An enzyme-linked immunosorbent assay (ELISA) for bovine LH capable of monitoring fluctuations in baseline concentrations

W. G. Abdul-Ahad and J. P. Gosling

Department of Biochemistry, University College Galway, Ireland

Summary. Using a Fab'–horseradish peroxidase conjugate, an ELISA for bovine LH was developed and validated. It had good specificity ($\leq 0.2\%$ cross-reactivity with FSH or TSH), was not susceptible to interference from plasma components and exhibited very low non-specific binding. It was carried out on microtitre plates and had incubation times totalling 4 or 18 h, not including the initial antibody coating step. The longer incubations gave a lower detection limit (10 pg, 300 amol) and were used when fluctuations in baseline concentrations were being monitored. The values determined were independent of the plasma volume employed, standard added to plasma samples was accurately determined and the results obtained from the analysis of plasma samples correlated closely with those obtained by radioimmunoassay. The assay was applied to the analysis of plasma samples taken from heifers with normal ovarian activity or treated in a number of ways, and in every case the results obtained were similar to those determined by radioimmunoassay and reported in the literature.

Introduction

The development of specific radioimmunoassays for LH in the peripheral circulation of cattle (Niswender *et al.*, 1969; Schams & Karg, 1969) has played a major role in the development of bovine reproductive physiology. These assays have been refined to make them capable not only of detecting the preovulatory release of LH (20–60 ng/ml) but also of monitoring baseline concentrations of LH which fluctuate regularly (0.1–5 ng/ml) in response to the pulsed release of gonadotrophin-releasing hormone from the hypothalamus (Rahe *et al.*, 1980). In addition, the measurement of LH concentrations has proved valuable in investigations of a wide range of normal, treatment-induced and pathogenic phenomena in female and male cattle.

However, the monitoring of bovine LH is still confined to larger laboratories with automatic gamma-counting equipment and facilities suitable for the radioiodination of proteins. In addition, the relatively short half-life of ^{125}I means that labelling must be carried out every month or so, which also mitigates against smaller facilities and can greatly increase costs, especially when small numbers of samples must be assayed.

We have previously reported an ELISA for bovine LH (Abdul-Ahad & Gosling, 1985) but that assay, which used whole antibody conjugated to horseradish peroxidase (HRP), was limited by high non-specific binding of conjugate to the solid phase, particularly in the presence of large concentrations of plasma proteins. We wish to report here the development of a convenient, high performance ELISA for bovine LH which overcomes all the above disadvantages with RIA and ELISA and should help to bring the ability to monitor LH in the peripheral circulation to smaller research facilities, even in underdeveloped regions.

Materials and Methods

Preparation of bovine anti-LH. Antigen (100 µg, EHC-bLH-1 or an uncharacterized bovine LH β preparation, both gifts from G. Hennen and J. Closset) was dissolved in 0.5 ml saline (0.9% (w/v) NaCl), emulsified with 1.5 ml Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) and injected intramuscularly (i.m.) at four sites, or intradermally (i.d.) or subcutaneously (s.c.) at multiple dorsal sites on each of 5 New Zealand White rabbits. After 13 and 15 weeks, each animal was boosted by injection in the same way with 50 µg LH in Freund's incomplete adjuvant. After 17 weeks the animals were bled out and the antisera were characterized with respect to titre, affinity and specificity using radioimmunoassay methods (Table 1); a suitable serum was selected (Rabbit 226) for further use.

Purification of IgG and preparation of conjugate. Anti-LH serum was passed through a Protein A Sepharose-4B CL column (Pharmacia, S-751 82, Uppsala, Sweden), the column was washed with 0.1 M-phosphate buffer pH 7.0, adsorbed IgG was eluted with 0.1 M-glycine-HCl buffer, pH 2.5, and the eluted fractions were neutralized with 0.5 M-Tris-HCl buffer, pH 8.0.

The following procedures for the preparation of Fab' and its conjugation to horseradish peroxidase were adapted from Ishikawa *et al.* (1983). The F(ab)₂ portion of the IgG was prepared by digestion with pepsin as follows: 40 mg IgG in 0.1 M-sodium acetate buffer pH 4.5 at 4°C were incubated with 0.8 mg pepsin (porcine stomach mucosa; Sigma, Poole, Dorset, U.K.) at 37°C for 15 h. After adjusting the pH to 8.0 the digest was applied to a Sephacryl S-200 column (Pharmacia; 1 × 85 cm) and eluted using 0.1 M-borate buffer, pH 8.0. The fractions containing the F(ab)₂ fragments were pooled and Fab' was prepared by reduction with 10 mM-2-mercaptoethylamine at pH 6.0. The number of thiol groups generated per Fab' molecule was about one.

Enzyme conjugated to Fab' was prepared as follows. One mg 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC, Pierce Chemical Co. Ltd, Cambridge, U.K.) in 30 µl N,N-dimethyl formamide was incubated with horseradish peroxidase (2 mg Type VI; Sigma) in 0.3 ml 0.1 M-phosphate buffer pH 6.0 at 30°C for 60 min. Unreacted SMCC was removed by gel filtration on a Sephadex G-25 column (Pharmacia) and the average number of maleimide groups introduced per peroxidase molecule was determined to be about 1.2. Then 1 mg of the maleimide-peroxidase was incubated with 1.5 mg Fab' in 0.4 ml 0.1 M-sodium phosphate, 5 mM-EDTA, pH 6.0 at 4°C for 20 h and the enzyme-Fab' conjugate was separated by chromatography on Sephacryl S-200 (Pharmacia; 1 × 85 cm column) using 0.1 M-phosphate buffer pH 6.5. Retention of enzymic and immunological activity (determined using ¹²⁵I-labelled bovine LH, see below) were monitored at every step and both were found to be about 100%. Conjugates prepared in this way were found to be stable for at least 1 year when stored in 1% bovine serum albumin (98–99%, cat. no. 7030, Sigma) in PBS at 20°C.

Radioimmunoassay. Bovine LH (LER-1716-2) was iodinated (Fraker & Speck, 1978) using iodogen (Sigma) and label with a specific activity of about 70 µCi/µg was obtained. The assay procedure was standard (Hanrahan *et al.*, 1977; 1:450 000 final dilution of antiserum from Rabbit 226), with label (15 000 c.p.m.) added after 24 h and separation of bound from free label carried out 24 h later. Separation was performed using protein A (insoluble, type II, Sigma), with centrifugation after incubation for 1 h, and the precipitate was counted in an automatic gamma counter (detection limit 125 pg/tube; ED-50 0.38 ng/tube; for specificity see Table 1). The performance of this assay was not optimized.

Enzyme-linked immunosorbent assay. The ELISA was carried out as follows. The wells of microtitre plates (Nunc Immunoplate 1 with certificate, DK 4000 Roskilde, Denmark) were coated with antibody by adding 200 µl of anti-LH IgG (1 µg/ml) in 0.05 M carbonate buffer, pH 9.6, incubating for 3 h at 37°C, emptying by inversion (with tapping on absorbent paper to drain) and washing the wells three times with 350 µl 0.01 M-phosphate-buffered saline (PBS) containing 0.05% Tween 20, pH 7.0. Standard LH (EHC-bLH-1, 150 µl in PBS with 0.1% bovine serum albumin [PBS/BSA]) or controls or samples (usually 15 µl of plasma diluted to 150 µl with PBS/BSA) were added to the wells

Table 1. Characterization of antisera prepared from blood taken from rabbits 17 weeks after initial immunization

Rabbit no.	Injection route	Hormone preparation	RIA titre ($\times 10^{-3}$)†	Affinity ($K_A \times 10^{-10}$ M)‡	% Cross-reactivity (RIA)§				
					bLH	bFSH	oLH	oFSH	oTSH
225	s.c.	EHC-bLH-1	1/182	3.57	100	0.7	178	4.7	12.5
226	i.m.	EHC-bLH-1	1/326	3.63	100	0.9	230	4.8	9.1
227	i.d.	EHC-bLH-1	1/115	2.22	100	0.5	177	3.5	8.1
228	s.c.	'bLH β '*	1/460	2.85	100	0.9	158	3.6	11.2
80	s.c.	'bLH β '*	1/269	1.66	100	0.8	250	7.8	15.8

*An uncharacterized sample of the β subunit of purified bovine LH.

†The antiserum dilution giving 50% binding of 15 000 c.p.m. of ¹²⁵I-labelled LH.

‡Determined by Scatchard analysis and, when the plots were concave upward, represents the higher affinity component.

§Fitting was 'by eye'.

§Bovine (b) or ovine (o) hormones and were the same preparations as those in Table 2.

and incubated for 90 min at 37°C or 4 h at room temperature. When samples containing very low concentrations of LH were assayed, samples of plasma up to 75 µl were assayed. Equal volumes of plasma, which had been stripped of endogenous LH by incubation with anti-LH IgG immobilized on Sepharose-4B, were also included in the standard solutions. After emptying and washing, 150 µl Fab'-HRP conjugate (17 ng/well) were added to each well and incubated for 90 min at 37°C or overnight at 4°C before the wells were emptied and washed as described above. The bound enzyme activity was assayed by addition of 150 µl substrate solution (1.5 mg *o*-phenylenediamine/ml and 4.4 mmol hydrogen peroxide in 0.05 M-phosphate-citrate buffer pH 5.0) and incubation at room temperature for 30 min. The reactions were stopped using 50 µl 4 M-sulphuric acid and the colour was measured at 490 nm. The plate reader (BioTek EL307B) was connected on line to a microcomputer (BBC model B, Acorn Computers Ltd, Cambridge, U.K.) and the LH concentrations were calculated using software (MELISA package written by J.P.G.) based on the 4-parameter fit of Rodbard *et al.* (1978).

For high precision analyses, including all of the data presented here, the outer wells of the microtitre plates were not used, because of the presence of systematic differences in the readings obtained in wells across plates which were tested. These differences were greatest at the edges and have been reported previously (Kricka *et al.*, 1980; Cleere *et al.*, 1985). Their usual effect is to reduce the accuracy of assay results.

The throughput using this assay was 600 samples assayed in duplicate and the results calculated per normal 5-day working week using the shorter incubation periods and including 9 standards and 3 controls in duplicate on each plate (only 60 wells used). The usage of antiserum was heavier than would be the case with an RIA but with the antiserum mainly employed in this assay (from Rabbit 226, RIA titre 1/326 000), 1 ml was sufficient to coat 40 000 plate wells (0.2 µg/well and 8 mg IgG obtained per ml). In addition, 1 ml antiserum processed to give Fab' and then to give enzyme conjugate yielded enough conjugate for about 40 000 wells. The overall productivity of 1 ml antiserum was, therefore, 20 000 wells.

We have prepared enzyme conjugates using antisera from Rabbits 225 and 226 and used them in combination with coated IgG from all of the antisera available (Table 1). The cross-reactivity using the conjugate with Rabbit 226 antiserum was <1% with bovine TSH or bovine FSH for all except antiserum from Rabbit 228 (1.3 and 1.1% respectively), and the 'sensitivities' of the standard curves obtained with suitable amounts of immobilized IgG were similar for all of the antisera.

Collection of blood samples. Blood samples were obtained from beef heifers, collected into heparinized or EDTA-containing evacuated tubes; the plasma was prepared immediately and stored frozen until assay. In the experiments with intensive sampling, blood samples were collected from indwelling jugular cannulae inserted 1 day previously. To monitor LH concentrations before ovulation, oestrus was synchronized by giving 8 µg cloprostenol (Estrumate: ICI, Macclesfield, U.K.) 36 h and 24 h before the start of sampling. The samples taken at 10-min intervals over 32 h from 4 normal heifers were provided by D. Ryan (Department of Agriculture, University College Dublin), and samples taken in connection with the administration of GnRH and ovariectomy were provided by J. M. Sreenan (An Foras Talúntais, Belclare, Co. Galway).

Results

Analytical and biological validation

Standard curve and precision profiles. Figure 1 shows the standard curve and the imprecision profile for the ELISA of LH. The non-specific binding has been subtracted from all points but was always less than an absorbance of 0.05, even with prolonged incubation periods and when up to 75 µl plasma were added per microtitre plate well. Antibody-enzyme conjugates prepared by other methods (two-step glutaraldehyde method or periodate method, both described by Ishikawa *et al.* (1983)) gave higher non-specific binding (about $\times 4$ and $\times 8$ respectively in the absence of plasma) and lower specific binding (unpublished results). The detection limit, as calculated from the error in the zero and the slope of the standard curve, was 10 pg (300 amol) per well. However, the range where the within-sample replicate CV was $\leq 10\%$ was 13.5–600 pg (0.45–20 fmol) per well. These performance parameters were determined when the assay was performed with longer incubation times (20 h total), but when LH concentrations in plasma were > 2 ng/ml shorter incubations (4 h total) gave good results, e.g. the standard range where the within-sample replicate CV was $\leq 10\%$ was 37.5–1200 pg (1.25–40 fmol) per well.

Specificity. Table 2 shows both the specificity of the antiserum as determined by RIA and the specificity of the ELISA which used the same antibodies, both to coat the plate wells and for the synthesis of the Fab'-HRP conjugate. The preparations of ovine FSH and ovine TSH used were, according to the NIAMDD, not purified to a stage sufficient for iodination and use as a ligand in RIA, and may, therefore, have contained significant traces of LH. The two-site ELISA displayed less cross-reactivity than the 'competitive' RIA in every case when the comparison was made.

Fig. 1. Composite standard curve (○) and precision profile (■) for LH ELISA carried out with incubations totalling 20 h. The standard curve was obtained by combining data from 10 curves carried out in duplicate. For the precision profile a range of plasma samples with previously determined LH concentrations were assayed 10 times in duplicate in different assay runs and the intra-assay coefficient of variation was calculated for each sample.

Table 2. Percentage cross-reactivity* of antiserum from Rabbit 226 when used in RIA or ELISA

Hormone	Species	Preparation	RIA	ELISA
Luteinizing hormone	Bovine	(EHC-bLH-1)	100	100
	Ovine	NIAMDD-0LH22	230	110
Follicle-stimulating hormone	Bovine	(G. Hennen)	0.9	0.1
	Ovine	NIAMDD-oFSH13	4.8	3.3
Thyroid-stimulating hormone	Bovine	NIAMDD-bTSH11	n.d.	0.2
	Ovine	NIAMDD-oTSH9	9.1	7.5
Chorionic gonadotrophin	Horse	(M. McNamara)	<0.01	<0.01

*Cross-reactivity is defined as: $100 \times \frac{(\text{mass of LH giving certain reading})}{(\text{mass of hormone giving same reading})}$ where the reading used was 50% displacement of bindable label (RIA) or an optical density of 0.5 (ELISA). Some of the RIA data are the same as in Table 1 and are shown again here to allow easy comparison.

Independence of volume and recovery. If ranges of dilutions of unknown samples are analysed a valid assay will give equivalent results for each dilution. Figure 2 shows that, for 3 plasma samples containing different concentrations of endogenous LH, the concentration measured was directly related to the volume of plasma added to the microtitre plate well. This indicates that for plasma volumes up to 15 μ l the standard curve was not influenced by plasma components and that over this volume range the readings obtained for both the standards and unknowns were parallel. To allow

Fig. 2. Demonstration that the amount of LH measured was independent of the volume assayed. Various amounts (1–15 μ l) of three plasma samples ($\circ$, $\bullet$, $\square$), previously shown to contain different amounts of LH, were diluted to 150 μ l and assayed.

Table 3. Recovery (ng/ml, % in parentheses) of LH added to plasma samples containing different concentrations of endogenous LH

Endogenous level	Amount of LH added (ng/ml)		
Overnight incubation	2.5	10	20
3.14	7.06(125)	14.1 (93.8)	22.8 (98.5)
11.6	13.8 (97.4)	19.3 (89.4)	28.3 (90.3)
40.7	45.8 (105)	55.9(110)	63.5(105)
3-h incubation	1.0	10	50
5.00	5.70 (95.0)	15.4 (98.0)	49.9 (90.7)
15.0	17.1 (106)	24.1 (96.6)	60.7 (92.3)
41.1	41.5 (97.2)	49.9 (96.5)	84.8 (92.8)

the assay of larger plasma volumes, the same volume of plasma stripped of endogenous LH was added to the standards and in this way volumes up to 75 μ l could be assayed accurately (see Table 3).

A valid assay will also measure accurately standard LH added to plasma samples and Table 2 gives results of an experiment to determine the recovery of three concentrations of standard added to 3 plasma samples containing different levels of endogenous LH, with overnight and short incubation periods. The recovery varied within reasonably narrow limits (89–125%; mean 98.8, s.d. 8.7).

Correlation with RIA. Figure 3 shows the results obtained when 56 samples were analysed by radioimmunoassay and by ELISA. The correlation coefficient was 0.98.

Within- and between-assay precision. Table 4 shows the between-assay coefficients of variation obtained for control plasma pools containing a wide range of LH concentrations. The within-assay variation for the assay with 20-h incubation periods is shown in Fig. 1.

Fig. 3. Correlation of LH concentrations determined by ELISA and by RIA for 56 plasma samples. The fitted line, determined by the unweighted least square method, had a slope of 0.95, an intercept of -2.3 ng/ml and a correlation coefficient of 0.98.

Table 4. Between-assay variation for control plasma samples: (A) lower level samples, 75 μ l assayed, total incubation periods 20 h, data obtained from 12 assays; (B) higher level samples, 15 μ l assayed, total incubation periods 4 h, data obtained from 12 assays

		Low	Medium	High
(A)	Mean LH (ng/ml)	0.47	2.29	5.14
	s.d.	0.05	0.15	0.31
	CV	10.4	6.59	6.0
(B)	Mean LH (ng/ml)	2.3	20.6	44.0
	s.d.	0.37	0.76	1.97
	CV	15.4	3.6	4.4

Clinical validation

The ELISA was applied to the determination of LH concentrations in plasma samples taken from heifers under a wide range of normal and experimental conditions.

Injection of GnRH. Blood samples were taken from normal heifers before and after they had been given injections of various amounts of synthetic GnRH. The maximum values of LH found were directly related to the amount of hormone given and were similar to those reported in the literature (see Fig. 4 and Webb *et al.*, 1977).

Ovariectomy. To examine the application of the ELISA to monitoring LH concentrations after removal of the feed-back inhibition by ovarian steroids on the pituitary, 3 heifers were ovariectomized. Figure 5 shows the LH concentrations in plasma samples taken over 22 days before and after treatment, and these patterns accord well with those reported in the literature (Butler *et al.*, 1983).

Mid-luteal phase fluctuations in LH concentrations. Blood samples were taken at 10-min intervals for 32 h from 2 normal heifers starting when they were at Day 10 of the ovarian cycle. The patterns of changes in LH concentration as determined by ELISA were similar in both animals and the results for one of them are shown in Fig. 6. This pattern is similar to those reported by Rahe *et al.* (1980).

Fig. 4. LH concentrations in a 2-year-old heifer injected with 40 µg GnRH.

Fig. 5. LH concentrations in 3 otherwise normal heifers (ear-tag numbers shown) before and after ovariectomy (arrows).

Fig. 6. Episodic release of LH during the luteal phase of the ovarian cycle. Blood samples were taken with the aid of an indwelling jugular cannula at 10-min intervals for 32 h from a normal heifer on Days 10 and 11 of the ovarian cycle.

Fig. 7. Episodic release and the preovulatory surge of LH during and after the follicular phase of the ovarian cycle in a heifer in which the cycle was regulated by injection of cloprostenol. The insert shows some of the same data with the ordinate axis expanded to show the pulsatile nature of the release before the main surge.

Preovulatory fluctuations in LH concentrations. Two heifers were treated to restart and synchronize their ovarian cycles and then subjected to intensive blood sampling for 32 h, from about 12 h before the preovulatory LH surges were expected. Analysis of the samples by ELISA showed that the majority of the LH surge was detected in the samples from one animal and the entire surge in the samples from the other (Fig. 7). The concentrations and periodicity for the fluctuations in baseline values and the concentrations and shape of the preovulatory surge corresponded closely to those reported by Rahe *et al.* (1980).

Discussion

The validation results presented above indicate that this rapid and convenient ELISA for LH has performance characteristics which are at least as good as those of the RIAs for LH which are in use at present. The standard curve, precision profile and data on within- and between-run precision indicate that the sensitivity and precision of the assay are satisfactory, while the data presented on specificity, ability to detect added standard and parallelism are consistent with the assay being capable of quantifying LH, without interference, in the presence of closely related hormones and plasma proteins. The results of the application of the assay in a wide range of clinical experiments are consistent with the assay being not just analytically valid but also clinically valid. Therefore, this ELISA for LH, which can be run in as little as 4 h from samples becoming available and which offers all the conveniences associated with the use of microtitre plates and associated equipment, is a valid alternative to the radioimmunoassays currently in use, which often take 4 days to run and many additional hours for the iodine-125 label to be counted with good precision. In co-operation with the Department of Agriculture, University College Dublin, this assay has already been applied to the analysis of samples from two field trials and the results will be published in due course.

On the negative side, this assay uses much more antiserum than radioimmunoassays, and the enzyme-antibody conjugate, while it has a much longer shelf-life than radioiodinated labels, is more complex to prepare. Therefore, of major relevance to the widespread use of assays such as

that described here is continuity of supply of suitable antisera for label preparation and coating of wells. In our experience raising polyclonal antibodies with good specificity and affinity and in adequate amounts was not difficult and required the investment of about 200 µg purified LH per rabbit to obtain antiserum sufficient for 800 000 plate wells (40 ml serum, 20 000 wells per ml; see 'Materials and Methods'). No LH is needed for labelling as in RIA, in which 5–10 µg LH must be iodinated every 4–6 weeks. However, the adoption of a suitable monoclonal antibody for well coating or labelling, or both, could be a good way to ensure long term continuity in the large scale operation of an ELISA for LH at a number of centres.

Moreover, the range of 'high performance' enzyme-linked immunoassays which can be carried out on microtitre plates and which have been validated for use with plasma and milk samples from domestic animals is growing (Munro & Stabenfeldt, 1984; Cleere *et al.*, 1985; Power *et al.*, 1985), and soon it will be feasible for a research group studying bovine reproductive physiology to analyse rapidly and conveniently a wide range of peptide and steroid hormones without the requirement for two automatic scintillation counters and laboratory facilities and practices essential for the use of radioisotopes.

This work was initially supported by a grant from An Foras Talúntais. We thank Professor P. F. Fottrell for help and advice; Mr D. Ryan and Dr J. M. Sreenan for samples; Professor G. Hennen, Dr J. Closset, Mr E. M. McNamara and NIAMDD for gifts of hormones; and D. Collins for skilled technical assistance.

References

- Abdul-Ahad, W.G. & Gosling, J.P. (1985) The development of an enzyme-linked immunosorbent assay for bovine luteinizing hormone. *Ir. J. med. Sci.* **154**, 284.
- Butler, W.R., Katz, L.S., Arriola, J., Milvae, R.A. & Foote, R.H. (1983) On the negative feedback regulation of gonadotropins in castrate and intact cattle with comparison of two FSH radioimmunoassays. *J. Anim. Sci.* **56**, 919–929.
- Cleere, W.F., Gosling, J.P., Morris, M.C., Charleton, M.F., Moloney, B.J., Sreenan, J.M. & Fottrell, P.F. (1985) A high performance, high throughput enzyme-immunoassay for the analysis of progesterone in plasma or milk. *Irish vet. J.* **39**, 6–14.
- Fraker, P.J. & Speck, J.C., Jr (1978) Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril. *Biochem. Biophys. Res. Commun.* **80**, 849–857.
- Hanrahan, J.P., Quirke, J.F. & Gosling, J.P. (1977) Genetic and non-genetic effects on plasma LH concentrations in lambs at 4 and 8 weeks of age. *J. Reprod. Fert.* **51**, 343–349.
- Ishikawa, E., Imagawa, M., Hashida, S., Yoshitake, S., Hamaguchi, Y. & Ueno, T. (1983) Enzyme-labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical staining. *J. Immunoassay* **4**, 209–327.
- Kricka, L.J., Carter, T.N.J., Burt, S.M., Kennedy, J.H., Holder, R.L., Halliday, M.O., Telford, M.E. & Wisdom, G.B. (1980) Variability in the adsorption properties of microtitre plates used as solid supports in enzyme immunoassay. *Clin. Chem.* **26**, 741–744.
- Munro, C. & Stabenfeldt, G. (1984) Development of a microtitre plate enzyme immunoassay for the determination of progesterone. *J. Endocr.* **101**, 41–49.
- Niswender, G.D., Reichert, L.E., Jr, Midgley, A. R., Jr & Nalbandov, A.V. (1969) Radioimmunoassay for bovine and ovine luteinizing hormone. *Endocrinology* **84**, 1166–1173.
- Power, M.J., Cleere, W.F., Gosling, J.P., Langley, O.J., Sreenan, J.M. & Fottrell, P.F. (1985) A direct, high throughput enzymeimmunoassay for oestrone sulphate in milk. *Irish vet. J.* **39**, 18–24, 1985.
- Rahe, C.H., Owens, R.E., Fleeger, J.L., Newton, H.J. & Harms, P.G. (1980) Pattern of plasma luteinizing hormone in the cyclic cow: dependence upon the period of the cycle. *Endocrinology* **107**, 498–503.
- Rodbard, D., Munson, P.J. & DeLean, A. (1978) Improved curve-fitting parallelism testing, characterization of sensitivity and specificity, validation, and optimisation for radioligand assays. In *Radioimmunoassay and Related Procedures in Medicine*, vol. 1, pp. 469–504. International Atomic Energy Agency, Vienna.
- Schams, D. & Karg, D. (1969) Radioimmunologische LH-Bestimmung im Blutserum von Rind unter besonderer Berücksichtigung des Brunstzyklus. *Acta endocr., Copenh.* **61**, 96–103.
- Webb, R., Lamming, G.E., Haynes, N.B., Hafs, H.D. & Manns, J.G. (1977) Response of cyclic and postpartum suckled cows to injections of synthetic LH-RH. *J. Reprod. Fert.* **50**, 203–210.

Received 21 January 1987