Hydrophobic-interaction chromatography and anion-exchange chromatography in the presence of acetonitrile

A two-step purification method for human prolactin

Steven C. HODGKINSON* and Philip J. LOWRY

The Pituitary Hormone Laboratory, Department of Chemical Pathology, St. Bartholomew's Hospital, 51–53 Bartholomew Close, London EC1A 7BE, U.K.

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Described is a two-chromatographic-step preparative-scale technique for the purification of human prolactin from a frozen pituitary homogenate. The method utilizes hydrophobic interaction chromatography on the mildly hydrophobic adsorbent phenyl-Sepharose CL-4B and anion-exchange chromatography on DEAE-cellulose in the presence of acetonitrile. Human prolactin was solubilized at pH 10.0 after a prior extraction of pituitaries at pH 4.0, the acid pH being ineffective at solubilizing human prolactin but capable of solubilizing large amounts of interfering protein. An 11-fold increase in the potency of the solubilized human prolactin was achieved in this manner. Prolactin could be adsorbed to phenyl-Sepharose at low ionic strengths ($I < 0.01$); few other proteins were adsorbed under these conditions. This is a demonstration of the hydrophobic nature of human prolactin. The amount of phenyl-Sepharose was limited to the minimum (35 mg of protein/g of phenyl-Sepharose) necessary to adsorb human prolactin, further reducing the uptake of other pituitary protein. Desorption was achieved by using an acetonitrile gradient (0–30%, v/v), resulting in a purification of human prolactin of 85-fold and recovery of 78%. Acetonitrile (20%, v/v) was also included in all buffers for DEAE-cellulose chromatography, increasing the resolution and recovery of human prolactin, apparently by minimizing non-ionic interactions with the matrix. Prolactin (10 mg) was recovered from 63 g of pituitaries, an overall recovery of 58%. It was homogeneous by gel filtration and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, contained less than 0.1% somatotropin (growth hormone), on iodination demonstrated more than 95% binding to excess anti-(human prolactin) serum and could be displaced from anti-(human prolactin) serum in a manner indistinguishable from the serum of a patient with a human prolactin-secreting adenoma.

The existence of a distinct lactogenic hormone (prolactin) in the human was not widely accepted until 1972 (Bewley *et al.*, 1970; Geschwind, 1972). Attempts to isolate a human prolactin by procedures similar to those used for bovine and ovine prolactin had been unsuccessful, and preparations of modified human somatotropin with increased prolactin activity and lowered growth-promoting activity had been reported (Wilhelmi, 1961; Apostolakis, 1965). However, the immunological, histological and clinical evidence for a separate prolactin was

increasing (Canfield & Bates, 1965; Kleinberg & Frantz, 1971; Guyda *et al.*, 1971; Friesen *et al.*, 1970) and has been summarized in a review (Friesen & Hwang, 1973). The isolation and characterization of human prolactin from frozen pituitary glands was achieved in 1971 (Lewis *et al.*, 1971; Hwang *et al.*, 1972) and from acetone-dried pituitary tissue in 1975 (Hwang *et al.*, 1975; Rathnam & Saxena, 1977).

The early methods for the purification of human prolactin involved a preliminary extraction of pituitary tissue in saline to remove blood protein and much somatotropin, followed by re-extraction at pH 10.0 to solubilize the prolactin (Lewis *et al.*,

* To whom correspondence and requests for reprints should be addressed.

1971). This system was modified (Lewis & Singh, 1972, 1977) by replacing the pH 10.0 extraction with one using ethanolic (60%, v/v) buffer, pH 9.5, followed by precipitation of solubilized material at pH 6.0 with increased ethanol (85%, v/v). The precipitate was resuspended in 0.01 M-NH₄HCO₃ buffer, pH 9.5, and chromatographed on Sephadex G-150, the human prolactin-bearing fractions being further chromatographed on DEAE-cellulose to remove somatotropin. A major problem with this system has been the poor recovery of human prolactin after solvent precipitation. Prolactin appears to associate irreversibly with itself or other insoluble material (Lewis *et al.*, 1972). We have noted similar losses on isoelectric or salt precipitation. In addition, enzymic proteolysis (Lewis & Singh, 1974) further lowers the recovery of human prolactin, and inhibitors must be included to decrease this tendency. Other purification techniques, including preparative isoelectric focusing, have been utilized in the purification of human prolactin (Rathnam & Saxena, 1977; Ben-David & Chrambach, 1977). Although capable of resolving the closely related isomorphous forms of human prolactin, the system is hindered by the 100-fold molar excess of somatotropin, which is physicochemically very similar to human prolactin, present in the pituitary.

Chromatography on DEAE-cellulose has been employed to separate human prolactin from somatotropin, but often fails to achieve this (Hwang *et al.*, 1972; Lewis & Singh, 1974), and we have noted poor recoveries of human prolactin from DEAE-cellulose. Similarly, CM-cellulose, though separating human prolactin from somatotropin, has been associated with low recoveries of human prolactin (S. C. Hodgkinson, unpublished work). Low recoveries on ion-exchange chromatography led us to suspect that human prolactin may be associating with the matrix by a non-ionic as well as an ionic mechanism as the increasing salt concentrations necessary for the recovery of human prolactin from charged groups on the matrix are the same as those that encourage its adsorption to uncharged hydrophobic groups.

The requirement of high pH to solubilize human prolactin, its self-association, poor resolution and low recovery on ion-exchange chromatography led us to suspect that human prolactin has some hydrophobic character, and we have developed a purification technique that exploits, rather than suffers from, this enhanced hydrophobicity compared with other pituitary proteins.

Materials and methods

Materials

Human pituitaries were collected *post mortem* and stored at -20°C for up to 3 months

before extraction. Phenyl-Sepharose CL-4B, octyl-Sepharose CL-4B, and Sephadex G-100 (40–60 µm particle size) were purchased from Pharmacia Fine Chemicals Ltd., Hounslow, Middx., U.K. DEAE-cellulose DE-52 was purchased from Whatman Biochemicals Ltd., Maidstone, Kent, U.K., and fractionated before use by the method of McMartin & Vintner (1969). 2-Mercaptoethanol, di-isopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride were purchased from Sigma London Chemical Co., Poole, Dorset, U.K. Acetonitrile (h.p.l.c. grade) was purchased from Rathburn Chemicals Ltd., Walkerburn, Peeblesshire, Scotland, U.K. The protein assay kit and reagents for polyacrylamide-gel electrophoresis were obtained from Bio-Rad Laboratories Ltd., Watford, Herts., U.K. All radioimmunoassay reagents were prepared in our laboratory, apart from a sheep anti-(rabbit second antibody) magnetizable-particle suspension, which was obtained from Seward Laboratories, London, E.C.1, U.K. Horse serum 3 (uninactivated) was obtained from Wellcome Research Laboratories, Beckenham, Kent, U.K. All other chemicals were of AnalaR grade and were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

The homogenization of pituitaries and all chromatographic manipulations were performed at 4°C and centrifugation was done in an MSE HS 21 refrigerated centrifuge set at 4°C and at 18 000 g for 30 min.

Total protein was determined with the Bio-Rad protein assay kit (Bradford, 1976) and was shown to correlate with A_{280} when a value for $A_{1\text{cm}}^{1\%}$ of 9.5 was applied. A Cecil CE 292 digital u.v. spectrophotometer (Cecil Instruments, Cambridge, U.K.) fitted with a quartz optical cell with a 1 cm path length was used to monitor A_{280} .

The human prolactin and somatotropin contents of each of the supernatants and of all the column eluate fractions were estimated by specific radioimmunoassays (Donald *et al.*, 1976; Jones *et al.*, 1979). The human prolactin and somatotropin antisera were in-house reagents of established low cross-reactivity that had been raised in rabbits. The radioimmunoassays had been desensitized by the use of 5-fold more antiserum than would be required for assays with sensitivity in the clinically useful range, facilitating decreased incubation times (3–4 h) and avoiding large dilutions of samples. The somatotropin used as standard was in-house material (Jones *et al.*, 1979); its immunological potency was 2.5 i.u./mg against the World Health Organisation International Reference Preparation (WHO IRP) no. 66/217. The human prolactin used as standard

was previously purified (Satgunasingam, 1977) in-house material with an immunological potency of 29 i.u./mg against WHO IRP no. 71/222. The range of standards for both human prolactin and somatotropin radioimmunoassays were 0–5 µg/ml, and separation of antibody-bound and free antigen was achieved by the addition of 25 µl of horse serum and poly(ethylene glycol) 6000 to a final concentration of 12% (w/v).

Extraction. Frozen human pituitary glands (63 g) were homogenized at 4°C in 100 ml of 0.14 M-phosphate/citrate buffer (0.061 M-citric acid and 0.079 M-disodium hydrogen orthophosphate with 0.01 M-Na₃, pH 4.0) using a Silver homogenizer (LA type 2MD; Peter Silver and Sons Ltd., Hampton, Middx., U.K.). The homogenate was diluted to 800 ml, stirred gently for 30 min, centrifuged and the supernatant (S1) discarded. The residue (R1) was minced, resuspended in buffer (800 ml), stirred for 30 min, re-centrifuged and the supernatant (S1a) discarded. The residue (R1a) was minced and dispersed in 800 ml of 0.05 M-ammonium acetate containing 0.01 M-Na₃, that had been adjusted to pH 10.0 by the addition of ammonia (17.5 ml, 23 M), stirred for 60 min, and centrifuged. The residue (R2) was minced and dispersed in 0.05 M-ammonium acetate (800 ml, pH 10.0) stirred for 60 min, centrifuged, the residue (R2a) discarded and the supernatant (S2a) retained. Samples of each supernatant were retained for radioimmunoassay and estimation of total protein.

The two pH 10.0 supernatants (S2 and S2a) were pooled, the pH adjusted to pH 8.5 by the addition of 10 M-acetic acid and di-isopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride added each to a final concentration of 0.15 mM. The supernatant was filtered through Whatman no. 1 paper (three layers) in a 14 cm pressure-filtration apparatus (model C2000; Chemlab Instruments Ltd., Ilford, Essex, U.K.) and then successively through 5 µm-, 1.2 µm-, and 0.8 µm-pore-size polycarbonate filters [Millipore (U.K.) Ltd., London N.W. 10, U.K.] in the same apparatus. The filtrate was concentrated from 1600 ml to 1000 ml by using a hollow-fibre apparatus fitted with a HIP10 cartridge (model CH4; Amicon Ltd., Woking, Surrey, U.K.) and with the apparatus in the dialysis mode the filtrate dialysed against 5 mM-ammonium acetate (10 litres, pH 8.5).

The non-diffusible material (S3) was used for the further purification of human prolactin.

Chromatography. Hydrophobic-interaction chromatography. Supernatant S3 was applied at 30 ml/h to a column (2.5 cm int. diam. × 17 cm) of phenyl-Sepharose CL-4B (equivalent to 35 mg of protein/g of phenyl-Sepharose; see the Discussion section) that had been prewashed with 500 ml of 5 mM-ammonium acetate, pH 8.5, containing 1 mM-

Fig. 1. Method for the fractionation and purification of human prolactin from pituitary glands

Na₃. After application of supernatant S3 the column was washed with further buffer (500 ml) and adsorbed material eluted with a linear gradient of acetonitrile (0–30%, v/v) in the same buffer. The total volume of the gradient was 2000 ml. Fractions (10 ml) were collected in glass tubes, monitored for A_{280} and assayed for human prolactin and somatotropin. Fractions containing immunoreactive human prolactin were pooled (S4).

Anion-exchange chromatography. Supernatant S4 was applied at 13.2 ml/h to a column (1.5 cm × 25 cm) of DEAE-cellulose that had been pre-equilibrated with 0.02 M-ammonium acetate containing 20% (v/v) acetonitrile, the pH of which had adjusted to pH 8.5 by the addition of NH₃. The column was washed with this buffer (100 ml) after sample application and adsorbed material eluted with a linear salt gradient from 0.02 to 0.25 M-ammonium acetate, pH 8.5, containing acetonitrile (20%, v/v). The total volume of the gradient was 600 ml, and 6 ml fractions were collected in glass tubes. The peak of human prolactin identified by radioimmunoassay and absorbance at 280 nm was pooled and freeze-dried (S5).

Polyacrylamide-gel electrophoresis. Freeze-dried samples of human prolactin (10 µg) were analysed by the Maizel (1972) method for acidic substances by using a discontinuous buffer system with the resolving-gel buffer at pH 8.9. A rod gel of 6.5% (w/v) acrylamide was used, with a spacer gel of 3%

(w/v) acrylamide. A potential of 100 V was applied for 180 min.

SDS/polyacrylamide-gel electrophoresis. Freeze-dried samples of human prolactin (7.5 μ g) were treated with SDS (1%, w/v) or with SDS (1%, w/v) containing 2-mercaptoethanol (1%, v/v) and placed in a boiling-water bath for 90 s before analysis by the method of Maizel (1972) for acidic substances. A slab gel of acrylamide (12.5%, w/v) and a spacer gel of acrylamide (3%, w/v) were used, together with a discontinuous buffer system. The resolving gel buffer was pH 8.9. A potential of 100 V was applied for 240 min.

The fixing and staining solution for all gels consisted of methanol/water/acetic acid (5:5:1, by vol.) containing Coomassie Blue R250 (0.1%, w/v). The gels were fixed and stained for 120 min and destained with methanol/water/acetic acid (1:8:1, by vol.).

Gel-filtration chromatography of the human prolactin preparation. Prolactin (3 mg) in 0.02 M-ammonium acetate (2 ml) was applied to a column (1 cm $\times$ 100 cm) of Sephadex G-100 and eluted at 3 ml/h with the same buffer; 1.25 ml fractions were collected and assayed for human prolactin immunoreactivity and A_{280} .

Iodination of human prolactin preparation. Iodination of human prolactin was achieved by a modification of the lactoperoxidase method of Marchalonis (1969), in which lactoperoxidase immobilized by coupling to a magnetizable-particle solid phase (Pourfarzaneh *et al.*, 1980) was used. The method involves addition of solid phase (0.2 mg) in 0.1 M-phosphate buffer, pH 7.4 (30 μ l) coupled to lactoperoxidase (5 μ g) to a vial containing human prolactin (0.5 nmol) and Na¹²⁵I (0.5 nmol, 1 mCi). The reaction was initiated by the addition of H₂O₂ (0.15 nmol) in water (10 μ l) to the vial, followed by two further additions, 10 min and 20 min after the first. The reaction was terminated after 30 min by sedimentation of the solid phase on a magnet and transfer of the liquid phase to 0.1 M-phosphate buffer, pH 7.4 (250 μ l). The mixture was then applied to a column (1 cm $\times$ 100 cm) of Sephadex G-100 and eluted at 3 ml/h with the above buffer containing (0.5%). Fractions (1.25 ml) were collected.

Human prolactin radioimmunoassay. A radioimmunoassay for human prolactin was developed by using radioiodinated human prolactin, prepared as described above, antiserum to human prolactin (P₄ 16/6/78) raised in the rabbit and standards diluted from the current International Reference Preparation for human prolactin (IRP 75/504). the immunological potency of which is 32.5 i.u./mg. A titre of anti-(human prolactin) serum was chosen that resulted in 60% binding of ¹²⁵I-labelled human prolactin (80 pg) in the absence of added unlabelled human prolactin and that would achieve 50% of

maximal displacement on addition of 25 μ i.u. of unlabelled human prolactin. The incubation time was 18 h at room temperature. The assay consisted of the simultaneous addition of standard (100 μ l, 0–4400 m.i.u./l) or sample (100 μ l), ¹²⁵I-labelled human prolactin (100 μ l, 80 pg) and anti-(human prolactin) serum (100 μ l, diluted 1:30 000), to labelled tubes. Separation of antibody-bound and free ¹²⁵I-labelled human prolactin was achieved by addition of a double-antibody magnetizable-particle suspension (0.5 mg/tube) (Kamel *et al.*, 1979) followed by 10 min incubation, sedimentation on a ferrite magnet and aspiration of the supernatant. In addition, dose-response curves were prepared by using human prolactin prepared as described above and the serum of a patient with an active-prolactin-secreting pituitary adenoma.

Contamination with other protein hormones. The somatotropin, follitropin, lutropin, thyrotropin, placental lactogen and α -foetoprotein contents of a known mass of purified prolactin were determined by using specific radioimmunoassays.

Bioassay. The biopotency of the prolactin preparation was estimated by a mouse mammary-gland bioassay *in vitro* (Forsyth & Myres, 1971) with C3H mice. The assay was kindly performed by Dr. I. Forsyth of the National Institute for Research in Dairying, Shinfield, Reading Berks., U.K. The second International Standard for Prolactin (Ovine) and the current National Institutes of Health Prolactin Standard (PS12) were used as standards, and eight mammary explants were treated at each of four different doses.

Results and discussion

Extraction

Preliminary observations of the insolubility of human prolactin at neutral and acid pH and the requirement of high pH to solubilize human prolactin confirmed the work of Lewis *et al.* (1972). At pH 4.0, 13 μ g of immunoreactive human prolactin/pituitary was solubilizable, in contrast with 190 μ g/pituitary at pH 10.0. The minimum solubility of human prolactin was at pH 4.0, and this was unexpected, because published isoelectric point(s) for human prolactin are close to pH 6.0 (Hummel *et al.*, 1975). When pituitaries were extracted at pH 10.0, 0.5 μ g of human prolactin were solubilized/mg of solubilized protein; however, a prior extraction of pituitaries at pH 4.0 was employed because large amounts of interfering protein could be removed, including 40% of recoverable somatotropin and all the glycoprotein hormones and corticotropin-related peptides. After solubilization of the residue at pH 10.0 an extract was obtained with a potency of 5.5 μ g of human prolactin/mg of solubilized protein, representing an enrichment of 11-fold. Two extractions of

the pituitaries at pH 4.0 were used before two extractions of the residue at pH 10.0. The solubilization of human prolactin from pituitary tissue was not affected by a prior extraction, the recovery of human prolactin at pH 10.0 was similar (170–190 μ g of human prolactin/pituitary) with and without prior extraction. The most efficient pH for solubilizing human prolactin was pH 12.0 (220 μ g of human prolactin/pituitary), but pH 10.0 was chosen to minimize possible deamidation and denaturation at high pH values. Omission of di-isopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride from the extraction resulted in a decrease in human prolactin immunoreactivity of 43% in 6 days, hence these inhibitors were always included in subsequent experiments.

A two-step solubilization of human prolactin from frozen pituitaries has been used, resulting in a preparation of increased potency, making the subsequent chromatography easier and allowing the processing of increased numbers of pituitaries. A yield of 22 mg of human prolactin was obtained from 115 glands (63 g).

Chromatography

Hydrophobic-interaction chromatography. Optimization. The adsorption of non-specific protein and human prolactin to the mildly hydrophobic adsorbent phenyl-Sepharose, and the more highly hydrophobic adsorbent, octyl-C Sepharose, was investigated. Adsorbent (1 g) was added to supernatant 3 (S3, 5 ml), the ionic strength of which had been adjusted to 0.1 mol/litre with NaCl. The tubes were mixed for 30 min, the adsorbent sedimented, and the supernatant assessed for total protein and human prolactin. The octyl-Sepharose had adsorbed 98% of immunoreactive human prolactin and 63% of total protein. The phenyl-Sepharose had adsorbed 93% of human prolactin and only 24% of total protein. Phenyl-Sepharose was therefore chosen for further investigation.

Hydrophobic interactions are known to increase with increasing ionic strength (Rosengren *et al.*, 1975; Pahlman *et al.*, 1977). The minimum ionic strength at which the bulk of prolactin could be adsorbed to phenyl-Sepharose was therefore established. Phenyl-Sepharose (1 g) was added to four 5 ml samples of S3, the ionic strengths of which had been adjusted to 0.01, 0.1, 1.0 and 2.0 mol/litre by the addition of NaCl. The samples were mixed by inversion for 30 min, the adsorbent sedimented and the percentages of original total protein and human prolactin remaining in the supernatant assessed. The results were: 0.01 mol/l: 85.9%, 9.3% (percentage of total protein and percentage of human prolactin respectively); 0.1 mol/l: 76.0%, 7.0%; 1.0 mol/l: 48.2%, 3.0%; and 2.0 mol/l: 27.0%, 1.9%. Final conditions of ionic strength were chosen that allowed

maximum adsorption of human prolactin with minimum contamination by interfering proteins. It can be seen that, at low ionic strength (1.0 M), human prolactin (90.7%) was adsorbed and there was little adsorption of interfering protein (14.1%). The bulk of protein present in S3 can therefore be separated from human prolactin with phenyl-Sepharose.

Adsorption of human prolactin to phenyl-Sepharose is also related to the proportions of adsorbent and total protein. Phenyl-Sepharose (4 $\times$ 1 g) was exposed to S3 protein in the proportions 1, 10, 100, and 200 mg of protein/g of adsorbent, and the samples mixed for 120 min. The adsorbent was sedimented and the percentages of total protein and human prolactin remaining in the supernatant estimated. The results were: 1 mg of protein/g of adsorbent: 22.1%, 1.1% (percentage of total protein and percentage of human prolactin respectively); 10: 78.9%, 1.1%; 100: 97.0%, 9.0%; 200: 99.0%, 24.0%. It can be seen that at 1 mg of protein/g of phenyl-Sepharose, the bulk of human prolactin and total protein were adsorbed, but at 10 mg of protein/g of phenyl-Sepharose, although the bulk of human prolactin (98.9%) was still adsorbed, 78.9% of the total protein remained in the supernatant. The final conditions were chosen to ensure maximum adsorption of human prolactin with minimum adsorption of interfering protein. These results suggest that it was possible to saturate the available sites on the adsorbent and, in the presence of excess protein, only the more hydrophobic species, including human prolactin, were adsorbed.

Chromatography. The ionic strength of S3 was decreased to 0.01 mol/litre and protein was applied to phenyl-Sepharose in the ratio of 35 mg of protein/g of phenyl-Sepharose. An 81 g column of phenyl-Sepharose was required for the application of S3. The column was washed after sample application with 5 mM-ammonium acetate (pH 8.5, 500 ml) and under these conditions 93% of human prolactin (20.5 mg), 7% of somatotropin (71 mg) and 10% of total protein (295 mg) were adsorbed.

Adsorbed materials are recovered by disruption of their hydrophobic bonding with the matrix (von Hippel *et al.*, 1969) and may be resolved by altering the elution conditions to give selective desorption based on the differing strengths of their hydrophobic interactions. We have found acetonitrile, which functions by depolarizing water, to be an effective means of recovering adsorbed material, and a linear gradient of acetonitrile (0–30%, v/v) in aqueous buffer resolved adsorbed human prolactin from somatotropin (Fig. 2). This method has proved a better means of resolving adsorbed material than the non-ionic detergent and ionic-strength gradient previously reported by us (Hodgkinson & Lowry, 1980).

Immunoreactive human prolactin was eluted in a

Fig. 2. *Chromatography of material adsorbed to phenyl-Sepharose from supernatant S3*
Bound material was eluted with a linear gradient (----) of acetonitrile (0–30%, v/v) in 5 mM-ammonium acetate, pH 8.5. The total volume of the gradient was 2000 ml. —, A_{280} ; ▨, human prolactin; ▩, somatotropin.

sharp peak at 15% (v/v) acetonitrile. A maximum purification of 86.7-fold was observed relative to S3 in the peak fractions, and the pooled human prolactin fractions had a specific activity of $191.2 \mu\text{g}$ of human prolactin/mg of protein. The recovery of human prolactin was 78% (17.2 mg) of that loaded. As well as this high recovery of human prolactin, a major resolution of human prolactin from somatotropin was achieved. The human prolactin 'pool' (S4) contained only an equimolar amount of somatotropin, a 59-fold decrease in the somatotropin/human prolactin ratio of the loaded sample. Hydrophobic interaction chromatography has therefore proved an ideal technique for resolving human prolactin from most of the contaminating somatotropin while enabling human prolactin to be recovered in high yield. It is noteworthy that a small amount (15.3%) of the adsorbed somatotropin was eluted from phenyl-Sepharose later than human prolactin and hence may be a strongly hydrophobic form of somatotropin.

Anion-exchange chromatography. DEAE-cellulose has been widely used in the purification of human prolactin and somatotropin (Jones *et al.*, 1979; Lewis *et al.*, 1971), but in our laboratory poor resolution and low recoveries of human prolactin have always resulted. This may be due to the operation of mixed hydrophobic and ionic effects on the matrix. Fig. 3(a) represents a DEAE-cellulose run, using the human prolactin peak (S4) from phenyl-Sepharose, in the absence of acetonitrile but otherwise as described under 'Methods'. It demonstrates the poor separation of human prolactin and somatotropin, and the recovery of human prolactin was only 29.7%.

We have included acetonitrile (20%, v/v) in aqueous buffer to minimize non-ionic interactions,

and the resulting anion-exchange chromatography (Fig. 3b) demonstrated much enhanced resolution. A sharp peak of immunoreactive human prolactin, which was completely separated from the remaining somatotropin present in supernatant S4, was eluted at 0.10 M-ammonium acetate. It was equivalent to that expected by absorbance at 280 nm. The recovery of human prolactin had substantially improved (74.3%, 10.1 mg). The overall recovery of human prolactin was equivalent to 58%. This value has been corrected for that portion of S4 which was 'sacrificed' on DEAE-cellulose as a demonstration of the poor recovery and resolution observed in the absence of acetonitrile.

The human prolactin peak was freeze-dried and stored at 4°C.

Polyacrylamide-gel electrophoresis

Analysis of $10 \mu\text{g}$ of the immunoreactive human prolactin peak as eluted from DEAE-cellulose (Fig. 3b), by polyacrylamide-gel electrophoresis gave a single band (Fig. 4). The relative mobility (R_f) of which was 0.44. The R_f of somatotropin in the same system was 0.50. There was no evidence of a faster-migrating deaminated band, and this may be due to the short exposure time (3 h) and mildly alkaline conditions (pH 10) employed here to solubilize human prolactin.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Prolactin ($7.5 \mu\text{g}$) migrated as a single band of apparent mol.wt. 24 000 as calculated from a set of calibrated molecular-weight standards. Analysis of further human prolactin ($7.5 \mu\text{g}$) after reduction with

Fig. 3. Anion-exchange chromatography of human prolactin peak from phenyl-Sepharose (S4) on DEAE-cellulose. The Figure shows chromatography of S4 in the absence of acetonitrile (a), and in the presence of acetonitrile (b). Supernatant S4 was applied directly (b) to a column of DEAE-cellulose and bound material was eluted by application of a linear salt gradient from 0.02 to 0.25 M-ammonium acetate, pH 8.5, containing acetonitrile (20%, v/v). The total volume of the gradient was 600 ml. (a) represents chromatography of S4 under the same conditions apart from the exclusion of acetonitrile from all buffers. —, A_{280} ; ▨, human prolactin; ▤, somatotropin; ----, [ammonium acetate] gradient.

mercaptoethanol also gave a single band of apparently higher molecular weight. This is due to the disruption of the tertiary structure of prolactin associated with reduction of its disulphide linkages and the resultant increase in Stokes radius. That the human prolactin migrates as a single band after reduction confirms that it consists of a single polypeptide chain. This is in contrast with a report by Mitra (1980) in which a post-translational cleavage of rat prolactin resulted in the identification of a two-polypeptide-chain protein.

Gel-filtration chromatography

Immunoreactive human prolactin was eluted from Sephadex G-100 in the amounts expected from monitoring of absorbance at 280 nm in a concise peak at K_{av} 0.484 (Fig. 5). Absorbance detected in fractions other than those bearing immunoreactive human prolactin accounted for less than 2.0% of the total, confirming the homogeneous analysis obtained by polyacrylamide-gel electrophoresis with and without sodium dodecyl sulphate. Gel filtration has been used here as a qualitative assessment of the product, since it was not used in the purification of the human prolactin.

Iodination of the human prolactin preparation

The incorporation of ^{125}I into human prolactin was 87% and the specific radioactivity of the preparation was $85 \mu\text{Ci}/\mu\text{g}$. The elution profile of the iodinated human prolactin on gel filtration demonstrated a small amount of radioactivity (10.2% of total iodinated material) in the position expected of dimerized human prolactin. This is thought to be a result of the iodination conditions employed here, as it was absent on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and from the elution profile of the uniodinated human prolactin on gel filtration. The major peak of radioactivity (79.3% of the total) was eluted in the position expected of monomeric human prolactin. This material demonstrated more than 95% human prolactin immunoreactivity, as judged by its binding to excess human prolactin antiserum (corrected for non-specific binding), and was used in the human prolactin radioimmunoassay.

Radioimmunoassay

The results obtained when the iodinated human prolactin preparation was used in a radioimmuno-

Fig. 4. Discontinuous polyacrylamide-gel electrophoresis of human prolactin ($10\mu\text{g}$) from DEAE-cellulose (Fig. 3b)

Migration was in the direction of the arrow, and the anode is at the bottom.

assay are given in Fig. 6. A standard curve was obtained by using WHO IRP no. 75/504 human prolactin diluted to the stated concentrations in horse serum (curve a). Curves b and c are arbitrary

Fig. 5. Gel-filtration chromatography of human prolactin on Sephadex G-100

Prolactin (3 mg, 2 ml), obtained from DEAE-cellulose (Fig. 3b) was applied to the column. †, Salt volume; —, A_{280} .

Fig. 6. Prolactin radioimmunoassay dose-response curves

● (curve a), IRP 75/504; □ (curve b), arbitrary dilutions of human prolactin prepared as described in the text; ○ (curve c), arbitrary dilutions of a hyperprolactinaemic serum.

dilutions of human prolactin prepared as described above, and a hyperprolactinaemic serum respectively. The iodinated human prolactin was displaced from the prolactin antiserum in a parallel fashion by each of the three preparations. We have detected no immunochemical differences in the preparations.

The contamination of the human prolactin with α -foetoprotein, human placental lactogen, thyrotropin, lutropin, follitropin and somatotropins were $<0.01\%$, $<0.01\%$, $<0.02\%$, $<0.01\%$, $<0.01\%$ and $<0.1\%$ respectively.

Bioassay

The human prolactin was calculated to have a potency of 20.8 i.u./mg (95% confidence limits 17.6–24.7) against the Second International Standard for Prolactin, the potency of which is stated as 22 i.u./mg. The preparation was also shown to be equipotent to the NIH PS12 standard. The potency

of this standard is quoted as 35 i.u./mg, but a more conservative estimate of its potency (23.5 i.u./mg) has been published (Forsyth *et al.*, 1978). The potency of our prolactin preparation compares favourably with those reported for other highly purified preparations (Hwang *et al.*, 1975; Satgunasingam, 1977) of the hormone.

The immunological and biological evidence, together with the analysis of human prolactin by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel filtration, suggests that the human prolactin suffered no deleterious effects by exposure either to the hydrophobic adsorbant or to aqueous acetonitrile.

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