Evidence that the bovine ovary secretes large amounts of monomeric inhibin α subunit and its isolation from bovine follicular fluid

P. G. Knight, A. J. Beard, J. H. M. Wrathall and **R. J. Castillo**

Department of Biochemistry & Physiology, University of Reading, Whiteknights, Reading RG6 2AJ

RECEIVED 16 November 1988

ABSTRACT

Analysis of bovine follicular fluid (FF) using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) coupled with a sensitive immunoblotting procedure resolved several components that were immunoreactive with an antiserum directed against the n-terminus of the α subunit of human inhibin (hIα(1–32)). Under non-reducing conditions, three intensely stained bands having apparent M_r values of 116 000, 44 000 and 25 000 were present, whilst under reducing conditions only two intensely stained bands (M_r 43 000 and 21 000) were detected. The M_r 44 000 and 25 000 immunoreactive forms (non-reducing conditions) were also demonstrated in bovine utero-ovarian vein and peripheral venous plasma after subjecting samples (40 ml) to immunoaffinity concentration using Sepharose beads coupled to anti-hIα(1–32), SDS-PAGE and immunoblotting. The same approach revealed the presence of the smaller (M_r 25 000) form in bovine granulosa cell-conditioned culture medium (GCCM). Gel-permeation chromatography (Sephacryl S-200), immunoaffinity chromatography (Sepharose-anti-hIα(1–32)) and reversed-phase high-performance liquid chromatography (RP-HPLC; C_{18} and C_8 columns) were employed to isolate from bFF (30 ml, 19·5 g protein) 750 μg protein which appeared essentially homogeneous by RP-HPLC and SDS-PAGE and had an M_r of 25 000 (non-reducing conditions)/21 000 (reducing conditions), identical to that of the immunoreactive component of lowest M_r found in bovine FF, utero-ovarian vein plasma, peripheral plasma and GCCM. The isolated material was highly immunoreactive with antisera against both hIα(1–32) and purified M_r 32 000 bovine inhibin but was devoid of biological activity when tested in a rat pituitary cell inhibin bioassay. Amino-terminal analysis revealed an amino acid sequence (residues 1–14) identical to that reported elsewhere for the α subunit (M_r 20 000/21 000) of bovine inhibin.

In conclusion, the present study has revealed that the bovine ovary secretes considerable quantities of monomeric inhibin α subunit. The unexpected presence of this material in peripheral blood is likely to hinder attempts to obtain physiologically relevant data on circulating levels of inhibin in cattle using conventional radioimmunoassays.

Journal of Molecular Endocrinology (1989) **2**, 189–200

INTRODUCTION

Inhibin is a gonadal glycoprotein hormone which selectively inhibits the production of follicle-stimulating hormone (FSH) by the anterior pituitary (for reviews see Findlay, 1986; de Jong, 1988; Ying, 1988). Within the last 3 years the chemical nature of inhibin has largely been resolved, the molecule being a heterodimer composed of two subunits (termed α and β) linked by disulphide bonds (for reviews see de Jong, 1988; Ying, 1988). In several species, including cattle, the smallest and most predominant form of biologically active inhibin identified has an M_r of approximately 31 000–32 000 (Ling, Ying, Ueno *et al.* 1985; Rivier, Spiess, McClintock *et al.* 1985; Miyamoto, Hasegawa, Fukuda *et al.* 1985; Fukuda, Miyamoto, Hasegawa *et al.* 1986; Robertson, de Vos, Foulds *et al.* 1986). Since the α and β subunits are generated by proteolytic cleavage of two independently synthesized high M_r precursor molecules (Mason, Hayflick, Ling *et al.* 1985; Forage, Ring, Brown *et al.* 1986; Mason, Niall & Seeburg, 1986) it is, perhaps, not surprising that inhibin forms of higher M_r have also been identified in ovarian follicular fluid (FF) from various species (Robertson, Foulds, Leversha *et al.* 1985; Hasegawa, Miyamoto, Fukuda *et al.* 1986; Miyamoto, Hasegawa, Fukuda & Igarashi, 1986).

  Printed in Great Britain
0952–5041/89/002–189 $02.00/0

Until very recently, attempts to measure endogenous inhibin concentrations in peripheral blood relied on bioassay procedures, the most reliable and sensitive of which involve detection of the dose-dependent suppression of basal FSH production by cultured rat (e.g. Scott, Burger & Quigg, 1980) or sheep (e.g. Tsonis, McNeilly & Baird, 1986) anterior pituitary cells. With few exceptions (Lee, McMaster, Quigg *et al.* 1981; Tsonis *et al.* 1986), however, these bioassays have proven too insensitive for this purpose. Within the last few years, several inhibin radioimmunoassays have been developed and applied to the measurement of immunoreactive inhibin concentrations in peripheral blood of various species, including man (McLachlan, Robertson, Healy *et al.* 1986*b*), rat (Rivier & Vale, 1987), sheep (Mann, McNeilly & Baird, 1988), cattle (McLachlan, Robertson, Burger & de Kretser, 1986*a*; Hasegawa, Miyamoto, Igarashi *et al.* 1987) and pig (Hasegawa, Miyamoto, Iwamura & Igarashi, 1988).

Whilst such radioimmunoassays are considerably more sensitive, precise and practicable than the bioassay methods, it is clearly imperative that rigorous validation is carried out to establish the nature of the immunoreactive material being measured. With regard to inhibin radioimmunoassays, the problem of specificity is compounded by the fact that the β subunit of inhibin shares extensive homology with several other proteins, including activin, transforming growth factor-β (TGF-β) and antimullerian hormone (for review see Ying, 1988). Moreover, dissociated α and β subunits of inhibin are reported to be devoid of biological activity (Ling *et al.* 1985; Miyamoto *et al.* 1985) but are likely to retain their immunoreactivity with most antisera.

In the course of validating an inhibin radioimmunoassay, which was developed in our laboratory and uses an antiserum specific for the α subunit of inhibin, we recently compared the biological and immunological potencies of a series of chromatographic fractions obtained during the routine purification of inhibin (M_r 32 000) from bovine FF (Knight, Castillo & Glencross, 1987). Unexpectedly, these studies revealed that two distinct zones of inhibin α-subunit immunoreactivity were resolved by the first gel-permeation chromatography step of our inhibin purification scheme. However, only the higher M_r zone, which eluted in the void volume of the column, was associated with significant biological activity. Since the immunoreactive material of lower M_r represented approximately 60% of the total inhibin immunoreactivity eluted from the column, it occurred to us that, should this material be released into the peripheral circulation, it would pose serious difficulties for investigators, including ourselves, intent on measuring circulating concentrations of inhibin in cattle using such radioimmunoassays.

The objectives of the present study were to clarify the nature of this low M_r form of immunoreactive inhibin and to establish whether it is a normal constituent of bovine FF or merely an artifact generated by the methods used to collect and process bovine FF for inhibin purification in our laboratory.

MATERIALS AND METHODS

Follicular fluid

Pooled bovine FF was routinely obtained by aspiration from several thousand cow ovaries which were collected at an abattoir and stored for <3 days at 4 °C. After centrifugation (2000 ***g*** for 15 min at 4 °C) to remove cellular debris, the fluid was stored at −20 °C until required. On several occasions, bovine FF was aspirated from individual cow follicles, immediately filtered through 0·22 μm membrane filters and snap-frozen in liquid nitrogen within 20 min of slaughter. Ovine FF was aspirated from the ovaries of two adult anoestrous ewes within 5 min of ovariectomy. The fluid was pooled, centrifuged to remove cellular debris and stored at −20 °C until required. Samples of pooled human (frozen) and porcine (freeze-dried) FF were kindly provided by Dr C. M. Howles (Bourn Hall Clinic, Bourn, Cambridge, U.K.) and Dr G. Bialy (NIH Contraceptives Development Branch, Bethesda, MD, U.S.A.) respectively.

Granulosa cell-conditioned media (GCCM)

Bovine granulosa cells were obtained from fresh ovaries collected at a local abattoir, and primary cultures established essentially as described by Henderson & Franchimont (1981). Briefly, cells were washed by centrifugation (180 ***g*** for 5 min) and resuspension in Dulbecco's Modified Eagle's Medium (DMEM) containing antibiotic/antimycotic solution (1%, v/v; Sigma, Poole, Dorset, U.K.) and bovine serum albumin (BSA; 0·3%, w/v), filtered through a nylon mesh (200 μm) and finally resuspended in complete culture medium at a concentration of 5×10^5 viable cells/ml. Complete culture medium consisted of a 50:50 mixture of DMEM and Ham's F12 medium supplemented with 10% (v/v) fetal calf serum, 2 mmol glutamine/l and stock antibiotic/antimycotic solution (1%). After culturing in Petri dishes (35 mm diameter) for 24 h at 37 °C under an atmosphere of 5% CO_2/95% air, media and non-attached cells were removed and the dishes washed three times with fresh media. After replacing with fresh media the cells were cultured for a further 2 days, and the GCCM was then harvested and stored at −20 °C until required.

Anti-inhibin sera

Antisera directed against inhibin α subunit were raised in sheep and rabbits by immunization with a synthetic peptide corresponding to the n-terminal sequence (1–32) of human inhibin α subunit (hIα(1–32); Peninsula Laboratories, St Helens, Merseyside, U.K.). The peptide (0·5 mg) was conjugated to tuberculin purified protein derivative (1·5 mg; MAFF, Central Veterinary Laboratory, Weybridge, Surrey) using gluteraldehyde (0·125%, v/v, final concentration) as the coupling agent. Primary immunizations (sheep: 100 μg conjugate, four s.c. sites in axillary regions; rabbits: 50 μg, six s.c. sites on back) prepared as emulsions with Freund's complete adjuvant were followed at 6-weekly intervals with booster injections (sheep, 50 μg conjugate; rabbits, 25 μg conjugate) emulsified with Freund's incomplete adjuvant; test blood samples were withdrawn 10–14 days after each booster. The titres of the antisera to hIα(1–32) used in this study, expressed as the final dilutions which bound 30% of ^{125}I-labelled M_r 32 000 bovine inhibin in a conventional double-antibody radioimmunoassay procedure, were 1:120 000 (sheep 114/2), 1:80 000 (sheep 119/2) and 1:16 000 (rabbit PPD2/4). An antiserum was also raised against native bovine inhibin by immunizing a laying hen with highly purified M_r 32 000 bovine inhibin isolated from bovine FF in this laboratory (Knight *et al.* 1987; Castillo, 1989). Inhibin (30 μg) was dissolved in phosphate-buffered saline (PBS), emulsified with 2·2 volumes of Freund's complete adjuvant and injected at four sites (i.m.) in the breast. Booster injections containing 15 μg inhibin, similarly emulsified with Freund's complete adjuvant, were given at 6-weekly intervals and blood samples withdrawn 10 days after each booster. The titre of the antiserum used in these studies (H95/2), expressed as the final tube dilution which bound 30% of ^{125}I-labelled M_r 32 000 bovine inhibin in a conventional double-antibody radioimmunoassay procedure, was 1:4000.

Radioiodination of inhibin

Purified M_r 32 000 bovine inhibin was radioiodinated using a modified chloramine T procedure in which 3 μg inhibin, 0·3 mCi Na^{125}I and 0·9 μg chloramine T were incubated in 40 μl phosphate buffer (0·25 mol/l; pH 7·3) for 5 min. Purification of ^{125}I-labelled inhibin from the reactant mixture was achieved by immunospecific adsorption onto Sepharose beads (Pharmacia-LKB, Milton Keynes, Bucks, U.K.) coupled to sheep anti-hIα(1–32) followed by elution at low pH. Briefly, the contents of the radioiodination vial were transferred to a 12 ml conical centrifuge tube containing 1 ml Sepharose–antibody (solid phase) and 2 ml PBS containing 0·1% (v/v) Tween 20 and BSA (PBS/Tween/BSA). After mixing by rotation for 30 min, the solid phase was washed three times with PBS/Tween/BSA by brief centrifugation (500 ***g***) followed by resuspension in fresh buffer. A final wash with sodium acetate (0·5 mol/l; pH 6·5) containing 20% (v/v) acetonitrile was followed by elution of the antibody-bound ^{125}I-labelled inhibin with 8 ml sodium acetate buffer (0·05 mol/l, adjusted to pH 3·0 with glacial acetic acid) containing 20% acetonitrile. After decanting from the solid phase, the supernatant was adjusted to pH 7·5 by dropwise addition of 3 ml saturated sodium bicarbonate solution and 1 ml 10× stock concentrated PBS solution containing 3% (w/v) BSA and 0·5% (w/v) sodium azide. Approximately 30% of the total radioactivity applied to the solid phase was eluted in the low pH fraction, and analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the presence of a single major peak of radioactively labelled protein, having an M_r of 32 000 (non-reducing conditions). Under reducing conditions the disappearance of this peak was associated with the appearance of two peaks of M_r 22 000 and 15 000, corresponding to the α and β subunits respectively. The tracer was capable of binding to the extent of >70% when incubated with an excess of antibody, and had a useful shelf-life of at least 6 weeks when stored at 4 °C.

SDS-PAGE and immunoblotting procedure

Samples of crude FF and of various partially and highly purified fractions were electrophoresed on 12·5 or 15% (total acrylamide concentration) slab gels (2·5 mm thick) under reducing or non-reducing conditions according to the procedure of Laemmli (1970). Samples were prepared for electrophoresis by heating (80 °C for 15 min) in SDS-PAGE sample buffer with (reducing conditions) or without (non-reducing conditions) 2-mercaptoethanol (10%, v/v). When reduced and non-reduced samples were run on the same gel, iodoacetate (10 mmol/l) was added to non-reduced samples before heating and to reduced samples after heating. Calibration of gels with respect to M_r was achieved using a mixture of marker proteins (kit VI; Sigma) which were visualized along with sample components by silver staining according to the method of Morrissey (1981). When gels were to be immunoblotted, however, either pre-stained (kit VIIB; Sigma) and/or biotinylated (kit VIB; Sigma) M_r marker protein mixtures were employed to facilitate M_r calibration of blots. Electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose sheets (0·45 μm pore

size) was carried out at 200 mA (50 V limit; overnight run) with Tris–glycine buffer (pH 8·3) containing 20% (v/v) methanol as the transfer buffer. Nitrocellulose sheets were incubated for 90 min with PBS containing 5% (w/v) non-fat dried milk and 1% (v/v) normal goat serum before immunostaining to eliminate non-specific binding of antibodies. After rinsing (three changes) with PBS containing 0·1% (v/v) Tween 20 (PBS/Tween), sheets were incubated for a further 90 min with either rabbit anti-hIα(1–32) serum (PPD2/4; 1:1000) or with chicken anti-bovine inhibin (M_r 32 000) serum (H95/2; 1:500) diluted in PBS/Tween. Control experiments involving incubation of sheets with preadsorbed antisera and with normal rabbit and chicken sera were also performed to confirm the specificity of immunostaining. The sensitive avidin–biotin–peroxidase complex (ABC) technique (Hsu, Raine & Fanger, 1981) was subsequently used to localize antigen–antibody complexes on the nitrocellulose sheets. Biotinylated anti-rabbit and anti-chicken IgG preparations and a standard ABC kit were purchased from Vector Laboratories, Peterborough, Cambs, U.K. and used according to the instructions supplied by the manufacturer. The colour-development reagent used was Tris–HCl buffer (0·1 mol/l; pH 7·3) containing diaminobenzidine (1 μg/ml), nickel chloride (0·4 mg/ml) and hydrogen peroxide (0·01%, v/v). On several occasions, bovine FF samples were incubated with ^{125}I-labelled inhibin before electrophoresis to assess the stability of the intact M_r 32 000 form of the molecule and also to provide some confirmation of the accuracy of the M_r calibration methods used. After immunostaining and photographing these nitrocellulose sheets, the distribution of radioactivity was revealed by cutting the sheets into 2 mm slices which were then counted in a gamma spectrometer.

Gel-permeation chromatography on Sephacryl S-200

Pooled bovine FF was mixed with 0·25 volumes of ammonium acetate (0·05 mol/l; pH 7·0), centrifuged (30 000 ***g*** for 30 min at 4 °C) and the supernatant (200 ml) applied to a 10 × 75 cm column of Sephacryl S-200 (Pharmacia-LKB) which was eluted with 0·05 ml ammonium acetate/l (pH 7·0) at a flow rate of 2·5 ml/min at 4 °C.

Immunoaffinity chromatography

A crude IgG fraction obtained by sodium sulphate fractionation of sheep anti-hIα(1–32) (S119/2) was coupled to cyanogen bromide-activated Sepharose 4B at a ratio of 10 mg IgG/ml swollen gel, according to the procedure recommended by the manufacturer (Pharmacia-LKB). After washing extensively with six cycles of high (9·0) and low (3·0) pH buffers to ensure removal of non-covalently bound protein, the beads were equilibrated with PBS/Tween containing sodium azide (0·1%, w/v) and stored at 4 °C. Affinity purification of the lower M_r zone of immunoreactive inhibin eluted from the gel-permeation column was achieved using a 2·2 × 20 cm column of antibody–Sepharose. Before applying the recombined fractions (approximately 900 ml) to the column, NaCl (9 g), sucrose (25 g) and Tween 20 (1 ml) were added to the sample to minimize non-specific adsorption to the column matrix. The sample was recycled through the column at 2 ml/min for 3 days at 4 °C before washing with PBS (approximately 500 ml) until the absorbance at 280 nm (A_{280}) of the eluant was <0·01 units. Antibody-bound material was eluted by passing through the column 100 ml ammonium acetate buffer (0·1 mol/l; pH 3·5) containing 20% (v/v) acetonitrile at the same flow rate. Fractions (10 × 10 ml) were collected and A_{280} values measured using a spectrophotometer. The column was then re-equilibrated with 200 ml PBS containing 0·1% sodium azide and stored at 4 °C until the next run.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

Affinity-purified material obtained from several successive runs was pooled and 10 ml aliquots were applied directly (using a sample injector fitted with a 10 ml loop) to a 7·8 × 300 mm RP-HPLC column (TSK-ODS-120T column; Anachem, Luton, Beds, U.K.). The column was eluted with a 50-min multilinear gradient of 10–60% acetonitrile in 0·1% (v/v) trifluoroacetic acid (TFA) at a flow rate of 3 ml/min at room temperature (see Fig. 6*a* for gradient profile). Fractions comprising the major peak of immunoreactive inhibin were pooled from multiple RP-HPLC runs, diluted with 4 volumes of 10% acetonitrile in 0·1% TFA, and 10 ml aliquots applied to a second RP-HPLC (C_8) column (6·2 × 80 mm; Zorbax PEP RP/1; Anachem) which was eluted with a 30-min gradient of 30–90% acetonitrile in 0·1% TFA at 1 ml/min (see Fig. 6*b* for gradient profile).

Inhibin bioassay

Inhibin-like biological activity was assessed using a rat pituitary cell bioassay based on the dose-dependent suppression of total FSH production by inhibin-containing preparations, full details of which are described elsewhere (Castillo, 1989). All samples referred to in this study were included in the same assay.

RESULTS

Bovine FF

As shown in Fig. 1, chromatography of crude bovine FF on Sephacryl S-200 resolved two distinct zones (peaks 1 and 2) of inhibin α-subunit immunoreactivity. Only peak 1, however, which eluted in the void volume of the column, was associated with inhibin-like biological activity as indicated by the suppression of basal FSH production by rat anterior pituitary cells *in vitro* (see Fig. 2). The retarded peak (approximate M_r of 20−50 000 contained about 60% of the total inhibin α-subunit immunoreactivity present in bovine FF, estimated by comparison of displacement curves in the radioimmunoassay.

As shown in Fig. 3*a*, analysis of pooled bovine FF by SDS-PAGE coupled with a sensitive immunoblotting procedure revealed that, under non-reduc-

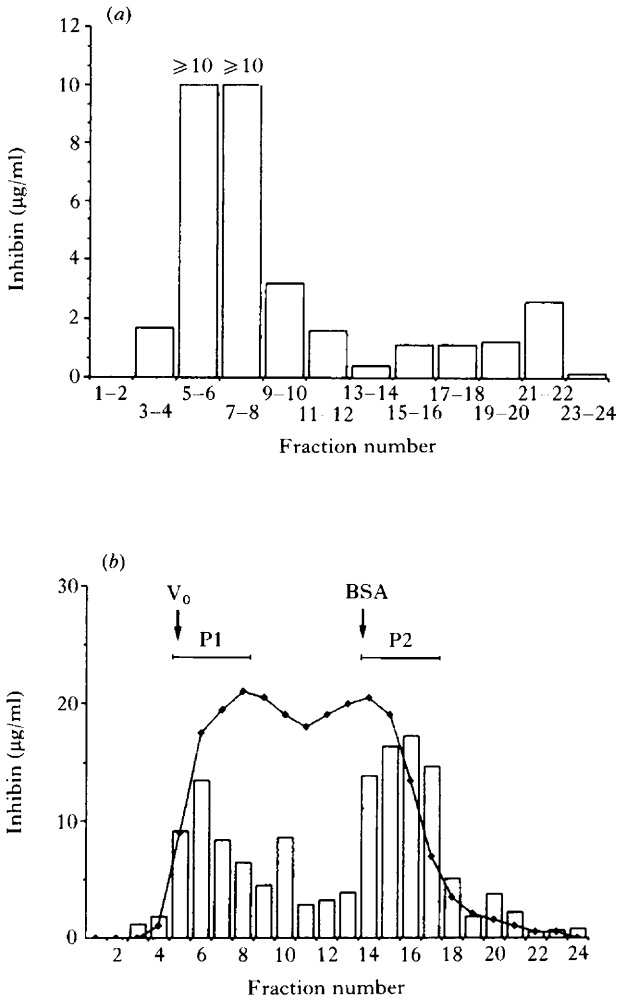

FIGURE 1. Gel-permeation chromatography of crude bovine follicular fluid (FF) on Sephacryl S-200. Eluate fractions were assayed for (*a*) inhibin-like biological activity using rat pituitary cells *in vitro* and (*b*) inhibin immunoreactivity using an antiserum raised against the n-terminal sequence of the α subunit of human inhibin. Biological and immunological activities are expressed in terms of a highly purified preparation of M_r 32 000 bovine inhibin prepared in this laboratory. The closed circles in (*b*) indicate the relative absorbance (at 280 nm) of the eluate in arbitrary units. V_0, void volume; BSA, bovine serum albumin (M_r 66 000). P1, immunoreactive inhibin peak 1; P2, immunoreactive inhibin peak 2. In (*a*) the upper assay limit was 10 μg/ml.

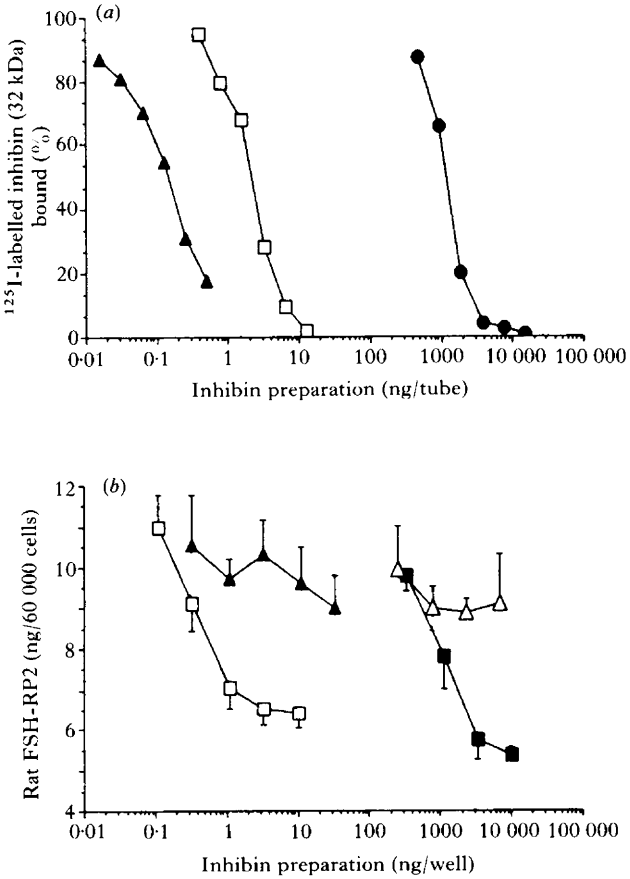

FIGURE 2. Comparison of (*a*) radioimmunoassay and (*b*) bioassay dose–response curves for different inhibin-containing preparations derived from bovine follicular fluid (FF). Highly purified M_r 32 000 bovine inhibin (□), highly purified α subunit of bovine inhibin (▲), crude bovine FF (●), peak 1 (■) and peak 2 (△) fractions were derived from a Sephacryl S-200 column (see Fig. 1). In (*b*) inhibin-like biological activity is associated with a dose-dependent suppression of basal FSH production by rat pituitary cells *in vitro*; values are means ± S.E.M. ($n = 4$).

ing conditions, three intensely stained bands of immunoreactive material were present (apparent M_r values of 116 000, 44 000 and 25 000) as well as several lightly stained bands (including bands of apparent M_r 56–58 000 and 30–32 000). Under reducing conditions (Fig. 3*b*) only two intensely stained bands (M_r values of 43 000 and 21 000) and two lightly stained bands (M_r 47 000 and 23 000) were evident. The specificity of immunostaining was confirmed by the absence of visible bands on 'control' portions of each blot incubated with antiserum preadsorbed with an excess (100 μg peptide/ml diluted (1:10) antiserum) of bIα(1–29)-Tyr^{30} (see Fig. 3*a*) or with normal rabbit serum (not shown). A comparison of immunoblots obtained for 'bulk-pooled' bovine FF (aspirated from ovaries stored for <3 days at 4 °C and used as starting material for inhibin purification in our laboratory) and for a bovine FF sample aspirated from a single follicle and snap-frozen within 20 min of slaughter revealed no detectable differences in the patterns of immunostaining between the two samples (compare Fig. 3*a* and *c*). Similarly, after incubation of portions of the latter bovine FF sample for 3 days at different temperatures (−20, +4 and +20 °C) in the presence of a trace amount (3 ng; 30 000 c.p.m.) of ^{125}I-labelled M_r 32 000 bovine inhibin, there were no detectable differences in either the pattern of immunostained bands or in the distribution of radioactivity on the nitrocellulose sheets. In each case only one major peak of radioactivity was observed at a position corresponding to an M_r value of 32 000 (Fig. 3*c*, *d* and *e*).

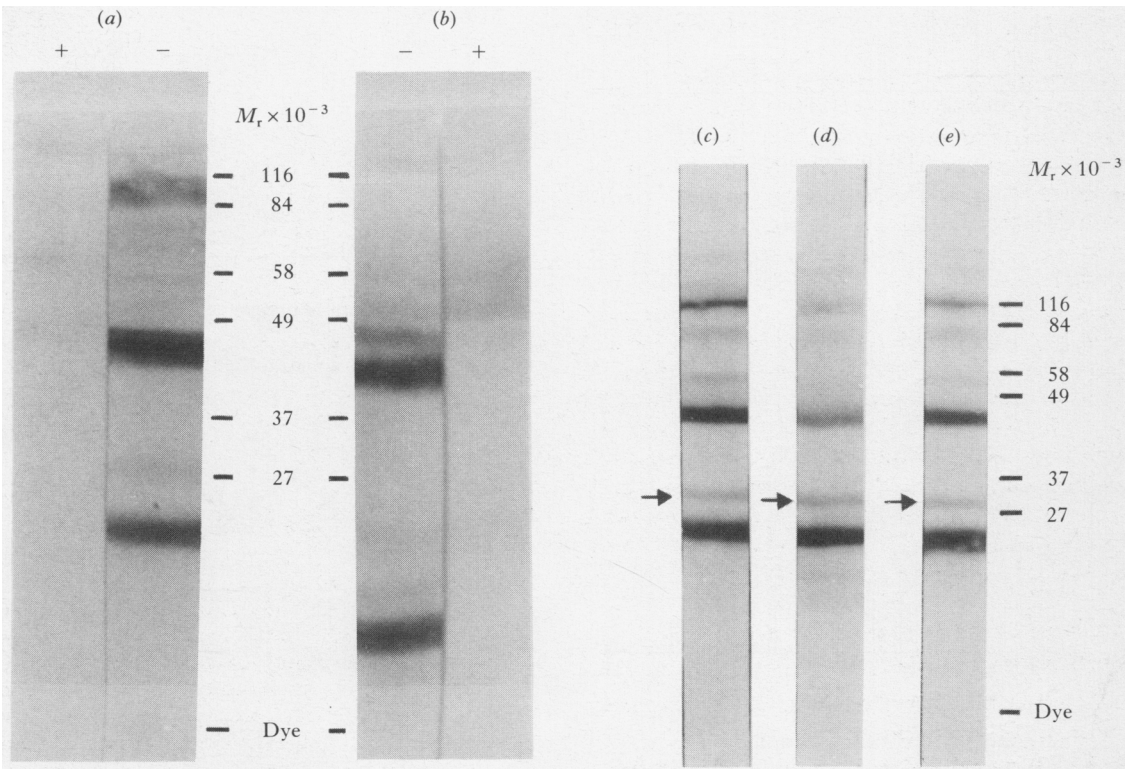

FIGURE 3. Patterns of immunostained bands on nitrocellulose sheets obtained following analysis of crude bovine follicular fluid (FF) samples by SDS-PAGE (15% gel) coupled with an immunoblotting procedure employing an antiserum raised against the n-terminal sequence (1–32) of the α subunit of human inhibin. The samples were (*a*) 'bulk-pooled' bovine FF under non-reducing conditions immunostained with antiserum in the presence (+) and absence (−) of an excess of bovine inhibin α subunit fragment (bIα(1–29)-Tyr^{30}, (*b*) 'bulk-pooled' bovine FF under reducing conditions immunostained with antiserum in the presence (+) and absence (−) of an excess of bIα(1–29)-Tyr^{30}, and (*c*, *d* and *e*) bovine FF aspirated from a single follicle electrophoresed under non-reducing conditions after incubation for 3 days with a trace amount of ^{125}I-labelled M_r 32 000 bovine inhibin at (*c*) −20, (*d*) +4 and (*e*) +20 °C. In each case an arrow indicates the position of the single major peak of radioactivity revealed by counting 2 mm slices of each nitrocellulose sheet. The positions of pre-stained molecular weight markers are indicated.

GCCM, utero-ovarian vein and jugular vein plasma

Immunoblotting of untreated samples of bovine GCCM (200 µl) and of utero-ovarian vein (15 µl) and jugular vein (15 µl) plasma after SDS-PAGE did not reveal the presence of any immunoreactive protein bands. Larger volumes of each sample (12 ml GCCM, 40 ml utero-ovarian vein plasma and 40 ml jugular vein plasma) were therefore subjected to immunoaffinity concentration by mixing for 3 days at 4 °C with Sepharose–anti-hIα(1–32) antibody (3 ml beads). After washing away unbound protein with 40 ml PBS (four 10 ml rinses) antibody-bound protein was eluted into 10 ml ammonium acetate buffer (0·1 mol/l; pH 3·5) containing 20% acetonitrile. After lyophilization, the residue was redissolved in 400 µl Tris–HCl (0·1 mol/l; pH 6·8) containing 10% (w/v) SDS, and portions (150 µl) were subjected to SDS-PAGE under both non-reducing and reducing conditions.

As shown in Fig. 4*a*, immunoblotting of affinity-purified bovine GCCM revealed the presence of a single intensely stained band with an apparent M_r of 25 000 under non-reducing conditions. Under reducing conditions (Fig. 4*b*) the apparent M_r of the band was 21 000. Several lightly stained bands were also evident, having apparent M_r values of 45 000 and 23 000 (non-reducing conditions) and 43 000 (reducing conditions). As shown in Fig. 4*c* and *d* respectively, immunoblotting of affinity-purified samples of both utero-ovarian vein and jugular vein plasma also revealed an intensely stained band of apparent M_r 25 000 under non-reducing conditions. Moreover, in both samples a second immunoreactive band (apparent M_r 45 000) was also clearly visible. In all cases the specificity of immunostaining was verified using antiserum preadsorbed with an excess of bIα(1–29)-Tyr^{30} peptide.

Comparison of bovine, porcine, ovine and human follicular fluids

Figure 5 shows the distribution of immunoreactive bands observed when samples (5 µl; approximately 300 µg protein) of human, ovine, bovine and porcine FF were analysed by SDS-PAGE under non-reducing conditions followed by immunoblotting. Whilst a relative paucity of immunoreactive material in human FF (confirmed by radioimmunoassay and bioassay; data not shown) precluded detailed comparison with the other FF samples, it was evident that an abundant M_r 42 000–45 000 component(s) was common to all four species, as were a number of larger components with M_r values ranging from 70 000 to 120 000. Notably, however, only bovine FF was associated with an intensely stained band of apparent M_r 25 000.

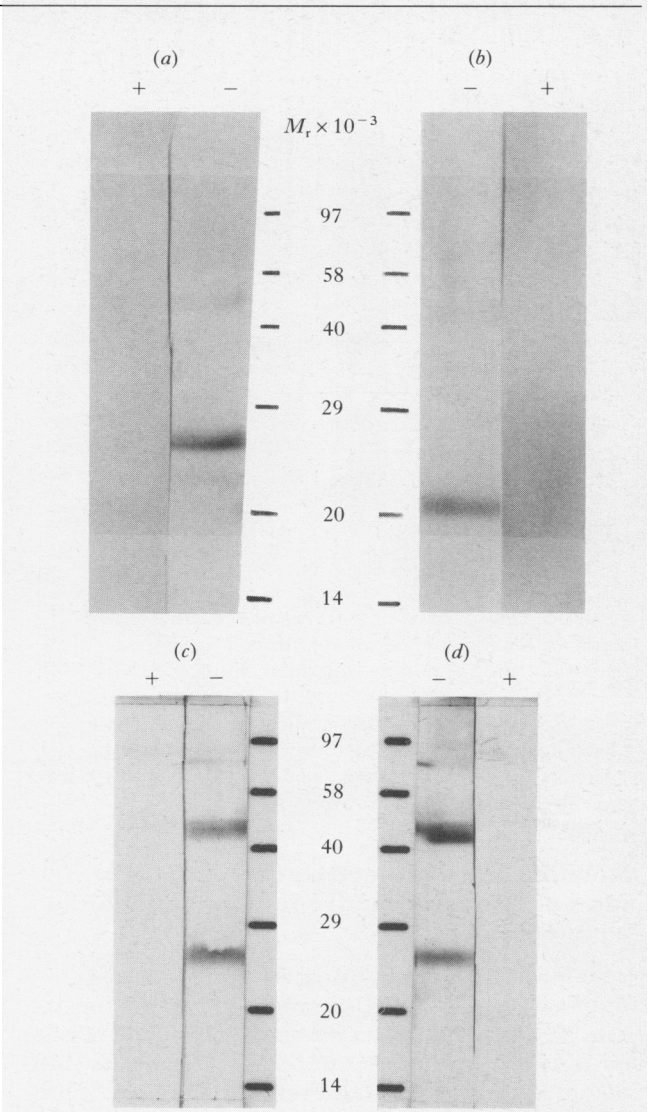

FIGURE 4. Patterns of immunostained bands on nitrocellulose sheets obtained following analysis of bovine granulosa cell-conditioned media (GCCM), utero-ovarian vein and jugular vein plasma by SDS-PAGE (15% gel) coupled with an immunoblotting procedure employing an antiserum raised against the n-terminal sequence (1–32) of the α subunit of human inhibin. Before electrophoresis, samples were subjected to immunoaffinity extraction to concentrate inhibin-like immunoreactivity. Samples were (*a*) GCCM extract under non-reducing conditions, (*b*) GCCM extract under reducing conditions, (*c*) utero-ovarian vein plasma extract under non-reducing conditions and (*d*) jugular vein plasma extract under non-reducing conditions. In each case, the specificity of immunostaining was assessed by incubating half-portions of each blot with antiserum in the presence (+) or absence (−) of an excess of bovine inhibin α subunit fragment (Inhibin-α(1–29)-Tyr^{30}). The positions of biotinylated molecular weight markers are indicated.

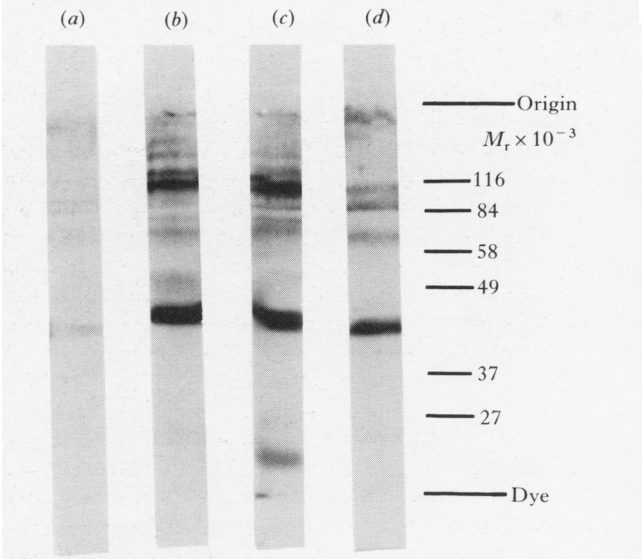

FIGURE 5. Comparison of patterns of immunostained bands following analysis of (*a*) human, (*b*) ovine, (*c*) bovine and (*d*) porcine follicular fluid by SDS-PAGE (12·5% gel; non-reducing conditions) coupled with an immunoblotting procedure employing an antiserum raised against the n-terminal sequence (1–32) of the α subunit of human inhibin. The positions of pre-stained molecular weight markers are indicated.

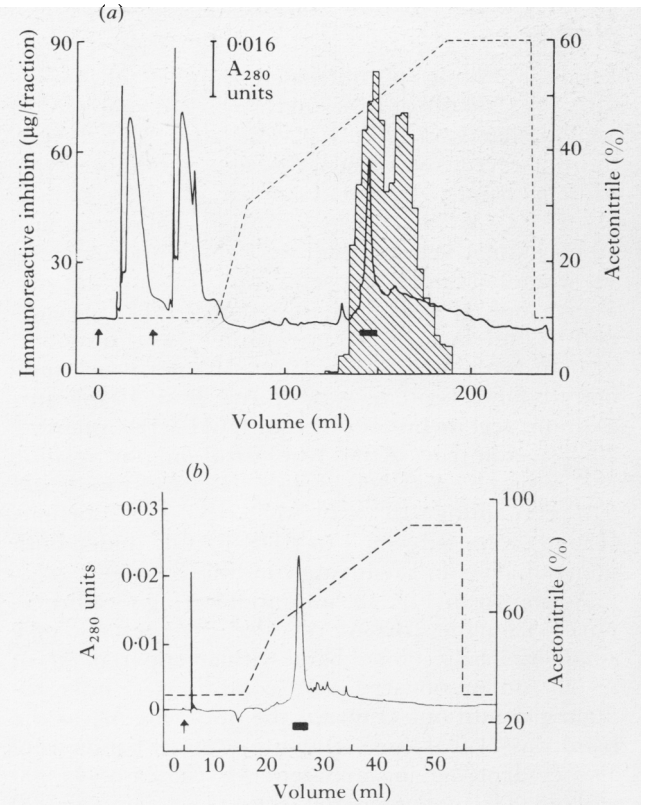

FIGURE 6. Purification by reversed-phase high-performance liquid chromatography (RP-HPLC) of an immunoreactive inhibin-containing fraction obtained from bovine follicular fluid after gel permeation and immunoaffinity chromatography. In (*a*) the immunoreactive fraction was applied (2 × 10 ml portions; arrows) to a 7·8 × 30 mm C_{18} column (TSK-ODS-120T) and eluted with a multilinear gradient of 10–60% (v/v) acetonitrile (broken line) in 0·1% (v/v) trifluoroacetic acid (TFA) at 3 ml/min. Immunoreactivity, expressed in terms of a highly purified preparation of M_r 32 000 bovine inhibin, is indicated by the hatched bars. Fractions comprising the major peak (solid horizontal bar) were mixed with 4 volumes of 10% acetonitrile in 0·1% TFA and applied to a second C_8 RP-HPLC column (Zorbax PEP RP/1; 6·2 × 80 mm) as shown in (*b*). The column was eluted with a multilinear gradient of 30–90% acetonitrile in 0·1% TFA at 1 ml/min. Immunoreactivity was confined to the single major protein peak eluting from the column, indicated by the solid horizontal bar. The solid lines in (*a*) and (*b*) indicate absorbance at 280 nm (A_{280}).

Isolation and characterization of a low M_r form of immunoreactive inhibin α subunit in bovine FF

The lower M_r immunoreactive fraction eluted from the Sephacryl S-200 gel-permeation column (peak 2, Fig. 1) was subjected to immunoaffinity purification on a column of Sepharose beads coupled to antibodies against hIα(1–32). From a total of 19·5 g protein applied to the affinity column (two runs) > 99% was not retained by the stationary phase and was eluted by washing the column with PBS (500 ml). Antibody-bound protein (approximately 0·1% of total; 23 mg) was eluted in a volume of 20–25 ml after passing through the column 100 ml ammonium acetate buffer (0·1 mol/l; pH 3·5) containing 20% acetonitrile. At least 80% of the total immunoreactivity applied to the column was recovered in this fraction which had an immunoreactive potency 460 times greater than that of crude bovine FF. On subjecting the affinity-purified fraction to RP-HPLC (C_{18} column) with acetonitrile gradient elution, several peaks of u.v. absorbing material were resolved as well as two peaks of immunoreactivity (Fig. 6*a*). The largest retarded protein peak, eluting at an acetonitrile concentration of 48%, contained the greatest amount of immunoreactivity (approximately 66% of total applied to column) and the immunoreactive potency of this fraction was about 3100 times greater than that of crude bovine FF. When fractions comprising this peak were pooled from several runs on the C_{18} column and applied to a second (C_8) RP-HPLC column, a single major protein peak (eluting at an acetonitrile concentration

of 63%) was found, representing approximately 63% of the total immunoreactivity applied to the column and having an immunoreactive potency 6200 times greater than crude bovine FF (Fig. 6*b*). Using this purification scheme, approximately 750 μg protein were recovered from 300 ml (19·5 g protein) crude bovine FF, with an overall yield of immunoreactive material of approximately 16%.

Analysis by SDS-PAGE (Fig. 7*a*) revealed that the purified protein was essentially homogeneous, appearing as a single major band of silver-stained material with an apparent M_r of 25 000 under non-reducing conditions and 21 000 under reducing conditions (i.e. identical to the respective M_r values for the dominant low M_r form of immunoreactivity demonstrated in crude bovine FF by immunoblotting). Furthermore, immunoblotting using antisera raised against highly purified M_r 32 000 bovine inhibin (Fig. 7*b*) and against hIα(1–32) (not shown) confirmed that all of the immunoreactivity present in the fraction was indeed associated with the M_r 25 000/21 000 protein. Amino-terminal sequencing of the isolated material (performed independently by Dr I. Blench, Department of Biochemistry, Imperial College, London) revealed the following sequence for the first 14 amino acid residues: NH_2-Ser-Thr-Pro-Pro-Leu-Pro-X-Pro-X-Ser-Pro-Ala-Ala-Leu (residues at positions 7 and 9 designated by X were not positively identified but were considered likely to be either Trp or Cys). This sequence is in complete accordance with that deduced from recombinant DNA studies by Forage *et al.* (1986) for the n-terminal sequence of bovine inhibin α subunit (M_r 21 000/22 000 form): NH_2-Ser-Thr-Pro-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu.

Despite its high degree of immunoreactivity (see Fig. 2*a*) when tested in the inhibin bioassay, the isolated material did not significantly suppress FSH production at doses as high as 30 ng/well and was judged to be at least 100 times less potent than highly purified M_r 32 000 bovine inhibin which suppressed FSH production with a medium effective dose of 0·45 ng/well (see Fig. 2).

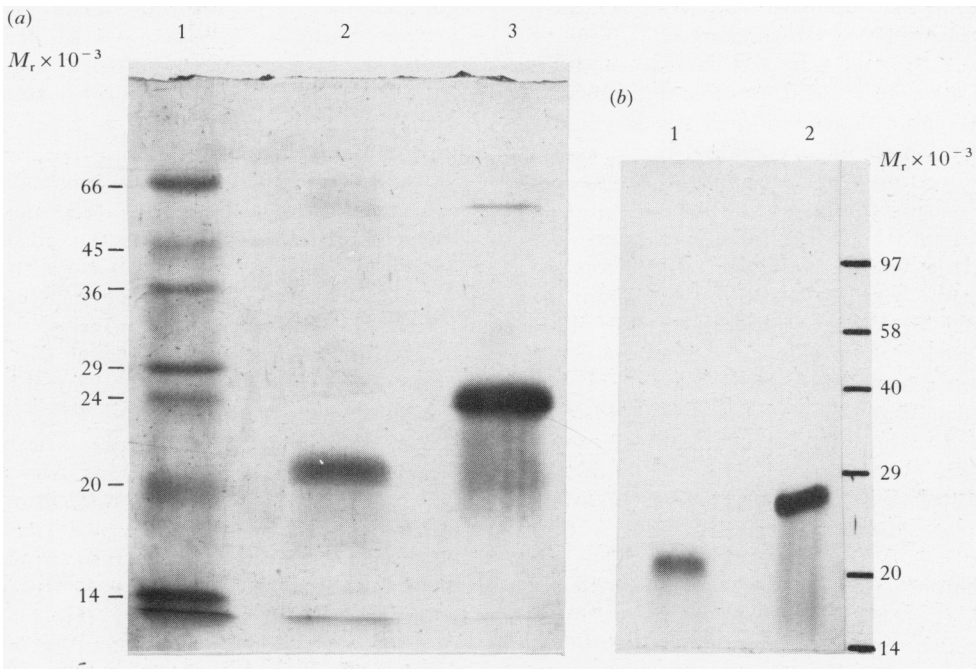

FIGURE 7. Analysis of the final immunoreactive fraction obtained after reversed-phase high-performance liquid chromatography by (*a*) SDS-PAGE (15% gel) and (*b*) SDS-PAGE (15% gel) coupled to an immunoblotting procedure. In (*a*) samples (5 and 10 μg respectively) were electrophoresed under reducing (track 2) and non-reducing (track 3) conditions in 2·5 mm thick slab gels calibrated with a mixture of molecular weight marker proteins (track 1), and protein bands visualized by silver staining. In (*b*) samples (2 μg) were similarly electrophoresed under reducing (track 1) and non-reducing (track 2) conditions, and after electrophoretic transfer to nitrocellulose sheet the distribution of inhibin immunoreactivity was revealed using an antiserum raised against highly purified M_r 32 000 bovine inhibin. The positions of molecular weight markers are indicated.

DISCUSSION

Whilst in general terms the present study confirms previous observations based on immunoblotting analysis that multiple M_r forms of immunoreactive inhibin are present in bovine FF (Miyamoto *et al.* 1986), marked discrepancies exist with respect to the apparent M_r values of the dominant immunoreactive forms identified. In contrast to the findings of Miyamoto *et al.* (1986) our data indicate that bovine FF contains substantial amounts of monomeric inhibin α subunit which, as judged by the relative intensities of immunostained bands, is apparently present in much greater concentrations than either M_r 58 000 or 32 000 forms of inhibin αβ dimer. Notably, two major bands of immunoreactive α subunit (apparent M_r values of 45 000 and 25 000 under non-reducing conditions), believed to correspond to the α subunits of M_r 58 000 and 32 000 forms of inhibin αβ dimer, were clearly resolved in both 'fresh' bovine FF (snap-frozen within 20 min of slaughter) and 'bulk-pooled' bovine FF which had been aspirated from ovaries stored at 4 °C for < 3 days after slaughter. The observation that incubation of 'fresh' bovine FF with a tracer amount of ^{125}I-labelled M_r 32 000 inhibin dimer for 3 days at −20, +4 or +20 °C did not affect either the pattern of immunostained bands or result in detectable degradation of the labelled M_r 32 000 inhibin dimer argues against the possibility that monomeric forms of α subunit were generated artifactually during the collection, storage and processing of bovine FF for inhibin purification in our laboratory. It is likely, therefore, that proteolytic processing of the high M_r α-subunit precursor molecule (Forage *et al.* 1986) to generate both M_r 45 000 and 25 000 forms of α subunit occurs before the granulosa cells release the material into FF. Indeed, it would appear that much of the processed α subunit is secreted without having been dimerized to a β subunit. These views accord with the findings of Bicsak, Cajander, Vale & Hsueh (1988) who studied the secretion of immunoreactive inhibin from rat granulosa cells *in vitro* and demonstrated that an α-subunit monomer (M_r 49 000) as well as an αβ-inhibin dimer (M_r 39 000) were the major forms secreted. They also revealed that most of the immunoreactive inhibin present within rat granulosa cells consisted of monomeric high M_r α-subunit precursor. No satisfactory explanation can be offered as to why Miyamoto *et al.* (1986) did not detect any α-subunit immunoreactivity of apparent M_r 45 000 (non-reducing conditions) in bovine FF. However, they did observe a minor band of M_r 26 000 which, upon reduction, migrated with an apparent M_r of 20 000. This latter component almost certainly corresponds to the substantially more abundant M_r 25 000 form of α subunit demonstrated in and subsequently isolated from bovine FF in this study. Since Miyamoto *et al.* (1986) performed their immunoblotting analyses on an immunoaffinity-isolated fraction of bovine FF rather than crude bovine FF as used by ourselves, it is possible, perhaps, that the immobilized monoclonal antibody used for their immunoaffinity extraction step did not bind appreciably to monomeric α subunit in its native state, thereby resulting in its depletion from the fraction subjected to immunoblotting analysis. The possibility of such artifactual alterations in the relative abundance of different M_r forms of immunoreactive inhibin was excluded in the present study by exploiting an immunoblotting procedure of enhanced sensitivity (ABC technique; Hsu *et al.* 1981) which permitted the analysis of untreated samples of bovine FF.

Whilst analysis of bovine GCCM in the present study revealed some immunoreactive material of M_r 45 000, the predominant immunoreactive species secreted by bovine granulosa cells *in vitro* was found to be a monomeric M_r 25 000 form of α subunit. A comparison of the immunological:biological (I:B) potency ratio for bovine GCCM (0·25) with that established for our highly purified preparation of M_r 32 000 bovine inhibin (I:B ratio normalized to 1·00; data not shown) revealed a fourfold excess of immunoreactivity over inhibin-like biological activity in bovine GCCM. This finding is consistent with immunoblotting data referred to above, since monomeric α subunit is essentially devoid of inhibin-like biological activity as demonstrated in this study (Fig. 2) and reported by others (Ling *et al.* 1985; Bicsak *et al.* 1988). A marked dissociation between immunoreactivity and biological activity was also observed during the first step of our inhibin isolation scheme in which crude bovine FF was fractionated using Sephacryl S-200 (Fig. 1). The observation is consistent with the finding of McLachlan *et al.* (1986*a*) that 12–18% of the total immunoreactivity eluted from their Sephacryl S-200 column was associated with a retarded fraction, which was devoid of biological activity. In the present study, however, a much greater proportion (60%) of the total immunoreactivity was associated with the retarded (peak 2) fraction, the I:B potency ratio for which was < 0·05 in comparison with values of 0·72 and 1·00 for the non-retarded (peak 1) fraction and highly purified M_r 32 000 bovine inhibin respectively. This quantitative discrepancy between our data and that of McLachlan *et al.* (1986*a*) with respect to the relative abundance of the retarded (lower M_r) immunoreactive fraction can be attributed to the characteristics of the different radioimmunoassays employed. Thus, whilst both assays utilize native M_r 32 000 bovine inhibin as radio-

iodinated tracer and standard, the antiserum used in the present study was raised against a synthetic peptide corresponding to the n-terminal region (1–32) of human inhibin α subunit and cross-reacted completely with free α subunit. In contrast, the antiserum used by McLachlan *et al.* (1986*a*) was raised against native M_r 58 000 bovine inhibin and was reported (Leversha, Robertson, de Vos *et al.* 1987) to show minimal (< 5%) cross-reaction with dissociated α or β subunits of inhibin. Since, on the basis of evidence presented in this report, we now believe that the lower M_r fraction (peak 2) eluted from the Sephacryl S-200 column contains mainly monomeric forms of α subunit, it is not surprising that our radioimmunoassay detected far higher levels than those reported by McLachlan *et al.* (1986*a*).

An essentially homogenous preparation of protein (apparent M_r 25 000 under non-reducing conditions) was isolated from the lower M_r fraction (peak 2) eluting from the Sephacryl S-200 column using a combination of immunoaffinity chromatography and RP-HPLC with an overall yield of 16%. The electrophoretic mobility of the isolated protein was identical to that of the lowest M_r band of immunoreactive material demonstrated in crude bovine FF and bovine GCCM and subsequently observed in bovine utero-ovarian and jugular venous plasma. Under reducing conditions the isolated protein migrated with an apparent M_r (21 000) identical to that shown by the reduced α subunit of highly purified M_r 32 000 bovine inhibin prepared in our laboratory (Knight *et al.* 1987; Castillo, 1989). The isolated protein was highly immunoreactive with antisera raised against the amino-terminal sequence of human inhibin α subunit (hIα(1–32)) and with an antiserum raised against highly purified native M_r 32 000 bovine inhibin but was devoid of inhibin-like biological activity (I:B ratio < 0·016 compared with 1·00 for highly purified M_r 32 000 bovine inhibin). The latter observation is consistent with previous reports (Ling *et al.* 1985; Miyamoto *et al.* 1985) that dissociated α or β subunits of inhibin do not suppress FSH production in rat pituitary cell inhibin bioassays.

Independent confirmation of the suspected identity of the isolated protein was provided by amino-terminal analysis which revealed an amino acid sequence (1–14) identical to the complementary DNA-derived sequence for the M_r 21 000 α subunit of bovine inhibin reported by Forage *et al.* (1986). The increased electrophoretic mobility of both the isolated protein and of the M_r 25 000 immunoreactive band visualized in bovine FF and bovine GCCM consistently observed upon sample reduction is probably attributable to an impaired binding of SDS to the protein in its non-reduced state. Since reliable estimates of M_r can be obtained only when both samples and molecular weight marker proteins are electrophoresed under reducing conditions (see Hames & Rickwood, 1981) it is most likely that the correct M_r value for the protein is 21 000 rather than 25 000. The monomeric nature and homogeneity of the isolated protein was confirmed during amino-terminal sequence analysis, since only one amino acid residue was identified at each successive cycle of Edman degradation.

Our initial suggestion that radioimmunoassayable forms of inhibin devoid of biological activity might be secreted by the bovine ovary to reach the peripheral circulation was confirmed by the demonstration of both M_r 45 000 and 25 000 forms (non-reducing conditions) of monomeric α subunit in bovine utero-ovarian and jugular venous plasma. Since the majority of reported inhibin radioimmunoassays (Bicsak, Tucker, Cappel *et al.* 1986; Ying, Czvik, Becker *et al.* 1987; Hasegawa *et al.* 1988), in common with our own unpublished assay, cross-react fully with inhibin α subunit, their potential use for the measurement of circulating levels of inhibin in cattle may be limited. Thus changes in plasma levels of immunoreactive and biologically active inhibin need not coincide, raising doubts about the physiological interpretation of such radioimmunoassay data alone. Finally, on the basis of our evidence from immunoblotting analysis (Fig. 5) it would appear that human, ovine and porcine FF also contain the higher M_r (45 000) form of monomeric α subunit but not the lower M_r (25 000) form identified in bovine FF. Whether, as seems likely, this immunoreactive material is also released into the peripheral circulation in these species is currently being investigated.

ACKNOWLEDGEMENTS

We are indebted to Dr I. Blench for carrying out the amino-terminal analysis, Drs G. Bialy and C. M. Howles for porcine and human FF, Drs B. J. McLeod and R. G. Glencross for bovine utero-ovarian vein plasma and to Mrs S. Dalton for typing the manuscript. The financial support of AFRC (Grant No. AG45/343) is gratefully acknowledged.

REFERENCES

Bicsak, T. A., Cajander, S. B., Vale, W. & Hsueh, A. J. W. (1988). Inhibin: studies of stored and secreted forms by biosynthetic labeling and immunodetection in cultured rat granulosa cells. *Endocrinology* **122**, 741–748.

Bicsak, T. A., Tucker, E. M., Cappel, S., Vaughan, J., Rivier, J., Vale, W. & Hseuh, A. J. W. (1986). Hormonal regulation of granulosa cell inhibin biosynthesis. *Endocrinology* **119**, 2711–2719.

Castillo, R. J. (1989). Bioassay and isolation of bovine ovarian inhibin and its effects on gonadotrophin secretion. *Ph.D. Thesis*. University of Reading.

Findlay, J. K. (1986). The nature of inhibin and its use in the regulation of fertility and diagnosis of infertility. *Fertility and Sterility* **46**, 770–783.

Forage, R. G., Ring, J. M., Brown, R. W., McInerney, B. V., Cobon, G. S., Gregson, R. P., Robertson, D. M., Morgan, F. J., Hearn, M. T. W., Findlay, J. K., Wettenhall, R. E. H., Burger, H. G. & de Kretser, D. M. (1986). Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 3091–3095.

Fukuda, M., Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K. & Matsuo, H. (1986). Isolation of bovine follicular fluid inhibin of about 32 kDa. *Molecular and Cellular Endocrinology* **44**, 55–60.

Hames, B. D. & Rickwood, D. (Eds) (1981). *Gel Electrophoresis of Proteins: A Practical Approach*, 290 pp. Oxford: IRL Press.

Hasegawa, Y., Miyamoto, K., Fukuda, M., Takahashi, Y. & Igarashi, M. (1986). Immunological study of ovarian inhibin. *Endocrinologia Japonica* **33**, 645–654.

Hasegawa, Y., Miyamoto, K., Igarashi, M., Yanaka, T., Sasaki, K. & Iwamura, S. (1987). Changes in serum concentrations of inhibin during the estrous cycle of the rat, pig and cow. In *Inhibin–Non-Steroidal Regulation of Follicle Stimulating Hormone Secretion*, Serono Symposia Publications, vol. 42, pp. 119–133. Eds H. G. Burger, D. M. de Kretser, J. K. Findlay & M. Igarashi. New York: Raven Press.

Hasegawa, Y., Miyamoto, K., Iwamura, S. & Igarashi, M. (1988). Changes in serum concentrations of inhibin in cyclic pigs. *Journal of Endocrinology* **118**, 211–219.

Henderson, K. M. & Franchimont, P. (1981). Regulation of inhibin production by ovarian cells *in vitro*. *Journal of Reproduction and Fertility* **63**, 431–442.

Hsu, S. M., Raine, L. & Fanger, H. (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *Journal of Histochemistry and Cytochemistry* **29**, 577–580.

de Jong, F. H. (1988). Inhibin. *Physiological Reviews* **68**, 555–607.

Knight, P. G., Castillo, R. J. & Glencross, R. G. (1987). Isolation from bovine follicular fluid (bFF) of a 32 kDalton molecule with potent inhibin-like biological activity (ILA). *Journal of Endocrinology* **112** (Suppl.), Abstract 52.

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

Lee, V. W. K., McMaster, J., Quigg, H., Findlay, J. & Leversha, L. (1981). Ovarian and peripheral blood inhibin concentrations increase with gonadotropin treatment of immature rats. *Endocrinology* **108**, 2403–2405.

Leversha, L. J., Robertson, D. M., de Vos, F. L., Morgan, F. J., Hearn, M. T. W., Wettenhall, J. K., Findlay, J. K., Burger, H. G. & de Kretser, D. M. (1987). Isolation of inhibin from ovine follicular fluid. *Journal of Endocrinology* **113**, 213–221.

Ling, N., Ying, S.-Y., Ueno, N., Esch, F., Denoroy, L. & Guillemin, R. (1985). Isolation and partial characterization of a M_r 32 000 protein with inhibin activity from porcine follicular fluid. *Proceedings of the National Academy of Sciences of the U.S.A.* **82**, 7217–7221.

McLachlan, R. I., Robertson, D. M., Burger, H. G. & de Kretser, D. M. (1986*a*). The radioimmunoassay of bovine and human follicular fluid and serum inhibin. *Molecular and Cellular Endocrinology* **46**, 175–185.

McLachlan, R. I., Robertson, D. M., Healy, D. L., de Kretser, D. M. & Burger, H. G. (1986*b*). Plasma inhibin levels during gonadotrophin-induced ovarian hyperstimulation for IVF: a new index of follicular function? *Lancet* i, 1233–1234.

Mann, G. E., McNeilly, A. S. & Baird, D. T. (1988). The source of ovarian inhibin secretion during the oestrous cycle of the sheep. *Journal of Reproduction and Fertility Abstract Series* **1**, 76.

Mason, A. J., Hayflick, J. S., Ling, N., Esch, F., Ueno, N., Ying, S.-Y., Guillemin, R., Niall, H. & Seeburg, P. H. (1985). Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. *Nature* **318**, 659–663.

Mason, A. J., Niall, H. D. & Seeburg, P. H. (1986). Structure of two human ovarian inhibins. *Biochemical and Biophysical Research Communications* **135**, 957–964.

Miyamoto, K., Hasegawa, Y., Fukuda, M., Nomura, M., Igarashi, M., Kangawa, K. & Matsuo, H. (1985). Isolation of porcine follicular fluid inhibin of 32 kDaltons. *Biochemical and Biophysical Research Communications* **129**, 396–403.

Miyamoto, K., Hasegawa, Y., Fukuda, M. & Igarashi, M. (1986). Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32k inhibin. *Biochemical and Biophysical Research Communications* **136**, 1103–1109.

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

Rivier, C. & Vale, W. (1987). Inhibin measurement and role in the immature female rat. *Endocrinology* **120**, 1688–1690.

Rivier, J., Spiess, J., McClintock, R., Vaughan, J. & Vale, W. (1985). Purification and partial characterization of inhibin from porcine follicular fluid. *Biochemical and Biophysical Research Communications* **133**, 120–127.

Robertson, D. M., Foulds, M. L., Leversha, L., Morgan, F. J., Hearn, M. T. W., Burger, H. G., Wettenhall, R. E. H. & de Kretser, D. M. (1985). Isolation of inhibin from bovine follicular fluid. *Biochemical and Biophysical Research Communications* **126**, 220–226.

Robertson, D. M., de Vos, F. L., Foulds, L. M., Burger, H. G., Morgan, F. J., Hearn, M. T. W. & de Kretser, D. M. (1986). Isolation of a 31 kDa form of inhibin from bovine follicular fluid. *Molecular and Cellular Endocrinology* **44**, 271–277.

Scott, R. S., Burger, H. G. & Quigg, H. (1980). A simple and rapid *in vitro* assay for inhibin. *Endocrinology* **107**, 1536–1541.

Tsonis, C. G., McNeilly, A. S. & Baird, D. T. (1986). Measurement of exogenous and endogenous inhibin in sheep serum using a new and extremely sensitive bioassay for inhibin based on inhibition of ovine FSH secretion *in vitro*. *Journal of Endocrinology* **110**, 341–352.

Ying, S.-Y. (1988). Inhibins, activins and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocrine Reviews* **9**, 267–293.

Ying, S.-Y., Czvik, J., Becker, A., Ling, N., Ueno, N. & Guillemin, R. (1987). Secretion of follicle-stimulating hormone and production of inhibin are reciprocally related. *Proceedings of the National Academy of Sciences of the U.S.A.* **84**, 4631–4635.