Size heterogeneity of rat pituitary prolactin

Michael WALLIS, Margaret DANIELS and Shirley A. ELLIS

School of Biological Sciences, University of Sussex, Falmer, Brighton, Sussex BN1 9QG, U.K.

(Received 21 February 1980)

The occurrence of multiple forms of rat prolactin with different molecular weights (size heterogeneity) was studied with anterior pituitary extracts, purified rat prolactin and ^{125}I -labelled rat prolactin. In each case, three main forms of the hormone were detected by gel filtration on Sephadex G-100: a major one (80–90%) corresponding to monomeric prolactin (mol.wt. 22 000–25 000), a peak (8–20%) that could be a dimer (mol.wt. 45 000–50 000) and a small quantity (1–5%) of a component of much greater molecular weight. On freezing and thawing of ^{125}I -labelled rat prolactin, there was little interconversion of monomer and 'dimer' peaks, but both were converted substantially to very high-molecular-weight material. All three peaks of ^{125}I -labelled rat prolactin could be precipitated by anti-(rat prolactin) serum and all three gave similar patterns of radioactive peptides after digestion with chymotrypsin followed by high-voltage paper electrophoresis. On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the monomer peak of ^{125}I -labelled prolactin migrated as a single component of mol.wt. 22 000, the very high-molecular-weight peak largely dissociated to a component running in the same position as the monomer, and the 'dimer' peak migrated partly as a component of mol.wt. 45 000 and partly as a component migrating with monomeric prolactin. No treatment was found that could dissociate the 'dimer' peak completely to monomeric prolactin.

Many protein and polypeptide hormones display size heterogeneity: multiple forms of the hormone occur, of different molecular weights. This is of interest both because of information that it may provide about precursors of the hormones, and because in many cases it has been shown to apply to the circulating hormones and therefore may have physiological significance.

Pituitary somatotropin (growth hormone), prolactin and placental lactogen all demonstrate high-molecular-weight forms, in addition to the monomeric molecules of mol.wt. approx. 20 000 (Berson & Yalow, 1971; Frohman *et al.*, 1972; Goodman *et al.*, 1972; Gorden *et al.*, 1973; Suh & Frantz, 1974; Schneider *et al.*, 1975; Lewis *et al.*, 1977). These large forms include dimers and species of greater molecular weight. They have been shown to occur in the circulation and in fresh pituitary extracts. The physiological significance of such forms is not yet understood, but it is clear that they occur in different proportions in the circulation according to the physiological (or pathological) state of the organism (von Werder & Clemm, 1974;

Rogol & Rosen, 1974; Guyda, 1975) and that they are secreted independently in some circumstances.

Most of our current information about high-molecular-weight forms of prolactin comes from studies on the human hormone. It was of interest therefore to try to assess the nature of such forms in the rat, a species in which many experimental studies on the secretion, metabolism and biological actions of the hormone have been carried out. The occurrence of multiple forms of prolactin in the rat pituitary and, in some circumstances, in the circulation has been reported by Stevens & Lawson (1977) and Lawson & Stevens (1978), but these forms have not been fully characterized. Since rat prolactin was only available to us in very small quantities, part of the study on size heterogeneity was carried out with ^{125}I -labelled rat prolactin, which allowed analysis of very small quantities of material. Information about the size heterogeneity of labelled prolactin is of interest because of the use of such material for biological studies and radioimmunoassays and because it may provide pointers to the nature of size heterogeneity of preparations of the hormone that have not been subjected to chemical modification in this way. A

Abbreviation used: SDS, sodium dodecyl sulphate.

brief report of some of this work has been presented elsewhere (Wallis, 1979).

Materials and methods

Materials

Rat prolactin for iodination (preparations NIAMDD-I-2 and I-3), rat prolactin for biological studies (NIAMDD-B1) and bovine prolactin (NIAMDD-P-B3) were gifts from Dr. A. Parlow and the National Institute of Arthritis, Metabolism and Digestive Diseases, N.I.H., Bethesda, MD, U.S.A. Antiserum and other reagents for use in radioimmunoassay of rat prolactin and rat somatotropin (growth hormone) were obtained from the same source. The antiserum used for immunoprecipitation of rat prolactin was raised in rabbits to rat prolactin preparation NIAMDD-B1, by using the procedure of Vaitukaitis *et al.* (1971).

Materials for gel filtration [Sephadex G-100 (Superfine grade) and Sephadex G-50 (fine grade)] were purchased from Pharmacia, Uppsala, Sweden. Marker proteins for gel electrophoresis, merthiolate (thimerosal), bovine serum albumin (Cohn fraction V), ovalbumin and sodium dodecyl sulphate were from Sigma (London), Poole, Dorset, U.K. Na^{125}I (carrier free; IMS 30) was from The Radiochemical Centre, Amersham, Bucks., U.K. The second antibody for immunoprecipitation (donkey anti-rabbit precipitating serum) was obtained from Wellcome Reagents, Beckenham, Kent, U.K. Poly(ethylene glycol) 6000, Chloramine-T and acrylamide were from BDH Chemicals, Poole, Dorset, U.K. α -Chymotrypsin ($3 \times$ crystallized) was from Worthington Biochemical Corp. (obtained through Cambrian Chemicals, Suffolk House, George Street, Croydon, U.K.).

Gel filtration

Gel filtration was carried out at 4°C on columns ($90\text{--}100\text{ cm} \times 0.78\text{ cm}^2$) of Sephadex G-100 Superfine grade, equilibrated and eluted usually with 'assay buffer' ($0.05\text{ M-NaH}_2\text{PO}_4$, adjusted to pH 7.6 with NaOH, containing 0.05% bovine serum albumin and 0.6 mM -merthiolate). In some cases different eluting buffers were used, and these are indicated in the text. Fractions collected from these columns were monitored for radioactivity with a Nuclear Chicago γ -radiation counter (model 1195); counting efficiency for ^{125}I was $50\text{--}55\%$. For molecular-weight calibration of these columns, bovine serum albumin (mol.wt. 66 000), ovalbumin (mol.wt. 45 000) and bovine prolactin (mol.wt. 22 700) were used.

Iodination of rat prolactin

The procedure used was based on that of Roth

(1975). Rat prolactin ($10\text{ }\mu\text{g}$) was dissolved in $30\text{ }\mu\text{l}$ of sodium phosphate buffer ($0.5\text{ M-NaH}_2\text{PO}_4$, adjusted to pH 7.6 with NaOH) and mixed with $10\text{ }\mu\text{l}$ (1 mCi) of Na^{125}I . Chloramine-T ($0.5\text{--}1\text{ }\mu\text{g}$; 1 mg/ml in sodium phosphate buffer, pH 7.6) was added and the mixture was left at room temperature (20°C) for 3 min. Incorporation of ^{125}I into protein was assessed by trichloroacetic-acid precipitation of approx. $0.1\text{ }\mu\text{l}$ of the reaction mixture (Roth, 1975); if incorporation was less than 40% , a further $0.5\text{--}1\text{ }\mu\text{g}$ of Chloramine-T was added and the incubation and the trichloroacetic acid precipitation were repeated.

Once incorporation of ^{125}I into protein was over 40% (usually achieved with one or two additions of Chloramine-T), labelling was stopped by addition of $100\text{ }\mu\text{l}$ of 0.1 M-KI in 'assay buffer' (see above), and the mixture was put on a column ($13\text{--}15\text{ cm} \times 0.78\text{ cm}^2$) of Sephadex G-50 (fine grade) equilibrated and eluted with the same 'assay buffer'. Fractions (1.0 ml) were collected and $5\text{ }\mu\text{l}$ of each was counted for γ radioactivity. The first peak to emerge represented ^{125}I -labelled rat prolactin. Incorporation of ^{125}I into prolactin with this procedure was $40\text{--}80\%$, and the product had a specific activity of $40\text{--}80\text{ }\mu\text{Ci}/\mu\text{g}$ of prolactin.

Radioimmunoassay of rat prolactin

All dilutions of standards, samples, ^{125}I -labelled rat prolactin and antiserum were carried out with 'assay buffer' (see above). Each assay tube contained 0.1 ml of antiserum [NIAMDD anti-(rat prolactin) serum 6; diluted $1:4000$], 0.1 ml of sample or standard and (added after 1 h) 0.1 ml of ^{125}I -labelled prolactin (the monomer peak from a Sephadex G-100 column; approx. 10 nCi). These were incubated for 44 h at $18\text{--}23^\circ\text{C}$ or for $70\text{--}90\text{ h}$ at 4°C and antibody-bound hormone was then precipitated with poly(ethylene glycol) (Desbuquois & Aurbach, 1971), by adding to each tube 0.3 ml of a cold (4°C) mixture containing 25% (w/v) poly(ethylene glycol), 0.15% bovine γ -globulin and 0.05 M-Tris , adjusted to pH 8.5 with HCl. After mixing, tubes were stood at 4°C for 1 h for completion of precipitation. They were then centrifuged (20 min , approx. 1000 g , 4°C), the supernatants were removed by aspiration and precipitates were counted for γ -radiation.

All estimates of rat prolactin are given in terms of the reference preparation (NIAMDD rat prolactin RP-1) provided by NIAMDD. The minimum detectable amount of rat prolactin in this assay was $0.1\text{--}0.2\text{ ng}$ of the standard and 50% displacement of ^{125}I -labelled prolactin was achieved with $0.4\text{--}0.7\text{ ng}$ of standard. Information provided with the assay reagents by Dr. Parlow indicated that there was no cross-reaction with rat follitropin, lutropin, thyrotropin or somatotropin in this assay.

Radioimmunoassay of rat somatotropin (growth hormone)

The assay was carried out in the same way as that for rat prolactin, with antiserum to rat somatotropin [NIAMDD anti-(rat growth hormone) serum, GHS3; diluted 1:30000], and ^{125}I -labelled somatotropin (N.I.A.M.D.D. rat GH-I-2) prepared as described for ^{125}I -labelled prolactin. All estimates of rat somatotropin are given in terms of the reference preparation (rat GH-RP-1) provided by the NIAMDD. The minimum detectable amount of this standard was 0.2–0.3 ng and 50% displacement of ^{125}I -labelled somatotropin was achieved with 0.4–0.7 ng of standard. Information provided with the assay reagents by Dr. Parlow indicated that there was no cross reaction with rat follitropin, lutropin or thyrotropin in this assay; rat prolactin gave approx. 1.6% cross reaction.

Extraction of pituitary glands

Female Sprague–Dawley rats (8–10 weeks old) from the laboratory colony were killed by decapitation, the pituitary gland was removed, and the posterior lobe was discarded. The anterior lobes were homogenized in $\text{NH}_4\text{HCO}_3/\text{NH}_3$ buffer (0.2 M- NH_4HCO_3 , adjusted to pH 8.5 with conc. aq. NH_3) and the homogenate was clarified by centrifugation before gel filtration.

Polyacrylamide-gel electrophoresis in SDS

Various fractions of iodinated rat prolactin were analysed by polyacrylamide-gel electrophoresis in the presence of SDS. Gels were prepared in glass tubes (0.6 cm $\times$ 9.0 cm) by the method of Weber & Osborn (1969) and contained 10% (w/v) polyacrylamide, with 2.7% crosslinking, 0.1% SDS and sodium phosphate buffer (28 mM- NaH_2PO_4 /72 mM- Na_2HPO_4 , pH 7.0). Samples were usually dissolved in sample buffer containing 1% SDS, 1% 2-mercaptoethanol, 0.005% Bromophenol Blue and 6 M-urea in the same phosphate buffer, and were heated at 37°C for 2 h. In some cases, this pretreatment was modified, as indicated in the text. Samples were applied to the tops of gels under the electrode buffer (0.1% SDS in the phosphate buffer detailed above) and electrophoresis was carried out at a constant current of 8 mA/gel until the Bromophenol Blue marker reached the bottom of the gel. After electrophoresis, gels were stained in 0.25% Coomassie Brilliant Blue R dissolved in methanol/acetic acid/water (9:2:9 by vol.) for 2 h and were destained electrophoretically. Samples of iodinated prolactin were usually mixed with carrier prolactin (20 μg of bovine prolactin) before electrophoresis, and/or with a mixture of marker proteins containing bovine serum albumin (mol.wt. 66 000), ovalbumin (mol.wt. 45 000), pepsin (mol.wt. 34 700), trypsinogen (mol.wt. 24 000), β -lactoglobulin

(subunit mol. wt. 18 400) and lysozyme (mol.wt. 14 300).

After staining, gels were drawn and then were cut into slices (1 or 2 mm) that were counted for γ radioactivity.

Immunoprecipitation

The immunological activity of various fractions of iodinated prolactin was determined by immunoprecipitation, with a rabbit antiserum against rat prolactin. Samples, antiserum and prolactin were dissolved in 'assay buffer' (see above). A sample (0.1 ml) of a fraction containing ^{125}I -labelled rat prolactin (2–20 nCi) was mixed with 0.1 ml of a 1:450 dilution of anti-(rat prolactin) serum in 'assay buffer' and 0.1 ml of buffer or rat prolactin solution (10 $\mu\text{g}/\text{ml}$). After incubation for 66 h at 20–25°C, 0.1 ml of second antibody (donkey anti-rabbit precipitating serum, diluted 1:60) was added and the tubes were incubated for a further 24 h at 4°C. The first antibody was used at a sufficiently high concentration for carrier serum to be unnecessary. The tubes were then centrifuged (20 min, 1000 g, 4°C) and the precipitates and supernatants were counted for γ -radioactivity. Radioactivity bound in the presence or absence of an excess (1 μg) of unlabelled rat prolactin indicated the non-specific and total binding respectively.

Preparation and fractionation of enzymic digests of ^{125}I -labelled prolactin

Samples of ^{125}I -labelled prolactin (20–70 nCi) dissolved in 'assay buffer' (see above) were mixed with 100 μg of bovine prolactin and heated at 100°C for 15 min to denature the protein. They were then cooled, 20 μg of chymotrypsin was added to each and the samples were incubated at 37°C for 20 h. The digests were dried *in vacuo* and then subjected to high-voltage paper electrophoresis in white spirit-cooled tanks (Michl, 1951) at pH 6.5 in pyridine/acetic acid/water (100:3:897, by vol.) at 40 V/cm for 90 min. After drying the paper, radioactive peptides were detected by radioautography or by cutting the paper into 1.0 or 0.5 cm strips and counting them for γ -radioactivity.

Results

Size heterogeneity of prolactin and somatotropin in the rat anterior pituitary gland

When a fresh extract of an anterior pituitary gland from a female rat was submitted to gel filtration on Sephadex G-100 (Superfine grade) and the eluate was analysed by radioimmunoassays for rat prolactin and somatotropin, size heterogeneity of both of these hormones was revealed (Fig. 1). In each case the major peak (peak M) of immunoreactivity detected migrated in a position corresponding to the

Fig. 1. Size heterogeneity of prolactin and somatotropin in the rat anterior pituitary gland

One hemipituitary gland from a female rat was extracted and half of the extract was fractionated by gel filtration on a column ($90.2\text{ cm} \times 0.78\text{ cm}^2$) of Sephadex G-100 (Superfine grade) using 'assay buffer' (see text) as eluant. Fractions (1.17 ml) were collected, and these were assayed for prolactin (a) and somatotropin (b) by radioimmunoassay.

monomeric hormone (a mol.wt. of approx. 25 000 for prolactin). The prolactin radioimmunoassay also revealed a substantial peak (peak D) that was eluted earlier, in a position corresponding to a mol.wt. of approx. 50 000; this could be a dimer, and corresponds to about 15% of the total immunoreactive prolactin. A much smaller peak of prolactin immunoreactivity (peak A) was detected close to the void volume of the column, presumably representing very high-molecular-weight material (aggregate). Small amounts of high-molecular-weight components were also detected by the somatotropin radioimmunoassay, but these were much less evident than for prolactin.

Size heterogeneity in a purified preparation of rat prolactin

A purified preparation of rat prolactin (NIAMDD rat prolactin I-3) was also subjected to gel filtration on Sephadex G-100 (Superfine grade) and the eluate was analysed by radioimmunoassay for rat prolactin. Again, size heterogeneity was demonstrated (Fig. 2). As with the fresh anterior pituitary homogenate, the monomeric

Fig. 2. Size heterogeneity of purified rat prolactin. Rat prolactin ($4.5\text{ }\mu\text{g}$) (NIAMDD rat prolactin I-3) was fractionated on a column ($91.4\text{ cm} \times 0.78\text{ cm}^2$) of Sephadex G-100 (Superfine grade), with 'assay buffer' as eluant. Fractions (approx. 0.6 ml) were collected and assayed for prolactin by radioimmunoassay. Recovery of immunoreactive prolactin from the column was 100%.

form (peak M) predominated, but there was 10–15% of a larger component migrating in the same position as peak D found for the pituitary extract (i.e. a mol.wt. of approx. 50 000) and a trace of prolactin-like material of higher molecular weight.

Size heterogeneity of ^{125}I -labelled rat prolactin

Since purified rat prolactin was in short supply, an investigation of size heterogeneity of the hormone after labelling with Na^{125}I was carried out. The increased sensitivity of detection provided by this permitted several types of investigation which would not otherwise have been possible.

Gel filtration of ^{125}I -labelled rat prolactin again revealed size heterogeneity, with a peak of radioactivity corresponding to peak D ('dimer') detected in unlabelled material, in addition to peak M, corresponding to monomeric prolactin (Fig. 3). A small amount of high-molecular-weight material migrating near the void volume of the column was also present, and this amount increased considerably if the preparation of labelled hormone was stored frozen before chromatography. The relative proportion of peak D was rather variable from one preparation of ^{125}I -labelled hormone to another, and in some cases a peak of higher-molecular-weight material (mol.wt. 60 000–70 000) was detected, which was shown (by SDS/polyacrylamide-gel electrophoresis) to represent ^{125}I -non-covalently associated with an unlabelled macromolecule (possibly serum albumin).

Fig. 3. Size heterogeneity of ^{125}I -labelled rat prolactin. ^{125}I -labelled rat prolactin (9 μCi ; 130 ng) (NIAMDD rat prolactin I-3) was fractionated by gel filtration on a column (96.1 cm $\times$ 0.78 cm 2) of Sephadex G-100 Superfine grade, with 'assay buffer' as eluant. Fractions (approx 0.7 ml) were collected and the radioactivity in each was determined by counting of γ radioactivity. Recovery of radioactivity for the column was 81%.

When the three main peaks obtained by gel filtration of ^{125}I -labelled rat prolactin were rechromatographed immediately after the original gel filtration they largely ran 'true' (Fig. 4). There was, however, some suggestion of conversion of 5–10% of the very high-molecular-weight peak to peak M, and vice versa. These peaks showed little sign of conversion to peak D, but there may have been some conversion of peak D to peak M or to high-molecular-weight material. The peaks seen in Fig. 3 thus appear to represent real, fairly stable, entities. When material corresponding to peaks M, D or A was stored for 1–2 weeks (at 4°C) before chromatography, interconversion of the various forms increased, though again there was little suggestion of conversion of the very high-molecular-weight component, or of peak M, into peak D.

The chromatographic separations described in the previous paragraphs were carried out with very small quantities of labelled prolactin in the presence of rather large amounts of serum albumin and merthiolate. It was possible that these components of the eluent were responsible for the interconversions of the various forms of the iodinated hormone, and various alternative buffers were therefore used. When serum albumin was left out of the buffer, no labelled material was eluted from the Sephadex G-100 column and a γ -radiation monitor showed that the radioactivity was bound to the column. A carrier protein (or equivalent) was thus essential for chromatography. When bovine serum albumin was

Fig. 4. Rechromatography of components of ^{125}I -labelled rat prolactin

Portions of peaks A (a; 140 nCi), D (b; 130 nCi) and M (c; 230 nCi) from a column similar to that shown in Fig. 3 were rechromatographed on Sephadex G-100 Superfine grade columns (96 cm $\times$ 0.78 cm 2), with 'assay buffer' as eluant. Fractions (approx. 1.0 ml) were collected and the radioactivity in each was determined by counting of γ radioactivity. Recovery of radioactivity from the columns was 68–81%.

replaced by horse heart myoglobin (chosen for its lack of cystine or cysteine) an elution pattern similar to that shown in Fig. 3 was obtained. Material from the various peaks again rechromatographed 'true' with slight interconversions, as observed previously in the presence of serum albumin. When the protein carrier was replaced by a casein hydrolysate an elution pattern similar to that seen in Fig. 3 was again seen, but the recovery of label from the column was much lower than that seen with serum albumin or myoglobin. Rechromatography of peaks obtained by using casein hydrolysate-containing

buffers again showed slight interconversion of peaks, but recovery of labelled material was now very poor. When gel filtration was carried out in buffer containing bovine serum albumin but without merthiolate, the elution pattern was very similar to that seen in the presence of this agent; however, this buffer was very susceptible to microbial contamination and could not be used for long-term experiments.

Effects of freezing and thawing of labelled prolactin

Repeated freezing and thawing of dilute solutions of iodinated or unmodified prolactin (and other protein hormones) is usually considered to be deleterious, and it was of interest to determine whether such treatment might affect the aggregation state of the labelled prolactin studied here. Samples from peaks D and M obtained after gel filtration of ^{125}I -labelled rat prolactin were frozen and thawed five times (by using an ethanol/solid CO_2 freezing mixture) and then rechromatographed. The results are illustrated in Fig. 5. Freezing and thawing caused

Fig. 5. Rechromatography of components of ^{125}I -labelled rat prolactin after freezing and thawing. Portions of peaks D (a; 70nCi) and M (b; 70nCi) from a column similar to that shown in Fig. 3 were frozen and thawed five times and then rechromatographed on Sephadex G-100 Superfine grade columns ($96\text{ cm} \times 0.78\text{ cm}^2$), with assay buffer as eluant. Fractions (approx 0.9 ml) were collected and the radioactivity in each was determined by counting of γ radioactivity. Recovery of radioactivity from the columns was 83–90%.

substantial aggregation of both peaks D and M. There was however no interconversion of peaks D and M.

Similar results were obtained when the initial fractionation of labelled prolactin was carried out in buffers containing myoglobin or casein hydrolysate instead of bovine serum albumin, suggesting that the aggregations caused by freezing and thawing were not due to interactions between labelled hormone and serum albumin in the buffer.

Electrophoresis of ^{125}I -labelled prolactin in the presence of SDS

Samples of the ^{125}I -labelled prolactin peaks from a Sephadex G-100 (Superfine grade) column similar to that shown in Fig. 3 were subjected to SDS/polyacrylamide-gel electrophoresis. Molecular weight markers were included in the samples, and each sample was heated with 2-mercaptoethanol and SDS. After electrophoresis the gels were stained, destained, sliced and counted for γ radioactivity. The results are shown in Fig. 6. The monomer peak (M) from gel filtration migrated as a single component of mol.wt. 22000 (Fig. 6c). A sample taken from the trailing edge of the monomer peak showed a similar electrophoretic pattern, but some lower molecular weight material was also present (Fig. 6d). The high molecular weight peak (A) migrated largely as a component of mol.wt. 21400, suggesting that it was dissociated to monomer by the conditions used (Fig. 6a). The 'dimer' peak (D) gave a more complex pattern on electrophoresis, migrating partly with a mobility similar to that of the monomer (mol.wt. 22100) and partly as a component with lower mobility and an apparent mol.wt. 45000 (Fig. 6b). In each case, the electrophoretic pattern was similar whether or not 2-mercaptoethanol was included in the starting sample.

It has been mentioned above that in some gel filtration fractionations of ^{125}I -labelled rat prolactin an additional component of mol.wt. 60000–70000 was detected. When this was subjected to SDS/polyacrylamide gel electrophoresis, a component of this size was not detected, but a large peak of labelled, very low-molecular-weight material was observed (before the gels were stained) with mobility similar to that of free $^{125}\text{I}^-$. The component of mol.wt. 60000–70000 thus seems to be a non-covalent association product between I^- (or possibly another low-molecular-weight component) and a macromolecule, possibly serum albumin.

Immunoprecipitation of fractions of ^{125}I -labelled prolactin

The immunological activity of each of the peaks from the Sephadex G-100 column shown in Fig. 3 was tested by immunoprecipitation with rabbit anti-(rat prolactin) serum, as described in the

Fig. 6. SDS/polyacrylamide-gel electrophoresis of the components of ^{125}I -labelled rat prolactin

Portions of peaks A (a; 1.8 nCi), D (b; 5.2 nCi), M (c; 49.0 nCi) and of the trailing edge of M (d; 4.7 nCi), were subjected to SDS/polyacrylamide-gel electrophoresis after addition of molecular weight markers and reduction with 2-mercaptoethanol. After electrophoresis, the gels were cut into 2 mm slices, which were counted for γ radioactivity. The top of the gel (cathode) is to the left.

Fig. 7. High-voltage electrophoresis of the components of ^{125}I -labelled rat prolactin after digestion with chymotrypsin

Portions of peaks A (a; approx. 17 nCi), D (b; approx. 55 nCi), M (c; approx. 59 nCi), the trailing edge of peak M (d; approx. 37 nCi) from the column shown in Fig. 3 and ^{125}I -labelled rat somatotropin (e; approx. 39 nCi) were digested with chymotrypsin. The digests were fractionated by high-voltage paper electrophoresis at pH 6.5 (90 min, 40 V/cm) and the electrophoretograms were then cut into 1 cm or 0.5 cm strips and counted for γ radioactivity. The arrows indicate the point of application. The anode is to the left.

Materials and methods section. The results are shown in Table 1. Each of the peaks was precipitated by the antiserum, precipitation of the monomer (peak M) being rather greater than that of the other components. The immunoprecipitation was specific, in that 89–94% of the radioactivity bound could be displaced by unlabelled rat prolactin. ^{125}I -labelled rat somatotropin showed slight binding to the antibody used, possibly due to contamination of the rat prolactin used to raise antibody or the rat somatotropin used for iodination. The high concen-

tration of antibody used in this experiment probably exaggerates the binding of ^{125}I -labelled somatotropin; no evidence for displacement of ^{125}I -labelled prolactin by rat somatotropin has been obtained with the much lower antibody concentration used for radioimmunoassay (which was also, of course, obtained from a different source).

Table 1. Immunoprecipitation of the components of ^{125}I -labelled rat prolactin

Portions of peaks A, D and M and of the trailing edge of peak M from the column shown in Fig. 3, and also ^{125}I -labelled rat somatotropin, were reacted with rabbit anti-(rat prolactin) serum; they were then precipitated with a second antibody and the radioactivity present in the precipitates was determined by counting of γ -radioactivity. The total radioactivity precipitated, the non-specific precipitation (that portion of the total not displaceable by $1\mu\text{g}$ of unlabelled rat prolactin) and the specific precipitation (the difference between total and non-specific precipitation) are all shown as a percentage of the total radioactivity added. All values are the means $\pm$ S.E.M. for three observations.

^{125}I -labelled sample	Precipitation of radioactivity (%)		
	Total	Non-specific	Specific
Peak A (^{125}I -labelled rat prolactin aggregate)	85.9 $\pm$ 2.2	9.4 $\pm$ 0.1	76.5
Peak D (^{125}I -labelled rat prolactin 'dimer')	82.3 $\pm$ 0.9	7.1 $\pm$ 0.2	75.2
Peak M (^{125}I -labelled rat prolactin monomer)	91.7 $\pm$ 0.8	5.5 $\pm$ 0.3	86.2
Trailing edge of peak M	85.3 $\pm$ 1.0	5.8 $\pm$ 0.1	79.5
^{125}I -labelled rat somatotropin	13.6 $\pm$ 1.8	8.2 $\pm$ 0.6	5.4

Enzymic digestion of ^{125}I -labelled prolactin

Portions of each of the prolactin peaks from the Sephadex G-100 column shown in Fig. 3 were digested with chymotrypsin, as described in the Materials and methods section. Digests were then fractionated by high-voltage electrophoresis, at pH 6.5, and the distribution of labelled peptides on the electrophoretograms was determined (Fig. 7). Each of the peaks of labelled prolactin gave a similar peptide pattern, which was clearly different from the pattern obtained with a corresponding digest of ^{125}I -labelled rat somatotropin (all samples showed a major peak moving slightly to the cathode, but this is the position to which neutral peptides move, and of minor significance for purposes of comparison). Undigested samples of the various labelled prolactin peaks did not move from the origin.

Fractionation of the digests by high-voltage electrophoresis at pH 2 also gave very similar patterns for the components of ^{125}I -labelled prolactin, but a clearly distinct one for a digest of ^{125}I -labelled rat somatotropin. Fractionation of tryptic digests led to a similar conclusion.

Discussion

The results described here demonstrate that several forms of immunoreactive prolactin with different molecular weights exist in extracts of female rat anterior pituitary glands, and that these different forms (or similar ones) also occur in a purified rat prolactin preparation and in ^{125}I -labelled rat prolactin. In fresh solutions monomeric material (mol.wt. 20 000–25 000) predominates (80–90%), but is accompanied by a peak of material of mol.wt. 45 000–50 000 which could be a dimer (8–20%) and by a small amount of very high-molecular-weight material (1–5%). In solutions that have been subjected to freezing and thawing the proportion of

very high-molecular-weight material increases. A similar result for rat pituitary extract was found by Stevens & Lawson (1977).

Since rat prolactin was in short supply, most of the studies described here were performed with ^{125}I -labelled rat prolactin (which could be detected with enhanced sensitivity). The results obtained are of direct interest, because of the widespread use of such labelled material for radioimmunoassays and radioreceptor assays. ^{125}I -labelled prolactin prepared by a version of the Chloramine-T technique by using more vigorous treatment than that used here is biologically active (Bullough & Wallis, 1977) and the modification appears to be confined primarily to tyrosine residues. It is likely also that the heterogeneity seen in iodinated prolactin reflects that occurring in the unmodified hormone, and probably that in the pituitary gland itself.

The three peaks of ^{125}I -labelled rat prolactin that were eluted from Sephadex G-100 (Superfine grade) were prolactin-like in that they were precipitated by antiserum to rat prolactin. They also gave similar patterns of labelled peptides when subjected to proteolytic digestion followed by high-voltage electrophoresis. This suggests extensive similarity in primary structure, despite the large differences in molecular weight, and tends to support the idea that the three peaks are all based on a prolactin monomer. Association of prolactin with another, non-prolactin-like, protein seems unlikely since there is no evidence for additional peptides in the digests of the larger components.

When the three peaks of ^{125}I -labelled rat prolactin were rechromatographed on Sephadex G-100 they re-ran 'true', though there appeared to be a slight conversion of high-molecular-weight material into monomer and vice versa. In the case of rechromatography of the 'dimer' (peak D), traces of monomer and high-molecular-weight material on rechromatography (Fig. 4b) may have been a

consequence of contamination of peak D from the first chromatogram, rather than conversion of 'dimer'.

Freezing and thawing of the monomer and 'dimer' peaks had a marked effect on the aggregation state as revealed by subsequent gel filtration. The procedure caused marked aggregation of both monomer and 'dimer' to high-molecular-weight (mol.wt. greater than 100000) forms, though there was no suggestion of formation of 'dimer' from monomer or vice versa by such treatment. Conversion of monomer to large aggregates was almost 90% after five cycles of freezing and thawing, but conversion of 'dimer' to large aggregates was only about 50%. It is not clear whether this last reflected a heterogeneity in the 'dimer' peak (only some of the material being aggregated) or a lower susceptibility to aggregation of the whole of the material in this peak. It has long been known that repeated freezing and thawing can denature some proteins. These results confirm that iodinated rat prolactin (and probably many other protein hormones) is aggregated by repeated freezing and thawing which should, therefore, be avoided in experimental manipulations.

The peaks of ^{125}I -labelled prolactin from a gel-filtration column were submitted to SDS/polyacrylamide-gel electrophoresis, after reduction of disulphide bridges with mercaptoethanol, to assess how components separated by gel filtration (in the absence of detergent) would behave under denaturing conditions. The monomer component migrated as a single peak, with mobility corresponding to mol.wt. 22000. The 'dimer' (peak D) from gel-filtration columns showed two main components after electrophoresis, one of which corresponded to mol.wt. 45000 (about 48% of the total radioactivity recovered) and the other to mol.wt. 22100 (about 37% of the total). SDS clearly caused considerable dissociation of this 'dimer' component, but the dissociation was incomplete, presumably because peak D is heterogeneous. A similar situation has been observed with the dimeric form of human somatotropin (Lewis *et al.*, 1977), where part of the mol.wt. 45000 component could be dissociated by 2-mercaptoethanol and was thought to be a disulphide-bridged dimer, whereas part of this component could not be dissociated in this way. On the other hand, the 'big' prolactin in human plasma can be almost completely converted to monomer by reduction with 2-mercaptoethanol (Benveniste *et al.*, 1979). The high-molecular-weight material (peak A) from gel filtration largely dissociated on SDS/polyacrylamide-gel electrophoresis to material with a molecular weight identical with that of monomeric prolactin, and also some material rather smaller than this.

Large aggregates and dimeric forms of prolactin have been detected previously in man and in the rat

and suggestions have been made by several authors that they are of physiological significance. The present study suggests that the very high-molecular-weight forms of prolactin are aggregates, which are readily formed by freezing and thawing (and perhaps by other experimental manipulations). Association between prolactin and soluble dextran may contribute slightly to the formation of such aggregates (Fang *et al.*, 1980), though the inclusion of carrier bovine serum albumin in eluting buffers used here should largely eliminate such association (Fang *et al.*, 1980). Appearance of such very high-molecular-weight forms of prolactin in plasma, pituitary extracts or purified prolactin preparations may often be due to such experimental manipulations rather than to physiological causes.

On the other hand, the (presumed) dimeric forms of iodinated rat prolactin were not formed from monomeric or aggregated prolactin under the experimental conditions we have studied; such dimers [which appear to be equivalent to the 'big' human prolactin described by Suh & Frantz (1974) and others] may thus represent a naturally occurring form of the hormone, a physiological role for which cannot be ruled out. It seems unlikely, however, that any such naturally occurring large form of prolactin can represent a biosynthetic precursor of the 'monomeric' prolactin, particularly in view of the fact that the primary translation product of prolactin mRNA (preprolactin) produced in cell-free protein synthesizing systems has a mol.wt. of only approx. 25000 (Maurer *et al.*, 1976; Evans & Rosenfeld, 1976; Lingappa *et al.*, 1977; Austin & Wallis, 1979).

We thank the Agricultural and Medical Research Councils for research support and Dr. A. Parlow and the National Institute of Arthritis, Metabolism and Digestive Diseases for gifts of rat prolactin, bovine prolactin and materials for radioimmunoassay of rat prolactin and rat somatotropin. We are grateful to Miss Joanna Harper for skilled preparation of the manuscript.

References

- Austin, S. A. & Wallis, M. (1979) *Biochim. Biophys. Acta* **564**, 534–545
- Benveniste, R., Helman, J. D., Orth, D. N., McKenna, T. J., Nicholson, W. E. & Rabinowitz, D. (1979) *J. Clin. Endocrinol.* **48**, 883–886
- Berson, S. A. & Yalow, R. S. (1971) in *Les Adénomes Hypophysaires Sécrétants: Endocrinopathies et Immunologie: Rapports de la XI^e Reunion des Endocrinologistes de Langue Française*, pp. 239–269, Masson et Cie, Paris
- Bullough, W. A. & Wallis, M. (1977) *Horm. Res.* **8**, 37–50
- Desbuquois, B. & Aurbach, G. D. (1971) *J. Clin. Endocrinol.* **33**, 732–738

- Evans, G. A. & Rosenfeld, M. G. (1976) *J. Biol. Chem.* **251**, 2842–2847
- Fang, V. S., Shian, L.-R. & Worsley, I. G. (1980) in *Molecular Endocrinology* (MacIntyre, I., ed.), Elsevier/North-Holland, Amsterdam, in the press
- Frohman, L. A., Burek, L. & Stachura, M. E. (1972) *Endocrinology* **91**, 262–269
- Goodman, A. D., Tanenbaum, R. & Rabinowitz, D. (1972) *J. Clin. Endocrinol.* **35**, 868–878
- Gorden, P., Hendricks, C. M. & Roth, J. (1973) *J. Clin. Endocrinol.* **36**, 178–184
- Guyda, H. J. (1975) *J. Clin. Endocrinol.* **41**, 953–967
- Lawson, D. M. & Stevens, R. W. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 637
- Lewis, U. J., Peterson, S. M., Bonewald, L. F., Seavey, B. K. & VanderLaan, W. P. (1977) *J. Biol. Chem.* **252**, 3697–3702
- Lingappa, V. R., Devillers-Thiery, A. & Blobel, G. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2432–2436
- Maurer, R. A., Stone, R. & Gorski, J. (1976) *J. Biol. Chem.* **251**, 2801–2807
- Michl, H. (1951) *Monatsh. Chem.* **82**, 489–493
- Rogol, A. D. & Rosen, S. W. (1974) *J. Clin. Endocrinol.* **38**, 714–717
- Roth, J. (1975) *Methods Enzymol.* **37**, 223–233
- Schneider, A. B., Kowalski, K. & Sherwood, L. M. (1975) *Endocrinology* **97**, 1364–1372
- Stevens, R. W. & Lawson, D. M. (1977) *Life Sci.* **20**, 1961–1968
- Suh, H. K. & Frantz, A. G. (1974) *J. Clin. Endocrinol.* **39**, 928–935
- Vaitukaitis, J., Robbins, J. B., Nieschlag, E. & Ross, G. T. (1971) *J. Clin. Endocrinol.* **33**, 988–991
- von Werder, K. & Clemm, C. (1974) *FEBS Lett.* **47**, 181–184
- Wallis, M. (1979) *J. Endocrinol.* **81**, 122P
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412