

A homologous radioimmunoassay for canine prolactin: plasma levels during the reproductive cycle

R. De Coster, J.-F. Beckers¹, D. Beerens and J. De Mey

*Department of Oncology, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium and
Department of Obstetrics and Pathology of Reproduction¹, Faculty of Veterinary Medicine, B-1070 Brussels*

Abstract. A method is described for the purification of canine prolactin, involving preparative isoelectrofocusing. Canine prolactin has a molecular weight of 23 000 daltons, an isoelectric point of 5.7 and exhibits a high degree of homogeneity in polyacrylamide gels stained by means of a silver method. A specific, homologous radioimmunoassay is described using the Bolton-Hunter method for preparation of the labelled ligand, with a sensitivity of 0.1 ng/tube. Basal plasma prolactin levels of 2-4 ng/ml obtained through the oestrous cycle remained fairly constant but a rise of 9 ng/ml was found at the end of dioestrus in non-pregnant bitches. Level also rose 30 days after mating to reach a peak of about 50 ng/ml near parturition and during early lactation.

Mammalian pituitary prolactin (Prl) is known to play a role in mammary gland development and in the initiation and maintenance of lactation. It also inhibits the normal release of gonadotrophins in lactational and pathological amenorrhoea and probably in lactational anoestrus in sheep and rodents (Mac Neilly 1980). Prl is also involved in some of the mechanisms of osmoregulation (Loretz & Bern 1982), of behaviour regulation (Ensor 1978) and in mammary gland tumourigenesis in rodents (Meites 1972). Since the dog is a useful model in biomedical research, a knowledge of the plasma levels of this hormone in various physiological and pathological states might be useful. Using homologous radioimmunoassays, Knight et al. (1977b), Gräf et al. (1977), and Gräf & El Etreby (1979) described the plasma Prl variations during the pregnant cycle of

the bitch but little is known about the plasma levels during the non-pregnant cycle. On the other hand, Jones et al. (1976), using an heterologous radioimmunoassay, described a rise of Prl at the end of pro-oestrus which was not observed by other investigators (Gräf & El Etreby 1979). This paper describes the development of a specific, homologous radioimmunoassay for canine Prl and the determination of plasma Prl levels during the oestrous cycle of the bitch.

Materials and Methods

Purification procedure

Canine pituitaries (637) were removed between 30 min and 5 h after death from dogs of both sexes and kept frozen until required. The purification procedure was performed at 4°C with some modifications, according to the methods of Hwang et al. (1973) and Gräf et al. (1977). Prolactin (Prl) and growth hormone (GH) activities were screened by radioreceptorassays, using rabbit mammary gland preparation (Shiu & Friesen 1974) or rabbit liver preparation (Tsushima & Friesen 1973), as described in detail by Beckers & Ectors (1981). The Prl activity of the various fractions was compared with a dilution curve of canine pituitary extracts, as canine Prl did not parallel the standard curve of bovine Prl. On the other hand, each fraction was tested by analytical isoelectrofocusing. In brief, after a first extraction in 0.05 M ammonium acetate, pH 5, the residue was stirred overnight in 0.05 M ammonium bicarbonate, pH 10. The pH was then adjusted to 8.5 with HCl 0.1 N and after centrifugation, the supernatant was concentrated and chromatographed on Sephadex G100 (Pharmacia Fine Chemicals, Sweden) in 0.01 M ammonium bicarbonate,

Requests for reprints: R. De Coster, Department of Oncology, Janssen Pharmaceutica, B-2340 Beerse, Belgium.

pH 9. After a second gel filtration on Ultrogel Aca 54 (LKB, Sweden) in the same buffer, the appropriate fractions were pooled, dialysed against ammonium bicarbonate 0.01 M, pH 8 and chromatographed on DEAE cellulose A32 equilibrated in this buffer. GH and PrI were successively eluted with a gradient of ammonium bicarbonate (0.01 M to 0.3 M). This was followed by preparative isoelectrofocusing in a horizontal electrophoretic cell, model 1415 (Biorad Laboratories, USA), using ampholines, pH 5–8. A current of 30 mA, 500 V was passed overnight. Strips of gels were cut off with a scalpel, washed with 1 ml of distilled water for pH determination, followed by 10 ml of ammonium bicarbonate 0.05 M, pH 9. The fractions containing PrI were lyophilized and passed through a column of Ultrogel Aca 54 to remove the major part of the ampholines.

Anodic discontinuous polyacrylamide gel electrophoresis in Tris-buffer pH 8.3 (PAGE, 8% gel) and discontinuous electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE, 12% gel) were performed in a vertical cell, model 220 (Biorad Laboratories, USA), respectively following the procedures of Davis (1964) and Laemmli (1970). Thin layer (0.4 cm) casting gel system with ampholines, pH 3–9 and 5–8 was used for analytical electrofocusing in a 5% polyacrylamide gel to follow the homogeneity of the protein fractions. Usually, 1 to 3 µg of protein was applied for each sample. The gels were stained following the silver procedure of Morrissey (1981), with some modifications during the fixation procedure. Isoelectrofocusing gels were fixed in a solution containing 50% methanol and 12% trichloroacetic acid. The next morning, they were successively washed in a solution of 30% methanol, 10% acetic acid for 30 min, and in a solution of 10% ethanol, 5% acetic acid for 20 min, two times. PAGE-SDS gels were fixed for at least 1 h in a solution of 50% methanol. The same solution, containing 10% SDS and 1% β-mercaptoethanol was used for PAGE gels to improve the sensitivity. All the types of gel were thereafter allowed to swell in water for at least 30 min and soaked in a solution containing 5 µg/ml dithiothreitol. Without rinsing, they were gently shaken in a solution containing 0.5% silver nitrate. After 30 min, they were rapidly rinsed with distilled water and soaked in the developer (50 µl of 37% formaldehyde in 100 ml 3% sodium bicarbonate).

The biological potency of PrI was determined by the pigeon-crop sac microassay of Lyons & Page (1935) using bovine PrI NIH B5 (32.2 IU/mg) as standard. Five groups of 5 pigeons received daily for 3 days 1.25, 2.5, 5, 10 and 20 µg canine or bovine PrI, respectively in the left or the right part of the crop. One day after the last injection, the animals were killed and the diameters of the reaction areas were measured and compared.

Radioimmunoassay

Four rabbits were immunized at 2 week intervals by multiple intradermal injections of 0.250 mg of PrI dis-

solved in 0.5 ml of NH_4HCO_3 0.05 M, pH 9.0 and emulsified with an equal volume of Freund's complete adjuvant. After 3 months, 2 of them gave antisera suitable for radioimmunoassay.

Ten µg of PrI was iodinated with the chloramine T technique ($n = 3$) (Hunter & Greenwood 1962), with the lactoperoxidase technique ($n = 2$) (Thorell & Johanson 1971) or with the Bolton-Hunter reagent (Amersham 1971) following the technique described by Bolton & Hunter (1973) with an incubation time of 1 h at 4°C ($n = 5$).

The radioiodinated hormone was separated from the free ^{125}I -reagent by chromatography on a Sephadex G100 column (1 × 28 cm) equilibrated with 0.05 M phosphate buffer containing 0.1% BSA or gelatin for the Bolton-Hunter method.

The buffer diluent of the assay procedure consisted of phosphate-buffer 0.05 M, 0.15 M sodium chloride, 0.01 M ethylenediaminetetraacetic acid, pH 7.6 containing 1% BSA (Cohn, Fraction V, Sigma). Plasma samples were assayed in duplicate at one or two dilutions, in two different assays. One hundred µl of antiserum were added at an initial dilution of 1:32 000 in 1:400 normal rabbit serum. After a pre-incubation of 30 h at 4°C, with a total volume of 400 µl, fresh [^{125}I]PrI was added (100 µl, about 12 000 CPM) and tubes were further incubated for 24 h at 4°C. Then free and bound hormones were separated by addition of 200 µl of a 1:100 dilution of goat antirabbit gamma-globulin. After 16 h of the second incubation time, the tubes were centrifuged for 20 min at 3200 g and the precipitates were counted in a γ-counter with an efficiency of 75%.

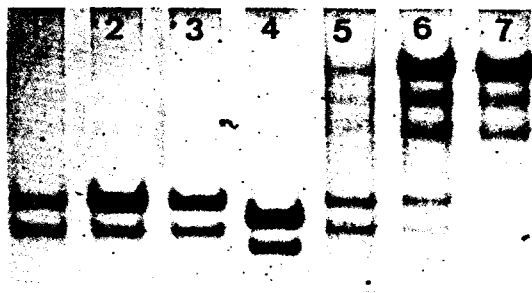


Fig. 1. PAGE of various canine pituitary preparations. 1 cPrI 4, 2 cPrI 5, 3 cPrI 6, 4 bPrI, 5 DEAE, PrI fraction, 6 DEAE, intermediate fraction, 7 cGH, DEAE fraction.

Experimental animals

Five Beagle bitches aged between 1 to 4 years were followed during 2 successive oestrous cycles. Between 8 and 9 a.m., once a week and every day during heat, 10 ml of blood was taken from the jugular vein in heparinized tubes and immediately centrifugated. Plasma was stored at -20°C until assayed. Five bitches were mated and whelped normally.

Results

Purification procedure

Two fractions of the preparative isoelectrofocusing gel contained Prl, at pH 5.65 (cPrl 5, 8 mg) and at pH 5.75 (cPrl 6, 6.4 mg). A third fraction (cPrl 4, 7 mg) was obtained at pH 5.68 with a second preparative gel. The electrophoretic mobility of these samples in PAGE is shown in Fig. 1. As for bovine Prl, two bands appear, which are probably aminated and deaminated Prl (Knight et al. 1976). In SDS-PAGE, only one band can be seen (Fig. 2). The molecular weight of canine Prl can be estimated to be 23 000, which is similar to that of bovine Prl. As determined by analytic and preparative isoelectrofocusing, the isoelectric point of dog prolactin is 5.7 (Fig. 3). Because of the higher homogeneity in this gel, cPrl 6 was selected as standard.

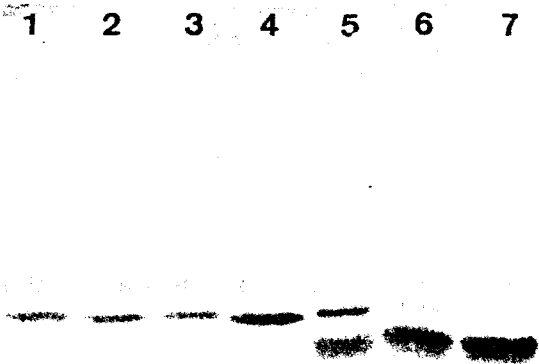


Fig. 2.

SDS-PAGE of various canine pituitary preparations. 1 cPrl 4, 2 cPrl 5, 3 cPrl 6, 4 bPrl, 5 DEAE, Prl fraction, 6 DEAE, intermediate fraction, 7 cGH, DEAE fraction.

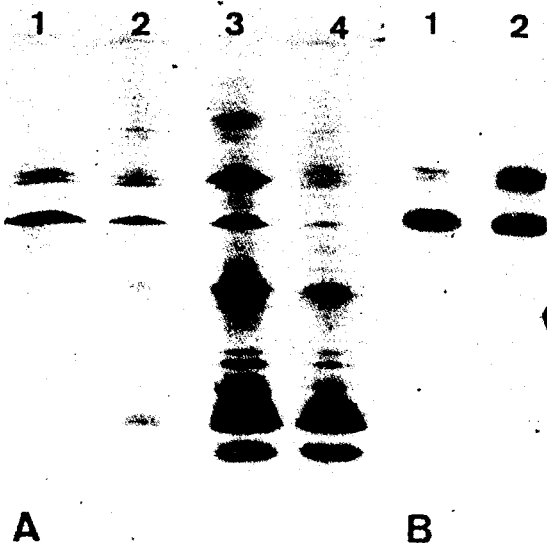


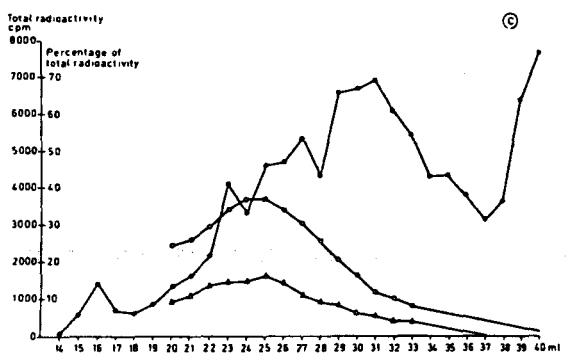
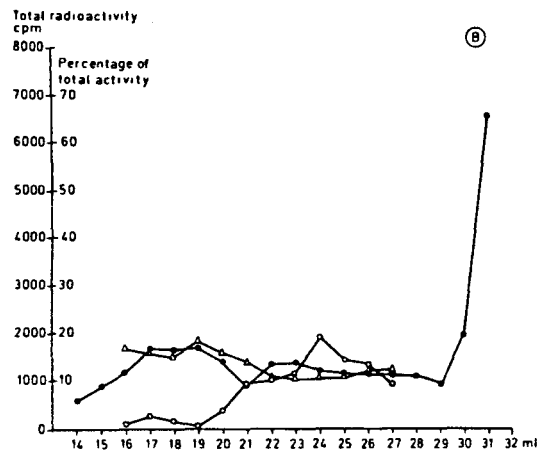
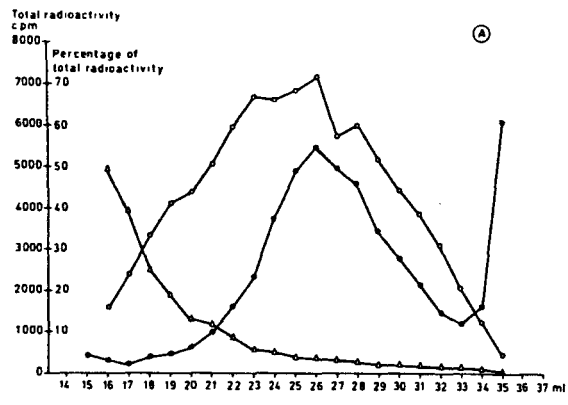
Fig. 3.

A) Analytical isoelectrofocusing gel, pH 3-8; 1 cPrl 5, 2 DEAE, Prl fraction, 3 DEAE, intermediate fraction, 4 cGH, DEAE fraction.
B) Analytical isoelectrofocusing gel, pH 5-8; 1 cPrl 6 (3 μg), 2 cPrl 5 (3 μg).

The biological potency of this preparation is 15.7 U/mg (9.7-30.0 IU/mg) relative to bovine Prl NIH B5.

Application of the radioimmunoassay

For the three iodination methods, one typical elution profile of radioiodinated cPrl on Sephadex G100 column and the specific and non-specific activity bindings are shown in Fig. 4. The best results were obtained with the Bolton-Hunter technique, which gives a major peak of labelled protein with a specific activity of $50 \pm 12 \mu\text{Ci}/\mu\text{g}$. Maximal binding reaches 78.9% with an excess of antibody. Competition studies with cLH (Stockell Hartree et al. 1972) do not show any displacement and only an incomplete displacement for cGH, bPrl and rat pituitary extract (pH 10). The sensitivity of the assay is 0.1 ng/tube and 50% binding of the tracer is obtained with 1 ng/tube (Fig. 2). The intra-assay coefficient of variation averages 13.6% ($n = 26$) and the inter-assay coefficient 6.4% ($n = 20$).



Figs. 4A, B and C.

Typical elution profile of radioiodinated cPrl, chromatographed on a Sephadex G-100 column (1 × 28 cm, fractions of 1 ml). A) Bolton Hunter; B) lactoperoxydase; C) chloramine T; ●—● CPM (× 10⁻³). ○—○ specific binding; △—△ non specific binding.

Dilution of lactating or normal canine sera, of crude pituitary extract and of standard curve is parallel (Fig. 5). Fig. 6 shows the mean variations of plasma Prl levels during the oestrous cycles of 5 mated, pregnant and 5 non-mated bitches.

Prl values are fairly constant during the non-pregnant cycle, with the exception of small fluctuations during prooestrus and oestrus, a slightly increased basal level in dioestrus and a rise between day 70 to 110, when plasma progesterone levels return to base-line (De Coster et al. 1979).

During pregnancy, Prl levels begin to rise 30 days after first mating and reach their highest values near parturition. During lactation, levels remain high but with marked fluctuations, probably related to periods of suckling. Plasma Prl returns to basal levels between 43 and 50 days after parturition.

Discussion

Preparative isoelectrofocusing improves the homogeneity of Prl preparation and eliminates any detectable contamination by GH. Electrophoretic data are in agreement with the findings of Gräf et al. (1977) and Jones et al. (1976). Saluja et al. (1973) reported an isoelectric point of 6.6 but the choice of another method, using a different gel concentration could explain this discrepancy.

The estimation of the molecular weight by SDS electrophoresis gives the same value as obtained by Papkoff (1976) using a gel filtration method.

The silver staining procedure can be used for all types of gel and shows a good sensitivity and reproducibility. Samples containing as little as 2 µg protein are sufficient to show the heterogeneity of fractions (Fig. 3), enabling the purification procedure to be monitored without using too much material.

The Bolton-Hunter iodination seems to give rather better results than the direct chloramine-T method since there is only one peak of iodinated Prl, without any aggregate and since the maximum binding with an excess of antibody is higher. These results agree with those of Knight et al. (1977a) and Gräf et al. (1977) who reported lower specific binding and some aggregation of labelled Prl with oxydative iodination methods.

Plasma Prl level remains fairly constant during the oestrous cycle, but rises dramatically during the

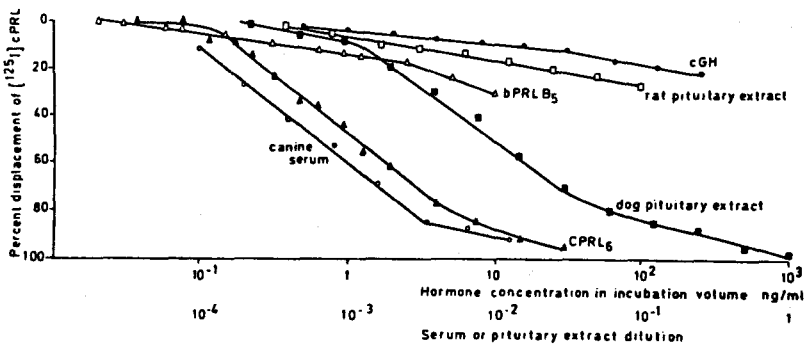


Fig. 5.

Typical standard curve of canine prolactin (mean of 7 assays), and competition studies with canine GH, bovine Prl, rat and canine pituitary extracts (pH 10), and canine serum.

second half of gestation and stays at a high level thereafter for some 40–60 days. The prooestrous surge described by Jones et al. (1976) could not be detected. These data are in agreement with the data of Gräf et al. (1977), although we did not find

a significant rise of plasma Prl between 1 and 2 weeks after oestrus. The frequency of the blood sampling was perhaps suboptimal in our study. Knight et al. (1977a,b) describes peak values in the same range, but a little higher basal level of 8–10

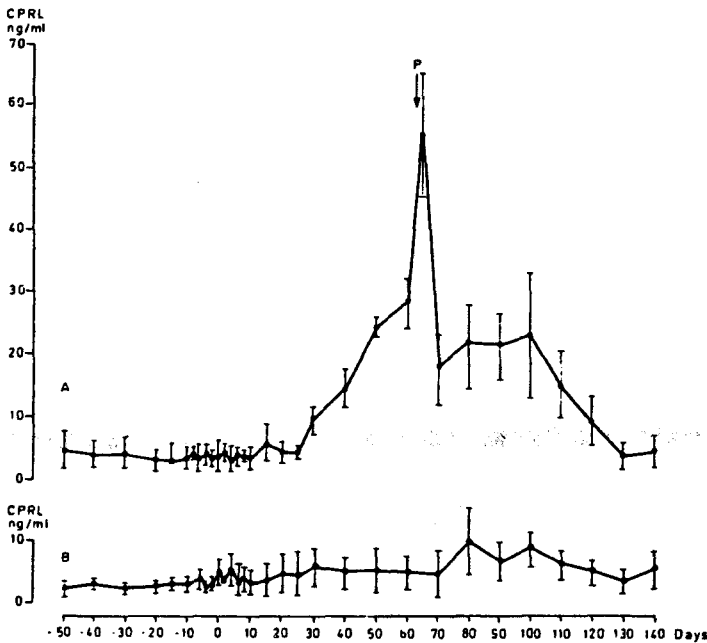


Fig. 6.

Variations of plasma prolactin levels during oestrous cycle of 5 mated (A) and 5 non-mated bitches (B) (mean \pm SD). Day 0 is the first day of copulation. P: parturition.

ng/ml. The explanation of this discrepancy needs further investigations.

The inverse relationship between Prl and progesterone levels described at the end of pregnancy by Gräf et al. (1977) is further confirmed by the small rise in Prl concentration at the end of dioestrus in non-mated bitches. This modification of Prl level could also be involved in some pathological conditions, such as false pregnancy or pyometra.

Acknowledgments

We are grateful to the NIH for the gift of bovine prolactin and growth hormone, to Mrs. Stockell Hartree for the gift of cLH, to the F.N.R.S. for financial support and to all the people who have helped us in collecting the pituitaries and realizing this work.

References

Beckers J F & Ectors F (1981): Dosage de l'hormone placentaire somatotrope et mammothrope bovine par liaison radio-compétitive aux récepteurs membranaires. *Ann Med Vet* 125: 311–319.

Bolton A E & Hunter W M (1973): The labelling of proteins to high specific radioactivities by conjugation to a ¹²⁵I containing acylating agent. *Biochem J* 133: 529–539.

Davies B J (1964): Disc electrophoresis: II. Method and application to human serum proteins. *Ann NY Acad Sci* 121: 404–407.

De Coster R, Beckers J F, Wouters-Ballman P & Ectors F (1979): Variations de la LH, de la FSH, du 17β oestradiol et de la progestérone au cours du cycle oestral de la chienne. *Ann Med Vet* 123: 177–184.

Ensor D M (1978): *Comparative Endocrinology*, p 187–189. Chapman and Hall Ltd, London.

Gräf K-J, Friedreich E, Matthes S & Hasan S H (1977): Homologous radioimmunoassay for canine prolactin and its application in various physiological states. *J Endocrinol* 75: 93–103.

Gräf K-J & El Etreby M F (1979): Endocrinology of reproduction in the female beagle dog and its significance in mammary gland tumorigenesis. *Acta Endocrinol (Copenh)*, Suppl 222: 1–33.

Hunter W M & Greenwood F C (1962): Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* 194: 495–496.

Hwang P, Robertson M, Guyda H & Friesen H (1973): The purification of human prolactin from frozen pituitary glands. *J Clin Endocrinol Metab* 36: 110–118.

Jones G E, Brownstone A D & Boyns A R (1976): Isolation of canine prolactin by polyacrylamide gel electrophoresis. *Acta Endocrinol (Copenh)* 82: 691–705.

Knight P J, Gronow M & Hamilton J M (1976): Purification of canine prolactin by preparative isotachopheresis. *J Endocrinol* 69: 127–132.

Knight P J, Hamilton J M & Scanes C G (1977a): Homologous radioimmunoassay for canine prolactin. *Acta Endocrinol (Copenh)* 85: 736–743.

Knight P J, Hamilton J M & Hiddleston W A (1977b): Serum prolactin during pregnancy and lactation in the beagle bitch. *Vet Rec* 101: 202–203.

Laemmli U K (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 227: 680–685.

Loretz C A & Bern H A (1982): Prolactin and osmoregulation in vertebrates. *Neuroendocrinology* 35: 292–304.

Lyons W R & Page E (1935): Detection of mammothrophin in the urine of lactating women. *Proc Soc Exp Biol Med* 32: 1049–1050.

Mac Neilly A S (1980): Prolactin and the control of gonadotrophin secretion in the female. *J Reprod Fertil* 58: 537–549.

Meites J (1972): Relation of prolactin to mammary tumorigenesis and growth in rats. In: Boyns A R & Griffiths K (eds). *Prolactin and Carcinogenesis*, p 54. Alpha-Omega-Alpha Publishers, Cardiff.

Morrissey J H (1981): Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal Biochem* 117: 307–310.

Papkoff H (1976): Canine pituitary prolactin: isolation and partial characterization. *Proc Soc Exp Biol Med* 153: 498–500.

Saluja P G, Gronow M & Hamilton J M (1973): Measurement of canine pituitary prolactin. *J Endocrinol* 56: 245–258.

Shiu R P C & Friesen H G (1974): Properties of a prolactin receptor from the rabbit mammary gland. *Biochem J* 104: 301–311.

Stockell Hartree A, Bell E T, Christie D W & Kirkham K E (1972): Partial purification of dog glycoprotein hormones. *J Endocrinol* 52: 403–404.

Thorell J I & Johanson B G (1971): Enzymatic iodination of polypeptides with ¹²⁵I to high specific activity. *Biochim Biophys Acta* 251: 363–369.

Tsushima T & Friesen H G (1973): Radioreceptor assay for growth hormone. *J Clin Endocrinol Metab* 37: 334–337.

Received on January 13th, 1983.