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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ISBN 0-315-55001-5

Canada

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

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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.

Acknowledgements

I wish to thank my Supervisor, Dr. David Idler for his advice and providing me with the necessary equipment and chemicals to perform this research. Also I would like to express my sincere gratitude to Dr. Melvin Weisbart for his advice, trust, friendship and help conferred on me prior to and during the thesis research.

This study was partly supported by NSERC grant A6732 to DRI, and A0781 to MW. Initial support (1985-88) to AM came from NSERC post-graduate scholarship.

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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.

Fig. 1.

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) 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) by granulosa cells of the ovarian follicle, the 17α,20β-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 17α,20β-DHP in salmonids (So, et al. 1976).

The MIS 17α,20β-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

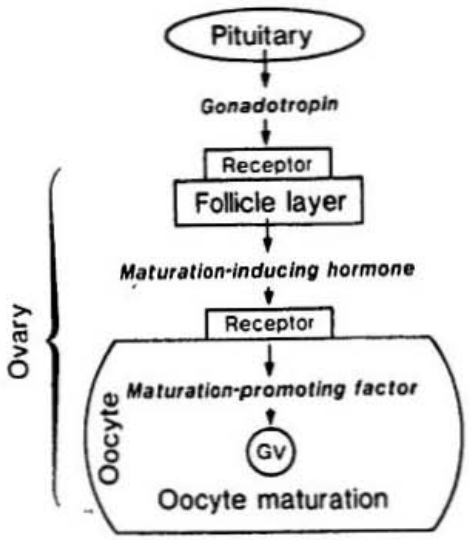
Various stages of oocyte during terminal maturation in salmonids.*

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.

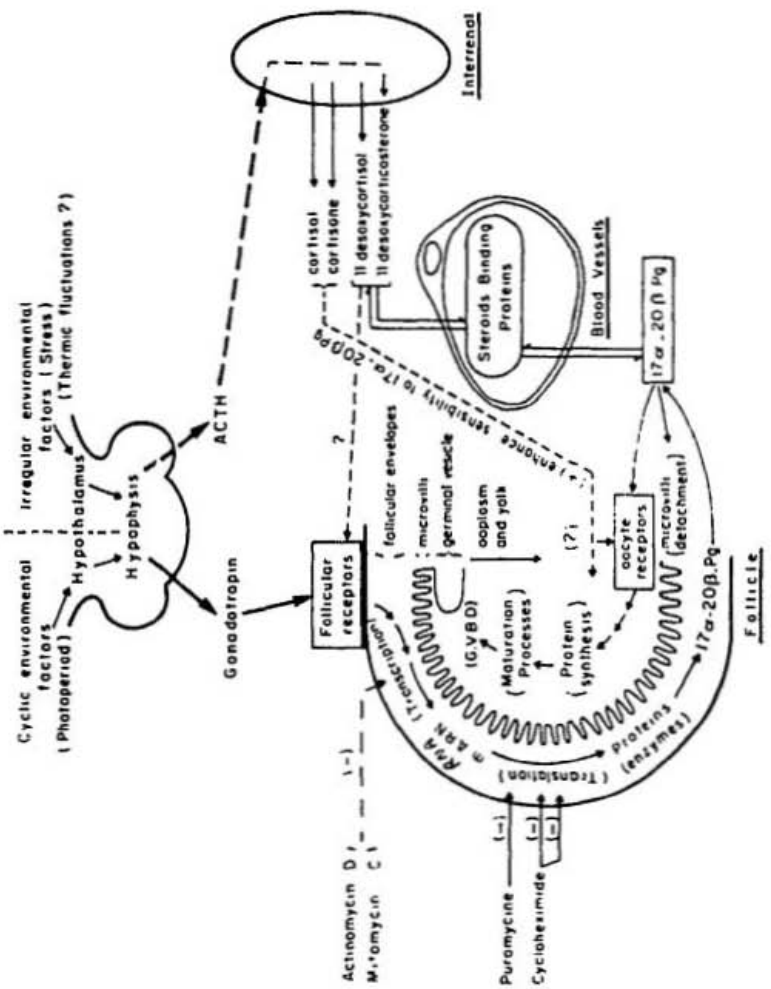

Pituitary
Gonadotropin
Receptor
Follicle layer
Maturation-inducing hormone
Receptor
Maturation-promoting factor
GV
Oocyte maturation
Oocyte
Ovary

by 17α,20β-DHP in trout, carp, and coho salmon (Jalabert, et al .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 salmonoids 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 salmonoids 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.


Cyclic environmental factors (Photoperiod)
Irregular environmental factors (Stress) (Thermic fluctuations ?)
Hypothalamus
Hypophysis
ACTH
Gonadotropin
Interrenal
cortisol
cortisone
11 desoxycortisol
11 desoxycorticosterone
Steroids Binding Proteins
Blood Vessels
17α.20β.Pg
Follicular receptors
follicular envelopes
microvilli
germinal vesicle
ooplasm and yolk
(G.V.B.D)
Maturation Processes
Protein synthesis
oocyte receptors
microvilli (detachment)
17α-20β.Pg
Follicle
RNA (Transcription)
mRNA
(Translation)
Proteins (enzymes)
Actinomycin D
Mitomycin C
Puromycine
Cycloheximide

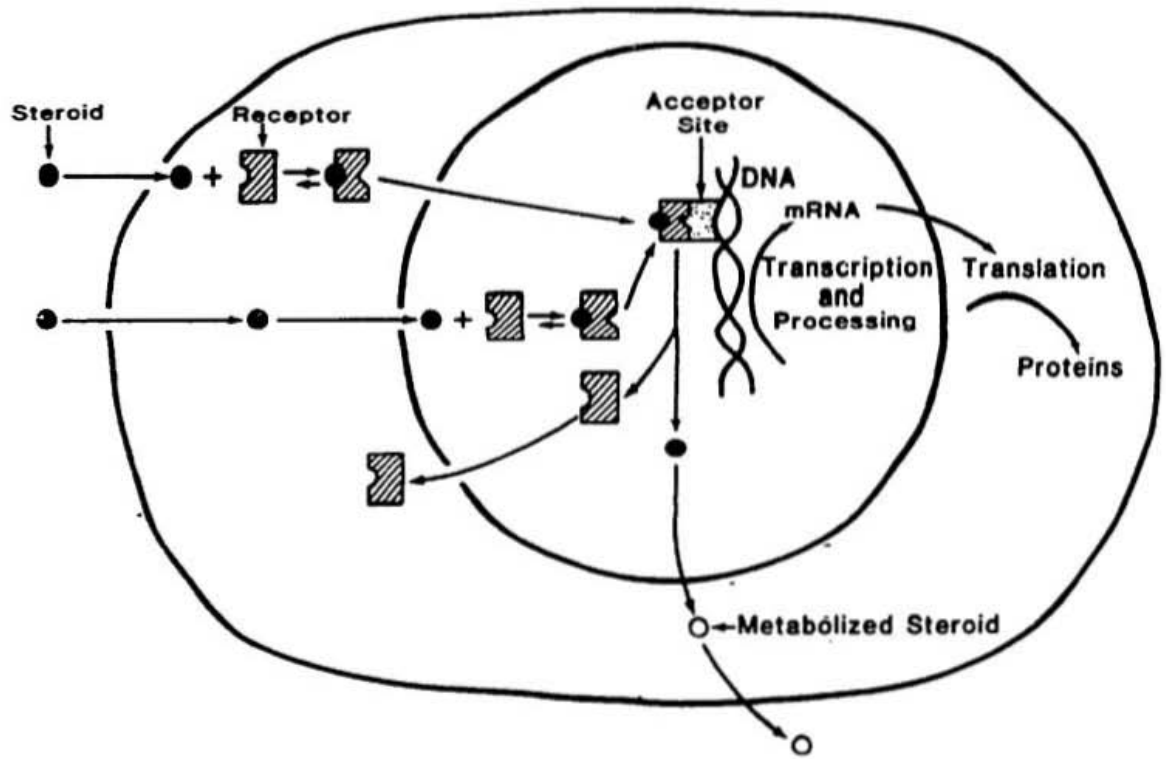
indicating that the mechanism of action involved in the steroid maturation of oocytes (GVBD) has a special characteristic which is different from the classical steroid mechanism of action via nuclei (Detlaff and Skoblina, 1969; Goswami and Sundararaj, 1973; Jalabert, 1976; Theojan and Goetz, 1981; DeManno 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 17α,20β-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 17α,20β-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.


Steroid
Receptor
Acceptor Site
DNA
mRNA
Transcription and Processing
Translation
Proteins
Metabolized Steroid

amphibian oocyte maturation in Xenopus 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 Xenopus . On the other hand Morril and collaborators have reported high affinity binding of progesterone in the cytosolic fraction of Rana oocytes (Kalimi, et al . 1979).

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

Maturation inhibiting substances

In sexually matured mammals, oocyte maturation in vivo 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, et al . 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 Fundulus heteroclitus 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 in vitro. 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.

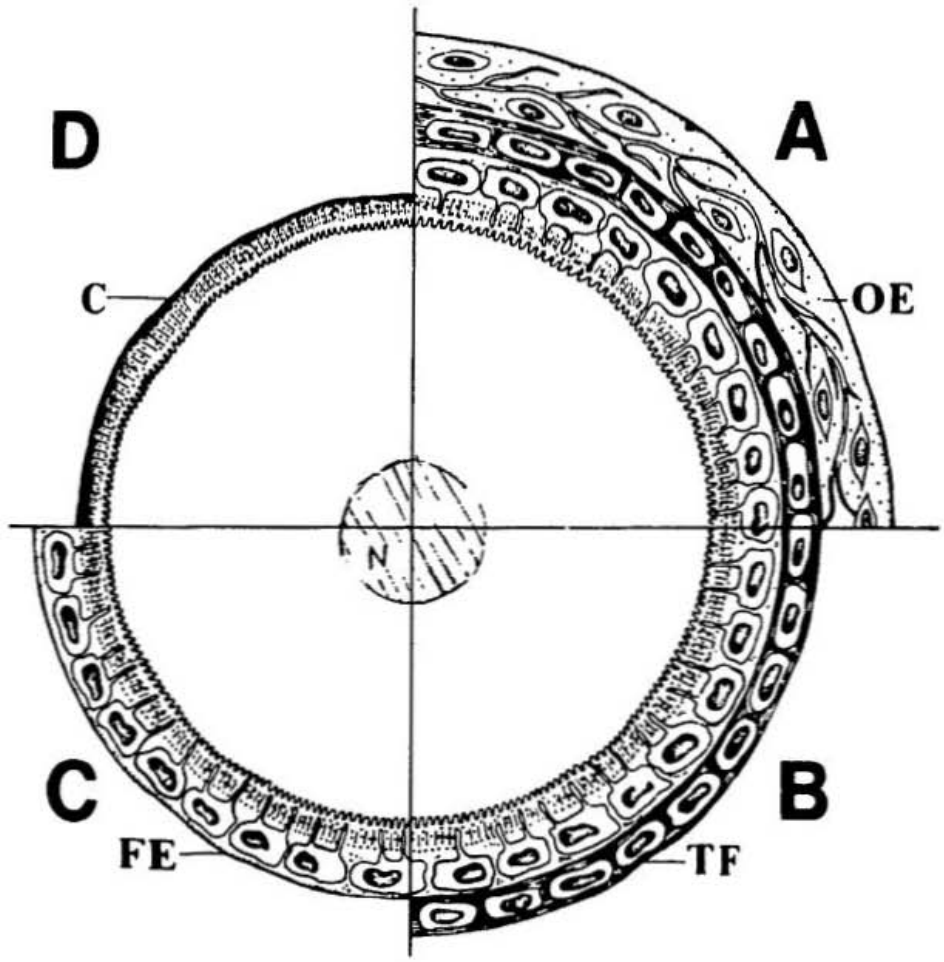
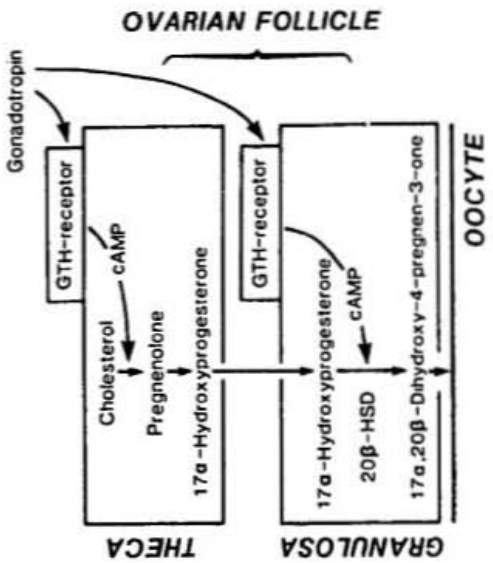


Fig. 1.2. Two-cell type model for the productiion of 17α,20β-DHP by salmonid ovarian follicles. 20β-HSD, 20β-hydroxy steroid dehydrogenase. Nagahama, Y. 1987. In Hormones and reproduction in Fishes, Amphibians and Reptiles. 171-193.(D.O. Norris and R.E. Jones, eds., Plenum Press, New York.).

Fig. 1.2.


OVARIAN FOLLICLE
Gonadotropin
GTH-receptor
cAMP
Cholesterol
Pregnenolone
17α-Hydroxyprogesterone
GTH-receptor
17α-Hydroxyprogesterone
cAMP
20β-HSD
17α,20β-Dihydroxy-4-pregnen-3-one
OOCYTE
THECA
GRANULOSA

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 17α,20β-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 17α,20β-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 :

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

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:

$$K_a = k_a / k_d$$

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 [^{3}H]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 17α,20β-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 17α,20β-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 17α,20β-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 equilibriation 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 i.. 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 17α,20β-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-^{3}H]Progesterone (specific activity 112 Ci/m mol), 17α-methyl [^{3}H]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 filteration 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 250ul 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 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 protcins, 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 et 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 $\ln \frac{B}{(B_{max}-B)}$ 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 :

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)}$$

, where V_e 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 ± 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 [^{3}H]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 [^{3}H] 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 ml 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 [^{3}H]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 $MgSO_4$), and incubated at 16 deg C. Samples (250 ul) were withdrawn every 30 min, then equilibrated immediately with [^{3}H]17α,20β-DHP $\pm$ 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 [^{3}H]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 equlibrated for 60 min at 1 deg C with [^{3}H]17α,20β-DHP $\pm$ inert steroid , and then assayed for specific binding.

2) In presence of [^{3}H]17α,20β-DHP in the cytosolic preparation.

Two aliquots (6.25 ml) of above cytosolic preparations were

added to vials containing [^{3}H]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 17α,20β-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

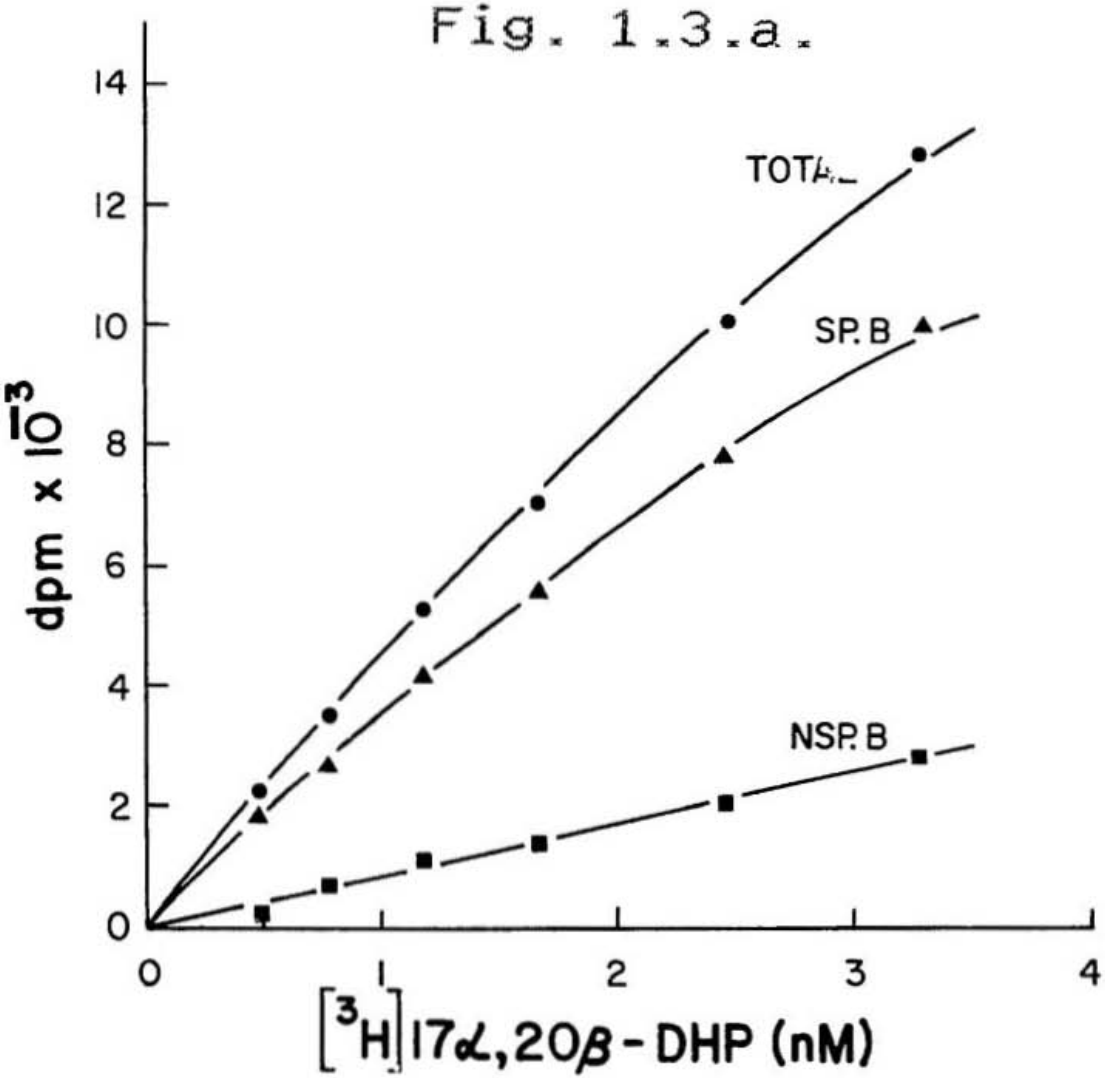
Steroid binding studies

Saturation curve and Scatchard analysis. [^{3}H]17α,20β-DHP and [^{3}H]progesterone were used in binding studies and for Scatchard analysis. The affinity constant (K_a) and maximum binding capacity (N_{max}) for 17α,20β-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 [^{3}H]17α,20β-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 [^{3}H]17α,20β-DHP and K_a was 8.04×10^7 M^{-1}. The blood plasma protein was later shown to be different from 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 17α,20β-DHP to cytosolic extract of brook trout ovarian tissue. Aliquots of ovarian tissue cytosol (250 µl) were incubated with increasing concentration of [^{3}H]17α,20β-DHP (0.5-3.5 nM) ± 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.



Competition studies

The specificity of [^{3}H]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-[^{3}H]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-[^{3}H]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 $1.28 \times 10^8\ M^{-1}$, the maximal concentration of binding sites was 1965 fmol/mg protein.

Fig. 1.3.b.

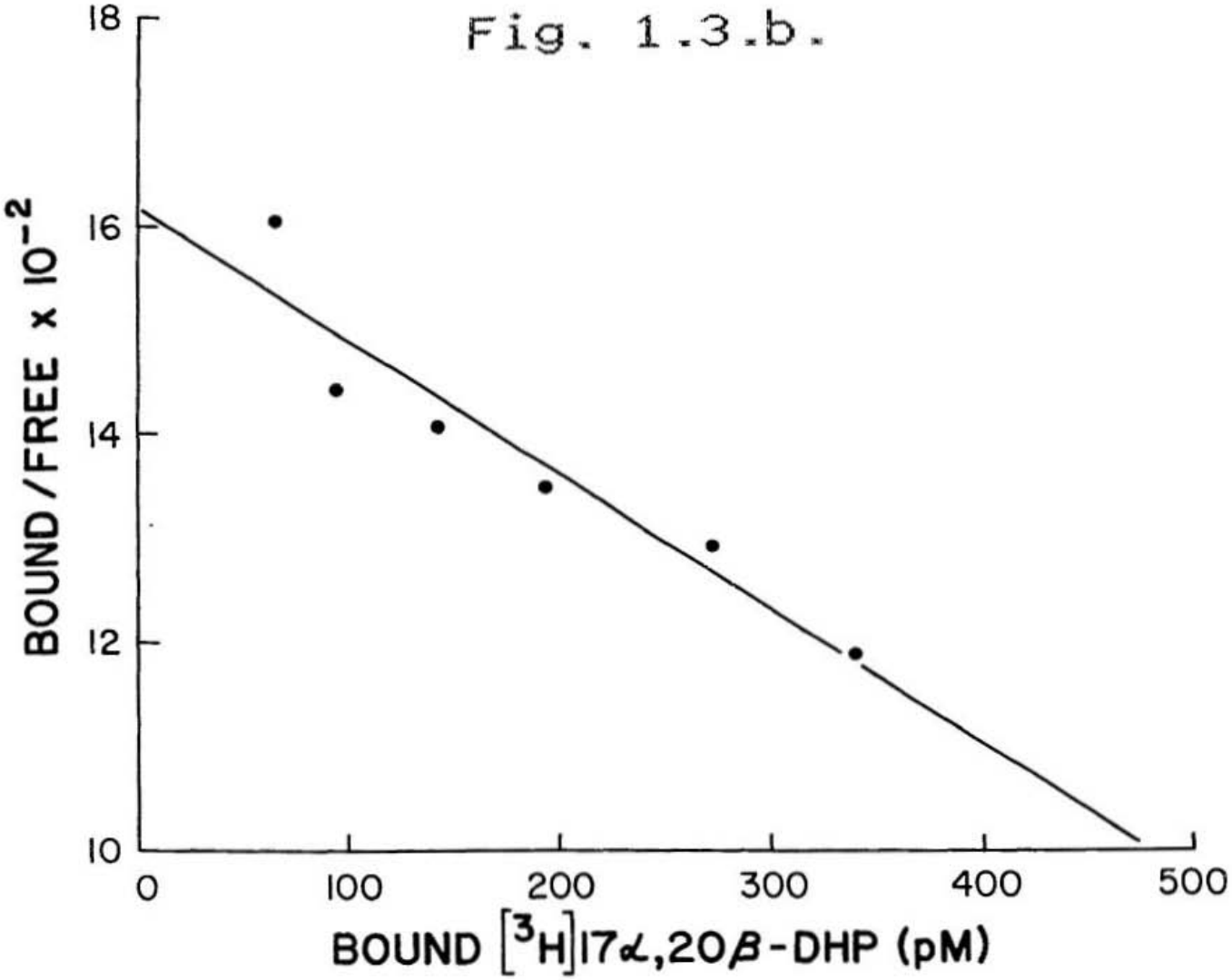

18
16
14
12
10
BOUND/FREE x 10^{-2}
0
100
200
300
400
500
BOUND [^{3}H]17α,20β-DHP (pM)

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.

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

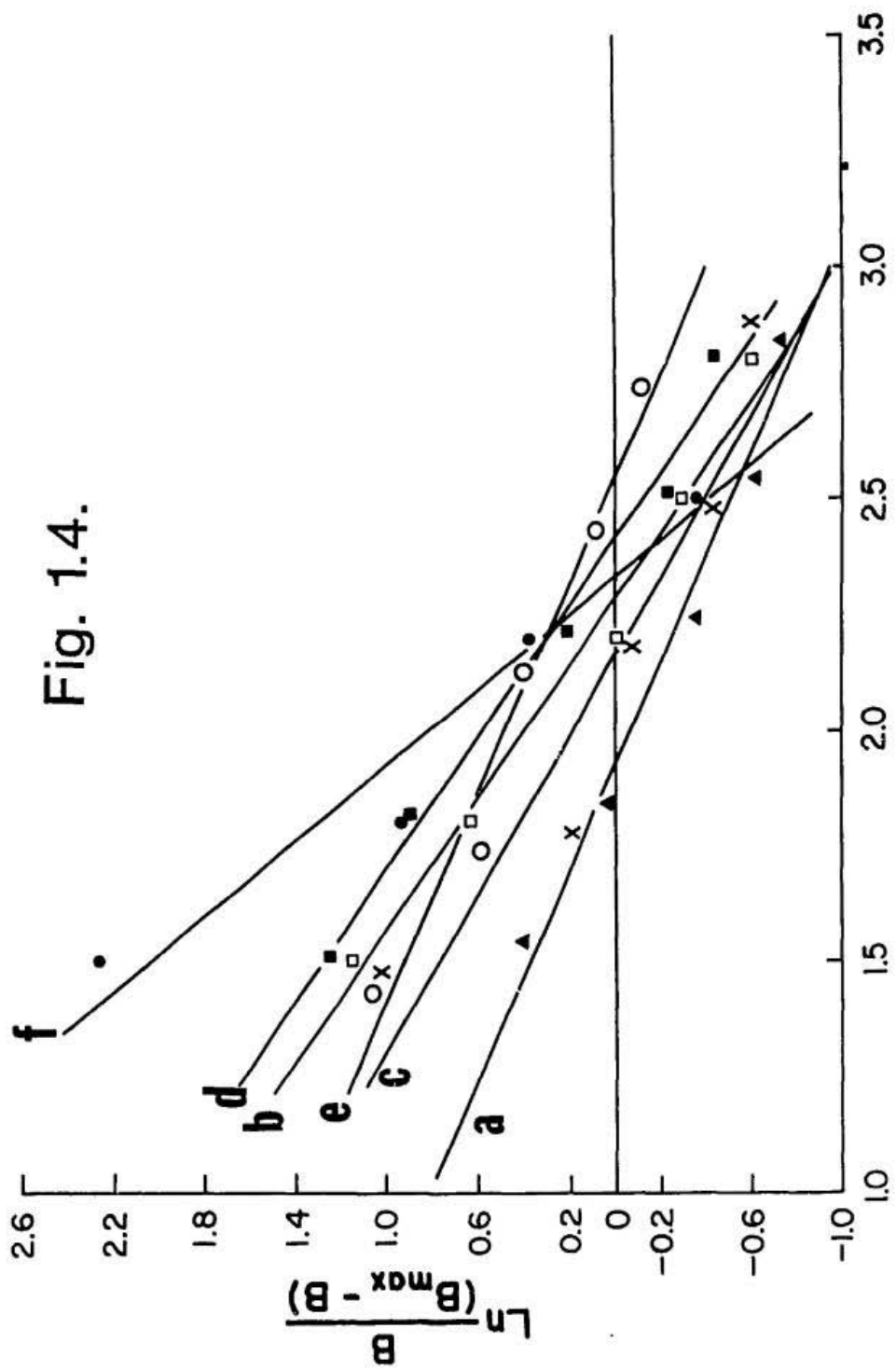


Fig. 1.4.
f
d
b
e
c
a
2.6
2.2
1.8
1.4
1.0
0.6
0.2
0
−0.2
−0.6
−1.0
1.0
1.5
2.0
2.5
3.0
3.5
LOG10 [I]
Ln B / (Bmax − B)

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,20ß-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,20ß-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 [^{3}H]17α,20β-DHP.

Fig. 1.5.a.

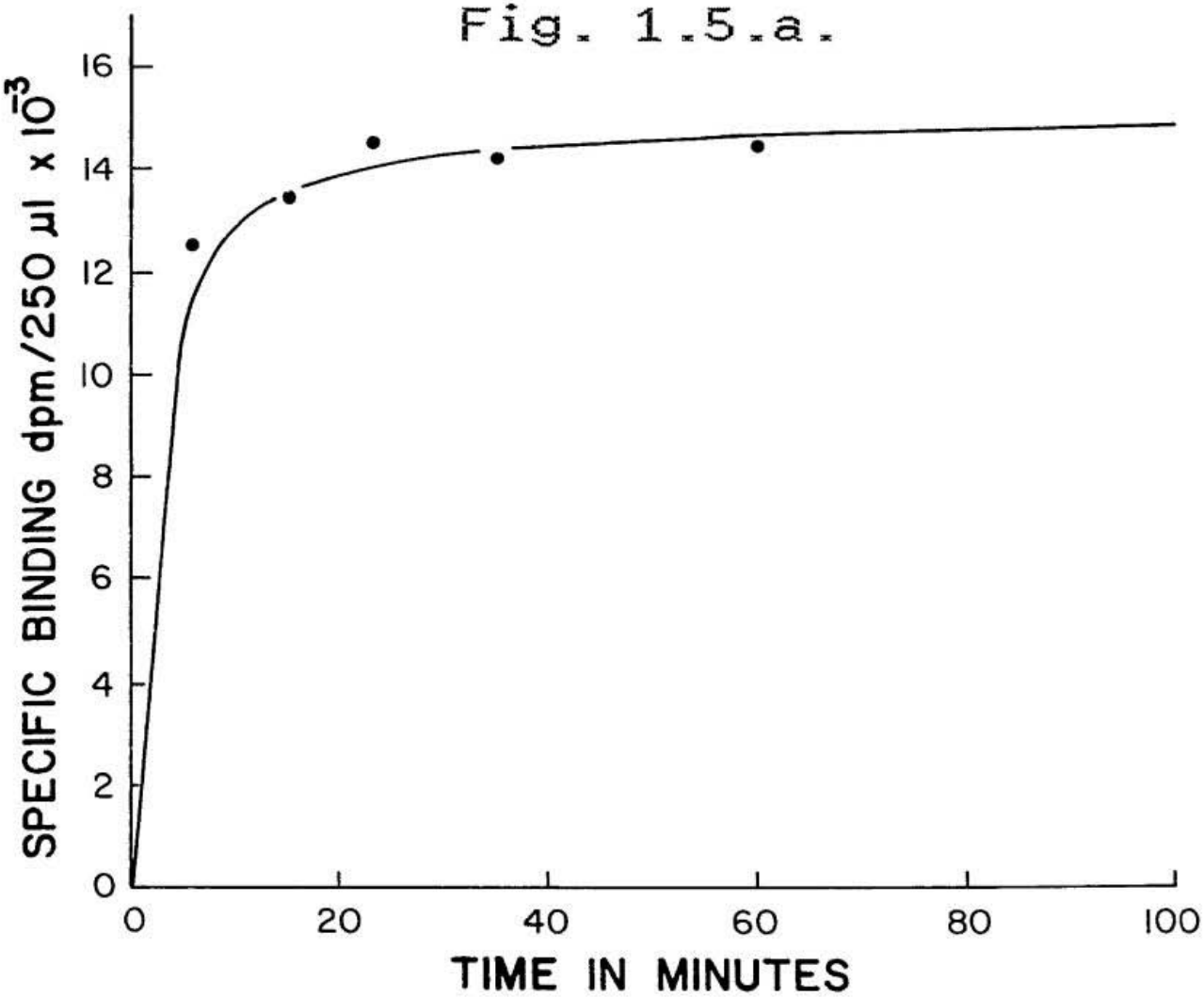

SPECIFIC BINDING dpm/250 µl x 10^{-3}
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
TIME IN MINUTES

TABLE 1.3

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

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

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

$$\frac{1}{T-S} \ln \frac{(T-X)}{(S-X)}$$

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.

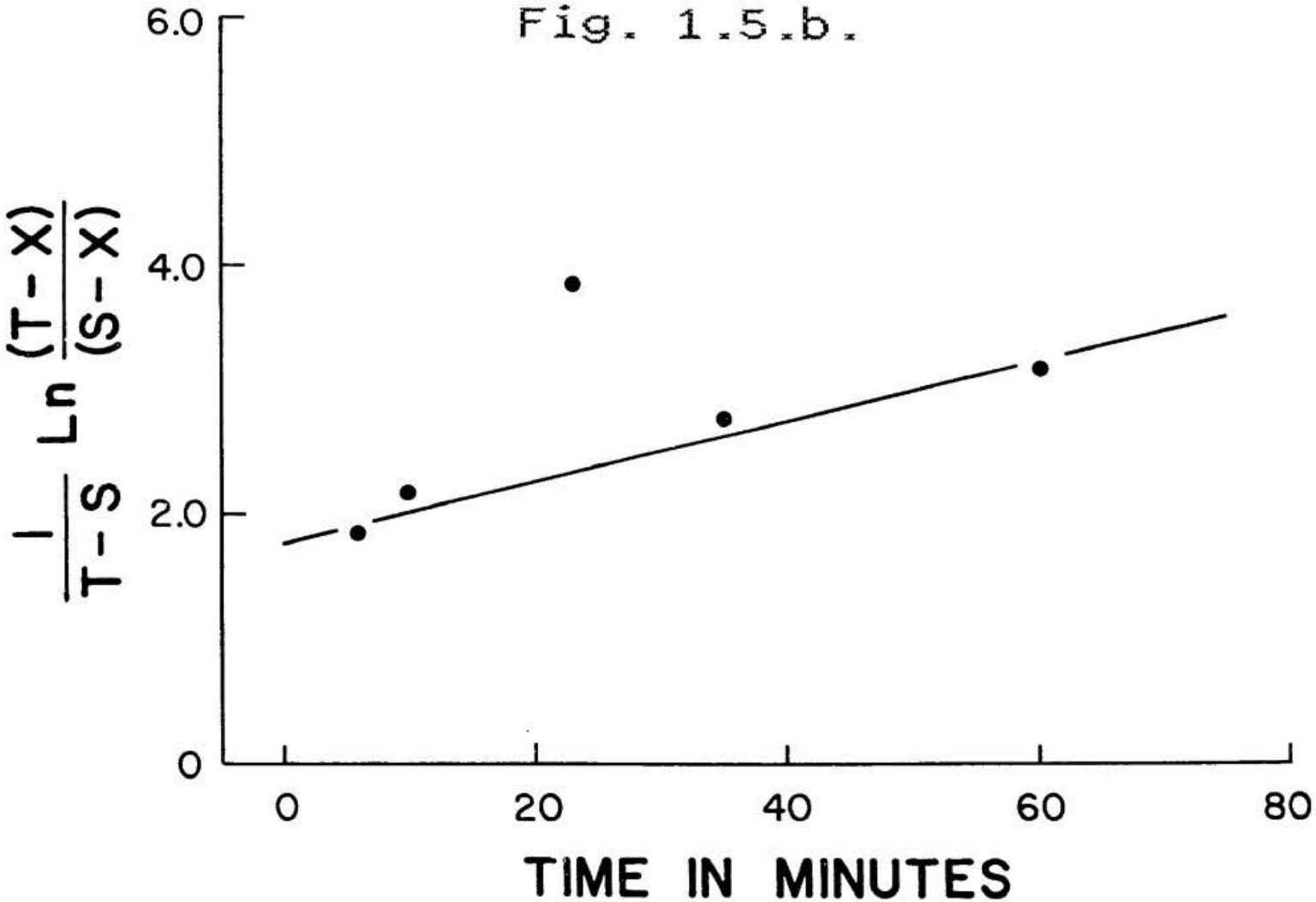

6.0
4.0
2.0
0
0
20
40
60
80
$\frac{1}{T-S} \text{Ln} \frac{(T-X)}{(S-X)}$
TIME IN MINUTES

Fig. 1.6. (a). Dissociation of the labelled [^{3}H]17α,20β-DHP receptor complex with time.

Fig. 1.6.a.

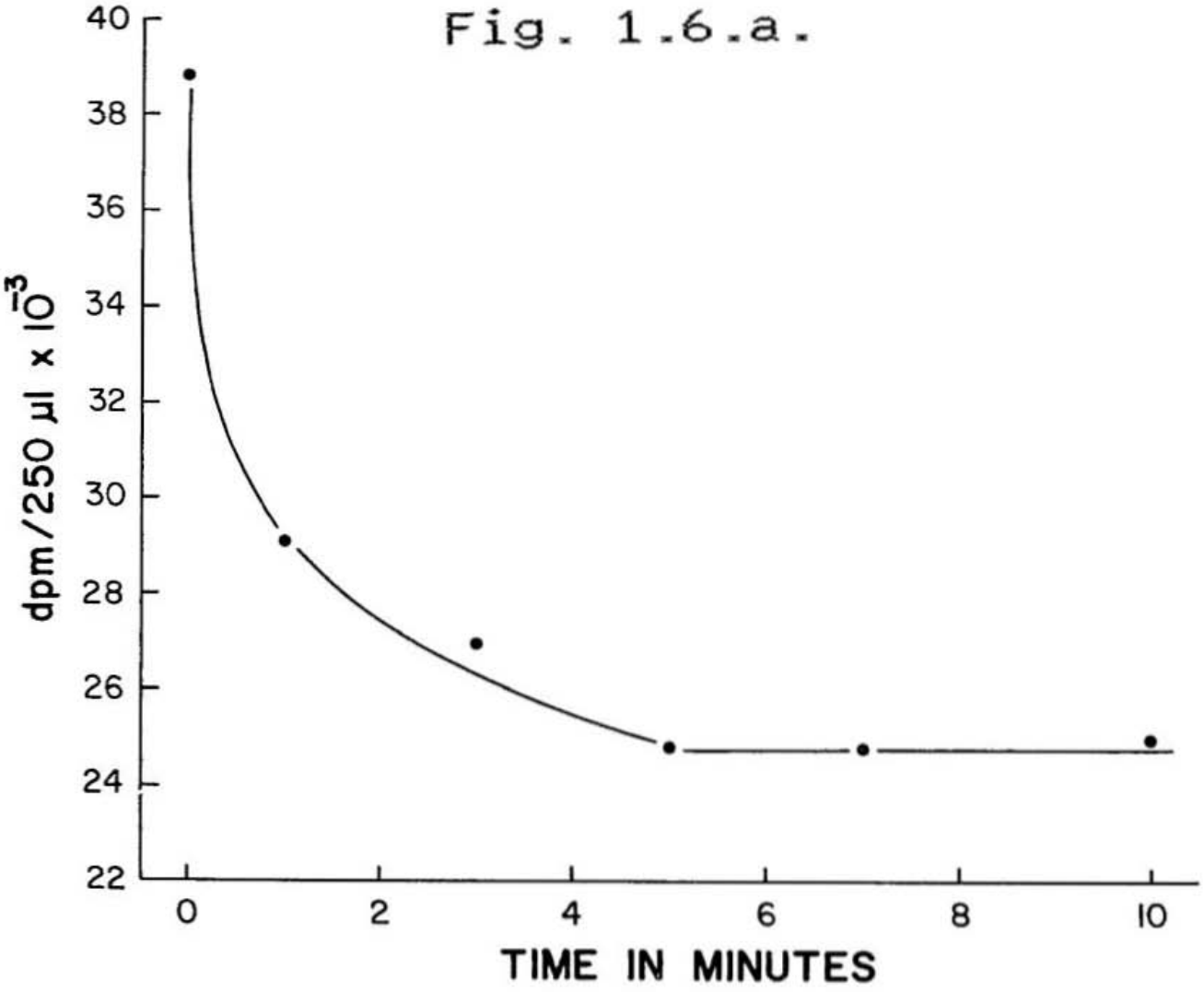
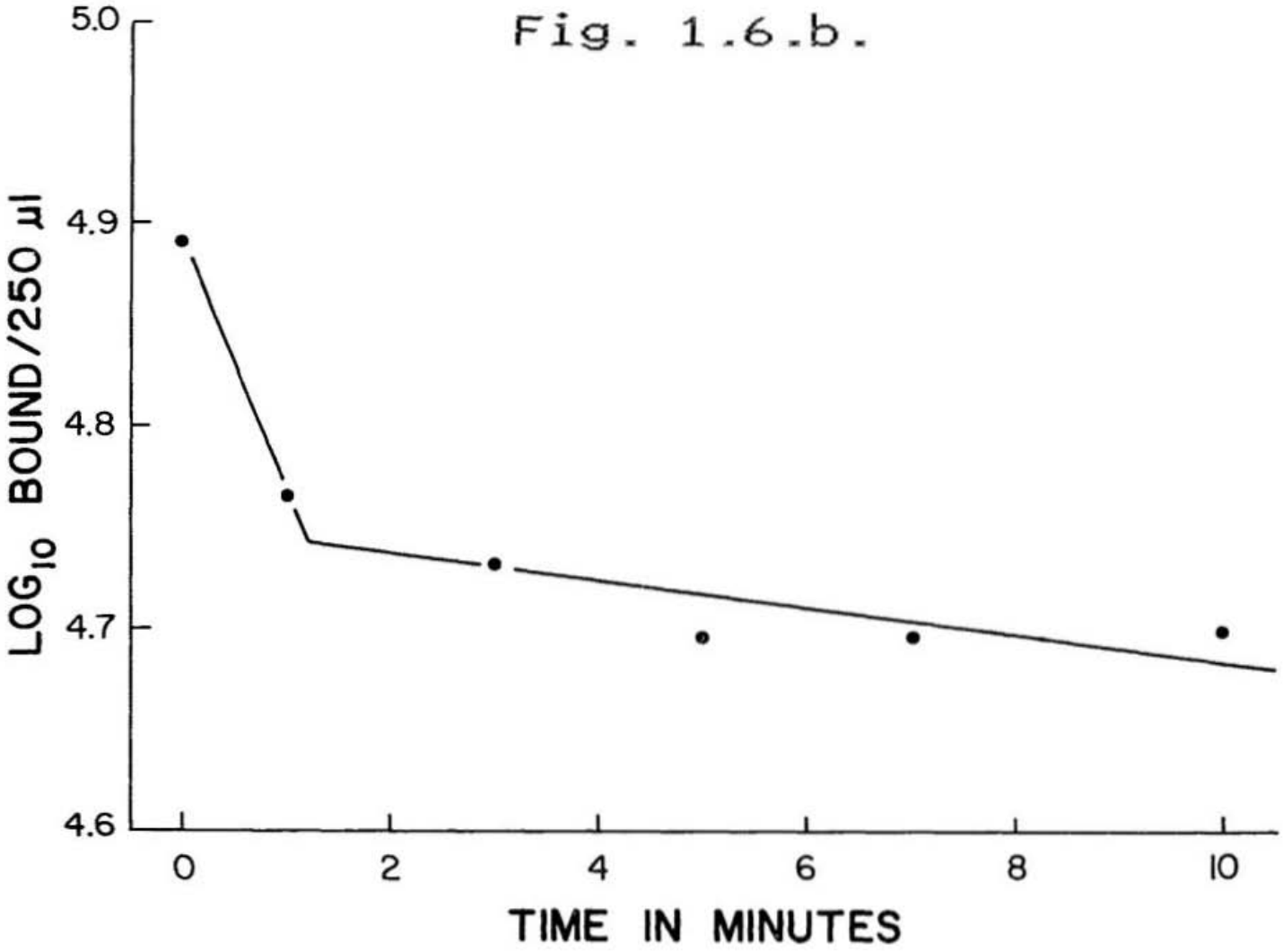

40
38
36
34
32
30
28
26
24
22
dpm/250 µl x 10^{-3}
0
2
4
6
8
10
TIME IN MINUTES

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

Fig. 1.6.b.



808a

$$\{ R + [^3H]17\alpha,20\beta\text{-DHP} \longleftrightarrow R\text{-}[^3H]17\alpha,20\beta\text{-DHP} \}$$

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 $[^3H]17\alpha,20\beta$-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 $[^3H]17\alpha$-,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' (Δ) 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 17α,20β-DHP ± 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.

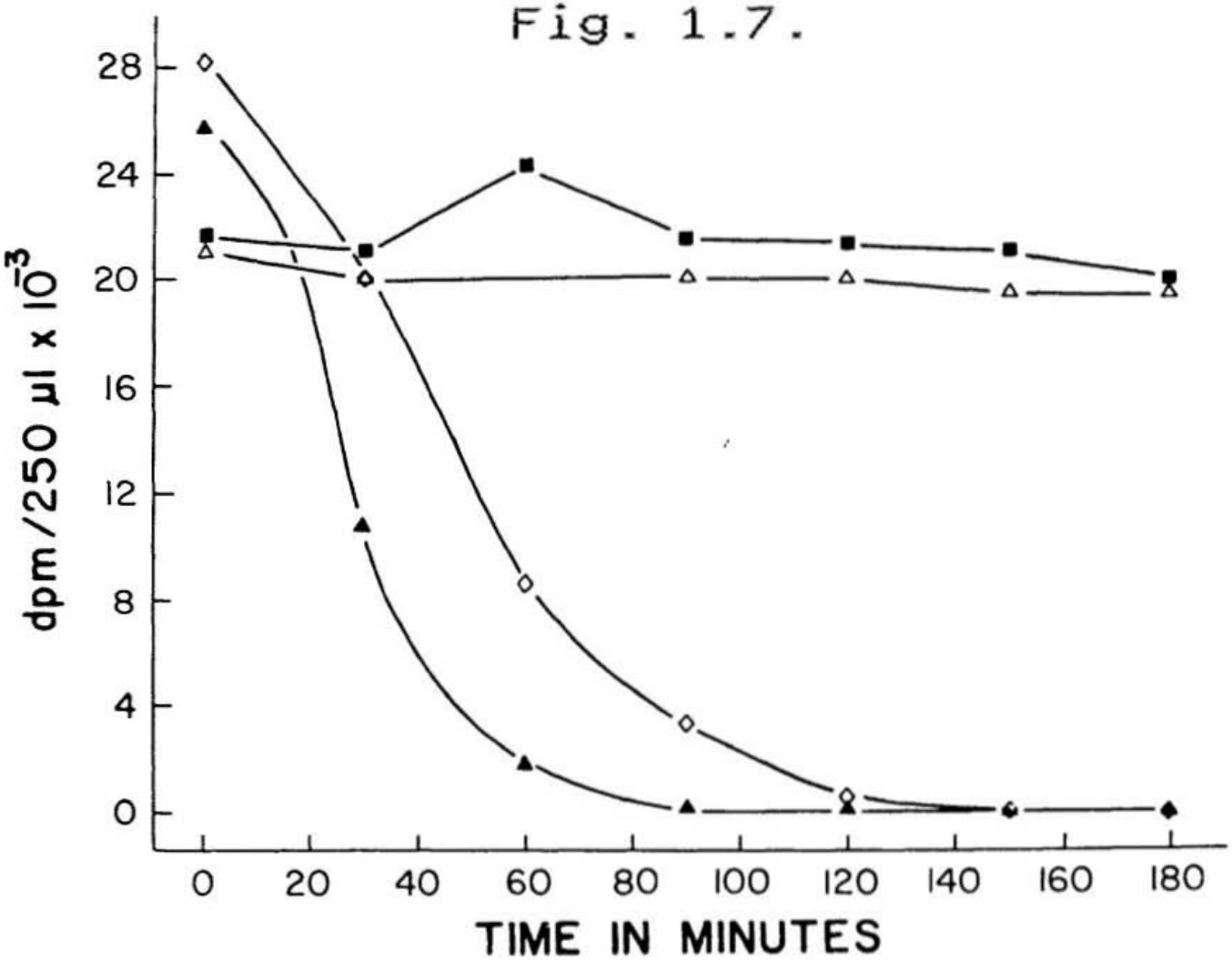
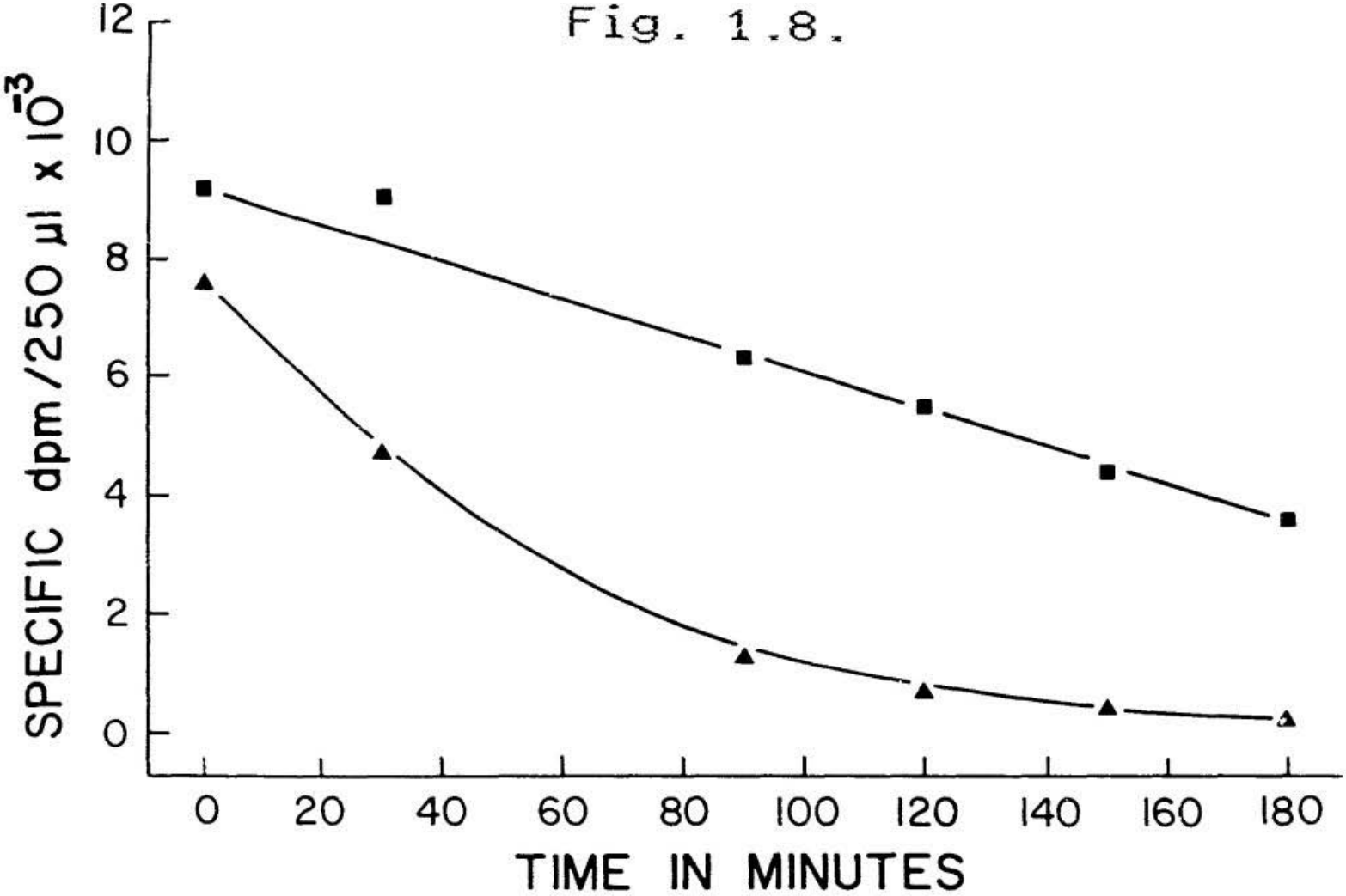

dpm/250 µl x 10^{-3}
28
24
20
16
12
8
4
0
0
20
40
60
80
100
120
140
160
180
TIME IN MINUTES

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).

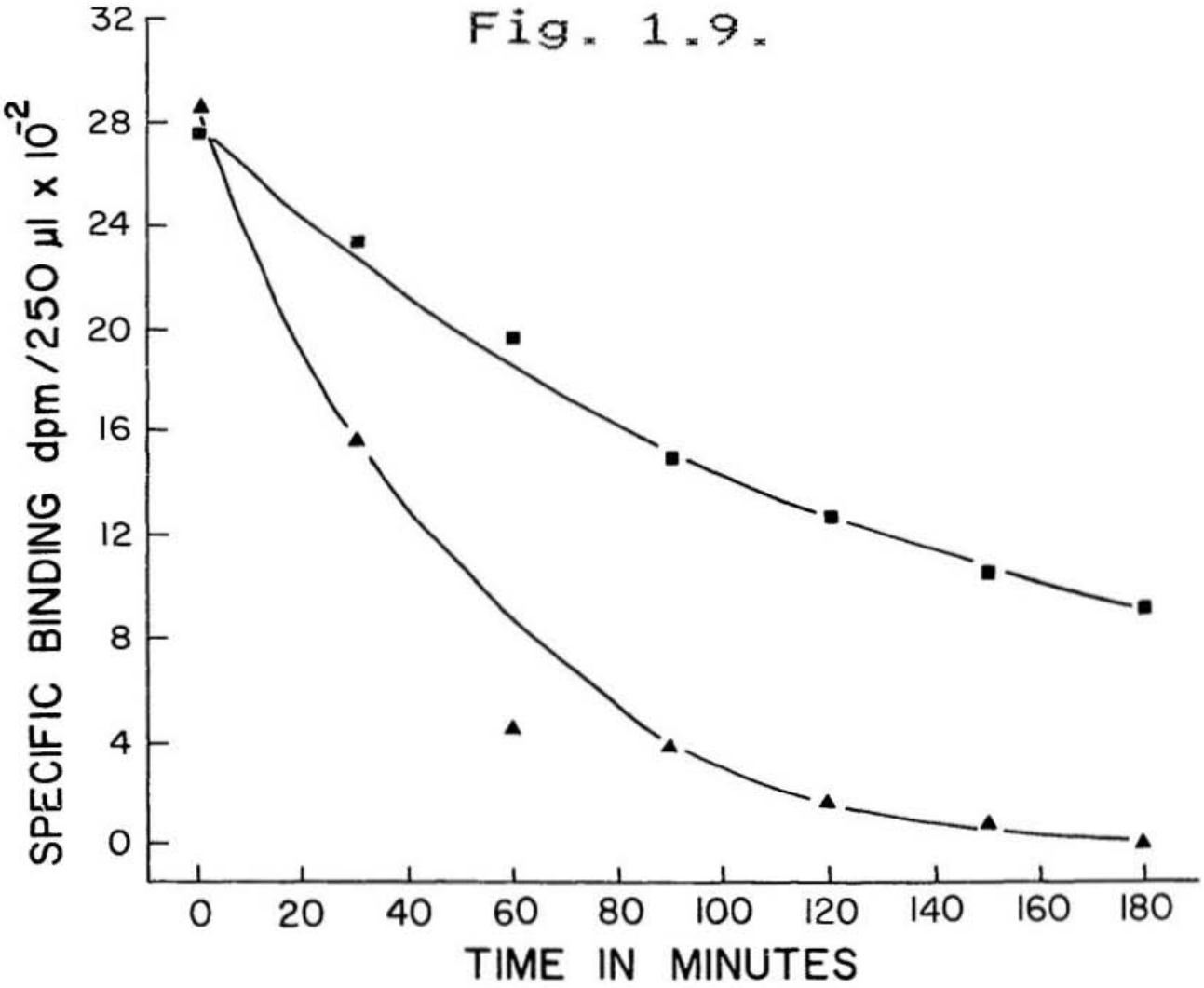
Fig. 1.8.


SPECIFIC dpm/250 µl x 10^{-3}
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
160
180
TIME IN MINUTES

83a.

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.


SPECIFIC BINDING dpm/250 µl x 10^{-2}
32
28
24
20
16
12
8
4
0
0
20
40
60
80
100
120
140
160
180
TIME IN MINUTES

brook trout cytosolic preparations as determined by paper chromatography.

Stability experiments with cytosolic preparation from brook trout ovarian tissue

In presence or absence of [^{3}H]17α,20β-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 [^{3}H]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*	$K_a \times 10^9$ + SEM (M^{-1})	N_{max} f mol/mg protein
1	5	0.21 ± 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.

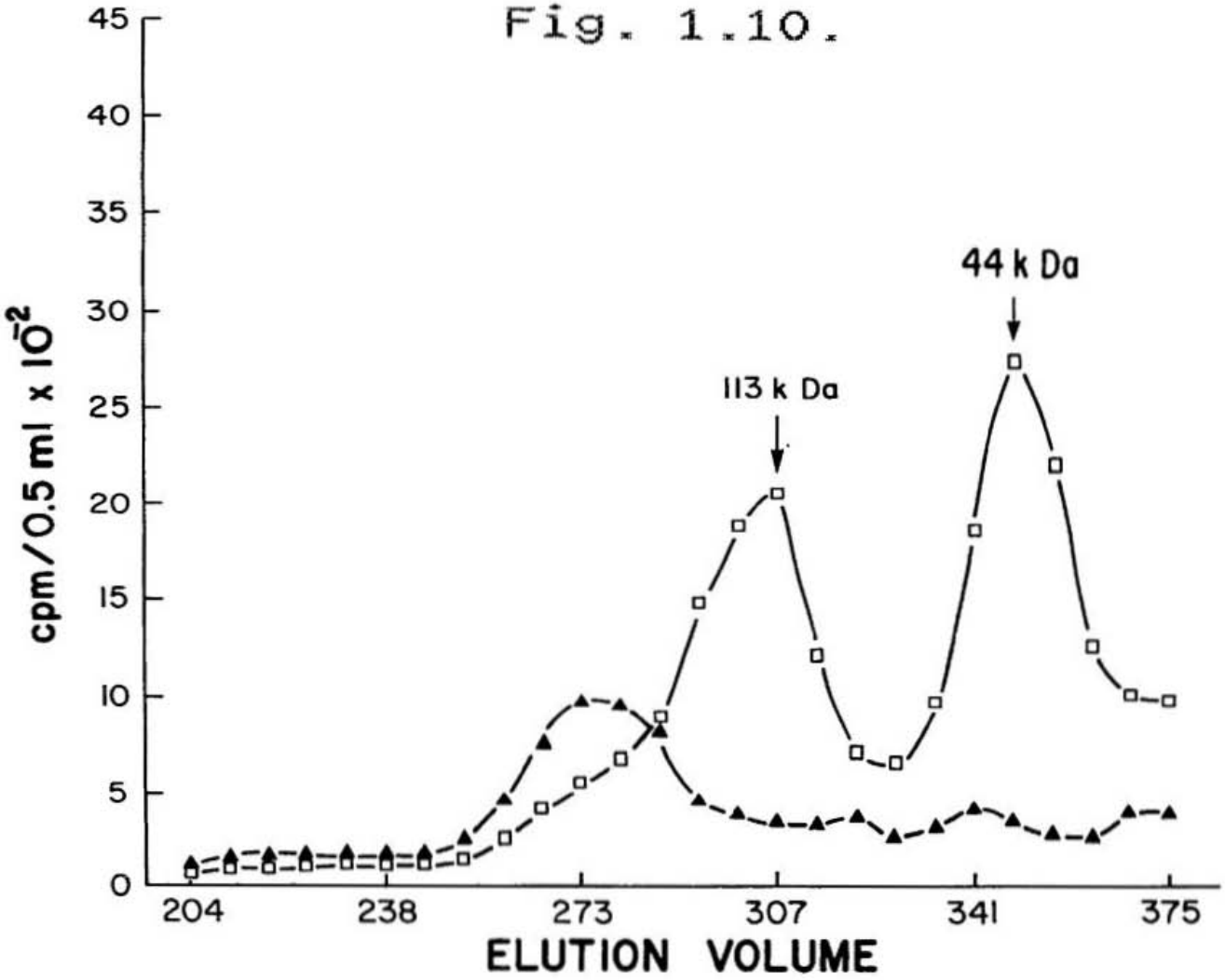
* number of fish used for determination
** mean ± SEM
† significantly different from each other, $P > 0.05$
++ no specific binding

for 120 min with [^{3}H]progesterone, [^{3}H]testosterone, and [^{3}H]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 [^{3}H]progesterone or [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]17α,20β-DHP were not as clearly defined as when using [^{3}H]progesterone and [^{3}H]testosterone.

The peak fractions from [^{3}H]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.


45
40
35
30
25
20
15
10
5
0
cpm/0.5 ml x 10^{-2}
44 k Da
113 k Da
204
238
273
307
341
375
ELUTION VOLUME

888

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.

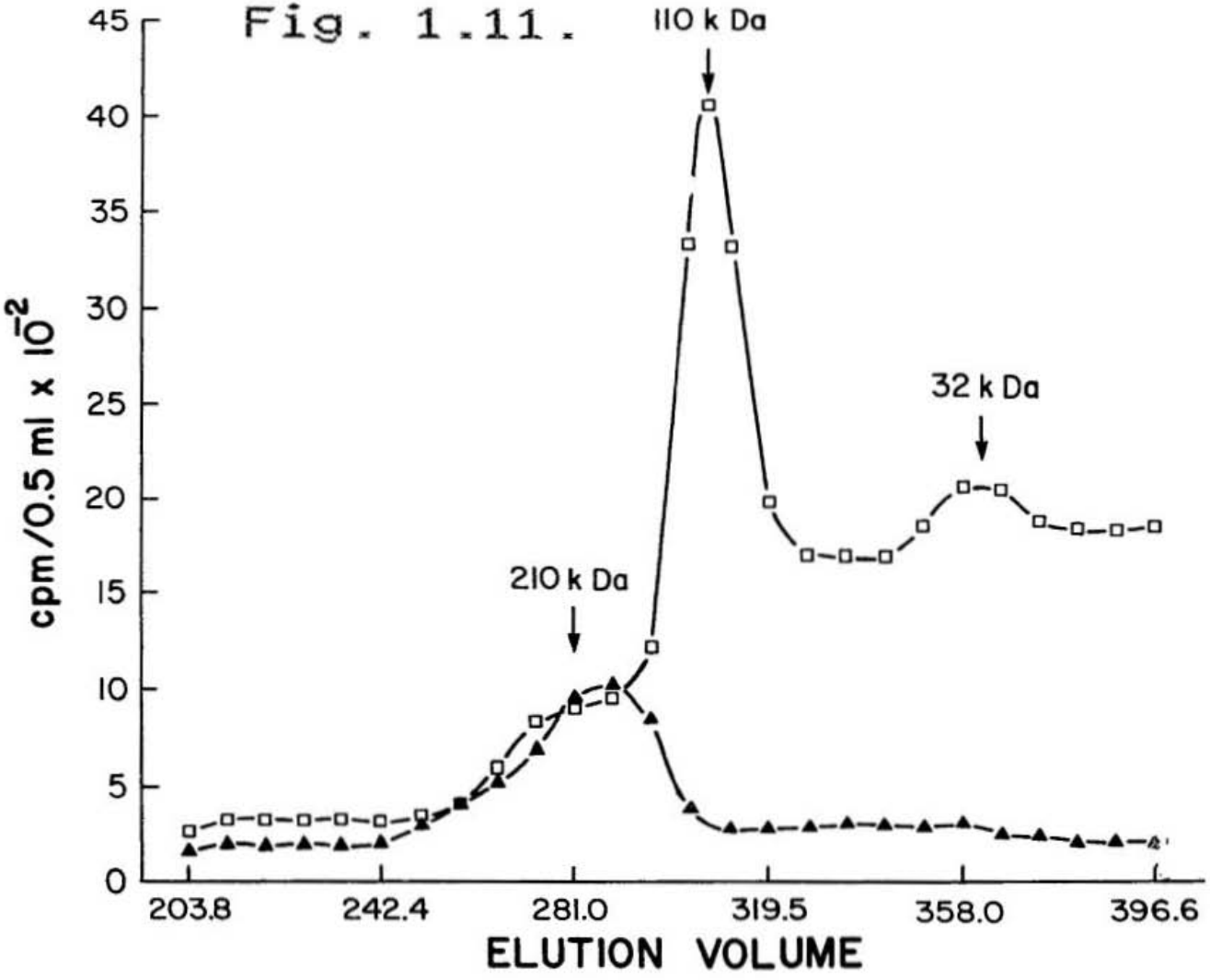
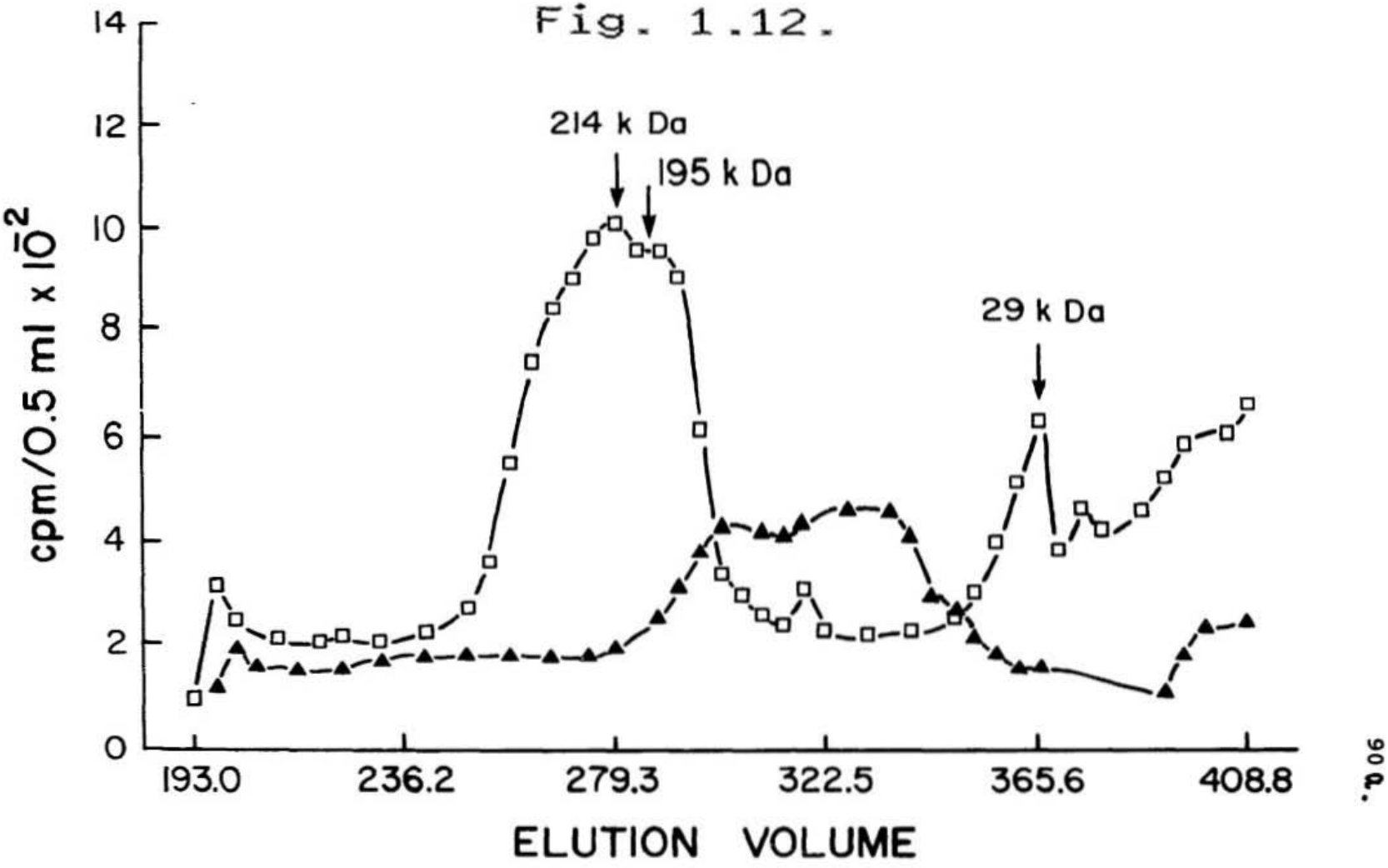

110 k Da
32 k Da
210 k Da
cpm/0.5 ml x 10^{-2}
45
40
35
30
25
20
15
10
5
0
203.8
242.4
281.0
319.5
358.0
396.6
ELUTION VOLUME

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.


214 k Da
195 k Da
29 k Da
cpm/0.5 ml x 10^{-2}
0
2
4
6
8
10
12
14
193.0
236.2
279.3
322.5
365.6
408.8
ELUTION VOLUME

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 [^{3}H]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 [^{3}H]progesterone prelabelled experiment two specific binding peaks were obtained at MW 263,000 and 132,000.

HPLC using Spherogel-TSK 3000SW column

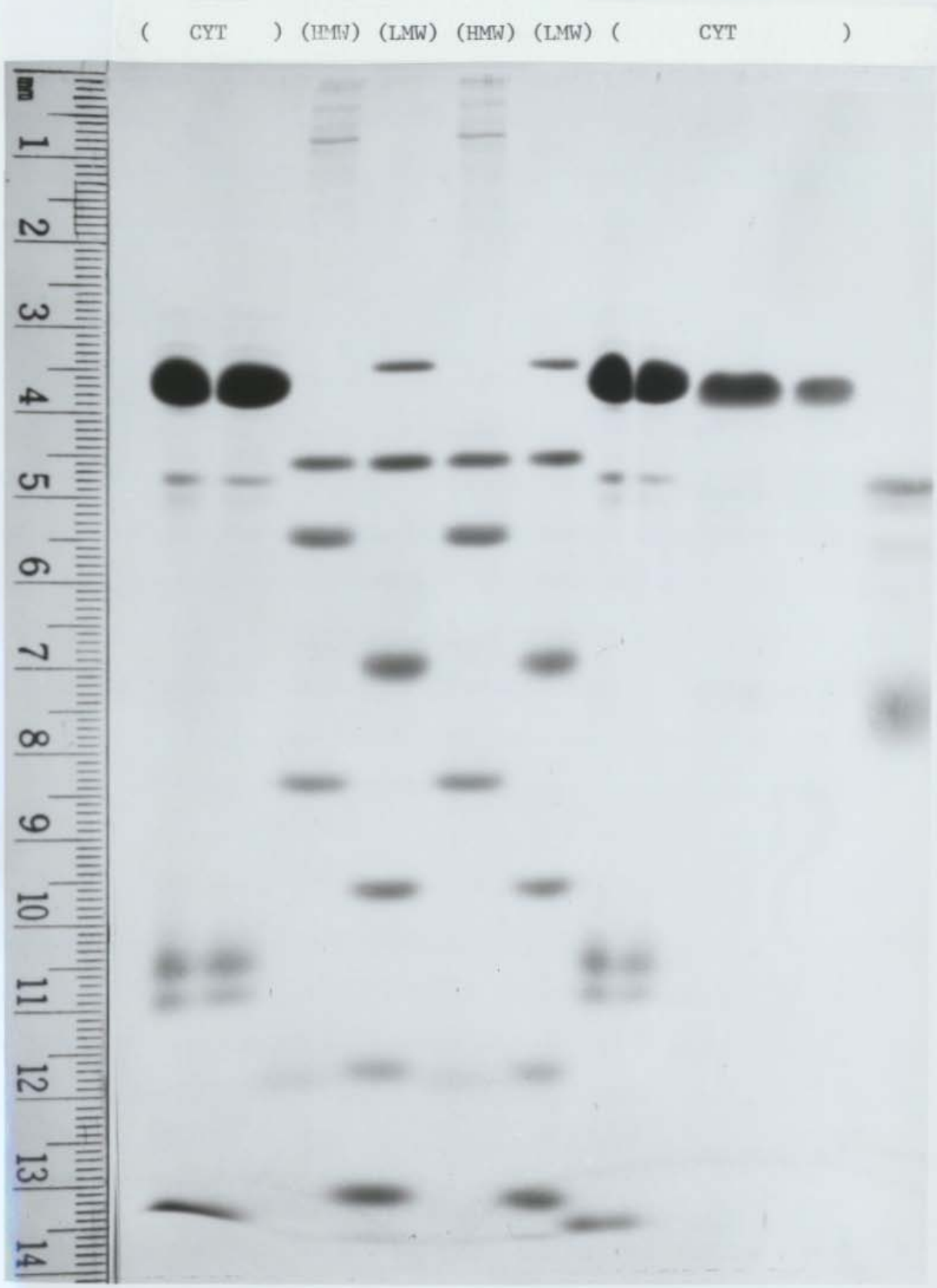
Pre-equilibration of brook trout cytosolic ovarian preparation with [^{3}H]progesterone gave specific binding peaks at MW 294,000, 233,000, 52,000, 34,000 and 21,000.

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.



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 17α,20β-DHP at this concentration. Testosterone and R5020 did not bring about maturation (Table 1.5). R5020 inhibited GVBD response due to 17α,20β-DHP by 10%. 17α,20β-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]17α,20β-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

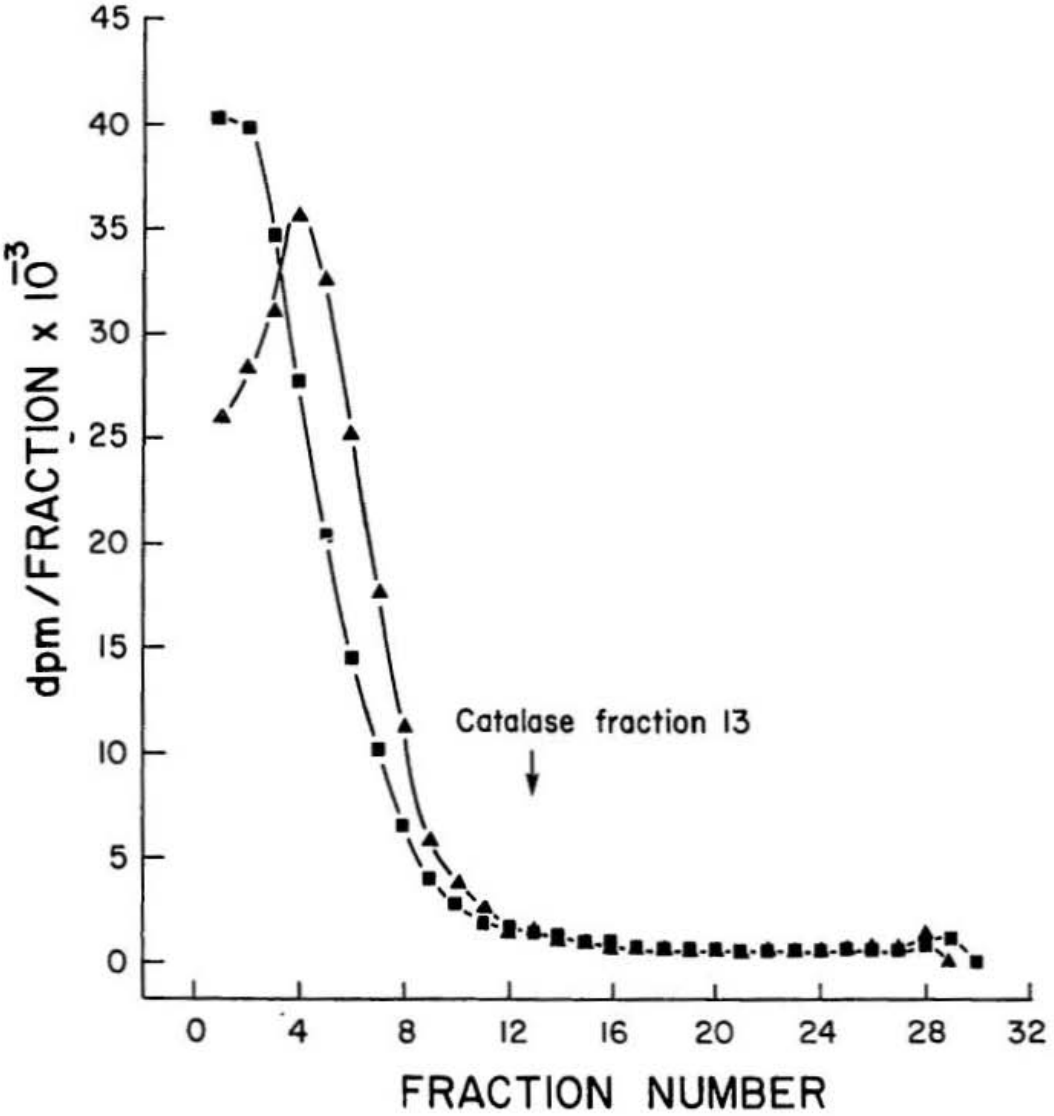
	n	Time to GVBD	GVBD (%)
17α,20β-DHP	3	44 - 48 hr	100%
17α,20β-DHP + testosterone	3	44 - 48 hr	100%
17α,20β-DHP + R5020	3	44 - 48 hr	90%
testosterone	3	no GVBD in 168 hr	Nil
R5020	3	no GVBD in 168 hr	Nil
no steroid control	3	no GVBD in 168 hr	Nil
testosterone + R5020	3	no GVBD in 168 hr	Nil
testosterone + 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 [^{3}H]17α,20β-DHP $\pm$ inert steroid and applied on to (5 - 30%) linear sucrose gradients total volume 11.6 ml (▲ sample + [^{3}H]17α,20β-DHP; ■ sample + [^{3}H]17α,20β-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.


45
40
35
30
25
20
15
10
5
0
dpm/FRACTION x 10^{-3}
Catalase fraction 13
0
4
8
12
16
20
24
28
32
FRACTION NUMBER

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 [^{3}H]17α,20β -DHP to cytosolic extracts using equilibrium dialysis (see text for experimental details).

Fig. 1.15.a. Saturation plot

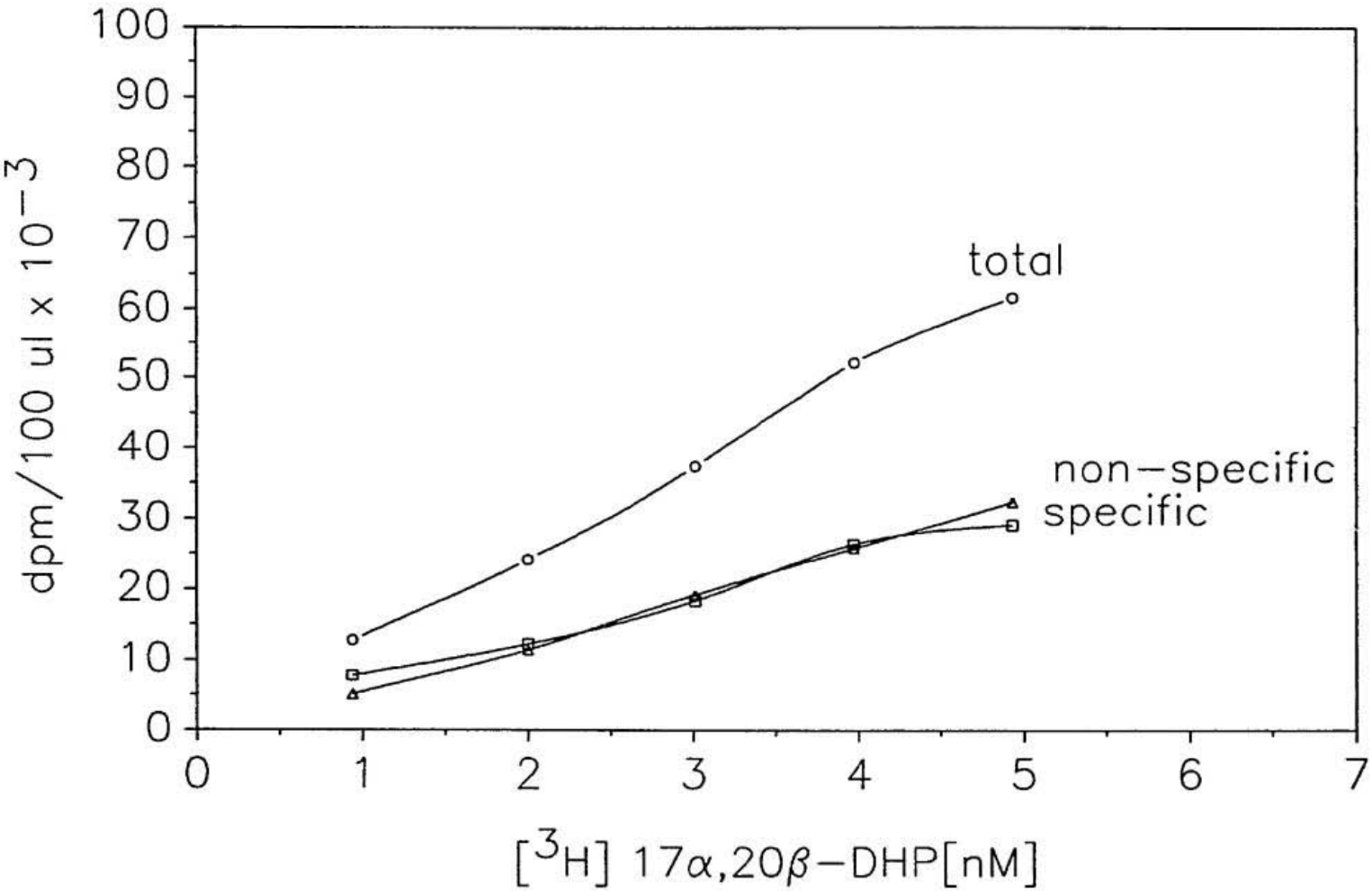
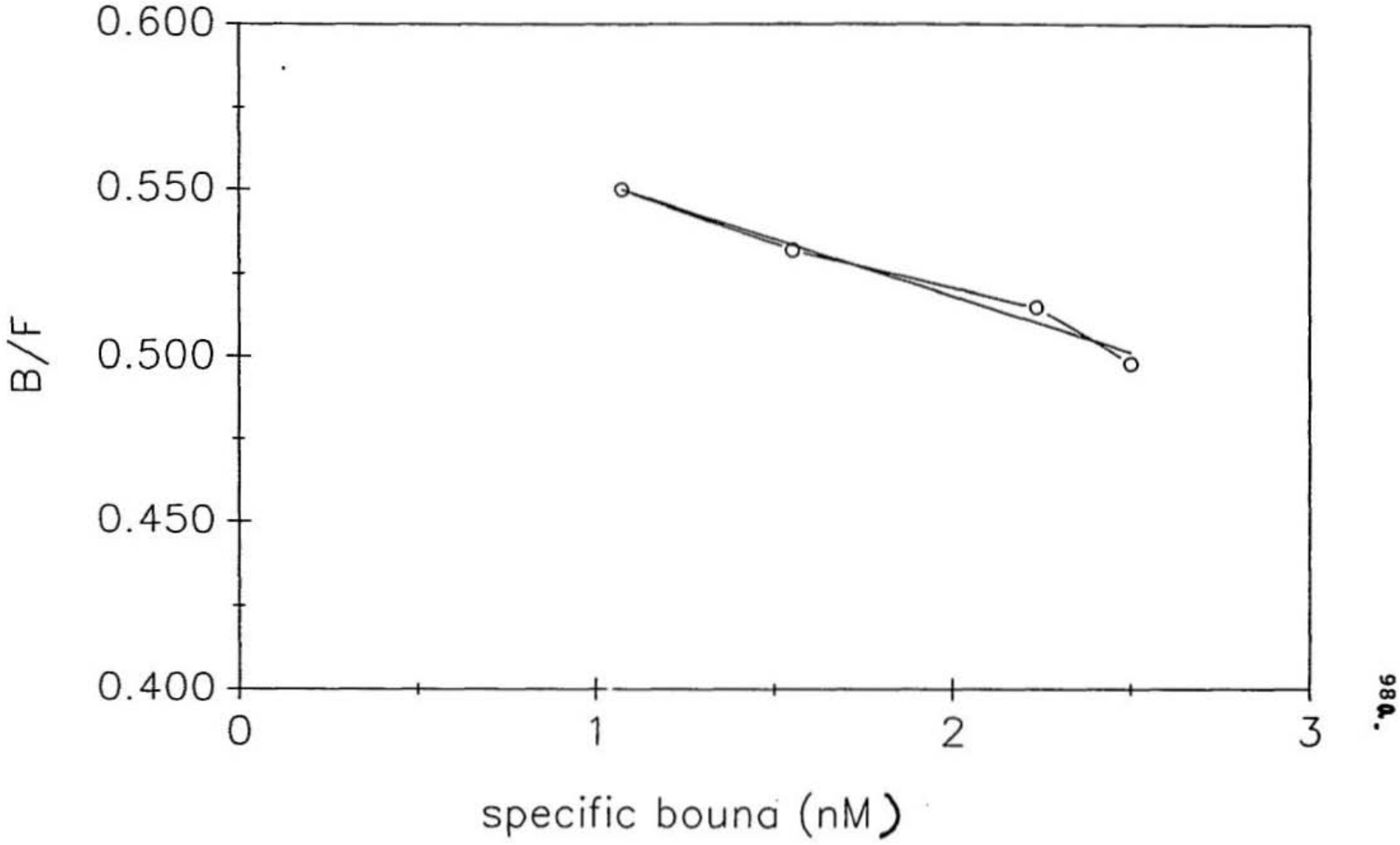


Fig. 1.15. (b). Linearization of specific binding by Scatchard analysis. The equilibrium association constant obtained from this graph was $0.34 \times 10^{-8} M^{-1}$, and the maximal concentration of binding sites was 992 f moles / mg protein.

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 17α,20β-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 [^{3}H]17α,20β-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 17α,20β-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 17α,20β-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 17α,20β-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 17α,20β-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 17α,20β-DHP did not suppress GVBD or maturation, since GVBD occurred within the same time period as with 17α,20β-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, 17α,20β,21-trihydroxy-4-pregnen-3-one; 3α,17α ,20β-trihydroxy-5-pregnane; 3β,17α,20β-trihydroxy-5 -pregnane; and 3α,17α,20β-,21-tetrahydroxy-5 -pregnane to be equipotent with 17α,20β-DHP. These results are in agreement with the non-specificity of the binding activity in the cytosolic preparations. The evidence for the presence of 17α,20β-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 17α,20β-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] 17α,20β-DHP receptor complex.

The classical somatic cell steroid receptors differ from brook trout 17α,20β-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 17α,20β-DHP receptors. The fast rate of dissociation indicates a different type of mechanism of action for the 17α,20β-DHP cytosolic receptors compared to the classical cytosolic steroid receptors.

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

The 17α,20β-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]17α,20β-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 discusssed in chapter 3. Although the precise biological action of the steroid-receptor complex remains to be elucidated.

Summary .

The presence of 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) oocyte receptor activity has been demonstrated in brook trout Salvelinus fontinalis . Scatchard analysis of the cytosolic fraction during various terminal stages of oocyte maturation gave a high equilibrium association constant (Ka) value of 1.39 ± 0.66 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 X 10^6 M^{-1}. The dissociation rate constant k_{-1} was 1.502 x 10^{-2} sec^{-1} for the first order dissociation reaction. The Ka = 1.52 x 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 > 17α-HP > 17α,20β-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 17α,20β-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

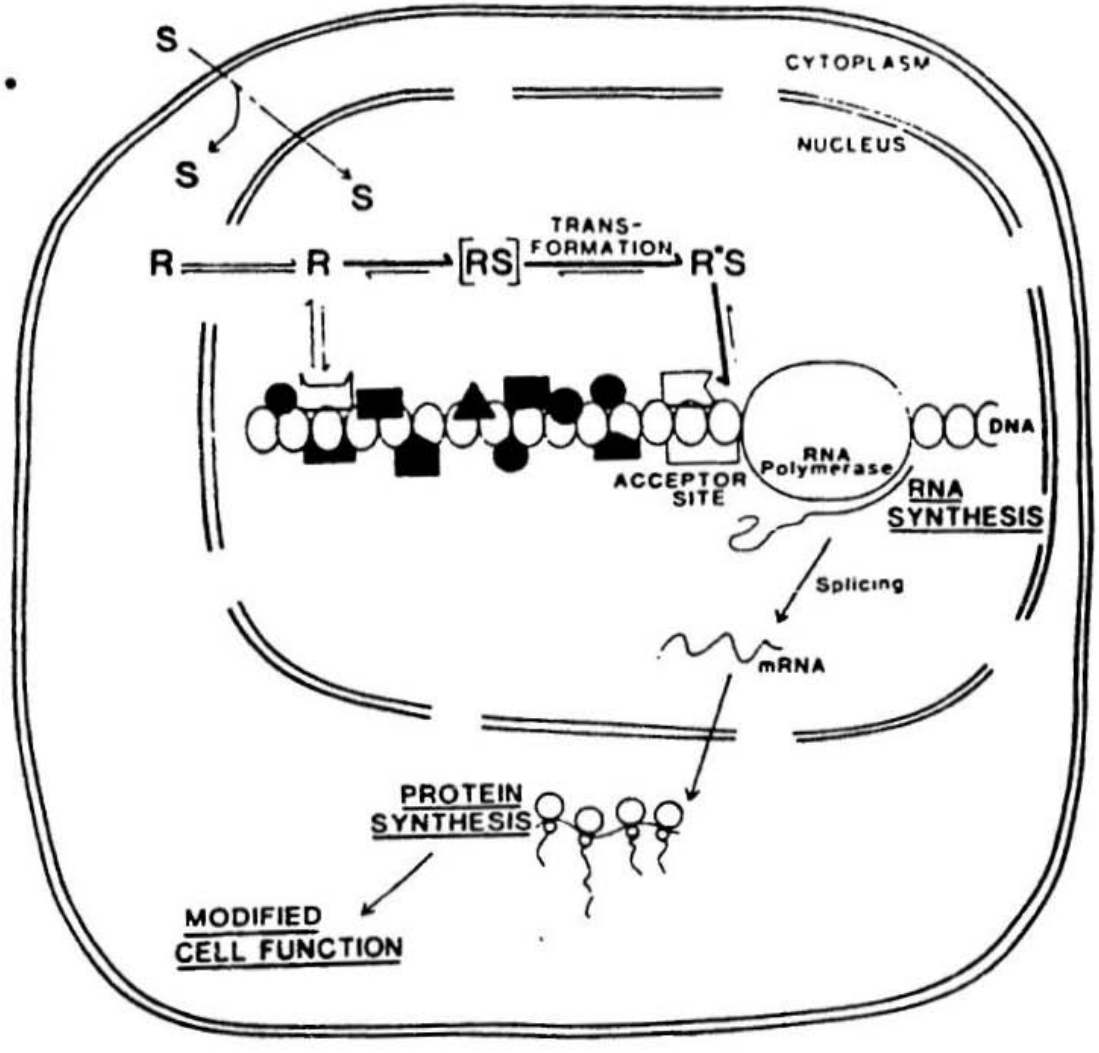
Hormone.	Tissue.	Reference.
Estrogens.	Squalus testis.	Callard and Mak, 1985.
	Breast cancer cells.	Garola and McGuire, 1977.
		Zava, et al . 1977.
		Geier, et al . 1982.
		Zava and McGuire, 1977.
		Panko and Macleod, 1978.
	Brain cells.	Sheridan, 1975.
		Meada, et al . 1983.
		Clark, et al . 1982.
	Chick oviduct.	Schimke, et al . 1975.
	Leydig tumor cells.	Meada, 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.


S
S
S
CYTOPLASM
NUCLEUS
R
R
[RS]
TRANS-
FORMATION
R*S
DNA
RNA
Polymerase
ACCEPTOR
SITE
RNA
SYNTHESIS
Splicing
mRNA
PROTEIN
SYNTHESIS
MODIFIED
CELL FUNCTION

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 follical 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 17α,20β-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 17α,20β-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 [^{3}H] standards efficiency curves were generated at least every 30 days. [^{3}H] 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 [^{3}H] 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.

DNA cellulose column chromatography

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 [^{3}H]17α,20β-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 $Mg(CH_3COO)_2$, 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

[^{3}H]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 [^{3}H]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 [^{3}H]R5020-nuclear

receptors from free [^{3}H]R5020 as described. The supernatants were aspirated, and the pelleted nuclei containing bound [^{3}H]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 [^{3}H]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 [^{3}H]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 17α,20β-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

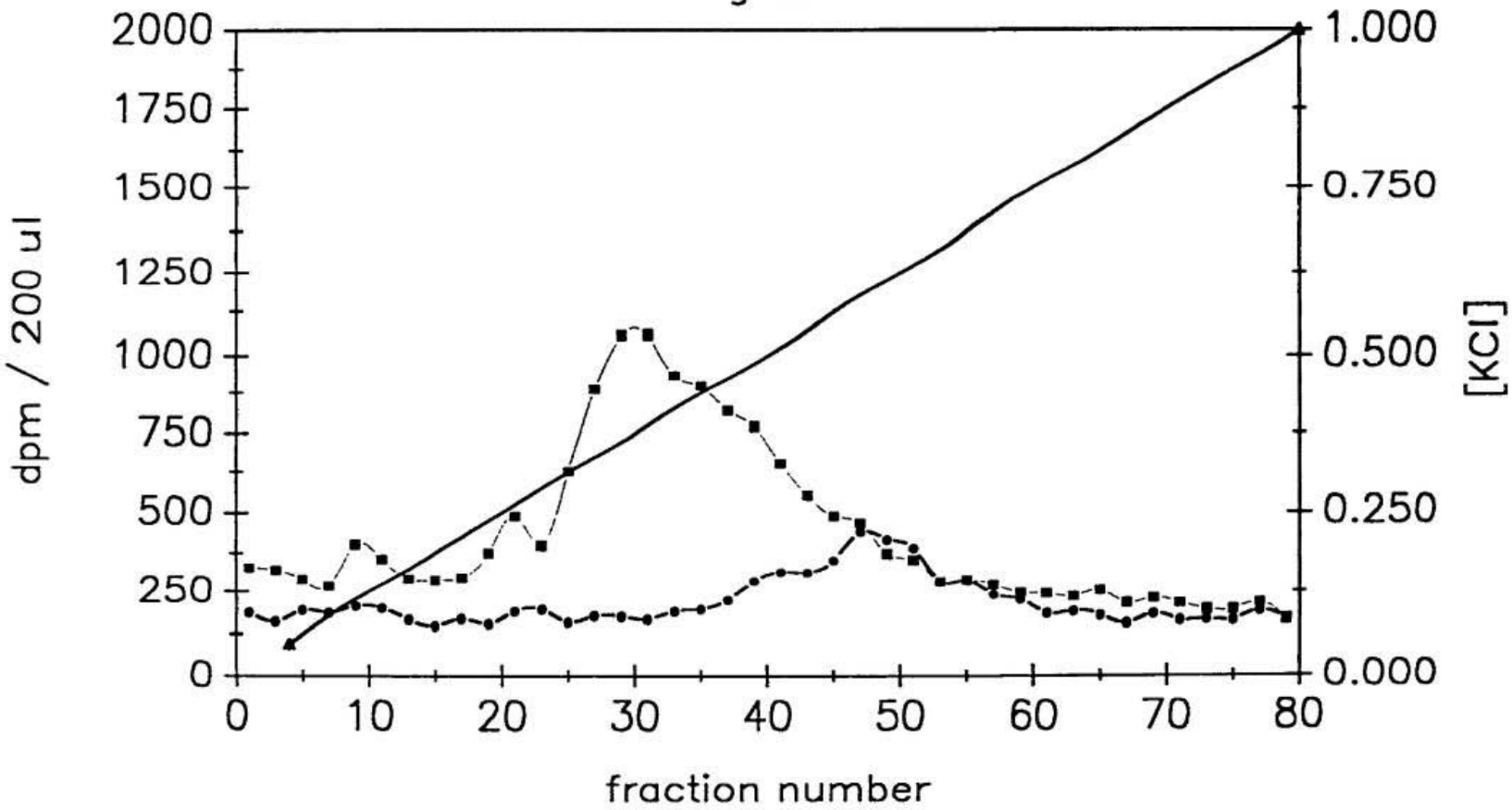

2000
1750
1500
1250
1000
750
500
250
0
dpm / 200 ul
1.000
0.750
0.500
0.250
0.000
[KCl]
0
10
20
30
40
50
60
70
80
fraction number

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

Fig. 2.3

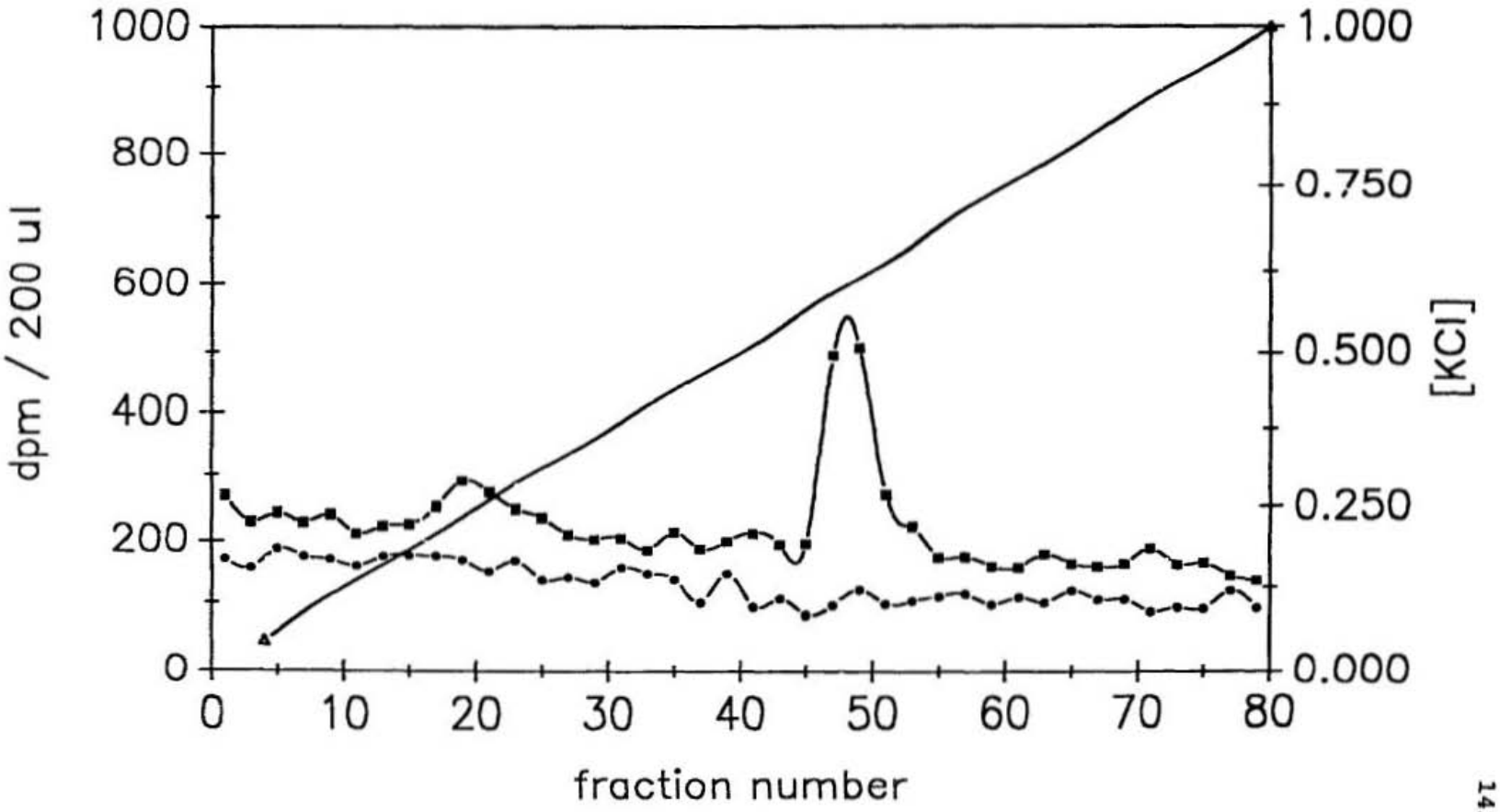

1000
800
600
400
200
0
dpm / 200 ul
1.000
0.750
0.500
0.250
0.000
[KCl]
0
10
20
30
40
50
60
70
80
fraction number

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

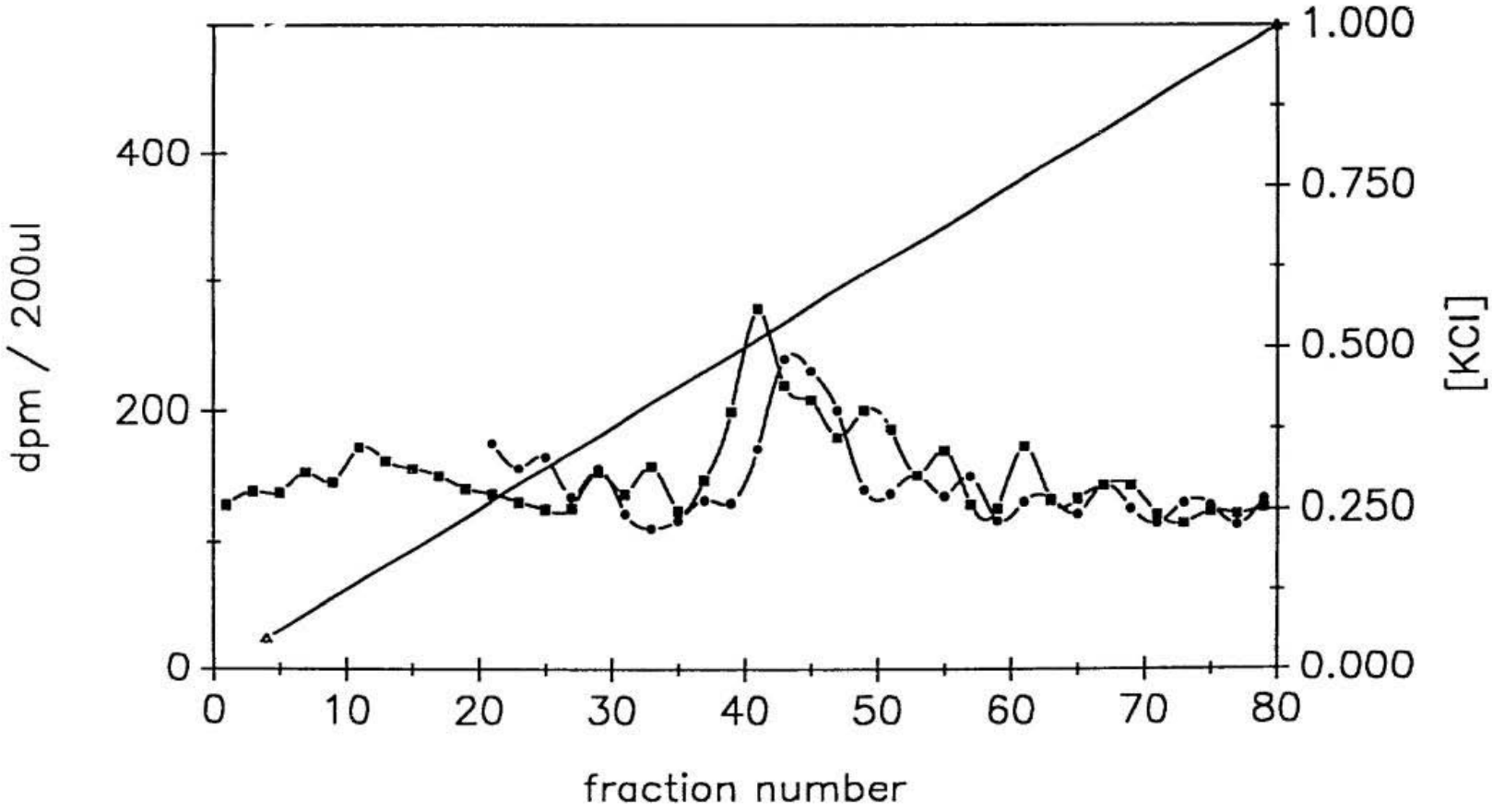
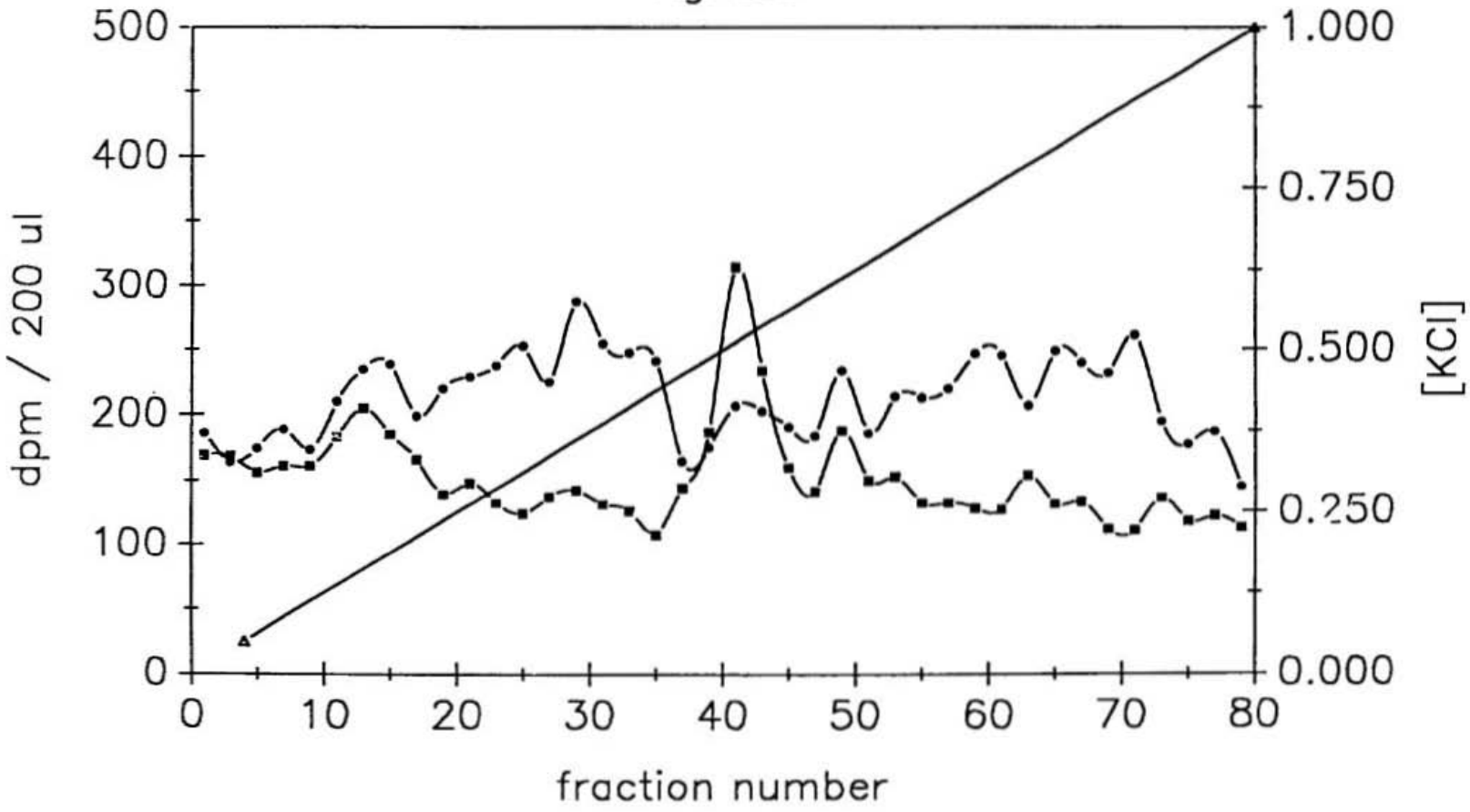

dpm / 200ul
400
200
0
1.000
0.750
0.500
0.250
0.000
[KCl]
0
10
20
30
40
50
60
70
80
fraction number

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

Fig. 2.5


500
400
300
200
100
0
dpm / 200 ul
1.000
0.750
0.500
0.250
0.000
[KCl]
0
10
20
30
40
50
60
70
80
fraction number

144 a.

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.

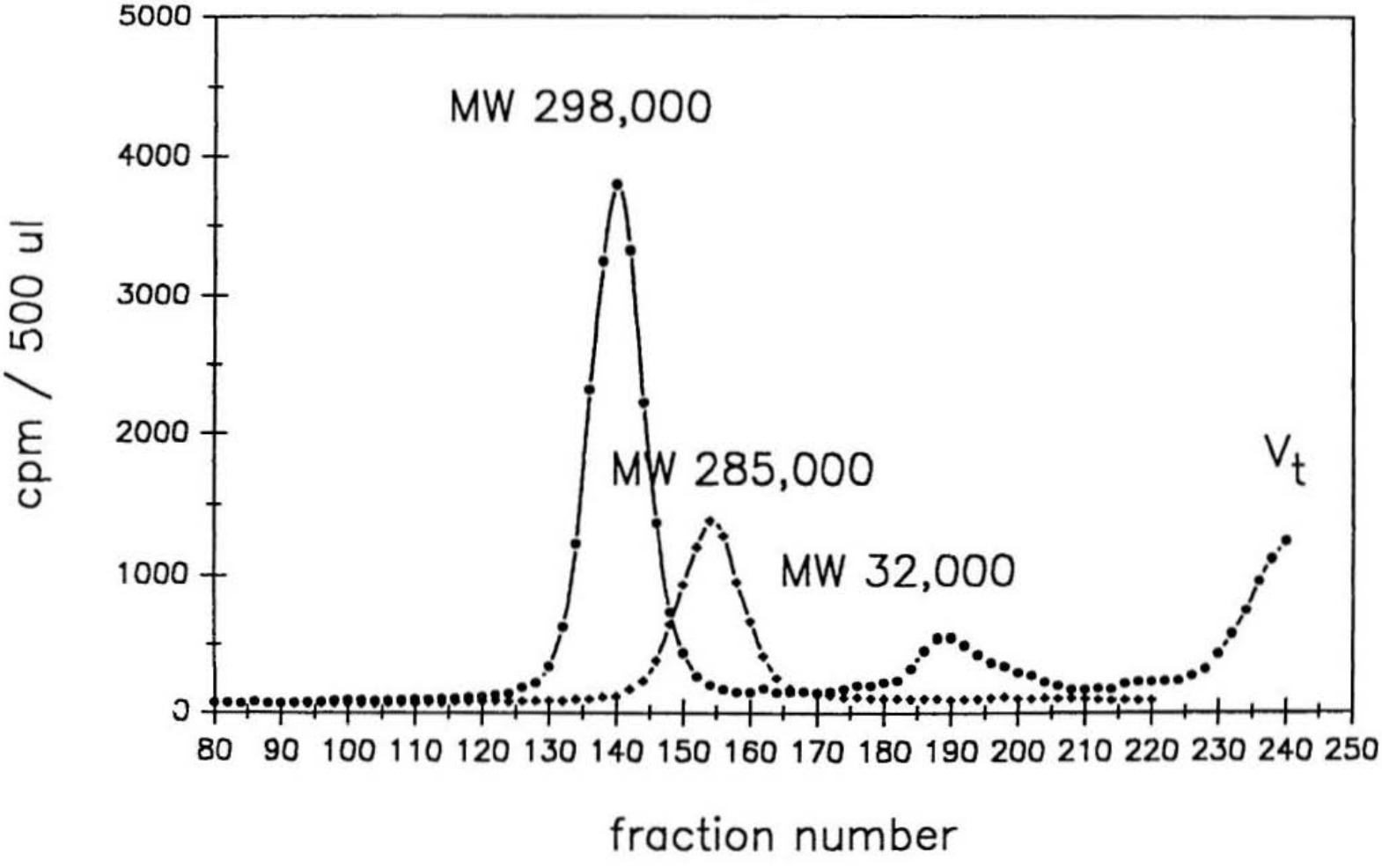
Very little binding to the nuclear material was observed in either the sample or the denatured receptor steroid complex control, ca. 2000 dpm/ 10^{6} erythrocyte nuclei. This indicates that a small amount of non-specific binding occurred in the nuclei.

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


5000
4000
3000
2000
1000
0
cpm / 500 ul
MW 298,000
MW 285,000
MW 32,000
V_t
80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250
fraction number

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 [^{3}H]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 [^{3}H]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.

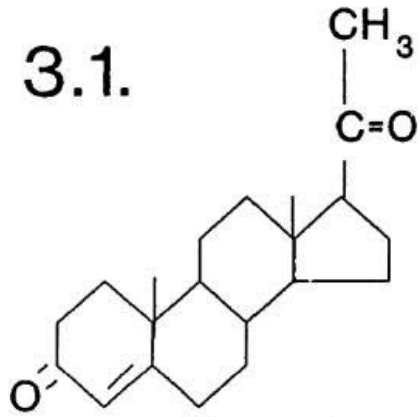
Plasma membrane bound 17α,20β-DHP receptors in brook trout oocytes

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.

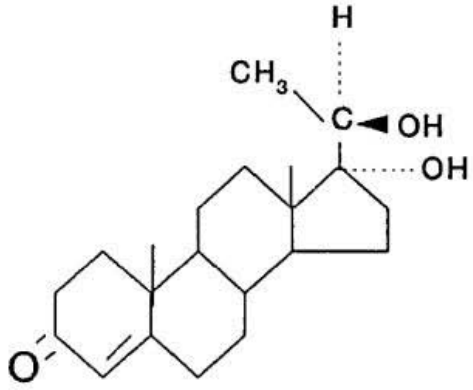
In Xenopus laevis oocytes progesterone binds to an oocyte membrane receptor protein (Sadler, et al . 1985; Blondeau and Baulieu, 1984; Sadler and Maller, 1982; Ishikawa et al. 1977). The possibility that a membrane receptor for the salmonoid progestin, 17α,20β-DHP may also exist was investigated. Nagahama and Kishimoto (1987), have demonstrated that micro injection of 17α,20β-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 17α,20β-DHP may be via membrane-bound receptors. Supportive evidence for a membrane-bound receptor was demonstrated by findings that physiological response (GVBD) to 17α,20β-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 17α,20β-DHP is different from the mechanism of classical steroids

Fig. 3.1.



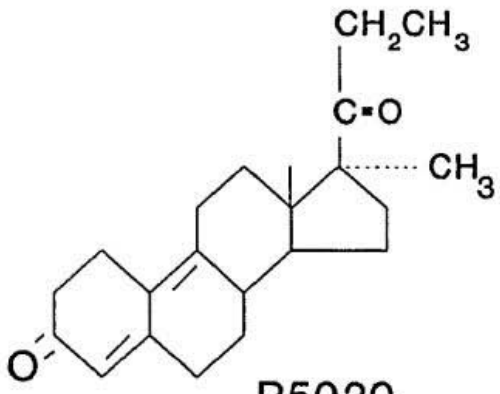
Progesterone

4-Pregnen-3,20-dione



17α,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 17α,20β-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 17α,20β-DHP as the ligand. Although the cytosolic preparation from previously frozen stage 6-7 oocytes demonstrated small amounts of binding activity with labelled 17α,20β-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 <--hν--> 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 labellled 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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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 labellled R5020.

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

glass tube containing vacuum evaporated [^{3}H]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 [^{3}H]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 [^{3}H]R5020 (5nM). The control membranes were not photoaffinity labelled but treated similarly with [^{3}H]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 were not photoaffinity labelled but treated with [^{3}H]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 radioactivity 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 [^{3}H]R5020 to the receptor protein. This binding was saturable with respect to time (Fig. 3.5). [^{3}H]R5020 photoaffinity labelling to the cytosolic receptor preparation is saturable below 5 nM [^{3}H]R5020, but above this concentration cooperativity in binding is observed (Fig. 3.6). This increased binding above 5nM [^{3}H]R5020 is also associated with a decrease in molecular weight of the receptor complex (see gel filtration chromatography below). [^{3}H]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.

Fig. 3.2.

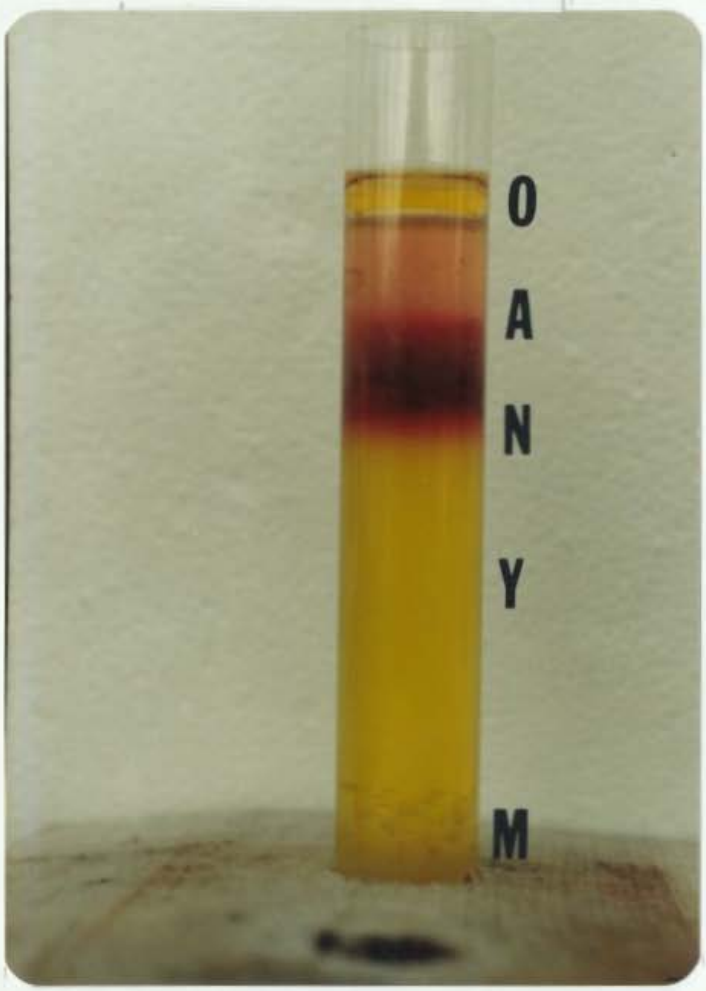


Fig. 3.3. Zona radiata membranes obtained from brook trout stage 6-7 oocytes, washed and then suspended in TETS buffer.

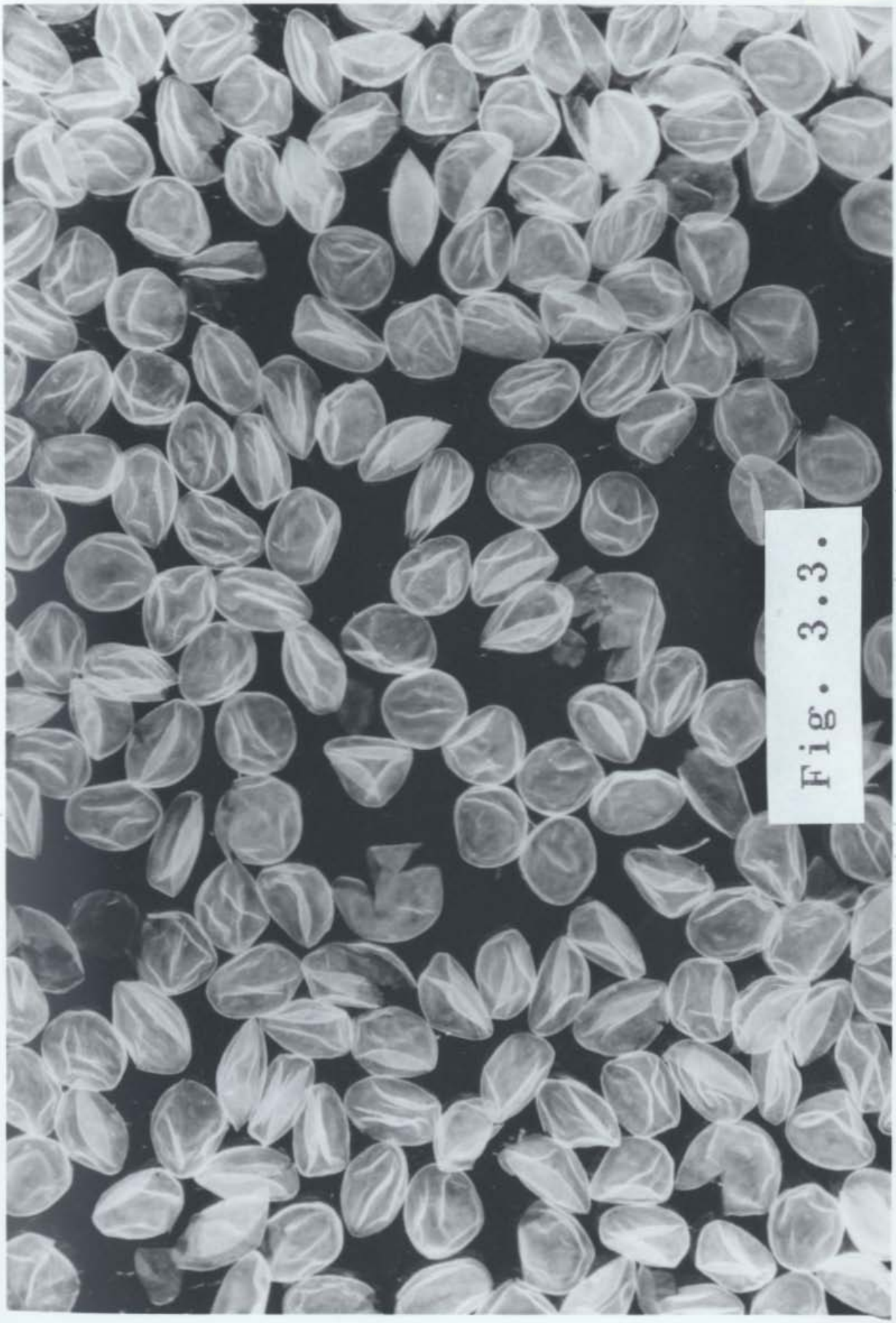


Fig. 3.3.

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.

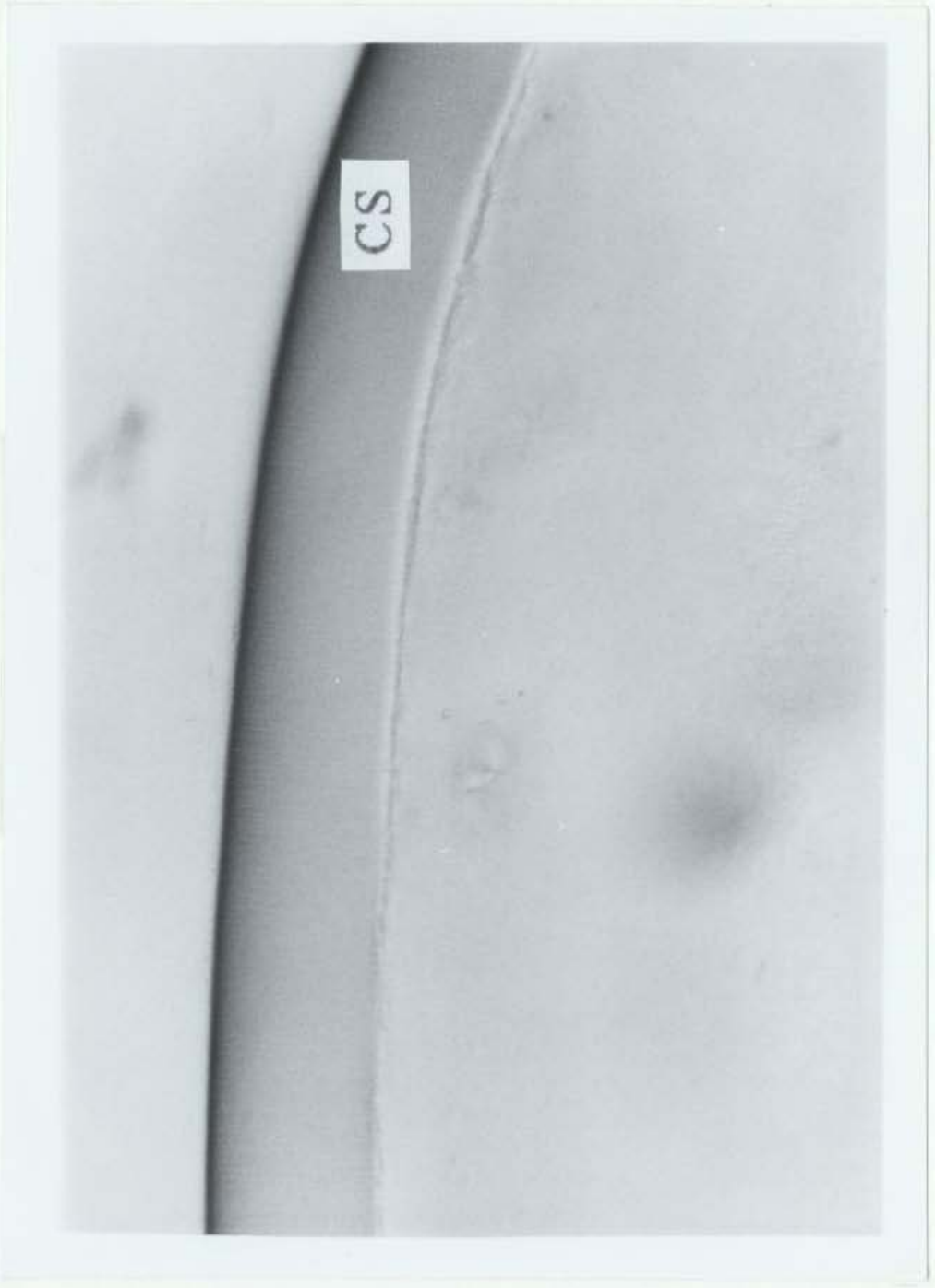


Fig. 3.4.

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

Fig. 3.5.

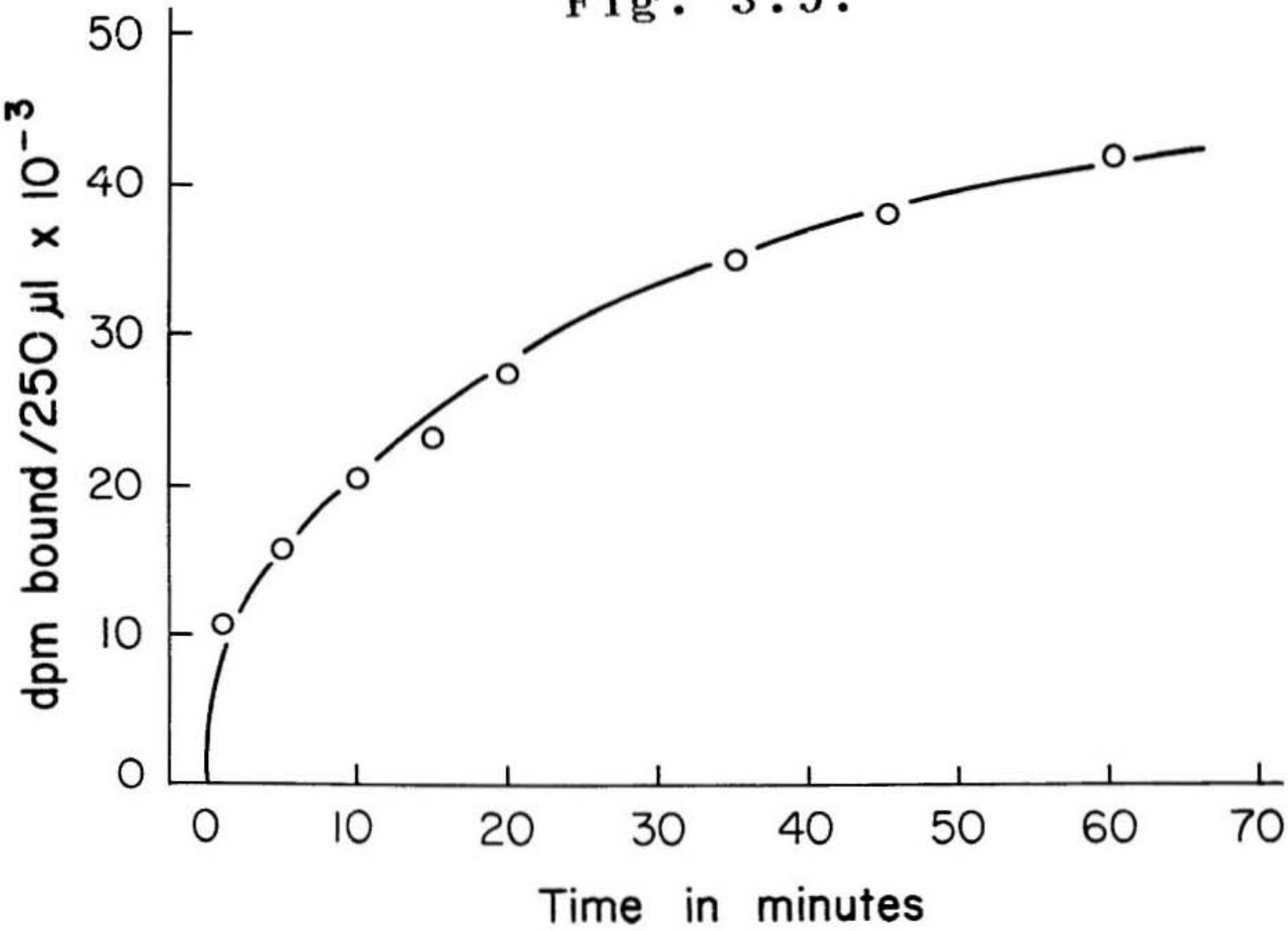

dpm bound /250 µl x 10^{-3}
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Time in minutes

Fig. 3.6. Photoaffinity labelling (2 min) of cytosolic receptor protein with [^{3}H]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.

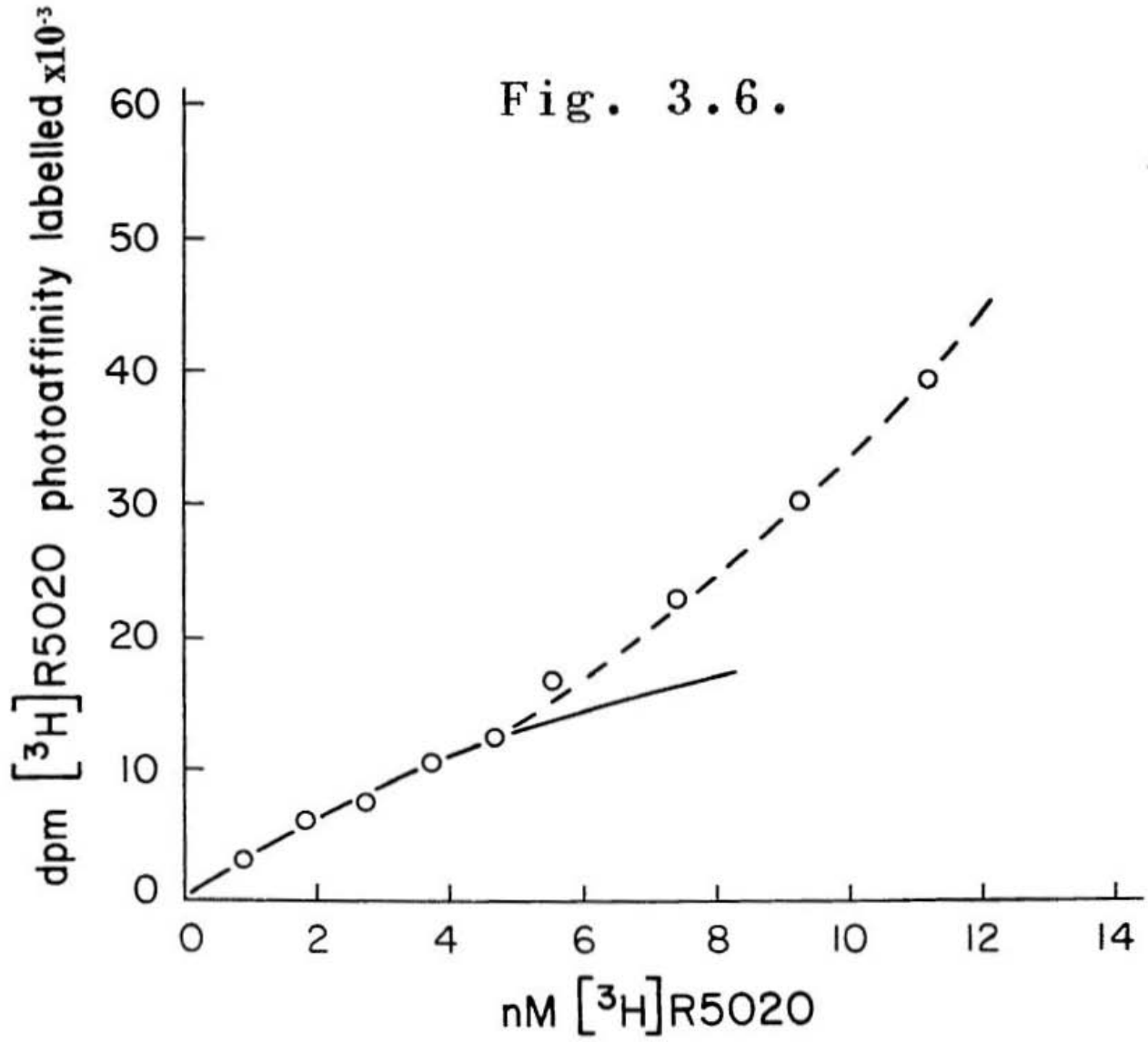

dpm [3H]R5020 photoaffinity labelled x10-3
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
nM [3H]R5020

Fig. 3.7. Inhibition of photoaffinity labelling of cytosolic receptors using inert 17α,20β-DHP (3μM) 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.

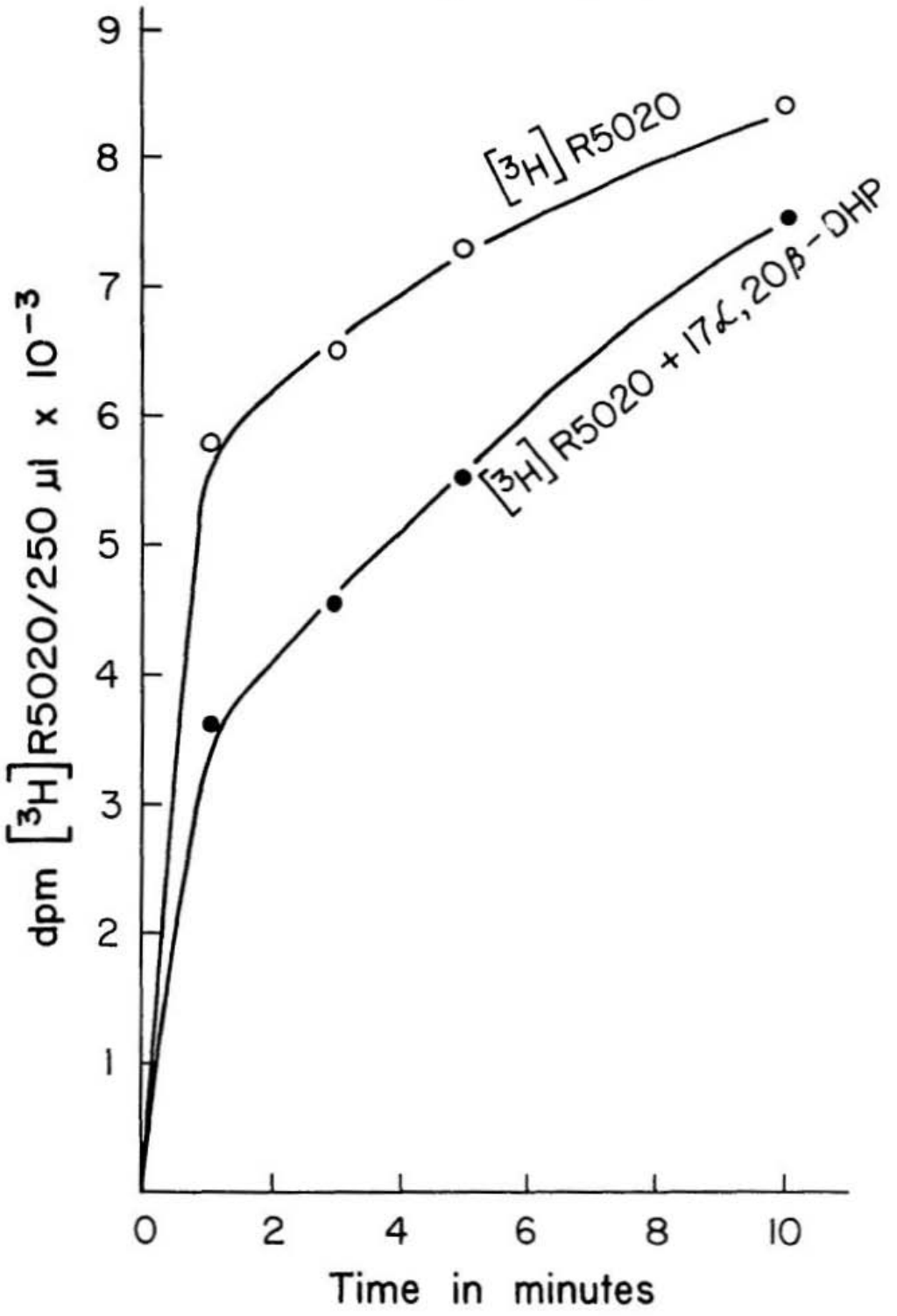
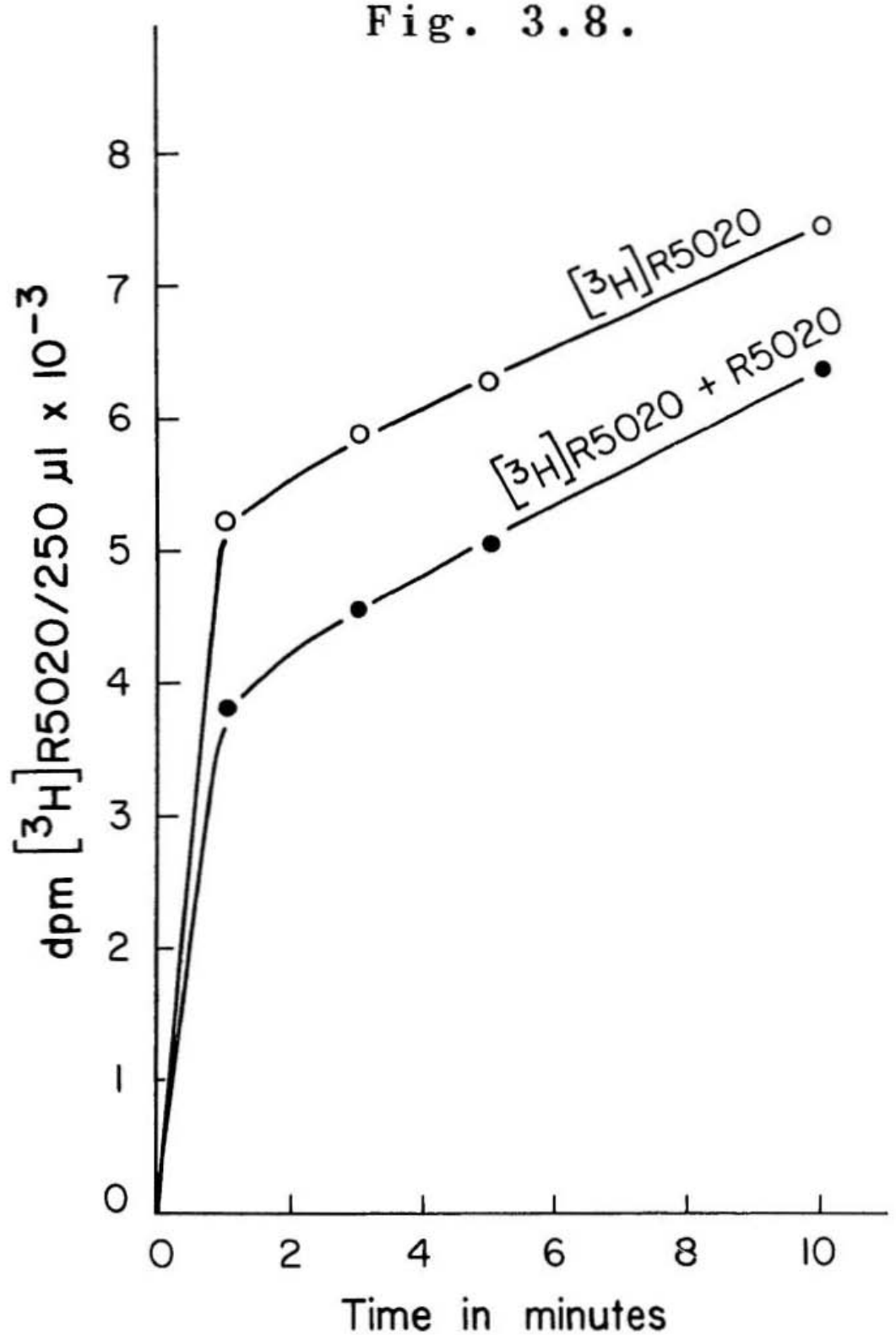

dpm [3H]R5020/250 µl x 10-3
9
8
7
6
5
4
3
2
1
0
2
4
6
8
10
Time in minutes
[3H] R5020
[3H] R5020 + 17α,20β-DHP

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.


dpm [3H]R5020/250 µl x 10-3
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
Time in minutes
[3H]R5020
[3H]R5020 + R5020

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

Demonstration of photoaffinity labelled receptors in zona radiata membranes . In brook trout stage 6-7 zona radiata isolated membranes the photoaffinity binding per gram of membranes was 139,000 ± 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 17α,20β-DHP concentration on the initial rate of photoaffinity labelling with [^{3}H]R5020. Time of photoaffinity labelling was 30 sec.

Fig. 3.9.

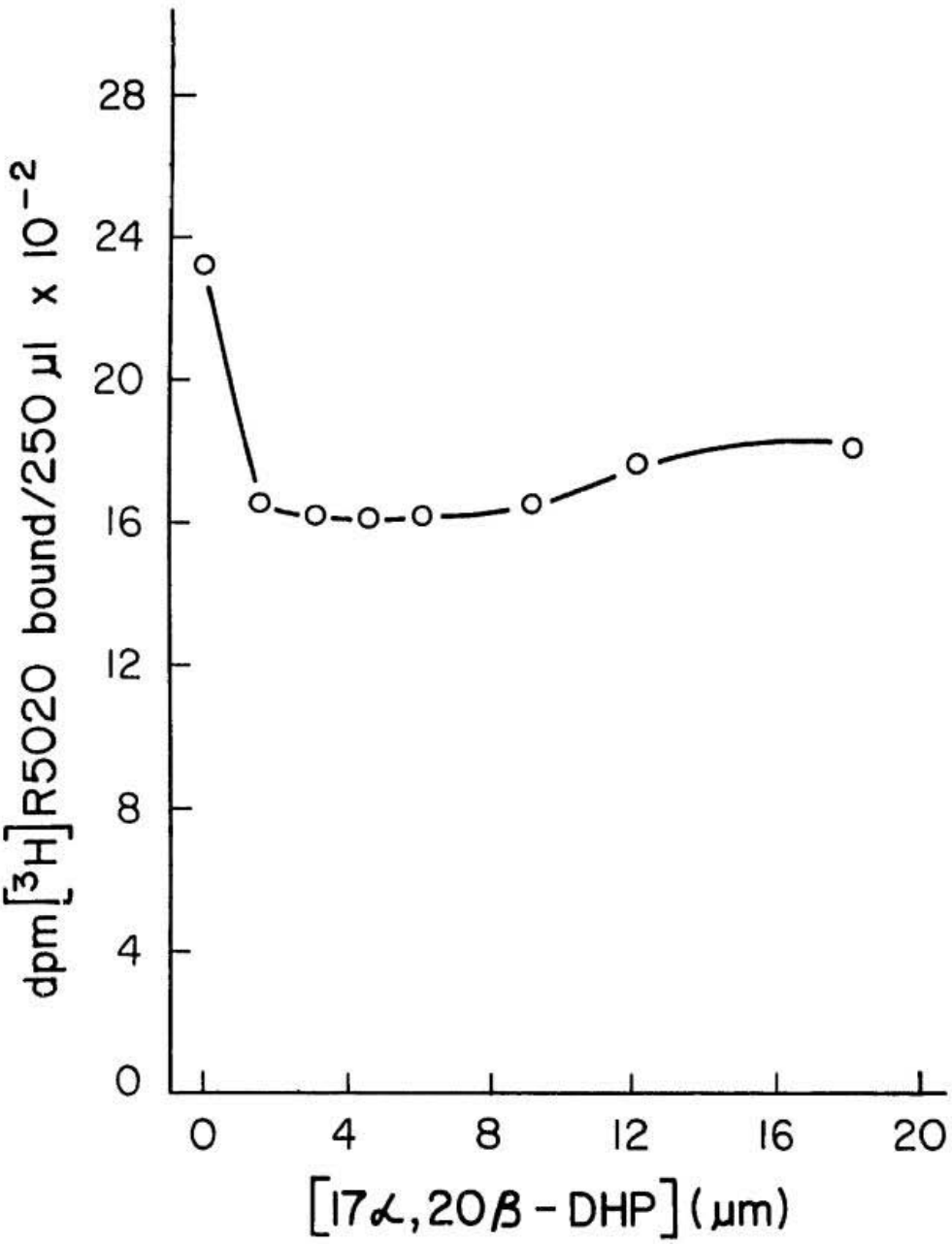

dpm[3H]R5020 bound/250 µl x 10-2
28
24
20
16
12
8
4
0
0
4
8
12
16
20
[17α,20β - DHP] (µm)

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.

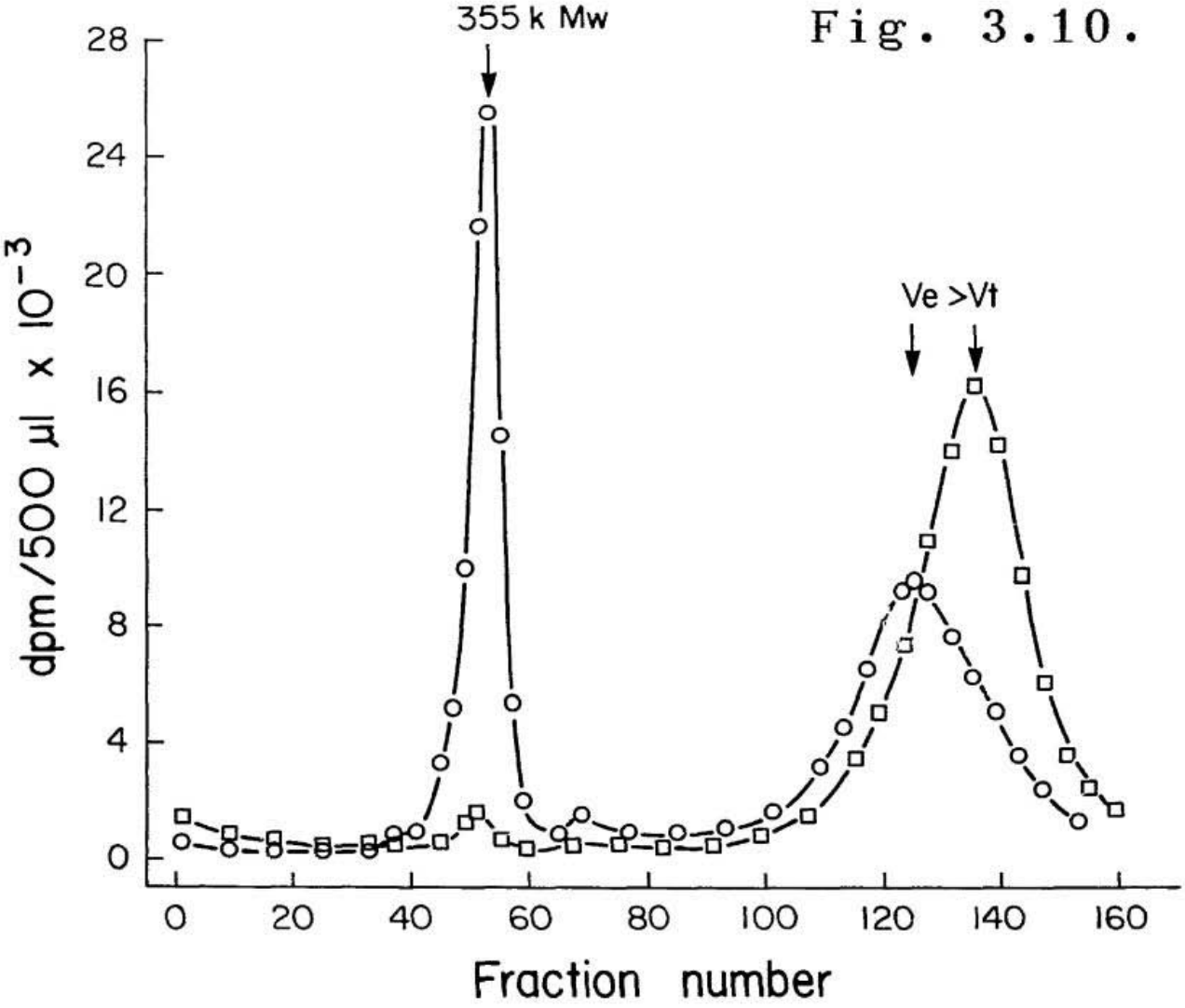

355 k Mw
Ve >Vt
dpm/500 µl x 10^{-3}
28
24
20
16
12
8
4
0
0
20
40
60
80
100
120
140
160
Fraction number

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.

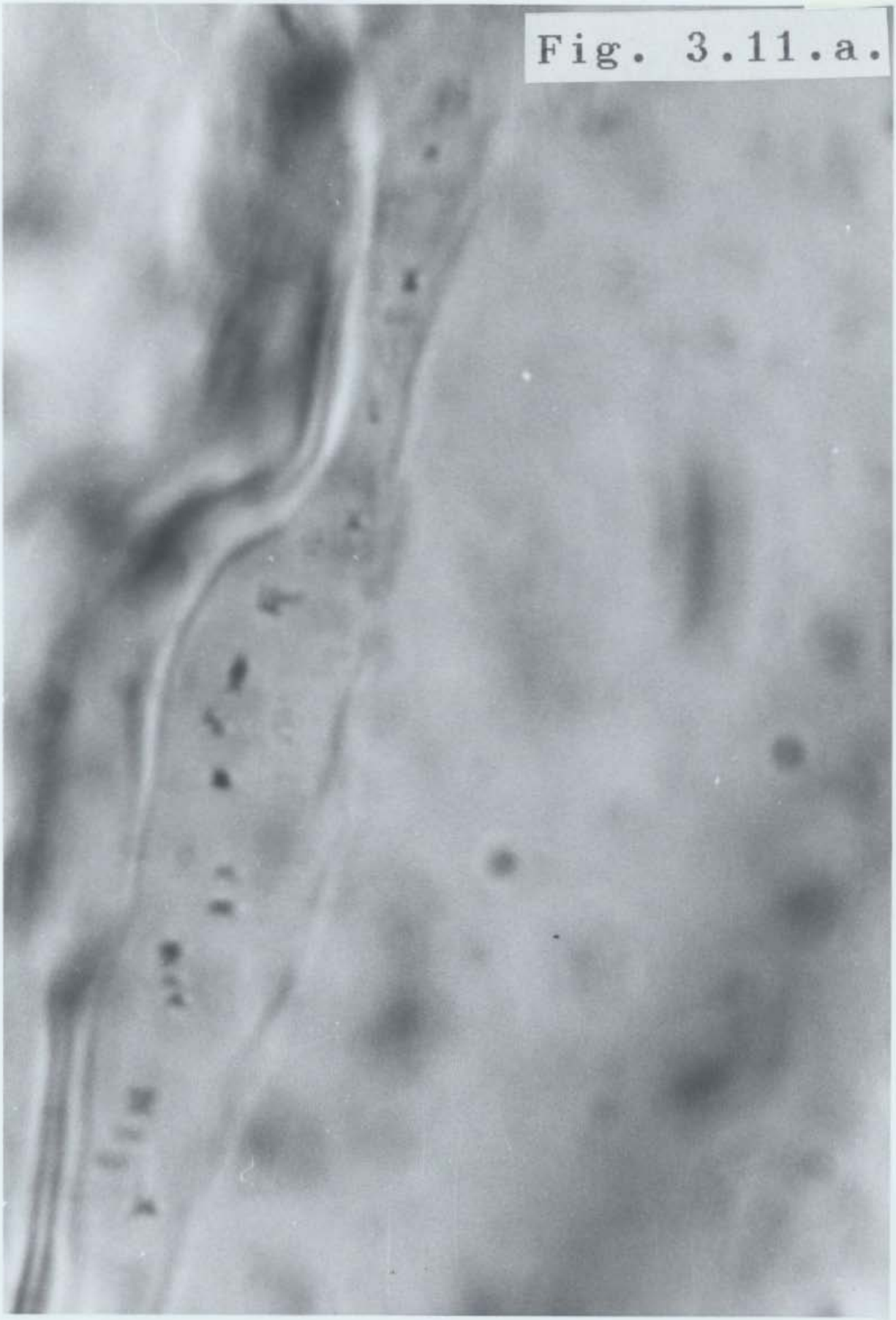


Fig. 3.11.a.

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.

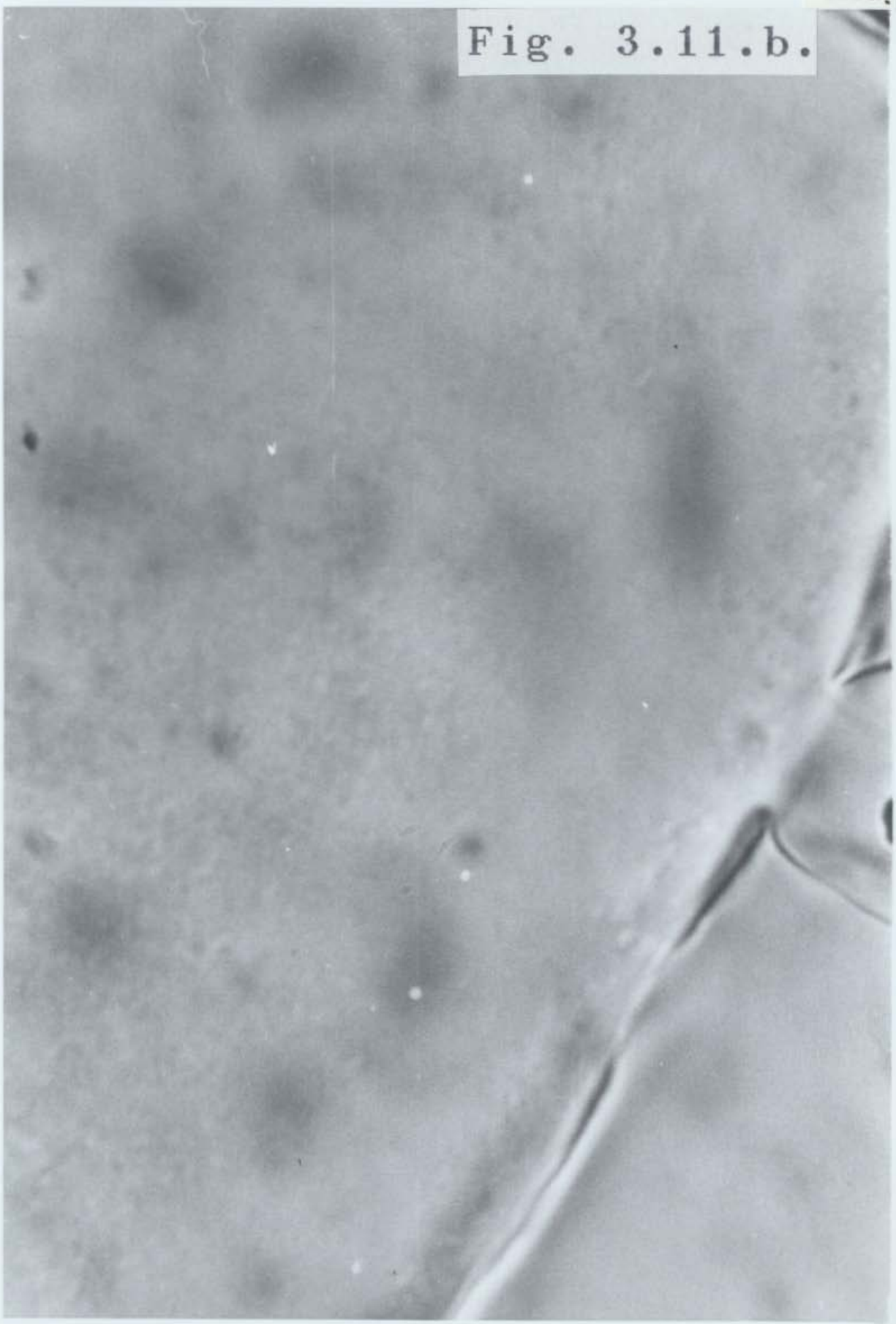


Fig. 3.11.b.

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

Sample *	Ka (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 Polyron 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

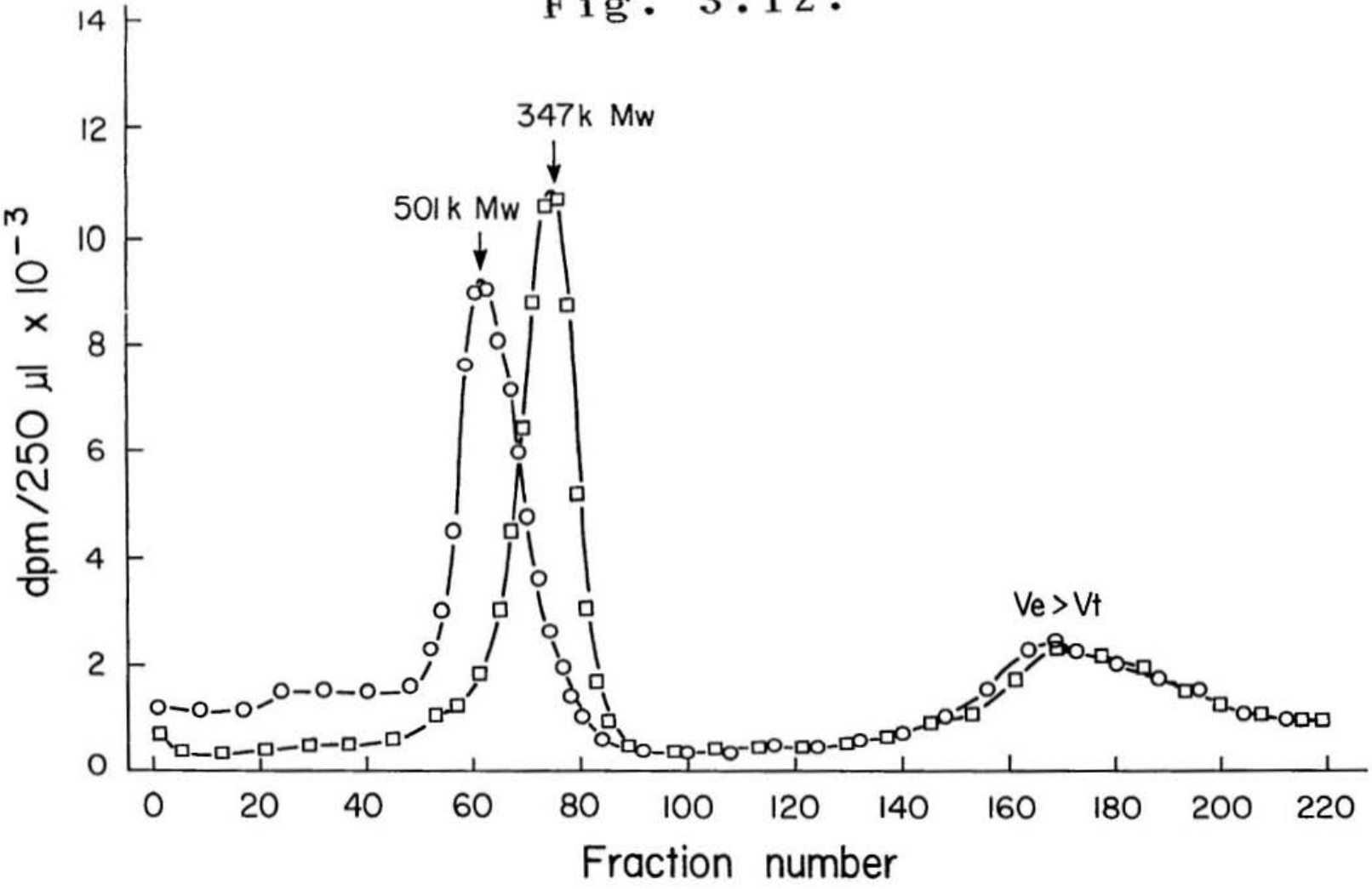
Ka - association constant

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.


347k Mw
501k Mw
Ve > Vt
dpm/250 µl x 10⁻³
Fraction number
0
2
4
6
8
10
12
14
0
20
40
60
80
100
120
140
160
180
200
220

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).

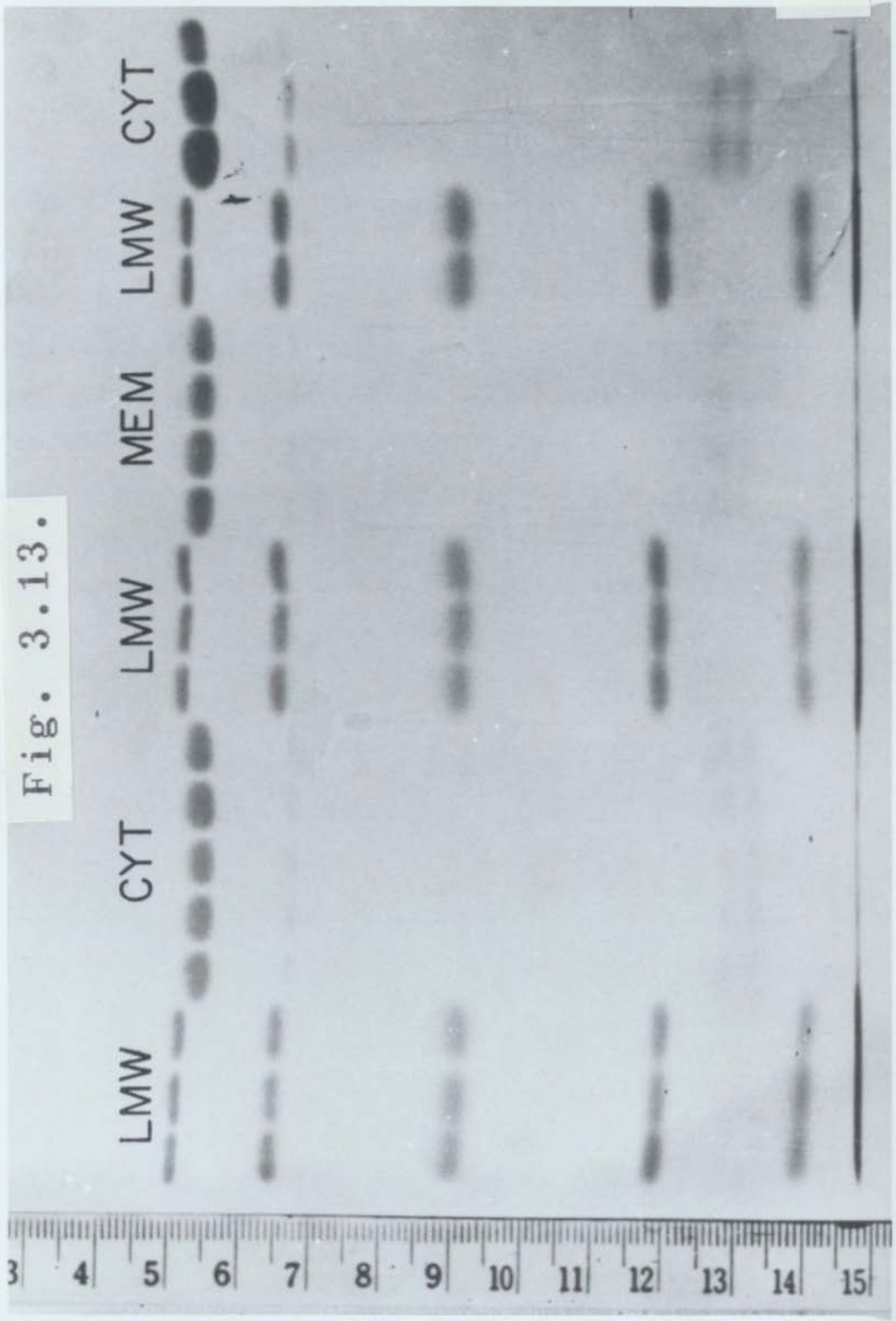


Fig. 3.13.

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).

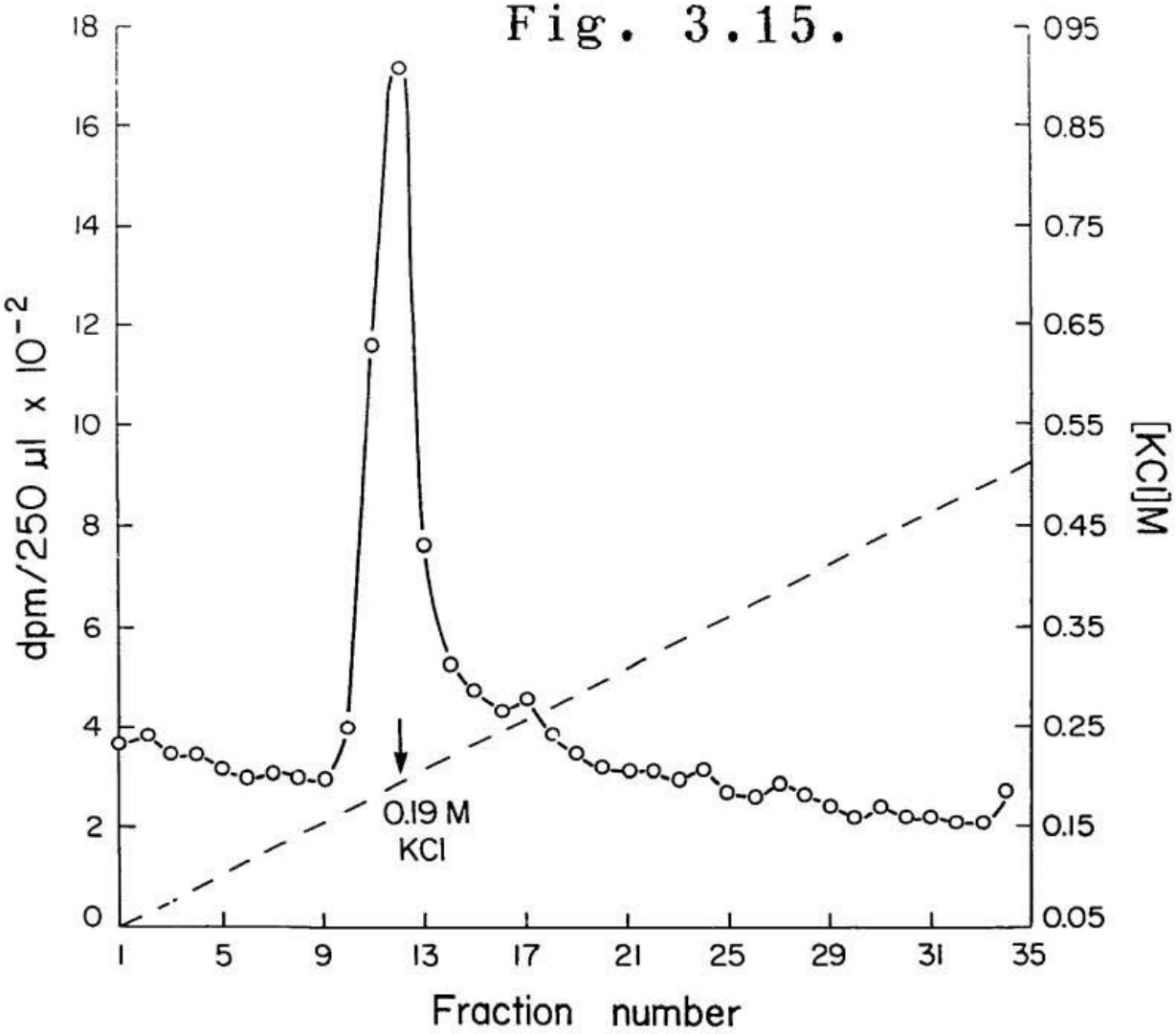
Ion exchange column chromatography of photoaffinity labelled cytosolic receptor preparation

a) Hydroxyapatite column chromatography. The [^{3}H]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 peristatic pump was used in conjunction with a linear gradient. [^{3}H]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 [^{3}H]R5020 is photoaffinity labelled mainly on the 84,000 MW subunit of the receptor protein.

b) DEAE Sephacel column chromatography. [^{3}H]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. [^{3}H]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.


dpm/250 µl x 10⁻²
[KCl]M
Fraction number
0.19 M
KCl
0
2
4
6
8
10
12
14
16
18
0.05
0.15
0.25
0.35
0.45
0.55
0.65
0.75
0.85
0.95
1
5
9
13
17
21
25
29
31
35

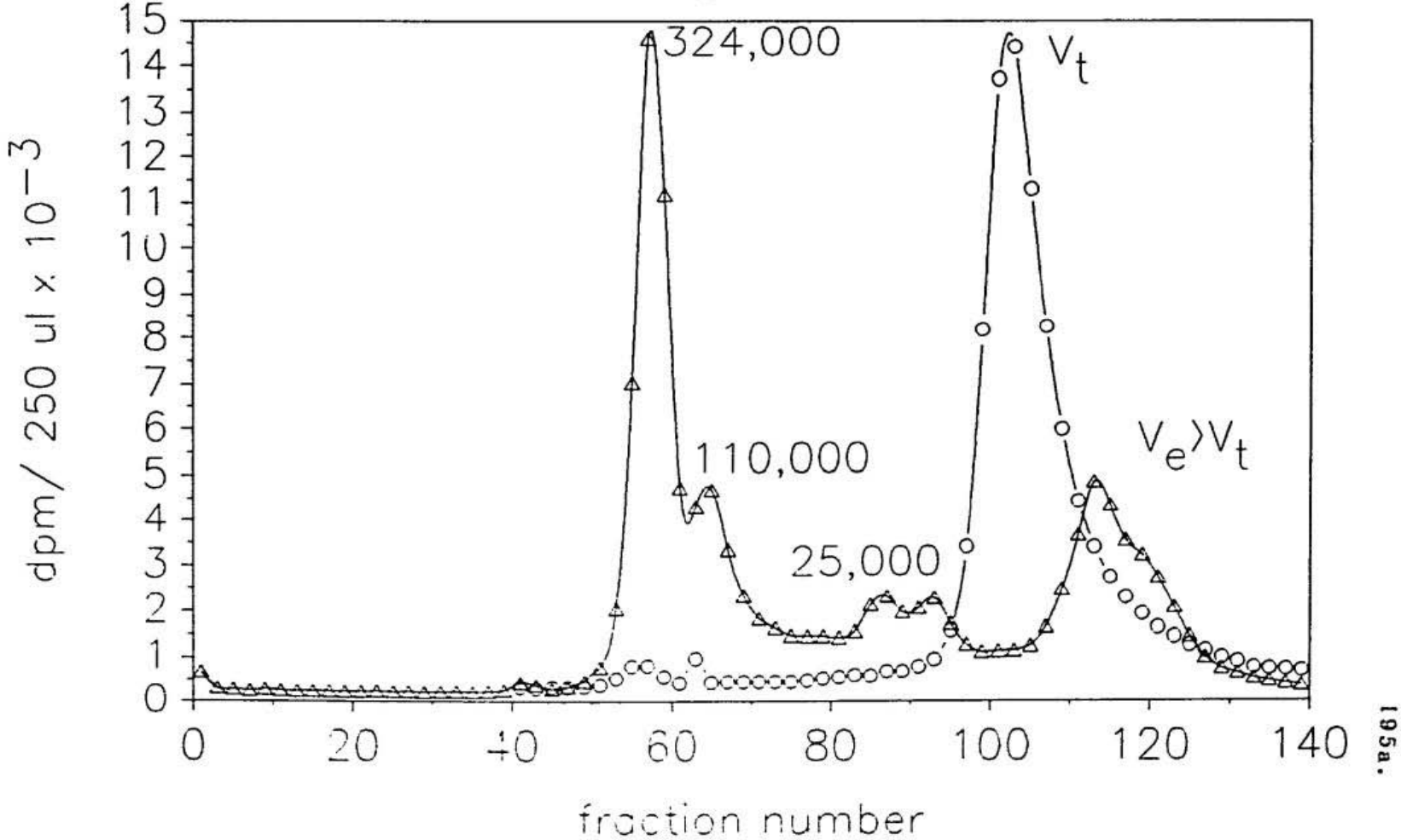
the radiolabelled tubes in Fig. 3.15 were combined and concentrated and then analysed by SDS-PAGE, all the four bands plus other contaminating bands were observed.

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, 20,000 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 (Δ-Δ-Δ). Control (5 ml) was also treated with labelled R5020 but not photoaffinity labelled (-O-O-).

Fig. 3.16


dpm/ 250 ul x 10^{-3}
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
20
40
60
80
100
120
140
fraction number
324,000
110,000
25,000
V_t
$V_e > V_t$

Discussion .

Goetz, et al . (1987), have demonstrated that plasma levels of MIS 17α,20β-DHP in brook trout Salvelinus fontinalis 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, et al . 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 17α,20β-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 1.99×10^7 M^{-1}, this value is 10 fold lower than observed for cytosolic receptors. Also membrane receptors showed cooperativity in binding to [^{3}H]17α,20β-DHP; cooperativity was not observed with cytosol receptors in binding studies with [^{3}H]17α,20β-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 [^{3}H]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 [^{3}H]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 [^{3}H]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 17α,20β-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 [^{3}H]R5020 photoaffinity labelling and SDS-PAGE. The K_a for the photoaffinity labelled protein was 1 x 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 measurments 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 (Maneckjee, *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 17α,20β-DHP levels (free + glucuronide) in the oocyte protoplasm from Atlantic salmon Salmo salar Quananiche 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 17α,20β-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). [^{3}H]17α,20β-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 [^{3}H]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 [^{3}H]17α,20β-DHP from incubation medium with time is shown in Fig.4. 1.a. The uptake of [^{3}H]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 [^{3}H] 17α,20β-DHP mode of transport .

When a double reciprocal plot of the uptake of labelled 17α-,20β-DHP against the concentration of [^{3}H]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 [^{3}H]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 [^{3}H]17α,20β-DHP from the incubation medium occurring with time.
The oocytes were incubated in the presence of [^{3}H]17α-,20β-DHP (5 nM) in TBSS at 10 deg. C, in the presence of moist oxygen with slow agitation.

Fig. 4.1.a.

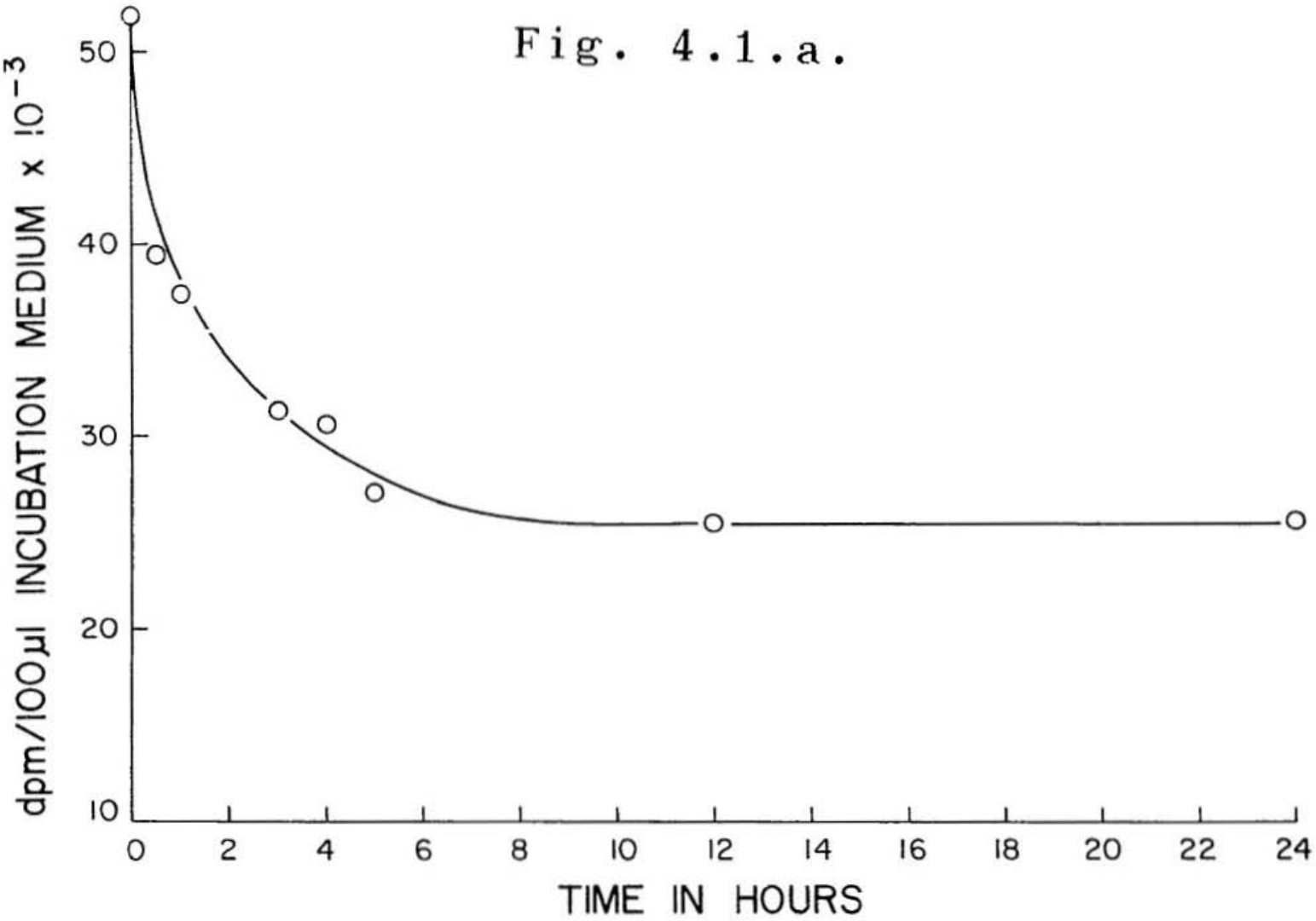
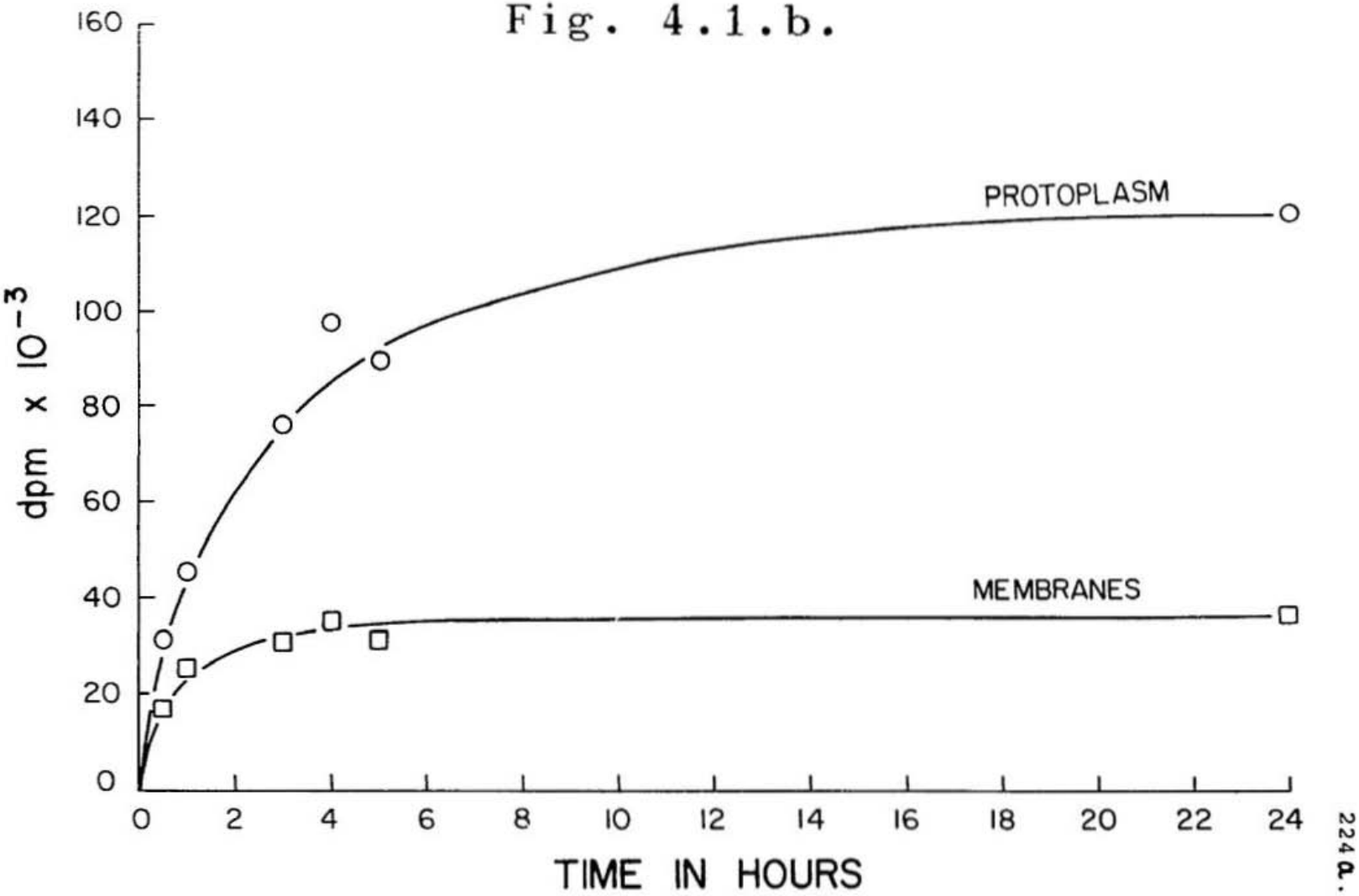

dpm/100µl INCUBATION MEDIUM x 10⁻³
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME IN HOURS

Fig. 4.1. b. Rate of uptake of [^{3}H]17α,20β-DHP into the protoplasm and the membranes of the oocytes with time.

Fig. 4.1.b.


160
140
120
100
80
60
40
20
0
dpm x 10^{-3}
PROTOPLASM
MEMBRANES
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME IN HOURS

224 a.

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 [^{3}H]17α,20β -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 [^{3}H]17α,20β-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.

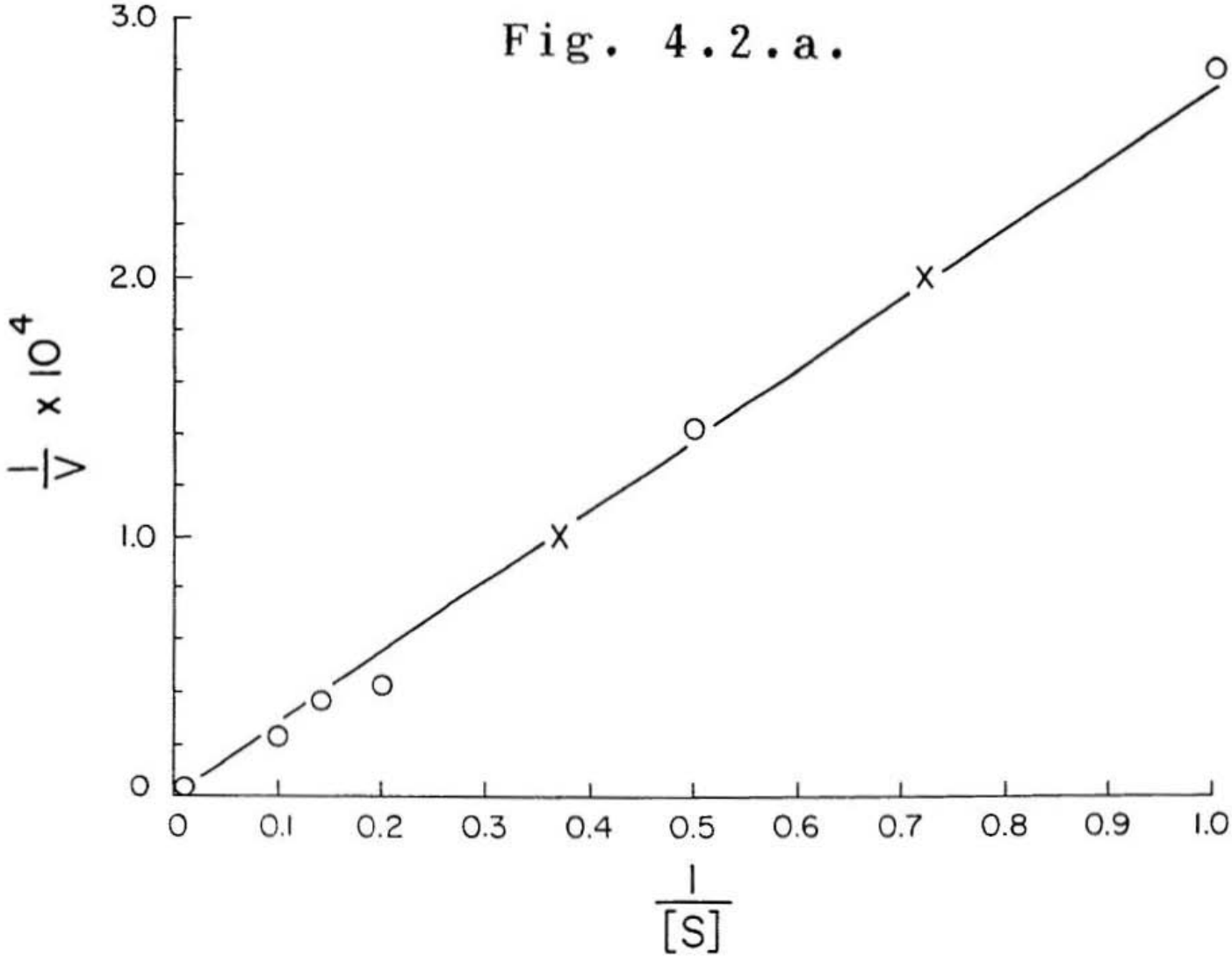


Fig. 4.2. b. Double reciprocal plot of uptake into the protoplasm of the oocytes (0.100 g) in dpm at various concentrations of labelled steroid (1-100 nM). The linear regression analysis gave regression coefficient of 0.9973, ($P < 0.01$). (X——X) denotes the linear regression fitted line.

Fig. 4.2.b.

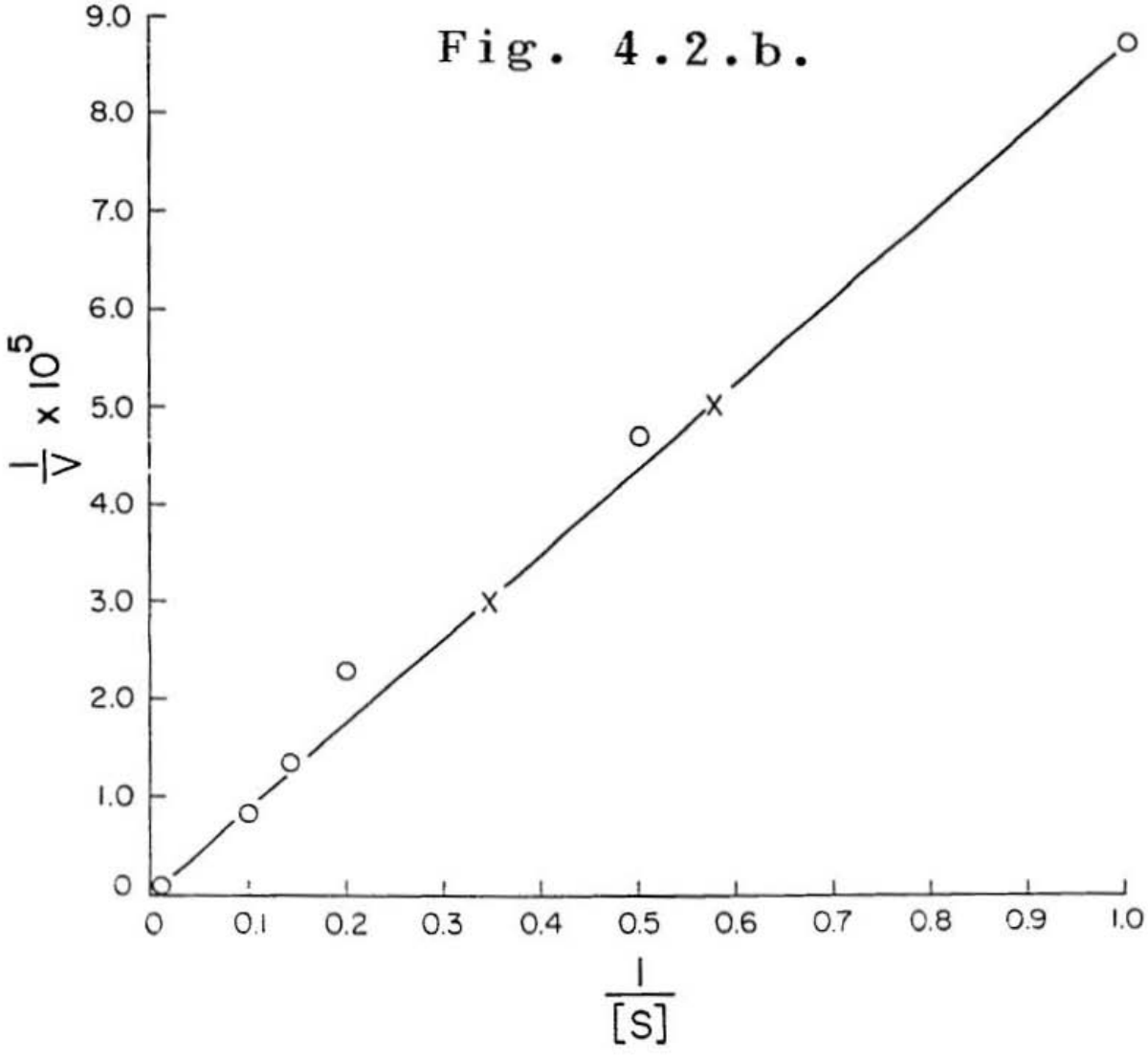

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
$\frac{1}{V} \times 10^5$
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
$\frac{1}{[S]}$

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.

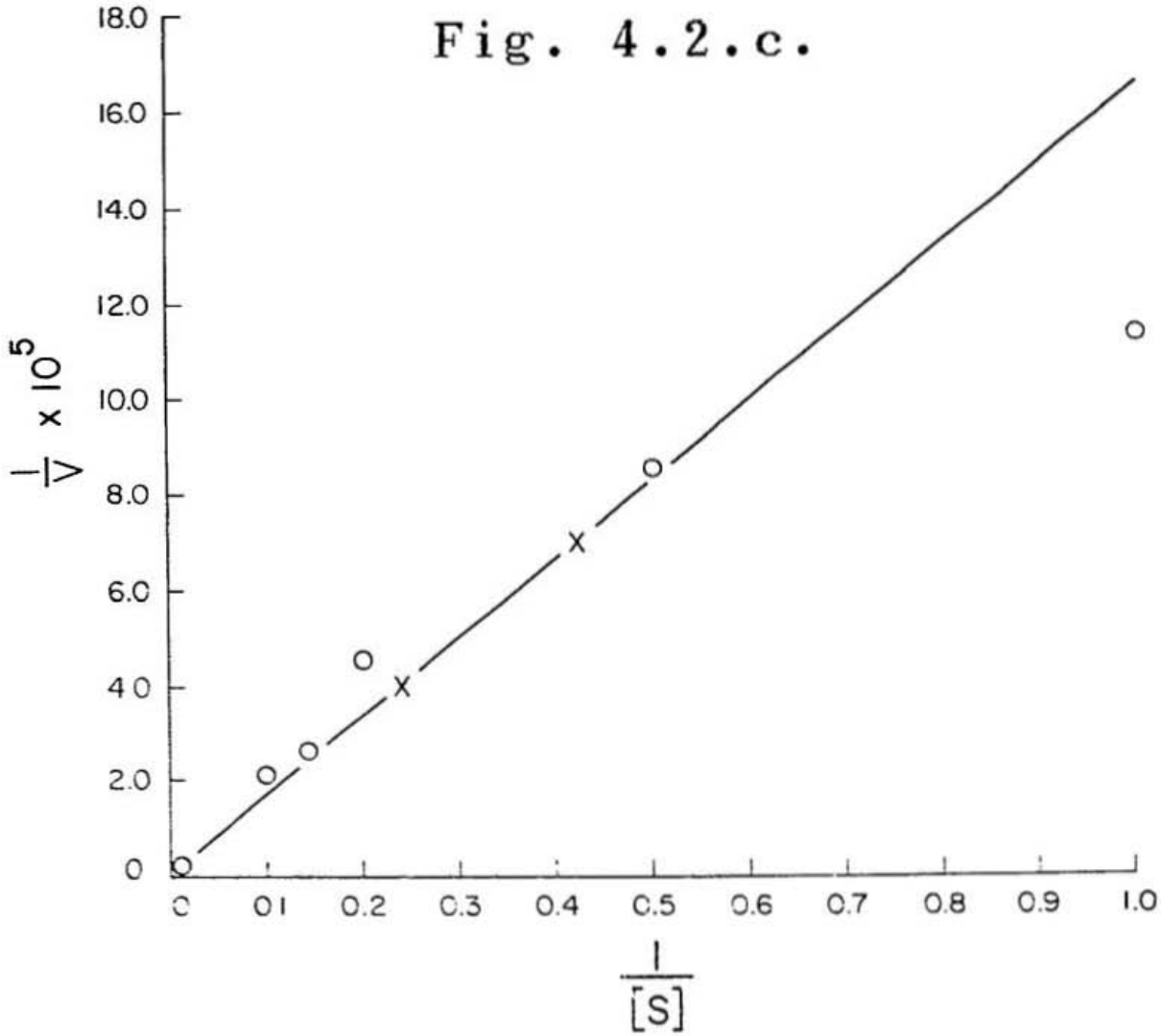

18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
$\frac{1}{V} \times 10^5$
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
$\frac{1}{[S]}$

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 17α,20β-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 17α,20β-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-17α,20β-triol and c) 5α-pregnane -3β,17α20β-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 17α,20β-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 rechromotographed 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]17α,20β-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 17α,20β-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 17α,20β-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 17α,20β-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 17α,20β-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 [^{3}H]17α,20β-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.

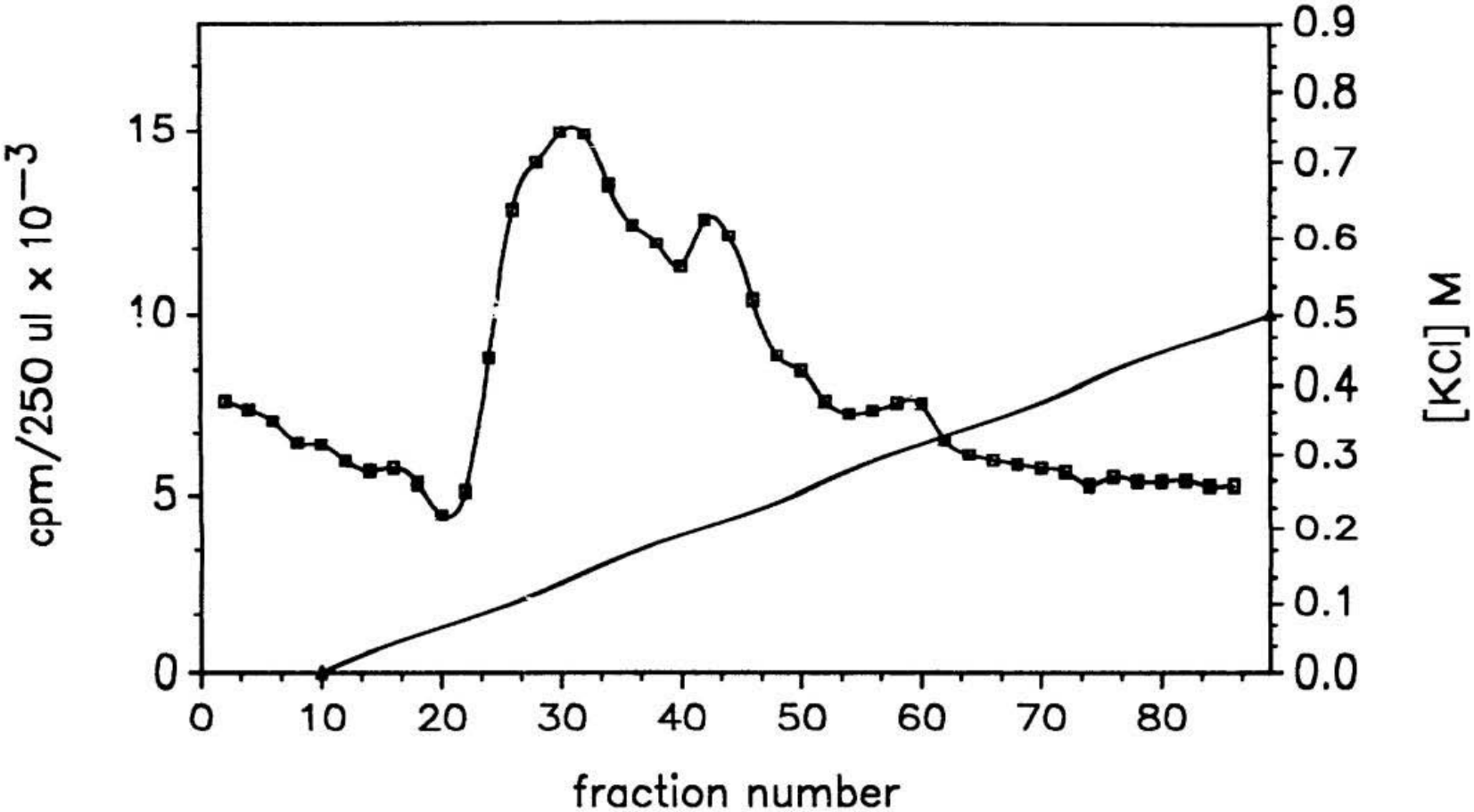
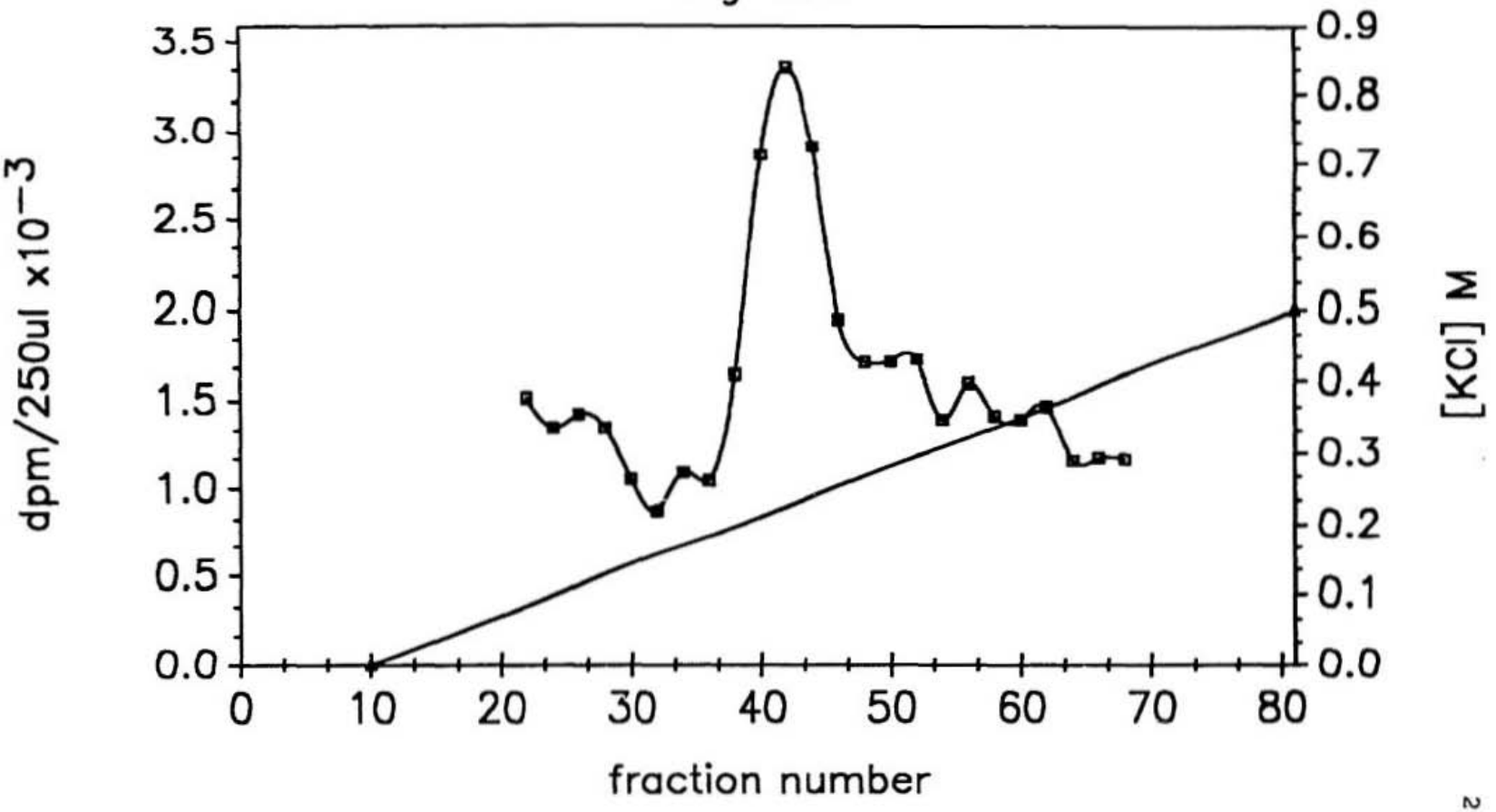

cpm/250 ul x 10⁻³
15
10
5
0
0
10
20
30
40
50
60
70
80
fraction number
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
[KCl] M

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.


dpm/250ul x10⁻³
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
80
fraction number
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
[KCl] M

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.

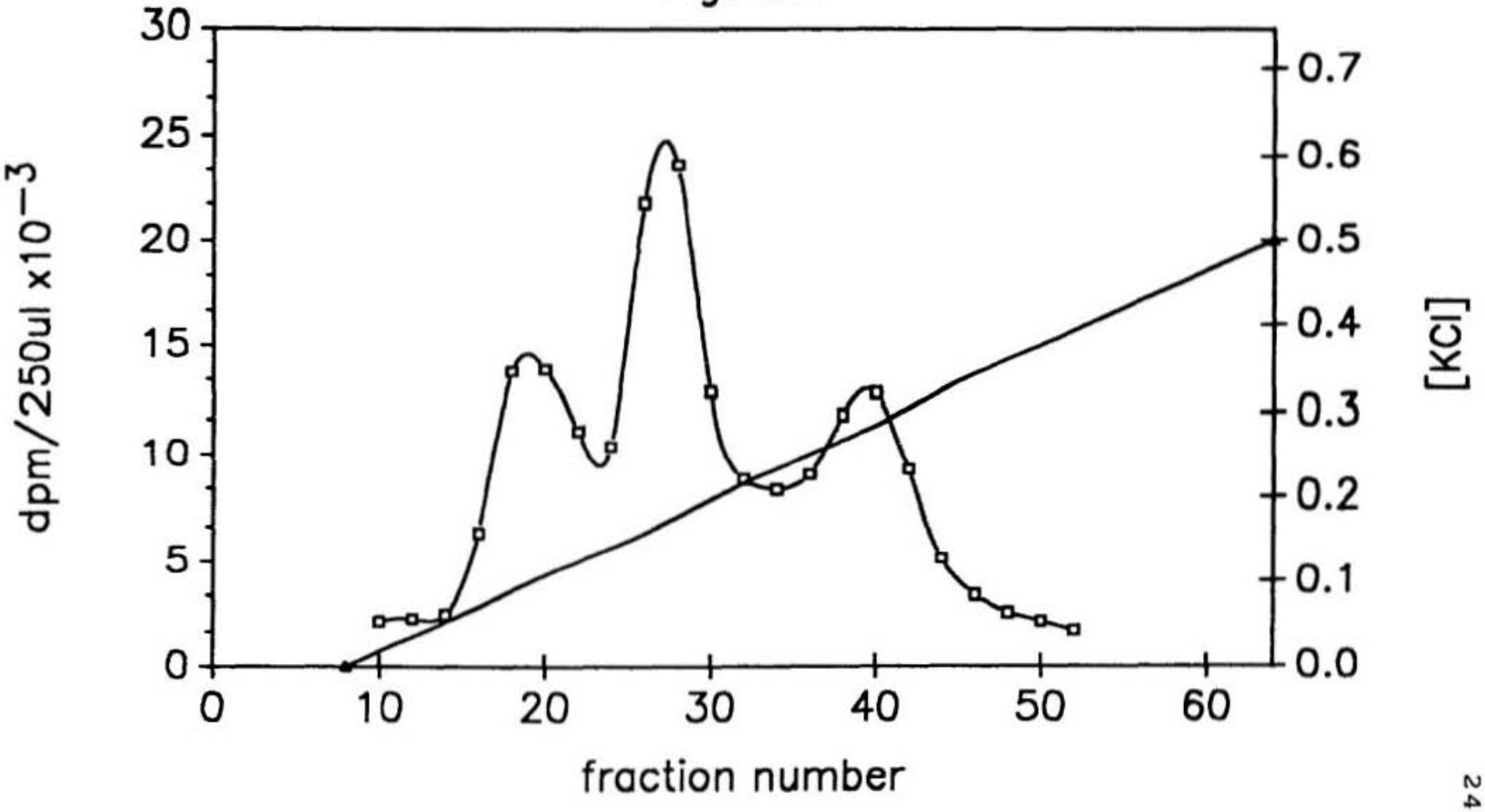

dpm/250ul x10−3
30
25
20
15
10
5
0
0
10
20
30
40
50
60
fraction number
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
[KCl]

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 17α,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).

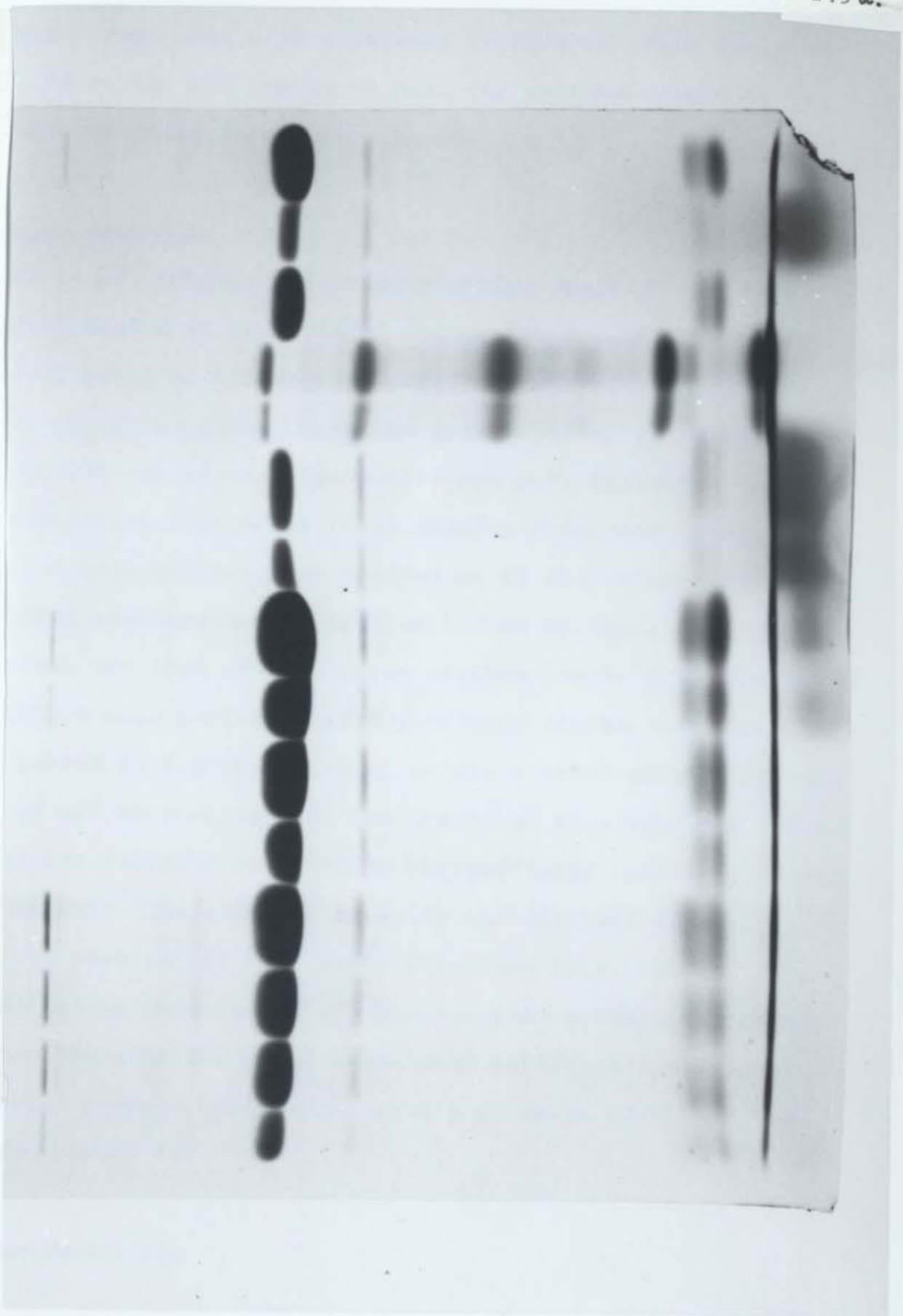


FIG 5.4

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.

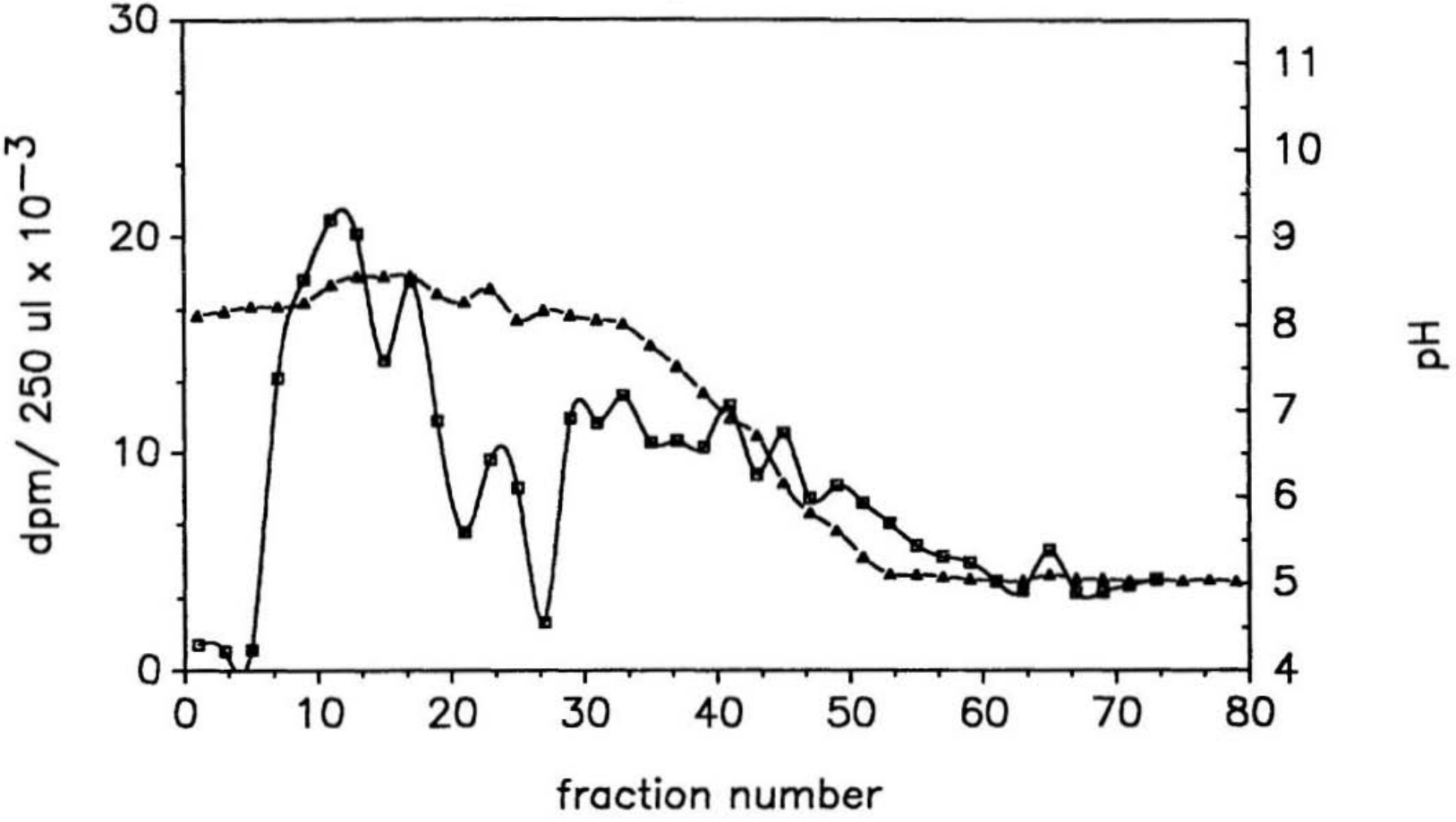
Experiment # 1.

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/CH_3COOH 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.


30
20
10
0
dpm/ 250 ul x 10−3
11
10
9
8
7
6
5
4
pH
0
10
20
30
40
50
60
70
80
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.

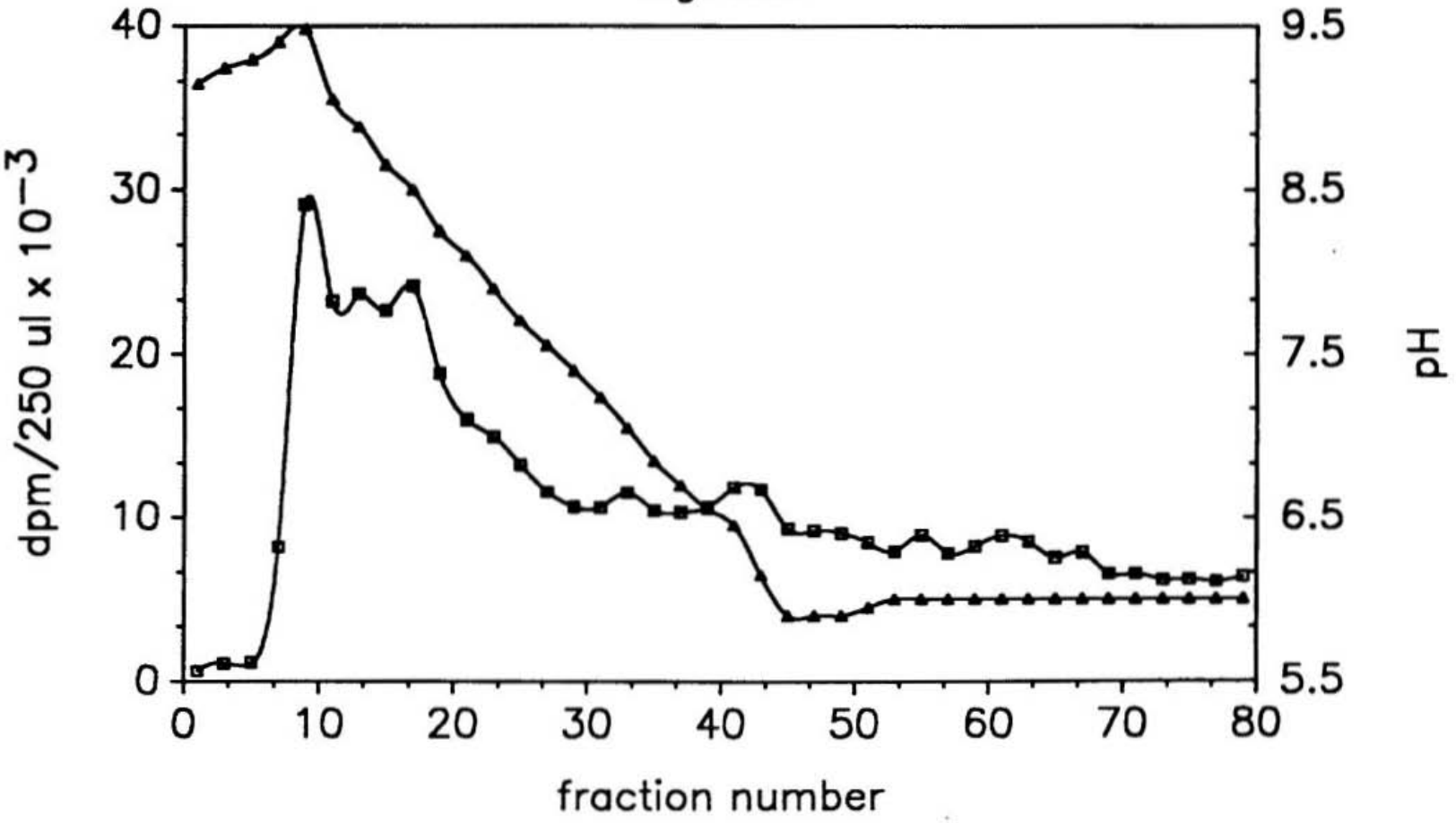
Experiment # 3.

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 (Δ-Δ).

Fig. 5.6.


dpm/250 ul x 10⁻³
40
30
20
10
0
0
10
20
30
40
50
60
70
80
fraction number
9.5
8.5
7.5
6.5
5.5
pH

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 [^{3}H]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 (o—o); pH (▲—▲).

Fig. 5.7.

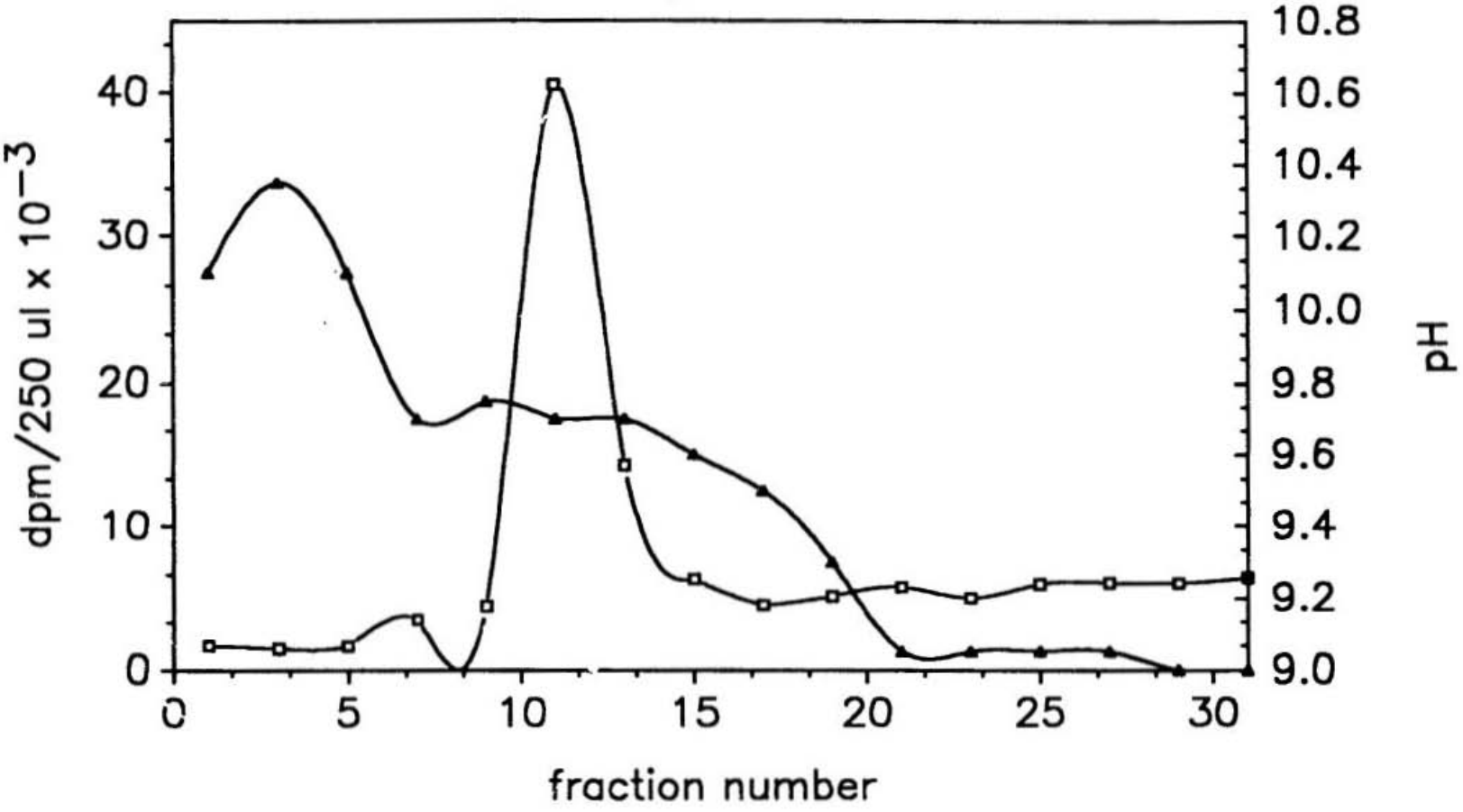

dpm/250 ul x 10⁻³
40
30
20
10
0
0
5
10
15
20
25
30
fraction number
10.8
10.6
10.4
10.2
10.0
9.8
9.6
9.4
9.2
9.0
pH

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

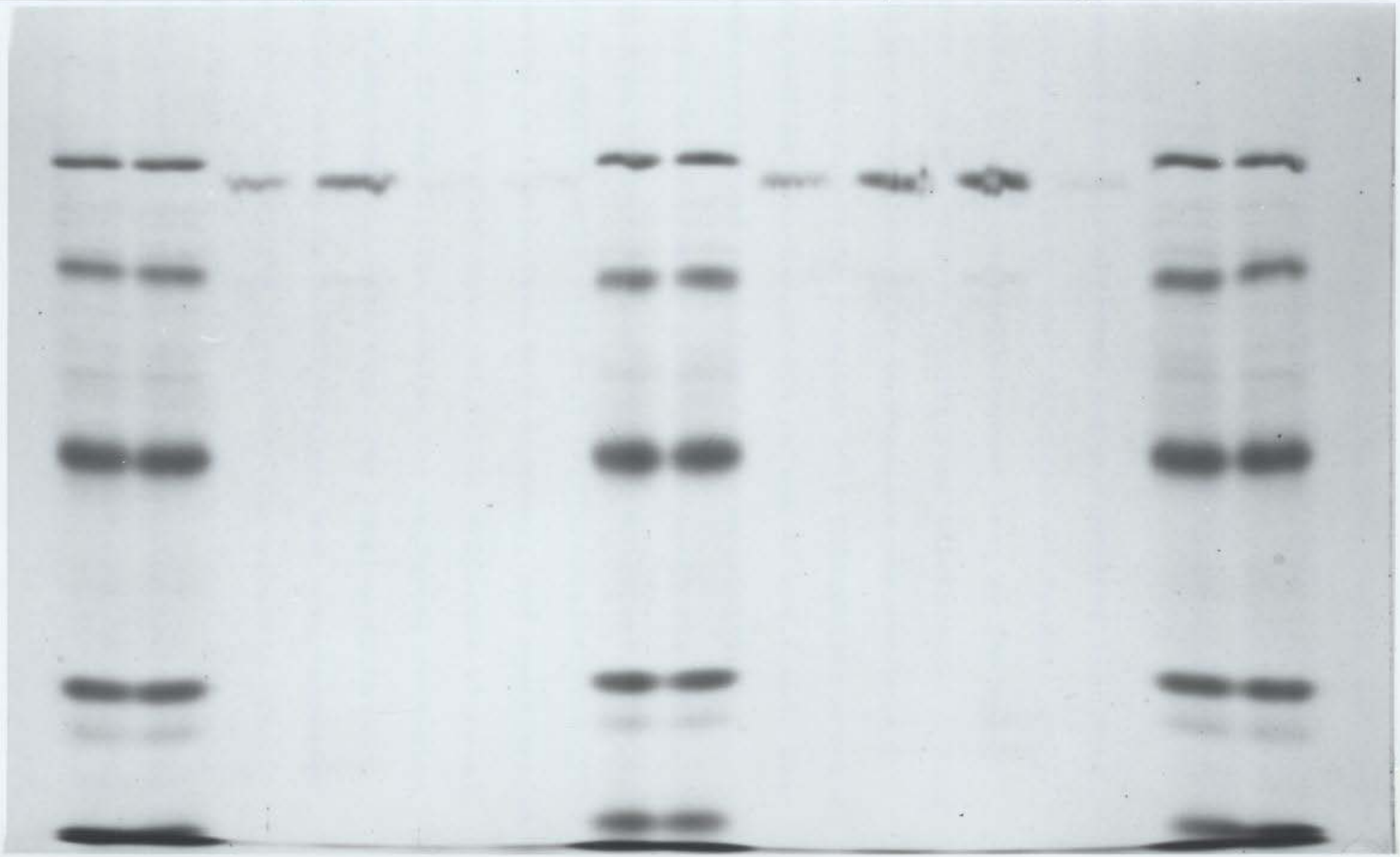

Lanes
1,2
LMW
3,4
F.10
5,6
F.11
7,8
F.12
9,10
F.13
11,12
F.14
13,14
F.15
15
LMW

256a.

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	3,4	5,6	7,8	9,10	11,12	13,14
	LMW stds.	1st peak Sep-300	2nd peak Sep-300	LMW	1st & 2nd peak 10 µl	1st & 2nd peak 25 µl	LMW stds.



257a.

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 l), 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 testoterone.

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 flurorescent 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_2H/NaH_2PO_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 [^{3}H] 17α,20β-DHP, by antibodies to the receptor protein .

[^{3}H]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 [^{3}H]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 mu 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

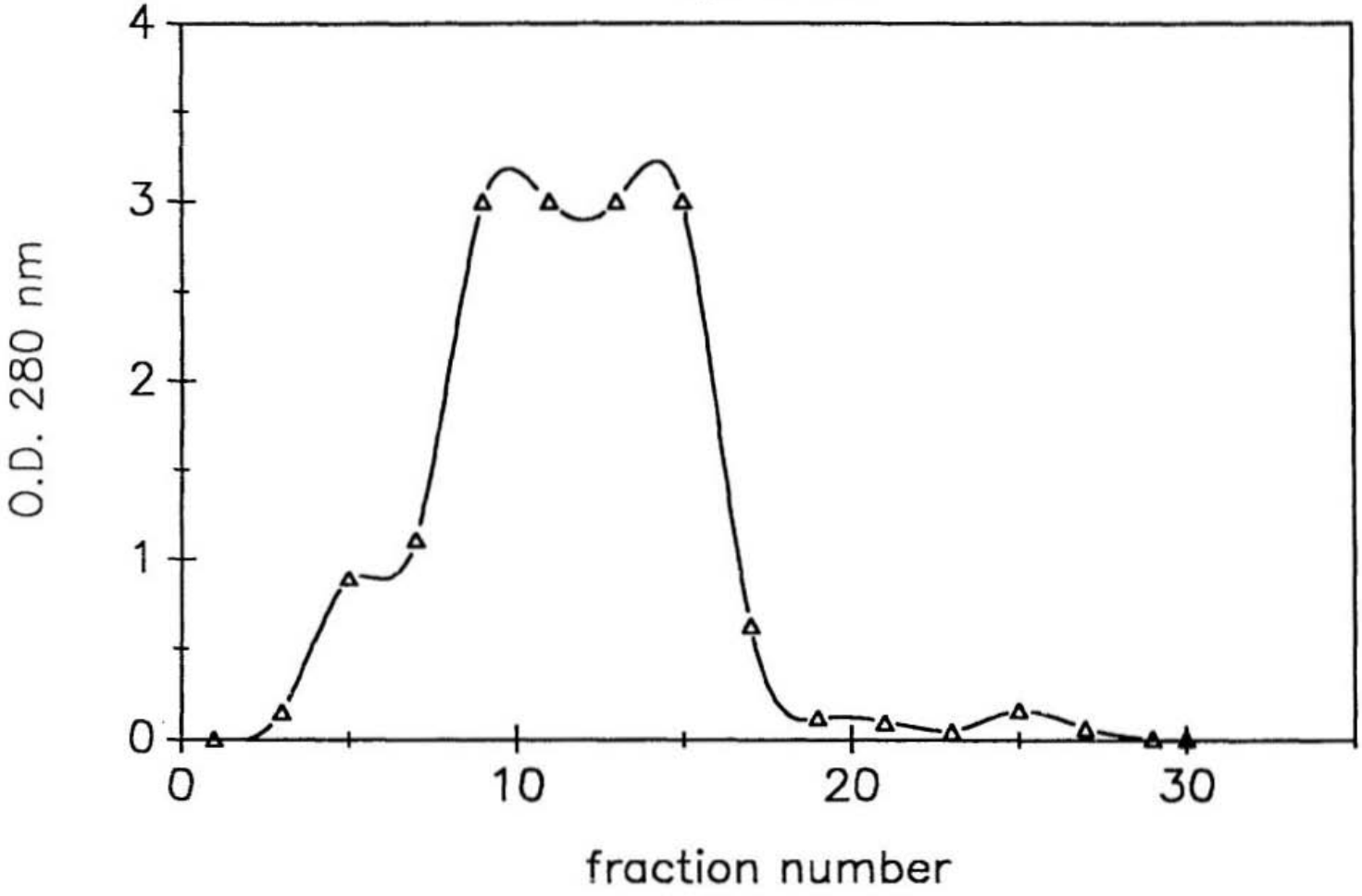
(1:10) dilution, gave positive precipitation reactions with crude, and Sephacryl S-300 purified cytosolic receptor preparations in the immunodiffusion test, suggesting the structural similarity between the cytosolic and membrane receptor activity. Also Protein A purified antibodies to the membrane-receptor 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.



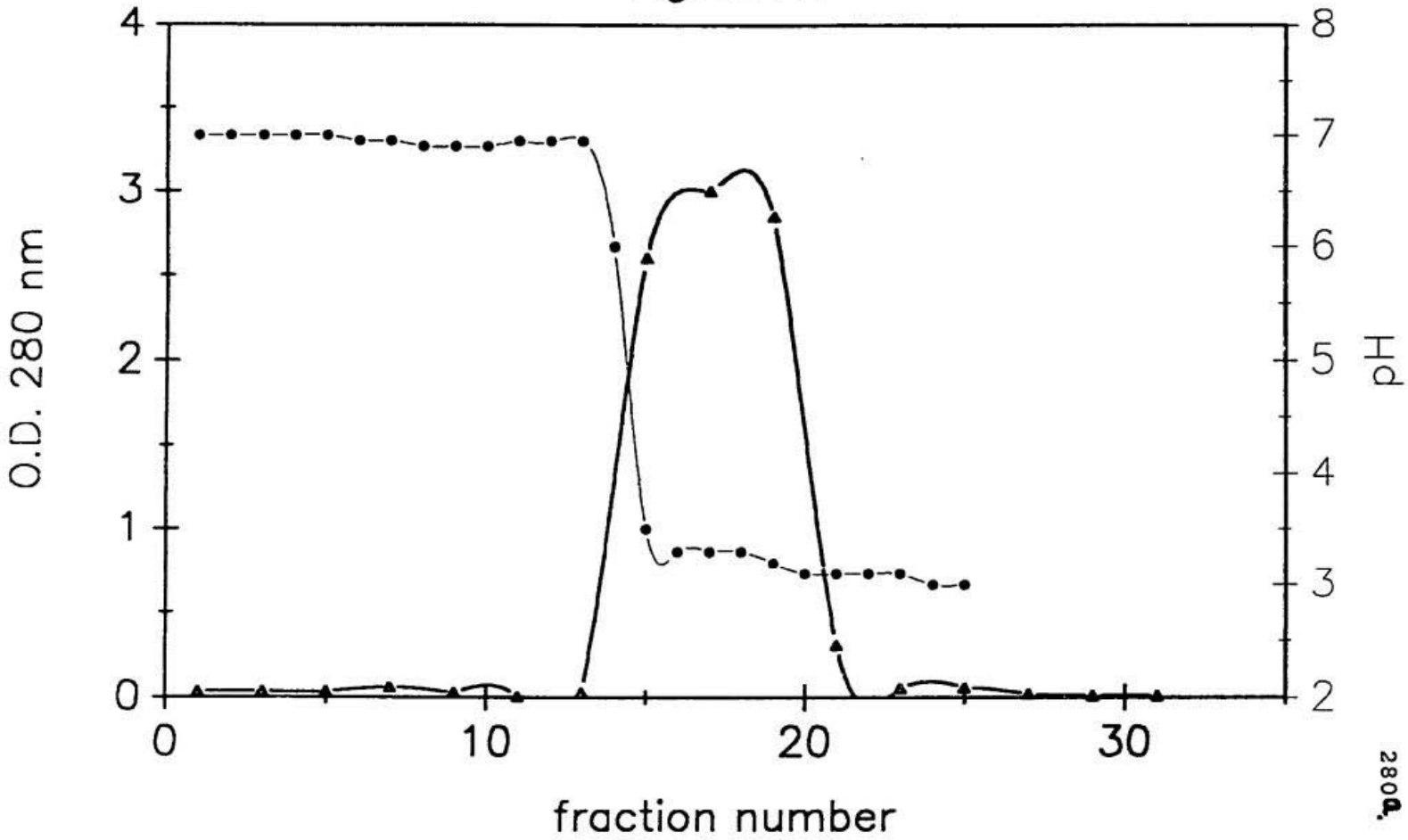
also obtained from the serum of a control injected rabbit (data not given).

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 17α,20β-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).

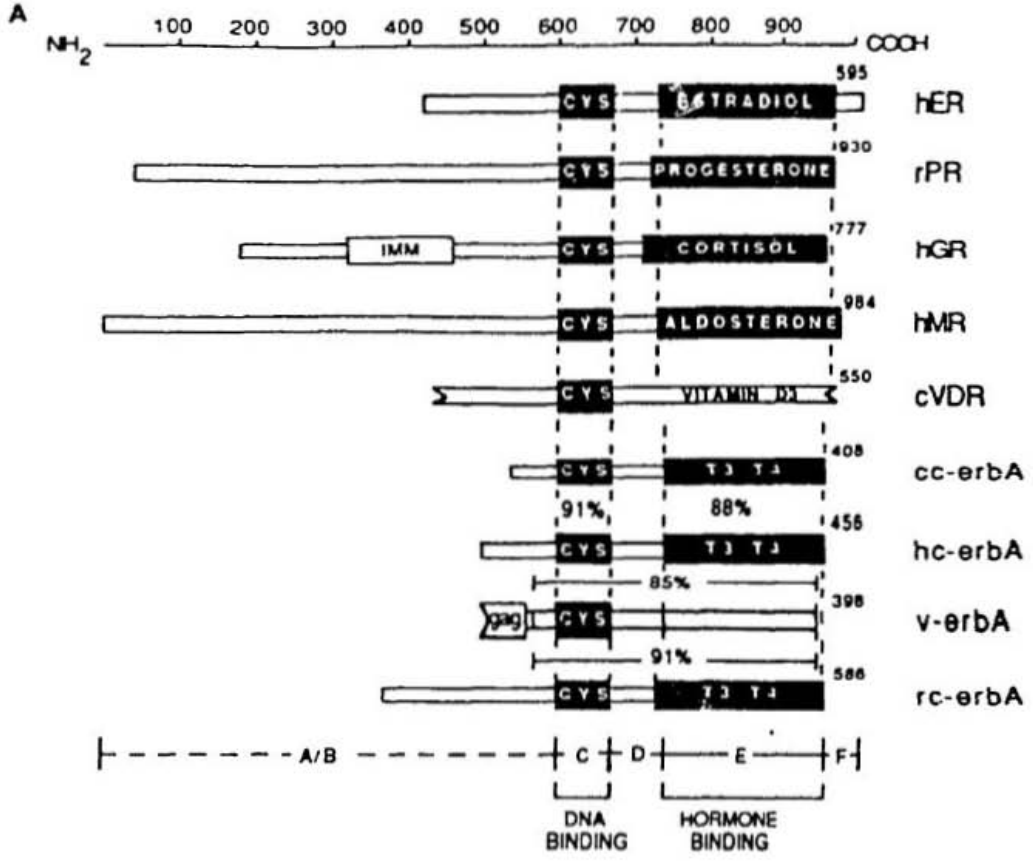
The nuclear steroid hormone receptors for cortisol, estradiol, progesterone, vitamin D3, aldosterone, ouabain, thyroid hormones and various oncogene products have been cloned and purified by affinity chromatography from various species. All of these proteins show similar structural/ functional relationship. 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.



B

	ER[a]	PR[b]	GR[c]	MR[d]	VDR[e]	erbA[f]
ER	–	58	61	56	46	53
PR	29	–	86	90	40	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.

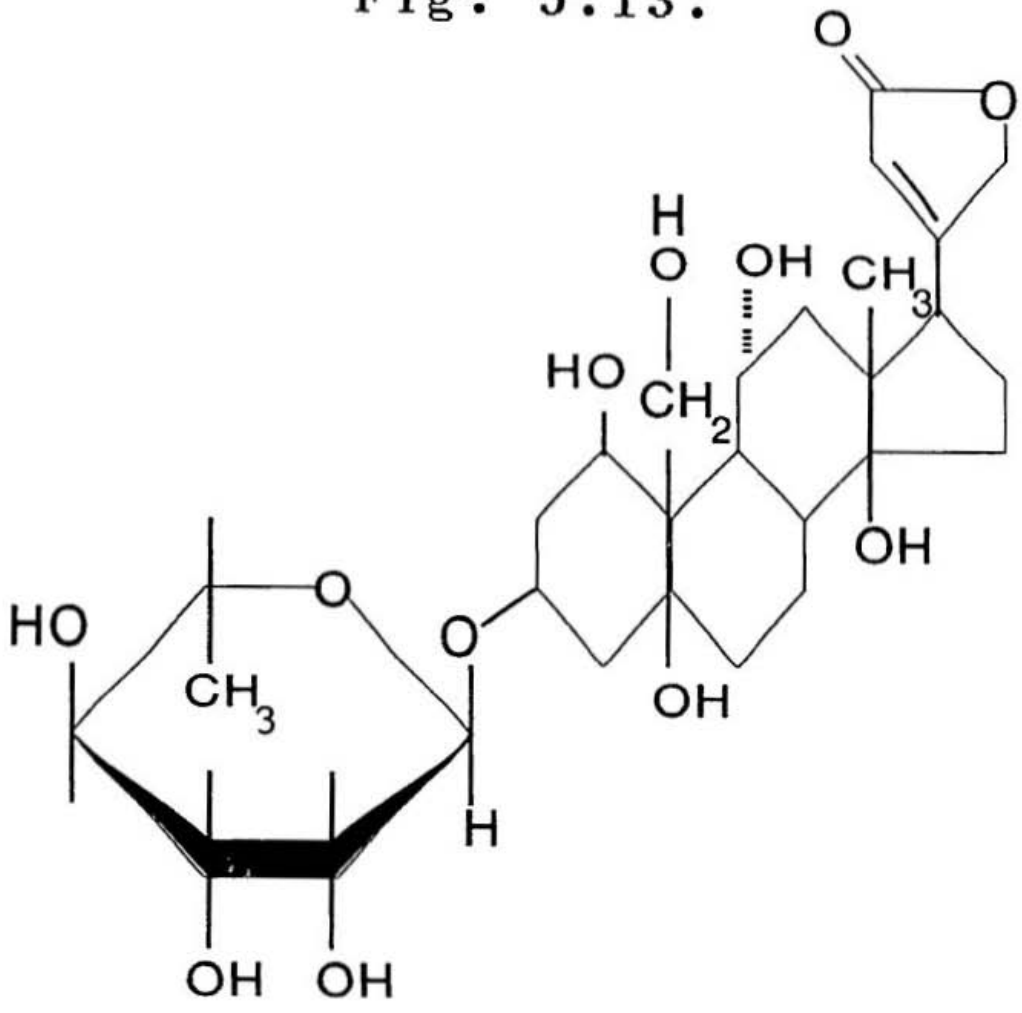
[d] Represents the human mineralocorticoid receptor.

[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 correponding 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 17α,20β-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 extr: 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

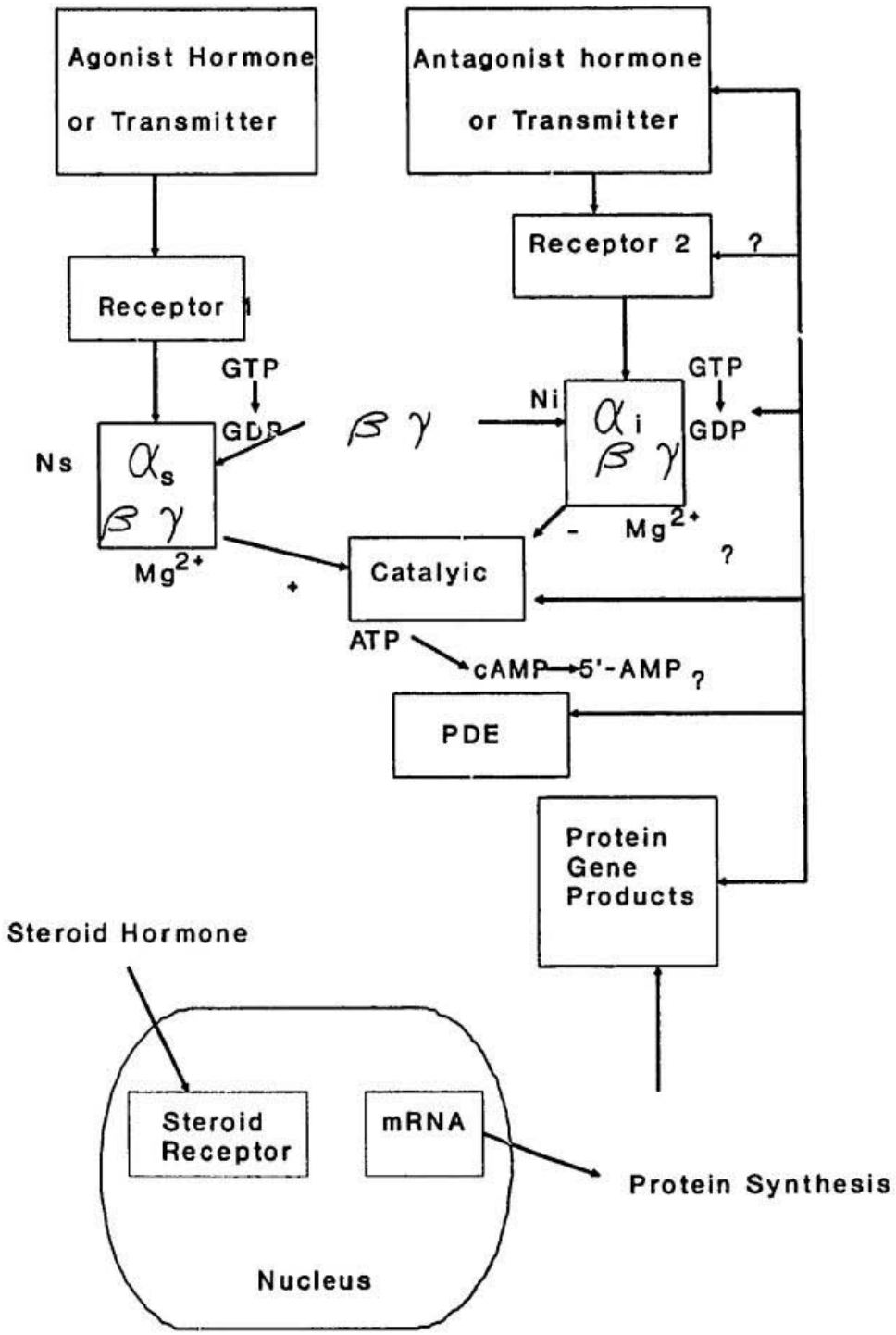
Harrelson and McEwen, (1987) quotes 59 published citations on the steroid modulation of cAMP metabolism.

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


Agonist Hormone or Transmitter
Antagonist hormone or Transmitter
Receptor 2
?
Receptor 1
GTP
GDP
Ns
α_s
$\beta\gamma$
Mg^{2+}
$\beta\gamma$
Ni
α_i
$\beta\gamma$
GTP
GDP
Mg^{2+}
-
?
+
Catalyic
ATP
cAMP → 5'-AMP ?
PDE
Protein Gene Products
Steroid Hormone
Steroid Receptor
mRNA
Protein Synthesis
Nucleus

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 cycle 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 17α,20β-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.) *Agonist-related desensitization and up-regulation*. 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 *in vivo* and *in vitro* 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 [γ-^{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_i) 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_i thereby rendering it inactive (Katada, et al . 1984). If the mechanism of inhibition of adenylate cyclase is via N_i, 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_i 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_i regulatory subunit of adenylate cyclase (Olate, et al . 1984; Sadler, et al . 1984; Goodhardt, et al . 1984).

After demonstrating the presence of N_i, 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

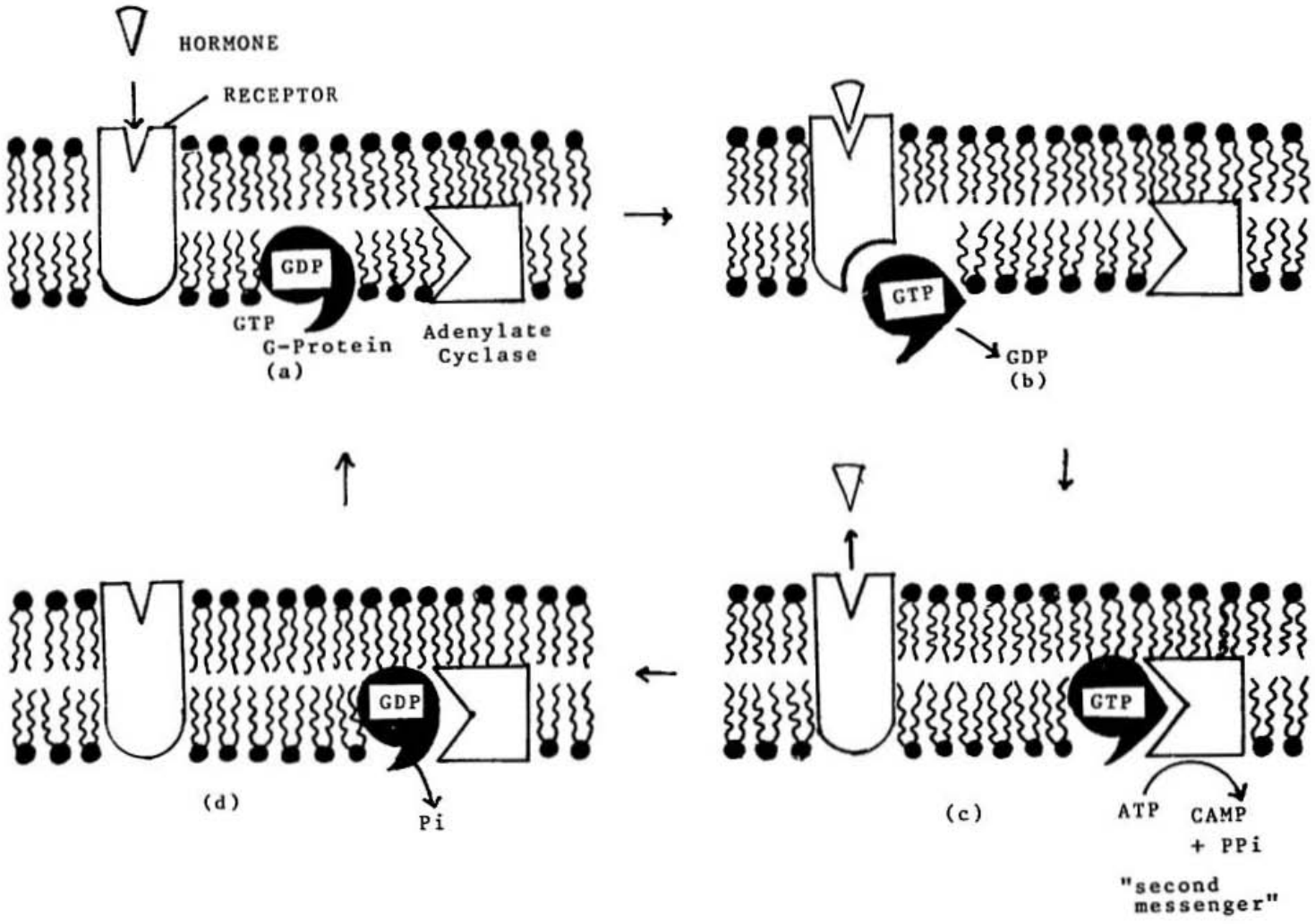
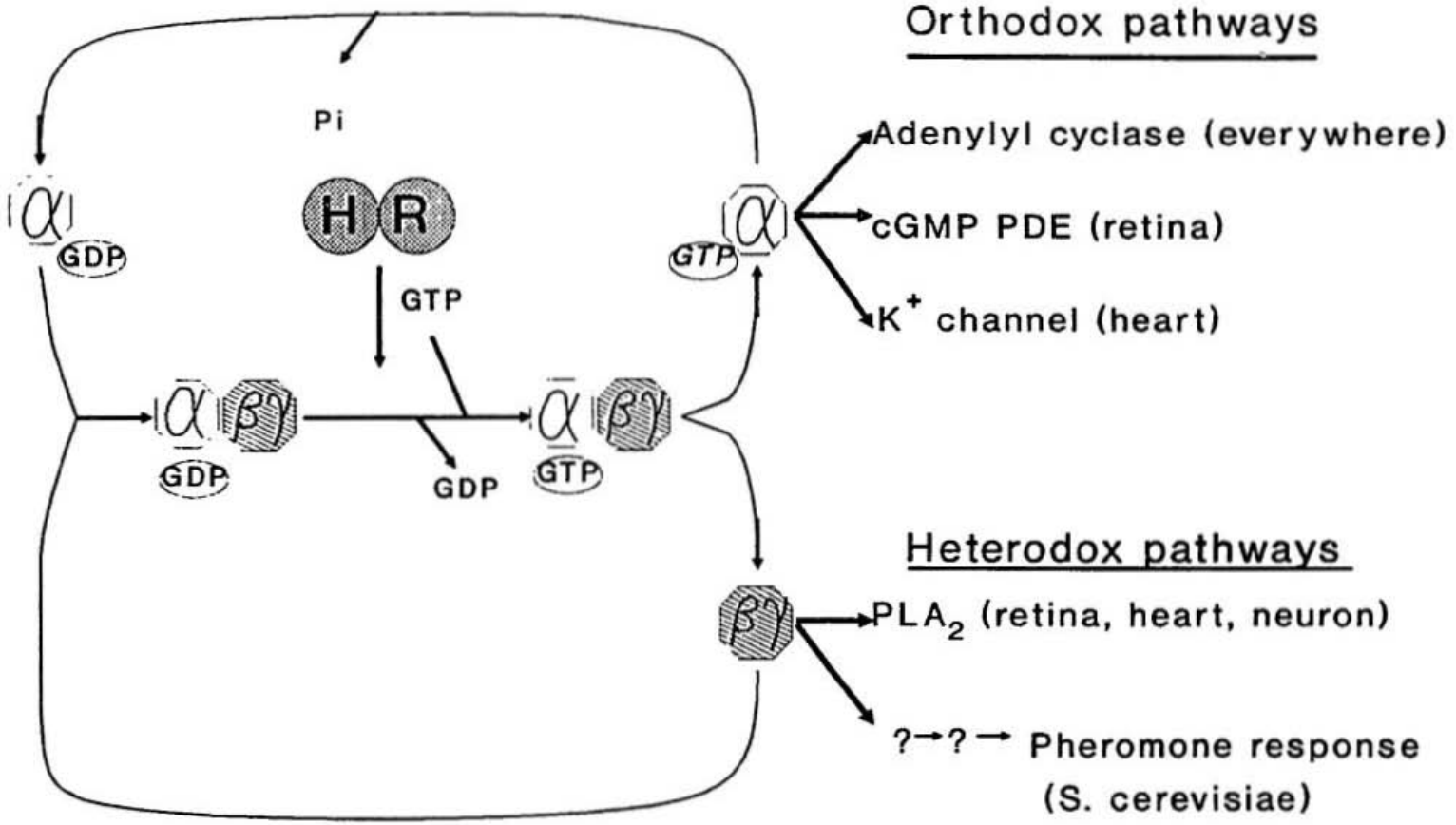

HORMONE
RECEPTOR
GDP
GTP
G-Protein
(a)
Adenylate
Cyclase
GTP
GDP
(b)
GTP
ATP
CAMP
+ PPi
"second
messenger"
(c)
GDP
Pi
(d)

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
Pi
Adenylyl cyclase (everywhere)
cGMP PDE (retina)
K+ channel (heart)
H R
GTP
GDP
GDP
GTP
GTP
Heterodox pathways
PLA2 (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 Wollf, 1977; Lodos, et al . 1979; Wollf, 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 ^{45}Ca 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:-

$$Ca^{++} + \text{calmodulin} \langle\text{-----}\rangle \text{Ca-calmodulin}$$

$$\text{Ca-calmodulin} + \text{PDE} \langle\text{-----}\rangle \text{Ca-calmodulin-PDE (activated).}$$

Injection of phosphodiesterase inhibitors into the oocytes

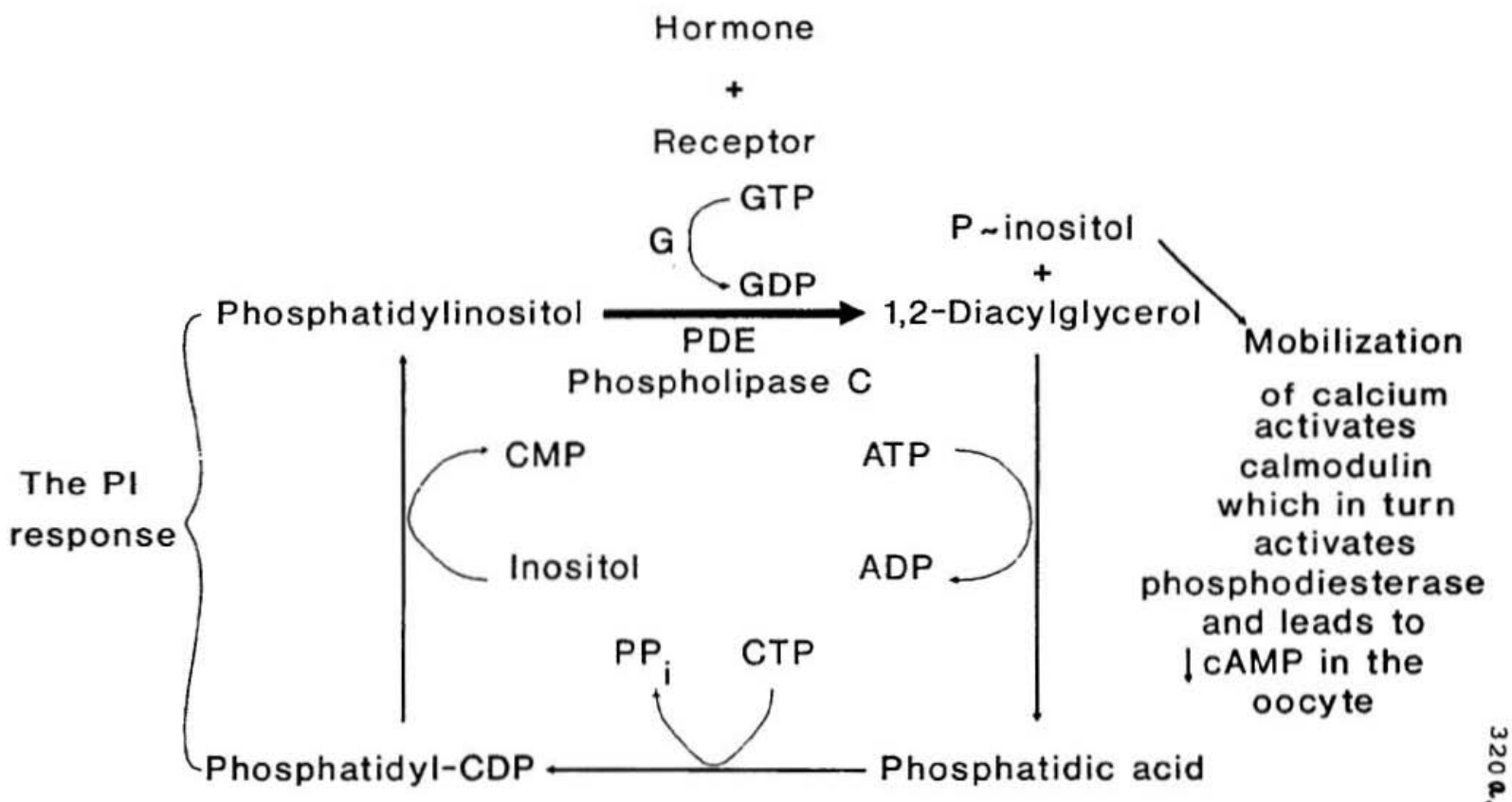
leads to inhibition of maturation, and it also prevents degradation of injected cAMP (Allende, et al. 1977; O'Conner and Smith, 1976). Also injection of calmodulin into the oocyte leads to maturation, and this effect is blocked in oocytes preinjected with catalytic unit of cAMP-dependent protein kinase (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
GTP
G
GDP
P~inositol
+
Phosphatidylinositol
PDE
Phospholipase C
1,2-Diacylglycerol
Mobilization
of calcium
activates
calmodulin
which in turn
activates
phosphodiesterase
and leads to
↓ cAMP in the
oocyte
The PI
response
CMP
Inositol
ATP
ADP
PP$_i$
CTP
Phosphatidyl-CDP
Phosphatidic acid

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 Fundulus heteroclitus 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 in vivo exposure to gonadotrophin. Normally these early stage ovarian follicles are not used for in vitro 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 in vivo 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 17α,20β-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 17α,20β-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 17α,20β-DHP, although much more work has to be done in order to establish the final mechanism of MIS 17α,20β-DHP action leading to maturation via cAMP.

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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).

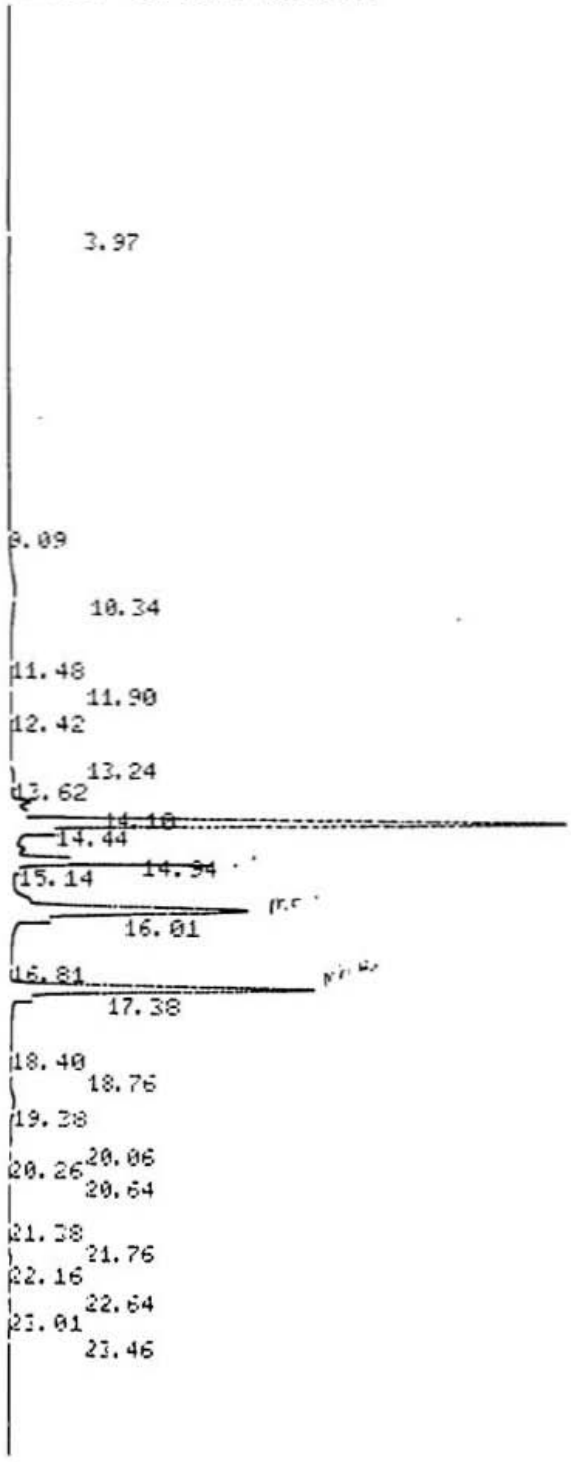
c) Using a column containing 18-carbon (C18) hydrophobic group separating medium (Beckman Ultra pore C-18 150 x 4.6 mm).
Trace 9, blank run. Trace 10, standard proteins. Traces 11 and 12, receptor samples (see text for complete details).

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



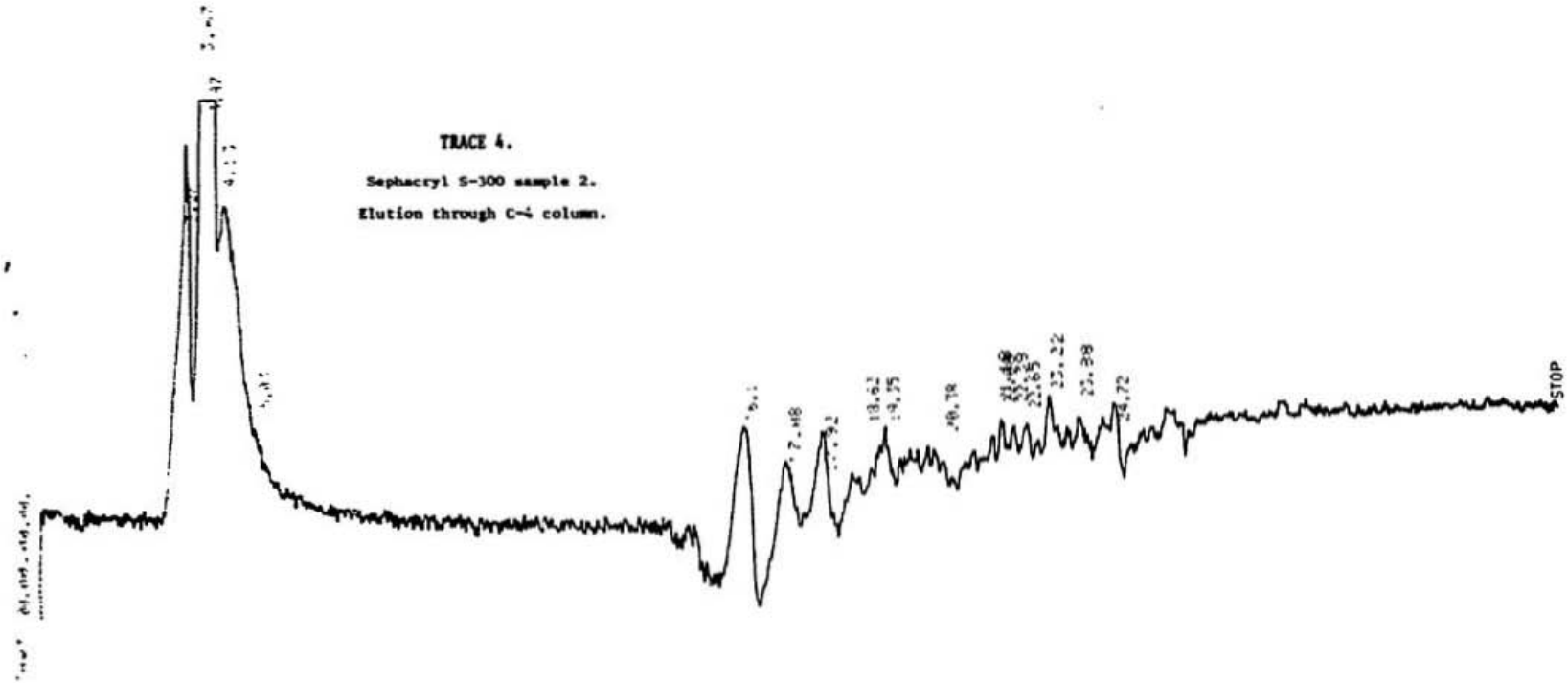
UNIT 1

TRACE 2.

RP molecular weight standards using C- 4 column.

TRACE 3.
Sephacryl S-300 sample 1.
Elution through C-4 column.

Run 2. Peak 2 Sephacryl S-300



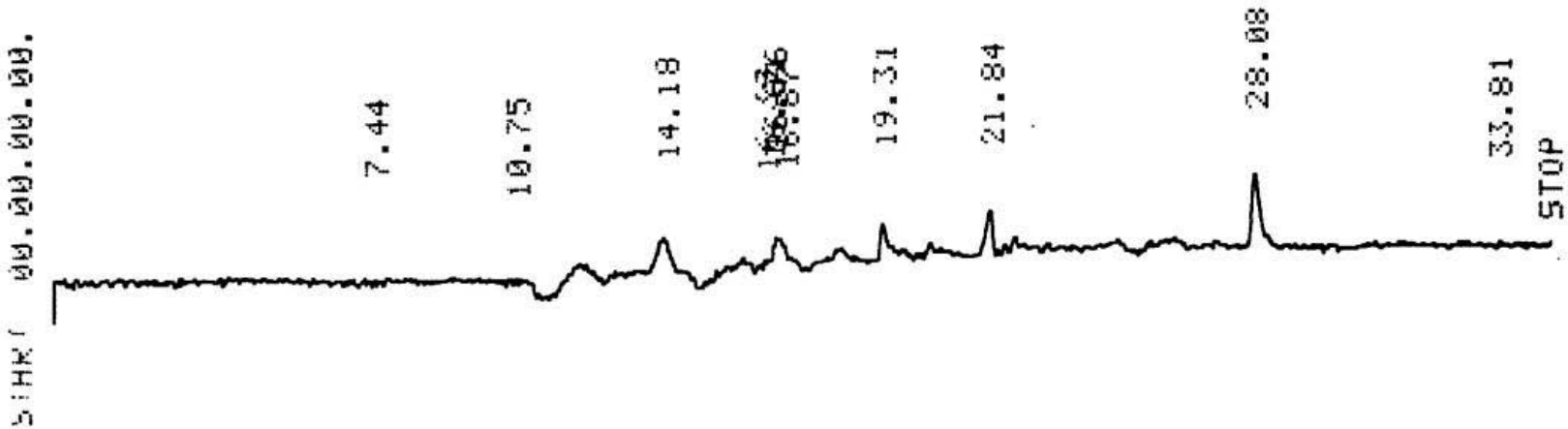
TRACE 4.

Sephacryl S-300 sample 2.

Elution through C-4 column.

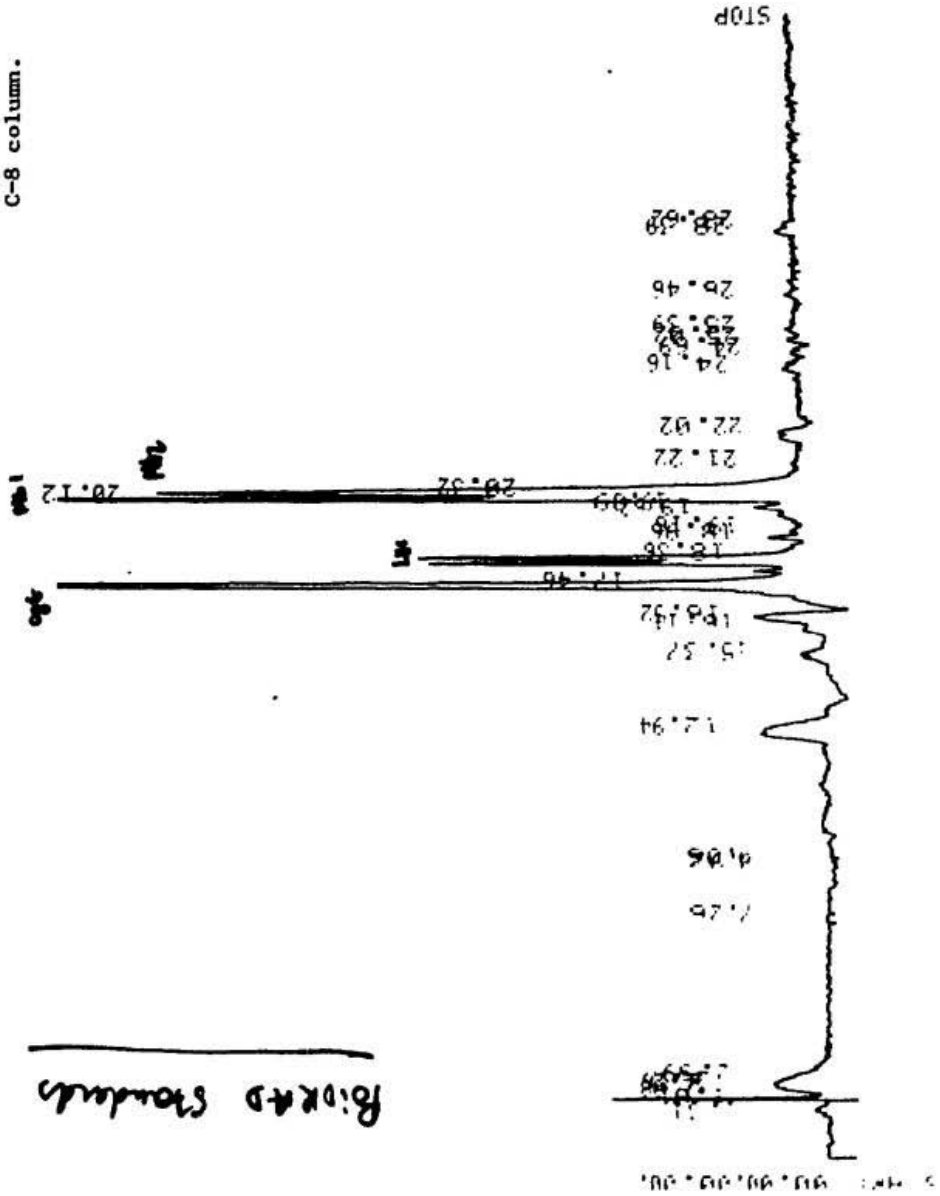
TRACE 5.

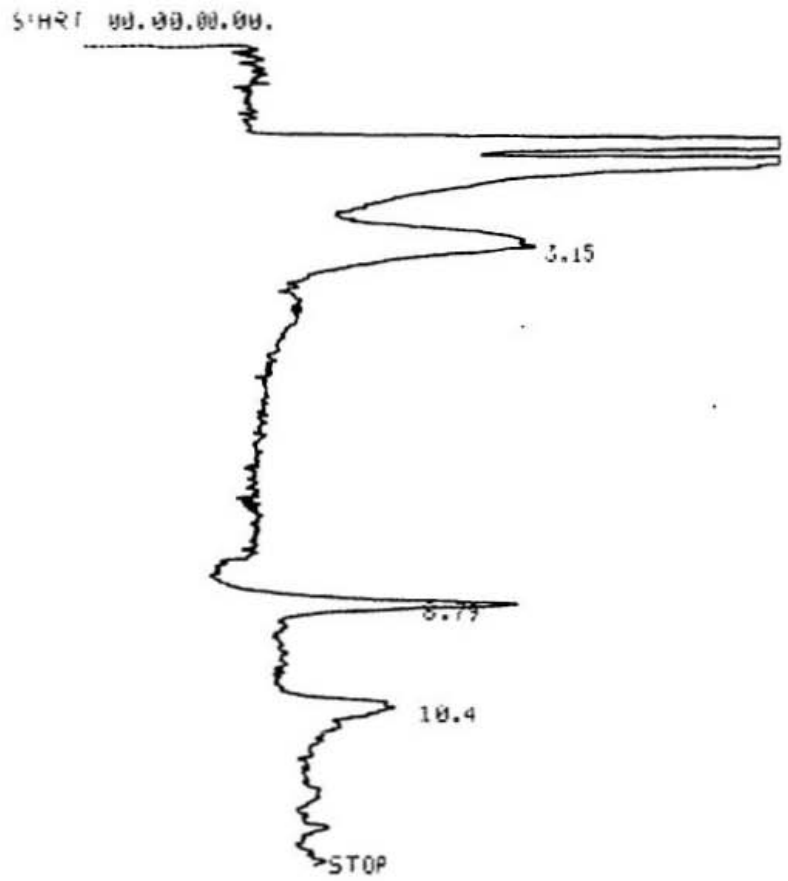
Control run through C-8 column.



TRACE 6.

RP molecular weight standards using C-8 column.

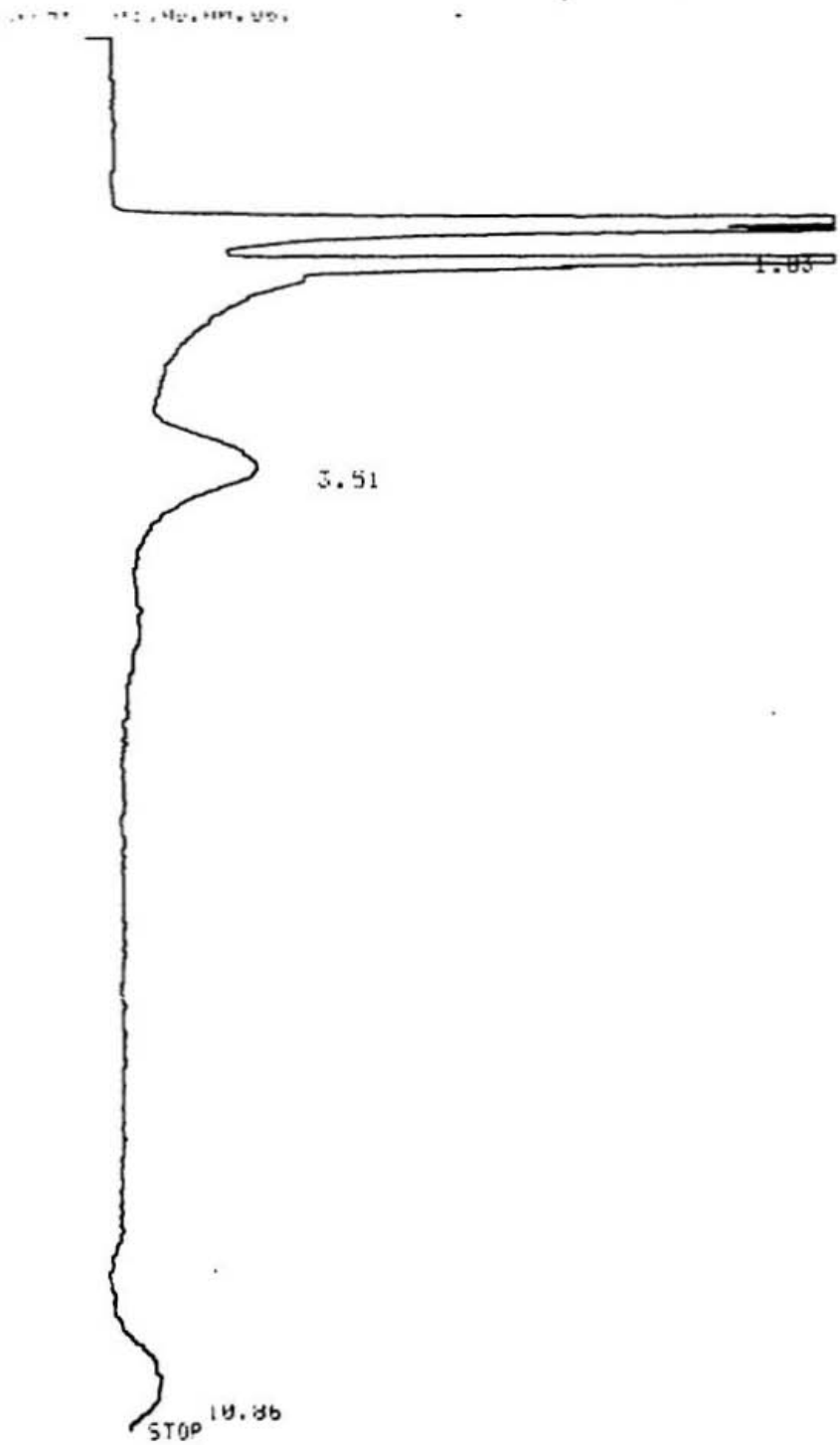




TRACE 7.

Sephacryl S-300 sample 1.

Elution through C-8 column.



TRACE 8.

Sephacryl S-300 sample 2.

Elution through C-8 column.

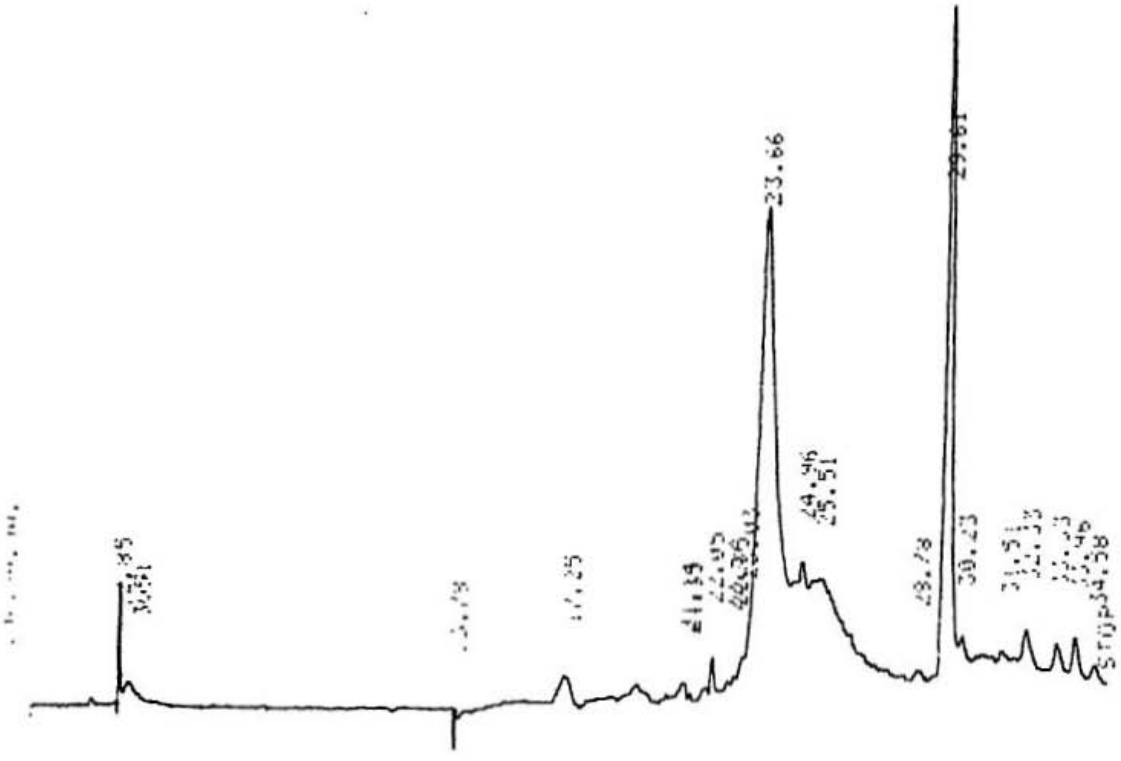
TRACE 9.

Control run through C-18 column.

TRACE 10.

RP molecular weight standards using

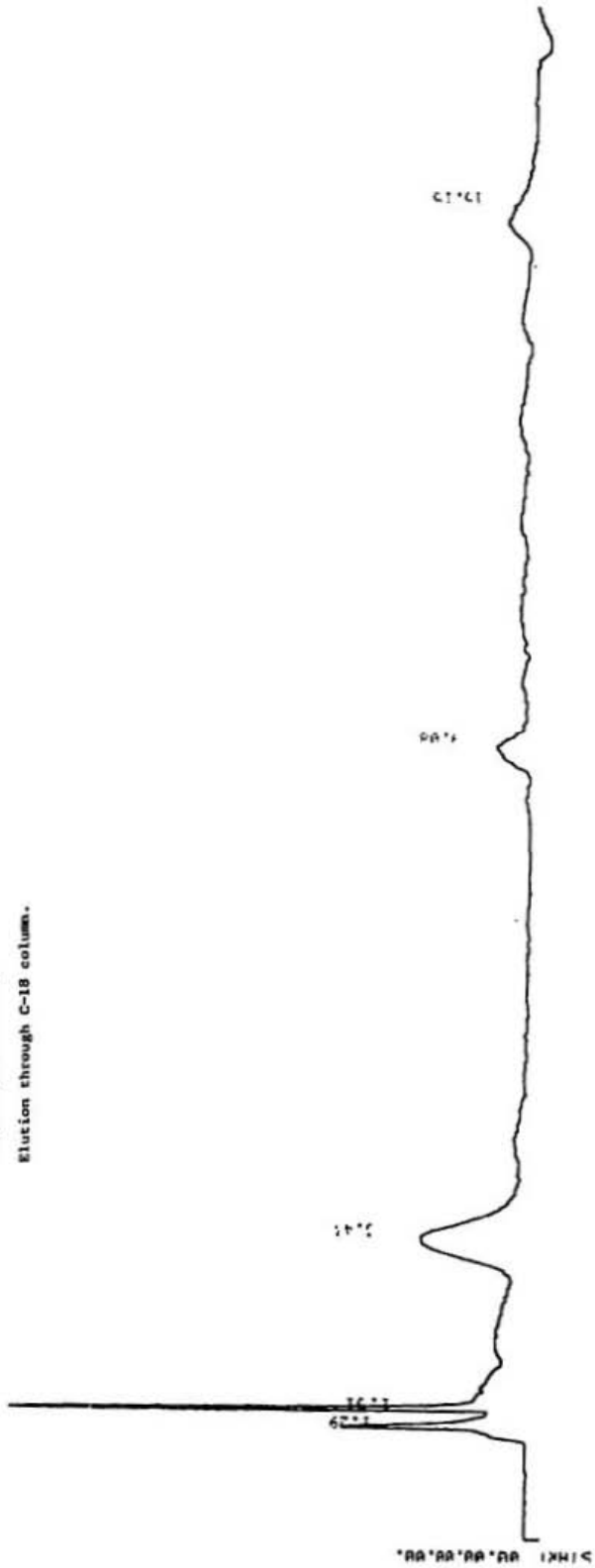
C-18 column.

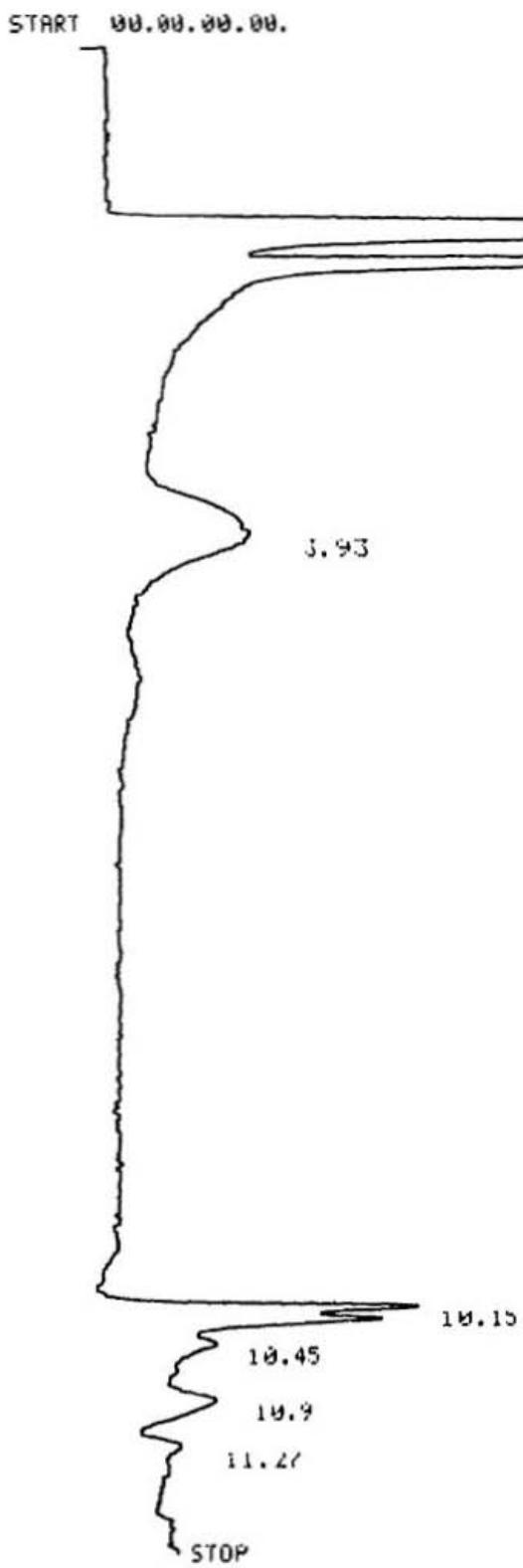


TRACE 11.

Sephacryl S-300 sample 1.

Elution through C-18 column.





TRACE 12.

Sephacryl S-300 sample 2.

Elution through C-18 column.

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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%
Comparative Physiology 2. 74%

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 17α,20β-dihydroxy-4-pregnen-3-one receptor activity in the ovary of brook trout Savelinus fontinalis during terminal stages of oocyte maturation. Fish Physiol. Biochem. 6, 19-39.

2. Beck, J., Donini, J., Maneckjee, A. 1983. The influence of sulphide and Cyanide on nerve function. Toxicology 26, 37-45.

3. Beck, J., Donini, J., Maneckjee, A. 1982. The effect of sulphide and cyanide on nerve function. Toxicology Letters 26, 182-193.

4. Smallman, B.N., and Maneckjee, A. 1981. The synthesis of acetylcholine by plants. Biochem. J. 194, 361-364.

5. Steele, R.W., and Maneckjee, A. 1979. Toxicological significance of acetylcholine esterase of the housefly thorax. Pesticide Biochemistry and Physiology 10, 322-332.

6. Morris, D., Maneckjee, A., and Smallman, B.N. 1976. Apparent 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.

PAPERS SUBMITTED .

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,1989, and after modifications resubmitted in July 1989.

2. Maneckjee, A., Idler, D., and Weisbart, M. 1989. Transport of 17 α,20β-dihydroxy-4-pregnen-3-one in brook trout Salvelinus fontinalis ovarian follicles. Sent to Journal of Fish Physiol. and Biochem. June 1989.

REFEREED CONFERENCE PROCEEDINGS

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 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. Supervisor's, Professor David R. Idler, and Professor Melwin Weisbart. Memorial University of Newfoundland. St. John's, Canada. Submitted 1989.

REFEREES .

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Research Scientist, Agriculture Canada.
University Sub Post Office
London, Ontario. Canada. N6A 5B7.
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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.

PROFESSONAL 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 an d Welfare, Ottawa.

EMPLOYMENT EXPERIENCE .

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

1980-83. Research associate with Dr's. J. Beck; and J. Donini. St. Frances Xavier University. Antigonish, N.S.

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.