Disappearance of Inhibin-like Activity in Bull Seminal Plasma Following Castration

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Ejaculates from normal and castrated bulls were evaluated for the presence of inhibin-like activity. Inhibin activity found in the seminal plasma of normal bulls became undetectable after castration. Crude protein concentrates prepared from the ejaculates of castrated bulls failed to produce inhibition of FSH secretion as measured by *in vivo* and *in vitro* tests: a) hCG-induced uterine weight test in mice and b) LH-RH-induced FSH secretion in 34-day-old male mouse pituitary. The concentrates also lacked the ability to inhibit the binding of iodine-125 (^{125}I)-labeled inhibin fraction from normal bull seminal plasma to ovine pituitary membranes. Chromatographic and electrophoretic analyses of the ejaculate from castrated bulls revealed the absence of the band corresponding to inhibin of normal bull seminal plasma.

Key words: inhibin, seminal plasma, castration.

The involvement of a nonsteroidal factor from the testis and the ovary in the regulation of pituitary gonadotropin secretion is now well recognized. Thus, new data reported during the last eight years have shown the presence of inhibin-like activity in different tissues or fluids related to the reproductive organs (see reviews, Setchell and Weir, 1979; Franchimont et al, 1979). Seminiferous tubules (Eddie et al, 1978) and Sertoli's cells from the rat testis (Steinberger and Steinberger, 1976) as well as granulosa cells (Erickson and Hsueh, 1978) maintained in culture can secrete FSH inhibitory substances into the medium. Based on this evidence, it has been assumed that these are the probable, if not the only, sites of origin of inhibin. The detection of a significant amount of inhibin-like activity in bull (Chari et al, 1978; Sairam et al, 1981a) and human (Franchimont et al, 1979; Thakur et al, 1978; Ramasharma et al, unpublished results) seminal plasma raises the question of its origin. It has been speculated (Franchimont et al, 1975) that the presence of inhibin-like material in seminal plasma may be due to a spillover caused by incomplete absorption in the epididymis, where, generally, most of the testicular secretions are absorbed. In a recent communication, we have reported the preparation and properties of an inhibin-like protein from bull seminal plasma (Sairam et al, 1981a). We now present evidence to show that this activity disappears from the ejaculate following castration. While these studies were in progress, Peek and Watkins (1979) reported that gonadotropin-inhibiting activity is also present in seminal plasma of vasectomized bulls.

Materials and Methods

Ejaculates from normal bulls were collected as described earlier (Sairam et al, 1980). Two of these adult Holstein bulls were bilaterally castrated, and ejaculates were collected after two to three months by electroejaculation. They were in good health before and after castration. As the volume of the ejaculate was greatly reduced after castration, collection over a six-week period was necessary to accumulate a sufficient

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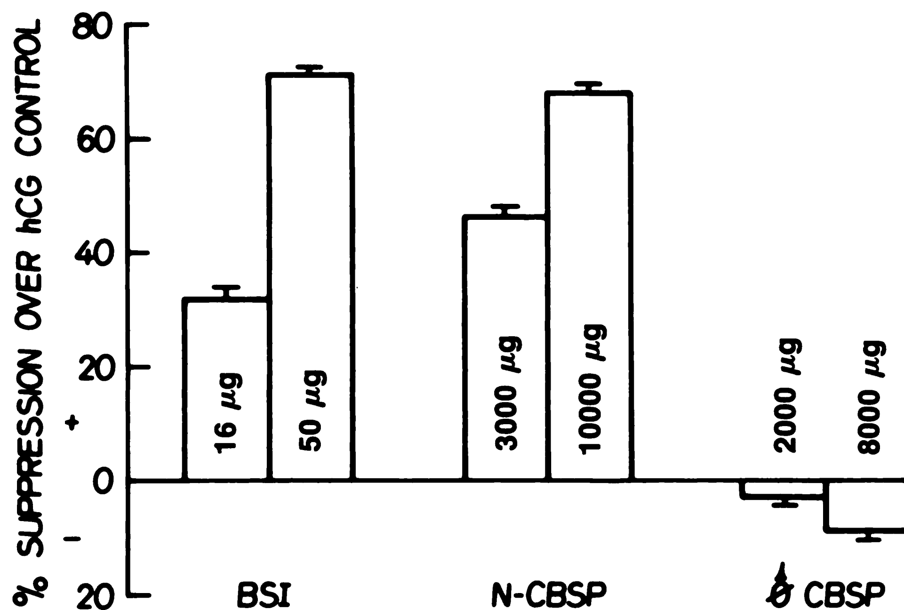

Fig. 1. Suppression of hCG-induced (10 IU) uterine weight increase in immature mice (24-day-old) by purified bovine seminal plasma inhibin (BSI), N-CBSP, and ⚨-CBSP. Bovine seminal plasma inhibin was purified according to Sairam et al (1981a); 10 mg N-CBSP represents 0.135 ml equivalent of seminal plasma; 8 mg ⚨-CBSP is the equivalent of 4 ml of ejaculate. In this indirect *in vivo* method of inhibition of FSH release, the uterine weights in completely suppressed animals would be equivalent to those of saline controls. Each group consisted of six mice. The degree of suppression was evaluated for significance by the Student's *t* test.

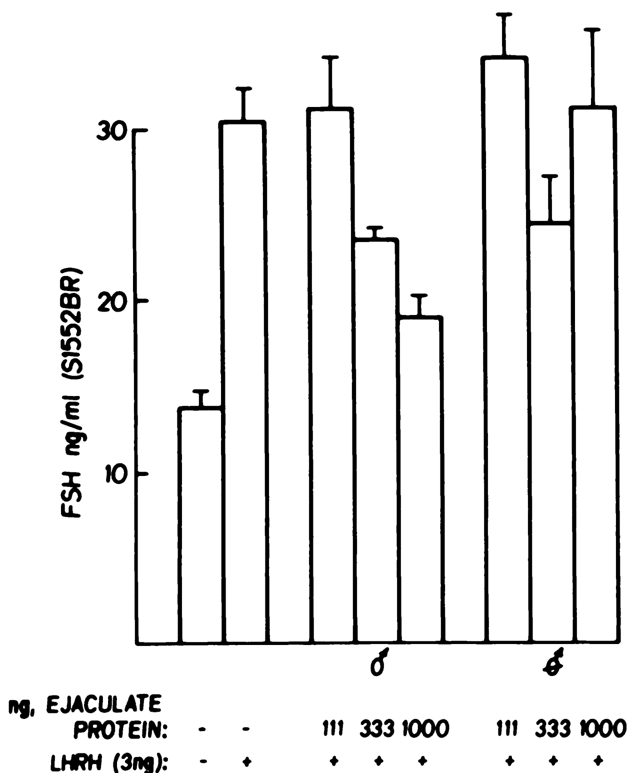

Fig. 2. Comparison of inhibition of LH-RH response *in vitro* produced by SP-C50 D fraction (Fig. 3) of N-CBSP (♂) and ⚨-CBSP. Whole mouse pituitaries were first exposed to the fractions for 1 hour at 37 C under O_2-CO_2 atmosphere. They were then incubated for 3 hours with synthetic LH-RH (3 ng, Ayerst Labs.) in a total volume of 1 ml in Dulbecco's modified Eagle's medium. After the fourth hour, the amount of FSH released into the medium was estimated by a specific radioreceptor assay using highly purified ovine FSH (S1552BR) tracer and a bull testicular receptor preparation. Inhibition caused by N-CBSP fraction was statistically significant ($P <$ 0.025). The decrease brought about by 333 ng ⚨-CBSP was not statistically significant from that of the LH-RH-treated control pituitary.

volume. The samples were stored frozen at −20 C. The ejaculate was clear and colorless as compared with the milky appearance of the normal ejaculate. The pooled ejaculate (~75 ml) was thawed and centrifuged at 4 C at 6340 g. Under microscopic examination, the small pellet that was obtained showed some cellular debris without any spermatozoa. A crude protein fraction was prepared from the supernatant by ethanol precipitation in a manner similar to that described earlier (Sairam et al, 1981a) for normal ejaculates. The total solids recovered after lyophilization were 1.87 mg/ml for the castrated ejaculate as compared with 74 mg/ml for normal bull ejaculates (Sairam et al, 1981a).

Ion Exchange Chromatography on SP-Sephadex-C-50

The chromatographic patterns of seminal plasma powder from the ejaculate of intact bulls (N-CBSP) and castrated bulls (♂-CBSP) were compared on the cation exchanger SP-Sephadex-C-50. We have used this ion exchanger to purify inhibin protein from bull seminal plasma. The conditions of the experiment were identical to those reported recently (Sairam et al, 1981a). The proteinaceous materials in the fraction eluted by 0.1 M Na_2HPO_4 (fraction D, Fig. 3A and B) were concentrated by ultrafiltration (PM-10 membrane, Amicon) and re-

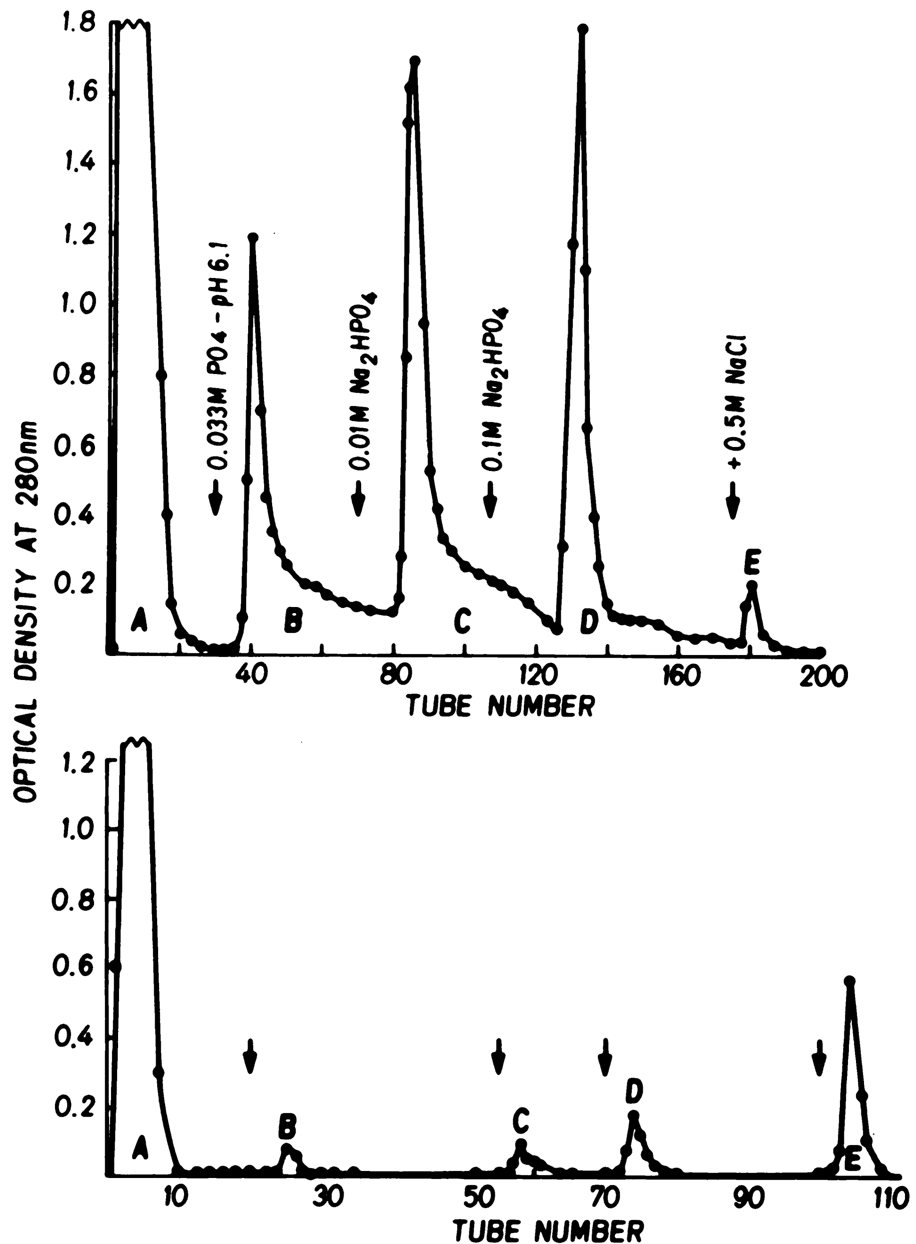

Fig. 3. Chromatography of seminal plasma powder on SP-Sephadex C-50. Columns were equilibrated in 0.033M acetate buffer, *p*H 5.4, and elution was carried out stepwise with buffer changes as indicated by arrows (A, top) Pattern obtained with 8 ml equivalent of N-CBSP (column 1.5 × 30 cm) at 4 C, 4.2 ml/tube. Inhibin activity is present in fraction D. (B, bottom) Chromatographic profile of 30 ml equivalent of ejaculate obtained after castration (column 0.8 × 13 cm), 1.5 ml/tube. No activity was found in any of the fractions.

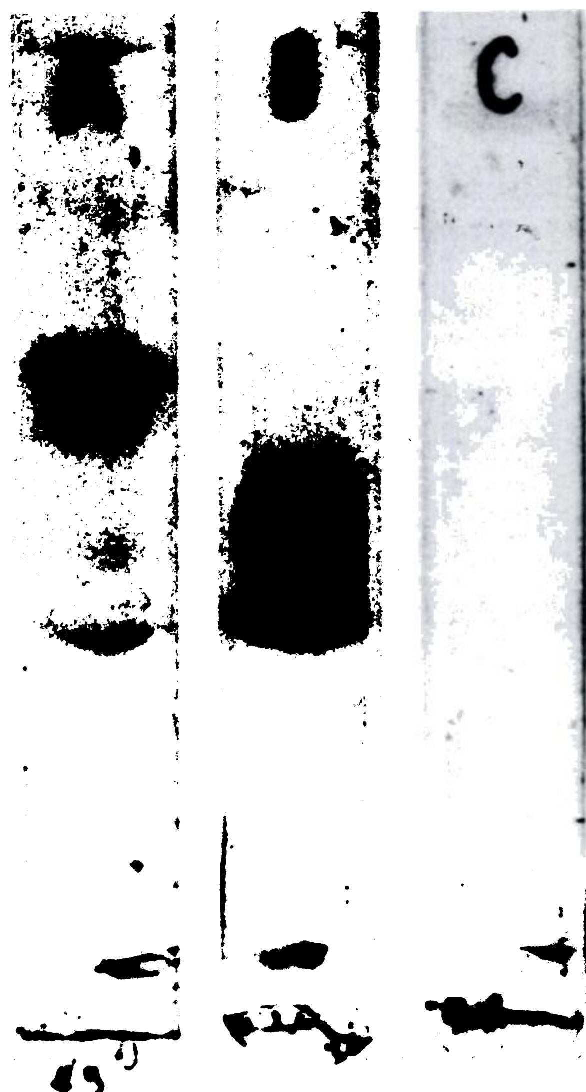

Fig. 4. Polyacrylamide gel electrophoresis (15%) at pH 4.5, 6 mA/tube, 1 hour; 50 μg protein/tube. From left to right: SP-C50D from Fig. 3A, purified BSI (G-100-Al step, Sairam et al, 1981a), and SP-C50D from Fig. 3B.

peated washings with distilled water followed by lyophilization. The fractions were kept at 4 C until use.

Analytic polyacrylamide gel (15%) electrophoresis was carried out at pH 4.5 (Sairam et al, 1981a). The gels were stained with Coomassie blue.

Biologic Activity. The ability of the N-CBSP, ♂̸-CBSP or their respective D fractions (Fig. 3) to inhibit FSH secretion was tested in the following *in vivo* and *in vitro* test models: a) The inhibition of hCG-induced mouse uterine weight increase in 23 to 24-day-old immature mice (Ramasharma et al, 1979); b) blockade of LH-RH action on whole mouse pituitaries incubated *in vitro*. The details of these methods have been given elsewhere (Sairam et al, 1981a). Briefly, this method consisted of incubating whole pituitaries from 34-day-old mice with the test material for 1 hour at 37 C under O_2-CO_2 atmosphere. They were then exposed to a single pulse of 3 ng synthetic LH-RH (Ayerst Labs., Montreal) in a total volume of 1 ml in Dulbecco's modified Eagles medium containing 0.1% BSA. At the end of 4 hours, the total amount of FSH released into the medium was estimated by a radioreceptor assay.

In addition, the activity of the fractions was also compared in a binding assay using ovine pituitary membrane preparations and ^{125}I eluted tracer (Sairam et al, 1981b). In this method, membrane preparations capable of binding ^{125}I-labeled bovine seminal plasma inhibin (BSI) were prepared from frozen ovine pituitary glands and stored at −70 C. The ^{125}I-labeled BSI bound to the membranes at pH 7.5 was subsequently eluted under acidic conditions (0.05 N HCl) and neutralized to obtain an eluted tracer that retained good binding characteristics to fresh pituitary membranes. The binding of eluted tracer to the membranes could be inhibited in a dose-dependent manner by unlabeled BSI. In this binding assay, we have recently shown that only those fractions of CBSP which have inhibin-like activity are also capable of preventing the binding of labeled inhibin-like material to ovine pituitary membranes (Sairam et al, 1981b).

Results

Inhibin Activity in N-CBSP and ♂̸-CBSP

a) In 24-day-old immature mice, 10 mg of N-CBSP (or 0.135 ml equivalent of seminal plasma) inhibited the increase in uterine weight induced by 10 IU of hCG. It may be noted that FSH inhibition in this assay is measured by a secondary response (Ramasharma et al, 1979). As compared with intact CBSP, administration of as much as 8 mg (or 4.3 ml equivalent of ejaculate) of ♂̸-CBSP had no effect whatsoever on the uterine weight. While there was no inhibition, a slight, insignificant increase in uterine weight was evident (Fig. 1). For comparison, the good inhibition obtained by 50 μg of the purified inhibin-like (BSI) material from seminal plasma of normal bulls is also shown, confirming previous results (Sairam et al, 1981a).

b) A partially purified inhibin fraction from N-CBSP (SP-C50 fraction D) blocked the action of LH-RH *in vitro* on whole mouse pituitaries during a 4-hour incubation (Fig. 2), and thus the release of FSH was inhibited. As shown elsewhere, bovine seminal plasma inhibin has no effect on LH release (Sairam et al, 1981a). A similar fraction from ♂̸-CBSP was without any effect in the same set of experiments, indicating the absence of the active principle.

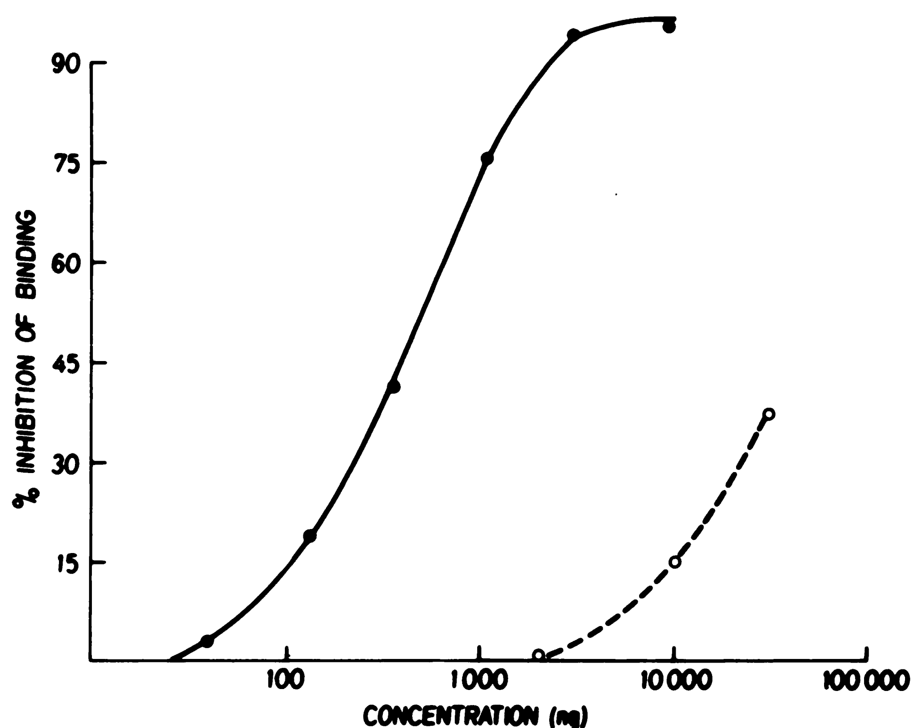

Fig. 5. Displacement of labeled inhibin in a binding assay by SP-C50D of N-CBSP (——) (Fig. 3A) and ♂̸-CBSP (O---O) (Fig. 3B). The binding assay was performed using ovine pituitary membrane fractions and ^{125}I-labeled purified bovine seminal plasma inhibin.

Ion Exchange Chromatography. The chromatographic patterns of N-CBSP and ♂̸-CBSP on SP-Sephadex C-50 (Fig. 3) were markedly different in quantitative aspects. When ♂̸-CBSP was chromatographed on the cation exchanger, fractions B, C, and D were obtained in very low yields (Fig. 3B). Inhibin activity is concentrated in the D fraction of N-CBSP. The recovery of lyophilized material in fraction D of ♂̸-CBSP was only 1.7 mg. This fraction was utilized for examining the presence of inhibin-like activity and for polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. At *p*H 4.5, the SP-C50 D fraction (Fig. 3A) from normal CBSP showed several protein bands (Fig. 4, gel A) that were completely absent in similar material obtained from castrated ejaculates (gel C). The gel B (Fig. 4) shows the mobility of a partially purified preparation of bovine seminal plasma inhibin (BSI) (Sairam et al, 1981a).

Binding Assay. In its ability to compete with ^{125}I-labeled inhibin of normal seminal plasma, the activity of fraction D from ♂̸-CBSP (Fig. 3B) was less than 1% as compared with the similar D fraction derived from N-CBSP (see Fig. 5).

Discussion

The data obtained in these studies show that inhibin-like activity, which is normally present in bull ejaculate, becomes nondetectable following castration of the animal. This was ascertained by indirect *in vivo* and direct *in vitro* tests. Such a result would be expected if seminal plasma inhibin indeed originated from the testes. A recent report (Peek and Watkins, 1979) has claimed that the inhibin activity in bull seminal plasma is not reduced following vasectomy, a procedure in which the direct outflow of testicular secretions, including spermatozoa, into the ejaculate would be blocked. No attempts were made, however, to compare the properties of the active material in normal seminal plasma and those of vasectomized bulls. If Peek and Watkins' report is confirmed, then it would indicate that the accessory male reproductive organs could either produce inhibin or somehow concentrate inhibin-like activity, which may eventually find its way into the ejaculate, such as in vasectomized animals. Thus, it may be argued that our results reflect a general decline of activity in reproductive tract function, which ensues after removal of the testis. The presence of

high levels of FSH in normal bull seminal plasma as compared with the low blood levels of this hormone may suggest the existence of some form of a concentrating mechanism (Sairam et al, 1980b). Whether or not this is true of other key physiologically active substances remains to be determined.

In contrast to the results in vasectomized bulls, studies with human seminal plasma from normal and azoospermic men show a marked reduction in inhibin-like activity in the latter group (Scott and Burger, 1980). These results indicate a direct correlation between the activity of seminiferous epithelium and the presence of inhibin in seminal plasma in men. Since testosterone levels were normal in the two groups (Scott and Burger, 1980), the secretory activity of the accessory organs were presumably unaffected.

There can be little doubt about the absence of detectable inhibin activity in bull seminal plasma after castration since three independent methods were employed to check for the presence of activity. Furthermore, the chromatographic studies as well as the disc electrophoresis provide firm evidence that the particular protein band, which we have designated as being due to inhibin (Sairam et al, 1981a) in bull seminal plasma, is completely absent in ejaculate of castrated bulls. An unequivocal answer to the question of origin of inhibin in bull seminal plasma must await its isolation in pure form and its detection by immunohistochemical procedures.

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