Radioimmunoassay for Ovine, Caprine and Bovine Prolactin in Plasma and Tissue Extracts

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1. A radioimmunoassay for ovine prolactin is described based on the inhibition of the reaction between ^{131}I -labelled ovine prolactin and guinea-pig or rabbit antiserum to ovine prolactin. The extent of the reaction after a 4-day incubation period is determined by chromatoelectrophoresis or by adsorption of unchanged ^{131}I -labelled ovine prolactin on charcoal. The sensitivity is equal to 5.9 ng. of prolactin/ml. of plasma with chromatoelectrophoresis, or 0.2 ng. of prolactin/ml. of tissue extracts with the charcoal separation. 2. A complete cross-reaction demonstrated between ovine prolactin and caprine pituitary extracts allows the assay to be used to measure caprine prolactin. The partial cross-reactions between ovine prolactin and bovine prolactin and between ovine prolactin and bovine pituitary extract differ, and an alteration in the immunological activity of bovine prolactin during its isolation is suggested. Bovine prolactin in plasma may be measured against a bovine pituitary extract as standard. No cross-reactions were demonstrated with pituitary extracts from a number of other species. The extent of the contamination of ovine and bovine growth hormone preparations by their respective prolactins is shown. 3. Dilutions of ovine and caprine plasma inhibit the reaction between ^{131}I -labelled ovine prolactin and antiserum with the same characteristics as ovine prolactin. 4. The immunoreactive material in plasma fractionates on Sephadex G-200 and in sucrose density gradients as a single peak similar to that shown by freshly dissolved ovine prolactin. There is no evidence that ovine prolactin is bound to a plasma protein. 5. By suppressing prolactin secretion and assaying serial samples of plasma thereafter it is shown that the immunological activity of the surviving hormone becomes progressively altered with time. It is suggested that this alteration is usually not detected but introduces an element of uncertainty into the quantitative but not the qualitative value of the measurements obtained by reference to standard ovine prolactin.

A sensitive measurement of prolactin concentrations in plasma from man, domestic animals and experimental animals or in cell-free systems from the pituitary gland would be of use in determining the factors that control the biosynthesis and secretion of prolactin. These approaches have proved valuable for a number of peptide hormones by using radioimmunological methods for assaying, in the main, the hormones in plasma (cf. Berson & Yalow, 1964; Greenwood, 1967*a*; Greenwood & Landon, 1968). Specific methods have been developed for rat (Kwa & Verhofstad, 1967*a*), mouse (Kwa, Verhofstad & van der Bent, 1967)

and sheep prolactin in plasma (Arai & Lee, 1967), and a number of factors influencing the plasma concentration have been noted (Kwa & Verhofstad, 1967*b,c*). The measurement of human prolactin awaits its isolation or separation from human growth hormone (cf. Greenwood, 1967*b*).

This paper describes a radioimmunoassay for prolactin in sheep plasma and pituitary extracts. Evidence for the limits of the specificity of these measurements and similar measurements in the related species, goat and ox, are presented. Preliminary reports of this work (Bryant & Greenwood, 1968) and of the physiological and experimental modification of amounts of plasma prolactin in the goat (Bryant, Greenwood & Linzell, 1968*a*) have been presented.

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MATERIALS

Buffers. Sodium salts were used in the preparation of buffers. Diluent buffer refers to a solution (0.5 mg./ml.) of bovine γ -globulin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) in 0.07 M-barbitone buffer, pH 8.6.

Ovine prolactin. A single preparation (NIH-P-S6), kindly supplied by the Endocrinology Study Section, National Institutes of Health, Bethesda, Md., U.S.A., was used as a standard and for the preparation of ^{131}I -labelled ovine prolactin. Electrophoresis in starch gel (Smithies, 1959) showed three components. Fractionation in Sephadex G-200 and on a sucrose density gradient showed one major component and a small proportion of polymerized material appearing only when fresh solutions were frozen and thawed. Electrophoresis in agar gel and subsequent diffusion against an antiserum to ovine prolactin showed a major arc with some evidence for a minor component closely related immunoelectrophoretically to the major component.

^{131}I -labelled ovine prolactin. This was prepared weekly by the method of Hunter & Greenwood (1962), except that [^{131}I]iodide (1 mc) obtained from Iso-Serve Inc., Cambridge, Mass., U.S.A., was made to react with the hormone (5 μg . in 0.01 ml.) and 6–50 μg . of chloramine-T. The least amount of chloramine-T consistent with a yield of reaction of more than 50% was preferred, to decrease immunological damage. These reaction conditions may, however, vary over a period of months depending on changes in the quality of the [^{131}I]iodide sample (Greenwood, 1967a). The ^{131}I -labelled ovine prolactin was separated from unchanged [^{131}I]iodide in a Sephadex G-75 column (1 g.), previously equilibrated with 1 ml. of 0.07 M-barbitone buffer, pH 8.6, containing 20 mg. of bovine γ -globulin. Eluates (15 $\times$ 1.5 ml.), in the 0.07 M-barbitone buffer alone, were collected into tubes containing 0.5 ml. of diluent. The percentage of the [^{131}I]iodide transferred to the peptide was $58 \pm 23\%$ (mean $\pm$ S.D., 57 experiments), yielding specific radioactivities of 70–160 $\mu\text{Ci}/\mu\text{g}$. The damage to the labelled peptide during its preparation was low (<10%) as judged by chromatoelectrophoresis (Berson, Yalow, Baumann, Rothschild & Newerley, 1956) and by the ability of the labelled peptide to bind to antiserum. The percentage damage was not increased by storage in diluent at 4° for up to 10 days, but if bovine serum albumin was used as the diluent protein a rapid increase in damaged components was noted on storage or incubation at 4°.

Guinea-pig and rabbit antisera to ovine prolactin. An antiserum to ovine prolactin (NIH-P-S4) obtained in a guinea pig was kindly supplied by Dr I. A. Forsyth, National Institute for Research in Dairying, Shinfield, Berks. Antisera to ovine prolactin were obtained in adult female rabbits by weekly subcutaneous injections of 1 mg. of ovine prolactin in Freund's complete adjuvant (Difco) for 3 weeks. At 3 weeks after the last injection the animals were injected intravenously with 1 mg. of ovine prolactin in 5 ml. of 0.15 M-NaCl. The animals were bled 7–10 days after the injection. Antisera were centrifuged at 8720 g_{av} . in a Spinco model L ultracentrifuge (no. 30 rotor) for 20 min. and samples (1:1, 1:4680 in diluent) were stored at –20° without preservatives. Latterly, ovine prolactin (100 μg . in 0.2 ml. of adjuvant) was injected into the spleen of rabbits under Nembutal anaesthesia by a technique kindly demonstrated to us by Dr G. Boyd, Medical Unit, St Mary's Hospital, London, W. 2 (G. Boyd & W. S. Peart, unpublished work).

Pituitary extracts. Sheep and ox pituitaries were obtained from M. W. Derrick Ltd., Hatfield, Herts. Goat, duck and pig pituitaries were kindly supplied by Dr J. L. Linzell, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambs., Dr P. Miahle, Institute of Physiology and Biochemistry, Strasbourg, France, and Mr B. Bates, Chelsea College of Science and Technology, London, S.W. 3, respectively. Mouse, rabbit and rat pituitaries were obtained by dissection of animals supplied by the animal house of this Laboratory. Extracts of pituitaries were prepared as described by Hunter & Greenwood (1964); the stock solutions contained 1 mg. of total solids/ml.

Pituitary fractions. Ovine and bovine growth hormones, having biological activities 0.79 and 1.53 U.S.P. units/mg. respectively, were kindly supplied by the Endocrinology Study Section, National Institutes of Health, Bethesda, Md., U.S.A.; bovine prolactin was a gift from Professor T. Winnick, University of Hawaii, Honolulu, Hawaii, U.S.A.

Animal plasmas. Blood samples were heparinized and centrifuged, and the plasma was stored at –20° and transported on solid CO₂. Samples were assayed within 1 month of collection. The assay of prolactin in several hundred plasma samples from goats was carried out in collaboration with Dr J. L. Linzell (Bryant *et al.* 1968a). Plasma samples from control and experimental groups of sheep formed part of a similar collaboration with Dr R. Short and Dr R. Connan, University of Cambridge, and Dr R. Denamur, Institut National de la Recherche Agronomique, Jouy-en Josas, France. Plasma samples from cows were kindly supplied by Mr V. Langley, Agricultural Research Institute, Fermoy, Co. Cork, Irish Republic.

METHODS

Radioimmunoassay for ovine prolactin by a chromatoelectrophoretic technique. Standard ovine prolactin (0.3 ml. of solutions containing 0, 0.8, 1.6, 3.2, 6.4 and 12.8 ng./ml. in diluent) or plasma (0.3 ml. of five dilutions selected from a range of doubling dilutions from 1:16 to 1:256 in diluent) was added to polystyrene incubation tubes (6 cm. $\times$ 1 cm.; Luckhams Ltd.). Antiserum to ovine prolactin (0.05 ml. of a 1:4680 dilution in diluent) was added; the contents of the tubes were mixed gently and the tubes, which were capped in bulk by a sheet of a Parafilm (Gallenkamp-Towers, London, E.C. 2), were incubated at 4°. The antiserum had previously been titrated against 50 pg. of the ^{131}I -labelled ovine prolactin as described below. After a 48 hr. incubation ^{131}I -labelled ovine prolactin (50 pg. in 0.05 ml. of diluent) was added, the contents were mixed gently, the tubes were capped again and the incubation was continued for a further 48 hr. Incubation damage to ^{131}I -labelled ovine prolactin in dilute plasma (1:16 dilution) was no greater than that in control tubes containing labelled peptide in diluent only.

The percentage of the radioactivity of the ^{131}I -labelled ovine prolactin bound to the immune γ -globulin after incubation was determined by chromatoelectrophoresis (Berson *et al.* 1956). The variables influencing the separation, the apparatus and the techniques were described for adrenocorticotrophin by Landon, Livanou & Greenwood (1967). For maximum separation between ^{131}I -labelled ovine prolactin and ^{131}I -labelled ovine prolactin bound to

Fig. 1. Standard assay curves for ovine prolactin: comparison of the amounts of prolactin required to decrease the amount of ^{131}I -labelled ovine prolactin bound to immune γ -globulin. Δ , ^{131}I -labelled ovine prolactin (50 pg.) treated with guinea-pig antiserum (1:37440 final dilution) and resolved by chromatoelectrophoresis; $\circ$, ^{131}I -labelled ovine prolactin (20 pg.) treated with rabbit antiserum (1:299520 final dilution) and resolved by adsorption on charcoal.

antibody the following modifications were used. Horse serum (Burroughs Wellcome, Beckenham, Kent) was added to each tube after incubation (0.1 ml. of a 1:4 dilution in diluent) and the contents were mixed, and 0.1 ml. of the final volume (0.5 ml.) was applied to a filter-paper strip (Whatman 3MM, not selected for chromatography, 46 cm. $\times$ 2.5 cm.) at a line 12 cm. from the cathode. Chromatoelectrophoresis was carried out at constant voltage (400 v) for 90 min. at room temperature with a bridge gap of 42 cm. and 0.1 M-barbitone buffer, pH 8.6, for the strips and the electrode buffers. The strips were air-dried, and two areas were removed and counted as scrolls for 10 min. in a Packard Autogamma counter. The area containing unchanged ^{131}I -labelled ovine prolactin was from 10 cm. to 16 cm. from the cathode end of the strip, and the area containing ^{131}I -labelled ovine prolactin bound to antibody, together with other serum proteins, was from 16 cm. to 22 cm. The latter radioactivity was calculated as a percentage (counts bound/counts bound+free) and plotted against the amounts of standard added by using a logarithmic/linear plot (Fig. 1). The amounts of ovine prolactin or their immunological equivalents in the test samples were calculated from this curve. The range of sensitivity of the assay is convenient for plasma prolactin concentrations generally found in the domestic animals studied (25–1500 ng./ml. of plasma). Unless otherwise specified all the results reported were obtained by using chromatoelectrophoretic separation.

Radioimmunoassay for ovine prolactin by a charcoal

separation technique. A more sensitive assay was devised to study the biosynthesis of prolactin in a cell-free system and its rate of discharge from pituitary slices. This used a higher-avidity antiserum obtained in a rabbit, and a charcoal separation technique (Herbert, Lau, Gottlieb & Bleicher, 1965). Samples (0.3 ml.) of standard ovine prolactin (0.2–1.6 ng./ml.) or dilutions of the test solutions were preincubated with rabbit antiserum (0.05 ml. of a 1:37440 dilution) as described above, and ^{131}I -labelled ovine prolactin (20 pg. in 0.05 ml. of diluent) was added before the second incubation. The tubes were then counted for 10 min. and horse serum (1:4 dilution in diluent) was added to each tube so that the total protein concentration was 3.5 mg./ml. The contents of the tubes were mixed gently and a suspension (1.0 ml.) of charcoal coated with dextran was then added [Norit A charcoal containing 0.5% (w/v) of Dextran 150, 5% (w/v) in 0.07 M-barbitone buffer, pH 8.6]. The contents of the tubes were mixed, allowed to stand at room temperature for 30 min. with further mixing at 20 min., and centrifuged. The supernatant was removed by aspiration (^{131}I -labelled ovine prolactin bound to antibody), and the residue was counted (^{131}I -labelled ovine prolactin adsorbed on the charcoal). The percentage of the total counts present in antibody-bound prolactin was calculated (Fig. 1).

The conditions necessary for the maximum separation with charcoal-dextran were determined for human growth hormone, thyroid-stimulating hormone, adrenocorticotrophin and angiotensin in our Laboratory by B. R. Webster, J. Landon & J. Girard (unpublished work). Similar variables were studied to achieve maximum separation in the ovine prolactin assay: the volume of the charcoal-dextran solutions, the ratio of charcoal to dextran and the total protein concentration. Maximum separation was judged by parallel separations with chromatoelectrophoresis.

The assay based on the charcoal separation technique was used only for the measurement of standard ovine prolactin in tissue-culture experiments and for the measurement of prolactin synthesis by bovine pituitary slices. The results of these experiments are not reported in the present paper.

Preliminary titration of antiserum. ^{131}I -labelled ovine prolactin (50 pg. in 0.05 ml. of diluent) was incubated with volumes (0.35 ml.) of antiserum at doubling dilutions from 1:3000 to 1:1536000 (final dilutions). Incubation was carried out for 48 hr. at 4° and the percentage of the ^{131}I -labelled hormone bound to the immune γ -globulin was determined by chromatoelectrophoresis as described above. A dilution of the antiserum such that 40–50% of the added labelled peptide was bound was chosen for the assay.

RESULTS

Separation technique and the detection of damage to ^{131}I -labelled ovine prolactin

Standard hormone or hormone in plasma or in other solutions is measured by its ability to inhibit the reaction between labelled peptide and the limited concentration of antibody in the system. This inhibition or competition is expressed as a fall in the percentage of the radioactivity bound to antibody as estimated by chromatoelectrophoresis

Table 1. *Damage to ^{131}I -labelled ovine prolactin on incubation with caprine plasma and bovine serum fractions*

^{131}I -labelled ovine prolactin (50 $\mu\text{g.}$ in 0.05 ml.) was incubated with 0.3 ml. of dilutions of caprine plasma, crystalline bovine serum albumin (0.5 mg. in 1 ml.) or bovine γ -globulin (0.5 mg. in 1 ml.) for 4 days at 4°. The percentage damage to the labelled peptide was measured by chromatoelectrophoresis.

Bovine serum			Dilutions of caprine							
protein	...	Albumin	γ -Globulin	plasma (v/v)	...	1/8	1/16	1/32	1/64	1/128
% damage (mean)		30.0	10.0			17.2	13.9	11.1	10.2	9.5
s.d.		± 7.0	± 3.0			± 1.2	± 1.2	± 1.4	± 2.0	± 1.4
No. of determinations		20	20			10	10	10	10	10

or by adsorption on charcoal-dextran. When antibody is present the separation methods do not distinguish between undamaged ^{131}I -labelled ovine prolactin bound to antibody and any of the labelled peptide that has become damaged by incubation with plasma, commonly encountered in the radioimmunoassay technique (Greenwood, 1967a). Since damaged labelled peptide is not adsorbed on filter paper or on charcoal-dextran under the conditions used to separate antibody-bound and free hormone, it is measured as an apparent increase in antibody-bound radioactivity. Suitable conditions or controls were found to minimize and measure this artifact. A series of control tubes were set up for each routine assay of prolactin in plasma. Dilutions of each plasma were incubated with ^{131}I -labelled ovine prolactin alone and the percentage damage was determined by chromatoelectrophoresis. The percentage damage increased with plasma concentration, but at dilutions greater than 1:16 it was acceptable and almost as low as that obtained on incubation with bovine γ -globulin solutions. Similar incubations of the labelled peptide and bovine serum albumin, commonly employed as a diluent protein, caused a more marked increase in damage (Table 1). ^{131}I -labelled ovine prolactin samples, containing less than 10% of damaged components after preparation and incubation, differed in their ability to bind to a constant dilution of antiserum. This was seen as a variation in the percentage of the labelled peptide bound in the absence of standard hormone ($41.2 \pm 7.1\%$, mean $\pm$ s.d. of 43 observations) and minor changes in the slope of the standard curve. Standard curves are run for each assay and the variation in slopes of these curves are paralleled by changes in the slope of the plasma measurements. The consequent changes in the precision of the assay form part of the observed precision of the procedure in routine use, described below. One factor decreasing the immunological activity of the ^{131}I -labelled ovine prolactin samples was found to be the amount of oxidizing agent, chloramine-T, used during radioiodination. Hence the least amount of this component in the reaction

Table 2. *Precision of the assay of prolactin in plasma expressed as estimates of the standard deviations of results from their means obtained by analyses of plasma at two concentrations*

Different samples of plasma were assayed at two convenient dilutions. The numbers of assays at two concentrations of plasma are given in parentheses. Values of s , an estimate of the standard deviation, are calculated in terms of ng. of prolactin/ml. of plasma. The mean concentrations of prolactin in the plasma samples obtained are grouped into the concentration ranges shown.

Concn. range of prolactin (ng./ml. of plasma)	Estimated S.D. (s)
10-50	± 3.0 (55)
50-100	± 7.0 (45)
100-300	± 53.0 (89)
300-500	± 50.0 (16)
500-1000	± 42.0 (10)

mixture was used (50-5 $\mu\text{g.}$), subject to variations in the quality of [^{131}I]iodide samples.

Precision and sensitivity of the assay

The precision of the assay in routine use is shown in Table 2. The lower limit of sensitivity calculated from the precision of the assay at low concentrations (Table 2) is better than 5.9 ng./ml. of plasma. At this low concentration the maximum percentage error ($P 0.05$) would be $\pm 50\%$ for determinations performed at two concentrations (Hunter & Greenwood, 1964). The precision data can also be used to calculate the significance of any differences observed between serial plasmas taken during experiments. However, in such experiments neither the sensitivity nor the precision was found to be limiting. A replication experiment was carried out to determine whether endogenous hormone was degraded on storage at -20° in plasma. A sample was assayed over a 3-month period on nine occasions. The S.D. (± 21.9 ng./ml.) of the mean concentration measured (715.0 ng./ml.)

Fig. 2. Time-course of the reaction between ^{131}I -labelled ovine prolactin and immune γ -globulin. ^{131}I -labelled ovine prolactin (50 pg. in 0.05 ml. of diluent) was incubated at 4° with antiserum (0.05 ml.; 1:37440 final dilution) and either (○) plasma from a hypophysectomized sheep (0.3 ml. of a 1:16 dilution in diluent) or (△) diluent (0.3 ml.). Values of percentage damage to labelled peptide incubated with plasma or diluent alone have been subtracted. The lines represent the best lines through the experimental points.

Fig. 3. Loss of precision with increased incubation time △, ^{131}I -labelled ovine prolactin (50 pg.) incubated for 4 days with antiserum (1:37440 final dilution) and various amounts of ovine prolactin. ○, ^{131}I -labelled ovine prolactin incubated for 2 days with a solution, preincubated for 2 days with antiserum (1:37440 final dilution) and various amounts of ovine prolactin.

was within the precision of routine measurements in this range given in Table 2. Although there was no systematic fall in the mean concentration with length of storage, samples were assayed within 1 month.

The sensitivity of the charcoal-dextran assay calculated from the first significant fall in the standard curve is 0.2 ng./ml. of tissue extract or pituitary fraction. Our more limited experience with this assay in routine use does not allow calculations of the sensitivity and precision in practice. In test samples, the absence of any effects of salt concentration, amino acids and sucrose on the immunological reaction in the assay was readily determined by suitable controls. In addition, amino acids labelled with ^{14}C or ^3H did not interfere with the counting of the ^{131}I -labelled ovine prolactin.

Incubation time and order of addition of reactants

The time of incubation of the labelled peptide and antiserum is a compromise between a practical time in which to obtain equilibrium, the rate at which labelled peptide is damaged and its initial specific radioactivity. Maximum sensitivity in the assay was obtained empirically, although mathematical models are available (Potts, Sherwood, O'Riordan & Aurbach, 1967; Ekins, Newman & O'Riordan, 1967), by the experimental protocol

shown in Fig. 2. ^{131}I -labelled ovine prolactin (50 pg.) was incubated for various times at 4° with antiserum in the presence either of plasma from a hypophysectomized sheep (0.3 ml. of a 1:16 dilution in diluent) or of diluent (0.3 ml.). Control tubes containing labelled peptide in diluent or in dilute plasma incubated in parallel showed an increase in damaged components of 3–4% by chromatoelectrophoresis over this period, and these values were subtracted. Binding between labelled peptide and antiserum was rapid and linear over the first 5 hr. of incubation, and thereafter showed a slight increase with time. The precision of the chromatoelectrophoretic analyses was not sufficient to demonstrate unequivocally any systematic difference in the rate of binding in diluent or plasma. Any such difference was of the order of 3%. Incubations over longer periods, up to 7 days, gave higher final binding, but damage to the labelled peptide became limiting. A small increase in chromatoelectrophoretic damage may reflect a greater degree of immunological damage to ^{131}I -labelled prolactin; this is suggested by the results shown in Fig. 3, where the addition of the labelled peptide was delayed (Samols & Bilkus, 1964). Addition of the labelled peptide after a preincubation of the standard peptide and antiserum gave a more sensitive and precise standard curve than the same total incubation time of the three reactants.

Fig. 4. Inhibition of the ^{131}I -labelled ovine prolactin-antiserum system. Antiserum to ovine prolactin (1:37440 final dilution) was incubated with ^{131}I -labelled ovine prolactin (50 pg.) and ovine prolactin ($\circ$), caprine pituitary extract (Δ) or ovine pituitary extract ($\square$). Pituitary extracts were diluted to the amounts shown from a stock solution containing 1 mg. of total solids/ml., and a volume of 0.3 ml. was used for the assay.

Specificity of the radioimmunoassay

The experimental approaches to this problem reported by Hunter & Greenwood (1964) and by Greenwood (1967a) were extended in the present study by applying the sucrose-density-gradient analysis used by Fontaine & Condliffe (1963) and O'Riordan, Condliffe, Potts & Aurbach (1967), and by a systematic search for fragmented hormone in plasma.

Effect of pituitary hormones, pituitary extracts and plasma in the assay. Standard ovine prolactin may be regarded as a specific inhibitor of the reaction between ^{131}I -labelled ovine prolactin and antibody to ovine prolactin. Fig. 4 shows that an immunologically identical inhibition is present in simple extracts of ovine and caprine pituitary glands. The results suggest that caprine prolactin is immunologically indistinguishable from ovine prolactin with the particular antiserum used and can be estimated in terms of an ovine standard.

On the other hand, a bovine prolactin preparation showed a partial cross-reaction, the slope of the inhibition curve being qualitatively different from that given by ovine prolactin at the same order of concentration (Fig. 5). However, a simple extract

Fig. 5. Inhibition of the ^{131}I -labelled ovine prolactin-antiserum system. ^{131}I -labelled ovine prolactin and antiserum, as in Fig. 4, were incubated with ovine prolactin ($\circ$), bovine prolactin (Δ) or bovine pituitary extract ($\square$). Pituitary extracts were diluted to the amounts shown from a stock solution containing 1 mg. of total solids/ml., and a volume of 0.3 ml. was used for the assay.

of a bovine pituitary gland was more similar immunologically to ovine prolactin than the purified bovine prolactin was. This suggests that some modification of bovine prolactin, or of this particular preparation, takes place during its isolation. Some additional evidence may be obtained by comparison with Fig. 7; bovine plasma inhibits the reaction with the characteristics of bovine pituitary extract rather than of bovine prolactin itself. Hence at the present time measurements of plasma bovine prolactin would be valid only with a laboratory standard of bovine pituitary extract.

Similar studies showed that extracts of pituitaries from the rat, mouse, guinea pig, rabbit, pig, duck and carp did not inhibit the reaction between labelled ovine prolactin and its antiserum.

Testing of the specificity of the assay with preparations of other hormones is limited by the homogeneity of these preparations. Ovine and bovine growth hormone preparations inhibit the reaction between ^{131}I -labelled ovine prolactin and its antiserum (Fig. 6), but the high concentrations required and the slopes of the inhibition curves, which were characteristic of ovine prolactin, suggest a low order of contamination of ovine prolactin (0.26% by wt.) in the ovine growth hormone samples. This interpretation is supported by biological assays of the ovine growth hormone for prolactin, which show that prolactin contamination

Fig. 6. Inhibition of the ^{131}I -labelled ovine prolactin-antiserum system. ^{131}I -labelled ovine prolactin and antiserum, as in Fig. 4, were incubated with ovine prolactin ($\circ$), ovine growth hormone (Δ) or bovine growth hormone ($\square$).

is not greater than 2.18% by wt. (A. E. Wilhelm, personal communication). A similar order of contamination of bovine growth hormone by bovine prolactin is suggested by Fig. 6, but quantitative estimates are not possible.

Inhibition by ovine and caprine plasma (Fig. 7) paralleled that given by standard ovine prolactin, whereas bovine plasma showed a cross-reaction. Plasma from a sheep after hypophysectomy showed no inhibition. The parallelism between standard ovine prolactin and ovine and caprine plasma samples was shown by the several hundred plasma samples since assayed at three or more dilutions. The exception to this finding is discussed below.

State of endogenous and exogenous prolactin in plasma. Plasma (2ml.) from a lactating sheep was fractionated on Sephadex G-200 (2g.) equilibrated with 0.05M-phosphate buffer, pH 7.4. The eluate (2ml. fractions) was assayed for prolactin, and the results are shown in Fig. 8. The major immunological activity was found as a single peak after the elution of the plasma proteins. In contrast, plasma from an animal injected with standard ovine prolactin showed an additional peak corresponding to the macroglobulin-globulin region. This suggested that the injected peptide had become aggregated before or after its exposure to plasma. Sucrose-density-gradient analysis (Fontaine & Condliffe, 1963) of freshly dissolved ovine prolactin, ovine prolactin after a cycle of freezing and thawing, and endogenous prolactin confirmed that aggregation of the standard hormone can take place, but

Fig. 7. Inhibition of the ^{131}I -labelled ovine prolactin-antiserum system. ^{131}I -labelled ovine prolactin and antiserum, as in Fig. 4, were incubated with ovine prolactin ($\circ$) in the amounts shown or with caprine (Δ), ovine ($\square$) or bovine ($\bullet$) plasmas at the dilutions given.

without apparent loss of immunological activity (Monjardino, Bryant, Stephenson & Greenwood, 1968).

A theoretical objection to the determination of a peptide hormone by immunological methods is that immunoreactive portions of the peptide, if present in plasma, would be detected as the intact hormone (Berson, Yalow, Glick & Roth, 1964; Greenwood, 1967a). By determining plasma at three to five dilutions it has been demonstrated experimentally that this situation can occur (Bryant, Huxster & Greenwood, 1968b). A moderately high concentration of endogenous caprine prolactin was decreased by the injection of insulin. The rate of fall in concentration was consistent with the half-life of survival of ovine prolactin in the goat (Bryant *et al.* 1968a). However, the slopes of the inhibition curves of samples after insulin injection (Fig. 9) were no longer characteristic of prolactin. The progressive decrease in immunoreactivity shown after insulin injection suggests that the intact peptide was no longer present but had become progressively altered. Insulin is without effect *in vitro* on prolactin but appears to cause an abrupt cessation of secretion from the pituitary (G. D. Bryant, J. L. Linzell & F. C. Greenwood, unpublished work).

Results obtained in plasma

Table 3 summarizes some of the results obtained in studies on goats and sheep. The acute changes over milking might have been expected for a

Fig. 8. Endogenous prolactin activity of plasma. Ovine plasma (2ml.) was fractionated on a Sephadex G-200 column. Fractions (2ml.) were collected, extinction at $280m\mu$ was measured (continuous line) and concentration of prolactin was assayed (solid vertical bars).

Fig. 9. Disappearance of endogenous prolactin of plasma. Caprine plasma, at the dilutions shown, was incubated in the ^{131}I -labelled ovine prolactin-antiserum system. The samples were obtained before ($\blacktriangle$), and at 28 min. ($\circ$), 58 min. ($\blacksquare$) and 206 min. ($\triangle$) after, secretion of prolactin was abolished by the intravenous injection of insulin (0.24 unit/kg. body wt.) into an adult female goat. ($\bullet$), Standard curve with ovine prolactin in the quantities shown.

concentrations are less satisfactory indices of specificity than the other evidence adduced, since changes were measured that are not expected from previous work on the control of prolactin secretion, namely a rise in plasma concentrations in response to stress, mating, visual stimuli, feeding and glucose injections, and the unexpected absence of prolactin after stalk-section in sheep.

DISCUSSION

With samples of a purified peptide hormone and an antiserum the development of a sensitive and specific radioimmunoassay for the immunological sequence of the peptide in plasma is possible. Problems in the development of an assay for ovine prolactin were limited to its unexpected high rate of damage in a commonly used diluent protein, bovine albumin, and in an occasional damaging batch of bovine γ -globulin. Routine radioiodination was no more of a problem than with other peptide hormones (McFarlane & Greenwood, 1966). The methods used to separate antibody-bound and free ^{131}I -labelled ovine prolactin were chosen for their ability to detect damaged peptide (chromatoelectrophoresis) or their speed (charcoal adsorption). A similar assay with ovine prolactin, ^{131}I -labelled ovine prolactin and a rabbit antiserum as reagents has been devised by Arai & Lee (1967). Starch gel rather than Sephadex was used to purify the labelled peptide, and its ability to bind to antibody rather than chromatoelectrophoresis was used to choose the least damaged fraction. Separation methods utilized the addition of a second antibody

galactopoietic hormone, and indirect evidence has also suggested that oxytocin and promazine stimulate prolactin secretion. Expected changes in

Table 3. *Serial estimations of ovine and caprine prolactin in plasma*

Concentrations of prolactin in plasma were assayed by the routine method and are expressed as ng./ml. of plasma.

Time	Concn. of prolactin (ng./ml.)
(a) Half-life of injected ovine prolactin in the goat. Ovine prolactin (10mg.) was given intravenously at time t_0 .	
$t_0 + 6$ min.	3699
$t_0 + 13$ min.	4659
$t_0 + 25$ min.	1539
$t_0 + 42$ min.	737
$t_0 + 60$ min.	368
$t_0 + 80$ min.	168
$t_0 + 100$ min.	122
(b) Samples from a lactating goat	
20 min. before milking	66.7
10 min. after milking	734.7
20 min. after milking	180.4
(c) Effect of oxytocin in a lactating goat. Oxytocin (1 i.u.) was injected intravenously at time t_0 . Blood was sampled from a jugular vein.	
$t_0 - 2$ min.	144
$t_0 - 1$ min.	187
$t_0 + 1$ min.	308
$t_0 + 2$ min.	191
$t_0 + 4$ min.	209
$t_0 + 8$ min.	179
(d) Effect of acetylpromazine in a ewe. Acetylpromazine (25 mg.) was injected intramuscularly at time t_0 .	
$t_0 - 1$ min.	4.0
t_0	4.0
$t_0 + 10$ min.	5.8
$t_0 + 20$ min.	127.1
$t_0 + 30$ min.	218.8
$t_0 + 40$ min.	267.1
$t_0 + 60$ min.	181.7

or chromatoelectrophoresis. Incubation damage at high plasma concentrations was observed and similar sensitivity and concentrations of plasma prolactin were obtained. Bovine serum albumin, used as a diluent, was not noted to cause damage to the labelled peptide.

The complete cross-reaction noted here between caprine and ovine prolactin and the partial cross-reaction between ovine and bovine prolactin (Arai & Lee, 1967) confirm the similar observations of Hayashida (1962) with immunodiffusion. No cross-reactions were detected here or by Hayashida (1962) with rat or mouse pituitary extracts. In contrast Rümke & Ladiges (1965) and Kwa *et al.* (1967) detected a partial cross-reaction between murine and ovine prolactin by immunofluorescence and radioimmunoassay methods respectively. Complete or partial cross-reactions are a function of the

particular antiserum and methods used (cf. Greenwood, 1967b), but operationally the results suggest that ovine, caprine and bovine prolactin can be measured with an anti(ovine prolactin) antiserum. On the other hand, successful radioimmunoassays for rat and mouse prolactin were based on material isolated from prolactin-producing tumours by Kwa & Verhofstad (1967a) and by Kwa *et al.* (1967) respectively.

The evidence adduced by comparing the characteristic inhibition of ovine prolactin with other pituitary hormones, with pituitary extracts and with different plasmas, together with the identical behaviour of exogenous and endogenous prolactin on Sephadex and in sucrose density gradients, suggests that the assay specifically measures ovine or caprine prolactin. More precisely the assay measures immunologically active sequences of amino acids of these peptides. It has already been noted that expected changes in plasma prolactin concentrations cannot be used as additional evidence since interesting but unexpected changes were also obtained.

The detection of immunoreactive material in plasma that is similar to but less reactive than intact ovine prolactin is an important qualification to the specificity of radioimmunological techniques. This material was detected under a particular set of conditions and then only by assaying multiple dilutions of plasma, but the material may be invariably present and measured as an additional immunological inhibitor to the more potent specific inhibitor, intact prolactin. The material under these conditions would assume the characteristics of the latter and be recorded quantitatively as ovine prolactin. The theoretical qualification of the specificity of immunoassays, the possible divergence of immunological and biological estimates of activity, is thus confirmed experimentally. The extent of this phenomenon cannot be measured, but the immunoreactive material measured at any one time may be the sum of newly secreted intact peptide together with degraded peptide from hormone secreted previously. The sensitive and rapid measurements are useful indices of prolactin secretion. However, when intact peptide is absent measurements in terms of the standard have little quantitative significance since the slopes of the inhibition curves will differ. Similar conclusions from a study of the specificity of a radioimmunoassay for adrenocorticotrophic hormone led Landon, Girard & Greenwood (1968) to propose that plasma adrenocorticotrophic hormone should be expressed more critically as pg.equiv. of standard/ml. of plasma rather than the convenient but erroneous short-hand formulation of pg. of adrenocorticotrophic hormone/ml. of plasma.

Although the major effort has been directed

towards the measurement of plasma concentrations after experimental or physiological stimuli by using the chromatoelectrophoretic method, some additional current uses of the charcoal assay are being made. During the stimulation by prolactin of the mouse mammary gland in organ culture (Prop, 1966) the uptake of prolactin from the culture medium may be measured and the appearance of fragments monitored. Similarly the synthesis and release of prolactin by bovine tissue slices, as judged by amino acid incorporation into prolactin fractions, can be expressed as net synthesis by radioimmunoassay. Finally, isolation procedures for prolactin devised by time-consuming biological assays can be rapidly amended by immunological assays.

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