PITUITARY HORMONES IN TELEOST FISH WITH EMPHASIS ON GONADOTHOPINS

TZI BUN NG

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PITUITARY HORMONES IN TELEOST FISH WITH EMPHASIS ON GONADOTROPINS

by

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ABSTRACT

This investigation on fish gonadotropins provides considerable evidence to support the concept of duality of gonadotropins in teleosts proposed by Campbell and Idler in 1976. Vitellogenic hormones were isolated from pituitaries of American plaice (Hippoglossoides platessoides) and winter flounder (Pseudopleuronectes americanus) and chum salmon (Oncorhynchus keta), devoid of oocyte maturational and androgenic activities, and they could be distinguished from the maturational hormones which were active in stimulating oocyte maturation and androgen production. The bioassays used to isolate the vitellogenic and maturational hormones utilized the hypophysectomized flounder as the assay animal, and this study represents one of the few investigations utilizing homologous bioassays to isolate fish gonadotropins.

Vitellogenic and maturational hormones are different in their amino acid compositions, carbohydrate contents, immunological activities and chromatographic behavior on Concanavalin A-Sepharose. An antiserum to vitellogenic hormone was capable of inhibiting incorporation of vitellogenin into the ovary, whereas an antiserum to maturational hormone depressed the plasma concentrations of estradiol and vitellogenin, indicating that the two gonadotropins play different roles in fish reproduction. Chromatography of maturational hormone on Concanavalin A-Sepharose did not lead to isolation of additional material unadsorbed on the immobilized lectin. All available evidence thus suggest that the vitellogenic hormone and the maturational hormone are different entities.

Thyroid stimulating hormone and gonadotropin were shown to be separate hormones. Two thyroid stimulating hormones could be isolated from pituitaries of both American plaice and winter flounder. The thyroid stimulating hormone unadsorbed on Concanavalin A-Sepharose stimulated mainly the production of

triiodothyronine in hypophysectomized flounder while the thyroid stimulating hormone adsorbed on the immobilized lectin enhanced mainly the production of thyroxine.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

CMI	-	fraction unadsorbed on carboxymethyl Bio-Gel A
CMII	-	fraction adsorbed on carboxymethyl Bio-Gel A
Con AI	-	fraction unadsorbed on Concanavalin A-Sepharose
Con AII	-	fraction adsorbed on Concanavalin A-Sepharose
cpm	-	counts per minute
DE I	-	first peak eluted from diethylaminoethyl Bio-Gel A
DE II	-	second peak eluted from diethylaminoethyl Bio-Gel A
DE III	-	third peak eluted from diethylaminoethyl Bio-Gel A
dpm	-	disintegrations per minute
DTT	-	dithiothreitol
EDTA	-	ethylene diaminetetracetic acid
FSH	-	follicle stimulating hormone
GAR	-	goat anti-rabbit immunoglobulin
HCG	-	human chorionic gonadotropin
LH	-	luteinizing hormone
M	-	molar
Monol	-	2-amino-2 methyl-1-propanol
MW 62K	-	a fraction with a molecular weight of 62,000 daltons obtained by chromatography on Ultrogel/Sephadex
NH_4OAC	-	ammonium acetate
PMSG	-	pregnant mare serum gonadotropin
Tris	-	Tris (hydroxymethyl) aminomethane
µCi	-	microcurie
µl	-	microliter
vol	-	volume

TERMINOLOGY

In this thesis, preparations with biological activity have frequently been named according to the method of isolation rather than according to biological activity. When the terms vitellogenic and maturational hormone are used it should be noted that the maturational hormone also is implicated in vitellogenesis, at least insofar as the synthesis of estradiol is concerned. In contrast, no maturational activity yet has been attributed to the vitellogenic hormone which functions to stimulate incorporation of lipophosphoprotein into the ovary.

The terms "FSH-like" and "LH-like" have not been used because the processes these hormones regulate in mammals are not always relevant to fish. Further we believe it would be premature to name preparations according to function until more information is available on their biological action in fish.

SUMMARY TABLE I

Species	Fraction No	Fraction	Purity	Biological Activity Vit[a]	Mat[b]	And[c]	Thy[c]
Salmon	1	Con A I	NT	NT	-	NT	NT
	2	Con A I MW 25K	0.4% cross reaction with Fraction 7	+	NT	NT	NT
	3	Con A I MW 25K DE III	1 major band and 1 minor band on electrophoresis. No cross reaction with Fraction 5 0.12% cross reaction with Fraction 6	+	NT	NT	NT
	4	Con A I MW 45K	0.4% cross reaction with Fraction 6	NT	NT	NT	NT
	5	Con A I MW 45K DE III	1 electrophoretic band 0.3% cross reaction with Fraction 6	+	NT	-	NT
	6	Con A II G-75	1 broad band 0.7% cross reaction with Fraction 3. No cross reaction with Fraction 5.	NT	+	NT	NT
	7	Con A II MW 40K	NT	+	+	+	NT
	8	Con A II MW 40K DE II CM I	1 broad band	NT	NT	+	NT
	9	Con A II MW 40K DE II CM II	NT	NT	NT	-	NT

a=Vitellogenic; b=Maturational; c=Androgenic; d=Thyrotropic
NT=not tested.

SUMMARY TABLE II

Species	Fraction No	Fraction	Purity	Biological Activity			
				Vit[a]	Mat[b]	And[c]	Thy[d]
Plaice	1	Con A I MW 62K	NT	+	-	NT	NT
	2	Con A I MW 62K DE III	2% cross reaction with Fraction 9	+	NT	NT	NT
	3	Con A I MW 62K DE III CM I	NT	+	NT	-	-
	4	Con A I MW 62K DE III CM II	NT	+	NT	-	+
	5	Con A I MW 28K	NT	+	NT	NT	+
	6	Con A I MW 28K DE III	2% cross reaction with Fraction 9	+	NT	NT	NT
	7	Con A I MW 28K DE III CM I	NT	+	NT	-	-
	8	Con A I MW 28K DE III CM II	NT	-	NT	-	+
	9	Con A II MW 62K	NT	+	+	+	+

a=Vitellogenic; b=Maturational; c=Androgenic; d=Thyrotropic
NT=not tested.

SUMMARY TABLE III

Species	Fraction No.	Fraction	Purity	Biological Activity Vit[a]	Mat[b]	And[c]	Thy[d]
Flounder	1	Con A I MW 62K	NT	NT	-	NT	NT
	2	Con A I MW 62K DE III	0.2% cross reaction with Fraction 8	+	NT	NT	+
	3	Con A I MW 62K DE III CM I	NT	+	NT	-	-
	4	Con A I MW 62K DE III CM II	NT	+	NT	-	+
	5	Con A I MW 28K	NT	+	NT	NT	+
	6	Con A I MW 28K DE III CM I	1 band no cross reaction with Fraction 9	+	NT	-	-
	7	Con A I MW 28K DE III CM II	5% cross reaction with Fraction 6. No cross reaction with Fraction 9.	-	NT	-	+
	8	Con A II MW 62K	NT	+	+	NT	-
	9	Con A II MW 62K DE I CM I	2% cross reaction with Fraction 6 1 broad band	NT	NT	+	-
	10	Con A II MW 62K DE I CM II	NT	NT	NT	+	+

a=Vitellogenic; b=Maturational; c=Androgenic; d=Thyrotropic
NT=not tested.

CHAPTER I

Pituitary Control of Gonadal Function

INTRODUCTION

Role of pituitary in vertebrate reproduction

The paramount importance of the pituitary gland in the control of vertebrate reproduction has been extensively reviewed in mammals (Gemzell and Roos, 1966), in birds and reptiles (Nalbandov, 1966), amphibians (Dodd, 1960) and fish (Hoar, 1969; Lam et al., 1978; Pickford and Atz, 1957). In mammals and birds, administration of pituitary extracts is capable of reversing the deleterious changes induced in gonads by hypophysectomy (see Greep, 1961; Nalbandov, 1966). Studies in reptiles including lizards (Licht, 1972), alligators (Forbes, 1937) and turtles (Hansen and Tabb, 1941) lead to the same conclusion. In frogs testicular functions can be restored after hypophysectomy by injections of purified pituitary gonadotropins (Lofts, 1961). The processes of spermatogenesis, vitellogenesis and ovulation can be induced in hypophysectomized goldfish by a partially purified salmon pituitary gonadotropin (Yamazaki and Donaldson, 1968b).

Two separate gonadotropic fractions, one follicle-stimulating and the other luteinizing in function, were identified in the pig pituitary many years ago (Fevold, Hisaw and Leonard, 1931). Gonadotropins have since been purified from numerous mammalian species including baboon (Shownkeen et al., 1973), man (Grasslin et al., 1970), pig (Anderson and O'Grady, 1972; Whitley et al., 1978) whale (Tamura-Takahashi and Ui, 1977), horse (Braselton and McShan, 1971), and cow (Grimek et al., 1979). Two types of gonadotropins similar in functions to the mammalian hormones were isolated from chicken (Furuya and Ishii, 1974; Stockell, Hartree and Cunningham,

1969) and turkey (Farmer et al., 1975). In reptiles (Licht and Papkoff, 1974a) and amphibians (Licht and Papkoff, 1974b) two types of gonadotropins could also be separated.

The role of the pituitary in controlling reproduction in fish has been well established (Pickford and Atz, 1957), but there has been much controversy as to whether one or two gonadotropins exist in the fish pituitary. Evidence from pituitary cytology is not conclusive because there is a disparity in the number of gonadotropins in the same species of fish reported by different workers (see De Vlaming, 1974). The bulk of the existing findings is in favour of an unique omnipotent gonadotropin wielding the control of diverse aspects of gonadal functions. Based on the results of studies on fish using mammalian gonadotropins the fish pituitary appears to secrete a single LH-like or HCG-like hormone. Mammalian LH is much more effective than FSH in stimulating vitellogenesis in the three-spined stickleback Gasterosteus aculeatus (Ahsan and Hoar, 1963), in re-initiating spermatogenesis in hypophysectomized Coeusius plumbeus (Ahsan, 1966), and in methallibure-treated Cymatogaster aggregata (Weibe, 1969) and Xiphophorus helleri (Baldwin and Li, 1942). Yolky oocytes in catfish can be prevented from undergoing atresia after hypophysectomy by HCG and LH but not by FSH (Anand and Sundararaj, 1974). However, due to phylogenetic specificity of the gonadotropin molecules, HCG is incapable of stimulating vitellogenesis in the hypophysectomized goldfish Carassius auratus (Yamazaki, 1965), and mammalian LH is inactive in stimulating trout gonadal cAMP production (Idler et al., 1975c). Various reports on the isolation of gonadotropins from teleosts including carp (Burzawa-Gerard, 1971;

Sundararaj et al., 1972) salmon (Schmidt et al, 1965; Donaldson et al., 1972; Yoneda and Yamazaki, 1976), sturgeon (Burzawa-Gerard et al., 1975) trout (Breton et al., 1976) and Tilapia (Farmer and Papkoff, 1977) gave no indications of the presence of two types of gonadotropins. Only two reports (Hattingh and du Toit, 1973; Haider and Blum, 1977) were indicative of duality of gonadotropins in mudfish and goldfish respectively.

Hormonal control of vitellogenesis and oocyte maturation in oviparous vertebrates

In oviparous vertebrates, reproduction in the female comprises the processes (a) vitellogenesis and (b) oocyte maturation and ovulation.

(a) Vitellogenesis

This is the process by which yolk is incorporated into the ovary which as a result grows in size. Yolk serves as a nutrient reserve for the early developmental stages of the embryo. The vitellogenic process occurs in both vertebrates and invertebrates. In the vertebrates it is controlled by the pituitary, whereas in the insects it is regulated by the corpora allata and in the crustaceans by secretions from the eyestalk (see Follett and Redshaw, 1974).

In the domestic fowl at the onset of lay, phosphoproteins which are precursors of ovarian yolk proteins, appear in the plasma. In the toad Xenopus laevis the precursor is called vitellogenin. The hepatic origin of these yolk precursors has been demonstrated by hepatectomy experiments and also by hepatic synthesis in vitro. The precursor role of these serum phosphoproteins can be followed with the use of radioactive phosphate. The label is first incorporated

into the liver yolk precursor protein, and then into the plasma and finally egg yolk (Follett and Redshaw, 1974).

In fish, vitellogenin has been induced in the circulation by estradiol. Plack et al. (1971) injected cod Gadus morhua with estradiol and observed an accumulation of vitellogenin in serum. De Vlaming et al. (1977) noted a similar phenomenon in the goldfish. Aida et al., (1973) related the appearance of vitellogenin in blood of Plecoglossus altivelis to the first appearance of yolk in oocytes. Emmersen and Petersen (1976) demonstrated the presence of vitellogenin in serum of vitellogenic female and estrogenized male flounder Platichthys flesus. Vitellogenin was first quantified by radioimmunoassay in the brown trout Salmo trutta (Crim and Idler, 1978) and Atlantic salmon (Idler et al., 1979). There is a correlation between the plasma titer of vitellogenin and ovarian development in trout (Crim and Idler, 1978) and Atlantic salmon (Idler et al., 1979). Idler et al. (1979) used a low molecular weight fraction (phosvitin) isolated from salmon egg yolk for radioiodination and as standard, and an antibody against salmon egg yolk proteins to set up a radioimmunoassay for plasma vitellogenin.

The synthesis of yolk precursors by the liver is under the control of estrogen and hence they are female specific proteins which are virtually absent in males (Emmersen and Petersen, 1976; Idler et al., 1979). Production of vitellogenin in cockerels (Jackson et al., 1977), male Xenopus (Follett and Redshaw, 1974) and male flounder (Emmersen and Petersen, 1976), similar to the female protein, can be induced specifically by estrogen.

In Xenopus (Follett and Redshaw, 1974) and cod (Plack et al., 1971), estrogen administration results in an accumulation of vitellogenin in

blood, but there is no vitellogenic growth of the ovary presumably because of a negative feedback effect of estrogen on pituitary gonadotropin secretion.

In Xenopus, mammalian gonadotropins stimulate estrogen production by the ovary and therefore vitellogenin synthesis by the liver, as well as incorporation of vitellogenin into the ovary (Emmersen and Kjaer, 1974; Follett and Redshaw, 1974). Xenopus vitellogenin is a lipophosphoprotein with a molecular weight of about 460,000 daltons. Once incorporated into the ovary, it is broken down into the yolk platelet proteins, phosvitin and lipovitellin. Phosvitin is a phosphoprotein, with a high phosphorus content due to the large number of phosphorylated serine residues and a low lipid content. Lipovitellin, on the other hand, contains much less phosphorus but much more lipid. It is postulated that vitellogenin is formed by the covalent complexing of two molecules of phosvitin (MW 40,000) with a lipovitellin dimer (MW 400,000) (Follett and Redshaw, 1974). They speculate that gonadotropins stimulate the incorporation of vitellogenin into the ovary with the following actions (i) recruitment of previtellogenic oocytes to the vitellogenic phase (ii) development of microvilli on the oolemma (iii) provision of vitellogenin binding sites to promote micropinocytosis (iv) dilation and development of ovarian thecal capillary network (v) molecular rearrangement of vitellogenin to form lipovitellin and phosvitin.

(b) Oocyte Maturation and Ovulation

In oogenesis, vitellogenesis occurs during an extended stage of the meiotic prophase. When vitellogenesis is completed the meiotic division is resumed to produce a mature egg, capable of

being fertilized after being extruded from the follicle (ovulation).

Throughout maturation, the germinal vesicle in the oocytes migrates from its original central position toward the periphery. After it has reached the periphery the germinal vesicle breaks down. Lipid droplets coalesce in the cytoplasm with the result that the oocyte increases in transparency. After the oocyte has undergone germinal vesicle breakdown and cleared, it is ovulated.

Steroids and gonadotropins are well-known agents for inducing oocyte maturation and ovulation. Among the steroids, estrogens are not effective in inducing germinal vesicle breakdown. Androgens are the second least active, followed by 11-oxycorticosteroids which, however, are very active in some fish. The most effective steroids are either 11-deoxycorticosteroids (Sundararaj and Goswami, 1977) or progestagens. In the rainbow trout, goldfish and pike, the progestagen 17α-hydroxy-20β-dihydroprogesterone is the most effective steroid both in vitro and in vivo (Fostier et al., 1973; Jalabert, 1976). The steroid was first identified by Schmidt and Idler (1962) in plasma of sexually mature salmon but not in immature fish. Another progestagen, 17α-hydroxyprogesterone was also discovered by Schmidt and Idler (1962). Campbell et al., (1976) found that winter flounder oocytes matured in donor plasma containing higher concentrations of 17α-hydroxyprogesterone and that maturing females had a higher concentration of this steroid in blood, suggesting a maturational role of the progestagen.

The mechanism of action of steroids on oocyte maturation and ovulation has been studied using afollicular oocyte (Jalabert, 1976). Results suggest that steroid interaction with the follicular

layer is necessary for final maturation in some species while in others it is not, but it is quite possible that removal of the follicular layer may affect other processes as well.

In most fish, mammalian FSH and HCG are either ineffective or only slightly effective, but gonadotropin preparations from salmon and carp pituitary glands are active (Jalabert et al., 1973; 1974), in inducing oocyte maturation.

Three mechanisms have been proposed to explain the actions of gonadotropins on oocyte maturation and ovulation.

(i) A gonadotropin - ovary axis

Gonadotropin first interacts with the follicular layer of an oocyte to produce a steroid which induces maturation. This is substantiated by the finding in some species that induction of maturation in afollicular oocyte has not been met with success.

(ii) A gonadotropin - interrenal - ovary axis

This mechanism was suggested by Sundararaj and Goswami (1977) to account for the observation that gonadotropins were unable to induce maturation of catfish ova in vitro unless interrenal tissue was added but were able to do so in vivo when administered to hypophysectomized catfish.

(iii) Steroid enhanced interrenal - ovary axis

Jalabert (1976) suggested that interrenal corticosteroids might enhance oocyte maturation induced by progestagens.

Hormonal control of testicular steroidogenesis and spermiation

The steroidogenic activities of various vertebrate gonadotropin preparations on diversevertebrate testicular tissues have been investigated (Farmer et al., 1977; Lance et al., 1977; Licht et al.,

1977b; Jenkins et al., 1978). In the goldfish, hypophysectomy leads to an atrophy of interstitial cells in the testis (Yamazaki and Donaldson, 1968a) and a decrease of 3β-hydroxysteroid dehydrogenase activity in the interstitium (Yamazaki and Donaldson, 1969). The interstitial hydroxysteroid enzyme activity can be restored by treatment with SG-G100, a partially purified preparation of salmon gonadotropin (Donaldson et al., 1972) but as Yamazaki and Donaldson (1968a) pointed out, measurement of 3β-hydroxysteroid dehydrogenase activity alone is not an adequate parameter of steroidogenic activity. There are also a few reports on the effect of teleost gonadotropin preparations on steroid production by various tetrapod testicular tissues. Tilapia LH is active in stimulating testosterone production by rat Leydig cells in vitro (Farmer and Papkoff, 1977). Gonadotropin preparations from salmon (Donaldson et al., 1972) and carp (Burzawa-Gerard, 1971) release androgen from testicular cells of Japanese quail in vitro (Jenkins et al., 1978), but the salmon gonadotropin preparation SG-G100 has no effect on male turtles in vivo (Lance et al., 1977). However, knowledge of the steroidogenic effects of purified fish gonadotropins in fish is completely lacking. Furthermore, androgen production in fish follows a pathway distinct from that in mammals (Idler et al., 1961; Idler and McNab, 1967).

Spermiation, or expulsion of spermatozoa, was completely inhibited in the goldfish after hypophysectomy but was initiated by treatment with a partially purified salmon gonadotropic preparation (Yamazaki and Donaldson, 1968b). The lack of an inhibitory effect of hypophysectomy on spermiation in plaice (Barr, 1963) and lake chub (Ahsan, 1966) was attributed to the usage of fish which had already

shown signs of spermiation before hypophysectomy (Yamazaki and Donaldson, 1968). Androgens were found to be effective in induction of spermiation (Yamazaki and Donaldson, 1968a).

Statement on the Research Problem

Until 1975, data from chemical fractionation studies and bioassays supported the concept that the teleost pituitary elaborated a single gonadotropin which controlled all phases of the reproductive cycle including vitellogenesis, oocyte maturation and ovulation, spermatogenesis, androgen production, and spermiation (Donaldson, 1973). However, histological studies on the teleost pituitary revealed more than one type of gonadotrope in some fish (see Reinboth, 1972) and Reinboth (1972) stressed that histological findings should not be overlooked. A more highly purified glycoprotein gonadotropin was obtained from chum salmon (O. keta) pituitaries by Idler et al. (1975 a,b) who first applied affinity chromatography on Concanavalin A (Con A)-Sepharose to isolate fish pituitary hormones. Pierce et al., (1976) employed affinity chromatography on Con A-Sepharose to purify further Donaldson's SG-G100 preparation and found substantial amounts of Con A I material in the hormone preparation, in accordance with the observation by Idler et al. (1975a) that ethanolic fractionation did not yield a Con A II gonadotropic fraction free of Con A I material.

A partially purified preparation of plaice vitellogenic hormone was achieved by Campbell and Idler (1976) using one-step affinity chromatography on Con A-Sepharose and chromatography on Sephadex G-75. Another gonadotropin was found in the fraction adsorbed on Con A-Sepharose. This fraction possessed no vitellogenic activity

(Campbell and Idler, 1976) but induced oocyte maturation and ovulation. A proposition for the elaboration of two types of gonadotropins by the plaice pituitary seemed opportune. However, these findings were complicated by the observation that the vitellogenic hormone preparation also contained an appreciable amount of maturational and ovulatory activities which were suggested to be due to contamination with the maturational gonadotropin (Campbell and Idler, 1977).

When the present work was initiated in 1976, there were available several techniques which could be developed into bioassays for gonadotropic activities. Campbell and Idler (1976, 1977) laid down the ground work for this thesis by developing a technique of hypophysectomizing the winter flounder, and bioassays for vitellogenic activity and oocyte maturational activity. The assay for vitellogenic activity is based on the work of Follett and Redshaw (1974) on amphibian vitellogenesis and on the work of Plack et al., (1971) on cod vitellogenesis. The assay for maturational activity is based on the methodology mentioned in Sundararaj and Goswami (1977). Idler et al., (1975 a,b) developed chromatographic techniques on Con A-Sepharose, Sephadex G-75, and DEAE Bio-Gel A. Without these techniques the present work would have been impossible because fish gonadotropins are very often inactive in mammalian bioassay systems (Donaldson et al, 1972; Idler et al., 1975). Mature winter flounder are usually available all year round. One of the goals of this investigation was to prepare the plaice gonadotropins in a purer form in order to facilitate interpretations of bioassay results and to further study both gonadotropins. Since the teleosts are a heterogeneous assemblage of piscine species, it was decided to purify gonadotropins

from the salmon and winter flounder pituitary glands as well, in order to ascertain whether the phenomenon of duality of gonadotropin could be extended to other species. The question of the number of gonadotropins in fish is an important one in comparative endocrinology since it has only been recently reported that two different types of gonadotropins, designated FSH and LH, can be isolated from the pituitaries of some, but not all, representative of the classes Mammalia, Aves, Reptilia and Amphibia (Licht et al., 1977b). The establishment of two types of gonadotropins depends on a comparison of chromatographic behaviour, physicochemical properties, biological and immunological activities of the gonadotropic fractions. Studies on the biological activities of the two types of gonadotropins would lead to a better understanding of the gonadotropic control of vitellogenesis, oocyte maturation, androgen production and spermiation because previous investigations utilizing partially purified gonadotropic preparations could not attribute the observed actions to one or other of the gonadotropins.

MATERIALS AND METHODS

Preparation of pituitary hormones

Pituitary glands of the American plaice Hippoglossoides platessoides were collected at Bonavista Cold Storage, Fermeuse, Newfoundland, and kept frozen at -80°C until they were processed. A batch collected in September was used for assay of vitellogenic activity, whereas hypophyses procured in June were utilized for maturation and ovulation studies. Pituitaries were collected in June from mature winter flounder Pseudopleuronectes americanus.

Chum salmon Oncorhynchus keta were caught in Johnstone Straits,

British Columbia, in October and were processed at Canadian Fishing Co., Vancouver, British Columbia. Gonadosomatic index of the male fish averaged about 4% and that of the female, about 12%. It is estimated that the fish were still a few weeks from spawning. The pituitaries were collected, frozen on dry ice, shipped, and stored at -80°C until extracted.

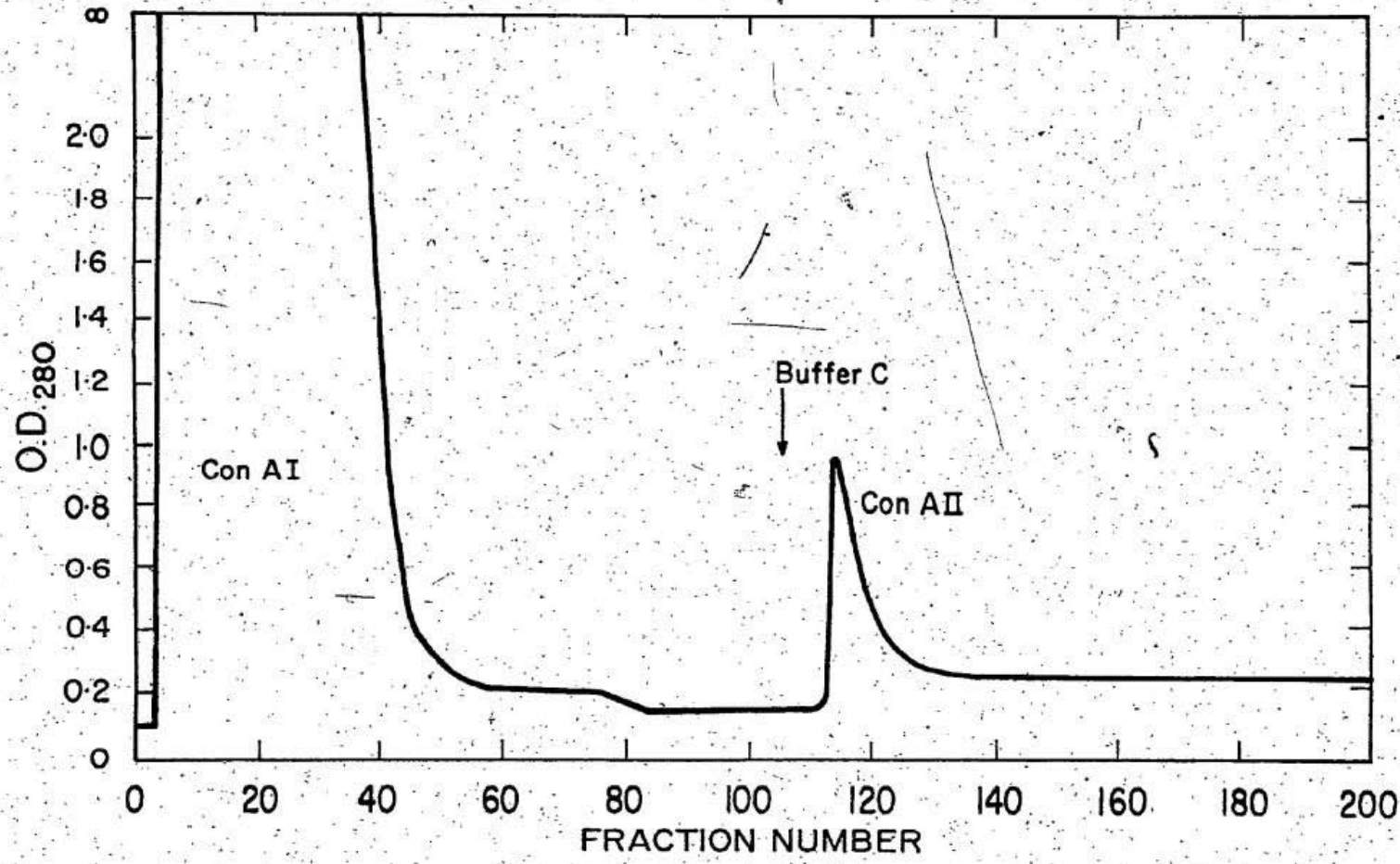
(a) Chromatography on Con A-Sepharose

Pituitaries were extracted and re-extracted with 4 vol. of Buffer B (0.05 M Tris-Cl, pH 7.8, 0.5 M NaCl, 0.2 mM dithiothreitol (DTT), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 3% Trasylol*) before being applied to a column containing Con A-Sepharose (Pharmacia) previously equilibrated with the same buffer. The column was eluted at a flow rate of 25 ml/hr., first with Buffer B and subsequently with Buffer C (0.05 M Tris-Cl, pH 7.8, 0.5 M NaCl, 0.2 mM DTT, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 3% Trasylol, 0.15 M α-methyl-D-glucoside) to yield the unadsorbed Con A I and the adsorbed Con A II fractions (Fig. 1), respectively, as described by Idler et al. (1975b). The Con A I fraction was rechromatographed on Con A-Sepharose to get rid of any contaminating Con A II material. The Con A II fraction which was held on Con A-Sepharose contains glycoproteins with α-D-glucopyranosyl end groups or internal 2-o-linked-D-mannopyranosyl residues, whereas the unadsorbed fraction designated as Con A I is comprised of non-glycoproteins and glycoproteins devoid of the appropriate residues (Goldstein et al., 1965).

(b) Gel filtration

*Trasylol was purchased from Boehringer Ingelheim Canada Ltd., Dorval, Quebec. It is a protease inhibitor. It has a molecular weight of 6512 and consists of a chain of 58 amino acid radicals representing 15 different amino acids.

FIG. 1 Fractionation of American plaice pituitary extract on Con A-Sepharose. Fraction size was 7 ml.


O.D.280
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0
20
40
60
80
100
120
140
160
180
200
FRACTION NUMBER
Con AI
Buffer C
Con AII

The Con A I and Con A II fractions were concentrated by ultrafiltration, dialyzed against the column buffer used in the next chromatographic step, and centrifuged before being applied on two Ultrogel AcA 54 or AcA 44 (LKB) columns (2.5 x 90 cm) connected in series and eluted in the ascending fashion at a flow rate of 18 ml/hr. For the Con A I fraction, eluates from the Ultrogel double column were pooled separately into three fractions A, B, and C with molecular weight ranges of $\geq 160 \times 10^3$, $160 \times 10^3 - 45 \times 10^3$, and $45 \times 10^3 - 10 \times 10^3$, respectively (Fig. 2). In later runs, Sephacryl S-200 Superfine (Pharmacia) was substituted for Ultrogel AcA 54 to achieve a fast preliminary separation at a flow rate of 75 ml/hr. The fractions were then ultrafiltered and chromatographed on Bio-Gel A-0.5 m (200-400 mesh) (Bio-Rad), Ultrogel AcA 44, or Ultrogel AcA 54 until peaks which appeared to be homogeneous were obtained (Figs. 3,4). For the Con A II fraction, eluates collected from the Ultrogel column with molecular weights falling in the range $20 \times 10^3 - 160 \times 10^3$ were pooled, ultrafiltered, and chromatographed on two Ultrogel AcA 54 columns connected in series (1.5 x 89 cm each). Rechromatography was carried out until symmetrical peaks were obtained.

Buffer G (0.05 M Tris-Cl, pH 7.8, 0.5 M NaCl, 0.2 mM DTT, 5 mM EDTA, 3% Trasylol) was used for gel filtration on Bio-Gel A-0.5 m (200-400 mesh, Bio-Rad). For chromatography on Ultrogel AcA 44 and Ultrogel AcA 54 Buffers D (0.05 M Tris-Cl, pH 7.8, 0.15 M NaCl, 0.1 mM DTT, 1 mM EDTA, 3% Trasylol). E (0.05 M Tris-Cl, pH 7.8, 1 M NaCl, 0.3 mM DTT, 1 mM EDTA, 3% Trasylol), and F (0.05 M Tris-Cl, pH 7.8, 0.5 M NaCl, 0.2 mM DTT, 10 mM EDTA, 3% Trasylol) were employed to investigate the effects of various concentrations of NaCl, DTT, and EDTA on the

FIG. 2 Profile of elution of plaice Con A I fraction from Ultrogel AcA 44. Fraction size was 8 ml.

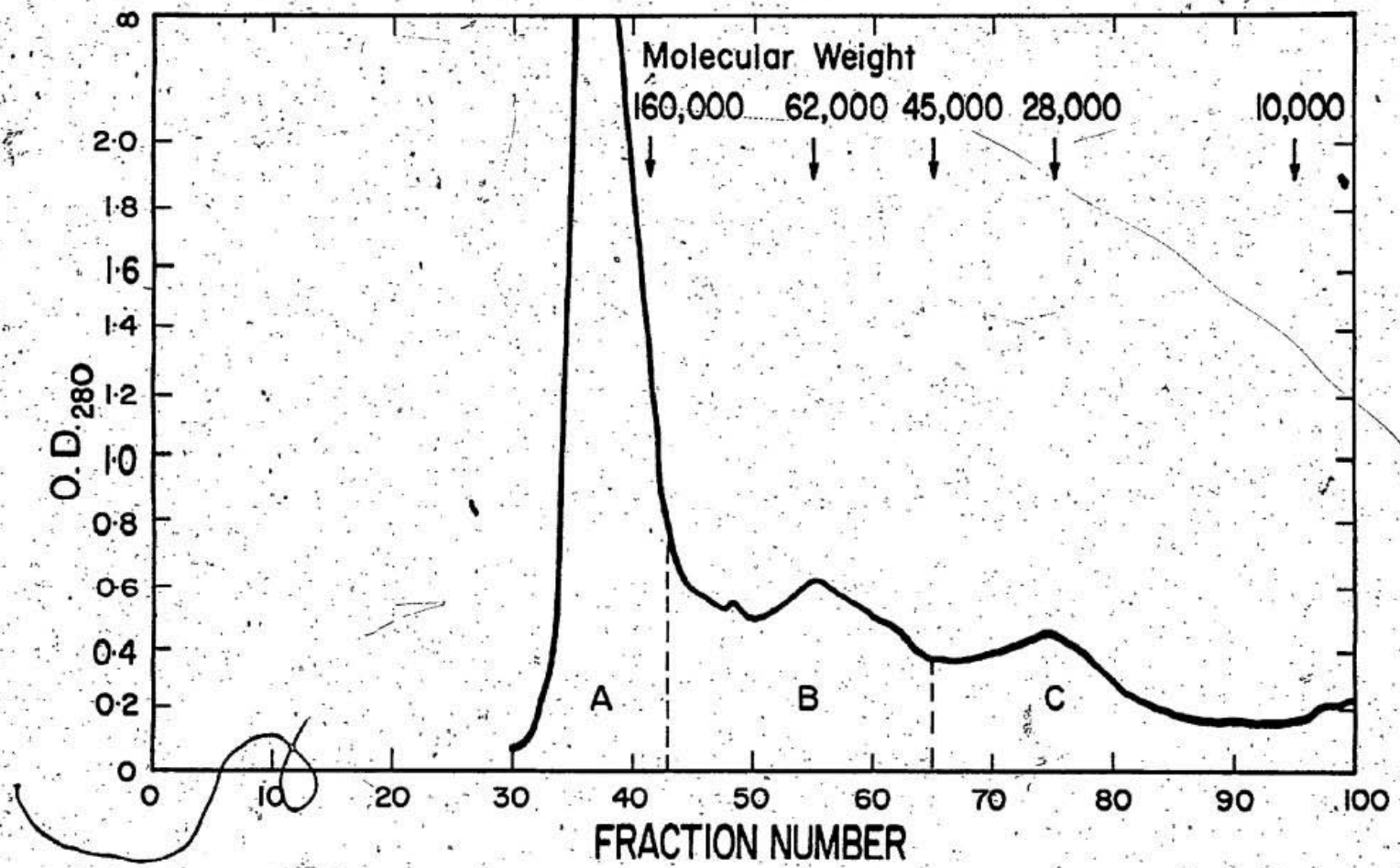

Molecular Weight
160,000
62,000
45,000
28,000
10,000
O.D.280
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
A
B
C
0
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

FIG. 3 Chromatography of plaice Con A I MW 62,000 fraction on Ultrogel AcA 44. Fraction size was 6 ml.

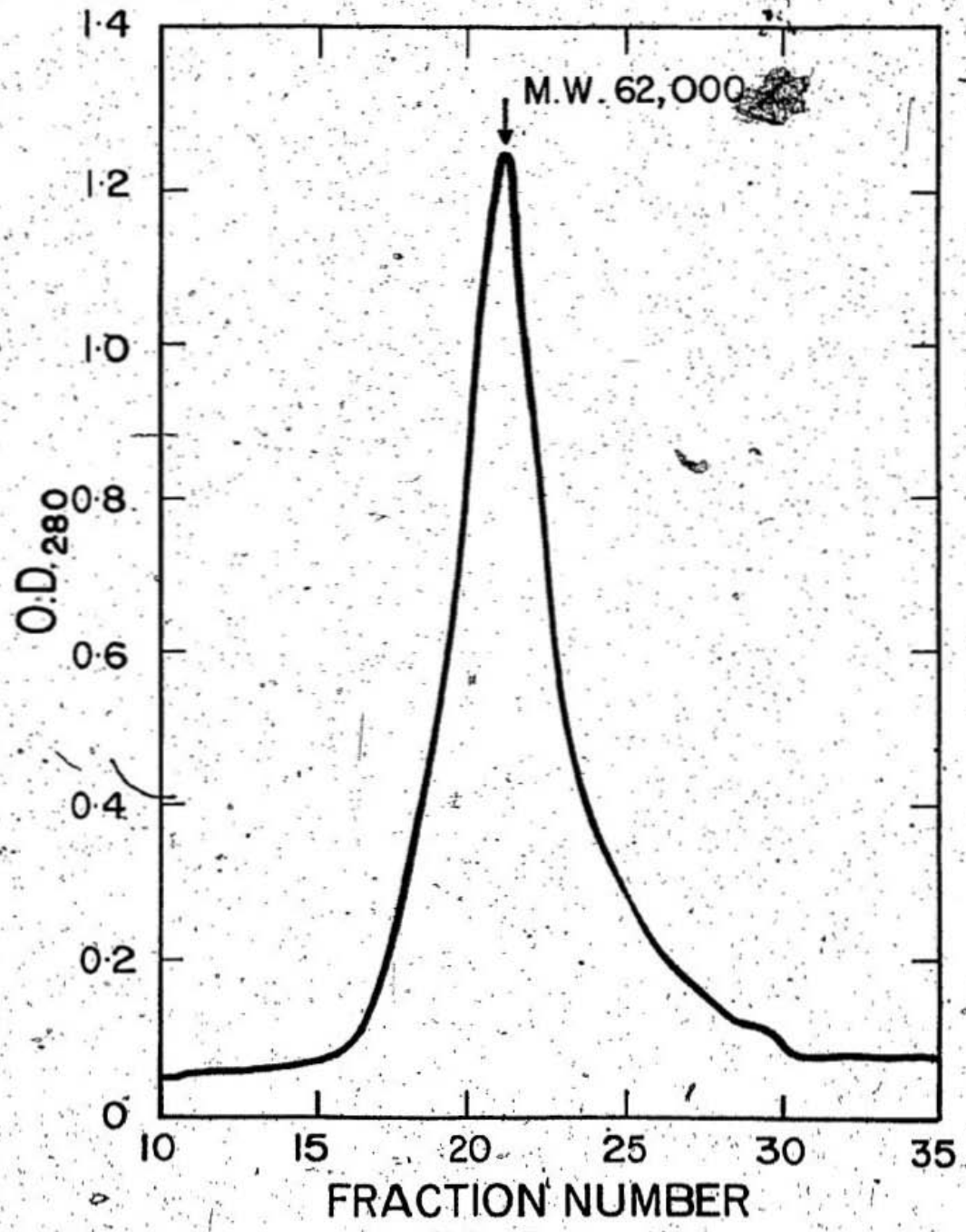
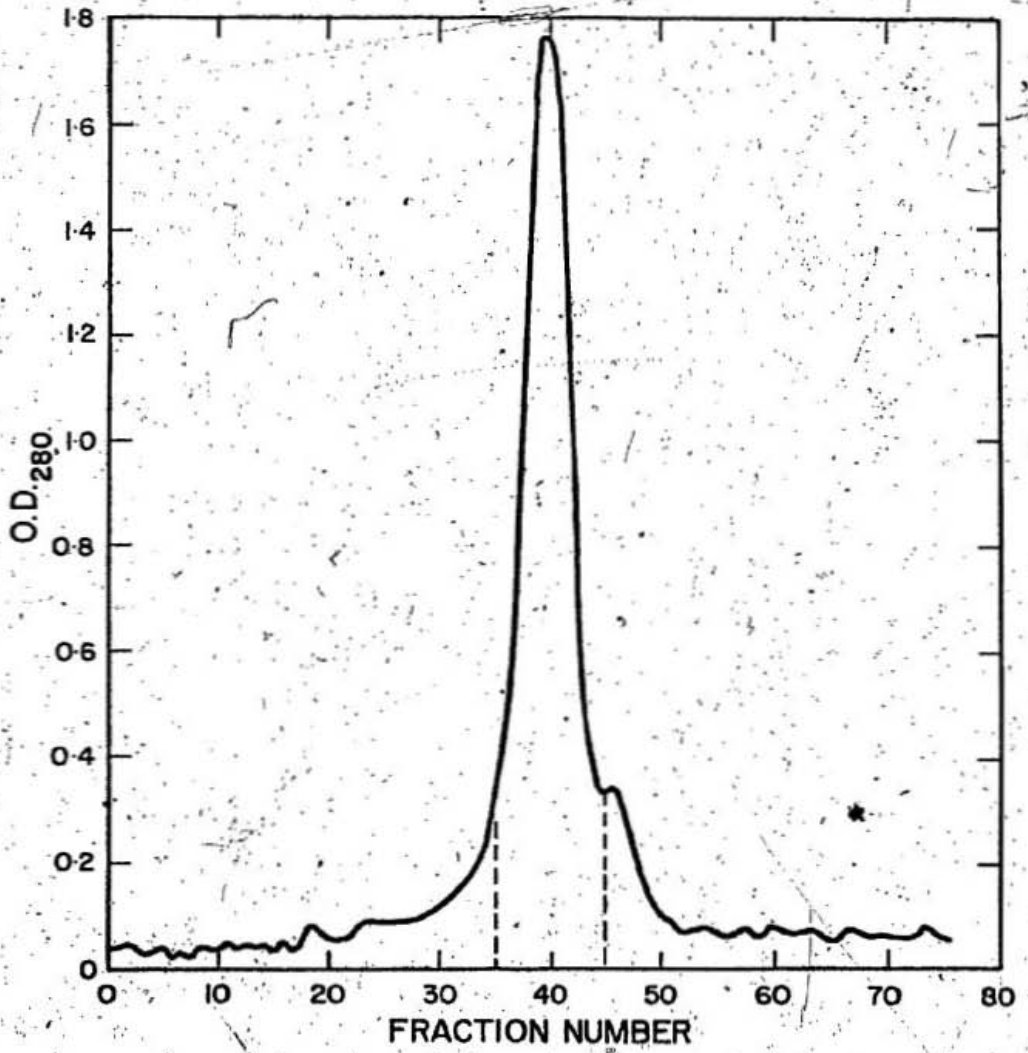

M.W. 62,000
O.D.280
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
10
15
20
25
30
35
FRACTION NUMBER

FIG. 4 . Chromatography of plaice Con A I MW 28,000 fraction on Bio-Gel A 0.5 m. Fraction size was 3 ml. The dotted lines represent the way the fractions were pooled for use in bioassay.


1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
O.D.280
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

possible interconversion of different molecular weight species.

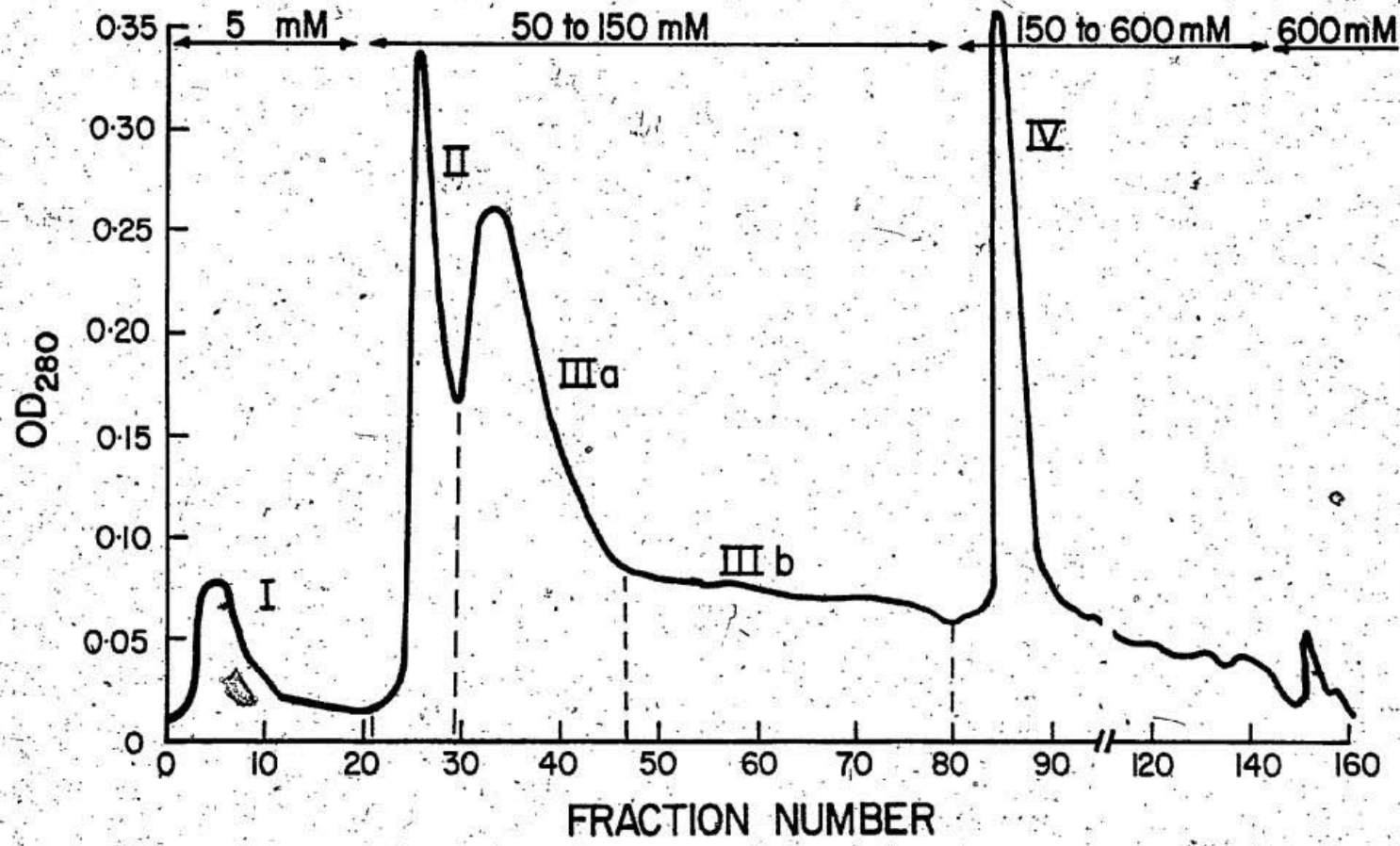
(c) Ion exchange chromatography

Subfractions of the Con A I fractions with appropriate molecular weight were pooled, ultrafiltered and extensively dialyzed against 5 mM NH_4HCO_3, pH 9, over a period of 3 days before being chromatographed on DEAE Bio-Gel A (Bio-Rad). The ion exchanger was fully regenerated with a saturated solution of NH_4HCO_3, followed by 1 M NH_4HCO_3, and then equilibrated with 5 mM NH_4HCO_3 over a period of several days before use. Unadsorbed proteins were eluted by 5 mM NH_4HCO_3. Adsorbed proteins were eluted by a linear gradient of 50 to 150 mM NH_4HCO_3 (mixing volume 250 ml : 250 ml) followed by a second gradient of 150 to 600 mM NH_4HCO_3 (mixing volume 250 ml : 250 ml). Strongly adsorbed proteins were eluted by 1 M NH_4HCO_3. All fractions were collected at 8 ml/hr from a 0.9 x 27 cm column (Figs. 5, 6).

The vitellogenic Con A I fractions (DE III fraction, Fig. 5) from the DEAE ion exchanger were further processed, after extensive dialysis against 3 mM ammonium acetate at pH 6, through a column of CM Bio-Gel A (Bio-Rad) equilibrated with the same buffer. Unadsorbed proteins were eluted with the starting buffer. Adsorbed proteins were eluted with 1M ammonium acetate (pH 6). The pattern of the elution profile is similar to that from Con A-Sepharose (Fig. 1) i.e., a big broad unadsorbed peak and a small sharp adsorbed peak.

The salmon maturational Con A II G-75 fraction II was prepared as described by Idler et al. (1975a). Fractions corresponding to the ascending half of the biologically active

FIG. 5 Profile of elution of 26 mg plaice Con AI MW 62,000 fraction from DEAE Bio-Gel A. Yields of DE I, II, III a, III b and IV were respectively 1.2, 1.83, 3.73, 3 and 4 mg. Fraction size was 3.5 ml.


5 mM
50 to 150 mM
150 to 600 mM
600 mM
0·35
0·30
0·25
0·20
0·15
0·10
0·05
0
OD280
I
II
III a
III b
IV
0
10
20
30
40
50
60
70
80
90
120
140
160
FRACTION NUMBER

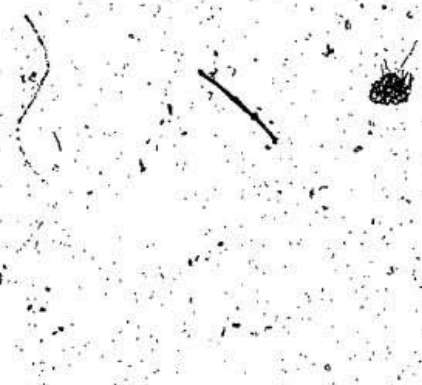
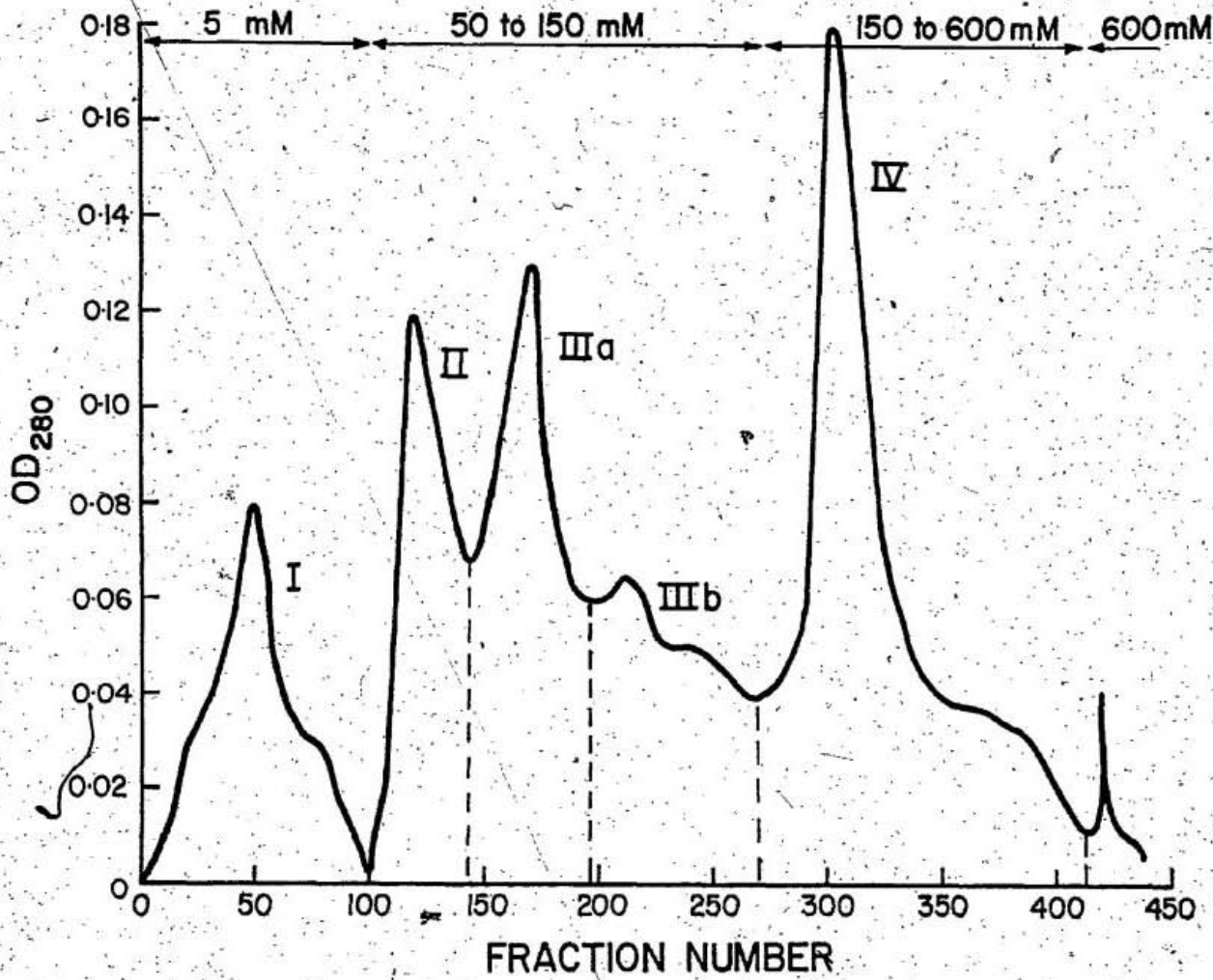


FIG. 6 Profile of elution of 21 mg plaice Con A I MW 28,000 fraction from DEAE Bio-Gel A. Yields of DE I, II, III a, III b and IV were respectively 1.2, 2.1, 2.6, 2.0 and 5.9 mg. Fraction size was 1.3 ml.


5 mM
50 to 150 mM
150 to 600 mM
600 mM
0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
OD280
I
II
IIIa
IIIb
IV
0
50
100
150
200
250
300
350
400
450
FRACTION NUMBER

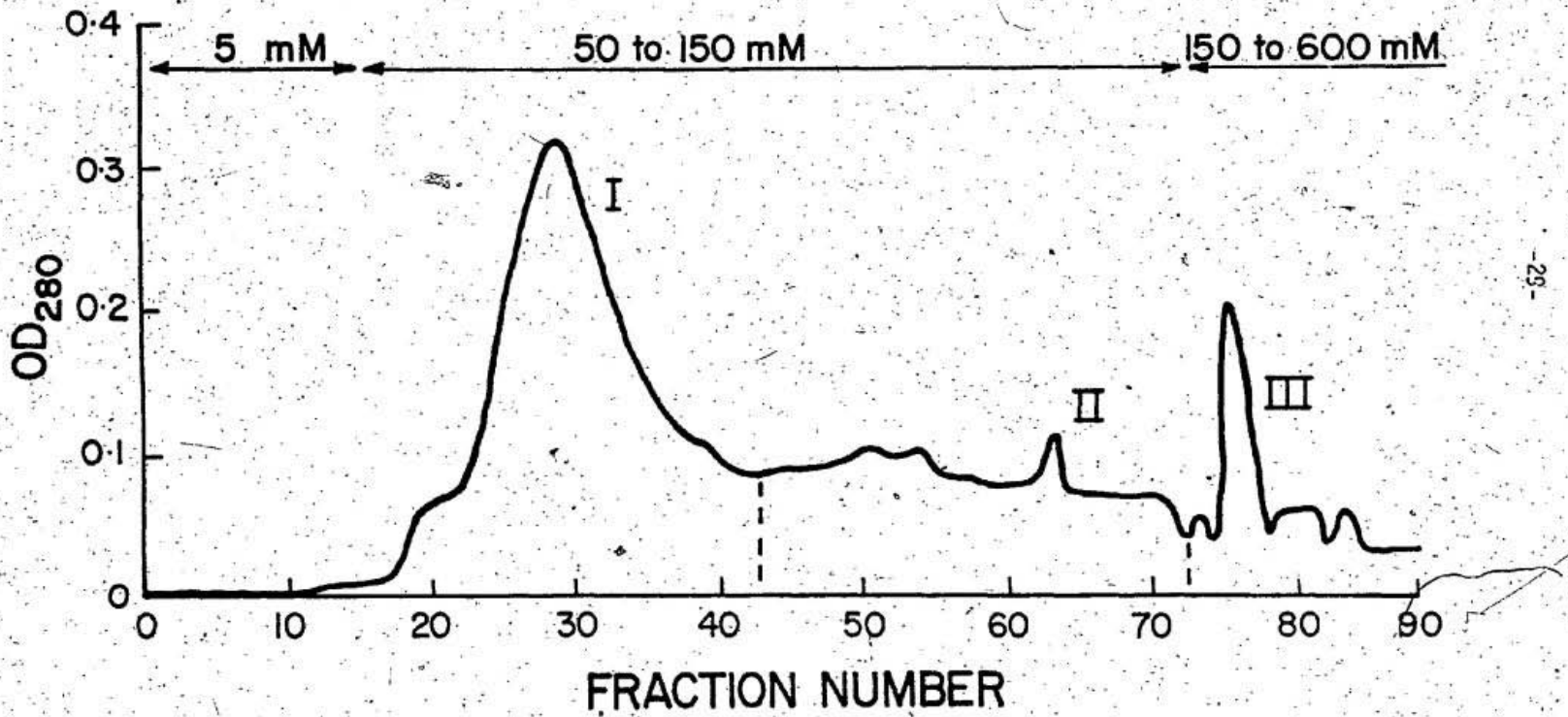
peak (MW 40,000 daltons) were pooled to form Con A II G-75 Fraction II as described by Idler et al. (1975a) and used for radioimmunological and chemical characterization studies without further purification on the DEAE ion exchanger, while the whole peak designated Con A II MW 40,000 fraction was used for vitellogenesis and oocyte maturation assays. Chromatography of salmon Con A II MW 40,000 fraction on DEAE Bio-Gel A to produce two peaks with gonadotropic activity, which were designated DE II and DE III in this study had been described by Idler et al. (1975b). When DE II and DE III were chromatographed on CM Bio-Gel A, adsorbed proteins were first eluted with 100mM ammonium acetate to obtain CM II and then with 2M ammonium acetate to yield CM III. In both cases CM I (unadsorbed fraction) constituted the bulk (≥ 90%) of the material in DE II and DE III fractions.

The plaice maturational Con A II MW 62,000 DE I fraction (Fig. 7) was prepared as described above for the vitellogenic hormone. The flounder maturational Con A II MW 62,000 DE I fraction was similarly purified.

Salmon Con A II MW 40,000 fraction, Con A I MW 25,000 fraction and Con A I MW 45,000 fraction were also chromatographed on Lentil lectin-Sepharose 4B (Pharmacia) which has a carbohydrate specificity similar to Con A-Sepharose (Allen et al., 1976) but a lower affinity (Stein et al., 1971). The column was eluted in the same manner as for Con A-Sepharose.

Salmon Con A I MW 25,000 fraction and Con A I MW 45,000 fraction were also chromatographed on wheat germ Lectin-Sepharose 6 MB (Pharmacia) and Helix Pomatia Lectin Sepharose 6 MB (Pharmacia). Unadsorbed proteins were eluted with Buffer B. Proteins adsorbed

FIG. 7 Profile of elution of 27 mg plaice Con A II MW 62,000 fraction from DEAE Bio-Gel A. Yields of DE I, II and III were respectively 11.2, 3.5 and 2.0 mg. Fraction size was 4.5 ml.


0·4
0·3
0·2
0·1
0
5 mM
50 to 150 mM
150 to 600 mM
I
II
III
OD280
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

on wheat germ Lectin-Sepharose were eluted with Buffer B containing N-acetylglucosamine at a concentration of 100 mg/ml. Proteins adsorbed on Helix Pomatia Lectin-Sepharose were eluted with Buffer B containing N-acetylgalactosamine (10 mg/ml).

Animals for bioassays

Mature female winter flounder Pseudopleuronectes americanus were collected by divers from September to January, the period during which the fish ovaries were actively undergoing vitellogenesis, and from February to March when the oocytes were maintained in a yolky state. These animals were used for studies on vitellogenesis while those caught in May and June, the normal spawning period, were used in the maturation and ovulation assay. Flounder were hypophysectomized in accordance with the technique described by Campbell and Idler (1976) and then allowed to recover in 33% seawater at 10°C.

Assay of vitellogenic activity in the pituitary hormone preparations

The assays were based on the work on amphibian vitellogenesis which revealed that mammalian gonadotropins could stimulate an ovarian uptake of lipophosphoprotein synthesized by the liver under estrogenic influence (Follett et al., 1968; Wallace and Jared, 1969).

Fish were used approximately 2 weeks after hypophysectomy. An intramuscular injection of estradiol benzoate in peanut oil (1.5 mg/kg) was given. Then an intraperitoneal injection of an aqueous solution of [^{3}H] leucine and $H_3{}^{33}PO_4$ (New England Nuclear) (2 µCi of each isotope/100 g of fish body weight) was administered on the first day. Three days later the fish received the first of a series of four daily injections of either the pituitary hormone preparation or buffer. They were sacrificed 72 hr after the last injection.

Duplicate 100- to 200-mg pieces of the ovaries were weighed out with a Cahn electrobalance; care was taken not to include the ovarian wall. They were digested in 2 ml of Protosol (New England Nuclear) at 55°C before counting for both ^{3}H and ^{33}P in 15 ml of Liquifluor (New England Nuclear) in a Packard Tri-Carb Model 2003 liquid scintillation spectrometer. Separate duplicate 100- to 200-mg pieces were homogenized directly in scintillation vials in 12% trichloroacetic acid with a Polytron homogenizer. The homogenates were incubated at 85°C for 20 min to eliminate any aminoacyl transfer RNAs from the precipitate (Roberts and Peterson, 1973). The precipitates which consisted of proteins and possibly some nucleic acids, were recovered on centrifugation at 2000 g for 45 min, subjected to Protosol digestion and then counted in Liquifluor as mentioned above. A 3- to 4-g piece was also excised from each ovary and homogenized in 4 vol of 0.5 M NaCl-5 mM EDTA (Plack et al., 1971), the yolk extraction medium. Centrifugation and re-centrifugation of the homogenates at 15,000 g for 2 hr in a Sorvall SS-3 automatic super-speed centrifuge yielded a supernatant, an aliquot of which was taken and dialyzed against three daily changes of 30 vol of 5 mM $CaCl_2$. Contents of the dialysis sacks were transferred into counting vials, the sacks were thoroughly rinsed with 5 mM $CaCl_2$ into the vials, and the combined non-diffusable material and washings in the vials were centrifuged at 2000 g for 45 min. The supernatant was aspirated off, and the phosphoprotein precipitate was dissolved in 3 ml of the 0.5 M NaCl-5 mM EDTA extraction medium before 10 ml of Aquasol (New England Nuclear) was added for scintillation counting. Disintegrations per minute per unit weight of ovary were calculated allowing for decay of

radioactivity, spillage of radioactivity across channels, and color quenching.

This assay was used to monitor vitellogenic activity of all fractions in the isolation of vitellogenic hormones from pituitaries of plaice, flounder and salmon.

Chromatography of phosphoprotein precipitate derived from flounder ovary

In the assay for vitellogenic activity of a hormone preparation, incorporation of leucine and phosphorus into the ovarian phosphoprotein precipitate was followed by radioactive tracers. Studies were made on the chemical characteristics of proteins in this precipitate in order to verify the validity of the assay method.

In each of the following runs the precipitate was dissolved in the buffer previously used to equilibrate the column, dialyzed against the same buffer, centrifuged and the supernatant was then applied to the column.

(i) Cellex T (TEAE cellulose, Bio-Rad)

The column was equilibrated with 2.5 mM citric acid - 15 mM Monol, pH 9.9. Unadsorbed proteins were eluted with the same buffer. Adsorbed proteins were then eluted with a linear gradient to 250 mM citric acid - 750 mM Monol, and finally with 250 mM citric acid - 750 mM Monol.

(ii) DEAE Bio-Gel A (Bio-Rad) and CM Bio-Gel A (Bio-Rad)

The columns were equilibrated with 50 mM Tris Cl-5 mM EDTA, pH 7.8. Unadsorbed proteins were eluted with the same buffer. Adsorbed proteins were eluted with a linear gradient of 50 mM-Tris Cl-5 mM EDTA to 50 mM-Tris Cl-5 mM EDTA-2 M NaCl.

(iii) Bio-Gel HTP (Hydroxyapatite, Bio-Rad) and Amberlite CG50 (BDH)

The columns were equilibrated with 10 mM potassium phosphate, pH 6.8. Unadsorbed proteins were eluted with the starting buffer. Adsorbed proteins were eluted with a linear gradient of 10 mM to 1 M potassium phosphate pH 6.8.

(iv) Con A-Sepharose (Pharmacia)

Conditions of the run were the same as described in (a).

(v) Sephacryl S200 Superfine (Pharmacia) followed by Ultrogel AcA 22 and Ultrogel AcA 54 (LKB)

Conditions of chromatography were as described in (b). Bovine gamma globulin (MW 205,000) urease (MW 490,000), thyroglobulin (MW 670,000) and blue dextran, were used to calibrate the Ultrogel AcA 22 column.

Determination of alkali-labile phosphorus content of flounder ovarian phosphoproteins

The method of Chen et al., (1956) was followed. Fractions from chromatographic method (v) were analyzed.

Assay for activity of incorporating serum phosphoprotein(s) into the water-insoluble (lipophosphoprotein) yolk fraction of the ovary

In January, mature male flounder were hypophysectomized. Five weeks after hypophysectomy each fish was given an injection of 15 mg estradiol-17β and 70 μCi $H_3{}^{33}PO_4$ (New England Nuclear). Eight days after the injection a pool of serum collected from all the fish was placed in a dialysis tubing (mol. wt. cut off 12,000-14,000) and dialyzed against a dilute saline solution with frequent changes to eliminate free $H_3{}^{33}PO_4$ and ^{33}P-labeled proteins of low molecular

weight, until the saline solution contained very little radioactivity. An aliquot of the dialyzed serum, and either saline or winter flounder Con A I 28,000 dalton DE III CM I fraction (100 µg/kg), were injected into hypophysectomized mature female flounder. Five days after injection the flounder were sacrificed and radioactivity in the water insoluble yolk fraction of the ovary was determined as described above in the assay for vitellogenic activity in pituitary hormone preparations.

Assay of maturational and ovulatory activities in the pituitary hormone preparations

Fish were used 3 to 4 days after hypophysectomy. Those which oviposited on stripping or which were in an advanced stage of maturation were excluded from the experiment. Hypophysectomized fish were injected daily with either the pituitary hormone preparation or buffer over a period of 5 to 9 days. Daily egg samples, from individual fish, each containing 100-150 ova were taken by means of a Silverman biopsy needle (14 gauge, 3 3/8 in., Becton, Dickinson & Co., U.S.A.) inserted through the genital pore. The eggs were first examined in teleost buffer* (pH 8.4) under a Nikon binocular microscope to ascertain the percentage of completely opaque clearing, and completely cleared eggs, and then again after fixation in an acetic acid:glycerol:teleost buffer mixture (4:6:90, v:v:v) to show up the germinal vesicle. Each egg in the biopsied sample was assigned a rating in accordance with

*The composition of teleost buffer is 11.19 g NaCl, 0.37 g KCl, 1.08 g glucose, 0.44 g $CaCl_2.2H_2O$, 0.41 g $MgCl_2.6H_2O$, 0.42 g $NaHCO_3$, 0.07 g $NaH_2PO_4.H_2O$, and 1.5 g caps (cyclohexylaminopropanesulfonic acid) in 1 liter of aqueous solution at pH 8.4

the stage of maturation as indicated below.

Rating	Stage of Maturation
1	Germinal vesicle in center
2	Germinal vesicle slightly off center
3	Germinal vesicle midway on its route of migration toward the periphery of the egg
4	Germinal vesicle migrated to a peripheral position
5	Germinal vesicle breakdown
6	Egg clearing and increasing in size
7	Egg translucent and had attained a large size; oozed out from the genital pore when a gentle pressure was applied along the abdominal region

The daily ratings (R) for individual fish were computed from the equation $R = \frac{n_1R_1 + n_2R_2 + n_3R_3 + \ldots n_n R_n}{n_1 + n_2 + n_3 + \ldots n_n}$ where n is the number of eggs with the rating R.

This assay was used to monitor maturational-ovulatory activities of all fractions in the isolation of maturational hormones from the pituitaries of plaice, flounder and salmon.

Assay for testicular uptake of radiophosphate and tritiated leucine and isotopic incorporation into proteins

Mature male flounder collected in January were injected 2 weeks after hypophysectomy with $H_3{}^{33}PO_4$ and ^{3}H-leucine (New England Nuclear), 20 µCi of each isotope for every kg body weight, together with the

hormone preparation or buffer. Hormone administration was continued for another 3 days, and the fish were autopsied 48 hours after the last injection. Radioactivity in Protosol digests of the entire testicular tissue, and of the trichloracetic acid-precipitable portion of the testis, was determined as described in the assay for vitellogenic activity, to assess the incorporation of the radioisotopes into the whole testis and testicular proteins respectively.

Assay for spermiation

Male winter flounder collected in March, with large testes but showing no signs of spermiation, were selected for the experiment. They were injected, on the 8th day after hypophysectomy, with either hormone or buffer.

Spermiation was checked starting from 48 hr post-injection by applying a slight pressure on the abdominal region. A positive response was detected by the free flow of milt from the genital orifice.

Assay for steroidogenic activities in hormone preparations

(a) In January mature male winter flounder were hypophysectomized and on the twentieth day after hypophysectomy they were injected with either buffer or hormone preparations. Twenty-four hr after the injection fish were bled, and the plasma samples were analyzed for testosterone and 11-ketotestosterone in radioimmunoassays. Throughout the experiment the flounder were kept in seawater at 10°C.

Radioimmunoassay of 11-ketotestosterone

The antiserum to 11-ketotestosterone was raised in a New Zealand white rabbit. 11-Ketotestosterone-(3-O-carboxymethyl)-oxime-bovine serum albumin conjugate (100 µg) was injected intradermally at multiple sites at the primary injection. Two months later a booster injection

of 2 mg was given intramuscularly into the hind limbs, followed by a booster every fortnight. An antiserum which showed 50% binding to tritiated 11-ketotestosterone at 1 : 30,000 dilution was utilized for radioimmunoassay.

The essential details for the assay were as described by Simpson and Wright (1977). Testosterone and 11β-hydroxytestosterone gave parallel displacements to the 11-ketotestosterone standard curve, and there was only 0.1% cross reaction from either androgen over the range of the assay. In view of the fact that 11-ketotestosterone was the predominant androgen in flounder plasma (Campbell et al., 1976) and there was little 11β-hydroxytestosterone in plasma of gonochoristic species like the flounder (Idler et al., 1976), all samples could be assayed directly without interference from testosterone.

Flounder plasma (25 µl) or landlocked salmon plasma (10 µl) was extracted with 2 ml dichloromethane and the extract was evaporated under nitrogen before being utilized for radioimmunoassay. Tritiated 11-ketotestosterone (1000 cpm/tube) was used both as recovery tracer during extraction and as labeled hormone (5000 cpm/tube) in the assay. For separating free from bound steroid, dextran charcoal was employed. After centrifugation, an aliquot of the supernatant was taken and counted in Riafluor (New England Nuclear).

Radioimmunoassay of testosterone

The antiserum to testosterone was obtained from Radioassay Systems Laboratories Inc., Carson, California, the antigen used being testosterone-3-oxime-human serum albumin conjugate. The radioimmunoassay using tritiated testosterone both as label and recovery tracer was conducted as described for 11-ketotestosterone. Both 11-

ketotestosterone and 11β-hydroxytestosterone gave parallel displacements to the testosterone standard curve. 11-Ketotestosterone showed 6-7% cross reaction while there was negligible (ca. 1%) cross reaction from 11β-hydroxytestosterone. Samples containing very small amounts of 11-ketotestosterone i.e., samples from all groups analyzed except the group treated with flounder Con A II MW 62,000 fraction (see Table 14) were assayed directly in the assay and the testosterone levels thus obtained were corrected for cross reactivity (6%) from 11-ketotestosterone. For samples from the group of flounder treated with flounder Con A II MW 62,000 fraction containing appreciable amounts of 11-ketotestosterone which would interfere significantly in the testosterone assay, testosterone was first separated from 11-ketotestosterone and 11β-hydroxytestosterone by thin layer chromatography on silicic acid with chloroform:ethanol (98:2).

Dichloromethane extracts of flounder plasma samples, after separation into 11-ketotestosterone and testosterone by thin layer chromatography showed, upon serial dilution, parallelism to the standard curve in the 11-ketotestosterone and testosterone assays respectively.

Treatment of assay data

Assay data were analyzed with Student's t test and reported as Mean ± Standard Error of the Mean.

Protein binding of steroids in plasma

The methodology was essentially the same as that described by Freeman and Idler (1966). A known volume of plasma sample was added to 2,000 - 3,000 cpm of tritiated 11-ketotestosterone. The plasmas were equilibrated for 15 min at room temperature and then overnight at 4C before being applied on a column of Sephadex G-25 fine

(Pharmacia) equilibrated with 0.9% saline. The column had previously been calibrated with blue dextran (Pharmacia) and tritiated 11-ketotestosterone; chromatography was performed at 4°C with 0.9% saline solution as the elution buffer, and 1 ml fractions were collected for counting in Riafluor.

Amino acid and carbohydrate analyses

Protein samples for amino acid analysis were hydrolyzed in 6 N HCl at 110°C for 24 hrs and were then applied to a Beckman Model 121 amino acid analyzer. Tryptophan was determined after 24 hr hydrolysis in 3N p-toluenesulfonic acid containing 0.2% tryptamine (Liu and Chang, 1971). Protein samples for amino sugar determination were hydrolyzed in 4 N HCl at 100°C for 8 hours and the hydrolysates were applied to the amino acid analyzer with a 15 cm Bio-Rad Aminex A-5 resin bed and a standard pH 5.28 citrate buffer for elution (Black et al., 1970). Hexose contents were estimated with the anthrone procedure (Roe, 1955). Optical density was read at 585 nm, an isosbestic point at which the absorbance by hexoses is independent of the amount of tryptophan. An equimolar mixture of mannose and galactose was used as the standard. Sialic acid was measured with the Warren method (Warren, 1959) in samples which had been incubated in 0.1 H H_2SO_4 at 80°C for one hr to release the bound carbohydrate.

Electrophoresis

Analytical polyacrylamide gel disc electrophoresis was carried out as described by Davis (1964) at pH 8.3. Gels were stained with 0.02% coomassie blue, 5% trichloroacetic acid, 5% sulfosalicylic acid, 18% methanol, and destained by diffusion in 7.5% acetic acid.

Reduction and S-carboxamidomethylation of salmon Con A II G-75 Fraction II

Twenty five microliters of an aqueous solution of 30 µg salmon Con A II G-75 Fraction II (Idler et al., 1975a) was allowed to react overnight at room temperature with 5 µl 2-mercaptoethanol and 100 µl 10M urea in Tris buffer (pH 8.5). Iodoacetamide (30 mg) was added and reaction was allowed to proceed in the dark for 20 minutes. The solution was then serially diluted with 0.08 M barbital buffer (pH 8.6) containing 0.5% bovine serum albumin and 0.01% thimerosal, (aprotinin), to yield a range of hormone concentrations to be assayed for immunological potency. The reaction medium was diluted and tested likewise in order to ascertain whether it had any effect on the antigen-antibody reaction. The double antibody technique (Crim et al., 1975) was used for radioimmunoassay. Con A II G-75 Fraction II (Idler et al., 1975a) was labeled with ^{131}Iodine (New England Nuclear) using the chloramine T method of Greenwood et al. (1963) and purified on a column of Bio-Gel P60 (Bio-Rad). The antiserum used was raised against SG-G100, the salmon gonadotropic preparations of Donaldson et al. (1972). A standard curve was set up using Con A II G-75 Fraction II as the standard.

Treatment of salmon hormones with neuraminidase

To 25 µl of an aqueous solution of 30 µg salmon Con A II G-75 Fraction II, or Con A I MW 25,000 DE III fraction, or Con A I MW 45,000 DE III fraction was added 60 µg Cl. perfringens neuraminidase (Type V, Sigma) in 0.1 M sodium acetate buffer pH 5.7 containing 5 mM $CaCl_2$. The enzymic reaction was allowed to proceed overnight at room temperature. The solution was then serially diluted with barbital

buffer to yield a range of hormone concentrations for radioimmunoassay. The reaction medium was likewise diluted to find out whether the antigen-antibody reaction was affected by the presence of the medium. Hormones used for bioassays were treated with neuraminidase under the same conditions except for the reduction of the enzyme : substrate ratio to 1 : 20. The efficacy of the enzyme preparation was checked by the detection of free sialic acid in the reaction mixture at the end of the enzymic reaction by the Warren method (Warren, 1959).

Production of antisera

For immunization, (a) salmon Con A II G-75 Fraction II, (b) salmon Con A I fraction, (c) salmon Con A I MW 45,000 fraction, (d) salmon Con A I MW 25,000 fraction, (e) plaice Con A II MW 62,000 fraction, (f) flounder Con A II MW 62,000 fraction, and (g) flounder Con A I MW 28,000 homogenized in Freund's complete adjuvant, were injected individually into New Zealand white rabbits. All rabbits received a primary intradermal injection at multiple sites on the back, followed two months later and monthly thereafter with booster intramuscular injections into the hind limbs until a reasonably high antibody titer was obtained. The antisera utilized in this study were as follows: (1) antiserum to (a), designated S-Con A II-ab, obtained after a primary injection of 200 µg and two booster injections of 1 mg, and which at a dilution of 1:80,000 showed 50% binding to salmon Con A II G-75 Fraction II (2) antiserum to (b), designated S-Con A I-ab, obtained after a primary injection of 1 mg salmon Con A I MW 45,000 fraction and 6 monthly booster (1 mg) injections of the same fraction. This series of booster injections had been interrupted by an injection of 3 mg of the entire Con A I fraction with the result that the

antiserum contained antibodies to both the MW 25,000 and the MW 45,000 fractions. However, when the antiserum was used at a dilution of 1:6,400 with the MW 25,000 DE III fraction as label, a radioimmunoassay for this fraction could be set up with a maximum binding of 40%. On the other hand, when the antiserum was used at a dilution of 1:6,400 with the MW 45,000 DE III fraction as label, an assay for this fraction could also be developed with a maximum binding of 40%. (3) Antiserum to (c), designated S-Con A I 45K-ab, obtained after a primary injection of 1 mg and 6 booster injections of 1 mg, and which at a dilution of 1:5,000 showed 50% binding to salmon Con A I MW 45,000 DE III fraction. (4) Antiserum to (d), designated S-Con A I 25K-ab, obtained after a primary injection of 1 mg and booster injections of 1 mg and which at a dilution of 1:5,000 showed 50% binding to salmon Con A I MW 25,000 DE III fraction. (5) Antiserum to (e), designated P-Con A II-ab, obtained after a primary injection of 1 mg and 6 booster injections of 500 µg, and which at a dilution of 1:6,400 showed 55% binding to plaice Con A II MW 62,000 fraction. (6) Antiserum to (f), designated F-Con A II-ab, obtained after a primary injection of 1 mg and 6 booster injections of 1 mg, and which at a dilution of 1:5,000 showed 50% binding to flounder Con A II MW 62,000 fraction. (7) Antiserum to (g), designated F-Con A I-ab, obtained after a primary injection of 1 mg and 6 booster injections of 1 mg and which at a dilution of 1:5,000 showed 50% binding to flounder Con A I MW 28,000 DE III fraction.

Testing of ability of salmon vitellogenic Con A I subfractions to bind to antisera raised against SG-G100 and Con A II G-75 Fraction II

Antiserum to SG-G100 was raised by Crim et al., (1973).

Preparation of antiserum to salmon Con A II G-75 Fraction II was described above. Five micrograms each of salmon Con A I MW 25,000 DE III fraction and Con A I MW 45,000 DE III fraction were treated with 2 mCi of ^{131}Iodine and purified on Ultrogel AcA 44 to get rid of free iodide.

Labeled hormone, 200 µl, containing 10,000 cpm, was added to 200 µl of a serial dilution of the antiserum and incubated with 100 µl of normal rabbit serum (1 : 40 dilution) at 4°C for 72 hr before addition of 100 µl of goat anti-rabbit globulin (1 : 20 dilution). Incubation at 4°C was continued for a further 24 hr before centrifugation and counting of the precipitate. Percent binding = $\frac{\text{(counts in precipitate - blank) x 100}}{\text{Total counts}}$ where blank = counts in precipitate in a tube from which the antibody is omitted during incubation and total counts = radioactivity in 200 µl of labeled hormone.

Relative percent binding =

$$\frac{\text{Percent binding in the presence of x pg cold hormone}}{\text{Percent binding in the absence of cold hormone.}}$$

Testing of ability of salmon Con A II G-75 Fraction II to bind to antisera raised against salmon vitellogenic Con A I subfractions

Serial dilutions of S-Con A I-ab, S-Con A I 45K-ab, and S-Con A I 25K-ab were tested for binding to iodinated salmon Con A II G-75 Fraction II as described above.

Testing of cross reactivity of antisera raised against flounder gonadotropic fractions

F-Con A I-ab was tested for binding to flounder Con A II MW 62,000 fraction. F-Con A II-ab was tested for binding to flounder Con A I MW 28,000 DE III fraction.

Testing of cross reaction from salmon Con A II G-75 Fraction II in radioimmunoassay for salmon Con A I MW 25,000 DE III fraction and Con A I MW 45,000 DE III fraction

In a radioimmunoassay for salmon Con A I MW 25,000 DE III fraction, 5 µg of the fraction was labeled with 2 mCi of ^{131}Iodine and then purified on Ultrogel AcA 44 to remove ^{131}Iodide. Labeled hormone, 200 µl, containing 10,000 cpm, was added to 200 µl S-Con A I -ab (1:6,400 dilution), 100 µl normal rabbit serum (1:40 dilution), and either 100 µl of the standard DE III fraction, or a serial dilution of salmon Con A II G-75 Fraction II. The mixture was incubated for 72 hr at 4C before addition of 100 µl goat anti-rabbit globulin (1:20 dilution). Incubation was continued for a further 24 hr before centrifugation and counting of the precipitate.

In a radioimmunoassay for salmon Con A I MW 45,000 DE III fraction, the fraction was labeled and the assay was set up using S-Con A I-ab at a dilution of 1:6,400. Cross reaction from salmon Con A II G-75 Fraction II was tested as described in the paragraph above.

Testing of cross reaction from salmon Con A I MW 25,000 DE III fraction and Con A I MW 45,000 DE III fraction in a radioimmunoassay for salmon Con A II G-75 Fraction II

The antiserum raised against SG-G100 by Crim et al., (1973), was used at a dilution of 1:100,000. Salmon Con A II G-75 Fraction II was iodinated and the Con A I subfractions tested for cross reaction.

Testing of cross reactivity from plaice Con A I MW 28,000 DE III fraction and Con A I MW 62,000 DE III fraction in a radioimmunoassay for plaice Con A II MW 62,000 DE I fraction

Plaice Con A II MW 62,000 DE I fraction (5 µg) was iodinated, and the assay was set up using P-Con A II-ab at a dilution of 1:6,400. Plaice Con A I MW 28,000 DE III fraction and Con A I MW 62,000 DE III fraction were tested for cross reaction.

Testing of cross reactivity from flounder Con A I MW 28,000 DE III fraction and Con A I MW 62,000 DE III fraction in a radioimmunoassay for flounder Con A II MW 62,000 fraction

Flounder Con A II MW 62,000 fraction (5 µg) was iodinated, and the assay was set up using F-Con A II-ab at a dilution of 1:6,400. Flounder Con A I MW 28,000 DE III fraction and Con A I MW 62,000 DE III fraction were tested for cross reaction.

Effect of gonadotropin antisera on vitellogenesis and oocyte maturation in landlocked salmon and winter flounder

(i) Experimental animals

Stunted landlocked salmon Salmo salar sebago averaging 5-6 years of age and 12 cm in length, were caught near St. John's, Newfoundland, in October 1977. The fish were stripped in December 1977 for artificial fertilization experiments and then held in the laboratory for one year before they were used for the present experiment. At autopsy the eggs were found to be well developed and yolky. It is an experience in this laboratory that fish held in captivity spawned within 2 weeks of fish caught in the pond at spawning time.

Winter flounder (Pseudopleuronectes americanus) with oocytes which were maintained in a yolky state but which had not undergone germinal vesicle migration were caught by divers two months before spawning and used immediately.

ii) Landlocked salmon experiments

Effects of antisera on (a) vitellogenesis and (b) oocyte maturation and ovulation:

(a) The fish were divided into three groups and were injected respectively with (i) 200 µl normal rabbit serum, (ii) 200 µl S-Con A I 25K-ab and (iii) 10 µl S-Con A II-ab + 190 µl normal rabbit serum (i.e., diluted to near equipotency with S-Con A I 25K-ab). Injections were given daily from November 10 to November 15, 1978. On November 13 the fish were injected with $H_3{}^{33}PO_4$ (40 µCi/kg), and on November 16 they were bled, samples of ovaries taken, and the incorporation of radioactivity into the water insoluble yolk fraction determined as described above. Plasma samples were assayed for vitellogenin as described by Idler et al., (1979). Estradiol in the plasma samples was determined with a radioimmunoassay following the procedure recommended by Dr. G. Abraham (Harbor General Hospital, California) and using an antiserum to estradiol (S310#5) supplied by him. Estradiol in the samples was extracted with ether and purified on Sephadex LH 20 as described by De Jong et al., (1973).

Since radioimmunoassay for the gonadotropins could not be used to show that blood samples, already containing antisera, did not have significant amounts of free hormone the blood samples were tested for an excess binding capacity for the appropriate hormone.

For each plasma sample, 2.5 µl, 10 µl and 25 µl were tested for binding to both chum salmon maturational and vitellogenic hormones. The samples were incubated with 100 µl iodinated hormone containing 10,000 cpm for 72 hr at 4C before addition of 100 µl GAR (1:5 dilution). The tubes were centrifuged 25 hr later and the precipitates were

counted. The counts represent binding of the labeled hormone by excess antibodies present in the fish plasma samples. (b) The dosages of antibodies were the same as in the first experiment, but injections were given on Nov. 21, Nov. 22, Nov. 24 and Nov. 26 in 1978 to fish which were close to spawning. Tritiated leucine and $H_3{}^{33}PO_4$ (40 µCi/kg of each isotope) were injected on Nov. 20. The fish were sacrificed on Dec. 11 and the maturational status of individual fish was determined. Plasma samples were assayed for vitellogenin and the presence of an excess of specific antibodies, and gonad samples for radioactivity in the water insoluble yolk fraction, as described in (a). Radioactivity in the water insoluble yolk fraction of plasma, representing newly synthesized vitellogenin was also determined by precipitating the fraction with distilled water and counting the precipitate.

(iii) Winter flounder experiment

The fish were randomly divided into three groups. The groups were injected with 100 µl of (i) normal rabbit serum, (ii) F-Con A I-ab, or (iii) F-Con A II-ab, once every two weeks over a period of six weeks. Two days after the final antiserum injection the fish were given ^{3}H-leucine and $H_3{}^{33}PO_4$ (20 µCi/kg) of each isotope intraperitoneally. Five days later the fish were killed and the maturational status of individual fish was determined. Pieces of ovaries were taken for assessment of incorporation of labeled yolk.

RESULTS

Preliminary purification of pituitary extracts on Con A-Sepharose and Ultrogel

(i) Chromatographic behaviour

The profiles of elution of pituitary extracts from plaice and flounder were similar to that for salmon pituitary extract: the bulk (≈90%) protein was in the unadsorbed Con AI fraction whereas the adsorbed Con A II fraction (≈ 10%) appeared as a sharp peak (Fig. 1).

The choice of Ultrogel AcA 44 and Ultrogel AcA 54 as the gel filtration media permitted clear-cut separation of the molecular weight marker proteins blue dextran (MW, 2000×10^3), bovine serum albumin (MW, 67×10^3), ovalbumin (MW, 45×10^3) and chymotrypsinogen a (MW, 25×10^3), from one another (Fig. 8). Chromatography of plaice Con A I fraction on Ultrogel AcA 44 yielded three peaks: a large one reaching an optical density of greater than 2 at the void volume region, and two smaller ones at 62,000 and 28,000 daltons, respectively. The peaks and tubes on either side of the peaks were pooled to form fractions A, B, and C with molecular weight ranges of $\geq 160 \times 10^3$, 160×10^3-45×10^3, and 45×10^3- 10×10^3, respectively (Fig. 2). The yield of fractions A, B and C are given in Fig. 9.

Rechromatography of fraction B on Ultrogel AcA 44 produced an apparently symmetrical peak with a molecular weight of 62,000 (Fig. 3). Chromatography of fraction C on Bio-Gel A 0.5 m (200-400 mesh) using Buffer G as the eluant gave a sharp symmetrical peak at 28,000 daltons (Fig. 4). However, when fraction C from several runs were pooled together, concentrated, and run on Ultrogel AcA 44 using an eluant Buffer D, a buffer with lower concentrations of NaCl, DTT, and EDTA than Buffer G, there was an apparent aggregation of the 28,000 MW species to form a 62,000 MW moiety (Fig. 10). That this was a process of aggregation rather than incomplete separation was inferred from the shape of the elution profile and the fine

Fig. 8 Profile of elution of blue dextran, aldolase, bovine serum albumin, ovalbumin and chymotrypsinogen a from Ultrogel AcA 44. Fraction size was 5 ml.

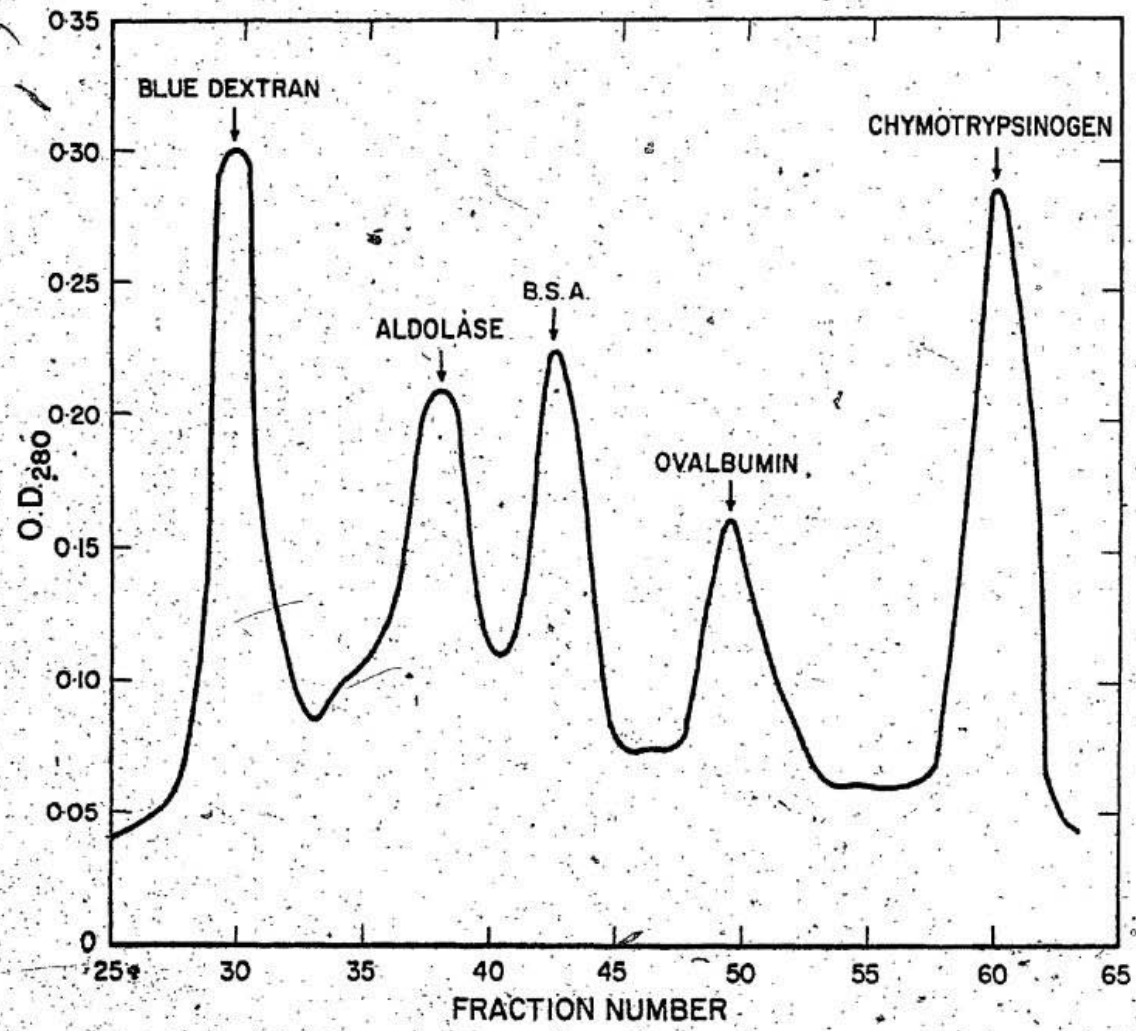

BLUE DEXTRAN
ALDOLASE
B.S.A.
OVALBUMIN
CHYMOTRYPSINOGEN
O.D.280
0·35
0·30
0·25
0·20
0·15
0·10
0·05
0
25
30
35
40
45
50
55
60
65
FRACTION NUMBER

Fig. 9 . Fractionation of plaice pituitary extract into Con A I fractions A, B, and C, and Con A II fraction

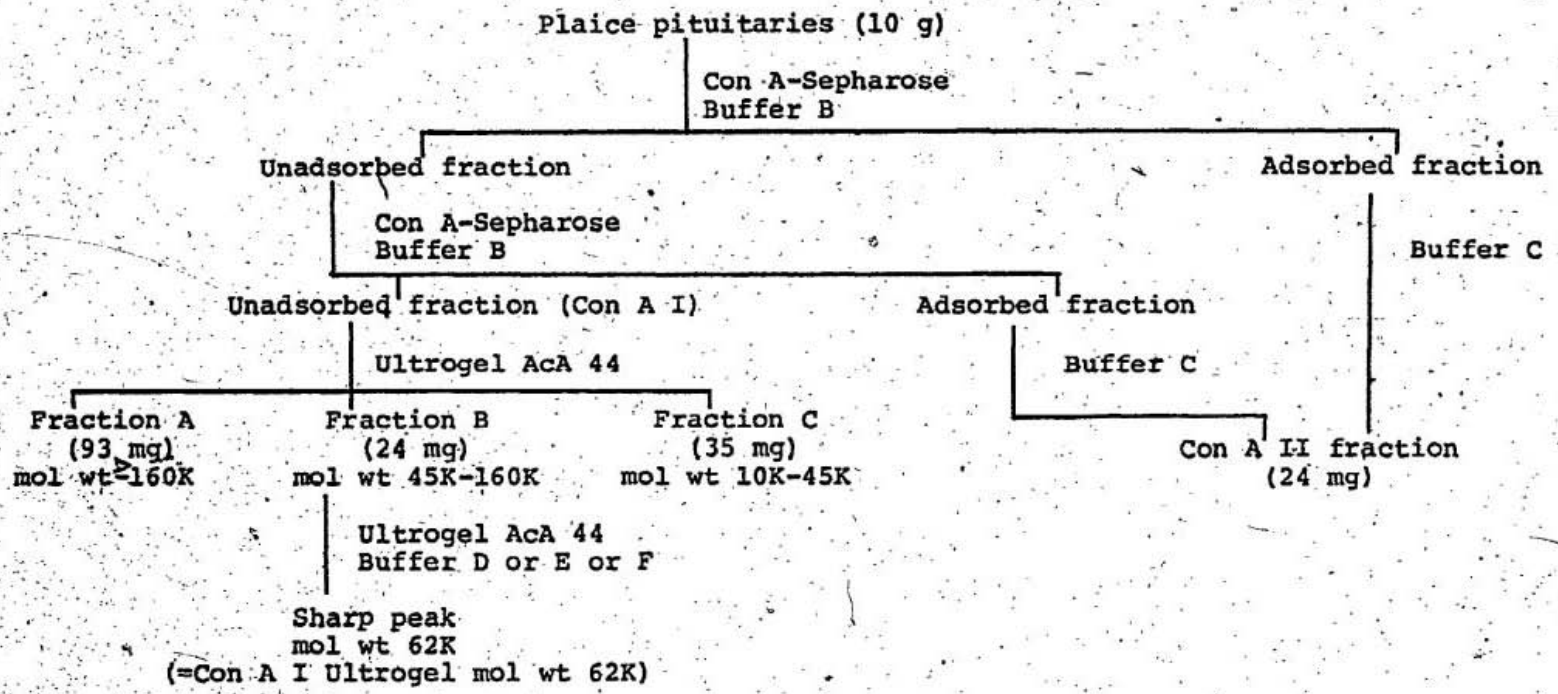
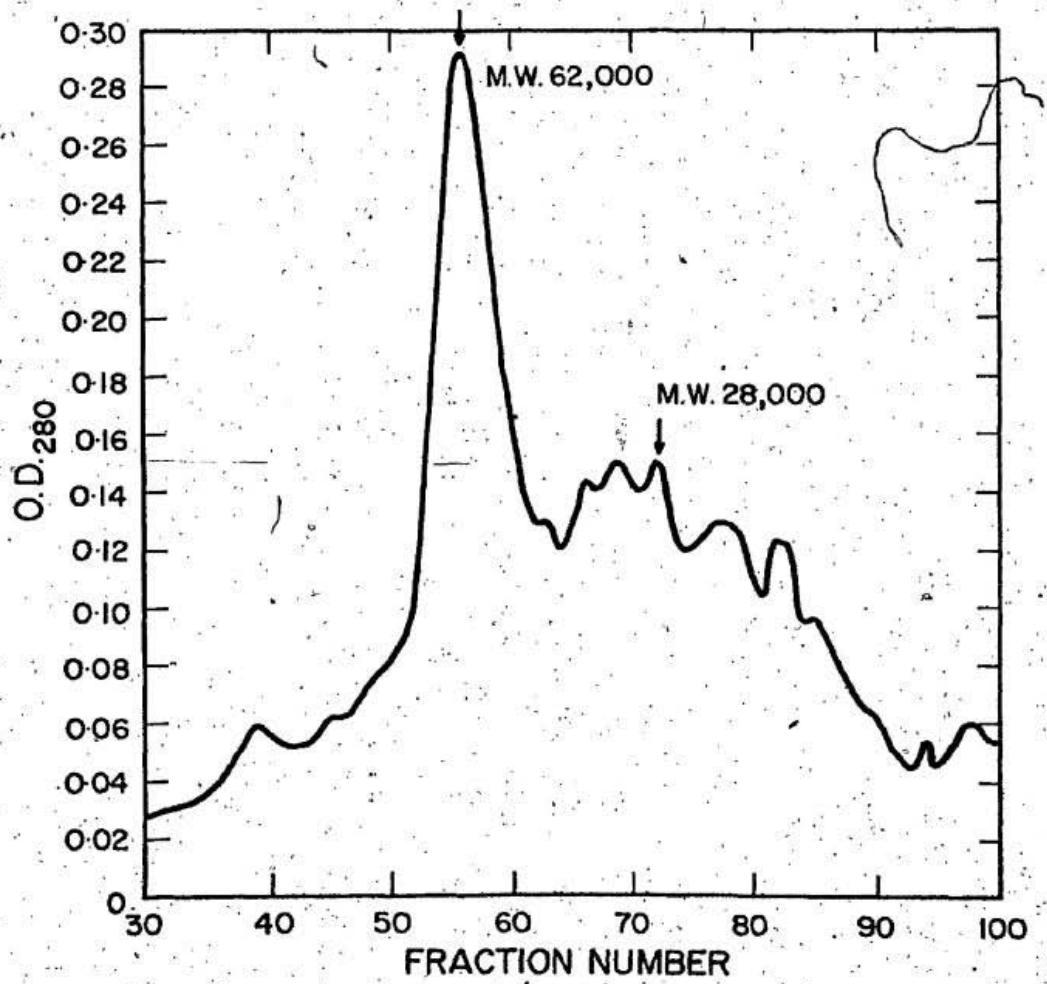

Plaice pituitaries (10 g)
Con A-Sepharose
Buffer B
Unadsorbed fraction
Adsorbed fraction
Con A-Sepharose
Buffer B
Buffer C
Unadsorbed fraction (Con A I)
Adsorbed fraction
Ultrogel AcA 44
Buffer C
Fraction A
(93 mg)
mol wt >160K
Fraction B
(24 mg)
mol wt 45K-160K
Fraction C
(35 mg)
mol wt 10K-45K
Con A II fraction
(24 mg)
Ultrogel AcA 44
Buffer D or E or F
Sharp peak
mol wt 62K
(=Con A I Ultrogel mol wt 62K)

Fig. 10 Rechromatography of a pool of plaice Con A I MW 28,000 fractions on Ultrogel AcA 44 equilibrated with buffer D. Fraction size was 4 ml.


M.W. 62,000
M.W. 28,000
O.D.280
0·30
0·28
0·26
0·24
0·22
0·20
0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
30
40
50
60
70
80
90
100
FRACTION NUMBER

resolving power on Ultrogel AcA 44 and Ultrogel AcA 54 in the molecular weight range 13.7 x 10^3-158 x 10^3. In order to ascertain whether this aggregation could be prevented or minimized by employing higher concentrations of NaCl and DTT in the buffer, Buffer E was used. Results revealed that aggregation still occurred to some, although a somewhat lesser extent (Fig. 11). The use of Buffer F, a buffer with a concentration of EDTA 10-fold that in Buffers D and E, prevented association altogether (Fig. 12). On the other hand, the molecular weight of fraction B was unaffected by the concentrations of NaCl, DTT, and EDTA in the elution buffer, with a very slight tendency to form high molecular weight aggregates and no signs of dissociating to form a 28,000 molecular weight species (Figs. 3,9).

Filtration of plaice Con A II fraction through Ultrogel AcA 54 yielded a sharp peak at the void volume (MW $\geq$ 160,000) plus a smaller one at 62,000 daltons (Fig. 13). Rechromatography on Ultrogel AcA 54 of the pool of fractions (MW 20-160 x 10^3) on either side of the MW 62,000 peak and the peak after concentration resulted in a peak at void volume, a sharp peak at 62,000 daltons, and a small peak at 28,000 daltons (Fig. 14). A symmetrical peak at 62,000 daltons was obtained by putting a concentrate of fractions on either side of the MW 62,000 peak and the peak through an Ultrogel AcA 54 column again (Fig. 15). However, when fractions on either side of the 28,000 peak were pooled, concentrated, and refiltered through Ultrogel AcA 44, aggregation to form a species of 62,000 MW was apparent (Figs. 16, 17).

Fig. 11 Rechromatography of a pool of plaice Con A I MW 28,000 fractions on Ultrogel AcA 44 equilibrated with Buffer E. Fraction size was 6 ml.

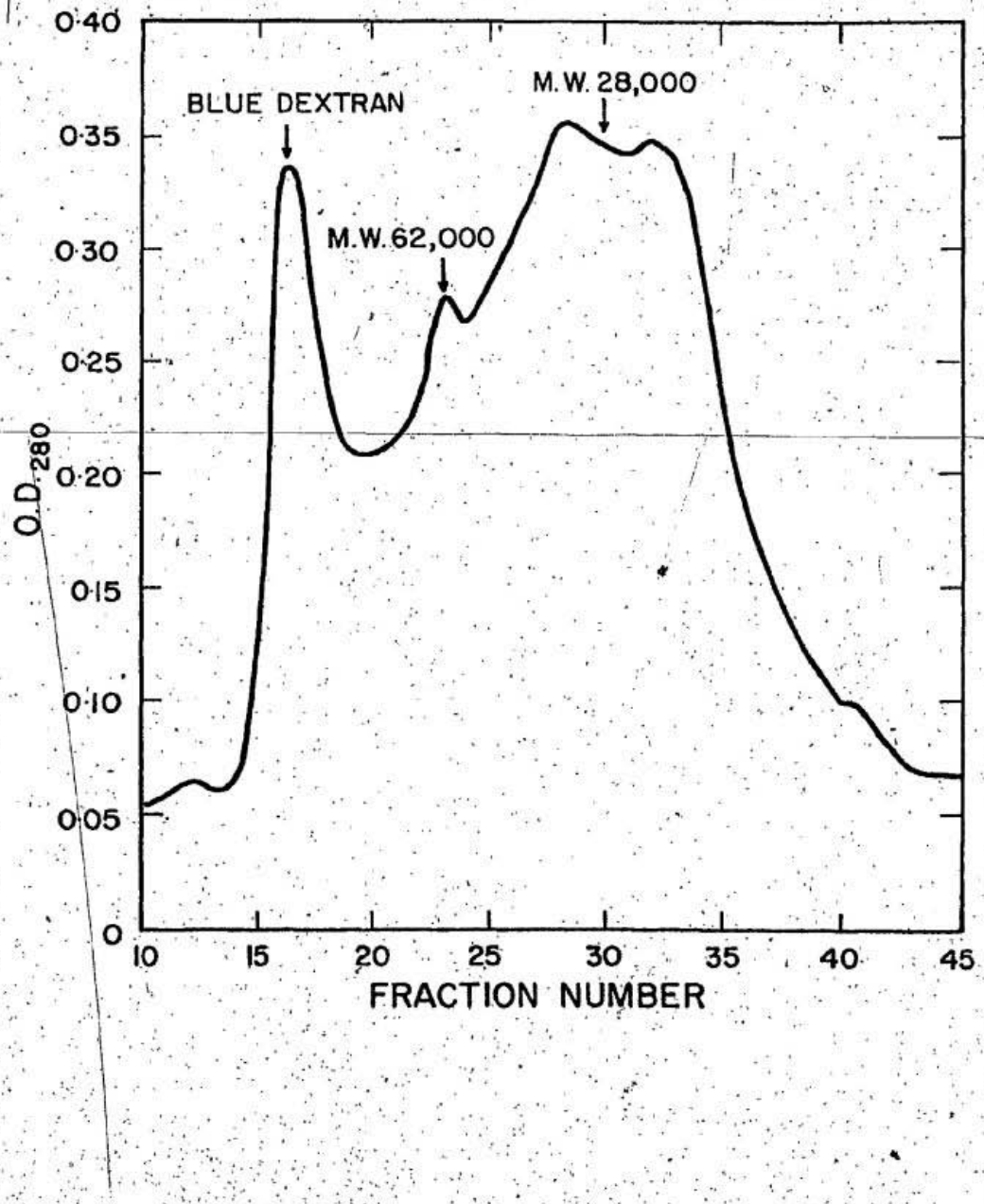

0·40
0·35
0·30
0·25
0·20
0·15
0·10
0·05
0
O.D.280
BLUE DEXTRAN
M.W. 62,000
M.W. 28,000
10
15
20
25
30
35
40
45
FRACTION NUMBER

Fig. 12 Further fractionation of plaice Con A I fraction C.

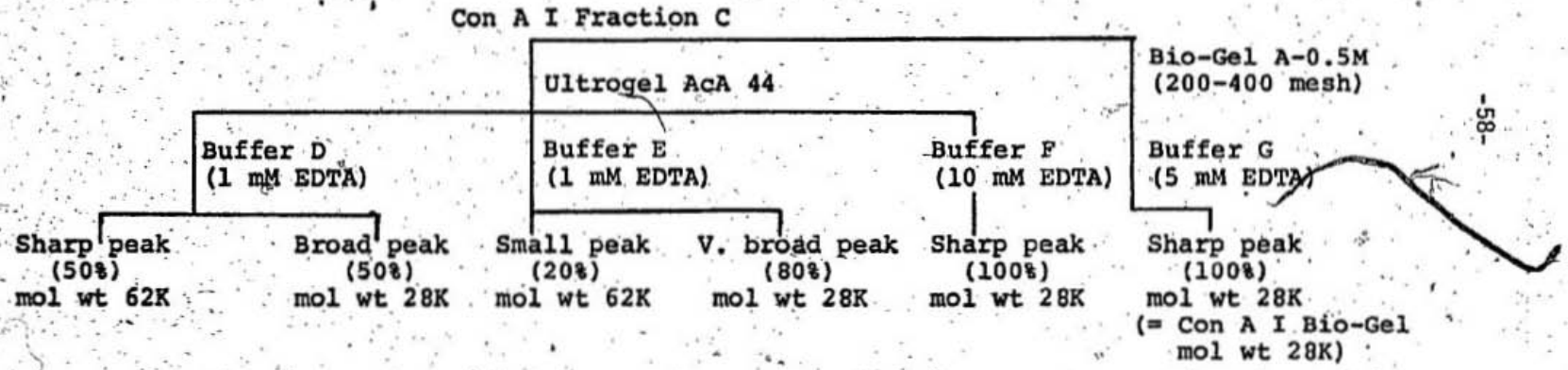
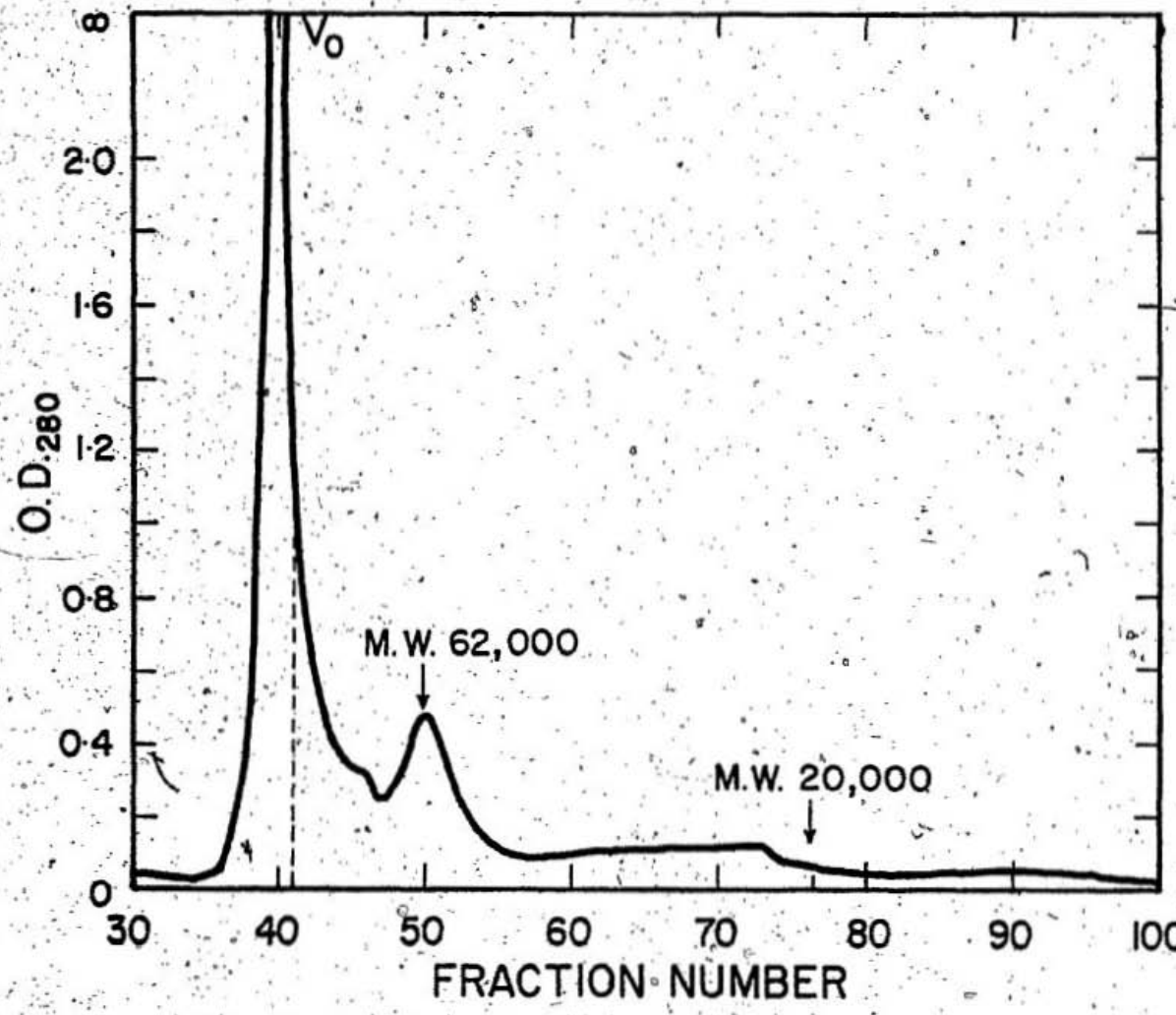

Con A I Fraction C
Ultrogel AcA 44
Bio-Gel A-0.5M
(200-400 mesh)
Buffer D
(1 mM EDTA)
Buffer E
(1 mM EDTA)
Buffer F
(10 mM EDTA)
Buffer G
(5 mM EDTA)
Sharp peak
(50%)
mol wt 62K
Broad peak
(50%)
mol wt 28K
Small peak
(20%)
mol wt 62K
V. broad peak
(80%)
mol wt 28K
Sharp peak
(100%)
mol wt 28K
Sharp peak
(100%)
mol wt 28K
(= Con A I Bio-Gel
mol wt 28K)

Fig. 13 Chromatography of plaice Con A II fraction on Ultrogel AcA 54. Fraction size was 8 ml.


V_0
O.D.280
2·0
1·6
1·2
0·8
0·4
0
M.W. 62,000
M.W. 20,000
30
40
50
60
70
80
90
100
FRACTION NUMBER

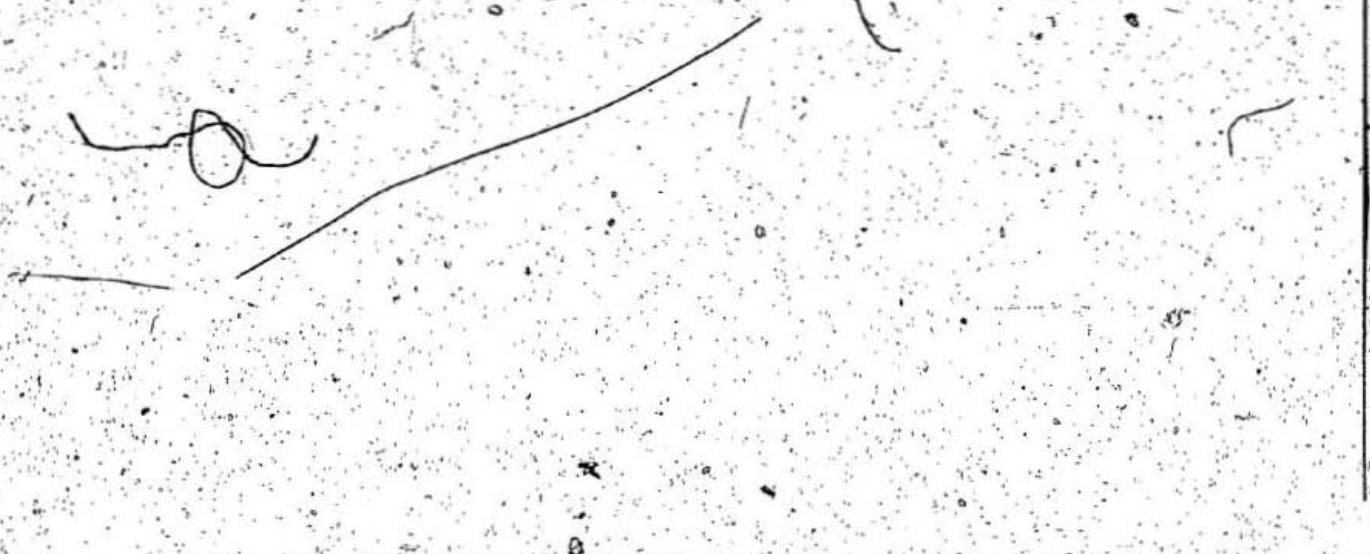


Fig. 14 Chromatography of a pool of fraction 41-80 (Fig. 13) on Ultrogel AcA 44. Fraction size was 6 ml.

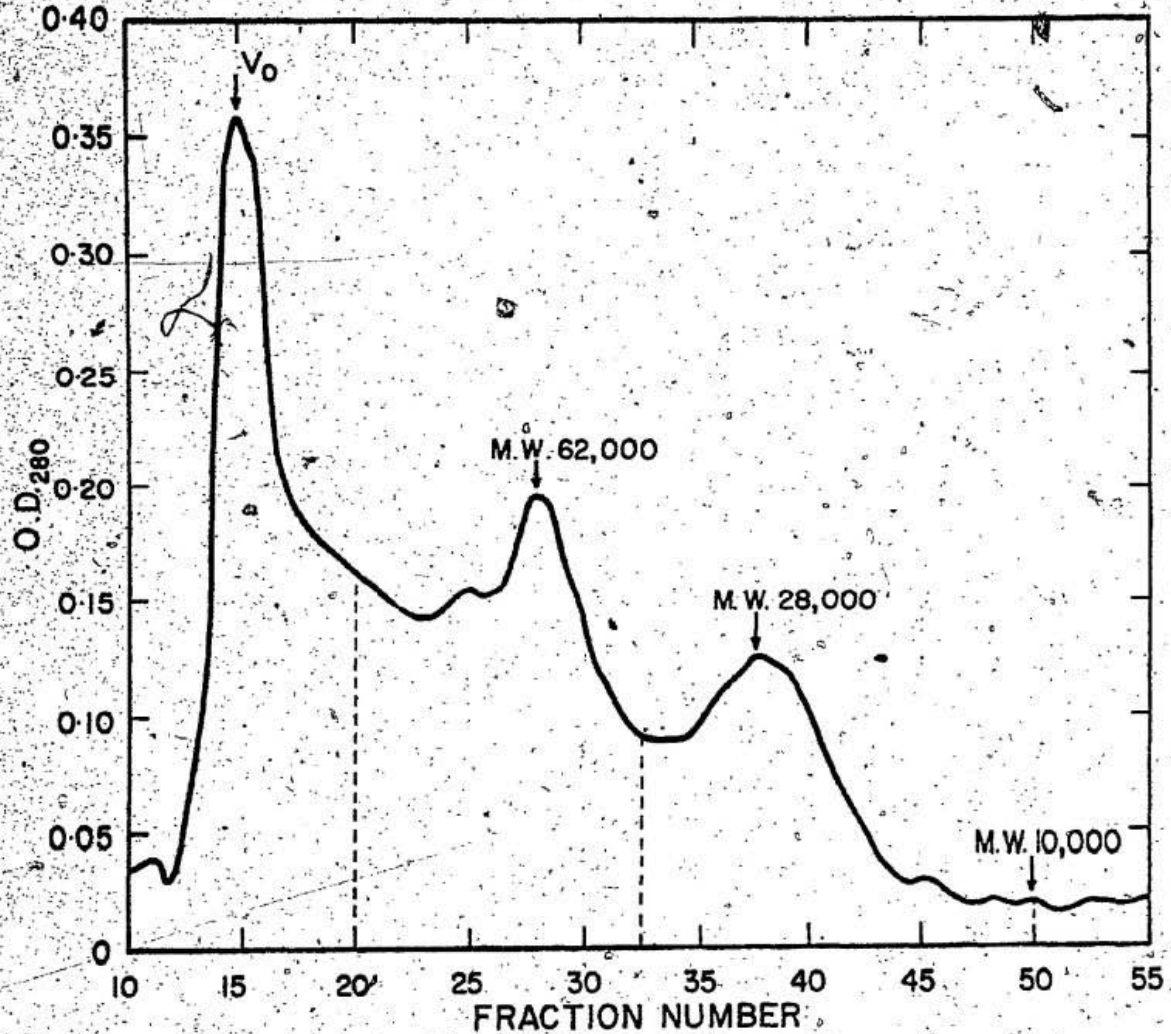

0·40
0·35
0·30
0·25
0·20
0·15
0·10
0·05
0
O.D.280
V_O
M.W. 62,000
M.W. 28,000
M.W. 10,000
10
15
20
25
30
35
40
45
50
55
FRACTION NUMBER

Fig. 15 Chromatography of a pool of fractions 20-32 (Fig. 14) on Ultrogel AcA 44. Fraction size was 4 ml.

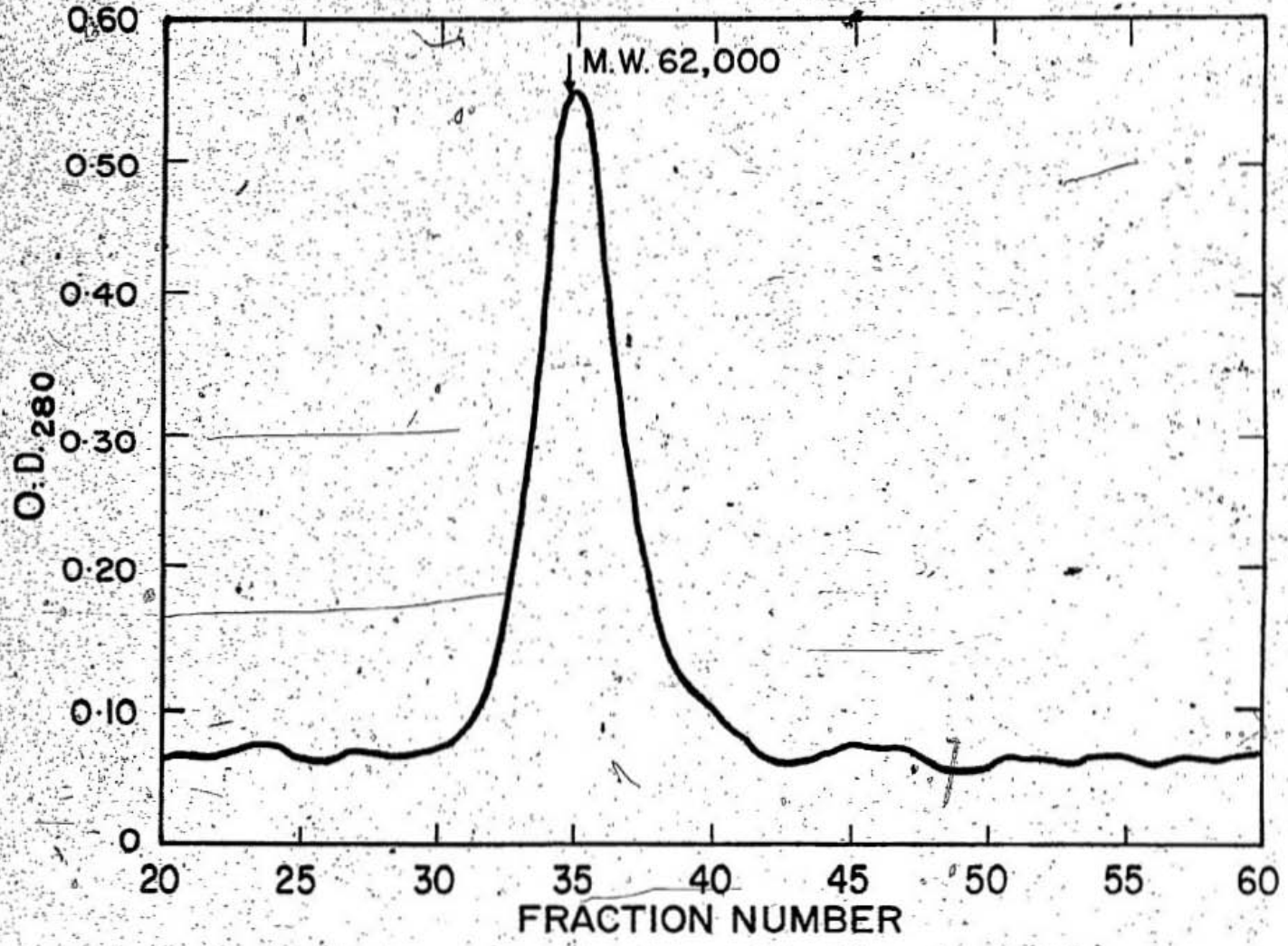

M.W. 62,000
0·60
0·50
0·40
0·30
0·20
0·10
0
O.D.280
20
25
30
35
40
45
50
55
60
FRACTION NUMBER

Fig. 16 Chromatography of a pool of fractions 33-50 (Fig. 14) on Ultrogel AcA 44. Fraction size was 4 ml.

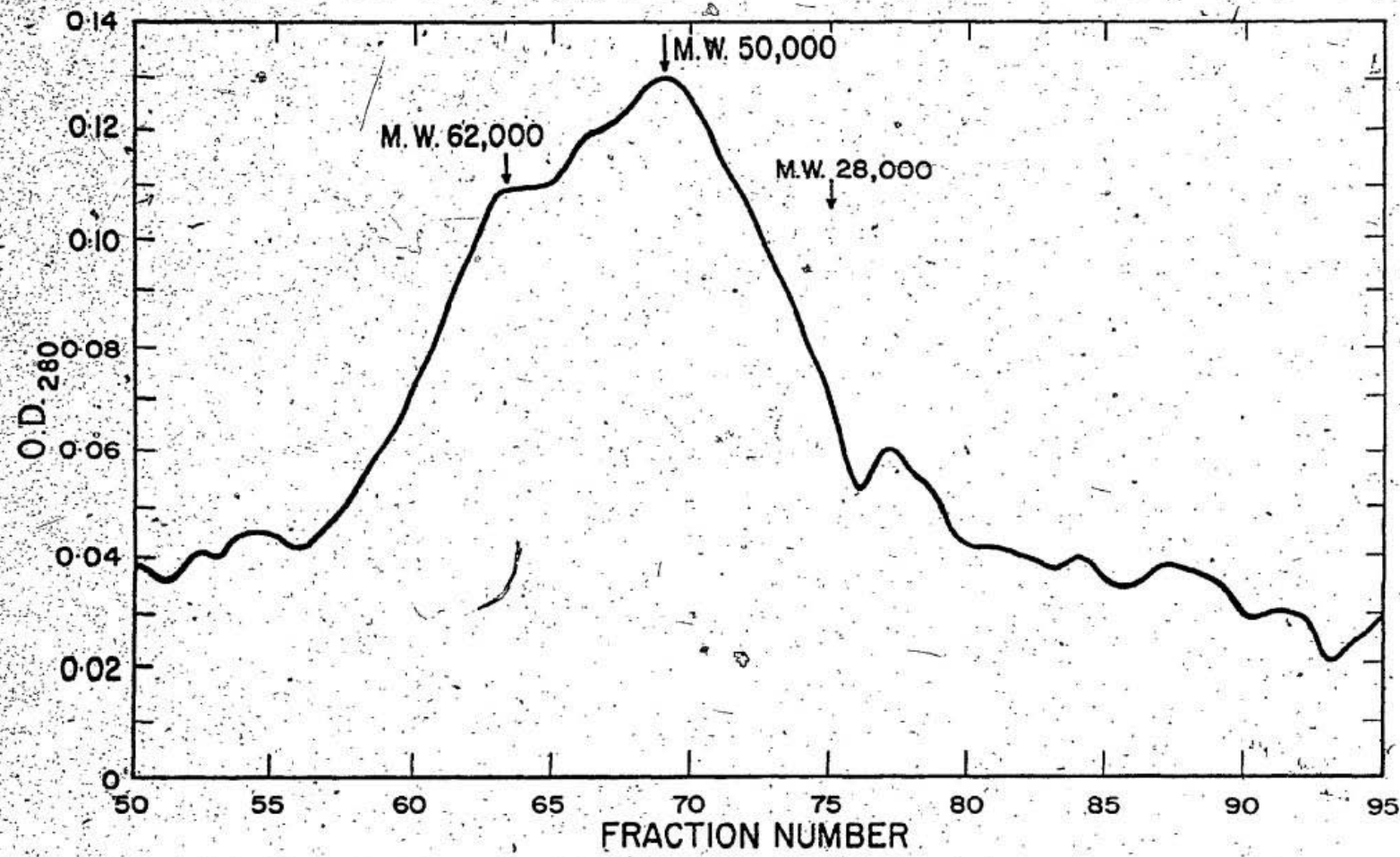
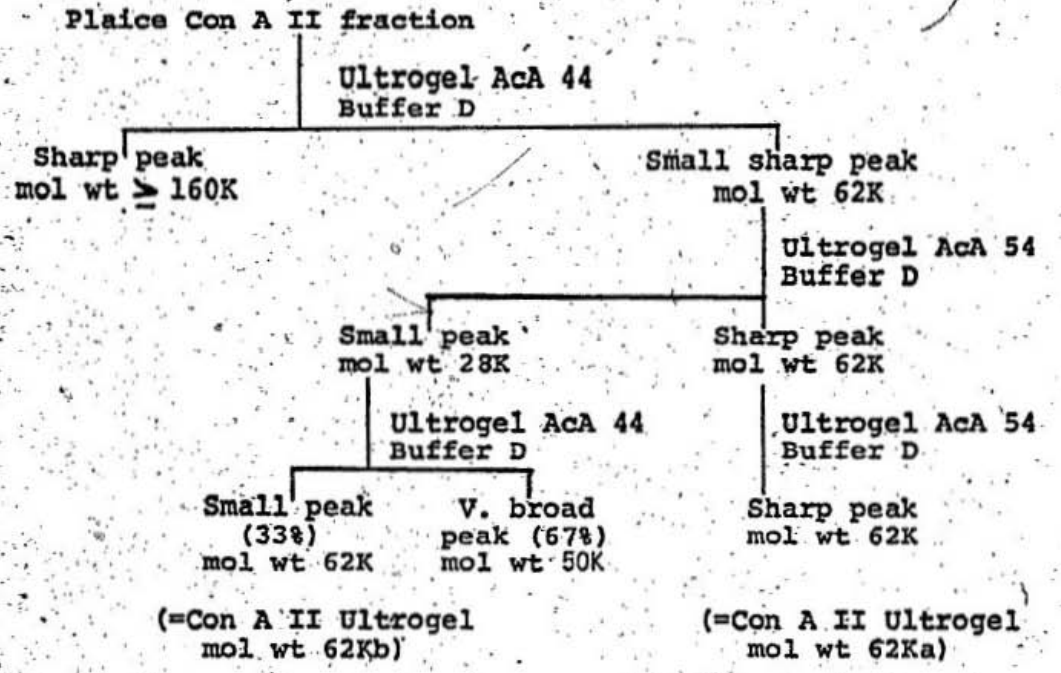

0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
O.D. 280
M.W. 50,000
M.W. 62,000
M.W. 28,000
50
55
60
65
70
75
80
85
90
95
FRACTION NUMBER

Fig. 17 Further purification of plaice Con A II fraction


Plaice Con A II fraction
Ultrogel AcA 44
Buffer D
Sharp peak
mol wt ≥ 160K
Small sharp peak
mol wt 62K
Ultrogel AcA 54
Buffer D
Small peak
mol wt 28K
Sharp peak
mol wt 62K
Ultrogel AcA 44
Buffer D
Ultrogel AcA 54
Buffer D
Small peak
(33%)
mol wt 62K
V. broad
peak (67%)
mol wt 50K
Sharp peak
mol wt 62K
(=Con A II Ultrogel
mol wt 62Kb)
(=Con A II Ultrogel
mol wt 62Ka)

The pituitary of the winter flounder, Pseudopleuronectes americanus also contains Con A I and Con A II fractions which are identical in molecular weight to those of its relative, the American plaice Hippoglossoides platessoides. Both fractions contain MW 28,000 and MW 62,000 forms (Fig.18) and the association of the MW 28,000 peak to form a MW 62,000 peak can be prevented by the inclusion of 10 mM EDTA in the elution buffer.

Results of chromatography of the chum salmon pituitary extract on Con A-Sepharose, Sephacryl S-200 Superfine, and Ultrogel AcA 44 in succession are presented in Figs. 19, 20a, 20b, and 20c. Two apparently homogeneous subfractions, with molecular weights of 25,000 and 45,000, respectively, were obtained from the Con A I fraction. In another run using pituitaries from the same batch, chromatography of the descending half of the MW 45,000 peak produced a peak with MW 45,000 daltons. The yields of the MW 25,000 and MW 45,000 fractions from 5 g of pituitaries were 15 and 20 mg, respectively (Fig. 19).

(ii) Biological activity

Fractions B and C from the plaice Con A I fraction increased the gonadosomatic index, the ovarian uptake of ^{33}P, and the incorporation of the ^{33}P label into the phosphoprotein fraction of the ovary very significantly above those of the control group, when administered at doses of 190 and 250 µg/100 g body weight/injection, respectively (Table 1). A much lower dose, 35 µg/100 g/body weight/injection, of fraction C which had been further purified on Bio-Gel A-0.5 m to give a homogeneous peak of 28,000 molecular weight was still effective in stimulating the uptake of ^{33}P and [^{3}H] leucine into the gonad. In addition, the incorporation of the two radioisotopes into the

Fig. 18 Profile of elution of winter flounder Con A I fraction from Ultrogel AcA 44. Fraction size was 3.6 ml.

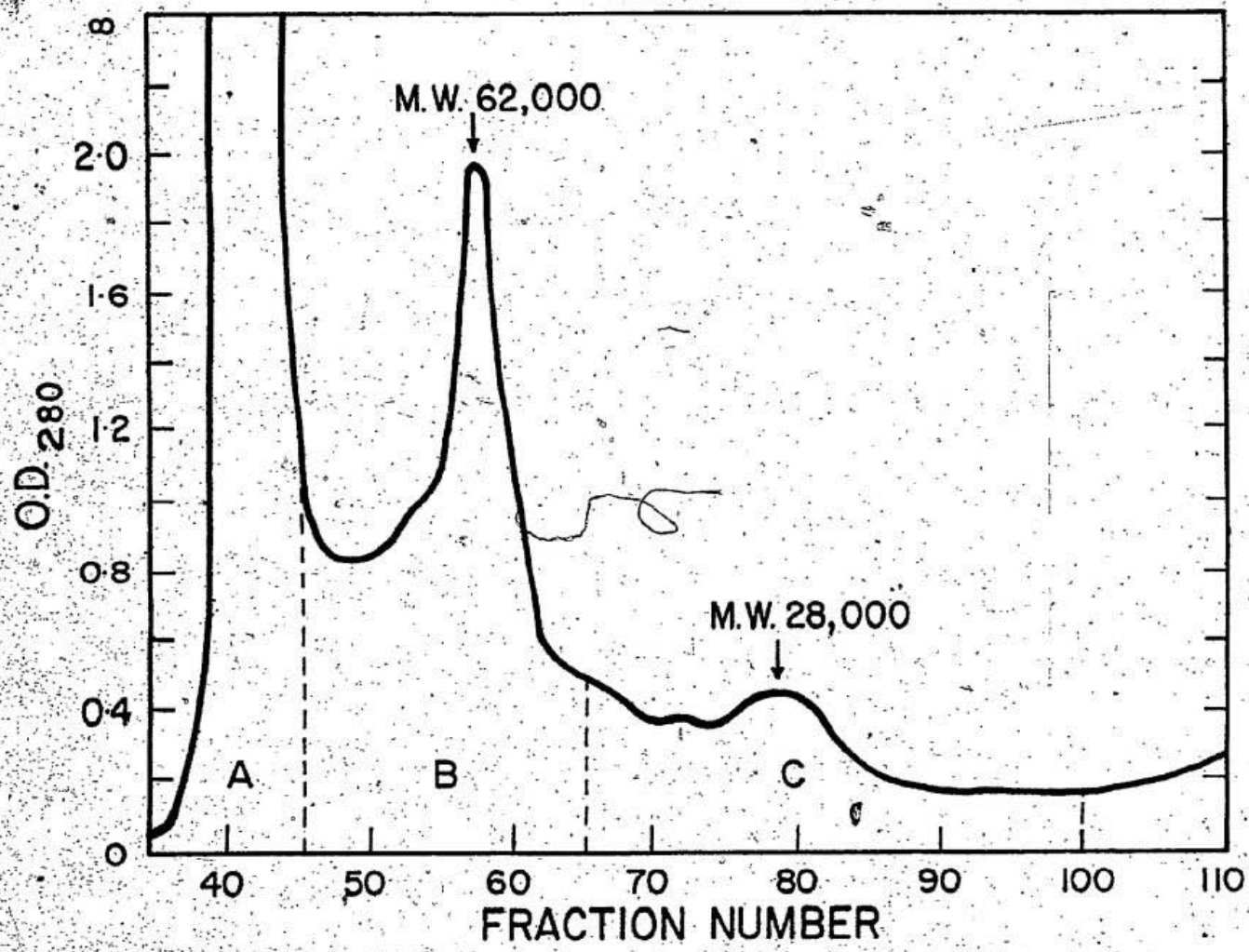

M.W. 62,000
M.W. 28,000
O.D. 280
2·8
2·0
1·6
1·2
0·8
0·4
0
A
B
C
40
50
60
70
80
90
100
110
FRACTION NUMBER

Fig. 19 Fractionation of salmon pituitary extract

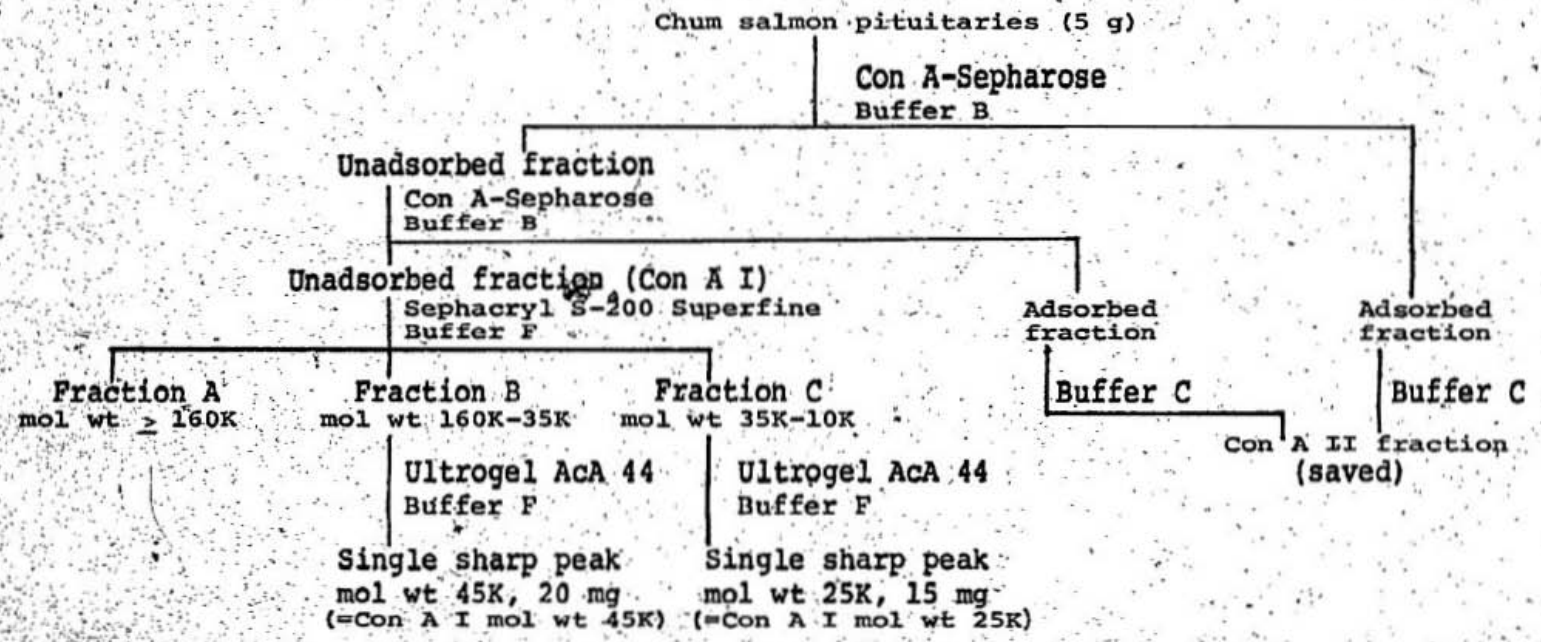
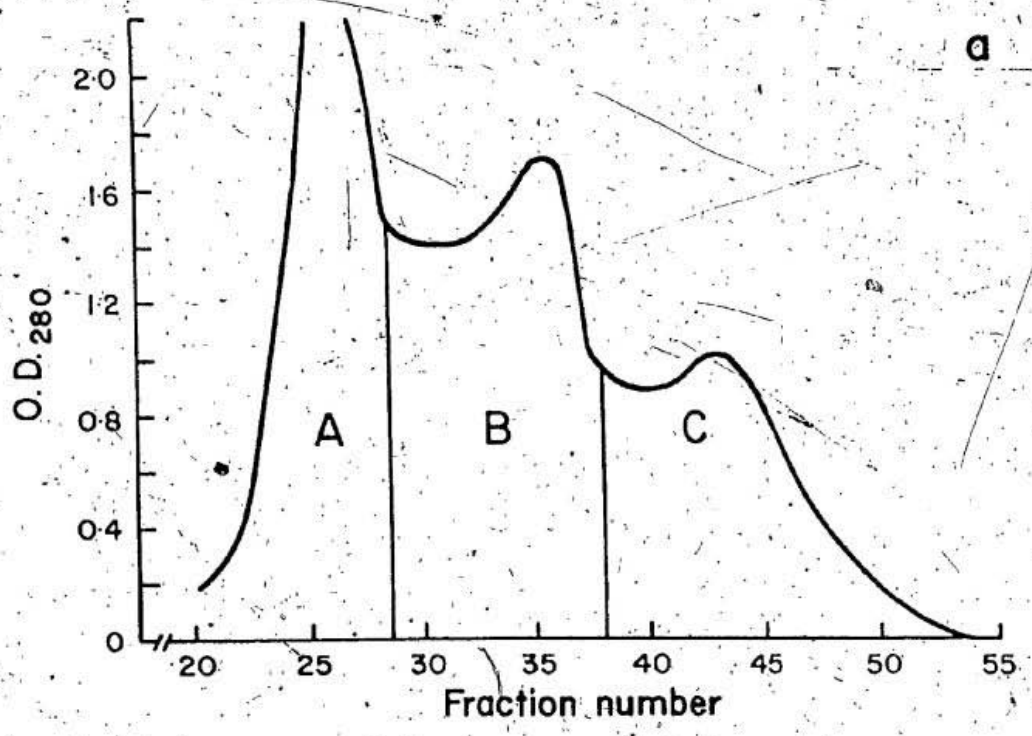
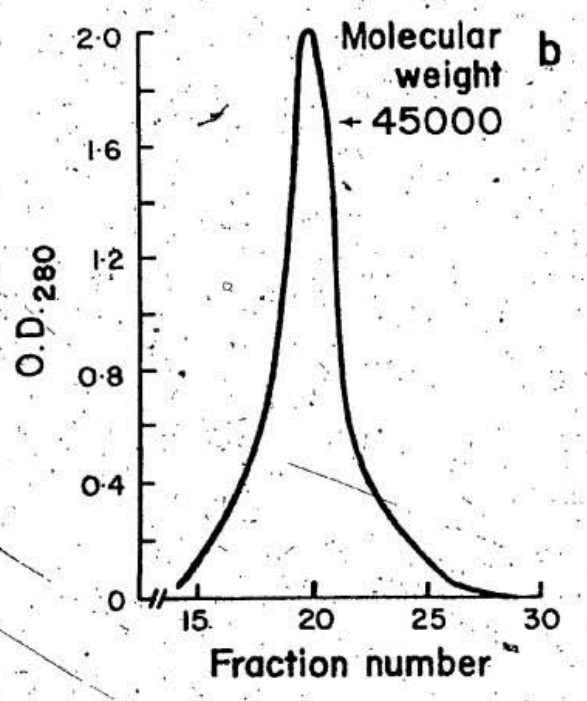

Chum salmon pituitaries (5 g)
Con A-Sepharose
Buffer B
Unadsorbed fraction
Con A-Sepharose
Buffer B
Unadsorbed fraction (Con A I)
Sephacryl S-200 Superfine
Buffer F
Adsorbed fraction
Adsorbed fraction
Fraction A
mol wt ≥ 160K
Fraction B
mol wt 160K-35K
Fraction C
mol wt 35K-10K
Buffer C
Buffer C
Con A II fraction
(saved)
Ultrogel AcA 44
Buffer F
Ultrogel AcA 44
Buffer F
Single sharp peak
mol wt 45K, 20 mg
(=Con A I mol wt 45K)
Single sharp peak
mol wt 25K, 15 mg
(=Con A I mol wt 25K)

Fig. 20 (a) Fractionation of the Con A I fraction of salmon pituitary extract on Sephacryl S-200 Superfine into subfractions A, B, and C. Column dimensions: 2.5 x 91 cm; fraction size: 7.5 ml.
(b) Rechromatography of subfraction B on Ultrogel AcA 44 to produce salmon Con A I (MW 45,000) fraction. Fractions 15-23 were pooled for the assay. Column dimensions: 1.6 x 90 cm; fraction size: 6 ml.
(c) Rechromatography of subfraction C on Ultrogel AcA 44 to produce salmon Con A I (MW 25,000) fraction. Fractions 23-30 were pooled for the assay. Column dimensions: 1.6 x 90 cm; fraction size: 6 ml.


a
O.D.280
2·0
1·6
1·2
0·8
0·4
0
A
B
C
20
25
30
35
40
45
50
55
Fraction number


b
Molecular weight
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O.D.280
2·0
1·6
1·2
0·8
0·4
0
15
20
25
30
Fraction number

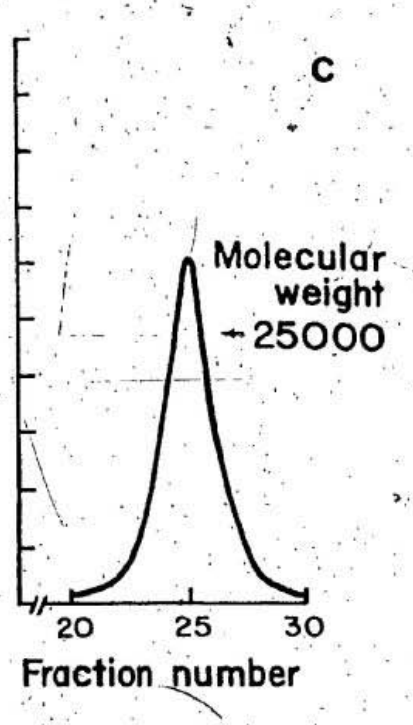

c
Molecular weight
25000
20
25
30
Fraction number

TABLE 1

EFFECTS OF FRACTIONS B AND C FROM PLAICE CON A I ON THE WEIGHT OF, THE UPTAKE OF ^{33}P INTO, AND THE INCORPORATION OF ^{33}P INTO THE YOLK FRACTION OF THE WINTER FLOUNDER OVARY

Group	Treatment	Number of fish	^{33}P label in 100-mg piece of ovary (cpm/mg)	^{33}P label in phosphoprotein fraction of ovary (cpm/g)	Ovary weight (g/100 g)
1	Buffer[a]	11	10.4 ± 0.95*	712 ± 47.5	9.23 ± 0.72
2	Fraction B[b]	10	21.4 ± 1.1	958 ± 44.0	12.3 ± 0.86
			(P < 0.001)	(P < 0.005)	(P < 0.025)
3	Fraction C[c]	14	30.2 ± 2.2	2103 ± 215	15.0 ± 0.62
			(P < 0.001)	(P < 0.001)	(P < 0.001)

[a]Buffer = (20 µCi of $H_3{}^{33}PO_4$ + 1.5 mg of EB[d]/kg + 0.5 ml of Buffer D/kg/injection.

[b]Fraction B = (20 µCi of $H_3{}^{33}PO_4$ + 1.5 mg of EB)/kg + 1.9 mg of fraction B/kg/injection.

[c]Fraction C = (20 µCi of $H_3{}^{33}PO_4$ + 1.5 mg of EB)/kg + 2.5 mg of fraction C/kg/injection.

[d]EB = estradiol benzoate.

*Mean ± Standard Error

trichloroacetic acid-precipitable (protein) and yolk fractions of the ovary was markedly enhanced (Table 2). Results of an assay of vitellogenic activity based on the three aforesaid criteria showed that the 62,000-dalton moiety in fraction B was also potent at a comparable dosage, 35 µg/100 g/injection (Table 3).

When salmon Con A I MW 25,000 and MW 45,000 fractions were tested in the hypophysectomized winter flounder at comparable dosages, both exerted a vitellogenic influence on the fish ovary as estimated by three slightly different parameters. Responses to the two fractions were not significantly different (Table 4).

Two fish which received daily injections of Buffer D exhibited no signs of oocyte maturation after 4 days, and two others had not matured when examined on the sixth day, the day on which the fish received their first injection being reckoned as Day 1. In contrast, the average rating for the group of seven fish treated daily with 200 µg of plaice Con A II MW 62,000 fraction (Fig. 17) which was derived from the 28,000 dalton fractions by aggregation rose from 1.6 to 3.5 after one injection, and attained a value of 5.4 on the fifth biopsy. One fish ovulated after four injections, and all of the other four survivors had ovulated by the eighth day. The maturational process had gone as far as the stage immediately preceding ovulation (a rating of 6) in two fish before they died (Fig. 21). Two fish in the control group subsequently injected with the hormone underwent maturation.

Seven fish injected with an equivalent dose of plaice Con A I MW 62,000 fraction were still at the same stage of maturation on Day 5 as they were on Day 1. Fifteen fish in the buffer-injected

TABLE 2

EFFECTS OF PLAICE CON A I MW 28,000 FRACTION ON THE OVARIAN UPTAKE OF ^{33}P AND [^{3}H] LEUCINE AND THE INCORPORATION OF THE RADIOISOTOPES INTO THE TCA-PRECIPITABLE (PROTEIN) AND YOLK FRACTIONS OF THE OVARY OF THE HYPOPHYSECTOMIZED WINTER FLOUNDER

Group	Treatment	Number of fish	100-mg ovarian piece (dpm/mg of ovary)		TCA precipitate (dpm/mg of ovary)		Phosphoprotein precipitate (dpm/g of ovary)	
			^{33}P label	^{3}H label	^{33}P label	^{3}H label	^{33}P label	^{3}H label
1	Buffer[a]	8	5.43 ± 0.85	15.6 ± 1.80	0.69 ± 0.19	8.64 ± 0.91	340 ± 50.3	750 ± 97.2
2	Hormone[b]	8	20.0 ± 5.13	92.4 ± 26.0	2.20 ± 0.33	34.5 ± 6.70	881 ± 16.4	4298 ± 663
			($P < 0.02$)	($P < 0.02$)	($P < 0.005$)	($P < 0.005$)	($P < 0.01$)	($P < 0.001$)

[a]Buffer = 20 µCi of $H_3{}^{33}PO_4$ + 20 µCi of [^{3}H]Leucine + 1.5 mg of EB[c])/kg + 0.5 ml of Buffer G/kg/injection.
[b]Hormone = (20 µCi of $H_3{}^{33}PO_4$ + 20 µCi of [^{3}H]Leucine + 1.5 mg of EB)/kg + 350 µg of hormone/kg/injection.
[c]EB = estradiol benzoate.

TABLE 3

EFFECTS OF PLAICE CON A I MW 62,000 FRACTION ON THE OVARIAN UPTAKE OF ^{33}P AND [^{3}H]LEUCINE AND THE INCORPORATION OF THE RADIOISOTOPES INTO THE TCA-PRECIPITABLE (PROTEIN) AND YOLK FRACTION OF THE OVARY OF THE HYPOPHYSECTOMIZED WINTER FLOUNDER

Group	Treatment	Number of fish	100-mg ovarian piece (dpm/mg of ovary)		TCA precipitate (dpm/mg of ovary)		Phosphoprotein precipitate (dpm/g of ovary)	
			^{33}P label	^{3}H label	^{33}P label	^{3}H label	^{33}P label	^{3}H label
1	Buffer[a]	5	2.48 ± 0.26	28.90 ± 5.47	0.73 ± 0.08	13.59 ± 1.70	154.92 ± 53.40	926.88 ± 300.04
2	Hormone[b]	5	5.73 ± 1.10	63.40 ± 8.84	1.88 ± 0.30	36.72 ± 6.53	647.04 ± 164.76	5011.20 ± 568.00
			($P < 0.02$)	($P < 0.01$)	($P < 0.01$)	($P < 0.01$)	($P < 0.02$)	($P < 0.001$)

[a]Buffer = (20 µCi of $H_3{}^{33}PO_4$ + 20 µCi of [^{3}H]Leucine + 1.5 mg of EB[c])/kg + 0.5 ml of Buffer D/kg/injection

[b]Hormone = (20 µCi of $H_3{}^{33}PO_4$ + 20 µCi of [^{3}H]Leucine + 1.5 mg of EB)/kg + 350 µg of hormone/kg/injection.

[c]EB = estradiol benzoate.

TABLE 4

EFFECTS OF SALMON CON A I MW 25,000 AND 45,000 FRACTIONS ON OVARIAN UPTAKE OF ^{33}P AND ^{3}H-LABELED-LEUCINE AND THE INCORPORATION OF RADIOISOTOPES INTO TCA-PRECIPITABLE (PROTEIN) AND LIPOPHOSPHOPROTEIN FRACTIONS OF THE OVARY OF THE HYPOPHYSECTOMIZED WINTER FLOUNDER

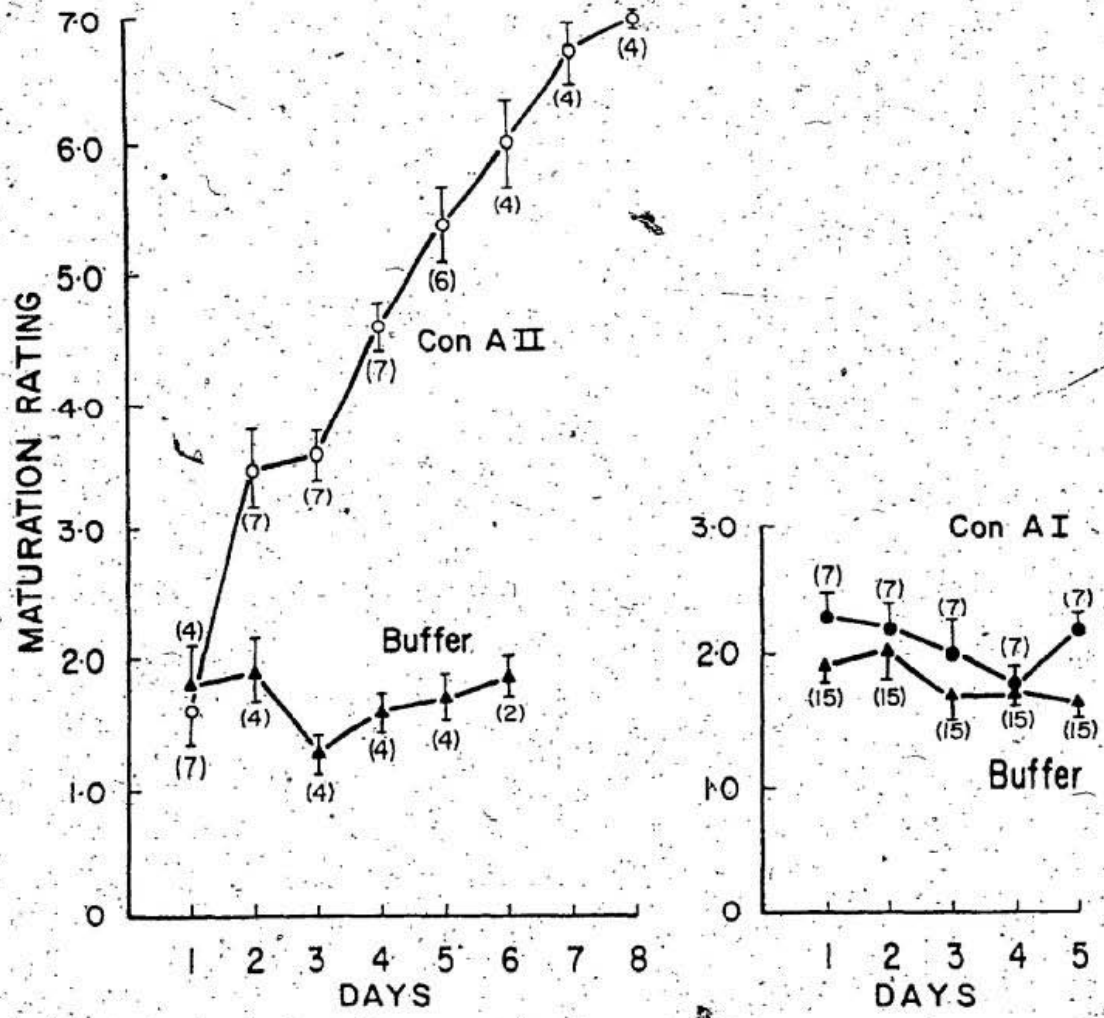
Group No.		Number of fish	100-mg Ovarian slice (dpm/mg of ovary)		TCA precipitate (dpm/mg of ovary)		Phosphoprotein precipitate (dpm/mg of ovary)	
			^{33}P	^{3}H	^{33}P	^{3}H	^{33}P	^{3}H
1	Buffer[a]	10	3.37 ± 0.68	24.8 ± 3.15	1.09 ± 0.09	8.98 ± 1.32	90.2 ± 13.2	411 ± 70
2	Con A I MW 25,000[b]	7	9.75 ± 1.78 ($P < 0.005$)	61.1 ± 11.3 ($P < 0.005$)	2.30 ± 0.35 ($P < 0.005$)	16.4 ± 2.30 ($P < 0.01$)	351 ± 35 ($P < 0.001$)	1326 ± 196 ($P < 0.001$)
3	Con A I MW 45,000[c]	7	11.2 ± 1.71 ($P < 0.001$)	48.3 ± 2.10 ($P < 0.001$)	2.97 ± 0.44 ($P < 0.001$)	32.5 ± 7.80 ($P < 0.005$)	251 ± 47 ($P < 0.005$)	1346 ± 350 ($P < 0.01$)

[a] Buffer = (20 µCi of $H_3{}^{33}PO_4$ + 20 µCi of [^{3}H]leucine + 1.5 mg of EB^+)/kg + 0.5 ml of Buffer F/kg injection.

[b] Con A I MW 25,000 = (20 µCi of $H_3{}^{33}PO_4$ + 20 µCi of [^{3}H]leucine + 1.5 mg of EB^+)/kg + 0.9 mg of Con A I MW 25,000/kg/injection.

[c] Con A I MW 45,000 = (20 µCi of $H_3{}^{33}PO_4$ + 20 µCi of [^{3}H]leucine + 1.5 mg of EB^+)/kg + 1.2 mg of Con A I MW 45,000/kg/injection.

Fig. 21 Effects of plaice Con A I MW 62,000 fraction and plaice Con A II MW 62,000 fraction on the process of oocyte maturation and ovulation in the flounder. Number of fish in parentheses. Day 1 is the day on which the fish received their first injection.


MATURATION RATING
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
Con A II
Buffer
(4)
(7)
(7)
(7)
(6)
(4)
(4)
(4)
(7)
(4)
(4)
(4)
(2)
1 2 3 4 5 6 7 8
DAYS
Con A I
Buffer
3·0
2·0
1·0
0
(7)
(7)
(7)
(7)
(7)
(15)
(15)
(15)
(15)
(15)
1 2 3 4 5
DAYS

group failed to proceed further along the process of maturation as evinced by the cessation of germinal vesicle migration (Fig. 21). Fish in these two groups subsequently responded to treatment with the Con A II MW 62,000a fraction (Fig. 17), the average rating had attained a value of 5.8 on the fifth daily biopsy.

Further purification of salmon gonadotropins on ion exchangers

(i) Chromatographic behaviour

When the MW 45,000 fraction was applied to a DEAE Bio-Gel A column 4 main peaks were discerned in addition to 2 minor ones (Fig. 22). The largest one (45 DE I) was eluted by 5 mM NH_4HCO_3. Two peaks of smaller size were eluted adjacent to each other when a gradient of 50 to 150 mM NH_4HCO_3 was applied, the molarity of elution being 54 and 66 mM respectively. Both peaks, designated MW 45,000 DE II and MW 45,000 DE III, were active in the vitellogenesis assay. The minor peaks and MW 45,000 DE I possessed no vitellogenic activity when tested at an equal dosage (Table 5).

Upon ion exchange chromatography on DEAE Bio-Gel A the MW 25,000 fraction was resolved into 4 peaks (Fig. 23). Only the third one (designated MW 25,000 DE III) possessed activity in the vitellogenesis assay (Table 5). It was eluted at a molarity of 68 mM. The other peaks tested at equal dosage were not active. The yields of MW 25,000 DE III and Con A II G-75 Fraction II (Idler et al., 1975b) from 1 g of pituitary were respectively 0.2 and 1.2 mg.

The maturational Con A II G-75 Fraction II content of chum salmon pituitaries used in these studies was 3.18 ± 0.17 (S.E.M.) mg per gram of pituitary. This result was based on the radioimmunoassay of extracts from ten separate pituitary collections averaging 130 g each

Fig. 22 - Profile of elution of 100 mg salmon Con A I MW 45,000 fraction from DEAE Bio-Gel A. Yields of DE I, II, III, IV, and V were respectively 20, 10, 28, 5.3, and 2.5 mg. Fraction size was 7 ml for DE I and 3.6 ml for all other fractions.

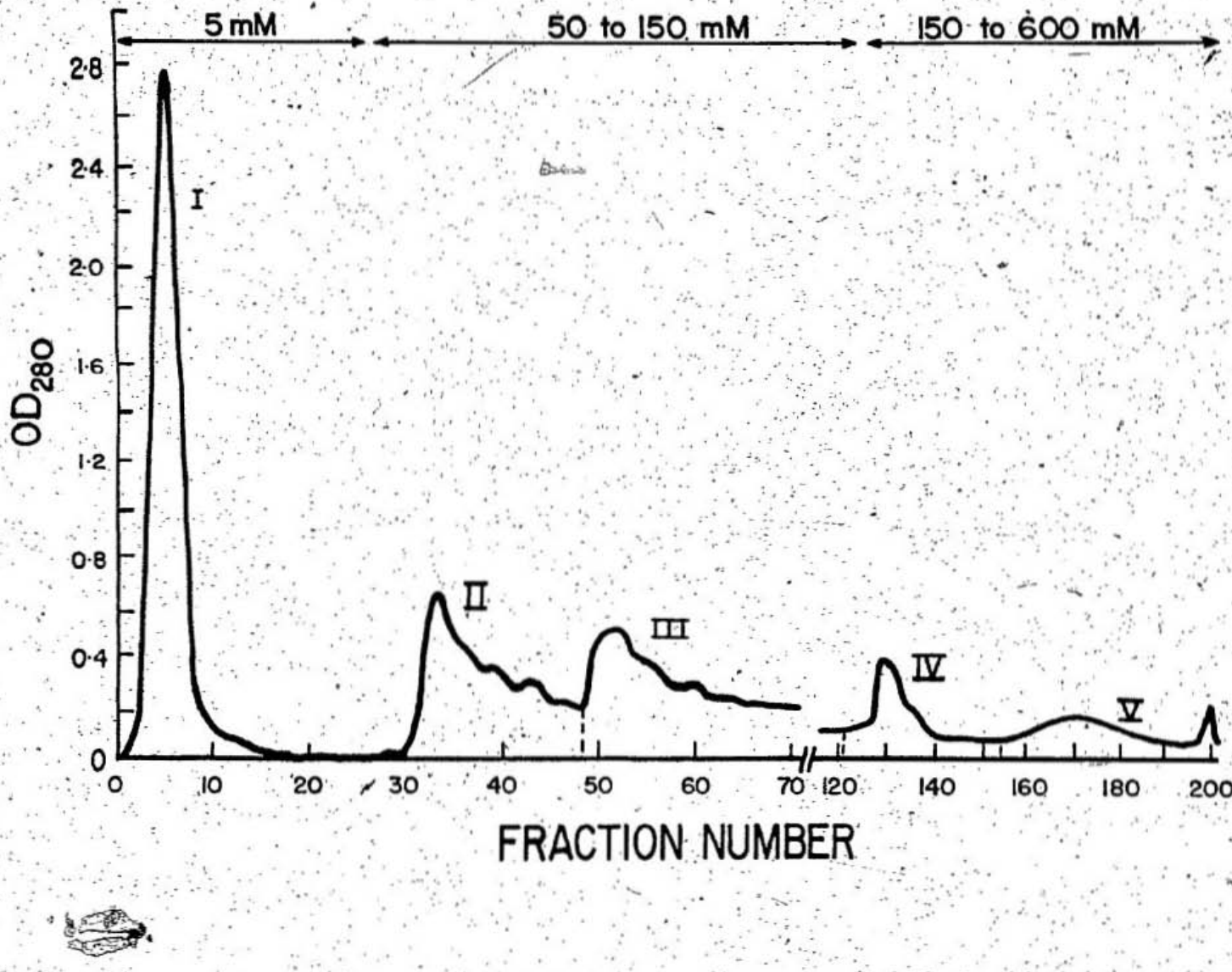

5 mM
50 to 150 mM
150 to 600 mM
OD280
2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
I
II
III
IV
V
0
10
20
30
40
50
60
70
120
140
160
180
200
FRACTION NUMBER

Fig. 23 Profile of elution of 44 mg salmon Con A I MW 25,000 fraction from DEAE Bio-Gel A. Yields of DE I, II, III and IV were 1.5, 10.5, 9.9, and 13.2 mg respectively. Fraction size was 3.6 ml.

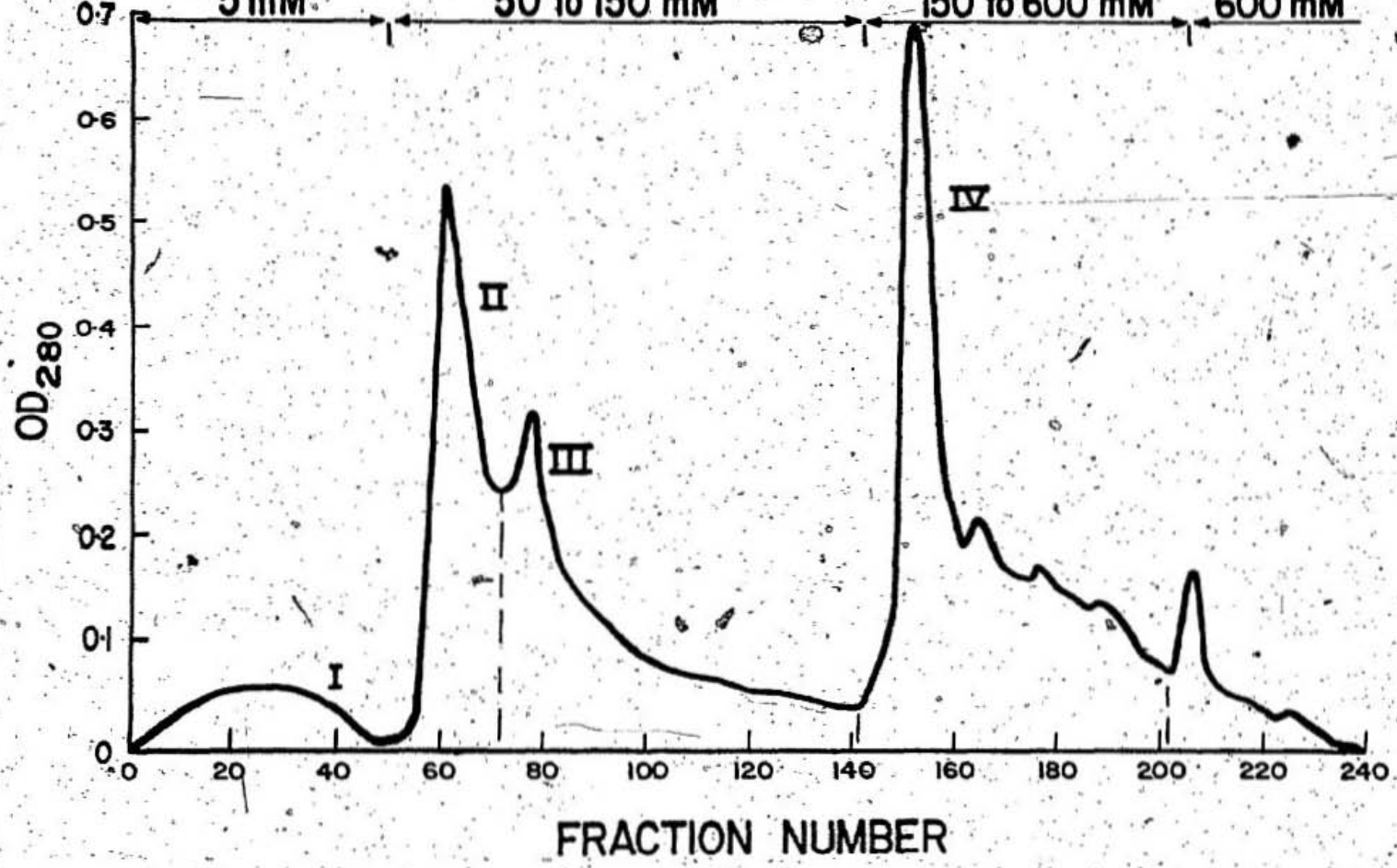

5 mM
50 to 150 mM
150 to 600 mM
600 mM
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
OD280
I
II
III
IV
0
20
40
60
80
100
120
140
160
180
200
220
240
FRACTION NUMBER

TABLE 5

EFFECTS OF VARIOUS GONADOTROPIN FRACTIONS ISOLATED FROM SALMON PITUITARIES ON OVARIAN FUNCTIONS INCLUDING VITELLOGENESIS.

Expt.	Group	Treatment	Dose (μg/kg)	Number of fish	100 mg Ovarian piece (dpm/mg of ovary)		TCA precipitate (dpm/mg of ovary)		Phosphoprotein precipitate (dpm/mg of ovary)	
					^{3}H label	^{33}P label	^{3}H label	^{33}P label	^{3}H label	^{33}P label
1	1	Buffer	-	12	2.4 ± 1.7	9.54 ± 0.22	7.98 ± 0.75	0.51 ± 0.04	1059 ± 16	96 ± 8.5
	2	Salmon Con A I MW 45,000 DE II	100	6	29.9 ± 2.0 ($P < 0.02$)	15.9 ± 1.2 ($P < 0.001$)	17.9 ± 1.5 ($P < 0.001$)	1.1 ± 0.1 ($P < 0.001$)	2113 ± 260 ($P < 0.001$)	215 ± 9.1 ($P < 0.001$)
	3	Salmon Con A I MW 45,000 DE III	100	7	31.2 ± 2.6 ($P < 0.02$)	15.1 ± 1.4 ($P < 0.001$)	23.1 ± 2.2 ($P < 0.001$)	1.3 ± 0.3 ($P < 0.05$)	1860 ± 173 ($P < 0.005$)	234 ± 37 ($P < 0.001$)
	4	Salmon Con A I MW 25,000 DE III	100	6	31.5 ± 1.4 ($P < 0.001$)	12.8 ± 0.2 ($P < 0.001$)	17.0 ± 1.2 ($P < 0.001$)	1.1 ± 0.3 ($P < 0.02$)	2731 ± 48 ($P < 0.001$)	256 ± 45 ($P < 0.001$)
2	1	Buffer	-	10	20.5 ± 2.6	19.8 ± 1.5	13.3 ± 1.5	3.0 ± 0.1	2114 ± 321	785 ± 63
	2	Salmon Con A II MW 40,000 fraction	400	6	37.6 ± 5.9 ($P < 0.01$)	38.9 ± 3.9 ($P < 0.001$)	34.0 ± 8.1 ($P < 0.01$)	7.1 ± 0.9 ($P < 0.001$)	5600 ± 1384 ($P < 0.005$)	2230 ± 244 ($P < 0.001$)
3	1	Buffer	-	7	20.8 ± 3.6	6.7 ± 1.3	9.0 ± 2.3	0.98 ± 0.22	831 ± 212	67 ± 9.3
	2	Salmon Con A I MW 45,000 fraction	400	5	20.9 ± 4.1 (NS)	6.4 ± 0.9 (NS)	24.6 ± 3.9 ($P < 0.01$)	2.4 ± 0.3 ($P < 0.005$)	2463 ± 146 ($P < 0.005$)	181 ± 25 ($P < 0.01$)
	3	Salmon Con A I MW 25,000 fraction	400	5	31.4 ± 3.5 (NS)	7.0 ± 2.1 (NS)	20.6 ± 2.9 ($P < 0.01$)	2.8 ± 0.4 ($P < 0.005$)	2503 ± 75 ($P < 0.001$)	203 ± 22 ($P < 0.001$)
	4	Salmon Con A II MW 40,000 fraction	400	6	63.5 ± 5.2 ($P < 0.001$)	18.4 ± 1.7 ($P < 0.001$)	40.7 ± 4.4 ($P < 0.001$)	2.4 ± 0.3 ($P < 0.005$)	2340 ± 515 ($P < 0.02$)	259 ± 58 ($P < 0.01$)

[a] All fish in experiments 1-3 received 20 μCi each of $H_3{}^{33}PO_4$ and [^{3}H]leucine and 1.5 mg estradiol benzoate/kg body weight on the first day of injection. Estradiol benzoate was omitted for fish in experiments 4 and 5.

made in September and October.

Chromatography of salmon Con A I MW 45,000 DE II and MW 45,000 DE III fractions on Ultrogel AcA 54 yielded a single peak at 45,000 daltons in each case. Salmon Con A I MW 25,000 DE III, Con A I MW 45,000 DE III and Con A II MW 40,000 fractions were largely unadsorbed ($\geq$ 90%) on CM Bio-Gel A equilibrated with 3 mM ammonium acetate buffer at pH 6. Salmon Con A I MW 45,000 DE III fraction was unadsorbed on SP-Sephadex equilibrated with 5 mM NH_4HCO_3 at pH 7.3.

(ii) Biological Activities

(a) Vitellogenic activities

Both Con A I MW 45,000 DE III and the Con A II MW 40,000 fractions stimulated the winter flounder testis to take up tritiated leucine and radiophosphate with a concomitant increase in incorporation into testicular proteins. However, interestingly only the Con A I MW 45,000 DE III fraction was able to elicit an increase in testis weight (Table 6).

The vitellogenic activity of Con A II MW 40,000 fraction was manifested in its ability to stimulate an increase in the incorporation of ^{3}H-leucine and $H_3{}^{33}PO_4$ into the water-insoluble yolk fraction of the ovaries of both estrogenized and non-estrogenized fish, similar to the action of the Con A I subfractions. Other parameters of gonadal activities, including uptake of the radioisotopes and their incorporation into ovarian proteins, and ovarian weight, were also stimulated (Table 5).

The maturational activity of the salmon Con A II MW 40,000 fraction showed a slight delay after neuraminidase treatment but

TABLE 6

EFFECTS OF SALMON GONADOTROPIC FRACTIONS ON THE UPTAKE OF [^{3}H]LEUCINE AND $H_3{}^{33}PO_4$ BY THE FLOUNDER FLOUNDER TESTIS AND ISOTOPIC INCORPORATION INTO TESTICULAR PROTEINS

Group	Treatment	Dose (µg/kg)	Number of fish	100 mg Testicular piece (dpm/mg of testis)		TCA precipitate (dpm/mg of testis)		Testis weight (g/100 g body wt)
				^{3}H label	^{33}P label	^{3}H label	^{33}P label	
1	Buffer	-	9	10.2 ± 1.0	5.5 ± 0.5	1.4 ± 0.1	0.5 ± 0.005	7.8 ± 0.4
2	Salmon Con A I MW 45,000 DE III fraction	150	9	21.6 ± 0.8 ($P < 0.001$)	10.4 ± 1.5 ($P < 0.005$)	3.9 ± 0.1 ($P < 0.001$)	1.4 ± 0.04 ($P < 0.005$)	10.3 ± 0.7 ($P < 0.001$)
3	Salmon Con A II MW 40,000 fraction	150	7	24.9 ± 2.9 ($P < 0.001$)	8.8 ± 0.6 ($P < 0.001$)	2.9 ± 0.5 ($P < 0.01$)	1.1 ± 0.15 ($P < 0.005$)	7.7 ± 0.6 (NS)

[a]All fish received 20 µCi each of $H_3{}^{33}PO_4$ and [^{3}H]leucine per kg body weight on the first injection.

inactivation was incomplete (Table 7).

(b) Ovulation- and spermiation-inducing activities

Despite the overlap in the biological activities outlined above, the actions of salmon Con A II MW 40,000 fraction in effecting ovulation were not paralleled by the salmon Con A I fraction (Table 7). Seven fish injected with the salmon Con A I fraction (10 mg/kg) did not spermiate, while seven other fish treated with buffer also showed no change, in contrast to six fish injected with Con A II MW 40,000 (0.1 mg/kg) which all spermiated.

(c) Steroidogenic activities

Hypophysectomy brought about a dramatic decrease in the plasma level of androgens in flounder (Table 8).

When the two adsorbed DEAE peaks derived from the salmon Con A II 40,000 dalton fraction (Idler et al., 1975) were chromatographed on CM Bio-Gel A, it was found that the large part (>90%) of each peak was unadsorbed (CM 1) on the ion exchanger. Steroidogenic activities resided in the CM 1 fractions (Table 8). When DE II CM I was injected into 5 hypophysectomized female winter flounder at a dosage of 50 µg/fish, all fish had ovulated when examined 5 days after the injection. Therefore steroidogenic and maturational activities were located in the same fraction.

(iii) Immunologic activities

The immunologic potencies of the salmon vitellogenic Con A I MW 25,000 DE III fraction, Con A I MW 45,000 DE III fraction and maturational Con A II G-75 Fraction II were unaltered after treatment with neuraminidase (e.g., Fig. 24). However, subsequent to reduction by 2-mercaptoethanol and blocking of the sulfhydryl groups

TABLE 7

EFFECTS OF SALMON GONADOTROPIC FRACTIONS ON THE PROCESS OF OOCYTE MATURATION AND OVULATION IN THE FLOUNDER

Group	Treatment	Dose (μg/kg)	Number of fish	Maturation Rating						
				Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	Buffer	-	9	1.6 ± 0.2	1.8 ± 0.3	1.8 ± 0.3	1.5 ± 0.2	1.7 ± 0.3	1.5 ± 0.3	-
2	Salmon Con A I Fraction	3000	7	2.1 ± 0.3	2.2 ± 0.3	1.9 ± 0.3	2.2 ± 0.2	1.9 ± 0.2	1.8 ± 0.3	-
3	Salmon Con A II MW 40,000 fraction	300	6	1.9 ± 0.2	3.2 ± 0.2	4.8 ± 0.3	6.5 ± 0.5	7.0	-	-
4	Salmon Con A II MW 40,000 fraction + NT[a]	300	6	1.5 ± 0.2	1.8 ± 0.2	1.6 ± 0.3	2.8 ± 0.3	3.8 ± 0.2	5.2 ± 0.5	7.0

[a] NT, after neuraminidase treatment.

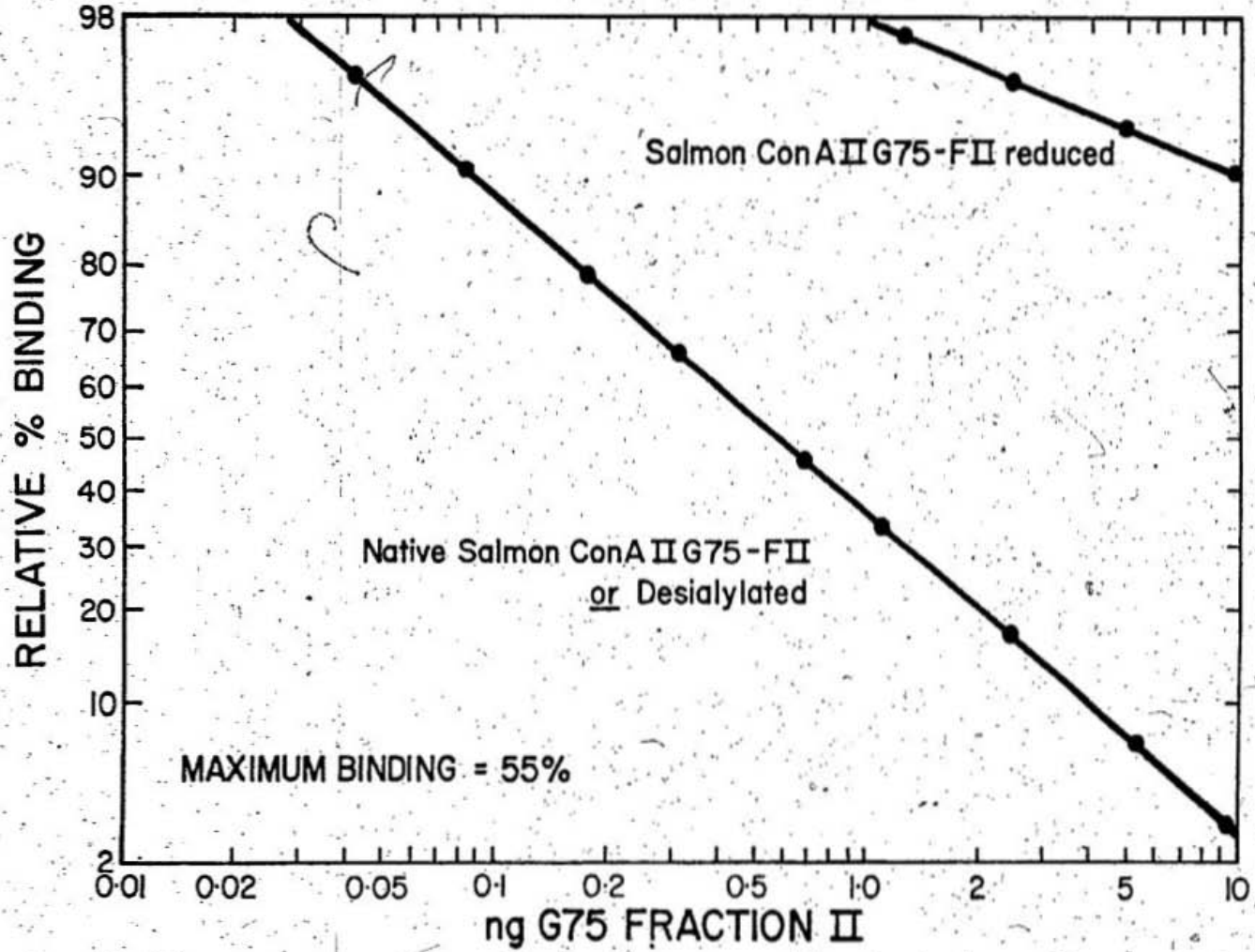
TABLE 8

EFFECTS OF SALMON PITUITARY GONADOTROPIC FRACTIONS ON PLASMA ANDROGEN LEVELS IN HYPOPHYSECTOMIZED MALE FLOUNDER

Expt.	Group	N	Treatment	Dose (µg/fish)	11-Ketotestosterone (ng/ml)	Testosterone (ng/ml
1	A	9	Buffer	-	5.5 ± 0.9	0.4 ± 0.2
	B	6	Intact	-	42.5 ± 5.5 (P < 0.001)	-
2	A	6	Buffer	-	1.5 ± 0.4	1.7 ± 0.2
	B	5	Con A I 45* DE III CM I	100	1.0 ± 0.5	1.5 ± 0.4
	C	5	Con A I 45* DE III CM II	100	3.1 ± 0.6	2.4 ± 0.4
	D	6	Con A II 40* DE II CM I	100	68 ± 12 (P < 0.001)	-
	E	5	Con A II 40* DE II CM II	100	2.8 ± 1.1	5.4 ± 0.8 (P < 0.009)
	F	5	Con A II 40* DE II CM III	100	1.4 ± 1.0	1.5 ± 0.6
	G	5	Con A II 40* DE III CM I	100	101 ± 14 (P < 0.001)	-
	H	5	Con A II 40* DE III CM II	100	2.8 ± 1.0	3.0 ± 1.4
	I	5	Con A II 40* DE III CM III	100	0.8 ± 0.1	2.4 ± 0.1 (P < 0.025)

*Molecular weight x 10^{-3}

Fig. 24 Radioimmunoassay of native, desialylated, and reduced S-carboxamidomethylated salmon Con A II G-75 Fraction II.


RELATIVE % BINDING
98
90
80
70
60
50
40
30
20
10
2
0·01
0·02
0·05
0·1
0·2
0·5
1·0
2
5
10
ng G75 FRACTION II
Salmon Con A II G75-F II reduced
Native Salmon Con A II G75-F II
or Desialylated
MAXIMUM BINDING = 55%

by iodacetamide, the ability of the modified Con A II G-75 Fraction II fraction to displace the labeled native hormone was lost (Fig. 24). The validity of the above conclusions was demonstrated by the lack of any effect of NaOAc, $CaCl_2$, neuraminidase, urea, and mercaptoethanol and iodoacetamide, on the binding of the antigen to the antibody.

The Con A I MW 45,000 and MW 25,000 fractions showed about 0.4% cross reaction in a radioimmunoassay for Con A II G-75 Fraction II (Fig. 25). The Con A I MW 25,000 DE III and MW 45,000 DE III fractions showed respectively 0.12% and 0.3% cross reaction in the radioimmunoassay.

Interference from Con A II G-75 Fraction II in radioimmunoassays for the Con A I MW 25,000 DE III (Fig. 26) and MW 45,000 DE III (Fig. 27) fractions was negligible, indicating that Con A II G-75 Fraction II had minimal contamination with the Con A I subfractions. Hence the biological activities found in each of these hormones were intrinsic characteristics. Results of these immunological studies also revealed that the salmon maturational hormone was probably more antigenic than the vitellogenic hormones and its antigenic determinants were different from those of the vitellogenic hormones.

Chemical compositions

The amino acid and carbohydrate analyses of salmon gonadotropins are presented in Table 9. Pronounced differences in a large number of amino acids and carbohydrates were found among the Con A I MW 25,000 DE III, Con A I MW 45,000 DE III fractions and Con A II G-75 Fraction II.

Electrophoretic characteristics

On electrophoresis salmon Con A I MW 45,000 DE III fraction showed a narrow band at Rf* 0.6. Con A I MW 25,000 DE III fraction showed a

* The tracking dye band was taken as the front. The gel was made by polymerizing a layer of large pore gel on top of small pore gel.

Fig. 25 Test of cross reactivity of salmon Con A I MW 25,000 and MW 45,000 fractions in a radioimmunoassay for salmon Con A II G-75 Fraction II.

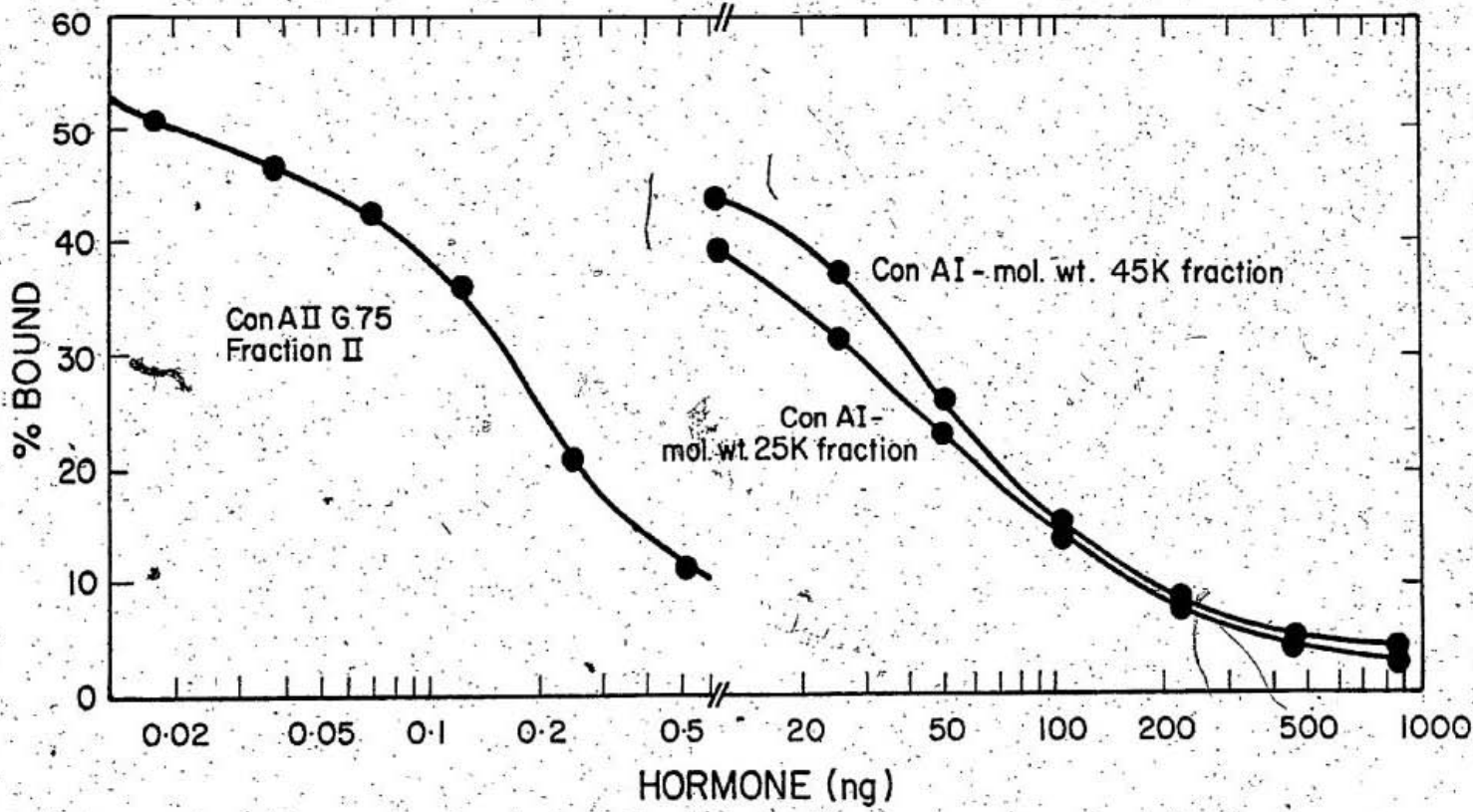

60
50
40
30
20
10
0
% BOUND
Con A II G 75
Fraction II
Con AI - mol. wt. 45K fraction
Con AI -
mol. wt. 25K fraction
0·02
0·05
0·1
0·2
0·5
20
50
100
200
500
1000
HORMONE (ng)

Fig. 26 Radioimmunoassay of salmon Con A I 25 DE III and testing of cross reactivity of salmon Con A II G-75 Fraction II and Con A I 45 DE III.

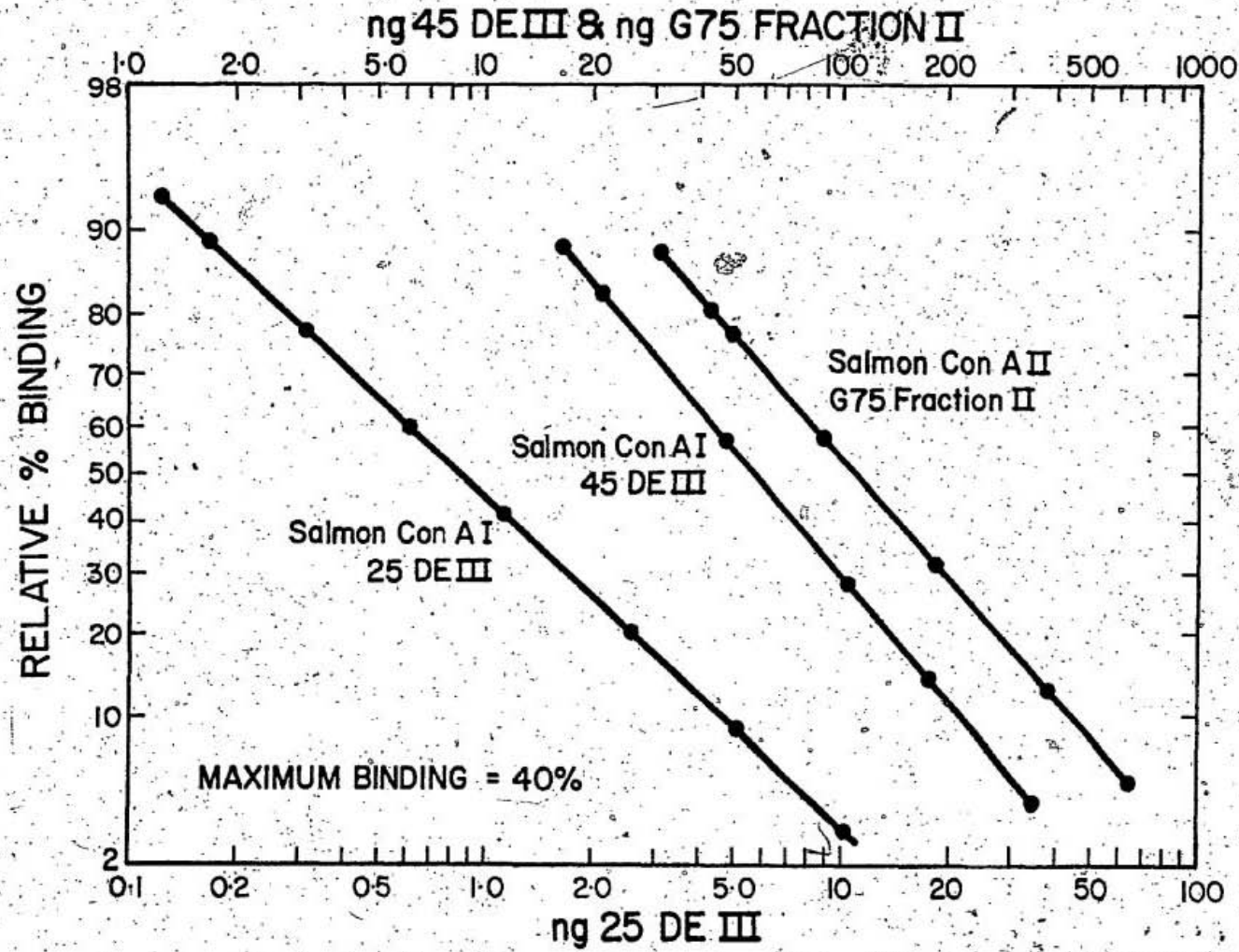

ng 45 DE III & ng G75 FRACTION II
1·0
2·0
5·0
10
20
50
100
200
500
1000
98
90
80
70
60
50
40
30
20
10
2
RELATIVE % BINDING
Salmon Con A II
G75 Fraction II
Salmon Con A I
45 DE III
Salmon Con A I
25 DE III
MAXIMUM BINDING = 40%
0·1
0·2
0·5
1·0
2·0
5·0
10
20
50
100
ng 25 DE III

Fig. 27 Radioimmunoassay of salmon Con A I 45 DE III and testing for cross reactivity of salmon Con A I 25 DE III and Con A II G-75 Fraction II.

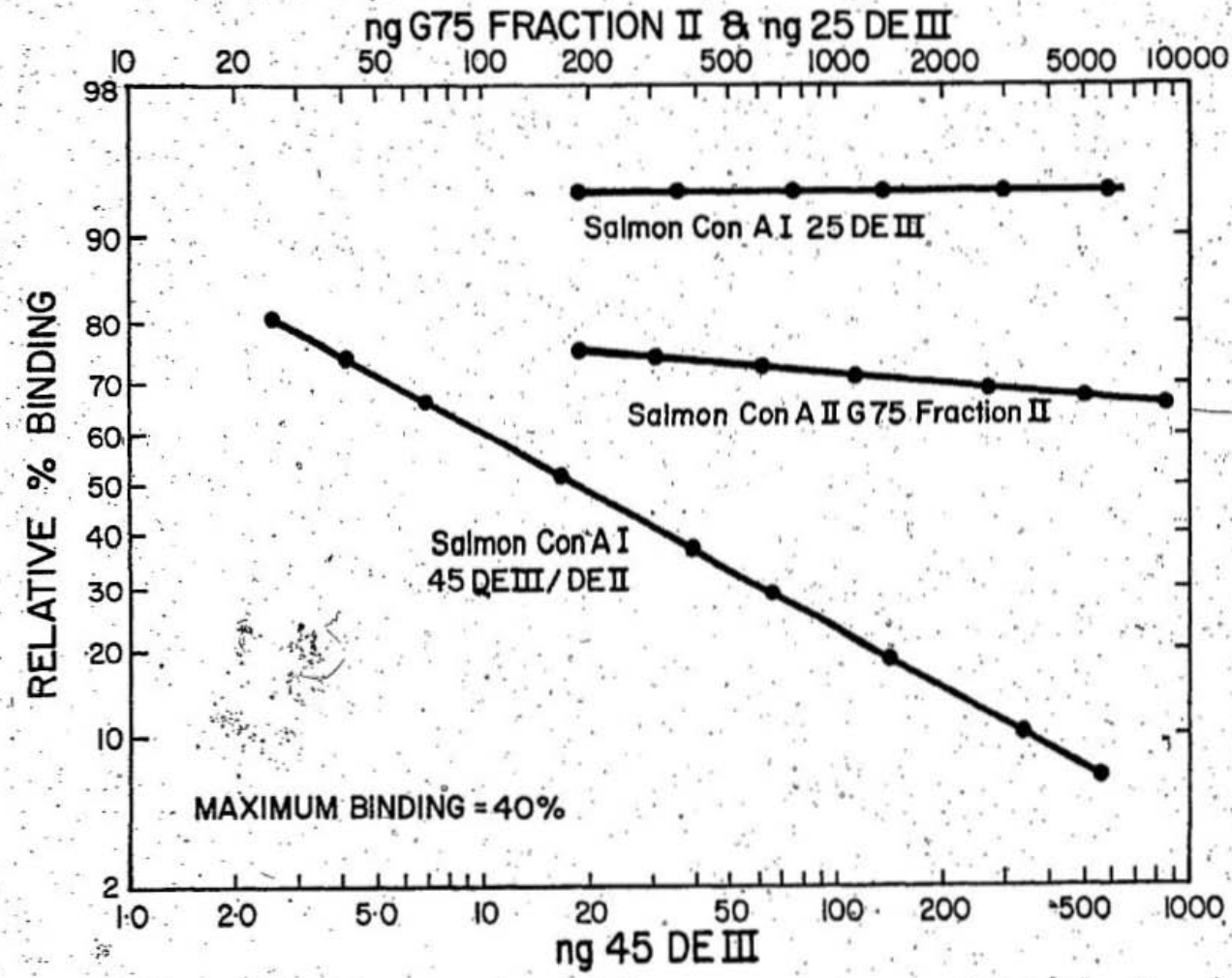

ng G75 FRACTION II & ng 25 DE III
10
20
50
100
200
500
1000
2000
5000
10000
98
90
80
70
60
50
40
30
20
10
2
RELATIVE % BINDING
Salmon Con A I 25 DE III
Salmon Con A II G75 Fraction II
Salmon Con A I
45 DE III / DE II
MAXIMUM BINDING = 40%
1·0
2·0
5·0
10
20
50
100
200
500
1000
ng 45 DE III

TABLE 9

AMINO ACID AND CARBOHYDRATE COMPOSITIONS OF SALMON GONADOTROPINS

	Salmon			
	Con A I MW 25,000 DE III fraction	Con A I MW 45,000 DE III fraction	Con A I MW 45,000 DE II fraction	Con A II G-75 Fraction II
Lysine[a]	6.8	17.4	14.6	13.0
Histidine	3.9	4.4	4.7	5.8
Arginine	8.6	7.7	7.3	6.4
Aspartic Acid	27.6	20.4	21.8	17.8
Threonine	5.2	11.9	12.1	29.3
Serine	23.6	12.9	12.8	19.4
Glutamic acid	28.6	17.2	22.5	10.6
Proline	17.8	9.5	10.6	17.7
Glycine	10.6	13.5	15.6	7.5
Alanine	6.0	18.2	17.8	4.2
1/2-Cystine	12.2	8.0	7.6	12.1
Valine	5.0	15.9	14.1	16.2
Methionine	3.2	4.2	1.3	3.0
Isoleucine	6.5	9.4	8.8	9.5
Leucine	22.8	16.8	17.4	12.9
Tyrosine	3.6	3.4	3.3	8.0
Phenylalanine	5.6	8.0	7.6	6.1
Tryptophan	2.2	1.1	0.2	0.5
Total hexoses[b]	1.8	2.0	1.8	6.0
Glucosamine	1.4	0.3	1.3	2.6
Galactosamine	0.1	0.2	0.3	0.1
Sialic acid	0.3	0.5	0.6	1.5

[a]Values for amino acid are expressed in number of residues for every 200 residues.
[b]Values for carbohydrates are expressed in g/100 g protein.
[c]Not determined.

major band at Rf 0.34 and a minor one at 0.4. Salmon Con A II G-75 Fraction II showed a broad band with Rf ranging from 0.48 to 0.61, and a very minor narrow band at Rf 0.69.

Further purification of American plaice hormones

(i) Chromatographic behaviour and biological activities

Results of chromatography on DEAE Bio-Gel A of, Con A I MW 28,000, Con A I MW 62,000, and Con A II MW 62,000 fractions from plaice pituitaries, are presented in Figs. 6, 5, and 7 respectively.

In the first two cases unadsorbed material (DE I) was eluted with 5 mM NH_4HCO_3 at pH 9. The patterns of elution for the Con A I fractions when the first concentration gradient (50 to 150 mM NH_4HCO_3) was applied were very similar, consisting of two adjacent peaks, designated DE II and DE III. Vitellogenic activity was found in both the main area (III a) of the DE III peak and its trailing portion, (III b) (Table 10). The other peaks were tested and found to be inactive. In the case of the plaice Con A I MW 62,000 fraction, only a single peak with a trailing end was eluted by the first concentration gradient. The main area of this peak (DE I) was very potent in inducing oocyte maturation and ovulation but the trailing portion (DE II) also had weak maturational activity (Table 11).

A single peak was eluted when the second concentration gradient (150 to 500 mM NH_4HCO_3) was applied, in all cases of Con A I and Con A II fractions studied.

Only plaice Con A II MW 62,000 fraction was able to reinitiate production of 11-ketotestosterone in the hypophysectomized flounder. The CM I and CM II subfractions derived from the vitellogenic DE III fraction unadsorbed on Con A-Sepharose did not have much

TABLE 10

EFFECTS OF PLAICE AND FLOUNDER GONADOTROPINS ON THE OVARIAN UPTAKE OF ^{33}P AND [^{3}H]LEUCINE AND ISOTOPIC INCORPORATION INTO THE TCA-PRECIPITABLE (PROTEIN) AND YOLK FRACTIONS OF THE OVARY OF HYPOPHYSECTOMIZED WINTER FLOUNDER

Expt.	Group	Treatment	Dose (ug/kg)	Number of fish	100 mg Ovarian piece (dpm/mg of ovary)		TCA precipitate (dpm/mg of ovary)		Phosphoprotein precipitate (dpm/g of ovary)	
					^{3}H label	^{33}P label	^{3}H label	^{33}P label	^{3}H label	^{33}P label
1	1	Buffer Plaice Con A I	-	6	28.2 ± 4.1	4.9 ± 0.2	24.6 ± 5.6	1.2 ± 0.3	586 ± 171	108 ± 20
	2	28[b] DE III a	50	6	63.0 ± 17.6	7.3 ± 1.2	55.2 ± 9.3 ($P < 0.02$)	2.3 ± 0.2 ($P < 0.02$)	2919 ± 740 ($P < 0.02$)	330 ± 40 ($P < 0.005$)
	3	28 DE III b	50	6	118 ± 35 ($P < 0.05$)	14.1 ± 3.5 ($P < 0.05$)	68.6 ± 13.0 ($P < 0.02$)	2.7 ± 0.3 ($P < 0.01$)	3243 ± 935 ($P < 0.02$)	412 ± 93 ($P < 0.005$)
2	1	Buffer Plaice Con A I	-	7	9.7 ± 1.3	1.9 ± 0.2	7.0 ± 0.6	1.0 ± 0.1	403 ± 63	172 ± 39
	2	62 DE III a	50	7	33.0 ± 2.3 ($P < 0.001$)	4.4 ± 0.2 ($P < 0.001$)	14.3 ± 1.5 ($P < 0.001$)	1.5 ± 0.1 ($P < 0.005$)	1012 ± 97 ($P < 0.001$)	493 ± 76 ($P < 0.005$)
	3	62 DE III b	50	11	23.4 ± 1.8 ($P < 0.001$)	6.0 ± 0.2 ($P < 0.001$)	18.4 ± 1.2 ($P < 0.001$)	1.7 ± 0.1 ($P < 0.001$)	1177 ± 70 ($P < 0.001$)	514 ± 75 ($P < 0.001$)
3	1	Buffer Flounder Con A I	-	10	20.6 ± 2.7	19.9 ± 1.6	13.3 ± 1.5	3.0 ± 0.1	2114 ± 321	785 ± 63
	2	62 DE III	100	6	72.1 ± 25.0 ($P < 0.02$)	55.9 ± 17.5 ($P < 0.02$)	66.8 ± 2.4 ($P < 0.02$)	9.5 ± 1.8 ($P < 0.001$)	4999 ± 1260 ($P < 0.02$)	2232 ± 345 ($P < 0.001$)
	3	62 DE IV	100	6	34.2 ± 6.6 ($P < 0.05$)	32.2 ± 6.4 ($P < 0.05$)	24.2 ± 3.3 ($P < 0.05$)	6.6 ± 0.5 ($P < 0.001$)	4026 ± 916 ($P < 0.05$)	1750 ± 234 ($P < 0.001$)
	4	Con A I 28	500	6	48.2 ± 7.4 ($P < 0.005$)	42.4 ± 6.5 ($P < 0.005$)	34.8 ± 4.5 ($P < 0.001$)	7.1 ± 1.3 ($P < 0.01$)	4724 ± 105 ($P < 0.001$)	2098 ± 72 ($P < 0.001$)
	5	Con A II 62	250	5	31.1 ± 2.0 ($P < 0.02$)	39.8 ± 3.5 ($P < 0.001$)	28.8 ± 2.1 ($P < 0.001$)	5.2 ± 0.5 ($P < 0.005$)	3402 ± 621 ($P < 0.05$)	1973 ± 134 ($P < 0.001$)

[a]All fish were injected with 20 μCi each of $H_3{}^{33}PO_4$ and [^{3}H]leucine and 1.5 mg estradiol benzoate/kg body weight. The other DEAE fractions not listed were inactive when tested at equal dosage.

[b]Values are molecular weight x 10^{-3}

TABLE 11

EFFECTS OF PLAICE AND FLOUNDER HORMONES ON THE PROCESS OF OOCYTE MATURATION AND OVULATION IN THE FLOUNDER

Expt. No.	Group	Treatment	Number of fish	Dose (µg/fish/day)	Maturational Rating			
					Day 1	Day 2	Day 3	Day 4
1	1	Buffer	15	-	1.95 ± 0.17	2.00 ± 0.19	1.68 ± 0.14	1.70 ± 0.10
	2	Plaice Con A II 62[b] DE I	9	40	1.87 ± 0.19	4.02 ± 0.26	4.78 ± 0.36	5.1 ± 0.32
	3	DE II	5	40	1.58 ± 0.19	2.36 ± 0.83	2.44 ± 0.91	4.04 ± 1.09
	4	DE III	5	40	1.64 ± 0.17	2.08 ± 0.11	1.98 ± 0.14	2.00 ± 0.14
2	1	Buffer	10	-	1.52 ± 0.17	1.43 ± 0.27	1.32 ± 0.19	1.42 ± 0.21
	2	Flounder Con A I 62[b]	7	320	1.76 ± 0.24	1.87 ± 0.31	1.62 ± 0.22	1.73 ± 0.11
	3	Con A II 62[b]	6	60	1.83 ± 0.26	3.78 ± 0.23	5.12 ± 0.78	6.3 ± 0.93

[a]A rating of 1 indicates no oocyte maturation while 7 indicates that all eggs from all fish ovulated.

steroidogenic activity (Table 12).

The yields of Con A I MW 62,000 DE III, Con A I MW 28,000 DE III, Con A II MW 62,000 and Con A II MW 28,000 fractions from 1 g of plaice pituitaries were 0.6, 0.5, 0.2 and 0.08 mg respectively.

(ii) Chemical composition

Studies on the carbohydrate compositions of the plaice vitellogenic (Con A I) and maturational (Con A II) hormones indicated that the latter hormones had a higher sugar content. The two types of gonadotropins differed in their amino acid compositions (Table 13). The half-cystine and methionine values might be lower than the actual values but unfortunately there was not enough material available for performic acid oxidation.

(iii) Cross reactivity in radioimmunoassay

It was evident from Fig. 28 that both the plaice Con A I MW 62,000 DE III and MW 28,000 DE III fractions cross-reacted to only about 2% in a radioimmunoassay for the Con A II MW 62,000 fraction, providing evidence that the two types of hormones were immunochemically dissimilar and that the former fractions had little contamination with the latter. The Con A II MW 28,000 fraction also did not interfere in the assay.

Purification of winter flounder hormones

(i) Chromatographic behaviour and biological activities

The elution profile of flounder Con A I MW 62,000 peak from DEAE Bio-Gel was similar to those of the plaice Con A I fractions (Fig. 29), and vitellogenic activity was located in the corresponding fraction (Table 10). The others peaks were tested and found to be inactive. The large parts ($\geq$ 90%) of plaice and flounder vitellogenic

TABLE 12

EFFECTS OF PLAICE PITUITARY GONADOTROPIC FRACTIONS ON PLASMA ANDROGEN LEVELS IN HYPOPHYSECTOMIZED MALE FLOUNDER

Expt.	Group	N	Treatment	Dose (µg/fish)	11-Ketotestosterone (ng/ml)	Testosterone (ng/ml)
1	A	9	Buffer	-	5.5 ± 0.9	0.4 ± 0.2
	B'	3	Con A II 62*	100	70 ± 5.9 ($P < 0.001$)	-
2	A	9	Buffer	-	1.7 ± 0.6	1.5 ± 0.7
	B	5	Con A I 62* DE III CM I	100	4.4 ± 1.4	0.2 ± 0.2
	C	5	Con A I 62* DE III CM II	100	3.5 ± 1.5	1.2 ± 0.5
	D	7	Con A I 28* DE III CM I	100	5.5 ± 1.3	1.0 ± 0.5
	E	6	Con A I 28* DE III CM II	100	1.9 ± 1.0	2.7 ± 0.4 ($P < 0.005$)

*Molecular weight x 10^{-3}.

TABLE 13

AMINO ACID AND CARBOHYDRATE COMPOSITIONS OF PLAICE GONADOTROPINS

	Con A I MW 62,000 DE III fraction	Con A II MW 62,000 DE I fraction	Con A I MW 28,000 DE III fraction
Lysine[a]	14.6	11.9	10.0
Histidine	7.0	5.6	5.6
Arginine	9.3	6.1	8.8
Aspartic acid	23.4	22.5	23.8
Threonine	12.5	13.7	10.1
Serine	13.7	15.4	17.0
Glutamic acid	20.8	17.9	23.6
Proline	9.4	8.7	12.7
Glycine	16.8	19.7	18.5
Alanine	14.0	13.5	13.3
1/2 Cystine	2.0	8.3	9.0
Valine	12.0	12.7	9.1
Methionine	4.0	14.7	5.1
Leucine	17.6	14.1	16.4
Tyrosine	5.4	4.7	4.5
Phenylalanine	9.1	8.1	6.4
Total hexoses[b]	1.5	15.0	2.0
Glucosamine	0.7	6.0	1.2
Galactosamine	0.4	0.3	0.7
Sialic acid	1.2	3.5	0.4

[a]Values for amino acids are expressed in number of residues for every 200 residues.

[b]Values for carbohydrates are expressed in g/100 g protein.

Fig. 28 Testing for cross-reaction from Con A I MW 62,000 DE III, Con A I MW 28,000 DE III, and Con A II MW 28,000 fraction from plaice pituitaries in a radioimmunoassay for plaice Con A II MW 62,000 DE I.

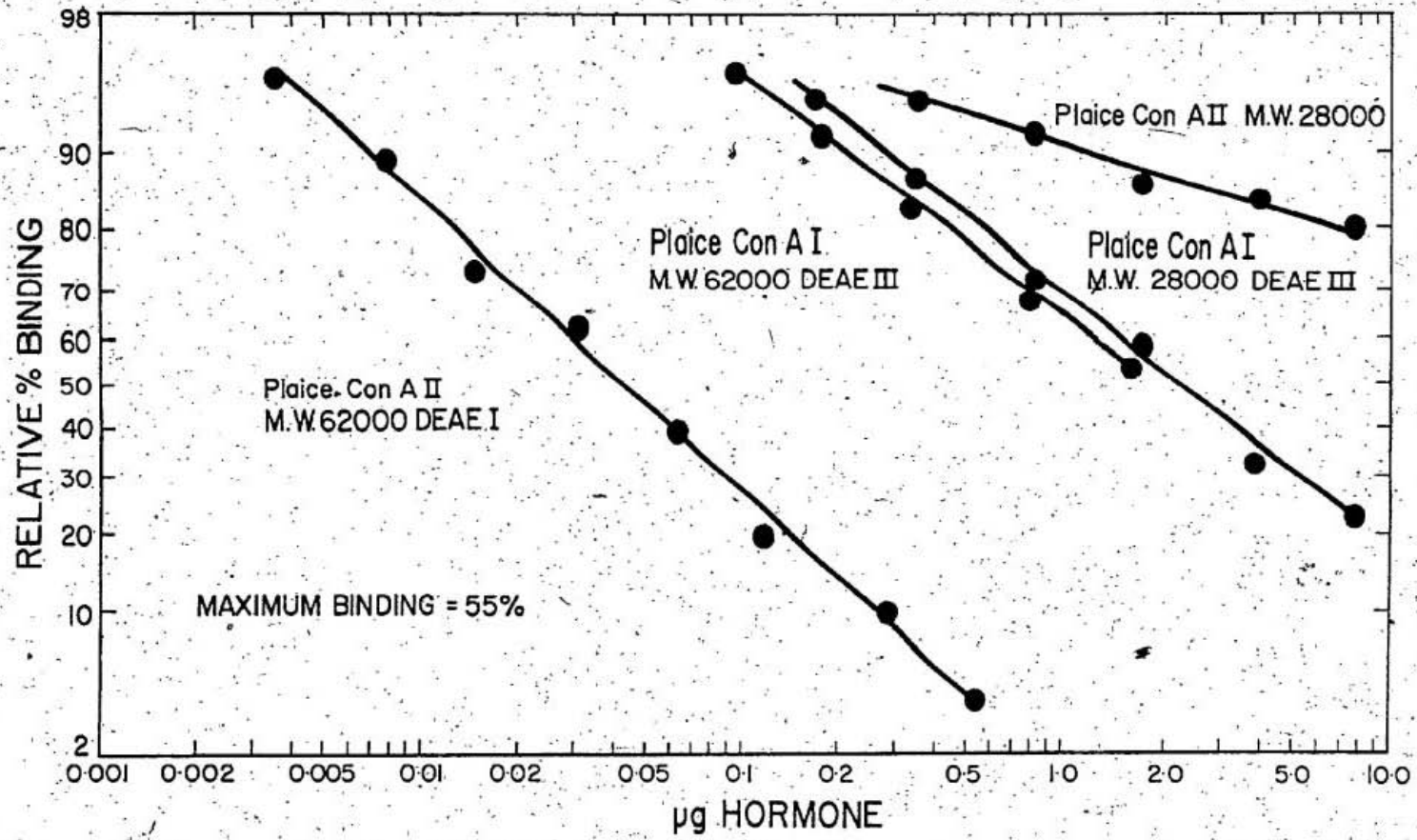
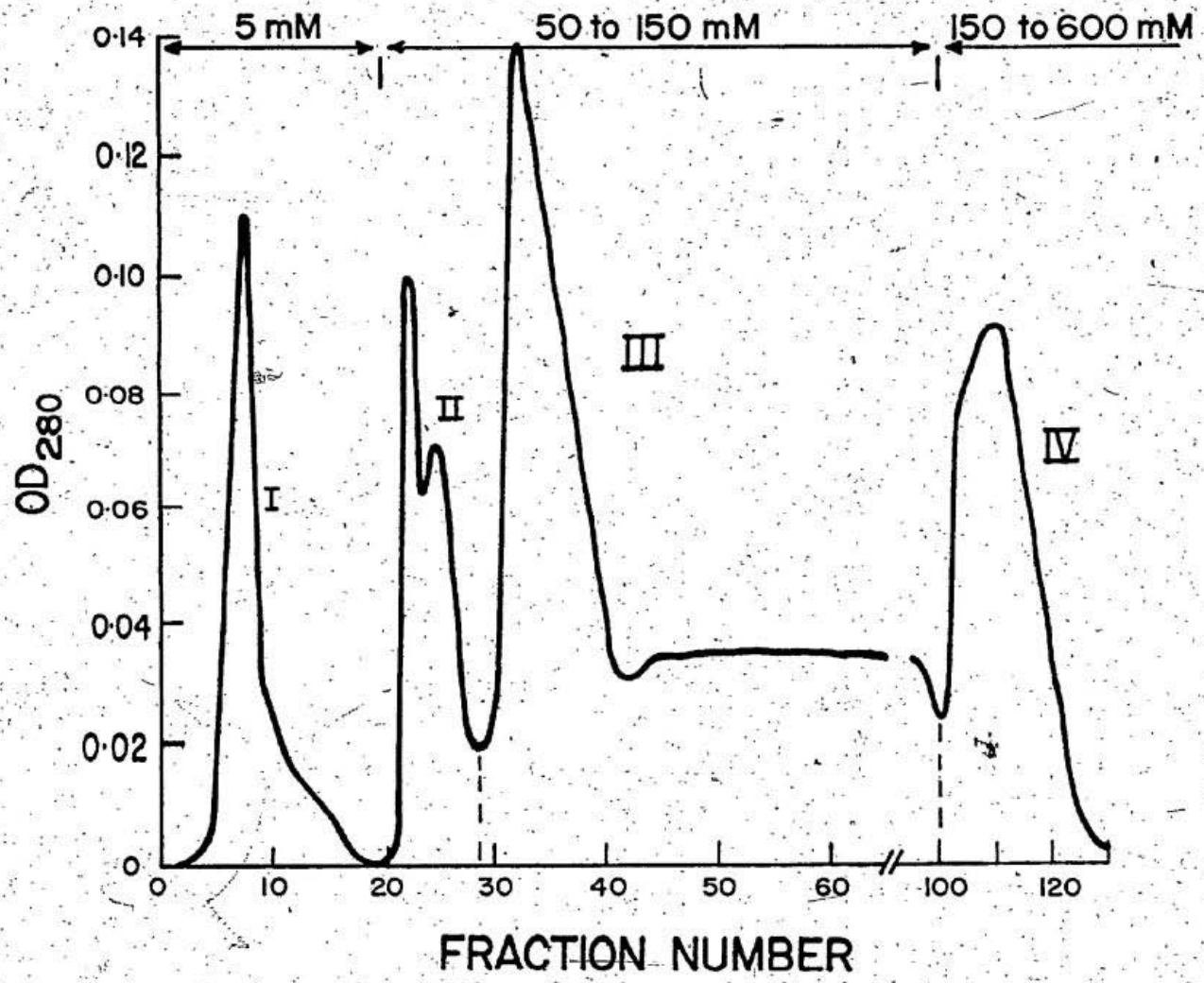

RELATIVE % BINDING
98
90
80
70
60
50
40
30
20
10
2
0·001
0·002
0·005
0·01
0·02
0·05
0·1
0·2
0·5
1·0
2·0
5·0
10·0
µg HORMONE
Plaice Con A II M.W. 28000
Plaice Con A I
M.W. 62000 DEAE III
Plaice Con A I
M.W. 28000 DEAE III
Plaice Con A II
M.W. 62000 DEAE I
MAXIMUM BINDING = 55%

Fig. 29 Profile of elution of 21 mg flounder Con A I MW 62,000 fraction from DEAE Bio-Gel A. Yields of DE I, II, III and IV were respectively 1.8, 1.9, 6.1 and 2.6 mg. Fraction size was 3.6 ml. Con A I MW 62,000 fraction, 21 mg and 10 mg Con A I MW 28,000 fraction were obtained from 15 g of flounder pituitaries.


5 mM
50 to 150 mM
150 to 600 mM
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
OD280
I
II
III
IV
0
10
20
30
40
50
60
100
120
FRACTION NUMBER

Con A I MW 62,000 DE III and MW 28,000 DE III fractions were found to be unadsorbed on CM Bio-Gel A equilibrated with 3 mM ammonium acetate at pH 6.

The flounder Con A I MW 62,000 fraction had no effect on the process of oocyte maturation and ovulation in the same species, consistent with the finding for plaice and salmon Con A I fractions while the flounder Con A II MW 62,000 fraction was active (Table 11).

Both Con A II MW 62,000 and Con A I MW 28,000 fractions were active in reinitiating vitellogenesis in hypophysectomized flounder (Table 10). When hypophysectomized female flounder were injected with plasma from estrogenized male flounder and saline, the ovaries incorporated 631 ± 71 cpm ^{33}P per gm ovary (N=9). Six fish injected with the plasma containing ^{33}P-labeled phosphoproteins and flounder Con A I MW 28,000 DE III CM I fraction incorporated 1221 ± 143 ($P < 0.005$ versus control) cpm ^{33}P per gm ovary.

The Con A II MW 62,000 fraction was able to reinitiate the production of 11-ketotestosterone and testosterone. The CM I and CM II subfractions derived from the vitellogenic DE III fractions unadsorbed on Con A-Sepharose did not have much steroidogenic activity (Table 14).

Hypophysectomy apparently led to a disappearance of the sex hormone binding proteins from circulation because tritiated 11-ketotestosterone equilibrated with plasma samples from hypophysectomized flounder was present entirely in the free form. Treatment of hypophysectomized fish with either Con A I or Con A II fractions resulted in a reappearance of the binding proteins (Table 14).

The yields of Con A I MW 62,000 DE III, Con A I MW 28,000, Con A

TABLE 14

EFFECTS OF FLOUNDER PITUITARY GONADOTROPIC FRACTIONS ON PLASMA ANDROGEN LEVELS IN HYPOPHYSECTOMIZED MALE FLOUNDER

Expt.	Group	N	Treatment	Dose (µg/fish)	11-ketotestosterone (ng/ml)	Testosterone (ng/ml)	B/F ratio+ of added ^{3}H 11-ketotestosterone
1	A	9	Buffer	-	5.5 ± 0.9++	0.4 ± 0.2	All free (0)
	B	6	Intact	-	42.5 ± 5.5 (P < 0.001)	-	1.8 ± 0.9
	B	6	Con A II 62*	100	79.5 ± 16 (P < 0.001)	6.5 ± 1.3 (P < 0.001)	0.9 ± 0.2
	C	7	Con A I 62* DE III CM I	100	3.1 ± 0.5	0.7 ± 0.2	8.5**
	D	5	Con A I 62* DE III CM II	100	7.4 ± 1.4	0.8 ± 0.2	3.6**
	E	6	Con A I 28* DE III CM I	100	2.9 ± 1.0	1.0 ± 0.4	3.5**
	F	5	Con A I 28* DE III CM II	100	7.2 ± 1.9	0.7 ± 0.3	2.6**

*Mollecular weight x 10^{-3}; ** Value determined from a pool of plasma samples in the group;
++Mean ± S.E.M.

+ Ratio of protein-bound steroid to free steroid.

II were respectively 0.58, 0.6, 0.12 and 0.1 mg.

(ii) Cross reactivity in radioimmunoassay

In a radioimmunoassay for the flounder Con A II MW 62,000 fraction it took 2 µg each of the flounder Con A I MW 62,000 DE III and MW 28,000 DE III fractions to give the same displacement as 4 ng and 8 ng of the Con A II MW 62,000 fraction respectively, indicating that the Con A I subfractions had minimal contamination with the Con A II MW 62,000 fraction.

Flounder Con A II MW62,000 fraction showed only 4% binding to the antiserum (diluted 1 : 5,000) against flounder Con A I MW 28,000 fraction illustrating immunologic unrelatedness between the maturational and vitellogenic hormones.

Chromatography of flounder ovarian phosphoproteins

When the ovarian phosphoproteins were chromatographed on Cellex T, DEAE Bio-Gel A, and Bio-Gel HTP, about 80% of the proteins were adsorbed. Over 90% of the proteins were unadsorbed on CM Bio-Gel A and about 80% on Amberlite CG-50. All of the proteins were unadsorbed on Con A-Sepharose.

Fractionation of the flounder ovarian phosphoproteins first on Sephacryl S-200 Superfine and subsequently on Ultrogel AcA 54 and AcA 22 revealed the presence of two principal peaks with molecular weight 30,000 (containing 4.5% alkali labile phosphorus) and 500,000 (0.8% alkali labile phosphorus), corresponding to phosvitin and lipovitellin respectively of the amphibian ovary (Redshaw and Follett, 1971).

Antibody inhibition of vitellogenesis and oocyte maturation in landlocked salmon and winter flounder

The specificity of the antisera used is shown in Table 15. The presence of an excess of specific antibodies in plasma of fish injected with antisera was demonstrated by the ability of the plasma to bind the appropriate labeled hormones (Table 16).

Landlocked salmon experiments

In the first experiment, vitellogenic fish treated with an antiserum to the maturational fraction had lower plasma levels of estradiol ($p < 0.001$) and vitellogenin than did the groups injected with normal rabbit serum or an antiserum to the vitellogenic fractions. Fish treated with S-Con A I-ab and fish treated with S-Con A II-ab showed less incorporation of yolk into the ovary compared with fish injected with normal rabbit serum (Table 17).

In the second experiment, of the eight pre-spawning fish injected with normal rabbit serum, oocytes of only one did not undergo germinal vesicle breakdown. More than half of the group treated with S-Con A I-ab matured. By contrast, oocytes in none of the fish injected with S-Con A II-ab underwent germinal vesicle breakdown.

Oocytes from the two fish which ovulated showed no incorporation of radioactive leucine and phosphorus into the ovarian yolk fraction. Ovaries in which germinal vesicle breakdown occurred contained less newly incorporated yolk than did those in which maturation had not started, although apparently there was no difference in plasma vitellogenin level (Table 18).

Winter flounder experiment

Despite some differences in the protocol from the landlocked salmon experiment, the results obtained were similar. Normal rabbit serum and F-Con A I-ab failed to inhibit the process of maturation

TABLE 15

SPECIFICITY OF ANTISERA

Iodinated Hormone	Percentage Binding			
	S-Con A I-ab	S-Con A II-ab	F-Con A I-ab	F Con A II-ab
Salmon Con A I MW 25,000 DE III CM I Fraction	50 (5×10^3)	1.5 (320)	-	-
Salmon Con A II G-75 Fraction II	4 (320)	50 (2×10^5)	-	-
Flounder Con A I MW 28,000 DE III CM I Fraction	-	-	50 (5×10^3)	3 (640)
Flounder Con A II MW 62,000 Fraction	-	-	4 (640)	50 (6.4×10^3)

*The number in parenthesis denotes the antiserum dilution.

TABLE 16

DEMONSTRATION OF EXCESS ANTIBODIES IN PLASMA OF LANDLOCKED SALMON INJECTED WITH ANTISERA

Group	Treatment	Binding (%) to salmon vitellogenic hormone			Binding (%) to salmon maturational hormone		
		2.5 µl	10 µl	25 µl	2.5 µl	10 µl	25 µl
1	Normal rabbit serum (NRS)	5-6	5-6	5-6	4-5	4-5	4-5
2	S-Con A II-ab	5-6	5-6	5-6	53	63	78
3	S-Con A I-ab	38	50	57	4-5	4-5	4-5
NRS Blank	None	5-6	5-6	5-6	4-5	4-5	4-5

TABLE 17

EFFECTS OF GONADOTROPIN ANTISERA ON VITELLOGENESIS IN LANDLOCKED SALMON

Group	N	Treatment	Estradiol ng/ml	Vitellogenin µg/ml	Water insoluble yolk ^{33}P dpm/g ovary
1	2	NRS	5.2 ± 0.05	3450 ± 1060	11120 ± 610
2	4	S-Con A II-ab	2.2 ± 0.1 ($p < 0.001$ vs Gp 1 and Gp 3)	1540 ± 50	5520 ± 340 ($P < 0.005$ vs Gp 1)
3	4	S-Con A I-ab	6.1 ± 0.3	5900 ± 2380	4910 ± 2140 ($P < 0.05$ vs Gp 1)

TABLE 18

EFFECTS OF GONADOTROPIN ANTISERA ON OOCYTE MATURATION IN LANDLOCKED SALMON AND WINTER FLOUNDER CHANGES IN VITELLOGENESIS DURING OOCYTE MATURATION

Expt. No	Species	Grp	N	Treatment	Maturational Status*(n)	Water Insoluble Yolk (dpm/g ovary) ^{3}H label	Water Insoluble Yolk (dpm/g ovary) ^{33}P label	Vitellogenin µg/ml	^{3}H-labeled serum yolk cpm/µl
A	Salmon	1	8	NRS	GV^{-}(6)	1430 ± 45 ($P < 0.001$ vs Grp 2)	490 ± 155 ($P < 0.005$ vs Grp 2)	3525 ± 570	490 ± 90
					OV (1)	0	0	1975	265
					GV^{+}(1)	16700	4500	2820	450
		2	6	S-Con A II-ab	GV^{+}(6)	13000 ± 1900	4245 ± 915	4650 ± 1120	465 ± 80
		3	7	S-Con A I-ab	GV^{-}(3)	780 ± 50 ($P < 0.005$ vs Grp 2)	540 ± 85 ($P < 0.005$ vs Grp 2)	4010 ± 720	340 ± 35
					OV (1)	0	0	1460	175
					GV^{+}(3)	11920 ± 50	4230 ± 1920	3135 ± 920	630 ± 130
B	Flounder	1	11	NRS	GV^{-}(6)	515 ± 115	160 ± 30	-	-
		2	11	F-Con A I-ab	GV^{-}(7)	450 ± 90	180 ± 30	-	-
					OV (4)	0	0	-	-
		3	8	F-Con A II-ab	GV^{+}(8)	1230 ± 150 ($P < 0.005$ vs Grp 1 & 2)	390 ± 50 ($P < 0.005$ vs Grp 1) ($P < 0.025$ vs Grp 2)	-	-

GV^{-} = Germinal vesicle absent. GV^{+} = Germinal vesicle present. OV = Ovulated.

and ovulation but F-Con A II-ab was effective. Ovulated eggs showed no incorporation of newly labeled yolk. Oocyte germinal vesicle breakdown was associated with an apparently lower incorporation of yolk (Table 18).

DISCUSSION

Chromatographic techniques

The present investigation utilized an improved version of the chromatographic procedures according to Idler et al., (1975a). Refinements of the technique entail the inclusion in all buffer systems of Trasylol, a protease inhibitor; the selection of Ultrogel AcA 44 and Ultrogel AcA 54, which offer fine resolution in the molecular weight range of interest, as the gel filtration media; and rechromatography of fractions to minimize intermingling of hormonal activities.

Assay animal

The testing of biological activities in the hormone preparations from plaice, flounder and salmon pituitaries in the hypophysectomized flounder allowed an insight into the normal physiological functions of the hormones in the fish itself. The well-defined separation of the vitellogenic season (September-January) and the period of maintenance of yolky oocytes (February-March) from the period of oocyte maturational and ovulatory activities (April-June), as well as the ease of hypophysectomy and good post-operational survival rate, make the winter flounder a very good experimental animal for the study of the hormonal control of reproduction.

The finding that the winter flounder pituitary produced two

types of gonadotropins with similar chromatographic behaviour and biological actions, to those of plaice and salmon, was significant because the winter flounder was used as the assay animal for the isolation of these fish gonadotropins and it possessed the appropriate gonadal receptors for the two types of gonadotropins. It was of considerable interest to note that tetrapod LHs were active in the lizard (Licht and Crews, 1975; Licht et al., 1977b) and were able to displace human FSH from binding to squamate gonadal tissue (Licht and Midgley, 1976) although the squamate pituitary was lacking in both LH-like biological (Licht, 1974) and immunological (Licht et al., 1974) activities. Gonadotropins might exert low or even no biological activity when tested in animals from another class (Farmer et al., 1975; Farmer and Papkoff, 1977). Utilization of winter flounder as the recipient of fish hormones thus minimized this problem of class specificity of protein hormones.

Bioassays

In the bioassay for vitellogenic activity, the ovarian uptake of ^{33}P and ^{3}H represents an incorporation of the isotopes into phospholipids, nucleotides, nucleic acids, peptides, phosphoproteins, and other proteins of the ovary. Labels in the trichloroacetic acid-precipitable fraction of the ovary after hydrolysis at 85C denote incorporation into the protein constituents and also some incorporation into nucleic acids. Radioactivity in the saline EDTA-extractable and $Ca\ 2^{+}$ - precipitable portion of the gonad is a measure of newly formed yolk proteins.

Fractionation of flounder ovarian phosphoproteins with molecular

exclusion chromatography revealed the presence of two principal peaks corresponding to phosvitin and lipovitellin respectively of the amphibian ovary (Redshaw and Follett, 1971). The chromatographic behaviour of these proteins on various ion exchangers, their solubility in concentrated salt solutions and insolubility in water, were characteristic of yolk platelet proteins (Mano and Lipmann, 1966; Redshaw and Follett, 1971). The presence of alkali labile phosphorus and little or no carbohydrate (indicated by non-absorption on Con A-Sepharose) in flounder yolk proteins was in agreement with findings for amphibian yolk (Wallace, 1963). Hence radioactivity due to the administered radioisotopes in the ovarian phosphoprotein precipitate represented a measure of newly formed yolk. However, $H_3{}^{33}PO_4$ injected into the fish concurrently with estradiol might be first incorporated into the hepatically synthesized yolk precursor 'vitellogenin' which was then taken up by the ovary and converted into ovarian yolk, or else $H_3{}^{33}PO_4$ might be taken up by the ovary and directly incorporated into any yolk synthesized within the ovary. This assay for vitellogenic activity would not distinguish between these possibilities. When plasma of estrogenized fish which should contain a high level of vitellogenin was dialyzed to eliminate $H_3{}^{33}PO_4$ and ^{33}P-labeled proteins of low molecular weight, ovarian yolk synthesis should be minimized.

Vitellogenesis in the plaice Pleuronectes platessa was shown to be under pituitary control but evidence pertaining to oviposition was less conclusive (Barr, 1963). In the present study it was found that ablation of the pituitary suppressed the process of maturation and ovulation in the winter flounder promptly because control fish injected with buffer over a period of several days after

hypophysectomy did not mature (Fig. 24). Examination of daily egg samples permitted the tracing of individual fish, initially at an early stage of maturation, throughout the whole process of oocyte maturation which utlimately culminated in ovulation.

Naming of hormones

The physiology of reproduction in the female fish consists of the processes of vitellogenesis, maturation, and spawning. These processes are not, strictly speaking, comparable to follicular growth and ovulation in the female mammal owing to different adaptations to external and internal fertilization. Results from bioassays using a mammal as the recipient (Otsuka, 1956; Farmer and Papkoff, 1977) do not establish the role that the fish hormones play in the regulation of reproductive processes in the fish itself. Mammalian hormones cannot mimic the full spectrum of biological activities fish gonadotropins display (see DeVlaming, 1974). A LH-like gonadotropin was finally isolated in addition to the FSH-like gonadotropin (Licht and Papkoff, 1974a) which had long been believed to be the only gonadotropin emanating from the reptilian pituitary based on results of injection experiments (Licht, 1970, 1972; Callard et al., 1972). Hence, the two piscine pituitary gonadotropins are best described as vitellogenic and maturational hormones rather than LH-like and FSH-like. The term vitellogenic hormone is used for the gonadotropin isolated from the Con A I fraction while the term maturational hormone is used for the gonadotropin isolated from the Con A II fraction, although it has to be borne in mind that the maturational hormone also plays a role in the proeess of vitellogenesis.

Biological activities

The activity of salmon (Donaldson et al., 1972) gonadotropic preparation in a variety of fish species had been amply cited (Donaldson, 1973; Fontaine, 1975). In the present study the two different types of hormones from salmon, plaice and flounder pituitaries showed some overlap in their spectra of biological activities.

General metabolism in gonads

Both types of gonadotropins were capable of stimulating general metabolism in testis and ovary as reflected in their effects on gonadal uptake of radiophosphate and tritiated leucine with subsequent isotopic incorporation into proteins, which represented typical actions of gonadotropins on gonads (McKerns, 1969; Janzen et al., 1977) and of hormones on their target organs in general (Riggs, 1964).

Vitellogenesis

Both types of piscine gonadotropins stimulated an increase in ovarian yolk. The flounder vitellogenic (Con A I) hormone stimulated incorporation of serum phosphoproteins into ovarian yolk. However the possibility of an ovarian synthesis could not at this stage be excluded.

The maturational hormone was also involved in the stimulation of ovarian estradiol production which led to an increase in vitellogenin level in the circulation. A mechanism of vitellogenesis similar to that in birds and amphibians (Flickinger and Rounds, 1956; Wallace and Jared, 1959), by which gonadotropins stimulated the ovary to incorporate vitellogenin, the yolk precursor, which was then transformed into yolk platelet proteins, had been proposed for the winter flounder (Campbell and Idler, 1976).

Incorporation of ^{3}H-leucine and $H_3{}^{33}PO_4$ into ovarian proteins other than lipovitellin and phosvitin

When the radioactivity due to ^{3}H-leucine in the lipophosphoprotein fraction is subtracted from that in the trichloroacetic acid precipitable fraction of the ovary, it can be seen that the piscine gonadotropins stimulate the synthesis of ovarian proteins other than lipophosphoproteins (lipovitellin and phosvitin).

Testicular activities: steroidogenesis and incorporation of ^{3}H-leucine and $H_3{}^{33}PO_4$ into testicular proteins

In the male, stimulation of incorporation of leucine and phosphate into testicular proteins was effected by both types of gonadotropins.

The maturational hormones from American plaice, winter flounder and chum salmon were able to reinitiate the production of 11-ketotestosterone and testosterone by hypophysectomized male winter flounder, but the stimulating effect on testosterone was apparently less than that on 11-ketotestosterone. By contrast, subfractions derived from the vitellogenic Con A I fractions of various piscine species did not manifest much steroidogenic activity. Thus it appears that the control of androgen production in the flounder is specific to the maturational hormone.

11-Ketotestosterone was first isolated by Idler et al., (1960). The androgen accelerated spermatogenesis and induced spermiation (Idler et al., 1971; Yamazaki and Donaldson, 1969). Testosterone was also capable of stimulating spermatogenesis (Sundararaj et al., 1971). In the male winter flounder, plasma titers of 11-ketotestosterone and testosterone increased gradually as maturation proceeded (Campbell et al., 1976) suggesting that these steroids

controlled the final stages of spermatogenesis.

Sex hormone binding proteins had been isolated from the skate by Freeman and Idler (1969). It was found in this study that the pituitary played a role in maintaining the binding proteins in circulation since they were absent only in hypophysectomized fish. Their reappearance in the circulation was not linked specifically to the maturational hormone which controlled steroidogenesis, because they were present in hypophysectomized flounder treated with Con A I fractions which exerted little effect on plasma levels of androgens. It is considered that protein-bound steroids are biologically inert while free steroids are physiologically active. It is thus important to note that the maturational hormone, which had steroidogenic activity, did not shift the ratio of bound to free androgens, in hypophysectomized flounder compared with intact flounder.

Oocyte maturation and spermiation

Oocyte maturation and spermiation in the winter flounder was suppressed by hypophysectomy. The lack of an inhibitory effect of hypophysectomy on spermiation in the plaice (Barr, 1963) and lake chub (Ahsan, 1966) was attributed to the usage of fish which had already shown signs of spermiation before hypophysectomy (Yamazaki and Donaldson, 1969).

The specific action of the piscine maturational Con A II hormone in controlling ovulation and spermiation correlated well with the abrupt surge in plasma hormone level with the advent of spawning season (Crim et al., 1973, 1975).

Antibody inhibition of vitellogenesis and oocyte maturation in landlocked salmon and flounder

The presence of an excess of antibodies in the plasmas of fish injected with antisera was demonstrated by binding of the appropriate iodinated hormone to the fish plasmas. The specificity of the antisera was established by lack of cross reactivity. It can be assumed that antibodies to the piscine vitellogenic and maturational hormones were able to bind to the appropriate hormones resulting in inhibition of the normal vitellogenesis and oocyte maturation *in vivo*.

Treatment of intact vitellogenic landlocked Atlantic salmon, with an antiserum to the maturational fraction prepared from chum salmon pituitaries, resulted in a decline of circulating levels of estradiol and vitellogenin, indicating that the salmon maturational hormone normally stimulated the production of estradiol and vitellogenin *in vivo*. The small number of fish in the group treated with normal rabbit serum resulted from an unexpected predominance of males. Notwithstanding this limitation on the data for this group these results are in agreement with the finding of Campbell and Idler (1979) that the salmon maturational hormone induced the production of estradiol and vitellogenin in juvenile rainbow trout. The decrease in the amount of yolk incorporated into the ovary, after treatment of the fish with an antiserum to the maturational Con A II fractions, could be due to the decrease in vitellogenin synthesis resulting from a fall in the plasma estradiol level. After treatment of the fish with an antiserum to the vitellogenic Con A I fraction, the decrease in the amount of yolk incorporated into the ovary might be explained by a decrease in the ability of the oocyte to incorporate vitellogenin since no change was detected in the

plasma levels of estradiol and vitellogenin. In a brief report Nath and Sundararaj (1977) stated that plasma vitellogenin level and ovarian weight in vitellogenic catfish decreased after treatment with an antiserum to a gonadotropic fraction. Because of differences in the methods of preparation it is not possible to attribute the observed action to one or other of the gonadotropic fractions (Con A I or Con A II) tested in the present study.

In both the landlocked salmon and winter flounder the onset of germinal vesicle breakdown was associated with a decrease in the incorporation of radioactive leucine and phosphorus into ovarian yolk. The plasma level of vitellogenin in the landlocked salmon did not change appreciably after germinal vesicle breakdown. Therefore the decrease in the incorporation of radioactivity into ovarian yolk was presumably due to a change in the ability of the maturing oocyte to incorporate vitellogenin.

In the present study treatment of the landlocked salmon and winter flounder, with an antiserum to the chum salmon vitellogenic Con A I fraction and an antiserum to the winter flounder vitellogenic Con A I fraction respectively, resulted in binding of any circulating vitellogenic hormone to its antibody but no suppression of oocyte maturation occurred. Thus it seems reasonable to suggest that, the flounder and salmon vitellogenic hormones are not involved with oocyte maturation. This information is important because mammalian FSH (see Grimek et al., 1976) and reptilian FSH (Licht and Crews, 1975) have been found to have intrinsic ovulatory activity in the homologous species, and mammalian FSH has been stated to have some oocyte maturation inducing activity in fish (Hirose,

1971; Goetz, 1976) although mammalian LH has often been shown to be more potent in fish (Sundararaj and Goswami, 1977). It was not certain whether the ovulatory activity present in a chicken FSH preparation was due to contamination from LH, and only in the amphibian was it established that LH was the gonadotropin controlling ovulation (see Licht et al., 1977b). The present study provides additional evidence that the vitellogenic hormone, which is not adsorbed on Concanavalin-A Sepharose, does not regulate the processes of oocyte maturation and ovulation. By contrast the maturational hormone, which is adsorbed on Concanavalin A-Sepharose, also plays a role in vitellogenesis.

The biological activities of each gonadotropin enumerated above were intrinsic properties rather than consequences of contamination with the other gonadotropin as evidenced by negligible cross-reactivity in radioimmunoassays, and by homogeneity upon rechromatography on Con A-Sepharose and chromatography on Lentil Lectin-Sepharose. The much weaker interaction of Lentil Lectin than Con A with carbohydrates (Stein et al., 1971), and the similar chromatographic behaviour of each gonadotropin on both types of lectins suggested that the common biological activities found in the two types of gonadotropins were not likely due to a component distributed between the Con A I and Con A II fractions. In this context it might be worth pointing out that it has recently been demonstrated that mammalian (Grimek et al., 1976) and submammalian tetrapod (Daniels et al, 1977; Licht et al., 1977a) gonadotropins possessed many common yet intrinsic biological actions.

Biochemical properties

The finding of two neighboring peaks with vitellogenic activity, when the salmon Con A I MW 45,000 dalton peak was purified on DEAE Bio-Gel A, was reminiscent of similar results obtained with salmon Con A II G-75 fraction II (Idler et al., 1975b). The 45 DE II and 45 DE III hormones were almost identical with regard to amino acid and carbohydrate compositions, and immunologically both were potent.

The immunologic difference between the large and small forms of salmon vitellogenic hormone (Figs. 26, 27) demonstrated that the vitellogenic activity found in each form was an intrinsic property and not due to contamination with the other form.

Following the isolation by Idler et al., (1975b) of two forms of chum salmon maturation hormones which were sex specific in stimulating cAMP production by immature trout gonad, Breton et al., (1978), employing similar chromatographic procedures, found that the male chinook salmon pituitary secreted a maturational hormone which differed from its female counterpart in biological specific activity in female trout. Chinook salmon gonadotropin had also been prepared from SG-G100 (Donaldson et al., 1972) with affinity chromatography on Con A-Sepharose followed by ion exchange chromatography (Pierce et al., 1976). The purity of both Idler's and Pierce's preparations was estimated to be comparable to that of the best mammalian gonadotropin preparations (Pierce et al., 1976). This argument was supported in the case of the former preparation by its high biological activity in the chick testicular radiophosphate uptake and trout gonadal cAMP assays (Idler et al., 1975a,b,c). The plaice and flounder maturational Con A II hormones were prepared with

the same procedure. Despite the high degree of purification attained, the salmon maturational hormone still exhibited microheterogeneity in polyacrylamide gel electrophoresis. Microheterogenity or polymorphism of glycoprotein hormones, revealed as broad bands or multiplicity of bands on electrophoresis, are very common among gonadotropins e.g., mammalian (Braselton and McShan, 1976; Courte et al., 1972), reptilian (Licht et al., 1976), amphibian (Papkoff et al., 1976a) and fish (Breton et al., 1976; Pierce et al., 1976) gonadotropins. The piscine vitellogenic hormones, however, because of their low carbohydrate contents, showed narrow bands upon electrophoresis. A comparison of the amino acid and carbohydrate compositions of the salmon vitellogenic hormones with the maturational hormones furnished data which could satisfactorily explain their immunologic unrelatedness. Differences in total hexose and glucosamine contents and possibly also the sequence of component carbohydrates between the two types of gonadotropins, endowed them with distinct chromatographic behaviour on Con A-Sepharose and Lentil lectin-Sepharose. Galactosamine (Goldstein et al., 1965) and sialic acid (Dufau et al., 1972) are not determinants for binding to the lectins owing to absence of the requisite α-D-mannopyranosyl configuration. The low hexosamine contents of the vitellogenic hormones accounted for their failure to be bound to Helix pomatia Lectin-Sepharose and Wheat Germ Lectin-Sepharose. The piscine gonadotropins all had a greater abundance of glucosamine than galactosamine as had been reported for mammalian, avian and reptilian gonadotropins (Jutisz and de la Llosa, 1972; Farmer et al., 1975; Papkoff et al., 1976b). The half-cystine and methionine values might be lower than the actual values but unfortunately there was not

enough material available for performic acid oxidation.

The molecular weights of the "little" forms of the plaice, flounder and salmon vitellogenic and maturational hormones are in close agreement with the values reported for gonadotropins of mammalian (see Jutisz and de la Llosa, 1972) origin.

It is worth noting that both vitellogenic and maturational hormones from plaice, flounder and salmon pituitaries are adsorbed on DEAE Bio-Gel A equilibrated with 5 mM NH_4HCO_3 at pH 9 and unadsorbed on CM Bio-Gel A equilibrated with 3 mM NH_4OAC at pH 6.

Although mammalian and avian FSHs and LHs are very similar in their molecular weights, they can be separated only by ion exchange chromatography on DEAE- or CM-cellulose (Furuya and Ishii, 1974; Rathnam and Saxena, 1974; Stockell and Hartree, 1975). Reptilian and amphibian gonadotropins can only be partially resolved from one another by gel filtration, but the separation can be improved by further chromatography on ion exchangers (Licht and Papkoff, 1974a,b). The remarkable similarities in chromatographic behaviour on Ultrogel, DEAE Bio-Gel A and CM-Bio-Gel A suggest that it would be very difficult or almost impossible to separate the two types of gonadotropic activities under mild conditions if the first step of affinity chromatography on Con A-Sepharose were omitted.

Affinity chromatography on Con A-Sepharose provides a rapid method to check the purity of Con A I and Con A II fractions. Donaldson's SG-G100 prepared by solvent fractionation (Donaldson et al., 1972) has been shown by Pierce et al., (1976) to contain a considerable amount of Con A I material. One step-chromatography of the chum salmon pituitary extract on Con A-Sepharose yielded a Con A I fraction

contaminated with 1-17% of the Con A II gonadotropin, which could be specifically detected by radioimmunoassay as well as by the immature trout gonadal cAMP augmentation assay (in vitro) (Idler et al., 1975c). Maturational and ovulatory activities in the plaice pituitary Con A I fraction due to contamination with the Con A II gonadotropin (Campbell and Idler, 1977) could be removed by rechromatography on Con A-Sepharose in the present study.

Application of the ethanolic extraction and precipitation procedure (Stockell Hartree, 1976) to prepare fish gonadotropin yielded material which could be further fractionated on Con A-Sepharose (Idler et al., 1975a; Pierce et al., 1976; Sumpter et al., 1978). It was found that crude preparations of mammalian gonadotropins, NIH-FSH-PI and NIH-LH-B5 (Ui et al., 1977) could be further purified on Con A-Sepharose, indicating the utility of this immobilized lectin as a tool in hormone isolation.

Vitellogenic hormones from salmon, plaice and flounder appear to exhibit the phenomenon of size heterogeneity. This phenomenon is common among mammalian protein and polypeptide hormones found both in the pituitary and in circulation; e.g., PMSG (see Papkoff, 1966), FSH (Gray, 1968), HCG (see Jutisz and de la Llosa, 1972), prolactin (Ben-David and Chambrach, 1974; Suh and Frantz, 1974), gastrin, insulin, ACTH, growth hormone, and parathyroid hormone (see Yalow, 1974). The "big" forms are speculated to be either aggregates of the monomeric forms or the results of association between the "little" forms and other proteins. The physiological significance of the "big" forms has not yet been elucidated; some may represent prohormones but the identities of enzymes involved in the conversion of these

suspected biosynthetic precursors to the active hormones are still obscure. Partial or full biological activity has been detected in proinsulin, big ACTH, and the multiple forms of gastrin, although the interrelationship among the different forms of a hormone has not yet been clarified in the majority of cases (see Yalow and Berson, 1973). The discovery of vitellogenic activity in two molecular entities is in harmony with the findings of comparable biological activity in the different molecular weight forms of human FSH (Gray, 1967), ovine FSH (Papkoff, 1973), alligator and turtle FSH (Licht et al., 1976). The present study is not adequate to provide a definite answer to the question of what relationship the big form bears to the small form in vivo. Potency in bioassays in vivo may not imply activity per se but may also be a result of an internal transformation of the inactive form into the active entity.

Freezing and thawing or prolonged storage result in a considerable conversion of the big human prolactin into the little component (Suh and Frantz, 1974), while lyophilization results in the preferential loss of the little human growth hormone (Gordon et al., 1973). However, there is no conversion of the monomeric growth hormone (Gordon et al., 1973) and prolactin (Suh and Frantz, 1974) to the dimer. In the case of plaice gonadotropins substantial aggregation of the "little" form to produce the "large" form took place in buffer when concentrations of NaCl, DTT, and particularly EDTA were low.

Dissociation of high molecular forms of human FSH and several steroid hormone receptors occurred in high concentrations of NaCl (Gray, 1968) and KCl (see King and Mainwaring, 1974).

Dithiothreitol and 2-mercaptoethanol minimize association of the

elasmobranch sex steroid binding protein (Freeman and Idler, 1969). However, high concentrations of NaCl and DTT did not prohibit association of the 28,000 MW plaice gonadotropins. On the other hand, the role that EDTA plays in maintaining the integrity of the 28,000 MW gonadotropins is worth noting. The chemical is a chelating agent, and divalent cations have been implicated in the association-dissociation phenomenon frequent among protein molecules. Calcium ions prevent aggregation of HCG (see Jutisz and la Llosa, 1972) and of the rat uterine cytoplasmic receptor for estrogen (see King and Mainwaring, 1974). Zinc ions are indispensable to the structure of the insulin hexamer (Blundell et al., 1971). In view of the lack of unanimity in opinions on the ability of different chemicals to prevent association of protein molecules, the mechanism by which EDTA prevents aggregation of the small forms of plaice and winter flounder gonadotropins has to wait clarification.

Most antisera to vertebrate FSHs required adsorption with LHs to eliminate cross reaction from the latter in radioimmunoassay, and vice versa (Daniels et al., 1977), and some antisera had a heterogeneous antibody population (Licht et al, 1977a). In a few cases the problem of cross reactivity could be quite serious (Cunningham and Herbert, 1973). It was thus of considerable interest to note the homogeneity of the antibody population with regard to the salmon vitellogenic and maturational gonadotropins in their respective antiserum and the specificity of radioimmunoassays set up employing these antisera.

The reduced S-carboxamidomethylated salmon maturational hormone was unable to compete with the iodinated native hormone for binding to the antibody. Pierce et al. (1976) also noted that no precipitin

arcs were formed between the carboxymethylated hormone and the antiserum in the immunodiffusion test.

The immunologic potencies of the salmon vitellogenic and maturational hormones were unaltered by desialylation. In this regard they were similar to HCG, human FSH and LH which either retained full or acquired enhanced immunoreactivity after desialylation (Braunstein et al., 1971; Van Hall et al., 1971). The preservation of activity in in vitro assay systems was attributed to the normal or even augmented binding affinity of the desialylated hormone preparation to its receptor (Catt et al., 1972); the role of sialic acid was to protect the hormone from metabolic clearance in vivo (Van Hall et al., 1971).

The extremely low sialic acid contents of the salmon vitellogenic hormones offers a plausible explanation for the absence of inactivation of vitellogenic activity after neuraminidase treatment, reminiscent of an analogous finding with ovine LH (Grimek et al., 1976) which has a similar sialic acid content (Jutisz and de la Llosa, 1972). The lack of complete destruction of vitellogenic and maturational activities of the salmon maturational hormones might well be due to its much lower sialic acid content compared with those of HCG, PMSG (Jutisz and de la Llosa, 1972) and ovine FSH (Grimek et al., 1971) which experienced a drastic decline in gonadotropic potency when deprived of sialic acid (Van Hall et al., 1971; Jutisz and de la Llosa, 1972; Grimek et al., 1976). Again, desialylation only partially cancelled the activity of SG-G100 in the chick testicular radiophosphate uptake assay (Donaldson, 1973) which measured the salmon maturational hormone in the preparation (Idler et al., 1975a). It had been found certain reptilian gonadotropins (Licht and Papkoff,

(1972) and enzymes (Jutisz and de la Llosa, 1972) were independent of sialic acid for biological activity, whereas some submammalian tetrapod gonadotropins were only partially inactivated by neuraminidase (Licht and Papkoff, 1972).

The profile of biological activities displayed by each type of piscine gonadotropin represents the potential physiological functions of the hormone. The process of spawning in the natural reproductive cycle of the salmonids is clearly under the control of the maturational hormone because it has been shown that there is a rapid rise in the maturational hormone level in blood prior to spawning (Crim et al., 1975), and only this gonadotropin can elicit oocyte maturation and ovulation. At this stage the relative physiological importance of the vitellogenic and maturational hormones in the control of vitellogenesis still awaits elucidation but the latter hormone is not readily detectable by radioimmunoassay when vitellogenesis is well under way (Crim et al., 1973, 1975).

Separate gonadotropic (anuran spermiation) and thyrotropic molecules are present in carp and eel pituitaries (Fontaine, 1969b). It remains to be determined whether TSH is a separate hormone from the vitellogenic and maturational gonadotropins discussed in the present studies.

The isolation of two different types of gonadotropins from chum salmon, American plaice and winter flounder pituitaries in this study suggests the phenomenon is not confined to only one or two species. Hopefully, this new finding will eventually lead to a better understanding of the physiology of fish reproduction.

CHAPTER 2

Pituitary Control of Thyroidal Function

INTRODUCTION

Two types of gonadotropins, vitellogenic and maturational in activities have now been isolated from fish pituitaries (Chapter I). The preparation of eel thyrotropin (TSH) in a form devoid of any gonadotropic activity in the anuran spermiation test has been reported by Fontaine and Condliffe (1963). However, the teleost thyroid has been implicated with reproduction (Sage, 1973; Hoar, 1973), and it responds to both gonadotropins and thyrotropin of mammalian origin (Fontaine, 1969a; Grau and Stetson, 1977). Mammalian TSH has a slight effect on the size of oocytes in the hypophysectomized eel (Fontaine, 1961). It is therefore of vital importance to determine whether TSH exists as a hormone separate from the vitellogenic and maturational hormones in the plaice and flounder pituitaries.

MATERIALS AND METHODS

Preparation of pituitary hormones

It was described in Chapter I. The CM I and CM II fractions derived from the gonadotropic DEAE peaks were tested for both gonadotropic and thyrotropic activities.

Testing of thyrotropic activity in the fish pituitary hormone preparations

Mature male winter flounder were used three weeks after hypophysectomy. They were not fed prior to and during the course of the experiment. Fish experimented on in the winter were kept in heated seawater at 10°C. Injections of hormones were given intraperitoneally at a site on the ventral surface just posterior to the pectoral fin and bleeding was done 24 hr after the injection. Plasma triiodothyronine (T_3) and thyroxine (T_4) were measured with

radioimmunoassay kits from the Micromedic Kit Inc., Philadelphia. The assay buffer contained 8-anilino-1-naphthalene sulfonic acid and barbital to free the two thyroid hormones from plasma protein binding sites. A serial dilution of flounder plasma showed parallelism to the standard curve in both T_3 and T_4 radioimmunoassay.

Bovine TSH (Sigma) was also tested for thyrotropic activity in intact and hypophysectomized flounder.

Effect of antisera to thyrotropic fractions on thyroid function in the flounder

Antisera to flounder Con A I MW 28,000 DE III CM II fraction (designated F-Con A I 28K TSH ab), flounder Con A I MW 62,000 DE III CM II fraction (designated F-Con A I 62K TSH ab) and flounder Con A II MW 62,000 DE I CM II fraction (designated F-Con A II TSH ab) were raised in New Zealand white rabbits. Two hundred micrograms were given intradermally at multiple sites on the back at the primary injection which was followed two weeks later with a booster injection of 200 µg and thereafter with a booster injection every fortnight. Antisera were harvested two weeks after the second booster.

In Experiment 1, flounder were divided into three groups and injected with 300 µl of (a) normal rabbit serum, (b) F-Con A I 28K TSH ab, or (c) F-Con A I 62K TSH ab, daily for a week. Fish were bled 24 hr after the last injection and plasma samples were assayed for T_3 and T_4.

In Experiment 2, flounder were divided into three groups, and injected with 300 µl of (a) normal rabbit serum (b) F-Con A I 28K TSH ab, or (c) F-Con A II TSH ab, and the same protocol was followed as in Experiment 1.

Testing of gonadotropic activities in the fish pituitary hormone preparations

(a) Steroidogenic activity

The flounder plasma samples assayed for T_3 and T_4 were also assayed for (i) 11-ketotestosterone and (ii) testosterone in radioimmunoassays. The results have already been presented and discussed in Chapter I.

(b) Vitellogenic and oocyte maturational activities

The tests were conducted as described in Chapter I.

Testing of cross reaction from flounder Con A II MW 62,000 DE I CM I and Con A I MW 28,000 DE III CM II fractions in a radioimmunoassay for flounder Con A I MW 28,000 DE III CM I fraction

An antiserum was raised against flounder Con A I MW 28,000 DE III CM I (vitellogenic) fraction by giving a New Zealand white rabbit a primary injection of 500 µg protein at multiple intradermal sites, followed by a booster every two weeks. An antiserum which showed 50% binding to the iodinated Con A I MW 28,000 DE III CM I fraction at 1:8,000 dilution was used for the study. A radioimmunoassay for this fraction was set up, and Con A II MW 62,000 DE I CM I (maturational) and Con A I MW 28,000 DE III CM II (T_3 stimulating) fractions tested for cross reaction.

Testing of cross reaction from flounder Con A I MW 28,000 DE III CM I and Con A I MW 28,000 DE III CM II fractions in a radioimmunoassay for Con A II MW 62,000 DE I CM I fraction

An antiserum was raised against flounder Con A II MW 62,000 DE I CM I fraction as described above for the Con A I MW 28,000 DE III CM I fraction. An antiserum which showed 50% binding at a dilution

of 1 : 10,000 to iodinated flounder Con A II MW 62,000 DE I CM I fraction, was used as a radioimmunoassay for the fraction. Conditions of the radioimmunoassay were as described in Chapter I. Flounder Con A I MW 28,000 DE III CM I and CM II fractions were tested for cross reaction.

Amino acid analysis, carbohydrate analysis and electrophoresis

Flounder Con A II MW 62,000 DE I CM I (maturational hormone) and Con A I MW 28,000 DE III CM I (vitellogenic hormone) fractions were analyzed for amino acid composition, hexose contents and electrophoretic behaviour, as described in Chapter I. The presence of glucosamine was also noted in the amino acid analysis.

Testing of mammalian TSH, mammalian FSH, T_3, and T_4 for vitellogenic activity in hypophysectomized flounder

Two bovine TSH preparations, one from Sigma and the other from Mann Research Company, were chromatographed successively on Cellex D (DEAE cellulose, Bio-Rad) and Cellex CM (CM cellulose, Bio-Rad) to minimize contamination, if any, with gonadotropins. The TSH fraction which was adsorbed on Cellex D in 5 mM sodium glycinate at pH 9.5 and subsequently adsorbed on Cellex CM in 3 mM ammonium acetate at pH 5.5 (Pierce et al., 1971) was tested for vitellogenic activity. The bovine TSH preparation from Sigma was then tested at a dosage of 0.2 mg/kg, while the preparation from Mann Research Company was tested at two doses, 0.1 mg/kg and 1 mg/kg. Porcine FSH (Sigma) was similarly purified. The FSH fraction which was adsorbed on Cellex D and subsequently unadsorbed on Cellex CM was tested for vitellogenic activity at a dosage of 1 mg/kg.

Mixtures of triiodothyronine (Sigma) in equal proportions were

administered intramuscularly to hypophysectomized flounder at two dosages, 250 µg/hormone/kg and 2.5 mg/hormone/kg.

Effects of T_3, T_4 and thiourea on vitellogenesis in intact flounder

Vitellogenic flounder collected in December were treated intramuscularly with (a) T_3, 4 mg/kg, or (b) T_4, 4 mg/kg, or intraperitoneally with (c) thiourea, 2 mg/kg, once every two days for three weeks. The fish were then given estradiol-17β (1.5 mg/kg) and tritiated leucine and $H_3{}^{33}PO_4$ (20 µCi/kg of each isotope). Three days after the injection they were sacrificed and pieces of ovaries were assayed for the incorporation of radioactivity into the water insoluble yolk fraction as described in Chapter I.

Testing of mammalian TSH for oocyte maturational activity in hypophysectomized flounder

The bovine TSH preparation from Sigma, purified on Cellex CM and Cellex D as described above, was tested at a dosage of 500 µg/kg/injection in the oocyte maturation assay as described in Chapter I.

RESULTS

Effect of hypophysectomy on plasma T_3 and T_4

Hypophysectomy in the flounder resulted in a great drop in the plasma titers of T_3 and T_4 (Table 19). Bovine TSH given to intact fish was able to elevate only the T_4 level, but when it was given to hypophysectomized flounder both T_3 and T_4 levels increased (Table 19).

When the MW 28,000 peak of the Con A I (unadsorbed on Con A-Sepharose) fraction of the winter flounder pituitary extract was chromatographed on DEAE Bio-Gel A, it was found that the elution profile was similar to that reported for the flounder Con A I MW 62,000 peak (Fig. 29). When the second adsorbed peak (DE III, eluted by the gradient of 50-150 mM NH_4HCO_3) was fractionated on CM Bio-Gel

TABLE 19

EFFECT OF HYPOPHYSECTOMY ON PLASMA TRIIODOTHYRONINE (T_3) AND THYROXINE (T_4) IN FLOUNDER. EFFECT OF BOVINE FSH ON THYROID FUNCTION IN INTACT AND HYPOPHYSECTOMIZED FLOUNDER.

Group	N	Treatment	IU/kg	T_3 ng/100 ml	T_4 ng/100 ml
1	10	Intact	-	243 ± 90	560 ± 140
2	12	Hypex	-	13 ± 11	56 ± 30
3	9	Intact + TSH	2.5	212 ± 91 NS[a]	1133 ± 33 (P < 0.005)[a]
4	5	Hypex + TSH	2.5	171 ± 6 (P < 0.001)[b]	841 ± 110 (P < 0.001)[b]

a : versus Group 1

B : versus Group 2

A, thyrotropic activity resided in the adsorbed CM II fraction which constituted about 5% of the DE III fraction. The flounder Con A I MW 62,000 DE III CM II fraction also had thyrotropic activity. Both fractions were able to elevate the plasma T_3 concentration of the hypophysectomized flounder, but not the plasma T_4 concentration (Table 20). When antisera generated against these two thyrotropic fractions were injected into intact flounder, a fall in plasma T_3 level ensued (Table 21). On the other hand, the Con A II MW 62,000 DE I fraction was able to reinitiate the production of T_4, without any effect on T_3. When this fraction was chromatographed on CM Bio-Gel A, only the CM II fraction retained the T_4 stimulating activity (Table 20). An antiserum to this Con A II thyrotropic fraction did not have effect on plasma T_3 level in the intact flounder but it depressed the T_4 level (Table 21)

The chromatographic behaviour of the thyrotropic fractions from the American plaice pituitaries was identical to that of the winter flounder. The Con A I thyrotropic fractions stimulated the production of T_3 only, whereas the Con A II thyrotropic fractions enhanced the synthesis of T_4 (Table 22).

The Con A I thyrotropic fractions from both winter flounder and American plaice were without effect on plasma 11-ketotestosterone level (Tables 12, 14). The Con A I MW 28,000 DE III CM II thyrotropic fractions did not possess any vitellogenic activity, although some vitellogenic activity was found in the corresponding MW 62,000 fractions (Table 23). The Con A I MW 28,000 DE III CM I and Con A I MW 62,000 DE III CM I fractions, on the other hand, were vitellogenic but not thyrotropic (Tables 20, 22, 23), providing evidence for separate vitellogenic and thyrotropic fractions in the Con A I fraction.

TABLE 20.

EFFECTS OF WINTER FLOUNDER PITUITARY FRACTIONS ON PLASMA TRIIODOTHYRONINE (T_3), THYROXINE (T_4), 11-KETOTESTOSTERONE (11-KETO), AND TESTOSTERONE (TEST.) IN HYPOPHYSECTOMIZED FLOUNDER

Expt.	Group	N	Treatment	Dose µg/kg	T_3 ng/100-ml	T_4 ng/100 ml	11-Keto. ng/ml	Test. ng/ml
1	1	12	Buffer	-	13 ± 11	56 ± 30	-	-
	2	10	Con A I 28K*	1000	136 ± 52[a]	36 ± 15	-	-
2	1	5	Buffer	-	24 ± 19	28 ± 6	-	-
	2	6	Con A II 62K*	400	19 ± 7	138 ± 42[a]		
	3	5	Con A I 62K* DE III CM I	200	57 ± 24	< 20	-	-
	4	5	Con A I 62K* DE III CM II	200	117 ± 23[b]	< 20	-	-
	5	7	Con A I 28K* DE III CM I	200	22 ± 15	< 20	-	-
	6	6	Con A I 28K* DE III CM II	200	148 ± 43[b]	< 20	-	-
3	1	6	Buffer		< 10	< 20	6 ± 1	-
	2	5	Con A II 62K* CM I		< 10	< 20	38 ± 5[c]	-
	3	5	Con A II 62K* CM II		< 10	88 ± 28	39 ± 12[b]	-

a = $P < 0.05$; b = $p < 0.025$; c = $p < 0.001$;

K = Molecular weight x 10^{-3}

The other DEAE fractions not listed were inactive when tested at an equal dosage.

TABLE 21

EFFECTS OF THYROTROPIN ANTISERA ON PLASMA TRIIODOTHYRONE (T_3) AND THYROXINE (T_4) IN INTACT FLOUNDER

Expt.	Group	N	Treatment	T_3 (ng/100 ml)	T_4 (ng/100 ml)
1	1	6	Normal Rabbit Serum	630 ± 90	2000 ± 550
	2	6	F-Con A I 28K TSH-ab	240 ± 70 ($P < 0.025$)	1200 ± 330
	3	5	F-Con A I 62K TSH-ab	170 ± 110 ($P < 0.025$)	900 ± 400
2	1	5	Normal Rabbit Serum	510 ± 140	900 ± 100
	2	6	F-Con A I 28K TSH-ab	76 ± 40 ($P < 0.01$)	700 ± 250
	3	5	F-Con A II TSH-ab	390 ± 64	400 ± 200 ($P < 0.05$)

TABLE 22

EFFECTS OF AMERICAN PLAICE PITUITARY FRACTIONS ON PLASMA TRIIODOTHYRONINE (T_3) AND THYROXINE (T_4) IN HYPOPHYSECTOMIZED FLOUNDER

Expt.	Group	N	Treatment	T_3 µg/kg	T_3 ng/100 ml	T_4 ng/100 ml
1	1	12	Buffer	-	13 ± 11	56 ± 30
	2	6	Con A I 28K*	1000	143 ± 52[a]	26 ± 10
	3	6	Con A I 62K* DE III	250	241 ± 90[c]	20 ± 8
2	1	5	Buffer	-	24 ± 19	28 ± 6
	2	5	Con A I 62K*	400	28 ± 16	76 ± 20[a]
3	1	11	Buffer	-	47 ± 8	< 20
	2	6	Con A I 62K* DE III CM I	200	47 ± 17	< 20
	3	5	Con A I 62K* DE III CM II	200	363 ± 130[b]	< 20
	4	6	Con A I 28K* DE III CM I	200	59 ± 20	< 20
	5	6	Con A I 28K* DE III CM II	200	278 ± 54[c]	< 20

[a] = $p < 0.025$, b = $p < 0.005$, c = $p < 0.001$.
K = Molecular weight x 10^{-3}
The other DEAE fractions not listed were inactive when tested at an equal dosage.

TABLE 23

EFFECTS OF WINTER FLOUNDER AND AMERICAN PLAICE PITUITARY CON A I FRACTIONS ON VITELLOGENESIS IN HYPOPHYSECTOMIZED FLOUNDER

Group	N	Treatment	Dose µg/kg	Water insoluble yolk (dpm/g ovary) ^{3}H label	Water insoluble yolk (dpm/g ovary) ^{33}P label
1	6	Buffer	-	1500 ± 300	800 ± 200
2	5	F* 28K** DE III CM I	100	4700 ± 1400[a]	2400 ± 500[c]
3	5	F 28K DE III CM II	100	2400 ± 1700 NS	1000 ± 300 NS
4	5	F 62K DE III CM I	100	4200 ± 100[b]	1700 ± 300[a]
5	5	F 62K DE III CM II	100	4300 ± 130[a]	1600 ± 400 NS
6	5	P*** 28K DE III CM I	100	3700 ± 400[c]	4400 ± 200[d]
7	5	P 28K DE III CM II	100	2200 ± 500 NS	1300 ± 200 NS
8	5	P 62K DE III CM I	100	12200 ± 460[b]	2600 ± 800[b]
9	5	P 62K DE III CM II	100	6100 ± 680[b]	1200 ± 100 NS

F* = winter flounder, K** = Molecular weight x 10^{-3}, P*** = American plaice

a = $p < 0.05$, b = $p < 0.025$, c = $p < 0.01$, d = $p < 0.001$, NS = statistically insignificant.

The chromatograph of flounder on Con A II MW 62,000 on DEAE Bio-Gel A was similar to the plaice Con A II MW 62,000 fraction (Fig. 7): no adsorbed material was found and the main DEAE fraction was designated DE I. The CM I fraction derived from this DE I fraction had only gonadotropic activity revealed in its ability to stimulate 11-ketotestosterone production, but no thyrotropic activity. The corresponding CM II fraction had both thyrotropic and gonadotropic (steroidogenic) activities; the presence of steroidogenic activity was probably due to contamination. However, the differential distribution of thyrotropic activity in the CM I and CM II fractions indicated the existence of separate gonadotropin and thyrotropin in the Con A I fraction (Table 20).

Duality of gonadotropins in flounder and plaice was revealed in the lack of steroidogenic activity in the vitellogenic DE III CM I fractions derived from the Con A I MW 28,000 and MW 62,000 peaks, and the presence of steroidogenic activity in the Con A II fractions (Table 12, 14, 20).

In the radioimmunoassay for flounder Con A I MW 28,000 DE III CM I fraction, flounder Con A II MW 62,000 DE I CM I fraction and Con A I MW 28,000 DE III CM II fraction showed respectively 2% and 5% cross reaction. In the radioimmunoassay for flounder Con A II MW 62,000 DE I CM I fraction, flounder Con A I MW 28,000 CM I and CM II fractions showed no cross reaction when tested up to 2 µg levels.

The amino acid compositions and hexose contents of flounder vitellogenic hormone (Con A I MW 28,000 DE III CM I fraction) and maturational hormone (Con A II MW 62,000 DE I CM I fraction) were presented in Table 24. Many differences could be noted which,

TABLE 24

AMINIO ACID AND CARBOHYDRATE COMPOSITIONS OF WINTER FLOUNDER GONADOTROPINS

	Con A II MW 62,000 DE I CM I fraction	Con A I MW 28,000 DE III CM I fraction
Lysine[a]	11	6
Histidine	5	4
Arginine	6	6
Aspartic acid	25	25
Threonine	22	8
Serine	14	21
Glutamic acid	17	32
Proline	11	14
Glycine	14	24
Alanine	12	18
Half-Cystine	5	8
Valine	14	4
Methionine	4	4
Isoleucine	8	3
Leucine	17	17
Tyrosine	5	2
Phenylalanine	10	4
Total hexoses[b]	15	2
Glucosamine	present	present

[a]Values for amino acids are expressed in number of residues for every 200 residues.

[b]Values for carbohydrates are expressed in g/100 g protein.

together with the immunological unrelatedness of the two hormones, suggested that the hormones were distinct. Flounder vitellogenic hormone showed one band at Rf = 0.76 in electrophoresis while maturational hormone showed a broad band at Rf = 0.6.

Mammalian TSH, FSH, T_3 and T_4 had no direct effect on yolk incorporation into flounder ovary (Table 25). Pretreatment of intact flounder with thiourea, T_3 and T_4 did not affect yolk deposition in the ovary (Table 26).

DISCUSSION

The present investigation demonstrates that in the winter flounder production of the thyroid hormones is under pituitary control because hypophysectomy results in a dramatic decline of the thyroid hormones to very low levels. The deleterious effect of hypophysectomy on the teleost thyroid could also be revealed by the histological technique (Pickford and Atz, 1957).

Thyroidal radioiodide uptake in the starved rainbow trout has been used to monitor thyrotropic activity during the isolation of eel TSH (Fontaine and Condliffe, 1963), but Chan and Eales (1976) find that a similar assay using the brook trout has some shortcomings which include a decrease in uptake at high TSH doses. However, the latter authors find that a linear relationship exists between log TSH dose and plasma T_4 of intact brook trout. Other histological and radiochemical bioassays have been suggested (see Chan and Eales, 1976). However, in view of the extremely disperse nature of the fish thyroid and possible differences in activity between different regions of the thyroid (see Gorbman, 1969), changes in plasma levels of thyroid hormones in the hypophysectomized flounder due to hormone treatment were used as

TABLE 25

EFFECTS OF MAMMALIAN TSH, MAMMALIAN FSH, T_3 AND T_4 ON VITELLOGENESIS IN HYPOPHYSECTOMIZED FLOUNDER

Group	N	Treatment	Dose ng/kg	Water insoluble yolk (dpm/g ovary)	
				^3H label	^{33}P label
1	11	Buffer	-	1010 ± 80	700 ± 80
2	5	TSH (Mann)	0.2	1350 ± 230	900 ± 95
3	5	TSH (Mann)	1	1270 ± 200	830 ± 180
4	5	TSH (Sigma)	0.2	1300 ± 180	700 ± 99
5	5	FSH (Sigma)	1	1060 ± 250	730 ± 170
6	6	$T_3 + T_4$	0.25	1250 ± 250	840 ± 200
7	5	$T_3 + T_4$	2.5	1420 ± 390	830 ± 210

TABLE 26

EFFECTS OF T_3, T_4 AND THIOUREA ON VITELLOGENESIS IN INTACT FLOUNDER

Group	N	Treatment	Dose ng/kg	Water Insoluble Yolk (dpm/g ovary)	
				^{3}H label	^{33}P label
1	6	Buffer	-	13600 ± 1500	2400 ± 300
2	6	T_3	4	10900 ± 1000	2200 ± 290
3	6	T_4	4	15800 ± 1000	5100 ± 1500
4	6	Thiourea	2	12400 ± 3000	4000 ± 570

an indicator of thyroid stimulating activity of the hormone preparation.

Con A I TSH fractions elevate plasma T_3 and Con A II TSH fractions elevate plasma T_4 in the hypophysectomized flounder. Furthermore, antisera to Con A I TSH depress plasma T_3 with no significant change in T_4, while Con A II TSH antiserum depresses plasma T_4 without effect on plasma T_3. These findings suggest that the Con A I TSH fraction has a major role to play in regulating T_3, while the Con A II TSH fraction regulates plasma T_4. The concept of separate control of plasma T_4 and T_3 in teleosts gains support from several recent reports of independent variation in plasma T_4 and T_3 in response to environmental or hormonal manipulations (Brown et al., 1977; Leatherland and Sonstegard, 1978; Leloup et al., 1976; Milne and Leatherland, 1978; Osborn and Simpson, 1971; White and Henderson, 1977). The more general concept that at least two distinct thyrotropic principles exist in the fish hypophysis is consistent with the histological observations of Kaul and Vollrath (1974) that two thyrotropes exist in the goldfish pituitary which differ in their innervation by A and B fibers.

It is of interest to speculate on the mechanism whereby Con A I TSH and Con A II TSH stimulate respectively, plasma level of T_3 and T_4. It is well known that T_4 and small amounts of T_3 are present in the teleost thyroid (Gorbman, 1969) and that in several species, including a pleuronectid (Osborn and Simpson, 1969, 1971), these hormones also occur in the blood. It is also known that in salmonids at least, from 40-70% of circulating T_4 may be extrathyroidally converted to T_3 (Eales, 1971 a,b), and that based on mammalian studies (Greer and Haibach, 1974) the possibility of intrathyroidal

T_4 to T_3 conversion exists. If T_4 is indeed the major hormone synthesized in the flounder thyroid, the Con A I TSH fraction must initially stimulate T_4 release. The fact that plasma T_3 is elevated rather than plasma T_4 could then be explained by rapid T_4 to T_3 conversion in the thyroprivic state resulting in elevation of plasma T_3 at expense of plasma T_4. This effect would be reinforced by the slower plasma clearance of T_3 relative to T_4 as demonstrated in salmonids (Eales, 1978; Higgs and Eales, 1977). The possibility also exists that Con A I TSH itself stimulates T_4 to T_3, conversion. This would be consistent with the observation that Con A II TSH administered alone to hypophysectomized flounder is capable of elevating plasma T_4 but not plasma T_3. Con A II TSH presumably causes thyroidal T_4 release, but, in the absence of Con A I TSH or some other pituitary factor, negligible T_3 formation takes place.

One apparently puzzling observation is that bovine TSH injected into hypophysectomized flounder elevates both plasma T_4 and T_3, whereas Con A II TSH, the presumed glycoprotein homolog of bovine TSH, elevates plasma T_4 only. Con A II TSH and bovine TSH would be expected to interact with the same receptor site and the different responses therefore seem anomalous. However, Con A II TSH fractions produced but mild thyroidal stimulation, whereas the high dose of bovine TSH caused massive stimulation that may have affected the thyroidal system in a different manner. In this regard it is of future interest to observe changes in plasma T_4 and T_3 in hypophysectomized flounder employing either low doses of bovine TSH or high doses of flounder TSH. It will also be worthwhile to measure plasma T_4 and T_3 at times other than 24 hr after injection

of assay materials.

Another point that remains to be resolved is why bovine TSH fails to elevate plasma T_3 in intact flounder despite massive elevations in plasma T_4. This pattern of response has been described previously for intact trout (Brown et al., 1978) and suggests that there is a mechanism for holding plasma T_3 in check. It is possible that this check does not exist in hypophysectomized flounder where bovine TSH stimulates both T_4 and T_3 levels, although it is important to note that even in hypophysectomized flounder the plasma T_3 does not exceed the control level. Without more detailed information on individual T_4 and T_3 negative feedback pathways to the hypothalmo-hypophyseal axis further speculation is dangerous. However, one possibility is that there is yet another hypophyseal factor awaiting discovery that is involved in the inhibition of plasma T_3 and which may function by suppressing T_4 to T_3 conversion.

Difference in biological activity between these two types of thyroid stimulating hormones, T_3 - stimulating versus T_4 - stimulating, argues against the likelihood of a single hormone distributed between the Con A I and Con A II fractions. It is perhaps pertinent to note that, whereas rechromatography on Con A-Sepharose removed most of the gonadotropic contamination due to the Con A II fraction from the Con A I fraction of the dogfish pituitary extract, the Con A I fraction still retained 31% of the total TSH activity whereas the Con A II fraction contained 69% (Sumpter et al., 1978).

The low carbohydrate content of the eel TSH prepared by Fontaine and Condliffe (1963) suggests that it may be a glycoprotein not adsorbed on Con A-Sepharose; the method of preparation involving

saline extraction, gel filtration and ion exchange chromatography would not likely separate Con A I proteins completely from Con A II proteins (Idler et al., 1975a). In Astyanax mexicanus the pituitary gonadotrops stain more intensely by the periodic acid-Schiff method than do the thyrotrops (Atz, 1953), suggesting that its TSH contained less carbohydrate than did its gonadotropin. However in other species the reverse seems to be the case (O and Chan, 1974). In the other vertebrate classes TSH was more closely associated during chromatography with LH than with FSH (Pierce et al., 1971; Scanes and Follett, 1972; Licht and Papkoff, 1974a,b). In the teleost the maturational hormone prepared from the Con A II fraction is comparable to LH in its biological activities (induction of oocyte maturation, ovulation and steroidogenesis), and it is interesting that the T_4 - stimulating hormone can be isolated from the Con A II fraction. It is possible that TSH assays utilizing thyroidal uptake of iodine (Fontaine and Condliffe, 1963) and phosphorus (Sumpter et al., 1978) cannot differentiate between T_3 - stimulating and T_4 - stimulating activities.

The chromatographic behaviour of the flounder and plaice thyroid stimulating hormone resembles that of mammalian TSH (Pierce et al., 1971), avian TSH (Scanes and Follett, 1972), and eel TSH (Fontaine and Condliffe, 1963) in that they are all adsorbed on diethylaminoethyl ion exchanger under alkaline conditions as well as on carboxymethyl ion exchangers under slightly acidic conditions. The piscine TSHs also run closely to the vitellogenic (Con A I) and maturational (Con A II) gonadotropins on Ultrogel and DEAE Bio-Gel A, and can only be separated from the gonadotropins on CM Bio-Gel A. Similarity in

chromatographic behaviour between gonadotropins and thyrotropin from both mammalian and avian sources accounts for the difficulty experienced in obtaining pure hormone preparations (Pierce et al., 1971; Scanes and Follett, 1972). A close association between gonadotropic and thyrotropic activities during chromatographic separation has also been observed in the amphibian and in the reptile (Licht and Papkoff, 1974 a,b). The low TSH content of the fish pituitary relative to the gonadotropin content (TSH content: GTH content approximately equals 1 : 20) agrees with the finding in mammals (Pierce et al., 1971).

The Con A I fraction of the flounder and plaice pituitary extracts has been shown to be devoid of any oocyte maturational and ovulatory activities (Chapter I). The preparation from the Con A I fractions of flounder and plaice pituitary extracts of thyrotropic CM II fractions without any vitellogenic or steroidogenic activity, and of vitellogenic CM I fractions without thyrotropic activity, furnishes evidence for the existence of separate vitellogenic hormone and T_3-stimulating hormone in the Con A I fraction. The flounder T_3-stimulating hormone has minimal contamination with the vitellogenic and maturational hormones as evidenced by low cross reactivity in radioimmunoassay. The presence of two distinct gonadotropins in the pituitaries of the winter flounder, the American plaice and the chum salmon (Chapter I) has been established. This finding is further supported by the observation that the hormone in the Con A II fraction which induces oocyte maturation also reinitiates androgen production in the hypophysectomized flounder, whereas the vitellogenic hormone in the Con A I fraction has neither activity. The existence of a T_4 -

stimulating hormone (CM II) in the Con A II fraction separate from the maturational hormone is suggested by the absence of thyrotropic activity in the gonadotropic CM I fraction. Gonadotropic activity in the thyrotropic CM II fraction could well be due to contamination with maturational hormone.

The inter-relationships among the pituitary, thyroid and ovary in fish reproduction are complex and there is no unanimous opinion on this issue (see Pickford and Atz, 1957; Sage, 1973; Eales, 1979), although interactions among thyroid hormones, gonadal steroids and pituitary gonadotropes and thyrotropes do exist (Sage and Bromage, 1970; Hurlburt, 1977).

In hypophysectomized flounder the thyroid hormones, bovine TSH and FSH (heterothyrotropin) did not influence vitellogenesis, and bovine TSH did not induce oocyte maturation and ovulation, though bovine TSH was thyrotropic as judged by plasma hormone levels. These findings agree with those for goldfish (Hurlburt, 1977) in indicating that thyroid hormones in the absence of pituitary hormones exert no effect on ovarian maturation. Sundararaj and Goswami (1966) and Anand and Sundararaj (1974) did observe marginal gonadotropic activity in the hypophysectomized Indian catfish due to mammalian TSH and FSH but this may be attributed to contamination with LH.

It is firmly established that thyrotrop activity can be increased or decreased respectively by thyroidal inhibition or thyroid hormone administration, and an attempt was made here to alter pituitary TSH production by injection of thiourea and T_3 or T_4 into intact flounder. None of the treatments with T_3, T_4, or thiourea

altered vitellogenesis indicating that T_3, T_4, thiourea or changes in endogenous TSH levels do not influence yolk synthesis. This is contrary to the findings of both Hurlburt (1977), who showed that T_4 in the presence of gonadotropins enhanced oocyte development, and Detlaff and Davydova (1974) who indicated a facilitative role of T_4 in gonadal development. The present negative results may be due to the use of a high level of T_4. Hurlburt observed that high levels of T_4 were less effective than low levels of T_4 and evidence from mammals suggests that extreme thyroid states lead to ovarian atrophy (Leatham, 1972).

CHAPTER III

General Discussion

Contrary to the conclusion arrived at by Fontaine and Burzawa-Gerard (1972) and by Donaldson (1973) that the fish pituitary elaborates only one gonadotropin, the results of this thesis strongly support the concept of duality of gonadotropins in the winter flounder, the American plaice and the chum salmon. The maturational hormone isolated from the pituitary Con A II fraction regulates the processes of androgen production, spermiation, and ovulation. It is also responsible for the first step in vitellogenesis i.e., the production of vitellogenin through stimulation of estrogen, although it is not too clear why the hormone is not readily detectable in the early phase of vitellogenesis (Crim et al., 1973, 1975).

The maturational hormone also appears to play a role in the second step of vitellogenesis i.e., incorporation of vitellogenin into the ovary and if any, ovarian yolk synthesis. On the other hand, the vitellogenic hormone prepared from the pituitary Con A I fraction does not have direct control over androgen production, spermiation or ovulation, but its gonadotropic activity is manifested by its ability to stimulate the second step of vitellogenesis, the incorporation of vitellogenin into the ovary. Whether the vitellogenic and maturational hormones stimulate this process through different mechanisms i.e., stimulation of incorporation of vitellogenin or ovarian yolk synthesis, awaits further studies.

The isolation from the Con A I fraction of plaice and flounder pituitary extracts, of thyroid stimulating hormones without vitellogenic activity, and of vitellogenic hormones without thyrotropic activity, is strong evidence for the existence of separate vitellogenic and thyrotropic hormones. This finding is important because of

implications of the teleost thyroid gland in reproduction (Hoar, 1973; Sage, 1973), and of the fact that the eel TSH was tested for gonadotropic contamination in an anuran bioassay and the possibility remains that there is a gonadotropin undetected in this assay.

The observation of T_3-stimulating activity in a pituitary Con A I fraction, and of T_4-stimulating activity in a pituitary Con A II fraction suggests that the production of thyroid hormones is controlled by two separate hormones. The relative physiological importance of these two thyroid stimulating hormones in the control of thyroid function is an interesting research topic for the the future.

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PUBLICATIONS ARISING FROM THIS WORK

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APPENDIX

Protocol for estradiol radioimmunoassay

Estradiol-[2,4,6,7,16,17]^{3}H with a specific activity of 130 μCi/m mole was purchased from New England Nuclear. Plasma sample (100 μl) and 1000 cpm of tritiated estradiol contained in 100 μl assay buffer were added to a test tube and the mixture was vortexed twice. Ether (3 ml) was added, the mixture was vortexed and then put on dry ice. The top ether layer was poured off into another tube. The bottom aqueous layer was re-extracted with ether before freezing on dry ice. The top ether layer is combined with the first ether extract before being evaporated to dryness under nitrogen. The extract was dissolved in 200 μl toluene:methanol (9:1) before being applied to a 6 mm x 5.5 cm Sephadex LH-20 column with a long drawn-out pipet. After the sample passed into the gel, another 200 μl of toluene:methanol (9:1) was added to the column followed by another 2.5 ml. A further 3.5 ml was used to rinse the column and the column effluent was collected and evaporated to dryness.

Tritiated estradiol was purified by chromatography on Sephadex LH-20 as described above before being used as label or tracer.

To the tube containing the extract of the sample 1 ml of assay buffer was added. The solution was vortexed vigorously and allowed to stand for 5 min before being vortexed again. Two aliquots of 175 μl each were transferred to counting vials for recovery estimates. Three aliquots of 200 μl each were added to three assay tubes. To each assay tube was added 100 μl estradiol antiserum (1:8000 dilution of the supplied stock), 100 μl tritiated estradiol containing 10,000 cpm and the mixture was incubated at 4°C for 2 hr before addition of 200 μl

dextran charcoal. The tubes were vortexed and 20 min later the tubes were centrifuged. Supernatant (400 µl) was taken and mixed with 2 ml Riafluor for counting.

The assay buffer (0.1 M phosphate, Ph 7) was prepared by dissolving 5.35 g NaH_2PO_4 H_2O, 8.66 g Na_2HPO_4, 9 g NaCl and 1 g sodium azide in 1 liter of distilled water. Gelatin (100 mg) was added to 100 ml of the above buffer and dissolved by gentle heating. The resulting solution was used as the assay buffer.

Protocol for triiodothyronine (T_3) radioimmunoassay

Sample (100 µl) was added to 950 µl of ^{125}I-labeled T_3 solution contained in a polypropylene assay tube coated with T_3 antibodies. The mixture was vortexed and incubated at 37°C for 90 minutes. The tube contents were then aspirated and the tubes were washed twice with distilled water before counting.

A series of T_3 standard solutions was provided in the Micromedic T_3 RIA kit.

Protocol for thyroxine (T_4) radioimmunoassay

A 50 µl aliquot of the standard solution was added to 450 µl distilled water in a glass tube. A 20 µl aliquot of the diluted material was added to 980 µl ^{125}I-labeled T_4 solution contained in a polypropylene assay tube coated with T_4 antibodies. The mixture was vortexed and incubated at 37°C for 60 minutes. The tube contents were aspirated and the assay tubes were washed twice with distilled water before counting.