valid only for standards when assayed in diluent solution. In these terms the present

assay has a formal sensitivity of 11 pg/ml if the formula, sensitivity = that amount of substance being assayed which gives a fall in percentage binding of tracer of $2.5 \times$ S.D. of percentage of tracer bound in the absence of unlabelled progesterone [as suggested by Borth (1970)], is used. The curve, shown as the broken line in Fig. 2, suggests that this value would be decreased from 11 pg/tube for the routine 1 ml incubation mixture to 5.5 pg/tube if 0.5 ml incubation volumes were used. The single disadvantage of these smaller volumes is a proportional increase in the radioactivity-counting time required to achieve identical precision. However, the slope of the standard curve is still shallow at the above formal detection limit and precision is therefore less than optimal. We have therefore preferred to discard values derived from responses which were less than 10% below the binding of tracer in the absence of unlabelled progesterone, and when considered in this way the working range for the routine assay (with 1 ml incubation volumes) is 33–600 pg. This provides for a range of 165–3000 pg/ml of plasma when 0.2 ml is extracted.

Progesterone-like activity in urine

Although the concentration of progesterone in urine is known to be low and to represent a very small proportion of the total progesterone secreted, it was decided to attempt its assay. Urine (0.5 ml) was extracted with 10 vol. of light petroleum on 24 h samples collected daily throughout complete menstrual cycles from three women. These extracts were evaporated to dryness and assayed by the standard procedure and yielded the results set out in Fig. 5. It was immediately obvious that the ratio between luteal-phase and follicular-phase urinary excretion rates was much smaller (4:1) than the comparable ratio in plasma from the same subjects (20:1). Very similar values were obtained and

the luteal-phase/follicular-phase ratios were maintained when these assays were repeated with a 6 β -hydroxyprogesterone-bovine serum albumin antiserum. Two explanations appeared possible: (a) that significant contributions to the apparent urinary progesterone values were in fact due to substances other than progesterone and that these provided a false base-line which naturally would be relatively more intrusive during the follicular phase, or (b) that the urinary excretion rate was disproportionately higher in the follicular phase. That the second suggestion was perhaps more probable was suggested by the finding that the progesterone-like activity from pools of follicular-phase and luteal-phase urine samples, which were run on Sephadex LH-20 columns as described above, gave values of 0.475 and 1.79 ng/ml, which were almost identical with values of 0.479 and 1.78 ng/ml obtained after simple extraction with light petroleum.

Discussion

Our results have confirmed the specificity to be expected of antisera raised to 11 α -hydroxyprogesterone-bovine serum albumin conjugates. The degree of cross-reactivity found in sera from all of the three rabbits yielding titres meriting detailed assessment was very similar to that first shown by Midgley & Niswender (1970), and later set out in greater detail (Niswender, 1973). The specificity of our antisera with respect to 3 β -hydroxypregn-5-en-20-one, 20 β -hydroxypregn-4-en-3-one, 11-deoxycorticosterone and cortisol was as good as any and better than most reported. For 20 α -hydroxypregn-4-en-3-one two antisera (Kutas *et al.*, 1972; Niswender, 1973) at 0.1 and 0.2% cross-reaction were somewhat better than ours with 1–2%. For 5 α -pregnane-3,20-dione, antiserum cross-reactions are very varied and ranged from 3% (Lindner *et al.*, 1972) to 80% (Youssefnejadian *et al.*, 1972a).

We report also detailed specificity studies on antisera raised to 6 β -hydroxyprogesterone-bovine serum albumin conjugates. These showed less ability to distinguish between progesterone and its 5 α and 5 β metabolites than was the case with the 11 α -hydroxyprogesterone antisera. Since they brought no compensatory improvement in specificity with respect to other closely related compounds which differ from progesterone elsewhere in its structure, the 6 β -hydroxyprogesterone-bovine serum albumin antisera were discarded in favour of the 11 α -hydroxyprogesterone-bovine serum albumin antisera for the routine assay.

The chances of obtaining satisfactory antisera by using the procedure described above may be judged from the following findings. The cross-reactivity of eight key compounds was tested on

each of six, four and two different samples respectively from each of three rabbits receiving 11 α -hydroxyprogesterone-bovine serum albumin over periods of 17, 11 and 7 months, and no significant alterations in specificity occurred. Six of the total of twelve rabbits produced antisera which permitted the establishment of assays of good sensitivity. They did so within 3–6 months and once this quality was achieved it was not later lost by any animal. Nine of our twelve rabbits gave antisera which could be used at final dilutions of 1/20000 or more when their sensitivity plateaus were first reached; eight of these animals later gave antisera which were used at 1/50000–1/60000, and again, once reached, such titres never subsequently decreased. The titres obtained by other workers have frequently been one order below this range (Bodley *et al.*, 1973; Spieler *et al.*, 1972; Kutas *et al.*, 1972; Youssefnejadian *et al.*, 1972a) and the improvement that we have obtained may have been due to our practice of giving injections at widely spaced time-intervals.

By choosing two general separation procedures, neither of which results in disruption of the equilibrium established for the primary reaction of antigen with antibody, we have obviated the necessity for investigating and optimizing a system purely for this particular application to progesterone. For routine assay purposes the use of antiserum which has been covalently linked to a solid particulate support medium (Wide, 1969) would appear to offer an ideal solution. The reagents required are inexpensive, the activation and coupling procedures are neither lengthy nor laborious, no special optimization is required for individual antisera, losses of effective titre are minimal and there was no loss of sensitivity when this system was used in place of the double-antibody procedure (Bolton & Hunter, 1973). When stored at 4°C batches of covalently coupled antisera were used for periods of up to 5 months without any apparent change in properties. Activated cellulose was also very stable if kept in ampoules under N₂. The separation procedure is manipulatively as simple as any, a single centrifugation being all that is required after the primary incubation. The precision obtained with this procedure was excellent, suggesting that misclassification error (Rodbard *et al.*, 1969) may be very small. The single disadvantage which is generally accepted as a necessary accompaniment to the use of antisera coupled to particulate media is the need for continuous agitation of the incubation mixtures. This can be accomplished for the short incubations required for steroid assays by using a reciprocating shaker and without stoppering the individual tubes. Preliminary experiments suggest that even this necessity can be avoided by the addition of sucrose in the incubation diluent, since

the covalently coupled antiserum then remains in suspension.

Finally, the common practice of distributing selected antisera between many laboratories could now be carried a stage further. If such antisera were first conjugated to the support medium the recipient would be provided with an antiserum and a separation system, with consequent savings in time spent on investigation and manipulation.

Previous methods have almost invariably involved two extractions and the inclusion of [^3H]progesterone tracer to all individual plasma samples in order to monitor and allow corrections for the variable recovery rates obtained. The reliability of the extraction procedure which we now report derives from the addition of ethanol to the plasma before extraction with light petroleum as first suggested by Holmdahl & Johansson (1972) for their competitive protein-binding method. This has yielded a sufficiently high and constant extraction rate as to call for only a single extraction, and virtually nothing remains to be gained by the tedious practice of monitoring and correcting for each individual recovery. We were surprised to find two different ways in which extracts of plasma (or of water) could interfere in the assay, since the literature provides little detailed evidence of such intrusion. There was much discussion of this problem with the earlier competitive protein-binding methods (Murphy, 1970). However, we have sought to devise a simple purification procedure for use on unselected batches of solvent rather than consider the alternative of dependence on a closely defined source of light petroleum (Johansson, 1969).

Of previously reported assays based on the more specific antibodies to 11α -hydroxyprogesterone, only those of Thorneycroft & Stone (1972) and Kutas *et al.* (1972) had sensitivities similar to those reported here. The concentrations and volumes of reagents used in our routine procedure seemed to be those most satisfactory for use in assaying progesterone in plasma from women of reproductive age. Thus a 200 μl portion of plasma permits accurate measurement in the follicular phase and therefore provides for the detection of the mid-cycle rise at its inception. Where a higher detection limit is acceptable, e.g. if only the magnitude and major part of the plasma concentrations during the luteal phase are to be measured, this can be accomplished either with the present procedure (with consequent economy of sample volume) or by scaling up the concentrations of all of the assay reagents, with a consequent saving of counting time. If on the other hand higher sensitivities were required, then the present concentrations of reagents would be retained while the overall incubation volume was decreased to 0.5 ml or even less. These smaller volumes provide for sensitivity increases of

twofold or more, the single expense being a commensurate increase in radioactivity-counting time.

We may speculate on why it remains necessary to include a solvent-extraction step in this (among all other) radioimmunoassays for individual steroid hormones. When assays were attempted on unextracted plasma the values obtained were spuriously high by about 2–5 ng of (apparent) progesterone/ml. The total calculated contribution of known steroid compounds to this response, at 200–300 pg/ml, was almost negligible. Whether the interference is due to steroids or other phenanthrene-containing compounds which have not been considered, to cross-reacting compounds having more tenuous structural relationships to progesterone or to non-specific inhibition of the primary antigen-antibody reaction cannot as yet be ascertained. It seems unlikely that the response of unextracted plasma was due to an effect on the separation system, since, unlike charcoal- and salt-precipitation systems, neither the double-antibody nor the solid-phase system used in these experiments is distorted by differences in the amount of plasma present in the incubation mixtures.

We thank Miss I. M. Marshall and Mr. I. McKenzie for their skilled technical assistance.

References

- Abell, L. L., Levy, B. B., Brodie, B. B. & Kendall, F. E. (1952) *J. Biol. Chem.* **195**, 357–366
- Abraham, G. E., Swerdloff, R., Tulchinsky, D. & Odell, W. D. (1971) *J. Clin. Endocrinol. Metab.* **32**, 619–624
- Abraham, G. E., Buster, J. E., Kyle, F. W., Corrales, P. C. & Teller, R. C. (1973) *J. Clin. Endocrinol. Metab.* **37**, 40–45
- Bodley, F. H., Chapdelaine, A., Flickinger, G., Mikhail, G., Yaverbaum, S. & Roberts, K. D. (1973) *Steroids* **21**, 1–16
- Bolton, A. E. & Hunter, W. M. (1973) *Biochim. Biophys. Acta* (1973) **329**, 318–330
- Borth, R. (1970) *Acta Endocrinol. (Copenhagen)* **64**, Suppl. **147**, 32–36
- Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065
- De Villa, G. O., Jr., Roberts, K., Wiest, W. G., Mikhail, G. & Flickinger, G. (1972) *J. Clin. Endocrinol. Metab.* **35**, 458–460
- Erlanger, B. F., Borek, F., Beiser, S. M. & Lieberman, S. (1959) *J. Biol. Chem.* **234**, 1090–1094
- Florensa, E. & Sommerville, I. F. (1973) *Steroids* **22**, 451–465
- Furuyama, S. & Nugent, C. A. (1971) *Steroids* **17**, 663–674
- Holmdahl, T. H. & Johansson, E. D. B. (1972) *Acta Endocrinol. (Copenhagen)* **11**, 743–754
- Hunter, W. M. (1973) in *Handbook of Experimental Immunology* (Weir, D. M., ed.), pp. 17.1–17.36, Blackwell Scientific Publications, Oxford, London, Edinburgh and Melbourne

- Johansson, E. D. B. (1969) *Acta Endocrinol. (Copenhagen)* **61**, 592–606
- Judd, H. L. & Yen, S. S. C. (1973) *J. Clin. Endocrinol. Metab.* **36**, 475–481
- Kutas, M., Chung, A., Bartos, D. & Castro, A. (1972) *Steroids* **20**, 697–716
- Lindner, H. R., Perel, E., Friedlander, A. & Zeitlin, A. (1972) *Steroids* **19**, 357–375
- Midgley, A. R., Jr. & Niswender, G. D. (1970) *Acta Endocrinol. (Copenhagen)* **64**, Suppl. **147**, 320–331
- Murphy, B. E. P. (1970) *Acta Endocrinol. (Copenhagen)* **64**, Suppl. **147**, 37–60
- Murphy, B. E. P. (1971) *Nature (London) New Biol.* **232**, 21–24
- Niswender, G. D. (1973) *Steroids* **22**, 413–424
- Rodbard, D., Cooper, J. A. & Rayford, P. L. (1969) *Program Meet. Endocrine Soc. 51st*, p. 111, J. B. Lippincott Co., Philadelphia, Pa.
- Spieler, J. M., Webb, R. L., Salderini, R. J. & Coppola, J. A. (1972) *Steroids* **19**, 751–762
- Thornycroft, I. H. & Stone, S. C. (1972) *Contraception* **5**, 130–147
- Wide, L. (1969) *Acta Endocrinol. (Copenhagen)* **63**, Suppl. **142**, 209–221
- Youssefnejadian, E., Florensa, E., Collins, W. P. & Sommerville, I. F. (1972a) *J. Steroid Biochem.* **3**, 893–901
- Youssefnejadian, E., Florensa, E., Collins, W. P. & Somerville, I. F. (1972b) *Steroids*, **20**, 773–788