The University of Manitoba

# CHARACTERIZATION AND SOLUBILIZATION OF GONADAL RECEPTORS FOR FOLLICLE-STIMULATING HORMONE

by

Vincent Wing-sang Tang

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

Department of Physiology

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To my loving parents, grandmother

and brother Bill

## ABSTRACT

Some properties of the specific receptor for FSH were characterized using partially purified plasma membranes from porcine testes and ovarian granulosa cells in culture. For testicular membranes, the association of $^{125}$I-labelled bFSH and the testicular receptor was maximal in 25 mM Tris-HCl buffer, pH 7.2, containing 10 mM $MgCl_2$. However, absence of the divalent ion $Mg^{++}$ or addition of the monovalent ion $Na^{+}$ decreased the specific binding of $^{125}$I-bFSH to receptor. After $^{125}$I-bFSH had bound to its receptor, only 60 % of the bound radioactivity was dissociated from testicular membranes after 14 hr at 37°C and the dissociation was not accelerated by adding excess amounts of unlabeled hFSH. Extremes of pH( $<3$ and $>11$), 2M $MgCl_2$, and n-butanol-saturated buffer effectively dissociated the receptor-bound $^{125}$I-bFSH; however only $MgCl_2$ did not cause irreversible denaturation of the receptor after treatment.

For ovarian granulosa cells in culture, $^{125}$I-bFSH associated maximally with the porcine cells after incubation at 37°C for 6 hr. After association, 70 % of the bound $^{125}$I-bFSH was dissociated from granulosa cells after 6 hr at 37°C and the presence of excess unlabeled hFSH failed to accelerate the dissociation of the receptor-bound hormone tracer. Pretreatment of cells with excess unlabeled hFSH for different time intervals did not decrease the uptake of the subsequently added $^{125}$I-bFSH by the granulosa cells. Furthermore, bovine granulosa cells

from large follicles (dia. >15 mm) bound significantly less $^{125}$I-bFSH but more $^{125}$I-bLH than cells from medium (dia. 7-15 mm) or small (dia. < 7 mm) ovarian follicles, indicating changes of levels of receptors for FSH and LH during follicle maturation.

Treatment of lyophilyzed porcine testicular membranes with organic solvents did not solubilize FSH receptor, but mostly destroyed receptor activity. An n-butanol/water system solubilized membrane extensively into the aqueous phase, but no receptor activity was recovered after solubilization. Similarly the non-ionic detergent Triton X-100 (0.1 %) destroyed most of the free FSH receptors in testicular membranes; however, the preformed particulate $^{125}$I-bFSH-receptor complex subsequently solubilized by Triton X-100 was relatively stable and was eluted as a major radioactive peak upon gel filtration on Sepharose 6B. Pre-incubation of $^{125}$I-bFSH and testicular membranes simultaneously with excess hFSH abolished the subsequent formation of solubilized $^{125}$I-bFSH-receptor complex. Further, addition of excess unlabeled FSH after the solubilization of $^{125}$I-bFSH-receptor complex failed to displace $^{125}$I-bFSH from the already solubilized hormone-receptor complex. Immunologically the solubilized hormone-receptor complex cross-reacted almost identically as the free $^{125}$I-bFSH tracer with an antiserum against bFSH. Upon polyacrylamide gel electrophoresis, the $^{125}$I-bFSH tracer was more acidic than the solubilized $^{125}$I-bFSH-receptor complex which is in turn more acidic than the $^{125}$I-bFSH-antibody complex. After reacting with the anti-bFSH antiserum, the $^{125}$I-bFSH-receptor complex became more basic in nature and migrated between the

$^{125}$I-bFSH-receptor and $^{125}$I-bFSH-antibody complexes, indicating the formation of a triple antibody-hormone-receptor complex.

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If I were to name a person who has influenced me most in science, it would be Dr. K.W. Cheng. Besides classifying him a supervisor constantly demanding for quality (and QUANTITY and TIME too!), I must acknowledge that his philosophy and aggressiveness toward science have re-shaped my attitude toward medical research. To him I owe my deepest thanks. I have also enjoyed the co-operation and friendship of all our colleagues in the Endocrine Section. Special thanks are due to Dr. Robert P.C. Shiu and Dr. May C. Robertson. Bobby's resourcefulness in "receptology" and May's friendliness are very much appreciated. Through Dr. Cheng I launched my studies on FSH receptor, but it was Bobby and May who introduced me to Humphrey Bogart and the fine Art of Ballet. The secretarial help of Janet Greer and Winnie Kum in typing this thesis is acknowledged.

## LIST OF ABBREVIATIONS

### Hormones

| | |
|---|---|
| FSH | follicle-stimulating hormone |
| LH | luteinizing hormone |
| TSH | thyroid-stimulating hormone |
| FSH $\alpha$ | the $\alpha$ subunit of follicle-stimulating hormone |
| FSH $\beta$ | the $\beta$ subunit of follicle-stimulating hormone |
| hCG | human chorionic gonadotropin |

### Prefix Denoting Species of Origin

| | |
|---|---|
| b | bovine |
| p | porcine |
| h | human |

### Assays and Activities

| | |
|---|---|
| specific binding | CPM bound (e.g. to receptor) that can be displaced by excess hormone |
| % specific binding | $\frac{\text{CPM specifically bound}}{\text{total CPM}} \times 100$ |

### Gel Filtration and Polyacrylamide Gel Electrophoresis

| | |
|---|---|
| $V_o$ | void volume |
| $R_f$ | electrophoretic mobility = $\frac{\text{distance of migration by protein}}{\text{total distance migrated by ion-front}}$ |

### Units of measure

| | |
|---|---|
| g | gram |
| mg | milligram |
| µg | microgram |
| ng | nanogram |
| ml | milliliter |

| | |
|---|---|
| µl | microliter |
| W/V | weight per unit volume (g/100 ml) |
| cm | centimeter |
| mm | millimeter |
| nm | nanometer |
| M | molar |
| mM | millimolar |
| N | normality |
| in | inch |

Reagents

| | |
|---|---|
| $^{125}I$ | prefix denoting radioactively iodide-labelled molecule (e.g. $^{125}I$-bLH) |
| PBS | phosphate buffer with saline (0.9% w/v) |
| BSA | bovine serum albumin |
| PEG | polyethylene glycol |

Miscellaneous

| | |
|---|---|
| g | unit of gravitational force |
| Ka | association constant of binding ($M^{-1}$) |

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# (I) INTRODUCTION

## (A) General Characteristics of Hormone Receptors

### (1) Definition of a Hormone Receptor

Different hormones are transported via the blood-stream to all parts of the body, but their biological effects are highly organ-specific. To early workers in the field of drug and hormone action, this simple observation suggested that organs which responded to a specific hormone must possess recognition-sites or receptors. The term "receptor" has been used rather loosely to describe an unknown structure or entity in the target cell of a hormone through which the biological effect of the hormone on the cell is mediated. However, many investigators have restricted the term "receptor" to be the recognition-function of the cellular structure(s), and the term "catalytic component" to be the structure(s) responsible for the transmission of hormonal signals from the external to internal milieu. It is necessary to distinguish the recognitive component from the catalytic component. First, different hormones bind to different and specific recognition-sites to initiate biological responses. In testicular plasma membranes, for example, FSH and LH bound specifically to different receptors (1,2), but stimulated presumably the same adenyl cyclase system because their effects were not additive (3,4). Secondly, in some cases where the responses are quite different, the recognition-specificity may be identical. For example, the action of acetylcholine on the intestine and heart where excita-

tory and inhibitory responses, respectively, were both mediated through an atropine-sensitive acetylcholine receptor (5).

A model of the multi-component receptor has widely been used for the hormone sensitive and membrane-bound adenyl cyclase system, in which an externally located recognition site is coupled to an internally located catalytic site as shown in Fig. 1. A number of experimental findings supported this model. First, fluoride ion, believed to activate directly at the catalytic site, did not activate intact cells but activated membrane fragments or inverted cells (6,7,8). Secondly, proteolytic enzymes could readily destroy hormonal sensitivity without affecting fluoride sensitivity (9,10,11).

(2) Criteria for Receptor Identification

It might be anticipated a priori that the presence of receptor binding would be restricted solely to tissues classically known to be sensitive to the hormone. In general, the presence of specific receptor binding closely parallels such a distribution, and indeed the logical place to establish the characteristics of the hormone-receptor interaction is in such recognized responsive tissues. Nonetheless, it is becoming increasingly evident that some tissues yet unrecognized as responsive to most peptide hormones can in fact either respond or bind with certain hormones with high affinity and specificity. For example, mouse spleen lymphocytes responded (increased production of cGMP and increased cytolytic activity) to insulin

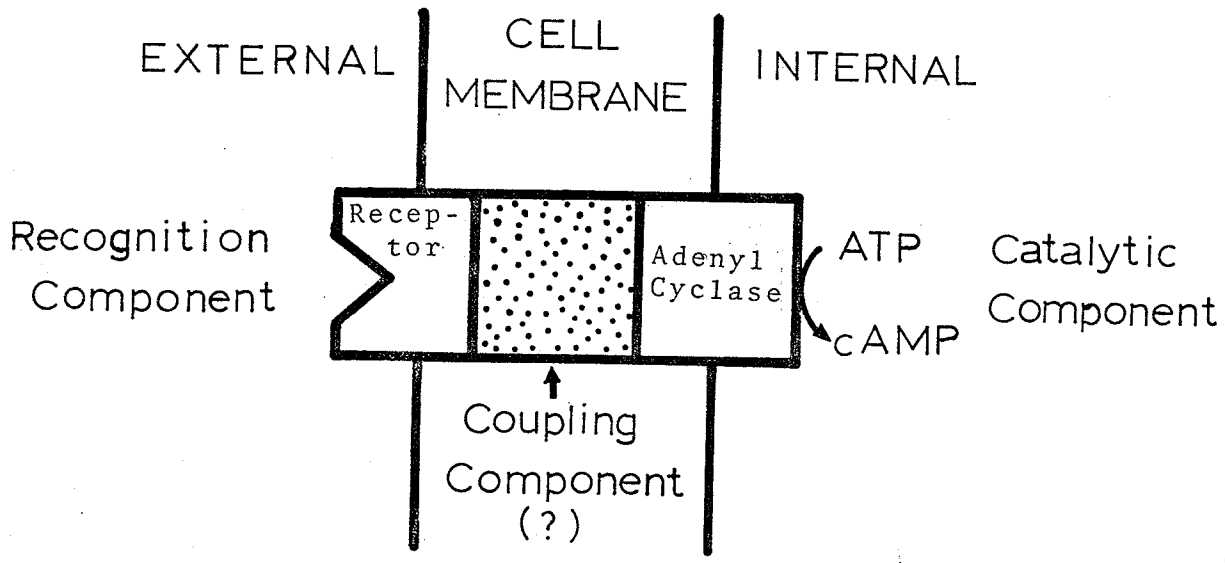



Figure 1: A multi-component receptor model for the hormone-sensitive and membrane-bound adenyl cyclase system in which an externally located recognition site is coupled to an internally located catalytic site through an as yet unidentified coupling component.

and cholinergic agents (12,13); and cultured human skin-derived fibroblasts responded to insulin at concentrations within the physiological range (14,15,16). Further, in some instances, a highly specific hormone-receptor interaction can be observed in a tissue for which a biological response has yet to be elucidated. For instance, insulin and epidermal growth factor bound to human placental tissues (17,18,19), and human prolactin bound with high affinity and specificity to adrenal tissues (20).

Although it is not always possible to identify hormone-binding sites as receptors, there are, however, some accepted operational characteristics for a hormone receptor:

(a) Specificity - The hormone of interest binds to its receptor and substances of unrelated biological activity do not bind, nor compete for binding to the same receptor. For example, FSH and LH bound specifically to different receptors in testicular membranes and the bindings were non-displaceable by each other or any other unrelated hormones (1,2).

(b) High Affinity - The interaction between a hormone and its receptor should have a high association constant (Ka), or a low dissociation constant (Kd), because of the low concentration of hormones in the blood under physiologic conditions. For example, the binding of human FSH to bovine testicular receptors has a Kd of $9.8 \times 10^{-11}$M (21).

(c) Saturability - The hormone-receptor interaction is a saturable process. In other words, there are a limited number of binding sites per unit of cell or tissue. Using Scatchard analysis (22), 1 mg of partially purified bovine testicular membrane could bind $5.9 \times 10^{-14}$ mol of human FSH (21).

(d) Correlation of binding with biological action - This is an important functional criterion of a hormone receptor. For example, the binding of hCG to the interstitial cells in rat testes has been correlated to the stimulation of testosterone production and cAMP synthesis at different doses of the hormone (23).

(3) Spare Receptors

In an *in vitro* study of the interaction of alkyl trimethylammonium ions and atropine with the guinea-pig ileum, Stephenson (24) observed in 1956 that occupancy of only a small amount of receptor was needed to elicit a maximum response. Using decapsulated rat testes, Catt and Dufau (23) showed that there were more hCG receptors than required for maximum activation of adenylate cyclase and for a maximum biological effect, such as testosterone secretion (Fig. 2). Similar data were obtained from studies on the effect of ACTH on isolated adrenal cells (25). These experimental findings suggested that if the receptors in a tissue are equivalent and indistinguishable from each other, most of the receptors are

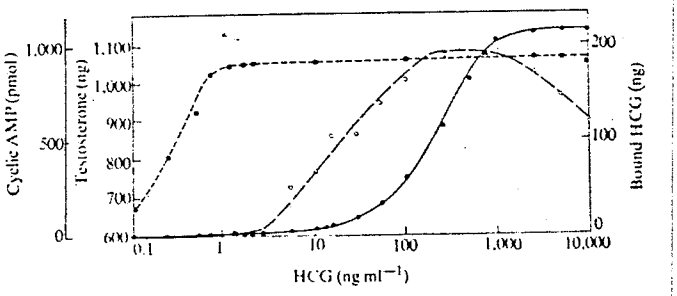



Figure 2: Dose-response relationships between hCG binding (•—•), cAMP release (∘—∘) and testosterone production (▪----▪) during in vitro incubation of decapsulated rat testes, reproduced from Catt and Dufau (23).

"spare" in the sense that there are more than enough receptors for a maximum response. The presence of spare receptors may help to increase the probability for interactions of the receptors with a fixed amount of the hormone. However, there are other plausible explanations for the apparent surplus of receptors. First, the hormone-binding sites may not be homogeneous and the majority of the observed binding sites may be unrelated to the biological response, such as storage-sites or degradation-systems for the hormone. Secondly, the same initial hormone-receptor interaction may generate a signal (e.g. elevated levels of cAMP) resulting in more than one response and the different responses may be achieved maximally by different levels of the signal. Thus the number of "spare" receptors observed may differ substantially, depending on the final response being monitored as the index of a biological effect.

(4) Negative Cooperativity

The Scatchard analysis (22), one of the most common methods for characterizing the binding of a hormone with its receptor, plots the bound/free (B/F) ratio of the hormone as a function of the total hormone bound to the receptor. If the hormone receptors are a homogeneous population of binding sites and the hormone-receptor binding is a second-order reaction, a linear plot should be obtained at equilibrium. However, non-linear plots are often obtained, ascribing to "two" independent hormone-binding sites, as it is known that hetero-

geneity of binding sites gives non-linear plots. There are also other reasons for these curvilinear plots, such as high non-specific binding at higher concentrations of the hormone, different binding affinities between labeled and unlabeled hormones to the receptor (26), and effects of interactions between receptors (27). The presence of site-site interactions between receptors (negative cooperativity) was evidenced by the increased dissociation rate of $^{125}$I-labeled insulin from its binding sites by incubating human lymphocytes in excess unlabeled insulin (27). Other hormones such as growth hormone, glucagon and calcitonin failed to accelerate the dissociation of $^{125}$I-labeled insulin from its receptor (27); therefore the cooperativity of receptors might be hormone-specific. It has further been suggested that all hormones which yield a curve by Scatchard analysis of the hormone-receptor binding are potential candidates for receptor cooperativity (27).

Interestingly, the binding of $^{125}$I-labeled insulin to non-receptor materials such as talc and various agarose-protein derivatives similarly exhibited negative cooperativity of receptor binding (28). Therefore, the interpretation of such data is open to question. Self-aggregation of hormone has been proposed by Cuatrecasas and Hollenberg (28) to account for the observed phenomena in the non-receptor systems, where there is no possibility for interactions between binding sites. For this reason, a similar yet unknown mechanism for hormone-receptor binding must be seriously

considered for the observations with cell membranes or cells. Since it is well-known that insulin formed dimers and aggregates (29,30), dimer formation might account for the enhanced "dissociation" of the receptor-bound $^{125}$I-labeled hormone *in vitro* provided that the receptor-bound monomer could still participate in dimerization and that dimerization altered the hormone conformation with a reduction of its affinity for the receptor.

(5) Desensitization (Down Regulation)

Theoretically, a cell could change its sensitivity to a hormone by altering the number of specific receptors. With higher concentration of receptors, the cell could respond more readily for a given amount of hormone. Several hormones, including insulin and growth hormone, have been shown to regulate the concentration of their own specific receptors on the surface of target cells (31-35). This phenomenon of self-regulation which generally leads to a loss of receptor sites with little or no change in the binding properties of the residual receptors, is termed "down-regulation". For instance, the desensitizing effects of LH and hCG upon ovarian adenylate cyclase (36-39) have been shown to be a result of loss of LH/hCG receptors (LH and hCG compete for the same receptor) (38,39). Furthermore, the down-regulation of testicular LH/hCG receptors by LH or hCG has been shown to be accompanied by reduced production of cAMP and testosterone in response to the hormones (40-45). Clinically, the inverse relationship

between receptor concentration in target tissues and the circulating levels of insulin, that is, the phenomenon of down-regulation, may be the basis for insulin resistance in hyper-insulinemic states (31).

Surprisingly, administration of prolactin in vivo has been shown to "up-regulate" rather than to decrease its own receptors in rat liver (46). Therefore it is possible that prolactin stimulated other tissues in vivo to produce certain substances which in turn caused the observed increase in prolactin receptors. In addition, receptors can either be increased or decreased in response to other non-homologous hormones. For example, FSH treatment in immature hypophysectomized rats has been shown to increase the level of LH receptors in testicular interstial cells (47),and androgens to reduce prolactin receptors in rat liver (46). Generally a high serum level of a hormone is often interpreted as an index of increased stimulation ( and increased biological response ) of its target tissue. Our recently acquired knowledge of receptor control by both homologous and non-homologous hormones has made it necessary to reconsider the classical way of interpreting fluctuations of hormone-levels. In order to assess the degree of stimulation of a target tissue by a hormone, it seems necessary to measure not only the hormone level in serum, but also the receptor level in the target tissue.

(6) Irreversibility and Internalization of Hormone-receptor complex

For studies of hormone-receptor interactions, it is generally assumed that the formation of a hormone-receptor complex is a reversible process. The Scatchard analysis (22), one of the most common methods for characterizing the binding of a hormone to its receptor, plots the bound/free (B/F) ratio of the hormone as a function of the total hormone bound to the receptor. The validity of this analysis depends on the achievement of an equilibrium in the hormone-receptor interaction. However, the reversibility of hormone-receptor interaction has recently been questioned.

Powell and Hollander (48) showed that the dissociation of the receptor-bound $^{125}$I-labeled ovine prolactin to rat mammary tumor membranes was less than 50% after 24 hours at 24$^{o}$C and that addition of standard unlabeled prolactin into the incubation mixture had little effect on the apparent rate of dissociation. Similarly, the binding of FSH to rat testicular membranes appeared to be irreversible (49). In contrast to the slow dissociation of radioactively labeled hormones bound to membrane preparations, the dissociation of bound hormone from living cells appeared to be rapid. For instance, 80-90% of $^{125}$I-labeled epidermal growth factor (EGF) bound to cultured fibroblasts could be dissociated at 37$^{o}$C after 2 hours (50). Similar phenomenon was observed for the dissociation of hCG bound to suspended Leydig tumor cells (51). In both cases, however, the dissociated radioactivity was

recovered in the culture medium as a low molecular weight component suggesting the breakdown of polypeptide hormones due to an energy-dependent process of "internalization" and degradation by lysozomal enzymes. Receptor-mediated endocytosis (internalization) of fluorescent analogs of EGF has also been directly visualized by fluorescent microscopy (52). These observations may explain the "down regulation" of hormone receptors mentioned previously (section A-5). It seems that the interaction of a hormone with its receptor leads to internalization of the hormone-receptor complex, followed by subsequent breakdown of the complex in the cell. As a result, less receptors are available on the cell surface for further interaction with the hormone and the sensitivity of this "down regulated" cell to stimulation by the hormone is decreased. Because the concentration of EGF for inducing down regulation (53) was nearly identical to that for the stimulation of DNA synthesis (54,55), Das and Fox (56) postulated that the effects of EGF on DNA synthesis might be mediated by a peptide fragment originated from proteolytic cleavages of the internalized hormone-receptor complex. On the other hand, Shechter et al (57) have recently suggested that even though internalization and degradation of hormones might be closely linked to the down-regulation of receptors, they might be irrelevant to the MECHANISM of hormonal stimulation. These investigators (57) demonstrated that in order to elicit a biological response, cell-bound EGF had to remain attached to the cell surface for protracted time periods

(at least 8 hours). Furthermore, selective intervention of the receptor-bound EGF by treatment with specific antisera after the major phases of down-regulation and internalization (after 8 hours) could still totally reverse the biological response. Therefore, significance of the observed internalization and degradation of hormones in the mechanism of hormone action remains to be clarified.

(7) The Mobile-receptor Hypothesis

The mobile-receptor hypothesis (58) has been advanced to conform with the recent concept of the dynamic and fluid nature of cell membranes (59). The hypothesis suggests that receptors and effectors (e.g. adenylate cyclase) are discrete and separate structures which acquire specificity and affinity for the formation of receptor-effector complex only after the receptor has been occupied by the hormone. After binding to hormone, the receptor molecule may change its conformation and become "activated" to combine with the effectors. These cellular structures are free to bind with each other because of the fluidity of the cell membrane. The hormone-binding sites of the receptor are on the external side, exposing to the aqueous medium; and the catalytic sites of the effector are facing inward exposing to the cytoplasm of the cell (Fig. 3).

The mobile-receptor hypothesis is attractive because it offers satisfying explanations for many of the properties of hormone receptors and effectors.

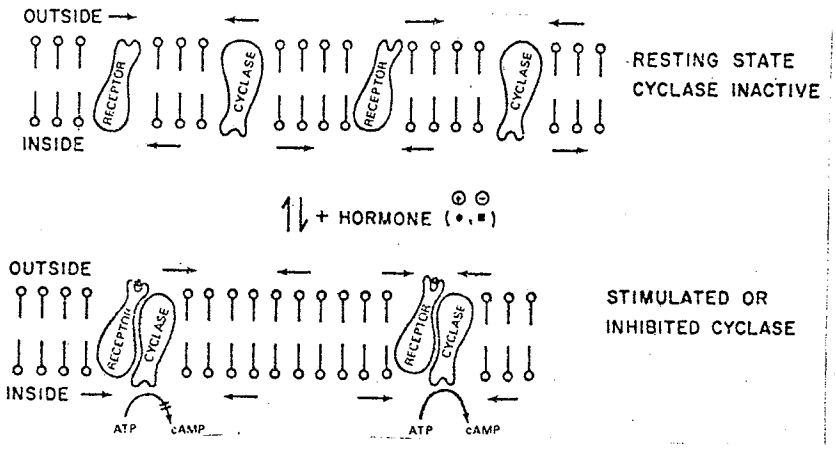



Figure 3: Mobile receptor hypothesis for the mechanism of modulation of adenylate cyclase activity of cell membranes by hormones (58). The central feature is that the receptors and the enzymes are discrete and separate structures that acquire specificity and affinity for complex formation only after the receptor has been occupied by the hormone. These structures can interact after the binding of the hormone to the receptor because of the fluidity of the cell membrane.

First, the concept of "spare receptors" fits into this mobile-receptor model satisfactorily. Thus, maximum cAMP-mediated response will occur when all the adenyl cyclase effectors are in contact with the occupied receptors; under this maximum stimulation, a large number of receptors may still remain unoccupied. The sensitivity of cell response to a hormone can also be altered by controlling the number of receptors on cell surface, without changing the number of effectors.

Secondly, the model can accomodate the observation that a number of hormones may work through the same effector. For example, in the fat cell at least eight different hormones (glucagon,ACTH, secretin, LH, catecholamines, prostoglandins, vasoactive intestinal polypeptide and insulin) appeared to act on the same adenyl cyclase, yet each of them acted by binding to its own receptor (58). Sharing of a common effector by different receptors simplifies the control of hormone response and helps to conserve genetic material because only the receptor concentration needs to be regulated in order to change the sensitivity of a biological response to a given hormone. The shared effectors remain unaltered, but still responsive to other hormones.

Finally, the site-site interactions between receptors (negative cooperativity, section A-4) and the internalization of hormone-receptor complexes (section A-6) can also be explained by the mobile-receptor hypothesis. Hormone-bound receptors floating in the "lipid sea" of a dynamic and fluid

cell membrane (59) can diffuse laterally, combine rapidly with their effectors, and form clusters for internalization.

## (B) The Gonadotropins

### (1) Biological Actions

The hormonal control of gonadal function in primates and mammals is predominantly exerted through two pituitary gonadotropins — FSH and LH.

The biological effects of FSH are upon maturation and function of the somatic cells immediately associated with gametogenesis, that is, the granulosa cells of the ovary and the Sertoli cells of the testis. In immature female rats, FSH treatment has been shown to stimulate a marked increase in LH receptor content without causing luteinization (60); therefore FSH may play a role in preparing the ovary for the mid-cycle LH surge during the female reproductive cycle. In granulosa cells, FSH stimulated the aromatization of androgen produced by theca cells to estrogen (61). During the follicular phase, human follicles containing FSH always contained more estradiol than those with undetectable FSH (62). These observations have suggested an important role of FSH in the control of steroidogenesis in the ovarian follicle.

In the testis, FSH is necessary for the development and maturation of the seminiferous tubule as well as the initiation of the process of spermatogenesis during puberty (63). In hypophysectomized rats, FSH enhanced markedly the

response of Leydig cells to LH for the synthesis and secretion of androgens (63). Whether this effect was secondary to the maintenance of the function of seminiferous tubule and the size of the testis, or a direct action of FSH upon the Leydig cells, has not yet been determined. Treatment of immature hypophysectomized rats (63) with FSH stimulated an increase in the LH receptors of the Leydig cell. Whether this effect of FSH upon Leydig cell function was exerted directly or indirectly through effects on the seminiferous tubule has not been clarified. The biochemical events following binding of FSH to plasma membrane receptors in Sertoli cells have been extensively studied in the immature rat (64). Hormone-receptor interaction was followed by activation of adenylate cyclase in the cell membrane and increased intracellular levels of cyclic-AMP. This led in turn to activation of cAMP-dependent protein kinase in the cytosol, and subsequent phosphorylation of various proteins which mediated the biochemical effects of FSH, including increases of RNA synthesis and cellular proteins (64), formation of a specific androgen-binding protein (65) and conversion of testosterone to estradiol (66).

LH in the testis and ovary acts predominantly upon steroidogenesis resulting in biosynthesis and secretion of androgens, estrogens, and progestins. In the ovary, LH has been shown to acutely increase serum progesterone levels during the luteal phase of the menstrual cycle in the rhesus monkey (67). Recently, granulosa cells have been found to

release plasminogen activator in substantial amounts, reaching a peak prior to ovulation. Since follicular fluid contains plasminogen at the same concentration as serum, and plasmin has been shown to act on follicle wall strips, this proteolytic system could be an important factor in follicular rupture. Such a possibility is supported by the finding that formation of plasminogen activator by granulosa cells was rapidly increased by LH *in vivo* and *in vitro* (68). The midcycle surge of LH is clearly essential for ovum maturation and ovulation. Both LH and FSH could cause ovulation when given systemically (69) or by intrafollicular injection (70). The preovulatory LH surge, however, causes ovum maturation, or resumption of the meiotic division that became arrested during fetal life. In mammals, ovum maturation *in vivo* has been shown to begin shortly before ovulation and could be reproduced *in vitro* by incubating follicles with LH, hCG, prostaglandin $E_2$ and cAMP (71).

In the testis, uptake of LH by Leydig cells was followed by stimulation of adenyl cyclase activity and elevation of intracellular cAMP levels (72). The increase in cAMP formation in Leydig cells led to activation of protein kinase in the cytosol with subsequent phosphorylation of cellular proteins which were believed to regulate the early steps in steroidogenesis (73).

(2) Chemical Structure

FSH, LH and hCG are glycoprotein hormones having a

molecular weight of approximately 30,000. Each has been shown to consist of two dissimilar polypeptide chains. One of the subunits, designated α, is common among the glycoprotein hormones (74). The other subunit, designated β, has a different amino acid sequence in each hormone and carries the information which dictates the specific hormonal activity to be expressed upon association with the α subunit (75). The function of the carbohydrate moieties in gonadotropins has not yet been defined, except that removal of the terminal sialic acid residues drastically shortened the half-life of the circulating hormone in the circulation (76). For this reason, preparations of hLH, hCG and FSH from which sialic acid has been removed showed reduced activity when measured by in vivo bioassays, but retained activity in short-term in vitro biologic assays (77,78). Furthermore carbohydrate residues might also influence the ability of the hormones to interact with their cellular receptor sites in the testis or ovary (79,80).

(C) The Gonadotropin Receptors

(1) Localization

In the testis, specific receptors for LH and hCG are present only in the interstitial cell compartment and are localized in the Leydig cells. No significant uptake of labeled LH or hCG by isolated seminiferous tubules, tubular cells, or homogenates was observed in in vivo or in vitro studies in rats (81,82,83). In the ovary, LH/hCG receptors were located

in the thecal and granulosa cells of the maturing follicle and in the luteal cells of the corpus luteum (84-89).

For FSH, testicular receptors appeared to be largely confined to the Sertoli cells of the seminiferous tubule (90,91,92). Ovarian receptors for FSH have been demonstrated in granulosa cells from follicles of immature, pseudopregnant, and pregnant rats (87).

(2) Solubilization of Gonadotropin Receptors

One of the objectives of receptor studies is to isolate the receptor molecule and to reconstitute an operational receptor system. However receptors may be classified as "integral" (59) or "intrinsic" (93) macromolecules because it has been very difficult to dislodge the receptor in a lipid-free form from the lipid matrix by manipulations of pH, ionic strength or chelating agents. In solubilizing receptors, it is critical to use those techniques which do not abolish the "recognition" function of the receptor. Not only the conformation of the receptor molecule must be retained, but also its binding sites to interact with the hormone must not be destroyed.

(a) LH/hCG Receptors - The use of non-ionic detergents has, to date, yielded the best success for extraction of receptors from membranes. During the past few years, many hormone receptors have been successfully solubilized with non-ionic detergents such as Triton X-100 and Lubrol PX. These including insulin (94,95), glucagon (96,97), nor-epinephrine

(98), acetylcholine (nicotinic) (99), angiotensin (100), prolactin (101) and nerve growth factor (102). LH/hCG receptors in rat testis (103,104) corpus luteum (105) and ovary (106) have also been solubilized with the non-ionic detergent Triton X-100 and partially purified by affinity chromatography.

Recently, a heat-labile "water soluble hCG-binding component" (SBC) has been obtained from Leydig cell membrane fractions by exposure to buffer systems of low ionic strength (107). The sedimentation coefficient of the $^{125}$I-hCG-SBC complex was determined to be 4.35S when the hormone was added to the SBC in the aqueous extract, or 5.0S if the hormone was first added to the particulate receptor followed by aqueous extraction of the soluble complex. The molecular weights of the $^{125}$I-hCG-SBC complexes were calculated from Kav-values and sedimentation coefficients to be 71,500 (4.35S) or 82,250 (5.0S) respectively, depending on whether the complex was formed with an already solubilized receptor or with a particulate receptor followed by solubilization. Thus the water soluble $^{125}$I-hCG-SCB complex ( M.W.82,250) was considerably smaller than the $^{125}$I-hCG-receptor complex ( M.W.228,000) solubilized by Triton X-100 (108). However, the association constant (Ka) calculated for $^{125}$I-hCG-SBC complex formation was 0.75-1.0 X $10^{10}$ $M^{-1}$, similar to those of membrane-bound and Triton X-100 solubilized hCG receptors.

(b) FSH Receptors - Specific receptors for FSH have been demonstrated in homogenates of rat testes (109,110) and ovaries

(111). For obtaining a large quantity of FSH receptors for solubilization, however, it would be more convenient to use homogenates of bovine testes (112). Recently, FSH receptors have been solubilized with different agents such as non-ionic detergents, ethanol and water.

(i) Non-ionic detergents - Using the non-ionic detergent Triton X-100, bovine (113), rat (114) and porcine (115) testicular receptors for FSH have recently been solubilized. Closset et al (115) reported that in pig testicular extracts approximately 20% of the total binding sites initially present in the particulate preparation was recovered in the soluble fraction and that less than 10% of the total receptors remained in the extracted pellet. They concluded that the solubilized porcine FSH receptor was relatively labile. The loss of FSH receptor during solubilization could not be prevented by various protease inhibitors such as trasylol, para-methylsulfonylfluoride, EDTA and trypsin inhibitors (97). Instability of Triton X-100 solubilized receptor has also been reported for testicular LH receptor (98) which was 50%-degraded during storage at 0-4$^{o}$C for 24 hr. or at 34$^{o}$C for 20 min. Besides, Dufau et al (114) have reported that the solubilized FSH receptor could not be characterized by gel filtration on Sepharose 6B in the free form. The properties of FSH receptors solubilized by Triton X-100 in general resembled the particulate FSH receptor, including hormone specificity and binding affinity (95-97).

In contrast to the solubilized free receptor, FSH

receptor-hormone complex solubilized by Triton X-100 has been shown to be relatively stable. By gel filtration on Sepharose 6B, the molecular weight of the FSH receptor-hormone complex has been shown to be between 183,000 (95) to 244,000 (97). A small peak of radioactivity, probably an aggregate of FSH-receptor complex, was detected in the void volume (96). Previous studies on ovarian and testicular LH/hCG receptors solubilized by Triton X-100 yielded similar elution profiles after gel-filtration on Sepharose 6B (108, 116). It appears that the molecular weights of receptors of different polypeptide hormones solubilized by Triton X-100 are very similar. For examples,solubilized receptors of LH/hCG (108,116), insulin (94,95) and prolactin (101) all appeared to have a molecular weight of approximately 200,000; and aggregates of larger molecular weight have also been observed (108).

(ii) Ethanol - In view of the problems of stability and aggregation in using non-ionic detergents such as Triton X-100 and Lubrol PX, other approaches to solubilize gonadotropin receptors have been attempted. Using 30% (v/v) ethanol in 0.01 M phosphate buffer at 37$^{o}$C, Bhalla et al (117, 118) reported with rat testes an "ethanol-soluble factor" (ESF) which could interact with FSH and LH. The active species was found to be stable to trypsin hydrolysis and heating at 100$^{o}$C (117). Fractionation of ESF by gel-filtration on Sephadex G-100 yielded two major active components, corresponding to molecular weights of approximately 67,000 and 22,500

(117). These fractions interacted with both FSH and LH. However similar ethanol extracts of non-gonadal tissues such as the liver, kidney and heart also interacted with $^{125}$I-hFSH (117). Therefore, the significance of these solubilized factors remains to be clarified.

(iii) Water - Using dextran-coated charcoal in their assay, Bhalla et al (118) reported that their "ethanol-soluble factor(s)" could also be extracted with water, but 10 to 25% ethanol (v/v) facilitated the solubilization. Reichert and Abou-Issa (119) have also extracted from rat testes a water-soluble "FSH binding inhibition factor" which was tissue-nonspecific and relatively heat-stable. This factor had a low molecular weight and inhibited the in vitro binding of $^{125}$I-hFSH to particulate testicular receptors. Heating at 100$^{o}$C for 10 min. destroyed 50% of the inhibitory activity. However, the low specific binding (1%) of their receptor assay and the reported interference of FSH-receptor interaction by various metalic salts have made the interpretation of their results difficult. Nevertheless, Dufau et al (114) also mentioned a water-soluble testicular fraction during their preparation of rat testicular membranes for detergent solubilization. This water-soluble fraction represented about 20% of the total FSH binding activity in the original testis.

(D) Solubilization of Membrane Proteins with Organic Solvents

Some organic solvents have been used successfully

to solubilize membrane proteins. For instance, the butanol extraction technique of Maddy (120) has been used widely to prepare erythrocyte membrane protein and glycoprotein components. Using a two-phase system of butanol and water, at least 90% of the total membrane protein can generally be extracted into the aqueous phase and 90-95% of the lipids into the butanol phase. The extraction conditions were relatively mild and antigenic and enzymic activities were not greatly affected (120). Solubilization is believed to be due to the fact that butanol competes effectively for the polar side-chains of the protein, displacing the lipids and dissociating lipid-protein interactions. The interactions between butanol and membrane proteins have been described by Roth and Seeman (121).

Other organic solvents such as phenol, pyridine, and chloroform-mixtures have also been used in solubilizing erythrocyte membrane proteins. These methods have recently been reviewed by Hughes (122).

## (II) SPECIFIC AIMS AND OBJECTIVES

It appears that a better understanding of the nature of the first step in hormone action, i.e. the hormone-receptor interaction, is essential to our understanding on the mechanism of hormone action. In this study , two lines of approach will be used to elucidate the nature of the hormone-receptor interaction:

(1) Interactions between FSH and its receptor in particulate preparations of testicular membrane, ovarian cell suspension and granulosa cell culture will be characterized and compared.

(2) As a first step to identify the chemical nature of the FSH receptor, porcine testicular membranes have been solubilized with various reagents such as non-ionic detergents and organic solvents. The efficacy of different chemical reagents on the solubilization of testicular FSH receptor will be evaluated.

# (III) MATERIALS AND METHODS

## (A) Materials

Purified bLH (potency:2.0 X NIH-LH-S-1) and bTSH were gifts from Dr. J.G. Pierce, UCLA, Los Angeles, Ca. Purified bFSH (potency: 164 X NIH-FSH-S-1) (104), hFSH, hLH, and hCG were prepared in this laboratory. Specific rabbit anti-sera against bFSH were also raised in this laboratory. Collagenase (207 U/mg), trypsin (204 U/mg) and lima bean trypsin inhibitor (1 mg inhibits 3.9 mg trypsin) were perchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Bovine serum albumin (fraction V), bovine-$\gamma$-globulin, and chloramine-T were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Lactoperoxidase was from Calbiochem, San Diego, Ca., U.S.A. $Na^{125}I$ (carrier-free) was obtained from the New England Nuclear Corp., Boston, Mass., U.S.A. Sephadex G-100 and Sepharose 6B were from Pharmacia, Dorval, Que., Canada. Triton X-100 (scintillation grade) was from Eastman Kodak Co., Rochester, N.Y., U.S.A. For cell culture, Dulbecco's Modified Eagle Medium (with 1000 mg glucose/liter and 110 mg/liter sodium pyruvate), fetal calf serum, 0.25% trypsin in $Ca^{++}$, $Mg^{++}$-free Hank's solution, and L-glutamine (200 mM) were perchased from GIBCO, Calgary, Alberta, Canada. All other reagents and chemicals were reagent grade from either Fisher Scientific Co., Fairlawn, N.J., U.S.A. or J.T. Baker Chemical Co., Phillipsburg, N.J., U.S.A. Porcine and bovine tissues were from Burns Meat Ltd., Winnipeg, Manitoba, Canada.

(B) Methods

(1) Protein Measurement

The protein concentration of membrane preparations was estimated by the method of Lowry et al (123) with slight modifications. Membrane preparations were diluted 1:2, 1:5, 1:10 and 1:20 with distilled water. Samples of 100 $\mu$l of each sample were pipetted into 15 X 150 mm pyrex test tubes followed by the addition of 300 $\mu$l of 1N NaOH. The samples were then boiled for 30 minutes to solubilize the membrane-bound proteins. A stock solution made up of 100 ml of 2% $Na_2CO_3$, 1 ml of 1% $CuSO_4$ and 1 ml of 2% Na/K tartrate was freshly prepared; 4 ml of this solution was then added to the reaction tubes containing the protein solution. After 10 minutes, 0.5 ml of 1N phenol solution was added to each tube followed by immediate mixing on a vortex. After 30 minutes, absorbance of the samples was read at 660 nm on the spectrophotometer. BSA standards prepared at concentrations of 2,5,10,15 and 20 mg/100 ml distilled water underwent the same treatment as the membrane preparations.

(2) Iodination

Bovine LH, hLH and hCG were prepared by the chloramine-T method of Greenwood, Hunter and Glover (124). All reactions were at room temperature. $Na^{125}I$ (0.5 mCi) in 25 $\mu$l of 0.5 M sodium phosphate buffer, pH 7.0, was pipetted into a 12 X 75 mm disposable glass culture tube containing 5 $\mu$g hormone in 25 $\mu$l of 0.1 M $NH_4HCO_3$ buffer. Chloramine-T

(50 μg) in 25 μl of 0.05 M sodium phosphate buffer, pH 7.5, as the oxidizing agent, was pipetted into the culture tube and allowed to react for 30 seconds. This was followed by the addition of 125 μg of sodium metabisulfate in 50 μl of 0.05 M sodium phosphate buffer, pH 7.5, in order to convert $^{125}$iodine into $^{125}$iodide. Potassium iodide (750 μg) in 75 μl of 0.05 M sodium phosphate buffer, pH 7.5, was then added to the reaction mixture to dilute the residual iodide. The reaction mixture was further diluted by the addition of 2.3 ml PBS, pH 7.4. The solutions of chloramine-T, sodium-metabisulfate and potassium iodide were freshly prepared and dissolved immediately before use.

Bovine and human FSH were iodinated by the lactoperoxidase method (125) with slight modifications according to the method of Cheng (126). All reactions were at room temperature. $Na^{125}I$ (1 mCi) in 25 μl of 0.5 M sodium phosphate buffer, pH 7.0, was added to a 12 X 75 mm disposable glass culture tube containing 5 μg bFSH or hFSH in 25 μl of 0.1M $NH_4HCO_3$ buffer and 25 μl of 0.05M sodium phosphate buffer, pH 7.0. Lactoperoxidase (5 μg), the oxidizing agent, in 5 μl of 0.05M sodium phosphate buffer, pH 7.0, was then added to the reaction tube. The lactoperoxidase was activated by two 10 μl additions of hydrogen peroxide (30% $H_2O_2$ diluted 1:15,000 with water)at 5 minute intervals. At the end of the second 5 minute period, 2.4 ml of 0.025 M Tris-HCl buffer, pH 7.4, was added to the reaction tube to stop the reaction, yielding a final volume of 2.5 ml.

Unreacted iodide and damaged hormones were separated from the iodinated hormone by gel filtration on a 2 X 45 cm column of Sephadex G-100 using PBS, pH 7.4, as the eluting buffer for the choramine-T method and 0.025 M Tris-HCl, pH 7.2 for the lactoperoxidase method. The column was pretreated with 2 ml of 2.5% BSA solution in the appropriate buffer in order to minimize the loss of iodinated proteins adhering to Sephadex G-100.

Fractions of 3-4 ml were collected in 15 X 50 mm glass pyrex test tubes. Radioactivity was monitored with a Chicago Nuclei geiger counter. Two peaks were usually obtained, corresponding to $^{125}$I-labeled hormone and free radioactive iodide. The peak of $^{125}$I-labeled hormone was used as tracer for radio-receptor assay.

For determination of the specific activity of the labeled hormone, to 10 $\mu$l of the reaction mixture before gel filtration, in duplicates or triplicates, acetone was added to a final concentration of 90%. The protein precipitate was washed 4-5 times with several volumes of acetone, and then counted in an autogamma spectrometer. By knowing the amount of hormone present in the 10 $\mu$l aliquots, the cpm in the 90% acetone precipitate and the cpm of 1 $\mu$Ci of Na$^{125}$I used for iodination, specific activity of the $^{125}$I-labeled hormone was estimated and expressed in $\mu$Ci per $\mu$g hormone.

(3) Preparation of Pocine Testicular Membranes

The procedure for preparation of the testicular membrane fraction was identical to the published method for bovine testes (126). Decapsulated testis was rinsed with cold 0.025M Tris-HCl buffer at pH 7.2, containing 0.3M sucrose, and then minced and homogenized with a Polytron homogenizer (Brinkman) type PT-10 at maximum speed for 30 sec at a concentration of 5 ml of buffer per g of tissue. The homogenate was first filtered through four layers of cheesecloth, and the filtrate was again filtered through eight layers of cheesecloth. The filtrate was then centrifuged at 12,000 X g (Beckman, J-21B refrigerated centrifuge, JA-14 rotor) for 30 min at $4^{o}$C. The pellet was discarded and the supernatant was further centrifuged at 100,000g (Beckman, L5-65 Ultra-centrifuge, 60 Ti rotor) for 1 hr at $4^{o}$C. The supernatant was discarded and the pellet was resuspended in cold 0.025 M Tris-HCl buffer at pH 7.2, containing 10 mM $MgCl_2$, at a concentration of 1 ml buffer per g of the original weight of the testis. The protein concentration at this stage was approximately 5-10 mg/ml. These isolated membranes which were defined as PTR (pig testicular receptor), were stored at $-70^{o}$C in aliquots of 5 ml per vial until use. The binding activity of the plasma membranes for FSH and LH was found to be stable for at least 4 months.

(4) Harvest of Granulosa Cells from Ovarian Follicles

Granulosa cells were harvested from bovine and porcine ovaries according to the method of Channing and Ledwitz-Rigby (127). Ovaries from 4 to 6 month-old pigs or cows at different ages were obtained from a local slaughter-house within 20 min of death. The ovaries were rinsed once with sterile Hank's buffer and kept in the same buffer on ice. Within 2-3 hr, the ovaries were rinsed with 80% alcohol followed by sterile Hank's buffer under aseptic conditions.Bovine granulosa cells and follicular fluid of the small follicles (diameter $<7$ mm) were aspirated with a 5 ml syringe and 20 gauge 1-inch needle into a 50 ml centrifuge tube. For medium (7-15 mm) or large ($>15$ mm) follicles, the follicular fluid and some loose granulosa cells were similarly transferred into separate 50-ml centrifuge tubes. Since granulosa cells residing in medium and large follicles adhered to the follicular wall, they had to be scraped out gently from the follicular wall. After aspiration, the "deflated" medium and large follicles were then slit in a V-formation with fine scissors and using a Pasteur pipette, the adhering granulosa cells within were removed by rinsing gently of the inner wall with a "complete medium". The "complete medium" was consisted of a Dulbecco's modified Eagle medium (with L-glutamine, glucose at 1000 mg/L and sodium pyruvate at 110 mg/L ) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 10 mg/L gentamicin. The apex of the V-incision in the follicle was held up with fine forceps while the inside of the follicle was rinsed 2-3 times with 1-2 ml

of "complete medium". The rinsings were pooled in a 50-ml graduated centrifuge tube. The cells in "complete medium" and in follicular fluid were centrifuged at 600 g for 5 min and the packed pellets of cells were pooled and resuspended in "complete medium" again. The concentration of cells was adjusted to 0.25-1.0 X $10^6$ cells/ml using a Coulter counter.

Porcine granulosa cells from follicles of different sizes (2-10 mm) were pooled together using the syringe-needle method for harvesting cells from small bovine follicles. The concentration of cells was also adjusted to a concentration of 0.25-1.0 X $10^6$ cells/ml in the "complete medium".

(5) Cell Plating

Two-ml suspensions of bovine or porcine granulosa cells containing 0.25-1.0 X $10^6$ cells/ml "complete medium" were plated in 35 X 10 mm Corning polystyrene culture-dishes and incubated at 37°C under humidified atmosphere of 5% $CO_2$ and 95% air. Granulosa cells became attached to the dishes within 1-2 days. The culture medium was replenished every two days. Cells grew to confluence in approximately 5 days.

(6) Specific Binding of $^{125}$I-bFSH to Cultured Cells

To assay the specific binding of $^{125}$I-bFSH to cultured granulosa cells, the medium of the culturing cells was aspirated and replenished with 1 ml of Dulbecco's modified Eagle medium containing 0.1% BSA. Unlabeled hFSH (0.1 ml, 50,000 ng/ml) dissolved in the same Eagle's medium with 0.1% BSA

was pipetted into each dish. The hormone solution was sterilized by passing through a filter with 0.45$\mu$ (diameter) pores. Finally, 0.1 ml of $^{125}$I-bFSH (approximately 1 X $10^6$cpm) was added to each dish, followed by incubation at 37°C for 6 hr under 5% $CO_2$ and 95% air. At the end of the incubation period, the incubation medium containing unbound $^{125}$I-bFSH was aspirated and the attached cells in the dish were rinsed twice with 2 ml of Hank's buffer, pH 7.4, containing 0.1% BSA. Total $^{125}$I-bFSH bound to the cells was estimated by solubilizing the cells with 1 ml of 3% SDS; and the solubilized solution of cells was then transferred to 12 X 75 mm glass culture tubes, and counted in an automatic gamma counter.

(7) Cell Counting

To estimate the number of cells in each 35 X 10 mm polystyrene culture-dish, the cells were first detached by incubating with 1 ml of 0.25% trypsin in $Ca^{++}$, $Mg^{++}$-free Hank's buffer, pH 7.4, at 37°C. After 1 hr incubation, 1 ml of "complete medium" (containing 10% fetal falf serum) was used to stop the trypsin digestion. The detached cells now appeared in clumps. They were further disrupted mechanically into single cells by being pipetted up and down a 5-ml disposable pipette. Then 0.5 ml (¼ of the total of 2 ml) of this cell suspension was transferred to a 20 ml Fisher Dilu-Vial and diluted to 10 ml with isotonic saline. After further pipetting the cells up and down with a 10 ml-pipette for 5 times, the number of cells in each vial was counted in an

automatic Coulter counter (Coulter Electronics, Inc. Hialeah, Florida, U.S.A.) By multiplying with a conversion factor of 4 due to dilution, the total number of cells in the original culture-dish was obtained.

(8) Effect of Organic Solvents on Testicular FSH Receptor

(a) One-phase system - To examine the effect of various organic solvents on the FSH receptor, an "one-phase system" was used to partially delipidate the testicular membranes. In a typical experiment, 1 ml aliquots of undiluted PTR in 15 X 85 mm test-tubes were lyophilized and extracted with 5 ml of different organic solvents such as benzene, pentane, and ether for 30 minutes at $4^{o}C$. The partially delipidated membranes were then spun down at 1,000 g for 15 minutes and were further extracted with another 5 ml of the same solvent. The delipidated residues of testicular receptors were then dried under vacuum overnight. To test the binding activity of the delipidated membranes for $^{125}I$-bFSH, the dried pellet was resuspended by sonication in 1 ml of 25 mM Tris-HCl, pH 7.2, buffer containing 10 mM $MgCl_2$ and 0.1 ml was assayed for $^{125}I$-bFSH binding activity using the $(NH_4)_2SO_4$-precipitation method (Section III-B-10).

(b) Two-phase system - The receptor activity of the testicular membranes (PTR) was treated also with a two phase system consisting of organic solvent and water.

The particulate preparation of PTR (5 ml) was diluted three-fold (15 ml) with ice-cold water. After adding 3 ml of organic solvents such as toluene, benzene, ether, or pentane to 3 ml aliquots of the 3 X diluted PTR, the mixture was vortexed vigorously for 30 seconds and kept in ice. After 15 min, the aqueous and organic phases were separated by centrifugation at 1,500 g for 15 min. The lipid phase was separated from the lower aqueous phase by a zone of denatured proteins. The organic solvents and denatured proteins were then aspirated and 0.1 ml of the aqueous phase was assayed for receptor activity by the $(NH_4)_2SO_4$-precipitation method (Section III-B-10).

For the two-phase extraction with n-butanol/water of integral proteins from testicular membranes, the method of Maddy and Kelly (128) was used. At 4°C, 10 ml of PTR was diluted to 30 ml with distilled and deionized water in a 2.87 X 10.3 cm (Beckman JA-20) centrifuge tube. After centrifugation at 22,000 g for 15 min., the resulting pellet was washed twice with 30 ml of distilled and deionized water to lower the ionic concentrations. The washed membranes were taken up in 19 ml of ice-cold water and 7.5 ml of n-butanol was added. The mixture was shaken briefly on a vortex. After standing for 20 min on ice, the mixture was separated into two phases by centrifugation at 22,000 g for 5 min. The lower (aqueous) phase was

removed with a hypodermic syringe and assayed for receptor-activity directly or dialyzed overnight against 8 liters of water at 4°C before assay.

(9) Solubilization of Testicular Membranes using Triton X-100

(a) Free FSH Receptor - For solubilization of free FSH receptor, 5 ml of PTR was centrifuged at 1,000 g for 15 min and 0.5 ml of 1% Triton X-100 in 25 mM Tris-HCl buffer, pH 7.2 was added to the pellet. After mixing in a vortex and stirring for 1 hr at 4°C, 4.5 ml of 25 mM Tris-HCl, pH 7.2, buffer was added to dilute the Triton X-100 to 0.1%. The suspension was centrifuged at 100,000 g for 1 hr, and the resulting supernatant was defined as the solubilized FSH receptor preparation. Solubilized receptor activity was assayed by the polyethylene glycol or $(NH_4)_2SO_4$ method (Section III-B-10).

(b) Prelabeled FSH-Receptor Complex - For extraction of prelabeled hormone-receptor complex, 5 ml of PTR was diluted to 20 ml with 25 mM Tris-HCl, pH 7.2, buffer containing 10 mM $MgCl_2$, and pre-incubated with 3 X $10^6$ cpm of $^{125}$I-bFSH in a 2.87 X 10.3 cm (Beckman JA-20) centrifuge tube at room temperature for 16 hr. The suspension was centrifuged at 11,000 g for 15 min and the pellet was washed three times with 20 ml of 25 mM Tris-HCl buffer containing 10 mM $MgCl_2$, 0.1% BSA to get rid of the unbound $^{125}$I-bFSH. The final pellet containing $^{125}$I-bFSH-receptor complex was solubilized as in the

previous section for solubilization of free FSH receptor.

(10) Assays for Particulate and Solubilized Receptor

For assays of both particulate and solubilized FSH receptors, 12/75 mm flint glass disposable culture tubes (Kimble Co.) were used. To each tube, 0.5 ml of 0.025 M Tris-HCl buffer at pH 7.2, containing 10 mM $MgCl_2$ and 0.1% BSA, 0.1 ml of the same buffer or 10,000 ng/ml unlabeled hFSH, 0.1 ml of $^{125}$I-bFSH tracer (50,000 cpm, approximately 1 ng) and 0.1 ml of particulate or solubilized testicular receptors of appropriate dilution (approximately 1-2 mg protein/ml) were added to make a final volume of 0.8 ml per tube. The mixture was then incubated at room temperature (25°C) for 16 hr. Following incubation, the binding of $^{125}$I-bFSH to particulate membranes was stopped by adding 2.2 ml of cold 25 mM Tris-HCl buffer containing 0.1% BSA whereas with solubilized receptors the reaction was stopped by adding 0.2 ml of a bovine-γ-globulin solution (5 mg/ml PBS), followed by 2 ml of 15% polyethylene glycol or 2.4 M ammonium sulfate in 25 mM Tris-HCl, pH 7.2. After vortexing, the tubes were centrifuged at 1,500 g for 30 min, the supernatant was aspirated and the precipitate was counted in a gamma-spectrometer.

The percentage of specific binding was calculated as the cpm displaced specifically by 10,000 ng/ml cold hormone divided by the total cpm in the reaction tube:

$$\% \text{ Specific Binding} = \frac{(\text{Bound CPM} - \text{Non-displacable CPM}) \times 100}{\text{Total CPM}}$$

(11) Column Chromatography

One ml of $^{125}$I-bFSH-receptor complex solubilized by Triton X-100 was chromatographed at 4°C on a 1.5 X 100 cm column of Sepharose 6B equilibrated in 25 mM Tris-HCl, pH 7.2, containing 10 mM $MgCl_2$, 0.1% BSA and 0.1% Triton X-100. Fractions of 1.0 ml (50 drops) were collected in 12 X 75 mm disposable glass culture tubes on a Buchler Instruments Fractomette 200 automatic fraction collector. The flow rate was 20ml/hr.

The void volume of the column was determined by chromatographing 1 ml of Blue Dextran (10 mg/ml) which was monitored at a wavelength of 660 nm in a spectrophotometer. The total elution volume of the column was determined by chromatographing free Na$^{125}$I.

(12) Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to the method of Davis (129). Gels of 6.0% (W/V) acrylamide with or without 0.1% Triton X-100 were used at pH 8.9. The electrode buffer was 0.012 M sodium glycinate, pH 9.2, containing 0.1% Triton X-100. To 50 µl of $^{125}$I-bFSH-receptor complex solubilized by Triton X-100, 1 drop of glycerol and 10 µl of 0.005% bromophenol blue were added. A current of 2 mAmp/gel was applied until the ion-front in the gel traveled a distance of 11 cm from the origin. The gels were then segmented in 5 mm slices and counted in an automatic gamma spectrometer to locate the $^{125}$I-bFSH-receptor peak.

(13) Interaction of Anti-bFSH Antiserum with bFSH-Receptor Complex Solubilized by Triton X-100

To examine the binding effect of specific FSH antibodies with the $^{125}$I-bFSH-receptor complex solubilized by Triton X-100, 100 µl of an anti-bFSH antiserum (diluted appropriately in PBS, pH 7.4, containing 0.1 % BSA) was added in duplicates to 100 µl (approximately 20,000 CPM) of $^{125}$I-bFSH or $^{125}$I-bFSH-receptor complex. The mixture was incubated for 24 hours at 25°C. Separation of antibody-bound and free radioactivity was achieved by adding 0.6 ml of PBS, 0.2 ml of bovine-γ-globulin in PBS (5 mg/ml) and 1 ml of 16 % polyethylene glycol in 25 mM Tris-HCl, pH 7.2, to each tube, followed immediately by vortexing. The tubes were centrigfuged at 1500 g for 30 min and the supernatant was aspirated. The radioactivity in the precipitate was counted in an automatic gamma counter.

# IV RESULTS

## (A) Characterization of the Binding of bFSH to Porcine Testicular Membranes

A crude preparation of plasma membrane (PTR) specific for binding of FSH and LH was obtained from porcine testes (section III-B-3). In a typical assay for FSH binding (section III-B-10) utilizing 50,000 CPM $^{125}$I-labelled bFSH (specific activity = 16-20 $\mu$Ci/$\mu$g) and 200 ug testicular membrane protein, 10-20% of the total $^{125}$I-bFSH added was bound to the 1500 g membrane pellet. Only 5-10% non-specific $^{125}$I-bFSH binding was observed which could not be displaced by 1000-fold excess of hFSH.

By using PTR, the interaction between $^{125}$I-bFSH and its testicular receptor was characterized.

### (1) Kinetics of Association

The association between $^{125}$I-bFSH and PTR was found to be dependent on incubation time. At room temperature (25$^{o}$C), duplicate assay tubes containing 100 $\mu$l of particulate receptor (200 $\mu$g membrane protein) and 100 $\mu$l of $^{125}$I-bFSH (approximately 50,000 CPM) in the presence or absence of 100 $\mu$l hFSH (10,000 ng/ml) were incubated at time intervals ranging from 15 minutes to 24 hours. Maximal specific binding (approximately 10-20%) was observed after 14 hours and remained high up to 24 hours (figure 4). Therefore, for assay purposes, an incubation period of 14-20 hours (overnight) at room temperature was routinely used.

### (2) Kinetics of Dissociation

To study the dissociation of specifically bound $^{125}$I-bFSH from crude testicular membranes, PTR (200 ug of protein) and $^{125}$I-bFSH were incubated under standard assay conditions (25$^{o}$C, overnight) to allow formation of $^{125}$I-bFSH-receptor complex, which was then separated from unbound hormone

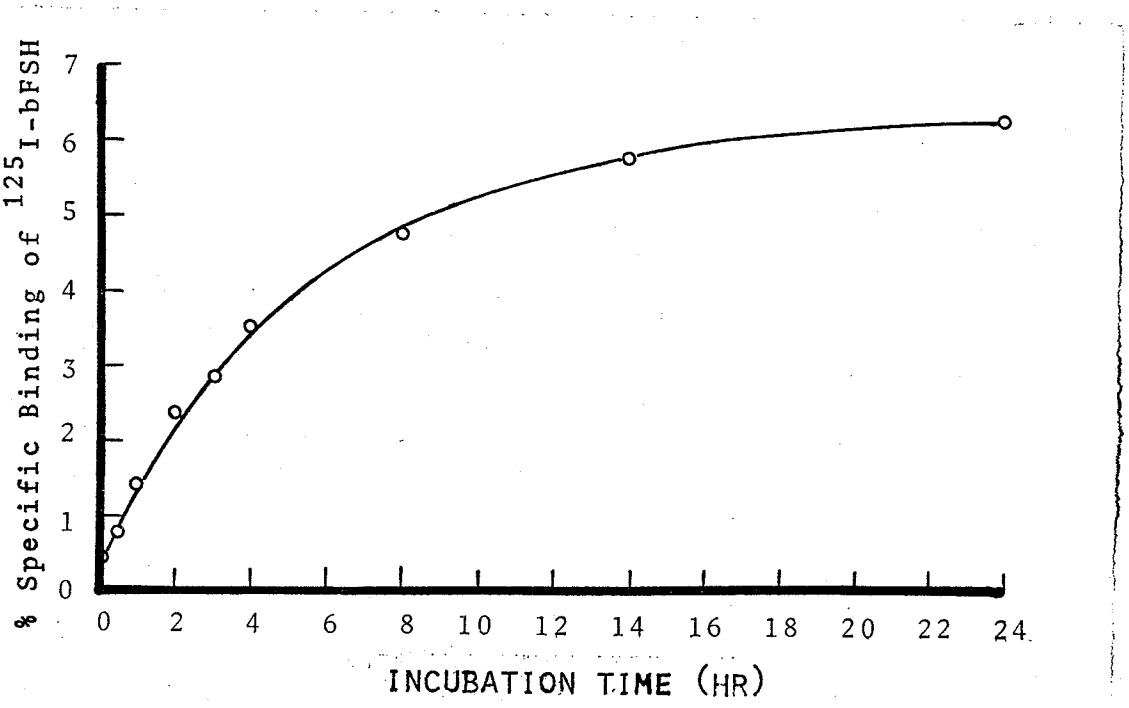



Figure 4: Effect of incubation time on the specific binding of $^{125}$I-bFSH to porcine testicular membranes (200 µg protein/ tube) at 25°C.

by centrifugation at 1500 g for 30 min. After washing the pellet for two times to remove traces of unbound radioactivity, the membrane pellet was resuspended in 1 ml of 25 mM Tris-HCl, pH 7.2, buffer containing 10 mM $MgCl_2$ and 0.1% BSA, with or without excess hFSH (1000 ng/ml). After incubation at 37°C for various periods of time up to 24 hr, the mixture was centrifuged and the remaining radioactivity in the pellet was measured.

The dissociation of specifically bound $^{125}$I-bFSH from testicular membranes was found to be time-dependent and approximately 60% of the total bound radioactivity was dissociated after 14 hr at 37°C (figure 5). Approximately 40% of the $^{125}$I-bFSH tracer appeared to be irreversibly bound to the membranes and could not be dissociated by incubating at 37°C up to 24 hr. It is noted that the presence of excess unlabelled hFSH (1000 ng/ml) did not increase the rate of dissociation of the membrane-bound radioactivity (figure 5). This situation is similar to that of the growth hormone-receptor system (27), but different to that of the insulin-receptor system (27). In the latter, the presence of excess insulin stimulated release of specifically bound insulin, and this was interpreted as reflecting site-site interaction and negative cooperativity. By analogy, therefore our results suggest that site-site interaction and negative cooperativity do not occur in the FSH-testicular membrane receptor system.

(3) Effect of pH

(a) Association of $^{125}$I-bFSH with receptor

To determine the effect of pH on the binding of $^{125}$I-bFSH to porcine testicular membranes, PTR (250 μg/tube) was added to 0.8 ml of buffer of various pH, 0.1% BSA and 50,000 CPM of $^{125}$I-bFSH. Binding was determined after incubation for 14 hr at 25°C. The buffers used were: 50 mM glycine-HCl (pH 3.0), 50 mM sodium acetate (pH 4.0, pH 5.0), 50 mM $NaH_2PO_4$-HCl (pH 6.0),

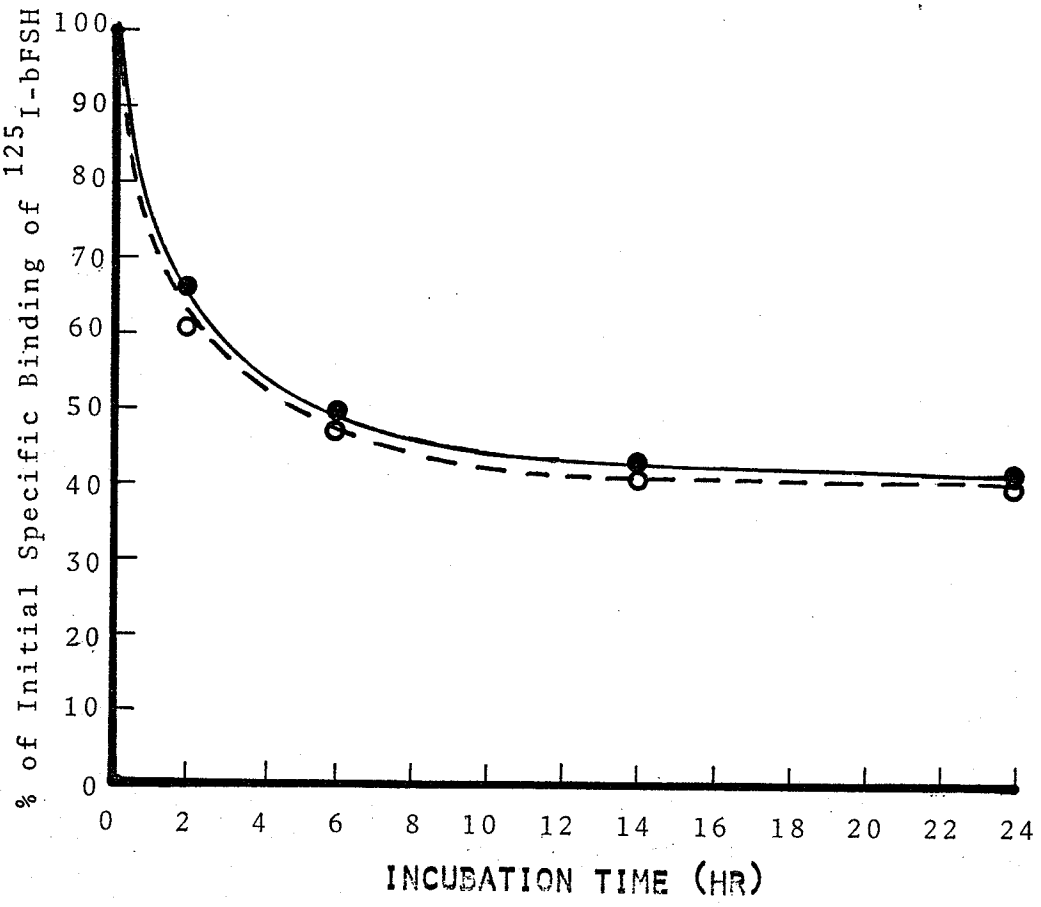



Figure 5: Effect of incubation time on the dissociation of specifically bound $^{125}$I-bFSH from porcine testicular membranes at 37$^{o}$C in the presence (O--O) or absence(●—●) of excess hFSH(1000 ng/ml).

50 mM Tris-HCl (pH 7.0, 8.0), 50 mM glycine-NaOH (9.0, 10.0) and 25 mM $Na_2HPO_4$-NaOH (pH 11.0). Significant binding of $^{125}$I-bFSH to receptor occurred from pH 5.0 to pH 9.0 (figure 6). Maximum binding occurred at pH 7.0 while 50% reduction in binding was observed at pH 6.0 and 33% at pH 8.0. No significant binding was observed at pH below 4.0 or above 10.0.

(b) Stability of receptor

The effect of pH on FSH receptor stability was tested by pretreating 250 µg PTR in 3 ml of buffers of different pH, ranging from 3.0 to 11.0. After incubation at 25°C for 1 hr, the pellet was obtained by centrifuging at 1500 g for 15 min, and the treated membrane was washed once with 3 ml of 25 mM Tris-HCl, pH 7.2, buffer containing 10 mM $MgCl_2$ and 0.1% BSA, followed by resuspending in the same Tris-HCl for assaying FSH binding activity. Figure 6 shows that the loss of FSH binding of pretreating receptor for 1 hr at various pH was similar to that of incubating the membrane at buffers of various pH. Therefore, the loss of FSH binding of the testicular membrane due to exposure of high or low pH was seemingly irreversible.

(c) Dissociation of $^{125}$I-bFSH-receptor complex

The effect of pH on the dissociation of specifically bound $^{125}$I-bFSH from preformed $^{125}$I-bFSH-receptor complex was also examined. Membrane pellets with bound $^{125}$I-bFSH after two washings was resuspended in 3 ml of buffers at different pH (3-11), followed by incubating at 25°C for 30 min. The tubes were then centrifuged at 1500 g for 30 min and the remaining radioactivity bound to the membrane pellets was counted in an autogamma counter. Figure 6 shows that the $^{125}$I-bFSH-receptor complex remained stable at a relatively wide range of pH, with greater than 80% dissociation only at extremes of pH (less than pH 3.0 or greater than pH 11.0).

(4) Effect of Salts

(a) Association of $^{125}$I-bFSH with receptor

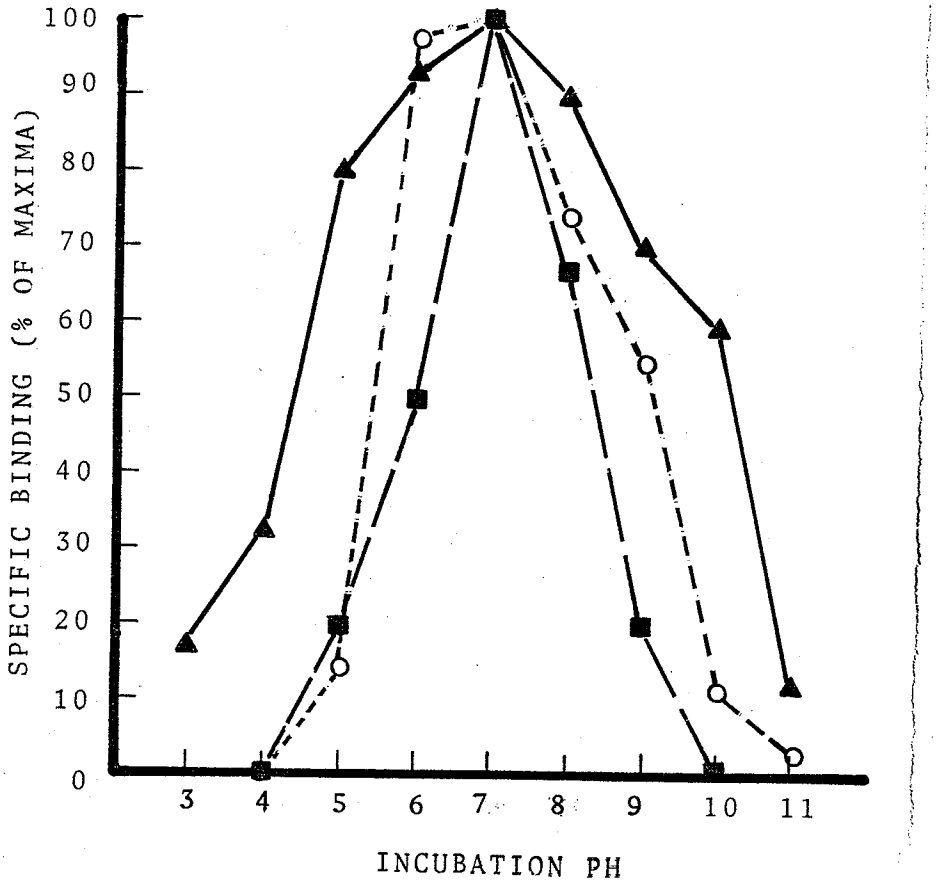



Figure 6: Effect of pH on the binding of $^{125}$I-bFSH to the testicular receptor(■—■),dissociation of $^{125}$I-bFSH from preformed $^{125}$I-bFSH-receptor complex(▲—▲), and stability of receptor(O---O).

The ionic requirement for optimal $^{125}$I-bFSH-receptor interaction was tested by monitoring the final concentration of monovalent (NaCl) and divalent ($MgCl_2$, $CaCl_2$) ions in a standard receptor assay. Figure 7 shows that only minimal $^{125}$I-bFSH could be specifically taken up by testicular membranes in the absence of added metallic ions (Incubation buffer was 25 mM Tris-HCl, containing no BSA). $CaCl_2$ or $MgCl_2$ at a final concentration of 10 mM was most effective in facilitating specific binding of $^{125}$I-bFSH to receptor; however, higher concentrations inhibited binding. Therefore 10 mM $MgCl_2$ was routinely used in all assays for FSH receptor.

The monovalent salt, NaCl, was much less effective in elevating specific binding of $^{125}$I-bFSH to receptor (figure 7). Additions of NaCl to the incubation medium in the presence of 10 mM $MgCl_2$, actually inhibited the binding (figure 8). Specific binding of FSH to its receptor was decreased 80% by NaCl at physiological concentration of 0.15 M.

(b) Dissociation of receptor-bound $^{125}$I-bFSH and $^{125}$I-bLH by $MgCl_2$

To study the ability of $MgCl_2$ to dissociate the bound $^{125}$I-bFSH or $^{125}$I-bLH from porcine testicular membranes, membrane pellets of either $^{125}$I-bFSH receptor or $^{125}$I-bLH-receptor complex obtained by standard incubation conditions was resuspended, after 2X washings, in 0.5 ml of 0M (Control), 1M, and 2M $MgCl_2$ solutions in 25 mM Tris-HCl buffer, pH 7.2. After an incubation period of 30 min at 25$^{o}$C, the $MgCl_2$ solution was diluted by adding 3.0 ml of 25 mM Tris-HCl, pH 7.2, buffer followed by centrifugation at 1500 g for 30 min to obtain the membrane pellet. Figure 9 shows that the total bound $^{125}$I-bFSH or $^{125}$I-bLH remaining associated with testicular membranes was decreased approximately 65% by 1M $MgCl_2$ and 80% by 2M $MgCl_2$.

(c) Stability of testicular FSH and LH receptors to $MgCl_2$

The stability and binding activity of testicular receptors for FSH

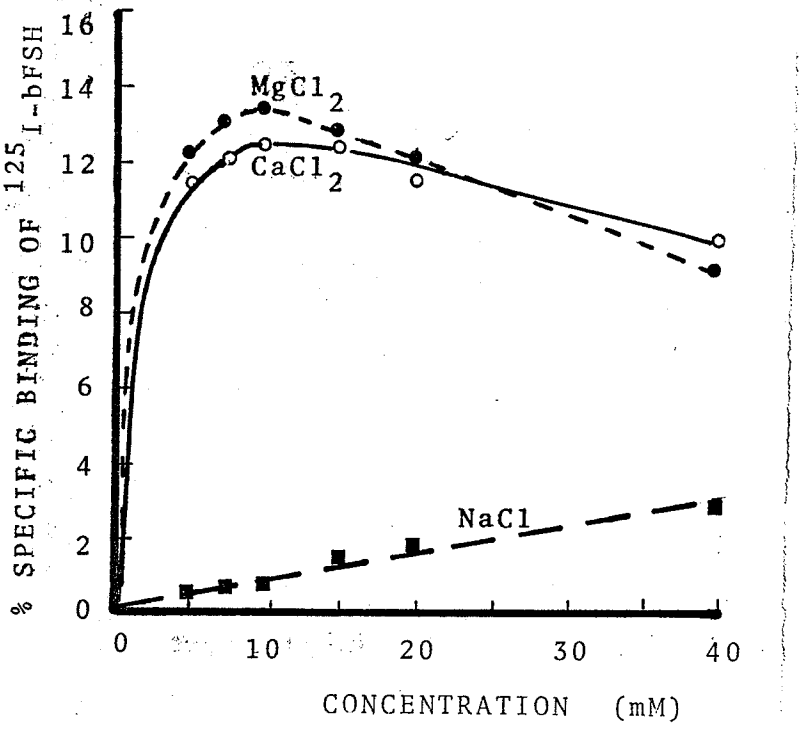



Figure 7: Effect of $MgCl_2$, $CaCl_2$, and NaCl on the specific binding of $^{125}$I-bFSH to testicular membranes. Incubation was carried out in 25 mM Tris-HCl buffer, pH 7.2, at 25°C for 14 hr.

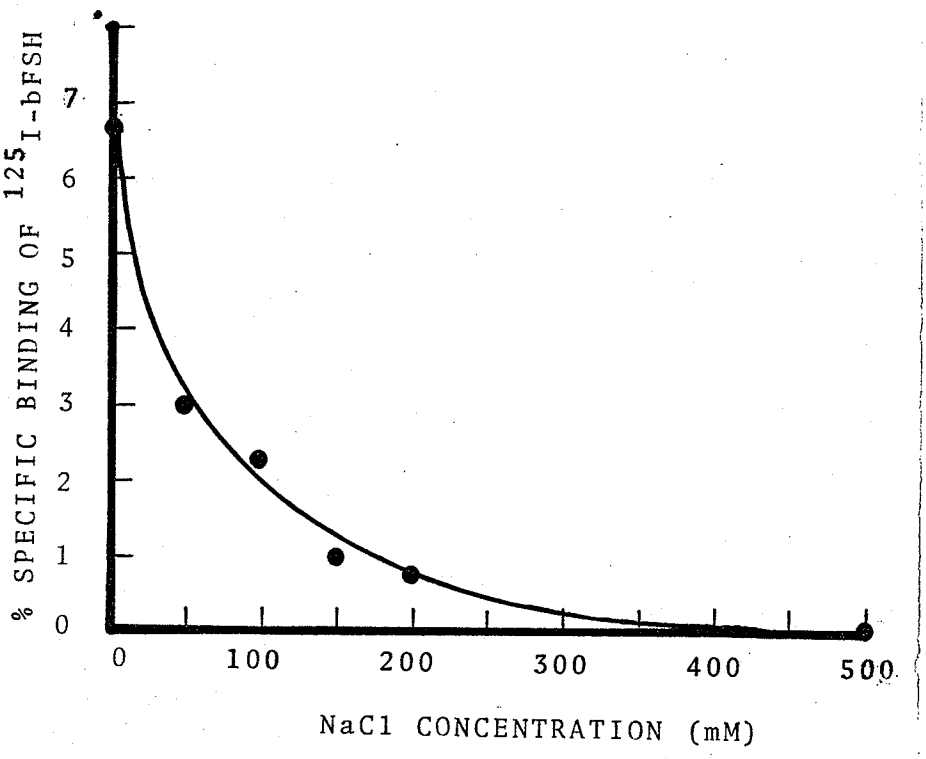



Figure 8: Inhibition of testicular receptor binding for $^{125}$I-bFSH by various concentrations of NaCl. Incubations were carried out in the presence of 10 mM $MgCl_2$ at 25°C for 14 hr.

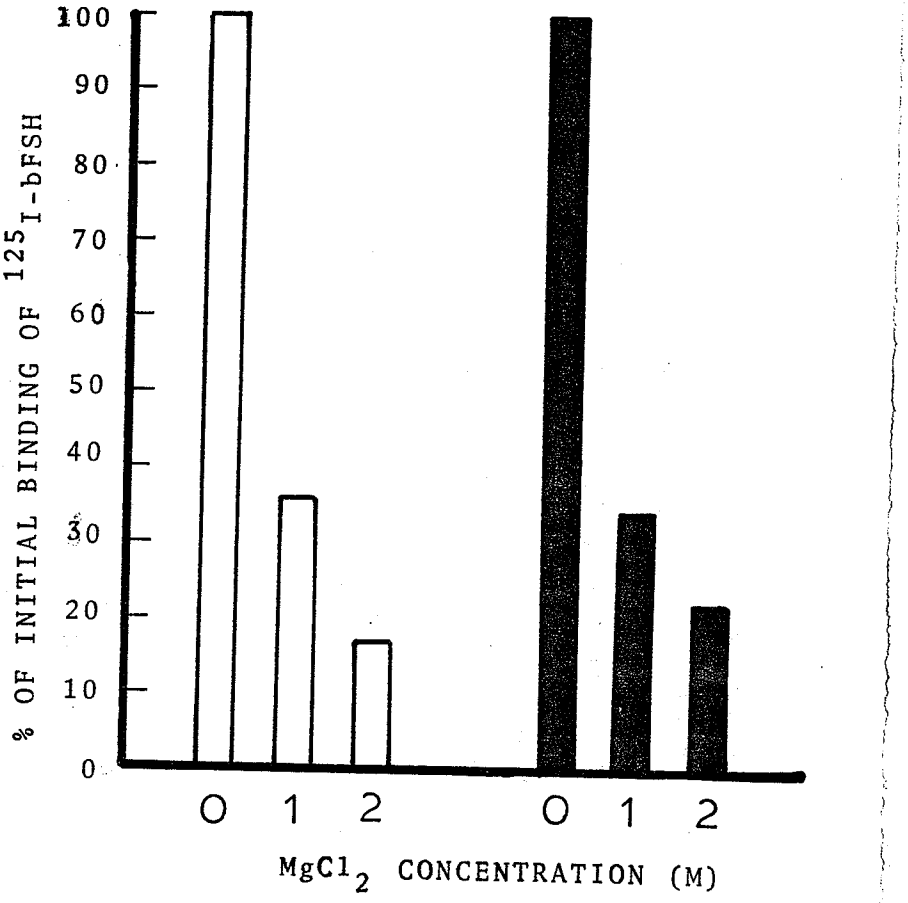



Figure 9: Effect of $MgCl_2$ on the dissociation of preformed $^{125}$I-bFSH-receptor(□) and $^{125}$I-bLH-receptor(■) complexes in porcine testicular membranes.

and LH after treatment with $MgCl_2$ were studied. $MgCl_2$ has been demonstrated to be an efficient reagent to dissociate bound hormones from receptor-hormone complexes (101). To a 2.87 x 10.3 cm centrifuge tube (Beckman JA-20), 5 ml of unlabelled hFSH (10,000 ng/ml) or bLH (5000 ng/ml) were incubated with 5 ml of PTR (10 mg protein/ml) in a final volume of 20 ml adjusted with standard assay buffer. After incubation at 25°C for 14 hr, the hFSH-receptor or bLH-receptor complex was pelleted after centrifugation at 5000 g for 15 min. After washing 2X with another 20 ml of Tris-HCl, pH 7.2 buffer, the pellet was resuspended in 5 ml of 25 mM Tris-HCl, pH 7.2 buffer. To 15 x 85 mm glass culture tubes, 1 ml aliquots each of the hormone receptor complex were treated with 1.0 ml of 0, 1, 2, 3 or 4 M $MgCl_2$ in Tris-HCl buffer for 1 hr at 4°C. At the end of incubation, each tube was centrifuged at 1500 g for 30 min and the pellet was washed with 10 ml Tris-HCl buffer before resuspension in 1 ml standard assay buffer and, 0.1 ml of the resuspension was assayed in triplicate for receptor activity.

For testing the effect of $MgCl_2$ on free-receptor, as control, PTR was incubated without previous addition of hFSH before $MgCl_2$ treatment, but treated identically through all the subsequent steps.

Figure 10 shows that testicular FSH and LH receptors were stable to pre-treatment of $MgCl_2$ up to a concentration of 1.5 M. At 2.0 M of $MgCl_2$, specific uptake of $^{125}I$-labelled bFSH and bLH were slightly decreased, indicating that the receptors might be damaged by 2.0 M $MgCl_2$.

Nonetheless, treatment of the preformed hFSH-receptor and bLH-receptor complexes with $MgCl_2$ increased the specific uptake of the labelled hormones by the treated receptors. Using 2.0 M $MgCl_2$, increases of 67% (Fig. 10a) and 700% (Fig. 10b) were observed for the FSH and LH receptor,

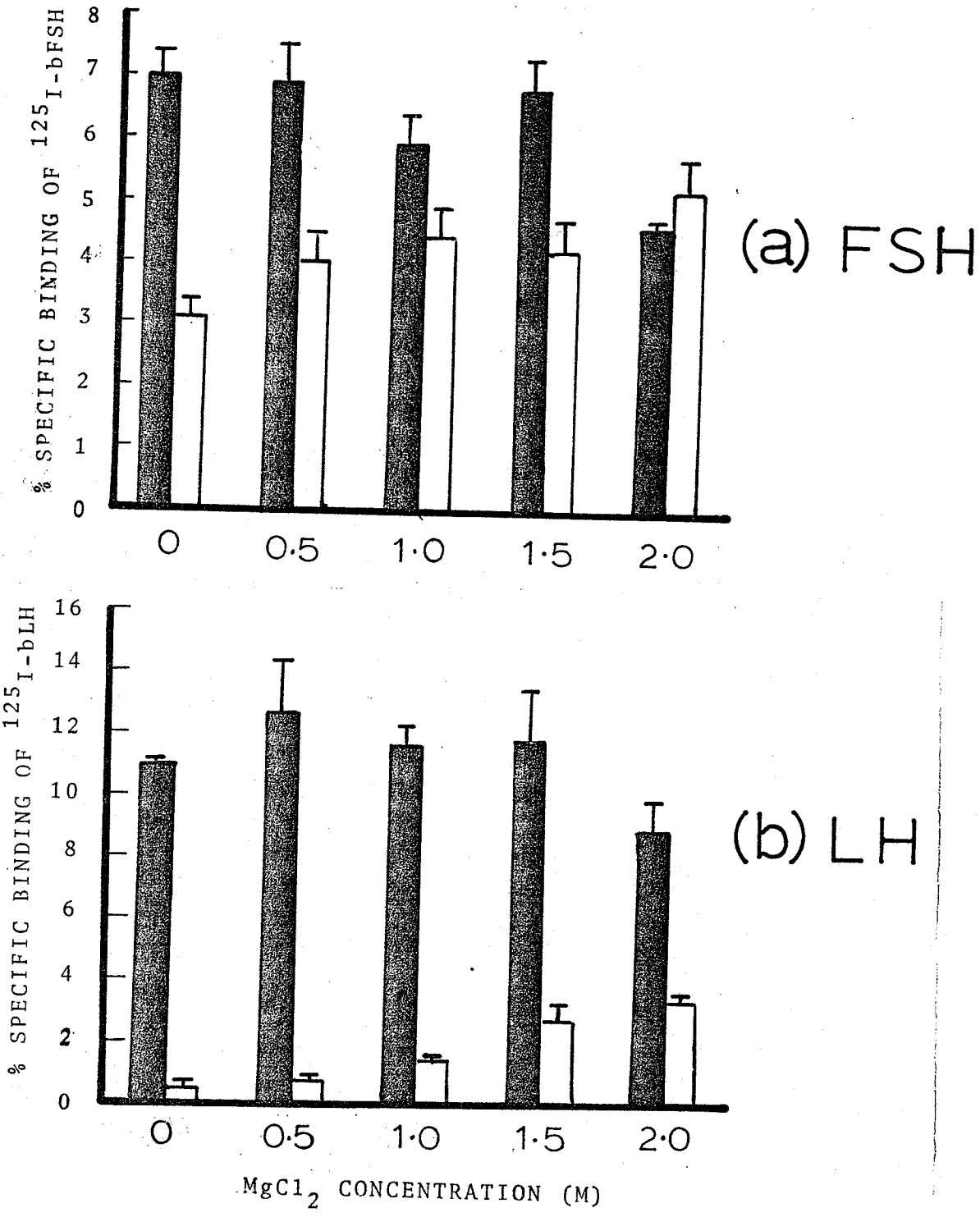



Figure 10: Effect of $MgCl_2$ on the (a) $^{125}$I-bFSH-binding and (b) $^{125}$I-bLH-binding activities of free receptors (solid columns) or receptors occupied by pre-treatment with excess unlabeled hFSH or bLH (open columns). Vertical bars indicate the range of triplicates.

respectively. The observed increase in receptor activity after $MgCl_2$ treatment could be due to either reversible dissociation of preformed hormone-receptor complexes or unmasking of previously unexposed binding sites.

(d) Stability of $^{125}$I-bFSH and $^{125}$I-bLH tracers to 2.5 M $MgCl_2$

The stability of $^{125}$I-bFSH and $^{125}$I-bLH in 2.5 M $MgCl_2$ was examined by treating 0.1 ml of labelled hormone (1.5 x $10^6$CPM) with 0.1 ml of 25 mM Tris-HCl, pH 7.2, with $MgCl_2$ (5M) at 25°C for 30 min. At the end of incubation, 4.8 ml of Tris-HCl buffer was added to lower the concentration of $MgCl_2$ to 100 mM. To another 0.1 ml of either $^{125}$I-bFSH or $^{125}$I-bLH, 4.8 ml of Tris-HCl buffer and 0.1 ml of 5 M $MgCl_2$ were added to bring the concentration of $MgCl_2$ also to 100 mM. Then appropriately diluted tracer was assayed with 250 ug of freshly prepared testicular membrane protein in $Ca^{++}$, $Mg^{++}$-free Tris-HCl, pH 7.2, buffer containing 0.1% BSA. Figure 11 shows that the binding of $^{125}$I-bFSH to the testicular receptors decreased 56% by pre-treatment of 2.5 M $MgCl_2$; whereas $^{125}$I-bLH was relatively stable under the same experimental conditions.

(5) Effect of Trypsin and Collagenase

To test the effect of proteolytic enzymes on testicular FSH and LH receptors, testicular membranes (300 µg protein) were treated with 3 ml of 25 mM Tris-HCl, pH 7.2, containing 0.01% trypsin (204 u/ml), or 0.01% trypsin plus 0.01% lima bean trypsin inhibitor (1 mg inhibits 3.9 mg trypsin). After incubation at 25°C for 30 min, testicular membranes were pelleted at 1500 g for 15 min and washed 2 X with 3 ml of 25 mM Tris-HCl buffer pH 7.2, containing 10 mM $MgCl_2$ and 0.1% BSA for assays of specific binding.

Figure 12 shows that treatment with 0.01% trypsin completely

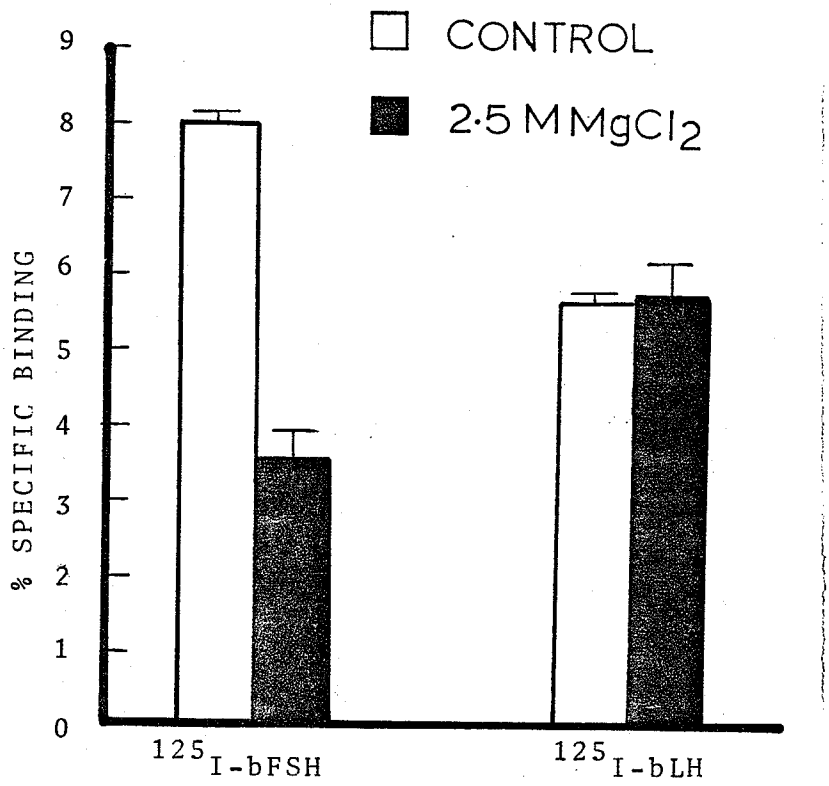



Figure 11: Effect of 2.5 M $MgCl_2$ on the integrity of $^{125}$I-bFSH and $^{125}$I-bLH tracers. The $^{125}$I-labeled hormones (0.1 ml) were treated with either 0.1 ml of 25 mM Tris-HCl buffer, pH 7.2(open columns) or 0.1 ml of 5M $MgCl_2$ in Tris-HCl buffer(solid columns) at 25°C for 30 min before appropriate dilution for assay with particulate porcine testicular membranes. Vertical bars indicate range of triplicates.

abolished the subsequent specific uptake of $^{125}$I-bFSH and $^{125}$I-bLH by PTR; whereas 0.01% collagenase decreased the specific uptake of $^{125}$I-bFSH and $^{125}$I-bLH by 59% and 64%, respectively. Addition of 0.01% lima bean trypsin inhibitor completely inhibited the proteolytic action of these enzymes (Fig. 12). Therefore contamination with trypsin in the collagenase preparations could account for the inhibitory effect of collagenase on FSH and LH receptors.

(6) Ability of $^{125}$I-bFSH to Bind Receptor after Treatment with bFSH Antibody

To 0.1 ml aliquots of $^{125}$I-bFSH (35,000 CPM) in duplicate 12 X 75 mm glass culture-tubes was added 0.1 ml of excess rabbit antiserum against bFSH, bLH, bFSH $\alpha$, bFSH $\beta$, or bTSH, all diluted at 1:1000 with PBS containing 0.1% BSA or just PBS buffer with 0.1% BSA (control). After 14 hr at room temperature, 0.1 ml of PTR (200 ug protein) and 0.5 ml 25 mM Tris-HCl, pH 7.2, buffer containing 10 mM $MgCl_2$ and 0.1% BSA were added to the tubes. For determination of nonspecific binding, 0.1 ml of hFSH (10,000 ng/ml) was included. The tubes were incubated for another 14 hr at room temperature before stopping the reaction by 2.2 ml ice-cold Tris-HCl buffer and centrifugation at 1500 g.

Figure 13 shows that preincubation of $^{125}$I-bFSH with bFSH antibody (Fig. 13) not only completely abolished the specific binding of $^{125}$I-bFSH to receptor, but also enormously increased the nonspecific binding. High nonspecific binding was also observed when testicular membrane-receptors were replaced by similarly prepared porcine thyroid membranes containing the same amount of protein (Fig. 13 c). However, even in the absence of any added membrane preparations (Fig. 13d), 12% of the total $^{125}$I-bFSH were precipitated by bFSH antiserum (1:1000). Therefore the $^{125}$I-bFSH-antibody complex might have formed

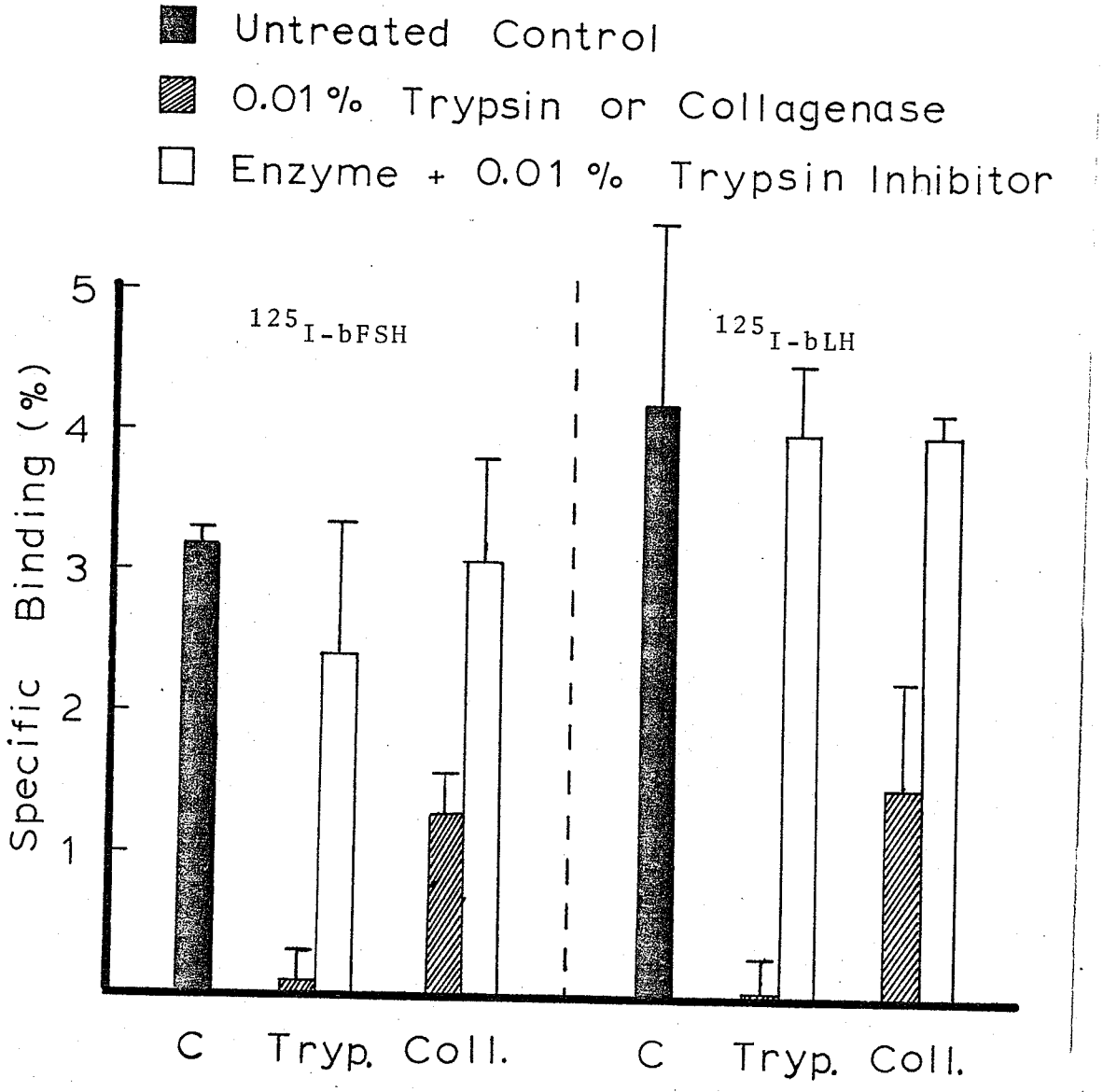



Figure 12: Effect of pre-treatment with trypsin or collagenase on the specific binding of porcine testicular membranes for $^{125}$I-bFSH and $^{125}$I-bLH. Vertical bars indicate the range of duplicate determinations.

heavy aggregates and the presence of membranes only served to "carry down" the aggregates during centrifugation. Pretreatment of $^{125}$I-bFSH with specific antiserum to bLH, bFSH$\alpha$ , bFSH$\beta$ or bTSH before incubation (Fig. 13 f - i) with testicular membranes also increased the nonspecific binding of $^{125}$I-bFSH, but only antisera against bLH (Fig. 13 f) and bFSH (Fig. 13g) significantly decreased $^{125}$I-bFSH specific binding, as compared to the control of pretreatment with PBS (Fig. 13a).

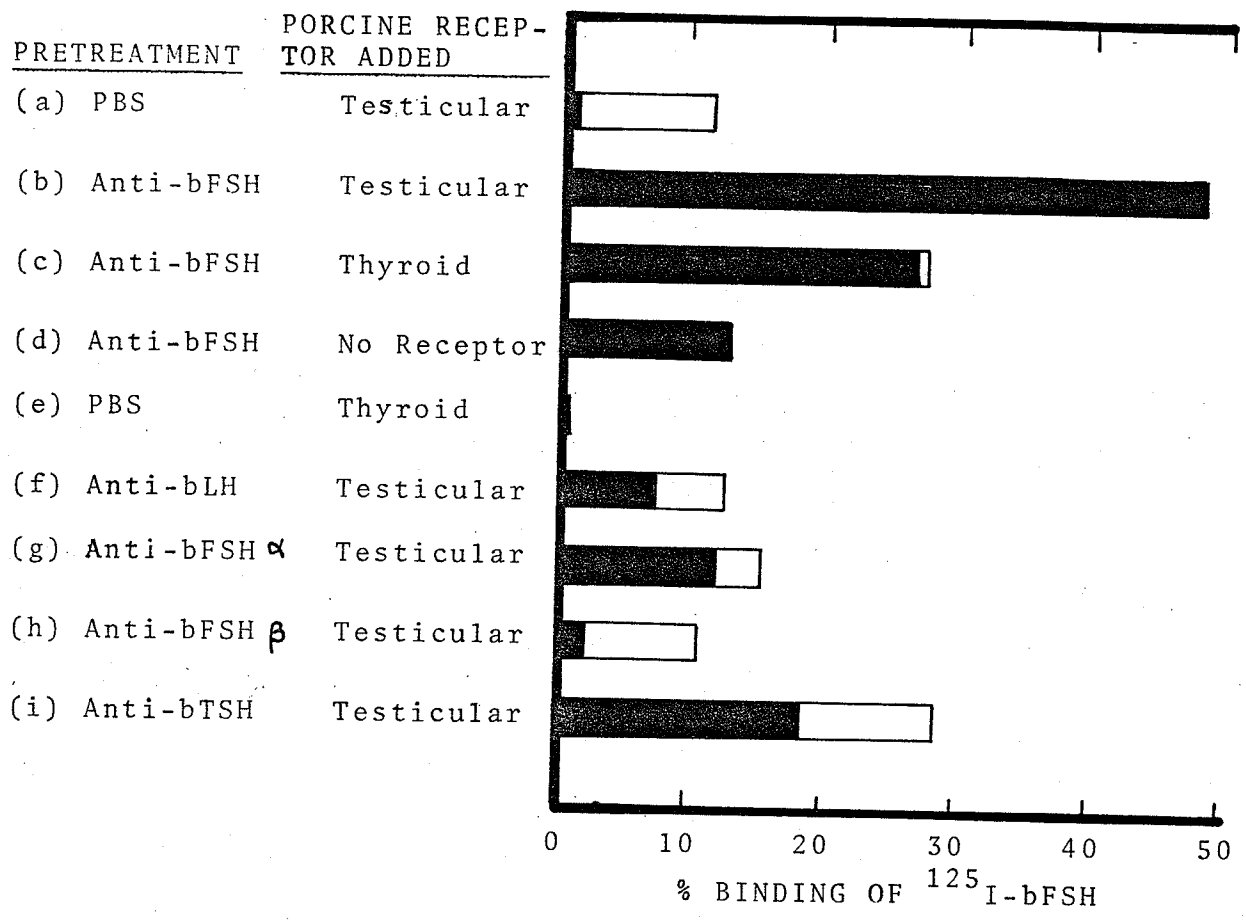



Figure 13: Effect of pretreatment (25°C, 14 hr) of $^{125}$I-bFSH (35,000 CPM) with various rabbit anti-sera(all diluted 1:1000) on its subsequent ability to bind specifically to porcine testicular receptor. The total binding of $^{125}$I-bFSH to the testicular membranes is represented by horizontal bars(mean of triplicates). The solid and open area in each bar represent respectively the non-specific and specific binding.

(B) Characterization of the Binding of $^{125}$I-bFSH to Ovarian Granulosa Cells

The interaction between $^{125}$I-bFSH and ovarian granulosa cells from bovine and porcine follicles were characterized with cells in culture and in suspension. In the cow, normally only one ovum is ovulated during each estrus cycle; therefore it is relatively easy to identify the dominant follicle or corpus and to determine the different stages of the reproductive cycle. However, for collections of large quantities of granulosa cells, it is more convenient to use porcine ovaries which usually contain many Graffian follicles and liberate several ova during ovulation.

(1) Kinetics of Association

(a) Cultured cells

Pig granulosa cells were harvested from ovarian follicles (section III-B-4) and 1.5 X $10^6$ cells were plated in 35 X 10 mm polystyrene culture-dishes (section III-B-5). After incubation at 37$^{o}$C under humidified atmosphere of 5% $CO_2$ and 95% air for 2 days, approximately 30% of the cells were attached to the dish for assays of FSH-receptor activity (section III-B-6). Routinely, 0.1 ml of $^{125}$I-bFSH (1-2 X $10^6$ CPM) and 1.0 ml of Dulbecco's modified Eagle medium containing 0.1% BSA were incubated with 0.5 - 1.0 X $10^6$

cells/dish in the presence of 0.1 ml unlabelled hFSH (500 ug/ml) Eagle-BSA medium.

The total and nonspecific uptake of $^{125}$I-bFSH by the cultured porcine granulosa cells (0.5 x $10^6$ cells/dish) are depicted in Fig. 14. The highest specific binding (6300 CPM) of $^{125}$I-bFSH to the cultured cells was observed after an incubation period of 5 to 7 hr at 37°C. Nonspecific binding amounted to 30% of the total $^{125}$I-bFSH uptake and stayed relatively constant after 1 hr.

(b) Suspended Cells

The uptake of $^{125}$I-bFSH was also studied with bovine granulosa cells in suspension. Bovine granulosa cells were harvested from follicles of different sizes (section III-B-4) and resuspended in Hank's buffer, pH 7.4, containing 25 mM Hepes and 0.1% BSA to obtain a concentration of 0.6 x $10^6$ cells/ml. In 12 x 75 mm glass culture tubes, 0.4 ml of the cell suspension (containing 240,000 cells) was incubated with 50 µl of $^{125}$I-bFSH (0.8 x $10^6$CPM) in the presence or absence of 50 µl hFSH (100 µg/ml). The tubes were incubated at 37°C for various time intervals of 1-6 hr. At the end of incubation the reaction was stopped by adding 2 ml of ice-cold Hank's buffer, pH 7.4, containing 25 mM Hepes and 0.1% BSA. The tubes were centrifuged at 1500 g for 10 min and the pellet was washed once with 2 ml of the same Hank's buffer and counted in an automatic gamma counter. Figure 15 shows that the total and nonspecific uptake of $^{125}$I-bFSH by bovine granulosa cells at 37°C were dependent on time, being maximal between 4-6 hr (Fig. 15).

(2) Kinetics of Dissociation

Porcine granulosa cells (2.0 x $10^6$ cells/dish at 0 time) were cultured for 2 days before assaying for FSH receptor activity. $^{125}$I-bFSH (0.1 ml, 2 x $10^6$CPM) was pre-incubated with the cultured cells for 6 hr with or without hFSH (0.1 ml, 100 µg/ml). Unbound $^{125}$I-bFSH was removed by

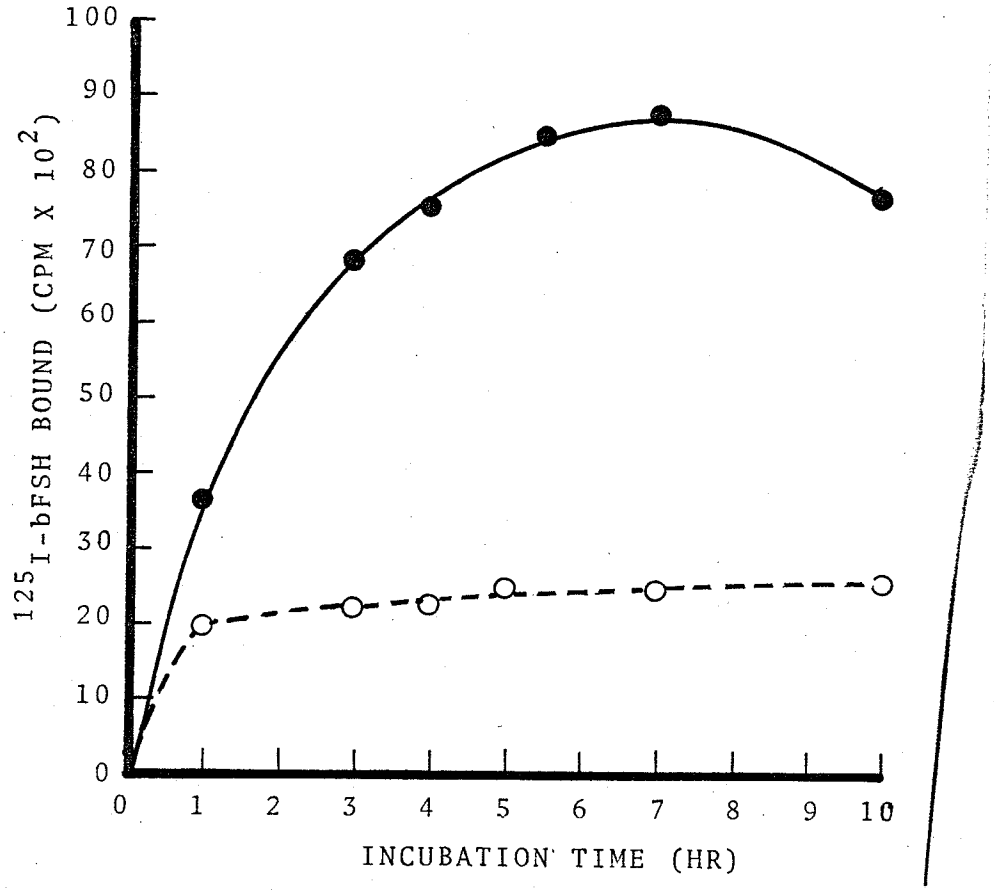



Figure 14: Effect of incubation time on the total(●——●) and nonspecific(○---○) uptake of $^{125}$I-bFSH by cultured porcine granulosa cells(0.5 x $10^6$ cells/dish).

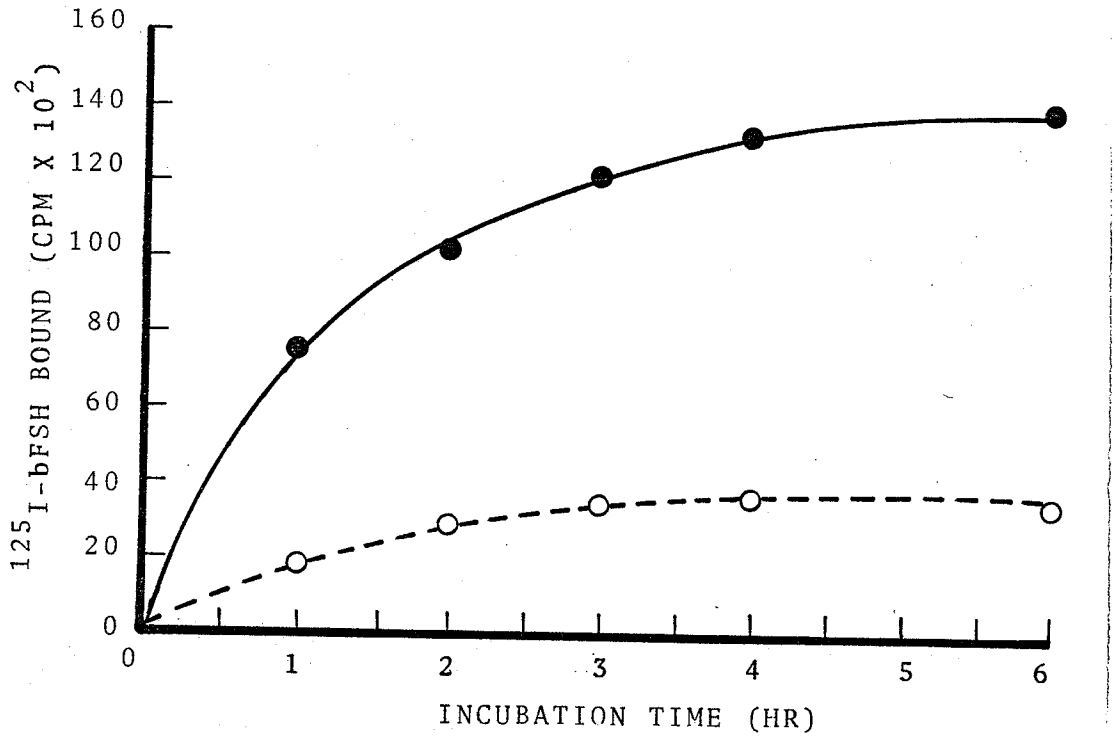



Figure 15: Effect of incubation time on the total (●—●) and nonspecific(○---○) uptake of $^{125}$I-bFSH by bovine granulosa cells in suspension(0.6 x $10^6$ cells/tube).

washing the cells twice with 2 ml Dulbecco's modified Eagle medium containing 0.1% BSA. The washed cells with bound radioactivity were again incubated at 37°C with 2 ml of medium in the presence or absence of 10 μg/ml hFSH. After incubating for various time intervals (1-12 hr), the medium in the culture-dish was aspirated and the cells washed once with medium. The remaining radioactivity bound to the cells was counted in an automatic gamma counter after solubilizing the cells with 1 ml of 3% SDS. Figure 16 shows that 70% of the $^{125}$I-bFSH bound to porcine granulosa cells could be dissociated at 37°C in 6 hr. Incubation up to 12 hr could not further dissociate the already bound $^{125}$I-bFSH. Furthermore the release of specifically bound radioactively labelled hormones could not be accelerated by incubating the prelabelled cells with large excess of unlabelled FSH.

(3) Effect of Culturing on FSH Receptor Level

Bovine granulosa cells were plated in 35 x 10 mm culture-dishes at a density of 0.3 x $10^6$ cells/dish. The culture medium used was Dulbecco's modified Eagle medium (with L-glutamine, glucose at 1000 mg/L and sodium pyruvate at 110 mg/L) suplemented with 10% fetal calf serum, 2 mM L-glutamate and 10 mg/L genatmicin. The medium was changed every two days. The binding activity of the cultured cells for $^{125}$I-bFSH and the number of cells per dish were determined on Days 1, 2, 3 and 6 after plating the cells for culture on Day 0. Fig. 17 shows that the specific binding of $^{125}$I-bFSH to bovine granulosa cells remained relatively constant over a period of 6 days, even though the cell number in each dish had increased approximately 10-fold, indicating that no new FSH receptor was synthesized by these cells in culture. This observation also shows that bovine granulosa cells could be maintained

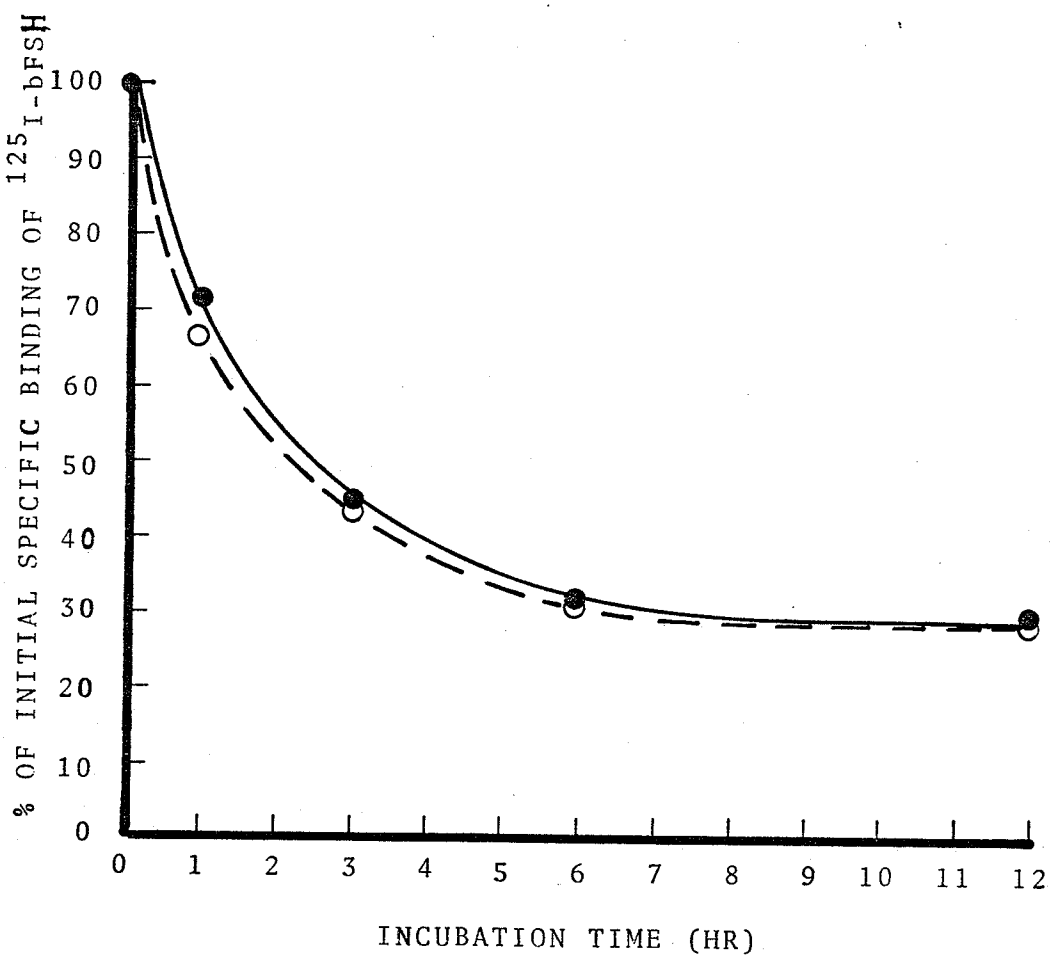



Figure 16: Dissociation of specifically bound $^{125}$I-bFSH from cultured porcine granulosa cells at 37°C in the presence(O---O) or absence(●——●) of excess hFSH (10 ug/ml).

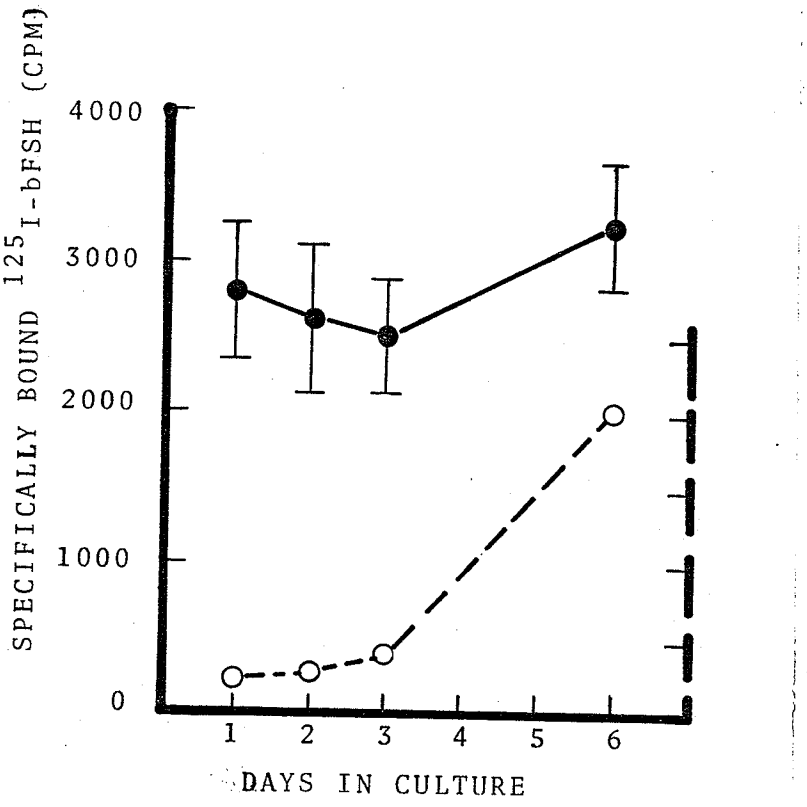



Figure 17: Effect of culturing on the binding ability of bovine granulosa cells for $^{125}$I-bFSH. ●——●, $^{125}$I-bFSH specifically bound; ○--○, cell number/culture dish. Each point represents the mean of triplicate determinations. Vertical bars indicate the range of triplicates.

in culture for 6 days without appreciable loss of ability to bind $^{125}$I-bFSH.

(4) Saturability of FSH Receptors to $^{125}$I-bFSH

Pig granulosa cells ($2.0 \times 10^{-4}$ cells/dish on Day 0) were cultured for 3 days before testing for receptor activity to bind increasing amoun of $^{125}$I-bFSH. Fig. 18 shows that the specific-binding of $^{125}$I-bFSH to pig granulosa cells was a saturable process. Using the same data, a linear Scatchard plot (22) was obtained (Fig. 19), indicating that a single homogeneous population of FSH receptor was involved. It also implied that no negative cooperativity was present in the FSH receptor system (see section I-A-4). Extrapolation of the linear plot to the X-axis showed that there were $6.5 \times 10^{-15}$ moles of FSH receptor per culture dish, assuming a one-to-one binding relationship between $^{125}$I-bFSH and receptor. As there were 659,000 cells/dish, the number of FSH receptors in each of the cultured porcine granulosa cells was calculated to be $5.9 \times 10^{3}$ (taking 1 mole = $6.023 \times 10^{23}$ molecules). The association constant (Ka) of the $^{125}$I-bFSH-receptor interaction was estimated to be $5.7 \times 10^{9} M^{-1}$.

(5) Effect of Homogenization and Incubation Buffer

Bovine granulosa cells were aspirated from small follicles ($< 6$ mm) and resuspended in 10 ml of Hank's buffer containing 0.1% BSA ($1.3 \times 10^{6}$ cells/ml). The cell suspension was separated into two 12 ml centrifuge tubes, each with 5 ml of cell suspension. The tubes were centrifuged at 600g for 5 min. One of the two cell-pellets was taken up in 5 ml of Hank's - 0.1% BSA buffer again, whereas the other pellet was resuspended in 25mM Tris-HCl, pH 7.2, containing 10 mM $MgCl_2$ and 0.1% BSA. Each of the cell suspensions was further split into two equal aliquots (2.5 ml each) one of which was homogenized manually with a glass homogenizer, while the

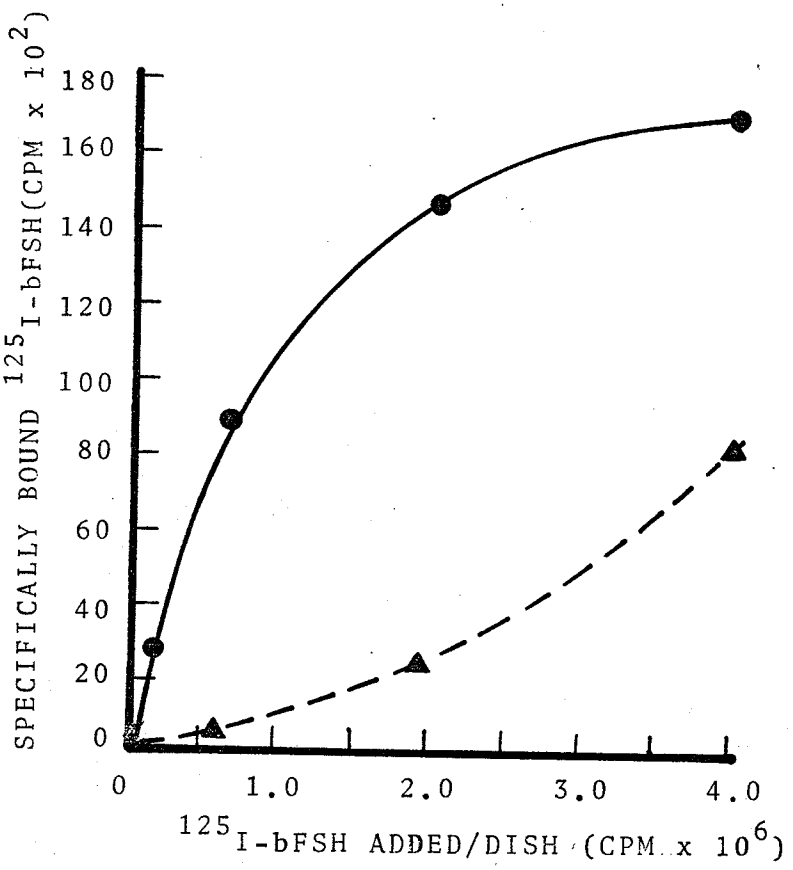



Figure 18: Effect of increasing concentration of $^{125}$I-bFSH on the specific binding to the 3 day-cultured porcine granulosa cells. ●——●, specific binding. ▲- - -▲, nonspecific binding. Each culture-dish contained 659,000 cells and incubations were carried out at 37°C for 6 hr in 1.2 ml of Eagle medium containing 0.1 % BSA, in the presence or absence of 10,000 ng hFSH.

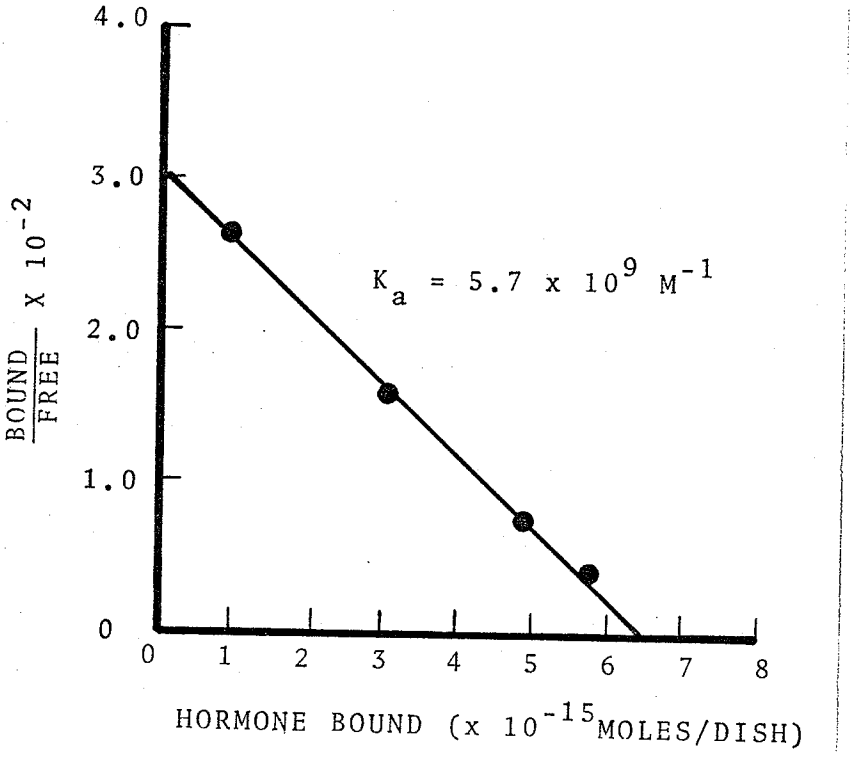



Figure 19: Scatchard plot of the specific binding of $^{125}$I-bFSH to 3 day-cultured porcine granulosa cells(data from Fig. 18).

other remained in cell suspension. Each of the fractions (0.4 ml) was assayed for binding of $^{125}$I-bFSH (50 µl, 150,000 CPM) in the presence or absence of a large excess of unlabelled hFSH (50 µl, 100 µg/ml). Incubation was done at 37°C for 4 hr and the reaction was stopped by adding 2.0 ml of ice-cold Tris-HCl buffer containing 10 mM $MgCl_2$, 0.1% BSA. After centrifugation, the pellet was washed once with 2.0 ml of the same buffer and counted in an automatic gamma counter.

Figure 20 shows that 25 mM Tris-HCl buffer pH 7.2, with 10 mM $MgCl_2$ was much more effective than Hank's buffer in facilitating the specific binding of $^{125}$I-bFSH to bovine granulosa cells or homogenates. Granulosa cells after homogenization seemed to bind $^{125}$I-bFSH slightly better as compared to the cells in suspension (Fig. 20).

(6) Effect of Follicular Size

Bovine granulosa cells were harvested from large, medium, and small follicles (section III-B-4). After centrifugation at 600 g for 10 min, the cells were resuspended in 25 mM Tris-HCl, pH 7.2, containing 10 mM $MgCl_2$. The number of cells in the suspension was adjusted to 0.4 x $10^6$ cells/0.4 ml. The cells were homogenized in the buffer and 0.4 ml of the homogenate was assayed for receptor-binding with 1 X $10^6$ CPM of $^{125}$I-bFSH or $^{125}$I-bLH in a final volume of 0.5 ml. After incubating at 37°C for 4 hr, the reaction was stopped by adding 2.0 ml of ice-cold Tris-HCl buffer. After centrifugation the pellet was washed once with another 2 ml of Tris-HCl buffer and the remaining radioactivity was counted. Figure 21 shows that homogenates of bovine granulosa cells harvested from large follicles bound specifically more $^{125}$I-bLH and less $^{125}$I-bFSH than the cells from small follicles. Similar observation was

reported by Channing and Kammerman (131) on the decrease of LH receptors during follicle-maturation in pig.

To examine changes of FSH-receptor binding during follicle maturation, homogenates of granulosa cells ($0.98 \times 10^6$ cells/tube) from large (> 15 mm), medium (7-15 mm) and small (< 7 mm) bovine follicles were incubated at 37°C for 4 hr, with increasing concentrations of $^{125}$I-bFSH in a final volume of 0.5 ml of 25 mM Tris-HCl containing 10 mM $MgCl_2$, pH 7.2. Figure 22 shows that the binding of $^{125}$I-bFSH to homogenates of granulosa cells was a saturable process and cells from small follicles bound more $^{125}$I-bFSH than cells from medium and large follicles. From the linear Scatchard plots of the same data (Fig. 23) the association constant (Ka) of the interaction between $^{125}$I-bFSH and granulosa cells from various sizes of follicles was similar, being $1.4 \times 10^9$ $M^{-1}$. The binding capacity for FSH of the granulosa cells from large follicles was approximately twice that of the small follicles.

(7) Effect of Pre-Treating Cells with Excess Unlabelled hFSH.

Two day-cultured bovine granulosa cells were pre-treated with unlabelled hFSH (5000ng/ml) in 2 ml of complete culture medium by incubating at 37°C for time intervals of 3, 6, 12 and 24 hr. After incubation, the cells were washed with 3 X 2 ml of sterile Hank's buffer containing 0.1% BSA. The binding activity for $^{125}$I-bFSH of these pretreated cells was assayed at 37°C for 6 hr either immediately or after further culturing for another 24 hr in 2 ml normal medium without the hormone.

Figure 24 shows that pre-treatment with excess hFSH (5000 ng/ml) did not significantly decrease the level of FSH receptor in cultured bovine granulosa cells. The slight decrease in specific binding for $^{125}$I-bFSH observed immediately after hFSH-pretreatment for 6, 12 and 24 hr might be

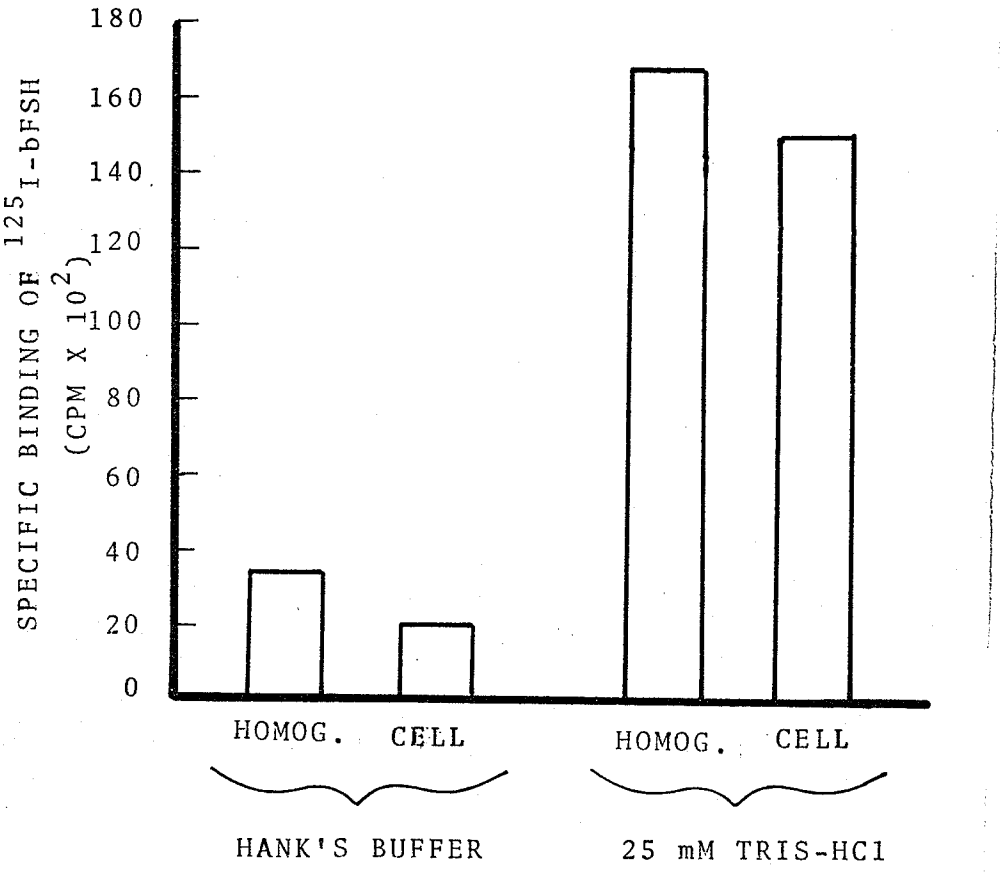



Figure 20: Effect of homogenization and incubation buffer on the binding activity of bovine granulosa cells ($0.5 \times 10^6$ cells/tube) for $^{125}$I-bFSH. Incubation was carried out at $37^{o}$C for 4 hr.

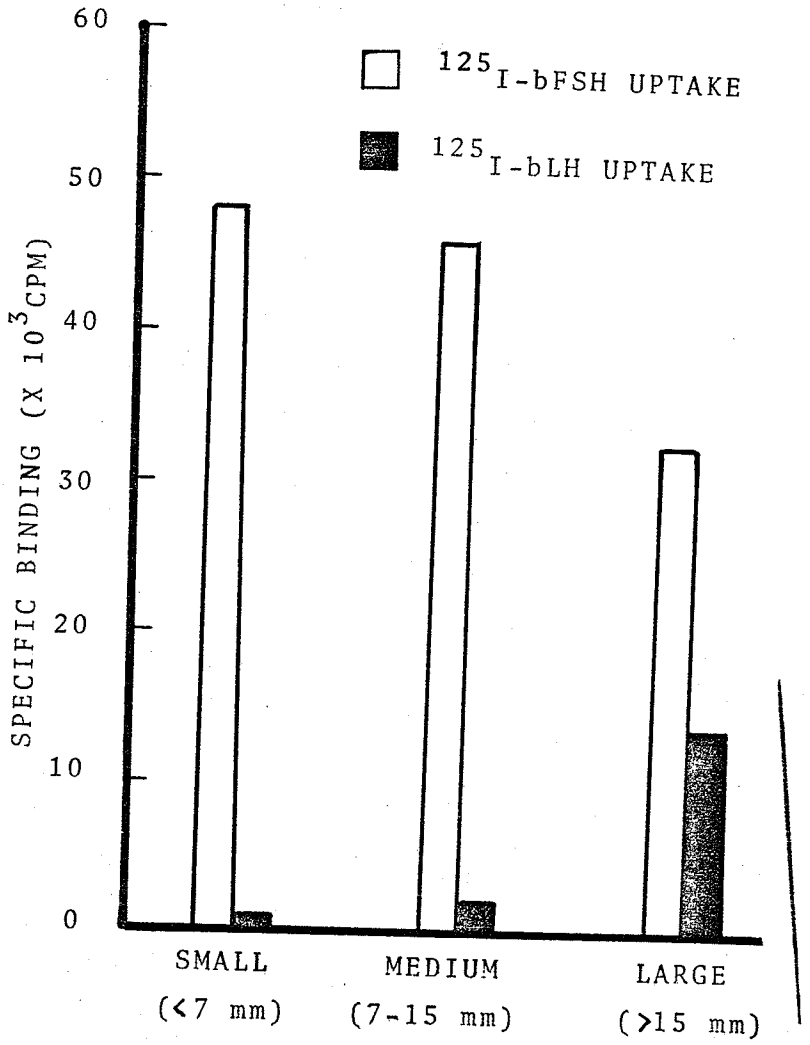



Figure 21: Effect of follicular size on the binding activity for $^{125}$I-bFSH and $^{125}$I-bLH of bovine granulosa cells from ovarian follicles of various sizes. Granulosa cells were homogenized in 25 mM Tris-HCl buffer for assay.

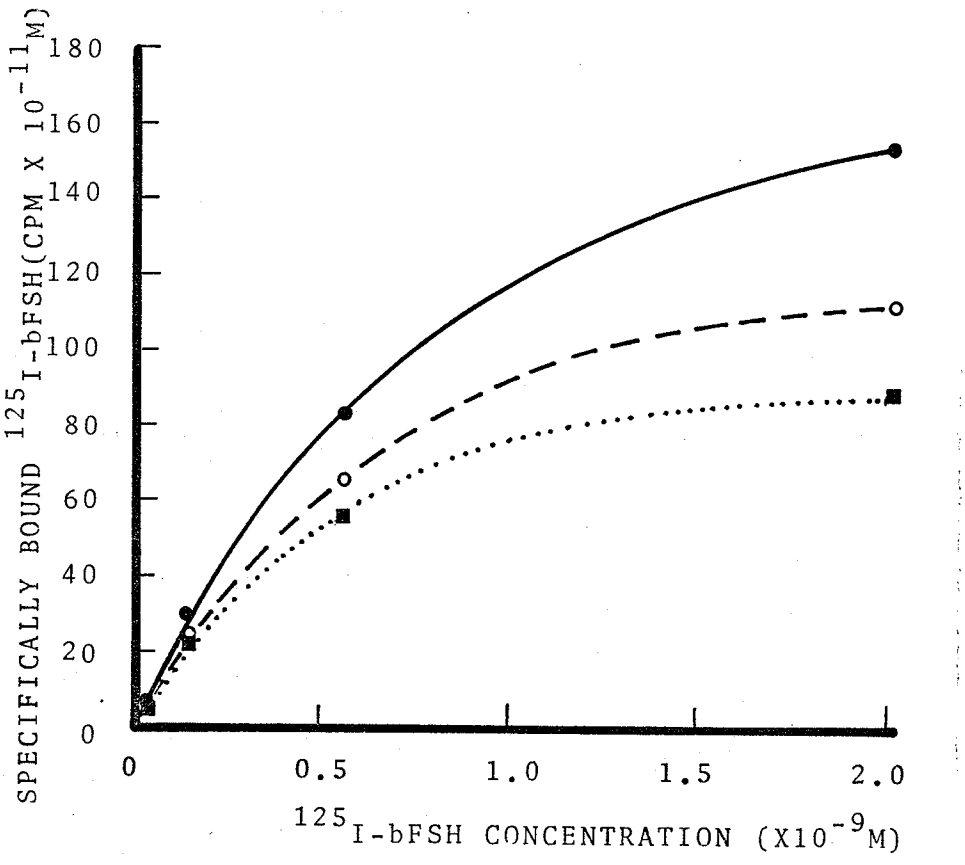



Figure 22: Specific binding of $^{125}$I-bFSH to bovine granulosa cells from large(■·····■), medium(o---o), and small (●——●) ovarian follicles as a function of increasing concentrations of $^{125}$I-bFSH. Incubations were carried out at 37°C for 4hr with 0.98 x $10^6$ cells in each tube(0.4 ml/tube).

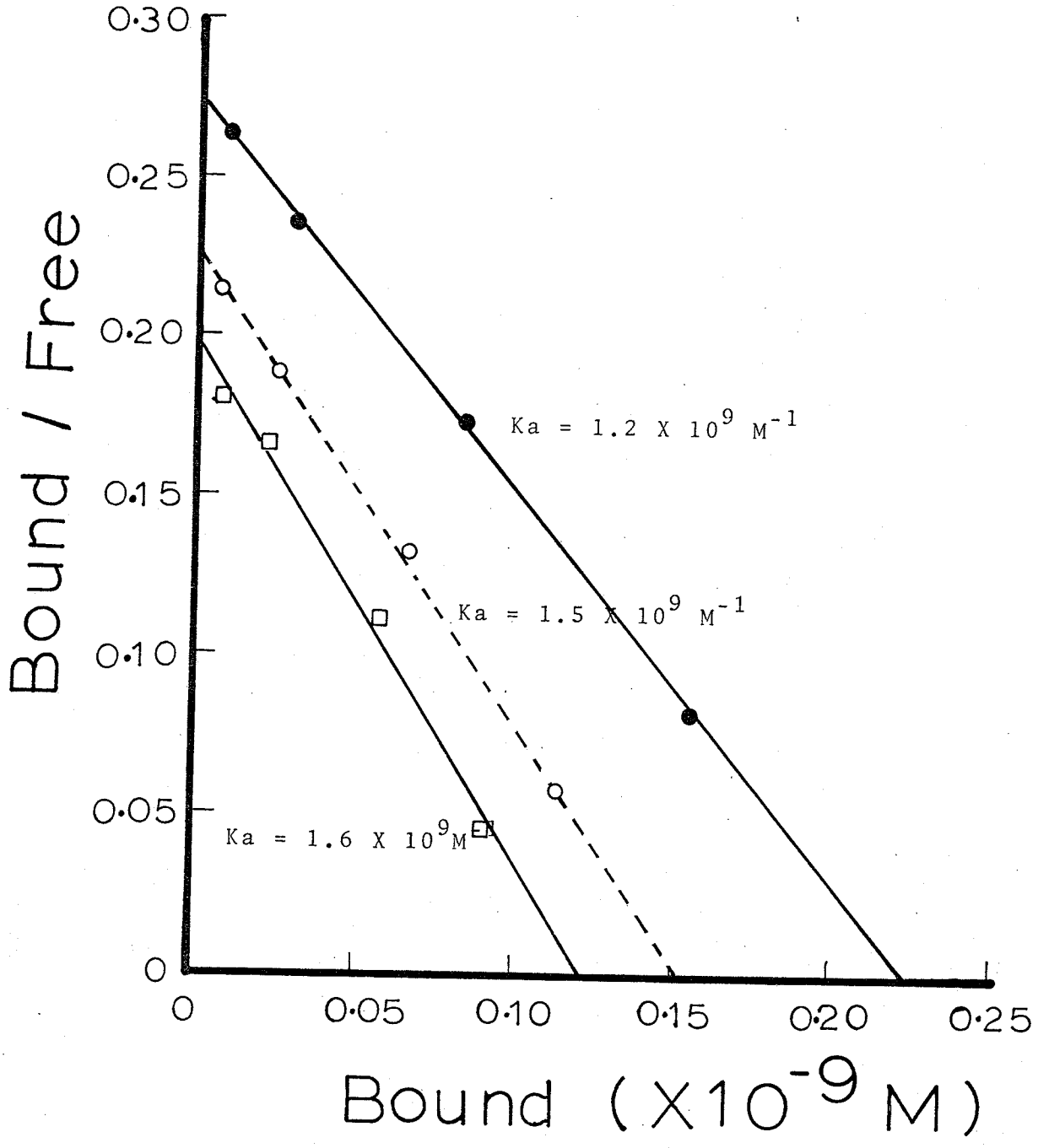



Figure 23: Scatchard analysis of the specific binding (data from Fig.22) of $^{125}$I-bFSH to bovine granulosa cells from large(□——□), medium(O---O), and small(●——●) ovarian follicles. Slope of the plot gives -Ka and the X-intercept provides the total binding capacity of the cells /tube.

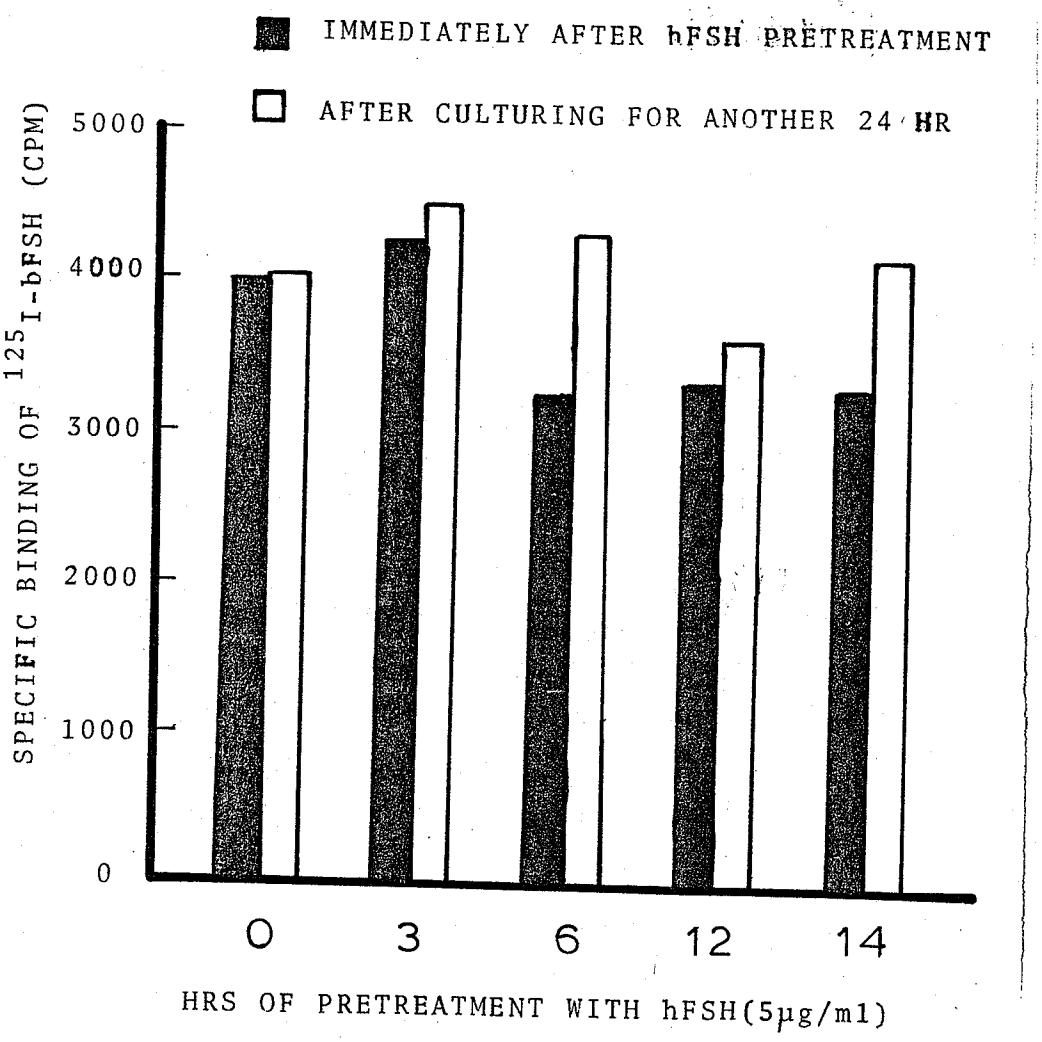



Figure 24: Effect of pretreating cultured bovine granulosa cells with excess hFSH(5000 ng/ml) on the subsequent specific uptake of $^{125}$I-bFSH by the cells. Specific uptake by the cells was assayed either immediately after washing (■) or after further culturing for another 24 hr in normal medium(□).

due to contamination of remaining hFSH in the cells after washing. This observation indicated that there was no "desensitization" or "down regulation" (section I-A-5) of FSH receptors in bovine granulosa cells by pre-treating the cells with a homologous hormone (hFSH).

(C) Studies on the Solubilization of Testicular FSH Receptors

(1) Effect of Sonication

The possibility to dislodge FSH receptors from porcine testicular membranes by sonication was examined by using a Megason ultrasonic disintegrator (Ultrasonic Instruments Intl. Ltd.). In an ice-cold 15 X 85 mm glass culture-tube 4 ml of PTR (200 mg protein/ml) was sonicated at maximum power for 2 min.

Figure 25 shows that sonification decreased 80% of the binding activity for $^{125}$I-bFSH in the particulate membranes obtained by centrifugation at 1500 g. However if 1.6 M $(NH_4)_2SO_4$ was used for precipitating the membranes, sonication increased the specific binding for FSH slightly.

It was found that sonication disrupted the testicular membrane into a very fine suspension which could not be precipitated out readily by low-speed centrifugation (1500 g). However, if ultracentrifugation (100,000 g, 1 hr) was used, the suspended membranes carrying the FSH receptors could be precipitated out completely. Therefore sonication was ineffective in solubilizing the FSH receptor.

(2) Effect of Organic Solvents

(a) One-phase system

PTR (10 mg protein/ml) was lyophilized in 0.5 ml aliquots and extracted twice with 5 ml of n-pentane, benzene, ether, amyl alcohol, n-butanol propanol, or ethanol (III-B-8-a). After extraction and being

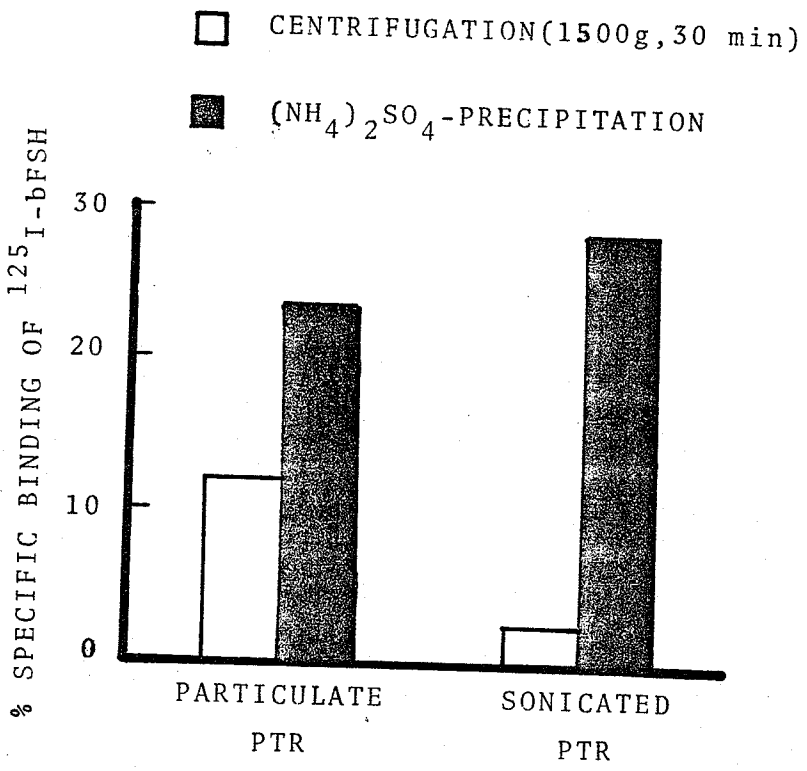



Figure 25: Effect of sonication on the binding activity of testicular membranes for $^{125}$I-bFSH. The specifically bound $^{125}$I-bFSH was obtained by centrifugation( ☐ ) or $(NH_4)_2SO_4$-precipitation( ■ ).

vacuum-dried, the entracted pellets were resuspended by sonication in 1 ml of 25 mM Tris-HCl, pH 7.2 containing 10 mM $MgCl_2$. For $^{125}$I-bFSH binding activity, 0.1 ml in triplicates of the resuspended pellet was used and the hormone-receptor complex was precipitated with 1.6 M $(NH_4)_2SO_4$ (section III-B-10).

Table I shows that integrity of the FSH receptor after treatment with organic solvent depended on the polarity of organic solvent used. For instance, the relatively non-polar organic solvents, n-pentane and benzene, were very "gentle" to the FSH receptor. In contrast, ethanol, which is relatively polar, totally denatured the FSH receptor (Table 1). These findings suggested that the more polar organic solvents disrupted the ionic-interactions (such as hydrogen bonds) between the more polar membrane lipids (e.g. phospholipids) thereby facilitating their extraction. Therefore, it is likely that the testicular FSH receptors contained or required some polar lipids in order to interact with the hormone. The more non-polar lipids such as cholesterol, which can be readily entracted by pentane and benzene, were probably not required for FSH-receptor interactions.

(b) Two-phase system

Table II shows that if the testicular membranes were treated with different organic solvents in the presence of equal volumes of aqueous buffer (Section III-B-8-b), the FSH receptor was much more labile than with a one-phase solvent-system. For instance, the FSH receptor was relatively stable to one-phase extraction of benzene (Table I), however, it was completely denatured by a two-phase extraction of the same solvent (Table II). This could be due to the effect of water which interferes with the ionic interactions between polar lipids in the membrane and facilitates the extraction by non-polar solvents such as pentane and benzene.

Table I: Effect of treatment with different organic solvents in a "one-phase system" on the binding activity of porcine testicular FSH receptor for $^{125}$I-bFSH.

| ORGANIC SOLVENTS | % SPECIFIC BINDING |
|---|---|
| CONTROL | 31.8 |
| n-PENTANE | 28.5 |
| BENZENE | 23.0 |
| ETHER | 11.0 |
| AMYL ALCOHOL | 10.2 |
| n-BUTANOL | 2.0 |
| PROPANOL | 0.6 |
| ETHANOL | 0.3 |

Table II: Effect of treatment with organic solvents in a two-phase system on the binding activity of porcine testicular FSH receptor for $^{125}$I-bFSH.

| ORGANIC SOLVENT | % SPECIFIC BINDING |
|---|---|
| CONTROL(Tris-HCl) | 10.5 |
| PENTANE/Tris-HCl | 3.9 |
| ETHER/Tris-HCl | 0.3 |
| TOLUENE/Tris-HCl | 0 |
| BENZENE/Tris-HCl | 0 |

Another two-phase system, n-butanol/$H_2O$ was also used to solubilize testicular membrane (Section III-B-8-b). After the extraction-mixture was separated into two phases by centrifugation, 0.1 ml of the lower aqueous phase was assayed for $^{125}$I- bFSH binding by $(NH_4)_2SO_4$-precipitation. No specific binding was observed in the aqueous phase after solubilization. Dialysis of the extract against water overnight at 4°C resulted large amounts of precipitates in the dialysis bag. No $^{125}$I-bFSH binding activity was found in the dialysate.

The effect of n-butanol on the association of testicular receptor and FSH was also studied. To a test tube 0.1 ml of PTR (2.5 mg protein/ml), 0.1 ml hFSH (10,000 ng/ml), 0.1 ml $^{125}$I-bFSH, and 0.1 ml of 80 mM $MgCl_2$ in 25 mM Tris-HCl (for adjusting the final concentration of $MgCl_2$ to 10 mM) were incubated with 0.4 ml of 25 mM Tris-HCl, pH 7.2, saturated with 100%, 50%, 25%, or 0% n-butanol. Incubation was carried at 25°C for 14 hr and reaction was stopped by adding 1.6 M $(NH_4)_2SO_4$ to precipitate the hormone-receptor complex. Table III shows that $^{125}$I-bFSH- receptor interaction was not affected by 12.5% saturated n-butanol (final conc.) during incubation, whereas 50% - saturated n-butanol completely abolished the binding activity. In addition, it was also found that short exposure (5 min) of FSH receptors to n-butanol- saturated Tris-HCl buffer abolished irreversibly the ability of receptor to bind the $^{125}$I-bFSH tracer.

The effect of 25%, 50%, and 100% - n-butanol-saturated 25 mM Tris-HCl buffer in the dissociation of preformed $^{125}$I-bFSH-receptor compelx was also examined. Figure 26 shows that Tris-HCl buffer 100% - saturated with n-butanol effectively released (80%) the already bound $^{125}$I-bFSH tracer from the preformed receptor-hormone complex. The integrity of the released radioactivity was tested by binding with fresh particulate receptors, and was found to be as good as or even 1½-fold better than the original $^{125}$I-bFSH

Table III: Effect of various saturations of n-butanol in the incubation buffer on the binding of $^{125}$I-bFSH to porcine testicular receptor.

| % SATURATURATION OF n-BUTANOL IN TRIS-HCl BUFFER | $^{125}$I-bFSH SPECIFIC BINDING (%) |
|---|---|
| 0 (CONTROL) | 14.3 |
| 12.5% | 14.5 |
| 25.0% | 5.0 |
| 50.0% | 0.1 |

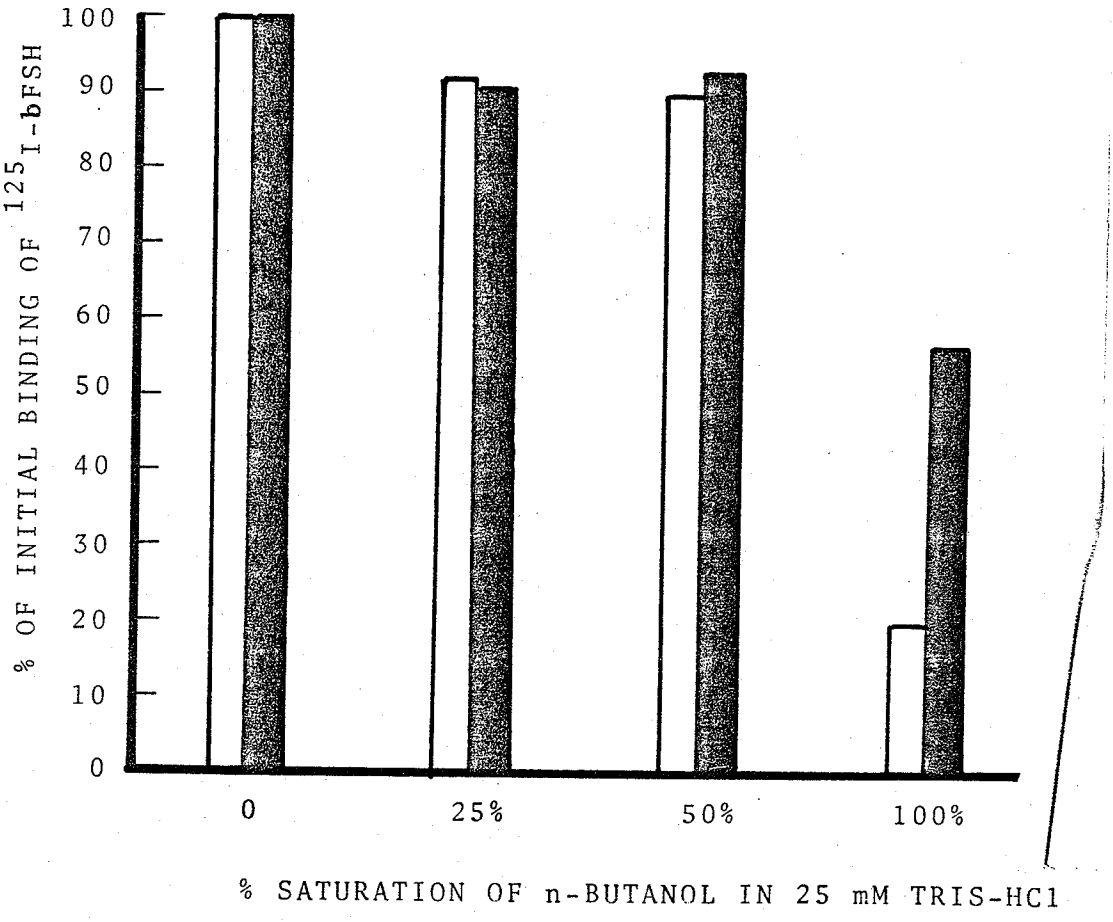



Figure 26: Dissociation of receptor-bound $^{125}$I-bFSH( □ ) or $^{125}$I-bLH( ■ ) by various saturations of n-butanol in Tris-HCl buffer.

tracer.

(3) Effect of Triton X-100

The non-ionic detergent, Triton X-100, was tested for solubilization of FSH receptors from porcine testicular membranes (section III-B-9). As shown in table V, extraction with 0.1% Triton X-100 destroyed all the binding activity of FSH receptors in the testicular membranes. The remaining pellet after extraction was also found to be devoid of FSH receptor. However, by prelabelling of the particulate membranes with $^{125}$I-bFSH, followed by extraction with 0.1% Triton X-100; 47% of the radioactivity, presumably the solubilized $^{125}$I-bFSH-receptor complex, was extracted into the 100,000 g supernatant.

(D) Characterization of $^{125}$I-bFSH-Receptor Complex

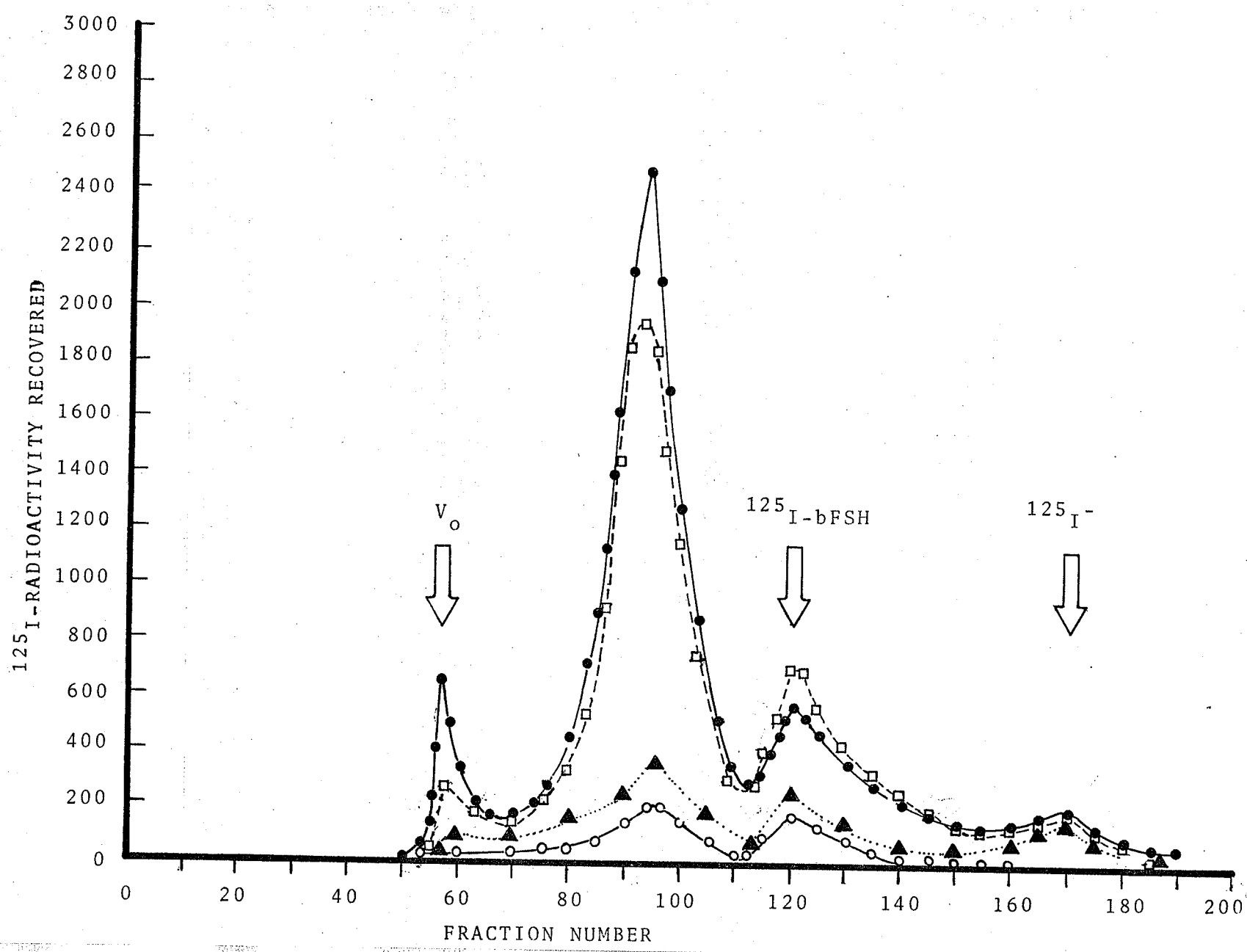
(1) Gel Filtration

Upon gel filtration on Sepharose 6B (Fig. 27) the solubilized $^{125}$I-bFSH-Receptor Complex eluted as one major and two minor radioactive peaks, corresponding to free $^{125}$I-bFSH, a major $^{125}$I-bFSH-receptor complex and a small peak at Vo. Using 10% polyethylene glycol, 6%, 57% and 86% respectively of the radioactivity in the three peaks were precipitated. Treatment of 1N HCl, pH 3, to denature the particulate FSH receptor before incubation with $^{125}$I-bFSH to form receptor-hormone complex diminished almost completely both the major $^{125}$I-bFSH-receptor peak and the peak at $V_o$ (Fig. 27). It appeared the radioactive peak at $V_o$ was an aggregate of $^{125}$I-bFSH-receptor complexes. The specificity of FSH-receptor complex was demonstrated by the dramatic decrease of the major radioactive peak by incubating the testicular receptor simultaneously with excess hFSH (1000 ng/ml) and the $^{125}$I-labelled tracer (Fig. 27). The radioactive $^{125}$I-bFSH-receptor complex, once it was formed and solubilized by Triton X-100, appeared to be non-

Table IV: Effect of Triton X-100 on testicular FSH receptor. For solubilizing free receptors, 10 mg of testicular membrane proteins were treated for 1 hr with 1 ml of 0.1% Triton X-100 in 25 mM Tris-HCl, pH 7.2. After 100,000g centrifugation for 1 hr, 0.1 ml of the suspension was assayed for specific binding for $^{125}$I-bFSH. $(NH_4)_2SO_4$ at 1.6M was used to precipitate the solubilized $^{125}$I-bFSH-receptor complex. For $^{125}$I-bFSH-prelabeled testicular membranes (10 mg protein), 1 ml of 0.1 % Triton X-100 in Tris-HCl buffer was added and after centrifugation at 100,000g for 1 hr, $^{125}$I-bFSH-receptor complex solubilized into supernatant was precipitated by 1.6M $(NH_4)_2SO_4$.

| | % $^{125}$I-bFSH BOUND | |
|---|---|---|
| | PART. MEMBR. BEFORE 0.1% TRITON X-100 | 100,000g SUPERN AFTER 0.1 % TRITON X-100 |
| SOLUBILIZATION OF MEMBRANES, FOLLOWED BY $^{125}$I-bFSH LABELING | 28.0 % | 0 % |
| PRELABELING PARTICULATE RECEPTOR WITH $^{125}$I-bFSH, FOLLOWED BY SOLUBILIZATION | 28.0 % | 12.6 % |

Figure 27: Gel filtration on Sepharose 6B (1.5 X 110 cm) of $^{125}$I-bFSH-receptor complex extracted with Triton X-100 from testicular membranes: (●—●), prelabeled with $^{125}$I-bFSH; (○—○), incubated simultaneously with $^{125}$I-bFSH and excess unlabeled hFSH; (▲····▲), pretreated with 1N HCl at pH 3 for 1 hr, followed by incubation with $^{125}$I-bFSH tracer; (□--□), the already solubilized $^{125}$I-bFSH-receptor complex incubated with excess unlabeled hFSH at 4°C for 24 hr. The column was eluted with 25 mM Tris-HCl, pH 7.2, buffer containing 10 mM $MgCl_2$, 0.1% BSA, and 0.1% Triton X-100. Arrows (⇩) indicate the relative elution volume of Blue Dextran ($V_o$), $^{125}$I-bFSH and Na$^{125}$I.


3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
$^{125}$I-RADIOACTIVITY RECOVERED
$V_o$
$^{125}$I-bFSH
$^{125}$I-
0
20
40
60
80
100
120
140
160
180
200
FRACTION NUMBER

displaceable by excess hFSH.

The Triton X-100-solubilized $^{125}$I-bFSH-receptor complex could be distinguished from free $^{125}$I-bFSH by precipitation with polyethylene glycol (Carbowax 6000) or $(NH_4)_2SO_4$ (section III-B-10). Fig. 28 and Fig. 29 show the effect of increasing concentrations of polyethylene glycol and $(NH_4)_2SO_4$, respectively, on precipitation of Triton X-100 -solubilized $^{125}$I-bFSH-receptor complex. It is observed that 10% polyethylene glycol or 1.6M $(NH_4)_2SO_4$ precipitated maximally the $^{125}$I-bFSH receptor complex. In both cases, approximately 80% of the radioactivity solubilized from $^{125}$I-bFSH-prelabelled membranes was precipitated. The $(NH_4)_2SO_4$ - precipitation method was found to be more convenient than the polyehtylene glycol method because 1.6 M $(NH_4)_2SO_4$ was less viscous so that mixing was easier. However, Triton X-100 was observed to be only slightly soluble in 1.6 M $(NH_4)_2SO_4$ and at concentrations ($>$ 1%), formed a greasy band sitting on the top of the $(NH_4)_2SO_4$ solution after centrifugation. Under these conditions, some radioactivity, presumably the $^{125}$I-bFSH-receptor complex, was carried by the Triton X-100 and prevented from precipitation.

(2) Stability of $^{125}$I-bFSH-receptor Complex

The stability of the solubilized $^{125}$I-bFSH receptor complex, was assessed by storage at -20$^{o}$C, 4$^{o}$C and 25$^{o}$C for 0, 1, 2, 4 and 8 days. The $^{125}$I-bFSH-receptor complex was precipitated by the polyethylene glycol method (Section III-B-10).

Figure 30 shows that the solubilized $^{125}$I-bFSH-receptor complex present in 0.1% Triton X-100 was more stable at a frozen state (-20$^{o}$C) than at 4$^{o}$C or 25$^{o}$C. It was 72%-precipitable by 11% polyethylene glycol on day 0.

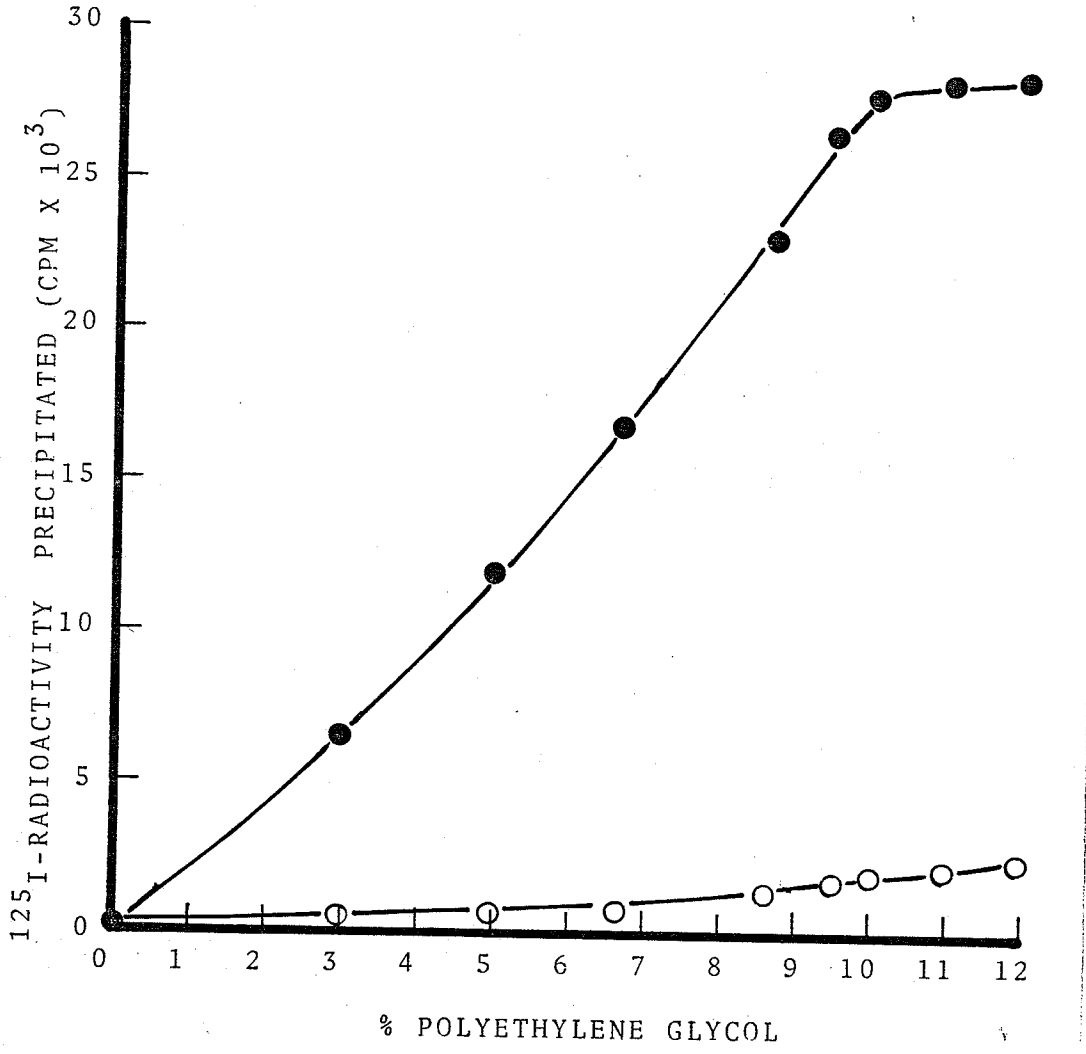



Figure 28: Precipitation of Triton X-100-solubilized $^{125}$I-bFSH-Receptor complex by increasing concentrations of polyethylene glycol(Carbowax 6000). ●—●, $^{125}$I-bFSH-Receptor complex; ○—○, free $^{125}$I-bFSH tracer.

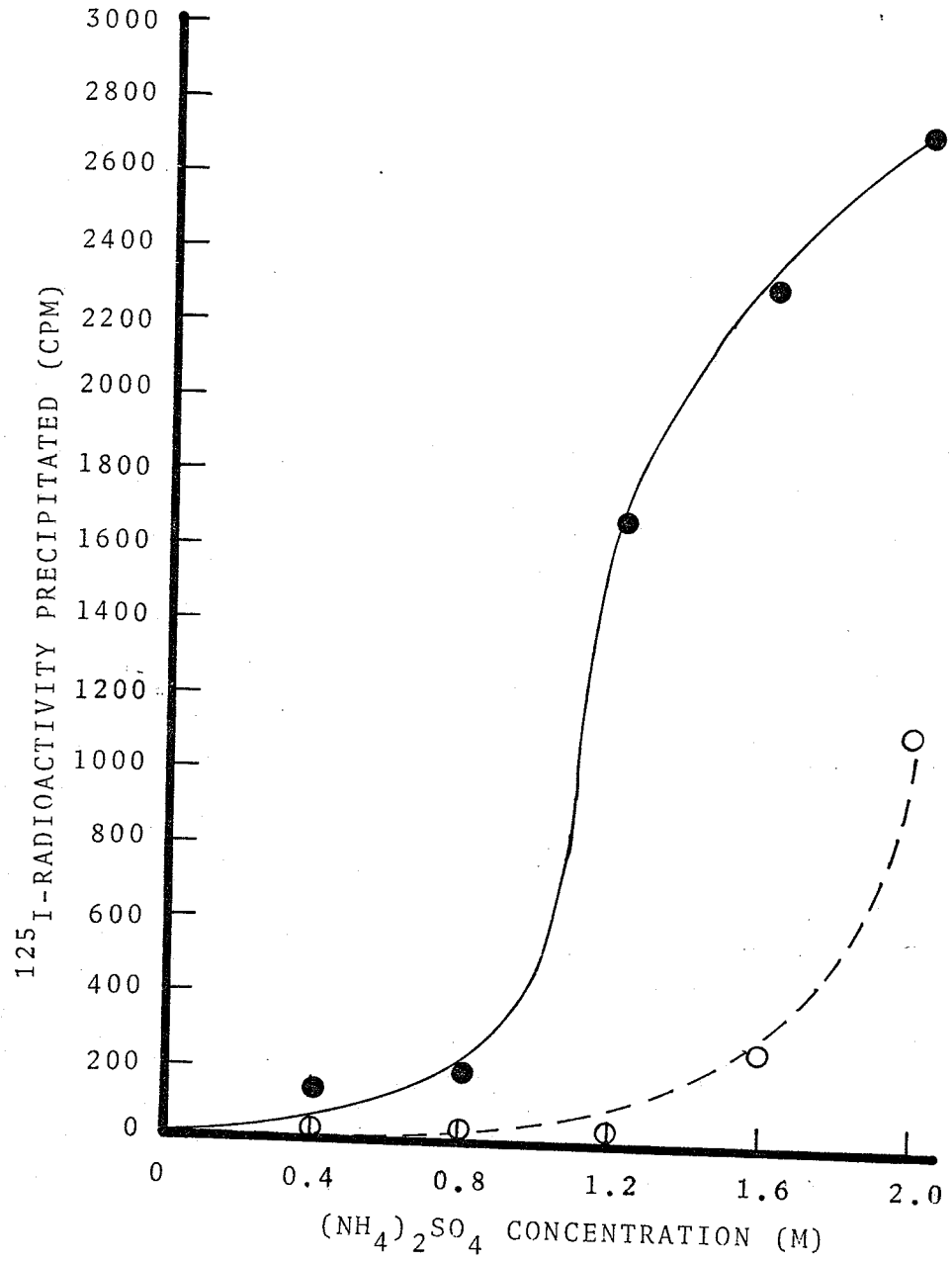



Figure 29: Precipitation of Triton X-100-solubilized $^{125}$I-bFSH-Receptor complex by increasing concentrations of $(NH_4)_2SO_4$. ●——●, $^{125}$I-bFSH-Receptor complex; ○- - -○, free $^{125}$I-bFSH tracer.

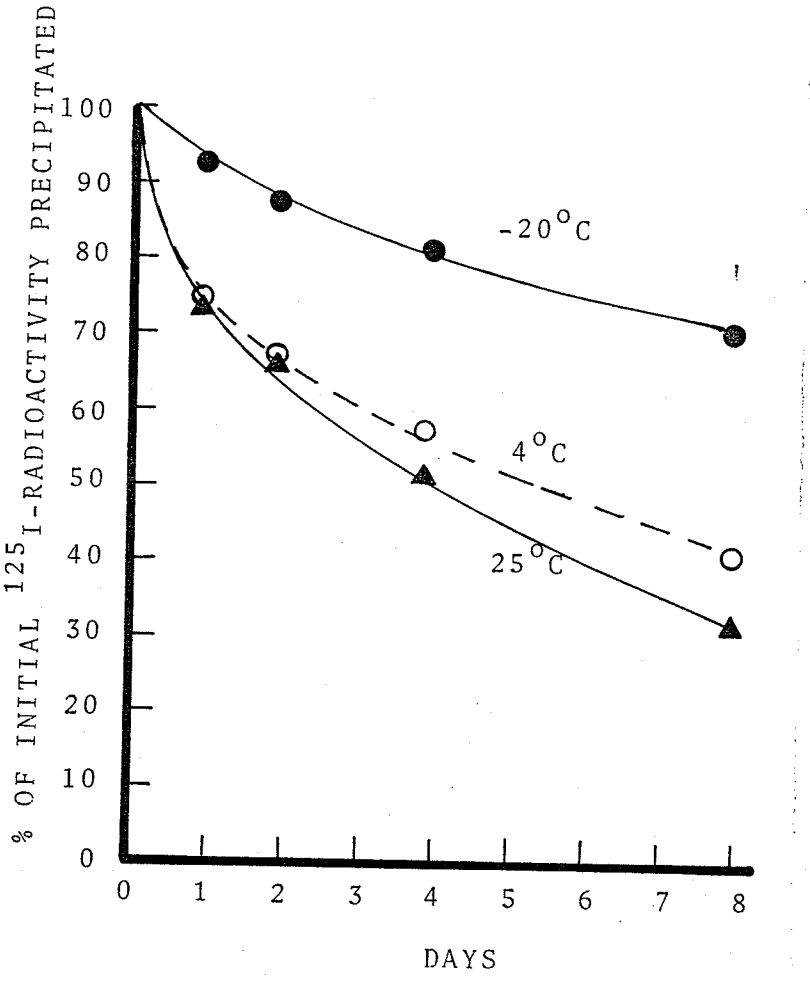



Figure 30: Stability of the $^{125}$I-bFSH-Receptor complex solubilized by Triton X-100 and stored at -20°C, 4°C, and 25°C.

(3) Interaction with bFSH- Antiserum

(a) Precipitation with polyethylene glycol

In order to examine whether the specific antiserum against bFSH still interacts with $^{125}$I-bFSH tracer after it has bound to the testicular receptors, 0.2 ml of Triton X-100 - solubilized $^{125}$I-bFSH-receptor complex or $^{125}$I-bFSH (20,000 CPM) in 0.1% Triton X-100 V was incubated with 0.1 ml of bFSH- antibody (1:50,000 or 1:1000) at 4°C for 2 days. Polyethylene glycol at different concentrations (3, 6, 9, 12, 15, 18%) were used to precipitate the "receptor-$^{125}$I-bFSH-antibody" or $^{125}$I-bFSH-antibody complexes, which were obtained by centrifugation. The effect of increasing concentrations of polyethylene glycol on the precipitation of free $^{125}$I-bFSH-antibody complex, $^{125}$I-bFSH-receptor complex and receptor-$^{125}$I-bFSH-antibody complex is shown in Fig. 31. Optimal precipitation of the $^{125}$I-bFSH-receptor complex was achieved at 10% polyethylene glycol; whereas the $^{125}$I-bFSH-antibody (1:50,000 or 1:1000 dilutions) complex was precipitated optimally by 6% polyethylene glycol. When the antiserum was used at 1:50,000 dilution to interact with the $^{125}$I-bFSH-receptor, substantial amounts of radioactivity were precipitated at 6% polyethylene glycol similar to that of the $^{125}$I-bFSH-antibody complex, but maximal precipitation was achieved only at 10% PEG (Fig. 31). In contrast, when the antiserum was used at 1:1000 dilution, optimal precipitation of radioactivity was accomplished at 6% PEG, almost identical to that of the $^{125}$I-bFSH-antibody complex. These observations suggested that at low concentrations of antibody (1:50,000 dilution) , a substantial amount of the $^{125}$I-bFSH receptor complex remained unbound to the antibody and was precipitated only as the concentration of PEG reached 10% (Fig. 31).

(b) Effect of various concentrations of bFSH antibody

To obtain the optimal concentration of the anti-bFSH-serum to

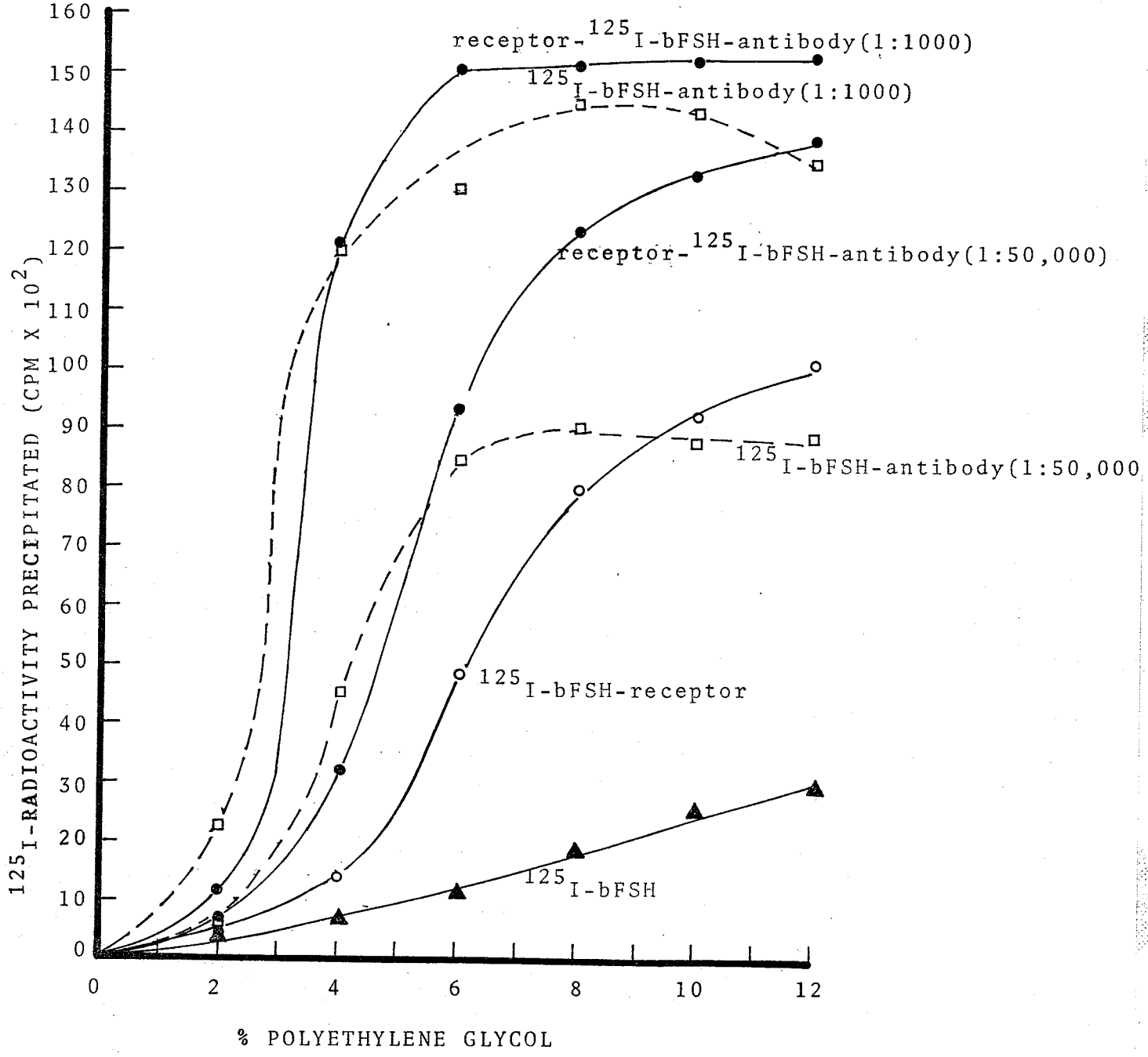



Figure 31: Effect of increasing concentrations of polyethylene glycol on the precipitation of free $^{125}$I-bFSH, $^{125}$I-bFSH-receptor, $^{125}$I-bFSH-antibody, and receptor-$^{125}$I-bFSH-antibody. The receptor- and antibody-complexes were solubilized by Triton X-100 and the reactions were carried out in Tris-HCl buffer containing 0.1% Triton X-100.

interact with the $^{125}$I-bFSH-receptor complex, the binding of the antiserum with the solubilized hormone-receptor complex was studied at various dilutions. To precipitate specifically the antibody bound radioactivity, a double-antibody system was used, in which the receptor - $^{125}$I-bFSH-antibody complex was precipitated by a second-antibody against rabbit γ-globulin raised in sheep. Figure 32 shows that the solubilized $^{125}$I-bFSH -receptor complex, similar to the free $^{125}$I-bFSH tracer, was bound by rabbit anti-bFSH serum and could be precipitated specifically by adding a second-antibody against rabbit γ-globulins. The precipitation curves for the free $^{125}$I-bFSH tracer and the $^{125}$I-bFSH-receptor complex at various concentrations of the antiserum were very similar, both being precipitated optimally by the antiserum at a dilution of 1:8000 (Fig. 32).

(c) Polyacrylamide Gel Electrophoresis

The electrophoretic mobility of free $^{125}$I-bFSH tracer, $^{125}$I-bFSH receptor complex (Section III-B-9-b), $^{125}$I-bFSH-antibody complex, and $^{125}$I-bFSH-receptor-antibody complex (section III-B-13, by excess anti-FSH antiserum at 1:000 dilution) were characterized by using 4% and 6% polyacrylamide gels at pH 8.9, containing 0.1% Triton X-100 (Section III-B-12). Upon polyacrylamide gel electrophoresis, the solubilized $^{125}$I-bFSH-receptor complex was resolved into two radioactive components, one which was quite similar to $^{125}$I-bFSH while the other was very much less acidic in nature, as reflected by its slower mobility toward the anode (Fig. 33a and b). For the $^{125}$I-bFSH-antibody complex, in both 4% and 6% gels, almost all the radioactivity stayed at the origin of application. For the "$^{125}$I-bFSH-receptor-antibody" complex, however, most of the radioactivity stayed near the origin in the 6% gel (Fig. 34a) but electrophoresed considerably into the 4% gel (Fig.33 b).

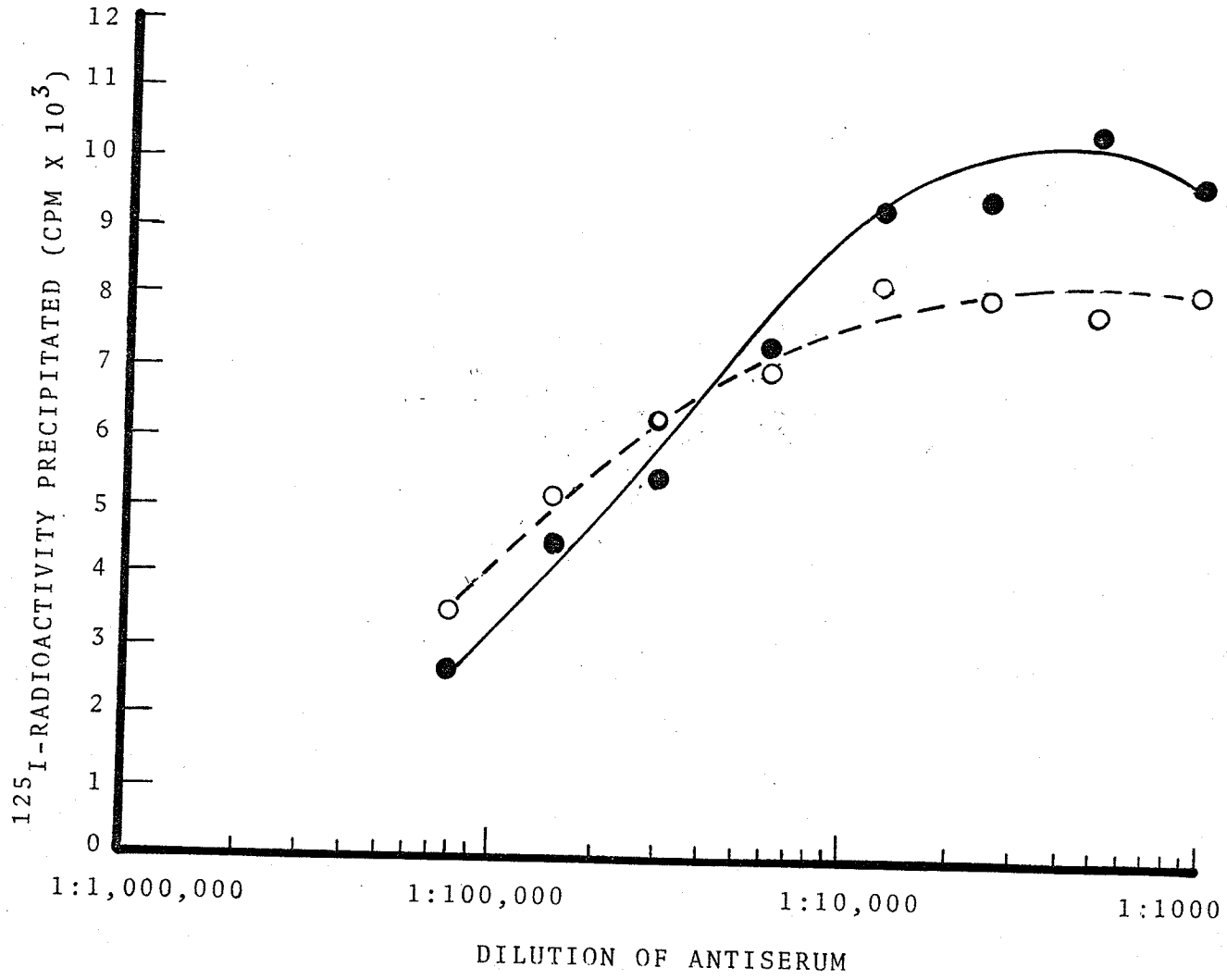



Figure 32: Reaction of specific anti-bFSH antiserum at various dilutions with solubilized $^{125}$I-bFSH-receptor(●—●) and free $^{125}$I-bFSH tracer(○---○).

These observations of differences in electrophoretic mobility between $^{125}$I-bFSH-antibody complex and $^{125}$I-bFSH-receptor-antibody complex upon polyacrylamide gel electrophoresis provided evidence that the interaction of anti-bFSH antiserum with the $^{125}$I-bFSH-receptor complex did not dissociate the already bound $^{125}$I-bFSH from the hormone-receptor complex but indeed transformed it into a bigger $^{125}$I-bFSH-receptor-antibody complex.

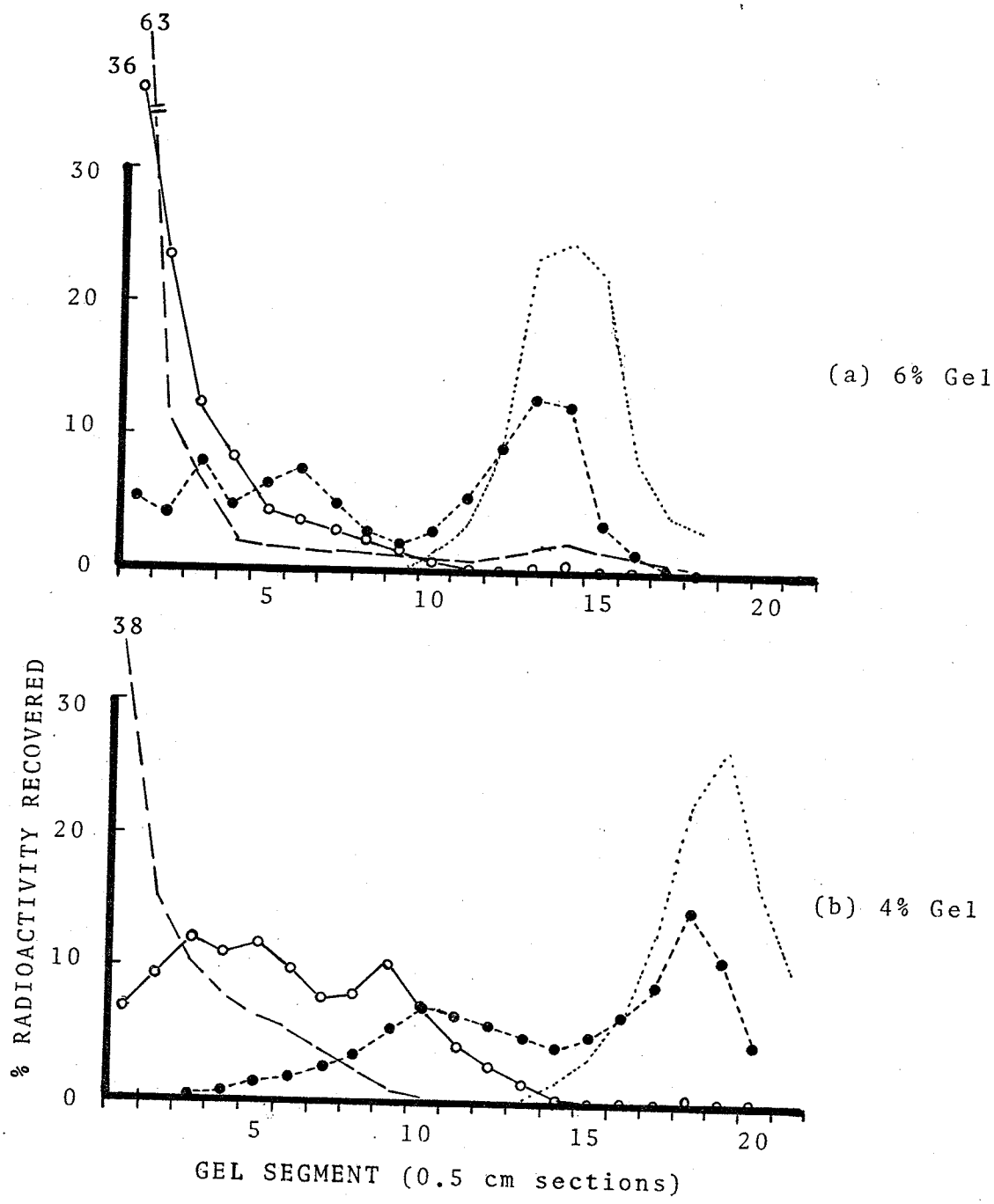



Figure 33: Electrophoretic mobilities of free $^{125}$I-bFSH(·····), $^{125}$I-bFSH-receptor(●-----●), $^{125}$I-bFSH-antibody(— — —), and receptor-$^{125}$I-bFSH-antibody(o——o) in (a) 6% and (b) 4% polyacrylamide gel.

## (V) DISCUSSION

### (A) Characterization of the Binding of $^{125}$I-bFSH to Porcine Testicular Membranes

Membranes prepared from porcine testes have been shown to bind specifically both bFSH and bLH. In comparing to the similarly prepared bovine testicular membranes (112), porcine testicular membranes often showed a higher capacity to bind bLH. The binding of bFSH to its receptor has been shown to be sensitive to divalent ions (Fig. 7, 8, 20), being maximal in 25 mM Tris-HCl buffer, pH 7.2, containing 10 mM $MgCl_2$; (or $CaCl_2$) however, high concentrations or absence of these ions ($Ca^{++}$, $Mg^{++}$) decreased the hormone binding. Monovalent ion ($Na^+$) was inhibitory for the binding of FSH to its receptor (Fig. 8).

Interference of hormone-receptor interaction by isotonic concentration of NaCl (0.15M) has also been reported in hCG-testis (132) and TSH-thyroid (133) systems; and 10 mM $MgCl_2$ approximately doubled the specific binding of $^{125}$I-labelled ovine prolactin to receptors prepared from pregnant rabbit mammary glands (20). At present, the physiological significance of these ionic effects is still not clear.

The dissociation of specifically receptor-bound $^{125}$I-bFSH from testicular membranes was incomplete after incubating in excess buffer for 14 hr at 37°C (figure 5). Approximately 40% of the $^{125}$I-bFSH tracer appeared to be irreversibly bound to the membrane. Whether this irreversibility was due to the damage of tracer and receptor during incubation or due to some yet undefined characteristics of a different class of FSH receptors remains to be clarified. The inability of excess unlabelled hFSH to accelerate the dissociation of bound $^{125}$I-bFSH from receptor (figure 5) indicated

that no negative cooperativity between FSH receptors was present in the testicular membrane. The insulin receptor-system suggested negative cooperativity (27); however, as the binding of $^{125}$I-labelled insulin to non-receptor materials such as talc and various agarose-protein derivates exhibited similar phenomena as the negative cooperativity of receptor binding (28), mechanisms other than "negative cooperativity", such as aggregation of insulin molecules, must be seriously considered.

(B) Characterization of the Binding of $^{125}$I-bFSH to Ovarian Granulosa Cells

FSH receptors in ovarian granulosa cells, similar to the receptors in testicular membranes, were also very sensitive to the presence of mono- and divalent ions as shown by the dramatic decrease of $^{125}$I-bFSH-uptake by cells suspended in Hank's buffer (containing 0.15 M NaCl) than those in a buffer of lower ionic strength, such as 25 mM Tris-HCl, containing 10 mM $MgCl_2$ and 0.1% BSA (Fig. 20). The observation that homogenization of bovine granulosa cells did not significantly increase the specific uptake of $^{125}$I-bFSH by the homogenate as compared to intact cells (Fig. 20) indicated that all of the membrane-bound receptors in intact cells were exposed on the cell surface, available for $^{125}$hormone binding.

It has been shown by Channing and Kammerman (131) that as ovarian follicles became mature they bound considerably more hCG than immature ones. Lee (134) has also shown that the hormone-sensitive adenyl cyclase system of granulosa cells from porcine ovarian follicles became increasingly responsive to LH and hCG than to FSH as the follicles matures. Our observation that bovine granulosa cells from large follicles bind less $^{125}$I-bFSH (50%) but approximately 20 X more $^{125}$I-bLH (Fig. 21) supports the hypothesis that as follicles are maturing, their granulosa cells become increasingly responsive to LH. In immature female rats, the increase in level of LH receptor

has been shown to be stimulated by FSH (60).

It is interesting that pretreatment of cultured bovine granulosa cells with unlabelled hFSH for 24 hr at 37°C did not significantly decrease their ability to bind freshly added $^{125}$I-bFSH tracer (Fig. 24). This has caused us to raise serious doubts on the existence of "down regulation" of hormone receptors. It appears that in the gonodal FSH-receptor system, the mechanism of down regulation (desensitization) to decrease the responsiveness of target cell to hormone does not operate. However, the possibility exists that even though the level of FSH-receptor was not decreased after treatment with excess FSH, the responsiveness of the granulosa cells to further FSH stimulation could have been decreased by other mechanisms, such as uncoupling of the FSH receptors from their effectors.

(C) Solubilization of Porcine Testicular FSH Receptor

The porcine testicular FSH receptor has not been solubilized in the free form without losing its recognition-function. Ultrasound sonication was gentle to the FSH receptor and dispersed testicular membranes into a very fine suspension, but under high speed centrifugation the suspended particles could be pelleted. By dispersing the testicular membranes, however, sonication may facilitate their solubilization by other solvents. Treatments with organic solvents, especially with two-phase systems, generally resulted in irreversible denaturation of the testicular FSH receptor. If the organic solvent employed was very hydrophobic (e.g. pentane), the binding integrity of the membrane appeared to remain undisturbed but the receptor remained insoluble. On the other hand, more ionic solvents such as ethanol or ether destroyed the receptor-activity either by precipitating the receptor-proteins or by extracting some polar lipids which were essential for supporting hormone-receptor interaction. Amphiphatic solvents were generally effective

in solubilizing membranes but the solubilized receptor often lost its ability to bind hormone again. The n-butanol/water system was very effective in solubilizing the membranes and could easily be dialyzed away. Unfortunately, the FSH receptor was also unstable in this system.

The non-ionic detergent Triton X-100 has been used frequently for solubilizing various hormone receptors. However there are limitations of using this detergent. First, it has a very low critical micelle concentration, and cannot be dialyzed away easily. Secondly, receptors solubilized by this solvent are often visualized as huge micelles consisting of a mixture of integral proteins, receptors, lipids, and detergents. These micelles, having a stable conformation, may prevent further purification of the specific receptor molecules from these mixtures of contaminants, and may also aggregate upon standing. The exact mechanism by which Triton X-100 solubilizes receptor is uncertain, but is probably due to the ability of its amphiphatic structure to form micelles with the receptor molecule. It appeared that the Triton X-100-solubilized receptors did not pellet upon ultracentrifugation because they became lighter in density after their association with the Triton X-100. The instability of FSH receptors in Triton X-100 could be due to the gradual unfolding of the receptor-molecule or masking of the FSH binding-sites by the detergent molecules.

(D) Characterization of $^{125}$I-bFSH-Receptor Complex Solubilized by Triton X-100

In contrast to the free FSH receptor, the FSH-receptor complex was found to be relatively stable after solubilization. Although $^{125}$I-bFSH was shown being bound to the solubilized receptor, yet its binding was not

readily reversible (Fig. 27). It is interesting to observe that the antigenicity of $^{125}$I-bFSH to rabbit anti-bFSH antiserum was not diminished after being bound to the receptor (Fig. 31). The question remains whether anti-FSH antibody did "strip off" the bound $^{125}$I-bFSH from the $^{125}$I-bFSH-receptor complex or the whole complex was attached to the anti-bFSH antibody.

Though not conclusive, results of the present study indicated that a large receptor - $^{125}$I-bFSH-antibody complex was formed. After gel electrophoresis on 4% polyacrylamide gel at an alkaline pH (Fig. 33b), the receptor- $^{125}$I-bFSH-antibody complex showed an electrophoretic mobility intermediate between $^{125}$I-bFSH-antibody and $^{125}$I-bFSH-receptor complexes. Together with the fact that the maximum precipitation of the receptor- $^{125}$I-bFSH-antibody complex by polyethylene glycol (Fig. 31) and second antibody (Fig. 32) was slightly higher than the $^{125}$I-bFSH-antibody complex, the formation of a large receptor - $^{125}$I-bFSH-antibody complex was suggested.

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