CHARACTERIZATION AND PHYSIOLOGICAL ROLE OF
17α,20β-DIHYDROXY-4-PREGNEN-3-ONE STEROID
RECEPTOR ACTIVITY IN THE CYTOSOLIC AND ZONA
RADIATA MEMBRANE FRACTION OF THE OVARIAN
FOLLICLES OF BROOK TROUT SALVELINUS FONTINALIS
DURING THE TERMINAL STAGES OF OOCYTE MATURATION

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Characterization and physiological role of 17α,20β-
dihydroxy-4-pregnen-3-one steroid receptor activity in the
cytosolic and zona radiata membrane fraction of the ovarian
follicles of brook trout _Salvelinus fontinalis_ during the
terminal stages of oocyte maturation.

BY

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A thesis submitted to the school of Graduate Studies in
partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry
Memorial University of Newfoundland
September, 1989

St. John's, Newfoundland

Good thoughts,

good words,

and good deeds.

zartushtra 5th century B.C.

This thesis is dedicated in memory of the Late Professor
Catherine O. Hebb (Institute of Animal Physiology, Babraham,
Cambridge. U.K.), a noted Canadian neuro-physiologist, whose
initial encouragement in the 1960's helped the author to
pursue university education. Also I would like to dedicate
this thesis to my late mother, father and my aunt Peroja
Mehta, for all they did for me.

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INTRODUCTION

In the female salmonoid fish, the ontogenetic development of
gametes (oocytes) occurs in the ovary through the process of
maturation or meiosis, as a result of which the oocytes
lose half of their chromosomes and become haploid. The
initial step in production of gametes is by proliferation of
oogonia by mitosis which gives rise to several hundred
oocytes in the ovary (Fig. 1). The period of growth of the
ovarian follicle is prolonged and the increase in size is
considerable. Growth takes place over a period of two to
three years depending on various factors with the major one
being availability of food. Many embryologists distinguish
two periods of oocyte development: the previtellogenesis
period and the vitellogenesis period. Growth is accelerated
during the vitellogenesis period, during which yolk is
deposited in the oocyte. Simultaneously with the growth of
the oocyte its nucleus (germinal vesicle) enters into
prophase 1 of meiotic division; the homologous chromosomes
pair together, but subsequent stages of meiosis are
postponed until the end of the period of vitellogenic
growth. The next phase of oogenesis involves the resumption
of meiosis or terminal oocyte maturation. This process is
accompanied by breakdown of the germinal vesicle(GVBD),

Fig. 1. A female brook trout *Salvalinus fontinalis* almost ready to spawn (stage 5) ovarian follicles, fork length 235 mm.

chromosomal condensation and extrusion of the first polar body. These changes occur prior to ovulation and are prerequisites for successful fertilization. The period between prophase 1 arrest and extrusion of the first polar body is called the period of maturation, and this process is under endocrine control in salmonids.

Three factors are involved in the endocrine control of the terminal maturation in salmonids (Fig. 2). The pituitary gonadotropin stimulates the synthesis of maturation inducing steroid (MIS) $17\alpha,20\beta$-dihydroxy-4-pregnen-3-one ($17\alpha,20\beta$-DHP) by granulosa cells of the ovarian follicle, the $17\alpha,20\beta$-DHP then interacts with the oocyte to bring about activation of maturation promoting factor (MPF) in the ooplasm which leads to GVBD (for review see Nagahama, 1987, 1988). During the period of terminal maturation the germinal vesicle (GV) migrates from the centre to the periphery of the oocyte. Seven stages of maturation are characterized according to the position of GV in the oocyte (Table 1). These stages have been used in conjunction with plasma levels of $17\alpha,20\beta$-DHP in salmonids (So, et al. 1976).

The MIS $17\alpha,20\beta$-DHP was first isolated and charcterized by Idler and co-workers from plasma of Sockeye salmon (Idler, et al .1960) and Atlantic salmon (Schmidt and Idler, 1962). Terminal oocyte maturation in vivo is accelerated

TABLE 1

Various stages of oocyte during terminal

maturation in salmonids.*

Stage Oocyte	Position of germinal vesicle (GV)
1	GV central
2	GV slightly off centre
3	GV midway between centre and periphery
4	GV peripheral
5	GV breakdown (GVBD)
6	Ovulated oocytes with oil droplets peripherally
7	Oocyte clear and translucent and free in body cavity

*As described by Ng and Idler (1978)

Fig. 2. Three major mediators of oocyte maturation in salmonids, 1) gonadotropin, 2) maturation inducing hormone and 3) maturation promoting factor (diagram from Nagahama, Y. 1987. Develop. Growth and Differ. 29, 1-12).

Fig. 2.

Pituitary
↓ *Gonadotropin*
Receptor
Follice layer
↓ *Maturation-inducing hormone*
Receptor
Maturation-promoting factor
GV
Oocyte maturation

Ovary
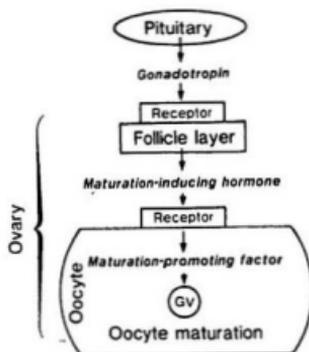
Oocyte

by 17α,20β-DHP in trout, carp, and coho salmon (Jalabert, et al [28] .1976, 1977, 1978). In vitro 17α,20β-DHP is the most effective steroid in stimulating GVBD in goldfish, rainbow trout, northern pike (Jalabert, 1976), brook trout and yellow perch oocytes (Goetz and Bergman, 1978; Goetz and Theofan, 1979). The highest plasma concentrations of 17α,20β-DHP occur in blood immediately before and/or around ovulation in salmonids and in goldfish (Campbell, et al . 1980; Fostier, et al . 1981; Scott and Baynes, 1982; Scott, et al . 1982; Wright and Hunt, 1982; Kagawa, et al . 1983; Stacey, et al . 1983; Nagahama, 1987 a and b). In a recent study on landlocked Atlantic salmon, 17α,20β-DHP plasma levels increased progressively from oocyte stages 1 to 6, with the glucuronide predominating in stages 1-4 and the free steroid in stages 5-7 (So, et al .1985). Since in vertebrates it is generally assumed that GVBD marks the resumption of meiosis (prophase 1 to metaphase 2), it is probable that 17α,20β- DHP in salmonids reinitiates meiosis at stage 5, when free plasma 17α,20β-DHP levels are the highest.

After establishing the functional role of 17α,20β-DHP in salmonoids, Jalabert, (1976) proposed its action via oocyte receptors, although the author questioned the mechanism of its action (Fig. 3), since steroid-induced GVBD is blocked by inhibitors of translation but not transcription

Fig. 3. Tentative scheme for the control of oocyte
maturation in trout (Jalabert, B. 1976. J. Fish. Res. Board
Can. 33: 974-988.). Jalabert questioned the presence of
classical steroid oocyte receptors, because maturation
(GVBD) was not blocked by inhibitors of transcription.

Fig. 3.

indicating that the mechanism of action involved in the steroid maturation of oocytes (GVBD) has a special characteristic which is different from the classical steroid mechanism of action via nuclei (Detlaff and Skoblina, 1969; Goswami and Sundararaj, 1973; Jalabert, 1976; Theojan and Goetz, 1981; DeManno and Goetz, 1986,1987). The classical steroid-receptor mechanism of action involves the entry of steroid into the cell cytoplasm by passive diffusion (Muller and Wotiz, 1979; Peck, _et_ _al_. 1973; Giorgi, 1980), and its binding with cytosol receptors causing transformation of the receptor. The transformed receptor-steroid complex then enters the nucleus and interacts with the acceptor site on the DNA which leads to transcription and processing of mRNA thereby causing translation of proteins related to the functional role of the steroid in the cytoplasm (Fig. 4).

Nagahama and Kishimoto, (1987) have shown that incubation of goldfish oocytes with $17\alpha,20\beta$-DHP results in GVBD, while the injection of the steroid into the oocyte does not result in GVBD. These results suggests that the action of $17\alpha,20\beta$-DHP is on the plasma membrane of the oocyte.

In amphibian _Xenopus_ _laevis_ oocytes, progesterone binds to an oocyte membrane receptor protein (Sadler, _et_ _al_. 1985; Blondeau and Baulieu, 1984; Sadler and Maller, 1982; Ishikawa, _et_ _al_. 1977). One of the surprising features of

Fig. 4. A classical model of steroid hormone action (diagram from Clark, et al. 1986. In "Laboratory Methods Manual for Hormone Action and Molecular Endocrinology" W. T. Schrader and B. W. O'Mally, eds., 10th ed., Chap.1. pp 1-55).

Fig. 4.
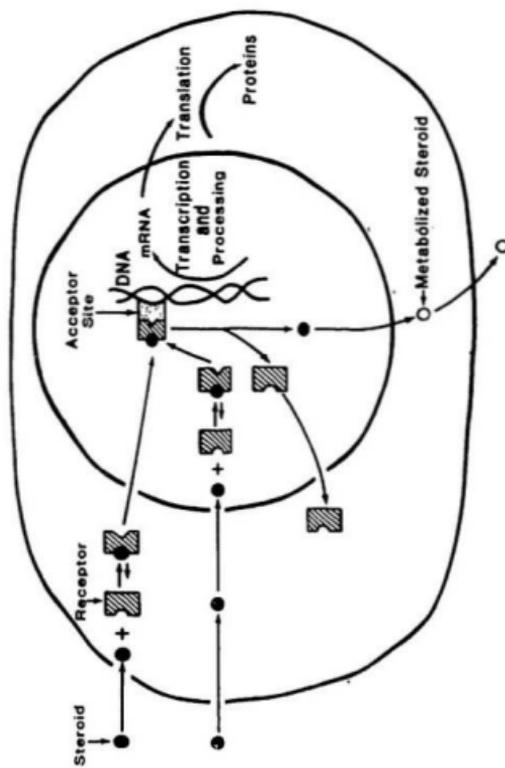
amphibian oocyte maturation in <u>Xenopus</u> has been the wide
variety of steroids and membrane active drugs that are
capable of stimulating the process. Also conventional
steroid receptor isolation methods have not revealed the
presence of a classical cytosol receptor in <u>Xenopus</u> . On the
other hand Morril and collaborators have reported high
affinity binding of progesterone in the cytosolic fraction
of <u>Rana</u> oocytes (Kalimi, <u>et al</u> . 1979).

In fish, recently Maneckjee, <u>et al</u> .(1987;1989 a and b)
reported $17\alpha,20\beta$-DHP receptor activity from oocytes. The
principal purpose of the present study was to establish the
presence of a target organ receptor for $17\alpha,20\beta$-DHP and to
begin to elucidate the mechanisms by which $17\alpha,20\beta$-DHP
brings about maturation.

Maturation inhibiting substances

In sexually matured mammals, oocyte maturation <u>in vivo</u>
occurs after a preovulatory surge of luteinizing hormone
(LH) or by administration of exogenous gonadotrophin (Gns)
(Tsafriri, 1978). Cell culture of the oocytes surrounded by
follicle cells do not spontaneously mature if the cells are
removed before the endogenous LH surge. Addition of LH
causes the follicles to initiate maturation (Linder, <u>et al</u>
. 1974); Masui and Clark, 1983). However, when

oocyte-cummulus cell complexes (oocyte + outer cummulus follicles) are removed from antral follicles and kept in culture medium, they spontaneously mature without addition of Gns or other stimulating agents (Edwards, 1965; Pincus and Enzmann, 1935). These results suggest that some component of the antral follicle inhibits oocyte maturation until that inhibition is overcome by the action of Gns. Follicular fluid of several mammalian species contains a meiotic-arresting substance called oocyte maturation inhibitor (OMI). Porcine OMI, a polypeptide, has been isolated and purified from granulosa cells with molecular weight < 2000 (Tsafriri, et al . 1976). OMI activity from mature human Graafian follicles was found to be inhibitory to progesterone induced meiotic maturation in Xenopus oocytes (Cameron, et al . 1983). The mechanism of action of OMI is not known, although it has been reported that follicular fluid from mature fertilizable human follicles contains significantly less OMI activity than similar fluid from immature oocytes (Channing, et al . 1983). Treatment of cumulus-enclosed and denuded mammalian oocytes with cAMP analogs or inhibitors of phosphodiesterase inhibited spontaneous maturation (Cho, et al .1974; Dekel and Beers,1978). Also Gns reversed the inhibitory action of dibutyryl cyclic AMP (dbcAMP), and 3-isobutryl-1 methylxanthine (IBMX) on the maturation of cumulus cell-enclosed rat oocytes but not the denuded oocytes (Dekel

and Beers, 1978, 1980). Thus meiotic arrest is maintained by
the passage of cAMP from the cumulus cells into the oocytes
through gap junctions, and that Gns trigger maturation by
physical disruption of intracellular communication between
cumulus cells and the oocyte (Gilula, et al .1978; Dekel
and Beers, 1980).

Recent findings of Eppig, et al .(1983), are at variance
with cAMP itself being OMI, since elevation of cAMP levels
in the cumulus cell-oocyte combination led to inhibition of
maturation while increased cAMP levels in denuded oocytes
had no effect. Furthermore sub-optimal doses of dbcAMP
caused greater inhibition of maturation on cumulus-cell
oocytes than on the denuded oocytes preparation. These
results suggest that a release of secondary factors from
cumulus-cell occured into the oocytes via cAMP interaction
(Downs and Eppig, 1985: Eppig and Downs, 1984). The
principal inhibitory component in porcine follicular fluid
and serum has been purified and characterized to be
hypoxanthine (Downs, et al .1985). These results are
different from earlier work of Tsafriri, et al . (1976), as
described above.

In a teleost fish Fundulus heteroclitus oocytes Greely,
et al .(1987), reported that the oocyte can resume meiosis
in the absence of their enveloping follicle cells and

exogenously supplied 17α,20β-DHP. The authors concluded that
the follicle cells of <u>Fundulus heteroclitus</u> may be
producing a substance that directly inhibits oocyte
maturation, or that the association of follicle cells and
oocyte may somehow contribute indirectly to the maintenance
of meiotic arrest.

Iwamatsu and Takama, (1980) demonstrated the presence of a
factor in rabbit serum which inhibits gonadotropin and 17α-
,20β-DHP induced maturation in follicle-enclosed medaka
oocytes. Similar activity was also confirmed in sera from
chicken and lizard as well as in several mammalian species
(Iwamatsu, 1981). It was later demonstrated that sera factor
also exhibited maturational activity when whole ovarian
follicles were exposed to the sera for 9-12 h and then
incubated in serum free medium for an additional 12 h
(Iwamatsu, 1983).

Certain steroid hormones also have been reported to block
hormone-induced oocyte maturation <u>in vitro</u> . In rainbow
trout, estradiol was shown to prevent gonadotrophin induced
maturation, but not 17α,20β-DHP induced maturation
(Jalabert, 1975). Further research demonstrated that the
inhibitory effect of estradiol was due to decreased
synthesis of 17α,20β-DHP in the follicular layer (Jalabert
and Fostier, 1984).

CHAPTER 1

Isolation of receptor activity for maturation inducing steroid 17α,20β-DHP

Most endocrinologist will agree with the principle that all steroid hormones act via specific receptors present in the target organs. Thus isolation and characterization of 17α,20β-DHP receptors from oocytes was the first step in the process of elucidating the mechanism of action of MIS 17α,20β-DHP. The ovarian follicle in salmonids consists of three layers of cells (Fig.1.1). A two cell model for synthesis of MIS 17α,20β-DHP has been demonstrated (Nagahama, 1987 a and b). Outer thecal cells synthesize the precursor steroid 17α-hydroxy progesterone in response to gonadotropin action, that traverses the basal lamina and is converted to MIS 17α,20β-DHP by the granulosa cell layer where gonadotropin acts to enhance the activity of 20β-hydroxy steroid dehydrogenase (Fig. 1.2). The MIS 17α,20β-DHP is then released directly on to the zona radiata membrane surrounding the oocyte, and the plasma membrane of the oocyte is intercalated with the zona radiata.

Various methods are used in the isolation

Fig. 1.1. Cross-section of stage 1 ovarian follicle from
brook trout shown diagramatically. Stepwise removal of outer
follicular layers are shown from A to D. A) All follicular
layers present. B) Outer epithelium (OE) removed, exposing
thecal cell layer (TF). C) Thecal cell layer removed
exposing granulosa cells (FE). D) Denuded oocyte after
removal of granulosa cells. The outer epithelium (C) is
called the chorion or zona radiata, the plasma membrane of
the oocyte is intercalated into the zona radiata from the
inside part of the oocyte (modified diagram from Lessman, C.
A. et al . 1985. Can. J. Fish Aquat. Sci. 42: 2053-2058).

Fig. 1.1.
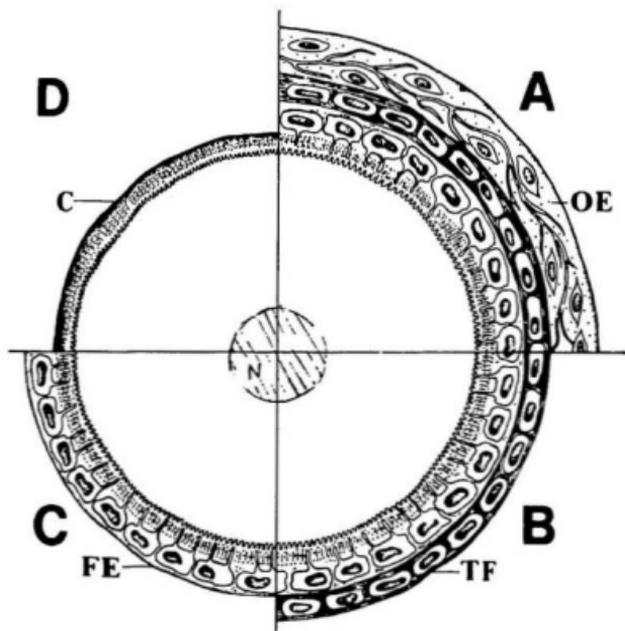
Fig. 1.2. Two-cell type model for the productiion of $17\alpha,20\beta$ DHP by salmonid ovarian follicles. 20β-HSD, 20β-hydroxy steroid dehydrogenase. Nagahama, Y. 1987. In Hormones and reproduction in Fishes, Amphibians and Reptiles. 171-193.(D.O. Norris and R.E. Jones, eds., Plenum Press, New York.).

Fig. 1.2.

OVARIAN FOLLICLE

Gonadotropin

THECA

GTH-receptor → cAMP

Cholesterol
↓
Pregnenolone
↓
17α-Hydroxyprogesterone

GRANULOSA

GTH-receptor

17α-Hydroxyprogesterone
↓ 20β-HSD ← cAMP
17α,20β-Dihydroxy-4-pregnen-3-one

OOCYTE
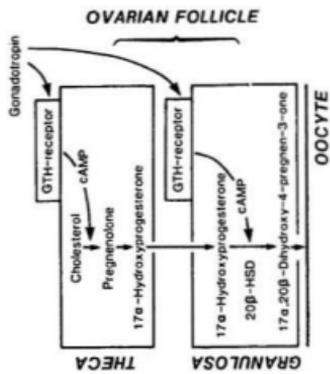
characterization of mammalian and fish cytosolic and nuclear
steroid receptors (Clark, et al . 1984; Sandor, et al .
1984; Callard and Mak, 1982; Alberts and Herrick, 1970).
These established methods were used with appropriate
modifications in order to isolate and characterize the
receptors for MIS $17\alpha,20\beta$-DHP.

Five criteria are used for characterization of steroid
receptors. 1) Finite binding capacity: this criterion is met
by demonstrating that the steroid binding system under study
can be saturated. This is seldom achieved completely with
fish steroid receptors due to lower affinity constant
(K_a) values, which leads to problems in the separation
of free from bound steroid. 2) High affinity: in mammalian
system steroid receptors possess high affinity, this is
expected because the circulating levels of steroid are
usually 10^{-10} to $10^{-8}M$ (Clark and Peck, 1979).
Thus the receptor must have affinity in the range of steroid
blood levels, otherwise the response would not occur;
although receptor interactions of low affinity are observed
if blood or tissue levels of steroids are high. 3) Steroid
specificity: receptors are expected to display high
affinities for the specific hormone or class of hormones to
avoid interference from other steroid hormones.
Nevertheless, receptor sites do not display absolute
steroid-specificity (Clark and Peck, 1984). 4) Tissue

specificity: since only specific cell types respond to given
hormones, and if the response is mediated _via_ receptors
then only the target organ responsive to steroid action
should possess receptors for that particular steroid. 5)
Binding correlated with biological response: the extent of
biological response should relate to some function of
receptor occupancy.

Cytosolic receptors

Cytosolic receptor isolation as described for somatic cells,
begins with homogenizing the minced target organ tissue
gently in low ionic strength buffer (to avoid breakdown of
nuclei). The low ionic strength buffer is used, because high
salt concentrations decrease receptor affinity (K_a) for
the steroid. Sodium molybdate is used in the isolation
buffer to stabilize the receptor-steroid complex from
transformation or activation. Sodium molybdate also keeps
the receptor in the aggregrated form, and acts as a protease
inhibitor. Also the MoO_4^{2-} ion is capable of
forming phosphomolybdate or sulphomolybdate complexes which
could prevent an irreversible loss of binding capacity. A
second component which has pronouced effects on the steroid
binding capacity of untransformed receptors is the reducing
environment. In the absence of a reducing environment (e.g.
DTT), the binding capacity is reversibly lost even in the

presence of the molybdate.

The nuclear pellet obtained after gentle hand homogenization
and centrifugation of the ovarian follicles showed that GV
of the oocyte had been disrupted. Although intact nuclei
from follicular cells were present. The GV of the oocyte is
larger than most somatic cells, and its disruption during
homogenization is thus difficult to avoid. Thus receptors
found in the cytosolic preparation can come from disrupted
GV (Callard and Callard, 1986). Although cytosolic
preparations obtained from the same batch of ovarian
follicles, when homogenized using gentle hand homogenizer,
or much more disruptive Polytron homogenization gave the
same amount of binding. Thus the receptor activity is
present in a very easily solubilized or in soluble form.
Nuclear receptors bound to the ligand steroid are found
associated with the chromatin in the nuclear pellet, and
thus are insoluble (Callard and Callard, 1986). It was
observed that dextran-charcoal treatment of stages 1-3
oocytes cytosolic preparations, did not increase its maximum
binding capacity (N_{max}) for the labelled steroid
ligands progesterone or $17\alpha,20\beta$-DHP, indicating that
receptor activity was present in the free form, or the
steroid exchange reaction was very fast during the binding
assay. Later studies indicated the absence of nuclear
receptor activity. Thus present evidence indicates that the

cytosolic receptor preparation consists of soluble form of
receptors most likely present in the cytoplasm of the
oocyte. The possibilty that receptor binding protein in the
cytosolic preparation from ovarian follicles is a blood
plasma steroid binding protein was also investigated. It was
demonstrated that the two proteins were different (see
section on blood plasma binding protein).

Steroid binding studies with cytosolic receptors

In the binding studies, the total amount of receptor
(R_t) is determined under equilibrium conditions by
adding steroid (S) to an aliquot of cytosolic receptor
preparation until saturation or near saturation is obtained.
The amount of bound ligand (R-S) which is observed in this
system is mathematically related to free ligand
concentration [S] by the equation :

$$[R-S] = \frac{[R_t][S]}{K_d + [S]}$$

Where K_d is the dissociation constant of the receptor
ligand complex. This equation is similar to the classical
Michaelis Menton equation and applies equally well to
studies of ligand binding as long as conditions of
equilibrium exist. From the saturation plot, the actual
point of saturation is equal to the number of receptor sites

(R_t). The dissociation constant (K_d) is the concentration of steroid at which 50% of the receptor sites (R_t) are bound. Although one can make reasonable estimates of (R_t) and (K_d) from saturation plot, these parameters can be obtained by 1) Scatchard analysis (Scatchard, 1949). In Scatchard plots specific bound/free hormone ratio is plotted as a function of bound steroid. The number of receptor sites (R_t) can be obtained directly from intercept on the x axis (bound steroid). The (K_d) can be calculated from the slope of the line or from intercept on the y axis. 2) K_d and R_t can also be obtained from double reciprocal plots of $1/[S]$ against $1/[R-S]$; the negative intercept on the $1/[S]$ axis equals – $1/K_d$, and intercept on the $1/[R-S]$ axis gives the value of R_t. 3) The affinity or equilibrium constant K_a for the receptor steroid complex is a function of the rate of association k_a and the rate of dissociation k_d of the steroid:

$$K_a \quad = \quad k_a / \, k_d$$

Thus by experimentally measuring k_a and k_d, value of K_a can be calculated. K_d is the reciprocal of K_a.

Experimental methods for cytosolic receptor assay

After equilibration of cytosolic receptors with labelled
steroid, the bound receptor-steroid complex separation from
free unbound steroid was attempted using various methods: 1)
charcoal-dextran absorption of free steroid (Koreman, et
al .1969; Mester, et al .1970; Sanborn, et al .1975;
Clark and Peck, 1984; Sandor, et al .1984. 2)
Hydroxy-apatite absorption of bound steroid-receptor complex
(Pavlik and Coulson, 1976; Peck, et al .1973; Waltor and
Clark, 1977). 3) Sephadex LH 20 gel filteration (Santi, et
al .1973). 4) Controlled pore glass bead absorption of
steroid- receptor complex (Clark and Gorski, 1969; Gormley,
et al .1985). 5) Ammonium sulphate/ calcium sulphate
precipitation of bound steroid-receptor complex (Sangalang
and Freeman, 1977,1987). 6) Protamine sulphate precipitation
of bound steroid-receptor complex (Steggles and King, 1970;
Chamness, et al .1975; Clark and Peck, 1984. 7) Equilibrium
dialysis, using teflon cells and low molecular weight
cut-off membranes (Spectrum Medical Industries, Los Angeles
USA.).

The charcoal-dextran separation assay described by Sandor,
et al .(1984) for glucocorticoid steroid receptors in fish
gills cytosolic preparation, was initially attempted in
order to separate free from bound steroid from cytosolic
preparation of ovarian follicles of brook trout using

[^{3}H]Progesterone and [^{3}H]17α,20β-DHP. Initially
very low binding was obtained, and Scatchard plots could not
be obtained. A literature search revealed that in mammalian
cytosolic progesterone receptor preparations, the
experimental conditions used for separation of free from
bound steroid was 30 sec (Clark and Peck, 1984). The longer
times of separation led to dissociation of receptor-steroid
complex, giving lower binding. Therefore the ovarian
cytosolic receptor assay was then modified, to optimise the
conditions for charcoal-dextran separation, and to obtain
maximum specific binding. A second experimental problem with
the assay using the modified optimised time of separation (5
min 15 sec), was that at high concentrations of steroid the
specific binding obtained was higher than the saturation
plot would have given. This was because in the original
method of receptor assay (Sandor et al . 1984), the
charcoal-dextran separation was carried out on 40 tubes per
each experiment (2 cytosolic receptor samples of 20 tubes
each; and each sample was assayed at 5 concentrations of the
steroid, and at each concentration total and non-specific
steroid binding was obtained in duplicate). Thus the
disparity in the observed experimental results was due to
differences in the time of charcoal-dextran treatment
received by the later (high [^{3}H]steroid concentration)
tubes, compared to the earlier tubes. The time of
charcoal-dextran treatment was measured from addition to the

first tube, therefore the later tubes got less and less time
respectively during charcoal-dextran treatment, since the
addition was carried out one tube at a time. It took about 2
min 20 sec to pipette and vortex charcoal-dextran solution
40 times consecutively; thus the last tube's exposure time
to charcoal-dextran was reduced by 2 min and 20 sec. The
assay procedure was modified, and a set of 8 tubes were
equilibrated at a time instead of 40 tubes and then
processed as before. The optimal time for charcoal-dextran
seperation using 8 tubes was experimentally measured and
was 5 min 15 sec, the modified procedure gave better
saturation curves, and linear Scatchard plots (P < 0.05 of r
value).

In the hydroxy-apatite batch method (Clark and Peck, 1984),
the cytosolic receptor preparation is equilibrated with
labelled steroid, and then hydroxy-apatite slurry is added.
The receptor-steroid complex binds the hydroxy-apatite and
the free steroid stays in solution. The tubes are then
centrifuged, and supernatant discarded. The hydroxy-apatite
pellet containing the labelled receptor-steroid complex is
then washed 3 times with ice cold equilibration buffer to
remove adsorbed free steroid from hydroxy-apatite. Then the
pellet is extracted with ethanol, and measured by
scintillation counting. With the cytosolic ovarian follicle
preparation from brook trout it was observed that

radioactivity was continously released during washing. Even after 7 washes radioactivity was being released in the washing buffer, indicating that receptor steroid complex was continously dissociating during washing. This method is suitable for receptors which have a slow rate of dissociation at 0-4 deg. C. The above method was also tried on glucocorticoid receptors from gill tissue, using [3H]cortisol, and after 3 washes very little radioactivity was observed in the wash. Gill cortisol receptors have lower rates of dissociation compared to ovarian 17α,20β-DHP receptor activity (Chakraborti, et al. 1987).

In the Sephadex LH 20 method using 12cm x 1/2cm columns (Bio-Rad Econo-Column 7370-0242), the separation of labelled receptor-steroid complex from free steroid gave low binding activity. The percentage of specific bound labelled receptor-complex recovered was only 3.6%, compared to values obtained using the charcoal-dextran separation assay. During elution through the Sephadex LH 20 the free steroid is held back in the gel and thus is separated from the receptor-complex, leading to dissociation of the complex. This method is lengthy and difficult to quantitate.

In the controlled pore glass bead (CPG) absorption method (Gormley, et al. 1985), the labelled receptor-steroid

complex is adsorbed on to the glass beads column (CPG beads
120/200 mesh, 544 A mean pore diameter purchased from
Electro-Nucleonics Fairfield, N J; USA). Excess labelled
steroid is washed away leaving the receptor steroid complex
bound to the beads. Bound labelled steroid is then eluted
using ethanol. With cytosolic preparations from ovarian
follicles equilibriated with labelled $17\alpha,20\beta$-DHP,
dissociation of labelled receptor-steroid complex occured
during washing procedure (20 ml washing buffer was used
according to the method), and no specific binding was
recovered in the ethanol.

Sangalang and Freeman, (1977,1987) used ammonium sulphate/
calcium sulphate, for separation of free and bound labelled
testosterone, and $17\alpha,20\beta$-DHP in their radioimmunoassays of
fish plasma. The authors measured the free steroid in
solution, after precipitation of antibody-steroid complex.
Extensive sets of experiments were carried out to determine
the conditions, and optimal time for precipitate formation,
after addition of ammonium sulphate/ calcium sulphate
solution to equilibriated cytosolic preparation from brook
trout. The initial results were promising, Scatchard plots
were obtained, but they were not linear ($P > 0.05$ of the r
value). In the receptor binding assay system, the amount of
free steroid is much greater than its bound counterpart.
Normally during Scatchard plots only 3-8% of total labelled

steroid binding is obtained as specific binding. Thus an error of 5% in measurement of free binding could lead to over 50% error in the bound value. In radioimmunoassays, the value of bound steroids are between 20-50%, and a similar 5% error in measurement of the free value does not lead to more than 20% error in the bound value. In receptor assays, another complication is introduced due to non-specific binding. This involves subtraction of non-specific values from total binding values. Since errors are additive, the results from ammonium sulphate/ calcium sulphate prepitation experiment were not significant, i.e. linear Scatchard plots were not obtained.

The method described by Clark and Peck (1984), was used in separation of free from bound steroid using protamine sulphate precipitation. The method involves, measurement of bound receptor-steroid complex, after precipitation with protamine sulphate. The precipitated complex is then washed twice, to remove adsorbed steroid. In the case of cytosolic $17\alpha,20\beta$-DHP receptors from brook trout ovarian follicles, the receptor complex dissociated during this washing period and low non-specific binding was obtained.

Extensive amount of work was carried out using Spectrum Medical Industries equilibrium dialyser, to obtain affinity constant (K_a), and maximum binding capacity

(N_{max}), using Scatchard analysis. Total binding is measured in a known volume of cytosolic receptor preparation, by placing the cytosolic preparation on one side of the teflon cell 1 separated by a semi-permeable membrane (Spectra/pore 2 ,132480; Spectra/pore 4,132496). Labelled steroid solution of same volume (made up in the same buffer as the sample cytosol preparation) is placed on the other side of the cell. The cell is then equilibriated by slow rotation at 4 deg C. Equilibration is reached within 6 h using #2 membrane, and within 12 h using #4 membranes. After equilibration sample aliquots, in triplicate are taken from both sides of the cell and counted by scintillation counting. Similarly for non-specific binding cell 2, the same volume of cytosolic solution is placed on one side of the cell and equivalent amount of labelled steroid plus 1000-fold excess inert (unlabelled) steroid solution is placed on the other side. In cell 1, the differerence between the amount of radioactivity obtained from the two sides gives total binding. Similarly, in cell 2, the difference between the amount of radioactivity obtained between the two sides, gives the value for non-specific binding. The specific binding value is the difference between total and non-specific binding values. A slight complication arises in obtaining the value for free steroid, since the semi-permeable membrane also binds the steroid. The membrane binding value is obtained as follows:-

Total amount of steroid added - total amount of steroid present after equilibriation from both sides of the cell = amount steroid bound to the membrane. This value was obtained for each cell. Although slight differences were observed, between membranes binding the steroid in the total and non-specific cells. The mean of these values was used in calculating the value for free steroid as follows:-

free steroid = Total steroid added - (specific steroid bound to cytosolic receptors + mean value of steroid bound to membrane).

In order to obtain K_a and N_{max} values from Scatchard plots, the equilibrium dialysis was performed using cytosol preparation from brook trout and various concentrations of labelled steroid. A typical saturation plot is shown in the results section of this chapter; the Scatchard plot obtained from this plot deviated at the lower concentration range (between 1 and 2 nM). If these lower concentration values are not taken into consideration during the linear regression analysis, the Scatchard plots were linear and significant ($P < 0.05$ of r value). The K_a and N_{max} values were very similar to that obtained using charcoal-dextran method. The deviation observed at the low concentrations, might be due to errors in calculation of mean value of steroid binding to the membrane, giving low values for free steroid and hence higher values for bound/free. This method is simple to carry out, but very

labour intensive and time consuming. Teflon cells are first washed in 1% count-off detergent, and then placed in 30% chromic acid over night and finally washed in distilled water 5 times. Membranes are prepared by placing them in solution of 10% EDTA at 50 deg for 3 h, and then washing them in distilled water at least 5 times. Before use, the membranes are equilibriated in experimental buffer for 2 h at 4 deg C. The time of equilibriation using the #4 (thicker) membranes was determined to be 12 h at 4 deg C, and receptors could denature during this period giving lower N_{max} value. The #2 (thinner) membranes were difficult to use, they curled during washing, and were difficult to place in between the cells, but required shorter equilibration times.

The modified charcoal-dextran method of separation of free from bound steroid gave the best results in terms of linear Scatchard plots. This method was further utilized in the measurement and characterization of the cytosolic $17\alpha, 20\beta$- DHP receptor activity from brook trout. The cytosolic receptor properties studied were specificity, molecular weights, kinetic parameters, stability, and protein nature.

Materials and methods

Fish .

Female, prespawning brook trout, __Salvelinus__ __fontinalis__
(about 35 cm and 0.5 kg) were provided by the department of
Fisheries and Oceans, Antigonish Fish culture Station,
Frasers Mills, Nova Scotia. The animals were exposed to
natural photoperiods, and housed in regular glass tanks of
recirculated, filtered dechlorinated fresh water topped up
daily with 10% addition of fresh water. The fish were kept
at 10 deg C.

Landlocked Atlantic salmon, __Salmo__ __salar__ Ouananiche, were
from a laboratory stock hatched in 1982 at the Marine
Sciences Research Laboratory, St. John's, Newfoundland.
Ouananiche were kept in running fresh water, and maintained
under natural seasonal conditions of photoperiod and
temperature. The fish were segregated by year class and used
when reproductively matured (2 + years, about 13 cm and
0.035 kg). Rainbow trout, __Salmo__ __gairdneri__ were purchased
from a fish hatchery in Hopeall, Newfoundland and used
within 48 h (about 35 cm and 0.67 kg).

Steroids

$[1,2,6,7-^3H]$Progesterone (specific activity 112 Ci/m
mol), 17α-methyl $[^3H]$Promegestone R5020

(17,21-dimethyl-19-nor-Pregn-4,9-diene-3-20-dione specific
activity 85 Ci/m mol) and [1,2-^{3}H]17α-HP (specific
activity 50 Ci/m mol) were purchased from New England
Nuclear Corporation (Canada), Lachine, Quebec.
[1,2-^{3}H]17α,20β-DHP (specific activity 50 Ci/m mol) was
synthesized from [1,2-^{3}H]17α-HP (Simpson, et al .
1964). [7-^{3}H]Pregnenolone (specific activity 10 Ci/m
mol) and [^{3}H]Org (16
-Ethyl-21-hydroxy-19-nor[6,7-^{3}H]pregn-4-en-3,20-dione,
specific activity 45 Ci/m mol) were purchased from Amersham
Corporation, Oakville, Ontario. The tritiated steroids were
purified by paper chromatography or thin layer
chromatography using standard methods.
Radioinert steroids progesterone, testosterone, cortisol and
triamcinolone acetonide (9-α-Fluro-11β,16α-
,17,21-tetrahydroxy-1,4-pregnadiene-3,20-dione) were
purchased from Sigma chemical company, St. Louis, Missouri.
U.S.A. Radioinert 17α,20β-DHP and 17α-HP were purchased from
Steraloids, Inc., Wilton, N.H. Inert Org 2058 and R5020 were
supplied by the manufacturers of the corresponding labelled
steroids. Pregnenolone was purchased from Ikapharm, Ramat,
Israel.

Chemicals

Trizma 7.4, sodium molybdate, glycerol, EDTA,
dithiothreitol, protease type 25 (pronase E), protease

(proteinase K), trypsin (T8253) and trypsin inhibitor (T9253) were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Sephacryl S-300 superfine, high molecular weight gel filtration kit, deoxyribonuclease 1 from bovine pancreas, ribonuclease 1A from bovine pancreas and electrophoresis calibration kits were purchased from Pharmacia (Canada) Inc., Dorval, Quebec. Common inorganic chemicals and organic solvents were purchased from Fisher Scientific, Caladon or BDH and were of highest purity available. Liquid scintillation fluid (Ready solve HP) was purchased from Beckman Instruments, Toronto, Canada.

Cytosolic ovarian follicle preparation

Fish were removed from the tanks and sacrificed by a blow on the head, and then placed on ice. A blood sample was withdrawn with a heparinized syringe from the caudal peduncle within 60 sec of introducing the hand net into the tank. The ovarian tissue was dissected out, weighed and then a sample of tissue (ca . 3.5 g) was used after the connective tissue was removed. The oocytes were then placed into ice cold TETS buffer (10mM Tris-HCl, 1mM EDTA, 5mM DTT, 10mM sodium molybdate at pH 7.4; Sandor, et al . 1984). The tissue was homogenized either with a Polytron PCU-2-110 instrument (Brinkmann Instruments, Rexdale, Ontario), or with a motor driven teflon-glass Potter-Elvehjem homogenizer (3 x 15 sec at 90 sec intervals in ice water). The

homogenate was then centrifuged at 750 x g for 30 min (IEC
Centra-7-R refrigerated centrifuge). The resulting
supernatant was further centrifuged after treatment with
charcoal-dextran to remove endogenous steroids at 15,000 rpm
(25,000 x g) for 120 min (IEC refrigerated centrifuge Model
B-20), or in a Beckman L5-50 ultracentrifuge at 35,000 rpm
(151,000 x g) for 60 min. The supernatant from the last
centrifugation step in both cases gave the same amount of
binding (N_{max}) with the radio-labelled steroids and was
used as a soluble cytosolic fraction. This fraction was
essentially free of hemoglobin indicating very little
contamination from blood plasma proteins. All centrifugation
and assays were performed at 0-2 deg C.

Steroid binding studies
Saturation curve and Scatchard plots with
[^{3}H]progesterone and [^{3}H]17α,20β-DHP. For the
determination of total ligand binding, 250 μl of cytosolic
receptor samples were pipetted into 12 x75 mm glass tubes
(CanLab T-1290-3) containing vacuum evaporated [^{3}H]
steroid to yield a concentration of 0.15 to 5nM. For the
determination of non-specific binding [^{3}H]steroid plus
1000 fold excess of radio-inert steroid was used. All
samples were incubated in duplicate. Incubation was started
in batches of 8 tubes every 20 min. After 120 min the
incubation was terminated by addition of 250μl of

charcoal-dextran suspension (0.5% charcoal, 0.05% dextran in TETS buffer at pH 7.4). The charcoal-dextran treatment for the separation of free steroid from bound fluid was carried out for 5 min and 15 sec, after which the batch of 8 tubes were immediately centrifuged at 2,500 rpm (750 x g) for 10 min. The supernatant, 250ul samples, were counted for radioactivity in 10 ml of scintillation fluid.

To determine steroid-binding to plasma proteins, blood was collected without the use of heparin and immediately centrifuged. The plasma was diluted (1:10) with TETS buffer and used after charcoal-dextran treatment exactly as for the cytosolic preparation except higher concentrations (15nM) of [^{3}H]17α,20β-DHP was required to effect saturation at this dilution of plasma.

Hormone binding kinetics

The association constant (k_{+1}) was determined on 250ul of cytosolic preparation incubated with [^{3}H]17α,20β-DHP in the presence and absence of inert steroid for 0-60 min. Bound and free ligand were separated by the charcoal-dextran method. The second order association constant k_{on} was calculated from the slope of the plot:

$$\frac{1}{(T-S)} \ln \frac{(T-X)}{(S-X)}$$ against time (Hansen $\underline{et}$ $\underline{al}$. 1976),

where T = total concentration of the steroid added, S =

total concentration of the binding sites, X = concentration
of bound ligand at time (t). T was 2.268nM and S was
0.495nM. Dissociation rate constant (k−1) was
determined after equilibration of the cytosolic preparation
with the [^{3}H]17α,20β-DHP for 120 min; 250ul of
equilibrated sample was added to the tubes containing 1000
fold excess steroid (vacuum evaporated), then 250ul of
charcoal-dextran was added at various times. The first order
dissociation rate constant (k−1) was calculated from
the log$_{10}$ concentration of the bound ligand against
time.

Competition studies with labelled progesterone using various
steroids

Cytosolic preparation samples were incubated with a constant
concentration of [^{3}H]progesterone (4.033nM) in the
presence or absence of increasing concentrations of
radio-inert steroids for 120 min at 0-1 deg C. The bound and
unbound fractions were separated using charcoal-dextran and
the bound fraction was counted as described previously.

The ln $\dfrac{B}{(B_{max}-B)}$ of the bound [^{3}H]progesterone was
plotted against the log of the relative molar excess of the
competing radioinert steroid.

Gel filtration chromatography

Cytosolic preparations of the ovarian tissue (5 ml; ca . 10 mg of protein per ml) were equilibrated with [^{3}H]progesterone, [^{3}H]testosterone, and [^{3}H]17α,20β-DHP at concentrations 5nM for 120 min at 1 deg C, and then applied to a Pharmacia K26/100 Sephacryl S-300 column equilibrated with TETS buffer containing 0.15M NaCl. The column bed volume was 493 ml and void volume was 194 ml. The constant flow rate was 26 ml per h. The column was calibrated with the following standard proteins, thyroglobulin molecular weight (MW) 660,000; ferritin MW 440,000; catalase MW 232,000; aldolase MW 158,000; bovine serum albumin MW 66,000. Following chromatography of the standard proteins, the distribution coefficient (K_{av}) for each standard was calculated :

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)}, \text{ where } V_e \text{ elution volume}$$

of the standard V_0= void volume of the column and V_t= total bed volume of the column. The MW of the cytosolic receptor was obtained from the linear plot of $\log_{10}$MW against K_{av} of the standard proteins (r = 0.99, P < 0.01).

[^{3}H]R5020 (5nM) photoaffinity labelled samples from blood plasma, cytosolic preparation, zona radiata membrane

solubilized protein, nuclear pellet extract were also applied to and eluted from the calibrated Sephacryl S-300 column (chapters 2 and 3).

Measurement of protein

Protein samples of cytosolic preparation were measured by the method of (Lowry, et al. 1951), with slight modification (Hatree, 1972) to have a linear photometric response.

Measurement of radioactivity

The radioactivity was measured using a modified Tracor 6892 liquid scintillation counter (Tracor Analytic incorporated, Elk Grove, Ill. U.S.A.) fitted with a Texas Instrument ASR733 terminal (Texas Instruments Inc., Houston, Texas). All samples were counted to at least 10,000 counts in each channel. Counts per minute were converted to absolute activity (dpm) by a computer program similar to that outlined by Carrol and Houser (1970).

Metabolism of radiolabelled 17α,20β-DHP, progesterone and testosterone during   equilibration binding incubation at 1-4 deg C.

Aliquots of cytosolic preparation in TETS buffer were separately incubated at 1-4 deg C for 120 min with the above labelled steroids [5nM]. The labelled steroids were then extracted and purified using paper chromatography and quantified (Peter, et al . 1984).

Calculations and statistics

Scatchard anlyses or other linear analysis were fitted by least square analysis and assessed for goodness of fit by examining the correlation coefficients. These analyses and the transformation of receptor binding data from dpm to n-moles were carried out using computer programs on the VAX 11/780 computer. Data are presented as the mean $\pm$ SEM. The data in table 1.4 were tested for homogeneity of variances and then analysed by one-way ANOVA. The differences between pairs of means were determined by Tukey's multiple range test.

Sucrose gradient centrifugation

Brook trout ovarian follicles (stage 1) were homogenized in TETS buffer without sodium molybdate in presence of 0.15M NaCl and then treated similarly as in the case of cytosolic

preparation. This modified cytosolic preparation (500µl) was equilibrated with [³H]17α,20β-DHP at 14.2 ± 100nM inert steroid for 120 min, then aliquots of 250µl were layered on 11.5ml of gradient solution (5-30% linear sucrose gradient in buffer containing 1mM EDTA; 10mM TRIS; 0.15M NaCl at pH 7.4). The polyallomer tubes (Beckman 331372) were centrifuged for 17h at 4 deg C at 30,000 rpm (111,000 x g) in a Beckman Model L5-50 ultracentrifuge, using SW41-Ti rotor. The gradients were formed using Searle auto Density Flow 2 pump and gradient maker. After centrifugation, the gradients were fractionated from the top of the tube using the same instrument in reverse. Thirty fractions were collected (16 drops, 0.4ml) and 200µl samples were counted. Catalase (250µl; 1mg/ml), was used separately in a gradient as a marker protein, and its O.D. measured at 254nm using ISCO type 6 optical unit during fractionation.

Further experiments were carried out using cytosolic preparation of brook trout in TETS buffer (containing molybdate), in the presence and absence of 0.15M NaCl. The sucrose gradients (5-15% linear gradients in buffers containing 1mM EDTA; 10mM TRIZMA pH 7.4; 10mM sodium molybdate; 5mM DTT) were prepared, and then ultracentrifuged. Gradient fractionation, and analysis were carried out under identical conditions as described above.

Trypsin digestion of cytosolic preparation

The cytosolic preparation from stage 1 ovarian follicles of
brook trout was prepared in TETS buffer (± molybdate) as
described. To 5.5ml aliquots was added 1.0ml of trypsin
(6.5 mg/ml in TETS buffer ± molybdate). The resulting
solution was incubated at 16 deg C with slow shaking
(Eberbach Shaker Bath, cat. # 6250, Eberbach Corporation,
Ann Arbor, Michigan. U.S.A.). Samples (250 μl) were removed
at various times, then immediately equilibrated with
[³H] 17α,20β-DHP ± inert steroid for 60 min at 1 deg C
and assayed for specific binding.

Effect of trypsin inhibitor (T-8253) on tryptic digestion of the receptor activity

To two separate 3.5ml aliquots of cytosolic preparation from
brook trout stage 1 ovarian follicles in TETS buffer were
added (a) 0.5 m³ trypsin (8 mg /ml) and (b) 0.5ml of trypsin
plus trypsin inhibitor (8 mg/ ml trypsin and 8 mg /ml
trypsin inhibitor). The cytosolic preparations were than
incubated at 25 deg C with slow shaking. Samples (250 ul)
were taken at various times, then equilibrated with
[³H]17α,20β-DHP ± inert steroid for 60 min at 1 deg C
and assayed for specific binding.

Effect of protease K, pronase E, DNase 1 and RNase 1'A' on the receptor activity

To cytosolic preparation from brook trout stage 1 ovarian
follicles (3.5 ml aliquot) was added 0.5 ml of DNase 1 and
RNase ≅'A' (1 mg /ml in TETS buffer containing 5.0mg /ml
MgSO₄), and incubated at 16 deg C. Samples (250 ul)
were withdrawn every 30 min, then equilibrated immediately
with [³H]17α,20β-DHP ± inert steroid for 60 min at 1
deg C, and assayed for specific binding. In the case of
protease K and pronase E, 0.5 ml of the enzyme solutions
(8mg /ml in TETS buffer pH 7.4) were added to aliquots of
cytosolic preparation (3.5 ml), so that the final
concentration of both proteases were 1mg /ml in the
cytosolic preparation.

Receptor stability experiments

1) In absence of [³H]17α,20β-DHP in the cytosolic
preparation.
Cytosolic preparation from stage 1 ovarian follicles from
brook trout in TETS buffer with and without molybdate in
aliquots of (3.0 ml) were incubated at 0, 16 and 25 deg C
with gentle shaking. Samples (250ul) were taken at various
times and immediately equilibrated for 60 min at 1 deg C with
[³H]17α,20β-DHP ± inert steroid , and then assayed for
specific binding.
2) In presence of [³H]17α,20β-DHP in the cytosolic
preparation.
Two aliquots (6.25 ml) of above cytosolic preparations were

added to vials containing [³H]17α,20β-DHP ± inert
steroid (vacuuo evaporated), so that final concentration of
labelled steroid added to vials were 5.67nM and inert 100nM.
The vials were then incubated at 16 deg C with gentle
shaking. Samples (250 μl) were pipetted out at various
times, and immediately cooled to 1 deg C and then assayed
for specific binding.

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)

SDS PAGE was carried out using vertical slab gel unit
(Studier slab gel SE 400, Hoefer Scientific Instruments, San
Francisco, CA 94107-9985), and discontinous system described
in the instruction manual. The acrylamide concentration of
stacking gel was 4% T, 2.7% C and resolving gel 10% T, 2.7%
C. The gels were scanned using model 1650
transmittance/reflectance scanning densitometer (Hoefer
Scientific Instruments, purchased through Bio-Rad
Laboratories). Procedures used for sample preparation,
buffers, fixing, staining and destaining were followed as
described in the instruction manual of the instrument.

Maturation studies in vitro using stage 3-4 brook trout oocytes and various steroids

The method used was essentially as described by So, et al .
(1985). The incubation was carried out at 10 deg C with

gentle agitation in the presence of moist oxygen.

High-performance liquid chromatography on an exclusion gel

The method described by (Pavlick, et al . (1982)) was used
to separate receptor activity on a Beckman Spherogel TSK
3000SW (7.5 X 60 cm) column, with a Beckman 114M pump and
340 injector, all located in a cold room at about 2 deg C.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out in
TRIS/glycine (pH 8.3) essencially as described by Davis,
(1964). Gels were polymerized in glass tubes 12.5 cm long
and 0.5 mm internal diameter (Bio-Rad 1653122). Both sample
and spacer gels were omitted since the samples were layered
directly on top of the separation gels. Disc electrophoresis
was carried out using Bio-Rad apparatus (Model 175 tube
cell). Gels were run at 5%, and 4% acrylamide concentrations
with 2.5% cross linking at 1mA/ tube for the first 30 min
and at 2 mA/ tube after 30 min for 3h at room temperature
(Bio-Rad Model 3000/300 power supply). Gels were removed
from the tubes and fixed in trichloroacetic acid (12%) for
15 min and then stained in Coomassie blue R-250 (10%
solution in 7% acetic acid) for 6-8 h, and then destained in
7% acetic acid for 12h using a gel destainer (Hoefer
Scientific Instruments DD-105).

Equilibrium dialysis

Equilibrium dialysis was carried out using Spectrum
Industries Equilibrium Dialyzer (Part no.132460, 20 cell
dialyzer with variable drive and semi-micro 1 ml cells).
Initially equilibration time for ligand $17\alpha,20\beta$-DHP between
the two sides of the cell was measured using Spectra/Por 2
and Spectra/por 4 membranes (47 mm, Part no's 132480 and
132496), as described in the instrument manual. Almost all
experiments were carried out using the thicker membranes
(Spectra/Por 4), although they took 12 h to equilibrate. The
thicker membranes were easier to handle and did not curl up
during washing and application stages. The thinner membranes
also did not form a good seal between the two parts of the
cell, and the cells leaked. TETS bufffer was used
throughout, and all the steroid solutions were made up by
first pipetting a known quantity of alcoholic solution of
steroid in a scintillation vial, and then vacuum evaporating
them. The evaporated steroid was then taken up in known
volume of TETS buffer by vortexing the solution for 1 min.
The experimental procedure for setting up the cells,
addition of the cytosolic preparation and steroid solution
to the cells, removal of samples from the both sides of the
cells at the end of the experiment were followed as
described in the instrument manual. The experiments were
carried out at 1 deg C, for 12 h in a refrigerated unit
(Foster Model GH-45-G-T, Drummondville, Quebec, Canada).

RESULTS

Steroid binding studies

Saturation curve and Scatchard analysis. $[^3H]17\alpha,20\beta$-
DHP and $[^3H]$progesterone were used in binding studies
and for Scatchard analysis. The affinity constant (K_a)
and maximum binding capacity (N_{max}) for $17\alpha,20\beta$-DHP and
progesterone were very similar (Table 1.1). A representative
saturation analysis plot is shown in (Fig. 1.3 a and b) for
brook trout ovarian tissue cytosolic preparation using
$[^3H]17\alpha,20\beta$-DHP. Ovarian tissue cytosolic preparation
from Atlantic salmon and rainbow trout also manifested
saturable binding of high affinity and low capacity (Table
1.1).

The blood plasma binding protein from stage 1 brook trout
after dilution (1:10) with TETS buffer gave a linear
Scatchard plot with $[^3H]17\alpha,20\beta$-DHP and K_a was
8.04×10^7 M^{-1}. The blood plasma protein was later
shown to be differen` om cytosolic receptor protein by
photoaffinity labelling, followed by gel chromatography and
SDS PAGE electrophoresis (chapter 3). The ovarian cytosolic
preparations were not red in colour, indicating the absence
of hemoglobin. Thus the binding observed in the cytosolic
preparation cannot be due to blood plasma binding steroid
protein contamination in the oocyte homogenates, although
trace quantities of contamination cannot be ruled out.

Fig. 1.3. a. Binding of $17\alpha,20\beta$-DHP to cytosolic extract of brook trout ovarian tissue. Aliquots of ovarian tissue cytosol (250 μl) were incubated with increasing concentration of [^{3}H]$17\alpha,20\beta$-DHP (0.5-3.5 nM) $\pm$ 1000 fold molar excess of radioinert steroid for 120 min at 0 deg C. SP.B, specific binding; NSP.B, non-specific binding.

Fig. 1.3.a.

TOTAL

SP.B

NSP.B

dpm x 10³

$[^3H]17\alpha,20\beta$-DHP (nM)
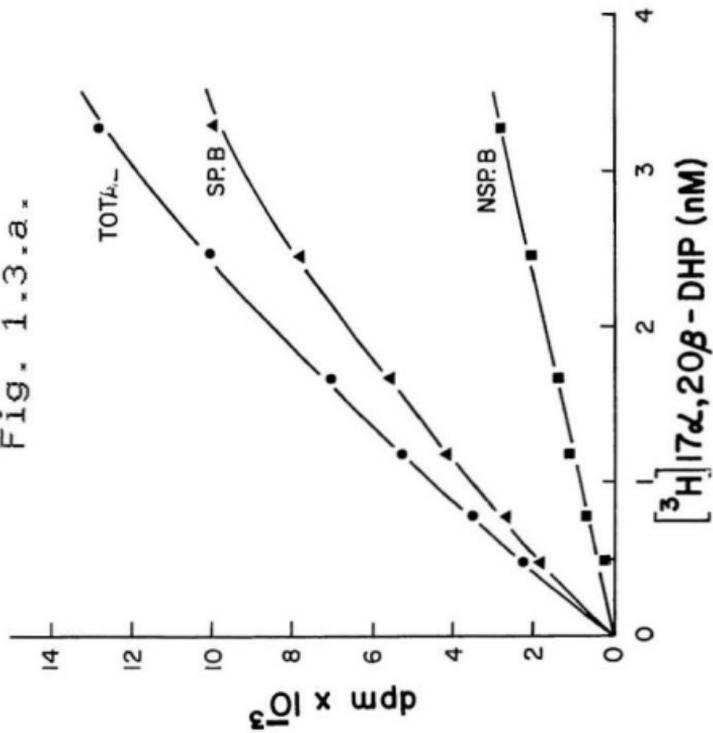
Competition studies

The specificity of [³H]progesterone binding to a brook
trout cytosolic preparation as measured by the competition
of radioinert steroids indicated a hierarchy of binding with
testosterone > 17α-HP > 17α,20β-DHP > R5020 > progesterone >
estradiol > pregnenolone (Fig. 1.4). Cortisol did not show
competitive inhibition (Table 1.2 a).

The hierarchy of binding in rainbow trout ovarian cytosolic
preparation was testosterone > R5020 > 17α,20β-DHP >
progesterone > 17α,-HP > ORG > pregnenolone. Cortisol and
triamcinolone acetonide showed very little inhibition (Table
1.2 b).

Hormone binding kinetics

The rate of association of receptor-[³H]17α,20β-DHP
complex for brook trout ovarian cytosolic preparation (Fig.
1.5 a and b) was higher in absence of sodium chloride in the
TETS buffer (Table 1.3).

The rate of dissociation of receptor-[³H]17α,20β-DHP
complex for brook trout ovarian cytosolic preparation (Fig.
1.6 a and b) was higher in absence of sodium chloride in
TETS buffer (Table 1.3).

The equilibrium association constant K_a for the
reaction:

Fig. 1.3. b. Linearization of the specific binding by Scatchard analysis. The equilibrium association constant obtained from this graph was 1.28×10^8 M^{-1}, the maximal concentration of binding sites was 1965 fmol/mg protein.

Fig. 1.3.b.

BOUND [^{3}H]17α,20β-DHP (pM)

BOUND/FREE x 10^{-2}
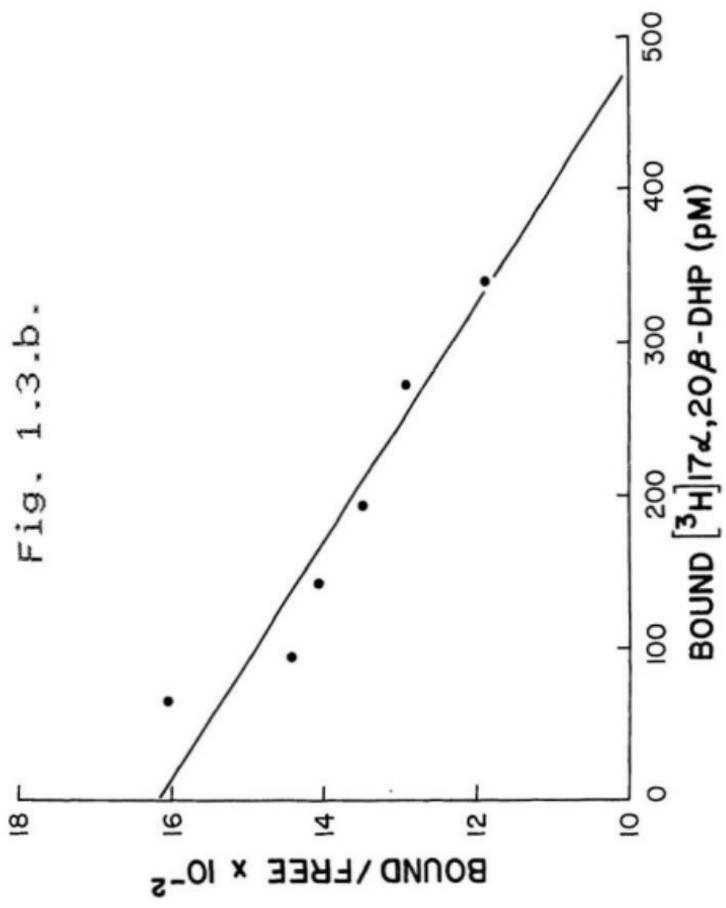
Fig. 1.4. Competition of radioinert steroids with

[^{3}H]progesterone for binding sites in brook trout

cytosolic extract. Samples of cytosolic extract (250 ul)

were incubated with 4.033 nM of [^{3}H]progesterone for

120   min at 0 deg C, in the presence or absence of 10, 20,

50, 100 and 200 fold molar excess of different radioinert

steroids.

$$\text{The ln} \frac{B}{(B_{max} - B)} \text{ was plotted against log}_{10} \text{ of the}$$

relative molar concentration of the competitors, where B is

the concentration of the bound ligand at different

concentrations of radio-inert steroids, and B_{max} is the

concentration of [^{3}H]progesterone bound in the absence

of non-labelled competitors. The molar concentration of the

competitor, read of the zero line of the 'Y' axis represents

the concentration of the competitor necessary to displace

50% of the [^{3}H]progesterone bound and is thus the

IC$_{50}$ of the competitor.

(A) ▲ Inert testosterone; (B) ▢ 17α,20β-DHP; (C) ✕ 17α

,OH-progesterone; (D) ◼ R5020; (E) ⊙ progesterone; (F) ●

estradiol.

Fig. 1.4.
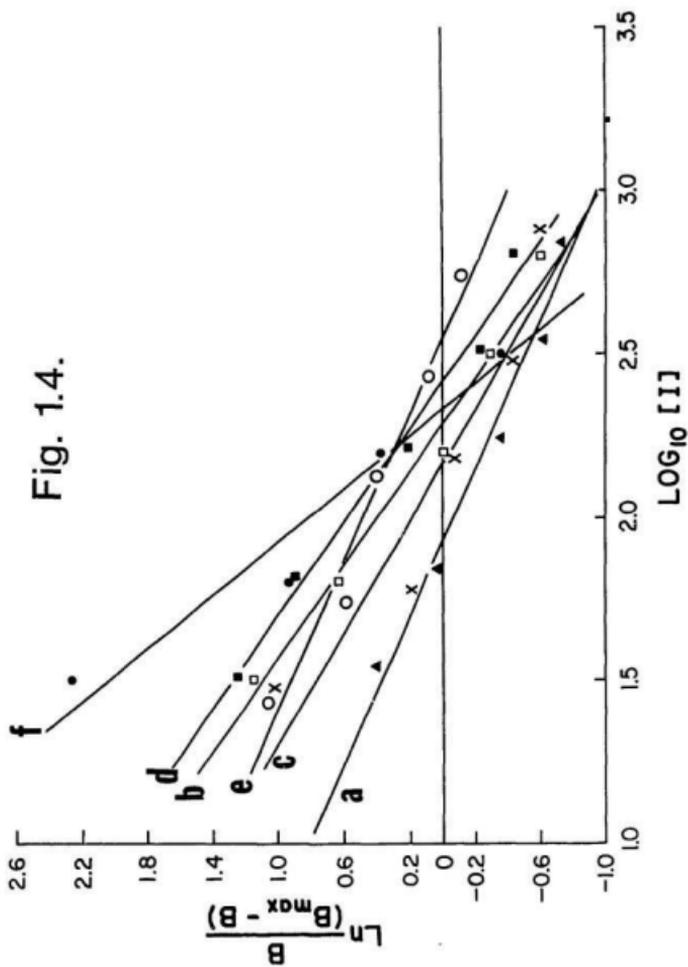
Table 1.1

Scatchard analysis results of ovarian tissue cytosol

Salmonid species	Binding ligand	$K_a \times 10^8$	$SEM(M^{-1})$	N_{max} fmol/mg protein
Brook trout	[^{3}H]17α,20β-DHP	1.4 ± 0.3 (n=7)		2090 ± 619*
	[^{3}H]Progesterone	2.2 ± 0.2 (n=5)		1928 ± 247**
Atlantic salmon	[^{3}H]Progesterone	2.5 ± 0.6 (n=2)		50.79 ± 4.7
Rainbow trout	[^{3}H]Progesterone	1.9 ± 0.2 (n=4)		2145 ± 835*

* mean values from various stages of maturation
** mean ± SEM for stage I oocytes
mean ± SEM for stages 5 and 6 oocytes

Table 1.2(a)

Effect of radio inert steroids on the binding of

[^{3}H]progesterone to cytosolic preparations of brook trout

Steroid	IC_{50} (nM)
Testosterone	82.7
17α-HP	146.7
17α,20β-DHP	196.6
R5020	266.1
Progesterone	317.1
Estradiol	363.1
Pregnenolone	6133
Cortisol	No competitive inhibition

*IC_{50} = Concentration of each competitor steroid necessary to decrease the [^{3}H]progesterone binding by 50%.

Table 1.2(b)

Effect of radio inert steroids on the binding of

[^{3}H]progesterone to cytosolic preparations of rainbow trout

Steroid	IC_{50} (nM)
Testosterone	67.6
R5020	109.6
17α,20β-DHP	132.7
Progesterone	137.6
17α-HP	142.0
Pregnenolone	3785
Cortisol	2.2×10^9
Triamcinolone acetonide	5.3×10^{11}

Fig. 1.5. (a). Plot of rate of association of cytosolic receptors with $[^3H]17\alpha,20\beta$-DHP.

Fig. 1.5.a.
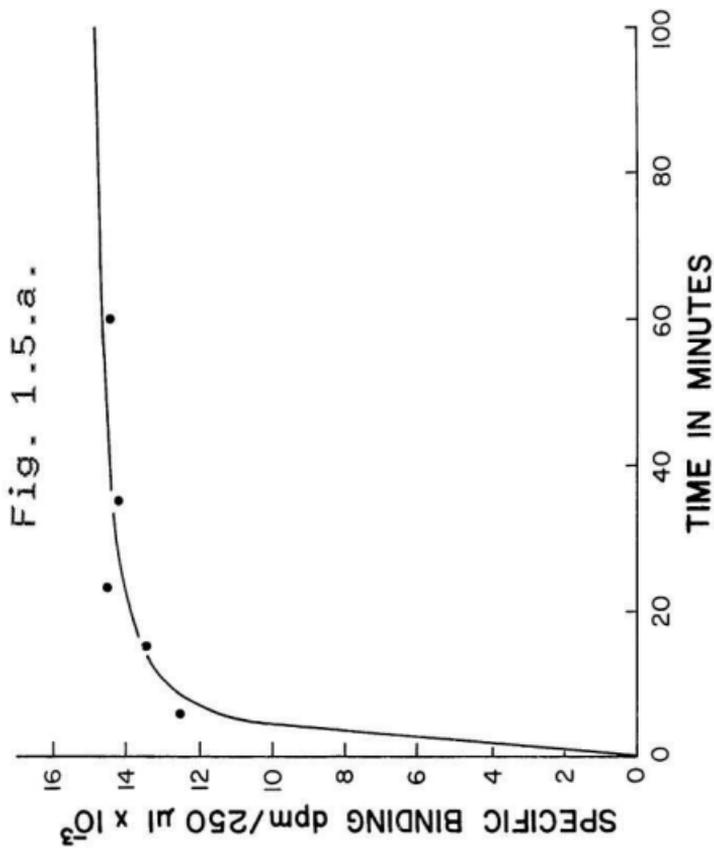
TABLE 1.3

Steroid binding kinetics using [^{3}H]17α,20β-DHP

	k_{+1} (M^{-1}sec^{-1})	k_{-1} (sec^{-1})	Ka (M^{-1})
Brook trout ovarian cytosol in TETS buffer	2.29 x 10^6	1.50 x 10^{-2}	1.53 x 10^8
Brook trout ovarian cytosol in TETS buffer containing 0.15 M NaCl	4.13 x 10^5	4.69 x 10^{-3}	0.88 x 10^8

Fig. 1.5. (b). Second order linear plot of

$$\frac{1}{T - S} \ln \frac{(T - X)}{(S - X)} \quad \text{against time,}$$

for the determination of k_{+1}. The point at time = 23 min was not used in the determination of slope of the line, and hence k_{+1}.

Fig. 1.5.b.
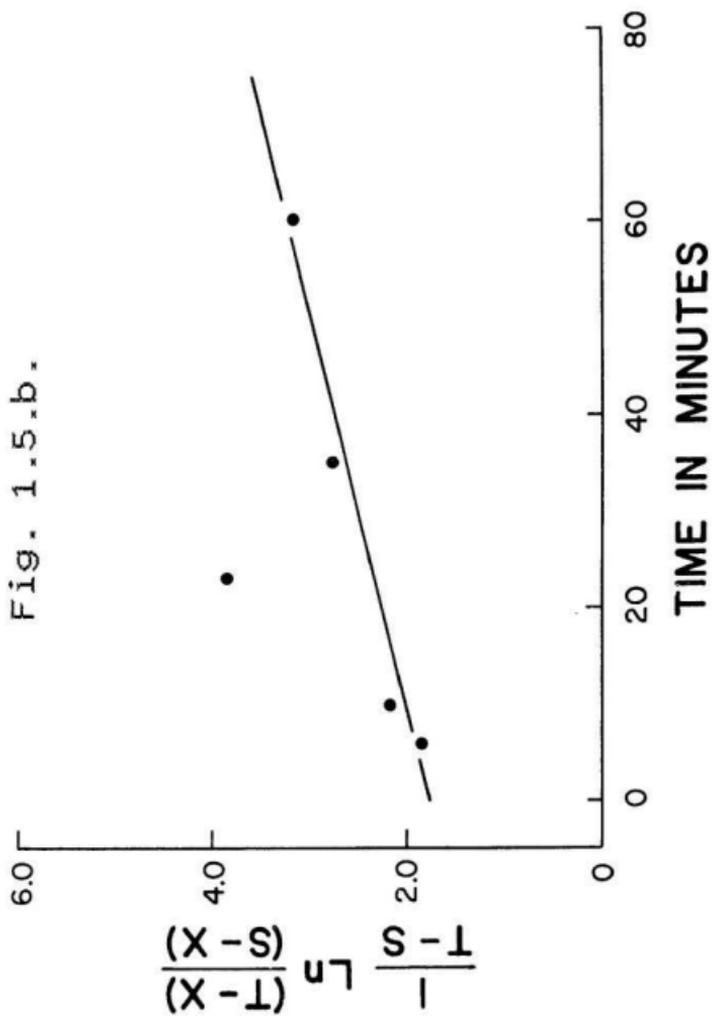
Fig. 1.6. (a). Dissociation of the labelled $[^3H]17\alpha,20\beta$- DHP receptor complex with time.
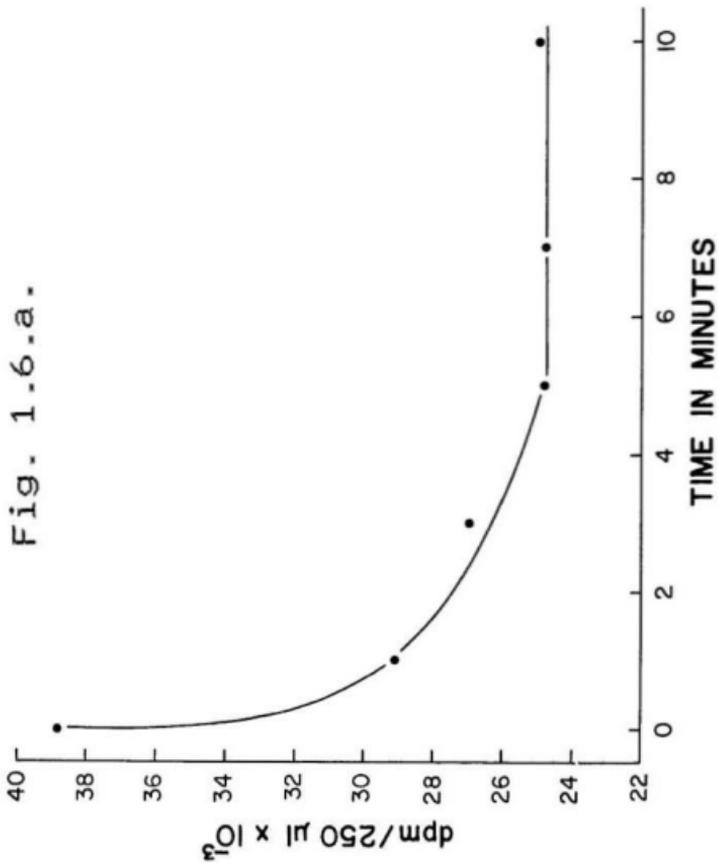
Fig. 1.6.a.

Fig. 1.6. (b). First order linear plot of $\log_{10}$ concentation of labelled receptor complex with time.

Fig. 1.6.b.

LOG₁₀ BOUND/250 μl

TIME IN MINUTES

{ R + [^{3}H]17α,20β-DHP <------> R-[^{3}H]17α,20β-DHP }
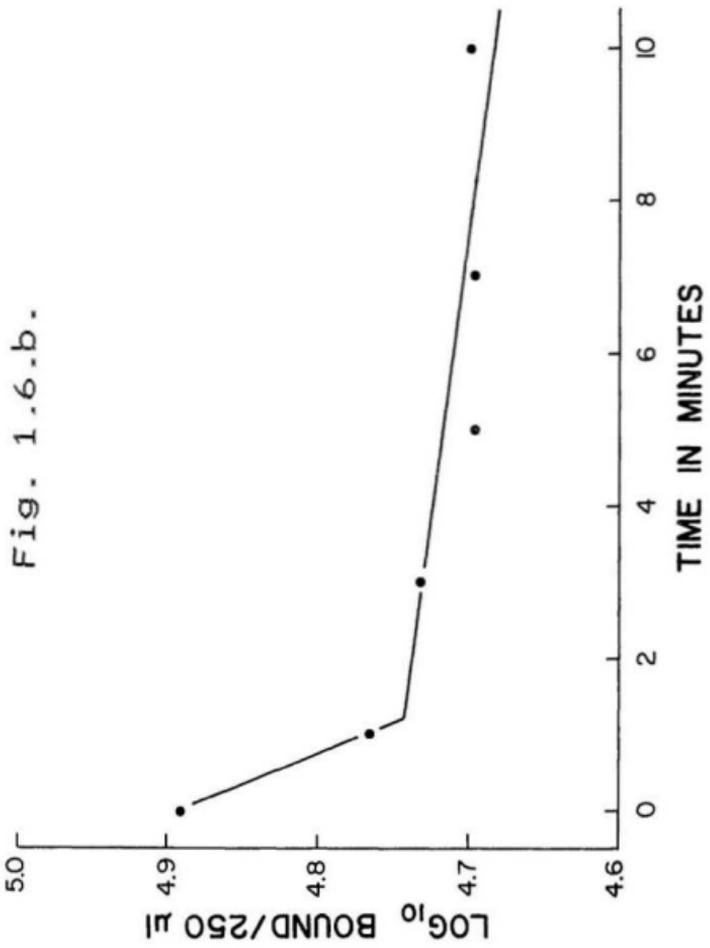
where $K_a = k_{on}/k_{off}$, was calculated and the
values are listed in Table 1.3. These values for K_a are
in close agreement with K_a obtained from Scatchard
analysis using [^{3}H]17α,20β-DHP.

Proteolytic digestion, and effect of DNase 1 and RNase 1'A'
on cytosolic preparation from brook trout ovarian tissue
DNase 1 and RNase 1'A' did not have a significant effect on
the receptor activity, but both proteases, protease K and
pronase E digested the receptor preparation activity in 180
min (Fig. 1.7). The rate of tryptic digestion of cytosolic
preparation in presence and absence of sodium molybdate was
negligible at 0-4 deg C, and substantial at 16 deg C (Fig.
1.8). In the presence of trypsin inhibitor, the rate of
tryptic digestion of receptor activity decreased (Fig.1.9).
These results indicate that the receptor activity is
associated with a protein component of the cytosolic
preparation.

Cytosolic metabolism of labelled steroids
No metabolic products of the labelled steroids [^{3}H]17α-
,20β-DHP, [^{3}H]progesterone and [^{3}H]testosterone
were isolated after incubation at 1 deg C for 120 min with

Fig. 1.7. Effect of protease K (▲), pronase E (◇), DNase 1 (■) and RNase 1'A' (△) on receptor activity. Aliquots of brook trout cytosolic extract were incubated at 16 deg C with the above enzymes at a concentration of 1mg/ml. Binding activity at various times was then measured by equilibration with labelled $17\alpha,20\beta$-DHP $\pm$ inert steroid at 0 deg C for 60 min. (Prior experiments had shown negligible effect of protease's on receptors at 0 deg C.

Fig. 1.7.
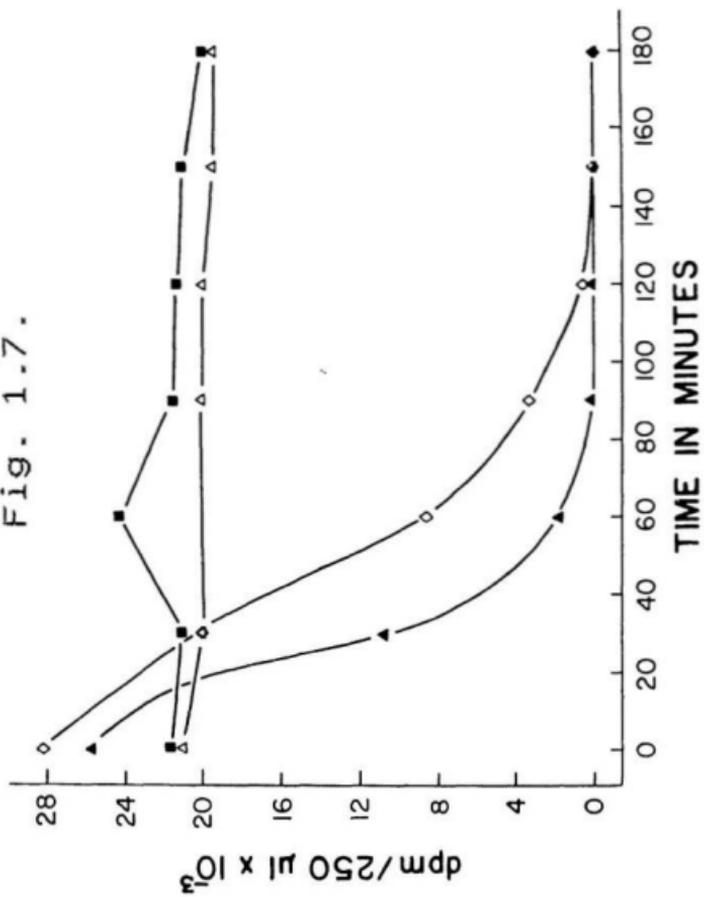
Fig. 1.8. Tryptic digestion of cytosolic receptors in presence (■) and absence (▲) of sodium molybdate (20 mM). Cytosolic extracts were incubated at 16 deg C with trypsin (1 mg/ml).

Fig. 1.8.

X-axis: TIME IN MINUTES

Y-axis: SPECIFIC dpm/250 µl x 10³
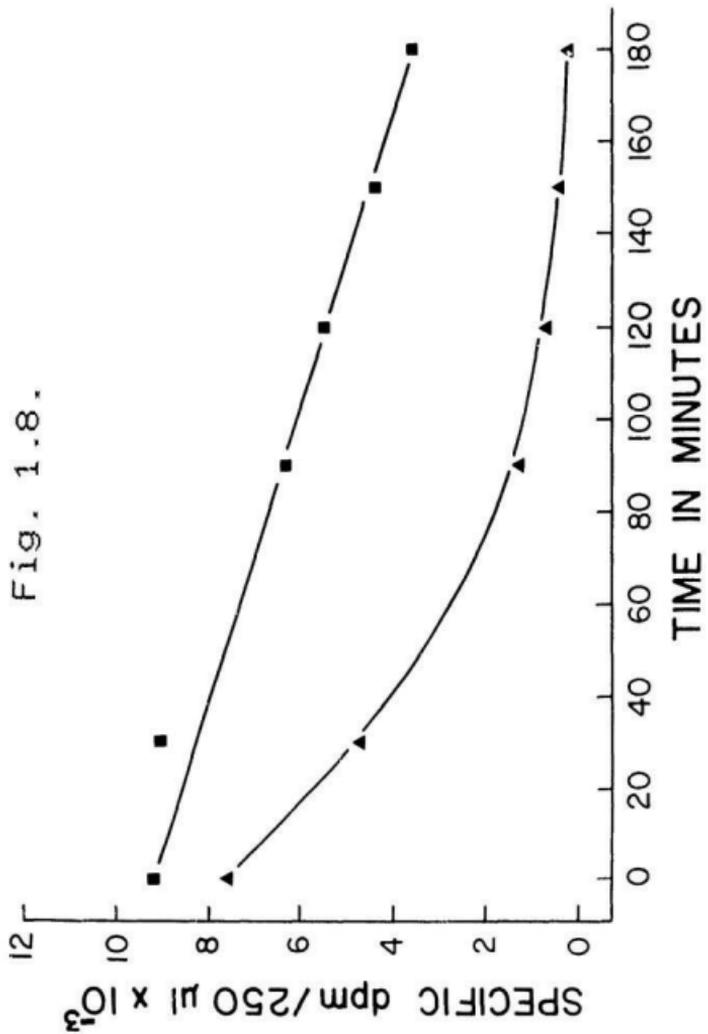
Fig. 1.9. Effect of trypsin inhibitor on tryptic digestion of cytosolic receptors. Aliquots of cytosolic extracts containing trypsin (▲ 1mg/ml) and trypsin + trypsin inhibitor (■ 1 mg/ml trypsin and 1 mg/ml inhibitor) were incubated at 25 deg C. Samples were taken at various times and binding activity measured at 0 deg C.

Fig. 1.9.
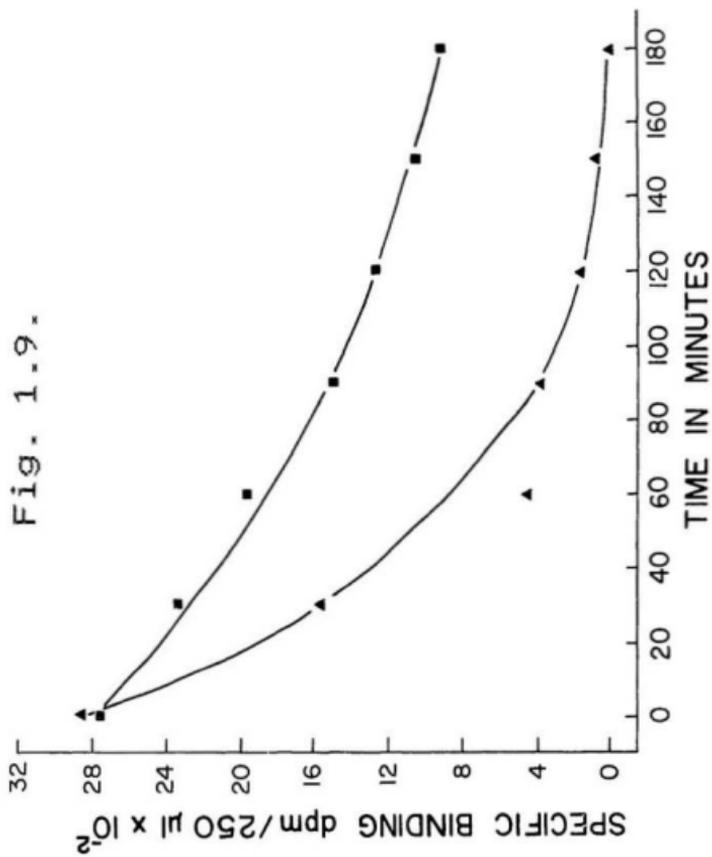
brook trout cytosolic preparations as determined by paper chromatography.

Stability experiments with cytosolic preparation from brook trout ovarian tissue

In presence or absence of $[^3H]17\alpha,20\beta$-DHP, the cytosolic preparation lost a maximum of 30% of the receptor binding activity at 25 deg C in 20 h of incubation. At 0 and 16 deg C the loss was ca. 15%. Sodium molybdate did not play any protective role.

Receptor levels during various stages of terminal oocyte maturation in the cytosolic preparation of brook trout

The receptor levels (N_{max}) in the cytosolic preparation decreased significantly during oocyte terminal maturation (Table 1.4). The K_a and N_{max} values were obtained from Scatchard analysis using $[^3H]$progesterone. The results were obtained using fresh ovarian follicles from the same batch of brook trout, sacrificed during various stages of terminal maturation during the same spawning season.

Sephacryl S-300 column chromatography of cytosolic preparation from brook trout ovarian tissue

The brook trout cytosolic preparations (5 ml) in TETS buffer containing 0.15M NaCl from stage 1 ovarian tissue were used for MW determination studies. The samples were equilibrated

Table 1.4

N_{max} values from brook trout cytosol at various stages

Stages oocytes	N^*	$K_a \times 10^9 \pm SEM \ (M^{-1})$	N_{max} f mol/mg protein
1	5	$0.21 \pm 0.019^{**}$	1928 ± 247.6
2	3	0.31 ± 0.008	1161 ± 70.5
3	5	0.20 ± 0.030	1180 ± 118.3 [†]
4 and 5	6	0.25 ± 0.048	514 ± 95.4 [†]
6 and 7	8	N.D. [††]	N.D.

* number of fish used for determination
** mean ± SEM
† significantly different from each other, $P > 0.05$
†† no specific binding

for 120 min with [³H]progesterone,
[³H]testosterone, and [³H]17α,20β-DHP at 5nM
concentration in the presence or absence of 1000 fold excess
inert steroid. After elution from the column only 3% to 5%
of the initial specific binding activity was recovered. This
loss was due to dissociation of the receptor steroid complex
during elution through the column. Experiments using either
[³H]progesterone or [³H]testosterone gave two main
peaks of specific binding activity at MW 110,000 and between
MW 30,000 and 40,000 (Figs. 1.10 and 1.11). When
[³H]progesterone equilibrated sample was frozen and
thawed before elution through the column a third main peak
of receptor activity at MW 210,000 was detected (Fig. 1.12),
although a shoulder peak at MW 210,000 was always present.
Slight variations in the MW and peak heights were observed
between experiments. When the cytosolic preparation was
equilibrated with [³H]17α,20β-DHP at 2.27nM
concentration, only one peak of specific binding activity
was obtained at MW 250,000. The same cytosolic preparation ,
after freezing and thawing and equilibration with
[³H]17α,20β-DHP (5.68nM) gave three specific binding
activity peaks at MW 240,000, 104,000 and 21,000. The peak
heights and resolution using [³H]17α,20β-DHP were not
as clearly defined as when using [³H]progesterone and
[³H]testosterone.

The peak fractions from [³H]17α,20β-DHP equilibrated

Fig. 1.10. Sephacryl S-300 elution pattern of freshly prepared brook trout cytosolic extract in TETS buffer containing 0.15M NaCl. The receptor preparation (5 ml) was pre-equilibrated with [^{3}H]progesterone (□), the molecular weights of the peaks obtained were 113,000 and 44,000. The receptor preparation (5 ml) was similarly pre-incubated with [^{3}H]progesterone + 1000 fold excess inert progesterone (▲), non-specific binding occurred at MW 220,000.

Fig. 1.10.

884.
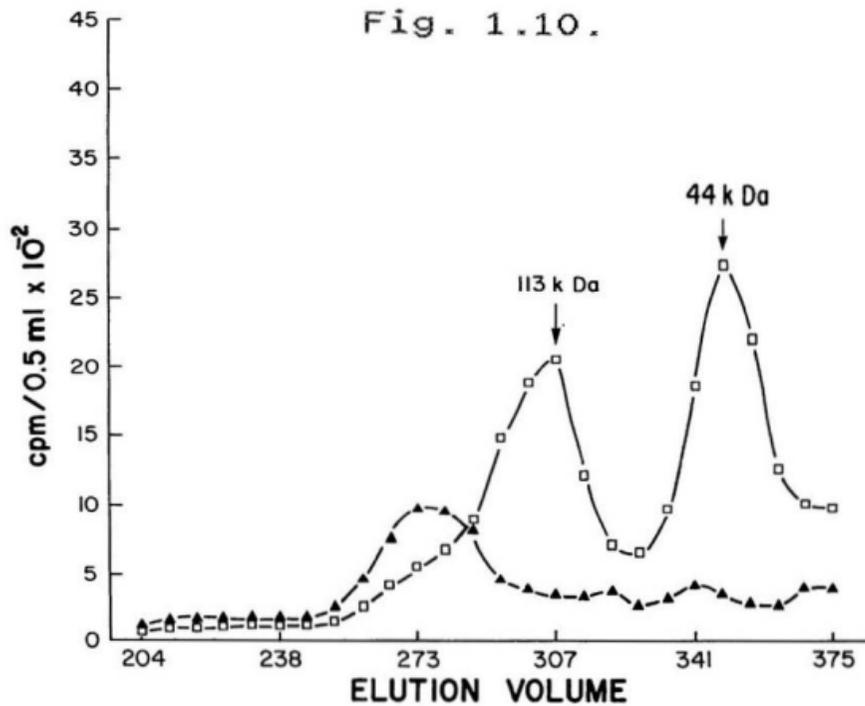
Fig. 1.11. Sephacryl S-300 elution pattern of freshly prepared brook trout cytosolic extract in TETS buffer containing 0.15 M NaCl. The receptor preparation (5 ml) was pre-incubated with [^{3}H]testosterone (□), the MW's of the peaks obtained were 110,000 and 32,000. Similarly receptor preparation was pre-incubated with [^{3}H]testosterone + 100 fold inert testosterone (▲), non-specific binding occurred MW 210,000.
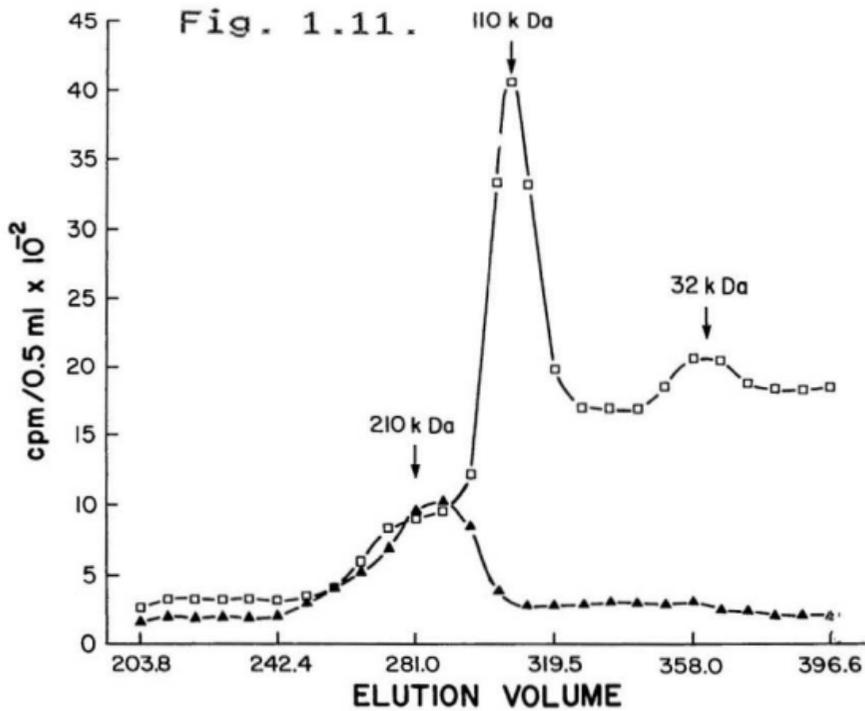
Fig. 1.11.

Fig. 1.12. Sephacryl S-300 elution pattern of brook trout cytosolic preparation in TETS buffer containing 0.15M NaCl. The receptor preparation (5 ml) was pre-incubated with [^{3}H]progesterone and then frozen at -18 deg C. After thawing and centrifugation the preparation was applied and then eluted from Sephacryl S-300 column (■), the MW's obtained were 214,000, 195,000 and 29,000. The receptor preparation was similarly incubated with [^{3}H]progesterone + 1000 fold inert progesterone (▲) and eluted.

Fig. 1.12.
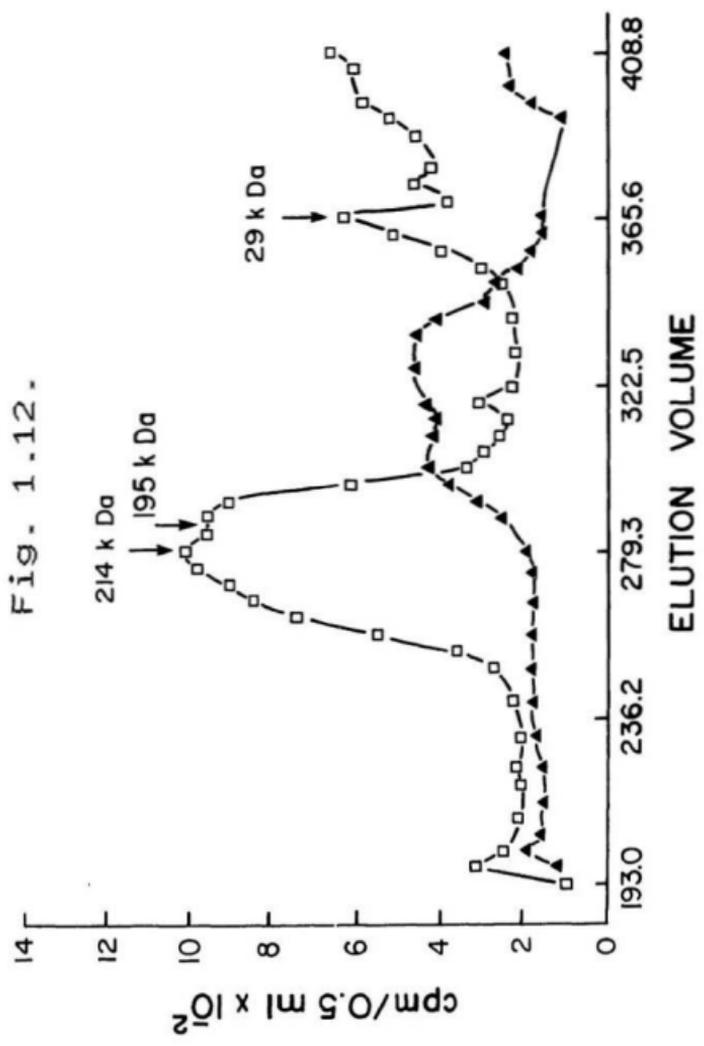
experiments, when re-equilibrated with the same labelled
steroid did not show any specific binding. Further, when the
peak tube fractions were pooled, concentrated and then
re-equilibrated with labelled steroid no specific binding
was obtained (both total and non-specific tubes gave about
the same amount of binding). Also when cytosolic preparation
in TETS buffer was applied on to the column and the
fractions post-labelled (equilibrated) with [³H]17α,20β-
DHP ± inert steroid and then assayed using charcoal-dextran
method, no specific binding was obtained (also pooled
concentrated fractions showed no specific binding activity,
although binding was present).

In rainbow trout cytosolic preparation using
[³H]progesterone prelabelled experiment two specific
binding peaks were obtained at MW 263,000 and 132,000.

HPLC using Spherogel-TSK 3000SW column

Pre-equilibration of brook trout cytosolic ovarian
preparation with [³H]progesterone gave specific binding
peaks at MW 294,000, 233,000, 52,000, 34,000 and 21,000.

SDS PAGE and PAGE electrophoresis

Following Sephacryl S-300 column chromatography of the
cytosolic preparation equilibrated with labelled 17α,20β-DHP
(2.27nM), the peak tube fraction obtained at MW 250,000 when
analyzed on SDS PAGE (under reducing and non-reducing

Fig. 1.13. SDS-PAGE analysis of peak tube of receptor activity obtained from Sephacryl S-300, after equilibration of cytosol with [^{3}H]17α,20β-DHP. The subunit MW's were 85,000, 67,000, 24,000 and 23,000. LMW, low molecular weights standards. HMW, high molecular weights standards.

Fig. 1.13.

(CYT) (HMW) (LMW) (HMW) (LMW) (CYT)
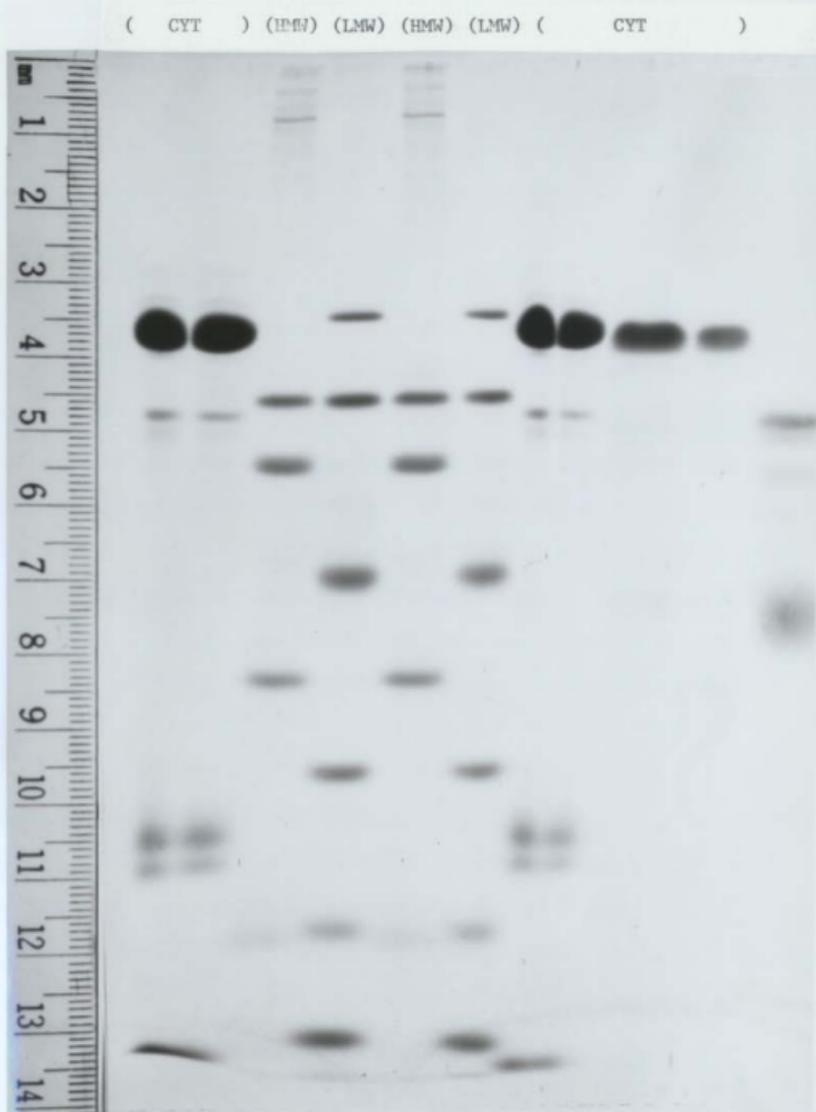
conditions), gave similar bands at MW 85,000, 67,000, 24,000 and 23,000. MW 67,000 band was broad, indicating a presence of very faint band at MW 65,000. The predominant band was at MW 85,000 (Fig. 1.13). On 5% and 4% PAGE gels a single band was obtained from the same cytosolic fractionated sample, using the Coomassie blue staining procedure.

Maturation studies

Concentration of steroids used were 50ng/ml. The only steroid effective in initiating maturation (GVBD) was $17\alpha,20\beta$-DHP at this concentration. Testosterone and R5020 did not bring about maturation (Table 1.5). R5020 inhibited GVBD response due to $17\alpha,20\beta$-DHP by 10%. $17\alpha,20\beta$-DHP also induced GVBD in ovarian follicles initially incubated for 168 h with either testosterone or R5020 (Table 1.5). It is important to note that testosterone binds the cytosolic receptor protein but does not bring about GVBD or maturation in the ovarian follicles.

Sucrose gradient density centrifugation

The brook trout cytosolic [^{3}H]$17\alpha,20\beta$-DHP receptor complex in absence of molybdate and DTT in the buffer sedimented on (5-30%) linear sucrose gradient at 3.05 S at 4 deg C (Fig. 1.14). A 500 fold molar excess of the radio inert ligand quenched the peak completely. The MW of the receptor complex was calculated to be 71,200, using catalase

Table 1.5

Maturation by various steroids in vitro at 10°C

	n	Time to GVBD	GVBD (%)
17α,20β-DHP	3	44 – 48 hr	100%
17α,20β-DHP + testosterone	3	44 – 48 hr	100%
17α,20β-DHP + R5020	3	44 – 48 hr	90%
testosterone	3	no GVBD in 168 hr	Nil
R5020	3	no GVBD in 168 hr	Nil
no steroid control	3	no GVBD in 168 hr	Nil
testosterone + R5020	3	no GVBD in 168 hr	Nil
testosterone + 17α,20β-DHP*	3	200 – 215 hr	100%
R5020 + 17α,20β-DHP*	3	200 – 215 hr	70%
control no steroid + 17α,20β-DHP*	3	200 – 215 hr	100%
testosterone + R5020 + 17α,20β-DHP*	3	200 – 215 hr	74%

* 17α,20β-DHP added after 168 hrs of incubation.

n = number of fish used, with each fish all samples were run in duplicate. On average 3% of oocytes were damaged due to mechanical shearing during incubation and were not scored.

Fig. 1.14. Sedimentation pattern of cytosolic receptor in
TETS buffer containing 0.15M NaCl. Samples (250 ul) were
equilibrated with $[^3H]17\alpha,20\beta$-DHP $\pm$ inert steroid and
applied on to (5 - 30%) linear sucrose gradients total
volume 11.6 ml (▲ sample + $[^3H]17\alpha,20\beta$-DHP; ■
sample + $[^3H]17\alpha,20\beta$-DHP + inert steroid). Catalase SW
10 was used as the external marker, the receptor complex
sedimented at 3.05 SW. units at 1 deg C.

Fig. 1.14.
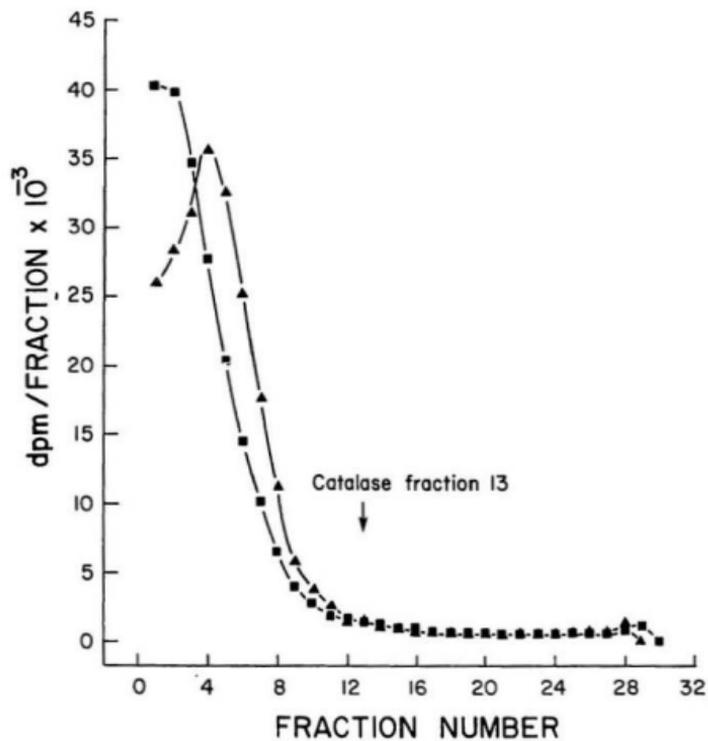
MW 232,000 as the standard protein.

In the experiment containing complete TETS buffer the SW value was 4.0, and MW value 92,000. In TETS buffer containing 0.15M NaCl the SW value was 5.0, and the calculated MW was 116,000.

Equilibrium dialysis

Initial experiments carried out using [^{3}H]17α,20β-DHP at concentrations below 1 nM gave variable binding results between the duplicate cells. This was due to variable amount of the steroid binding to both SpectraPor 2 and 4 membranes at the lower concentrations, this altered the amounts of steroid available for binding in solution. At steroid concentration above 1nM the binding obtained between duplicate cells was not as variable, and significant Scatchard plots were obtained. Although binding to membranes increased with increased concentration of the labelled 17α-,20β-DHP above 1 nM, indicating that it was not simply saturation of binding sites on the membranes above 1nM which gave more reliable results. The K_a values obtained between three different stage (1-2) ovarian cytosolic preparations in TETS buffer without molybdate but containing 0.15M NaCl, showed 10 fold variability. A typical saturation and Scatchard plot is indicated in (Fig.1.15 a and b), the K_a value was 0.34 x 10^{8}M^{-1}. The values for N_{max} was (992 fmoles/ mg protein), which is _ca._ twice

Fig. 1.15. (a). Binding of [³H]17α,20β -DHP to
cytosolic extracts using equilibrium dialysis (see text for
experimental details).

Fig. 1.15.a. Saturation plot
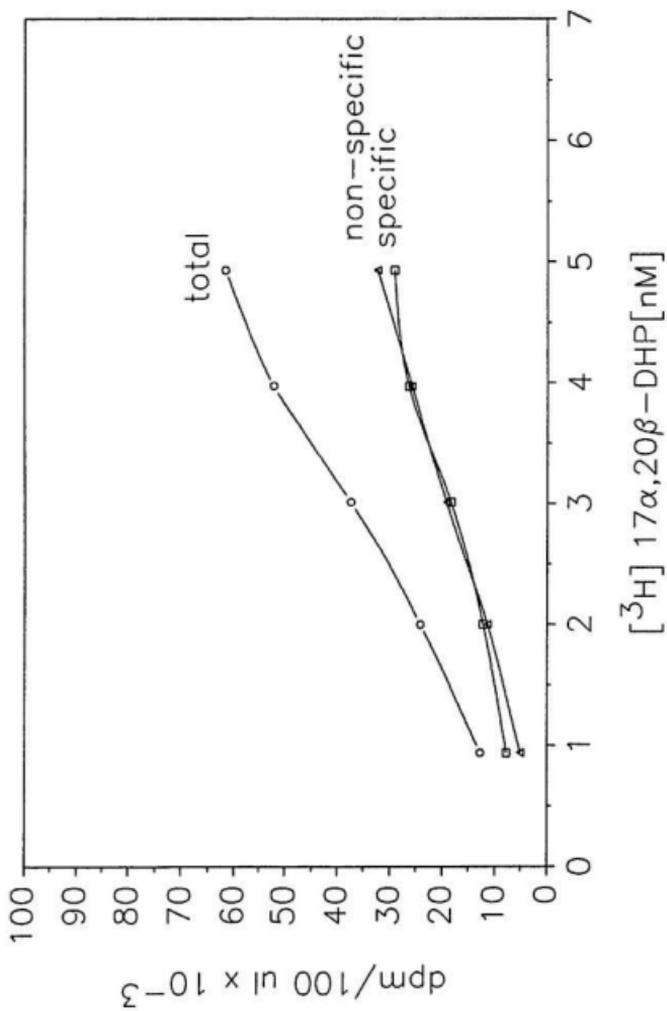
Fig. 1.15. (b). Linearization of specific binding by Scatchard analysis. The equilibrium association constant obtained from this graph was $0.34 \times 10^{-8} M^{-1}$, and the maximal concentration of binding sites was 992 f moles / mg protein.

Fig. 1.15.b. Scatchard plot
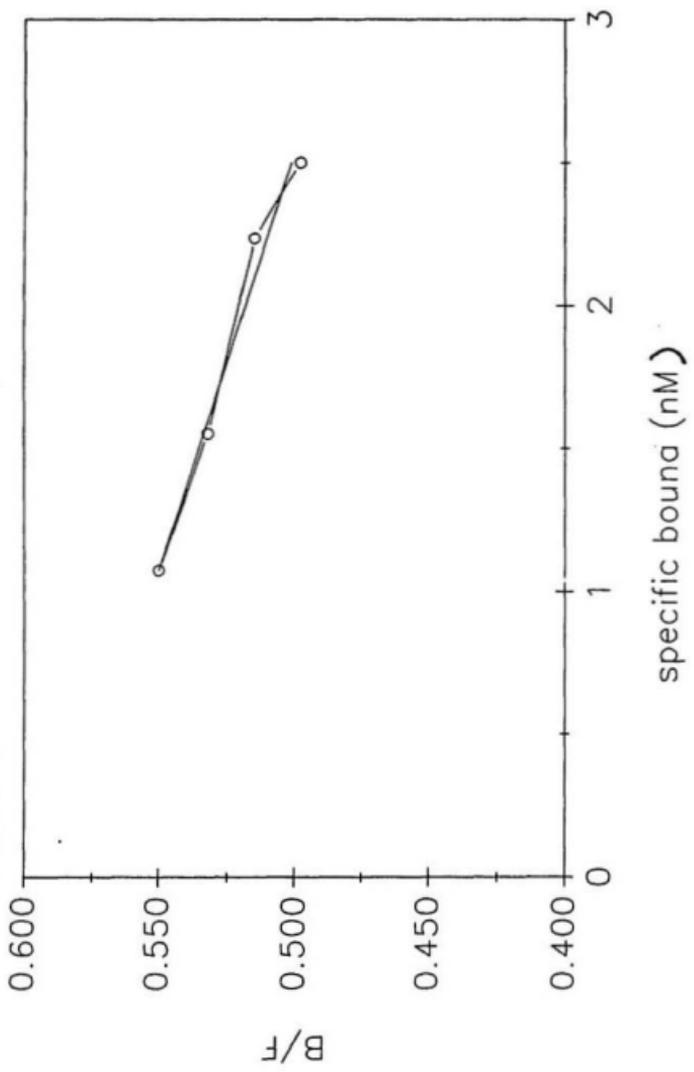
that obtained using charcoal-dextran separation method from
frozen brook trout stage 1 ovarian follicles in TETS buffer.
During equilibrium dialysis all the proteins present in the
cytosolic preparation which bind $17\alpha,20\beta$-DHP even with very
low affinities will contribute towards binding. While using
charcoal-dextran method of separation only the high affinity
receptors binding is taken into account, because the low
affinity sites are stripped off by dextran charcoal. This is
indicated by the shape of the saturation curve in case of
equilibrium dialysis (Fig. 1.15 a), which indicates multiple
binding (slight 'S'curvature). Points below 1nM steroid
concentration do not fit in the Scatchard plots which also
indicates that high affinity sites are present in the
preparation and are separate from the lower affinity sites.
Although both sites are contributing towards the final
binding, the affinities are not very different to give two
separate slopes in the Scatchard plot. The K_a obtained
from equilibrium dialysis were also lower than that obtained
from frozen brook trout tissue using $[^3H]17\alpha,20\beta$-DHP as
the ligand and charcoal-dextran method of separation, which
is in agreement with multiple binding hypothesis during
equilibrium dialysis. The equilibrium dialysis method is
highly recommended for use with purified protein
preparations, or with proteins with K_a which are
different enough to give separate slopes in the Scatchard
plot. In mammalian system the K_a for receptor proteins

are 100-1000 fold higher than in case of fish receptors and
thus multiple binding due to the presence of low affinity
cellular or extracellular proteins is easier to resolve from
the Scatchard plots.

Discussion.

Steroid receptors are proteins with high affinity, high
specificity and low capacity for the naturally occurring
steroid (Clark, et al . 1981). When the steroid binds to
the receptor a specific biological response ensues. The
results attained for $17\alpha,20\beta$-DHP cytosolic receptors from
brook trout does not satisfy all of the above criteria, but
are consistant with the presence of receptor activity
associated in a protein with relatively high affinity
(K_a= 1.39 $\pm$ 0.253 10^8 M^{-1}) and saturable
low capacity (N_{max} = 2090 $\pm$ 619 f mol/ mg protein).

Specificity.

The cytosolic $17\alpha,20\beta$-DHP receptor activity in brook and
rainbow trout did not demonstrate absolute specificity as
shown by competitive inhibition experiments. In salmonoids
the granulosa cells which synthesize $17\alpha,20\beta$-DHP are in
direct contact with the oocytes and the steroid hormone is
locally released and probably present at high concentrations
at the membrane site. This condition may eliminate the
necessity of the receptor having very high affinity and
steroid specificity, since competing steroids probably never
reach the oocyte membrane at high concentrations in vivo

during the physiological action of $17\alpha,20\beta$-DHP. Testosterone showed the highest affinity in the binding studies with the cytosolic receptors although it was not biologically active in bringing about GVBD. Also testosterone in the presence of $17\alpha,20\beta$-DHP did not suppress GVBD or maturation, since GVBD occurred within the same time period as with $17\alpha,20\beta$-DHP. These results indicate that cytosolic receptors may not be the active species involved in bringing about maturation. The decrease in the level of cytosolic receptors during the final stages of maturation, and the presence of plasma membrane receptors from the later stages of maturation also indicates that cytosolic receptors may become functional only after its integration into the plasma membrane of the oocyte (chapter 3).

Recently Canario and Scott (1988) have reported structure-activity relationships of C21 steroids in vitro oocyte maturation bioassays in rainbow trout Salmo gairdneri . They found four steroids, $17\beta,20\beta,21$-trihydroxy-4-pregnen-3-one; $3\alpha,17\alpha,20\beta$-trihydroxy-5-pregnane; $3\beta,17\alpha,20\beta$-trihydroxy-5 -pregnane; and $3\alpha,17\alpha,20\beta,21$-tetrahydroxy-5 -pregnane to be equipotent with $17\alpha,20\beta$-DHP. These results are in agreement with the non-specificity of the binding activity in the cytosolic preparations. The evidence for the presence of $17\alpha,20\beta$-DHP is overwhelming in the blood of female salmonoids undergoing maturation. This evidence includes detection of the steroid

by radioimmunoassay (review Goetz, et al .1987), double
isotope dilution assay (Campbell, et al . 1980) bioassay,
and mass spectrometry (Nagahama and Adachi, 1985). There is
virtually no evidence for the presence of the above four
steroids in either blood plasma or incubates from ovarian
follicles stimulated with gonadotrophin in salmonoids. The
lack of of high affinity and specificity for progesterone
receptors in reinitiating meiosis has also been reported in
Xenopus laevis oocytes (Baulieu, et al . 1978). Steroids
described as very active were testosterone,
deoxycorticosterone acetate and cortisol. Although the
authors conclude that the above steroids (other than
progesterone) are not physiologically related to maturation.
Similarly, in the Pleurodeles waltlii 'melanosome'
fraction of the oocytes, deoxycorticosterone, testosterone
and various other steroids interfered with specific binding
of labelled progesterone (Ozon and Belle, 1973).

Molecular weight.

The results obtained from Sephacryl S-300 column
chromatography, and HPLC suggests that the cytosolic $17\alpha,20\beta$-
DHP receptor protein is present in various molecular forms.
These molecular forms might be composed of a combination of
different subunits as demonstrated by SDS gel
electrophoresis of the main peak fraction from Sephacryl

S-300. The progesterone receptors from fresh human uterine
tissue show polymorphism with various synthetic ligands (Van
der Walt and Wittliff, 1986). Similar polymorphism has also
been reported for corticosteroid receptors in gills of brook
trout (Chakraborti, et al. 1987). The Sephacryl S-300
elution of the cytosolic preparation using
[^{3}H]progesterone, [^{3}H]testosterone and [^{3}H]17α-
,20β-DHP as binding ligands also showed polymorphism in the
corresponding radiolabelled eluted peaks. Wide variations in
molecular weights of cytosolic receptors has been reported
under various conditions. However, in the presence of sodium
molybdate striking similar structures has been observed
between MW's 280,000 to 330,000 for several classes of
steroid receptors. These structors are oligomeric, from
which the smaller receptor forms are derived by
dissociation. Differences in methodology, salt
concentration, freezing and thawing and temperature all
contribute to the wide variety of structures detected
(Gaubert, et al. 1986; Sherman, et al. 1983). The gel
filtration and HPLC results for 17α,20β-DHP cytosolic
receptors are similar to those of classical steroid
receptors. After elution from Sephacryl S-300 column the
ovarian cytosolic 17α,20β-DHP receptors did not bind
specifically to [^{3}H]17α,20β-DHP, indicating a change in
conformation (also known as receptor transformation).
Transformation, a common property of classical cytosolic

steroid receptors, is brought about by various procedures such as prolonged storage, dilution, gel filtration, warming and exposure to high concentrations of salts (Sherman and Stevens, 1984).

Rate of dissociation of the [^{3}H] $17\alpha,20\beta$-DHP receptor complex.

The classical somatic cell steroid receptors differ from brook trout $17\alpha,20\beta$-DHP oocyte cytosolic receptors in having a very slow rate of dissociation (k_{-1}) with the half life for the receptor complex in the order of hours compared to minutes for the ovarian $17\alpha,20\beta$-DHP receptors. The fast rate of dissociation indicates a different type of mechanism of action for the $17\alpha,20\beta$-DHP cytosolic receptors compared to the classical cytosolic steroid receptors.

Modulation of cytosolic receptor activity during various stages of final maturation.

The $17\alpha,20\beta$-DHP receptor activity in the cytosolic preparation from fresh ovarian follicles decreased from stage 1 to 5, and no receptor activity was obtained in stage 6-7. However, frozen stage 6-7 oocytes, manifested a small amount of binding activity. Since no nuclear receptors for [^{3}H]$17\alpha,20\beta$-DHP were demonstrated (chapter 2), one possible explanation is that the receptor activity, which has been shown to reside on the oocyte plama membrane

(chapter 3), becomes progressively less capable of being solubilized from the membrane during stages 1 to 7. The physiological significance of these observations will be discussed in chapter 3. Although the precise biological action of the steroid-receptor complex remains to be elucidated.

Summary .

The presence of $17\alpha,20\beta$-dihydroxy-4-pregnen-3-one ($17\alpha,20\beta$-DHP) oocyte receptor activity has been demonstrated in brook trout <u>Salvelinus fontinalis</u> . Scatchard analysis of the cytosolic fraction during various terminal stages of oocyte maturation gave a high equilibrium association constant (Ka) value of $1.39 \pm 0.66 \times 10^8$ M^{-1} (n = 7) and low maximum binding capacities (N_{max}). The association kinetics of the receptor was second order k_{+1} 2.29×10^6 M^{-1}. The dissociation rate constant k_{-1} was 1.502×10^{-2} sec^{-1} for the first order dissociation reaction. The $Ka = 1.52 \times 10^8$ M^{-1}, when it was determined from k_{+1}/k_{-1} a value close to that found from Scatchard analysis. Competition studies showed that receptor binding was not steroid-specific, and following binding affinities were obtained testosterone $> 17\alpha$-HP $> 17\alpha,20\beta$-DHP $>$ Progmegestone $>$ progesterone $>$ estradiol $>$ pregnenolone; cortisol showed no competitive inhibition. Cytosolic extracts when pre-equilibrated with various labelled steroids and eluted from a Sephacryl S-300 column gave multiple specific binding peaks. On sucrose density gradient centrifugation specific binding was obtained at 3.05 S in a cytosolic preparation containing 0.15 NaCl buffer. The receptor lost binding activity when incubated with various proteases, but DNase

and RNase had no effect. The receptor levels in cytosolic preparation from fresh tissue decreased progressively during final maturation (stages 1-5), and no receptor binding was observed in the late stage (6-7) ovulated oocytes.

The target organ receptor in fish for $17\alpha,20\beta$-DHP has not been reported. This is the direct first evidence of such receptor activity in the ovarian tissue of brook trout.

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CHAPTER 2

Nuclear receptors

Introduction.

The concept that steroid hormone action is mediated by
interaction with specific target tissue receptor proteins
has been accepted by endocrinologists, but changes in the
classical steroid receptor model proposed over 25 years ago
by Jensen and Jacobson (chapter 1) have taken place, and
subcellular localization of unoccupied receptors is a recent
controversy. Recent experimental evidence strongly suggests
that localization of a large proportion of the unoccupied
receptors occurs in the nuclei (Table 2.1.). Various models
of cytosolic/ nuclear receptor distribution have been
described as follows:- 1) Two-step model of steroid hormone
action proposed independently by Gorski, et al . (1968) and
Jensen, et al . (1968). According to this model, the
receptors initially interact with the steroid ligands in the
cytoplasm of the target cells, and are transformed, and then
translocate to the nuclei (see chapter 1.). 2) Equilibrium
model was proposed by Williams and Gorski (1972). The
cytosolic and the nuclear receptor binding sites are in a
rapidly reversible equilibrium. Binding of the hormones in
the cytoplasm, appears to shift this equilibrium, such that
a large percentage of the filled binding sites becomes

Table 2.1.

Reports on experimental demonstration of nuclear unoccupied
steroid hormone receptor

Hormone.	Tissue.	Reference.
Estrogens.	_Squalus_ testis.	Callard and Mak, 1985.
	Breast cancer cells.	Garola and McGuire, 1977.
		Zava, _et al_. 1977.
		Geier, _et al_. 1982.
		Zava and McGuire, 1977.
		Panko and Macleod, 1978.
	Brain cells.	Sheridan, 1975.
		Meada, _et al_. 1983.
		Clark, _et al_. 1982.
	Chick oviduct.	Schimke, _et al_. 1975.
	Leydig tumor cells.	Mcada, _et al_. 1983.
	Liver.	Mester and Baulieu, 1972.
		Defer, _et al_. 1974.

Ozon and Bell, 1973.

Pituitary tumor cells. Sonnenschein, _et_ _al_ . 1977.

Uteri. Giannopouios, _et_ _al_ . 1980.

Carson and Gorski, 1980.

Geier, _et_ _al_ . 1980.

Levy, _et_ _al_ . 1980.

White, _et_ _al_ .1981.

Androgens. Brain cells. Sheridan, 1975.

1,25 $(OH)_2D_3$

Intestinal mucosa Waltors, _et_ _al_ .1980.

Kream, _et_ _al_ . 1976.

Lawson and Wilson, 1974.

Pituitary Gelbard, _et_ _al_ . 1980.

Ecdysteroid Imaginal dics Yund, _et_ _al_ . 1978.

Progestins Not found.

associated with the nuclear fraction. 3) Distribution model was proposed by Sheridan, et al .(1979). The free steroid can enter both the cytoplasm and the nuclei of the cell. The unbound receptors are in equilibrium, partitioned between nucleus and the cytoplasm, according to the free water content of these intracellular compartments. 4) The affinity model was proposed by Walters, et al .(1981 a and b), and Walters, (1985). This model is a slightly modified model of that of Sheridan, et al . (1979), where the final distribution of receptors between the cytoplasm and the nuclei, is further dependent on the affinity (K_N) of the untransformed receptors for the nuclear acceptor sites. This model is schematically presented in Fig. 2.1. 1) The lipophilic steroid hormones (S) are distributed within both cytoplasmic and nuclear compartments. 2) The unoccupied receptors (R) migrate between both the cytoplasmic and nuclear compartments, and are in an equilibrium state defined by the partitioning within the free volumes of each compartment, and the affinity (K_N) of the untransformed receptors for the nuclear acceptor sites. The unoccupied receptors are believed to be primarily concentrated in the nuclei in a reversible equilibrium binding state with the nuclear matrix and /or chromatin. 3) Steroid binding to the receptor results in the conversion of what is probably a transient ([RS]) occupied receptor species into the biologically active occupied transformed ([RS*]) receptor

Fig. 2.1. A revised model of steroid-receptor interaction and induction of cellular response. Reproduced from Endocrine Reviews, 1985. vol. 6. pp.512-543. "Steroid hormone receptors and the nucleus." Author Marian R. Walters.
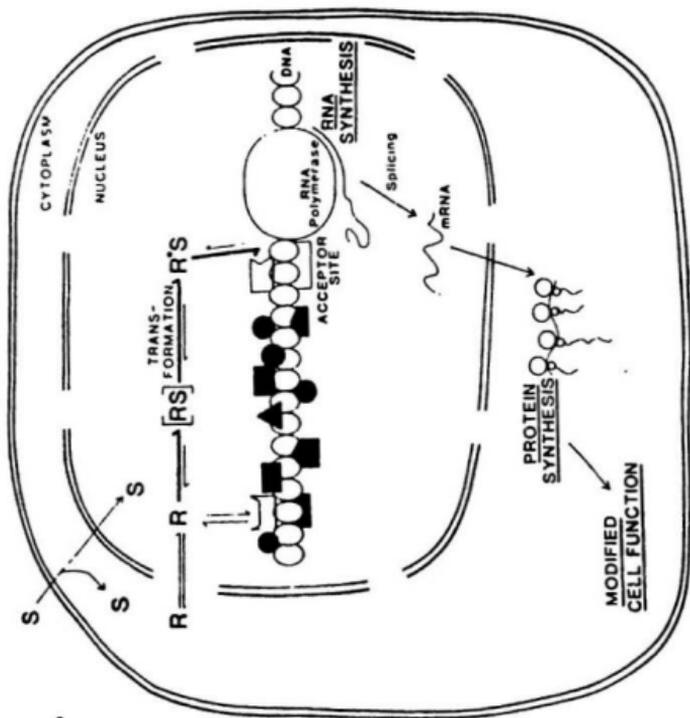
Fig. 2.1.

form. 4) On dilution of the cytoplasm, the cytosolic
concentration in vitro of biologically active receptors
which contain weakly bound steroids suggests that most of
these steps are reversible to some extent. 5) The affinity
(K_N) of the transformed receptor for the nuclear
acceptor sites is elevated, favoring receptor binding to the
acceptor sites and resulting in the induction (or
occasionally repression) of mRNA transcription, altered
protein synthesis, and regulation of cell function. These
models based on experimental results, have been useful in
providing a simple understanding of the different
subcellular localizations of the receptor species, whether
occupied or unoccupied, both in vitro and in vivo .

Thus according to the revised model of steroid-receptor
interaction (Walters, 1985), it is possible that the
receptors obtained during the cytosolic preparation of brook
trout ovarian follicles as described in chapter 1, could
have come from the oocyte nucleus or germinal vesicle (GV)
during the homogenization process mainly due to breakdown of
the GV and dilution of the cytoplasm. If such were the case
more unoccupied receptors should be found in the nuclei
pellet fraction of the ovarian follicular preparation during
earlier stages of maturation. This possibility was
investigated initially, by gently homogenizing the stage 1
ovarian follicles in low ionic strength buffer without

molybdate, and then measuring the nuclear receptor activity
by 1) binding studies as described in chapter 1, and 2) by
DNA cellulose column chromatography (Callard and Callard,
1987; Alberts and Herrick, 1970; Fox and Pardee, 1971). Only
non-specific binding was obtained from the nuclear pellet
assays, using the above methods. The possibility that the
high non-specific binding (due to the contamination of
cytosolic and membrane associated proteins) could have
masked a small specific activity in the nuclei was
investigated by isolation of whole intact nuclei. Various
methods have been used for isolation of somatic cells nuclei
(Lawson, et al .1984). Ideally isolated nuclei should
contain all of the sub-nuclear components and functional
activities initially present (Simard, (1970); Bouteille, et
al .1975; Puvion and Bernhard, 1975), and also be free from
contamination by cytoplasmic material. There are three main
considerations to take into account during nuclear
extractions from cells 1) the disruption of cells to avoid
nuclear breakdown, 2) solution used to stabilize nuclear
structure and/or composition, and 3) separation of nuclei
from solubilized extranuclear material. Generally nuclei
have higher densities (1.32) compared to other cell
organelles, therefore nuclei can be separated from other
cell components by isopycnic density centrifugation. The
method used for oocyte nuclei separation was initially used
in obtaining nuclei from erythrocytes of brook and rainbow

trout. The nuclei were then used in binding studies with radiolabelled $17\alpha,20\beta$-DHP, and photoaffinity labelling with labelled R5020.

A direct association of steroid-receptor complexes with the chromatin in target cell nuclei has been observed for estrogen (Hamilton, 1968; Steggles, et al .1971), testosterone (Steggles, et al .1971), aldosterone (Swaneck, et al .1970), and progesterone (Steggles, et al .1971). In the case of progesterone in chick oviduct system, it has been demonstrated that receptor binding is associated with non-histone proteins of the nuclei, which when complexed with DNA constitute the chromatin acceptor sites (Spelsberg, et al .1971). The presence of acceptor binding sites, in the isolated oocyte and erythrocyte nuclei for the $17\alpha,20\beta$-DHP-receptor complex, was also investigated using covalently bound [^{3}H]R5020-receptor complex, obtained after photoaffinity labelling and purification of cytosolic preparations.

Materials and methods

FISH

Female, prespawning brook trout, __Salvelinus fontinalis__
(length 22-28 cm, weight 0.12- 0.23 kg) were obtained from
Long Pond, St. John's, Newfoundland using gill nets by the
Ocean Sciences Centre / Marine Lab personnel. The fish were
caught during the week of September 3, 1987, and were
exposed to natural photoperiods and housed in a fiberglass
round tank of 2250 l capacity at ambient temperature in
filtered pond water. Rainbow trout __Oncorhynchus mykiss,__
were purchased from a fish hatchery in Hopeall, Newfoundland
and used immediately.

Steroids

Promegestone, [17α-methyl [^{3}H]R5020 (specific activity
85 ci/m mol) and [1,2-^{3}H]17α-HP (specific activity 50
ci/m mol) were purchased from New England Nuclear
Corporation (Canada), Lachine , Quebec. [1, 2-^{3}H]17α,20β-
DHP (specific activity 50 ci/m mol) was synthesized from
[1,2-^{3}H]17α-HP (Simpson, et al . 1964). The tritiated
steroids were purified by paper chromatography or thin layer
chromatography using standard methods. Radioinert 17α,20β-
·DHP was purchased from Steraloids, Inc., Wilton, N.Y. Inert
R5020 was supplied by New England Nuclear Corporation
(Canada), Lachine, Quebec.

Chemicals

Trizma pH 7.4, sodium molybdate, glycerol, EDTA, activated charcoal, dithiothreitol, MOPS, HEPES, polyoxyethylene 23-Lauryl ether (Brij 35) and common inorganic and organic chemicals were purchased from Sigma Chemical Company, St. Louis Missouri, U.S.A. Common organic solvents were purchased from Fisher Scientific, Caledon, BDH and were of highest purity available. Liquid scintillation fluid (Ready safe) was purchased from Beckman Instruments, Toronto, Ontario. Millipore MilliQ, 18 mega ohm water was used throughout (Amicon Canada Ltd. Oakville, Ontario).

Measurement of protein

Protein samples were measured using the Bio-Rad micro dye-binding procedure with gamma globulin as the protein standard (Bio-Rad protein assay kit 1).

DNA isolation and measurement

The isolated nuclei preparation was taken up in 5% perchloric acid by heating at 70 deg C for 15-20 min, and the supernatant used for DNA measurement with diphenylamine reagent (Schneider, 1945; Burton, 1956).

Measurement of radioactivity

Liquid scintillation counter Model 300C TRI-CARB United Technologies/Packard Instruments, fitted with a DPM option program was used. The [3H] standards efficiency curves were generated at least every 30 days. [3H] efficiency

check was carried out at each count using automatic
efficiency control. All samples were counted at 0.2%
statistical precision or 20 min whichever came first.

Attempted nuclear binding assays

Fish were sacrificed by a sharp blow on the head, and the
ovaries were dissected out. The ovarian epithelium, and
attached connective tissue was removed and the ovarian
follicles were weighed (3.5 g), and then carefully
homogenized in 10 ml of TED buffer (10 mM TRIZMA, pH 7.4;
1.5mM EDTA; and 1mM dithiothreitol) in a Potter-Elvehjem
homogenizer (glass/teflon) with a mechanical drive. The
speed of the homogenizer was increased slowly till breakage
of the minced ovarian follicles occurred. The homogenate was
then centrifuged at 2,500 rpm (750 x g) for 30 min. The
pellet was quickly washed with TED buffer containing 0.1%
Triton X-100 (5 ml), and after centrifugation rewashed with
TED buffer (5 ml). The pellet was then suspended in 0.5M KCl
in TED buffer using a glass rod. The resulting nuclear
suspension was then centrifuged at 10,000 rpm (25,000 x g)
for 10 min, the supernatant separated and diluted (1:5) with
TED buffer and then treated with charcoal-dextran to remove
endogenous steroids for 30 min. All the experimental
procedures were carried out between 1-4 deg C.

Binding studies using [3H] 17α,20β-DHP

The procedure as described in chapter 1 for binding studies

using cytosolic receptor preparation was followed. a) The
free from bound steroid separation was carried out using
charcoal-dextran solution at time intervals of 5 and 10 min
as previously described. b) Free steroid was separated from
bound using protamine sulphate (250 µl of 1 mg/ml in TED
buffer) in each assay tube containing 250 ul of nuclear
extract. Protamine-precipitated protein was incubated in
duplicate with increasing concentrations of labelled steroid
(1-10 nM) for 12 h in the presence (non-specific) or absence
(total) of 1000 fold excess inert steroid. Bound and free
steroid were separated, after addition of 250 ul of
hydroxy-apatite slurry to the samples (to aid in washing of
the protamine sulphate precipitated nuclear proteins). The
tubes were centrifuged, and the pellet washed with 3x 1ml of
ice cold TED buffer, and then extracted with 1 ml of ethanol
at room temperature for 1 h. Aliquots (500 ul) of ethanol
extracts were then counted with scintillator.

<u>DNA cellulose column chromatography</u>

DNA cellulose (1.5 g, Lot 33F-80301 from Sigma Chemicals,
capacity 5.7 mg double stranded DNA/g) was mixed with 1.5 g
of Avicel microcrystalline cellulose (Brinkman Instruments),
and suspended in TEDG buffer (50mM TRIZMA 7.4, 0.25mM EDTA,
0.5 mM DTT and 10% glycerol). After removal of suspended
'fines' the DNA cellulose matrix was packed into a Pharmacia
C10/10 column. The column was coupled with an AC10 adapter

to a Gilson minipuls 2 pump and Gilson micro fractionator.
Total bed volume of the column matrix was 6.7 ml. Two
columns were run simultaneously, and used under identical
conditions.

DNA cellulose column chromatography of nuclear pellet extract.

Frozen brook trout ovarian tissue from stage 1 and stage 6-7
was used. Ovarian tissue (5.0 g) was gently homogenized
(after mincing with scissors), using glass/teflon
homogenizer for 3x10 sec at 60 sec intervals in 20 ml of
TEDG buffer pH 7.4. The homogenate was then centrifuged at
2,500 rpm (750 x g) for 30 min. After separating the
supernatant from the pellet, the latter was quickly washed
with 5.0 ml of buffer and recentrifuged for 5.0 min. The
supernatant was discarded, and the pellet was resuspended in
5.0 ml of TEDG buffer containing 0.5M KCl or NaCl and
centrifuged at 15,000 rpm (25,000 x g).

The above nuclear pellet extract was diluted (1:10) using
TEDG buffer, then treated with charcoal-dextran for 60 min
to remove endogenous steroids. The nuclear extract
containing charcoal-dextran was then recentrifuged at 15,000
rpm (25,000 x g) for 10 min. Two 5.0 ml aliquots of the
above charcoal-dextran treated supernatents were then
equilibrated with $[^3H]17\alpha,20\beta$-DHP (5 nM) with or
without inert steroid (100nM) for 12h, and then applied to

two separate but identical DNA cellulose columns. The
samples were equilibrated on the columns for 60 min at 4-6
deg C, and then washed with TEDG buffer till all the unbound
radioactivity was removed (50 ml); the column was then
further eluted using 0.05 to 1.0M linear KCl or NaCl
gradient in TEDG buffer. Eighty fractions of 0.6 ml were
collected, and 200μl samples were counted for radioactivity
in 10 ml of scintillation fluid; the rate of flow through
both columns was 9 ml/h.

Isolation of nuclei from erythrocytes of brook and rainbow
trout

Fish were removed from tanks and sacrificed by a blow on the
head, and then placed on ice. A blood sample was taken,
without heparin using a syringe, from the caudal artery in
the caudal peduncle within 60 sec of introducing the hand
net and catching the fish. The blood was immediately
centrifuged (1.0 ml) in duplicate samples in 1.5 ml
polypropylene micro test tubes (Bio-Rad Laboratories,
catalog 223-9505), using a IEC Micro-MB centrifuge for 2 min
at room temperature. The serum was aspirated, and to the red
blood cells precipitate was added 0.5 ml of fish
homogenization buffer (FHB) (20 mM (3-[N-N-morpholino]
propane sulphonic acid) MOPS, 0.5% Triton X-100, 2 mM Mg
$(CH_3COO)_2$, 3mM $CaCl_2$, 0.1mM EDTA, 2mM DTT),
the tubes were vortexed to hemolyze the cells, and their
contents applied on to the surface of

fish cushion buffer (FCB) (2M sucrose, 2mM
Mg(CH$_3$COO)$_2$, 3mM CaCl$_2$, 0.1mM EDTA, 20 mM
MOPS pH 7.2, 2mM DTT) (10 ml) contained in 'ultra-clear'
tubes (Beckman 344059) and centrifuged in a Beckman model
L5-50 ultra-centrifuge using a SW 41-Ti rotor at 25,000 rpm
(77,100 x g) for 60 min at 5 deg C. The supernatant was
carefully aspirated and to the pellet (nuclei) at the
bottom of the tube was added 0.5 ml of nuclear storage
buffer (NSB) (50% w/w glycerol, 5mM DTT, 30mM HEPES
(N-2-Hydroxy ethyl piperazine-N'-2-ethanol sulphonic acid)
pH 7.55, 10mM potassium acetate, 10 mM magnesium acetate,
and gently mixed.

Binding of erythrocyte nuclei to [^{3}H] R5020-receptor complex

Cytosol preparation after photoaffinity labelling with
[^{3}H]R5020 (chapter 3) was fractionated on a Sephacryl
S-300 column and the peak tube containing covalently
labelled receptor complex (500 μl) was equilibrated with the
above nuclei suspensions for 8h at 1 deg C. The control
tubes contained denatured [^{3}H]R5020-receptor complex
obtained by warming the tube at 60 deg C for 60 min. After
equilibration the samples were applied to FCB and
centrifuged to separate out the free radiolabelled receptor
complex from that bound to the nuclei as described above.
The supernatant was aspirated out and the pellet mixed with

500 ul of NSB and transferred to the scintillation vial, 2.0
ml of Protosol was then added and the capped vials kept
overnight at 50 deg C. After cooling, 20 ml of scintillation
fluid was added and samples counted after 6 h adaptation in
the dark or until constant counts were obtained. The
experiment was repeated three times. Rainbow trout
erythrocytes nuclei were also used in binding studies with
brook trout [^{3}H]R5020-receptor complex.

Photoaffinity labelling of brook trout erythrocyte nuclei in
an attempt to demonstrate the presence of nuclear binding
sites

The nuclei were prepared in duplicate tubes from 0.5 ml of
blood as described in the last section, and suspended in 1.0
ml of TETS buffer containing 0.1% Brij 35. Samples 250 ul,
were than added to 12 x 75 mm glass tubes containing vacuum
evaporated [^{3}H]R5020 (3.7 nM) and photoaffinity
labelled for 20 min under standard conditions as described
in chapter 3. The control tubes were not photoaffinity
labelled but treated similarly as the samples. The reaction
was terminated by addition of charcoal-dextran (250 ul), and
samples left at 1 deg C for 60 min. The tubes were
centrifuged at 3,500 rpm (1650 x g) for 10 min, and then 250
ul of supernatent counted. Furthermore, nuclei from 0.5 ml
blood were suspended in FHB (1 ml) and 250 ul in triplicate
were photoaffinity labelled as above. The control nuclei
were not photoaffinity labelled but treated with

[³H]R5020 and kept in the dark. The separation of free steroid from steroid bound to the nuclei was carried out by isopycnic centrifugation in FCB as described. The pelleted nuclei were then counted for radioactivity after solubilization in Protosol as described.

Isolation of nuclei from stage 1 and 3 ovarian follicles of brook trout, and photoaffinity labelling of nuclei

Brook trout ovarian follicles (10 g) after separation from ovarian epithelium, were placed in a 30 ml syringe and gently squeezed out, the cell sap was pressed out through size 50 nylon mesh to remove membranes and connective tissue. About 7.0 ml of filtrate was recovered, to which was added 28 ml of FHB and gently stirred using a glass rod until a homogeneous solution was obtained. The above homogenate (5.0 ml) was then applied into six tubes containing FCB (6 ml) and ultracentrifuged to obtained pelleted nuclei as described for erythrocytes. The presence of nuclei was demonstrated by measurement of protein and DNA. The nuclei were suspended in NSB (2 ml) and added to a scintillation vial containing vacuum evaporated [³H]R5020 (3.7 nM). After mixing three samples were photolyzed for 30 min and 3 control vials were treated similarly but kept in the dark. Aliquots (1.0 ml) of photolyzed and non photolyzed nuclei were applied on FCB (9 ml) and ultracentrifuged to separate [³H]R5020-nuclear

receptors from free [3H]R5020 as described. The
supernatants were aspirated, and the pelleted nuclei
containing bound [3H]R5020-receptors were suspended in
500 ul of NSB and transferred to a scintillation vial,
digested in Protosol and then counted as described
previously.

Gel filteration chromatography of nuclear pellet extract

The nuclear pellet extract from frozen stage 3 brook trout
ovarian follicles was prepared in 0.5M KCl containing TED
buffer as described. The nuclear extract was then diluted
with TED buffer (3 ml to 10 ml), and a 5 ml aliquot
equilibrated with [3H]progesterone (20 nM) for 22 h at
1 deg C. The equilibrated sample was then applied to a
Sephacryl S-300 column previously equilibrated with 0.15 M
KCl in TED buffer at pH 7.4. and eluted using the same
buffer. The experiment was then carried out using nuclear
extract from the same batch of frozen ovarian follicles
equilibrated with [3H]progesterone (20nM) in the
presence of inert progesterone (3uM). The complete
experimental protocol for Sephacryl S-300 column
chromatography has been described in chapter 1.

Results

Nuclear pellet extract assays.

As mentioned in the introduction section of this chapter, no
specific binding was demonstrated in the nuclear pellet
extracts, using either charcoal-dextran or protamine
sulphate methods for separation of bound steroid from free
after equilibration either with labelled progesterone or MIS
$17\alpha, 20\beta$-DHP.

DNA cellulose elution chromatography of nuclear extracts.

The elution pattern from DNA cellulose column chromatography
for brook trout gill nuclear cortisol receptors is
represented in Fig. 2.2. The cytosolic preparation from the
same fish did not bind DNA cellulose as depicted in Fig.
2.4. Oocyte (stage 6) nuclear receptors, elution pattern
from DNA cellulose column is indicated in Fig. 2.3, the
cytosolic extract also bound DNA cellulose (Fig. 2.5). The
specific binding obtained with nuclear extracts of brook
trout ovarian follicles was very small and not reproducible.
The peak receptor binding activity did not elute at the same
KCl concentration even from the same nuclear pellet
preparation. Similar results were also obtained from nuclear
extracts of stage 1 ovarian follicles.

Fig. 2.2. KCl gradient elution pattern from DNA cellulose column chromatography of brook trout gill nuclear pellet extracted receptors, equilibrated with [^{3}H]cortisol (5 nM) in the absence (■), and the presence of inert (3 μM) steroid (●).

Fig. 2.2
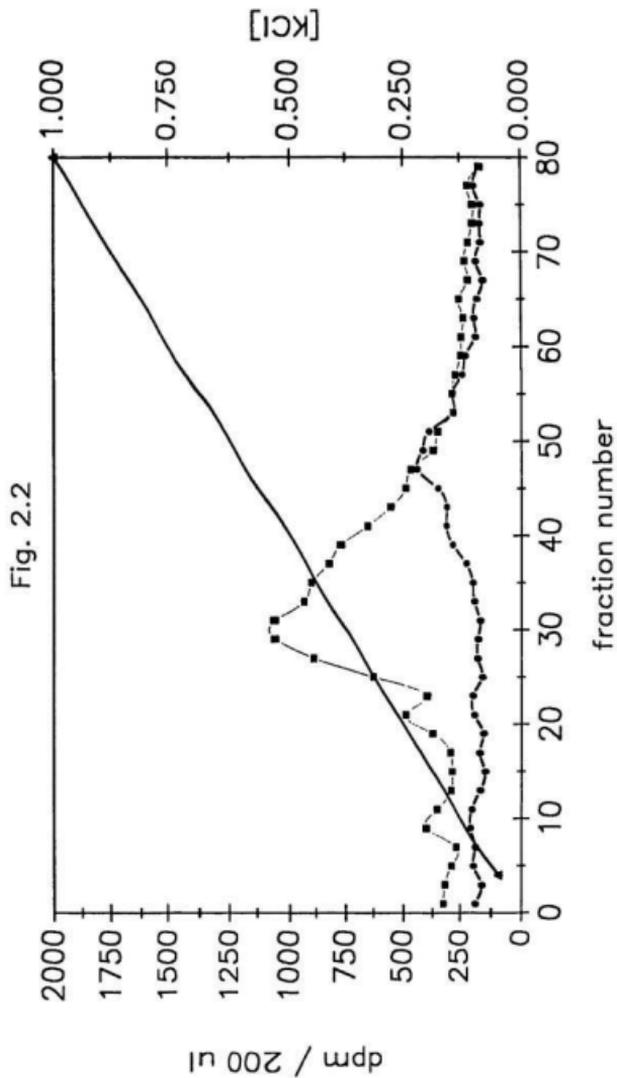
Fig. 2.3. KCl gradient elution pattern from DNA cellulose column chromatography of brook trout stage 6 nuclear extracted receptors, equilibrated with $[^3H]17\alpha,20\beta$-DHP (5nM) in the absence (�), and the presence of inert (3μM) steroid (●).

Fig. 2.3
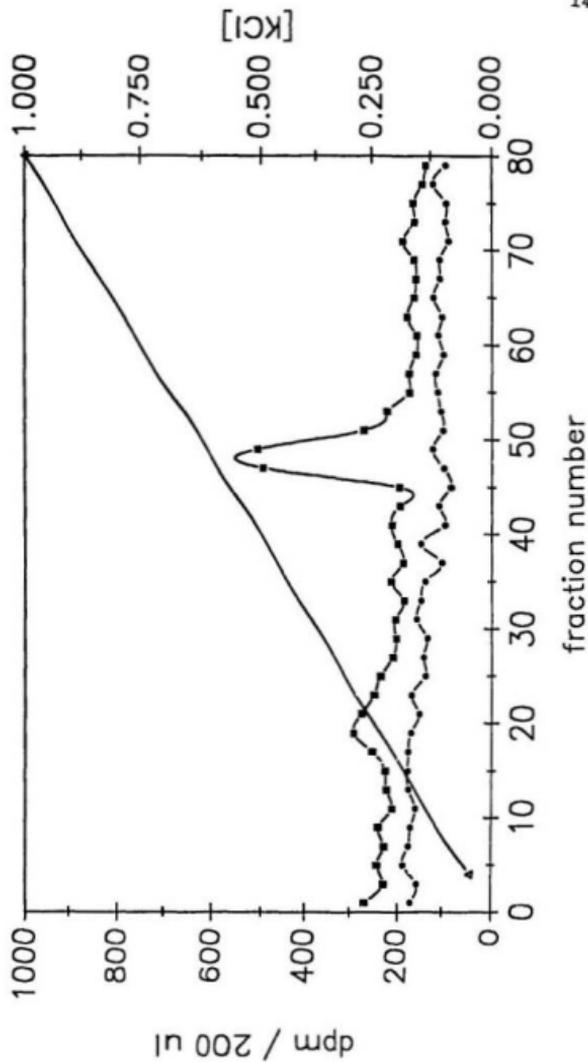
Fig. 2.4. KCl gradient elution pattern from DNA cellulose column chromatography of brook trout gill cytosolic receptors, equilibrated with [^{3}H]cortisol (5nM) in the absence (■), and the presence of inert (3μM) steroid (●).

Fig. 2.4
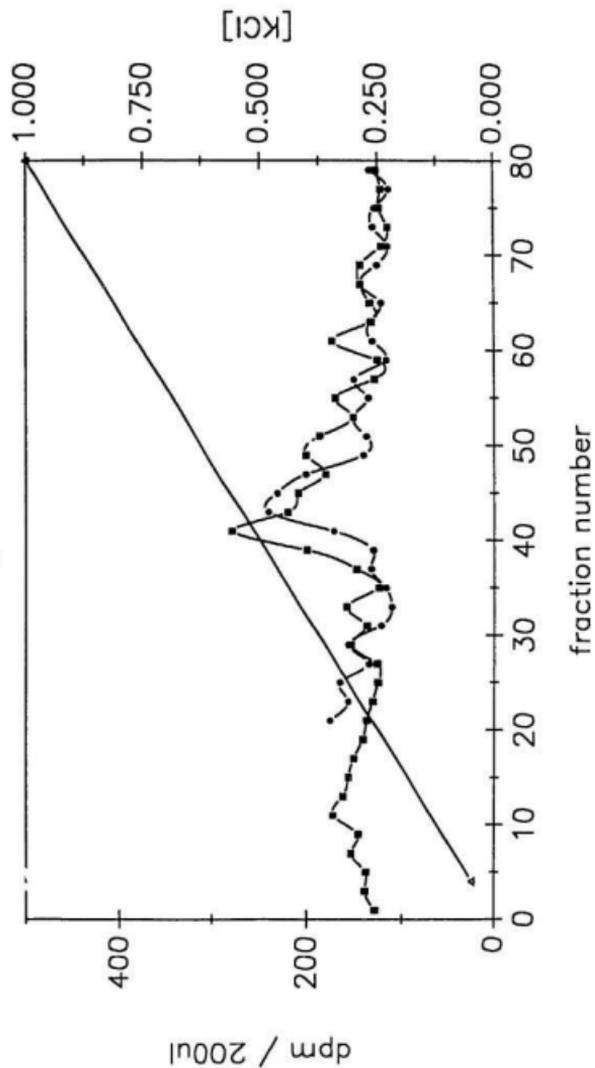
Fig. 2.5. KCl gradient elution pattern from DNA cellulose column chromatography of brook trout stage 1 cytosolic receptors, equilibrated with $[^3H]17\alpha,20\beta$-DHP (5nM) in the absence (■), and the presence of inert (3μM) steroid.

fig. 2.5
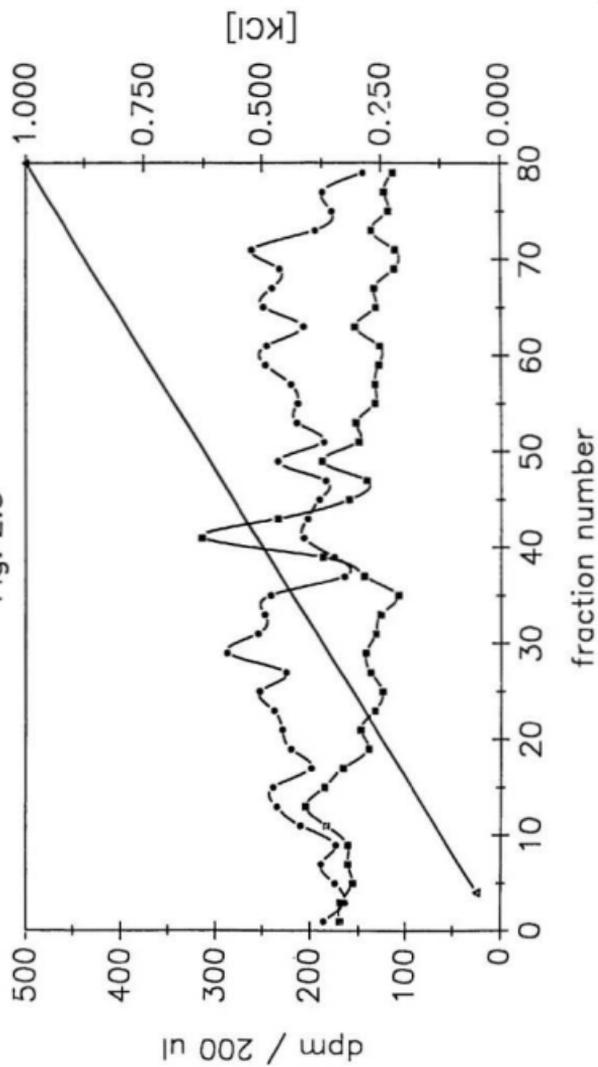
Photoaffinity labelling of brook trout oocytes and
erythrocyte nuclei.

The photoaffinity labelled and control nuclei, both gave
binding activity. Control levels were equivalent or even
higher than photoaffinity labelled sample values. The
experiment was then repeated using denatured nuclei (alkali
treated at pH 12 for 60 min at 40 deg C, and then
reacidified to pH 7), and the results obtained were similar.
These results suggested non-specific steroid binding to the
nuclei. The separation of free steroid from photoaffinity
labelled nuclear receptor was also carried out using the
isopycnic density method, and similar results were obtained.
The time of photoaffinity labelling was then reduced to 60
sec, and the results obtained were similar. Using
erythrocyte nuclei, the binding obtained was higher than
with the oocyte nuclear preparation, supporting the
hypothesis that non-specific hydrophobic binding of steroid
to DNA bases might be occurring.

[^{3}H] R5020-receptor complex binding to erythrocytes
nuclei.

Very little binding to the nuclear material was observed in
either the sample or the denatured receptor steroid complex
control, ca. 2000 dpm/ 10^6 erythrocyte nuclei. This
indicates that a small amount of non-specific binding
occurred in the nuclei.

Gel filteration chromatography of ovarian nuclear pellet extract.

Sephacryl S-300 elution pattern from brook trout nuclear pellet extract equilibrated with [^{3}H]progesterone (20 nM) gave MW of 298,000 and 32,000 (Fig. 2.6).

Fig. 2.6. Sephacryl S-300 elution pattern of brook trout stage 3 nuclear extract. The nuclear extract in 0.5M NaCl was diluted to 0.15M NaCl, and then 5 ml equilibrated with [^{3}H]progesterone (20 nM) in the presence and absence of inert steroid (3μM). The specific binding peak occurred at MW 298,000 and 32,000 (●). Non-specific binding peak activity occurred at MW 285,000 (◆).

Fig. 2.6

147 a.
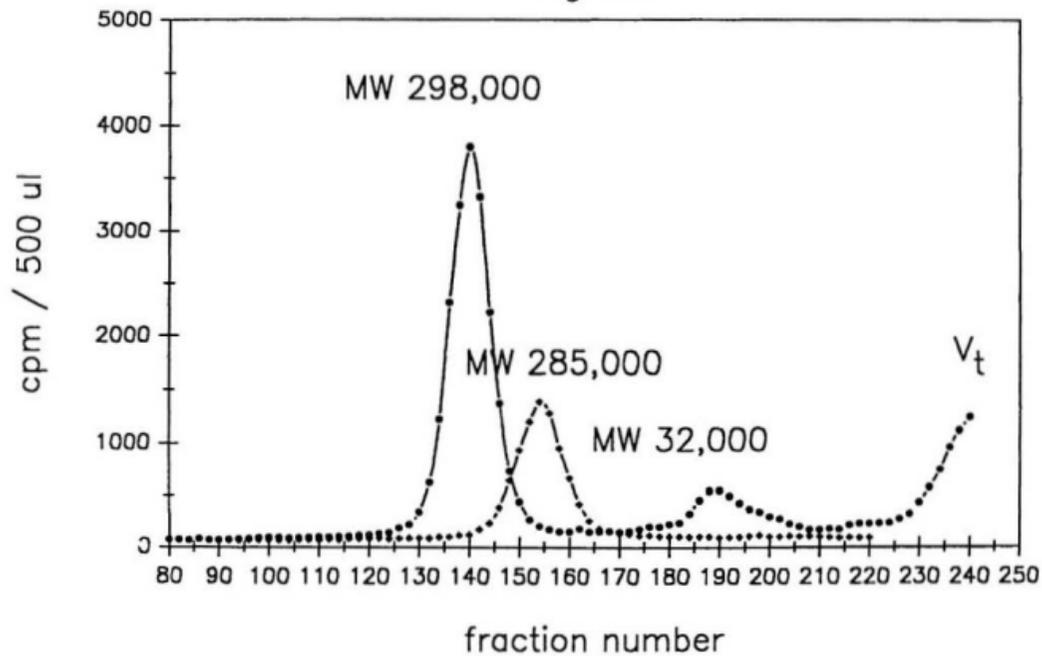
Discussion.

The results obtained from the nuclear pellet assay, and from
isolation and binding experiments of intact nuclei from
oocyte protoplasm demonstrated that non-specific binding
occurred for [³H]17α,20β-DHP.

DNA cellulose column chromatography has been used by various
researchers, to isolate receptors quantitatively from the
nuclear pellet extract. Transformed receptors which are
normally present in the nuclei, also have high affinity for
DNA cellulose and phosphocellulose, while unbound receptors
do not bind DNA cellulose or phosphocellulose. Also steroid
binding proteins from blood or tissues do not bind DNA
cellulose. DNA cellulose column chromatography indicated
very little specific binding occurred for [³H]17α,20β-
-DHP, and this binding was not reproducible since the
receptor labelled steroid complex was eluted at different
KCl concentrations through the DNA cellulose column. The
cytosolic preparation from brook trout ovarian follicles
also specifically bound to DNA cellulose, but the amount of
binding was very small compared to the specific binding
observed from cytosolic dextran-charcoal assay. An attempt
was made to identify the nature of the eluted labelled
complex from DNA cellulose column by PAGE (5% gel). The peak
tubes from 3 runs after concentration by lyophilization from
the nuclear and cytosolic ovarian follicles preparation did

not give any bands on the gel when stained with Coomassie blue. It is probable that the very small amount (50-100 dpm) of [^{3}H]17α,20β-DHP or a labelled impurity is adsorbed on to the cellulose moiety of the column and is released during elution.

A nuclear pellet assay for glucocorticoid receptors, carried out as reported by Chakraborti, et al . 1987, and DNA cellulose column chromatography of gill tissue nuclear extracts from brook trouts, previously injected with cortisol (to induce cortisol nuclear receptors in the nuclei), both gave reproducible specific binding activity, using [^{3}H]cortisol as the binding ligand (samples of gill tissue nuclear extracts were provided by Professor Weisbart, St. Frances Xavier University, Antigonish, N.S.). The same methods and reagents were utilized with the ovarian nuclear extracts and gave no specific binding. This rules out the possibility that any of the reagents, e.g. DNA cellulose, hydroxy apatite, protamine sulphate etc. or the conditions of the assay contributed to the failure to detect nuclear receptors from ovarian follicles.

The frozen ovarian follicular nuclear extract, once frozen and thawed, after dilution and equilibration with [^{3}H]progesterone (20 nM) was eluted through a Sephacryl S-300 column and gave specific binding peaks at MW's 298,000 and 32,000. These MW's values are close to those obtained

from cytosolic preparations. Since all of the above reported experimental evidence indicates the absence of nuclear receptors, these solubilized receptors must have come from the sub-cellular organelles precipitated with the nuclear pellet. The evidence for the presence of plasma membrane bound receptor activity from the zona radiata membrane of the oocyte is given in chapter 3.

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Chapter 3

Introduction.

Plasma membrane bound 17α,20β-DHP receptors in brook trout
oocytes

The primary action of steroids is on gene transcription
(Yamamoto, 1985). The range of steroid actions is immense;
to quote a few, steroid hormones affect various
physiological systems and processes e.g. intermediary
metabolism, immune system, ionic transport in intestinal and
renal epithelia, hypothalamic and pituitary hormone
synthesis and secretion, and also certain aspects of
behavior. Thus it is difficult to perceive a common cellular
mechanism of gene transcription for all the above steroid
actions, and it is becoming increasingly clear that steroid
hormones can act non-genomically either through specific
receptors in the cytoplasm or in the plasma membrane (Szego
and Pietras, 1981; Baulieu, 1983). In the last decade
experimental data has been obtained by various researchers
(Pietras and Szego, 1977,1979 a and b,1980; Blondeau and
Baulieu, 1985; Savart and Cabillic, 1986; Smith and Ecker,
1971; Suyemitsu and Terayama, 1975; Rao, et al . 1976,1977;
Fant, et al . 1979; Alllera, et al . 1980; Strel'chyonoc,
et al . 1984; Savart and Cabillic, 1986; Miguel, et al .
1987; Sadler and Maller, 1982; Sadler, et al . 1985;

Ozegovic, _et_ _al_ . 1977; Koch, _et_ _al_ . 1978) which
indicate the presence of plasma membrane receptors for
various steroid hormones, and their action via non-genomic
mechanisms.

In _Xenopus_ _laevis_ oocytes progesterone binds to an oocyte
membrane receptor protein (Sadler, _et_ _al_ . 1985; Blondeau
and Baulieu, 1984; Sadler and Maller, 1982; Ishikawa _et_ _al_.
1977). The possibility that a membrane receptor for the
salmonoid progestin, $17\alpha,20\beta$-DHP may also exist was
investigated. Nagahama and Kishimoto (1987), have
demonstrated that micro injection of $17\alpha,20\beta$-DHP into full
grown immature gold fish oocytes was ineffective in inducing
GVBD, while external application was effective. These
results suggests that the action of $17\alpha,20\beta$-DHP may be via
membrane-bound receptors. Supportive evidence for a
membrane-bound receptor was demonstrated by findings that
physiological response (GVBD) to $17\alpha,20\beta$-DHP was blocked by
cAMP (DeManno and Goetz, 1987, 1986; Jalabert and Finet,
1986). Further evidence for membrane bound receptor is
provided from studies using translational and
transcriptional inhibitors of protein synthesis; it has been
demonstrated that transcription of RNA is not a requirement
for steroid induced final maturation, although translation
is (Goetz, 1983), substantiating that this action of $17\alpha,20\beta$-
DHP is different from the mechanism of classical steroids

Fig. 3.1.
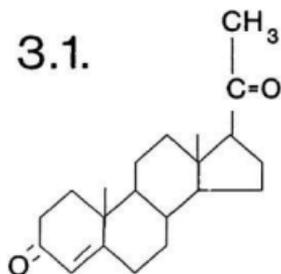
Progesterone

4-Pregnen-3,20-dione

17 α,20β-DHP
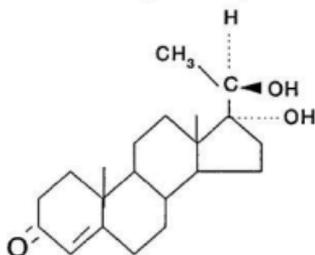
17 α,20β-dihydroxy-4-Pregnen-3-one
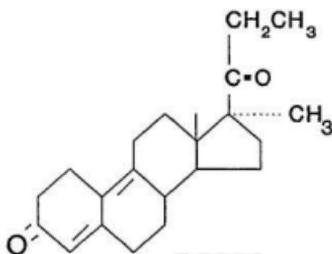
R5020

17,21-dimethyl-19-nor-Pregn-4,9-diene-3-20-dione

action in somatic cells where transcription of RNA from DNA
is the preliminary mode of steroid action.

The presence of a protein with receptor activity for $17\alpha,20\beta$-
DHP was demonstrated from fresh ovarian follicles of brook
trout cytosolic preparation (Maneckjee, et al . 1987, 1988,
and chapter 1). The levels of receptor activity decreased
from stage 1 to 5 and the receptor activity was not detected
in stage 6-7 cytosolic preparation. In Atlantic salmon
Salmo salar Ouananiche cytosolic preparation from fresh
stage 6-7 oocytes demonstrated no receptor binding activity
with either tritiated progesterone or $17\alpha,20\beta$-DHP as the
ligand. Although the cytosolic preparation from previously
frozen stage 6-7 oocytes demonstrated small amounts of
binding activity with labelled $17\alpha,20\beta$-DHP and progesterone.
Similar results were also obtained from brook trout
cytosolic preparations. Since no nuclear receptor binding
activity was obtained from either fresh or frozen ovarian
follical preparations (chapter 2), the above results
indicate that partial solubilization of plasma membrane
bound receptors occurred in stage 6-7 oocytes during
freezing/thawing cycle due to breakdown of membrane
structure by ice crystals.

Since photoaffinity (PA) labelling using labelled synthetic
progestin 17,21-dimethyl-19-nor-pregn-4, 9-diene-3-20-dione
(R5020) has been utilized for the isolation and

characterization of the membrane-bound oocyte progesterone receptors in _Xenopus_ _laevis_ , this method was applied to demonstrate the presence of MIS 17α,20β-DHP receptors in the isolated zona radiata membrane preparations from stage 6-7 oocytes. The plasma membrane of the oocyte is intercalated with the zona radiata membrane of the oocyte.

Affinity labelling of the binding sites for small ligand molecules with protein molecules has in numerous instances been helpful to identify the functional group components of such sites or to identify which protein in a complex mixture of proteins contains the site of interest. Usually it is not possible to use natural ligand as its own affinity reagent since it may not contain a suitable chemically reactive group. The structures of progesterone, MIS 17α,20β-DHP and synthetic progestin R5020 are shown in Fig. 3.1. Both progesterone and R5020 are competetive inhibitors of MIS 17α-,20β-DHP in binding with brook trout cytosolic receptor activity (chapter 1, Table 1.2). It is probable that progesterone and R5020 bind at the same site on the receptor as the natural steroid 17α,20β-DHP. R5020 contains a keto group in conjunction with a conjugated double bond, and is more readily excited by light of 280-320 nm to electronically excited states in chemically reactive species. The photoaffinity (PA) labelling of receptors by R5020 is a three stage process.

1) protein + ligand <--------> protein.ligand

2) protein.ligand <--$\overset{h\nu}{\text{-------}}$--> protein.ligand*   `

3) protein.ligand* ---------> chemically modified
protein

1) non-covalent binding of the ligand to the appropriate
site on the receptor molecule; 2) absorbtion of light by the
bound ligand resulting in its electronic excitation; 3)
reaction of the excited ligand with a functional group(s) in
its binding site (Martyr and Benisek, 1973).

Materials and methods

Isolation of zona radiata membrane fraction.

The stage (6-7) ovulated oocytes which were loose in the
abdominal cavity were centrifuged at 35,000 rpm (151,000 x
g) for 90 min at 1 deg. C in a Beckman Model L5-50
ultracentrifuge using a SW 41-Ti rotor and "ultra-clear"
tubes (Beckman 344059). The concept used involves rupture of
the oocytes by centrifugal force and separation of cell
organelles by differential and isopycnic centrifugation,
where yolk proteins act as the medium of separation. The
bottom of the tubes contain the zona radiata membrane
fraction which was separated by removing the upper fractions
and then cutting the tube just below the surface of the
membrane fraction to avoid contamination by the upper layer
during removal. The membranes were gently resuspended in
TETS bufffer using a glass rod and then vortexed and left in
ice water to settle out; the upper aqueous layer was
aspirated out, and the treatment repeated four times. At
this point there was no bound [^{3}H]R5020 receptor
complex after photoaffinity labelling of the supernatant.
After the final wash the membranes were centrifuged at
10,000 rpm (15,000 x g) for 10 min at 5 deg C.

Photoaffinity labelling using labelled R5020.

Cytosolic preparation. zona radiata membranes suspended in

TETS buffer, and Brij 35 (0.1%) solubilized extract of the zona radiata membranes in TETS buffer were all photoaffinity labelled using [^{3}H]R5020. The [^{3}H]R5020 solution was added to the glass scintillation vials or 12 x 75 mm disposable tubes and evaporated using a vacuum oven (Napco model 5381, Fisher Scientific). Samples were added and then vortexed and photoaffinity labelled on ice using two Westinghouse lamps (FS20; lamda max. 320 nm) positioned 5 cm above the sample, control samples contained [^{3}H]R5020 but were kept in the dark under similar conditions.

Photoaffinity labelling of brook trout oocyte cell sap (protoplasm).

The fresh ovarian follicles (stage 2) were quickly separated from connective tissue and placed in TBSS AT 1 deg C. The separated follicles (3 g) were placed on a 50 micron nylon screen and gently dabbed with tissue to remove TBSS adhering to the screen and the follicles. The follicles were then individually cut with a scalpel blade on the screen and the protoplasm of the oocyte gently squeezed out and collected. The follicular cells attached to the zona radiata remained intact and stayed on the nylon screen. The protoplasm (0.12 g) was mixed with TETS buffer containing Brij 35 0.1% (10 ml) at 1 deg C and then centrifuged (151,000 x g) for 60 min at 4 deg C. The resulting supernatant (5 ml) was then photoaffinity labelled (4.13 nM, [^{3}H]R5020) using

standard method as described above. The photoaffinity labelled sample was then applied on to a Sephacryl S-300 column, and eluted using TETS buffer containing Brij. Aliquots (250 μl) of alternate fractions were added to scintillation fluid (10 ml) and then counted for radioactivity. The control 151,000 x g supernatant was also treated with [3H]R5020 but not photoaffinity labelled, and fractionated on the Sephacryl S-300.

Saturation and Scatchard plots of zona radiata membrane receptor activity using labelled 17α,20β-DHP.

For the determination of ligand binding, 250 μl of solubilized zona radiata receptor preparation were pipetted into 12 x 75 mm glass tubes (Canlab T-1290-3) containing 0.15 - 5 nM [3H]R5020. All samples were incubated in duplicate. Incubation was started in batches of 8 tubes every 20 min. After 120 min the incubation was terminated by addition of 250 μl of charcoal-dextran suspension (0.5% charcoal, 0.05% dextran in appropriate TETS buffer used for extraction of zona radiata preparation). The charcoal-dextran treatment for the separation of free from bound was carried out for 5 min and 15 sec, after which the batch of 8 tubes was immediately centrifuged at 3000 rpm (1650 x g) for 10 min. The supernatant 250 μl was counted for radioactivity in 10 ml of scintillation fluid.

Inibition of photoaffinity labelling of cytosolic
preparation by excess inert R5020 and 17α,20β-DHP.

Cytosolic preparation (250 μl) was pipetted into 12 x 75 mm
glass tubes (CanLab T-1290-3) containing vacuum evaporated
[3H]R5020 to yield 0.19 nM steroid. The concentration
of inert 17α,20β-DHP and R5020 used was 3 μM. The initial
reaction rates of covalent binding to receptor protein were
measured at 1,3,5 and 10 min, the reaction was terminated by
the addition of 250 ul of charcoal-dextran, and the
separation time for free and bound steroid was 30 min. The
conditions of photoaffinity labelling and centrifugation
step for the separation of charcoal-dextran from bound
steroid were the same as in above section.

Saturation of photoaffinity labelling with time.

Cytosol preparation (250 μl) was pipetted into 12 x75 mm
glass tubes containing vacuum evaporated [3H]R5020 at a
concentration of 3.705 nM. The samples were photoaffinity
labelled for various times and reaction terminated by
addition of charcoal-dextran. The conditions of
photoaffinity labelling and separation of free steroid that
covalently bound to receptor were as described above.

Saturation of photoaffinity labelling of cytosol, with
regards to concentration of labelled R5020.

Cytosolic preparation (250 μl) was pipetted into 12 x 75 mm

glass tube containing vacuum evaporated [³H]R5020 to yield 0.1 to 12.5 mM steroid. Time for photoaffinity labelling was 2 min, the reaction was terminated by addition of charcoal-dextran. The conditions of photoaffinity labelling and separation of free from covalently bound to the receptor were as described above.

Effect of inert 17α,20β-DHP concentration on initial rate of photoaffinity labelling to cytosolic preparation

Cytosolic preparation (250 μl) was pipetted into 12 x 75 mm glass tubes containing vacuum evaporated [³H]R5020 (0.19 nM) and inert 17α,20β-DHP at various concentrations. Time of photoaffinity labelling was 30 sec. The reaction was terminated by addition of charcoal-dextran. The conditions of photoaffinity labelling and separation of free steroid from covalently bound to the receptor were as described above.

Demonstration of membrane bound receptors from zona radiata using four different methods.

1) The membrane fraction was prepared as described, and photoaffinity labelled in TETS buffer (5 ml) for 60 min with [³H]R5020 (5nM). The control membranes were not photoaffinity labelled but treated similarly with [³H]R5020. After photoaffinity labelling the membranes were centrifuged at 10,000 rpm (15,000 x g) for 10 min, the

supernatant was decanted and membranes re-suspended in 10 ml of TETS buffer by gentle mixing. The washing procedure was repeated three times to remove the free steroid. The membranes were then removed from the centrifuge tube and dabbed with tissue-paper to remove adhering aqueous medium, and membrane samples in triplicate were weighed and then solubilised in 'Protosol' (2 ml), plus distilled water (1ml) by heating the membranes in capped scintillation vials at 70 deg for 8 h. After cooling, 20 ml of scintillation fluid was added and vials counted after being ca. 24 h in the dark till constant counts were obtained.

2) After photoaffinity labelling the brook trout zona radiata membranes were homogenized in TETS + 0.1% Brij 35 (5ml) buffer using a Polytron PCU-2-110 homogenizer for 3 x 5 sec at 60 sec intervals at 0 deg C. The homogenate was centrifuged at 10,000 rpm (15,000 x g) for 10 min. The supernatant was then applied on to a Sephacryl S-300 column and eluted using TETS buffer containing 0.1% Brij 35. Radioactivity in alternate fractions was measured by scintillation counting. The peak radioactive tubes were frozen and stored at -70 deg C. The control membranes were not photoaffinity labelled but treated with [3H]R5020 under similar conditions and eluted through the Sephacryl S-300 column and fractions counted.

3) The membranes were isolated, photoaffinity labelled and washed as described previously, the control membranes were

not photoaffinity labelled but treated similarly with
[^{3}H]R5020. The photoaffinity labelled and control
membranes were then step wise dehydrated in 30%; 50%; 70%;
90% and 100% ethyl alcohol (EtOH) (5 min at each step; twice
in 100% EtOH). The membrane samples were than placed in
xylene for 5 min, and the xylene removed by placing the
membranes twice in melted wax (Paraplast plus; MonoJect
Scientific, Division of Sherwood Medical, St. Louis, MO
USA). The membranes were then embedded in wax using Fisher
Histo-Center (Fisher Scientific company, Don Mills, ONT).
The blocks were trimmed and sections cut (15 microns thick)
using a microtome ("820" Spencer Microtone, American optical
corporation, U.S.A.). The sections were placed onto gelatin
coated slides with distilled water and slowly dried on a
slide warmer (Fisher Scientific). The wax was removed by
dipping the slides in xylene and then dipped in EtOH to
remove the xylene. The membranes were rehydrated in the
reverse order in EtOH-aqueous solutions. The slides were
dipped into photographic emulsion (Kodak Nuclear Tract
Emulsion, type NTB2, Catalog # 165 4433, Kodak Company,
Rochester, New York), in a dark room and left at -70 deg in
the dark for three weeks. The emulsion coated slides were
developed and fixed using standard methods. The
autoradiographic sections of the membranes were photographed
using a camera attached to the microscope.

4) Zona radiata membranes after washing, were solubilized in

TETS buffer containing 0.1% Brij 35 by homogenization in a
Polytron PCU-2-110 instrument for 3 x 5 sec at 60 sec
intervals at 0 deg C. The homogenate was centrifuged at
10,000 rpm (ca. 15,000 x g) for 10 min and supernatant (5
ml) photoaffinity labelled with [^{3}H]R5020 as described
previously for 60 min. The photoaffinity sample was then
fractionated on a Sephacryl S-300 column. Alternate
fractions were counted for radioacctivity by scintillation
counting. The main [^{3}H]R5020 protein binding tubes were
then frozen at -70 deg C. Control sample was not
photoaffinity labelled, but treated similarly as the
photoaffinity labelled sample in every aspect.

Ion exchange chromatography of cytosolic preparation after photoaffinity labelling with labelled R5020.

a) Using hydroxyapatite column chromatography: cytosolic
preparation (1 ml) was added to a scintillation vial
containing vacuum dried [^{3}H]R5020 (5nM) and
photoaffinity labelled for 30 min under conditions described
previously. The photolysed sample was then applied to an
hydroxyapatite column (Biogel HTP) total volume 2.5 ml
equilibrated with TETS buffer pH 7.4. The column was washed
with 2.5 ml of TETS buffer initially to remove free
[^{3}H]R5020. The [^{3}H]R5020-receptor complex was then
eluted from the column using a stepwise gradient (5 ml) of
KH_2PO_4/K_2HPO_4 buffers (0.05- 0.5M) pH

7.4.

b) DEAE Sephacel (Pharmacia Fine Chemicals, Lot No. 3020 wet particle size 40-150 microns) was mixed with TETS buffer pH 7.4. After removal of the suspended 'fines' the DEAE Sephacel matrix was packed into a Pharmacia C10/10 column. The column was coupled with an AC10 adaptor to a Gilson Minipuls 2 pump and a Gilson micro fractionator. Total bed volume of the column matrix was 7.8 ml. The column was washed with 50 ml TETS buffer. Cytosolic preparation (1 ml) from stage 1 brook trout oocytes was added to a scintillation vial containing vacuum dried [^{3}H]R5020 (5 nM) and photoaffinity labelled for 60 min under conditions described previously. The photolyzed sample was then applied on the DEAE Sephacel column. The column was washed with 50 ml of TETS buffer to remove free [^{3}H]R5020. The labelled receptor complex was then eluted from the column using linear 50 ml KCl gradient (0.0- 0.5M KCl) in TETS buffer pH 7.4 using a Bio-Rad model 385 gradient former (Bio-Rad Laboratories). Thirty-six 50 drops fractions were collected (1.39 ml/ fraction), and radioactivity measured using scintillation counting.

Results

Isolation of zona radiata.

Ultracentrifugation of whole intact stage (6-7) oocytes
leads to separation of various fractions as shown in Fig.
3.2. After suspension of the membrane fraction in TETS
buffer the zona radiata membranes take up a spherical shape
from fresh oocytes (Fig. 3.3). A cross-sectional view of the
zona radiata membrane shows the absence of follicular cells
(Fig. 3.4).

Photoaffinity labelling of cytosolic preparation using labelled R5020.

Photoaffinity labelling leads to covalent binding of
[3H]R5020 to the receptor protein. This binding was
saturable with respect to time (Fig. 3.5). [3H]R5020
photoaffinity labelling to the cytosolic receptor
preparation is saturable below 5 nM [3H]R5020, but
above this concentration cooperativity in binding is
observed (Fig. 3.6). This increased binding above 5nM
[3H]R5020 is also associated with a decrease in
molecular weight of the receptor complex (see gel filtration
chromatography below). [3H]R5020 photoaffinity
labelling to the cytosol receptor was inhibited by inert 17α-
,20β-DHP or inert R5020 during initial reaction rates (Figs.

Fig. 3.2. Various fractions obtained after ultracentrifugation of whole brook trout oocytes. The concept involves breakage of oocytes by centrifugal force, and separation of organelles by differential and isopycnic centrifugation, where the york protein act as the medium of separation. M, zona radiata; Y, yolk proteins; N, nuclei; A, aqueous layer; O, oil layer.

174a.

Fig. 3.2.
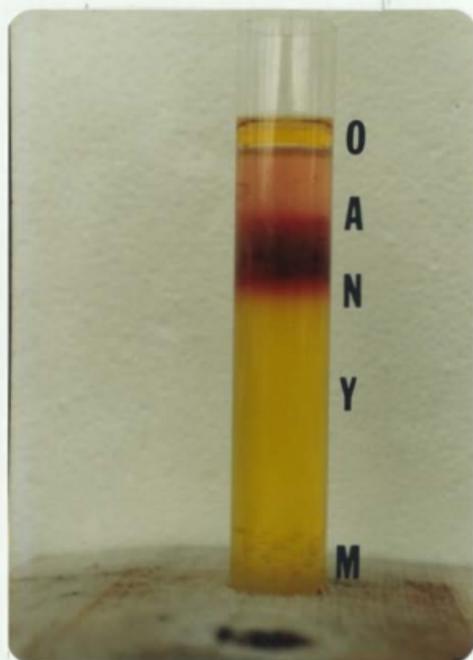
Fig. 3.3. Zona radiata membranes obtained from brook trout stage 6-7 oocytes, washed and then suspended in TETS buffer.
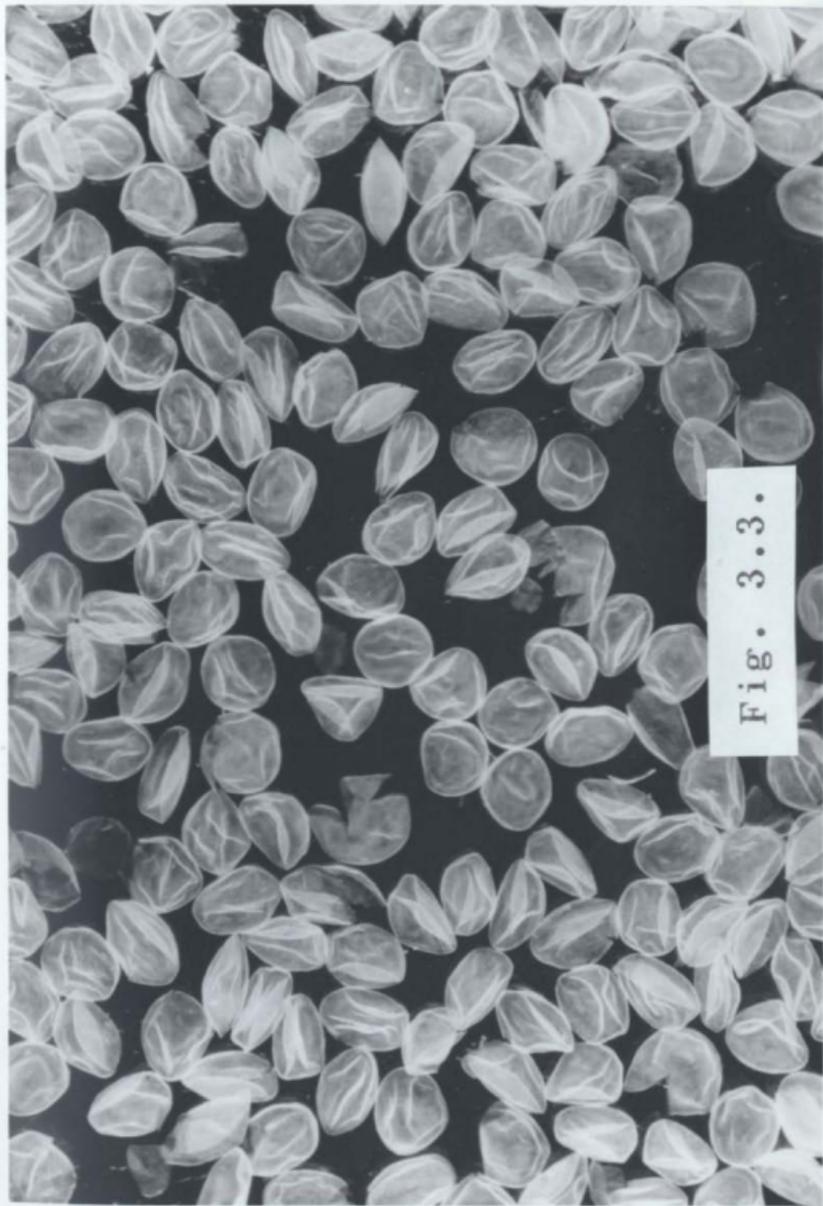
Fig. 3.3.

Fig. 3.4. Cross-sectional (CS) view of the zona radiata membrane from stage 6-7 oocytes. Note the absence of granulosa and thecal cells on the outer edge of the membrane.

Fig. 3.4.
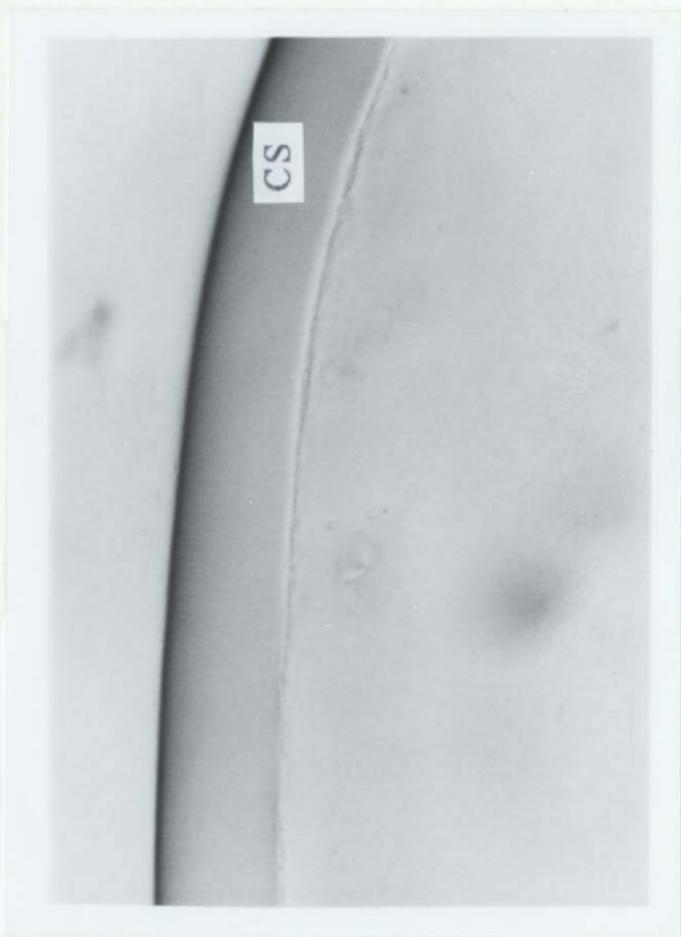
Fig. 3.5. Photoaffinity labelling of the cytosolic receptor protein using $[^3H]R5020$ as a function of time.

Fig. 3.5.
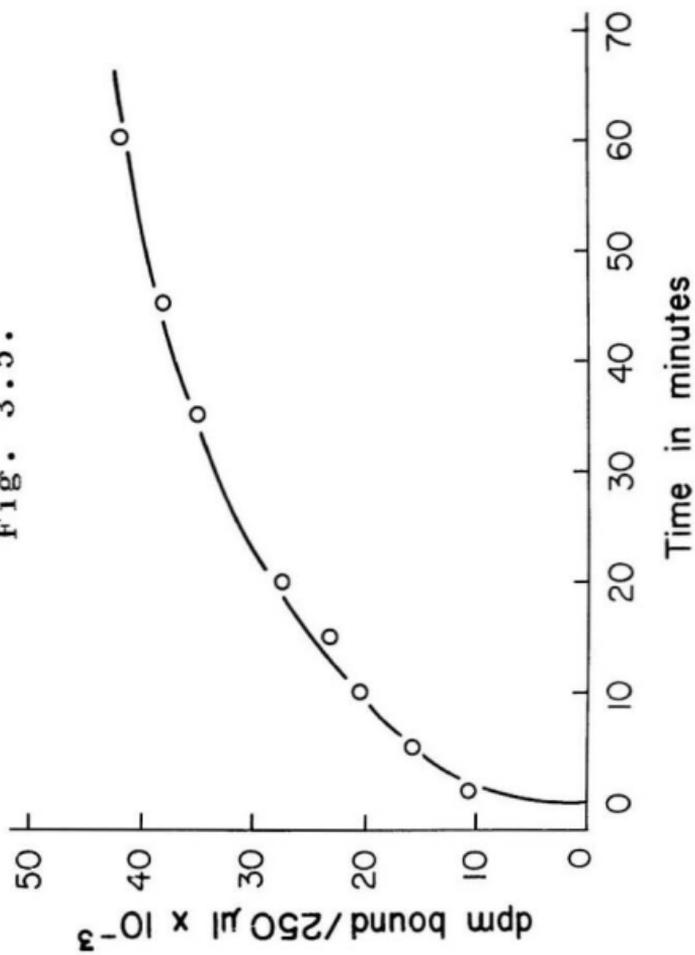
Fig. 3.6. Photoaffinity labelling (2 min) of cytosolic receptor protein with $[^3H]R5020$ was saturable below ca. (5nM), as indicated by the extended solid line. Above (5nM), cooperativity in binding was observed as shown by dotted line joining the experimental points.
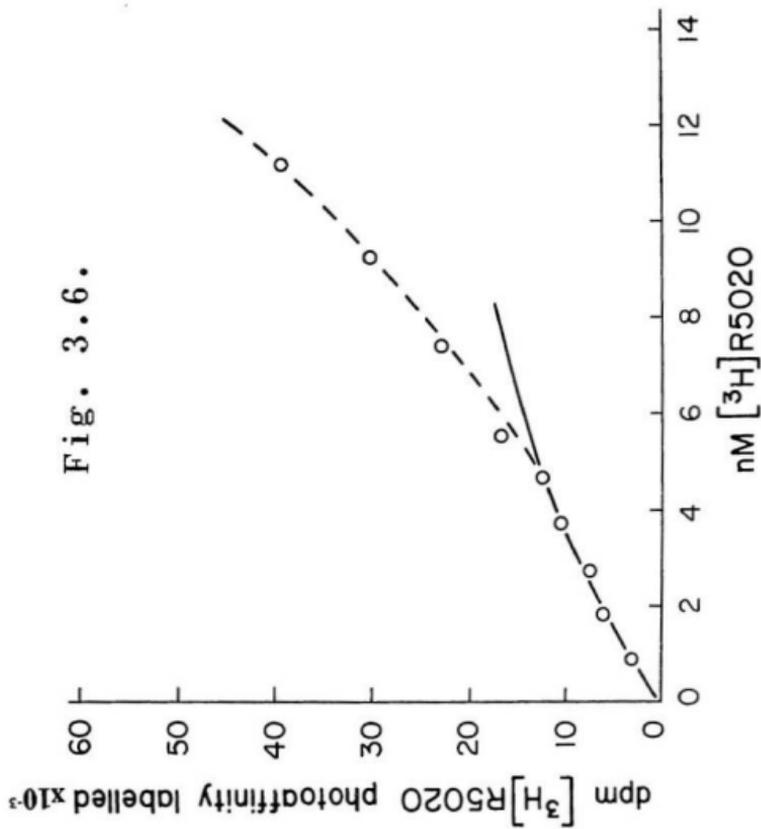
Fig. 3.6.

Fig. 3.7. Inhibition of photoaffinity labelling of cytosolic receptors using inert $17\alpha,20\beta$-DHP ($3\mu M$) during initial reaction rates. The concentration of [^{3}H]R5020 used was 1.85 nM. At higher concentrations of [^{3}H]R5020 the inhibition was less pronounced due to the fast initial rate of photoaffinity reaction.

Fig. 3.7.
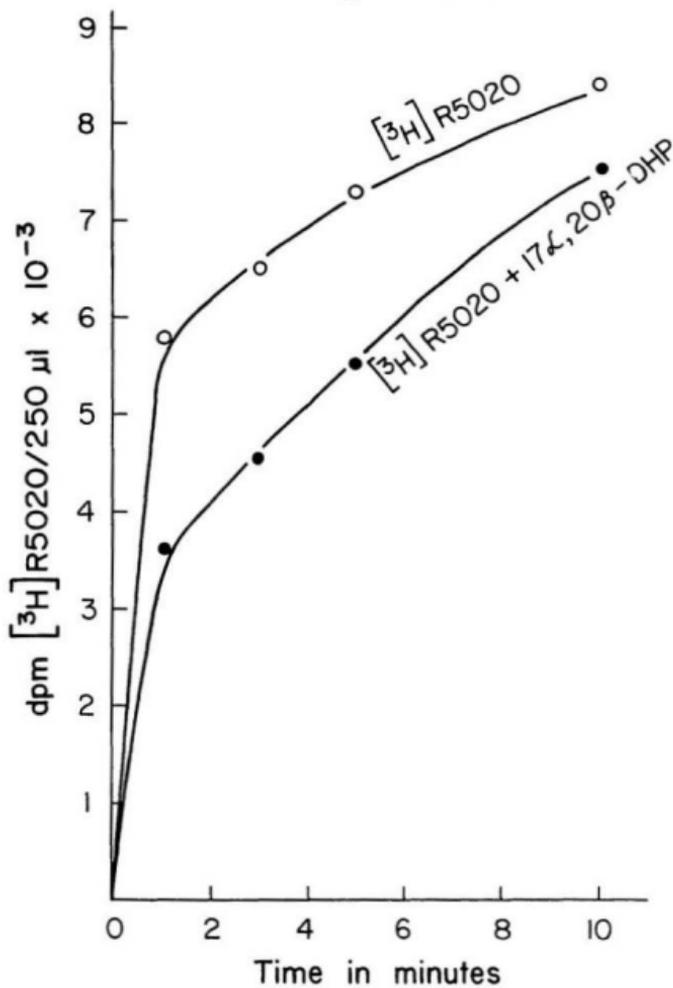
Fig. 3.8. Inhibition of photoaffinity labelling of cytosolic receptors using inert R5020 (3 μM). The concentration of [^{3}H]R5020 used was 1.85 nM. At higher concentrations of [^{3}H]R5020 the inhibition was less pronounced due to the very fast initial rate of the photoaffinity reaction.

Fig. 3.8.
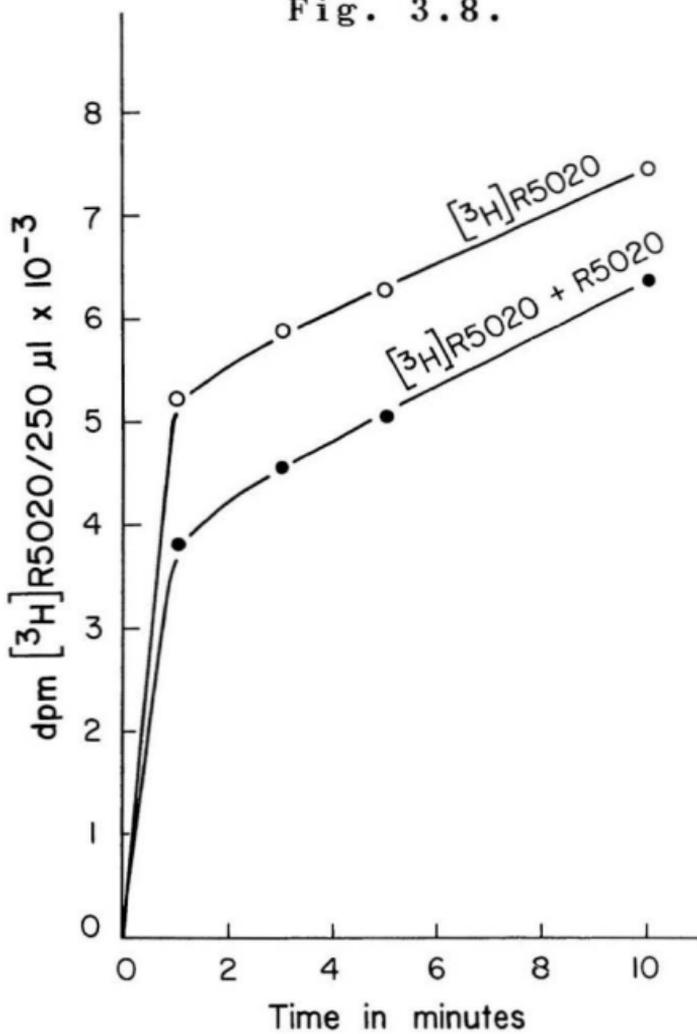
3.7 and 3.8). Effect of increasing $17\alpha,20\beta$-DHP concentration on the initial reaction rates of [^{3}H]R5020 photoaffinity binding to cytosol is shown in Fig. 3.9.

<u>Demonstration of photoaffinity labelled receptors in zona radiata membranes</u> . In brook trout stage 6-7 zona radiata isolated membranes the photoaffinity binding per gram of membranes was 139,000 $\pm$ 15,000 dpm (n=3), which is equivalent to 0.727 nmol of [^{3}H]R5020 binding sites per gram of membranes. In rainbow trout stage 4-5 membranes the photo affinity binding per gram of membranes was 122,000 dpm, which is equivalent 0.633 nmol of [^{3}H]R5020 binding sites per gram of membranes.

The molecular weight (MW) of the [^{3}H]R5020-receptor complex was 355,000 (Fig. 3.10), the second peak was due to the free [^{3}H]R5020 and was eluted at (Ve) slightly greater than the total volume of the column. In the control run, the receptor preparation was not photoaffinity labelled but was treated similarly with [^{3}H]R5020 but kept in the dark. Similar results were obtained when the zona radiata membranes were first solubilized and then photoaffinity labelled with [^{3}H]R5020, and eluted from a Sephacryl S-300 column.

The plasma membrane of the oocyte is intercalated within the zona radiata membranes. The [^{3}H]R5020 photoaffinity labelled zona radiata membranes after autoradiography showed

Fig. 3.9. Effect of increasing $17\alpha,20\beta$-DHP concentration on the initial rate of photoaffinity labelling with [³H]R5020. Time of photoaffinity labelling was 30 sec.

Fig. 3.9.

Figure axes: y-axis "dpm [^{3}H] R5020 bound/250 μl × 10^{-2}" (0, 4, 8, 12, 16, 20, 24, 28); x-axis "[17α, 20β – DHP] (μm)" (0, 4, 8, 12, 16, 20)
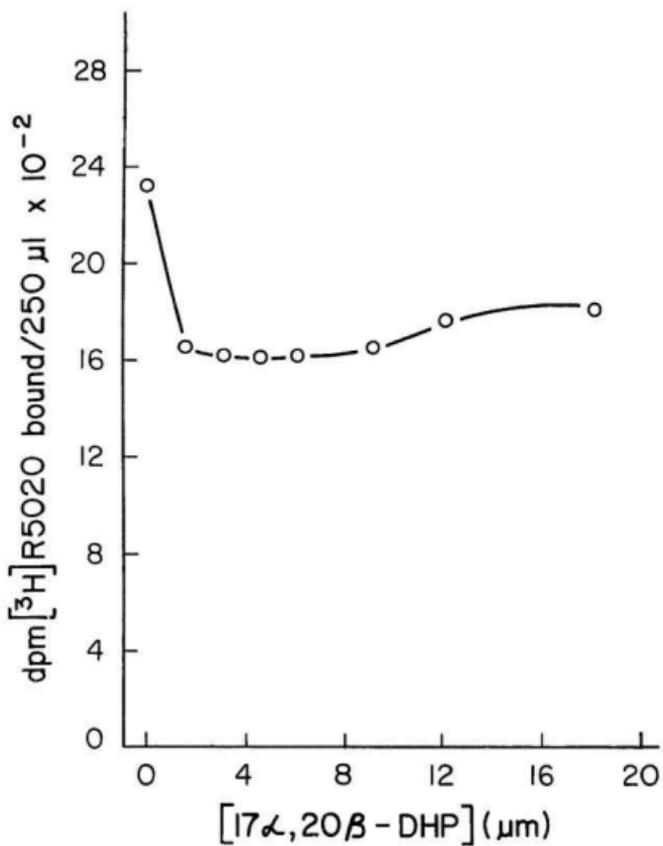
Fig. 3.10. Sephacryl S-300 elution pattern of the solubilized zona radiata membranes after photoaffinity labelling with [^{3}H]R5020. The membranes were isolated from brook trout stage (6-7) oocytes, then solubilized in TETS buffer containing 0.1% Brij 35, and a sample (5 ml) was photoaffinity labelled [^{3}H]R5020 (5 nM) for 50 min and eluted from the column (O). Control preparation contained [^{3}H]R5020 and was treated similarly, but was not photoaffinity labelled (◘).
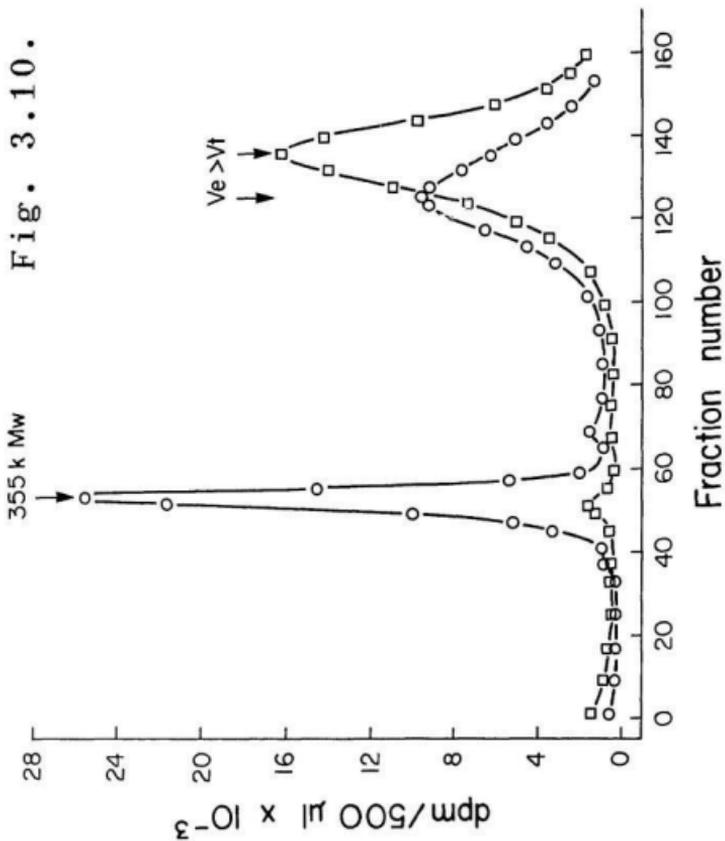
Fig. 3.10.

Fig. 3.11.a. Zona radiata photoaffinity labelled membrane section after autoradiography. Clusters of black spots indicate presence of [^{3}H]R5020 covalently bound to the receptor protein in the cross-sectional view of the membrane section.

Fig. 3.11.a.
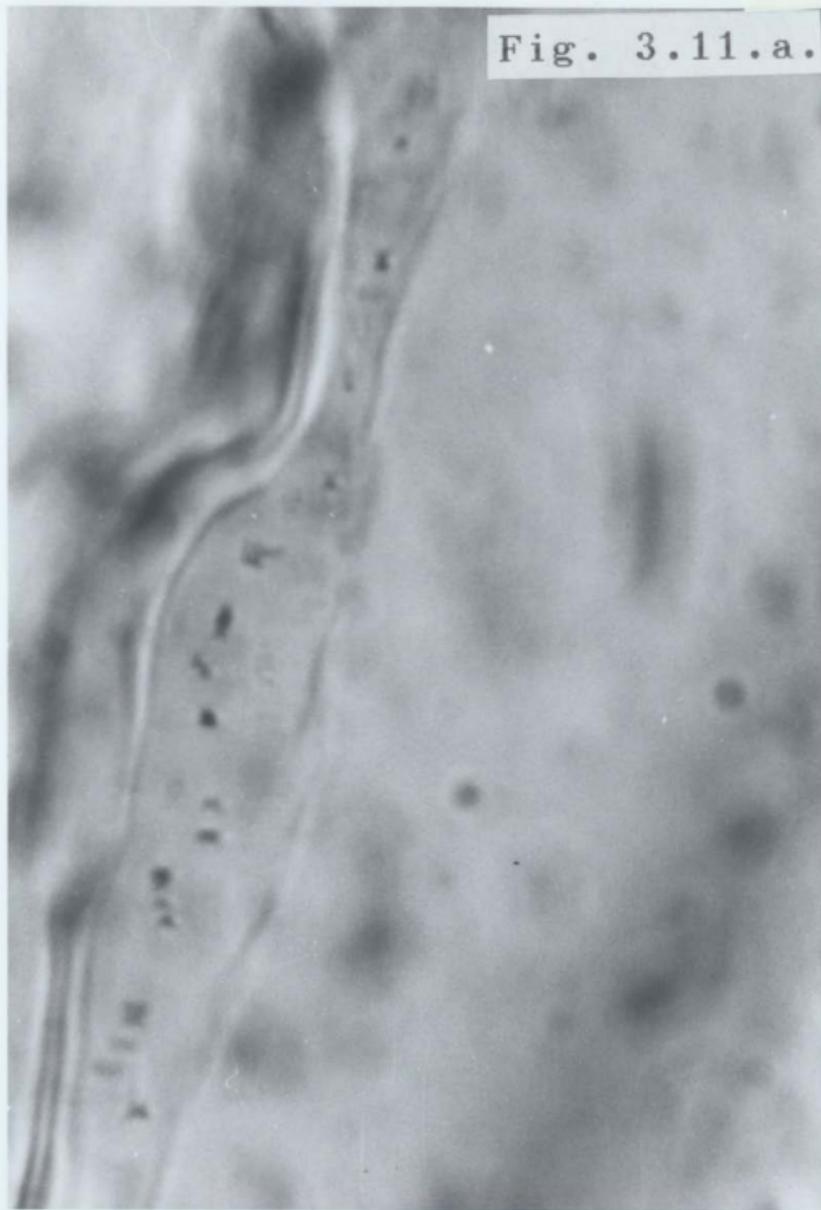
Fig. 3.11.b. Control membrane section was treated similarly with [^{3}H]R5020 but not exposed to 320 nm radiation, and autoradiographed as discussed in the text.

Fig. 3.11.b.
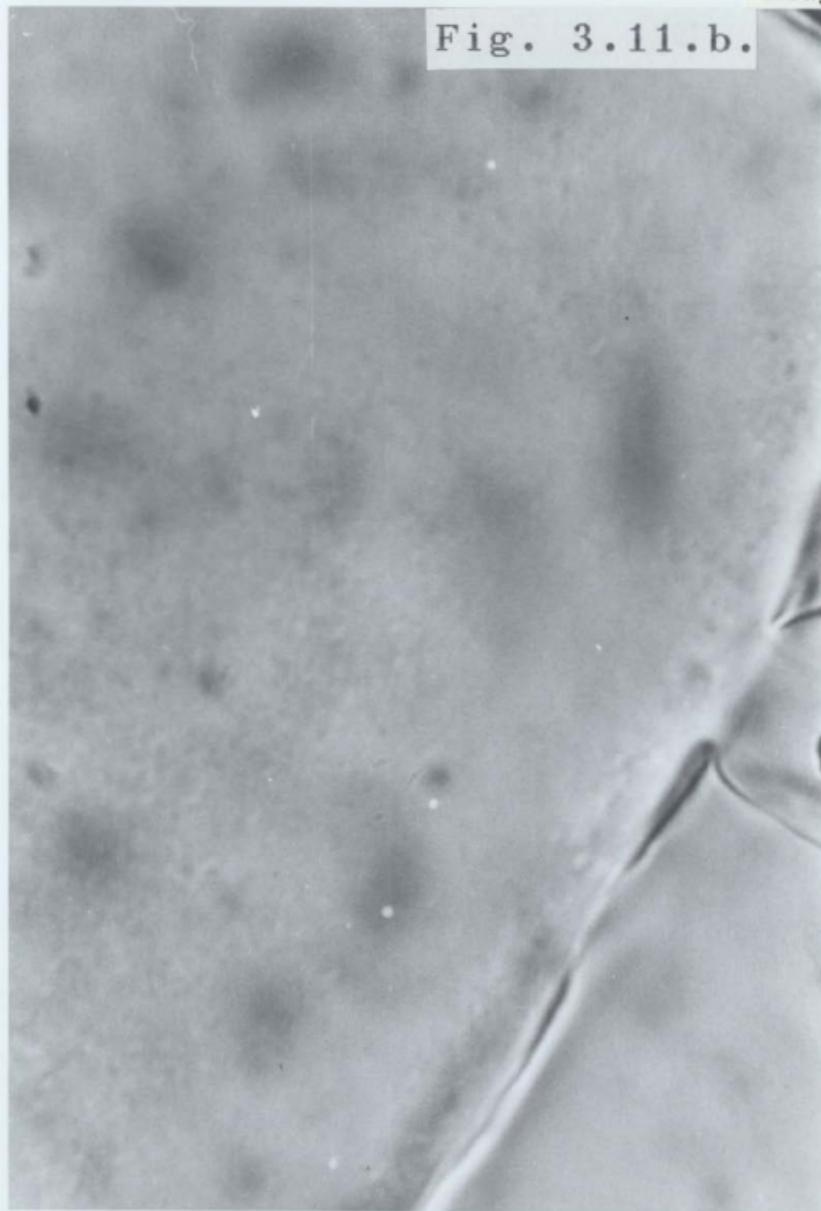
clusters of black spots within the cross-sectional view (Fig. 3.11a), indicating the presence of [^{3}H]R5020-receptor complex. In the non-photoaffinity labelled membranes these spots were not present (Fig. 3.11b).

Steroid binding studies using labelled 17α,20β-DHP with solubilized zona radiata membranes.

The zona radiata membrane extracted receptor protein from brook trout stage 6-7 oocytes demonstrated cooperativity of binding to [^{3}H]17α,20β-DHP in the presence of excess inert 17α,20β-DHP (3 μM), higher binding to [^{3}H]17α,20β--DHP was observed in the presence of inert 17α,20β-DHP than when the inert was absent at all concentrations of labelled steroid. When binding of [^{3}H]17α,20β-DHP to the membrane receptors was obtained in the absence of inert steroid, the binding showed saturation, and Scatchard plots were highly linear (p <.05). Values obtained for association constant (K$_a$) and maximum binding capacity (N$_{max}$) for the zona radiata solubilized receptors in TETS buffer, TETS + 0.15M NaCl and TETS + 0.1% Brig 35 are reported in Table 3.1.

Gel filtration chromatography using Sephacryl S-300.

Cytosolic preparation from stage 1 brook trout ovarian follicles photoaffinity labelled with [^{3}H]R5020 (5nM)

TABLE 34 Binding parameters of brook trout oocytes (stage 6-7) membranes

Sample *	K_a (M^{-1})	$N_{(max)}$ nM	Protein mg/ml	N_{max} f moles/mg protein
Membranes solubilized in TETS buffer S1	4.75×10^7	0.88	12.0	73.7
Membranes solubilized in TETS buffer S2	4.79×10^7	1.25	13.3	93.7
Membranes solubilized in TETS buffer + 0.15M NaCl	8.17×10^7	1.12	17.4	64.3
Membranes solubilized in TETS buffer + 0.1% Brig 35	1.99×10^7	9.87	23.1	427.2

* zona radiata membranes were isolated, then homogenized in the above

buffers using Polytron PCU-2-110 instrument for 3 x 5 sec (on position

6) at 60 sec intervals in ice water. Homogenates were left for 30 min

at 1°C and then centrifuged at 35,000 rpm (151,000 x g) for 60 min.

The supernatants were used for binding studies using [^{3}H]17α,20β-DHP.

K_a - association constant

N_{max} - maximum binding capacity

S1 - sample 1; S2 - sample 2

Fig. 3.12. Sephacryl S-300 elution pattern of the cytosol preparation from frozen stage 1 oocytes of brook trout after photoaffinity labelling with [^{3}H]R5020 (5nM) (O), and with [^{3}H]R5020 + inert R5020 (3μm) (□).

Fig. 3.12.
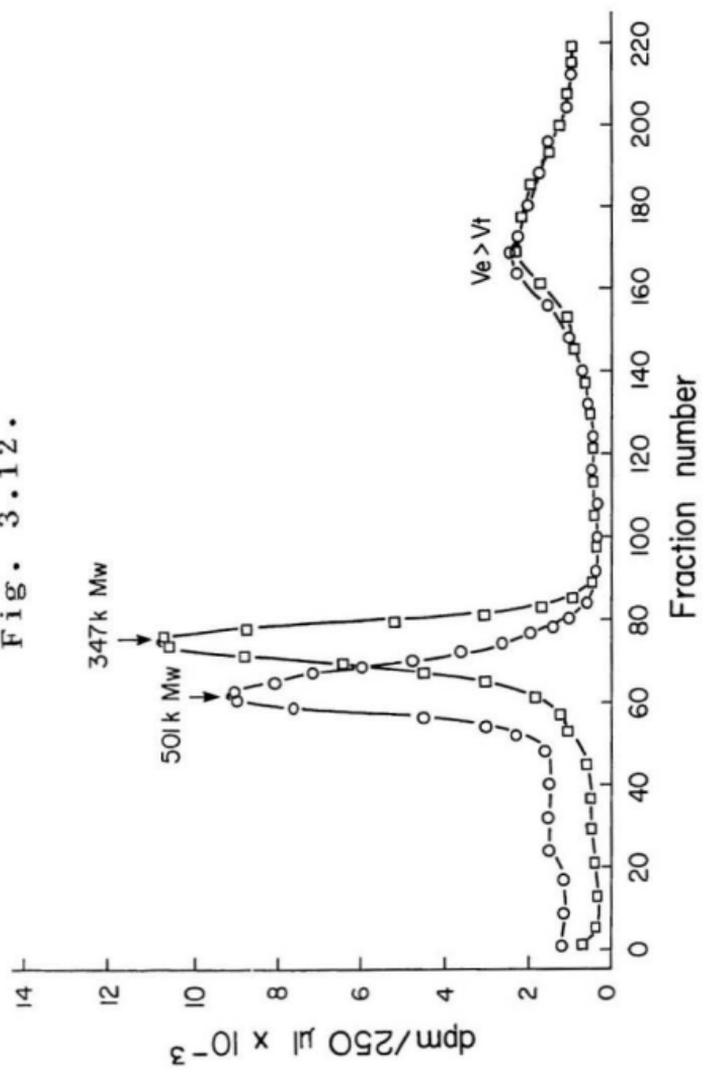
gave MW at 501,000, and in the presence of inert R5020 (2 uM) the MW decreased to 347,000. This change in MW is also associated with increased binding (Fig. 3.12). Also compare Fig. 3.6 where receptor binding to [^{3}H]R5020 above 5nM leads to cooperativity in photoaffinity labelling. This cooperativity in binding was also observed using [^{3}H]R5020 and inert progesterone (3μM) instead of inert R5020. Zona radiata membrane solubilized from previously frozen stage 6-7 oocytes after photoaffinity labelling with [^{3}H]R5020 (5nM) gave MW of 355,000, a value very close to the cytosolic receptor preparation after transformation (Fig. 3.10).

SDS-PAGE analysis

a) Sub-unit MW of [^{3}H]R5020 photoaffinity labelled protein from cytosolic preparation of stage 1 brook trout ovarian follicles. Four bands were detected at MW 83,000, 67,000, 24,000 and 23,000 from the 501,000 MW peak tube fraction of Sephacryl S-300 (Fig. 3.13). b) Sub-unit MW of the [^{3}H]R5020 photoaffinity labelled protein from zona radiata membranes. Similar bands to cytosolic photoaffinity labelled protein were obtained with MW 355,000 peak fraction of zona radiata solubilized receptor complex (Fig 3.13), indicating that sub-unit structure of cytosolic and membrane receptors are same. c) Sub-unit MW of [^{3}H]R5020 photoaffinity labelled protein from previously frozen plasma

Fig. 3.13. SDS-PAGE analysis of [^{3}H]R5020 photoaffinity labelled receptors from cytosol and zona radiata membranes. The cytosolic preparation was photoaffinity labelled for 50 min with [^{3}H]R5020 (5 nM) and then fractionated on Sephacryl S-300 column, the peak radioactive tube containing the [^{3}H]-receptor complex (MW 501,000) was analyzed on SDS PAGE. Membrane receptor (MEM); low MW standards from pharmacia (LMW); cytosolic receptors (CYT).

Fig. 3.13.
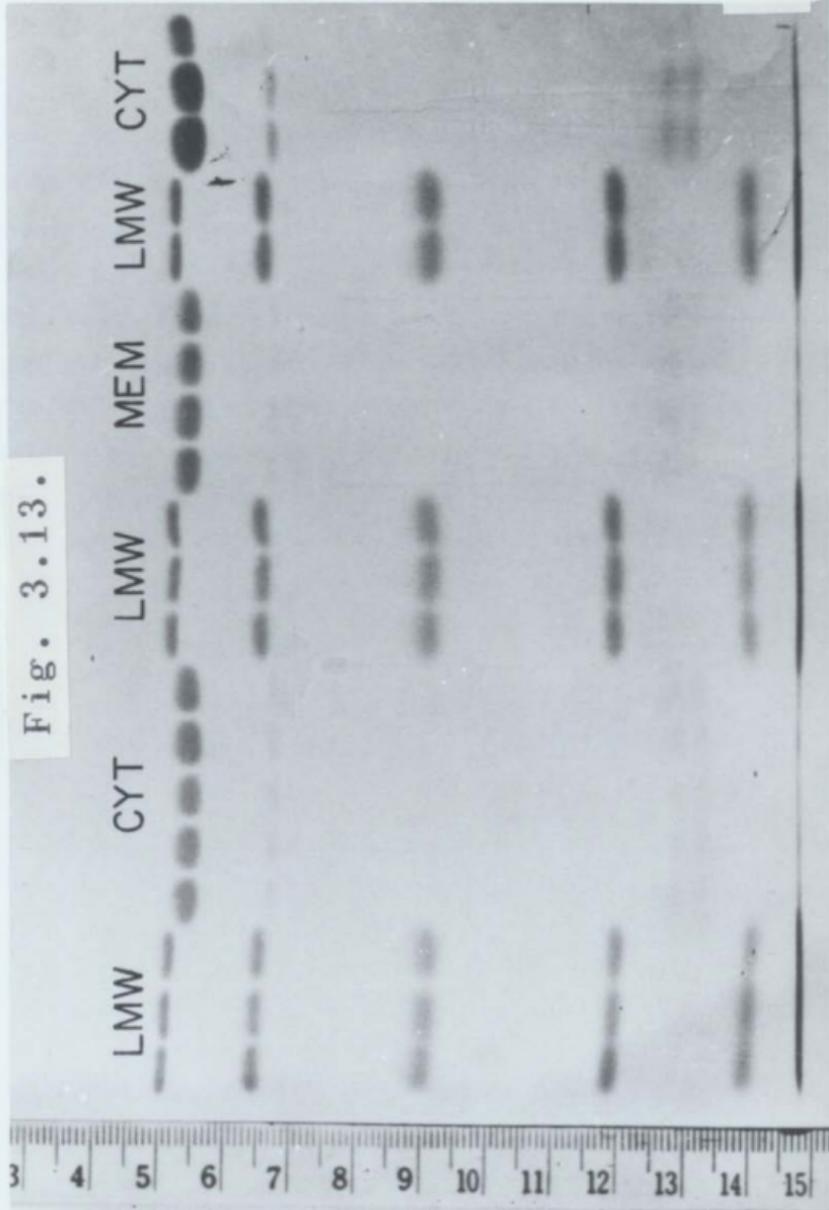
Fig. 3.14. SDS-PAGE analysis of [^{3}H]R5020 photoaffinity labelled protein from previously frozen plasma of brook trout. Blood plasma (1:5) diluted in TETS buffer was photoaffinity labelled for 50 min with [^{3}H]R5020 (5nM) and then fractionated on a Sephacryl S-300 column, the peak radioactive tube containing [^{3}H]R5020-receptor complex (MW 589,000) was analyzed on SDS-PAGE.

[^{3}H]R5020-receptor complex was also run in parallel.

Plasma binding protein (PL.); low molecular weight standards from pharmacia (LMW); membrane receptors (MEM).

Fig. 3.14.
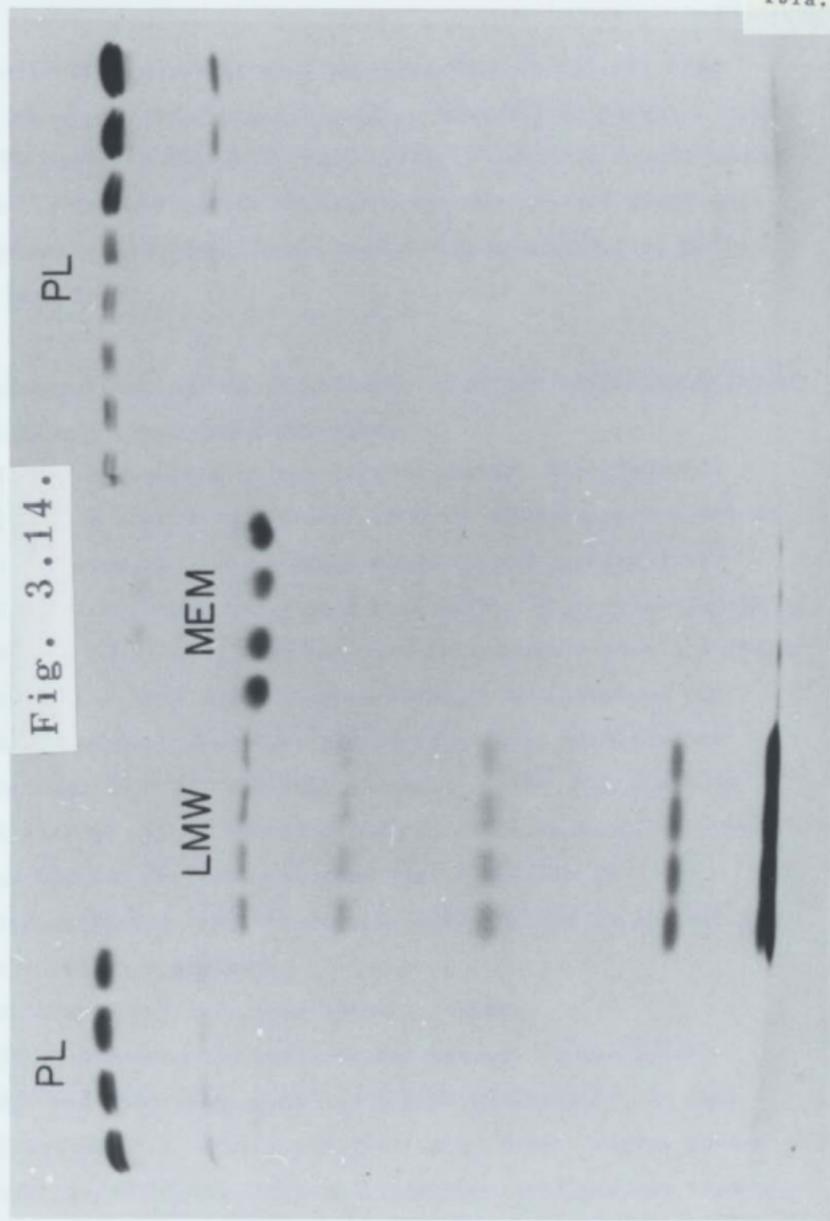
of brook trout. The peak tube fraction MW 589,000 from
Sephacryl S-300 when analyzed on SDS-PAGE in parallel with
the membrane extracted photoaffinity labelled sample showed
no correspondence in MW between plasma protein bands and
membrane receptors bands, indicating that they are different
(Fig. 3.14).

Ion exchange column chromatography of photoaffinity labelled cytosolic receptor preparation

a) Hydroxyapatite column chromatography. The [³H]R5020
receptor complex was eluted from the hydroxyapatite column
using step wise gradient (0.05-0.5M) and gravity flow.
Step-wise gradient was used because the flow rate diminished
when a peristaltic pump was used in conjunction with a linear
gradient. [³H]R5020-receptor activity was obtained in
0.2M and 0.3M phosphate buffer fractions, with maximal
binding in 0.3M. SDS-PAGE analysis of the 0.2M and 0.3M
fractions after concentrating by freeze drying gave a band
at 84,000 MW, demonstrating that [³H]R5020 is
photoaffinity labelled mainly on the 84,000 MW subunit of
the receptor protein.

b) DEAE Sephacel column chromatography.
[³H]R5020-receptor activity was present in the 0.19M
KCl fraction (Fig. 3.15). SDS-PAGE analysis of the peak
fraction (12) after concentrating by freeze drying gave a
band at MW 84,000, although when the total protein from all

Fig. 3.15. [³H]R5020-receptor complex elution profile from DEAE Sephacryl column. Linear gradient KCl (0.05 - 0.5 M) in TETS buffer was used for the elution of the column. The peak activity of the complex was eluted in the 0.19M KCl containing fraction.
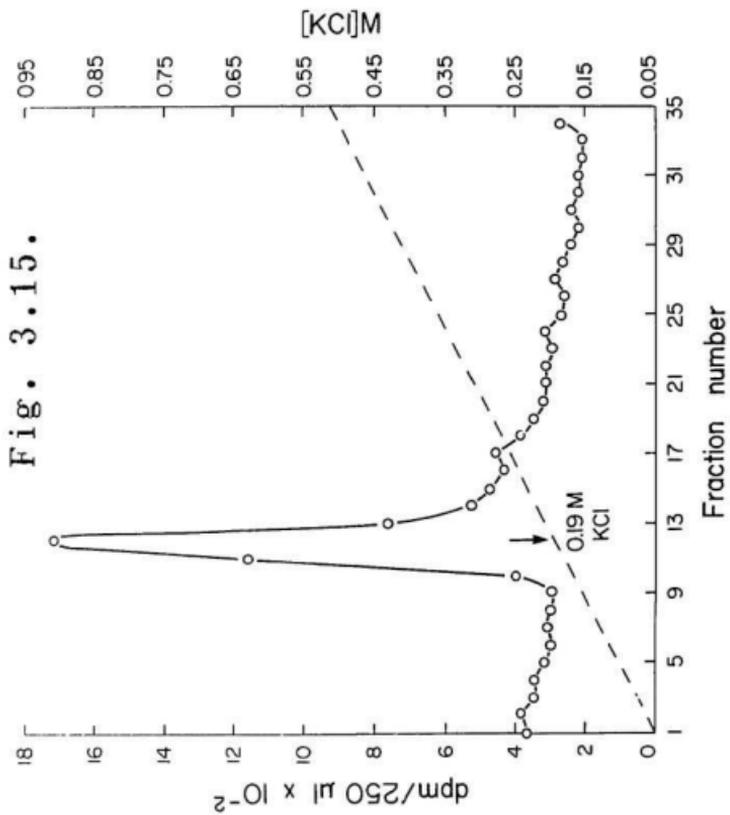
Fig. 3.15.

the radiolabelled tubes in Fig. 3.15 were combined and
concentrated and then analysed by SDS-PAGE, all the four
bands plus other contaminating bands were observed.

Photoaffinity labelling of oocyte cell sap (protoplasm) for
isolation of receptor activity.

Sephacryl S-300 elution pattern of the photoaffinity
labelled oocyte protoplasm and control sample in TETS
containing 0.1% Brij buffer gave a major peak of receptor
activity at MW 324,000, minor peaks of activity were also
obtained at MW 110,000, 26,000 and 12,000 (Fig. 3.16). The
peak tube at MW 324,000 on SDS PAGE gave similar subunits as
the purified cytosolic and membrane extracted photoaffinity
labelled proteins.

Fig. 3.16. Sephacryl S-300 elution pattern of the diluted protoplasm from brook trout stage 2 ovarian follicles after photoaffinity labelling with [^{3}H]R5020. Protoplasm (0.12g) was diluted to 10 ml with TETS containing 0.1% Brig, and then centrifuged. Supernatant (5 ml) was then photoaffinity labelled and eluted through the column (△-△-△). Control (5 ml) was also treated with labelled R5020 but not photoaffinity labelled (○-○-○).

Fig. 3.16
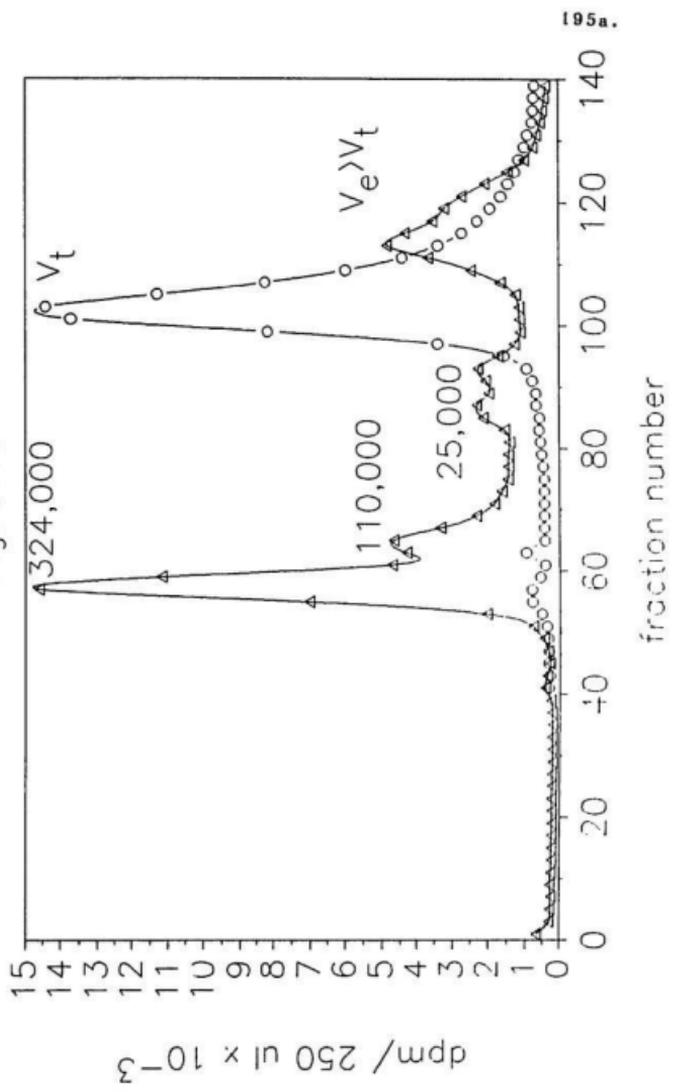
Discussion .

Goetz, <u>et al</u> . (1987), have demonstrated that plasma
levels of MIS 17α,20β-DHP in brook trout <u>Salvelinus
fontinalis</u> are very low prior to GVBD (0.70 ng/ml), and
increase dramatically at GVBD (148 ng/ ml). The cytosolic
receptor activity in brook trout ovarian follicles fell from
stage 1 to 5 and no activity was present in cytoplasm during
stages 6-7 (chapter 1; Maneckjee, <u>et al</u> . 1987,1989). Also
the presence of nuclear receptor activity was not
demonstrated either in stage 1 or stage 6-7 ovarian
follicles (chapter 2). The goal of this study (chapter 3)
was to demonstrate the presence and location of MIS 17α,20β-
DHP receptor activity in brook trout oocytes during final
stages of maturation (stages 6-7), when the plasma levels of
MIS are the highest.

The method as described in the text for the isolation of
zona radiata membranes by ultracentrifugation of whole
oocytes has not been described in the literature. The plasma
membrane of the oocyte is intercalated with the zona
radiata. That the 17α,20β-DHP receptor activity is
membrane-bound or associated with protein was demonstrated
by solubilizing the zona radiata membrane fraction.
Initially 0.1% Triton X-100 in TETS buffer was used for

solubilizing the receptors without success because of
micelle formation. A literature search revealed that a 0.1%
solution of Brij. 35 was used for solubilizing amphibian
progesterone receptors (Sadler, et al . 1985). When TETS
buffer containing 0.1% Brij 35 was used for solubilizing the
zona radiata membrane bound receptors, an increase in
maximum binding capacity was obtained from Scatchard
analysis using labelled $17\alpha,20\beta$-DHP; an increase in total
soluble protein was also observed (Table 3.1).

The affinity constant (K_a) for the zona radiata
membrane bound receptors after solubilization in TETS buffer
containing 0.1% Brij. 35 was 1.99 x 10^7 M^{-1}, this
value is 10 fold lower than observed for cytosolic
receptors. Also membrane receptors showed cooperativity in
binding to [^{3}H]$17\alpha,20\beta$-DHP; cooperativity was not
observed with cytosol receptors in binding studies with
[^{3}H]$17\alpha,20\beta$-DHP during Scatchard analysis. Due to its
lower K_a value the membrane receptors could not be
isolated from a Sephacryl S-300 column chromatography using
labelled steroid, as the binding ligand because of complete
dissociation of receptor ligand complex during column
elution. [^{3}H]R5020 a synthetic progestin with
conjugated double bonds binds progesterone receptors
covalently when photoaffinity labelled at 320 nm.
[^{3}H]R5020 photoaffinity labelling was ideally suited to

labelling the zona radiata receptors for isolation and
characterization. Initial experiments were carried out on
cytosol preparations to show that photoaffinity labelling
using [^{3}H]R5020 was saturable with time and
concentration of [^{3}H]R5020. The initial rate of
photoaffinity labelling was partially inhibited by inert 17α-
,20β-DHP and R5020 (3μM).

[^{3}H]R5020 photoaffinity labelled cytosolic protein
after fractionation on the Sephacryl S-300 column gave peak
binding activity at MW 501,000. The subunits of this
photoaffinity labelled cytosol protein on SDS-PAGE under
reducing and non-reducing conditions were similar (agrees
with subunits) to the cytosol [^{3}H]17α,20β-DHP-receptor
complex from peak activity tube after Sephacryl S-300
elution (MW 250,000). These results demonstrated that
[^{3}H]R5020 binding in cytosol has the same subunit
composition as [^{3}H]17α,20β-DHP receptors, although the
MW of [^{3}H]R5020 binding moiety is twice as large. The
cytosol photoaffinity labelled receptor complex obtained
after chromatography on hydroxyapatite or DEAE Sephacel,
gave on SDS-PAGE analysis a single band corresponding to MW
84,000. The low MW bands of the subunit were not present,
indicating that only 84,000 MW subunit of the receptor binds
to [^{3}H]R5020 at or below 5nM concentration.

The zona radiata membrane solubilized protein in TETS
buffer containing 0.1% Brij 35 after photoaffinity labelling

and fractionation on Sephacryl S-300 gave a MW of 355,000.
SDS-PAGE analysis of this peak fraction gave the same
subunit structure as the cytosol [³H]R5020
photoaffinity labelled component. The above evidence
suggests that the membrane component may be derived from the
cytosol receptors. It is of interest to note that when
cytosolic preparation was photoaffinity labelled with
[³H]R5020 (5nM) in the presence of inert R5020 (3 μM)
for 50 min, higher binding was obtained (cooperative effect
above 5nM) than in the absence of inert steroid, and also
the MW of the steroid-receptor complex decreased to 347,000.
Thus the cytosolic receptor protein dissociates from MW
501,000 to MW 347,000 in order to incorporate more binding
sites (Fig's. 3.6. and 3.12.). Also the membrane receptors
showed cooperative binding with [³H]17α,20β-DHP during
Scatchard analysis; in the presence of inert 17α,20β-DHP,
the membrane receptors gave higher binding. Thus it is
possible that 17α,20β-DHP enters the oocytes during early
stages of its synthesis from granulosa cells and interacts
with the cytosolic receptors which leads to transformation
of the cytosolic receptors and its incorporation into plasma
membrane where it becomes a functional receptor during the
later stages of maturation when MIS 17α,20β-DHP levels are
highest. This hypothesis postulates that transformation /
translocation phenomenon involving 17α,20β-DHP regulates the
functional receptor levels in the plasma membrane of the

oocyte.

Labelled MIS 17α,20β-DHP is readily taken up by the oocytes, the uptake into the oocyte membranes and protoplasm is by passive diffusion and not carrier mediated (chapter 4.). This data rules out the possibility that membrane bound 17α-,20β-DHP receptors are carrier proteins for 17α,20β-DHP.

Supportive indirect experimental evidence for membrane bound / associated receptors for MIS 17α,20β-DHP during resumption of maturation in fish oocytes comes from recent work of several researchers, and was briefly mentioned in the introduction part of this chapter. Nagahama and Kishimoto reported that micro injection of 17α,20β-DHP into full grown immature goldfish oocyte was ineffective in inducing GVBD, while external application brought about maturation (Nagahama, 1987). Steroid induced GVBD is blocked by inhibitors of translation but not transcription indicating that the mechanism of action involved in the steroid maturation of oocytes has special characteristics which is different from classical steroid mechanism of action via nuclei (Detlaff and Skobilina, 1969; Goswami and Sundararaj, 1973; Jalabert, 1976; Theojan and Goetz, 1981; DeMenno and Goetz, 1986; 1987).
Inhibitors of phosphodiesterase such as

3-isobutyl-1-methylxanthine (IBMX), theophylline,
1-ethyl-4-hydrazino-1-pyrazolo [3, 4-6]
pyridine-5-carboxylic acid ethyl ester hydrochloride (SQ
20,006 Squibb) and activators of adenyl cyclase such as
forskolin and cholera toxin which increase the intracellular
levels of cyclic adenosine 3',5'-monophosphate (cAMP)
inhibit steroid-stimulated maturation (GVBD) _in_ _vitro_ in
several species (Goetz and Hennessey, 1984; Jalabert and
Finet, 1986; DeManno and Goetz, 1986,1987. These results
indicate that cAMP is involved in the mechanism of action of
$17\alpha,20\beta$-DHP induced maturation.

In amphibians, progesterone is considered to be the MIS.
Smith and Ecker, (1971); Masui and Market, (1971),
demonstrated that progesterone induced GVBD in _Xenopus_
oocytes only when the steroid was added to the incubation
medium. When the progesterone was injected directly into the
oocyte it had no effect. Ishikawa, _et_ _al_ . (1977), induced
GVBD in _Xenopus_ oocytes by incubation of denuded oocytes
with deoxycorticosterone bound to amino-ethylated agrose
beads. These results indicate that the action of the steroid
is on the outer oocyte surface. Sadler and Maller (1982),
identified a single protein (MW 110,000) from the outer
vitelline envelope containing the plasma membrane of
Xenopus oocytes using [³H]R5020 photoaffinity
labelling and SDS-PAGE. The K_a for the photoaffinity
labelled protein was 1×10^6 M^{-1}, which was in

agreement with the concentration of R5020 required to bring about 50% maturation. R5020 after photoaffinity labelling inhibited adenyl cyclase activity in the membranes, and inhibition was proportional to the level of R5020 bound. Sadler, et al . (1985), further supported the physiological significance of the R5020 plama membrane receptor (MW 110,000) by inhibition studies using synthetic progestin RU486, and showed that photoaffinity labelling was specific to progesterone receptors on the plasma membrane. Blondeau and Bauliau (1984), reported a 30,000 MW protein binding [^{3}H]R5020, after photoaffinity labelling. These authors homogenized defolliculated whole oocytes and established that a 10,000 x g pellet contained the plasma membrane fraction. They also found [^{3}H]R5020 photoaffinity labelled protein in various other fractions. Vitelline envelope fraction and vitelline membrane platelets fractions had binding activity corresponding to MW 98,000, which is close to MW 102,000 reported by Sadler, et al . 1985. In chapter 1 on cytosolic receptors, binding activity was also shown to be associated with MW 29,0000 to 44,000 protein. It is possible that this protein is either a precursor or a breakdown product of the receptor protein, since it was not observed from the solubilized membrane fraction. Alternatively it is also possible that conditions of photoaffinity labelling used by Blondeau and Bauliau disrupts the receptor protein, since they found a decrease

in photoaffinity labelling after a very short time of
photoaffinity interaction.

Maller 1985, has proposed the following hypothesis for the
interaction of progesterone with the Xenopus oocytes:
progesterone interacts with the oocyte plasma membrane
receptors which leads to a decrease in cAMP mediated
entirely or in part by an inhibition of adenyl cyclase.
Subsequent events include an increase in intracellular pH
and the phosphorylation of ribosomal protein S6; the later
playing a role in translation control of maturation. Late
events in maturation involve the appearance of the
maturation promoting factor (MPF), a cytosolic protein
responsible for GVBD. There is also evidence for increased
levels of Ca^{2+} ion after MIS action on the oocytes,
which indicates involvement of membrane phospholipids in the
action of the steroid via phosphatidylinositol (Baulieu, et
al . 1978; Morril, et al . 1981; Schorderet-Slakine, et al
. 1982).

Initially there was controversy surrounding the mechanism of
action of MIS progesterone in amphibians, since various
researchers were unable to demonstrate with certainty the
decrease in levels of cAMP after incubation with
progesterone, although adenyl cyclase was shown to be
inhibited. The various factors contributing towards the

measurement of cAMP levels leading to the above results were due to 1) variability in total content of cAMP in the oocyte 2) small magnitude of the decrease in cAMP compared to the total oocyte cAMP, and 3) limitations on the precision of the available assay methods. In rainbow trout, cAMP levels decreased significantly after 17α,20β-DHP treatment within 7 hours of incubation (Jalabert and Finet, 1986). These authors were able to show the decrease in cAMP because of the large number of oocytes used per sample, and large number of analysis per sample. Similar measurements of cAMP levels have also been made on brook trout oocytes, but significant decreases have not been detected (DeManno and Goetz, 1987). Jalabert and Finet (1986), also demonstrated increased levels of cAMP after administration of phosphodiesterase inhibitor IBMX (10^{-5}M), and IBMX (10^{-5}M) + forskolin (5×10^{-5}M), forskolin is an activator of adenyl cyclase. The above authors demonstrated increased levels of cAMP in late stage oocytes, and oocyte sensitivity to 17α,20β-DHP positively correlated with cAMP concentration. They also showed direct inhibition of maturation by cAMP.

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Chapter 4 .

Transport of 17α,20β-DHP into the brook trout Salvelinus
fontinalis ovarian follicles.

Introduction

MIS 17α,20β-DHP receptor activity was demonstrated on zona
radiata membrane preparations of the stage (6-7) oocytes
(Maneekjee, et al . 1989 a and b). It was decided to
measure the levels of MIS in the oocyte protoplasm
(cytoplasm + nucleus), because if this MIS action was
similar to classical steroid action via nuclei, the levels
of MIS within the protoplasm should correlate with the
number of MIS-receptor complexes present in the protoplasm
during the various stages of maturation due to receptor MIS
interaction.

When extremely low levels of MIS were found in the oocyte
protoplasm, it was important to determine if MIS uptake was
occurring into the oocyte after its synthesis from the
granulosa cells. Since it is possible that the physiological
action of MIS is at the plasma membrane level via receptors,
uptake of MIS into the oocyte protoplasm is not
physiologically necessary, and may not occur. After

establishing _in_ _vitro_ uptake of labelled MIS into the
ovarian follicles, the mechanism of uptake was also
investigated.

Methods and Materials

Chemicals

N-2-Hydroxyethyl piperazine-N′-2-ethane sulfonic acid
(HEPES), sodium chloride, potassium chloride, magnesium
sulphate $7H_2O$, magnesium chloride. $6H_2O$, calcium
chloride. $2H_2O$, penicillin G and streptomycin sulphate
were obtained from Sigma Chemical Company, St. Louis,
Missouri, U.S.A. Common organic solvents, hydrochloric acid,
and sodium hydroxide were purchased from Fisher Scientific,
Caledon and BDH, and were of highest purity available.
MilliPore Milli Q 18 mega ohm water was used throughout the
experimental procedure.

Measurement of total $17\alpha, 20\beta$-DHP levels (free + glucuronide) in the oocyte protoplasm from Atlantic salmon Salmo salar Ouananiche during final stages of maturation .

The frozen ovarian follicles stored at -70 deg C were thawed
at 0 deg C, and individually cut. The protoplasm of the
oocyte was separated from the ovarian follicle outer layers
(zona radiata plus outer follicular layers and some
connective tissue) by aspiration. To a known volume of
oocyte protoplasm was added labelled $17\alpha, 20\beta$-DHP as an
internal standard for recovery calculations and then

dialyzed against 25% ethanol/aqueous solution in a dialysis bag. The ethanol/aqueous dialyzed extracts (3 x 100 ml) containing the steroid were freeze-dried, and the freeze-dried powder was taken up in aqueous solution and then extracted with ether to remove the free steroid. The aqueous extract contained the glucuronide, and the sample was incubated with bovine liver beta-glucuronidase (EC 3.2.1.31, Sigma chemical Co. Ltd.) at 1000 Fishman U/ml in 0.1 M sodium acetate buffer pH 4.5 at 37 deg C for 24 h to convert the glucuronide to free steroid. The free steroid was then extracted and evaporated under nitrogen, put on a paper chromatogram, and developed with toluene-hexane-methanol-water (33:66:80:20) for 6 h at 23 deg C. Appropriate areas of the paper strips, as indicated by radioscan of adjacent reference strips of tritiated 17α-,20β-DHP were eluted with ethanol. An aliquot of the eluate was removed to measure recovery; the remainder was evaporated to dryness and redissolved in buffer for assay. Radioimmunoassays were set up using the buffers and charcoal procedure described by Simpson and Wright, for 11-oxotestosterone (1977). The initial ether extracts of free steroid samples were also similarly chromatographed and after extraction radioimmunoassayed. An antiserum to 17α,20β- DHP was produced in rabbits as described by Idler and Ng, (1979). The cross reactivity with various steroids has been described previously (So, et al. 1985).

Labelled MIS 17α,20β-DHP uptake experiments .

Fish were removed from the tank, and sacrificed by a blow on the head and then immediately placed on ice. The ovarian tissue was dissected out, and the connective tissue was removed from the ovarian follicles using forceps. The ovarian follicles were placed in TBSS buffer pH 7.5 (20 mM HEPES, 0.15 M NaCl, 3 mM KCl, 0.3 mM $MgSO_4$ $7H_2O$, 1 mM $MgCl_2$ $6H_2O$, 3.5 mM $CaCl_2$ $2H_2O$, penicillin 10^5 units/ l, steptomycin sulphate 100 mg/ l). $[^3H]17\alpha,20\beta$-DHP to yield 5nM solution was pipetted into a scintillation vial and then evaporated to dryness in a vacuum oven (NAPCO model 5831, Fisher Scientific Co.). TBSS buffer (5ml) was then added to the vial and vortexed, and to the resulting steroid solution at 10 deg was added brook trout ovarian follicles (20). The vials containing the ovarian follicles were incubated at 10 deg C in the presence of moist oxygen with slow agitation in a water bath shaker (Eberbach Corporation, Ann Arbor, Michigan, USA) connected to an RTE-8 refrigerated cooling unit (NESLAB Instruments, USA) for various time periods. The incubation was terminated by separation of ovarian follicles from incubation mixture, and a known amount of incubation mixture counted for radioactivity. The ovarian follicles were then quickly washed with 5 ml of TBSS at 1 deg C on a filter of nylon

mesh size 50. The ovarian follicles were then squeezed
through a glass syringe (1 ml), and oocyte protoplasm
separated from oocytes outer membranes using nylon mesh size
50. The membranes were suspended in TBSS and gently vortexed
to solubilize the cell protoplasm and the supernatant was
aspirated off, after which the membranes were dabbed with
tissue, aliquots weighed, and then digested in 'Protosol'
(2ml) plus water (1ml) at 50 deg C for 8 h. The digested
samples were then cooled and counted using scintillation
fluid (20 ml) after dark adaptation. A known aliquot of
oocyte protoplasm was also weighed and treated similarly.
The 'Protosol' digestion was carried out in capped
scintillation vials.

Initial rates of labelled 17α,20β-DHP uptake into the
oocytes, and oocyte outer membranes at various steroid
concentrations.

The [^{3}H]17α,20β-DHP uptake experiments were carried out
at various concentrations of the steroid under initial rate
conditions. The incubation and experimental conditions were
the same as in the previous experiment. The amount of
radioactivity was measured in the protoplasm and the outer
membranes of the oocytes as described previously. Double
reciprocal plots of initial rates of uptake against
concentration of steroid were plotted to establish the

steroid mode of transport.

Metabolism of labelled 17α,20β-DHP after uptake into stage (4-5) ovarian follicles at 10 deg C .

Brook trout ovarian follicles (20) were incubated in the presence of oxygen and [³H]17α,20β-DHP (100 nM) in TBSS (5 ml) at 10 deg C till GVBD occurred (47 h, 15 min). The incubation medium was extracted with 35 ml (7 x vol) of dichloromethane and then evaporated to dryness using rotary evaporator under vaccum below 40 deg C (Buchi HB-140), the resulting extract was then extracted in ethanol (3 x 3 ml) and made up to 10 ml. Aliquots of ethanol extracts were counted by scintillation counting, and 600,000 dpm (known values) of ethanol extracts were chromatographed on paper using toluene:hexane:methanol:water (33:66:80:20) system. Marker steroids (possible metabolites of 17α,20β-DHP, and inert 17α,20β-DHP were applied on the paper, 20 μg/spot) were also run simultaneously on adjacent strips for detection in UV or by phosphomolybdic acid technique. The sample chromatograph strips were cut and scanned for radioactivity using Packard model 7201 Radiochromatogram scanner and recording ratemeter. The ovarian follicles were homogenized in 10 ml TBSS, and centrifuged at 10,000 rpm (15,000 x g) for 10 min. The supernatant was extracted with dichloromethane (35 ml x 2), also the precipitate was mixed with dichloromethane (10 ml x 2) and extract separated by

centrifugation. The dichlomethane extracts were combined and rotary evaporated to dryness. The flask contents was extracted with methanol (3 ml x 3) and counted for radioactivity. The methanol extract and the marker steroids were then chromatographed on paper using heptane:methanol (80:20) system, and the strips scanned for radioactivity.

Results .

Uptake of labelled 17α,20β-DHP into the oocyte protoplasm and outer membranes

The decrease in [³H]17α,20β-DHP from incubation medium with time is shown in Fig.4. 1.a. The uptake of [³H]17α-,20β-DHP by the oocyte protoplasm and outer membranes (zona radiata + outer follicular cells) is indicated in Fig.4. 1. b. These results directly indicate that oocyte membranes are permeable to 17α,20β-DHP and that the uptake reaches equilibrium within 12 h of incubation.

Demonstration of [³H] 17α,20β-DHP mode of transport .

When a double reciprocal plot of the uptake of labelled 17α-,20β-DHP against the concentration of [³H]17α,20β-DHP (1-100 nM) was plotted using the data obtained by sampling the incubation medium. The plot yielded a stright line passing through the origin (Fig.4. 2. a.) demonstrating that the transport of [³H]17α,20β-DHP into the ovarian follicle is by passive diffusion and is not carrier mediated. In carrier mediated transport (saturable) the straight line cuts the abscissa (V_{max} finite), while in simple diffusion the line passes through the origin (V_{max} is infinity; 1/ V_{max} = 0) (Jacobson, and Saier, 1984).

Fig. 4.1. a. The decrease in $[^3H]17\alpha,20\beta$-DHP from the incubation medium occurring with time.

The oocytes were incubated in the presence of $[^3H]17\alpha$-,20β-DHP (5 nM) in TBSS at 10 deg. C, in the presence of moist oxygen with slow agitation.

Fig. 4.1.a.
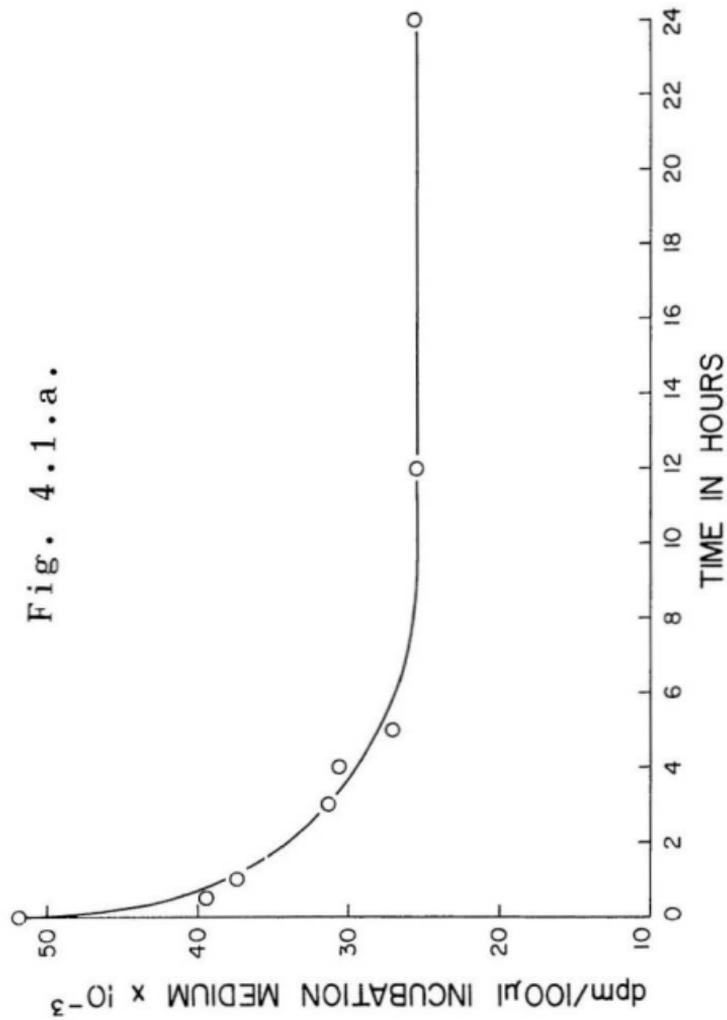
Fig. 4.1. b. Rate of uptake of $[^3H]17\alpha,20\beta$-DHP into the protoplasm and the membranes of the oocytes with time.

Fig. 4.1.b.
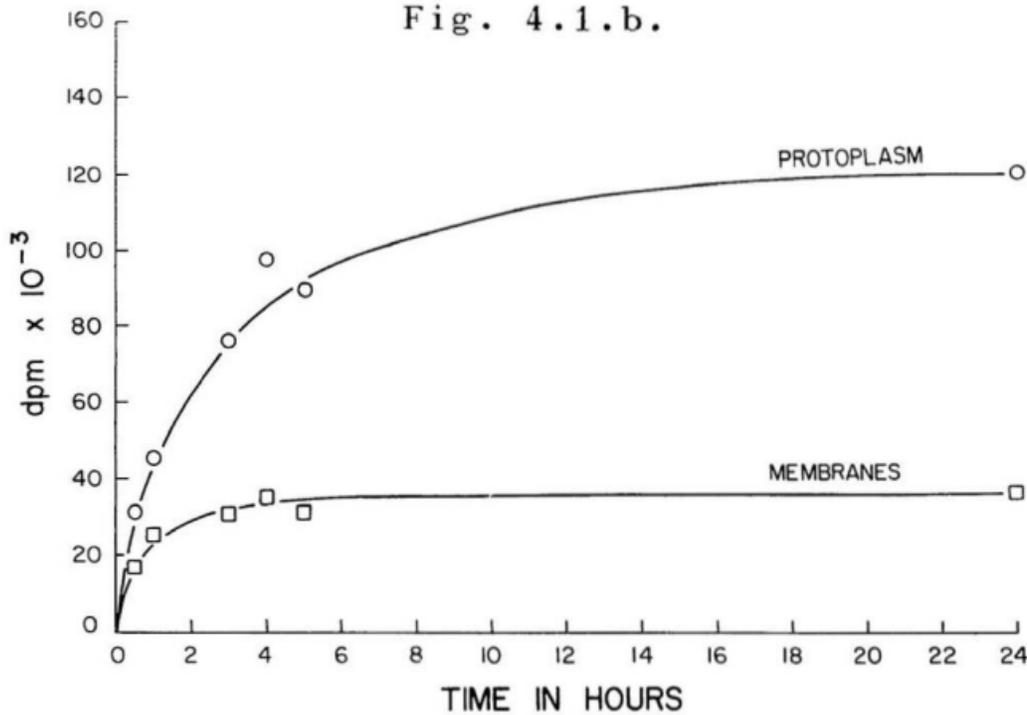
Similarly double reciprocal plots of uptake into the oocyte protoplasm gave a straight line passing through the origin, indicating passive diffusion as the mode of transport (Fig. 4. 2. b.). The deviation from linearity was observed in the case of transport into the membranes at lower concentration of the steroid mainly at 1 nM where the value for the initial rate of uptake was higher than anticipated thereby giving a lower value for $1/V_{max}$ (Fig. 4. 2. c.). This higher uptake indicates binding of the labelled steroid to the receptor sites in the zona radiata part of the membrane. The double reciprocal plot was linear at higher concentrations 2 nM and above, suggesting that the amount of binding occurring to the receptor protein is limiting and becomes negligible compared to the total uptake of steroid at higher concentrations.

Levels of 17α,20β-DHP in the ovarian follicles .

The levels of MIS 17α.20β-DHP measured by radioimmunoassay were very low and are given in Table 4.1.

Metabolism of labelled MIS .

After GVBD (47 h. 15 min) the incubation medium contained 31.7% of initial radioactivity added. 68.3% was taken up by the ovarian follicles. The incubation medium contained 48% organic soluble radioactivity, and 52% aqueous soluble radioactive material (probably glucuronide). The organic

Fig. 4.2. a. Double reciprocal plot of $[^3H]17\alpha,20\beta$-DHP decrease from the incubation mixture/(100 ul) into the oocytes at various concentrations of labelled steroid (1-100 nM). V = initial rate of decrease in $[^3H]17\alpha,20\beta$-DHP from incubation mixture per hour in dpm. S = concentration of labelled steroid in nM. (X——X) indicates the linear regression fitted line.

Fig. 4.2.a.

$\frac{1}{V} \times 10^4$

$\frac{1}{[S]}$
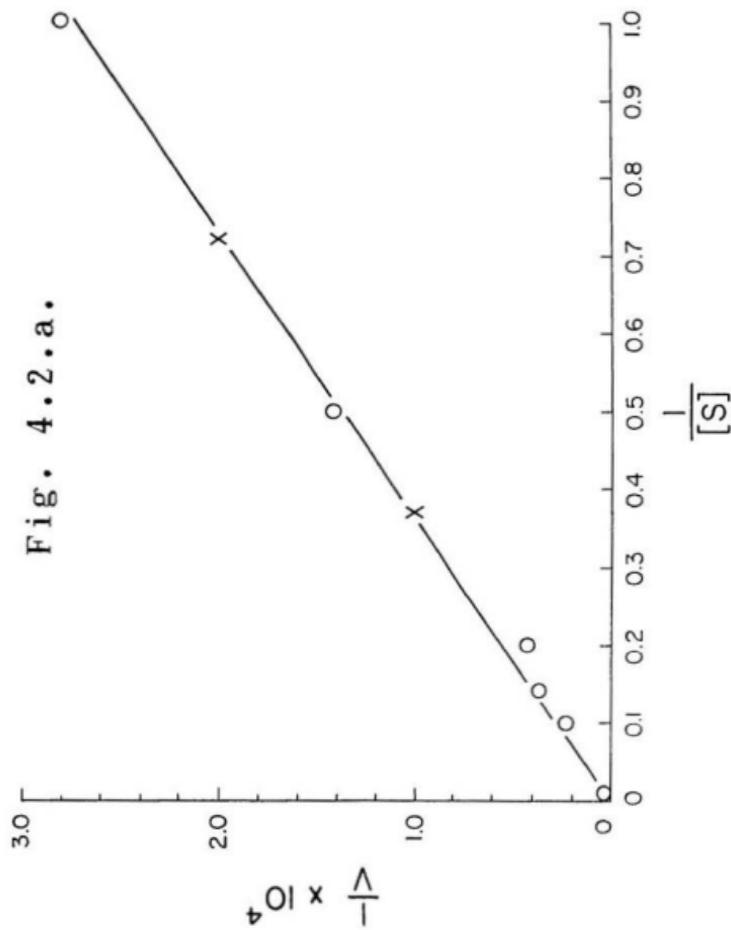
Fig. 4.2. b. Double reciprocal plot of uptake into the protoplasm of the oocytes (0.100 g) in dpm at various concentrations of labelled steroid (1-100 nM). The linear regression analysis gave regression coefficient of 0.9973, (P < 0.01). (X——X) denotes the linear regression fitted line.
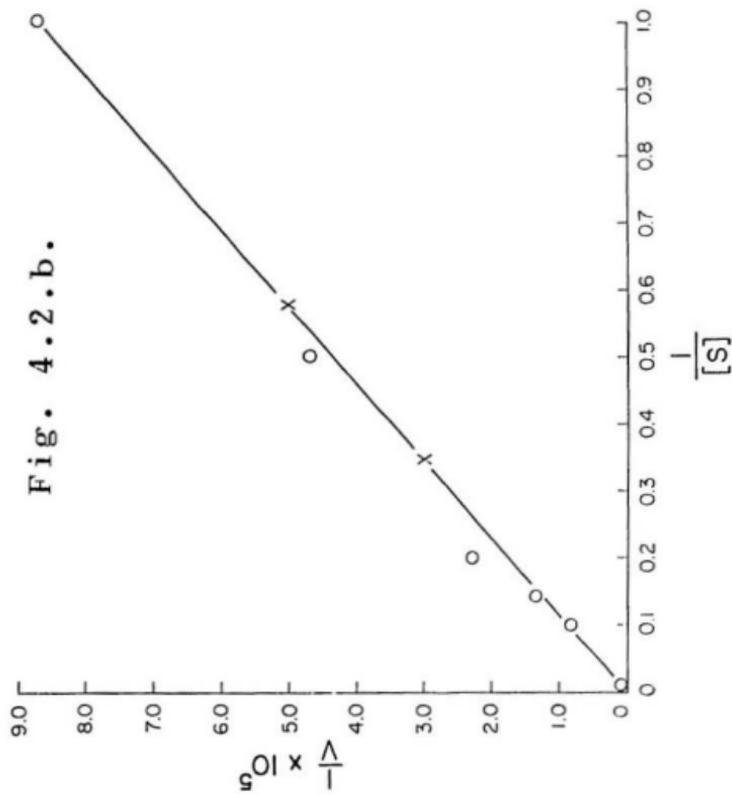
Fig. 4.2.b.

Fig. 4.2. c. Double reciprocal plot of uptake into the membrane fraction of the oocytes (0.030 g) in dpm at various concentrations of labelled steroid (1-100 nM). The linear regression analysis gave regression coefficient of 0.9896 using the first five points (P < 0.010).

Fig. 4.2.c.
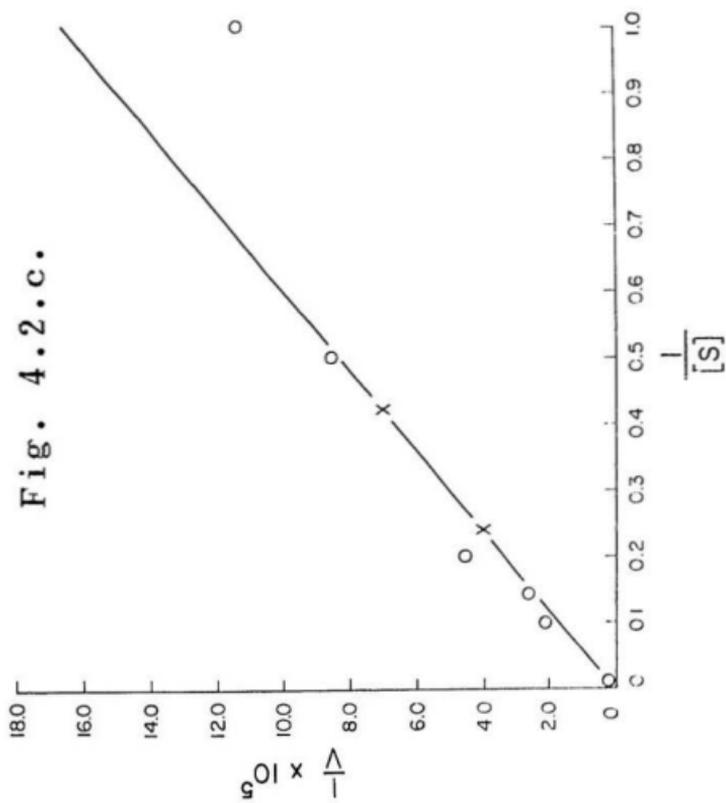
Table 4.1.

Concentration of 17α,20β-DHP in protoplasm and membranes of oocytes in different stages of maturation (n = 2).

Stage oocyte	Total 17α,20β-DHP (pg/ml protoplasm)	Total 17α,20β-DHP (pg/g membranes)
0-1	310, 266	163, 93
1-2	118, 80	205, 145
4	100, 86	213, 156
6-7	344, 172	513, 249

extract of incubation mixture after paper chromatography
demonstrated the presence of labelled $17\alpha,20\beta$-DHP (49.2% of
organic extract). Also 33.5% of the organic extract
radioactivity stayed at the origin, indicating the presence
of steroids more polar than $17\alpha,20\beta$-DHP, and 17.3% of
organic extract radioactivity ran off the paper, indicating
the presence of non polar steroids. The presence of marker
steroids used during paper chromatography a) 5β-pregnane -3β-
,17α, 20β-triol, b) 5α-pregnane-3α-$17\alpha,20\beta$-triol and c) 5α-
pregnane -$3\beta,17\alpha 20\beta$-triol were not observed during
radiochromatogram scanning of the paper strips. The organic
extract of ovarian follicles on paper chromatography
(heptane 80: methanol 20) gave 3 peaks of radioactivity, the
peak at origin constituted 40-50% of radioactivity, the
second the peak was $17\alpha,20\beta$-DHP and constituted 40% of
radioactivity and the furthest peak from the origin
contained 5-10% of radioactivity. The peak at the origin was
extracted out in ethanol, concentrated using N_2 and
rechromatographed using (toluene:hexane:methanol:water
33:66:80:20) system, with the above marker steroids, the
radioactivity peak after scanning did not correspond with
the marker steroids.

Discussion.

Levels of MIS 17α,20β-DHP from plasma of brook trout
Salvelinus fontinalis during final stages of maturation
have been recently reported by Goetz, _et al_ . (1987). The
MIS level during stage 2 (GV, just off centre) were 0.7 ng/
ml plasma, and level of steroid at stage 5 (GVBD) were
reported to be 148 ng/ml plasma. The levels of MIS present
in the oocyte protoplasm and the outer membranes as given in
Table 4.1 are at least 1000 fold smaller than the plasma
values. These results led into the investigation of uptake
of MIS 17α,20β-DHP occurring into the oocyte protoplasm and
the outer membranes. After establishing that MIS uptake was
occurring into the oocyte protoplasm, the mode of uptake or
transport of MIS was investigated. The results demonstrated
passive diffusion as the mode of transport into the oocyte,
and also indicated the presence of a binding protein for MIS
in the outer membranes of the oocyte. The presence of
receptors for MIS 17α,20β-DHP in the zona radiata membranes
of brook trout oocytes has been reported (Maneckjee, _et al_
. 1989 a and b, and chapter 3), and the results reported in
this chapter further support the evidence for the presence
of the membrane bound receptors for the MIS 17α,20β-DHP. The
very low levels of MIS 17α,20β-DHP found in the oocyte
protoplasm indicates the absence of MIS bound receptors
complexes in the oocyte cytoplasm and nuclei. Thus the

action of MIS 17α,20β-DHP is not directly on the nuclei, as in the classical steroid mechanism of action.

Since _in_ _vitro_ the uptake of MIS 17α,20β-DHP does occur into the oocyte protoplasm, but the levels of MIS 17α,20β-DHP observed in protoplasm of oocytes are atleast 1000 fold lower than reported in plasma. These results indicate that either _in_ _vivo_ uptake of MIS does not occur into the oocyte or after its _in_ _vivo_ uptake into the oocyte protoplasm the MIS 17α,20β-DHP is metabolised. The paper chromatography of the organic extract of the oocyte protoplasm (after labelled MIS 17α,20β-DHP uptake), indicated a presence of a major and a minor labelled metabolites of MIS 17α,20β-DHP. These metabolites were not characterized in this study. Although it is possible that these metabolites may have secondary roles during maturation.

High affinity binding to glucocorticoids has been demonstrated in the membranes of rat liver cells (Suyemitzu and Terayama, 1975; Terayama, _et_ _al_ . 1976), and rat hypophysis (Koch, _et_ _al_ . 1976,1977,1978). Also high affinity binding to estrogens has been demonstrated on the surface of the endometrial and liver cells (Pietras and Szego, 1977, 1978). High affinity steroid membrane binding proteins in liver cells have been implicated in the

transport of steroids (Rao, et al . 1977 b). The
experimental evidence from the double reciprocal plots of
initial rates of labelled steroid uptake against the
concentration of MIS 17α,20β-DHP clearly demonstrated a
passive diffusion mode of transport into the protoplasm of
the oocyte at and above the physiological concentration of
the steroid. The 17α,20β-DHP activity is present on the
oocyte plasma membrane (intercalated with zona radiata) and
does not seem to be involved in the transport of the
steroid. Jackson and Chalkey, (1974) hypothesised that
estradiol receptors in the uterine endometrial cells
originally bound to the plasma membrane, undergo
conformational change after binding the steroid and are
transferred into the cytoplasm and then into the nucleus.
This possibility can be ruled out in the case of 17α,20β-
DHP membrane bound receptors because 1) the levels of
cytosolic receptors decreases during final maturation, when
physiological action of 17α,20β-DHP occurs, 2) the presence
of nuclear receptors could not be demonstrated with any
certainty, 3) the physiological action of 17α,20β-DHP does
not involve the classical steroid mechanism i. e.
transcription of mRNA from DNA in the nucleus, since no
specific nuclear binding to MIS was observed (Maneckjee, et
al . 1989 a and b).

In the initial rates of [^{3}H]17α,20β-DHP uptake

experiments into the oocytes, the labelled steroid could have been metabolized to glucuronide and other labelled metabolites inside the oocyte protoplasm, and efflux of these metabolites of [^{3}H]17α,20β-DHP could have occurred into the incubation medium. Since the concentration of these metabolic products would be higher inside the oocyte than outside in the incubation medium at any given time. It is not possible during the initial rate 'time period' that uptake of these metabolites reoccuring into the oocytes from the incubation medium, unless the metabolites uptake occurs by active transport. After a 48h incubation with [^{3}H]17α,20β-DHP, the presence of labelled steroid as [^{3}H]17α,20β-DHP was demonstrated on the outside incubation medium as well as in the ooplasm by paper chromatography. Thus the measurements of radioactivity as indicator of 17α,20β-DHP uptake during the initial rates can be justified.

The uptake of [^{3}H]17α,20β-DHP was saturated within 6-12 h into the ooplasm, which is very slow compared to uptake of steroids into somatic cells eg. liver and endometrial cells (Rao, et al 1977; Muller and Wotiz, 1979; Peck, et al. 1973). The initial rate was chosen within 10% of this value which is standard practice in enzyme kinetics. This slow rate of uptake into the ooplasm is due the presence of outer layers of cells and membranes i.e. thecal cells, granulosa

cells, basal membrane and zona radiata. In Fig. 4.2.C., the
rate of uptake at low concentration of [^{3}H]$17\alpha,20\beta$-DHP
mainly at (1nM) into the outer layers of ovarian follicles
is higher than anticipated, which leads to lower value for
(1/V) on the graph. This could be explained in terms of the
presence of MIS $17\alpha,20\beta$-DHP receptors in the zona radiata
membrane binding to the steroid and giving total higher
uptake values. At higher concentrations of steroid this
receptor binding value is much smaller than the total uptake
of labelled steroid and becomes negligible. The steroid
concentrations for the uptake experiments were chosen with
consideration of the amount of steroid present under
physiological conditions. During final maturation plasma
levels of steroid $17\alpha,20\beta$-DHP have been reported in
salmonoids to reach 50-500 ng/ml (150-1500 nM), Goetz, et
al . 1987. Also the association constant (K_a) for the
$17\alpha,20\beta$-DHP zona radiata membrane bound receptor activity
was found to be 2.0 x 10^7 M^{-1} which is within the
range of values used for the study of the initial rates of
uptake.

The results obtained for $17\alpha,20\beta$-DHP uptake into the oocytes
is in agreement with the generally accepted concept of
steroids transport into animal cells occurring by passive
diffusion (Giorgi, 1980).

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Chapter 5.

Introduction.

This chapter is divided into two sections. The first section, describes the purification of cytosolic receptors from stage 1 brook trout ovarian follicles, where the cytosolic receptors are most abundant. The purpose of purification, was to try and isolate the 4 subunits of the purified cytosolic receptor in order that the sequence of the amino acids of each subunit can be carried out for further studies at a later date. The amino acid, and the gene sequences for the various steroids receptors are known, and similarities between the sequences have been observed (Evans, R. M., 1988; Green et al . 1986; Shepel and Gorski, 1988); it would be of interest to establish, whether the genes for the MIS receptor have any common features, with other steroid receptor genes. The purification of the cytosolic receptor is important, because it is the unmodified form of the receptor which is initially synthesized in the cytoplasm, and the amino acid sequences of the subunits may reflect more closely to the DNA sequences than that of the membrane receptor. The membrane receptor subunits might have been modified before its insertion into the plasma membrane. The purification of the membrane associated receptor was carried out for the preparation of antibodies and has been described in the

second section of this chapter (section b.)

Chapter 5b. also discusses the production, isolation, characterization, purification, properties and various applications of the antibodies produced in rabbits to MIS 17α-,20β-DHP receptor protein from the zona radiata, containing the plasma membrane of the oocyte. The antibodies towards the membrane receptors were produced because they are the most likely final functional form of the receptor protein involved in the physiological action of the MIS 17α,20β-DHP in salmonoids.

Chapter 5a.

Purification of brook trout cytosolic receptor preparation .
The purification was attempted using various biochemical
methods, and using frozen tissue from stage 1 ovarian
follicles of brook trout.

a.) Ammonium sulphate precipitation, of cytosolic receptor
preparation.

The receptor activity from cytosolic preparation
precipitated out maximally, in the the 0-30 % ammonium
sulphate fraction. After solubilization and dialysis, of the
precipitated receptor in TETS buffer, only 30% of initial
specific binding activity was recovered. SDS PAGE pattern of
the ammonium sulphate precipitated solubilized preparation,
did not show any significant improvement over the initial
cytosolic preparation, during this purification step. Also
specific activity (specific binding to $[^3H]17\alpha,20\beta$-DHP
per mg of protein), was about the same as the initial
cytosolic preparation, thereby indicating no improvement in
purity during this purification step. This was due to a loss
of receptor binding activity, during the ammonium sulphate
precipitation.

b.) Sephacryl S-300 column chromatography, of cytosolic
preparation.

After equilibration with labelled 17α,20β-DHP, the cytosolic preparation from brook trout stage 1 ovarian follicles was eluted from Sephacryl S-300 column in TETS buffer containing 0.15M NaCl (details on Sephacryl-S300 gel chromatography in chapter's 1 and 3 of thesis). Only 3-5 % of specific binding activity was recovered after elution. The loss of specific binding activity was due to two factors: 1) dissociation of receptor-ligand complex, and 2) transformation of the receptors during elution through the column. Dissociation alone does not explain the fact that the combined active peak fractions containing the binding activity from the Sephacryl S-300 eluate when re-equilibrated with labelled 17α-,20β-DHP, and assayed, showed no specific binding activity (equivalent amount of binding was obtained in both the total and the non-specific tubes). Also when the cytosolic preparation (without pre-equilibration) was eluted from the Sephacryl column, and the eluted fractions post-equilibrated with labelled 17α,20β-DHP and assayed, no specific binding was obtained (equivalent amount of total and non-specific binding was present in the active fractions). But when the cytosolic preparation was equilibrated with labelled 17α,20β-DHP, and then treated with charcoal-dextran for 30 min, to remove both the bound and free steroid, and the resulting receptor supernatant solution re-equilibrated with labelled steroid there was no loss in specific binding activity. Therefore the elution through the Sephacryl S-300 column and

loss in specific binding activity (transformation) may be
due to removal of other proteins, cofactors, or metal ions
present in the crude cytosolic preparation, and not only due
to dissociation or separation of the binding steroid ligand
during elution through the column. The peak receptor
activity tube fraction (after elution from the Sephacryl
S-300) on electrophorsis (page 4% gel), gave a single broad
band. On SDS-PAGE four subunits were obtained as described
in chapter 1 using the coomasic blue staining procedure.
This method has its limitation, due to the very small amount
of protein that can be purified each time through the
column, and due to the cytosolic receptor transformation
during elution.

c.) DEAE Sephacel column chromatography.

[^{3}H]R5020 photoaffinity labelled brook trout cytosolic
preparation, when applied on to DEAE Sephacel column, and
then eluted using KCl gradient in TETS buffer gave peak
receptor activity at 0.19 M KCl. The peak activity tube
on SDS PAGE gave a single band at 84,000 MW (chapter 3, Fig.
3.15). When the main tubes containing the photoaffinity
labelled receptor activity were pooled, and then
concentrated (freeze-dried),and then applied on to the SDS
PAGE gave the usual four bands at MW 84,000, 65,000, 24,000
and 23,000. Also when the cytosolic preparation was
equilibrated with 17α, 20β-DHP, and then applied on to the
DEAE-Sephacel column, similar results were obtained.

Although when cytosolic preparation was applied on to the
DEAE Sephacel column (without photoaffinity labelling or
equilibration with labelled 17α,20β-DHP), three peaks of
receptor activity were detected on post photoaffinity
labelling the various fractions (Fig. 5.1.). The ratio of
the peaks varied from preparation to preparation, and was
dependent on the protein concentration (weight of ovarian
follicles used per ml of buffer during initial
homogenization of cytosolic preparation). Better resolution
of peaks was acheived, when the protein concentration of the
cytosolic preparation was kept low (less than 100 μg/ml). At
higher protein concentrations, the peaks merged and gave
rise to a single broad peak. The peak activity tube from
this single broad peak of receptor activity did not give the
normal four bands on SDS PAGE, but was contaminated by other
proteins. When the tubes containing the two peak fractions
(fractions 24 through 48, Fig. 5.1.), were pooled,
concentrated by freeze-drying (lyophilization), and
fractionated on a Sephacryl S-300 column, a single peak of
receptor activity was obtained at MW 182,000 (assayed by
post photoaffinity labelling the fractions). When the peak
receptor activity tube fractions (MW 182,000) from Sephacryl
S-300 column were pooled, concentrated, equilibrated with 17α-
,20β-DHP (3 μM), and then eluted from the the DEAE Sephacyl
column (.05-.5M KCl), only peak of receptor activity was
obtained at 0.225M KCl (the receptor activity was measured

Fig. 5.1. DEAE Sephacel column chromatography of cytosolic
preparation from stage 1. brook trout ovarian follicles
(53g/60 ml in TETS buffer). Cytosolic preparation (50 ml),
was applied on to a DEAE Sephacel column (23 cm x 1.5 cm
dia.), washed with TETS buffer (50 ml), and eluted with a
linear KCl gradient (0.0-0.5M in TETS buffer; total volume
120 ml). Eighty nine fractions collected each _ca._ 1.4 ml;
250 μl aliquots assayed using photoaffinity labelling under
standard conditions as described in chapter 3. The peak
receptor activity was obtained at 0.125M, 0.195M and 0.325M
KCl.

Fig. 5.1.
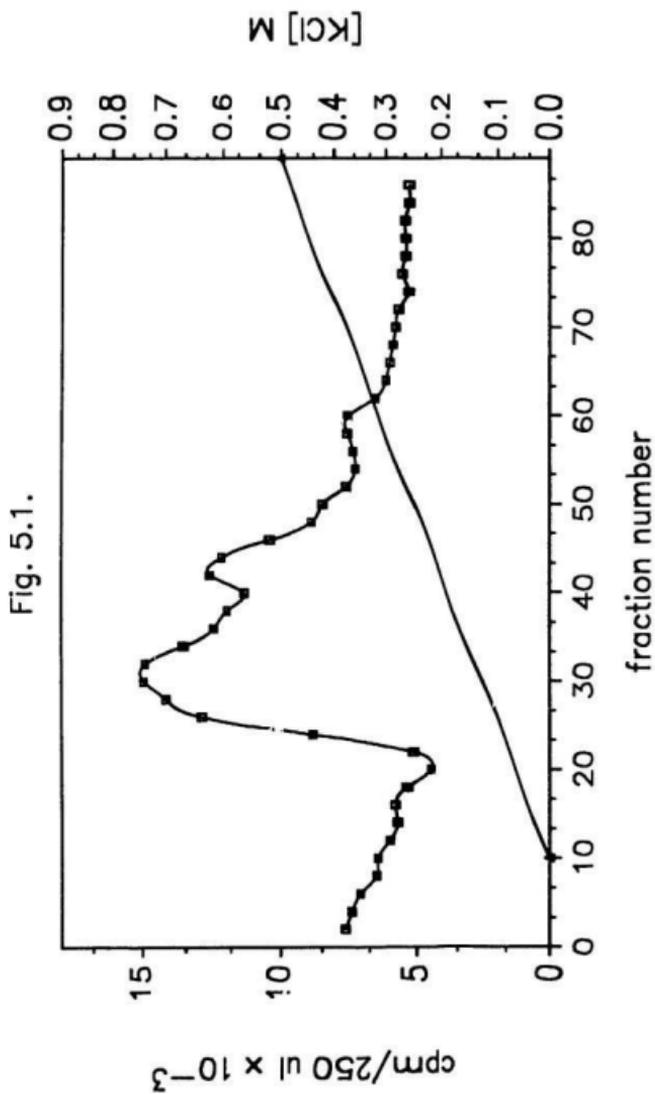
Fig. 5.2. DEAE Sephacel column chromatography of receptor preparation previously partially purified (see text), and then equilibrated with 17α,20β-DHP (3 μM). 15 ml of the equilibrated sample was applied on to a DEAE Sephacel column (22 cm x 1.5 cm dia.), and then washed with 60ml of TETS buffer, and then eluted using linear KCl gradient (0.0-0.5M in TETS buffer; total volume 120ml). Fractions (81) were collected, and 250 μl aliquots were photoaffinity labelled using labelled R5020. The peak receptor activity was obtained at 0.225M KCl.

Fig. 5.2.

246a.
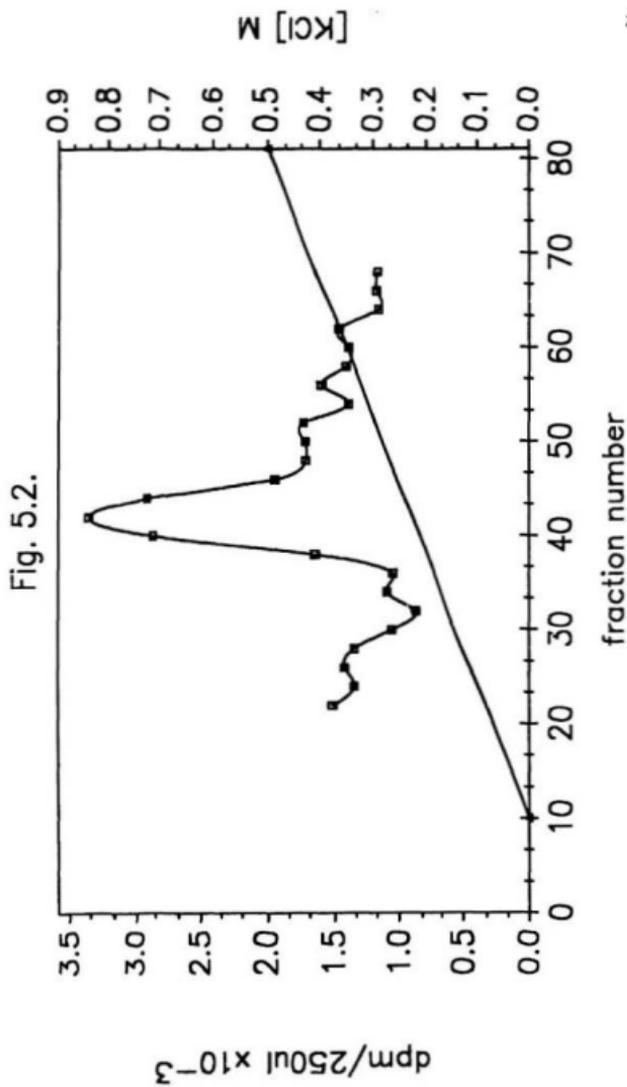
by post photoaffinity labelling the fractions). (Fig. 5.2.).

In one attempt at large scale purification brook trout stage 1 ovarian follicles (10.5 g), were homogenized in TETS buffer (60 ml at pH 7.4). After centrifugation, the supernatant was equilibrated with inert 17α,20β-DHP (3 μM), and applied to a DEAE Sephacel column and eluted using a KCl gradient. Sixty-four fractions were collected, and 250 μl aliquots assayed by photoaffinity labelling. Three peaks of receptor activity were obtained (Fig. 5.3.), after concentrating the three peak fractions by lyophilysation, the SDS PAGE from all the three peak tubes of receptor activity gave the usual 4 bands at MW 84,000, 67,0000, 24,0000 and 23,0000. Various other bands at high MW were indicated the presence of contaminating proteins in these fractions (Fig. 5.4.).

d.) Chromatofocusing .

Chromatofocusing separates proteins, through differences in their pI values with high resolution and high capacity. Chromatofocusing consists of a special exchanger gel (PBE exchanger, Pharmacia), with various charged groups. The gel is adjusted to one pH and eluted with a special eluent (Polybuffer, Pharmacia) at a second pH; thereby forming a pH gradient on the column, just as if two buffers at different pH were gradually mixed in a mixing chamber of a gradient

Fig. 5.3. DEAE Sephacel column chromatography of cytosolic preparation of brook trout ovarian follicles (10.6g/60 ml of TETS buffer). Cytosolic preparation (60 ml), was applied to a DEAE Sephacel column (24.75cm x 1.5cm dia.), washed with 60 ml TETS buffer, and eluted using linear KCl gradient (0.0-0.5M in TETS buffer; total volume 120 ml). Fractions (64) were collected, and 250 μl aliquots photoaffinity labelled using [^{3}H]R5020. Three peaks of receptor activity were observed at 0.11M, 0.18M and 0.285M KCl concentration.
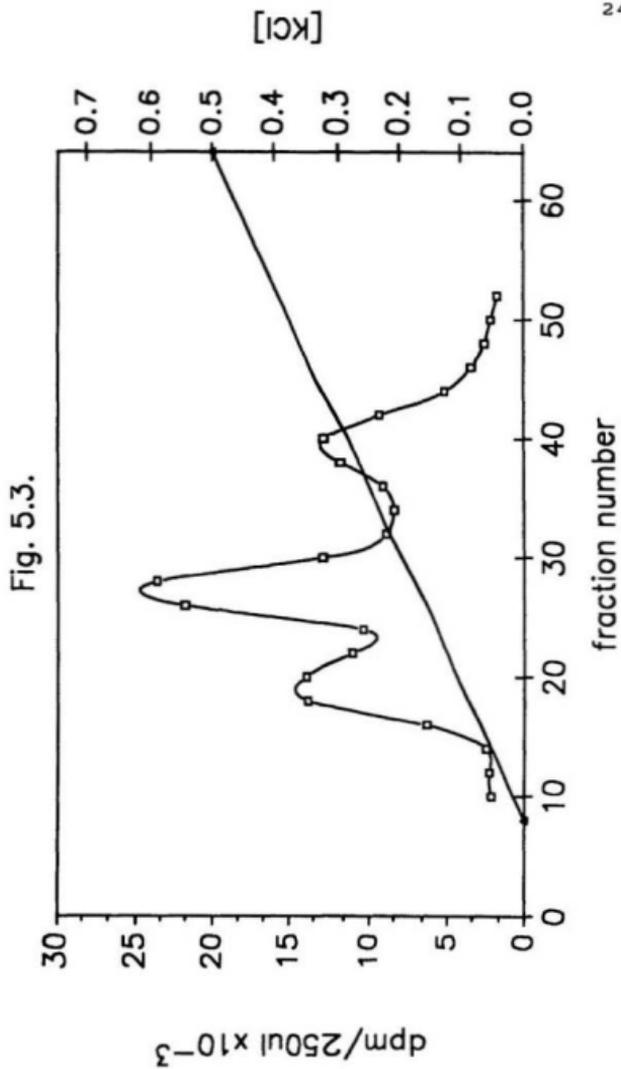
Fig. 5.3.

Fig. 5.4. SDS gel electrophoresis of the peak tube fractions
obtained from DEAE Sephacel column chromatography of
cytosolic preparation, of brook trout ovarian follicles
equilibrated with 17,20β-DHP (Fig. 5.3.). From left to
right, tube 20 (10,20,30 and 50 μl of sample), tube 28
(110,20,30, and 50 μl), tube 40 (10,20,μl), low molecular
weight standards (LMW), and samples from each peak tube (20
μl).

FIG 5,4

Tube 40   LMW Stds   20, 28, 40

Tube 28

Tube 20

249 a.
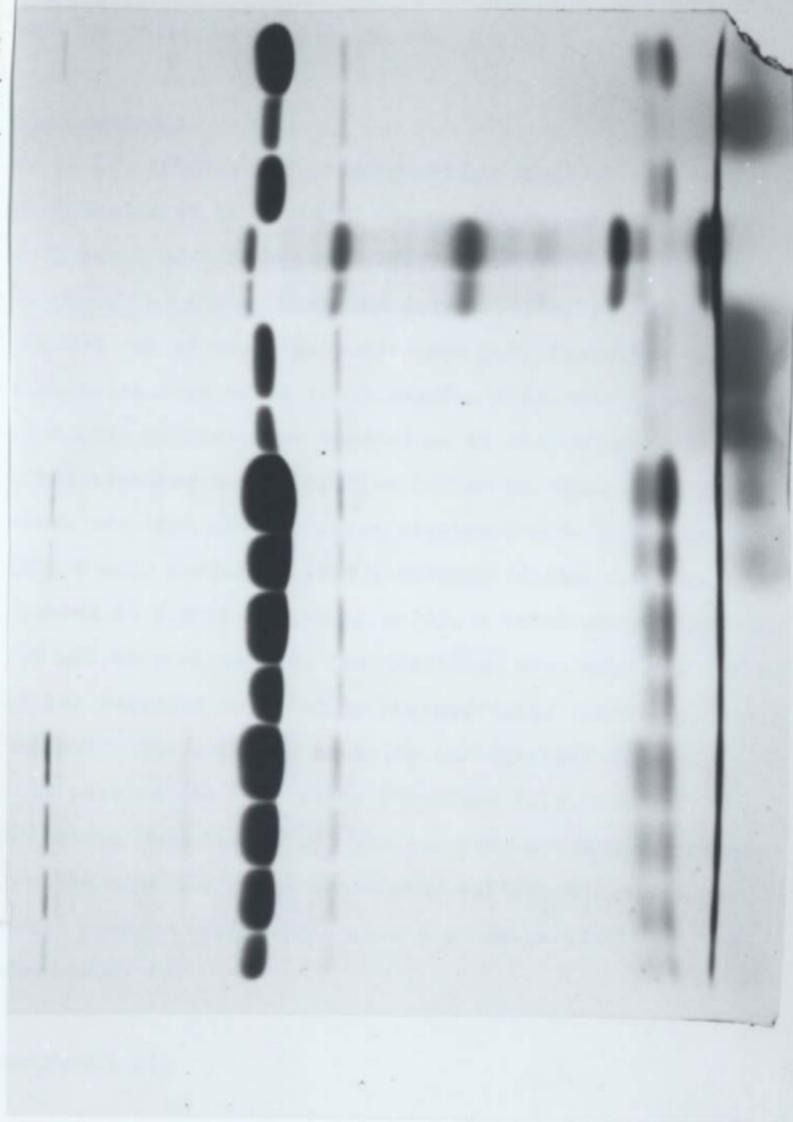
maker. When such a pH gradient, is used to elute proteins
bound to the PBE exchanger gel, the proteins elute in the
order of their isoelectric points.

Experiment # 1.

PBE 94 gel (Pharmacia), was suspended, packed, and then
equilibrated in to a C10/40 column (23 x 1 cm, Pharmacia),
using starting buffer (TRIS/CH3COOH pH 8.3, 0.025M).
The pH of the eluent from the column, after equilibrating
with 500 ml of starting buffer was 8.0. Cytosolic
preparation from brook trout ovarian follicles (stage 1,
3.5g/ 20ml buffer), was applied on to the column (after
buffer exchange in the elution buffer by dialysis). The
column was then eluted, using eluting buffer [poly buffer 96
(30%) + poly buffer 74 (70%), diluted (1:10), and pH
adjusted to 5.0 using acetic acid]. A total of 80 fractions
(2.5 ml) were collected. The fractions were measured for pH
and for receptor activity by photoaffinity labelling, using
[^{3}H]R5020. The receptor activity was obtained as a
broad peak in the very early fractions (Fig. 5.5.),
indicating that the pI of the receptor protein was higher
than the starting pH of the eluted buffer(pH 8.0). The
marker protein hemoglobin, gave a pI value of 6.7 in the
tube number 48.

Experiment #2.

Fig. 5.5. Chromatofocusing elution pattern obtained from cytosolic preparation of brook trout ovarian follicles. The cytosolic preparation (after buffer exchange), was applied on to a PBE 94 gel exchanger, equilibrated at pH 8.0, and then eluted using elution buffer at pH 5.0. The receptor activity did not bind the gel, as indicated by the initial broad elution peak, because the pI of the receptor activity, was higher than the pH of the initial eluted solution. dpm (◻–◻); pH (▲–▲).

Fig. 5.5.

pH

dpm / 250 ul × 10⁻³

fraction number
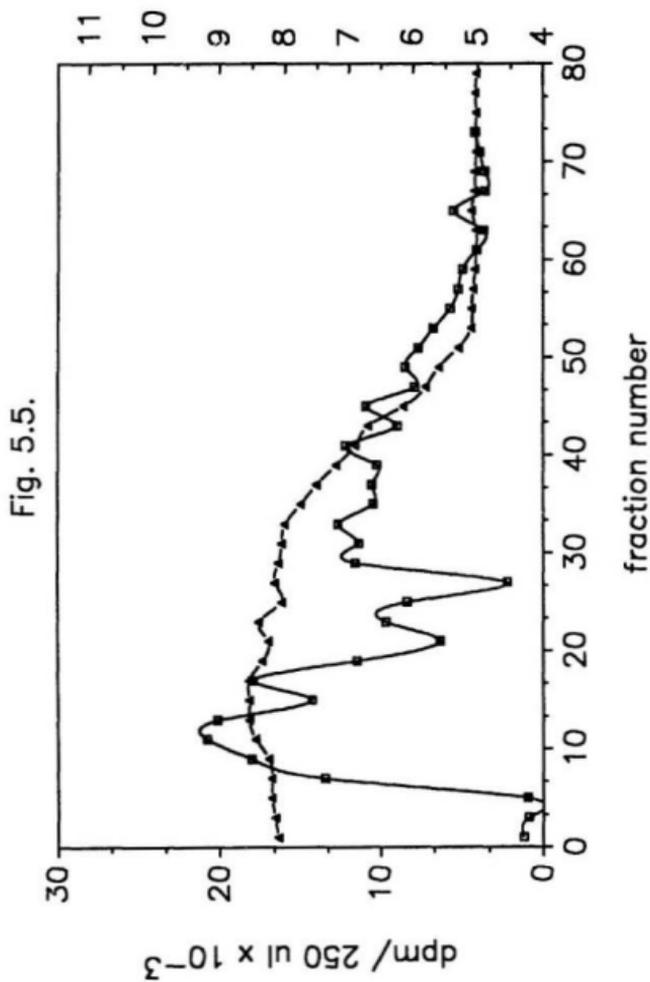
The above PBE 94 gel column, was initially washed with NaCl (1M, 100ml), and then equilibrated with starting buffer (ethanolamine/acetic acid pH 9.4, 0.025M, 500ml). The cytosolic preparation from stage 1 ovarian follicles in TETS buffer was dialysed (buffer exchange) with Polybuffer 96, at pH 6.0 (Polybuffer (1:10) diluted after adjustment to pH 6.0 with acetic acid), and then applied to the PBE 94 column. The column was eluted using the Polybuffer 96 (1:10 at pH 6.0). The pH and receptor activity were measured in the 80 fractions (3.35 ml) collected (by photoaffinity labelling, using [^{3}H]R5020). A broad peak of receptor activity was obtained between pH 9.48- 8.50, during initial elution through the column indicating that pI of the receptor protein, was higher than the pH of the initial eluted solution (Fig. 5.6.). The marker protein hemoglobin gave a pI value of 6.9.

Experiment # 3.

PBE 118 gel (Pharmacia) for higher pH range (11 - 7), was suspended, packed, and than equilibrated in a C10/40 column (26.5 x 1 cm, Pharmacia), using starting buffer (triethylamine/HCl pH 11.0, 0.025M). The eluent, from the column after equilibrating with 400 ml of starting buffer had a pH of 11.0. Cytosolic preparation, from brook trout ovarian follicles stage 1 (3.5g/20 ml), was used after buffer exchange in elution buffer by dialysis. After

Fig. 5.6. Chromatofocusing elution pattern, obtained from cytosolic preparation of brook trout ovarian follicles. The cytosolic preparation (after buffer exchange), was applied on to a PBE 94 gel exchanger equilibrated at pH 9.6, and then eluted using elution buffer at pH 6.0. The receptor activity did not bind the gel, indicating that the pI of the receptor is higher than the the pH of the initial eluted buffer solution.

dpm (□-□); pH (▲-▲).

Fig. 5.6.
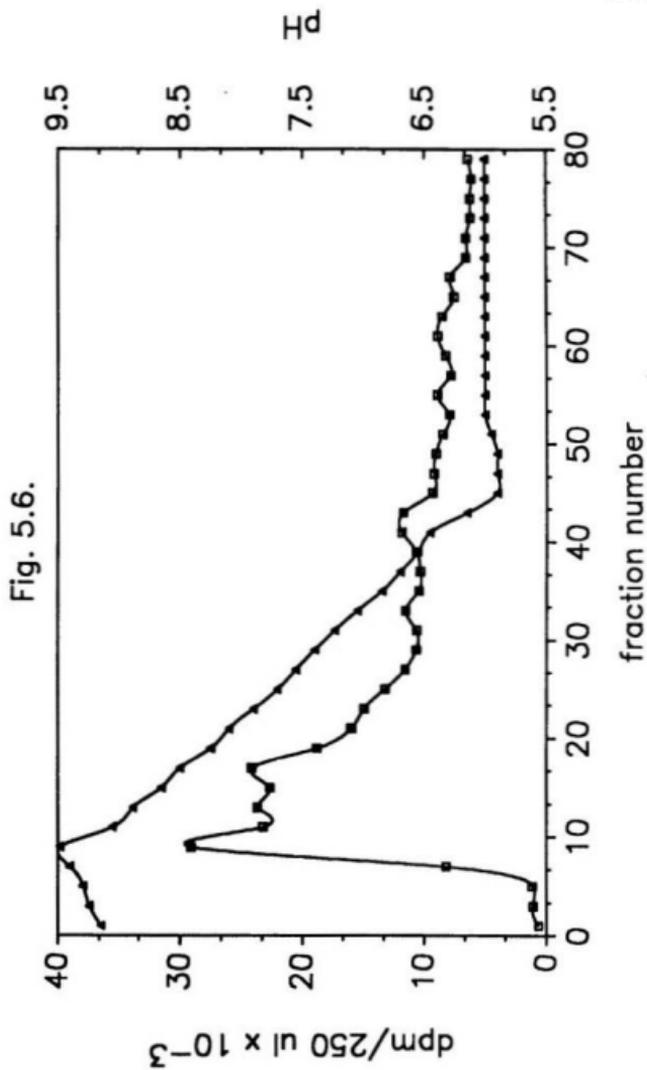
application of the sample, the column was eluted with pH 9.0
elution buffer (Pharmacyte 8-10.5, diluted 1:45). Eighty
fractions were collected, and measured for pH and receptor
activity (by photoaffinity labelling using [³H]R5020).
The receptor activity was obtained in fractions with pH
9.75-9.70 (Fig. 5.7.). The pH in fractions (30-80) remained
at 9.0. The column was then further eluted using the same
eluent buffer as above, after adjusting its pH to 7.0. No
receptor activity was observed in the next 80 fractions
(final pH 7.0). The fractions containing the receptor
activity (10,11,12 and 13), were concentrated (freeze
dried), and then taken up in 1 ml of TETS buffer. On SDS
PAGE analysis of these fractions, only 4 bands at MW 84,000,
65,000, 24,000 and 23,0000 were observed, with maximum
staining obtained in fraction 11 (Fig. 5.8.). The
concentrated fractions 10,11,12 and 13 were pooled, and then
applied on Sephacryl S-300 column (to remove endogenous
ampholines), and then eluted using TETS buffer containing
0.15m NaCl. The fractions were assayed for receptor activity
by photoaffinity labelling using labelled R5020. Two peaks
of activity were obtained, the active fractions from each
peak were combined, and freeze dried. The freeze dried
powders, were taken up in ammonium acetate solution (0.05 M,
pH 7.0), and than dialyzed in the same solution for 24 h
with three changes of 1.5 l each. The dialyzed samples were
freeze dried, and a small sample of each used for SDS PAGE

Fig. 5.7. Chromatofocusing elution pattern obtained from cytosolic preparation of brook trout ovarian follicles. The cytosolic preparation (after buffer exchange), was applied on to a PBE 118 gel exchanger equilibrated at pH 11, and then eluted using elution buffer at pH 9.0. The receptor activity, was obtained in fractions whose pH were 9.75- 9.70 (fractions 9,10,11,and 12). Thus the receptor protein pI is between 9.75-9.70.

dpm (□–□); pH (▲–▲).
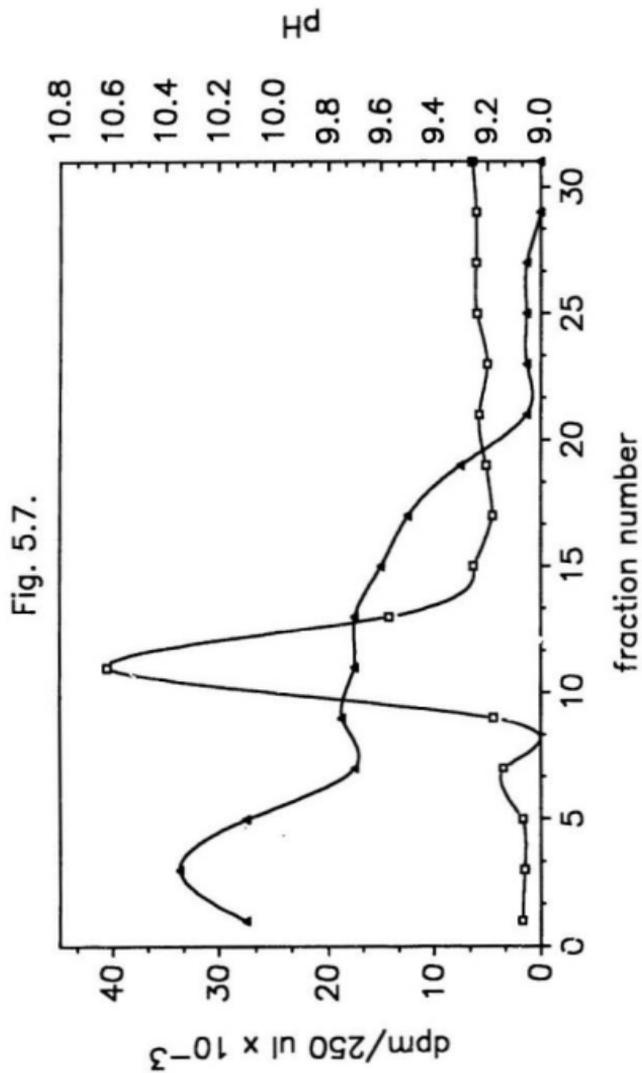
Fig. 5.7.

Fig. 5.8. SDS gel electrophoresis pattern obtained (after chromatofocusing) from active receptor fractions 10,11,12,13,14 and 15. The maximum activity was obtained in the fraction 11 (Fig. 5.7), which corresponds to the maximum staining obtained in fraction 11. Low molecular weight standards LMW. Fractions (F).

FIG, 5.8

256 a.
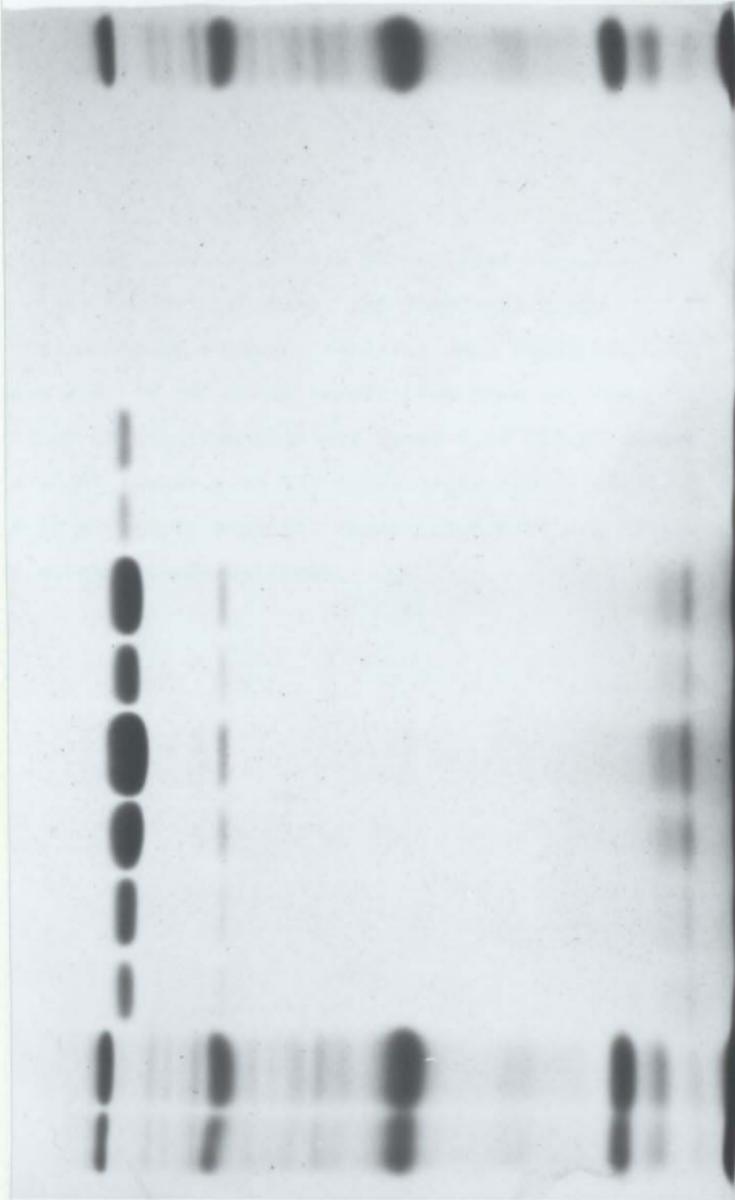
Fig. 5.9. SDS gel electrophoresis of purified receptor samples after chromotofocusing, and Sephacryl S-300 elution. Two peaks of receptor activity were obtained, see text. Lanes 3,4 (10 and 20 μl sample from peak 1); lanes 5,6 (10 and 20 μl sample from peak 2); lanes 9,10 (10 μl sample peak 1, + 10 μl sample peak 2); lanes 11,12 (25 μl sample peak 1, + 25 μl sample peak 2); lanes 1,2,7,8,13 and 14 low molecular weight standards (LMW).

FIG. 5.9

Lanes

1,2 LMW stds.

3,4 1st peak Sep-300

5,6 2nd peak Sep-300

7,8 LMW

9,10 1st & 2nd peak 10 µl

11,12 1st & 2nd peak 25 µl

13,14 LMW stds.

2570.
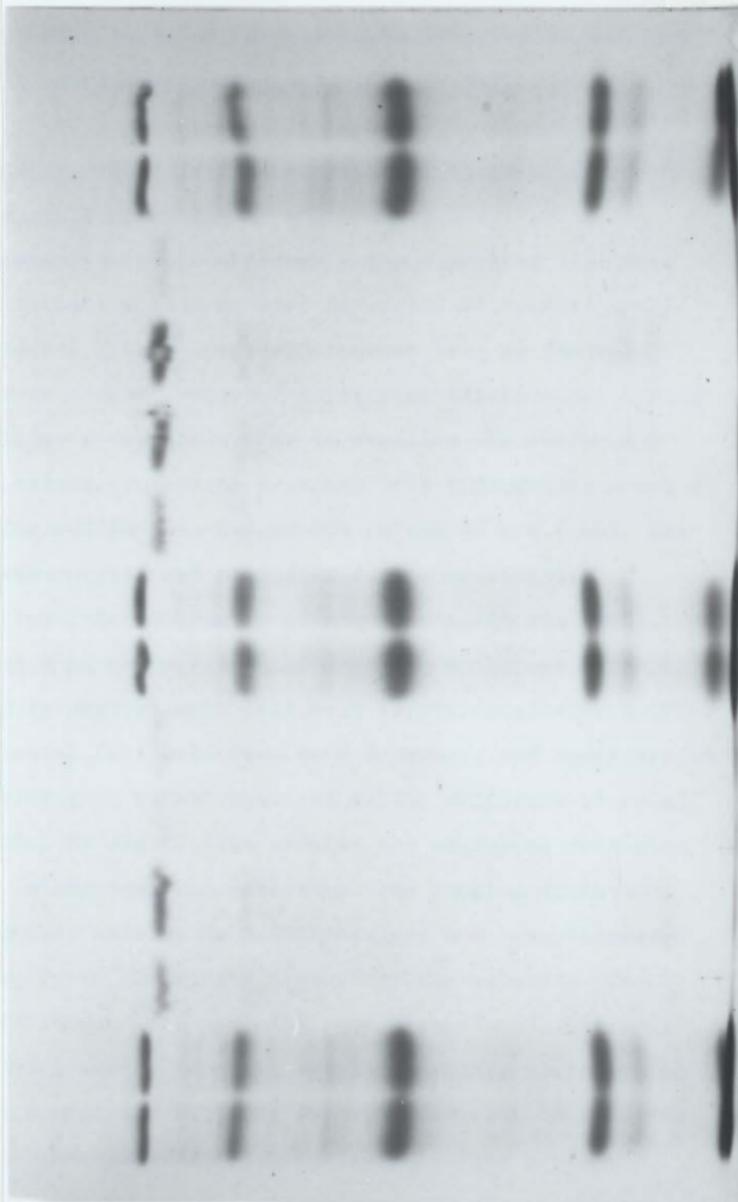
analysis. Four bands of MW 84,000, 65,000, 24,000 and 23,000
from both samples were observed. (Fig. 5.9.).

Attempted separation of the subunits of receptor protein, using reversed phase HPLC.

The chromatography was perfomed using a Beckman 110A pump
system, connected to a Beckman 332 gradient former, and a
Hitachi model 110-40 spectrophotometer (set at 280 nm),
attached to a chart recorder-integrator (Altex model C-R1A).
Initially an attempt was made to seperate the sub-units,
using a column containing 4-carbon (C4) hydrophobic group
separating medium (Bio-Rad RP 304 column 25 x 4.6 mm). The
column was conditioned according to the manufacturer
instructions, by washing it with 90% acetonitrile (ACN), and
then with HPLC grade water (18 mega-ohm Millipore water),
and finally equilibrated with 0.1% trifluroacetic acid (TFA)
in HPLC water (all solutions were degassed, and equilibrated
with Helium gas, before use, and during HPLC runs at room
temperature 22 deg C). The samples for analysis, were also
made up in the degassed solutions. The samples to be
analysed were made up in 0.1%TFA-water, and then incubated
at 37 deg C for 120 min to dissociate the subunits. The
incubated sample were injected, and then eluted initially
with 0.1%TFA-water (10 min), and then finally eluted using a
gradient containing 0.1% TFA in 0-95% ACN over 30 min. The
flow rate throughout the run was 1ml/min. The protein

concentration in the two samples were measured by the bicinchoninic acid procedure (Smith, et al . 1985; Olson, et al . 1985). The reagents for the protein assay were purchased from Pierce Chemical Company. The protein concentrations from the two peaks of receptor activity obtained after Sephacryl S-300 column chromatography were: peak 1. 380 μg/ml; total 950 μg, and peak 2. 410 μg/ml; total 1025 μg. The HPLC column was then tested for proper functioning, by performing several blank runs (by injecting the solution in which the sample was prepared). Also, Bio-Rad reverse phase column calibration standards were used for checking the separation capabilities of the column (consisting of cytochrome C, lysozyme and myoglobin). A typical result obtained, for blank and standard proteins, are shown in appendix (Trace 1 and 2). The receptor samples, from peak 1, and peak 2 (Sephacryl S-300), were then applied on to the column (10 μg protein/injection), and the typical results obtained are shown in appendix (trace 3 and 4). Two main peaks, and two minor peaks were obtained from both samples. The retention times were low, but reproduceable. The samples were eluted during the column washing period, with 0.1%TFA in water, and not during the actual gradient elution. All runs were carried out at least in triplicate.

The protein elution occurred very early during the run, indicating that very little hydrophobic binding was

occurring between the sample protein, and the C-4 hydrophobic groups of the column matrix. The possibility, that increased column retention times could be achieved, by increasing the size of the hydrophobic binding groups on the column matrix was investigated: a) by using C-8 (Beckman RPMC Ultra pore, series 7RPMC26, 75 x 4.6 mm), and b) C-18 (Beckman Ultra pore, 150 x 4.6 mm) columns . After conditioning the C-8 column, a typical control run (containing the 0.1%TFA in HPLC water) is depicted in Appendix (trace 5). Trace 6 in Appendix shows, the elution pattern obtained using the Bio-Rad RP molecular standards. The elution times of the standard proteins were increased, during the elution through the C-8 column, although, the background was not as stable, and reproduceable as when using the C-4 column. Also the retention times for both of the Sephacryl S-300 samples were lower, than with results obtained from C-4 column (Appendix; Traces 7, and 8), and separation not as well defined. In most runs, two main peaks, and a small broad peak were observed from both Sephacryl S-300 samples, although, it is possible that the first peak could have been composed of two proteins (Appendix; Traces 7, and 8). The possibility, that dissociation of the subunits of the receptor protein in 0.1% TFA, may have not been complete was also investigated, by incubating the two samples in propionic acid final concentration [1M] for 36 h at 3⁷ deg C (Burzawa-Gerard, _et_

al . 1976). But similar results were obtained as with 0.1% TFA method as described above. The three peak fractions obtained during the various runs, were collected (Gilson model 203 fraction collector, using the peak tube collection mode). Since the results obtained from the C-8 column, were not as satisfactory as the results from the C-4 column, further work was carried out in anticipation of getting better results using a C-18 column (Beckman Ultra pore RPMC, 150 x 4.6 mm). The typical results obtained for the control, and the reverse phase protein standards using C-18 column are shown in the Appendix (Traces 9, and 10). Due to the high hydrophobicity of this column, not all of the RP standard proteins eluted from the column completely (under the conditions very similar to that used as described above). Although the two samples from the Sephacryl S-300, were eluted, as shown in the Appendix (Traces 11, and 12). The results from all of the three reverse phase columns were similar, and gave between 3 to 4 peaks.

The SDS gel electrophoresis of the eluted subunits (separated peaks, after concentrating) from both Sephacryl S-300 samples was carried out, under the same conditions as described in chapter 1, in order to estimate the molecular weights of the purified subunits. The gels were stained by Coomassie blue, and silver nitrate (Morrissey, 1981) methods. The gels were stained with Coomassie blue for 24 h (instead of 60 min). After destaining very faint bands were

observed (not strong enough for photography). Peak 1 from both samples, displayed a very faint band near 84,000 MW band position. The silver nitrate staining method was then attempted in order to get better staining of the bands, but the method did not stain these protein subunits, or the low molecular weight standards used on the gels. The silver staining method was repeated twice, without any success.

Attempted affinity purification of cytosolic receptors.
Synthesis of Thiopropyl-Sepharose 6B derivatives of MIS 17α-.20β-DHP and testosterone, using ^{60}Co
gamma-radiation.

Thiopropyl-Sepharose 6B, lot number 03304 was purchased from Pharmacia Canada, and (6 gm) washed to remove the stabilizing additives, using freshly prepared Milli-Q 18 mega ohm degassed, helium saturated water (the Milli-Q water was degassed by sonication under vacuo, and saturated with helium gas, prior to use). The free -SH groups of the purchased gel, were protected by thiopyridyl groups (to prevent them from oxidation), and the protecting groups had to be removed prior to use. These groups were removed by treating the gel with a solution (200 ml) containing 0.3M sodium bicarbnate, 1mM disodium EDTA, and 1% DTT at pH 8.4.(prepared in Milli-Q degassed, helium saturated water). The gel was further washed, with a solution containing 0.1M acetic acid, 0.5M NaCl, and 1 mM disodium EDTA (2 1), followed by, degassed, helium saturated Milli-Q water, and

then with double distilled degassed, helium saturated ethyl
alcohol. The gel was filtered, and then divided into two
equal parts by weight, and placed into scintillation vials.
A solution of testosterone (75 mg/5 ml) in double distilled
degassed helium saturated ethyl alcohol was added to one of
the vials. A solution of MIS 17α,20β-DHP (75 mg/5 ml) was
added to the second vial containing the gel. The vials were
flushed with nitrogen gas, and sealed and sent to Dr. Kazi
Shamsuzzaman at the Radiation Applications department of
Atomic Energy of Canada, Research Company, Pinawa, Manitoba,
Canada. The ^{60}Co radiation of the above vials
containing the thiopentyl-Sepharose 6B and the steroids were
carried out according to the method of Brandt, et al.
(1977).

b) Affinity column chromatography using the thio-Sepharose
6B derivatives of 17α,20β-DHP and testosterone.
The covalently bound thio-Sepharose derivatives of 17α,20β-
-DHP and testosterone, obtained after gamma radiation were
washed several times with ethanol (200 ml previously
degassed and saturated with helium). The extracts were then
evaporated to dryness using a Buchi rotatory evaporator to
test the presence of free steroids. Each derivative on the
gel matrix was then treated with 0.1M iodoacetamide (100 ml)
for 5 min, to block any free -SH groups present, and then
washed with MilliQ 18 M ohm water. The 17α,20β-DHP

derivative was then packed into a Pharmacia C10/10 column.
The column was coupled via a AC10 adapter to a Gilson
minipuls pump, and a Gilson micro fractionator; total bed
volume of the column was 3.3 ml. The column was first
equilibrated with TRIS buffer (10 mM Trizma 7.4, 1 mM EDTA)
at pH 7.4. The soluble (151,000 x g) receptor fraction
prepared from frozen stage 1 ovarian follicles (7.0 g / 40
ml of TRIS buffer) was then slowly pumped through the column
(10 ml/h). The column was further washed with TRIS buffer
(10 ml), and then eluted with 0.1M glycine/HCl buffer at pH
3.0. Fractions (50 drops) were collected, the pH adjusted to
7.0 and then assayed for protein, and receptor activity by
photoaffinity labelling as previously described in chapter
3. Receptor activity was observed in the washing buffer and
also in the initial eluate from the column. Protein and
receptor activity were not observed in the glycine/ HCl
eluate from the column. The same experimental procedure was
then repeated using testosterone thio-Sepharose 6B
derivative.

Chapter 5b.

Production, isolation, characterization, purification and properties of rabbit antibodies to 17α,20β-DHP membrane receptor protein.

Introduction.

Production of antibodies to receptor protein from the membrane receptors of MIS 17α,20β-DHP was attempted to block in vitro the physiological action (GVBD) of the steroid. The in vitro application of antibodies to denuded oocytes would provide evidence of steroid action at the plasma membrane of the oocyte via the receptors, if the application inhibited the action of 17α,20β-DHP on GVBD. At present the evidence from the maturation studies, indicates specific GVBD response to 17α,20β-DHP which is partly inhibited by competetive inhibitor R5020 (chapter 1.; Maneckjee, et al . 1989.). Other application aspects of antibodies to the membrane receptors were also considered and partly attempted: 1) Immunocytochemical localization (detection) of receptor activity on the zona radiata membrane with antibodies using fluorescent anti rabbit gamma globulins produced in goat. 2) Covalant binding of the receptor antibodies to CNBR-activated Sepharose 4B, for batch affinity purification of the receptors. 3) Radio labelled antibodies could be used, for radioimmuno localization of the receptors by combined histochemical/autoradiography method, and for quantitative estimation of receptors by

various methods, e.g. sucrose gradient sedimentation, column chromatography, and gel electrophoresis.

This part of chapter 5, describes the experimental aspects of production, isolation, characterization, purification and some properties of rabbit antibodies made from purified receptor protein isolated from the zona radiata membrane fraction of stage 6-7 oocytes of brook trout.

Methods and materials.

Fish.

The source of receptor activity used in the preparation of antibodies to the membrane receptor protein, was from the same batch of brook trouts as reported and described in Methods and Materials section of chapter 2. The stage (6-7) oocytes, were collected during late October and early November 1987. The oocytes (loose in the abdominal cavity), were scooped up and placed into plastic sample bags (Fisherbrand catalog # 01-816C size 9 x 18 in) in a single layer, and immediately frozen flat at -70 deg C.

Isolation, and purification of membrane receptors for antibodies production.

The frozen oocytes (stage 6-7) from brook trout were carefully unfrozen at 0 deg C, and the zona radiata membrane fraction isolated by ultracentrifugation as described in

chapter 3 (23 g of oocytes were used in a single batch
purification procedure). The membranes were washed in TETS
buffer (3 x 5ml), and then homogenized in TETS buffer
containing 0.1% Brij 35 (10 ml). After ultracentrifugation
of the homogenate (151,000 x g, for 60 min), the supernatant
was applied on to a calibrated Sephacryl S-300 column, and
fractionated using TETS buffer containing 0.1% Brij. About
3.5 ml fractions were collected, and 250µl aliquots from
alternate fractions of the 160 fractions collected were
photoaffinity labelled, and assayed for receptor activity as
described in chapter 3. The peak tube containing the
receptor activity was then immediately frozen at -70 deg C.
The above receptor isolation and purification procedure was
repeated (10 times). The peak activity receptor tubes from
each of the 10 runs were combined, and then freeze-dried.
Small quantity of the freeze-dried powder was taken up in
water, and samples analyzed on PAGE (4% gel) and by SDS PAGE
as described previously. Also protein analysis was carried
out on the sample.

Preparation of rabbit antibodies, to membrane-isolated
receptor protein.

Initially, a single New Zealand white male rabbit was used
in the production of antibodies. Intradermal injection at
multiple sites on the back, consisted of 105 mg of freeze
dried powder (90 ug of receptor protein) dissolved in water

(2 ml), and to it added monophosphoryl lipid (MPL) plus thehalose dimycolate (TDM) emulsion (2.0 ml consisting of 0.5 mg MPL, 0.5 mg TDM in 2% Tween 80, Ribi ImmunoChem Research, Inc. Hamilton ONT). Also a single subcutaneous injection (1 ml), of killed mycobacteria (Pertusis vaccine strain 18334) was given. Two months after the primary injection, booster injections were given every month intramuscularly to the hind limbs consisting of 65 μg of receptor protein in 2 ml of water, plus 2 ml Freund's adjuvant (Difco Laboratories). Blood was removed from the artery in the rabbit ear and serum antibody titer was monitered using the immunodiffusion technique (Immunodiffusion gel box, catalog 170-4168; Agarose immunodiffusion tablets, catalog 170-3002. BIO-RAD Laboratories, Richmond, California). A second rabbit was also injected after 5 months following the first rabbit. The receptor protein was similarly prepared, although only 75 μg of receptor protein was initially injected. Booster injections were given thereafter every month using 50 μg of receptor protein as described above (Chard, 1987).

Purification of antibodies from serum proteins .

Immunoglobulins containing the antibodies to the membrane receptor protein (IgG subclasses), were purified from crude rabbit serum by affinity chromatography using Staphylococcal Protein A bound to Agarose (BRL). Protein A column (30 x 1

cm), containing 23.56 ml of gel was first washed with 0.1M
glycine-HCl buffer pH 3.0 (200 ml), by elution through the
column. The column was than washed with 0.1M
Na$_2$H/NaH$_2$PO$_4$ buffer pH 7.0 (200 ml). A serum
sample (7 ml, rabbit #2 date 21.9.88) was applied to the
column, and then washed with the above phosphate buffer
until all the unbound serum proteins other than the gamma
globulins (IgG) were eluted from the column. Thirty-five
fractions of 100 drops were collected, and its optical
density (O.D.) measured at 280 nm. The column was further
eluted using the glycine-HCl buffer at pH 3.0. The pH, and
the O.D. of the eluted fractions were then measured. The
fractions containing the IgG were combined, the pH adjusted
to 7.0, and then divided into 1 ml aliquots and frozen at
-70 deg C. Similarly, the experiment was repeated using
serum (7 ml)
from a control rabbit injected with carrier medium only (not
containing the receptors), and fractions containing gamma
globulin were also purified.

Inhibition of receptor binding to MIS [³H] 17α,20β- DHP, by antibodies to the receptor protein.

[³H]17α,20β-DHP binding with cytosolic receptor
preparation were carried out in the presence of receptor and
control antibodies as described in chapter 1 (steroid
binding studies). The concentration of [³H]17α,20β-DHP

was 4.3 nM, and antibodies solutions were at (1:8) dilution
of plasma. During Protein A purified antibodies inhibition
experiments, the cytosolic receptor preparation was
preincubated for 90 min with the antibodies, prior to the
addition of the [^{3}H]17α,20β-DHP.

Attempted in vitro inhibition of oocyte maturation using antibodies to the membrane receptor protein .

The fish were killed by blow on the head and kept on ice.
The ovary was dissected out and placed in TBSS at 4 deg C.
The individual follicles were separated out, by removal of
connective tissue under dissecting microscope using fine
tweezers. The separated ovarian follicles were then
incubated in TBSS (20 follicles/ 5 ml TBSS in a
scintillation vial), at 10 deg C in a thermostatically
controlled shaking water bath. The vials were gently shaken
at 5 times per min. The experimental protocol was as
follows:-

Vial #	Description
1,2	20 O.F.* in TBSS
3,4	20 O.F. + 17α,20β-DHP, 50 ng/ml
5,6	20 O.F. + purified antibodies (AB)
7,8	20 O.F. + purified control antibodies (CAB)
9,10	20 O.F. pre-incubated for 2h with AB, + 17α,20β-DHP

11,12   20 O.F. pre-incubated for 2h with CAB + 17α,20β-DHP

* stage 4 brook trout ovarian follicles

The vials were removed from the water bath, at least twice
within each 24 h period and placed on ice, and the ovarian
follicles were examined visually under a dissecting
microscope for GVBD. The experiment was continued for total
of 70.5 h, when the visual examination indicated 100% GVBD
in the vials 3 and 4. Then each vial was re-examined, after
addition of egg clearing solution (chapter 1) for GVBD and
scored. The experiment was repeated using stage 4 ovarian
follicles from a different fish.

Removal of outer follicular layers from the ovarian
follicles .

1) From early stage 1 Salmo salar Ouananiche ovarian
follicles: it was very difficult to remove the granulosa
cells completely from the zona radiata membrane of the stage
1 oocytes. The removal of the outer connective tissue and
the thecal layers, was accompanished after the ovarian
follicles were incubated for a minimum of 4-6 h in calcium
free TBSS, containing 5 mM EDTA at 4 deg C. The connective
tissue and the thecal layers were both removed
simultaneously, under the dissecting microscope by holding
the connective tissue by fine tweezers (#N5 INOX), and

slowly lifting the ovarian follicle from the solution. Then
a cut was made below the thecal layer using micro scissors
(#24 IREX), after which the cut was expanded, and the oocyte
containing the granulosa cells, got detached from the outer
layers. The presence of granulosa cells on the zona radiata
was demonstrated by staining the oocytes with ethidium
bromide (Greeley, et al . 1987). An attempt was made to
remove the granulosa cells attached to the zona radiata by
stirring the oocytes containing the granulosa cells over a
nylon mesh (250 mμ pore size) using a gyro-rotator (at 75
rpm), in a cold room at 6 deg C without success (as
indicated by the dye test). Partial removal of the granulosa
cells did occur during this process.

2) Brook trout stage 4 ovarian follicles: the ovarian
follicles were separated by removal of the connective tissue
as described above, and then placed in calcium free Cortland
buffer for 48 h, with several changes of buffer. The outer
connective tissue and the thecal layers were removed under
dissecting microscope by holding the connective tissue layer
by two fine forceps (#5 INOX), and then pulling in the
opposite direction to tear the tissue, whereby the oocyte
containing the granulosa cell layer burst forth from the
slit created. The granulosa cells were then detached from
the outer zona radiata membrane by placing the above oocytes
in a 125-ml conical flask containing 25 ml of calcium free
Cortland buffer, and gyro-rotating it at 100 rpm for 10 min

at 1-4 deg C. The oocytes were separated from the granulosa
cells, by placing the oocytes on a large pore nylon mesh and
washing them with Cortland buffer. The oocytes were then
placed in Cortland buffer for 60 min prior to use.
3). Brook trout stage 4 oocytes, can also be removed from
the outer thecal and connective tissue layers, by simply
holding the follicles in flat tweezers and pressing. The
granulosa cells were then also detached from the oocytes by
gyro-rotation as described above in method (2).

Attempted inhibition of maturation, by membrane receptor
antibodies using 1.) denuded oocytes, and 2.) oocytes
containing granulosa cell layer as obtained from methods (2)
and (3), without the gyrorotatory step .
The denuded oocytes, or the oocytes containing the granulosa
cell layer were first conditioned in Cortland buffer, for at
least 60 min before incubation with antibodies. The
experimental protocol was the same, as described above for
the whole ovarian follicles, but only 10 oocytes were used
per each vial.

Identification of MIS receptors on zona radiata membrane
(after incubation with receptor antibodies), using
fluorescent anti-rabbit gamma globulins produced in goat .
Zona radiata membrane fraction was prepared from fresh brook
trout stage (6-7) oocytes by ultra-centrifugation as

described in chapter 3. The membranes were washed (3 x 5 ml), and then divided into two parts. One part was incubated with Protein A purified antibodies (1 ml), and the second part with Protein A purified control antibodies (1 ml) for 12 h at 10 deg C. The membranes were separated by filtration (nylon mesh), and then washed with TBSS (3 X 5ml) and frozen at -70 deg C. The membranes were then thawed at 0 deg, and placed in Bouin fixative at 4 deg C for 3 days. The Bouin solution was removed, and the membranes dehydrated using ethanol solutions (30%, 50%, 70%, 90% and 100%; 10 min at each stage, and twice in 100%). The ethanol was removed by decantation and the membranes placed in xylene. The xylene was removed by transferring the membranes in paraffin wax. Finally the membranes were embedded in paraffin blocks using Fisher Histo-Center (Fisher Scientific Company).

Results

Preparation of the membrane receptor for antibodies production .

The total weight of freeze-dried powder obtained after combining the peak tubes from the ten Sephacryl S-300 runs was 0.78 g. The freeze-dried powder contained 0.670 mg of purified receptor protein. The SDS PAGE analysis gave 4 bands at MW 84,000, 65,000, 24,000 and 23,000. A single band at high molecular weight was obtained on 4% PAGE gel. In both SDS, and PAGE analysis, the gels were stained using Coomasie blue method.

Antibody titers .

The injection schedule of membrane receptor protein, and antibodies as detected by immunodiffusion method is given below. In the immunodiffusion method, using agar plate, the purified receptor preparation was placed in the centre well (.01g of freeze dried power/60 μl of H_2O), and 30 μl used per plate. Serum containing the antibodies to the receptor (30 ul), and also serum after dilution of 1:4, 1:10, 1:40, 1:100 and 1:400 were placed in the periphery wells.

Crude antibodies titre in rabbit number 1 .

```
Date      Injection # and amount  Titre by immunodiffusion
----------------------------------------------------------
22.1.88   1st.        90 µg
21.3.88   2nd.        65 µg
24.4.88   3rd.        50 µg   positive with serum
26.5.88   4th.        50 µg   positive with (1:4) dil serum
21.6.88   5th.        50 µg   positive with (1:10) dil serum
21.7.88   6th.        50 µg   positive with (1:40) dil serum
21.8.88   7th.        50 µg   positive with (1:100) dil serum
21.9.88   Rabbit disposed off by animal care, after
          misplacement by an animal care worker.
```

Crude antibodies titre rabbit number 2.

```
26.5.88   1st.        75 µg
21.6.88   2nd.        50 µg
21.7.88   3rd.        50 µg   positive with serum
21.8.88   4th.        50 µg   positive with (1:4) dil serum
21.9.88   5th.        50 µg   positive with (1:10) dil
serum
21.10.88  6th         50 µg   positive with (1:40) dil
serum
21.12.88  7th         50 µg       not tested
27.02.89  8th         50 µg       not tested
15.05.89  9th         50 µg       not tested
```

Also serum antibodies to the membrane receptor protein at

(1:10) dilution, gave positive precipitation reactions with crude, and Sephacryl S-300 purified cytosolic receptor preparations in the immunodiffusion test, suggesting the structural similarity between the cytosolic and membrane receptor activity. Also Protein A purified antibodies to the membrane-receptor protein, gave a positive test with both purified membrane, and cytosolic preparations. Crude (serum) and Protein A purified antibodies from non injected control rabbit, did not give antigen-antibody precipitation reactions either with purified membrane, or crude and purified cytosolic preparations. Thus, specific antibodies to the receptor protein, were only present in the serum of the membrane-receptor injected rabbits.

Protein A affinity purification of the serum containing the receptor antibodies .

Elution pattern from Protein A column, after application of the serum from receptor protein injected rabbit, is represented in Fig. 5.10. The column was eluted using phosphate buffer pH 7.0, and the protein peak represents proteins other than IgG class of proteins present in the serum. Elution pattern after application of glycine HCl buffer pH 3.0, is shown in Fig. 5.11. IgG class of protein fractions containing the antibodies to the receptor protein are eluted at about pH 3 from the Protein A column, as indicated in the figure. Similar IgG protein profiles were

Fig. 5.10. Purification of antibodies to the receptor protein. Elution profile from protein A affinity column, of serum (7 ml) from rabbit injected with receptor protein. The serum was applied on to the column, and the column then washed with phosphate buffer as described in the text. The protein elution peak represents the serum proteins not bound to the protein A.

Fig. 5.10.
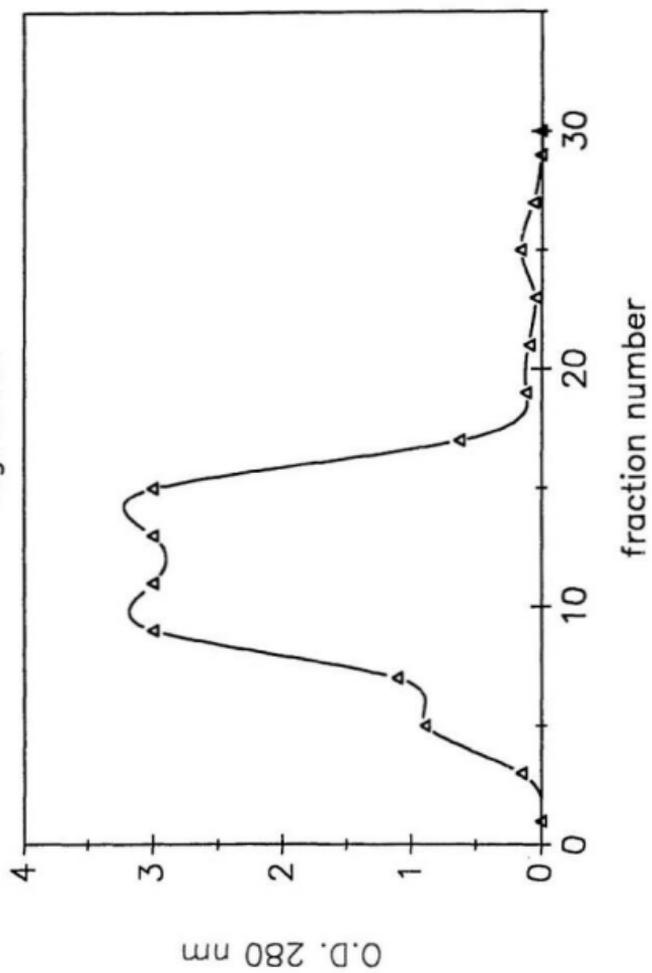
also obtained from the serum of a control injected rabbit
(data not given).

Inhibition of receptor binding to MIS [^{3}H] 17α,20β-DHP. by antibodies .

Crude antiserum at 1:8 dilution from receptor-injected
rabbit, inhibited the cytosolic receptors binding to
[^{3}H]17α,20β-DHP (4.3 nM) by 72%. Crude control
antiserum from non injected rabbit under similar
experimental conditions did not inhibit cytosolic receptors
binding to [^{3}H]17α,20β-DHP. On the other hand, Protein
A purified serum from receptor injected rabbit did not
inhibit cytosolic receptors binding to [^{3}H]17α,20β-DHP
(under similar experimental conditions used with the crude
antiserum). Further binding studies were then carried out
with the cytosolic receptors using the Protein A purified
receptor antiserum from injected rabbit (at various
dilutions), where the antiserum was preincubated with
cytosolic preparation, for 90 min, prior to the binding
equilibration step with [^{3}H]17α,20β-DHP. But no
inhibition of binding of receptors to [^{3}H]17α,20β-DHP
was observed. It is interesting to note that, the purified
antiserum from the receptor injected rabbit showed specific
binding with [^{3}H]17α,20β-DHP under the experimental
conditions used for binding studies.

Fig. 5.11. Purification of antibodies to the receptor protein. Elution profile from protein A affinity column after washing with phosphate buffer. The protein A column was further eluted with glycine/ HCl buffer at pH 3.0. The protein O.D. profile at 280 nm (▲▲) represents, total gamma globulins fraction of serum proteins containing antibodies to the membrane receptor protein for MIS. The changes in pH (●●) with fraction number is also indicated.

Fig. 5.11.

Maturation studies in vitro, attempted inhibition by
antibodies .

1). Using stage 4 whole ovarian follicles: the results
obtained from brook trouts # 45 and # 48 are given in Table
5.1. Some of the ovarian follicles also gave ovulation
response after GVBD.

Table 5.1.

sample description	% GVBD after 70.5 h	
	fish #45	fish # 48
O.F. + TBSS	15.4%	20%
O.F. + 17α,20β-DHP	100%	100%
O.F. + (AB)*	15%	---
O.F. + (CAB)**	36.4%	---
O.F. pre-incubated (AB) + 17α,20β-DHP	100%	100%
O.F. pre-incubated (CAB) + 17α,20β-DHP	93.5%	100%

* purified antibodies to the receptor protein.

** purified antibodies from control rabbit.

Ovarian follicles (O.F.)

2). Using stage 4 denuded oocytes, and also stage 4 oocytes
containing granulosa cell layers.

The defolliculated stage 4 oocytes, obtained by either method 2 or 3 as described in the method section, all manifested spontaneous maturation and ovulation (100%) within 1 - 2.5 h, after removal of the follicular layers. The presence of the granulosa cell layer did not prevent spontaneous maturation from these late stage oocytes. Thus a definite conclusion can not be reached from these inhibition experiments using antibodies. The stage 1 oocytes from Atlantic salmon did not lose the granulosa cell layer completely during gyro-rotatory treatment, under the experimental conditions described. Also these early stage oocytes, did not exhibit spontaneous maturation and ovulation on removal of the outer follicular cells, as did the late stage 4 oocytes. Experiments using earlier stage oocytes from brook trout were not carried out, due to the unavailability of the ovarian follicles from these stages (see discussion).

Discussion

MIS receptor subunits

The purified MIS $17\alpha,20\beta$-DHP cytosolic receptors obtained after gel chromatography, chromatofocusig, DEAE cellulose, and hydroxy-apatite ion exchange column chromatography, all demonstrated four sub-units of receptor activity on SDS gel electrophoresis, under reducing and non-reducing conditions. The MW's of the subunits were 84,000, 65,000, 24,000 and 23,000 (total monomeric MW 196,000, if each of the subunits present per molecule). Although the actual receptor species present in solution was oligomeric, and slightly variable in MW's from preparation to preparation (see chapter 1 and 3.). Sadler, et al . 1985, reported that the MIS progesterone receptor from plasma membrane of Xenopus oocytes, had a MW of 102,000, while Blondeau and Baulieu,(1984), indicated MW value of 30,000. These authors, homogenized defolliculated whole oocytes and demonstrated that the 10,000 x g pellet contained the plasma membrane fraction. They also found [^{3}H]R5020 photoaffinity labelled protein in various other fractions. Vitelline envelope fraction, and vitellin membrane platelets fractions had binding activity corresponding to MW of 98,000. In chapter 1, the data presented on cytosolic receptor MW's from Sephacryl S-300 column chromatography indicated the presence of a low MW

specific binding activity protein to be between 29,000 to
44,000 MW. It is possible that this protein is either a
precursor or breakdown product of the receptor protein (mero
receptor), present in the cytosolic preparation. This low MW
protein was not observed from the solubilized membrane
extracts (chapter 3). It is interesting to note that when
fresh ovarian follicles were used to prepare the cytosolic
receptor preparation, the MW's obtained from the Sephacryl
S-300 column chromatography were ca. 110,000. Although
when frozen tissue was used the MW's were ca. 210,000
(chapter 1). Thus the receptor preparation from frozen
tissue leads to the oligomeric form of receptor due to
protein-protein interactions between the four tightly bound
subunits, leading to total MW of ca. 210,000. The subunits
which make up the MW of ca. 110,000, are possibly the
active components of the receptor (84,000 + 24,000 =
108,000), and the other two subunits are possibly copurified
with the actual receptor. Chick oviduct progesterone
receptor complex is reported to be composed of 2-hormone
binding subunits with MW's 79,000 and 105,000 (O'Malley,
B., et al . 1988), and it is possible that the MIS 17α,20β-
DHP receptor complex also contains these two subunits of
MW's 84,000 + 24,000 =108,000, and 65,000 + 23,000 = 88,000.
It is also interesting to note that on sucrose gradient
ultracentrifugation of the cytosolic preparation containing
0.15M NaCl in absence of molybdate and DTT, the MW obtained

for the receptor complex was 71,000, in the presence of
complete TETS bufffer the MW obtained was 92,000, and in
TETS buffer containing 0.15M NaCl, the MW value was 116,000
(see discussion chapter 1).

The nuclear steroid hormone receptors for cortisol,
estradiol, progesterone, vitamin D3, aldosterone, ouabain,
thyroid hormones and various oncogene products have been
cloned and purified by affinity chromatography from various
species. All of these proteins show similar structural/
functional relationship. Thus they are grouped into a family
of proteins, known as transcriptional response elements
(Shepel and Gorski, 1988; Evans, 1988; Green, and Chambon,
1988; O'Malley, et al . 1988). This family of
transcriptional response elements have three funtional
sites, 1) hormone binding domain, 2) DNA binding domain and
3) transactivating domain. The comparision of these domains
for various receptors has been indicated in Fig. 5.12.

Ouabain binding receptors

Ouabain is a member of a class of compounds called
cardiotonic steroids , the structure of which is depicted
in Fig. 5.13. Ouabain receptors can also be classified
within the family of transcriptional response elements. From
its structure, the ouabain binding site on the receptor in
terms of size and sequences of amino acids is very similar

Fig. 5.12.(A). Amino acid comparisons of steroid and thyroid hormone receptor sequences. Shown at the bottom is the division of receptors into six regions (A-F). Region 'C' corresponds to the putative DNA binding region, and is rich in cystein, and is highly conserved between receptors. Region 'E' correspond to hormone binding domain, this region also shows major similarities. Other regions of the receptors contain little or no significant similarities. (B). Percent sequence similarity between various receptors. Each row contains average values as described below the table. Numbers in the right half of the table (bold print), represents similarity in region 'C' of the receptor, while those below the diagonal (plain print) represent similarity in region 'E'. NA indicates a value which is not available. (Diagram from Shepel, L.A., and Gorski, J. 1988. BioFactors 1, 71-83).

Fig. 5.12.

A

B	ER[a]	PR[b]	GR[c]	MR[d]	VDR[e]	erbA[f]
ER	-	58	61	56	46	53
PR	29	-	86	90	46	41
GR	30	54	-	94	40	46
MR	21	56	57	-	43	48
VDR	NA	NA	NA	NA	-	46
erbA	17	18	18	17	NA	-

[a] Represents average of values for human and chicken ER.

[b] Represents average of values for human, rabbit, and chicken progesterone receptors.

[c] Represents average of values for human and rat glucocorticoid receptors.

[d] Represents the human mineralocorticoid receptor.

[e] Represents average of values for chicken and rat vitamin D receptor.

[f] Represents average of values for v-erbA, human c-erbA (two forms), chicken c-erbA, and rat c-erbA.

Fig. 5.13. The structure of ouabain, a cardiotonic steroid which inhibits the Na^+-K^+ ATPase indirectly, and causes an increase in intracellular Ca^{++}, which stimulates contraction of the cardiac muscle cells. This explains the clinical use of digitalis in treating such conditions as congestive heart failure. The receptor protein for ouabain, has been characterized in the cytoplasm and plasma membrane of the cardiac muscle cells, similar to the receptors for MIS 17α,20β-DHP.

Fig. 5.13.
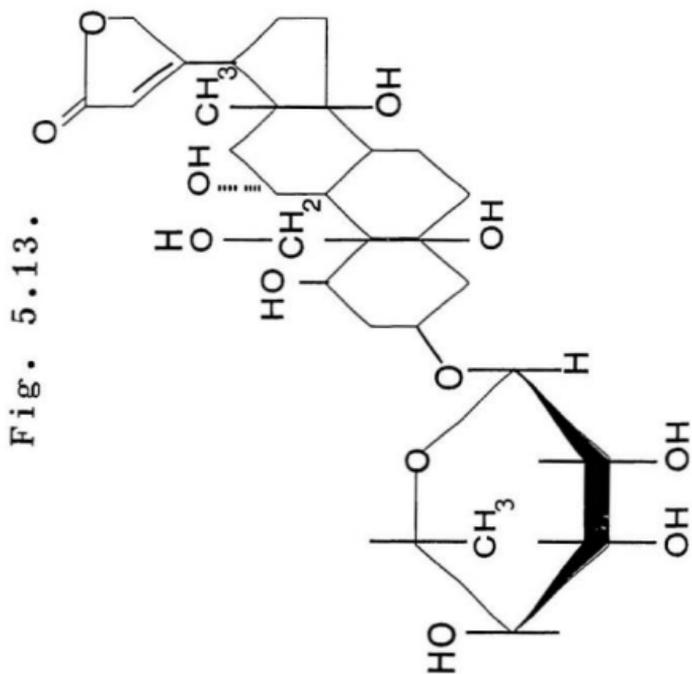
to the corresponding glucocorticoid or estrogen receptor
binding sites. The ouabain receptor is a plasma membrane
bound protein, and couples ATP hydrolysis with Na+ and
K+ transport across the plasma membrane. Ouabain
receptor has been termed the alpha subunit of the Na+/
K+ ATPase (Kawakami, et al . 1985; Shull, et al .
1985; Shepel and Gorski, 1988). Although the ouabain
receptor does not completely fit into the category of
transcriptional response elements since it is a
membrane/cytoplasmic protein, and does not have nuclear
function, the sequence similarities in the steroid binding
region supports the contention that the protein could have
evolved from the same gene as the other transcription
response elements. Thus it is possible that MIS 17α,20β-DHP
receptors could have also evolved in a similar way. The
amino acid sequence analysis of the 4 subunits will help to
resolve this question.

Antibodies inhibition.

Theoretically it seems simple to inhibit the maturation
response (GVBD) by incubating the denuded oocytes (oocytes
free from outer cell layers) with the antibodies to the
plasma membrane receptors, prior to exposure to MIS 17α,20β
DHP. Experimentally it is very difficult to achieve, due to
following considerations:- 1) After denuding the ovarian
follicles, antibodies access to the receptor sites on the

plasma membrane of the oocyte (which is intercalated from
the inside of the oocyte surface in the zona radiata) must
be achieved.2) Antibodies may also trigger the maturation
response, since they may also interact with the receptor
sites (if the antibodies are specifically directed to
receptor domain which binds $17\alpha, 20\beta$-DHP). 3) Various
researchers have reported ovarian follicles exhibiting
spontaneous maturation and ovulation when denuded by
surgical procedures (Greeley, et al . 1987). Despite all
these anticipated problems, an attempt was made to
physiologically inhibit maturation, using 'Staphylococcal
Protein A' (Golding, 1978) purified antibodies to membrane
receptor protein. The main difficulty encountered during the
oocyte maturation period in 1988, was the unavailability of
brook trout in early stages of maturation (stages 1-3). Due
to fresh water shortages in 1987 (very dry and warm weather
conditions), the brook trout population in Fraser Mills
hatchery (Antigonish, N.S) was transferred to a nearby lake.
Due to a shortage of food in the lake, the oocytes in these
fish did not develop, when the fish were brought back later
in the year to the hatchery. During 1988, the same fish kept
in the hatchery, matured about 6 weeks earlier than the
anticipated period in November (other environmental cues
might have also contributed towards early maturation in
1988). Although many antibody inhibition experiments were
carried out on denuded ovarian follicles of stage 4 fish,

none were successful, due to the spontaneous maturation and
ovulation response occuring within (1-3) h of removal of the
outer follicle layers.

The crude rabbit serum polyclonal antibodies, and the
Protein A purified antibodies at various dilutions, gave a
positive precipitation reaction during immunodiffusion test
with crude and purified cytosolic and membrane extra
receptors. The similarly prepared crude and Protein A
purified control antibodies gave negative precipitation
reaction. The Protein A purified antibodies to the receptor
protein also bound MIS [^{3}H]17α,20β-DHP, the similarly
prepared protein A purified control antibodies did not bind
the labelled steroid. These tests indicate the presence of
specific antibodies to the membrane receptors. The cross
reactivity between the cytoplasmic and membrane associated
receptors, also indicate the presence of similar sequences
of amino acids in the various domains of both forms of
receptors. Polyclonal antibodies to the estrogen receptors
from calf uterus also bind labelled estradiol, and this
binding to the steroid does not prevent the antigen
(receptors) binding to the antibodies (Jensen, et al.
1981).

The antibodies titre obtained for the membrane receptor, was
not as high as observed for various other antibodies

produced by Idler (private communication; e.g. vitellogenin,
and antibodies against various steroids). This might have
been due two reasons, 1) due to the loss of first rabbit the
injection schedule was too short. 2) The amount of antigen
injected into the rabbit might not have been enough. Idler
and his co-workers inject between 0.5-2 mg of antigen in
rabbits initially, 50% of the initial dose at bimonthly
intervals, and within a year's time obtain high titres of
antibodies. Chard, (1987), whose protocol for the
preparation of antibodies was followed, warns against using
more than 100 ug of antigen in rabbits, since the rabbits
become refractory to the production of antibodies at higher
doses, and also the impurities in the purified antigen will
tend to increase with the amount of antigen injected, and
this could also lead to the production of antibodies to the
impurities. Chard's protocol was followed mainly because of
unavailability of high amounts of purified receptor protein.
It is known that antibodies production in rabbits is
variable, depending on the antigenicity of the antigen, the
protocol used for the injection, and the various components
of the injection medium also contribute towards the
antigenicity of the antigen (Rudbach, et al . 1988.). The
latest molecularly engineered microbial immunostimulators
were used in the production of the MIS 17α,20β-DHP receptor
antibodies. The purified antibodies were not coupled to the

CNBR activated Sepharose, for the use in affinity
purification of the receptors, because of low titre present
at the time of writing, but booster injections every two
months are still in progress. Also labelled antibodies
(Iodine) could be used for the detection and quantitative
estimation of receptors, once the titre gets higher.

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Chapter 6.

Recent advances in steroid receptors localization, and mode of action of steroids via cAMP .

The concept of receptors was first postulated by Paul
Ehrlich, in a Croonian lecture at The Royal Society meeting
(U.K.) in 1900. Elrlich's concepts of specific ligands
binding cell surface recognition sites leading to
modification of the cellular properties, may be responsible
for the development of concepts for peptide hormone
receptors action via cell plasma membrane (Sutherland, 1972;
Kahn, 1976; Catt and Dufau, 1977). Nonetheless, quite a
conflicting and contradictory view has prevailed in the
localization of receptor macromolecules of steroid hormones.
It has been widely accepted, despite certain evidence to the
contrary that steroid hormones freely and indiscriminately
enter cells (Peck, et al . 1973; Muller and Wotiz, 1979;
Giorgi, 1980), and affect cytoplasmic receptors. The
cytosolic receptor after binding to the steroid hormone, may
then undergo transformation or activation thereby rendering
it capable of migration into the nuclei, and interaction
with the chromatin (classical steroid receptor model was
discussed in chapter 1, and recent modifications discussed
in chapter 2).

Early experimental evidence (after injection _in_ _vivo_ , and perfusion or uptake _in_ _vitro_) for association of various classes of steroids (estrogens, androgens, mineralocorticoids, glucocorticoids, cardiac glycosides and aglycones), with microsomal and mitochondrial:lysosomal particulate fractions containing cellular membranes has been extensively summarized in the review article by Szego and Pietras, 1980 (31 published citations). Futhermore in the same article, the authors have also cited (33 published citations) from various reseachers regarding direct experimental evidence for specific interactions (binding) of various classes of steroids, with surface plasma membranes of the target cells. Also in chapter 3 of the thesis, work of various researchers in the last decade, related to the presence of plasma membrane receptors for various classes of steroids hormones, and their actions via non-genomic mechanisms was briefly mentioned.

Various classes of steroid hormones, alter the levels of cAMP in the target cells via specific receptors. The cAMP levels are modulated by activation or inhibition of adenylate cyclase and phosphodiesterase. Numerous reports have been published in recent years on cAMP, adenylate cyclase and phosphodiesterase, and their modulation by various classes of steroid hormones. A review article by

Harrelson and McEwen, (1987) quotes 59 published citations on the steroid modulation of cAMP metabolism.

The mechanism by which steroids regulate cAMP metabolism is rather complex. More recent aspects of the adenylate cyclase system is briefly described below (review articles: Smigel, et al . 1984; Birnbaumer, et al . 1985).

Enzyme adenylate cyclase : cAMP is synthesized from adenosine triphosphate (ATP) by adenylate cyclase. The primary component of the adenylate cyclase complex is the so called catalytic subunit, a large protein that resides in the inner side of the plasma membrane and is responsible for the actual hydrolysis of ATP (Fig. 6.1). The catalytic subunit has been partially purified and has approximate molecular weight of 135,000-150,000 (Coussen, et al . 1985; Yeager, et al . 1985). Also the genes coding for catalytic subunit from Escherichia coli and yeast have been cloned and the primary structure of the protein deduced (Aiba, et al . 1984; Casperson, et al . 1985; Katoka, et al . 1985). When purified from brain tissue of rat, the protein has been found to contain a calmodulin-binding site. Adenylate cyclase complex also consists of two regulatory units which are heterotrimeric proteins known as guanyl nucleotide-binding stimulatory coupling protein (Ns) and the guanyl nucleotide-binding inhibitory coupling protein (Ni).

Fig. 6.1. Simplified schematic diagram indicating the
hormone action, leading to regulation of cAMP levels in
eucaryotic cells. The agonist hormones act through N_s
activate adenylate cyclase, and antagonist hormones act
through N_i inhibiting adenylate cyclase. The cAMP
generated is broken down by phosphodiesterase (PDE). Steroid
hormones acting via cytoplasmic receptors that affect
genomic activity, induce or repress gene products. These
products may either be part of the adenylate cyclase system
e.g. receptors, PDE, the regulatory proteins N_s and
N_i. In addition, a few direct effects of steroids have
been identified, via membrane receptors e.g. progesterone
action in _Xenopus_ oocytes (reference Harrelson and McEwen,
1987).

Fig 6.1
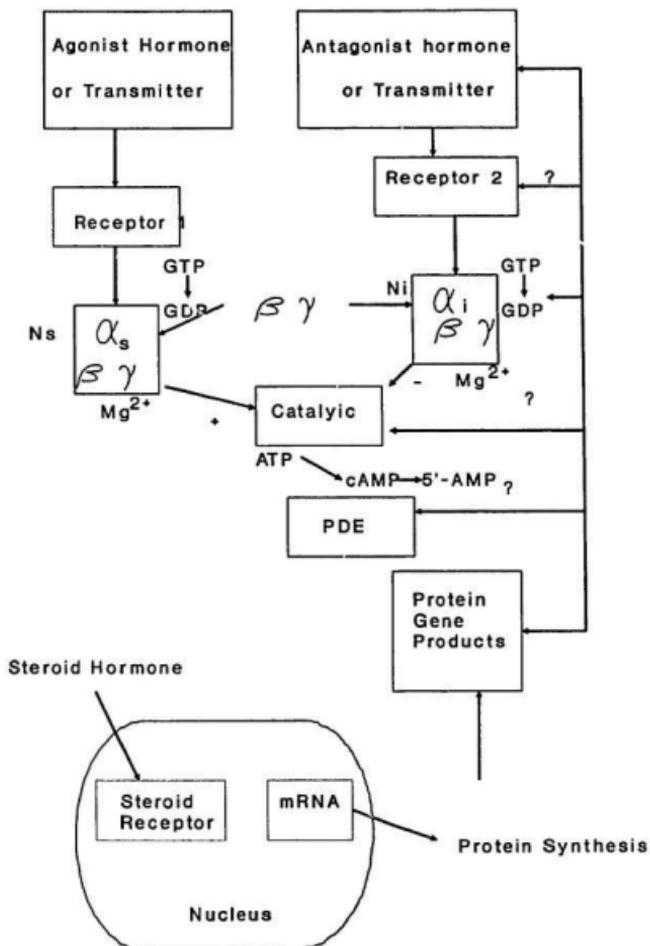
The basal adenylate cyclase activity of the catalytic subunit is fairly low, and it is Ns that is responsible for stimulating that activity to significant levels. The magnitude and efficiency of that stimulation can in turn be inhibited by Ni. Both Ns and Ni consists of α, β, and γ subunits. The β and γ subunits seem to be identical in both Ns and Ni, whereas the α subunits seem to be different, which leads to Ns and Ni having different properties. In order to be functional the guanyl nucleotide-coupling proteins require Mg^{++}, and the binding and subsequent hydrolysis of guanosine triphosphate (GTP). Activation of the catalytic subunit seems to involve binding of GTP and Mg^{++} to N_s, followed by dissociation of the α subunit from the β and γ subunits; and it is the α subunit of Ns which interacts with the catalytic subunit. Hydrolysis of GTP and the release of the α subunit from the catalytic subunit terminate the activation cycle, at which time the α, β and γ subunits reunite. A similar inhibition occurs for Ni. Thus, in the unstimulated state, adenylate cyclase activity reflects the steady state between the activation cycle of Ns and the inhibition cyc e of Ni. Activation or inhibition of adenylate cyclase by hormones acting via cell surface receptors accelerates either the activation cycle or the inhibition cycle, depending upon the particular type of receptor activated (there are exceptions to this general scheme). Thus the hormone binds the receptor, which causes a

conformational change in the receptor that causes it to
interact with the appropriate GTP-binding protein (Fig.
6.1.).

Based on the model discussed above, one can make several
generalizations and speculations (based on further
supporting experimental evidence) about how steroids
modulate cAMP levels. a.) _Steroids affect directly on_
adenylate cyclase, via membrane specific receptors (without
nuclear intervention) : only a few cases of this mode of
action of steroids has been reported, where there is no
apparent mediation or requirement of gene transcription. The
evidence for such a mechanism is most persuasive in the case
of _Xenopus_ oocyte maturation by progesterone. This mode
of action will be discussed later in the chapter in more
detail, since a parallel mechanism could also apply in the
case of oocyte maturation in brook trout via MIS $17\alpha,20\beta-$
DHP. Briefly, progesterone (1 uM) acts via a membrane bound
receptor (Sadler and Maller, 1982 ; Finidori-Lepicard, _et_
_al_ . 1981; Jordana, _et al_ . 1981). The precise mechanism
does not seem to involve alterations in the activities of Ni
(Olate, _et al_ . 1984; Goodhart, _et al_ . 1984; Sadler, _et_
_al_ . 1984), but rather, the response to progesterone
involves N_s. Phosphorylation of the 48,000 MW protein
has been implicated in oocyte maturation, but further work
has not been reported (Blondeau and Baulieu, 1985). The
steroid action may also involve calcium effects on adenylate

cyclase, as progesterone causes a redistribution of membrane Ca^{++} with inhibition of adenylate cyclase. There have been a few other reports of direct steroid membrane-receptor effects on adenyl cyclase (reported in review article, Harrelson and McEwen, 1987). In one of these reports Bergamini, et al. (1985), reported that in vitro addition of estradiol to membranes from human endometrium caused a three fold increase in adenylate cyclase activity. Thus in a limited number of cases steroids alter adenylate cyclase directly at the membrane level, without an intermediate step of steroid induced gene transcription. b.) Gene transcription mechanism : In almost all other cases the initial action of the steroid hormone takes time to develop and may occur at the level of gene transcription. The time lag is usually several hours. The steroids first cause alterations in levels of gene products, which subsequently lead to changes in adenylate cyclase activity. This conclusion is supported by the fact that steroids are inactive on the adenylate cyclase system when they are tested in isolated membrane preparations, and also steroids effects are blocked by inhibitors of protein synthesis (except in the above cases mentioned in a.) and c.) Alterations in adenylate cyclase due to other second messengers and agonist . It is possible that some of the effects of steroids on the adenylate cyclase system may not involve changes in the amounts of the constituent proteins

of the adenylate cyclase system, but instead involve
modification of their activities, for example, by
interactions with other agonist or second messenger systems.
In such instances, the possible involvement of gene
expression and the nature of gene products involved, may not
be of importance. For example, in cultured rat brain cells
(astrocytes) somatostatin inhibits β-adrenergic receptors–Ns
coupling, and in dog and rat myocardium, muscurinic agonists
modulate catacolamine receptor–Ns coupling (Watanabe, et al
. 1978; Yamada, et al . 1980; Rougon, et al . 1983;
Niehoff and Mudge, 1985). A second possibility of steroid
regulation is via other agonists that activate the adenylate
cyclase system. Londos, et al . (1985) have shown that
endogenous adenosine has a powerful influence on basal and
hormone stimulated adenylate cyclase activity, and thus it
may influence the effectiveness of steroid and other
hormones that are adenylate cyclase agonists. A third
possibility is that steroid actions first affect
second-messenger systems other than adenylate cyclase, and
that these messengers subsequently alter the ability to
generate cAMP, e.g., by phosphorylation of receptors,
coupling proteins, or adenylate cyclase itself. Numerous
reports suggest that two second-messenger systems adenylate
cyclase and hormone stimulated phosphotidylinositol (PI)
cycle may interact, even to the point of sharing as a
component the inhibitory guanyl nucleotide-binding protein

Ni (Gilman, 1985). For example, in mast cells, Ca^{++} mediated histamine release and the stimulation of inositol triphosphate breakdown are blocked by islet-activating protein, a specific modifier of Ni (Nakamura and Ui, 1985). Conversely, Bell et al . (1985) reported that in S49 lymphoma cells, phorbol esters, activators of C kinase increase β-adrenergic-stimulated adenylate cyclase by enhancing the interaction between the α-subunits of Ns and the catalytic subunit of adenylate cyclase. This suggests that some of the steroid effects on adenylate cyclase discussed above may actually derive from steroid action on parts of the PI cycle, whose products subsequently exert their effects on adenylate cyclase. d.) <u>Agonist-related desensitization and up-regulation</u> . The steroid effects may be on the production and release of agonists that activate adenylate cyclase. Such action might also involve genomic regulation and take time to appear. Steroids tend to modify the sensitivity of target cell populations to hormones, which are responsible in the production of these steroids. For example, corticotrophic cells in the anterior pituitary, which are responsible for synthesizing adrenocorticotrophic hormone and thus controlling the synthesis of glucocorticoids, are themselves targets for glucocorticoid regulaton of their sensitivity to corticotropin-releasing factor. Glucocorticoids modulate cAMP levels <u>in vivo</u> and <u>in vitro</u> both cortisone and dexamethasone decrease cAMP

accumulation in the anterior pituitory gland elicited by corticotropin-releasing factor (CRF) (Bilezikjan and Vale, 1983; De Souza, et al .1985), while CRF-stimulatd adrenocorticotrophic release is dependent on increased cAMP synthesis. This pattern of steroid feedback to alter their own production can also be seen for PTH and vitamin D in bone forming cells and for the gonadal steroids and pituitary sensitivity to gonadotropins.

Oocyte maturation in amphibians.

The action of MIS progesterone, with the isolated oocytes of Xenopus appears to be sufficient to trigger the normal maturational response of GVBD and other cytological changes (Smith, et al . 1968). To trigger the maturation response the progesterone must act on the external cellular membrane instead of entering the cell to bind a cytosolic receptor, since microinjecction of progesterone into the Xenopus laevis oocytes fails to induce the process (Drury and Schorderet-Slatkine, 1975). Also, since enucleated oocytes respond to progesterone, interaction with the nuclear chromatin is not involved in the process (Masui and Clark, 1979).

Oocyte maturation has been divided into early events, and late events. GVBD is considered as the morphological end point for many studies of oocyte maturation, and $GVBD_{50}$

refers to the time required after progesterone
administration for 50% of the oocytes to manifest GVBD. On
relative time scale if progesterone administration is
considered to be at time 0.0, and $GVBD_{50}$ refers to
relative time period of 1.0 (Wasserman and Masui, 1975),
within relative time scale of 0.1 $GVBD_{50}$, a rapid drop
in the level of cAMP occurs to about 40-60% of the basal
level in the oocyte (Maller et al . 1979; Morril et al
. 1977; Schorderet-Slatkine et al . 1982; Speaker and
Butcher, 1977).

In eukaryotic cells, cAMP is believed to exert its effects
exclusively through the activation of a protein kinase. The
mechanism of activation involves the dissociation of an
inactive complex of regulatory and catalytic subunits, as
indicated:

$$R_2C_2 + 4cAMP \longleftrightarrow \{R(cAMP)_2\}_2 + 2C$$
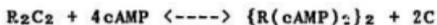
Thus the regulatory subunit (R) is an inhibitor of the
catalytic subunit (C), when bound to it; cAMP relieves the
inhibition by binding to the (R), which causes dissociation
of the R_2C_2 complex giving rise to a catalytic
subunit of the protein kinase. The catalytic and regulatory
subunits from different tissues or different species are
highly conserved and can interact with each other. Thus an
injection of regulatory subunit into the oocyte (which

would bind the endogenous cAMP, lowering its levels) leads
to maturation in the absence of progesterone. On the other
hand injection of catalytic subunit totally blocks
progesterone action (the equilibrium in the above equation
is shifted in favor of release of cAMP). These results
indicate that the decrease in cAMP is necessary for oocyte
maturation (Maller and Krebs, 1977). On injection of an
inhibitor of the catalytic subunit reduced phosphorylation
occurred, which led to maturation (Boyer,1980). Mulner, et
al . (1979) have shown that progesterone treatment of
oocytes reduces in vivo synthesis of cAMP from
microinjected [Y-^{32}P]ATP. Further, Sadler and Maller
(1981) have demonstrated that treatment of the intact
oocytes with progesterone prior to membrane isolation
reduces the adenylate cyclase in these fractions. Also
Finidori-Lepicard et al . (1981) have shown that a
fraction of the oocyte adenylate cyclase can be inhibited by
physiological concentrations of progesterone. These results
were supported by the work of Jordana et al . (1981) who
also demonstrated the requirement for guanine nucleotides
for the hormone inhibition. These findings suggest a model
in which the prophase arrest of the resting oocyte is
maintained by a phosphoprotein (substrate of cAMP-dependent
protein kinase). Dephosphorylation of this protein is
necessary to cause oocyte maturation (Maller and Krebs,
1977). Dephosphorylation of this protein can also be carried

out by a phosphatase protein, whose activity might be under
progesterone control. Foulkes and Maller, (1982) and Huchon,
et al . (1981) demonstrated that the activity of protein
phosphatase-1 can have an effect on oocyte maturation.
Thus a concerted model postulates that a high steady-state
level of a phospho protein is necessary and sufficient to
maintain the prophase block of the oocyte, and that the
level of protein is maintained by the rate of
phosphorylation by cAMP-dependent protein kinase and the
rate of dephosphorylation by protein phosphatase (Maller,
1985).

Molecular mechanisms of cAMP decrease .

Three ways in which cAMP levels could be lowered by the
progesterone in the oocyte are: 1.) by inhibition of
adenylate cyclase, 2.) by activation of phosphodiesterase
(or combination of 1. and 2.), and 3.) by excretion of cAMP
from the cell. Several laboratories has indicated that cAMP
does not appear in the incubation medium after the
progesterone treatment (Maller, 1985). Cholera toxin an
irreversable activator of adenylate cyclase, which blocks
the progesterone mediated oocyte maturation in a dose
dependent manner (Maller, et al . 1979; Sadler and Maller,
1981). These results suggested that the activity of
adenylate cyclase can affect the oocyte maturation process.
The direct evidence of progesterone action on adenylate

cyclase activity was demonstrated after injection and conversion of γ-[^{32}P-ATP] into cAMP by the oocyte in the presence and absence of progesterone. Progesterone decreased the rate of cAMP synthesis (Mulner, et al . 1979). These results were further supported by in vitro experiments using isolated membrane preparations, where progesterone inhibited the adenylate cyclase activity in a dose dependent manner (Sadler and Maller, 1981; Jordana, et al . 1981; Finidori-Lepicard, et al . 1981). Also the half maximal concentration at which the progesterone inhibited the adenylate cyclase, was similar to the concentration of progesterone required for 50% GVBD, a result that supports the physiological analogy. The next step in the resolution of the mechanism is how progesterone inhibits adenylate cyclase. The various components of hormone action leading to the adenylate cyclase activation and/or inhibition in somatic cells was discussed earlier in the chapter and was depicted in Fig. 6.1.

In the adenylate cyclase complex from Xenopus oocyte membranes, the presence and role of catalytic (C) and regulatory N_s subunits were characterized by the experimental procedures involving cholera toxin as described previously. Hormone mediated inhibition of adenylate cyclase almost always involves the activation of regulatory subunit N_i (Londos, et al . 1981; Jakobs, et al . 1981;

Bokoch, et al . 1983; Codina, et al . 1983). Whether, the progesterone acts by similar mechanism (via N_1) in amphibians was investigated using islet-activating protein (IAP) (also known as Bordetella pertussis toxin). This IAP protein ribosilates ADP, in the presence of $[^{32}P]NAD^+$ the α-subunit of N_1 thereby rendering it inactive (Katada, et al . 1984). If the mechanism of inhibition of adenylate cyclase is via N_1, the progesterone action of maturation of the oocyte should be blocked by IAP. On preincubating the oocytes with the IAP and labelled NAD, a labelled NAD ribosilated membrane protein was isolated (MW 41,000, which had the same MW as the α-subunit from N_1 of human erythrocytes), but the IAP protein did not inhibit progesterone mediated oocyte maturation. Thus the progesterone induced inhibition of adenylate cyclase activity was not via N_1 regulatory subunit of adenylate cyclase (Olate, et al . 1984; Sadler, et al . 1984; Goodhardt, et al . 1984).

After demonstrating the presence of N_1, but non-involvement in the mechanism of inhibition of adenylate cyclase via progesterone in the Xenopus oocyte membrane preparation, it was important to show that progesterone might possibly inhibit the N_s regulatory protein which is involved in keeping the basal activity of the adenylate cyclase in the oocyte membrane. Fig. 6.2. indicates schematically the various steps involved in the stimulatory

action of agonist hormones (via membrane bound receptors),
on the activity of adenylate cyclase via N_s regulatory
protein. The hormone receptor is embedded in the lipid
bilayer of the membrane, and the hormone binding site faces
the outside of the cell. When the receptor binds the
agonist, it undergoes a molecular conformational change that
allows it to activate the GTP-binding protein (G) or N_s
to exchange GDP bound to the protein with GTP. Since GTP is
present in the cytoplasm, the GTP exchange site possibly
faces the inside of the cell. GTP binds to the α-subunit of
the N_s, which leads to dissociation of N_s, and the α-
-subunit bound to GTP then interacts with the catalytic
subunit of the adenylate cyclase leading to activation and
synthesis of cAMP (Fig's. 6.2. and 6.3). The α-subunit of
N_s also has GTPase activity that prevents it from being
permanently activated. When the hormone receptor complex
activates N_s to bind GTP, there is a reciprocal
inhibitory effect of N_s on the receptor that lowers the
affinity of the receptor for the agonist hormone. Both the
negative effect of the GTP binding protein N_s (possibly
via $\beta\gamma$ complex ?) on hormone binding and the hydrolysis of
GTP serve to limit the extent of adenylate cyclase
activation, when the hormone is not at saturating
concentrations. There are two ways in which the basal
functional metabolic activity of adenylate cyclase in the
oocyte membranes can be inhibited via N_s regulatory

Fig. 6.2. The agonist hormone-receptor complex stimulates N_s (G protein) to exchange GDP bound to the protein with GTP, which allows it to activate the adenylate cyclase. Binding of GTP may also dissociate the receptor from the G protein. Hydrolysis of GTP inactivates G, and also leads to dissociation of the G protein from the adenylate cyclase (reference Palmiter, 1984).

Fig. 6.2
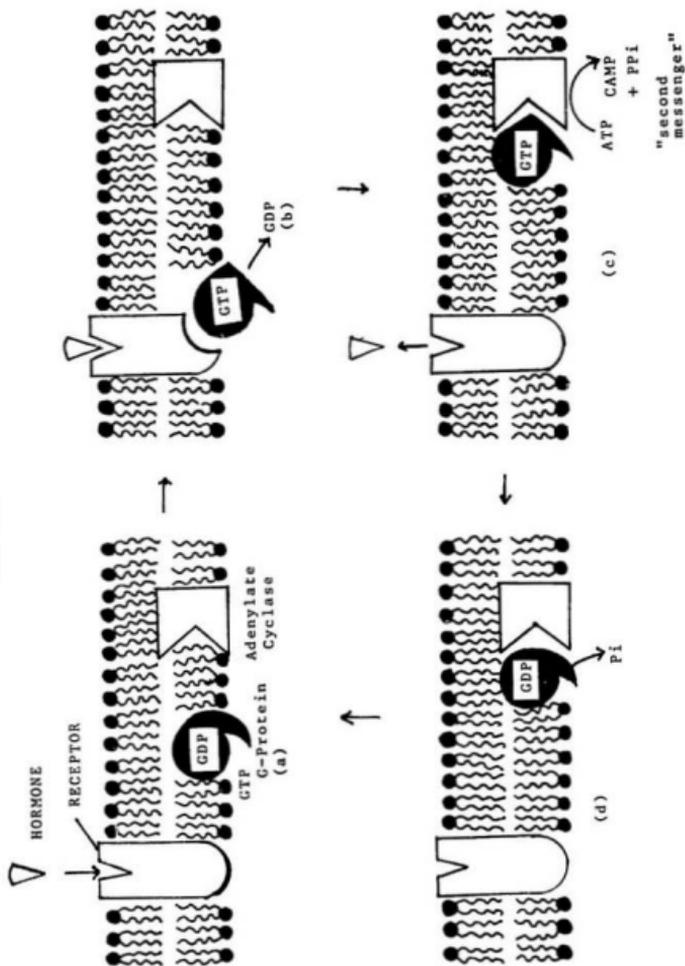
Fig. 6.3. Demonstrates the subunit interactions of regulatory protein N_s in hormone receptor mediated actions of adenylate cyclase (reference Bourne, 1989). (HR) hormone receptor; (α, β, γ) subunits of Ns regulatory protein.

Fig 6.3

Orthodox pathways

→ Adenylyl cyclase (everywhere)

→ cGMP PDE (retina)

→ K$^+$ channel (heart)

Heterodox pathways

→ PLA$_2$ (retina, heart, neuron)

? → ? → Pheromone response (S. cerevisiae)
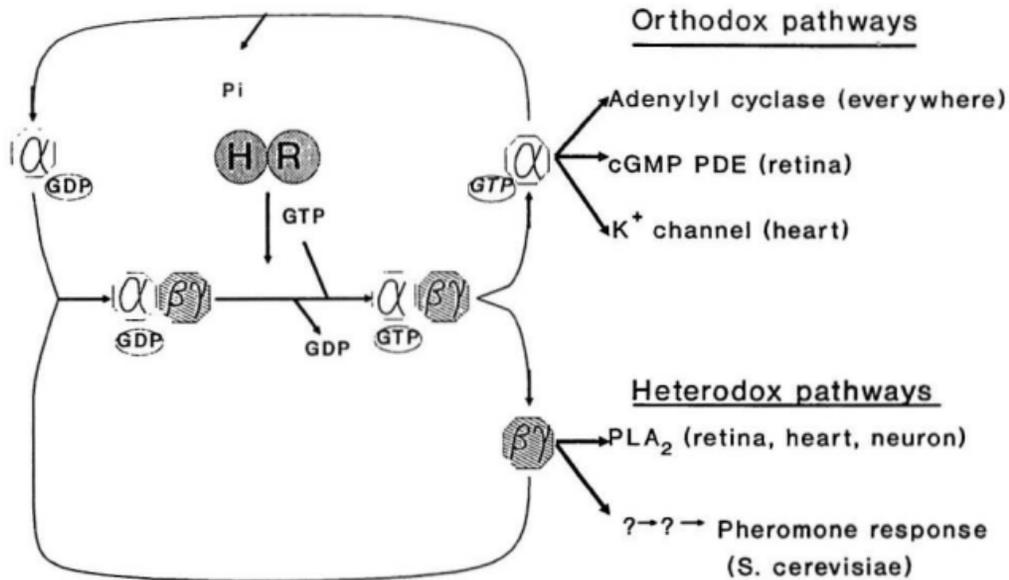
protein: 1.) by decreasing the rate of GDP exchange with GTP, after progesterone activation of the N_s via the receptor action, 2) by decreasing the rate of GTP hydrolysis, which would lead to decrease in rate of cAMP synthesis by the catalytic subunit of the adenylate cyclase. The molecular mechanisms by which progesterone can intervene during these steps are not known.

In Xenopus oocyte, the adenylate cyclase activity after stimulation with Gpp(NH)p a non-hydrolyzable derivative of GTP, can be inhibited by progesterone. In most mammalian systems as yet studied, Gpp(NH)p activated adenylate cyclase activity is not inhibited by the various hormones, and GTP is required for inhibition (Jakobs, et al . 1981; Wolff, et al . 1981). Although a group of adenosine analogs called P-site agonists, has been shown to inhibit Gpp(NH)p-stimulated adenylate cyclase activity (Lodos and Wolff, 1977). Two of the potent P-site agonist are 2',5'-dideoxyadenosine (2',5'-DDA), and 9-β-D-arabinofuranosyladenine. These compounds were shown to inhibit the oocyte adenylate cyclase (Sadler and Maller, 1983), and the P-site action was shown to be similar to other mammalian systems (Lodos and Wolff, 1977). Also it was shown that the P-site agonist and progesterone acted by a similar mechanism (Sadler and Maller, 1983). Although a major difference between the action of progesterone and

2',5'-DDA was encountered in the presence of Mn^{++}.
These Mn^{++} ions potentiated the inhibition by
2',5'-DDA, while it abolished the inhibition caused by
progesterone in the _Xenopus_ oocytes. The effect of
Mn^{++} is similar to that observed in conjunction with
P-site agonist in other mammalian systems (Lodos and Wolff,
1977; Lodos, _et al_ . 1979; Wolff, _et al_ . 1981). The
ability of Mn^{++} to diminish the inhibitory effect of
progesterone suggested that the steroid action is receptor
mediated and involves N$_s$ regulatory protein (Sadler and
Maller, 1985), since P-site agonists do not act via external
receptor mediated mechanism, but are natural constituents of
the cells (Lodos, _et al_ .1979).

GDP exchange by GTP has been indicated as the rate-limiting
step in the activation of adenylate cyclase in avian cell
systems (Selinger and Cassel, 1981). In mammalian cell
systems, the interaction between the hormone and the
receptor causes increased release of isotopically labelled
nucleotide, regardless of whether the hormone induces
activation or inhibition of adenylate cyclase activity. Thus
the nucleotide exchange may not be of importance in
mammalian systems (Michel and Lefkowitz, 1982). When
adenylate cyclase is stimulated by the non-hydrolyzable
guanine nucleotide Gpp(NH)p, there is a characteristic lag
period before the adenylate cyclase can be activated. This

lag period is attributed to the time required for replacement of GDP bound to the α-subunit of N_s regulatory protein with Gpp(NH)p, or to the transition from an inactive to an active adenylate cyclase enzyme conformation (Cassel and Selinger, 1978; Abramowitz, et al . 1980). When the time lag of Gpp(NH)p activation with oocyte adenylate cyclase was measured in the presence and absence of progesterone, an increase in time lag was observed. This indicates that progesterone decreases the rate of Gpp(NH)p exchange, which can be interpreted as slowing of GDP exchange by GTP in the presence of progesterone in the oocyte, resulting in the inhibition or decrease in adenylate cyclase activity. Similar results were observed with P-site agonist 2',5'-DDA (Sadler and Maller, 1983). These results, and the direct demonstration of decrease in exchange of GDP with GTP in the presence of progesterone and 2',5'-DDA (Sadler and Maller, 1983; 1985) indicate that the mechanism of adenylate cyclase inhibition involves a decrease in rate of exchange between the two forms of the guanine nucleotides at the first step of the N_s regulatory protein cycle (Fig's. 6.2. and 6.3.).

Role of Ca++ in oocyte maturation.

Ca++ is released intracellularly after the treatment of oocytes with progesterone. The increase in free

intracellular Ca++ was demonstrated by increased in
aequorin luminescence after injection of the dye into the
albino oocytes, and the increase in free Ca++
paralleled in time the decrease in the cAMP levels
(Wasserman, et al . 1980). Also an increased efflux of
45Ca ion was observed after progesterone incubation of
oocytes preloaded with labelled calcium (O'Connor, et al .
1977). Furthermore microinjection of Ca++, or the
ability of divalent cation ionophore A2387 to cause
maturation after Ca++ addition to the incubation
medium, indicated a role of Ca++ in maturation (Maller
and Krebs, 1980, for review).

The decrease in cAMP that followed in parallel with
increased Ca++ levels intracellularly suggest that
phosphodiesterase (PDE) might also be activated by
progesterone. Calmodulin is an acidic low-molecular weight
heat stable protein that activates many enzymes in target
cells, and its action follows increase in free Ca++ ion
intracellularly after hormone action. Phosphodiesterase is
one of the main enzyme activated by calmodulin (Lin and
Chung, 1980). The activation of PDE by calmodulin occurs as
follows:-

Ca++ + calmodulin <-----> Ca-calmodulin

Ca-calmodulin + PDE <-----> Ca-calmodulin-PDE

(activated).

Injection of phosphodiesterase inhibitors into the oocytes

leads to inhibition of maturation, and it also prevents degradation of injected cAMP (Allende, et al. 1977; O'Conner and Smith, 1976). Also injection of calmodulin into the oocyte leads to maturation, and this effect is blocked in oocytes preinjected with catalytic unit of cAMP-dependent protein kinase (Maller and Krebs, 1980; Wasserman and Smith, 1981). The presence of several calmodulin target enzyme systems has been demonstrated in the oocytes (Wasserman and Smith, 1980; Echeverria, et al. 1981; Foulkes and Maller, 1982; Maller, 1983) including phosphodiesterase, which might be potentially important in decreasing the cAMP levels leading to maturation. Recent work indicates that a calmodulin activated phosphodiesterase mechanism plays a major role in oocyte maturation brought about by insulin, insulin like growth factor 1 and [Val^{12}, Thr^{59}] Ha-ras Protein (Sadler and Maller, 1987, 1989). These compounds may have a very minor role in progesterone mediated oocyte maturation. Furthermore, microinjection of inositol-1,4,5-trisphosphate (IP$_3$) into the oocyte increased the rate of progesterone or insulin mediated maturation (Stith and Maller, 1987). IP$_3$ is produced in cells following polyphosphatidylinositol breakdown, which occurs in many systems where receptors are linked to the release of Ca^{++} ions. The IP$_3$ functions as an intracellular calcium ionophore (Berridge and Irvin, 1984). The Ca^{++} ions released in response to IP$_3$ could

activate calmodulin leading to activation of phosphodiesterase, thereby decreasing the cAMP levels (Fig. 6.4.).

The effect of Ca^{++} in the external incubation medium during maturation has been studied by various researchers (Ecker and Smith, 1970; Merriam, 1971; Baltus, et al. 1977; Masui, et al. 1977). Maturation using progesterone was inhibited when Mg^{++} and Ca^{++} ions were not present in the incubation medium, and maturation response reoccured in the presence of 2mM Mg^{++} and was partially inhibited at 2mM Ca^{++} (Merriam, 1971a; Masui, et al. 1977). The presence of EDTA (10 mM) in the incubation medium also prevented progesterone mediated maturation (Marot, et al. 1976), or delayed maturation (Moreau, et al. 1980). While the presence of EGTA (10mM) did not have any effect (Marot, et al. 1976; Moreau, et al. 1980). Furthermore maturation could be obtained by incubating the oocytes in oil solution (Merriam, 1971b). These studies indicate the necessity for Ca^{++} in the external medium during progesterone induced in vitro oocyte maturation. The maturation induced by ionophore A23187 in the presence of Ca^{++} and Mg^{++} above 5mM (Wasserman and Masui, 1975) indicates that calcium uptake into the oocyte can lead to maturation under non-physiological conditions. It is more than likely that the increased Ca^{++} ion levels observed in the oocyte after progesterone action is due to uptake

Fig. 6.4. The PI cycle or response. Hormone receptor
interaction leads to activation of a G protein, which in
turn activates phospholipase C and leads to degradation of
phosphatidylinositol. The degradation product inositol
triphosphate binds to a specific receptor on the endoplasmic
reticulum to stimulate calcium mobilization in the cytoplasm
by mechanism not yet known. The increase in Ca^{++} ion
stimulates various proteins and enzymes and may stimulate
calmodulin, which stimulates phosphodiesterase and leads to
decrease in cAMP levels (see text). The phosphatidylinositol
is resynthesized as shown in the diagram.

Fig. 6.4

Hormone
+
Receptor

Phosphatidylinositol

The PI response

CMP

Inositol

PP$_i$ CTP

Phosphatidyl-CDP ←——— Phosphatidic acid

G GTP
   GDP

PDE
Phospholipase C

P~inositol
+
1,2-Diacylglycerol

ATP

ADP

Mobilization of calcium activates calmodulin which in turn activates phosphodiesterase and leads to ↓cAMP in the oocyte
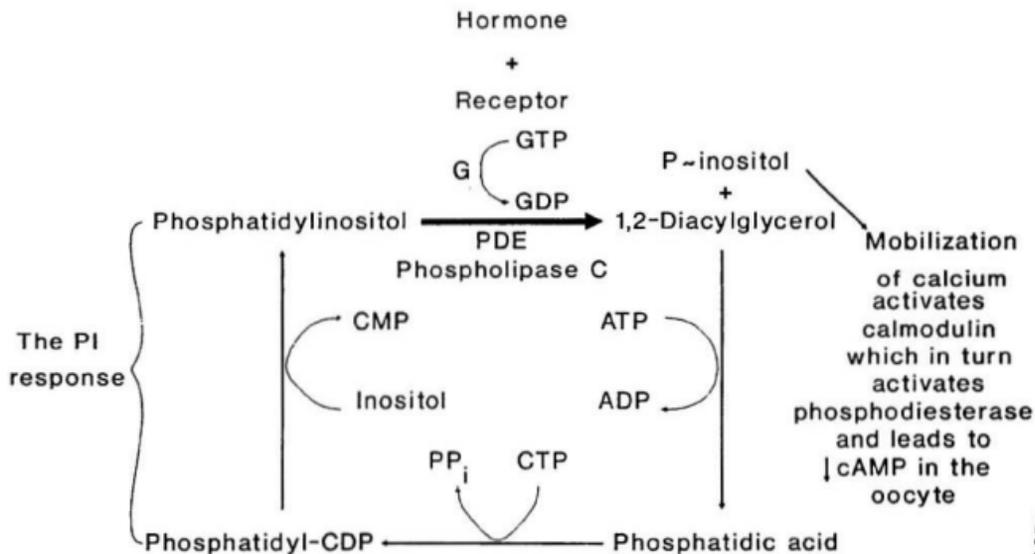
from the medium, than endogenously released from bound
protein form. Also it has been reported that endogenous
increase or surge of Ca^{++} did not occur after
progesterone action during maturation (Bell'e, _et al_.
1977; Robinson, 1985). Robinson used microelectrode
technique to measure internal Ca^{++} concentration. Thus
it is possible that Ca^{++} ions may not be involved in
the physiological mechanism _in vivo_ leading to oocyte
maturation, since it is unlikely that the same progesterone
receptor can activate both the adenylate cyclase and also
the PI cycle leading to calcium release (both via same, or
different G proteins). The insulin maturation action under
non-physiological conditions (via insulin receptors as yet
not isolated in oocytes) is mainly involved in calcium
mediated PI mechanism leading to activation of calmodulin
and phosphodiesterase, thereby leading to decreased levels
of cAMP and maturation (Fig. 6.4.).

17α,20β-DHP mediated mechanism of maturation in fish oocytes.

Current status of research .

Goetz (1983), in a review suggested that the action of 17α-
,20β-DHP in fish oocyte maturation might be similar to
progesterone action in _Xenopus_ oocyte maturation. The
basis on which this conclusion was drawn, was the
similarities observed during the steroid induced maturation,

and the effect of protein transcription and translation
inhibitors during oocyte maturation in both the species of
vertebrates. Furthermore Goetz and Hennessy (1984)
demonstrated that MIS 17α,20β-DHP-induced ovarian follicle
maturation can be blocked by inhibitors of phosphodiesterase
(which leads to increased levels of cAMP in the oocyte).
These results supported the analogous results obtained in
amphibian _Xenopus_ oocytes, where the progesterone-induced
meiotic maturation is mediated by lowering of the
intra-oocyte level of cAMP. Further work by French
researchers Jalabert and Finet (1986), in rainbow trout
Oncorhynchus mykiss ovarian follicles also supported the
amphibian model. The 17α,20β-DHP-induced meiotic maturation
was blocked by cAMP (> 1mM) or dibutyl cAMP (>2mM),
phosphodiesterase inhibitors theophylline (>.2mM) and
3-isobutyl-1- methylxanthine (IBMX > .1 mM), and adenylate
cyclase activators cholera toxin (> 100nM) and forskolin (>
.03mM). Also combined effect of IBMX (1mM) and forskolin
(.01 and.05 mM) on the intra ovarian levels of cAMP was
investigated. These researchers also measured the cAMP
levels in the ovarian follicles during various stages of
final maturation. Significant positive correlation between
oocyte cAMP concentration and the follicular weight was
observed, also a significant negative correlation between
cAMP and the median dose of 17α,20β-DHP required to elicit
maturation was observed. Finally, the authors demonstrated

directly, that the levels of cAMP significantly decreased during the first 10 h of incubation with maturation-inducing concentration of MIS 17α,20β-DHP. Iwamatsu, et al . (1987) further demonstrated that forskolin increased the *in vitro* synthesis of MIS 17α,20β-DHP in the outer follicular granulosa cells of the ovarian follicles via cAMP mediated mechanism in Oryzias latipes , and forskolin also inhibited the *in vitro* maturation in naked oocytes induced by MIS 17α,20β-DHP, indicating the presence of adenylate cyclase in the fish oocytes. In 1987, the evidence for the MIS 17α,20β-DHP receptors in the brook trout ovarian follicles cytosolic preparation was presented, the levels of cytosolic receptors during the final stages of maturation reported and no receptor activity was observed in the cytosolic preparation during the final stages (6-7) of maturation (GVBD) (Maneckjee, et al . 1987), when the MIS 17α,20β-DHP levels are the highest in blood plasma (Goetz, et al . 1987). No specific binding to nuclear pellet extracts utilizing either labelled MIS 17α,20β-DHP or progesterone was obtained. Also photoaffinity labelling (using labelled R5020) of the isolated nuclei preparations from brook trout ovarian follicles did not demonstrate specific binding, although non-specific binding was present in both the above cases. The presence of receptors in the isolated zona radiata membrane preparation containing the plasma membrane of the oocyte (from the late stage oocytes)

was also demonstrated (Maneckjee, et al. 1987; 1989a.;
1989b.).

Finet, et al. (1988), have proposed a slightly different
mechanism of MIS 17α,20β-DHP action in rainbow trout
oocytes. The authors suggest that MIS action leads to
activation of a phosphatase (by an as yet unknown mechanism
involving Mg^{++}), as the major enzyme involved in the
breakdown of endogenous cAMP levels, and not due to the
inhibition of adenylate cyclase. However the authors do not
provide any direct evidence for the phosphatase activation,
nor have they presented any evidence on adenylate cyclase
inhibition. Their speculation regarding the mechanism is
based on the measurement of endogenous cAMP levels in
rainbow trout from defolliculated oocytes during incubation
with time. They observed increased levels of cAMP
synthesized during incubation (4.5 fold in 6 h) in the
oocyte. The defolliculation was carried out enzymatically
using collagenase (.32 mg/g of ovarian follicles), but the
defolliculation medium also contained epinephrine-bitartrate
(10 ug/ml); polyvinylpyrolidone (2 mg/ml); hyaluronic acid
(.05 mg/ml); dextran sulfate (1 mg/ml) in the IM/280 medium.
No control experiments were carried out where the ovarian
follicles were incubated with the above medium without the
collagenase. A control was used in which the ovarian
follicles were incubated in the medium IM/280 during the

period of cAMP measurement. It is possible that epinephrine
could have been taken up by the oocyte during the
defolliculation process, and then released into the medium
during the incubation. Epinephrine could then activate
adenylate cyclase (via epinephrine membrane α or β -
receptors), leading to the synthesis of cAMP. Epinephrine
is known to stimulate the _in_ _vitro_ ovulation in rainbow
trout (Jalabert, 1976), and also in yellow perch, brook
trout and carp ovarian follicles (Stacy and Goetz, (1982);
Goetz, unpublished results), suggesting the presence of
epinephrine receptors on the oocyte membrane. Thus it is
possible that the reported increased levels of cAMP, could
be due to epinephrine action leading to increased cAMP
levels via G protein stimulation of adenylate cyclase. The
authors also report similar increase in cAMP levels in
oocytes defolliculated by mechanical means (pressure). These
mechanically denuded oocytes also contained certain numbers
of granulosa cells (demonstrated by electron microscopy). It
is extremely difficult to speculate on the mechanism's
leading to increased cAMP under such non-physiological
conditions. The above results are at variance with other
published data. In amphibians the cAMP levels remained
unchanged after defolliculation (Maller and Krebs, 1980).
Spontaneous maturation occurs after defolliculation with
fall in intraoocyte cAMP levels (Schultz, _et_ _al_ . 1983;
Aberdam, _et_ _al_ . 1987). Also the results of Greeley, _et_ _al_

. (1987), on <u>Fundulus</u> <u>heteroclitus</u> oocytes, and results
reported in chapter 5 of the thesis, demonstrate that
spontaneous maturation occurs after defolliculating the
oocytes. The above authors did not report spontaneous
maturation occuring, which would suggest that ovarian
follicles used for the study must have been from early
stages of maturation, at least prior to its <u>in</u> <u>vivo</u>
exposure to gonadotrophin. Normally these early stage
ovarian follicles are not used for <u>in</u> <u>vitro</u> MIS mediated
maturation studies, but rather stage (3-4) follicles are
used (Goetz, 1983). Thus the intraoocyte cAMP synthesized in
these denuded oocytes may not play a role in final
maturation. Also because of the high levels of cAMP observed
due to its synthesis in these denuded oocytes, the authors
claim that in the intact ovarian follicles either
testosterone or low levels of MIS 17α,20β-DHP produced by
the granulosa cells (prior to the main surge of MIS),
inhibit the intraoocyte cAMP synthesis (most likely by
inhibition of phosphodiesterase ?). This explanation was
supported by experimental evidence, but its physiological
role <u>in</u> <u>vivo</u> does not seem to be reasonable, since it is
the lowering of intraoocyte cAMP which leads to maturation.
And because of this there is no need for a maturation
inhibitory factor or a signal from follicular cells to keep
the intraoocyte cAMP levels low.

Recently membrane associated receptor for MIS 17α,20β-DHP

has been demonstrated in the membrane of the nasal passage
in gold fish (Rosenblum, et al . 1988). Also a receptor for
MIS 17α,20β, 21-trihydroxy-4-pregnen-3-one in spotted
seatrout has been characterized from plasma membrane
preparations of the ovarian follicles (Patino and Thomas,
1988).

Future perspectives .

a.) Inhibition of oocyte maturation by antibodies to the
receptor protein . As already mentioned in chapter 5, the
antibodies inhibition of maturation in vitro should be
attempted using denuded follicles from stage (2-3) oocytes.

b.) Demonstration of MIS receptors on the zona radiata
membranes . After in vitro incubation of the zona
radiata membranes with the MIS receptor antibodies, the
presence of MIS receptor-antibody complexes should be
detected using anti rabbit gamma globulin goat fluorescent
antibodies.

c.) Direct measurement of adenylate cyclase activity on the
isolated zona radiata membrane preparation of the stage
(3-4) oocytes in the presence and absence of MIS 17α,20β-DHP
against time of incubation . This experiment will
demonstrate if adenylate cyclase activity is inhibited by

MIS. Also since this type of inhibition does not invoke nuclear protein synthesis, the direct action of steroid on the adenylate cyclase would be indicated.

d.) **Mechanism of forskolin inhibition of maturation** . Although it has been demonstrated that forskolin inhibits oocyte maturation induced by MIS $17\alpha,20\beta$-DHP (Jalabert and Finet, 1986), it is also important to demonstrate that the activity of the adenylate cyclase is responsible for this inhibition of maturation. Thus, in the presence of forskolin and the MIS $17\alpha,20\beta$-DHP during incubation, the zona radiata associated adenylate cyclase activity should not be decreased or inhibited so that it becomes lower than the intra-oocyte basal levels leading to maturation.

e.) **Does the N_i regulatory subunit inhibit adenylate cyclase after MIS action** . The next step in the MIS induced mechanism of maturation would be to demonstrate if the MIS induced inhibition of adenylate cyclase is via N_s subunit of the adenylate cyclase (in anology with the findings in amphibians). This should readily be demonstrated by attempting the maturation studies in the presence and absence of islet-activating protein (IAP), also known as **Bordetella pertussis** toxin, the N_i subunit of the adenylate cyclase is inhibited by the toxin. Thus if the toxin does not inhibit maturation induced by the MIS (as is

the case with amphibians), the action of MIS is via the
N_s subunit of adenylate cyclase.

f.) **Does the N_s regulatory subunit inhibit adenylate**
cyclase after MIS action . If the MIS action is not via
N_i, it would be of interest to investigate the action
of MIS on the N_s. This can be acheived by measuring the
MIS inhibition of maturation (and also adenylate cyclase
levels), in the presence of Gpp(NH)p. If it is not inhibited
(as in the case of amphibians), the mechanism of inhibition
is not via GTP hydrolysis or GTPase activity of the N_s,
but possibly via rate of exchange of GDP with GTP step of
the mechanism. This could be directly or indirectly tested
as discussed in the earlier part of the chapter.

g.) **Effect of microinjected monoclonal antibodies to** ras
gene product (adenylate cyclase) on MIS 17α,20β -DHP induced
maturation . The rate of MIS induced maturation should be
increased by the antibodies to the ras gene product
(Sadler, et al . 1986).

h.) **Inducement of maturation in stage (3-4) denuded oocytes**
of brook or rainbow trout using MIS 17α,20β-DHP covalently
bound to BSA, and thiopropyl Sepharose-6B derivative of MIS
17α,20β-DHP . This experimental procedure would test whether
the action of MIS is via outer plasma membrane receptors of

the oocyte.

The experimental procedure developed by the author for the
isolation of intact zona radiata membrane preparation
containing the plasma membrane of the oocyte will facilitate
future research in fish and amphibians. The experimental
procedures (as described above in (a-g) future perspectives
on research), should be easier to carry out than in the
past. In amphibians, the adenylate cyclase measurements were
carried out on a membrane fraction after homogenization of
the oocyte (10,000 x g pellet obtained after
homogenization). The results obtained were variable,
depending on the conditions of homogenization. The research
described in this thesis has made a small contribution
towards establishing membrane receptors for $17\alpha,20\beta$-DHP,
although much more work has to be done in order to establish
the final mechanism of MIS $17\alpha,20\beta$-DHP action leading to
maturation via cAMP.

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Xenopus oocytes. Proc. Natl. Acad. Sci. U.S.A. 79, 850-854.

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activity. Adv. Cyclic Nucleotide Protein Phosphorylation
Res. 17, 1-18.

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Appendix

Reverse phase (HPLC) separation of the subunits of receptor protein .

a) Using a column containing 4-carbon (C4) hydrophobic group separating medium (Bio-Rad RP 304 column 25 x 4.6 mm). Trace 1, blank run. Trace 2, standard proteins. Traces 3 and 4, receptor samples (see text for complete details).

b) Using a column containing 8-carbon (C8) hydrophobic group separating medium (Beckman RPMC Ultra pore column 75 x 4.6 mm). Trace 5, blank run. Trace 6, standard proteins. Traces 7 and 8, receptor samples (see text for complete details).

c) Using a column containing 18-carbon (C18) hydrophobic group separating medium (Beckman Ultra pore C-18 150 x 4.6 mm). Trace 9, blank run. Trace 10, standard proteins. Traces 11 and 12, receptor samples (see text for complete details).

TRACE 1.

Control run through C-4 column.

FILE 1 VIAL 1.01 INJ 1 OF 1 VOL 1030 TRAY 1
EQUIL. TIME .00 GRAD. DELAY .00

CHANNEL A   INJECT 01/06/89 02:51:57

TRACE 2.

RF molecular weight standards using

C- 4 column.
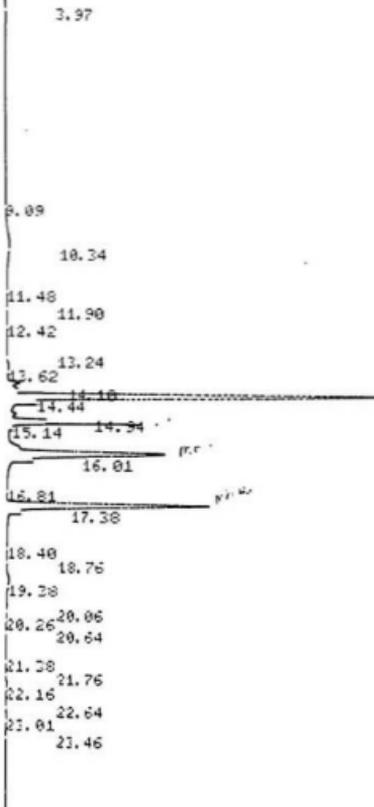
3.97

9.09

10.34

11.48
11.90
12.42

13.24
13.62
14.10
14.44
15.14   14.94

16.01

16.81

17.38

18.40
18.76

19.28

20.26   20.06
20.64

21.38
21.76
22.16
22.64
23.01

23.46

UNIT 1

TRACE 3.
Sepharcyl S-200 sample 1.
Elution through C-4 column.

TRACE 4.
Sephacryl S-300 sample 2.
Elution through C-4 column.

TRACE 5.

Control run through C-8 column.

STOP
33.81
28.08
21.84
19.31
16.82
14.18
10.75
7.44

.355 .
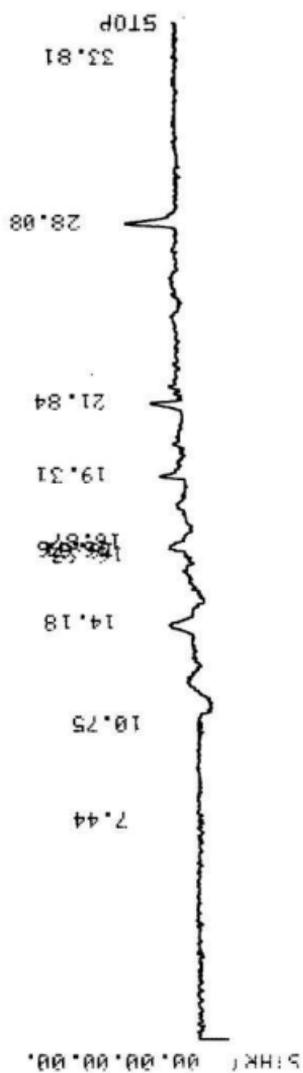
5:HR: 00.00.00.00.

TRACE 6.

RP molecular weight standards using

C-8 column.

BioRAD Standards

STOP

28.49
28.08

26.46

25.93
25.33

24.16

22.02

21.22
20.72
20.32

19.89

18.79
18.31

17.46

16.04

15.52

13.94

9.86

7.97

START 00.00.00.00.

1.40
1.76

5.15

8.79

10.4

STOP
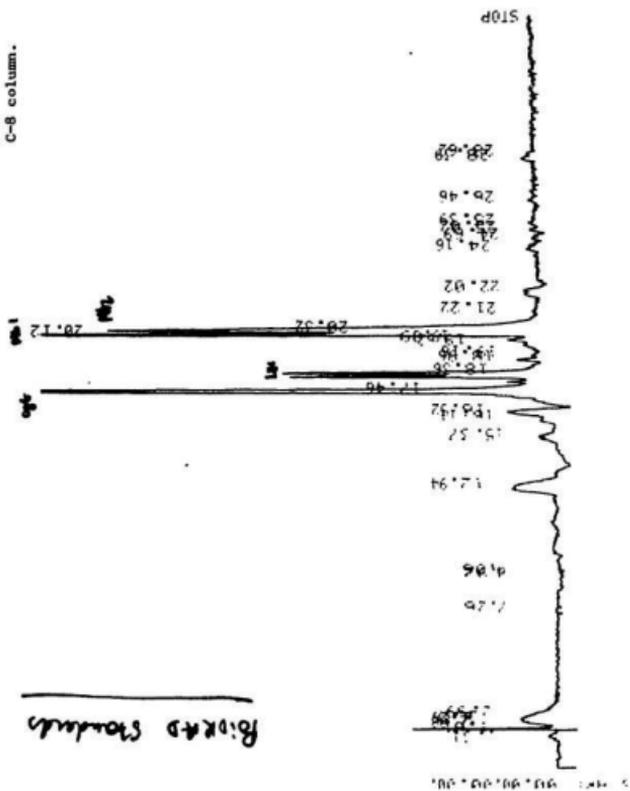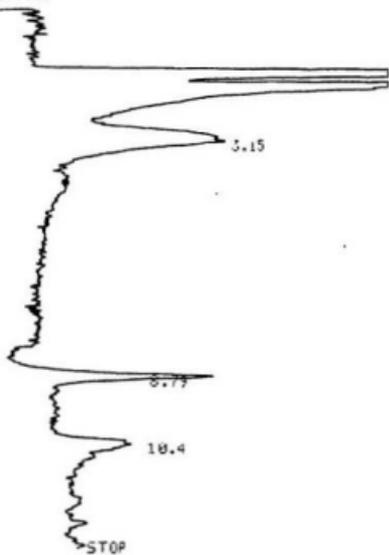
TRACE 7.
Sephacryl S-300 sample 1.
Elution through C-8 column.

TRACE 8.
Sephacryl S-300 sample 2.
Elution through C-8 column.
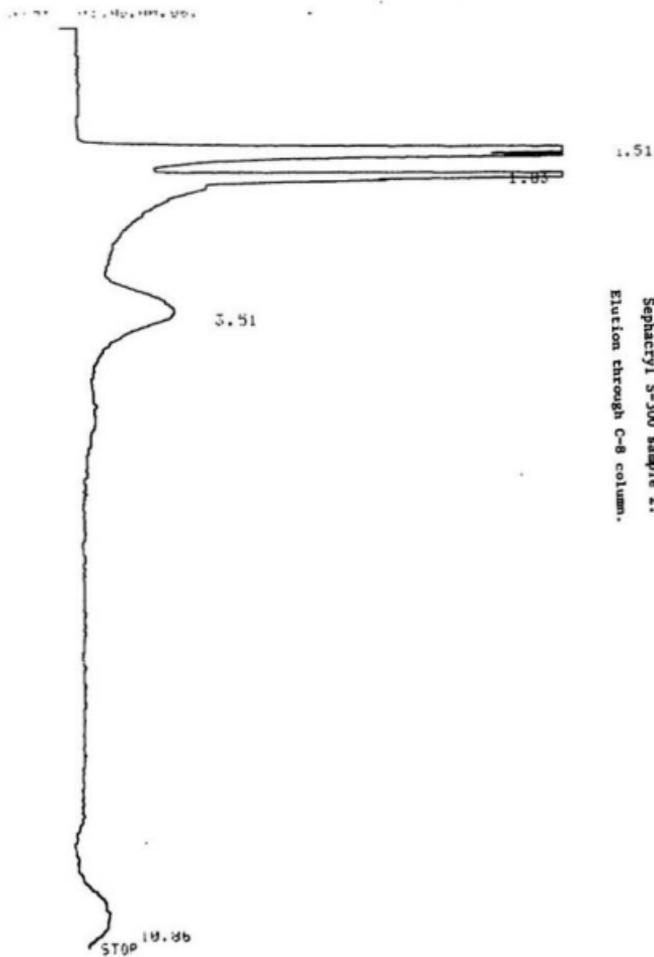
TRACE 9.
Control run through C-18 column.

TRACE 10.

RP molecular weight standards using C-18 column.
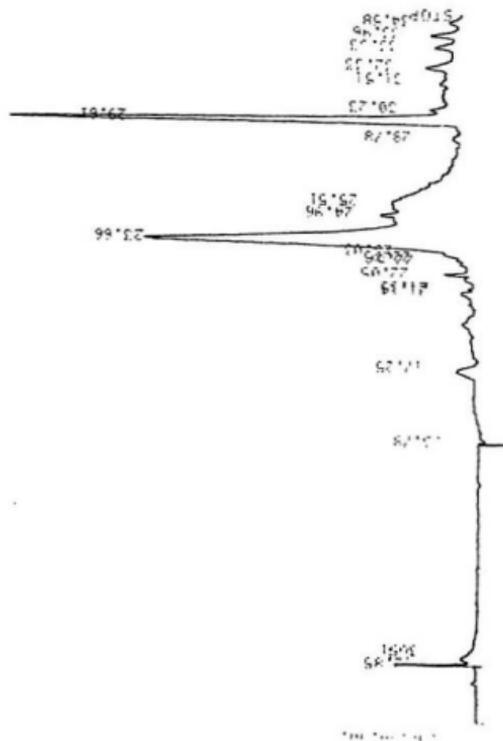
TRACE 11.

Sephacryl S-300 sample 1.
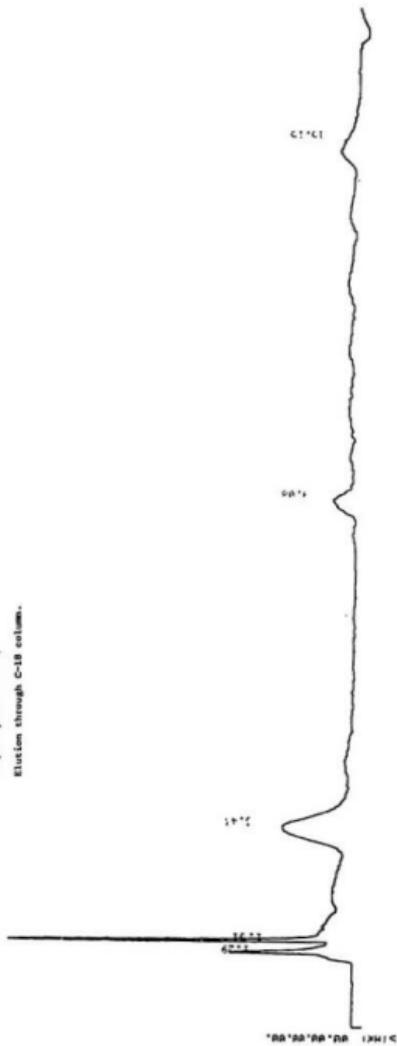
Elution through C-18 column.

START 00.00.00.00.

1.03

1.44

1.74

3.93

TRACE 12.

Sephacryl S-300 sample 2.

Elution through C-18 column.

10.15

10.45

10.9

11.27

STOP
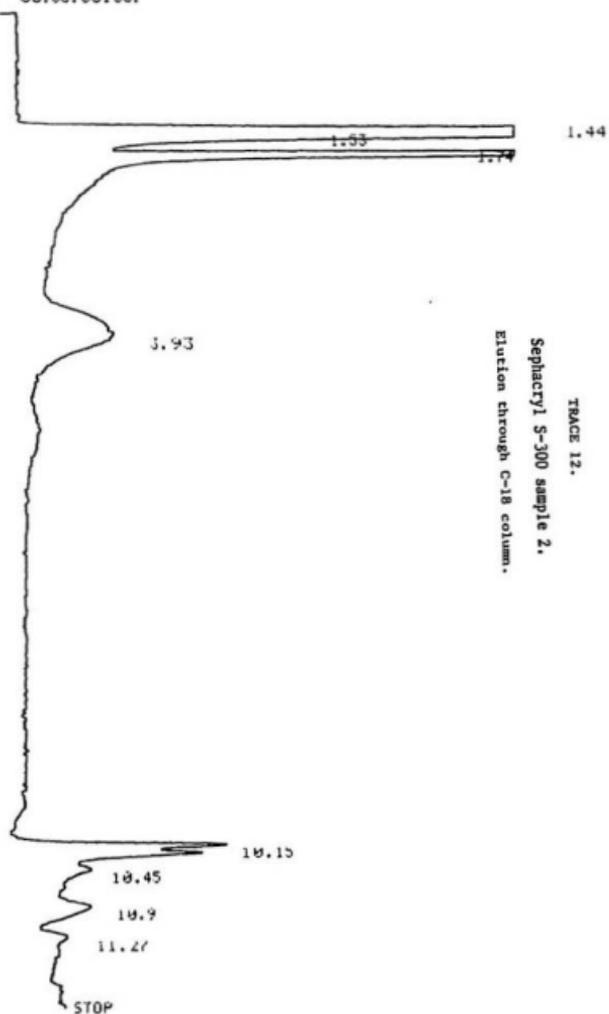
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MARITAL STATUS:	Married.
CITIZENSHIP:	Canadian.
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EDUCATION .

Ph.D. Biochemistry.	Memorial University of Newfoundland, Canada. 1989.
M.Sc. Chemistry.	St. Francis Xavier University, Canada. 1985.
B.Sc. Molecular Sciences.	University of Warwick. U.K. 1969.
H.N.C. Chemistry.	Royal Institute of Chemistry and Department of Education. U.K. 1966.
University of London. U.K.	General Certificate of Education Examinations. 'A' levels, and 'O' levels.
Board of Secondary Education. Karachi.	School leaving examinations.

GRADUATE COURSES .

 Memorial University of Newfoundland .
Passed Ph.D. final comprehensive examinations.

Proteins (401).   80%
Molecular Biology (400).   76%

St. Francis Xavier University .

Advance analysis and Instrumentation 1.(Chemistry 461) 95%
Comparative Physiology 1.   84%
Bio-organic Chemistry.   78%
Advance analysis and Instrumentation 2.(Chemistry 462) 73%
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Queen's University .

Medical Pharmacology (Pharm. 800).   81%
Medical Biochemistry (Biochem. 800).   74%
Isotopes in Biology. (Biology 910).   78%
Structured Programming. (Computing 2210W) Audit.
Microbiology (810). Audit.

University of Ottawa .

Medical Physiology (Phs. 5521)   68%
Neuroanatomy and Neurophysiology (Ana. 5422)   61%
Elementry French (Institute of second language)   Passed.

PUBLICATIONS .

FULL JOURNAL PAPERS:

1. Maneckjee, A., Weisbart, M., and Idler, D.I. 1989. The
presence of $17\alpha,20\beta$-dihydroxy-4-pregnen-3-one receptor activity
in the ovary of brook trout _Savelinus fontinalis_ during
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2. Beck, J., Donini, J., Maneckjee, A. 1983. The influence of
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4. Smallman, B.N., and Maneckjee, A. 1981. The synthesis of
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5. Steele, R.W., and Maneckjee, A. 1979. Toxicological signi-
ficance of acetylcholine esterase of the housefly thorax.
Pesticide Biochemistry and Physiology 10, 322-332.

6. Morris, D., Maneckjee, A., and Smallman, B.N. 1976. Appar-
ent synthesis of acetyl-β-methylcholine by _Celerio
euphorbiae_ . Insect Biochem. 6, 247-250.

7. Hardina, P, Ling, G.M., and Maneckjee, A. 1971. Desimpramine: Effects on level of acetylcholine in whole brain and in striatum of rats. European J. of Pharmacol. 15, 223-229.

8. Morris, D., Maneckjee, A., and Hebb, C.O. 1971. The kinetic properties of human placental choline acetyltransferase. Biochem. J. 125, 857-863.

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1. Maneckjee, A., Idler, D. R., Weisbart, M. 1989. Demonstration and studies of membrane and cytosol steroid receptors for 17α,20β-dihydroxy-4-prenen-3-one in brook trout Salvelinus fontinalis oocytes by photoaffinity labelling using synthetic progestin 17,21-dimethyl-19-nor-pregn-4,9-diene-3-20-dione (R5020). Sent to the Journal of Fish Physiol. and Biochem. January,19 89, and after modifications resubmitted in July 1989.

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1. Maneckjee, A., Idler, D.R., and Weisbart, M. 1989. Evidence for 17α,20β-dihydroxy-4-pregnen-3-one steroid receptors on the zona radiata membrane of brook trout oocytes. 32nd Annual meeting of Canadian Federation of Biological Socities. Calgary, Alberta.

2. Maneckjee, A., Idler, D.R., and Weisbart, M. 1989. Purification and molecular characteristics of cytosolic and membrane associated receptors, for maturation inducing steroid 17α-,20β-dihydroxy-4-pregnen-3-one from brook trout Savelinus fontinalis ovarioan follicles. 28th Annual meeting of the Canadian Socities of Zoologist. York, Ontario.

3. Maneckjee, A., Weisbart, M., Idler, D.R. 1987. Isolation and characteristics of 17α,20β-dihydroxy-4-pregnen-3-one receptor activity in ovarian tissue of brook trout Savelinus fontinalis . (D. R. Idler, L. W. Crim. and J.M. Walsh, eds.). Proceedings Third International Symposium on Reproductive Physiology of Fish. Memorial University of Newfoundland, St. John's.

4. Beck, J., Donini, J., Maneckjee, A. 1983. The interaction between sulphide and calcium ion. Presented at CIC conference, Calgary, Alberta.

5. Beck, J., Donini, J., Maneckjee, A. 1982. Toxicology of hydrogen sulphide. 25th Annual meeting of Canadian Federation of Biological Socities. Edmonton, Alberta.

6. Hrdina, P., Singhal, R., Peters, J., Ling, G.M., Maneckjee, A. 1971. Acetylcholine and dopamine in rat straitum duri ng acute DDT poisoning. 14th Annual meeting of Canadian Federation of Biological Societies. Toronto, Ontario.

COMMUNICATIONS .

Hrdina, P., Maneckjee, A. 1971. "Free and Bound" acetylcholine concentrations in rat brain. J. Pharm. and Pharmacology. 23, 540-541.

THESIS .

B.Sc. The effects of cross-linking agents on stability and a activity of proteases used with detergents. Supervisor professor D. W. Griffiths. University of Warwick. Coventry, U.K. 1969.

M.Sc. Hydrogen Sulphide Toxicity: In vitro effects on cholinergic nervous system components, acetylcholine esterase and nicotinic receptor sites. Supervisor Professor John Donini. St Francis Xavier University. Antigonish, Nova-Scotia. Canada. 1985.

Ph.D. Characterization and Physiological role of $17\alpha,20\beta$-dihydroxy-4-pregnen-3-one steroid receptor activity in the cytosolic and zona radiata membrane fraction of the ovarian follicles of brook trout Salvelinus fontinalis during the terminal stages of oocyte maturation. Supervisor's, Professor David R. Idler, and Professor Melwin Weisbart. Memorial University of Newfoundland. St. John's, Canada. Submitted 1989.

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PERSONAL INTERESTS .

Founder and President of the International Society at University of Warwick.

Played Competition Tennis (1960-1969).

Physical fitness: Jogging five times a week, also participated in the noon hour fitness program at Queen's University.

Organic gardening and farming to acheive self-sufficiency in production of vegetables, meat, eggs, milk and dairy products (1978-1986).

Enjoy outdoor activities, boating,cross-country skiing.

Enjoy working with small motor's, and maintenance of automobile and house.

PROFESSIONAL SOCIETIES

Member of Canadian Zoological Society.
Member of the Chemical Institute of Canada.

SCHOLARSHIPS :

Awarded National Sciences and Engineering Research Council Canada post-graduate scholarship (1985-1988), for doctoral study at Memorial University of Newfoundland.

Awarded National Sciences and Engineering Research Council of Canada post-doctoral fellowship (1989-1990), at Health and Welfare, Ottawa.

EMPLOYMENT EXPERIENCE .

1983-85. Research associate with Dr. M. Weisbart. Chairman, Department of Biology. St. Frances Xavier University. Antigonish, N.S.

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1972-80. Research assistant to Dr. B. N. Smallman. Professor and Head of the department of Biology, Queens University. Kingston, Ontario.

1970-72. Research assistant to Dr's. P. Hardina, and G. Ling. University of Ottawa. Ontario.

1963-66. Assistant Experimental Officer, ARC Institute of Animal Physiology. Babraham, Cambridge. U.K. Professor C.O.Hebb, and Dr. David Morris Supervisor's.

1960-63. Technologist to Dr. John Harris, Fison's Pest Control LTD. Cambridge, U.K. Research division.