STUDIES OF A SPECIFIC ANDROGEN-BINDING PROTEIN IN BOVINE FOLLICULAR FLUID

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY, FACULTY OF MEDICINE

THE UNIVERSITY OF MANITOBA

WINNIPEG, MANITOBA

JULY, 1983

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

A specific androgen binding protein has been identified in bovine follicular fluid. In cattle, androgen binding activity has been observed to be higher in follicular fluid aspirated from mature ovarian follicles than in serum or extracts of epididymis. The follicular fluid androgen binding activity has been shown to have higher affinities for dihydrotestosterone and testosterone than for estrogen. Partially purified preparations of androgen binding activity from bovine follicular fluid appeared to be unstable below pH 7.0 and very heat labile, but became relatively stable at $4^{\circ}C$ in Tris-HCl buffer, pH 7.4 in the presence of 10% glycerin. Analyses by DEAE-cellulose chromatography and polyacrylamide gel electrophoresis of partially purified active fractions revealed differences in protein patterns and amount of binding activity in follicular fluid and serum. These observations indicated the presence of a specific androgen binding protein in the follicular fluid.

Purification of bovine ff-ABP, has been found to be successful by a combination of 7 steps of different experimental conditions, including ammonium sulfate precipitation, DEAE-cellulose chromatography, affinity chromatography on Sepharose-4B conjugated with Concanavalin A, and gel filtration on Sephadex G-200. Highly purified ff-ABP has been prepared from 400 ml bovine follicular fluid with a yield of 0.536 mg.

Purified bovine ff-ABP has been shown to be a homogenous protein with high affinity binding for ^{3}H-DHT upon steady state polyacrylamide

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gel electrophoresis. The isoelectric point (pI) of ff-ABP has been demonstrated to be pH 4.75 by analytical isoelectrofocusing in polyacrylamide gel. The molecular weight of ff-ABP has been estimated to be 110,000 by gel filtration on Sephadex G-200, and 80,000 by polyacrylamide gel electrophoresis in the presence of SDS. No subunit-structure for ff-ABP has been observed. Biologically, purified ff-ABP appeared to have high binding affinities for dihydrotestosterone and testosterone, but negligible binding for estradiol-17β. These findings indicate that ff-ABP is a specific androgen binding protein in the follicular fluid, and is definitely chemically, and possibly functionally, different from testicular ABP and serum TeBG.

ACKNOWLEDGEMENTS

Sincere appreciation is extended to my thesis advisor, Dr. K.W. Cheng, who has provided critical supervision and invaluable advice throughout this investigation as well as constructive comments during the preparation of this thesis. Sincere thanks are also extended to Dr. R.P.C. Shiu for his helpful suggestions.

I would also like to express my appreciation to Drs. M. Robertson, J. Klindt and I. Worsley for their cooperation and helpful discussions. The mutual support and encouragement of V.W.S. Tang and S. Schellenberg have also been a source of inspiration.

The technical assistance from H. Sy, E. Ng and G. Lagadi is also a part of this successful investigation. The preparation of this thesis would not be possible without the help of A. Kwan and R. Sanii.

Finally, special thanks are extended to my mother and my sister for their financial and moral support throughout my study in Canada.

LIST OF ABBREVIATIONS

PREFIX FOR SPECIES

b = bovine

c = canine

h = human

r = rat

rb = rabbit

UNITS OF MEASUREMENT

g = gram

mg = milligram

µg = microgram

ng = nanogram

ρg = picogram

ml = milliliter

µl = microliter

mm = millimeter

min = minute

M = molar

mM = millimolar

cpm = counts per minute

Ka = association constant

CHEMICAL REAGENTS

SDS = sodium dodecyl sulfate

PAGE = polyacrylamide gel electrophoresis

TEG = 20 mM Tris-HCl, 2 Mm EDTA and 10% glycerin

TG = 20 mM Tris-HCl and 10% glycerin

SS-PAGE = steady state polyacrylamide gel electrophoresis

HTP = hydroxylapatite

DEAE = diethyl aminoethyl cellulose

Con A= Concanavalin A

GENERAL TERMS

ff = follicular fluid

ABP = androgen binding protein

TeBG = testosterone binding globulin

SBG = steroid binding globulin

SHBG = sex hormone binding globulin

DHT = dihydrotestosterone

T = testosterone

E = estrone

E_2 = estradiol-17β

E_3 = estriol

DHA = dihydroepiandrosterone

DIOL = androstanediol

P = progesterone

DMS = dexamethansone

Ald = aldosterone

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INTRODUCTION

A binding protein is defined as a protein for the transport of a specific hormone(s) from the site of its synthesis to its target cells to facilitate its association with cellular receptors for the expression of its biological activity.

At present, four serum proteins have been identified as binding proteins to interact with steroid hormones: albumin (69K), α_1-acid glycoprotein (41K), corticosteroid binding globulin (CBG or transcortin, 52K) and testosterone binding globulin (TeBG, 70K)(8). Since the discovery of thyroxine binding globulin (58), a whole class of related hormone binding globulins in plasma has become recognized. For glucocorticoid, higher vertebrates have been shown to possess corticosteroid binding globulin, which varies somewhat in structure and properties from species to species (35,36,165,200). For human CBG, cortisol and progesterone bind with high affinities but testosterone binds poorly. The existance of a separate sex hormone binding globulin (hSBG, SBP, TeBG and 17β-hydroxy-steroid binding globulin) in man was suggested by the experiments of Daughaday (36) on testosterone binding in plasma and was clearly demonstrated by Mercier et al. (113). Later, several other steroid binding plasma proteins have been demonstrated, including the ones for progesterone in pregnant guinea-pigs, estradiol in rats and sex hormone binding globulin in rabbits, and similarly in human plasma (148, 203). Similar sex hormone binding protein has also been identified in most primates (104).

Apart from binding proteins in plasma, an androgen binding protein (ABP) has also been demonstrated in the epididymis and testis of the rat (12,154,192), rabbit (33,152) and other species such as the bull (66), the monkey (83) and the ram (23,64,83). ABP has different physical and chemical properties and functions from cytosolic receptors (189).

I. ANDROGEN BINDING PROTEIN IN TESTIS AND EPIDIDYMIS

Studies in several laboratories have shown that rat epididymis contains androgen binding proteins both in cytoplasm and nuclei (12,60, 63,70,154). The cytosolic fraction contains at least two different 5α-DHT binding proteins (65). One of these appears quite similar to the androgenic receptor found in the cytosolic fraction of the ventral prostate. The other androgen binding protein (ABP) resembles human testosterone binding globulin (TeBG)(60,65,66) and has subsequently been shown to be located in the epididymal lumen (53). The disappearance of ABP from the ipsilateral epididymis cytosolic fraction 3 weeks after hemicastration suggests the testicular origin of ABP (53). Vernon et al. (192) have demonstrated that epididymal and testicular cytosol contains a heat stable binding protein (ABP) with high affinity for androgens and estrogens but not antiandrogens. Tindal et al. (189) have reported the production of ABP by Sertoli cells, and this observation has been confirmed by Sertoli cell cultures (120). Although ABP has been most extensively studied in the rat, a similar protein has also been identi-

fied in the epididymis or testis of several other species (162).

1. PHYSIOLOGY OF ANDROGEN BINDING PROTEIN

ABP has been localized in the lumen of the epididymis. It has also been measured in high concentrations in testicular efferent duct fluid, and ligation of these ducts causes an increase of ABP in the testis and a decrease in the epididymis (65,66). French and Ritzen (102) have suggested that ABP is either degraded in the lumen of the caput or taken up by a particulate fraction of the caput epithelial cells. In both the rat and rabbit the concentration of ABP is much higher in the efferent duct fluid than the epididymis and the testis (73). Within the epididymis, the amount in the caput is higher than that in the cauda, but the segmental differences in rabbit are much greater than those in the rat (73). The concentration of ABP gradually decreases from the proximal caput to the cauda, indicating that ABP loses binding activity as it passes through the lumen, where it is taken up and degraded by the epithelium.

The concentration of ABP in the testis and epididymis appeared to correlate with the age of the animal (69). ABP was found to increase in the caput epididymis at 5 weeks of age with the highest level at the 8^{th} week, then it subsequently decreased to a stable level in adult rats of 16-32 weeks old. This peak concentration of ABP occurs at the age when mature sperm were first observed in the testis and shortly before the age when spermatogenesis was fully developed (185). The high con-

centration of ABP in the caput epididymis in early puberty was caused by a high production rate of ABP in the testis and not due to a lower rate of elimination in the epididymis. At this age the seminiferous tubular epithelium was undergoing extremely rapid growth and the level of FSH in plasma was concomitantly greatly elevated (185). A similar increase of ABP in caput epididymis has been demonstrated in developing rabbits (170).

Hypophysectomy of 5 week-old rats causes a complete disappearance of ABP from the testis by eight weeks of age (69), indicating that the production of ABP in the testis was controlled by the pituitary gland. The dramatic changes of ABP after hypophysectomy have prompted investigations on the possible role of the gonadotropins in the regulation of ABP production. FSH causes a dramatic increase of ABP both in the testis and caput epididymis, while LH treatment has no significant effect. Hypophysectomized rats likewise show an increase in ABP following treatment with FSH but not with LH or testosterone propionate (69).

It is well established that gonadotropic hormones are of central importance for the initiation and maintainance of spermatogenesis in mammals. It is also known that testosterone administration can maintain spermatogenesis in hypophysectomized rats (181). This suggests that testosterone is able to substitute for FSH regarding its trophic actions on the seminiferous tubules. Hansson et al. (69) have suggested that in the intact rat FSH induces synthesis of ABP in the seminiferous

tubule, and that ABP provides the basis for accumulation of androgenic hormones in the seminiferous tubule necessary for normal spermatogenesis. This might be particularly important for the initiation of spermatogenesis at puberty when the plasma testosterone has not reached the adult level (69).

2. PHYSICOCHEMICAL PROPERTIES OF ANDROGEN BINDING PROTEIN

ABP has a Stokes radius of about 47^{o}A and a sedimentation coefficient of about 4.5-5 S (60), indicating a molecular weight of approximately 90,000 and a fractional ratio of 1.6 ($\bar{V}$ = 0.725 cm^{3}/g). The mean molecular radius of ABP has been calculated to be 2.92 nm and a molecular weight of about 90,000 upon polyacrylamide gel electrophoresis (151), but upon SDS-PAGE rat ABP has been revealed to consist of two major bands of protein with apparent molecular weights of 41,000 and 45,000 rather than 90,000 as observed for native rat ABP (128). The rabbit ABP has been claimed to be a single chain of 84,000 daltons (199); however, recent studies of Chen et al. (27) have demonstrated rabbit ABP to be a dimeric protein with subunits of 43,000 and 47,000 daltons. The binding activity of ABP has been shown to be rapidly destroyed below pH 5; however, complex of ^{3}[H]DHT-ABP could be focused at pH 4.6-4.7. The binding activity of ABP is unstable above 50^{o}C. The epididymal ABP binds to 5α-DHT, testosterone and estradiol-17β but not progesterone or estradiol-17α (66). From his studies, Vigersky (196) has suggested that ABP is not distinguished from hTeBG; however, Hsu and Teven (79)

have reported that testicular hABP is distinguished from hTeBG by charge, binding kinetics, pH for specific binding, and adsorbtion by Concavanalin A for separating ABP from TeBG.

3. PHYSIOLOGICAL SIGNIFICANCE OF ANDROGEN BINDING PROTEIN

Cyproterone acetate has been shown to inhibit androgen binding to the cytoplasmic receptor in the ventral prostate (49) and epididymis (189), but has minimal effect on the binding of androgen to ABP. It is found that in the epididymis and the prostate, cyproterone acetate inhibit the nuclear uptake to the same extent as the cytoplasmic receptor binding for androgen (189). This finding therefore indicates that epididymal cytoplasmic receptor, rather than ABP, is directly involved in the nuclear uptake of androgen. Tindal (189) has suggested that the uptake of androgen by epididymal nuclei involves a rate-limiting transfer from the cytoplasm, indicating that a cytoplasmic receptor plays a direct role in the transfer process. ABP, on the other hand, does not appear to be involved directly in the nuclear transport of 5α-DHT; however, ABP has been reported to increase the amount of radioactive labelled DHT bound to the intracellular receptor (189).

Reports from several laboratories have shown the importance of androgen for normal maturation of spermatogenesis in the epididymis (13,45). It is suggested that the FSH induced stimulation of ABP synthesis and the secretion of ABP by the Sertoli cells enhance the

transport of androgens from the testis to the epididymis by way of the testicular fluid, and therefore concentrate androgens within the lumen of the caput epididymis. ABP appears to be of significant importance for spermatogenesis in the testis as well as for sperm maturation in the epididymis (67).

4. DIFFERENCE BETWEEN ANDROGEN BINDING PROTEIN AND ANDROGEN RECEPTORS

The testis as the androgen target organ has gained further support from the findings of cytoplasmic and nuclear androgen receptors in the testis (68,175,181). The cytoplasmic receptor in the testis cytoplasmic fraction has been shown to be similar to that in the epididymis and the ventral prostate, but clearly different from ABP. The epididymal cytosol fraction contains at least two different androgen-binding-protein components (65). The larger component has physicochemical properties similar to those of the intracellular receptor in the ventral prostate gland, and the smaller component is similar to ABP (65). Both binding proteins are highly specific for DHT. The large component has been located intracellularly in epithelial cells since unilateral orchiectomy for 4 weeks has no influence on the presence of this protein but ABP disappears (65). The large receptor upon centrifugation displays aggregates of an 8-10S component and an 3-4S component; and the larger species dissociates into 3-4S components at high ionic strength (65). This intracellular receptor could be separated from ABP by polyacrylamide electrophoresis (190), and the presence of ABP appears

to facilitate the binding of 5α-DHT to the cytoplasmic receptor. Androgen receptors could also be distinguished from ABP by the rate of dissociation of the bound 5α-^{3}[H]DHT. At 0°C, bound 5α-^{3}[H]DHT could be almost completely displaced from rat ABP within 30 min ($t_{\frac{1}{2}}$=6 min at 0°C); whereas less than 10% of bound DHT could be displaced from the cytoplasmic receptor even after 18 hrs (189). This difference in dissociation rate strongly suggest the possibility of different binding functions of ABP and the cytoplasmic receptors with regard to the binding of androgens. ABP seems to function as an extracellular carrier protein; whereas the receptor acts for intracellular transport of the hormone from cytoplasm to the nucleus against a concentration gradient of the hormone. The very low dissociation of bound hormone from cytoplasmic receptor stresses the concept that this protein probably enters the nucleus as a complex with 5α-DHT (92, 189).

II. TESTOSTERONE BINDING GLOBULIN IN SERUM

Only a very small fraction of the biologically active steroid hormones in the plasma exists in the unbound state. In serum about 5-10% of cortisol exists in the free form; whereas about 1-3% of testosterone (T) and E_2, are in unbound forms (18,55). T is also bound to albumin (47,164), which has a low binding affinity (Ka = $6X10^{4}M^{-1}$) but is important because of its very high capacity. Small amounts of T are also bound to CBG: however, most of the T in plasma is bound to TeBG

(113,164). This binding is of high affinity ($Ka=10^{9}M^{-1}$), readily reversible at 37°C. In man the binding is nearly saturated since the molar concentration of TeBG with respect to binding sites is only marginally greater than the molar concentration of T in serum (139). In the female, the plasma concentration of TeBG is higher and T is lower (113,139) and therefore most of the binding sites are unoccupied. Compared to T, E_2 binds less well to TeBG, but better to albumin, and does not bind significantly to CBG (113).

Despite the small amounts, the free steroids, not the bound fractions, are the biologically important hormones (121,142,156). Being freely interchangeable between the plasma, the extracellular and intracellular compartments, this biologically active free form of steroids determines the intracellular concentration of the hormones (143). Studies with albumin, α_1-acidic glycoprotein and CBG have shown that protein-bound progesterone is biologically inactive (24). Large doses of E_2 have shown to produce no effect on uterine weight in young rats when estrogen binding protein (EBG) is still high; but the steroid becomes extremely potent in older animals when the binding protein for estrogen has fallen (147). This indicates therefore that steroid binding proteins have an important physiological role in controlling the amounts of free steroids in the blood circulation. TeBG appears to be involved in the mechanism of action of sex steroid hormones by controlling or regulating the availability of unbound hormones for the target tissues (18).

1. PHYSIOLOGY OF TESTOSTERONE BINDING GLOBULIN

The presence of sex hormone binding globulins (SHBG) in human serum has been suggested by the experiments of Daughaday (37) on testosterone-binding and was more conclusively demonstrated by Mercier et al. (113). Subsequent investigations have shown that this binding protein is distinct from cortisol binding globulin (140,180) and binds estrogen as well as androgen (30,180), and the name Testosterone-Estrogen binding globulin (TeBG) is given to this protein. Other names have also been used for this protein: Testosterone-binding globulin, Estradiol binding globulin, Sex steroid binding β-globulin (SBβG), Sex steroid binding protein (SBP) and 17β-hydroxy steroid binding globulin (1).

There are few studies on the species distribution of TeBG. Murphy (126) has reported androgen binding activity in the serum of humans, cows and frogs but not rats, rabbits, dogs or ducks. More recently, Corvol and Bardin (29), have identified TeBG in the plasma of 13 out of 21 animal species studied, including primates, rabbits, goats, cows, sheep, reptiles and amphibians, but not in birds, cats, dogs and rodents. Later, by the technique of steady state PAGE, TeBG was detected in dogs but with binding affinity for androgen and estrogen 20-80 fold lower than those observed from hTeBG (186). A low affinity TeBG has also been identified in the rat (157). These observations have suggested that TeBG in various species has a wide range of steroid binding affinities. The divergence of TeBG among species is also suggested by the lack of common immunodeterminant in this protein since antibodies to the human protein cross-reacts with TeBG from other

primates but not with those of cat, dog, sheep, goat or cow (157). Antibodies against rabbit TeBG cross-react only with the rabbit protein and not with those of other species (205). For most species TeBG is believed, though not well established, to be synthesized in the liver (8). Anticonvulsant drugs, notably phenobarbitone and phenytoin, have been postulated to induce hepatic synthesis of SHBG (1).

2. PHYSICOCHEMICAL PROPERTIES OF TESTOSTERONE BINDING GLOBULIN

TeBG has been isolated in many laboratories (80,81, 117,134, 144,178). The sex steroid plasma protein from late pregnancy human plasma has been purified by ammonium sulfate precipitation, followed by affinity chromatography and preparative electrophoresis (108). A molecular weight of 92,000 daltons has been estimated for the pure SBP by native PAGE; but in the presence of sodium dodecyl sulfate a molecular weight of 67,000 daltons was observed. Rosner (158) has reported that homogeneous hTeBG has a molecular weight of 94,000 with no apparent subunit structure. Other investigators (114,118) have reported the presence of dimeric structures for hTeBG with a minimum subunit molecular weight of 36,000-67,000 daltons as determined under denaturing conditions. Recently, Iqbal and Johnson (81) have reported that highly purified hTeBG consists of 4 subunits. Even though the chemical composition and subunit structure of TeBG has not been established conclusively yet, it has been generally agreed that TeBG is a glycoprotein containing one binding site per native molecule (81).

Ion exchange chromatography on DEAE cellulose has been used effectively to distinguish radioactively labelled steroids bound to albumin, TeBG, or Transcortin (CBG)(62). T binds to TeBG as well as to CBG; whereas 5α-DHT binds exclusively to TeBG. The binding of T to CBG is particularly high at low temperature. At $37^{o}C$ it has been shown that CBG is of minor importance for binding androgens in serum (153). The affinity of 5α-DHT for hTeBG is high. Its dissociation constant (Kd) at $0^{o}C$ has been determined to be $8x10^{-10}M$, and the amount of high-affinity binding sites in male serum is about $4x10^{-8}M$ (72). Binding activity of hTeBG has been shown to be relatively stable at $50^{o}C$ for 30 minutes but completely destroyed at $60^{o}C$ for 30 minutes (32).

Homogeneous preparations of TeBG from rabbits (117), dogs (186) and cows (177) have been reported. The molecular weight, subunit structure, and amino acid composition of rabbit TeBG (rb TeBG) have been reported to be similar to those reported for hTeBG by the same laboratory (118). The steroid binding affinity for rbTeBG is much lower (157). Canine TeBG, similar to rbTeBG, has a much lower affinity for androgens than hTeBG, and is also composed of dimers (186). However, the molecular weight of the native and subunits of bovine TeBG (bTeBG) have been reported to be 89,500 and 28,000 respectively, suggesting that this bovine binding protein is composed of three subunits (177). Thus TeBG of various species so far examined are large glycoproteins (M.W of 80-90,000) with subunit-structures and a high affinity for androgens and to a lesser degree for estrogens. Isofocusing in sucrose gradient or acrylamide gel of rTeBG has demonstrated a major peak of activity at

pH 5-5.5; however, rbTeBG has been observed to be rather stable and precipitated easily at its isoelectric point. Binding activity of rbTeBG becomes markedly reduced at 50°C for 30 minutes. Rabbit TeBG, as compared to hTeBG, appears to be smaller in size, more negatively charged and less stable to changes of temperature and pH. Futhermore, the rate of dissociation of bound 5α-DHT from rb TeBG at 0°C ($t_{½}$ = 5-6 min.) is much faster than that from hTeBG ($t_{½}$ = 70 min.)(74). The similarities and differences between rbTeBG and rbABP are summarized in table 1.

3. FUNCTIONAL SIGNIFICANCE OF TESTOSTERONE BINDING GLOBULIN

The effect of various concentrations of SHBG on the relative binding of T and E_2 in plasma has been studied by Burke and Anderson (18). Changes of SHBG concentration produce a much greater alteration to the percentage of unbound T than of E_2. This is partly because E_2 binds less well than T to SHBG, and partly because E_2 binds better than T to albumin. In the same study it has been found that changes of T concentration over the physiological range does not significantly increase the unbound fraction of E_2. These findings, together with the fact that E_2 stimulates and androgen inhibits SHBG production (139,193) have suggested the possible role for SHBG in regulating the relative concentrations of unbound T and E_2 in serum.

Serum levels of TeBG have been shown to change with age and sex. At infancy TeBG level is low in serum with no difference between sexes. However, in the male its level increases pregressively up to 3

TABLE 1. COMPARATIVE PHYSICOCHEMICAL PROPERTIES OF RABBIT ANDROGEN BINDING PROTEIN AND TESTOSTERONE BINDING GLOBULIN*

	ABP	TeBG
$S_{20,w}$	4.7S 4.5S	4.4S
Stokes radius (rs)	43°A	44°A
M.W. ($S_{20,w,rs}$)	74,000d	76,000d
Molecular radius (PAGE)	2.74 nm	2.74 nm
f/f_0(M.W.,rs)	1.61	1.55
M.W. (PAGE)	75,000d	75,000d
M.W. (SDS-PAGE)	68,000	-
M.W. (equilibium ultra centrifugation)	65,500	-
pI	4.7	5.4
Ka^{DHT} at 0°C	$1.6x10^{9}M^{-1}$	$1.9x10^{9}M^{-1}$
Stability binding destroyed by heat above	50°C	50°C
Dissociation rate DHT protein complex ($t_{½}$°C)	6 min	5.2 min.
Steroid specificity	DHT, Adiol,T, P, androstenediol cortisol	-

* Weddington et al., 1975 Nature 258:257.

months of age and then declines to a low adult level, but in the female its level remains relatively unchanged. In adults, the level of TeBG is lower in males and higher in females (97). These observations have suggested the possible role of TeBG as a regulator for the serum concentration of sex steroids for sex maturation. These changes of TeBG levels in serum might be due to changes in the production of endogeneous sex steroid. TeBG has been shown to be decreased by endogeneous T (127,143,160) but increased by estrogen (40,119,191,193).

The physiological role of TeBG has still been uncertain; however, it has been suggested that this protein is involved in the mechanism of action of steroid hormones by keeping steroids in the circulation and therefore controlling the amount of unbound hormones for target tissues. For instance, an inverse relationship between the metabolic clearance of T and the serum level of TeBG in various species, including man (176,193) and rhesus monkey (193), has further supported the notion that this protein regulates the concentrations of unbound steroids available for biological action.

III. FOLLICULAR FLUID

Follicular fluid is a liquid accumulating in the extracellular space within ovarian follicles. It appears in spaces between granulosa cells at early stages of follicular development and finally surrounds the oocyte and its associated epithelial cells in fully mature follicles. Follicular fluid is present in many mammalian species. The amount of

this fluid appears to correlate with body weight, as does the size of the mature follicle at ovulation (17). The volume of fluid in mature follicles varies between species from 0.005 ml in mice to 4 ml in women and up to 65 ml in mares.

Follicular fluid consists mainly of a transudate of plasma, although it also contains secretory products from follicular cells. Follicular fluid consists of the plasma proteins and a high concentration of steroids synthesized by follicular cells including theca, granulosa and stroma cells. In addition, it also contains mucopolysaccharides and some proteins derived from granulosa cells (11,25,179). The concentration of proteins, steroids, carbohydrates and mucopolysaccharides in the fluid are not constant throughout follicular growth (2,45,109).

The presence of follicular fluid in many species appears to have great importance in ovarian physiology (108). The fluid of a particular follicle provides a relatively specific local environment that becomes quite different from adjacent follicles (108). Changes in physiological and endocrine properties of follicular fluid are necessary for follicular steroidogenesis,oocyte maturation, ovulation and transport of the oocyte to oviduct, in addition to preparing the follicle for subsequent formation of a corpus lutem (108). Despite the unique importance of the follicular fluid, relatively few studies have been carried out in several species, including primates, rats, rabbits and cows. Furthermore, studies have been carried out mostly only on changes of concentrations of gonadotropins and steroid hormones.

1. PHYSICOCHEMICAL PROPERTIES OF FOLLICULAR FLUID

Follicular fluid is a slightly viscous liquid. The density of cow follicular fluid is 1.021 at room temperature with a pale yellow color (108). In general, the color of follicular fluid varies greatly from colorless to a tinted yellow. The pH of the fluid is generally above 7.0 and either similar to or slightly lower than that of serum (52,96,208). According to Shalgi et al. (167) the pCO_2 is probably the major factor regulating the follicular pH. The concentrations of Na^+ and K^+ are important determinants of the osmotic pressure of the fluid. The concentrations of Na^+ and K^+ ions have been observed to be similar to those in blood in women, but slightly variable in cows (45, 132,136,168).

2. PHYSIOLOGY OF FOLLICULAR FLUID

Follicular fluid appears to come from two sources: plasma and cells of the follicle. According to Luschka (95) and Waldeyer (198) the follicular fluid derives from cytoplasmic proteins of granulosa cells in a fluid transudated from blood vessels, the so called para-albumin being thus considered as transformed cytoplasm. Zondek and Askehun (210) have stated that the liquid portion of follicular fluid derives from the blood or lymph; however, they have maintained that the follicular fluid as a whole should be considered as a secretion of the cells of theca interna. According to Selye (166) the production of follicular

fluid is a function of the granulosa cells as its production fails to occur in those conditions where granulosa cells have been destroyed, such as after x-irradiation. A similar view is also held by Sainto, Simonnet and Brouhs (162). The granulosa cell is also reported to be the source of mucopolysaccharides (15,59,182,208).

The accumulation of fluid within larger follicles appears to depend on the proliferating granulosa cells, which in turn depend on the exposure to FSH and estrogen (57). For hypophysectomized immature rats, FSH alone is not enough to stimulate follicular growth or antrum formation; whereas estrogen induces granulosa cell proliferation without the formation of an antrum. Only addition of FSH together with estrogen could induce proliferation of granulosa cells and the concurrent development of a fluid-filled antrum (108). Peckham and Kiekhofer (141) have shown that the antral follicles are highly permeable to tritiated water and concluded that the rate at which capillary water diffuses into the follicular antrum is limited only by the vascularity of the tissues and not by any membranes effects. Changes in the composition of the fluid within follicles are partly related to the permeability of the follicular wall and also partly related to the composition of the interstitial fluid, which in turn is related to the blood supply of the enlarging follicle (149). It is clear that the increased blood flow and permeability of capillaries of the enlarging follicle enhance the tranfer of plasma substances into the surrounding extravascular antrum. Changes in blood flow to the enlarging follicle also influence the biosynthetic capacity of the follicle, which in turn influence the composition and concentration of hormones within the follicular antrum (108).

The transfer of substances from serum into the intercellular space around granulosa cells and the antrum has been studied by many investigators (31,45,74,83,169). It has been reported that radioactively labelled proteins injected intravenously appear in ovarian lymph within a few minutes (125). Proteins of different sizes could pass into follicles with different rates (101). Most serum proteins could enter intrafollicular fluid via thecal capillaries, the intercellular space of theca and granulosa cells, and then into the antrum (31). It has been reported (2,31,138) that substances with molecular weights less than 1,000,000 could gain entrance into follicles from serum.

3. COMPOSITION OF FOLLICULAR FLUID

Follicular fluid contains numerous substances, including most of those found in serum. Some are slightly similar but some are quite different in concentration as compared to those in serum.

a. Carbohydrate

The carbohydrate content has been measured in follicular fluid obtained from cows, sows, mares and women (132,136,170,208). In cow follicular fluid, 80% of the total carbohydrate content is glucose, and the highest level is found during estrus (135). Very little fructose has been observed in follicular fluid (170).

b. Mucopolysaccharide

Two distinct types of mucopolysaccharides have been found in both rabbit and bovine follicular fluids. They consist of either hyaluronic acid or chondroitin sulfuric acid (29,84,209). These might

be the substances responsible for local changes in viscosity of extracellular fluid within the follicle (172). Hyaluronic acid is the most commonly found in the intercellular fluid around granulosa cells; while the less vicous chondroitin sulfuric acid is the most commonly found in the antral fluid (183). The source of mucopolysaccharides in follicular fluid is yet uncertain. Most of them are probably derived from the protein polysaccharides in plasma, since hexose-bound proteins have been identified in the fluid of cows and mares (22,208). Sulfate mucopolysaccharides might originate from granulosa cells (131,208). Heparin is another polysaccharide present in follicular fluid. It is physiologically important to prevent follicular fluid from clotting and to assure the passage of the oocyst during ovulation.

c. Protein.

The composition of proteins in follicular fluid has been examined in women, cows, sows, rabbits and rats (2,38,45,85,105,168). The total concentration of proteins in the fluid is either similar to or slightly lower than that in serum (Table 2). The composition of proteins in the follicular fluid varies with different stages of the ovarian cycle (137), but appears to be similar in all follicle at a certain stage of the cycle sizes (2). Most serum proteins have been identified in the follicular fluid but the concentration of individual proteins often differs greatly from those in the serum (Table 3).

Hormones, including prolactin, LH, FSH and their subunits have been measured in human (108) and porcine (24) follicular fluids. In both species, concentrations of FSH and LH are generally lower than those in plasma, but prolactin is highly variable from 8-180% of that in

TABLE 2. CONCENTRATION OF TOTAL PROTEIN IN FOLLICULAR FLUID EXPRESSED AS A PERCENTAGE OF SERUM PROTEIN (LIT. REF.)

Woman	53.0 (99), 74.3 (87), 77.8 (142), 98.7 (167)
Cow	77.8-79.8 (43), 64.1-77.6 (137), 79.8 (22), 86.4 (2)
Pig	74.7-77.5 (170), 83.8-88.7 (105), 103.0 (86),
Rabbit	100 (38)
Rat	80.8 (9)

TABLE 3. CONCENTRATION OF INDIVIDUAL PROTEIN IN HUMAN AND BOVINE FOLLICULAR FLUID EXPRESSED AS A PERCENTAGE OF SERUM

PROTEIN	HUMAN (85)	HUMAN (169)	COW (2)
albumin	58	133	113.5
α-glycoprotein	50	-	-
β,A-globulin	45	-	-
haptoglobin	-	29.7	-
transferrin	41	96	-
IgG	45	74	81.2
IgA	36	-	29.7
IgM	12	-	4.0

plasma. The ratio of follicular fluid to plasma concentration of these protein hormones varies greatly, possibly due to differences in size of follicles and stages of the ovarian cycle.

The presence of steroid binding proteins in the follicular fluid has been offered as an explanation for the retention of high concentrations of steroids within this fluid (3, 45,56). In human follicular fluid, an estrogen binding protein has been reported to bind DHT, T and estradiol specifically and appears to be indistinguishable from ABP (195). Furthermore, androgen binding proteins have also been reported in human follicular fluid obtained from patients with polycystic ovarian disease (PCO) (196) or hydatidiform mole vesicles (130). The binding protein appears to be very similar to the previously reported estrogen binding protein (196), and whether or not this protein is a contaminant of serum TeBG is not clear. Albumin has also been reported to bind estrogen with low affinity but high capacity (195).

There are at least two sources for proteins in the follicular fluid: the plasma transudate and the secreted products of cells within the follicle. There is considerable evidence to indicate that granulosa cells are engaged in protein biosynthesis (6,151). However, specific proteins of granulosa cell origin in follicular fluid have not been determined.

d. Hormone

Follicular fluid is dependent on circulating FSH and LH (3,44, 103). The concentration of FSH in the fluid increases progressively as the follicle enlarges; however, this increase of the FSH level in the follicle is not dependent on the FSH level in plasma, and follicular

fluid FSH generally does not exceed 60% of the plasma FSH level (110). LH is not detectable in all follicles, and is found in follicular fluid only when the plasma LH level is elevated (110). Concentrations of LH in follicular fluid generally do not exceed 30% of the peripheral plasma level (110). Prolactin has also been detected in follicular fluid. In general, the concentration of prolactin varies greatly, being highest in small follicles and lowest in large follicles.

The concentration of various steroid hormones changes through out the different stages of follicular growth in all species examined (45,78,90,172,205). During the growth phase, the androgen and estrogen are highly elevated, and just before ovulation progesterone becomes elevated as well. During the luteal phase, large amounts of progestin and androgen are found in the follicles (112,173). Progesterone concentration varies with different stages of follicle maturation (110). Levels of progesterone in follicular fluid during the luteal phase are about 50-200 times lower than tissue levels in the corpus luteum but are similar to levels in follicles in the early follicular phase (184). This suggests that the secretions of the corpus luteum does not alter the progesterone levels in adjacent follicles. The elevation of progesterone in the fluid coincidently with hypertrophy of granulosa cells and appearance of LH in the fluid, suggesting that the source of preovulatory progesterone in follicular fluid is the granulosa cell (5,42,76).

The major androgen in follicular fluid is androstenediol (173), which in human is 100-500 times higher than that in peripheral plasma (39). The main source of androstenedione is probably the theca cell (112). Testosterone in human follicular fluid has been estimated to be 10-3,000

times higher than that in peripheral plasma (112). The cellular source of testosterone is probably thecal cells (112).

Estrogen has been found 40-40,000 times higher in concentration than that in peripheral plasma. In the middle and late follicular phase, large follicles contain high concentrations of estradiol as compared to small follicles. The fact that estradiol in follicles decreases just before ovulation is probably a reflection of the decline in secretion of estrogen by follicles following the preovulatory surge of LH (124). The main source of follicular estradiol appears to be granulosa cells (112). Estrone has been found to be 5-300 times higher in concnetration than that of peripheral plasma (112).

McNatty et al. (112) have demonstrated a close relationship between the presence of FSH and concentrations of estradiol and androstenediol in human follicular fluid. Follicles with undetectable amounts of FSH contain high levels of androstenediol but low estradiol. On the other hand, follicles containing high FSH concentration have low levels of androstenediol but high estradiol (172). During the middle and late follicular phases, concentrations of estradiol are higher than androstenediol (49,112,207), suggesting that estradiol secretion is dependent on a functional relationship between the theca and the granulosa cells. The main pathway of biosynthesis of estradiol appears to be via the androstenediol, most of which is secreted by the theca cells (4). The high level of estradiol is probably due to an increased aromatization by granulosa cells being exposed to FSH (44,48,161), leading to the conclusion that the granulosa cell is the major source of estrogens. Similarly, a relationship between estradiol and prolactin has also been

demonstrated. Follicles with high levels of estradiol contain lower concentrations of prolactin. A relationship between concentrations of LH and progesterone has also been reported (110)such that higher concentrations of progesterone are found in follicles containing LH.

4. FUNCTIONAL SIGNIFICANCE OF FOLLICULAR FLUID

The endocrine microenvironment of the human antral follicle has been shown to be correlated with the viability of the oocyte, the ability of the oocyte to resume meiosis, the mitotic activity of the granulosa cells and the biosynthetic capacity of the granulosa cells to produce progesterone (106,111). The endocrine microenvironment of a developing follicle is of considerable importance for the growth of the follicle and for secretory activity of its granulosa cells (106,109,112). The formation of a fluid filled space within the follicle provides the means by which the hormonal environment of an individual follicle becomes unique. The exposure of granulosa cells and the oocyte within each developing follicle to a specific and unique hormonal environmental condition, which is different from that in adjacent follicles, makes it possible to explain the fate of individual follicles. Some become fully developed for ovulation and others undergo atresia after exposure to the same circulating levels of gonadotropin (74,106,109,110). The follicular fluid is in fact a medium for local control between granulosa cells and the oocyte.

AIM OF STUDIES

1. To establish the experimental conditions for assaying androgen binding activity in bovine follicular fluid.

2. To demonstrate the existence of a specific binding protein of high affinity for androgen in bovine follicular fluid.

3. To establish a procedure for the purification of the bovine follicular fluid androgen binding protein.

4. To characterize the physiocochemical and biological properties of the purified bovine follicular fluid androgen binding protein.

MATERIALS AND METHODS

A. MATERIALS

1. BIOLOGICAL MATERIALS

Bovine ovaries, blood samples and epididymal tissue were obtained from a local slaughter house, either the Burns Meat Company or the Canada Pakers Company. Rat epididymis tissue was a gift from Dr. May Robertson.

2. HORMONE

^{3}H-DHT and ^{3}H-E_2 were obtained from New England Nuclear. DHT, T, E, E_2, E_3, DHA, DIOL, P, DMS and Ald were obtained from Sigma Chemical Company.

3. CHEMICAL REAGENTS

Sephadex G-25 and G-200 were obtained from Pharmacia Fine Chemical. HTP, acrylamide, Bis-acrylamide, ammonium persulfate, SDS, coomassie brilliant blue G-250, crocein scarlet were obtained from Bio-Rad Limited. Spectrofluor, NCS and Omnifluor were obtained from Amersham Corp. α-methyl-glucoside and all protein markers were obtained from Sigma Chemical Company. DE-32 was obtained from Whatman Company. Ampholine was obtained from LKB.

All other standard laboratory reagents were obtained from either Fisher Scientific Company, Sigma Chemical Company or BDH Chemical.

B. METHODS

1. PREPARATIONS OF FOLLICULAR FLUID, SERUM AND EPIDIDYMAL EXTRACTS

a) Follicular fluid. Fresh bovine ovaries were obtained from a local slaughter house within 30 minutes of sacrifice. The ovaries were kept in an ice bath, and follicular fluid was aspirated within 2-3 hours, and was separated from follicular cells by centrifugation at 3,000 RPM for 30 minutes at 4°C. For some comparative studies, follicular fluids were obtained separately from small (diameter <4mm) medium (diameter 4-6 mm) and large (diameter >6mm) follicles. For bulk preparations, only follicular fluid from large follicles was used. After centrifugation, the fluid was either stored at -20°C or processed immediately.

b) Serum. Female bovine blood samples were collected in the slaughter house and allowed to clot at 4°C for 1-2 hours. Serum was obtained after centrifugation at 3,000 RPM at 4°C for 30 minutes and was kept frozen at -20°C or processed immediately.

c) Epididymis. The membranous capsule of fresh epididymis was peeled off and the tissue was minced and then homogenized in TEG buffer with a Polytron homogenizer (Brinkman type PT10) at maximum speed for 1 minute. The homogenate was filtered by passing it through layers

of cheese cloth. The filtrate was then centrifuged at 10,000 RPM at $4^{o}C$ for 30 minutes and the supernatant was collected and diluted appropriately with TEG for binding assays.

2. REMOVAL OF STEROIDS FROM FOLLICULAR FLUID, SERUM AND EPIDIDYMAL EXTRACTS.

Steroids in follicular fluid, serum or extracts of epididymis were removed by treatment with pre-washed charcoal at 1 mg of charcoal per ml of sample overnight at $4^{o}C$ with slow stirring as described by Ritzen (151). After treatment, charcoal was removed by centrifugation first at 10,000 RPM at $4^{o}C$ for 30 minutes, and the supernatant was further centrifuged at 40,000 RPM at $4^{o}C$ for 60 minutes. Finally, the supernatant was filtered through Whatman's filter paper to remove the fine charcoal particles.

3. MEASUREMENT OF SPECIFIC BINDING OF ^{3}[H]-LABELLED DIHYDROTESTOSTERONE.

Approximately 20,000 cpm of ^{3}[H]-DHT (0.07ng) with or without 500ng of unlabelled DHT in 0.02 ml of ethanol was added to a 1.2x7.5 cm disposable glass culture tube and was dried by nitrogen. Charcoal treated samples of 0.1-0.5 ml were added individually to each tube containing the dried ^{3}[H]-DHT with or without unlabelled DHT and the mixture was then incubated at $4^{o}C$ for 2 hours. All samples were assayed in duplicate. If the amount of protein in the sample was lower than 0.03 mg, 1 mg of γ-

globulin in 0.1 ml of water was added to the mixture as "carrier" and the volume of the mixture was made up to 1.2 ml. After the addition of ammonium sulfate to 60% saturation, the incubated samples were vortexed and left at $4^{o}C$ for 30 minutes for complete precipitation of proteins. The precipitate was collected by centrifugation at 3,000 RPM at $4^{o}C$ for 30 minutes. The pellet was redissolved in 3 ml of 60% (w/v) of saturated ammonium sulfate solution and the precipitate was obtained again by centrifugation. The final pellet was dissolved in 0.1 ml of water and was then transferred to a counting vial containing scintillation cocktail and was counted for 10 minutes in a scintillation β-counter. The scintillation cocktail was prepared by adding 3 g of Omnifluor to 1 liter of Toluene.

Specific binding was calculated as the following:

$$\% \text{ Specific binding} = \frac{\text{Bound cpm} - \text{nondisplacable cpm} \times 100}{\text{Total cpm}}$$

where "Bound cpm" = cpm in pellet of sample incubated with ^{3}[H]-DHT.

"nondisplaceable cpm" = cpm in pellet of sample incubated with ^{3}[H]-DHT and 500 ng unlabelled DHT.

"Total cpm" = cpm of total ^{3}[H]-DHT used in sample.

4. MEASUREMENT OF PROTEINS

a) Absorbance at 278 nm. Protein concentrations in fractions collected at different steps of purification were estimated by absorbance at 278 nm.

b). Lowry's method. More accurate determinations of protein concentration in most of the experiments involving the characterization of follicular fluid, serum and epididymal extracts were carried out by the method of Lowry (94) with slight modifications.

c). Bradford's method. For estimations of protein concentrations at various steps or purification, a less time consuming and accurate protein assay was carried out according to the method of Bradford (16).

5. CONCENTRATION OF SAMPLE SOLUTIONS

At various steps of purification, pools of appropriate fractions collected were concentrated by ultrafiltration either in an Amicon stirred cell using a PM 10 filter or a Millipore immersible CX-30 concentrator.

6. REMOVAL OF SALTS.

a) Dialysis. In several steps of the purification procedure, one buffer had to be changed to another or salts had to be removed for further studies. Dialysis was carried out with 10-20 volume of appropriate buffer with slow stirring at $4^{o}C$ overnight. Completion of dialysis was measured for appropriate conductivity and pH of the dialysed sample.

b) Desalting by Sephadex G-25. For samples of small volumes, desalting was carried out by gel filtration on small columns of Sephadex G-25, equilibrated in TEG buffer.

7. ISOLATION OF FOLLICULAR FLUID ANDROGEN BINDING PROTEIN (ff-ABP)

a) Fractionation of proteins at different saturations of ammonium sulfate. Saturated ammonium sulfate solution was prepared by warming 800 g of ammonium sulfate in 1 liter of distilled water to 65°C and then allowed to cool slowly to room temperature for use.

To fractionate proteins, appropriate volumes of saturated ammonium sulfate solution were added to a known volume of follicular fluid to obtain a 28% saturation of ammonium sulfate. The mixture was stirred at 4°C for 60 minutes and the precipitate was pelleted by centrifugation at 10,000 RPM for 30 minutes. More saturated ammonium sulfate solution was added to the supernatant until it was 55% saturated of ammonium sulfate and the precipitate was again pelleted by centrifugation. The 28-55% ammonium sulfate precipitate was dissolved in 3 volumes of TEG and dialysed against 10-20 volumes of the same buffer overnight with 2 changes. The conductivity and pH of the dialysed protein solution were checked and applied onto a DEAE-cellulose ion exchange column for further fractionation of proteins.

b) Stepwise gradient elution on DEAE cellulose chromatography. A Whatman DE-32 column was equilibrated in TEG buffer at

pH 7.4. The dialysed 28-55% ammonium sulfate precipitate sample of follicular fluid was loaded onto the DE-32 column. After washing with 3 volumes of TEG, the proteins absorbed on the column were eluted stepwise with 3-4 volumes of 0.05, 0.125 and 0.2 M NaCl in TEG. The fractions were monitored for proteins by absorbance at 278 nm and for ABP by binding of ^{3}[H]-DHT. The appropriate ABP fractions were pooled and dialysed in TEG buffer for the next fractionation step.

c) Continuous gradient elution on Hydroxylapatite.

(i) Hydroxylapatite was packed in a column and equilibrated in TG at pH 7.4. The pooled fractions after DE-32 chromatography were loaded onto the HTP column at a ratio of approximately 1.5 mg of protein/mg of HTP. After washing with 3 volumes of TG, the adsorbed proteins in the column were eluted with a continuous gradient of 0, 3, 1.5, 5 mM phosphate in TG buffer. The remaining proteins were eluted by 5mM and 400 mM phosphate in TG buffer in a stepwise manner. The fractions were monitored for protein by absorbance at 278 nm and for ABP by binding of ^{3}[H]-DHT. The appropriate ABP fractions were pooled and dialysed in TG buffer for a second HTP column.

(ii) At a ratio of 1 mg of protein/mg of HTP, the pooled fractions of high ABP activity in TG buffer, from the previous HTP column, after diaysis were loaded onto a second HTP column. After the unadsorbed fractions were eluted with TG buffer, a gradient of 3 chambers, containing 0, 1.5, 1.5 mM phosphate, respectively, in TG buffer, was used to elute the absorbed proteins. The distribution of proteins and binding activity were monitored, and the appropriate

fractions were pooled and dialysed in TEG for further fractionation.

d) Continuous gradient elution on DEAE-Cellulose chromatography. The dialysed sample from the second HTP column was loaded onto a DE-32 column, equilibrated in TEG buffer. After the unadsorbed fractions, the adsorbed proteins were eluted with a continuous gradient of 8 chambers, containing 0, 0.2, 0, 0, 0.25, 0.1, 0.125, 0.15 M NaCl in TEG buffer, pH 7.4 After elution, the distribution of proteins and ABP activity were monitored and appropriate fractions were pooled.

e) Affinity chromatography on Sepharose-Concanavalin A. The appropriately pooled fractions from the previous compound gradient DE-32 column were loaded onto a Concanavalin A column, equilibrated in 50 mM Tris-HCL, containing 0.5 M NaCl, 1 mM $MgCl_2$. 1 mM $MnCl_2$ and 1 mM $CaCl_2$ at pH 7.4 After the unadsorbed fractions, the column was extensively washed with the same buffer. The adsorbed glycoproteins were then eluted with 0.2 M α-methyl-D-glucoside in the same buffer. The eluted glycoproteins were pooled and concentrated for the final step of gel filtration on Sephadex G-200.

f) Gel filtration on Sephadex G-200. Gel filtration was carried out on Sephadex G-200 equilibrated in TEG. The concentrated protein sample was loaded on top of the Sephadex G-200 column and the same TEGbuffer was used for elution. Protein concentrations and binding activities were monitored and the appropriate fractions were pooled and concentrated for further studies.

8. CHARACTERIZATION OF FOLLICULAR FLUID ANDROGEN BINDING PROTEIN.

a) Polyacrylamide gel electrophoresis. Analytical PAGE was performed on a 10% separating gel and a 3% stacking gel at pH 8.9 (133). To the protein sample, 15 µl of 0.005% Bromophenol Blue was added as tracking dye. The electrode buffer was 0.15 M Tris glycinate buffer at pH 8.3. Electrophoresis was carried out at a current of 10 mA/cm^2 of gel surface. At the end of electrophoresis, the gel was removed and stained with 1% amidoblack in 7% acetic acid overnight to visualize proteins.

The ratio of the mobility of protein to the mobility of dye front was determined as relative mobility (Rf) of the protein. This regular basic polyacrylamide gel was used to compare proteins in various fractions of the purification procedure.

To demonstrate the binding activity of ff-ABP, a steady state polyacrylamide gel electrophoresis was used. The SS-PAGE was performed similarly as the regular basic polyacrylamide gel, except that the gel itself contained ^{3}H-DHT (151). As the binding protein moved down the gel during electrophoresis ^{3}H-DHT became associated with the binding protein. To determine the distribution of ^{3}H-DHT after electrophoresis, the gel column was segmented into 2 mm slices, and soaked in the scintillation cocktail overnight. The radioactivity in each segment was then counted in an automatic beta-counter.

b) Estimation of molecular weight

(i) Gel filtration on Sephadex G-200.

A Sephadex G-200 column was calibrated with protein markers: γ-globulin (160K), BSA (68K), ovalbumin (45K) and chymotrypsinogen (23K). The purified ff-ABP was then applied onto the same Sephadex G-200 column for the estimation of molecular weight. The size of ff-ABP was estimated from its elution volume against those of the marker proteins of known molecular weight.

(ii) Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The molecular weight of ff-ABP was also estimated by polyacrylamide gel electrophoresis in the presence of SDS (174). Gels containing 1% SDS were allowed to polymerize overnight for use. The protein sample was denatured by heating in boiling water for 5 minutes in the presence of β-mercaptoethanol. Electrophoresis was carried out at 8 mA per gel using 0.06 M Tris glycenate as electrode buffer. After electrophoresis, the gel was removed and stained with 2.5% Coomassie brilliant blue G-250 in 50% TCA overnight and destained in 10% glacial acetic acid and methanol.

The relative mobility (Rf) of the protein markers: transferrin (85K), BSA (68K), ovalbumin (45K), chymotrypsinogen (23K) and myoglobulin (17K) were plotted against their molecular weights. The molecular weight of ff-ABP could be estimated by plotting its Rf and comparing to those of the protein markers.

c) Gel Isoelectric focusing. The isoelectric point (pI) of the purified ff-ABP was estimated by the polyacrylamide gel iso-focusing method (204). Gels of 10% polyacrylamide containing 4% Ampholyte were prepared and allowed to polymerize for 30 minutes.

The gel was prerun for 8 hours using 0.2% H_2SO_4 in the lower chamber and 0.4% monoethanolamine in the upper chamber. The protein sample in 2.5% Ampholyte and 10% sucrose was then loaded on top of the gel. Isoelectric focusing was carried out by using 1.5 watt per gel for 24 hours. At the end, the gel was removed and fixed with 50 volumes of 10% TCA overnight with 3 changes, and then stained for 8 hours in 0.04% coomassie brilliant blue, 0.5% crocein scarlet and 0.5% cupric sulfate in 22% isopropanol and 20% glacial acetic acid. The gel was then destained in 10% glacial acetic acid and 20% isopropanol with several changes.

To measure the pH gradient in the gel, another blank gel focusing at the same time and identical conditions but without staining was segmented into 2 mm slices. The pH of each gel slice was measured after soaking each segment individually in 1 ml distilled water for 3 days. The pI of ff-ABP was determined by matching the electrophoretic mobility of the protein after staining and the pH gradient formed in the gel after isoelectrofocusing.

RESULTS

I. CONDITIONS FOR THE ANDROGEN BINDING ASSAY.

An effective and reliable assay for binding of ^{3}H-DHT for studies of androgen binding proteins is essential. Appropriate experimental conditions for a reproducible assay have to be established.

1. EFFECTS OF INCUBATION TIME.

Crude bff, after removal of steroids, was incubated with ^{3}H-DHT in the presence or absence of unlabelled DHT at different time intervals. Bound and free ^{3}H-DHT were separated by a 60% saturated ammonium sulfate solution.

Figure 1 shows that the binding of ^{3}H-DHT to crude bff is relatively rapid, and an equilibium is reached after 2-4 hours of incubation at 4°C. Thereafter, the binding stays constant even up to 22 hours at 4°C.

2. EFFECTS OF AMMONIUM SULFATE AT VARIOUS SATURATION.

Crude bff was incubated with ^{3}H-DHT in the presence or absence of 500 ng DHT for 2 hours at 4°C. Protein bound ^{3}H-DHT was then precipitated with ammonium sulfate at various saturations, ranging from 30-80% final concentration.

Figure 2 shows that ammonium sulfate at saturations between 55-65% was the most appropriate concentration to precipitate the protein bound ^{3}H-DHT for assaying binding activities.

3. EFFECTS OF γ GLOBULIN ON PRECIPITATION OF PROTEIN BOUND ^{3}H-DIHYDROTESTOSTERONE.

The binding of ^{3}H-DHT to a diluted partially purified

Figure 1 Time course for the binding of DHT to bovine follicular fluid. Sample of 20 µl of charcoal treated bFF were incubated with 140 ρg ^{3}H-DHT (20,000 cpm) in the presence or absence of unlabelled DHT for 0.5, 1, 3, 7.5 and 22 hours. Protein bound ^{3}H-DHT was separated from unbound ^{3}H-DHT by precipitating with 60% saturation of ammonium sulfate and was counted in a liquid scintillation counter. (■) Total binding; (□) non-specific binding and (●) specific binding.

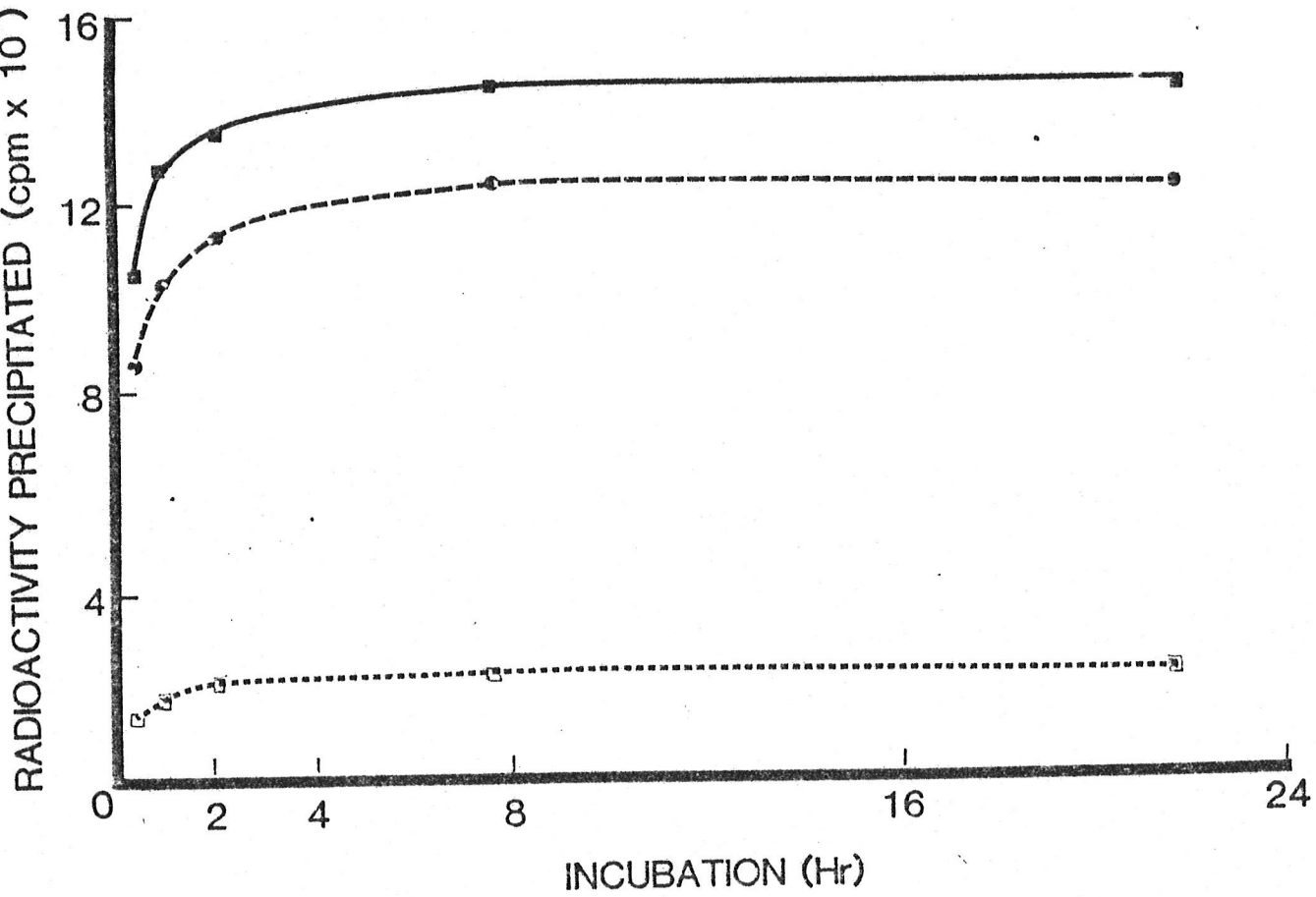
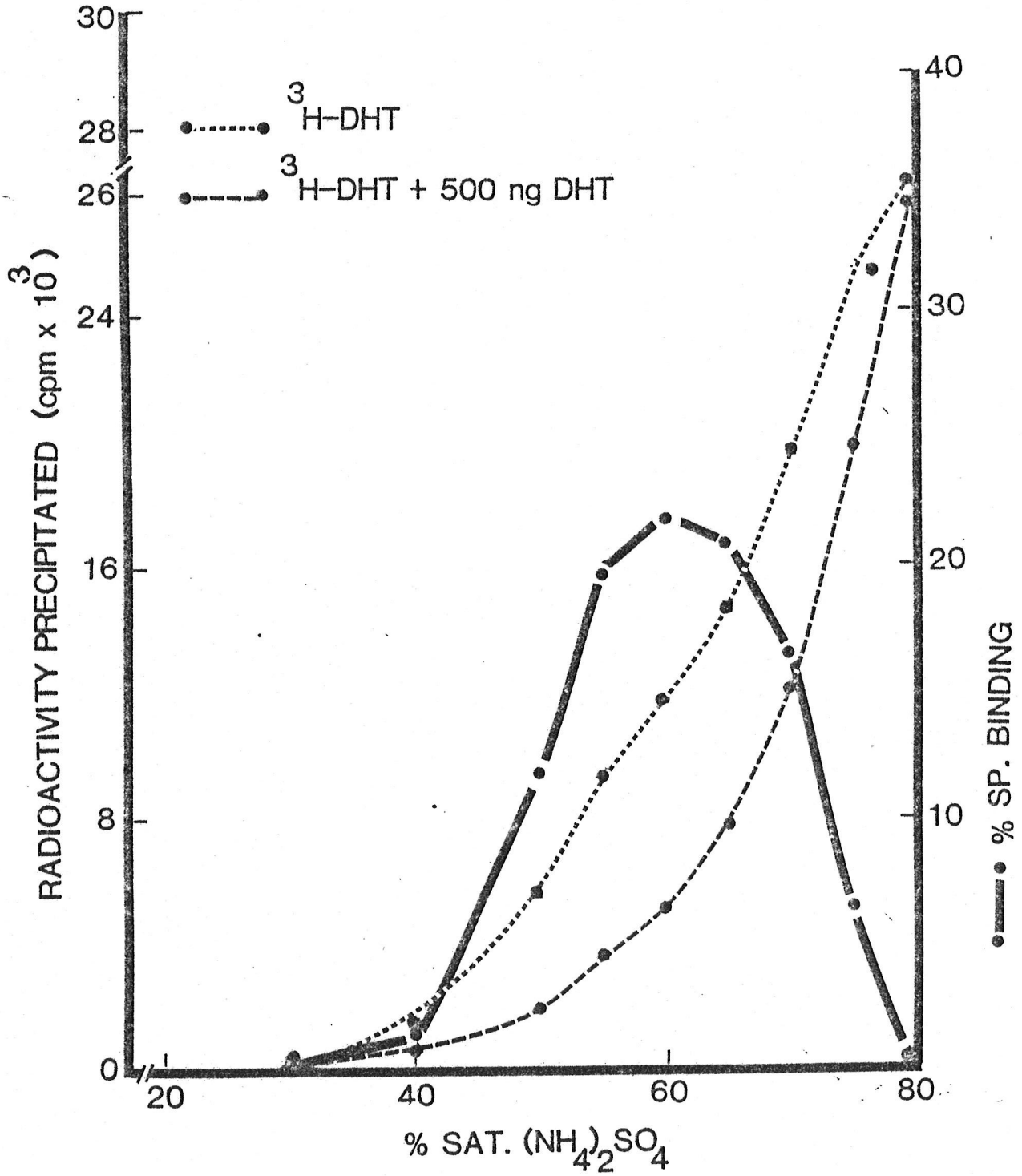

RADIOACTIVITY PRECIPITATED (cpm x 10^3)
16
12
8
4
0
2
4
8
16
24
INCUBATION (Hr)

Figure 2 Precipitation of bound ^{3}H-DHT by various saturation of ammonium sulfate. Samples of 50 µl charcoal treated bff were incubated with 210 ρg ^{3}H-DHT (29,000 cpm) with or without unlabelled DHT at 4°C for 2 hours. Protein bound ^{3}H-DHT was precipitated in the presence of 1 mg γ-globulin by 35,40,50,55,60,65,70, and 80% saturation of ammonium sulfate. (●--●) ^{3}H-DHT; (●--●) ^{3}H-DHT + 500 ng unlabelled DHT; (●—●) specific binding for ^{3}H-DHT.


30
28
26
24
16
8
0
40
30
20
10
20
40
60
80
^{3}H-DHT
^{3}H-DHT + 500 ng DHT
RADIOACTIVITY PRECIPITATED (cpm x 10^3)
% SP. BINDING
% SAT. $(NH_4)_2SO_4$

bff-ABP at a low protein concentration was carried out, and the bound ^{3}H-DHT was precipitated by ammonium sulfate in the presence or absence of 1 mg γ-globulin.

Results in Figure 3 show that addition of γ-globulin as a "carrier" is important for the precipitation of protein bound ^{3}H-DHT.

II. PRELIMINARY STUDIES ON THE PROPERTIES OF THE ANDROGEN BINDING ACTIVITY IN BOVINE FOLLICULAR FLUID.

Preliminary studies were carried out to study some of the properties of the binding activity in the follicular fluid, such as the binding affinities of various steroid hormones and the stability of the androgen binding activity.

1. ANDROGEN BINDING ACTIVITY IN BOVINE FOLLICULAR FLUID.

The androgen binding activity was investigated in follicular fluid obtained from bovine ovaries. Follicular fluid samples were aspirated and pooled according to follicular size, and assayed for ^{3}H-DHT binding.

Results in Figure 4 show that androgen binding activity was present in bff at all stages of follicular maturation, and the binding was higher in the fluid of follicles with diameters more than 4 mm. This binding activity is in fact relatively high since only a very small amount of fluid was used (0.01 ml) in the assay.

Futhermore, the androgen binding activities in bovine follicular fluid, serum and epididymis were compared by assaying at different dilutions of protein for binding of ^{3}H-DHT.

Figure 5 shows that follicular fluid had the highest binding acti-

Figure 3 Effects of γ-globulin on the precipitation of protein bound ^{3}H-DHT by ammonium sulfate. Samples of 100 µl (1.2 mg/ml) of fractions after HTP chromatography were incubated with ^{3}H-DHT (45,000 cpm) at 4°C for 2 hours. Protein-bound ^{3}H-DHT was precipitated by various saturations of ammonium sulfate in the presence (▼) or absence (▽) of 1 mg of γ-globulin.

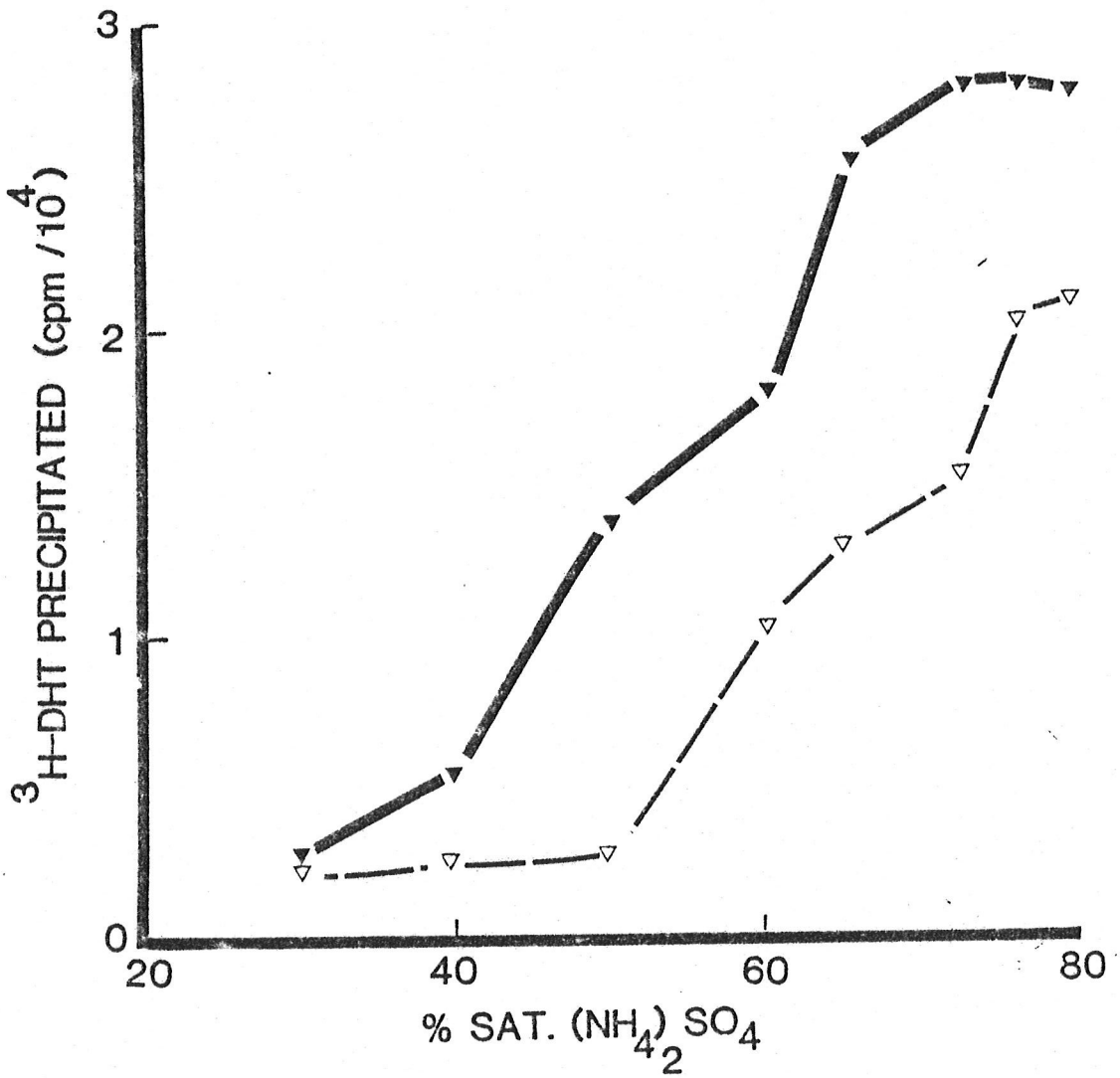

3
2
1
0
20
40
60
80
^{3}H-DHT PRECIPITATED (cpm /10^4)
% SAT. $(NH_4)_2 SO_4$

Figure 4 Androgen binding activity in follicular fluid from ovarian follicles of different sizes. Follicular fluid was aspirated and pooled according to size of follicles with diameters of < 2, 2-4, 4-6, and > 6 mm. After removal of free steroids by charcoal treatment, samples of 10 µl each were tested for specific binding for ^{3}H-DHT. Each column represents a mean of 2 experiments induplicate, and the vertical bar indicates the range of values.

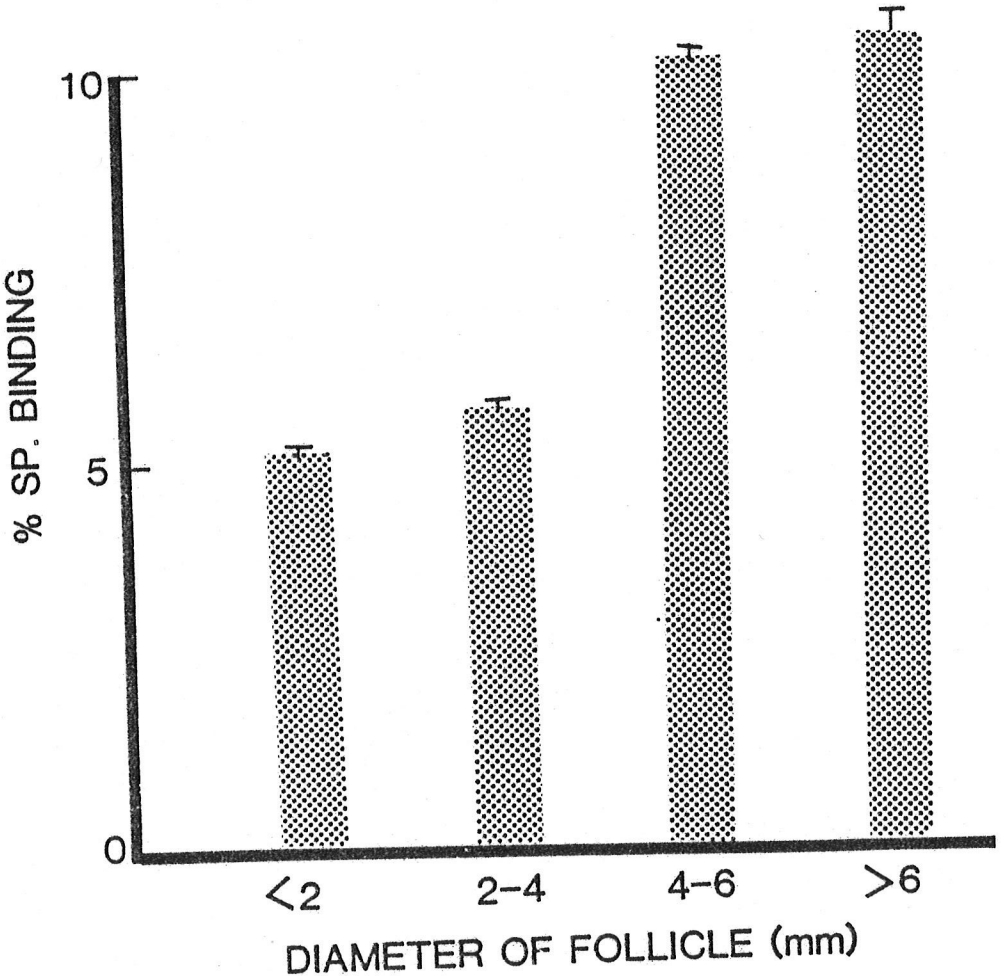

10
5
0
% SP. BINDING
<2
2-4
4-6
>6
DIAMETER OF FOLLICLE (mm)

Figure 5 Comparison of the androgen binding activities in bovine follicular fluid, serum and epididymal extracts. Samples of bovine follicular fluid (FF), serum and epididymal extracts were tested at various concentrations for specific binding of ^{3}H-DHT.

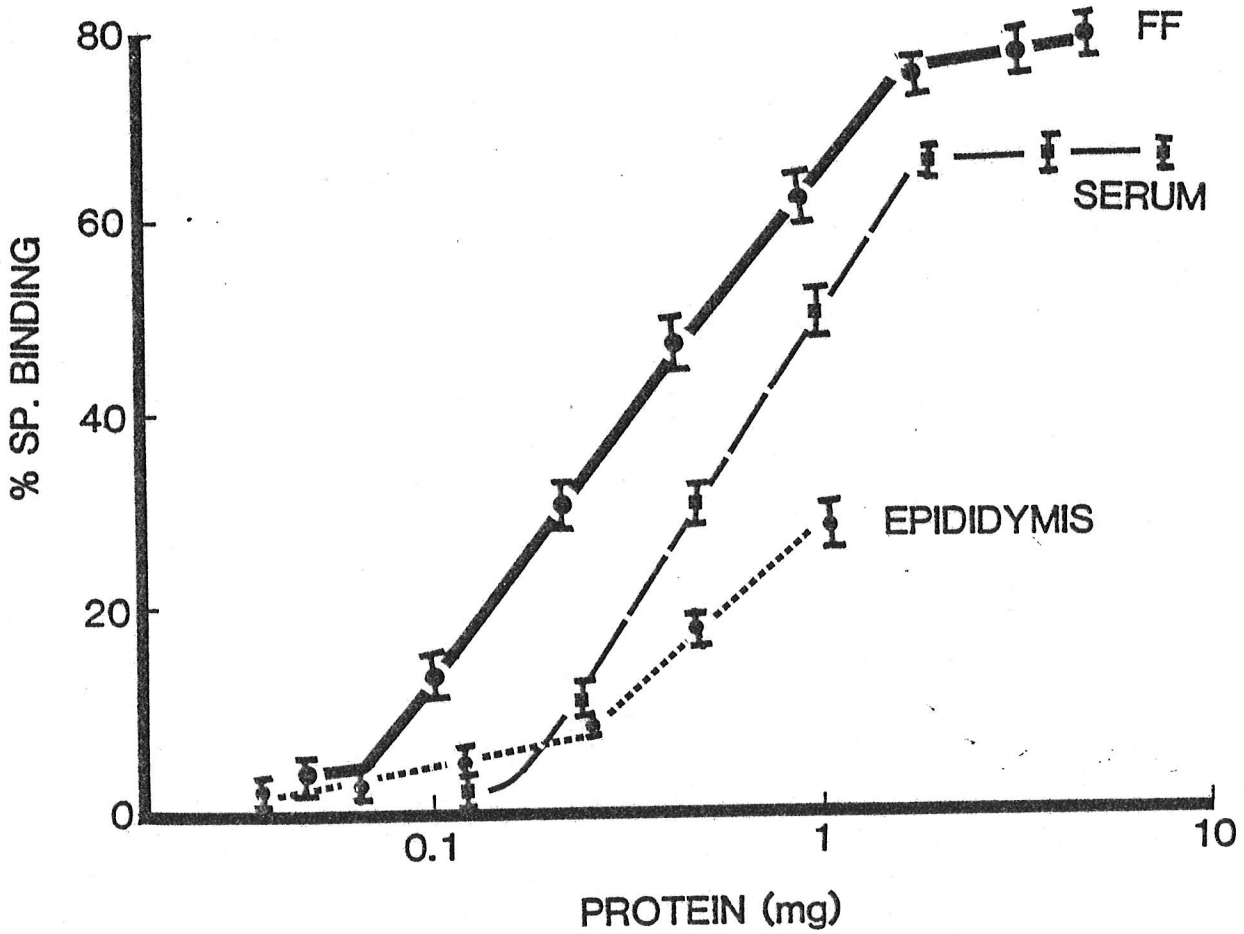
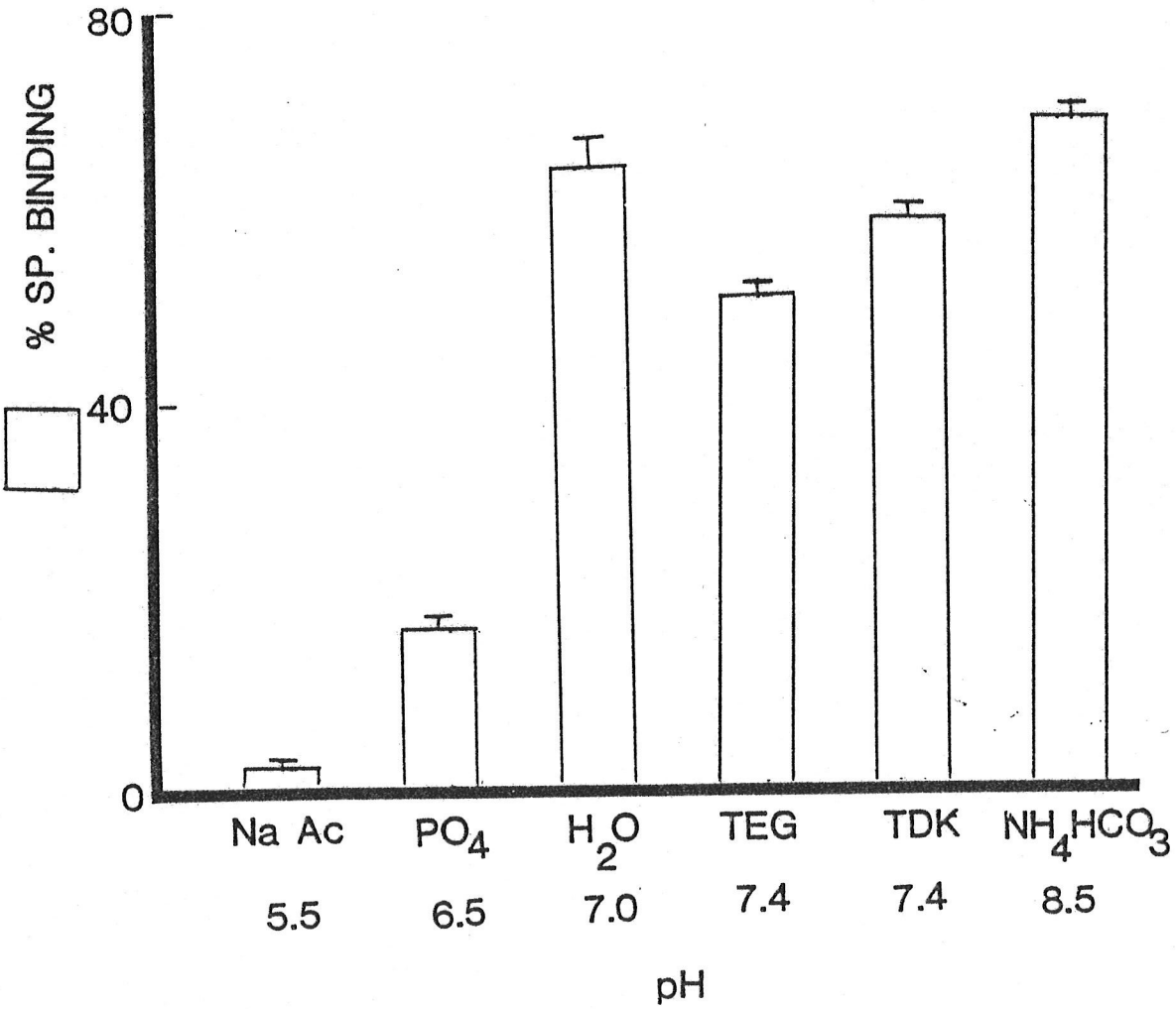

80
60
40
20
0
% SP. BINDING
0.1
1
10
PROTEIN (mg)
FF
SERUM
EPIDIDYMIS

Figure 6 Effects of pH on the androgen binding activity in bovine follicular fluid. Samples of 1 ml of fractions after HTP chromatography were dialysed in 0.1 M potassium phosphate (pH 6.5), distilled water (pH 7.0), TEG (pH 7.4), TDK (pH 7.4) and 0.5% ammonium bicarbonate (pH 8.5) for 8 hours, and 0.1 ml of each sample was tested for specific binding activity with ^{3}H-DHT (20,000 cpm) in the presence or absence of unlabelled DHT. Each column represents a mean of 2 experiments in duplicate, and the vertical bar indicates the range values.


80
40
0
% SP. BINDING
Na Ac
PO4
H2O
TEG
TDK
NH4HCO3
5.5
6.5
7.0
7.4
7.4
8.5
pH

vity for androgen and that the slope of the dilution curve for bff was similar to that for serum but nonparallel to that for epididymis.

2. STABILITY OF THE ANDROGEN BINDING ACTIVITY.

The stability of the androgen binding activity in follicular fluid was tested at different pH's, temperatures and storage time intervals.

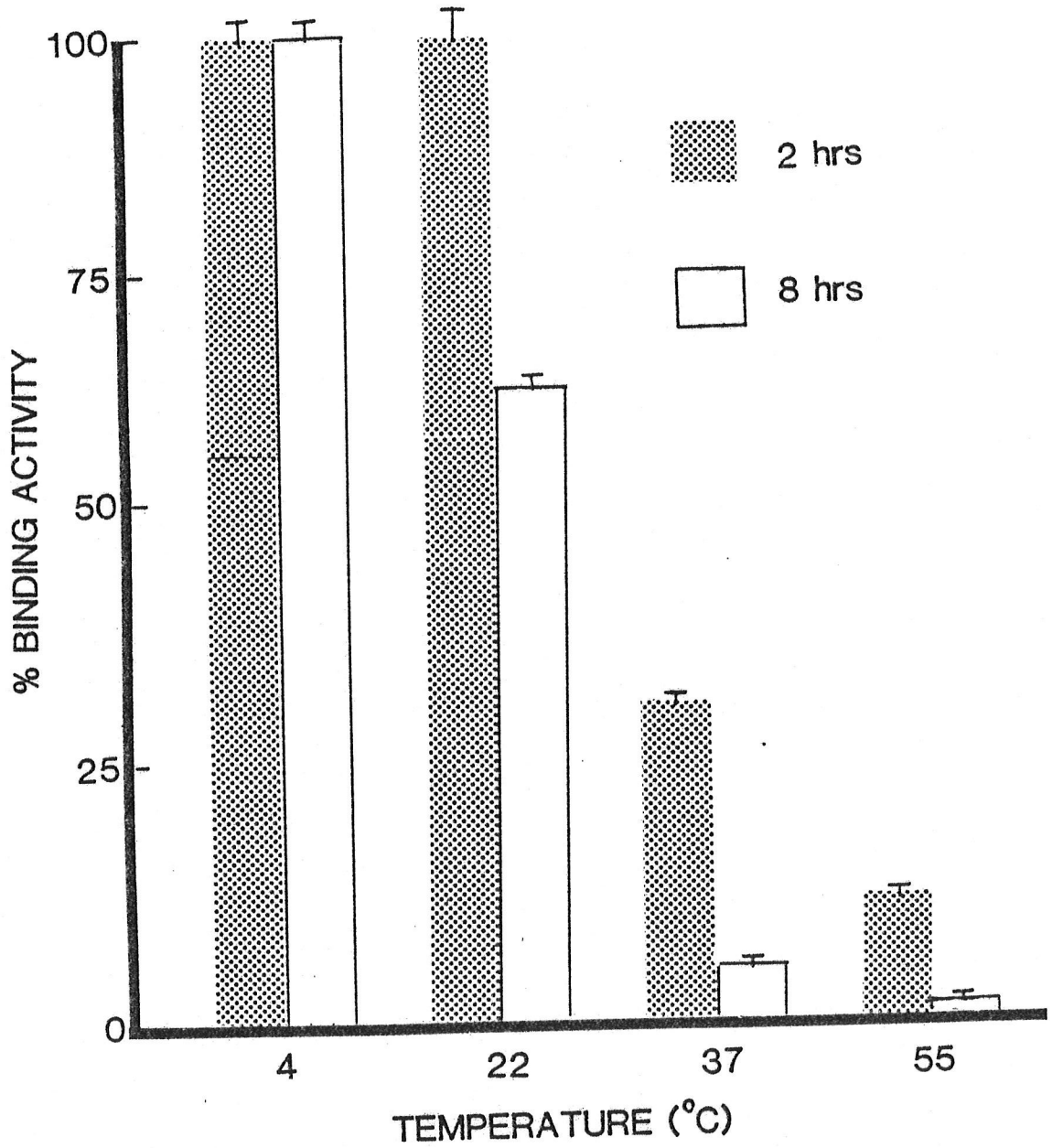
a) Effect of pH on the ^{3}H-DHT binding activity of follicular fluid. One ml each of bff was dialysed in 10 volumes of sodium acetate buffer at pH 6.5, water at pH 7.0, DTK solution at pH 7.4, TEG at pH 7.4 and 0.05% ammonium bicarbonate at pH 8.5 for 8 hours, and tested for binding of ^{3}H-DHT.

The results are shown in Figure 6 indicating that the androgen binding activity in follicular fluid was declined below pH 7, but relatively stable at a pH range of 7.0-8.5.

b) Effect of temperature on the ^{3}H-dihydrotestosterone binding activity in follicular fluid. The binding activity of bff was measured after incubating at temperatures of 4°, 22°, 37°, 55° for 2 or 8 hours.

Figure 7 indicates that after 2 hours of incubation bff showed no difference in binding activity at 4°C of 22°C but decreases sharply at 37°C and becomes very low at 55°C; and after 8 hours of incubation at 22°C, the binding activity of bff decreased considerably compared to that at 4°C, and became very low at 37°C and almost undetectable at 55°C. The results indicate that the androgen binding activity in bff declined at room temperature (22°C) and was very labile at temperature above 37°C.

Figure 7 Effect of temperature on the androgen binding activity of bovine follicular fluid. Samples of 1 ml of charcoal treated bff were kept at 4^{o}, 22^{o}, 37^{o} and 55^{o}C for 2 or 8 hours in TEG, and 0.1 ml of each sample was tested for specific binding to ^{3}H-DHT. Each column represents a mean of 2 experiments in duplicate, and the vertical bar indicates the range of values.


100
75
50
25
0
% BINDING ACTIVITY
2 hrs
8 hrs
4
22
37
55
TEMPERATURE (°C)

c) Effect of incubation at 4°C on the specific binding of follicular fluid for DHT. Samples of bff were stored at 4°C in TEG or distilled water up to 5 days, and the binding activity was measured using freshly diluted bff in TEG and distilled water as controls.

Results are shown in Figure 8 indicating the activity of bff decline considerably when stored in water but is relatively stable when stored in TEG. It appears that the presence of glycerol in the buffer helps to stabilize the binding activity.

3. DISPLACEMENT OF BOUND ^{3}H-DIHYDROTESTOSTERONE BY VARIOUS STEROID HORMONES.

The specificity of the androgen binding activity in bff was examined by displacement of bound ^{3}H-DHT by various steroid hormones. Samples of bff were incubated for 2 hours with ^{3}H-DHT in the presence or absence of varying quantities of 5α-dihydrotestosterone (DHT), testosterone (T), estrone (E), 17β-estradiol (E_2), estriol (E_3), dehydroepiandrosterone (DHA), androstanediol (DIOL), progesterone (P), dexamethasone (DMS) and aldosterone (Ald). The % specific binding plotted against the amount of steroid hormone used for displacement is show in Figure 9. The binding activities for DHT and T were similar and of high affinities since the slopes of both dose-response curve were very similar. Estrogens also bound to bff but with much lower affinity when compared to DHT and T. Other androgenic steroids, DHA and DIOL, showed only slight binding, and no binding was observed for progesterone, DMS and aldosterone. These results differ slightly from those reported for TeBG in bovine serum (1,178).

Figure 8 Stability of the androgen binding activity in TEG buffer. Samples of 1 ml of bff were diluted 1:10 with TEG or water and stored at 4 °C for 0 or 5 days, and were then tested for specific binding for ^{3}H-DHT. Each column represents of 2 experiments in duplicate, and the vertical bar indicates the range of values.

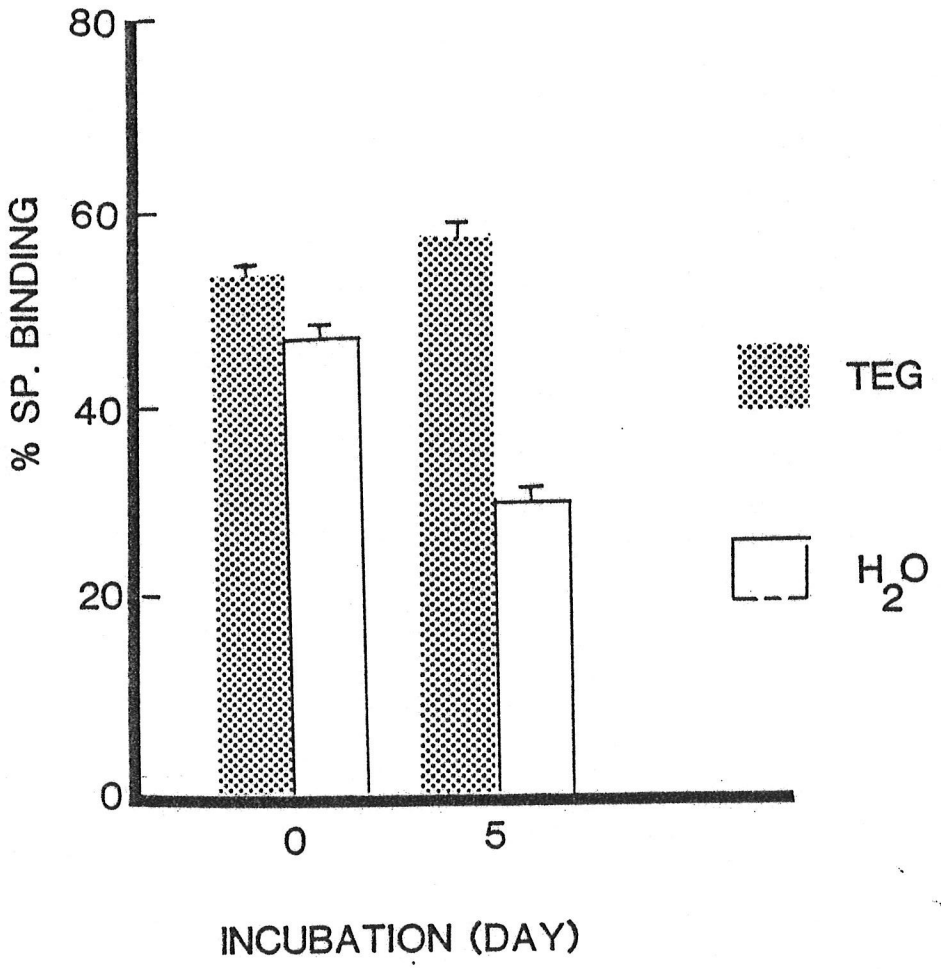
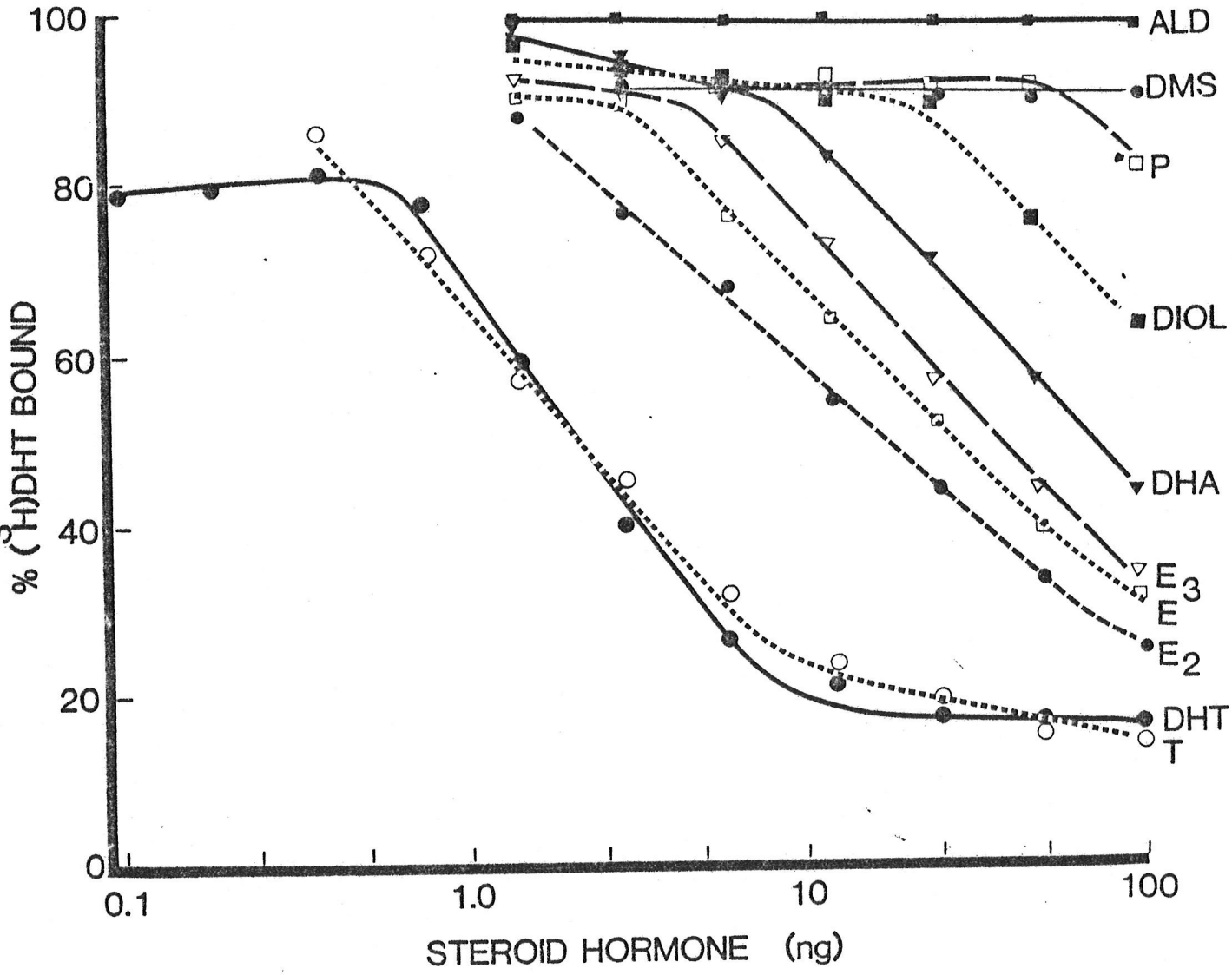

80
60
40
20
0
% SP. BINDING
0
5
INCUBATION (DAY)
TEG
H2O

Figure 9 Displacement curves of various steroid hormones for the specific binding of ^{3}H-DHT by bovine follicular fluid. Samples of 30 µg protein of charcoal treated bff were incubated with ^{3}H-DHT (20,000 cpm) in the presence or absence of various amounts of unlabelled 5 α-dihydrotestosterone (DHT), testosterone (T), 17β-estradiol (E_2), estrone (E), estriol (E_3), dihydroepiandrosterone (DHA), androstanediol (DIOL), progesterone (P), dexamethasone (DMS) and aldosterone (Ald). Incubation was carried out at 4°C for 2 hours.


100
80
60
40
20
0
% (^{3}H)DHT BOUND
0.1
1.0
10
100
STEROID HORMONE (ng)
ALD
DMS
P
DIOL
DHA
E3
E
E2
DHT
T

III. COMPARATIVE STUDIES ON THE ANDROGEN BINDING ACTIVITIES IN BOVINE FOLLICULAR FLUID AND SERUM.

In order to answer the question of whether the androgen binding component(s) in bovine follicular fluid and serum are the same or not, partially purified preparations of these materials were processed and their properties were analysed and compared.

1. EXPERIMENT I.

The androgen binding activities in bff and serum were compared upon partial purification after ammonium sulfate precipitation, DEAE-cellulose chromatography, HTP chromatography and a second DEAE-cellulose chromatography upon continuous gradient elution.

Samples of 50 ml each of bff and serum were precipitated by 25-55% saturation of ammonium sulfate to concentrate most of the androgen binding activities, and then processed separately but under identical experimental conditions. After dialysis in TEG, the sample from ammonium sulfate precipitation was loaded onto a DEAE-cellulose column. The adsorbed proteins were eluted stepwise with 0.05, 0.125 and 0.2 M NaCl in TEG. The results in Figure 10 for bff and Figure 11 for serum show that the distributions of proteins and androgen binding activities for both sample were very similar, except a slightly lower binding activity in the 0.125 M NaCl fraction for serum.

The dialysed bff and serum samples after DEAE chromatography (Figure 10 and 11) were then individually applied to HTP columns equilibrated in TG buffer. After the unadsorbed fractions, the adsorbed proteins were eluted by a continuous gradient of 0, 3, 1.5, and 5 mM potassium phos-

Figure 10 DEAE- cellulose chromatography of bovine follicular fluid. The precipitate of 25-55% saturation ammonium sulfate of 50 ml bovine follicular fluid, after dialysis, was loaded onto a DE-32 column (3 x 7 cm) in TEG pH 7.4. After the unadsorbed fraction, the adsorbed proteins were eluted stepwise by 0.05, 0.125 and 0.2 M NaCl in TEG. Fractions 0-79, 80-159 and 160-200 were collected in 7,2 and 4 ml per tube respectively, at a flow rate of 75 ml per hour. Aliquots of 50 µl of each fraction were assayed for binding activity.

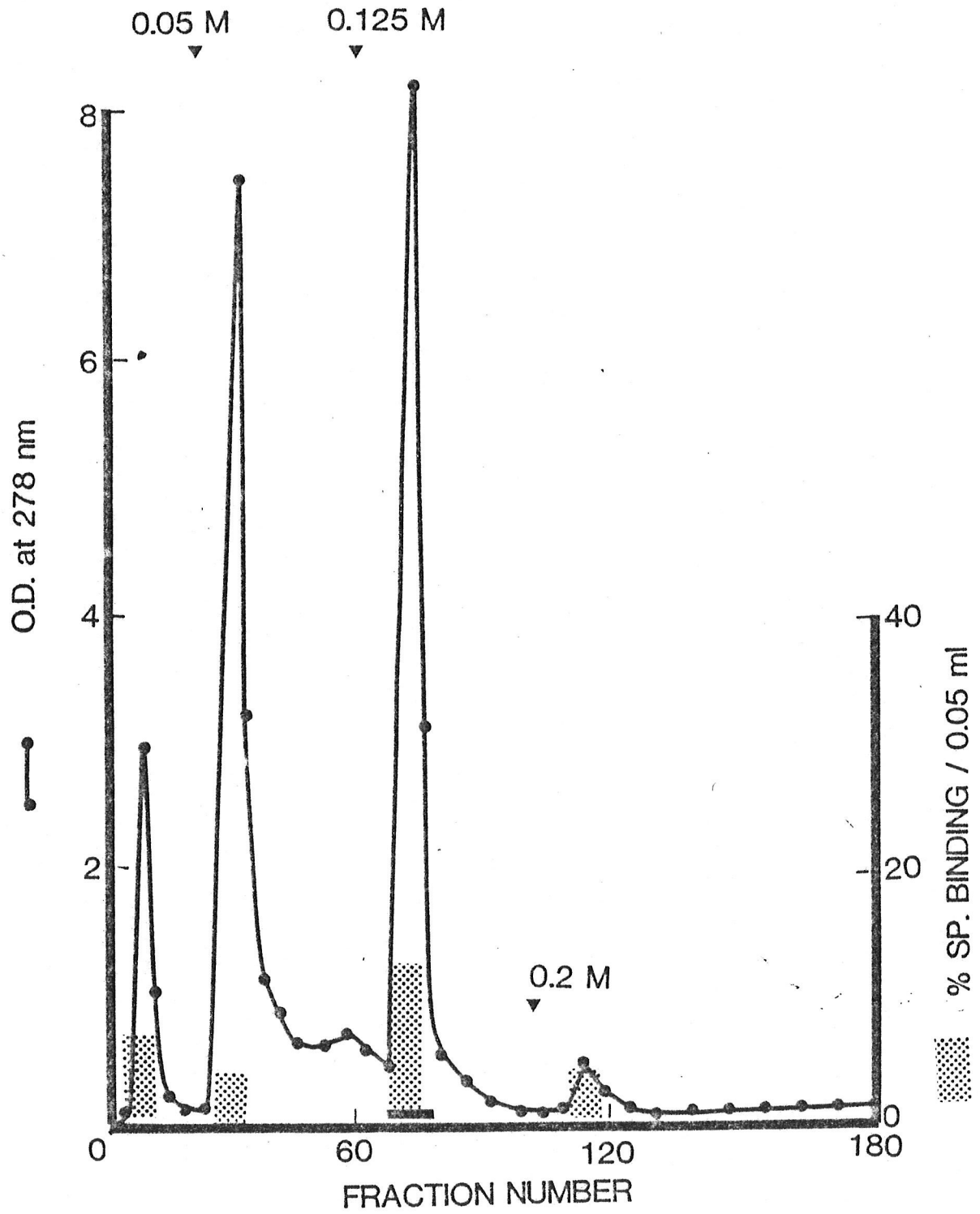
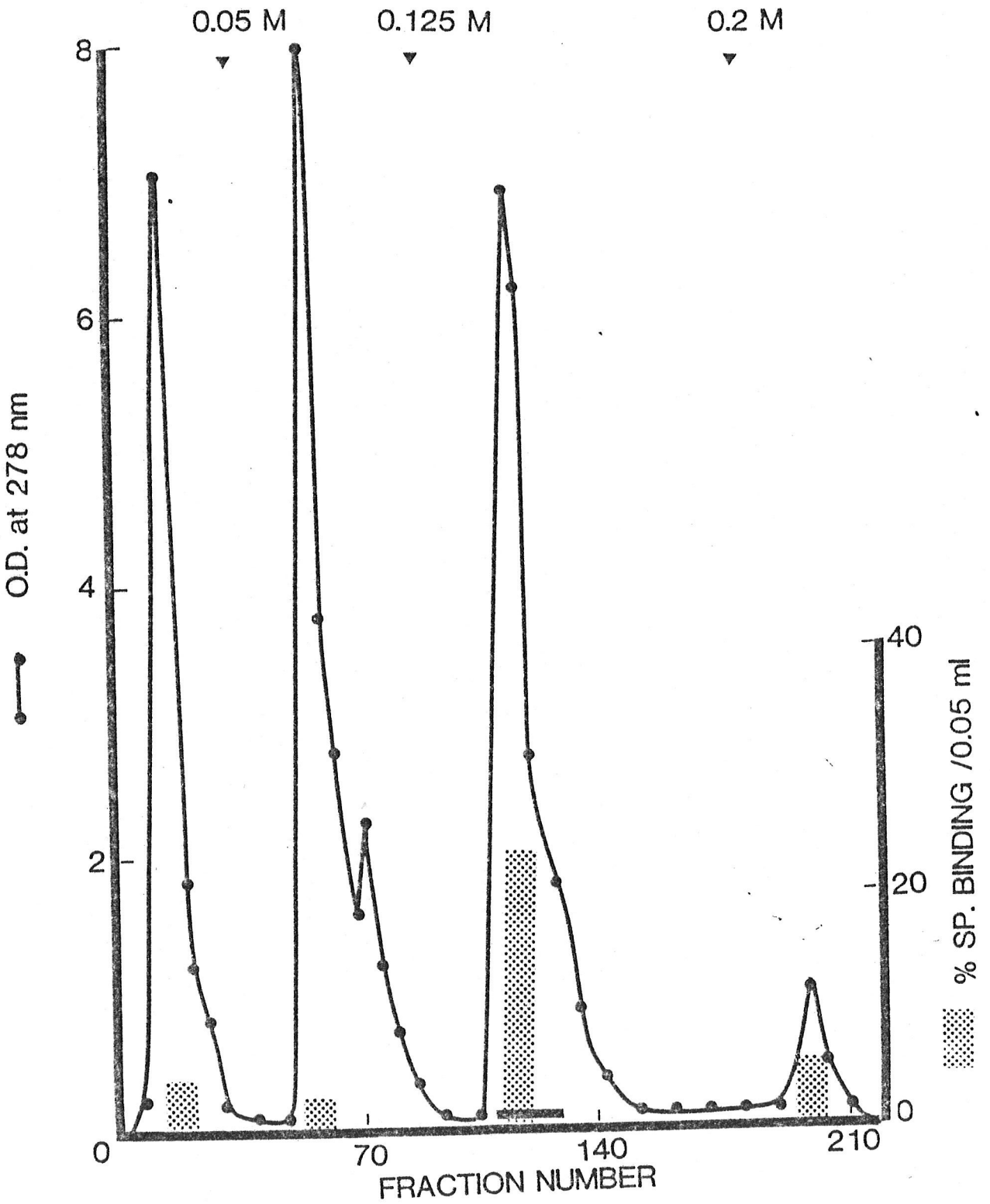

0.05 M
0.125 M
0.2 M
O.D. at 278 nm
% SP. BINDING / 0.05 ml
FRACTION NUMBER
0
2
4
6
8
0
20
40
0
60
120
180

Figure 11 DEAE-cellulose chromatography of bovine serum. The precipitate of 25-55% saturated ammonium sulfate of 50 ml non-pregnant cow serum, after dialysis, was loaded onto a DE-32 (3 x 7 cm) in TEG, pH 7.4. After the adsorbed fractions, the adsorbed protein were eluted stepwise by 0.05, 0.125 and 0.2 M NaCl in TEG. Fractions 0-64, 65-109 and 110-180 were collected in 7, 2 and 4 ml respectively, at flow rate of 75 ml/hour. Aliquots of 50 µl of each fraction were assayed for binding activity.


0.05 M
0.125 M
0.2 M
8
6
4
2
0
O.D. at 278 nm
40
20
0
% SP. BINDING /0.05 ml
70
140
210
FRACTION NUMBER

phate in TG buffer. Results in Figur 12 for the bff and Figure 13 for the serum sample show that profiles for elution of proteins and binding activities in bff and serum samples were relatively similar; but a considerably higher binding activity was observed in the bff fraction (Figure 12). The binding activities were eluted from the HTP column at approximately 1.2 mM of potassium phosphate. The appropriate fractions were pooled and dialysed against TEG, pH 7.4. The dialysed samples after HTP chromatography (Figure 12 and 13) were then applied to another DEAE-cellulose column in TEG buffer. The adsorbed fractions were eluted with a continuous gradient of 0, 0.2, 0, 0, 0.25, 0.10, and 0.15 M NaCl in TEG. The results in Figure 14 for bff sample and Figure 15 for serum sample show that the profiles of proteins were relatively similar, though not the same, but the distributions of androgen binding activities were distinctly different between the bff and serum samples. A small but distinct peak of proteins with high binding activity was eluted at 0.08-0.10 M NaCl in the bff sample and was in significantly low in the serum sample (Figure 14 and 15). These 0.08-0.10 M NaCl fractions of bff and serum were pooled separately and analysed by PAGE. Figure 16 shows that the electrophoretic patterns of these fractions were not identical and that the bff fraction contatined an additional protein band. This stage of the experiment, there were very small amounts of partially purified fractions available for further characterization to be more conclusive on the identities of the binding proteins in follicular fluid and serum.

2. EXPERIMENT II.

In the second series of experiments, the bff and serum

Figure 12 Hydroxylapatite chromatography of the binding activity fraction from follicular fluid after DEAE-cellulose chromatography. The pooled actived fractions of bff (512 mg) eluted by 0.125 M NaCl upon DEAE-cellulose chromatography (Figure 10), after dialysis, was loaded onto a HTP column (3 x 9 cm) in TEG, pH 7.4. After the unadsorbed fraction, the adsorbed proteins were eluted by continuous gradient of 0, 3, 1.5 and 5 mM phosphate in TG. Fractions were collected in 3 ml/tube at a flow rate of 75 ml/hour. Aliquots of 200 µl from fraction were assayed for binding activity.

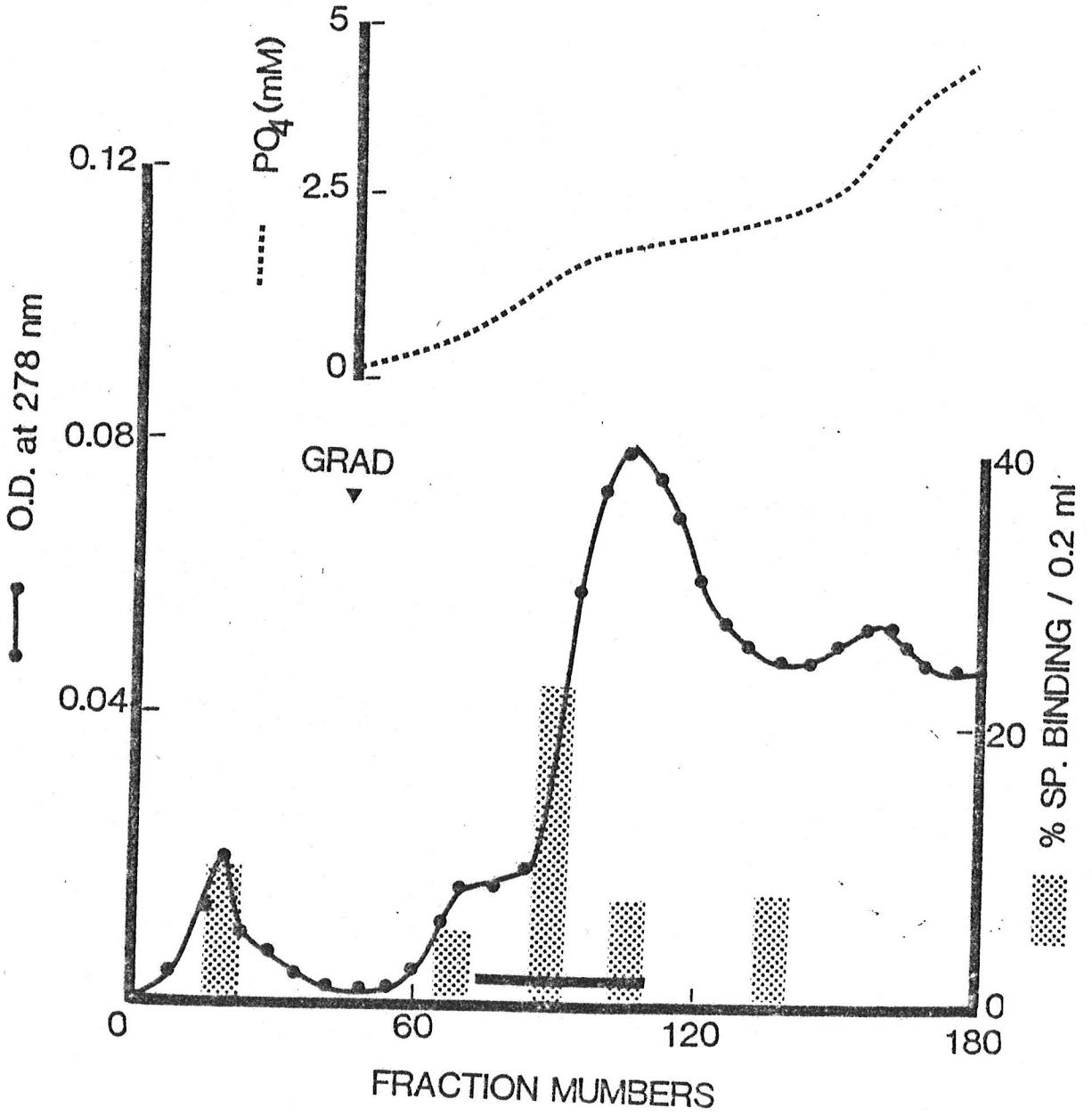

5
2.5
0
PO4 (mM)
0.12
0.08
0.04
O.D. at 278 nm
GRAD
40
20
0
% SP. BINDING / 0.2 ml
0
60
120
180
FRACTION MUMBERS

Figure 13 Hydroxylapatite chromatography of the androgen binding fraction from bovine serum after DEAE-cellulose chromatography. The pooled active fraction of serum from non-pregnant cow (256 mg) eluted by 0.125 M NaCl upon DEAE-cellulose chromatography (Figure 11), after dialysis, was loaded onto a HTP column (3 x 9 cm) in TG, pH 7.4. After the unadsorbed fractions, the adsorbed proteins were eluted by a continuous gradient of 0, 3, 1.5 and 5 mM phosphate in TG. Fractions were collected in 3 ml/tube at a flow rate 75 ml/hour. Aliquots of 200 µl from each fraction were assayed for binding activity.

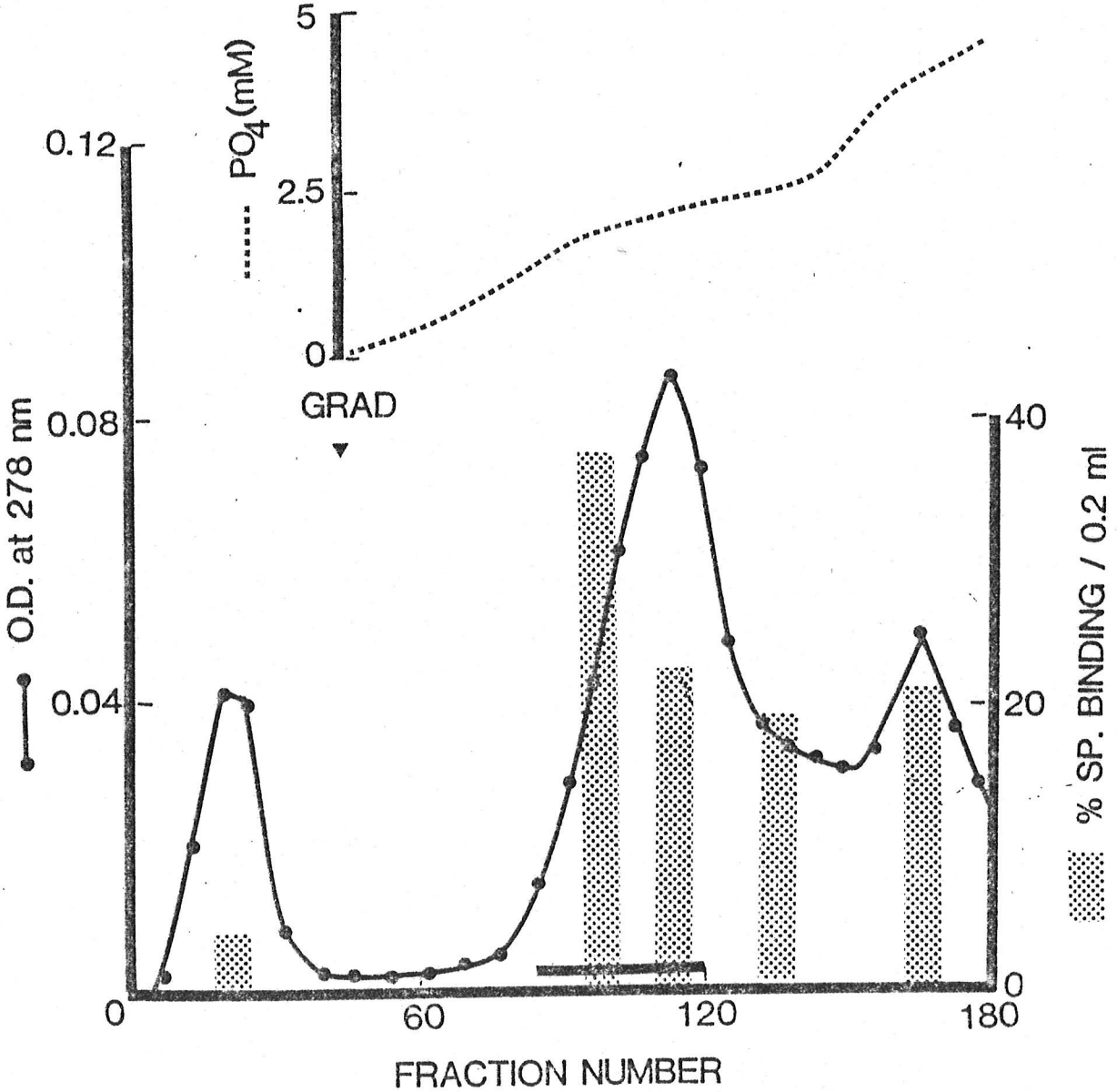

5
2.5
0
PO4(mM)
GRAD
0.12
0.08
0.04
0
O.D. at 278 nm
40
20
0
% SP. BINDING / 0.2 ml
0
60
120
180
FRACTION NUMBER

Figure 14 DEAE-cellulose chromatography of the active fraction from bovine follicular fluid after hydroxylapatite chromatography. After dialysis, the pooled active fraction of bff (12 mg) after HTP chromatography (Figure 12) was loaded onto a DE-32 column (1.4 x 7 cm) in TEG pH 7.4. The adsorbed proteins were eluted by a continuous gradient of 0, 0.2, 0, 0, 0.25, 0.1 and 0.15 M NaCl in TEG. Fractions were collected in 4.5 ml/tube at a flow rate 75 ml/hour. Aliquots of 200 µl of each fraction were assayed for binding activity.

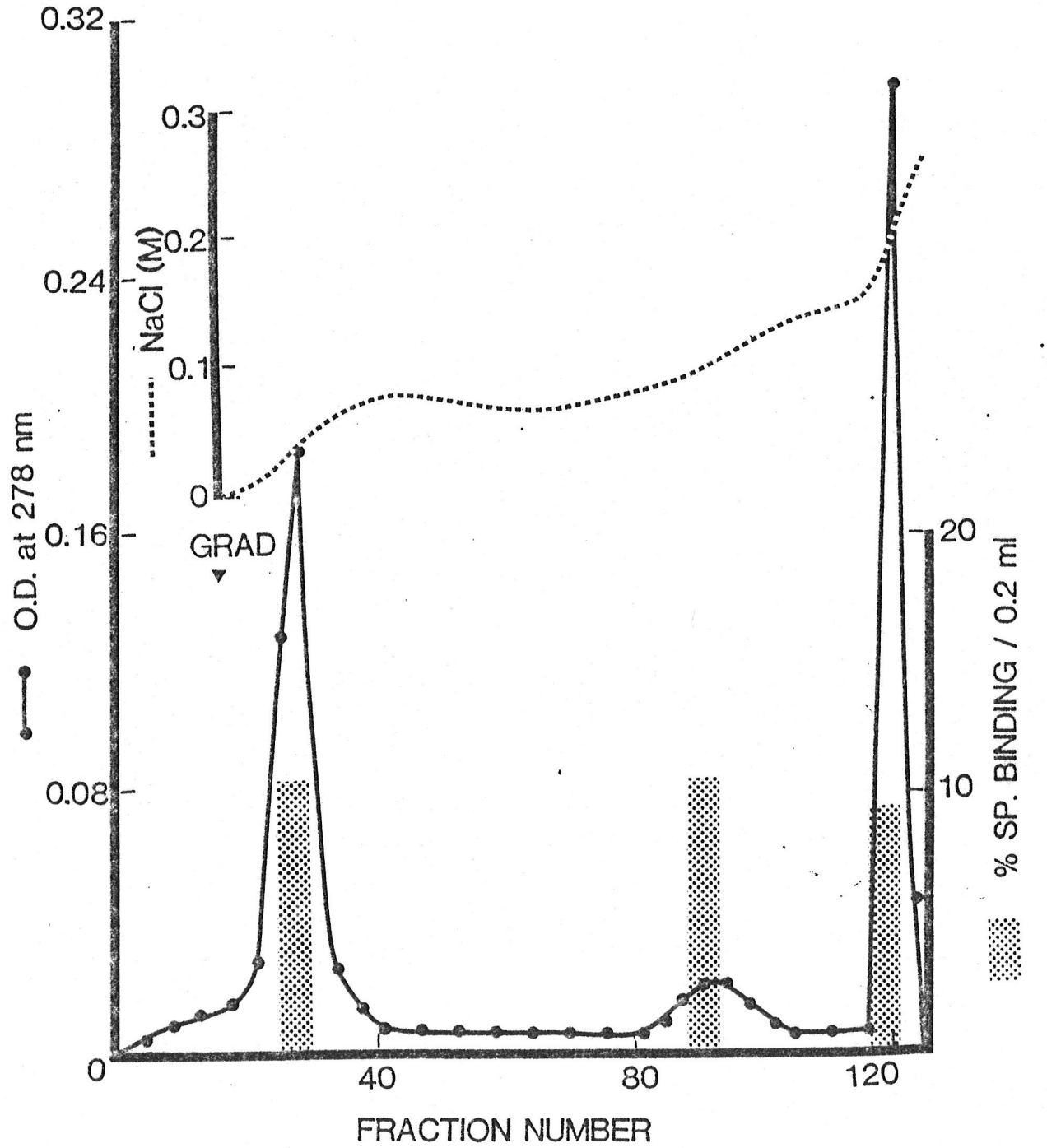

0.32
0.24
0.16
0.08
0
O.D. at 278 nm
0.3
0.2
0.1
0
NaCl (M)
GRAD
20
10
% SP. BINDING / 0.2 ml
0
40
80
120
FRACTION NUMBER

Figure 15 DEAE-cellulose chromatography of the active fraction from bovine serum after hydroxylapatite chromatography. The pooled active fraction of serum from non-pregnant cow (7.5 mg) after HTP chromatography (Figure 13), after dialysis, was loaded onto a DE-32 column (1.4 x 7 cm) in TEG pH 7.4. The adsorbed proteins were eluted by a continuous gradient of 0,0.2,0,0,0.25, 0.1 and 0.15 M NaCl in TEG. Fractions were collected in 4.5 ml per tube at a flow rate of 75 ml per hour. Aliquots of 200 µl of each were assayed for binding activity.

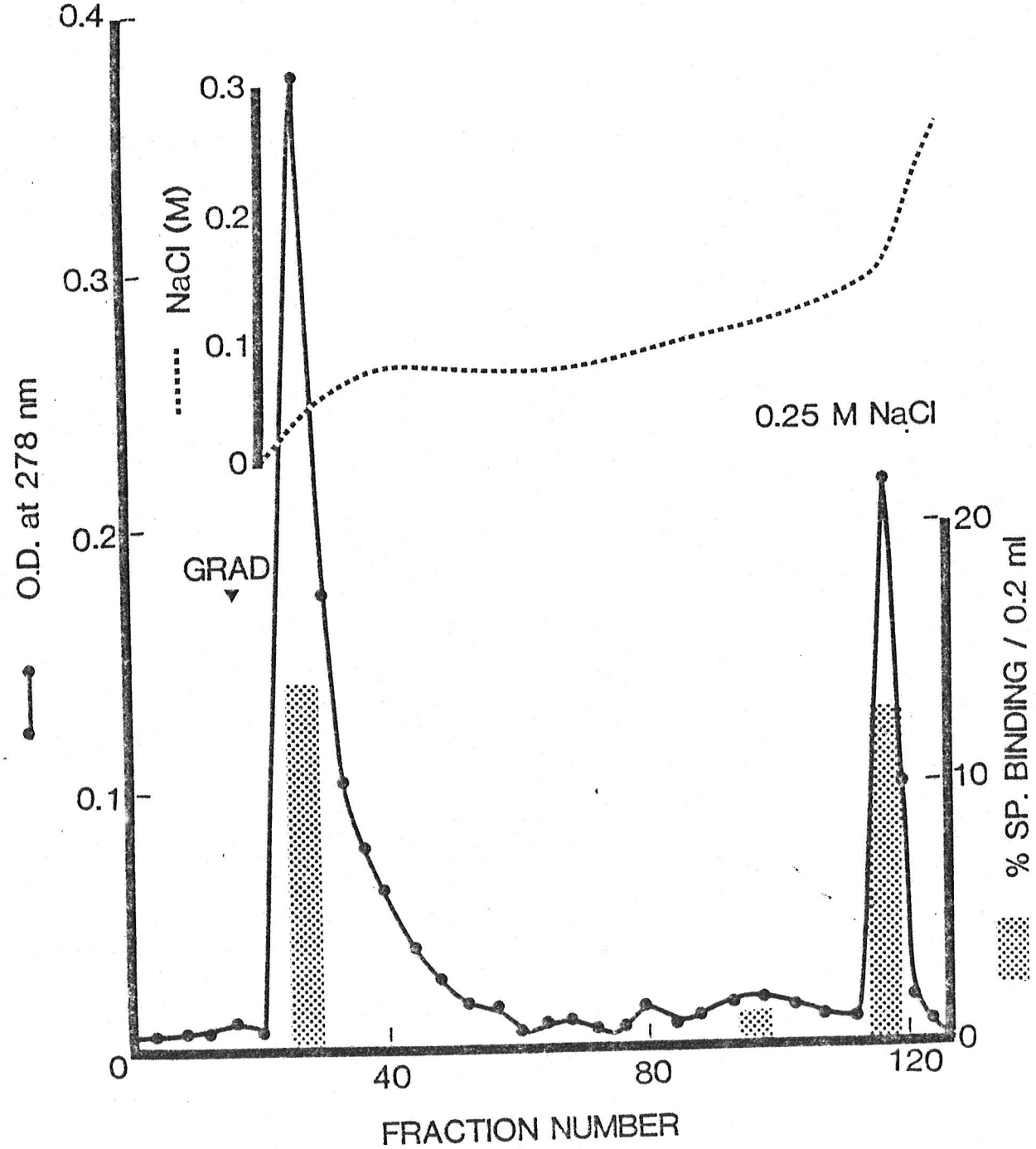
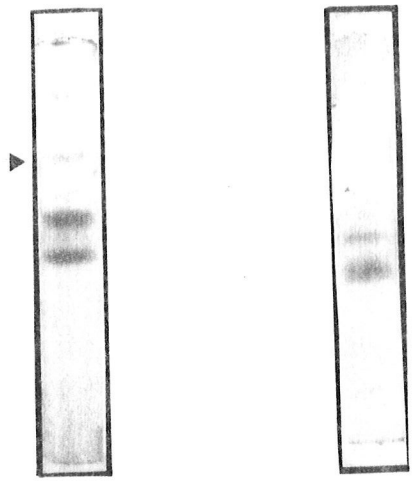

0.4
0.3
0.2
0.1
0
O.D. at 278 nm
0.3
0.2
0.1
0
NaCl (M)
GRAD
0.25 M NaCl
20
10
0
% SP. BINDING / 0.2 ml
0
40
80
120
FRACTION NUMBER

Figure 16 Comparison of protein patterns upon polyacrylamide gel electrophoresis of partially purified binding activities from follicular fluid and serum. Samples of 20 µl each of pools of 0.08-0.1 mM NaCl protein fractions with high DHT-binding activities from bovine follicular fluid (Figure 14) and bovine non-pregnant serum (Figure 15) after DEAE-cellulose chromatography were analysed by PAGE and stained for protein. ▶ indicates the presence of an additional protein band in the bff sample.



FOLLICULAR FLUID SERUM

samples were partially purified by ammonium sulfate precipitation, DEAE-cellulose chromatography, and 2 different conditions of HTP chromatographies followed by SS-PAGE for comparison.

The binding activities for androgen in bff and serum were precipitated by 25-55% saturation of ammonium sulfate as described previously. The dialysed sample after ammonium sulfate precipitation was applied to a DEAE-cellulose column and the adsorbed proteins were eluted stepwise with increasing concentrations of NaCl. Results show relatively similar patterns of distributions for proteins and binding activities of bff and serum samples and are in good agreement with those of previous experiment (Figure 10 and 11). Fractions of the 0.125 M NaCl elution were appropriately pooled and dialysed.

The dialysed sample after DEAE-cellulose chromatography was then applied to an HTP column as described in the previous experiment. Results confirm the results of the previous experiment (refer to Figure 12 and 13) that high affinity binding activities were eluted at approximately 1.5 mM potassium phosphate. Appropriate fractions were pooled and dialysed in TG buffer at pH 7.4.

The dialysed samples after HTP chromatography were further fractionated by rechromatography onto another HTP column, but eluting with a finer compound gradient of 0, 1.5, 1.5 mM potassium phosphate in TG buffer. Figure 17 (bff) and Figure 18 (serum) show that in both cases a peak of binding activity was eluted at approximately 0.8-1 mM potassium phosphate in TG, and that the bff sample contained significantly higher binding activities than the serum sample. The appropriate fractions were pooled and concentrated by ultrafiltration.

Figure 17 Recycling upon hydroxylapatite chromatography of the activity fraction from follicular fluid after previous hydroxylapatite chromatography. The pooled active fraction of bff (112 mg) after HTP chromatography, after dialysis, were applied onto another HTP column (2 x 10 cm) in TG, pH 7.4. The adsorbed proteins were eluted by a continuous gradient of 0,1.5,1.5 mM phosphate buffer. Fractions of 5 ml/tube were collected at a flow rate of 60 ml/hour. Aliquots of 50 µl from each fraction were assayed for binding activity.

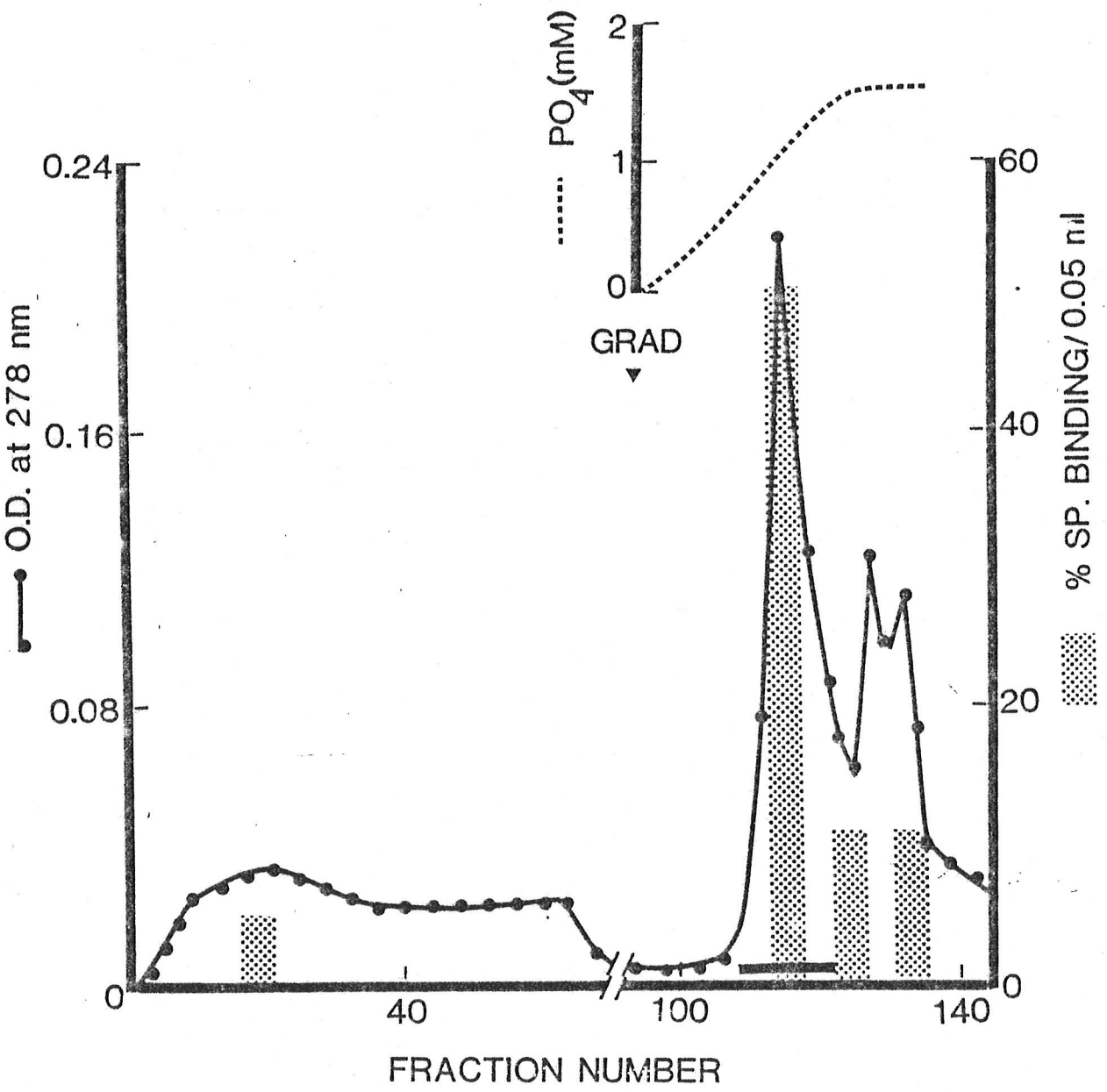
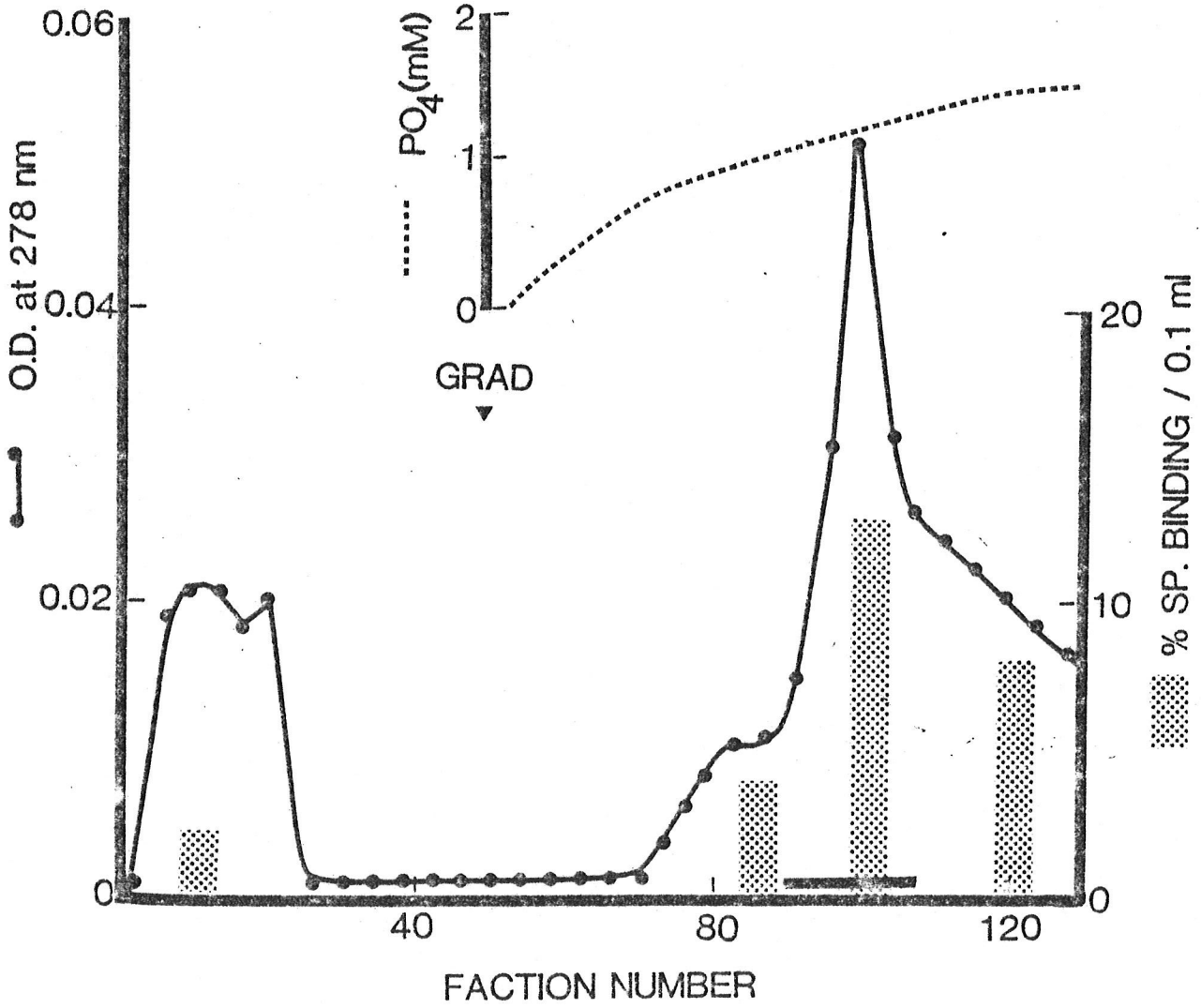

PO4 (mM)
2
1
0
GRAD
0.24
0.16
0.08
0
O.D. at 278 nm
60
40
20
0
% SP. BINDING/0.05 ml
40
100
140
FRACTION NUMBER

Figure 18 Recycling upon hydroxylapatite chromatography of the active fraction from bovine serum after previous hydroxylapatite chromatography. The pooled active fractions of non-pregnant cow serum (36 mg) after HTP column, after dialysis, were applied onto another HTP column (2 x 3 cm) in TG, pH 7.4. The adsorbed proteins were eluted 5 ml/tube at a flow rate of 60 ml/hour. Aliquots of 100 µl from each fractions were assayed for binding activity.


0.06
0.04
0.02
0
O.D. at 278 nm
2
1
0
PO4(mM)
GRAD
20
10
0
% SP. BINDING / 0.1 ml
40
80
120
FACTION NUMBER

The concentrated samples of partially purified androgen binding activities from bff and serum after the second HTP column were characterized by SS-PAGE to examine the electrophoretic mobilities of the binding protein (s) in these preparations. Gel of 10% polyacrylamide at pH 8.9 were used. Samples of 20 µg of the bff fraction of high binding activity (Figure 17) and 200 µg of the serum fraction of relatively low binding activity (Figure 18) after the second HTP chromatography were studied by SS-PAGE for proteins and ^{3}H-DHT binding activities. (From preliminary experiments it was observed that 20 µg of the serum fraction was not enough to show ^{3}H-DHT binding activity after electrophoresis). The results in figure 19 indicate that the electrophoretic mobilities of the ^{3}H-DHT binding proteins in these partially purified preparations of bff and serum were clearly different: Rf = 0.2 (segment 5) for bff and Rf = 0.5 (segment 12) for serum.

IV. PURIFICATION OF ANDROGEN BINDING PROTEIN IN FOLLICULAR FLUID.

1. FRACTIONATION BY AMMONIUM SULFATE PRECIPITATION.

Charcoal treated bff was fractionated by ammonium sulfate at various saturations by adding different amounts of saturated ammonium sulfate solution. The first step in isolating the bff-ABP was to concentrate the binding activity by precipitation of protein at 28-55% saturation of ammonium sulfate. Figure 20 shows the results of concentrating the androgen binding activity of bff by ammonium sulfate precipitation that the 28-55% fraction was comparatively low in protein but high in binding activity. This precipitate was redissolved in TEG and dialysed extensively against TEG for the next step of purification by ion-exchange chromatography.

Figure 19 Comparison by steady state polyacrylamide gel electrophoresis of the active fraction from bovine follicular fluid and serum after recycling on hydroxylapatite chromatography. SS-PAGE was prepared by mixing ^{3}H-DHT in 10% polyacrylamide gel at pH 8.9. Samples of 40 and 300 µg respectively, contained high binding activities after recycling on an HTP column of bff (Figure 17) and serum (Figure 18) were analysed by SS-PAGE in duplicate. One gel was stained for protein pattern and the other was segmented and counted for ^{3}H-DHT. The distribution of ^{3}H-DHT in each gel segment was plotted as percent of the total radioactivity in all segments of the gel. From the stained protein bands, the major binding activity of the bff sample was located at a Rf at 0.2 and those of the serum were located at Rf's of 0.5 and 0.67.

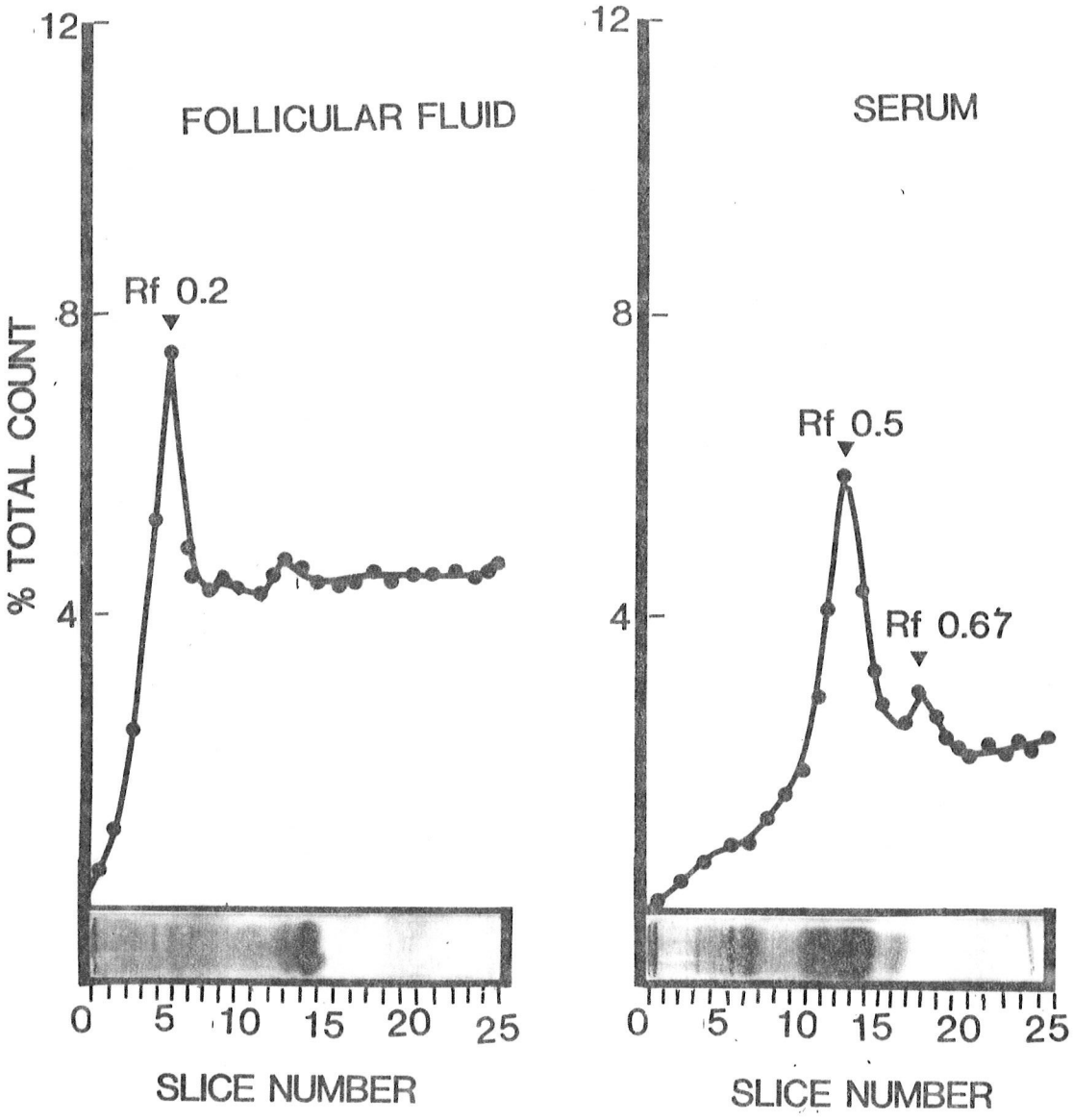
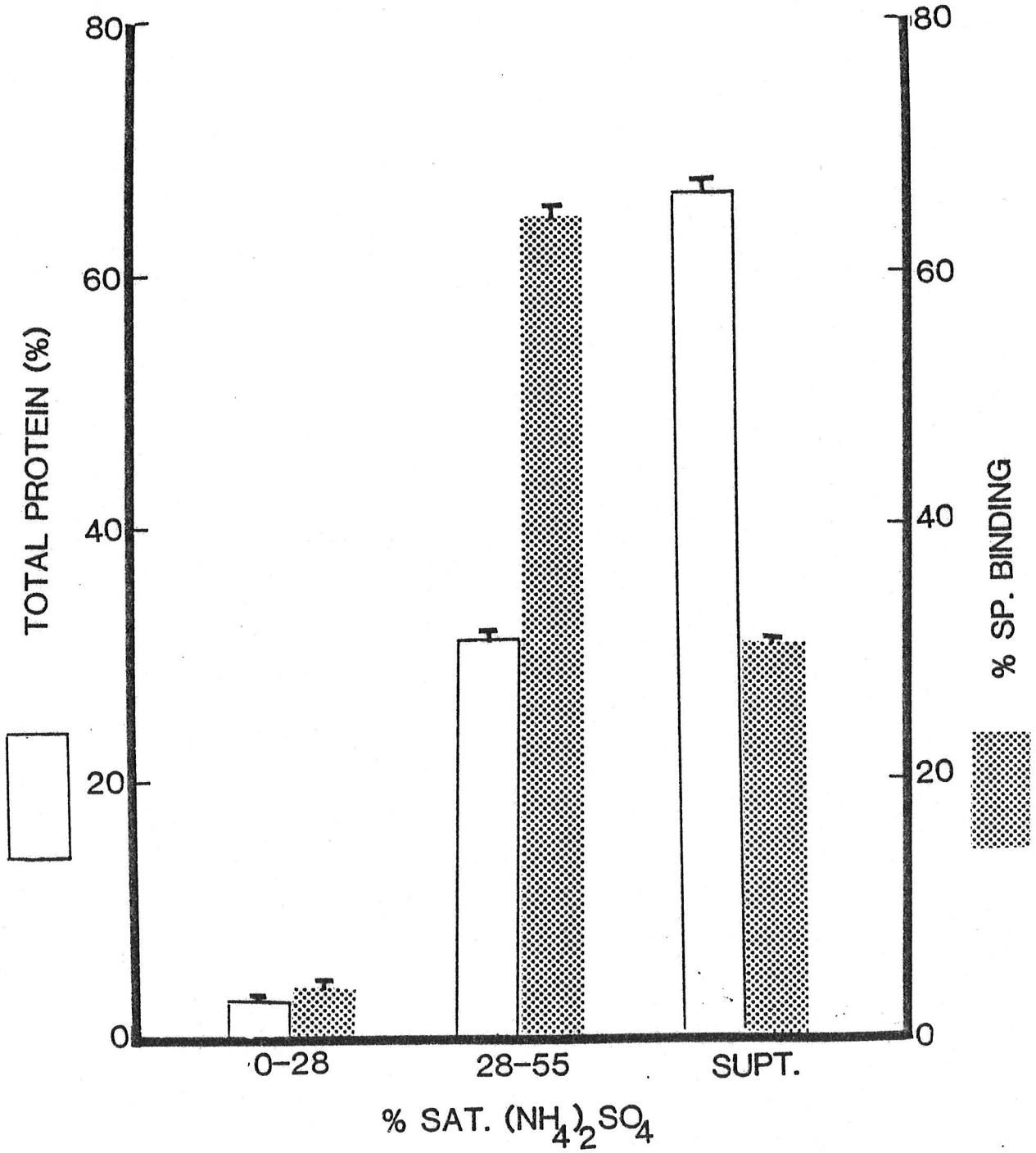

FOLLICULAR FLUID
Rf 0.2
% TOTAL COUNT
12
8
4
0
5
10
15
20
25
SLICE NUMBER
SERUM
Rf 0.5
Rf 0.67
12
8
4
0
5
10
15
20
25
SLICE NUMBER

Figure 20 Precipitation of the androgen binding activity in follicular fluid by ammonium sulfate saturation. Preparative scale of 400 ml charcoal treated bff was precipitated by adding saturated ammonium sulfate slowly to 28% and then to 55% saturation in order to precipitate protein with binding activities. After stirring for 30 minutes at 4°C, precipitated proteins were harvested by centrifugation at 10,000 RPM at 4°C for 30 minutes. The redissolved precipitates and supernatants were dialysed and assayed for binding activity.


80
60
40
20
0
TOTAL PROTEIN (%)
80
60
40
20
0
% SP. BINDING
0-28
28-55
SUPT.
% SAT. (NH4)2SO4

2. DEAE-CELLULOSE CHROMATOGRAPHY.

The dialysed concentrate of the 28-55% ammonium sulfate fraction was applied to DEAE-cellulose column, equilibrated in TEG, pH 7.4 . After the unadsorbed fraction, most of the adsorbed proteins with relatively low binding activity were eluted with 0.05 M NaCl in TEG, and most of the binding activity was then eluted with 0.12 M NaCl in the same buffer as shown in Figure 21. It was observed that the protein peak of the 0.12 M NaCl fraction was relatively small, compared to the unadsorbed and 0.05 M NaCl fractions, but contained high androgen binding activity. The 0.12 M NaCl fractions were pooled and dialysed against TG buffer for the next step of isolation.

3. ADSORBTION CHROMATOGRAPHY ON HYDROXYLAPATITE.

The HTP column was equilibrated in TG at pH 7.4. The dialysed sample after DEAE-cellulose chromatography (Figure 21) was loaded onto the HTP column. After the unadsorbed fractions, the adsorbed proteins were eluted by a continuous gradient of 0, 3, 1.5 and 5 mM potassium phosphate in TG buffer, follow by subsequent stepwise elution by 50 mM and 400 mM potassium phosphate in TG. Various elutions were monitored for protein distribution and androgen binding activities as summarized in Figure 22. A relatively small protein peak of high binding activity was eluted at approximately 1 mM of phosphate, and large peaks of adsorbed proteins with substantial binding activities were also eluted by 50 mM and 400 mM phosphate (Figure 22). Appropriate fractions were pooled accordingly and characterized for the presence of a high affinity binding protein for ^{3}H-DHT.

Figure 21 DEAE-cellulose chromatography of bovine follicular fluid proteins after ammonium sulfate precipitation. The redissolved precipitate of 28-55% saturation of ammonium sulfate of 400 ml bovine follicular fluid (Figure 20), after dialysis , was loaded onto a DE-32 column (8 x 6 cm) in TEG, ph 7.4. After the unadsorbed fractions, the adsorbed proteins were eluted stepwise by 0.05, 0.12 and 0.2 M NaCl in TEG. Fractions were collected in 25 ml/tube at a flow rate of 150 ml/hour. Aliquots of 100 µl from each fraction were assayed for binding activity.

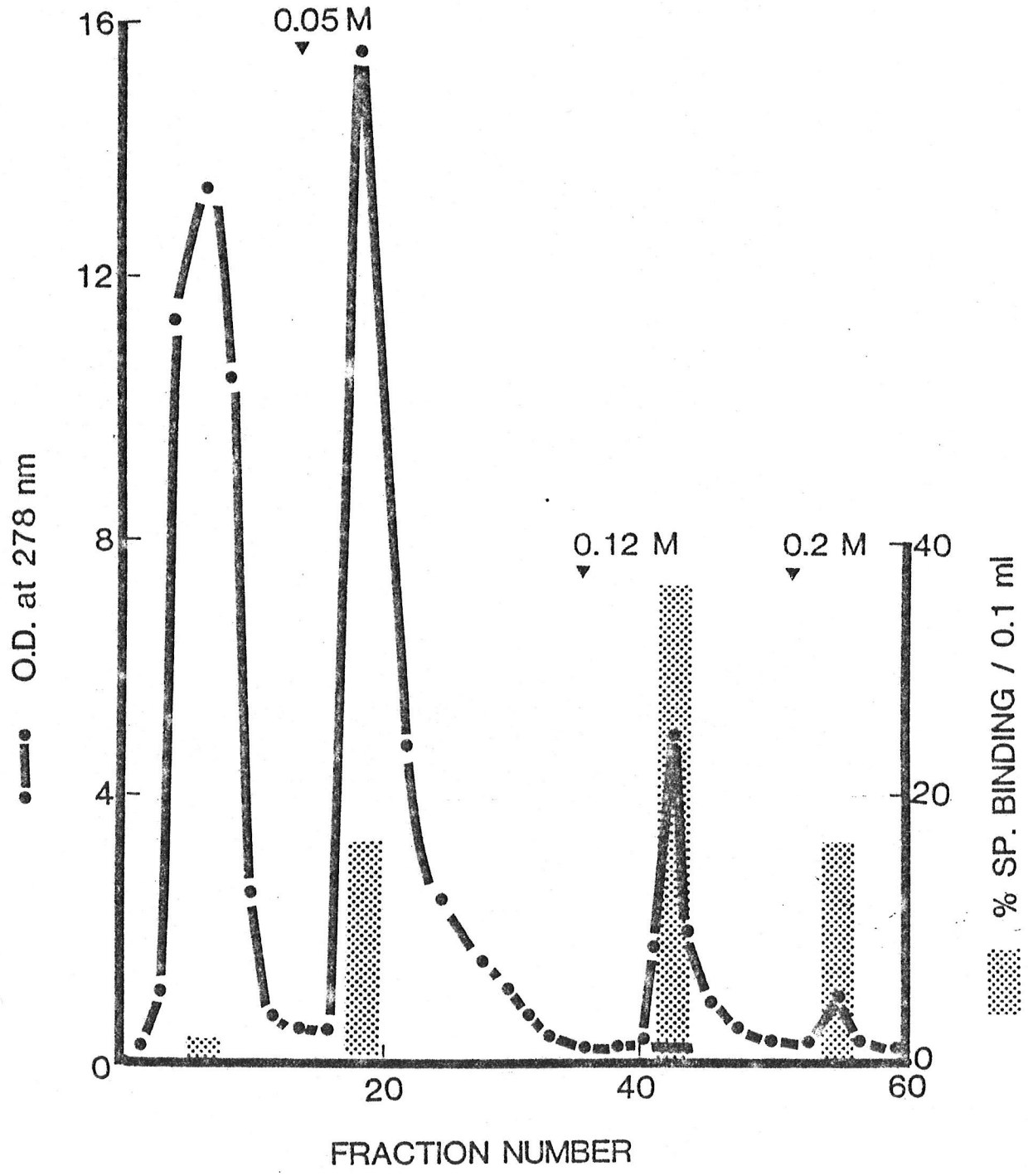
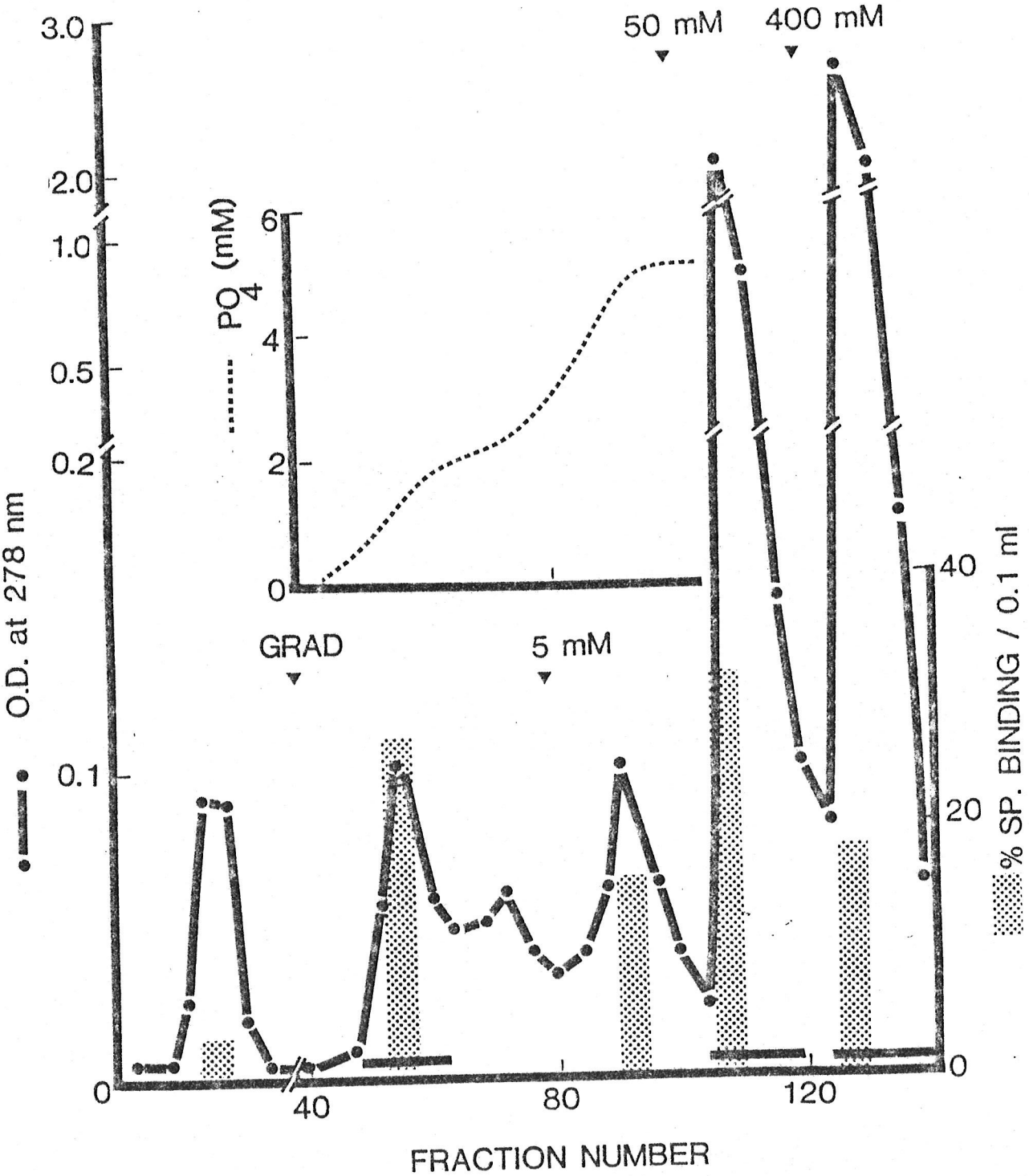

16
12
8
4
0
0.05 M
0.12 M
0.2 M
40
20
0
20
40
60
FRACTION NUMBER
O.D. at 278 nm
% SP. BINDING / 0.1 ml

Figure 22 Hydroxylapatite chromatography of the active fraction from follicular fluid after DEAE-cellulose chromatography. Pooled active fractions of bff (1908 mg) eluted by 0.12 M NaCl upon DEAE-cellulose chromatography (Figure 21), after dialysis, was loaded onto a HTP column (6 x 6 cm) in TG, pH 7.4. After the unadsorbed fractions, the adsorbed proteins were eluted by a continuous gradient of 0, 3, 1.5, and 5 mM, followed by stepwise elution of 0.05 and 0.4 M phosphate in TG. Fractions were collected 20 ml/tube at a flow rate of 120 ml/hour. Aliquots of 100 µl from each fraction were assayed for binding activity.


50 mM
400 mM
3.0
2.0
1.0
0.5
0.2
0.1
0
O.D. at 278 nm
PO4 (mM)
6
4
2
0
GRAD
5 mM
40
20
0
% SP. BINDING / 0.1 ml
40
80
120
FRACTION NUMBER

The 1 mM fraction (Figure 22) was examined for the presence of androgen binding protein since the amount of protein was relatively low in that fraction. Figure 23 shows that this 1 mM fraction contained several components of protein, and the major binding activity was coincident with a minor protein component (Rf = 0.2), which was very similar to the electrophoretic mobility of the specific bff androgen binding activity as characterized previously (refer to figure 19). This 1 mM fraction appeared to be the appropriate fraction, and was dialysed in TG buffer for the next step of isolation.

Since substantial binding activity was also observed in the fractions eluted stepwise by 50 mM and 400 mM phosphate (Figure 22), these fractions were also characterized further and examined for suitability and usefulness for purification of bff-ABP. Before SS-PAGE could be applied to examine the binding activities, further fractionation of these fractions by DEAE-cellulose chromatography had to be initiated because they contained relatively large amounts of other proteins.

Results in Figure 24 for the 50 mM fraction and Figure 25 for the 400 mM fraction show that both fractions could be resolved essentially into 3 protein peaks by DEAE-cellulose chromatography upon a continuous gradient of 0, 0.2, 0, 0, 0.25, 0.1 and 0.15 M NaCl in TEG buffer,except that the 50 mM fraction had a relatively large middle peak at 0.08-0.1 M NaCl (Figure 24) and the 400 mM fraction a large first peak at 0.03-0.06 M NaCl (Figure 25). Low binding activities were observed in these fractions. From data of previous studies (refer to Figure 14) that specific bff-ABP was eluted by 0.08-0.1 M NaCl upon DEAE-cellulose chromatography, the binding activity in the middle

Figure 23 Analysis by steady state polyacrylamide gel electrophoresis of the binding activity in the 1 mM phosphate fraction of hydroxylapatite chromatography. SS-PAGE was prepared by mixing ^{3}H-DHT in 10% polyacrylamide gel at pH 8.9. Samples of 40 µg containing high binding activity from 1 mM phosphate fraction were analysed by SS-PAGE in duplicate for ^{3}H-DHT binding. The distribution of ^{3}H-DHT in each gel segment was plotted as percent of the total radioactivity recovered in all segments of the gel. From the stained protein bands, the binding activity of the sample was located at Rf of 0.2.

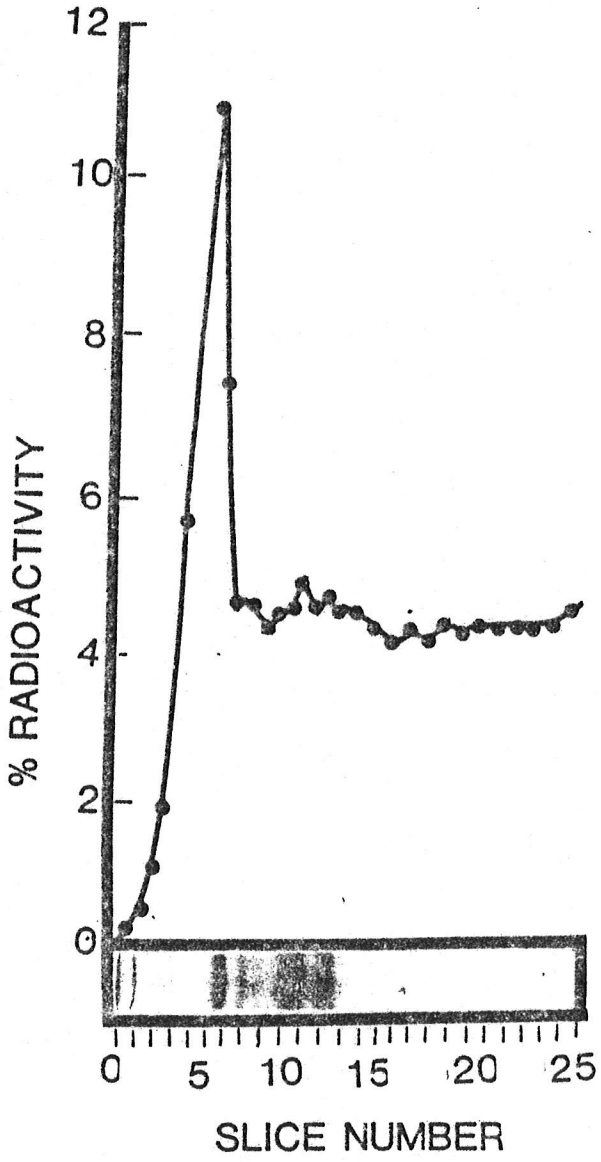

12
10
8
6
4
2
0
% RADIOACTIVITY
0
5
10
15
20
25
SLICE NUMBER

Figure 24 DEAE-cellulose chromatography of the 50 mM phosphate fraction from bovine follicular fluid after hydroxylapatite chromatography. The pooled 50 mM phosphate fractions of bff (737 mg) after dialysis, was loaded onto a DE-32 column (3 x 3 cm) in TG, pH 7.4. The adsorbed proteins were eluted by a continuous gradient of 0, 0.2, 0, 0, 0.25, 0.1 and 0.15 M NaCl in TEG. Fractions were collected in ml/tube at a flow rate 25 ml/hour. Aliquots of 200 µl of each fraction were assayed for binding activity.

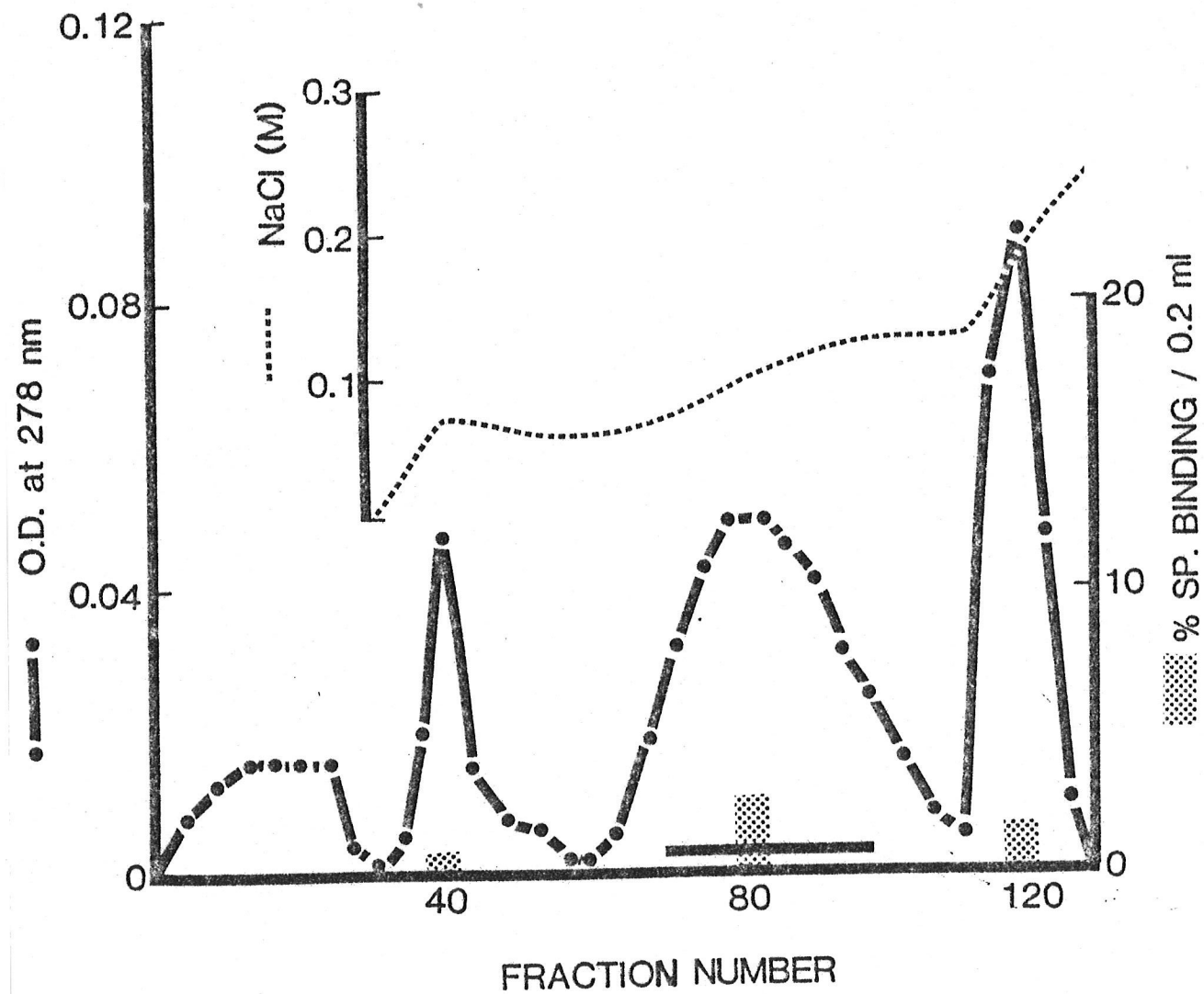
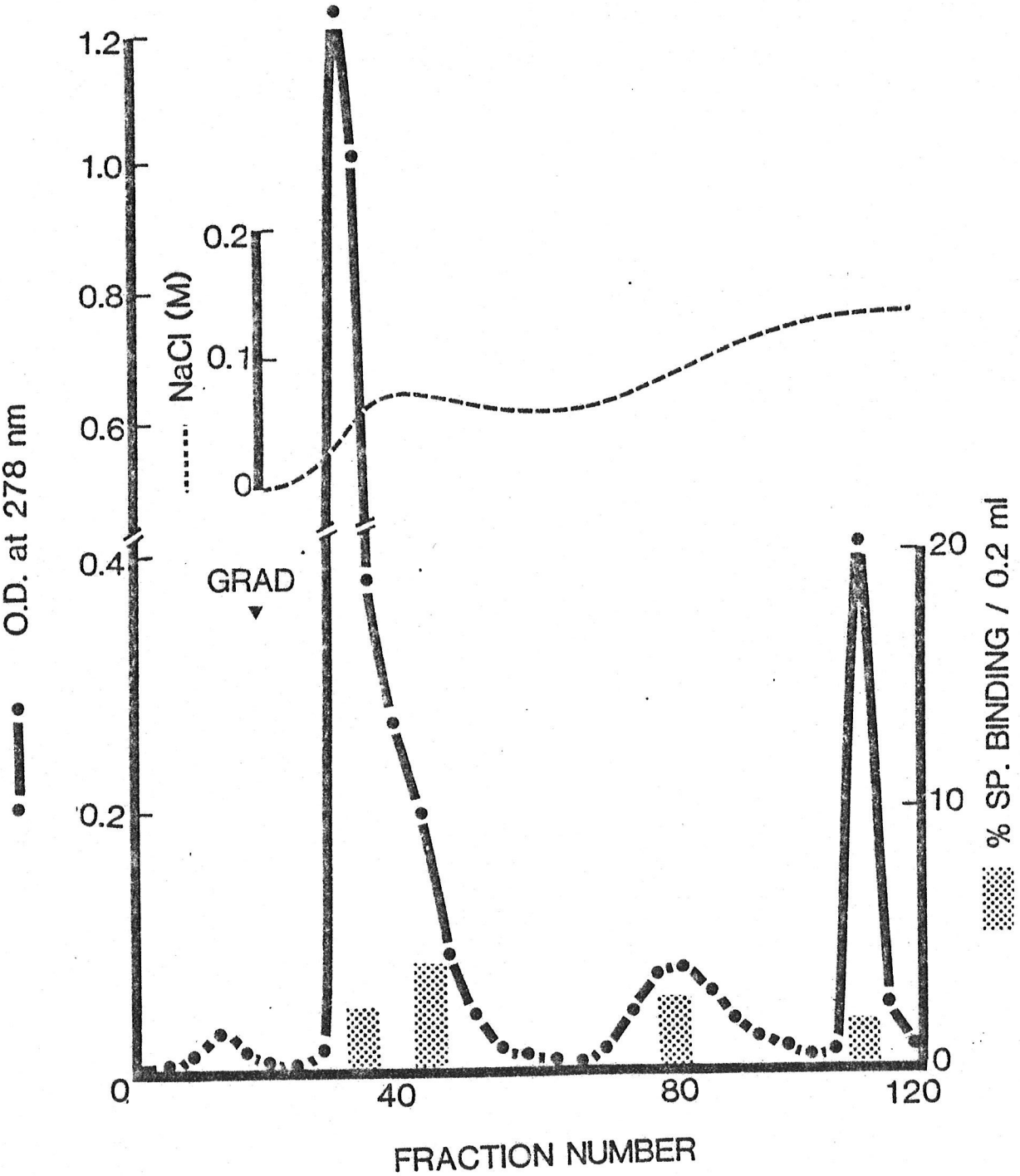

0.12
0.08
0.04
0
O.D. at 278 nm
0.3
0.2
0.1
NaCl (M)
20
10
0
% SP. BINDING / 0.2 ml
40
80
120
FRACTION NUMBER

Figure 25 DEAE-cellulose chromatography of the 400 mM phosphate fraction from follicular fluid after hydroxylapatite chromatography. The pooled 400 mM phosphate fraction of bff (918 mg) after HTP chromatography (Figure 22), after dialysis, was loaded onto a DE-32 column (4 x 6 cm) in TEG, pH 7.4. The adsorbed proteins were eluted by a continuous gradient of 0, 0.2, 0, 0, 0.25, 0.1 and 0.15 M NaCl in TEG. Fractions were collected in 25 ml/tube at a flow rate of 25 ml/hour. Aliquots of 200 µl of each fraction were assayed for binding activity.


O.D. at 278 nm
NaCl (M)
% SP. BINDING / 0.2 ml
GRAD
FRACTION NUMBER
1.2
1.0
0.8
0.6
0.4
0.2
0
0.2
0.1
0
20
10
0
40
80
120

protein peak of the 50 mM fraction (Figure 24) was therefore pooled and characterized further by SS-PAGE. Results in Figure 26 show that this fraction contained several protein components, of which three had binding for ^{3}H-DHT, and the follicular fluid specific binding protein was quantitatively only a very minor component (segment 8). It was concluded that these fractions (50 and 400 mM phosphate), containing only minute amounts of bff-ABP but large amounts of many other proteins, were of no practical value for further steps of isolation.

4. RECYCLING ON HYDROXYLAPATITE CHROMATOGRAPHY.

Data in Figure 22 indicated clearly that the high affinity binding activity protein upon HTP chromatography was eluted by approximately 1 mM phosphate. Recycling of the 1 mM fraction onto another HTP column with a more refined gradient of phosphate was initiate in order to get rid of further contaminating proteins. The extensively dialysed 1 mM fraction after the first HTP column (Figure 22) was loaded onto another HTP column in TG and the adsorbed proteins were eluted by a continuous gradient of 1, 1.5 and 1.5 mM phosphate in the same buffer. Figure 27 shows that small but significant amounts of contaminating protein were separated in unadsorbed and adsorbed fractions eluted by 2.5 mM phosphate, and the one major peak of proteins with high binding activity was again eluted by 1 mM phosphate. The appropriate fractions were pooled and dialysed in TEG for the next step of purification.

5. RECYCLING ON DEAE-CELLULOSE CHROMATOGRAPHY.

Data in Figure 24 indicated that chromatography on DEAE-cellulose eluted with a continuous gradient of 7 chambers was very valuable

Figure 26 Analysis by steady state polyacrylamide gel electrophoresis of the binding activity in the 50 mM phosphate fraction of hydroxylapatite column after DEAE-cellulose chromatography. SS-PAGE was prepared by mixing ^{3}H-DHT in 8% polyacrylamide gel at pH 8.9. A sample of 40 µg containing high binding activity after DEAE-cellulose chromatography (Figure 25) was analysed by SS-PAGE in duplicate. One gel was stained for the protein pattern and the other was segmented and counted for ^{3}H-DHT. The distribution of ^{3}H-DHT in each gel segment was plotted as a percentage of the total radioactivity recovered in all segments of the gel. From the stained protein bands, the major binding activity of the sample was located at Rf of 0.34.

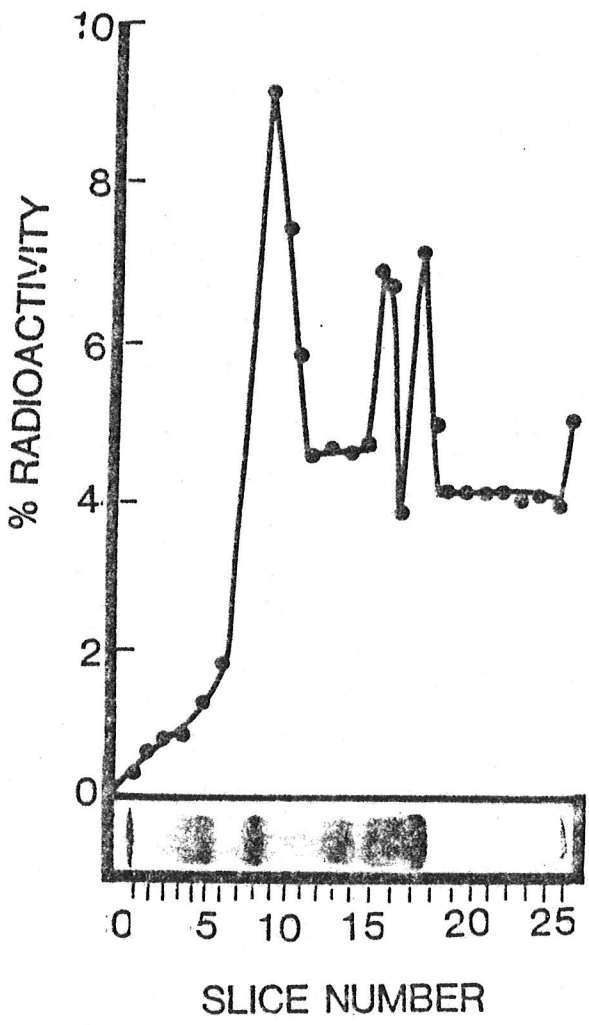
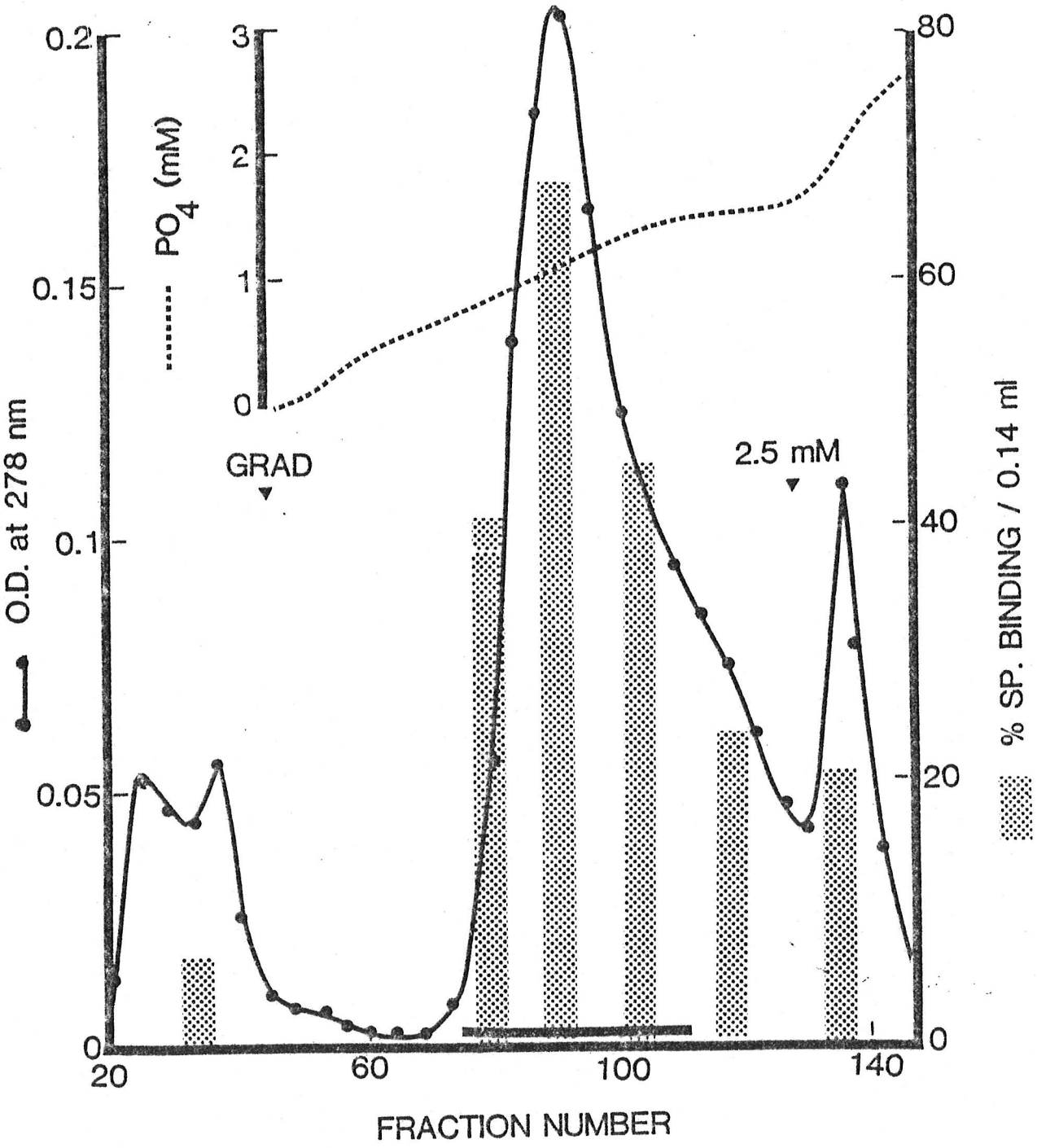

SS-PAGE (8% GEL)
% RADIOACTIVITY
10
8
6
4
2
0
0
5
10
15
20
25
SLICE NUMBER

Figure 27 Recycling upon hydroxylapatite chromatography of the active fraction from follicular fluid after hydroxylapatite chromatography. The pooled active-continuous gradient fraction of bff (132 mg) after HTP chromatography (Figure 22), after dialysis, was applied onto another HTP column (3 x 3 cm) in TG, pH 7.4. The adsorbed proteins were eluted by a continuous gradient of 0, 1.5 and 1.5 mM phosphate buffer. Fractions were collected at 3 ml/tube at a flow rate of 75 ml/hour. Aliquots of 140 µl from each fraction were assayed for binding activity.


0.2
0.15
0.1
0.05
0
O.D. at 278 nm
3
2
1
0
PO_4 (mM)
GRAD
2.5 mM
80
60
40
20
0
% SP. BINDING / 0.14 ml
20
60
100
140
FRACTION NUMBER

for the separation of some major contaminant proteins from bff-ABP. Therefore another step of column chromatography on DEAE-cellulose was initiated for further purification of bff-ABP after the second HTP-chromatography. The dialysed sample after the HTP column (Figure 27) was applied onto a DEAE-cellulose column, equilibrated in TEG, pH 7.4, and the totally adsorbed proteins were eluted by a compound gradient of 0, 0.2, 0, 0, 0.25, 0.1, 0.125 and 0.15 M NaCl in the same buffer. Figure 28 shows that more than 50% of protein with low binding activity was eluted by 0.06 M NaCl, and a major peak of protein with relatively high binding activity at 0.085 M NaCl. The step of ion-exchange chromatography proved to be very useful in separating a major portions of contaminating proteins of low binding affinity. The appropiate fractions of binding protein were pooled for the next step of purification by affinity chromatography.

6. AFFINITY CHROMATOGRAPHY ON SEPHAROSE-4B CONJUGATED WITH CONCAVANALIN A.

A Sepharose-Con A column was equilibrated in 50 mM Tris buffer, pH 7.4, containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.5 M NaCl. After the last step of DEAE-cellulose chromatography, active fractions were pooled and loaded onto the Sepharose-Con A. After extensive washing with the same buffer, the adsorbed glycoproteins were eluted with a solution of 0.2 M α-methyl-D-glucoside in the same buffer as shown in Figure 29. Most of the sample was eluted in the unadsorbed fraction and only a very small peak of glycoproteins was eluted by α-mthyl-D-glycoside. However, binding activities were observed in both unadsorbed and adsorbed fractions (Figure 29).

Figure 28 Recycling upon DEAE-chromatography of the active fraction from follicular fluid after recycling hydroxylapatite chromatography. The pooled active fraction of bff (824 mg) after recycling HTP chromatography (Figure 27), after dialysis, was loaded onto another DE-32 column (2 x 6 cm) in TEG, pH 7.4. The adsorbed proteins were eluted by a continuous gradient of 0, 0.2, 0, 0, 0.25, 0.1, 0.125 and 0.15 M NaCl in TEG. Fractions were collected in 1 ml/tube at a flow rate 60 ml/hour. Each fraction was monitored for protein by absorbance at 278 nm and activity by binding assay with 200 µl aliquots.

The inset shows the protein patterns after PAGE of fractions 1, 2 and 3 as indicated in this figure. Arrow (◀) indicates the protein band of bff-ABP.

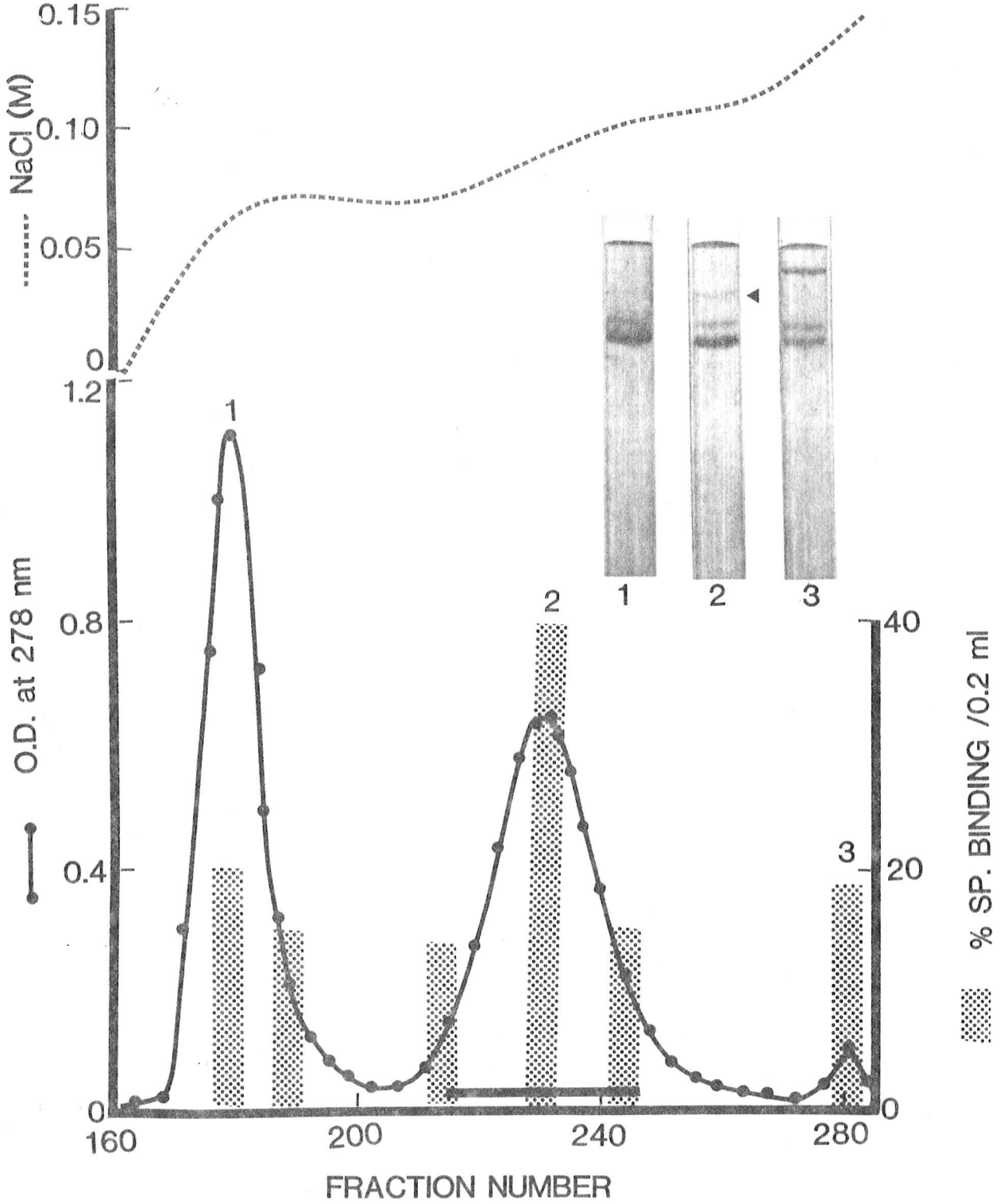
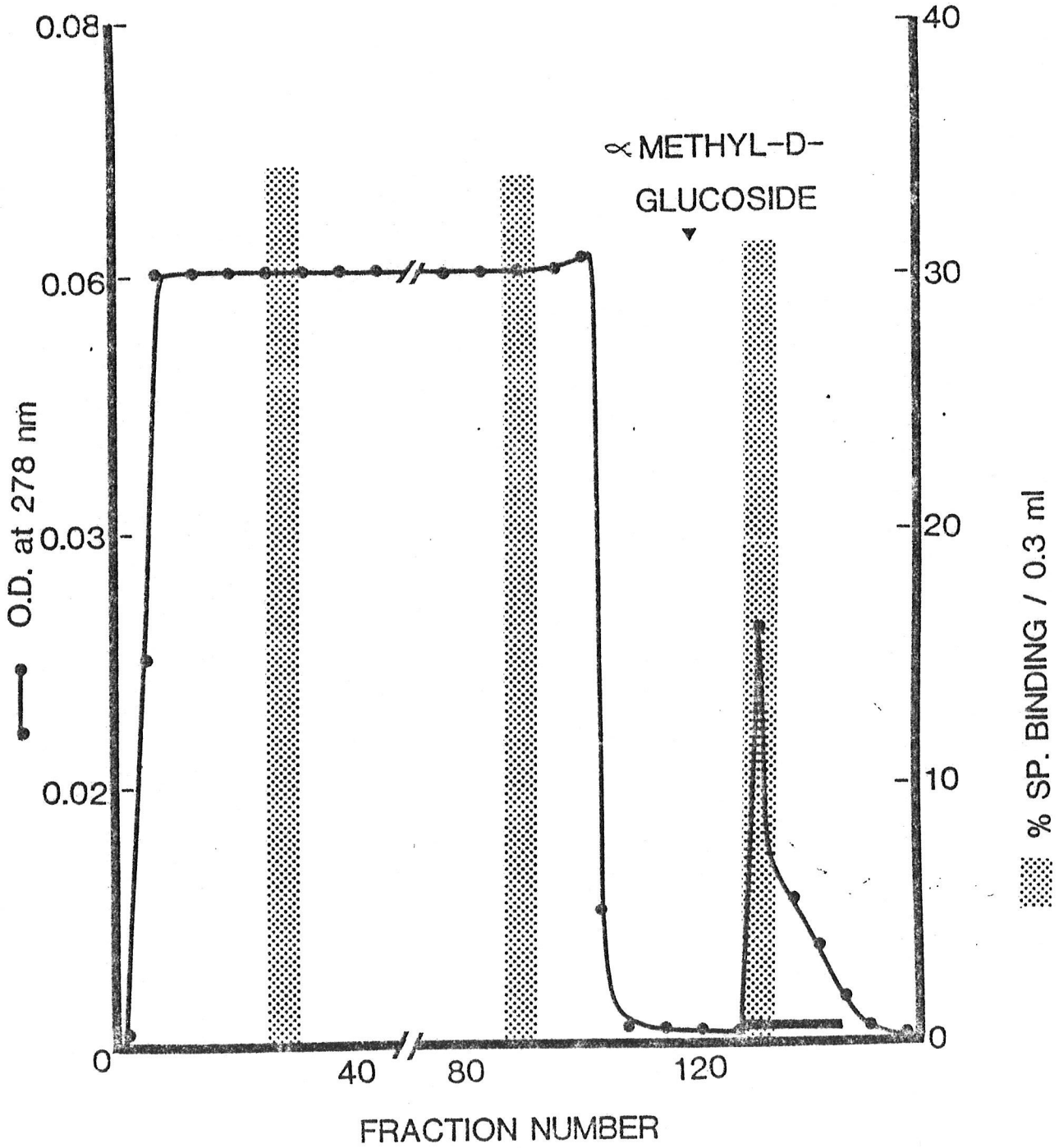

0.15
0.10
0.05
0
NaCl (M)
1.2
0.8
0.4
0
O.D. at 278 nm
1
2
3
40
20
0
% SP. BINDING /0.2 ml
160
200
240
280
FRACTION NUMBER
1
2
3

Figure 29 Affinity chromatography on Sepharose-Concanavalin A of the active fraction from follicular fluid after recycling DEAE-cellulose chromatography. A Sepharose-Con A column (2 x 10 cm) was equilibrated in 50 mM Tris-HCl containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.4. The pooled fractions of high binding activity (48.14 mg) after DE-32 column chromatography (Figure 28) were applied onto the Sepharose-Con A column. After the unadsorbed fractions, the column was washed extensively with the same equilibrating Tris-HCl buffer, and the specially bound glycoproteins were then eluted by 0.2 M solution of α-methyl-D-glycoside in the same buffer. Fractions were collected in 25 ml/tube at a flow rate of 120 ml/hour. Proteins were monitored by adsorbance at 278 nm and activities by binding assay with 300 µl aliquots.


0.08
0.06
0.03
0.02
0
40
30
20
10
0
∝METHYL-D-
GLUCOSIDE
O.D. at 278 nm
% SP. BINDING / 0.3 ml
40
80
120
FRACTION NUMBER

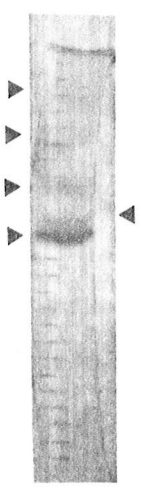
Both fractions were pooled and concentrated by ultrafiltration. Upon PAGE, it was observed that the unadsorbed fraction contained at least 3 protein components, of which the binding protein was one of the minor bands (Figure 30). The adsorbed fraction consisted of only two protein bands, of which the binding protein was the major component (Figure 30). Affinity chromatography on Sepharose-Con A thus proved to be one of the most useful steps in isolating this binding protein.

7. GEL FILTRATION ON SEPHADEX G-200.

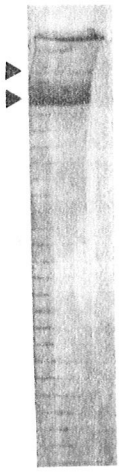
Finally, the concentrated sample of relatively pure bff-ABP in adsorbed fraction after Sepharose-Con A was subjected to gel filtration on Sephadex G-200 in TG buffer. Figure 31 shows that the two proteins were only partially separated (P_1 and P_2) and that the distribution of binding activities, though found in both peaks, appeared to slightly favor the more retarded peak (P_2). Both protein peaks were pooled and concentrated by ultrafiltration.

Both P_1 and P_2 materials were recycling individually by the same Sephadex G-200 column. Figure 32 shows that P_1 was partially fractionated into two proteins, of which only the more retarded one bound ^{3}H-DHT. Recycling of P_2 under the same conditions is shown in Figure 33, depicting that most of this fraction was eluted as one symmetrical peak of protein, coincident with the binding activity. Accordingly, the appropriate fractions were pooled and concentrated by ultrafiltration as the final product of purified bff-ABP.

Figure 30 Polyacrylamide gel electrophoresis of the unadsorbed and adsorbed fractions after Concanavalin A affinity chromatography. Arrows (▶) indicate the stained protein bands.



UNADSORBED
FRACTION



ADSORBED
FRACTION

Figure 31 Gel filtration on Sephadex G-200 of the active fraction from follicular fluid after Sepharose-Con A affinity chromatography. The concentrated adsorbed fractions (2.97 mg) after Sepharose-Con A column chromatography (Figure 29) were loaded onto a Sephadex G-200 column (1 x 110 cm) equilibrated in TG buffer, pH 7.4 and eluted with the same buffer. Fractions were collected in 1 ml/tube at a flow rate of 15 ml/hour. Proteins were monitored by absorbance at 278 nm and activities by binding assay with 100 µl aliquots.

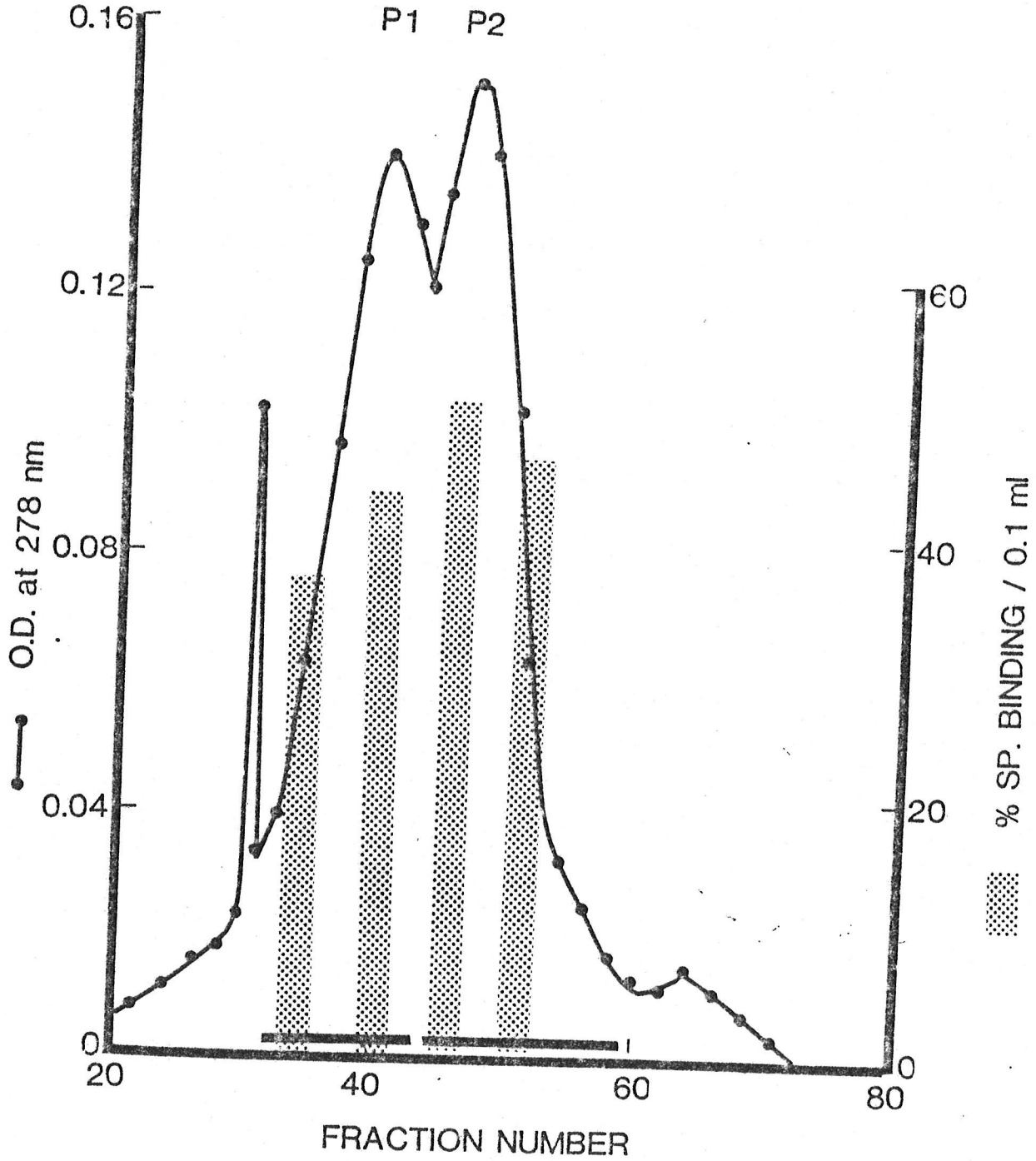

P1
P2
0.16
0.12
0.08
0.04
0
O.D. at 278 nm
60
40
20
0
% SP. BINDING / 0.1 ml
20
40
60
80
FRACTION NUMBER

Figure 32 Recycling on Sephadex G-200 of the first protein peak (P_1) of the binding protein obtained after gel filtration on Sephadex G-200. The concentrated sample of peak 1 after Sephadex G-200 column chromatography (Figure 31) was loaded onto the same Sephadex G-200 column (1 x 110 cm) and eluted with TG buffer, pH 7.4. Fractions were collected in 1 ml/tube at a flow rate of 15 ml/hour. Proteins were monitored by absorbance at 278 nm and activities by binding assay with 100 µl aliquots.

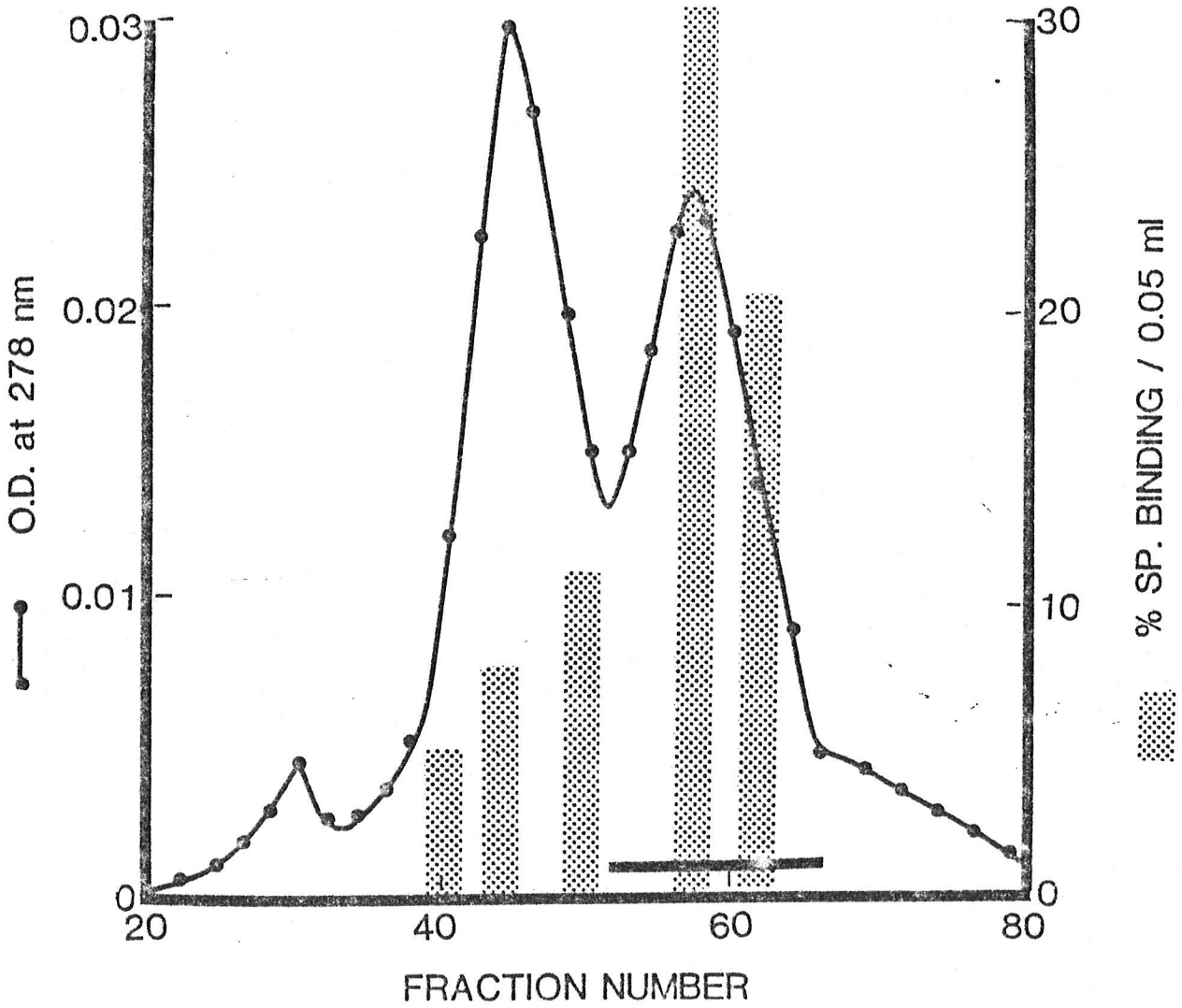
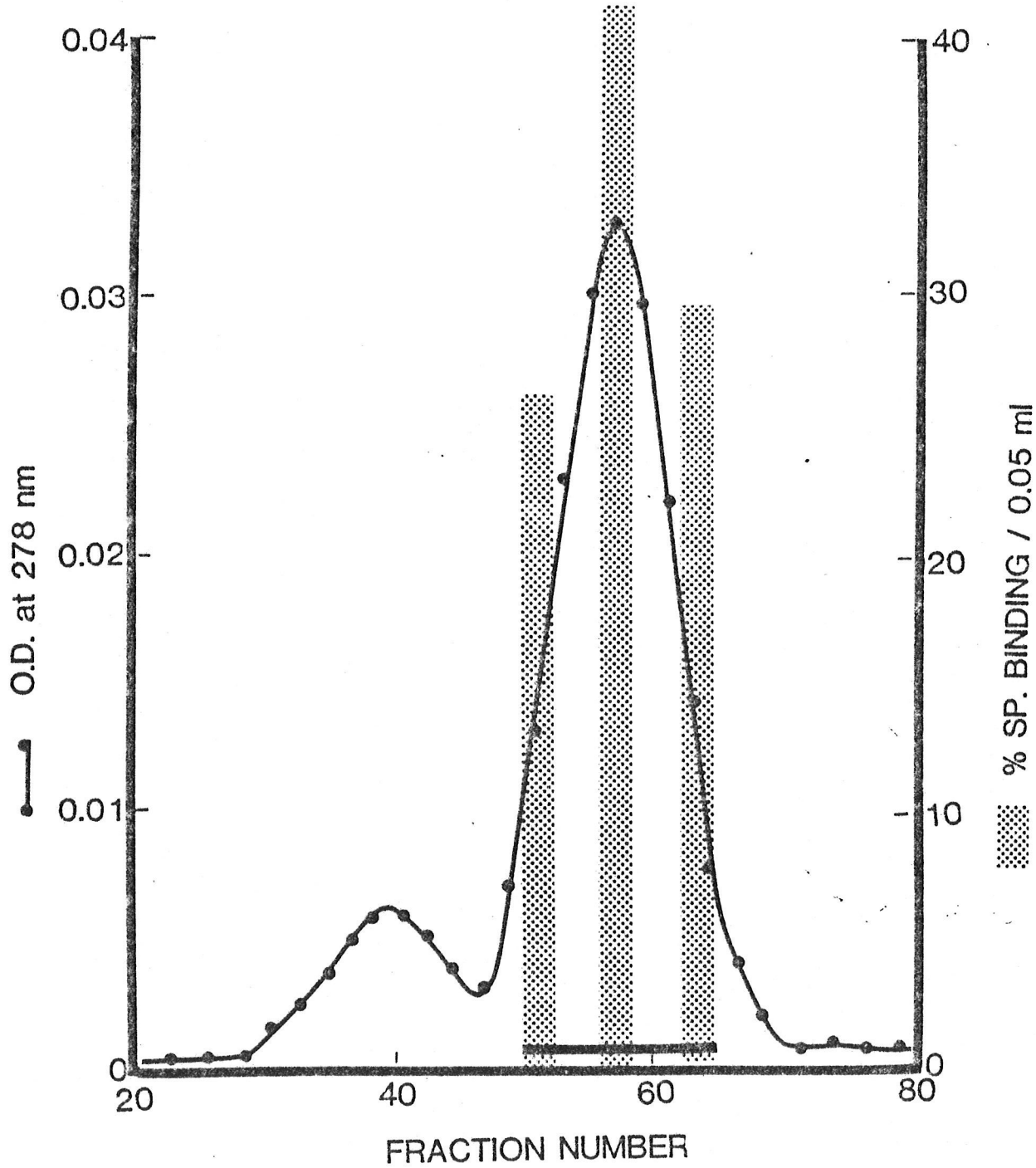

0.03
0.02
0.01
0
O.D. at 278 nm
30
20
10
0
% SP. BINDING / 0.05 ml
20
40
60
80
FRACTION NUMBER

Figure 33 Recycling on Sephadex G-200 of the second protein peak (P_2) of the binding protein obtained after filtration on Sephadex G-200 column. The concentrated sample of peak 2 after Sephadex G-200 column chromatography (Figure 31) was loaded onto the same Sephadex G-200 column (1 x 110 cm) and eluted with TG buffer, pH 7.4. Fractions were collected in 1 ml/tube at a flow rate of 15 ml/hour. Proteins were monitored by absorbance at 278 nm and activities by binding assay with 50 µl aliquot.


0.04
0.03
0.02
0.01
0
O.D. at 278 nm
40
30
20
10
0
% SP. BINDING / 0.05 ml
20
40
60
80
FRACTION NUMBER

SUMMARY OF THE PURIFICATION PROCEDURE FOR FOLLICULAR FLUID ANDROGEN BINDING PROTEIN.

The procedure for the purification of bff-ABP is shown in Table 4, and the yields and recoveries of materials and activities of the binding protein at different steps of purification are summarized in Table 5. From 400 ml of bovine follicular fluid, after 7 steps of isolation procedure, a preparation of 0.536 mg of highly purified bff-ABP was achieved.

Figure 34 summarizes the electrophoretic patterns of proteins in various fractions at different steps of purification.

V. CHARACTERIZATION OF THE FOLLICULAR FLUID ANDROGEN BINDING PROTEIN.

The highly purified bff-ABP was characterized for its physico-chemical and biological properties.

1. POLYACRYLAMIDE GEL ELECTROPHORESIS.

Purified bff-ABP after gel filtration on Sephadex G-200 (Figure 33) was subjected to SS-PAGE (10% gel, containing ^{3}H-DHT) for electrophoretic mobility and specific binding of DHT. Samples of 2-5 µg were analysed in duplicate. At the end of electrophoresis, one gel was segmented and counted in a liquid scintillation counter for characterization of binding proteins.

Figure 35 shows that the preparation of bff-ABP after Sephadex G-200 consisted of only one protein upon PAGE and one peak of ^{3}H-DHT coincident with the stained band. It was concluded that bff-ABP was purified to homogeneity after various steps of chromatography on DEAE-cellulose, HTP, Sepharose-Con A, followed by gel filtration on Sephadex G-200.

TABLE 4. FLOW CHART FOR THE PURIFICATION OF BFF-ABP.

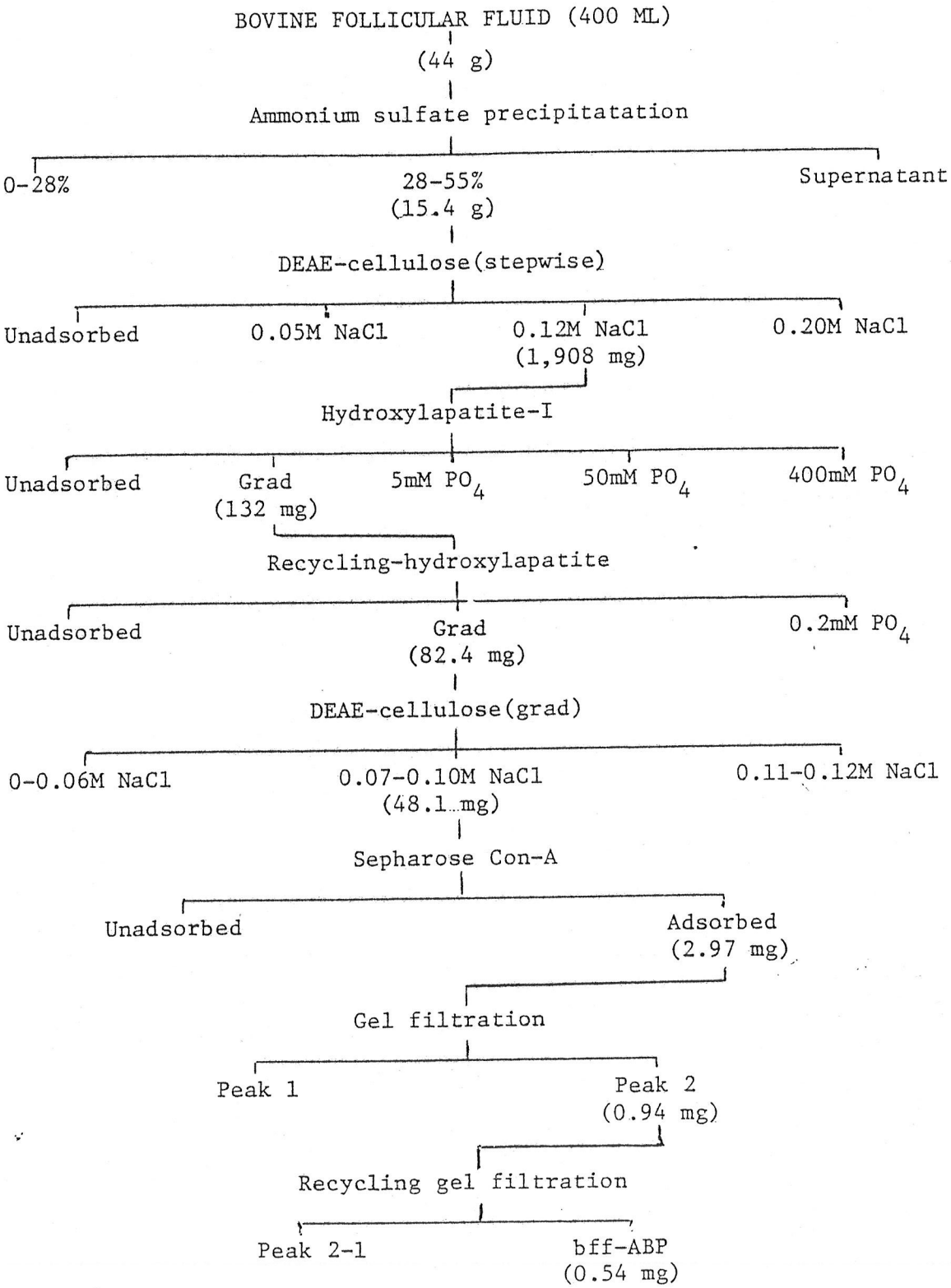


TABLE 5. SUMMARY OF BOVINE FOLLICULAR FLUID ANDROGEN BINDING PROTEIN PURIFICATION.

	TOTAL PROTEIN (mg)	RECOVERY PROTEIN[1] (%)	ACTIVITY[2] (ρg DHT/mg PROTEIN)	TOTAL ACTIVITY[3] (ng DHT)	RECOVERY ACTIVITY[4] (%)	PURIFICATION FACTOR[5]
bovine follicular fluid (400 ml)	44,000.00	100.000	19.1	840.0	100.0	1
Amm.Sulf.ppt.	15,400.00	35.000	36.8	567.2	67.5	2
DE-32 (stepwise)	1,907.80	4.340	224.5	428.3	51.0	12
HTP (grad-I)	132.00	0.300	1,533.3	202.4	24.1	80
HTP (grad-II)	82.40	0.187	1,791.7	147.6	17.6	94
DE-32 (grad)	48.14	0.109	2,530.0	121.8	14.5	133
Con-A column	2.97	0.006	19,089.0	56.7	6.7	1,000
G-200	0.95	0.002	40,197.0	42.3	5.0	2,106
G-200 (recycling)	0.54	0.001	46,900.0	25.1	3.0	2,456

1 RECOVERY PROTEIN = protein in each step of purification calculated in percent of total bff protein.

2 ACTIVITY = an ability of all binding protein in bff bind to DHT/mg protein. The actual ability of bff-ABP might be different depend on how much of bff-ABP in that step.

3 TOTAL ACTIVITY = total of ability of all binding protein in each step bind to DHT. The actual bff-ABP activity might different.

4 RECOVERY ACTIVITY = total activity of all binding protein in each step of purification calculated in percent of total ability of bff protein bind to DHT.

5 PURIFICATION FACTOR = how many fold of binding activity increase in each step of purification.

Figure 34 Polyacrylamide gel electrophoresis of the proteins in various fractions at different steps of purification of the bovine follicular fluid androgen binding protein.

1 = bovine follicular fluid.

2 = 28-55% saturated ammonium sulfate precipitation.

3 = 0.12 M NaCl eluted fraction of DEAE cellulose chromatography.

4 = gradient phosphate fraction of hydroxylapatite chromatography.

5 = recycling hydroxylapatite chromatography.

6 = 0.085 M NaCl eluted fraction of recycling DEAE-cellulose chromatography.

7 = adsorbed fraction of Concanavalin A chromatography.

8 = second peak of Sephadex G-200 (P_2).

9 = recycling of second peak of Sephadex G-200 on the same column (bff-ABP).

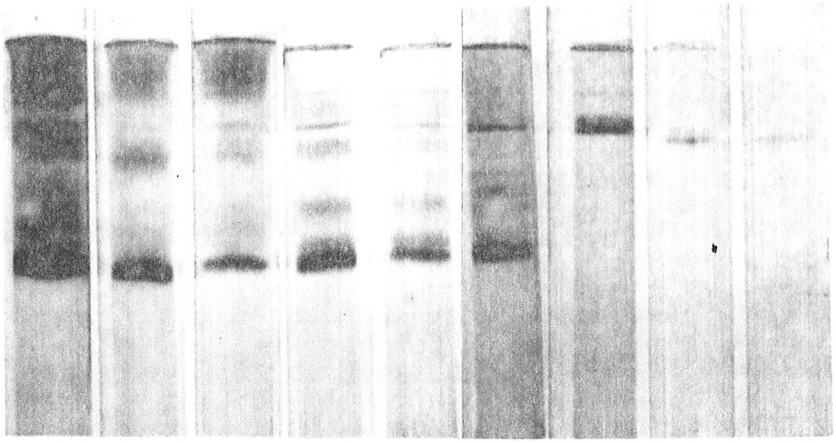
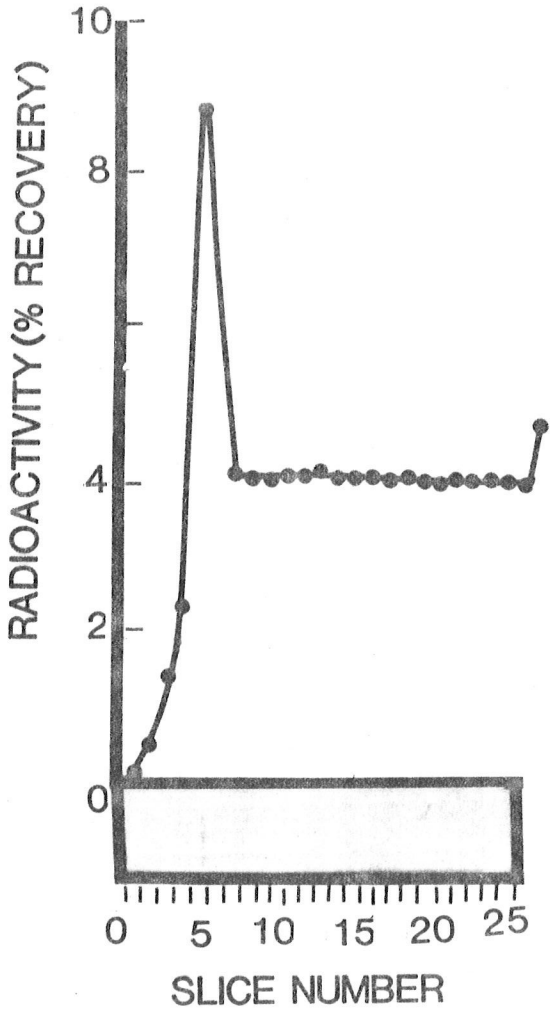

1
2
3
4
5
6
7
8
9

Figure 35 Analysis of purified bovine follicular fluid androgen binding protein by steady state polyacrylamide gel electrophoresis. Sample of 1.2 µg were analysed in duplicate by 10% polyacrylamide gels, pH 8.9. One gel was stained for protein and the other gel was sliced into 2 mm segments and counted for distribution of ^{3}H-DHT.


RADIOACTIVITY (% RECOVERY)
10
8
4
2
0
0
5
10
15
20
25
SLICE NUMBER

2. GEL ISO-ELECTRIC FOCUSING.

Analytical isoelectric focusing was performed in 10% polyacrylamide gel.A sample of 20µg of highly purified bff-ABP was focused for 24 hours and stained for protein. Another blank gel was run at the same time under identical conditions to determine the pH gradient formed upon focusing.

Figure 36 shows purified bff-ABP had only one stained protein band, and was focused at pH 4.7-4.8. Upon gel isoelectric focusing, highly purified bff-ABP was confirmed to be homogeneous and the isoelectric point (pI) of bff-ABP appeared to be 4.75.

3. DETERMINATION OF MOLECULAR WEIGHT.

a) Gel filtration on Sephadex G-200. The molecular weight of highly purified bff-ABP was estimated by the method of gel filtration on Sephadex G-200. The elution volume of bff-ABP was compared with those of protein markers of known molecular weight, BSA(68K), ovalbumin(45K) and chymotrypsinogen(23K), calibrated in the same column under identical experimental conditions.

Figure 37 shows the elution profiles of bff-ABP and other protein markers after gel filtration on Sephadex G-200. The molecular weight of bff-ABP was estimated by plotting the elution volumes of these proteins against their molecular weights as shown in figure 38. By this method of gel filtration on Sephadex G-200, the molecular weight of bff-ABP was determined to be 110,000 (Figure 38).

b) Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The molecular weight of purified bff-ABP was also determined by the method of PAGE in the presence of 1% SDS. Figure 39 shows

Figure 36 Analysis of purified bovine follicular fluid androgen binding protein by polyacrylamide gel isoelectrofocusing. A sample of 10 µg of purified bff-ABP was focused in a 10 % polyacrylamide gel for 12 hours. After focusing, the gel was stained for protein; another blank gel was sliced into 2 mm segments and each soaked in distilled water for measurement of pH.

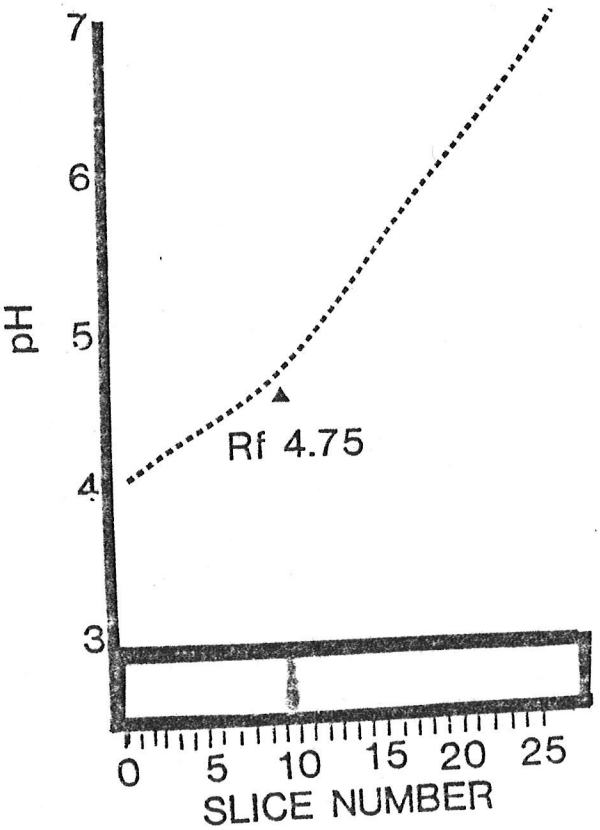

7
6
5
4
3
pH
Rf 4.75
0
5
10
15
20
25
SLICE NUMBER

Figure 37 Determination of molecular weight for purified bovine follicular fluid androgen binding protein by gel filtration on Sephadex G-200. A column of Sephadex G-200 (1 x 110 cm) in TG buffer was used. Sample bff-ABP = bovine follicular fluid androgen binding protein; BSA = bovine serum albumin (68K); OVAL = ovalbumin (45K) and CHYM = chymotrypsinogen (23K).

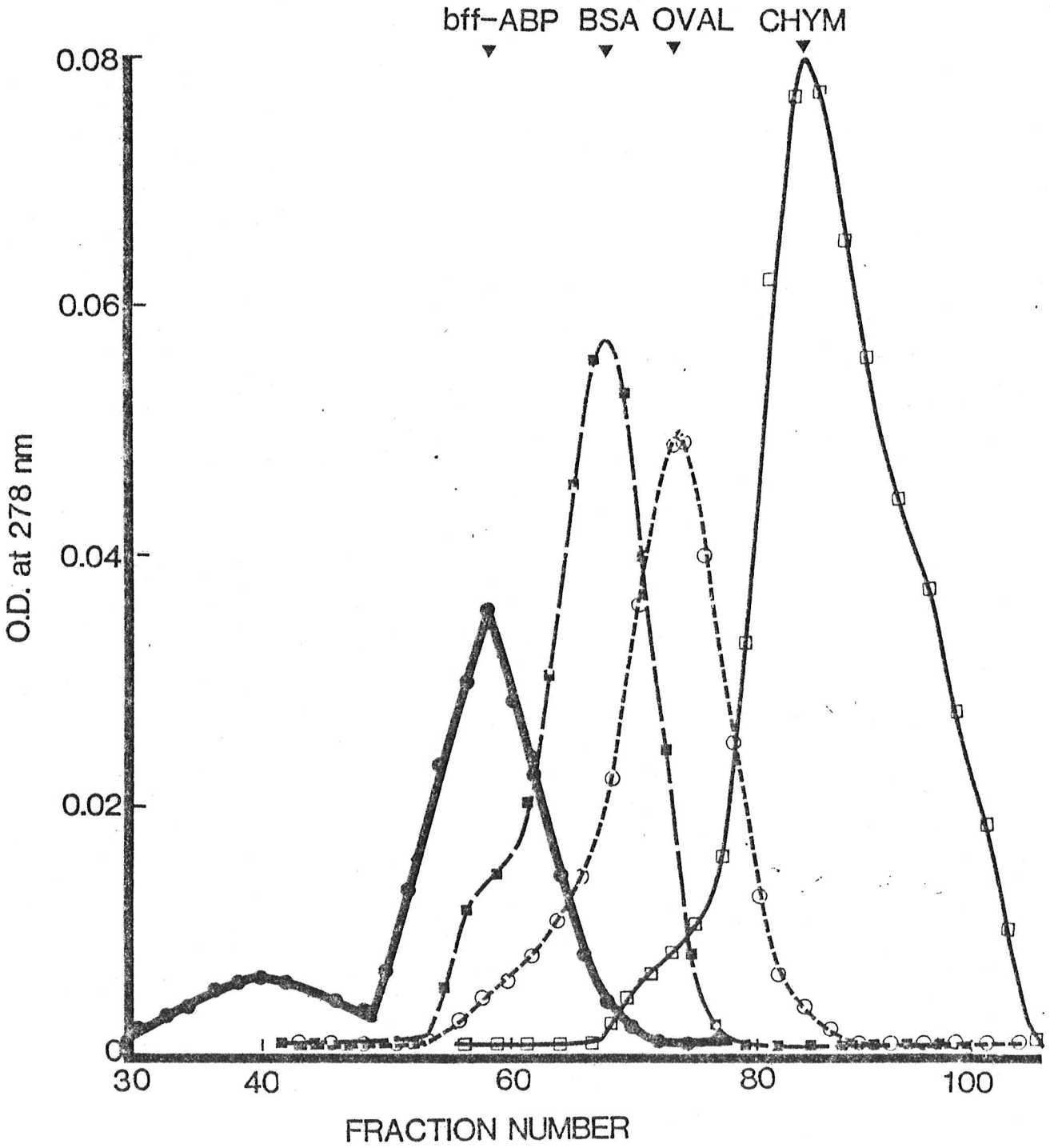

bff-ABP
BSA
OVAL
CHYM
0.08
0.06
0.04
0.02
0
O.D. at 278 nm
30
40
60
80
100
FRACTION NUMBER

Figure 38 Estimation of molecular weight for purified bovine follicular fluid androgen binding protein by gel filtration on Sephadex G-200. A column of Sephadex G-200 (1 x 110 cm) in TG buffer was used. Sample bff-ABP = bovine follicular fluid androgen binding protein; BSA = bovine serum albumin (68K); OVAL = ovalbumin (45K) and CHYM = chymotrypsinogen (23K).

M.W. BY GEL FILTRATION

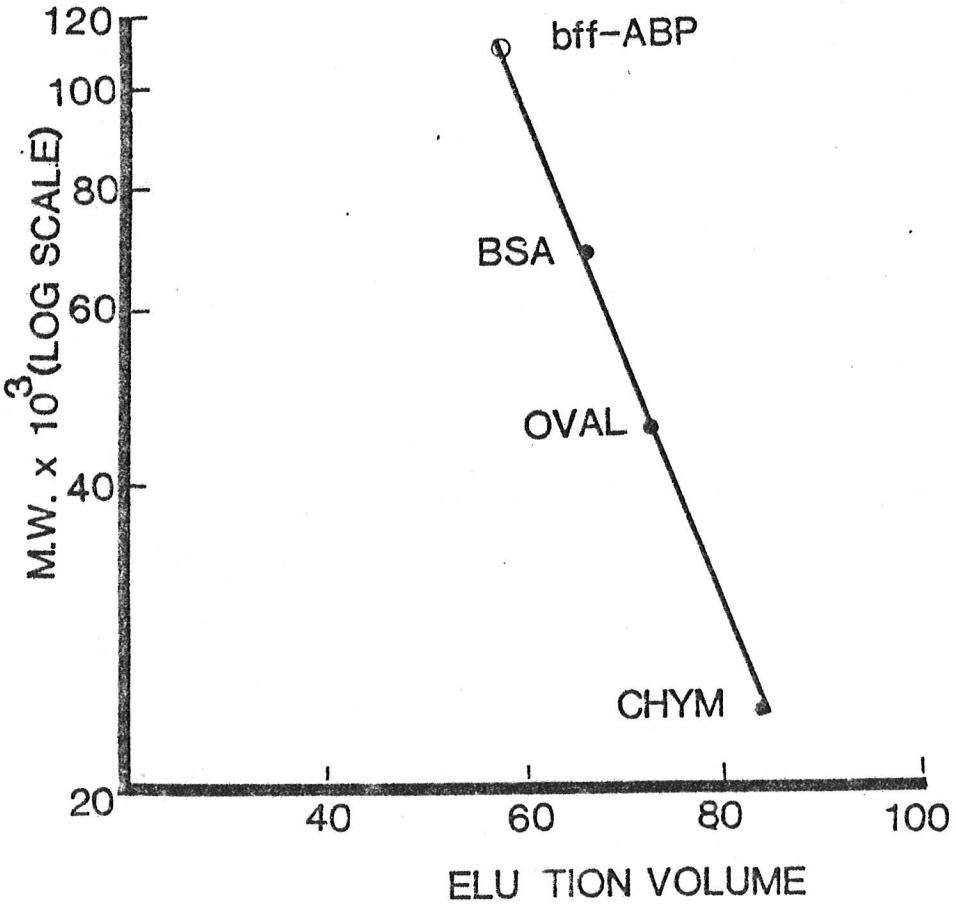


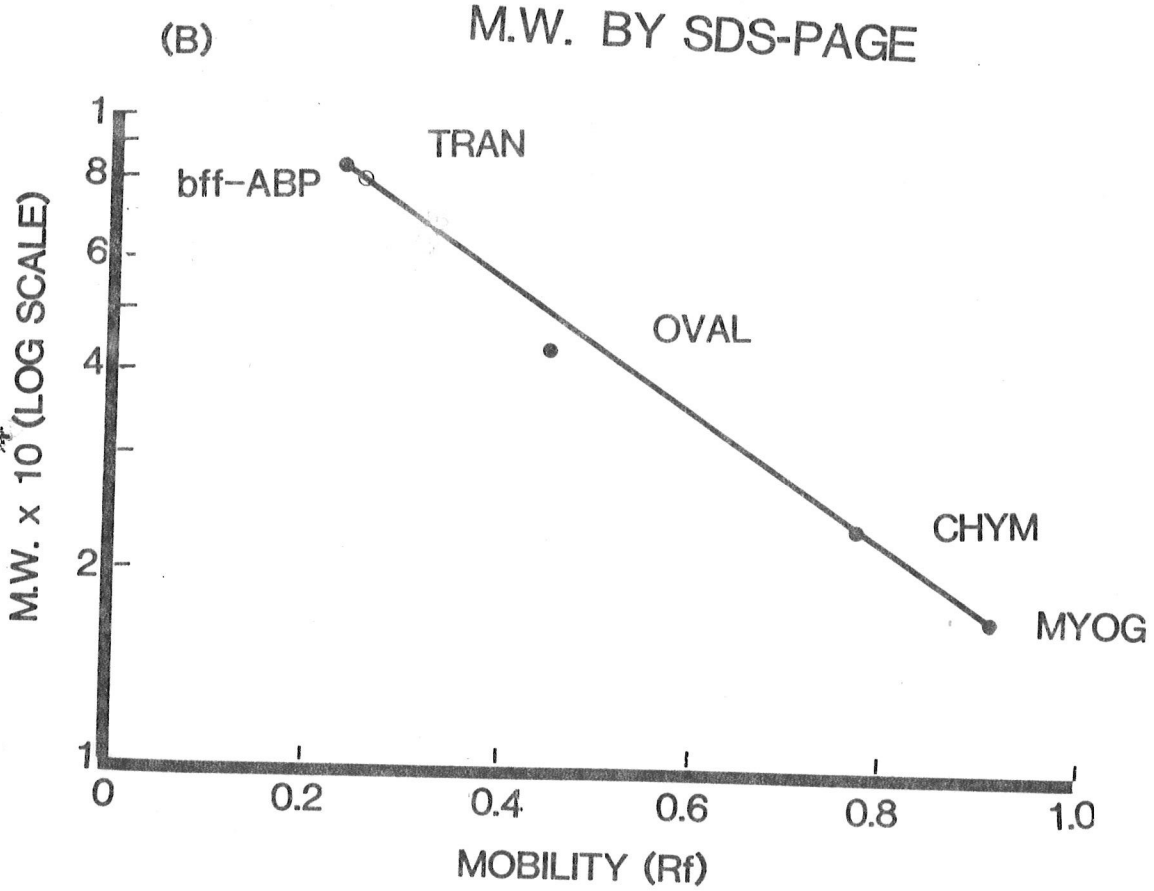
Figure 39 (A) Analysis of purified bovine follicular fluid androgen binding protein by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Sample : 1 = myoglobin; 2 = chymotrypsinogen; 3 = ovalbumin; 4 = transferrin and 5 = bff-ABP.

(B) Estimation of molecular weight for puirfied bovine follicular fluid androgen binding protein by SDS-PAGE. The Rf values of the proteins are plotted against their molecular weights on a logarithmic scale. Sample: bff-ABP = purified bovine follicular fluid androgen binding protein; TRAN = transferrin (85K); OVAL = ovalbumin (45K); CHYM = chymotrysinogen (23K) and MYOG = myolglobin (17K).

(A)


1
2
3
4
5

(B)

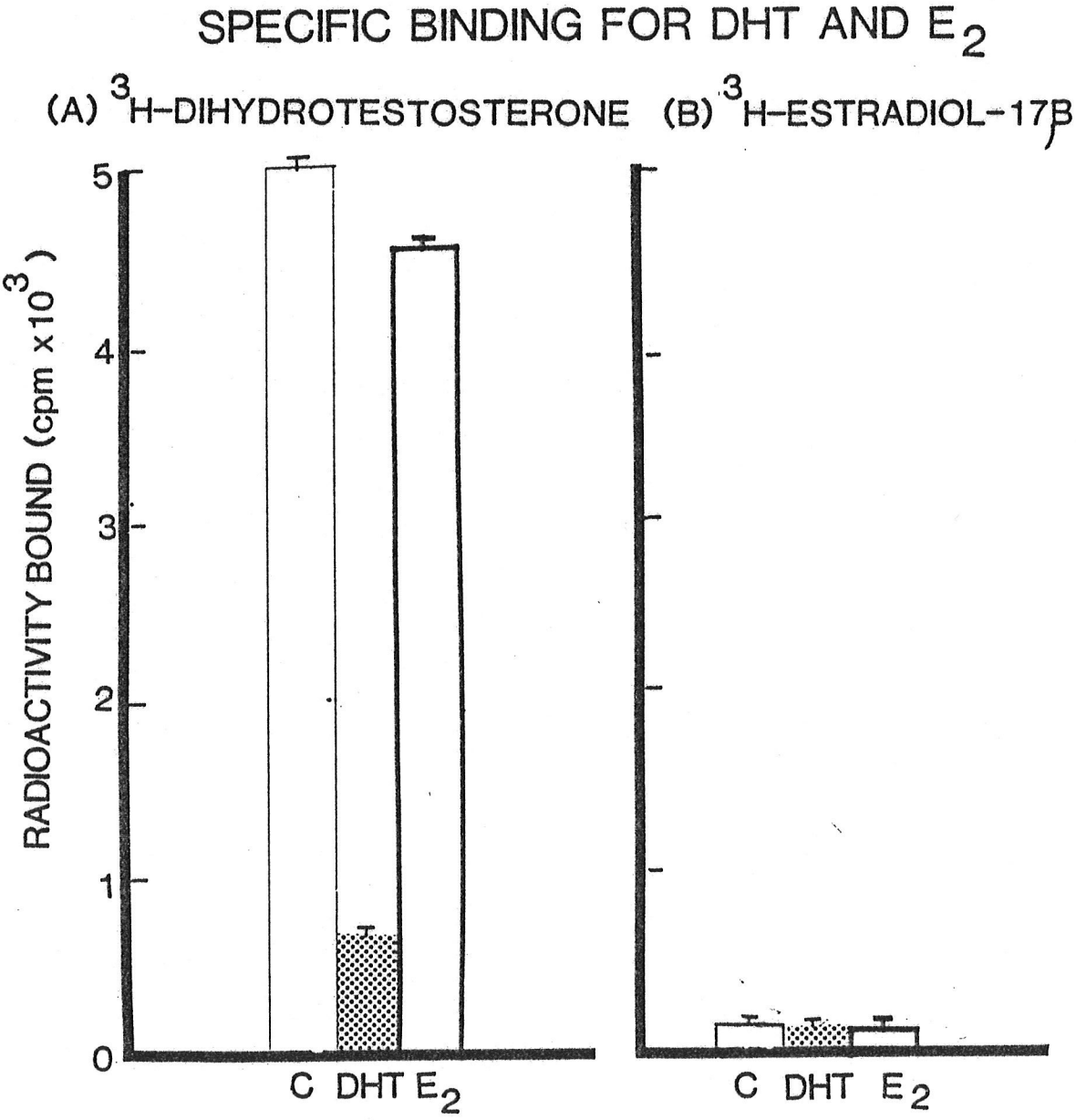

M.W. BY SDS-PAGE
M.W. x 10⁴ (LOG SCALE)
1
8
6
4
2
1
bff-ABP
TRAN
OVAL
CHYM
MYOG
0
0.2
0.4
0.6
0.8
1.0
MOBILITY (Rf)

the stained protein pattern of purified bff-ABP and the mobilities of protein markers of transferrin(85K), ovalbumin(45K), chymotrypsinogen(23K) and myoglobin(17K). Figure 39 shows the determination of molecular weight by plotting the electrophoretic mobilities (Rf) of the protein markers and their molecular weights. The highly purified bff-ABP appeared to be a monomeric protein with no subunit structures since there was only one major band of protein upon SDS-PAGE (Figure 39A). The molecular weight of bff-ABP was determined to be 80,000 by the method of SDS-PAGE (FIgure 39B), and was therefore slightly smaller than but similar to that obtained as 110,000 by the method of gel filtration on Sephadex G-200 (Figure 38). The follicular fluid androgen binding protein appears to be a relatively large protein with no subunit structure and differs markedly in structure from serum TeBG (178) and epididymis ABP (199), which consist of two subunits each .

4. BINDING OF DIHYDROTESTOSTERONE AND ESTRADIOL TO THE FOLLICULAR FLUID ANDROGEN BINDING PROTEIN.

The binding of bff-ABP for DHT and E_2 was examined by incubating highly purified bff-ABP with either ^{3}H-DHT or ^{3}H-E_2 in the presence or absence of 10 ng unlabelled DHT or E_2. Results in Figure 40 shows that bff-ABP had a very high binding affinity for ^{3}H-DHT, which was displaced almost entirely by excess amounts of unlabelled DHT but only slightly by E_2 (Figure 40A), and further that the binding of ^{3}H-E_2 to purified bff-ABP was negligeble (Figure 40B). This finding that bff-ABP binds specifically to DHT with high binding indicates again that bff-ABP is different from serum TeBG (158), which has been reported to have relatively high binding affinity to both DHT or E_2.

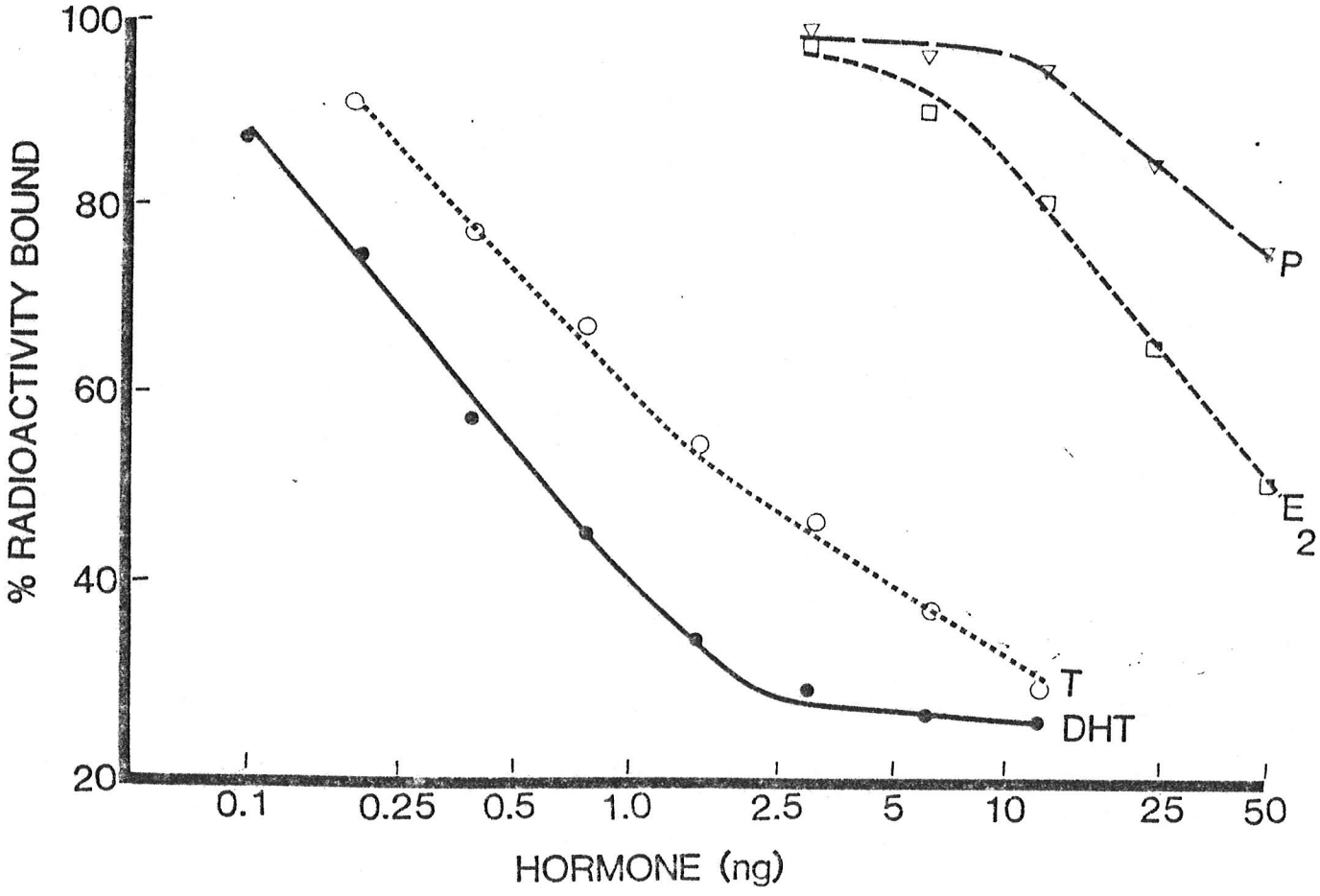
Figure 40 Specific binding of purified bovine follicular fluid androgen binding protein for dihydrotestosterone and estradiol-17β. Sample of 1 μg each of purified bff-ABP were incubated with ^{3}H-DHT (5,000 cpm) or ^{3}H-E_2 (5,000 cpm) with or without unlabelled DHT or E_2 for 2 hours at 4°C. After adding 1 mg of γ-globulin, the protein-bound ^{3}H-DHT or ^{3}H-E_2 was precipitated by 60% ammonium sulfate saturation. Each column representes a mean of two experiments in duplicate, and the vertical bar indicate the range of values.


SPECIFIC BINDING FOR DHT AND E_2
(A) ^{3}H-DIHYDROTESTOSTERONE
(B) ^{3}H-ESTRADIOL-17β
RADIOACTIVITY BOUND (cpm x 10^3)
5
4
3
2
1
0
C DHT E_2
C DHT E_2

5. COMPETITIVE BINDING OF VARIOUS STEROID HORMONES FOR THE FOLLICULAR FLUID ANDROGEN BINDING PROTEIN.

The specific binding of ^{3}H-DHT to the purified bff-ABP was further characterized by competitive displacement of various steroid hormones. Figure 41 shows the dose-response curve for the displacement of bound ^{3}H-DHT by unlabelled dihydrotestosterone, testosterone, estradiol-17β and progesterone. The binding of bff-ABP was relatively specific for DHT and that T though relatively effective was significantly lower in binding affinity for displacement of bound ^{3}H-DHT. Estradiol-17β was 100 fold less effective as compared to DHT for displacement of bound ^{3}H-DHT. Progesterone had minimal binding to purified bff-ABP.

Figure 41 Dose-response curve for the purified bovine follicular fluid androgen binding protein bound ^{3}H-dihydrotestosterone by various steroid hormones. Samples of 1 μg of purified bff-ABP were incubated with ^{3}H-DHT with or without unlabelled DHT, T, E_2, or P at various concentrations for 2 hours at 4°C. After adding 1 mg γ-globulin, the bff-ABP bound ^{3}H-ABP bound ^{3}H-DHT was precipitated by 60% saturation of ammonium sulfate.


100
80
60
40
20
% RADIOACTIVITY BOUND
0.1
0.25
0.5
1.0
2.5
5
10
25
50
HORMONE (ng)
P
E 2
T
DHT

DISCUSSION

I. ANDROGEN BINDING ACTIVITIES IN FOLLICULAR FLUID, SERUM AND EPIDIDYMIS.

The best conditions for assaying the androgen binding activity in follicular fluid appear to be precipitation with 55-65% saturation of ammonium sulfate after incubation for 2-4 hours at $4^{o}C$ (Figure 1). These assay conditions are different from those established for the androgen receptor assay (197), which has been reported to be precipitated optimally at 35-45% saturation of ammonium sulfate. It is also important to point out that addition of γ-globulin to the reaction mixture as carrier-protein is essential for precipitation of protein-bound androgen, especially in samples of low protein concentrations (Figure 3).

The androgen binding activity in follicular fluid is relatively heat labile, and has been observed to decline spontaneously even at room temperature (Figure 7). This labile property of androgen binding activity in follicular fluid is in contrast with that of the androgen binding activity in Crystic Ovary (194), which has been reported relatively heat stable even above $55^{o}C$. Further, the androgen binding activity in follicular fluid is very unstable at below pH 7 (Figure 6). However, we have observed that the follicular fluid androgen binding activity is relatively stable (for at least 1 month) in pH 7.4 at $4^{o}C$ in the presence of 10% glycerine (Figure 8). This observation agrees very well with the report of Weddington *et al.* (199) that 10-20% glycerin in Tris-HCl buffer maintains the stability of epididymal

androgen binding protein.

Studies on binding specificity for various steriod hormones (Figure 9) indicate that follocular fluid contains binding components with high affinity for androgen but relatively low affinity for estrogen. These results are in contrast to the data reported on human and bovine TeBG, which have been shown to have similar affiniities for testosterone and estradiol-17β (158). However, androgen binding activity in follicular fluid is in fact a combined effect of several binding proteins. It is therefore difficult to distinguish the extent of effects of various individual binding proteins in follicular fluid contributing to the binding affinities for androgens and estrogens. Binding activities for androgen and estrogen in follicular fluid appear to be similar to those reported for the trophoblastic androgen binding activity (130), but different from those for polycystic ovarian androgen binding activity (169), which has been shown to bind androgen and estrogen in a similar manner to TeBG.

II. PRESENCE OF A SPECIFIC ANDROGEN BINDING PROTEIN IN FOLLICULAR FLUID.

The fact that serum proteins are present in follicular fluid has demonstrated the possibility that proteins are interchangeable between follicular fluid and serum (107). Infact, several steroid binding proteins have been reported in follicular fluid of the pig, cow, sheep (28) and human (130,193). In the present studies, we have observed a specific androgen binding protein in follicular fluid, and reported some

of its properties (89) as well as a procedure for the purification of this high affinity binding protein for androgens. In comparison, the androgen binding activities in bovine serum and extracts of the epididymis have been observed to be significantly lower than that in the follicular fluid (Figure 5). This interesting observation has prompted us to further study the androgen binding activity in the follicular fluid and hence the purification and characterization of bff-ABP (90).

It has been well established that follicular fluid contains serum proteins (22) and that the serum binding protein TeBG is present in follicular fluid (130). The high androgen binding activity in the follicular fluid is probably contributed mostly by the serum binding proteins, such as TeBG and BSA. The amount of proteins in general and BSA in particular in the follicular fluid have been shown to be similar to those in serum (167). The observation of higher androgen binding activity quantitively in the follicular fluid than in serum has led to the speculation of the existence of a specific binding protein with high affinity, though minor in quantity, for androgens in the follicular fluid. Futhermore, an androgen binding protein has been identified in human follicular fluid aspirated from follicles of ovaries from patients with polycystic ovarian disease (PCO) and choriocarcinoma (196). This protein has been shown to have high affinity but low capacity for DHT and 17β-estradiol. Studies on the comparison of this binding protein with plasma TeBG by polyacrylamide gel electrophoresis and equilibium dialysis did not reveal any significant difference in molecular weight, heat stability, steroid

specificity or binding affinity (196). Results of those studies suggested that the androgen binding activity in follicular fluid was only due to the presence of serum TeBG, and especially the presence of serum proteins in follicular fluid has been well established. Little attention has been given to elucidate further the identity of the androgen binding activity in the follicular fluid.

Our newly identified androgen binding protein in bovine follicular fluid is clearly different from the previously reported PCO-ABP, which has been demonstrated to bind with similar affinities to DHT, T and E_2 and is relatively stable at 55°C (196). Our studies on bff-ABP have shown that this specific binding protein has high affinity to bind DHT and T but neglible binding for E_2 (Figure 40) and is relatively labile (Figure 7). Interestingly, the properties of bff-ABP appear to resemble, to a large extent, those of a recently reported ABP in Hydatidiform mole vesicle (130). This trophoblastic androgen binding protein has been shown to be different from serum TeBG by its isoelectric point, affinity for DHT and interaction with Concanavalin A. The pI of the trophoblastic ABP has been estimated to be 4.6 as compared to 5.2 for TeBG (130); and the trophoblastic ABP has a significantly higher affinity for binding DHT and T than E_2 (130). In general, we believe that ff-ABP is a specific androgen binding protein in the follicular fluid and is different from the serum TeBG and the PCO-ABP but appears to be relatively similar, though not identical, to reported trophoblastic ABP in physicochemical properties.

III. ISOLATION OF THE BOVINE FOLLICULAR FLUID ANDROGEN BINDING PROTEIN.

The purification of ff-ABP to homogeneity from bovine follicular fluid is made possible by seven steps of isolation procedure, including five major different methods of protein fractionation: ammonium sulfate precipitation, DEAE-cellulose chromatography, HTP chromatography, affinity chromatography on Sepharose Concanavalin A and gel filtration on Sephadex G-200. Several steps of isolation are found necessary since bovine follicular fluid contains only small amounts of ff-ABP, but relatively large amounts of BSA, TeBG and other proteins. At least, TeBG and BSA are both binding proteins for T and DHT: and the presence of these binding proteins in follicular fluid complicates the problem of purifying bff-ABP by a simple onestep scheme of affinity chromatography on Sepharose-DHT-HA, which has been used successfully for the purification of rat epididymal ABP (129). Furthermore, TeBG and ff-ABP appear to have very similar molecular weights upon gel filtration on sephadex G-200. However, DEAE-cellulose chromatography has been reported to be able to separate to some extent human TeBG and ABP (116).

One major problem in the purification of bff-ABP has been the fractionation and recovery of the small amounts of bff-ABP from relatively large quantities of TeBG and BSA. A very small percentage of these binding proteins remaining in a fraction of small amounts of ff-ABP recovered in a purification step becomes a major contaminant of binding proteins in the fraction. Accordingly, several steps of different experimental conditions have been used to isolate bff-ABP free from

contamination by TeBG and BSA. The initial step of salt fractionation of 28-55% saturation of ammonium sulfate precipitation eliminated more than 50% of contaminating proteins with no androgen binding activity (Figure 20), and this was also a very useful step for concentrating the androgen binding activity from large volumes of follicular fluid. Another useful step in separating bulk of contaminating proteins from bff-ABP was the DEAE-cellulose column chromatography. About 50% of inactive proteins remaining after ammonium sulfate precipitation was removed as an unadsorbed fraction upon a DEAE-cellulose column chromatography (Figure 21). The adsorbed bff-ABP and other proteins is then eluted with 0.12 M NaCl and separated further from other protein impurities. Under these experimental conditions, the bulk fraction of bff-ABP was still contaminated with substantial amounts of TeBG and BSA, which were present in large quantities in follicular fluid. It has been reported (116) that under similar experimental conditions of DEAE-cellulose chromatography human TeBG could be eluted by 0.06 M NaCl or below, but human ABP need concentrations of 0.07-0.1 M NaCl. Our data on preliminary studies to characterize the androgen binding activity in follicular fluid and serum also indicate that serum TeBG was not eluted by NaCl at concentrations of 0.07-0.1 M upon DEAE-cellulose chromatography (Figure 15). The remaining TeBG contaminant was eliminated mostly by another step of DEAE-cellulose chromatography on a compound gradient of NaCl (Figure 28). This approach to separate bff-ABP from TeBG using a compound gradient of NaCl has proved most successful and is adopted from the established methods of successful purifications of

several structurally similar pituitary hormones (26). It is also important to point out that hydroxylapatite chromatography has also been found very useful to eliminate the bulk of protein impurities; almost 90% of proteins with no binding activity including BSA in the fraction after the first DEAE-cellulose column become fractionated in this step of hydroxylapatite chromatography (Figure 22). The recycling of this material through another hydroxylapatite column helped to eliminate some more TeBG in the unadsorbed fractions.

After two steps each of hydroxylapatite and DEAE-cellulose chromatographies, the remaining relatively small amounts of BSA can be eliminated by affinity chromatography on Sepharose-Concanavalin A; BSA and small amounts of other minor contaminants are in the unadsorbed fractions, and most of the bff-ABP remains adsorbed and is eluted by a solution of α-Methy-D-glucoside (Figure 29). Androgen binding activities were found in both the unadsorbed and adsorbed fractions. This observation is not surprising since several reports have demonstrated that ABP is only partially retained by Concavanalin A (116,91). The binding activity in the unadsorbed fractions is probably due to leakage of small amounts of bff-ABP and presence of other contaminating proteins, such as BSA. More than half of the binding activity has been recovered in the adsorbed glycoprotein fractions eluted by the α-methyl-D-glucoside containing buffer.

The final purification of bff-ABP involved gel filtration on Sephadex G-200, a step of separation according to the molecular weights of the proteins (Figure 31). A contaminating glycoprotein with no

binding activity was separated by recycling on Sephadex G-200 (figure 33), and a homogeneous preparation of bff-ABP has thus been acheived. The established procedure for the isolation of bff-ABP has employed different criteria of protein separation, including charge heterogeneity, molecular size, interaction with carbohydrate residues and adsorption to hydroxylapatite.

Finally, it is worth noting that the yield of homogeneous bff-ABP is 0.356 mg from 400 ml of follicular fluid with an estimated 2,456-fold of purification on the basis of the recovered binding activity (Table 5). It is fair to point out, however, that with the estimation of 50-80% of the original total binding activity in crude follicular fluid is due to TeBG and BSA, the purification-factor for bff-ABP from follicular fluid becomes approximately 5,000-10,000 fold (Table 5). However, at present, we do not have data on the original concentration of bff-ABP in crude bovine follicular fluid to support this notion.

IV. PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES OF THE FOLLICULAR FLUID ANDROGEN BINDING PROTEIN.

Upon electrophoresis in 10% polyacrylamide gel at pH 8.9, bff-ABP is a homogeneous protein, and the androgen binding activity is coincident with the protein band (Figure 35). Bovine ff-ABP is a relatively basic protein, having a Rf of 0.20. The Rf value for testicular ABP and serum TeBG from various species has been reported,

and appears to be rather confusing. The Rf has been demonstrated to be 0.25 for human polycystic ovarian ABP (196), 0.46 or 0.47 for rat ABP in 6,5% or 6.0% gel, respectively (51,129), and 4.0 for rabbit ABP in 6.5% gel (119). Similarly, the Rf value has been estimated to be 0.32 for human TeBG in 5% gel (116), 0.3 for human serum binding globulin (115), 0.34 for monkey SBG (196), 0.34 for canine SBG (115), 0.40 for rabbit TeBG in 5% gel (157), 0.32 and 0.34 for female and male dogs respectively (186). Apparently, the Rf value of 0.20 of bovine ff-ABP is relatively similar only to that of polycystic ovarian ABP, and is quite different from those of testicular ABP and serum TeBG of various species. However, it is important to point out that the Rf value of the protein depends, to a large extent, on the purity of material and the experimental conditions, such as the pH and the percentage of polyacrylamide gel, being employed for the analysis.

By analytical polyacrylamide gel isofocusing, the pI of bff-ABP has been determined to be 4.75 (Figure 36). It is worth noting that the isoelectric point of bff-ABP is almost identical to that reported for trophoblastic ABP of 4.6 (130) and rat testicular ABP of 4.6-4.7 (51, 129) but differs from that of human serum TeBG of 5.2 (130). These findings indicate possible similarities between follicular fluid ABP and testicular ABP but differences between serum TeBG.

The molecular weight of highly purified bff-ABP has been estimated to be 110,000 by the method of gel filtration on Sephadex G-200 (Figure 38), and 80,000 by polyacrylamide gel electrophoresis in the presence of SDS (Figure 39B). Infact, the molecular weight of

bff-ABP appears to be relatively similar to that of native testicular ABP and serum TeBG. The molecular weight has been reported to be 100K for rat ABP (199), 65.5K (128) and 90K (199) for rabbit ABP, and 89.5K for bovine TeBG (177) and 76K for canine TeBG (178).

In contrast to testicular ABP and serum TeBG, purified bovine ff-ABP has no subunit structures upon SDS-polyacrylamide gel electrophoresis (figure 39A). The molecular weights have been reported to be 41K and 47K for subunits of rat ABP (199), 41K and 45K (128) and 43K and 47K (27) for rabbit ABP, and 28.4K for subunits of bovine TeBG, and 40K for subunits of canine TeBG (178). This observation that bff-ABP has no subunit-structures provides further evidence that follicular fluid ABP is not serum TeBG or testicular ABP. These three binding proteins might share similar functions in binding androgen, follicular fluid ABP is structurally very different from testicular ABP and serum TeBG. However, the exact chemical relationship between these androgen binding proteins remains to be clarified.

Biologically, purified bff-ABP has been demonstrated to have affinity binding for DHT and T, but negligible binding for E_2 (Figure 40). This finding is not surprising, and in fact agrees well with my earlier observation that crude follicular fluid binds both T and E_2, but with a much higher binding for androgen as compared to serum (Figure 40). This functional specificity of ff-ABP for binding of T and DHT indicates the possibility of an important physiological role for ff-ABP in follicular fluid, which contains high concentrations of both T and E_2 (1). There are at least 3 androgen binding proteins in follicular fluid:

BSA, TeBG and ff-ABP. O'Brien et al. (130) have reported the presence of TeBG together with trophoblastic ABP in hydaditiform mole vesicles. The presence of a specific ff-ABP with high affinity for DHT and T, may play a very important regulatory role in follicular physiology. BSA has been shown to bind T and E_2 with high capacity but low affinity (108). Similarly, TeBG also binds both T and E_2 eventhough with higher affinities. These hormone-binding proteins play an important role in regulating the concentrations of protein-bound and unbound T and E_2 in the follicular fluid effecting the differentiation of the granulosa cells. For example, TeBG in the follicular fluid may regulate to a large extent the unbound T, but its ability to bind T is limited by the amount of E_2 in the follicular fluid since high concentrations of E_2 will compete for the binding of TeBG and thus release free T (Burke and Anderson, 1972). These binding proteins play a regulatory role for the balance of T and E_2 in follicular fluid. The ff-ABP, on the other hand, might be the finer physiological regulator of bound and free T in the follicular fluid since it binds mostly T and not E_2 (Figure 41), and its binding to T will be influenced mostly by the concentrations of free T and not E_2 in the follicular fluid. However, the biological role of this specific binding protein, and the physiological relationship between these hormone binding proteins in ovarian physiology remains to be elucidated.

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