INCORPORATION OF PLASMA PROTEINS DURING OOCYTE GROWTH AND ITS HORMONAL CONTROL IN WINTER FLOUNDER, PSEUDOPLEURONECTES AMERICANUS (WALBAUM)

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Incorporation of plasma proteins during oocyte growth and its hormonal control in winter flounder, *Pseudopleuronectes americanus* (Walbaum)

by

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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March 1986

St. John's Newfoundland

ISBN 0-315-36960-4

Wherever is Krishna, the end of Yoga, wherever is Arjuna, the master of bow, there is beauty, victory, wealth and the height of righteousness. This is my faith.

From Bhagavadgita.

To my parents

ABSTRACT

Direct evidence is provided for ovarian incorporation of plasma proteins other than vitellogenin in winter flounder Pseudopleuronectes americanus. Two major polypeptides of molecular weights 70 K and 28 K from Peak A protein(s) of the plasma were demonstrated to be incorporated into ovarian proteins. Both the polypeptides were structurally different from vitellogenin and were found to exist in the oocyte as polypeptide fragments of molecular weights 28 K, 70 K and 76 K. Vitellogenin was shown to occur as 96 K and 86 K fragments in the oocyte. The ovarian polypeptides of molecular weight 96 K fragment from vitellogenin, and 28 K fragment from Peak A protein(s) appear to be complexed together and are classified here as lipovitellin I and lipovitellin II. This is similar to the molecular organization of yolk in Xenopus laevis [Berridge, M.V., and Lane, C.D. (1976). Cell, 8, 283-297]. In addition, the presence of the 70 K and 28 K polypeptides of Peak A protein(s) in the testicular proteins has been established.

Carbohydrate-poor pituitary proteins stimulated the ovarian incorporation of vitellogenin and the non-vitellogenin plasma proteins, Peak A protein(s) and Peak E protein(s). A biologically active peptide (Rf 0.72 protein) was obtained from the carbohydrate-poor proteins; this has a molecular weight of 14.3 K by polyacrylamide gel electrophoresis containing sodium dodecyl sulphate and elutes in the region of 28 K on Ultrogel AcA 44 together with prolactin and growth hormone. A regulatory role of this gonadotropin in the ovarian uptake of Peak A protein(s) was established. These findings were in agreement with the earlier studies on the duality of gonadotropins in many species of teleosts, reported from this laboratory (see Idler and Ng, 1983).

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to Dr. David R. Idler for his supervision, unfailing help and continued support during my program of study and while preparing this thesis. My thanks are also due to Drs. V.S. Ananthanarayanan and J.M. Green for the useful discussions, to Mr. D. Hall for performing the amino acid and amino sugar analyses, to Mr. G. Chaisson and his diving team for their assistance in collecting flounders, to Mrs. G. Dyer for assisting in bioassays and to Mr. J. Martin and Mr. G. Hillier for their assistance in photography.

I wish to tender grateful acknowledgements to Dr. R. Garcia, Health Sciences Centre, Memorial University, for allowing me to use his various equipment and also for his very valuable suggestions and advice as a friend.

My special thanks are also due to Mr. S.J. Hwang for raising the antibody to one of the plasma proteins and the help received while doing the preparative PAGE. The assistance offered by Dr. Y.P. So in hypophysectomy, labelling of proteins, and much helpful advice and discussions are greatfully acknowledged.

I am indebted to Dr. P. Lobel and Mark for their company. The help from M.S.R.L. Custodians, Mike, Walter, Wayne and Jim, maintenance staff Tony, Bruce and John, is gratefully acknowledged.

Preparation of this thesis would not have been possible in a short time without the unfailing help and encouragement from my friend, Trudy.

The blessings of my parents and my brother were the greatest encouragement during this study.

Mrs. Maureen James deserves special thanks for her very careful typing of this thesis.

The award of a Memorial University graduate fellowship and a fellowship from the International Centre for Ocean Development is gratefully acknowledged.

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LIST OF ABBREVIATIONS

approx.	approximately
BSA	bovine serum albumin
c	centri, 10^{-2}
C	degrees Celsius
Ci	currie (s)
CM I	Pituitary fraction which is unadsorbed on carboxymethyl cellulose column
cpm	counts per minute
Con AI	pituitary fraction which is unadsorbed on a concanavalin A-Sepharose (Con A-Sepharose) column
Con AII	pituitary fraction which is adsorbed on a concanavalin A-Sepharose column
DTT	dithiothreitol
disc electrophoresis	polyacrylamide gel electrophoresis in a disc electrophoresis apparatus (tube gels)
DE I (DEAE I)	Con AI fraction which is unadsorbed by DEAE Bio-Gel A anion exchanger
DE II-IV (DEAE II)	Con AI fraction which is adsorbed by DEAE Bio-Gel A anion exchanger
EDTA	ethylenediaminetetracetic acid
g	grams(s)
GAR	goat anti-rabbit immunoglobulin
GSI	gonadosomatic index
hr	hour(s)
HPLC	high-performance liquid chromatography

[^{131}I]	Iodine-131 (^{131}Iodine)
[^{125}I]	Iodine-125 (^{125}Iodine)
IgG	immunoglobulin G
K	1000
28 K fraction	a fraction of molecular weight 28,000 on a gel filtration column
28 K fragment	a fragment of molecular weight 28,000 on a SDS-PAGE
µL	microliter
M	molar
µ	micro, 10^{-6}
mol	mole(s)
min	minute(s)
MW	molecular weight
m	milli
mL	milliliter
mM	millimole
mm	millimeter
nm	nanometer
N	normal
NRS	normal rabbit serum
µg	microgram(s)
ng	nanogram(s)
n	number(s)
OD	optical density
P	probability

PAGE	non-denaturing polyacrylamide gel electrophoresis under basic conditions
Peak A protein(s)	plasma protein(s) eluted immediately after the void volume on a Sephacryl S-300 gel filtration column
Peak B + C	the second peak from gel filtration of plasma on a Sephacryl S-300 column
Peak D	the third peak from gel filtration of plasma on a Sephacryl S-300 column
Peak E protein(s)	the fourth peak from gel filtration of plasma on a Sephacryl S-300 column
PMSF	phenylmethylsulfonyl fluoride
prep. PAGE	preparative polyacrylamide gel electrophoresis
RT	retention time in a HPLC gel filtration column
RNA	ribonucleic acid
Rf	relative mobility to a marker dye in a PAGE
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate PAGE under denaturing conditions
[^{35}S]	sulphur-35
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
Vg	vitellogenin
UV	ultraviolet
(V/V)	volume/volume
(W/V)	weight/volume
X	times
<	less than
>	greater than

CHAPTER I

General Introduction

Processes such as maturation and fertilization depend on the synchronised growth of the gametes during the reproductive phase. Schuetz (1985) noted in his recent review that the female gamete is the primary or only cellular link between the present and successive generations. The cellular and molecular events that occur within the cytoplasm and nucleus of an oocyte during its development are of fundamental interest and importance to developmental biology.

In this thesis, I have addressed the question of the functional role of plasma proteins in oocyte growth in a teleost, Pseudopleuronectes americanus, with emphasis on non-vitellogenin proteins. The work also deals with the gonadotropic regulation of uptake of these plasma proteins into the ovary during the reproductive cycle.

Role of plasma proteins in non-mammalian vertebrate reproduction

Efforts to understand the role of plasma proteins in non-mammalian vertebrate ovarian growth have been almost entirely concentrated on vitellogenin, also known as the female-specific plasma protein (Aida et al., 1973a). The subject of vitellogenin and vitellogenesis has been extensively reviewed in non-mammalian vertebrates with respect to oocyte growth (Wallace, 1978; 1985), expression of the vitellogenin genes in eukaryote organisms and the relationship between vitellogenin and egg yolk proteins (Tata and Smith, 1979), stages of ovarian growth

(Wallace and Selman, 1981; Wiegand, 1982) and teleost yolk formation and differentiation (Ng and Idler, 1983). Inducibility of this lipophosphoglycoprotein (Emmersen and Petersen, 1976; Hori et al., 1979) with estrogen treatment has been well established in all the classes of non-mammalian vertebrates. Its chemical and immunological characteristics with respect to yolk proteins have also been well studied (see Wallace, 1978).

The history of the role of circulating plasma proteins in the synthesis of yolk in non-mammalian vertebrates dates back to some 90 years ago, when Klemperer (1893) observed that antibody-like activity could be found in yolk derived from eggs of hens previously immunized with specific antigens. Immunological and biochemical similarities between certain serum proteins of the laying hen and the hen's egg yolk proteins have also long been recorded (see Wallace, 1978). Later studies on the isolation of the yolk proteins (Cook, 1961), and demonstration of the synthesis of some yolk precursor by liver slices (Heald and McLachlan, 1965), reinforced the long standing conviction among workers in avian reproductive biology that all macromolecule materials of the developing oocyte, including the yolk proteins, are derived from the maternal bloodstream (see Wallace, 1978).

Earlier, Laskowski (1936) had observed plasma phosphoprotein appearing during the breeding season of a number of female oviparous vertebrates. Flickinger and Rounds (1956) reported the cross reactivity of an antibody prepared against a yolk component with female serum as well as with a variety of other extracts. However, these studies were either incomplete or not consistent (Wallace, 1978) and, moreover, Kaster and Schechtman (1957) failed to obtain evidence for

the transfer of injected protein to the ovary. Later, Glass (1959) demonstrated that proteins from the maternal bloodstream could apparently be incorporated antigenically intact into developing amphibian oocytes. In addition, Ulrist and Schjeide (1961) found that components with biochemical and ultracentrifugal properties similar to those of yolk proteins appeared in the serum. Outstanding studies by Wallace and co-workers first with Xenopus and more recently with fish provided conclusive evidence on oocyte growth in relation to the plasma protein vitellogenin and defined the gonadotropin-estrogen relationship to vitellogenin synthesis. Its existence as phosphoglycolipoprotein, in circulation and its conversion to yolk proteins, lipovitellin and phosvitin, has also been well documented.

As in birds and amphibians, vitellogenin is very important in teleost yolk synthesis (see reviews by Wallace and Selman, 1981; Wiegand, 1982; Ng and Idler, 1983). Female specific proteins have been detected in teleosts by immunological, electrophoretic, chromatographic or ultracentrifugal methods (see Wallace and Selman, 1981; Idler et al., 1979; So et al., 1984).

Autoradiographic studies by Korfsmeier (1966) after injection of tritiated amino acids into adult female zebra fish Brachydanio rerio, which breed continuously, indicated the transfer of protein from the blood to yolk spheres within the oocyte. Korfsmeir (1966) demonstrated the appearance of labelled protein first in the liver before incorporation into yolk. Electrophoretic studies by te Heesen and Engels (1973) showed the ontogenesis of female specific proteins in the ovaries of the zebra fish.

Apart from the following indirect studies, one on Xenopus and two

others on teleosts, no other published study exists concerning the specific role of other plasma proteins in non-mammalian reproduction.

Wallace and Jared (1969), in order to provide proof for the role of the ovary in removing vitellogenin from the circulation, injected equivalent amounts of two serum protein fractions S_1 and S_2 and vitellogenin, all labelled with ^{3}H-leucine, into three groups of human chorionic gonadotropin treated Xenopus females. From this experiment, they concluded that vitellogenin was rapidly removed from the circulation and became associated with ovarian protein, whereas the other two serum protein fractions did so to a much lesser extent; also, the incorporation of vitellogenin into ovarian protein appeared to be about 50 times more rapid than the incorporation of either of the other serum protein fractions. They reported that the process was selective but the other serum proteins were also incorporated to a limited extent. This study on Xenopus was the first experimental evidence indicating a possible role of other serum proteins in the ovarian growth. However, based on quantitative analyses performed with the oocytes of Xenopus laevis, Wallace et al. (1972) reported that > 99% of the yolk protein was derived from vitellogenin.

In teleosts, evidence for the incorporation of non-vitellogenin macromolecules by oocytes was shown by Wegmann and Gotting (1971). Through ultrastructural studies on the swordtail Xiphophores helleri, they showed the incorporation of an electron-dense molecule into the yolk bodies of vitellogenic oocytes.

The remaining evidence in teleost comes from an in vitro study on vitellogenin incorporation by the ovarian follicles of rainbow trout, Salmo gairdneri (Campbell and Jalabert, 1979). The authors reported

that a protein from the serum of S. gairdneri, induced by estrogen treatment, was incorporated into vitellogenic follicles to a greater extent than other serum proteins of S. gairdneri or bovine serum albumin (BSA) which served as controls. They reached no conclusion concerning these non-vitellogenic proteins as possible sources of oocyte proteins and suggested that a large proportion of the incorporated BSA and other non-vitellogenic proteins could be associated with follicular tissues other than the oocytes.

Yolk proteins

Whether it is vitellogenin or non-vitellogenin proteins, their relevance to oocyte growth is mostly reflected in terms of yolk proteins. Molecular organization of the yolk in oocyte becomes pertinent here. In teleosts yolk occurs as fluid-filled spheres (Grodzinski, 1954, 1973) within the peripheral ooplasm (Droller and Roth, 1966; Anderson, 1968; Ulrich, 1969; Gupta and Yamamoto, 1972; Shackley and King, 1977), whereas in amphibians or in other non-mammalian vertebrates, yolk occurs as crystalline granules (see Wallace 1978). However, crystalline yolk has also been described in several distantly related teleosts (Yamamoto and Oota, 1967; Gupta and Yamamoto, 1972; Riehl, 1978), which indicates some homology between teleost and amphibian yolk. Yolk spheres in teleosts are known to maintain their integrity throughout oocyte growth (Yamamoto, 1957b) or fuse centripetally to form a continuous mass of fluid yolk, which confers on eggs their characteristic transparency (Wallace and Selman, 1981).

It is well established that the yolk proteins of oviparous verte-

brates are composed of lipovitellin and phosvitin (see Follett and Redshaw, 1974; Ohlendorf et al., 1975). A number of investigators have attempted to isolate lipovitellin-like and phosvitin-like yolk proteins from the ovaries of a variety of teleost species (see Ng and Idler, 1983). In teleosts, lipovitellins are frequently heterogenous, they contain very low amounts of protein-phosphorus (see Wallace, 1978) and are soluble in solutions of low ionic strength (Jared and Wallace, 1968). The phosvitins are also frequently heterogenous (Mano and Lipmann, 1966 a,b; Jared and Wallace, 1968), have lower molecular weights and contain widely divergent amounts of protein-phosphorus (see Wallace, 1978). Phosvitins appear to sometimes be completely absent from yolk preparations in some marine teleosts (Jared and Wallace, 1968).

Lipovitellin monomer (glycolipophosphoprotein) in amphibians has a molecular weight in its native form of about 200 K and is composed of 20% lipid and of about 0.5% protein-phosphorus (Wallace, 1978). Under denaturing conditions, it can be resolved further into two peptides with a 1:1 molar ratio and molecular weights of 31 K and 120 K (Wallace, 1978). Only the smaller peptide is phosphorylated (Bergink and Wallace, 1974). On the contrary, phosvitin has a molecular weight of about 35 K - 40 K under both native and denaturing conditions with a total protein-phosphorus content of 9.5% (Redshaw and Follett, 1971).

Different values of molecular weights for lipovitellin have been reported among teleost species. A molecular weight of 300 K for trout (Hara and Hirai, 1978; Campbell and Idler, 1980), 500 K for winter flounder (Ng and Idler, 1979) were noted. Ando (1965) indicated that trout lipovitellin has a molecular weight of 240 K in its native form

and has two subunits. deVlaming et al. (1980) reported two subunits for lipovitellin of goldfish by sodium dodecyl sulfate polyacrylamide gel electrophoresis having molecular weights of 105 K - 110 K and 19 K - 25 K, respectively. Plack and Fraser (1970) claimed the presence of two types of lipovitellin in the cod ovary.

On the other hand, the molecular weight of trout phosvitin was reported to be 43 K by Campbell and Idler (1980) and 19 K by Schmidt et al. (1965) and Mano and Yoshida (1969); Ng and Idler (1979) reported a value of 30 K for winter flounder. In general, molecular weights of teleost phosvitins were reported to be lower than that of other vertebrate phosvitins (see Ng and Idler, 1983).

Apart from these reported major yolk proteins, yolk components distinct from lipovitellin and phosvitin were also reported in the teleost ovary (Jared and Wallace, 1968). One of these, the "component" found in salmonids contained no lipid or proteinphosphorus and was reported to have a molecular weight of about 30 K; it was derived from the serum of sexually maturing females (Markert and Vanstone, 1971; Campbell and Idler, 1980); was extractable with 0.5 M NaCl (see Ng and Idler, 1983). Isolation of livetin, another ovarian protein was reported by Markert and Vanstone (1968) and by Jared and Wallace.

A quote from Wallace (1978) is relevant to the present investigation: "In view of recent information on (1) the nature and origin of the β-component in salmonids, (2) the extreme susceptibility of at least one teleost vitellogenin to proteolysis (Hickey and Wallace, 1974) and (3) the derivation of yolk proteins from vitellogenin by proteolysis (Bergink and Wallace, 1974), it seems more reasonable to postulate at this time that teleost vitellogenins are proteolytically

cleaved at alternate and/or more numerous sites during their conversion to yolk proteins".

Gonadotropic regulation of ovarian growth

In non-mammalian vertebrates, the major event during the process of ovarian growth is represented by the massive synthesis of yolk by the oocytes, which is indirectly controlled by the pituitary hormones (see Redshaw and Follet, 1971). As indicated earlier, yolk is synthesized by incorporating the plasma protein(s), principally vitellogenin (see Wallace, 1978). The hormonal control of ovarian growth in teleosts has been addressed in several reviews (Dodd, 1972; Reinboth, 1972; Donaldson, 1973; deVlaming, 1974; Fontaine, 1975; Fontaine, 1976; Lam et al., 1978; Idler and Ng, 1983; Ng and Idler, 1983). Akin to other non-mammalian vertebrates, in teleosts, gonadotropins were found to enhance the transfer of vitellogenin from the blood into vitellogenic oocytes (Campbell and Idler, 1976; Campbell, 1978; Ng and Idler, 1978a,b); apparently by stimulating micropinocytotic activity at the oocyte membrane (Droller and Roth, 1966; Anderson, 1968; Wegmann and Gotting, 1971; Upadhyay et al., 1978).

The existence of two separate gonadotropins in fish was a controversial issue until 1975. Various reports on the isolation of gonadotropins from teleosts gave no indication of the presence of two biochemically distinct types of gonadotropins (see Ng, 1980). Studies by Idler and co-workers (Idler et al., 1975 a,b; Campbell and Idler, 1976, 1977; Ng and Idler, 1978 a,b, 1979; Idler and Ng, 1979; Idler and Hwang, 1978) illustrated the existence of two distinct types of

gonadotropins in several teleost species. They called these two gonadotropins Con AI gonadotropin(s) which exhibit no affinity to concanavalin A, "ie" carbohydrate-poor GtH) and Con AII gonadotropin(s) which exhibit affinity to Con A, ie carbohydrate-rich GtH). The Con AI GtH controls ovarian development by stimulating the incorporation of yolk precursor from the blood and the Con AII GtH stimulates oocyte maturation and ovulation (see Idler and Ng, 1983; Ng and Idler, 1983). The possibility that each type of gonadotropin may have other functions in regulating ovarian development is not excluded.

Statement on the research problem

Wallace and Jared (1976) reported a very high specificity of incorporation of vitellogenin by isolated amphibian oocytes, although Wallace and Selman (1981) noted that the available evidence for teleosts indicates a reduced specificity (see Campbell and Jalabert, 1979). The earlier findings on the incorporation of nonvitellogenin plasma proteins were rather indirect evidence since the experiments were conducted to establish the specificity of vitellogenin uptake by oocytes. In addition, the serum proteins that were tested may not be the protein species required by the oocytes. Factors like stage of oocyte development could be very important in the regulation of protein uptake. Further, the study conducted by Campbell and Jalabert (1979) was on isolated ovarian follicles *in vitro* and this may not reflect the situation *in vivo*. Thus the available evidence either emphasized the predominant role of vitellogenin in oocyte growth or provided inconclusive evidence for the possible participation of other plasma

proteins.

Hence, there is a gap in our knowledge concerning the functional selection of non-vitellogenin plasma proteins in oviparous vertebrates during oocyte growth. Before this investigation was started, the preliminary experiment conducted in this laboratory (Idler and So, personal communication) indicated that at least two plasma protein(s), in addition to vitellogenin were also taken up by flounder ovary. Their study also indicated that the uptake was enhanced by the Con AI fraction from the pituitary. These results provided the groundwork for this study.

The purpose of this investigation initiated in 1984 was:

1. To answer the question: Is vitellogenin the dominant protein or do other plasma proteins also play an important role in teleost oocyte development?
2. To elucidate the structural and functional relatedness or unrelatedness to vitellogenin of the plasma proteins, which were found to be taken up by the oocytes.
3. To define more clearly the influence of the carbohydrate-poor gonadotropic fraction on the uptake of non-vitellogenin plasma proteins and to compare the uptakes at different times during the reproductive cycle.

CHAPTER II

WINTER FLOUNDER PLASMA PROTEINS

Introduction

Although the study of mammalian, and especially human, plasma has been intense, less information is available on the plasma proteins of other vertebrate classes. Recently the protein moieties of fish lipoproteins have received increased attention (McKay et al., 1985). Like those of mammals, fish plasma lipoproteins contain a heterogenous collection of apoproteins associated with particular density classes (Nelson and Shore, 1974; Chapman et al., 1978; Chapman, 1980). In higher vertebrates, the plasma proteins are best known for the transport of dietary and endogenously synthesized lipids (McKay et al., 1985) and cardiovascular diseases (Goldstein and Brown, 1977; Green and Glickman, 1981) but among the oviparous vertebrates, the best undestood function of one of the plasma lipoproteins, vitellogenin, is its role in the synthesis of yolk which is universal among the oviparous vertebrates. The reports indicating the uptake of other plasma proteins by the ovary (General Introduction) and the preliminary study conducted in this laboratory on the ovarian uptake of other lipoproteins left many avenues open to pursue. Vitellogenin having a defined functional role in oocyte growth, the question of functional and structural relatedness of these non-vitellogenic plasma proteins to vitellogenin has been addressed in this Chapter. This can be done by comparing the polypeptides, primary structure by peptide mapping, and the immunological cross reactivities. Such an attempt was made in this part of the work using winter flounder, Pseudopleuronectes americanus (Walbaum) as the experimental animal.

Materials and Methods

Isolation and analysis of winter flounder plasma proteins

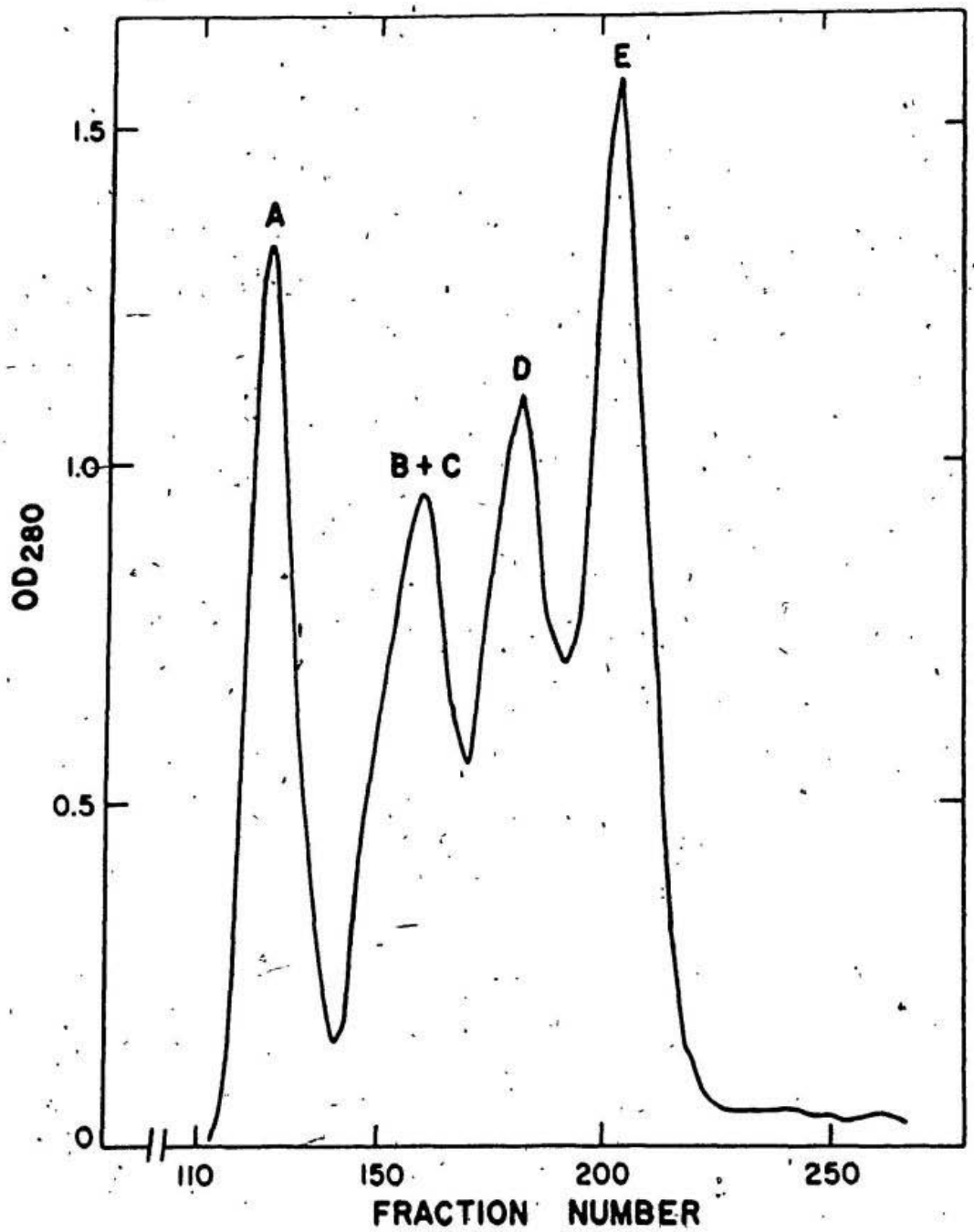
Blood collection

Blood was collected from female and male winter flounder Pseudopleuronectes americanus at different times of the year in 1 volume of ice-cold PMSF solution [(50% 0.2 M phosphate buffer saline, pH 7.8; 3 mM PMSF), DeVlaming et al., 1980] for every 4 volumes of blood and centrifuged at 8000 g for 20 min in a Sorvall centrifuge at 0 C to obtain plasma. The plasma was immediately diluted with an equal volume of vitellogenin (Vg) buffer (150 mM Tris-base, pH 8.0; 0.5 M NaCl; 10 mM EDTA, 0.25% PMSF) modified from Idler et al. (1979) and centrifuged again at 8000 g for 20 min at 0 C. The supernatant was either chromatographed or kept frozen at -80 C until used.

Sephacryl S-300 chromatography

An equivalent of 2.0 mL of the original plasma was chromatographed at 4 C with ascending elution on a 2.6 x 86 cm column of Sephacryl S-300 superfine (Pharmacia) which had been equilibrated with Vg buffer. The column was eluted at a flow rate of 15 mL/hr and 1.4 mL/tube fractions were collected. The optical density of the fractions was read at 280 nm in a spectrophotometer and the readings were plotted on graph paper to obtain major peaks, designated as: Peak A, Peak B + C, Peak D, and Peak E (Fig. 1). The names A, B + C, D and E for these peaks are

Figure 1. Chromatography of plasma from female flounder. A plasma equivalent of 2.0 ml was chromatographed on a 2.6 x 86 cm Sephacryl S-300 Superfine column. Fractions of 1.4 ml were collected at a flow rate of 15 ml/hr and protein was monitored at OD_{280}.


A
B + C
D
E
1.5
1.0
0.5
0
OD280
110
150
200
250
FRACTION NUMBER

based on their gel filtration profile. However, Peak B + C was identified earlier as vitellogenin based on its electrophoretic mobility and inducibility with estrogen in Atlantic salmon (So et al., 1984) and winter flounder (Idler and So, unpublished).

Non-denaturing polyacrylamide gel electrophoresis (PAGE) of the plasma proteins

To obtain evidence on the protein species present in individual Sephacryl S-300 peaks, PAGE under non-denaturing conditions was performed. Disc electrophoresis (Davis, 1964) in 5% running gel (5% acrylamide, 2.6% cross linkage), pH 8.9 and 4% stacking gel, pH 6.8 was performed on the plasma and on the eluted peaks from the Sephacryl S-300 gel filtration column for the peak tubes, tubes representing different parts of the peaks and also on the pooled peaks, at a constant current of 2.0 mA/tube for 3 hr. Proteins were stained with 0.1% Coomassie blue G-250 (Bio-Rad), while fixing in 12% TCA for 30 min. The bands were then intensified with 0.1% Coomassie blue R-250 (Bio-Rad) in 7% acetic acid, overnight, and destained in 7% acetic acid in a tube gel destainer (Bio-Rad). For identification of lipoproteins, the gels after electrophoresis were stained with acetylated Sudan black B (BDH) as described by Prat et al. (1969).

Pooling of the fractions

After visualising the electrophoretic band pattern by Coomassie blue G-250 staining, which indicates apparent homogeneity of the

preparations, the protein peaks from Sephacryl S-300 column were pooled. Only 4 to 5 tubes representing the top of the peaks were pooled separately and aliquots were kept for protein estimation and PAGE. The remaining protein preparations were aliquoted and thereafter kept frozen at -80 C.

Protein estimation

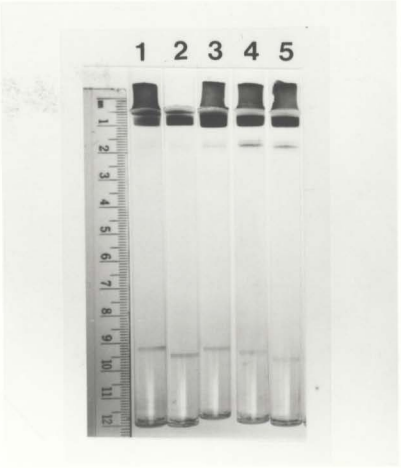
Proteins in different samples were estimated by the method of Lowry et al. (1951) after precipitating the proteins overnight with 12% TCA. The method described by Bradford (1976) was used to estimate proteins in samples used for amino acid and amino sugar analyses.

Sodium dodecyl sulphate (SDS) PAGE

An SDS-PAGE (10% acrylamide) was performed on Peak A protein(s) and vitellogenin by the Dreyfuss et al. (1984) procedure. The samples were electrophoresed initially at 20V, and then at 50V for 12 hr. The gels were fixed in 50% methanol, 10% acetic acid and 40% distilled water for 30 min and stained in 0.6 g Coomassie blue R-250, 150 mL methanol, 30 mL acetic acid and 120 mL distilled water for 30 min. Destaining was done in 10% methanol and 10% acetic acid in distilled water.

Since Peak A protein(s) showed a very minor band of Rf 0.18 with a major band (Fig. 2) in a 5% PAGE, a different approach was tried in SDS-PAGE to identify the polypeptides contributed by the major band. PAGE (5%) in a disc electrophoresis (Davis, 1964) apparatus was

Figure 2. Non-denaturing polyacrylamide gel electrophoresis (5% disc gel) of Peak A protein(s) from Sephacryl S-300 chromatography. Lanes 1 to 5 contain samples from left trailing part of the peak. Lanes 4 and 5 contain equal amounts of samples from peak tubes. Gels were stained with Coomassie blue R-250.


1 2 3 4 5
1 2 3 4 5 6 7 8 9 10 11 12

performed for Peak A protein(s) and the gels were stained with 0.1% Coomassie blue G-250 while fixing in 12% TCA for 20 min. The major band from gels was cut (2 mm length) with a razor blade. The slices were then equilibrated for 30 min at room temperature with occasional swirling in 10 mL of a buffer containing final concentrations of 0.125 M Tris-HCl, pH 6.8; 0.1% SDS and 1 mM EDTA (Cleveland et al., 1977). The slices were then boiled in 70 µL of 2X SDS sample buffer for 5 min, cooled for 15 min in an ice bath and then each gel slice was pushed with a spatula to the bottom of the well of a second SDS gel (10%) filled with the above buffer. Spaces around the slices were filled by overlaying each slice with 10 µL of the above buffer containing 20% glycerol, followed by 10 µL of the same buffer containing 10% glycerol and finally 30 µL of 2X SDS sample buffer. Electrophoresis was performed in the normal manner.

In another approach, ^{125}I labelled Peak A protein(s) was electrophoresed as described above. Instead of staining the gel, 2 mm long slices were cut and counted in a gamma counter. The slice representing the highest counts was electrophoresed on a second denaturing gel as described above. After the electrophoresis, the gel was fixed, stained, destained, and then dried in a slab gel dryer, Model 1125B (Bio-Rad) for 2 hr and then autoradiography was done using Kodak X-OMAT AR X-ray film (XAR-5) at -70 C. The films were developed, and fixed in Kodak chemicals.

Pre-stained high molecular weight markers from BRL and high molecular weight markers from Pharmacia were used in the molecular weight determination. In some cases, RNA polymerase II, histone and actin were used.

Labelling of the proteins

The recently developed Iodogen method modified from Fraker and Speck (1978) and Salacinski et al. (1979) was adopted for the iodination of flounder Peak A protein(s), vitellogenin (So et al., 1984) and Peak E protein(s). Ten µL of iodogen [(1,3,4,6-tetrachloro-3α,6β-diphenyl glycoluril); Pierce], at a concentration of 1 mg/mL of dichloromethane, was allowed to dry on the bottom of a disposable borosilicate glass culture tube (6 x 50 mm). Fifteen µg of flounder plasma Peak A protein(s) or vitellogenin or Peak E protein(s) in 25 µL of sodium phosphate buffer (0.5 M, pH 7.4) were added. After transferring 1.5 mCi of ^{131}Iodine (New England Nuclear) to the mixture, they were allowed to react for 12 min. The reaction was terminated by further addition of 600 µL of 0.05 M phosphate buffer. The radioiodinated protein was then cleaned up on a disposable Sephadex G-25 M column (Pharmacia column PD-10), before being used in bioassays, electrophoresis, checking antibody titre and antibody binding assays. The same procedure was followed while labelling Peak A protein(s) with ^{125}Iodine (Amersham).

Polyacrylamide gel analysis of Peak A protein(s)

PAGE (5%) was done for the ^{131}I labelled Peak A protein(s). The purpose of this analysis was to find out the level of radioactivity contributed by the minor contaminant. About 2 x 10^6 cpm in 20 µL were loaded onto the top of a tube gel. Disc electrophoresis was performed for 3 hr. The gel was then cut into 5 mm slices and counted in

a gamma counter. The same procedure was followed for Peak A protein(s) labelled with ^{125}I.

High performance liquid chromatography (HPLC) of plasma proteins by gel filtration

Among many isolation methods employed in separating plasma proteins, HPLC has been widely utilised for the separation and fractionation of many biological substances from the plasma because of its inherent speed and excellent reproducibility (Okazaki and Hara, 1984). Recently, Hara et al. (1980), developed a method using HPLC with an aqueous gel permeation column (TSK-GEL) to separate serum lipoproteins. The technique has been successfully used to study human serum proteins by Hara et al. (1980, 1982), Okazaki et al. (1982), Ohno et a . (1981), Busbee et al. (1981) and by many other workers for both quantitative and qualitative analyses. Swergold and Rubin (1983) used the gel permeation HPLC TSK-G3000-PW column to determine the molecular weights of proteins and peptides in their native forms. In this study, gel permeation (gel filtration) HPLC was used to isolate the protein species from Peak A protein(s), to purify Peak E protein(s) and to determine their molecular weight in native form.

A Spectra-Physics three pump high performance liquid chromatograph system, equipped with Spectroflow 757 UV detection and Spectra-Physics integrator was used in HPLC gel filtration of the plasma proteins. The following combinations of gel filtration columns were used unless otherwise mentioned.

1. A 300 x 7.5 mm Bio-Gel TSK-50 G 5000 PW column connected to a 21.5

x 7.5 mm Bio-Sil TSK preparative guard column or 21.5 x 7.5 mm Bio-Gel TSK guard column.

2. A 300 x 7.5 mm Bio-Sil TSK-250 G 3000 SW column connected to a 21.5 x 7.5 mm Bio-Sil TSK preparative guard column. All the columns were purchased from Bio-Rad.

The main columns used in this investigation were Bio-Gel TSK-50 G 5000PW which has a separation range of 4 K - 8000 K (polyethylene glycol) and 10 K - 2000 K (Dextran) and Bio-Sil TSK-250 G 3000-SW, which has a separation range of 1000 - 300 K. These ranges were suited for the molecular weight ranges of the plasma proteins of interest here.

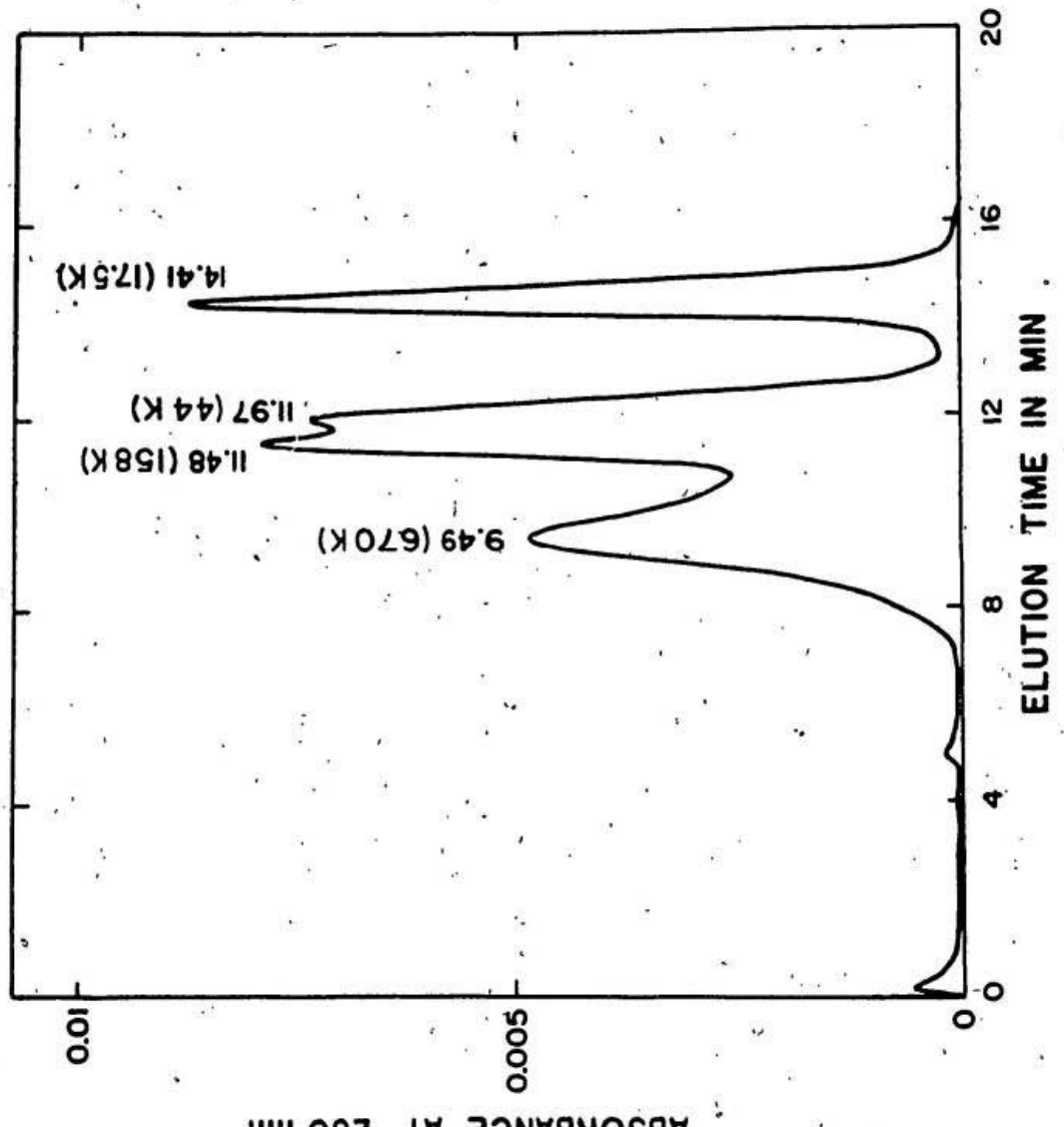
The buffer system used in all the chromatograms was 0.1 M sodium sulfate (Na_2SO_4) and 20 mM sodium dihydrogen phosphate (NaH_2PO_4), pH 6.8 and the samples were eluted at a flow rate of 1 ml/min. Detection was at 280 nm with sensitivity 0.01 AUFS, in all the runs.

Gel filtration

A Bio-Gel TSK-50 G 5000 PW column connected to a Bio-Sil guard column was used for the gel filtration of the samples. The columns were calibrated using the following molecular weight markers from Bio-Rad: Thyroglobulin (670 K); bovine gamma-globulin (158 K); chicken ovalbumin (44 K) and bovine myoglobin (17.5 K). A total of 36.0 µg of the standards were eluted through the column (Fig. 3).

To determine the interferences and/or contributing peaks by the Vg buffer, 2 µL of Vg buffer was eluted through the column. Further, the

Figure 3. Calibration of HPLC gel filtration column with 5 µg of high molecular weight standards (Bio-Rad). HPLC column system used: Bio-Sil TSK guard column + Bio-Gel TSK-50 G 5000 PW main column. Sensitivity: 0.01 AUFS. Elution buffer: 0.1M Na_2SO_4 + 0.02M NaH_2PO_4, pH 6.8; Flow rate 1 ml/min. Detection at 280 nm.


ABSORBANCE AT 280 nm
0.01
0.005
0
0
4
8
12
16
20
ELUTION TIME IN MIN
9.49 (670 K)
11.48 (158 K)
11.97 (44 K)
14.41 (17.5 K)

individual components of Vg buffer, 80 µL of 10 mM EDTA and 90 µL of 0.025% PMSF were eluted separately.

Preparation of the samples

The plasma proteins from Sephacryl S-300 eluted in Vg buffer were used. To eliminate the interferences from Vg buffer, the samples were ultrafiltered in an Amicon concentrator using YM5 25 mm diameter membrane (Spectrapore) with HPLC grade distilled water.

A. Gel filtration of Peak A protein(s)

In the above system, 0.6 mg sample in 100 µL was eluted at a flow rate of 1 mL/min. A peak with RT 8.83 min was resolved along with the peaks from Vg buffer. Peak A protein(s) from one of the Sephacryl S-300 runs had the additional protein band in almost equal quantity (40%) in tubes representing the right trailing part of the peak. This sample was run through a HPLC gel filtration column to separate the contaminant. Only one peak of RT 8.83 min was resolved, with the other peaks representing Vg buffer, indicating adsorption of the contaminant protein to the column. The RT 8.83 min peak was collected from several runs and re-chromatographed to confirm the above results.

Also, a few chromatograms were performed at pH 3.0, 4.0, 5.0 and 8.0 of the above elution buffer. Samples were also eluted with 36% acetonitrile in the presence or absence of 0.1% trifluoro acetic acid (TFA) (Swergold and Rubin, 1983). A combination of columns was tried to confirm the adsorption of the second protein to columns and to

confirm that protein is not eluted with the buffer peaks. In a combination of Bio-Sil TSK preparative guard column, Bio-Sil TSK 250 G 3000 SW column and Bio-Gel TSK 50 G 5000 PW column, 0.6 mg sample in 100 µL was eluted as described above.

B. Gel filtration of vitellogenin

A Bio-Gel TSK-50, G 5000 PW main column connected to a Bio-Sil TSK guard column was used to elute vitellogenin. Vitellogenin sample used here for HPLC gel filtration was isolated from the plasma of estrogenised female flounders where, the estrogen treatment dramatically enhances the synthesis of vitellogenin by the liver (Plack et al., 1971· Aida et al., 1973b; Campbell and Idler, 1976, 1980; Emmersen and Petersen, 1976; Idler and Campbell, 1980) and hence yields a homogeneous preparation by Sephacryl S-300 chromatography (So et al., 1984). Sample preparation and gel filtration were carried out as described for Peak A protein(s).

C. Gel filtration of Peak E protein(s)

A Bio-Sil TSK 250 G 3000 SW column connected to a Bio-Sil TSK preparative guard column was used; sample was prepared as previously described. The column was calibrated with the same Bio-Rad standards used to calibrate the Bio-Gel TSK-50 column. The rest of the procedure was as described earlier for gel filtration of Peak A protein(s).

All the peaks from several chromatograms for Peak A protein(s) and Peak E protein(s) in each combination were pooled separately,

concentrated and a 5% PAGE was performed. Protein was quantified by the Lowry et al. (1951) method. These procedures were not performed for peaks from vitellogenin gel filtration.

Molecular weight determination

An attempt to determine the molecular weights of plasma proteins in native form were done using HPLC gel filtration columns.

Con A-Sepharose affinity chromatography of Peak A proteins

Peak A protein(s) sample from HPLC was re-chromatographed on a Con A-Sepharose column. The preparation from HPLC was dialysed for 2 days against Buffer B (0.05 M Tris-HCl, pH 7.8, 0.5 M NaCl, 0.2 mM DTT, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% PMSF) and then applied to a Con A-Sepharose column (2 x 15 cm). The sample was eluted first with Buffer B and subsequently with buffer C (0.05 M Tris-HCl, pH 7.8, 0.5 M NaCl, 0.2 mM DTT, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% PMSF, 0.15 M β-methyl-D-glucoside) to yield the unadsorbed and adsorbed fractions respectively.

Preparation of the antiserum to Peak A protein(s)

New Zealand white male rabbits were used for raising the antiserum. Initially they received intradermal injections at multiple sites on the back with 1 mg of flounder Peak A protein(s) from Sephacryl S-300 emulsified with Freund's complete adjuvant and a

primary subcutaneous injection of 0.1 mL pertussis. Half of the primary dose was given intramuscularly to the limbs in subsequent booster doses, every month, until a reasonably high antibody titer was obtained. Assay titer of the antibody was determined at the dilution that gave 50% binding of ^{131}Iodine labelled Peak A protein(s) to antibody.

Preparation of Peak A protein(s) IgG

Rabbit IgG against flounder Peak A protein(s) was prepared from one mL of antiserum using a protein A agarose (BRL) affinity column as reported by So et al. (1984) to isolate vitellogenin IgG. The eluted fractions from the affinity column were checked as follows for the binding to ^{131}I labelled Peak A protein(s). An aliquot of each fraction representing the eluants was diluted 100,000 fold and 200 µL of these samples were incubated with 200 µL ^{131}I Peak A protein(s) (approximately 10,000 cpm/200 µL) and 100 µL assay diluent (barbital buffer, 80 mM, pH 8.6, containing 0.5% BSA and 0.01% thimersol) at 4 C. After three days, normal rabbit serum (1:80 dilution) and goat anti rabbit β-globulin antibody (1:20 dilution) was added to the samples and incubated at 4 C overnight. The tubes were then centrifuged at 4000 g for 15 min. The supernatant was aspirated off and the precipitates were counted in a gamma counter. Flounder vitellogenin IgGs were isolated in a similar manner in this investigation.

Checking the cross reactivity of Peak A protein(s) antibody with the other plasma proteins vitellogenin, Peak D and Peak E

Competitive radioimmunoassay was performed to check the cross reactivity of Peak A protein(s) antibody to other plasma proteins; this would also provide additional information on the structural relatedness or unrelatedness of Peak A protein(s) to vitellogenin and two other protein(s) preparations. Five µg of Peak A protein(s), vitellogenin, Peak D, and Peak E were diluted to 100, 10,000 and 100,000 times. Triplicates of 100 µL of samples from these three different dilutions were incubated with 200 µL of radiolabelled Peak A protein(s) (about 10,000 cpm) and 200 µL of Peak A protein(s) antibody (1:100,000, 50% binding titer) at 4 C for three days. Then 100 µL each of normal rabbit serum (1:80 dilution) and goat anti rabbit ß-globulin antibody (1:20 dilution) were added. After 24 hr, the tubes were centrifuged at 4000 g for 15 min, the supernatant was aspirated off and, the precipitate complex was counted in a gamma counter. Percent binding was calculated as follows. Percent binding = $\left(\frac{\text{Counts in the precipitate-Blank}}{\text{Total counts}}\right) \times 100$, where, blank = counts in precipitate in a tube from which the antibody is omitted during incubation; total counts = radioactivity in 200 µL of labelled proteins.

Peptide mapping of Peak A protein(s) and vitellogenin

To obtain additional evidence to corroborate the structural relatedness or unrelatedness of Peak A protein(s) and vitellogenin, peptide mapping by partial proteolysis by Staphylococcus aureas V8

protease enzyme (Cooper) was done, for Peak A protein(s) and vitellogenin, according to the procedure described by Cleveland et al. (1977). Peptide mapping was also done for radioiodinated Peak A protein(s) (^{125}I) where gel slice (2 mm long) having peak counts from a 5% PAGE (represents the major band) was cut and the protein in the slice was digested in a SDS gel (10%) with V8 enzyme as described by Cleveland et al. (1977). Autoradiography was done as described on page 19.

Amino acid and carbohydrate analysis

Protein samples for amino acid analysis were hydrolysed in 6 N HCl at 100 C for 24 hr and were then applied to a Beckman Model 121 amino acid analyzer. Protein samples for amino sugar determination were hydrolyzed in 4 N HCl at 100 C for 8 hr 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 was used for elution (Black et al., 1970).

Results

Isolation of plasma proteins

When the plasma from female flounder was chromatographed on Sephacryl S-300 column, four major peaks were resolved. The molecular weights of these peaks were: Peak A, 1,000,000; Peak B + C, 600,000 - 500,000; Peak D, 300,000; and Peak E, 200,000, estimated by comparison

with the standards. In case of plasma from male flounder, the second Peak (B + C) was considerably smaller with an optical density of less than 0.7. The other peaks were comparable to peaks from female plasma.

Variations in the Sephacryl S-300 chromatography elution profiles of flounder plasma collected during different times of the year

(a) Samples from female flounder

In the sample collected during the first week of October, when the fish was actively incorporating vitellogenin into oocytes (Chapter IV), Peak B + C was major peak. Peaks D and E were almost indistinguishable from Peak B + C. Peak A was comparatively smaller but was quite distinct (Fig. 4).

Peak A was the major peak in the plasma collected during mid February when the fish had stopped active incorporation of vitellogenin into oocytes, but not the incorporation of Peak A protein(s) (Chapter IV). Peak B + C was comparatively a smaller peak. However, Peak E was a major peak (Fig.5).

Peak A was the major peak in the plasma collected during early June, when the fish was ready to spawn. Peak E was almost the same intensity as Peak A. Peak B + C was a minor peak, not very much different from the February sample (Fig. not shown).

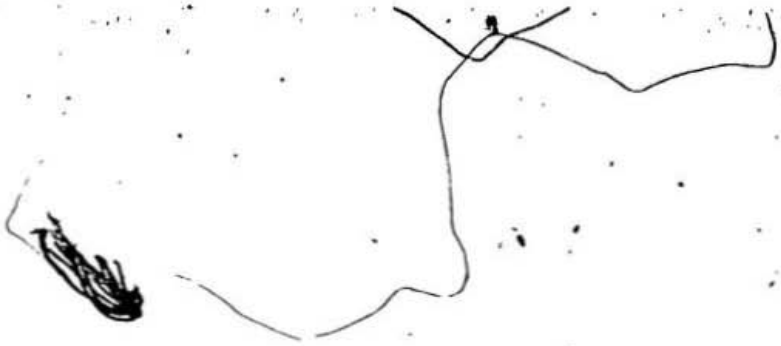
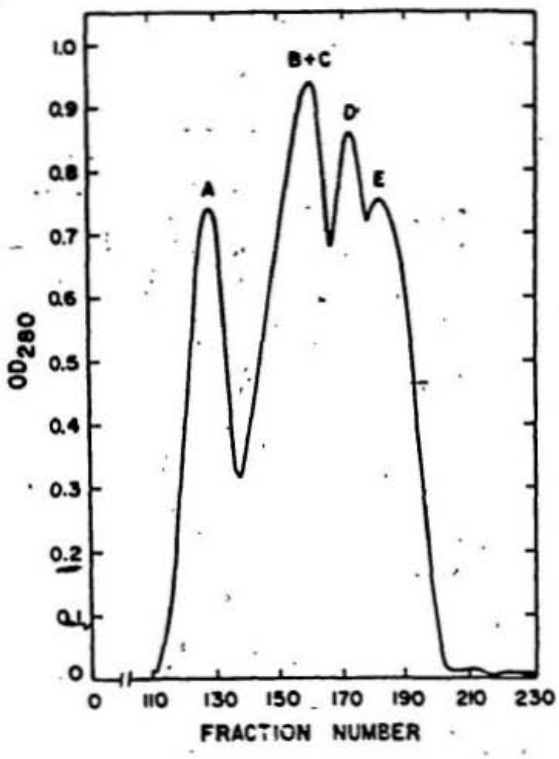
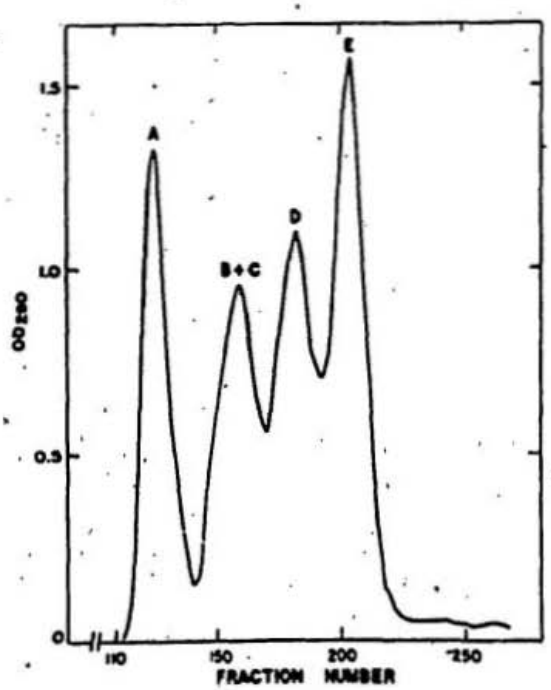


Figure 4. Chromatography of plasma from female flounder collected during October. A 1.8 ml equivalent of plasma was chromatographed on a 2.6 x 86 cm Sephacryl S-300 Superfine column. Fractions of 1.4 ml were collected at a flow rate of 15 ml/hr and protein was monitored by OD_{280}.

Figure 5. Chromatography of plasma from female flounder collected during February. A plasma equivalent of 2.0 ml was chromatographed on a 2.6 x 86 cm Sephacryl S-300 Superfine column. Fractions of 1.4 ml were collected at a flow rate of 15 ml/hr and protein was monitored at OD_{280}.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD280
A
B+C
D
E
0
110
130
150
170
190
210
230
FRACTION NUMBER


1.5
1.0
0.5
0
OD280
A
B+C
D
E
110
150
200
250
FRACTION NUMBER

(b) Samples from male flounders

Peak A was a very major peak reaching an optical density of 3.0 in the sample collected during March. Peak E was the next major peak. Female equivalent of Peak B + C was almost undetectable (Fig. 6).

In the sample collected during early June (early gametogenesis), Peak A was a very minor peak of optical density < 0.4. Peak B + C was also a very minor peak. However, Peak E was a major peak followed by Peak D (Fig. 7).

Polyacrylamide gel analyses of the plasma proteins

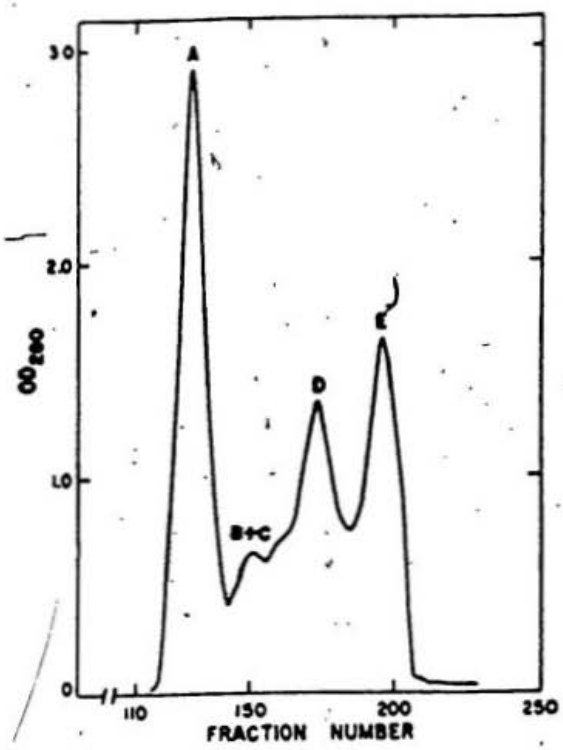
PAGE

Peak A protein(s) had a major band which did not move beyond 0.7-0.8 cm under non-denaturing conditions in a 5% gel. This major band stained with Sudan black, indicating the presence of lipids (Fig. 8). However, there was another band of Rf 0.17-0.18. The proportion of this band seemed to be higher in the fractions from the right part of the peak. The question I addressed here was whether the major band in Peak A protein(s) constitutes one single protein or more than one protein. An attempt to separate any additional proteins migrating together was done by performing PAGE for a longer duration; this indicated no additional bands from the major band, however, the minor band separated into three closely moving components.

The second peak, B + C from female plasma, had several minor bands and a major Sudan black stainable band of Rf 0.38. Peak B + C in males

Figure 6. Chromatography of plasma collected from male flounder during March. A plasma equivalent of 2.0 ml was chromatographed on a 2.6 x 86 cm Sephacryl S-300 Superfine column. Fractions of 1.4 ml were collected at a flow rate of 15 ml/hr and protein was monitored by OD_{280}.

Figure 7. Chromatography of plasma collected from post spawned male flounder (June). An equivalent of 2.0 ml plasma was chromatographed on a 2.6 x 86 cm Sephacryl S-300 column. Fractions of 1.4 ml were collected at a flow rate of 15 ml/hr and protein was monitored by OD_{280}.


A
B+C
D
E
OD_{280}
3.0
2.0
1.0
0
110
150
200
250
FRACTION NUMBER

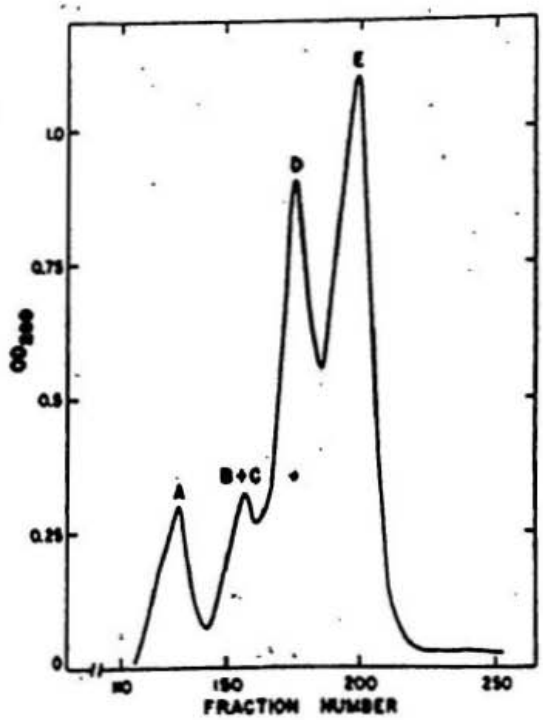
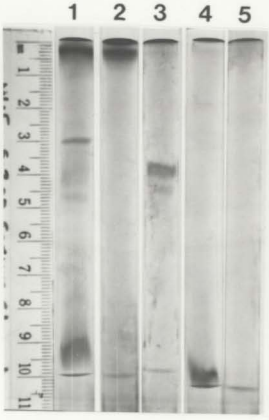

A
B+C
D
E
OD_{280}
1.0
0.75
0.5
0.25
0
110
150
200
250
FRACTION NUMBER

Figure 8. Non-denaturing gel analysis (5% disc gel) of the plasma protein peaks from Sephacryl S-300 chromatography illustrates lipoproteins stained with Sudan black. Lane 1 contains 10 µl flounder plasma, lanes 2 to 5 represent the peak tubes from the four peaks: Peak A, Peak B + C (vitellogenin), Peak D and Peak E respectively.


1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11

gave a band of Rf 0.30.

Peak D from males and females had a very fast moving band of Rf 0.93 along with few minor bands. Peak E in both sexes had several bands between Rf 0.50 to 0.62 and 0.85 to 0.90, and all the bands were more or less of the same intensity. The band pattern did not differ between male and female flounders very much (Fig. 9).

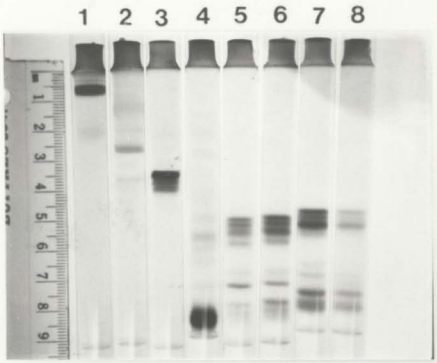
The band patterns obtained for these peaks were very consistent for samples from every Sephacryl S-300 chromatograms.

SDS-PAGE

Peak A protein(s) preparation on a SDS-polyacrylamide gel (10%) migrated as two major polypeptides of molecular weights 70 K and 28 K. However, the 28 K fragment did not stain very well with Coomassie blue (Fig. 10). There were a few minor bands associated with these polypeptides. SDS-PAGE of the slice having highest radioactivity (which represents the major band), cut from a non-denaturing disc gel, gave two fragments of MW 70 K and 28 K; the other minor fragments were absent (Fig. 11). This indicated that these two polypeptides were from a single protein or from more than one protein having same mobility under nondenaturing conditions.

Band patterns for radiolabelled Peak A protein(s) and vitellogenin on a 10% gel were similar to the above results. However, vitellogenin upon radiolabelling, gave more fragments when compared to non- radiolabelled protein.

Figure 9. Comparison of the band patterns on 5% polyacrylamide gel electrophoresis (disc gel) under non-denaturing conditions for female (top) and male (bottom) plasma Sephacryl S-300 eluants. Female: Lane 1 contains sample from the peak tube of Peak A, lane 2 represents the sample between Peak A and Peak B+C at the trailing region, lane 3 represents the peak tube of Peak B+C, lane 4 contains Peak D peak tube and lanes 5 to 8 contain samples from different locations on Peak E, lane 7 being the peak tube. Male: Lane 1 represents the tube between the Peak A and the Peak B+C at the trailing region, lanes 2 to 6 contain the samples from Peak A at different locations on the peak, lanes 7 and 8 represent the two samples from Peak B+C, lane 8 being the peak tube. Lanes 9 and 10 represents Peak D, lane 10 being the peak tube. Lanes 11 and 12 contain Peak E samples.


1 2 3 4 5 6 7 8

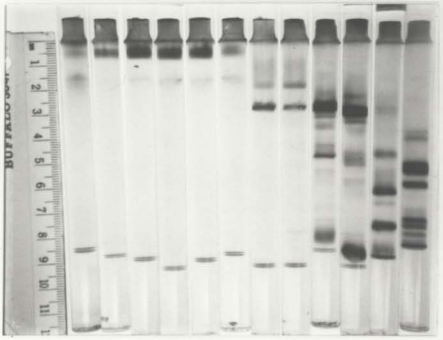

1 2 3 4 5 6 7 8 9 10 11 12

Figure 10. Polyacrylamide gel (10%) analysis (SDS-PAGE) of Peak A protein(s) and vitellogenin. Lane 1 represents 30 µg Peak A protein(s), lane 2 contains 20 µg vitellogenin and lane 3 represents 2 µl of the ovarian extract. Lane 4 contains about 20 µg of the molecular weight markers, RNA polymerase II, actin, and histones. The molecular weight x 10^{-3} is shown on the right.

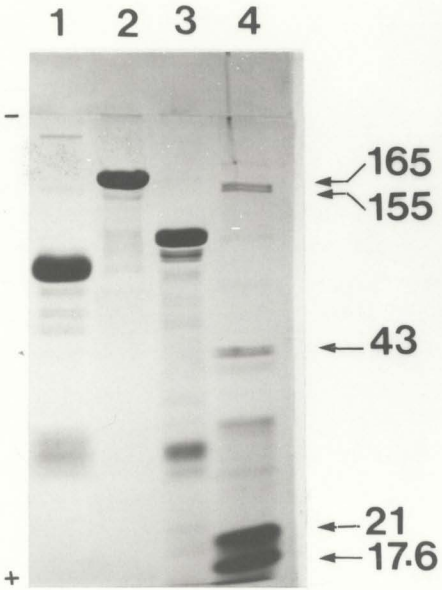
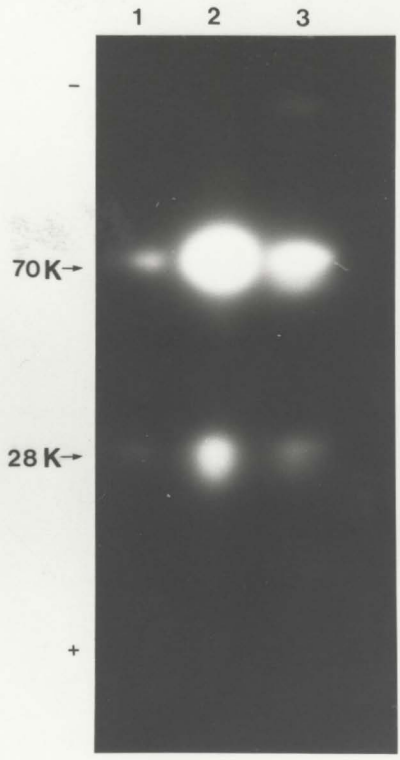

1
2
3
4
−
+
165
155
43
21
17.6

Figure 11. Polyacrylamide gel (10%) analysis (SDS-PAGE) of Peak A protein(s) major band from a 5% non-denaturing tube gel. Labelled Peak A protein(s) was first electrophoresed on tube gels and the slices representing the peak radioactivity were re-electrophoresed on a SDS gel as described under Materials and Methods. Lane 1 contains 20,000 cpm, lane 2 contains 150,000 cpm and lane 3 contains 100,000 cpm. Autoradiography was done at -70 C for 40 hr using Kodak XAR-5 film.


1
2
3
-
70K→
28K→
+

Labelling of the proteins

All three of the proteins were labelled successfully with ^{131}I by the Iodogen method, with a high recovery of 62.3 ± 3.2% (n = 12) for Peak A protein(s), 51 ± 0.9% (n = 6) for vitellogenin and 60.1 ± 2.2% (n = 5) for Peak E protein(s). Peak A protein(s) labelled very well with ^{125}I by the Iodogen method with a recovery of 53%. Also, the incorporation of the radioactivity to the protein was greater when compared to ^{131}I labelling.

PAGE (5%) analysis of radioiodinated Peak A protein(s)

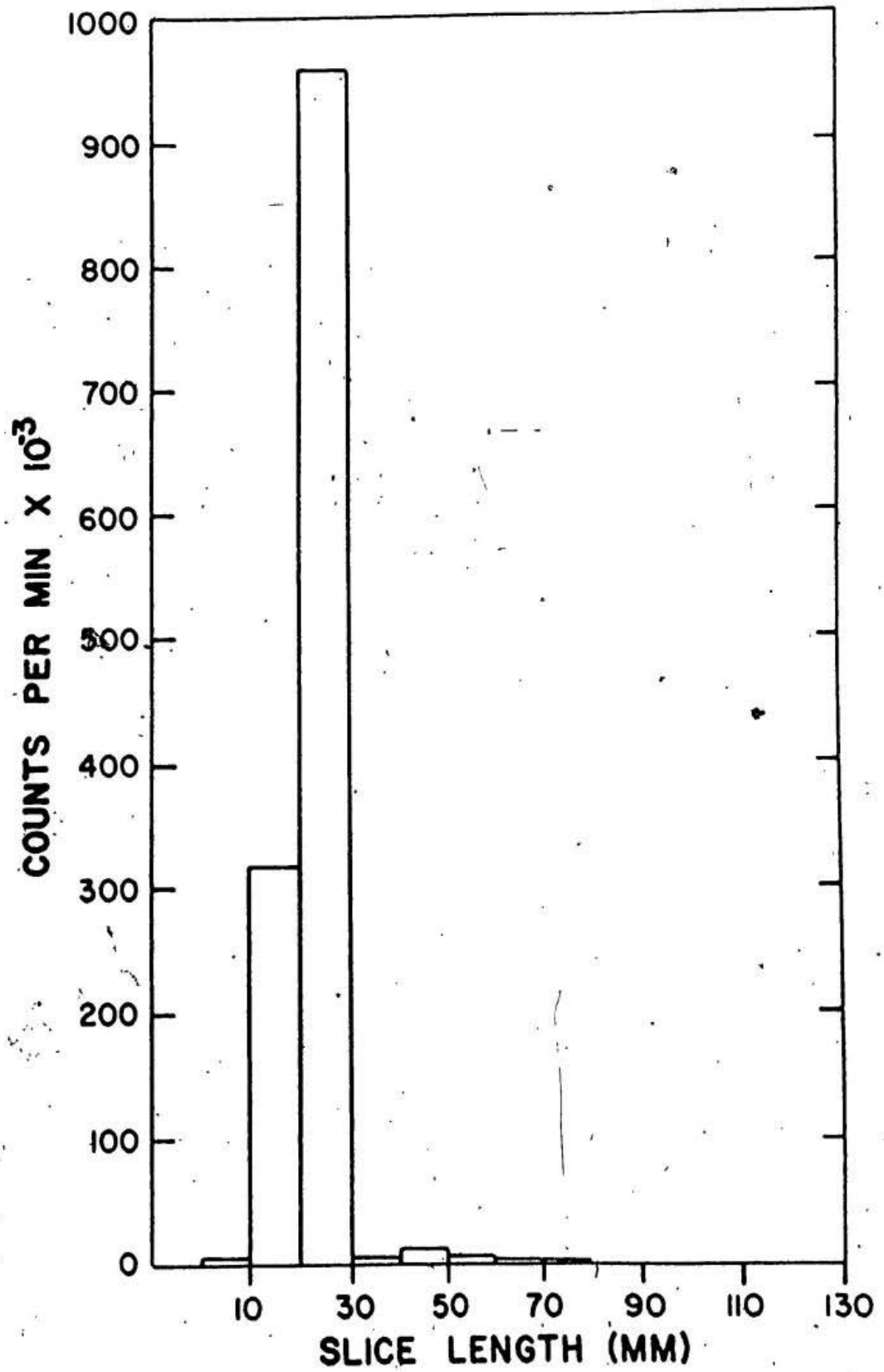
PAGE (5%) analysis of Peak A protein(s), performed in order to find out the specific radioactivity contributed by the minor contaminant, indicated that the counts concentrated entirely in the major band, excluding any possibility of contribution by the minor contaminating protein to the total counts (Fig. 12). A similar result was obtained with ^{125}I labelled Peak A protein(s).

HPLC-GEL FILTRATION OF THE PLASMA PROTEINS

Vg buffer

When Vg buffer was chromatographed on a Bio-Gel TSK 50 G 5000 PW column connected to a Bio-Sil TSK guard column, a large, broad peak of RT 13.45 ± 0.03 min was resolved. However, when individual components of Vg buffer, 10 mM EDTA and 0.025% PMSF were chromatographed separa-

Figure 12. Histogram of the radioactivity from a non-denaturing gel (disc gel, 5% PAGE) of ^{131}I labelled Peak A protein(s). The gel was sliced manually into 5 mm pieces beginning at the boundary between the stacking gel and the separating gel and the radioactivity was counted in a gamma counter.


1000
900
800
700
600
500
400
300
200
100
0
COUNTS PER MIN X 10^3
10
30
50
70
90
110
130
SLICE LENGTH (MM)

tely, a single broad peak of RT 13.47 min was resolved for EDTA (Fig. 13) and three smaller peaks were resolved for PMSF with RT 12.68, 13.46 and 14.78 min, respectively, which were otherwise hidden within the EDTA peak (Fig. 14).

Gel filtration of Peak A protein(s)

When Peak A protein(s) was chromatographed on a Bio-Gel TSK-50 G 5000 PW column connected to a Bio-Sil TSK guard column, a very symmetrical peak was resolved with a RT 8.83 (RT 8.87 ± 0.04 min), along with buffer peaks of RT 12.73 min and RT 13.43 min (Fig. 15). There were variations in these peaks in terms of percentage area which depended on the extent of ultrafiltration of the sample.

The peak A protein(s) sample, selected to be rich in the contaminating protein, also gave similar results. There was no additional peak which would correspond to the second band on a 5% gel. Also, percentage of recovery was very poor (<10%) indicating adsorption to the column. When a Bio-Gel guard column was used with a Bio-Gel main column, the buffer peaks came out immediately after the protein peak.

When the pH of the elution buffer was lowered to below 6.0, there was no elution of the proteins from the column, even when 1 mg of protein in 100 µL was chromatographed. The same result was observed when the elution buffer was changed to 36% acetonitrile in the presence or absence of TFA.

In a combination of Bio-Sil TSK-250 and Bio-Gel TSK-50 columns, Peak A protein(s) resolved with a RT 14.43 min (Fig. 17). The Bio-Sil gel matrix seemed to retain EDTA and PMSF much longer in the column

Figure 13. HPLC gel filtration of 80 µl of 10 mM EDTA solution. HPLC column system used: Bio-Sil TSK guard column + Bio-Gel TSK-50 G 5000 PW main column. Sensitivity: 0.01 AUFS. Elution buffer: 0.1M Na_2SO_4 + NaH_2PO_4, pH 6.8; Flow rate 1 ml/min. Detection at 280 nm.

Figure 14. HPLC gel filtration of 80 µl of 0.025% PMSF solution. HPLC column system and other conditions were as above.

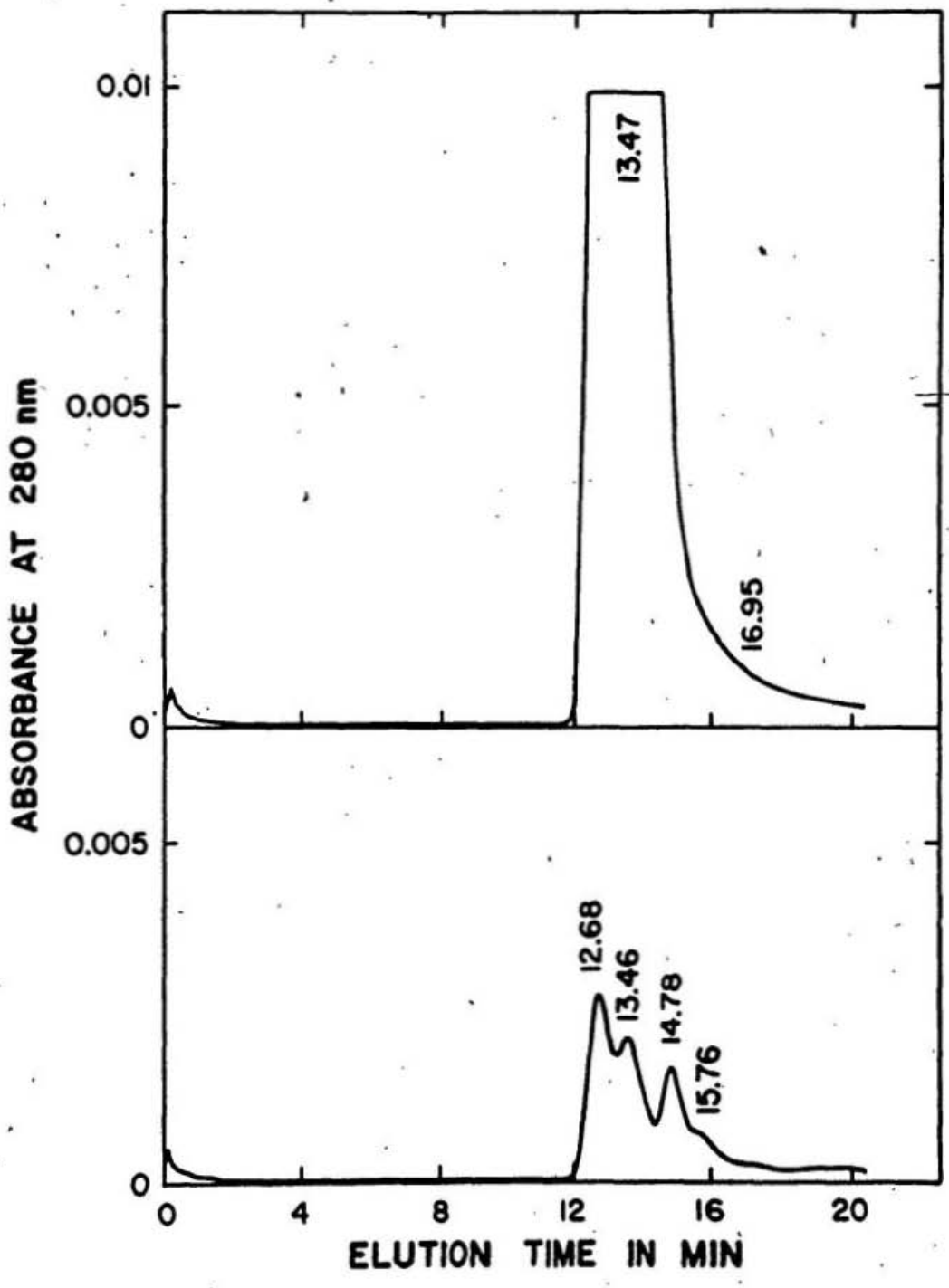

0.01
0.005
0
13.47
16.95
ABSORBANCE AT 280 nm
0.005
0
12.68
13.46
14.78
15.76
0
4
8
12
16
20
ELUTION TIME IN MIN

Figure 15. HPLC gel filtration of 170 µg of Peak A protein(s) prepared on a Sephacryl S-300 column. HPLC column system used: Bio-Sil TSK guard column + Bio-Gel TSK-50 G 5000 PW main column. Sensitivity: 0.01 AUFS. Elution buffer: 0.1M Na_2SO_4 + 0.02M NaH_2PO_4, pH 6.8; Flow rate 1 ml/min. Detection at 280 nm.

Figure 16. HPLC gel filtration of 75 µg of Peak A protein(s) RT 8.83 min peak from the above gel filtration. RT 8.83 min peak from several chromatograms were pooled, concentrated and chromatographed again on the above system under identical conditions.

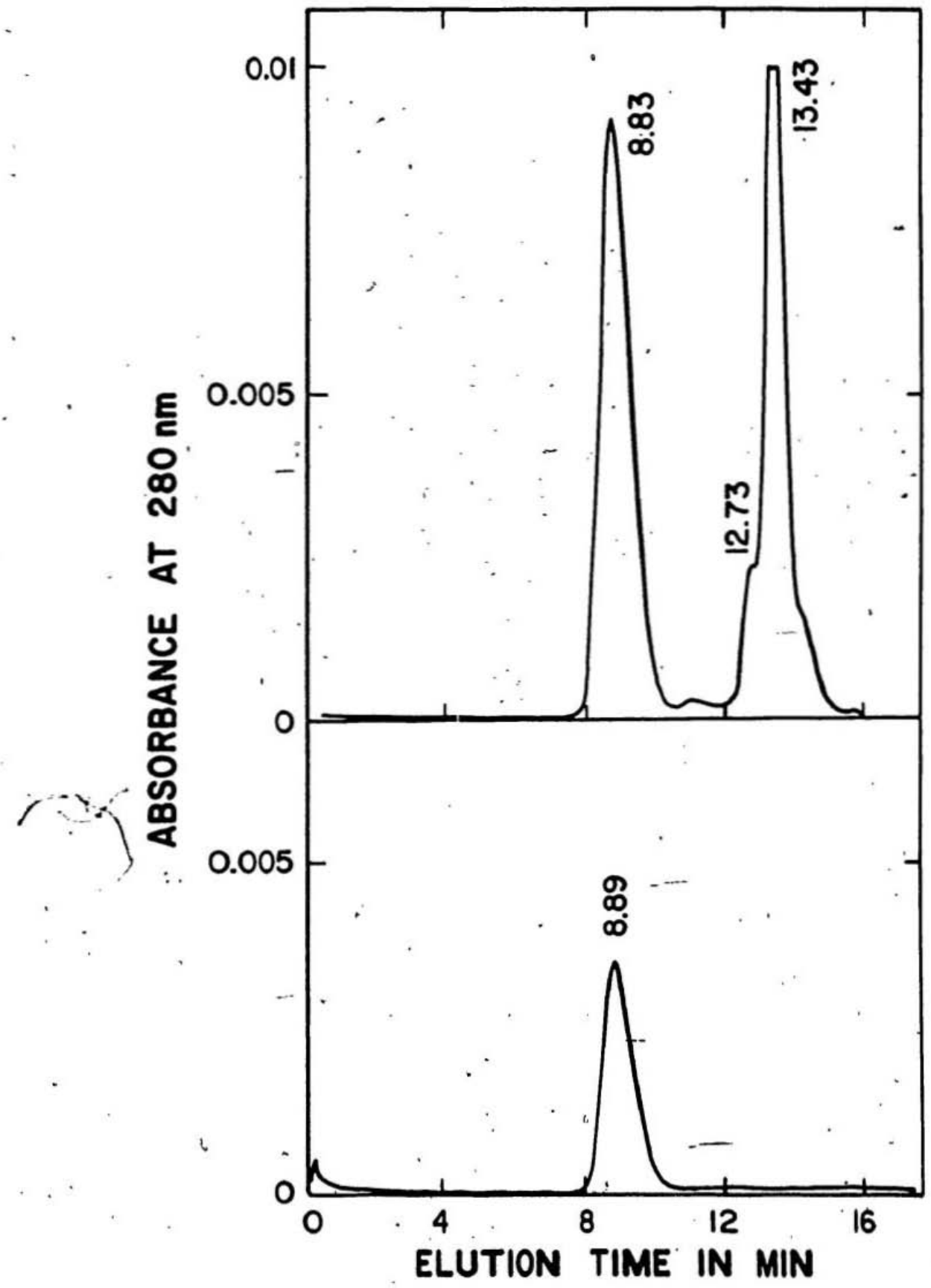
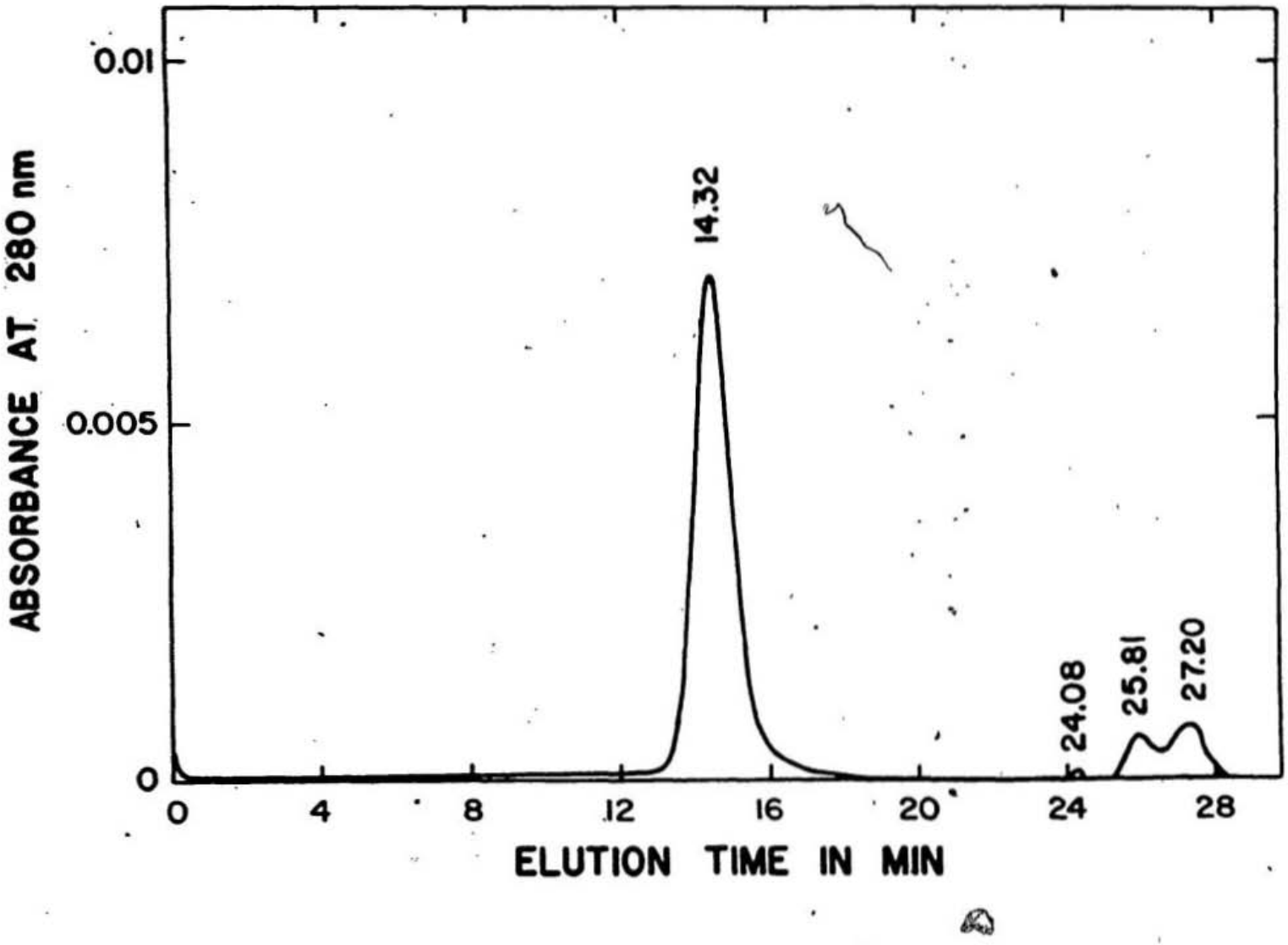

0.01
0.005
0
0.005
0
8.83
12.73
13.43
8.89
ABSORBANCE AT 280 nm
0
4
8
12
16
ELUTION TIME IN MIN

Figure 17. HPLC gel filtration of 0.8 mg of Peak A protein(s) prepared on a Sephacryl S-300 column. HPLC column system used: Bio-Sil TSK guard column + Bio-Gel TSK-50 G 5000 PW main column + Bio-Sil TSK-250 G 3000 SW main column. Sensitivity: 0.01 AUFS. Elution buffer: 0.1M Na_2SO_4 + NaH_2PO_4, pH 6.8; Flow rate 1 ml/min. Detection at 280 nm.


ABSORBANCE AT 280 nm
0.01
0.005
0
14.32
24.08
25.81
27.20
0
4
8
12
16
20
24
28
ELUTION TIME IN MIN

when compared to Bio-Gel.

When the pooled RT 8.87 ± 0.04 min peak was re-chromatographed on a Bio-Gel TSK-50 with Bio-Sil TSK guard column, a single peak with RT 8.83 min was resolved (Fig. 16).

Gel filtration of vitellogenin

Gel filtration of vitellogenin in a Bio-Gel TSK-50 column with a Bio-Sil TSK guard column resolved a symmetrical peak with RT 10.29 min along with the buffer peaks (Fig. 19). The recovery was very low, similar to Peak A protein(s), indicating adsorption to the column.

Gel filtration of Peak E protein(s)

Gel filtration of Peak E protein(s) on a Bio-Sil TSK-250 column connected to a Bio-Sil TSK guard column yielded a symmetrical peak with RT 10.23 ± 0.04 min, followed by the buffer peaks of RT 14.01 and 15.95 min. Peak E protein(s) in a Bio-Gel TSK-50 column came out with an RT 11.12 min (Fig. 18). The recovery was low but a little higher than Peak A protein(s) and vitellogenin.

PAGE (5%) performed on pooled peaks from Peak A protein(s) and Peak E protein(s) chromatograms gave a single band for RT 8.83 min peak from Peak A protein(s) (Fig. 20) and four bands for RT 11.04 or 10.23 from Peak E protein(s). The remaining peaks from Peak A protein(s) or Peak E protein(s) elution did not show any bands confirming that no proteins are eluted with the buffer peaks. Protein determination also did not indicate any proteins in these peaks.

Figure 18. HPLC gel filtration of 160 µg of Peak A protein(s) and Peak E protein(s) together prepared on a Sephacryl S-300 column. HPLC column system used: Bio-Sil TSK guard column + Bio-Gel TSK-50 G 5000 PW main column. Sensitivity: 0.01 AUFS. Elution buffer: 0.1M Na_2SO_4 + 0.02M NaH_2PO_4, Ph 6.8; Flow rate 1 ml/min. Detection at 280 nm.

Figure 19. HPLC gel filtration of 60 µg of flounder vitellogenin prepared on a Sephacryl S-300 column. HPLC column system and conditions of chromatography were as above.

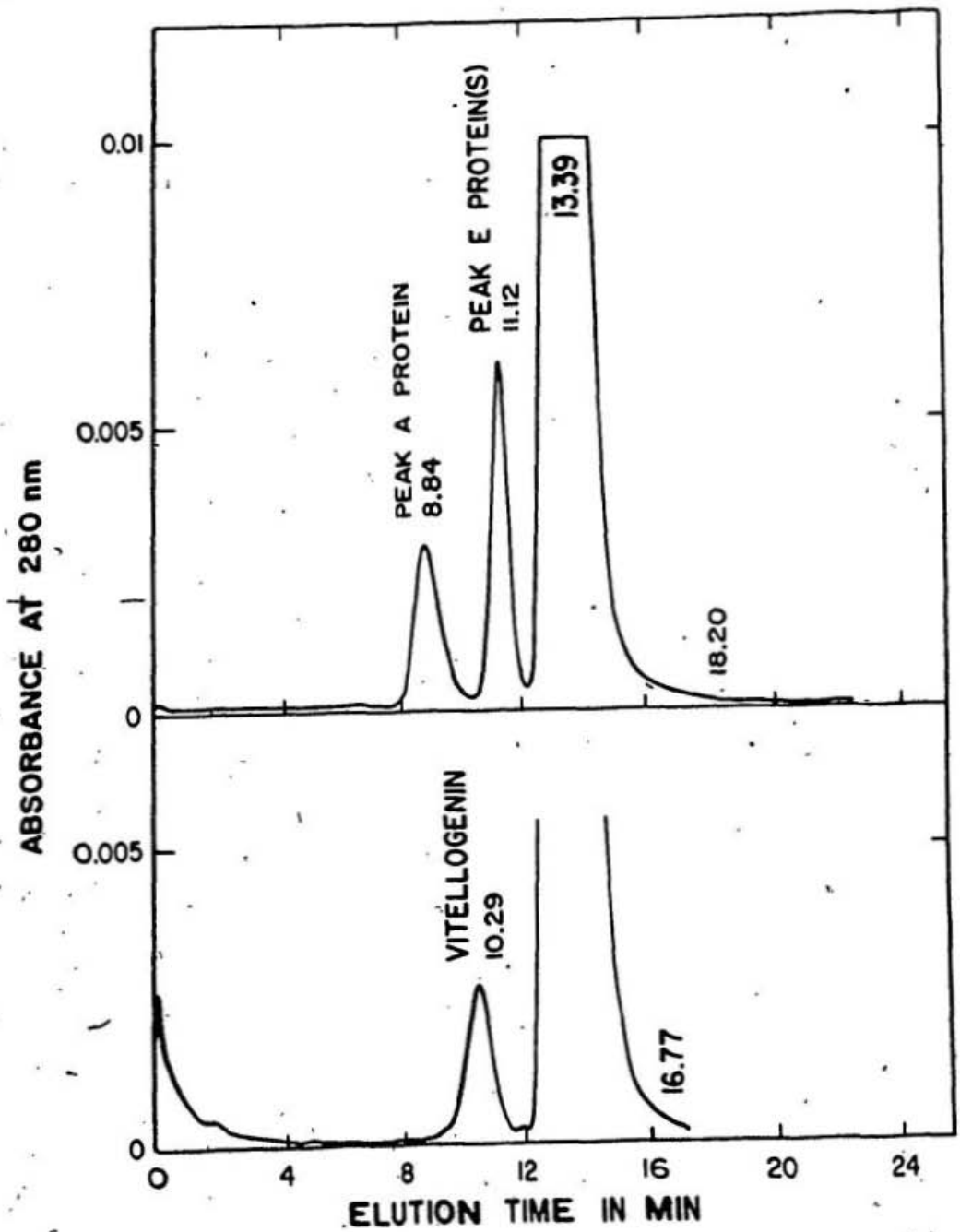
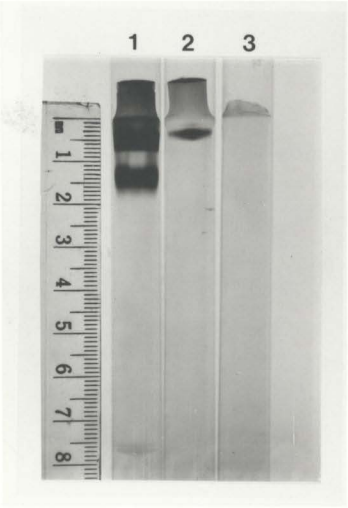

ABSORBANCE AT 280 nm
0.01
0.005
0
PEAK A PROTEIN
8.84
PEAK E PROTEIN(S)
11.12
13.39
18.20
0.005
0
VITELLOGENIN
10.29
16.77
0
4
8
12
16
20
24
ELUTION TIME IN MIN

Figure 20. Non-denaturing polyacrylamide gel analysis (5% disc gel) of the samples from HPLC gel filtration of Peak A protein(s). Lane 1 represents the original Peak A protein(s) (100 µg) used for the HPLC gel filtration. Lane 2 contains 20 µg of RT 8.83 min peak from HPLC and lane 3 represents the pooled, concentrated peaks of RT 12.47 and 13.46 min. The gels were stained with Coomassie blue R-250.


1
2
3
1
2
3
4
5
6
7
8

Molecular weight determination of Peak A protein(s), vitellogenin and Peak E protein(s) by HPLC gel filtration procedure

By comparing the RT of Peak A protein(s) and vitellogenin and Peak E protein(s) to the standards, the molecular weights were determined. Peak A protein(s) molecular weight was found to be 1000 K, vitellogenin being 450 K and Peak E protein(s) had a molecular weight of 200 K (Fig. 21). These values represent the molecular weights of the proteins in their native forms.

Con A-Sepharose affinity chromatography of Peak A protein(s)

To obtain evidence on the glycosylation of Peak A protein(s), the preparation from HPLC was re-chromatographed. Peak A protein(s) was adsorbed by Con-A Sepharose which indicated this protein(s) is a glycoprotein (Fig. not shown).

Checking Peak A protein(s) antiserum titer and isolation of Peak A protein(s) IgG

Antiserum raised in New Zealand white male rabbits against Peak A protein(s) gave an antibody titer value of 47% binding in three months at a dilution of 1:100,000.

When rabbit anti-Peak A protein(s) serum was chromatographed on a protein A Agarose column, the fraction eluted with 0.1 M glycine-HCl (pH 3) contained the specific IgG as shown by its binding capacity to radioiodinated Peak A protein(s) (Fig. 22). The total protein in the

Figure 21. Molecular weight determination of Peak A protein(s), vitellogenin and Peak E protein(s) by Bio-Gel TSK-50 HPLC gel filtration chromatography as described under Materials and Methods. The retention time (RT) for the standards and for the samples were determined from several chromatograms and the mean values were plotted against the molecular weights.

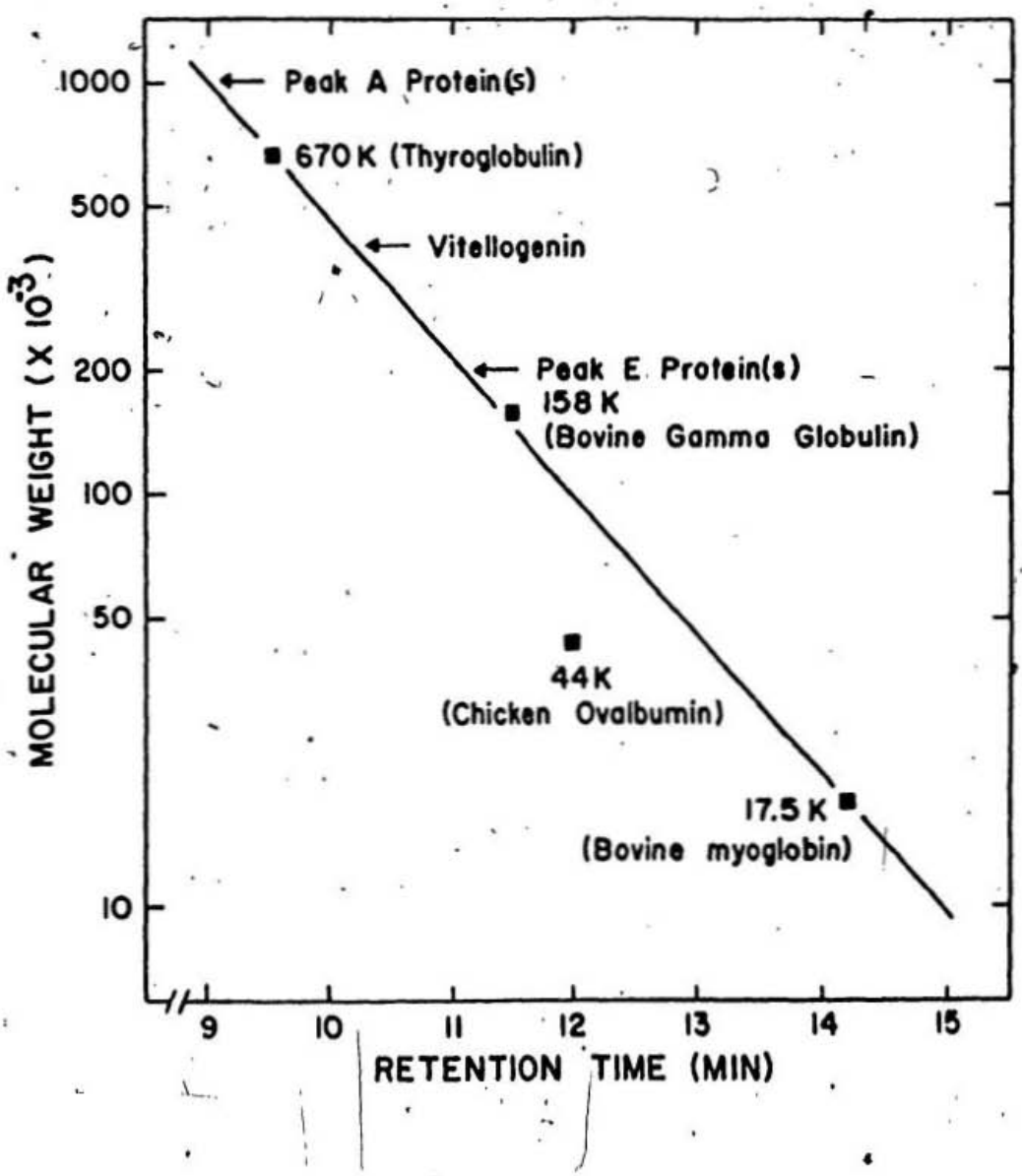
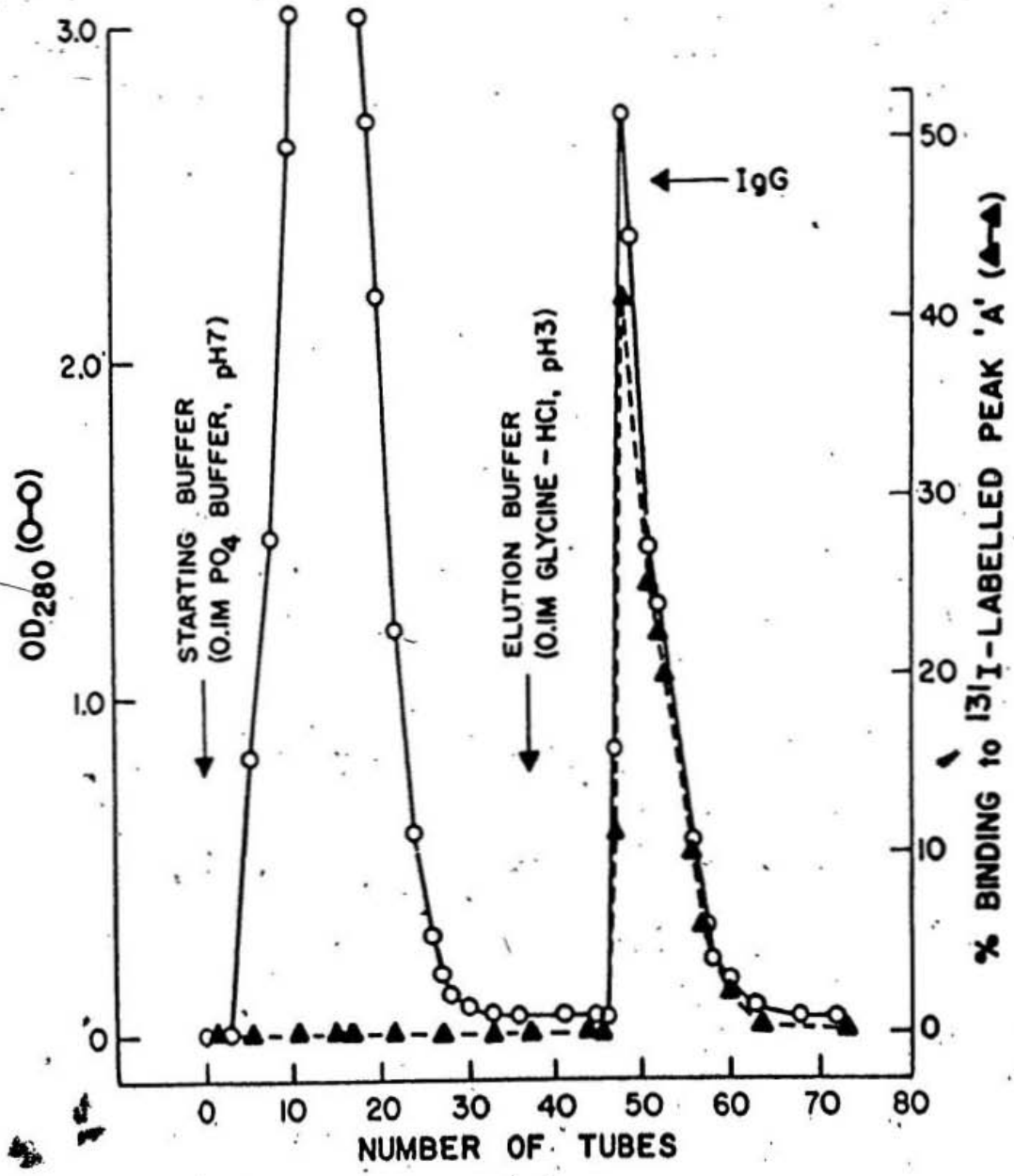

Peak A Protein(s)
670 K (Thyroglobulin)
Vitellogenin
Peak E Protein(s)
158 K
(Bovine Gamma Globulin)
44 K
(Chicken Ovalbumin)
17.5 K
(Bovine myoglobin)
MOLECULAR WEIGHT (X 10^-3)
1000
500
200
100
50
10
9
10
11
12
13
14
15
RETENTION TIME (MIN)

Figure 22. Affinity chromatography for the isolation of Peak A protein(s) IgG from 1 ml of rabbit antiserum against flounder plasma Peak A protein(s) on a protein A Agarose column. Fractions were collected at 1.5 ml/tube. An aliquot of each fraction was diluted 100,000-fold for checking the binding capacity to ^{131}I labelled Peak A protein(s) as described under Materials and Methods.


3.0
2.0
1.0
0
OD280 (O—O)
STARTING BUFFER
(0.1M PO4 BUFFER, pH7)
ELUTION BUFFER
(0.1M GLYCINE – HCl, pH3)
IgG
50
40
30
20
10
0
% BINDING to 131I-LABELLED PEAK 'A' (▲—▲)
0
10
20
30
40
50
60
70
80
NUMBER OF TUBES

IgG fraction was 5.2 mg from one mL antiserum.

Cross reactivity of Peak A protein(s) antibody with the other plasma proteins vitellogenin, Peak D and Peak E

Other plasma proteins, vitellogenin, Peak D and Peak E did not displace the binding of Peak A protein(s) to Peak A protein(s) IgG in a competitive radioimmunoassay performed as described in Materials and Methods. On the other hand, Peak A protein(s) displaced the binding of radiolabelled Peak A protein(s) to IgG, even at a dilution of 100,000X, when compared to binding in the tubes where only radiolabelled Peak A protein(s) was incubated with the IgG. Blank counts were only 3%.

Peptide mapping of Peak A protein(s) and vitellogenin

To obtain additional evidence for structural unrelatedness between Peak A protein(s) and vitellogenin, one dimensional peptide maps were compared. Peptide maps generated by partial proteolysis with Staphylococcus aureas V8 protease enzyme were entirely different for Peak A protein from vitellogenin. Upon analysis of the digest by SDS-PAGE (15%) the 74 K - 70 K fragment of Peak A protein was completely digested at an enzyme concentration of 15 ng/100 µg of the sample. However, the 28 K fragment was intact even at a concentration of 30 ng/100 µg. There were several fragments of molecular weights less than 28 K. Proteolytic digestion of vitellogenin produced numerous fragments, no single fragment being prominent. The major fragment of MW 180-200 K was totally broken down into minor fragments and none of the

peptides co-migrated with the peptides from Peak A protein(s) (Fig. 23).

Peptide mapping of radioiodinated Peak A protein(s) major band performed as described in Materials and Methods gave similar results. The 28 K fragment was intact (Fig. 24).

Amino acid and amino sugar analyses

The amino acid and amino sugar compositions of Peak A protein(s) from male and female flounder are listed in Table 1.

Discussion

Isolation procedures

Several techniques such as gel filtration (Rudel et al., 1974), affinity chromatography (Wicham, 1979), selective precipitation with polyanion/divalent cation reagents (Burnstein et al., 1970) are currently used in the clinical and investigative laboratory for separation and quantification of human serum lipoproteins, which can also be used to isolate fish lipoproteins. The isolation of plasma proteins from fish has been done using DEAE-cellulose chromatography and TEAE cellulose chromatography (deVlaming et al., 1980). Gel filtration procedure was used in this investigation to isolate plasma proteins, because this is a very gentle method of separating proteins on the basis of their molecular weights.

Figure 23. One-dimensional V8-generated peptide maps of Peak A proteins(s) and vitellogenin. Samples of 10 µg, 20 µg and 30 µg Peak A protein(s) (lanes 1, 2 and 3) and 10 µg and 20 µg vitellogenin (lanes 4 and 5) were digested each with 0.5 µg V8 enzyme. The digests were separated in the presence of SDS on a 15% polyacrylamide gel. Lane 6 contains the molecular weight markers (Pharmacia). The gel was stained with Coomassie blue R-250.

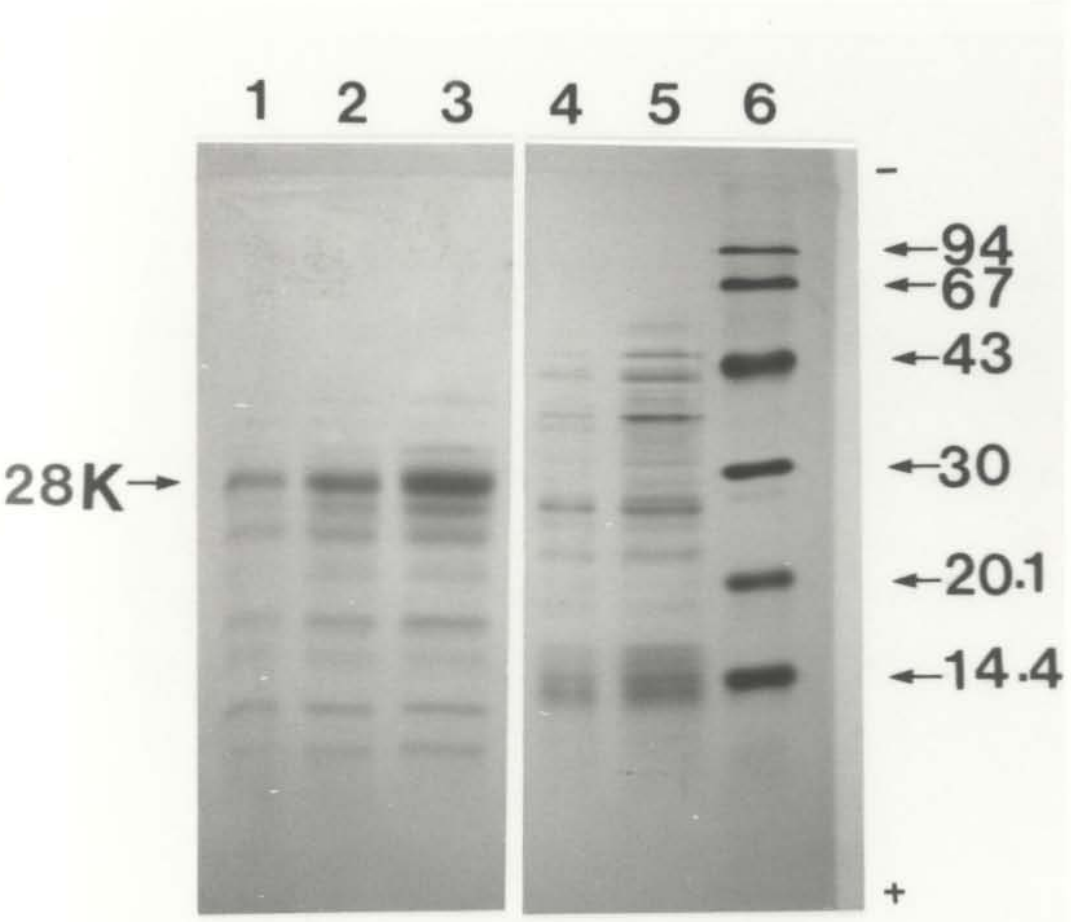

1
2
3
4
5
6
28K
-
94
67
43
30
20.1
14.4
+

Figure 24. One-dimensional V8-generated peptide map of Peak A protein(s). Lane 1 represents the labelled Peak A protein(s). Two 10 µl of labelled Peak A protein(s) were digested with 6 ng- and 3 ng V8 protease (lanes 2 and 3) and the digests were separated in the presence of SDS on a 10% polyacrylamide gel. Lanes 4 and 5 represent V8 digestion on the gel. Labelled Peak A protein(s) was first electrophoresed under non-denaturing conditions on a 5% disc gel and the gel slice containing the peak radioactivity was introduced to a second SDS gel in the presence of V8 enzyme as described under Materials and Methods. Lane 4 represents digestion with 3 ng and lane 5 represents digestion with 0.5 ng. The slice digested in lane 5 had lower counts than the slice in lane 4. Autoradiography was done overnight at -70 C using Kodak XAR-5 film.


1
2
3
4
5
–
70 K→
28 K→
+

TABLE 1. COMPARISON OF AMINO ACID AND AMINO SUGAR COMPOSITION OF PEAK A PROTEIN(S) FROM MALE AND FEMALE WINTER FLOUNDER

Amino acid	Mole %	
	Male	Female
Alanine	6.8	6.1
Arginine	1.8	2.2
Aspartic acid	11.6	10.9
Glutamic acid	10.2	10.5
Glycine	11.2	11.0
Histidine	1.8	1.8
Isoleucine	1.7	2.1
Leucine	7.2	7.3
Lysine	6.6	6.5
Phenylalanine	3.3	3.3
Proline	6.9	7.0
Serine	13.7	13.1
Threonine	8.7	9.4
Tyrosine	4.4	4.3
Valine	4.1	4.5
Amino sugars		
Galactosamine	2.3	2.7
Glucosamine	97.7	97.3

Variation in the Sephacryl S-300 chromatography elution profiles of flounder plasma collected during different times of the year

In females, the observation of high levels of Peak B + C during October was in agreement with the other studies on many teleosts (So et al., 1984), coinciding with the ovarian uptake pattern. A high level of Peak A protein(s) during February, and a low level of vitellogenin, was also in agreement with the vitellogenin uptake pattern (Chapter IV). In males, the two elution profiles showed a very distinct difference with respect to Peak A protein(s) levels in the plasma. A very low level in post spawned fish, but a high level during March, indicated that this protein could be involved in testicular growth also.

Analyses of the plasma proteins

As indicated by the results from PAGE and SDS-PAGE, Peak A protein(s) preparation from Sephacryl S-300 gel filtration was not entirely homogeneous. The first question was whether the minor band (Rf. 0.17-0.18) observed in PAGE, was a part of Peak A protein(s), a contaminating protein or was a proteolytic fragment from the main protein. The electrophoretic mapping of fractions across the peak showed an increased concentration of this band towards the right trailing edge of the peak. In addition, the use of protease inhibitor in blood collection and isolation buffers made it likely that this minor band was another protein species. It could not be part of major protein species in the Peak A protein(s) preparation, because its

concentration was not the same across the peak.

The next question I addressed was whether the major band in Peak A protein(s) preparation constitutes one protein or more than one protein. The results from SDS-PAGE analysis of the major band suggested two possibilities. 1) Both the polypeptides, the 70 K and the 28 K are from a single protein; 2) If the 28 K polypeptide is a separate protein, it most assuredly becomes tightly associated with the 70 K polypeptide. Either of the polypeptides was not separated by PAGE performed for a longer duration, which however, separated the other protein band (Rf 0.17-0.18) into three components; electrophoresis for a shorter duration also did not reveal any additional bands associated with the major band. Re-chromatography of Peak A protein(s) on Concanavalin A-Sepharose column indicated that the protein which represents the major band in a 5% PAGE, was held by Con A-Sepharose and hence a glycoprotein. On the other hand, the minor contaminants did not exhibit any affinity to Con A-Sepharose and were eluted. The procedure, however, did not provide any evidence on the homogeneity of the major [redacted] Due to very high molecular weight of Peak A protein(s) (major band [redacted] isoelectric focusing on a lowest possible acrylamide concentration and cross linkage (5% acrylamide, 2.6% cross linkage) followed by 2-dimensional gel electrophoresis was not successful.

Analysis of Peak A protein(s) preparation labelled with ^{131}I indicated that the radioactivity was entirely represented by the 70 K and 28 K polypeptides. Hence the ovarian uptake of labelled Peak A protein(s) (Chapter III and IV) represents the uptake of these two polypeptides. Also, the relationship to ovarian proteins was established for both the polypeptide fragments (Chapter III). Hence

their identity as one protein or two separate proteins, and the minor contaminants in Peak A protein(s) preparation do not make much difference in the context of this study.

However, Peak E protein(s) preparation indicated a group of proteins of Rf ranging from 0.4 to 0.95. Hence, this protein(s) preparation was not studied in greater detail at this time.

Vitellogenin reportedly has two main polypeptide subunits of about 200 K each in Xenopus laevis (Bergink and Wallace, 1974; Clemens et al., 1975; Berridge et al., 1976). Two polypeptide subunits of 176 and 127 K have been reported for Atlantic salmon vitellogenin (So et al., 1984), estimated under denaturing conditions. In this study, flounder vitellogenin gave a single fragment of approximately 180 K - 200 K and can be separated into two polypeptides of approximately 180 K and 190 K (data not shown). Also, it should be noted that Peak A protein(s) and vitellogenin did not yield any polypeptide fragments of the same size, which indicated their structural unrelatedness. Even if two polypeptides, 70 K and 28 K were to be considered individual proteins, all the three polypeptides appeared structurally different.

HPLC gel filtration studies on Peak A protein(s) indicated that the peak with RT 8.87 $\pm$ 0.04 min was from the major protein band of Peak A protein(s). This conclusion was based on following observations. The two later peaks of RT 12.68 min and 13.47 min were found to be contributed by EDTA and PMSF from Vg buffer. The possibility of any protein peaks within these peaks was ruled out based on the following evidence. Protein estimation by the Lowry method failed to detect any proteins in later peaks after pooling and concentration. Only the peak with RT 8.87 min indicated protein. Also, only this peak gave a band

corresponding to Peak A protein(s) on PAGE and SDS-PAGE. One more conclusion drawn from these results was that the contaminating protein, as well as the major protein in Peak A protein(s), was adsorbed to the gel matrix; however, the major protein was partially eluted but the recovery was very low (< 10%).

The low recovery of protein due to adsorption to the column limited the use of this procedure for isolating the protein. The recoveries were also low when vitellogenin and Peak E protein(s) were chromatographed. Vitellogenin, as well as Peak E protein(s), gave single peaks along with the buffer peaks. Surprisingly, Bio-Sil TSK-250 column which had a separation range of 1000-300,000 daltons, did not separate the different protein species in Peak E. This was confirmed by 5% PAGE, which indicated that all the bands in the original sample were present. Also, the use of combination of columns connected in series did not resolve any more protein peaks from Peak A protein(s) and Peak E protein(s).

The low recovery due to adsorption is not uncommon depending on the nature of the proteins in the gel filtration columns. An essential requirement for size-exclusion chromatography is that solute must have minimal attractive or repulsive interactions with the stationary phase. This is achieved when the stationary and mobile phases are of similar polarity (Kato et al., 1980; Mori, 1978). Under such conditions, partitioning occurs only between the solvent serving as the mobile phase and the solvent trapped inside the solid support as the stationary phase (Swergold and Rubin, 1983). Systems employing buffers containing high concentrations of salts or denaturing agent were used to minimize solute interactions with the stationary phase (Kato et al., 1980;

Rivier, 1980; Hashimoto et al., 1978; Hefti, 1982). In this study, the requirement for obtaining the proteins of interest under non-denaturing conditions limited the use of such modifications. In addition, these plasma proteins are lipid containing high molecular weight proteins; hence having both hydrophilic and hydrophobic groups. Considering the possibility of a hydrophobic interaction in an aqueous buffer system, the elution system was changed to 36% acetonitrile with or without 0.1% TFA, a system reported by Swergold and Rubin (1983). The complete adsorption of proteins to the column with this elution system confirmed an incompatible polarity.

Interestingly, Blewitt et al. (1985) had reported the effect of pH on the conformation of diptheria toxin and its implications on membrane penetration, based on HPLC Sepharogel-TSK 3000 SW column elution studies apart from electrophoresis, circular dichroism, and binding to non-ionic detergents. They had proposed that exposure to low pH triggers a rapid change in diptheria toxin conformation; the transition occurs over a narrow pH range and below the transition pH, buried tryptophans become exposed and the toxin becomes hydrophobic, favouring membrane penetration. Their findings were in agreement with several other studies (Sandvig and Olsnes, 1980, 1981; Blewit et al., 1984). These observations seem to apply to Peak A protein(s) as well for the following reasons:

1. The adsorption of the proteins onto the column and very low recovery when aqueous buffer system was used at pH 6.8.
2. Gradual decrease in recovery with the above buffer system when the pH was lowered from 6.8 to 6 and complete adsorption to the column at pH below 6.0.

3. Complete adsorption in a volatile buffer system (acetonitrile).

Based on the above observations, it is probable that a conformational change of Peak A protein(s) could occur before entry into oocytes by micropinocytosis. Also evidence obtained in this investigation regarding the role of the pituitary gland and the hormone involved in the uptake of both Peak A protein(s) and vitellogenin (Chapter IV) suggests a common mechanism of their ovarian uptake.

Molecular weight determination of Peak A protein(s), vitellogenin and Peak E protein(s) by HPLC gel filtration

The molecular weights determined by this procedure corresponded with the molecular weights determined by Sephacryl S-300 gel filtration reported by So et al. (1984). A molecular weight of 1,045,000 for Peak A protein(s) from Atlantic salmon was reported in its native form. Owing to its very high molecular weight, the sensitivity of Sephacryl S-300 and HPLC gel filtrations seemed to be poor. The molecular weight of 400 K for flounder vitellogenin, determined by HPLC gel filtration in this study was much lower than the reported value of 550 K (Emmersen and Petersen, 1976). The molecular weight was lower when compared to other species: namely Atlantic salmon, 495 K by gel filtration and 520 K by gradient gel electrophoresis (So et al., 1984); rainbow trout, 500 K (Sumpter, 1981), 600 K (Hara and Hirai, 1978), 450 K (Campbell and Idler, 1980); higher than coho salmon, 390 K (Markert and Vanstone, 1971), and goldfish, 326 K (Hori et al., 1979), 380 K (deVlaming et al., 1980). The molecular weight of 200 K for Peak E protein(s) was a little lower than the reported value for this protein(s) in Atlantic

salmon (220 K, So et al., 1984).

Isolation of Peak A protein(s) IgG

The high titer value obtained within three months indicated that this protein is highly antigenic. Peak A protein, being a glycoprotein (held by Con A-Sepharose) could be quite antigenic. The 70 K fragment of Peak A protein(s), which stained positive with periodic acid Schiff reagent, confirmed this observation and indicated that these fragments could be possessing antigenic determinants. This was later confirmed by immunoblotting (Western blotting) (Chapter III).

The protein A agarose affinity chromatography procedure (Bethesda) seemed to be well suited for the isolation of Peak A protein(s) IgGs from rabbit antiserum. The same procedure was used earlier to isolate IgG against vitellogenin (So et al., 1984).

Structural unrelatedness of Peak A protein(s) to vitellogenin

Besides confirming the specificity of Peak A protein(s) IgGs, the cross-reactivity of IgG was checked to determine whether Peak A protein(s) shares any properties with vitellogenin and other plasma proteins in antigenicity (antigenic determinants), and to confirm that the IgG could be used to detect Peak A protein(s) in yolk by immunoblotting and immunoprecipitation (in Chapter III). The absence of cross reactivity of Peak A protein(s) IgG with other proteins confirmed its high specificity and provided additional information that vitellogenin and Peak A protein(s) are structurally different and have

separate antigenic determinants.

To obtain further evidence to corroborate the structural unrelatedness of Peak A protein(s) and vitellogenin and to characterise the peptide structures, one-dimensional V8-generated peptide maps were compared. The peptide maps indicated that the peptides generated from Peak A protein(s) and vitellogenin have different electrophoretic mobilities. Considering these results it can be stated that Peak A protein(s) is structurally different from vitellogenin and is not a dimer of vitellogenin. At the same time it was quite interesting that the 28 K fragment of Peak A protein(s) remained undigested by V8 enzyme. S. aureus V8 protease enzyme cleaves at the COOH-terminal side of aspartic acid and glutamic acid residues (Houmard and Drapeau, 1972) and has proven to be eminently suitable for generating digests which contain many appropriately sized fragments (Cleveland et al., 1977). Apparently, the 28 K fragment did not possess any aspartic acid or glutamic acid residues. However, the 70 K fragment was completely digested into low molecular weight fragments. On the other hand, vitellogenin produced numerous fragments of molecular weights higher than the Peak A protein(s) fragments. This finding corroborated the results reported by Hickey and Wallace (1974) in that catfish, Ictalurus nebulosus, vitellogenin was composed of high molecular weight peptides but was highly susceptible to proteolysis. The implications of the results from peptide mapping of Peak A protein(s) and vitellogenin are discussed in relation to ovarian proteins (yolk synthesis) in Chapter III of this thesis.

Based on the results from PAGE, SDS-PAGE, HPLC gel filtration studies, immunological studies and peptide mapping, Peak A protein(s)

appears to be structurally different from vitellogenin. In addition, estrogen treatment of female flounder did not enhance the plasma level of Peak A protein(s) (Idler and So, personal communication) and taken together with the fact that this protein(s) is also present in the plasma of male flounder, confirm the above inference made based on biochemical studies.

Amino acid and amino sugar analyses

The amino acid compositions of Peak A protein(s) from female and male flounders were essentially the same, supporting the 5% PAGE band patterns that the protein(s) was the same in females and males. Also, the higher aspartic acid and glutamic acid contents supported the V8 enzyme digestion, which cleaves at the COOH terminal of aspartic acid and glutamic acid.

Amino sugars in Peak A protein(s) samples from both males and females were also essentially the same. In addition, the high amino sugars content corroborated Peak A protein(s) affinity towards Concanavalin A-Sepharose.

CHAPTER III

WINTER FLOUNDER OVARIAN PROTEINS

Introduction

The characterization of lipovitellin and phosvitin as principal yolk proteins in oviparous vertebrates such as amphibians hen and teleosts has been reported by many workers (Karasaki, 1963; Redshaw and Follet, 1971; Follet and Redshaw, 1974; Wallace and Jared, 1969; Ng and Idler, 1983; Wallace, 1978). At the same time, the relationship of vitellogenin to these yolk components was established on the basis of immunological cross reactivity (see Wallace, 1978; Wittlift and Kenney, 1972; Plack et al., 1971). Redshaw and Follett (1971) had reported that vitellogenin has essentially the same amino acid profile as the combined yolk platelet proteins. However, the yolk proteins isolated from teleosts were reported to be generally atypical (Wallace, 1978). Studies by various workers also indicated that there are a number of anomalies in teleost yolk components (Jared and Wallace, 1968; Markert and Vanstone, 1968).

Even though considerable evidence was reported regarding the autosynthetic activity of an oocyte, before maturation and after maturation (see Wallace, 1978), the sources of many yolk components are not known, whether they are heterosynthetic or autosynthetic in nature have not been confirmed. Lipovitellin, being such a big molecule, could be made up of more than one species of proteins apart from vitellogenin; the molecular organization of this yolk component is not known. Also,

except for vitellogenin, ovarian proteins have not been studied from a heterosynthetic point of view with respect to other non-vitellogenin plasma proteins. The preliminary results obtained in this laboratory showed that at least two non-vitellogenin plasma proteins were also incorporated by the oocytes.

As indicated in the General Introduction of this thesis, the main objective of this part of the investigation is to resolve the relationship of plasma proteins, vitellogenin and the 70 K and 28 K polypeptides from the Peak A protein(s) preparation to ovarian proteins. This would help to understand ovarian protein synthesis, especially the yolk proteins, from a heterosynthetic viewpoint. The other question addressed in this chapter is whether the 70 K and 28 K polypeptides of Peak A protein(s) are also present in the testicular extracts.

Materials and Methods

ISOLATION AND ANALYSIS OF OVARIAN PROTEINS FROM FLOUNDER OVARIES

Extraction of ovarian proteins

Mature ovaries were excised in December 1985 and ovarian proteins were extracted in 0.5 M NaCl - 5 mM EDTA solution (Plack et al., 1971) and stored at -80 C until used.

Polyacrylamide gel analyses of the ovarian extract

In order to establish a possible relationship of plasma proteins

to ovarian proteins, the following procedures were followed.

Non-denaturing 5% PAGE and SDS-PAGE (8% and 10%) were performed for the ovarian extract. PAGE (Davis, 1964) was performed in a disc electrophoresis apparatus under basic conditions in 5% gels (Chapter II). For SDS-PAGE, the ovarian proteins after extraction were dialysed for 24 hr in a buffer (0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 1 mM EDTA), before electrophoresis. In another preparation, lipovitellin and phosvitin were precipitated from the ovarian extract by dialyzing for two days with 5 mM $CaCl_2$ and re-dissolving the precipitate in the above buffer. SDS-PAGE (8% and 10%) was performed also on the lipovitellinphosvitin precipitates. Vitellogenin and/or Peak A protein(s) was electrophoresed along with these samples.

In another experiment, 5% PAGE (disc gel) was performed for the ovarian extract and the major band from these tube gels was cut and re-electrophoresed on a SDS-PAGE (10%), as previously described (Chapter II).

Tracing the injected radioiodinated Peak A protein(s) in the ovarian extract by direct autoradiography

About 4 x 10^6 cpm/0.1 µL/500 g body weight of ^{131}I Peak A protein(s) was injected to an intact female flounder during December, 1985. After 3 days, the fish was killed and the ovarian proteins were extracted and prepared for SDS-PAGE as described above. Amounts varying from 25 µL to 60 µL were electrophoresed on a SDS-polyacrylamide gel (10%). Electrophoresis and autoradiography were done as described in Chapter II.

Immunoprecipitation of ovarian proteins with Peak A protein(s) antibody and analysis of the precipitate by SDS-PAGE

Immunoprecipitation with IgG against Peak A protein(s) was done for (a) ovarian proteins extracted from normal flounder and (b) ovarian proteins extracted from ^{125}I Peak A protein(s) injected flounder.

(a) Two dilutions of antigen-antibody were incubated: (i) 10 µL of the sample was incubated with 100 µL antibody (1:10); (ii) 400 µL of the sample was incubated with 100 µL of antibody (4:1).

After incubation at 4 C for 24 hr, the precipitates were recovered by centrifugation in a Beckman microfuge for 20 min and the supernatant was separated. The precipitates were washed with 70% ethanol and then with 100% ethanol. The supernatant and the precipitates were electrophoresed on a SDS polyacrylamide gel (10%) as described previously.

In another immunoprecipitation experiment, the following scheme was adopted: (i) 40 µL of ovarian extract was diluted to 200 µL with 0.5 M Tris-base, pH 7.0, and then centrifuged for 30 min in a Beckman microfuge. The supernatant was again centrifuged for 15 min and 200 µL of Peak A protein(s) antibody was added to 15 µL, 30 µL, and 60 µL of the supernatant from the above preparation and incubated at 4 C; (ii) 25 µL of ovarian extract was centrifuged for 15 min and 2 L of supernatant was incubated with 100 µL of antibody. The rest of the procedure was as described in (a).

(b) In this immunoprecipation experiment, 1.0 mL of ^{125}I ovarian extract (approx. 1250 cpm) was incubated with 500 µL of antibody (1:2) for 12 hr at 4 C. After centrifugation, the precipitates were

recovered, counted in a gamma counter and then electrophoresed (approx. 300 cpm) as described previously.

The gel was treated with 'Enhance' solution (New England Nuclear) according to the supplier's instructions and then autoradiography was done using intensifying screens (Du-Pont).

Immunoblotting

Immunoblotting (Western blotting) was done for the ovarian extract according to the procedure supplied by Amersham, with suitable modifications. Antibodies against Peak A protein(s) and vitellogenin were used to probe the blot. In brief, the procedure was as follows:

A. Peak A protein(s)

(i) Ovarian protein extraction

Ovarian proteins were extracted from mature ovaries as described previously.

(ii) SDS-PAGE

SDS-PAGE (10% and 8%) was done for the ovarian extract according to the Dreyfuss et al. (1984) method described earlier. The gel was not stained before transferring (transblotting) the proteins from the gel to a Hybond-N (Nylon) membrane (Amersham).

(iii) Transblotting

The gel was equilibrated for 90 min in protein transfer buffer (0.025 M Tris-HCl, pH 8.3, 0.15 M glycine). Transblotting was done in an electroblotting unit (Hoeffer Scientific) for 8 hr at 0.6A with

cooling. The membrane was washed with transfer buffer after transblotting to remove residual acrylamide and allowed to dry for 15 min.

(iv) Blocking the protein blot

Blocking was done to minimize the non-specific binding of protein probes to the membrane. The membrane was washed for 15 hr at 50 C in 10X phosphate buffered saline [0.1 M sodium phosphate, pH 7.5, 1.3 M NaCl, (PBS)], containing 1% gelatin and 10% BSA.

(v) Probing the blot

(a) The probe, in this case, Peak A protein(s) antibody, was diluted in diluting buffer (PBS containing 1% BSA). A 1:200 dilution of the antibody was used. The membrane was incubated with the diluted antibody in a heat-sealed plastic bag using 10 mL per 100 cm^2 of the membrane. Incubation was at room temperature for 1 hr on a rotary shaker. The membrane was washed in 200 mL of washing buffer [PBS containing 0.1% tween 20 (Sigma] by incubating at room temperature for 15 min in an orbital shaker. Washing was repeated two more times.

(b) Species-specific second antibody, anti-rabbit IgG (Amersham), was used as reporter molecule. Anti-rabbit IgG was diluted in diluting buffer (1:200) and added to the blot. The rest of the procedure was as described in step (a).

(vi) Detection

Biotin-streptavidin system (Amersham) was used to detect the specific binding in the blot. After washing the membrane in step (b), the membrane was incubated with 3 mCi of (0.1 mCi/mL) biotinylated ^{35}S-Streptavidin for 20 min at room temperature on a rotary shaker. ^{35}S-Streptavidin was diluted in diluting buffer in the volume used in step (v). The membrane was washed five times for 4 hr in a large

volume of washing buffer and then was blot dried, wrapped in Saran wrap and autoradiography was done at -70 C as described on page 19.

B. Vitellogenin

The blot which had been previously probed with Peak A protein(s) antibody as described above was used again to probe with vitellogenin antibody. The procedure was as follows:

(i) The previous blot was first de-probed with a buffer (0.005 M sodium phosphate, pH 7.5, 0.002 M 2-mercaptoethanol, 2% (W/V) SDS by incubating the blot for 30 min at 60 C. The blot was air dried.

(ii) The dried blot was then incubated in a heat sealed plastic bag with 0.1 mCi of ^{131}I labelled vitellogenin antibody (IgG) in 30 ml of incubation buffer for 30 min on a rotary shaker (labelling of vitellogenin IgG was done by the Iodogen method as described under Materials and Methods in Chapter II, for the plasma proteins). The blot was then washed with 300 mL washing buffer for 6 hr, changing the washing buffer 4 times. The membrane was then blot dried, wrapped in Saran wrap and autoradiography was done as previously described.

Determination of antigenic determinant of Peak A protein(s)

Western blotting was done to determine the antigenic determinant of Peak A protein(s). Peak A protein(s) was electrophoresed on a SDS gel (10%), transblotted onto a Hybond-N membrane, and the blot was probed as described earlier in Step A, page 85.

ISOLATION AND ANALYSES OF TESTICULAR PROTEINS

Extraction of testicular proteins

Samples of testes (2.0 g) from October and February were extracted with 5,0 mL of 0.5 M NaCl - 10 mM EDTA solution as described previously for the extraction of ovarian proteins.

Immunoprecipitation of testicular proteins with Peak A protein(s) antibody and analysis of the precipitate by SDS-PAGE

Immunoprecipitation with IgG against Peak A protein(s) was done as follows:

(i) Testicular extracts from October and February were centrifuged for 30 min in a Beckman microfuge. Fifteen µL of supernatants were then incubated with 50 µL of Peak A protein(s) antibody at 4 C for 24 hr.

(ii) To serve as control, 20 µL of testicular extract supernatant from October and February samples was incubated with 50 µL normal rabbit serum (NRS) (1:4 dilution).

After 24 hr, the above samples were centrifuged and analysed on a SDS-PAGE (10%).

RESULTS

Polyacrylamide gel analyses of the ovarian extract

The ovarian extract on 5% PAGE gave a broad and very intense band,

the Rf extending from 0.25 to 0.32. A few narrow bands of Rf 0.23, 0.56 and 0.76 were also observed (Fig. 1). The main band however, seemed to have more than one component moving together under basic non-denaturing conditions of the gel. There were no bands which would correspond to Peak A protein(s) or vitellogenin.

SDS-PAGE for the phosphoprotein precipitates and for the supernatant did not show any difference in band pattern from the total ovarian extract in 8% and 10% gels. In SDS-PAGE, ovarian extract gave three major bands of molecular weights 96 K, 86-76 K, and 28 K (Fig. 2). The 96 K and 28 K bands were very broad and strong, however, the 86-76 K band had three components. Also, there were bands of molecular weights, 70 K and 40 K, along with several minor bands. Re-electrophoresis of the major band of Rf 0.25-0.32 on a SDS gel (10%) yielded fragments of molecular weights, 96 K, 86 K, 76 K, and 28 K (Fig. 3).

Direct tracing of injected radioiodinated Peak A protein(s) in the ovarian extract by autoradiography after SDS-PAGE

As a starting point for investigating a possible relationship between Peak A protein(s) and polypeptides in the ovarian extract, I asked if injected radioiodinated Peak A protein can be traced to ovarian polypeptides by autoradiography. But one problem in using this procedure was the precipitation of ovarian proteins in the gel when the sample volume was greater than 60 µL, even after the samples were prepared in the way described in Materials and Methods. This amount of ovarian extract contains very low counts which is not enough to give a

Figure 1. Non-denaturing polyacrylamide gel analysis (5% disc gel) of the ovarian extract prepared from flounder ovaries. Lane 1 contains about 200 µg and lane 2 contains about 125 µg of the sample. The gels were stained with Coomassie blue R-250.

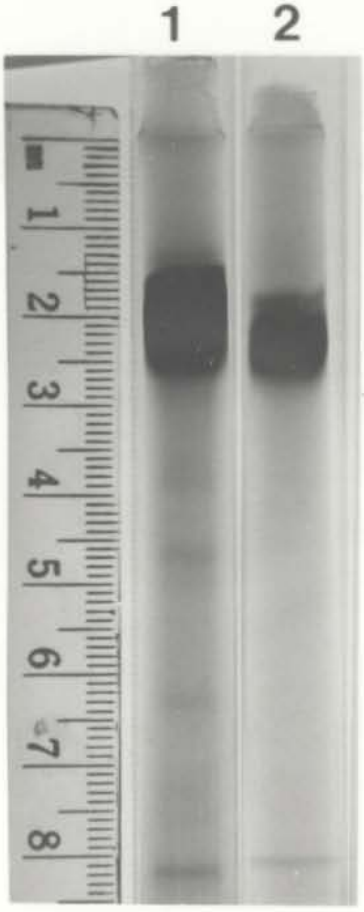

1
2

Figure 2. Polyacrylamide gel (10%) analysis (SDS-PAGE) of the ovarian extracts. Lane 1 represents the supernatant (5 µl) after 5 mM $CaCl_2$ dialysis of the total ovarian extract. Lane 2 contains $CaCl_2$ precipitable portion of the ovarian extract, dialysis was done for 5 days. Lanes 3, 4 and 5, 6 represent the supernatants and the precipitates from 3 days and 1 day dialyses. Lanes 7 and 8 contain 30 µg Peak A protein(s) and 20 µg vitellogenin. Lane 10 represents the molecular weight markers, RNA polymerase II, actin and histones (MW x 10^{-3}).

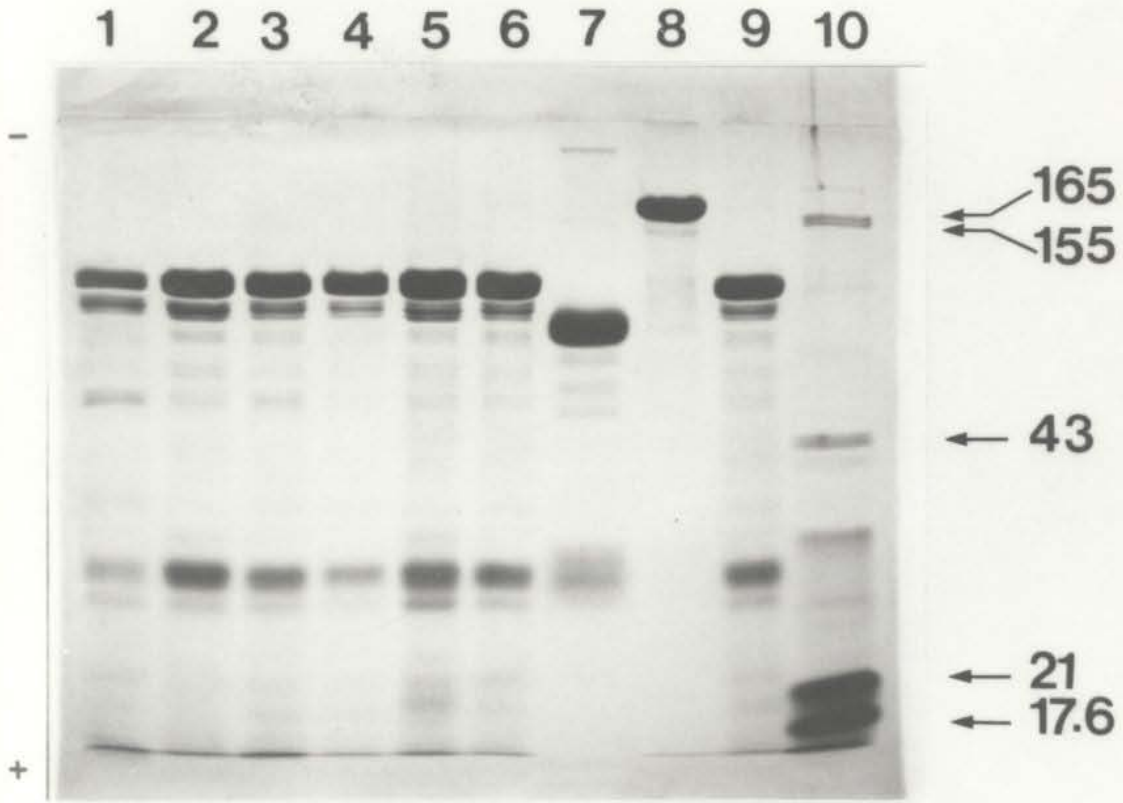
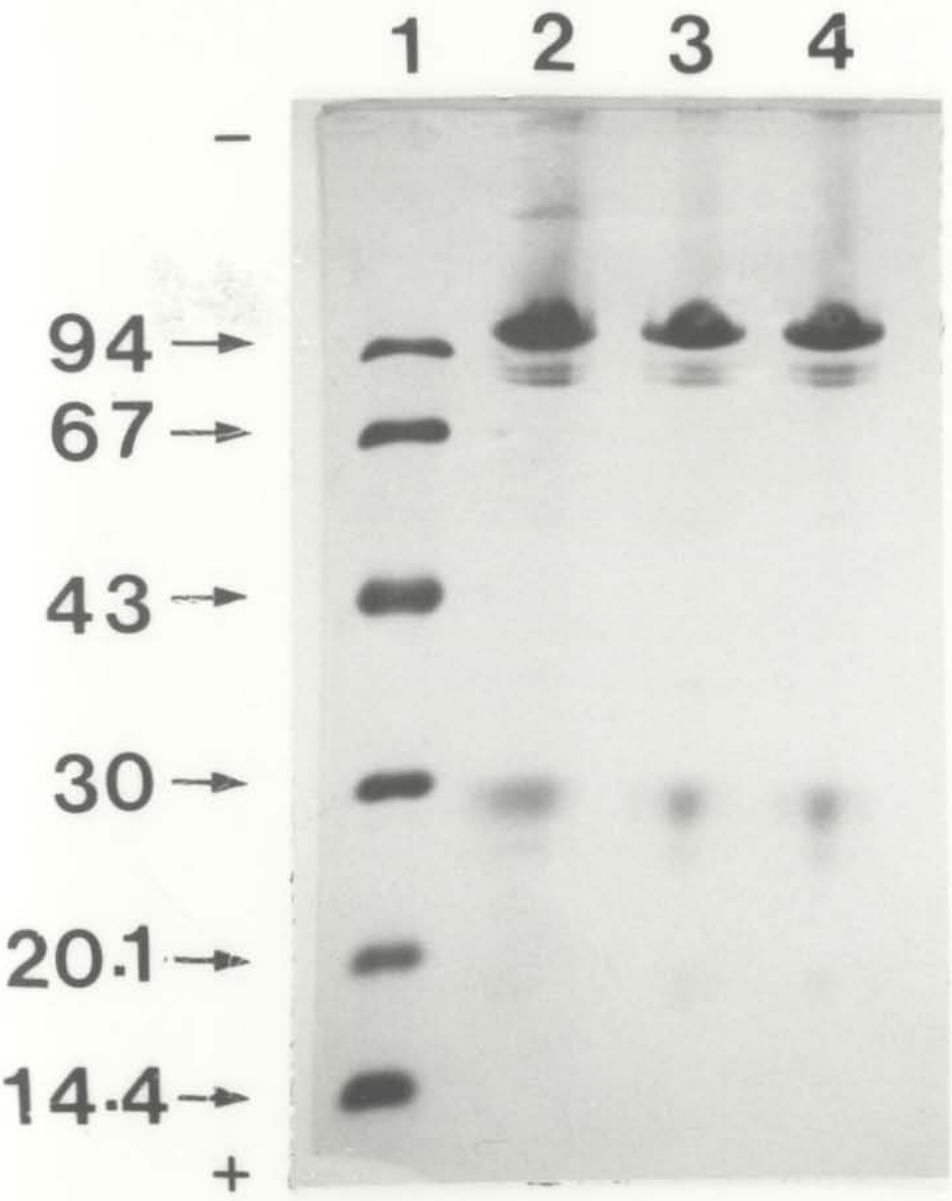

1 2 3 4 5 6 7 8 9 10
−
+
165
155
43
21
17.6

Figure 3. Polyacrylamide gel (10%) analysis (SDS-PAGE) of the major band sliced from a non-denaturing gel (5% disc gel) electrophoresis of the ovarian extract. About 100 μg of the total ovarian extract was first electrophoresed (5% PAGE) and the slice was re-electrophoresed on a SDS gel as described under Materials. The gel was stained with Coomassie blue R-250. The MW $\times 10^{-3}$ are shown on the left.


1
2
3
4
–
94
67
43
30
20.1
14.4
+

proper signal in an autoradiogram. However, on one occasion, enough counts were obtained to give a reasonably good signal in the autoradiogram.

There were two bands of MW 70 K and 28 K after one month exposure to Kodak XAR-5 X-ray films, (Fig. 4) from an original 40 cpm loaded onto the gel. Seven days exposure, however, indicated a narrow band of MW 70 K, but the 28 K band had not yet appeared in the autoradiogram.

Immunoprecipitation of the ovarian extract by Peak A protein(s) antibody and identification of precipitates by SDS-PAGE

The occurrence of Peak A protein(s) as 70 K and 28 K polypeptides in the ovarian extract made the interpretation difficult. There was a possibility that these bands were from the intact Peak A protein(s) polypeptides. Hence, further analyses of the ovarian extract were done with the mono-specific antibody to Peak A protein(s).

(a) Analysis of the immunoprecipitate of ovarian proteins extracted from normal flounder

Immunoprecipitation analysis of ovarian proteins, extracted from normal flounder, in two dilutions in one set of experiments, and four dilutions in another set of experiments, gave the same band patterns by SDS-PAGE for the precipitates as well as for the supernatant. There were major protein bands of molecular weights 96 K, 86-76 K, 28 K and 70 K in the immunoprecipitates as well as in the supernatants (Fig. 5). The 28 K band and the minor 70 K band corresponded to the 28 K and 70 K bands of Peak A protein(s). There was an additional band from the free antibody to the supernatant. A proper antibody-antigen ratio to obtain

Figure 4. Autoradiogram indicating the presence of ^{131}I labelled Peak A protein(s) in the ovarian extract as 70 K and 28 K. Lane 1 contains about 200 cpm of ^{131}I-Peak A protein(s). Lanes 2 and 3 contain about 40 cpm and 30 cpm of the ovarian extract prepared as described under Materials and Methods. The samples were electrophoresed on a 10% polyacrylamide gel in the presence of SDS and exposed to Kodak XAR-5 film at -70 C for 27 days.

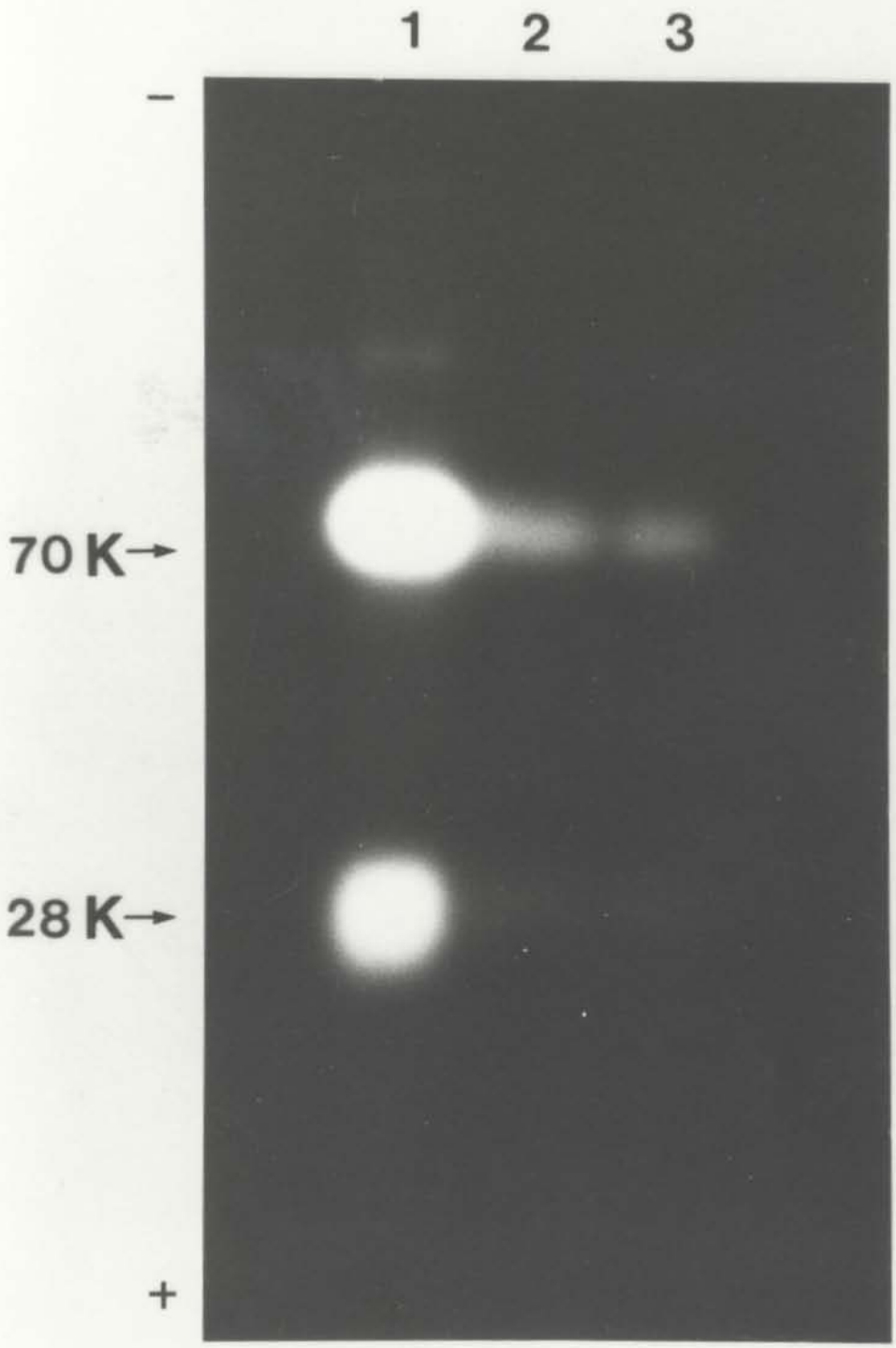
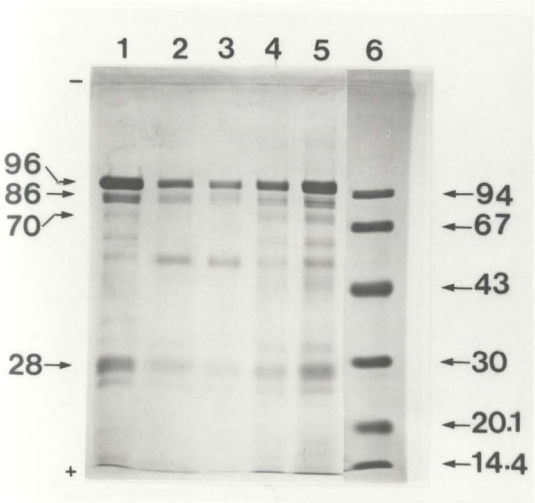

1
2
3
–
70 K→
28 K→
+

Figure 5. Polyacrylamide gel (10%) analysis (SDS-PAGE) of the ovarian extract immunoprecipitated with Peak A protein(s) antibody. Lane 1 contains the total ovarian extract (5 µl). Lanes 2 and 5 contain supernatants from the two incubations (1:10 and 4:1 antigen to antibody) and lanes 3 and 4 contain immunoprecipitates from the above two incubations. Lane 6 contains the high molecular weight markers (Pharmacia). The gel was stained with Coomassie blue R-250.


1
2
3
4
5
6
–
+
96
86
70
28
94
67
43
30
20.1
14.4

complete precipitation was not achieved in several other combinations.

(b) Analysis of the immunoprecipitate of ovarian proteins extracted from flounder injected with ^{125}I Peak A protein(s)

The immunoprecipitation of ovarian proteins extracted from normal flounder in the previous experiment indicated that most of the ovarian fragments were precipitated. All these fragments could not be from Peak A protein(s). One possibility for the immunoprecipitation of all these ovarian polypeptides could be that the 70 K and 28 K fragments from Peak A protein(s) were associated with other polypeptides in the oocyte. Hence, immunoprecipitation of ovarian proteins extracted from ^{125}I-Peak A protein(s) injected flounder was done; this would indicate the labelled Peak A protein(s) in the ovarian polypeptides.

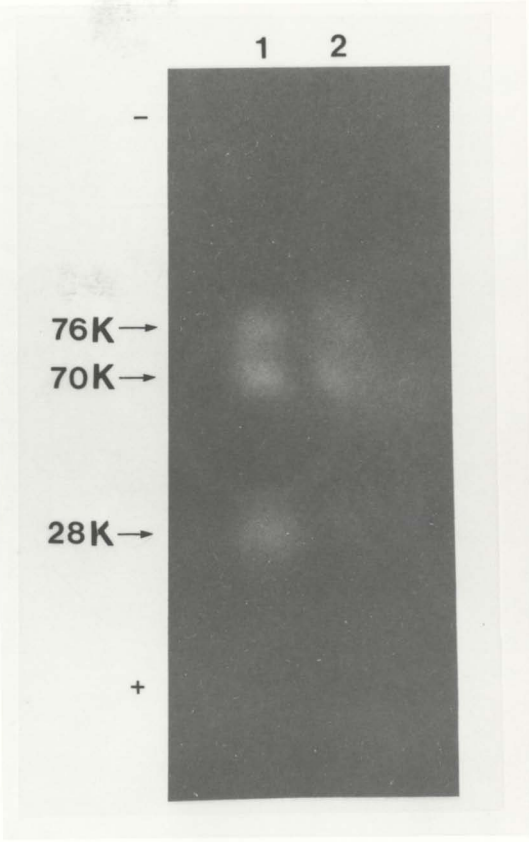
The autoradiogram of SDS gel gave bands of molecular weights 76 K, 70 K and 28 K in the precipitate and these corresponded to the 70 - 76 K and 28 K fragments of Peak A protein(s). The supernatant also gave 3 fragments of size 76 K, 70 K and 28 K indicating incomplete precipitation but establishing that no other radioactive component was detectable (Fig. 6).

Analysis of the ovarian extract by immunoblotting

Peak A protein(s)

Immunoblotting of the ovarian extract, when the ovarian proteins were probed with Peak A protein(s) antibody after separation on a SDS-PAGE, gave two recognition signals. This indicated that the 76 K and 70 K fragments of the ovarian extract are from Peak A protein(s)

Figure 6. Autoradiogram indicating Peak A protein(s) in the ovarian extract as 76K, 70K and 28K fragments, ^{125}I ovarian proteins were immunoprecipitated with Peak A protein(s) antibody and electrophoresed on a SDS-polyacrylamide gel as described under Materials and Methods. The gel was treated with 'Enhance' solution (NEN) and exposed to Kodak XAR-5 film at -70 C.


1
2
–
76K
70K
28K
+

(Fig. 7). There was no radioactivity detected at the 28 K region. At the same time, immunoblotting of plasma Peak A protein(s) indicated two antigenic determinants for Peak A protein(s) at the 74 K and 70K regions (Fig. 8). The 28 K fragment of plasma Peak A protein(s) did not react with the antibody and hence no radioactivity was detected in this region.

VitelTogenin

The protein blot which was already probed with Peak A protein(s) antibody was used to re-probe with vitellogenin antibody. The radiogram showed detection signals for vitellogenin at the 96 K and at about 86 K region. The 96 K signal was very strong (Fig. 9). There were no detection signals from any of the fragments from Peak A protein(s) or from ovarian extract which showed positive detection with Peak A protein(s) antibody.

Immunoprecipitation and analysis of the testicular extracts

SDS-PAGE (10%) analysis of the testicular extracts from October and February indicated a quantitative and qualitative difference in their protein species. A polypeptide fragment of molecular weight 28 K was a minor fragment in the October extract (Fig. 10, lane 2).

Immunoprecipitates of the testicular extracts from February with Peak A protein(s) antibody, on SDS-PAGE (10%), yielded a fragment of molecular weight 76 K and another major fragment of MW 28 K. Immunoprecipitation of the October extract had also precipitated the 28 K

Figure 7. Autoradiogram of a Western blot indicating Peak A protein(s) in the ovarian extract as 76K and 70K polypeptide fragments. Duplicate ovarian extract from December was electrophoresed on 8% polyacrylamide gel in the presence of SDS, transblotted on to a Hybond-N membrane and hybridized to Peak A protein(s) antibody as described under Materials and Methods. Autoradiography was done at -70 C for 7 days using AGFA-GEVAERT RP 1 film.

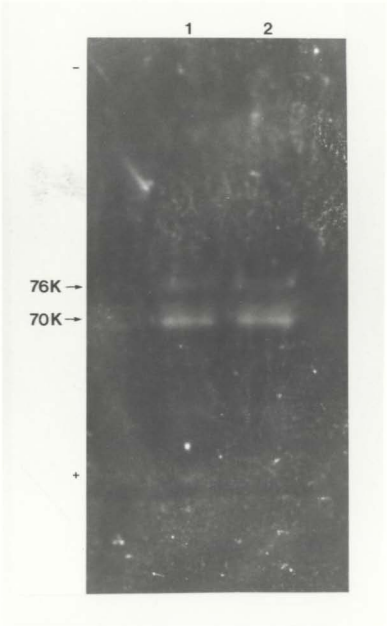

1
2
-
76K
70K
+

Figure 8. Western blotting of Peak A protein(s) indicating antigenic domains at the 74K and 70K regions. Peak A protein(s) was electrophoresed in the presence of SDS on a 10% polyacrylamide gel, transblotted on to Hybond-N nylon membrane and hybridized with Peak A protein(s) antibody (IgG) as described under Materials and Methods. Autoradiography was done for 7 days at -70 C using Kodak XAR-5 film.

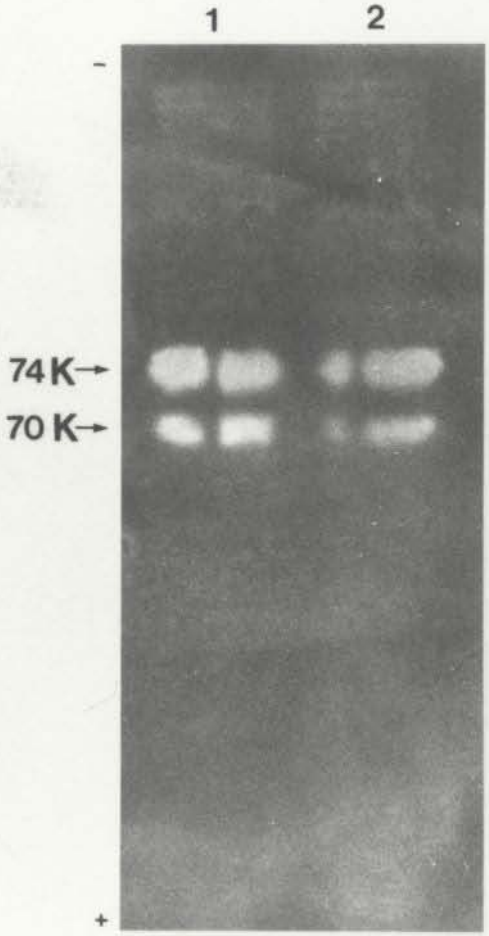
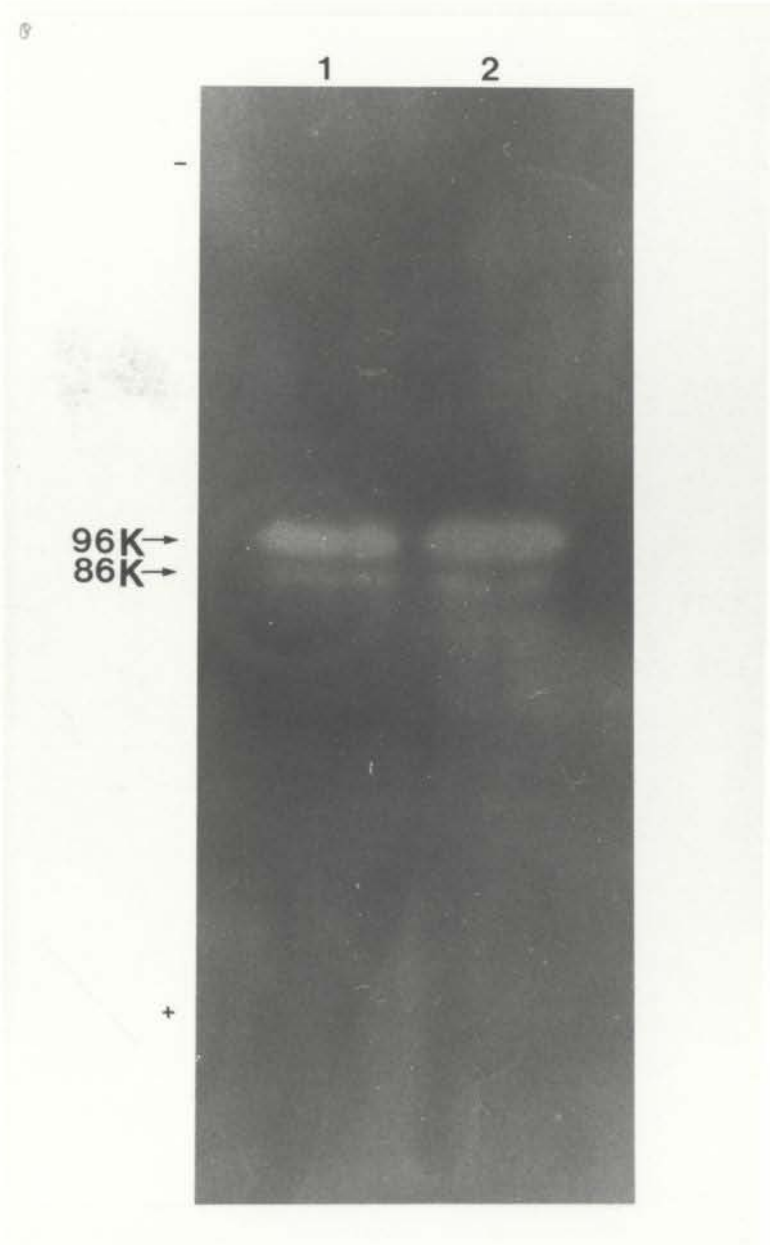

1
2
-
74K
70K
+

Figure 9. Autoradiogram of a Western blot indicating vitellogenin in the ovarian extract as 96K and 86K polypeptides. The blot which was previously probed with Peak A protein(s) antibody was de-probed and used again to probe with the radioiodinated vitellogenin antibody (IgG) as described under Materials and Methods. The membrane was exposed to AGFA-GEVAERT RP 1 film for 3 days.


1
2
-
96K
86K
+

fragment (Fig. 10, lane 2), but this was a minor fragment during October as observed in SDS-PAGE on the total extract. Immunoprecipitation of the October and February testicular extracts and also the ovarian extract with normal rabbit serum did not precipitate any of these fragments (Fig. 11).

DISCUSSION

Isolation procedure

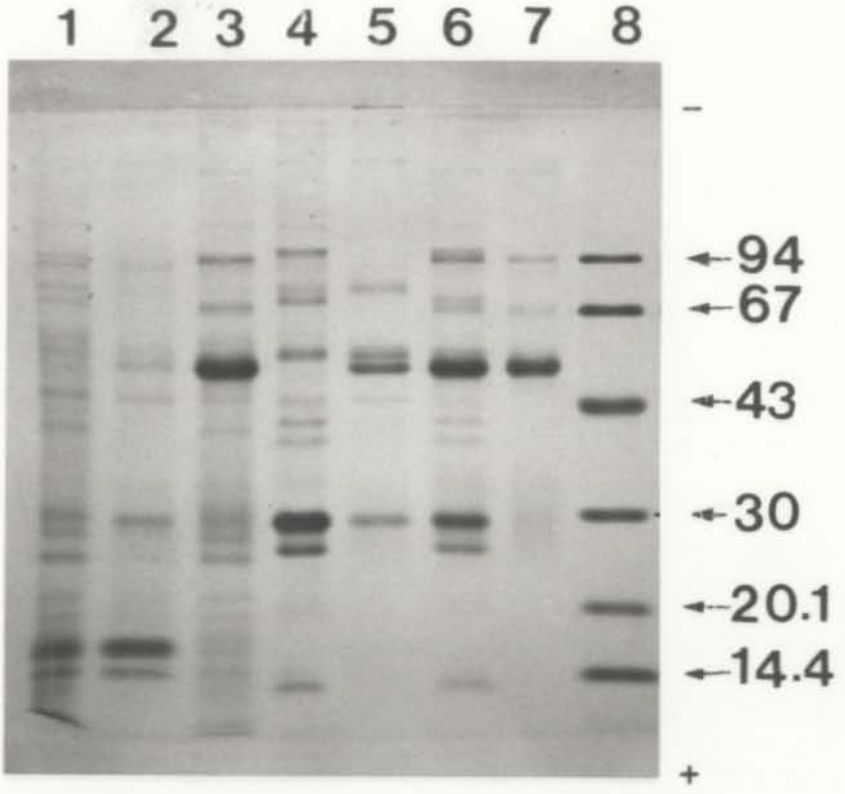
A number of investigators have attempted to isolate lipovitellin-like and phosvitin-like yolk proteins from the ovaries of a variety of teleost species (see Ng and Idler, 1983). Extraction with 0.5 M NaCl has been adopted by many investigators (Wallace et al., 1966; Jared and Wallace, 1968; Markert and Vanstone, 1968; Plack et al., 1971; Campbell and Idler, 1976, 1980; Idler et al., 1979). The purpose of this study was to characterise the ovarian proteins extractable with 0.5 M NaCl in relation to plasma Peak A protein(s) and vitellogenin. Accordingly, the ovarian extraction was done using the yolk extraction medium reported by Plack et al. (1971).

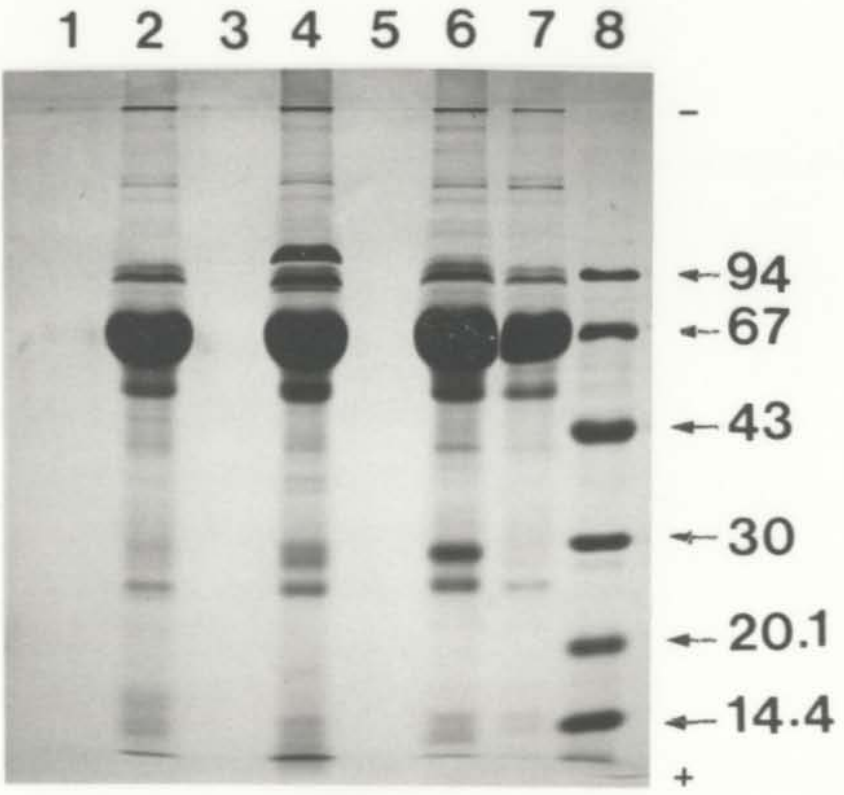
Ovarian extract analyses

In this study, the 70 K and 28 K polypeptides from Peak A protein(s) were shown to be incorporated into ovarian as well as testicular proteins. The structural unrelatedness of these polypeptides to vitellogenin was demonstrated in the previous chapter.

Fig. 10. SDS-polyacrylamide gel (10%) analysis of immunoprecipitates of the testicular extracts from October and February precipitated with Peak A protein(s) antibody as described under Materials and Methods. Lane 1 contains total testicular extract from October (5 µl), lanes 2 and 3 contain the immunoprecipitate and the supernatant from October extract. Lane 4 contains the total testicular extract from February (5 µl), lanes 5 and 6 contain the immunoprecipitate and the supernatant from the February extract. Lane 7 represents Peak A protein(s) antibody (IgG). Lane 8 represents the high molecular weight markers (Pharmacia). The gel was stained with Coomassie blue R-250.

Fig. 11. SDS-polyacrylamide gel (10%) analysis of the control incubations of the testicular extracts and the ovarian extract with normal rabbit serum (1:4 dilution). Lane 1 represents the precipitates and lane 2 represents the supernatant from the October testicular extract incubation. Lanes 3 and 4 are the precipitates and the supernatant from the ovarian extract. Lanes 5 and 6 are precipitate and the supernatant from the February testicular extract. Lane 7 contains the normal rabbit serum and lane 8 is the high molecular weight markers (Pharmacia). The gel was stained with Coomassie blue R-250.


1 2 3 4 5 6 7 8
-
94
67
43
30
20.1
14.4
+


1 2 3 4 5 6 7 8
-
94
67
43
30
20.1
14.4
+

The PAGE pattern for the ovarian extract indicated that there were no protein bands which would correspond to Peak A protein(s) and vitellogenin as such, suggesting a rearrangement of these plasma proteins in the oocyte. Such heterosynthetic modifications (or conversions) of the plasma proteins inside the oocyte has been suggested by many investigators (see Wallace, 1978). However, in locusts (Chen and Wyatt, 1981) and in Drosophila (see Postlethwait and Giorgi, 1985) yolk platelet proteins were known to be the same molecular weight polypeptides in the oocyte and in the plasma.

The preliminary study conducted in this laboratory on the uptake of ^{131}I-Peak A protein(s) indicated that the protein(s) becomes associated with the oocytes. Based on direct evidence obtained in this investigation, the incorporation of this protein(s) to ovary (and to testis) was concluded to be specific.

Comparison of the polypeptides from Peak A protein(s) and from vitellogenin to those from ovarian extract by SDS-PAGE was the first step in establishing a possible relationship between these plasma proteins and ovarian proteins. In SDS-PAGE, the major fragments of MW 96 K and MW 28 K appeared to be lipovitellin I and lipovitellin II, similar to the lipovitellin organization in Xenopus. This conclusion was based on the results reported by Berridge and Lane (1976). Their study on the translation of liver mRNA in Xenopus oocytes and vitellogenin synthesis and conversion to yolk proteins was very valuable here. They had shown that the source of yolk platelet proteins, lipovitellin I (MW 100 K) and phosvitin, is vitellogenin. But their study did not indicate vitellogenin as the source of another major fragment, lipovitellin II (MW 28-30 K). The results from SDS-PAGE of flounder

ovarian extract, which yielded a polypeptide of MW 28 K as did the Peak A protein(s) is significant here.

The direct tracing of injected ^{131}I-Peak A protein(s) in the ovarian extract by SDS-PAGE and autoradiography indicated that Peak A protein(s) occurred as 70 K and 28 K polypeptides in the oocyte. However, there was a problem in this experiment. The radioactivity/unit volume of the ovarian extract was low and there was a large amount of proteins/unit volume of extract. This limited the volume of extract which could be electrophoresed and also the cpm which could be electrophoresed. Thus there was a possibility that more than two ^{131}I polypeptides from Peak A protein(s) were present in the ovarian extract. The results from immunoprecipitation and immunoblotting analyses of the ovarian extract with Peak A protein(s) antibody confirmed this possibility.

The results from immunoprecipitating the ovarian protein(s) extracted from flounder injected with ^{125}I-Peak A protein(s) complemented the earlier results and provided firm evidence for the functional incorporation of this protein(s) into the oocyte. The results indicated that the ovarian fragments of molecular weights, 76 K, 70 K and 28 K were from Peak A protein(s). However, the 76 K fragment was weaker and hence was not detected in direct tracing by autoradiography.

Analysis of the ovarian extract by immunoblotting

The protocol supplied by Amersham was used with modifications for immunoblotting or Western blotting of the yolk extract. These

modifications entail the use of BSA and gelatin (10% and 1% respectively) to block the non-specific binding sites in the Hybond-N membrane after transblotting; blocking was done at 50 C for 15 hr. The blot was then washed after probing with ^{35}S Streptavidin for 4-6 hr in a large volume of washing buffer. These modifications reduced the background and ruled out the possibility of any false signals. SDS was omitted from the transfer buffer while transblotting. Though SDS facilitated the transfer of proteins from gel to the nylon membrane, as reported earlier (Erickson et al., 1982; Nielsen et al., 1982; Vaissen et al., 1981), subsequent probing failed to detect any signals. By omitting SDS from the transfer buffer, the voltage had to be increased to effectively transfer the high molecular weight polypeptides; also, the radioactive bands were very well detected. The technique, first accomplished by Renart et al. (1979), termed Western blotting (Burnette, 1981) reportedly has high sensitivity and innate versatility (see Gershoni and Palade, 1983). This procedure made it possible in this study to detect the 76 K as well as the 70 K polypeptides in the ovarian extract. The 28 K polypeptide detected by direct scanning was not detected by this method. The most likely explanation is that the 28 K polypeptide is not precipitated by Peak A antibody except when it is associated with Peak A protein(s) or the ovarian proteins. The immunoblotting of Peak A protein(s) with the antibody had indicated that this plasma 28 K polypeptide does not cross react with the antibody.

The results from partial proteolysis of Peak A protein(s) (Chapter II) was particularly important here. There was a fragment of MW 28 K which was resistant to V8 enzyme digestion. However, at this point, it

is not known that the 28 K fragment, observed in the peptide map, is the same 28 K polypeptide from Peak A protein(s). The possibility that the 28 K fragment after proteolysis is from the 70 K polypeptide of Peak A protein(s) can not be ruled out at this point. In either case, it is important to note that there is a fragment of MW 28 K, which is apparently resistant to proteolytic cleavage in Peak A protein(s); this has direct relevance to ovarian proteins since Peak A protein(s) also give rise to a 28 K polypeptide fragment in the oocyte.

Vitellogenin in ovarian extract was represented by the major 96 K fragment and another fragment of MW 86 K. There were other fragments in the ovarian extract and they may be derived from plasma proteins or autosynthetic proteins or chromosomal proteins. It has been reported as indicated in the Introduction that vitellogenin occurs in the ovary as lipovitellin, a major yolk protein and also as phosvitin. The 96 K fragment appears to be lipovitellin (Lipovitellin I), however phosvitin fragment was not recognized in this study with vitellogenin antibody, probably the recognition was not possible for lack of any antigenic determinant in phosvitin. The other fragment which gave a positive signal with vitellogenin antibody had a higher molecular weight than reported for phosvitin.

Further, it should be noted that the relative strength of signals in autoradiograms should not be interpreted quantitatively, as indicated by Gershoni and Palade (1983). The affinity of a ligand to an immobilized protein may be considerably less than to its native counterpart. For instance, the isolated, immobilized, α-subunit of the acetylcholine receptor binds α-bungarotoxin with an affinity 10^3-10^4 times less than that shown by the non-denatured

receptor (Gershoni et al., 1982).

Immunoprecipitation of the testicular extracts

The testicular samples from two different reproductive phases were selected for immunoprecipitation, i.e., from October, when the testis was immature and from February, when the testis was fully matured. The reason for this was that when Peak A protein(s) was tested for uptake in males during January (see Chapter IV) there was no uptake; however, uptake was observed before December in a few males while testing the uptake in females. One conclusion was that by December, the uptake had stopped, owing to the fact that the testes were fully matured and sperm were activable. If the Peak A protein(s) is present in the testis in February, this conclusion would be confirmed. In addition, the experiment was done to determine the protein pattern of the testicular extract and to note differences, if any, in two different phases and to compare the pattern with the ovarian extract.

In this study, the difference between the two phases of testicular development were quite obvious, both in the number of protein fragments present and in the amount. Specific changes in the pattern of protein synthesis during meiotic maturation of amphibians and mammalian oocytes has been shown by many workers (Smith and Ecker, 1969; Ecker and Smith, 1971; Schultz and Wassarman, 1977). But no work was reported with respect to testicular proteins, other than histones and protamines, from fish. The presence of several polypeptides in the February testicular extract (note that the second meiotic division was completed by this time in this species) notably a very major fragment of MW 28 K and

another fragment of about 26 K molecular weight, both were absent or very low (undetectable) in the October extract, demonstrates the specific change. The 76 K fragment in the February extract of testes seems to be the homologous fragment to the ovarian 76 K fragment from Peak A protein(s). The results from immunoprecipitation of the testicular extract with Peak A protein(s) antibody confirmed the likely origin of the 28 K fragment, both in males as in females, from Peak A protein(s). Since vitellogenin is not present in males, the 28 K fragment could not be from vitellogenin. Since the testis has not yet incorporated Peak A protein(s) during October in appreciable amounts, the 28 K fragment was still a minor polypeptide.

The specific incorporation of Peak A protein(s) into both ovary and testis, demonstrated in this investigation, appears to be very significant in gonadal growth during the reproductive cycle of winter flounder. Comparison of this aspect among species may enhance our understanding of relative importance of this protein(s) to vitellogenin and other plasma proteins in oocyte growth.

CHAPTER IV

GONADOTROPIC REGULATION OF OVARIAN UPTAKE OF YOLK PRECURSORS

Introduction

The existence of two gonadotropic hormones in fish has been the subject of controversy for many years. However, Idler and co-workers defined two distinct gonadotropic preparations and their biological activities. Con AII stimulates the gonadal synthesis of estrogen (see Ng and Idler, 1983) while Con AI stimulates the incorporation of vitellogenin (Campbell and Idler, 1976). However, in hypophysectomised mature flounders, Con AII also stimulates vitellogenin uptake (Idler and Ng, 1979). The stimulation of vitellogenin incorporation into salmon or flounder oocytes could be attributed to Con AI fraction based on the following experimental results:

1. In salmon, Con AII becomes undetectable in the circulation during the active vitellogenic stage (Crim et al., 1975).

2.(a) An antiserum to Con AI prevents the incorporation of vitellogenin, indicated by the follicular atresia in maturing females; antiserum to Con AII does not have any such appreciable effect on the histological appearance of the ovary (Ng et al., 1980).

(b) Wiegand and Idler (1984) reported the failure of Con AII antibody to inhibit rapid ovarian growth in vitellogenic landlocked Atlantic salmon but it did inhibit in earlier stages and in immature fish.

3. The immunofluorescent localization of the hormones in the ovary (Ng et al., 1980) corroborated the above findings, ie Con AI was located in

the ooplasm of both large immature and growing oocytes and in follicular envelopes of growing oocytes implying that the hormone primes the oocyte for the ovarian uptake of yolk precursor protein(s). Con AII was localized in follicular envelopes, in interstitial tissues, and large immature oocytes, suggesting a steroidogenic role.

One of the primary goals in this investigation was to isolate a single active component from Con AI fraction which would stimulate the ovarian uptake of plasma proteins; this would provide additional information on the basic mechanism and the influence of pituitary hormones on the process of oocyte growth.

Flounder Con AI fraction was shown to influence the ovarian uptake of non vitellogenin plasma proteins (Idler and So, unpublished). Hence, the possibility of narrowing down this biological activity of Con AI to a single gonadotropic component, which would stimulate the ovarian uptake of these proteins, is addressed in this chapter. This would extend the biological function of this gonadotropin from stimulating the ovarian uptake of vitellogenin to stimulating the uptake of other plasma proteins also.

Materials and Methods

Preparation of pituitary hormones

In order to find out whether there are any detectable differences in the principal hormone and biological activity, the carbohydrate-poor fraction was prepared and analysed from pituitaries collected during the active vitellogenic phase (September) and near to spawning (May).

A total of 80 g of pituitary glands from approx. 10,000 fish (winter flounder) were collected at Margaree, Newfoundland, from fish caught in St. Georges Bay during May 1980 and September 1985. They were kept frozen at -80 C until processed.

Processing of pituitary glands to obtain various hormone preparations was done according to published methods (Idler et al., 1975a,b; Ng and Idler, 1978a,b) with minor modifications. Briefly, the methods were as follows:

(a) Chromatography on Concanavalin A-Sepharose

Pituitaries were extracted 2 times with 4 volumes of Buffer B (0.05 M Tris-HCl, pH 7.8, 0.5 M NaCl, 0.2 mM DTT, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% PMSF and then applied to a Con A-Sepharose (Pharmacia) column (5 x 30 cm) previously equilibrated with the same buffer. The column was loaded at a flow rate of 30 mL/hr and eluted at 50 mL/hr, first with Buffer B and subsequently with Buffer C (0.05 M Tris-HCl, pH 7.8, 0.5 M NaCl, 0.2 mM DTT, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% PMSF, 0.15 M β-methyl-D- glucoside) to yield the unadsorbed Con AI and the adsorbed Con AII fractions respectively. Con AI fraction which contained non-glycoproteins and glycoproteins devoid of the appropriate residues (Goldstein et al., 1965) is used in this investigation.

(b) Gel filtration

Ng and Idler (1978a,b) and Idler and Ng (1979) Had earlier reported the fractionation of Con AI based on molecular weights by gel filtration on 2 Ultrogel AcA 54 or AcA 44 columns connected in series, into 3 major peaks: A, B and C with molecular weight ranges of > 160 x 10^3, 160 x 10^3 - 45 x 10^3; 45 x 10^3 - 10 x 10^3 respectively.

Ng and Idler (1978) reported big and little forms of maturational (Con

AII, carbohydrate rich) and vitellogenic (Con AI, carbohydrate poor) hormones of 62 K and 28 K from Peak C of Con AII and Con AI fractions in American plaice. Further, they reported that the biological activity with respect to vitellogenin uptake by the gonads resides in the 28 K fraction. This fraction was isolated for further fractionation in this investigation.

The high molecular weight proteins (> 100K) and very low molecular weight proteins (< 10K) were removed from Con AI fraction by Minitan ultrafiltration system (Millipore) using >100 K and then <10 K molecular weight cut off Minitan plates. This also concentrated the Con AI fraction to a convenient volume for gel filtration. This preparation was dialysed against Buffer D (0.05 M Tris-Cl, pH 7.8, 0.15 M NaCl, 0.4 mM DTT, 1.0 mM EDTA, and 0.1% PMSF), centrifuged and applied on 2 Ultrogel AcA 44 (LKB) columns (5 x 165 cm) connected in series and eluted using Buffer D in the ascending mode at a flow rate of 60 mL/hr to obtain three peaks. Peak C defined in terms of molecular weight range was the major peak.

Re-chromatography of peak C after concentration in Minitan ultrafiltration system of <10 K cut off plates was done on AcA 44 double columns to obtain a broad and symmetrical peak. The pooled peak C was concentrated in an Amicon concentrator using YM 5, 43 mm diameter membrane (Spectrapore) of molecular weight cut off > 5,000 to a convenient volume. To remove any contaminating residual glycoproteins (Con AII), this preparation was again eluted with Buffer D through a Con A-Sepharose column. The tubes containing eluted proteins were pooled, concentrated and dialysed extensively in a 6-8 K molecular weight cut off dialysis bag (Spectrapore) against 5 mM NH_4HCO_3, pH 9.0 with

two changes daily for three days, before being chromatographed on a DEAE Bio-Gel A (Bio-Rad) ion-exchange column.

(c) Ion-exchange chromatography

The anion 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 for several days before use.

The unadsorbed proteins, DEAE I (DE I) fraction was first eluted with 5 mM NH_4HCO_3 in descending mode and adsorbed proteins DEAE II, III and IV (DE II-IV) were collectively eluted by 0.15 M NH_4HCO_3, at a flow rate of 10 mL/hr by reversing the flow to ascending mode. Tubes representing DE II-IV were pooled together and concentrated in an Amicon concentrator using YM5 membrane to obtain a protein concentration of 8-10 mg/mL. All the procedures were done at 4 C. Protein was estimated according to Lowry et al. (1951).

(d) Preparative polyacrylamide gel electrophoresis

Preparative polyacrylamide gel electrophoresis (prep. PAGE) was done on pooled concentrated DE II-IV preparation, to isolate different protein species based on their relative mobility (Rf) on a 7.5% PAGE.

Polyacrylamide gel (7.5%), pH 8.9 was polymerised overnight at 4 C in an Isotachophoresis (LKB) column, the gel dimensions being 60 x 5 mm for separating gel and 5 x 5 mm for 4% stacking gel, pH 6.8. The gel was pre-electrophoresed for 4 hr at 320 volts before loading the sample. The sample DE II-IV was dialysed for 2 days against Prep. PAGE upper buffer (0.1073 M Tris-base, pH 8.7, 0.08 M Glycine) with frequent changes. 14-16 mg of DE II-IV in 2.0 mL with a final concentration of 10% sucrose were loaded onto the top of the stacking gel. The electrophoresis was performed at 320 volts for 15 hr and the electroeluted

proteins were eluted from the column with lower buffer (0.125 M Tris-base, pH 7.7) at a flow rate of 15 mL/hr and 0.6 mL fractions were collected. The eluted proteins were read in Spectrophotometer 240 (Gilford) at OD_{230} and the eluants representing each peak were pooled, and kept frozen at -80 C.

Polyacrylamide gel analyses of the pituitary hormones

PAGE and SDS-PAGE in the presence of 2-mercaptoethanol as reducing agent was performed to identify the proteins in each fractionation procedure, starting from Con A-Sepharose chromatography to prep. PAGE.

(a) PAGE

Non-denaturing 7.5% PAGE [Disc electrophoresis (Davis, 1964)], pH 8.9 was performed on Con AI, Con AII, DE I, DE II-IV and eluants from Prep. PAGE, as described previously.

(b) SDS-PAGE

SDS-PAGE (15%) was performed for all the preparations from Con AI fraction to prep. PAGE eluants according to the procedure published by Dreyfuss et al. (1984) to determine the purity of the preparations and the molecular weights of different species of proteins. High molecular weight and low molecular weight markers (BRL) were used to determine the molecular weights of the protein species. The details of the procedure is given under Materials and Methods, Chapter II.

Bioassays

In biological activity studies, in terms of uptake of vitellogenin,

Figure 1. Fractionation of flounder pituitary extract to obtain various hormone preparations.

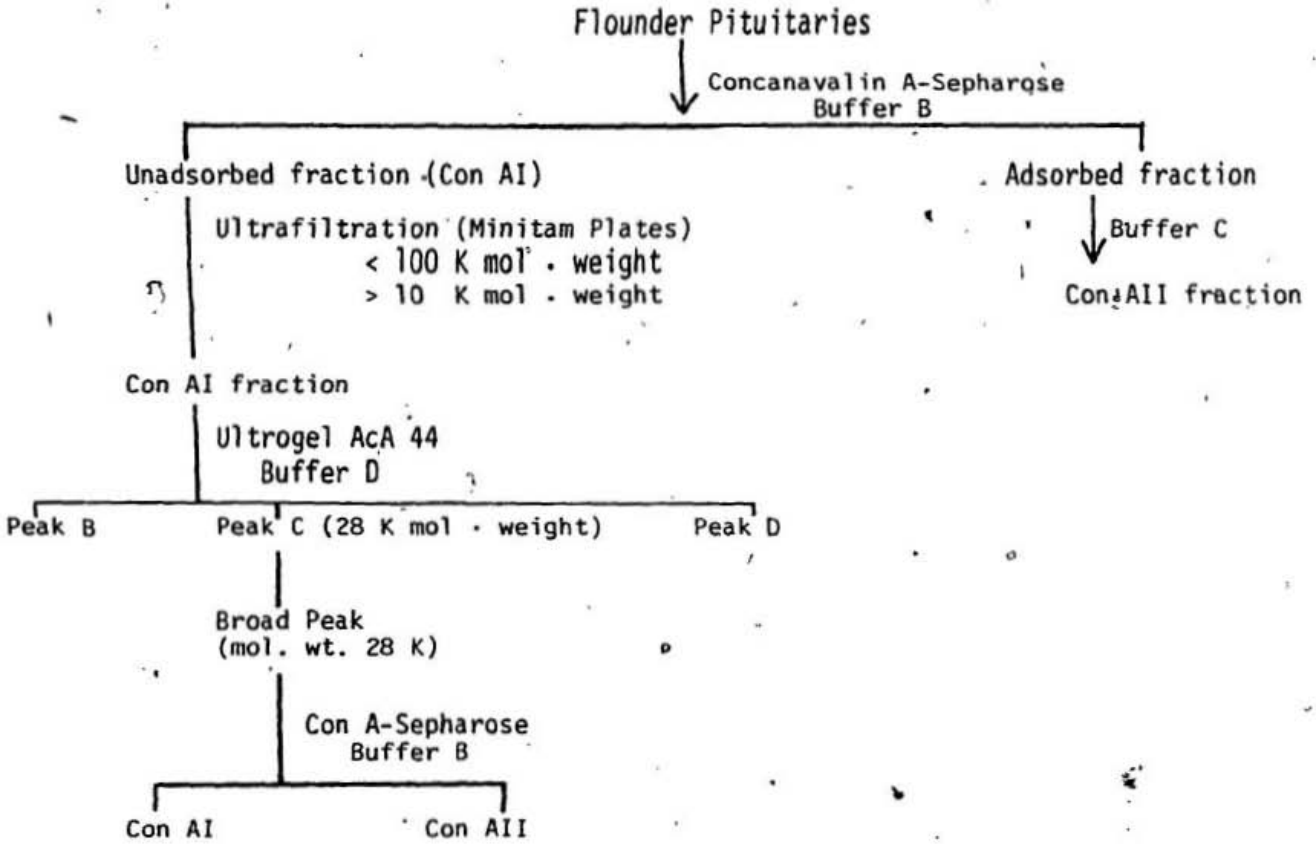
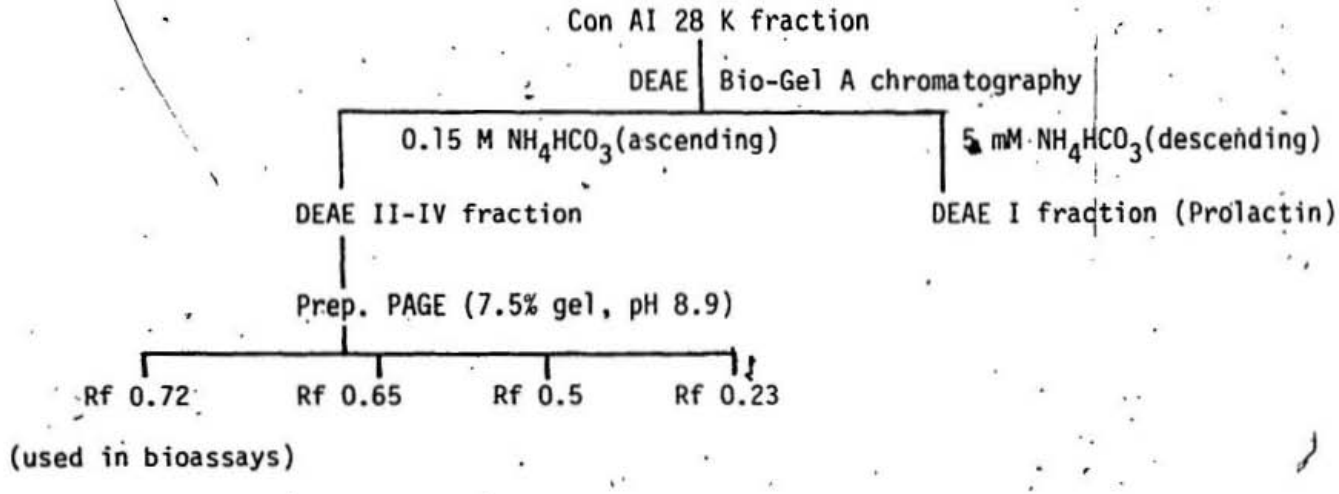

Flounder Pituitaries
Concanavalin A-Sepharose
Buffer B
Unadsorbed fraction (Con AI)
Adsorbed fraction
Buffer C
Con AII fraction
Ultrafiltration (Minitam Plates)
< 100 K mol . weight
> 10 K mol . weight
Con AI fraction
Ultrogel AcA 44
Buffer D
Peak B
Peak C (28 K mol . weight)
Peak D
Broad Peak
(mol. wt. 28 K)
Con A-Sepharose
Buffer B
Con AI
Con AII

Figure 2. Further fractionation of flounder pituitary Con A I 28K fraction.


Con AI 28 K fraction
DEAE Bio-Gel A chromatography
0.15 M NH_4HCO_3(ascending)
5 mM NH_4HCO_3(descending)
DEAE II-IV fraction
DEAE I fraction (Prolactin)
Prep. PAGE (7.5% gel, pH 8.9)
Rf 0.72
Rf 0.65
Rf 0.5
Rf 0.23
(used in bioassays)

Peak A protein(s) and Peak E protein(s), DE II-IV and Rf 0.72 protein from DE II-IV fractionated by prep. PAGE were used.

Animals for bioassay

Mature female and male winter flounder Pseudopleuronectes americanus were collected by M.S.R.L. divers from September to May, representing the two reproductive phases: active vitellogenesis and vitellogenin uptake by gonads, from September to January, and mature, from February to April, when the oocytes were maintained in a yolky state, till spawning. Fish from both phases were used in uptake and bioassay studies.

Flounder were hypophysectomised as described by Campbell and Idler (1976) and/or sham operated and then allowed to recover in 33% seawater at 8 C for at least 10 days.

In four different experimental approaches, animals were assayed for uptake of vitellogenin, and other non-vitellogenin plasma proteins [Peak A protein(s) and Peak E protein(s)] and to study pituitary influence on the uptake of these proteins.

1. To compare the uptake of vitellogenin with Peak A protein(s), intact female flounders were injected with 2×10^6 cpm/0.1 mL/500 g body weight of ^{131}I labelled Peak A protein(s) and ^{131}I labelled vitellogenin, intravenously at the caudal vein. The fish were sacrificed after 48 hr.

2(a) To study the influence of the pituitary gland on the uptake of these proteins, intact, sham operated and hypophysectomised female flounders were injected with 2×10^6 cpm/0.1 mL/500 g body weight of

^{131}I labelled Peak A protein(s), Peak E protein(s) and vitellogenin, and sacrificed after 48 hr.

(b) Sham operated and hypophysectomised male flounders were injected with 2 x 10^6 cpm/0.1 mL/500 g body weight of ^{131}I labelled Peak A protein(s).

3. Two or more groups of hypophysectomised female flounders were used. One group was injected with Tris buffer (pH 7.7) as control and other groups were injected with various Con AI preparations, DE II-IV and Rf 0.72 protein and once, sockeye salmon Con AI fraction in different experiments. Four injections on alternate days were given intraperitoneally to these fish. Also, each fish was given estradiol benzoate at a rate of 10 µg/kg body weight with the first hormone injection. With the fourth hormone injection, 2 x 10^6 cpm/0.1 mL/500 g body weight of ^{131}I labelled, Peak A protein(s) or vitellogenin or Peak E protein(s) were injected intravenously. The fish were sacrificed after 48 hr.

In all the above experiments, ovaries and livers were excised and weighed on a Mettler analytical balance. A blood sample was collected from each fish before autopsy. The animals were checked for the absence or the presence of the pituitary gland. Samples from fish which were partially hypophysectomised were rejected. The total radioactivity in the ovaries, liver and plasma was measured in a gamma counter. Methods of by Ng and Idler (1978a,b) were followed to calculate the incorporation of injected Peak A protein(s) into TCA precipitable proteins and yolk proteins as phosphoprotein precipitates. Duplicate 100-200 mg pieces of the ovaries were weighed out on a Cahn electrobalance taking care not to include the ovarian wall. These

pieces were homogenised directly in scintillation vials in 5% TCA in a polytron homogenizer. The homogenates were incubated at 85 C for 20 min to eliminate any aminoacyl transfer RNA from the precipitate (Roberts and Peterson, 1973). The precipitates were recovered upon centrifugation at 2,000 g for 45 min and counted in a gamma counter.

A 2-3 g piece was also exised from each ovary and homogenised in four volumes of yolk extraction medium (0.5 M NaCl - 5 mM EDTA; Plack et al., 1971) and incubated overnight at 4 C. Centrifugation and re-centrifugation of these homogenates at 15,000 g for 2 hr in a Sorvall centrifuge at 4 C, yielded a supernatant, which was taken and dialysed against three daily changes of 30 volumes of 5 mM $CaCl_2$, as described by Ng and Idler (1978a,b). The contents of dialysis bags along with the washings of the bags were centrifuged at 2000 g for 45 min. The supernatant was aspirated off and the protein precipitates were counted directly in a gamma counter; cpm/g of ovary was calculated.

The blood samples from each fish were centrifuged at 8000 g for 20 min and one mL serum was counted in a gamma counter. All the values were corrected for ^{131}I decay.

The same assay procedure was used when determining the testicular uptake of Peak A protein(s). However, the phosphoprotein precipitation was omitted owing to very low counts/g of testis.

4. To study the uptake of Peak A protein(s) by the ovary over a 6-day period of time (time course study), another bioassay procedure was followed. Sham operated and hypophysectomised female flounders were injected with 2 x 10^6 cpm/0.1 mL/500 g body weight of ^{125}I labelled Peak A protein(s). Biopsy was done for every 48 hr by introducing a cannula into the ovary by directly puncturing the ovary from

the ventral side of the body and sucking the eggs using a 20 cc syringe connected to the cannula by a 18 gauze needle. Duplicate 100-200 mg of eggs were sucked out, weighed in a Cahn electrobalance and counted in a gamma counter. Three such biopsies were done over a period of six days.

Treatment of assay data

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

Results

ISOLATION OF CARBOHYDRATE-POOR FRACTION FROM THE PITUITARY EXTRACTS ON CON A-SEPHAROSE AND ULTROGEL

Chromatographic behaviour

The elution profiles of pituitary extracts from the May batch pituitaries were similar to those of pituitaries collected during September on a Con A-Sepharose column: the bulk of proteins (90%) was in the unadsorbed Con AI fraction, whereas the adsorbed Con AII fraction accounted for only 10%.

The use of minitan ultrafiltration system (Millipore) fitted with minitan plates, which are tangential flow filter units, in the ultrafiltration of Con AI fraction yielded a concentrated protein preparation free of high molecular weight proteins (>100 K) and very low

molecular weight proteins (<10 K) before passing through Ultrogel AcA 44 gel filtration column.

Ultrogel AcA 44 gel filtration of Con AI fraction gave three peaks: a small peak, one large and broad peak reaching an optical density of greater than 1.5 and another small peak (Fig. 3). The small peak (Peak B) was at the region of 62 K molecular weight, and the major peak (Peak C) being at 28 K region. Tubes representing Peak C were pooled as shown in Figure 3 to give fraction C. Peak A, representing high molecular weight proteins, was absent.

Re-chromatography of Peak C on Ultrogel AcA 44 double columns produced an apparently symmetrical, broad peak with a peak tube molecular weight of 28 K (Fig. 4).

FURTHER FRACTIONATION OF FRACTION C FROM ULTROGEL

DEAE Bio-Gel A Chromatography

DEAE Bio-Gel A chromatography yielded an unadsorbed DE I fraction and an adsorbed DE II-IV fraction. This DE II-IV fraction was used for further fractionation.

Prep. PAGE

The protein species present in the DE II-IV preparation were separated by prep. PAGE (7.5% gel). The electroeluted fractions from the gel gave several peaks (Fig. 5). The first major peak, where the OD_{230} was greater than 3.0, was contributed by sucrose and

Figure 3. Chromatography of Con AI fraction of flounder pituitary extract on Ultrogel AcA 44 double columns connected in series. Fractions of 4.5 ml were collected at a flow rate of 60 ml/hr and protein was monitored by OD_{280}. The dotted line indicates the pooling of Peak C (Fraction 2).

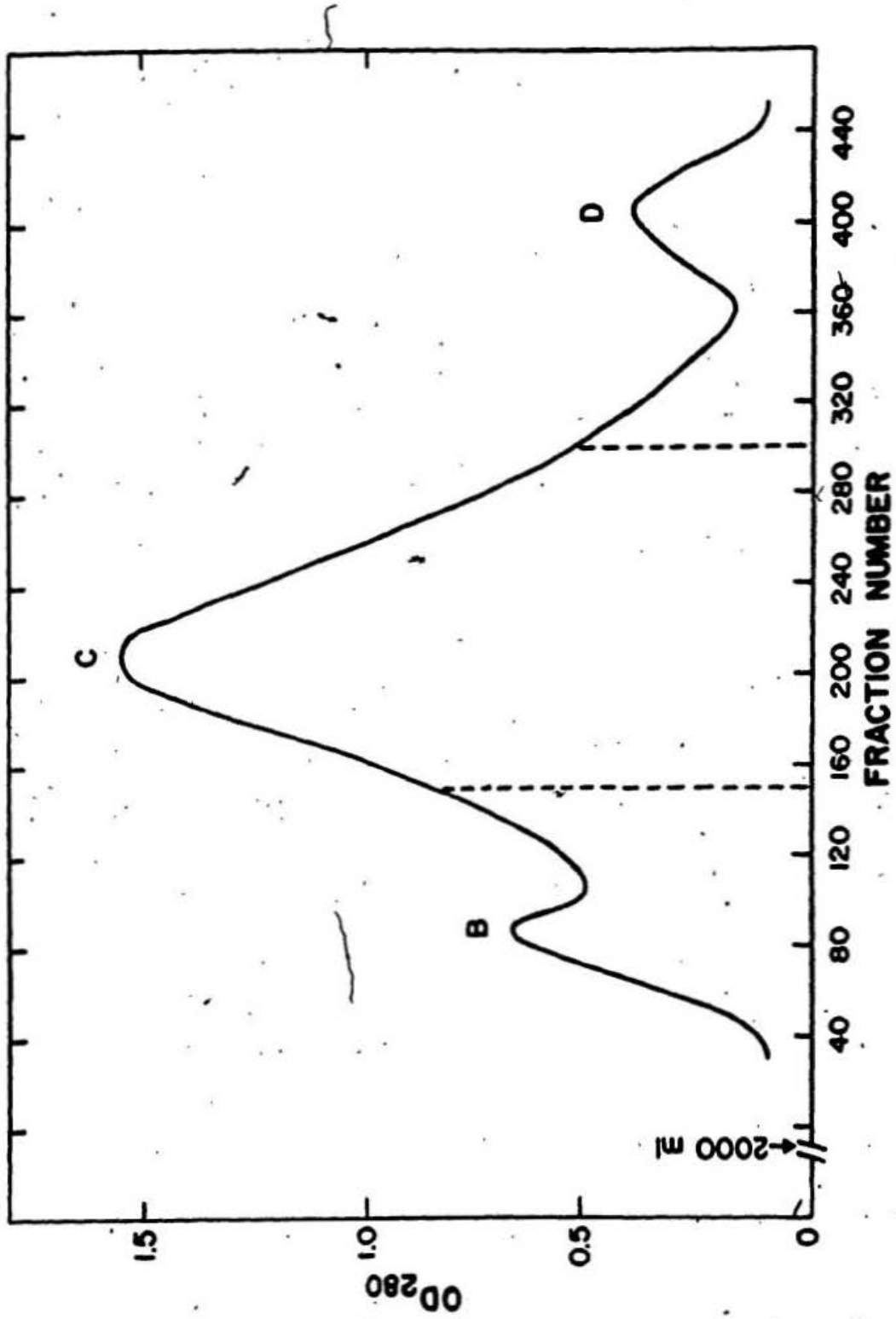

OD280
1.5
1.0
0.5
0
2000 ml
40
80
120
160
200
240
280
320
360
400
440
FRACTION NUMBER
B
C
D

Figure 4. Re-chromatography of Peak C (Fig. 3) on Ultrogel AcA 44 double columns connected in series. Fractions of 4.5 ml were collected at a flow rate of 60 ml/hr and protein was monitored by OD_{280}.

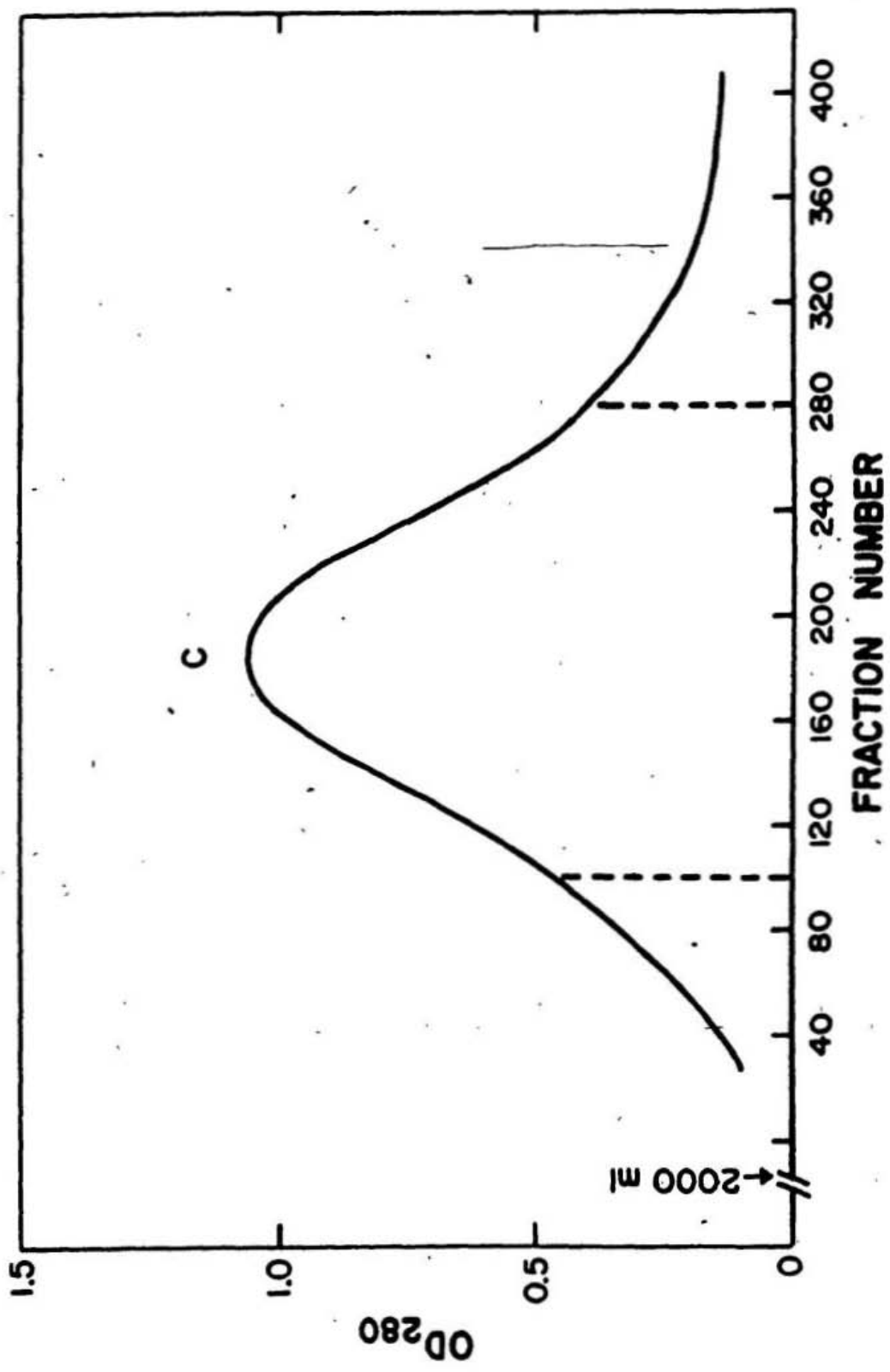
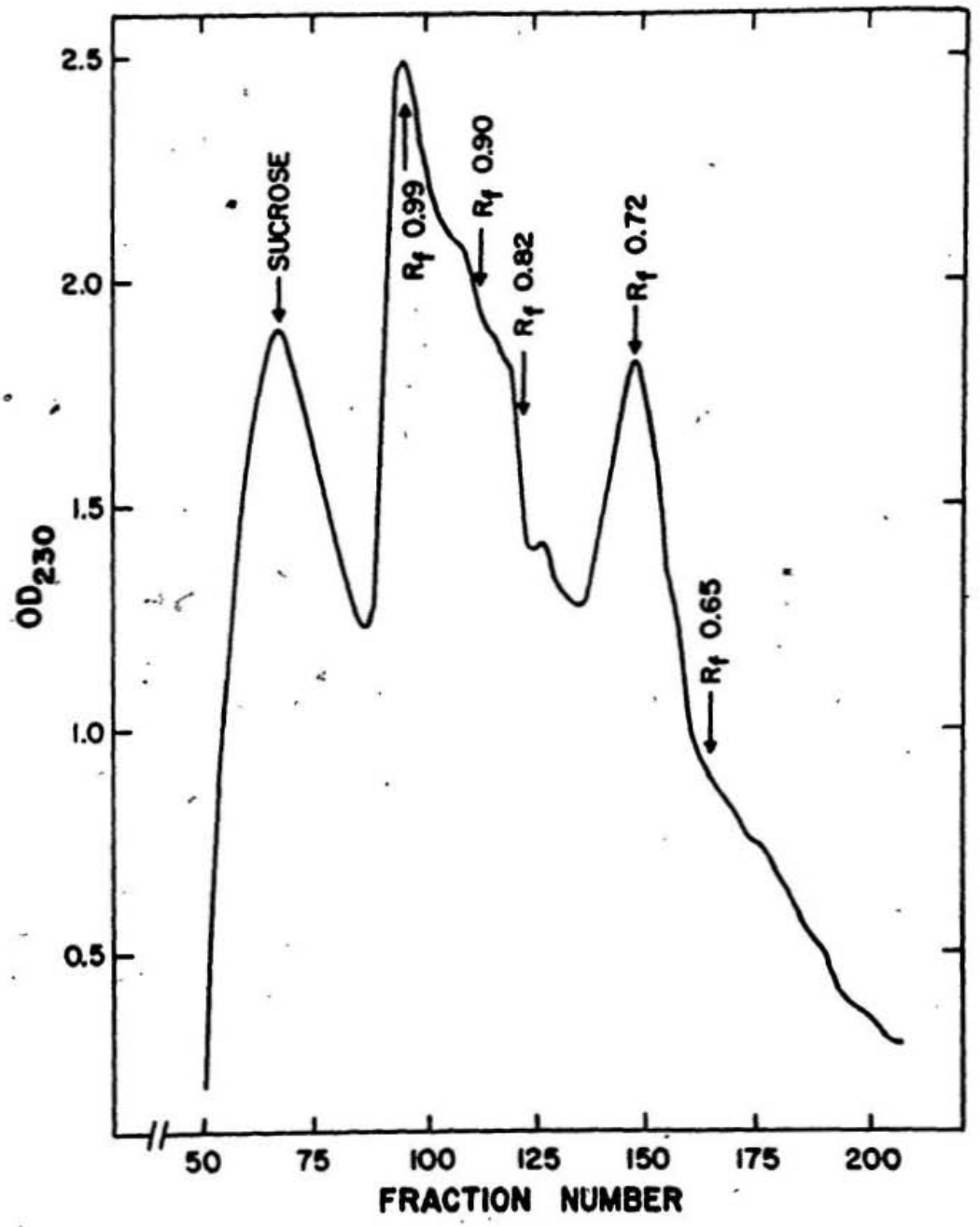

C
OD280
1.5
1.0
0.5
0
2000 ml
40
80
120
160
200
240
280
320
360
400
FRACTION NUMBER

Figure 5. Preparatory polyacrylamide gel electrophoresis (prep. PAGE) of flounder Con AI DEAE II-IV prepared from the May batch pituitaries. 7.5% non-denaturing gel, pH 8.9 was polymerised overnight with a 4% stacking gel, pH 6.8 and 14.8 mg of the sample in 2 ml containing a final concentration of 10% sucrose was electrophoresed at 320 volts for 15 hr. The electro-eluted proteins were eluted from the column with the lower buffer at a flow rate of 15 ml/hr and fractions of 0.7 ml were collected. Protein was monitored by OD_{230}. The entire process was carried out at 4 C.


2.5
2.0
1.5
1.0
0.5
OD230
SUCROSE
Rf 0.99
Rf 0.90
Rf 0.82
Rf 0.72
Rf 0.65
50
75
100
125
150
175
200
FRACTION NUMBER

un-polymerised acrylamide gel residues. The later peaks represented different proteins and particularly one peak was sharp and symmetrical. This peak had a Rf of 0.72 on 7.5% PAGE.

Polyacrylamide gel analyses of Con AII fraction and Con AI fraction components

PAGE (7.5%) and SDS-PAGE (15%) done at each stage of fractionation indicated the specific proteins in various preparations.

(a) PAGE (7.5%)

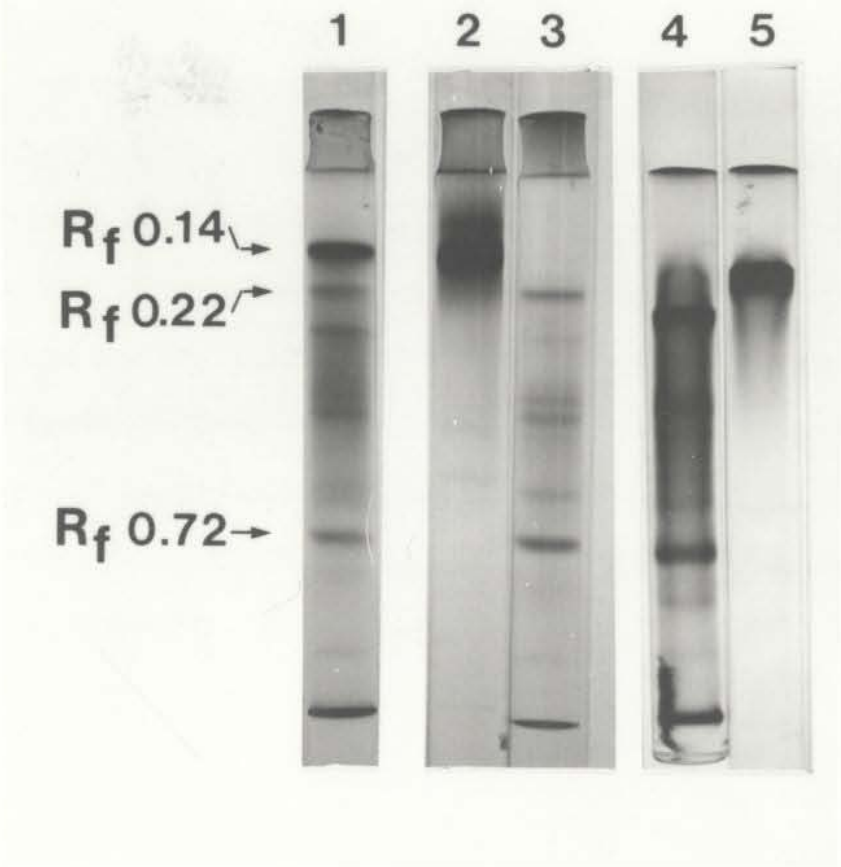
(i) Con AI

Band pattern for Con AI fraction was essentially the same for the May batch when compared to the September batch pituitary preparations, except that a band of Rf:0.22-0.23 was very minor for the September batch pituitaries when compared to the May batch. Remaining bands were the same in both the preparations. The Rf of these bands were as follows: 0.15-0.16 which is a major band; 0.22-0.23; 0.27-0.28; several minor bands between 0.37 to 0.45; 0.60-0.64; 0.72-0.73, a major band; 0.88; and 0.99 (Fig. 6).

(ii) DE I, DE II-IV

Band patterns on 7.5% PAGE done at subsequent stages of fractionation corresponded very well to the original Con AI fraction bands (Fig. 4), which allowed to monitor the presence and purity of the different preparations. DEAE Bio-Gel A chromatography separated the major protein species of Rf 0.15-0.16 (DE I). The remaining collective fraction, DE II-IV, had the remaining protein species, Rf 0.72-0.73 being the major. However, DE I fraction had other minor bands from DE II-IV

Figure 6. Non-denaturing polyacrylamide gel electrophoresis (7.5% disc gel) of various fractions from flounder pituitaries. Lane 1 contains 40 µg Con AI fraction, lane 2 represents 40 µg DEAE I and lane 3, 15 µg DEAE II-IV. These preparations were from the September batch pituitaries. Lanes 4 and 5 contain 60 µg DEAE II-IV and 40 µg DEAE I, prepared from the May batch pituitaries. The gels were stained overnight with Coomassie blue R-250. Note the quantitative difference in growth hormone (Rf 0.22) from the September and the May DEAE II-IV preparations.


1
2
3
4
5
Rf 0.14
Rf 0.22
Rf 0.72

indicating an incomplete separation on DEAE Bio-Gel chromatography.

(iii) prep. PAGE eluants

The electroeluted fractions from prep. PAGE indicated that the different proteins present in DE II-IV were well separated (Fig. 7). The main protein species in DE II-IV was well separated and gave a single band of Rf 0.72.

(iv) Con AII fraction

PAGE pattern for Con AII fraction was completely different from Con AI fraction. Con AII appeared to have more acidic and/or high molecular weight proteins being held at the stacking gel, when compared to Con AI fraction. Con AII had a major band of Rf 0.07-0.08, a minor band of Rf 0.10, several bands between 0.4-0.57, and another major band at 0.66 (Fig. 8).

(b) SDS-PAGE

SDS-PAGE (~~15%~~) for various preparations of pituitary extract gave a band pattern which corresponded to 7.5% PAGE and indicated the molecular weights of different species of proteins and purity of different preparations.

Con AI fraction had two main protein species of molecular weights, 24 K and 14.3 K. There were several minor bands representing high molecular weight and low molecular weight proteins (Fig. 9). DE I had 3 major components, 24 K, 21 K and a 14.3 K. DE II-IV revealed that the 24 K band in Con AI fraction had actually two protein species of molecular weights, 24 K and 23 K, one from Rf 0.15-0.16 (DE I) and the other from Rf 0.22-0.23. The band at 14.3 K, corresponding to Rf 0.72 on 7.5% PAGE, was another major component in DE II-IV. The Rf 0.72 protein preparation from prep. PAGE gave a major band on SDS-PAGE of

Figure 7. Non-denaturing polyacrylamide gel analysis (7.5% disc gel) of flounder DEAE II-IV prep. PAGE eluants representing the fractions from different peaks. Lanes 5 to 11 represent the samples from Rf 0.72 protein peak, lane 8 being the peak tube. The gels were stained with Coomassie blue G-250 while fixing in 12% TCA for 30 min and then stained overnight with Coomassie blue R-250.

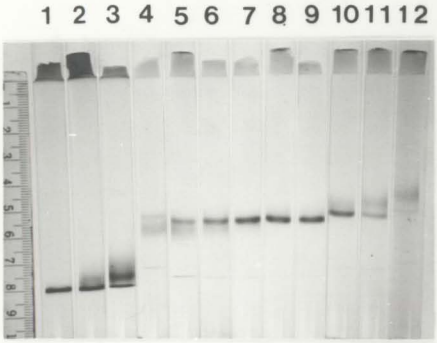
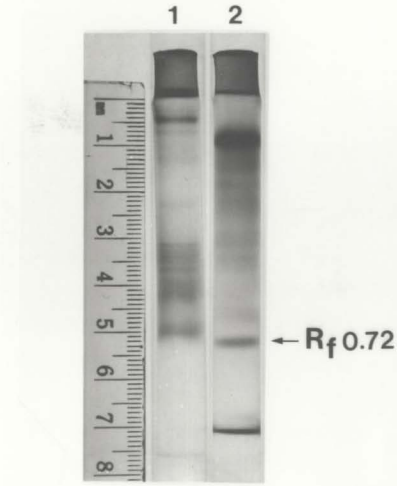

1 2 3 4 5 6 7 8 9 10 11 12

Figure 8. Non-denaturing polyacrylamide gel (7.5% disc gel) analysis of flounder Con AI and Con AII preparations from the September batch pituitaries. Lane 1 contains 30 µg Con AII and lane 2 contains 40 µg Con AI. Gels were stained with Coomassie blue G-250 while fixing in 12% TCA and then the bands were intensified by staining with Coomassie blue R-250, overnight.


1
2
1
2
3
4
5
6
7
8
← Rf 0.72

molecular weight 14.3 K (Fig. 9). There were two other minor bands of higher molecular weights in this preparation.

BIOASSAYS

1. Effect of hypophysectomy on the gonadal uptake of plasma proteins

(i) Peak A proteins(s)

Hypophysectomy significantly decreased the ovarian uptake of ^{131}I Peak A protein(s) when compared to sham operated animals. The incorporation into TCA precipitable (protein) and phosphoprotein fraction of the ovary was markedly decreased in hypophysectomised animals. However, in the case of males, the uptake in hypophysectomised animals was not different from the sham operated and the uptake was too low to be of any significance (Table 1). This experiment was done in December-January, when the males were fully matured and the sperm were motile.

(ii) Vitellogenin

In a bioassay experiment done during January-February, hypophysectomy significantly decreased the ovarian uptake of ^{131}I vitellogenin, when compared to sham operated or intact animals. There was a significant difference between hypophysectomised and sham operated or intact animals in the TCA precipitable (protein) fraction. However, there was no significant difference between these groups in phosphoprotein precipitate fraction of the yolk (Table 2).

Figure 9. Polyacrylamide gel (15%) analysis (SDS-PAGE) of various pituitary preparations from the September batch pituitaries. Lane 9 contains 37 µg Con AII fraction, lanes 8 and 7 contain 37 µg and 75 µg Con AI fraction, lane 6 contains 35 µg DEAE I, lanes 5 and 4 contain 35 µg and 45 µg DEAE II-IV and lanes 3 and 2 represent 8 µg and 10 µg of Rf 0.72 protein from prep. PAGE. Lane 1 contains the high molecular weight markers (BRL). The gel was stained with Coomassie blue R-250.

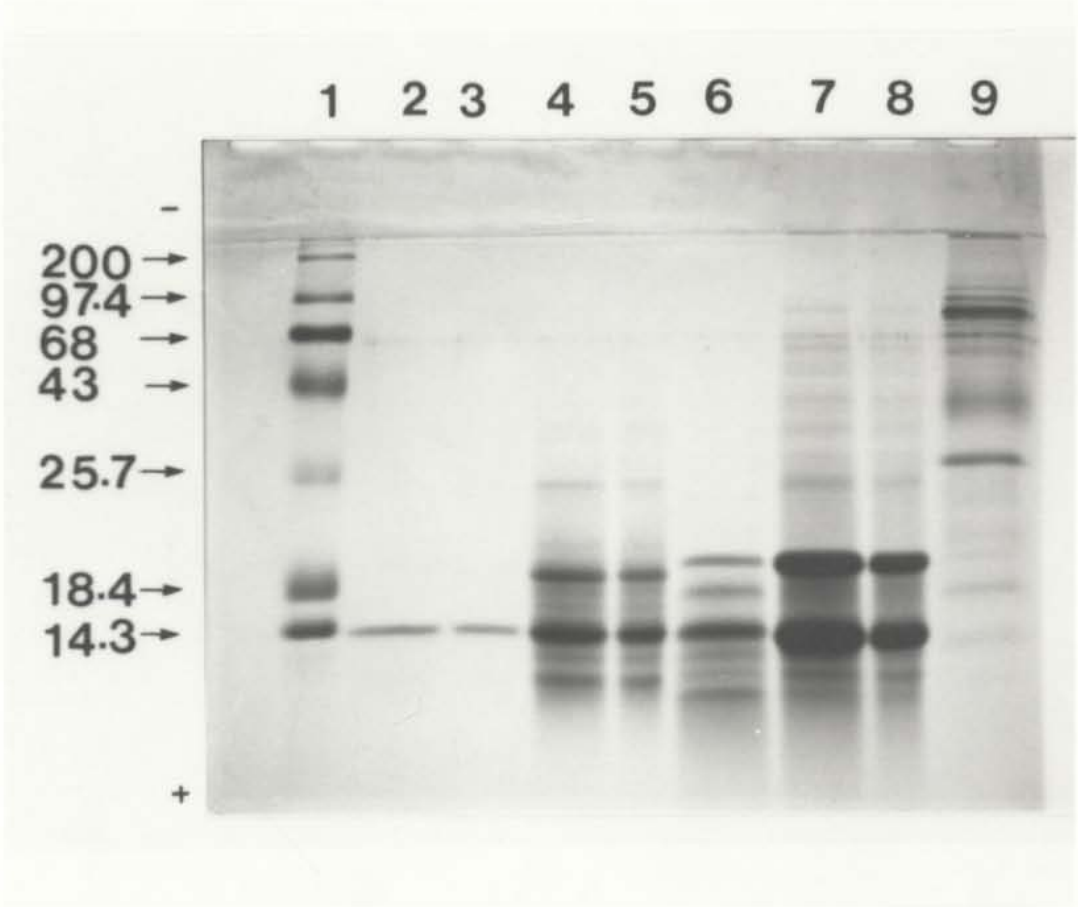

1 2 3 4 5 6 7 8 9
-
200
97·4
68
43
25·7
18·4
14·3
+

TABLE 1. EFFECT OF HYPOPHYSECTOMY ON THE GONADAL UPTAKE OF PEAK A PROTEIN, ISOLATED FROM FLOUNDER PLASMA

Experiment	Sex	Date	Group	Animal	Number of fish	Total counts (cpm/g of gonad)	TCA precipitate (cpm/g of gonad)	Phosphoprotein precipitate (cpm/g of gonad)
1	Female	4/3/85-25/3/85	1	Sham operated	7	1611 ± 95	844 ± 49	87 ± 3.9
			2	Hypophysectomised	8	1204 ± 46	552 ± 22	81 ± 3.9
						($P < 0.01$)	($P < 0.001$)	(NSD)
2	Female	28/3/85-16/4/85	1	Sham operated	8	3736 ± 272	1518 ± 86	274 ± 12.6
			2	Hypophysectomised	8	2787 ± 135	1063 ± 39	186 ± 10
						($P < 0.01$)	($P < 0.001$)	($P < 0.001$)
3	Male	21/12/85-5/1/86	1	Sham operated	8	274 ± 13	216 ± 13	-
			2	Hypophysectomised	8	256 ± 18	220 ± 9	*
						(NSD)	(NSD)	

All fish received 2×10^6 cpm/0.1 mL/500 g body weight.
All values are corrected for ^{131}Iodine decay.
*Phosphoprotein precipitation from yolk extract was not done owing to very low counts in the testis.

TABLE 2. EFFECT OF HYPOPHYSECTOMY ON THE OVARIAN UPTAKE OF VITELLOGENIN ISOLATED FROM FLOUNDER PLASMA.

Experiment	Sex	Date	Group	Animal	Number of Fish	Total counts (cpm/g of ovary)	TCA precipitate (cpm/g of ovary)	Phosphoprotein precipitate (cpm/g of ovary)
1	Female	26/1/85-8/2/85	1	Hypophysectomised	7	2150 ± 80	675 ± 40	40 ± 4
			2	Sham operated	9	2730 ± 135 ($P < 0.01$)	765 ± 10 ($P < 0.05$)	36 ± 7 (NSD)
			3	Intact	7	2460 ± 120 ($P < 0.05$)	780 ± 40 ($P < 0.1$)	45 ± 2 (NSD)

All fish received 2×10^6 cpm/0.1 ml/500 g body weight.
Values are corrected for ^{131}Iodine decay.

(iii) Peak E protein(s)

Hypophysectomy significantly decreased the ovarian uptake of ^{131}I Peak E protein(s) and also the TCA precipitable (protein) and phosphoprotein fractions of the ovary, when compared to sham operated animals (Table 3).

2. Effect of Con AI DE II-IV and Rf 0.72 protein of DE II-IV on the gonadal uptake of Peak A protein(s)

DE II-IV and Rf 0.72 protein increased the ovarian uptake of ^{131}I Peak A protein(s) significantly above those of the control group when administered at doses of 800 µg and 200 µg/kg body weight/ injection of DE II-IV and 110 and 100 µg/kg body weight/ injection of Rf 0.72 protein. In addition, the incorporation of injected protein into TCA-precipitable (protein) and phosphoprotein fractions of the ovary was markedly enhanced (Table 4

However, when the fish were ready to spawn (during June), the difference between the Rf 0.72 protein treated and control animals were very low in terms of ovarian uptake, TCA-precipitable (protein) and phosphoprotein fractions.

Effect of sockeye salmon Con AI fraction and flounder Con AI DE-II-IV on the gonadal uptake of vitellogenin

In the bioassay conducted during November (31/10/85-20/11/84), the ovarian uptake of ^{131}I vitellogenin was significantly higher in sockeye salmon Con AI fraction and flounder DE II-IV treated animals,

TABLE 3. EFFECT OF HYPOPHYSECTOMY ON THE OVARIAN UPTAKE OF PEAK E PROTEIN(S) ISOLATED FROM FLOUNDER PLASMA

Experiment	Date	Group	Animal	Number of fish	Total counts (cpm/g of ovary)	TCA precipitate (cpm/g of ovary)	Phosphoprotein precipitate (cpm/g of ovary)
1	4/4/85-25/4/85	1	Sham operated	7	3560 ± 75	2530 ± 80	570 ± 10
		2	Hypophysectomised	5	2915 ± 130 ($P < 0.01$)	1990 ± 20 ($P < 0.001$)	415 ± 30 ($P < 0.001$)

All fish received 2×10^6 cpm/0.1 ml/500 g body weight.
Values are corrected for ^{131}Iodine decay.

TABLE 4. EFFECT OF CARBOHYDRATE-POOR GONADOTROPIC HORMONE FRACTIONS ISOLATED FROM FLOUNDER PITUITARIES ON THE OVARIAN UPTAKE OF PEAK A PROTEIN(S) ISOLATED FROM FLOUNDER PLASMA IN HYPOPHYSECTOMISED FEMALE FLOUNDERS

Experiment	Date	Group	Treatment	Dose (µg/kg)	Number of fish	Total counts (cpm/g of ovary)	TCA precipitate (cpm/g of ovary)	Phosphoprotein precipitate (cpm/g of ovary)
1	30/11/84-19/12/84	1	Buffer	-	6	2605 ± 260	1765 ± 120	390 ± 50
		2	DEAE II-IV	800	6	4270 ± 295	2565 ± 120	545 ± 60
						(P < 0.01)	(P < 0.001)	(P < 0.1)
2	4/1/85-22/1/85	1	Buffer	-	8	2005 ± 150	1345 ± 80	197 ± 10
		2	DEAE II-IV	800	9	2680 ± 125	1635 ± 73	210 ± 15
						(P < 0.1)	(P < 0.02)	(NSD)
3	14/5/85-3/6/85	1	Buffer	-	5	1620 ± 410	565 ± 40	130 ± 20
		2	DEAE II-IV Rf 0.72	110	5	3090 ± 335	725 ± 65	165 ± 15
						(P < 0.05)	(P < 0.1)	(P < 0.2)
*4	2/10/85-30/10/85	1	Buffer	-	6	460 ± 35	345 ± 20	80 ± 5
		2	DEAE II-IV	200	5	620 ± 25	490 ± 35	125 ± 20
		3	Rf 0.72	100	7	655 ± 55	515 ± 55	125 ± 9
						(P < 0.02)	(P < 0.02)	(P < 0.01)
5	12/11/85-4/12/85	1	Buffer	-	8	1510 ± 80	920 ± 45	435 ± 45
		2	DEAE II-IV Rf 0.72	100	7	1900 ± 100	1180 ± 65	595 ± 30
						(P < 0.01)	(P < 0.01)	(P < 0.02)

All fish received 2 x 10^6 cpm/0.1 mL/500 g body weight except in experiment, *4 fish received 0.8 x 10^6 cpm/0.1 ml/500 g body weight.
Values are corrected for ^{131} Iodine decay.
All fish were injected with estradiol benzoate at a rate of 10 µg/500 g body weight with the first hormone/buffer injection.

compared to control animals, when administered at doses of 1000 µg and 800 µg/kg body weight/injection respectively. The incorporation into TCA-precipitable (protein) and phosphoprotein fraction of the ovary was also markedly increased in the hormone treated groups. However, bioassay experiments for flounder DE II-IV, conducted during December, January and February failed to give any significant differences between the hormone treated animals and control animals, when tested at 800 µg/kg body weight/injection (Table 5). Sockeye salmon Con AI fraction was not tested in these experiments for biological activity nor was the biological activity of Rf 0.72 protein of DE II-IV for the uptake of vitellogenin by the ovary.

Effect of flounder Con AI DE II-IV on the ovarian uptake of Peak E protein(s)

Only one bioassay experiment was conducted for the uptake of Peak E protein(s) (in December). The ovarian uptake of ^{131}I Peak E protein(s) was significantly higher in DE II-IV injected animals compared to buffer injected control animals, when tested at a dose of 800 µg/kg body weight/injection. Incorporation into ovarian TCA-precipitable (protein) and phosphoprotein fraction were also significantly higher in the hormone injected group than the control group (Table 6).

3. Comparison of ovarian uptake of Peak A protein(s) and vitellogenin

In two experiments conducted during December and February, ovarian

TABLE 5. EFFECT OF CARBOHYDRATE-POOR GONADOTROPIC HORMONE FRACTIONS ISOLATED FROM FLOUNDER PITUITARIES ON THE GONADAL UPTAKE OF FLOUNDER VITELLOGENIN IN HYPOPHYSECTOMISED FEMALE FLOUNDERS

Experiment	Date	Group	Treatment	Dose (µg/kg)	Number of fish	Total counts (cpm/g of ovary)	TCA precipitate (cpm/g of ovary)	Phosphoprotein precipitate (cpm/g of ovary)
1	31/10/84-20/11/84	1	Buffer	-	6	5170 ± 340	2870 ± 575	225 ± 16
		2	Sockeye salmon Con AI	1000	7	8465 ± 1095 ($P < 0.02$)	5800 ± 386 ($P < 0.01$)	400 ± 22 ($P < 0.001$)
		3	Flounder Con AI DEAE II-IV	800	7	7790 ± 345) ($P < 0.001$)	5800 ± 135 ($P < 0.001$)	415 ± 28 ($P < 0.001$)
2**	2/12/84-22/12/84	1	Buffer	-	5	14920 ± 2550	8015 ± 2285	1670 ± 145
		2	Flounder Con AI DEAE II-IV	800	6	19165 ± 2420 (NSD)	8450 ± 1285 (NSD)	1715 ± 140 (NSD)
3	14/1/85-5/2/85	1	Buffer	-	9	1645 ± 165	835 ± 50	90 ± 9
		2	Flounder Con AI DE II-IV	800	9	1590 ± 125 (NSD)	770 ± 25 (NSD)	75 ± 16 (NSD)
4	26/1/85-8/2/8585	1	Buffer	-	7	2150 ± 80	675 ± 40	40 ± 4.0
		2	Flounder Con AI DE II-IV	800	6	2100 ± 100 (NSD)	645 ± 30 (NSD)	40 ± 3.0 (NSD)

All fish received 2 x 10^6 cpm/0.1 mL/500 g body weight, except experiment #2* where fish received 4 x 10^6 cpm/0.1 ml/500 g body weight.
Values are corrected for ^{131}Iodine decay.
All fish were injected with estradiol benzoate at a rate of 10 µg/500 g body weight with the first hormone injection.

TABLE 6. EFFECT OF CARBOHYDRATE-POOR GONADOTROPIC HORMONE FRACTIONS ISOLATED FROM FLOUNDER PITUITARIES ON THE OVARIAN UPTAKE OF PEAK E PROTEIN(S) ISOLATED FROM FLOUNDER PLASMA IN HYPOPHYSECTOMISED FEMALE FLOUNDER

Experiment	Date	Group	Treatment	Dose (µg/kg)	Number of fish	Total counts (cpm/g of ovary)	TCA precipitate (cpm/g of ovary)	Phosphoprotein precipitate (cpm/g of ovary)
1	2/12/84-24/12/84	1	Buffer	-	4	1695 ± 175	1305 ± 95	165 ± 10
		2	Flounder Con AI DEAE II-IV	800	5	5260 ± 730 ($P < 0.01$)	3640 ± 650 ($P < 0.01$)	450 ± 75 ($P < 0.01$)

All fish received 2×10^6 cpm/0.1 mL/500 g body weight.
Values are corrected for ^{131}Iodine decay.

uptake of ^{131}I Peak A protein(s) was significantly higher than the ^{131}I vitellogenin uptake, when tested in intact flounders. The significance markedly increased between the two groups, in ovarian TCA-precipitable (protein) and phosphoprotein fractions; Peak A protein(s) incorporation was higher (Table 7).

Interestingly, the liver and plasma counts were much lower in the vitellogenin injected group than in the Peak A protein(s) injected group (Table 7).

4. Time course study of ovarian uptake of Peak A protein(s) over a period of six days in hypophysectomised and sham operated flounders

When ^{125}I labelled Peak A protein(s) was injected into hypophysectomised and sham operated flounders (January), the ovarian uptake was considerably higher in sham operated fish compared to the hypophysectomised fish (Table 8). The initial rate of uptake in hypophysectomised flounder was quite high, however, in the second and third biopsies (four and six days after injection), the rate of uptake had reached a plateau indicating no more uptake after two days. In sham operated flounders, the uptake increased exponentially in the first four or five days after the injection. However, the subsequent rate of uptake declined considerably (Fig. 10). Values from later biopsies failed to show any significant correlation to the above uptake trend, due to rupturing of the ovarian wall caused by repeated biopsies.

The drop in plasma counts of ^{125}I Peak A protein(s) for

TABLE 7. COMPARISON OF OVARIAN UPTAKE OF PEAK A PROTEIN(S) AND VITELLOGENIN IN INTACT FLOUNDERS

Experiment	Date	Group	Injected I labelled protein	Number of fish	Total counts (cpm/g of ovary)	TCA precipitate (cpm/g of ovary)	Phosphoprotein precipitate (cpm/g of ovary)	Liver counts (cpm/g of liver)	Plasma counts (cpm/ml of plasma)
1	4/12/85-11/12/85	1	Flounder Peak A protein(s)	8	1794 ± 101	1180 ± 41	260 ± 13	11349 ± 1841	61172 ± 2377
		2	Flounder vitellogenin	8	1528 ± 107 ($P < 0.1$)	766 ± 45 ($P < 0.001$)	95 ± 6 ($P < 0.001$)	8091 ± 1427 ($P < 0.1$)	4881 ± 348 ($P < 0.001$)
2	15/2/85-23/2/85	1	Flounder Peak A protein(s)	8	3176 ± 201	2315 ± 100	370 ± 21	1400 ± 1331	34400 ± 1815
		2	Flounder vitellogenin	7	2360 ± 119 ($P < 0.01$)	750 ± 40 ($P < 0.001$)	56 ± 2 ($P < 0.001$)	1085 ± 985 (NSD)	8010 ± 183 ($P < 0.001$)

All fish received 2×10^6 cpm/0.1 ml/500 g body weight.
Values are corrected for ^{131}I decay.

TABLE 8. OVARIAN UPTAKE OF ^{125}IODINE PEAK A PROTEIN(S) OVER A SIX DAY TIME PERIOD IN HYPOPHYSECTOMISED AND SHAM OPERATED FLOUNDERS

Experiment	Group	Animal	Number of fish	Sampling number	Date	Total counts (cpm/g of gonad)	Plasma counts (cpm/ml)
1	1	Sham operated	2	1	16/1/86	6710	159288
				2	18/1/86	7786 ± 113	127250
				3	20/1/86	8600 ± 146	92097
	2	Hypophysectomised	2	1	16/1/86	4730	167437
				2	18/1/86	5442 ± 173	108266
				3	20/1/86	5108 ± 200	77396

All fish were injected with 2.04×10^6 cpm/0.1 ml/500 g body weight on 14.1.86.
Values are not correced for ^{125}Iodine decay.

every two day interval in hypophysectomised and sham operated female flounders is presented in Figure 11.

Discussion

Polyacrylamide gel analyses

PAGE and SDS-PAGE analyses of flounder Con AI and Con AII preparations indicated that they have entirely different protein species and this is in agreement with the original findings of Idler et al. (1975a) based on Con A chromatographic behavior, Ng and Idler (1978 a,b; 1979), Idler and Ng (1979) in terms of chemical composition and biological activities, in many teleosts. By polyacrylamide gel analyses, it was possible to identify the three major protein species in Con AI: Prolactin (DE I, Idler et al., 1978) growth hormone (Idler et al., 1978; Komourdjian and Idler, 1979) and another protein species of Rf 0.72. As observed in this investigation, the low level of growth hormone in September coincides with the feeding behavior of flounder. They stop feeding when they enter the active reproductive phase, (September) and the synthesis of growth hormone would not be necessary. However, the level of prolactin and Rf 0.72 protein appeared to be the same in both phases; this complemented the biological activity of DE II-IV and Rf 0.72 protein, prepared from pituitaries of both the phases of ovarian growth.

BIOASSAYS

Hormone preparations and plasma proteins

Flounders were used from two phases (May and September) of their reproductive cycle. This was to find out the biological activity of various Con AI fractions prepared in terms of uptake of Peak A protein(s), vitellogenin and Peak E protein(s).

The successful labelling of plasma proteins by the Iodogen method provided a direct measure of the ovarian uptake of these proteins and their subsequent incorporation into ovarian proteins. This was an improvement over the indirect method, which utilized leucine and phosphate (Ng and Idler, 1978 a,b; 1979).

Naming of hormones

The term Con AI fraction or carbohydrate-poor gonadotropic hormone has been used in this study instead of vitellogenic hormone, as proposed by Campbell and Idler (1976). Vitellogenesis rather refers to synthesis of vitellogenin by the liver under the influence of estrogens (Plack and Fraser, 1970; Plack et al., 1971; Idler and Campbell, 1980). The incorporation of this protein in winter flounder occurs for a shorter period of time, from August to January. One of the Con AI components, Rf 0.72 protein, studied here stimulates the uptake of Peak A protein(s), for a longer period of the reproductive cycle. The term vitellogenic implies that only vitellogenin is taken up by the oocyte and this study shows that this is not the case. In addition, the Rf

0.72 protein was found in both the reproductive phases and was not restricted to the active vitellogenin incorporation phase alone. The term Rf 0.72 protein applied to this component is based on its electrophoretic mobility on a non-denaturing 7.5% gel.

Biological activity and uptake studies

1. Influence of hypophysectomy on the gonadal uptake of plasma protein.

The reduced ovarian uptake of plasma proteins in hypophysectomised female flounder compared to sham operated or intact controls clearly provided evidence for the role of certain pituitary hormone(s) in stimulating their uptake. The uptake of Peak A protein(s) obtained with male flounders in the experiment conducted during January was very valuable in this context. Significantly low uptake with no difference between hypophysectomised and sham operated males when compared to females during and after December-January suggested that the uptake in males stops by this time. It has been observed in this study that before January, there was uptake in males comparable to that of females, when a few males were present in the experiments with females. The immunoprecipitation studies (Chapter III) of the testicular extracts confirm the above finding. During January, the sperm were motile in seawater. Earlier, Burton and Idler (1984) recorded a maximum increase in GSI from mid-October to mid-November, and by January activable sperm were present. Following the second meiotic division of the spermatocyte, the relatively unspecialized spermatid commences a remarkable nuclear and cytoplasmic transformation,

spermiogenesis, resulting in formation of the mature sperm (Grier et al., 1981). This may mark the time limit of Peak A protein(s) uptake in males. Females reach their final stage of maturity only just before spawning and this could explain the continued uptake of Peak A protein(s), until spawning. The uptake of vitellogenin and Peak E protein(s) was not tested in males.

2. Influence of Con AI - DE II-IV and Rf 0.72 protein from DE II-IV on the ovarian uptake of plasma proteins.

Campbell and Idler (1976) for the first time illustrated the ability of the Con AI fraction to stimulate the ovarian uptake of $H_3{}^{33}PO_4$ and ^{3}H-leucine. Con AI contains four major components: DE I, DE II, DE III, and DE IV (Ng and Idler, 1978a,b). Ng and Idler (1978a,b; 1979) and Idler and Ng (1979) narrowed down the stimulatory effect of Con AI fraction to DE III. They also observed that the ovarian uptake of serum proteins labelled 'in vivo' with $H_3{}^{33}PO_4$ and ^{3}H leucine was stimulated by Con AI 28 K (Peak C) DE III CM I fraction and there was no activity in the DE I fraction. The DE I fraction identified as prolactin from salmon was reported to be active in maintaining sodium levels in hypophysectomised Poecilia latipinna (Idler et al., 1978). Similarly DE II was identified as growth hormone and its somatotropic activity has been demonstrated (Idler et al., 1978; Komourdjian and Idler, 1979). Con AI fraction from flounder has another protein species, the Rf 0.72 protein. The stimulatory effect of DE II-IV fraction and Rf 0.72 protein in the ovarian uptake of plasma protein observed in this study confirmed that this protein is the gonadotropin. Although the effect of Rf 0.72 protein in facilitating vitellogenin and Peak E protein(s) uptake was

not tested, the stimulatory effect of DE II-IV on their uptake and the results obtained with Peak A protein(s), suggest that Rf 0.72 protein would influence the uptake of these proteins also. It was reported that tetrapod LHs were active in the lizard (Licht and Crews, 1975; Licht et al., 1977) 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. Hence the stimulatory effect of sockeye salmon Con AI fraction in ovarian uptake of vitellogenin by flounders was not surprising, in spite of a species difference.

The failure of Rf 0.72 protein to increase the ovarian uptake of Peak A protein(s) significantly during June could be due to a slower rate of Peak A protein(s) uptake since the fish were ready to spawn. With respect to vitellogenin, DE II-IV fraction failed to increase the ovarian uptake during December, January and February. This suggests the possibility that the fish had already assimilated vitellogenin and the active uptake had stopped. The dosage of DE II-IV given was quite potent which rules out the possibility of dosage below the minimum effective dose.

In some experiments, significant differences were not obtained in phosphoprotein precipitation of the ovarian extract between the two groups. Unlike the TCA precipitates, phosphoprotein precipitates represent the protein that would precipitate with $CaCl_2$ solution. Results from analysis of this precipitation on SDS-PAGE indicated that such a precipitation was not quantitatively complete (Chapter III). Hence, the results from $CaCl_2$ precipitation are not always a good

indicator of the stimulatory effect of gonadotropin on the incorporation of plasma proteins.

3. Ovarian uptake of Peak A protein(s) and vitellogenin during late February.

In all the bioassay and uptake studies, vitellogenin, having a well established ovarian uptake and function, served as a positive control with respect to ovarian uptake of other plasma proteins. The higher ovarian uptake of Peak A protein(s) compared to vitellogenin during late December and late February in normal flounders and the higher uptake of vitellogenin during October-November (unfortunately, a direct comparison was not made between these two proteins, when the GSI of the ovary was rapidly increasing) suggested a definite pattern between these two proteins in their ovarian uptake from the circulation. Sephacryl S-300 elution profiles of plasma from female flounders at different times support this possibility by indicating that relative differences in circulating levels of these plasma proteins coincide with the uptake pattern.

In the above experiment, in addition to other experiments on biological activity, the high plasma and liver counts in groups injected with Peak A protein(s) suggest a slower metabolic clearance rate (mcr) for this protein compared to vitellogenin.

4. Variable rates of ovarian uptake of Peak A protein(s) between sham operated and hypophysectomised flounders.

Continued ovarian uptake after four days in sham operated as opposed to hypophysectomised flounders, where the uptake reached a plateau, explained the high uptake in controls of previous bioassay experiments. The autopsy was done in those experiments after 48 hr and

the difference between the groups had not yet reached a maximum. However, further study is needed to interpret the ovarian uptake pattern observed in this experiment.

Figure 10. Time course study of the ovarian uptake of ^{125}I Peak A protein(s) for a period of 6 days. (o) indicates the uptake in hypophysectomised fish and (●) in sham operated fish. Biopsy was done on alternate days.

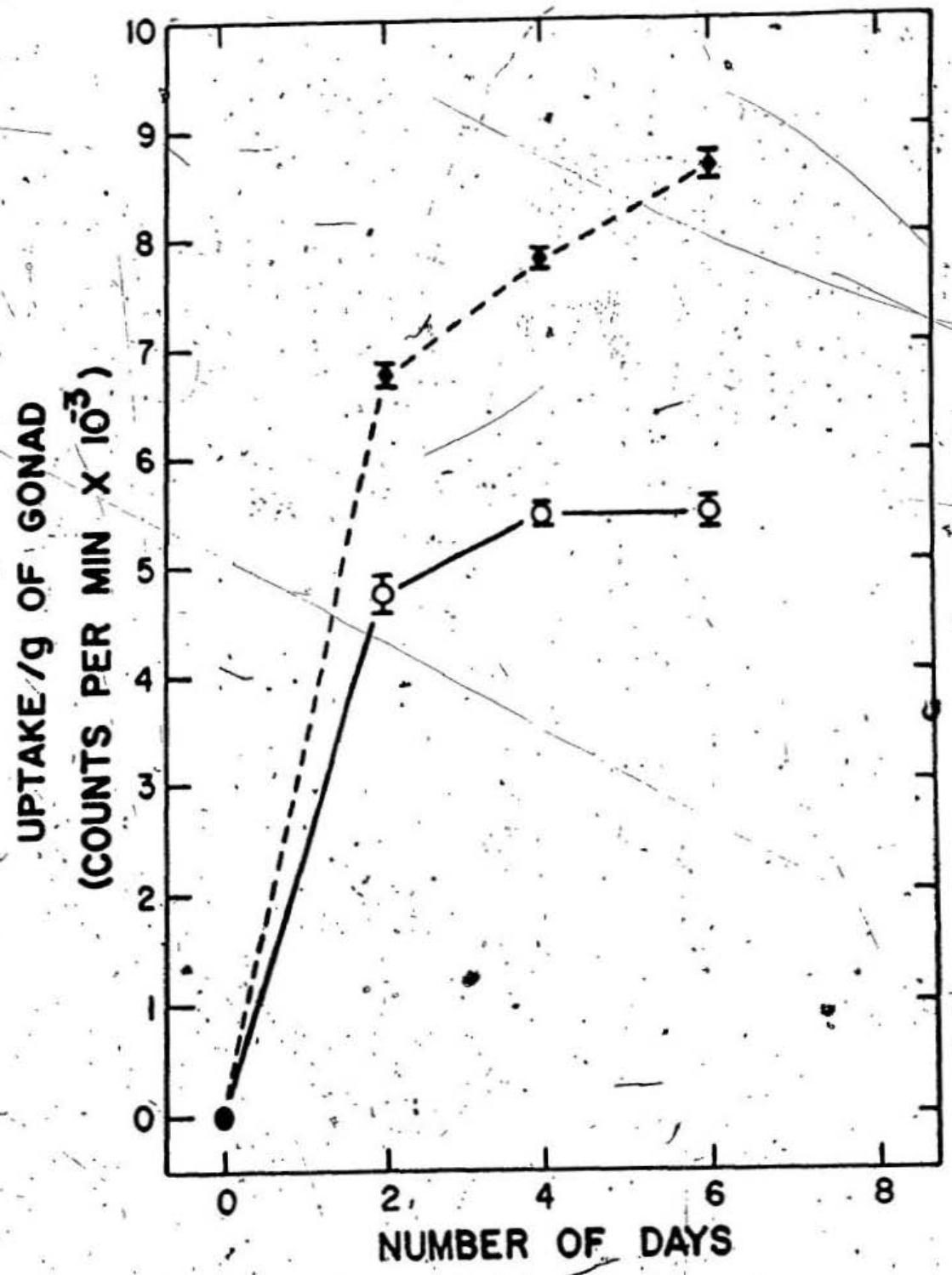
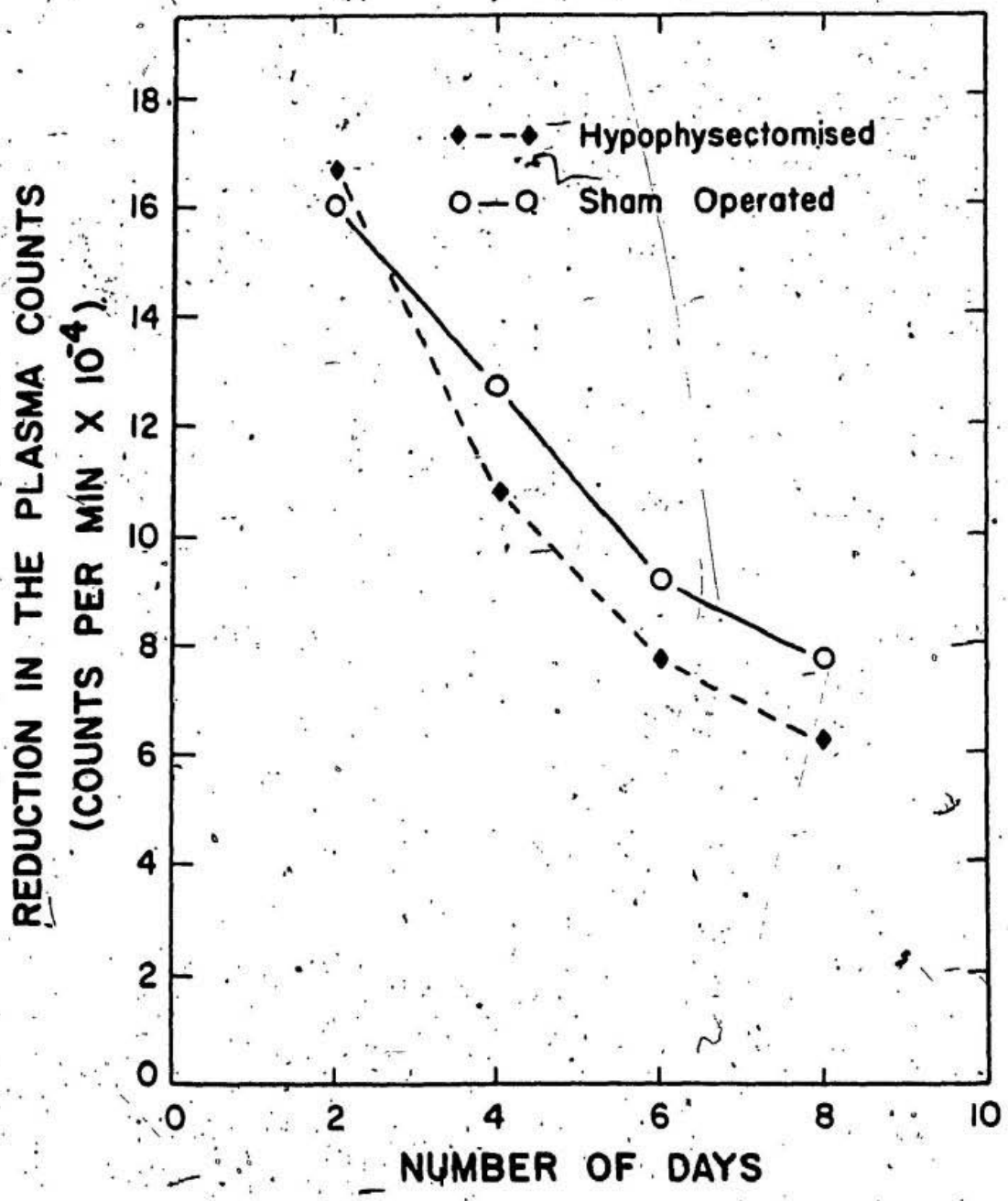

UPTAKE/g OF GONAD
(COUNTS PER MIN X 10^{-3})
NUMBER OF DAYS
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8

Figure 11. Loss of ^{125}I labelled flounder Peak A protein(s) from the plasma in hypophysectomised and sham operated fish. About 2 10^6 cpm/0.1 ml/500 g body weight of labelled protein was injected intravenously. One ml of the plasma was counted each time.


Hypophysectomised
Sham Operated
REDUCTION IN THE PLASMA COUNTS
(COUNTS PER MIN X 10^{-4})
NUMBER OF DAYS
0
2
4
6
8
10
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CHAPTER V

Summary

In teleosts, the importance of an enhanced understanding of ovarian biochemistry during oocyte growth is underlined by the observation that hormonal acceleration of the reproductive cycle of Atlantic salmon can yield eggs with highly variable viability (Crim and Glebe, 1984). Female winter flounder spawn generally from the middle of May to the middle of July, but the active ovarian uptake of vitellogenin stops in December-January. However, this investigation indicated the continued uptake of another plasma protein(s), Peak A protein(s), until May (Chapter IV) and also demonstrated that this plasma protein(s) is a yolk component. This implies that the process of ovarian development continues until spawning and an induced early spawning using hormones like LHRH might yield eggs of poor viability. The testicular protein analyses during the two phases of testicular growth indicated that Peak A protein(s) is the major testicular component (Chapter III). The presence of Peak A protein(s) in the circulation of male flounders was also demonstrated (Chapter II).

The isolation of a biologically active peptide component (Rf 0.72 protein) from the carbohydrate-poor gonadotropins (Chapter II) supports the original findings of the duality of gonadotropins in teleosts, reported from this laboratory (see Idler and Ng, 1983). In addition, the results from the bioassays indicated the stimulatory effect of this pituitary protein on incorporating Peak A protein(s) into oocytes and possibly testes. Although the biological activity of this protein in

stimulating the uptake of vitellogenin was not tested in this investigation, the stimulatory effect of the carbohydrate-poor fraction, and the DEAE. II-IV fraction on incorporating vitellogenin into oocytes observed in this investigation as well as in earlier studies reported from this laboratory, could be attributed to the Rf 0.72 peptide component.

These results extend the preliminary findings of Campbell and Jalabert (1979) that the ovarian uptake of vitellogenin in teleosts may not be highly selective. In addition, this investigation also indicated a common mechanism of gonadal growth in terms of synthesizing the ovarian and testicular proteins derived from the parental blood stream (heterosynthetic). The functional selection of different plasma proteins by the ovary and testis, regulated by a single gonadotropin, distinct from the more common carbohydrate-rich gonadotropin in a representative from the early vertebrate class is quite fascinating.

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