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A new homologous radioimmunoassay for ovine follicle stimulating hormone : development and characterization

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Summary. The development and characterization of an homologous radioimmunoassay for ovine FSH is described in detail. Two antisera obtained by immunization of rabbits either with « native » FSH or by periodate oxidized FSH have been compared. These two antisera were chosen for their low cross-reactions with plasma proteins in the presence of highly purified labelled FSH. After saturation with a purified ovine LH preparation, these antisera measured similar FSH activities in the plasma. Cross-reactions with pituitary proteins were low (1-2 p. 100 TSH, 0.02 p. 100 LH and lower for other pituitary hormones tested : GH, Prol. and ACTH) and probably result from FSH contamination of these preparations. With each antiserum, inhibition curves with ovine plasma, crude pituitary extracts or purified preparations of FSH were parallel. The total recovery of exogenous FSH added to plasma demonstrated that plasma proteins do not interfere with the assay. Activities of purified FSH preparations by bioassay or by radioimmunoassay were similar for some and different for others. This observation is discussed. Lastly, a study of the effect of incubation volumes on the precision and the sensitivity facilitated a complete automatisation of the pipetting phase of the assay. Under these conditions, the intra and inter-assay precision were 5.5 to 8 p. 100 and 17.5 to 19.7 p. 100 within the range of 25 to 70 p. 100 B/Bo and 46 to 60 p. 100 B/Bo respectively and the sensitivity was 2.4 ng NIH-FSH-S_3/ml plasma. These results suggest that the present assay, using either antiserum, can be used for specific quantitation of ovine FSH.

In the sheep, the lack of information on the mechanism of regulation of the secretion of follicle stimulating hormone (FSH) can be attributed to difficulties in assaying FSH in plasma and serum by radioimmunoassay (RIA). These difficulties can be classified into three main groups : the existence of cross-reactions with luteinizing hormone (LH) and thyroid stimulating hormone (TSH) (Kerdelhué *et al.*, 1972 ; Hopkinson and Pant, 1973 ; Cunningham and Hebert, 1973 ; Lincoln *et al.*, 1977), the poor antigenicity of ovine FSH (oFSH) even with highly purified preparations (L'Hermite *et al.*, 1972) and cross-reactions with plasma proteins in homologous systems (albumin or a related protein : Bailly du Bois *et al.*, 1970 ; Salamonsen *et al.*, 1973 ; orosomucoid : Dubois *et al.*, 1973). To circumvent the last problem, heterologous RIA's have been proposed using anti oFSH sera with labelled human FSH (hFSH) (L'Hermite *et al.*, 1972 ; Hopkinson and Pant, 1973) or labelled rat FSH (rFSH) (Kerdelhué *et al.*, 1972 ;

Lincoln *et al.*, 1977), anti hFSH sera with labelled oFSH or rFSH (Salamonsen *et al.*, 1973 ; McNeilly *et al.*, 1976) and anti rFSH sera with labelled oFSH (Dobson and Ward, 1977). Some of these heterologous RIA's are specific and accurate and can apparently be used to measure circulating oFSH levels. Also, satisfactory homologous oFSH RIA's have been described (Crim and Geschwind, 1972 ; Kragt and Cons, 1973). The present paper describes in detail the development and characterization of a highly sensitive and specific homologous RIA for oFSH using antisera raised against either « native FSH » or periodate oxidized FSH and its application in the measurement of plasma levels.

Material and methods.

Material.

Purified oFSH preparations (table 5) of varied biological activities were used. Ovine orosomucoid was prepared from plasma using the techniques of Weimer *et al.* (1950) and Weimer and Winzler (1955).

Iodination procedure.

Highly purified « native » oFSH preparations (CNRS FSH P67C, CNRS FSH P26 and HG FSH 225) were iodinated using a modification of the technique of Greenwood *et al.* (1963). Ten μl containing 10 μg FSH in phosphate buffer (0.5 M, pH 7.2) was added to 1 mCi (5 or 10 μl) $Na^{125}I$ (IMS 300, The Radiochemical Centre, Amersham, GB). Freshly prepared chloramine T (30 μg in 10 μl phosphate buffer 50 mM, pH 7.2) was allowed to react with this mixture for 10 sec. Sodium metabisulphite (50 μg in 10 μl) and potassium iodide (500 μg in 50 μl) dissolved in the same buffer were then added immediately. The total reaction volume was then transferred onto a Sephadex G 50 fine column (0.9 $\times$ 30 cm, diameter $\times$ length). Radioactivity was eluted with barbitone buffer A (barbitone : 25 mM, pH 8.5, human serum albumin (HSA) : 0.25 p. 100, sodium azide : 0.1 p. 100). The most radioactive fraction containing labelled FSH, which was also the most immunoreactive, was eventually purified on a Sephadex G 100 column (0.9 $\times$ 60 cm, diameter $\times$ length). If 5 or 2 μg FSH were iodinated, the concentrations of reagents were reduced proportionately to ensure a constant reaction volume.

For comparison purposes HG FSH 225 was also enzymatically iodinated and separated from free iodine as described by Ketelslegers and Catt (1974).

Specific radioactivity was calculated according to the ratio of the radioactivity not retained by the Sephadex G 50 to that of the total radioactivity eluted from this column.

Antisera.

An initial series of antisera (AS) were obtained on rabbits and guinea pigs using purified oFSH preparations (CNRS FSH P67C, CNRS FSH M 2) as previously described (Dubois *et al.*, 1973). A second series of antisera were raised in rabbits following the same immunization procedure but with oxidized oFSH : CNRS FSH P28a or CNRS

FSH M3 treated with potassium periodate (Kennedy and Butt, 1969) (1.5 mM KIO_4 per mg FSH, 4 °C, 16 hrs, darkness).

To determine the ability of the AS (39 from 13 rabbits and 36 from 11 guinea-pigs) to bind iodinated oFSH preparations, each serum (50 µl) was added in increasing dilutions from 1:1 000 to 1:512 000 (final) in duplicate tubes containing 400 µl of labelled FSH (10 000 cpm) and 50 µl barbitone buffer B (barbitone : 25 mM, pH 8.5, HSA : 0.25 p. 100) containing, if appropriate, a quantity of a purified FSH preparation. Dilutions of the AS were made in barbitone buffer B containing 1 p. 100 normal rabbit serum (final dilution) and 0.1 p. 100 sodium azide. The tubes were incubated 2 days at 4 °C. Separation of antibody bound and free hormone was achieved by addition of 150 µl of a second antibody solution (sheep γ-globulin anti rabbit γ-globulin, barbitone 25 mM, pH 8.5, calcium chloride — 0,54 M — 1:3:8 v/v) and overnight incubation at 4 °C. One point eight ml buffer B without HSA was then added to the tubes which were then centrifuged at 4 000 g, 20 °C for 25 min. The supernatant was discarded and bound ^{125}I-FSH was counted in a well-type gamma spectrophotometer counter (Packard or LKB-Rackgamma 1270).

Assay procedure.

All dilutions were made in buffer B. For assays, polystyrene tubes (11 × 55 mm, Labo Expres Service, France) were used. Three volume systems were tested. In the first, 50 µl appropriately diluted AS (1:60 000 final dilution for AS 4 P_3 and AS 5 BB) and 100 µl standard oFSH or unknown sample were added to 50 µl buffer B in each tube. The quadriplicate (standard) or duplicate (unknown) samples were then incubated for 4 days at 4 °C. Three hundred µl of labelled FSH (12 500 cpm) were added and tubes were incubated for a further 2 days. The second and third volume systems were designed with a view to an eventual use of an automatic sampling apparatus (LKB Ultro RIA ^{R}2071 sample processor) : 300 µl of diluted AS were added to 100 µl standard oFSH or unknown samples and incubated for 4 days at 4 °C. Labelled FSH (300 µl or 150 µl : second and third volume system respectively) was added and incubated as described above. The end volumes in the three systems (500, 550 or 700 µl) contained the same final dilution of AS, 0.1 p. 100 normal rabbit serum and 0.01 p. 100 sodium azide added with the AS aliquot.

Separation of antibody-bound and free hormone was achieved as mentioned above.

Validation of the assay.

The pituitary hormone preparations used to assess the specificity of the FSH assays were the following : CNRS LH M_3 (1.8 times NIH LH S_1), NIH TSH S_2 (1), TSHb (Dr. John Pierce), NIH GH S_6, NIH Prol. S_6, ACTH (Byla). All these preparations were of ovine origin except TSH b (bovine) and ACTH (porcine). Specificity with plasma proteins was examined using either hypophysectomized ewe plasma or ovine orosomucoid at a concentration (2 mg/ml) known to exist in the plasma (Putnam, 1965). Antiserum saturated with oLH were prepared just before use in the assay

(1) This preparation contains 25 to 30 p. 100 of biologically inactive LH.

by incubation (90 min., 37 °C) of the AS with appropriate quantities (0.5 to 2 mg/ml pure AS) of CNRS LH M_3. No precipitate was observed.

Parallelism between the dose-response curve for oFSH standards diluted in buffer and dilutions of ovine pituitary extracts or serum samples containing various levels of endogenous oFSH was assessed. Recovery was measured by comparison between known amounts of standard FSH added to a plasma sample and amounts assayed. The precision measured at different levels of B/Bo ([1]) and expressed as the intra- or inter-assay coefficient of variation of the concentrations (ng/ml) was assessed by repeated measures of a series of sheep plasma samples during different assays. Sensitivity, « defined as the smallest amount of unlabelled hormone which can be distinguished from no hormone » (Midgley *et al.*, 1969) was calculated on the basis of the mean standard curve (obtained by averaging those obtained in various assays) and compared in two assay systems.

Calculation of hormone concentration in a sample.

Two methods of calculation have been successively used : the first one made use of logit B/Bo-log dose transformation, while the second used a B/Bo-log dose plot between each segment of two doses of the standard as proposed in the RIA 01 S8B program (program obtained from LKB including corrections for radioactivity decay).

FSH *plasma levels in the sheep.*

Two cryptorchid rams were blood sampled every 15 min. for 25 hrs. They received 40 ml of HSA in 2 injections, 20 ml at the 5th and 20 ml at the 6th hour after the beginning of sampling as described elsewhere (Blanc *et al.*, 1978). LH and FSH plasma levels were measured (Pelletier *et al.*, 1968 and this paper, respectively) in each sample and the correlation between each FSH and LH value in the same sample was calculated to clarify any cross-reaction by LH in the FSH assay.

Throughout a complete œstrous cycle, one ewe was blood sampled once a day initially then every two hours from the beginning of the behavioural œstrous for 36 hrs. FSH and LH were measured in the plasma samples.

Results.

Iodination. — The mean specific activity observed was 76.7 $\pm$ 1.8 (mean $\pm$ s.e.m., n = 16) μCi/μg for CNRS FSH P26 and 77.4 $\pm$ 1.1 (n = 6) μCi/μg for HG FSH 225. A higher chloramine T concentration or a longer reaction time did not increase the iodination yield which, in the conditions used, seemed to be limited by the molar ratio of iodide to hormone. Under the labelling conditions chosen, the binding activity of labelled HG FSH 225 to ovine or porcine testis cell membranes was preserved (respectively 9.3 and 13.6 p. 100 specific binding of total labelled FSH added with receptor excess) (Combarnous and Peyrat, personnal communication).

([1]) B = amount of labelled hormone bound to AS in the presence of unlabelled hormone expressed as cpm/tube ; Bo = amount of labelled hormone bound to AS in the absence of unlabelled hormone expressed as cpm/tube.

Iodination by lactoperoxidase rather than chloramine T did not influence the characteristics of the assay (specificity or sensitivity).

Antisera against native and oxidized FSH. — Of the 7 rabbits and 11 guinea pigs immunized with a native FSH preparation, only one animal (rabbit 4) responded with a titer (Bo/T) higher than 1:8 000 (final dilution) in the presence of labelled CNRS FSH P26. However, of the 6 rabbits immunized with oxidized FSH, 5 responded with a titer higher than 1:8 000. Rabbit 5 was one of these 5.

Role of antisera and labelled FSH on the plasma protein specificity of the assay. — The respective roles of AS and purification grade of the labelled FSH in plasma protein specificity were analyzed under identical experimental conditions (fig. 1). AS 1 (fig. 1*a*) had a high titer with labelled CNRS FSH P67C in the absence of orosomucoid

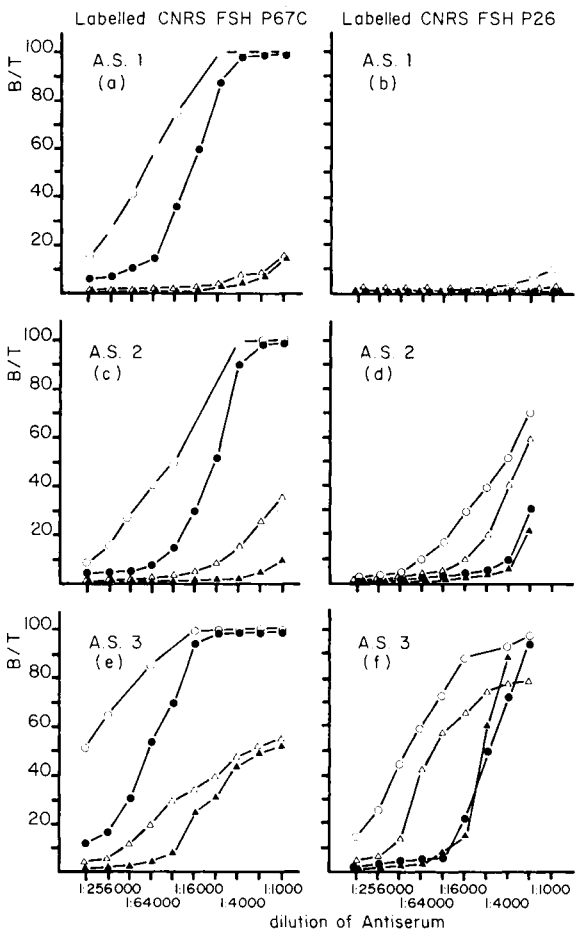

FIG. 1. — *Effect of* 3 *antisera* (AS 1, 2 or 3) *and* 2 *labelled FSH preparations* (CNRS P67C : a, c, e ; CNRS P26 : b, d, f) *on plasma protein specificity.* Without orosomucoid : AS alone (o——o), AS + 6 ng CNRS FSH M_3 per tube (●——●) ; with orosomucoid (100 µg per tube) : AS alone (△——△), AS + 6 ng CNRS FSH M_3 per tube (▲——▲). Final dilutions of antiserum are quoted.

but a very low one in presence of it. In contast, the titer was low with labelled CNRS FSH P26, whatever the conditions (fig. 1*b*). The titer of AS 3 (fig. 1e) with labelled CNRS FSH P67C was elevated (Bo/T [1] = 50 p. 100 for 1:512 000 final dilution) but a cross-reaction still existed with orosomucoid although less than with AS 1. The titer of AS 3 was lower with CNRS FSH P26 (Bo/T = 50 p. 100 for 1:90 000 final dilution, fig. 1*f*) than with CNRS FSH P67C, but the cross-reaction with orosomucoid was considerably lower. The curves obtained with unlabelled FSH were almost identical with and without orosomucoid. The behaviour of AS 2 with the two labelled preperations (fig. 1*c* and *d*) was intermediate between AS 1 and AS 3.

AS 4 P_3 and AS 5 BB were chosen : 1) for their low cross-reactions (B/Bo = 10 and 5 p. 100 respectively) with orosomucoid or hypophysectomized ewe plasma and oLH when used at the appropriate dilution in the presence of labelled HG FSH 225 or CNRS FSH P26, 2) for the high sensitivity that was obtained.

Specificity and pituitary hormones. — The only problem of specificy observed was with LH and TSH. All of the AS prepared cross-reacted with LH and TSH, although to differing degrees. Low doses of LH had a similar effect to those of FSH (fig. 2*a*) : the upper part of the standard curve (B/Bo $\geqslant$ 40 p. 100) was not specific for FSH. After saturation of the same AS with LH (fig. 2*b*) the cross-reaction observed (0.02 p. 100)

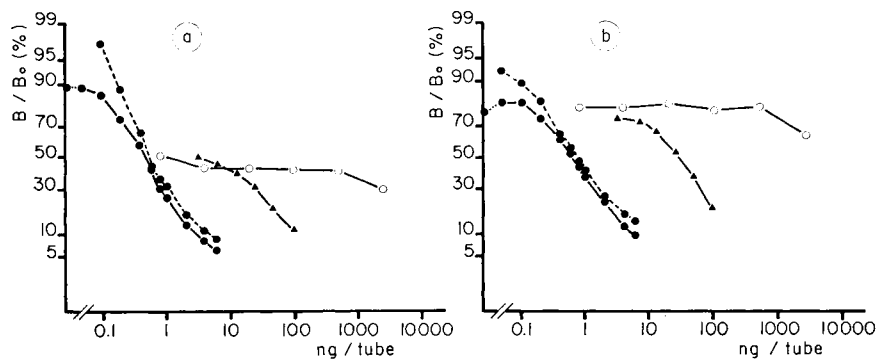

FIG. 2. — *Specificity of the RIA for LH and TSH using labelled CNRS FSH P26 and AS 4 P_3 before (a) or after saturation by LH (b).* Displacement observed with CNRS FSH M_3 (●), CNRS LH M_3 (○) or NIH TSH S_2 (▲), without (- - -) and with (——) hypophysectomized ewe plasma (50 µl per tube). A log-logit plot was used. Bo/T was 54.7 p. 100 for (*a*) and 28.3 p. 100 for (*b*).

was compatible with FSH contaminant in LH. Furthermore no effect was observed with 0.8 to 500 ng LH (B/Bo > 98 p. 100). Cross-reactions with pituitary hormones are listed in table 1. The absence of a noticeable cross-reaction with TSH (either NIH TSH S_2 or TSHb) after saturation of the AS by LH (1-2 p. 100) was confirmed by the lack of a rise in « FSH-like » activity in the plasma of three ewes blood sampled after thyroidectomy [2].

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[1] T = total labelled hormone added to each tube expressed as cpm per tube.

[2] According to NIH specifications NIH TSH S_2 contains 1.6 p. 100 FSH as measured by the ovarian augmentation assay.

With AS 5 BB, a partial cross-reaction with LH was similarly eliminated by a saturation pre-treatment. The cross reaction with LH was then negligeable (0.02 p. 100).

TABLE 1

Cross-reactions with pituitary hormones in the RIA *using* LH *saturated* AS 4 P_3 *and labelled* CNRS FSH P26 *expressed as weight for weight. Standard* FSH *is* CNRS FSH P26

Purified hormone preparation	Cross-reaction (p. 100)
CNRS LH M_3	0.02
NIH TSH S_2	2
TSH b	1
NIH Prol S_6	ND (1)
NIH GH S_6	0.06
ACTH (Byla)	ND (2)

(1) Not detected in 2 500 ng ; (2) Not detected in 50 ng.

Parallelism. — Specificity was assessed by analyzing parallelism between standard curves and those obtained by dilution of an ovine plasma sample (table 2). The concentrations in ng/ml obtained from various volumes were not significantly different. Furthermore, the curves obtained using dilutions of standard preparations (CNRS FSH P26, and HG FSH 225) were parallel with those obtained by dilution of crude ovine pituitary extracts or ovine plasma. Between 70 and 50 p. 100 B/Bo values (table 3), the recovery was demonstrated to be within the precision of the assay.

TABLE 2

Endogenous FSH *plasma concentrations measured in different volumes of the same sample. The* RIA *used* LH *satured* AS 5 BB *and labelled* HG FSH 225

Volume of sample (µl)	Amount measured per tube (1)	Concentration (ng) per ml plasma (1)	Corresponding B/Bo (p. 100)
20	1.052 ± 0.007	52.6 ± 0.4	17.6
10	0.492 ± 0.038	49.2 ± 3.8	34.0
5	0.236 ± 0.004	47.2 ± 0.9	53.9
2.5	0.120 ± 0.004	47.9 ± 1.5	71.6
1.25	0.068 ± 0.008	54.2 ± 6.1	82.8
0.625	0.037 ± 0.007	58.4 ± 11.0	90.6
		$\bar{x}$ = 51.6 ± 2.1 (2)	

(1) Results are expressed as ng HG FSH 225, mean ± s. e. m. (n = 4) ; (2) Mean ± s. e. m. (n = 24).

Accuracy. — Accuracy was analyzed by comparing the measurements of 50 different samples collected from two ram lambs and assayed by the two assay systems described previously (AS 5 BB vs AS 4 P_3 with hypophysectomized plasma added to

the standard curve). Linear regression analysis revealed a highly significant correlation coefficient : $r = 0.91$, $P < 0.001$. The slope was 1.7 ± 0.1, y intercept was 0.1. The first assay gave rise to slightly higher values than the second one.

TABLE 3

Recovery by radioimmunoassay of various amounts of a highly purified ovine FSH *added to* 50 µl *plasma samples of an intact ram. The* RIA *used* LH *saturated* AS 5 BB *and labelled* HG FSH 225

Ovine FSH added (1)	Ovine FSH recovered (1) (2)	Total amount calculated (3)	Corresponding B/Bo (p. 100)
0	127.0 ± 7.2	—	70
25	161.5 ± 2.2	152	68
50	172.2 ± 8.6	177	66
100	222.7 ± 9.5	227	60
200	331.0 ± 17.8	327	50

(1) Expressed as pg HG FSH 225 per tube ; (2) Mean ± s. e. m. (n = 4) ; (3) Calculated as amount measured with no FSH added + amount FSH added.

Sensitivity. — To take into account variability between assays, sensitivity was calculated from standard curves obtained in various assays repeated with the two assay systems mentioned above. For each dose (n = 8, range 25 to 2 500 pg) of the same standard preparation (HG FSH 225), the mean B/Bo was calculated. For the two assay systems, the logit B/Bo log. dose linear regression equation was $Y = -(2.42 \pm 0.04) X + 5.90$ (n = 14 assays, r = 0.999) and $Y = -(2.49 \pm 0.11) X + 6.72$ (n = 4 assays, r = 0.999) for AS 5 BB and AS 4 P_3 respectively (fig. 3).

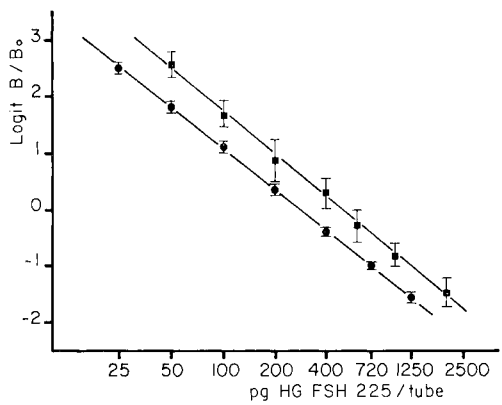

FIG. 3. — *Mean standard curves obtained from various assays using HG FSH* 225 *as standard and labelled hormone and LH saturated anti « native » FSH* (AS 4 P_3 : ■——■, n = 4 assays) *or LH saturated anti-oxidized* FSH (AS 5 BB : ●——●, n = 14 assays). Each point represents the logit of the mean values of the B/Bo observed in the different assays. Error bars represent s.e.m.

Twenty five pg HG FSH 225 per tube (250 pg HG FSH 225/ml or 3.5 ng NIH FSH S_3/ml) and 50 pg of the same standard in the two systems were different from 0 ($P < 0.001$) (B/Bo = 92 p. 100 in each case). In the most sensitive system Y = Log 95 p. 100 was equivalent to X = 17 pg HG FSH 225 per tube i.e. 2.4 ng NIH FSH S_3/ml.

Effect of incubation volumes. — The use of a higher incubation volume (200 + 300 µl, volume system 1 ; 400 + 300 µl, volume system 2 ; 400 + 150 µl, volume system 3) resulted in a parallel standard curve but with a decrease in sensitivity (respectively 22 and 22 vs 32 ng/ml). Variability at different levels of B/Bo was lower with volume system 3 than with 1 or 2 (table 4).

TABLE 4

FSH *measurements in 3 plasma samples assayed with different final volumes in the same assay system* (AS 5 BB-labelled HG FSH 225)

Final volumes (1)		Plasma sample 1 (50 µl) n = 30	2 (100 µl) n = 50	3 (50 µl) n = 20
« 200 + 300 » µl (2)	Mean ± s. e. m. (4).....	1.71 ± 0.04	1.43 ± 0.02	9.5 ± 0.22
	CV (p. 100).....	12.3	8.3	10.1
	B/Bo (p. 100)	76	65	25
« 400 + 300 » µl (3)	Mean ± s. e. m. (4)	1.99 ± 0.05	1.48 ± 0.07	11.1 ± 0.19
	CV (p. 100).....	10.8	32.0	7.8
	B/Bo (p. 100)	83	72	35
« 400 + 150 » µl (3)	Mean ± s. e. m. (4)	2.40 ± 0.03	1.69 ± 0.02	11.3 ± 0.15
	CV (p. 100).....	7.6	8.0	6.1
	Mean ± s. e. m. (5)	2.55 ± 0.04	1.78 ± 0.02	12.4 ± 0.15
	CV (p. 100).....	8.0	7.0	5.5
	B/Bo (p. 100)	70	60	25

(1) Final concentration of the 1st AS is identical in the final volume (500, 700 and 550 µl).

(2) Samples were manually pipetted (Eppendorf pipette).

(3) Samples were automatically pipetted (Ultro RIA(R) system 2 071 sample processor LKB). Sample (100 or 50 µl) was pipetted and « pushed » by 300 µl AS.

(4) Expressed as ng HG FSH 225 per ml plasma. Calculated from logit-log. plot.

(5) Expressed as ng HG FSH 225 per ml plasma. Calculated from B/Bo-log. plot (see text for detail).

Precision. — Three plasma samples resulting in 3 different levels of B/Bo were repeatedly pipetted using the different volume systems (table 4). With volume system 3, the coefficient of variation (CV) varied from 6.1 to 8 p. 100 within the range of 25 to 70 p. 100 B/Bo (n = 20 to 50) and was lower than the CV obtained with the other volume systems (8.3 to 12.3 and 7.8 to 32 with volume systems 1 and 2 respectively). However when the same samples were measured in different assays (n = 7 to 8)

the CV's were 19.7 and 17.5 p. 100 for 2.75 and 4.86 ng HG FSH 225 respectively (B/Bo averaging 60 and 46 p. 100 respectively).

Relationship between biological and immunological activity. — When various FSH preparations were tested in doubling dilutions, parallel standard curves were obtained. All these curves were also parallel with those obtained by dilution of crude ovine pituitary extracts or ovine plasma. An immunological activity ratio was calculated by comparison with NIH FSH S_3 (table 5). The immunological to biological activity ratio varied from 0.17 to 1.20. These ratios did not depend on the presence of hypophysectomized ewe plasma or orosomucoid in the incubation medium. The RIA activity ratio between two purified preparations within the same system varied with the system used (table 5) : for instance, between CNRS FSH P26 and NIH FSH S_3 it was 25 in the RIA using AS 4 P_3 *versus* 14 in the RIA using AS 5 BB.

TABLE 5

Relation between radioimmunological and biological activities (RIAa and Ba respectively) *of various purified* FSH *preparations*

	RIAa I (1)	Ba (2)	RIAa/Ba ratio	RIAa II (3)	RIAa III (4)
NIH FSH S_3	1.0	1.1	0.91	0.07	0.07
HG FSH 225	35.0	150	0.23	3.1	2.6
CNRS FSH P26	25.0	40	0.63	1.00	1.00
CNRS FSH M4	—	11.9	—	1.2	0.9
CNRS FSH M3	19.2	22.8	0.84	—	—
CNRS FSH P67E	15.0	19	0.79	—	—
CNRS FSH P65F1	9.3	18	0.52	—	—
CNRS FSH P67C	4.8	29	0.17	—	—
CNRS FSH P28a	3.8	7.9	0.48	—	—
CNRS FSH M2	3.3	6	0.55	—	—
CNRS FSH P15b	1.1	1.1	1.00	—	—
CNRS FSH P14a	0.8	1.0	0.80	—	—
CNRS FSH De 65	0.6	0.5	1.20	—	—

(1) RIA activity using LH saturated AS 4 P_3, labelled CNRS FSH P26 and NIH FSH S_3 as a standard.
(2) Biological activity (ovarian augmentation assay) using NIH FSH S_1 as a standard.
(3) and (4) RIA activity using LH saturated AS 5 BB, labelled CNRS FSH P26 (3) or labelled HG FSH 225 (4) and CNRS FSH P26 as a standard.

FSH plasma levels in the sheep. — In the cryptorchid rams (fig. 4), FSH plasma levels did not, incontrast to LH plasma levels, show marked variations throughout the day (CV's of the sampling period : 8 and 8.1 p. 100 for the 2 rams as compared to 7 p. 100 for CV of the assay). The correlation between FSH and LH in the same sample was not significantly different from 0 ($r = -0.11$ and 0.16, $P > 0.05$) for the two rams respectively.

During the œstrus cycle, the plasma concentrations of FSH underwent two major changes : an augmentation concomitant with the preovulatory LH surge and another

rise some 24 hrs later which took place when LH concentrations had returned to the basal level (fig. 5).

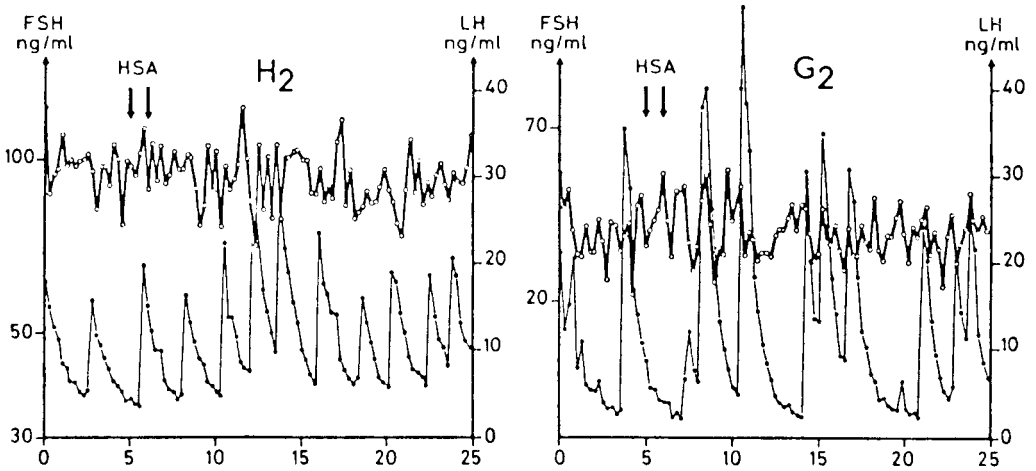

FIG. 4. — *Plasma levels of FSH* (o——o) *and LH* (●——●) *over a* 25 *hr period in* 2 *cryptorchid rams* (H_2 and G_2) *injected with human serum albumin* (HSA). The FSH RIA used LH saturated AS 4 P_3, labelled HG FSH 225 and CNRS FSH P26 as a standard.

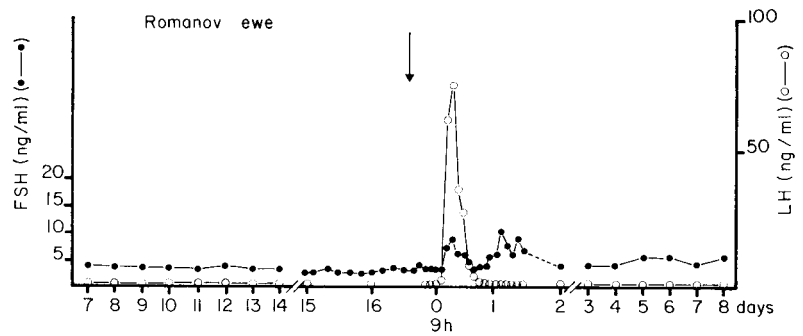

FIG. 5. — *Plasma levels of FSH* (●——●) *and LH* (o——o) *of one Romanov ewe during an œstrous cycle.* O is the day of the behavioural œstrous. The FSH RIA used LH saturated AS 5 BB, labelled HG FSH 225 and CNRS FSH P26 as a standard.

Discussion.

The present paper describes in detail an homologous assay for oFSH using either an anti-native FSH serum (AS 4 P_3) or an anti-oxidized FSH serum (AS 5 BB). Antisera raised against oxidized FSH can recognize the native antigen as shown by the high correlation coefficient between measurements of endogenous FSH using AS 4 P_3 and AS 5 BB. Furthermore, the fact that cyclic variations of FSH plasma levels in the ewe observed using AS 5 BB (this paper) or AS 4 P_3 (Bindon *et al.*, 1979) are similar

to those described by other authors (L'Hermite *et al.*, 1972 ; Salamonsen *et al.*, 1973 ; Hopkinson and Pant, 1973) points to the validity of this assay.

As previously described (L'Hermite *et al.*, 1972), native oFSH has been found to be a poor immunogen (1 rabbit responding among 7 rabbits and 11 guinea pigs immunized). In contrast, although the experimental protocol makes it difficult to say whether periodate treatment increase immunogenicity (see p. 2), it seems likely that this periodate oxidation has this effect on oFSH (5/6 immunized rabbits responding) probably through the modification of the carbohydrate moeity (Neuberger and Marshall, 1966 ; Kennedy and Butt, 1969). To our knowledge, such a phenomenon has never been described previously. However, desialilation of a sialoglycoprotein has been reported to augment the capability of the orosomucoid to raise antibodies which give the same arc of precipitate in double diffusion tests with native and asialoprotein (Athineos *et al.*, 1962). Although the phenomenon by which immunogenicity of the molecule is raised remains obscure, periodate oxidation could perhaps be used to obtain antibodies against other glycoproteins.

The two AS tested showed partial cross-reactions with LH which were completely suppressed by saturation of the AS with LH. After saturation low amounts of LH did not inhibit the binding of the AS to labelled FSH and the cross-reaction observed with higher quantities of LH (0.02 p. 100) can be accounted for by FSH contamination of this LH preparation. These results are very similar to those obtained by L'Hermite *et al.* (1972). However, in a number of assays (Kerdelhué *et al.*, 1972 ; Hopkinson and Pant, 1973 ; Cunningham and Hebert, 1973 ; Lincoln *et al.*, 1977) a significant cross-reaction with LH was found (8.5 to 17 p. 100). In spite of the low value of these figures, a cross-reaction with LH may be more serious if low amounts of LH are able to inhibit the binding of the labelled FSH with the antiserum. In such cases, the interference by LH would be high especially with low concentrations of FSH (e. g. intact rams) and the amount of LH in the assayed sample could not be taken in account to correct the assayed FSH value. However, it appears that the herein reported RIA is not influenced by the pituitary hormones tested other than FSH. The absence of any correlation between LH and FSH plasma levels in the cryptorchid ram during the sampling period reinforce this statement.

The relative effects of the AS and purification grade of the labelled FSH on plasma protein specificity clearly indicate the AS to be used and FSH preparation to be labelled. In the presence of the interfering plasma protein (orosomucoid : Dubois *et al.*, 1973) the behaviour of a specific antiserum (AS 3) with labelled CNRS FSH P67C and CNRS FSH P26 was quite different in spite of the similar biological potencies of the two preparations (30 and 40 times NIH FSH S_3 respectively) while the behaviour of the same antisera with CNRS FSH P26 and HG FSH 225 was similar in spite of the differing biological potencies to these hormones (40 and 150 times NIH FSH S_3 respectively). In fact immunological potencies ranged in accordance with these results : CNRS FSH P67C had a low activity as compared to that of CNRS FSH P26 and HG FSH 225 (4.8 vs 25 and 35 respectively). The residual cross-reaction found with AS 4 P_3 or AS 5 BB with orosomucoid or hypophysectomized ewe plasma in the presence of labelled HG FSH 225 did not seem to affect the specificity of the system since : 1) dilution of plasma from entire or cryptochid rams, or of pituitary extracts were parallel to dilutions of purified FSH preparations ; 2) recovery of exogenous FSH added to

plasma was found to be satisfactory over the working range of the curve. These results are in agreement with those of Kragt and Cons (1973) who found it necessary to purify the labelled FSH (biological potency : 20 times NIH FSH S_3) to avoid cross-reactions with plasma proteins. However, they are in complete disagreement with those of Cunningham and Hebert (1973) who apparently found no cross-reaction with plasma using labelled NIH FSH S_4.

When immunological potencies were calculated for various purified FSH preparations a wide range of biological to immunological potency was observed (0.17 to 1.20). Other authors have observed ranges of 0.57 to 1.22 (L'Hermite *et al.*, 1972) and 1.22 to 7.7 (Salamonsen *et al.*, 1973). Similarly, McNeilly *et al.* (1976) have found immunological potencies of 0.4 and 2.2 for two purified FSH preparations using two different heterologous assays. The discrepancy between biological and immunological activity in some purified preparations is difficult to explain. However the discrepancy does not exist in all purified preparations which would seem to indicate that purification procedures affect biological activity more than immunological activity (ratio < 1). Besides, a ratio higher than 1 could be explained if, during the time elapsed between biological and immunological assays (sometimes several years), the purified preparations had lost some of their biological activity without any loss of immunological potency.

The sensitivity of the assay system (2.4 ng NIH FSH S_3/ml plasma) was higher than that observed in other specific RIA's (L'Hermite *et al.*, 1972 : $\neq$ 6 ng NIH FSH S_4/ml ; Salamonsen *et al.*, 1973 : 13 ng NIH FSH S_6/ml ; McNeilly *et al.*, 1976 : 15 ng NIH FSH S_8/ml) and is compatible with precise measurement ($70 \geqslant B/Bo \geqslant 30$ p. 100) of the low concentrations found in the intact ram.

Comparisons between assays are avoided in routine work as the coefficient of inter-assay variation observed is high (17.5-19.7 p. 100). The values for inter-assay variation coefficient calculated on the basis of the concentrations of a series of pool plasmas (5.5 to 8 p. 100) are considered as satisfactory.

The FSH plasma levels observed in the cycling ewe, before or after œstrous (this paper ; Bindon *et al.*, 1979 ; Cahill *et al.*, 1979 : 60-110 ng NIH FSH S_3) using the assay described here are in good agreement with previously observed values (L'Hermite *et al.*, 1972 : 100-160 ng NIH S_4/ml ; Hopkinson and Pant, 1973 : 40-90 NIH S_9/ml) ; Salamonsen *et al.*, 1973 : 13-100 NIH S_6/ml ; McNeilly *et al.*, 1976 : 50-120 NIH S_8/ml ; Dobson and Ward, 1977 : 40-90 ng NIH S_9/ml). Values observed at œstrous using this and other assays cited previously also show good agreement. Similarly, FSH plasma levels observed in adult rams using this assay (10-90 ng NIH FSH S_3/ml : Ortavant *et al.*, 1977 ; Blanc, Courot and Ortavant, unpublished) are in agreement with those of Lincoln, Peet and Cunningham (1977) (20-150 ng FSH NIH S_{10}/ml). However, the values found for ram lambs using this assay (14-40 ng NIH FSH S_3/ml, Blanc and Terqui, 1976) were different from those observed by Crim and Geschwind (1972) (150 ng NIH FSH S_1/ml). This late discrepancy could be accounted for by the different antisera used.

As previously described (Blanc *et al.*, 1978), FSH in contrast to LH is not secreted in pulses in the cryptorchid ram. This could mean that if LH pulses are due to LRH pulses, the gonadotrophs of the cryptorchid ram do not release FSH after LRH under these conditions. Periovulatory changes of FSH plasma levels were similar to those

observed by other authors (L'Hermite *et al.*, 1972 ; Hopkinson and Pant, 1973 ; Salamonsen *et al.*, 1973 ; Dobson and Ward, 1977 ; Bindon *et al.*, 1979 ; Cahill *et al.*, 1979). Due to their restricted amplitude and duration, these changes can only be seen if an appropriate sampling interval is used (McNeilly *et al.*, 1976).

The highly specific and sensitive homologous RIA described here is being used to examine the regulation of FSH secretion in the sheep.

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Résumé. La mise au point d'un dosage radioimmunologique homologue de la FSH ovine est décrite en détail. Deux antisera obtenus par immunisation de lapin, l'un avec une préparation purifiée de FSH « native », l'autre avec une préparation de FSH oxydée par le periodate ont été comparés. Ces deux antisera ont été choisis car lorsqu'ils réagissent avec certaines préparations très purifiées de FSH, marquées à l'iode 125, ils ne donnent que des réactions croisées très faibles avec les protéines plasmatiques. Après saturation par une préparation purifiée de LH ovine, ces antisera permettent de mesurer des activités plasmatiques FSH analogues. Les réactions croisées avec les protéines hypophysaires sont faibles (1 à 2 p. 100 avec TSH, 0.02 p. 100 avec LH et des taux inférieurs pour les autres hormones antéhypophysaires éprouvées GH, Prol. et ACTH) et mesurent probablement les contaminants FSH de ces préparations. Avec chaque antisérum, les courbes d'inhibition de liaison par des dilutions de plasmas ovins, d'extraits hypophysaires ou de préparations purifiées de FSH sont parallèles à celle de l'étalon. La récupération totale de différentes quantités de FSH rajoutées à du plasma indique que les protéines plasmatiques n'interfèrent pas avec le dosage. Si les mesures de FSH par dosage biologique ou radioimmunologique de différentes préparations purifiées de FSH sont concordantes, elles peuvent aussi diverger. Cette observation est discutée. Enfin, une étude de l'effet des volumes d'incubation sur la précision et la sensibilité du dosage a permis l'automatisation complète de la phase de pipettage du dosage. Dans ces conditions, la variabilité à l'intérieur d'un même dosage est de 5,5 à 8 p. 100 pour des valeurs de B/Bo comprises entre 25 et 70 p. 100 ; entre différents dosages, elle est de 17,5 à 19,7 p. 100 pour des valeurs de B/Bo comprises entre 46 et 60 p. 100. La sensibilité du dosage est de 2,4 ng NIH FSH S_3/ml de plasma. Les résultats suggèrent que le dosage présenté utilisant l'un ou l'autre des antiséra décrits peut être utilisé pour quantifier d'une manière spécifique la FSH ovine.

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