

Low Molecular Weight FSH Binding Inhibitor in Bovine Testis

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Purification of low M_r (< 5000) inhibitors of FSH binding to receptors has been hampered by their low concentration in tissue extracts and physiologic fluids. The calf testis represents an accessible and economic source of large quantities of tissue, and was therefore studied as a source of FSH binding inhibitors. Supernatants ($27,000 \times g$) of calf testis homogenates inhibited binding of [125 I]-hFSH as well as [125 I]-hCG to membrane-bound receptor from the same source. FSH binding inhibitor was concentrated from large volumes of testis supernatant by precipitation of inert material with metaphosphoric acid, concentration/desalting of the resulting supernatant by ultrafiltration (Amicon UM-05 membranes) and lyophilization. Separation of FSH binding inhibitor and LH(hCG) binding inhibitor activities could be achieved by molecular sieving on Sephadex G-25. A partially purified fraction with inhibitors of FSH binding activity ($ED_{50} = 44 \mu\text{g}$ protein) and free of LH(hCG) binding inhibitor activity (no activity at $800 \mu\text{g}$ protein) emerged with a V_e/V_o of 1.9, reflecting an apparent M_r of about 1500. Inhibitors of LH(hCG) binding activity emerged with the column outer volume. Rechromatography of the FSH binding inhibitor fraction on G-25 indicated two closely associated peaks of activity. These could be further resolved by gel filtration through BioGel P2, to give a salt-free fraction with an FSH binding inhibitor activity (ED_{50}) of $24 \mu\text{g}$ protein. The inhibitor was heat-labile, losing 80% of its activity after 2 hours at 100°C . The testicular low molecular weight FSH binding inhibitor is similar to bovine follicular fluid and serum FSH binding inhibitor by several parameters. Bovine testis should prove a useful source for binding inhibitor purification.

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Substances capable of inhibiting FSH binding to receptor have been identified in a number of tissues and physiologic fluids, including rat testis (Reichert and Abou-Issa, 1977; O'Shaughnessy, 1979), human or bovine serum (Reichert et al, 1979, 1981; Sanzo and Reichert, 1982; Andersen et al, 1983); human or sheep ovary (Krishnan et al, 1983); and human, bovine, or porcine follicular fluid (Daume et al, 1981; Darga and Reichert, 1978; Sato et al, 1982; Sluss and Reichert, 1984a; Fletcher et al, 1982). These binding inhibitors differ with respect to apparent molecular weight, charge, hormone specificity, and possibly hydrophobicity (Sluss and Reichert, 1984a). Purification and chemical characterization of FSH binding inhibitors have been hampered by lack of available starting material and its low concentration in tissue extracts and physiologic fluids. FSH binding inhibitors with a reported molecular weight of less than 5000 are of particular interest, since they have been associated with inhibin-like activity (Moodbidri et al, 1980) and correlated with physiologic parameters of follicular function in the bovine (Sluss et al, 1983). The bovine testis represents a ready source of tissue, and recently has been shown to possess a large M_r form (M_r about 33,000) of FSH binding inhibitor (Dias and Reichert, 1984). We report the presence of

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a low M_r inhibitor of FSH binding (about 1500) in calf testis, and describe a convenient method for its concentration from large amounts of tissue extract, as well as its separation from inhibitors of LH binding activity, partial purification, and characterization.

Materials and Methods

Highly purified human follicle stimulating hormone (hFSH, LER-1781-2, 4000 IU/mg) and highly purified hCG (CR-119, 12,000 IU/mg) were used as the radioligands in this study. The details of the radioiodination procedure have been described elsewhere (Maghuin-Rogister et al, 1978). The only modification of the radioiodination procedure was the use of a Sephadex G-25 (0.7 × 50 cm) column equilibrated and eluted with 0.05 M phosphate buffer, pH 7.0, for separation of radioiodinated hormone from free iodine. Specific activity of each radioligand was determined by the self-displacement method (Catt et al, 1974). The specific radioactivity of the [125 I]-hFSH was 28 mCi/ μ g, and that of the hCG, 69.9 mCi/ μ g.

Calf (between 10 to 15 weeks of age) testes were obtained from a local abattoir and stored at -20°C until they were thawed for use. Homogenates were prepared in Tris-HCl buffer (10 mM Tris-HCl buffer, pH 7.4, 1 mM MgCl_2 , 0.01% NaN_3) containing 15% sucrose, and then centrifuged at $7000 \times g$ for 45 minutes at 4°C . This and all subsequent operations were carried out at 4°C . The $7000 \times g$ supernatant was concentrated by ultrafiltration through an Amicon DC-10 unit using a hollow fiber H5P-50-43 (exclusion limit $M_r = 50,000$). The concentrate was suspended in an equal volume of Tris-HCl buffer (but with no sucrose) and concentrated again. The reconcentrated material was stored in 4-ml aliquots containing 50% (v/v) glycerol until needed for the radioreceptor assay. At that time, the suspension was centrifuged at $31,000 \times g$, washed one time with assay buffer, and then recentrifuged. The final pellet was utilized for the receptor assay as described by Sluss and Reichert (1984a). Nonspecific binding was determined using a 300-fold molar excess of unlabeled FSH and a 1200-fold molar excess of unlabeled LH. Assay results were evaluated as described earlier (Sluss and Reichert, 1984a).

Frozen calf testes were detunicated, freed from epididymis, and made into a 40% homogenate with 0.05 M ammonium acetate buffer, pH 6.5, using a Brinkman polytron tissue grinder (Westbury, NY) at 4°C . The homogenate was subjected to sequential centrifugation at $2000 \times g$ and $27,000 \times g$ for 15 minutes and 60 minutes, respectively. Freshly pre-

pared 6 N metaphosphoric acid was added dropwise to the supernatant, with constant stirring, to a final pH of 4.0 (Moodbidri et al, 1976). It was then centrifuged at $27,000 \times g$ for 15 minutes to remove inert protein precipitate. The resulting supernatant was neutralized with 1 M KOH. KOH is used for this purpose instead of NaOH since sodium ions are more inhibitory to FSH binding (Andersen and Reichert, 1982). The supernate was then concentrated by ultrafiltration using an Amicon cell fitted with an Amicon UM-05 membrane (exclusion limit: 500 M_r) and desalted isovolumically by diafiltration with a 6-volume turnover of deionized water. The desalted UM-05 retentate was lyophilized and stored at -20°C until used for further characterization.

The UM-05 retentate described above was further purified by gel filtration chromatography through Sephadex G-25 (Pharmacia, Piscataway, NJ). Samples were dissolved in deionized water, clarified by centrifugation at $27,000 \times g$ for 15 minutes and loaded on a G-25 column (1.5 × 90 cm) that had been equilibrated with 0.05 M ammonium acetate buffer, pH 6.5. The column was developed (4 C) with the same buffer at a flow rate of 15 ml/hr and 1.5-ml fractions were collected. The outer volume (V_o) of the column was determined using blue dextran 200. The eluant was continuously monitored for ultraviolet absorbance at 254 nm, and fractions were tested for inhibition of FSH binding activity.

The Sephadex G-25 active fractions containing inhibitors of FSH binding activity were further purified by gel filtration on BioGel-P2 (1 × 60 cm). The column was equilibrated and developed with deionized water at a flow rate of 20 ml/hr and 1-ml fractions were collected. The V_o of the column was determined using bovine serum albumin and the inner volume (V_i) was determined by conductivity measurements, using 1 M ammonium sulfate as a reference. FSH binding inhibitor activity was determined by radioligand receptor assay and the protein content of each fraction was measured by the method of Lowry et al (1951) using bovine serum albumin as a standard.

Results

The procedure used for partial purification of FSH binding inhibitor activity and its separation from LH binding inhibitor activity is summarized in Fig. 1. The UM-05 retentate had both FSH binding inhibitor ($\text{ED}_{50} = 2500 \mu\text{g}$) and LH binding inhibitor ($\text{ED}_{50} = 16,000 \mu\text{g}$) activity. Lyophilized UM-05 retentate was dissolved in deionized water and sieved through

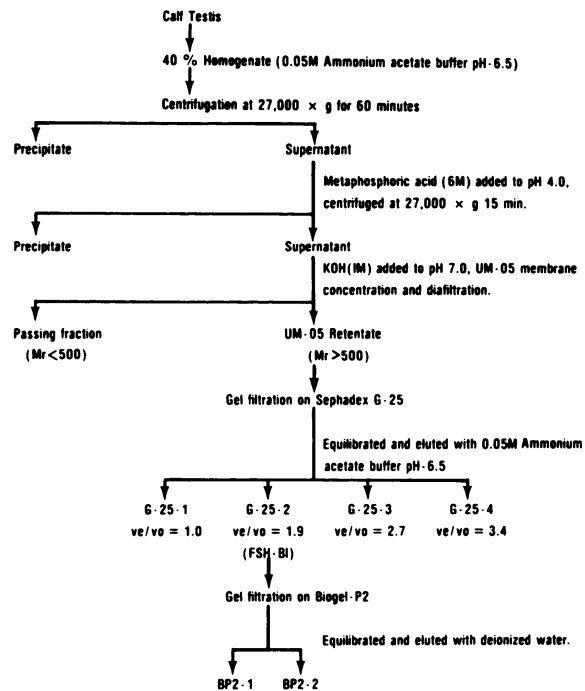


Fig. 1. Outline of the procedure for isolation of low molecular weight FSH binding inhibitor from calf testis. Kilogram quantities of decapsulated testis were used to prepare a 40% homogenate in 0.05 M ammonium acetate buffer, pH 6.5, and further fractionated as indicated. Active fractions from Sephadex G-25 gel filtration (described in the legend to Fig. 2) were pooled and further purified by BioGel P-2 gel filtration as shown in Fig. 5.

a superfine Sephadex G-25 column (1.5 × 90 cm) equilibrated and developed with 0.05 M ammonium acetate buffer, pH 6.5, yielding four fractions (Fig. 2). The fraction emerging at the column V_0 (G-25-1) contained both LH binding inhibitor ($ED_{50} = 7800 \mu\text{g}$) and FSH binding inhibitor ($ED_{50} = 5200 \mu\text{g}$) activity. Fraction G-25-2, which was retained by the column ($V_e/V_0 = 1.9$), was enriched with regard to FSH binding inhibitor activity ($ED_{50} = 44 \mu\text{g}$ protein), but was devoid of LH binding inhibitor activity at 800

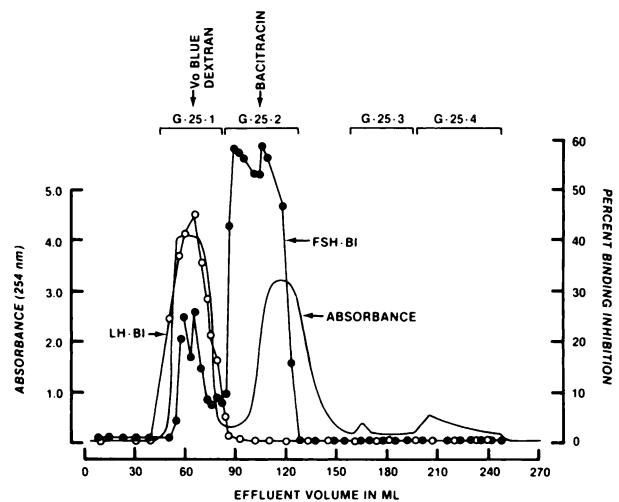


Fig. 2. G-25 gel filtration of UM-05 retentate. Lyophilized samples of UM-05 retentates were reconstituted to 1 g/ml with deionized water and clarified by centrifugation at 27,000 × g for 15 minutes at 4 C. Two-ml aliquots (approximately 2 g dry weight) were applied to a 1.5 × 90 cm column of Sephadex G-25 equilibrated with 0.05 M ammonium acetate buffer, pH 6.5. Samples were eluted with the same buffer at a flow rate of 15 ml/h and 1.5-ml fractions were collected every 6 minutes.

μg , suggesting that a separation of the two factors had been achieved. Recovery of FSH binding inhibitor activity in G-25-2 was 62%. Fractions from several replicate runs were pooled as indicated in Fig. 2, lyophilized, reconstituted in deionized water, and assayed at multiple doses to quantitate the FSH binding inhibitor and LH binding inhibitor activity (Fig. 3). The most potent fraction with respect to FSH binding inhibitor activity was G-25-2, which inhibited [^{125}I]-hFSH binding in a dose-related manner, with a 50% inhibition at 44 μg /protein (Fig. 3). Fraction G-25-1 was rechromatographed on the same column used in the preparative runs (Fig. 4B) and, as before, the LH binding inhibitor emerged with the column outer volume, although FSH binding inhibi-

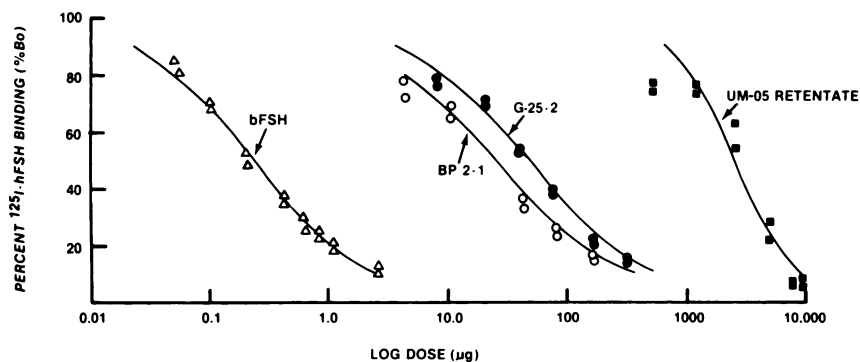


Fig. 3. Inhibition of [^{125}I]-hFSH binding to calf testis receptor by graded doses of bovine FSH (bFSH, 0.50 NIH FSH-S1 units/mg, Δ), fractions G-25-2 (\bullet) UM-05 retentate (\blacksquare), and BP-2-1 (\circ). The data were analyzed by a nonlinear, four-parameter logistic curve fitting program (Faderi et al, 1980). Calculated BI_{50} values for bFSH, G-25-2, BP-2-1 and UM-05 retentate were $240 \pm 11 \text{ ng}$, $44 \pm 3 \mu\text{g}$, $23 \pm 2 \mu\text{g}$ and $2481 \pm 249 \mu\text{g}$, respectively.

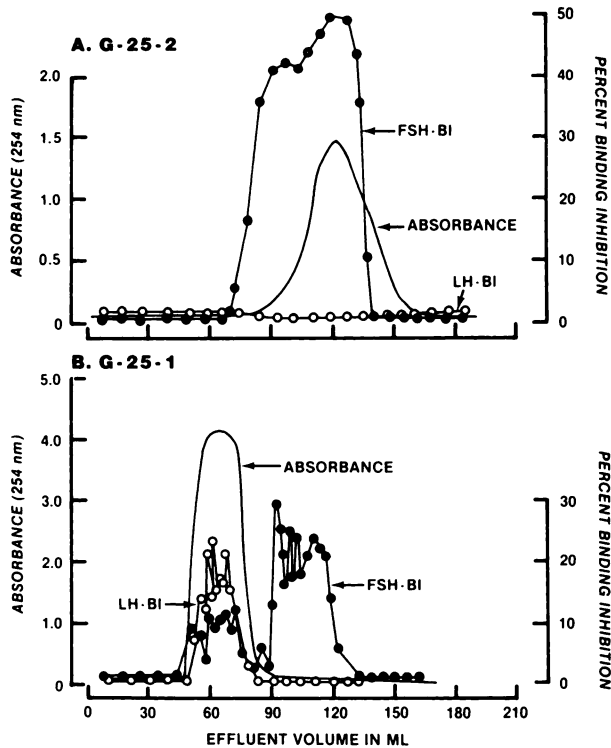


Fig. 4. Rechromatography of the LH binding inhibitor fraction G-25-1 (LH-BI) and the FSH binding inhibitor fraction G-25-2 (FSH-BI) on Sephadex G-25 (1.5 × 90 cm). Lyophilized samples of G-25-1 and G-25-2 (Fig. 2) were reconstituted to 2 ml, clarified by centrifugation at 27,000 × g for 15 minutes, and then rechromatographed separately through the same column of Sephadex G-25. The column was equilibrated and developed with 0.05 M ammonium acetate buffer, pH 6.5, at a flow rate of 15 ml/h and 1.5-ml fractions were collected every 6 minutes. Aliquots (1-ml) of the fractions were used to determine the absorbance profile at 254 nm and 250- μ l aliquots were assayed to identify FSH-BI or LH-BI activity.

tor activity also was detected in a retained fraction emerging with a V_e/V_o ratio of 1.9. These results indicate that the inhibition of FSH binding activity present in the outer volume probably resulted from overloading the column. The presence of a large molecular weight form or aggregated form of FSH binding inhibitor activity, which dissociated upon refiltration under more dilute conditions, cannot, however, be ruled out. Rechromatography of fraction G-25-2 confirmed elution of FSH binding inhibitor activity in the region of M_r 1500. The "split peak" of FSH binding inhibitor activity seen in the initial chromatography was seen again when gel filtration was performed on an analytic scale (Fig. 4A).

In an attempt to more completely resolve these closely associated components, fraction G-25-2 was further fractionated by gel filtration through BioGel

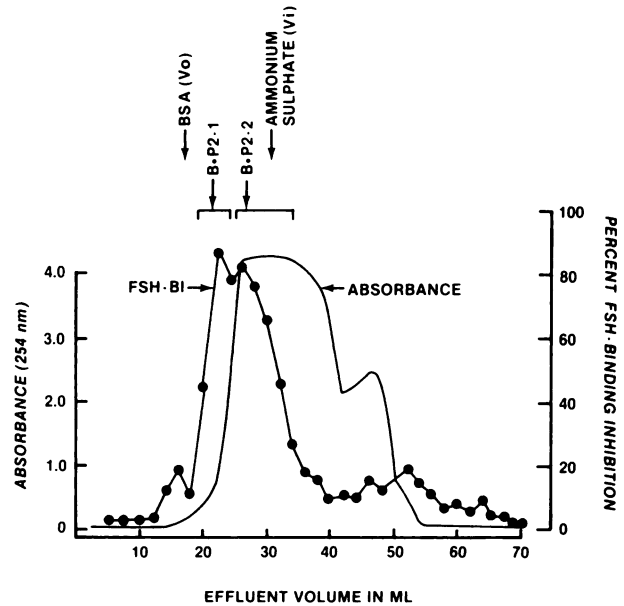


Fig. 5. BioGel-P-2 gel chromatography of the FSH binding inhibitor fraction G-25-2 (FSH-BI). Column size 1.0 × 60 cm. The column was equilibrated and developed with deionized water at 4 C. Lyophilized fraction G-25-2 (17.6 mg protein) was reconstituted with 1 ml of deionized water and, after clarification by centrifugation, was applied to the column. Samples were eluted at a flow rate of 20 ml/h and 1.0-ml fractions were collected every 3 minutes. Aliquots (1-ml) of the fractions were used to determine the absorbance profiles at 254 nm and 250- μ l aliquots were assayed to identify FSH-BI activity. Based on this data, fractions were pooled as shown in the figure to obtain fractions B-P2-1 and B-P2-2. The outer volume (V_o) and inner volume (V_i) shown were determined by BSA absorbance and ammonium sulfate electrical conductance, respectively.

P2 ($V_o = M_r$ about 2000). The results are summarized in Fig. 5. The most potent fraction, fraction BP2-1, had an ED_{50} of 24 μ g based on protein determination by the Lowry method with bovine serum albumin as the reference standard. We were concerned that the apparent FSH binding inhibitor activity in this fraction might not have been due to its protein content, but rather to nonprotein constituents, such as salt, that had not been completely removed by the various filtration steps previously described. To examine this point, fraction BP2-1 was ashed at 600 C for 24 hours. At the end of this procedure, there was a loss of 39% in mass, so that from a starting weight of 7.4 mg, 4.5 mg was recovered. When this ashed material was tested at 1.6 mg, no significant binding inhibition was observed, which indicated that FSH binding inhibitor activity in fraction BP2-1 could not be explained on the basis of contamination with residual salt.

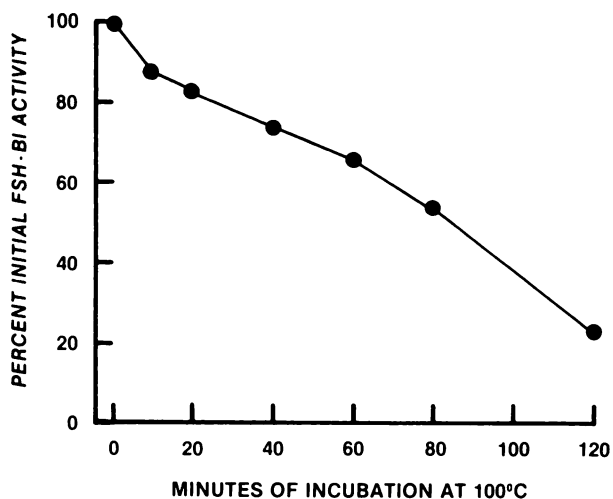


Fig. 6. Heat lability of low M_r FSH binding inhibitor (FSH-BI). Concentrations of the low M_r FSH-BI fraction G-25-2 were selected based on data obtained from assays summarized in Fig. 3 to give 70% FSH-BI activity. The samples then were heated in a water bath at 100 C for the period indicated. After heating, insoluble material was removed by centrifugation and the supernatant tested for FSH-BI activity. Results are expressed as percent activity present in G-25-2 at time 0.

Since the goal of this study was to develop a rapid method for processing large quantities of tissue that are available in virtually unlimited amounts, we were prepared to accept large losses in activity and low yields in order to prepare sufficient quantities of the highly active inhibitor fraction for final purification and chemical characterization. In our laboratory, the estimated recovery was approximately 50 mg of active fraction, measured as protein, per kilogram of testis.

Earlier studies (Reichert and Abou-Issa, 1977; Sanzo and Reichert, 1982) showed that low molecular weight FSH binding inhibitors in homogenates of rat testis and human serum lost significant activity upon heating. The effect of heating FSH binding inhibitor fraction G-25-2 at 100 C for various periods of time is summarized in Fig. 6. As shown, testicular FSH binding inhibitor activity was heat-labile, losing 80% of initial activity after 120 minutes. Similar heat lability has been reported for low M_r FSH binding inhibitor from the other sources (*vide supra*).

Discussion

We have reported previously the presence of low molecular weight FSH binding inhibitors in homogenates of immature rat testis (Reichert and Abou-

Issa, 1977). Recently, high molecular weight FSH binding inhibitors have been reported in ovine and bovine testis (Papkoff et al, 1983; Dias and Reichert, 1984), as well as in human seminal plasma (Dias et al, 1981). In this report, we describe the presence of low molecular weight FSH binding inhibitors in calf testis. Bovine testis apparently contains both high and low molecular weight species of FSH binding inhibitors. As referenced earlier (see Introduction), high and low molecular weight species of FSH binding inhibitor also have been reported in extracts of human or ovine ovary; in human and bovine serum; and in human, bovine, and porcine follicular fluid. An understanding of the physiologic role and possible relatedness of FSH binding inhibitors from these different sources awaits final purification and characterization of the inhibitor. This enterprise has been hampered seriously by the very low levels of binding inhibitor in all of the sources tested. However, the calf testis represents an easily obtained and economical source of large quantities of tissue, and therefore of FSH binding inhibitor. The approach that we have used in these studies, that is, precipitation of inert protein with metaphosphoric acid, allows easy handling of extremely large volumes of testis homogenate, and should facilitate purification of the binding inhibitor. The FSH binding inhibitor prepared in this study had a binding inhibitory potency of $ED_{50} = 24 \mu\text{g}$ protein, representing a 104-fold purification relative to the inhibition of FSH binding activity in the starting testis homogenate. Although the initial testis homogenate also contained LH-hCG binding inhibitor activity, this activity was separated from FSH binding inhibitor activity by gel filtration. Several characteristics of the low molecular weight testis FSH binding inhibitor are similar to those reported for FSH binding inhibitor from other sources. These include molecular weight ($M_r \approx 1500$), separation of FSH binding inhibitor from LH binding inhibitor by gel filtration, and heat lability. A particular problem in purifying low molecular weight FSH binding inhibitor from homogenate and other physiologic fluids is the surprising difficulty encountered in separating this activity from residual salt, even when applying techniques such as gel filtration and diafiltration that are generally considered to be highly effective in this regard. In our hands, even the most highly purified FSH binding inhibitor fractions, prepared by diafiltration and gel filtration techniques, contained a significant amount of inorganic salt, as determined by ashing experiments. The amount of inorganic salt present in the highly purified fractions, however,

could not account for the FSH binding inhibitor activity noted.

Low (500–5000) molecular weight substances that inhibit FSH binding to receptor are of particular interest, since similar fractions derived from bovine follicular fluid have been correlated with physiologic parameters related to follicular development (Sluss et al, 1983). Materials of slaughterhouse origin have been shown to contain bacteria (Sluss and Reichert, 1983) capable of producing FSH binding inhibitor activity. In the present studies, the testes used also were of abattoir origin, and we cannot exclude unequivocally the possibility that bacterial contaminants could be responsible for at least a portion of the observed inhibitory activity. We believe, however, that this is an unlikely possibility for several reasons. First, as with follicular fluid, the number of contaminating bacteria originally present in the calf testis was low and therefore, by analogy with studies on bacteria-contaminated follicular fluid (Sluss and Reichert, 1983), several days of incubation at 4 C would be required before significant numbers of bacteria or increases in bacteria-derived FSH binding inhibitor activity would be observed. Also, as discussed below, an advantage of the use of metaphosphoric acid is that it allows a rapid (within 2-day) recovery of the low molecular weight FSH binding inhibitors. It seems improbable that significant quantities of bacterial products could be generated during this time. In addition, although the low molecular weight FSH binding inhibitor lost 50% of initial activity after 80 minutes of incubation at 100 C, bacteria-generated FSH binding inhibitor loses 50% of its activity after only 10 minutes under similar conditions (Sluss and Reichert, 1984b). Finally, as we reported earlier (Sluss and Reichert, 1984b) FSH binding inhibitor from the bacteria *Serratia* appears to have a relatively high molecular weight, in excess of 30,000 (Sluss et al, 1985), whereas the low molecular weight FSH binding inhibitor from testis reported here has a molecular weight of about 1500.

The methodology previously used with human serum (Sanzo and Reichert, 1982) or porcine follicular fluid (Sluss and Reichert, 1984a) requires long periods of time (several days) to separate low molecular weight FSH binding inhibitor from large (> 5000) molecular weight components. Besides limiting the volume of material that could be processed conveniently, such procedures expose low molecular weight inhibitors to endogenous proteases and to bacteria if nonsterile collection systems have been employed. The present methodology is advantageous in this

regard, since metaphosphoric acid is used to rapidly remove large molecular weight substances under conditions of low pH, where protease activity of the trypsin or chymotrypsin types should be minimal.

Gonadotropin binding to specific receptors generally is accepted as the first step in gonadotropin stimulation of target cell function. Thus, endogenous inhibitors of FSH binding may function as physiologic regulators of FSH action (Fletcher et al, 1982). However, unambiguous verification of this will require homogeneous preparations and chemical identification of the inhibitor. Purification has been impeded by the difficulty in obtaining large amounts of material, such as follicular fluid or serum, that are required in deriving sufficient quantities of partially purified inhibitor for the final purification attempts. We have demonstrated that kilogram quantities of calf testis can be processed rapidly to obtain highly potent preparations of low molecular weight FSH binding inhibitor. Since the availability of bovine testis is essentially unlimited, the procedure described here should be useful in developing procedures for final purification of the binding inhibitor.

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Human Materials for Animal and Human Research

1. hGH, hFSH, hLH, hTSH, hPRL now are available for animal and human research. This is possible because the priorities for human clinical research are under temporary suspension.
2. Human pituitary fractions and human pituitaries also are available for research, as has been the case in prior years.

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