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Bovine follitropin.

Isolation and characterization of the native hormone and its α and β subunits.

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Summary. — 1) A reproducible procedure was developed for the purification of bovine follitropin.

2) The method involved ammonium sulfate precipitation, ion exchange and adsorption chromatography, concanavaline-A-Sepharose chromatography and gel filtration.

3) A specific radioligand receptor assay was used to monitor each chromatographical step.

4) The potency of highly purified bovine follitropin as measured by Steelman and Pohley bioassay was 62 times the NIH-FSH-B1 standard preparation.

5) Contaminations of bovine follitropin by other glycoprotein hormones such as thyrotropin and lutropin amounted to 3 and 0.45 per cent by weight respectively as measured by specific radioimmunoassays and radioligand receptor assays.

6) The subunits α and β of bovine follitropin were obtained by incubation in acidic urea, the chains being then separated by anion exchange chromatography.

The subunits were submitted to complete characterization. The amino-terminal residue of the α subunit is phenylalanine while a half cystine residue was found at the amino-terminal end of the β chain.

8) Cross-contamination of the α and β subunit preparations was measured by specific radioimmunoassays and amounted to 0.02 and 0.1 per cent by weight respectively.

INTRODUCTION.

The study of the hormones involved in the mechanisms of reproduction in cattle is of considerable scientific and economical implication.

Follitropin (FSH : Follicle Stimulating Hormone) activity was first demonstrated in bovine pituitary crude extracts by Nalbandov and Casida [1] in 1940. Since then, histological studies [2, 3] and preparations of pituitary extracts [4, 5] have shown that the content of follitropin in bovine pituitary gland is very low when compared to the amount present in the pituitary of other species. The limited hormone content in the tissue combined with lability of the biological activity of the hormone in purified form explain easily why only two reports [6, 7] have been published so far on the purification of bovine follitropin.

Indeed Cheng in 1976 [7] described a procedure for the isolation of bovine follitropin in highly purified form which possesses a biological activity similar to that described for follitropin in other species. Using a crude pituitary extract obtained from Organon (Oss, The Netherlands) as starting material, we found the procedure described by Cheng poorly reproducible and giving an extremely low yield in purified bovine follitropin.

Consequently we have applied a method previously described by our group, for the isolation of porcine [8, 9] and human follitropin [10]. In this paper, we shall described modifications of our procedure to specifically isolate bovine follitropin.

A radioligand-receptor assay for follitropin [11] was used to monitor hormonal activity during chromatographical steps of the purification procedure. Taking advantage of previous experiments on the isolation of porcine follitropin subunits [8, 9] we have purified the subunits of bovine follitropin using dissociating buffers and ion exchange chromatography. As those glycoproteins had never been characterized, we present here data on their biological, physical and chemical properties.

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MATERIAL AND METHODS.

A number of selected modifications, which resulted in greater yield and better separation of the bovine hormone from its contaminants, were introduced in the initial procedure and are described in detail below.

A crude bovine pituitary extract (60 g) obtained from Organon (Oss, The Netherlands) was dissolved in 2 liters of 0.3 M potassium chloride adjusted at pH 5.5 by hydrochloric acid and fractionated by precipitations after addition of ammonium sulfate from 0 to 40 per cent, 40 to 60 per cent and 60 and 80 per cent saturation at 4°C. The bulk of the follicle stimulating activity was recovered in the fraction precipitated by ammonium sulfate at 60 per cent saturation as measured by binding inhibition of ^{125}I-porcine follitropin on porcine testis receptors by bovine follitropin compared to a standard curve established with porcine follitropin. The resulting sediment was redissolved in 0.001 M phosphate buffer pH 6.2 and chromato-

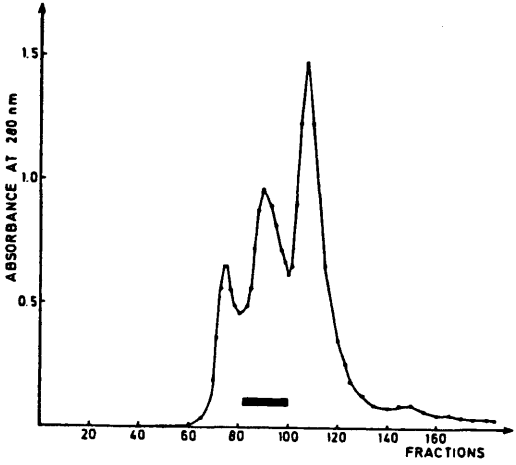

FIG. 1. — *Gel filtration on Sephadex G-75.*
Column (5 × 300 cm) ; developing buffer : 0.05 M pyridine acetate pH 5.0 ; flow rate 60 ml/h. ; sample : 1,000 mg dissolved in 35 ml of the buffer ; fraction size : 25 ml ; temperature : 4°C.
The area indicated by solid bar contains the hormone.

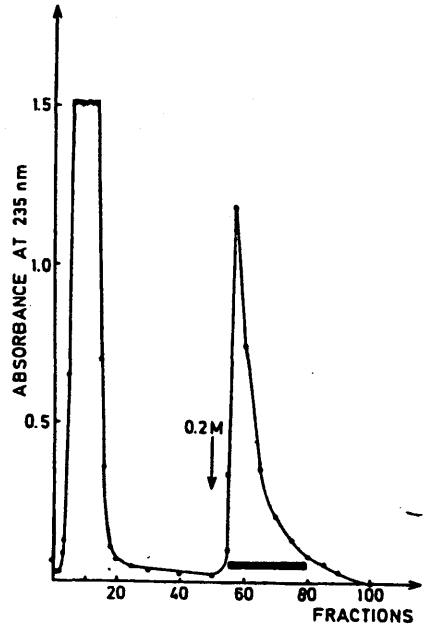

FIG. 3. — *Chromatography on Concanavaline-A-Sepharose.*
Column (3.5 × 12 cm) equilibrated in 0.05 M Tris-chloride buffer pH 7.4 ; sample : 310 mg dissolved in 50 ml of the buffer. Elution with 0.2 M methylmannoside in the initial buffer is indicated by the arrow. The solid bar indicates the pooled fractions.

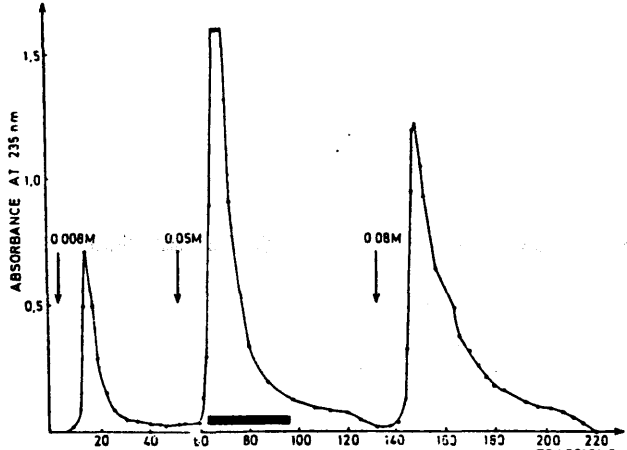

FIG. 2. — *Chromatography on hydroxylapatite (Biogel HTP).*
Column (3.5 × 10 cm) equilibration in 0.001 M potassium phosphate buffer pH 6.8 ; fraction size : 10 ml ; the sample (780 mg) was applied in 45 ml of the buffer. Stepwise elution with buffer of increasing phosphate concentration is indicated by the arrows.

graphed on CM-Sephadex G-25 (Pharmacia) under conditions already described [12]. Further purifications including DEAE-Sephadex A-25 and Sephadex G-75 (Pharmacia) chromatography were performed under conditions already used for porcine follitropin purification [8, 9].

The active fraction eluted from Sephadex G-75 (fig. 1) was first concentrated by ultrafiltration on the Amicon-Unit and equilibrated in 0.001 M phosphate buffer pH 6.2 by gel filtration on Sephadex G-25. The non retarded material was chromatographed on hydroxylapatite (Biogel HTP) under conditions described in figure 2.

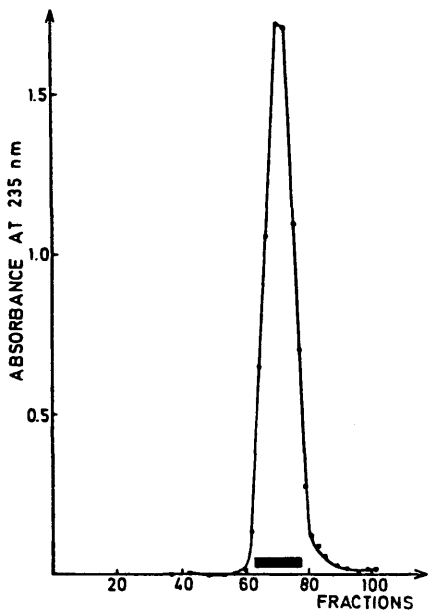

FIG. 4. — *Gel filtration on Sephadex G-100 of bovine follitropin.*

Column (1 × 95 cm) developing buffer 0.05 M Tris-chloride buffer pH 7.4 ; fraction size : 2 ml ; flow rate ; 12 ml/h. ; sample : 34 mg dissolved in 2 ml. Temperature : 4°C. The pooled fractions indicated by solid bar contains the hormone.

The fraction eluted with 0.05 M potassium phosphate contained the bulk of the hormone.

This fraction was then equilibrated in 0.05 M Tris-chloride buffer pH 7.4 by gel filtration and chromatographed on Concanavaline-A-Sepharose 4 B (Pharmacia) under conditions described in figure 3. The material eluted by the methyl mannoside (Fluka) containing buffer was concentrated and equilibrated against 0.05 M pyridine acetate buffer pH 5.0. Final purification was achieved by gel filtration on Sephadex G-100 in the same pyridine acetate buffer (fig. 4).

Dissociation of the hormone.

The hormone (25 mg) was dissolved in 3 ml of 0.05 M hydrochloric acid, 8 M urea (Merck P. A. grade) pH 2.8 and kept for 24 hours at room temperature. The isolation of the subunits was performed as follows : the incubation medium was first diluted by 6 ml of distilled water and 3 ml of 0.02 M sodium glycinate buffer pH 9.5 ; pH was then adjusted to pH 9.5 with 0.1 N sodium hydroxyde. This solution was immediately applied to a DEAE-Sephadex A-25 column.

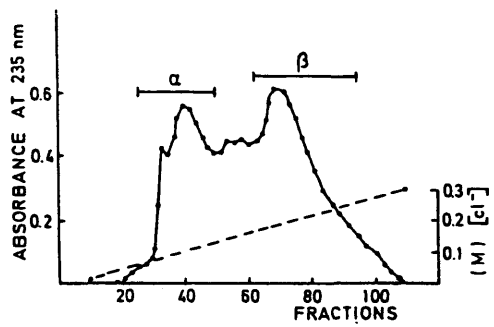

FIG. 5. — *Isolation of the subunit of bovine follitropin.*

Chromatography at 4°C on DEAE-Sephadex A-25. Column (1 × 10 cm) equilibrated in 0.02 M sodium glycinate buffer pH 9.5 ; fraction size : 2 ml ; sample : 23 mg in a total volume of 12 ml. The slope of linear gradient is indicated by broken line.

After washing the column with 0.02 M sodium glycinate buffer a linear gradient of increasing ionic strength was started, from 0.02 M sodium glycinate to 0.25 M sodium chloride in the same glycinate (fig. 5). Final purification of the subunits was achieved by gel filtration on Sephadex G-100 in 0.05 M ammonium bicarbonate as described in figure 6 A and B.

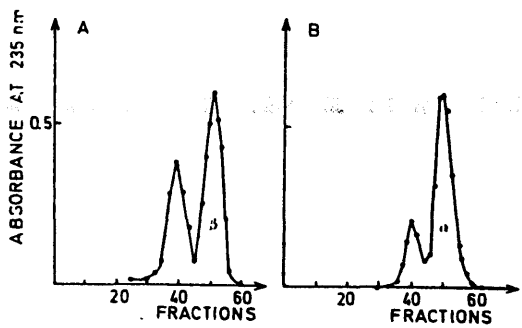

FIG. 6. A and B. — *Gel filtration on Sephadex G-100 of the α subunit (B) and β subunit (A).*

Column (1 × 95 cm) developing buffer 0.05 M Tris, buffer pH 7.4 ; fraction size : 2 ml.

Analytical methods.

The methods used for the amino acid analyses, determination of sialic acid content, amino-terminal amino acids and polyacrylamide gel electrophoresis have been described elsewhere [12].

Bioassays.

Follicle stimulating activity was measured according to Steelman and Pohley [13] with the National Institutes of Health Follicle-stimulating Hormone B1 as standard.

This standard B1 is the last reference preparation distributed by the National Institutes of Health for bovine follitropin.

Radioimmunoassays.

Methods used for labelling bovine thyrotropin have been previously described [11]. Purification of the labelled antigen was performed on small cellulose columns (Whatman CF-11) [14]. The radioimmunoassay developed for bovine thyrotropin was essentially performed according to Vandalem *et al.* [15] using the International Bovine Thyrotropin Standard and a highly purified bovine thyrotropin [16] prepared in our laboratory as reference preparations.

Contamination of our follitropin preparation by lutropin was measured by a specific radioimmunoassay described by Ketelslegers *et al.* [17] using the purified bovine lutropin prepared in our laboratory.

Cross-contamination of our subunit preparations were measured by specific radioimmunoassays for lutropin α subunit [18] and for follitropin bovine β subunit.

The antiserum against follitropin β chain were obtained by intradermal immunization according to the method described by Ross [19]. Labelling of the β chain were performed according to Ketelslegers and Catt [20]. The assays were performed in conditions previously described [15].

Radioligand receptor assays.

Porcine follitropin and bovine lutropin were labelled with the lactoperoxidase method according to Ketelslegers and Catt [20].

Purification of labelled porcine and bovine follitropin was performed on Sepharose-Concanavaline-A as described by Dufau *et al.* [21].

^{125}I labelled bovine lutropin was purified by gel filtration on Sephadex G-25.

The radioligand receptor assay for porcine follitropin were performed according to Maghuin-Rogister *et al* [11] ; briefly :

— 100 µl of standard porcine follitropin preparation (0,5 to 20 ng) or 100 µl of unknown amounts of bovine follitropin fraction in 0.05 M Tris chloride buffer pH 7.4 ;

— 50 µl of ^{125}I porcine follitropin (20,000 cpm/tube) in Tris buffer ;

— 100 µl of porcine testis homogenate (about 130 µg of protein) were incubated for 16 hours at 25°C.

Bound and free hormone were separated by centrifugation at 3000 g during 20 min.

RESULTS AND DISCUSSION.

Yields in weight and follitropin activity at the different steps of purification are summarized in table I.

TABLE I.

Recovery of follitropin from 60 g of bovine pituitary crude extract.

Fraction	Yield of protein (mg)	Total activity (mg)	Recovery per cent
Crude extract	60,000	110	100
40-60 percent saturation $(NH_4)_2$ SO_4 precipitation	24,000	92	84
CM-I	10,800	60	55
DEAE 0.12	3,750	45	41
G-75 II	784	39	35
HTP 0.05	310	34	31
Con-A II	34	29	26

The protein yields are the amount measured by the Lowry assay. The follicle stimulating activity is determined by radioligand receptor assay with reference to porcine follitropin [8, 9]. The percentage of follitropin recovery in each fraction was calculated with reference to the total amount present in the crude extract.

Behaviour of bovine and porcine follitropin during the various purification steps were similar excepted for ammonium sulfate precipitation where the bulk of bovine follitropin was recovered between 40 per cent - 60 per cent saturation.

Due to the low content in follitropin present in bovine pituitary extract, an additional chromatography on Sepharose-Concanavaline-A (fig. 3) had to be added to the procedure described for the porcine hormone in order to achieve purification of bovine follitropin. The final yield in bovine follitropin was about 25 per cent the amount initially

present in the crude extract. This is significantly higher than the yield obtained by the alternative procedure [7].

As seen in figure 4 our bovine follitropin preparation was finally eluted from Sephadex G-100 as an homogeneous fraction with an elution volume similar to that of the porcine follitropin.

In comparing data from Cheng [7] and our laboratory (table II) no significant differences are observed in amino acid compositions of preparations.

TABLE II.

Compositions of bovine and porcine follitropin.

Aminod acid	Bovine	Porcine (8,9)
	residue/100 residues	
Lysine	6.5	7.7
Histidine	5.1	3.0
Arginine	3.3	3.1
Aspartic acid	7.2	7.7
Threonine	9.5	10.1
Serine	7.2	6.2
Glutamic acid	9.8	8.7
Proline	6.6	4.9
Glycine	6.8	5.4
Alanine	7.4	8.2
Half-cystine	8.4	9.3
Valine	6.7	4.5
Methionine	1.6	1.9
Isoleucine	3.5	3.5
Leucine	4.4	4.9
Tyrosine	5.0	6.7
Phenylalanine	3.5	3.7
Tryptophane	n.d.	0.8
Sugars	µmol/mg	
Sialic acid	0.057	0.55

Nevertheless, it must be noted that bovine follitropin possess 10 times less sialic acid than its porcine counterpart.

This must be correlated with the lower biological activity of *in vivo* bioassays of the bovine hormone as compared to the porcine follitropin.

Figure 5 shows that the native hormone dissociated into two fractions well separated by chromatography on DEAE-Sephadex. Amino acid analyses of both fractions clearly demonstrated that the material eluted at the end of the linear gradient of ionic strength contained the β chain. The amino acid compositions of our bovine α and β subunits are compared to their porcine counterparts in table III.

Chromatography on Sephadex G-100, as described in figure 6 A and B was necessary to minimize both the cross-contaminations between subunits and the contamination of the subunits by the undissociated hormone. Consequently, our subunit preparation possessed follicle stimulating activities amounting to less than 0.5 per cent of the native hormone as measured by radioligand receptor assay. The electrophoretic analyses of the intact hormone and its subunits are shown in figure 7. Bovine follitropin gave a rather diffuse protein band as is commonly observed for follitropin of other species [8, 9, 22, 23]. The more cathodic character of the α subunit compared to the β chain at alkaline pH is consistant with the respective chromatographic behaviour of the subunits on DEAE-Sephadex.

TABLE III.

Compositions of bovine and porcine follitropin subunits.

Amino acid	Bovine		Porcine	
	α subunit	β subunit	α subunit	β subunit
	residue/100 residues			
Lysine	7.6	4.4	8.7	7.2
Histidine	2.9	2 1	4.2	3.7
Arginine	3.2	3.6	3.6	2.8
Aspartic acid	6.2	9.3	5.5	8.9
Threonine	8.5	11.1	9.6	10.7
Serine	7.2	6.8	6.5	5.5
Glutamic acid	9.3	10.3	8.4	8.9
Proline	6.8	7.1	6.4	4.7
Glycine	7.0	6.1	5 8	5.5
Alanine	8.5	6.1	9.0	5.7
Half-cystine	8.2	8.2	8.5	9.7
Valine	7.1	6.3	5.0	7.2
Methionine	3.0	0.0	3.0	0.3
Isoleucine	2.3	4.6	2.5	4.2
Leucine	3.6	5.6	4.5	5.0
Tyrosine	4.2	5.5	5.1	6.8
Phenylalanine	3.9	3.1	3.9	3.7
Tryptophane	n.d.	n.d.	0.0	0.7
Sugars	µmol/mg			
Sialic acid	0.024	0.095	0.48	0.50

End group analysis revealed phenylalanine as the sole terminal amino acid residue for the α chain while CM-Cysteine was detected at the amino-terminal end of the reduced and carboxymethylated β subunit.

The biological activity of our purified bovine follitropin were found to be 62 times that of the National Institutes of Health Follicle Stimulating Hormone B1 (bovine) preparation. Cheng [7] gave a figure of 164 times the NIH-FSH-S1 (ovine) preparation. This reference substance (S1) was distributed such a long time ago that a significant loss of its specific activity is most probable. In addition, in the Steelman and Pohley's assay, bovine and ovine follitropins give non parallel response curves.

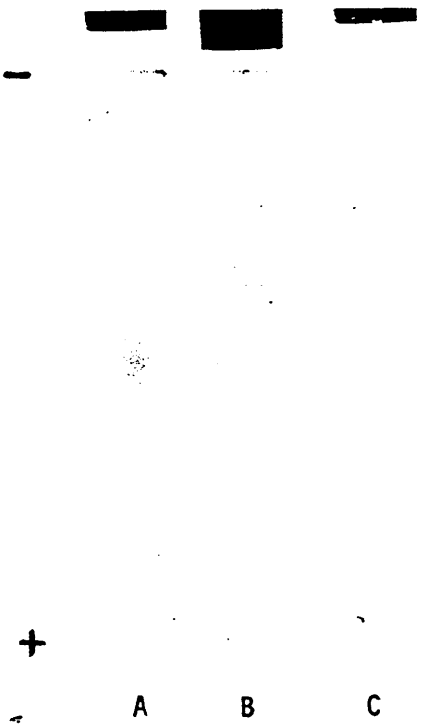

FIG. 7. — *Gel electrophoresis in 12 per cent polyacrylamide of bovine follitropin (A) and its α (B) and β (C) subunits.*
Temperature : 4°C and 3 mA/tube for 3 hours.

Calibration by a bovine standard is thus compulsory. By radioligand receptor assay, our purified follitropin was found to be 55 times more potent than bovine NIH-FSH-B1 standard a value in close agreement with the activity measured by bioassay. Our bovine follitropin preparation thus exhibits activity in the range of that of other purified follitropin preparations [11] in the radioligand receptor assay. The minute differences observed are probably due to slight variations in the degree of purity of the various preparations or to species specificity of the porcine testicular receptor.

Our preparation was also assessed for contamination by other pituitary glycoprotein hormones by specific radioimmunoassays and radioligand receptor assays.

Tested in the bovine thyrotropin assay system, bovine follitropin exhibited a contamination by thyroid-stimulating hormone of 2.8 per cent by weight.

Tested in the bovine lutropin radioimmunoassay system, the bovine follitropin exhibited a contamination by lutropin of about 3.0 per cent by weight.

Measured by homologous radioligand receptor assay the contamination of bovine follitropin by lutropin amounted to 0.45 per cent by weight.

Such discrepancy between the contaminations measured by radioligand receptor assay and immunoassay could be explained by the presence in the preparation of degraded forms of lutropin retaining immunoactivity only.

Concerning the subunit preparations, the cross-contaminations have been measured with specific radioimmunoassays. They amounted to 0.02 and 0.1 per cent by weight for the α and β subunit respectively.

CONCLUSIONS.

The procedure described above for bovine follitropin purification can be employed for the hormone from other species likes pig [8] and man [10].

Monitoring the entire purification procedure by a specific radioligand receptor assay which permits selection of follicle stimulating activity lead us to obtain bovine follitropin essentially devoid of luteinizing and thyrotropin activities and possessing a specific biological activity similar to that of purified follitropin of other animal species. Using our preparations specific immunoassays have now been developed for both bovine follitropin and its subunits. They are being used together with the specific radioligand receptor assays for unequivocal physiological and biological experiments in the physiology of reproduction.

The α and β subunits of bovine follitropin were isolated and fully characterized in this study. The purity of these reagents is such that they could be used for primary structure determination.

RÉSUMÉ.

1) Un procédé reproductible d'isolement de la follitropine de bœuf est décrit.

2) Ce procédé utilise la précipitation fractionnée au sulfate d'ammonium, les chromatographies échangeuses d'ions et d'absorption ainsi qu'une séparation sur Concanavaline A et une filtration sur gel Sephadex.

3) L'utilisation d'un dosage basé sur l'inhibition de la liaison sur récepteur testiculaire de la follitropine porcine radioactive par l'hormone bovine nous a permis de repérer la follitropine bovine au cours des différentes étapes de purification.

4) Mesurée dans le test *in vivo* de Steelman and Pohley, la follitropine bovine possède une activité biologique 62 fois supérieure à la préparation de référence B1 du National Institute of Health.

5) La contamination de la follitropine bovine par la thyrotropine est de 3 p. cent mesurée en dosage radioimmunologique spécifique. La contamination en lutropine mesurée par dosage sur récepteur testiculaire est de 0,45 p. cent.

6) La follitropine bovine se dissocie en ses sous-unités α et β par incubation en urée à pH acide. Celles-ci sont alors séparées par chromatographie sur DEAE-Sephadex.

7) La phenylalanine est l'acide aminé terminal de la chaine α tandis que la carboxyméthylcystéine se trouve à l'extrémité amino-terminale de la chaine β réduite et carboxyméthylée.

8) Les sous-unités α et β de la follitropine bovine sont de grande pureté puisque leurs contaminations croisées respectives sont seulement de 0,02 et 0,1 p. cent en poids mesurées par leurs dosages radioimmunologiques spécifiques.

Acknowledgments.

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