

Radioimmunoassays and ruminant endocrinology

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To try and cover comprehensively all aspects of radioimmunoassays in relation to ruminant endocrinology in one paper is an impossible task. I will therefore outline the general principles and techniques involved in these assays and show how the results obtained from using them have increased our knowledge in the field of ruminant reproductive physiology. A better understanding of the factors controlling the reproductive cycles in domestic animals may well lead to better methods for the control of animal production and maximize the availability of our own food requirements.

The principles and techniques involved in radioimmunoassays

The 1960s saw what can only be termed as a revolution in the measurement of protein and polypeptide hormones. This revolution was the development of radioimmunoassays (RIA) or, as they were and sometimes still are called, saturation analyses or competitive assays. The credit for the pioneering work will be associated with the names of Berson and Yalow (Yalow & Berson, 1959, 1960) from the United States and with Ekins (1960) from England, working on human insulin and thyroxine respectively. After insulin and thyroxine it was the turn of larger molecules such as human growth hormone and thyroid-stimulating hormone (TSH) and more

Table 1. *Substances being measured by radioimmunoassay applicable to ruminant endocrinology*

PEPTIDE HORMONES	NON-PEPTIDE HORMONES	NON-HORMONAL SUBSTANCES
Growth hormone	Steroid hormones	Cyclic nucleotides
Adrenocorticotrophic hormone	Thyroid hormones	Serum proteins
Thyroid-stimulating hormone	Prostaglandins	Vitamins
Follicle-stimulating hormone (FSH)		Enzymes
Luteinizing hormone (LH)		
Prolactin		
Vasopressin		
Oxytocin		
Insulin		
Glucagon		
Parathyroid hormone		
Calcitonin		
Gastrin		
Angiotensins		
Bradykinins		
Thyrotrophin releasing factor		
LH-FSH releasing factor		

recently the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Subsequently, smaller molecules were given attention and as a result RIA have been developed for substances such as adrenocorticotrophic hormone, glucagon, vasopressin, gastrin, steroid hormones and most recently of all many drugs (digoxin, morphine, lysergic acid diethylamide and barbiturates), cyclic nucleotides, vitamins and prostaglandins. Table 1 shows some of the hormones and other substances currently being measured by RIA which are applicable to ruminant endocrinology. The reader is referred to the following recent general articles which cover the subject of RIA (Margoulies, 1969; Van Cauwenberge & Franchimont, 1970; Kirkham & Hunter, 1971; Cotten, 1973; Sönksen, 1974).

Many RIA were initially developed for the measurement of hormones in human biological fluids, but adaptations to measure the same hormones in ruminants have followed, and in fact many assays set up to measure a particular human hormone have been used directly for the ruminants where no structural difference occurs, for example, the steroids and prostaglandins.

In theory, the principles and techniques involved in all RIA's are simple. Providing a specific antibody can be raised against the compound to be measured and the compound can also be labelled with a radioisotope then the basic requirements for setting up an assay have been established. As with most things, however, the practice is never as simple as the theory. Nonetheless, RIA represent one of the most sensitive and precise methods available for visualizing any antigen-antibody reaction. Under standard conditions the amount of labelled antigen which remains free or the amount which combines with antibody after incubation, or both, is determined. This competition between standard amounts of labelled antigen and antibody will be altered by the presence of unlabelled antigen. By introducing known amounts of unlabelled antigen into the system, a standard calibration curve can therefore be obtained for use in determining the amount of antigen in unknown samples. The higher the concentration of unlabelled antigen in the system, the lower the percentage binding of the known amount of labelled antigen will be to the antibody. Before any RIA can be performed it is therefore necessary to establish a standard curve showing a decrease in the ratio antibody-bound:free labelled antigen, as a function of increasing known quantities of unlabelled antigen.

There are, therefore, three basic requirements for the setting up of any particular RIA: (1) a specific antiserum raised against the compound (antigen) to be measured; (2) a pure labelled antigen (and also a pure preparation of unlabelled antigen for use as a standard); (3) a technique for separating antibody-bound and free antigen after the competitive reaction has reached equilibrium so that either or both may be counted for the radioisotope used as a label.

1. *Antibody production*

The production of a specific antibody is achieved by injecting the compound (antigen) into usually a rabbit or guinea-pig at multiple sites over a period of weeks. Serum samples are taken at intervals and the affinity or 'titre' of the antiserum to

radioactive antigen tested. Generally polypeptide hormones of molecular weight greater than 4000 are sufficiently immunogenic when the hormone is administered as an emulsion in Freund's adjuvant. It is not necessary for the antigen to be the purest preparation available, as often slight impurities render it more antigenic. For substances such as steroids and other low-molecular-weight compounds the first step is to make them antigenic by coupling to a large protein or polypeptide (bradykinin: Talamo, Haber & Austen, 1968; steroids: Exley, Johnson & Dean, 1971; Kuss & Goebel, 1972; prostaglandins: Levine & Van Vunakis, 1970). With the steroid group of molecules, particularly the oestrogens, an initial problem was the cross-reactivity of antisera raised against steroid-protein conjugates where conjugation was at the C₃(phenolic) or C₁₇ functional group. Antisera raised against such conjugates were able to recognize and react with a wide range of other steroids. With conjugation at the C₆ position, however, free access to the C₃ and C₁₇ functional groups remained open and higher-specificity antisera were obtained (Exley *et al.* 1971).

Purification of antisera raised against protein conjugates can be achieved by absorption with the protein used for conjugation. Increased specificity of antisera may also be achieved by absorption with hormones or sub-units of hormones that cross-react with the one under study. LH, FSH and TSH all contain an α -subunit which has a common structure, hence most antisera raised against these hormones have a certain degree of cross-reactivity. It must also be mentioned here in the context of antiserum specificity that many components of biological fluids, unrelated to the antigen being measured, can interfere with the antigen-antibody reaction and must be considered in establishing any RIA.

2. Labelling of antigen

All RIA require a pure form of labelled antigen although an 'immunoradiometric' assay method has been described which uses radio-iodinated antibody eluted from its antigen (Miles & Hales, 1969).

The limit of detection of any RIA is a function of the specific activity of the immunoreactive labelled hormone (Ekins & Newman, 1970). Many tritiated and ¹⁴C-labelled non-protein hormones and compounds are now commercially available at a high enough specific activity for use in RIA.

For the measurement of protein and polypeptide hormones, however, radio-iodination, using either ¹³¹I or ¹²⁵I, is a prerequisite step. The chloramine-T method of Greenwood, Hunter & Glover (1963) was the method of choice until more recently an enzymic method using lactoperoxidase and hydrogen peroxide (Marchalonis, 1969) has been found to cause less structural damage to a number of polypeptide hormones (Miyachi & Chrambach, 1972). Purification of iodinated protein hormones is essential for achieving maximal conditions of specificity and sensitivity in RIA although it is worthwhile mentioning that RIA do not require that labelled and unlabelled antigens be identical. A necessary condition for the validation of a RIA procedure is that the antigen concentration measured in an unknown sample be

independent of the dilution assayed. This requires immunochemical identity of standards and unknown. This can be tested for by assaying multiple dilutions of an unknown sample and determining whether the competitive inhibition curve is superimposable on the standard curve used for the assay.

3. *Separation techniques*

Various systems have been proposed for the separation of the free labelled antigen from the antigen-antibody complex. Yalow & Berson (1960) used an electrophoretic technique. The principle of this technique is that the free antigen remains at the point of origin while the antigen-antibody complex migrates with the γ -globulins. As RIA became more widely used, however, simpler techniques developed. Adsorption of free antigen to solid-phase material such as charcoal (Herbert, 1969), silicates (Rosselin, Assan, Yalow & Berson, 1966), and ion-exchange resins (Lazarus & Young, 1966) has been applied to the assay of many hormones. Precipitation of antigen-antibody complexes is also another widely used separation technique. In this procedure the antigen-antibody complex is precipitated by anti- γ -globulin antibodies and separated from the free antigen by filtration (Hales & Randle, 1963) or centrifugation (Morgan & Lazarow, 1962). This assay utilizing a second antibody for separation is referred to as the double-antibody method. The antigen-antibody complex may also be precipitated by a semi-saturated solution of ammonium sulphate or by various organic solvents such as alcohol and dioxane (see Hunter & Ganguli, 1971).

One of the most recently developed separation techniques for use in RIA has been the adsorption or complexing of antibody to solid-phase materials such as plastic tubes, dextran and glass. Catt & Tregear (1969) described a very simple technique employing polystyrene tubes. The inner surfaces of such tubes are coated with a layer of the specific antibody. The competitive reaction between unlabelled and labelled antigen is then allowed to take place in the tube and at the end of the reaction the contents are aspirated and the bound labelled antigen determined by counting the tube. Despite its simplicity and claimed reproducibility this method has yet to receive as wide attention as earlier separation techniques. Laboratories tend to develop and perfect one set of techniques for RIA and, providing accurate and reproducible results are being obtained, there is no need to change these conditions.

Application of RIA to ruminant endocrinology

The application of RIA to ruminant research is adding knowledge to our understanding of the various endocrine glands, and their activities and secretions. Their use as diagnostic tools for determining endocrine malfunctions or abnormalities has not yet become routine, as has happened in many clinical laboratories now diagnosing abnormal pituitaries, thyroids, gonads and pancreases from blood hormone assays. It would perhaps be surprising anyway if this situation ever arose with our domestic animals. Naturally their health and well-being is of utmost importance to us, but the human endocrine diseases are of little economic importance in ruminants.

The application of RIA to the assessment of nutritional deficiencies (i.e. vitamins) may well prove to be useful in the ruminant species but at present one of the fields where advances have been made since the introduction of RIA has been in reproductive endocrinology. Man has for decades been trying to control and maximize reproductive efficiency in the cow and sheep. With every new hormone or controlling step involved in reproduction that is discovered, efforts are renewed.

Since the adaptation of RIA to measuring ruminant reproductive hormones, an almost overwhelming volume of literature has been written on the subject. Because of the relatively small volume of plasma or serum needed for a RIA (0.1–1.0 ml), frequent sampling of any one animal has been possible and detailed patterns of hormonal changes during the oestrous cycle have emerged. LH levels in the ewe have been reported by, amongst others, Wheatley & Radford (1969) and Goding, Catt, Brown, Kaltenbach, Cumming & Mole (1969). Scaramuzzi, Caldwell & Moor (1970) reported on levels of LH and oestrogen during the oestrous cycle of the ewe; Bjersing, Hay, Kann, Moor, Naftolin, Scaramuzzi, Short & Younglai (1972) reported levels of gonadotrophins and ovarian steroids in sheep around oestrus and Crighton, Foster, Holland & Jeffcoate (1973) reported levels of LH and LH-releasing factor (LH-RH) in jugular venous blood of the sheep at oestrus. FSH levels have been measured by L'Hermite, Niswender, Reichert & Midgley (1972) and progesterone levels most recently by McNatty, Revfeim & Young (1973). McCracken, Carlson, Glew, Goding, Baird, Green & Samuelsson (1972) have also reported on the involvement of prostaglandin $F_{2\alpha}$ as the luteolytic hormone in the sheep.

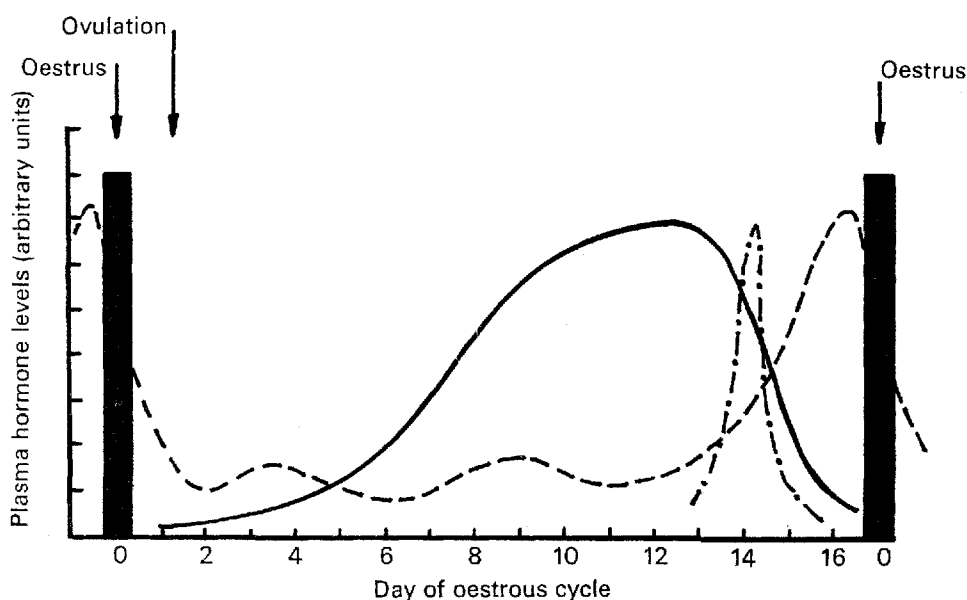


Fig. 1. Diagrammatic representation of the hormonal changes associated with the oestrous cycle of the sheep: —, progesterone; - - -, oestrogen; - · - ·, prostaglandin $F_{2\alpha}$; ■, luteinizing hormone.

From these studies a composite picture of the hormonal changes during the oestrous cycle of the sheep can be made (see Fig. 1). The cycle is normally 16–19 d in length. The trigger for the hormonal events leading to ovulation appears to be the falling progesterone levels around day 15 of the cycle (day 0 = day of oestrus), coinciding with regression of the corpus luteum. This event is followed by a rise in oestrogen secretion from the ovary, which, through its positive feedback effect on the hypothalamus-pituitary, leads to the pre-ovulatory surge of LH. This surge of LH lasts for approximately 10 h and is closely associated with the behavioural signs of oestrus, which may last for up to 24 h. Ovulation normally occurs about 36 h after the LH peak. Maximum peripheral levels of LH are 200–300 ng/ml plasma, compared to basal levels of approximately 1 ng/ml. The smaller peaks of oestrogen seen during the luteal phase of the cycle are thought to be due to follicular development in the ovary.

It has long been recognised that the release of the gonadotrophic hormones from the anterior pituitary was under the control of the hypothalamus but it was not until an active porcine hypothalamic releasing hormone was isolated and characterized by Schally and co-workers (Matsuo, Baba, Nair, Arimura & Schally, 1971) and then synthesized (Geiger, König, Wissmann, Geisen & Enzmann, 1971) that detailed studies were possible. The decapeptide isolated by Schally was shown to release LH and FSH in a number of species (Schally, Arimura, Kastin, Matsuo, Baba, Redding, Nair, Debeljuk & White, 1971), although there is still debate as to whether one hypothalamic hormone controls both pituitary gonadotrophs (Bowers, Currie, Johansson & Folkers, 1973). With the availability of the decapeptide and RIA for all reproductive hormones, work began on the possible use of the compound in both clinical and veterinary fields. The possibility existed that the administration of the decapeptide (LH-RH, as it became designated) could trigger off the release of LH and therefore may induce oestrous cycles in non-cycling or anoestrous animals.

Initial *in vitro* work with ovine pituitary tissue (Crighton, Hartley & Lamming, 1973) showed that LH-RH was capable of releasing LH and FSH. *In vivo* studies in sheep (Symons, Cunningham & Saba, 1973, 1974) also showed that administration of LH-RH was capable of releasing LH and FSH but behavioural signs of oestrus were not induced. However, the fact that RIA have been developed and applied to all the work so far mentioned perhaps indicates how rapidly results are being obtained in reproductive endocrinological research.

As can be seen from Fig. 1, most of the hormonal changes associated with the oestrous cycle of the sheep are short-lived, needing frequent sampling to detect them. The one exception to this is the change in progesterone levels throughout the cycle. The application of RIA to the measurement of progesterone levels every couple of days in the plasma of post-partum cows could, if performed efficiently, lead to endocrine profiles being quickly available. This may help in distinguishing sub-fertile animals and could also give a more accurate indication of when the next oestrous was expected. Routine applications such as this, however, could be costly and would need to be based on sound correlations between say, infertility and endocrine profiles.

Perhaps a more useful and simpler application of progesterone determinations in the ruminants is for pregnancy diagnosis (Shemesh, Ayalon & Lindner, 1973). In theory, a progesterone assay performed one cycle length after mating or insemination should show high levels of progesterone (above 2 ng/ml plasma) if the animal is pregnant and an active corpus luteum is being maintained. If the animal is not pregnant, progesterone levels will have fallen to a nadir one cycle length later. Preliminary results using this test are encouraging, and the finding by Heap, Gwyn, Laing & Walters (1973) that progestagens can be measured by RIA in bovine milk samples makes the method even more attractive.

It may therefore be said, in conclusion, that radioimmunoassays have contributed and are contributing extensively to our knowledge of ruminant endocrinology but, as yet, their direct application to problems of economic importance remains limited.

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