PRIMATE TESTICULAR GONADOTROPIN RECEPTORS: CHARACTERIZATION AND FUNCTIONAL STUDIES

by

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D E D I C A T I O N

TO MY PARENTS,
SISTER (MARILYN) AND
BROTHER (WARREN)

ABSTRACT

This investigation was primarily concerned with the interaction of the ^{125}I-labeled human gonadotropins with testicular tissue from the human and from nonhuman primate species. The binding of the ^{125}I-hFSH and ^{125}I-hCG (or ^{125}I-hLH) to a particulate fraction (P1) of the primate testis was highly specific. Gonadotropin binding was competitively displaced by the synthetic estrogens and an inhibitory factor present in testicular extracts (140,000 x g supernatant). The biochemical properties of the gonadotropin receptor (testicular) interaction of the different primate species were similar in most respects. All tissues had a greater FSH than LH binding capacity with an apparent dissociation constant in the range of $10^{-10} - 10^{-11}$ M.

An FSH responsive adenylate cyclase was characterized in human testicular membranes. In the presence of a chemically deglycosylated derivative of FSH it was possible to uncouple the FSH responsive adenylate cyclase system. The synthetic estrogens were also effective inhibitors of the human testicular adenylate cyclase.

SOMMAIRE

Nous avons étudié principalement l'interaction des gonadotropines humaines marquées à l'iode^{125} avec le tissu testiculaire d'humains et de quatre espèces de primates non-humains. Nous avons observé que la liaison de la hFSH et de l'hCG (ou hLH) marquées à l'iode^{125} à une fraction sous-cellulaire de testicules de primate était hautement spécifique. La liaison de la gonadotropine est déplacée de façon compétitive par les estrogènes synthétiques et par un facteur inhibant que l'on retrouve dans les extraits de testicules (140,000 x g supernageant). L'interaction récepteur (testiculaire) gonadotropine possède des propriétés biochimiques similaires dans presque toutes les espèces de primates. Tous les tissus ont une capacité de liaison de la FSH supérieure à celle de la LH avec une constante de dissociation apparente située entre 10^{-10} - 10^{-11} M.

Dans les membranes testiculaires humains, nous avons caractérisé l'adenylate cyclase qui répond à la FSH. Nous avons pu découpler le système adenylate cyclase qui répond à la FSH en ajoutant un dérivé de la FSH déglycosylé chimiquement. Nous avons de plus observé que les estrogènes synthétiques étaient d'efficaces inhibiteurs de l'adenylate cyclase de testicules humains.

STATEMENT OF OBJECTIVES

The purpose of this study is to identify and directly compare several of the properties of the testicular gonadotropin receptors of various primate species. In addition, the properties of the human gonadotropin-adenylate cyclase system will be investigated.

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ABBREVIATIONS

Hormones:

A	Androstendione
ACTH	Adrenocorticotropic hormone
CG	Chorionic gonadotropin
*Cyclic AMP	Adenosine 3':5' cyclic monophosphate
DES	Diethyl stilbestrol
DES-NaP	Diethyl stilbestrol sodium phosphate
E2	Estradiol
E2-β	Estradiol benzoate
FSH	Follicle stimulating hormone or follitropin
GH	Growth hormone
ISO	Isoproterenol
LH	Luteinizing hormone or lutropin
LHRH	Luteinizing hormone releasing hormone
P	Progesterone
PMSG	Pregnant mare serum gonadotropin
PRL	Prolactin
PRO	Propranolol
T	Testosterone
TSH	Thyroid stimulating hormone or thyrotropin

Prefix to hormones:

b	bovine
e	equine
h	human
o	ovine
p	porcine
r	rat

*also referred to as cAMP

Monkey species:

Mfl	Macaca fascicularis or crab eating monkey
Mm	Macaca mulatta or rhesus monkey
Mn	Macaca nemestrina or pig tailed monkey
Pc	Papio cynocephalus or yellow baboon

Salts

$CaCl_2$	Calcium chloride
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
$MnCl_2$	Manganese chloride
KCl	Potassium chloride
NaCl	Sodium chloride

Units of measurement:

Ka	Apparent affinity or association constant
Kd	Apparent dissociation constant
n	Binding capacity
C	Centigrade
cpm	Counts per minute
Ci	Curie(s)
g	Gram(s)
x g	Gravitational force
h	Hour(s)
l	Liter(s)
m	Meter
min	Minute(s)
M	Molar
MW	Molecular weight
N	Normal
%	Percent
yr	Year(s)

Prefix to units of measurement:

C	Centi
f	Femto
u	Micro
m	Milli
n	Nano
p	Pico

Additional abbreviations:

ATP	Adenosine 5'-triphosphate
B	Bound
BSA	Bovine serum albumin
BTR	Bovine testicular receptor
CHO	Carbohydrate
C^1	Catalytic subunit
cDNA	Complimentary deoxyribonucleic acid
C.L.	Confidence limit
CK	Creatine kinase
CRP	Creatine phosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol or Cleland's reagent
EDTA	Ethylenediaminetetraacetic acid
EC_{50}	Effective concentration (50% displacement)
fig	Figure
ex	For example
FSK	Forskolin
F	Free
>	Greater than
GDP	Guanine diphosphate
GTP	Guanine 5'-triphosphate
GMP-P(NH)P	5'-guanylimido-diphosphate
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A reductase
$t_{\frac{1}{2}}$	Half-life
^{125}I	Iodine-125

<	Less than
MgATP	Magnesium adenosine 5'-triphosphate
mRNA	Messenger ribonucleic acid
MK	Myokinase
#	Number
^{32}P	Phosphorus-32
PEG	Polyethylene glycol
RTR	Rat testicular receptor
V	Rate or velocity of reaction
R	Receptor
RBI	Receptor binding inhibitor
N	Regulatory protein
NaF	Sodium fluoride
SB	Specific binding
SD	Standard deviation
SEM	Standard error mean
ie	That is
TLCK	1-chloro-3-tosylamido-7-amino-L-2-heptanone
Univ.	University
H_2O	Water

CHAPTER 1

GENERAL INTRODUCTION

1.1 Hormones

The importance of hormones in the regulation of the bodily functions of multicellular organisms was first defined by Claude Bernard (1). He used the term internal secretions to describe how the endocrine system adjusts and correlates the activities of the various body systems. The internal secretions functioned to maintain the constancy of the internal milieu (homeostasis). It remained for Bayliss and Starling to define the term hormone "as a substance produced in one part of the body and carried by the blood or lymph to some other part, the activity of which is thereby modified" (2).

1.2 Discovery of the Gonadotropin Hormones

The pituitary, a tiny organ surrounded by the sphenoid bone and covered with the sellar diaphragm, lies in a long cavity, the sella turcica, near the hypothalamus and optic chiasm (3). The dependence of the reproductive system upon the pituitary was conclusively demonstrated by the effects of ablation and/or replacement of the pituitary gland on gonadal growth and function. Smith demonstrated that hypophysectomy (ie. removal of the pituitary gland) resulted in gonadal atrophy accompanied by a complete loss of secretory function, as evidenced by regression of the accessory sexual structures. Implantation of rat pituitary tissue or administration of saline extracts of bovine anterior pituitary tissue were successful in restoring the deficiencies of the rat reproductive system (4). Pituitary tissue transplants were also capable of bringing normal immature mice and rats to precocious puberty (5, 6). These results suggested the presence of biologically active substances in the pituitary gland capable of promoting growth and of maintaining gonadal function.

Subsequent work led to the discovery and partial purification of two distinct and separate gonadotropic hormones from the anterior lobe of the pituitary gland (7). These hormones were named for their effects on the ovary (8). One of the gonadotropic hormones, luteinizing hormone (LH), is involved in the maintenance of the local and peripheral concentrations of the gonadal steroidal hormones which are essential for normal reproductive function and promotes the growth and maintenance of the corpora lutea. The second hormone, known as follicle stimulating hormone (FSH), regulates the processes concerned with germ cell development (9). A third gonadal stimulating material was discovered in the urine of pregnant women. This hormone, of placental origin, was recognized as being different from pituitary preparations and was termed human chorionic gonadotropin or hCG (10-12). Human chorionic gonadotropin is normally produced only by the female, not the male, at the time of pregnancy. It is necessary for maintaining the corpus luteum and stimulating steroidogenesis in the corpus luteum (13).

1.3 The Gonadotropic Hormones

The anterior pituitary hormones LH, FSH, and TSH (thyroid stimulating hormone) as well as the placental hormone hCG are closely related in structure in spite of their diverse physiological functions. The above hormones are glycoproteins consisting of a protein core with branched carbohydrate side chains. The protein backbone is composed of two nonidentical polypeptide chains held together by noncovalent interactions - hydrogen bonding and Van der Waals forces. One of the peptide chains, designated as the alpha or common subunit, is essentially identical for each hormone, and is highly conserved from species to species. The other subunit, designated as the beta subunit, is unique to each hormone and confers hormonal specificity to the molecule (9, 14). Thus subunit recombination, both interspecies and intraspecies, results in hybrids which assume the biological activity of the chosen beta chains. Both subunits are needed for binding and the full biological expression of the molecule (15, 16). These hormones are found in all mammals studied and hormones with similar properties have been observed in lower vertebrates (17).

1.4 Polypeptide Structure of the Alpha Subunit

The human alpha subunit is composed of 89 amino acids with two carbohydrate moieties attached to an asparagine amino acid at position #56 and #82 by an N-glycosidic linkage. The amino acid sequences of the alpha subunits of the gonadotropic hormones from several mammalian species are compared in Figure 1. While the amino acid sequences of the different gonadotropic hormone beta subunits within a given species diverge significantly (Fig. 2), the sequence of the alpha subunits are nearly identical with the exception of a two amino acid inversion and amino terminal heterogeneity. Heterogeneity at the amino terminus accounts for the presence of more than one form of the gonadotropic hormones. The gonadotropins exist as combinations of peptide chains of either 89, 90 or 92 amino acids. In the case of human LH, an 89 amino acid form predominates (95%). Human FSH and hCG exist primarily (60%) as a 92 amino acid peptide chain (14, 18). Among the different mammalian species, there is approximately 60% homology of the alpha subunits (Fig. 1). The majority of differences in amino acid residues at the various positions are due to a single base change in the codon (14). Recently, a single hCG alpha subunit gene was detected in human placental tissue. Using complimentary DNA (cDNA) clones encoding this alpha subunit, it was found that only a single gene is transcribed to make the alpha subunits for LH, FSH, hCG and TSH. The alpha subunit gene for hCG has been localized specifically on the short arm of chromosome 18 in tumor derived human JEG cells and normal lymphocytes (19). Whether the gene is also present on chromosome 6 must await confirmation of the data (19, 20). The existence of a single alpha subunit gene which has been conserved through evolution would explain the highly conserved structure of the different mammalian gonadotropin alpha subunits (21, 22).

As noted earlier, studies on subunit recombination indicated that the potency of the recombinant was primarily dependent on the beta subunit. It is now clear that the alpha subunit also contributes to the biological activity of the recombinant. Recombinants with a common beta subunit but different alpha subunits exhibited altered steroidogenic and receptor

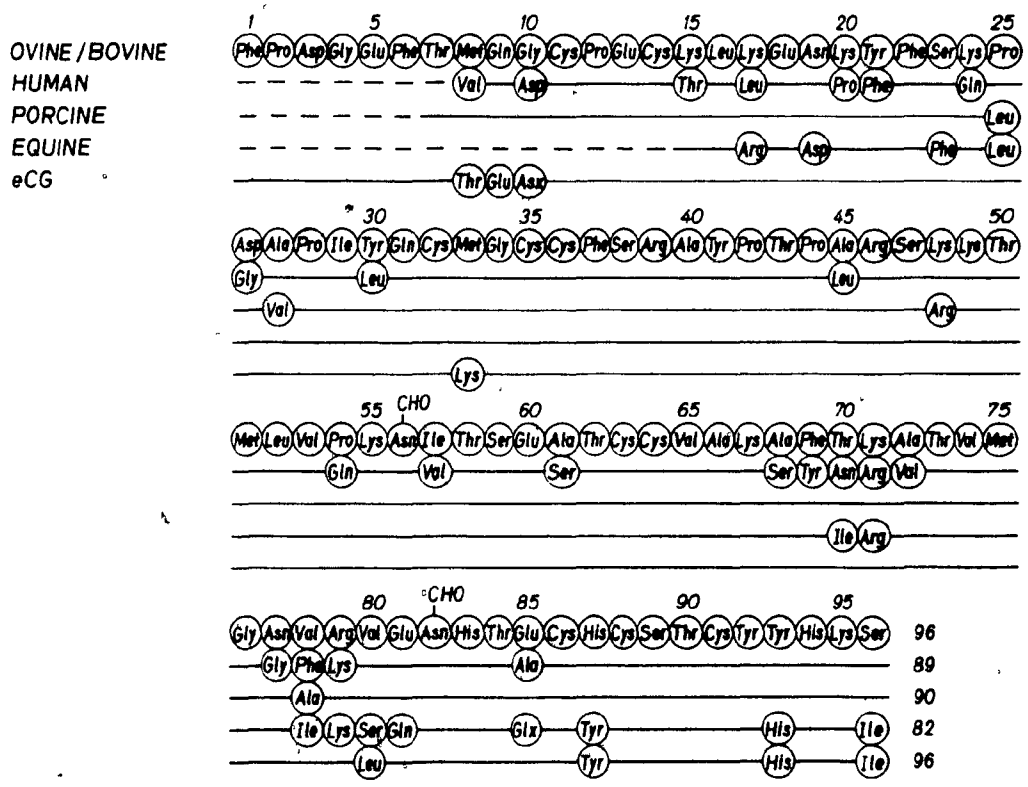


FIG. 1

Amino acid sequence of the alpha subunit of either LH and/or FSH from different species. CHO stands for carbohydrate moiety. The numbering of residues corresponds to the ovine/bovine hormones. Discontinuous lines show that the chains are shorter in the species as compared to ovine; solid lines show that the sequences are identical to ovine subunit. Only those residues that are different from the ovine are shown in the circles. (14)

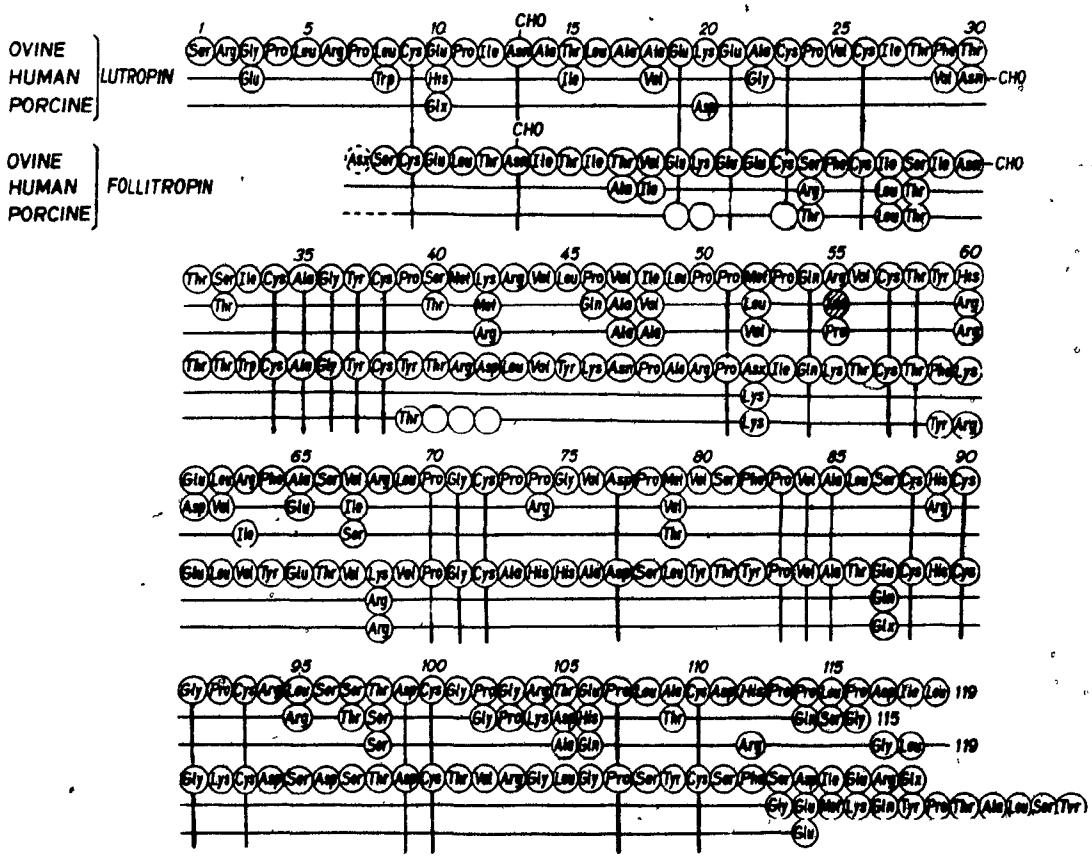


FIG. 2

Comparison of the structures of LH beta and FSH beta subunits of three species (14). The constant (C) and variable (V) regions (27) are as follows: V1 is 1-15, V2 is 39-55, V3 is 101-COOH terminus; C1 is 16-38, C2 is 56-100. The numbers correspond to ovine LH beta sequence.

binding activity (15, 16). Chemical modification of the amino acid or carbohydrate portion of the alpha subunit was capable of changing the potency of the hormone (14). Further, monoclonal antibodies to specific regions of the individual subunits of hCG interfered with hormone-receptor complex formation and blocked the biological activity of hCG (23, 24). These studies indicate that specific regions of the alpha and/or beta subunits are important for subunit interaction and/or receptor interaction. A summary of the amino acids which are known to be involved in subunit-subunit interaction and hormone-receptor binding is given in Table 1.

1.5 Polypeptide Structure of the Beta Subunit

The gene structure for the beta subunit is more complex than that of the alpha subunit gene. At least seven separate and distinct beta hCG or hCG like genes were identified in placental tissue. An eighth gene, which appeared similar to the hCG genes, turned out to code for the LH beta subunit (25). Whether all these genes are active in synthesizing the beta subunit is unknown at the present time. The similarity of the nucleotide sequence of the alpha and beta genes suggests that these two genes are related and probably have evolved from a common ancestor (26).

The human LH beta subunit is longer than the alpha subunit, being composed of 115 amino acids with the carbohydrate moieties attached to an asparagine residue at position #30 by an N-glycosidic linkage. There is considerable homology among the beta subunits. Human LH shares 50% homology between itself and the ovine, bovine, and porcine beta subunits and 82% homology with the beta subunit of hCG (Fig. 3). The beta subunit of hCG differs from that of human LH in having an extra 30 amino acids at the carboxy end and carbohydrate moieties attached to positions #13 and #30 at asparagine residues. Furthermore, the hCG beta subunit has four more points of carbohydrate attachment to serine residues near the extended carboxy terminal portion (14).

The human FSH beta subunit shares 85% homology with the beta subunits of ovine, bovine and porcine FSH (Fig. 4). The second half of the FSH

TABLE 1

MAPPING OF INTERSUBUNIT AND RECEPTOR BINDING SITES IN GONADOTROPINS[a] (14)

Residue[b]	Subunit interaction	Receptor binding	Subunit interaction	Receptor binding
Tyrosine/ phenylalanine	α-30, α-41, α-69	α-92	β-37, Cys-Ala-Gly-Tyr Tyr-59, Phe-82	—
Lysine	α-67	α-55, 95	None required	Not essential
Lysine/arginine	α-71	—	—	β-94
Methionine	—	α-51 or α-75	—	β-41
Carboxylic groups	Essential	—	—	—
Cystine	—	α-11-35	β-93-100	—
Histidine	—	α-94	—	—
Tryptophan	—	—	β-33 (follitropin)	—

[a] Most of the data are derived from studies on lutropin.

[b] Numbering corresponds to sequence of α and β subunits in ovine lutropin.

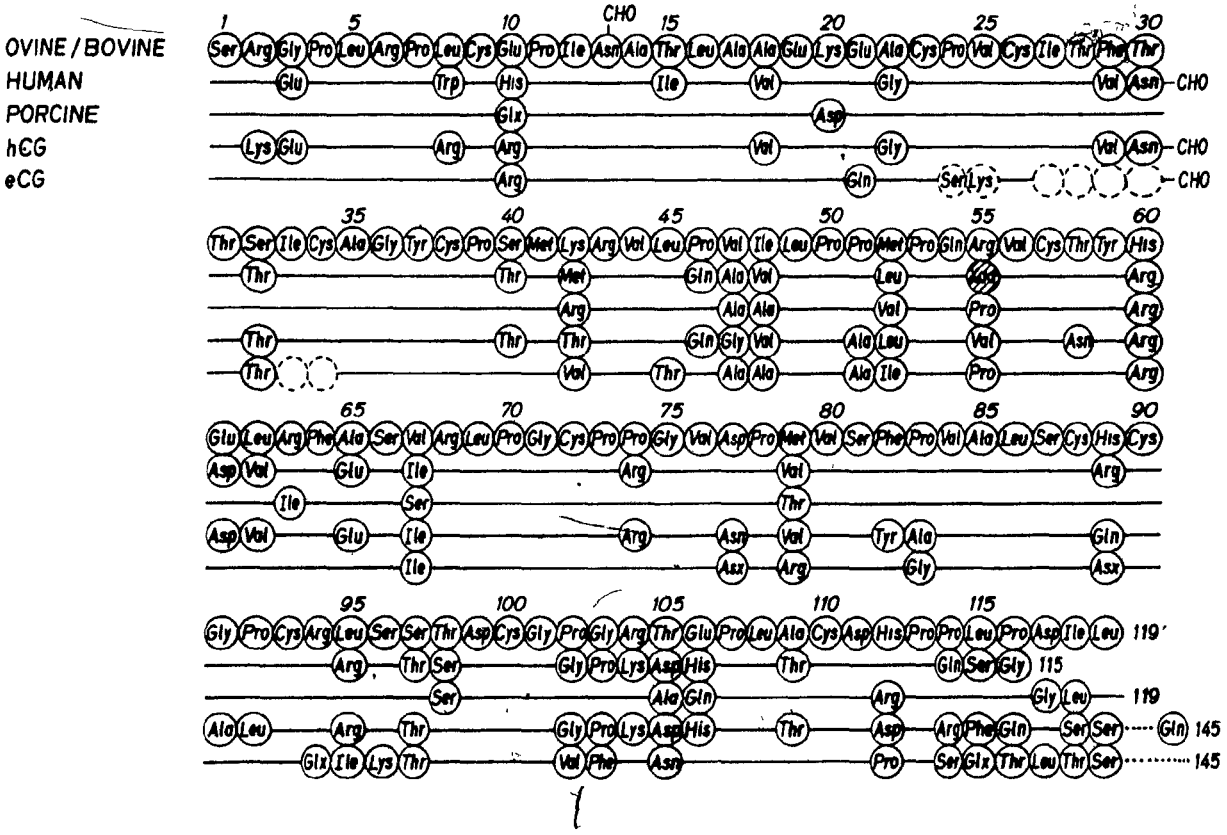


FIG. 3

Comparison of the LH β subunit sequences (14). Xaa indicates a gap in the sequence; CHO is a carbohydrate moiety. Some segments of the eCG β sequence are incomplete and shown by dotted lines. The linkages and the number of oligosaccharide units in the extended C-terminal piece of eCG are unknown at present. Most LH β subunits have a CHO moiety at position 13 but in human this is at 30; hCG β and eCG β have CHO units attached at this site also.

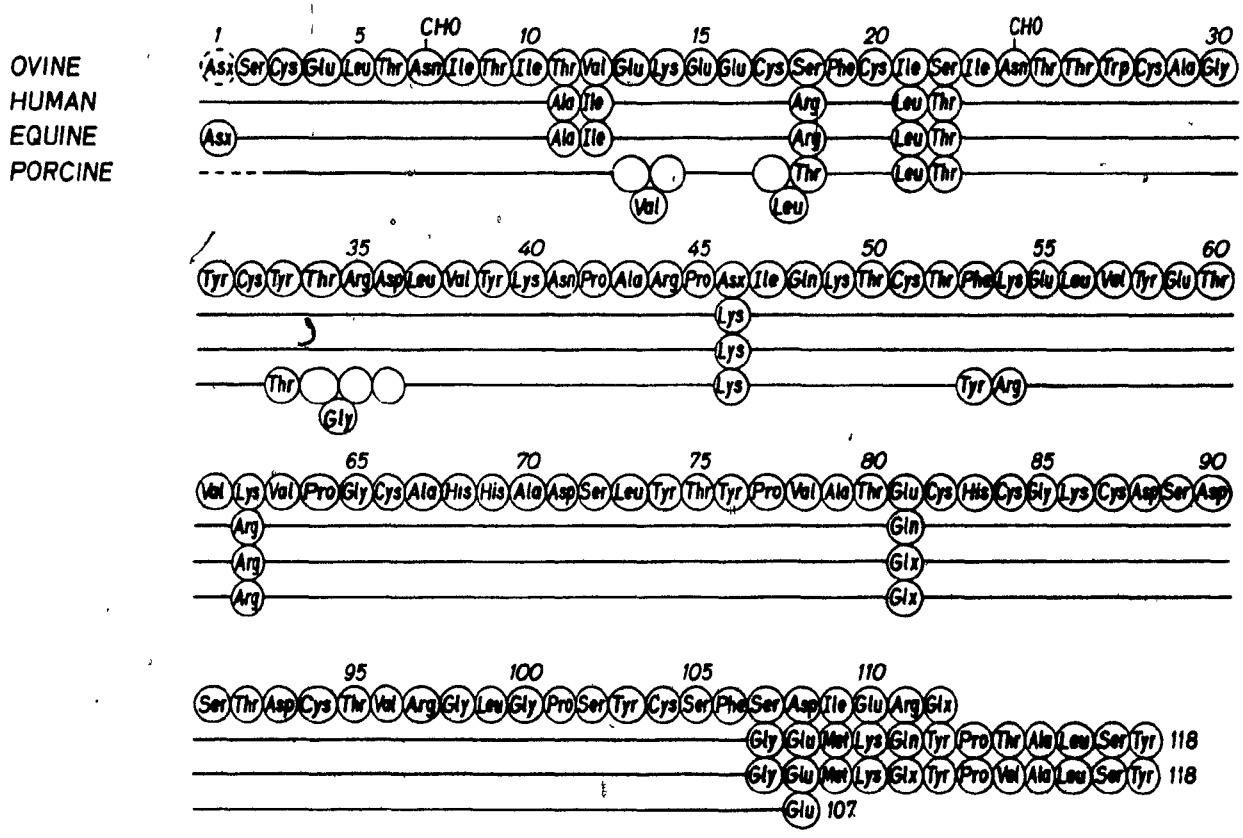


FIG. 4

Comparison of FSH β of different species (14). The reported porcine sequence contains three extra amino acid residues that are placed just below the main sequence. The amide assignments are not fully clarified in the ovine, porcine, and equine hormones. In those positions where their identity is known in at least two species, it is assumed that such residue positionsare the same in the unclarified sequence. Linkage of the two carbohydrate moieties is identical in all sequences.

beta subunit, except for the carboxy terminal portion, is identical in all four mammalian species. Human FSH is composed of 118 amino acids with two carbohydrate moieties attached to an asparagine residue at positions #7 and #24 (14).

The human alpha and beta subnits are highly crosslinked internally with five and six intrachain disulfide bridges respectively. There are no interchain disulfide bridges. The twelve half cysteine residues of the beta chain of LH, FSH, TSH and hCG occupy identical positions suggesting that the disulfide bridges in these molecules are very similar. The high degree of crosslinking of the gonadotropin beta subunits is thought to be responsible for preserving the structural and/or conformational integrity of the active site (14). Recently, two constant and three variable regions have been described in the amino acid sequence of the gonadotropic hormones. The two constant or highly homologous regions have been considered responsible for the subunit - subunit interactions, which in turn confers the structural specificity required for the interaction of the alpha subunit with the receptor. The adjacent variable or heterologus regions could be responsible for the differences in the hormone specific conformation of the constant regions and so determine receptor binding specificity. When the beta subunits of various species of LH (human, bovine, ovine and porcine) were compared, an increased amount of conservation of sequence was found in the second variable region (V2) (Fig. 2). It was observed that nine out of a possible twelve variable positions became conserved (27). Thus the V2 region of the different LH beta subunit sequences is likely a region responsible for determining the specificity of the hormone's interaction with the receptor. Another hypothesis, the determinant loop hypothesis, suggests that receptor specificity is determined by the nature and charge of the amino acid residues in an octapeptide determinant loop formed by a disulfide bond between cystine #93 and #100 of the beta subunit of ovine LH (28). This loop has been seen in the beta subunit of bovine LH and TSH and in human chorionic gonadotropin. Further investigation will be needed to resolve the question of which sites in the gonadotropic hormones are necessary for determining receptor specificity.

1.6 Carbohydrate Moieties

The gonadotropic hormones contain various amounts of carbohydrate covalently attached to the polypeptide chains. The amount of carbohydrate moieties varies from a low of 16% in bovine LH to a maximum of 45% in pregnant mare serum gonadotropin. The oligosaccharide (carbohydrate) portion of the gonadotropin hormones are made up of the following sugars: D-mannose, D-galactose, L-fucose, N-acetyl neuraminic acid (sialic acid), N-acetylated D-glucosamine and N-acetylated D-galactosamine. The exact structure of the oligosaccharide portion of the molecule is known for a few, but not all the gonadotropic hormones. As indicated in Figure 5, the number and arrangement of the carbohydrate moieties varies for each hormone. The alpha subunit of all gonadotropins contains two N-linked oligosaccharide units attached to an asparagine residue. Depending on the hormone, the beta subunit contains one or two oligosaccharide units bound to asparagine by an N-glycosidic linkage. However, in the case of hCG there are six oligosaccharide units. Two of them are N-glycosidic linkages to asparagine residues and the remainder are O-glycosidic linkages to serine residues (14). The N-linked oligosaccharide moieties of the pituitary hormone LH differs from those of placental hCG by their composition (14), greater resistance of LH to glycosidases (29), and the presence of sulfate linked to the nonreducing termini in LH but not hCG (29, 30). It has been proposed that the presence of sulfate is to compensate for the lower sialic acid content of LH. The sulfate is thought to protect the terminal hexosamine residues from chemical and enzymatic degradation (30). The functional role of the carbohydrate moieties has been assessed primarily by the effect of removal of various carbohydrate residues on hormonal functions (14). These studies have shown that the structure of the oligosaccharide chain is important in determining the circulating half-life (31, 32), solubility in aqueous solutions (33, 34), and biological responses (35-39) to the gonadotropins. There is no doubt that the carbohydrate moiety plays an important part in gonadotropic hormone activity and further assessment of the function of the carbohydrate moieties will lead to a greater understanding of the mode of action of the gonadotropins.

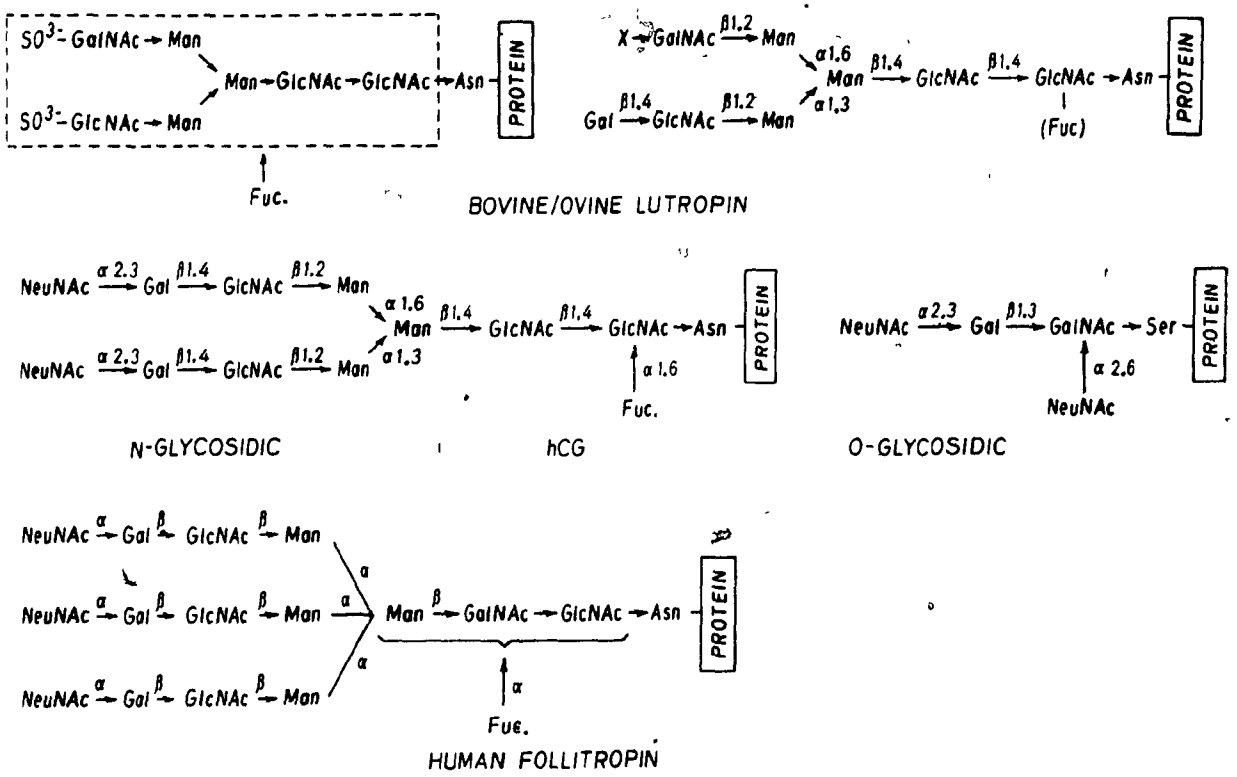


FIG. 5

Proposed arrangement of the monosaccharide units in the carbohydrate moiety of the glycoprotein hormones. Although not every glycopeptide in the α and β subunits of the different hormones has been extensively studied, it is believed that the basic structural unit is the same. This cannot explain the difference in the carbohydrate content between hormones such as LH and FSH from within the same species (14).

1.7 Receptors

Cellular recognition is a process by which cells interact with and respond to molecular signals in their environment. The ability of target cells to receive and translate signals is dependent on the existence of specific binding sites known as receptors. The "receptor concept" was introduced by Langely (40), at about the same time that Starling (2) introduced the term hormone, to explain the action of nicotine and curare on skeletal muscle. Langely defined receptors as "substances which are acted upon by chemical bodies and in certain cases by nervous stimuli." The term receptor was coined by Ehrlich as the hypothetical specific chemical groupings of protoplasm upon which chemotherapeutic drugs were assumed to act (41). The next significant contribution was the formulation of the occupancy theory by Clark and Gaddum (42, 43). This theory stated that the intensity of the pharmacological effects is directly proportional to the number of receptors occupied by the drug. Therefore the effect of the drug increases proportionally to the number of occupied receptors and becomes maximal when all the receptors are occupied. This was an important concept is establishing the receptor theory on a more firm quantitative basis and stimulated further experimental testing and refinements.

Much of our early knowledge of receptors was based on pharmacological studies into the selective action of drugs because purified hormones were not available. Even with the isolation of the pituitary gonadotropic hormones in high yield and quality (44-46), little progress was made in the characterization of the gonadotropin receptors. The discovery of a gentle labeling procedure for peptide hormones which did not destroy the hormone's biological activity (47, 48) and the development of the radioimmunoassay technique (49, 50) provided a means for the direct biochemical characterization of receptors. The application of these techniques has enabled investigators to make great strides in the isolation and purification of receptors and led to a better understanding of the mechanism of receptor regulation.

1.8 Receptor Function

The receptor serves two main functions. The first is to recognize and distinguish a particular signal, for example LH, from the mixture of hormones and other molecules in the circulation which impinge on the target cell. Subsequent to binding, the second function of the receptor is to transfer the signal in such a way that it leads to the appropriate physiological response. A number of criteria have been established to define hormone receptors (51). These criteria are listed below.

1) The binding sites only interact with a specific hormone or class of hormones. Substances, such as LH, which have no effect on a particular cell (ex: the Sertoli cell) will not bind to its receptors (ex: the FSH receptor) or competitively displace the native hormone (ex: FSH) from its receptor at normal physiological concentrations. Significant numbers of binding sites are only found in tissues which respond to the hormonal signal (52). These tissues are referred to as target tissues.

2) The binding sites have a high affinity for their respective hormones. The blood levels of many hormones are normally very low, usually 10^{-11} - 10^{-9} M for the gonadotropins, so the receptor must have a similar affinity in order to detect these substances (53).

3) Presence of a finite number of binding sites. The biological response (ex: testosterone production) to a hormone (ex: LH) is a saturable phenomenon (53, 54). Therefore if the formation of hormone receptor complexes is obligatory for the production of a biological response, then the number of receptors should be limited. This can be demonstrated by saturating the receptor with its respective hormone (53).

4) Interaction of the hormone with its binding site should be rapid and is usually reversible. This is assessed by analyzing the kinetics of association and dissociation of a radiolabeled hormone with its receptor (53).

The gonadotropin binding sites in the testis and ovary satisfy the above criteria to be classified as receptors. There are a finite number of binding sites and binding is specific, reaching equilibrium within

1-2 hours, and reversible. Gonadotropin receptors have been studied in a variety of species, and although species related differences exist, they share many binding characteristics (53). Cellular receptors can be subdivided broadly into two main types - intracellular receptors and membrane bound (extracellular) receptors, both of which are discussed in the next section.

1.9 Intracellular Receptors

Intracellular receptors are located in the soluble intracellular compartment of the cell, ie. both in the cytoplasm and the nucleus. Steroid hormones and amino acid derivatives (ex: thyroid hormones) enter target, as well as nontarget tissues, most likely by the process of passive diffusion (55, 56). The mechanism of action of progesterone in the chick oviduct is illustrated in Figure 6. Once inside the cell, the hormone is rapidly bound by its cytoplasmic receptor. Formation of the hormone receptor complex leads to a temperature dependent activation of the complex. If the complex is not activated or transformed, it will not be capable of binding to the nuclear receptor in the nuclear chomatin. Inside the nucleus, interaction of the activated hormone receptor complex with specific nuclear acceptor sites allows the hormone to directly modify transcription and regulate the levels of specific messenger RNA's (mRNA's). The new mRNA's are released into the cytoplasm where they regulate new protein synthesis (55, 56). It is the newly synthesized proteins which exert the physiological hormone's actions leading to altered cell function. Thus glucocorticoids and progesterone alter cell function through the synthesis of new cell products - aminotransferases and avidin respectively (56, 57).

1.10 Cell Membrane Receptors

Polypeptide hormones, free fatty acids, prostaglandins and catecholamines, unlike steroid hormones, cannot penetrate the cell plasma membrane. Instead they bind to specific sites (receptors) located on the plasma membrane of the target cell. For most polypeptide hormones

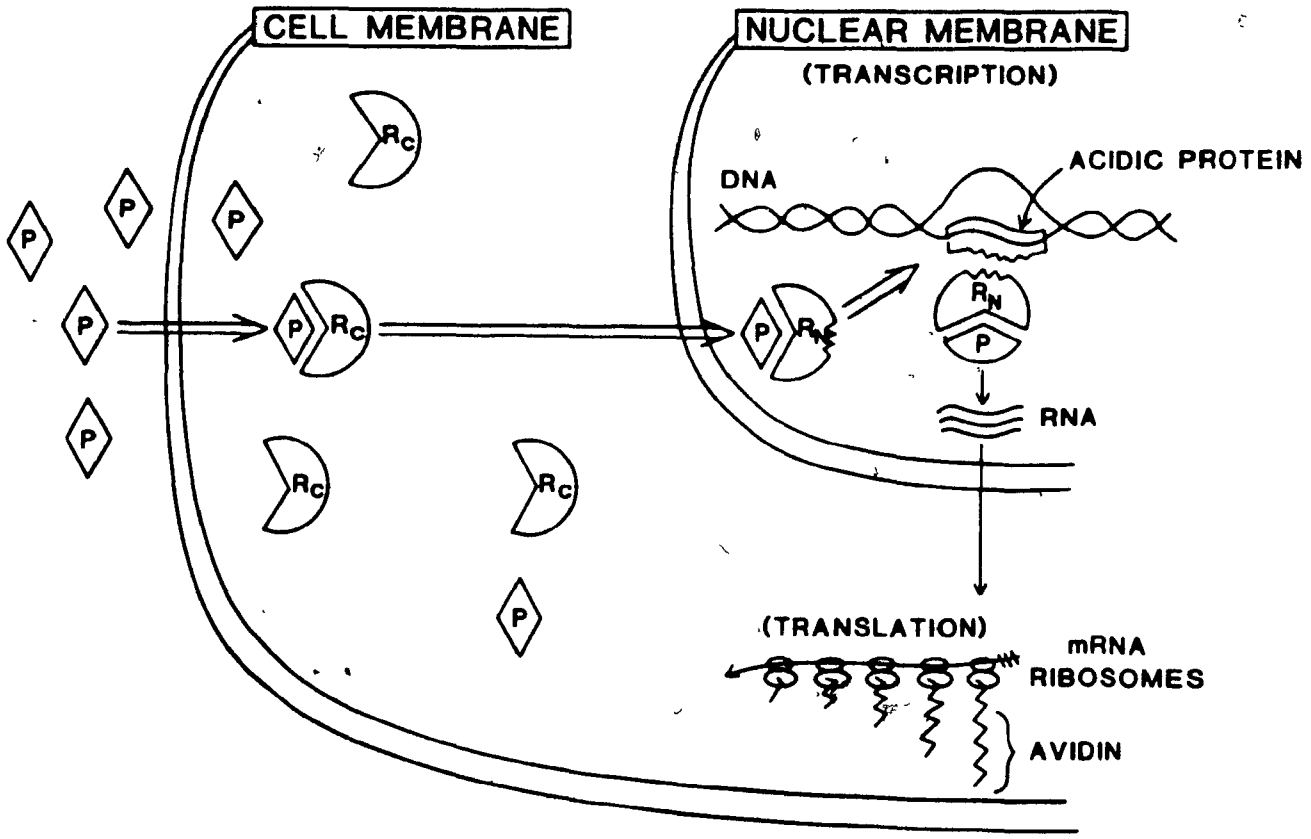


FIG. 6

General model of steroid hormone action, showing binding of hormone [in this case progesterone (P)] to cytoplasmic receptor (R_c), translocation (arrow), and induction of gene transcription by nuclear receptor (R_N) to produce messenger RNA (mRNA), which is translated on cytoplasmic ribosomes to synthesize new protein [in this case avidin, a protein whose synthesis is induced by progesterone in the chick oviduct (56)].

(ex: gonadotropins), formation of the hormone-receptor complex results in the activation of a membrane bound enzyme, adenylate cyclase. Adenylate cyclase catalyzes the conversion of adenosine 5'-triphosphate (ATP) to adenosine 3'-5' cyclic monophosphate (cyclic AMP). Cyclic AMP acts as a second messenger for the hormone's action (primary message). The catalytic component of cyclic AMP binds to the regulatory subunit of the enzyme protein kinase. The cyclic AMP regulatory subunit dissociates leaving activated catalytic subunits of the enzyme. These protein kinases phosphorylate specific serine and threonine residues of specific proteins within the cell to produce a hormone induced response characteristic of that cell (58) (Fig. 7). For other hormones, such as insulin and prolactin, cyclic AMP is not involved and they are presumed to work through the action of an as yet unidentified second messenger. Preliminary work suggests this factor is a low molecular weight peptide (approximately 2000 daltons) which works through dephosphorylation of regulatory enzymes and nuclear proteins (59-61).

1.11 Gonadotropin Receptors

The idea that the testes are the primary site of biological action for the gonadotropic hormones in the male was suggested by the ability of purified gonadotropin preparations to cause morphological changes or to increase hormone secretion of testicular cells (62, 63). A direct interaction between the gonadotropins and testicular cells was demonstrated using fluorescent and histochemical techniques. This group of investigators were able to show that labeled ovine LH and ovine FSH were preferentially localized in the Leydig cells and Sertoli cells respectively of the rat testes (64, 65). These in vivo studies on the cellular distribution of the gonadotropin binding sites in the male gonad were subsequently confirmed by a variety of techniques such as radioautography and binding studies (66-70). The presence of specific testicular FSH (71-76) and LH (75-80) receptors have now been described in a wide variety of laboratory and domestic animal species. Spermatocytes, like Sertoli cells, have also been found to contain FSH receptors (69). In the

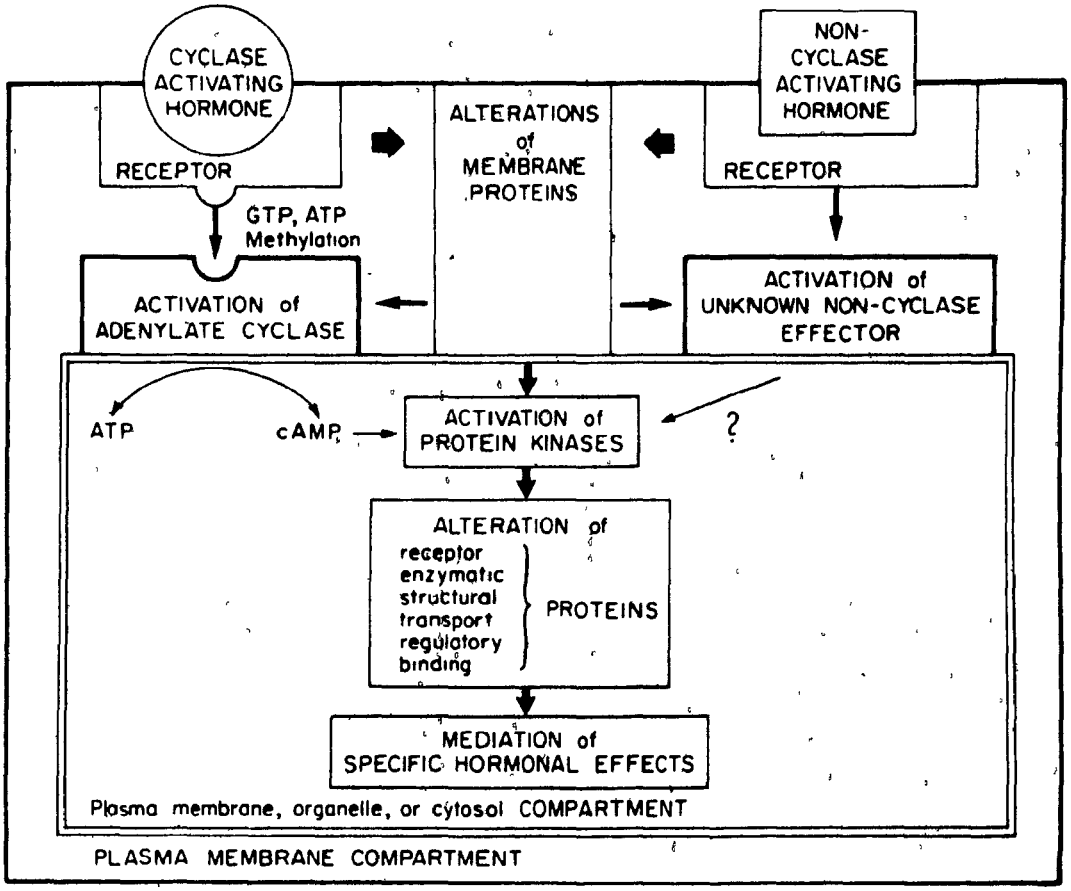


FIG. 7

A scheme of the mechanisms of hormone actions in cells (58).

female, LH receptors have been demonstrated on ovarian thecal cells, ovarian interstitial cells, granulosa cells of large follicles and the corpus luteum. Receptors for FSH are localized on granulosa cells of small follicles (81). The LH receptor does not distinguish a difference in the gonadotropic hormones LH and hCG. Both gonadotropins are bound to the same site in testicular (82) and ovarian (83) tissue with essentially identical binding affinities. This is consistent with their similar molecular structures and biological effects (9). As stated earlier, the gonadotropin receptors fulfill the criteria of functional receptors. Gonadotropin binding capacity changes with increasing age and physiological state of the animal.

Detailed characterization studies of the gonadotropin receptor have been hampered by the small quantities of starting material available for investigation, low yields and poor stability of the isolated receptor fractions. Early studies on the testes and ovary suggested that the molecular weight (MW) of the detergent solubilized gonadotropin receptor was approximately 200,000 daltons (84-86). Over the years a greater amount of progress has been made on the elucidation of the structure of the LH(hCG) receptor than the FSH receptor. The rat testicular LH receptor (87-89) is thought to be composed of a dimer of two subunits of identical molecular weight (79,000-90,000 daltons). Similarly in rat ovarian extracts, a 90,000 dalton subunit of the LH receptor has been detected after purification by affinity chromatography (90). The presence of three hormone (hCG) binding components in porcine ovarian tissue suggests a greater complexity to the subunit nature of the LH(hCG) receptor (91). The differences in the reported subunit composition and molecular weight of the LH(hCG) receptor could be a reflection of a species or sex difference, differences in solubilization techniques, receptor isolation techniques, or methods of radiolabeling the hormone-receptor complexes. The possibility that these binding components were generated due to proteolytic enzyme cleavage can not be excluded. Recently, the availability of a large and constant source of extracts

of bovine corpus luteal tissue has enabled Dattatreyamurty et al. to publish the most extensive study on the structure of the LH(hCG) receptor to date (92). The extracts were initially separated by a discontinuous sucrose density gradient ultracentifugation. Fractions were then solubilized in detergent (Triton X-100) and purified on a series of gel chromatography columns. The final (active) hormone binding fraction was resolved by zone electrophoresis on a cellulose column as a single band of 5.9 million daltons. Pretreatment of this material with SDS and a disulfide reducing agent (mercaptoethanol or dithiothreitol) separated it into various fragments. The authors suggest that the bovine LH/hCG receptor exists as a 5.9 million dalton complex composed of an aggregate of a 280,000 dalton species. Each 280,000 dalton species is thought to be made up of two identical 120,000-140,000 dalton units, each of which contains two functional subunits of 85,000 and 35,000 daltons linked by disulfide bonds. This model accounts for the fractions isolated in their study and those of previous investigators (87-91). Amino acid and carbohydrate analysis (Table 2) of the 5.9 million dalton species indicated that it is a glycoprotein containing approximately 10% carbohydrate and rich in the amino acids glutamic acid and aspartic acid. The low cysteine content of the LH(hCG) receptor would seem to indicate that bovine LH, which contains a greater amount of cysteine (Fig. 1 and 3), is not a contaminant of the receptor preparation. The glycoprotein nature of the gonadotropin receptor had been suggested by earlier studies demonstrating an alteration in hormone binding ability after chemical and/or enzymatic modification of the gonadotropin receptors. For example, treatment with proteolytic enzymes and neuraminidase, which affect the protein and carbohydrate portion of the gonadotropin receptor respectively, led to a change in hormone binding ability (92, 93). In addition, phospholipids, such as phosphatidylserine and phosphatidylethanolamine, and gangliosides are thought to be important directly as components of the gonadotropin receptor or indirectly by preserving the conformational structure of the receptor (93-95).

TABLE 2

AMINO ACID AND CARBOHYDRATE ANALYSES OF THE BOVINE CORPUS LUTEUM LH/hCG RECEPTOR (GRAMS/100 G OF PROTEIN)

	Zone electrophoresis purified LH-hCG receptor
Amino acid	
Aspartic acid	9.1
Threonine	5.3
Serine	5.8
Glutamic acid	12.1
Proline	5.2
Glycine	4.5
Alanine	4.8
Valine	8.3
Cysteine	
Cysteic acid	2.4[b]
Methionine	2.3
Isoleucine	4.4
Leucine	8.7
Tyrosine	4.9
Phenylalanine	5.0
Lysine	7.0
Histamine	2.8
Arginine	7.4
Carbohydrate	
Fucose	ND[a]
Mannose	1.1
Galactose	1.6
N-Acetylglucosamine	3.7
N-Acetylgalactosamine	2.6
Sialic acid	1.9

[a] ND, not detectable

[b] Determined on a separate aliquot after performic acid oxidation.

1.12 Soluble Receptors

Various investigators have reported the presence of gonadotropin binding components in the supernatant fraction of low ionic strength buffer extracts of gonadal tissues (96-98). These soluble fractions or receptors specifically bound ^{125}I-radiolabeled gonadotropins and shared many of the physicochemical properties of particulate and detergent solubilized receptors (99, 100). Soluble receptors have also been reported for insulin (101), growth hormone (102), and thyroid stimulating hormone (103). Solubilization in the absence of detergent has several advantages. Many detergents disrupt protein conformation and possibly hormone binding (104). Detergents can form a complex with the labeled hormone which can be mistaken as a hormone receptor complex (105). The drawback to nondetergent solubilization is the low yield (98) of material (5-10%) and the presence of proteolytic enzymes in the soluble fraction (92). In the luteinized rat ovary, five molecular components (12,000-160,000 daltons) of the LH(hCG) receptor were resolved after water extraction (98). Unlike previous reports of only a 90,000 dalton component of the LH(hCG) ovarian receptor after detergent solubilization (85, 90), these investigators detected all five forms in ovarian tissue extracted either by water or detergent (98). Both the water and detergent solubilized components were bound to concanavalin A sepharose and eluted as four well defined peaks of activity (99). This suggested that the LH(hCG) receptor is a glycoprotein, with a marked degree of micro-heterogeneity. The distribution of binding activity per individual peak was different probably due to carbohydrate differences. For example in fraction two and five the 12,000 MW receptor species had the greatest binding activity, while the 81,000 MW receptor species had the highest binding activity in fractions three and four. This study proposed that the rat ovarian LH(hCG) receptor exists as a polymer of the 12,000-81,000 molecular weight species. The soluble receptor could be derived from the cytoplasm, a part of or attached to the cell membrane and released upon homogenization, products of synthesis, or products of degradation. Two water soluble components of different sizes were released by the rat

testes (52,000 and 24,000 daltons) and rat ovary (46,000 and 24,000 daltons). The release of these components could be inhibited with thiol-group blocking agents and EDTA (106). Therefore it's possible that these components were generated by proteolysis of the native receptor as has been suggested for other receptor systems (107-109). If true, proteolysis may play an important role in the regulation of receptor function. Further investigation is needed to clarify the structure and biological relevance of the solubilized gonadotropin receptor and prove it is not an artifact of tissue preparation or proteolysis.

1.13 Intracellular Receptors

Subcellular fractionation of bovine corpora lutea and analysis of fractions for binding with radiolabeled ^{125}I-hCG revealed the presence of intracellular binding sites for hCG (110). The specific binding of ^{125}I-hCG to the plasma membranes, rough endoplasmic reticulum, and Golgi apparatus indicated a single population of gonadotropin binding sites. Nuclear membranes and lysosomes appeared to contain two populations of independent binding sites. The highest concentration of LH(hCG) receptors was found in the plasma membrane (135 fmol/mg protein) and heavy region of the Golgi apparatus (164 fmol/mg protein). The various subcellular organelle LH(hCG) glycoprotein receptors were of the same size (200,000 daltons) and shared many ^{125}I-hCG binding characteristics such as pH optimum, and binding affinity. This would seem to indicate that the same LH(hCG) receptor is present in these subcellular organelles. The slight differences in their binding properties is probably a consequence of the local environment imposed by the surrounding membrane macromolecules and/or alteration of the binding site during organelle processing. The validity of these intracellular gonadotropin binding sites was verified by measuring various marker enzyme activities to ensure there was no plasma membrane contamination or cross-contamination among the different organelles. A similar intracellular distribution of LH(hCG) receptors has been found in the human (111) and rat ovary (112). Intracellular receptors have also been reported for other hormones such as insulin (113, 114), growth hormone (114), and insulin-like growth factor (115).

The function of these intracellular receptor sites is unknown but they may play a role in the biosynthesis and subsequent transport of receptors to the cell surface, degradation of receptors, recycling of receptors from the cell surface to intracellular sites and vice versa, or to recognize hormones at these sites (111, 116).

1.14 Mechanism of Action of Gonadotropins

As mentioned previously, the primary event in the action of the gonadotropic hormones is to interact with specific target cell surface receptors. This interaction results in the activation of the gonadal membrane bound enzyme adenylate cyclase and inhibition of the cytosolic enzyme phosphodiesterase. These two enzyme responses lead to the generation and maintenance of high intracellular cyclic AMP levels (117-120). Acting as a second messenger, cyclic AMP activates specific enzyme systems to produce the characteristic biological target cell response (Fig. 8). The hormone-sensitive adenylate cyclase complex is composed of at least three components - a hormone receptor (R), a catalytic subunit (C^1), and a regulatory protein (N) capable of binding GTP or its analogues (121). Although both the receptor and the enzyme, adenylate cyclase, are located in the plasma membrane, they are two separate and distinct entities. This has been shown by their separate elution on gel chromatography (122, 123), the ability to transfer receptors to a cell lacking that particular receptor by cell fusion to generate a functionally active receptor-cyclase system (123, 124) and desensitization (rat ovary) which caused a reduction of receptor activity independently of any affect on adenylate cyclase activity (122). The regulatory component has also been purified by gel chromatography as a separate entity composed of two subunits of 42,000-52,000 daltons and 35,000 daltons (121, 125). The regulatory protein links the hormone-receptor interaction to activation of adenylate cyclase. Guanine nucleotides function as regulators of the receptor-coupled adenylate cyclase system. It is proposed (Fig. 9A) that the main function of the hormone receptor complex is to promote the exchange of guanine

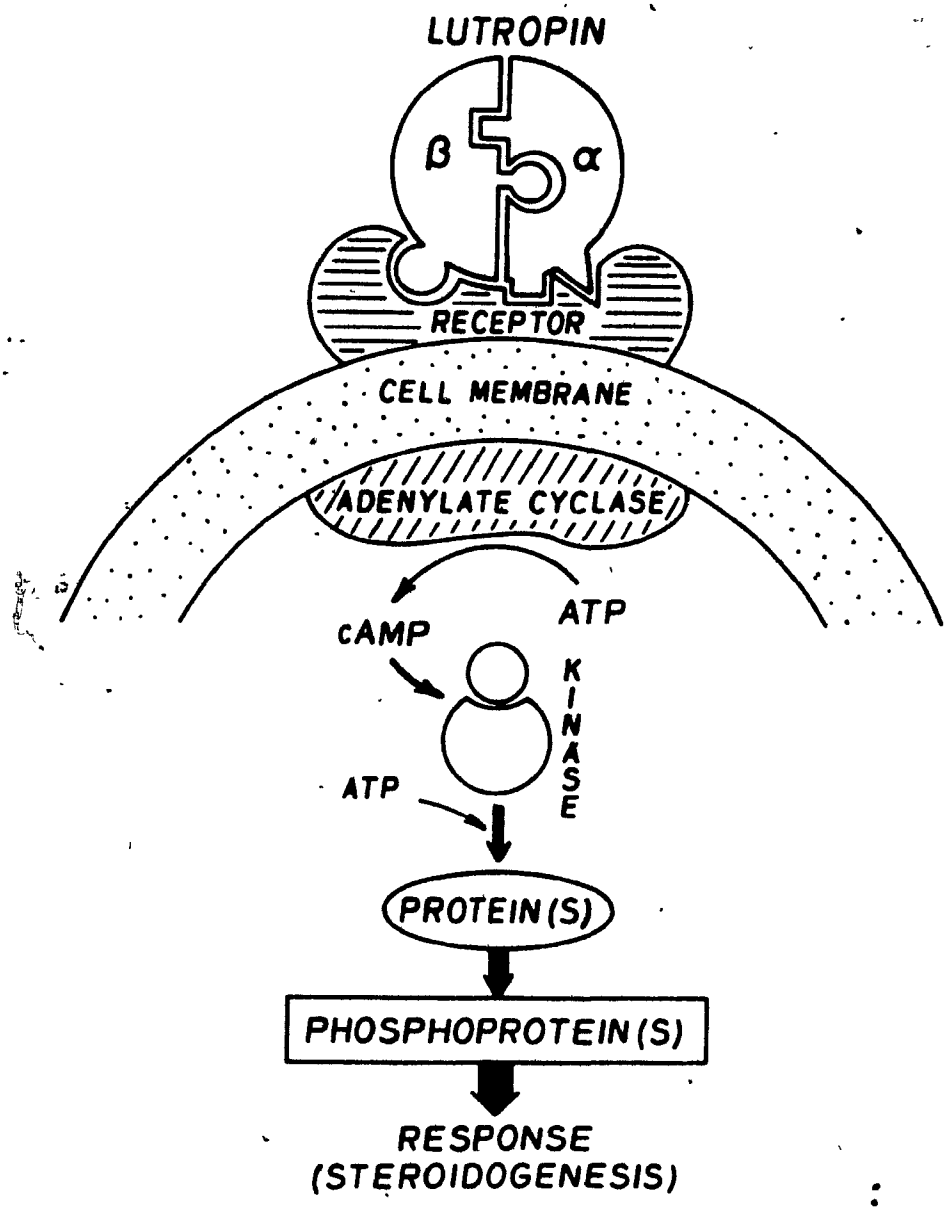


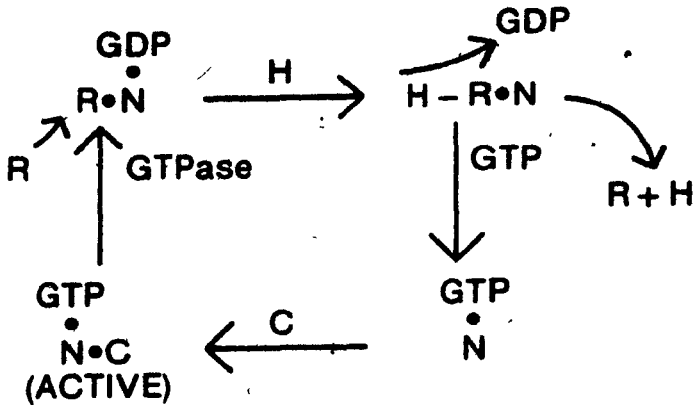
FIG. 8

Action of LH on the Leydig cell (14). The hormone is depicted as having three binding sites (two in α and one in β) in its interaction with the receptor. The increased size of the arrows signifies amplification of the initial hormone signal at each step. A similar mechanism of action has been proposed for FSH.

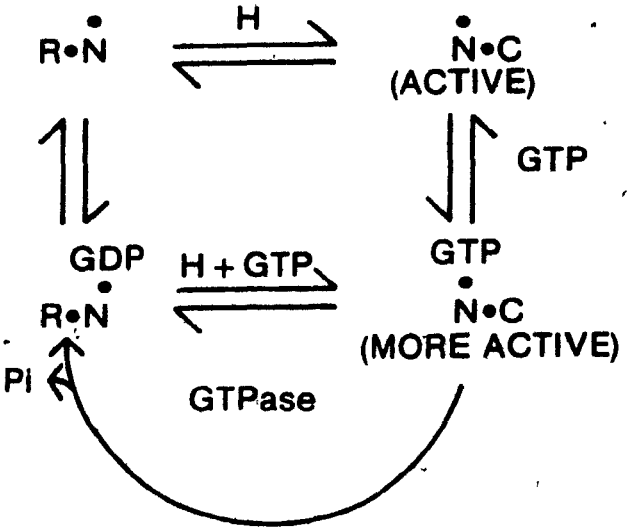
FIG. 9

Proposed mechanisms for activation of adenylate cyclase (128). A. Serial model. Active state of enzyme dependent on guanosine triphosphate bound form of G unit which associates with catalytic unit (C). Hormone binding to receptor functions merely to facilitate exchange of guanine nucleotide on G unit. B. Equilibrium model. All forms of enzyme are in equilibrium (equilibrium constants not necessarily unity). Hormone influences conversion of inactive enzyme to active enzyme whether guanine nucleotide bound or not. The bound form nevertheless is postulated to be more active than the form not complexed with guanine nucleotide.

A


GDP
R•N
H
H – R•N
GDP
R
GTPase
GTP
R + H
GTP
N•C
(ACTIVE)
C
GTP
N

B


R•N
H
N•C
(ACTIVE)
GTP
GDP
R•N
H + GTP
GTP
N•C
(MORE ACTIVE)
Pi
GTPase

triphosphate (GTP) for guanine diphosphate (GDP) on the regulatory protein. Binding of GTP leads to activation of the catalytic unit, whereas hydrolysis of GTP terminates activation of the enzyme complex (126). An alternative mechanism has been proposed for hormonal regulation of adenylate cyclase. This model by Abramowitz et al. (Fig. 9B) maintains that the enzyme exists in two conformations, active and inactive, and that the equilibrium between the conformations is influenced by the hormone. Therefore the hormone can influence the equilibrium of the enzyme from an inactive to an active state independently of the binding of an activated guanine nucleotide (127, 128). Resolution of the correct model will depend on whether there is a natural endogenous hormone independent rate of conversion of inactive adenylate cyclase to an active state bearing no bound effective guanine nucleotide.

Generation of cyclic AMP leads to the stimulation of specific protein kinases in the Leydig cell (129) and Sertoli cell (117). These cyclic AMP dependent protein kinases phosphorylate proteins whose specific function and location is still unknown (130, 131). The phosphorylated enzymes or structural proteins presumably alter the function of the cell in such a way as to generate the characteristic biological response of the target cell to the hormone (132).

1.15 Biological Actions of the Gonadotropins

The biological function of the pituitary gonadotropins is to stimulate the maturation and function of the testes and ovary and to regulate gonadal gametogenesis and steroidogenesis (9). In the testes, the gonadotropins LH and FSH exert part of this control through specific compounds secreted, under appropriate conditions, from the Leydig cell and Sertoli cell respectively (9, 130). Figure 10 illustrates the site of production of the steroidal sex hormones-testosterone (133) and estradiol (134, 135), inhibin (136), and specific proteins such as androgen binding protein (117), and plasminogen activator (137) from the two testicular cells. These hormones modulate the action of the gonadotropic hormones through direct interactions upon the testicular

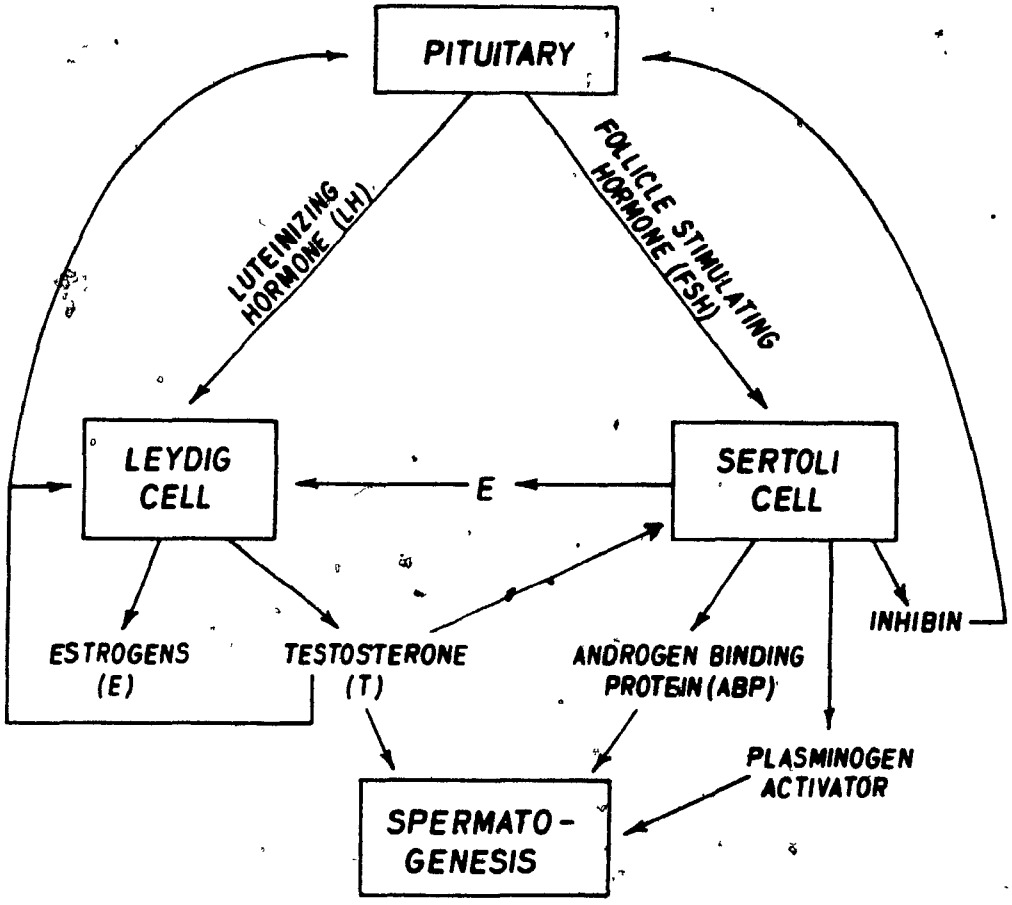


FIG. 10

Schematic view of the interrelationships between the pituitary and the testicular Leydig and Sertoli cells involved in the regulation of testicular steroidogenesis and spermatogenesis.

cells and by feedback mechanisms, both positive and negative, that regulate the hypothalamic-pituitary secretion of the gonadotropins (9).

The paucity of data on the regulation of testicular FSH receptors precluded a detailed description of its regulation. Therefore the following section concerning gonadotropin receptor regulation will deal primarily with the LH(hCG) receptor and where possible, examples will be given on the FSH receptor.

1.16 Disparity Between Hormone Binding and Generation of the Biological Response

In the Leydig cell (138), the full steroidogenic response occurs with only partial receptor occupancy, ie. 1% of the total receptor population. The presence of an additional number of receptors or spare receptors in excess of those required for the generation of the maximal biological response has also been reported for several other systems. For example the effect of FSH on the seminiferous tubules (132), insulin on fat cells (139), and adrenocorticotropic hormone (ACTH) on the adrenal cells (140). The presence of these spare receptors could serve as a mechanism to enhance the probability of a hormone-receptor interaction, thereby increasing the sensitivity of the target cell to low hormone levels (138). Alternatively the receptor site may be thought of as a quantal unit so that a cell would respond in a maximal all or none fashion if the number of sites filled exceeded a given threshold (141). An excess of receptors could function as a reserve of sites. This would permit the replacement of damaged or lost receptors without significantly changing the concentration of receptors at the cell surface and so ensure a cellular response (138).

1.17 Regulation of Gonadotropin Receptors

Administration of a single dose of LH or hCG to male rats resulted in an alteration of the number of LH(hCG) receptors of the Leydig cells. Initially a rapid upregulation of receptors was observed. That is there was an increase in LH(hCG) binding capacity with no change in the

equilibrium association constant (Ka). A maximum increase in binding was seen approximately one hour after injection of ovine LH (142) and within six hours after injection of hCG (143). However, a loss, not an increase, in receptor levels is seen if too large a dose of hormone (ex: 1,000 I.U. hCG) is administered (143). It is possible that any increase of receptor numbers is masked by the rapid, pronounced decrease of receptors. Treatment with cytochalasin B, an inhibitor of microfilament function, abolished the ability of LH to increase its own receptor levels. Cyclohexamide, a protein synthesis inhibitor, and colchicine, a microtubule inhibitor, had no effect. These results suggested that the increased LH/hCG binding capacity was due to the rearrangement of previously sequestered or masked receptor sites and is dependent on an intact microfilament (142, 143). Upregulation of binding sites is observed in a variety of tissues such as the ovary (144, 145) and liver (146). The increase in receptor numbers was followed by a progressive decline or downregulation of the LH/hCG receptors which was maximal by 24-48 h (143, 147, 148). It has been reported that desensitization of the rat ovary is accompanied by a major reduction of two of the five receptor species of the LH(hCG) receptor (99). Whether downregulation of the testicular LH(hCG) receptor involves all or only some of its molecular components remains to be assessed. Restoration of receptor levels was a slow process, commencing on day 2 or 3 and complete by day 6-10 (143, 147, 148). The reduction in the number of LH/hCG binding sites is not completely due to receptor occupancy since the loss of receptors was in excess of the level of occupany caused by the administered hormone (147). Indeed binding could be detected when plasma hCG levels were extremely high and was undetectable when plasma levels were very low and receptor levels were falling (149, 150). The reduction in receptor numbers from the surface of the cell is thought to be due to internalization of the hormone receptor complex and adjacent unoccupied receptor sites (150-153) or shedding of receptors from the cell surface into the surrounding fluid (106, 154). Recovery of the LH(hCG) receptors is dependent on the dose of hormone administered. The greater the hormone concentration, the greater the loss of receptors (143, 147).

Cycloheximide prevented this loss indicating a dependence on protein synthesis (143). New protein synthesis would also explain the necessity to wait at least six days to completely restore receptor levels (149). A hormonal dependency was indicated by the ability of PMSG, FSH, growth hormone and prolactin to prevent, and testosterone and estrogens to enhance the hCG/LH induced loss of receptor levels (155-158). Down-regulation, which is important in a number of cell systems, is thought to be a mechanism for preventing overstimulation of the target cell (159-162).

1.18 Desensitization of the Biological Response to Gonadotropins

An acute dose of hCG or LH not only results in an alteration of LH(hCG) receptor levels, but can induce a change in the biological response of the testis and ovary (147, 163, 164). During the time of increased receptor occupancy in the Leydig cell, LH(hCG) caused an increase in the secretion of testosterone for 1-2 h followed by a period of declining cyclic AMP and testosterone production, in spite of increased hormone levels (142, 147, 149). The decline in cyclic AMP levels is due to a decrease in adenylate cyclase activity which precedes the loss of LH/hCG receptors and lags behind the reappearance of LH/hCG receptors (142, 165). This period of desensitization or loss of responsiveness of the Leydig cell to gonadotropic stimulation continues for about three days before a return of responsiveness occurs. The development of desensitization does not seem to correlate with the loss of receptors because cycloheximide which prevents the loss of receptors does not prevent desensitization of the Leydig cell to hCG (166) and the Leydig cells can recover from desensitization after three days while receptor levels are still low (147). Although a defect in the gonadotropic hormone-receptor-adenylate cyclase coupling system (165, 167, 168) must play some role in desensitization, the inability of dibutyryl cyclic AMP to overcome the steroidogenic lesion indicates an additional (post-receptor) lesion (150, 167). Recent studies have demonstrated an early, estrogen independent, steroidogenic lesion of the

biosynthetic enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA), which is the rate limiting enzyme for cholesterol biosynthesis (169). A second, estrogen dependent, late steroidogenic lesion exists at the enzymes 17α-hydroxylase and 17,20-desmolase, which are responsible for the conversion of progesterone to androgens (170). The decreased Leydig cell response has been attributed to the effects of several hormones besides the gonadotropins. The hormones testosterone (171), the estrogens (172) and luteinizing hormone releasing hormone (158) are known to decrease hCG stimulated testosterone production. A direct inhibitory effect on the steroidogenic enzymes has been demonstrated for the estrogens (172) and luteinizing hormone releasing hormone (158). The mechanism by which gonadotropins cause and maintain desensitization is not yet fully resolved and probably is the result of the interplay of more than one hormone acting at various levels of the steroidogenic pathway.

1.19 Receptor Dysfunction as a Cause of Disease State

A better understanding of the structure and function of the gonadotropin receptor should prove to be a great asset for designing improved hormonal agonists and antagonists. Measurements of human gonadal gonadotropin receptor numbers and analysis of their structure from biopsy samples will provide a useful tool for the early diagnosis of disease states. Early detection of alterations in receptor numbers as occur in certain disorders such as polycystic ovarian disease (173), infertility (174), and insulin resistant states (58) would allow quicker diagnosis and possibly more effective therapy. Recently, circulating inhibitory factors have been detected in the serum of autoimmune diseased patients with gonadal dysfunction (175-177). These factors, which could represent anti-receptor antibodies, might contribute to gonadal dysfunction. Anti-receptor antibodies are not unique to the gonadotropin-receptor system. They have also been described for TSH (178), insulin (58) and acetylcholine (179) receptors. Although circulating anti-receptor antibodies have been demonstrated in several disease states

(179-182), the reverse is not always true (177, 178, 180). Undoubtedly, it would be clinically useful in the human testes and ovary to be able to distinguish between fluctuations in receptor numbers, alterations of receptor structure and physical blocking or masking of an otherwise functional receptor molecule in prescribing the best possible treatment of gonadal dysfunction.

CHAPTER 2

NONHUMAN PRIMATE GONADOTROPIN RECEPTORS

2.1 Introduction

The mechanism of action of the gonadotropic hormones in laboratory animals has been extensively investigated by in vivo and in vitro methods. Consistent with the idea that the first step in the mechanism of action of peptide hormones is their binding to the cell membrane, the presence of surface receptors for gonadotropins have been demonstrated in ovarian and testicular tissue from many species (183, 184). Several recent investigations have shown the presence of specific binding sites (receptor(s)) for hCG(LH) in monkey (185) and human testes (174, 185-188). These receptors have been shown to be similar in many respects to the LH(hCG) receptor from animals (185). Data on the biochemical properties of the primate LH(hCG) receptor (174, 185) are meager, while reports on the presence of specific FSH binding sites (receptors) in this mammalian order are nonexistent. Much of our present knowledge of the testicular FSH receptor comes from studies carried out with the rat (52, 68, 93) and a few domestic species such as the bull (72, 86, 189, 190) and the pig (74). In marked contrast to the adult rat, the essential role of FSH in the spermatogenic process of the adult monkey has been recently demonstrated (191-193). Fundamental to our understanding of the control of FSH action in man is the knowledge of the properties of the cellular receptors. Therefore we have initiated investigations aimed at the elucidation of the characteristics of primate gonadotropin receptors. This chapter presents data pertaining to the demonstration and preparation of high affinity specific binding sites for human FSH and hCG in the following four different nonhuman primate species - the rhesus monkey (Macaca mulatta, Mm), the pig tailed monkey (Macaca nemestrina, Mn), the crab eating monkey (Macaca fascicularis, Mf1),

and the yellow baboon (Papio cynocephalus, Pc). The biochemical properties of the primate testicular gonadotropin receptors were investigated principally with the rhesus monkey, a model which has been widely employed for research into human reproduction. The scarcity of tissue from the other species precluded a detailed study of their biological properties. Preliminary results of this study have been reported (194-196).

2.2 Materials and Methods

2.2.1 Hormones

Highly purified human FSH (hFSH) (197), ovine FSH (oFSH) (198) and ovine-LH (oLH) (199) were prepared in this laboratory. hCG (CR119) (iodination grade) was supplied by the National Hormone and Pituitary Program, National Institutes of Health, Bethesda, Maryland. Human LH(hLH) was purified in this laboratory (200).

2.2.2 Primate testes

Testes from four different species of nonhuman primates (Macaca mulatta, Macaca nemestrina, Macaca fascicularis, Papio cynocephalus) collected at surgery were frozen on dry ice with minimum delay and shipped to us from the Regional Primate Research Center at the University of Washington, Seattle, Washington. Frozen testes of adult rhesus monkeys removed at castration were also obtained from Emory University, Atlanta, Georgia. After arrival in the laboratory they were stored at -70C until use. The approximate ages of the monkeys and identification were provided by the institutions which sent us the samples.

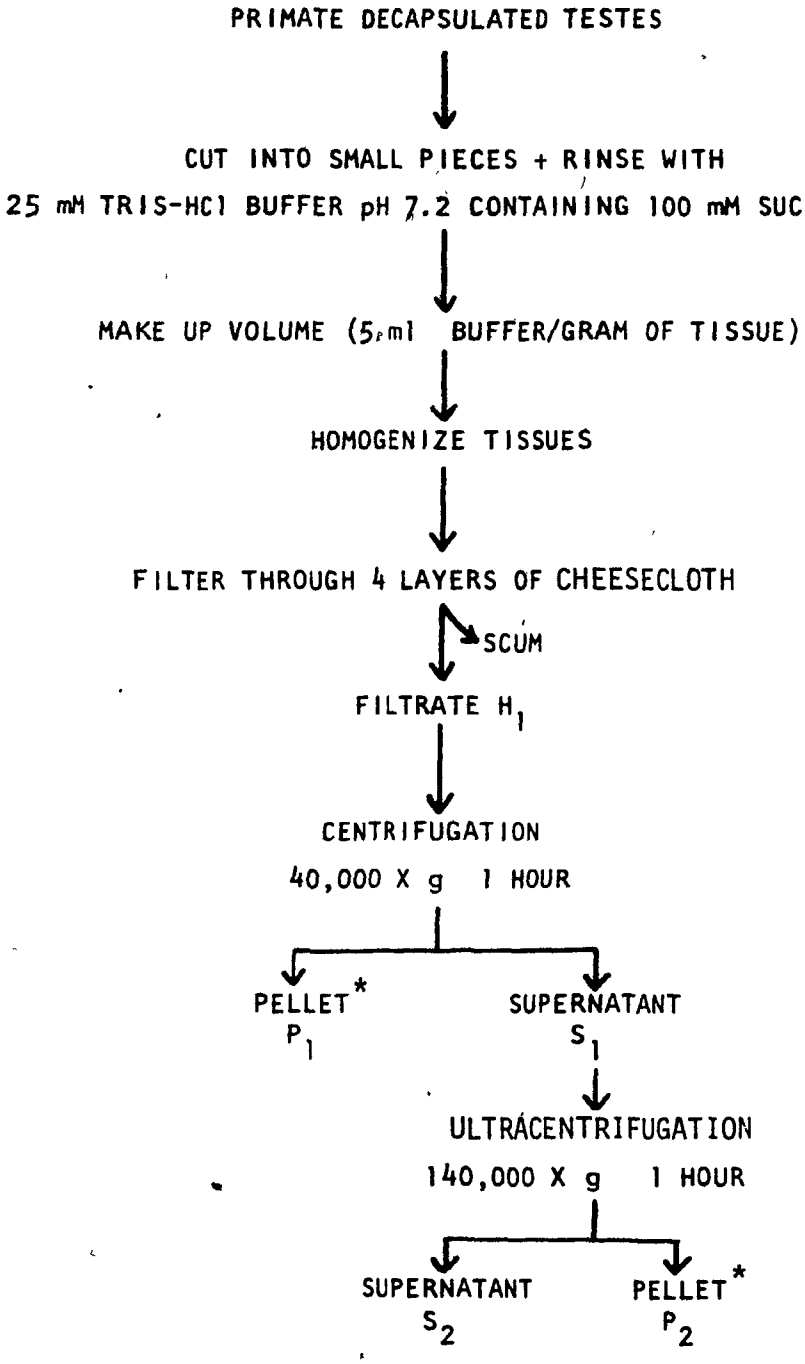
2.2.3 Other materials

The following enzymes or reagents obtained from different sources were used for the characterization of the gonadotropin receptor fractions. Bovine pancreatic ribonuclease A (Sigma, St. Louis, Mo., 64K units/mg), bovine pancreatic trypsin (Sigma, St. Louis, Mo.), neuraminidase from

clostridium perfringens (Böehringer-Mannheim, Montreal, Que., 0.6 units/mg), bovine pancreatic deoxyribonuclease (Worthington, Freehold, N.J., 1200 units/mg), phospholipase C (Calbiochem, San Diego, Ca., 1.5 units/mg), mercaptoethanol (Eastman, Rochester, N.Y.) and lactoperoxidase (Sigma Chemical Co., Montreal, Que.). The protein assay kit was purchased from Bio-Rad (Richmond, Ca.). All other chemicals were of reagent grade and used without further purification.

2.2.4 Preparation of subcellular fractions from primate testes

Testicles from the four adult nonhuman primate species (Mm, Mn, Mf1, Pc) were used in the preparation of the particulate LH and FSH receptors. The decapsulated testes were thawed under a stream of blowing cold air and all subsequent steps were performed at 4C (Fig. 11). The testes were minced with scissors, rinsed and suspended in 25 mM Tris-HCl buffer, pH 7.2 containing 100 mM sucrose (5 ml per g of tissue). The addition of a hypotonic solution of sucrose aids cellular disruption by causing cell lysis. One testis from each of the four species was homogenized by a hand held ground glass tissue grinder using 8 strokes up and down). The other tissues were individually homogenized mechanically using a Tekmar tissuemizer, set at low speed using 2-3 short pulses of 10-15 seconds duration. The homogenate was filtered through 4 layers of cheesecloth and the filtrate was centrifuged at low speed (600 x g) in a Beckman J21B centrifuge to remove cellular debris. As the amount of debris eliminated was usually small in the first few experiments, this step was omitted in the subsequent studies without significantly affecting the results. The filtered homogenate (H1) was then centrifuged at 40,000 x g for 1 h and the supernatant from this was further subjected to ultracentrifugation at 140,000 x g for 1 h. The resultant pellets designated P1 and P2 respectively were resuspended by mechanical dispersion using 8 strokes in a tight fitting glass Dounce homogenizer (1 g per 2 ml of 25 mM Tris-HCl buffer, pH 7.2 containing 10 mM $MgCl_2$). These were stored in small aliquots at -70C. Prior to assay, the fractions were thawed at room temperature and again gently homogenized



*Fractions P_1 and P_2 resuspended by mechanical dispersion in 25 mM Tris-HCl, pH 7.2 buffer containing 10 mM $MgCl_2$ (1 ml buffer/gram of tissue).

FIG. 11

Scheme for the preparation of gonadotropin receptors.

in the assay buffer (as above with 1 mg/ml bovine serum albumin added) to obtain a uniform suspension (see below).

For purposes of comparison with the primate receptor a FSH receptor preparation from adult bull testes (BTR) was prepared (201). A LH-FSH receptor preparation was also prepared by homogenizing adult CRCD rat testes (RTR) in 25 mM Tris-HCl buffer, pH 7.2 containing 10 mM sucrose (5 ml/g). The homogenate was centrifuged at 20,000 x g and washed and resuspended in 25 mM Tris-HCl buffer, pH 7.2 with addition of 10 mM $MgCl_2$ (2 ml/g).

2.2.5 Protein estimation

To assess protein content, 100 µl aliquots of each subcellular fraction was mixed with 100 µl of 0.5 N NaOH and kept in a boiling water-bath. After 30 min, 100 µl of 0.5 N HCl was added and the protein content was determined using the Bio-Rad protein determination kit (Bio-Rad, Richmond, Ca.) employing bovine serum albumin as standard (202).

2.2.6 Iodination of hormones

The hormones hFSH, hCG, hLH, oLH, oFSH and hPRL were labeled with ^{125}I by the lactoperoxidase method (203) as previously described (201) using carrier free Na-^{125}I (Amersham Inc., Chicago, Ill.). To 5 µg of the desired hormone to be iodinated (ex. 5 µg/25 µl hFSH), the following reagents were added. First 81.5 µg EDTA (163 µg/100 µl water) was added, followed by 10 µl Na-^{125}I, and 10 µg lactoperoxidase (50 µg/250 µl 10 mM potassium phosphate buffer, pH 7.4). The reaction was started by the addition of 100 µl hydrogen peroxide (0.00028%) and stopped after a 5 min incubation at room temperature by application of all but 10 µl of the reaction mixture (235 µl) on a Sephadex G-100 column (0.7 cm x 33 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.4 containing 0.1% BSA and 0.2% sodium azide. The 10 µl aliquot was used in the determination of the specific activity of the labeled hormone. Gel filtration at room temperature on Sephadex G-100 resolved

the labeled hormone from the free Na-^{125}I (Fig. 12). The radiolabeled hormone eluted from the column in the same position as the unlabeled native hormone indicating the absence of any gross changes being induced in the hormone by the iodination procedure. As a further precaution against using damaged radiolabeled hormone, only the peak tubes of the labeled hormone (first peak) were preserved in aliquots at -70C until use. The radiolabeled hormone was generally used within two weeks of preparation. The integrity of the labeled hormones were verified by electrophoresis, gel chromatography, radioreceptor assay, radioimmunoassay and specific bioassays.

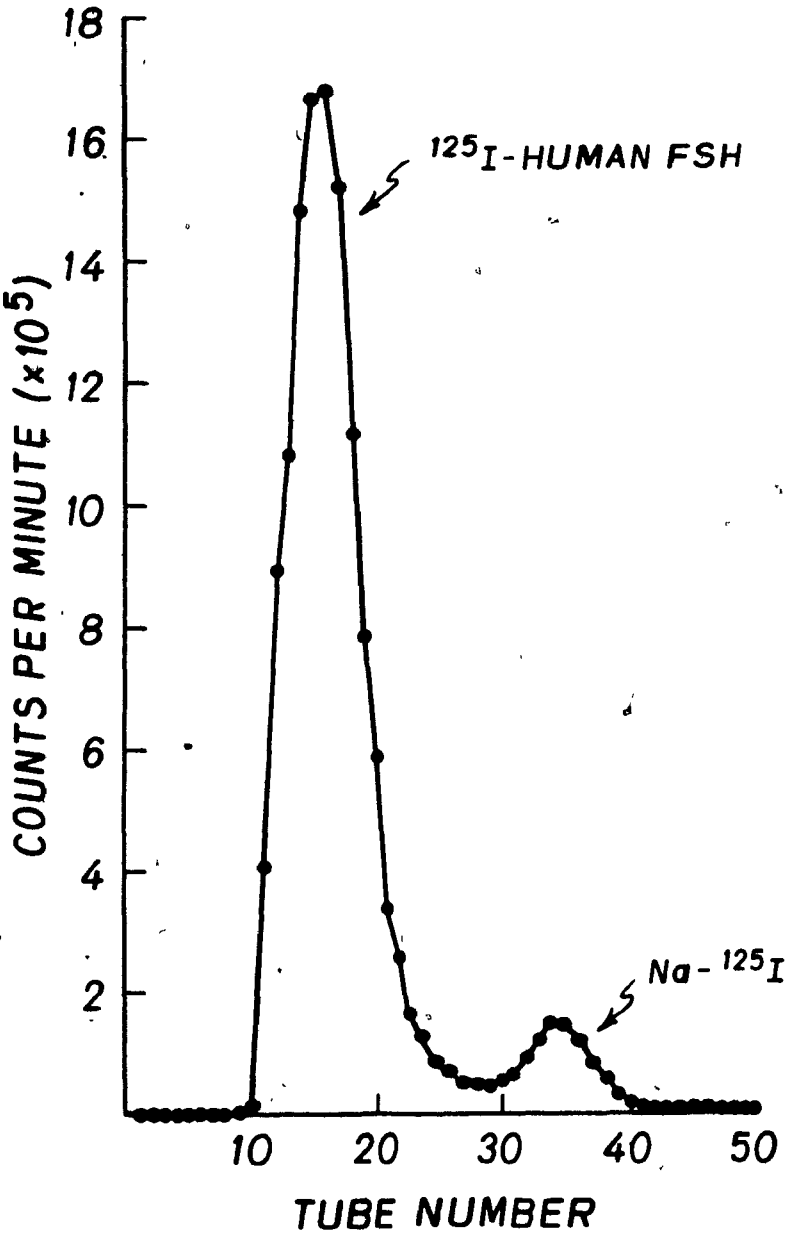
The specific activity of the labeled hormone, in triplicate, was assessed by precipitating the hormone in 2 ml acetone from a known aliquot of the reaction mixture (425 pg/20 µl). After standing at room temperature for 30 min, the tubes were spun in an IEC clinical centrifuge at 2900 x g for 5 min at 4C. The supernatant containing the free iodine was removed by suction and the pellet again washed with 2 ml acetone to remove any residual free Na-^{125}I. The precipitated radioactivity in the tubes was assumed to represent only hormone bound radioactive iodine. The tubes were counted in an LKB rackgamma II counter (70% counting efficiency) and the specific activity, ie. the amount of radioactivity incorporated, calculated from the counts per minute (cpm)/known quantity of hormone. The specific activities of the labeled hormone used in this study were in the range of 60-100 µCi per µg.

2.2.7 Binding of ^{125}I-labeled gonadotropins to testicular fractions

Specific binding of ^{125}I-labeled hormones to each of the primate testicular subcellular fractions were assessed. The assays were performed in triplicates in 10 x 75 mm disposable polystyrene tubes. Unless otherwise indicated each tube contained approximately 50,000 cpm (400 pg) ^{125}I-labeled hormone, 100-300 µg protein equivalent of the testicular fractions and 100 µl of the unlabeled hormone and/or assay buffer to constitute a final volume of 250 µl per tube. The assay buffer consisted of 25 mM Tris-HCl buffer, pH 7.2 containing 0.1% bovine

FIG. 12

Resolution of ^{125}I-labeled hFSH from the free iodine peak by gel filtration on a Sephadex G-100 column (0.7 x 33 cm, bed height = 29 cm). Fractions (0.8 ml/tube) were eluted from the column with 10 mM potassium phosphate buffer, pH 7.4 containing 0.1% BSA and 0.2% sodium azide. The specific activity of ^{125}I-labeled hFSH was 99 uCi/ug. Similar separation patterns were obtained for the other labeled hormones.


18
16
14
12
10
8
6
4
2
COUNTS PER MINUTE (x10^5)
125I-HUMAN FSH
Na-125I
10
20
30
40
50
TUBE NUMBER

serum albumin (BSA) and 10 mM $MgCl_2$). The tubes were vortexed and incubated in a continuously shaking Dubnoff water bath for 6-8 h at 34C. The reaction was terminated by the addition of 2 ml of chilled assay buffer followed by centrifugation at 2,900 x g for 15 min at 4C in a table top IEC clinical centrifuge. The presence of $MgCl_2$ tends to cause aggregation of the membrane particles allowing pelleting at low speed centrifugation. The supernatant was removed by aspiration under vacuum and the radioactivity in the pellet was determined in an LKB rackgamma II counter. Centrifugation under these conditions was adequate to sediment all of the hormone-receptor complex(es). In the initial studies, precipitation of the supernatant by agents such as polyethylene glycol did not reveal any additional specific binding. Hence this step was not essential in other routine experiments. In all cases, non-specific binding was determined in the presence of a 1000 fold excess of respective unlabeled hormone. The difference between the total radioactivity bound and nonspecific binding was defined as the amount specifically bound and expressed as a percentage of the total counts put into the tubes. All variations in this procedure with respect to handling, temperature, medium, and processing are indicated at appropriate sections in the results.

2.2.8 Dissociation reaction

The testicular receptor-hormone complex was first formed by incubating 100 µg protein equivalent of the subcellular P1 fractions with 0.4 ng of ^{125}I-labeled hFSH (^{125}I-hFSH) in a total volume of 250 µl for 16 h at 25C with continuous shaking. The reaction was terminated by the addition of 2 ml cold assay buffer, and the tubes centrifuged at 2900 x g for 15 min at 4C. The supernatant containing the free hormone was removed by suction. The volume of each tube was reconstituted with 250 µl of the assay buffer of desired pH and molarity in the presence or absence of unlabeled hFSH. The tubes were then vortexed to ensure dispersion of the pellet and incubated further at the desired temperature (see results). At specific intervals, samples were removed, diluted

with 2 ml of assay buffer at 4C and centrifuged. The supernatant was removed and radioactivity in the pellet was determined.

2.2.9 Effects of pH, salt concentration and enzyme digestion on the receptor

Testicular fraction P1 (125 µg protein per tube) was preincubated at 37C in the presence of the agent to be tested. After 30 min, 2 ml of ice cold assay buffer was added to dilute the reagent while terminating the reaction at the same time. After centrifugation at 2900 x g for 15 min at 4C, the supernatant was removed and the pellet containing the receptor was then resuspended in 250 µl of the assay buffer containing about 0.4 ng ^{125}I-hFSH in the presence or absence of 100 ng of unlabeled hFSH. The binding reaction was then initiated by placing the tubes in the shaking water bath at 37C. After 2 h, the reaction was then terminated by the addition of 2 ml cold assay buffer and the tubes were processed as above. Specific binding was calculated from the radioactivity bound to the pellet in the presence and absence of unlabeled hFSH.

2.2.10 Detection of soluble hormone binding (receptor) activity

Aliquots of supernatants derived from the 140,000 x g centrifugation of testicular tissues were incubated for 16 h at room temperature (25C) with 50,000 cpm (400 pg) of ^{125}I-labeled hormone in a final volume of 250 µl. In the case of ^{125}I-hPRL, incubations were for 2 hr at 34C. Nonspecific binding was assessed by adding an excess of unlabeled hormone (500 fold). The assay buffer consisted of 25 mM Tris-HCl buffer, pH 7.2 containing 1 mM bacitracin, 0.1% BSA and 0.2% $MgCl_2$. Bacitracin, an antibiotic polypeptide, was added to suppress any proteolytic enzyme activity in these fractions. The reaction was stopped by adding 200 µl of bovine gamma-globulin (5 mg/ml), which acts as a carrier, followed by 1 ml of a 15% polyethylene glycol solution (MW 6000). Bovine gamma globulin and polyethylene glycol (PEG) were prepared in 25 mM Tris-HCl

buffer, pH 7.2 containing 154 mM NaCl. The tubes were vortexed, allowed to stand for 10-15 min at 4C to allow maximum precipitation and then centrifuged at 4C for 10 min at 2900 x g. The supernatant containing the unbound hormone was removed by suction and the pellets counted in an LKB rackgamma II counter.

2.2.11 Leydig cell bioassay

Dispersed interstitial cells from testes of 180-200 gram rats were prepared by collagenase digestion (199, 204). Approximately 100-200,000 cells per ml were incubated in Dulbecco's modified Eagles medium containing 0.1% bovine serum albumin and 0.1% lima bean trypsin inhibitor for 2 h at 37C in the presence of 95% O_2-5% CO_2. The total volume of the incubation was 0.6 ml. The cells were incubated with various doses of radiolabeled or unlabeled LH or hCG. After the incubation, the reaction was stopped by adding 4 ml cold assay buffer and testosterone was measured by radioimmunoassay (199).

2.2.12 Statistical analysis

Data were analyzed for significance by Student's 't' test. Scatchard plots were done on a programmable Hewlett-Packard desk top calculator.

2.3 Results

2.3.1 Distribution of FSH binding sites

The distribution of protein and specific binding of ^{125}I-labeled hFSH (^{125}I-hFSH) in the different subcellular fractions prepared from adult rhesus monkey testes (13 yr) was first investigated. The amount of protein in the homogenate (H1) resulting from either homogenization by hand or by mechanical dispersion was approximately the same. The distribution in the pellet P1 and P2 were also similar. Operationally mechanical

homogenization was easier and faster than manual glass homogenization and the former was routinely used in more detailed characterization of the rhesus monkey FSH receptor (195) and investigations of the other species of monkeys (see below). By either method of handling (Table 3) approximately 40% of the homogenate protein was recovered in the subcellular fraction sedimenting at 40,000 x g (designated fraction P1). Centrifugation of the supernatant of this fraction at 140,000 x g for 1 h did not result in significant recovery of protein (approximately 2%) in the small pellet fraction P2.

The specific binding of ^{125}I-labeled hFSH as assessed by incubation at 34C for 8 h (Fig. 13) was proportional to the amount of the testicular protein fraction. Binding to the homogenate (H1) was low with maximum specific binding (8%) occurring in the presence of about 400 µg protein. However, binding to the 40,000 x g pellet P1 was about 2- to 3-fold greater than the homogenate. Little or no FSH binding activity was detectable in the small amount of fraction P2.

The specificity of binding of various ^{125}I-radiolabeled hormones (human and nonhuman) to testicular fractions derived from the adult rhesus monkey, bull and rat is illustrated in Fig. 14. Binding of ^{125}I-labeled hFSH and oFSH was observed with both rhesus monkey testis fractions, with the P1 fraction showing greater binding. However, the rhesus monkey receptor preparations bound very little of labeled primate LH (^{125}I-labeled hCG or ^{125}I-labeled hLH) or nonprimate LH (^{125}I-labeled oLH). Incubation of ^{125}I-labeled hCG with up to 900 µg of fraction P1 did not result in more than 1% specific binding of the hormone. This should be compared with 16-20% specific binding of ^{125}I-labeled hFSH observed with 300-400 µg of the P1 fraction. Our inability to detect LH receptors in adult monkey tissue preparations is unlikely due to inactive radiolabeled LH. This is because these same radiolabeled hormones were bound by LH receptors in rat testicular tissue and were as potent as the native unlabeled hormone in stimulating testosterone production by rat Leydig cells (Fig. 15).

TABLE 3

FRACTIONATION OF ADULT RHESUS MONKEY TESTES FOR GONADOTROPIN BINDING SITES

Subcellular fractions	Yield of Protein (mg of protein/g of tissue)				% protein yield
	I	II	III*	avg	
Homogenate (H1) (after filtration)	29.6	38.2	37.8	35.2	100
40,000 x g pellet (P1)	10.9	19.3	14.4	14.9	43.8
140,000 x g pellet (P2)	0.5	0.7	0.5	0.6	1.8
Testicular weight (g)**	20	23	22	22	

Data represent the distribution of protein obtained from three separate extractions of mature 13-year-old rhesus monkey testicles. Binding data are shown in Figures 13 and 14.

* Hand homogenization;

** Weight of testes after decapsulation.

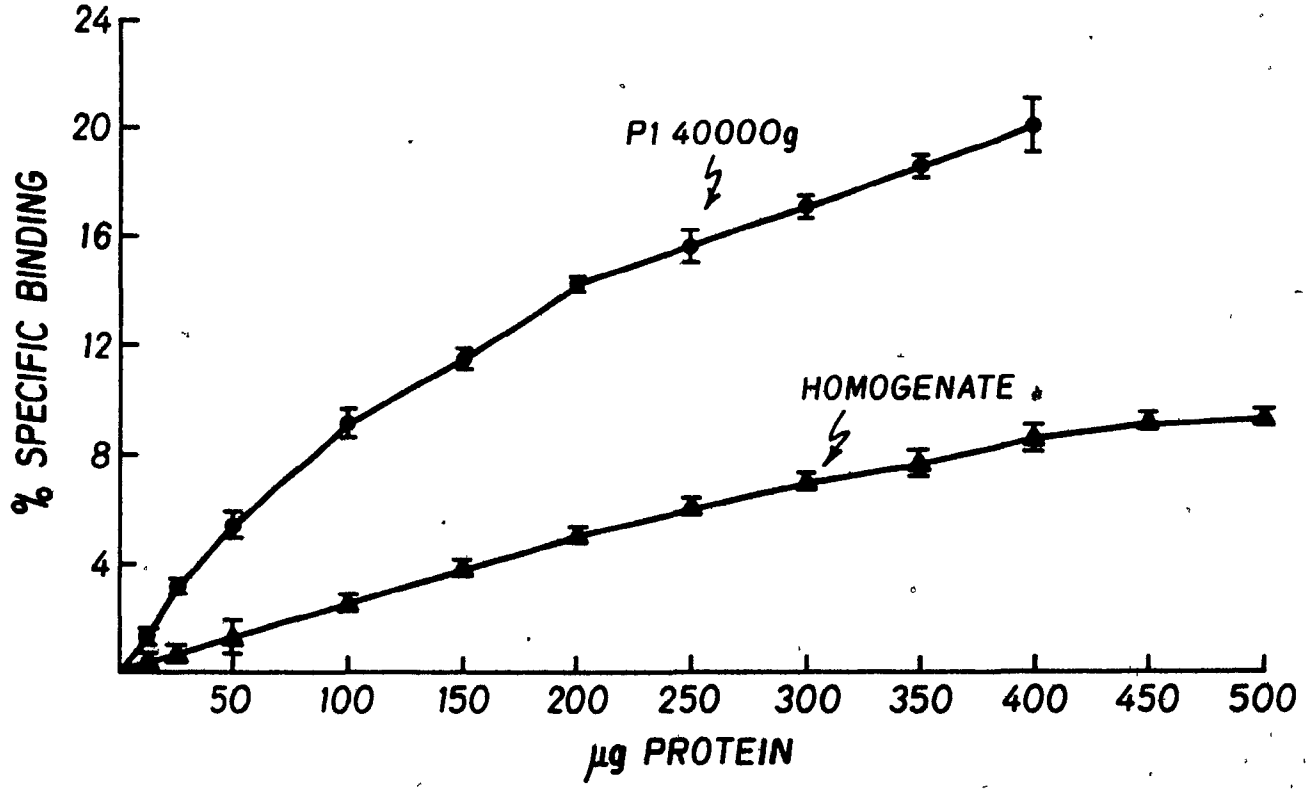


FIG. 13

Specific binding of ^{125}I-labeled hFSH (^{125}I-hFSH) to varying amounts of testicular fractions obtained from a 13 yr old rhesus monkey. Similar results were obtained with testicular tissue from the other three nonhuman primate species. Unless stated otherwise, the reaction mixture consisted of approximately 50,000 cpm (400 pg) of labeled hormone and 100 ul of testicular tissue. The reaction volume was made up to 250 ul with the assay buffer (25 mM Tris-HCl buffer, pH 7.2 containing 10 mM $MgCl_2$ and 0.1% BSA). Nonspecific binding was determined in the presence of 100-500 ng of unlabeled hormone. Incubations were for 8 h at 34C. Experimental values were done in triplicate and represent the mean ± SEM of at least duplicate experiments.

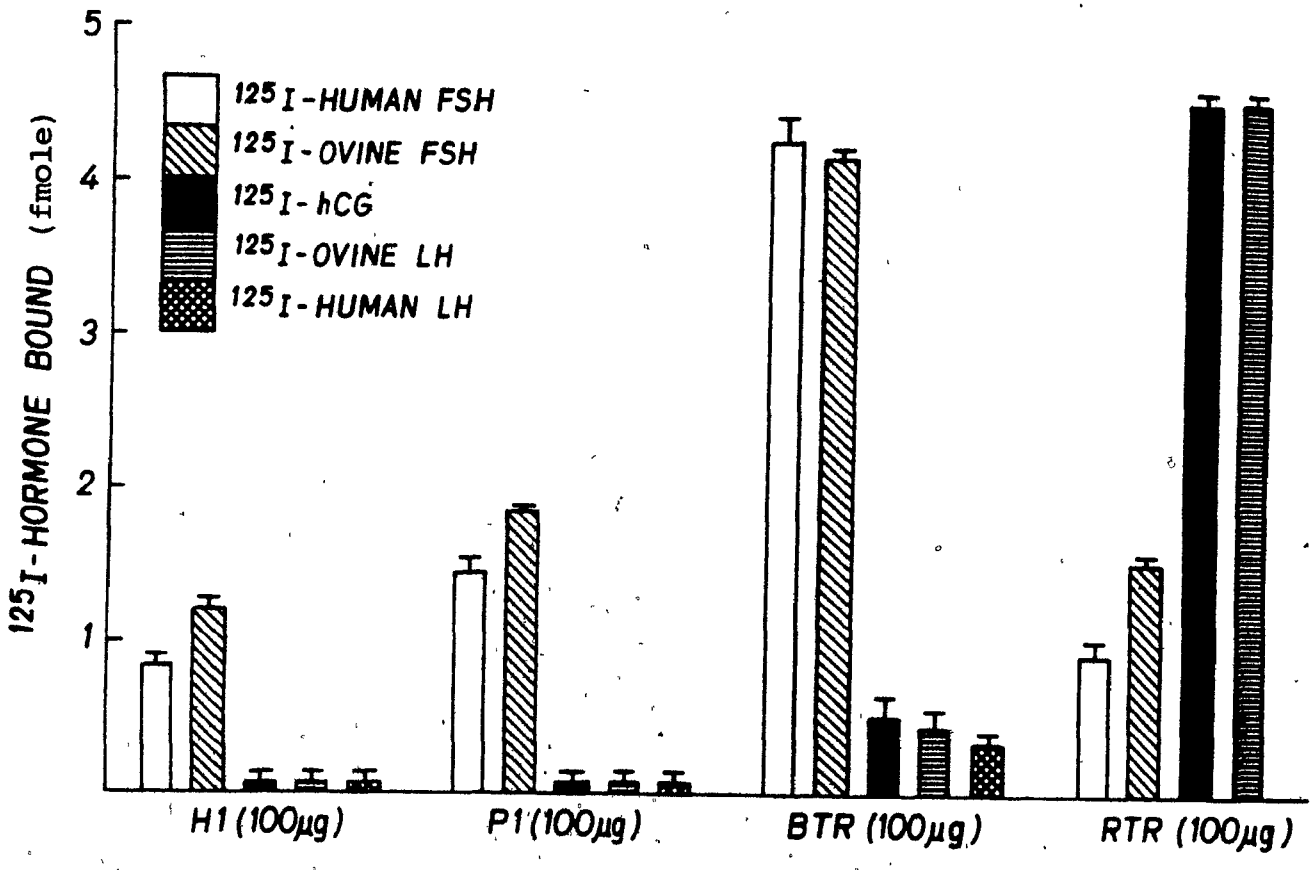


FIG. 14

Relative binding ability of ^{125}I-labeled human gonadotropins (hCG, hLH, hFSH) and sheep gonadotropins (LH and FSH) to testicular fractions from the rhesus monkey (13 yr), adult bull (BTR) and adult rat (RTR). In each test an equivalent of 100 µg protein was incubated with the labeled hormones for 3 h at 34C. The specifically bound hormone is represented as fmol (for the calculations the molecular weight of hCG was taken to be 36,700 daltons and that of LH and FSH to be 30,000 daltons each). The binding patterns of labeled hormones to fractions from the other 3 species of monkeys were similar to the rhesus testes (data not shown). Unless stated otherwise, data represents the mean ± SEM of triplicate values of at least two separate experiments. H1 = Homogenate; P1 = 40,000 x g pellet fraction; BTR = bovine testicular receptor; RTR = Rat testicular receptor.

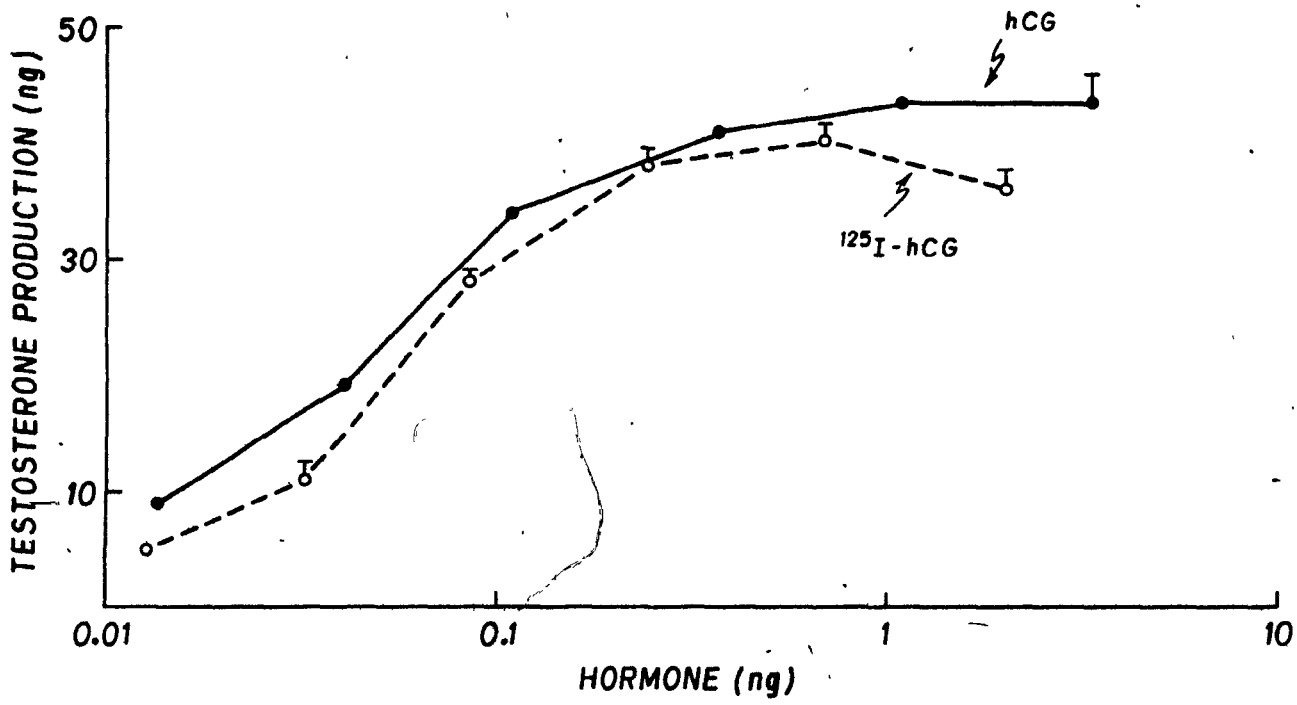


FIG. 15

Comparison of the effects of ^{125}I-labeled hCG and unlabeled hCG on collagenase dispersed rat interstitial cells in vitro. Similar results were obtained using labeled and unlabeled oLH and hLH. Control incubations with no hormone added had a testosterone value of 5.57 ± 0.29 ng/tube. Data shown are the mean ± SEM of triplicate values of one of two experiments.

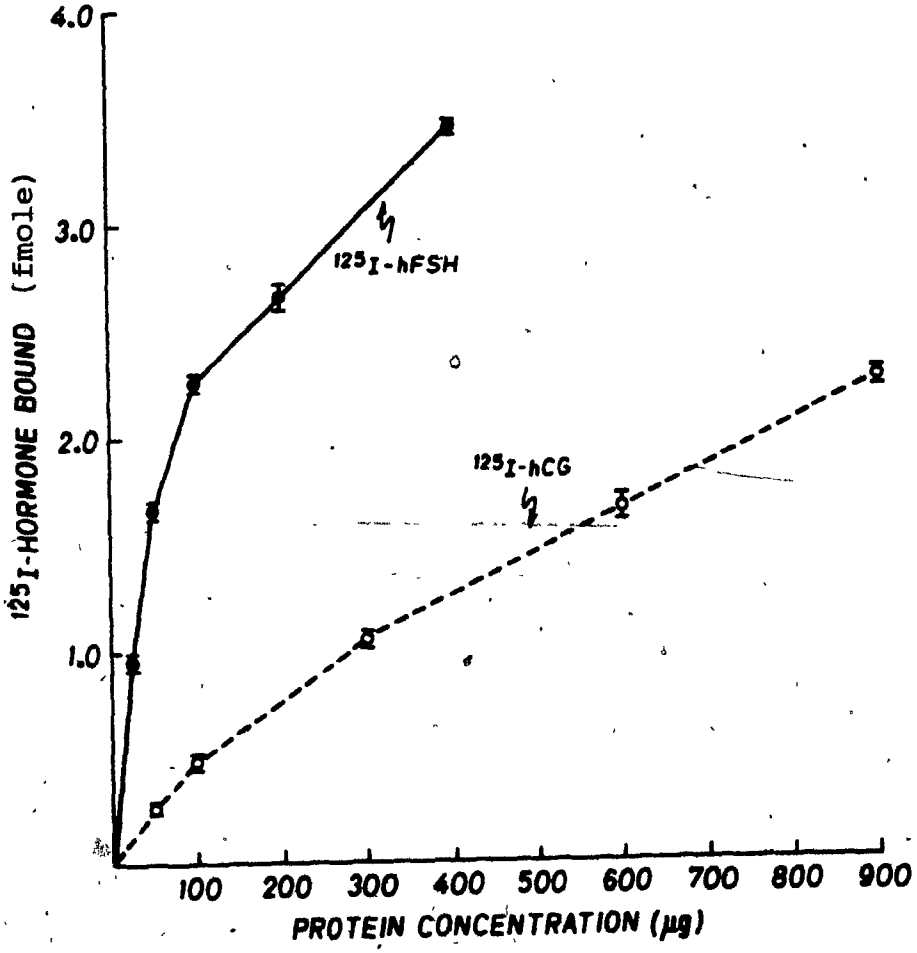
It is possible that our inability to detect significant LH binding sites in the adult rhesus monkey tissues used was due to our method of membrane preparation. Therefore, we took a pair of testicular tissues from a 13 yr old rhesus monkey and subjected one to a gentle homogenization by hand in a tissue grinder and the other to the usual rough cellular dispersion by the Tekmar tissuemizer (see methods). Gentle hand homogenization had previously been shown to yield particulate fractions of primate testis with some hCG binding ability (185). However, while both techniques gave approximately the same amount of ^{125}I-labeled hFSH binding, no significant ^{125}I-labeled hCG or hLH binding was again observed. To exclude the possibility that LH(hCG) hormone-receptor complex's were being formed but were not sedimenting at the speed of centrifugation utilized in this study, samples were precipitated with polyethylene glycol (see section 2.2.10). There was no significant increase in the specific binding of the radiolabeled LH or hCG again suggesting that there are few if any LH receptors present in adult rhesus monkey testes investigated in the current studies.

2.3.2 Subcellular distribution of gonadotropin binding sites in young rhesus monkeys

Subcellular fractions were prepared from the testes of a 5.8 yr old rhesus monkey. No change in the protein yield was observed. In contrast to adult tissue, there was specific binding of ^{125}I-hCG to homogenates and fraction P1, with a two fold enhancement of hCG and hFSH binding in fraction P1. A comparison of ^{125}I-hCG and ^{125}I-hFSH binding to fraction P1 is illustrated in Fig. 16. Note that for equivalent amounts of protein, a preferential uptake of ^{125}I-hFSH was observed. As a consequence of its greater ^{125}I-hCG binding, the characterization studies of the rhesus LH receptor were done on testicular fractions derived from young monkeys (less than 6 years old).

FIG. 16

Comparison of the specific binding of ^{125}I-hCG and ^{125}I-hFSH to the subcellular testicular fraction P1, obtained from a 5.8 yr old rhesus monkey, as a function of protein concentration. Similar results were obtained with testicular tissue from the other three nonhuman primate species. The specific binding of 1 fmol of labeled hormone represents 36.7 pg of hCG and 30 pg of hFSH. Of the counts associated with the pellet, up to 60% of the ^{125}I-hCG and 80% of the ^{125}I-hFSH represented specifically bound hormone.


4.0
3.0
2.0
1.0
^{125}I-HORMONE BOUND (fmole)
^{125}I-hFSH
^{125}I-hCG
100
200
300
400
500
600
700
800
900
PROTEIN CONCENTRATION (µg)

2.3.3 Subcellular distribution of gonadotropin binding sites in four nonhuman primate species

We have been able to study testes from three other species and the subcellular distribution of protein in the different fractions is compared in Table 4. The pattern of protein yield for these nonhuman primates was approximately the same as in the testes of adult rhesus monkeys, with about 35.0 ± 1.5 mg of homogenate and 12.4 ± 0.6 mg of fraction P1 obtained per gram of tissue. Comparable age groups of the nonhuman primates were utilized for the binding studies. The relative ^{125}I-hFSH and ^{125}I-hCG binding of the P1 fraction was 2-3 fold greater (data not given) than the respective homogenates, a result identical to that observed for the rhesus monkey testes (Fig. 16).

2.3.4 Effects of time and temperature on the association of ^{125}I-labeled gonadotropins to the primate testicular receptor

The specific binding of the radiolabeled gonadotropins to the subcellular testicular fraction P1 prepared from the nonhuman primates is dependent on both the duration and temperature of the incubation. A comparison of the association pattern of ^{125}I-hFSH and ^{125}I-hCG to the P1 fraction derived from the different primate species is illustrated in Figures 17A and 17B respectively. At 34C, their physiological temperature, significant and rapid binding occurred during the first two hours of incubation, reaching an equilibrium by about 6-10 h. While a similar pattern of association of the radiolabeled gonadotropins to the four primate species was observed, the binding equilibrium for ^{125}I-hFSH and ^{125}I-hCG binding to the bull and rat testes was reached earlier, within 2 h and 4 h respectively.

A more detailed study of the temperature dependence of the binding of the radiolabeled gonadotropins was performed using the rhesus monkey (Fig. 18A and 19) and the yellow baboon testes (Fig. 18B). The rate of association of the hormone to the receptor was similar at both 34C and 37C. At 25C, the interaction of the gonadotropin with its receptor was

TABLE 4

COMPARISON OF THE SUBCELLULAR PROTEIN DISTRIBUTION AMONG FOUR DIFFERENT PRIMATE SPECIES

Subcellular Fraction	Yield of Protein (mg of proteins/g of tissue) (Mean ± SEM)			
Primate species*	Mm (>5)	Mn (>5)	Mfl (>5)	Pc (>5)
Average Testicular Weight (g)**	18.3±2.0	15.1±1.6	12.6±1.0	14.1±3.7
Homogenate (Hl)	39.3±0.57	30.4±4.31	30.0±0.30	32.7±1.46
40,000 x g pellet (Pl)	11.8±0.67	11.6±0.97	10.9±1.73	11.2±1.32
140,000 x g pellet (P2)	0.6±0.06	0.5±0.08	0.5±0.06	0.3±0.05

* Number in parentheses represents the age of the primate in years;

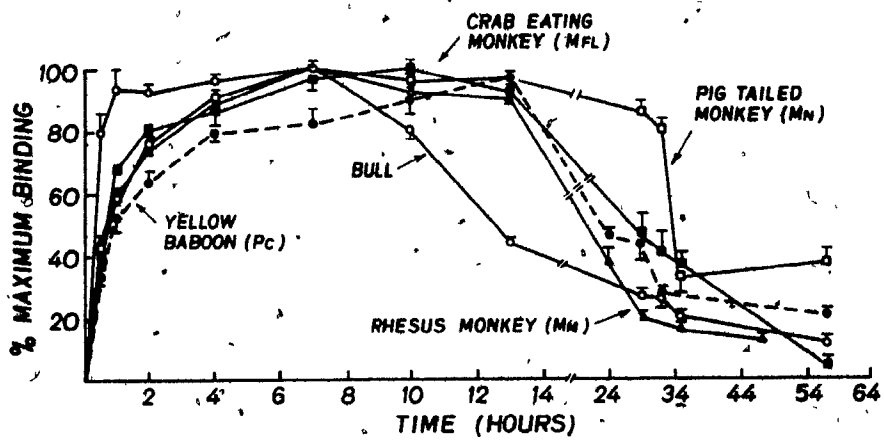
** Weight of testis after decapsulation.

Values shown above (n = 4 to 6 tissues) were obtained by mechanical disruption of the tissues. Comparable values were obtained using hand homogenization (not shown).

Mm -- Macaca mulatta (rhesus monkey); Mn -- Macaca nemestrina (pig tailed monkey); Mfl -- Macaca fascicularis (crab-eating monkey); Pc -- Papio cynocephalus (yellow baboon).

FIG. 17

Time dependence of the association of ^{125}I-hFSH (A: top panel) and ^{125}I-hCG (B: bottom panel) to the subcellular fraction P1 obtained from adult animals of various species. Aliquots of 100-300 µg protein of fraction P1 were incubated with the ^{125}I-labeled hormone in the presence and absence of the respective unlabeled hormone for various lengths of time at 34C. Specific binding was determined at the indicated time intervals. The maximum binding of ^{125}I-hFSH (Mfl = 16% S.B., Mm = 8% S.B., Mn = 10% S.B., Pc = 16% S.B., and bull = 20% S.B.) and ^{125}I-hCG (Mfl = 2% S.B., Mm = 8% S.B., Mn = 2% S.B., Pc = 3% S.B., and rat = 34% S.B.) was considered as 100%.


CRAB EATING MONKEY (MFL)
PIG TAILED MONKEY (MN)
BULL
YELLOW BABOON (PC)
RHESUS MONKEY (MM)
% MAXIMUM BINDING
100
80
60
40
20
2
4
6
8
10
12
14
24
34
44
54
64
TIME (HOURS)

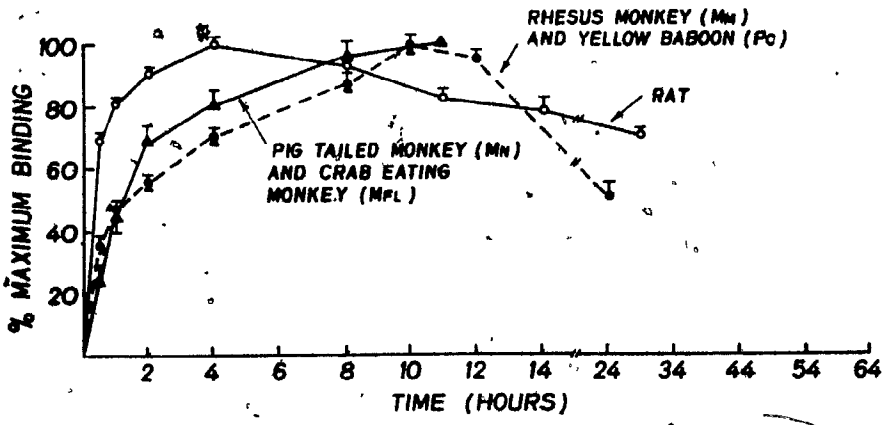
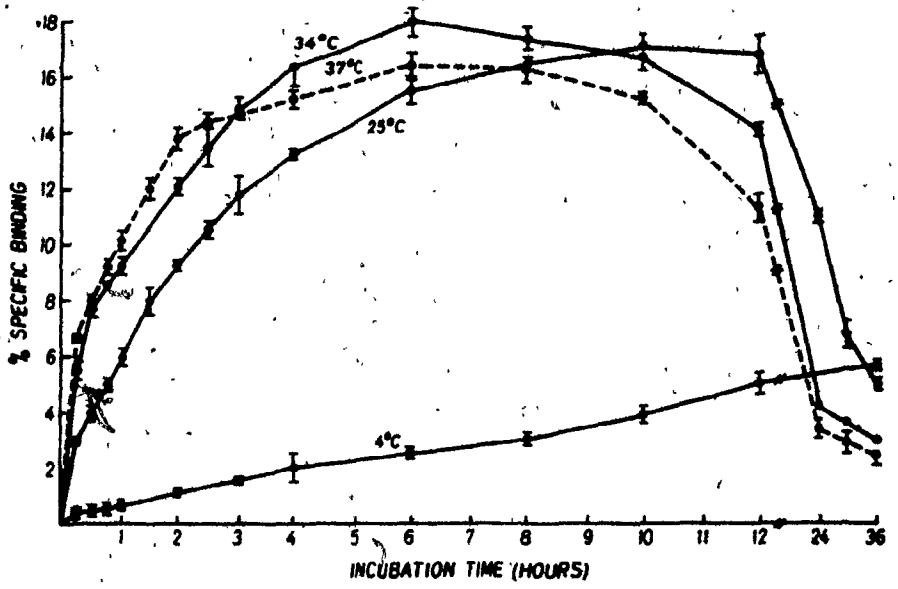
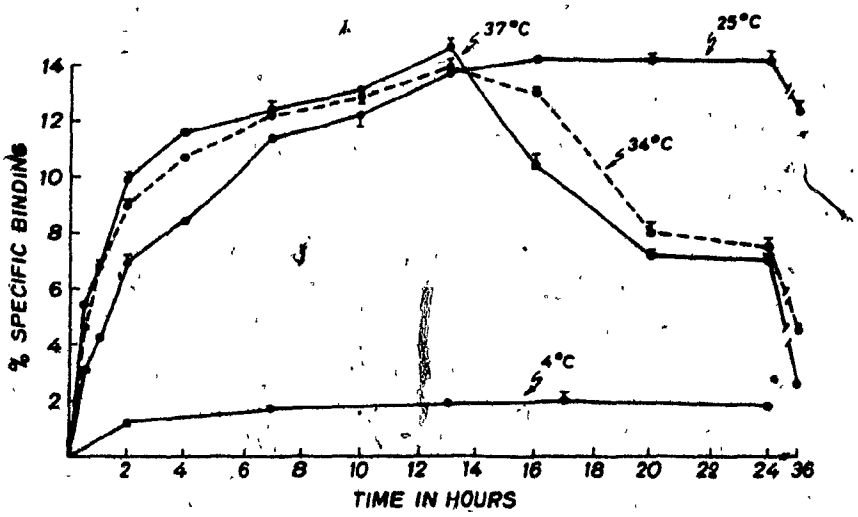

RHESUS MONKEY (MM) AND YELLOW BABOON (PC)
RAT
PIG TAILED MONKEY (MN) AND CRAB EATING MONKEY (MFL)
% MAXIMUM BINDING
100
80
60
40
20
2
4
6
8
10
12
14
24
34
44
54
64
TIME (HOURS)

FIG. 18

Temperature dependence of the association of ^{125}I-hFSH to the subcellular fraction P1 obtained from a 13 yr old rhesus monkey (A: top panel) and an 8 yr old yellow baboon (B: bottom panel). Specific binding was assessed at the indicated time intervals to 100-150 µg protein of fraction P1. Data represents the mean ± SEM of triplicate values of one of at least two experiments.


% SPECIFIC BINDING
INCUBATION TIME (HOURS)
34°C
37°C
25°C
4°C


% SPECIFIC BINDING
TIME IN HOURS
37°C
25°C
34°C
4°C

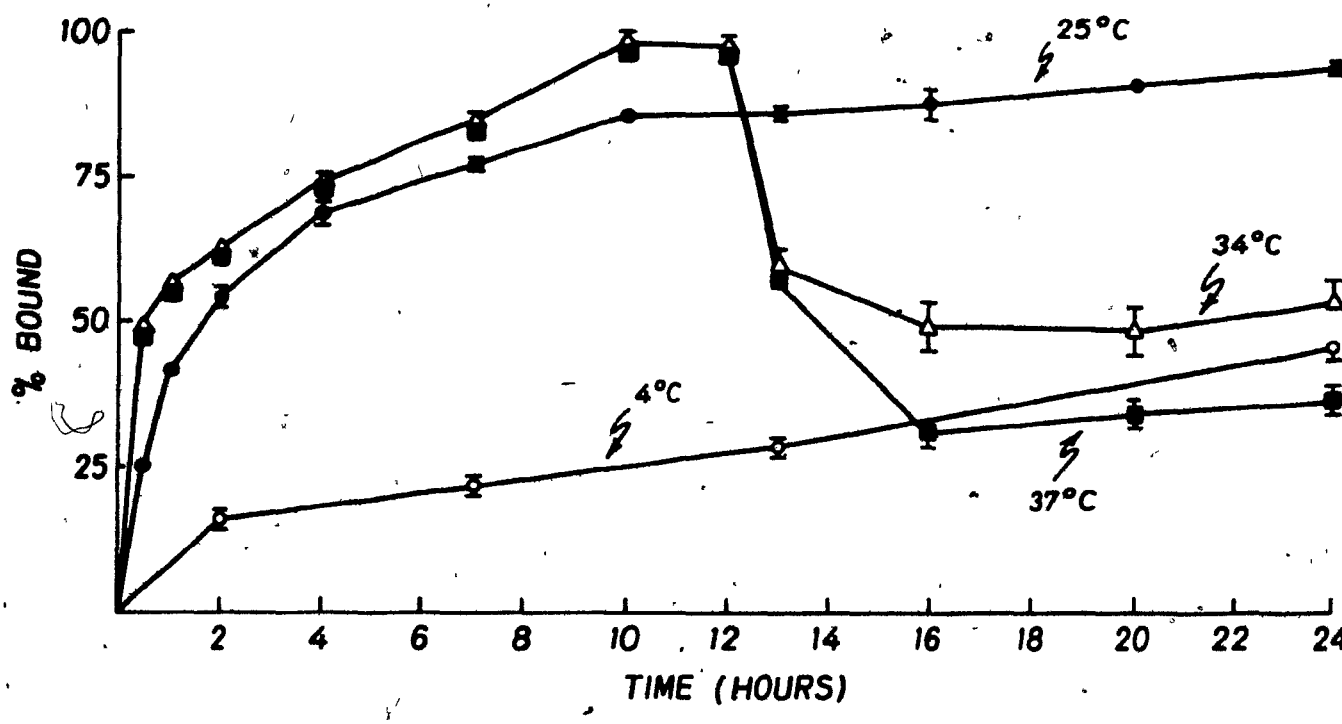


FIG. 19

Temperature dependence of the association of ^{125}I-hCG to 210 ug of the testicular subcellular fraction P1 obtained from the rhesus monkey (5.8 yr). The maximum binding of ^{125}I-hCG (6% S.B.) was considered as 100%. Data represents the mean ± SEM of triplicate values of one of two experiments.

much slower, requiring about 12 h of incubation to attain equilibrium. The binding at 4C increased very slowly and had not reached equilibrium even after 24 h of incubation. The specific binding gradually decreased when the incubation was prolonged beyond 12 h at 37C and 34C. A similar phenomenon was evident at 25C, but at a slower rate. In the case of the rhesus monkey, the hCG hormone-receptor complex appeared to be more stable than the FSH hormone-receptor complex at 25C. After 24 h, the binding of ^{125}I-hCG was near optimal levels while ^{125}I-FSH binding had declined by 60%. The decline in binding in these instances may have been due to the denaturation or degradation of the receptor and/or the ligand (see below).

2.3.5 Temperature stability of the receptor and radiolabeled hormone

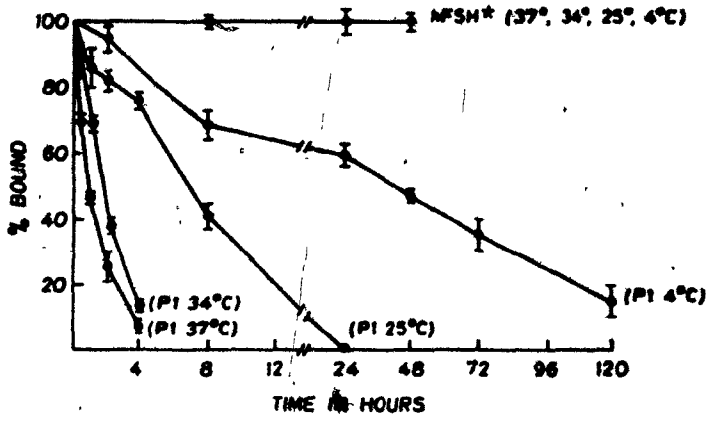
In order to distinguish if the drastic decline in specific binding of ^{125}I-hFSH and ^{125}I-hCG to the receptor at 37C, 34C or 25C was due to the lability of the hormone and/or the receptor, the following experiments were performed. The receptor fraction P1 and labeled hFSH were separately incubated at the different temperatures for various time periods. At the time intervals as shown in Fig. 20 and 21, binding ability of the pretreated radiolabeled hormone or the receptor fraction was evaluated by incubation with their (untreated) respective counterparts. A 4-h binding assay was performed at 37C to determine specific binding (see section 2.2.7). The radiolabeled hFSH and hCG showed no significant change in their ability to bind to the monkey receptor following incubation at all three temperatures for as long as 48 h. Indeed, the labeled hormone kept at 4C for 4 days showed no change in its binding ability as compared to zero time (data not shown). In contrast to the stability of the labeled hormone, the receptor fraction showed a marked decrease in its binding ability after preincubation. A comparable pattern of decline was observed for the testicular fractions derived from the rhesus monkey and yellow baboon (Fig. 20 and 21). At 34C, a 50% loss of ^{125}I-hFSH binding ability occurred by 1.5 h and 5 h and a 50% loss of ^{125}I-hCG

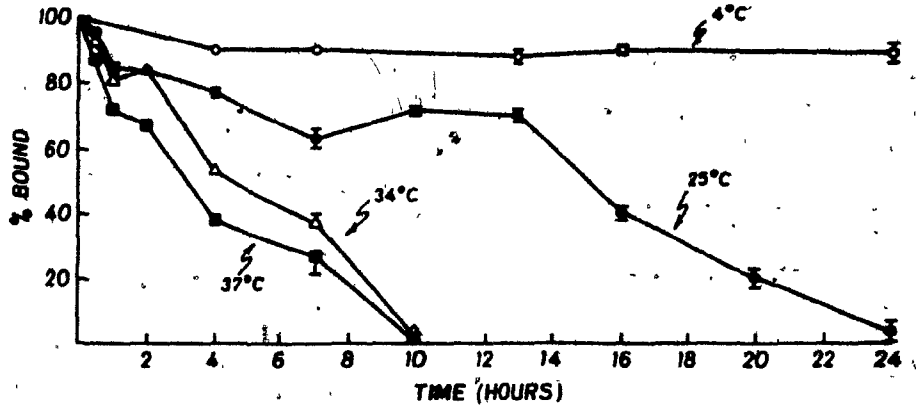
FIG. 20

Effect of preincubation on the integrity of the adult rhesus monkey testicular receptor.

A (top panel): ^{125}I-hFSH binding to 150 µg protein of subcellular fraction P1 from a 13 yr old rhesus monkey.

B (bottom panel): ^{125}I-hCG binding to 200 µg protein of subcellular fraction P1 from a 5.8 yr old rhesus monkey. The receptor fraction P1 and the ^{125}I-labeled hormones were separately incubated in the assay buffer at different temperatures for various durations of time and their ability to bind to the unincubated labeled hormone or the receptor respectively was assessed in a subsequent 4 h incubation at 37C. The maximum specific binding of ^{125}I-hFSH (10% specific binding) and ^{125}I-hCG (6% specific binding) was considered as 100% for all the calculations. The labeled hormones, ^{125}I-hFSH (top panel) and ^{125}I-hCG (data not shown), did not suffer any inactivation at 4C, 25C, 34C or 37C for 24-48 h. Data represents the mean ± SEM of triplicate values of one of two experiments.


100
80
60
40
20
% BOUND
NFSH* (37°, 34°, 25°, 4°C)
(P1 4°C)
(P1 34°C)
(P1 37°C)
(P1 25°C)
4
8
12
24
48
72
96
120
TIME IN HOURS


100
80
60
40
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% BOUND
4°C
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TIME (HOURS)

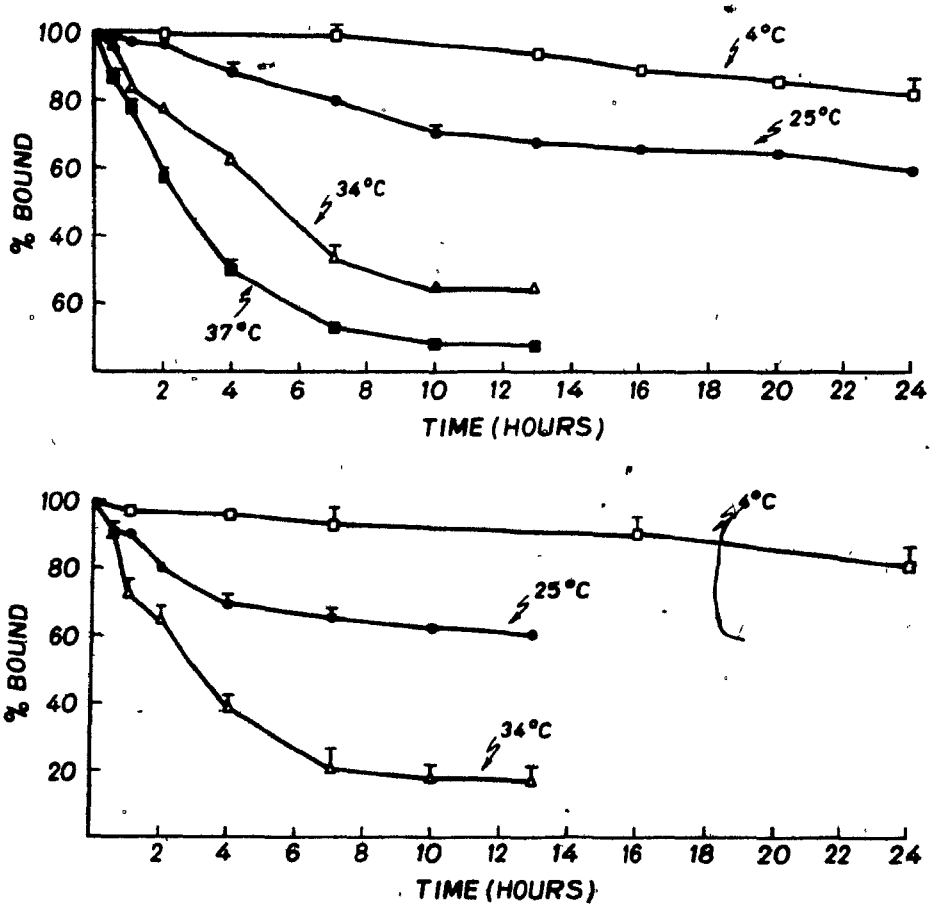


FIG. 21

Effect of preincubation on the integrity of the adult yellow baboon testicular receptor. A (top panel): ^{125}I-hFSH binding to 200 ug protein of subcellular fraction P1 from a 10 yr old yellow baboon. B (bottom panel): ^{125}I-hCG binding to 350 ug protein of subcellular fraction P1 from an 8 yr old yellow baboon. The maximum specific binding of ^{125}I-hFSH (14% specific binding) and ^{125}I-hCG (3% specific binding) was considered as 100% for all the calculations. Data represents the mean ± SEM of triplicate values of one of two experiments.

binding ability occurred by 4 h and 3 h for the rhesus monkey and yellow baboon respectively. The decline in hormone binding ability was clearly related to the temperature of preincubation. For the rhesus monkey (Fig. 20A), the receptor binding ability for ^{125}I-hFSH was rapidly lost at 37C showing a 50% decay, after only 1 h of preincubation. The rate of loss was slower at 34C, 25C and 4C. At 34C, 25C and 4C, a 50% loss of binding ability occurred after 1.5, 7 and 48 h of preincubation, respectively. The difference in stability of the receptor at 37C and 34C was reproducible. Data from this experiment clearly show that the exposed binding sites are susceptible to degradative processes in the absence of the hormone. However, if the ligand was included in the incubation at 37C, 34C or 25C, binding could be demonstrated up to about 10-12 h (Fig. 18 and 19). The decrease in specific binding that occurred beyond this period even in the presence of the ligand (Fig. 18 and 19) may again be attributed to inactivation of the receptor.

2.3.6 Effect of pH on the binding of ^{125}I-labeled hFSH to the receptor

The specific binding of radiolabeled hFSH to rhesus testicular receptor fraction P1 occurs over a fairly wide range of pH (Fig. 22) with optimal binding between pH 7.0-7.5. At the extremes of pH (4 and 10), the degree of specific binding was either small or insignificant. Table 5 illustrates the similar effects of pH on the binding of ^{125}I-hFSH by the rhesus monkey and yellow baboon. In addition to the pH, binding was apparently dependent on the type of buffer used. Although the same concentration of the buffer was used (25 mM in all cases), at every pH studied, specific binding in the presence of Tris-HCl was significantly higher than that obtained using phosphate buffers. The reduced binding of the hormone to the receptor at extremes of pH could be a result of a loss of the integrity of the particulate receptor or instability of the hormone itself. To investigate this effect further, the particulate receptor fraction from the rhesus monkey was exposed to pH 5.5 and pH 9.0 for 30 min at 37C followed by washing, resuspension and incubation with

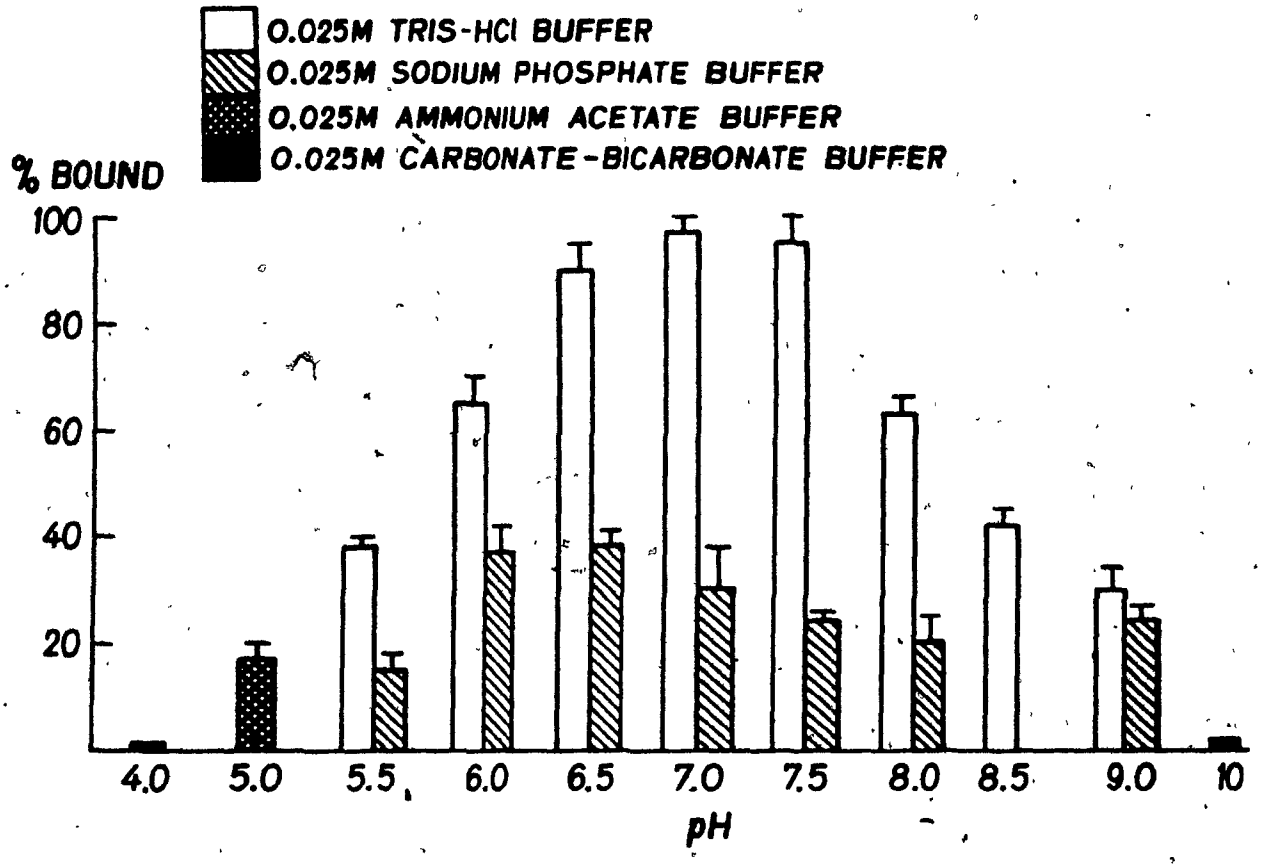


FIG. 22

Effect of pH on the interaction of ^{125}I-hFSH with 100 µg of testicular fraction (P1) from the rhesus monkey (13 yr). Each set of incubations were carried out at 34C for 8 h in the respective buffers indicated, in the presence of 10 mM $MgCl_2$ and 0.1% BSA. Other conditions of incubation are the same as shown in legend to Fig. 13. The maximum specific binding (10% specific binding) obtained with 25 mM Tris-HCl at pH 7.0 was set as 100% for comparison.

TABLE 5

INFLUENCE OF INCUBATION MEDIA pH ON THE SPECIFIC BINDING OF ^{125}I-hFSH TO THE NONHUMAN PRIMATE FSH RECEPTOR (TESTICULAR)

BUFFER	pH	RHESUS MONKEY (Mm)	YELLOW BABOON (Pc)
Acetate	4	4.89 ± 2.09	2.86 ± 1.83
	5	3.02 ± 0.78	1.31 ± 0.75
	5.5	31.13 ± 2.13	41.09 ± 2.61
Tris-HCl	6	78.90 ± 6.36	76.93 ± 3.31
	6.5	97.05 ± 4.65	77.96 ± 2.31
	7	100.50 ± 2.89	100.00 ± 1.93
	7.5	95.96 ± 3.85	88.74 ± 1.13
	8	70.05 ± 3.34	60.65 ± 2.14
	8.5	46.05 ± 1.52	55.78 ± 2.96
	9	33.36 ± 1.95	52.71 ± 1.27
	9.5	N.D.	43.74 ± 0.90
Sodium phosphate	6	42.41 ± 2.51	39.90 ± 3.37
	6.5	40.40 ± 1.26	N.D.
	7	30.02 ± 1.58	33.39 ± 2.41
	7.5	22.78 ± 9.09	N.D.
	8	23.56 ± 2.95	23.41 ± 2.09
Carbonate-bicarbonate	9	6.00 ± 1.07	N.D.
	9.5	3.02 ± 1.64	6.41 ± 0.38
	10	2.67 ± 1.92	2.59 ± 0.20
	10.5	1.24 ± 0.31	0.58 ± 0.19

Subcellular fractions were prepared from the rhesus monkey (13 yr) and yellow baboon (8 yr) testes. Aliquots of testicular fraction P1 (100-200 µg protein) were incubated at 34C for 8 h in the respective buffers indicated, in the presence of 10 mM $MgCl_2$ and 0.1% BSA. Other conditions of incubation are as shown in legend to Fig. 13. The maximum specific binding, 12% and 14% specific binding for the rhesus monkey and yellow baboon respectively, at pH 7.0 was set as 100% for comparison. Data represents the mean ± SEM of triplicate determinations of at least two separate experiments. N.D. = not determined.

^{125}I-hFSH in the usual assay buffer (25 mM Tris-HCl, pH 7.2) for 2 h. This procedure led to about 50-60% restoration of the specific hormone binding ability of the receptor (data not shown) indicating partial reversibility of the pH effect at least over the short interval studied.

2.3.7 Effect of ionic environment on the interaction of labeled hFSH with the monkey receptor

Exposure of the primate testicular receptor fraction to increasing concentrations of salts included in the assay buffer (25 mM Tris-HCl, pH 7.2) had profound effects on the binding of ^{125}I-labeled hFSH (Table 6). A typical result is shown in Figure 23A using the rhesus monkey as an example. Optimum specific binding of hFSH occurred in the presence of 5-10 mM $CaCl_2$ or $MgCl_2$. At equivalent concentrations of Na^+ or K^+, the degree of specific binding was very poor and only at higher concentrations of these ions was the binding significant. The highest binding observed at 50-100 mM of NaCl or KCl was still substantially lower than that seen with 5-10 mM $CaCl_2$ or $MgCl_2$. Using these values as the optimal molarity of monovalent and divalent cations necessary for maximum specific binding of ^{125}I-hFSH, the ionic strength of the incubation medium was calculated. A similar ionic strength of 0.02-0.05 was found for monovalent and divalent cations to result in maximal specific binding of ^{125}I-hFSH.

The effect of pretreating the particulate receptor fraction P1 of the rhesus monkey for 30 min at 37C with various ionic concentrations of $MgCl_2$ followed by washing and resuspension in 25 mM Tris-HCl buffer, pH 7.2 containing 10 mM $MgCl_2$ is shown in Figure 23B. It may be noted in this experiment that the manipulation process itself diminished the total amount of radioactivity bound at the 10 mM $MgCl_2$ concentration (compare Fig. 23B, open and hatched bars). This should now be viewed as the standard for comparing the effects of preincubation at all other $MgCl_2$ concentrations. Taking this into consideration, it becomes clear that there was restoration of hFSH binding in tubes preincubated with a suboptimal concentration of $MgCl_2$ such as 0.5 and 1 mM and then

TABLE 6

EFFECT OF VARIOUS SALTS ON THE SPECIFIC BINDING OF ^{125}I-hFSH TO THE NONHUMAN PRIMATE FSH RECEPTOR

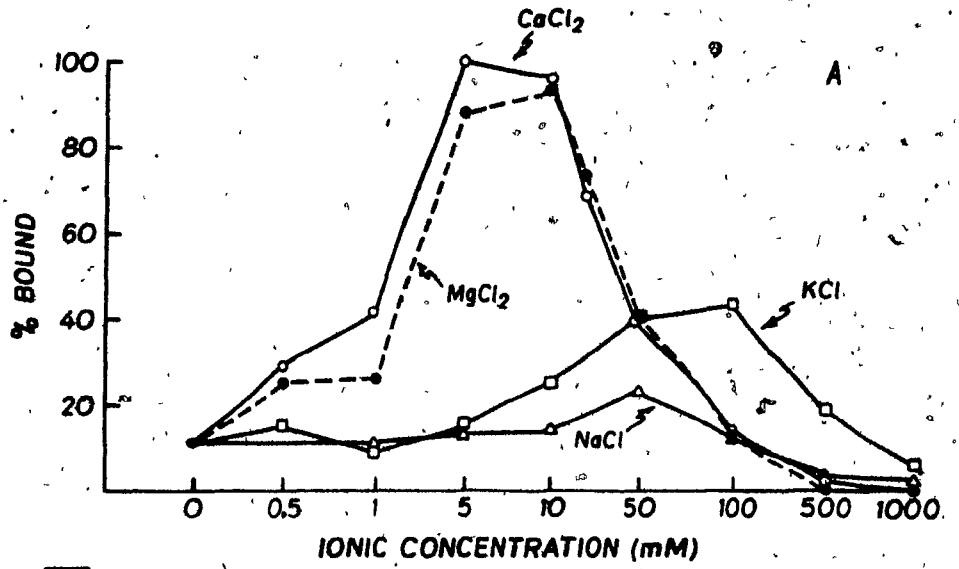
Species	Ionic Concentration (mM)							
	1000	500	100	50	10	5	1	0.5
$MgCl_2$ (mM)								
rhesus monkey (Mm)	1.60±0.98	1.68±0.71	14.68±1.40	53.09±2.64	100.00±1.34	88.37±2.33	38.10±3.69	28.73±1.43
yellow baboon (Pc)	0	1.54±0.74	20.35±1.21	62.47±0.64	100.00±1.26	89.93±2.20	53.49±1.64	N.D.
pig tailed monkey (Mn)	N.D.	0	12.66±0.39	47.35±3.26	100.00±3.18	86.53±3.07	54.58±2.07	N.D.
$CaCl_2$ (mM)								
rhesus monkey (Mm)	1.24±0.59	2.58±1.25	14.03±0.83	41.32±1.35	102.13±2.63	102.20±2.76	45.08±1.83	30.97±1.16
yellow baboon (Pc)	0	1.74±0.82	17.03±1.01	55.08±1.02	109.89±3.52	98.48±3.65	63.71±3.60	51.76±3.72
pig tailed monkey (Mn)	N.D.	0	11.12±0.67	44.82±0.46	113.71±1.67	102.63±1.79	70.89±2.47	50.40±2.58
$MgSO_4$ (mM)								
rhesus monkey (Mm)	N.D.	6.76±0.47	25.11±1.95	41.56±1.78	86.84±3.45	61.55±1.86	69.27±2.58	35.00±3.63
yellow baboon (Pc)	0	12.51±1.20	50.69±1.50	72.00±1.12	74.93±2.93	72.73±4.07	45.43±2.34	43.50±2.24
pig tailed monkey (Mn)	N.D.	4.21±0.21	41.32±1.64	66.50±4.02	80.47±1.54	72.36±1.22	43.14±1.81	33.71±1.15
NaCl (mM)								
rhesus monkey (Mm)	2.27±0.40	2.72±0.50	11.65±2.09	22.66±2.62	14.01±0.58	13.04±2.01	11.89±1.86	N.D.
yellow baboon (Pc)	0.39±0.24	0.78±0.68	14.65±1.15	22.05±1.13	23.49±0.47	22.01±0.45	22.47±0.84	26.11±1.32
pig tailed monkey (Mn)	N.D.	1.09±0.49	12.52±0.64	18.83±0.82	20.91±1.34	15.75±2.78	20.67±1.91	19.96±0.55
KCl (mM)								
rhesus monkey (Mm)	3.10±1.05	19.67±1.99	45.91±1.28	45.02±3.13	27.55±3.15	17.15±2.91	9.86±1.56	14.55±2.85
yellow baboon (Pc)	1.04±0.60	7.46±3.70	61.96±2.04	63.41±2.98	48.17±3.22	36.33±1.97	30.96±3.23	29.60±1.70
pig tailed monkey (Mn)	N.D.	2.98±1.46	54.58±3.77	54.86±1.06	44.97±2.01	31.75±1.86	20.80±3.08	26.10±4.04

Subcellular fractions were prepared from the rhesus monkey (13 yr), yellow baboon (8 yr) and pig tailed monkey (10 yr) testes. Aliquots of testicular fraction P1 (100-200 µg protein) were incubated at 34C for 8 h in the regular assay buffer at pH 7.2 but with varying concentrations of the salts. The maximum specific binding (Mm=10% S.B., Pc=12% S.B., and Mn=10% S.B.) obtained in the presence of 10 mM $MgCl_2$ was set as 100% for comparison. In the absence of salt only 20±2% specific binding was observed. Data represents the mean±SEM of triplicate determinations of at least two separate experiments. ND=not determined.

FIG. 23

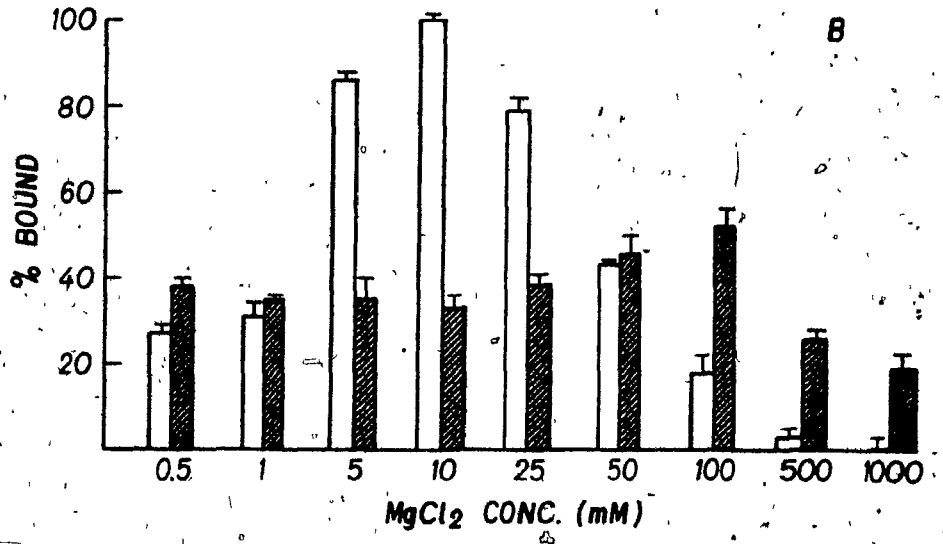
(A) Effect of ionic environment on the equilibrium binding of ^{125}I-hFSH to 100 µg testicular fraction (P1) from the rhesus monkey (13 yr). All incubations were carried out at 34C for 8 h in the regular assay buffer at pH 7.2 but with varying concentrations of the salts as indicated in the figure. The maximum specific binding (9% specific binding) obtained in the presence of 10 mM $MgCl_2$ was set as 100% for comparison. Each point was done in triplicate with the bars omitted from the figure for clarity. Data represents the mean of 3 separate experiments.

(B) Effect of pre-exposure of 125 ug of testicular fraction P1 from the rhesus monkey (13 yr) to a varying $MgCl_2$ environment at pH 7.2 on its subsequent ^{125}I-hFSH binding ability. The receptor was preincubated for 30 min at 37C in 25 mM Tris-HCl pH 7.2 with the indicated concentration of $MgCl_2$. After centrifugation and washing of the pellet, the receptor was re-incubated in the regular assay buffer (i.e. 25 mm Tris-HCl, pH 7.2, 10 mM $MgCl_2$) for 2 h at 37C in the presence of ^{125}I-hFSH for evaluation of binding. The maximum binding (7% specific binding) obtained with 10 mM $MgCl_2$ without the first 30 min preincubation was set as 100%. Note that a 30 min preincubation of the receptor without the ligand under these optimum conditions results in loss of binding ability in the subsequent incubation.



□ INCUBATION AT THE INDICATED $MgCl_2$ CONCENTRATION

▨ PREINCUBATION AT THE INDICATED $MgCl_2$ CONCENTRATION, WASHING, AND REINCUBATION IN THE PRESENCE OF 10mM $MgCl_2$



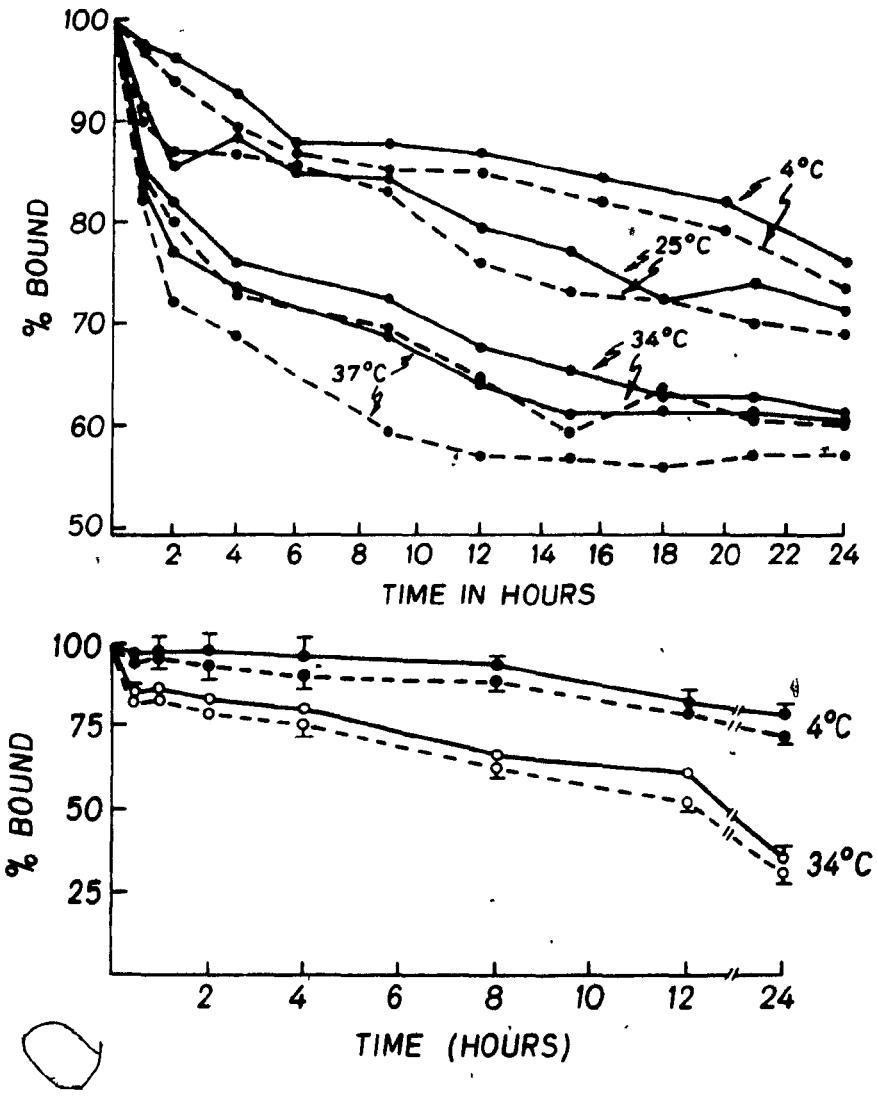
transferred to a 10 mM $MgCl_2$ environment. Tubes in which the receptor was preincubated in 25-1000 mM now showed enhanced binding when subsequently incubated. While removal of the higher salt concentration did not restore optimal binding in all cases, there was nonetheless a significant increase in original binding. The tendency for binding higher than that obtained at 10 mM $MgCl_2$, becomes clear upon examination of the bars showing binding before and after in the tubes preincubated with 25, 50 and 100 mM $MgCl_2$ concentrations. Failure to obtain optimum binding following treatment at the very high salt concentrations during preincubation could be due to the disorganization of the FSH receptor either by solubilization or other processes. At intermediate concentrations, such as 50 and 100 mM $MgCl_2$, the displacement of endogenous hormone by the salt and subsequent removal by the washing and centrifugation could lead to the exposure of more sites capable of binding the added labeled hormone in the second incubation.

2.3.8 Temperature and time dependence of the dissociation of the preformed hormone-receptor complex

Similar to the association reaction (Fig. 18 and 19), the dissociation reaction is also dependent on the incubation time and temperature. The dissociation of the hFSH receptor complex in the rhesus monkey and yellow baboon testis is shown in Figure 24. For reasons of clarity, only the effects of 34C and 4C are included for the yellow baboon (Fig. 24B). Dissociation of the labeled hFSH from the preformed ^{125}I-hFSH-receptor complex was a very slow process at all temperatures studied in the presence of a hormone free assay buffer. The addition of a 1000-fold molar excess of unlabeled hFSH was unable to significantly enhance the dissociation of the complex. After 24 h at 34C, only about 40-50% of total specifically bound radioactivity was lost (displaced) from the pellet of the rhesus monkey and yellow baboon. This is in comparison to about a 20-25% decrease observed at 4C after 24 h for the rhesus monkey and yellow baboon.

FIG. 24

Effect of temperature and unlabeled hormone on the dissociation of ^{125}I-hFSH from 100-200 ug testicular fraction P1 obtained from a 13 yr old rhesus monkey (A: top panel) and an 8 yr old yellow baboon (B: bottom panel). The preformed ^{125}I-hFSH-receptor complex obtained by incubation for 16 h at 25C, was washed and re-incubated in the same volume at different temperatures for various times in the presence (broken lines) or absence (solid lines) of a 1000-fold excess of unlabeled hFSH. The binding obtained at the beginning of the second incubation, 12% and 14% specific binding for the rhesus monkey and yellow baboon respectively, was set as 100%. The nonspecific binding in this experiment amounted to 20% and 30% of the total counts bound to the FSH receptor of the rhesus monkey and yellow baboon respectively. Data represents the mean ± SEM of triplicate determinations of at least three experiments. In panel A the error bars were omitted from the figure for clarity. Each point in panel A is the mean of triplicate determinations which varied less than 5%.


100
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2 4 6 8 10 12 14 16 18 20 22 24
TIME IN HOURS
100
75
50
25
% BOUND
4°C
34°C
2 4 6 8 10 12 24
TIME (HOURS)

2.3.9 Susceptibility of the hormone-receptor complex to pH and salt concentration

It was seen from data in Figure 22 that optimum binding of labeled human FSH to the receptor occurs between pH 7.0-7.5. The influence of extremes of pH such as pH 5 or pH 10, which were not conducive to hormone binding, also had marked effects on the dissociation of the preformed hFSH-receptor complex (Fig. 25A). Incubation at these pH values (5.0, 5.5, 10.0) resulted in a rapid dissociation of most of the specifically bound ^{125}I-hFSH from the rhesus monkey receptor within 1 h at 37C (about 85% decrease). Following this rapid loss the rate declined more slowly and by the end of 12 h all of the specifically bound hormone was stripped off the receptor.

The presence of a high salt concentration in the incubation medium also led to enhanced dissociation of the labeled hormone from the preformed complex (Fig. 25B). In the presence of 500 mM $MgCl_2$, nearly all of the specifically bound ^{125}I-hFSH was dissociated. It may also be interesting to note that significant dissociation of the hormone from the hormone-receptor complex can also be achieved by simply omitting $MgCl_2$ in the subsequent incubation (Fig. 25B). As noted in Figure 23A, a low (5-10 mM) concentration of divalent cation is required for maintenance of the efficient binding of the hormone to the receptor. In this series of experiments (Fig. 25B) the addition of a 1000 fold excess of unlabeled FSH failed to increase the dissociation of the bound hormone from the receptor.

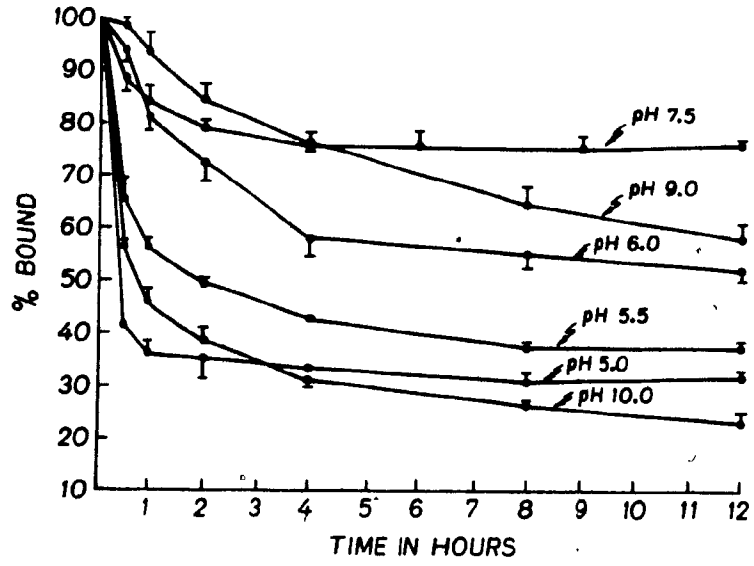
2.3.10 Nature of the receptor

The chemical nature of the rhesus monkey FSH receptor was studied in a limited fashion by incubating the particulate fraction with various enzymes or reagents. These results are shown in Figure 26. The exposure of P1 receptor fraction to enzymes such as ribonuclease or deoxyribonuclease or to 0.1% ethanol did not affect the specific binding of ^{125}I-hFSH. However, treatment with the proteolytic enzyme trypsin, phospholipase C

FIG. 25

(A) Dissociation of the preformed ^{125}I-hFSH receptor complex as a function of pH of the incubation medium. The complex was formed from 100 µg testicular fraction P1 obtained from a 13 yr old rhesus monkey as detailed in Fig. 24 and incubated at the indicated pH for varying time intervals in sets of triplicates. The following buffers (all at 25 mM containing 0.1% BSA and 10 mM $MgCl_2$) were used: acetate buffer pH 5.0 and 5.5; Tris-HCl buffer pH 6.0-9.0 and carbonate bicarbonate buffer pH 10.0. The binding obtained at the beginning of the second incubation, 8% specific binding, was set as 100%. The nonspecific binding in this experiment amounted to 25% of the total counts bound to the receptor pellet.

(B) Influence of the ionic environment on the dissociation of the preformed ^{125}I-hFSH receptor complex. The complex was formed from 100 µg testicular fraction P1 obtained from a 13 yr old rhesus monkey as detailed in Fig. 24 and incubated at 37C in 25 mM Tris HCl pH 7.2 with 0.1% BSA in the presence of the indicated concentration of $MgCl_2$. The binding obtained at the beginning of the second incubation, 8% specific binding, was set as 100%. The nonspecific binding in this experiment amounted to 25% of the total counts bound to the receptor pellet.


100
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80
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% BOUND
1 2 3 4 5 6 7 8 9 10 11 12
TIME IN HOURS
pH 7.5
pH 9.0
pH 6.0
pH 5.5
pH 5.0
pH 10.0

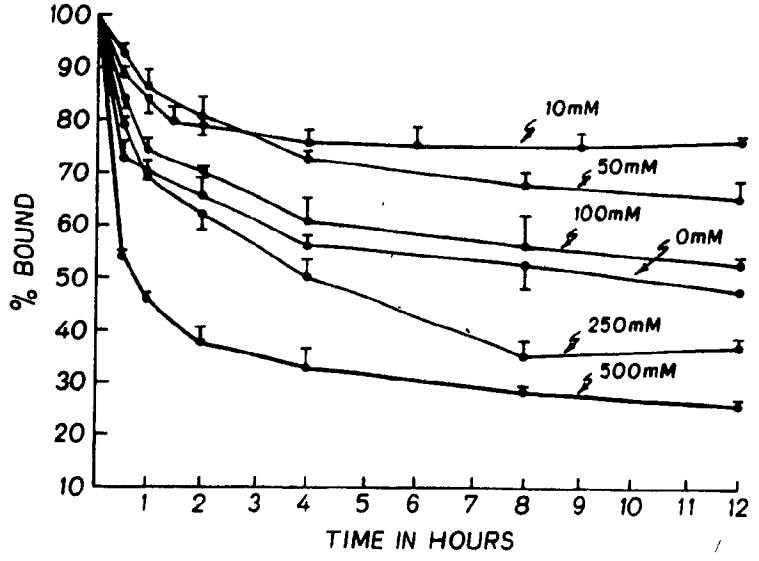
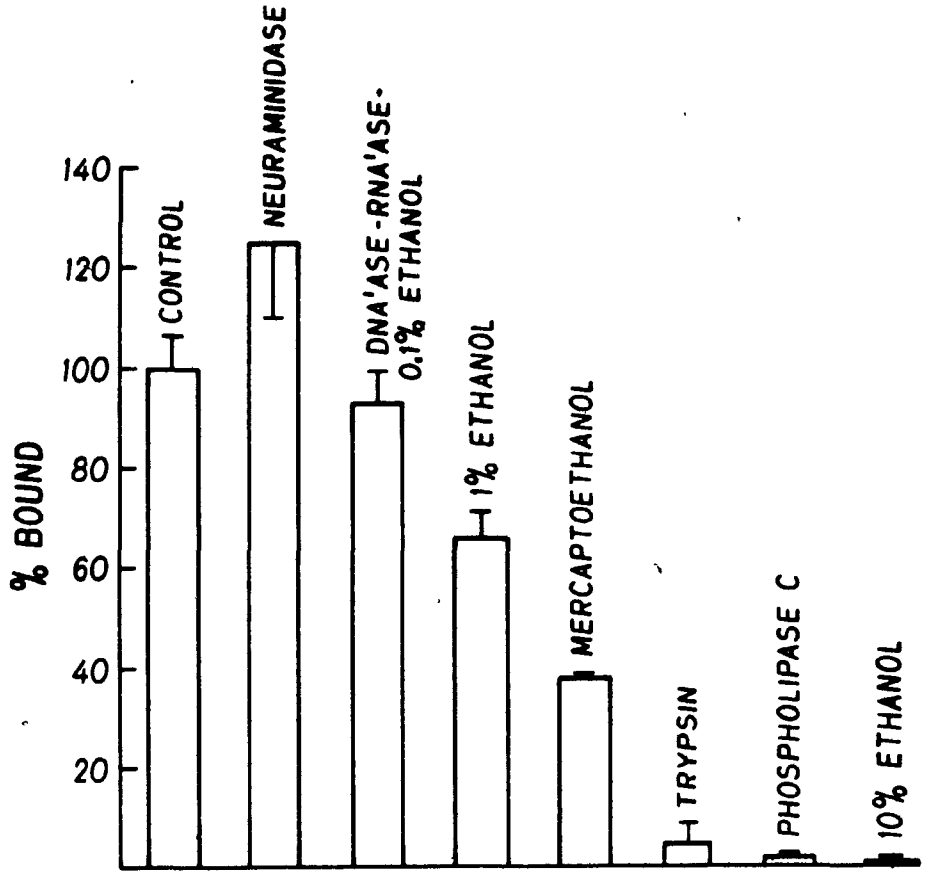

100
90
80
70
60
50
40
30
20
10
% BOUND
1 2 3 4 5 6 7 8 9 10 11 12
TIME IN HOURS
10mM
50mM
100mM
0mM
250mM
500mM

FIG. 26

Stability of the monkey testicular FSH receptor to enzymes and chemicals. The testicular fraction P1 (100 µg) from a 13 yr old rhesus monkey was exposed to the various treatments for 30 min at 37C. Phospholipase C treatment was done in 25 mM Tris-HCl buffer, pH 7.2 containing 0.1% $CaCl_2$ and neuraminidase action was effected by incubation in 25 mM ammonium acetate buffer pH 5.5 containing 0.2% $MgCl_2$. All other treatments were in 25 mM Tris-HCl buffer, pH 7.2 containing 0.2% $MgCl_2$. The second incubation which examined the ability of the control or treated receptor to bind ^{125}I-hFSH was performed at 37C for 2 h in the regular assay buffer (see Fig. 13). All values have been normalized to the appropriate controls. Data represent the mean ± SEM of triplicate values of one of two separate experiments.


% BOUND
140
120
100
80
60
40
20
CONTROL
NEURAMINIDASE
DNA'ASE-RNA'ASE-
0,1% ETHANOL
1% ETHANOL
MERCAPTOETHANOL
TRYPSIN
PHOSPHOLIPASE C
10% ETHANOL

and 10% ethanol almost completely eliminated the binding of labeled hormone indicating that a protein and phospholipid component of the particulate fraction may play an important role in the control of the FSH-receptor interaction in the primate testis. The significant decrease in the presence of a reducing agent such as mercaptoethanol suggests that disulfide bonds are also of structural importance to receptor integrity. Treatment of the receptor fraction with neuraminidase which removes accessible terminal sialic acid residues from glycoproteins did not reduce binding of ^{125}I-hFSH. On the other hand, a small increase in binding was observed. Therefore, sialic acid of the membrane does not appear to be essential for hormone uptake.

2.3.11 Effect of freezing and lyophilization on the receptor

The binding ability of ^{125}I-labeled hFSH and hCG to the gonadotropin receptor (P1 fraction) was assessed immediately after preparation and following freezing at -70C for 2 days. In all the four species studied, the degree of ^{125}I-hFSH binding to the receptor was slightly decreased (about 20%) after 2 days of freezing at -70C and thawing (Fig. 27). The decrease appeared to be greater (30%) in a species which had low initial binding, ex. pig-tailed monkey. With others such as the yellow baboon, the frozen and thawed receptor still showed very good (20%) specific binding. In the same tissues, the loss of ^{125}I-hCG binding was slightly greater (20-40%) probably reflecting the two-four fold lower hCG binding capacity of these tissues. Again the decrease was greatest in tissues with low hCG binding (less than 2% specific binding). Storage of these tissues for longer periods of time (up to one year) did not result in any further significant loses of hormone binding ability. This suggested that repeated freezing and thawing of tissues were detrimental to binding. This proved to be true, so fractions were only refrozen a maximum of two times. After storage for 2½ years at -20C, a lyophilized fraction of the rhesus monkey FSH receptor (P1 fraction) was as active as a fraction kept frozen at -70C. This should prove to be a more convenient method for long term storage of large quantities of the gonadotropin receptors.

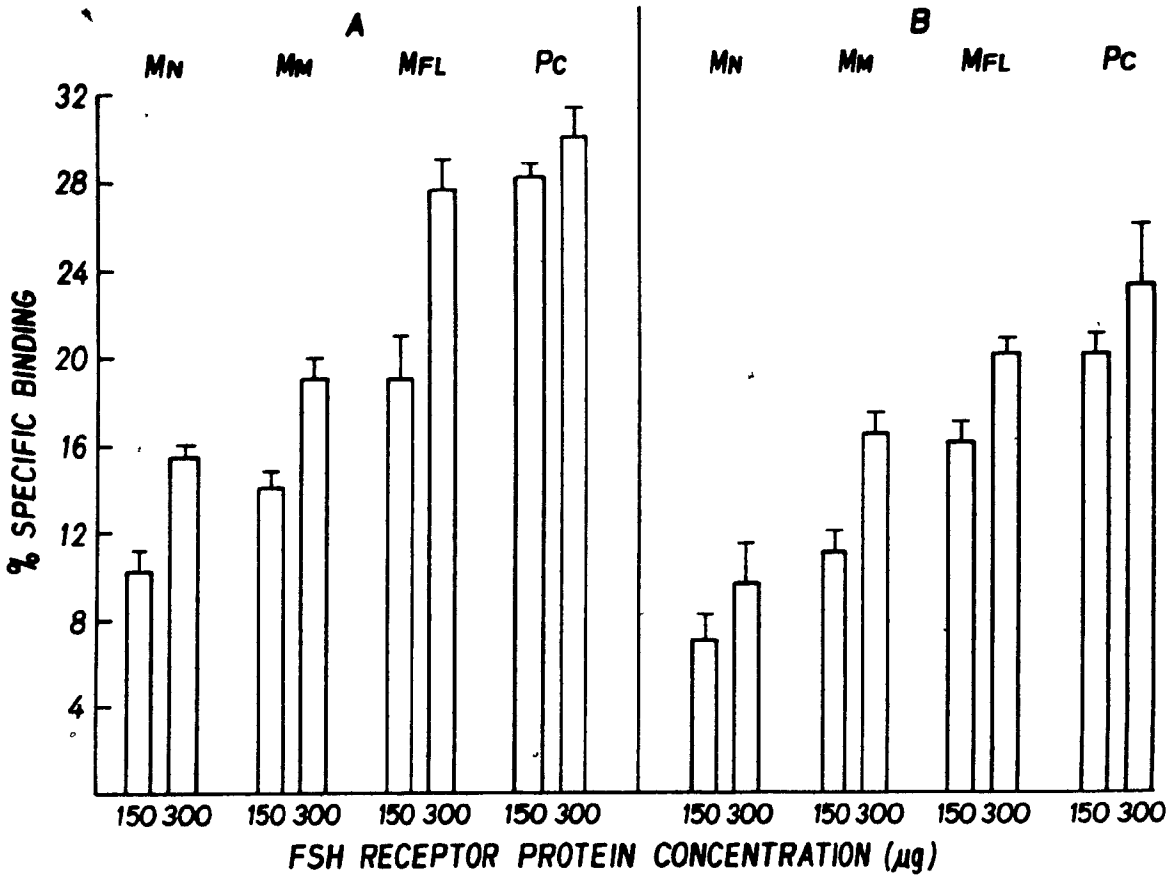


FIG. 27

Comparison of the binding ability of ^{125}I-hFSH to the monkey receptor (P1) fraction on the day of its preparation (panel A) and after two days of freezing at -70C (panel B). In the latter case, the frozen receptor was allowed to gradually thaw at room temperature. Non-specific binding (20% of added radioactivity) was determined in each case in the presence of 100 mg hFSH. The percentage reduction in panel B was 30, 18, 22 and 22 in Mn (15 yr), Mm (13 yr), Mfl (8 yr) and Pc (10 yr) respectively.

2.3.12 Specificity of the primate FSH receptor

In a binding assay using receptor fraction P1 from the four different species of monkeys, the binding of ^{125}I-labeled hFSH or hCG was effectively inhibited by their respective unlabeled hormones in a dose dependent manner. The nature of the displacement curves obtained with the receptor from all the four species were similar. A typical result is shown in Figure 28. Unlabeled LH did not displace ^{125}I-hFSH except at very high, non-physiological concentrations. Similarly unlabeled FSH did not displace ^{125}I-hCG.

2.3.13 Quantification of testicular primate gonadotropin receptors

The specific binding of ^{125}I-hFSH and ^{125}I-hCG to the P1 fraction derived from a 5.8 yr old rhesus monkey was proportional to the amount of labeled hormone added to the incubation medium (Fig. 29). Similar gonadotropin binding patterns were obtained using the subcellular fractions (P1) prepared from the other nonhuman primates. Specific binding of ^{125}I-labeled hFSH and hCG to the testes of the four nonhuman primates was linear at low hormone concentrations. Saturation of binding sites by ^{125}I-hFSH and ^{125}I-hCG occurred between 2-30 fmol and 0.05-1.6 fmol per 100 µg protein respectively. Binding data were analyzed by Scatchard plot (205) using the formula:

$$B/F = Ka\ (n-B)$$ where:

B = amount of hormone specifically bound

F = amount of free hormone

n = hormone binding capacity

Ka = apparent association constant

If the hormone receptor interaction is a simple bimolecular reaction and is at equilibrium, then the plot of B/F versus B should give a straight line (see insert Fig. 29). Thus one can determine the apparent dissociation constant (Kd) from the inverse of the slope of the line and the number of binding sites or binding capacity from the X intercept (n). For these calculations, the molecular weights of hFSH and hCG were considered to be 30,000 and 36,700 daltons respectively.

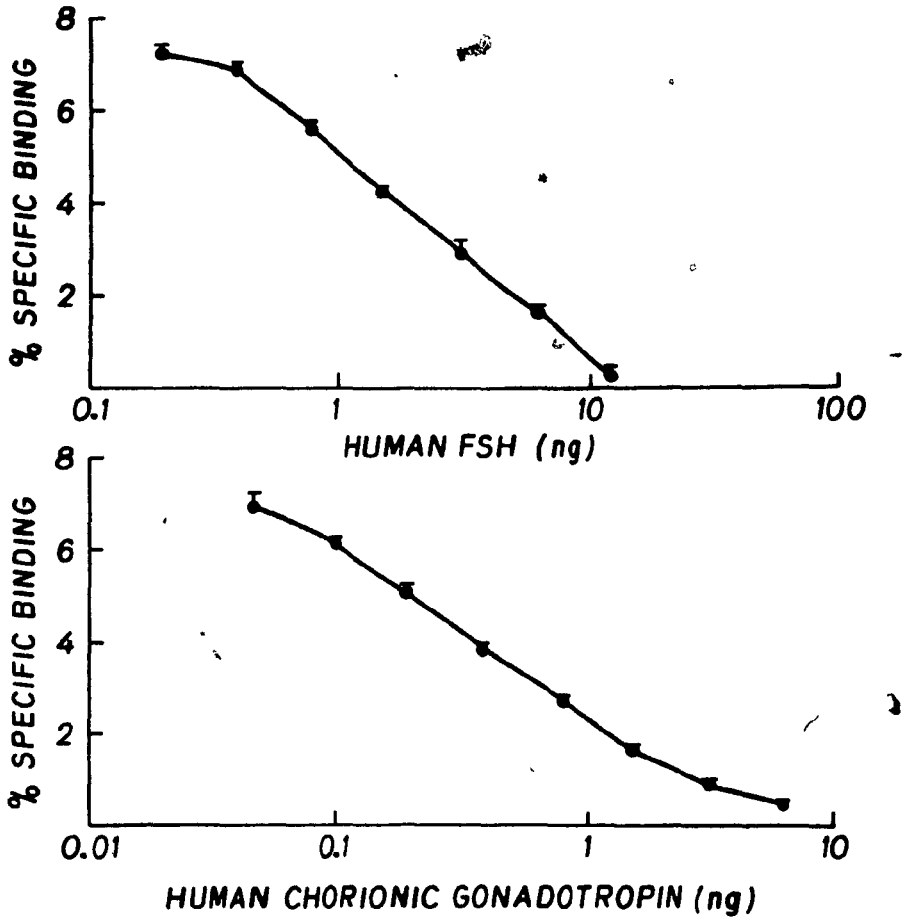
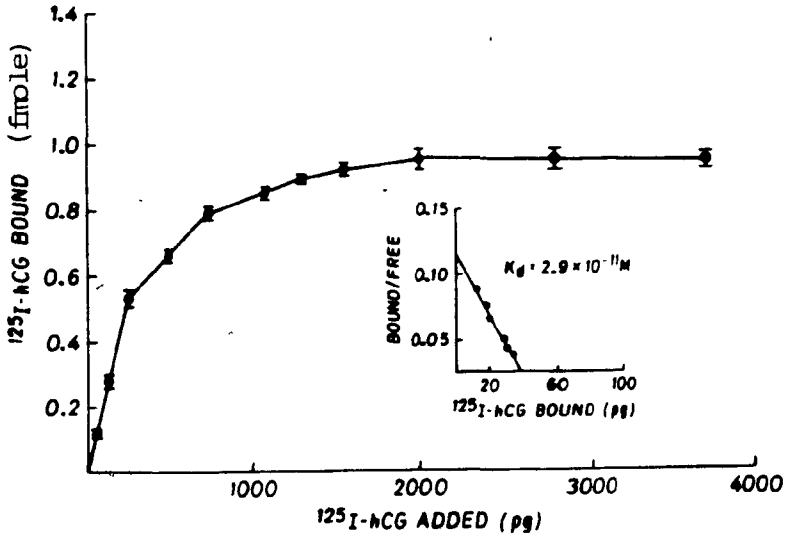


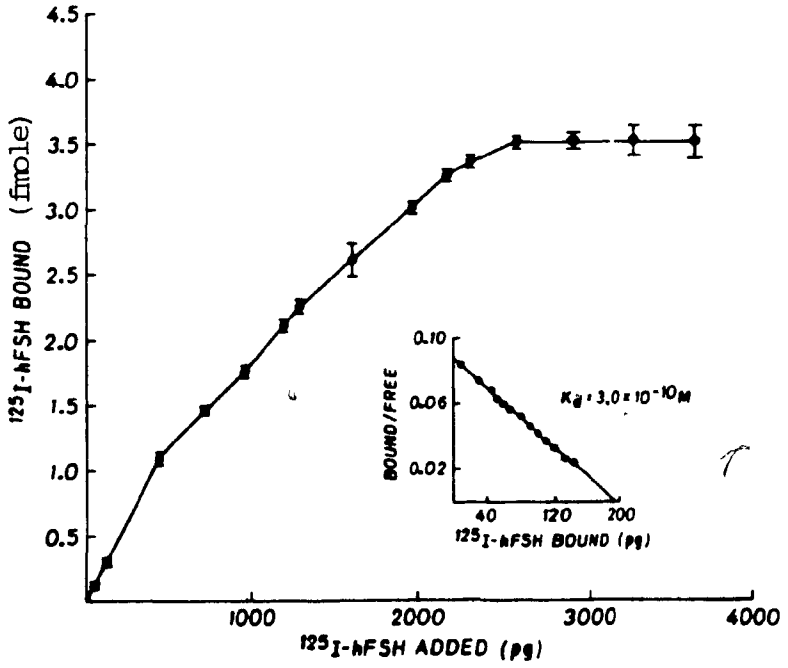
FIG. 28

Competition of ^{125}I-hFSH (A: top panel) and ^{125}I-hCG (B: bottom panel) by their respective unlabeled hormones for binding sites on the testicular fraction P1 (100-200 µg) obtained from the rhesus monkey (5.8 yr). Data represent the mean ± SEM of triplicate determinations of one of at least two separate experiments.

FIG. 29

Specific binding of increasing concentrations of labeled gonadotropins to a testicular fraction (P1) prepared from a 5.8 yr old rhesus monkey. A (top) ^{125}I-hCG with 165 µg protein equivalent of receptor (P1). B (bottom) ^{125}I-hFSH with 50 µg protein equivalent of receptor (P1). Nonspecific binding for each point was determined in the presence of a 500 fold molar excess of the unlabeled hormone. Inset in each panel shows the Scatchard plot of the binding data. The reciprocal of the negative slope of the line yields the apparent dissociation constant (Kd) and the intercept on the abscissa yields the total amount of labeled hormone bound in picograms (pg), which is used to express the number of receptors. The number of binding sites for hCG and hFSH were 0.64 and 13.1 fmol / 100 µg protein respectively, which is equivalent to 0.071 and 1.45 pmol/g of testes.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
^{125}I-hCG BOUND (fmole)
1000
2000
3000
4000
^{125}I-hCG ADDED (pg)
0.15
0.10
0.05
BOUND/FREE
K_d = 2.9 x 10^{-11}M
20
60
100
^{125}I-hCG BOUND (pg)


4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
^{125}I-hFSH BOUND (fmole)
1000
2000
3000
4000
^{125}I-hFSH ADDED (pg)
0.10
0.06
0.02
BOUND/FREE
K_d = 3.0 x 10^{-10}M
40
120
200
^{125}I-hFSH BOUND (pg)

The effect of increasing age on the binding capacities and affinities of the rhesus monkey LH(hCG) and FSH receptors were computed and then tabulated (Table 7). With one exception, the 5.8 yr old tissues, all tissues were surgically removed during the summer months, ie. during the nonbreeding season. Although the few number of tissues available to us limited the age range which could be studied, a marked increase in the testicular binding capacity for the gonadotropic hormones was observed with advancing age (Fig. 30). Taking into consideration only tissues taken from monkeys during the nonbreeding season, a progressive increase was observed in the number of LH(hCG) receptors up to 4.0 yr of age (Fig. 30A). FSH receptors increased up to 5.2 yr, with a marked increase in receptor numbers (4.2X) occurring between ages 4.0 and 5.2 yr (Fig. 30B). At the next age group available for study (13 yr), there was a pronounced decline in LH receptors (50%) and no significant change in FSH receptors. A distinct difference in the number of gonadotropin receptors was noted between the testes removed from a 5.8 yr old monkey during the breeding season (January-September) and the closest age matched monkey tissue taken during the nonbreeding season (5.2 yr). There was a greater number of LH receptors but a lower number of FSH receptors in the 5.8 yr old monkey testes. Whether this is a general phenomenon or something peculiar only to this animal is unknown. If the number of gonadotropin receptors is expressed as pmol hormone bound per gram of testes, then younger tissues are found to have a greater quantity of LH(hCG) receptors than older tissues. The period of increasing binding capacity (3.0-5.2 yr) is accompanied by a 50-100 fold increase in testicular size. Thus the increased testicular gonadotropin binding capacity of older animals was most likely a consequence of an increase in testicular size. Every tissue studied contained a greater number of FSH receptors than LH(hCG) receptors (10-100 times greater). While no age related change in the LH affinity constant was observed, there was a significant decrease in the affinity for hFSH with increasing age. The cause of the increased binding capacity and accompanying lower affinity for hFSH in the rhesus monkey testes is as yet unexplained.

TABLE 7

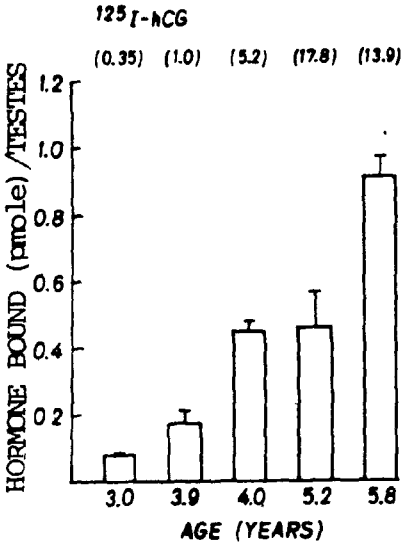
EFFECT OF AGE ON THE BINDING CAPACITY AND APPARENT BINDING AFFINITY OF THE TESTICULAR FSH AND LH(hCG) RECEPTORS OF THE RHESUS MONKEY

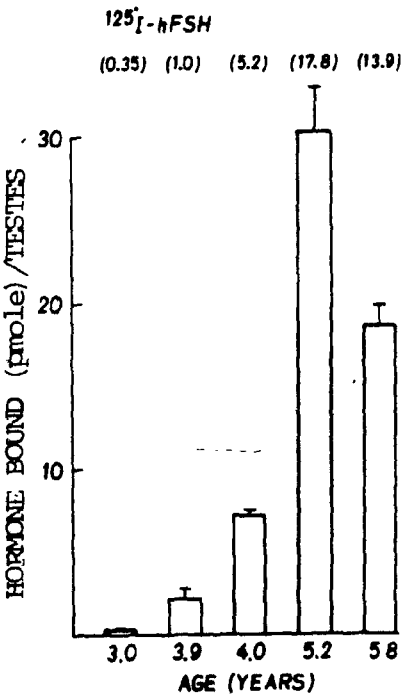
Age (yr)	Testicular weight (g)	Hormone binding capacity				Apparent binding affinity (Ka)	
		FSH receptor		LH(hCG) receptor		FSH receptor	LH(hCG) receptor
		pmol/g testis	pmol/testis	pmol/g testis	pmol/testis	($10^{10}M^{-1}$)	($10^{10}M^{-1}$)
3.0	0.35±0.01	0.91±0.09	0.32±0.03	0.230±0.001	0.082±0.042	1.49±0.30	3.23±0.15
3.9	1.02±0.19	2.18±0.19	2.26±0.61	0.174±0.001	0.177±0.032	1.15±0.19	2.45±0.13
4.0	5.25±0.75	1.41±0.27	7.20±0.36	0.086±0.007	0.447±0.028	0.73±0.001	3.94±0.28
5.2	17.80±0.50	1.71±0.20	30.34±2.71	0.026±0.007	0.450±0.121	0.29±0.01	2.47±0.46
5.8	13.85±0.15	1.34±0.10	18.55±1.19	0.067±0.005	0.921±0.053	0.35±0.02	2.87±0.58
13.0	20.85±0.85	1.85±0.31	32.20±3.52	0.021±0.005	0.434±0.086	0.40±0.21	2.17±0.24

Data was derived from Scatchard analysis of tissues utilizing ^{125}I-hFSH and ^{125}I-hCG. With the exception of the monkeys aged 13 yr (n = 4 animals, 1 tissue each) data represents individual animals (4 determinations each).

FIG. 30

Influence of age of testicular tissue on the binding of ^{125}I-hCG (A: top panel) and ^{125}I-hFSH (B: bottom panel) to the rhesus monkey gonadotropin receptor. Testicular weight is indicated in parenthesis. All tissues, except the 5.8 year old, were surgically removed during the non-breeding season. The number of receptors were determined by Scatchard analysis of the binding data (see Fig. 29) for each tissue. Each value represents the mean ± S.D. of Scatchard analysis of individual animals (4 determinations each).


^{125}I-hCG
(0.35) (1.0) (5.2) (17.8) (13.9)
HORMONE BOUND (pmole)/TESTES
1.2
1.0
0.8
0.6
0.4
0.2
3.0 3.9 4.0 5.2 5.8
AGE (YEARS)


^{125}I-hFSH
(0.35) (1.0) (5.2) (17.8) (13.9)
HORMONE BOUND (pmole)/TESTES
30
20
10
3.0 3.9 4.0 5.2 5.8
AGE (YEARS)

A comparison of the binding capacities and affinity constants for the interaction of ^{125}I-hCG and ^{125}I-hFSH with the different nonhuman primates is shown in Tables 8 and 9 respectively. The few tissues studied, did not permit a correlation between tissue age and receptor numbers. The binding affinities for both gonadotropins were in the range of 10^{9} - 10^{10} M, consistent with their blood levels. However all the nonhuman primate tissues exhibited a significantly higher affinity for ^{125}I-hCG. The binding capacities of the nonhuman primates for the gonadotropins were similar when expressed as pmol hormone bound per gram of intact tissue. The crab eating monkey and rhesus monkey exhibited the greatest FSH and hCG binding capacity respectively. All tissues from each species had a greater quantity of FSH receptors than LH(hCG) receptors, with this ratio being age and species dependent.

2.3.14 Detection of buffer soluble FSH binding components in testicular extracts

The presence of buffer soluble gonadotropin binding components was investigated in the 140,000 x g supernatant fraction of extracts of the bull, rat, human, and the four nonhuman primate testes. Various aliquots of the supernatant fractions (up to 2 mg of protein) were incubated overnight at room temperature with either ^{125}I-hCG or ^{125}I-hFSH (see section 2.2.10). Any hormone-receptor complex(es) formed was precipitated using polyethylene glycol. No significant binding (less than 1% specific binding) of either ^{125}I-hFSH or ^{125}I-hCG was detected in 5 individual testicular extracts from the rat (aged 34 days) or the human (aged 16-70 yr). Only the testicular supernatant fractions derived from the four nonhuman primates or the bull specifically bound ^{125}I-hFSH. These fractions bound ^{125}I-hFSH and ^{125}I-oFSH equally well. None of the fractions demonstrated a capability to specifically bind ^{125}I-hPRL, ^{125}I-hCG or ^{125}I-oLH (less than 1% specific binding). A typical result for ^{125}I-hFSH binding is shown in Figure 31A using the rhesus monkey as an example. Binding of ^{125}I-hFSH was proportional to the amount of

TABLE 8

COMPARISON OF THE BINDING CAPACITIES OF THE FSH AND LH RECEPTORS IN THE NONHUMAN PRIMATE TESTES

Species	Testis weight mean (g)	Hormone binding capacity FSH pmol/g testis	FSH pmol/testis	LH(hCG) pmol/g testis	LH(hCG) pmol/testis	Ratio of FSH/LH receptors/ testis
Macaca fascicularis (3)	11.1±8.8	1.58±0.29	20.07±0.13	0.011±0.002	0.13±0.03	154.4
Macaca mulatta (9)	12.6±1.4	1.25±0.15	11.60±2.85	0.075±0.02	0.37±0.09	31.4
Macaca nemestrina (3)	15.1±3.2	0.82±0.18	11.85±1.73	0.021±0.004	0.32±0.05	37.0
Papio cynocephalus (4)	15.7±7.7	1.08±0.16	14.56±2.11	0.028±0.007	0.30±0.04	48.5

Numbers in parentheses represent the number of animals utilized for the study (two testes/animal). These values are for animals greater than 2.9 yr of age. Data was derived from Scatchard analysis of tissues utilizing ^{125}I-hFSH and ^{125}I-hCG. Values represent the mean ± SEM of at least two Scatchard plots performed per tissue.

TABLE 9

ESTIMATION OF THE APPARENT DISSOCIATION CONSTANT (Kd) OF THE GONADOTROPIN RECEPTORS IN THE NONHUMAN PRIMATE TESTES

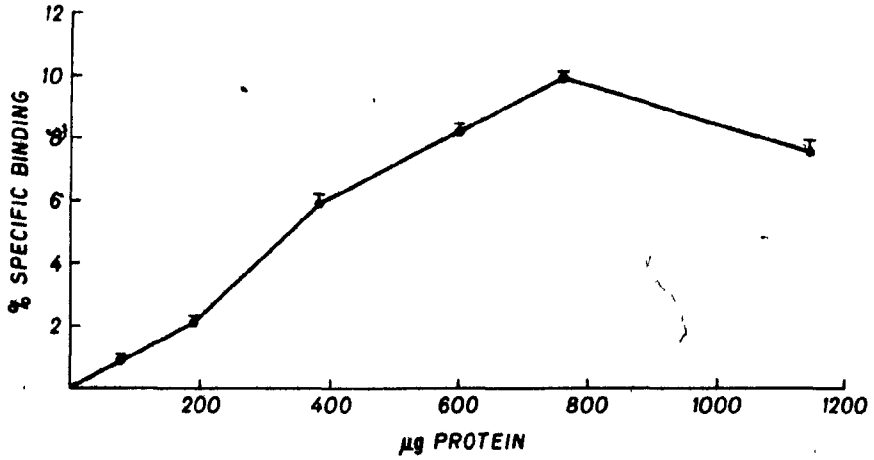
Species	Apparent Dissociation Constant (Kd)	
	FSH receptor (10^{-10}M)	LH receptor (10^{-10}M)
Macaca fascicularis (3)	2.51±0.36	0.58±0.14
Macaca mulatta (9)	2.09±0.49	0.44±0.08
Macaca nemestrina (3)	2.17±0.36	0.29±0.08
Papio cynocephalus (4)	2.37±0.35	0.46±0.03

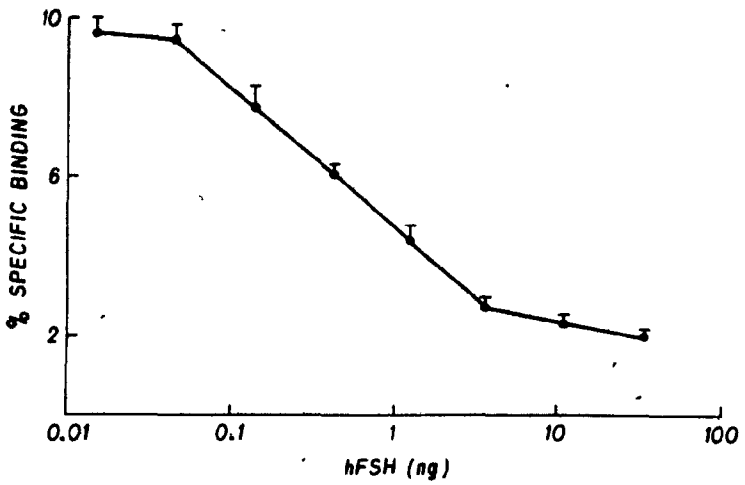
The numbers in parentheses represents the number of animals utilized for the study (two testes/animal). Values were derived from Scatchard analysis of the tissues using ^{125}I-hFSH and ^{125}I-hCG. Data represents the mean ± SEM of at least two Scatchard plots per tissue (two testes/animal). The differences in Kd values between the FSH and LH(hCG) receptors were statistically significant for all species. Within the FSH or LH(hCG) receptors, the Kd values were not statistically significant.

FIG. 31

(A) Specific binding of ^{125}I-hFSH to varying amounts of the testicular supernatant fraction obtained from a 5.2 yr old rhesus monkey. Data represents the mean ± SEM of triplicate values of one of two separate experiments.

(B) Displacement of ^{125}I-hFSH binding to the soluble FSH receptor-like component (760 µg protein) by unlabeled hFSH. The soluble FSH receptor-like component was derived from the testes of a 5.2 yr old-rhesus monkey. Unlabeled hCG did not displace ^{125}I-hFSH binding. Data represents the mean ± SEM of triplicate values of one of two separate experiments.


% SPECIFIC BINDING
12
10
8
6
4
2
200
400
600
800
1000
1200
µg PROTEIN


% SPECIFIC BINDING
10
6
2
0.01
0.1
1
10
100
hFSH (ng)

protein added, with saturation occurring at approximately 760 µg protein (equivalent to 20 mg testes). The specific binding of ^{125}I-hFSH was competitively displaced by unlabeled hFSH in a dose dependent manner (Fig. 31B). Addition of an excess of hCG (1 µg) was ineffective in displacing ^{125}I-hFSH binding. Specific binding of ^{125}I-hFSH to the soluble binding component was variable. The supernatant fractions from the nonhuman primates were not all capable of binding ^{125}I-hFSH. Approximately 2-3% specific binding of ^{125}I-hFSH per 20 mg equivalents of testicular tissue was observed, with 10% specific binding the best result obtained. The supernatant fraction derived from the young bull testes was more active than that prepared from two adult bull testes suggesting an age dependent difference. This fraction also demonstrated the higher specific binding of ^{125}I-hFSH, 13% per 2 mg of protein (equivalent to 160 mg testes). The activity of the soluble binding component from the bull or rhesus monkey testes was lost when boiled for 50 min at 100C suggesting a protein nature of this component.

2.4 Discussion

The elucidation of the properties of the hormone-membrane bound receptor interaction is an essential prerequisite to gaining a full understanding of the relationship between the phenomenon of hormone binding and the intricate process of the activation of cellular events. At the time we initiated our studies, no data were available in the literature on the interaction of primate testes with FSH at the cellular level. We chose the rhesus monkey as our model because of all the nonhuman primates it is the best characterized in terms of testicular morphology and function (206, 207). The high cost and rarity of the rhesus monkey for laboratory studies, impose serious constraints on the choice and quantity of tissues available for receptor studies. These constraints which have forced investigators to use other species of nonhuman primates as models of human reproductive function led us to

investigate three additional species of nonhuman primates - the pig-tailed monkey (Mn), the crab-eating monkey (Mfl) and the yellow baboon (Pc) as alternative models of the gonadotropin-hormone receptor interaction.

In the present study, we have identified a particulate fraction in the monkey testis which can specifically bind hFSH or hCG. The ^{125}I-hFSH bound well to the particulate fraction from all the four species investigated. In the rhesus monkey, ^{125}I-hCG bound better to tissues prepared from younger animals aged 3-6 yr (3-12% specific binding) than to older adult animals aged 13 yr (less than 1% specific binding). The failure to detect significant hCG binding in adult tissues utilizing up to 900 µg of protein could not have been due to the loss of integrity of the labeled hormone because these preparations showed good specific binding to a rat testicular preparation (Fig. 14).

As demonstrated in the present work, frozen tissues are suitable for the preparation and characterization of primate LH and FSH receptors. As there may be some small initial loss after freezing, our estimates of the gonadotropin binding capacity of the different species may have been slightly underestimated. Our inability to demonstrate significant binding of ^{125}I-hCG to the rhesus monkey (13 yr) testes could be due to the loss of the very low amount of the LH(hCG) receptors in the mature testes during the period when they were kept frozen. While studies with fresh tissues, especially for the LH receptor, would clearly be preferable, this is difficult as they are not easily available.

Preliminary studies on gonadotropin binding capacities of the nonhuman primates was limited by the number and age of the testicular tissues provided to us by the various institutions. While it was possible to investigate the changes in testicular gonadotropin receptors with increasing age in the rhesus monkey, definite conclusions of the age dependency is restricted by the limited age range studied. Nonetheless certain trends were observed which are consistent with studies on non-primates and which will hopefully stimulate further work on the subject.

A comparable gonadotropin binding capacity was found for all four nonhuman primate species. For the rhesus monkey we determined an FSH and hCG(LH) binding capacity of 1250 ± 154 fmol/g testes and 75 ± 20 fmol/g testes respectively. Our value for the overall hCG binding capacity of the rhesus monkey is in agreement with those reported by Davies et al. (185) for animals greater than 6 yr (23-146 fmol/g) and by Zaidi et al. (208) for animals aged 3.5-5.0 yr (198-355 fmol/g). However Zaidi et al (208) observed a 10 fold higher hCG binding capacity and binding affinity for their 5 yr old monkeys. Besides the fact that we studied frozen tissues, other factors such as age and variation in reproductive activity could have contributed to the differences in these results. Examination of adult female rhesus monkeys of unspecified age revealed a similar binding affinity to the testicular LH receptor but a 30-100 fold higher LH(hCG) binding capacity (209, 210). The LH binding capacity in these female monkeys depended on the stage of the menstrual cycle, being highest during the midluteal phase (8.9 ± 0.75 pmol/g) and lowest at menses (2.3 ± 0.3 pmol/g).

The rhesus monkey is reported to be a seasonal breeder even under laboratory conditions showing cyclic variations in hormonal profiles, testicular size and activity (207). The dependence of the gonadotropin binding capacity, but not binding affinity, on the particular season is reflected by the increased number of testicular LH receptors and decreased number of FSH receptors by a tissue prepared near the end of the breeding season (January). In another seasonal breeder, the sheep, the stage of sexual activity and the number, but not affinity, of the gonadotropin receptors is dependent on the particular time of year (211). The concentration and total number of gonadotropin receptors per testis was highest before and decreased during the breeding season. Therefore it is important to note that most of the tissues we have processed, except the baboon, were removed from animals in the summer months, a time when the seasonal cycle is apparently at a lower ebb. If this was related to the low amount of LH(hCG) receptors the decrease might be exaggerated by freezing.

The four nonhuman primates are similar to the bull (79, 212), sheep (211, 213), and pig (78, 79) in having more FSH receptors than LH receptors and differ from the mature rat (71, 77, 214) which shows a reverse trend in this respect (Fig. 14). The rhesus monkey testicular FSH receptor is similar to that of the bull (212) and rat (71, 214) in that the total number of FSH receptors increased as the animal matured. The increase in FSH receptors was due to the increased testicular weight. This is better visualized when the number of receptors is expressed as fmol/g testes. The concentration of FSH receptors showed a significant rise from 3.0-3.9 yr (Table 7) commensurate with the development of puberty in the rhesus monkey (215). Beyond 4 yr of age the concentration of FSH receptors remained fairly constant. A similar variation is seen in the rat (214) where puberty is accompanied by a rapid fall, followed by a constant testicular FSH receptor level up to 100 days. This is dramatically different from the bull (212) where the concentration of testicular FSH receptors progressively declines from birth. Rhesus monkeys can live as long as 27 yr so it is possible that studies of older monkeys might reveal additional age-related changes in the concentration of gonadotropin receptors.

Huhtaniemi et al (216) reported a value of 2780 fmol/g testes for a fetal tissue of 140-160 days gestational age. Although it is not wise to draw conclusions from one value, a higher hCG binding capacity by prepubertal testes would be consistent with data from studies on laboratory animals. The increased LH receptor content/testes in the prepubertal sheep (213), pig (78), and rat (214, 217) is due to the increased number of Leydig cells and increased number of LH receptors per Leydig cell. In the rhesus monkey (Table 7) an increase in the total number of LH receptors is observed until the attainment of puberty (4 yr), again probably due to the increase in testicular size. However, unlike the FSH receptor, attainment of puberty was followed by the maintenance of a constant level of hCG receptors which only decreased at 13 yr. This is unlike the pig (78) or rat (214, 217) where the testicular LH receptor level increases after puberty. However, the rat is similar in having

a slower rate of rise between 50 and 110 days of age (217). The concentration of LH receptors in the rhesus monkey is highest at puberty, declines after puberty and then remains constant up to 13 yr. A similar pattern is observed in the pig (78) but not the rat (214, 217) although again the number of LH receptors seems to level off between 50-100 days of age (217).

There was no significant change in the affinity constant of the LH receptor at different stages of sexual maturation of the rhesus monkey (Table 7). On the other hand, as large as a 4-5 fold decrease in the binding affinity of the FSH receptor occurred with advancing age. This is partly analogous to the situation in the rat (214) where no change in the LH receptor binding affinity but an approximately two fold increase in the FSH receptor binding affinity was observed during sexual maturation. However, these authors point out that this difference might not be significant. A species related difference in binding affinity is suggested by the failure to note a change in the binding affinity of the bull testicular FSH receptor during sexual maturation (212). Furthermore, in the pig (78) and sheep (213) no apparent age dependent variation in the binding affinity of the testicular gonadotropin receptors was noted. At all ages, the binding affinity of the LH receptor was greater than that of the FSH receptor in the rhesus monkey, analogous to the situation in the sheep (213) and rat (214). The difference in gonadotropin binding affinities of the rhesus monkey is unlikely due to storage or handling procedures because the majority of tissues were processed and frozen on the same day under identical conditions.

A specific FSH but not hCG(LH) binding component was detected in the soluble fraction of the bull and four nonhuman primate testes. Although we have not characterized this fraction in detail, it appears to be similar to the FSH soluble binding component of immature bovine testes (96, 100). This tissue specific calf FSH receptor-like component shared many features in common with the particulate and detergent solubilized receptor such as species specificity and salt dependency.

As yet is it not possible to state that the FSH soluble binding component of the nonhuman primate testes is a solubilized receptor, a receptor component or an antibody. The source of these soluble binding components is also unknown but they could have arisen from the cell cytosol or cell membrane (intracellular or extracellular). The low yield and lack of correlation between the quantity of FSH membrane bound receptors and soluble FSH receptor-like components in the supernatant fraction (data not shown) argue against the cell membrane being the sole source of the soluble FSH binding components. Whereas primate testes with a large quantity of LH receptors did not possess any soluble LH binding components, testes with comparable numbers of FSH receptors did possess a soluble FSH binding component. This is consistent with an intracellular source. Incubation of membrane extracts may be required to release this soluble LH binding component (97). Unlike the rhesus monkey, the amount of the soluble FSH binding component is reported to be age dependent in the bull and rat and would explain the absence of these components (less than 1% specific binding) in adult rat (34 days) and human (16-70 yr) testicular extracts (96).

Investigations of younger human testicular supernatants may yield a FSH soluble binding component. While these FSH receptor-like components would be expected to be present and interfere with hormone binding in tissue homogenates, for most tissues their concentration is insufficient to be responsible for the two fold lower binding ability of homogenates versus fraction P1. Other factors such as proteolytic enzymes and receptor binding inhibitors are thought to play a major role in this inhibition (92, 218).

Data from the present studies indicate that the properties of the adult monkey LH(hCG) and FSH receptors are in many ways similar to those of the bovine and rodent species. Although the study of an interaction of homologous monkey LH and FSH with its testis would have been highly desirable, the nonavailability of purified gonadotropins made this difficult. Hence, highly purified hFSH and hCG were used in these studies.

The interaction of hFSH and hCG with monkey testis, like that of many hormones with their receptor is both time and temperature dependent (Fig. 17). The time course of association of the ^{125}I-gonadotropins with the monkey receptor at 34C is very similar to the receptor from the various nomprimate testes, but considerably slower. For example in the rat system, maximum binding of FSH (52, 68) and LH (79) have been reported to be within 3 and 8 h respectively. The monkey LH(hCG) testicular receptor is very similar to that of the bull and pig, whose interaction with hCG is slow, requiring 8-12 h of incubation at 34C for attainment of equilibrium (79).

The monkey testicular LH(hCG) and FSH receptors appear to be quite sensitive to temperature. The rapid inactivation of the receptor at 34C and 37C (Fig. 20 and 21) could either be caused by some proteolytic enzyme still associated with the semi-purified membranes or by the disruption of receptor structure in the free (exposed) state by thermal perturbations. These degradative phenomena are apparently operative at 25C and 4C, although to a markedly lesser degree (Fig. 20 and 21). The presence of the hormone increases the stability of the receptor (Fig. 17). Occupancy of the binding sites by either ^{125}I-hFSH or ^{125}I-hCG renders the complex more stable at the same temperature (34C). This is clearly shown by data in Figure 24, in which there was only about 30% dissociation of ^{125}I-hFSH in 2 h and this loss only marginally increased to 45% after 24 h at 34C. The monkey FSH receptors, with the exception of the pig tailed monkey (Mn) (Fig. 17), are unlike the human testicular hCG receptor, which yielded significant specific hormone binding during 24 h of incubation at 37C (186).

The binding of ^{125}I-hFSH to the monkey receptor is not readily reversible as revealed by data in Figure 24. The failure of a large excess of unlabeled hormone added after the formation of labeled hormone-receptor complex to influence specific binding is clearly indicative of this. While we have noted a similar low degree of irreversibility with the adult bovine testicular FSH receptor (219), other reports indicate

up to 50% dissociation in the same species (72) by unlabeled hFSH. The reasons for the differences noted in bovine are not clear at present. However, in marked contrast to either the monkey testis or bovine testis nearly all of the bound hormone dissociates from the rat testicular FSH receptor in a much shorter time at 37C (190) even in the absence of unlabeled hormone. These data indicate differences in the properties and nature of the FSH-testis receptor interaction in the different species. The irreversibility of the ^{125}I-hFSH binding to the monkey testicular receptor is similar to recent data from other laboratories which have investigated the nature of the ^{125}I-hCG interaction with rat testes (220, 221).

The observations of marked reduction in the specific binding of ^{125}I-hFSH to the monkey receptor following treatment with trypsin and phospholipase C but not with nucleases are similar to the bovine (72) and rat (190) systems. These data are suggestive of a lipoprotein nature of the binding sites. Since we have only used partially purified membranes in these studies, confirmatory evidence as to the precise nature of the primate FSH receptor must await its solubilization.

With respect to pH, the monkey FSH receptor appears to be much more stable than the human testicular hCG receptor (186) which exhibited a sharp optimum of about pH 7.8. In addition, the effects of low (pH 5.5) and high (pH 9.0) pH's on the rhesus monkey FSH receptor are partially reversible. It is also interesting to note that the monkey FSH receptor exhibited a preference for Tris-HCl buffers rather than sodium phosphate buffers (Table 5). The significance of this is not clear at the present time. The rat FSH receptor is also reported to give varying degrees of binding depending upon different buffers (52) but not with the same preference as the monkey receptor.

Optimum specific binding of ^{125}I-hFSH to the monkey receptor occurred in the presence of 5-10 mM $MgCl_2$ or $CaCl_2$. The divalent cations are thought to neutralize the negative charge of the membrane thus enabling the binding of the physiologically negatively charged hFSH (222).

Induction of a conformational change of the membrane receptor would allow unmasking of previously sequestered binding sites. This would explain the ability of Mg^{+2} to increase the binding capacity but not binding affinity of the calf FSH receptor (222). A biphasic effect of metal ions on the interaction of gonadotropins with their receptors has been reported for the bovine (222, 223) and rat (190) gonads. In the immature pig (74) and adult bull (72), graded concentrations of various salts did not affect FSH binding except at high concentrations, when an inhibitory effect was observed. The inability to observe stimulation at low ionic concentrations could be due to the presence of high endogenous levels of salt in the membrane preparations.

The nature of the preformed hormone-receptor complex was also studied by evaluating its ability to undergo dissociation under a variety of conditions. While the unlabeled hFSH was at best marginally effective in dissociating the complex (see Fig. 24), the latter was more susceptible to variation in pH (Fig. 25A) and $MgCl_2$ concentration (Fig. 25B). About 85% of the bound ligand was released from the rhesus monkey receptor within the first 30-60 min of incubation at 37C, when the pH was altered (to pH 5 or 10, see Fig. 25A) or in the presence of 0.5 M $MgCl_2$ (Fig. 25B). The ability of $MgCl_2$ to dissociate bound hCG or LH from rat testicular receptors has been reported (221, 224). Various metal ions besides $MgCl_2$ are important for stable hormone receptor interaction. Monovalent ions, such as Na^{+} are reported to alter the binding affinity of the gonadotropin receptor thereby enhancing dissociation of the hormone-receptor complex (100). EDTA, a metal ion chelator, caused rapid dissociation of the hormone receptor complex (100).

Similar to the interaction of hCG with rat Leydig cells (220, 221), a part of the hFSH-primate receptor complex is also resistant to dissociation, at physiological pH and temperature. This is clearly evident from Figure 24 in which the remaining 50% of the bound hFSH could not be released even after incubation for as long a period as 12-24 h at 37C. Whether or not the tightness of the hormone binding to the receptor is a time dependent phenomenon has not been ascertained in the present

study. The implications of such a tight binding of the hormone to the receptor is not clear and requires further investigation. It is worthy of note that the interaction of several protein hormone ligands such as hGH (225), prl (226) and TSH (227) with their respective receptors have also been shown to be not completely reversible.

CHAPTER 3

HUMAN TESTICULAR GONADOTROPIN RECEPTORS

3.1 Introduction

The molecular mechanisms involved in hormone action can be clearly defined by elucidating the structural features of the hormone as well as the cellular processes in the target tissue. The initial event of a peptide-protein hormone action is mediated by interaction with specific high affinity sites on the plasma membrane of the cell. The identification of surface receptors for gonadotropins in ovarian and testicular tissue from many species (183, 184) is consistent with this concept. During the last decade, major advances have been made in our understanding of the structural organization of the human pituitary gonadotropic hormones LH, FSH and placental hCG, including their amino acid sequence and arrangement of the carbohydrate units (14). Unfortunately, very few studies have been done with human gonadotropin receptors. However, the basic properties of the gonadotropin-gonadal receptor interaction, have been well characterized in many laboratory and domestic animal species (183, 184). While these data are valuable in designing appropriate methodology for study of primate gonadotropin receptors, it is difficult to know how much of the nonprimate data may be relevant to man. Several recent investigations have shown the presence of specific binding sites for hCG(LH) in monkey (185) and human (174, 186-188) testes. Specific FSH binding sites (receptors) in human testis have not previously been studied. In an attempt to understand the nature of the primate gonadotropin receptors in the testis, a comparative study was launched using tissue from humans and several species of monkeys (194-196). Preliminary results of this investigation were recently presented (228, 229).

3.2 Materials and Methods

3.2.1 Hormones

All the hormones used in these investigations were highly purified. Human FSH, its subunits, ovine FSH, ovine LH, bovine LH, hCG, its subunits, human growth hormone (hGH) and human thyroid stimulating hormone (hTSH) were prepared in our laboratory (197-200). hCG (CR119), hLH (iodination grade), rat FSH (iodination grade) and rat LH (iodination grade) were all supplied by the NIADDK, NIH, Bethesda, Md. Ovine prolactin (35 IU/mg) was from Dr. C.H. Li, Univ. of California, San Francisco, Ca; human prolactin from Dr. H.G. Friesen, Univ. of Manitoba, Winnipeg, Man; equine FSH (100 x NIH-FSH-S10) from Dr. T. Landefeld, Univ. of Michigan, Ann Arbor, Mi; and porcine FSH (12.4 x NIH-FSH-S10) was a gift of Dr. R.J. Ryan, Mayo Clinic, Rochester, Mn. Synthetic luteinizing hormone releasing hormone (LHRH) was obtained from Ayerst Laboratories, Montreal, Que.

3.2.2 Chemicals

Lactoperoxidase, bacitracin, 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK), and bovine serum albumin (BSA), were purchased from Sigma, St. Louis, Mo. All other chemicals were of reagent grade from Fisher Scientific Company, Montreal, Que.

3.2.3 Testicular tissues

Fresh human testicular tissue was obtained from patients undergoing orchidectomy in local hospitals. Tissue was also collected from individuals of various ages within 12 h post mortem. No significant differences in binding properties were noticeable between surgical or autopsy material. Monkey testes were collected at surgery, frozen on dry ice and sent to us from the Regional Primate Research Center at the University of Washington, Seattle, USA. Testicular tissue from adult rats was collected in the laboratory and testes from mature bulls was obtained from the local abbatoir. The hospitals which supplied us with

pieces of human testicular tissue did not possess the facilities to quick-freeze the tissues in liquid nitrogen or store them at -70C. As tissues became available they were used immediately or stored at -20C until they could be picked up (maximum of 5 days). The number of FSH and LH receptors as assessed in the P1 fractions showed no dramatic decrease during the first 5 days of freezing. Routinely when the testicular tissue was not immediately used, it was frozen at -70C. After 1 year of freezing at -70C or lyophilization, the subcellular fraction P1 did not differ significantly in gonadotropin binding capacity or binding affinity from when it was first prepared. Losses in gonadotropin binding ability are mainly due to repeated freezing and thawing of the samples so this was kept to a minimum (less than 2 times). Human tissues were processed within one month of their collection.

3.2.4 Preparation of subcellular fractions from primate testes

Frozen individual testes of known weight were thawed under a stream of blowing cold air and decapsulated. All subsequent steps were performed at 4C. To facilitate homogenization, the testes were minced with scissors, rinsed and suspended in 25 mM Tris-HCl buffer, pH 7.2 containing 100 mM sucrose (4 ml/g of tissue). The minced tissues were mechanically homogenized using a Tekmar tissuemizer set at a low speed setting using 2-3 pulses of 10-15 seconds duration. As preliminary experiments revealed no difference between hand and Tekmar mechanical homogenization, the latter method of handling was used for all the studies. The homogenate (H1) in each case was filtered through 4 layers of cheesecloth and the filtrate was centrifuged at 40,000 x g for 1 h. The resultant pellet (P1) was resuspended in the buffer by dispersion using 8 strokes in a tight fitting glass Dounce Homogenizer (1 g per 2 ml of 25 mM Tris-HCl buffer, pH 7.2 containing 10 mM $MgCl_2$). Centrifugation of the supernatant at 140,000 x g for 1 h did not significantly increase the protein yield. Samples were stored in aliquots at -70C. Prior to assay, the fractions were again gently homogenized in the assay buffer to obtain a uniform suspension. Protein content was assessed as previously described in Chapter 2 (see section 2.2.5).

3.2.5 Iodination of hormones

Hormones were labeled with ^{125}I by the lactoperoxidase method (201, 203) using carrier free $Na^{125}I$ (Amersham Inc., Ill.) as previously described in Chapter 2 (see Section 2.2.6). The labeled hormones were purified by gel filtration on Sephadex G-100 and preserved in aliquots at -70C until use. They were generally used within two weeks of preparation. The specific activities of the labeled hormones were in the range of 60-100 µCi per µg.

3.2.6 Binding of ^{125}I-labeled gonadotropins to testicular fractions

Tests for specific binding of ^{125}I-labeled hormones to the gonadotropin receptor were carried out in duplicates or triplicates in disposable 10 x 75 mm polystyrene tubes. Each tube contained approximately 50,000 cpm (approximately 400 pg) ^{125}I-labeled hormone, various amounts (µg protein equivalent) of the testicular fractions and 100 µl of the unlabeled hormone and/or assay buffer to constitute a final volume of 250 µl per tube. The assay buffer consisted of 25 mM Tris-HCl buffer, pH 7.2 containing 0.1% BSA, 10 mM $MgCl_2$ and 0.6 mM bacitracin. Following the various additions, the tubes were vortexed and incubated in a continuously shaking Dubnoff water bath for 8 h at 34C or 16 h at 25C. The reaction was terminated by the addition of 2 ml of chilled assay buffer or 200 µl bovine gamma globulin (5 mg/ml) plus 1 ml polyethylene glycol solution (MW 6000) (see section 2.2.10). Pelleting was accomplished by centrifugation at 2900 x g for 15 min at 4C in a table top IEC clinical centrifuge. The supernatant was removed by aspiration under vacuum and the radioactivity in the pellet was determined in an LKB rackgamma 11 counter (counting efficiency 70%). In all cases, nonspecific binding was determined in the presence of a 1000 fold excess of respective unlabeled hormone. The difference between the total radioactivity bound and nonspecific binding was defined as the amount specifically bound and expressed as a percentage of the total counts put into the tubes. All variations in this procedure with respect to handling, temperature, medium, and processing not mentioned in the methods section are indicated at appropriate sections in the results.

3.2.7 Prolactin binding

The integrity of the ^{125}I-labeled human prolactin (hPRL) was assessed using a liver homogenate from pseudopregnant rabbits (obtained from Dr. M. Kahn, McGill University, Montreal, Que.) and a homogenate of granulosa cells from pig follicles (prepared in this laboratory) which were both rich in specific prolactin receptors. The presence of prolactin binding sites was investigated in the testicular subcellular fraction P1 of the human, rhesus monkey and yellow baboon. Briefly, approximately 50,000 cpm of ^{125}I-hPRL was incubated with 100-300 µg protein, in the presence and absence of unlabeled hPRL, in a final incubation volume of 250 µl. After an incubation of 3 h (34C) the reaction was stopped by adding 2 ml of chilled assay buffer, centrifuged and counted.

3.2.8 Dissociation reaction

The testicular receptor hormone complex was first formed by incubating 100-300 µg protein equivalent of the subcellular fraction P1 with ^{125}I-hFSH or ^{125}I-hCG in a total volume of 200 µl for 15 h at 25C. To each tube was then added 50 µl of the assay buffer of desired pH and molarity in the presence or absence of unlabeled hormone. The tubes were then vortexed to ensure proper dispersion of the pellet and incubated further at the desired temperature (see results). At specific intervals, samples were removed, diluted with 2 ml of assay buffer at 4C and centrifuged and processed as above. The supernatant was removed and radioactivity in the pellet was determined.

3.2.9 Temperature stability of the receptor and radiolabeled hormone

A 100 µl aliquot of the receptor fraction P1 (100-300 µg protein) was preincubated at different temperatures for various periods of time in the presence or absence of 1 mM bacitracin or TLCK. At specific time intervals radiolabeled hCG or hFSH was added in the presence or absence of a 500 fold excess of the respective unlabeled hormone. The volume was

made up to 250 µl with the assay buffer and the binding ability of the pretreated receptor fraction evaluated by performing a 4-h binding assay at 34C. Tubes were processed by centrifugation or by polyethylene glycol precipitation (see section 3.2.6). The reason for the decreased binding of the gonadotropic hormones, i.e. inactivation of the receptor and/or radiolabeled hormone, was also studied. In these experiments, the testicular receptor fraction P1 was preincubated in a volume of 150 µl at 34C for either 8 h or 15 h in order to achieve at least a 50% loss of ^{125}I-labeled hCG and hFSH binding respectively. As a control, an identical set of tubes was preincubated for the same length of time at 4C. The tubes (set #1) were then centrifuged at 2900 x g for 10 min at 4C. The supernatant (100 µl), containing any degradative enzyme activity, was removed and added to another set of tubes (set #2). Tubes from set #1 were resuspended with 200 µl assay buffer containing ^{125}I-hFSH or ^{125}I-hCG in the presence or absence of a 500 fold excess of the respective unlabeled hormone. To the 100 µl of the supernatant fraction in set #2 was added 50 µl of the original receptor (fresh) and 100 µl of ^{125}I-hFSH or ^{125}I-hCG in the presence or absence of a 500 fold excess of the respective unlabeled hormone. Both sets were vortexed to resuspend the pellet and incubated for 4 h at 34C. At the end of the second incubation, only the reaction in set #1 was stopped by adding 2 ml chilled assay buffer. The two sets of tubes were centrifuged and the supernatant removed from set #1 by aspiration. An aliquot of the supernatant (200 µl) was removed from the second set of tubes and added to a third set. Tubes of set #2 were then washed with 2 ml chilled assay buffer, centrifuged, aspirated and counted. Set #3 tubes received fresh bull (FSH assay) or rat (hCG assay) testicular receptor and ^{125}I-hFSH or ^{125}I-hCG in the presence or absence of a 500 fold excess of the respective unlabeled hormone (final volume 300 µl). After a third incubation of 2 h at 34C, tubes were centrifuged, aspirated and counted. All incubations were done in triplicate with the appropriate controls.

3.2.10 Assessment of the gonadotropin binding inhibitory activity of testicular extracts (140,000 x g supernatant)

Briefly, aliquots of the testicular supernatant fraction were assessed for their ability to inhibit ^{125}I-hCG binding to a rat testicular LH receptor and ^{125}I-hFSH binding to a bull testicular FSH receptor. Incubations were for 2 h at 34C in a final volume of 250 µl. The effects of preincubation of the gonadotropin receptor with the testicular supernatants on gonadotropin binding was also determined. In these experiments the rat or bull gonadotropin receptor preparation was first incubated with the desired testicular supernatant fraction for 2 h at 34C. The tubes were then washed once with assay buffer to remove the supernatant fractions and the membrane pellet recovered by centrifugation. The membranes were resuspended in assay buffer in the presence of ^{125}I-hCG or ^{125}I-hFSH to reconstitute the original volume (250 µl). A second incubation of 2 h at 34C was then performed to assess the effects of this treatment on the receptor.

3.2.11 Assessment of the gonadotropin binding capacity of the human testes

The binding capacity of the human testicular tissues were determined by Scatchard analysis (205) and/or saturation analysis (212, 230). Briefly, for saturation analysis individual tissues (200-600 µg) were incubated for 15 h at 25C with a saturating concentration of the radiolabeled hormone (1 ng). Nonspecific binding was assessed by the addition of a 500 fold excess of unlabeled hormone (250 µl final volume). The amount of radiolabeled hormone specifically bound was then compared to a standard curve. This standard curve was generated by incubating the radiolabeled hormone with increasing concentrations of human testis (P1) of known receptor concentration. The binding capacity of the standard tissue was determined by Scatchard analysis. Using either method, comparable values for the number of unoccupied binding sites was obtained. Saturation analysis allowed the processing of a large number of tissues with only a minor expenditure of time and tissues.

3.2.12 Statistical analysis

Data were analyzed for significance by Student's 't' test. Displacement curves were tested for parallelism and Scatchard plots performed using a programmable Hewlett-Packard desk top calculator.

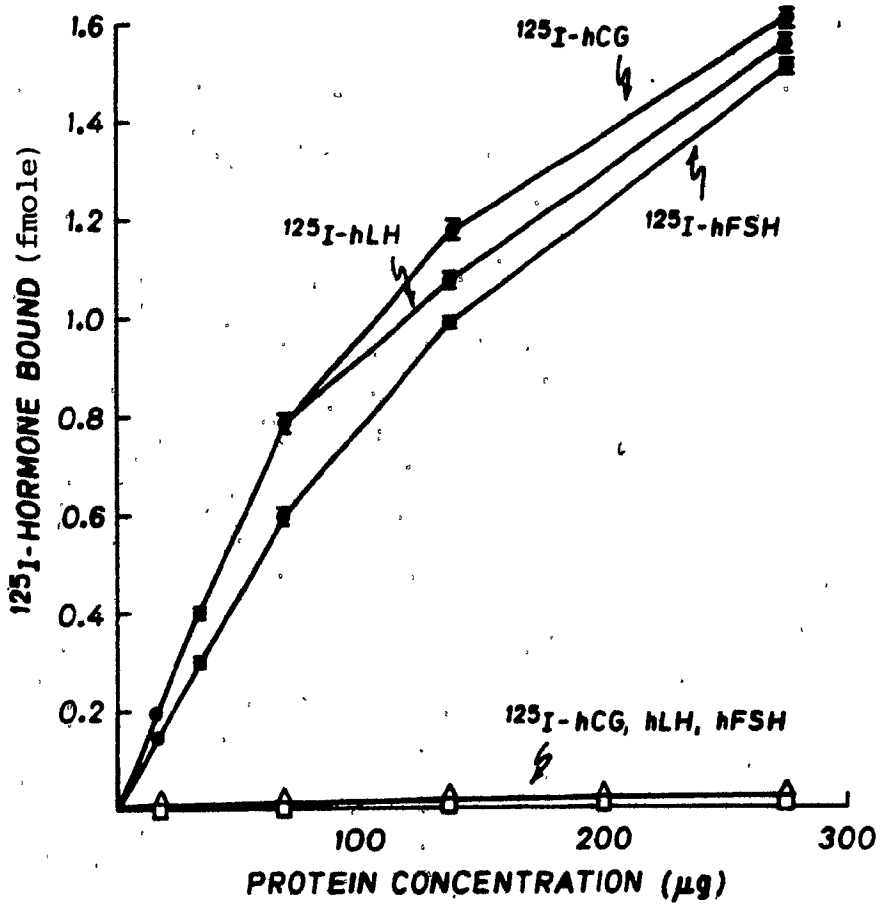
3.3 Results

3.3.1 Distribution of gonadotropin binding sites in human testis and other tissues

The presence of gonadotropin receptors was assessed in testicular and nontarget tissues obtained from a 50-yr old man. The tissues were processed (see section 3.2.4) and diluted to yield approximately 30 mg fraction H1 and 16 mg fraction P1 per gram of intact tissue. Significant specific binding of ^{125}I-labeled hCG, hLH and hFSH was detected in both homogenates and a 40,000 x g subcellular fraction prepared from the testes. Gonadotropin binding was proportional to the amount of testicular protein (fraction P1, see Fig. 32) added. About 70-80% of the radiolabeled hFSH and hCG associated with the pellet represented specifically bound hormone but with radiolabeled hLH only about 50% could be displaced by the unlabeled hormone. This is different from the rat testicular receptor(s) where approximately 70-80% specific binding was obtained with all the three labeled hormones. Testicular fraction P1 showed a two fold enhanced binding activity over the crude homogenate for all three gonadotropins. The very low and often undetectable binding of all three radiolabeled gonadotropins by subcellular fractions prepared from other tissues (liver, spleen, lung, pancreas, intestine, muscle, thyroid and kidney) suggests localization of the gonadotropin receptors only in the testis. Further addition of up to 700 µg of protein (equivalent to 49 mg wet weight of intact tissue) only resulted in increased nonspecific binding, without showing an increase in specific binding of the gonadotropins.

FIG. 32

Specific binding of radiolabeled human gonadotropins (hFSH, hLH, hCG) to particulate fractions (P1) of adult human (50-70 yr) testis (●——●), liver (□——□) and spleen (△——△). Various amounts of tissues were incubated with approximately 0.4 ng (50,000 cpM) of ^{125}I-labeled hormone in the absence or presence of the respective unlabeled preparations (100 ng) for 8 h at 34C. The incubation was done in 250 µl of 25 mM Tris-HCl buffer, pH 7.2 containing 10 mM $MgCl_2$, 0.1% BSA and 0.6 mM bacitracin. The specific binding of 1 fmol of labeled hormone represents 36.7 pg of hCG and 30 pg of hLH and hFSH. In this and all other figures the data points show the mean ± SEM of triplicate incubations of at least two separate experiments.


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
^{125}I-HORMONE BOUND (fmole)
^{125}I-hCG
^{125}I-hLH
^{125}I-hFSH
^{125}I-hCG, hLH, hFSH
100
200
300
PROTEIN CONCENTRATION (µg)

3.3.2 Optimum gonadotropin binding conditions

At 34C, specific binding of the gonadotropins occurred rapidly during the first two hours of incubation, reaching a maximum in 8-10 h (Fig. 33). Maximum hormone-receptor complex formation required more time at 25C (12 h) and did not attain a maximum after 24 h at 4C (fig. 34). The LH and FSH receptors showed similar binding characteristics at 37C and 34C. Further incubation beyond 12 h at 34C (fig. 33 and 34) resulted in decreased specific hormone binding possibly due to inactivation or degradation of the receptor by temperature and/or enzymes. Specific binding of ^{125}I-hFSH to the human testicular FSH receptor was also salt and pH dependent (fig. 35 and 36). Optimum binding occurred in the presence of 5-10 mM $MgCl_2$ or $CaCl_2$ at pH 6.5-7.5.

3.3.3 Dissociation of the preformed hormone-receptor complex

The dissociation of either ^{125}I-hFSH or ^{125}I-hCG from their human testicular receptor site(s) was dependent on the time, temperature, pH, and ionic concentration of the incubation medium (fig. 37 and 38). Dissociation at all temperatures studied, in the presence or absence of a 1000 fold molar excess of unlabeled hormone was a slow process just as was observed for the rhesus monkey (fig. 25). While 1/4 of the hormone receptor complex dissociated within the first 2 h, prolongation of the incubation up to 4-8 h was necessary to achieve an additional 20-25% decrease at 34C (fig. 37). Increasing the ionic strength or pH of the incubation medium markedly enhanced the dissociation of ^{125}I-hCG and ^{125}I-hFSH from the preformed complex (fig. 38). With either gonadotropin receptor, a high salt concentration (400 mM $MgCl_2$) was the most effective agent for enhancing dissociation of the hormone receptor complex. Approximately 80% of the hormone was specifically dissociated from the receptor within 2 h. Interestingly, the FSH receptor appeared less susceptible to the effects of low pH (pH 5.0) than the LH(hCG) receptor.

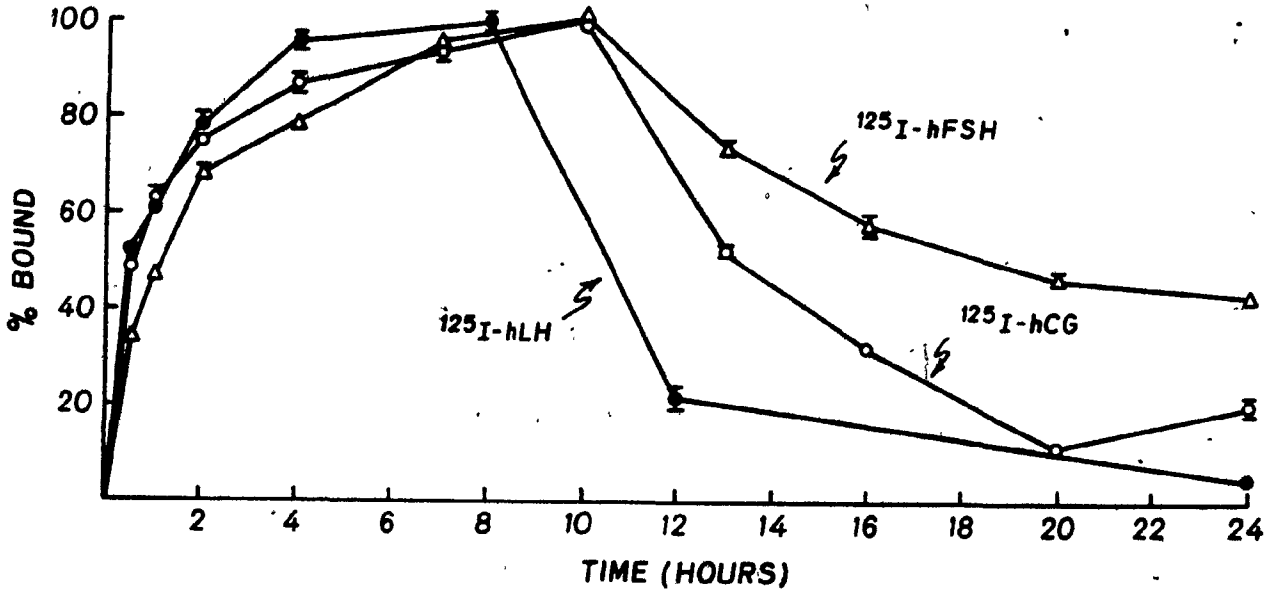


FIG. 33

Time course of the binding of labeled hLH, hCG and hFSH to human testicular fraction P1 obtained from adult males (20-70 yr). The incubation conditions except for time were the same as in Fig. 32. The maximum binding attained in each instance was set as 100%. Thus 100% binding represents 4%, 8% and 11% specific binding of radiolabeled hLH, hCG and hFSH to 20.5, 11.2 and 12.9 mg equivalents of intact testicular tissue respectively (100-300 µg protein). The differences between ^{125}I-hLH, ^{125}I-hCG and ^{125}I-hFSH binding patterns after 8 h were reproducible.

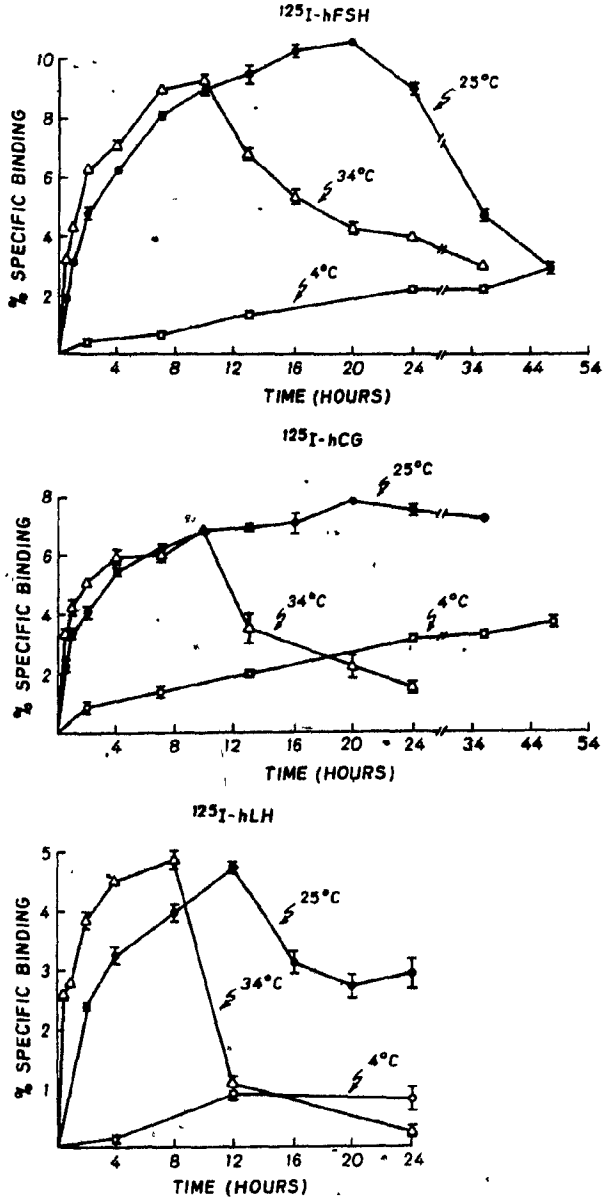


FIG. 34

Temperature dependence of the association of ^{125}I-hFSH (A: top panel), ^{125}I-hCG (B: middle panel) and ^{125}I-hLH (C: bottom panel) to 100-300 µg protein of testicular fraction P1 obtained from a 46 yr, 50 yr and 20 yr old man respectively. Specific binding was determined at the indicated time intervals.

FIG. 35

Effect of the ionic environment on the interaction of ^{125}I-hFSH with 150 µg of testicular fraction P1 obtained from a 73 yr old man. All incubations were carried out at 34C for 8 h in the regular assay buffer at pH 7.2 but with varying concentrations of the salts as indicated in the figure. The maximum specific binding (8% specific binding) obtained in the presence of 10 mM $MgCl_2$ was set as 100% for comparison.

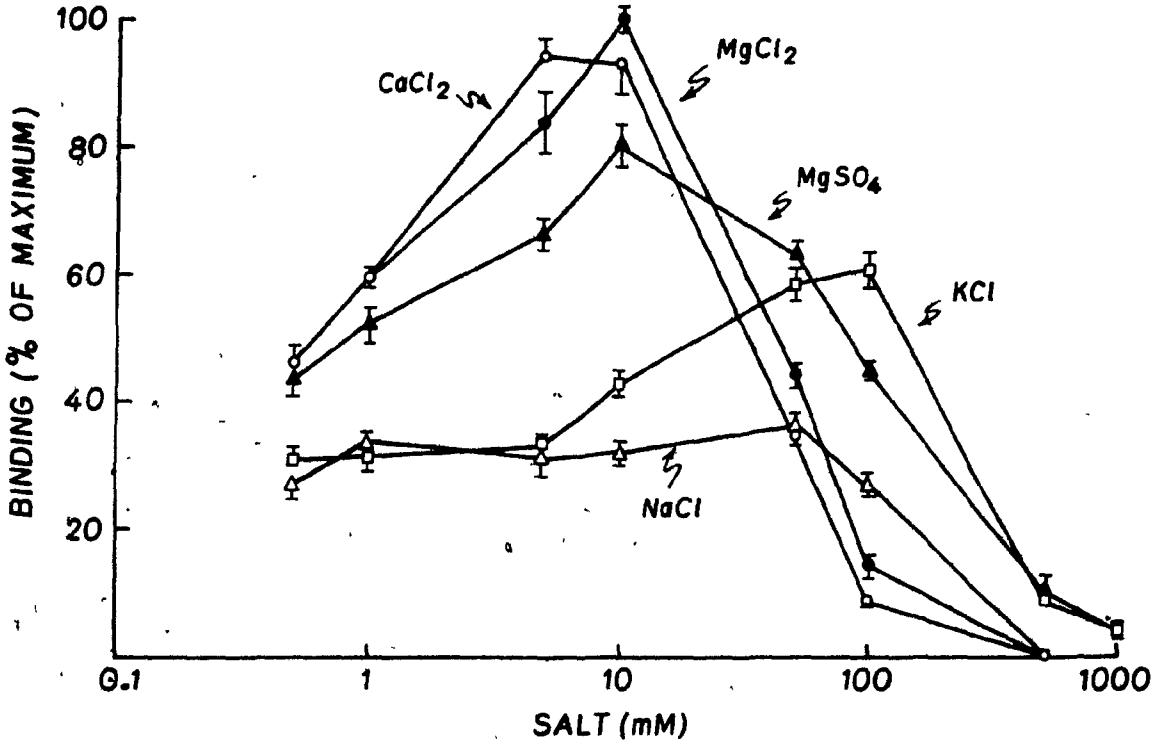

100
80
60
40
20
BINDING (% OF MAXIMUM)
CaCl2
MgCl2
MgSO4
KCl
NaCl
0.1
1
10
100
1000
SALT (mM)

FIG. 36

Effect of pH of the incubation medium on the interaction of ^{125}I-hFSH with 50-100 µg testicular fraction P1. Each set of incubations were carried out at 34C for 8 h in the respective buffers indicated, in the presence of 10 mM $MgCl_2$ and 0.1% BSA. The maximum specific binding (8% specific binding) obtained with 25 mM Tris-HCl at pH 7.0 was set as 100% for comparison. Data represents the mean ± SEM of triplicate determinations of two separate experiments on testicular fraction P1 from a 46 and 62 yr old individual.

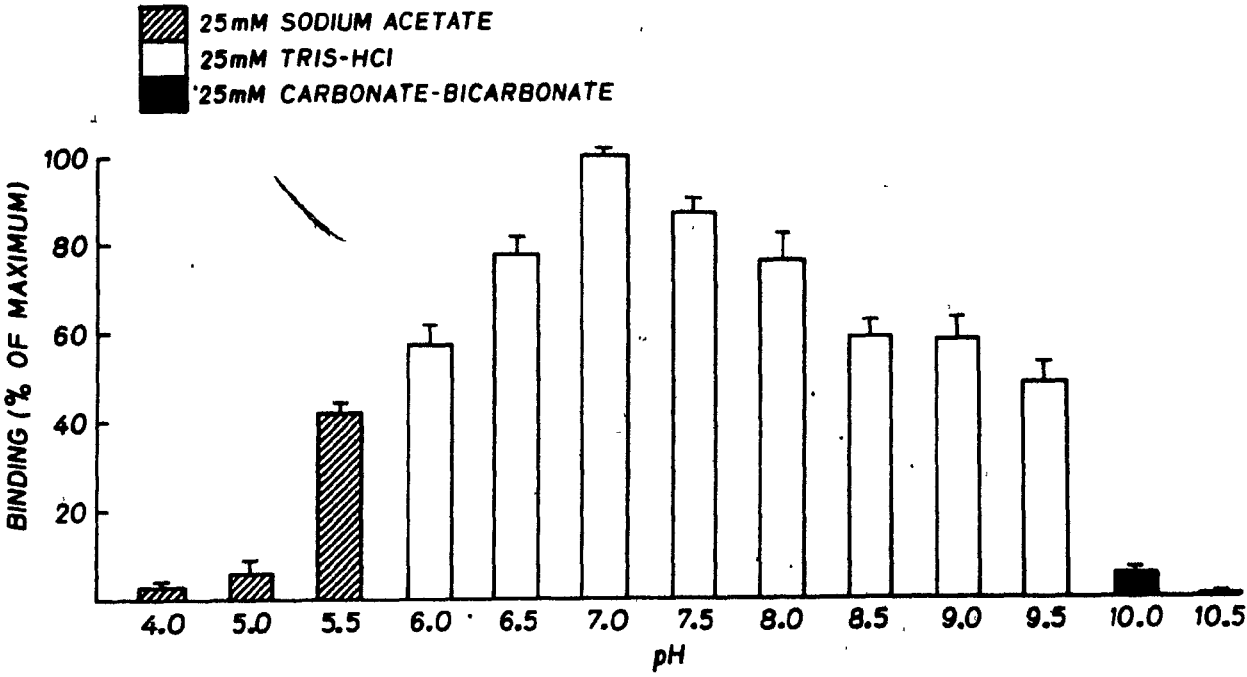
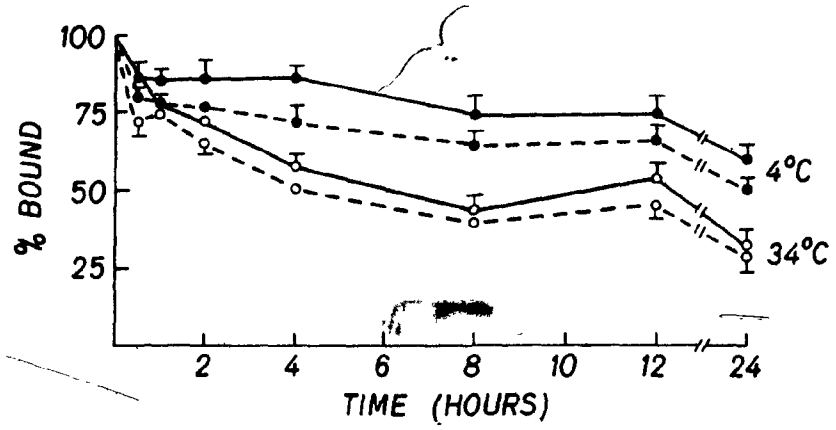

25mM SODIUM ACETATE
25mM TRIS-HCl
25mM CARBONATE-BICARBONATE
BINDING (% OF MAXIMUM)
100
80
60
40
20
4.0
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
pH

FIG. 37

Effect of temperature and unlabeled hormone on the dissociation of ^{125}I-hCG (A: top panel) and ^{125}I-hFSH (B: bottom panel) from 45 and 85 µg respectively of testicular fraction P1 obtained from two adult men (50 and 46 yr respectively). The preformed ^{125}I-hFSH-receptor complex formed by incubation for 15 h at 25C was re-incubated at different temperatures for various times in the presence (broken lines) or absence (solid lines) of a 500-fold excess of unlabeled hormone. The binding obtained at the beginning of the second incubation, 4% and 6% specific binding for ^{125}I-hCG and ^{125}I-hFSH respectively, was set as 100%. The nonspecific binding of ^{125}I-hCG and ^{125}I-hFSH amounted to 60% and 23% respectively of the total counts bound to the gonadotropin receptor. Data represents the mean ± SEM of triplicate determinations of one of at least two separate experiments.


100
75
50
25
% BOUND
2
4
6
8
10
12
24
TIME (HOURS)
4°C
34°C

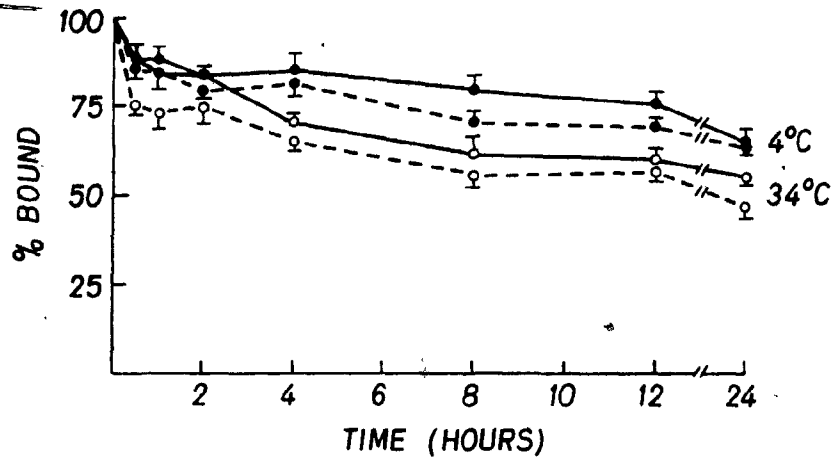
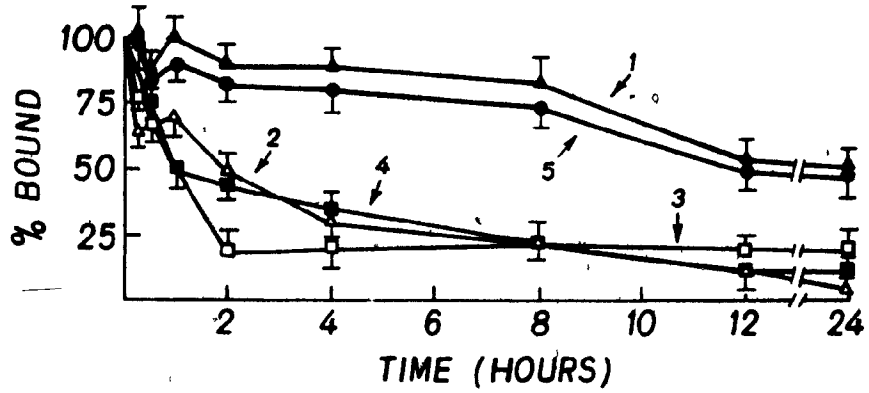
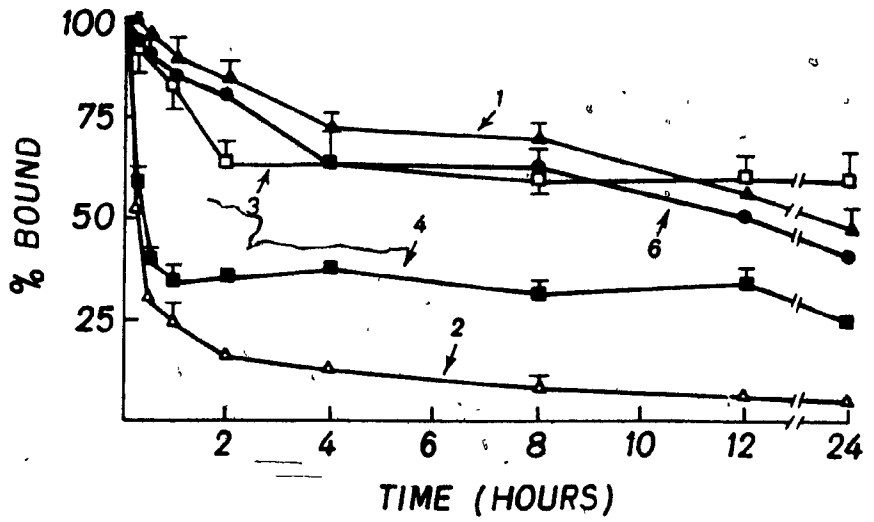

100
75
50
25
% BOUND
2
4
6
8
10
12
24
TIME (HOURS)
4°C
34°C

FIG. 38

Dissociation of the preformed hormone receptor complex as a function of the pH and ionic concentration of the incubation medium. The ^{125}I-hCG (A: top panel) and ^{125}I-hFSH (B: bottom panel) receptor complex was formed with 100-200 µg testicular fraction P1 from an individual aged 52 and 54 yr respectively as indicated in Fig. 37. The binding obtained at the beginning of the second incubation, 4% and 14% specific binding for ^{125}I-hCG and ^{125}I-hFSH respectively, was set as 100%. The non-specific binding of ^{125}I-hCG and ^{125}I-hFSH amounted to 75% and 20% respectively of the total counts bound to the gonadotropin receptor. Data represents the mean ± SEM of triplicate determinations of one of at least two separate experiments. The following buffers (all at 25 mM containing 0.1% BSA and 0.6 mM bacitracin) were used: (1) Tris-HCl buffer, pH 7.2 + 10 mM $MgCl_2$, (2) Tris-HCl buffer, pH 7.2 + 400 mM $MgCl_2$, (3) Acetate buffer, pH 5.0 + 10 mM $MgCl_2$, (4) Carbonate-bicarbonate buffer, pH 9.5 + 10 mM $MgCl_2$, (5) Tris-HCl buffer, pH 7.2 + 10 mM $MgCl_2$ + 500 ng unlabeled hCG, (6) Tris-HCl buffer, pH 7.2 + 10 mM $MgCl_2$ + 500 ng unlabeled hFSH.


100
75
50
25
% BOUND
1
2
3
4
5
2
4
6
8
10
12
24
TIME (HOURS)


100
75
50
25
% BOUND
1
2
3
4
6
2
4
6
8
10
12
24
TIME (HOURS)

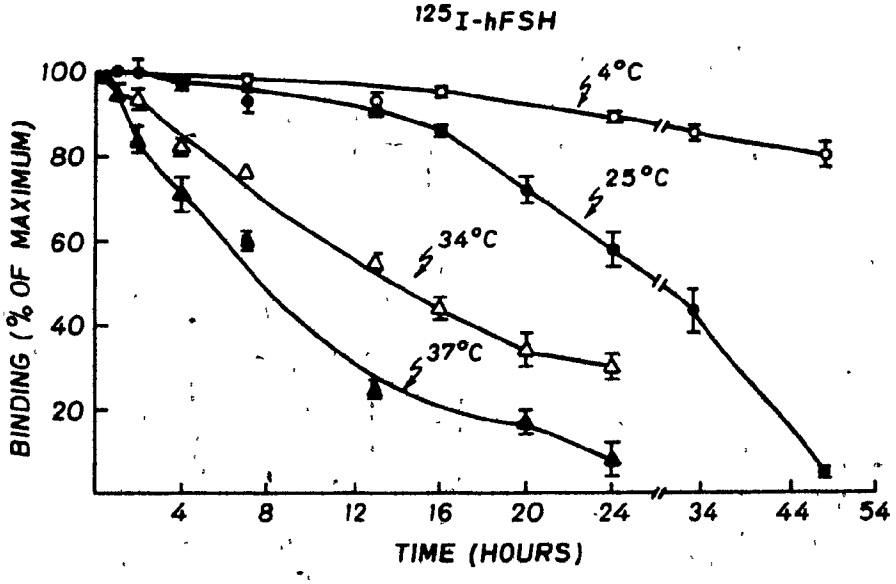
3.3.4 Temperature lability of the gonadotropin receptors

The lability of the gonadotropin receptors were first tested by incubating the P1 fraction at different temperatures. At various time intervals (fig. 39A and 39B), hormone binding was evaluated by a second incubation of 4 h at 34C with fresh labeled hormone. Both FSH and LH receptors were stable at 4C for about 1-2 days. At other temperatures there was a marked difference in stability of the FSH and LH receptors. The $t_{\frac{1}{2}}$ of the FSH and LH receptors at 25C, 34C and 37C were 30 h, 14 h, 9 h and 11 h, 4 h, 2 h respectively.

If the decreased binding of the ^{125}I-labeled gonadotropins is due to the release of degradative enzymes into the incubation medium, then removal or neutralization of these enzymes should restore hormone binding. Inclusion of bacitracin (MW 1411), an antibiotic polypeptide produced by Bacillus subtilis, or TLCK (MW 369), an inhibitor of trypsin activity, at a dose (1 mM) which does not interfere with gonadotropin binding (fig. 40) did not prevent the loss of binding at 34C of ^{125}I-hCG or ^{125}I-hFSH by the subcellular fraction P1. Precipitation with polyethylene glycol did not enhance gonadotropin binding indicating that the decreased binding was not due to temperature dependent solubilization of the receptors. Removal and replacement of the incubation medium after preincubation of the subcellular fraction P1 (first incubation) also did not restore gonadotropin binding. If the supernatant fraction from this first incubation was added to a fresh human testicular FSH receptor preparation, the supernatant fraction of the 34C, but not the 4C incubation, possessed some FSH binding inhibitory activity. This inhibitory activity was low and variable (5-20% inhibition). Though this inhibitory factor might play some role in the decreased binding of the gonadotropins to the gonadotropin receptor, it cannot completely account for the observed 60-80% loss of gonadotropin binding. Possibly the best evidence for a specific inactivation of the gonadotropin receptor is that the same radiolabeled hormone (^{125}I-hCG or ^{125}I-hFSH) which failed to bind to the pretreated receptor fraction of the human, rhesus monkey or yellow baboon testes was capable of interacting with a fresh receptor preparation (90-100% original activity).

FIG. 39

Effect of preincubation on the integrity of the human gonadotropin receptor. ^{125}I-hFSH (A: top panel) binding to 200 µg protein of testicular fraction P1 (equivalent to 13 mg wet weight of testis) from a 46 yr old individual. ^{125}I-hCG (B: bottom panel) binding to 100 µg protein of testicular fraction P1 (equivalent to 8 mg wet weight of testis) from a 50 yr old individual. The P1 fraction of the testis was incubated in the assay buffer at different temperatures and at various intervals the ability of the P1 fraction to specifically bind the radiolabeled gonadotropins was assessed in a second incubation carried out at 34C for 4 h. The maximum specific binding of 8% in A (top panel) and 5% in B (bottom panel) was considered as 100% for all the calculations. The ^{125}I-labeled hormones do not suffer any inactivation at 37C for 24-48 h (not shown).


^{125}I-hFSH
BINDING (% OF MAXIMUM)
100
80
60
40
20
4°C
25°C
34°C
37°C
4
8
12
16
20
24
34
44
54
TIME (HOURS)

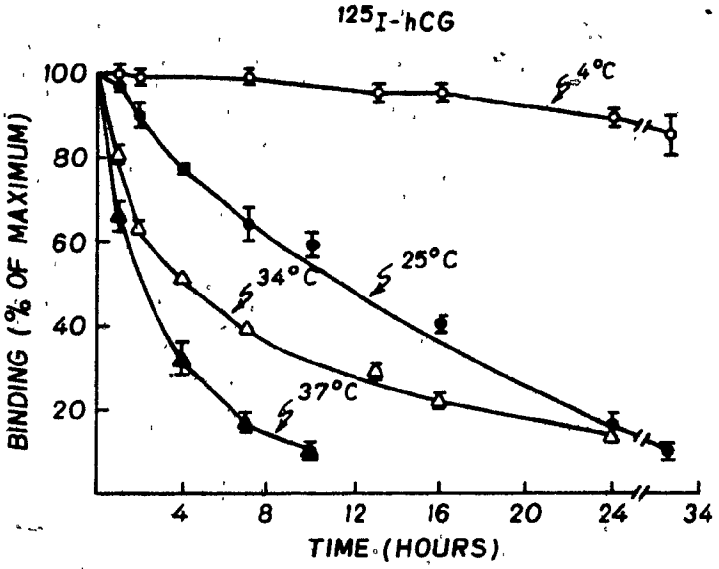
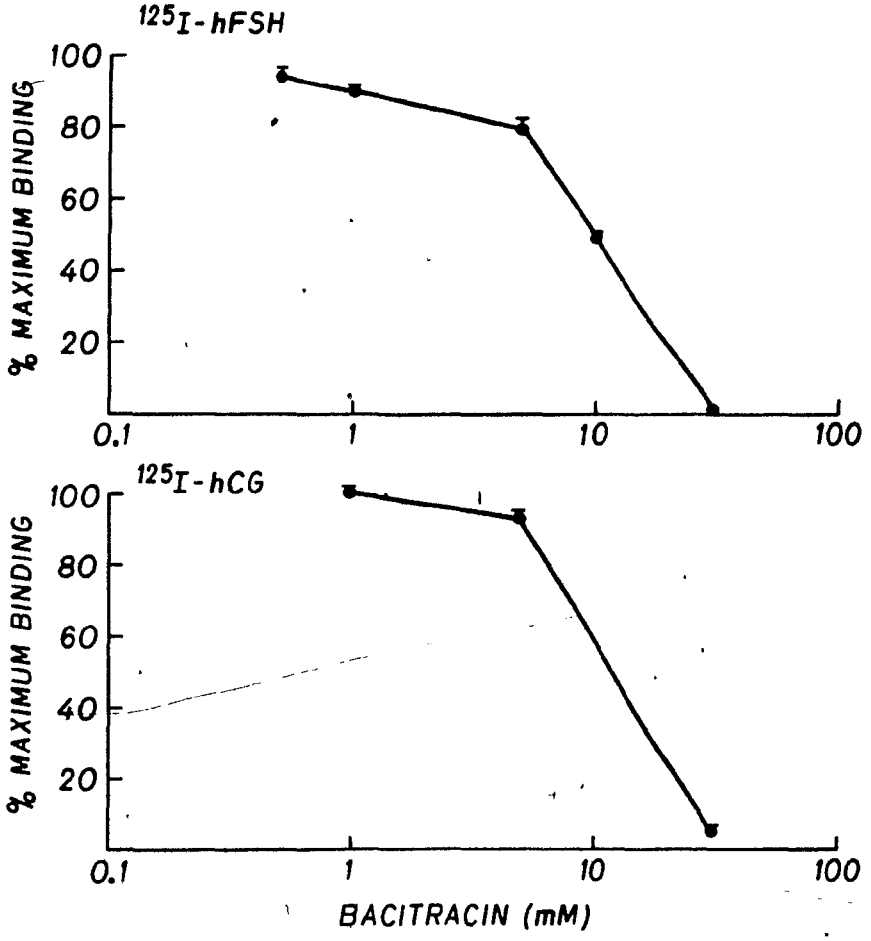

^{125}I-hCG
BINDING (% OF MAXIMUM)
100
80
60
40
20
4°C
25°C
34°C
37°C
4
8
12
16
20
24
34
TIME (HOURS)

FIG. 40

Displacement of ^{125}I-hFSH (A: top panel) and ^{125}I-hCG (B: bottom panel) binding to 100 µg and 200 µg respectively of human testicular fraction P1 by increasing concentrations of bacitracin. The maximum binding (11% and 10% specific binding for ^{125}I-hFSH and ^{125}I-hCG respectively) was set as 100%. Testicular tissues were derived for ^{125}I-hFSH and ^{125}I-hCG binding from two individuals aged 51 yr and 76 yr respectively. Similarly, TLCK at a concentration of up to 1 mM did not interfere with ^{125}I-labeled gonadotropin binding.


^{125}I-hFSH
% MAXIMUM BINDING
100
80
60
40
20
0.1
1
10
100
^{125}I-hCG
% MAXIMUM BINDING
100
80
60
40
20
0.1
1
10
100
BACITRACIN (mM)

3.3.5 Gonadotropin receptor binding inhibitors

The ability of the human testicular subcellular fraction (P1) upon incubation to release an FSH binding inhibitory factor into the incubation medium (section 3.3.4) prompted an investigation of the supernatant fraction (140,000 x g) of testicular extracts to determine if they possessed a similar factor. Aliquots of the testicular supernatant fraction from several adult species (rhesus monkey, human, rat, bull) inhibited the binding of ^{125}I-hCG and ^{125}I-hFSH to their respective receptors (fig. 41) in a dose dependent manner. Though it would have been preferable to assess the inhibitory activity of the supernatant fractions of the primate testes with a primate receptor this was not possible due to the limited quantity of primate gonadotropin receptor material available. At equivalent concentrations of the rhesus monkey or human testicular supernatant fractions comparable inhibition of ^{125}I-hCG and ^{125}I-hFSH binding was observed. At the highest concentration tried, a 40% inhibition of gonadotropin binding was obtained (fig. 41A and 41B). With the rat and to some extent the bull, there appeared to be a disparity between the extent of inhibition of ^{125}I-hCG and ^{125}I-hFSH binding (fig. 41C and 41D). Preincubation of the two testicular receptor preparations with the supernatant fractions of the various species, followed by their removal upon washing the testicular membranes, abolished the inhibitory effect of the supernatants. Thus the inhibition of gonadotropin binding by the supernatant fractions appears to be reversible, occurs without damaging the receptor, and is dependent on the continued presence of this unknown inhibitory factor.

3.3.6 Assessment of the number of gonadotropin receptors

Specific binding of ^{125}I-labeled gonadotropins to fraction P1 from a 50-yr old man was proportional to the amount of labeled hormone added to the incubation medium (fig. 42). The similarity in the pattern of binding for ^{125}I-hLH and ^{125}I-hCG as well as their displacement by either hormone suggests that they share the same receptor(s). Saturation of binding sites occurred at 1.0-2.0 ng of hormone added. Analysis of data

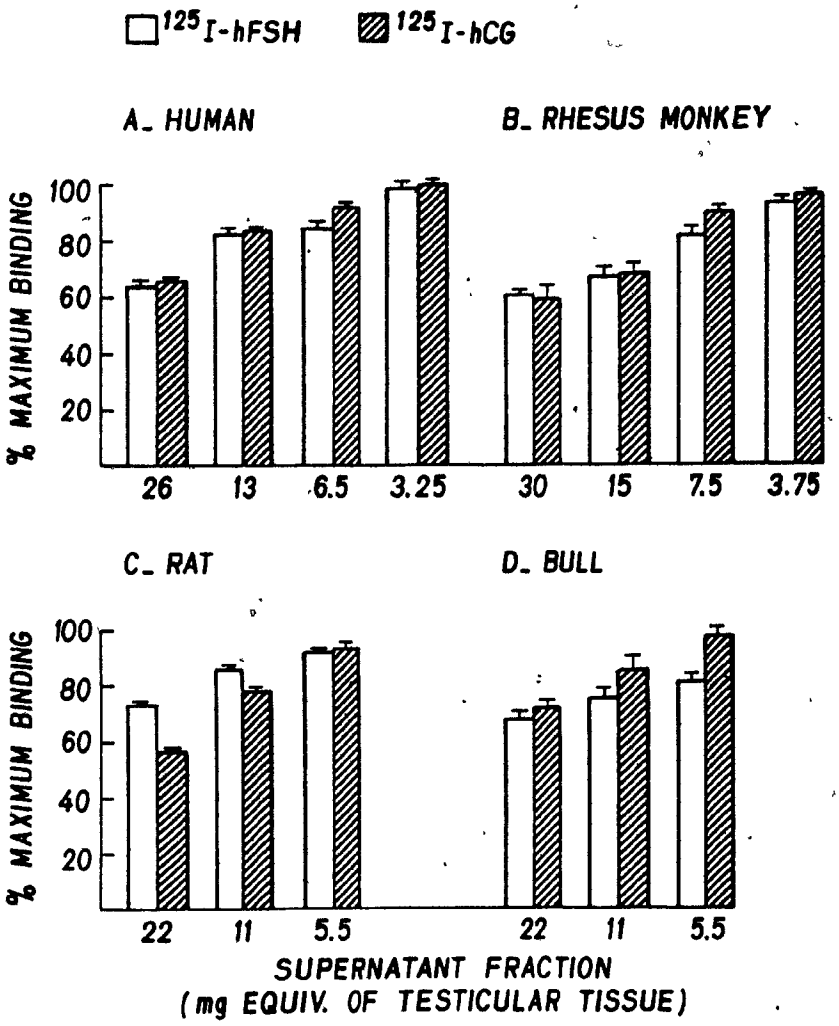
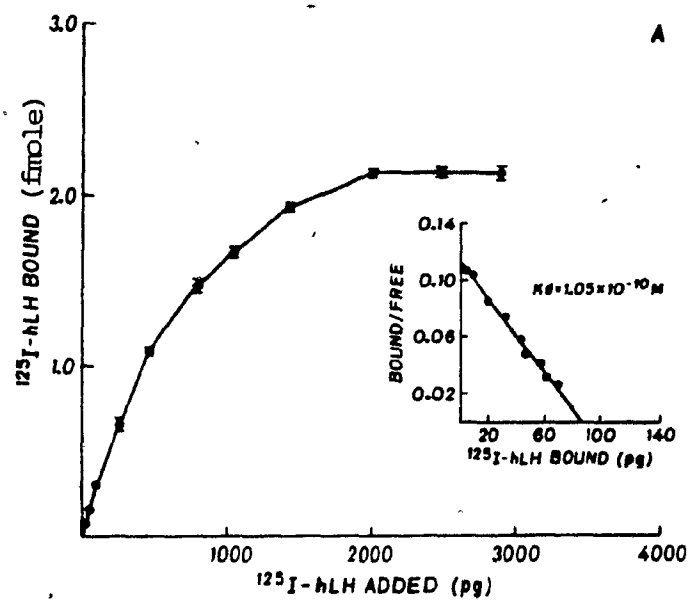


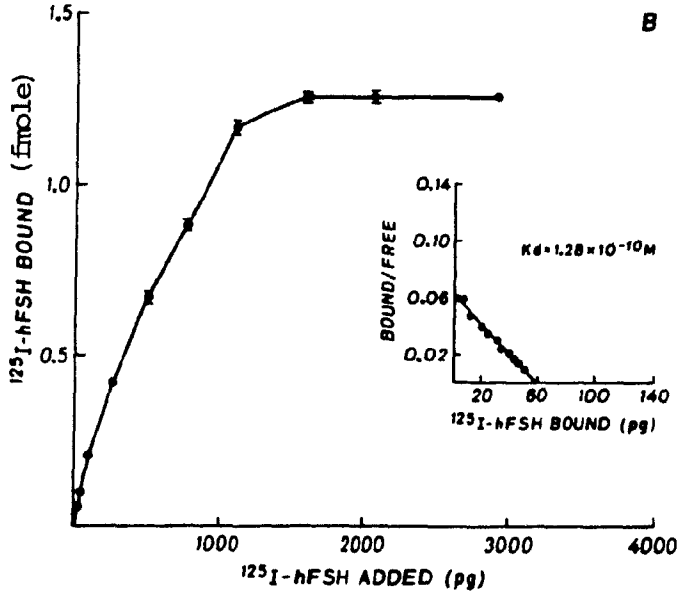
FIG. 41

Effect of varying amounts of the 140,000 x g testicular supernatant from the human, rhesus monkey, rat and bull on the binding of ^{125}I-hCG and ^{125}I-hFSH to the rat and bull testicular receptor respectively. The maximum binding (25% specific binding for ^{125}I-hCG and ^{125}I-hFSH to 150 and 75 µg protein respectively) was set as 100%.

FIG. 42

Specific binding of increasing concentrations of radio-labeled gonadotropins to a testicular fraction (P1) prepared from a 50 yr old man. ^{125}I-hLH (A: top panel) with 110 µg protein equivalent of receptor (P1). ^{125}I-hFSH (B: bottom panel) with 55 µg protein equivalent of receptor (P1). Nonspecific binding for each point was determined in the presence of a 500 fold molar excess of the unlabeled hormone. Inset in each panel shows the Scatchard plot of the binding data. The reciprocal of the negative slope of the line yields the apparent dissociation constant (Kd) and the intercept on the abscissa yields the total amount of labeled hormone bound in picograms (pg), which is used to express the number of receptors. The number of binding sites for LH and hFSH were 2.7 and 3.6 fmol/100 µg protein respectively, which is equivalent to 210 and 283 pmol/g of testes.


A
3.0
2.0
1.0
^{125}I-hLH BOUND (fmole)
1000
2000
3000
4000
^{125}I-hLH ADDED (pg)
0.14
0.10
0.06
0.02
BOUND/FREE
Kd=1.05×10^{-10}M
20
60
100
140
^{125}I-hLH BOUND (pg)


B
1.5
1.0
0.5
^{125}I-hFSH BOUND (fmole)
1000
2000
3000
4000
^{125}I-hFSH ADDED (pg)
0.14
0.10
0.06
0.02
BOUND/FREE
Kd=1.28×10^{-10}M
20
60
100
140
^{125}I-hFSH BOUND (pg)

by Scatchard plots provided the number of receptors (n) and the apparent dissociation constant (Kd) (see insert fig. 42). For these calculations, the molecular weight of hFSH and hLH was considered to be 30,000 and that of hCG was 36,700. The Scatchard plots for hLH and hCG in this individual were nearly identical, with the number of receptors being 2.7 and 2.9 fmol per 100 µg protein, respectively (equivalent to 210 and 231 fmol per gram of testes). The number of FSH receptors was 3.6 fmol per 100 µg protein or 283 fmol per gram of testis. The dissociation constants calculated for all three gonadotropins were similar (hCG: Kd = 1.02×10^{-10}M, hLH: Kd = 1.05×10^{-10}M, hFSH: Kd = 1.28×10^{-10}M).

Comparison of the number of testicular gonadotropin receptors in a larger sample population (17 tissues) yielded no significant difference in the number of LH receptors using either ^{125}I-hCG (115.41±15.74 fmol per gram of testes) or ^{125}I-hLH (122.29±13.09 fmol per gram of testes). As before there was a significantly greater number of FSH receptors (349.94±32.15 fmol per gram of testes). The dissociation constants (Kd) calculated for all three gonadotropins were again similar (hCG: Kd = $1.21 \pm 0.12 \times 10^{-10}$M, hLH: Kd = $1.34 \pm 0.07 \times 10^{-10}$M, hFSH: Kd = $1.37 \pm 0.08 \times 10^{-10}$M).

In all, the FSH and LH binding capacity was determined in 71 and 60 testicular tissues respectively utilizing ^{125}I-hFSH and ^{125}I-hLH. All tissues were from individuals greater than 16 yr of age (postpubertal). From an analysis of the binding data, the tissues were divided into eight major age groups. With the exception of group one, groups were of 10 yr duration (fig. 43 and Table 10). The number of FSH receptors was highest in the first age group studied (16-20 yr). Although there was a tendency for the number of FSH receptors to decrease with advancing age, no significant difference was observed between groups 2-7. The sharp decline in FSH receptors observed in group 2 (21-30 yr) and group 8 (81-90 yr) could be a reflection of the small sample size (less than 4 tissues). Similar to the FSH receptor, the number of LH receptors remained constant up to 60 yr if the drop in LH receptor levels in group 2 is again assumed to reflect the small sample size. Following

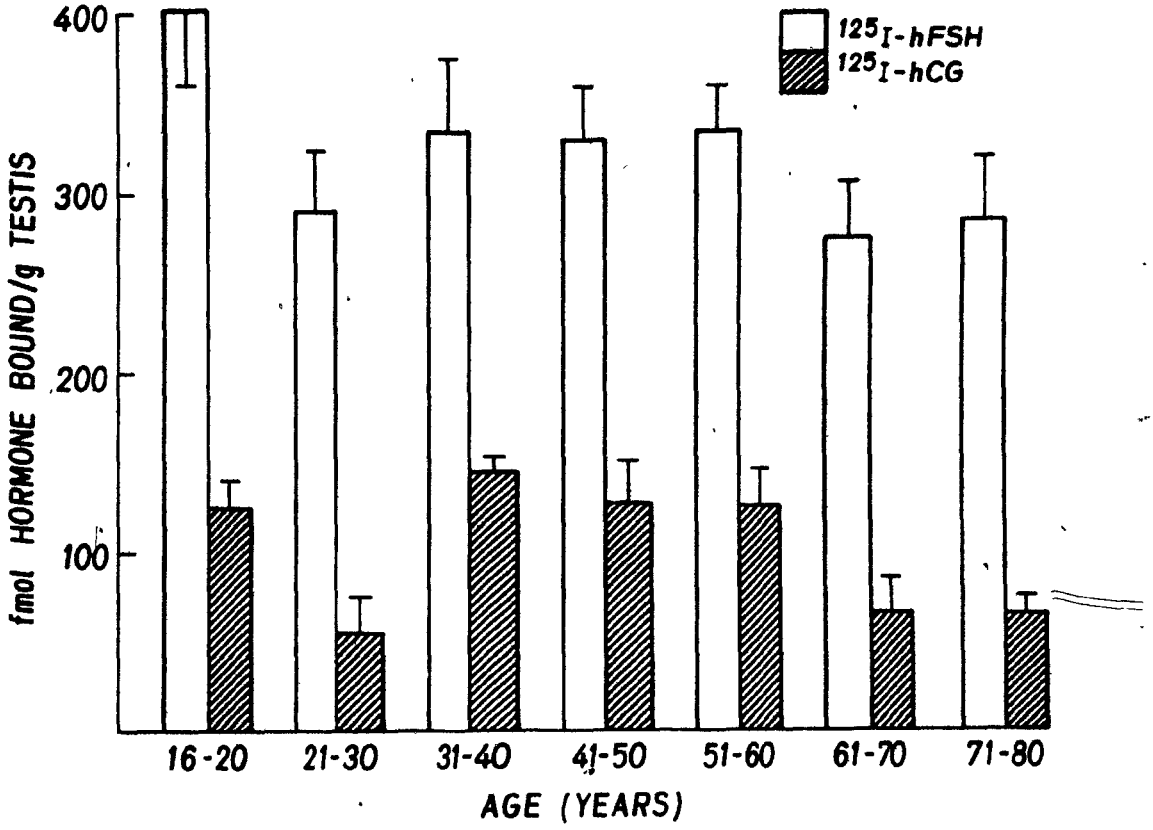


FIG. 43

Influence of age of testicular tissue on the binding of ^{125}I-hCG and ^{125}I-hFSH to the human gonadotropin receptor. Similar values for the number of LH receptors were obtained using either ^{125}I-hCG or ^{125}I-hLH. The number of gonadotropin receptors were determined by Scatchard and saturation analysis of the binding data for each tissue. Each bar represents the mean ± SEM.

TABLE 10

ESTIMATION OF THE BINDING CAPACITY AND APPARENT DISSOCIATION CONSTANT (Kd) OF THE FSH AND LH RECEPTORS OF THE HUMAN TESTES

GROUP	AGE (yr)	FSH receptor (fmol/g testis)	LH receptor (fmol/g testis)	FSH receptor Kd (10^{-10} M)	LH receptor Kd (10^{-10} M)	Ratio of FSH/LH receptors
1	16-20	402.25±41.49 (8)	123.75±10.55 (8)	1.47±0.13	1.20±0.07	3.25
2	21-30	290.00±34.65 (3)	56.00±18.04 (3)	1.25±0.17	1.35±0.28	5.18
3	31-40	336.00±40.20 (3)	143.50± 6.50 (3)	1.45±0.10	1.60±0.15	2.34
4	41-50	329.00±29.99 (11)	126.78±22.82 (9)	1.47±0.09	1.26±0.16	2.60
5	51-60	335.84±23.73 (19)	125.43±19.83 (14)	1.47±0.08	1.31±0.14	2.68
6	61-70	274.87±30.91 (15)	65.58±15.40 (12)	1.39±0.10	1.61±0.25	4.19
7	71-80	284.36±36.08 (11)	65.73±11.05 (11)	1.60±0.20	1.16±0.35	4.33
8	81-90	167.00 (1)	32.00 (1)	1.74	1.81	5.22

FSH receptor - in all groupings, except 1 vs. 2, 1 vs. 6, and 1 vs. 7, the differences were not statistically significant. Note the small size of groups 2 and 3 could be responsible for the observed differences.

LH receptor - in all groupings except 1 vs. 2, 6 or 7, 3 vs. 2, 6, or 7, 4 vs. 2, 6 or 7 and 5 vs. 2, 6 or 7; the differences were not statistically significant. Note the small sample size of groups 2 and 3 could be responsible for the observed differences.

FSH Kd and LH Kd - the differences among all groupings were not statistically significant.

Data were derived from Scatchard and saturation analysis of tissues using ^{125}I-hFSH and ^{125}I-hLH. Similar results were obtained using either ^{125}I-hLH or ^{125}I-hCG. The numbers in parentheses represent the number of tissues analyzed. Values represent the mean ± SEM. The differences between the LH and FSH receptor groups were statistically significant ($p < 0.05$). The differences between the apparent dissociation constants of the LH and FSH receptors were not statistically significant.

this, the LH receptors significantly decreased to levels below the 16-60 yr age group. The number of FSH receptors was greater than the number of LH receptors at all ages. However, the ratio of FSH/LH receptors was considerably reduced in the 31-60 yr age group. The binding affinities for both gonadotropins were similar (Table 10) and did not differ significantly at different ages.

3.3.7 Hormonal specificity of the human testis

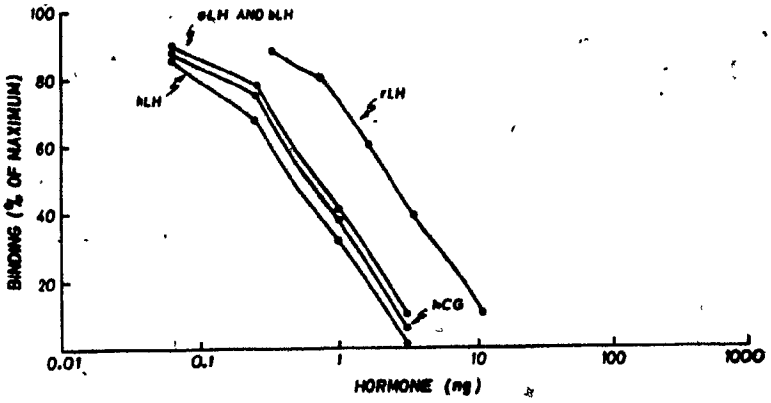
The hormonal specificity of the human testicular subcellular fraction P1 as assessed by its interaction with various radiolabeled hormones (human and nonhuman) is similar to that of the young adult rhesus monkey. Radiolabeled human and ovine FSH were both bound well by the human testicular tissue. In contrast to the FSH receptor, the human LH receptor interacted better with the radiolabeled primate hormones, ^{125}I-hCG or ^{125}I-hLH (8% specific binding) than with the radiolabeled nonprimate hormone ^{125}I-oLH (1-2% specific binding). Subcellular fraction P1 of the adult rhesus monkey and human testes showed very poor binding of ^{125}I-hPRL (1-2% specific binding). The integrity of the hormone (^{125}I-hPRL) was verified by its ability to bind to a rabbit liver (10% specific binding) and porcine ovarian (4% specific binding) prolactin receptor.

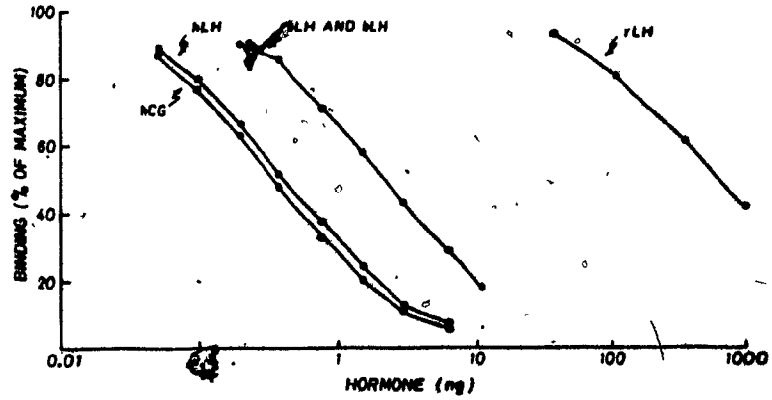
3.3.8 Specificity of hormone binding in the primate vs the nonprimate testis

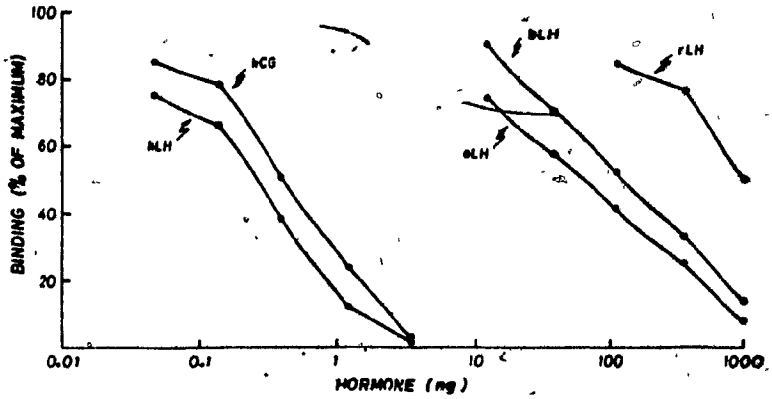
The competition between ^{125}I-hCG and various concentrations of unlabeled LH from different species for specific binding to the primate and nonprimate LH receptor is illustrated in Figure 44. The human LH receptor (fig. 44C) discriminated between human and nonhuman hormones, showing a definite preference for LH of human origin. Alteration of the radioligand, i.e. substitution of ^{125}I-hLH for ^{125}I-hCG, did not change the marked species selectivity of the human LH receptor. Further, ^{125}I-oLH was a poorer ligand when tested with the human (1-3% specific binding) than with the rat testis (20% specific binding). The rhesus monkey and rat testicular LH receptor(s) recognized the heterologous

FIG. 44

Comparison of the displacement pattern of the binding of ^{125}I-hCG by unlabeled LH of different species to the particulate fraction P1 (150 µg protein) prepared from the adult rat (A: top panel), young rhesus monkey, (B: middle panel), and human (C: bottom panel) testes. Similar results were observed in the human testes with ^{125}I-hLH as the ligand. The amount of hormone specifically bound (26%, 5% and 9% specific binding to the rat (34 days), rhesus monkey (4 yr) and human (50 yr) testes respectively) was set as 100% for all calculations. In the experiment shown for the monkey LH receptor, the relative potency of the oLH and bLH was about 20% of hLH, whereas in another (not shown) the two nonprimate hormones were as potent as hLH. With the LH receptor from all the three species, the displacement pattern was not altered after freezing and thawing.


oLH AND bLH
bLH
rLH
hCG
BINDING (% OF MAXIMUM)
HORMONE (ng)
0.01
0.1
1
10
100
1000
20
40
60
80
100


bLH
bLH AND bLH
hCG
rLH
BINDING (% OF MAXIMUM)
HORMONE (ng)
0.01
1
10
100
1000
20
40
60
80
100


hCG
bLH
oLH
rLH
BINDING (% OF MAXIMUM)
HORMONE (ng)
0.01
0.1
1
10
100
1000
20
40
60
80
100

hormones (Fig. 44A and B). Some differences were apparent between monkey and human LH receptors in their ability to interact with oLH and bLH. The estimated potencies of oLH and bLH in displacement assays with the human and rhesus monkey receptors were 0.0042 x hCG (0.0037 - 0.0061, 95% CL) and 0.242 x hCG (0.20 - 0.39, 95% CL) respectively. The monkey LH receptor was similar to the human with respect to its poor ability to bind rat LH (potency 0.00014 - 0.00077 x hCG). The species specificity of the human testicular LH receptor was not lost by freezing, i.e. heterologous hormones were unable to displace ^{125}I-hLH/^{125}I-hCG when frozen and thawed human receptor was used. Due to the small amount of tissues available, the species specificity of the yellow baboon LH(hCG) receptor could only be evaluated once in a single tissue. The estimated potencies of bLH 0.045 x hCG (0.041 - 0.049, 95% CL) and oLH 0.068 x hCG (0.061 - 0.076, 95% CL) were similar and greater than that of rat LH 0.00016 x hCG (0.00014 - 0.00020, 95% CL) (fig. 45). While the LH (hCG) receptors of this baboon (10 yr) appear to resemble those of the adult human (fig. 44C) in their ability to recognize nonprimate LH preparations, this data needs to be confirmed before proposing that the yellow baboon might be a better model for studies on the human than the rhesus monkey.

The binding of ^{125}I-hFSH to the human testicular receptor is competitively displaced by intact FSH preparations from human as well as nonprimate pituitaries (fig. 46). Bovine and several nonhuman primate receptors show similar behavior (Table 11). The displacement curves for all the five species of FSH preparations investigated were not significantly nonparallel. Equine FSH was the most potent in the human competition assay, showing about ten times the activity of hFSH. Rat FSH was the least active species of FSH.

While the human testicular LH receptor differed from the FSH receptor in being species specific with respect to the ligand, both gonadotropin receptors were hormone specific. Hormone subunits and most nongonadotropic hormones (ex. hPRL, hGH, LHRH) had very low activity in the human and nonhuman primate gonadotropin receptor assays, ie. less than 0.5%

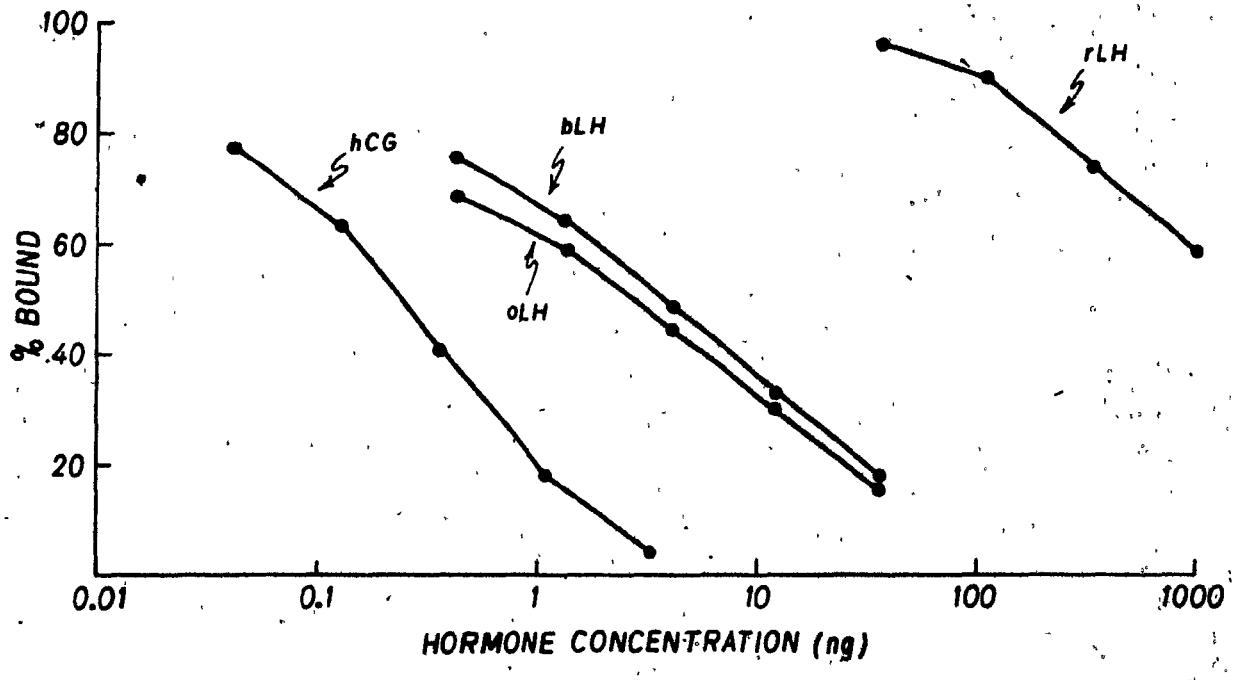


FIG. 45

Displacement of ^{125}I-hCG binding to 700 µg of testicular fraction P1 of the yellow baboon (10 yr) by unlabeled LH of various species. Due to the limited amount of tissue this experiment was only performed once. The maximum binding (7% specific binding) was set as 100%.

FIG. 46

Interaction of the human testicular FSH receptor with highly purified heterologous FSH preparations. The amount of hormone specifically bound (11% specific binding) to 150 µg fraction P1 was set as 100%. Note that the human FSH receptor from the same tissue (50 yr) as the human LH receptor (see Fig. 44C) is unlike the LH receptor which was more species selective. Identical results were obtained with fresh or frozen and thawed receptor. The average potency was calculated from the amount of hormone (ng) required to displace 50% of the radioactivity from receptor. Setting hFSH as 1, the activities of the hterologous FSH were as follows: equine FSH 11 x (9.6 - 12.5, 95% CL), ovine FSH 1.55 x (1.40 - 1.61, 95% CL), porcine FSH 0.57 x (0.48 - 0.65, 95% CL), rat FSH 0.23 x (0.13 - 0.31, 95% CL). Using ^{125}I-oFSH as the ligand, the displacement obtained with the various FSH preparations showed an identical pattern.

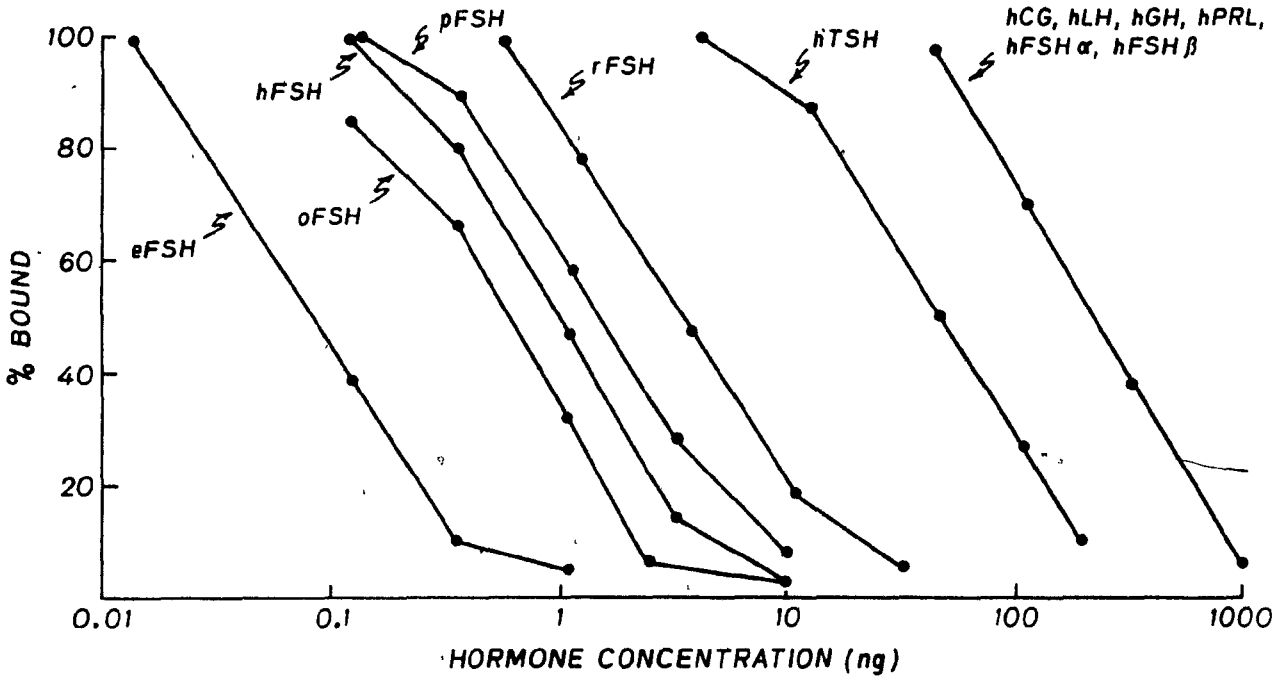

100
80
60
40
20
% BOUND
0.01
0.1
1
10
100
1000
HORMONE CONCENTRATION (ng)
eFSH
oFSH
hFSH
pFSH
rFSH
hTSH
hCG, hLH, hGH, hPRL,
hFSH α, hFSH β

TABLE 11

SPECIFICITY OF ^{125}I-hFSH BINDING TO THE FSH TESTICULAR RECEPTOR OF VARIOUS ADULT SPECIES

Species	hFSH	eFSH	oFSH	pFSH	rFSH	hFSH sub-units, hCG and hGH
Crab eating monkey (Mfl)	1.0	10.25 (9.14-11.49)	1.30 (1.12-1.52)	1.13 (0.98-1.31)	0.42 (0.33-0.53)	<0.005
Pig tailed monkey (Mn)	1.0	12.53 (10.79-14.54)	1.85 (1.55-2.21)	1.75 (1.43-2.14)	0.44 (0.37-0.53)	<0.005
Rhesus monkey (Mm)	1.0	9.71 (8.65-10.88)	1.88 (1.54-2.26)	0.47 (0.40-0.55)	0.24 (0.21-0.27)	<0.005
Yellow baboon (Pc)	1.0	9.12 (7.53-11.03)	1.19 (0.99-1.42)	1.04 (0.89-1.21)	0.40 (0.34-0.47)	<0.005
Human	1.0	11.14 (10.20-12.15)	1.55 (1.49-1.61)	0.57 (0.53-0.62)	0.23 (0.20-0.26)	<0.005
Bull	1.0	7.48 (6.76-8.29)	2.02 (1.84-2.21)	0.85 (0.77-0.94)	0.33 (0.30-0.37)	<0.005

The average potency was calculated from the amount of unlabeled hormone (ng) required to displace 50% of the radioactivity (^{125}I-hFSH) from the receptor. The relative potency of unlabeled hFSH was set as 1. Data represents the mean potency value of at least two separate experiments. Values in parentheses represent the 95% confidence limits. (< = less than).

gonadotropin activity. Human TSH was about 3% as active as the ligand in the human LH, human FSH and nonhuman primate FSH receptor assays. This could be because of some intrinsic activity or trace contamination. With the rhesus monkey P1 fraction, after 7 months of freezing at -70C or lyophilization, an identical pattern of displacement of ^{125}I-hFSH by the different species of FSH preparations was observed.

3.4 Discussion

The present study was aimed at a better understanding of the interaction of human gonadotropins with homologous receptor preparations. By carrying out simultaneous studies on FSH and LH/hCG binding within the same laboratory, we hoped to gather data on the characteristics of the testicular receptors as well as their relative age distribution. Various investigators (174, 186-188, 231) have employed differing experimental conditions resulting in significant differences in the estimates (Table 12) of LH receptors in human testis. Therefore, in order to more reliably compare data on the LH and FSH testicular receptors it seemed important to first establish the optimum conditions for handling of the tissues and hormone binding to the particulate fraction (P1). Although there have been a few reports in the literature on the detection of LH receptors in human testis (174, 186-188, 231, 234) not all the factors mentioned above have been considered in these studies.

All the tissues that we have examined showed good binding of the labeled gonadotropins. The 6-12% total specific binding of ^{125}I-hCG or ^{125}I-hLH to the P1 fraction is much higher than that previously reported (174, 186-188, 231, 234) by other investigators who have used a total homogenate after preliminary filtration. Analysis of the homogenate, the partially purified pellet (P1 fraction) and the 140,000 x g supernatant fraction in our experiments revealed that the low binding is clearly due to some interfering substance(s) present in the 140,000 x g supernatant. Indeed the 140,000 x g supernatant fraction of both human

TABLE 12

COMPARISON OF THE LH RECEPTOR DATA FROM DIFFERENT LABORATORIES

Age (yr)	Type of fraction analyzed	Kd dissociation constant Mean ± SEM (10^{-10}M)	n binding sites fmol/g testis Mean ± SEM	References
17-80	40,000 x g pellet	1.49±0.11 (60)[a]	101.87±7.98 (60)[c]	This study
17-80	40,000 x g pellet	1.42±0.14 (38)[b]	99.19±8.00 (60)[d]	This study
75-90	Low speed pellet	5.0 (N.D.)	62 (N.D.)	186
60-75	20,000 x g pellet	0.3-0.5 (8)	23-146 (8)	185
53-93	Homogenate	1.64±0.21 (21)	716.6±0.16 (21)	231
56-85	Homogenate	0.71±0.16 (5)	570.0±140.0 (13)	232
23-72	Testicular pieces	---	239.8-711.2 (13)	174
Fetal (14-20 wk)	Homogenate	0.935±0.12 (5)	698-1150 (5)	187
Fetal (10-24 wk)	Homogenate	0.4-5.5 (N.D.)	500-6500 (N.D.)	233

ND = value not determined
a = Derived by Scatchard analysis using ^{125}I-hLH
b = Derived by Scatchard analysis using ^{125}I-hCG
c = Derived by Scatchard and saturation analyses using ^{125}I-hLH.
d = Derived by Scatchard and saturation analyses using ^{125}I-hCG.

The value in parentheses in our study refers to the number of observations using hLH or hCG. The value in parentheses in other studies refers to the number of observations using hCG. Data represents the mean ± SEM or the range of values.

and monkey testes inhibits the binding of radiolabeled gonadotropins to their respective receptors of nonprimate testicular tissue.

Similar activity has already been detected in serum (235, 236), seminal plasma (235), follicular fluids (237-239), and aqueous extracts of a variety of tissues such as the testes (218, 236), corpus luteum (240-242), liver (218, 236) and kidney (218, 236). Gonadotropin binding in this study could be restored by removal of the testicular supernatant fractions by washing. The discrepancy with previous reports (218, 236) on the irreversibility of the receptor binding inhibitory activity (RBI) in the rat testes could be due to the use of particulate receptor fractions of different purities. The human testicular supernatant fractions did not contain any significant amount of soluble receptor-like binding components (see Chapter 2, section 2.3.14), as do the bull (96), rat (96, 127) and nonhuman primate (see Chapter 2, section 2.3.14) testes. Thus at least for the human, the presence of RBI activity and the absence of a soluble-like binding activity in the supernatant fraction would suggest that these two activities are due to at least two separate and distinct molecules. Very little is known or understood about the nature of the gonadotropin RBI's. It is possible that the inhibitory activity is due to the presence of proteolytic enzymes or endogenous gonadotropins. The gonadotropin RBI's might play a role in the age dependent loss of gonadotropin activity possibly by impairing the coupling of the gonadotropin-receptor-adenylate cyclase system (237, 239, 242).

This study is the first report on the description of FSH receptors in human testis. The interaction of radiolabeled gonadotropins with their respective human receptors was not only specific but also time, temperature, pH and salt dependent in a manner similar to that of the nonhuman primates (see results, Chapter 2) and laboratory animals (52, 72, 79, 243). Optimal binding to the human and nonhuman primate FSH receptors occurred in the presence of 5-10 mM $MgCl_2$ or $CaCl_2$ (Table 13) at pH 6.5-7.5 (Table 14). The binding characteristics of radiolabeled hCG and hFSH with the testicular P1 fraction also resembles ^{125}I-hCG binding to homogenates of the human testes (186, 231, 244). However,

TABLE 13

EFFECT OF VARIOUS SALTS ON THE SPECIFIC BINDING OF ^{125}I-hFSH TO THE PRIMATE FSH TESTICULAR RECEPTOR

Species	Ionic Concentration (mM)							
	1000	500	100	50	10	5	1	0.5
$MgCl_2$ (mM)								
rhesus monkey (Mm)	1.60±0.98	1.68±0.71	14.68±1.40	53.09±2.64	100.00±1.34	88.37±2.33	38.10±3.69	27.83±1.43
yellow baboon (Pc)	0	1.54±0.74	20.35±1.21	62.47±0.64	100.00±1.26	89.93±2.20	53.49±1.64	N.D.
pig tailed monkey (Mn)	N.D.	0	12.66±0.39	47.35±3.26	100.00±3.18	86.53±3.07	54.58±2.07	N.D.
human	0	0.56±0.45	14.63±0.64	44.38±1.74	100.00±2.05	83.45±4.77	59.26±0.96	N.D.
$CaCl_2$ (mM)								
rhesus monkey (Mm)	1.24±0.59	2.58±1.25	14.03±0.83	41.32±1.35	102.13±2.63	102.20±2.76	45.08±1.83	30.97±1.16
yellow baboon (Pc)	0	1.74±0.82	17.03±1.01	55.08±1.02	109.89±3.52	98.48±3.65	63.71±3.60	51.76±3.72
pig tailed monkey (Mn)	N.D.	0	11.12±0.67	44.82±0.46	113.71±1.67	102.63±1.79	70.89±2.47	50.40±2.58
human	0	0	8.78±0.20	35.31±2.21	93.09±5.82	94.07±3.40	58.89±3.82	46.35±3.05
$MgSO_4$ (mM)								
rhesus monkey (Mm)	N.D.	6.76±0.47	25.11±1.95	41.56±1.78	86.84±3.45	61.55±1.86	69.27±2.58	35.00±3.63
yellow baboon (Pc)	0	12.51±1.20	50.69±1.50	72.00±1.12	74.93±2.93	72.73±4.07	45.43±2.34	43.50±2.24
pig tailed monkey (Mn)	N.D.	4.21±0.21	41.32±1.64	66.50±4.02	80.47±1.54	72.36±1.22	43.14±1.81	33.71±1.15
human	5.44±0.50	9.81±3.49	44.79±1.13	63.14±1.98	79.89±3.60	62.57±2.23	52.67±3.12	43.57±1.74

TABLE 13 (cont'd)

NaCl (mM)								
rhesus monkey (Mm)	2.27±0.40	2.72±0.50	11.65±2.09	22.66±2.62	14.01±0.58	13.04±2.01	11.89±1.86	N.D.
yellow baboon (Pc)	0.39±0.24	0.78±0.68	14.65±1.15	22.05±1.13	23.49±0.47	22.01±0.45	22.47±0.84	26.11±1.32
pig tailed monkey (Mn)	N.D.	1.09±0.49	12.52±0.64	18.83±0.82	20.91±1.34	15.75±2.78	20.67±1.91	19.96±0.55
human	0	1.10±0.81	26.69±1.36	36.25±1.38	32.12±2.16	31.06±1.13	34.53±0.71	27.01±2.26
KCl (mM)								
rhesus monkey (Mm)	3.10±1.05	19.67±1.99	45.91±1.28	45.02±3.13	27.55±3.15	17.15±2.91	9.86±1.56	14.55±2.85
yellow baboon (Pc)	1.04±0.60	7.46±3.70	61.96±2.04	63.41±2.98	48.17±3.22	36.33±1.97	30.96±3.23	29.60±1.70
pig tailed monkey (Mn)	N.D.	2.98±1.46	54.58±3.77	54.86±1.06	44.97±2.01	31.75±1.86	20.80±3.08	26.10±4.04
human	4.37±1.13	8.95±0.58	60.73±3.08	58.44±2.65	42.79±2.11	32.86±2.37	27.26±1.38	31.14±2.52

Subcellular fractions were prepared from the rhesus monkey (13 yr), yellow baboon (8 yr), pig tailed monkey (10 yr) and human (73 yr) testes. Aliquots of testicular fraction P1 (100-200 µg protein) were incubated at 34C for 8 h in the regular assay buffer at pH 7.2 but with varying concentrations of the salts. The maximum specific binding (Mm=10% S.B., Pc=12% S.B., Mn=10% S.B., and Man=9% S.B.) obtained in the presence of 10 mM $MgCl_2$ was set as 100% for comparison. In the absence of salt only 20±2% specific binding was observed. Data represents the mean ± SEM of triplicate determinations of at least two separate experiments. ND = not determined.

TABLE 14

INFLUENCE OF INCUBATION MEDIUM pH ON THE SPECIFIC BINDING OF ^{125}I-hFSH TO THE PRIMATE FSH TESTICULAR RECEPTOR

Buffer	pH	Species		
		Rhesus monkey (Mm)	Yellow baboon (Pc)	Human
Acetate	4	4.89±2.09	2.86±1.83	2.78±1.00
	5	3.02±0.78	1.31±0.75	8.31±1.73
	5.5	31.13±2.13	41.09±2.61	42.09±2.58
Tris-HCl	6	78.90±6.36	76.93±3.31	57.55±4.26
	6.5	97.05±4.65	77.96±2.31	77.94±3.44
	7	100.50±2.89	100.00±1.93	100.00±1.49
	7.5	95.96±3.85	88.74±1.13	87.10±2.85
	8	70.05±3.34	60.65±2.14	76.34±6.22
	8.5	46.05±1.52	55.78±2.96	58.92±3.49
	9	33.36±1.95	52.71±1.27	58.85±4.26
	9.5	N.D.	43.74±0.09	48.48±4.20
Sodium phosphate	6	42.41±2.51	39.90±3.37	49.27±5.23
	6.5	40.40±1.26	N.D.	N.D.
	7	30.02±1.58	33.39±2.41	40.57±2.68
	7.5	22.78±9.09	N.D.	N.D.
	8	23.56±2.95	23.41±2.09	29.79±3.26
Carbonate-bicarbonate	9	6.00±1.07	N.D.	N.D.
	9.5	3.02±1.64	6.41±0.38	8.44±1.15
	10	2.67±1.92	2.59±0.20	5.67±0.94
	10.5	1.24±0.31	0.58±0.19	0.68±0.10

N.D. = not determined.

Subcellular fractions were prepared from the rhesus monkey (13 yr), yellow baboon (8 yr) and human (46 yr) testes. Aliquots of testicular fraction P1 (100-200 µg protein) were incubated at 34C for 8 h in the respective buffers indicated, in the presence of 10 mM $MgCl_2$ and 0.1% BSA. Other conditions of incubation are as shown in legend to Fig. 13: The maximum binding, 12%, 14% and 8% specific binding for the rhesus monkey, yellow baboon and human respectively, at pH 7.0 was set as 100% for comparison. Data represents the mean ± SEM of triplicate determinations of at least two separate experiments.

the loss of ^{125}I-hCG specific binding to the 40,000 x g pellet (fig. 34B) at 34C is faster than that reported for testicular homogenates (244). This difference could be due to the poor specific binding (1.5-3.0%) obtained in their studies, which is close to the lower limit of resolution of the assay.

The most marked difference between the human and nonhuman primate FSH receptors appears to be the greater stability of the human receptor. The half time of inactivation ($t_{\frac{1}{2}}$) of the human FSH receptor (fig. 39A) at 34C is about 14 h as against 2-5 h for the rhesus monkey (fig. 20A) and yellow baboon (fig. 21A) testicular FSH receptor. The human FSH receptor was more stable to heat (34C) than the LH receptor of the human (fig. 39B), rhesus monkey (fig. 20B) and yellow baboon (fig. 21B) testes ($t_{\frac{1}{2}}$ = 3-4 h). When the relative stability of the human testicular FSH and hCG(LH) receptor in the unoccupied state is considered, our data clearly show that the former is more stable at all the three temperatures (25C, 34C and 37C). The temperature dependent loss of activity is a result of inactivation of the gonadotropin receptor, not the hormone. Degradation of unoccupied receptors may play a role in the physiological regulation of cellular receptors.

As there is no loss in binding at 4C for up to 36-48 h (fig. 39), it may be feasible to carry out receptor determinations on tissues kept at 4C for this length of time. It is also important to note that the FSH receptor suffers little or no inactivation at 25C for up to 12-16 h, a finding which adds reliability to the estimated binding sites in tissues that we have collected up to 12 h after death. As the LH/hCG receptor undergoes about 30-40% loss in the same interval at 25C, the values that we have shown (Table 10), for the different ages may represent an underestimate. Our estimates of the LH receptors are lower than that reported by investigators from Finland (231) but within the range of values from other laboratories (see Table 12).

hCG is a placental hormone of pregnancy with chemical and biological properties similar to pituitary LH, but is not found in the circulation of normal men. All the studies reported in the literature on human

testicular receptors have used placental hCG rather than pituitary LH as the ligand, perhaps because of reasons of easier availability and stability of the former. As the rat testis, which is the most widely studied model, exhibits differential affinities for hCG, hLH or oLH (82) it was considered appropriate to examine the human testicular receptor with labeled hCG and hLH. The concentration dependent competition by unlabeled hLH for binding of ^{125}I-hCG to the receptor or vice versa indicated that the two hormones occupy the same binding sites. The estimated binding constants and capacities (Table 12) were the same in both cases, thus validating the use of hCG for studying the human testicular LH receptor. However, there appears to be some differences between ^{125}I-hCG and ^{125}I-hLH in the stability of the hormone-receptor complex as seen from our kinetic data shown in Figure 33. More detailed studies are necessary to examine if these are due to differential rates of inactivation of the ligands or dissociation rates.

In rodents, prolactin is involved in the induction and maintainance of testicular LH receptors (245). Prolactin treatment is accompanied by potentiation of LH stimulated spermatogenesis and testosterone production (246, 247). Sleep-related changes in male plasma testosterone are related to serum LH and prolactin concentrations suggesting that prolactin influences human testicular function (248). Prolactin secreting human pituitary adenomas are often associated with impotence and lower testosterone production (249). In addition, induction of hyperprolactinaemia in men leads to partial inhibition of estrogen production possibly by modifying testicular aromatase enzyme activity (250). The actions of prolactin in the rodent have been attributed to a direct effect on the testes via specific prolactin receptors located on the Leydig cell (251, 252). It was therefore of physiological relevance to examine human testicular tissue for the presence of prolactin receptors.

Analysis of five individual human testicular tissues (aged 50-70 yr) failed to yield significant binding of ^{125}I-hPRL (less than 1% specific binding). In contrast to the poor binding of ^{125}I-hPRL, these tissues bound ^{125}I-hCG and ^{125}I-hFSH (greater than 5% specific binding). The discrepancy in the number of prolactin receptors for the primate

and nonprimate testes is not unique to this hormone. Clayton et al. (253) have reported that receptors for LHRH are present in the rat but not human testes. Possible reasons for the poor binding of ^{125}I-hPRL in the human testes are the use of suboptimal binding conditions, a small receptor population for prolactin, or occupancy of prolactin receptors by endogenous prolactin. Absence of prolactin receptors in adult tissues does not preclude their existence in younger individuals (less than 50 yr) (251).

The greatest impediment to the characterization of the age dependency of gonadotropin binding to the primate testes is the scarcity of normal tissues. Though this study is of limited magnitude, utilization of autopsy tissue samples has allowed the most detailed study to date of the age dependent changes in the number of human testicular gonadotropin receptors. With the exception of tissues at the extreme ends of the age groups investigated, there was a tendency for the gonadotropin binding capacity (fmol/g testis) to remain relatively constant in the postpubertal human testes with advancing age (Fig. 43). A similar gonadotropin binding pattern was observed for the rhesus monkey (Table 7) and rat testes (FSH receptor only) (214). The gonadotropin binding capacities to testis in several species of animals is listed in Table 15. The binding affinities for all species for FSH and LH/hCG are in the range of 10^{9}-10^{10} M^{-1}. Among the primates, the human and rhesus monkey testes had the greatest LH and FSH binding capacity (fmol/g testis) respectively. In all the species except the rat, the number of FSH receptors was greater than that for LH. In the rat, an opposite pattern is evident despite the lower number of Leydig cells. Data on the gonadotropin receptor patterns of seven of the listed species - man, nonhuman primates, bull and rat - are directly comparable because the determinations were all done in our laboratory using similar techniques and identical ligands.

The divergence of testicular FSH and LH receptors between rat and other species including primates (Table 15) points out the inadequacy of the rat model for receptor studies. There appear to be significant

TABLE 15

COMPARISON OF THE FSH AND LH RECEPTORS IN TESTIS OF VARIOUS ANIMALS[a]

Species	Testis weight Mean (g)	Hormone binding capacity FSH pmol/g testis	FSH pmol/ testis	LH(hCG) pmol/g testis	LH(hCG) pmol/ testis	Ratio of FSH/LH receptor/g testis	Reference
I Primate							
Man	9±2	0.31±0.02 (71)[b]	2.77±1.32 (11)	0.10±0.01 (60)[b]	1.32±0.65 (11)	3.1	This study
Rhesus monkey	18±4	1.51±0.46 (10)	14.59±10.95(10)[c]	0.16±0.09 (10)[d]	0.42±0.30 (10)[c]	9.4	This study
II Non-Primate							
Bull	290±50	0.62±0.02	197.7 ± 7.5	0.04±0.01	12.89±0.98	15.3	This study (72, 212)
Rat	1.5±0.4	0.404±0.055	0.61 ± 0.08	1.320±0.04	1.98±0.06	0.3	This study (71, 77)
Pig	1-300 (various ages)	2.650[b]	ND	3-9	90-240	ND	(74,78)
Sheep	206 6	0.240±0.04[b]	49.5 ±8.3	0.03±0.004[b]	6.1±0.8	8.0	(73)

[a] Only selected data from the literature has been considered for species such as bull and rat. Only those studies which permitted calculation of data on a uniform basis (pmol/ g testis) were evaluated. Data represents the mean ± SEM or a range of values.

[b] Denotes use of homologous hormone for receptor determinations.

[c] The wide range of values arises from the steep increase in testicular weight that occurs with increasing age.

[d] These are values for animals of about 3-6 years of age because adult tissues (greater than 12 yr) have low or undetectable LH(hCG) binding.

ND = Not determinable.

Data from our study was derived by Scatchard and/or saturation analysis of ^{125}I-labeled gonadotropin binding to the adult bull (>5 yr), rat (34-44 days), rhesus monkey (3-6 yr) and human (16-80 yr) testes. Data represents the mean ± SEM of at least 5 animals.

differences in the relative distribution of receptors within primates. In the rhesus monkey, a species so widely used as a model in studies on reproduction, we have detected little hLH/hCG binding in the adult animal as compared to man. It is possible that in the adult monkey testis, the LH receptor may be more susceptible to degradation (194-196). If this is true, it appears to be more stable in the young monkey testis as LH receptors are easily detectable (Table 7 and 8). Age dependent changes in testicular inactivation of FSH and FSH receptors in the rat testis have been speculated to have a role in the control of hormone action (254). Whether similar phenomena may operate in primates remains to be investigated.

Another significant difference between the human and monkey testicular LH receptor lies in their ability to recognize nonprimate LH. The human receptor was more selective because several highly purified nonprimate LH preparations which were effective in the monkey or rat system had extremely low activity in competitive binding assays using ^{125}I-hCG/^{125}I-hLH and the human receptor. It may be pointed out that our data (Fig. 44B) on the rhesus monkey are strikingly different from the observations reported by Davies et al. (185). Our highly purified oLH and bLH preparations were about 25% as active as hCG or hLH in one experiment (Fig. 44B) and almost 100% active in another, while the rat LH was consistently low in activity. The reasons for the discrepancy in the data are not clear at present.

The human testicular FSH receptor is clearly different from the LH receptor in its behavior as the former can efficiently interact with many heterologous FSH preparations (Fig. 46). A nonhuman hormone such as equine FSH was more active than hFSH itself in the displacement assay. ^{125}I-oFSH bound as efficiently as ^{125}I-hFSH to the receptor. These results are identical to the situation in monkey testis (196). Unlike the reported species specificity of the LH/hCG receptor in the primate testis (185) and human (255) and monkey (256) corpus luteum, the FSH receptor from the human testis was clearly different. Not only did many unlabeled heterologous FSH preparations (equine, ovine, porcine,

rat) compete as effectively as or better than hFSH for binding sites (Fig. 46, Table 11) but ^{125}I-oFSH also bound well to the human and different monkey receptors. While the cause of the pronounced difference in species specificity of the LH and FSH receptors remains a mystery, we may speculate that the very high degree of homology in the structure of the hormone specific β subunits of different species of FSH (fig. 4) (257) may render them suitable for interaction with the human receptor. The degree of structural conservation among the hormone specific β subunits of different species of LH (fig. 3) (14) is not as high as in FSH (fig. 2 and 4) (257).

The human testicular LH receptor is unlike that of the corpus luteum's in that it maintains its preference for the homologous hormone after freezing and thawing. It has been reported that human luteal LH receptors while maintaining high specificity for the human hormone in the fresh state apparently lose this characteristic after freezing (-20C for 3-21 months) and begin to recognize nonhuman hormones (255). Such a change is unlikely with the monkey testis because a particulate fraction from freshly removed testes from the bonnet monkey (Macaca radiata) interacts with FSH from several species (Sairam, M.R., Rao, A.J., Moudgal, N.R. & Berman, M.I., unpublished results). It is possible that the primate FSH receptor is different from the LH receptor as far as its species specificity is concerned, with the latter exhibiting a more strict requirement for primate hormones. Our data, on the interaction of heterologous FSH with the primate FSH receptor, agree well with data reported in the literature. Several studies have shown that adenohypophyseal extracts derived from a variety of species (sheep, pig, horse, human) were capable of successfully inducing an increase in the size of the human and nonprimate ovaries, due to an enormous enlargement of the follicles with little or no trace of luteinization (258-262). While FSH receptors have been demonstrated in the primate ovary (263) its species specificity has not been determined. Data on the effects of heterologous gonadotropins in monkey testis are scanty. Reports such as that of Greep (1937) indicated enlargement of gonads of immature

animals following treatment with nonhuman pituitary fractions containing FSH (264). The marked enlargement of seminiferous tubules and Sertoli cell cytoplasm noted in immature rhesus monkeys following oFSH or PMSG treatment (265), clearly indicates a lack of species specificity in hormone interaction, consistent with our observations (Fig. 46 and Table 11).

CHAPTER 4

HUMAN TESTICULAR ADENYLATE CYCLASE ACTIVITY

4.1 Introduction

Hormone-target cell interaction is known to modulate the biological activity of the target cell through the activation of the adenylate cyclase system (266-268). The membrane bound hormone responsive adenylate-cyclase system is composed of at least three separate and distinct components. These are the hormone receptor (R), a catalytic subunit (C), and a regulatory protein (N) capable of binding GTP or its analogues. The regulatory protein provides a functional linkage between the hormone-receptor interaction (at the outer membrane surface) and activation of the catalytic unit of adenylate cyclase (at the inner membrane surface), which results in the formation of cyclic AMP. Cyclic AMP, acting as a second messenger, mediates the intracellular response of the cell to the hormone (266-268). In the rat testis and ovary, specific binding of the gonadotropin hormones led to the direct stimulation of the enzyme adenylate cyclase (269). During sexual maturation, changes in testicular receptor numbers resulted in parallel changes in adenylate cyclase responsiveness to gonadotropins. There was an increase in LH responsive adenylate cyclase activity and LH receptors with advancing age while the FSH responsive adenylate cyclase activity and FSH receptors were decreasing (270,271). Priming of female rats with PMSG resulted in a parallel rise and fall of gonadotropin binding sites and adenylate cyclase activities (272). These studies suggest that the number of available gonadotropin receptors is correlated with the ability of gonadotropins to stimulate adenylate cyclase. Desensitization, which results in a loss of gonadotropin receptors, also leads to a loss of responsiveness of the adenylate cyclase system to gonadotropins (165,168). This is consistent with a

loss of target cell responsiveness due to impaired receptor-adenylate cyclase coupling.

Chapter 3 described the age dependent changes in gonadotropin binding to the human testes. Gonadotropin stimulation of human testicular tissue has been shown to result in the accumulation of cyclic AMP (273, 274). It was therefore of interest to determine if the human testicular receptors are coupled to adenylate cyclase and if maturation alters hormone-receptor coupling or adenylate cyclase responsiveness to gonadotropin stimulation. The mechanism involved in coupling receptor binding to enzyme activation was studied using highly specific gonadotropin agonists and antagonists prepared in this laboratory. Preliminary results of this study have been reported (275).

4.2 Materials and Methods

4.2.1 Hormones

Highly purified human FSH (hFSH), ovine FSH (oFSH) and ovine LH (oLH) were prepared in this laboratory. The hCG (CR119 - iodination grade) and human LH (hLH 1_4 - iodination grade) were supplied by the National Hormone and Pituitary Program, National Institute of Health, Bethesda, Maryland. Human prolactin (hPRL) and equine FSH (eFSH) were provided by Dr. H.G. Friesen, University of Manitoba, Winnipeg, Canada and Dr. T. Landefeld, University of Michigan, Ann Arbor, Mi. Isoproterenol (ISO), propranolol (PRO), estradiol-17β (E2), β-estradiol-3-benzoate (E2β), diethyl stilbestrol (DES), progesterone (P), testosterone (T), and androstendione (A) were purchased from Sigma Chemicals, St. Louis, Mo. A clinically useful derivative of DES, Honvol (diethyl stilbestrol sodium phosphate, DES-NaP) was donated by Horner Laboratories, Montreal, Que., Canada.

4.2.2 Chemicals

Lactoperoxidase, bacitracin, guanosine 5'-triphosphate (GTP) sodium salt from equine muscle, 5'-guanylimido-diphosphate (GMP-P(NH)P) sodium salt, adenosine 5'-triphosphate (ATP) disodium salt from equine muscle, adenosine 3':5' cyclic monophosphate (cyclic AMP) sodium salt and

creatine phosphate (CRP) disodium salt were purchased from Sigma, St. Louis, Mo. Forskolin (FSK) from Coleus forskohlii and dithiothreitol (DTT) or Cleland's reagent were purchased from Calbiochem-Behring Corp., San Diego, Ca., Creatine kinase (CK) from rabbit muscle and myokinase from rabbit muscle were obtained from Boehringer Mannheim, Montreal, Que., Canada. Sodium fluoride was obtained from Aldrich Chemical Co., Montreal, Que., Canada. Radiolabeled adenosine 5'-triphosphate, tetra (triethylammonium) salt, [α-^{32}P] or [α-^{32}P] ATP was purchased from Amersham Inc., Arlington Heights, Il.

4.2.3 Dowex 50 columns

These columns were prepared by adding a suspension of Biorad AG 50 W-8X, 100-200 mesh, H^+ form, into 0.7 x 4 cm polypropylene columns (10 ml) to give a final bed height of 3.5 cm. After use columns were regenerated by addition of 5 ml of 1N HCl followed by three successive washings of 8 ml deionized water.

4.2.4 Aluminum oxide (alumina) columns

The aluminum oxide was suspended in 100 mM Tris-HCl, stirred, allowed to settle for about 1 min, decanted and resuspended. This process was repeated 4-5 times to remove any fine particles which would retard the column's flow rate. Columns (0.7 x 4 cm) were packed to give a bed height of 3.5 cm. One wash of 8 ml 100 mM Tris-HCl is the only requirement to regenerate these columns.

4.2.5 Preparation of testicular tissues

Human testes collected at surgery or within 12 h post mortem from healthy individuals were utilized for the study. Tissues were cut into small pieces and homogenized in 25 mM Tris-HCl buffer pH 7.2 containing 100 mM sucrose and 1 mM EDTA. The solution was then filtered through cheese-cloth and centrifuged at 40,000 x g. The resultant P1 pellet was resuspended (1 g per 2 ml) in 25 mM Tris-HCl buffer pH 7.2

containing (1 mM EDTA). EDTA (1 mM) was utilized to preserve the activity of adenylyl cyclase during homogenization and prolonged storage. This concentration of EDTA does not interfere with ^{125}I-labeled gonadotropin binding to the human testicular gonadotropin receptor. Similarly fractions were prepared from 34 day old CRCD rat testes. Protein content was assessed as described previously in Chapter 2 (see Section 2.2.5).

4.2.6 Iodination of hormones

The gonadotropic hormones, hCG, DGhCG and hFSH were labeled with ^{125}I by the lactoperoxidase method as previously described in Chapter 2 (see Section 2.2.6). The specific activities of the labeled hormones were in the range of 50-80 µCi per µg.

4.2.7 Deglycosylation of the gonadotropins

The gonadotropic hormones, hCG, oLH and oFSH were separately subjected to deglycosylation by exposure to anhydrous HF for 60 min at 0C as previously described (33). This procedure removes approximately 80% of the carbohydrate residues without damaging the protein backbone.

4.2.8 Binding studies

The compounds estradiol (MW 272.4), diethyl stilbestrol (MW 268.3), testosterone (MW 288.4), androstendione (MW 286.4), and progesterone (MW 314.5) were dissolved in ethanol to give a final concentration of 6 mg/ 400 µl. Preheating at 40-50C for one minute was required for samples to dissolve. Diethyl stilbestrol sodium phosphate (50 mg/ml) (MW 428.3) and the deglycosylated gonadotropic hormones are water soluble and were directly diluted with assay buffer. Assays were performed in triplicates in 10 X 75 mm disposable polystyrene tubes. Unless otherwise indicated, each tube contained approximately 50,000 cpm (400 pg) of ^{125}I-labeled hormone, 100-300 µg protein equivalent of the testicular subcellular fraction P1, 50 µl of the agent to be tested and 100 µl of the unlabeled

hormone (500 fold excess) and/or assay buffer to constitute a final volume of 250 µl/tube. In the case of the steroid hormones, suitable controls receiving the same concentration of ethanol or assay buffer were always employed. The assay buffer consisted of 25 mM Tris-HCl buffer, pH 7.2 containing 0.1% BSA, 0.2% $MgCl_2$ and 0.6 mM bacitracin. The tubes were vortexed and incubated in a continuously shaking Dubnoff water bath for 15 h at 25C. The reaction was terminated by the addition of 2 ml of chilled assay buffer followed by centrifugation at 2900 x g for 15 min at 4C in a table top IEC clinical centrifuge. The supernatant was removed by aspiration under vacuum and the radioactivity in the pellet was determined in an LKB rackgamma II counter (counting efficiency 70%). The difference between the total radioactivity bound and nonspecific binding was defined as the amount specifically bound and expressed as a percentage of the total counts put into the tubes.

4.2.9 Reversibility of synthetic steroid hormone action

The human testicular subcellular fraction P1 was first incubated with the compound being tested for 2 h at 34C in a final incubation volume of 250 µl. The tubes were then washed once with 2 ml of chilled assay buffer, the membranes recovered by centrifugation at 2900 x g for 10 min at 4C and the supernatant removed by aspiration. Tubes were resuspended to the original volume with the assay buffer and either ^{125}I-hCG or ^{125}I-hFSH. A second incubation of 15 h at 25C was then performed. Specifically bound radioactivity in the pellet was determined and expressed as a percentage of control tubes.

4.2.10 Adenylate cyclase assay

Adenylate cyclase activity was determined by measuring [^{32}P] cyclic AMP formation from [α^{32}P] ATP as described previously (276, 277) with minor modifications. Unless stated otherwise, the assay medium consisted of 25 mM Tris-HCl buffer, pH 7.2, 0.5 mM MgATP, [α^{32}P] ATP (1.2-1.8 x 10^6 CPM), 5 mM $MgCl_2$ (in excess of the ATP concentration), 0.5 mM cyclic AMP, 1 mM DTT, 10 µM GTP and an ATP regenerating system consisting of

5 mM creatine phosphate, 0.1 mg/ml creatine kinase and 0.1 mg/ml myokinase in a final volume of 200 µl. The above conditions resulted in optimal stimulation of adenylate cyclase activity by the gonadotropic hormones. Incubations were initiated by the addition of rat or human testicular tissue (50-100 µg of protein) to the reaction mixture which had been thermally equilibrated for 15 min at 34C. Reactions were terminated, after a 15 min incubation at 34C, by addition of 0.6 ml of a zinc acetate-cyclic AMP solution (43 mg cyclic AMP/250 µl 120 mM zinc acetate) and 0.5 ml of 144 mM sodium carbonate (Na_2CO_3). This step results in the partial purification of cyclic AMP by coprecipitation of other nucleotides. Precipitation was aided by centrifugation at 2900 x g for 10 min at 4C. Cyclic AMP was further purified by subsequent chromatography on a double column system as described previously (276, 277) with minor modifications.

4.2.11 Separation of [^{32}P] cyclic AMP from [α^{32}P] ATP derived [^{32}P] labeled compounds

As stated in the previous section, cyclic AMP is initially purified by coprecipitation of contaminants. Following centrifugation, a 1 ml aliquot of the clear supernatant from each tube was applied to a separate Dowex 50 column and the effluents discarded. These columns were washed with 3 ml deionized water and the effluents again discarded. The Dowex 50 columns were subsequently placed on top of a set of aluminum oxide columns (piggyback) and cyclic AMP eluted from the Dowex 50 columns into the aluminum oxide columns by applying 8 ml deionized water to the Dowex 50 columns. Eluates from the Dowex 50 columns are allowed to penetrate completely into the aluminum oxide columns, whose effluents are discarded. [^{32}P] cyclic AMP is then eluted from the aluminum oxide columns directly into 20 ml scintillation vials by addition of 8 ml 100 mM Tris-HCl to the aluminum oxide columns. Chromatography on the double column system results in the removal of more than 99% of the contaminating ^{32}P. The samples were analyzed

for [^{32}P] cyclic AMP by 10 min counting in a Beckman LS-350 liquid scintillation counter. Overall recovery of [^{32}P] cyclic AMP per tube as assessed by reading the optical density at 259 nm was 70-90%. Assays were carried out in triplicate and results expressed as the mean of at least two experiments. Activity was expressed as pmol cyclic AMP formed per 15 min per mg protein.

4.2.12 Statistical analysis

Data were analyzed for significance by Student's 't' test. Displacement curves were tested for parallelism and Scatchard plots performed using a programmable Hewlett-Packard desk top calculator.

4.3 Results

4.3.1 Gonadotropin-sensitive adenylate cyclase

The presence of a gonadotropin sensitive adenylate cyclase was investigated in 40,000 x g membrane fractions derived from human testicular tissue which demonstrated significant ^{125}I-hCG and ^{125}I-hFSH binding. Each tissue (3-20 mg equivalent of original testes weight) was simultaneously assessed for FSH and LH responsive adenylate cyclase activity using a saturating dose of the gonadotropic hormones (1 µg) under standard assay conditions (see Section 4.2.10). A wide fluctuation of FSH stimulated adenylate cyclase activity was observed. Of the 44 tissues procured from the clinique, only 27 of these tissues (61%) had significant FSH responsive adenylate cyclase activity. Of these 27 tissues, 41% (11/27 tissues) responded well to hFSH (stimulation was greater than 1.6 times basal activity), 44% (12/27 tissues) were 1.3-1.6 times as active and the remaining 15% (5/27 tissues) were poor responders, i.e. less than 1.3 times as active as the basal activity. Typical results are summarized in Table 16. A maximum of 2.0 fold stimulation over the basal values was obtained. Subsequent experiments utilized tissues from the first group (1.7 x basal activity) to ensure maximization of the response and reliability of the recorded data.

TABLE 16

RELATIONSHIP OF FSH STIMULATED ADENYLATE CYCLASE ACTIVITY TO THE NUMBER OF GONADOTROPIN RECEPTORS IN THE HUMAN TESTES

FSH response	Number of tissues	% FSH stimulated adenylate cyclase activity	FSH receptors fmol/g testes	LH receptors fmol/g testes
FSH responsive	12	60-120	280.00±43.08	58.55±15.06
FSH responsive	11	30- 60	269.25±46.12	101.70±24.73
FSH responsive	4	20- 30	359.25±88.65	75.25±32.09
FSH non-responsive	17	—	229.94±38.47	59.94±11.85

Testicular tissue (50-100 µg fraction P1) was obtained from individuals aged 16-70 yr. Adenylate cyclase activity was measured under standard assay conditions (see methods, section 4.2.10). Gonadotropin binding capacity was assessed by Scatchard and saturation analysis using ^{125}I-hFSH. Data represents the mean ± SEM. Note the subdivision of FSH responsive tissues is arbitrary. There was no statistically significant difference in the number of FSH or LH receptors. In all groups, the number of FSH receptors was significantly greater than the number of LH receptors.

In response to 1 µg hCG or hLH (data not shown), very little, if any, significant enzyme stimulation was observed in spite of good ^{125}I-hCG binding and the presence of sufficient accessible binding sites (Table 17). Only 8 out of 36 tissues (22%) had LH responsive adenylate cyclase activity.

No significant difference was observed in the FSH or LH(hCG) binding capacity of the gonadotropin adenylate cyclase responsive and non-responsive testicular tissues. Among the three FSH adenylate cyclase responsive tissues, no correlation between gonadotropin receptor numbers and adenylate cyclase activity was detected. Indeed, tissues with the same number of FSH receptors exhibited various degrees of FSH responsive adenylate cyclase activity, from no detectable response to a two fold stimulation of adenylate cyclase activity. The few tissues with significant adenylate cyclase activity precluded an age dependent study. Nonetheless, FSH responsive adenylate cyclase activity was observed in tissues from individuals aged 20-60 yr. This seems to suggest that FSH stimulation of adenylyl cyclase is age independent.

The low enzyme response to hCG (0-40% above basal) might reflect the use of a suboptimal concentration of hormone. Whereas increasing concentrations of hFSH (1-5000 ng) led to a well defined dose dependent increase in FSH-sensitive adenylate cyclase activity (Fig. 47), little or no change in the LH(hCG) sensitive enzyme activity was observed. Therefore failure to obtain large stimulation of adenylate cyclase activity by hCG was not due to insufficient hormone. The first increase in adenylate cyclase activity occurred in the presence of 1.67 nM hFSH. The EC_{50} for hFSH stimulation of adenylate cyclase was 8.33 nM with saturation occurring at 83.3 nM hFSH. In agreement with the poor binding ability of ^{125}I-hPRL to the human or rhesus monkey testes (less than 1% specific binding), hPRL (1 µg) did not significantly stimulate the human testicular adenylate cyclase enzyme. Human growth hormone (1 µg hGH) and isoproteronol (5-500 µM) also did not significantly increase the activity of adenylate cyclase.

TABLE 17

RELATIONSHIP OF GONADOTROPIN STIMULATED ADENYLATE CYCLASE ACTIVITY TO THE NUMBER OF GONADOTROPIN RECEPTORS IN THE HUMAN TESTES

Gonadotropin response	Number of tissues	% gonadotropin stimulated adenylate cyclase activity	FSH receptors fmol/g testes	LH receptors fmol/g testes
FSH responsive	27	20–120	286.96±29.34	78.48±13.05
FSH non-responsive	17	—	222.94±38.47	59.94±11.85
hCG responsive	8	7–41	338.13±54.39	81.33±18.10
hCG non-responsive	28	—	290.89±26.35	74.96±12.32

Testicular fraction P1 (50–100 µg) was obtained from individuals aged 16–70 yr. Adenylate cyclase activity was measured under standard assay conditions (see methods, section 4.2.10). Gonadotropin binding capacity was assessed by Scatchard and saturation analysis using ^{125}I-hFSH and ^{125}I-hCG. Data represents the mean ± SEM. Among the various groups, there was no statistically significant difference in the number of FSH or LH receptors. In all groups, the number of FSH receptors was significantly greater than the number of LH receptors.

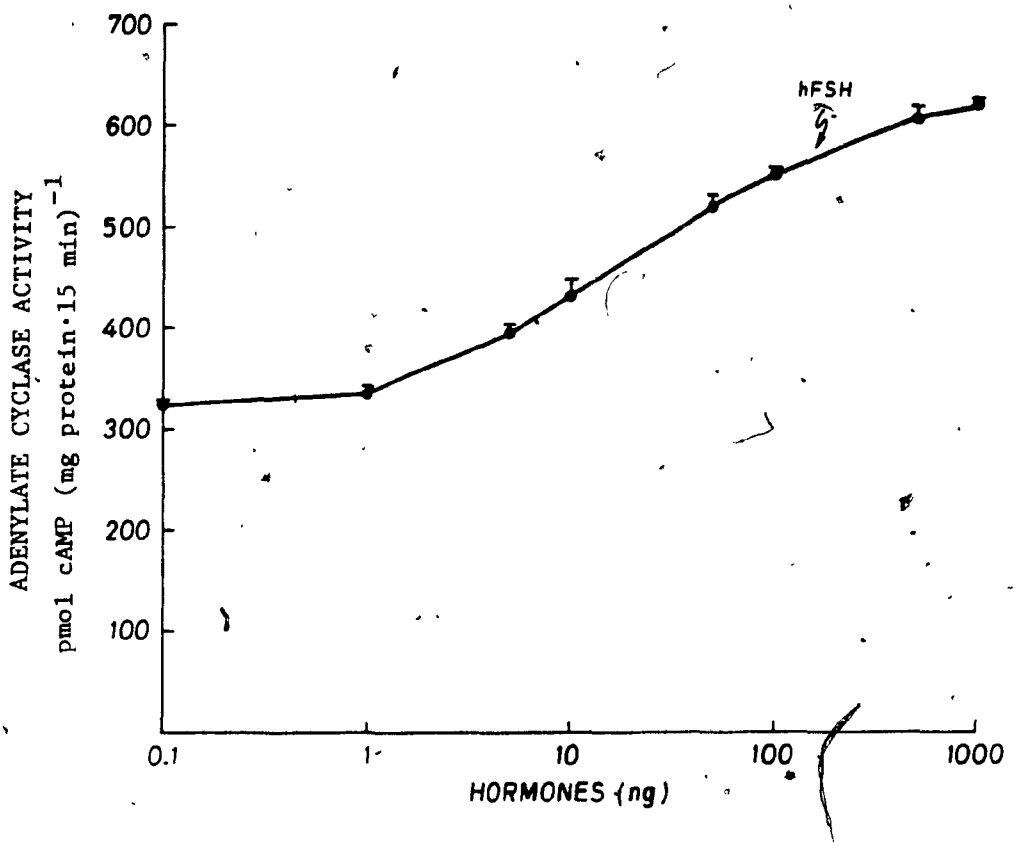


FIG. 47

Stimulation of human testicular (20 yr) adenylate cyclase activity by various concentrations of hFSH. Adenylate cyclase activity in this and subsequent figures was assessed as stated in methods (section 4.2.10). Basal enzyme activity was 130.81 ± 0.84 pmol cAMP (mg protein·15 min)$^{-1}$. Unless stated otherwise, data represents the mean ± SEM of triplicate determinations of one of at least two separate experiments.

4.3.2 Optimum conditions for adenylate cyclase activation by hFSH

Stimulation of adenylate cyclase by hFSH (1 µg) was proportional to the amount of testicular protein added (Fig. 48). There was a linear increase in the production of cyclic AMP between 50-200 µg of protein. At equivalent protein concentrations, no significant stimulation of adenylate cyclase activity by hCG (1 µg) was obtained with this tissue. A comparison of the ability of FSH to bind to the testicular receptor and stimulate adenylate cyclase is shown in Figure 49. Optimal binding of FSH to the FSH receptor at 34C occurred within 10 h (Fig. 49A). A linear activation of adenylate cyclase activity was observed for up to 20 min at 34C (Fig. 49B). Note that maximal activation of FSH stimulated adenylate cyclase activity does not correspond to maximal binding of ^{125}I-hFSH. In the presence of low binding, we observe optimal adenylate cyclase activity. At 20 min when cyclase activity is maximal, only 20% binding of ^{125}I-hFSH to its receptor is observed. Prolongation of the incubation, led to a progressive fall in adenylate cyclase activity in spite of increased binding of ^{125}I-hFSH. Under standard assay conditions (see Methods, section 4.2.10), optimal stimulation of adenylate cyclase activity occurred in the presence of 5 mM creatine phosphate (CRP). Dithiothreitol (1 mM DTT is used to protect adenylyl cyclase from oxidation or loss of free sulfhydryl groups. While this concentration of DTT is not detrimental, larger concentrations inhibited the FSH responsive adenylate cyclase activity.

4.3.3 Metal ion dependency of testicular adenylate cyclase

Metal ions have been shown to play an important role in the activation of adenylate cyclase in various tissues such as the brain, liver and heart (278-280). In the present studies, hFSH stimulation of adenylate cyclase was also dependent on the concentration of metal ions (Fig. 50). Although basal enzyme activity increased with increased metal ion concentration of Mg^{2+} or Mn^{2+}, the degree of stimulation (V_{FSH}/V_{BASAL}) was greatest at low ionic concentrations. A two fold stimulation of adenylate cyclase activity by hFSH was observed in the presence of 0.5 mM $MgCl_2$, which was completely abolished at high

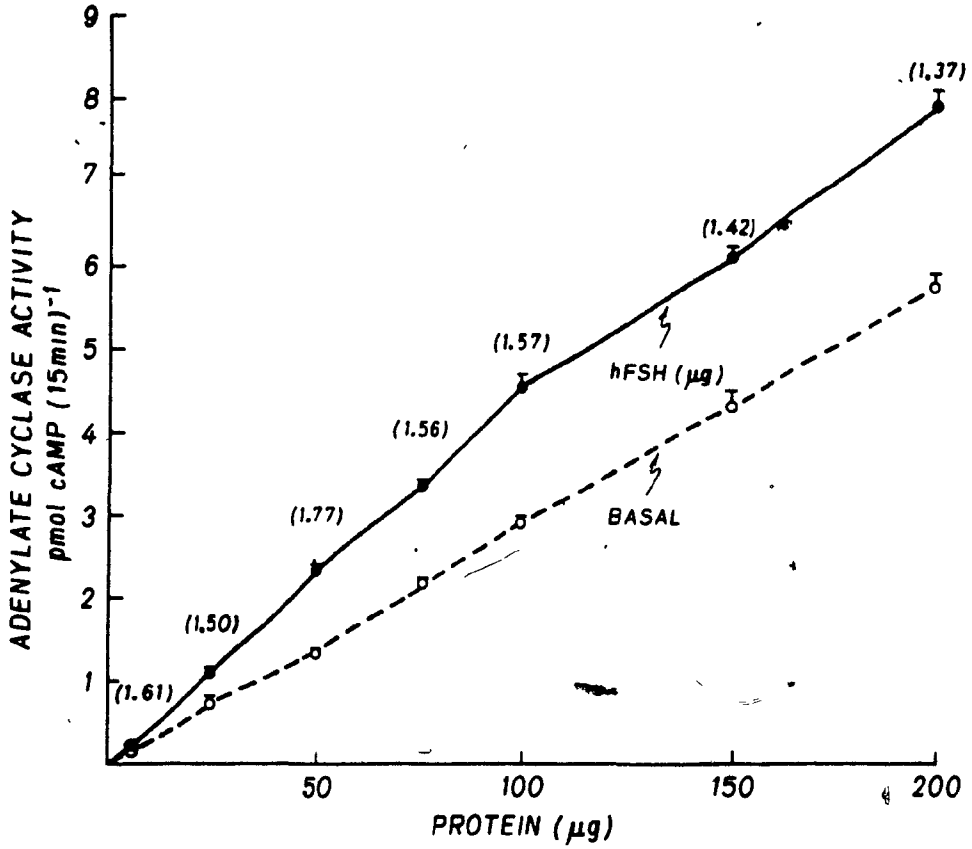
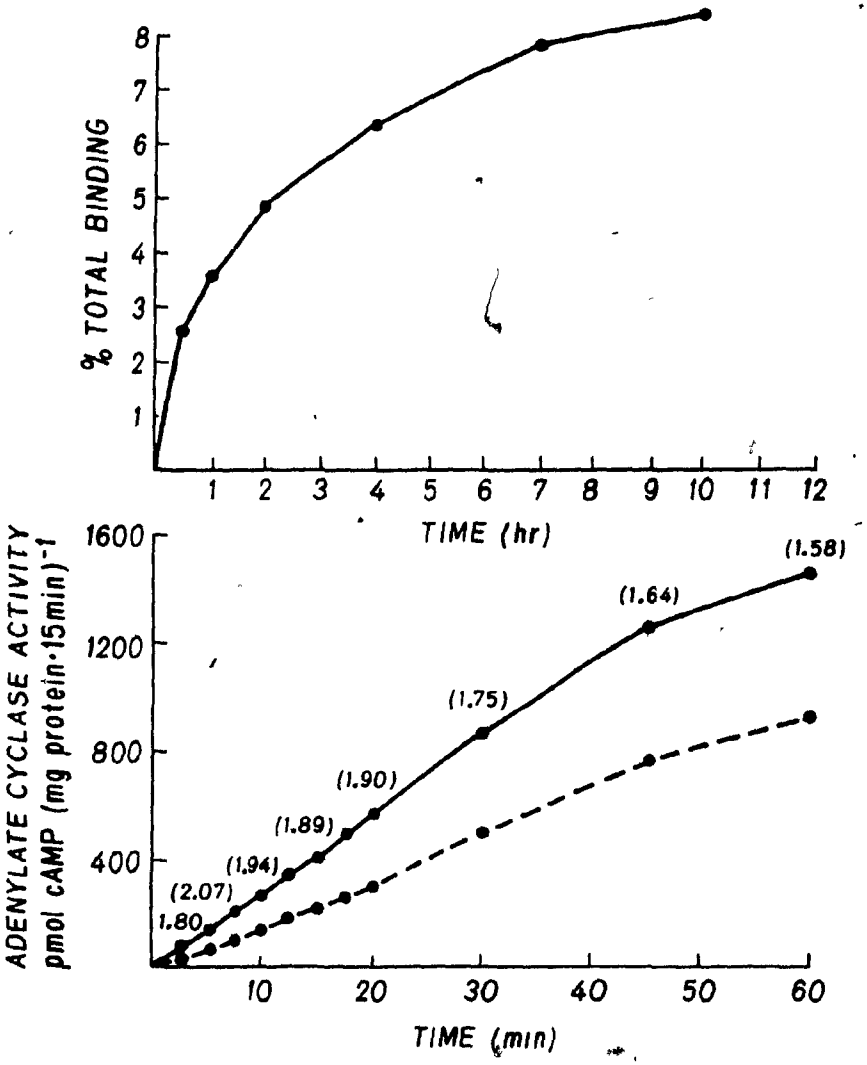


FIG. 48

The effect of increasing concentrations of fraction P1 on basal (dashed line) and hFSH (1 µg) stimulated (solid line) human testicular adenylate cyclase activity. Testicular tissue was obtained from a 52 yr old individual. Basal enzyme activity was set as 1 for comparison.

FIG. 49

Time dependence of ^{125}I-hFSH binding (A: top panel) and of FSH stimulation of adenylyl cyclase (B: bottom panel) at 34C. Human testicular tissue (50-150 µg) was obtained from 2 different individuals aged 73 yr (panel A) and 69 yr (panel B). Adenylate cyclase activity was determined in the presence (solid line) or absence (dashed line) of 1 µg hFSH. Basal enzyme activity was set as 1 for comparison. In this and subsequent figures, where error bars are not shown the error is within the point.


% TOTAL BINDING
TIME (hr)
ADENYLATE CYCLASE ACTIVITY
pmol cAMP (mg protein·15min)⁻¹
TIME (min)
(1.58)
(1.64)
(1.75)
(1.90)
(1.89)
(1.94)
(2.07)
1.80

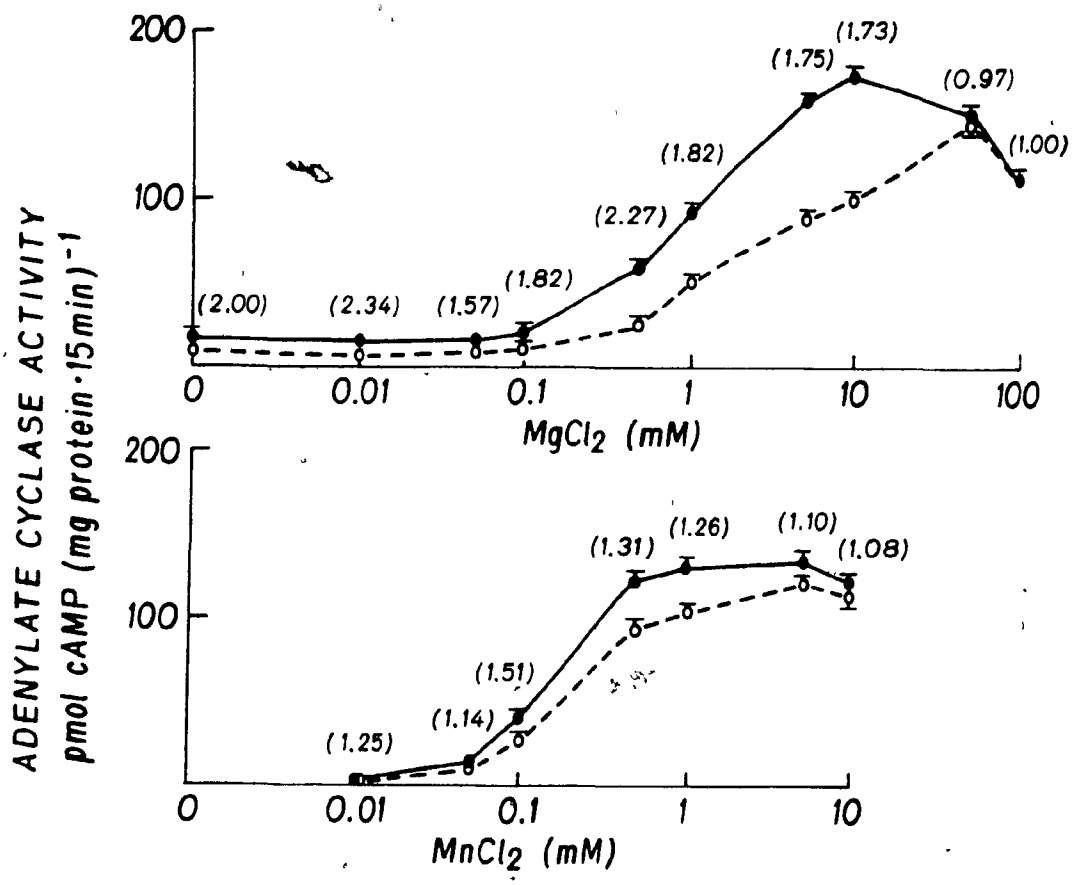


FIG. 50

Effect of various concentrations of $MgCl_2$ (A: top panel) and $MnCl_2$ (B: bottom panel) on the human testicular adenylate cyclase activity. Testicular tissue was obtained from two different individuals aged 20 yr (panel A) and 58 yr (panel B). Adenylate cyclase activity was determined in the presence (solid line) or absence (dashed line) of 1 μg hFSH. The salts, $MgCl_2$ (panel A) and $MnCl_2$ (panel B), were omitted from the assay mixture and added separately at the indicated concentrations. Basal enzyme activity was set as 1 for comparison.

concentrations (>10 mM $MgCl_2$). In the case of $MnCl_2$, maximal stimulation was observed at 0.1 mM $MnCl_2$. The FSH responsive adenylate cyclase in the human testes is also influenced by the concentration of MgATP (Fig. 51). Both the basal and hFSH stimulated adenylate cyclase activity increased with increasing concentrations of MgATP at a fixed concentration of Mg^{+2} (0.5 mM). The relative response to hFSH was optimal at 0.5-1.0 mM MgATP. These data suggest that FSH, like other hormones, binds to the metal binding site in this system (267, 268).

4.3.4 Effect of guanine nucleotides

Guanine nucleotides have been shown to be required for the expression of hormone responsive adenylate cyclase activity (280-283). The effects of guanosine 5'-triphosphate (GTP) or 5'-guanylimido-diphosphate [GMP-P(NH)P], a nonhydrolyzable derivative of GTP, on the human testicular adenylate cyclase activity in the presence and absence of FSH was investigated utilizing a particulate fraction P1. As indicated in Table 18, GTP and GMP-P(NH)P at various concentrations, 0.1-100 μM, did not significantly change the basal adenylate cyclase activity. Indeed, the FSH stimulated adenylate cyclase activity (1.70 x) was also not significantly altered by the presence of GTP or GMP-P(NH)P, whether a homogenate or particulate membrane fraction was utilized. The lack of a requirement of GTP for stimulation of the human testicular adenylate cyclase might be due to the presence of endogenous GTP in the tissue extracts (284).

4.3.5 Specific binding of native and deglycosylated gonadotropins to the human testicular gonadotropin receptor

The ability of several gonadotropic hormones and their deglycosylated derivatives to competitively displace radiolabeled ^{125}I-hFSH and ^{125}I-hCG from their respective human testicular receptors is illustrated in Figure 52. The displacement curves of the native and deglycosylated hormones were not significantly nonparallel. Removal of approximately 80% of the carbohydrate moieties from the native hormones did not impair

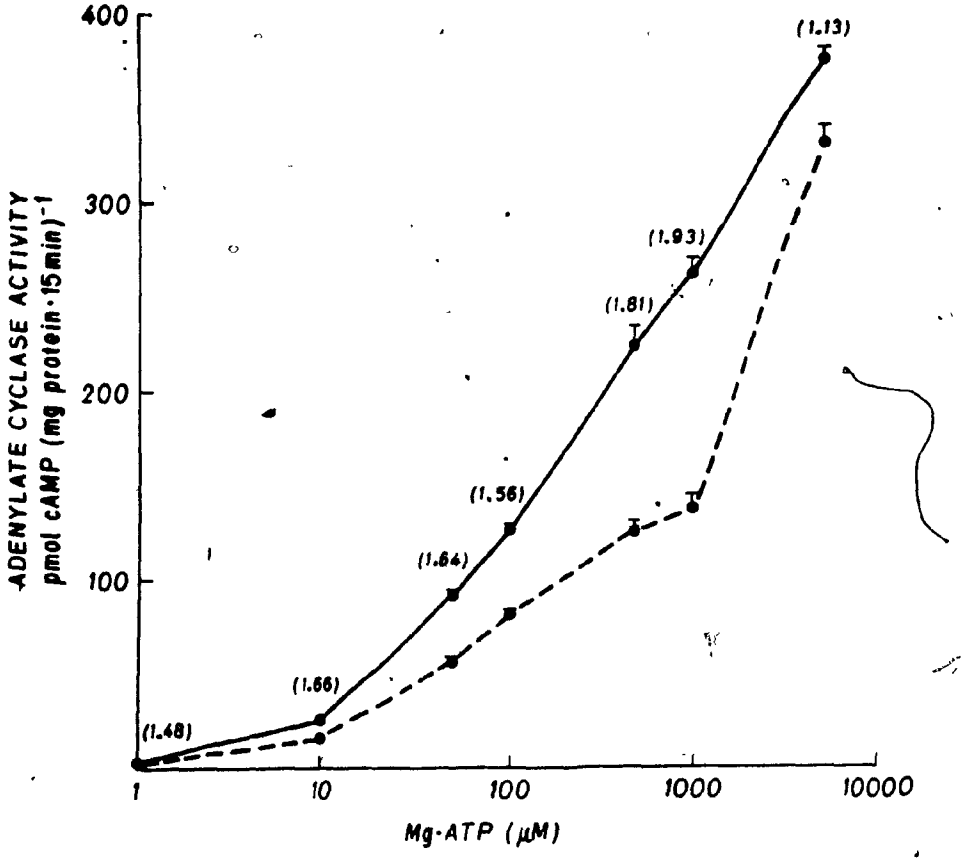


FIG. 51

Dependence of adenylate cyclase of the human testes (60 yr) on the concentration of MgATP. Adenylate cyclase activity was assessed in the presence (solid line) or absence (dashed line) of 1 µg hFSH. The MgATP was omitted from the assay mixture and added separately at the indicated concentrations. Basal enzyme activity was set as 1 for comparison.

TABLE 18

EFFECT OF GUANINE NUCLEOTIDES ON BASAL AND FSH STIMULATED HUMAN TESTICULAR ADENYLATE CYCLASE ACTIVITY

Additions	Concentration (μM)	Basal	hFSH (1 μg)	% Stimulation
None	—	81.71±4.51	137.99±2.84	1.69
GTP	0.1	91.14±3.62	158.04±1.90	1.73
	1.0	98.03±3.28	161.19±4.71	1.64
	10.0	94.89±2.23	159.61±3.71	1.68
	100.0	101.82±4.41	169.43±3.55	1.66
GMP-P(NH)P	0.1	85.23±5.13	151.94±5.56	1.78
	1.0	93.78±4.09	152.09±2.98	1.62
	10.0	96.49±3.43	163.43±4.50	1.69
	100.0	104.11±3.22	179.65±8.23	1.73

Adenylate cyclase activity was assessed as stated in methods (section 4.2.10) except that GTP was omitted from the assay mixture. Testicular fraction P1 (60 μg) was obtained from a 60 yr old man. The values represent the mean ± SEM of triplicate determinations of at least two separate experiments. Basal enzyme activity was set as 1 for comparison.

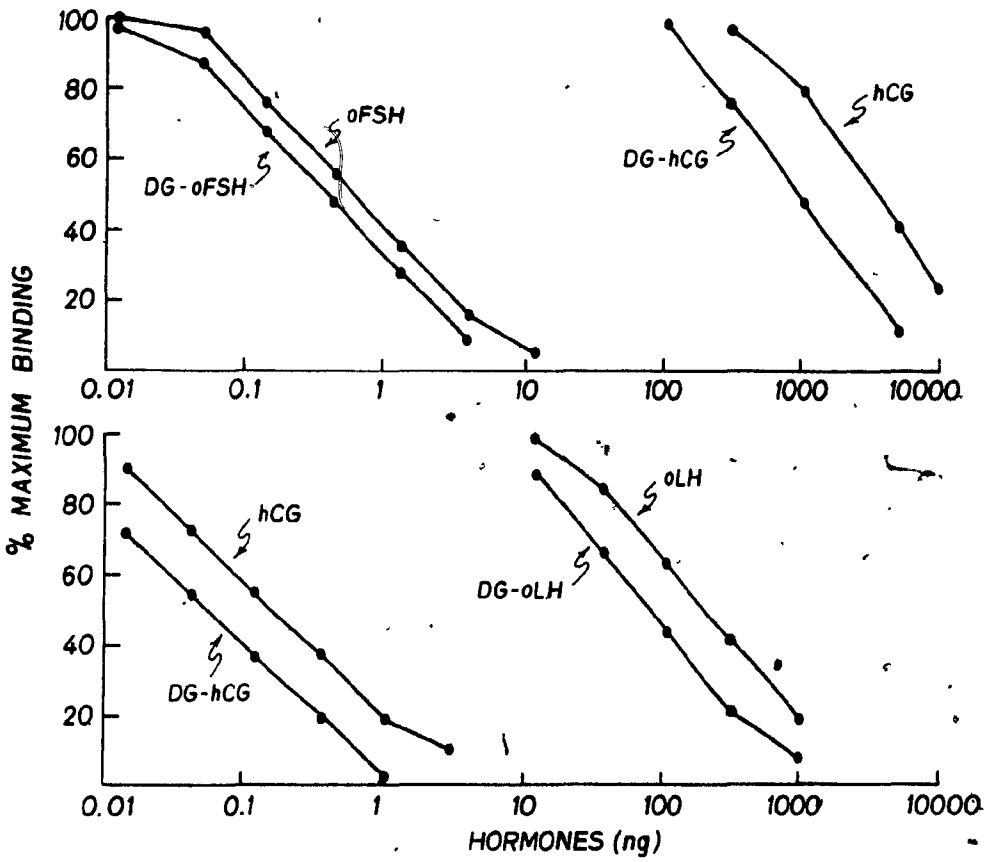
FIG. 52

(A) TOP PANEL

Comparison of the ability of the gonadotropic hormones and its derivatives to displace ^{125}I-hFSH from the human testicular (51 yr) FSH receptor (P1). The amount of hormone specifically bound (8% total binding) was set as 100% for all calculations. The average potency was calculated from the amount (ng) of hormone required to displace 50% of the radioactivity from the receptor. Setting hFSH as 1, the potency of DGoFSH was 1.26 x (1.17-1.36, 95% C.L.), while oLH, DGoLH, hCG, and DGhCG were less than 0.001 x as active.

(B) BOTTOM PANEL

Comparison of the ability of the gonadotropic hormones and its derivatives to displace ^{125}I-hCG from the human testicular (50 yr) hCG receptor (P1). The amount of hormone specifically bound (8% total binding) was set as 100% for all calculations. Setting hCG as 1, the potency of DGhCG was 3.34 x (2.29-4.80, 95% C.L.), while oLH, DGoLH, oFSH, and DGoFSH were less than 0.001 x as active.


% MAXIMUM BINDING
100
80
60
40
20
0.01
0.1
1
10
100
1000
10000
oFSH
DG-oFSH
hCG
DG-hCG
hCG
DG-hCG
oLH
DG-oLH
HORMONES (ng)

the dose dependent interaction of these deglycosylated derivatives with the human receptor. On the contrary, the binding potencies of DGoFSH (1.17-1.36, 95% C.L.) and DGhCG (2.29-4.80, 95% C.L.) to their respective receptors was enhanced. The specificity of this reaction was demonstrated by the inability of DGoFSH and DGhCG to respectively displace ^{125}I-hCG and ^{125}I-hFSH, except at very high (nonphysiological) concentrations. Although deglycosylation of oLH enhanced its potency in the rat LH radioreceptor assay, DGoLH displayed only a slight enhancement of activity in the human LH radioreceptor assay. Radiolabeled ^{125}I-DGhCG was specifically bound by the human and rat testes (data not shown). This confirms the above observations that deglycosylation does not impair the hormone receptor interaction. This is the first demonstration that deglycosylation does not change the species specificity requirement of the receptor.

4.3.6 Antagonistic effect of deglycosylated FSH on FSH-stimulated adenylate cyclase activity

The human testicular adenylate cyclase enzyme can be equally activated by either 1 µg of the nonprimate hormones, ovine and equine FSH, or the primate hormone, human FSH. As illustrated in Figure 53A, oFSH and eFSH gave identical dose response curves for stimulation of adenylate cyclase activity in man. Low, but significant stimulation was obtained with DGoFSH. However, addition of an excess of DGoFSH (5 µg DGoFSH) was ineffective in stimulation of adenylate cyclase activity to levels obtained with an optimal dose of native FSH (500 ng) in the human (Fig. 53A) or rat testes (Fig. 53B). DGoFSH was a potent antagonist of the action of the native hormone. Addition of DGoFSH led to a dose dependent inhibition of FSH stimulated adenylate cyclase activity in the human or rat testes (Fig. 53). At a concentration of 1 µg DGoFSH, the effects of a saturating dose of oFSH (1 µg) were completely abolished. This inhibition was specific as demonstrated by the absence of any effect of DGhCG (1 µg) or propranolol (100 µM), a catecholamine inhibitor, on FSH stimulated adenylate cyclase activity (Table 19).

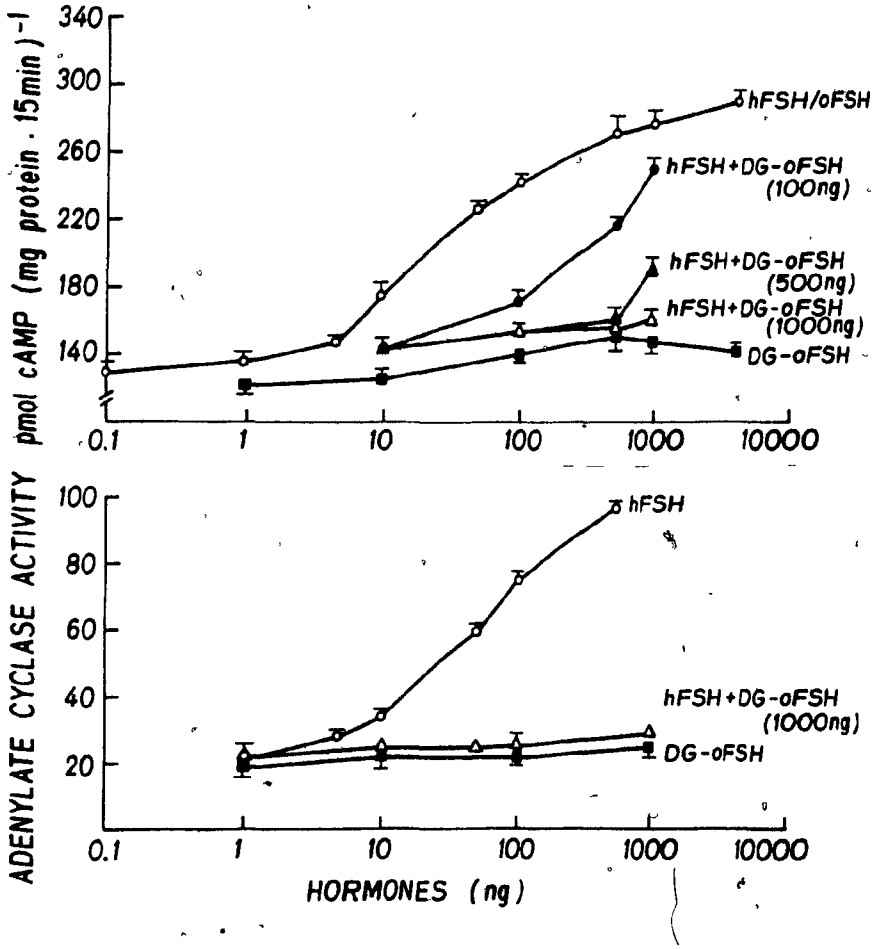


FIG. 53

Inhibition of FSH stimulated adenylate cyclase activity by various concentrations of its deglycosylated derivative (DGoFSH) in the human (A: top panel) and rat (B: bottom panel) testes. Testicular tissue was obtained from an adult human (20 yr) and rat (34 days). Activity was determined in the presence of FSH (o-o), and DGoFSH (■-■) alone or in various combinations. Basal enzyme activities were 130.81 ± 0.84 and 23.14 ± 2.49 pmol cAMP (mg protein - 15 min)$^{-1}$ for the human and rat respectively.

TABLE 19

EFFECT OF VARIOUS AGENTS ON BASAL AND FSH STIMULATED ADENYLATE CYCLASE ACTIVITY OF THE ADULT HUMAN TESTES (20 YR)

Addition	Adenylate Cyclase Activity pmol cAMP (mg protein · 15 min)$^{-1}$	
	None	hFSH (167 nM)[a]
None	82.63 ± 2.91	171.81 ± 1.65
DGoFSH (208 nM)	95.89 ± 3.95	100.33 ± 3.16
DGhCG (184 nM)	75.10 ± 1.47	168.60 ± 3.49[c]
hCG (136 nM)	90.26 ± 0.92	176.03 ± 2.03[c]
PRO (100 μm)	76.95 ± 2.11[b]	169.26 ± 6.72[c]
NaF (1 mM)	327.64 ±10.83	396.30 ± 6.66[d]
NaF (10 mM)	1055.93 ±51.22	1028.20 ±20.00
FSK (1 μM)	281.75 ±12.86	392.02 ± 6.74[d]
FSK (50 μM)	739.78 ±20.56	856.75 ±30.23[d]
NaF (10 mM) + FSK (50 μM)	2181.63 ±42.76	—

[a] Hormone and the compounds to be tested were added together to the membranes. Values represent the mean ± SEM of triplicate determinations of one of three experiments. Adenylate cyclase activity was determined in the presence of 10 μM GTP.

[b] Denotes not significantly different from basal value.

[c] Denotes not significantly different from FSH stimulation.

[d] Denotes addition + FSH value is significantly different from addition + basal value.

DGoFSH = Deglycosylated ovine FSH (MW = 24,000); DGhCG = Deglycosylated hCG (MW = 27,200); PRO = Propranolol; NaF = Sodium fluoride; FSK = Forskolin.

4.3.7 Effect of sodium fluoride and forskolin on adenylate cyclase activity

Forskolin (FSK) and sodium fluoride (NaF) stimulated the human testicular adenylate cyclase activity 8 and 12 fold respectively, in a concentration dependent manner (Fig. 54). Maximal stimulation of adenylate cyclase activity occurred in the presence of either 10 µM forskolin or 5-10 mM sodium fluoride. Forskolin was more potent, with an EC_{50} value of 2.5 µM compared to an EC_{50} value of 2.0 mM for sodium fluoride. In the testes, as in other tissues (285), GTP suppressed forskolin and sodium fluoride stimulation of adenylate cyclase. Forskolin has been shown to directly potentiate hormonal stimulation of adenylate cyclase in a number of tissues (285, 286). Therefore, it was of interest to study their effects in the human testis. The hormonal response of the testicular adenylate cyclase to a saturating dose of hFSH (1 µg) was augmented by forskolin. However, sodium fluoride only augmented the FSH stimulated adenylate cyclase activity when given as a subsaturating dose. Combination of an optimal saturating dose of forskolin (50 µM) and sodium fluoride (10 mM) resulted in a doubling of adenylate cyclase activity (Table 19). This suggests that these two agents stimulate adenylate cyclase by distinct mechanisms.

Forskolin, which can activate adenylate cyclase by a receptor independent mechanism (285) stimulated adenylate cyclase activity in the gonadotropin unresponsive tissues. Thus the unresponsiveness to gonadotropins by these tissues was unlikely due to enzyme inactivation, but most probably caused by an uncoupling of the catalytic subunit of adenylate cyclase.

4.3.8 Effect of steroids on the binding of ^{125}I-hCG and ^{125}I-hFSH to the human testes

The ability of several estrogenic compounds (Fig. 55) to directly interfere with the gonadotropin-receptor interaction was investigated by incubating the adult human testicular fraction P1 with either ^{125}I-labeled hCG or hFSH in the presence of these estrogenic compounds.

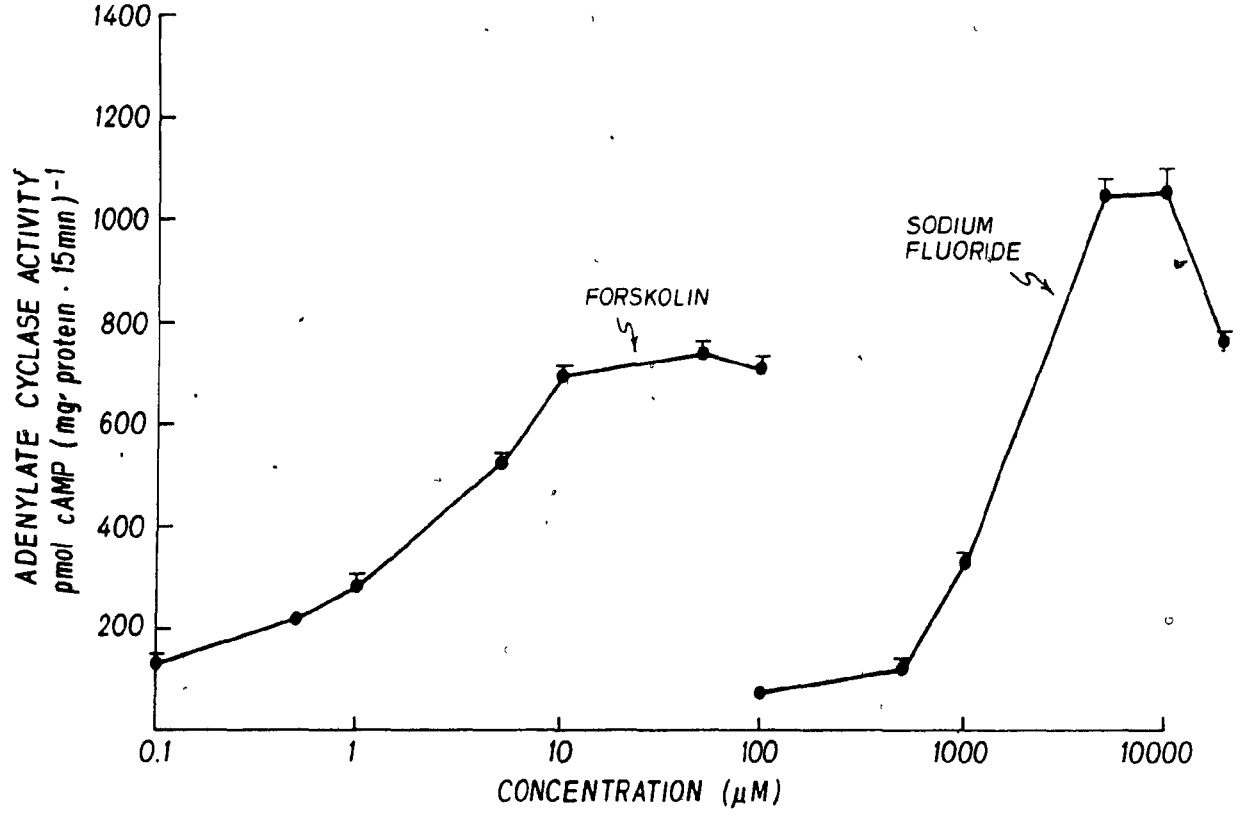


FIG. 54

Dose dependent stimulation of human testicular (20 yr) adenylate cyclase by forskolin and sodium fluoride. Basal enzyme activity was 7.83 ± 0.28 pmol cAMP (mg protein · 15 min)$^{-1}$.

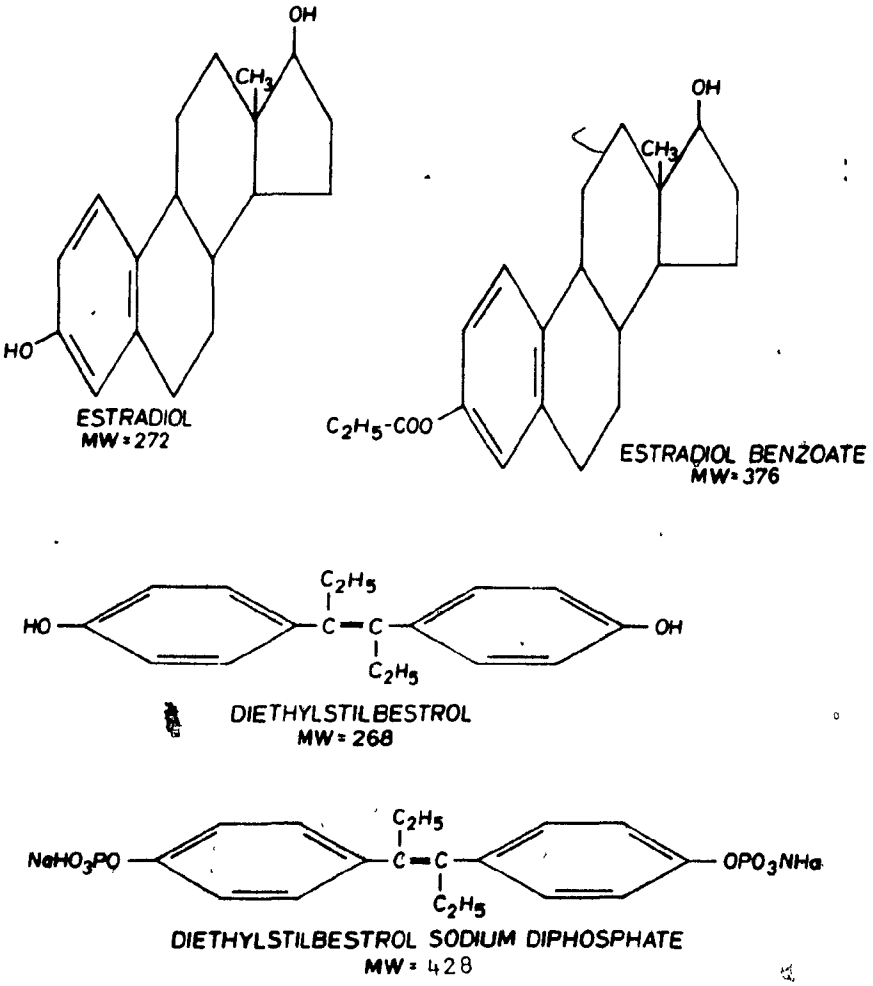


FIG. 55

Structure of the estrogenic compounds used in this study.

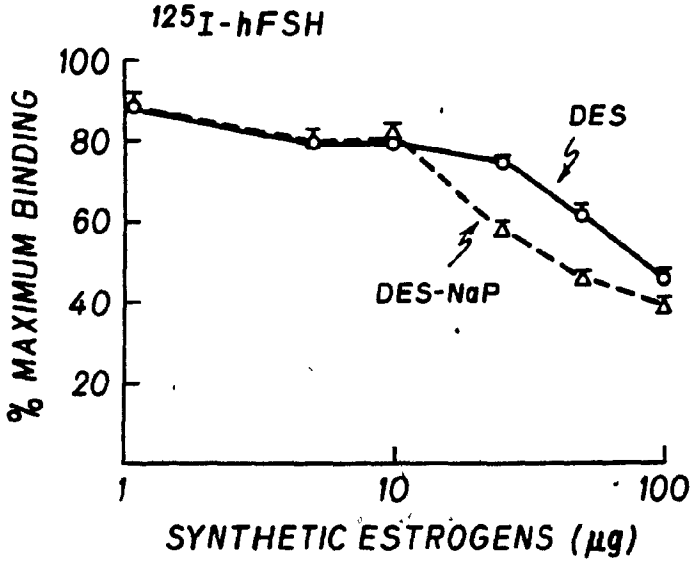
Among the different compounds tested only the synthetic estrogen diethyl stilbestrol (DES) and its water soluble clinically used derivative, diethyl stilbestrol sodium phosphate (DES-NaP) significantly interfered with the binding of the labeled gonadotropins (Fig. 56). FSH binding to the human receptor was affected to a greater degree than the ^{125}I-hCG receptor interaction (60% vs 30% inhibition) suggesting a preferential inhibitory effect of the synthetic estrogens. Of the two synthetic estrogens, DES-NaP was the most potent. For example, a concentration of 364 µM DESNaP was as effective as a much larger concentration of DES (1193 µM) in inhibiting ^{125}I-hFSH binding by 50%. The steroids testosterone (T), androstendione (A), and progesterone (P) were without effect at a concentration of 400 µg/ml. Preincubation of the human FSH receptor with DES and DES-NaP, followed by washing, prevented subsequent binding of the radiolabeled hFSH, indicating a strong effect which is not reversed by the washing procedure (Fig. 57). Since DES-NaP is soluble in aqueous buffers, the loosely bound component would be effectively removed by washing. The water solubility of DES-NaP, and the negligible effects of ethanol (1.2% ethanol) on gonadotropin binding confirms that the interference of binding is due to the synthetic estrogens and not ethanol. The greater inhibition observed when the human FSH receptor was preincubated with these synthetic estrogens suggests an effective alteration of gonadotropin binding sites on the membranes.

4.3.9 Interference of hFSH stimulation of adenylyl cyclase by synthetic estrogens

In agreement with their ability to interfere with hFSH binding, DES and DES-NaP competitively inhibited adenylate cyclase activity of the human and rat testes (Fig. 58, Tables 20 and 21). At high concentrations (greater than 100 µg) both synthetic estrogens were capable of causing almost complete suppression of basal and FSH stimulated adenylate cyclase activity. At lower concentrations (less than 10 µg) the FSH stimulated adenylate cyclase activity of the human testes appeared to be more sensitive to the effects of the synthetic estrogens. For example,

FIG. 56

Effect of DES and DES-NaP on ^{125}I-hFSH (A: top panel) and ^{125}I-hCG (B: bottom panel) binding to 100-200 µg of the human testicular fraction P1. The maximum binding (10% specific binding) to testicular tissue from adults (20-60 yr) was set as 100%. Data represents the mean ± SEM of triplicate determinations of at least three separate experiments.


125I-hFSH
100
80
60
40
20
% MAXIMUM BINDING
DES
DES-NaP
1
10
100
SYNTHETIC ESTROGENS (µg)

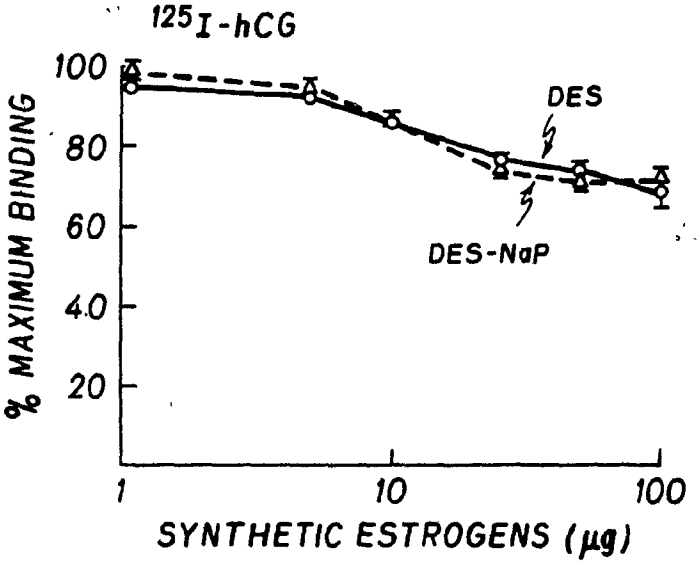

125I-hCG
100
80
60
40
20
% MAXIMUM BINDING
DES
DES-NaP
1
10
100
SYNTHETIC ESTROGENS (µg)

FIG. 57

Reversibility of the inhibitory effects of DES (A: top panel) and DES-NaP (B: bottom panel) on the binding of ^{125}I-hFSH to the human testicular fraction P1. The maximum binding (10% and 7% specific binding before and after washing respectively) to the human testes (20-60 yr) was set as 100%. Data represents the mean ± SEM of triplicate determinations of two separate experiments.

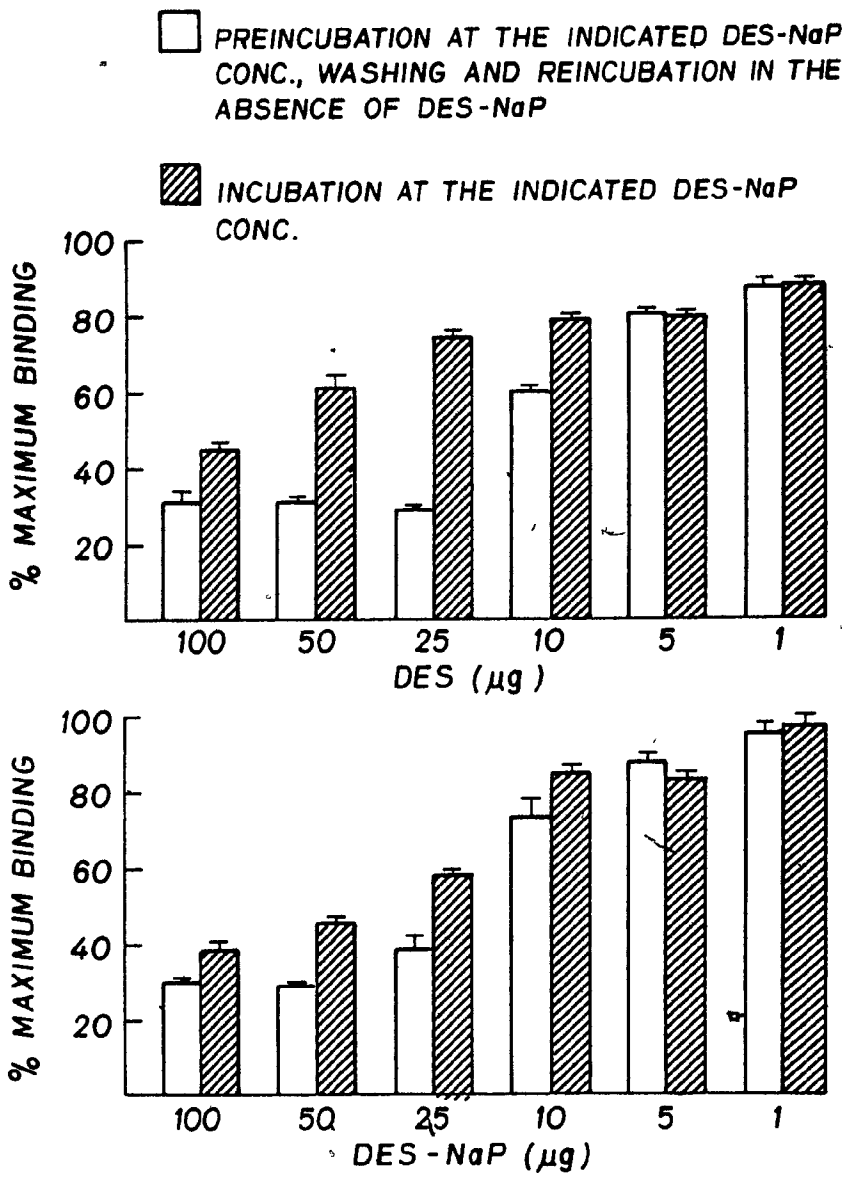

PREINCUBATION AT THE INDICATED DES-NaP CONC., WASHING AND REINCUBATION IN THE ABSENCE OF DES-NaP
INCUBATION AT THE INDICATED DES-NaP CONC.
% MAXIMUM BINDING
100
80
60
40
20
100
50
25
10
5
1
DES (µg)
% MAXIMUM BINDING
100
80
60
40
20
100
50
25
10
5
1
DES-NaP (µg)

FIG. 58

Influence of varying concentrations of DES-NaP on the adenylate cyclase activity of the human (A: top panel) and rat (B: bottom panel) testes. Adenylate cyclase activity in the human (69 yr) and rat (34 days) testicular fraction P1 was assessed in the presence (solid line) or absence (dashed line) of 1 µg of hFSH. Basal enzyme activity of the human and rat testes were 114.0 ± 2.0 and 10.2 ± 0.9 pmol . cAMP (mg protein · 15 min)$^{-1}$ respectively. The FSH stimulated adenylate cyclase activity of the human and rat testes were 214.0 ± 4.8 and 37.3 ± 0.3 pmol cAMP (mg protein · 15 min)$^{-1}$ respectively.

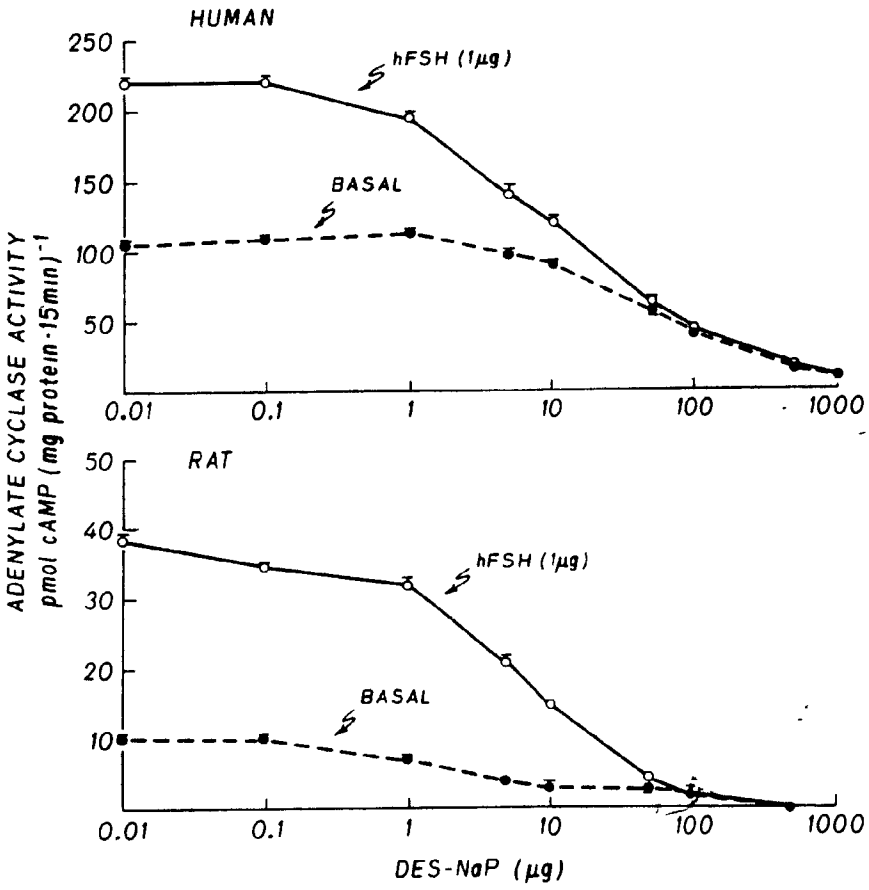

HUMAN
hFSH (1µg)
BASAL
250
200
150
100
50
0.01
0.1
1
10
100
1000
RAT
hFSH (1µg)
BASAL
50
40
30
20
10
0.01
0.1
1
10
100
1000
ADENYLATE CYCLASE ACTIVITY
pmol cAMP (mg protein·15min)⁻¹
DES-NaP (µg)

TABLE 20

INFLUENCE OF ESTROGENIC COMPOUNDS ON BASAL AND FSH STIMULATED HUMAN TESTICULAR ADENYLATE CYCLASE ACTIVITY

Addition	Concentration (μg)	% basal activity	%hFSH activity	% relative stimulation
None	-	100.00	100.00	100.00
DES-NaP	0.1	99.24±2.02	98.26±1.42	97.48±2.52
	1.0	99.17±1.15	82.95±3.80	82.55±3.86
	5.0	86.09±4.87	59.87±5.19	67.96±3.12
	10.0	80.69±2.15	53.97±1.58	65.89±0.48
	50.0	47.13±1.85	29.04±2.07	60.73±3.04
	100.0	36.47±0.25	21.65±1.21	58.06±4.06
	500.0	14.68±2.84	6.76±0.80	56.98±5.27
	1000.0	8.34±3.12	5.25±2.65	60.50±5.34
DES	0.1	95.50±1.73	93.51±4.48	92.86±2.37
	1.0	92.44±2.68	70.87±3.03	68.10±3.42
	10.0	83.51±1.35	40.59±4.77	55.24±3.15
	50.0	39.37±4.77	22.51±3.46	65.06±4.86
	100.0	11.33±4.31	12.64±4.63	N.D.
E2	0.1	98.44±1.49	93.33±1.30	92.45±5.18
	1.0	93.95±0.65	99.72±0.52	105.63±1.57
	10.0	93.90±1.48	82.95±6.58	98.17±5.26
	100.0	92.57±1.52	89.10±0.93	95.85±1.30
E2β	0.1	99.66±0.35	102.65±2.33	105.54±1.73
	1.0	100.09±1.74	97.48±0.75	94.55±3.30
	10.0	92.38±7.44	97.00±4.12	95.76±3.62
	50.0	91.59±3.61	98.36±3.92	107.88±5.47

Des-NaP = Diethyl stilbestrol sodium phosphate
Des = Diethyl stilbestrol
E2 = Estradiol-17β
E2β = Estradiol benzoate
N.D. = Not determined

Adenylate cyclase activity was assessed in 50-100 μg fraction P1 obtained from the human testes (27-70 yr). The basal adenylate cyclase activity, hFSH (1 μg) stimulated adenylate cyclase activity and percent relative stimulation were set as 100% for comparison. Data represents the mean ± SEM of triplicate determinations of four separate experiments.

TABLE 21

EFFECT OF ESTROGENIC COMPOUNDS ON BASAL AND FSH STIMULATED RAT TESTICULAR ADENYLATE CYCLASE ACTIVITY

Addition	Concentration (μg)	% basal activity	% hFSH activity	% relative stimulation
None	—	100.00	100.00	100.00
Des-Nap	0.1	100.10±2.75	93.01±2.51	93.15±4.67
	1.0	68.85±5.98	86.17±3.56	125.14±6.06
	10.0	38.59±3.74	40.57±3.33	144.26±4.41
	100.0	12.74±2.75	6.06±2.73	43.39±4.43
DES	100.0	14.07±5.01	7.34±2.19	52.16±5.47
E2	100.0	98.79±2.15	75.50±6.54	76.51±4.74
E2β	50.0	104.20±3.89	95.19±3.65	91.38±5.69

DES-NaP = Diethyl stilbestrol sodium phosphate

DES = Diethyl stilbestrol

E2 = Estradiol-17β

E2β = Estradiol benzoate

Adenylate cyclase activity was assessed in the rat testes (34 days). The basal adenylate cyclase activity, hFSH (1 μg) stimulated adenylate cyclase activity and percent relative stimulation were set as 100% for comparison. Data reprsents the mean ± SEM of triplicate determinations of two separate experiments.

5 µg DES-NaP caused a pronounced decrease (approximately 40%) in hFSH stimulated adenylate cyclase activity with only a slight inhibition (about 15%) of basal enzyme activity (Table 20). DES-NaP was a more potent inhibitor of adenylate cyclase activity than DES in the human testes. Whereas the percent relative stimulation of the human testicular adenylate cyclase declined with increasing DES-NaP concentration, a significant increase in the percent relative stimulation of the rat adenylate cyclase activity was observed at 1-10 µg DES-NaP. This increase was due to a greater decline in basal than FSH stimulated adenylate cyclase activity (Table 21). Whether this result was peculiar to this experiment or represents a difference between these two species awaits confirmation of the data. It should be noted that ethanol itself at high concentrations (greater than 1.5%) leads to a stimulation of both basal and FSH stimulated adenylate cyclase activity. This necessitated the addition of appropriate experimental controls when using ethanolic solutions. The steroids E2, E2β, T, A and P were less active than the synthetic estrogens and only demonstrated inhibitory activity at supraphysiological concentrations.

4.4 Discussion

The present study demonstrates the existence of a functional gonadotropin receptor coupled to the enzyme adenylate cyclase in the human testes. The hormones human, equine and ovine FSH were all capable of binding to the human testicular FSH receptor and activating the enzyme adenylate cyclase indicating that the human testes is biologically responsive to primate and nonprimate hormones. However, a disparity was observed between FSH binding to its receptor and FSH activation of adenylate cyclase activity. Consistent with the spare receptor theory (138), maximal activation of adenylate cyclase occurred with low hormone binding. It is not known if the failure of additional FSH binding to increase FSH stimulated adenylate cyclase activity is due to uncoupled receptor-cyclase units or a consequence of having attained the full (maximal) biological response.

Although hLH and hCG were bound well by the human testes, as evidenced by significant binding of ^{125}I-labeled hCG and hLH and the presence of specific binding sites, they were poor stimulators of adenylate cyclase. In contrast to the two fold stimulation seen with FSH, the response to hCG was low and inconsistent. In all but two of the 29 gonadotropin-adenylate cyclase responsive tissues, FSH was the more potent stimulator of adenylate cyclase activity. Whether the increased FSH responsiveness is due to the greater number of FSH receptors, the higher proportion of Sertoli cells or the greater susceptibility of the Leydig cell adenylate cyclase enzyme to damage and/or inactivation is unknown. Such a disparity in the response of adenylate cyclase to LH and FSH is not unique to man (273), but has been reported in the rat testes and ovary (270, 287). In the present studies, the responsiveness of the adenylate cyclase enzyme to FSH stimulation was greater in the rat (3-4 times basal activity) as compared to the human (2 times basal activity). Huhtaniemi et al. (274) have also reported that hCG stimulated cyclic AMP production is greater in the rat than human testes suggesting a species related difference.

Gonadotropin stimulated adenylate cyclase activity has been shown to be age dependent in the testes and ovary of a number of laboratory animals (270, 271). The small number of gonadotropin-adenylate cyclase responsive tissues precluded a detailed study of the dependence of adenylate cyclase activity on changes in age. The FSH responsive adenylate cyclase activity was quite variable with enzyme activity being detected in the full range of tissues studied (20-70 yr). No age group appeared to be more responsive to gonadotropin stimulation. The wide fluctuations in adenylate cyclase responsiveness is probably a reflection of the clinical history of the patient. Damage of the enzyme during tissue preparation or the assay procedure is unlikely since all tissues were handled similarly and tissues assayed on the same day gave variable responses to gonadotropin stimulation.

Sodium fluoride and forskolin directly activate the adenylate cyclase system by a receptor independent mechanism (285, 288). The

ability of gonadotropin responsive and nonresponsive tissues to react identically to a saturating dose of forskolin suggests the presence of a functional catalytic subunit. The impairment in the gonadotropin unresponsive tissues could be due to an altered regulatory protein (N) or an uncoupling of the regulatory protein (N) and the catalytic subunit. Such an impairment is thought to account for the decreased adenylate cyclase activity of desensitized Leydig cells (289).

The human testicular adenylate cyclase enzyme shares many of the characteristics of the adenylate cyclase enzyme of the nonprimate testes (290, 291) and other tissues (292, 293). The enzyme activity was dependent on temperature, substrate concentration and metal ions. The particulate testicular adenylate cyclase activity is distinct from the soluble adenylate cyclase activity reported in the supernatant fraction of rat and human testicular extracts (294, 295). The soluble enzyme being different with respect to its dependence on Mn^{+2} and its unresponsiveness to gonadotropic hormones. In the present studies GTP or GMP-P(NH)P increased basal and FSH-stimulated adenylate cyclase activity of the human testicular subcellular fraction P1 but did not change the degree of stimulation. A similar phenomenon was observed utilizing testicular and corpora luteal tissue of the rat or pig (270, 296, 297). In these systems, GTP could not enhance hormonal stimulation of adenylyl cyclase but did stimulate the catalytic efficiency of the system. Utilizing immature rat testicular extracts, Reichert et al. (298) reported that GMP-P(NH)P augmented FSH stimulation of adenylate cyclase activity. This discrepancy in results from the findings of other investigators could be due to differences in membrane preparations, variations in assay techniques or better coupling in their system. The poor responsiveness of the human adenylate cyclase system to GTP or its analogue may be due to the presence of saturating levels of endogenous GTP in the human testes.

Modification of the carbohydrate portion of the gonadotropins hCG and oFSH enhanced their binding to the human testes. Previous studies have shown that deglycosylation of gonadotropic hormones does not impair

subunit interaction or receptor binding to testicular or ovarian tissues (26, 299-302). Removal of 80% of the carbohydrate residues from these gonadotropins, without damage or modification of the protein moiety, did not change the hormone specificity exhibited by their respective receptors. The human LH receptor, with its marked preference for primate hormones provided us with a unique opportunity to assess the role of the carbohydrate moiety in determining its species specificity. Using oLH, we found that removal of most of its carbohydrate moieties did not alter the species selectivity of the human LH receptor. These results suggest that specificity is determined not only by the hormone but by the receptor as well. The protein backbone and/or the innermost carbohydrate moieties of the beta chain are the factors which most likely determine hormone specificity.

Although deglycosylation did not interfere with binding, a drastic change occurred in the ability of the deglycosylated derivatives to initiate the cellular response, i.e. activate adenylate cyclase. Deglycosylation of oFSH sharply decreased its ability to activate either the human or rat testicular adenylyl cyclase above basal values. This could explain the inability of DGoFSH and DGoLH to stimulate cyclic AMP production in isolated rat seminiferous tubules (303) and Leydig cells (35) respectively. Deglycosylated hCG similarly did not stimulate adenylate cyclase in the rat testes (304) or ovary (305). The inability of DGhCG to increase adenylate cyclase activity in the human testes was not due to a failure to bind to the tissue (data not shown) but likely due to a defect in coupling the receptor to adenylate cyclase. Deglycosylated oFSH was a potent and specific inhibitor of FSH stimulation of adenylate cyclase in both the human and rat. The data suggest that DGoFSH by blocking the FSH receptor sites can prevent FSH activation of adenylate cyclase.

It has long been recognized that administration of gonadal steroids in intact animals results in an inhibitory effect on the reproductive system of male animals and that the mammalian testis can synthesize

estrogens, albeit in smaller quantities than the ovary (306-308). At first estrogens were thought to exert their effects solely by suppression of gonadotropin secretion by the anterior pituitary (307-309). Several recent studies have now shown that estradiol administration to intact males could result in decreased serum testosterone levels by directly inhibiting several testicular enzymes in the steroidogenic pathway (310-312). However, very few studies have been carried out regarding the influence of estrogenic compounds on hormonal binding and hormonal activation of adenylyl cyclase.

This study demonstrates that structurally unrelated hormones, such as the synthetic estrogens DES and DES-NaP, can directly affect the specific interaction of LH and FSH with their respective human testicular receptors. It is possible that DES or DES-NaP may bind to membrane (receptor) components (313-315) and subsequently induce conformational changes resulting in decreased binding ability of the gonadotropin receptors and loss of basal and FSH stimulated adenylate cyclase activity. Reports of estrogen receptors in the human testes need to be confirmed (315-317). Preincubation of the human or nonprimate (219) gonadal membrane preparations with the synthetic estrogens, followed by washing, effectively blocked subsequent gonadotropin binding suggesting a direct effect on the human gonadotropin receptor. The synthetic estrogens might also hinder formation of stable hormone-receptor complexes (219). Although effective inhibitors of testosterone biosynthesis by rat (318, 319) or human (320, 321) Leydig cells, equivalent concentrations of E2 or E2β did not interfere with the gonadotropin receptor interaction or suppress adenylate cyclase activity of the subcellular P1 fraction of the rat or human testes. The suppressive effects of E2 and E2β thus appear to be distal to the activation of cyclic AMP formation. Alternatively, greater concentrations or longer term incubations might be required to observe any inhibitory effects. Exposure of gonadal tissues in vivo to E2 for longer periods (greater than 3 days) resulted in suppression of the hCG binding capacity and LH stimulated adenylate cyclase activity (322, 323). Kirchick et al. (323) suggest that both the loss of LH receptors and alteration in the number or structure of

the regulatory protein (N) contribute to the loss of gonadotropin stimulated adenylate cyclase activity.

It may be noted that pharmacological concentrations of the synthetic estrogens were required for inhibition of gonadotropin binding and adenylate cyclase activity (308). It is not known if high local concentrations of estrogens sufficient to affect testicular function could be achieved. Conclusions regarding the role of estrogens in the regulation of testicular cell function must take these into consideration. It remains to be conclusively established whether estrogens in man regulate testosterone production directly at the testes or indirectly at the hypothalamic-pituitary level.

CHAPTER 5

SUMMARY

5.1 Summary

The purpose of this investigation was to gain a better understanding of the molecular mechanisms involved in the action of the gonadotropic hormones. Utilizing testicular tissue obtained from the human and four nonhuman primate species, it was possible to identify and directly compare the properties of the testicular gonadotropin receptors of the various primate species. As discussed in the first two chapters the primate and nonprimate gonadotropin testicular receptors shared many biochemical features. In the last chapter the properties of the human gonadotropin-adenylate cyclase system were investigated. The data showed that at least in the case of the human FSH receptor, one is dealing with a functional receptor-adenylate cyclase unit.

5.2 Claims to Originality

1) Specific FSH binding sites (receptors) were identified in the primate testes. The characterization of both FSH and LH(hCG) receptors in the same tissues allowed direct comparison of the biochemical properties of the gonadotropin receptors. The use of homologous hormones in the study of binding characteristics of the human gonadotropin receptor makes the data physiologically relevant.
2) In the primate, age dependent changes in the testicular gonadotropin binding capacity and apparent binding affinity have been correlated.
3) Specific differences have been shown between primate and nonprimate gonadotropin testicular receptors with respect to their species specificity. In both the human and yellow baboon, the FSH receptor could recognize heterologous hormones but the LH receptor was highly specific to primate hormones.
4) Detection of an FSH soluble receptor-like component and a receptor binding inhibitory factor in the cytosolic fraction of testicular extracts of primate as well as nonprimate species.
5) Characterization of an FSH responsive adenylate cyclase from human testes.
6) The carbohydrate portion of the gonadotropins is not essential for binding to the human testes. It is however important for the activation by FSH of the human and rat testicular adenylate cyclase. Deglycosylated FSH uncouples the FSH receptor-adenylate cyclase system providing reproductive endocrinologists with an effective and specific antagonist.
7) The synthetic estrogens, DES and DES-NaP, effectively interfered with gonadotropin binding and activation of adenylate cyclase. The use of high nonphysiological concentrations precludes it being a specific or physiologically relevant effect.

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