SYMPOSIUM REPORT No. 10 September 1977

JOURNAL OF
REPRODUCTION
AND
FERTILITY

NEWER ASSAY TECHNIQUES

TWENTIETH SYMPOSIUM
OF THE SOCIETY FOR
THE STUDY OF FERTILITY
SHEFFIELD, JULY 1976

Published by the Journals of Reproduction and Fertility Ltd

Journal of Reproduction & Fertility

SYMPOSIUM REPORT No. 10

NEWER ASSAY TECHNIQUES

Proceedings of the twentieth Symposium

of the

Society for the Study of Fertility

Sheffield, July 1976

Convener: K. Griffiths

Chairman: L. Rees

Edited by

K. Griffiths and Barbara J. Weir

Journals of Reproduction and Fertility Ltd.

1977

Journal of Reproduction & Fertility

The Journal of Reproduction and Fertility is published every 2 months, two issues forming one volume. The subscription rate for 1976 is £65.00 (US $150.00) per annum, post free, by surface mail to overseas addresses. The price for single issues is £13.00 (US $30.00). The agent for distribution is the Biochemical Society Book Depot and all correspondence and remittances relating to subscriptions, back numbers (including the cumulative index), supplements and offprints should be sent to a bookseller or direct to:

Journal of Reproduction and Fertility, P.O. Box 32, Commerce Way, Colchester CO2 8HP, U.K.

Under its Memorandum of Association the Journals of Reproduction and Fertility (Limited by Guarantee) must apply all its resources towards the promotion of this or similar journals or of knowledge relevant thereto.

Editorial office:

Journal of Reproduction and Fertility, 7 Downing Place, Cambridge CB2 3EL, U.K.

Second-class postage paid at New York, N.Y. 10022, U.S.A.

CONTENTS

J. Reprod. Fert. (1977) **51**, 267–272

Recent developments in steroid radioimmunoassays

S. L. Jeffcoate

Department of Chemical Pathology, St. Thomas's Hospital, London SE1, and National Institute for Biological Standards and Control, Hampstead, London NW3, U.K.

The rapid progress of steroid radioimmunoassays since their introduction in 1969 has resulted in their widespread application to mammalian physiology and experimental and clinical medicine. The technical aspects of this progress up to the middle of 1974 have been covered in a number of books and reviews (see, for example, Abraham, 1974, 1975; James & Jeffcoate, 1974; Cameron, Hillier & Griffiths, 1975; Gupta, 1975). This review will be a somewhat personal assessment of current developments (up to mid-1976) and their possible place in the future; it will be neither exhaustive nor, I hope, exhausting.

The technique of steroid radioimmunoassay (RIA) has, like the ages of Man, seven stages and these will be considered in turn: antisera, tracers, sample preparation, reagent dispensing, separation of free and bound radioactivity, counting and data processing, quality control and standardization.

Steroid antisera

In the past 2 years, there have been no significant developments in the production, assessment or treatment of antisera for steroid RIA (apart from separation methods, discussed later). It was established by mid-1974 that the nature of the immunogen, i.e. the steroid hapten, the chemistry, site and number of linkages, and the nature of the protein carrier, governed the properties of the resulting antisera. The site of linkage to the steroid nucleus was found to be particularly important and chemists were induced to try all the possible sites. For testosterone, for instance, virtually every carbon atom has been tried in an attempt to limit the cross-reaction with 5α-dihydrotestosterone (DHT). Even the most specific antisera for testosterone still show, however, a 15% cross-reaction with DHT which is little improvement on the antisera raised against the easily prepared 3-linked conjugates.

Affinity chromatography has been used in attempts to improve the specificity and affinity of steroid antisera. In my view, these attempts are doomed to failure, primarily because the cross-reaction from closely-related steroids is not the result of a separate population of antibodies which can be removed but is an inherent property of the binding site of the antibody. As regards the selecting out of the high affinity antibodies, this is made difficult by the fact that it is these antibodies that bind most avidly to the solid-phase ligand and are the most difficult to elute without damage.

Steroid tracers

Tritium-labelled steroids are most widely used as tracer ligands in the assay and also for recovery checks. Indeed, other tracers cannot be used for the latter purpose since they will not behave identically to the unlabelled steroid in partition and chromatographic systems. With the move toward less preparation of the sample before assay this use of [^{3}H]steroids will become less necessary. The advantages and disadvantages of [^{3}H]steroids have been discussed elsewhere (Jeffcoate, Edwards, Gilby & White, 1975) and are summarized in Table 1. The need to ensure radiochemical purity is most important; it should not be taken for granted that manufacturers have provided the correct steroid in the stated amount and with the desired degree of purity. In one study, wide ranges of purity were found (Manlimos & Abraham, 1975) and attributed to the shipment of the steroids from the manufacturer in solution at ambient temperatures. Stability was improved by shipping in the dry state after addition of carrier cholesterol (1 μg/1 μCi) which should not cross-react with the antiserum. Purity should be checked frequently, e.g. every 3 months.

The need to check the radiochemical purity of [^{3}H]steroids to some extent negates the main

Table 1. A comparison of the advantages and disadvantages of ^{3}H and ^{125}I-labelled tracers

^{3}H	^{125}I
Advantages	
1. Available commercially	1. Easier counting
2. Chemically defined	2. Higher specific activity
3. Long half-life	3. Higher affinity
Disadvantages	
1. Expense	1. Preparation
2. Lower specific activity	2. Higher affinity
3. Isotope effects	3. Cost of iodine

disadvantages of ^{125}I-labelled tracers, the short half-life and the need for frequent preparation. The use of ^{125}I-labelled tracers has been reviewed elsewhere (Jeffcoate, 1975) and in my view their advantages outweigh their disadvantages. The routine plasma testosterone assay at St. Thomas's Hospital has utilized a testosterone–histamine-^{125}I tracer without trouble for the past 3 years and the results are comparable to those obtained using [^{3}H]labels when collaborative assays are carried out between laboratories (M. J. Wheeler, personal communication). Another gamma emitter, ^{75}Se with a half-life of 120 days, has been used in a competitive protein-binding assay for cortisol (Chambers, Glover & Tudor, 1975), but this isotope has not yet been used successfully in an RIA. At present the specific activity of ^{75}Se is low compared with that of ^{125}I.

Non-isotopic tracers for RIA are being developed: such markers include:

(a) viruses, e.g. bacteriophage;
(b) enzymes, e.g. alkaline phosphatase;
(c) fluorescent;
(d) spin-labelling;
(e) immunological FETs

and some of the potential advantages are:

(a) a long shelf life;
(b) cheapness of materials and equipment;
(c) no separation step;
(d) automation;
(e) simultaneous assays;
(f) no isotope health hazard and disposal problem.

The prospect of cheap automation is the main attraction of these methods, although at present the price of lower sensitivity has to be paid. Future developments in this area should be expected.

Sample preparation

The extent to which a biological sample has to be purified before steroid RIA is proportional to the amount of error (losses, blanks, transcriptional) introduced and inversely proportional to the number of samples that can be processed in unit time. For both these reasons, there is a gradual move to less and less pre-assay preparation. This approach obviously carries its own potential danger, that of loss of specificity because of the presence in the sample of cross-reacting steroids or other interfering, 'non-specific' substances.

The ultimate goal, that of no sample treatment at all, is only possible for steroids present in biological fluids in very high concentration (μmol/l), e.g. cholesterol and cholesterol esters, dehydroepiandrosterone (DHA) sulphate or steroid glucuronides (Kellie, 1975).

For steroids like cortisol, which are present in moderate concentration (nmol/l), non-extraction methods are also possible but in this case only after cortisol-binding globulin has been inhibited.

This can be done by (a) heating to 60°C for 1 h, (b) treatment with ANS (8-anilino-naphthalene sulphonic acid) as used in thyroid hormone assays or (c) precipitation of plasma proteins with ethanol. These methods could also be applied to sex hormone-binding globulin (SHBG).

For most steroids a solvent extraction is required. Although there has been little recent development in this area, four points are worth making. First, the purity of the solvent should be initially high and frequently checked: a few ml evaporated and run as a sample in the RIA will soon reveal if pseudosteroid 'blank' material is being added. Secondly, the choice of solvent can add specificity to the assay. This can be illustrated with respect to progesterone: it is unnecessary to extract with a polar solvent like ether when a much 'cleaner' extract can be obtained with a less polar solvent, such as hexane, with a minimum reduction in recovery. The logical extension of this view is that each steroid has its own extraction recipe tailored to its polarity so as to produce the cleanest extract with maximal recovery.

This conclusion does however militate against the third consideration, that of assay standardization (see also the final section). The more solvents in use in a laboratory and within a group of laboratories, in which attempts are being made to achieve comparability of assay results, the more difficult it is to control the quality of the reagents. It may thus be best to select one (e.g. ether) which will extract all the steroids of interest, and to specify its purity and mode of use in as much detail as possible.

Finally, there have been developments in speeding up the rate-limiting step in steroid RIA, the separation and drying of the organic phase. A neat method of separation is to freeze the aqueous subnatant either in solid CO_2/acetone or with a cryoprobe (use of a deep-freeze is not recommended if ether is the solvent) and then to decant the organic supernatant without fear of contamination with the water phase. Evaporation machines are becoming commercially available. These will hold up to 100 tubes and will shake or warm them in a flow of nitrogen or air.

Chromatographic separation of steroids from biological fluids has been a traditional approach of steroid assayists but with the gradual improvement of reagents and the increasing pressure of sample numbers, it is becoming less necessary and less popular. It still has two important places. First, non-chromatographic methods need to be validated against those in which chromatography has been used. The second application is in obtaining multiple steroid analyses of a single sample. This may be of value when several related steroids are of pathophysiological interest in a specific situation. Although other systems have been used, Abraham has developed this approach using extracts of 1 ml plasma on celite columns, e.g. for 4 androgens (Abraham, Manlimos, Solis & Wickman, 1975) or for 4 progestins (Abraham, Manlimos, Solis, Garza & Maronlis, 1975). Celite column chromatography was also used by Parker, Ellegood & Mahesh (1975) for assay of 7 steroids of interest in reproductive endocrinology after separation (in < 2 h) from a single 1–2 ml sample of plasma.

Reagent dispensing

The move toward increased mechanization of steroid RIA is occurring within the context of increasing automation of RIA and clinical chemistry in general. There has been a rash of dilutors, dispensers and pipetting stations on the market in addition to the relatively simple hand-held repeating pipettes. All these have their place, as do the completely automated machines under development which take in a sample at one end and, after a suitable delay, push out an answer at the other end.

Separation of free from bound radioactivity

After the incubation of the reagents for an appropriate time the next important phase in steroid RIA is the separation of free from bound radioactivity. Many methods have been used (Ratcliffe, 1974) and perhaps the aims of these methods should be stated before discussing how developments might advance steroid RIA. These are: no mis-classification error (i.e. all the bound and none of the free is in the bound fraction); cheapness and simplicity; potential for automation; ability to be standardized within and between laboratories.

Adsorption of the free fraction onto activated charcoal, uncoated or coated with dextran, albumin or gelatin is the most widely used method in steroid RIA. It has the advantage of cheapness and simplicity and can be fairly well standardized: one large batch of charcoal can be purchased to serve a number of laboratories for many years. Its disadvantages are (i) the dependence on time between addition and centrifugation; there is an increase in the radioactivity adsorbed to the charcoal caused either by dissociation of the bound complex and adsorption of the freed tracer or, more likely, adsorption of the bound complex itself, and (ii) the difficulty of using charcoal in automatic equipment.

Polyethylene glycol (PEG) is easier to handle in these respects (Schiller & Brammall, 1974, 1976) and recently has been widely used. One disadvantage of PEG is the need for higher centrifugation speeds (at least 1500 *g*) compared with dextran-coated charcoal (500 *g*). This may limit its universal applicability.

The double-antibody methods widely used in peptide assays can also be applied to steroids but they seem to be unnecessarily expensive and time-consuming when other techniques are available.

Although the first steroid RIA published was a solid-phase (coated-tube) method, these techniques have been slow to gain ground. They appear to have many advantages particularly in terms of cost, ease and potential automation. The routine plasma testosterone RIA at St. Thomas's Hospital mentioned above has employed antibody-coated tubes for several years. Temperature control at the coating stage is the only critical factor.

Other solid-phase methods that seem to be increasingly used are those in which antibody is covalently bound to particles which can be easily centrifuged for separation (e.g. Sepharose or glass beads). A novel approach (Nye & Landon, 1976) is the use of antibodies coupled to magnetic particles which can be simply and automatically removed from the incubation medium with a magnet.

In summary, developments in this area of steroid RIA are in the direction of methods that are either cheap, automated or easily standardized and preferably all three.

Counting and data processing

The counting of the radioactivity in either the antibody-bound or free fractions (or both) requires automatic counters. These machines are expensive and becoming more so. Two tendencies are discernible however: first, in the direction of more complicated counters that will do a certain amount of data processing in addition to counting; and secondly in the direction of cheaper, simpler bench-top counters with, for example, a single channel and limited sample capacity. Different users will have different needs and this will govern the choice of counter.

Scintillation-counting of β-emitters such as [^{3}H]steroids has been advanced by the use of polypropylene inserts which are produced by several manufacturers. The sample plus scintillation fluid is put in the insert which is then placed in a standard counting vial. Three benefits accrue: less scintillant is used; the waste scintillant and the insert can be easily disposed of by incineration thus reducing the health hazard; washing of counting vials is reduced to a minimum.

Computers are used in RIA for two major purposes: optimal assay design and the calculation and evaluation of results. The theoretical basis and practical application of these have been extensively discussed. In 1974, Rodbard wrote that "the literature on RIA statistics and data processing is large and growing rapidly." This is still true in 1976. The reviews of Rodbard (1974) and Cook (1975) summarize the situation admirably. As regards standard curve-fitting, the logit–log linear transform appears to have gained widest currency and this and other programmes are readily available for use on relatively simple programmable calculators. It is an inefficient use of resources to have an on-line computer system for data calculation.

Quality control and assay standardization

Although the least glamorous aspect of steroid RIA, in my view this is the area in which the most significant developments are taking place in 1976. It has been recognized in public health laboratories (and is even permeating into the thinking in 'pure' research laboratories) that the quality of the

assays performed is at least as important as the number of assays performed. Many of the principles and practices adopted by bioassayists for decades are only reluctantly used by immunoassayists and yet they are essential if the results are to have any meaning.

Quality control is aimed at two interrelated problems, internal and external. Within a laboratory, the assay needs to be as near 'correct' as possible with errors limited and with a mechanism for detecting real or potential breakdown in the assay. This is done by incorporating relevant samples in each assay. Thus the maximal binding (B_o), minimal (non-specific) binding and slope of the standard curve can be assessed. Quality control samples can be included and should preferably cover the range to be expected in the unknown samples which they should resemble as closely as possible. Thus it is better to use the endogenous steroid rather than to 'strip' (e.g. with charcoal) the sample and add known amounts of standard steroid. Cumulative charts showing within-assay and between-assay variance should be kept and make good laboratory wall-paper. Rodbard (1974) and Rodbard, Lenox, Wray & Ramseth (1976) have summarized the use of internal quality control schemes.

External quality control schemes are necessary because (i) many laboratories do not run adequate internal quality control schemes, (ii) results between laboratories need to be comparable so that, in the first place, clinicians and others can use data from physiological and pathological situations without having to readjust to different normal ranges, and secondly, multi-centre research programmes obtain uniform results.

A number of external quality control schemes for steroid RIA are under development. In the United Kingdom, a national scheme has arisen out of the work of the Supra-regional Assay Service (SAS) and all the steroids of major biological interest are covered. In general, about four coded samples are sent out at monthly intervals and the results returned and processed centrally. These schemes give information regarding between and within-laboratory variance and reproducibility. A trouble-shooting element is an essential feature with the organizing quality control laboratory being able to give advice on reagents, assay protocols, etc.

On a world-wide scale, the Human Reproduction Unit of WHO has instituted an external quality control scheme for four steroid hormones in an attempt to increase the quality and comparability of the results obtained by the research laboratories participating in its research programmes in fertility control.

Those who have experienced the results of external quality control schemes for RIA are well aware of the need for assay standardization. International organizations have seen the need for this and laid down guide-lines very clearly (IEAE, 1974; WHO, 1975). The 26th report of the WHO Expert Committee on Biological Standardization is a milestone in this area and covers the need to provide well-characterized, matched reagents for immunoassay and the organization of hormone assay services at district, regional, national and international levels.

The U.K. is ahead of the world here with the SAS scheme and a developing network of regional schemes. The standardization of RIA within the SAS and within the WHO programmes involves a package of matched reagents (well-characterized, in sufficient supply to last for 3–5 years and in ampoules available for distribution) plus detailed assay protocols (buffers, incubation and separation procedures, etc.) and an external quality control scheme. Using such a package, the achievement of the goal of assay standardization and universal comparability of high quality RIA results is brought immeasurably closer.

References

Abraham, G.E. (1974) Radioimmunoassay of steroids. *Acta endocr., Copenh., Suppl.* **183**, 1–42.

Abraham, G.E. (1975) The application of steroid radioimmunoassay to gynecologic endocrinology. In *Progress in Gynecology*, Vol. VI, pp. 111–144. Eds M. L. Taymor & T. H. Green. Grune and Stratton, New York.

Abraham, G.E., Manlimos, F.S., Solis, M., Garza, R. & Margoulis, G.B. (1975) Combined radioimmunoassay of four steroids in one ml of plasma. I. Progestins. *Clin. Biochem.* **8**, 369–373.

Abraham, G.E., Manlimos, F.S., Solis, M. & Wickman, A.C. (1975) Combined radioimmunoassay of four steroids in one ml of plasma. II. Androgens. *Clin. Biochem.* **8**, 374–378.

Cameron, E.H.D., Hillier, S.G. & Griffiths, K.

(Eds) (1975) *Steroid Immunoassay*. (Proc. 5th Tenovus Workshop.) Alpha Omega Alpha Publishing Ltd, Cardiff.

CHAMBERS, V.E.M., GLOVER, I.S. & TUDOR, R. (1975) (^{75}Se)—radio ligands in steroid immunoassay. In *Steroid Immunoassay*, pp. 177–182. Eds E. H. D. Cameron, S. G. Hillier & K. Griffiths. Alpha Omega Alpha Publishing Ltd.

COOK, B. (1975) Automation and data processing for radioimmunoassays. In *Steroid Immunoassay*, pp. 293–310. Eds E. H. D. Cameron, S. G. Hillier & K. Griffiths. Alpha Omega Alpha Publishing Ltd, Cardiff.

GUPTA, D. (Ed.) (1975) *Radioimmunoassay of Steroid Hormones*. Verlag Chemie, Weinheim.

IAEA (1974) Standardisation of radioimmunoassay procedures. Report of an International Atomic Energy Agency panel. *Int. J. appl. Radiation and Isotopes* **25**, 145–160.

JAMES, V.H.T. & JEFFCOATE, S.L. (1974) Steroid radioimmunoassays. *Br. med. Bull.* **30**, 50–54.

JEFFCOATE, S.L. (1975) Use of ^{125}iodine tracers in steroid radioimmunoassays. In *Radioimmunoassay of Steroid Hormones* pp. 185–195. Ed. D. Gupta. Verlag Chemie, Weinheim.

JEFFCOATE, S.L., EDWARDS, R., GILBY, E.D. & WHITE, N. (1975) The use of [^{3}H]-labelled ligands in steroid radioimmunoassays. In *Steroid Immunoassay*, pp. 133–140. Eds E. H. D. Cameron, S. G. Hillier & K. Griffiths. Alpha Omega Alpha Publishing Ltd, Cardiff.

KELLIE, A.E. (1975) Radioimmunoassay of steroid glucuronides. In *Radioimmunoassay of Steroid Hormones*, pp. 165–183. Ed. D. Gupta. Verlag Chemie, Weinheim.

MANLIMOS, F.S. & ABRAHAM, G.E. (1975) Chromatographic purification of tritiated steroids prior to use in radioimmunoassay. *Analyt. Letters* **8**, 403–410.

NYE, L. & LANDON, J. (1976) Automated hormone radioimmunoassays based on the use of antibodies linked to ferro-magnetic particles. *J. Endocr.* (In press).

PARKER, C.R., ELLEGOOD, J.O. & MAHESH, V.B. (1975) Methods for multiple steroid radioimmunoassay. *J. Steroid. Biochem.* **6**, 1–8.

RATCLIFFE, J.G. (1974) Separation techniques in saturation analysis. *Br. med. Bull.* **30**, 32–37.

RODBARD, D. (1974) Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Clin. Chem.* **20**, 1255–1270.

RODBARD, D., LENOX, R.H., WRAY, H.L. & RAMSETH, D. (1976) Statistical characterisation of the random errors in the radioimmunoassay dose-response variables. *Clin. Chem.* **22**, 350–358.

SCHILLER, H.S. & BRAMMALL, M.A. (1974) A radioimmunoassay of plasma estradiol-17β with the use of polyethylene glycol to separate free and antibody-bound hormone. *Steroids* **24**, 665–674.

SCHILLER, H.S. & BRAMMALL, M.A. (1976) Non-chromatographic radioimmunoassay of unconjugated estriol in plasma with polyethylene glycol as precipitant. *Clin. Chem.* **22**, 359–363.

WHO (1975) Twenty-sixth report of the World Health Organisation Expert Committee on Biological Standardisation (26th ECBS). WHO, Geneva.

J. Reprod. Fert. (1977) **51**, 273–286

The measurement of human gonadotrophins by radioimmunoassay

G. V. Groom

Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath Park, Cardiff CF4 4XX, U.K.

Introduction

The study of the gonadotrophins may be considered to have begun, perhaps, with the observation by Crowe, Cushing & Homans (1910) that gonadal atrophy invariably followed hypophysectomy in dogs. A decade elapsed, however, before Evans & Long (1921) demonstrated the effect of intraperitoneal administration of anterior pituitary tissue on oestrus and ovulation. Zondek (1930), after a series of papers on the biological properties of these pituitary factors, first used the terms Prolan A, now called follicle-stimulating hormone (FSH), and Prolan B, now called luteinizing hormone (LH). Forty years passed before the existence in man of the third gonadotrophin, prolactin, was finally established.

During this period many great developments occurred in methodology which were to revolutionize endocrinology. The transition from bioassay to the more sensitive and, supposedly, more precise radioimmunoassays (RIA) was probably one of the most significant of these changes. With the advent of this technique a reproducible, sensitive, specific and relatively simple method was available for the rapid monitoring of these hormones in biological fluids; a technique which became one of the most widely used methods in routine and research endocrine departments.

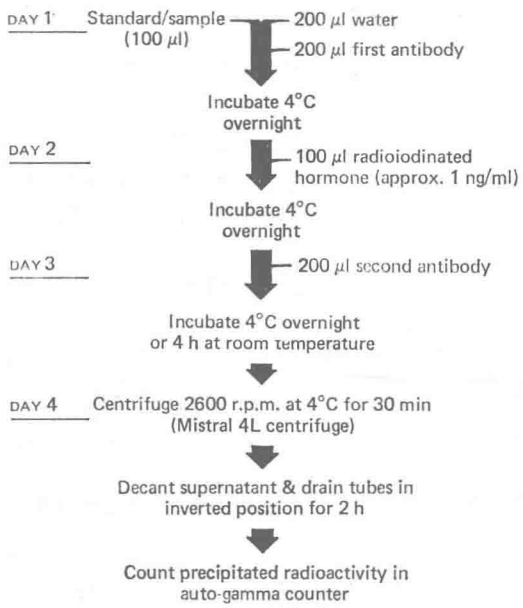
In recent years, few major developments have occurred in the technique of protein hormone RIA. The innovations have rather been in the use of hormone fragments or subunits for the production of new antisera. I will consider the use of some of these antisera, with particular reference to the difficulty of interpretation of data and the conflict between bioassay and RIA results. Evidence will be presented to demonstrate some of the limitations of RIA and suggestions for the future development of these assays put forward. Reference will be made to the RIA determination of human LH, FSH, chorionic gonadotrophin (HCG) and prolactin.

Methods

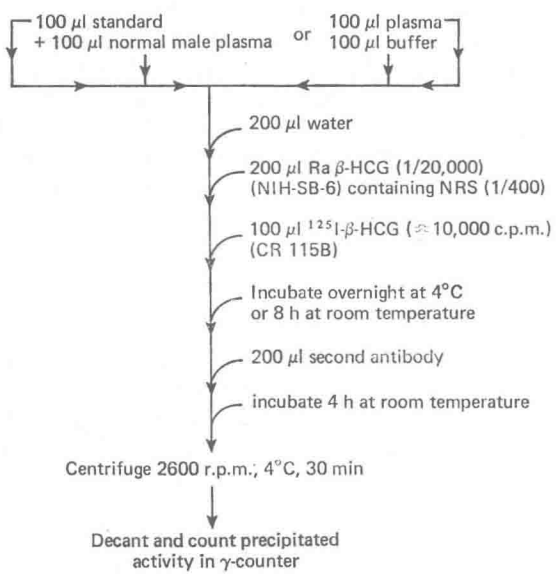
A simplified buffer system suitable for the RIA of a number of protein hormones, including those discussed herein, consists of 0·2 M-phosphate, pH 7·5, 1% (w/v) human or bovine serum albumin, 0·05 M-EDTA and 0·1% (w/v) merthiolate. The solution can be prepared at × 5 concentration and stored at −20°C until use when thawing and diluting in deionized water are the only operations required. Batches of fresh buffer concentrate need only be prepared at 3–4 month intervals depending on use, thus being economical on technician time and providing better continuity between assays over this period.

Only two additions need be made to this basic buffer. Normal rabbit serum (NRS, 0·25% v/v) is added to the buffer used to prepare first antibody solutions, when the dilution of this antiserum is greater than 1:1000 (v/v). The NRS serves as carrier in the double-antibody RIA method utilizing a first antibody raised in rabbits. When the first antibody is used at dilutions of less than 1:1000 the NRS concentration is adjusted to maintain a total rabbit globulin concentration of 0·25% (v/v) in the first antibody solution.

The second antibody solution is supplemented with Triton X-100 (5% v/v) which has been shown to have no deleterious effect on the performance of the assays but does expedite draining of the assay tubes after centrifugation and decanting, thus allowing counting of the radioactivity after a much shorter delay. In addition, considerable improvement in non-specific binding to less than 1% of counts added is obtained with the use of Triton.



Text-fig. 1. The basic radioimmunoassay procedure employed for the determination of LH, FSH, GH or prolactin.



Text-fig. 2. Radioimmunoassay procedure employed for the determination of β-HCG.

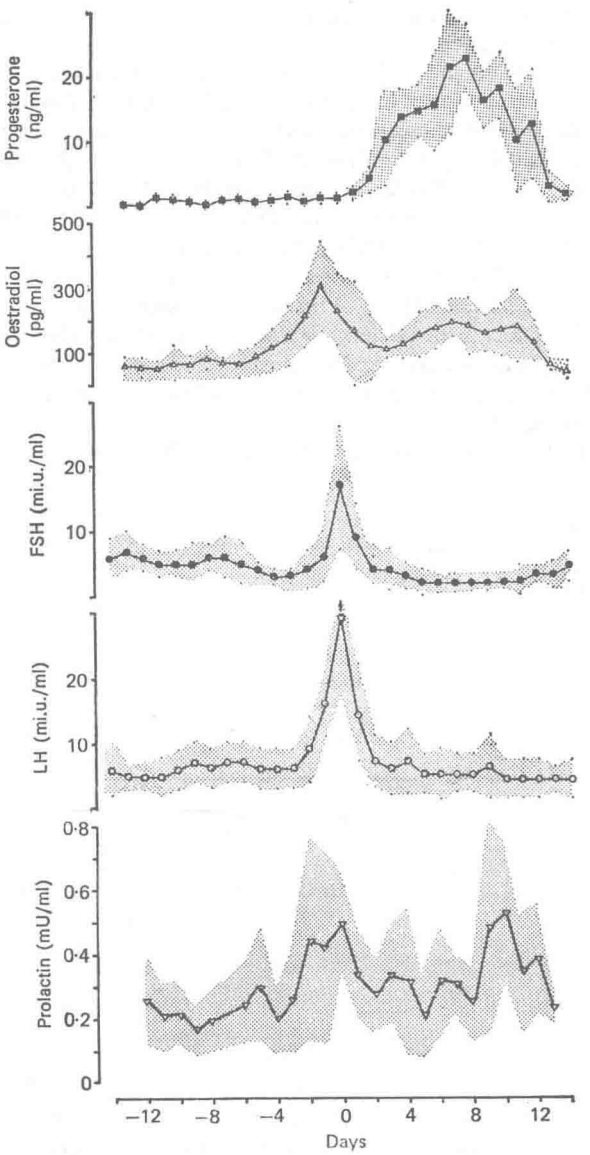
The current procedure used for the assay of LH, FSH and prolactin is shown in Text-fig. 1. The method for the measurement of LH and FSH was modified from that described by Groom, Groom, Cooke & Boyns (1971). Antisera to LH (F 87) and FSH (M 93) and purified FSH for labelling (CPDS 13), generously donated by Dr W. Butt, are used at final dilutions of 1:240,000 and 1:500,000 respectively. Dr A. S. Hartree kindly provided the LH for labelling (IRC–2).

The assay of human prolactin with antiserum raised to a pituitary extract (antiserum 7110; final dilution 1:60,000) is as shown in Text-fig. 1. The assay of human prolactin with antiserum raised to

an amniotic fluid extract (antiserum R47; final dilution 1:1200) is that of Cole & Boyns (1973) as modified by Cole, Groom, Link, Flanagan & Seldrup (1976). Purified prolactin preparations for labelling and used in both assays were kindly provided by Dr U. J. Lewis. Further purified prolactin samples were kindly donated by Dr P. Lowry.

Materials for the assay of the β-subunit of HCG (Text-fig. 2) were generously provided by the National Institutes for Arthritic, Metabolic and Digestive Diseases (NIAMDD), Bethesda, Maryland.

Human growth hormone (GH) is assayed as outlined in Text-fig. 1, by using antisera obtained from Burroughs Wellcome Ltd, Beckenham, Kent (RD 16, final dilution 1:300,000), and purified growth hormone for labelling kindly donated by the M.R.C., National Institute for Medical Research, London.



Text-fig. 3. Mean (±S.D.) plasma levels of LH, FSH, prolactin, oestradiol and progesterone during the menstrual cycle (N = 6).

All iodinations were carried out with chloramine T after the method of Greenwood, Hunter & Glover (1963) with subsequent purification on Biogel P–60 as described by Groom *et al.* (1971).

Results and Discussion

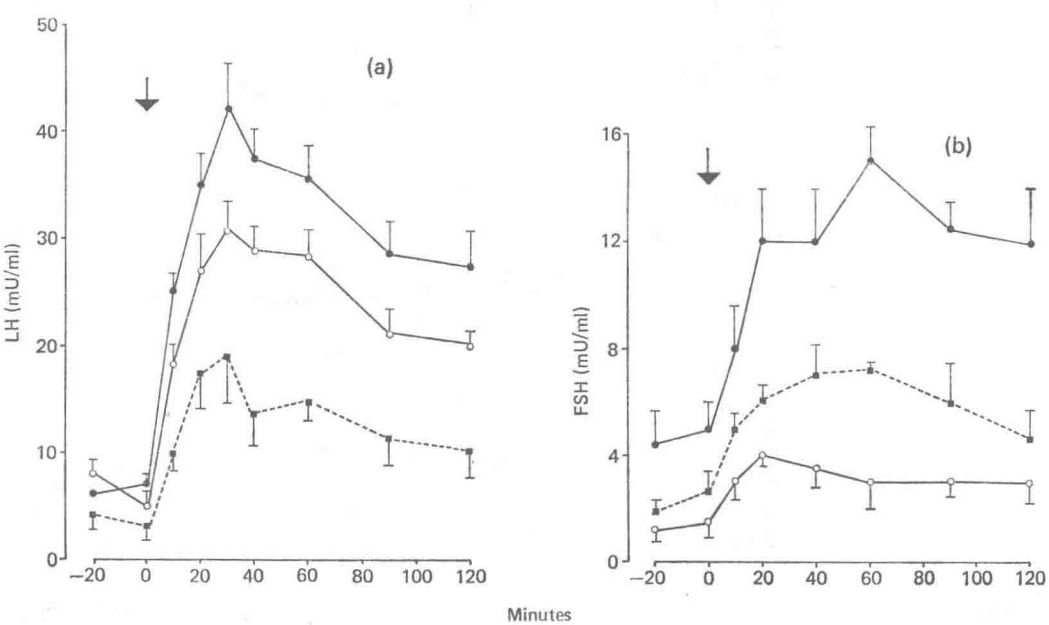
Radioimmunoassay of LH and FSH

Text-figure 3 shows the mean levels of LH and FSH in the plasma of 6 normal women bled daily throughout a menstrual cycle and relates these hormones to concomitant levels of prolactin, oestradiol and progesterone. The parallel secretion pattern of LH and FSH is clearly seen with the single major peak occurring at mid-cycle, after the oestradiol peak, and probably responsible for ovulation within the succeeding 24–72 h (Stevens & Vorys, 1967). While the release of the egg is the classical role for LH no direct role for FSH has been described during mid-cycle. The simultaneous release of both hormones is, therefore, taken to be merely a necessary consequence of the dual-acting LH-releasing hormone (LH-RH).

The classical role of FSH, of course, is the development of the follicle and the secretion of oestradiol. The lack of any substantial peak in FSH levels during the early follicular phase of the cycle is difficult to understand. This is particularly so in view of the fact that earlier workers using bioassay techniques had found equivalent peaks of urinary FSH at mid-cycle and during the early follicular phase (Stevens & Vorys, 1967). Follicular-phase levels of FSH determined by RIA are slightly higher than in the luteal phase but the conflict between RIA and bioassay observations of a distinct FSH peak during the early follicular phase remains to be resolved.

The early report of Schally *et al.* (1971) of a single releasing hormone responsible for the hypothalamic control of both LH and FSH was at first regarded with some doubt. However, no evidence has yet been produced to indicate the existence of a separate FSH-releasing hormone (Shahmanesh & Jeffcoate, 1976). Furthermore, attempts to determine specific LH- and FSH-releasing moieties of the LH-RH decapeptide molecule have proved fruitless (Schally, Arimura, Debeljuk, Redding & Reeves, 1973; Groom & Boyns, 1973).

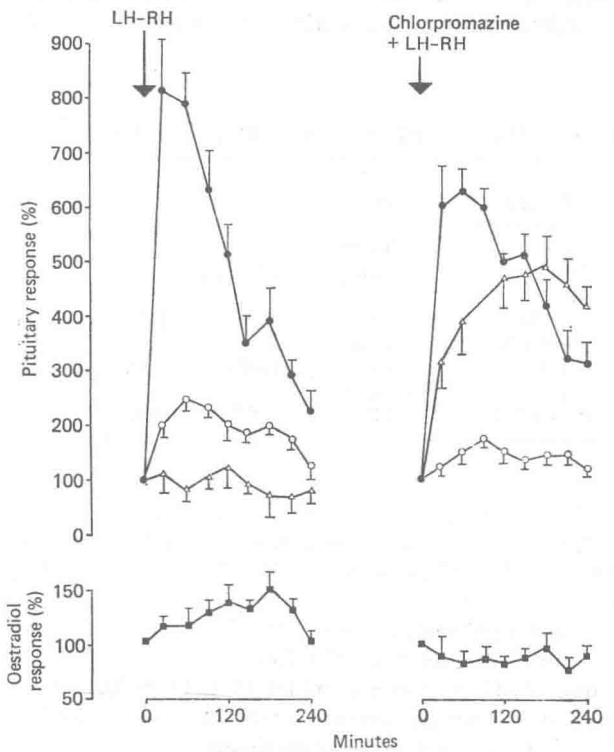
The LH-RH stimulation test has thus become an established and reliable test of both LH and



Text-fig. 4. Individual serum responses (mean ±S.D.) of (a) LH and (b) FSH to intravenous administration of LH-RH (arrow) on three separate occasions to 3 volunteers. ●, Subject A, 100 μg; ○, Subject B, 100 μg; ■, Subject C, 50 μg. The response to the same dose was reproducible in each patient. (Modified from Shahmanesh *et al.*, 1975.)

FSH pituitary reserve. While there is variation between individuals given the same dose of the releasing hormone, the LH response to a given dose is very reproducible in the same subject (Text-fig. 4a). Similarly, when one considers FSH release (Text-fig. 4b), there is a somewhat lower response to any dose of LH-RH and the time-course differs from the LH release pattern, but the same reproducibility is present. The response of the two hormones commences simultaneously and the discrepancies in time-course may reflect differences in metabolic clearance or half-life of the two hormones.

The LH-RH test is equally useful in men and women and can be extended to test ovarian/testicular response to the gonadotrophin stimulation. Great care must be taken in interpreting these data, however, since the action of other hormones may be limiting. For example, the normal oestradiol response after LH-RH stimulation is abolished when LH-RH is administered with chlorpromazine which stimulates prolactin secretion (Text-fig. 5). Elevated prolactin levels have been implicated in many cases of infertility, probably acting by a direct block on LH and FSH action at the ovary (Thorner, McNeilly, Hagan & Besser, 1974).



Text-fig. 5. Effect (mean ± S.E.M.) of chlorpromazine (50 mg i.m.) on the LH (●), FSH (○), prolactin (△) and oestradiol (■) response to LH-RH (100 μg i.m.) of 4 normal men. Results are expressed as a percentage of the value at zero time. (Modified from Cole *et al.*, 1976.)

The presence of a common hypothalamic releasing hormone is but one aspect of the close relationship between LH and FSH. Considerable apparent redundancy has been shown between the biological activities of the two molecules. This topic was reviewed by Greep (1961) and Schwartz & McCormack (1972) and will not be further dealt with here. However, the various biological activities attributed to the two hormones could be accounted for by a single molecule producing effects which may be modified by, or depend upon, the state of the target tissue. Furthermore, LH- and FSH-like material have been reported to be present in the same pituitary cell (Nakane, 1970), contrary to the generally held maxim of one cell–one hormone. Phifer, Midgley & Spicer (1973), using extensively

characterized, highly specific antisera, also observed both gonadotrophins in cells in human pituitary sections and supported their findings by biological staining techniques.

The specificity of antiserum to the gonadotrophins has always been a problem owing to the close chemical similarites of LH, FSH and the third pituitary glycoprotein, thyroid-stimulating hormone (TSH), and HCG. Human LH, FSH and TSH consist of two non-identical subunits each of approximately 100 amino acids (Reichert & Midgley, 1968; Liao & Pierce, 1970; Ryan, Jiang & Hanlon, 1971). The primary sequence of the α-subunit is identical for the three hormones in any species, while the hormone-specific β-units still exhibit considerable homology. This similarity of structure leads to problems of chemical purification while, in addition, the normally covalently bound subunits can readily dissociate and re-associate. This recombination of subunits has been shown to be possible not only between different hormones within a species, but also between subunits from hormones of different species (Table 1). The dimeric nature of these hormones with its inherent lability creates further problems to the chemist attempting to purify the molecules with as little structural damage as possible. Furthermore, excessively harsh conditions of iodination of this purified hormone by the RIA technician could lead to further alteration and polymerization of the molecule. Unless suitable purification steps are subsequently taken, spurious results could be obtained with this damaged tracer in an RIA.

Table 1. Recovery of biological activity after recombination of glycoprotein hormone subunits

α-Subunit	β-Subunit	Activity recovered	Reference
LH	TSH	TSH	Liao & Pierce, 1970
TSH	TSH	TSH	
TSH	LH	LH	
Rat LH	Rat LH	LH	Ward *et al.*, 1971
Ovine FSH	Ovine FSH	FSH	Papkoff & Samy, 1967
Ovine LH	Ovine FSH	FSH	
Ovine FSH	Ovine LH	No activity	
Human LH	Bovine LH	LH	Reichert, Midgley, Niswender & Ward, 1970

Pituitary glycoprotein subunits have been shown to exist in normal plasma (Franchimont, Gaspard, Reuter & Heynen, 1972; Hagen & McNeilly, 1975) and can be released from the pituitary in response to LH-RH and TRH in certain clinical conditions (Kourides, Weintraub, Ridgway & Maloof, 1973; Benveniste, Frohman, Bell, Spitz & Rabinowitz, 1975). Their properties, however, are completely unknown. Virtually none of the biological activity of the intact hormone remains in either subunit but almost complete activity returns after recombination (Table 1). McKerns & Ryschkewitsch (1974) showed that the subunits of LH can compete and inhibit LH activity at the corpus luteum. To date, however, the search for subunit activity has been largely short-sighted and much work remains here in searching for activity in the many possible target tissues.

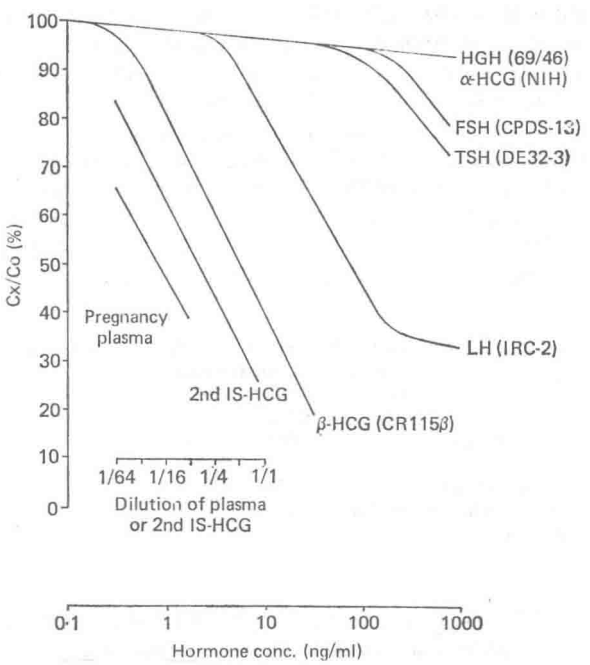
Several workers have shown the existence of 'big' precursor hormones for insulin, parathyroid hormone and adrenocorticotrophin, and even 'big-big' antecedents of these molecules. Could it be that LH is the 'big' precursor of β-LH while the α-subunit is only a carrier, as suggested by Franchimont *et al.* (1972)? Conversely, could the LH–FSH complex be a 'big-big' gonadotrophin? What biological activity, if any, would such a molecule possess and what changes in pituitary secretion rates of this material would be measured if a suitable assay existed? Young, Harsoulis, Kuku & Fraser (1975) reported that the major form of LH in human plasma and pituitary was of molecular weight in excess of 70,000 while that in urine was of 30,000 or less. Since the molecular weight of each subunit, calculated from its primary sequence, is of the order of 15,000 it would appear that plasma and pituitary LH is largely present in the form of a tetramer molecule.

Many questions remain to be answered concerning the nature of gonadotrophins. The weight of evidence suggests, however, that because they have largely parallel secretion patterns, a single

hypothalamic releasing hormone, exist in the same pituitary cell, and exhibit such overlapping chemical and biological activity, LH and FSH may well be fragments of a larger molecule with, perhaps, still other biological properties.

Radioimmunoassay of β-*HCG*

The β-subunit of HCG differs from that of LH merely in the composition of a dozen amino acids through the molecule, the presence of additional carbohydrate moieties, and a further sequence of 32 amino acids at the C-terminus (Shome & Parlow, 1973; Bahl, Carlsen, Bellisario & Swaminathan, 1972), while the α-subunits of the two molecules are identical. It is because of this considerable structural homology, that antisera raised to one hormone show a great degree of cross-reactivity with the other. With the availability of purified subunits and subunit fragments, however, antisera have been produced to give much reduced cross-reaction. Text-figure 6 illustrates the lack of cross-reaction with LH, FSH, TSH and the α-subunit in a β-HCG assay. With this assay, pregnancy can be detected at a very early stage when previous non-specific assays could not have distinguished it from a rise in pituitary gonadotrophin (Text-fig. 7).



Text-fig. 6. Effect of various pituitary and placental hormone preparations and pregnancy plasma on the assay for β-HCG.

The presence of HCG in the plasma of non-pregnant women is indicative of a pathogenic condition and work is underway in many centres to determine the usefulness of the β-HCG assay as a tumour marker determinant (Rees, 1975). The assay is of proven worth in detecting trophoblastic tumours (Vaitukaitis, Braunstein & Ross, 1972) and has been found in the plasma of approximately 50% of patients with ovarian or testicular tumours, as well as, to a lesser extent, in those with gastrointestinal or lung tumours (Vaitukaitis, 1975a).

As part of our studies into tumour markers in breast and prostatic cancer, a series of samples from control populations was assayed. These samples were derived from three sources: young, healthy laboratory technicians, or a random collection of plasma samples acquired by the MRC Epidemiology Unit from the South Wales area, or a collection of samples from patients hospitalized for non-