INHIBIN
purification and characterization

INHIBINE
zuivering en karakterisering

PROEFSCHRIFT
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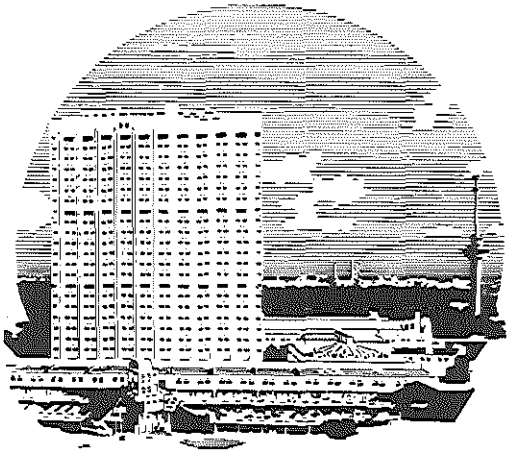

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CONTENTS

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CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF THE THESIS

Gonadal function in mammals is regulated by two glycoprotein hormones which are synthesized and secreted by the anterior pituitary gland: the gonadotrophins lutropin (luteinizing hormone, LH) and follitropin (follicle-stimulating hormone, FSH). These hormones act on the different cell types of the testes in male and the ovaries in female animals. Release of LH and FSH from the pituitary gland is triggered by the decapeptide gonadotrophin releasing hormone (GnRH) (for reviews, see Schally et al., 1972, and Fink, 1976), which is secreted by hypothalamic neurones into the portal veins connecting the hypothalamus with the anterior hypophysis. It is generally accepted that a single hypothalamic releasing hormone exists which stimulates the secretion of both gonadotrophins (Wise et al., 1979). The gonadotrophins released by the anterior pituitary gland enter into the circulation and influence in the gonads the morphology and function of gonadal cells. Luteinizing hormone induces ovulation in the ovary and can influence the functions of the corpus luteum in a number of species. In the testis LH stimulates Leydig cells to produce testosterone, while FSH stimulates the development of the ovarian follicles in the female and initiates spermatogenesis in the male. The gonadal steroid hormones, in turn, influence the secretion of hypothalamic releasing factors and pituitary secretion of FSH and LH in addition to their effects on the development of accessory sex organs and secondary sex characteristics.

For a long time steroid hormones were believed to be the only gonadal factors which played a role in the inhibition of gonadotrophin secretion from the anterior hypophysis. With the development of more specific and sensitive detection methods it became clear that the release of FSH was not only inhibited by steroids, but that other nonsteroidal gonadal factors were involved. The existence of this hypothetical hormone was suggested by Mottram and Cramer (1923). This hormone was named "inhibin"*) by McCullagh (1932) and described as a water-soluble hormone that

"...prevents the cellular changes from appearing in the pituitary gland after castration of rats and also completely inhibits the hyperfunction of the pituitary gland". Inhibin was later redefined as "... a water-soluble, gonadal substance, which exerts a specific inhibition of the release of FSH from the pituitary gland" (de Jong, 1979). The possible role and physiological significance of inhibin have been studied extensively with crude and partially purified gonadal inhibin-containing preparations in a variety of in vivo and in vitro assay systems. However, its exact role and function can be elucidated only after inhibin has been purified and its biological activity evaluated in well-defined assay systems.

The aims of the studies described in this thesis were to purify and characterize inhibin from ovarian follicular fluids. The first objective was an extension of the studies by Jansen et al., (1981) who had partially purified inhibin from bovine ovarian follicular fluid (bFF) and made some preliminary observations on its physico-chemical characteristics, mainly based on its behaviour in chromatographic systems used in the purification studies. From the point of view of studying the role of inhibin in human reproduction and for its possible clinical application, it appeared more interesting to purify and characterize inhibin from human ovarian follicular fluid (hFF). The partial purification of inhibin from bFF and hFF represents a major section of the work described in this thesis. These studies are described in Chapter 3 and Appendix papers 1 and 2. We have used partially purified preparations from bFF to obtain preliminary data on an inhibin radioimmunoassay system and to characterize crude and partially purified inhibin-containing preparations using immunological techniques. These experiments have been described in Chapter 4

*) Only the purified and unequivocally characterized hormone should be referred to as "inhibin", whereas all other biological activities, which fulfil the criteria of the definition should be referred to as "inhibin activity" or "inhibin-like activity". We will use the latter designation only for biological activities of questionable specificity; activity in inhibin containing preparations of well-defined specificity will be referred to as "inhibin" or "inhibin activity".

and Appendix paper 3. In Chapter 5 a comparison is made with recently published data on inhibin from other comparable sources (i.e. ovarian follicular fluids). The introductory Chapter 2 will deal with the historical development of the inhibin concept, the assay systems employed by various laboratories, the putative physiological significance of inhibin and a summary of the literature on the purification and characterization of inhibin from male and female gonadal fluids and tissues of male and female animals.

CHAPTER 2

INHIBIN: SIGNIFICANCE AND CHARACTERIZATION

2.1 DEVELOPMENT OF THE INHIBIN CONCEPT

The early studies on the regulation of pituitary function revealed functional interactions between the hypophysis and gonads. Moore and Price (1932) showed a reciprocal relationship between hormonal activities of the pituitary gland and the gonads. These authors concluded from experiments involving castration, hypophysectomy and injection of ovarian and testicular extracts that "...gonad hormones have a depressing effect upon the hypophysis, which results in a diminished amount of the sex-stimulating factor available to the organism...". The decrease in circulating levels of these gonadal hormones after castration leads to the appearance of highly vacuolized basophilic cells ("castration cells") in the anterior hypophysis. In 1923 Mottram and Cramer noticed the appearance of these castration cells and a severe atrophy of the seminiferous tubules with complete cessation of spermatogenesis after gonadal irradiation with radium. They concluded that the changes observed after castration or irradiation of the testes were due to the absence of an internal secretion of the "seminal epithelium". The observation that the morphological changes in the pituitary gland were not observed after resection of the vas deferens was considered as support for an internal secretion of the seminiferous tubules which caused the change in the pituitary gland; this made it unlikely that pituitary function was affected by an external secretion of the testis.

Using parabiosis (surgical union of intact and castrated male and female rats via the abdominal wall) Martins and Rocha (1931) postulated the existence of two gonadal hormones, one which stimulated the growth of the accessory sex organs and a second, which prevented the appearance of castration cells in the pituitary gland of males and precocious maturation of the intact immature females. Injection of testicular homogenates in castrated male rats produced the former effects in castrated male rats. In

contrast, when testicular homogenates were injected in the ovariectomized female parabiont of a pair of female rats, the precocious maturation of the intact immature female parabiont was not prevented. McCullagh (1932) observed that injection of aqueous extracts of testes into castrated male rats prevented the appearance of castration cells and hypertrophy of the hypophysis but had no effect on the atrophy of the accessory sex glands. He named the hormone present in these extracts "inhibin", to distinguish it from the benzene-soluble hormone "androtin", which prevented atrophy of the accessory sex organs but did not influence pituitary hypertrophy in male rats after castration. The structure of "androtin" was elucidated when David et al. (1935) isolated and characterized testosterone from bovine testes. In the following years experiments were published which did not confirm McCullagh's original observations and which were conflicting with the inhibin hypothesis (Nelson, 1934; Nelson and Gallagher, 1935; Vidgoff and Vehrs, 1940; Rubin, 1941). It was then concluded that only steroids exert the feedback action from the testis on the hypophysis. The possible existence of inhibin in female animals was not investigated at that time, although McCullagh and Schneider (1940) showed that injection of aqueous testicular extracts could disrupt the oestrous cycle in female rats.

The development of bioassays for the measurement of the urinary gonadotrophic hormones, FSH and LH, offered possibilities to study the gonadal control of gonadotrophin secretion in men. It was observed that a positive correlation existed between urinary FSH concentrations and spermatogenic damage (Klinefelter et al., 1942; Heller and Nelson, 1945; del Castillo et al., 1947; McCullagh and Schaffenburg, 1952), while normal FSH concentrations were found when Leydig cells were damaged or absent (McCullagh and Schaffenburg, 1952). While these observations suggested a dual endocrine regulation of the pituitary secretion of FSH by the testes, they did not result in the concept of regulation of pituitary LH secretion by androgens from the Leydig cells and regulation of FSH secretion by inhibin from the seminiferous tubules, but instead gave rise to the "utilization-hypothesis" (Heller and Nelson, 1948; Heller et al, 1952), which postulated that damaged germinal epithelium was not able to utilize or inactivate the

circulating FSH, which caused increased urinary FSH levels. After this hypothesis was discredited by Howard et al. (1950), the identification of an increasing number of testicular steroids seemed to offer new possibilities for the separate regulation of LH and FSH secretion from the hypophysis; in particular the role of oestrogens in the regulation of FSH secretion was emphasized (McCullagh and Schaffenburg, 1952; Sherins et al., 1982; see also reviews by Baker et al., 1976, and Setchell et al., 1977). Finally, Lincoln (1979) indicated that changes in the frequency of GnRH-pulses had differential effects on secretion of FSH and LH.

In the beginning of the 1970's Setchell and Jacks (1974) observed that an FSH-suppressing activity was present in ram rete testis fluid. This observation caused a renewed interest in the inhibin hypothesis, which led to the detection of inhibin activity in a large number of gonadal fluids and tissues, the development of reliable bioassays for the detection and measurement of inhibin activity, and to the purification and characterization of the biochemical entity that is responsible for the specific suppression of FSH release from the pituitary gland.

2.2 PHYSIOLOGICAL ROLE OF INHIBIN

The physiological significance of inhibin in reproductive biology is not yet completely understood, mainly because measurements of peripheral concentrations of inhibin activity using adequate methods have only recently been reported (Lee et al., 1982; Lee and Gibson, 1985; Channing et al., 1985a). Reviews covering the physiology of inhibin have been published by Channing et al. (1982a, 1985b), Grady et al. (1982), Hermans (1982), de Jong et al. (1985) and in the monograph edited by Sairam and Atkinson (1984).

2.2.1 Importance of inhibin in male animals

Initially, data on inhibin were collected from experimental male animals (mainly rats) and from clinical observations on male patients with impaired testicular function. At present, however, the role of inhibin in adult males is less clear than in adult females (see section 2.2.2).

Another important factor was the observation of de Jong and Sharpe (1977), de Jong et al. (1978) and Hermans et al. (1980) that the effects of gonadal manipulations on FSH were largest in the prepubertal male. This suggested that inhibin was more important in the regulation of FSH in the immature than in adult animals. Hermans et al. (1980) showed that 8 h after castration the change of FSH was largest in 15 day old male rats and that the relative rise of FSH levels decreased with increasing age of the animal. The postcastration rise of FSH could be completely prevented by administration of steroid-free bFF (i.e. exogenous inhibin activity). This rise of FSH could not be attributed to a decrease in testosterone levels (Hermans et al., 1980). Hemicastration of male rats early in life resulted in compensatory testicular hypertrophy, which was thought to be a result of higher peripheral FSH levels due to impaired feedback of the remaining testis on gonadotrophin secretion (Cunningham et al., 1978). After neonatal hemicastration of male rats FSH levels were increased on day 21 of age, but by days 42 and 63, FSH had returned to control levels (Ultee-van Gessel et al., 1985). These authors demonstrated that FSH released from the pituitary gland of neonatally hemicastrated rats was increased at day 21 of age and at control levels at days 42 and 63, which indicated that inhibin may played a major role in FSH feedback in immature rather than in mature male animals. Neonatal irradiation of rat testes resulted in decreased numbers of Sertoli cells in the testes of 21, 51 and 81 days old rats and in significantly increased levels of serum FSH at 21 days but not at 81 days of age (de Jong and Sharpe, 1977). These observations indicated the importance of Sertoli cells and inhibin in FSH feedback regulation in the immature animal.

Efferent duct ligation causes intratesticular accumulation of

tubular fluid and intratubular increase of inhibin activity without affecting FSH and LH levels in serum within 72 h. At longer time intervals cessation of spermatogenesis was noted (Main et al., 1978; Au et al., 1984). After hypophysectomy secretion of inhibin activity is reduced in bilaterally ligated adult male rats. Secretion of inhibin activity can be stimulated under these conditions by FSH administration, whereas testosterone and hCG had no effect (Au et al., 1985, 1986). These authors observed 7 days after the ligation a decline in intratesticular inhibin activity concomitant with a rise in FSH and LH, presumably caused by irreversible damage to the tubules and the germinal epithelium. Lipner and Dhanarajan (1984) reported suppression of secretion of FSH and LH by the pituitary gland after administration of porcine follicular fluid (pFF) to male rats which were subjected to efferent duct ligation and anti-androgen treatment. Their conclusion, that inhibin regulates pituitary secretion of both FSH and LH, but only pituitary synthesis of FSH, might be incorrect because the anti-androgen treatment could influence the hypothalamic control of gonadotrophin secretion.

More direct indications for the significance and regulation of production of inhibin activity came from in vitro cultures of Sertoli cells. In cultures of Sertoli cells of 21 days old rats inhibin activity was increased by the addition of FSH (Steinberger, 1981; Le Gac and de Kretser, 1982; Ultee-van Gessel et al., 1986) and decreased by the addition of testosterone (Ultee-van Gessel et al., 1986). On the other hand, Verhoeven and Franchimont (1983) reported stimulation of secretion of inhibin activity by the addition of testosterone and no effects of FSH in rat Sertoli cell cultures. The reasons for these differences are not clear: the presence of foetal calf serum in the medium obtained from Sertoli cell cultures (spent culture medium), which may have toxic effects on pituitary cells (Le Gac and de Kretser, 1983) or the presence or absence of peritubular cells (Skinner and Fritz, 1986) might play a role. Furthermore, the amount of inhibin activity produced per Sertoli cell is larger in immature testes than in mature testes (Massicote et al., 1984; Ultee-van Gessel and de Jong, unpublished results).

In castrated adult male rats the postcastration rise of FSH

and LH could be prevented by testosterone administration, whereas injection of steroid-free pFF suppressed FSH only (Jones et al., 1985). However, in long-term castrated adult male rats, testosterone had no effect on FSH levels (Jones et al., 1985). These authors concluded that, at least in the adult male rat, inhibin functions as a secondary regulator, the effects of which are superimposed on, but independent of, steroid feedback mechanisms.

The above-mentioned observations indicate that the possibility of feedback regulation of FSH secretion by inhibin activity is more important and more pronounced in the immature male rat than in the adult animal. A direct comparison between the in vivo and the in vitro regulation of inhibin production in immature and mature male rats should be made with caution because the in vivo situation is far more complex due to mutual interactions of the testicular compartments and compensatory mechanisms which might influence both pituitary and testicular function.

2.2.2 Importance of inhibin in female animals

The physiological significance of inhibin in female animals became of major interest after the demonstration of the presence of inhibin activity in bovine ovarian follicular fluid (de Jong and Sharpe, 1976). Inhibin might play a role in the regulation of FSH secretion under circumstances where the patterns of peripheral concentrations of FSH and LH diverge. Such situations occur near the time of pro-oestrus/oestrus in the rat and after unilateral or bilateral ovariectomy, as will be discussed below.

During the early hours of the day of oestrus in rats, peripheral concentrations of LH decline, while a secondary peak of FSH concentration in plasma is found. At this time the number of large antral follicles is greatly reduced (Welschen et al., 1980). Injection of follicular fluid prevents the secondary FSH peak (Schwartz and Channing, 1977; Welschen et al., 1980) which cannot be suppressed by the administration of physiological concentrations of steroid hormones (Welschen et al., 1980). After unilateral ovariectomy of rats on the second day of di-oestrus a rise in serum FSH was suppressed within 24 h, when the number of

large antral follicles in the remaining ovary reached the number which was present in both ovaries before operation. No changes in serum LH were detected during this period (Welschen et al., 1978). The immediate rise of FSH can be suppressed by injection of steroid-free follicular fluid but not by the administration of steroids (Welschen et al., 1978). Bilateral ovariectomy, on the other hand, resulted in a rise of serum FSH which can be partly suppressed by a physiological dose of exogenous steroids, but administration of steroid-free bFF suppressed FSH to baseline levels (Marder et al., 1977; Welschen et al., 1977). Bilateral ovariectomy in immature female rats resulted in an increase in peripheral FSH concentrations from 25 days of age onwards, but not at 15 days of age, suggesting that a short-term negative feedback regulation of FSH secretion does not exist at that age (Hermans et al., 1980). Sander et al. (1985) could only measure low levels of endogenous inhibin activity ovarian homogenates in rats 18 days of age and older.

With the onset of puberty, peak levels of inhibin activity were observed on the day of first ovulation. Studies in adult rats revealed an inverse relationship between the number and size of antral follicles and peripheral FSH levels (Welschen et al., 1980). These authors suggested that the number of healthy granulosa cells was maintained at a constant level due to the negative feedback signal of inhibin, produced by the granulosa cells themselves, on the pituitary FSH secretion. Studies on the size of the follicles, numbers of granulosa cells per follicle and the inhibin content of the follicular fluid showed an increase in inhibin activity with follicular fluid volume in ovine (Scott et al., 1980; Tsonis et al., 1983), human (Channing et al., 1981a), bovine (Henderson and Franchimont, 1981, 1983) and porcine (van de Wiel et al., 1983) follicles. The amount of inhibin activity found per follicle was, however, dependent on the viability of the follicle: Henderson et al. (1984a) observed that atretic follicles contained little or no inhibin activity and that atretic follicles could only be discerned from healthy follicles by culturing granulosa cells for a period of 24 h.

Taken together these data suggested that in adult female animals the number of healthy granulosa cells could influence the

peripheral levels of FSH through the secretion of inhibin activity. A decrease in secretion of inhibin activity resulted in an increase of serum FSH, which in turn stimulated the proliferation of granulosa cells (Welschen et al., 1980; Lee and Gibson, 1985).

This hypothetical regulation system may be substantiated by the observations on the Booroola Merino ewes. In this species low follicular fluid inhibin levels, high serum FSH levels and high fecundity are observed (Cummins et al., 1983; McNatty et al., 1985a). Lowering the peripheral levels of inhibin activity by immunization of ewes with partially purified inhibin-containing preparations from bFF increased the number of developing follicles and the ovulation rate (Bindon, 1984; Henderson et al., 1984b). Paradoxically, treatment of ewes in the luteal phase with steroid-free bFF also increased the ovulation rate, probably due to a rebound in FSH secretion in the following follicular phase after an initial suppression (Wallace and McNeilly, 1985; Wallace et al., 1985). Simultaneous administration of bFF and FSH during the follicular phase also resulted in an increased ovulation rate (McNatty et al., 1985b; McNeilly, 1985) as a result of increased levels of circulating exogenous FSH. These observations are in agreement with the observations of Welschen et al. (1980), Hermans et al. (1980, 1981, 1982a) and de Jong et al. (1982a) in female rats. These authors reported on the effects of bFF administration throughout the oestrous cycle and observed a significant reduction of FSH within 4 h and an ensuing "overshoot" of serum FSH levels 16-20 h after the last injection of bFF.

Using pregnant mare serum gonadotrophin (PMSG), which exhibits both FSH- and LH-like activities, Lee et al. (1982) observed in immature female rats a gradual increase of inhibin activity in peripheral plasma and ovarian tissue and a decrease in serum FSH concentrations. In PMSG-treated immature mice a positive correlation between follicular growth and inhibin activity was observed with a concomitant decrease of peripheral FSH levels (Lee and Gibson, 1985). In the latter study the ovulation rate increased, indicating the growing periovulatory follicle as the most likely site of inhibin production.

The role of inhibin in the regulation of FSH secretion in human females is not as clearly established, since most of the

studies were performed as part of a clinical programme with subjects with pathophysiological endocrine conditions. The following clinical evidence is suggestive for a role for a nonsteroidal factor in the control of FSH secretion in women: Sherman (1976) suggested that elevated levels of FSH in menopausal women could be caused by reduced amounts of an inhibin-like substance. Thomas et al. (1977) observed highly elevated levels of FSH in response to GnRH challenges after treatment of patients with cytotoxic drugs which could have caused damage to the ovaries, possibly resulting in disturbed synthesis and secretion of inhibin activity.

The amount of inhibin activity in hFF varies with the stage of the menstrual cycle: in the preovulatory follicle it is highest, while no inhibin activity can be detected in the luteal stage of the cycle (Channing et al., 1981a, 1984c, 1985a; Chappel et al., 1980; Lefevre et al., 1981). Treatment of women with human menopausal gonadotrophin (hMG) resulted in an increase of inhibin activity in the follicular fluid and in the ovarian venous plasma (Channing et al., 1984e, 1985b; diZerega et al., 1984). Similar results were obtained with Rhesus monkeys (Schenken et al., 1984). Cultures of granulosa cells from human infants showed detectable levels of inhibin activity that could be modulated by addition of gonadotrophins (Channing et al., 1984c). The amount of inhibin activity in these infant ovaries decreased with increasing age of the children. The significance of the presence of inhibin activity in these ovaries was not clear.

Another interesting phenomenon is seen in polycystic ovary (PCO) disease, in which patients have numerous small follicular cysts. Low basal peripheral FSH levels are detected in conjunction with elevated LH levels (Tanabe et al., 1983). Intrafollicular levels of inhibin activity were normal but the number of granulosa cells per follicle was reduced. Apparently, under these conditions granulosa cells secrete more inhibin. Moreover, excessive androgen secretion was also observed. Androgens can be converted to oestrogens, which can influence the pituitary sensitivity to GnRH, resulting in increased secretion of LH in comparison with FSH secretion. A possible explanation is that the elevated LH levels cause hypertrophy of theca cells, which may result in stimulation of inhibin secretion from the granulosa cells

through increased androgen levels. Low serum FSH levels, caused by increased secretion of inhibin activity into the ovarian vein, and a relative insensitivity of granulosa cells to LH may result in poor follicular growth (Tanabe et al., 1983; Channing et al., 1985b).

In vitro studies with cultured bovine granulosa cells showed that testosterone and 5α-dihydrotestosterone stimulated production of inhibin activity, whereas progesterone inhibited production of inhibin activity (Henderson and Franchimont, 1981). Furthermore, these authors observed an increased progesterone production and a decreased production of inhibin activity by cultured bovine granulosa cells as luteinization progressed. Testosterone and oestradiol were also shown to stimulate inhibin production in PMSG-stimulated rat granulosa cells, whereas progesterone was inhibitory (Lee, 1984). An increase in intra follicular inhibin activity in human patients was positively correlated with intrafollicular oestrogens and androgens, but negatively with progesterone (diZerega et al., 1984; Marrs et al., 1984). At the onset of luteinization, when progesterone levels rose, inhibin activity concentrations decreased dramatically (Channing et al., 1981). After treatment with hMG the positive correlation between inhibin activity and oestrogens and androgens and the negative correlation between inhibin activity and progesterone were no longer observed (Channing et al., 1984e; Marrs et al., 1984).

It appears that inhibin is one of the factors involved in the regulation of fertility by destining the number of follicles that are to ovulate. Therefore, inhibin seems to be a regulator of major importance in the mature female animal.

2.3 ASSAYS OF INHIBIN

Much of the progress in inhibin research has been obstructed by the lack of reliable bioassays. The controversies with respect to the existence and characteristics of inhibin have been largely due to the often poorly characterized methods for its detection

and quantification. A number of *in vivo* and *in vitro* bioassays for inhibin activity have been described, while a limited number of reports described radioimmunological methods. Inhibin assays and their contributions to inhibin research have been reviewed by Hudson et al. (1979), de Jong (1979) and Baker et al. (1981). The assay methods used to detect inhibin activity are based on either the indirect biological effects of FSH suppression, the direct suppression of FSH, the suppression of FSH release or cellular FSH content of cultured pituitary cells or displacement of labelled inhibin in radioligand assays. With ill-defined assay systems any material that interferes with the functioning of pituitary cells and as such disturbs production and secretion of FSH might be considered erroneously as inhibin activity. The most important criterion for a reliable assay of inhibin activity lies in its capacity to discriminate between specific FSH suppression and nonspecific effects. As will be described below, only few of the published methods meet this criterion.

2.3.1 *In vivo* methods

The early methods used in the detection of inhibin-like activity made use of parabiotic union of castrated adult male and intact immature female rats (Martins and Rocha, 1931), in which steroid hormones were metabolized before entering the peripheral circulation of the parabiotic partner, whereas proteins were not (Baker et al., 1981). An increase in ovarian weight was observed in the immature female parabiont, presumably as a result of the increased FSH secretion in the castrated male parabiont; inhibin-like activity administered to the castrated male parabiont prevented the ovarian weight increase (Fachini et al., 1963; Lugaro et al., 1969, 1973; Baker et al., 1981). A quantitative evaluation of this method has never been made, with respect to a dose-response relationship between the injected amount of inhibin-like activity and the reduction of ovarian weight increase. Moreover, it is questionable whether the response of the ovary reflected only changes in FSH concentrations or whether other factors were involved (see below). A final objection was that endogenous inhi-

bin production in the enlarged ovaries of the female parabiotic partner could affect the FSH secretion in the castrated male partner (Johnson, 1981; Hermans, 1982).

A summary of the currently used *in vivo* inhibin bioassays and reported precision indices is given in Table 2.1. A widely used *in vivo* inhibin assay makes use of the involvement of FSH in the human chorionic gonadotrophin (hCG)-induced augmentation of ovarian weight in immature female rats (Steelman and Pohley, 1953). When inhibin-containing material is administered, the amount of circulating FSH decreases and a suppression of the hCG-induced increase in the weight of the ovary and of the uterus is observed (Setchell and Wallace, 1972). This so-called "reversed Steelman-Pohley assay" has been used by Setchell and Sirinathsinghji (1972), Ramasharma et al. (1979) and Shashidhara Murthy et al. (1979), who measured the suppression of hCG-induced increase in uterine weight of immature female mice, and by Chari et al. (1976) who measured the suppression in hCG-induced increase of ovarian weight in immature female rats. The main objection against this method is its poor reproducibility (Setchell et al., 1977; Davies et al., 1978b; Hudson et al., 1979; de Jong et al., 1979b) which is presumably related to differences in the age of the experimental animal, the timing of injections and the dose of hCG used. The precision, expressed as the precision index λ (the

TABLE 2.1: Inhibin bioassays, based on the suppression of FSH in *in vivo* systems

Method	Precision index λ	References
Inhibition of hCG-augmentation on:		
uterine weight in rat	-	Setchell and Sirinathsinghji, 1972
mouse	-	Setchell and Sirinathsinghji, 1972
mouse	0.104	Shashidhara Murthy et al., 1979
ovarian weight in rat	0.21	Chari et al., 1976
mouse	-	Sheth et al., 1979
FSH-suppression in rats	2.77	Hudson et al., 1979
Inhibition of postcastration rise of FSH in rats	-	Nandini et al., 1976
	1.37	Hudson et al., 1979

ratio of the standard deviation and the slope of the curve), and sensitivity of this type of assay are low when compared with those of *in vitro* techniques (Tables 2.1, 2.2 and 2.3). A λ-value higher than 0.3 is considered unsatisfactory for bioasays (Hudson et al., 1979). Another objection against these indirect *in vivo* assays is that FSH-binding inhibitors, which may also be present in gonadal fluids, can exert inhibin-like effects on ovarian and uterine weight by competition with FSH for its receptor (Fletcher et al., 1982; Reichert et al., 1982; Sluss and Reichert, 1983). The presence of these inhibitors in partially purified inhibin-containing preparations has been reported (Mohapatra et al., 1985).

A more direct *in vivo* method for detection of inhibin activity consists of measurement of specific suppression of plasma FSH after administration of inhibin-containing preparations to intact or castrated, immature or mature, male or female animals. Irrespective of the source of inhibin activity, FSH levels are never

TABLE 2.2: Comparison of the sensitivity of different bioassay systems for inhibin

Method	ED_{50} (units oTLP)*
FSH-suppression in: - rat	> 5,700
- monkey	5,000
- sheep	35,000
Inhibition of postcastration rise of FSH in: - rat	> 150
- mouse	> 2
Augmentation of: rat ovarian weight	> 70
mouse uterine weight	100-150
In vitro rat pituitary cells: FSH-release	0.5
FSH-content	1.2
in vitro rat pituitary halves	1,000

Data from Hudson et al. (1979).

* ED_{50} = dose needed to achieve a half-maximal effect, expressed in units oTLP. 1 unit = the activity of 1 mg ovine testicular lymph protein (Eddie et al., 1979).

suppressed to values lower than 25 % of pretreatment control values (de Jong, 1979). Generally, the suppression of FSH by inhibin activity becomes evident within a few hours after injection of inhibin and is returning to control values within 6-16 h after the last injection (Nandini et al., 1976; de Jong et al., 1978; Hermans et al., 1980, 1981, 1982a). The specificity of the assay depends on the possibility to exclude effects of factors other than inhibin (for reviews, see Baker et al., 1981, and Hermans, 1982). The precision of the method is unsatisfactory (Table 2.1). Further disadvantages of these in vivo systems are the much larger amounts of inhibin-containing preparations, which have to be administered compared to the amounts used in in vitro assays (Table 2.2), and the possibility that compensatory or immune responses will develop when the treatment with crude or (partially) purified inhibin-containing preparations extends over a long time period (Baker et al., 1981, Channing et al., 1982b).

2.3.2 In vitro methods

The in vitro bioassays of inhibin activity are based on the suppression of FSH production and secretion by pituitary gonadotrophic cells. These effects can be measured either in a culture of dispersed pituitary cells or during short-term incubations of whole or hemisected pituitary glands (Table 2.3). The advantages of in vitro bioassays, when compared to in vivo methods, consist of the direct effects of inhibin activity on FSH production and release, an improved assay precision (Table 2.3), a higher sensitivity (Table 2.2) and the use of a smaller number of experimental animals. The most widely used in vitro bioassays are based on the suppression of FSH release from cultured pituitary cells under basal or GnRH-stimulated conditions. Precision and sensitivity of these assays are high, although interassay variation is not completely satisfactory: 15-38 % (Eddie et al., 1979; Hermans et al., 1982b; Au et al., 1985).

Inhibin activity from various sources specifically suppresses basal release of FSH from cultured rat pituitary cells (Steinberger and Steinberger, 1976; de Jong et al., 1979a; Hermans et

TABLE 2.3: Inhibin bioassays, based on suppression of FSH in *in vitro* systems.

Method	Precision index λ	References
Incubation of hemipituitaries		
-Inhibition of GnRH-stimulated FSH-release	-	Davies et al., 1978b
	0.901	Hudson et al., 1979
Rat pituitary cell cultures		
-Inhibition of GnRH-stimulated FSH-release	-	Labrie et al., 1978
	0.026-0.281	Eddie et al., 1979
	0.048	Hudson et al., 1979
	0.07-0.11	de Jong et al., 1979a
	0.051-0.120	Scott et al., 1980
-Inhibition of spontaneous FSH-release	-	Steinberger and Steinberger, 1976
	0.17-0.22	de Jong et al., 1979a
-Suppression of cell content	0.032-0.098	Scott et al., 1980
Hamster pituitary cell cultures		
-Inhibition of GnRH-stimulated FSH-release	-	Chappel et al., 1979
Ovine pituitary cell cultures		
-Inhibition of spontaneous FSH-release	-	Huang and Miller, 1984

al., 1982b). After a preculture period standard and test substances are added; after an additional culture period of several days FSH is measured in the culture medium. Alternatively, FSH can be estimated in medium collected after an additional culture period of 6 h in the presence of GnRH (Baker et al., 1976; Eddie et al., 1979; Hudson et al., 1979). In the latter case a significant dose-dependent suppression of LH is also observed. Finally, measurement of FSH and LH content of lysed cultured pituitary cells, which have been precultured for 18 h and cultured for 72 h in the presence of inhibin-containing substances, has been claimed to offer a more specific and faster bioassay system for inhibin activity than the estimation of release of FSH into the medium (Scott et al., 1980). Precision and sensitivity of suppression of FSH release and FSH content are comparable (Tables 2.2 and 2.3).

Hamster (Chappel et al., 1979), rabbit (Goodman, 1984) and

ovine (Huang and Miller, 1984) pituitary cell cultures have also been utilized to detect inhibin activity.

When pituitary halves were incubated in the presence of GnRH a dose-dependent suppression of FSH was observed using short-term incubations in the presence of gonadal extracts (Davies et al., 1978a,b, 1979; Hudson et al., 1979). Jenner et al. (1982a,b) showed that hemipituitaries secrete considerable amounts of FSH, even under basal conditions, that was suppressed after addition of steroid-free bFF. Precision and sensitivity of this system appeared to be unsatisfactory (Tables 2.2 and 2.3; Hudson et al., 1979; Baker et al., 1981). Inconsistency in results of the various *in vitro* bioassays may be due to nonspecific effects on the pituitary cells of the added, putative inhibin-containing preparations: cytotoxic effects, enzymatic degradation of gonadotrophins and GnRH, and inhibitory effects on pituitary protein synthesis have been described (de Jong et al., 1979b; Baker et al., 1981). If these effects are associated with a reduction in release or cellular content of FSH, they might be erroneously ascribed to the presence of inhibin activity in the added preparations. It is therefore mandatory to check the specificity of the response which is observed. Parallelism of dose-response curves of standards and unknown preparations, measurement of LH and other pituitary hormones, e.g. growth hormone or prolactin, in the culture and unchanged morphology of the pituitary cells have been used to indicate a specific response of FSH release to inhibin activity (Scott et al., 1980; Baker et al., 1981). Another method for directly measuring cytotoxicity of test substances is based on the estimation of the release of ^{51}Cr into the culture medium of pituitary cells (Robertson et al., 1982). These authors showed that release of ^{51}Cr was independent of cell- and ^{51}Cr-concentration and of culture time. Using this technique it was shown that crude seminal plasma was highly cytotoxic to pituitary cells, probably accounting for its observed "inhibin-like activity". The dose-dependent decrease of FSH release was accompanied a concomitant dose-dependent decrease of LH release and increase of ^{51}Cr release (Robertson et al., 1982; Scott et al., 1982). The cytotoxicity of human seminal plasma was associated with components smaller than 10 kDa. In contrast, ovine testicular lymph,

ovine rete testis fluid and hFF all caused specific suppressing effects on cellular content of FSH without affecting ^{51}Cr release or cellular content of LH (Scott et al., 1982).

2.3.3 Radioligand assays

Several inhibin assays, based on the use of radioactively labelled ligands, have been described. Two types of assays have been reported: radioimmunoassays, based on the use of antibodies raised against preparations with putative inhibin activity (human seminal plasma inhibin: Franchimont et al., 1977; Sheth et al., 1978; Vaze et al., 1979; bovine follicular fluid inhibin: de Jong et al., 1983b; McLachlan et al., 1986) and radioreceptor assays, based on the binding of radioactively labelled inhibin-like preparations to putative inhibin receptors on pituitary cell membranes (Sairam et al., 1981b; Steinberger et al., 1982; Dighe et al., 1984; Seethalakshmi et al., 1984). The results obtained with the radioimmunological techniques described by Franchimont et al. (1977), Sheth et al. (1978) and Vaze et al. (1979), who employed seminal plasma inhibin and who measured inhibin concentrations in various samples, showed no correlation with data obtained in well-characterized inhibin bioassays. Recent data on the structure and function of proteins, considered to represent seminal plasma inhibins, raise considerable doubt about the specificity of the inhibin-like effect (see section 2.4.2). De Jong et al. (1983b) and McLachlan et al. (1986) described radioimmunoassays employing antibodies raised against (partially) purified inhibin from bFF; these authors satisfactorily described correlations between bio- and immunopotencies of several inhibin-containing preparations over a wide range of biological activities. The existence of several subunits of follicular fluid inhibin (Miyamoto et al., 1985, 1986; McLachlan et al., 1986; Robertson et al., 1986b) demands a careful evaluation of the antisera against inhibin, especially in view of the possible differences in biological activities of the various forms of inhibin and the specificity of the antibodies in immuno- and bioassay systems.

The radioreceptor assay described by Sairam et al. (1981b) em-

ployed a radioiodinated protein preparation purified from bovine seminal plasma. This radiolabelled material was bound to ovine pituitary membranes and was displaced by an excess of the same unlabelled protein and by a fraction of bFF. Both of the latter preparations showed inhibin-like activity when tested in in vivo bioassays employing the hCG-induced augmentation of uterine weight in immature mice and suppression of the postcastration rise of FSH in castrated male rats (Sairam et al., 1981a,b). With respect to the possible presence of inhibin in seminal plasma, we refer to the data discussed in section 2.4.2.

Steinberger et al. (1982) observed that internally labelled Sertoli cell proteins also bound to rat anterior pituitary membranes and were displaced after addition of nonlabelled Sertoli cell proteins and by protein preparations from rat testes, ovine rete testis fluid and ovine testicular lymph, containing inhibin activity, whereas "non-inhibin substances" did not displace the labelled material (Dighe et al., 1984; Seethalakshmi et al., 1984). These radioreceptor assays have not been sufficiently characterized and evaluated in comparison with other, well-defined, bioassay systems.

Finally, binding of radioiodinated inhibin-like material, purified from bovine seminal plasma (Vaze et al., 1979), to either rat anterior pituitary membranes (Vanage and Sheth, 1982), pituitary LHRH-receptors (Sheth et al., 1982), rat spermatids (Dandekar et al., 1983) and rat ventral prostate membranes (Vanage et al., 1985) has been described. However, correlations between inhibin activity determined in in vitro bioassays using cultured pituitary cells and in the latter binding assays have not been studied.

2.3.4 Immunological assays

A faster and more sensitive assay system for inhibin activity has been developed with specific antibodies, raised against purified inhibin (McLachlan et al., 1986). Several authors described the production and use of antibodies against inhibin activity (Jansen et al., 1981; Channing et al., 1982b; de Jong et al., 1982a,b,

1983a,b, 1984; Rivier et al., 1985; van Dijk et al., 1986; Lee et al., 1986; McLachlan et al., 1986; Miyamoto et al., 1986). A quantitative and qualitative evaluation of an antiserum raised in rabbits against inhibin activity from bFF was given by de Jong et al. (1983b), who showed a good correlation between bio- and immunopotency in a number of inhibin-containing preparations. These authors showed in further studies that their antisera were capable of neutralizing inhibin activity obtained from different stages of purification of inhibin. Furthermore, they showed that inhibin activity in gonadal fluids and extracts from several species could be neutralized as well (de Jong et al., 1984; van Dijk et al., 1986). Lee et al. (1986) used monoclonal antibodies directed against inhibin activity obtained from rat ovarian homogenates to neutralize inhibin activity in bovine, ovine, porcine and rat ovarian follicular fluid, which is suggestive of similarities in the immunological determinants of inhibin from several species.

2.3.5 General remarks on reliability of inhibin assays

Most studies on inhibin assays emphasize specificity of the assay as the most important factor for the reliability of the method. Moreover, many of these studies state that the described systems are specific for the estimation of inhibin without estimating aspecific effects on the release or production of FSH. The observation that the physicochemical characteristics of inhibin-containing preparations show such great diversity in apparent molecular weight, isoelectric point, amino acid composition, biological activity and apparent site of production (see section 2.4), raises doubts on the specificity of a number of the published assay systems for inhibin activity. The discrepancy between the results of inhibin assays may be explained by the fact that effects of preparations with inhibin-like activity have not been compared with standard preparations in a dose-dependent relationship using parallel line statistics (Baker et al., 1981). Many authors agree on the specificity and reliability of the *in vitro* assay using cultured rat anterior pituitary cells (Eddie et al.,

1979; Hudson et al., 1979; de Jong et al., 1979a; Scott et al., 1980, 1982; Baker et al., 1981; Robertson et al., 1982; de Jong and Robertson, 1985). Cytotoxic effects can be recognized in this type of assay (Robertson et al., 1982), the sensitivity of the method is sufficiently high to detect inhibin activity in crude and partially purified preparations and the precision and inter-assay variability are acceptable (Tables 2.2 and 2.3; Baker et al., 1981, 1982a,b). This type of method was used to detect inhibin activity in the isolation of ovarian inhibin (Rivier et al., 1984, 1985; Ling et al., 1985; Miyamoto et al., 1985; Robertson et al., 1985, 1986b; Fukuda et al., 1986). Based on their high specificity for inhibin, methods employing the reduction of FSH release or cellular FSH content of cultured rat anterior pituitary cells represent the most reliable bioassays for inhibin. In these types of assays parallelism of dose-response suppression curves has been demonstrated between female and male gonadal fluids, tissues and spent culture media of Sertoli and granulosa cells, thus suggesting a similar biological specificity for inhibin from female and male animals.

2.4 SOURCES AND PHYSICOCHEMICAL CHARACTERIZATION OF INHIBIN

2.4.1 Sources of inhibin in male animals

Inhibin activity appears to be present in fluids and tissues of gonadal origin in male animals of several species. The early observations of Mottram and Cramer (1923) suggested a relationship between seminiferous epithelium and function of the hypophysis (see section 2.1). McCullagh (1932) suggested that the disappearance of castration cells in the hypophysis of castrated male rats after injection of an aqueous extract of testes was caused by inhibin.

Inhibin-like activity was found in bovine (Keogh et al., 1976; Nandini et al., 1976), ovine (Shashidhara Murthy et al., 1979; Sheth et al., 1979) and rat (Davies et al., 1978b; Au et al.,

1983) testicular extracts.

Setchell and Sirinathsinghji (1972) showed that ram rete testis fluid suppressed the hCG-induced increase in uterine weight of mice and rats. Setchell and Jacks (1974) demonstrated that ram rete testis fluid lowered FSH levels in long-term castrated rats without influence on LH levels; these authors concluded that this action was most likely due to the presence of inhibin activity in rete testis fluid. Baker et al. (1978) detected inhibin activity in ovine testicular lymph using an in vitro bioassay; Cahoreau et al. (1979) and Davies et al. (1979) used in vivo assays to demonstrate inhibin activity in ovine rete testis fluid.

Inhibin-like activity has been demonstrated to be present in human and bovine seminal plasma (Franchimont et al., 1975, 1979a,b; Chari et al., 1978; Sairam et al., 1981b; Mohapatra et al., 1985). A fraction from bovine seminal plasma obtained by ethanol precipitation suppressed plasma FSH levels in castrated male rats (Franchimont, 1972; Franchimont et al., 1972, 1975). In in vitro inhibin bioassays this inhibin-like effect is most likely related to nonspecific cytotoxic effects (Robertson et al., 1982; Scott et al., 1982).

Spermatogenic cells have been reported as the site of production of inhibin by several authors (Fanchini et al., 1963; Lugaro et al., 1969, 1973, 1974; Franchimont et al., 1972) who employed suppression of FSH levels in chronically castrated male rats as an assay system, although clinical (reviewed by Setchell et al., 1977) and experimental (de Jong and Sharpe, 1977) evidence was obtained against spermatogenic cells as the source of inhibin.

Direct evidence for the site of production of inhibin in the testis came from experiments in which testicular cell types were isolated and cultured and assayed for inhibin activity. Steinberger and Steinberger (1976) showed that cultured rat Sertoli cells secrete a substance, which suppresses FSH release from cultured rat pituitary cells. These authors referred to this substance as "Sertoli cell factor". The production of an inhibin activity by Sertoli cells was amply confirmed by others (de Jong et al., 1978; Labrie et al., 1978; Le Gac and de Kretser, 1982; Verhoeven and Franchimont , 1983; Ultee-van Gessel et al., 1986).

2.4.2 Purification and characterization of inhibin from male origin

Numerous reports have appeared on the detection, purification and characterization of testicular inhibin. After the observations of Setchell and Jacks (1974) on the presence of inhibin activity in rete testis fluid much work was directed at the purification of the active principle from this source. Data on reported molecular weights of testicular inhibin have been summarized in Table 2.4. The work of Davies et al. (1978b, 1979) suggested association of inhibin activity with a high molecular mass protein of 90 kDa. However, after gel filtration in the presence of urea inhibin activity also appeared to be associated with a 5 kDa protein. Cahoreau et al. (1979) reported on the association of inhibin activity with a >100 kDa protein from ovine rete testis fluid,

TABLE 2.4: Reported molecular mass of testicular inhibin

Source	Molecular mass (kDa)	Estimated by*	Measurement of inhibin activity	References
Ovine rete testis fluid	90, 20, < 5	Gel filt.	Uterine weight FSH in vivo FSH in vitro	Davies et al., 1978a,b, 1979b
	>100	Gel filt.	FSH in vivo	Cahoreau et al., 1979
	>100 < 5	Gel filt.	FSH in vivo FSH in vitro	Franchimont et al., 1979b
	90, 60 30	Gel filt. SDS-PAGE	FSH in vitro	Baker et al., 1982a,b, 1985
Testis				
Ovine	< 1.5	Gel filt.	Ovarian weight FSH in vivo	Vijayalakshmi et al., 1980
	20	Gel filt.	Uterine weight	Moudgal et al., 1984
Bovine	-	Gel filt.	FSH in vivo	Sahni et al., 1981
Human	"low"	Gel filt.	FSH in vivo Ovarian weight	Krishnan et al., 1982
Rat	50-60	Gel filt.	FSH in vitro	Au et al., 1983

* Gel filt.: gel filtration column chromatography
SDS-PAGE: sodium dodecylsulphate-polyacrylamide gel electrophoresis

whereas Franchimont et al. (1979a,b) obtained active fractions from ram rete testis fluid with proteins of >100 kDa and <5 kDa. Baker et al. (1982a,b, 1985) found different molecular weights for inhibin activity in ovine rete testis fluid, depending on the experimental conditions used: gel filtration under basic conditions revealed a molecular mass of >90 kDa, but under acidic conditions <60 kDa; after sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) a molecular mass of approximately 30 kDa was found. Inhibin activity from rete testis fluid was partially retained on a column of immobilized lectins, indicating a glycoprotein character and possible heterogeneity for testicular inhibin (Baker et al., 1982a,b).

Inhibin purified by gel filtration chromatography and ultrafiltration from ovine testicular homogenates exhibited a molecular mass <1.5 kDa (Vijayalakshmi et al., 1980). Moudgal et al. (1984), however, detected an inhibin containing preparation from ovine testicular extracts with a molecular mass of 20 kDa. An inhibin preparation was obtained by gel filtration and ion exchange chromatography of an extract of the testis of the Indian water buffalo and considered pure on basis of gel electrophoresis (Sahni et al., 1981) although a molecular weight was not reported. Inhibin activity from rat testicular extracts was found in a broad area from 17-100 kDa with maximal bioactivity between 50-60 kDa after fractionation on Sephacryl S-200 and elution with saline (Au et al., 1983).

A purification procedure for inhibin-like activity from bovine seminal plasma was reported (Chari et al., 1978) consisting of acetone precipitation, gel filtration and ion exchange chromatography. The final product, which reduced the increase in weight of ovaries of immature female rats after administration of hCG, had an apparent molecular mass of 19 kDa. These findings were confirmed by Mohapatra et al. (1985) who described the purification of two seminal plasma peptides of 18 and 20 kDa with inhibin-like activity based on the mouse uterine weight assay. Only the 18 kDa protein suppressed the postcastration rise of FSH in castrated male and female rats, while the 20 kDa protein inhibited FSH-binding. Sairam et al. (1981a) reported the purification of an 18 kDa protein from bovine seminal plasma using in

vivo suppression of circulating FSH as a measure for inhibin activity. Also low molecular weight components with FSH-suppressing activity in vivo were detected (Ying et al., 1981, Lugaro et al., 1984). The latter authors also mentioned that these inhibin-like activities displayed FSH-binding inhibitor activity. Proteins with inhibin-like activity have also been purified from human seminal plasma (Thakur et al., 1978; Franchimont et al., 1979a). More recently, several reports described the purification and subsequent characterization of the amino acid sequence of "inhibin" from human seminal plasma (Johansson et al., 1984; Ramasharma et al., 1984; Seidah et al., 1984a,b; Sheth et al., 1984; Arbatti et al., 1985). Two peptides with inhibin-like activity in various in vivo assays were described: one of 31 amino acids ("α-inhibin") and one of 94 amino acids("β-inhibin"). No sequence homology was found between these two forms, nor between putative precursor forms of the 31 amino acid peptide and the 94 amino acid peptide, which were isolated by Li et al. (1985). Synthetic peptides, identical to the 31 amino acid peptide (Ramasharma et al., 1984; Yamashiro et al., 1984), however, did not possess inhibin activity when tested in an in vitro bioassay based on rat anterior pituitary cells in culture (de Jong et al., 1985; Liu et al., 1985). The 31 amino acid peptide showed partial sequence homology with yeast enolase (Seidah et al., 1984b) and has been shown to be identical to the N-terminal of a basic protein which was present as a structural protein in the coagulate of ejaculated semen and which originates from the seminal vesicles (Lilja and Jeppsson, 1985).

The 94 amino acid "β-inhibin" and its 28 amino acid C-terminal fragment also lack inhibin activity in an in vitro bioassay (Kohan et al., 1986). The 94 amino acid peptide was identified as a sperm-coating antigen (Johansson et al., 1984; Akiyama et al., 1985) and as a part of PDC-109, a peptide of prostatic origin (Esch et al, 1983a,b). Moreover, it has been shown that PDC-109 is identical to structural domains of bovine fibronectin and human plasminogen activator (Baker, 1985). Taken together, these observations make it highly unlikely that the so-called "seminal plasma inhibins" had any structural and functional relationship to gonadal inhibin.

2.4.3 Sources of inhibin in female animals

Inhibin activity is relatively abundant in ovarian follicular fluid from several species. Initially, de Jong and Sharpe (1976) demonstrated inhibin activity in bFF employing a test system based on suppression of the postcastration rise of FSH levels in male rats. Likewise, Schwartz and Channing (1977) demonstrated inhibin activity in porcine follicular fluid (pFF) by suppression of the secondary rise in serum FSH in pro-oestrous female rats. Since then, inhibin activity has been detected using a number of *in vivo* and *in vitro* assay systems in bovine (Welschen et al., 1977; de Jong et al., 1979a,b; Jansen et al., 1981; Ireland et al., 1983; van Dijk et al., 1984; McNeilly, 1984; Robertson et al., 1985), porcine (Marder et al., 1977; Welschen et al., 1977; Bronson and Channing, 1978; Williams et al., 1979; Shander et al., 1980; Channing et al., 1982b, 1984b; Stillman et al., 1983; Goodman, 1984; Huang and Miller, 1984; Lumpkin et al., 1984; Rivier et al., 1984; Sairam et al., 1984; Schenken et al., 1984; Thomas and Nikitovitch-Winer, 1984; Redmer et al., 1985), hamster (Chappel et al., 1979), horse (Miller et al., 1979), human (Chari et al., 1979, 1982; Chappel et al., 1980; Channing et al., 1981a, b, 1984a,e, 1985a; Lefevre et al., 1981; Marrs et al., 1984; van Dijk et al., 1985a,b), ovine (Dobos et al., 1983; Tsonis et al., 1983), and rat (Fujii et al., 1983) follicular fluids. Furthermore, inhibin activity was demonstrated to be present in bovine (Hopkinson et al., 1977), hamster (Chappel, 1979; Chappel et al., 1979), monkey (Channing et al., 1984d), ovine (Vijayalakshmi et al., 1980) and rat (Sander et al., 1985) ovarian extracts. Ovaries transplanted under the kidney capsule of ovariectomized mice (Bronson and Channing, 1978) or ovariectomized rats (Uilenbroek et al., 1978) secreted substances that suppressed the postcastration rise of FSH and LH. Ovaries transplanted into the spleen also suppressed elevated FSH levels in ovariectomized rats, whereas no effects on the postcastration rise of LH was observed (Uilenbroek et al., 1978), indicating that a nonsteroidal factor produced by the ovary regulates FSH levels.

Analogous to the Sertoli cell as the possible site of production of inhibin activity in the male, the granulosa cells of the

ovary have been suggested as the source of inhibin activity in the female (de Jong and Sharpe, 1976, 1977). Direct evidence for the granulosa cell as the site of production of inhibin activity came from the studies of Erickson and Hsueh (1978) using cultured rat granulosa cells. Their observations were confirmed and extended by Hermans et al. (1982b) and de Jong et al. (1982b) in long-term cultures of rat and bovine granulosa cells and by Sander et al. (1984) in rat granulosa cells under basal, nonstimulated conditions. Hermans et al. (1982b) observed inhibition of release of FSH from cultured rat anterior pituitary cells by spent culture medium of rat granulosa cells without significant changes in LH release. In contrast, Erickson and Hsueh (1978) observed in addition to inhibition of FSH release, a small, but significant decrease of LH release. In spent medium of cultured bovine ovarian tissue (Franchimont et al., 1981; Henderson and Franchimont,1981, 1983) and rat ovarian cells (Croze and Franchimont, 1984a,b) inhibin activity could also be detected. In these studies inhibin activity was demonstrated to be present in spent culture medium from granulosa cells and not in the spent medium from theca, stroma and luteal cells (Franchimont et al., 1981; Henderson and Franchimont, 1983) indicating that the granulosa cell is the most likely source of inhibin activity. Similarly the presence of inhibin activity in spent culture medium of porcine (Anderson and DePaolo, 1981; Anderson and Hoover, 1982), monkey (Channing et al., 1982a), rabbit (Goodman, 1984) and human (Channing et al., 1982a, 1984a,c) granulosa cells has been demonstrated.

2.4.4 Purification and characterization of inhibin from female origin

Many attempts to purify inhibin from ovarian follicular fluid from various species have been reported (for reviews, see: Channing et al., 1985b; de Jong and Robertson, 1985). Data on reported molecular weights of follicular fluid inhibin have been summarized in Table 2.5. Chari et al. (1979, 1982) reported the isolation of a 23 kDa protein from hFF with inhibin-like activity in the rat ovarian weight assay. Rechromatography of the 23 kDa pro

TABLE 2.5: Reported molecular mass of ovarian inhibin

Source	Molecular mass (kDa)	Estimated by*	Measurement of activity	References
Follicular fluid				
bovine	65	SDS-PAGE	FSH in vitro	Jansen et al., 1981
	65	SDS-PAGE	FSH in vitro	van Dijk et al., 1984
	105	SDS-PAGE	FSH in vitro	Godbout and Labrie, 1984a
	56	SDS-PAGE	FSH in vitro	Robertson et al., 1985
	31	SDS-PAGE	FSH in vitro	Robertson et al., 1986b
	96, 55, 32	SDS-PAGE	FSH in vitro	Fukuda et al., 1986
	120, 108, 88 65, 55, 32	SDS-PAGE	FSH in vitro	Miyamoto et al., 1986
human	23, <1	SDS-PAGE Gel filt.	Ovarian weight FSH in vivo	Chari et al., 1979, 1982
	65	SDS-PAGE	FSH in vitro	van Dijk et al., 1985a
ovine	<1.5	Gel filt.	Ovarian weight FSH in vivo	Vijayalakshmi et al., 1980
	80	Gel filt.	FSH in vitro	Dobos et al., 1983
porcine	10-35	Gel filt.	FSH in vitro	Williams et al., 1979
	194	Radiation-inactivation	FSH in vitro	Ward et al., 1983
	160, 65	Gel filt.	FSH in vitro	Channing et al., 1984b
	140	SDS-PAGE	FSH in vitro	Godbout and Labrie, 1984a,b
	10-12	Gel filt.	FSH in vitro	Rivier et al., 1984
	43, 25-30	Gel filt.	FSH in vitro	Sairam et al., 1984
	32	SDS-PAGE	FSH in vitro	Ling et al., 1985
	100, 80, 55, 32	SDS-PAGE	FSH in vitro	Miyamoto et al., 1985
	32	SDS-PAGE	FSH in vitro	Rivier et al., 1985

* Gel filt.: gel filtration column chromatography
SDS-PAGE: sodium dodecylsulphate-polyacrylamide gel electrophoresis

tein on Sephadex G-10 yielded a <1 kDa peptide, which possessed inhibin-like activity, FSH-binding inhibitor activity and oocyte maturation inhibitor activity (Chari et al., 1982). Williams et al. (1979), using a purification procedure based on dye-ligand affinity chromatography on immobilized Cibacron Blue F3GA and immunoaffinity chromatography on immobilized antibodies against barrow serum, obtained a bioactive fraction from pFF as determined in a dispersed pituitary cell assay. The biological activi-

ty was associated with 10-35 kDa proteins with an isoelectric point (IEP) between pH 5.0 and pH 5.8.

Using a similar approach, Jansen et al. (1981) reported the partial purification of inhibin from bFF by dye-ligand affinity chromatography on immobilized Procion Red 3B, hydrophobic interaction chromatography on Phenyl-Sepharose and ω-aminohexyl agarose and affinity chromatography on immobilized Concanavalin A. The purified material contained predominantly a 65 kDa protein. Antibodies raised in rabbits against this preparation neutralized inhibin activity in the in vitro bioassay. When these antibodies were immobilized on cyanogenbromide(CNBr)-activated Sepharose, inhibin activity from bFF was specifically retained (de Jong et al., 1983a,b). Using the column of immobilized antibodies prepared against inhibin, de Jong et al. (1982a) isolated internally labelled inhibin obtained after in vitro culture of bovine granulosa cells in the presence of radiolabelled amino acids and sugars. In these procedures inhibin activity was associated with a 65 kDa glycoprotein (de Jong et al., 1982a, 1983a). Van Dijk et al. (1984) isolated a 65 kDa protein from bFF with inhibin activity using Fast Protein Liquid Chromatography (FPLC). Robertson et al. (1985, 1986b) purified a 56 kDa and a 31 kDa inhibin from bFF which consisted of subunits of 44 and 14 kDa and 20 and 14 kDa, respectively, linked by interchain disulfide bridges, using gel filtration chromatography, reversed phase high pressure liquid chromatography (RP-HPLC) and preparative SDS-PAGE. Fukuda et al. (1986) and Miyamoto et al. (1986) reported the presence of proteins with inhibin activity in bFF, the main bioactive form, which was isolated, being a 32 kDa protein.

Vijayalakshmi et al. (1980) observed inhibin-like activity in ovine follicular fluid (oFF) with an apparent molecular mass <1.5 kDa, as estimated by gel filtration chromatography. On the other hand, Dobos et al. (1983) obtained in the fractionation of oFF by RP-HPLC a fraction with inhibin activity associated with a molecular mass of approximately 80 kDa as assessed by gel permeation HPLC.

Ward et al. (1983) reported the apparent molecular mass of inhibin in pFF to be 194 kDa as estimated by the technique of radiation inactivation, whereas Godbout and Labrie (1984a,b) observed

a molecular mass of 140 kDa for porcine inhibin after SDS-PAGE. Sairam et al. (1984) reported molecular masses of >60 kDa by gel filtration and 43 and 25-30 kDa after SDS-PAGE of pFF fractions. Channing et al. (1984b) found inhibin activity in pFF to be associated with 160 kDa and 65 kDa proteins, depending on the elution conditions used in gel filtration chromatography. Recently, inhibin from pFF was purified to homogeneity employing HPLC-techniques (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985). The molecular mass was reported to be 32 kDa, whereas Miyamoto et al. (1985) also found proteins with higher molecular mass (55, 80 and 100 kDa), associated with inhibin activity. The observations by Ward et al. (1983), Channing et al. (1984b), Godbout and Labrie (1984a,b) and Miyamoto et al. (1985) might indicate that inhibin associates readily, e.g. by hydrophobic interactions, with other proteins or with itself, forming higher molecular weight complexes.

The complementary DNA (cDNA) nucleotide sequences of porcine (Mason et al., 1985), human (Mason et al., 1986) and bovine (Forage et al., 1986) inhibin have been elucidated. Interestingly, the nucleotide and derived amino acid sequence of the 14 kDa chain of porcine inhibin shows sequence homology with transforming growth factor-beta (TGF-β, Mason et al., 1985) and with Müllerian inhibiting substance (MIS, Cate et al., 1986), whereas dimers of the 14 kDa β-chain exhibit FSH-releasing activity (Ling et al., 1986; Vale et al., 1986). In this respect inhibin seems to be part of a putative multimember family of proteins regulating the gonadal-hypophyseal system. The structure of the several forms of inhibin will be discussed more extensively in Chapter 5.

2.5 MECHANISM OF ACTION OF INHIBIN

From the data presented in sections 2.2 and 2.3 it appears that inhibin acts on the pituitary gland by suppression of the production and secretion of FSH. Its biochemical mode of action is, however, far from clear. Scott and Burger (1981) report that inhibin activity from ovine testicular lymph suppresses both FSH

release and cell content of pituitary cells in culture in a time- and dose-related manner. These authors postulated the following model for the action of inhibin: 1) inhibin has a long term inhibiting effect on the synthesis of FSH, which results in reduction of the cellular content of FSH available for release; and 2) inhibin has an acute inhibiting effect on FSH release. Inhibition of the GnRH-stimulated release of LH might be caused by the same release-inhibiting effect (Scott and Burger (1981).

Jenner et al. (1982a,b) studied the regulation of cellular content and release of FSH using incubated hemipituitaries. These authors observed that basal as well as GnRH-stimulated release of FSH was dependent on protein synthesis (Jenner et al., 1982a). When hemipituitaries were incubated in the presence of steroid-free bFF, synthesis and basal release of FSH were inhibited; synthesis appeared to be inhibited at an earlier time than release (Jenner et al., 1982b). This suggests that inhibin activity acts primarily on the synthesis of FSH rather than on its release. Jenner et al. (1985) suggested that an increase in cyclic adenosine-3',5'-monophosphate (cAMP) levels suppressed synthesis and secretion of FSH, while stimulating release of LH. Preliminary experiments with forskolin, a compound which stimulates adenylate cyclase activity, have shown that adenylate cyclase is probably not involved in the action of inhibin, making it unlikely that cAMP acts as a second messenger in the transduction of the inhibin signal (de Jong et al., 1985). On the other hand, Franchimont et al. (1983) indicated that inhibin-like activity from ram rete testis fluid suppressed cAMP levels and stimulated cyclic guanosine-3',5'-monophosphate (cGMP) levels in cultured pituitary cells under basal and GnRH-stimulated conditions. When the phosphodiesterase inhibitor, 1-methyl-3-isobutyl-xanthine, was added to GnRH-stimulated pituitary cells, cAMP and cGMP levels were increased compared to the control values. In the presence of inhibin-like activity no change in cAMP levels is observed, whereas cGMP levels increase with a concomitant decrease in FSH and LH levels (Franchimont et al., 1983). These authors concluded that inhibin might mediate its effect via stimulation of the production of cGMP.

It might be expected that the exact mechanism of action of

inhibin will be resolved in the near future now that purified preparations are available and experimental conditions can be established in which only the specific effects of inhibin can be measured.

2.6 CONCLUDING REMARKS

1) The existence of inhibin has long been a matter of speculation, because of the lack of reproducibility and specificity of the assays for inhibin activity, and the confusion surrounding the necessity to postulate the existence of inhibin in order to explain physiological observations. The demonstration that testicular and ovarian protein preparations exerted a specific suppressive effect on FSH release from the pituitary gland proved an important step in inhibin research. Proof of its existence was obtained with the purification of the biologically active inhibin molecule from ovarian follicular fluid.
2) Most of the data concerning the physiology of inhibin have been gathered by circumstantial evidence, because it was, until recently, not possible to measure inhibin activity directly in the peripheral circulation. With the recently developed radioimmunoassay for inhibin it is possible to measure inhibin activity in bovine and human serum.
3) The presumed physiological significance of inhibin in males is most likely restricted to prepubertal animals. During this period the feedback regulation of peripheral FSH concentrations by inhibin is most important for the development and division of Sertoli cells. Disturbance of this feedback mechanism early in life may result in the impairment of spermatogenesis and reduced fertility.
4) Inhibin plays a major role in the regulation of FSH levels in the mature female. The peripheral levels of FSH influence the development of the antral follicles while inhibin secretion by the granulosa cells provides a feedback mechanism to control synthesis and secretion of FSH from the hypophysis and in that way the follicular development.
5) Inhibin activity has been tested in a variety of *in vivo* assay

systems. In vivo methods for inhibin using reduction of organ weight lack specificity and sensitivity. Suppression of FSH in the peripheral circulation can be used, but this method is also rather insensitive.

6) The most reliable assay system for inhibin is based on the reduction in FSH release or cellular FSH content of cultured rat anterior pituitary cells. Using either assay system it has been possible to monitor the purification of inhibin from ovarian follicular fluid.

CHAPTER 3

PURIFICATION OF INHIBIN FROM OVARIAN FOLLICULAR FLUID

3.1 INTRODUCTION

During the last ten years research on inhibin has been concerned mainly with the three following topics: 1) the development of a reliable bioassay for inhibin from several sources, which proved the existence of inhibin as a clearly defined activity; 2) the explanation of the role and physiological significance of inhibin in the regulation of the hypothalamo-hypophyseal-gonadal system in mammals, and 3) the purification and characterization of the biochemical entity which is responsible for the inhibin effect in female and male mammals.

After it was shown that inhibin activity is present in ovarian follicular fluid (de Jong and Sharpe, 1976) many attempts were made to purify inhibin from follicular fluid from several species (see Chapter 2.4). The results of the studies of Jansen et al., (1981) and de Jong et al., (1982a,b, 1983a,b, 1984) on the purification and characterization of inhibin from bFF will be reviewed in more detail in this chapter, since part of the work described in this thesis is an extension of their investigations.

The "classical" approach for purification of proteins, using ammonium sulfate precipitation, acid-ethanol precipitation, ultrafiltration, ion exchange chromatography on DEAE- and CM-cellulose and gel filtration on various types of Sephadex was not very successful in the purification of inhibin as the degree of purification was limited and the loss of biological activity was high (de Jong et al., 1979b, 1981; Jansen et al., 1981). The introduction of dye-ligand affinity chromatography on immobilized Procion Red 3B resulted in a 20-fold purification with a nearly 100 % recovery of activity (Jansen et al., 1981). Further purification steps included several hydrophobic interaction matrices and affinity chromatography on immobilized antibodies and immobilized lectins (de Jong et al., 1981; Jansen et al., 1981). A purification sequence employing immobilized Procion Red 3B, ω-

aminohexyl agarose and Concanavalin A Sepharose resulted in an 81-fold purified preparation with a recovery of approximately 14%. Antibodies raised in rabbits against this preparation were immobilized on CNBr-activated Sepharose and used as an immunoaffinity column. This procedure yielded the same purification factor for inhibin from bFF (de Jong et al., 1983b). Based on SDS-PAGE, association of bovine inhibin activity with a 65 kDa protein was postulated (Jansen et al., 1981). The use of Procion Red 3B in combination with immobilized desoxycholic acid also yielded an 80-fold purified preparation (de Jong et al., 1983a).

The development of HPLC-techniques, such as reversed-phase chromatography, gel permeation and ion pair chromatography as well as improved ion exchange chromatographic matrices (Regnier, 1982, 1983), proved to be a major step forward for the purification of many biologically interesting proteins, including inhibin. Reversed phase (RP)-HPLC was employed to purify ovarian inhibin from bovine (Robertson et al., 1985, 1986b; Fukuda et al., 1986) and porcine (Rivier et al., 1984, 1985; Ling et al., 1985; Miyamoto et al., 1985) follicular fluid. The FPLC-system using columns prepacked with rigid microspheres of a uniform diameter, which was introduced in 1982, seemed to offer the combined advantages of mild chromatographic procedures, compatible with labile biologically active compounds and high resolution. We have employed this FPLC-system as part of our purification procedure, using the anion exchange column Mono Q, the chromatofocusing column Mono P (Sluyterman and Elgersma, 1978; Sluyterman and Wijdenes, 1978) and the gel filtration column Superose-12.

In this chapter we will first describe in section 3.2 some theoretical aspects of column chromatography. The application of these techniques in the partial purification and characterization of inhibin from bovine and human follicular fluid will be described in sections 3.3 and in Appendix papers 1 and 2. Finally, a comparison between our results and the results of other investigators has been made in sections 3.4 and 3.5.

3.2 SOME THEORETICAL ASPECTS OF COLUMN CHROMATOGRAPHY

Successful separation of proteins in a complex mixture can be achieved by differential adsorption and desorption of proteins in solution to a solid phase. The application of adsorption techniques in column chromatography has created powerful methods for separation and purification of proteins in a variety of systems, especially in ion exchange and affinity chromatography.

The principles of chromatography can be described by a simple mathematical model, which describes the distribution behaviour of a substance between a liquid and a solid phase. The partition coefficient α can be defined as the fractional amount of protein adsorbed to the solid phase at any instant (see Scopes, 1982). If the protein of interest is adsorbed completely to the column, $\alpha = 1$; if the protein is not adsorbed at all, $\alpha = 0$. When the protein is adsorbed partially ($0 < \alpha < 1$), it moves down the column and elutes when $1/(1-\alpha)$ column volumes of liquid have passed through.

A more efficient way to elute adsorbed proteins from the column is to change the composition of the buffer during the elution procedure, which causes differential changes in the partition coefficient of the adsorbed proteins.

3.2.1 Ion exchange chromatography

Proteins can be separated by ion exchange chromatography on the basis of differences in their net surface charges at a certain pH. The ion exchange stationary phase carries covalently bound electrically charged groups, usually diethylaminoethyl (DEAE)- or carboxymethyl (CM)-moieties, bound to cellulose, dextran or agarose beads. The DEAE-types are anion exchangers, carrying a weak positive charge, whereas the CM-types are cation exchangers, carrying a weak negative charge. Separation of proteins on these type of exchangers is based on the acid-base behaviour of proteins in solution. The acidic and basic groups in the proteins can be ionized: carboxyl-groups can carry a negative charge, amino groups a positive charge. The protein either carries an overall negative, positive or no charge, depending on the pH of

the buffer solution. The dissociation constant at neutral pH, K', reflects the ratio of the concentrations of the ionized and nonionized forms of the protein. Therefore, proteins can be considered as weak acids or weak bases. They will bind to ion exchange matrices with opposite charge. The charges on the column beads are counterbalanced by salt- and buffer ions, which can be displaced by protein molecules, which then become attached. Some cautionary remarks have to be made. Binding of the protein displaces counterions from the charged column and this event, if it happens rapidly, can result in a change of the ionic composition of the buffer in the column in front of the band of adsorbed proteins. To avoid these changes of pH and ionic strength, which disturb the adsorption and therefore the resolution of the chromatographic process, the applied protein concentration should not be too high.

The adsorption capacity of an ion exchanger depends partly on the molecular size of the proteins. Very large proteins are only bound to the surface of the ion exchange particles due to a size exclusion effect, resulting in lower binding capacity for this category of proteins. The capacity of the column material for proteins of a certain molecular mass in $mg.cm^{-3}$ shows an inverse linear relationship with the log of the molecular mass. Columns "saturated" with very large proteins are still capable of binding an appreciable amount of smaller protein molecules.

The processes that take place in a chromatographic column also necessitate appropriate buffering in a buffer with a pH close to its pKa. The pH of the microenvironment of an ion exchanger is not the same as the pH of the applied buffer, because protons can be repelled from, or attracted to the column matrix due to the Donnan potential (Scopes, 1982). As a result, in anion exchange matrices the pH is in general 1 unit higher and in cation exchange matrices 1 unit lower than in the buffer solution. With decreasing ionic strength the differences in pH will increase. This may have considerable consequences for the pH of the buffering system to be used, because this difference can result in denaturation and loss of biological activity of adsorbed proteins, especially under mild acidic conditions. Because most proteins have an IEP between pH 7 and pH 4 and because acidic

treatment of proteins often results in denaturation, proteins tend to be more stable at weak alkaline conditions. Anion exchangers, which normally operate between pH 7-10, usually do not result in denaturation.

Proteins can be desorbed from ion exchange matrices by two methods: a) by increasing the ionic strength of the elution buffer, thereby weakening the electrostatic interactions between protein and adsorbent, and b) by changing the pH of the elution buffer to a value where binding is less strong, for example a lower pH for anion exchangers and a higher pH for cation exchangers. Changing the ionic strength is the method of choice in conventional ion exchange chromatography. The effect of salts can be explained in two ways. One explanation is that an increasing gradient of salt ions can displace the protein from the column and prevent reattachment of the protein. The other explanation considers the system as in equilibrium: even strongly adsorbed proteins with a high α value will spend some time in solution. The salt ions weaken the attraction between protein in solution and adsorbent and so proteins will become unattached. Proteins then will be eluted at a fixed salt concentration (isocratic elution). As salt concentrations increase, the partition coefficient decreases. Protein bands will become sharpened because they pass slower through the column than the salts and thus will be subjected to a continuously increasing salt concentration. Therefore, the trailing edge of the protein band will speed up and eventually run very close to the leading edge of the band.

The second method of desorption of proteins from an ion exchange matrix is changing the pH of the elution buffer. This method is usually not very successful in conventional ion exchange techniques, because high capacity buffering systems are used in ion exchange chromatography in order to prevent sudden large pH changes as proteins become desorbed, resulting in minimal resolution. The development of the "chromatofocusing" technique by Sluyterman and coworkers (1978), employing anion exchange matrices, resolved these complications. In this technique a pH gradient is formed using a buffer of the ampholyte type with high buffering power, but low ionic strength. The charged groups on the anion exchanger are continuously titrated through a large pH

range within the column by the acidic elution buffer, resulting in a steady pH gradient emerging from the column at low ionic strength. Proteins elute at or slightly below their isoelectric points because of the effects caused by the Donnan potential (Sluyterman and Elgersma, 1978; Sluyterman and Wijdenes, 1978). The resolving power is reported to be very high: the optimum elution of each component of a protein mixture can be achieved within a pH range of 0.05 units.

However, not all proteins behave ideally and sometimes will be adsorbed on the "wrong side" of their IEP. "Sticky" proteins can be adsorbed to both types of exchangers in the same buffer and "slippery" proteins do not bind to any adsorbent at all within a stable pH range. These effects can be partly caused by non-electrostatic interactions, such as hydrophobic and van der Waals forces and partly by uneven distribution of surface charges on the protein molecules.

3.2.2 Affinity chromatography

Affinity chromatography is another form of adsorption chromatography. Two stages of affinity chromatography can be discerned: affinity elution and affinity adsorption.

Affinity elution involves the presence of a ligand in the elution buffer, which can reduce the partition coefficient α. It does not necessarily require any specific property of the adsorbent: affinity elution from e.g. ion exchangers has been used extensively to purify enzymes. In principal, the basic steps in affinity elution are the following: an enzyme is adsorbed to an ion exchanger at such a pH that the partition coefficient is close to or equals 1. Introduction of a ligand with a charge opposite to the net charge of the enzyme in the elution buffer and subsequent binding of the ligand decreases the net charge on the enzyme and lowers its electrostatic interaction with the adsorbent. This can result in specific elution of the enzyme. The mechanism of this process presumably involves the charged residues near the active site of the enzyme, which is likely to be at or near the surface of the molecule. These charges apparently

have a more pronounced influence than randomly distributed charges. Alternatively, binding of the ligand can introduce a conformational change, which can weaken the interactions with the adsorbent and results in elution. Because practically all ligands, with the exception of metal ions, are negatively charged, affinity elution is restricted to neutral or basic proteins that adsorb to a cation exchanger at neutral pH.

Affinity adsorption is the technique which is commonly referred to as affinity chromatography. The adsorbent has a specific affinity for the protein because of the specificity of its ligand binding properties. General features are that the ligand is covalently bound to the matrix in such a way that binding of a protein to the ligand is not disturbed. A "spacer arm" is required to bind the protein. Nonspecific interactions have to be minimal (see below). Examples of ligands that can be coupled include enzymes and enzyme substrate analogues, inhibitors and cofactors, lectins, nucleotides, hormones, vitamins, antigens and antibodies and even complete cells.

The degree of adsorption is dependent on the length and the structure of the spacer arm. However, access of the ligand to the protein is not the only factor involved in the process of binding. Nonspecific, hydrophobic interaction between protein and spacer arm also enhance binding of the protein to the ligand by weak electrostatic forces. A possible explanation for this phenomenon is that only a small percentage of the ligands have the correct spatial orientation to bind proteins properly. Additional nonspecific interactions could help to adsorb the protein and increase binding. These deviations from ideal behaviour can be exploited to improve specific separations (Scopes, 1982).

Elution of adsorbed proteins can be achieved by free ligand in the buffer, increase in ionic strength, disruption of binding by chaotropic agents, changing the pH, and decreasing temperature or ionic strength to decrease hydrophobic interactions. The best method is usually empirically determined and depends to a great extent on the type of interaction between ligand and protein.

3.2.3 Gel filtration chromatography

Not all proteins withstand the exchanges between liquid and solid phase, which occur during adsorption chromatography, without change of their molecular characteristics. A more gentle approach is offered by gel filtration in which proteins remain in solution and are separated according to their molecular size.

Gel filtration, also referred to as "gel permeation" or "size exclusion" chromatography, employs an open, cross-linked three dimensional molecular network, usually cross-linked dextran beads, cross-linked polyacrylamide beads, agarose gels, agarose gels with polyacrylamide cross-links or dextran with bisacrylamide cross-links. For gel permeation-HPLC silica-based beads are usually employed because of their greater rigidity. The pores within the beads are accessible to small molecules and inaccessible to the larger ones. The exclusion limit for the different kind of gel filtration matrices depends on the average pore size. The behaviour of molecules of a particular size can be related to the total column volume V_t and the void volume V_o (volume outside the beads) by the expression:

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

where V_e is the elution volume of the molecules and K_{av} the fraction of pores which are accesible for the molecules. The elution volume is not directly related to the molecular size of the molecule under investigation but depends, theoretically, on the Stokes radius, which describes a perfect sphere with a hydrodynamic behaviour equivalent to that of an irregularly shaped particle (Scopes, 1982).

Nonideal behaviour of proteins during gel filtration chromatography can be caused by several factors. Gel filtration materials are not completely inert, causing adsorption of proteins under specific conditions. This adsorption can have the characteristics of that observed with ion exchange matrices, which can be avoided by using high ionic strength buffers. Alternatively, this adsorption may display a hydrophobic character which can be avoided by using low ionic strength buffers. Some gel filtration materials,

like the Sephacryl types, contain aliphatic chains which enhance adsorption effects at low pH and high ionic strength. Therefore, gel filtration is usually performed at neutral pH and at moderate ionic strength. Of course, the nonideal behaviour of proteins on gel filtration matrices can be used to resolve certain separation problems (Kopaciewicz and Regnier, 1982; Regnier, 1982, 1983).

The actual resolution of proteins in a mixture during gel filtration depends on the extent of nonideal behaviour and diffusion in the column. The front of a protein band moving down a gel filtration column is usually wider than can be explained by diffusion alone, because of two complicating factors. Firstly, the more dense particles tend to run ahead of their theoretical position in the protein front due to gravitational effects. Secondly, turbulence in the column in which the buffer is squeezed around the gel beads causes additional spreading of the protein bands. Turbulent flow can be reduced by decreasing the flow rate, but at a very low flow rate molecular diffusion can result in larger band broadening than the turbulent flow effects observed at higher flow rates. Both nonideal behaviour effects can be decreased by using smaller beads, which results in disturbances over a shorter distance. Furthermore, with smaller beads equilibration is reached faster, so flow rates can be increased without losing equilibrium, but the backpressure of the system increases as well and may eventually result in distortion of the beads and blocking of the column. In HPLC small (5-10 µm) particles, which can withstand high pressure, are used. However, this technique can only be applied on an analytical scale with a few milligrams of protein. Larger amounts of protein reduce resolution because of the limited loading capacity of the columns and increase separation times because of the larger size of the columns (Scopes, 1982; Regnier, 1983). It should be remembered that an important factor in resolution is the column volume and the volume of protein applied to the column. With spherical beads V_o equals 30-35 % of V_t. The useful fractionation range lies within about 80 % of the remaining volume ($V_t - V_o$), i.e. about 55 % of V_t. Optimal resolution is obtained when the volume of applied protein sample equals 1-3 % of the column volume V_t. Below 1 % of V_t diffusion and nonideal behaviour will result in

spreading of protein peaks. Usually the first peaks eluted from the column will be sharp because of short retention times, whereas later peaks will tend to trail and spread because of diffusion effects. The choice of the fractionation range of the gel filtration material depends to a great extent on the characteristics of the proteins to be separated. As mentioned before, finer, more rigid materials allow faster separation under pressure. Moderate pressure techniques, as represented by FPLC, might fill the gap between low pressure separation and high pressure HPLC-techniques.

3.3 PURIFICATION AND MOLECULAR CHARACTERIZATION OF OVARIAN INHIBIN

3.3.1 Purification procedures

Inhibins from bFF and hFF have been partially purified by a series of chromatographic procedures, which are summarized in Fig. 3.1. The materials and methods used in the purification procedures have been described elsewhere (Appendix papers 1 and 2; van Dijk et al., 1985b). Technical details of unpublished experiments will be given in the legends to the figures. Results of the experiments described in Appendix papers 1 and 2 will be briefly dealt with here, whereas results obtained from unpublished experiments (e.g. gel filtration and preparative gel electrophoresis) will be discussed in greater detail.

Follicular fluid was chromatographed on immobilized Procion Red 3B and inhibin activity was eluted with 1.2 M KCl (Jansen et al., 1981; van Dijk et al., 1984, 1985a,b). After the subsequent desalting step on Sephadex G-25 the purification factor or relative specific activity (RSA) was approximately 20-30 with a recovery of 100 % (bFF) and 70 % (hFF) (Table 3.1). Anion exchange chromatography of follicular fluid on the FPLC Mono Q column at pH 7.9 resulted in RSAs of 50 - 100 (bFF; n = 20) and 15 - 50 (hFF; n = 5). Fractionation on the chromatofocusing column Mono P with a pH gradient from pH 7.1 to pH 4.0 resulted in RSAs of 18 - 45 (bFF; n = 3) and 50 - 175 (hFF; n = 16).

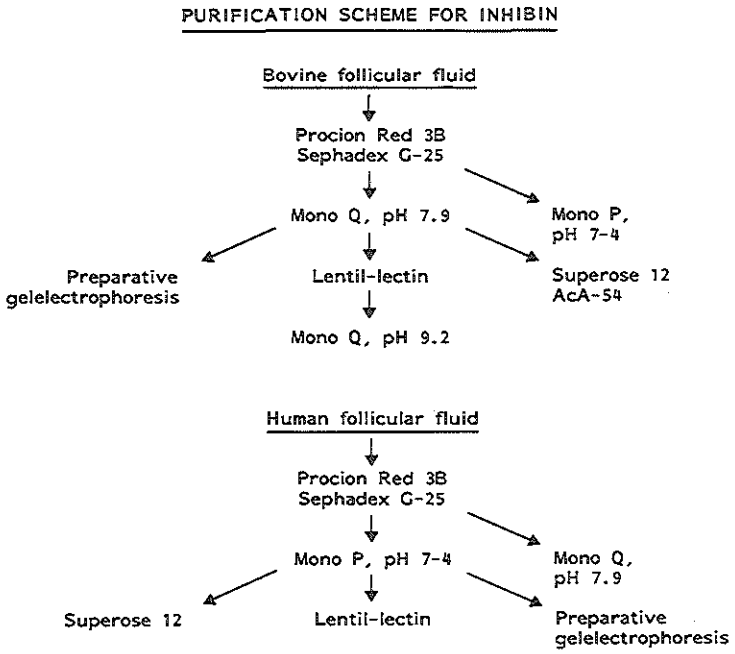


Figure 3.1

Summary of steps used in the purification of inhibin from bovine and human follicular fluid.

Recovery of bovine inhibin activity was somewhat higher when compared with human inhibin using the chromatofocusing procedure (Table 3.1). Inhibin activity from bFF as well as hFF was retained on immobilized Lentil lectin and Concanavalin A. The activity could be eluted with methyl-α-D-mannopyranoside, resulting in purification factors of 330 for bFF and 480 for hFF. Part of the inhibin activity from hFF did not bind to Concanavalin A and was recovered in the nonretained fraction with a RSA of approximately 720 in the rat anterior pituitary cell assay. Reduction of FSH levels might have been caused by Concanavalin A that was not bound to the column matrix and was washed from the column. When these preparations with Concanavalin A were tested for inhibin activity, Concanavalin A might reduce FSH release from pituitary cells as shown by Ponsin et al., 1981. Alternatively, separation of inhibin activity and FSH-releasing factors (see Chapter 5.6) could result in higher specific activities in

inhibin-containing fractions and increase FSH release in other fractions. A final chromatographic purification step for bovine inhibin on the anion exchange column Mono Q at pH 9.2 resulted in a purification factor of approximately 425-870 in the most active fractions with a total recovery of 0.75 % (Table 3.1).

3.3.2 Chromatofocusing

Chromatofocusing of the Procion Red 3B fraction of hFF on Mono P with a pH gradient from pH 6.3 to pH 3.5 yielded preparations with RSA between 50-175, which could be eluted between pH 5.6 and pH 5.0 (Fig. 3.2). Results of separation of the proteins present in these fractions on SDS-polyacrylamide (SDS-PAA) gels indicated that a 68 kDA protein was always present in the fractions with the highest RSA (Fig. 3.2, lower panel), of which the relative abundance was decreased when the RSA of the column

TABLE 3.1: Relative specific activities (RSA) and recoveries of inhibin from bovine and human follicular fluid after various chromatographic procedures.

	bovine follicular fluid		human follicular fluid	
Purification step	RSA	Recovery (%)	RSA	Recovery (%)
Follicular fluid	1	100	1	100
Procion Red 3B + Sephadex G-25	30	100	10-30	60-75
Mono Q, pH 7.9	50-100	11-15	15-50	18-27
Mono P, pH 7 - 4	18-45	34	50-175	20-25
Lentil lectin	330	2	-	-
Concanavalin A	-	-	480 720	2.5[a] 10 [b]
Mono Q, pH 9.2	425-870	0.75	-	-

[a] Retained fraction, eluted by methyl-α-D-mannopyranoside
[b] Eluted fraction, not bound to Concanavalin A
Data from van Dijk et al. (1985a,b).

fractions became lower and which was absent in non-active fractions. No other proteins with the same distribution pattern were observed. These observations suggest that human inhibin activity was associated with a protein with a molecular mass of approximately 68 kDa and an IEP between pH 5.1 and pH 5.6 (Fig. 3.2 and Appendix paper 2).

3.3.3 Gel filtration chromatography

An estimation of the molecular weight of human and bovine inhibin has been made by gel filtration chromatography on Ultrogel AcA-54 (fractionation range 5-70 kDa, exclusion limit 90 kDa; LKB, Bromma, Sweden) and on Superose-12 (fractionation range 1-300 kDa, exclusion limit 2,000 kDa; Pharmacia, Uppsala, Sweden). When inhibin-containing fractions from bFF obtained after ion exchange chromatography on Mono Q at pH 7.9 were chromatographed on the AcA-54 column inhibin activity was recovered in the first protein peak (Fig. 3.3a) with a RSA of approximately 10-15. After analysis of the three peaks eluted from the column on SDS-PAA gels, the protein patterns of the fractions showed a remarkable similarity (Fig. 3.3b): in all three fractions proteins of approximately 50-55 kDa and 25-30 kDa were present and both fraction A and C contained proteins in the 65-70 kDa range, which were not present in fraction B. Only fraction A contained biological activity in the pituitary cell assay. It seemed that proteins were separated by a mechanism different from that based on molecular size.

Inhibin activity from hFF was fractionated on Superose-12. Biologically active fractions were eluted in the molecular weight range between the bovine serum albumin (BSA; 68 kDa) and ovalbumin (43 kDa) marker proteins (Fig. 3.4a). Inhibin activity from bFF (RSA 20-50) was eluted in the same fractions using this column (Fig. 3.4b). SDS-PAGE of human inhibin-containing fractions from the Superose-12 column showed that one particular fraction with a RSA of approximately 250 contained a single 68 kDa protein (Fig. 3.5, lane B). After reduction with β-mercaptoethanol protein bands were observed only in the molecular mass range <15 kDa (data not shown).

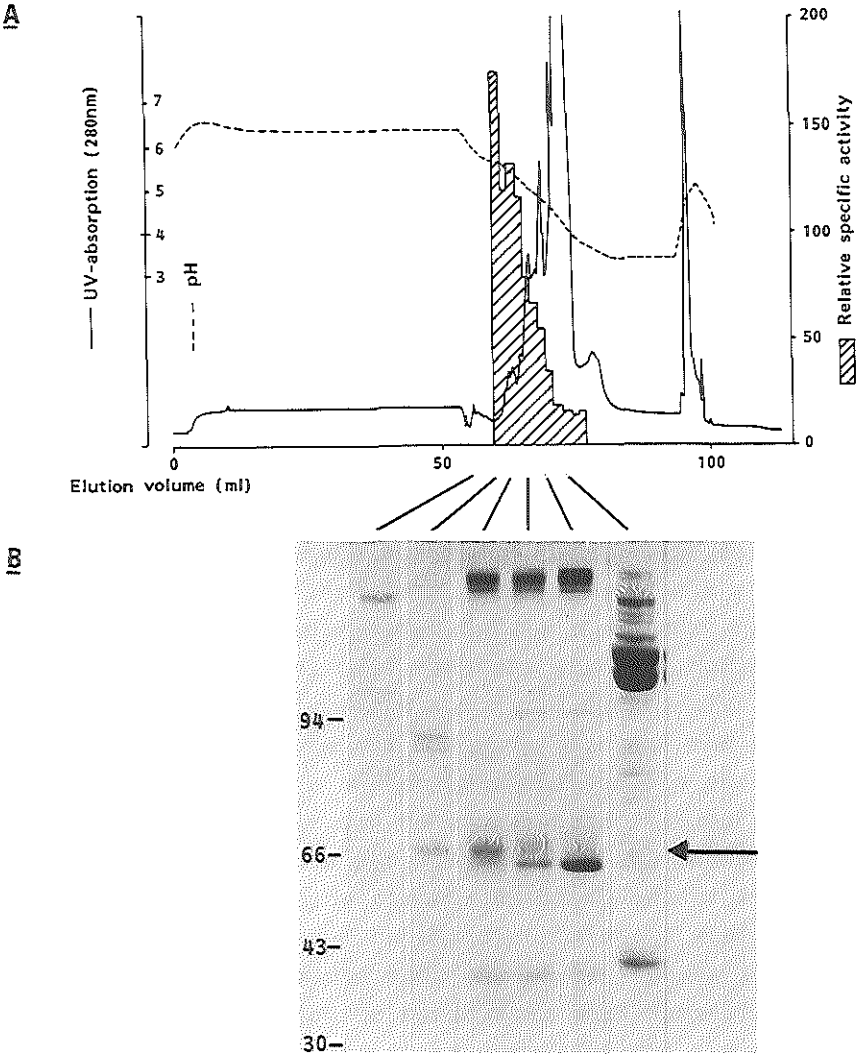
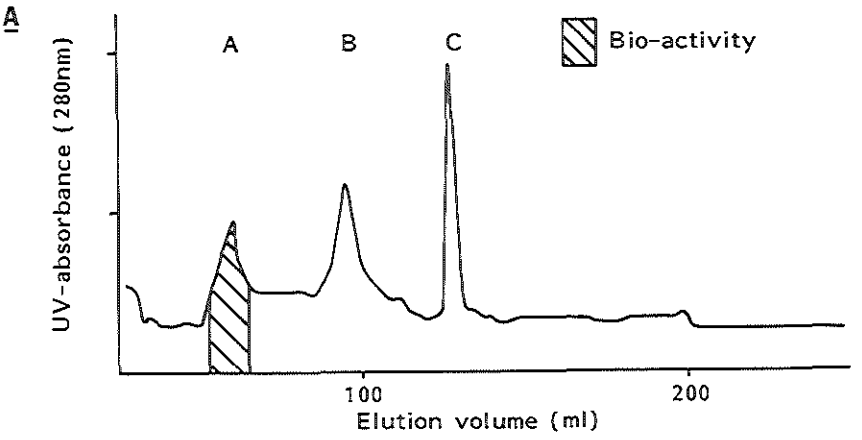


Figure 3.2

A) Protein pattern after chromatofocusing of an inhibin-containing fraction of hFF on a Mono P column. Proteins were eluted by applying a pH gradient from pH 6.3 to pH 3.5. The starting material was obtained after chromatography on immobilized Procion Red 3B and Sephadex G-25. The column was equilibrated in 20 mM Bistris, pH 6.3, and eluted with 10% (v/v) Polybuffer 74, pH 3.5.
B) Proteins in the indicated fractions (panel A) were electrophoretically separated on SDS-polyacrylamide gels (according to Laemmli, 1970) under nonreducing conditions and stained with Coomassie Brilliant Blue (0.1% (w/v) in 10% (v/v) acetic acid, 45% (v/v) methanol, 45% (v/v) H_2O). Positions of molecular weight markers are indicated; the arrow indicates the position of the 68 kDa protein.



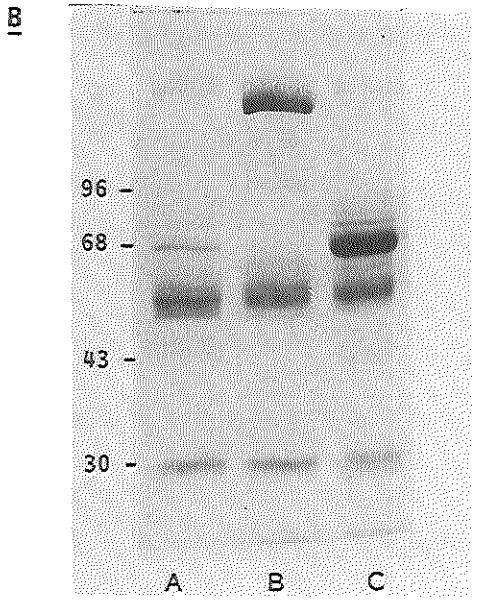


Figure 3.3

A) Protein pattern after gel filtration chromatograpy of an inhibin-containing fraction of bFF on AcA-54 (column size 50 x 1.6 cm). The starting material was obtained after chromatography of bFF on immobilized Procion Red 3B, Sephadex G-25 and Mono Q at pH 7.9. Proteins were eluted with 20 mM ethanolamine, pH 9.2, containing 4 M urea.
B) SDS-polyacrylamide gel electrophoresis (according to Laemmli, 1970) of the peak fractions from the AcA-54 column. Proteins were stained with Coomassie Brilliant Blue (0.1%(w/v) in 10% (v/v) acetic acid, 45% (v/v) methanol, 45% (v/v) H_2O). The positions of the molecular weight markers are indicated.

3.3.4 Immunoaffinity chromatography of bovine inhibin

Antisera, raised in sheep against bovine inhibin (see Appendix paper 3), were chromatographed in phosphate buffered saline (PBS), pH 7.0, on a column of an immobilized fraction of plasma proteins obtained from an ovariectomized cow. The nonretained fraction, which contained the antibodies, was subsequently adsorbed to immobilized rabbit-anti-sheep immunoglobulins, covalently cross-linked to Protein A Sepharose by dimethyl pimelimidate (Schneider et al, 1982). The presence of antibodies in the eluted fractions was confirmed by the Ouchterlony double immunodiffusion technique. The bound ovine immunoglobulins directed against bovine inhibin were either coupled to a substituted Protein A Sepharose column (Schneider et al, 1982) or eluted with 50 mM diethylamine, pH 11.5, and subsequently immobilized on CNBr-

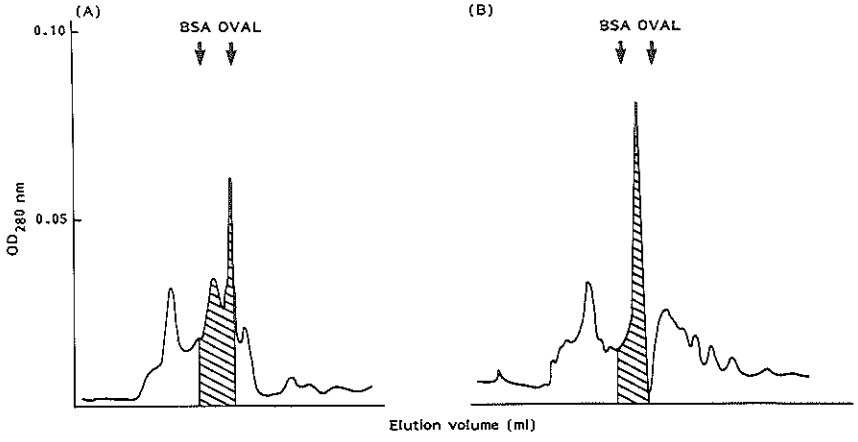


Figure 3.4

Protein patterns after chromatography of inhibin-containing protein preparations on a Superose-12 gel filtration column.
A) Fraction of hFF obtained after chromatography on immobilized Procion Red 3B, Sephadex G-25 and chromatofocusing on Mono P as described for Figure 3.2.
B) Fraction of bFF obtained after chromatography on immobilized Procion Red 3B, Sephadex G-25 and Mono Q at pH 7.9. Proteins were eluted in 25 mM Tris-HCl, pH 7.9.
The elution positions of BSA and ovalbumin have been indicated.
Hatced area: inhibin activity.

activated Sepharose according to directions of the manufacturer (Pharmacia, 1979). The former method usually produced immunoaffinity matrices with a higher efficiency (Schneider et al., 1982).

Inhibin-containing fractions obtained from the Mono Q anion exchange column at pH 7.9 were fractionated on this column. The column was eluted by a stepwise increase of the pH of the elution buffer and the inhibin activity was detected in the retained fraction. The retained fractions contained a protein of 66-68 kDa (Fig. 3.5, lane A) with an RSA of approximately 22.5. In addition high molecular weight material was found under nonreducing conditions, which might have contributed to the low specific activity in the pituitary cell assay. In the presence of β-mercaptoethanol

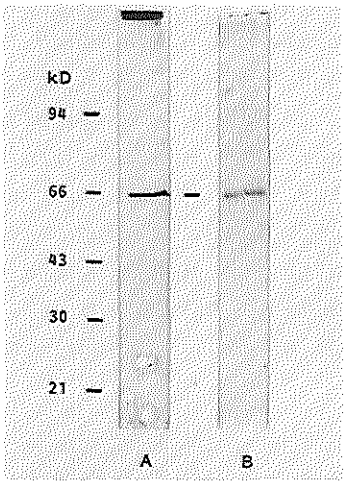


Figure 3.5

SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) of purified inhibin preparations fom bFF (A) and hFF (B).
Preparation A was obtained after immunoaffinity chromatography on immobilized antibodies, raised in sheep against bovine inhibin, and eluted with 50 mM diethylamine, pH 11.5.
Preparation B was obtained after gel filtration on Superose-12. See text for details. Proteins were made visible by silver staining according to Wray et al. (1981). The positions of molecular weight markers are indicated.

a cluster of protein bands between 65 and 70 kDa was observed, while the high molecular weight material was no longer observed (data not shown).

3.3.5 Preparative gel electrophoresis

Inhibin-containing fractions from bFF and hFF were fractionated using preparative gel electrophoresis on 3 mm thick SDS-PAA gels (see Fig. 3.1). After electrophoresis gels were cut in 2.5 mm slices and proteins were recovered from these slices by electrophoresis (Strålfors and Belfrage, 1983; Appendix paper 1) or by diffusion. The bioactive fractions from hFF, obtained after chromatofocusing (cf. Fig. 3.2), were pooled, loaded onto the preparative SDS-PAA gel, separated electrophoretically and washed out of the gel slices as described in the legend to Fig. 3.6. Inhibin activity was recovered in the 30-32 kDa molecular weight range of the gel (Fig. 3.6). These findings were surprising in view of our earlier experiments (see also sections 3.3.6 and 3.5) which indicated inhibin activity from bFF as well as hFF to be associated with a 68 kDa glycoprotein. On the other hand, this observation is in agreement with the observations of Miyamoto et al., (1985), Ling et al., (1985), Rivier et al., (1985), Fukuda et al., (1986) and Robertson et al., (1986b) on bovine and porcine inhibin.

3.3.6 Proteolytic activity in follicular fluid

The comparison of our results with data in the literature suggested that the 30-32 kDa protein could be derived from the 68 kDa protein by proteolysis. Using a procedure to detect proteases by digestion of a nonspecific substrate after electrophoresis (Heussen and Dowdle, 1980; Channing et al., 1985b) proteolytic activity was detected in bFF, hFF (data not shown; cf. Beers, 1975), and in fractions obtained after chromatofocusing of hFF (Fig. 3.7; details of the experiment are given in the legend). The lighter bands on the dark background, which can be made visible

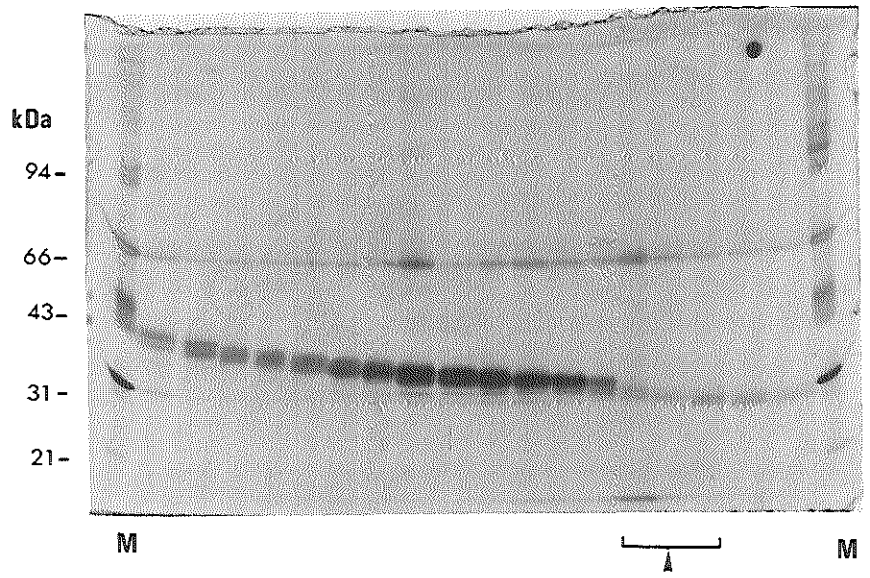


Figure 3.6

SDS-polyacrylamide gel electrophoresis of proteins from an inhibin-containing fraction of hFF obtained after chromatofocusing on Mono P, followed by SDS-polyacrylamide gel electrophoresis on a 3 mm thick preparative gel. After electrophoresis gels were cut in 2.5 mm slices and proteins were washed out of the gel by diffusion in 25 mM Tris-HCl, pH 7.5. Electrophoresis was performed according to Laemmli (1970) without β-mercaptoethanol. Fractions with inhibin activity have been indicated by the arrow. The positions of molecular weight markers proteins (M) are indicated. Proteins were stained with silver according to Wray et al. (1981).

after electrophoresis and staining for undigested substrate, indicate the presence of proteolytic enzymes. Because putative protease inhibitors could have been removed during electrophoresis we also looked for proteolytic activity in the column fractions after diffusion in agarose containing the substrate gelatin (Fig. 3.8). Proteolytic activity was found as a lighter ring around the wells. The results of these experiments indicate that active proteases are present in the inhibin preparations, obtained by chromatofocusing. The action of these proteases might generate a 30-32 kDa protein from the 68 kDa protein, which still possesses inhibin activity.

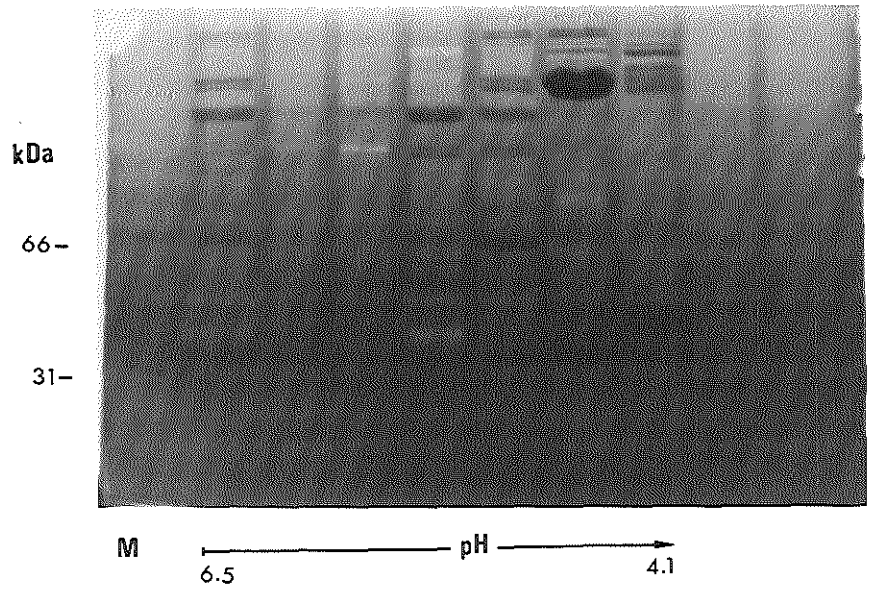


Figure 3.7

Detection of proteases in inhibin-containing fractions of hFF obtained after chromatofocusing on Mono P. After electrophoresis in SDS-PAA gels containing 0.1% (w/v) gelatin, SDS was removed by washing with 2.5% (v/v) Triton X-100 in water (1 h at 22 °C). Subsequently, gels were incubated at 37 °C in 0.1 M Glycine-NaOH, pH 8.3, for 5 h. Gels were stained with Amido Black (0.1% (w/v) in 10% (v/v) acetic acid, 30% (v/v) methanol, 60% (v/v) H_2O) and destained. Proteases appear as light bands on a coloured background.
The positions of molecular weight markers are indicated.

3.4 COMPARISON OF SPECIFIC ACTIVITIES OF INHIBIN FROM SEVERAL SPECIES

The specific activity of hFF is approximately 100-fold lower than that of a standard bFF preparation (see Appendix paper 2). The low level of inhibin activity in hFF may be a result of either an intrinsically low inhibin concentration or a reduced biological effect of human inhibin in the rat pituitary cell bioassay system due to species differences. Human follicular fluid was collected during oocyte aspiration as part of an *in vitro* fertilization programme. Because patients were stimulated with hMG and hCG,

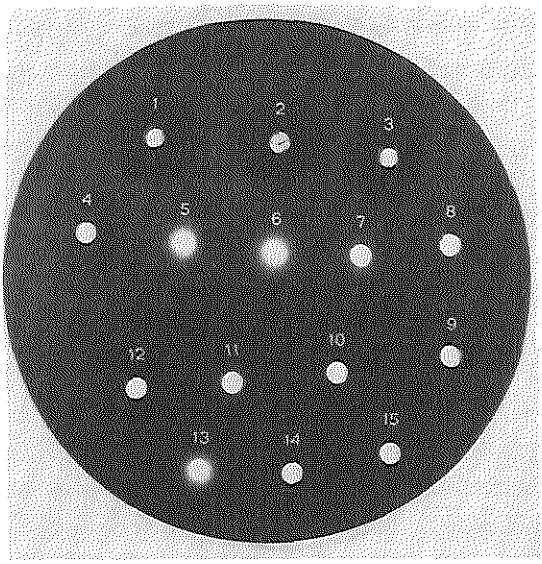


Figure 3.8

Detection of proteolytic activity in inhibin-containing preparations. Preparations were allowed to diffuse into agarose (1% (w/v) containing 0.1% (w/v) gelatin) at 37 °C for 8 h. The agarose was stained and destained as described in the legend to Figure 3.7. Proteolytic activity appears as a light diffuse area around the wells in the agarose. Preparations are: 1) bFF; 2) bovine plasma; 3) hFF; 4) 2% ovalbumin; 5–11) Subsequent fractions of hFF, eluting from the chromatofocusing column between pH 6.5 and pH 4.1; 12) 1% BSA; 13) 0.1% pepsin in HCl; 14, 15) empty wells as controls.

inhibin levels might have been influenced by this treatment. Channing et al. (1984e) demonstrated increased inhibin activity in the follicular fluid of normal women, who were treated with hMG/hCG, which would support the reduced biological activity of hFF in the bioassay as a result of species differences rather than a low concentration of inhibin in hFF.

To compare the purification factors which we have obtained with those described in other reports it is not sufficient to consider the purification factors as such. It is also important to examine the specific activity of the starting material. Unfortunately, these quantitative comparisons have rarely been made. In the present study specific activities of inhibin-containing preparations were expressed in relation to a standard bFF prepa-

ration (de Jong et al., 1979a) using parallel line statistics (Finney, 1964). Robertson et al. (1985, 1986b) and McLachlan et al. (1986) expressed their results, using an *in vitro* bioassay based on the reduction of FSH content in pituitary cells, in units/mg protein determined in terms of a standard ovine testicular lymph protein (oTLP) preparation, which was arbitrarily given the potency of 1 unit per mg protein (Eddie et al., 1979; Scott et al., 1980). Our standard bFF preparation (de Jong et al., 1979a, 1981) has been compared with this standard oTLP preparation in an inhibin bioassay in which the bFF standard had an activity of 320 oTLP units/mg protein (de Jong et al., 1981). This is 2.4 to 5.1 times more potent than the starting material used by Robertson et al. (1985, 1986b) and McLachlan et al. (1986) (cf. Table 3.2), although differences between the assay systems may account in part for the observed differences in inhibin activity. Miyamoto et al. (1985) and Fukuda et al. (1986) expressed their results as ED_{50} values (dose to achieve half-maximal suppression of FSH release from cultured pituitary cells) for bFF and pFF (Table 3.2). An approximation of ED_{50} values, derived from suppression of FSH release in a pituitary cell

TABLE 3.2: Comparison of specific activities of bFF, hFF and pFF between laboratories using different units to express specific activity.

Source	oTLP-units* U/mg	U/ml	ED_{50}** (µg/ml)	References
bFF	63	4095		Robertson et al., 1985
	(80)	5200		McLachlan et al., 1986
	135	9060		Robertson et al., 1986b
	320	20900***	2.65***	de Jong et al., 1981
			6.4	Fukuda et al., 1986
hFF		168		McLachlan et al., 1986
		approx. 205***		
pFF			6.8	Miyamoto et al., 1985

* oTLP-units are defined as the activity of 1 mg ovine testicular lymph protein (Eddie et al., 1979; Scott et al., 1980).

** ED_{50}: dose to achieve half-maximal suppression of FSH release from cultured pituitary cells.

*** Calculated as described in the text.

culture by the Rotterdam standard bFF preparation gives 2.65 ± 0.07 µg protein/ml (n = 15, SEM), which is 2.4-2.6 times as potent as the follicular fluids employed by Miyamoto et al. (1985) and Fukuda et al. (1986). To compare purification factors between laboratories these differences must to be taken into account.

3.5 DISCUSSION

In this chapter the partial purification of inhibin from bFF and hFF was described as well as some physico-chemical characteristics of inhibin from both sources (see also Appendix papers 1 and 2). The characteristics of bovine and human inhibin, as derived from our experiments, described in this chapter and in Appendix papers 1 and 2 are summarized in Table 3.3.

Comparison of the purification procedures used for bovine and human inhibin indicates that the two molecules probably have a similar structure. Inhibin activity from bFF and hFF bound to Procion Red 3B (Appendix paper 2) and could be eluted with a high salt concentration. It has been reported (Huang and Miller, 1984; Miyamoto et al., 1985), that inhibin from pFF also binds to Procion Red 3B, but purification factors were lower with recovery of inhibin activity of approximately 80-90 % (Miyamoto et al., 1985; Fukuda et al., 1986). The lower recovery might be due to loss of inhibin in the elution procedure.

Inhibin activity from bFF and hFF was eluted from the anion exchange column Mono Q at pH 7.9 between 0.21 and 0.26 M NaCl (Appendix paper 2). Using anion exchange FPLC in the purification of inhibin from pFF, Sairam et al. (1984) obtained several bioactive fractions between 0.1 and 0.2 M NaCl in 25 mM Tris, pH 7.5, whereas ion exchange chromatography on DEAE-Sepharose of bFF and pFF resulted in elution of inhibin activity between 0.15 and 0.20 M NaCl at pH 7.5 (Miyamoto et al., 1985; Fukuda et al., 1986). These elution conditions do not differ very much from the conditions that have been established for bovine and human inhibin. The small differences in ionic strength when eluting bovine and human inhibin from the Mono Q column (Appendix paper 2) could

TABLE 3.3: Molecular characteristics of bovine and human ovarian inhibin

	bovine inhibin	human inhibin
Molecular mass	65-68 kDa	65-68 kDa
Bioactive fragment	n.d	30-32 kDa
Isoelectric point	4.9-5.3	5.1-5.6
Glycoprotein	+	+
Relative specific activity*	1	0.009-0.011

* Relative specific activity compared to a standard bFF preparation in the rat anterior pituitary cell culture as described by de Jong et al. (1979a) and Hermans et al. (1982b).
n.d. not determined.

still represent differences in the net charge of the respective molecules. When the chromatographic behaviour of bovine and human inhibin activity on the chromatofocusing column is compared (pI for bovine inhibin between pH 4.9 and pH 5.3, pI for human inhibin between pH 5.1 and pH 5.5; Appendix paper 2), differences in charge become more evident. The differences in elution pH, and consequently in apparent pI, are most probably the result of differences in amino acid sequence or degree of glycosylation. The recently published nucleotide sequences and derived amino acid sequences of bovine and human ovarian inhibin (Forage et al., 1986; Mason et al., 1986) indeed show differences between species. These data will be discussed in more detail in Chapter 5.

The reported differences in molecular weight of inhibin isolated from bFF, hFF and pFF are intriguing. Sairam et al. (1984) estimated a molecular mass of 40 kDa for inhibin from pFF using gel filtration chromatography, whereas Miyamoto et al. (1985) and Fukuda et al. (1986) noted molecular masses of approximately 32 kDa for porcine inhibin and for bovine inhibin, respectively. Our own results (Appendix paper 1) confirmed the results of Jansen et al. (1981) and showed that inhibin activity from bFF is associated with a 68 kDa glycoprotein, whether purified by anion exchange chromatography at high pH, by elution from SDS-PAA gel slices (Appendix paper 1) or by immunoaffinity chromatography (Fig. 3.5). Results obtained with gel filtration indicated a

similar molecular mass (Fig. 3.4b).

Initially, studies on the purification of inhibin from hFF indicated a molecular mass of approximately 68 kDa, as assessed by SDS-PAGE analysis of fractions obtained by chromatofocusing (Fig. 3.2) and by gel filtration (Fig. 3.5, lane B). When, however, in a different set of experiments fractions containing inhibin activity from a chromatofocusing run were pooled and subsequently separated by SDS-PAGE and eluted from the gels, inhibin activity could be attributed mainly to a 30-32 kDa protein (Fig. 3.6). The latter observation is in accordance with the observations of Ling et al. (1985), Miyamoto et al. (1985), Rivier et al. (1985), Fukuda et al. (1986) and Robertson et al. (1986b) for porcine and bovine ovarian inhibin. A common feature in these studies was, that all authors used purification conditions of low pH to obtain their 32 kDa protein. These conditions may also be present during the elution of the chromatofocusing column using a decreasing pH gradient. Proteases, which are present in follicular fluid preparations (Fig. 3.7) and can be activated by acidic purification conditions (Beers, 1975), might have cleaved the 68 kDa (pro)-hormone to a 32 kDa active fragment. The presence of active proteases has been confirmed by the results summarized in Figs. 3.7 and 3.8. The observation of Hermans et al. (1980) that pronase, but not trypsin destroys inhibin activity in bFF, would suggest the presence of specific protease inhibitors in follicular fluid but could also be an indication for processing of a larger inhibin precursor molecule. This hypothesis will be discussed in more detail in Chapter 5 in conjunction with recently published data on nucleotide sequence and predicted amino acid sequence of ovarian inhibin.

3.6 CONCLUSIONS

1) Inhibin activity from bFF and hFF can be partly purified using a purification sequence involving chromatography on immobilized Procion Red 3B and Sephadex G-25, FPLC anion exchange and gel filtration chromatography and chromatofocusing, affinity chromatography on immobilized lectins and immunoglobulins

and preparative SDS-PAA gel electrophoresis, resulting in 500-800 fold purified inhibin preparations.

2) Inhibins from bFF and hFF are characterized as glycoproteins with apparent molecular masses of 65-68 kDa. At low pH during chromatofocusing inhibin activity from hFF was associated with a 30-32 kDa protein, presumably caused by proteolytic processing. These observations indicate that inhibin apparently exists as a larger precursor molecule of 65-68 kDa with a smaller biologically active form of 30-32 kDa.
3) The isoelectric point of inhibin from bovine and human follicular fluid, as assessed by chromatofocusing of partially purified preparations, lies in between pH 4.9 and pH 5.3 (bovine inhibin) and pH 5.1 and pH 5.6 (human inhibin). Differences in amino acid sequences and the degree of glycosylation of human and bovine inhibin could account for the differences in pI.
4) The main problem in the purification of inhibin was the low recovery after each step, probably due to nonspecific interactions with the column materials and denaturation of inhibin with concomitant loss of biological activity.
5) The relative specific activity of crude hFF is approximately 100-fold less than the activity of a standard bFF preparation in the rat pituitary cell assay. No indications were obtained that this difference is due to a 100-fold lower concentration of inhibin in hFF compared to bFF. The correlation between purification factors and protein composition of bioactive fractions, determined by SDS-PAGE, indicated that species differences are more likely.

CHAPTER 4

IMMUNOLOGICAL CHARACTERIZATION OF INHIBIN

4.1 INTRODUCTION

Immunological methods are among the most frequently used techniques in biochemistry and related fields, whether used as a detection method in different kinds of immunoassays or as a means of purification and characterization of proteins and peptides.

The development and use of polyclonal antisera to inhibin has been described by several authors. Channing et al. (1982b) raised an antiserum to pFF in monkeys, which neutralized inhibin activity in hFF and pFF *in vivo* as well as *in vitro*. Williams et al. (1979) used an antiserum raised in sheep against barrow plasma in a purification procedure for inhibin from pFF. McLachlan et al. (1986) and Robertson et al. (1986b) reported the development of a radioimmunoassay for inhibin from bFF which showed cross-reactivity with inhibin activity in hFF and human serum. The antiserum neutralized the biological activity of inhibin-containing preparations from gonadal fluids of cows, humans, rats and sheep (McLachlan et al., 1986). Lee et al. (1986) reported the production of monoclonal antibodies directed against rat ovarian inhibin, that cross-reacted with inhibin activity from bovine, ovine, porcine and rat follicular fluid. Monoclonal antibodies against bovine inhibin were raised by Miyamoto et al. (1986).

In our own laboratory polyclonal antisera have been used in: 1) studies on purification of inhibin (de Jong et al., 1982a,b, 1983a,b, 1984), 2) immunoneutralization of inhibin activity from gonadal tissues and fluids of various species (de Jong et al, 1982a,b; van Dijk et al., 1986), 3) a radioimmunoassay for inhibin (de Jong et al., 1983b), and 4) the characterization of inhibin preparations obtained during purification (de Jong et al., 1984). Part of these studies has been published elsewhere (de Jong et al., 1984). The studies on the radioimmunological characterization of inhibin will be described in this chapter (see also Appendix paper 3).

4.2 MATERIALS AND METHODS

ANTISERA. Antisera were raised in rabbits against an approximately 80-fold purified inhibin-containing fraction from bFF as described by Jansen et al., (1981). An antiserum against a fraction of plasma from an ovariectomized cow (bPcas), which was obtained after chromatography on immobilized Procion Red 3B, was raised in rabbits as described by de Jong et al. (1982b). Inhibin antisera were incubated overnight at 4°C with bPcas and precipitated by centrifugation (15 min at 2270 x g). The supernatants were used in the subsequent experiments.

Antisera against a bovine inhibin-containing fraction were also raised in ovariectomized ewes as described in Appendix paper 3.

RADIOLABELLING. Inhibin containing preparations were radiolabelled with ^{125}iodine using the chloramine-T method (Greenwood et al., 1963) or the iodogen method (Fraker and Speck, 1978).

RADIOIMMUNOASSAY (RIA) OF INHIBIN. An inhibin-containing preparation, obtained from bFF after chromatography on immobilized Procion Red 3B, Sephadex G-25, immunoaffinity chromatography on a column of immobilized antibodies, raised against the Procion Red fraction of bPcas, followed by chromatography on a column of immobilized antibodies, raised in rabbits against bovine inhibin (de Jong et al., 1982a,b), was radioiodinated with chloramine-T. The labelled proteins were further purified on Lentil lectin Sepharose. The retained fraction, which was eluted with methyl-α-D-mannopyranoside, was used in a double-antibody RIA, as described by de Jong et al. (1982a,b, 1983a,b), employing the preadsorbed rabbit antiserum against bovine inhibin and a donkey antiserum against rabbit immunoglobulins.

STAPHYLOCOCCUS AUREUS RADIOIMMUNOASSAY (SaRIA). In the radioimmunoassay, heat-killed and fixed Staphylococcus aureus Cowan I was used as immunoprecipitating agent. S. aureus (donated by Dr. G.H.Vos, Biochemical Laboratories, University of Groningen, the Netherlands) was cultured, harvested and prepared as a bacterial immunoadsorbent essentially as described by Kessler (1975) and Goding (1978). The radioiodinated proteins were incubated overnight, essentially as described by Kessler (1975), in phosphate

buffered saline (PBS), pH 7.0, containing 0.5 % (v/v) Nonidet P-40 (Sigma, St. Louis, MO), with the antisera raised against inhibin or bPcas and various amounts of bFF or bPcas at 22°C. Immune complexes were incubated with the S. aureus immunoadsorbent in the above buffer for 1.5 h at room temperature, precipitated by centrifugation for 5 min at 2270 x g, washed and counted. Bacterial pellets were dissolved in SDS-PAGE sample buffer and proteins were separated on SDS-PAA gels (Laemmli, 1970). Subsequently, gels were cut in 2 mm slices and radioactivity in these slices was counted.

4.3 RADIOIMMUNOLOGICAL CHARACTERIZATION OF BOVINE INHIBIN

4.3.1 Radiolabelling

Several inhibin-containing preparations obtained during the purifications described in Chapter 3 were radioiodinated and tested for their ability to bind to the rabbit antiserum against inhibin. Differences were observed between preparations iodinated with chloramine-T or iodogen. In both procedures the incorporation of iodine was approximately 20-30 %, but the binding efficiency of the iodogen-labelled preparations was approximately 50-70 % higher than for the chloramine-T labelled preparations (data not shown). The same observations were made for radioiodinated FSH and LH. This confirms the observation that considerable destruction of protein may occur with the chloramine-T method and that the iodogen method is a milder way of labelling proteins with ^{125}iodine (Fraker and Speck, 1978; Salacinski et al., 1981).

4.3.2 Radioimmunoassay

Specificity of binding of partially purified bovine inhibin containing preparations to the inhibin antiserum in rabbits was studied by incubation of the radioiodinated inhibin-containing preparation with the antiserum and bFF or bPcas. Bovine follicular fluid displaced the labelled proteins from the antiserum in a

dose-dependent manner, whereas bPcas did not displace the radiolabel (Fig. 4.1). It was concluded that the radiolabel was displaced by ovary specific proteins in the follicular fluid. Several inhibin-containing preparations were tested in the inhibin bioassay and in the immunoassay system. Comparison of relative specific bioactivities with radioimmunological results indicated a good correlation between bio- and immunopotencies (Fig. 4.2; de Jong et al., 1983b).

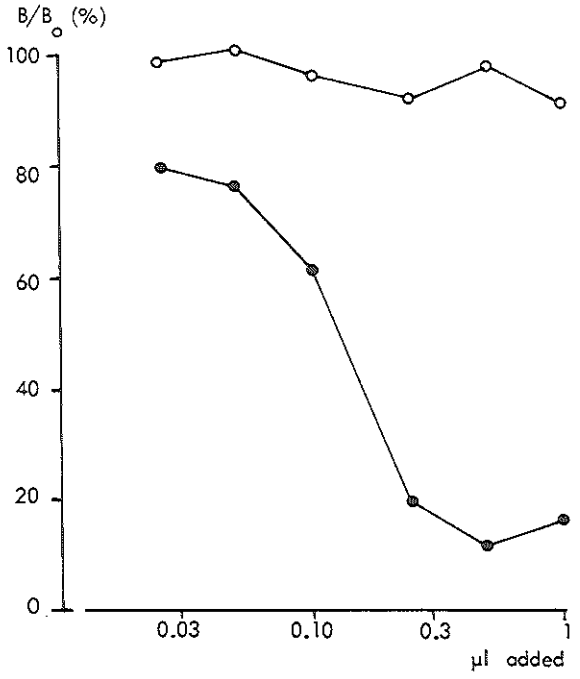


Figure 4.1

Displacement of ^{125}I-bovine inhibin activity from bovine plasma (preadsorbed with rabbit antibodies against bovine inhibin) expressed as the percentage bound radioactivity in the absence (B_0) or in the presence (B) of bFF (●) or bovine castrate plasma (○). See text for details on purification and iodination of the antigen and the radioimmunoassay.
(From: de Jong et al., 1983b).

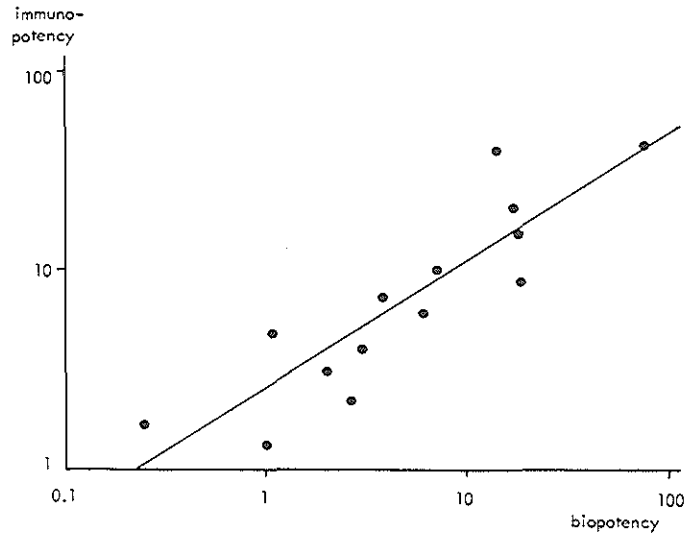


Figure 4.2

Relationship between relative bio- and immunopotencies of inhibin- containing preparations from bFF at different stages of the purification procedure ($n = 14$, $r = 0.88$, $p < 0.001$).
(From: de Jong et al., 1983b).

4.3.3 Immunoprecipitation

A ^{125}I-labelled, highly purified bovine inhibin preparation obtained after chromatography on Mono Q at pH 9.2 (see Appendix paper 1) and radioiodination, contained after SDS-PAA gel electrophoresis and counting of the radioactivity in the slices of the gel one major labelled band at approximately 65-68 kDa (Fig. 4.3a, arrow) and some minor labelled proteins > 200 kDa, at 50 kDa and 30 kDa. The labelled proteins were incubated with the rabbit antiserum against bovine inhibin and subsequently displaced from the antiserum with Procion Red fractions from bFF or bPcas. After precipitation of immune complexes with *S. aureus*, SDS-PAGE of the immunoprecipitates and slicing of the gel, radioactivity in these slices was counted. The difference in radioactivity caused by displacement of labelled proteins from the antiserum after addition of bFF and bPcas corresponded to a peak at approximately 68 kDa (Fig. 4.3b). Because in the bPcas no

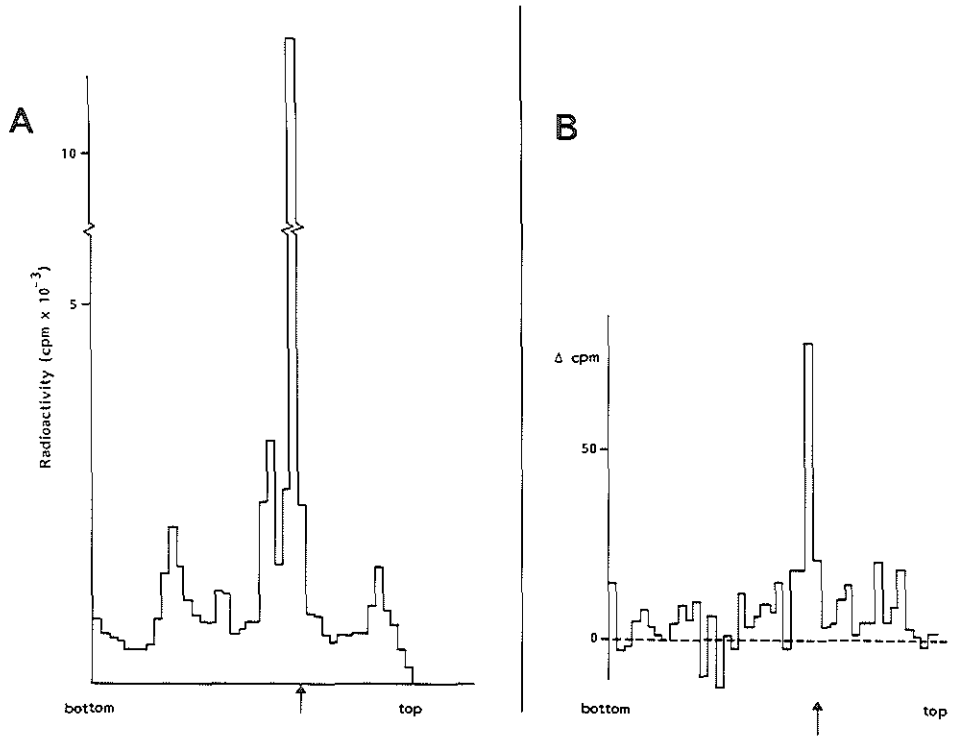


Figure 4.3

A) SDS-polyacrylamide gel electrophoresis of a radioiodinated inhibin-containing preparation from bFF obtained after chromatography on immobilized Procion Red 3B, Sephadex G-25, Mono Q at pH 7.9 and Mono Q at pH 9.2.
B) Differences in immunoprecipitated radioactivity after displacement of the radioiodinated inhibin-containing preparation from the inhibin antiserum by bFF and bovine castrate plasma in the SaRIA. After SDS-PAA gel electrophoresis the gels were cut in 2 mm slices and radioactivity was counted.
The arrow indicates the BSA molecular marker.

inhibin activity could be determined, it was concluded that this 68 kDa peak represented an ovarian protein, most likely bovine inhibin.

4.3.4 *Staphylococcus aureus* radioimmunoassay

With a different type of RIA, in which *S. aureus* was used as an

immunoadsorbent, various radioiodinated bovine inhibin-containing preparations, obtained after ion exchange chromatography on the FPLC at pH 9.2 and from the AcA-54 gel filtration column, were incubated with the rabbit antisera against bovine inhibin or against bPcas. The radioiodinated proteins bound partially to both antisera. Subsequently, the labelled proteins were incubated with the antisera in the presence of equal volumes of bFF or bPcas. The differences in binding after incubation with bFF or bPcas are shown in Fig. 4.4. When fractions, which contained inhibin bioactivity (Mono Q fractions and AcA-54 fraction A) were used as a tracer, significantly more label was displaced from the antiserum against bovine inhibin by addition of bFF than by addition of bPcas (Fig. 4.4, open bars). Use of a nonactive radiolabelled fraction (AcA-54, fraction C) or the rabbit antiserum against bPcas proteins (Fig. 4.4, hatched bars) did not

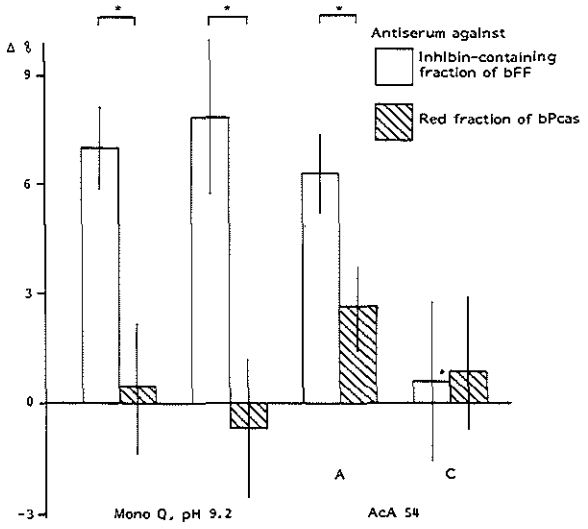


Figure 4.4

Difference (%) between binding of various radioiodinated protein preparations after displacement by Procion Red 3B fractions of bFF and bovine plasma (bPcas) from inhibin antiserum and plasma antiserum in the SaRIA. See text for the origin of the radioiodinated fractions. Values are expressed as $\bar{x} \pm$ SEM (n = 7-14, *$p < 0.05$).
(From: de Jong et al., 1984).

result in such difference. The latter observation indicated that no binding of ovary specific radiolabelled proteins occurred to the antiserum against bPcas.

4.4 IMMUNONEUTRALIZATION OF INHIBIN FROM SEVERAL SPECIES

The ovine antiserum against bovine inhibin was used to study the in vitro immunoneutralization of inhibin bioactivity in gonadal fluids from several species and the possible differences between inhibin activity obtained from male and female animals (Appendix paper 3). For the latter study gonadal preparations from rats and sheep were used.

We observed that the ovine antiserum against bovine inhibin restored bFF-suppressed basal as well as GnRH-stimulated FSH release from pituitary cells in vitro to the levels found in the media of cells cultured in the absence of bFF. The suppression of GnRH-stimulated release of LH from pituitary cells in culture caused by addition of bFF (de Jong et al., 1979; Scott and Burger, 1981) was also restored to control levels after addition of the antiserum. The immunoneutralization of the inhibin-induced suppression of both the spontaneous release of FSH and the release of FSH and LH in the GnRH-stimulated system followed the same pattern. These observations might imply that the suppression of the GnRH-stimulated release of LH by follicular fluid is an intrinsic effect of inhibin. The possibility of another ovarian factor, which modulates LH release, is co-purified with inhibin and has antigenic properties similar to those of inhibin, is less likely.

The ovine antiserum against inhibin-containing fractions of bFF appeared to neutralize inhibin bioactivity from human, porcine and ovine follicular fluid, ovine testicular lymph and rete testis fluid, spent medium from rat granulosa and Sertoli cell cultures and extracts from rat ovaries and rat testes. The degree of immunoneutralization and cross-reactivity was higher for the inhibin-containing preparations obtained from ewes and female rats than from rams and male rats (Appendix paper 3: Table 2). These observations suggest a difference between the molecular

structure of inhibin from female and male animals.

4.5 DISCUSSION

Using conventional polyclonal antisera raised in rabbits and sheep against bovine inhibin-containing preparations we have been able to study several characteristics of inhibin. A rabbit inhibin antiserum that was active in neutralizing inhibin activity *in vitro* (de Jong et al., 1982a) and that bound inhibin activity after immobilization of the antiserum on CNBr-activated Sepharose (de Jong et al., 1983a,b), reacted specifically with a 68 kDa ovarian protein in bovine follicular fluid (Fig. 4.3b). Because the material, which was radioiodinated, contained inhibin activity and because of the immunoneutralizing ability of the antiserum against inhibin, it was likely that the 68 kDa peak, that remained as a difference in radioactivity after displacement of the label from the antiserum by bFF and bPcas and subsequent gel electrophoresis (Fig. 4.3b), represented inhibin.

The rabbit antiserum against inhibin was used in an RIA, incorporating either immunoprecipitation with a second antibody or immunoadsorption with *S. aureus*. The correlation between bio- and immunopotencies in the double-antibody RIA with various bovine inhibin-containing preparations was good (Fig. 4.2). In the SaRIA, inhibin-containing preparations showed a significant difference in binding to the inhibin antiserum and the plasma antiserum (Fig. 4.4). A preparation without inhibin activity bound equally well to both antisera (Fig. 4.4).

The *in vitro* immunoneutralization of inhibin activity from hFF by the rabbit antiserum directed agaqinst bovine inhibin and the cross-reactivity of hFF in the RIA using this rabbit antiserum were rather poor (data not shown). In contrast, the *in vitro* immunoneutralization of inhibin from various species with the inhibin antiserum that was raised in sheep as described in Appendix paper 3, showed cross-reactivity with inhibin-containing preparations from various species (Appendix paper 3). The ovine inhibin antiserum was, in this respect, more suitable for immunological studies of inhibin.

Crude or partially purified inhibin-containing preparations caused a marked decrease in GnRH-stimulated release of LH into the culture medium of rat pituitary cells (de Jong et al., 1979; Scott and Burger, 1980; Scott et al., 1981; Appendix paper 3). On the other hand, Huang and Miller (1984) reported an increase in LH release by an inhibin-containing fraction of pFF in an GnRH-stimulated ovine pituitary cell culture. Our conclusion, based on immunoneutralization experiments, that the inhibin-induced suppression of GnRH-stimulated release of LH is an intrinsic effect of inhibin is in contradiction to the conclusion of Huang and Miller (1984), who stated that inhibin might be responsible for a GnRH-induced increase in LH release. It can not be excluded that these two opposing conclusions reflect characteristics of the different assay systems rather than characteristics of bovine or porcine inhibin. In this respect, it is interesting that Robertson et al. (1986a) used purified 58 kDa inhibin in a rat pituitary cell assay and observed only suppression of FSH release under basal and GnRH-stimulated conditions. A possible explanation for these opposing observations could be the difference in biological specificity between the 58 kDa inhibin and the putative precursor 68 kDa inhibin (see also Chapter 5). Other factors, present in follicular fluid, might also play a role.

Furthermore, using monoclonal antibodies raised against bovine inhibin, Miyamoto et al. (1986) detected several high molecular weight forms of inhibin. It will be interesting to study the in vitro biological activity of these different forms, using both suppression of basal FSH release and GnRH-stimulated FSH and LH release and suppression of cellular content of FSH as response parameters.

4.6 CONCLUSIONS

1) An antiserum, which was raised in rabbits against a partially purified inhibin-containing preparation from bFF and which neutralized inhibin bioactivity in an in vitro bioassay, reacted specifically with a 68 kDa protein in bFF.
2) The 68 kDa protein could be displaced from the antiserum by

bFF, but not by bPcas. It is likely that this ovary specific protein represents bovine inhibin.

3) A radioimmunoassay was developed using the antiserum raised in rabbits against inhibin from bFF. A good correlation was observed between biological activities and immunopotencies of inhibin containing preparations from bFF obtained in various stages of purification.
4) An antiserum, which was raised in sheep against a partially purified inhibin-containing preparation from bFF showed *in vitro* cross-reactivity with inhibin activity from several species.
5) *In vitro* neutralization of inhibin bioactivity from rat and ovine male and female gonads by preadsorbed inhibin antiserum raised in sheep indicates a sexual dimorphism which might arise from differential processing of inhibin in gonadal fluids and tissues.
6) Suppression of LH release by bFF in GnRH-stimulated pituitary cells might be an intrinsic effect of inhibin.

CHAPTER 5

GENERAL DISCUSSION: INHIBIN, A 68 kDa PROHORMONE

5.1 INTRODUCTION

Since McCullagh (1932) postulated the existence of a water-soluble gonadal hormone, inhibin, which selectively suppresses secretion of FSH from the hypophysis, investigators have tried to isolate and to identify this link in the gonadal-hypophyseal regulation system. The older literature on inhibin shows much confusion about the very existence, physiological significance and physicochemical characteristics of inhibin, but as reviewed in Chapter 2, some major advances in the field of inhibin research have occurred during the past few years. With improved biochemical separation methods it has been possible to purify and characterize inhibin from bovine (Robertson et al., 1985, 1986b; Fukuda et al., 1986) and porcine (Rivier et al., 1984, 1985; Ling et al., 1985; Miyamoto et al., 1985) ovarian follicular fluid. From the partial amino acid sequences of these inhibins it was possible by cloning and DNA sequencing techniques to elucidate the amino acid sequence and structure of inhibin from bovine (Forage et al., 1986), porcine (Mason et al., 1985) and human (Mason et al., 1986) follicular fluid. A summary of reported molecular masses of inhibin and subunits of ovarian inhibin is given in Table 5.1. In the following sections of this chapter we will discuss the results described in the preceding chapters and in the Appendix papers in relation to the data in the above-mentioned reports.

5.2 MOLECULAR CHARACTERIZATION OF OVARIAN INHIBIN

Since the paper by Jansen et al. (1981) on the partial purification of inhibin from bFF and its tentative characterization as a 65 kDa glycoprotein, our efforts were directed to the purification and characterization of this protein from bFF and hFF. As

described in Chapter 3 and Appendix paper 2 we succeeded in obtaining an approximately 800-fold purified inhibin preparation from bFF using a combination of chromatographic procedures. When the protein composition of highly purified inhibin preparations was analysed using SDS-PAGE, heterogeneous protein staining patterns were observed. Of the several protein bands present in these preparations a 65-68 kDa protein became more dominant with increasing purification of the biological activity. Some of the preparations exhibited a single 68 kDa protein band after chromatographic purification steps (Fig. 3.5 and Fig. 4 in Appendix paper 1) or after elution of proteins from SDS-polyacrylamide gels (Fig. 5 in Appendix paper 1).

Characterization of bovine inhibin preparations after radiolabelling in a radioimmunoassay system as described in Chapter 4.3 resulted in the specific immunoprecipitation of a 68 kDa protein (Fig. 4.1b). Furthermore, inhibin secreted by cultured bovine granulosa cells was also associated with a 65-68 kDa protein (de Jong et al., 1982a). In conclusion, our results indicate that, at least with the purification procedures which we employed, inhibin activity from bFF is associated with a 68 kDa glycoprotein (Chapters 3.3 and 4.3, Appendix paper 1).

The characteristics of inhibin from hFF as described in Chapter 3 and Appendix paper 2 are similar to those of bovine inhibin. A small difference was observed between the pH-ranges in which inhibin activity from bFF and hFF were eluted from the chromatofocusing column (Table 3.3; Appendix paper 2). This suggests a difference in charge of the molecules and thus in amino acid composition or the degree of glycosylation (see also section 5.4). Robertson et al. (1986b) reported a pI between pH 6.9 and pH 7.3 for their 58 kDa bovine inhibin, in contrast to the values that we have observed: pH 4.9 to pH 5.3 for bovine inhibin activity and pH 5.1 to pH 5.6 for human inhibin activity. The differences between the observations of the former authors and our data might be caused by structural differences between the 58 and the 68 kDa forms of inhibin. Alternatively, it might rather reflect the pI of (a) carrier protein(s) than the pI of inhibin itself.

Our observations on human inhibin also show another interes-

ting feature with respect to the molecular mass of the molecule. Several observations reflect an approximate molecular mass of 65-68 kDa for human inhibin (Chapter 3). These indications were obtained from gel filtration chromatography (cf. Fig. 3.5) and SDS-PAGE analysis after chromatofocusing (cf. Fig. 3.2). When, however, a preparation obtained after chromatofocusing was subjected to preparative gel electrophoresis and eluted from these gels, inhibin activity was associated with a 30-32 kDa protein (Fig. 3.6).

5.3 68 kDa INHIBIN IS PROTEOLYTICALLY CLEAVED TO 32 kDa INHIBIN

The apparent discrepancy between our observations of a 68 kDa form of inhibin from bFF and hFF and the 58 kDa protein from bFF (Robertson et al., 1985) and the 32 kDa proteins from porcine and bovine follicular fluid (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985, Fukuda et al., 1986; Robertson et al., 1986b; see Table 5.1) might be explained in terms of cleavage of the large form (58-68 kDa) of inhibin to a 32 kDa form without marked loss of biological activity. Investigators who have purified a 32 kDa protein included in their purification procedures an acid treatment of their material, whether it was an acid precipitation or elution at low pH. Beers (1975) showed that proteases in follicular fluid can be activated by acid treatment. We have shown that active proteases are present in inhibin containing preparations from hFF (Figs. 3.7 and 3.8) after chromatofocusing. These observations suggest that the 32 kDa form of inhibin from ovarian follicular fluid could be a purification artifact due to acidic proteolysis. Processing of a large (58-68 kDa) form to a small (30-32 kDa) form of inhibin could be of physiological importance, as is suggested by the observations of McLachlan et al. (1986). These authors observed inhibin from bFF was split into a 31 kDa form in the presence of bovine and human serum at 4 °C and at 30 °C, whereas in the presence of bFF such cleavage of the 58 kDa form did not occur. This suggests that ovarian inhibin could be processed to a smaller form *in vitro* by the action of acidic proteases and *in vivo* after entering the circula-

tion.

The difference between the 58 and 31 kDa inhibin (table 5.1), as described by Robertson et al., (1985) and the 68 kDa inhibin as described in this thesis may be explained on basis of the suggested precursor and subunit structure of inhibin (Fig. 5.1). The

Table 5.1: Molecular mass and subunit structures of ovarian follicular fluid inhibin and related proteins

Source	Molecular mass (kDa) Native	Subunits	Detection method*	References
Ovarian follicular fluid inhibin				
Bovine	65	-	SDS-PAGE	Jansen et al., 1981
	65-68	-	SDS-PAGE	van Dijk et al., 1984
	56	44 14	SDS-PAGE	Robertson et al., 1985
	31	20.2 14.8	SDS-PAGE	Robertson et al., 1986b
	58	32.3 (A) 13.0 (B)	cDNA	Forage et al., 1986
	31	20 (Ac) 15 (B)	SDS-PAGE	
	32	20 (A) 13 (B)	SDS-PAGE	Fukuda et al., 1986
Porcine	32	18 (α) 14 (β_A, β_B)	SDS-PAGE	Ling et al., 1985
		14.5 (α) 14 (β_A, β_B)	cDNA	Mason et al., 1985
	32	20 13	SDS-PAGE	Miyamoto et al., 1985
	32	18 (A) 14 (B)	SDS-PAGE	Rivier et al., 1985
Human	68, 32	-	SDS-PAGE	van Dijk et al., 1985a, this thesis
	32	18 (α) 14 (β_A, β_B)	cDNA	Mason et al., 1986
Related proteins				
TGF-β	25	12.5	SDS-PAGE cDNA	Derynck et al., 1985
FRP	24	13.8-14.7 (β_A, β_B)	SDS-PAGE	Ling et al., 1986
	28	15 (β_A)	SDS-PAGE	Vale et al., 1986
MIS	140	70, 74 58	SDS-PAGE cDNA	Cate et al., 1986

* SDS-PAGE: sodium dodecylsulphate-polyacrylamide gel electrophoresis
cDNA: nucleotide sequence of complementary DNA.

nucleotide sequences of the cDNAs derived from the mRNAs, which contain the coding region(s) for inhibin and which were extracted from porcine (Mason et al., 1985), bovine (Forage et al., 1986) and human (Mason et al., 1986) granulosa cells have been published. Based on the predicted amino acid sequences and on the SDS-PAGE data from the purified inhibins it was postulated that bovine and porcine 32 kDa inhibin consist of two subunits, a 18-20 kDa A (or α) subunit and a 13-15 kDa B (or β) subunit, which are linked by disulfide bridges (Table 5.1) and which each separately do not exhibit inhibin activity (Ling et al., 1985; Miyamoto et al., 1985).

5.4 THE RELATIONSHIP BETWEEN THE 68 kDa AND THE 58 kDa INHIBIN

The 58 kDa bovine inhibin appears to consist of a 43 kDa A subunit and a 15 kDa B subunit (Fig. 5.1a). Robertson et al. (1985, 1986b) have shown that after acidic treatment the 43 kDa A subunit is cleaved to a 20 kDa Ac subunit. The nucleotide sequence of the cDNA encoding the bovine A subunit (Forage et al., 1986) shows that upstream of the 43 kDa precursor the cDNA still contains an additional 60 amino acid N-terminal extension following the putative Met-start codon. If the complete sequence were translated *in vivo* it would give rise to an inhibin subunit of 360 amino acids with 2 putative glycosylation sites and a molecular mass of approximately 51 kDa (Fig. 5.1a). The nucleotide sequence data for the α-subunit of human inhibin are consistent with this model (Fig. 5.1b; Mason et al.,1986). It can be postulated, on basis of this model, that inhibin exists in the form of a larger precursor hormone. This (pre-)prohormone presumably consists of an A and a B subunit with a combined molecular mass of approximately 66-67 kDa, which is in close agreement with our observations of 65-68 kDa, as assessed by SDS-PAA gel electrophoresis. The difference between the 68 kDa and the 58 kDa forms of bovine inhibin might reflect hydrolytic cleavage at the 3 Arg-Arg sites around His-60, the putative first N-terminal amino acid of the 58 kDa inhibin (Fig. 5.1a; Forage et al., 1986). This position could be equivalent to His-31 in human inhibin (Fig. 5.1b;

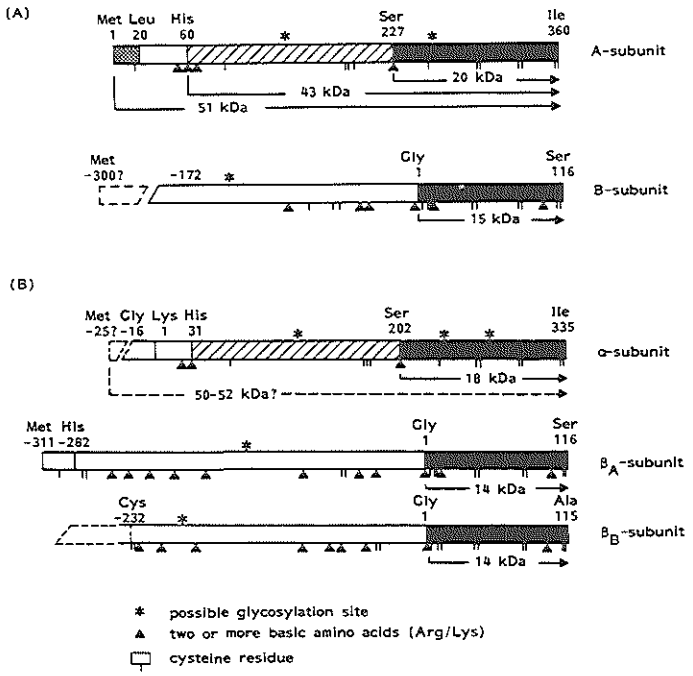


Figure 5.1

Possible precursor and subunit structure of bovine (A) and human inhibin (B), predicted from the nucleotide sequence of the cDNAs derived from the mRNAs encoding bovine inhibin.
Black area: purified and sequenced subunit; Hatched area: N-terminal extension of the A-subunit; Dotted area: possible leader sequence; Dashed area: postulated N-terminal extension of the prohormone.
(Data from Forage et al., 1986, Mason et al., 1986, and Robertson et al., 1986c).

Mason et al., 19869. These short stretches of basic amino acids are likely to be susceptible to proteolytic enzymes. The first 20 amino acids of the A subunit of bovine inhibin show an amino acid sequence which is typical for a signal sequence (Watson, 1984; Forage et al., 1986).

The reason that we find a 68 kDa inhibin and Robertson and collaborators a 58 kDa inhibin in bFF might reflect differences in handling of the follicular fluid before and during the first purification steps. Temperature, pH, buffer composition and removal of protease inhibitors could be factors responsible for these

discrepancies.

The two forms of inhibin from hFF that we have observed are in good agreement with the described model for bovine inhibin. The recently published nucleotide sequences of the cDNAs encoding human inhibin (Mason et al., 1986) show a high degree of homology with the nucleotide sequences of cDNA encoding for bovine inhibin (Forage et al., 1986). The differences found in the amino acid sequence in the α-subunit are mainly due to base substitutions, resulting in a possible third glycosylation site (Fig. 5.1b). Mason et al. (1985, 1986) found two forms of the β-subunit, both for porcine and human inhibin. The differences between those two chains suggest that they were transcribed from two different genes (Mason et al., 1985, 1986). For bovine inhibin only one B-subunit was found (Forage et al., 1986). In a recent report, Miyamoto et al. (1986) proposed a three-subunit model for bovine inhibin to explain 88 kDa, 102 kDa and 120 kDa forms of bovine inhibin. Their data did not clearly explain whether their putative third subunit actually differs from the B subunit of inhibin and whether it is necessary to postulate a third subunit to explain their observations. However, if the model of Miyamoto et al. (1986) is proven to be correct, then the processing of inhibin may be even more complex.

5.5 DIFFERENCES BETWEEN INHIBIN FROM MALE AND FEMALE ANIMALS

In the experiments described in Chapter 4.4 and Appendix paper 3 we have shown by neutralization of inhibin activity in an *in vitro* inhibin bioassay with preadsorbed inhibin antiserum, that a difference can be demonstrated between inhibin activity from male and female gonadal sources. Because these experiments were carried out with crude inhibin-containing preparations, the exact origin of the differences could not be established. The antiserum specifically neutralized inhibin activity from both male and female gonadal fluids and tissues from different species in the *in vitro* inhibin assay. A difference in the molecular structure of inhibin from male and female sources could account for the differences in *in vitro* immunoneutralization. Data on the molec-

ular weight of inhibin from rat testicular extracts obtained after gel filtration indicated an apparent molecular weight of of 50-60 kDa (Au et al., 1983). Baker et al. (1985) reported a variability in molecular mass for inhibin activity from ovine rete testis fluid, depending on the purification procedure employed. Gel filtration of rete testis fluid proteins under acidic conditions resulted in a protein band on SDS-PAA gels of 30 kDa associated with inhibin activity (Baker et al., 1985), confirming earlier observations (Baker et al., 1982) and in close agreement with data on ovarian inhibin (see section 5.3 and 5.4).

Taking these data on male and female inhibin into account, it could be speculated that male and female inhibin are encoded by the same gene(s), have similar subunit structures and that differences as described in Chapter 4.4 and Appendix paper 3 might reflect either differences in in vivo post-translational processing or differences in in vitro proteolytic processing of the native prohormone. The real character of the sexual dimorphism as observed (Chapter 4.4 and Appendix paper 3) still remains to be elucidated.

5.6 RELATIONSHIP BETWEEN INHIBIN AND OTHER REGULATORY PROTEINS

The β-chain of porcine inhibin shows sequence homology with several regulatory proteins (Table 5.1): transforming growth factor-β (TGF-β; Mason et al.,1985); FSH-releasing proteins (FRP; Ling et al.,1986, Vale et al., 1986); and Müllerian inhibiting substance (MIS), a glycoprotein that causes regression of the Müllerian duct during development in the male embryo (Cate et al., 1986). The interrelationship between these proteins is puzzling.

TGF-β, a homodimeric protein of approximately 25 kDa (Table 5.1), which, depending on the cell type and the physiological conditions, stimulates or inhibits cell proliferation (Derynck et al., 1985), has been shown to stimulate FSH-release by cultured pituitary cells (Ying et al., 1986a) and to enhance the FSH-induced secretion of oestradiol by cultured granulosa cells (Ying et al., 1986b). These actions of TGF-β have been reported to be antagonistic to the effects of inhibin (Ying et al., 1986a,b).

The FRPs are reported to be heterodimers of the two forms of the β-subunit (Ling et al., 1986) or homodimers of the β_A-subunit (Vale et al., 1986) of porcine inhibin (Table 5.1). Both forms of FRP stimulate the release of FSH by pituitary cells in vitro (Ling et al., 1986; Vale et al., 1986), whereas the homodimer enhances production of FSH in pituitary cells in vitro (Vale et al., 1986). These authors also reported that porcine inhibin and FRP added together to pituitary cells in culture have antagonistic effects on the release of FSH. FRP and GnRH act synergistically on the FSH release from pituitary cells, but by a different mechanism of action. Moreover, FRP does not influence LH release by pituitary cells (Vale et al., 1986). The biological and structural similarities between TGF-β and FRP raise the following questions: 1) Does FRP have a paracrine effect on granulosa cells comparable to TGF-β (Ying et al., 1986b); 2) Does FRP have similar effects on the Sertoli cells in the testis; 3) Is there an inhibin-FRP antagonism in granulosa and Sertoli cells; and 4) Can FRP be detected only in follicular fluid or is it, comparable to TGF-β, ubiquitously found in body fluids and tissues, and if so, what is the role of FRP?

The sequence homology of the β-chain of porcine inhibin and TGF-β with bovine and human MIS is restricted to the conserved C-terminal region (Cate et al., 1986). It has been suggested that these proteins are products of a gene family with important regulatory functions in the hypophyseal-gonadal system (Mason et al., 1985; Cate et al., 1986; Vale et al., 1986; Ying et al., 1986a,b). This protein family could be based on multiple subunit rearrangements of dimeric proteins, linked with disulfide bridges, all derived from a limited number of genes. In this respect, the inhibin β-chain could represent an important regulatory protein, whereas the biological specificity might be attributed to the α-chain. The role and distribution of this class of (hetero)-dimeric regulatory proteins might be studied by expression of recombinant DNA sequences of these proteins in various cell types. As described for MIS (Cate et al., 1986) conditioned medium of transfected cell cultures might have biological effects on putative target cells in vitro, indicating that bioactive gene products have been synthesized and secreted.

5.7 CONCLUSIONS

1) Inhibins from bovine and human follicular fluid have been purified as 68 kDa glycoproteins, consistent with the unprocessed prohormone form, as established from the cDNA sequence of bovine, human and porcine inhibin. *In vitro* a 30-32 kDa form can be generated, presumably by proteolytic cleavage, that exhibits inhibin activity in a pituitary cell culture assay. It is suggested that a similar processing mechanism may act *in vivo*.
2) Differences in the molecular mass between 68 and the 58 kDa inhibin might be the result of a purification artefact. The relationship between the reported higher molecular weight forms of inhibin and the 68 kDa form of inhibin is not clear.
3) Data from other laboratories indicate that inhibin has a subunit structure with an A- or α-subunit of 18-20 kDa and one B-subunit (bovine inhibin) of 14 kDa or two different β-subunits (human and porcine inhibin) of 14-15 kDa, connected by disulfide bridges.
4) The β-subunit(s) of inhibin show sequence homology with transforming growth factor-β (TGF-β), FSH-releasing proteins and Müllerian inhibiting substance (MIS). These proteins might be members of a family of regulatory proteins in the hypophyseal-gonadal system.

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SUMMARY

The work described in this thesis concerns the purification and characterization of the gonadal hormone inhibin.

The development and the action of the gonads are regulated by two hormones, the gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are secreted by the pituitary gland. The gonadotrophins stimulate different cell types: in the testis LH acts on the Leydig cells and FSH on the Sertoli cells, whereas in the ovary LH acts on the theca cells, while both hormones act on the granulosa cells. The secretion of the gonadotrophins is modulated by a feedback mechanism from the gonads, which controls synthesis of FSH and LH in and secretion of these hormones from the gonadotrophic cells in the hypophysis. Steroid hormones, which are produced in the Leydig cells and in the theca and granulosa cells, mainly influence the production of LH, whereas FSH release is not only regulated by steroids, but also by a nonsteroidal factor, the protein hormone inhibin. Although the existence of inhibin has been suspected for more than 60 years, the need for another gonadal factor in the hypophyseal-gonadal feedback system has been vehemently disputed. Two main causes for the denial of the existence of inhibin were the inability to detect and quantitate an inhibin effect and the belief that another steroidal factor would be discovered, which might be responsible for part of the feedback on FSH secretion. As has been described in Chapter 2, observations in the early 1970s that administration of steroid-free gonadal fluids influenced the levels of FSH in the peripheral circulation of experimental animals revived the interest in the inhibin hypothesis.

The role of inhibin in reproductive biology has been extensively investigated for several animal species with a variety of *in vivo* and *in vitro* systems. Most of these studies were performed in rats. Reliable bioassays for inhibin were developed, in which the specific suppression of the release of FSH from cultured rat anterior pituitary cells was measured after addition of inhibin-containing preparations to the culture medium. It became clear that inhibin is produced in the Sertoli cells in the testis

and in the granulosa cells in the ovary. The function of inhibin appeared to be most pronounced when the role of FSH is most important. In prepubertal male animals FSH influences the development of the Sertoli cells and in that way, indirectly, spermatogenesis, whereas the main target for FSH in the female animal is the stimulation of follicular growth just before and after puberty. Inhibin provides an important feedback signal in these situations. In the immature female and the mature male animal inhibin feedback of FSH secretion probably plays a minor role.

Attempts to purify inhibin from gonadal fluids have been manifold and reported molecular masses ranged from < 1 kDa to approximately 200 kDa. In Chapter 3 and Appendix papers 1 and 2 the partial purification of inhibin from bovine and human ovarian follicular fluid (bFF and hFF, respectively) has been described. Using a sequence of purification steps, involving dye-ligand affinity chromatography, FPLC anion exchange and gel filtration chromatography and chromatofocusing, affinity chromatography on immobilized lectins and immobilized antibodies and subsequent preparative sodium dodecylsulphate polyacrylamide gel electrophoresis, inhibin was characterized as a 68 kDa glycoprotein. After submission to a low pH during chromatofocusing and subsequent gel electrophoresis, a 30-32 kDa form of inhibin from hFF was generated.

Immunological studies on inhibin are described in Chapter 4 and Appendix paper 3. Antibodies against partially purified bovine inhibin were raised in rabbits. Using these antibodies a radioimmunoassay for bovine inhibin was developed. The results of this radioimmunoassay showed a good correlation with biological activities of several inhibin-containing preparations of bovine origin. Using the same antibodies it was possible to precipitate specifically a 68 kDa protein of ovarian origin.

Antibodies raised against partially purified bovine inhibin in sheep, appeared able to neutralize inhibin activity *in vitro* from several bovine, human, ovine, porcine and rat gonadal preparations (Chapter 4 and Appendix paper 3). *In vitro* neutralization of inhibin activity from ovine and rat female and male gonadal preparations, using this ovine antiserum, suggested a sex-related dimorphism of inhibin molecules, which might be caused by diffe-

rential processing of the putative prohormone form of inhibin. Furthermore, in pituitary cells stimulated *in vitro* with gonadotrophin releasing hormone suppression of release of FSH as well as LH after administration of bFF was observed. Both FSH and LH release were restored to control levels, when ovine antiserum against bovine inhibin was added to the pituitary cells in the presence of bFF (Appendix paper 3). The observed suppression of LH might therefore reflect an intrinsic effect of inhibin.

In Chapter 5 the relationship between the different forms of inhibin and the homology with other regulatory proteins is discussed. Data from several laboratories on the cDNA sequences of bovine, human and porcine inhibin indicate that inhibin consists of two subunits. The results obtained in our laboratory, as described in Chapter 3 and Appendix papers 1 and 2, are consistent with these data. A model for inhibin is proposed (Chapter 5), in which inhibin is synthesized as a 68 kDa prohormone, which might be processed *in vivo* to a 30-32 kDa bioactive form. *In vitro* cleavage to a bioactive 30-32 kDa form can be achieved by proteolytic activity. The sequence homology of the inhibin β-subunits with transforming growth factor-β and Müllerian inhibiting substance and the detection of FSH-releasing proteins which consist of a dimer of inhibin β-subunits, suggest that inhibin might be part of a more complicated regulation system.

SAMENVATTING

In dit proefschrift worden zuivering en karakterisering van het hormoon inhibine, dat geproduceerd wordt door de testis (zaadbal) en het ovarium (eierstok), beschreven.

De ontwikkeling en het functioneren van de gonaden (geslachtsklieren) worden geregeld door een samenspel van de gonadotrofinen (gonade-stimulerende hormonen) FSH (follikel-stimulerend hormoon) en LH (luteïniserend hormoon), beide afkomstig uit de hypofyse, een klier aan de onderkant van de hersenen. De gonadotrofinen stimuleren verschillende celtypen in de testis en in het ovarium, die daardoor steroïdhormonen produceren, die op hun beurt de aanmaak en afgifte van de gonadotrofinen door de hypofyse afremmen. Het aldus ontstane evenwichtssysteem zorgt voor een functionele ontwikkeling van de geslachtsorganen.

Op grond van dierexperimenteel en klinisch onderzoek heeft men vastgesteld, dat de aanmaak van steroïdhormonen voornamelijk gestimuleerd wordt door LH en dat steroïdhormonen voornamelijk de produktie en afgifte van LH beïnvloeden. De aanmaak en afgifte van FSH wordt slechts ten dele door steroiden geregeld; een andere factor, inhibine (afgeleid van het Latijnse werkwoord "inhibere", dat remmen of verhinderen betekent), speelt hier waarschijnlijk ook een belangrijke rol. Afwijkingen in de spermatogenese (ontwikkeling van de zaadcellen) en de ontwikkeling van de eicel, die eventueel kunnen leiden tot een verminderde vruchtbaarheid, kunnen mogelijkerwijs verklaard worden door een verstoring van dit regelsysteem.

De discussie over het bestaan van inhibine startte rond 1920, toen men ontdekte dat na bestraling of verwijdering van de testes een bepaald celtype in de hypofyse in omvang toenam. McCullagh liet in 1932 zien, dat deze zogenoemde "castratiecellen" weer kleiner werden na inspuiten van testisextracten in de gecastreerde dieren. Deze resultaten konden echter niet door anderen bevestigd worden, terwijl tevens duidelijk werd, dat steroïdhormonen een remmende werking op de afgifte van gonadotrofe hormonen uit de hypofyse konden hebben. Deze ontwikkelingen hebben de inhibine-hypothese tijdelijk naar de achtergrond gedrongen. Dit had tot

gevolg dat tot in het begin van de jaren '70 ernstig getwijfeld werd aan het bestaan en de mogelijke rol van inhibine in de voortplantingsfysiologie. Voortgaande onwikkelingen maakten echter duidelijk dat naast steroïden nog een factor actief moest zijn in de negatieve terugkoppelingsregulatie van de afgifte van gonadotrofinen; dit resulteerde in een hernieuwde interesse voor inhibine.

De rol van inhibine in de voortplantingsbiologie is het meest uitgebreid onderzocht bij ratten. De resultaten van deze studies laten zien dat inhibine in mannelijke dieren een belangrijke rol speelt voor de puberteit, terwijl het belang van inhibine bij vrouwelijke dieren vooral wordt gevonden tijdens en na de puberteit. Er is een betrouwbare bepalingsmethode voor inhibine ontwikkeld, waarin de vermindering van de afgifte van FSH door gekweekte hypofyse cellen van ratten onder invloed van inhibinebevattende preparaten wordt gemeten.

In hoofdstuk 2 van dit proefschrift worden de studies beschreven die betrekking hebben op de fysiologische rol van inhibine, de verschillende bepalingsmethoden voor inhibine en de pogingen inhibine te isoleren uit verschillende gonadale vochten.

Ons onderzoek heeft zich toegespitst op het isoleren van inhibine uit de vloeistof van follikels uit ovaria. Deze vloeistof, het follikelvocht, blijkt een zeer rijke bron van inhibineactiviteit te zijn. Inhibine is gedeeltelijk gezuiverd uit menselijk en runderfollikelvocht (hoofdstuk 3 en de bijlagen -Appendix papers- 1 en 2). Met behulp van een serie kolomchromatografische zuiveringstechnieken, zoals affiniteitschromatografie, ionenwisselingschromatografie, gel-filtratie chromatografie en chromatofocusering, gevolgd door gel-elektroforese, werd inhibine uit deze follikelvochten gekarakteriseerd als een glycoproteine met een molecuulmassa van 68 kDa. Na chromatofocusering bij lage waarden van de pH (zuurgraad) en daaropvolgende gel-elektroforese, werd uit humaan follikelvocht een vorm van inhibine met een molecuulmassa van 30-32 kDa geïsoleerd.

Studies betreffende de immunologische karakterisering van inhibine worden beschreven in hoofdstuk 4 en de bijlage -Appendix paper- 3. Antilichamen tegen gedeeltelijk gezuiverd runderinhibine werden opgewekt in konijnen. Met behulp van deze antilicha-

men werd een radio-immunologische bepalingsmethode ontwikkeld. De resultaten verkregen met behulp van deze bepalingsmethode toonden een goede correlatie met resultaten van bepalingen waarin de biologische activiteit van verschillende inhibine-bevattende preparaten, afkomstig uit runderfollikelvocht, werd gemeten. Met behulp van deze antilichamen was het mogelijk een specifiek uit het ovarium afkomstig eiwit met een molecuulmassa van 68 kDa aan te tonen.

Antilichamen, gericht tegen een gedeeltelijk gezuiverd runder-inhibine, werden eveneens opgewekt in schapen. Deze antistoffen neutraliseerden in een systeem van gekweekte hypofysecellen (in vitro) de effecten van inhibine afkomstig van koeien, mensen, ratten, schapen en varkens. In vitro neutralisatie van inhibine activiteit, afkomstig uit de geslachtsklieren van mannelijke en vrouwelijke ratten en schapen, suggereert dat er een verschil bestaat in de moleculaire structuur van inhibines afkomstig van vrouwelijke en mannelijke dieren. De afgifte van FSH en LH door hypofysecellen in kweek wordt in aanwezigheid van "gonadotrofine stimulerend hormoon" door toevoegen van runderfollikelvocht onderdrukt. Wanneer in een dergelijk experiment de inhibine activiteit geneutraliseerd wordt door de toevoeging van bovengenoemde antilichamen tegen inhibine, herstelt niet alleen de afgifte van FSH zich, maar ook de afgifte van LH. Dit zou kunnen betekenen dat de waargenomen vermindering van LH afgifte, evenals de verminderde FSH afgifte, een effect van inhibine is.

In hoofdstuk 5 worden tenslotte de relaties tussen de verschillende vormen van inhibine op grond van recente literatuurgegevens, bediscussieerd. Tevens wordt de relatie tussen inhibine en een aantal andere regulerende eiwitten toegelicht. Uit de gepresenteerde gegevens blijkt dat inhibine waarschijnlijk is opgebouwd uit twee subeenheden. Een model voor inhibine wordt voorgesteld, waarin inhibine aanvankelijk gesynthetiseerd wordt als een prohormoon met een molecuulmassa van 68 kDa, dat omgezet kan worden naar een biologisch actieve vorm met een molecuulmassa van 30-32 of 58 kDa.

De overeenkomst in structuur tussen inhibine en een aantal andere regulatie-eiwitten suggereert dat inhibine deel is van een uitgebreidere familie van eiwitfactoren, die een functie ver-

vullen in de ontwikkeling en het functioneren van de geslachtsorganen en de hypofyse. Nader onderzoek is nodig om deze hypothese te bevestigen.

LIST OF ABBREVIATIONS

bFF	bovine follicular fluid
bPcas	bovine castrate plasma
BSA	bovine serum albumin
cAMP	cyclic adenosine-3',5'-monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine-3',5'-monophosphate
CNBr	cyanogen bromide
FPLC	fast protein liquid chromatography
FRP	follicle-stimulating hormone releasing protein
FSH	follicle-stimulating hormone, follitropin
g	gravitational constant
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
hFF	human follicular fluid
hMG	human menopausal gonadotrophin
HPLC	high pressure liquid chromatography
IEP	isoelectric point
kDa	kilodalton
LH	luteinizing hormone, lutropin
MIS	Müllerian inhibiting substance
mRNA	messenger ribonucleic acid
oFF	ovine follicular fluid
oTLP	ovine testicular lymph protein
PCO	polycystic ovary
pFF	porcine follicular fluid
PMSG	pregnant mare serum gonadotrophin
RP-HPLC	reversed phase high pressure liquid chromatography
RIA	radioimmunoassay
rGCCM	rat granulosa cell culture medium
RSA	relative specific activity
rSCCM	rat Sertoli cell culture medium

SaRIA	*Staphylococcus aureus* radioimmunoassay
SD	standard deviation
SDS	sodium dodecylsulphate
SDS-PAA	sodium dodecylsulphate polyacrylamide
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
TGF-β	transforming growth factor-β
v/v	volume/volume

DANKWOORD

Het ontstaan van dit proefschrift zou onmogelijk zijn geweest zonder een aantal mensen, die ik op deze plaats wil bedanken voor hun inzet en hun bijdragen. Zonder te suggereren compleet te zijn wil ik met name noemen:

Henk van der Molen, mijn promotor, die ondanks zijn drukke bezigheden elders het onderzoek altijd op kritische wijze heeft gevolgd en gestimuleerd;

Frank de Jong, mijn co-promotor, die met zijn schier encyclopedische kennis van en zijn bijkans onverwoestbare geloof in inhibine dit onderzoek heeft begeleid en mogelijk gemaakt;

Cobie Steenbergen, die, naast de soms noodzakelijke hand- en spandiensten, een belangrijk deel van het immunologische werk met grote zorg voor haar rekening heeft genomen;

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mijn ouders, die mij de mogelijkheden tot zelfontplooiing hebben geboden;

Mineke, voor alles van de afgelopen jaren.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 31 oktober 1953 te Berlikum. In Leeuwarden behaalde hij in 1973 het getuigschrift Gymnasium B aan het Stedelijk Gymnasium. In datzelfde jaar werd begonnen met de studie chemie aan de Rijksuniversiteit van Groningen, waar hij in 1977 het kandidaatexamen scheikunde (S1) behaalde. Hierna werd de studie voortgezet met het doctoraalvak biochemie en het bijvak fysiologische chemie. In deze periode heeft hij onderzoek verricht bij Prof. Dr. B. Witholt (Biochemische Laboratoria, Rijksuniversiteit Groningen) aan het hittelabiele enterotoxine van Escherichia coli en bij Prof. Dr. A.M. Kroon (afdeling Fysiologische Chemie van dezelfde universiteit), waar hij de localisatie en nucleotidenvolgorde van het mitochondriële gen voor cytochroom c oxidase van Neurospora crassa heeft onderzocht. In deze periode werd tevens de onderwijsbevoegdheid voor schei- en natuurkunde behaald. In 1981 werd de Groninger periode "afgesloten" met het behalen van het doctoraalexamen scheikunde.

Vanaf november 1981 is hij werkzaam geweest op de afdeling Biochemie II (Chemische Endocrinologie) van de Erasmus Universiteit Rotterdam, waar hij heeft gewerkt aan de zuivering en karakterisering van het eiwithormoon inhibine. Dit proefschrift is hiervan het resultaat.

APPENDIX PAPERS

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USE OF FAST PROTEIN LIQUID CHROMATOGRAPHY IN THE PURIFICATION OF INHIBIN FROM BOVINE FOLLICULAR FLUID

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Inhibin from bovine follicular fluid was partly purified using affinity chromatography on immobilized Procion Red 3B, gel filtration on Sephadex G-25 and ion-exchange chromatography on the fast protein liquid chromatography system. Inhibin was subsequently characterized using preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroelution. Biological activity was associated with a protein with an apparent molecular weight of approximately 65 kD. © 1984 Academic Press, Inc.

The existence of inhibin as a testicular hormone has been postulated by Mottram and Cramer in 1923 (1), but there is still confusion about the exact molecular nature and physiological significance of inhibin. It is generally accepted, however, that inhibin is a protein or polypeptide which specifically suppresses follitropin (FSH)-release by the pituitary gland without affecting lutropin (LH)-release (2). The apparent lack of success in the purification of inhibin from various sources as reported by a number of authors (3-8) may be partly attributed to the a-typical behaviour of inhibin when classical purification techniques were used (5). Another difficulty in comparing results from different authors is caused by the various bioassays used with their large differences in sensitivity and specificity (9). The introduction of the fast protein liquid chromatography system (FPLC), using improved ion-exchange resins with a high resolution, offered new possibilities for separating proteins while maintaining their

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biological activity. Here we report the partial purification of inhibin from bovine follicular fluid (bFF) using FPLC and electroelution of biologically active proteins from sodium dodecyl sulfate polyacrylamide (SDS-PAA) gels.

MATERIALS AND METHODS

bFF was obtained at a local slaughterhouse by aspiration of fluid from medium and large ovarian follicles. Inhibin-like activity was determined using the inhibition of FSH-release by cultured anterior pituitary cells obtained from male rats (10). Potencies were expressed relative to a standard bFF preparation which was given the arbitrary potency of 1 using a computer programme for parallel line statistics (11).

bFF, adjusted to pH 7.0, was applied to a column of immobilized Procion Red 3B (Matrex Gel Red A; Amicon, Lexington, Mass.) and proteins were eluted stepwise using 50 mM Tris, pH 7.0, containing 100 mM KCl; 50 mM Tris, pH 7.5, containing 350 mM KCl and 0.5 M urea; 50 mM Tris, pH 7.0, containing 1.2 M KCl and 0.5 M urea, essentially as described by Jansen et al. (5). The last fraction, which contains inhibin activity, was desalted on Sephadex G-25 (Pharmacia, Uppsala, Sweden) in 20 mM Tris, pH 7.9, containing 0.5 M urea. After centrifugation at 200,000 x g for 1 hour) or filtration over 0.22 µm filters, the protein samples were further fractionated on a prepacked Mono Q HR 5/5 anion-exchange column (Pharmacia, Sweden) using a salt gradient from 0-1 M NaCl in 20 mM Tris, pH 7.9, in the presence of 0.5 or 4 M urea. The inhibin containing fractions were pooled and rechromatographed on Mono Q in 20 mM ethanolamine, pH 9.2 with 0.5 M urea using a salt gradient from 0-1 M NaCl. Protein concentrations were estimated by UV-absorption at 280 nm assuming $OD^{1\%}_{280} = 10$. Protein samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12) using a 8-15% acrylamide gradient. Gels were stained either with Coomassie Brilliant Blue R 250 or with silver nitrate using the method described by Wray et al. (13).

Before preparative SDS-PAGE on 3 mm thick gels, protein samples were incubated in SDS-PAGE sample buffer (12) for 30 minutes at 37°C. Proteins were recovered from gel by electroelution (14).

RESULTS

A bioactive fraction of bFF was obtained after chromatography on immobilized Procion Red 3B and Sephadex G-25 (relative specific activity (RSA) 10-18, recovery 50-80%, n=5). This fraction was chromatographed on the Mono Q column at pH 7.9. Protein elution pattern and distribution of bioactivity are shown in fig. 1. Two main protein peaks were obtained: the first while loading the column in the presence of 0.15 M NaCl to prevent protein denaturation, and the second which was eluted with the actual

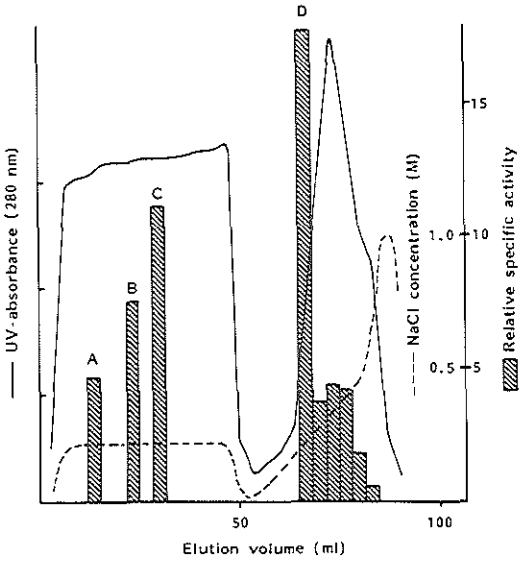

Figure 1
Protein-elution profile of an inhibin-containing fraction of bFF, which was obtained after chromatography on Matrex Gel Red A and Sephadex G-25, on Mono Q at pH 7.9 in the presence of 4 M urea.

salt gradient. Three fractions (fig. 1, A-C) from the first peak contained increasing amounts of bioactivity, whereas further bioactivity could be eluted in a sharp band at an NaCl-concentration between 0.18 and 0.25 M (fig. 1, D). When these fractions were analyzed on SDS-PAGE, the protein composition of fractions A-C appeared to be identical apart from an increase in the intensity of a 65 kD protein (fig. 2, arrow). In fraction D the 65 kD protein was more distinctly present, while the further protein composition of this fraction was different from the fractions A-C.

In subsequent experiments, where the desalted fraction from the immobilized Procion Red 3B column was brought onto the Mono Q column in the absence of 0.15 M NaCl, but in the presence of 0.5

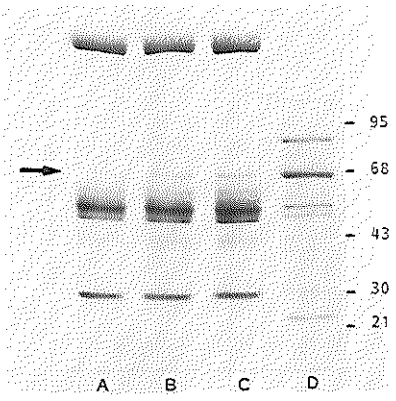

Figure 2
SDS-PAA-gel analysis of inhibin-containing fractions of bFF after Mono Q at pH 7.9 in the presence of 4 M urea (cf. Fig. 1); lanes A-C: fractions loaded in the presence of 0.15 M NaCl; lane D: fraction eluted with NaCl; position of molecular weight markers (in kD) as indicated. Arrow indicates protein, of which the relative amount is correlated with increase in bioactivity (see text).

M urea, all bioactivity was eluted in a single peak at a salt concentration between 0.1 and 0.2 M NaCl. RSA for these fractions varied between 25 and 45, with an overall recovery between 5 and 20% (n=5).

Rechromatography of these latter bioactive fractions on Mono Q at pH 9.2 resulted in the elution of inhibin-like activity at a NaCl-concentration between 0.26 and 0.32 M (fig. 3). The bioactive fraction contained a single 65 kD protein (fig. 4, lane B). Recovery and RSA of this fraction were 3% and 20, respectively.

Finally, when the proteins in the bioactive fraction from the Mono Q run at pH 7.9 were electrophoretically separated on a preparative SDS-PAA-gel, bioactivity (RSA>35) was again found to be associated with a protein with an apparent molecular

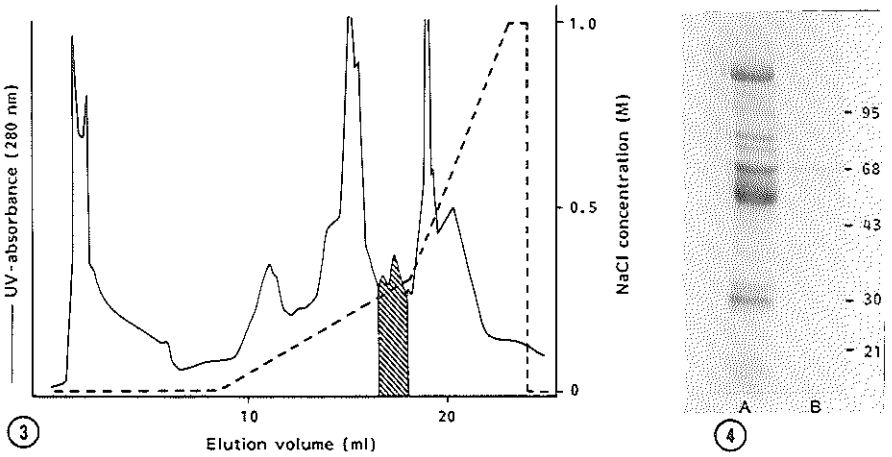

Figure 3
Protein-elution profile of an inhibin-containing fraction of bFF on Mono Q at pH 9.2 in the presence of 0.5 M urea after Matrex Gel Red A, Sephadex G-25 and Mono Q at pH 7.9. Hatched area: bioactivity.

Figure 4
SDS-PAA-gel analysis of inhibin-containing fractions of bFF after Mono Q at pH 7.9 (lane A) and pH 9.2 (lane B); position of molecular weight markers (in kD) as indicated.

weight of 65 kD (fig. 5, lane D) after electroelution of the various gel slices.

DISCUSSION

Studies on the purification of inhibin from different sources using conventional methods for protein purification have resulted in claims that inhibin-like activity is associated with polypeptides of molecular weight ranging from less than 1,500 D (15) to more than 100,000 D (4). Our experiments indicate that ion-exchange chromatography using FPLC can be used in the purification of a protein with an apparent molecular weight of 65 kD, which contains inhibin-like activity. The main advantage of FPLC in the purification of inhibin, when compared to e.g. HPLC as reported earlier (7, 16-18), is that biological activity is

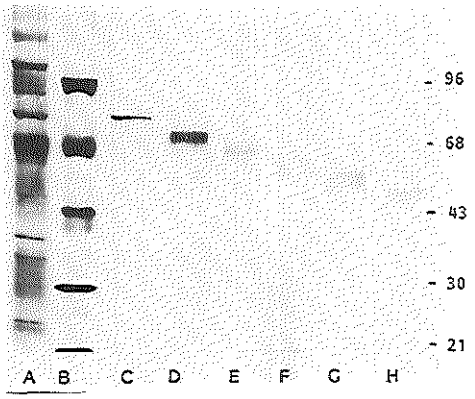

Figure 5
SDS-PAA-gel analysis of proteins electroeluted from preparative Gel Red A, Sephadex G-25 and Mono Q at pH 7.9 (lane A); lane B: marker proteins; lane D-H: electroeluted proteins; lane D contains bioactivity.

not lost during chromatography. Dialysis and lyophilization techniques, as employed after reversed phase-HPLC, may result in a large loss of bioactivity. Specific activities relative to bFF were between 25 and 45 after Mono Q at pH 7.9, with recoveries of activity of about 20%. The relatively low recovery during this step might be caused by denaturation and subsequent loss of protein during storage of desalted fractions, filtration or centrifugation.

The increase of bioactivity (fig. 1), associated with the increased presence of a 65 kD protein in the eluted fractions, (fig. 2) supports the apparent molecular weight of 65 kD for inhibin. After chromatography at pH 9.2 the RSA was increased 20-fold with a recovery of only 3%. This might reflect a pH-dependent inactivation of the biologically active molecule, apart from the factors mentioned above. Moreover, although only a single protein band appears on SDS-PAA gel (fig. 4, lane B), the chromatographic elution pattern suggests the presence of at

least two proteins in this fraction with the same apparent molecular weight, one of which could be inactive.

Finally, electroelution of proteins from an SDS-PAA gel containing a purified inhibin-preparation showed that bioactivity was associated again with a protein (or proteins) with a molecular weight of 65 kD. The observation that the RSA is much lower than expected for a purified protein might be due to denaturation during SDS-PAA gel electrophoresis and electroelution. No estimation of protein content of the electroeluted fraction could be made due to UV-absorption of the glycerol-containing buffer; therefore no data on actual RSA and recovery can be given.

The molecular weight of inhibin as reported here agrees with earlier reports on bovine ovarian inhibin (5, 19), while for inhibin from ovine ovarian follicular fluid and rat testicular extracts apparent molecular weights of 80 kD (7) and 50-60 kD (20) have been reported. For inhibin from human seminal plasma molecular weights of 14,000 D (16) and 5,000 D (17,18) have been reported recently. No indication for "low molecular weight"-inhibin was obtained in the experiments described here.
In conclusion, the experiments presented in this report show that it is possible, using dye-ligand affinity and ion-exchange chromatography, to isolate a 65 kD protein from bovine follicular fluid possessing inhibin-like activity.

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Comparison between inhibin from human and bovine ovarian follicular fluid using fast protein liquid chromatography

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Summary

Inhibin from human and bovine ovarian follicular fluid was purified 700–900-fold using affinity chromatography on immobilized Procion Red 3B, desalting on Sephadex G-25, ion-exchange chromatography on the fast protein liquid chromatography (FPLC) columns Mono Q and Mono P and chromatography on immobilized lectins. Isoelectric points for inhibin from human and bovine origin were between 5.1–5.7 and 4.75–5.25, respectively. Inhibin from both sources was retained by immobilized lectins, indicating its association with a glycoprotein. Overall recoveries of inhibin activity after these chromatographic procedures were approximately 1%.

The function of the gonads is regulated by the hypophyseal hormones lutropin (LH) and follitropin (FSH). The secretion of both LH and FSH is stimulated by the hypothalamic decapeptide LH-releasing hormone, while the secretion of LH is inhibited by gonadal steroids. The secretion of FSH is only partly inhibited by steroids. Another gonadal factor, inhibin (McCullagh, 1932), also plays a role in the negative feedback regulation of the secretion of FSH (Setchell et al., 1977; De Jong, 1979; Grady et al., 1982).

The source, function and physicochemical characteristics of inhibin are not completely elucidated. Ovarian follicular fluid (De Jong and Sharpe, 1976) and testicular rete testis fluid (Setchell, et al., 1977; Baker et al., 1982) are rich sources of inhibin activity. Ovarian granulosa cells (Erickson and Hsueh, 1978; Hermans et al., 1982) and testicular Sertoli cells (Steinberger and Steinberger, 1976; Le Gac and De Kretser, 1982) are capable of producing inhibin activity in vitro. Seminal plasma (Ramasharma et al., 1984; Seidah et al., 1984) and prostatic tissue (Krishnan et al., 1982) also have been reported to contain inhibin-like activity. Part of the observed differences in physicochemical characteristics of inhibin purified from the fluids and tissues that have been studied may be attributed to the various assays used in detecting inhibin activity (De Jong and Robertson, 1985). These assays show large differences in sensitivity and specificity (Baker et al., 1981). Another obstacle in the purification of inhibin from various sources is the apparent atypical behaviour of the hormone (Jansen et al., 1981) using 'classical' protein purification techniques.

Results of partial (Van Dijk et al., 1984) or complete (Robertson et al., 1985) purification of inhibin from bovine ovarian follicular fluid (bFF) using the fast protein liquid chromatography system (FPLC) or HPLC techniques, respectively,

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have been described recently. In the present communication we compare results obtained in the purification of inhibin from human ovarian follicular fluid (hFF) with those obtained with inhibin from bFF.

Materials and methods

Fluid from bovine ovarian follicles (bFF) was collected at a local slaughter-house; fluid from human ovarian follicles (hFF) was collected by Drs. Alberda and Zeilmaker (Rotterdam, The Netherlands) and by Dr. Briët (Meppel, The Netherlands) during oocyte aspiration for in vitro fertilization after treatment with human menopausal gonadotrophins or during diagnostic laparoscopies. bFF and hFF were stored at −20°C without addition of protease inhibitors because of the apparent stability of inhibin activity in follicular fluid (De Jong et al., 1979, 1981). After adjustment to pH 7.0 with 1 M HCl, bFF and hFF were chromatographed on Matrex Gel Red A (immobilized Procion Red 3B; Amicon, Lexington, MA) and the inhibin-containing fractions were desalted on Sephadex G-25 (Pharmacia, Uppsala, Sweden), essentially as described previously (Jansen et al., 1981; Van Dijk et al., 1984).

Ion-exchange chromatography was performed using FPLC (Pharmacia, Uppsala, Sweden) on the anion-exchange column Mono Q at pH 7.9 and 9.2 as described before (Van Dijk et al., 1984); 0.5 M urea and 0.001–0.01% (w/v) Triton X-100 were added to the buffers in order to prevent precipitation of protein and aspecific binding of inhibin to column materials.

The FPLC Mono P chromatofocussing column was equilibrated in 20 mM Bistris-iminodiacetic acid, pH 7.1, containing 0.5 M urea and 0.001% Triton X-100. After loading the sample in this buffer, proteins were eluted with a descending pH gradient developed by Polybuffer 74 (Pharmacia, Uppsala, Sweden) adjusted to pH 4.0 with iminodiacetic acid.

Fractions which contained inhibin activity, collected either during anion-exchange chromatography or during chromatofocussing, were further fractionated using Lentil Lectin-Sepharose or Concanavalin A-Sepharose (Pharmacia, Uppsala, Sweden), which were packed in 0.8 × 5.0 cm columns and equilibrated in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, with or without 0.5 M urea, 0.001% Triton X-100, 0.7 mM $MgCl_2$, 0.7 mM $MnCl_2$ and 0.7 mM $CaCl_2$. Glycoproteins were eluted with the same buffer, containing 0.2 M methyl-α-D-mannoside (Sigma, St. Louis, MO).

Protein content of all fractions was estimated assuming $OD^{1\%}_{280\ nm} = 10$. Inhibin activity was determined using the suppression of spontaneous FSH release from cultured dispersed anterior pituitary cells obtained from male rats (Hermans et al., 1982). Potencies were calculated using a computer program for parallel line statistics (Finney, 1964). The inhibin activity in bovine material was expressed relative to a standard bFF preparation which was given the arbitrary potency of 1. Relative specific activities of human inhibin were expressed relative to the potency of a standard hFF preparation, which was approximately 100 times less potent than the standard bFF preparation.

Suppression of FSH release by standard preparations of bFF and hFF showed no significant divergence from parallelism.

Results

Bovine follicular fluid

The first step used in the purification of inhibin from bFF was chromatography of the fluid on Matrex Gel Red A, followed by desalting on Sephadex G-25. The relative specific activity (RSA) of

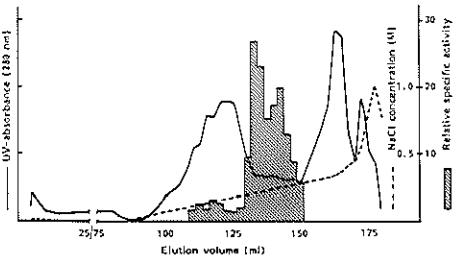

Fig. 1. Protein elution profile of an inhibin-containing fraction from bFF during chromatography on Mono Q at pH 7.9. The starting material was obtained after chromatography on Matrex Gel Red A and Sephadex G-25. The specific activity indicated is relative to bFF = 1.

TABLE 1

RELATIVE SPECIFIC ACTIVITY (RSA) AND RECOVERIES OF INHIBIN FROM bFF AFTER VARIOUS CHROMATOGRAPHIC PROCEDURES

	RSA [a]	Recovery (%)	ng/ml required for maximal suppression of FSH [b]
bFF	1	–	7300
Matrex Gel Red A + Sephadex G-25	30	100	250–500
Mono Q, pH 7.9 (2×)	50–100	11–15	100
Lentil Lectin	330	2	25
Mono Q, pH 9.2	425–870	0.75	16
Matrex Gel Red A + Sephadex G-25	30	100	250–500
Mono P, pH 7.4	18–45	34	

[a] Relative to bFF = 1.

[b] Maximal suppression of FSH release in cultured rat pituitary cell assay (Hermans et al., 1982).

the bioactive fraction was 30 (Table 1). This fraction was chromatographed on the FPLC Mono Q column at pH 7.9 using a shallow Cl^- gradient (Fig. 1). Bioactivity was recovered at a salt concentration of 0.21–0.25 M, while most of the proteins eluted at a lower or higher ionic strength (Fig. 1). Recovery of bioactivity was 20–25%.

Rechromatography of the pooled bioactive fractions on the Mono Q column resulted in an overall 2–3-fold increase in RSA (Table 1); the bioactivity was eluted again at a salt concentration between 0.21 and 0.25 M (data not shown). The inhibin-containing fraction was subsequently loaded onto a Lentil Lectin-Sepharose column and, after extensive washing, bioactivity was eluted with 0.2 M methyl-α-D-mannoside. No bioactivity could be detected in the eluate of the column before addition of methyl-α-D-mannoside. After this step the RSA of the bioactive fraction increased to 330 (Table 1). Salt was removed from the bioactive fraction using Sephadex G-25 and the resulting desalted preparation was chromatographed on FPLC Mono Q at pH 9.2 (Fig. 2). Inhibin activity was recovered at a salt concentration of 0.25–0.29 M with an average RSA of 550 (870 in fraction 51,

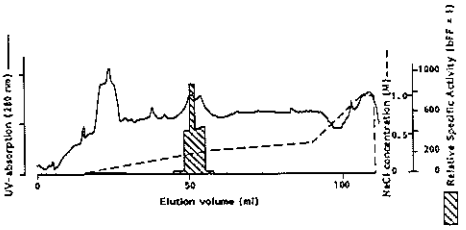

Fig. 2. Protein elution profile during chromatography on Mono Q at pH 9.2 of an inhibin-containing fraction from bFF, which was obtained after chromatography on Matrex Gel Red A, Sephadex G-25, Mono Q (pH 7.9) and Lentil Lectin-Sepharose. Fractions of 1 ml were collected.

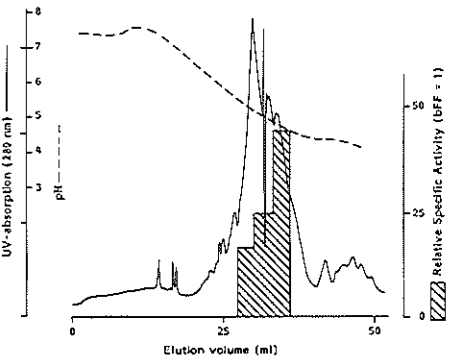

Fig. 3. Protein elution profile after FPLC chromatofocussing from bFF of an inhibin-containing fraction, which was obtained after chromatography on Matrex Gel Red A and Sephadex G-25.

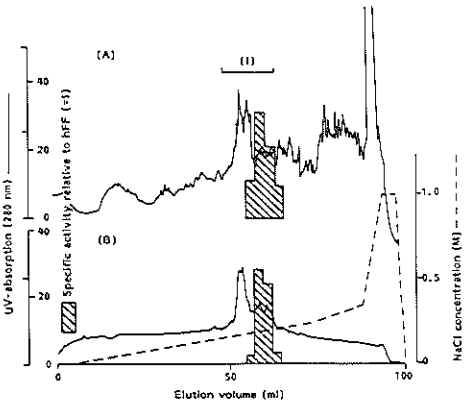

Fig. 4. Protein elution profile of an inhibin-containing preparation from hFF, which was obtained after chromatography on Matrex Gel Red A and Sephadex G-25. *A*: Chromatography on FPLC Mono Q at pH 7.9. *B*: Rechromatography of fraction I on Mono Q at pH 7.9 under identical conditions after desalting on Sephadex G-25.

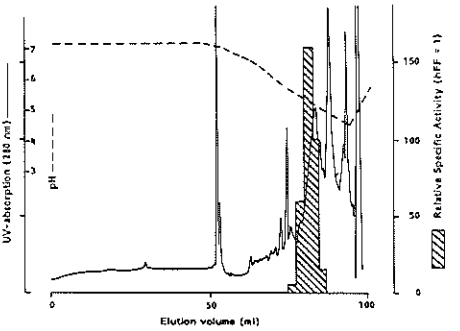

Fig. 5. Protein elution profile after chromatofocussing of an inhibin-containing fraction from hFF, which was obtained after chromatography on Matrex Gel Red A and Sephadex G-25.

425–450 in the fractions 50, 52 and 53; Fig. 2 and Table 1).

When the initial bioactive fraction of bFF, obtained after chromatography on Matrex Gel Red A and subsequent desalting on Sephadex G-25, was fractionated on the Mono P chromatofocussing column with a pH gradient between 7 and 4, the RSA increased only slightly (Fig. 3; Table 1). The isoelectric point of bovine inhibin appeared to be in the range between 4.75 and 5.25.

Human follicular fluid

Inhibin activity from hFF was eluted from Matrex Gel Red A under the same conditions as described above for bovine inhibin (data not shown). The bioactive fraction was desalted on Sephadex G-25 and ion-exchange chromatography was performed on Mono Q at pH 7.9. Bioactivity could be eluted in a narrow zone at an ionic

TABLE 2

RELATIVE SPECIFIC ACTIVITY (RSA) AND RECOVERIES OF INHIBIN FROM hFF AFTER VARIOUS CHROMATOGRAPHIC PROCEDURES

	RSA [a]	Recovery (%)	μg/ml required for maximal suppression of FSH [b]
hFF	1	–	650
Matrex Gel Red A			
+Sephadex G-25	10–30	60–70	20–65
Mono Q, pH 7.9	15–50	27	10–20
Mono Q, pH 7.9			
(rechromatography)		18	
Matrex Gel Red A			
+Sephadex G-25	10–30	60–70	20–65
Mono P, pH 7.4	50–150	20	5
Concanavalin A	720 (eluted)	10	1–1.5
	480 (retained)	2.5	1–1.5

[a] Relative to hFF = 1.

[b] Maximal suppression of FSH release in cultured rat pituitary cells (Hermans et al., 1982).

strength of 0.22–0.26 M NaCl (Fig. 4). The RSA of the fractions varied between 15 and 50 (Fig. 4; Table 2). When the bioactive fractions were pooled, desalted on Sephadex G-25 and rechromatographed on Mono Q at pH 7.9, bioactivity was eluted at the same ionic strength (Fig. 4B). However, no increase in RSA was observed, while the recovery of bioactivity was low (Table 2).

Chromatofocussing on Mono P of an inhibin-containing fraction from the Matrex Gel Red A column resulted in a better resolution than obtained with anion-exchange chromatography on Mono Q and the RSA after Mono P was increased to 50–150 (Fig. 5; Table 2). Inhibin activity eluted between pH values of 5.1 and 5.7.

Finally, chromatography of the inhibin-containing preparation from the Mono P column on Concanavalin A-Sepharose resulted in 2 bioactive fractions: most of the inhibin activity was not bound to the column, while a small fraction was retained. RSAs in these fractions were 720 and 480, respectively (Table 2), with an overall recovery of 10 and 2.5% of the bioactivity.

When inhibin-containing preparations obtained from bFF and hFF after chromatography on immobilized lectins and ion-exchange chromatography at pH 9.2 were analysed on sodium dodecyl sulphate-polyacrylamide gels under non-thiol-reducing conditions, several protein bands with apparent molecular weights between 35 000 and 150 000 could be visualized using a silver staining technique as described previously (Van Dijk et al., 1984). Correlation between bioactivity and the presence of a 65 kDa protein in the same fractions (data not shown) suggests that inhibin is associated with a protein of this apparent molecular weight.

Discussion

Ovarian follicular fluid is the richest source of inhibin. Therefore, many attempts have been made to purify inhibin from bovine (Jansen et al., 1981; Godbout and Labrie, 1984; Van Dijk et al., 1984; Robertson et al., 1985), porcine (Williams et al., 1979; Godbout and Labrie, 1984; Rivier et al., 1984), ovine (Dobos et al., 1983) and human (Chari et al., 1982) follicular fluid. The apparent atypical behavior of inhibin during the application of 'classical' protein purification techniques (Jansen et al., 1981) is one of the reasons that there is no consensus with regard to physicochemical and physiological characteristics of inhibin from follicular fluid: even authors who claimed to have obtained purified inhibin preparations report completely different characteristics for the active molecule from human (Chari et al., 1982), porcine (Rivier et al., 1984) and bovine (Robertson et al., 1985) origin.

Purification of inhibin from bFF using affinity chromatography on Matrex Gel Red A, ion-exchange chromatography on the FPLC system and chromatography on Lentil Lectin-Sepharose resulted in a fraction with an RSA of 870 and an overall recovery of approximately 1% (Table 1). When ion-exchange chromatography on Mono Q was applied to a bioactive preparation of hFF, the increase in RSA was smaller than found for the comparable preparation from bFF (cf. Table 1 and Table 2). Bioactivity from both preparations was eluted at comparable ionic strength (0.21–0.25 and 0.22–0.26 M NaCl for inhibin from bFF and hFF, respectively), indicating similarities in charge distribution at pH 7.9.

Inhibin activities from bFF and hFF were eluted from the Mono P chromatofocussing column at overlapping pH values between 4.75–5.25 and 5.1–5.7, respectively. This again indicates physicochemical similarities between inhibin from both species and is in agreement with values for isoelectric points as reported earlier for inhibin from porcine follicular fluid (5.0–5.8, Williams et al., 1979; 5.5–5.6, Godbout and Labrie, 1984), bovine follicular fluid (5.0–5.7, Jansen et al., 1981; 4.6–5.1, Godbout and Labrie, 1984) and human follicular fluid (5.2–5.4, Chari et al., 1982).

Inhibin activity from bFF can be bound to Lentil Lectin-Sepharose (Table 1). This is in agreement with the reported glycoprotein character of inhibin from bovine (Jansen et al., 1981; De Jong et al., 1982a; Godbout and Labrie, 1984) and porcine (Godbout and Labrie, 1984) follicular fluid. However, inhibin activity from hFF is only partly retained by Concanavalin A-Sepharose. This might indicate heterogeneity of the supposed carbohydrate moiety of the hormone to such an extent that only a small fraction is bound to concanavalin A. An alternative explanation could

be the observed leakage of concanavalin A monomers from Concanavalin A-Sepharose (De Jong et al., 1982b).

The overall recovery of bioactivity from both bFF and hFF after chromatography on immobilized lectins is rather low; this could indicate aspecific interactions between the hormone and the column matrices, as observed earlier (Jansen et al., 1981), despite the presence of urea and Triton X-100.

The correlation between presence or absence of bioactivity with the presence or absence of a 65 kDa protein in various fractions obtained during this study is in agreement with earlier observations (Jansen et al., 1981; De Jong et al., 1982a, b, 1984; Van Dijk et al., 1984). A protein band with an apparent molecular weight of 56 000, as recently purified from bFF by Robertson et al. (1985), was not present in these preparations. Whether the bioactive material purified by a number of authors from several origins represents possible subunits or proteolytic degradation products of the same parent molecule will only be clarified when the amino acid sequences of the reported inhibin-like molecules can be compared.

The present results provide the first data on the purification of inhibin from hFF using high-performance liquid chromatography techniques without any precipitation, dialysis and lyophilization as employed in reversed-phase high-performance liquid chromatography. This is also the first comparison between the behaviour of inhibin activities from bFF and hFF using similar chromatographic purification techniques. The similarities in chromatographic behavior of human and bovine inhibin suggest similarities in molecular structure. A final comparison between human and bovine inhibin awaits the further purification of the bioactive hormone from both species.

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SEXUAL DIMORPHISM IN IMMUNONEUTRALIZATION OF BIOACTIVITY OF RAT AND OVINE INHIBIN

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Running title: Sexual dimorphism of inhibin

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ABSTRACT

Inhibin was partially purified from bovine follicular fluid using chromatography on immobilized Procion Red 3B and anion-exchange chromatography. Ovariectomized Texel ewes were immunized against the inhibin-containing fraction from the Procion Red 3B column and the immune response was subsequently boosted with similar fractions or with the preparation obtained from the anion-exchange column. The potencies of the resulting antisera were evaluated in an in-vitro bioassay system for estimating inhibin activity, using dispersed rat pituitary cells. The antisera were found to inhibit the bioactivity of inhibin preparations from ovarian follicular fluid of either bovine, porcine, ovine or human origin, as well as inhibin activity in ovine testicular lymph and rete testis fluid, in culture media from rat granulosa and rat Sertoli cells and in homogenates of rat ovaries and testes. These results indicated that the inhibin molecules from several species contain a common bioactive moiety. The results also showed that the antiserum was more effective in neutralizing inhibin activity from ovarian than from testicular sources in both sheep and rat, indicating a sex-related difference in the inhibin molecules within a species.

INTRODUCTION

Inhibin, a protein hormone which specifically suppresses the pituitary secretion of follicle-stimulating hormone (FSH), has been detected in several gonadal fluids (de Jong & Robertson, 1985) and in medium of cultured Sertoli (Steinberger & Steinberger, 1976) and granulosa (Erickson & Hsueh, 1978) cells. It is not clear, however, whether the demonstrated activities from male and female gonads represent the same molecule or a set of related or even completely unrelated molecules.

Antibodies have been used in immunochromatographic procedures for the purification and characterization of inhibin (Williams, Rush & Lipner, 1979; de Jong, Jansen, Steenbergen et al., 1983). Antibodies raised against porcine follicular fluid (pFF)(Channing, Tanabe, Turner & Hodgen, 1982) or against partially purified inhibin from bovine follicular fluid (bFF) (de Jong, Jansen, Hermans & van der Molen, 1982 a,b) were shown to neutralize inhibin activity in vivo in rhesus monkeys and in vitro in porcine, human (Channing et al., 1982) and bovine (de Jong et al., 1982a) follicular fluids.

For the present studies we have raised antibodies against partially purified inhibin from bFF in sheep. This report describes the use of this antiserum to neutralize inhibin activity from male and female sources.

MATERIALS AND METHODS

Inhibin-containing preparations

Rat Sertoli cells were isolated and cultured as described by Ultee-van Gessel, Leemborg, de Jong & van der Molen (1985); rat testicular homogenates were prepared according to the method of Au, Robertson & de Kretser (1983). Rat granulosa cells were isolated and cultured as described by Sander, van Leeuwen & de Jong (1984); rat ovarian homogenates were prepared as described by Sander, Meijs-Roelofs, Kramer & van Leeuwen (1985). Bovine, porcine and ovine follicular fluids were aspirated from ovaries collected at a slaughterhouse. Human follicular fluid (hFF) was collected during oocyte aspiration for in-vitro fertilization. The concentrations of protein in follicular fluids was 65 g/l. Follicular fluids were centrifuged for 30 min at 5,000 x g to remove cellular debris. Ovine testicular lymph (oTL; 40 g protein/l) and ovine rete testis fluid (oRTF; 1.5 g protein/l) were gifts of Prof. B.P. Setchell (Waite Research Institute, Adelaide, Australia). All inhibin preparations were stored at -20 °C until used. No protease inhibitors were added because of the reported stability of inhibin-containing preparations (de Jong, Jansen and van der Molen, 1981; Baker, Eddie, Higginson, Hudson and Niall, 1982).

Immunization

Four ovariectomized Texel ewes were immunized with 1 mg protein consisting of an approximately 20- to 30-fold purified inhibin-containing preparation obtained after chromatography of bFF on immobilized Procion Red 3B (Amicon, Lexington, MA, USA) (Jansen, Steenbergen, de Jong and van der Molen, 1981). This preparation was emulsified in Freund's complete adjuvant and injected subcutaneously. Booster doses were administered 7 and 12 weeks later using the same preparation in GNE-adjuvant (Intervet, Boxmeer, The Netherlands). After 6 months the two ewes with the highest serum titre of antibodies were boosted again with 350 µg protein consisting of an approximately 80- to 100-fold purified inhibin-containing preparation, obtained after chromatography of bFF

on Procion Red 3B, Sephadex G-25 and anion-exchange chromatography (van Dijk, de Jong and van der Molen, 1984; van Dijk, Steenbergen, de Jong and van der Molen, 1985). Blood was collected in vacutainers from the jugular vein of ewes before immunization and at regular time intervals after boosting. After clotting of the blood and centrifugation, the serum was stored at -20°C until assayed for the presence of antibodies against inhibin.

Immunoneutralization

The presence of immunoneutralizing antibodies in these ovine sera was evaluated by addition of the antisera, together with bFF, to cultured dispersed rat pituitary cells (Hermans, van Leeuwen, Debets et al., 1982). After a 3-day preculture period, culture media were changed and increasing amounts of inhibin-containing preparations added. In parallel culture wells, increasing amounts of the antisera or pre-immune serum were added to the amount of inhibin activity which had a maximum FSH-suppressing effect.

In a second set of experiments, dose-response curves of inhibin-containing preparations were made in the absence and presence of fixed amounts of the antiserum. After a 3-day culture period media were collected for assay of FSH and luteinizing hormone (LH). The same test substances were added to the culture wells, together with 80 nmol LH-releasing hormone (LH-RH)/l (Hoechst, Frankfurt, FRG) and the incubation was continued for 6 hours. These media were also collected and assayed for FSH and LH. The potencies of the inhibin preparations obtained in the presence of antiserum were expressed as the "apparent relative potencies" as compared with the dose-response curve without addition of antiserum, for which the potency was defined as 1. The apparent relative potency was estimated using parallel line statistics (Finney, 1964). The precision index of the bioassays was 0.152 ± 0.021 (SEM, n=10; range 0.059 - 0.249); no significant deviation from parallelism or linearity was observed.

RESULTS

Sera from two of the four ewes that were immunized against bovine inhibin could be shown to immunoneutralize bovine inhibin *in vitro* (Fig. 1). After several booster injections, however, only one ewe continued to give a positive response (Fig. 1, solid line). Our studies were continued with antisera from

this particular ewe.

Addition of 25 µl non-immune serum of ovariectomized ewes to the pituitary cell culture did not significantly affect the release of either FSH or LH irrespective of the presence or absence of bFF (data not shown). When 5 µl antiserum were added to the culture medium in the absence of bFF, FSH levels were 105 ± 20% (SD, n=4) and LH levels were 85 ± 15% (n=4) of control values. Data on the inhibin-neutralizing activity of this antiserum are summarized in Fig. 2. Addition of different volumes of the antiserum together with the maximally suppressing amount of bFF caused a dose-dependent increase of FSH-release. Subsequently, similar immunoneutralization experiments were performed using inhibin-containing preparations from other species. These preparations were added to the pituitary cells in amounts which caused a significant, although not maximal, suppression of FSH release, in the presence of increasing amounts of the antiserum against bovine inhibin (Figs 3A and B). Addition of the antiserum neutralized inhibin activity from several species and sour-

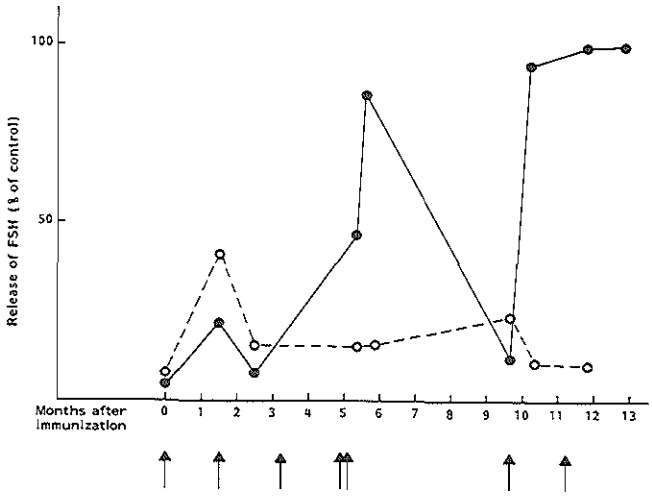

Figure 1
Effect of the addition of 25 µl serum of two different ewes (● and o) which were immunized against preparations containing bovine inhibin together with a maximally suppressing dose of bovine follicular fluid (bFF; 112.5 nl), on FSH-release by cultured rat anterior pituitary cells. Sera were collected at regular time-intervals after the primary injection and subsequent boosting of the ewes (indicated by the arrows). FSH values are expressed as percentage of the differences between concentrations of FSH in control dishes and in dishes with a maximally suppressing dosis of bFF.

ces. However, the amount of antiserum required to increase FSH release to the control level varied considerably: 0.25 µl antiserum was sufficient to completely neutralize a maximally suppressing amount of bovine inhibin activity, but 0.5-1.0 µl of antiserum was needed for neutralization of inhibin activbity in ovine follicular fluid (oFF) and 1-2 µl for immunoneutralization of inhibin activity in pFF, hFF, oTL and oRTF.

To obtain more quantitative data on the immunoneutralization of inhibin from various species, dose-response curves of the suppression of FSH release from the pituitary cells were made using various amounts of bFF, oFF, oTL and oRTF in the presence or absence of a fixed amount of antiserum (Fig. 4). The shift of the suppression curves towards higher doses of inhibin-containing materials corresponds with the decrease in apparent relative potency, as sum-

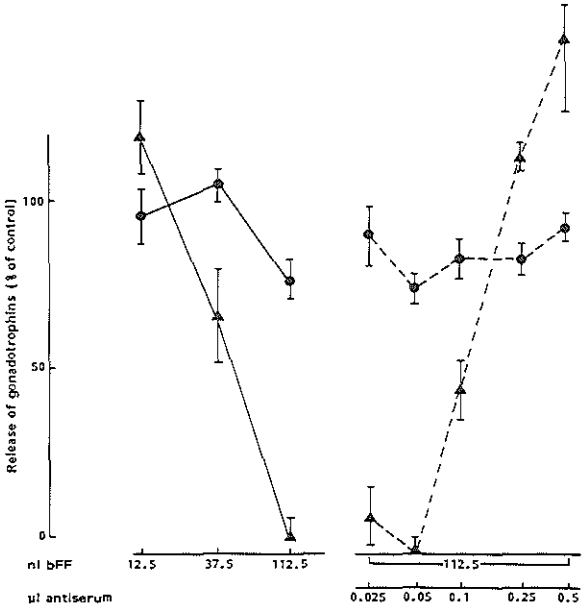

Figure 2
Effect of addition of (a) different amounts of bovine follicular fluid (bFF) or (b) inhibin antiserum in the presence of a maximally FSH-suppressing amount of bFF (112.5 nl) on the release of FSH (▲) and LH (●) from cultured rat anterior pituitary cells. Values are means ± SEM (n=4) and are expressed as percentage of the differences between concentrations of FSH in control dishes and in dishes with a maximally FSH-suppressing amount of bFF. Values for LH were expressed as percentage of control dishes. Actual concentrations were: FSH, 718 ± 26 ng/ml (control) and 321 ± 22 ng/ml (with maximal amount of bFF); LH, 660 ± 35 ng/ml (control) and 509 ± 42 ng/ml (with maximal amount of bFF).

marized in Table 1 for bFF. Doubling the amount of antiserum added to the cells in the presence of bFF caused a 50% decrease in relative potency. In LHRH-stimulated pituitary cell cultures, not only a suppression of FSH release by bFF (to 36 ± 2% (SEM, n=4) of release of FSH without any addition) was observed, but also LH release was suppressed to 65 ± 5% (SEM, n=4) of LH-levels without treatment. The inhibin antiserum interfered with the suppression of FSH and LH release as shown by the results in Table 1 for the apparent relative potency in the presence of the antiserum in LHRH-stimulated pituitary cell culture medium.

The apparent relative potency of oFF decreased to 0.23 in the presence of 1 µl of the inhibin antiserum, whereas addition of the same amount of antiserum suppressed the apparent relative potencies of oRTF and oTL to significantly higher values (Table 2).

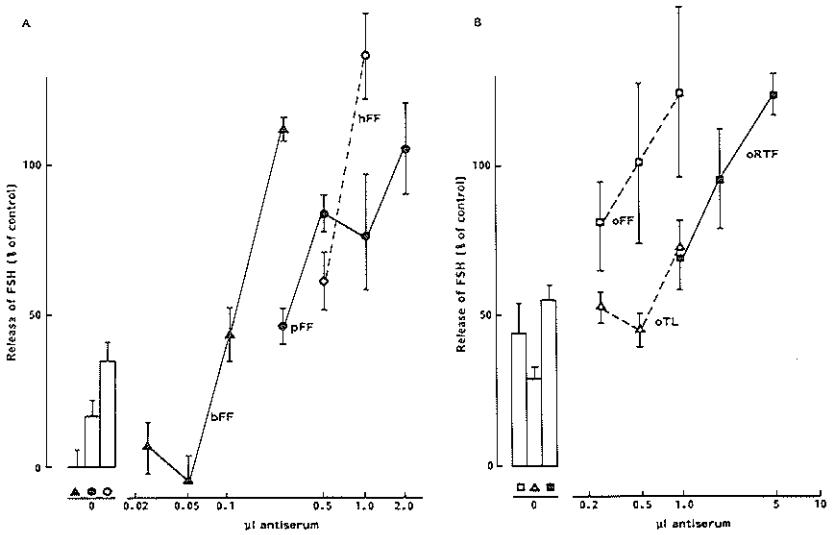

Figure 3
Effect of addition of inhibin antiserum on the suppression of FSH release from cultured rat anterior pituitary cells caused by (a) follicular fluid (FF) of various species and (b) inhibin-containing gonadal fluids from sheep. Bars represent FSH release in dishes to which the inhibin preparation was added without the antiserum. Values are means ± SEM (n=4) and are expressed as percentage of the differences between concentrations of FSH in control dishes and in dishes with a maximally FSH-suppressing amount of bovine FF. The inhibin preparations added to 1 ml of culture medium were bovine FF (▲, 7.2 µg protein), porcine FF (●, 6.5 µg protein), human FF (○, 650 µg protein), ovine FF (□, 1.6 µg protein), ovine testicular lymph (△, 800 µg protein) and ovine rete testis fluid (■, 37.5 µg protein).

Using a similar experimental protocol the capacity of theantiserum to neutralize the inhibin activity in culture media of rat granulosa and Sertoli cells and in homogenates of rat ovaries and testes was investigated using 5 µl inhibin antiserum. Results of this experiment are presented in Fig. 5.

The apparent relative potency of inhibin in rat granulosa cell culture medium (rGCCM) after addition of 5 µl inhibin antiserum was significantly lower than the apparent relative potency of inhibin in Sertoli cell culture medium (rSCCM) in the presence of the same amount of antiserum (Table 2). The apparent relative potency of inhibin in rat ovarian homogenate (rOH) in the pre-

Table 1: Apparent relative potencies of bovine follicular fluid (bFF) after addition of ovine inhibin antiserum. Potencies for FSH release were estimated in the absence or presence of LHRH in the culture medium; for LH release only values in the presence of LHRH are given. Potencies are expressed relative to the FSH or LH-suppressing activity of the same amount of bFF without antiserum; 95% confidence limits are given in parentheses.

	Apparent Realative Potency	
Treatment	FSH	LH
antiserum (0.1 µl)	0.59 (0.53 - 0.67)	-
antiserum (0.2 µl)	0.29 (0.26 - 0.33)	-
antiserum (0.2 µl) + LHRH	0.25 (0.19 - 0.33)	0.25 (0.15 - 0.43)

sence of antiserum was lower than for inhibin activity in rat testicular extracts (rTE) (Table 2).

Apart from these data on apparent relative potencies after immunoneutralization of inhibin from various sources using the antiserum against bovine ovarian inhibin, calculated cross-reactivities of inhibin from various species are shown in Table 2.

DISCUSSION

The present results show that it is possible to raise antibodies against bovine inhibin in ovariectomized ewes. The resulting antiserum neutralizes

bovine inhibin activity in a rat anterior pituitary cell assay and shows cross-reactivity with inhibin activity of porcine, ovine, human and rat origin.

The number of reports on immunoneutralization of inhibin activity, using the in-vitro bioassay with dispersed rat pituitary cells, is limited. Channing pFF in ovariectomized rhesus monkeys which counteracted the effect of both pFF and hFF in rat anterior pituitary cell cultures, without affecting LH release. de Jong et al. (1982a) raised an antiserum in rabbits against a partially

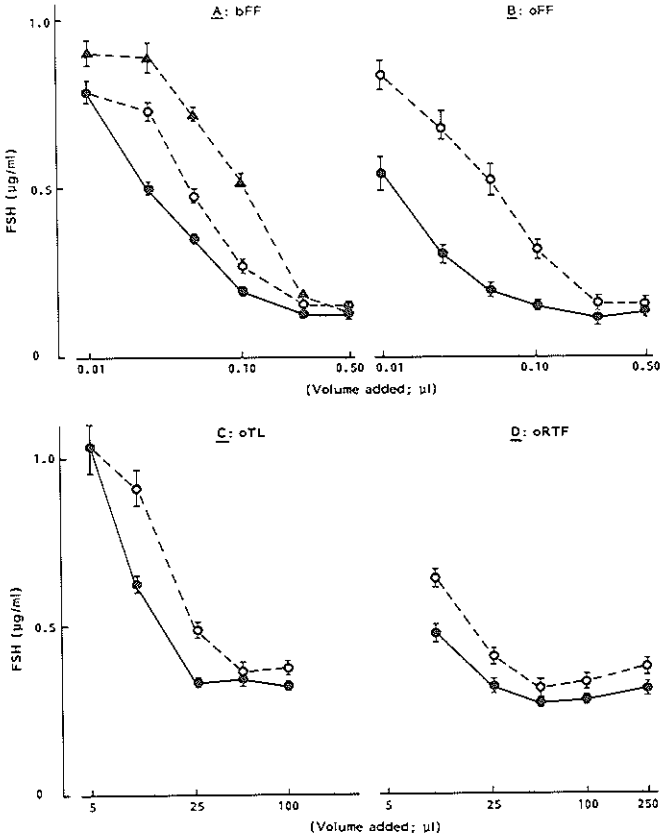

Figure 4
Effect of addition of inhibin antiserum on the suppression of release of FSH from cultured rat anterior pituitary cells caused by addition of various amounts of inhibin-containing preparations.
A: effect of various amounts of bovine follicular fluid (bFF) alone (●) and bFF with 0.1 (○) and 0.2 (▲) µl antiserum.
B-D: effect of the addition of 1.0 µl of antiserum to ovine follicular fluid (oFF) (B), ovine testicular lymph (oTL) (C) and oRTF (D).
Solid line: without antiserum; broken line: with antiserum. Values are mean ± SEM, n=4.

Table 1: Apparent relative potencies, immunoneutralization and cross-reactivity of inhibin from various species and sources after addition of ovine inhibin antiserum

	Apparent relative potency	Volume of antiserum (µl)	INA	INA/µl	Percentage cross-reactivity
Preparation					
bFF	0.29 (0.26-0.33)	0.2	0.71	3.55	100
hFF	0.61	0.5	0.39	0.78	22
pFF	0.75/0.43	0.25/0.5	0.25/0.57	1.07	30
oFF	0.23 (0.15-0.32)	1.0	0.77	0.77	22
oRTF	0.58 (0.45-0.74)	1.0	0.42	0.42	12
oTL	0.57 (0.48-0.68)	1.0	0.43	0.43	12
rGCCM	0.07 (0.03-0.13)	5.0	0.93	0.19	5.3
rSCCM	0.24 (0.14-0.39)	5.0	0.76	0.15	4.2
rOH	0.28 (0.19-0.39)	5.0	0.72	0.14	3.9
rTE	0.48 (0.34-0.68)	5.0	0.52	0.10	2.8

Apparent relative potency is expressed relative to the preparation without antiserum; when available, 95% confidence limits are given in parentheses. INA, immunoneutralizing activity, expressed as 1 - apparent relative potency. Cross-reactivity is expressed as a percentage ratio of (INA/µl preparation of interest): (INA/µl bovine follicular fluid, bFF). hFF: human FF; pFF: porcine FF; oFF: ovine FF; oRTF: ovine rete testis fluid; oTL: ovine testicular lymph; rGCCM: rat granulosa cell culture medium; rSCCM: rat Sertoli cell culture medium; rOH: rat ovarian homogenate; rTE: rat testicular extract.

purified bovine inhibin preparation. They used this antiserum in an additional purification step and in immunological characterization of bovine inhibin (de Jong et al., 1982b; de Jong, van Dijk, Steenbergen and van der Molen, 1984).

The present ovine antiserum against partially purified bovine inhibin was most potent in blocking inhibin activity from bFF, while inhibin activity from hFF, pFF, ovine inhibin-containing preparations (Figs 3 and 4) and rat inhi-

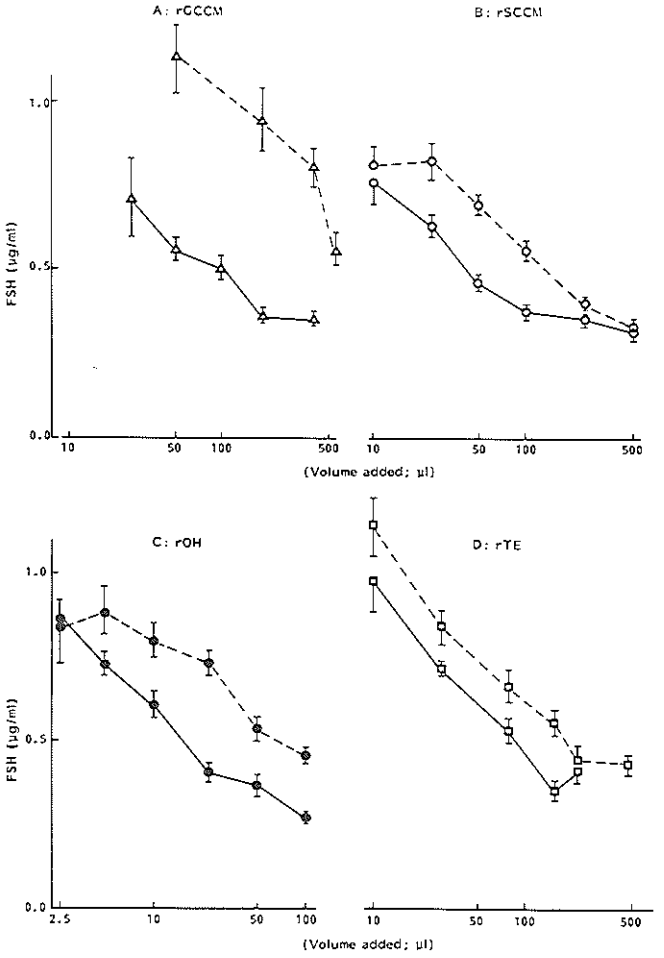

Figure 5
Effect of the addition of 5 µl inhibin antiserum on the suppression of the release of FSH from cultured rat anterior pituitary cells caused by the addition of different amounts of inhibin-containing gonadal preparations from rats.
Panel A: rat granulosa cell culture medium (rGCCM). Panel B: rat Sertoli cell culture medium (rSCCM). Panel C: rat ovarian homogenate (rOH). Panel D: rat testicular extract (rTE). Solid line: preparations without antiserum; broken line: preparations with antiserum. Values are means ± SEM, n=4.

bin-containing preparations (Fig. 5) were less effectively neutralized. These observations indicate that the antiserum is effective in blocking the bioactivity of the inhibin molecule from various species and suggest that the specific structure responsible for the bioactivity of inhibin molecules is immunologically similar in the species used in our study. In this respect the neutralization of inhibin activity from hFF is surprising: approximately 100 times more hFF than bFF protein was required to obtain suppression of FSH in the in vitro bioassay. On the other hand, only two-four times the amount of antiserum needed to block the activity of bovine inhibin was necessary to immunoneutralize human inhibin activity. We have suggested that a 100-fold excess of hFF was needed due to species differences in the in-vitro assay (van Dijk et al., 1985). However, the behaviour of human inhibin towards immunoneutralization might also be explained by assuming a 25- to 50-fold lower concentration of inhibin in the hFF preparations. An alternative explanation could be that the antiserum is not directed to a highly conserved bioactive centre, but to a well-conserved receptor-binding site in the inhibin molecule. In this latter case the difference between the bioactive response and immunoneutralization may reflect species differences.

de Jong, Welschen, Hermans et al. (1979) have shown that in LHRH-stimulated pituitary cell assays, bFF suppressed FSH as well as LH release, although the latter was suppressed to a lesser degree. The suppression of LH was attributed to a low molecular weight factor which had no influence on spontaneous FSH release, but had a slightly stimulating effect on spontaneous LH release. Scott, Burger & Quigg (1980), using oTL, demonstrated that in an LHRH-stimulated pituitary cell culture, FSH and LH secretion were suppressed, and the FSH content in the cell was reduced, while the LH content was not affected. Our observations indicate that LHRH-induced LH suppression can be immunoneutralized eith the present antiserum (Table 1). These results suggest that the LHRH-induced suppression of LH release into the culture medium of rat pituitary cells is an intrinsic effect of inhibin, unless it is postulated that two different gonadal effector molecules are neutralized by antibodies with the same titer and affinity in this antiserum.

The results of the experiments depicted in Figs 4 and 5 indicate that the antiserum neutralizes inhibin activity from the ovary more effectively than inhibin activity from the testis in the same species. In the presence of antiserum, the relative shift of dose-response curves was greater when inhibin activity from female sources was used than with inhibin from the corresponding male source. This shift was reflected in a smaller apparent relative potency

of inhibin preparations from female sources in the presence of antiserum (Table 2), resulting in a lower cross-reactivity of inhibin from testicular sources. Furthermore, it is evident that the inhibin activity in the culture media is more effectively neutralized than in the gonadal homogenates, resulting in a lower apparent relative potency (Table 2) and higher cross-reactivity, suggesting differences between secreted and non-secreted forms of inhibin.

These differences in immunoneutralization may reflect a heterogeneity and sexual dimorphism of inhibin-like activity within one species. This heterogeneity may involve differences in glycosylation (Franchimont, Lecomte-Yerna, Henderson et al., 1983; de Jong et al., 1983; Godbout & Labrie, 1984a,b; Lecomte-Yerna, Hazee-Hagelstein, Charlet-Renard & Franchimont, 1984; Sairam, Kato, Manjunath & Ramasharma, 1984; van Dijk et al., 1985). Deglycosylation of LH and human chorionic gonadotrophin (hCG) (Sairam & Schiller, 1979; Manjunath & Sairam, 1982) can influence the biological activity of the hormone without affecting immunological properties. Differences in glycosylation of FSH have been found to correspond with differences in receptor binding and in-vitro and in-vivo biological activity (Blum & Gupta, 1985; Foulds & Robertson, 1985). Alternatively, different post-translational processing of the pro-inhibin molecule, which contains a number of possible hydrolytic sites (Mason, Hayflick, Ling et al., 1985), in male and female gonads may be the cause of these differences; in view of the stability of inhibin at room temperature (de Jong et al., 1981; Baker et al., 1982), it is unlikely that differences between male and female inhibin have appeared as a result of differential proteolytic degradation during storage at -20°C. More detailed structural studies will be necessary to decide between these possibilities.

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