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Variability in the Apparent Molecular Weight of Inhibin

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Abstract

An *in vitro* bioassay based on suppression of GnRH-stimulated FSH secretion by pituitary cells in culture was used to monitor inhibin activity after dialysis, gel filtration or polyacrylamide gel electrophoresis of protein preparations from a variety of gonadal secretions and extracts under native and dissociating conditions. The suggestion that inhibin is a peptide of molecular weight less than 5000 was not confirmed. Although some fractions of low molecular weight suppressed FSH secretion, the amount of activity was low and the dose response curves were not parallel with a standard preparation of inhibin. Under most conditions, inhibin eluted with an apparent molecular weight of about 90 000. However, gel filtration of rete testis fluid protein in 1 M acetic acid resulted in elution of inhibin activity with a lower apparent molecular weight and with polyacrylamide gel electrophoresis in 0·1% (w/v) sodium dodecylsulfate, the apparent molecular weight was 30 000. It is concluded that inhibin is a protein which tends to aggregate and coelute with larger molecules.

Introduction

Inhibin is believed to be a non-steroidal hormone produced by the gonads to regulate the secretion of FSH by the pituitary gland (Baker *et al.* 1982*a*, 1983). Purification is proceeding in several laboratories and a number of different apparent molecular weights have been reported. Material with inhibin-like activity and a molecular weight of approximately 20 000 has been demonstrated in bovine and human seminal plasma, ovine rete testis fluid (RTF), bovine and ovine testicular extracts and porcine ovarian follicular fluid (Baker *et al.* 1982*a*; Chari *et al.* 1982; Ying *et al.* 1982; Sheth *et al.* 1984). However, inhibin-like activity has also been found in material of lower and higher molecular weight. Several groups (e.g. Davis *et al.* 1978; Franchimont *et al.* 1979; Vijayalakshmi *et al.* 1980; Chari *et al.* 1982; Williams and Lipner 1982; Ramasharma *et al.* 1984) have suggested that a peptide of molecular weight less than 5000 exists or can be dissociated from a larger peptide by repeated gel filtration or treatment with urea. The larger protein (mol. wt $\geqslant 100\,000$) has been demonstrated by gel filtration and X-ray inactivation (Cahoreau *et al.* 1979; Ward 1981; Baker *et al.* 1982*a*; Williams and Lipner 1982).

This wide variation in reported findings may indicate that inhibin exists in several forms but is more likely to be due to the use of different methods for detecting inhibin-like activity. Interference by material which blocks the action of gonadotrophins on the gonads is possible in some of the indirect methods, such as the inhibition of augmentation of hCG on ovarian or uterine weights in immature rodents (Baker *et al.* 1981). Other techniques, in which suppression of the secretion of FSH is measured

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directly, may also lack specificity particularly if they are used as single-point detection methods. To date, few of the reported studies on the purification of inhibin have involved monitoring by classical parallel-line bioassays.

An *in vitro* bioassay for inhibin has been developed in this laboratory (Eddie *et al.* 1979). It is based on the suppression of FSH secretion by cultures of rat anterior pituitary cells maximally stimulated with GnRH. A standard is included in each assay and potencies of unknown samples are determined with parallel-line bioassay statistics. There is close agreement between potency estimates of samples assayed with this method and FSH-suppressing activity *in vivo* tested by infusion of the samples into castrated male sheep and measurement of serum FSH levels (Baker *et al.* 1981). This assay permits quantitative monitoring of purification steps for changes in specific activity and yield, and also includes a check for non-specificity through examination for parallelism between the unknown and standard preparations.

When this assay was used to survey inhibin activity in a variety of gonadal secretions and extracts, crude preparations of ovine RTF were found to have a high specific activity and thus RTF was chosen as the main source for further purification of inhibin (Eddie *et al.* 1979; Baker *et al.* 1982*a*, 1982*b*). However, when it became apparent that the inhibin-like activity in RTF was associated with proteins of high molecular weight, attempts were made to detect inhibin activity in dissociated smaller moieties, and other sources of inhibin were investigated to see if activity was present in fractions of lower molecular weight.

Materials and Methods

Sources of Inhibin

Ovine RTF was obtained by the method of Voglmayr *et al.* (1966). The fluid was collected in refrigerated flasks (4°C) which were changed once a day. Sperm were removed by centrifugation, the supernatant frozen and later pooled into 10-litre batches, sterilized by filtration (0·22 μm) and stored at −20°C in 1-litre lots. Ovine testicular lymph was collected by cannulation of lymphatic vessels in the spermatic cord (Morris and McIntosh 1972). Bovine seminal plasma and human seminal plasma came from specimens of semen provided for analysis which had normal sperm concentration, motility and morphology. Culture of mature rat seminiferous tubules was performed as previously described and the medium stored after centrifugation and filtration (Eddie *et al.* 1978). Sow ovaries were obtained in an abattoir and placed on dry ice immediately after removal from the carcass. Porcine follicular fluid was aspirated from follicles of diameter greater than 0·5 cm. Whole ovaries were homogenized for the acid-urea extraction. Ovine follicular fluid was collected from follicles between 3 and 10 mm in diameter. All liquid preparations were stored at −20°C. Lyophilized powders were stored in a desiccator at room temperature.

Assays

Inhibin activity was measured by bioassay (Eddie *et al.* 1979). Lyophilized materials were dissolved in Dulbecco phosphate-buffered saline (DPBS) and 100 μl added to the medium of randomly ordered culture dishes. Liquid samples were added either directly or after dilution to 100 μl with DPBS. The inhibin standard was prepared from ovine testicular lymph (OTLS) and arbitrarily assigned a potency of one unit per milligram (1 u/mg; Eddie *et al.* 1979). A new standard was prepared from ovine RTF (RTFS) and has been used since February 1982. RTFS has a potency of 416 (95% confidence limits: 370–460) OTLS u/mg. In most assays the standard was used at five doses in triplicate over the range 0·1–3 u/ml and unknowns in three doses in duplicate. Chromatography effluents were assayed for zones of activity by using one or more doses singly or in duplicate from each fraction or pool of fractions. Multiple doses from zones of activity were assayed to check for parallelism with the standard. Minimal detectable activities were 0·1 u/ml with a single point or 0·3 u/ml with a dose response line. Indices of precision (λ values) were less than 0·200 and averaged 0·074 (Baker *et al.* 1981). Repetitive measurements of the same sample gave an intra-assay coefficient of variation of 5·9% and a between-assay coefficient of variation of 17%. Specific activities were expressed with respect to the mass of protein determined by absorbance at 280 nm. FSH in the culture medium was

measured by radioimmunoassay using reagents donated by the National Institutes of Health, which had intra-assay and between-assay precisions of 3·2 and 8·1% (coefficient of variation) respectively.

Dialysis and Gel Filtration

Dialysis was performed with cellulose dialysis casing with nominal molecular weight cut-offs of 1000 or 2000 (Spectrapor 6), 6000–8000 (Spectrapor 1) or 12 000–14 000 (Spectrapor 2) at 4°C against several changes of DPBS or 10 mM NH_4HCO_3 until the pH and conductivity of the sample was equal to that of the buffer. Dialysis of samples of RTF against water results in formation of a precipitate which contains inhibin activity. Diafiltration was performed in an Amicon hollow-fibre dialyser–concentrator (DC2) with a hollow-fibre cartridge (H1 P5) with nominal molecular weight cut-off of 5000. Samples were concentrated 5- to 10-fold and then diafiltered with a 10- to 20-fold volume of 10 mM NH_4HCO_3. RTF was also concentrated 4- to 5-fold over an Amicon UM2 membrane with nominal cut-off 1000.

Gel filtration was performed at 4°C on Biogel P60, Sephacryl S200 or S300 under various conditions. The effluents were monitored at 280 or 230 nm. The columns were calibrated with globular proteins of known molecular weight obtained from Pharmacia.

Gel permeation in 0·1 M NH_4HCO_3 (pH 8·0) or 1 M acetic acid (pH 2·5) was performed on a 4 mm by 250 mm Lichrosorb DIOL 5 μm column supplied by Merck with Altex high-performance liquid chromatography equipment. Partially purified samples or standard proteins were dissolved (10 mg/ml), filtered (0·22 μm) and injected in 10- or 500-μl volumes. Fractions (three drops) were collected by hand and where 10-μl volumes were used, four runs were pooled, lyophilized and redissolved in DPBS for assay.

Polyacrylamide Gel Electrophoresis (PAGE)

Cylindrical 7·5% (w/v) gels with stacking gels were prepared according to the method of Davis (1964) and 200 μg of RTF protein dissolved in sample buffer layered on each of 12 75-mm gels and electrophoresis performed at 3 mA per gel for 2 h. Two gels were stained with amido black and the remainder cut into 5-mm sections, corresponding sections pooled, crushed and eluted with 0·1 M Tris . HCl (pH 8·75) for 60 h and the eluents assayed.

Preliminary experiments showed that inhibin samples exposed to 0·1% (w/v) sodium dodecylsulfate (SDS) retained activity, but were inactivated by exposure to mercaptoethanol or dithiothreitol (Baker *et al.* 1982*b*). Prolonged heating of RTF protein will inactivate inhibin, but exposure to 65°C for 1 h has no effect (Baker *et al.* 1982*b*). For SDS PAGE using the method of Laemmli (1970) 1 mg protein was dissolved in 100 μl of sample buffer without mercaptoethanol and heated at 40°C for 15 min and layered across the central area of a stacking gel. Protein standards of low molecular weight from Pharmacia were placed in slots at the edges of the stacking gel. Separating gels of various acrylamide plus bisacrylamide concentrations (total polymer concentration 7·5–15%) were cast in 2 by 50 by 100 mm slabs and electrophoresis conducted at 4 mA for 14 h at 4°C. The sides of the gel containing the standard protein tracks and the edges of the sample tract were cut and stained with 0·2% Coomassie brilliant blue in 50% (w/v) trichloroacetic acid and destained in 7% (v/v) acetic acid. The central area was sliced into 2- or 5-mm strips and the proteins eluted into 2 ml water at 4°C for 24 h and the eluents assayed.

Results

Dialysis Studies

Recovery of inhibin activity was virtually complete (≥84%) following dialysis of RTF in cellophane membranes with nominal molecular weight cut-off of 1000, 2000 or 6000–8000 against DPBS, diafiltration with a hollow-fibre cartridge (mol. wt cut-off 5000) against 10 mM NH_4HCO_3 followed by lyophilization or concentration over a UM 02 membrane. When RTF was exposed to dissociating conditions—Triton X-100, 8 M urea, 2 M guanidine hydrochloride or low pH, and then dialysed through membranes retaining molecular weights of 1000–2000 or 12 000–14 000, there were some losses of activity, but recoveries were similar with both membranes, suggesting that large amounts of inhibin-like material with a molecular weight between 1000

and 14 000 were not generated by these procedures as indicated in the following tabulation:

Conditions	Recovery (%) of inhibin activity for membrane nominal molecular weight cut-off of:	
	1000–2000	12 000–14 000
Pooled RTF	106	104
Formic acid (pH 1·9)	73	80
2 M guanidine . HCl, 20°C, 24 h	77	67
8 M urea, pH 8·0, 40°C, 60 min	92	96
1% (v/v) Triton X-100	104	106

There was, however, some FSH-suppressing activity in the lyophilized dialysates after acid treatment but this did not lower FSH secretion in parallel with the standard (Fig. 1).

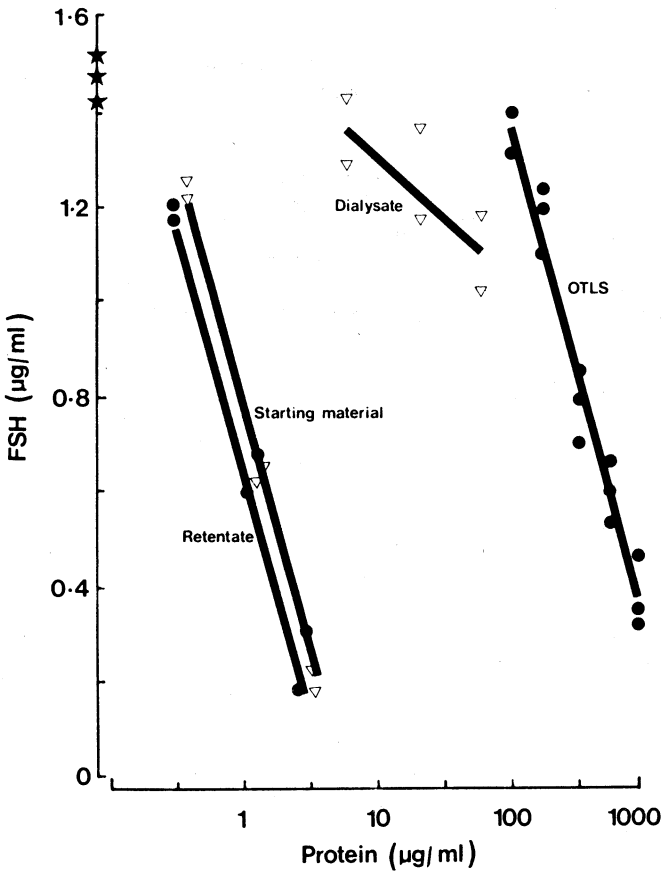

Fig. 1. Inhibin assay of RTF protein following acidification (pH 1·9) with formic acid, dialysis in a membrane (mol. wt cut-off 12 000–14 000) against 10 mM NH_4HCO_3 and lyophilization. Concentration of FSH in pituitary cell culture medium was measured by radioimmunoassay. Concentration of protein in culture medium added with standard or unknown samples was calculated from measurements of absorbance at 280 nm. The standard (OTLS) was used at five doses in triplicate and the unknowns at three doses in duplicate. The concentration of FSH in medium of cells exposed only to GnRH is indicated (*). The specific activity of the retentate was the same as that of the starting material. There was some FSH-suppressing activity in the lyophilized dialysate but this was not parallel with the standard.

Gel Filtration of RTF Protein

When fresh RTF was gel-filtered on Sephacryl S300, material with inhibin activity eluted with proteins of high molecular weight, there being none detectable in more retarded zones (Fig. 2). Material with inhibin activity and with an apparent molecular weight of 90 000 also eluted when a 55% (w/v) ammonium sulfate precipitate of RTF protein was chromatographed on Sephacryl S300 (Table 1). Exposure of RTF protein to a variety of dissociating and extraction procedures aimed at producing a dissociated form of inhibin was unsuccessful (Table 1). Although FSH-suppressing activity was found in salt volume eluates from columns after treatment with guanidine hydrochloride, magnesium chloride and potassium thiocyanate, this did not suppress FSH levels in parallel with the inhibin standard and was probably due to a toxic effect of the salts

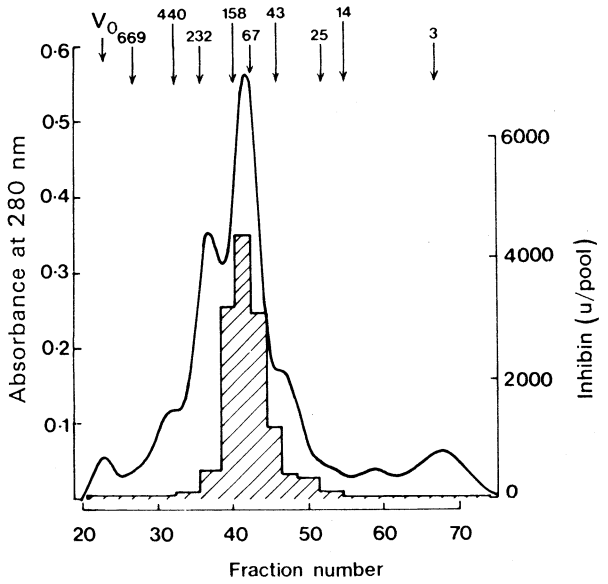

Fig. 2. Gel filtration of 4 ml fresh rete testis fluid on Sephacryl S300, 1·6 by 83 cm column equilibrated with Dulbecco phosphate-buffered saline (pH 7·4) and eluted at 12 ml/cm^2 h with 4-ml fractions. The fractions were assayed at two doses and the starting material at three doses in duplicate. The recovery of ultraviolet-absorbing material in the column effluent was 102% and of inhibin activity 76%. Specific activities were 100 (80–130) u/mg for the starting material and 430 (300–600) u/mg for the most active fraction. The void volume (V_0) and positions of elution of standard proteins (mol. wt $\times$ 10^{-3}) are indicated by arrows.

on the pituitary cells. Gel filtration of RTF on Biogel P60 in 1 M acetic acid resulted in two peaks of inhibin activity, a small one in the void volume and a retarded peak separated from the bulk of the protein. However, apparent molecular weights could not be estimated as the standard proteins ran anomalously: trypsin inhibitor (21 500) and pepsin (35 000) eluting late (Fig. 3).

Similar results were obtained with gel-permeation chromatography on Lichrosorb DIOL. In 0·1 M NH_4HCO_3 inhibin eluted in the void volume of the column (mol. wt $\geqslant$67 000) whereas in 1 M acetic acid the activity was retarded. Standard proteins ran anomalously under both conditions; for example, in 0·1 M NH_4HCO_3, pepsin and

Table 1. Gel filtration of RTF protein

Pretreatment	Specific activity of starting material (u/mg)	Gel filtration conditions Gel	Column[A]	Buffer[B]	10^{-3} × Apparent mol. wt of inhibin	Yield (%)
$(NH_4)_2SO_4$ precipitation	400	S300	1	1	90	66
6 M urea	89	P60	2	2	⩾60	144
8 M urea[C]	61	S200	3	3	90	89
2 M guanidine . HCl[D]	200	S200	3	3	90	⩾39
5 M guanidine . HCl[D]	79	S200	3	3	90	122
5 M $MgCl_2$	200	P60	2	4	⩾60	23
4 M potassium thiocyanate	200	S300	1	1	90	56
Heat[E]	140	S200	3	3	90	⩾87
Acid-ethanol supernatant	540	S200	3	3	90	87
0·1% (w/v) SDS[F]	280	S300	1	1	90	36
0·2% (v/v) Tween 80	305	S300	2	3	90	96

[A] Column dimensions (cm): 1, 2·5 by 63; 2, 2·5 by 90; 3, 1·6 by 85.
[B] Buffers: 1, 0·1 M NH_4HCO_3, pH 8·0; 2, 6 M urea in 0·1 M Tris . HCl, pH 8·75; 3, DPBS, pH 7·4; 4, 0·1 M ammonium acetate, pH 5·5.
[C] RTF was adjusted to pH 5·5 with 0·1 M HCl, urea added to 8 M and incubated in a 40°C water-bath for 60 min.
[D] RTF with 2 or 5 M guanidine . HCl was incubated at 20°C for 24 h. The 2 M sample was applied to the column directly. The 5 M sample was dialysed with a membrane of molecular weight cut-off = 1000 and lyophilized before gel filtration.
[E] RTF was adjusted to pH 5·5 with 0·1 M HCl, heated in a boiling water-bath for 60 min, centrifuged to remove the precipitate and the supernatant dialysed with a membrane of molecular weight cut-off = 1000 and lyophilized. This resulted in a 50% loss of inhibin activity.
[F] Lyophilized RTF protein was dissolved in 0·1 M NH_4HCO_3 with 0·1 (w/v) SDS and applied directly to the column.

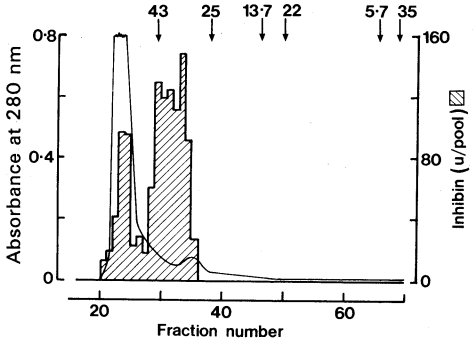

Fig. 3. Gel filtration of RTF protein in 1 M acetic acid on Biogel P60. RTF was diafiltered with a hollow fibre cartridge (mol. wt cut-off 5000) and lyophilized, 20 mg dissolved in 5 ml 1·0 M acetic acid and applied to a 2·5 by 90 cm column of Biogel P60 equilibrated in 1 M acetic acid and eluted at a rate of 20 ml/cm^2 h. The 5-ml fractions were diluted 1 : 2 and lyophilized. The starting material was assayed at three doses in duplicate and the fractions at one or two doses. The specific activity of the starting material was 87 (73–100) u/mg and the peak fractions were 28 u/mg in the void volume and 590 u/mg in the retarded peak. Total recovery of activity from the column was 85%. Material from salt volume fractions suppressed FSH levels in the pituitary cell culture medium but not in parallel with the inhibin standard. Note standard proteins (mol. wt × 10^{-3}) did not elute in order of molecular weights.

trypsin inhibitor eluted near the void volume and chymotrypsinogen (mol. wt 25 000) and trypsin (22 000) were retarded and eluted after ribonuclease (13 700). In 1 M acetic acid, ovalbumin (43 000) and catalase (232 000) eluted after ribonuclease (13 700).

Polyacrylamide Gel Electrophoresis of RTF Proteins

Material showing inhibin activity was eluted from sections of 7·5% polyacrylamide gel cylinders in a broad zone which migrated more slowly than albumin (Fig. 4). With 0·1% (w/v) SDS PAGE. 20–76% of the inhibin activity applied to the gel was recovered, most being in one section corresponding to a molecular weight of approximately 30 000 (Fig. 5). When SDS PAGE was performed with gels of different concentrations (7·5, 10, 12·5, 15% total polymer concn) the Fergusson plots for the standards did not extrapolate to the origin but inhibin eluted consistently in the 30 000 region.

Table 2. Gel filtration of other sources of inhibin

Source	Specific activity of starting material (u/mg)	Gel filtration conditions Gel	Column[A]	Buffer[B]	10^{-3} × Apparent mol. wt of inhibin	Yield (%)
Ovine testicular lymph	1·2	P60	1	1	⩾60[C]	99
Rat seminiferous tubule culture medium	2·2	P60	1	1	20[C]	81
Human seminal plasma	0·66	S300	2	2	90[C]	83
Porcine follicular fluid	6·7	P60	1	1	⩾60	42
Ovine follicular fluid	200	S200	3	2	90	85
Acid-urea extract of pig ovaries	3·8	S300	2	2	90[C]	58

[A] Column dimensions (cm): 1, 2·5 by 90; 2, 2·5 by 63; 3, 1·6 by 85.
[B] Buffers: 1, 0·1 M ammonium acetate, pH 5·5; 2, 0·1 M NH_4HCO_3, pH 8·0.
[C] FSH-suppressing activity in salt volume (see text).

Gel Filtration of Other Sources of Inhibin

The specific activity of inhibin in other gonadal secretions or extracts was considerably lower than for ovine RTF and follicular fluid (Table 2). Apart from seminiferous tubule culture medium, the inhibin activity eluted either in the void volume of Biogel P60 or with an apparent molecular weight of 90 000 on Sephacryl S300. The seminiferous tubule culture medium displayed a major peak of activity in the 20 000 zone although there was also a small amount of inhibin activity in the void volume and some FSH-suppressing activity in the salt volume. FSH-suppressing material was also present in the salt volumes of the elution patterns of several other sources (Table 2). However, the activity was either very low, did not suppress FSH levels in parallel with the inhibin standard, or was toxic, causing detachment of the pituitary cells from the culture dishes.

Discussion

This search for a low molecular weight peptide with inhibin activity was undertaken because such a molecule might be more stable, less liable to aggregation and easier to purify than a molecule of higher molecular weight. Also, it is possible that such a peptide

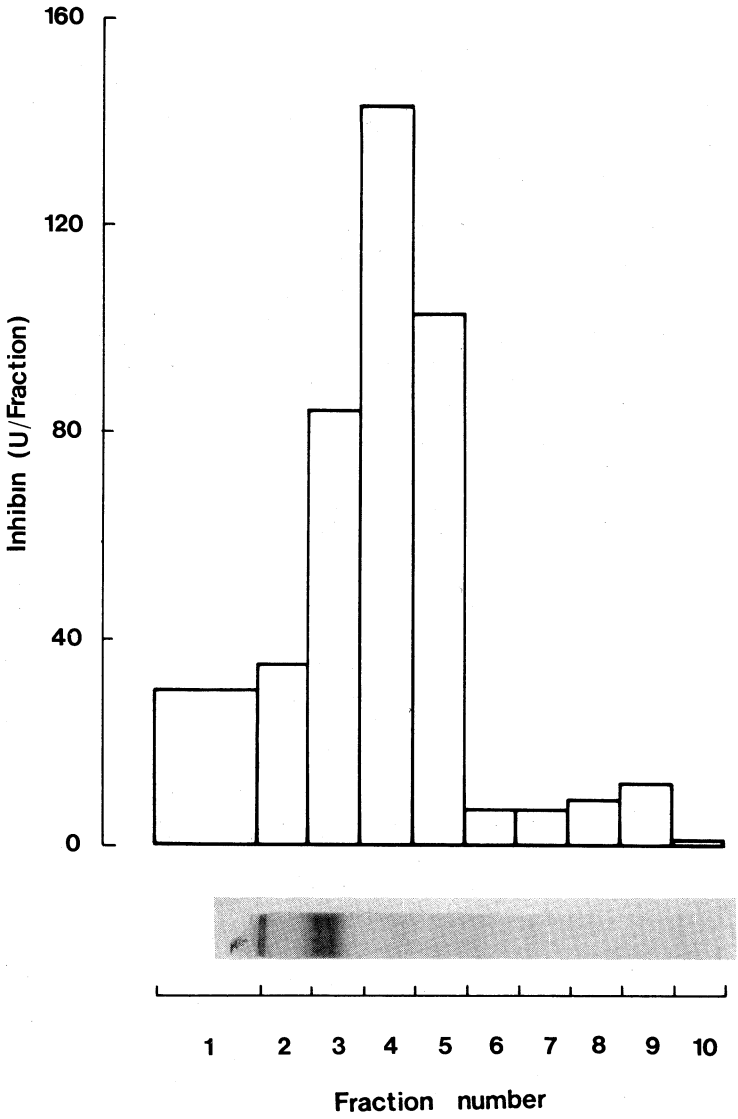

Fig. 4. Discontinuous 7·5% (w/v) polyacrylamide gel electrophoresis. Inhibin was measured in eluates from pooled sections of gel cylinders. Recovery of inhibin activity was 86%. The position of migration serum albumin was between fractions 5 and 6. Samples were applied to the top of the gels (left side of figure).

could be synthesized. However, in contrast to the findings of others, there was no evidence in these studies for the existence of a peptide of molecular weight less than 5000 with inhibin-like activity. Insignificant amounts of inhibin were lost following

dialysis of RTF and other sources of inhibin with membranes of molecular weight cut-offs up to 12 000–14 000 or following diafiltration in a hollow-fibre cartridge with a molecular weight cut-off of 5 000. On gel filtration of most sources of inhibin, the activity eluted with proteins of molecular weight greater than 60 000. Procedures such as extraction with acid urea (useful for obtaining proteins of low molecular weight such as parathormone—Rasmussen *et al.* 1964), or treatment with 6–8 M urea, 2 M guanidine hydrochloride, 5 M magnesium hydrochloride or 4 M potassium thiocyanate (useful for dissociating proteins into subunits—de la Llosa and Jutisz 1969; Sawyer and

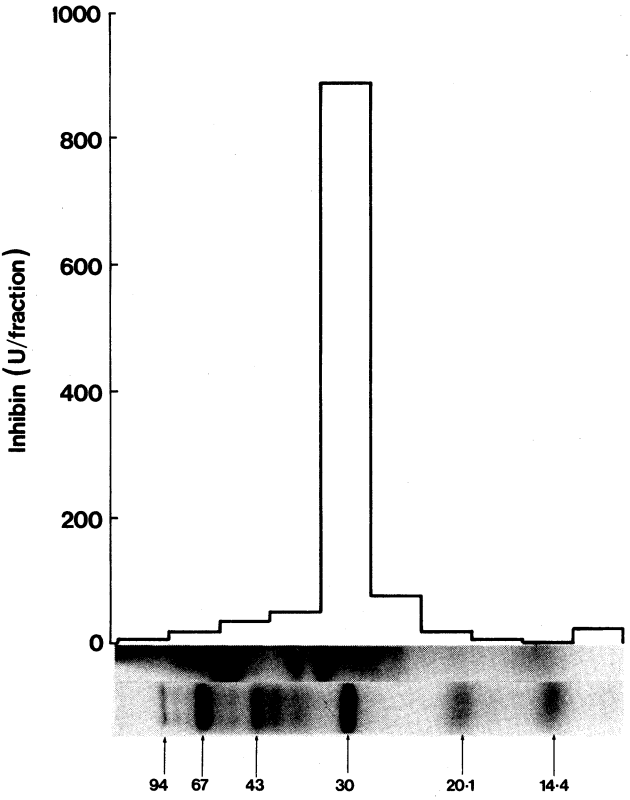

Fig. 5. SDS (0·1% w/v) polyacrylamide (12% w/v) slab gel electrophoresis of 1 mg RTF protein. The central area of the slab was sectioned (5 mm), eluted and assayed for inhibin activity and the edges of the gel stained to show the RTF and standard proteins (mol. wt $\times 10^{-3}$). Total recovery of inhibin was 76%.

Puckridge 1973), or heating, or extraction with acid, acid ethanol, SDS, Triton or Tween (useful for freeing peptides from binding proteins—Jacob *et al.* 1968) did not generate a molecule of low molecular weight with inhibin-like activity which would have been lost on dialysis or eluted in retarded zones on gel filtration. In general, recovery of inhibin activity was complete except when buffers with pH 5·5 were used where poor recoveries are probably accounted for by precipitation of inhibin at its isoelectric point (Baker *et al.* 1982*b*).

Although there were small amounts of material with FSH-suppressing activity in dialysates and salt volumes of some gel filtration runs, this material was not inhibin. It was either toxic to the pituitary cells or did not suppress FSH levels in parallel with

the inhibin standard. In some instances this activity may have been due to testosterone or other steroids in the samples. For example, in the dialysis experiment (Fig. 1), calculations from the measured content of testosterone in the lyophilized powder indicate the concentration of this steroid in the culture medium would have been between 0·6 and 6 nM. As shown previously these concentrations of testosterone may produce a slight inhibition of FSH secretion by the cultured rat pituitary cells (Eddie *et al.* 1979).

While material with inhibin activity eluted in the void volume of Biogel P60 (mol. wt $\geqslant$60 000) or with an apparent molecular weight of 90 000 from Sephacryl S200 and S300 for most sources investigated, material with inhibin activity and an apparent molecular weight of approximately 20 000 was obtained from the culture medium of rat seminiferous tubules. Previously, Eddie *et al.* (1978) found that most of the material with FSH-suppressing activity in similar culture medium had an apparent molecular weight of less than 20 000 on gel filtration of Sephadex G100. The reason for this difference in behaviour of inhibin preparations of *in vivo* and *in vitro* origin is not clear. Gel filtration of bovine seminal plasma was attempted on three occasions. Material with FSH-suppressing activity eluted as a broad peak from Sephacryl S200 and S300 but the starting material and fractions eluted in the lower molecular weight zones (less than 20 000) were toxic in the inhibin bioassay. Recently inhibin has been purified to homogeneity from bovine follicular fluid by Robertson *et al.* (1985). From this work bovine inhibin has a subunit structure and a molecular weight of 56 000. In our hands preliminary gel filtration studies of crude bovine follicular fluid produced results similar to those for ovine RTF (Baker *et al.* 1983).

The only conditions found to alter the relative position of elution of inhibin in RTF were gel filtration under acid conditions and PAGE in the presence of SDS. The finding that gel filtration in 1 M acetic acid produces a more retarded zone of activity separated from the bulk of other protein has been reproduced with other acids such as 3 mM HCl and 0·1% (v/v) trifluoroacetic acid. However, the peaks are more spread out, particularly if more purified inhibin preparations are submitted to gel filtration (Baker *et al.* 1984).

No estimates of apparent molecular weight could be made from gel filtration under acid conditions because of the anomalous behaviour of standard proteins. There was also a lack of relationship between molecular weight and elution volume on Lichrosorb DIOL under both acidic and basic conditions. This has been attributed to a combination of ionic and hydrophobic interactions between the proteins and the column packing (Schmidt *et al.* 1980).

In 0·1% (w/v) SDS PAGE, inhibin activity could be recovered in reasonable yield for analytical studies by elution of strips of gel. The estimate of apparent molecular weight of 30 000 is tentative because the Fergusson plots did not pass through the origin. This imperfect behaviour of proteins on PAGE could result from the low concentration of SDS (0·1% w/v) or the absence of a reducing agent. However, the stained RTF and standard protein patterns did not appear to be different in the presence or absence of 5% (v/v) mercaptoethanol in the sample buffer.

These results indicate that inhibin from most sources is a medium-sized protein which aggregates so that it has a higher molecular weight on gel filtration. They also suggest that estimation of molecular weight may be unreliable and that the true molecular weight of inhibin may only be defined after purification. Finally, gel filtration under acid conditions and PAGE in the presence of SDS may be useful steps for purification of inhibin from a variety of sources.

Acknowledgments

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