SEQUESTRATION OF VITELLOGENIN BY THE DEVELOPING OVARY OF THE RAINBOW TROUT, *SALMO GAIRDNERI*

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DECLARATION

The work described in this thesis was carried out between 1984-1987 at the University of Aston in Birmingham. It has been done independently and has not been submitted for any other degree.

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Abstract

An investigation was made into the nature and control of VTG sequestration into the developing ovary of the rainbow trout, *Salmo gairdneri*. It includes (1): *in vivo* studies which examined the uptake and subsequent processing of VTG, and selectivity of VTG deposition, and (2) *in vitro* experiments which established a method for culturing vitellogenic follicles and subsequently investigated the effects of VTG concentration, temperature, follicle size and hormones on the rate of VTG sequestration.

Gel-filtration chromatography in conjunction with a specific RIA for VTG confirmed that VTG, a glycolipophosphoprotein, (440 000 Daltons) was a major circulating protein in the blood during exogenous vitellogenesis. Incorporation of VTG into vitellogenic follicles and its subsequent processing to yolk proteins was monitored using $^{32}P.^{3}H$-VTG. $^{32}P.^{3}H$-VTG was processed in the oocytes into ^{3}H-lipovitellin (390 000 Daltons) and ^{32}P-phosvitin (40 000 Daltons). Once sequestered these proteins underwent no further proteolysis, for experimental periods up to 4 weeks. Quantitative analyses showed that VTG was sequestered at rates of up to 300 $ng.mm^{-2}$ follicle surface.$^{-1}$ hr^{-1}.

The uptake of VTG was highly selective ^{3}H-VTG being sequestered up to 60 times more rapidly than ^{14}C-BSA. Furthermore the BSA sequestered was degraded into an array of smaller peptides and subsequently exocytosed from the follicle. These results suggest that VTG entered the follicles by receptor mediated endocytosis.

Results from the *in vitro* studies showed that the surface epithelium was fairly impervious to VTG transport and had to be removed by dissection for subsequent protein uptake studies. Kinetic theory applied to the sequestration of VTG *in vitro* showed that labelling VTG with ^{125}Iodine had no pronounced effects on the structural integrity and uptake of VTG. The lability of the ^{125}I on the VTG molecule, however, limited its use to short term cultures (up to 8 hours). By contrast $^{32}P.^{3}H$-VTG proved to be an excellent ligand for *in vitro* studies; it was taken up avidly by the cultured follicles and was processed *in vitro* in a similar, albeit reduced, manner to that seen *in vivo*. Increases in temperature induced higher rates of VTG sequestration. The trout follicles were still able to sequester VTG below 5^{o}C. The concentration of VTG in the medium was perhaps the single most important parameter, of those investigated, controlling VTG sequestration, higher concentrations of VTG producing greater sequestration rates. The maximum rate of VTG uptake *in vitro* (~ 170 $ng.\ mm^{-2}.hr^{-1}$) was attained at a VTG concentration of 40-50 $mg.ml^{-1}$.

Follicles of different sizes sequestered VTG at different rates. Below a diameter of approximately 1mm, no VTG was sequestered. Above 1mm in diameter increasing follicle size led to an increased rate of VTG uptake, both per follicle and also when the rate of uptake was related to surface area. However, follicles approaching full-size, i.e. near ovulation, had reduced rates of VTG sequestration.

Investigations on the hormonal control of VTG sequestration by vitellogenic follicles in culture showed that both CHO-rich (maturational) GtH and insulin were capable of enhancing the rate of VTG uptake. Of the hormones investigated GtH was the most potent, increasing the rate of VTG sequestration by 34% at a dosage of 100ngml^{-1} (this GtH also induced ovarian steroidogenesis in a dose-dependent manner). The degree of stimulation of VTG uptake at the doses adopted suggested that GtH may not have a strong, driving influence on follicle growth throughout the vitellogenic cycle, as has frequently been implied in the literature.

Keywords: Vitellogenin, vitellogenesis, follicle, sequestration, trout.

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GLOSSARY

ANOVA	analysis of variance
apo-Lv-1	apolipovitellin 1
apo-Lv-2	apolipovitellin 2
BSA	bovine serum albumin
^{14}C BSA	methylated ^{14}C bovine serum albumin
Ca^{++}	calcium cations
μCi	Curies (2.2×10^6 dpm)
E_2	17ß-oestradiol
EDTA	diaminoethane tetracetic acid
EGTA	1,2-di (2-aminoehoxy ethane-N', N', N') N-tetra acetic acid
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
GH	growth hormone
GSI	gonadosomatic index
GtH	gonadotropin
CHO-poor GtH	carbohydrate-poor gonadotropin
CHO-rich GtH	carbohydrate-rich gonadotropin (maturational GtH)
^3H	tritium
^3H-leu	tritiated leucine
HCl	hydrochloric acid
HRP	horse-radish peroxidase
IGF	insulin-like growth factor
^3H leu lipovitellin	tritiated leucine lipovitellin
LDF	low density fraction
LDL	low density lipoprotein
LH	luteinizing hormone
Mg^{++}	magnesium cations
mRNA	messenger ribonucleic acid
^{32}P	^{32}phosphorus
PE	pituitary extract
^{32}P phosvitin	^{32}phosphorus phosvitin
PMSF	phenyl methyl sulphonyl fluoride
T	testosterone
T_3	tri-iodothyronine
T_4	thyroxine
TSH	thyrotropin (thyroid stimulating hormone)
VLDL	very low density lipoprotein
V_o	void volume
V_T	total volume
VTG	vitellogenin
^3H.VTG	tritiated leucine vitellogenin
^{125}I.VTG	iodinated vitellogenin
$^{32}P.^3H$-VTG	^{32}phosphorus.^3H leucine vitellogenin

CHAPTER ONE

GENERAL INTRODUCTION

Rainbow trout, *Salmo gairdneri*, originate from the Pacific coastal areas of North America but widespread introductions over the last hundred years have now produced self-sustained populations in all continents except Antartica. The species lends itself well to domestication, which together with its popularities as a sporting and table fish, make it the most important farmed fish in North America, Western Europe and Japan. The reproductive cycle of the rainbow trout has received considerable attention in recent years because knowledge of the timing and the mechanisms of control of gonadal development are important in both the fish farming industry, where aquaculture requires that eggs and fry are available outside the normal spawning time in order to maintain continuity of production, and in understanding the general physiological and endocrinological processes accompanying teleost reproduction.

Rainbow trout in the wild usually spawn in the spring over a short period of 6 to 8 weeks each year, but as a result of artificial selection and photoperiodic manipulations a large number of strains now exist with spawning seasons covering the range from august to april (Sumpter*et al.*, 1984), providing advantages for the industry, who can obtain eggs and fry over an extended period, and also for researchers, who can get fish in any stage of sexual development during most of the year.

Fish, like other animals, sexually propagate by producing specialized cells, the haploid gametes. Generally eggs and sperm are produced by separate sexes, as in the case in all salmonids, and after fusion of these gametes a fertilized egg or zygote is created. Fertilized eggs have been described as being at the 'crossroads of development' in that they represent both the origin and the biological goal of each organism (Postlethwaite and Giorgi, 1981). Eggs provide two important functions for the embryo they enclose; firstly they contain developmental instructions given by the mother to direct the initial phases of embryogenesis, and secondly they are usually 'well-provided' with niutritive substances to support the embryo until it can obtain its own food. In oviparous vertebrates, relatively large oocytes with large niutritive stores are necessary because of the demand this form of reproduction has on supplying an adequate energy store for the subsequent independent development of the young. These nutrient reserves, generically termed 'yolk' (a rather loose term since in chemical structure there are considerable species differences), include complex carbohydrates, lipids and proteins. Generally conjugated lipoproteins predominate and may comprise more than 80% of the eggs dry weight (Callen *et*

al., 1980). The process by which food supplies are built up in the egg is called vitellogenesis or yolk deposition. In vertebrates yolk deposition broadly involves two mechanisms; autosynthetic vitellogenesis, in which nutrient products are synthesised from within the oocyte, and heterosynthetic vitellogenesis, whereby yolk-precursors are synthesised by the liver and transported to the ovary via the maternal blood stream. Although many aspects of vitellogenesis in invertebrates and vertebrates are similar, others including the site of yolk protein production, are very different. In oviparous invertebrates the yolk is usually produced in either fat bodies (as in most insects) and subsequently transported to the developing ovary or, as in all crustaceans, synthesised by the ovary itself. In the trout yolk deposition produces an increase in the gonadosomatic index (GSI: (total gonad weight/body weight) x 100) from 1% or less of body weight up to about 20% and, as it occurs over a relatively short period of time, consequently it involves a dramatic re-organisation of the animal's metabolism. This study concentrates on the area of yolk deposition, and investigates what storage materials are incorporatedinto trout eggs, the dynamics of this deposition and how it is controlled.

In the rainbow trout, as in all seasonally-breeding teleosts, egg production is a complex process involving a number of individual stages which, although distinct from yolk-protein production, are all essential for the successful development of the ovary. An awareness of these developmental stages is important both in understanding the picture of how vitellogenesis 'fits' into the ovarian cycle and in determining how particular phases affect proximal stages of development which are referred to and discussed further later in the text.

Maturing female rainbow trout have paired trough-like ovaries situated in the dorsal part of the coelomic cavity; they produce a single batch of eggs each year and spawn several times during their lifetime. The annual reproductive cycle of the salmonids can be broadly classified into four periods or phases according to the stage of oocyte growth. Recognised by their structural and functional differences, these phases comprise: oogonial proliferation, oogenesis and folliculogenesis, endogenous vitellogenesis and exogenous vitellogenesis, and maturation proceeded by ovulation (Vakaet, 1955; Yamamoto *et al.*, 1965; Braekevelt and McMillan, 1967; Khoo, 1979; Hurk and Peute, 1979; Kagawa *et al.*, 1981; Wallace and Selman, 1981; Nagahama, 1983). A flow diagram and detailed drawings of the stages involved in follicle development in the trout are provided in Figures 1.1 and 1.2, respectively. It should be stressed, however,

Figure 1.1. Flow diagram showing the cytological events associated with follicle development in the rainbow trout. (Taken from Cumaranatunga,1985).

Figure1.2. Developmental stages of rainbow trout follicles showing both the cytological and follicular tissue changes that occur. (Taken from Cumaranatunga, 1985).

that although the nomenclature implies that these phases of egg development follow on from one another ths is not so and often 2 or 3 phases are occurring simultaneously in different follicles within an ovary. Indeed it is likely that even within a single follicle there are periods when these growth phases overlap.

The first phases of the reproductive cycle encompassing oogonial proliferation, oogenesis and folliculogenesis, collectively make up 'pre-vitellogenesis'. It is unclear exactly when this period of ovary development occurs in the rainbow trout but in the brown trout *Salmo trutta fario* it may reach this stage within 3 months of hatching, 2 years or more before they mature (Billard, 1987). Oogonial proliferation initiates the pre-vitellogenic stage of gonadal development during which time the ovaries consist of a network of connective tissue, the stroma, and rounded cells comprising mainly a nucleus with scant cytoplasm, called oogonia. The oogonia occur either singularly or in small nests; both undergo mitotic proliferation and can be found throughout the reproductive cycle of the trout (Hurk and Peute, 1979). Franchi *et al.* (1962) suggested that trout, as in the majority of species with circumscribed breeding cycles, show a limited period of peak oogonial division, during or just after spawning each year, however, Wallace (pers. com.) suggests that this conclusion may be due to the difficulty in observing this process in a rapidly developing vitellogenic ovary and in fact oogonial proliferation occurs throughout ovarian growth.

Oogonia subsequently undergo meiotic transformation to give rise to primary oocytes (Tokarz, 1978; Khoo, 1979; Hurke and Slof, 1981; Forberg, 1982). This transformation has not been well documented for teleosts, although it is generally accepted that the chromosomes become arrested at diplotene stage of the first meiotic prophase (Wallace and Selman, 1981). Growth during the three sub-stages of the meiotic prophase, i.e. the chromatin nucleous, early perinucleolar and late perinucleolar stages, is largely attributable to the increase in electron dense cytoplasm (Lambert, 1970a; Wallace and Selman, 1981; Forberg, 1982) called 'ciment' (cement) when associated with the mitochondria and 'nuage', yolk nuclei, or 'Balbiani bodies' when independent from mitochondria. Towards the end of this pre-vitellogenic growth phase, concomittant with the loss of the basophilic character of the cytoplasm, the Balbiani bodies migrate to the periphery of the ooplasm (Yamamoto *et al.*, 1965; Hurke and Peute, 1979; Bruslé, 1980; Wallace and Selman, 1981). By the end of this growth phase the oocyte lies within, and has an intimate association with four follicular layers

(Wallace and Selman, 1981, Figure 1.2). In order of progression from the ooplasm outwards the follicular layers comprise an acellular zona radiata (also known as the zona pelluciuda, vitelline membrane or chorion) surrounded by 3 cellular layers; a single layer of squamous cells, the granulosa (Braekevelt and McMillan, 1967), a layer of thecal cells and a surface epithelium covered by connective tissue.

The detailed structural development and functional significance of these follicular layers, especially their role in protein sequestration, are investigated and discussed in more detail in Chapter 5. After ovulation all the follicular layers are left within the ovary itself except the zona radiata or chorion which forms the outer limiting wall of the egg.

There is confusion in the literature regarding the terms oocyte and follicle, both are used to describe the developing female gamete. Furthermore the term follicle is sometimes used to describe just the follicular tissues surrounding the developing oocyte or even more specifically just the granulosa. Throughout these studies an intact follicle refers to an oocyte surrounded by the granulosa, theca and surface epithelium. An oocyte has none of the 3 cellular follicular layers and comprises only the yolk surrounded by the acellular zona radiata, i.e. an oocyte consists of only that which is maintained in an ovulated egg. The physical distinction between an oocyte and follicle is illustrated in Figure 1.3. The components surrounding the oocyte are described as the follicular layers. This terminology is in accordance with the definitions for a follicle and an oocyte in both mammals and non-mammalian oviparous vertebrates as proposed by Harrison and Weir (1977) and Dodd (1977) respectively.

During pre-vitellogenesis the follicle volume increases a few hundred fold. This growth phase appears to occur independently of a pituitary influence (Khoo, 1979) however after hypophysectomy follicles do not grow beyond a 'critical size' (Fostier *et al.*, 1983; Ng and Idler, 1983) and do not enter vitellogenesis indicating that development beyond this phase is dependent on pituitary hormone(s) (Upadhyay *et al.*, 1978; Wallace and Selman, 1981).

"Lipid yolk" accumulation commences prior to that of protein yolk (Raven, 1961) and its appearance can be considered to mark the start of the pituitary dependent growth phase, vitellogenesis (Shackley and King, 1977). These lipid inclusions are distinct from vitellogenin-derived protein yolk granules discussed below, although critical details of their origin are unknown. Lipid bodies appear initially in the perinuclear cytoplasm of the follicle. They

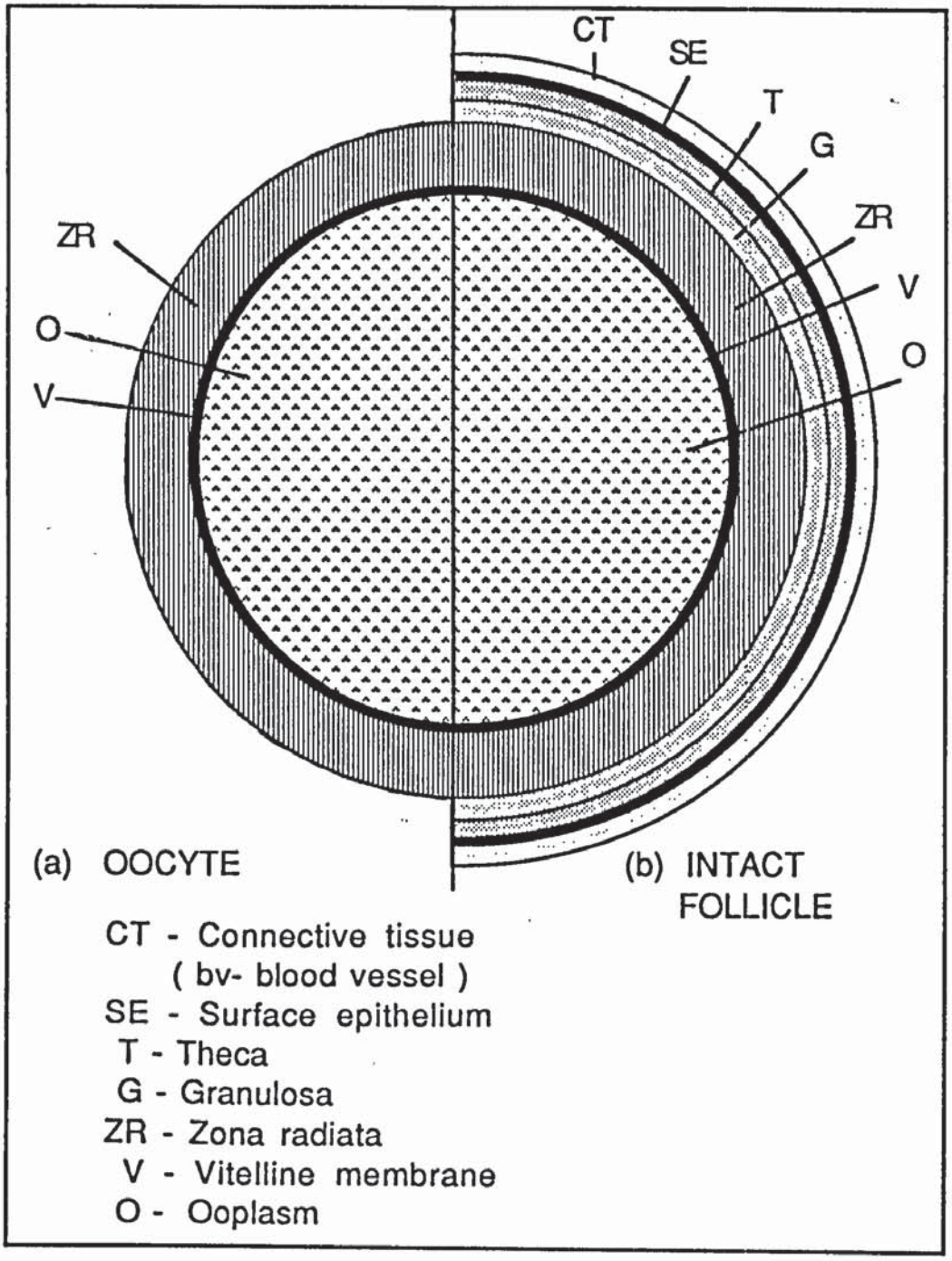


Figure1.3. A diagramatic representation of the distinction between an oocyte and an intact ovarian follicle .

proliferate during the development and migration of Balbiani bodies and occur frequently in association with these bodies (Chopra, 1958; Guraya, 1963, 1965; Droller and Roth, 1966; Beans and Kessel, 1973). A general classification of early lipid inclusions, consisting predominantly of phospholipids (Guraya, 1963, 1965; Shackley and King, 1977), as true yolk is of questionable accuracy as they have been observed to disappear in some species prior to or during, subsequent exogeneous vitellogenesis (Chopra, 1958; Guraya, 1963, 1965) but may persist in others (Shackley and King, 1977). Detailed analysis of follicle cytoplasmic lipids and a direct demonstration of the capacity of follicles to synthesise them remain to be performed. Lipid inclusions described in more advanced follicles during exogenous vitellogenesis consist primarily of triglycerides (Leger *et al.*, 1981). The capacity of trout ovaries to synthesise triglycerides at a time when vitellogenin uptake is occurring suggests an endogenous source for some of this yolk (Wiegand and Idler, 1982). An exogenous contribution to the triglycerides pool from plasma lipoproteins including vitellogenin is also likely (Leger *et al.*, 1981, See Chapter 3).

Vitellogenesis, including endogenous and exogenous vitellogenesis, comprises the major processes of follicle growth. Endogenous vitellogenesis, usually occurring naturally during the spring and early summer, is characterized by the appearance of yolk vesicles. These structures contain an endogenously synthesised glycoprotein called 'primary' or intravesicular yolk (Korfsmeier, 1966). When almost all of the cytoplasm is full of vesicles they migrate to the periphery of the follicle and lie adjacent to the oolemma (Yamamoto, 1956a, 1956b, 1957; Khoo, 1979; Wallace and Selman, 1981, see Figure 1.2). These vesicles appear to be the same structures termed cortical alveoli seen in more developed follicles (Selman *et al.*, 1987). Towards the end of vitellogenesis as the follicles enter final maturation, the cortical alveoli fuse with the vitelline membrane and at the time of fertilization release their glycoprotein contents into the perivitelline space (Wallace and Selman, 1981; Selman *et al.*, 1987). The significance of these cortical alveoli with their 'putative yolk' in ovarian development is still poorly understood and it is questionable whether the contents of these vesicles should be called 'yolk' because it probably bears no relationship to true yolk which provides nutritive substances for the subsequent embryo.

Furthermore describing both the production of yolk vesicles (endogenous) and the aquisition of 'true' yolk (exogenous) as 'vitellogenesis' is confusing because they are entirely distinct processes. The term vitellogenesis

should therefore perhaps be reserved to describe the events involved in the aquisition of true yolk during exogenous vitellogenesis, described below. The diameter of follicles of rainbow trout during the yolk vesicle stage of development varies between 0.4-1.1mm (Hurke and Peute, 1979).

Exogenous vitellogenesis occurs in summer and autumn and is the principal event responsible for the enormous growth of the oocyte. During this growth phase a large amount of an extra-ovarian proteinaceous material, termed vitellogenin (VTG) is produced by the liver in response to oestrogens from the ovary, it accumulates in the blood, and is subsequently sequestered by the developing follicles (Dumont, 1972; Wallace, 1978). Histologically the onset of exogenous vitellogenesis is marked by the appearance of yolk granules in the periphery of the ooplasm, and as more VTG is sequestered they increase in number and size until they completely fill the ooplasm (Figure 1.2). The fluid-filled yolk granules eventually fuse centripetally to form a continuous mass of fluid yolk, conferring the characteristic transparency to the ovulated egg. Trout follicles grow up to approximately 3.4mm in diameter during this yolk granular stage of development (Hurke and Peute, 1979).

In the model proposed for vitellogenesis in the *Amphibia* and *Aves*, VTG, once sequestered by the follicle, is degraded to the yolk protein products, including lipovitellin and phosvitin (Wallace and Dumont, 1968; Bergink *et al.*, 1974). Support for a similar model in teleosts is now accumulating. In amphibian eggs yolk platelets, composed almost entirely of lipovitellin and phosvitin, constitute 80-90 percent of the total protein (Benbow *et al.*, 1975; Callen *et al.*, 1980); however, quantification of the proportion of yolk materials derived directly from VTG has not been performed in fish (Wallace and Selman, 1981).

After completion of yolk deposition, and with the appropriate hormonal stimulation, oocytes undergo enlargement by hydration to reach their full size (Wallace & Selman, 1979). In rainbow trout this is usually between 4-5mm in diameter. During this process of maturation chromosomes resume meiosis and enter the second meiotic division. This period of development is marked by the migration of the nucleus from a central position within the ooplasm to a peripheral one. The nucleus eventually lies adjacent to a specialized opening, "the micropylar cell", through which the sperm will enter at fertilization and fuse with the migrated material nucleus (see Figure 1.2). Follicular maturation is under the control of pituitary gonadotropin(s) which induce the follicular

layers to synthesise specific steroids, some of which in turn act directly on the follicle to promote maturation. 17α20ß-dihydroxyprogesterone as well as other progestagens play an important role in initiating maturation and in turn are modulated by other circulating steroids (Jalabert, 1976); this control is discussed further in Chapter 7. After maturation is complete the oocyte or rather egg, is ovulated into the ovarian lumen and under natural conditions is ready for oviposition. In captivity however, where the appropriate series of environmental and/or behavioural stimuli are not found, ovulated eggs are not released to the environment but have to be manually stripped from the body cavity.

Follicles during any stage of their development can undergo degeneration or 'atresia' (Ryan, 1981) and this commonly occurs among all vertebrate groups (Brambell, 1956; Byskov, 1978; Saidapur, 1978). The function of atresia and the factors involved in controlling its level are not well understood. The incidence of atresia is believed to be influenced by age, the stage of the reproductive cycle, nutrition and the individual's hormonal status (Ingram, 1962; Cumaranatunga and Bromage, 1986). Atresia in all vertebrates may be involved in the initiation of follicular growth, in the selection of follicles for ovulation and in some may play a steroidogenic role.

The reproductive phases detailed above are synchronised by internal (endogenous) and environmental rhythms. The significant energy costs associated with salmonid reproduction (as previously mentioned a mature ovary can comprise 20 percent of the body weight) emphasise the importance of the physiological mechanisms that ensure these costs are expended at ecologically appropriate times. It is now well recognised that the reproductive cycle of salmonid fish is controlled by modifications in levels of hormones from the hypothalamic-pituitary-gonadal axis timed by cues from the external environment. Much work suggests that of all the factors known to affect reproductive habits, including photoperiod, temperature, rainfall, pheromones, nutrition and salinity, it is photoperiod (changes in seasonal daylength) which exerts the major influence in salmonids (Henderson, 1963; Kunesh *et al.*, 1974; de Vlaming *et al.*, 1974; Whitehead *et al.*, 1978a, b & c; McQuarrie *et al.*, 1979; Bromage *et al.*, 1982a, 1982b; Scott and Sumpter, 1983b). In general cultured rainbow trout spawn in the autumn and winter months under a decreasing or short photoperiod and it is a long or increasing daylength that provides the stimulus for gonadal recrudescence (Henderson, 1963; Whitehead and Bromage, 1980;

Bromage *et al.*, 1982b; Scott and Sumpter, 1983). Recent data from Duston & Bromage (1987), however, supports the hypothesis that photoperiod entrains an endogenous rhythm of maturation rather than having a direct, driving influence on the control of reproduction.

This thesis deals only with exogenous vitellogenesis and as briefly mentioned focuses specifically on the deposition of the yolk proteins, the dynamics of their sequestration and how this process is controlled. Some aspects of exogenous vitellogenesis have been thoroughly investigated in birds and amphibians (reviewed by Wallace, 1985) but not in fish. In the domestic fowl, *Gallus domesticus* (Cutting and Roth, 1973; Roth *et al.*, 1976) and the South African clawed toad, *Xenopus laevis*, follicles selectively sequester VTG by receptor mediated endocytosis and subsequently process this serum precursor to the yolk proteins including lipovitellin and phosvitin. This receptor mediated intake of VTG from the array of circulating proteins in the blood gives a degree of control on the proteins sequestered by the maturing follicles. There is very little information available on the hormonal control of yolk deposition amongst any of the oviparous vertebrate and invertebrate groups, however, in *Xenopus laevis* VTG sequestration has been shown to be mediated by gonadotropin (Wallace, 1978).

Our knowledge of the dynamics of vitellogenesis is thus based almost entirely on birds and amphibians. Nearly all the information available on vitellogenesis of fish concerns either the hormonal control of VTG synthesis or the purification of VTG and its measurement in the blood (reviewed by Ng and Idler, 1983); little is known about the uptake of VTG into maturing follicles and its relationship to the yolk proteins and the control of this uptake process which is surprising in view of the commercial importance of teleost egg size and quality (especially in salmonid species). In fish the processing of VTG to lipovitellin and phosvitin has not been clearly demonstrated and while there is no reason to doubt that the principles so elucidated may not be applied to oviparous vertebrates in general they still remain to be described for fish. Available evidence for teleosts shows a reduced specificity for VTG uptake (Campbell and Jalabert, 1979). Furthermore precise quantifications of protein uptake have not been performed in fish and they are said to sequester a series of macromolecules quite distinct from VTG (Campbell and Jalabert, 1979; Wallace and Selman, 1983). The number and nature of hormones involved in the regulation of VTG incorporation in teleosts is the subject of much controversy (Idler, 1982; Burzawa-Gerard, 1982) and a

direct hormonal control of yolk protein precursor uptake into vitellogenic follicles has yet to be demonstrated either *in vivo* or *in vitro*. The array of teleost hormones implicated in the stimulation of VTG uptake include gonadotropin(s) (Campbell and Idler, 1970; Burzawa-Gerard, 1974; Ng *et al.*, 1980) thyroid hormones (Lewis and Dodd, 1974; Hurlburt, 1977) corticosteroids (although of limited physiological significance individually they may act in conjunction with pituitary gonadotropin(s)), prolactin (Ensor, 1982) and testosterone (Young and Ball, 1982). Since hormonal treatments may directly control or influence the production and assimilation of yolk proteins it is surprising that the application or use of hormones in the uptake of VTG has not been widely researched.

The following studies are broadly divided into two sections the first adopts an *in vivo* approach (Section I) and the second an *in vitro* one (Section II). Section I, encompassing Chapters 3 and 4, investigates the origins of the sequestered yolk proteins during exogenous vitellogenesis and determines their relationship to the serum protein, VTG. This section further investigates the rates and selectivity of VTG sequestration into vitellogenic follicles. These studies provided the 'basic model' for trout vitellogenesis and were subsequently used to validate the *in vitro* experiments conducted in Section II. An *in vivo* approach was adopted in these initial studies primarily because it incurred fewertechnical problems.

In vivo studies, however, can be cumbersome and at times prone to difficulties of interpretation, because the involvement of particular parameters in VTG uptake may be either direct, for example by affecting follicle receptivity and competence, or indirect, their actions being relayed by other physiological and endocrinological processes. Section II, which includes Chapters 5, 6 and 7 establishes an *in vitro* method for culturing individual vitellogenic trout follicles and investigates the factors involved, particularly follicle size and hormones, in the control of VTG uptake.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

This chapter describes the materials and methods used in more than one experiment. Materials and methods specific to particular experiments are given in the relevant chapters.

2.1 Fish maintenance, identification, anaesthesia, blood sampling and serum preparation

2.1.1 Fish maintenance

The experimental animals used throughout this study were female rainbow trout (Genus: *Salmo gairdneri*, Family: Salmonidae). Fish of several different stocks, strains and age groups were used, however, care was taken to ensure that fish from the same sources were used in related experiments. The majority of fish were purchased from commercial farms and subsequently held in a purpose built aquarium at Aston University (see Appendix I). Each tank system had a separate supply of recirculating water maintained at a constant $12^{o}\pm1^{o}C$. Twenty-four hour light regimes were adopted in an attempt to induce a twice-yearly spawning cycle (cf. Bromage *et al.*, 1982 a & b). Photoperiodic manipulations in association with strain differences in spawning times produced an annual continuity in the supply of vitellogenic follicles. The fish were fed daily at a rate of 0.5% of their body weight with a commercially available pelleted trout feed, (Mainstream, BP Nutrition UK Ltd, Witham, UK).

2.1.2 Identification of fish

All experimental fish were marked with one of 2 identification systems; either plastic tags or alcian blue dye. Plastic numbered tags (Charles Neal Finchley Ltd, East Finchley, UK) of different colours were fixed to the basal-anterior margin of the dorsal fin using a tagging gun. The tagging device (Kimbal Systems Ltd, Leicester, UK) is generally supplied to the clothing industry for attaching labels, and operates by punching plastic pins through holes in the tag and through the object to be tagged, in this case the fish's fin.

The second identification system using alcian blue involved using a panjet (FH Wright, Dental MFG Co Ltd, West Dundee, Scotland), a dentistry instrument that fires a fine stream of loadent dye under high pressure. The dye penetrated the fish's skin and scales and the use of different numbers and placements of dye applications on the fish's flanks enabled many fish held in the same tank system to be identified individually. The markings remained visible upto the time when the fish were sacrificed, often a year later. The alcian blue dye proved more successful

than the plastic tags because the latter often became entangled in the net and consequently detached when fish were captured.

2.1.3 Anaesthesia

All blood sampling, hormone and isotope injections, and operations were carried out under anaesthesia to avoid scale damage and consequent microbial infections, as well as to reduce stress to a minimum. 2-phenoxyethanol (Sigma Chemical Company, Poole, Dorset, UK), a liquid anaesthetic readily soluble in water was used at a concentration of 1:3000 in water. Fish were considered to be sufficiently anaesthetised when a firm squeeze along the caudal peduncle failed to induce the normal 'kicking' reflex of the tail fin. After performing the various procedures fish were transferred into a tank with a rapid flow of fresh water in order to aerate the gills and aid their recovery. Generally fish recovered well within 5 minutes and post-sampling mortalities rarely occurred.

2.1.4 Blood sampling and serum preparation

Blood samples were collected from the Cuverian sinus into chilled 5ml monovettes (Sarstedt, Leicester, UK). Essentially these containers are combined syringes and centrifuge tubes containing glass beads that create a large surface area to enhance blood clotting. The monovettes were fitted with 21G (1 1/2 inch) sterile hypodermic needles (Gillette UK Ltd, Middlesex, UK) and a protease enzyme inhibitor, either aprotinin (Sigma Chemical Company) or phenylmethyl sulphonyl fluoride (PMSF, Sigma Chemical Company) added at 1000 Kallikrein units per ml or 10 mM concentrations, respectively, to avoid, or at least reduce, proteolysis (see Hickey and Wallace, 1974; de Vlaming *et al.*, 1980). The blood samples were placed in the refrigerator at 5^{o}C during the clotting process to further minimise proteolysis. The samples were subsequently centrifuged in a MSE chillspin (Fisons plc, Scientific Equipment, Bishop Meadow Rd, Loughborough, Leics, UK) for 15 minutes at 2500g and the resulting serum supernatant withdrawn, pipetted into clean plastic tubes (LP3 and LP4, Luckham Ltd, Sussex, UK) and stoppered ready for use. If the serum samples were not analysed or used immediately they were subsequently frozen at -20^{o}C until required.

2.2 Assessment of the degree of ovarian development by measurement of serum calcium

As described more fully later in the text, total serum calcium is a fairly accurate and easily measured indicator of ovarian development in *Salmo gairdneri*, its increase in the blood during the period of exogenous vitellogenesis paralleling the levels of VTG. Figure 2.1, containing data obtained from 50 vitellogenic fish, shows a strong positive correlation between serum VTG (determined by radioimmunoassay, see section 2.7) and serum calcium, with a regression coefficient (r) of 0.9. The figure further illustrates that when calcium levels exceeded 13mg.100 ml^{-1} a female could be considered to be actively synthesising VTG. Thus by measuring calcium profiles throughout the year in individual fish a specific animal could be used for experimentation at an appropriate time, i.e. when the ovary contained a full complement of vitellogenic follicles.

Calcium was measured using a Coring E1 calcium analyser (Coring Instruments, Medfield, Massachusetts, USA). The analytical procedure for this analyser was based upon the quenching of the fluorescence from the non-dissociative complex formed between calcien dye with calcium ions in an alkaline medium, by chelating ions with the titrant ethyleneglycol-bis N'N-tetra acetic acid (EGTA); (Schmidt and Reilley, 1957). All serum samples were assayed immediately after extraction. Inter and intra-assay coefficients of variation were 0.02% and 1.27% respectively.

2.3 Ovarian sampling

2.3.1 Determining total vitellogenic follicle numbers

On completion of the *in vivo* protein uptake studies animals were killed by a sharp blow to the head, both ovaries removed, washed in saline and weighed on a Mettler HIOT spring loaded balance. The number of vitellogenic follicles was counted in a weighed portion of the ovary (usually about 20% of the whole ovary) and from this was derived the total number of follicles in the ovary, as described below.

O - total weight of both ovaries

F - weight of ovarian portion removed for counting

N - number of vitellogenic follicles in F

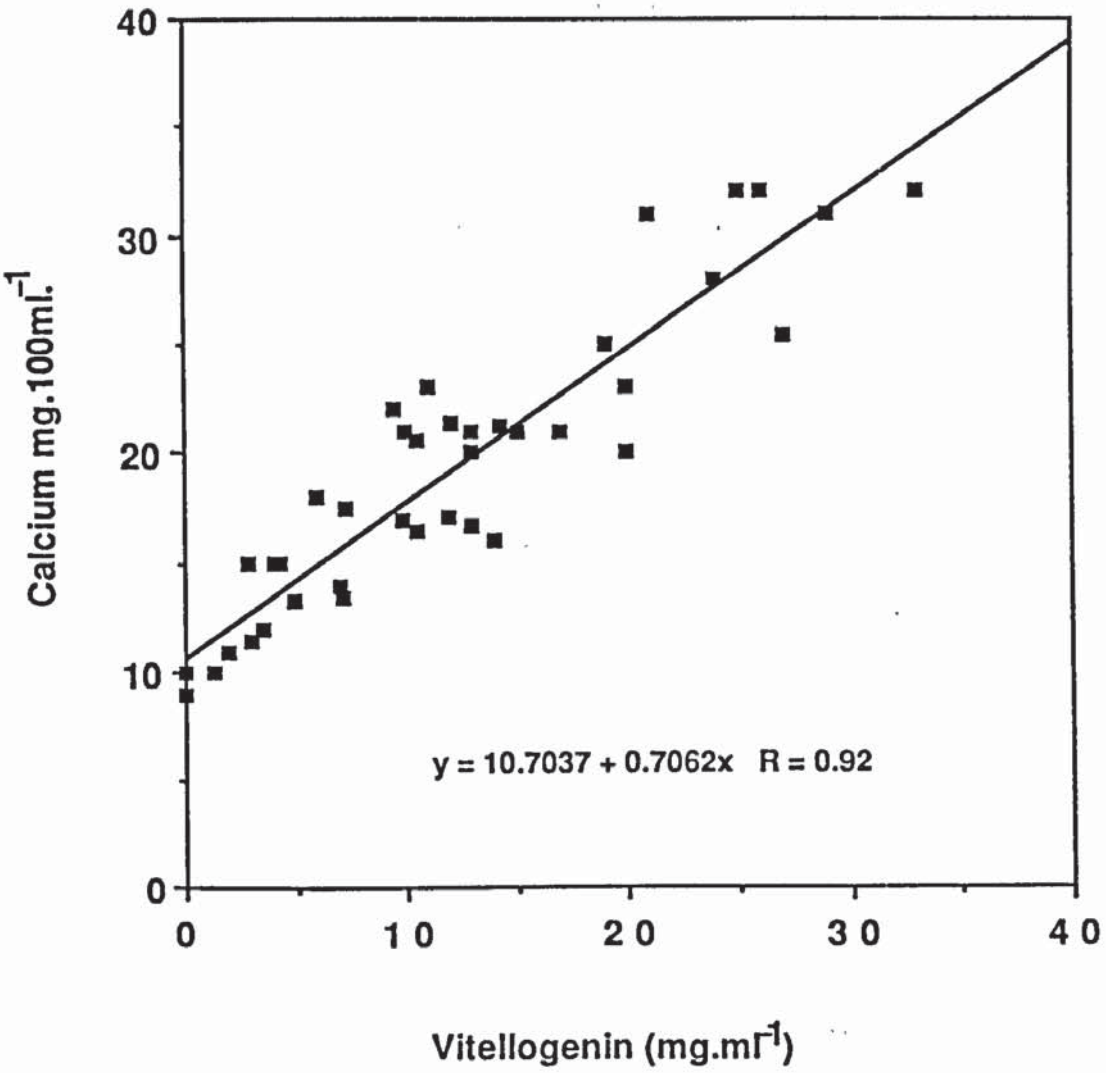


Figure 2.1. Correlation between serum vitellogenin and total serum calcium.

$$\text{Total maturing follicles} = \frac{O}{F} \times N$$

2.3.2 Determination of follicle diameter and surface area

In all experiments the diameters of vitellogenic follicles, dissected from the ovary for sampling or culture, were carefully measured to the nearest 0.1mm. Large vitellogenic follicles, greater than 2.5mm in diameter, were measured using hand calipers (KWB Plastic Instruments, Switzerland) follicles below this size were measured at x10 magnification using a binocular microscope fitted with a micrometer eye-piece graticle.

Rates of protein incorporation into follicles from one vitellogenic female were compared to those of others by standardizing the protein uptake and expressing it as ng of protein sequestered per mm^2 follicle surface per hour, ($ng.mm^2$ follicle $surface^{-1}.\ hr^{-1}$. or ng. $mm^{-2}.hr^{-1}$). Trout follicles are roughly spherical and therefore the surface area available for protein sequestration was calculated using the formula $4\pi\ r^2$, where $\pi = 3.142$ and r is the radius of the follicle. It should be stressed, however, that the calculated area of a follicle's surface is only an approximation of its true surface for, as detailed in the text of Chapter 6, the membrane of a follicle possesses many folds and crypts which serve to greatly increase the surface area.

2.4 Gel filtration procedures

Gel filtrations were performed on 1.5 x 100 cm chromatographic columns (Pharmacia Fine Chemicals, Milton Keynes, UK) packed with either Sepharose 6B (fractionation range 1×10^4 - 4×10^6 Daltons) or Sephadex G100 (fractionation range 4000-150 000 Daltons) (Pharmacia Fine Chemicals). Each column was carefully poured over a period of 2 days to ensure homogeneous packing and to avoid the formation of air pockets. Both columns were equilibrated with 0.15M phosphate buffer (Na_2HPO4 and $Na\ H_2\ PO_4$) ph 7.8, containing 0.2g per litre sodium azide (added as an antimicrobial agent) and the protease enzyme inhibitor PMSF at a concentration of 10mM. Filtrations were performed under refrigerated conditions (9 ± 1^oC) to further minimize proteolysis. The Sepharose 6B column was run at 6 $ml.hr^{-1}$ using a peristaltic pump and fractions between 1.5 and 2.4 ml collected, and similarly the Sephadex G100 column at 12 $ml.hr^{-1}$ with the collection of 2ml fractions. Due to the softness of the Sephadex G100 gel this column was run in reverse, i.e. from the bottom upwards, to prevent further

settling and packing of the gel-bed. The eluents passed through a spectrophotometer (Pharmacia UK Instruments Ltd, Pharmacia House, Milton Keynes, UK) containing a 1cm pathlength silica flow cell and were monitored for protein at 276 nm.

Both chromatography columns were calibrated using molecular weight calibration kits (Pharmacia Fine Chemicals), Sepharose 6B using a high molecular weight (HMW) kit and Sephadex G100 using a low molecular weight (LMW) kit. The HMW kit included thyroglobulin (669 000 Daltons), ferritin (440 000 Daltons) and catalase (232 000 Daltons). The LMW kit included bovine albumin (66 000 Daltons), albumin (45 000 Daltons) and chymotrypsin (24 000 Daltons). The void volumes (Vo) of the columns were determined using Blue Dextran 2000 (molecular weight 2000 000 Daltons). Between 2 and 10 mg of each molecular weight marker was sufficient to produce a distinct elution peak. The elution volumes of the protein markers were measured from time of sample application to the centre of the elution peaks, the latter being determined by the intersection of the 2 tangents drawn to the sides of the peak. The elution volumes were then used to prepare calibration curves (detailed below) from which the molecular weights of other proteins could be calculated.

A molecular weight calibration curve defines the relationship between the elution volume of a set of standard proteins and the logarithm of their respective molecular weights. The procedure for calculating the calibration curves was as follows:

(a) Kav values (representing the fraction of the gel volume which is available for diffusion of a given solute species) of standard protein markers of known molecular weights (calibration kits) were calculated using the equation

$$Kav = Ve - Vo/V_T - Vo$$

where

Ve = elution time of solute

Vo = column void volume

V_T = total gel bed volume

(b) Using semi-logarithmic paper, Kav values for each standard protein were plotted, on the linear scale, against their respective molecular weights, on the logarithmic scale.

(c) Finally, after a regression analysis, a straight line was plotted through these points.

The sizes of unknown proteins were then determined by reading their respective molecular weights from the calibration curve using their calculated Kav values.

The elution profiles and respective calibration plots for the HMW kit run on Sepharose 6B and and LMW kit run on Sephadex G100 are shown in Figures 2.2 and 2.3 respectively. The regression line on the calibration plot for Sepharose 6B is represented by Kav = 6.76 - 1.14 log mol wt with a regression coefficient (r) of 0.95. The corresponding line on Sephadex G100 is represented by Kav = 3.52-1.141 log mol wt. with a regression coefficient (r) of 0.99.

2.5 Protein labelling

Vitellogenin was labelled both using ^{125}Iodine (^{125}I) and *in vivo* using 3,4,5-^{3}H leucine (^{3}H leu.) and ^{3}H leu. in combination with ^{32}phosphorus (^{32}P). All isotopes were supplied by Amersham International plc (Amersham, UK). The *in vivo* production of ^{3}H leu. and ^{32}P; ^{3}H leu.-VTG is described in detail in Chapter 3.

2.5.1 Iodination of VTG

VTG was iodinated using Iodogen as the oxidizing agent, as described in Salacinski *et al.*, (1982). The materials and protocol are briefly described below.

2.5.1.1 Materials

(a) 4 µg rainbow trout VTG (purified by Norberg and Haux; molecular weight approximately 440 000 Daltons) dissolved in 10 µl 0.5M sodium phosphate buffer.

(b) 15 µl 0.5M sodium phosphate, pH 7.4.

(c) 5-10 µl IMS 30 (≡ 500 - 1000 µCi ^{125}I)

(d) 2 µg Iodogen

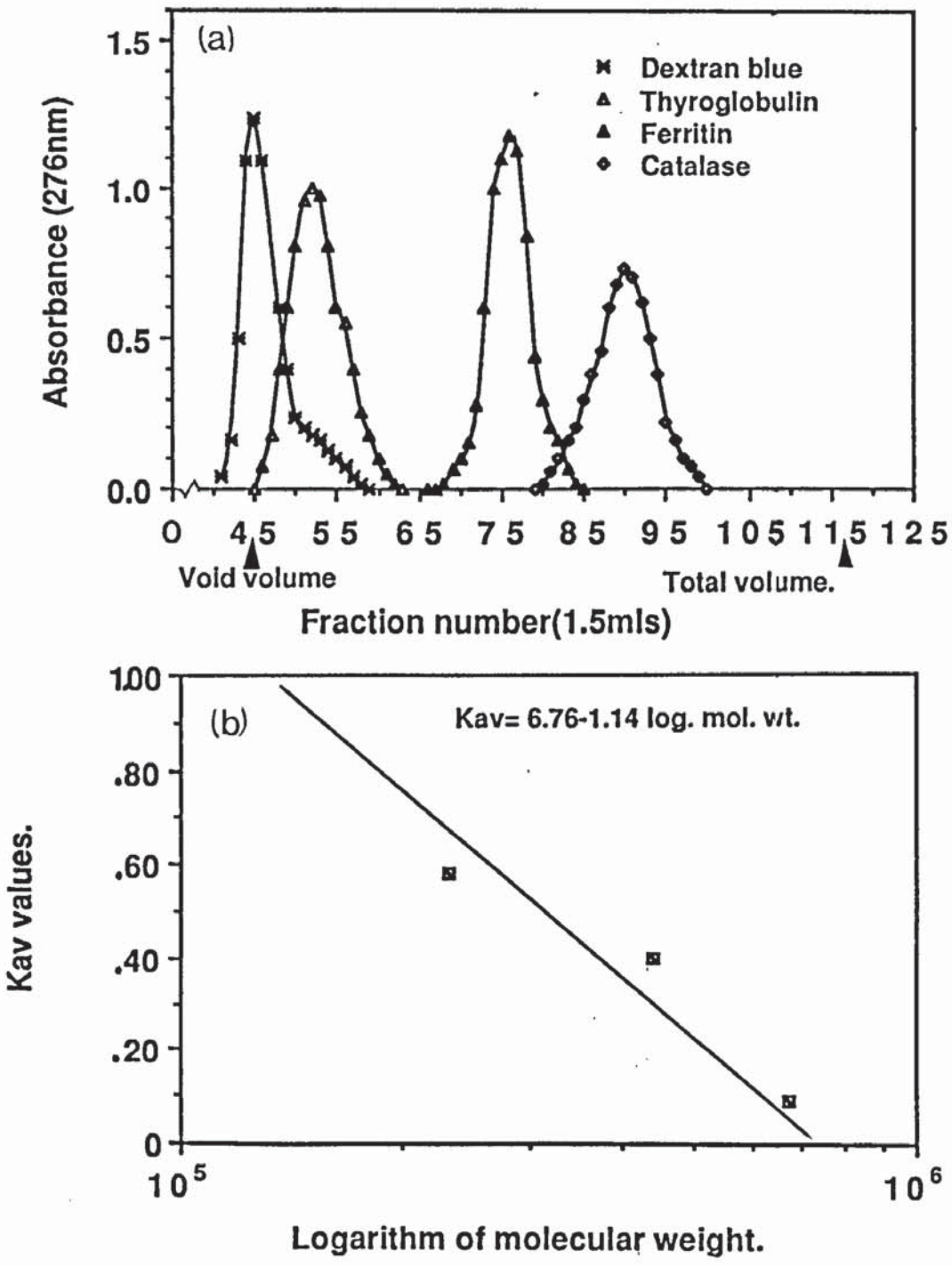


Figure 2.2. Calibration of the column of Sepharose -6B. (a) Absorbancy profiles of the high molecular weigh calibration kit proteins. (b) Semi-ıogarithmic plot of the known molecular weights of the protein markers against their respective Kav values. Using plot b the molecular weights of unknown proteins were determined from their calculated Kav values.

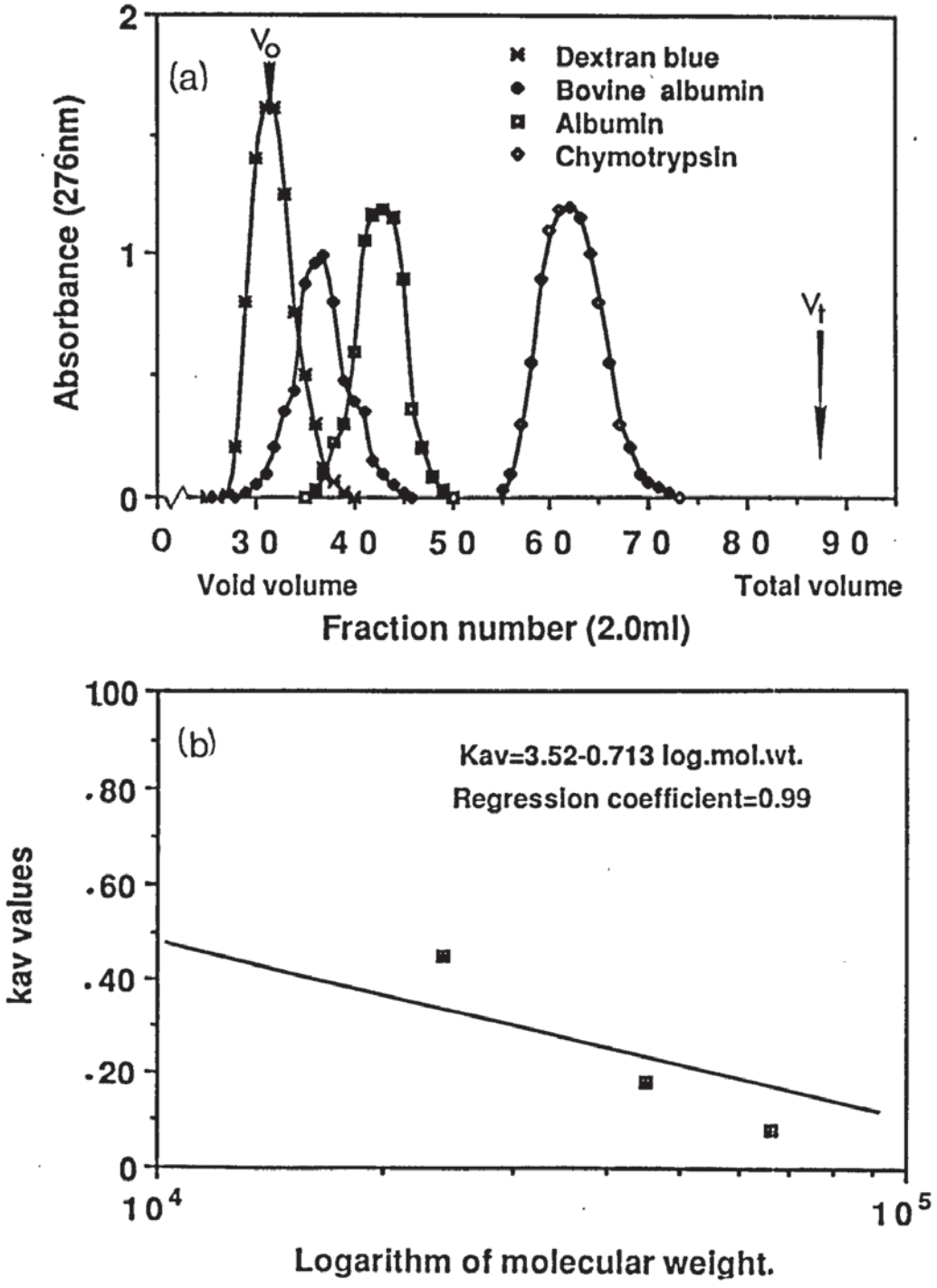


Figure 2.3. Calibration of the column of Sephadex -G100. (a) Absorbancy profiles of the low molecular weight calibration kit proteins. (b) Semi-logarithmic plot of the known molecular weights of the protein markers against their respective Kav values. Using plot b the molecular weights of unknown proteins were determined from their calculated Kav values.

2.5.1.2 Protocol

(a) The trout VTG, buffer and IMS 30 were added together in an LP3 tube (Luckhams Ltd) and gently mixed.

(b) The Iodogen was added on to the sides of a separate LP3 tube and allowed to dry.

(c) The VTG solution was added to the iodogen and left for 15 minutes.

(d) The ingredients were then transferred to a Sephadex G25 column (1 x 2cm) to separate the labelled ^{125}I-VTG from the free iodine.

(e) The labelled VTG, eluting first from the column, usually incorporated approximately 50% of the ^{125}I and had an average specific activity of approximately 100 μ Ci.μg^{-1}.

Each stock of ^{125}I-VTG was made separately just prior to its use in an incubation.

2.6 Measuring isotopic activity

A number of different radiolabelled compounds were used. The ß-emitters, which included ^{3}H leucine, ^{32}phosphorus (^{32}P) and ^{14} carbon (^{14}C; Amersham International), were counted in a Packard Tricarb 2660 liquid scintillation counter. ^{3}H leu. was counted singularly or in conjunction with either ^{32}P or ^{14}C. The low emission energy of tritium and the very high emission energies of both ^{32}P and ^{14}C meant that ^{3}H could be counted simultaneously with either of the other two labels using dual label programmes.

Tissue samples counted for tritium only and tritium in conjunction with ^{14}C were placed in glass scintillation vials and incubated with 1ml 0.5M. Protosol tissue solubilizer (Dupont (UK) Ltd, Biotechnology Systems Division, NEN Research Products, Stevenage, Herts, UK) for 24 hours at 37^{o}C. Column eluents counted for the same isotopes were similarly treated after an overnight incubation at 37^{o}C to evaporate the liquid phase. After completion of the protein digests each vial received 9ml of Protosol scintillation fluid (Dupont (UK) Ltd). Tissue samples and column eluents counted for ^{3}H together with ^{32}P received similar treatments to

those above using 1M 'Scintran' tissue solubilizer (BDH Chemicals Ltd, Atherstone, Warwickshire) in conjunction with the compatible Optiphase 'safe' scintillation fluid (LKB). All samples were placed in the ß-counter at least 2 hours before counting commenced to equilibrate the samples to the environment of the counter and standardize the background counts for each vial. Each vial was counted for 10 minutes. The counting efficiences for ^{3}H, ^{32}P and ^{14}C, using the cocktails above were 23-31.4%, 50.2% and 75.6% respectively (Quench curves are shown in Appendix II).

Radioactive phosphorus, unlike ^{3}H and ^{14}C (whose half-lives are 12.3 and 5730 years, respectively) has a relatively short longevity with a half-life of 12.9 days. Therefore in order to allow comparisons to be made of phosphorus levels at different sampling intervals, each count was multiplied by a factor related to the amount of decay it had undergone. The specific factor used in each case was obtained from a decay curve.

The ^{125}Iodine used to iodinate VTG is a gamma (γ) emitter and was counted in a Gamma set 500 gamma counter (ICN Instruments, UK). Samples for γ-counting, either whole follicles or liquids, were placed in LP4 tubes (Luckhams Ltd), capped and counted for 3 minutes. ^{125}Iodine has a half life of 60 days, which is long enough to avoid the use of a decay curve, especially as the time between the first and last samples measurement was less than 2 hours. The counting efficiency of ^{125}I was approximately 70%.

2.7 Vitellogenin radioimmunoassay

Vitellogenin levels were measured by radioimmunoassay (RIA), as described by Sumpter *et al.*, 1985, in the serum of maturing females and also on column eluent fractions taken from chromatographed serum and ovary samples. It is a homologous rainbow trout VTG RIA which has been widely used to estimate blood VTG levels under both natural (Scott and Sumpter, 1983; Sumpter *et al.*, 1984; Copeland *et al.*, 1986) and artificial (Elliott *et al.*, 1984) conditions. As the antibody is directed towards the lipovitellin moiety of the VTG molecule the RIA was also used to monitor the presence of lipovitellin after gel filtration of ovary homogenates. All the VTG RIAs were performed by Dr Sumpter at Brunel University, however, a brief outline of the protocol of the RIA is given below.

2.7.1 Protocol

Day 1

(a) Prepare 8, 50 µl pure-rainbow trout VTG standards from 1400-11 ng. ml^{-1}.

(b) 50 µl unknown sample (diluted with phosphate buffered saline to fit within standard range).

(c) Add 50 µl VTG antibody (R2B8), approximately 1:100 000 made up in 1:400 normal rabbit serum to standard (a) and unknowns (b).

(d) Add 50 µl label (approximately 25000 cpm ^{125}I-VTG) to all tubes and incubate at room temperature for 6 hours.

(e) Add 50 µl 1:10 anti-rabbit gamma-globulin and incubate at room temperature overnight.

Day 2

(f) Centrifuge tubes at 3000g for 30 minutes.

(g) Aspirate supernatents.

(h) Count remaining precipitates.

(i) Plot standard curve (log ng VTG against % maximum binding).

(j) Read unknown from standard curve to give ng.ml^{-1}, corrected to account for dilution undergone to fit standard curve.

The level of VTG in both the blood and ovarian samples was usually very high (mg.ml^{-1}) and hence samples had to be diluted considerably to get them onto the standard curve. The assay has a sensitivity of 1 ng.ml^{-1}, however, it was generally run in an insensitive mode in order to reduce the number of dilution steps. The intra assay coefficient of variance was 5± 1.5% and the inter assay

coefficient of variance 10%.

2.8 Histological methods

Removal of specific follicular tissues by dissection (Chapter 5) and assessments of the stages of vitellogenic development (Chapter 4) were determined using histological methods.

2.8.1 Materials

(a) Bouins fixative

(b) 70% ethanol

(c) LKB Histo resin kit (Pharmacia/LKB Instruments Ltd):
- Glycolmethacrylate (GMA)
- Hydroquinone
- Polyethyleneglycol 400 (PEG 400)
- Water 5-8%

Glycomethacrylate is a water soluble ester of methacrylic acid. Hydroquinone prevents the self polymerization of the plastic monomer. The softener PEG 400 is used to improve the sectioning properties of the final resin.

(ii) Activator, consisting of:
- Benzoyl peroxide
- A plasticizer

Benzoyl peroxide, a strong oxidizing agent, reactivates the glycomethacrylate. The plasticizer secured the benzoyl peroxide which is explosive as a pure dry powder.

(ii) Hardener, consisting of:
- A derivative of barbituric acid
- Dimethyl sulfoxide (DMSO)

The derivative of barbituric acid and initiates the polymerisation reaction when added to the infiltration solution.

(c) Polyethylene histomoulds

(d) Historesin mounting medium, consisting of 2 component methacrylate polymers:

- Polymethylmethacrylate (PMMA)
- Benzoyl peroxide with 50% stabilized plasticizer

(ii) Liquid component, consisting of:

- Methylmethacrylate (MMA)
- Dimethyl paratoluidin

(e) Wooden mounting blocks

(f) Staining materials: Erhlich's haematoxylin and eosin.

2.8.2 Protocol

Fixation: Follicles were fixed in Bouins fixative for 24 hours, then washed and stored in 70% ethanol.

Infiltration: the infiltration solution was prepared by mixing 50 ml of basic resin with 0.5g of activator. Follicles were transferred from 70% ethanol into a 1:1 mixture of 70% ethanol and infiltration solution and left overnight at 4°C before being placed in 1:20 mixture of 70% ethanol and infiltration solution, respectively. The intermediate infiltration step ensured the complete penetration of the polymer. The temperature was maintained at 4°C in order to slow down the rate of polymerization and therefore attain a 'complete' follicle fixation. The specimens were kept in the 95% infiltration medium for at least 1 week before they were embedded for sectioning. When the infiltration was complete the follicles appeared slightly translucent and sank to the bottom of the glass vial.

2.8.3 Embedding and mounting

The embedding solution was prepared by adding 15ml of infiltration solution and adding 1ml of hardener, taking care to avoid air bubbles. The embedding solution begins to polymerize within 5 minutes and after 15-30 minutes it becomes too thick to handle at room temperature; therefore embedding procedures were caried out on ice to extend the working time. Large volumes of solution polymerize more rapidly than small volumes due to the exothermic nature of the reaction; thus at all times small batches of the embedding solution were prepared.

The lower depression of an embedding mould was filled with embedding medium, the specimen immersed, and the medium them allowed to polymerize for at least 4 hours at room temperature.

The embedded samples were mounted onto wooden blocks using the mounting medium (prepared by adding 2 parts of powder to 1 part liquid, by weight) and allowed to set for at least 1 hour before sectioning.

2.8.4 Microtomy

2-4 μ thick sections were cut using a retracting rotary microtome (rotary-one; Pharmacia-LKB Instruments). Sections were floated on a beaker of distilled water to expand and flatten the sections before being mounted on glass slides. Sectioning was aided by periodically breathing over the block surface. Slides mounted with sections were placed on a hot plate at 45-50^{o}C and dried before staining.

2.8.5 Staining

All sections were stained using haematoxylin and eosin (Pantin, 1960).

2.9 Photography

Stained follicle sections and whole follicles for morphological examination (for viability assessments; see section 2.10.2) were photographed under a Zeiss photomicroscope).

2.10 *In vitro* cultures of vitellogenic follicles

2.10.1 Culture medium

Follicles were cultured in Leibovitz L-15 complete growth medium (Flow Laboratories, Rickmansworth, Herts., UK, see Table 2.1). Sachets containing 14.8 grams of powdered medium were dissolved in 1 litre of Hi-per solv purified water (BDH Chemials Ltd). Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) was added as a buffer at a concentration of 5mM and the medium adjusted to pH 7.8 using 0.5M sodium hydroxide. Both Penicillin G (Benzyl penicillin) and streptomycin sulphate (Sigma Chemical Company) were added at 100 000 International units (IU) per litre. The culture mixture was finally sterile-filtered using 0.22 μm 'Sterivex' filtration units (Millipore Corporation, Bedford, Massachusetts). Before the follicle incubations were started the volume of culture

Leibovitz L-15 Medium (Modified)

Ingredient	$mg.l^{-1}$	Ingredient	$mg.l^{-1}$
L-Alanine	225.0	Choline chloride	1.0
L-Arginine	500.0	Folic acid	1.0
L-Asparagine H_2O	250.0	i-Inositol	2.0
L-Cysteine	120.0	Nicotinamide	1.0
L-Glutamine	300.0	Pyridoxine HCl	1.0
Glycine	200.0	Riboflavin phosphate sodium salt	0.10
L-Histidine	250.0		
L-Isoleucine	125.0	Thiamine monophosphate $2H_2O$	1.00
L-Leucine	125.0		
L-Lysine HCl	93.70	$CaCl.2H_2O$	185.0
L-Methionine	75.0	KCl	400.0
L-Phenylalanine	125.0	KH_2PO_4	60.0
L-Serine	200.0	NaCl	8000
L-Threonine	300.0	$MgSO_4.7H_2O$	400.0
L-Tryptophan	20.0	Na_2HPO_4	190.0
L-Tyrosine disodium salt	372.9	D-Galactose	900.0
		Phenol red sodium salt	10.0
L-Valine	100.0		
D-Calcium pantothenate	1.00		

Table 2.1. The constituents of Leibovitz (L-15) culture medium.

medium required was added to vials containing the labelled VTG concentrate, placed in a thermostatically controlled water bath at 18 ± 1°C, and allowed to equilibrate.

2.10.2 Follicle cultures

Follicles, divested of their surface epithelium and surrounding connective tissue (see Chapter 5, experiment 1) were maintained in the protein free culture medium for 1 hour before the incubations were initiated. After this time only those maintaining the appearance of freshly dissected follicles were used in the subsequent experiments. Abnormal follicles, probably damaged during their dissection from the ovary, showed 2 main visual differences; firstly there was the appearance of a transparent ring around the periphery of the ooplasm that expanded as the follicle deteriorated and, secondly, therewas a polar coalescence of yolk lipid globules (see plate 2.1). Similar visual examinations for follicle viability were performed at the end of the cultures and only the 'normal-looking' follicles retained for counting and subsequent analysis.

2.10.3 Assessing the rates of vitellogenin uptake

Viable follicles received 2, 10 minute washes in protein free culture medium after incubation before being counted for radiolabel content. Follicles incubated with ^{3}H-VTG or ^{125}I-VTG were prepared and counted as previously described (section 2.6).

Rates of VTG uptake were expressed as ng VTG sequestered.mm^{2} follicle surface.$^{-1}$ hr.$^{-1}$ (ng. mm.$^{-2}$ hr.$^{-1}$) and were calculated from the formula below:

$$\left(\frac{\text{Counts per follicle}}{\text{Total counts in culture medium}}\right) \times \frac{\text{Number of hours in culture}}{\text{Follicle surface area } (4\pi \text{ radius of follicle}^2)} \times \text{Total VTG in the culture medium}$$

2.11 Statistical methods

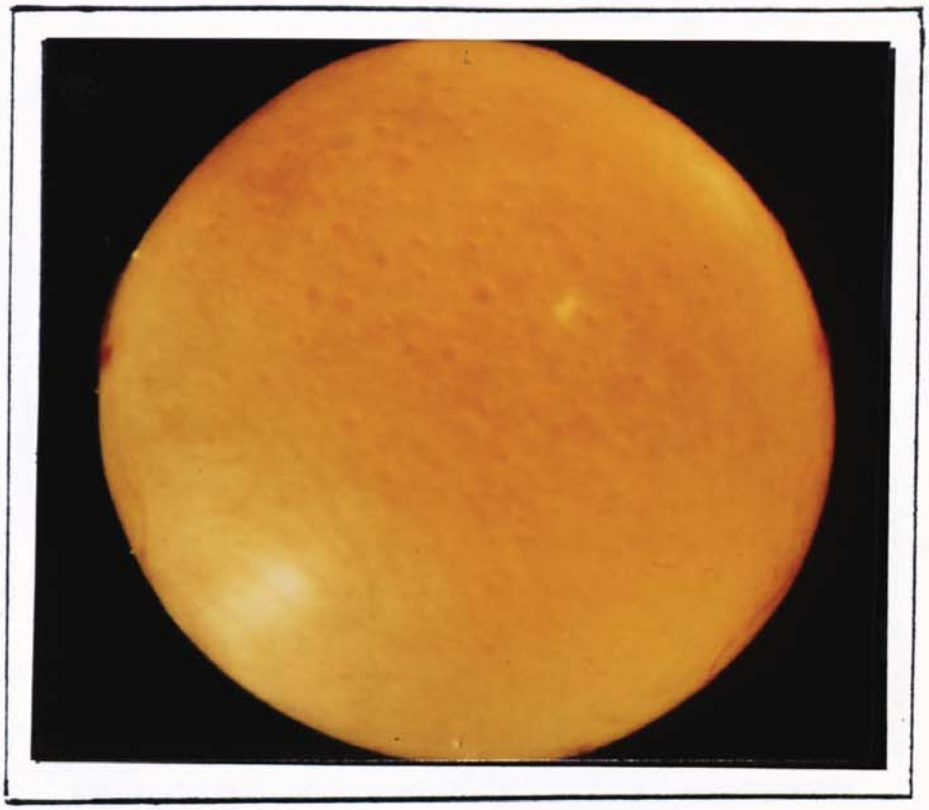
The statistical techniques used to analyse the data in the proce ding studies are listed below. More detailed descriptions of the tests employed are given in Sokal and Rohlf (1981).

Plates 2.1. Gross morphological differences of "viable follicles (used in incubations and subsequently for radio-label counting) and "inviable" (disguarded) follicles.

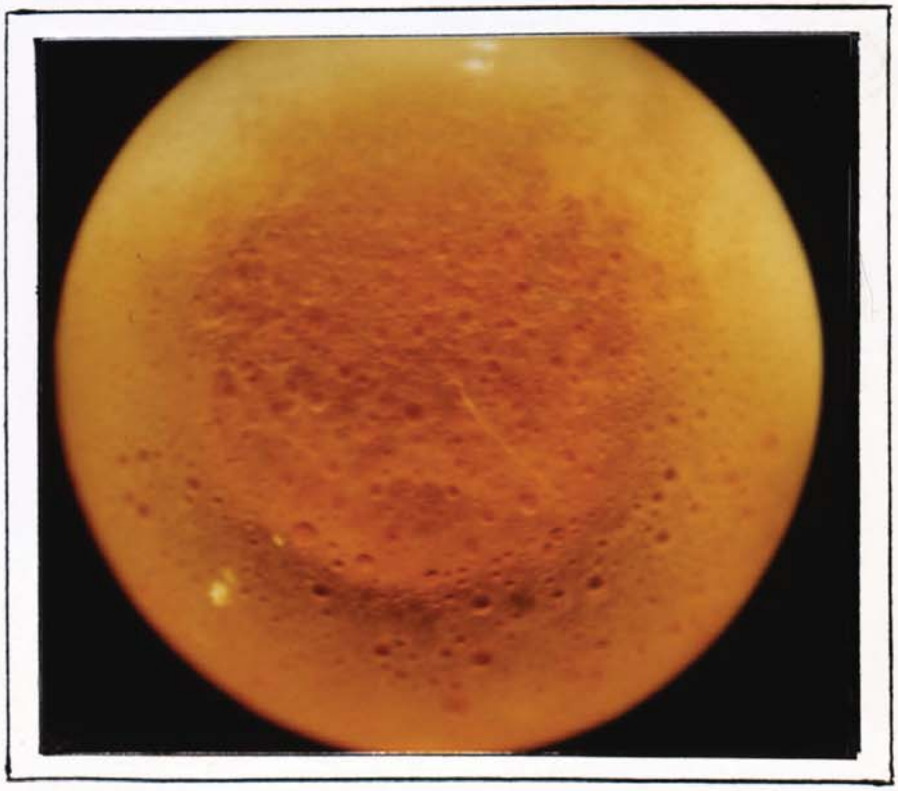
2.1a Viable follicle showing the homogenous pattern of yolk and lipid globule distribution, similar to that of a freshly dissected follicle. Magnification ×30

2.1b Inviable follicle showing the development of a pale ring at the periphery of the ooplasm (possibly caused by a contraction of the yolk protein mass) and a polar coalescence of the lipid globules. Magnification ×30

2.1a



2.1b



2.11.1 Estimation of the mean

The arithmetic mean of a sample represents an estimate of the true population mean (μ) and is calculated by summing all the individual observations of items of a sample and dividing the sum by the number of items in the sample. An estimate of the population mean throughout this thesis is written as the arithmetic mean ± one standard deviation ($Y \pm sd$).

$$\text{Arithmetic mean} = Y = \sum^{n} \frac{Y}{n}$$

where n = total number of sampling items
Y = independent variables
$\sum^{n} Y$ = sum of independent variables
$\sum^{n} (\bar{Y} - Y)^2$ = variance of sample

$$\text{Standard deviation} = \sqrt{\frac{\sum^{n} (\bar{Y} - Y)^2}{n}}$$

2.11.2 Comparison of more than two samples

One way analysis of variance (ANOVA) was used to compare data from more than 2 follicle groups subjected to different treatments. ANOVA calculations were carried out using a 'Minitab' statistical package (Ryan *et al.*, 1981) on a Harris mainframe computer. The test compares the mean values of the different treated groups and determines whether there is a difference between them. Sample sizes, of equal or unequal numbers of items (follicles) were analysed in this way.

An 'F' value is calculated, along with the degrees of freedom among the sample groups and the degrees of freedom within the sample groups.

$$\text{F value} = \frac{\text{Mean squares (MS) groups}}{\text{MS within}}$$

With degrees of freedom (df); between sample groups = a-1, within sample groups=

$\sum^{a} n - a$

where; MS groups =

$$\left[\Sigma^{a} \frac{(\Sigma Y)^2}{n} - \frac{(\Sigma \Sigma Y)^2}{\Sigma n} \right] \Big/ (a-1)$$

MS within =

$$\left[\Sigma \Sigma Y^2 - \frac{(\Sigma \Sigma Y)^2}{\Sigma n} - \left(\Sigma^{a} \frac{(\Sigma Y)^2}{n} - \frac{(\Sigma \Sigma Y)^2}{\Sigma n} \right) \right] \Big/ \Sigma n - a$$

a = number of sample groups
n = number of items or observations in each group
Y = observations

Using statistical tables and the 3 calculated values, F, df (a-1) and df (Σ-a) a probability value (P) is obtained. This P value determines whether the Null hypothesis (Ho; that there is no difference between the sample groups (treatments)) is correct. For example if the tabulated value of P was less than 0.05 one can be 95% confident that the group sample means are different.

2.11.3 Multiple Comparisons of the Means

Although ANOVA determines whether there are differences between a set of sample groups it does not determine which group (or groups) are responsible for the variation (i.e. which of the treatments are having an effect). To determine this a multiple comparisons of the means test had to be conducted, either the T-method when group items (n) were equal or the GT2-method (modified by Gabriel, 1978) when n was unequal.

2.11.3.1 T Method

(a) A minimum significant difference value is calculated (MSD).

MSD = (Critical value) x standard error (SE)

The critical value is obtained from calculated values in the studentized-range tables using Q α [k, v] where k = number of observations or items (i.e. n); v = df of the MS within and α = probability testing value.

The SE for the T-method is:

$$S_y = \sqrt{\frac{\text{MS within}}{n}}$$

(b) A pair of means, Yi and Yj, is declared significantly different at the experimentwise error rate α only if the difference between the sample means equals or exceeds the critical difference, i.e. if $(Yi - Yj) \geq MSD$.

(c) A convenient method for systematically testing all a (a-1)/2 pairs of means and employed throughout these studies is to compute upper and lower comparison limits for each mean such that 2 means are significantly different if and only if their limits do not overlap. The limits (Gabriel, 1978) are;

Li = Yi - 1/2 MSD (lower limit)
Ui = Yi + 1/2 MSD (upper limit)

Throughout the succeeding studies employing this test the MSD values were calculated for $\alpha = 0.05$ and therefore the subsequent comparison limits were for an experimentwise 0.05 error rate.

2.11.3.2 <u>The GT2 Method</u>

(a) A MSD value was again calculated = (Critical value) x SE. The critical value m α (k*,v) was obtained from 2-tailed studentized maximum modulus tables, where k = a (number of groups) and k* = k (k-1)/2, v = df of the $MS_{within.}$ and α = probability testing value (again throughout these studies 0.05).

The standard error for the GT2 test is:

$$SEij = \sqrt{\frac{2}{SYi} + \frac{2}{SYj}} = \sqrt{\frac{\text{MS within}}{ni} + \frac{\text{MS within}}{nj}}$$

(b) A pair of means (Yi, Yj) is declared significantly different at the experimentwise error rate of α (0.05) if and only if their difference equals or exceeds the critical difference, MSDij, that is, if (Yi - Yj) $\geq$ MSDij.

(c) Either a (k x k) table can now be prepared that lists these differences and MSD values or upper and lower comparison limits can be computed in a similar manner described in the T-method. Throughout these studies comparison limits were determined which avoided the tedium of table preparations, however, it should be stressed that comparison limits are an approximate method (Gabriel, 1978). Two means are declared significantly different only if their intervals do not overlap. For the i^{th} mean the limits are simply:

Li (lower limit) = Yi - $\sqrt{1/2}$ m α (k* v) SYi

Ui (upper limit) Yi + $\sqrt{1/2}$ m_{α} (k*, v) SYi

SECTION I - *IN VIVO* STUDIES

CHAPTER THREE

THE OVARIAN UPTAKE AND PROCESSING OF VITELLOGENIN AND ITS RELATIONSHIP TO THE YOLK PROTEINS

3.1 Introduction

An avian follicle performs one of the most dramatic examples of cellular growth aquiring 99% of its size during the 7 days before laying (Romanoff and Romanoff, 1949). An uptake of 1g plus of protein per day during this period, consisting predominantly of very low density lipoprotein (VLDL) and VTG, produces an increase in follicle diameter from 8 to 37mm (Cutting and Roth, 1973). *Xenopus* follicles similarly show rapid growth immediately prior to ovulation and here it is almost exclusively attributable to VTG accumulation (Wallace, 1983). Only in birds and amphibians have the processes relating to vitellogenic growth of the ovary been thoroughly investigated. Briefly, in these groups VTG is produced by the liver in response to oestrogens and on entering the circulatory system it is transported to the ovary where it is sequestered and processed into two major groups of distinct yolk proteins; the lipovitellins and the phosphoproteins, composed predominantly of phosvitins (Follett and Redshaw, 1974; Wallace, 1978).

The growth of a trout follicle from less than 1mm in diameter to 4mm and above within the few months prior to ovulation, although perhaps less spectacular as a cellular event compared to the process in the domestic fowl, is even more dramatic when one considers the number of eggs finally ovulated, approximately 2500 in a 1kg fish. In teleosts however, as mentioned in Chapter 1, quantification of the uptake of VTG into developing follicles has not been performed and the processing of VTG into the yolk proteins lipovitellin and phosvitin has not been clearly demonstrated. This chapter investigates the dynamics of VTG deposition and its relationship to the yolk proteins in vitellogenic trout follicles. Our knowledge of fish VTG and vitellogenesis, as briefly described above, is based on the models provided by the studies on amphibians and birds, and therefore this introductory passage as well as discussing the available information on fish vitellogenesis, documents the well established characterizations of ovarian and amphibian VTG and yolk proteins and details the dynamics of their deposition.

3.1.1 Production and isolation of vitellogenin

Vitellogenin was first extracted in a crude form in 1935, when Laskowski diluted the serum of laying hens with water and obtained a precipitate which he called serum vitellin. During the subsequent decade the view prevailed that chicken yolk proteins were found in the blood as a non-covalent complex. What were originally thought to be individual yolk protein precursors were

subsequently extracted and identified as part of this complex (McCully and Common, 1961; Mok *et al.*, 1961; Heald and Machlachlan, 1963, 1964; Beuving and Grober, 1971; Culbert and McIndoe, 1971).

Similarly in *Xenopus laevis* a lipoglycophosphoprotein was identified and isolated from the blood of oestrogen-treated males and shown to be composed of a number of smaller more readily characterized, yolk proteins (Wallace, 1970). The name vitellogenin (VTG), which originated as a generic term for the female specific blood borne yolk precursors in insects (Pan *et al.*, 1969), was adopted to describe this complex protein in *Xenopus* and similar molecules have subsequently been identified from nearly all oviparous (egg producing) vertebrate groups including fish.

All groups of non-mammalian vertebrates, from hagfish (Yu *et al.*, 1981), sharks (Craik, 1978a) and other fish (Wiegand, 1982) to turtles (Ho *et al.*, 1981) and birds (Giggins and Robinson, 1982) have been shown to synthesize and secrete VTG in response to oestrogens. In general 17-ß-oestradiol has been found to be the most effective steroid (Redshaw *et al.*, 1969; Sundararaj and Nath, 1981). Using 17-ß-oestradiol VTG production can be induced in both immature and male teleosts (Aida *et al.*, 1973; Emmersen and Petersen, 1976; Campbell and Idler, 1980; Nath and Sundararaj, 1981; Bromage and Cumaranatunga, 1987) and may also be augmented in maturing females (Bailey, 1957; Campbell and Idler, 1980). *In vitro* cultures of liver tissue have led to a greater understanding of the processes involved in the production of VTG. After induction by oestrogens, VTG is synthesised in membrane-bound ribosomes, subsequently undergoes a number of post-translational modifications which include; glycosylation, lipidation and phosphorylation, before being packaged in the Golgi-apparatus prior to its secretion into the blood (Waugh and Knowland, 1975; Green and Tata, 1976; see Figure 3.1). In birds and amphibians, once VTG has been released into the blood, either as a result of oestrogen induction, or by using females in the vitellogenic phase of their reproductive cycle, it can be isolated and purified using several different procedures (Wiley *et al.*, 1979; Wallace, 1985). Generally a precipitation step, involving the divalent cations calcium (Ca^{2+}) or magnesium (Mg^{2+}) and 1,2-di(2-aminoethoxy ethane-N',N',N'N-tetra acetic acid (EGTA) or diaminoethane tetracetic acid (EDTA), is employed followed by a gel filtration and/or ion-exchange chromatography. Divalent ions cannot alone precipitate VTG when other serum proteins are present probably because they form soluble complexes with the VTG

Figure 3.1. Scheme summarizing the major events triggered by 17β- oestradiol leading to the secretion of vitellogenin into the blood (after Tata,1978).

divalent cation salts. This mechanism probably serves to keep the VTG, which is normally present as the calcium salt (Wallace, 1970), soluble in the blood. The bifunctional chelating agents EGTA and EDTA apparently displace such proteins and effectively cross link and precipitate the divalent salts of VTG. Using this method *Xenopus* VTG may be obtained at high yields and at purities of up to 99% (Wallace and Selman, 1982).

Vitellogenin, however, is particularly difficult to obtain in a pure and unaltered form from many teleosts. As described later teleost VTG's are generally less phosphorylated than those of other vertebrates (de Vlaming *et al.*, 1980) so that the established methods for VTG isolation, which rely on its high surface charge, such as EDTA-Mg^{2+} precipitation or chromatography on DEAE-Cellulose (Wiley *et al.*, 1979), either fail to precipitate VTG or do not resolve it adequately from other blood proteins. Furthermore, teleost VTG's are said to be highly susceptible to proteolysis during isolation and divalent cations such as Ca^{2+} may activate proteolytic enzymes. Bjornsson and Haux (1985), working on rainbow trout, further demonstrated that VTG concentrations of at least 7-8g.100ml^{-1} were required for effective precipitation. One of the primary objectives of this study was to establish a protocol for the purification of intact trout VTG.

3.1.2 Physio-chemical structure of vitellogenin

3.1.2.1 Birds and amphibians

The molecular weight of serum VTG in *Xenopus* and the white leghorn chicken have been shown to be approximately 400 000 Daltons (Wallace and Bergink, 1974; Wallace, 1978) and 450 000 Daltons (Deeley *et al.*, 1975; Christman *et al.*, 1977) respectively. Both VTG's exist as dimers in the blood and have monomeric subunit molecular weights of between 200-240 000 Daltons (Figure 3.2). In both the domestic fowl and *Xenopus* each monomer of VTG contains lipovitellin and phosvitin moieties. The lipovitellin and phosvitin substructures of the VTG molecule are identical to the corresponding yolk protein products and are therefore described in more detail in the later section on yolk proteins (see Figure 3.2). The amino acid composition of *Xenopus* and chicken VTG is very similar both being rich in the non-polar, aliphatic amino acids, alanine and leucine and the carboxylic amino acids aspartic acid and glutamic acid. Hydroxylic amino acids have considerable representation in the form of serine and threonine as does the amino group in lysine and arginine. The sulphur containing amino acids notably, cysteine and methionine comprise the smallest component

Figure 3.2. Proposed structural compositions of amphibian (Xenopus laevis) and avian (Gallus domesticus) vitellogenins. Amphibian; (a)- Bergink and Wallace (1974), (c) - Wiley and Wallace (1981). Avian; (b) Christmann et al.,(1977).

(reviewed in Campbell and Idler, 1980).

The carbohydrate fraction of VTG is incorporated in to the molecule within a smooth-membrane compartment during the post translational modifications of VTG (Wallace, 1985). This fraction consists of 3 mannose, 3 galactose 5-N-acetylglucosamine and 2 sialic acid residues arranged in a branched structure and attached to the protein via one asparagine residue (Callard and Ho, 1986). Yamasaki (1974) and Gellisson *et al.*, (1976) have both suggested that the carbohydrate fraction may play a role in yolk precursor and yolk protein recognition, an idea that is reinforced by observations that in the ayu, *Plecoglossos altevelis* the carbohydrate fraction remains unchanged from its incorporation in VTG until the processing of VTG into the yolk protein product lipovitellin (Aida *et al.*, 1973). Lipidation of VTG in the liver is difficult to study because of the noncovalent nature of the association, therefore little is known about the mechanism or site of VTG lipidation in the liver or the extent to which lipid exchange or sequestration can occur once VTG is secreted into the bloodstream. *Xenopus* and chicken VTG are rich in phospholipids but also contain triglycerides and cholesterol.

The high degree of phosphorylation places VTG as the most acidic of the serum proteins (Deufal *et al.*, 1979). Data indicating that 94-98% of the intracellular VTG in the liver is unphosphorylated (Wang and Williams, 1982), together with the observation that ^{32}P.VTG appears in plasma with a lag of only several minutes after ^{32}P-orthophosphate administration to oestrogen treated roosters (Bergink *et al.*, 1973), argue for VTG phosphorylation occurring immediately before secretion in the chicken. In *Xenopus* approximately 30% of the phosphate residues are covalently attached to VTG in the roughendoplasmic reticulum after termination of polypeptide synthesis; the remainder of the phosphate is incorporated during intracellular translocation through membrane compartments comprising the smooth microsome subfraction (smooth endoplasmic reticulum, Golgi apparatus, secretory elements). The principle sites of phosphoprotein phosphorylation are the serine-hydroxyl groups. The functional role of phosphoprotein is not clear, but it has been suggested that the high binding energy could be utilized during embryonic development. Alternatively the phosphorylation may play a part in the binding of VTG to its receptor in the ovary.

Once hepatically synthesised and post-translationally modified, VTG becomes loosely complexed with calcium before its secretion into the blood. Functionally the divalent cation is thought to solubilize the VTG complex in the

serum (Follett and Redshaw, 1974) as well as providing a source of calcium for the developing embryo. Wallace and Bergink (1974) suggested that the calcium ions neutralize the negative charges on the VTG molecule, which are a consequence of its high phosphorus content. Although it is not certain whether all or a portion of the calcium enters the follicle when VTG is taken up, it may represent a mechanism for producing a calcareous shelled egg, which is widely found in oviparous vertebrates. Exposure of *Xenopus* follicles to a calcium-free solution causes a reversible loss of sequestering ability, an effect that Wallace *et al.*, (1973) ascribed to the temporary loss of receptor sites for VTG from the oocyte surface. The calcium ion may thus be involved in receptor-binding of VTG and/or its transport across the oocyte membrane (Wallace *et al*, 1973). Studies on both the domestic fowl and *Xenopus* have provided evidence for a multiplicity of VTG molecules. The occurrence of more than 1 type of VTG in these oviparous species has been shown to be determined, at least in part, by the number of genes coding for VTG. The fowl, which is diploid (having paired chromosomes and thus 2 alleles for each gene) has 2 major and 1 minor VTGs, encoded by distinct genes (Wang *et al.*, 1983) which may be differentially expressed at different stages of the reproductive cycle (Wang and Williams, 1983). Similarly extensive work by Wahli and associates (1976, 1978, 1979) has demonstrated that *Xenopus*, which is also diploid, VTG is encoded by a small family of related genes, comprised of 2 major gene groups, A and B, which differ in their sequences by 20% containing 2 subgroups, A_1 and A_2 and B_1 and B_2, which differ by 5%. Genes A_1 and B_1 are linked (Wahli *et al.*, 1982) suggesting that the A and B sequences arose by duplication of an ancest ral gene and have subsequently diverged. Each gene in this 'family' is transcribed by a 6.3 kilo-base mRNA. The monomers created by the multiplicity of VTG genes have molecular weights of 197,000, 188 000 and 182 000 Daltons, are produced in a 2:2:1 ratio, respectively (Wahli *et al.*, 1978). Tata *et al.* (1980) have discovered further VTG-like genes in *Xenopus* that are non-expressible showing that care should be taken when drawing parallelisms between gene numbers and VTG types however, and that a multiplicity in VTG genes does not necessarily mean a multiplicity in VTG molecules.

Clearly VTG is a molecule of high complexity and its intricate structure along with the multiplicity of production make it difficult to thoroughly characterize even in animals that have received extensive study. In almost all

oviparous vertebrate species studied the nature of the chemical bonds that maintain the VTG complex intact in the serum and the mechanism responsible for the macromolecule conversion within the follicle are not yet fully understood.

3.1.2.2 Fish

Vitellogenin(s) have been demonstrated in a wide variety of teleosts (LeMenn, 1978; Wallace, 1978; Tata and Smith, 1979; Nath and Sundararaj, 1981; Wallace and Selman,1981) including the rainbow trout (Hara and Hirai, 1978; Campbell and Idler, 1980; Sumpter, 1985) and in many cases their hepatic origins confirmed (Plack and Frazer, 1971; Campbell and Idler, 1976; de Vlaming *et al.*, 1977; LeMenn and Lamy, 1977; Larsen, 1978; Idler and Campbell, 1980; Sundararaj and Nath, 1981). Attempts to determine the molecular structure of fish VTGs have indicated both inter- and intra- species differences. The large intraspecific variations are most likely due to the use of different isolation procedures as well as different methods for molecular weight determinations. Studies on rainbow trout VTG using gel-filtration have produced estimates of the molecular weight for the intact molecule of between 440 000 Daltons (Campbell and Idler, 1980; Copeland, 1984; Norberg and Haux, 1985) and 600 000 Daltons (Hori *et al.*, 1979). Using native gel electrophoresis two groups of investigators (Hori *et al.*, 1979; de Vlaming *et al.*, 1980) have both found monomeric and dimeric forms of VTG in the goldfish, *Carassius auratus*. Studies on the rainbow trout have also demonstrated a monomeric form of VTG with a molecular weight of 170 000 Daltons (Chen, 1983). In the trout it is apparently the non-covalently bound dimeric form that is present in the serum (Chen, 1983; Norberg and Haux, 1985).

The isolation and partial characterization of rainbow trout VTG has shown that about 80% of the molecule is protein (Fremont *et al.*, 1984; Norberg and Haux, 1985). Analysis of the amino acid composition shows a similar pattern to that of other oviparous vertebrates (Campbell and Idler, 1980) with alanine, leucine and glutamic acid being the most abundant (Hara and Hirai, 1978; Campbell and Idler, 1980).

Further chemical studies of trout VTG have confirmed its glycolipophosphoprotein nature (Hori *et al.*, 1979; Campbell and Idler, 1980). To date, however, the carbohydrate fraction has not been characterized. Rainbow trout VTG has a lipid content of approximately 20% (Fremont *et al.*, 1984; Norberg and Haux, 1985) and in general fish VTGs have been found to contain

much higher levels of lipid than reported for *Xenopus* VTG (Redshaw and Follett, 1971) and those of other oviparous species (Wallace, 1970). The largest proportion of lipid in trout VTG, approximately 61%, consists of phospholipid (Norberg and Haux, 1985). This fraction probably plays an important role in embryogenesis both as an energy source and as a precursor of structural elements in embryonic tissues. The remaining lipids include tryglycerides (22%) and cholesterol (11%) (Norberg and Haux, 1985). The origin of these lipids is believed to be fat depots in the muscle and around the intestine (Kaitaranta and Ackman, 1981). Although VTG is likely to be the major source of polar-lipids to the developing follicle there are 2 alternative pathways for the accumulation of lipids; the first is the endogenous synthesis of lipids (Wiegand and Idler, 1982) and the second the selective uptake of lipids, mainly triglycerides, (Leger *et al.*, 1980). Both processes are said to occur prior to the onset of exogenous vitellogenesis (Raven, 1961; Guraya, 1965). The precise contributions of each of these pathways together with the uptake of lipids as integral components of the VTG complex, has not been established.

In contrast to *Xenopus* VTG, teleost VTGs are relatively poor in serine and consequently contain a lower level of protein phosphorus. The phosphorus content of teleost VTG ranges from 0.5-0.8% by weight (Wallace, 1978; Norberg and Haux, 1985). Campbell and Idler (1980), assigned a phosphorus content of 0.63% for rainbow trout VTG, although van Bohemen *et al.*, (1981) suggested that trout VTG exists in a number of forms with different degrees of phosphorylation. The suggestion of more than one type of VTG in the rainbow trout is discussed below.

In trout, as in other oviparous vertebrates, VTG on passing into the blood becomes looselycomplexed with calcium. It is well documented in teleosts that an induction of VTG production using oestrogens causes a concomittant rise in serum calcium (Elliott *et al.*, 1979; de Vlaming *et al.*, 1980) and by separating the free and bound fractions of plasma calcium Bailey (1957) demonstrated in oestrogenized carp, *Cyprinus carpio*, that this increase was entirely due to the binding of calcium to protein. Ultrafiltration of trout plasma (Björnsson and Haux, 1985) confirmed the binding of calcium to VTG and further demonstrated that the physiologically important free fraction of this ion remained unchanged. The calcium content of rainbow trout VTG is 0.5-0.6% by weight (Bjornsson and Haux, 1985; Sumpter, 1985).

The origin of VTG-bound calcium in fish varies with the degree of organisation of the bone. Cellular boned fishes, for example the eel, *Anguilla anguilla*, and the rainbow trout derive the calcium from bones (Lopez *et al.*, 1976). Acellular boned fishes on the other hand, for example the killifish, *Fundulus heteroclitus*, obtain calcium from scales (Mugiya and Watabe, 1977).

Some teleost VTGs appear to be highly susceptible to proteolysis and are easily denatured during isolation procedures (Hickey and Wallace, 1974; de Vlaming *et al.*, 1980). This feature may be due to the different chemical composition and structure of fish VTGs compared to those of other oviparous vertebrates (Norberg and Haux, 1985). Hickey and Wallace (1974) suggested that serine protease inhibitors prevented the disintegration of teleost VTG, however, in a thorough characterization of goldfish VTG, de Vlaming and co-workers (1980) showed that even when these precautions are taken proteolysis cannot be entirely prevented.

Multiple high molecular weight VTG polypeptides, apparently not generated by proteolysis, as well as being present in birds and amphibians, have also been observed in the goldfish (Hori *et al.*, 1979; de Vlaming *et al.*, 1980) and these authors suggest that they arise as a result of different post-translational modifications. Furthermore all members of the family Salmonidae are considered to be tetraploid (Allendorf and Thorgaard, 1984) and thus it seems likely that they could produce multiple VTG's. Indeed multiple VTG's would explain the conflicting data amongst teleosts regarding the size and number of the different components of the VTG complex. That such a large and heterogenous molecule should be presented in more than one form suggests that different VTG's may have different physiological roles either in their nutritional value to the egg, in their speed of uptake or in their availability at different stages of the reproductive cycle. It should be realized, however, that to date only one VTG mRNA has been detected in the liver of rainbow trout (Chen, 1983; Valatoire *et al.*, 1984) and a fuller understanding of the nature of teleost VTG(s) is required to determine the number(s) of VTG(s). Clearly the possibility of multiple forms of VTG and/or their susceptibility to undergo denaturation are complicating factors in structural studies of the intact protein. It should be noted, however, that although the detailed structure of VTG is of scientific interest it is not the primary concern of this chapter, which is concerned more with VTG's relationship to the yolk proteins.

3.1.3 Measurement of serum vitellogenin

A number of techniques have been developed for the determination of serum levels of VTG in fish, including; immunoagglutination (Le Bail and Breton, 1981), densitometry following electrophoresis (van Bohemen *et al.*, 1980), radial immunodiffusion (Hara, 1975) and radioimmunoassay (Idler *et al.*, 1979a; Campbell and Idler, 1980; Sumpter, 1985). Indirect methods for assessing serum levels of VTG include measurement of calcium and phosphoprotein phosphorus (Laskowski, 1936; Wallace, 1970; Whitehead *et al.*, 1978a, 1978b; Elliott *et al.*, 1979; Hori *et al.*, 1979). Each of these techniques has distinct advantages and disadvantages. Phosphorus and calcium measurements are less sensitive than the direct methods of VTG measurement because, as previously mentioned, VTG may exist in forms with different degrees of phosphorylation and possibly calcium content. The blood calcium level in a male and immature female trout is around 10 mg^{-1}.100ml (when VTG levels are in $ng.ml^{-1}$or less) and not until the blood VTG rises above 5 mg^{-1}.ml in a maturing female does the calcium value significantly increase; when blood VTG levels are below 5 mg^{-1}.ml the calcium attached to the VTG is a very small percentage of the basal serum level (10 $mg.^{-1}$ 100 ml) and hence is difficult to measure. Data derived using such techniques have led to suggestions of quiescent phases when really they don't exist; it is only due to the relatively insensitive technique. However, calcium and phosphorus measurements are quick and simple to perform and are useful in the middle and later stages of exogenous vitellogenesis.

Radioimmunoassay for VTG, the major technique used in this study, is much more sensitive than other techniques of quantifying VTG concentrations. Usng this method VTG can be measured down to a concentration of 1ng. ml^{-1}(Sumpter, 1985; Sumpter *et al.*, 1984) using the same assay showed that VTG could be detected in the blood of female rainbow trout and shown to undergo changes in production, 2 or more years before uptake of VTG begins; in female fish of less than a year old, weighing between 50-100g, VTG levels may be only $10ng.ml^{-1}$. Blood levels increase a million fold during sexual maturation. At the start of exogenous vitellogenesis serum levels are usually around $0.5mg.ml^{-1}$ (van Bohemen and Lambert, 1981) and this rises up to the time of spawning when levels may exceed 50 $mg.ml^{-1}$ (Scott and Sumpter, 1983; Elliott *et al.*, 1984; Fremant *et al.*, 1984), making VTG the major blood protein. Care has to be taken in the use of this assay, however, for the lability of VTG could mean that there is an increase in the number of antigenic sites, leading to overestimations of the true

VTG level. Indeed Elliott (1984), using this assay, found that serum samples showed an increase in the calculated VTG content with increasing periods of freezing; VTG: calcium contents in one serum sample were 1.3:1 and 1.9:1 after 1 and 9 months in storage respectively. Radioimmunoassay for VTG may also be used to detect and measure components of the VTG molecule conferring a cross reactivity to antigens to the intact molecule. In the VTG assay used in this study, as described in Chapter 2 (section 2.7) the antibody is directed to the lipovitellin moiety of the VTG molecule and this feature clearly has useful implications in determining the relationships between serum VTG and yolk protein products.

3.1.4 Yolk protein

3.1.4.1 Birds and amphibians

In birds and amphibians VTG is sequestered by the vitellogenic follicle as two major cleavage products; the lipovitellins and the phosphoproteins (Follett and Redshaw, 1974; Wallace, 1978). In *Xenopus* these yolk proteins are complexed in an orthorhombic lattice (Ohlendorf *et al.*, 1978) to form yolk platelets. These crystalline yolk platelets, which are relatively insoluble, have now been found in a number of other amphibians (Lange and Richter, 1981). By contrast the yolk of chicken eggs does not have a crystalline structure (Bellairs, 1967) but rather is packaged in yolk spheres (Grodzinski, 1951), which are membrane limited and generally contain numerous osmiophilic subdroplets or granules (Bellairs, 1961, 1967); these granules contain the cleavage products of VTG. The matrix of the yolk sphere in which the subdroplets are embedded presumably is derived from blood-borne proteins other than VTG, particularly the very low density lipoproteins (VLDL); these will be discussed later in this section.

Avian and amphibian lipovitellins are lipoglycoproteins and in the amphibians comprise the bulk of the yolk proteins (Wiley and Wallace, 1980). Most of the lipids consist of phospholipids with small amounts of neutral lipid, present as esters of cholesterol. In their native state lipovitellins in both birds and amphibians are large dimers with an approximate molecular weight of 400 000 Daltons (Bernardi and Cook, 1960; and Wallace *et al.*, 1966, respectively). The monomeric subunits have molecular weights of around 200 000 Daltons (Wallace, 1965) i.e. each monomer of VTG gives rise to a monomer of lipovitellin. The lipovitellin monomer in the domestic fowl may be further subdivided into 135 000 Dalton apolipovitellin 1 (apo-Lv-1) and 30 000 Dalton

apolipovitellin 2 (apo-Lv-2) units (Christmann *et al.*, 1977). Similarly, in *Xenopus* the monomer may be divided into 120 000 Dalton apo-Lv-1, and 31 000 Dalton, apo-Lv-2, subunits (Bergink and Wallace, 1974). Wiley and Wallace (1981) in a more extensive study on the yolk platelet proteins in *Xenopus*, using higher resolution analytical procedures found 3 apo-Lv-2 polypeptides (121 000, 116 000 and 111 000 Daltons) and 3 apo-Lv-2 polypeptides (34 000, 31 500 and 30 500 Daltons, see Figure 3.2). The lipovitellins in each class may be derived either from the same parent molecule by differential intra-follicle cleavage or from separate VTG species as discussed earlier.

In the domestic fowl the yolk phosphoprotein derivatives of the VTG dimer are 2 phosvitin molecules which have slightly different amino acid compositions (Connelly and Taborsky, 1961; Clark, 1970) and sizes (40 000 and 36 000 Daltons, Taborsky and Mok, 1967). In *Xenopus*, however, as shown in Figure 3.2, the follicle phosphoproteins are divided into 2 categories each containing 2 proteins; the phosvitins (34 000 and 33 000 Daltons) and the smaller phosvettes (19 000 and 13 500 Daltons; Wiley and Wallace, 1981). Ohlendorf *et al*, (1978) and Wiley and Wallace (1981) proposed that the phosvitins arise from the differential cleavage of 2 of the VTG polypeptides whereas the phosvettes are formed from an additional cleavage site in the phosvitin domain of a third VTG. The phosvitins are phosphoglycoproteins and contain little or no lipid. The unique feature of all characterized phosvitins is their high phosphorus content which is in turn linked to the protein's high proportion of serine residues. Phosphorus, joined by ester-linkages to these residues, may comprise 10% of the phosvitin's weight, creating one of the most highly phosphorylated proteins in nature (Levene and Alsberg, 1901; Mecham and Olcott, 1949).

In *Xenopus* and the majority of oviparous species studied the yolk protein products of VTG comprise, by far, the bulk of the follicle's reserves. However, this is not the case in birds and in the domestic fowl where VTG products comprise only 20% of the yolk solids (Burley and Cook, 1961). The major part of chicken yolk is composed of a so-called low density fraction (LDF), this comprises 70% of the yolk solids (Martin *et al.*, 1963). The low density fraction is a unique component of avian yolk and has been fractionated into several partially characterized peptides (Burley, 1957). The LDF is derived from a 'very low density' lipoprotein in the blood (VLDL). Originally detected in the serum of egg-laying hens (Schjeide, 1954) VLDL contains 82-88% lipid associated with protein (Hillyard *et al.*, 1956) and is synthesised and secreted in large amounts

in response to oestrogens (Urist and Schjeide, 1961; Cham *et al.*, 1976). Serum VLDL is comprised of only 2 polypeptide species (Williams, 1979); however, it is not known whether the more numerous peptides found in the LDF apoprotein are formed by proteolytic processing within the follicle or during the lengthy manipulations of isolation.

Another group of proteins found in significant quantities in the developing chicken follicle are the livetins. These make up approximately 10% of the total yolk solids and are a heterogenous collection of proteins quite distinct from lipovitellin, phosvitin, or the phosvettes. Livetins have been shown conclusively to be derived and related to the normal serum proteins (Williams, 1962; Mok and Common, 1964). Albumin, α_2-glyco-proteins, transferrin and τ-globulins are the most abundant components of the livetin fraction. Other serum proteins such as the vitamin-binding proteins have also been shown to selectively enter the follicle during vitellogenic growth in the domestic fowl. These are considered in more detail in Chapter 4.

Although fewer non-vitellogenin protein compounds appear to enter amphibian follicles during vitellogenesis, over the years researchers have documented the accumulation of other materials such as lipids, glycogen, histones and maternal RNA transcripts, which although present at much lower levels are nevertheless indispensible for embryonic development and survival.

3.1.4.1 Fish

In cyclostomes and a few teleost species the yolk proteins are complexed together as crystalline platelets in much the same way as for *Xenopus* although the lattice pattern differs in being monoclinic as opposed to orthorhombic. However, in the majority of teleosts, particularly the marine species, the yolk protein is not found in relatively insoluble platelets; rather it accumulates in fluid-filled yolk spheres or yolk globules (Grodzinski, 1954; Wallace and Selman, 1981). These structures either maintain their integrity throughout follicle growth (Yamamoto, 1957) or, as described earlier for the rainbow trout, fuse centripetally eventually forming a continuous mass of fluid yolk.

Attempts have been made to isolate 'lipovitellin-like' and 'phosvitin-like' proteins from the ovaries of a variety of teleost species, these species include: the herring, *Clupea harengus* (Barman *et al.*, 1979), cod, *Gadus morhua* (Plack *et al.*, 1971), Pacific salmon (Markert and Vanstone, 1968), Atlantic salmon,

Salmo salar, (Idler *et al.*, 1979) and trout species (Ando, 1965; Ito *et al.*, 1966; Wallace *et al.*, 1966; Campbell and Idler, 1980). Although electrophoretic studies on teleost yolk proteins have yielded results consistent with data obtained for lipovitellin and phosvitin in other species (Hara and Hirai, 1978; de Vlaming *et al.*, 1980; Lange *et al.*, 1983) the yolk proteins isolated from teleosts generally show atypical properties. Molecular weight estimations for trout lipovitellin(s) range from 390 000 Daltons (Copeland, 1984) down to 130 000 (Campbell and Idler, 1980). Trout lipovitellin appears to be composed of 2 subunits (Ando, 1965), a similar pattern to that previously described for *Xenopus* (Wallace *et al.*, 1966). The high lipidation, little or no protein phosphorus and high solubility in low-ionic strength solutions (Jared and Wallace, 1968) however, are unlike all other characterized lipovitellins. Lipovitellin lipids in teleosts consist of 58-66% phospholipid (Markert and Vanstone, 1971; Plack *et al.*, 1971) and small amounts of neutral lipid (Market and Vanstone, 1971). Campbell and Idler, (1980) calculated an alkali-labile phosphorus content of 0.007% for trout lipovitellin, whereas undetectable levels were found in the cod lipovitellin by Plack *et al.*, (1971). Teleost lipovitellins exhibit greater physio-chemical heterogeneity than corresponding molecules from the hen, frog, dog-fish and cuttle fish, a difficulty which has complicated attempts to resolve the structure and derivation(s) of this molecule (Wallace and Jared, 1968).

Information on phosvitin, the yolk phosphoprotein from teleost eggs, is extremely diverse and indeed more puzzling than the more established data from other vertebrate groups (Wallace, 1978). Phosvitins are said to be more numerous (Mano and Lipmann, 1966; Jared and Wallace, 1968) and appear to have lower molecular weights than found in both birds and amphibians (Schmidt *et al.*, 1965; Wallace *et al.*, 1966; Market and Vanstone, 1971; de Vlaming *et al.*, 1980). Reported molecular weights for rainbow trout phosvitin range from 19,400 (Ando, 1965) to 45 000 Daltons (Campbell and Idler, 1980). Mano and Yoshida (1969) and de Vlaming *et al.* (1980) working on the rainbow trout and goldfish, respectively, both suggested that phosvitin exists as a dimer. Teleost phosvitins contain a widely divergent levels of phosphoprotein phosphorus. The variability in phosphoprotein phosphorus content may in part be due to the differences in the proportion of serine residues in different teleosts; from 42% in the rainbow trout (Mano and Lipman, 1966) up to 75% in the herring (Inoue *et al.*, 1971). Mano and Lipman (1966) have suggested that there are specific

protein kinases present within the developing follicle which differentially phosphorylate and dephosphoprylate the phosvitin molecules. Furthermore the specific phosvitin content of fish ovaries appears to be quite variable, varying from about 3% in the ovaries of the rainbow trout (Campbell and Idler, 1980) to negligible levels in several marine fish (Jared and Wallace, 1968; Craik, 1982). Jared and Wallace (1968) proposed that developing eggs from marine species could obtain the phosphorus they require from the environment.

Other native proteins have been isolated from yolk preparations in fish which have neither the characteristics of lipovitellin nor of phosvitin (Jared and Wallace, 1968) One such protein is the 'ß' component, isolated from the yolk of *Oncorhynchus kisutch* (Markert and Vanstone, 1971) and the rainbow trout (Campbell and Idler, 1980). It contains neither lipid nor protein-phosphorus and has a molecular weight of approximately 30 000 Daltons. The 'ß' component is derived from the blood of sexually maturing females and oestrogenised animals and is not a livetin or normal blood protein (Jared and Wallace, 1968). However, studies by Campbell and Idler (1980) suggest that the 'ß component' is immunologically related to VTG, implying it is derived from this serum precursor. Until the structure(s) of the teleost VTG(s) are fully resolved it would not be appropriate to designate the 'ß component' as a distinct non-vitellogeninprotein.

3.1.5 Aims of Study

It should be stressed that the processing of VTG to the yolk proteins lipovitellin and phosvitin has not yet been clearly demonstrated in fish, although there is some evidence that this relationship does exist. A number of studies have illustrated a clear immunological relationship between teleost VTG and egg yolk proteins (Suzuki and Suyama, 1979; de Vlaming *et al.*, 1980) and similarities in chemical compositions between VTG and yolk proteins have been demonstrated in the catfish using peptide mapping and SDS gel electrophoresis (Sundararaj, 1981). Extensive studies on the rainbow trout have shown strong similarities between the immunological, biochemical and electrophoretic properties of serum VTG and the yolk proteins (Norberg and Haux, 1985). The amino acid composition of trout VTG and yolk proteins are also remarkably alike further reinforcing the closeness of the relationship (Hara and Hirai, 1978; Campbell and Idler, 1980). Furthermore histological studies on vitellogenic follicles confirm the appearance of yolk granules beneath the vitelline membrane coincident with high VTG

concentrations ($mg.ml^{-1}$) in the serum (Aida *et al.*, 1973).

Although the available evidence suggests that the mechanism for vitellogenesis is similar in teleosts to that already documented in birds and amphibians, the information reviewed here clearly shows that fish VTG and yolk proteins are structurally and physiologically different from other oviparous vertebrates, and appear to hold a position of uniqueness (Wallace, 1978).

The objectives of the experiments in this chapter were 3 fold; firstly to gain an understanding of the relationship between serum VTG and the major yolk proteins, secondly to establish a protocol for the isolation and purification of intact VTG in order to provide the large titres of labelled and non-labelled VTG required in subsequent experiments on vitellogenesis in this thesis, and, thirdly, using the purified VTG, to determine the rates of uptake and processing of VTG into vitellogenic follicles and therefore the importance of VTG in ovarian growth.

3.2 Experiment 1:The uptake and processing of vitellogenin in the ovary of the rainbow trout

This first experiment was modelled on work previously performed by Wallace and Jared (1969) on *Xenopus laevis* and attempted to describe the uptake and processing of VTG into the yolk proteins. *Xenopus* lipovitellin and phosvtin are known to contain 98% of the yolk platelet leucine and 79% of the phosphorus, respectively. Therefore, by injecting a double labelled (^{3}H-leucine and ^{32}Phosphorus) VTG into a maturing female *Xenopus* Wallace and Jared were able to monitor the progression of VTG from its initial uptake into the follicle until its subsequent cleavage into ^{3}H-leucine-lipovitellin and ^{32}Phosphorus-phosvitin. In rainbow trout VTG, the leucine and phosphorus components of lipovitellin and phosvitin are thought to parallel those of *Xenopus* (Campbell and Idler, 1980) and thus by injecting free ^{32}Phosphorus and ^{3}H-leucine into females undergoing exogenous vitellogenesis it should be possible to observe their incorporation into VTG, and the subsequent processing of this dual-labellel VTG into single-labelled yolk proteins. During this experiment the identities of VTG and major yolk proteins were confirmed by both radioimmunoassay (VTG and lipovitellin; see earlier) and by amino acid analysis.

Throughout this study proteins were separated using gel-filtration in preference to using ion-exchange chromatography on TEAE-cellulose (used by Wallace and Jared in their studies on *Xenopus*) because the former separates proteins on the basis of molecular weight and the sizes (and hence elution

positions) of rainbow trout VTG and yolk proteins are fairly well documented (see the introduction to this chapter) whereas the latter separates proteins on the basis of their charges and the charge, and hence elution sequences from an ion-exchange gel of VTG and yolk proteins are at present not well defined.

3.2.1 Part A

Tracing the uptake of ^{32}P and ^{3}H leu. from their injection into the peritoneal cavity into the blood and subsequently their incorporation into the ovary of vitellogenic females

3.2.1.1 Materials and methods

Two female trout, both undergoing exogenous vitellogenesis (as determined by their serum calcium levels), were each injected with 100 μ Curies and 50 μ Curies (μC; 1 μC = 2.2×10^6 dpm) of ^{32}Phosphorus and ^{3}H leucine respectively. The labels were diluted in physiological saline to give an injection volume of 1ml and administered intraperitoneally using 1 ml syringes (Sabre International Products Ltd, Berkshire, U.K.) fitted with 19G hypodermic needles (Sabre International Products).

One of the 2 experimental fish (Fish I) was blood sampled 3 hours after the labels had been administered and a 1ml serum sample extracted, chromatographed on a column of Sepharose 6B and 1.5ml fractions analysed for protein and radio labels as described previously in Chapter 2. Similar procedures for blood analysis were adopted using the same fish at 2 and 7 days post-injection. An immature female was also blood sampled and this serum run on a Sepharose 6B column to provide a non-vitellogenic serum protein trace.

After 7 days Fish I was sacrificed and its ovaries removed and washed in physiological saline. Fifty follicles were carefully dissected out, measured to the nearest 0.1mm and again washed in physiological saline to remove any contaminating blood. The follicles were added to 1ml of column buffer and macerated using a hand homogeniser. The homogenate was centrifuged at 2500g for 15 minutes to remove connective tissue debris and fatty materials. After centrifugation the ovarian homogenate separated into 3 distinct layers: a pellet, probably consisting of cell membranes and connective tissues; a surface layer, consisting primarily of lipid globules; and an aqueous supernatant, containing the follicle yolk proteins which was used for column analyses. The ovarian supernatant was applied to the Sepharose 6B column and the resulting fractions

analysed for protein and radio-labels.

The second fish (Fish II) was sacrificed 3 weeks after the initial label injection and blood and ovarian samples analysed on Sepharose 6B in the same way as with Fish I. A further group of 100 follicles, dissolved in 2ml column buffer, was applied to a column of Sephadex G100 and 2ml fractions analysed for protein and radio labels.

Estimations of the levels of labels incorporated into the ovary of Fish I and II, 1 and 3 weeks post injection, respectively, were determined by counting further groups of 25 follicles (after 2 pre-washes in physiological saline) and extrapolating the mean values to the whole ovary using the formula in Chapter 2. The protocol and timings for tissue analyses are summarized in Figure 3.3.

3.2.1.2 Results

The Sepharose 6B chromatographic profile of serum proteins present in an immature, non-vitellogenic, female rainbow trout is shown in Figure 3.4. There is not enough information in the literature to be able to identify the various proteins, but by analogy with higher vertebrates it seems likely that the protein eluting at the void volume (Vo, fractions 40-50) represent macroglobulins and those eluting in the middle of the profile (mainly fractions 80-105) globulins. These proteins were also present in the serum of mature vitellogenic females (Figure 3.5).

The succeeding chromatographic protein profiles and corresponding label traces in this experiment are plotted on the same graphs except for the analyses on Sepharose 6B of the blood and ovarian homogenates 3 hours and 7 days respectively, after injection of radio labels; these analyses also included a VTG RIA on the column eluents and for the purpose of clarity the protein profiles are plotted with the VTG RIA on a separate graph to the radio label traces. The figures are arranged in an attempt to illustrate the progression of labels (^{32}P and ^{3}H) from their injected free state in the blood to incorporation into blood protein(s) and subsequently their passage into the follicle.

The first series of chromatographic separations, Figures 3.5a-e represent the protein absorbancy profiles of vitellogenic blood serum run on Sepharose 6B at 3 hours, 48 hours and 7 days (168 hours) post-injection in Fish I and 21 days (504 hours) in Fish II. In each of these profiles the appearance of a major new protein peak eluting just before the presumptive globulins and centred around fraction 74 can be clearly seen. This elution

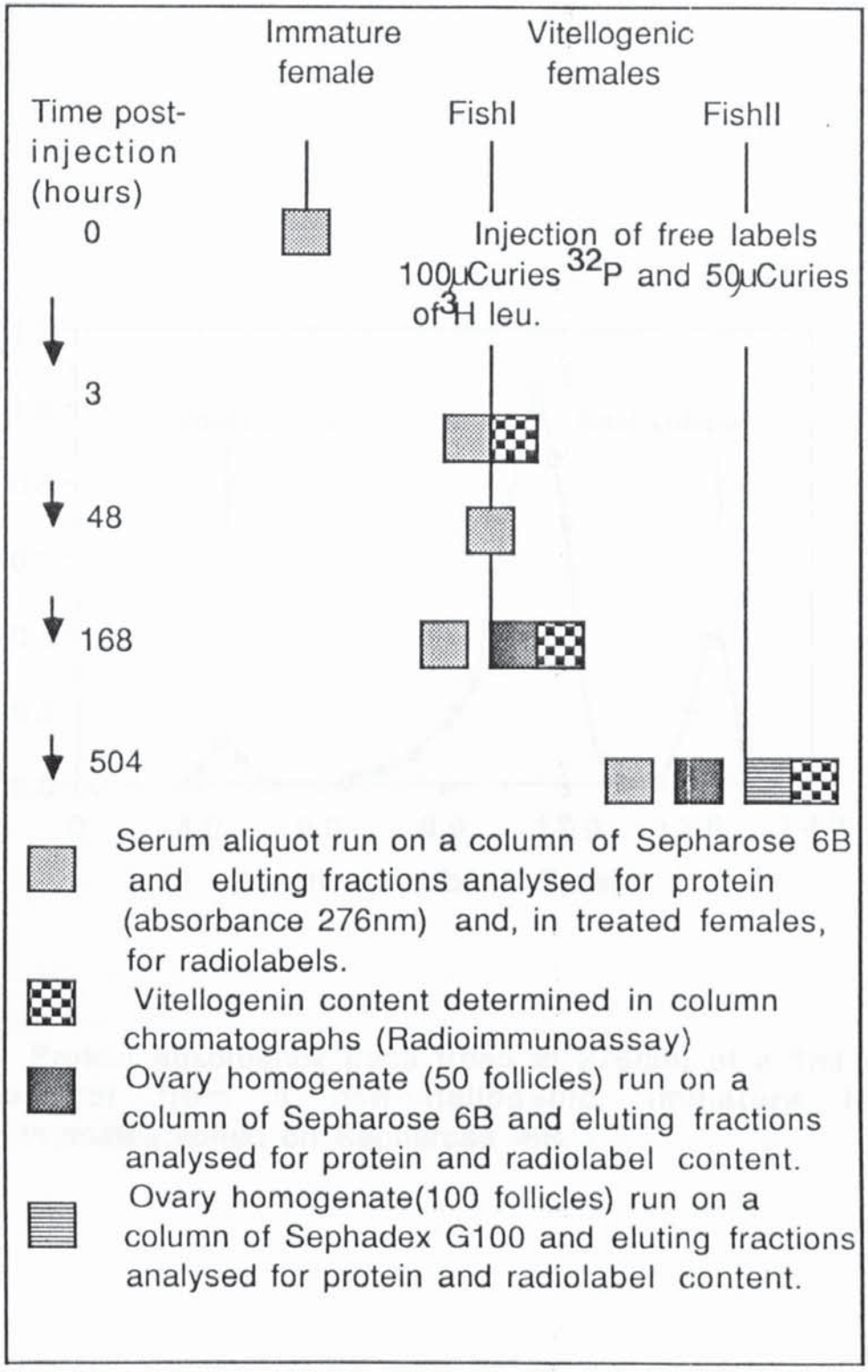


Figure 3.3. Outline for the protocol of experiment 1, investigating the incorporation of ^{32}phoshorus and ^{3}H-leucine into newly synthesised serum vitellogenin followed by their yolk-processing pathways in the vitellogenic ovary.

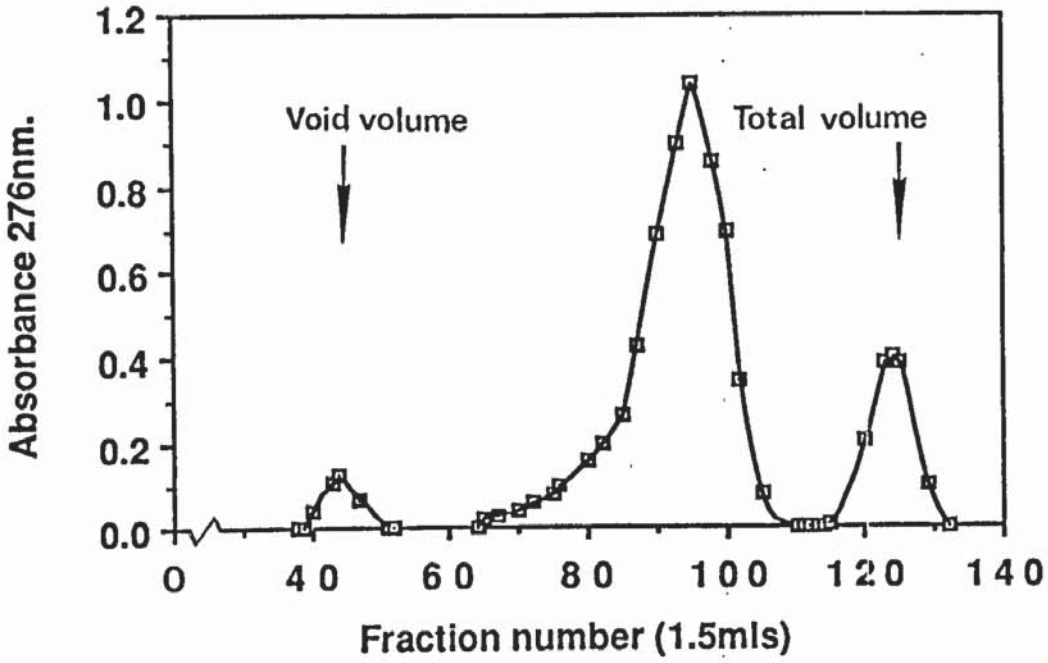


Figure 3.4. Protein absorbancy trace (read at 276nm) of a 1ml serum aliquot from a non-vitellogenic, immature female chromatographed on Sepharose -6B.

position gives this 'new' protein a Kav value of 0.32 and, extrapolating from the molecular weight calibration chart in Figure 2.2, a size of 440 000 Daltons. This protein peak is never observed on column chromatograms of 'control' male serum (data not shown). Radioimmunoassay for VTG of the eluting column fractions confirmed that VTG was present in this protein peak (Figure 3.5a). Further, the concentration of VTG in these fractions relative to their protein concentration suggested that much, if not all, of the protein in this peak was VTG. The similarity in the serum protein profiles of the same fish taken at different time intervals (compare Figures 3.5a, c d and e) showed that the column separations were consistent and indicated that the radiolabel traces across the sampling periods could be compared directly.

The initial traces of radiolabel activity from the serum show that after 3 hours the majority of both ^{32}P and ^{3}H-leu. present in the blood eluted at the V_T (Figure 3.5b). The labels were present in the blood in approximately equal proportions to those originally injected indicating equivalent absorption rates from the peritoneal cavity into the blood. The presence of a second much smaller peak of radioactivity was observed around the VTG peak.

After 2 days there was a significant decrease in the amount of labels eluting at the V_T and a concomittant increase at the VTG peak. In the serum profiles at days 2 and 7 there was a second, less well defined ^{32}P peak at and around fraction 83; however, in subsequent experiments it was not observed and in-part was probably linked to the labile nature of phosphorus on the VTG complex.

After 2 days approximately 75% of the ^{3}H leu. and 85% of the ^{32}P in the blood were associated with the VTG peak, the remaining fractions eluted at the V_T. The newly synthesised VTG had a ^{32}P:^{3}H ratio of 3:2.

After 1 week there was a significant reduction in the amount of labels at both fraction 74 and the V_T and this progressed further in week 3, suggesting a clearance from the blood.

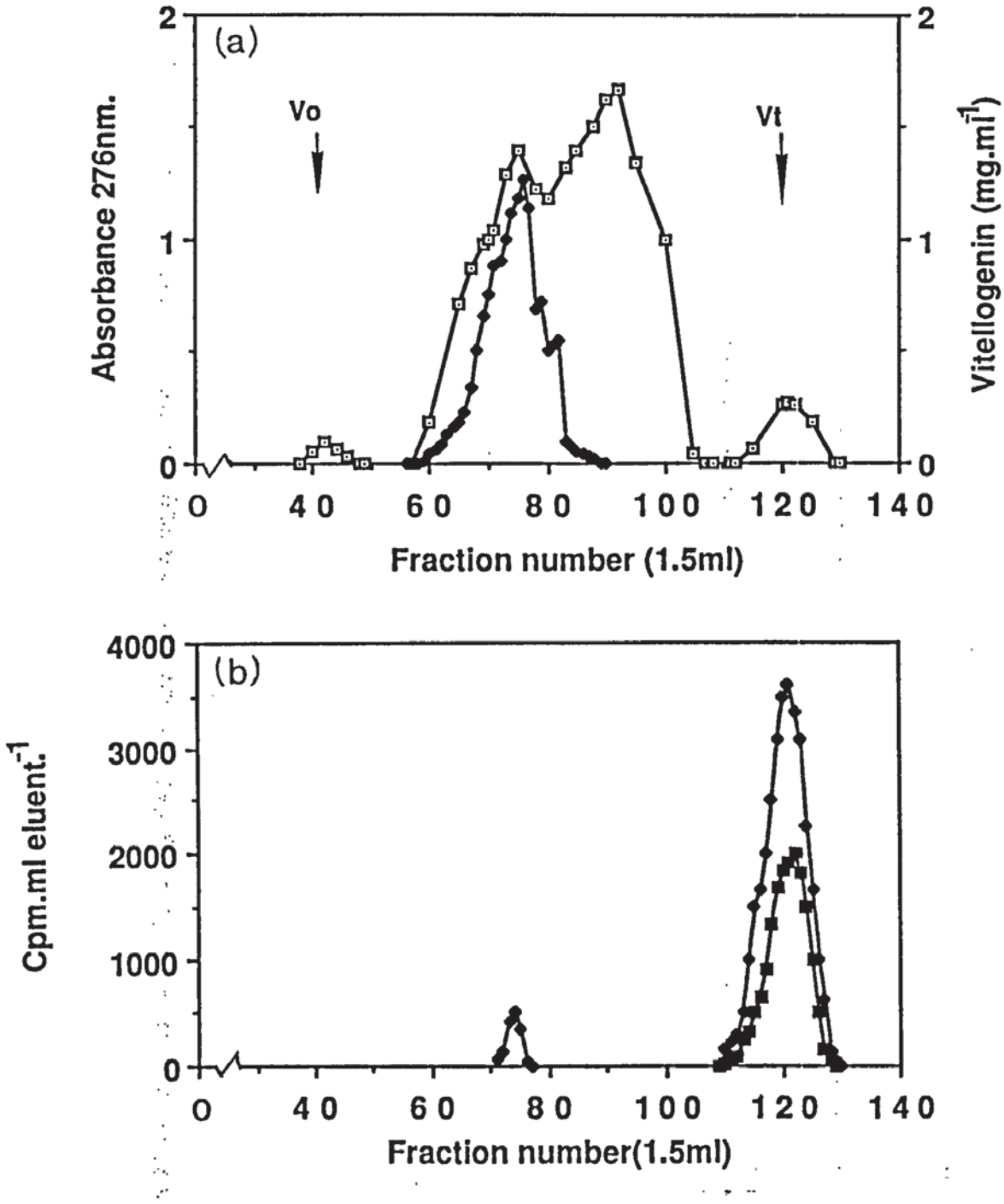


Figure 3.5. Gel-filtration on a column of Sepharose -6B of a 1ml serum aliquot from a vitellogenic female (Fish I) 3hours after intraperitoneal injection of 50µCi of ^{3}H-leucine and 100µCi of ^{32}phosphorus. Eluent fractions were monitored for absorbance at 276nm (open symbols: □) and cross-reactivity in a vitellogenin radioimmunoassay (closed symbols: ◆, profile a) and for ^{3}H(■) and ^{32}P(◆) (b). The column void(Vo) and total volumes(Vt) are marked.

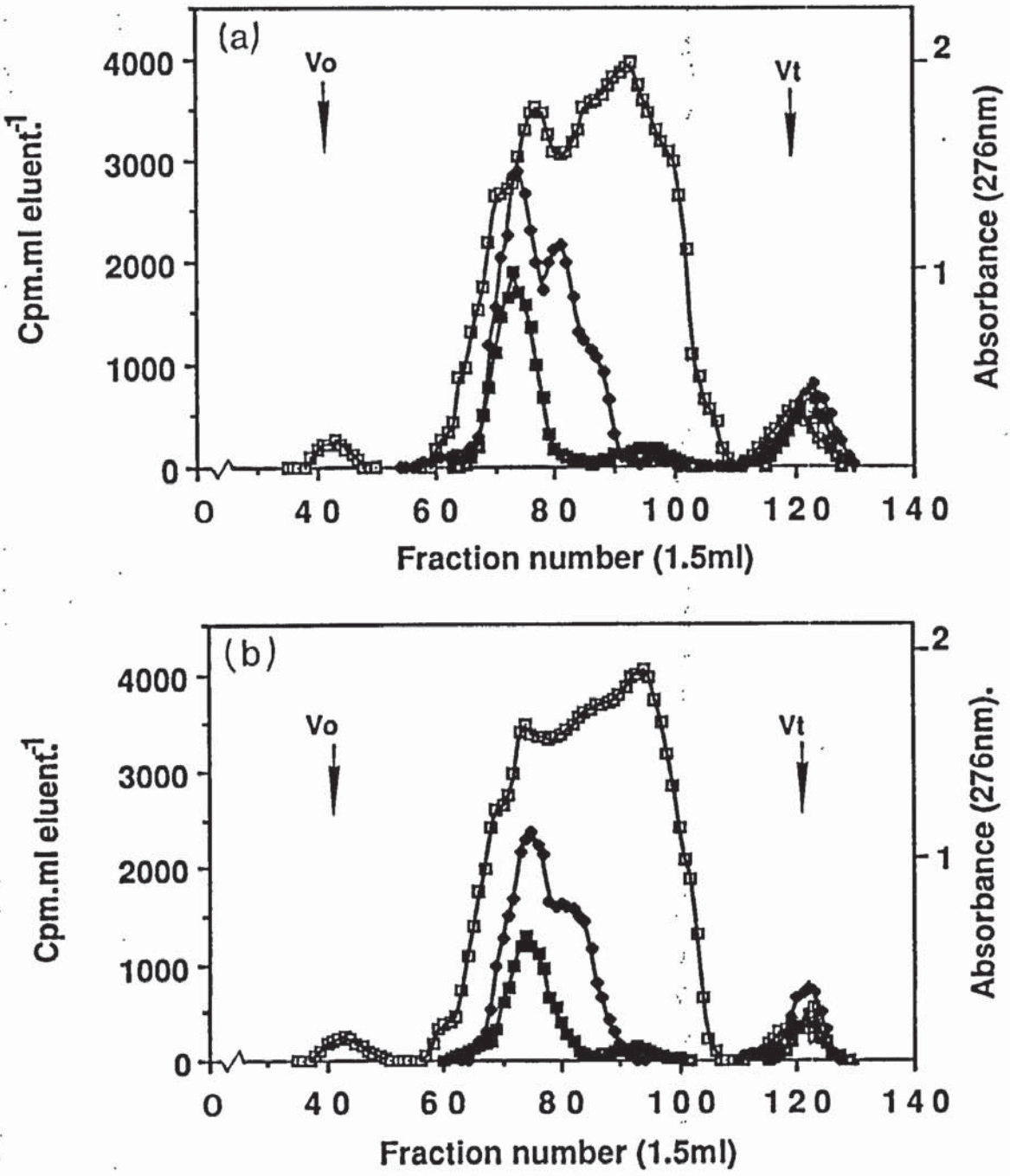


Figure 3.5 cont. Gel-filtration on a column of Sepharose -6B of1ml serum samples from a vitellogenic female (Fish I) 48 hours (profile a) and 7 days (profile b) after intraperitoneal injection of 50µCi of ^{3}H-leucine and 100µCi of ^{32} phosphorus. Eluents were monitoured for absorbance at 276 nm (open symbols:□) and for ^{3}H (■) and ^{32}P (◆). The void (Vo) and total (Vt) volumes are marked.

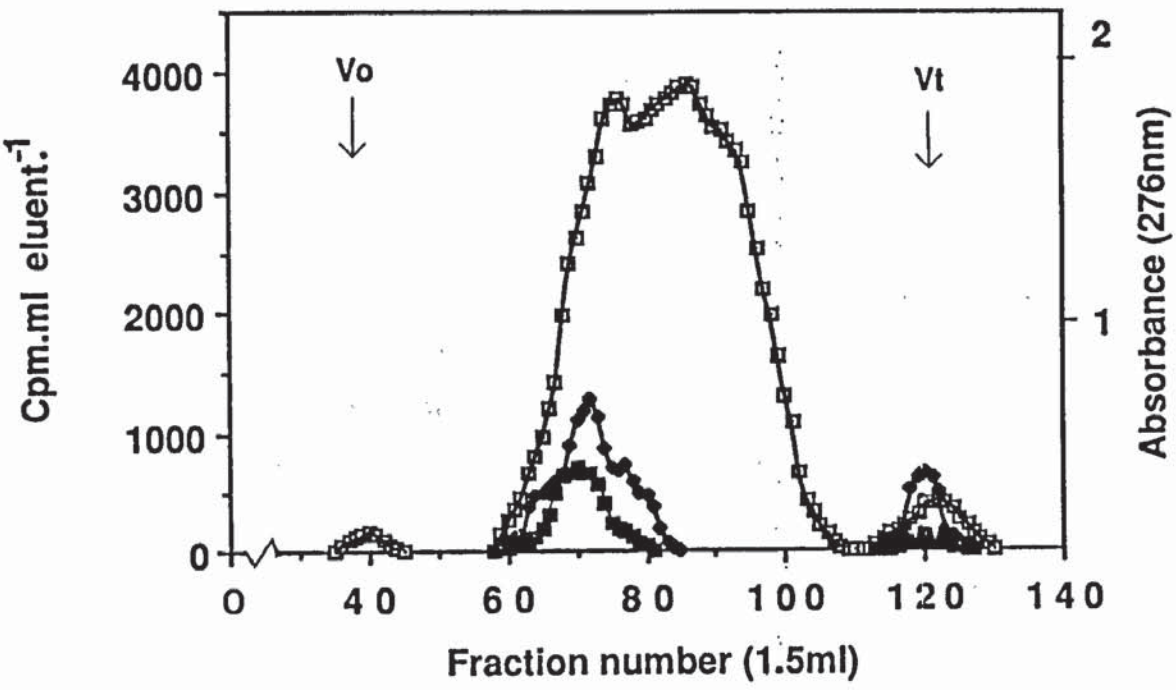


Figure 3.5 cont. Gel- filtration on a column of Sepharose -6B of a 1ml serum sample from a vitellogenic female (Fish II) 21 days after intraperitoneal injection of 50μCi of ^{3}H-leucine and 100μCi of ^{32}phosphorus. Eluents were monitored for absorbance at 276nm (open symbols:□) and for ^{3}H (■) and ^{32}P (◆). The column void (Vo) and total (Vt) volumes are marked.

The chromatograms of ovary homogenates run on Sepharose 6B produced a completely different protein profile with 4 distinct protein absorbancy peaks (Figure 3.6). The peak of protein at Vo represents components of the follicular cell layers because it is not present when yolk alone, rather than a homogenate of all the ovarian tissues, is run on the column (data not shown). The other 3 protein peaks eluting at fractions 82, 102 and V_T represent the major yolk proteins. The protein eluting at fraction 82 had a molecular weight of approximately 390 000 Daltons and was the only yolk protein to demonstrate a strong cross reactivity in the VTG radioimmunoassay (Figure 3.6a). This protein peak 1 week post-injection included approximately 95% of the ovary-incorporated ^{3}H-leu. and, somewhat cautiously, was termed the 'lipovitellin complex'. The phosphorus, however, was divided almost equally between the three yolk protein peaks. The ratio of ^{32}P:^{3}H leu. in the lipovitellin complex was almost 1:2, thus the lipovitellin had become enriched with ^{3}H leu. relative to ^{32}P compared to the VTG originally synthesised.

An ovarian homogenate was run on Sephadex G100 to determine more accurately the molecular weights of the smaller yolk proteins. The protein profile (Figure 3.7a) produced three peaks, eluting at the Vo, corresponding to the lipovitellin peak on Sepharose 6B, the V_T and fractions 40-50. The protein eluting at fractions 40-50 appeared to correspond to the second yolk protein peak eluting on Sepharose 6B, at and around fraction 102, for when either fractions were placed on the other gel-filtration column they fractionated at the appropriate position (data not shown). This small phosphoprotein had an approximate molecular weight of 40 000 Daltons and was tentatively designated 'phosvitin'. The labels eluting at V_T on Sepharose 6B also eluted at V_T on Sephadex G100, corresponding to a molecular weight of less than 4 000 Daltons. When this fraction (the V_T) was treated with TCA (10% final concentration) the labels remained in solution whereas most proteins would be expected to precipitate. This further suggests that the labels were 'free', rather than protein bound.

After 3 weeks the ovary profile of radio labels in Fish II run on Sepharose 6B, was much the same as that observed in Fish I after 1 week (compare Figures 3.6 b and 3.6c). Approximately 65% of the protein bound phosphorus was associated with the low molecular weight phosphoprotein and in excess of 90% of the ^{3}H leu. eluted with the 'lipovitellin complex'.

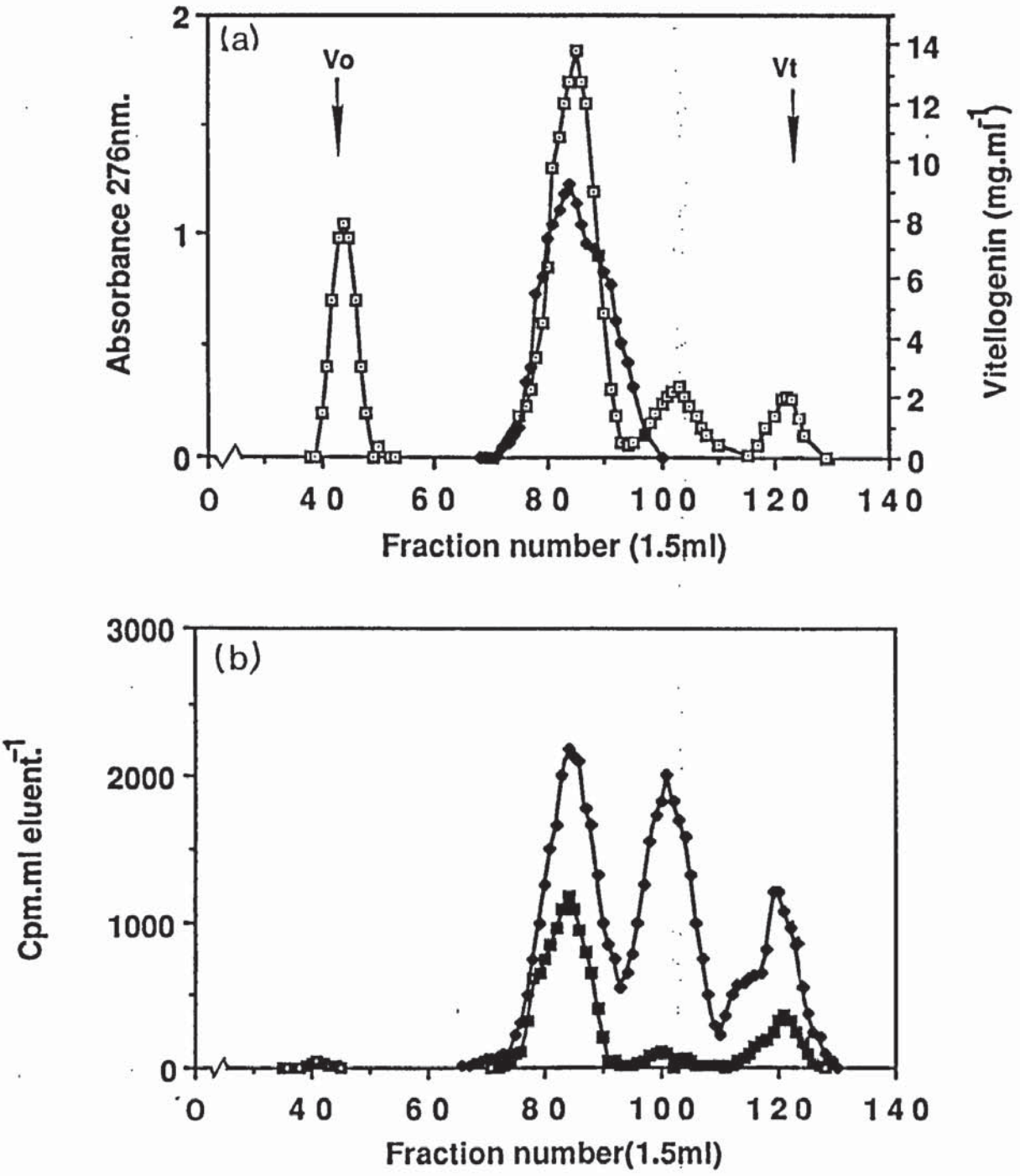


Figure 3.6. Gel-filtration on a column of Sepharose -6B of an ovarian homogenate (derived from 50 follicles) from a vitellogenic female (Fish I) 7 days after intra- peritoneal injection of 50μCi of ^{3}H-leucine and 100μCi of ^{32}phosphorus. Eluent fractions were monitored for: (a) absorbance at 276nm (open symbols:□)and cross reactivity in a vitellogenin radio- immunoassay (closed symbols:◆) and; (b) for ^{3}H.(■) and ^{32}P.(◆, profile b). The column void (Vo) and total (Vt) volumes are marked.

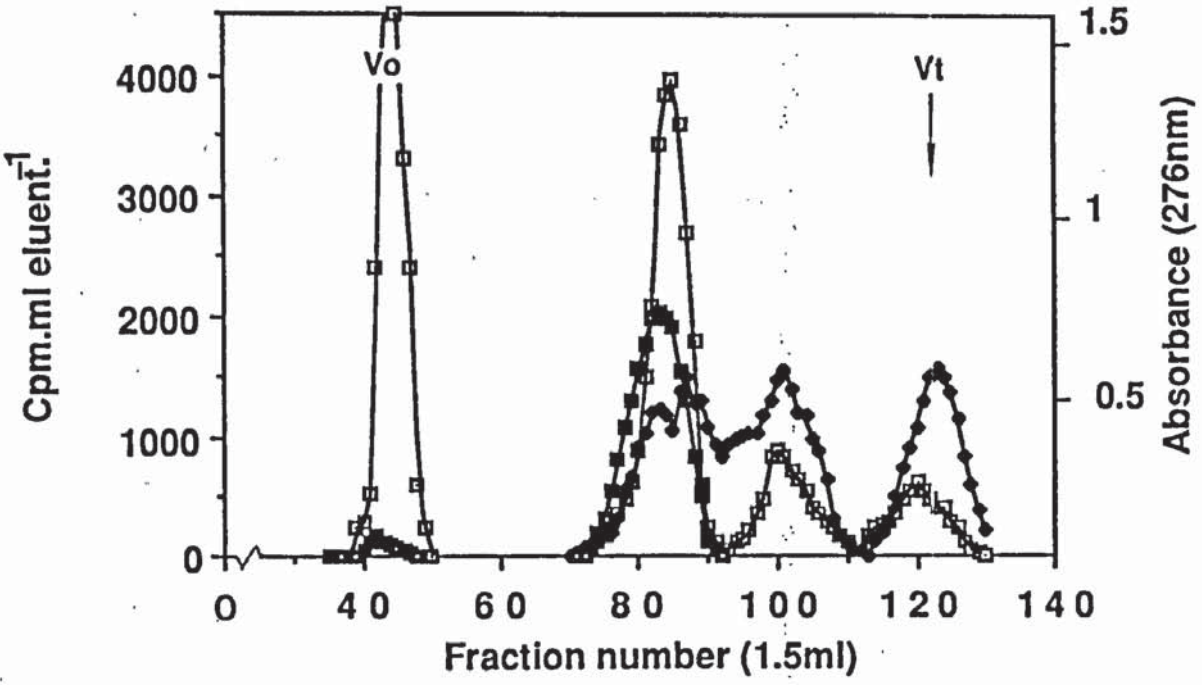


Figure 3.6 cont. (c) Gel-filtration on a column of Sepharose -6B of an ovarian homogenate (derived from 50 follicles) from a vitellogenic female (Fish II) 21 days after intraperitoneal injection of 50μCi of ^{3}H-leucine and ^{32}phosphorus. Eluent fractions were monitored for absorbance at 276nm (open symbols:□) and for ^{3}H.(■) and ^{32}P.(◆).The column void (Vo) and total (Vt) volumes are marked.

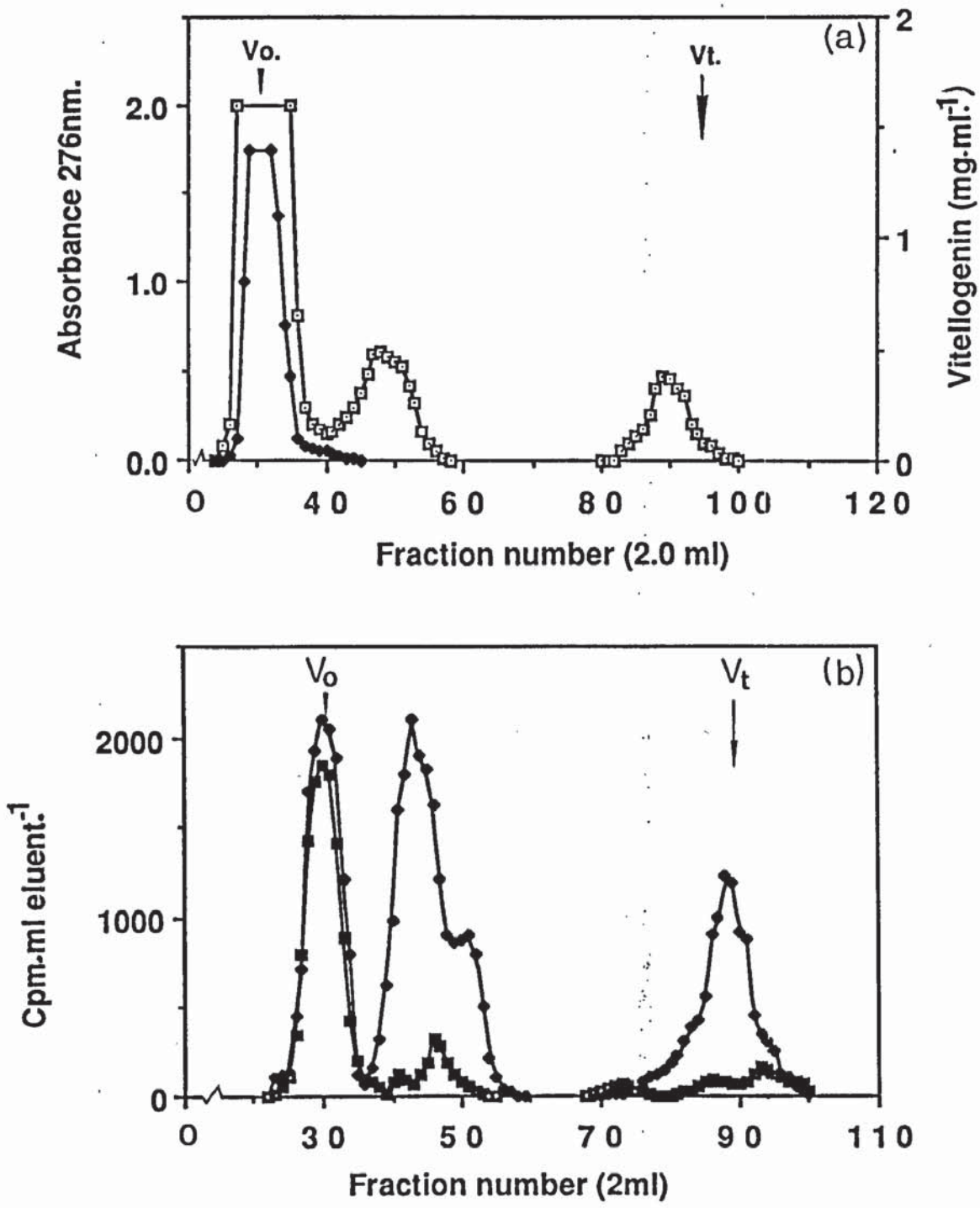


Figure 3.7. Gel-filtration on a column of Sephadex -G100 of an ovarian homogenate (derived from 100 follicles) from a vitellogenic female (Fish II) 21 days after intra-peritoneal injection of 50μCi of ^{3}H-leucine and 100μCi of ^{32}phosphorus. Eluent fractions were monitored for absorbance at 276nm (open symbols: □) and cross reactivity in a vitellogenin radio-immunoassay (closed symbols:◆) and; (b) for ^{3}H.(■) and ^{32}P.(◆). The column void (Vo) and total (Vt) volumes are marked.

One week post-injection approximately 12% and 21% of the originally injected ^{3}H leu. and ^{32}P, respectively, had become incorporated into the maturing ovary of Fish I and these values increased to 20% and 23% for ^{3}H leu. and ^{32}P respectively, in Fish II after 3 weeks.

3.2.2 Part B - Amino acid analyses of vitellogenin and its yolk protein products

Samples of the column eluents fractionating at the protein peaks corresponding to serum VTG, 'lipovitellin' and fraction 102 on Sepharose 6B and fraction 49 on Sephadex G100 ('phosvitin') in part A of this experiment were subjected to amino acid analyses for further characterisation.

3.2.2.1 Materials and methods

The analyses were performed on a custom built automatic amino-acid analyser based on molecular high pressure liquid chromatography (HPLC) components. The system was controlled by a Nascom III computer providing ease of operation. Briefly, the proteins were initially hydrolysed in constant boiling hydrochloric acid (HCl) at 100^{o}C for 24 hours, the solutions degassed to remove oxygen (which can destroy some of the amino acids) and then treated with 10% sulphosalicyclic acid to precipitate protein that would otherwise irreversibly bind to the ion exchange resin. The amino acid sample was then injected onto a column of strong action exchange resin and the amino acids eluted by a stepwise gradient of increasing pH and ionic strength. The protein hydrolysates were then eluted by a series of lithium citrate buffers. The detection methodology adopted was colormetric detection by ninhydrin (detailed methodology of 'Macromolecular analysis' issued by the Department of Chemistry, University of Birmingham, Birmingham, England). Tryptophan, which is often totally destroyed by HCl hydrolysis, was not measured in these analyses.

3.2.2.2 Results

The amino acid analyses of the blood serum vitellogenin and 'yolk' proteins are illustrated in Table 3.1. The vitellogenin primarily shows a predominance of the non-polar, aliphatic amino acids alanine and leucine and the carboxylic amino acids aspartic acid and glutamic acid. Hydroxylic amino acids are well represented in the form of serine and threonine as is the amino group in lysine and arginine. The lowest amino acid group found in vitellogenin is the sulphur-containing amino acids notably, cysteine and methionine.

Species studied	VITELLOGENIN				'LIPOVITELLIN'			
	Salmo gairdneri			*Xenopus laevis*	*Salmo gairdneri*			*Xenopus laevis*
Amino acid	a	b	c	d	a	b	c	d
aspartic acid	9.2	8.8	8.4	8.4	7.3	7.6	7.7	8.8
threonine	6.2	5.1	5.0	5.5	5.9	5.4	5.3	5.6
serine	7.3	7.4	7.5	11.5	5.3	5.0	4.8	8.0
proline	4.6	4.8	5.2	4.8	5.3	5.4	5.4	5.0
glutamic acid	11.1	9.2	11.5	13.2	9.8	9.3	11.3	13.8
glycine	5.1	4.1	4.2	5.1	4.1	4.0	4.1	5.3
alanine	11.5	11.3	11.7	8.5	12.7	12.7	14.2	10.2
½cysteine	1.0	1.6	1.2	1.3	0.8	1.2	-	0.9
valine	6.6	7.9	7.1	4.6	7.0	8.3	7.7	5.1
methionine	2.0	2.6	2.6	2.3	2.6	2.8	2.6	2.0
isoleucine	5.0	5.7	5.5	3.6	5.7	6.0	5.9	4.1
leucine	9.5	9.1	9.5	8.1	9.5	9.8	10.4	8.5
tyrosine	2.9	2.9	3.0	3.0	3.1	2.8	2.9	3.0
phenylalanine	4.0	4.0	4.0	3.6	4.9	4.4	4.6	4.2
lysine	7.1	7.6	7.1	7.9	7.0	7.0	6.3	7.3
histidine	2.9	2.3	2.1	2.9	2.8	2.3	2.2	2.9
arginine	4.5	4.3	4.5	5.3	5.4	4.3	4.4	5.6

Table.3.1.

Species studied	PHOSVITIN							'b- COMPONENT' E2 OV-6B PEAK 3		
	S .gairdneri		*S. trutta*		*O. keta.*	*X. laevis*		*S. gairdneri*		
	a	b	f	g	h	i	d	b	c	a
Amino acid										
aspartic acid	14.9	9.6	10.0	8.0	10.0	9.0	5.0	15.3	15.7	14.5
threonine	4.2	1.8	2.0	2.0	2.0	1.0	0.7	4.5	4.0	4.3
serine	18.8	42.0	54.0	57.0	50.0	57.0	56.0	7.9	10.9	18.5
proline	2.9	4.6	3.0	3.0	5.0	3.0	3.0	3.1	3.3	2.6
glutamic acid	9.0	3.7	3.0	3.0	4.0	3.0	3.0	3.1	3.2	8.9
glycine	5.7	4.2	4.0	3.0	3.0	3.0	2.8	6.0	5.7	5.9
alanine	3.8	4.0	3.0	2.0	3.0	3.0	2.2	3.5	3.5	4.1
½cysteine	2.2	0.2	-	-	-	-	-	0.9	2.7	2.0
valine	5.5	0.2	-	-	-	-	0.6	9.4	7.8	5.8
methionine	2.5	0	-	-	-	-	-	2.7	3.0	2.2
isoleucine	4.2	2.3	2.0	2.0	3.0	3.0	0.3	5.8	5.0	4.4
leucine	6.2	0.2	0.4	-	-	-	1.4	8.2	7.0	6.5
tyrosine	2.9	1.0	2.0	1.0	1.0	1.0	0.9	6.0	3.9	2.8
phenylalanine	1.6	-	1.0	-	-	-	0.7	2.1	1.7	1.6
lysine	9.0	4.9	3.0	4.0	4.0	4.0	7.6	12.2	10.6	9.4
histidine	2.5	-	-	-	-	-	2.8	2.6	2.3	2.2
arginine	5.2	15.6	10.0	14.0	12.0	12.0	6.0	2.3	3.2	4.8

Table 3.1. cont. Amino acid compositions of rainbow trout blood vitellogenin and egg-yolk proteins compared to published analyses on several salmonid species and Xenopus laevis.
(a) - this study. (b) - Campbell and Idler (1980) (c) - Hara and Hirai (1978)
(d) - Redshaw and Follett (1971) (f) - Ito et al.,(1966) (g) - Mano and Yoshida (1969)
(h) - Schmidt et al.,(1965) (i) - Clark and Jalabert (1974).

The 390 000 Dalton 'yolk' protein (the lipovitellin complex) largely contains an amino acid profile similar to that of the blood vitellogenin; however, there are specific differences. In the first instance, there is a marked reduction in the level of serine, indeed this amino acid represents the largest residue difference between the two proteins. The carboxylic amino acids, aspartic acid and glutamic acid are significantly reduced in lipovitellin. The aromatic amino acids, tyrosine and phenylalanine show slightly elevated levels in the 'lipovitellin' protein but the remaining residues illustrate little change.

The second follicle 'yolk' protein, eluting at fractions 98-110 on Sepharose 6B and designated 'phosvitin', has an amino acid profile completely at variance to the above two proteins. The most notable feature is the high level of serine, occupying approximately 19 percent of the total residues. This figure is nearly four times the level in both the vitellogenin and lipovitellin. Aspartic acid and lysine similarly have a significantly higher content in 'phosvitin' than the other protein analysed. In contrast alanine, an amino acid of high proportions in both VTG (11.5%) and lipovitellin (12.5%), is exceptionally reduced, to a level of 3.8%. Although little difference in the aromatic amino acid tyrosine was observed between the proteins, the phenyalanine content in 'phosvitin' was less than half that of both lipovitellin and serum VTG. The amino acid analysis of the small phosphoprotein eluting on Sephadex G100 produced a composition almost identical to the 'phosvitin' peak eluted from Sepharose 6B. The analyses revealed that the content of none of the individual residues varied by more than 4% between the two protein fractions and confirms that fraction 102 on Sepharose 6B and fraction 49 on Sephadex G100, although apparently conferring different molecular weights, represent the same yolk protein, 'phosvitin'.

3.3 Experiment 2 - The production and purification of vitellogenin

Further experiments described later in this thesis on vitellogenesis, both *in vivo* and *in vitro*, required large titres of labelled and unlabelled VTG. As teleost VTG is difficult to obtain in a pure and unaltered form using established methods for VTG isolation, this study therefore aimed to produce a reliable protocol for the production and extraction, and purification (using gel-filtration) of high titres of intact VTG. The study also investigated methods for supplying radiolabel(s) in the fish for their subsequent incorporation into VTG.

3.3.1 Material and methods

3.3.1.1 Vitellogenin induction

Vitellogenin synthesis was induced in immature females using 17 ß-oestradiol (see Elliott *et al.*, 1979). Fish were injected intraperitoneally with 10 µg of oestrogen per $g.^{-1}$ body weight suspended in an emulsion of ethanol and oil of arachis (BDH Chemicals Ltd) in a ratio of 1:3 respectively. The injection volume was 1ml. Immature female fish were used for VTG production because unlike males (which can also be induced to synthesise VTG using oestrogens, see the introduction to this chapter), they did not require an initial 'priming' dose and therefore, requiring only a single hormone injection, responded more quickly to oestrogen treatment. When the serum calcium exceeded 20 mg.100 ml^{-1} (a level considered indicative of a high rate of VTG production, see Figure 2.1) either the serum was extracted for the purification of 'cold' VTG or radiolabels were administered into the fish for the production of labelled VTG.

3.3.1.2 *In vivo* radiolabelling of vitellogenin

Isotopes were administered into the peritoneal cavity of pre-oestrogenised immature females either by injection in a suspension of oil of arachis, or contained within a surgically implanted mini-osmotic pump (Alza Palo Alo, California, USA).

3.3.1.2.1 The radio-labelling of vitellogenin using an intraperitoneal injection of ^{32}P and ^{3}H leu.

A pre-oestrogenised female (Fish I) with a serum calcium of 24 mg. 100 ml^{-1} (12 days post injection) was injected with 1m Curie and 0.5m Curie (37 M $Bqml^{-1}$) of ^{32}P and ^{3}H leu., respectively (Amersham International plc) suspended in 0.5ml of oil of arachis. The final injection volume (2ml) was administered in two equal aliquots into the peritoneal cavity from either side of the fish in an attempt to reduce the amount of label leakage from the injection site. Six days after isotope injections the trout was drained of blood from the Cuverian sinus and the serum extracted (see Chapter 2).

3.3.1.2.2 The radiolabelling of vitellogenin using implanted mini-osmotic pumps

An oestrogenised female (Fish II) with a serum calcium of 21 mg. 100 ml^{-1} was implanted intraperitoneally with a mini-osmotic pump (model 2001: volume capacity 200 µl) containing 0.5m Curies of ^{3}H leu. (185 $MBq.ml^{-1}$). The isotope volume (100 µl) was insufficient to fill the pump and was therefore initially diluted with an equal volume of 0.9% physiological saline. The fish was anaesthetised and the pump inserted into the peritoneal cavity through a small incision made on the flank of the fish. The incision was subsequently closed with 2 stitches and the wound 'dusted' with a 'dental cement' containing wide spectrum antibiotics to reduce the chance of microbial infection. The aim of using these pumps was to provide a continuous and 'regulated' supply of free label for incorporation with newly synthesising VTG and also to reduce the incidence of label leakage seen using the direct injection of labels.

Alzet mini-osmotic pumps

Specifications for model 2001 (see Figure 3.8)

Reservoir volume:	200 µl
Dimension, overall: Length 3.0cm; Diameter:	0.7cm; Weight (empty) 1.1g
Total volume	1 ml

Pump body materials:
Outer membrane - cellulose, ester blend
Drug reservoir- thermoplastic hydrocarbon elastomer

Alzet osmotic pumps are miniature self-powered pumps that continuously deliver test agents, in our case radio-label, at controlled rates. The pump consists of a collapsable reservoir of flexible, impermeable material, surrounded by a sealed layer containing an osmotic agent - all of which is contained within a semipermeable membrane (Figure 3.8). When the filled pump is put into an aqueous environment, the osmotic agent imbibes water at a rate that is controlled by the semipermeable membrane. The imbibed water generates hydrostatic pressure on the flexible lining of the reservoir, gradually compressing it, producing a constant flow of its contents through the delivery portal. Only the material placed into the reservoir leaves the pump; the osmotic agent cannot pass

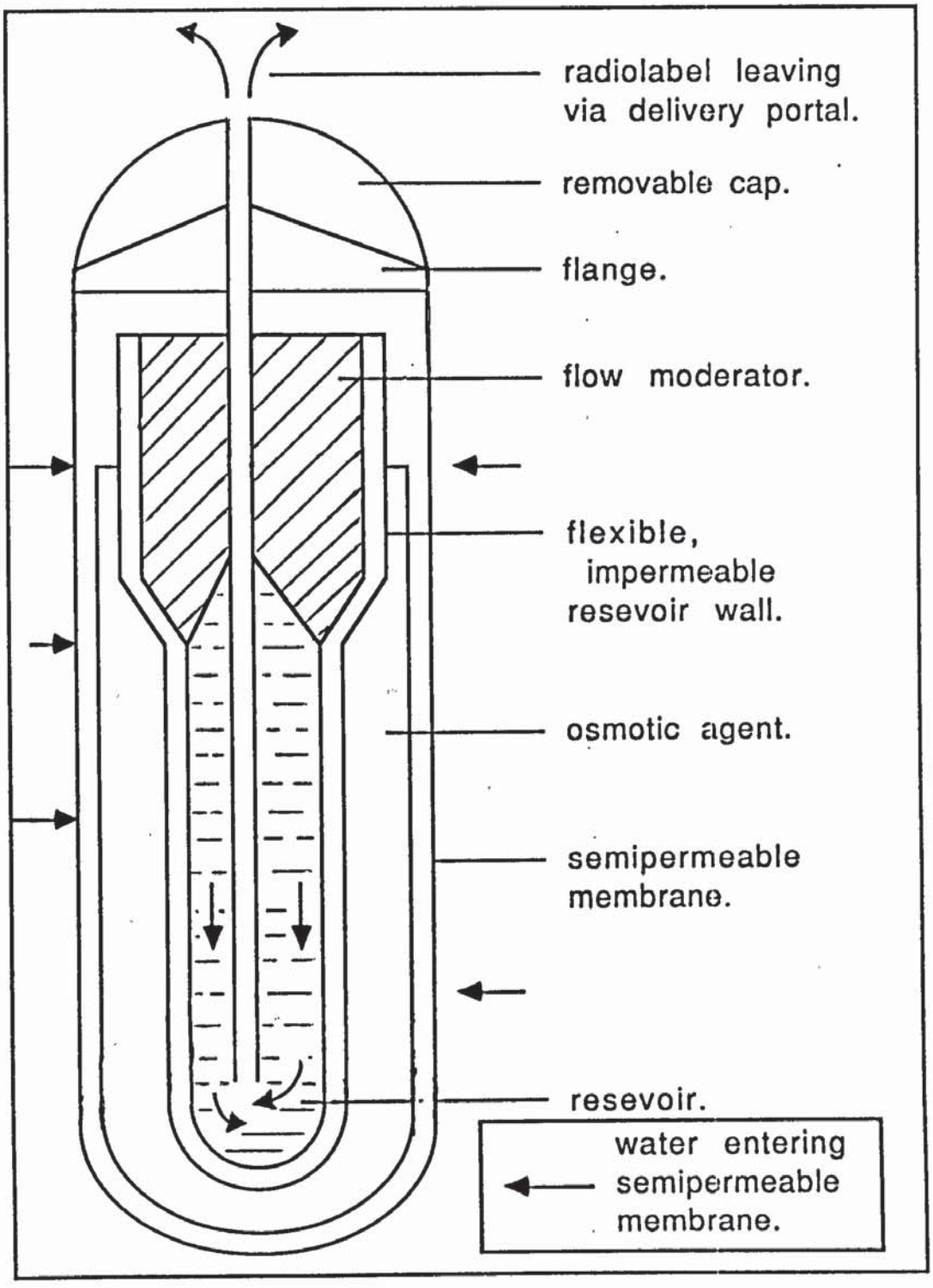


Figure 3.8. The structure and functioning of an Alzet mini-osmotic pump, used for the administration of radio-labels in the production of labelled vitellogenin.

into the reservoir or out through the semipermeable membrane. The pumps delivery rate of vehicle is fixed and predetermined at manufacture. Pump rates are provided by the manufacturers for usage in mammals where the operative temperature is 37°C. However, in these experiments with trout the operative temperature was 12°C ± 1°C and the approximate pump rate had to be calculated by using an equation provided by the company for predicting the pumping rate in heterothermic (poikilothermic) animals: $Q = Qo\ [0.141 \exp (0.051\ T) - 0.007\ \pi + 0.2]$, where Q is the expected pumping rate in an environment having an osmotic activity of π (atmospheres) and a temperature of T (°C), and Qo is the nominal label value of 10 $\mu l.hr.^{-1}$. The equation was believed to be predictive within ± 10%. Calculations, at 12°C produced an approximate pumping rate of 1.3 $\mu l.day^{-1}$ and therefore a time interval to empty the pump of 28days. The pump was left in the experimental fish for 30 days to ensure all the radio-label had been released to the surrounding cavity before the fish was blood sampled and the serum extracted. Examination of the pump after sampling indicated there was no fluid and therefore label, remaining.

3.3.1.3 Vitellogenin extraction and purification

Five ml aliquots of 'hot' (radiolabelled) or 'cold' (un-labelled) vitellogenic serum were applied to a column of Sepharose 6B run at $6ml.hr^{-1}$ and 2.2ml eluent fractions collected. The 10 fractions eluting at and around the peak concentration of VTG (fractions 53-63; determined in the production of labelled VTG by the activity profiles) were pooled and centrifuged in centricon C30 microconcentrators (Amicon, Stonehouse, Gloucester, UK) to remove the majority of liquid and thus concentrate the VTG.

Centricon C30 microconcentrators

Centricon centrifugal microconcentrators provide a fast efficient concentration of macromolecule-containing solutions by ultrafiltration through a low-absorption, hydrophilic Amicon 'YM' membrane. The membrane use in this experiment, C30, has a molecular weight cut off of 30 000 Daltons. The concentrators (illustrated in Figure 3.9) provided enrichment of the sample, with minimal loss of solute by absorption. This method of VTG concentration also has the convenient advantage of microsolute removal.

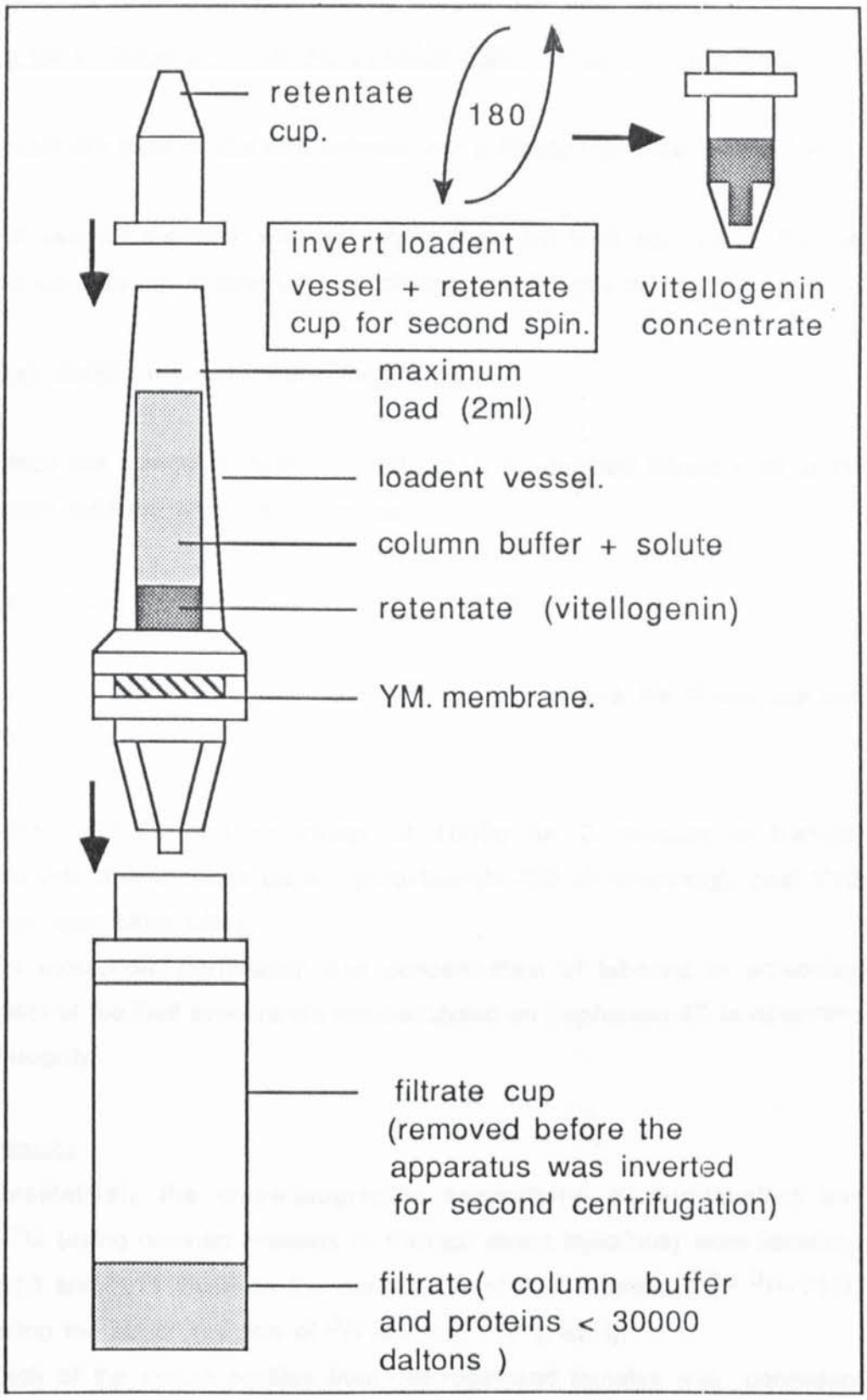


Figure 3.9. Structure and working protocol of Centricon C30 microconcentrators, used for the extraction and concentration of purified vitellogenin. .

Procedure for vitellogenin concentration using C30 microconcentrator tubes

1 Insert the base of the concentrator into a filtrate cup (see Figure 3.9).

2 Fill sample reservoir with an aliquot from the VTG pool up to the 2ml marking taking care not to touch the membrane with the pipette tip.

3 Cap sample reservoir with retentate cup.

4 Place the device (capped concentrator with attached filtrate cup) in the rotor; counter balance with similar device.

5 Spin 2500g in Chillspin centrifuge at 4°C for 1 hour.

6 Take the assembly out of the centrifuge and remove the filtrate cup and discard the filtrate.

7 Invert sample and centrifuge at 1000g for 2 minutes to transfer concentrate into the retentate caps (approximately 400 µl remaining); pool VTG concentrates from other tubes.

On extraction, purification and concentration of labelled or unlabelled VTG, aliquots of the final concentrate were analysed on Sepharose 6B to determine protein integrity.

3.3.2 Results

Qualitatively the chromatographic separations of non-labelled and labelled VTG (using osmotic implants or through direct injections) were identical. Figures 3.10 and 3.11 illustrate the purification of dual labelled, ^{32}P ^{3}H-VTG, labelled using the direct injection of ^{3}H leu. and ^{32}P (Fish I).

Each of the serum profiles from oestrogenised females was consistent with that reported in experiment 1 for vitellogenic females; they showed a large high molecular weight protein peak; VTG. In many of the oestrogenised females the protein peaks eluting at V_T and Vo were significantly reduced compared to immature animals prior to treatment.

Figure 3.10 shows that the majority of administered radio labels eluted with VTG, the remaining ^{32}P and ^{3}H leu. eluting at V_T. The labels at V_T were not precipitated by the addition of an equal volume of 20% trichloracetic acid (TCA) suggesting they were free and not protein bound, probably representing label not yet incorporated into VTG.

The chromatographed VTG concentrate (Figure 3.11) showed that at the end of the extraction and purification procedure VTG was intact, eluting over approximately 15 fractions with an approximate molecular weight of 440 000 Daltons (i.e. identical to that found in the serum of vitellogenic females in experiment 1).

The final labelled VTG concentrates produced by direct injection and pump insertion of ^{3}H leu., had specific activities of 2.87 and 3.95 x 10^4 cpm.mg^{-1} VTG, respectively.

3.4 Experiment 3 - The rates of uptake and processing of ^{32}P.^{3}H vitellogenin by maturing vitellogenic follicles in vivo

This experiment analysed the dynamics of both VTG uptake and VTG processing into the yolk proteins. Extensive studies on *Xenopus* ascribe a half-life of serum VTG in maturing females of approximately 2 days at 20^{o}C, whereas it is almost 30 days in males (Wallace *et al.*, 1970). Furthermore, once endocytosed *in vitro* observations suggest that VTG is processed to its yolk protein products within a few hours (Wallace and Jared, 1969). Most of the studies in *Xenopus* which have investigated the dynamics of VTG uptake, however, have been carried out on individuals that have been stimulated with gonadotropin, specifically human chorionic gonadotropin (hCG). Demonstrations on this uptake process in oviparous vertebrates unstimulated by hormone treatments are comparatively few. Studies on the dynamics of VTG uptake in fish are even fewer. Craik (1978b), working on the lesser spotted dogfish (*Scyliorhinus canicula*) demonstrated a blood half-life for VTG of 9 days at 7^{o}C. However, this species spawns over 6-9 months of the year (Sumpter and Dodd, 1979) and does not show any seasonal variation in blood VTG levels, remaining at approximately 0.4 mg. ml^{-1} throughout the year (Craik, 1978b). Rainbow trout, unlike elasmobranchs, ovulate at a specific time of the year and show quite pronounced seasonal cycles in blood VTG and ovarian growth (Scott and Sumpter, 1983), and therefore the dynamics of vitellogenesis are likely to differ considerably. Using dual radiolabelled (^{32}P.^{3}H) VTG this experiment investigated, *in vivo*, the rate of

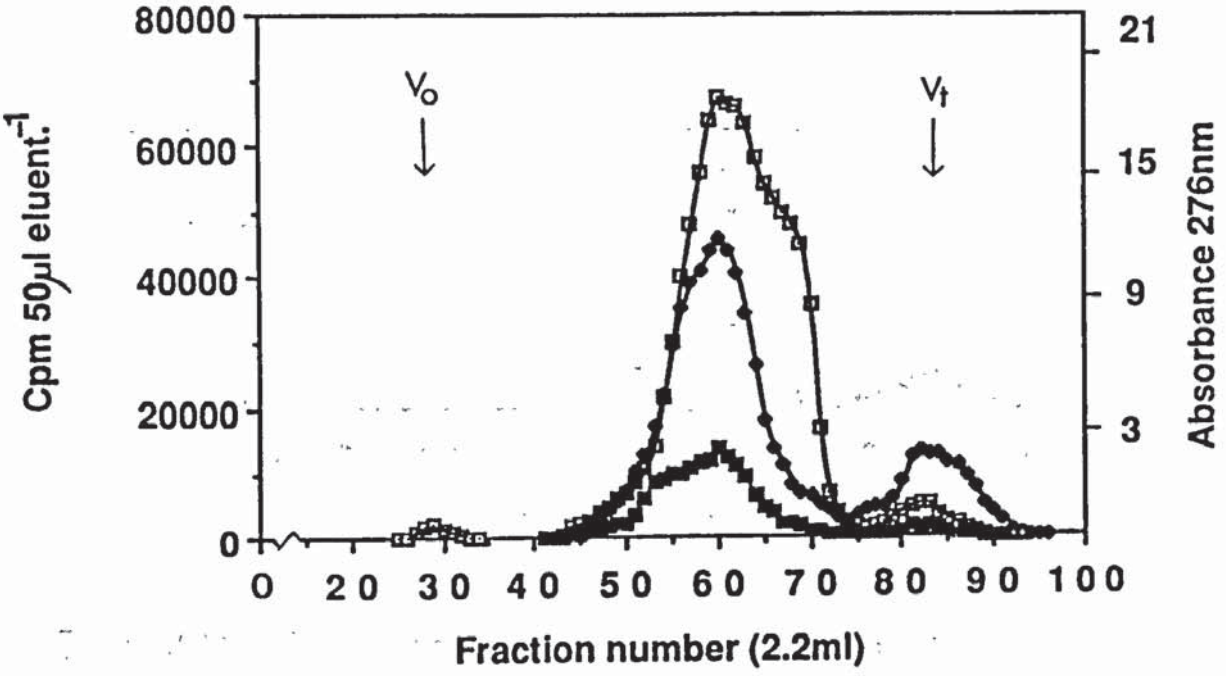


Figure 3.10. Gel-filtration on a column of Sepharose -6B of a 1ml serum sample from an oestrogenized immature female 10 days after intraperitoneal injection of 1mCi of ^{32}phosphorus and 0.5mCi of ^{3}H-leucine. Eluent fractions were monitored for absorbance at 276nm (open symbols:□) and for ^{3}H (■) and ^{32}P (◆). The column void (Vo) and total (Vt) volumes are marked.

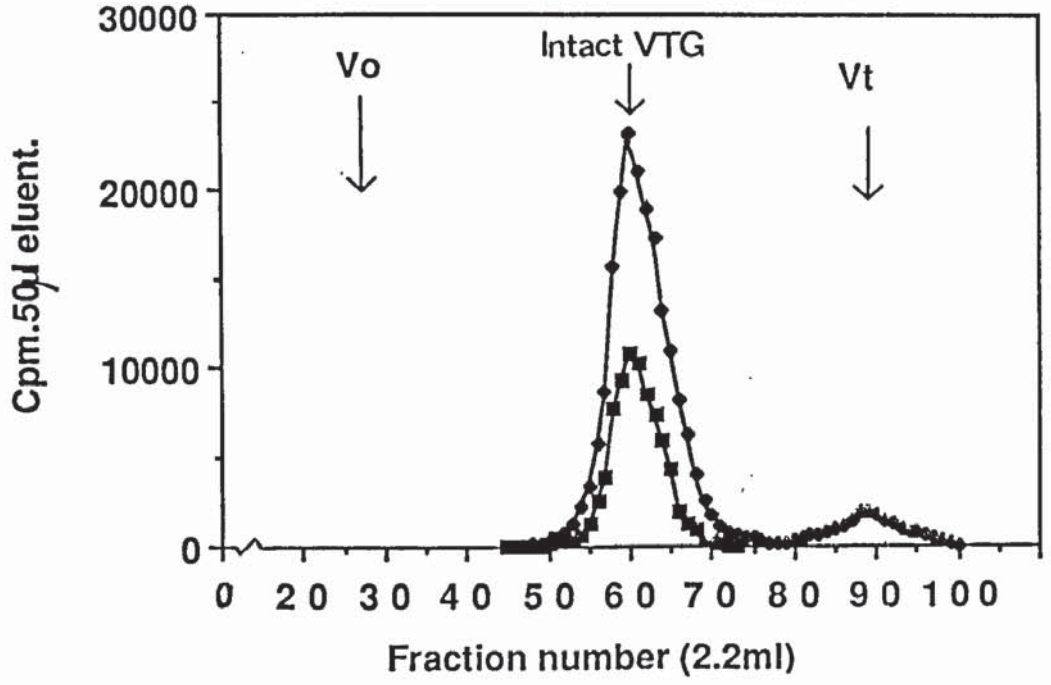


Figure 3.11. Gel-filtration in a column of Sepharose -6B of an aliquot of purified $^{32}P.^{3}H$.VTG. Eluent fractions were monitored for ^{3}H (■) and ^{32}P (◆). The column void (Vo) and total (Vt) volumes are marked.

uptake and processing of VTG in maturing trout follicles in females unstimulated by hormonal treatments.

3.4.1 Materials and methods

The protocol for this experiment is summarized in the flow diagram in Figure 3.12. Three exogenously vitellogenic female trout, weighing 1.1, 1.2 and 1.5 kilograms were injected intraperitoneally with $^{32}P.^{3}H$-VTG with a specific activity of 2.87×10^4 cpm.mg^{-1} VTG. Each fish received a concentrated solution of VTG containing 1.37×10^6 and 4.52×10^6 cpm of ^{3}H and ^{32}P, respectively. Subsequently, each fish was blood sampled and follicles dissected out at 2 time intervals; Fish I at 4 and 8 hours post injection, Fish II at 24 and 48 hours post-injection and Fish III at 1 and 2 weeks post-injection. At the first sampling period the fish was anaesthetised and individual follicles were carefully removed through a small anterior-posterior incision in the flank of the fish. The incision was closed using 2-3 suture stitches and dental cement containing wide spectrum antibiotics was applied to aid healing and reduce the chance of infection.

Serum samples (100 µl) and individual follicles (after 2 pre-washes in physiological saline) were digested and counted for radioactivity. At the second sampling point the fish were killed and their ovaries removed. The diameters of 25 follicles were measured using calipers and their mean size calculated. The total number of vitellogenic follicles was determined and serum VTG levels were measured by radioimmunoassay.

One ml aliquots of serum from Fish I, at 8 hours post injection and ovary homogenates from Fish I, II and III at 8, 24 and 336 hours post-injection, respectively, were run on Sepharose 6B at 6ml hour^{-1} and 2.2ml fractions collected. The fractions were analysed for protein and radiolabel content, as detailed above, to determine the rate of VTG uptake and processing.

3.4.1.1 Determination of the rates of vitellogenin uptake into the developing follicles

The rates of VTG uptake from the serum into both the whole ovary and individual follicles were determined using the ^{32}phosphorous label, the higher energy label of the two radiolabels, for each of the three fish injected with $^{32}P.^{3}H$-VTG. The rate of VTG uptake, including both the labelled and non-labelled protein, was calculated using the formula below and expressed as ng of VTG

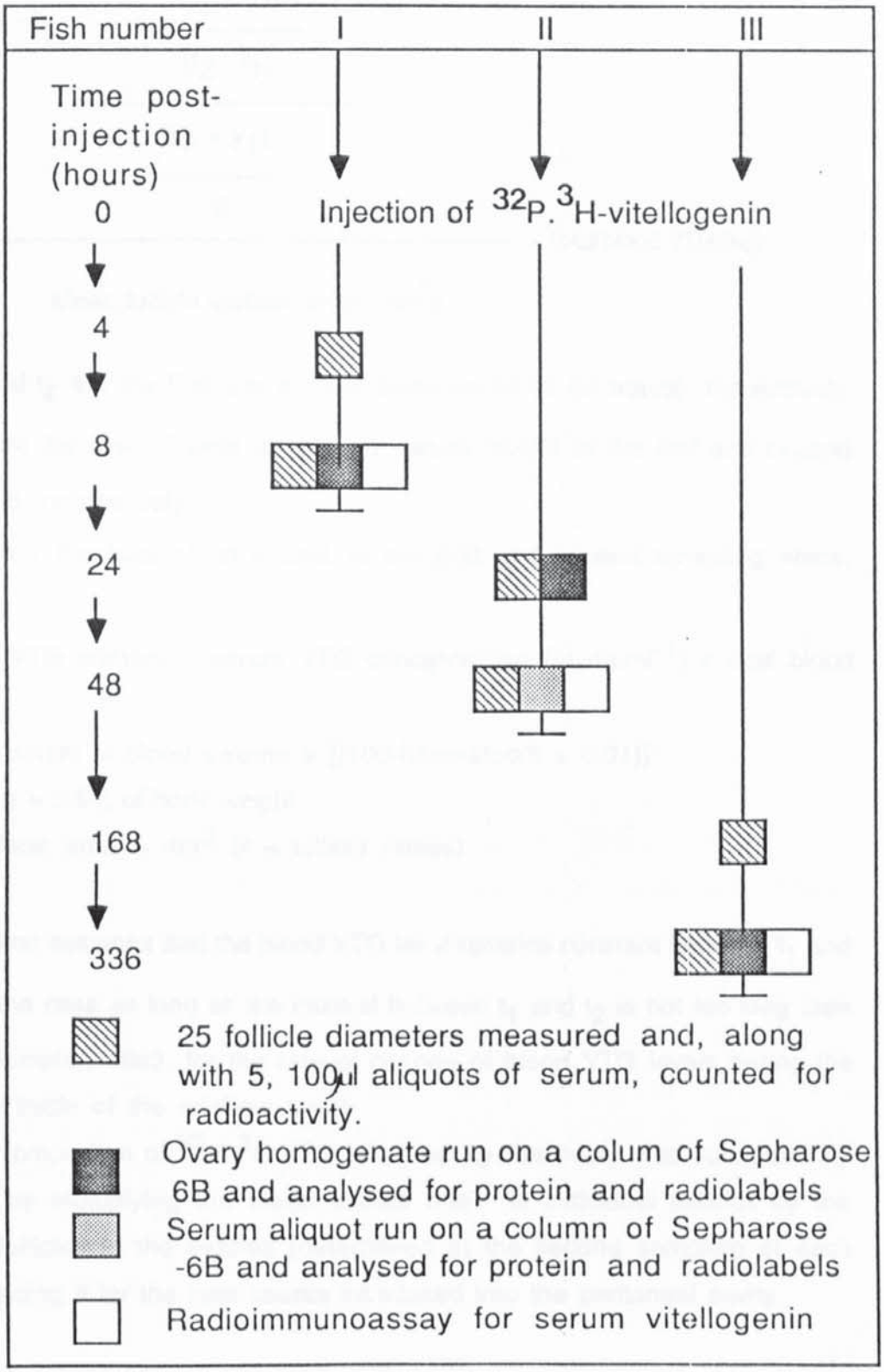


Figure 3.12. Outline of the protocol for experiment 3, investigating the rates of uptake and yolk-protein processing of $^{32}P.^{3}H$.VTG. in vitellogenic females.

sequestered per mm^2 of follicle surface per hour (ng VTG.mm^{-2}.hr^{-1}).

$$\text{Uptake of VTG } (ng.mm^{-2}.hr.^{-1}) = \frac{\dfrac{(x_2 - x_1)}{(t_2 - t_1)}}{\dfrac{(y_2 + y_1)}{2}} \times \frac{\text{Total blood VTG (ng)}}{\text{Mean follicle surface area } (mm^2)}$$

where: t_1 and t_2 are the first and second sampling times (in hours), respectively

x_1 and x_2 are the mean follicle counts per minute (n=20) at the first and second sample times, respectively

y_1 and y_2 are the total blood counts at the first and second sampling times, respectively

Total blood VTG content = serum VTG concentration (in $ng.ml^{-1}$) x total blood serum

Total blood serum = blood volume x [(100-haematocrit x 0.01)]

Blood volume = 5.5% of body weight

Follicle surface area = $4\pi r^2$ (r = follicle radius)

This calculation assumes that the blood VTG level remains constant between t_1 and t_2. This is the case as long as the interval between t_1 and t_2 is not too long (see Scott and Sumpter, 1983, for the rate of change of blood VTG levels during the reproductive cycle of the rainbow trout).

The proportion of $^{32}P.^{3}H$-VTG taken up by the ovary was subsequently determined by multiplying the mean counts min^{-1} in individual follicles by the number of follicles in the ovaries (determined at the second sampling of each fish) and dividing it by the total counts introduced into the peritoneal cavity.

3.4.2 Results

Figure 3.13, a serum chromatogram run 48 hours after $^{32}P.^{3}H$-VTG injection, showed that the labelled VTG eluted as a single discrete protein peak and that the ratio of $^{32}P.^{3}H$ leu. it contained was equal to that of the VTG administered

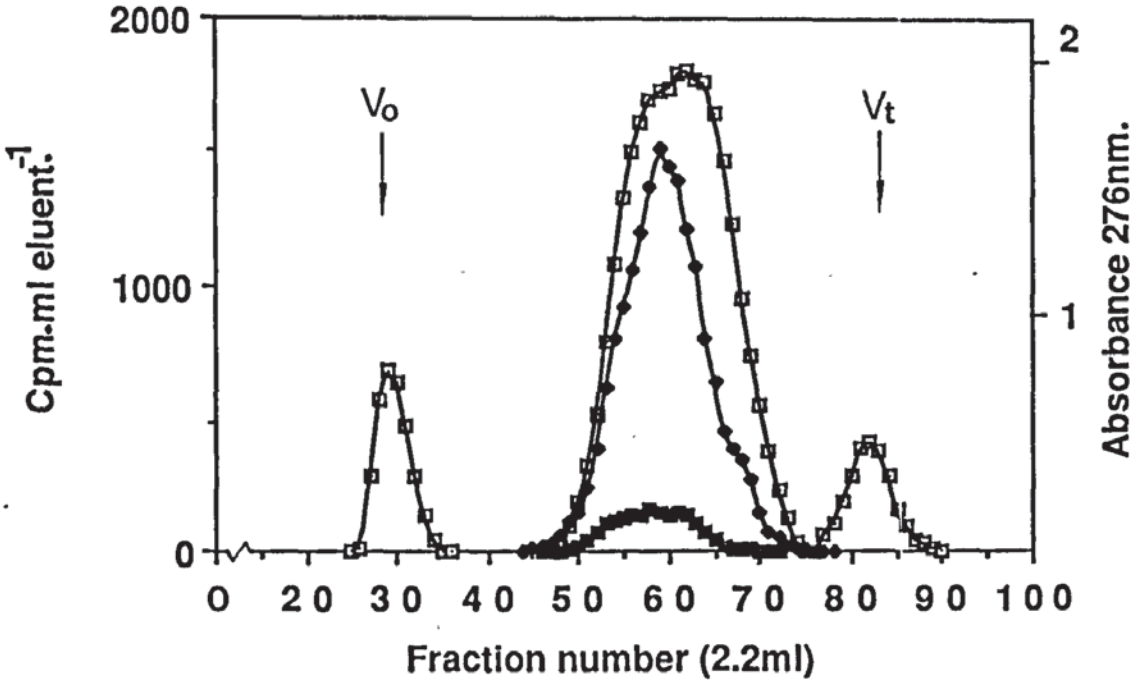


Figure 3.13. Gel-filtration on a column of Sepharose -6B of a 1ml serum sample from a vitellogenic female (Fish I) 48 hours after intraperitoneal injection of ^{32}P. ^{3}H.VTG. Eluent fractions were monitored for absorbance at 276nm (open symbols: □) and for ^{3}H (■) and ^{32}P (◆). The column void (Vo) and total (Vt) volumes are marked.

(approximately 3.4:1 respectively). This showed that even after purification, freezing and its subsequent passage from the peritoneal cavity into the blood $^{32}P.^{3}H$-VTG was in its intact form, rather than being present as degradation products, and hence was available as such for sequestration by the maturing follicles.

Figures 3.14 a-c chart the protein absorbancy profiles and corresponding labelling patterns of ovarian extracts run on Sepharose 6B from Fish I, II and III at 8, 24 and 336 hours post-injection, respectively, detailing the rate of VTG processing. Each ovarian protein profile contained the 3 distinctive yolk protein peaks illustrated in experiment 1. The labelling pattern of proteins 8 hours after $^{32}P.^{3}H$-VTG administration demonstrated one major peak of activity centred around the expected position of VTG at fraction 58, and not coincident with the 3 peaks of yolk proteins, a second smaller peak at the Vo, eluting in the same position as the follicle wall components and smaller ill-defined peaks of radioactivity associated with the yolk proteins. The amount of radioactivity present in the ovarian homogenates only 8 hours after injection of the $^{32}P.^{3}H$-VTG was a small factor which probably accounts for the somewhat ill-defined label profiles.

After 24 hours considerably more radioactivity was present in the follicles which enabled a more precise description of the distribution of the radiolabels. Although a peak of radioactivity still eluted at fraction 58, the position of intact VTG, it no longer constituted the major peak of activity. Instead there was discrete peaks of activity centred on the elution positions of lipovitellin, phosvitin and the V_T of the column (Figure 3.14b). As in experiment 1 the ratio of $^{32}P:^{3}H$ in lipovitellin was significantly less than that of serum VTG (2:1 versus 3.4:1 respectively, i.e. almost half of that of the intact VTG injected).

After 2 weeks the profiles of radiolabels in the ovary were much the same as that observed after 24 hours (compare Figures 3.14b and c). However, all of the radioactivity within the follicle was centred around the 3 yolk protein peaks, showing that of all the internalised $^{32}P.^{3}H$-VTG had been processed, none eluted at the position of intact VTG. The lipovitellin and phosvitin contained the majority of the protein bound ^{3}H leucine and ^{32}P in the follicles, approximately 85% and 65% respectively. These values are similar to that found when free labels were injected into vitellogenic fish and followed into the follicles (experiment 1).

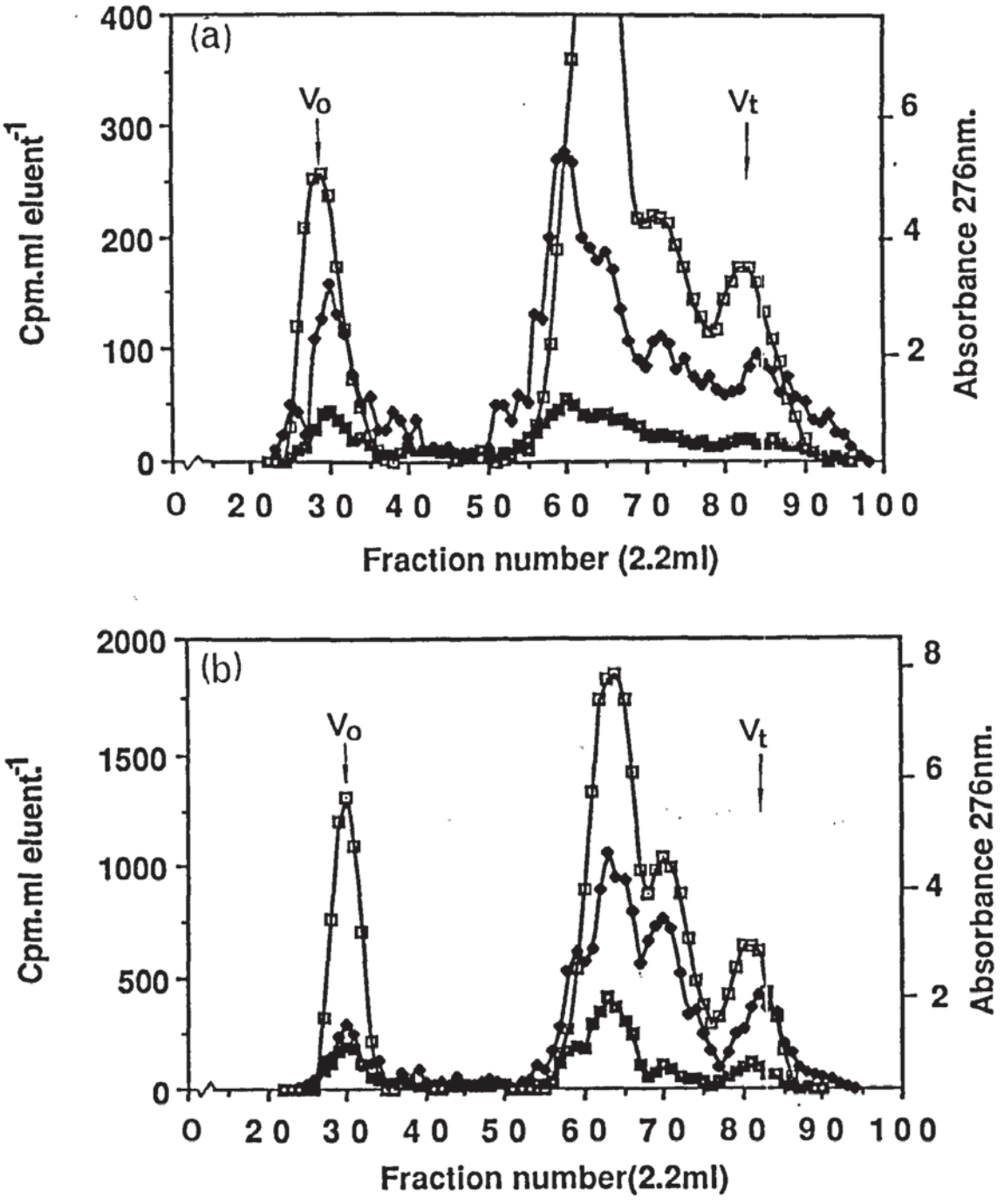


Figure 3.14 Gel-filtration on a column of Sepharose -6B of ovarian homogenates from vitellogenic females, (a) 8 hours (Fish I) and, (b) 24 hours (Fish I) after intraperitoneal injection of ^{32}P. ^{3}H.VTG. Eluent fractions were monitored for absorbance at 276nm (open symbols: □) and for ^{3}H (■) and ^{32}P (◆). The column void (Vo) and total (Vt) volumes are marked.

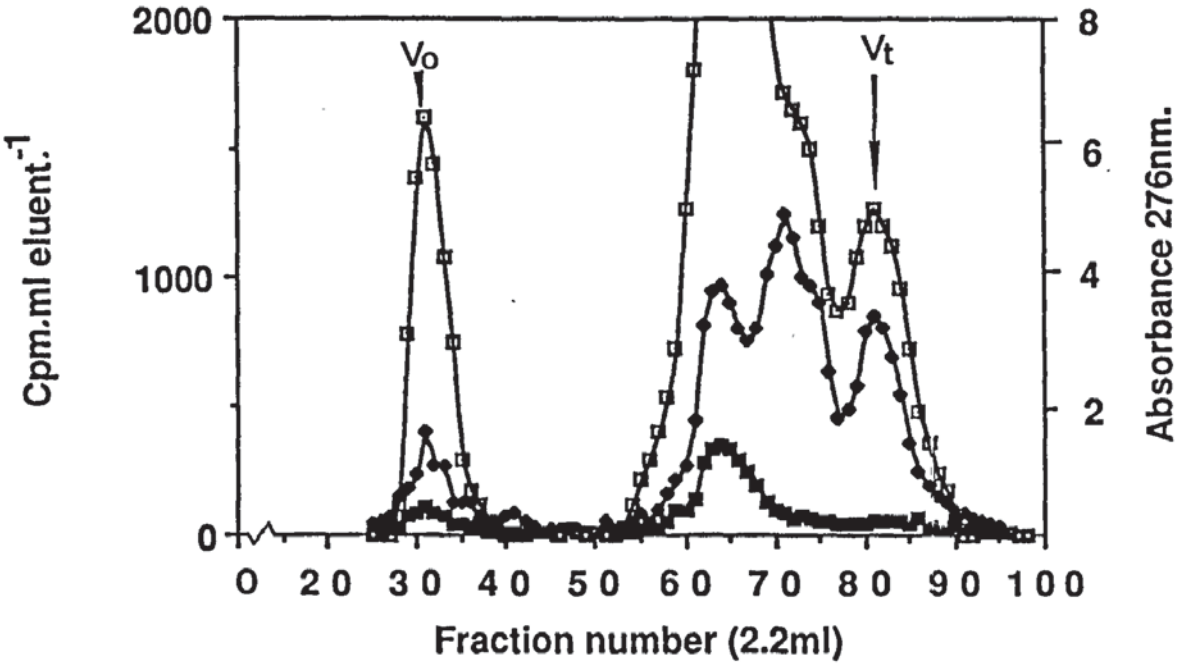


Figure 3.14 cont. (c). Gel-filtration on a column of Sepharose -6B of an ovarian homogenate from a vitellogenic female (Fish III) 14 days after intraperitoneal injection of $^{32}P.^{3}H$.VTG. Eluent fractions were monitored for absorbance at 276nm (open symbols:□) and for ^{3}H (■) and ^{32}P (◆). The column void (Vo) and total (Vt) volumes are marked.

The quantitative data obtained on the rates of $^{32}P.^{3}H$-VTG uptake into the blood from the peritoneal cavity, followed by its uptake into the vitellogenic follicles, are presented in Table 3.2. These data must be interpreted carefully because, at least during the early sampling times, absorption from the peritoneal cavity was occurring in parallel with uptake into the follicles. Nevertheless, the figures do allow many aspects of VTG uptake to be quantified. Within 4 hours of injection almost 38% of the labelled VTG had been absorbed into the blood from the peritoneal cavity, and within 8 hours 45%. Although it is not clear exactly how much of the labelled VTG entered the blood, in Fish III the figure must have exceeded 70% of that initially injected because this amount was sequestered by the ovaries of this fish 336 hours after injection. After this rapid absorption from the peritoneal cavity the level of labelled VTG in the blood fell rapidly, largely because it was sequestered equally rapidly by the follicles. The blood VTG level was positively correlated with both follicle diameter and the rate of uptake of VTG into the follicle (Table 3.2). Vitellogenic follicles of 2.5 ± 0.2, 3.0 ± 0.1 and 4.5 ± 0.1 mm sequestered VTG at rates of 10.7 ± 3.9, 78.5 ± 12.6 and 114.8 ± 35.8 ng VTG mm^2 follicle $surface^{-1}$. $hour^{-1}$, respectively.

Fish number	Counts per min. ^{32}P injected	Sampling time (hours)	^{32}P in blood (% of injected)	^{32}P in ovary (% of injected)	Serum VTG ($mg.ml^{-1}$)	Follicle diameter (mm)	Rate of VTG uptake between t1 and t2 ($ng.mm^{-2}hr^{-1}$)
I	$4*10^6$	t1=4 t2=2	37.8 45.4	0.1 6.7	6.1	3.0 ∓ 0.1	78.5 ∓ 12.6
II	$4*10^6$	t1=24 t2=48	32.8 12.4	24.9 28.28	3.9	2.5 ∓ 0.2	10.7 ∓ 3.9
III	$4*10^6$	t1=168 t2=336	9.3 0.2	34.8 15.0	15.0	4.5 ∓ 0.1	114.8 ∓ 35.8

Table 3.2. Uptake of labelled vitellogenin into the rainbow trout ovary. Three vitellogenic females were injected with $^{32}P.^{3}H$.VTG and its passage from the peritoneal cavity into the blood and ovary monitored.

3.5 Discussion

The chromatographic separations of serum proteins in vitellogenic females followed by their specific RIA for VTG showed that VTG is probably the major blood protein during this phase of reproductive development. Its elution indicated a molecular weight of 440 000 Daltons. This value corresponds well with a number of other gel-filtration studies on rainbow trout VTG which have provided sizes of 455 000 Daltons (Campbell and Idler, 1980) and 440 000 Daltons (Norberg and Haux, 1985; Copeland, 1985). Of all the many different characteristics attributed to VTGs in different oviparous species a large molecular weight is the feature common to all. The VTG eluting in the serum chromatogram of Fish I, in experiment 1 was shown as a distinct peak by radioimmunoassay (Figure 3.7a), suggesting that the molecule was intact and had not undergone protein proteolysis. This showed that rainbow trout VTG, when handled at low temperatures and in the presence of protease enzyme inhibitors, was less susceptible to proteolysis than has been reported for other teleost species; both sea trout (*Salmo trutta*, Norberg and Haux, 1985) and goldfish (de Vlaming *et al.*, 1980) VTG's were shown to be highly susceptible to degradation even in the presence of enzyme inhibitors. This finding corroborates some of the results in a previous study on rainbow trout however, where isolated VTG was reported to be intact (Campbell and Idler, 1980). The separation of VTG in its intact form avoided complications when later in this study the molecule was further characterized and the nature of its processing pathway determined. However, gel-filtration separations do not allow subtle differences in molecular weights to be distinguished and consequently one cannot confirm from these data whether rainbow trout have a single or a number of VTG molecules. The evidence in favour of a single species of VTG in the rainbow trout include: that only one VTG mRNA has been found (Chen, 1983; Valatoire *et al.*, 1984); Babin (1987) in extensive physiochemical studies on VTG reported only 1 VTG type and; to date, no one has reported more than 1 VTG in salmonids. The evidence against there being a single species of VTG are: firstly, a number of higher oviparous vertebrates have been shown to have multiple VTGs (Wahli *et al.*, 1976, 1978, 1979; Wang *et al.*, 1983, see the Introduction to this chapter) and secondly all members of the family Salmonidae are considered to be tetraploid and therefore expected to have at least 2 genes coding for VTG (though they may not necessarily be functional).

The first part of experiment 1, tracing the passage of ^{3}H.leu. and ^{32}P into newly synthesised VTG, demonstrated that even 3 hours after injection,

although the majority of radiolabels absorbed into the blood resided still in their free state, eluting at the V_T, radiolabelled VTG had already been synthesised and secreted by the liver into the blood. The rates of VTG production in vitellogenic females parallel results for *Xenopus* (Clemens *et al.*, 1975), the domestic fowl (Beuving and Gruber, 1971) and the invertebrate, *Locusta migratoria* (Rohrkasten and Ferenz, 1985). Sundararaj and Nath (1981), working on the catfish *Heteropruestes fossilis*, similarly demonstrated a rapid incorporation of radiolabel into serum VTG, showing an inclusion of radiolabelled phorphorus as early as 1 hour post-injection.

The amino acid analysis of rainbow trout VTG, determined in part B of experiment 1 shows a composition that is almost identical to that established by Hara and Hirai (1978) and Campbell and Idler (1980, see Table 3.1). Primarily this demonstrates that the pool of VTG from the gel -filtration column was relatively pure. Comparison of the VTG amino acid content in this study with the composition elucidated by Redshaw and Follett (1971) for *Xenopus laevis* VTG (Table 3.1) shows a number of differences between rainbow trout and amphibian VTG. Several non-polar amino acids, such as alanine, leucine and isoleucine, are relatively more abundant in rainbow trout than in *Xenopus* VTG and rainbow trout VTG has a much lower serine content than *Xenopus*, 7.3 versus 11.5%, respectively. This latter feature of rainbow trout VTG, as mentioned earlier, is obviously reflected by its lower level of phosphorylation.

The ovarian homogenates run on Sepharose 6B showed that 'lipovitellin' constitutes the majority of the yolk protein in the follicle. The ^3H leu. content and cross reactivity of this protein with the VTG RIA, suggests that it was derived from its serum precursor, VTG. It should perhaps be stressed that antibodies for the RIA were raised against a VTG purified from the serum and subsequently shown to cross- react with the yolk proteins, whereas most other immunological assays conducted to date have raised antibodies to the yolk proteins (usually lipovitellin), using these as a probe for serum VTG (Olivereau and Ridgeway, 1962; Ridgeway *et al.*, 1962; Markert and Vanstone, 1971; Plack *et al.*, 1971; Aida *et al.*, 1973; Le Menn, 1979; Fine and Drilhon, 1964). The molecular weight of 390 000 Daltons determined for rainbow trout lipovitellin here is higher than that ascribed by Campbell and Idler (1980) and Hara and Hirai (1978, 300 000 Daltons) but identical to the values reported by Markert and Vanstone (1968) and Copeland (1985). A value of 400 000 (Wallace and Jared,

1960) is also very similar and it is suggested that the modest differences in molecular size have, at least in part, resulted from the use of gel-filtration (Rhobard, 1976).

The lipovitellin showed a similar amino acid composition to that reported in 2 other studies on rainbow trout, and in *Xenopus* (Table 3.1). The amino acid composition therefore indicates that despite the evolutionary distance between the teleost and amphibian follicle lipovitellin these molecules have been strongly conserved between the 2 distinct phylogenic groups, a feature also found with other lipoprotein and apolipoprotein structures throughout the vertebrates.

The low molecular weight phosphoprotein within the follicle appears to elute as a single protein, a result that corresponds well with the single low molecular weight phosphoprotein found in salmon follicles. Preliminary separations on SDS-electrophoresis appeared to confirm the presence of a single molecular weight form of phosvitin (data not shown) although more detailed studies on rainbow trout follicles throughout the vitellogenic development of the ovary would need to be conducted to confirm this. Indeed recent evidence from a number of teleosts report that the number and types of phosvitin(s) change throughout the vitellogenic growth period (Wallace *et al.*, 1987; Norberg, 1987). The phosvitin molecule in this study had a molecular weight of approximately 40 000 Daltons a value similar to the 43 000 Daltons reported for rainbow trout phosvitin by Campbell and Idler (1980). The high phosphorus content of phosvitin (and consequently the high ^{32}P content in the dual-labelled VTG), and low content of aromatic amino acids were reflected in the chromatograms of ovarian homogenates, where the phosphoprotein ^{32}P values did not parallel the absorbance at 276mm. Rainbow trout phosvitin's lack of absorbance of ultra-violet light is a feature well recognised in other teleosts and higher vertebrate phosvitins. Unlike lipovitellin, phosvitin showed no cross-reactivity in the VTG RIA, showing that antibodies were indeed raised to the lipovitellin moiety of the VTG complex. The serine content of phosvitin, although high was significantly lower than reported for rainbow trout phosvitin by both Campbell and Idler (1980) and Ito *et al.* (1966) being approximately 19% versus 42 and 54%, respectively. A repeat analysis on a further phosvitin fraction produced a similar value for serine to that obtained before; the reason for this discrepancy is unknown. However, an analysis of the structure of rainbow trout VTG by Hara and Hirai (1978) found a similar sized protein (30 000 Daltons) conferring a similar amino acid content, which they termed the E2

component (see Table 3.1), and it is suggested that both the E2 component and the small molecular weight phosphoprotein peak separated in this study, although both containing phosvitin, also include other small molecular weight yolk protein(s), such as the 'ß' component discussed earlier, i.e. the gel filtration procedures adopted to separate proteins did not resolve phosvitin completely.

The qualitative data presented in experiment 1 show, for the first time in teleosts, that VTG follows a similar pathway of processing within the follicle to that observed in higher oviparous vertebrates; in all cases the serum VTG gave rise to a lipovitellin moiety and a highly phosphorylated, phosvitin. It should be stressed, however, that yolk lipovitellin and phosvitin are not necessarily the only derivatives of VTG and that other rainbow trout yolk proteins such as the 'ß' component, mentioned above and shown to be immunologically related to VTG (Campbell and Idler, 1980), may enter the follicle as a component of the VTG complex. The radiolabel profiles in the ovarian homogenates in Fish I and II in experiment I at 1 and 3 weeks post-injection, respectively, showed a difference between the levels of ^{3}H leu. present in the lipovitellin, which simply reflected an increase in the amount of sequestered VTG into the follicle with time. The different levels of ^{32}P eluting at the V_T (probably as free label) in the ovarian homogenates from Fish I and II after similar time intervals was less clear, however, it probably reflects individual differences and not differences in the degree of phosvitin phosphorylation. Indeed Wallace (pers. comm.) strongly suspects that phosvitin has a specific degree of phosphorylation within any one species and does not lose the high energy bonding until embryonic development is initiated. The overall profile pattern of the radiolabels within the rainbow trout follicles remained constant over at least a 3 week period (the sampling interval), indicating that no further processing or degradation of the yolk proteins occurred after initial sequestration. Opresko *et al.*, (1980) demonstrated that in *Xenopus* the lipovitellin and phosvitin remained intact until fertilization occurred and the embryo started its development.

In experiment 2 oestradiol's induction of VTG synthesis was both rapid and specific. Vitellogenin was the only 'new' blood protein to be seen on the chromatogram and to contain the injected ^{32}P and ^{3}H-leu. labels (Figure 3.10), though small amounts of other induced proteins may have remained undetected. During the production of labelled VTG heavily oestrogenised fish showed a reduction in the amount of protein eluting at the V_T (as illustrated by the

absorbancy profiles) and often a loss of protein eluting at Vo compared to non-oestrogenised individuals, indicating that nutrient demands created by the production of large quantities of VTG could only be met by reducing the synthesis of other serum proteins. Plack *et al.*, (1971) working on the cod, demonstrated a 20-50% decrease of serum proteins other than VTG in fish which were undergoing vitellogenesis. Norberg and Haux (1985), suggested that oestradiol affects various extra-hepatic proteins in order to supply the necessary precursors and energy for VTG synthesis in the liver. Furthermore, recent evidence from *in vitro* studies on the liver of rainbow trout shows that the albumin gene is turned off as the VTG gene is turned on (Maitre *et al.*, 1986). An alternative explanation to the alteration in the levels of some non-VTG serum proteins in oestrogenised individuals is that the reduction may be linked to the physiological stress imposed by the hormones themselves; Thurston (1967) and Plack *et al.* (1971) both document immediate and dramatic effects on serum protein concentrations in stressed rainbow trout and cod, respectively.

The *in vivo* approach to radiolabelling VTG meant that the ^{32}P and ^{3}H leu. became integral parts of the protein molecule during its synthesis; ^{3}H leu. incorporated as part of the proteins amino acid backbone and ^{32}P added during VTG's hepatic post-translational phosphorylation. The success of this labelling method was demonstrated in experiment 3 and subsequently in the experiment in Chapter 4, when even after its purification from serum and its injection and subsequent uptake into the serum of experimental fish, the label is still associated with intact VTG (Figure 3.13); there was little if any degradation. Other approaches to VTG labelling involve oxidizing or alkylating agents and these chemicals can structurally alter the protein molecule such that it may not be handled and sequestered by follicles in the normal fashion. The incorporation of free radiolabels into VTG appeared to be more effective using surgically implanted mini-osmotic pumps than by a direct intraperitoneal injection; with the resulting purified VTG concentrates having specific activities of 287 cpm and 395 $cpm.\mu g^{-1}$ protein, respectively. However, a number of variables including; possible differences in the rate of VTG synthesis between the 2 oestrogenised immature females and the higher specific activity of the ^{3}H leu. label administered using the osmotic pumps meant that a direct comparative quantification of these 2 methods could not be conducted. In the production of labelled VTG subsequently mino-osmotic pumps were used to administer radio labels.

The third experiment investigated the dynamic relationship between serum VTG and the follicle yolk proteins using the purified $^{32}P.^{3}H$-VTG prepared in experiment 2. Qualitative studies on protein transport into the developing follicles of teleosts have been reported for the sheepshead minnow (Selman and Wallace, 1982) using the electron-dense marker horseradish peroxidase (HRP); (see Introduction to Chapter 4). However, there are no quantitative data on the rates of VTG uptake and follicle processing in any species of fish. The Sepharose 6B chromatograms (Figures 3.13 and 3.14) in experiment 3 clearly demonstrated a rapid uptake of the intraperitoneally injected $^{32}P.^{3}H$-VTG into the blood and subsequently the follicles, followed by its specific cleavage to lipovitellin and phosvitin. The pattern of labelling in the ovarian homogenate 8 hours after injection of $^{32}P.^{3}H$-VTG showed that a large proportion of VTG was present in its unprocessed, native form. Furthermore, although most of the labelled VTG had been processed after 24 hours, and the majority of the ^{32}P and ^{3}H-leu. eluted with the yolk proteins, consistent with the results in experiment 1, small amounts of intact VTG were still present. Wallace and Jared (1969) in a similar study with *Xenopus* showed that after 8 hours most of the radioactivity present in the yolk extract was associated with lipovitellin and phosvitin, with only a little eluting with intact VTG. Thus it appears that *Xenopus* which are usually maintained between 20-25^{o}C, may process VTG rather faster than rainbow trout maintained at 10^{o}C. Subsequent studies on follicle yolk extracts, rather than ovarian homogenates in this laboratory have failed to demonstrate appreciable quantities of intact, labelled VTG in yolk, although small amounts are sometimes seen. It is probable then that the small amounts of intact $^{32}P.^{3}H$-VTG found in the ovarian homogenates in the present work was associated with the follicular layers of the follicle which was released during the preparation of homogenates. However, the presence of small amounts of labelled VTG even in follicle extracts would also suggest that the follicle does contain a little intact VTG, probably material that has been recently sequestered. In *Xenopus* the processing of VTG of lipovitellin and phosvitin takes place within an endocytotic compartment and although follicles incorporate VTG into membrane bound vesicles within 15 minutes, follicle processing of VTG does not take place until between 1-2 hours after uptake (Opresko *et al.*, 1980). As a result of the time required for sequestered VTG to reach the appropriate endocytotic compartment, there is a period after uptake during which VTG is in the follicle in its intact form. The presence of intact VTG in the follicle extracts of the rainbow trout probably

reflects a similar model of membrane compartmentalization during VTG sequestration and processing to that observed in *Xenopus*, an idea investigated and discussed further in Chapter 4. Once $^{32}P.^{3}H$-VTG had been endocytosed and cleaved to its yolk protein products, as with the processing of labelled VTG after an initial injection of 'free' ^{32}P and ^{3}H leu. in experiment 1, the overall labelling pattern remained relatively constant over the sampling period (2 weeks), indicating that the yolk proteins are stable. The long term stability of the sequestered proteins represents a unique and experimentally convenient feature for further studies conducted in this project which investigated aspects of protein uptake, transport and sequestration by developing vitellogenic rainbow trout follicles.

In all of the females sacrificed in experiments 1 and 3 all the vitellogenic follicles within a particular ovary were at a similar stage of development, indicated in experiments by a homogeneity in both follicle size and uptake of labelled VTG (Table 3.2). This feature is generally not seen in vitellogenic ovaries containing follicles less than 2.5mm in diameter where there is often a considerable size disparity (own observations and Sumpter pers. comm.). The follicle size disparity in the early phases of vitellogenic development and the question of how the synchrony in follicle size is achieved in later vitellogenic growth so that all are of similar size at ovulation is investigated and discussed further in Chapter 5. The rates of VTG uptake, although varying from approximately 11 to 114 $ng.mm^{-2}.hr^{-1}$ with different sizes of follicles in different fish measuring 2.5-4.5mm in diameter respectively, parallel the value of 49 $ng.mm.^{-2}.hr.^{-1}$ obtained in *Xenopus* follicles cultured *in vitro* (Wallace and Jared, 1969). The higher uptake rates in the larger follicles may be a reflection of the greater availability of blood VTG (see Table 3.2), i.e. the rate of VTG uptake is not saturated in the smaller follicles, because generally there is less VTG available in the blood at this stage of follicle development (Scott and Sumpter, 1983; Sumpter *et al.*, 1984). Alternatively, as the follicle progresses through the vitellogenic cycle there may be an increased ability to endocytose VTG. These questions are investigated further in Chapters 5 and 6.

Rainbow trout VTG clearly follows a parallel pathway of processing within follicles and at similar rates to those observed in other oviparous vertebrates.

CHAPTER FOUR

THE SELECTIVITY OF PROTEIN SEQUESTRATION INTO VITELLOGENIC FOLLICLES

4.1 Introduction

The reproductive success of a species is dependent on the ability of the female to provide her offspring with specific substances essential to meet their nutritional requirements early in life. In many oviparous vertebrates the most important of these nutrient reserves is VTG and therefore its acquisition by the growing follicle is of paramount importance. In the domestic fowl (Cutting and Roth, 1973) *Xenopus* (Wallace, 1978) and the oviparous invertebrate, *Locusta migratoria*, (Ferenz *et al.*, 1981) VTG is selectively removed from the pool of blood proteins by the process of receptor mediated endocytosis; however, in fish even though there are over 20 000 species of oviparous teleosts alone, very little is known about the transport of VTG and other serum proteins from the blood to the maturing ovary.

Receptor mediated endocytosis involves the binding of the protein to a specific receptor located on the apical surface of a cell. This binding induces the formation of coated pits (concentrations of the receptor complexes) which pinch off internally giving rise to endocytotic coated vesicles containing the invaginated proteins. The protein is subsequently degraded by lysosomes while the receptor is recycled.

In *Xenopus* follicles receptor mediated endocytosis selectively sequesters VTG *in vivo* at least 25 times more rapidly than other serum proteins (Wallace *et al.*, 1970) and, *in vitro*, up to 50 times faster than a number of heterologous macromolecules (Wallace and Jared, 1976). VTG sequestration accounts for 99% of the stable yolk proteins in a mature *Xenopus* follicle and accounts for approximately 80% of its vitellogenic growth (Wallace *et al.*, 1972).

Extensive studies on *Xenopus* follicles using pulse-chase experiments with labelled proteins followed by cell fractionation and gel electrophoresis have revealed the dynamic aspects of the endocytotic system of protein transport (Opresko *et al.*, 1980) and together with histological studies, providing the pathways morphological framework, have produced a 'working model' of the endocytotic pathway for VTG sequestration, which is detailed below and in Figure 4.1.

The follicular layers of a follicle are the initial site of selection for proteins and other nutrients. In *Xenopus* when VTG becomes available for uptake, at the onset of exogenous vitellogenesis, the granulosa cells decrease their interactions with adjacent granulosa cells but increase their contact with the oocyte.

Figure 4.1. Current working model for post-endocytotic compartmentation of protein in Xenopus laevis follicles (adapted from Opresko et al., 1980)

Furthermore large intercellular spaces form between adjacent follicular cells to facilitate the protein transport to the oocyte surface; this process is called patency, meaning that VTG can now reach the surface of the oocyte, and hence be sequestered. As these processes occur at the follicular layer interfaces 2 important changes take place at the surface of the oocyte; firstly the oocyte sends numerous finger-like microvillus projections across the peri-vitelline space to interdigitate with similar structures emerging from the granulosa cells, and secondly coated pits develop, these are localized depressions in the plasma membrane that protrude towards the cortical ooplasm. Coated pits are found widely among animal cells that are actively pinocytosing: generally they consist of 3 layers; a hexagonal lattice meshwork on the cytoplasmic side of the pit, a fibrous coat on the luminal side, and between these a unit that is continuous within the oocyte membrane. The lattice forms a basket consisting of the protein clathrin (Whyte and Ockleford, 1980) around the pit which helps both to anchor the specific receptors and later to form endocytotic vesicles. The total area occupied by these coated pits is difficult to determine, for in oviparous vertebrates generally the plasma membrane is made up of many deep folding and tortuous crypts that greatly increase the surface area (Perry *et al.*, 1978; Roth *et al.*, 1976). However, these coated pits are extremely numerous in oocytes of both *Xenopus* (Wallace, 1985) and rainbow trout (Upadhyay *et al.*, 1978).

Vitellogenin reaches the oocyte's plasma membrane from the capillary plexus via the interstitial spaces between the granulosa cells (Selman and Wallace, 1982) and then passes along the surface of the microvilli to the oolemma surface (Abraham *et al.*, 1982). Here the VTG then binds to the specific receptors. Scatchard plot analyses of *Xenopus* oocytes revealed that there are between 2-28 x 10^{10} surface binding sites for VTG. There is only a single class of receptor with an affinity of 1.3 x 10^{-6}M at 22°C and 2-4 x 10^{-6}M at O°C and high specificity, showing less than 5% non-specific binding at 2 x 10^{-6}M (Opresko and Wiley, 1987a). The specific internalization of the VTG receptor is first order and highly variable (Opresko and Wiley, 1987a) and at around 2 x 10^{-3} s^{-1} is very rapid relative to mammalian cell-receptor systems at similar temperatures. The VTG receptors appear to be continuously internalized regardless of occupancy (Opresko *et al.*, 1981), illustrating that the presence of the ligand does not induce the formation of endocytotic vesicles but instead they are internalized at preformed sites (the coated pits). *Xenopus* follicles have very large intracellular pools of receptors and although surface receptors are internalized on the time scale of

minutes, the intracellular pool is recycled on the time scale of hours (Opresko and Wiley, 1987a). The actual VTG receptor has not been characterized in *Xenopus* but in the domestic fowl it elutes on SDS gel electrophoresis with a molecular weight of 116 000 Daltons (Woods and Roth, 1979, 1980). Direct binding studies indicate that in the domestic fowl both VTG and phosvitin bind to the oocyte plasma membrane with essentially similar affinities, with dissociation constants of approximately 6 x 10^7 (Woods and Roth, 1980). These authors suggested that phosvitin confers the receptor-recognition site in the VTG molecule, an idea reinforced by the argument that lipovitellin, in view of its extreme hydrophobicity would be burried in the interior of the VTG molecule. However, in contrast to the chicken, studies conducted on *Xenopus* suggest that the phosvitin moiety does not confer the receptor recognition site in the VTG molecule. Opresko and Wiley (1987a) demonstrated that phosvitin was needed at 50 times the concentration of VTG to inhibit VTG binding. Wallace (1983) proposed that lipovitellin 2 was the site of oocyte binding. The VTG receptor is probably developmentally regulated since in *Xenopus* neither stage I or II (pre-endogenously vitellogenic) follicles appear capable of incorporating VTG (Dumont, 1972; see Chapter 6).

After binding in *Xenopus* VTG enters the ooplasm in clathrin coated vesicles (see Figure 4.1). These coated vesicles subsequently give rise to uncoated vesicles or endosomes, a process taking between 5 and 60 minutes (Dumont, 1978). When the VTG receptors are unoccupied the endosomes fuse directly with the yolk platelets, whereas endosomes containing occupied VTG receptors associate with one another and condense, giving rise to transitional yolk bodies. A corollary of this observation is that receptor occupancy can act as a transmembrane signal that directs the post-endocytotic compartmentalization of VTG (Opresko *et al.*, 1980). In the transitional yolk body VTG becomes uncoupled from the receptors, a process possibly induced by the compartments low pH (Geuze *et al.*, 1983). In most receptor mediated processes the endocytotic internalization of external protein leads to digestive sequelae mediated by lysosomes (Kaplan, 1981). However, although acid hydrolases have been found in full grown *Xenopus* follicles and eggs (Decroly *et al.*, 1979; Steinert and Hancq, 1979), typical lysosomes have not been detected in healthy vitellogenic oocytes (Dumont and Wallace, 1973; Wallace and Hollinger, 1979) and sequestered VTG is not digested (Opresko *et al.*, 1979). Proteolytic processing of VTG into yolk peptides occurs only in the transitional yolk bodies and is probably mediated by a specific VTG processing hydrolase; the transitional yolk bodies thus represent modified secondary lysosomes (Opresko *et al.*, 1980). The

transitional yolk bodies subsequently fuse with the yolk platelets and the yolk proteins aggregate to form crystalline lattices. These crystalline structures are fairly insoluble and by this agency avoid further proteolytic action. The insoluble yolk proteins are unavailable to any membranous elements that pinch off from the yolk platelets in the proposed process of receptor recycling during the receptors translocation back to the surface of the oocytes plasma membrane (Telfer *et al.*, 1982; see Figure 4.1). Opresko *et al.*, (1981) demonstrated that once formed the yolk proteins in *Xenopus* remain stable upto the time of ovulation. Recent studies on a number of marine teleosts, however, in contrast to that observed in *Xenopus*, suggest that yolk proteins undergo degradation prior to ovulation. Studies on the cod, Atlantic halibut, *Hippoglossus hippoglossus*, and plaice, *Pleuronectes platessa*, show that further hydrolysis of yolk proteins occurs in maturing follicles and consequently hydrated eggs contain proteins of lower molecular weight than in non-hydrated eggs (Norberg, 1987). Polyacrylamide gel electrophoresis on follicles from the mummichog, *Fundulus heteroclitus*, indicated that proteolysis of yolk proteins occurred prior to germinal vesicle breakdown (McPherson *et al.*, 1987).

In contrast to the fate of sequestered VTG, that injected directly into the ooplasm of *Xenopus* follicles is inaccessible to the endocytotic compartments and is simply degraded, with a half-life of 6-14 hours (Wallace and Hollinger, 1979). Opresko *et al*, (1980) demonstrated that in *Xenopus* there is only one micropinocytotic mechanism and that other serum proteins and niutrients enter the ooplasm adventitously in the fluid phase during the receptor mediated endocytosis of VTG. Unlike VTG, however, but in a similar way to micro-injected proteins, adventitiously incorporated macromolecules undergo turnover (Wallace and Hollinger, 1979; Opresko *et al.*, 1979, 1980). Detailed studies carried out on the non-specific incorporation of ^{125}Iodine labelled bovine serum albumin (BSA) into cultured *Xenopus* follicles demonstrated that 40% of that sequestered was rapidly lost, with a half-life of 3 hours, but the rest appeared to be stable (Opresko *et al.*, 1980). The processing of invaginated non-specific proteins has not been determined and the BSA undergoing degradation, sequestration in a stable form, and/or secretion from the ooplasm may do so along a number of theoretical pathways (see Figure 4.1). BSA taken into the ooplasm in vesicles with unoccupied VTG receptors, which as previously mentioned cannot fuse together and condense to form endosomes, may either be directly exocytosed, in an intact or degraded form, along one of the possible routes of receptor recycling, or alternatively it may enter the

yolk protein complex in vesicles fusing directly with the yolk platelets. BSA entering vesicles containing occupied VTG receptors passes through the same endocytotic compartments as sequestered VTG. In this pathway BSA is degraded in the transitional yolk bodies, probably non-specifically by the VTG-hydrolase. In either event fragmented or intact BSA entering the yolk platelets, along either of the translocative routes, and residing in the platelets fluid phase remains susceptible to protein proteolysis and exocytosis during receptor recycling. The BSA remaining stable with the follicle, like other non-vitellogenic serum derived proteins, may escape proteolysis by intercalating with the hydrophobic yolk crystals (Opresko *et al.*, 1980). Although receptor-mediated endocytosis fulfils an important nuitritive role, enabling *Xenopus* follicles to ingest VTG specifically and in large amounts, the fluid phase engulfment during this endocytotic process also serves a number of other important roles. As seen by the uptake and processing of BSA it may regulate both the composition and quantity of other cellular constiuents. It is suggested that endocytosis provides a link between the ooplasm and circulating blood hormones and furthermore it may also serve to remove substances from the extracellular fluid for degradation (Opresko *et al.*, 1980).

Unlike in *Xenopus* follicles where, at least to date, only VTG has been shown to enter selectively, in the domestic fowl there are other yolk proteins specifically sequestered by receptor-mediated endocytosis. These proteins include very low density lipoprotein and low density lipoprotein (Holdsworth *et al.*, 1974), Immunoglobulin G (Patersen *et al.*, 1962; Cutting and Roth, 1973) and biotin-binding protein (White *et al.*, 1974). Other proteins present in avian follicles are thought to enter by concurrent adventitious engulfment during these receptor-mediated processes and/or by non-specific pinocytotic mechanisms. Thus although it appears that a considerable amount is known about protein uptake and subsequent compartmentilization in follicles in fact our knowledge has been derived from just the two species described above, the domestic fowl and *Xenopus*.

In fish precise quantification of both the rate and specificity of protein uptake have not been performed although it has been suggested that teleost follicles can sequester a heterologous collection of macromolecules quite distinct from VTG. The passage of significant quantities of non-vitellogenic serum proteins into maturing follicles has been demonstrated *in vitro* in the rainbow trout (Campbell and Jalabert, 1979) and *in vivo* in the ayu, (Aida *et al*, 1973). Electron microscopy studies on the follicles of the swordtail, *Xiphophorus helleri*, have charted the incorporation of an electron dense marker into the forming yolk bodies

(Wegman and Gotting, 1971) and Selman and Wallace (1983) have shown the sequestration of both trypan blue and horse-radish peroxidase into the developing follicles of *Fundulus heteroclitus*. However, studies using markers such as horse radish peroxidase and trypan blue are of limited physiological significance to the understanding of how and what proteins are transported into teleost follicles; heterologous molecules may not necessarily be transported into the ooplasm in a similar manner to that of vitellogenic and non-vitellogenic yolk protein precursors. Furthermore teleost VTG is around 10 times larger in size than horse-radish peroxidase (40 000 Daltons) and 40 times the size of trypan blue (960.8 Daltons).

Studies carried out to date on VTG uptake into teleost follicles are ambivalent on the question of protein selectivity; in one case VTG uptake into trout follicles appeared to be fairly specific (Campbell, 1978) where as in another there was little, if any selectivity, BSA being sequestered almost as rapidly as VTG (Campbell and Jalabert, 1979). These studies conducted *in vitro*, were further complicated by the comparatively low rates of VTG sequestration compared to that seen in this study *in vivo* and *in vitro* in *Xenopus* (review Wallace, 1985; see Chapters 5, 6 and 7). Wallace, 1985, reviewing the selectivity studies concluded that "something is clearly amiss in the trout system". The present investigation addresses the question of protein selectivity in teleosts by comparing *in vivo* the relative rates of uptake of radiolabelled VTG and BSA in vitellogenic rainbow trout follicles. The processing pathways and respective stabilities of the sequestered proteins were determined using column chromatography.

4.2 Materials and methods

4.2.1 Labelled proteins

Methylated ^{14}C bovine serum albumin (^{14}C-BSA) with a specific activity of 0.026 mCi mg^{-1} was purchased from Dupont.

Vitellogenin was labelled *in vivo* using L-[4-5-^{3}H] leucine (Amersham International) which had a specific activity of 0.000018 m Ci mg^{-1} protein.

4.2.2 Experimental protocol

The cost of obtaining sufficient labelled proteins limited this study to 3 vitellogenic female fish; however, there is no reason to think that the results would not apply to all individuals. Fish number I and II were injected intraperitoneally with 4 x 10^6 cpm of both ^{3}H-VTG and ^{14}C-BSA, and Fish III with 3 x 10^6 cpm of ^{3}H-VTG and ^{14}C-BSA. Both labelled proteins were administered at the same specific activity (27.3 mg protein for 10^6 cpm); the labelled BSA being equated with the labelled VTG's activity by dilution with cold BSA (Sigma Chemical Company). Forty-eight hours after the labelled proteins were injected, Fish II was blood sampled and its ovaries, liver, spleen, and some muscle tissue dissected out, washed thoroughly in physiological saline, and weighed, before approximately 10 mg samples (n=5 for each tissue) and 100 µl aliquots of serum (n=5) were prepared for scintillation counting as described previously in Chapter 2, Section 2.6. Finally the diameter of 25 ovarian follicles was measured.

Fish I and III were blood sampled and 25 follicles analysed at each of two separate time intervals: Fish I at 48 and 72 post-injection, and Fish III at 312 and 624 hours post-injection. At the initial sampling follicles were removed from each fish through a small incision made in the flank, which was subsequently closed with 2 to 3 stitches. Wide spectrum antibiotics were added to reduce the chance of microbial infection. At the second sampling tissues from both Fish I and III were collected and analysed as detailed above for Fish II. The total amount of the two radio labels in the particular tissues sampled was determined by multiplying the tissue weight by the fraction the 10mg sample counted represented as a proportion of the total tissue weight.

A 1ml aliquot of serum from Fish II, obtained 48 hours post-injection, and ovarian homogenates from Fish I and III, prepared from samples collected 72 hours and 312 hours post-injection, respectively, were subjected to gel-filtration on Sepharose 6B, with the eluents being analysed for both absorbance at 276mm and radiolabel content. The outline for the complete protocol for this experiment is

summarized in Figure 4.2.

4.3 Results

The serum chromatogram of Fish I, a maturing vitellogenic female sampled 48 hours after the injection of ^{14}C-BSA and ^{3}H-VTG (Figure 4.3) shows that the protein profile (described by the absorbancy of the eluent at 276 nm) is consistent with profiles reported previously for serum from female trout undergoing exogenous vitellogenesis in Chapter 3. The ^{3}H-VTG and ^{14}C-BSA, injected intraperitoneally but now present in the blood, eluted as single, distinct peaks, centred around fractions 53 and 67, with apparent molecular weights of approximately 440,000 and 70,000 Daltons, respectively. Only small amounts of the two labels were found at the V_T of the column, where 'free' label would be expected to elute. This confirmed that the ^{14}C-BSA and the ^{3}H-VTG were both present in the blood as intact molecules.

Both labelled proteins were rapidly absorbed from the peritoneal cavity into the blood. At the first sampling, 48 hours after injection of the labelled proteins, the serum concentration of ^{14}C-BSA in vitellogenic fish was approximately 3 times that of ^{3}H-VTG (Table 4.1). This may reflect a faster uptake of ^{14}C-BSA into the blood (it is a smaller protein) or, alternatively, a more rapid clearance of ^{3}H-VTG from the blood. In Fish II, however, although the blood concentrations of ^{14}C-BSA after 48 hours was the same as that observed in the vitellogenic fish, the blood concentration of ^{3}H-VTG was 3 times higher, i.e. the same as that of ^{14}C-BSA (Table 4.1). This strongly suggests that both proteins were absorbed into the blood at equal rates, and that the differences in blood levels of ^{3}H-VTG and ^{14}C-BSA observed in vitellogenic fish were due to a rapid clearance of ^{3}H-VTG from the blood. As the experiment progressed, the blood concentrations of both labelled proteins fell in the vitellogenic fish, though this fall was much more pronounced for ^{3}H-VTG than for ^{14}C-BSA. After 26 days there was very little ^{3}H-VTG left in the blood, but still appreciable amounts of ^{14}C-BSA (about 10% of that injected).

This progressive fall in the blood concentration of ^{3}H-VTG was accompanied by rising levels of this labelled protein in the ovary. After 48 hours approximately one third of the ^{3}H-VTG injected initially was already in the ovary. This value had risen to 49% by 72 hours. In Fish III 45% of the ^{3}H-VTG was present within the ovary after 26 days, at which time very little (< 0.1%) was left

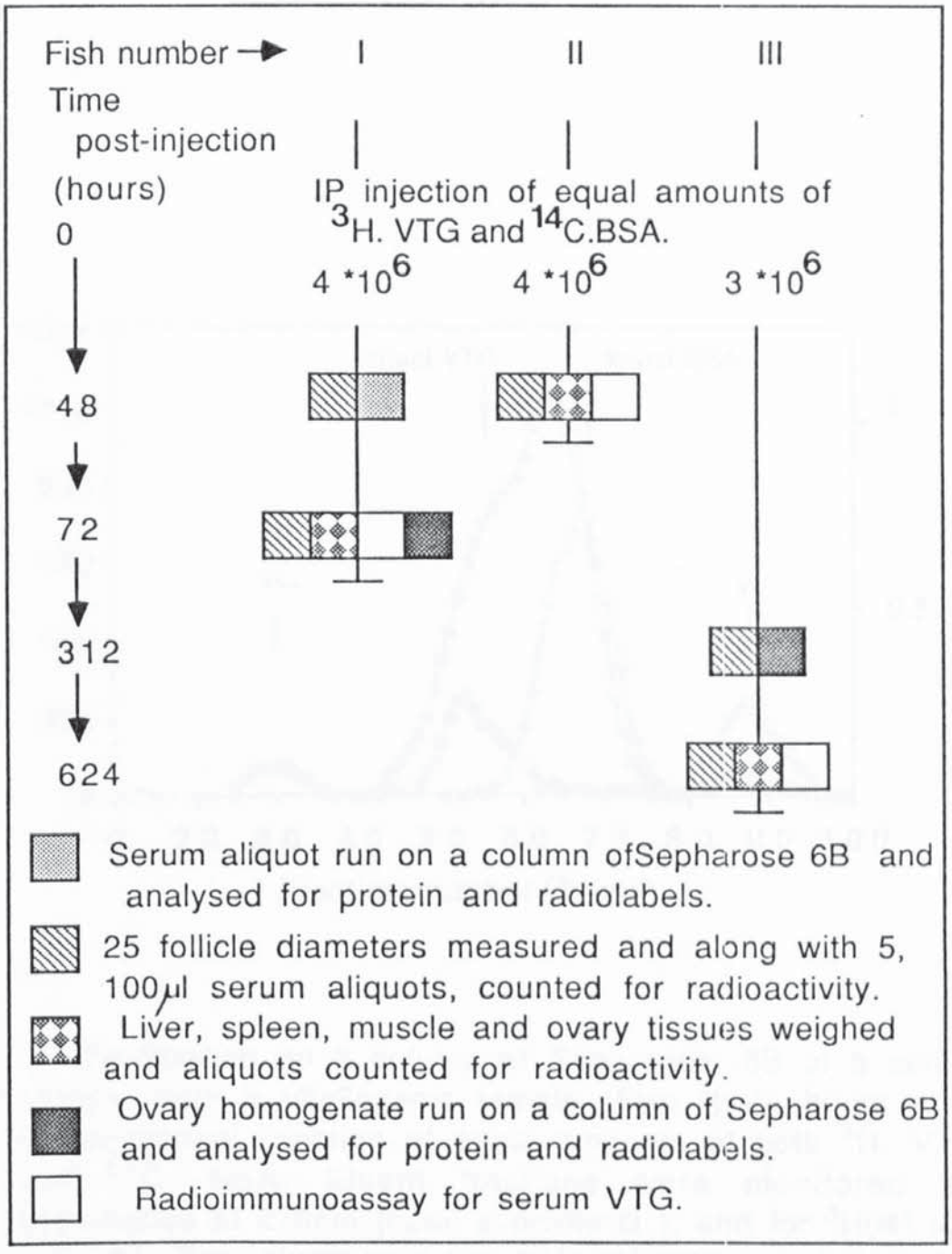


Figure 4.2. Outline for the protocol investigating the selectivity of protein sequestration by vitellogenic follicles.

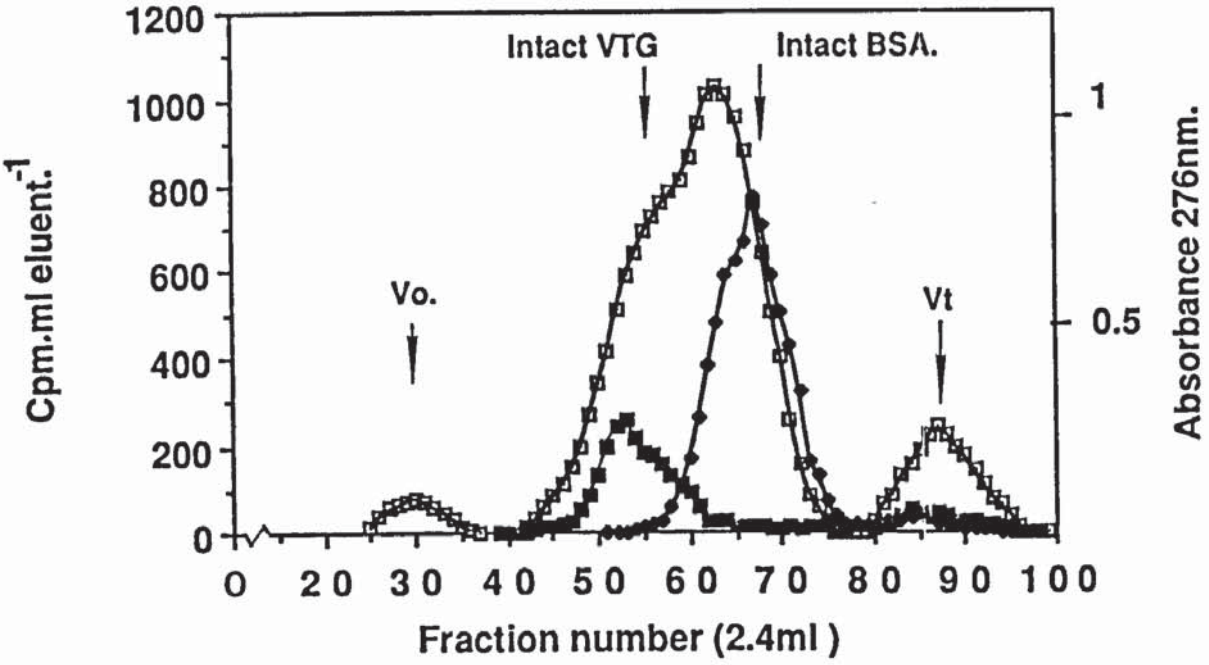


Figure 4.3. Gel-filtration on a column of Sepharose -6B of a serum sample from a vitellogenic female (Fish I) 48 hours after intraperitoneal injection of equal amounts of both ^{3}H. VTG and ^{14}C. BSA. Eluent fractions were monitored for absorbance at 276nm (open symbols: □), and for ^{3}H(■) and ^{14}C (◆). The column void and total volumes are represented by Vo and Vt, respectively.

Fish number	Counts per min. injected		Sample time (hour)	Counts per min. in 100µl serum		Uptake in ovary (% of injected)		Serum VTG ($mg.ml^{-1}$)	Follicle diameter (mm)	Rate of VTG uptake into follicle ($ng.mm^{-2}.hr^{-1}$)
	^{3}H-VTG	^{14}C-BSA		^{3}H	^{14}C	^{3}H	^{14}C			
I	$4*10^{6}$	$4*10^{6}$	t1=48	731 ∓19	2206∓12	32	1.9	6.5	2.75 ∓ 0.1	300
			t2=72	503 ∓23	1894 ∓27	49	2.5			
II	$4*10^{6}$	$4*10^{6}$	t1=48	1861∓29	2112 ∓31	2.3	0.6	1.8	1.0 ∓ 0.05	not determined
III	$4*10^{6}$	$4*10^{6}$	t1=312	318 ∓16	1096 ∓25	33	2.5	10.0	4.5 ∓ o.1	59
			t2=624	12∓5	498 ∓17	45	1.3			

Table 4.1. Selectivity of ovarian uptake of ^{3}H.VTG and ^{14}C.BSA. .
Fish were injected intraperitoneally with both ^{3}H.VTG and ^{14}C.BSA , and the passage of these labelled proteins into the blood and ovary assessed at varying times. Fish I and III were clearly vitellogenic, whereas Fish II was not as advanced reproductively, possessing follicles that had only recently entered the phase of exogenous vitellogenesis.

in the blood. The rate of VTG uptake in this fish was considerably slower than that observed in Fish I (59 and 300 ng.mm^2 follicle surface.$^{-1}$ hr.$^{-1}$ respectively, Table 4.1), which might explain, at least in part, why 49% of the ^{3}H-VTG was found in the follicles of Fish I after 3 days, whereas only 45% was similarly located after 26 days in Fish III.

Compared to ^{3}H-VTG, ^{14}C-BSA was not sequestered to any significant extent by the ovary in any of the fish (Table 4.1). In the vitellogenic fish, ^{3}H-VTG was taken up approximately 15 times as rapidly as ^{14}C-BSA. The data also provide some evidence that the ^{14}C-BSA sequestered by the follicles was subsequently exocytosed. In Fish III there were 40 ± 8 cpm ^{14}C per follicle after 13 days, but this had fallen to 20 ± 3 after 26 days, whereas the amount of ^{3}H-VTG in the follicles rose over this period (Table 4.1). This rise in level of ^{3}H-VTG, and fall in ^{14}C-BSA, meant that 26 days after injection there was 35 times as much ^{3}H-VTG as ^{14}C-BSA in the follicles.

Whereas the vitellogenic fish avidly and selectively sequestered ^{3}H-VTG, this was not observed in Fish II. The follicles in this fish had only recently begun to endocytose VTG, confirmed by a histological examination of the ovary which showed that the most advanced follicles had only reached an early yolk granule stage of development (Plate 4.1, see Chapter 1) and showed a much reduced and considerably less selective ^{3}H-VTG uptake (Table 4.1).

Although the ovary of vitellogenic fish sequestered ^{3}H-VTG avidly, no other tissue did so. Neither the liver, spleen, nor muscle contained appreciable amounts of ^{3}H-VTG after 26 days (Table 4.2). Expressing the results as cpm.g^{-1} tissue showed that after 26 days there were 13,000 cpm in the ovary but only approximately 350 cpm ^{3}H in liver, spleen or muscle. Furthermore, whereas the ovary selectively sequestered ^{3}H-VTG, in contrast there were approximately equal amounts of ^{3}H-VTG and ^{14}C-BSA in the other tissues (Table 4.2).

Ovary homogenates from Fish I, 72 hours after injection of the labelled proteins, and Fish III, 13 days after injection, were subjected to gel-filtration on Sepharose 6B (Figures 4.4 and 4.5, respectively). After 72 hours almost all of the ^{3}H eluted as a single peak coincident with lipovitellin. Rainbow trout phosvitin contains very little leucine (Campbell and Idler, 1980; own data Table 3.1) and consequently was not significantly labelled with ^{3}H-leucine. There was no evidence of labelled VTG, which elutes before lipovitellin (Chapter 3; Figure 4.4). There was only a small amount of ^{14}C present throughout the chromatogram, but, unlike the labelled VTG sequestered, this was present in its unprocessed, native state,

Plates 4.1. Sections of follicles from Fish II, showing that this female had only recently entered the phase of exogenous vitellogenesis. Preparations were stained with haematoxylin and eosin.

4.1a. Section of an ovary. (refer to Fig.1.2.)
Magnification × 60

St.2c.-stage 2c follicle- 'Balbiani bodies' at periphery of ooplasm. Oocyte surrounded by granulosa and thecal layers.

St.3.-stage 3 follicle- 'Balbiani bodies have dissapeared.

St.4.-stage 4 follicle- Follicles readily identified by the presence of yolk vesicles.

St.4a.- yolk vesicles appearing at the periphery of the ooplasm.

St.4b. - yolk vesicles fill ooplasm.

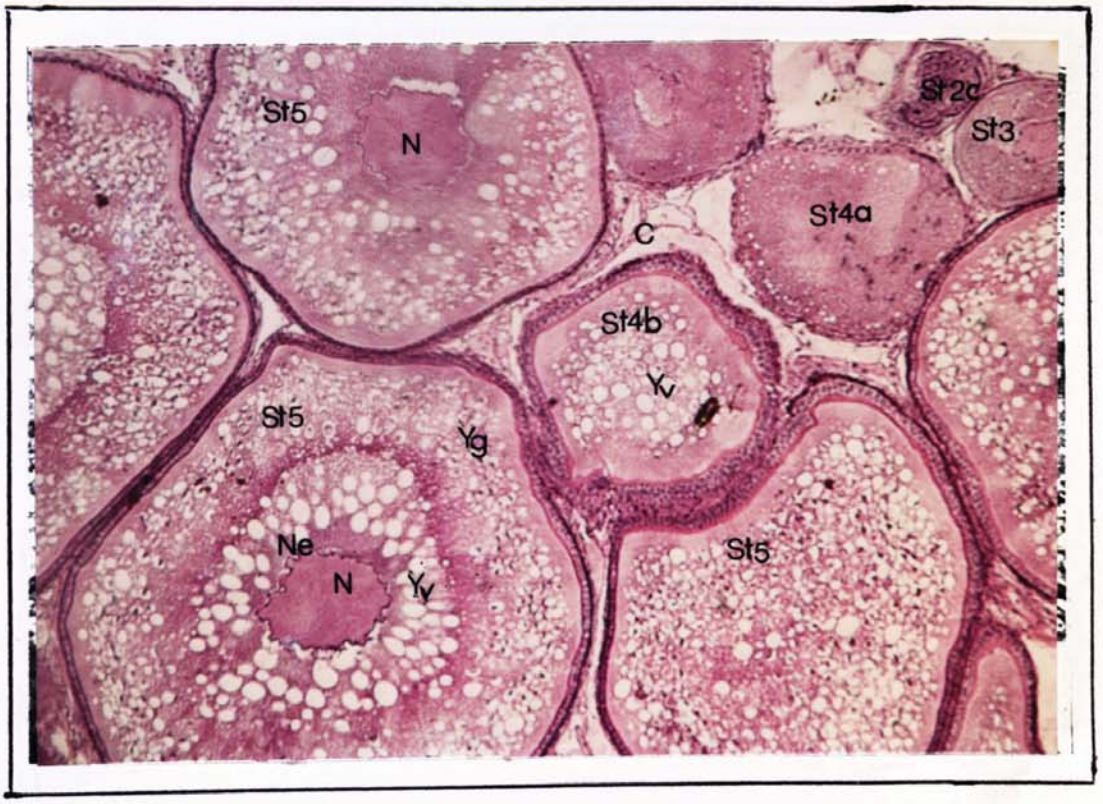
St.5. - stage 5 follicle- Easily distiguished from stage 4 follicles by the presence of exogenous yolk granules in the ooplasm adjacent to the microvilli of the zona radiata. Approximate diameter = 1000µm.

4.1b. Section of a follicle used for analyses.
Magnification × 140

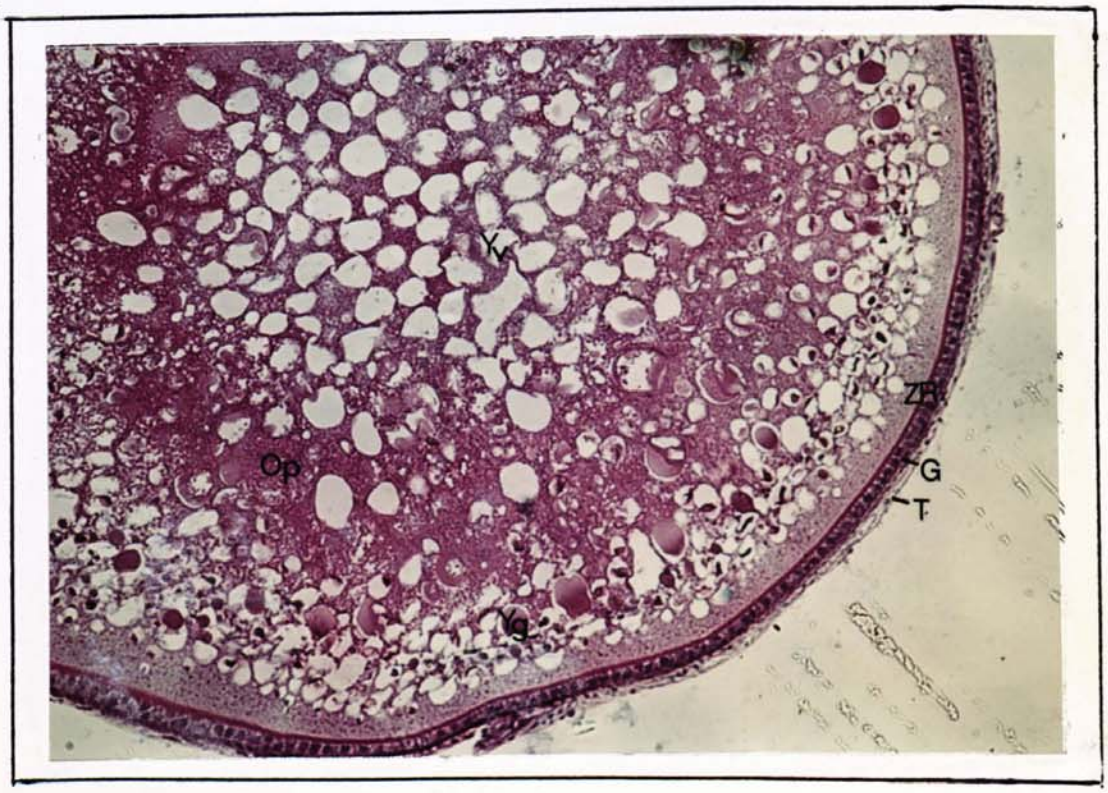
Only the largest follicles in the heterogenous group of developing follicles within the ovary of Fish II were used for analysing the uptake of radiolabelled proteins, mean diameter1.0∓ 0.05mm. The light micrograph shows the appearance of yolk granules at the periphery of the ooplasm indicating that the follicles analysed were just entering exogenous vitellogenesis (stage 5).

C-Connetive tissue, T-Theca, G-Granulosa, ZR-Zona radiata, N-Nucleus, Ne- Nuclear envelope, Op-Ooplasm, Yv-Yolk vesicle, Yg-Yolk granule.

4.1a



4.1b



Fish number	Time post-injection (hours)	Percentages of injected labels in varios tissues							
		Ovary		Liver		Spleen		Muscle	
		^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
I	72	49	2.5	3.8	1.1	0.1	0.1	10.4	11.0
III	624	45	1.3	0.2	0.2	0.01	0.01	4.3	4.9

Table 4.2. Distribution of ^{3}H.VTG and ^{14}C.BSA in the various tissues of vitellogenic females. Fish were injected intraperitoneally with both ^{3}H.VTG and ^{14}C.BSA simultaneously, killed at times indicated, and aliquots (n=5) of various tissues counted to assess the amounts of each isotope present. Results are expressed as percentages of the total amount injected.

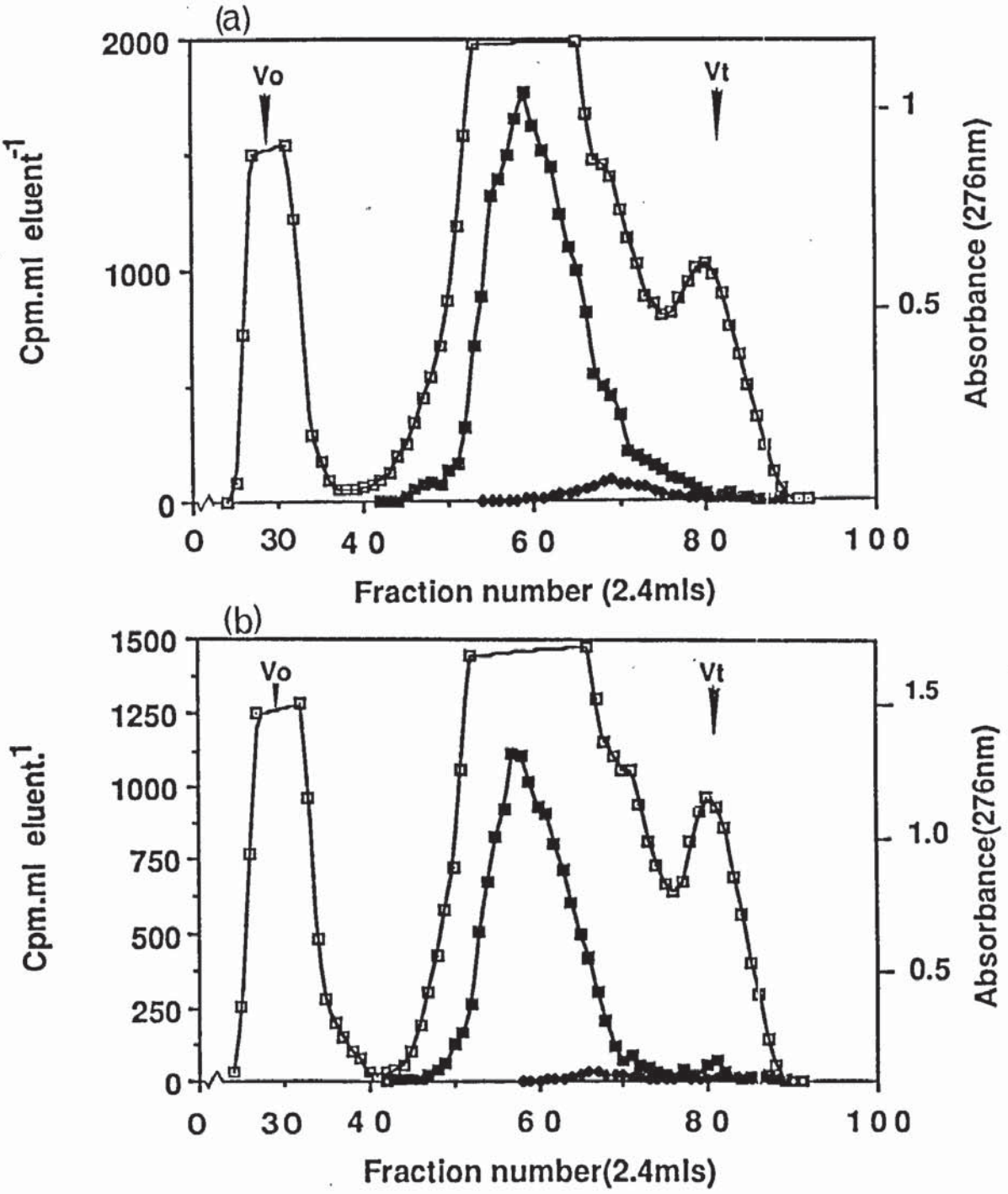


Figure 4.4. Gel-filtration on a column of Sepharose -6B of ovarian homogenates from vitellogenic females 72 hours (FishI, profile a) and 13 days (Fish III, profile b) after intraperitoneal injection of equal amounts of ^{3}H.VT and ^{14}C.BSA. Eluent fractions were monitored for absorbance at 276nm (open symbols:□) and for the radio-labels, ^{3}H (▮▮) and ^{14}C (◆). The columns void(Vo) and total(Vt) volumes are marked.

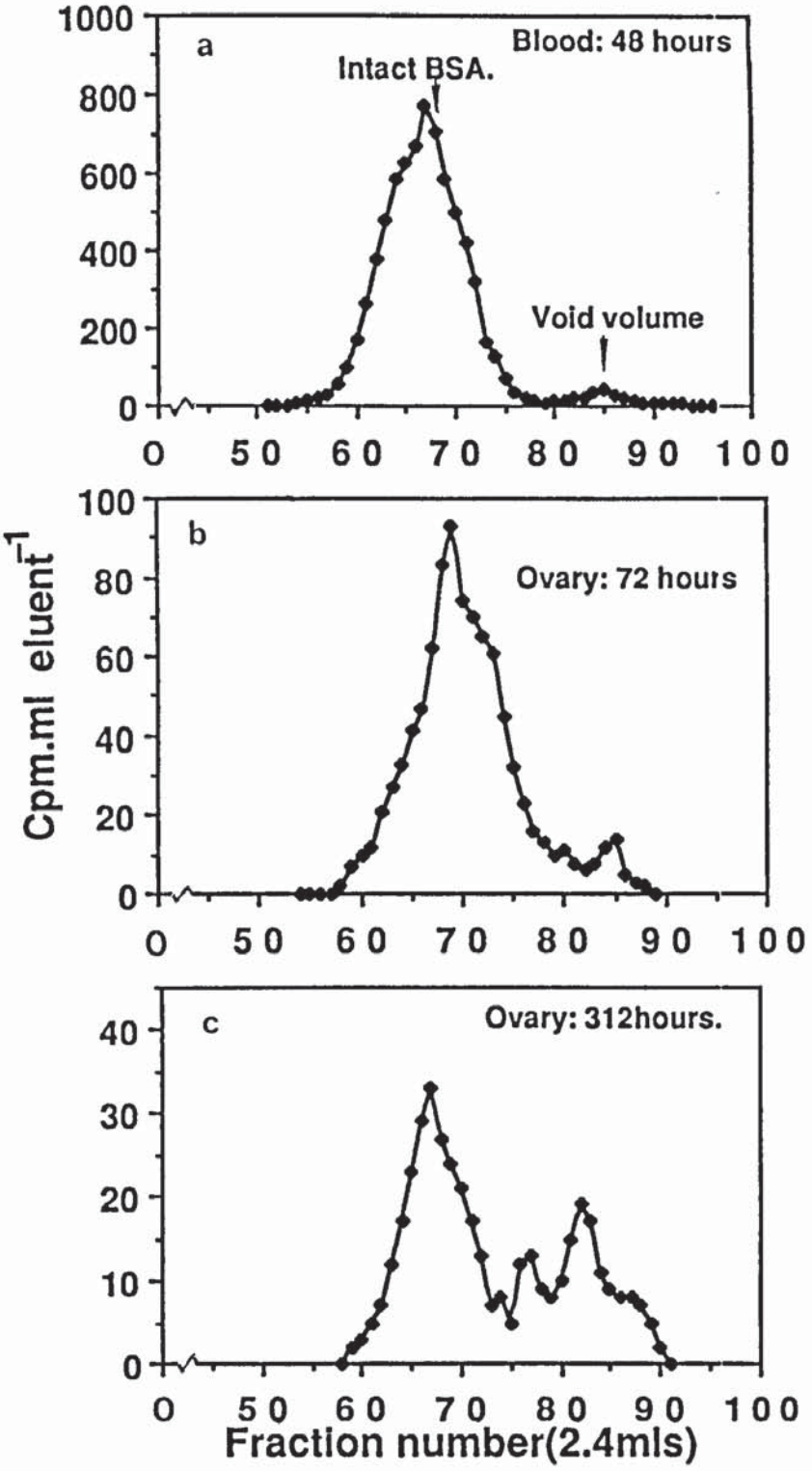


Figure 4.5. Gel-filtration on a column of Sepharose -6B of a serum aliquot and ovarian homogenates at different times after intraperitoneal injection of ^{3}H. VTG an ^{14}C. BSA. (a) Serum aliquot after 48 hours (Fish I).(b) Ovarian homogenate after 72 hours (Fish I). (c)Ovarian homogenate after 13 days (Fish III). Only part of the chromatograms are shown, and the results plotted on different scales to allow comparisons of the distribution of ^{14}C across the eluent fractions. The absorbancy and ^{3}H profiles to these chromatographs are shown in Figures 4.3 and 4.4.

eluting as a single, discrete peak at an identical position to serum ^{14}C-BSA (compare Figures 4.2 and 4.4).

The distribution of labelled proteins was examined again after 13 days to assess whether further processing had occurred (Figure 3). Nearly all of the ^{3}H eluted with lipovitellin, again showing that this yolk protein was stable within the follicle. However, the ^{14}C did not elute as a discrete peak, as it had done after 72 hours, but instead eluted in a more heterogeneous manner, suggesting that there had been some proteolysis of the labelled BSA. These results are difficult to visualize in Figure 4.4 and are redrawn in Figure 4.5 on an expanded scale. By calculating the areas under the graphs in Figure 4.5 it was possible to estimate the degree of degradation. Over 97% of the ^{14}C present in the blood was associated with intact BSA (Figure 4.5a). Seventy-two hours post-injection the vast majority (93%) of the ^{14}C in the ovary was associated still with intact BSA (Figure 4.5b), suggesting that it was taken up initially as intact BSA. After 13 days this figure had fallen to 59%, the remaining 41% eluting near or at the V_T of the column, suggesting that it was of smaller molecular weight than BSA (Figure 4.5c).

4.4 Discussion

The present results show clearly that vitellogenic follicles of the rainbow trout sequester VTG much more avidly than BSA. Although a selective sequestration of VTG has previously been demonstrated for the oocytes of the chicken (Cutting and Roth, 1973) and *Xenopus* (Wallace *et al.*, 1970; Wallace and Jared, 1976), it has not been clearly demonstrated in fish; in fact the limited literature is contradictory, some results suggesting that there is little, if any, selectivity of protein uptake (Campbell and Jalabert, 1979). It is difficult to reconcile our results with those of Campbell and colleagues obtained using cultured rainbow trout follicles. In one study Campbell (1978) reported selective uptake, with VTG being sequestered faster than other serum proteins and BSA, although interpretation of these results is complicated by the fact that the follicles may have been previtellogenic. In another study, in which the follicles used were definitely vitellogenic, there was little if any selectivity, and BSA was sequestered almost as fast as VTG (Campbell and Jalabert, 1979). These results are surprising when one considers that large rainbow trout oocytes are largely comprised of yolk derived from VTG (see Chapter 3) and it seems far more likely that uptake is selective, as shown in this study.

The differing amounts of ^{3}H and ^{14}C present in the ovaries 72 hours after injection of the labelled proteins showed that VTG left the circulation and became associated with the ovarian yolk proteins between 15 and 35 times faster than BSA. In fact, this estimate probably understates the degree of selectivity. This is because the plasma already contained appreciable amounts of "cold" VTG, which would have effectively diluted the ^{3}H-VTG injected, whereas obviously the blood would not have contained any BSA. Thus, although the two proteins injected were of approximately equal specific activity, they would not have circulated in the recipient fish at similar specific activities. For example, in the case of Fish I about 100 mg of labelled VTG was injected into a fish which already had about 240 mg "cold" VTG in the blood circulation. Assuming all of the injected ^{3}H-VTG reached the blood,it was diluted by a factor of 3.4, suggesting that our estimate of a 15 to 20-fold selectivity shown by Fish I ought to be nearer 60. Whatever the absolute value, our data indicate that VTG is selectively sequestered *in vivo* by vitellogenic trout follicles at rates at least as fast or possibly rather faster to those previously reported for *Xenopus in vitro* (Wallace*et al.*, 1970; Wallace and Jared, 1976).

Within 72 hours the ovaries of Fish I had incorporated approximately 50% of the injected ^{3}H-VTG, giving a maximal physiological half-life for blood VTG

of 3 days in this vitellogenic fish. This estimate of the half-life may be longer than it actually is, because both the percentage of labelled VTG passing from the peritoneal cavity into the blood and the time period during which this passage occurred are not known. However, a more reliable estimate of the half-life of VTG would require a study using many fish. Further they would need to all be at the same stage of follicle development, because the half-life may well change during the vitellogenic phase of the reproductive cycle. Again, this rapid sequestration of VTG is parallelled in *Xenopus*, where the half-life of VTG in the blood was shown to be less than 2 days at 20°C (Wallace and Jared, 1968). It should also be noted that the fish in the present experiment were maintained at a temperature of exactly half that in which the studies were performed on *Xenopus* follicles, further emphasising the similarities in sequestration rates of VTG between the two vertebrate groups.

Fish II, exhibited a lower degree of selectivity in protein sequestration. Presently, it is not known which developmental parameter is impairing the selective sequestration of VTG, but it may include lack of the necessary hormonal stimulus, the attainment of a full complement of VTG receptors, or some other feature dictated to or by the developing follicle. The VTG receptor is believed to be developmentally regulated in *Xenopus* and as detailed in the introduction pre-vitellogenic follicles appear incapable of incorporating VTG (Dumont, 1972). If teleost VTG receptors are similarly developmentally regulated it is likely that low levels of these receptors are impairing the selective sequestration of VTG in Fish II (see Chapter 6 for a further discussion).

Although the specificity for VTG uptake in vitellogenic fish was marked, it was not absolute and the ^{14}C-BSA present in the blood was also sequestered by the follicles. This observation is in agreement with the uptake of heterologous macromolecules into the growing oocytes of *Xenopus* (Wallace and Jared, 1976) and *Fundulus* (Selman and Wallace, 1983) and suggests that many, if not all, macromolecular components of the blood are taken up in varying amounts by the recrudescing ovary. It appears that the ^{14}C-BSA entered the trout in the current work in a similar non-selective manner to that reported for *Xenopus* (Opresko *et al.*, 1980) and probably occurred as a result of adventitious engulfment during VTG uptake.

After uptake into the vitellogenic ovary ^{3}H-VTG was specifically cleaved to yield ^{3}H-lipovitellin, which accumulated within the follicles and underwent no further processing over the 26 day period of study. Unlike the VTG incorporated by the ovary, the BSA underwent turnover within the follicles, and 13 days

post-injection approximately 40% of the ^{14}C present in the ovary eluted near or at the V_T, not in the position of intact BSA, indicating that some proteolysis of the sequestered ^{14}C-BSA had occurred. The stability of the sequestered VTG, as its yolk protein product lipovitellin, and the turnover of the heterologous BSA are both in accordance with the studies conducted in *Xenopus* (Opresko *et al.*, 1980) and suggests that a similar series of protein processing pathways exist in the rainbow trout as occur in the toad (see Figure 4.1).

Further study on the trout showed that the ^{3}H-lipovitellin formed a fairly insoluble matrix when treated with distilled water (data not shown) and as in *Xenopus* (Opresko *et al.*, 1980) by this agency may have reduced its susceptibility to degradation. On the other hand over 85% of the ^{14}C-BSA remained in the soluble phase and as such would have been readily susceptible to degradation.

Between days 13 and 26 there was a 50% decrease in ^{14}C content of the ovary, indicating that BSA and/or its degradation products are exocytosed from the follicle. This loss from the follicle of incorporated BSA was also observed in *Xenopus* by Opresko *et al.*, (1980) who ascribed the loss to secretion during the receptor mediated endocytosis of VTG. The turnover of BSA, and possibly other non-specific proteins incorporated into follicles, along with the selective uptake of VTG, explains why proteins other than VTG and its cleavage products account for only a small proportion of the follicle's reserves both in *Xenopus* and in the rainbow trout (as indicated by the yolk protein chromatograms in Chapter 3). In *Xenopus* 80-90% of the total protein content of full-grown follicles is found in the crystalline yolk platelets (Wallace, 1978) which contain almost exclusively the VTG-derived yolk proteins lipovitellin and phosvitin (Karasaki, 1963; Redshaw and Follett, 1971). Trout follicles appear to have adopted a similar mechanism for the selective accumulation of extra-ovarian precursors of yolk proteins to that already established for *Xenopus*. The data presented in Chapters 3 and 4 show clearly that VTG accounts for the major part of ovarian growth during vitellogenesis.

SECTION II: *IN VITRO* STUDIES

CHAPTER FIVE

THE DEVELOPMENT OF AN *IN VITRO* CULTURE SYSTEM FOR PROTEIN SEQUESTRATION STUDIES IN TROUT FOLLICLES

5.1 Introduction

Almost 20 years ago, a procedure was developed for growing amphibian follicles *in vitro*, which involved placing follicles dissected from their follicular layers into a nutrient medium containing VTG (Jared and Wallace, 1969; Wallace *et al.*, 1970; Wallace and Jared, 1976; Wallace and Misulovin, 1978; Wallace *et al.*, 1978, 1980b). Vitellogenic follicles obtained from gonadotropin-stimulated females were found to grow *in vitro* in the absence of added serum or gonadotropin at rates similar to or which exceeded those found for the most rapidly growing follicles in intact gonadotropin-stimulated females *in vivo*. The addition of serum increased the growth rate by about 30%. A much more important variable, however, proved to be the external VTG concentration with growth rates reduced to zero simply by lowering the concentration to less than 2 $mg.ml^{-1}$. The *in vitro* studies established that VTG sequestration in *Xenopus laevis* was both rapid, often exceeding 50 $ng.mm.^{-2}\ hr^{-1}$ (Wallace *et al.*, 1970; Wallace and Jared, 1976), and specific (Chapter 4). Vitellogenin was incorporated 25 times more rapidly than other serum proteins (Wallace *et al.*, 1980) and up to 50 times faster than a group of heterologous macromolecules (Wallace and Jared, 1976). *Xenopus* follicles cultured for periods of up to 4 weeks and more showed that follicles divested of the theca obtained from unstimulated females grew equally as well as similarly treated follicles from gonadotropin-stimulated females (Wallace and Misulovin, 1978; Wallace *et al.*, 1980b) suggesting that the follicular layers constrain the follicles growth. This hypothesis was confirmed when full-grown follicles divested of the theca resumed growth to remarkable levels (even doubling in volume in 3 weeks, Wallace, 1983) when placed in culture, showing that the maximum size reached by follicles in the ovary is not an intrinsic property of the follicle but is imposed extrinsically by the ovarian environment (Wallace *et al.*, 1981). Combined *in vivo* and *in vitro* studies on *Xenopus* show that the follicular layers control oocyte growth in a number of ways; firstly they produce oestrogens in response to circulating gonadotropins and subsequently these steroids induce the production of the yolk-protein precursor, VTG, secondly they direct VTG to reach the oocyte surface thus controlling the levels available for incorporation; and thirdly they alter steroid synthesis and produce progesterone to bring about maturation of the full-grown oocyte (Wallace, 1983). However, although gonadotropin levels have been shown to regulate the amount of steroid synthesis,

the manner in which the theca and granulosa cells respond to circulating gonadotropin(s) appears to be ultimately determined by the developing oocyte. Furthermore follicles divested of both the theca and granulosa (oocytes) growing *in vitro* canacquire the competence to resume meiosis and become an egg (Wallace, 1985). Extensive studies with cultured *Xenopus* follicles have further established the transport and processing pathways of incorporated proteins. Pulse-chase experiments with labelled proteins followed by fractionation and gel electrophoresis have provided the detailed model of VTG endocytosis described in Chapter 4 (Opresko *et al.*, 1980, 1981). Clearly the ability to culture amphibian follicles has enabled a number of parameters involved in VTG sequestration to be examined; this has rapidly advanced our knowledge relating to the cellular and dynamic aspects of follicle growth in oviparous vertebrates.

In vitro studies do not incur some of the problems of *in vivo* experiments, where the involvement of a particular factor may be either direct or indirect (being relayed by other physiological or endocrinological processes). This allows one to determine the actions of specific factors on a distinct cellular event. Furthermore tissues in culture are provided with a 'controlled' environment unlike *in vivo*, where the environment is dynamic. The success of an *in vitro* approach to studying vitellogenic growth of follicles seen in the studies on *Xenopus*, described above, has yet to be applied to other oviparous vertebrate groups.

Salmonid follicles would appear to be favourable candidates for *in vitro* studies relating to vitellogenic growth; firstly salmonid follicles are large (in the rainbow trout vitellogenic follicles measure between 1 and 5mm in diameter, depending on their stage of development, see Chapter 1) and therefore are easily handled and; secondly, the follicles develop synchronously, an enormous advantage for biochemical studies since a large number of follicles all at the same stage of development can be obtained. In the rainbow trout approximately 2000 eggs kg^{-1} body weight are produced each breeding season, and the provision of such a large number of homogenous follicles allowsmany features relating to follicle growth to be tested using the ovary of a single animal. This saves resources and also eliminates any 'inter-animal' variation that would otherwise be brought about using more than 1 fish.

Various follicle preparations have been cultured from a number of salmonids, including the amago salmon, brook trout and rainbow trout [reviewed by Nagahama, 1987). Generally these have been used to investigate ovarian steroidogenesis, the results providing detailed information on the actions of

gonadotropin on both 17ß-oestradiol and 17α, 20ß-dihydroxyprogesterone production by the follicular tissues. Indeed it is now well established that the ovarian thecal and granulosa layers co-operate for the production of these 2 steroid hormones. In both cases, the thecal layer is the site of precursor synthesis and the granulosa layer is the site of conversion of the precursors to the final products (Nagahama, 1987; see Chapter 7). Further studies on cultured follicles of *Fundulus heteroclitus* have investigated the process of hydration (Wallace and Selman, 1978; McPherson *et al.*, 1987) and shown that this phase of maturation is triggered hormonally by 17α, 20ß-dihydroxyprogesterone. *In vitro* studies on VTG uptake in fish however, have proved far less fruitful than either the steroidogenic studies or the vitellogenic growth studies in *Xenopus*. The few studies conducted on rainbow trout follicles have produced conflicting data. For example, work by Campbell and Jalabert (1979), although showing that BSA was unable to inhibit VTG uptake (as would be expected for a receptor mediated endocytotic process), suggested that there was little specificity for VTG sequestration, a result that is clearly at variance with their initial observations (Campbell, 1978; see Chapter 4), although the follicles in the first experiments of Campbell may have been pre-vitellogenic, and with our own *in vivo* data. Furthermore, as briefly mentioned in Chapter 4, their studies achieved poor rates of VTG uptake; VTG uptake was never more than 9 ng.mm.$^{-2}$ hr^{-1} and often much less. These rates are significantly below those established in *Xenopus in vitro* and the present *in vivo* studies in the rainbow trout (see Chapters 3 and 4) suggesting that the follicles were not functioning properly in culture.

The aim, therefore of the work described here was to develop a culture system for rainbow trout follicles that produced rates of both uptake and processing of VTG that parallelled our previous *in vivo* data. Validation of the culture system for use in the subsequent uptake studies (in experiments 5 and 6 and Chapters 6 and 7) was performed in the first 4 experiments: the first experiment examined the permeabilities of the outer follicular layers, the surface epithelium and theca, to VTG transport and investigated whether their removal(s) were pre-requisites for protein uptake studies *in vitro*; the second investigated the effects of labelling VTG *in vivo* (using ^{3}H.leucine) and *in vitro* (using ^{125}Iodine) on the rate of VTG uptake, the third determined the importance of serum supplements on VTG sequestration and follicle survival and the fourth monitored the yolk protein processing of ^{32}P.^{3}H-VTG in the cultured follicles and

compared it to that occurring *in vivo* (Chapters 3 and 4). The last 2 experiments (experiments 5 and 6) in this chapter used the validated culture system to investigate the importance of both VTG concentration and temperature on both VTG uptake and to determine their influences in the ovarian cycle of the intact animal.

The teleost ovarian follicle is a complex structure consisting of somatic cells and a single germ cell or oocyte (Chapter 1; Guraya, 1978). The somatic cells in the rainbow trout are arranged into distinct layers; an external epithelium (a single layer of squamous cells closely associated with the ovarian connective tissues), a layer of elongated cells constituting the theca and an underlying layer of cuboidal cells forming the granulosa. Beneath the granulosa there is an acellular layer, the zona radiata, part of which, immediately adjacent to the ooplasm, forms the vitelline envelope (basement membrane: Hurke and Peute, 1979; see Figure 1.2). The individual follicle layers undergo various changes during the development of the follicle to accommodate their known structural and physiological functions.The zona radiata, appearing before the onset of exogenous vitellogenesis, forms betweeen the vitelline envelope and granulosa and reaches a thickness of up to 16 µm during maturation (Hurke and Peute, 1979). As mentioned in Chapter 1 the zona radiata (chorion) is the outer-most layer in an ovulated egg; the remaining follicular tissues being retained within the spent ovary. It is believed that surrounding the granulosa layer is responsible for the production of the zona radiata with which it remains closely associated by adherent desmosomal junctions throughout the entire period of follicle growth. At the same time the zona radiata is intimately associated with the ooplasm, both physically and functionally. Radial striations (Forberg, 1982) through which cytoplasmic micro-villi from both the granulosa and the ooplasm penetrate, extend the entire width of the zona radiata and are involved in the exchange of metabolites, including VTG, between the follicular layers and the ooplasm (Flugal, 1967). At the time of ovulation these 'passageways' no longer mediate macromolecular transport and efficiently seal the ooplasm off from the surrounding environment. One major pathway of transport through the chorion remains, at least in the short-term, however; it is the micropyle, created by a specialized granulosa cell. This channel remains open to allow the sperm to enter and fertilize the ovulated egg.The granulosa originally forms around the oocyte as a simple layer of squamous cells which at the end of the pre-vitellogenic phase of development become cuboidal (Braekevelt and McMillan,1967 ; Kagawa *et al.*,

1981). In mature follicles it again becomes squamous due to the increase in size the oocyte (Kagawa *et al.*, 1981). In these mature follicles intercellular channels develop, although the cells still remain connected to each other by special protoplasmic attachments (Kagawa *et al.*, 1981). These spaces may be involved in the passage of VTG from the capillaries to the ooplasm (Abraham *et al.*, 1982; Selman and Wallace, 1982) and therefore have an important role in controlling the rate of VTG uptake. The granulosa cells are responsible for the production of some phospholipids. Although these lipids are transported to the oocyte (Hoar and Nagahama, 1978) their functional significance is unclear. The granulosa also exhibits a prominent steriodogenic activity in maturing females. The cells contain aromatases which are enzymes capable of producing 17ß-oestradiol from precursors supplied by special thecal cells. Nearer ovulation they cease 17ß-oestradiol production, and are involved in 17α 20ß dihydroxy-progesterone production (review Nagahama, 1987; see Chapter 7). Both these steroidal secretions can be induced in appropriate follicles *in vitro* by treatment with gonadotropins (Nagahama *et al.*, 1982; Nagahama, 1987).

The theca first appears at the end of the early perinucleolar stage of the first growth phase of the follicle (Forberg, 1982). On further development the theca may arbitrarily be divided into 2 layers, a theca interna and theca externa. These layers have a smooth muscle-like appearance, both containing fibroblasts and collagen fibres that confer strength to the growing follicles. The capillary plexus contained within the thecal tissues supplies the macromolecules for sequestration by the developing oocyte. The thecal cells are linked by tight junctions, suggesting that they may provide a barrier against VTG uptake, or at least inhibit to a degree, in culture conditions. The surface epithelium is closely connected to the ovary connective tissues and, often difficult to view using light microscopy (Wallace pers. comm.); it is penetrated by capillaries that terminate in the underlying theca.

Under *in vitro* conditions VTG contained within the culture medium does not have access to the capillary network that normally supplies nutrients to the developing follicle and therefore VTG has to penetrate the outer follicular epithelium and theca through an alternative route. In *Xenopus* studies have been conducted on VTG uptake into follicles both invested and divested of their follicular layers and the results indicated that *in vitro* the theca and overlying follicular tissues inhibit protein sequestration (Wallace *et al.*, 1973a, Wallace pers.

comm.). Similar studies on the rainbow trout, however, produced results that were less clear (Campbell and Jalabert, 1979) and the importance of the follicular layers to protein transport in cultured follicles remains to be established. Removal of the follicular layers, or 'denuding' as it is sometimes referred, may be conducted using chemical, enzymatic or dissection procedures, each of which has a number of advantages and disadvantages. Chemical treatment using EGTA or EDTA in association with vigorous shaking can remove the granulosa, but such treatments severely reduce VTG uptake in *Xenopus* (Wallace *et al.*, 1973a). EGTA and EDTA chelate calcium, which is known to play important roles in membrane structure and function (Wallace *et al.*, 1973). Also determining the importance of the gra nulosa cells in VTG uptake *in vitro* is further complicated by the fact that once divested of the theca the granulosa cells tend to 'slough' off to a variable extent during the incubations (Wallace *et al.*, 1973a). The granulosa is very difficult to remove by manual techniques. Results from studies on *Xenopus* (Wallace *et al.*, 1973) and the rainbow trout (Campbell and Jalabert, 1979; own observations) however, clearly showed that the granulosa cells do not inhibit VTG uptake and the physiological importance of this follicular layer received no further study.

In the removal of the outer follicular layers (the theca and surface epithelium), a variety of proteolytic enzymes, particularly trypsin have frequently been used to dissociate cells. In some of their experiments on the rainbow trout, Campbell and Jalabert (1979) used the collagenase, pronase, to facilitate the dissociation of adhering follicular cells. However, although such enzymatic treatments effectively remove follicular layers they significantly reduce protein sequestration activity (Wallace *et al.*, 1973a); these enzymes in some manner interfere with the integrity of the oocyte membrane(s), possibly damaging the receptors for VTG.

The alternative approach to removal of the outer ovarian follicular layers, down to and including the theca, is by dissection using watchmaker forceps. Nagahama *et al.*, (1982a and b), demonstrated that the large size of salmonid follicles facilitated this approach and demonstrated that follicles so treated maintained their normal physiological integrity, at least with regards to their steroidogenic activities. Indeed their *in vitro* studies elucidated the relative contributions of the theca and granulosa in the production of 17ß-oestradiol and 17α 20ß dihydroxyprogesterone in both the amago salmon

and the rainbow trout (see Chapter 7).

In experiment 1, due to the possible problems created by chemical and enzymatic treatments in the removal of follicular layers the permeabilities of the surface epithelium and theca to VTG transport were determined by removing these follicular layers by dissection.

Preliminary investigations and the results from all subsequent cultures showed that a low level of the total available VTG became incorporated by each follicle (usually below 0.02%). This low level of protein sequestration, in association with the relatively low specific activity of the ^{3}H-VTG, meant that high titres of labelled protein were required in each culture to obtain sufficient levels of uptake for accurate quantification. Furthermore the *in vivo* production of ^{3}H-VTG was both time consuming and expensive.

Vitellogenin labelled *in vitro* using ^{125}Iodine, however (routinely prepared by J Sumpter as a tracer for the VTG radioimmunoassay), provides, at least in theory, a suitable alternative to ^{3}H-VTG for use in cultures. ^{125}I-VTG is relatively inexpensive and considerably less time consuming to produce than ^{3}H-VTG. The VTG can be purified and characterized prior to its labelling and, perhaps most importantly, it can be produced with a very high specific activity (averaging 100 $\mu Ci.\mu g^{-1}$VTG). However, there are potential problems in studying VTG uptake using ^{125}I-VTG. Firstly the production of the labelled protein involves the use of an oxidizing agent, Iodogen, and this can affect the structural integrity of the VTG molecule such that sequestration is impaired. Ferenz and co-workers (1978, 1981) showed that VTG uptake rates *in vitro* in locust follicles were 8 times slower than those occurring *in vivo* using ^{125}I-VTG but only 3 times slower using ^{3}H-VTG; that is the uptake of ^{3}H-VTG was over twice as fast as the uptake of ^{125}I-VTG. These authors suggested that the binding sites for the VTG receptors were partially blocked by the iodine, therefore inhibiting selective incorporation. This is unlikely, however, for a protein with a molecular weight of 440 000 Daltons and specific activity of around 100 $\mu Ci.\mu g^{-1}$ would have only 5 to 10 iodides per molecule, i.e. an extra mass of approximately 280-560 Daltons, which is unlikely to have altered its steric configuration substantially. It is more likely that the VTG was damaged by the iodination procedure with iodogen and it is this damage which will have reduced its binding capacity. The second potential problem in using ^{125}I-VTG for uptake studies lies in the stability of ^{125}I-VTG. The stability of ^{125}I-VTG during the

culture periods of up to 24 hours at 18°C was unknown and should the iodine prove labile it would clearly complicate the VTG uptake studies.

Experiment 2 set out to determine the suitability of ^{125}I-VTG in uptake studies *in vitro*. The structural integrity of the ^{125}I-VTG was assessed by comparing its rate of sequestration to that using ^{3}H-VTG. Wallace *et al.* (1976) in a series of experiments on the incorporation of BSA into *Xenopus* follicles, analysed the resulting data in accordance to the classic concepts developed for enzyme catalysed reactions, assigning the protein in culture as "substrate" and the incorporated protein as "product"; they compared the V_{max} (the maximum velocity of the reaction measured in units of quantity of substrate transformed (sequestered/unit time) and Km values (the value of the substrate concentration(s) at which the rate of the reaction equals 1/2 V_{max}) between the different treatments. Both variables were determined from a linear Lineweaver-Burk (1974) plot (1/rate of VTG uptake (ordinate) versus 1/substrate concentration (abscissa); the intersections of the ordinate and abscissa representing $1/V_{max}$ and 1/Km respectively. A similar approach was adopted in this study to compare the rates of uptake of ^{125}I-VTG and ^{3}H-VTG. The criteria which allow this treatment are: (1) under these *in vitro* conditions only a small fraction of the protein in the medium is incorporated into the follicles (subsequent results of all *in vitro* cultures), (2) the amount of protein bound to the cell surface at any given time is low and probably considerably less than 10% of the total incorporation measured (see results in this chapter), (3) the uptake of VTG is saturable, temperature dependent (see succeeding experiments 4 and 5) and essentially linear with time during the incubation period and (4) as established *in vivo* (Chapters 3 and 4) VTG incorporated into the follicle is processed into a stable form which is neither lost from the follicle nor broken down and re-incorporated into other proteins. It should be stressed that this type of approach to uptake analysis, although convenient, probably represents a simplistic approach to an undoubtedly much more complicated process.

The stabilities of ^{125}I- VTG and ^{3}H.VTG were assessed at the end of the culture period by running aliquots of the culture media through a column of Sepharose 6B, which separated labelled, intact protein from any 'free' iodine or leucine and thus allowed an assessment of the degree of breakdown of the ^{125}I-VTG and ^{3}H.VTG to be made.

Serum is frequently used when various mammalian and non-mammalian cell lines are cultured. However, its precise role is still not fully understood. Serum components are required by particular cells for both growth and survival. Although preliminary investigations on cultured trout follicles showed that VTG was sequestered without a serum supplement experiment 3 investigated whether a supplement could enhance VTG uptake and/or increase the survival of cultured follicles.

In preliminary cultures the viability of follicles appeared to fall as the culture period was extended (data not shown). Later culture studies in Chapter 7, however, required longer incubation periods than the short-term sequestration studies in this chapter. In order to better assess the viability of follicles in culture it was decided in experiment 4 to examine the rate of processing of internalized labelled VTG, and to compare this rate with that observed *in vivo* (Chapters 3 and 4); a similar rate would suggest that the cultured follicles were fully competent, whereas a significantly reduced rate would suggest that the culture conditions were not optimal.

During the sexual development of female trout serum levels of VTG may increase a million fold (Sumpter *et al.*, 1984). Serum VTG is found in $ng.ml^{-1}$ levels in immature animals, but rapidly increases at the onset of exogenous vitellogenesis, to reach concentrations of 50 $mg.ml^{-1}$ or more at ovulation (Scott and Sumpter, 1983; Elliott *et al.*, 1984). The changes in VTG levels parallel the growth rate of the developing ovary, where as previously mentioned the gonadosomatic index (GSI) increases from less than 1% up to 20% or more in a few months prior to spawning. It therefore appears that not only a selectivity for VTG incorporation is required to account for this inordinate growth of the ovaries, but also a high blood titre of VTG needs to be maintained. Experiment 3 investigated the effect of VTG concentration on the rate of incorporation and saturation kinetics of the process.

One of the major factors influencing reproductive development in many species of fish is temperature (Kime, 1982). The effects of temperature on steroidogenesis in reproductive organs are well documented; the action may be either indirect, acting via the hypothalamic thermoreceptors which induce the release of pituitary gonadotropins to stimulate gonadal steroid production, or direct, acting on steroidogenic enzymes to give maximal stimulation of steroid production at temperatures which are environmentally the most suitable for

gonadal development (Kime, 1979). The involvement of temperature in other enzymatic and transport pathways involved in gonadal development of teleosts, including VTG uptake has, however, received little attention, which is surprising in view of the potential effects in poikilotherms.

Populations of rainbow trout, either living naturally, or maintained on farms, can experience fluctuating water temperatures during the vitellogenic growth phase of the ovary (which usually occurs in the autumn and winter in the UK), for example, water temperatures can reach 19°C in late summer/early autumn and 1-2°C in winter. If the rate of VTG sequestration shows a strong temperature dependence then the rate of ovarian growth will be affected and possibly also the time of spawning. The final experiment in this chapter investigates the effects of temperatures between 0-25°C (those within the normal physiological range experienced by rainbow trout) on VTG uptake.

5.2 Experiment 1: Vitellogenin sequestration in intact and partially denuded cultured follicles

5.2.1 Materials and methods

Exogenously vitellogenic follicles were dissected from 3 females and prepared for incubation after one of the following manipulations:

(a) The follicles were left intact or
(b) divested of their surface epithelium and associated connective tissue or
(c) divested of their surface epithelium and theca.

The manipulations described above are more clearly illustrated in Figure 5.1. The dissections were performed under a microscope using watchmaker forceps. The completeness of the various procedures were verified histologically (see Chapter 2, section 2.10). The removal of the theca during 'denuding' proved difficult and follicles were often burst. To avoid exposing successfully prepared follicles to the lytic enzymes released on puncture of other follicles during unsuccessful attempts each follicle was handled in a separate vial. All follicle preparations were incubated for 1 hour before the experiments began and only those which maintained the appearance of freshly dissected follicles were used for the subsequent experiments (see Chapter 2, section 2.10.2). For each treatment groups of 20 follicles were incubated for 8 hours with ^{3}H-VTG at concentrations of 3, 4.5 and 19 mg.ml^{-1} for Fish 1, 2 and 3, respectively. Follicle diameters in Fish 1, 2 and 3 were 3.1, 4 and 3.5 mm, respectively. In parallel to the incubations of follicles from Fish 1, a group of 20 ovulated eggs, obtained from a further fish which had undergone ovulation were cultured as controls.

The percentage of 'viable' follicles under each treatment were recorded before 10 of these were prepared and subsequently counted for radioactivity. Intact follicles, after completion of the incubations, were divested of their 'wispy' connective tissue to remove any associated ^{3}H-VTG before they were counted for radioactivity.

Finally the distribution of the radio-labelled VTG within the cultured follicles divested of their surface epithelium was assessed by rupturing the follicle and counting the yolk contents separately from the remaining follicular tissues. This was performed to determine the proportion of measured radioactivity that had become incorporated into the yolk and therefore that which

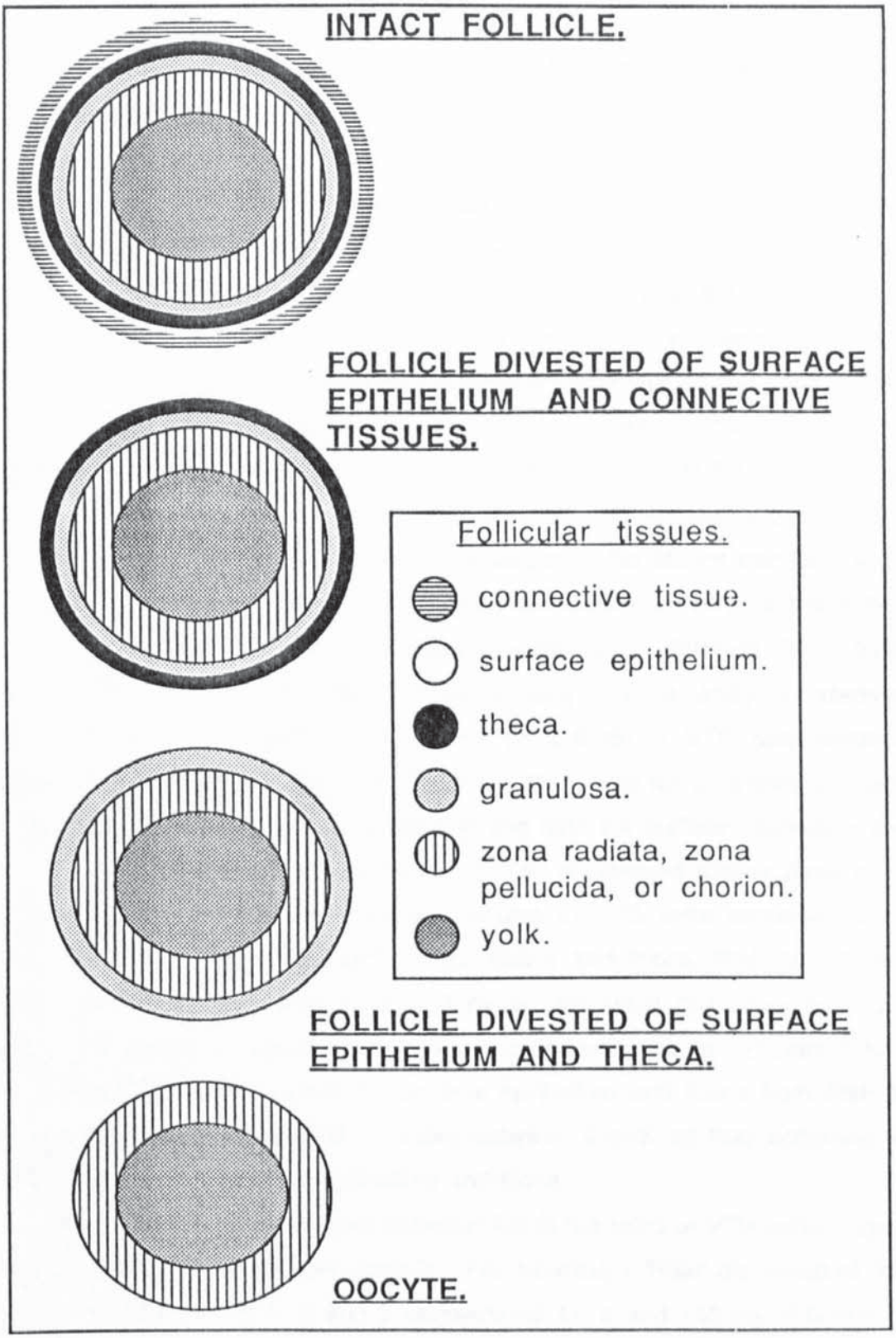


Figure 5.1. Diagramatic representation of the stepwise denuding of vitellogenic follicles.

was, either specifically or non-specifically, associated with the follicular tissues. This study was carried out on 20 follicles undergoing exogeneous vitellogenesis from Fish 1, again incubated with ^{3}H-VTG at a concentration of 3 mg.ml^{-1} for 8 hours.

5.2.2 Results

Plates 5.2a-c show sections of intact and partially denuded follicles. The plates illustrate that the manual dissections were successful: intact follicles were indeed intact, possessing all the follicular tissues; those divested of the surface epithelium retained the zona radiata, granulosa and theca; the follicles divested of both the surface epithelium and theca retained only the granulosa and zona radiata.

The rates of VTG uptake in the different types of follicles and their survival rates over the incubation period are presented in Table 5.1. In each fish the removal of follicular layers significantly affected VTG sequestration (ANOVA; $p < 0.05$ in each fish). Using a Multiple Comparison of the Means test (with equal numbers of observations, n=10; see Chapter 2, section 2.11.3) all the follicle groups in both Fish 1 and 2 sequestered significantly different ($P < 0.05$) amounts of VTG from one another. In Fish 3, using a similar analysis, however, although there was a significant difference ($P < 0.05$) in VTG sequestration between intact follicles and the other 2 groups, there was no difference between follicles dissected of their surface epithelium and both the surface epithelium and theca. The 3 follicle preparations from each fish all showed similar patterns in their rates of VTG uptake; the greatest amounts of VTG were sequestered by follicles divested of both their surface epithelium and theca, then by follicles divested of their surface epithelium and by far the least quantities by intact follicles. The maximum rate of VTG uptake recorded was 150 ng VTG.mm.$^{-2}$ hr^{-1} in the follicles dissected of both the surface epithelium and theca from Fish 3. Intact follicles incorporated VTG at rates between 0-25% of that occurring in follicles divested of the surface epithelium and theca.

There were clear differences between fish in the rates of VTG uptake even though the follicles were treated similarly. For example follicles dissected of the surface epithelium in Fish 1, 2 and 3 sequestered 11, 8 and 143 ng VTG.mm.$^{-2}$ hr^{-1} respectively.

Analysis of the distribution of ^{3}H-VTG within the follicles divested of their surface epithelium from Fish 1 showed that 85 ± 5% was present in the yolk and 15% ± 4.9 associated with the remaining follicular tissues (theca,

Plates 5.1. Sections of intact and partially denuded vitellogenic follicles. Stained with haematoxylin and eosin.

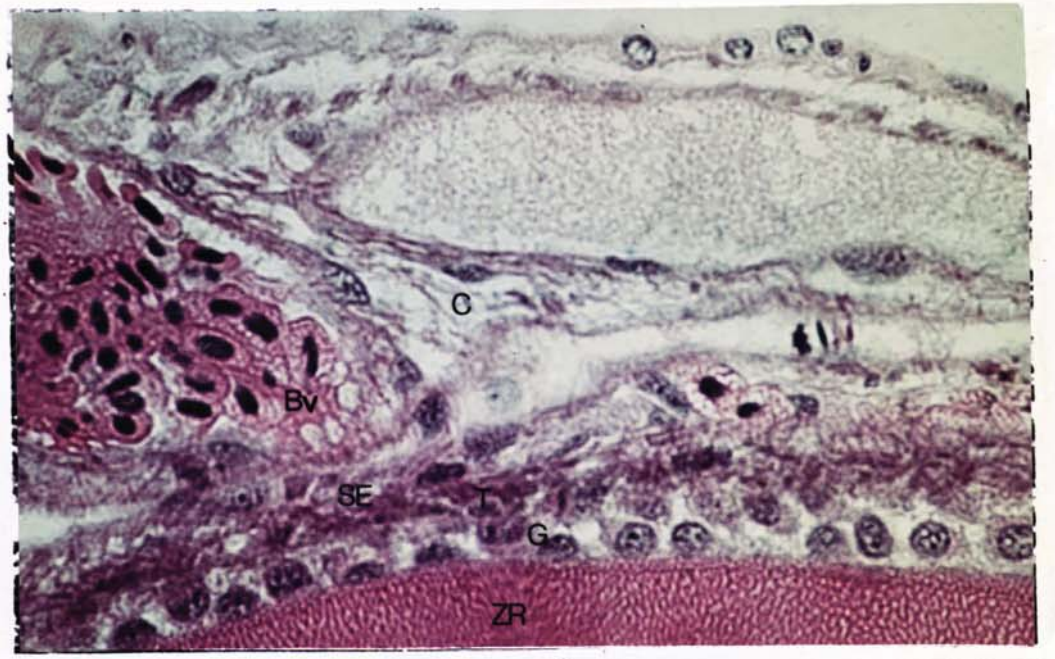
(a) Intact follicle- possessing all the follicular tissues. Magnification×200

(b) Follicles divested of the outer surface epithelium and associated connective tissues but retaining the zona radiata, granulosa and theca. Magnification× 200

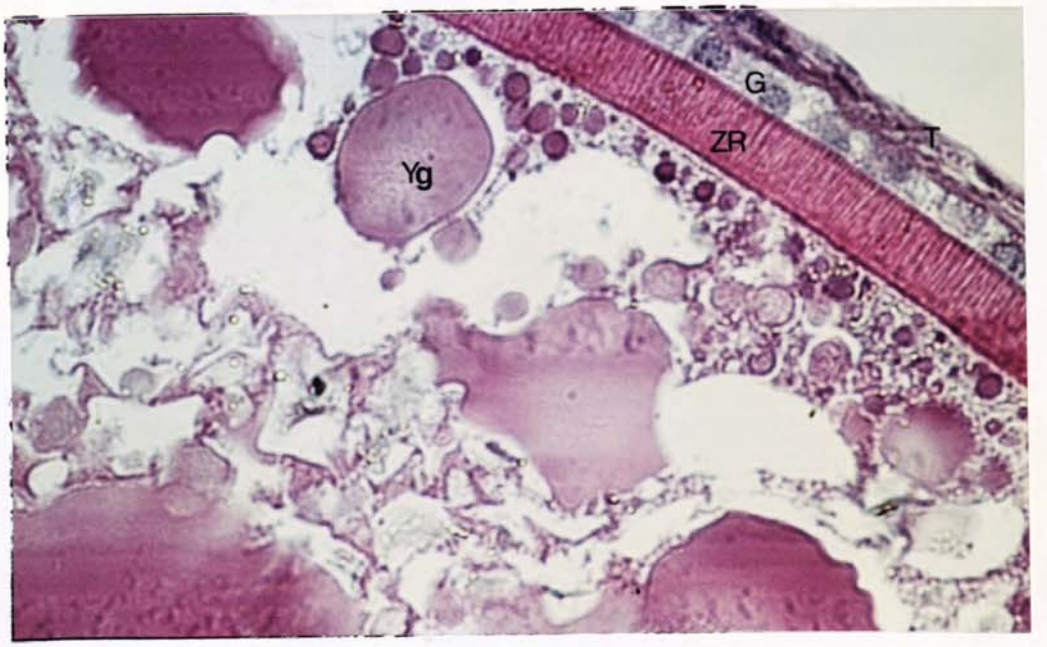
(c) Follicles denuded of the surface epithelium and thecal layers, retaining only the granulosa and zona radiata.Magnification×200

C - Connective tissue, SE.- Surface epithelium
T - Theca, G - Granulosa, ZR. - Zona radiata.
Bv- Blood vessel Yg - Yolk granule

5.1a



5.1b



5.1c

Fish	Vitellogenin uptake ($ng.mm^{2}$ follicle $surface^{-1}.hr^{-1}$)			
	Follicle treatment.			
	Intact	-surface epithelium	- surface epithelium and theca	ovulated
1	4.94 ∓ 2.3 (90)	11.19 ∓ 2.6 (70)	19.25 ∓ 4.7 (50)	0.27 ∓ 0.03 (100)
2	2 ∓ 1.2 (100)	8.28 ∓ 2.5 (75)	16.24 ∓ 3.5 (50)	
3	19 ∓ 6.4 (95)	143 ∓ 19 (60)	154 ∓ 24 (55)	

Table 5.1. Comparative rates of vitellogenin uptake into intact and partially denuded vitellogenic follicles. Mean uptake rates are given ∓ 1 standard deviation (n=10) Numbers in parentheses represent survival percentages from each group of 20 cultured follicles.

granulosa, zona radiata and vitelline membrane).

The survival rates amongst the pre-ovulated follicles a fter 8 hours in culture were highest in the intact follicles in each of the 3 fish (90% and above) and the lowest in the groups denuded of both the surface epithelium and thecal layers (between 50-55%).

The very low level of counts associated with the ovulated eggs (Table 5.1) was subsequently found to be entirely contained within the remaining zona radiata and not within the yolk, showing that there had been no VTG sequestration. It is probable that the counts in the ovulated eggs resulted from ^{3}H-VTG non-specific absorption to the chorion.

5.3 Experiment 2: The comparative uptake rates of vitellogenin labelled either with ^{3}H leucine or ^{125}iodine

5.3.1 Materials and methods

5.3.1.1 Vitellogenin labels

^{125}I-VTG was produced with a specific activity of approximately 80 μ Ci μg^{-1} protein. ^{3}H-VTG was produced *in vivo* with a specific activity of 100 cpm.μg^{-1} VTG.

5.3.1.2 Follicle incubations

Follicles of 3 ± 0.15mm in diameter which were undergoing exogenous vitellogenesis were divested of the outer surface epithelium and cultured for 6 hours with ^{125}I-VTG or ^{3}H-VTG at a range of VTG concentrations. ^{3}H-VTG concentrations ranged from 2-10 mg.ml^{-1}. Follicles cultured with ^{125}I-VTG were made up to concentrations of 0.89 to 25 mg.ml^{-1} using 'cold' purified VTG.

5.3.1.3 Calculations

The rates of VTG uptake using ^{125}I-VTG and ^{3}H-VTG as labels were plotted on Lineweaver-Burk graphs and the Km and V_{max} values calculated.

5.3.1.4 Chromatography

Aliquots of the culture media containing ^{125}I-VTG and ^{3}H-VTG were run on a column of Sepharose 6B at the end of the follicle culture period (6 hours) and the eluents analysed for protein and radioactivity. Similar analyses were

performed on the culture media held at 18°C for a further 18 hours, i.e. 24 hours after the incubation had been started. The latter analyses were carried out to determine the stabilities of the labelled VTG's over longer incubation periods, time periods for example that would be required to determine hormonal effects on VTG uptake investigated in Chapter 7.

5.3.2 Results

The double reciprocal (Lineweaver-Burk) plots (Figures 5.2a and b) of 1/VTG uptake against 1/substrate concentration produced regression coefficients of r = 0.99 and 0.94 for ^{125}I-VTG and ^{3}H-VTG, respectively. The intercepts on the ordinate for iodinated and tritated VTG yielded V_{max} values of 49.0 ng.mm^{-2} hr^{-1} and 49.5 ng.mm^{-2} hr^{-1}, respectively. Extrapolating the line plots to the negative section of the abscissa produced Km's of 4.4mg(5 x 10^{-7}M) for ^{3}H-VTG and 3·28mg(6.8 x 10^{7}M) for ^{1} I-VTG.

The Sepharose 6B chromatograms (Figures 5.3a and b) show that 6 and 24 hours after the cultures were set up ^{3}H-VTG eluted with an approximate molecular weight of 440 000 Daltons and all the tritium was associated with the protein absorbancy peak. The chromatograms for culture media containing ^{125}I-VTG (Figures 5.4 a and b) showed that the 'cold' VTG eluted with an identical molecular weight to ^{3}H-VTG (only ng amounts of iodinated VTG were added to each culture and such amounts would not register at the detection sensitivity adopted). The elution pattern of the iodine label provided a different picture to the tritium. After 6 hours (Figure 5.4a) the majority of ^{125}I eluted with the 'cold' VTG peak. However, 10% of the remainder eluted at V_T and this value increased to 35% after 24 hours (Figure 5.4b).

5.4 Experiment 3: The effect of serum on vitellogenin uptake into, and survival of, cultured follicles

5.4.1 Material and methods

Sera vary from animal to animal and in particular with the age, sex and nutritional state of the donor. In this study a group of 3 immature female trout were blood sampled and the resulting serum pooled before use in the ensuing cultures.

Twenty follicles (mean diameter 3.5 ± 0.15mm) were cultured with serum concentrations of 0, 10, 20, 30, 40 and 50%. The follicles were

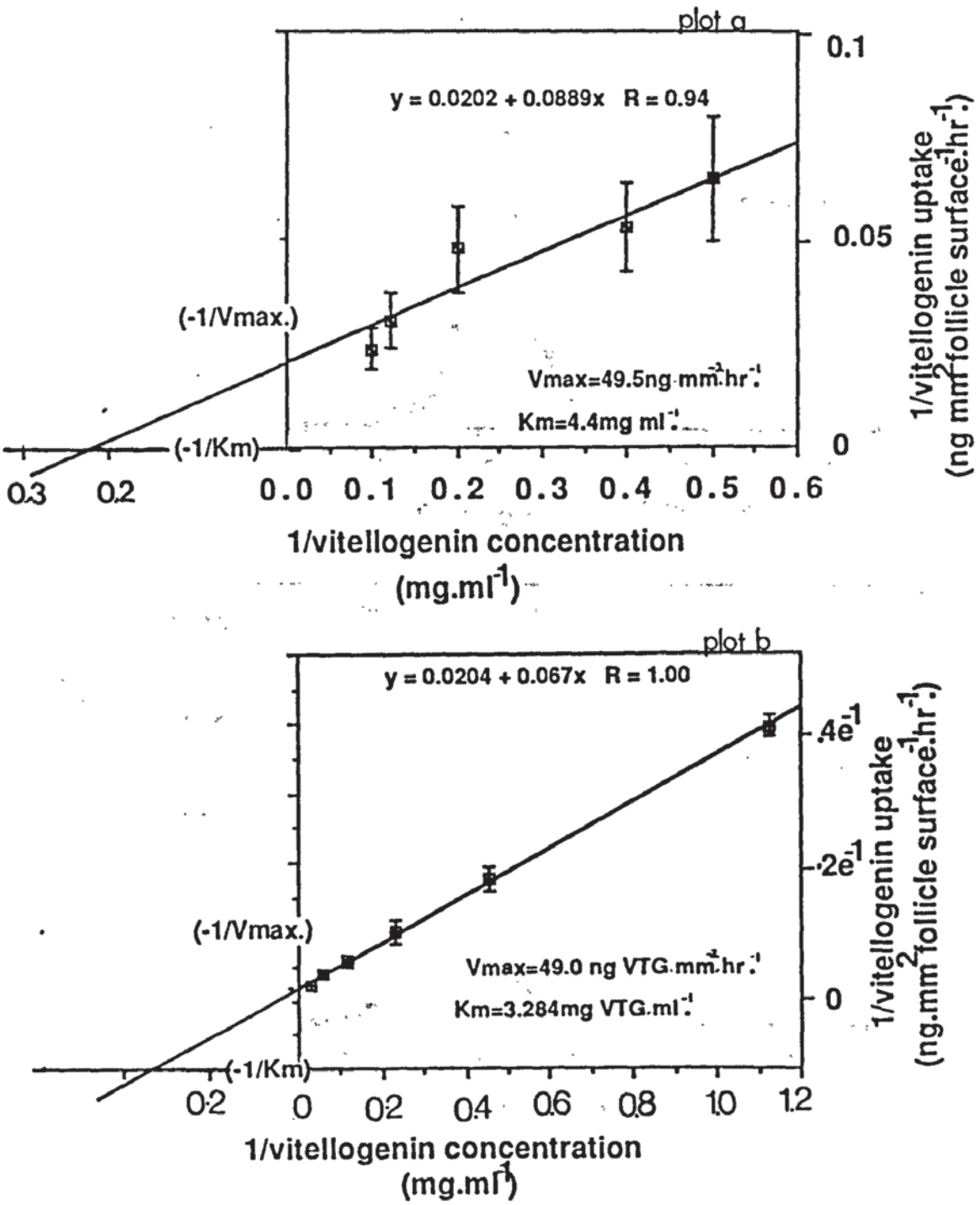


Figure 5.2. Lineweaver-Burk plots of vitellogenin uptake versus substrate concentration using ^{3}H-VTG (plot a) and ^{125}I-VTG (plot b). The regression line interceptions on the ordinate and abscissa represent 1/Vmax and 1/Km, respectively. Each point represents the mean uptake from 10 follicles. Vertical bars denote standard deviations.

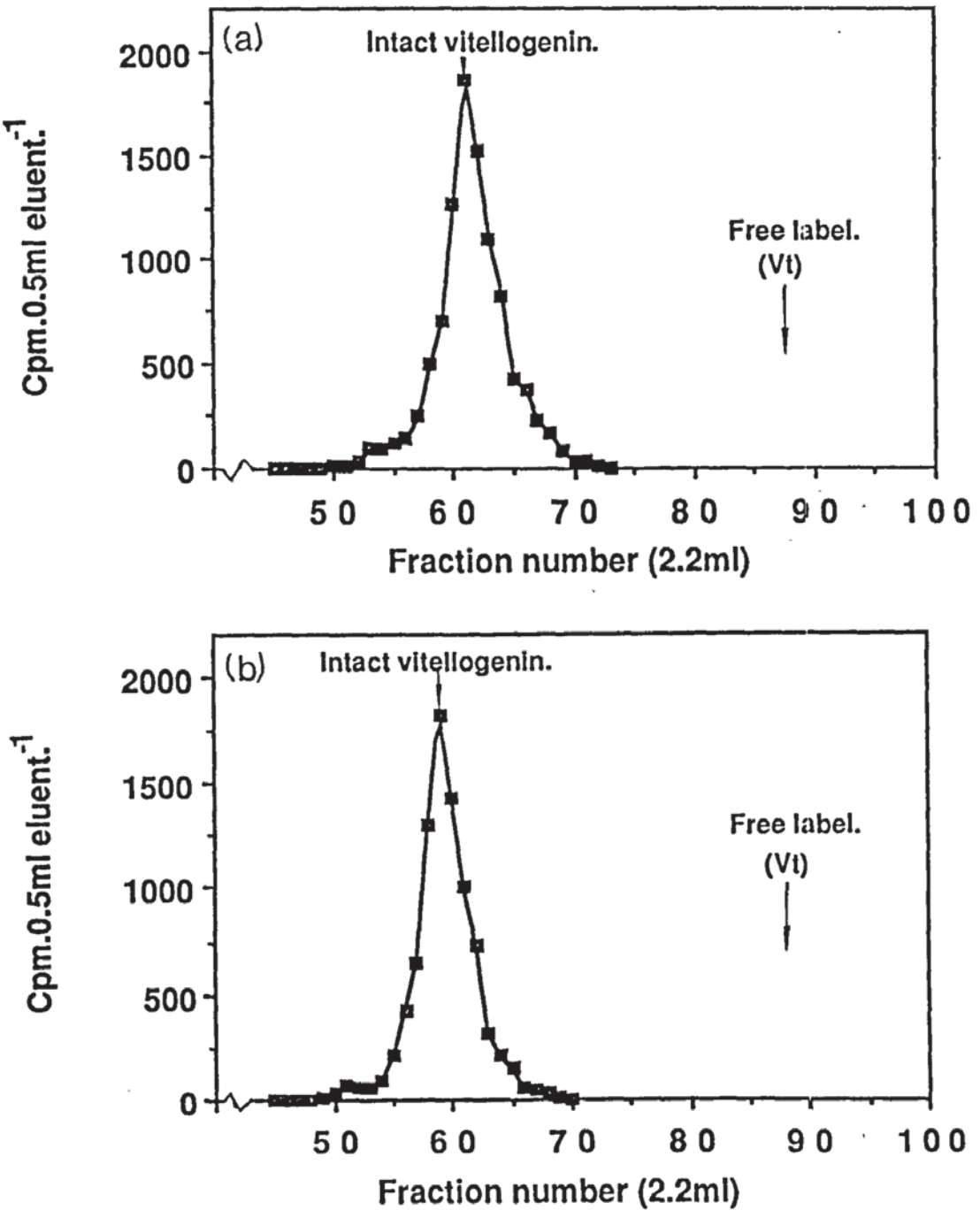


Figure 5.3. Sepharose -6B column chromatographs of the culture medium containing ^{3}H.VTG. (a) 6 hours after the incubations were started. (b) 24 hours after the incubations were started. Eluent fractions were monitored for ^{3}H only.

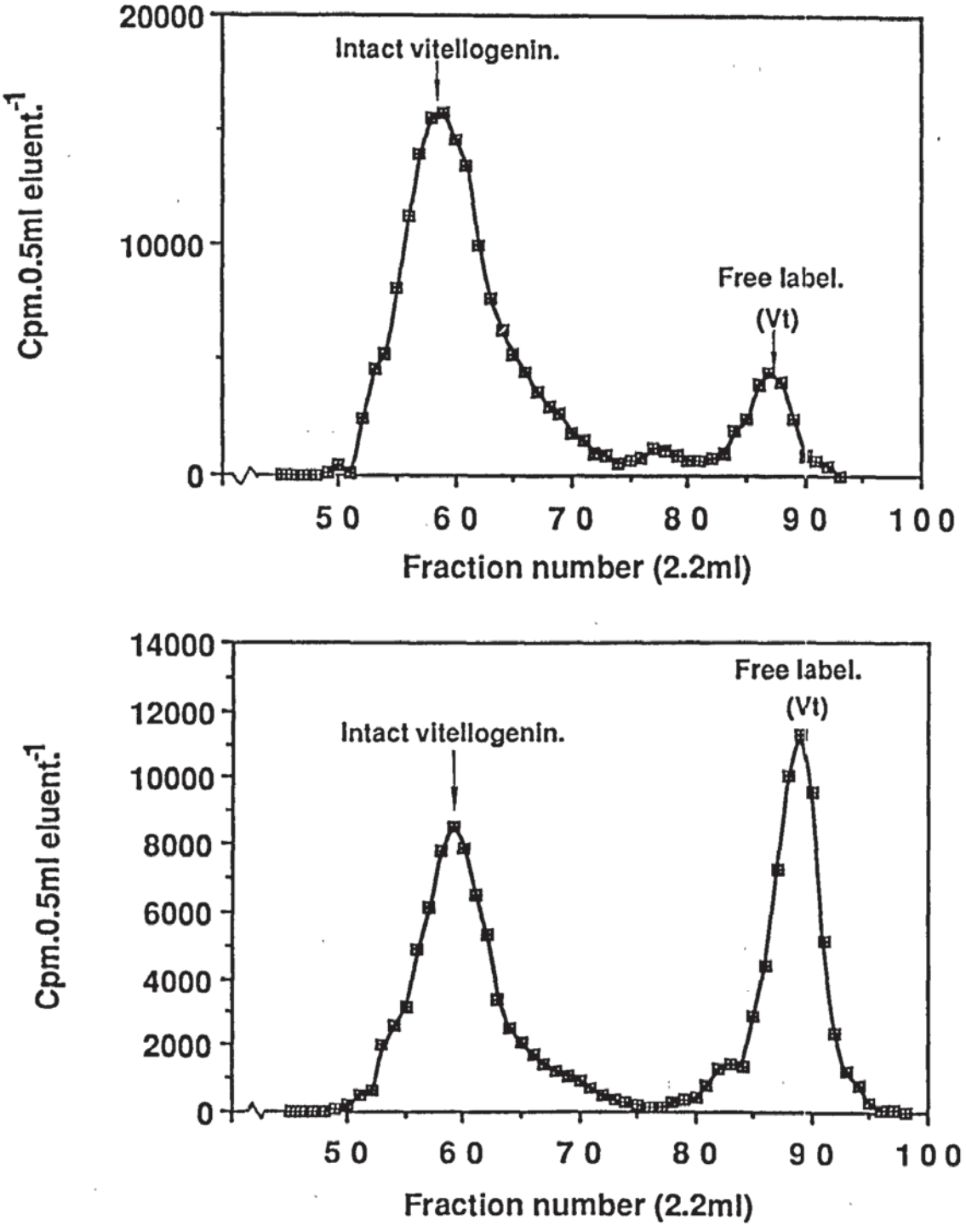


Figure 5.4. Sepharose -6B column chromatographs of the culture medium containing ^{125}I. VTG. (a) 6 hours after the incubations were started. (b) 24 hours after the incubations were started. Eluent fractions were monitored for ^{125}I only.

incubated with ^{125}I-VTG at a a total VTG concentration of 4.5 mg.ml^{-1} for 6 hours.

5.4.2 Results

Table 5.2 illustrates that there were no significant differences (ANOVA; $P < 0.05$) in the rate of VTG sequestration between follicles recieving a serum supplement and those that did not. Increasing serum concentrations did not enhance VTG uptake. Furthermore the survival rates of cultured follicles in either of the treatments were very similar, ranging from 75-85%.

5.5 Experiment 4: Yolk processing of ^{32}P^{3}H -vitellogenin in cultured follicles

5.5.1 Materials and methods

One hundred follicles (4.0 mm in diameter) were cultured in 10ml of medium containing 3.2mg ^{32}P.^{3}H-VTG.ml^{-1}. After 24 hours 50 follicles were prepared for column chromatography on Sepharose-6B. The eluents were monitored for protein and counted for radiolabels as previously described. A 1ml aliquot of the culture medium was similarly analysed to assess the stability of the labelled VTG over the incubation period.

5.5.2 Results

Figure 5.5a shows that even after 24 hours in culture the ^{32}P.^{3}H-VTG remaining in the medium was still intact, (similar to that of the *in vivo* labelled ^{3}H-VTG in experiment 2 above), eluting with a molecular weight of approximately 440 000 Daltons. All the ^{3}H-leucine and over 80% of the ^{32}phosphorus eluted with the intact VTG molecule. The instability of the ^{32}phosphorus attached to the VTG molecule, rather than degradation of VTG, probably accounts for the ^{32}phosphorus label at the V_T, because there was no protein absorbancy associated with "free" ^{32}phosphorus eluting at the V_T.

The yolk protein labelling pattern (Figure 5.5b) shows that almost 50% of the internalized VTG remained intact after 24 hours, while the other half of the sequestered ^{32}P.^{3}H-VTG had been processed to the yolk protein products ^{3}H-lipovitellin and ^{32}P-phosvitin as observed in the *in vivo* model (see Chapters 3 and 4).

Serum concentration.	Vitellogenin uptake ($ng \cdot mm^{-2} hr^{-1}$)	Survival percentage
0	17.4 ∓ 4.3	75
10	19.2 ∓ 3	85
20	19.7 ∓ 3.1	80
30	21.3 + 4.1	80
40	18.5 ∓ 2.3	80
50	14.7 ∓ 3.7	75

Table 5.2 The effects of increasing amounts of serum suppliments on the rates of vitellogenin uptake and survival in cultured follicles.

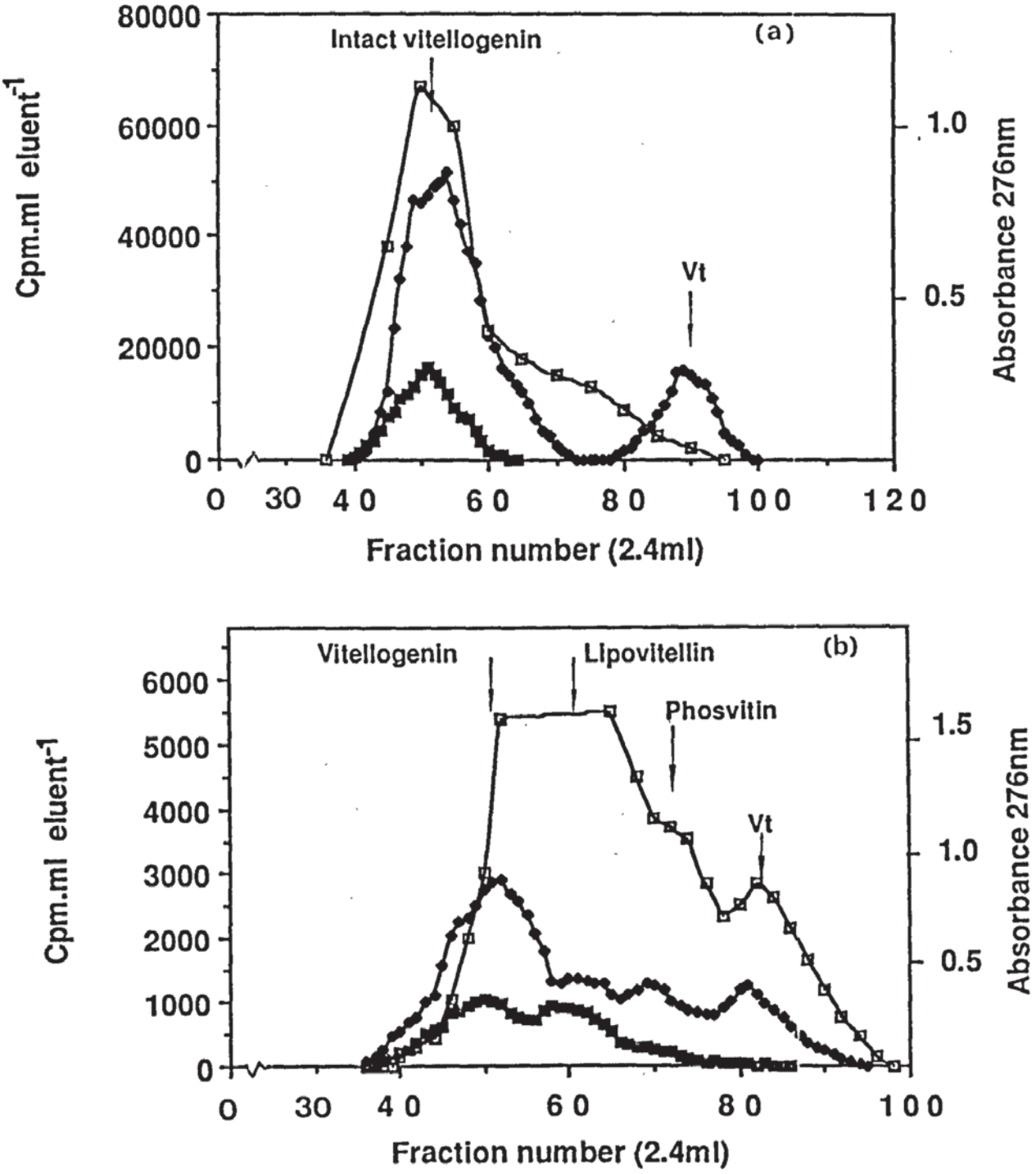


Figure 5.5. Sepharose -6B column chromatograms of: (a) the incubation medium used for follicle cultures, containing $^{32}P.^{3}H.VTG$, and; (b) an homogenate of 50 cultured follicles. Both analyses were performed after an incubation period of 24 hours. Eluents were monitored for absorbance at 276nm (open symbols:□) and for ^{3}H (■) and ^{32}P (◆). The void (Vo) and total (Vt) columns are marked.

5.6 Experiment 5: The effect of vitellogenin concentration on the rate of uptake of vitellogenin into cultured follicles

5.6.1 Materials and methods

Two sets of exogenously vitellogenic follicles were cultured in VTG concentrations ranging between 0.5 and 50 $mg.ml^{-1}$; set 1 from Fish I (follicle diameter 3.9 ± 0.16mm) at levels between 0.5-17 $mg.ml^{-1}$; and set 2 from a second fish (Fish II; follicle diameter 3.2 ± 0.1mm) at levels between 20-49.5 $mg.ml^{-1}$. A further group of follicles from Fish 2 were cultured at 13 mg VTG ml^{-1}, similar to one of the sets from Fish I to determine any differences in VTG uptake between the fish. Twenty follicles were cultured in each treatment at 10°C for 8 hours. The lower incubation temperature was adopted in order to observe the saturation kinetics of VTG incorporation; at higher temperatures (18°C) preliminary studies indicated that very high VTG levels may be necessary to obtain saturation (see also Experiment 6) and these are not easily obtained in culture.

5.6.2 Results

The rate of VTG uptake was positively correlated with increasing VTG (Figure 5.6); concentrations in the medium ranging from 0.5-49.5 $mg.ml^{-1}$ produced uptake rates of 1.2 to 178 $ng.mm^{-2}\ hr^{-1}$, respectively. The rates of VTG uptake at 13 mg VTG ml^{-1} in Fish 1 and 2 were 23.2 ± 2.7 and 19 ± 4.9 ng VTG. $mm.^{-2}hr^{-1}$, respectively, showing no significant differences ($P > 0.05$) in sequestration. At VTG concentrations between 40 and 50 $mg.ml^{-1}$ the saturation curve levels off, indicating that the maximal rate of incorporation at 10°C had been reached.

5.7 Experiment 6: The effect of temperature on vitellogenin sequestration

5.7.1 Materials and methods

Three sets of vitellogenic follicles, mean diameters 1.4 ± 0.5mm, 1.8 ± 0.1mm and 3.9 ± 0.15mm were cultured at temperatures of 0, 5, 10, 15, 20 and 25 ± 1°C. Follicles cultured at 0 and 5°C were placed in a thermostatically controlled refrigerator maintained at 5°C. A temperature of 0°C was achieved by placing ice around the refrigerated incubation vessels. The remaining temperatures were achieved in thermostatically controlled water baths. Each group of 20 follicles was incubated with ^{3}H-VTG at a concentration of 4 $mg.ml^{-1}$

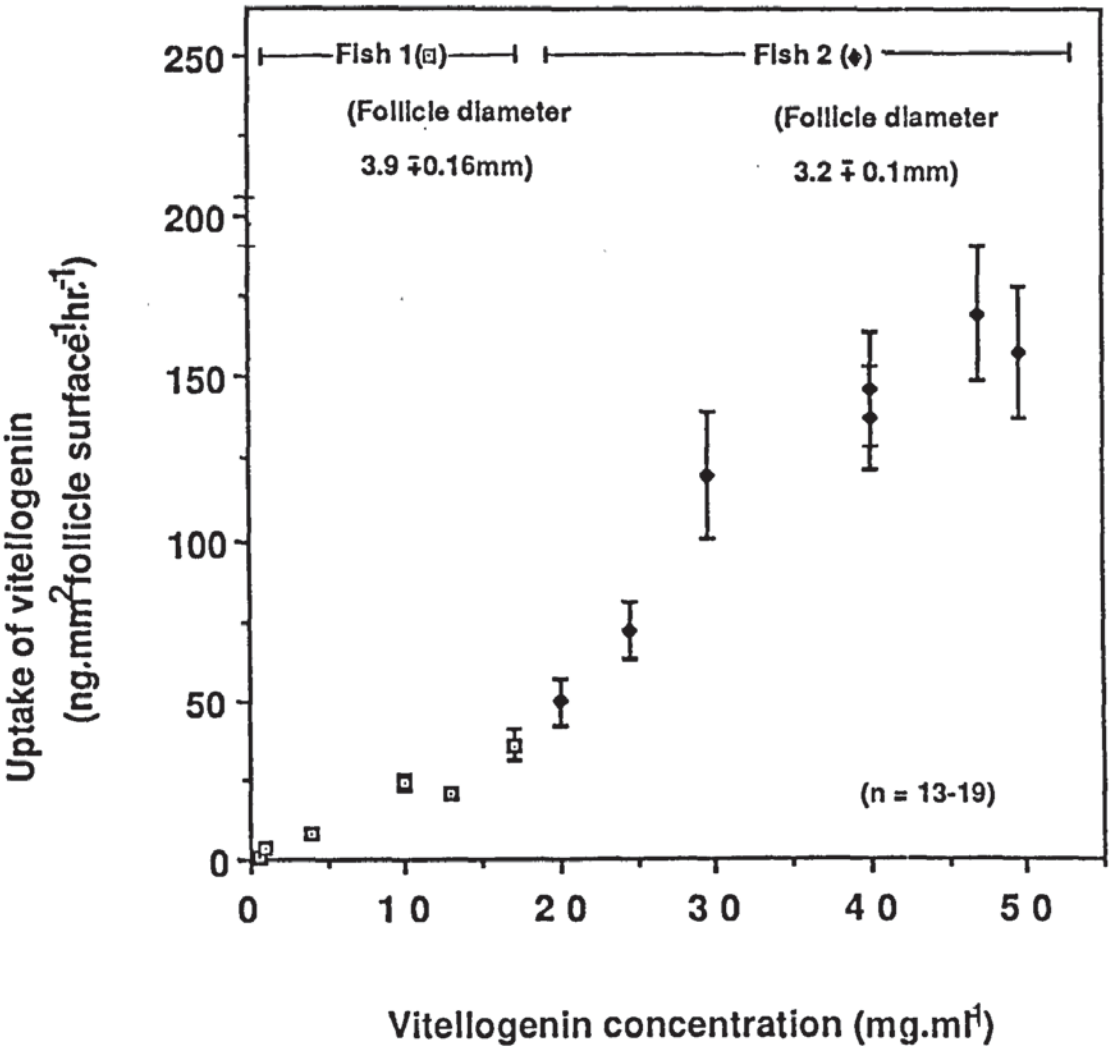


Figure 5.6. Rates of vitellogenin uptake into vitellogenic follicles cultured in different concentrations of vitellogenin.The graph is a composite of 2 sets of follicles (each set obtained from separate fish): set 1(follicle diameter 3.9∓0.16mm) were cultured in vitellogenin concentrations between 0.5-17mg.ml^{-1} and; set 2 (follicle diameter 3.2∓0.1mm) were cultured in vitellogenin concentratios between 20-49.5mg.ml^{-1}. Vertical bars denote standard deviations.

for 8 hours.

5.7.2 Results

Vitellogenin uptake showed a clear temperature dependence, with greater amounts of VTG sequestered with increasing temperature up to 25°C (Figure 5.7a). Rates of VTG uptake in the 3 sets of vitellogenic follicles ranged from 4.5-7 $ng.mm.^{-2}\ hr^{-1}$ at 5°C to 10-23 $ng.mm.^{-2}\ hr^{-1}$ at 25°C. In most cases over this temperature range an increase in 10°C doubled the rate of VTG incorporation.

Follicles at 0°C appeared to sequester small amounts of VTG (in all cases less than 3 $ng.mm.^{-2}\ hr^{-1}$); the low levels of associated radioactivity may in part be attributable to VTG binding to the follicle surface and/or to VTG incorporated immediately after the initial transfer of follicles from the medium which they were maintained during dissection, which was at 12 ± 1°C.

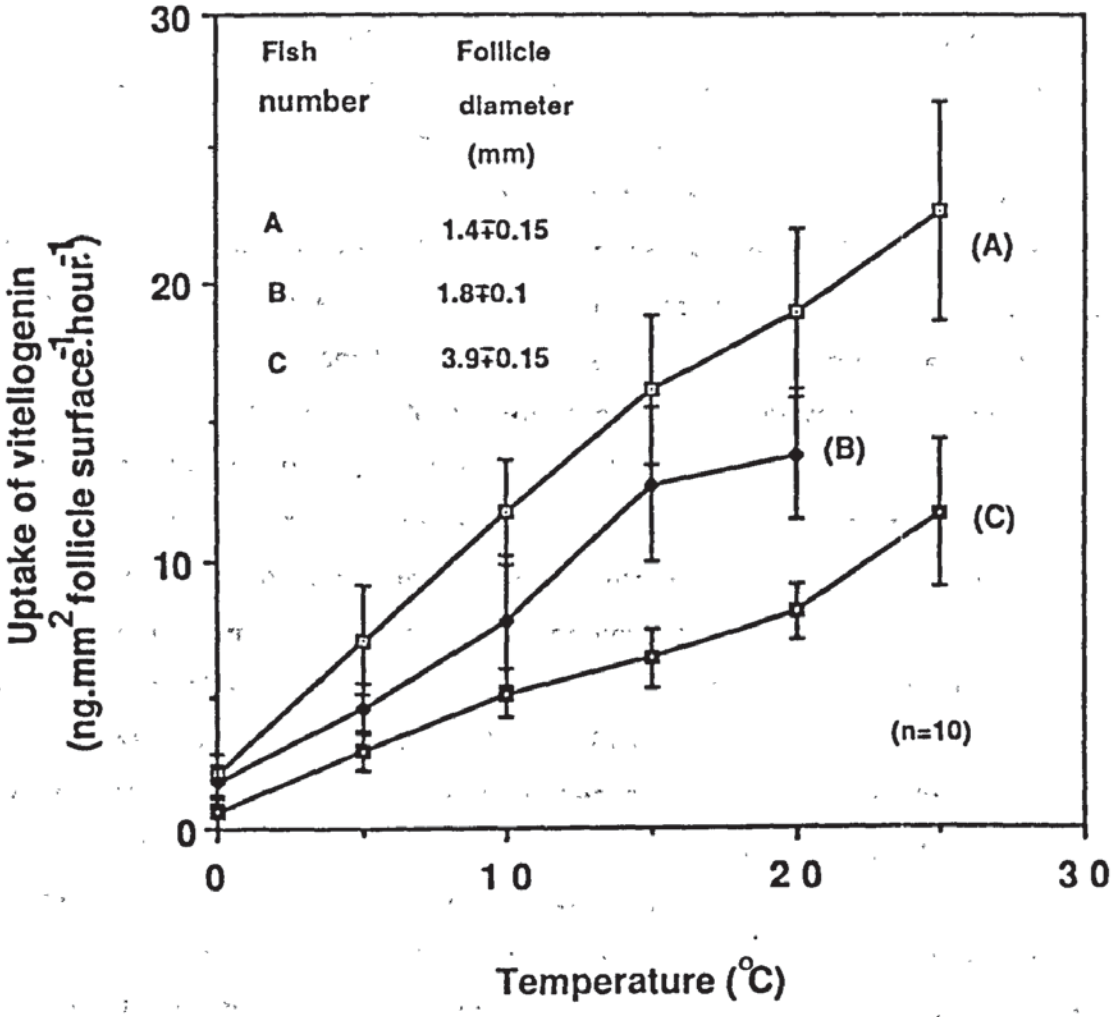


Figure 5.7. The effect of temperature on vitellogenin uptake into 3 sets of vitellogenic follicles obtained from separate fish. Each point represents the mean uptake rate from 10 follicles. Vetical bars denote standard deviations.

5.8 Discussion

The results from experiment 1 showed that all the follicle types: intact, those divested of the surface epithelium and follicles di ted of both the surface epithelium and theca, were capable of sequestering VTG, although their rates of uptake differed significantly. The intact follicles incorporated the least amount of VTG showing that under culture conditions the combined surface epithelium and connective tissues severely restrict VTG transport and therefore have to be removed to allow appreciable amounts of VTG to be sequestered. Jared and Wallace (1969), working on *Xenopus laevis*, similarly demonstrated that the outer surface membranes of the amphibians follicle were relatively impermeable to protein as opposed to small molecular weight substances. Furthermore it is probable that the surface epithelium in the rainbow trout follicle is even more impervious to VTG than suggested by the results; it is likely that during the dissection of the follicles from the ovary the outer epithelial and connective tissue layers are scored and become torn, thus exposing the more permeable thecal layer. Indeed Wallace (pers. comm.) suggests that at least in *Xenopus* it is difficult not to cause some damage to the follicles during their manual dissection from the ovary. In the intact animal the impermeability to protein transport of the surface epithelium probably serves to prevent the VTG, which is supplied to the follicle via capillaries in the underlying theca, from leaching outwards away from the oocyte.Follicles divested of their surface epithelium in all 3 vitellogenic fish sequestered significantly higher quantities of VTG than intact follicles ($P < 0.05$, Multiple Comparison of the Means). However, these quantities in Fish 1 and 2 (but not in 3) were significantly lower than the follicle groups divested further of the theca, suggesting that the theca also restricts VTG uptake under culture conditions, but to a far lesser degree than the surface epithelium. Although the thecal layer is a barrier to VTG uptake in cultured follicles it does not play a major limiting role like the surface epithelium, and its removal is not a pre-requisite to study protein uptake *in vitro*. The diffusion of VTG across the theca, although suggesting the theca plays a passive role in VTG transport does not preclude its participation in the initiation and/or cessation of VTG incorporation.

The analysis of the distribution of labelled materials within the follicles divested of their surface epithelium clearly demonstrated that the majority of label ($85 \pm 15\%$) was associated with the yolk proteins. Labelled protein therefore, is not appreciably incorporated by the follicular layers and does not accumulate at

the surface of the follicle. Instead it is continuously incorporated into the yolk. It should be stressed, however, that the percentage of labelled protein associated with the yolk proteins is only an approximation due to the inherent problems in separating labelled materials in the yolk from those contained in the follicular tissues; under certain conditions some of the ^{3}H-VTG associated with the follicular tissues would become physically dislodged during the preparative procedures and subsequently counted with the yolk proteins; this would cause overestimations in the yolk levels and under estimations in the follicular tissue content, whilst on other occasions yolk proteins with associated labels may adhere to the follicular tissues causing under estimations in the yolk contents and over estimations in the label contained in the follicular tissues.

Follicles divested of the surface epithelium and surface epithelium with theca incorporated VTG at rates that parallel those observed *in vivo* (Chapters 3 and 4) indicating that the culture method provides a suitable system for observing the uptake of protein. However, despite a similar treatment of follicles there were clear differences in the rate of VTG uptake between fish. From the first experiment it is not clear what parameters are dictating the differing rates of sequestration between the similarly treated follicles but they may include; the external concentration of VTG (the data from this experiment indicated that higher VTG concentrations induce higher sequestration rates) and/or follicle size, these factors are discussed further below and in Chapter 6, respectively. Alternatively some other feature related to the female fish from which the follicles were removed may dictate these differing rates of VTG sequestration; for example follicles exposed to a particular stimulus, such as a hormonal one (see Chapter 7), prior to their dissection for culture may continue to be stimulated during a period of the incubation and consequently sequester higher levels of VTG.

The 'control' group of ovulated eggs were impermeable to VTG in accordance with their normal physiological function. As described in the introduction to this chapter the radial striations or channels that mediate VTG transport through the zona radiatain vitellogenic follicles are closed at ovulation and subsequently terminate protein sequestration. Indeed once the micropyle closes, generally after the sperm has entered, the chorion creates an impervious seal between the yolk constituents and the surrounding environment.

The data obtained from experiment 1 emphasise that the interpretation of *in vitro* studies must take into account what kind of ovarian components are incubated and that clearly defined morphological criteria must be employed to

define the state of denuding. All subsequent cultures utilized follicles divested only of their outermost epithelial layer because the rates of VTG sequestration paralleled that seen *in vivo* (Chapters 3 and 4) with the minimum preparative handling.

In experiment 2, kinetic theory applied to the sequestration of VTG, showed that the rates of uptake of iodinated and tritiated VTG were similar. The results indicated that the V_{max} for ^{125}I-VTG (49.0 mg.mm.$^{-2}$ hr^{-1}) was almost identical to that of "native" ^{3}H-VTG (49.5 mg.mm^{-2}.hr^{-1}) and that the relative Km's for the 2 proteins were within 25% of each other (5 x 10^{-7} and 6.8 x 10^{-7}M, respectively). Thus, no pronounced alterations in the structural integrity and uptake of iodinated VTG by follicles were indicated and the iodinated product appeared to provide a favourable alternative for VTG uptake studies *in vitro*. The Km's in the order of 10^{-7}M reflect a fairly strong affinity of the receptor for the ligand (VTG), a feature one would expect in a receptor mediated transport system. *Xenopus* VTG receptors have been shown to have similar binding affinities; 13 x 10^{-6}M at 22^{o}C and 2-4 x 10^{-6}M at 0^{o}C (Opresko and Wiley, 1987a). However, this affinity is not as high as often seen in receptor-ligand binding studies (often up to 10^{-12}M) and it is suggested that this is due to both the high availability of VTG in the circulation during exogenous vitellogenesis (in the rainbow trout up to 50 mg.ml^{-1}) and the rate of VTG-receptor complex dissociation that probably occurs to allow the receptors a rapid replacement to the follicles binding surface (thus allowing the rapid rates of VTG uptake seen *in vivo*).

The Sepharose 6B column chromatograms of the ^{3}H.VTG in experiment 2 (Figures 5.3a and b) confirmed that the labelled protein was stable for up to 24 hours after the incubations were started; after 24 hours all the tritium was associated with the intact VTG which eluted with a mobility of approximately 440 000 Daltons. This observation was reinforced in experiment 4 when after a similar culture time period all the ^{3}H and in excess of 80% of the ^{32}P of ^{32}P. ^{3}H-VTG eluted with the intact molecule. Iodinated VTG appeared fairly stable after a 6 hour culture period (over 90% of the iodine eluting with the intact VTG molecule); however, this was not so after 24 hours and approximately 35% of the label eluted at V_T, the latter arising either from the degradation of ^{125}I.VTG into small iodinated fragments or from the iodine becoming detached from the iodinated VTG molecule. The protein absorbancy profiles of the 'cold' VTG,

(chromatographed with the ^{125}I-VTG), showed that VTG remained intact after 24 hours and therefore the latter explanation is the more likely. Further incubations of follicles undergoing exogenous vitellogenesis with free ^{125}I (data not shown) demonstrated that ^{125}I was readily incorporated, probably through pinocytosis and/or fluid phase engulfment during the receptor-mediated endocytosis of VTG, showing that follicle cultures over long periods of time could lead to overestimations in the rate of VTG uptake. The data from experiment 2 shows that ^{125}I-VTG has limitations as a marker for VTG uptake and this restricted its use in subsequent cultures to short term incubations of 6 hours or less. Longer term cultures throughout this and subsequent chapters utilized the more stable ^{3}H-VTG.

The addition of serum to the culture medium in experiment 3 did not significantly enhance the rates of ^{125}I-VTG sequestration; in fact at the highest serum concentration (50%) there appeared to be a slightly reduced rate of incorporation. It is possible that the increased viscosity of the medium produced by this concentration of serum may have inhibited the sequestration process. Furthermore no improvement in survival rates were observed in the follicles cultured with serum. Together these results demonstrated that follicle cultures, at least in the short term, did not require a serum supplement. Indeed serum supplements can add unknown substances (such as hormones) to the culture medium which could confuse the interpretation of subsequent experiments.

In contrast to these data with rainbow trout follicles, Wallace (1978) working on *Xenopus* follicles showed that the rate of VTG uptake was enhanced in a media supplemented with serum. In his studies a 10% serum supplement increased the rate of VTG uptake by 32%. However, it should be stressed that data in experiment 3 were obtained from short-term cultures and VTG uptake was used as the criterion for analysis; Wallace conducted his studies over a period of many days and used growth *per se* to assess the benefits of added serum. Possibly, serum addition might have advantages for longer term trout follicle incubations.

The first three experiments clearly showed that cultured follicles sequestered VTG at rates similar to that observed *in vivo* and demonstrated that the system developed provided an ideal model for short term sequestration studies. However the protein processing of ^{32}P.^{3}H-VTG in experiment 4 over a longer time period of 24 hours showed that although the pattern of label processing was similar to that occurring *in vivo* (Chapters 3 and 4) demonstrating that the cultured follicles were capable of degrading VTG specifically, the rate of

processing was considerably reduced to that occurring *in vivo*. After 24 hours *in vivo* the Sepharose 6B chromatograms illustrated that in excess of 95% of the labels were associated with the yolk protein products, whereas *in vitro* almost 50% were still associated with intact VTG. The limited capacity of the follicles in culture to process VTG may have some bearing on their survival capabilities over long periods of incubation. Recent findings by Wallace and co-workers (pers. comm.) have shown that in *Xenopus* the ionic environment of the cell and the regulation of cation flow across the cell membrane can have pronounced effects on the internal physiological processes. It is possible in our system adapted for culturing trout follicles that an adjustment in the medium's osmolarity is required to produce a more isotonic environment such that energies are not expended by the follicle on maintaining ionic balance but instead are directed to the yolk processing of VTG. This question has yet to be investigated.

The rate of VTG uptake into trout follicles was clearly dependent on the concentration of VTG in the surrounding medium, with higher titres inducing greater rates of sequestration. In *Xenopus* VTG levels below 2 $mg.ml^{-1}$ reduced uptake rates *in vitro* to almost zero (Wallace and Jared, 1976). Similarly in experiment 5 trout follicles cultured in VTG levels below 2 $mg.ml^{-1}$ had significantly reduced incorporation rates, as low as 6 $ng.mm.^{-2}hr^{-1}$. These data emphasise the importance of a high VTG titre in culture studies. Vitellogenin uptake was saturated at a VTG concentration between 40-50 $mg.ml^{-1}$ at 10°C, and although this value is considerably higher than that reported for *Xenopus* follicles (3-4 $mg.ml^{-1}$) at 20°C, it parallels the physiological levels of VTG found in the blood of maturing females during the latter stages of vitellogenic growth (Scott and Sumpter, 1983; Elliott *et al.*, 1984). Trout follicles also reach a final diameter of 4.5-5mm, which is 3-4 times that in *Xenopus* (1.3-1.4mm maximum) and therefore must incorporate higher quantities of VTG. This feature, together with the fact that a few thousand follicles grow simultaneously in the trout, probably accounts for the higher saturation kinetics of VTG sequestration in trout follicles compared with *Xenopus* follicles.

Similar kinetics for VTG sequestration in the trout are seen in the locust where VTG uptake is saturated at 40 $mg.ml^{-1}$ at 22°C (Rohrkastein and Ferenz, 1985). Locust follicles increase in diameter from 1.5 to 6.5mm (locusts possess oval follicles and measurements were taken from along the longitudinal axis) over an 8 day period. This rapid growth emphasizes that it is the type of ovarian growth pattern which dictates the dynamics of VTG sequestration.

It is possible however, that the saturation value for VTG sequestration in trout changes during exogenous vitellogenesis as the follicles increase in size. The value of 40-50 $mg.ml^{-1}$ in this experiment was calculated for follicles which were towards the end of the exogenous vitellogenic growth phase, i.e. follicles that were nearing their maximum size. Although little is known about the number and dynamics of the VTG receptor in trout follicles, it is likely that smaller follicles possess fewer receptors and therefore have a lower saturation concentration for VTG sequestration (see also Chapters 4 and 6); this would fit with the lower blood levels of VTG reported during the initial phase of exogenous vitellogenesis (Scott and Sumpter, 1983; Sumpter *et al.*, 1984).

The *in vitro* studies show that vitellogenic rainbow trout follicles sequester VTG at rates comparable to those observed *in vivo* in the absence of any external hormones and stimuli, the culture media being devoid of hormones and serum supplements. Furthermore the rate of VTG uptake (and subsequently the rate of follicle growth) appears to be dictated by the surrounding VTG concentration. The availability of VTG clearly has a major controlling influence on the rate of ovarian growth during exogenous vitellogenesis.

Uptake of VTG into rainbow trout follicles is clearly adapted to low temperatures and although significantly reduced experiment 6 shows it still persisted at 5°C and below. In contrast *Xenopus* and locust follicles are unable to incorporate VTG at temperatures below 4°C (Wallace, 1983) and 10°C (Rohrkastein and Ferenz, 1985), respectively. However, these species do not normally encounter such temperatures during their regular cycles of ovarian recrudescence. Although not fully understood, it is believed that cold temperatures depolymerize microtubular structures (Porter, 1966) which play an important role in protein uptake and transport (Wallace and Ho, 1972) and thus inhibit VTG sequestration.

Ordinary temperature curves can usually be interpreted as a composite of 2 general physiological processes involving physiological function and, at higher temperatures, inactivation. Temperatures up to 25°C did not appear to inactivate VTG sequestration. However, the inflexion in the temperature curve of 1.8mm follicles, which occurred between 15 and 20°C, indicated for trout and other temperature species that temperatures above 20°C should perhaps be avoided.

There is no evidence that temperature acts as a trigger for gonadal maturation in salmonids, as it does in cyprinids (Billard *et al.*, 1978). However, the data presented here shows it does have a direct effect on VTG uptake and

therefore ovarian growth. Morrison and Smith (1986) found that rainbow trout broodstock maintained in 10 ± 1°C spring water spawned in December to January, well in advance of those held in creek water (which reached temperatures as low as 2°C), where spawning occurred between March and April. Together these data suggest that the temperature influences on ovarian growth may in turn indirectly affect the time of ovulation.

Rainbow trout induced to spawn twice yearly (to extend the season of egg availability) using 'long-day' photoperiods (16 hours light or more) produce smaller eggs at their second spawning (Duston and Bromage, pers. comm.). Larger eggs however, produce larger alevins (Springate and Bromage, 1985) and tend to be commercially more acceptable. By maintaining female bloodstock on long photoperiods and constantly high temperatures (15-18°C) it may be possible to produce annually 2 batches of large eggs (4.5mm plus).

CHAPTER SIX

SEQUESTRATION OF VITELLOGENIN BY FOLLICLES OF DIFFERENT SIZES

6.1 Introduction

Experiments in the preceeding chapters indicated that the rate of VTG uptake was, at least in part, determined by the size of the vitellogenic follicle, with larger follicles sequestering greater amounts of VTG. It was not possible, however, to quantify these observations in either the *in vivo* or the *in vitro* studies due to the numbers of other variables involved. This chapter addresses this question and investigates the rate(s) of VTG uptake into different sized follicles.

Overall by far the most information on follicle growth among oviparous species is available for *Xenopus*. In this species ovarian follicles develop asynchronously; follicles of all stages are present, without dominant populations, throughout the breeding season. The asynchronous follicle development and the knowledge that feeding in laboratory maintained animals provides, at least in part, the stimulus for breeding, enables follicles of all sizes to be obtained for study through almost the entire year. Furthermore, follicle asynchrony means responses to particular hormonal or physiological parameters can be determined for all stages of follicle development using a single female.

Studies on VTG uptake in *Xenopus in vivo*, using ^{32}P.VTG, showed that as the vitellogenic follicle increases in size greater quantities of VTG were sequestered (Keem *et al.*, 1979). However, these authors suggested that the increase in uptake was merely a function of the increased surface area, rather than a change in the level of stimulation. Wallace *et al.*, (1970) further demonstrated that maturing follicles, with a diameter greater than 1.1mm, subsequently displayed a diminished capacity for VTG incorporation, indicating that follicular growth in *Xenopus* slows down before the maximum size of 1.3-1.4 mm is reached.

Vitellogenic growth of follicles has also received considerable attention in the marine teleost, *Fundulus heteroclitus*. Like *Xenopus* the ovarian follicles in this species develop asynchronously and follicles of all sizes may be found throughout the breeding cycle, which usually extends from early May to late June, including eggs which have been ovulated into the ovarian lumen (approximately 1.8mm in diameter, Selman and Wallace, 1983). In their studies on *Fundulus* Selman and Wallace showed that the rate of macromolecule incorporation into the different sizes of vitellogenic follicles, using both homologous ^{32}P.VTG and heterologous ^{3}H.VTG (isolated from *Xenopus*) was bimodal, with peaks of sequestration occurring when the follicles were between 1.0-1.1mm and 1.5-1.7mm in diameter. The peak in VTG sequestration by follicles between 1.0-1.1mm probably reflects the maximal

rate of vitellogenic growth. The reason for the higher rates of uptake in maturing follicles between 1.5-1.7mm however, was less clear, and was interpreted by Selman and Wallace (1983) as being a reflection of an increase in blood availability to the oocyte during maturation. They hypothesised that maternal macromolecules (especially VTG) made readily available to the oocyte during vitellogenesis gradually begin to be denied access as the follicle grows to its maximum vitellogenic size (1.3-1.4mm) until, with the onset of maturation, a change in follicular layer architecture temporarily renders VTG more readily available to the hydrating follicle. Sequestration is terminated during final maturation only by cessation of the capacity for micropinocytosis concomittant with germinal vesicle breakdown, which in *Fundulus* occurs when follicles reach 1.6-1.7mm in diameter (Wallace and Selman, 1978). In *Fundulus heteroclitus* during the final maturation of the follicle, however, at least 80% of the final enlargement is attributed to hydration, and VTG sequestration therefore accounts for less than 20% of the normal growth during this period (Wallace and Selman, 1978). Unlike *Fundulus*, however, in many other marine teleosts and the majority of freshwater spawning teleosts, including the salmonids, hydration during final maturation accounts only for a small increase in follicle size, and throughout the follicle's development, from the onset of exogenous vitellogenesis right up to the time of ovulation, enlargement is almost solely attributable to macromolecule sequestration.

In their studies on *Fundulus*, Selman and Wallace (1983) showed that follicles equal to or less than 0.7mm in diameter (pre-vitellogenic follicles) did not sequester appreciable amounts of either homologous or heterologous VTG. However, these follicles did take up trypan blue, a small molecular weight molecule used as a marker for pinocytotic activity, indicating that all the follicle sizes they investigated, from 0.4-1.8mm in diameter (encompassing pre-vitellogenic, vitellogenic and hydrating follicles), were capable of incorporating exogenous materials.

In the rainbow trout, unlike both *Xenopus* and *Fundulus*, the follicles develop in synchrony and one group of vitellogenic follicles grow and are subsequently all ovulated at one time (see Chapter 1). When the developing follicles reach the vitellogenic phase of development they appear to show uninterrupted growth until attaining their final size at ovulation. Little is known about the dynamics of follicle growth during vitellogenesis, in particular whether different sized follicles grow at different rates. A study of this type in an animal where all

the vitellogenic follicles at any one time fall within a limited size range is more difficult to conduct than that in asynchronous spawners where a full complement of follicle sizes may be obtained from a single animal. Comparative studies on the rates of growth between different sized vitellogenic follicles in synchronous spawners therefore requires that a number of females at different stages of follicular development are sacrificed. In the rainbow trout a number of strains exist and therefore it is possible to obtain a range of ovaries at different phases of the reproductive cycle at one sampling interval. Alternatively a range of different sized vitellogenic follicles can be obtained by sampling a single population at different time intervals during the period of vitellogenic development, an approach adopted in the second experiment in this study.

Although follicles in the rainbow trout broadly develop in synchrony and are ovulated together generally there is a size disparity between follicles within a single ovary during the early part of vitellogenic development; follicles may range from less than 1mm in diameter to a maximal size of approximately 2.5mm. As exogenous vitellogenesis progresses, however, the smaller vitellogenic follicles 'catch up' with the larger ones and a homogeneity in follicle size results, which is maintained until ovulation. In ovaries containing vitellogenic follicles of 3mm in diameter and above the sizes rarely ranged by more than 0.5mm. As yet it is unclear how this 'normalizing' effect on follicle size is brought about during the vitellogenic development of the ovary, but it might include a variation in the rate of VTG uptake with follicle growth. The first experiment in this chapter capitalized on the observations of disparity in follicle size observed during early vitellogenic development in the rainbow trout and investigated the rates of VTG uptake *in vitro* in follicles of a range of different sizes. Within this study the ability of pre-vitellogenic follicles to sequester VTG was also investigated. This experiment, however, only allowed follicles up to a certain size to be examined and, therefore, a second experiment was conducted to include follicles of later vitellogenic development. In experiment 2 a single population of maturing females was sampled on a number of occasions and the rates of VTG uptake for follicles ranging from the initial stages of exogenous vitellogenesis to follicles having completed development were investigated *in vitro*.

6.2 Experiment 1: Uptake of vitellogenin in follicles of different sizes within a single ovary

6.2.1 Materials and methods

A 1kg female rainbow trout at an early stage of vitellogenic development, with a GSI of 3%, was sacrificed and 100 follicles ranging in size from 0.5mm to 2.3mm (the largest in the ovary) in diameter dissected out for incubation. An attempt was made to obtain a sample of follicles of all different sizes rather than a sample strictly proportional to the distribution of sizes present in the ovary. Ten groups of 10 follicles of similar sizes were cultured in ^{3}H.VTG at a concentration of 13 mg ml^{-1} for 8 hours. At the end of the incubation period follicles were assessed for viability (see Chapter 2) and those maintaining the appearance of freshly dissected follicles were carefully measured, using a micrometer fitted into the eyepiece of a dissecting binocular, and placed in separate vials for radio-label counting.

The rates of VTG sequestration by the different sizes of follicles were calculated both for the whole follicle and per unit (mm^2) of follicle surface. Rates of VTG incorporation expressed in relation to surface area were determined using the formula given in Chapter 2 (section 2.10.3). Rates of VTG uptake into complete follicles were assessed using the following formula:

$$\text{Uptake of VTG per follicle (ng. follicle.}^{-1}\text{ hr.}^{-1}\text{)} = \frac{\text{cpm . follicle.}^{-1}\text{ hr.}^{-1}}{\text{Total cpm in culture medium}} \times \text{Total VTG in incubation medium}$$

6.2.2 Results

Figures 6.1 and 6.2 show the rates of VTG sequestration into 72 surviving follicles, which encompass all the sizes isolated from the ovary. Both figures show that isolated follicles below approximately 1.0mm in diameter did not incorporate ^{3}H.VTG. All follicles above 1.0mm in diameter sequestered VTG. Figure 6.1 which shows the rate of VTG uptake into whole follicles, demonstrates that greater amounts of VTG were sequestered with increasing follicle size. A regression analyses on follicles sequestering VTG produced a correlation coefficient (r) of 0.93. Uptake rates of VTG per follicle ranged from 4 ng hr^{-1} in 1.1mm follicles to 220 ng.hr^{-1} in the largest follicles, 2.3mm diameter.

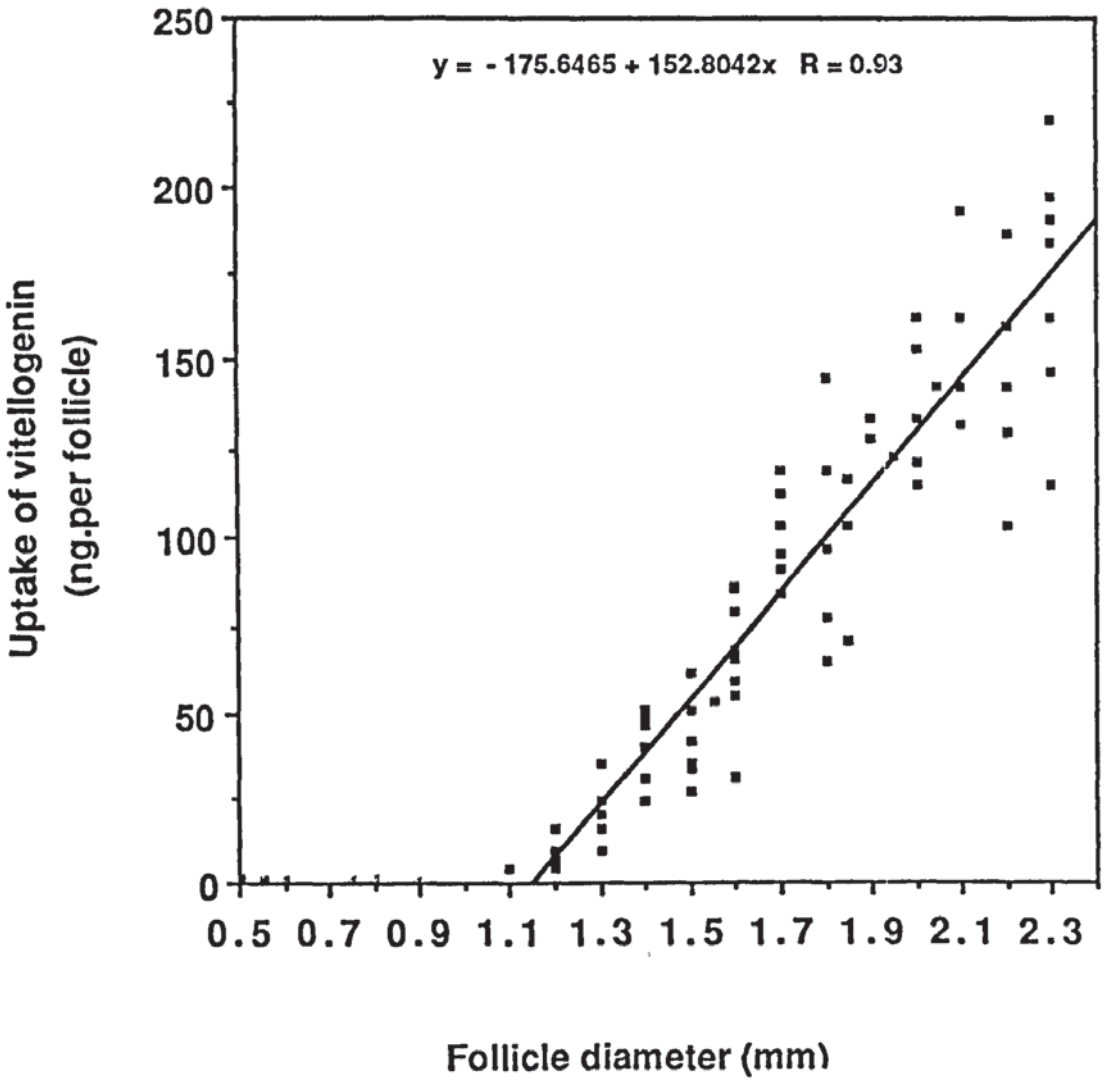


Figure 6.1. Incorporation of ^{3}H.VTG into cultured follicles of various sizes obtained from a single vitellogenic female.

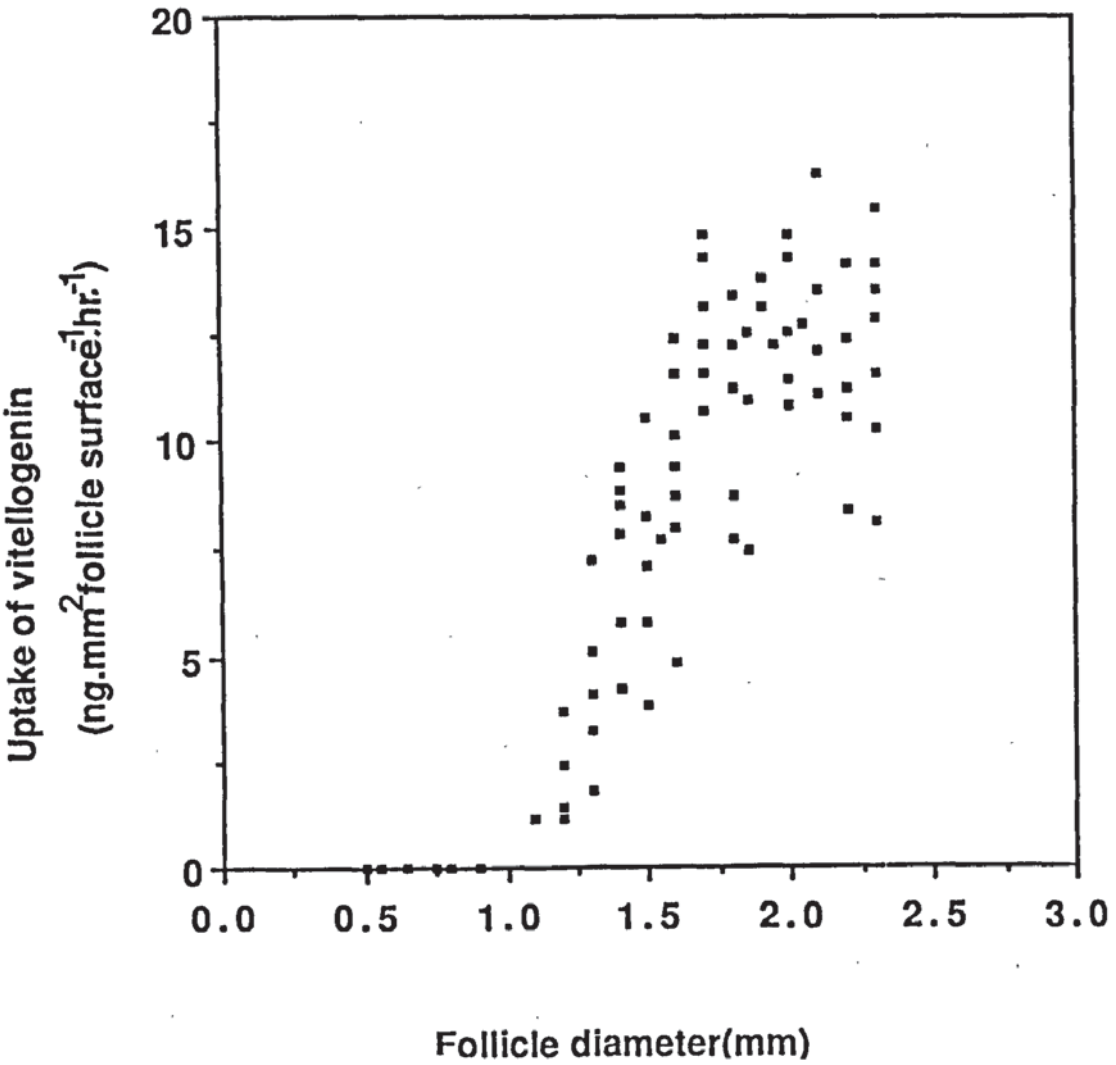


Figure 6.2. Rates of vitellogenin uptake (expressed as uptake per unit follicle surface) into various sized follicles obtained from a single vitellogenic female.

Figure 6.2 which expresses the uptake of VTG relative to follicle surface area, gives a slightly different pattern of sequestration in the different sized follicles to that shown in Figure 6.1. The data can broadly be divided into 3 sections: (1) those follicles which did not sequester VTG (below 1.0mm in diameter); (2) follicles between 1.1 and 1.7mm in diameter which incorporated increasing amounts of VTG with increasing follicle size and (3) follicles between 1.8-2.3mm which did not show any further increase in the rate of uptake as the diameter increased but rather their rate plateaued at around 12ng.mm.$^{-2}$ hr^{-1}. Within the second data group the smaller follicles (1.1-1.2mm in diameter) sequestered 1.5-4 ng.VTG mm.^{2} follicle surface^{-1} hr^{-1} whilst the larger ones, measuring 1.7mm sequestered 10.5-15 ng.VTG mm.$^{-2}$ follicle surface.$^{-1}$ hr^{-1}. A regression analysis on this group of follicles produced a regression coefficient (r) of 0.85. Within the groups of larger follicles (1.8-2.3 mm) incorporation rate of VTG. varied only between 8-15 ng.VTG mm.$^{-2}$ hr^{-1}.

6.3 Experiment 2: Changes in vitellogenin uptake during exogenous vitellogenesis with increasing follicle size

To obtain a more complete picture of VTG sequestration with follicle growth, especially with respect to the rates of sequestration in larger vitellogenic follicles, a second experiment was conducted investigating VTG uptake into follicles over the entire period of vitellogenic ovarian development in a single population of maturing females.

6.3.1 Materials and methods

A single population of 1 year plus 'Caribou' strain rainbow trout were used throughout this study. This strain spawns from mid-october onwards and sampling times were chosen to include the initial, middle and late (approaching ovulation) stages of exogenous vitellogenic development.

The population was sampled on 6 July, 26 August and 4 October. On each occasion 5-8 females were sacrificed and the body and ovary weights measured for the determination of the GSI. From each female 20 follicles were dissected out and prepared for culture. Isolated follicles were cultured in ^{3}H.VTG at a concentration of 13 mg.ml^{-1} for 8 hours. The culture conditions at each sampling interval were standardized as much as possible, except the follicle size, the parameter under investigation. The same stock of radio-labelled VTG was used throughout all the

incubations. At the end of each incubation the follicles were prepared and counted as detailed in Chapter 2. The rates of VTG uptake both into whole follicles and per unit follicle surface were determined as described above for experiment 1.

6.3.2 Results

Vitellogenic follicles isolated for culture from individual fish on 6 July, 26 August and 4 October measured between 1-1.3mm, 1.9-2.7mm and 3.0-3.9mm in diameter respectively. The gonadosomatic indices in these groups of fish increased from between 0.19-0.295 to 1.93-5.01 to 6.42-11.92 on the successive sampling dates respectively (Table 6.1). Ovaries sampled on 6 July were in the initial stages of exogenous vitellogenic development and consequently included a wide range of follicle sizes. In these ovaries only follicles greater than 1.0mm in diameter were isolated for culture, ie. those known to be able to sequester VTG (see Chapter 4). Figure 6.3 and Table 6.1 showing VTG uptake into whole follicles of various sizes, present a similar pattern of sequestration to that seen in follicles of different sizes isolated from a single ovary (compare to Figure 6.1) with larger follicles sequestering larger quantities of VTG. A regression analysis on these data produced a correlation coefficient (r) between VTG uptake and follicle size of 0.92. All vitellogenic follicles cultured from the first sampling date, measuring less than 1.4mm in diameter, sequestered less than 75ng.VTG follicle^{-1} hr^{-1}. Follicles of mean diameters between 1.9-2.7 incorporated 227-916 ng.VTG follicle.$^{-1}$ hr^{-1}.

Figure 6.4 shows that the larger vitellogenic follicles also sequestered proportionally larger quantities of VTG on the basis of surface area than their smaller counterparts. Uptake rates of VTG at the 3 sampling dates into follicles of 1.1-1.3mm, 1.9-2.7mm and 3.0-3.9mm in diameter ranged between 7.3-16.5, 20-40 and 17-43 ng.mm^2 follicle.$^{-1}$ surface.$^{-1}$ hr^{-1} respectively. Sequestration of VTG per unit surface area appeared to increase more rapidly between the follicles sampled on the first and second dates than that occurring between the second and third sampling dates, when follicles were in the later stages of development. Thus, an increase in the mean follicle diameter between the first and second samplings of approximately 1mm (1.15 to 2.36mm) almost tripled (300% increase) the mean rate of VTG uptake. However, a similar mean increase in follicle diameter of 1mm between the second and third (final) sampling dates (mean follicle size increases from 2.36 to 3.35mm) increased the mean rate of VTG

Date	Fish	Gonadosomatic index	Size range of follicles cultured (mm)	Rate of VTG uptake ng. follicle^{-1} hr.$^{-1}$	Rate of VTG uptake ng. mm^{-2} hr.$^{-1}$
6 July	1	0.295	1 ± 0.05	43.9 ± 11.3	14 ± 3.6
	2	0.231	1 ± 0.05	42.4 ± 15	13.5 ± 4.8
	3	0.191	1 ± 0.05	32 ± 9.7	10.2 ± 3.1
	4	0.521	1 ± 0.1	51.8 ± 21.9	16.5 ± 7
	5	0.247	1 ± 0.05	27.3 ± 12.4	8.7 ± 3.95
	6	0.270	1 ± 0.1	22.9 ± 8.5	7.3 ± 2.71
	7	0.51	1 ± 0.05	43.67 ± 16.7	13.9 ± 5.3
26 Aug.	1	2.24	2.4 ± 0.1	524 ± 94	29 ± 5.2
	2	2.73	2.3 ± 0.1	349 ± 82	21 ± 4.9
	3	1.93	1.9 ± 0.15	226 ± 53	20 ± 4.7
	4	5.01	2.7 ± 0.2	916 ± 167	40 ± 7.3
	5	4.90	2.5 ± 0.1	667 ± 155	34 ± 7.9
4 Oct.	1	8.3	3.2 ± 0.1	949 ± 151	29.5 ± 4.7
	2	6.01	3.3 ± 0.1	896.5 ± 135	26.2 ± 3.9
	3	9.21	3.7 ± 0.1	731 ± 234	17 ± 5.2
	4	11.92	3.9 ± 0.1	942 ± 301	19.7 ± 6.3
	5	7.05	3.2 ± 0.1	1216 ± 151	37.8 ± 4.7
	6	9.41	3.1 ± 0.1	1298 ± 178	43 ± 5.9
	7	7.95	3.4 ± 0.1	1056 ± 172	29 ± 4.75
	8	6.42	3.0 ± 0.1	898 ± 183	31.7 ± 6.5

Table 6.1 Rates of vitellogenin uptake in different sizes of follicles sampled at different times during exogenous vitellogenesis.

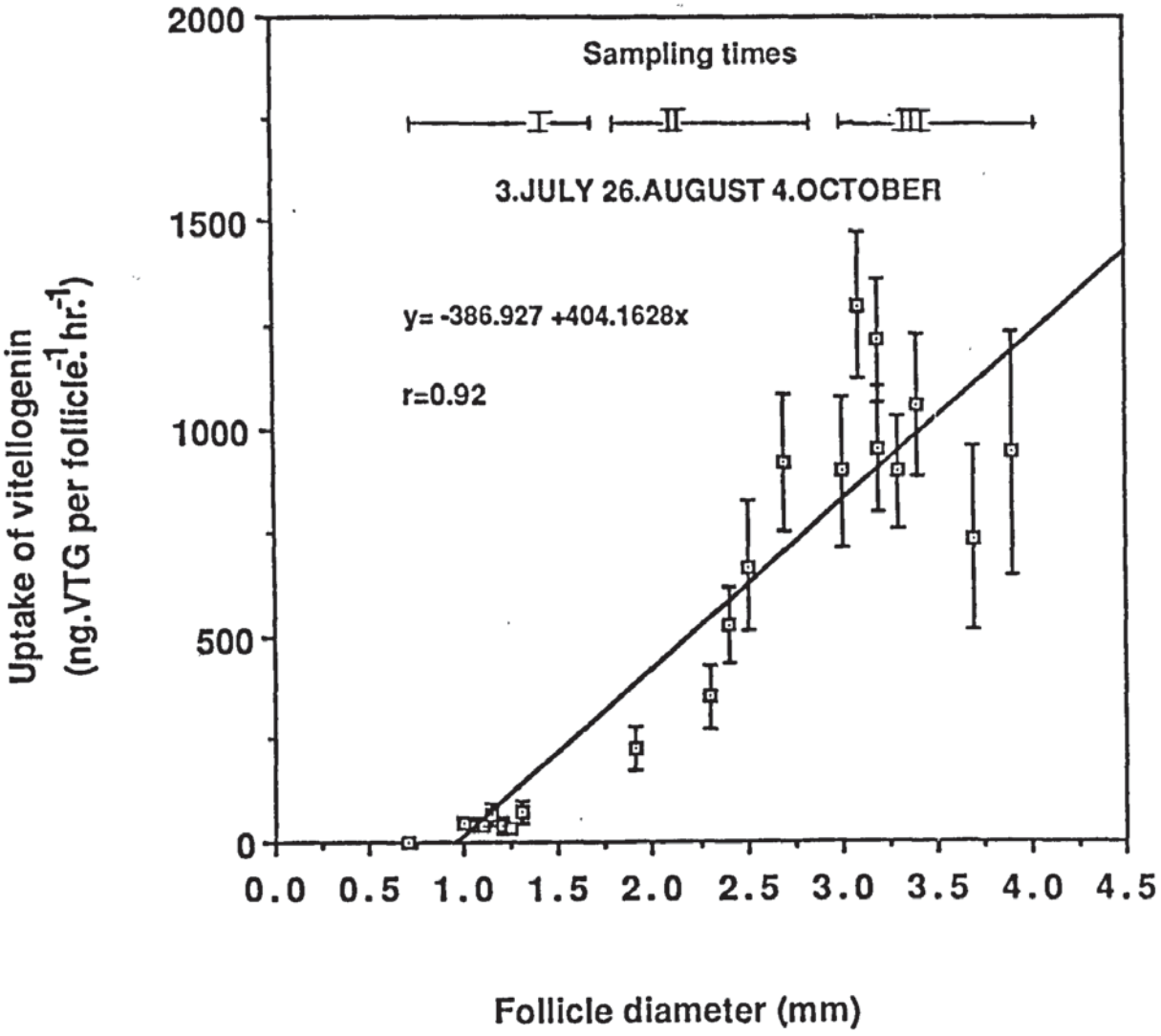


Figure 6.3. Incorporation of ^{3}H.VTG into sets of cultured follicles of various sizes obtained from a number of females at different time intervals over the period of vitellogenic growth. Each point represents the mean uptake from 10 follicles. Vertical bars denote standard deviations.

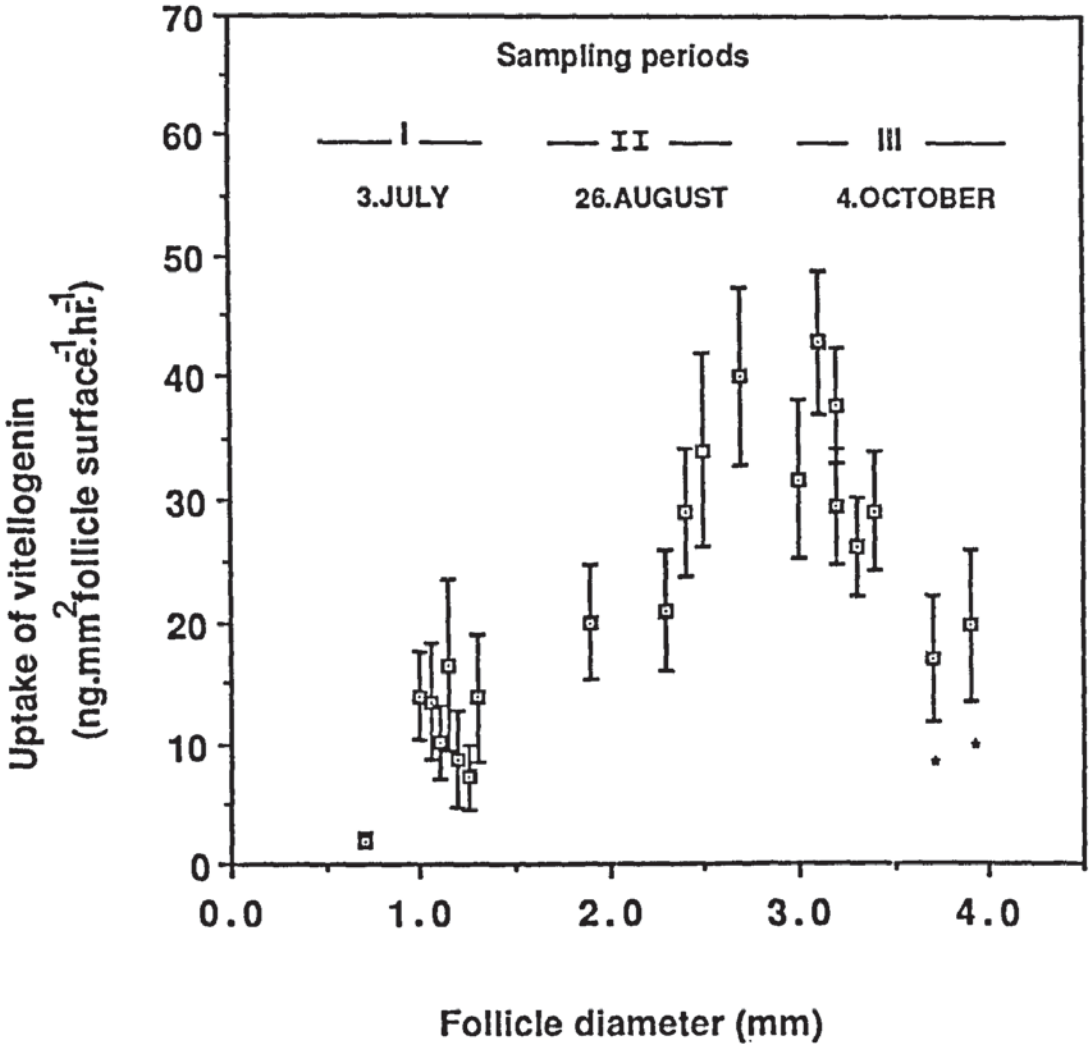


Figure 6.4 Rates of vitellogenin uptake (expressed as uptake per mm^2 follicle surface) in various sized follicles. Follicles were obtained from a number of vitellogenic females at 3 sampling intervals during the vitellogenic growth of the ovary. Each point represents the mean uptake from 10 follicles. Vertical bars denote standard deviations.

uptake by only 17%. The two largest follicle sizes cultured, mean diameters 3.7 and 3.9mm, which were sampled on 4 October, showed a considerable reduction in the relative rate of VTG sequestration (uptake VTG.mm^2 follicle surface.$^{-1}$ hr.$^{-1}$) incorporating 17-20 ng VTG mm^2 follicle surface^{-1} hr^{-1}, a value approximately half that seen in the follicles of mean diameter 3.0-3.2mm (29-43 ng.mm.$^{-2}$ hr.$^{-1}$).

6.4 Discussion

Figures 6.1 and 6.2 in experiment 1 show that rainbow trout follicles of less than 1mm in diameter were unable to sequester VTG. Histological examinations of these follicles were not performed but reference to the studies reported in Chapter 4 (and Sumpter *et al.*, 1984 and Bromage and Cumaranatunga (1987)) illustrated that exogenous vitellogenesis commences when rainbow trout follicles are approximately 1mm in diameter, suggesting that follicles below this size are pre-vitellogenic. Similar studies conducted on *Fundulus heteroclitus, in vivo*, using both heterologous (derived from *Xenopus*) and homologous VTG, also indicated that pre-vitellogenic follicles sequestered negligible amounts of VTG compared to vitellogenic follicles (Selman and Wallace, 1983). The inability of pre-vitellogenic follicles to sequester appreciable quantities of VTG in either of these two teleosts is possibly due to the absence of the specific receptor system for VTG which must develop in vitellogenic follicles (see Chapter 4). Alternatively the receptor system for VTG may be present in pre-vitellogenic follicles but is not active, awaiting the appropriate stimulus, which may include the follicle reaching a particular size and/or receiving a chemical stimulus. It is also possible that the receptor system for VTG is present and active in the pre-vitellogenic follicles but the granulosa and/or theca are preventing access of VTG to the oocyte surface; possibly the channels between these cell layers are not yet present, or open. Pre-vitellogenic follicles are, however, capable of pinocytosis (Selman and Wallace, 1983) and it is unclear why there has been no uptake at all of VTG into the trout follicles in the present study; one would expect that some VTG would have been incorporated adventitiously. This observation may in part be due to the low specific activity of ^{3}H.VTG (see Chapter 5, experiment 2) such that significant quantities of VTG (ng) need to be sequestered before it can be clearly detected. Little is known about pinocytosis in teleost follicles, however, and there may be a size limit on the macromolecules that are incorporated; VTG is a very large protein, approximately 440 000 Daltons in the rainbow trout (see Chapter 3) and may therefore be excluded from this transport mechanism. By contrast Campbell (1978), investigating the specificity of VTG uptake into cultured follicles of the rainbow trout, demonstrated that follicles, reported to be pre-vitellogenic were capable of sequestering VTG. However, the follicles used measured up to 1.2mm in diameter, a size that in both this study (see Chapter 4) and in more detailed works by Bromage and Cumaranatunga (1986) were considered to be in the initial stages of exogenous

vitellogenesis.

Follicles which were able to sequester VTG, (i.e. those larger than 1mm in diameter) incorporated increasing quantities of VTG with increasing follicle size, both in terms of total uptake and uptake per unit surface area (relative uptake). Studies on both *Xenopus* (Keem *et al.*, 1979) and *F.heteroclitus* (Selman and Wallace, 1983) similarly showed that larger vitellogenic follicles sequestered larger total amounts of VTG. A re-assessment of the data presented by Selman and Wallace (1983) on *F.heteroclitus* (which reported total VTG uptake in relation to follicle size) further indicates that the relative rate of VTG uptake also changes with follicle growth. Unlike the situation in the rainbow trout, however, where the relative rate of uptake of VTG appeared to continue increasing over much of the period of exogenous vitellogenesis (see Figure 6.4) the corresponding rates in *Fundulus* showed a bimodal peak occurring in follicles between 1.0-1.1mm and 1.5-1.7mm in diameter. *Xenopus* follicles, in contrast to those of rainbow trout and *F.heteroclitus*, show a fairly constant rate of VTG uptake in relation to surface area throughout the major part of vitellogenic growth (follicles between 0.7mm and 1.1mm in diameter (Keem *et al.*, 1979). The ability of the larger vitellogenic follicles of rainbow trout to sequester VTG faster may be the result of an increase in the number of VTG receptors per mm^2 of follicle surface and/or an increase in the reaction rates of processes involved in the endocytosis of VTG.

The increased rate of VTG sequestration per unit follicle surface, together with the high degree of specificity for VTG incorporation (see Chapter 4) and the steadily increasing, high concentrations of VTG in the blood that accompany vitellogenic growth, together explain how the rapid rate of follicle growth is brought about in the rainbow trout.

It could be argued that using follicle surface area to quantify VTG uptake has a limited value, for as mentioned earlier although the function of surface area allows comparisons in VTG uptake rate to be made between follicles of different sizes, the surface area calculation as used here ($4\pi r^2$) assumes a smooth surface, whereas the true surface area of the vitelline membrane in both amphibians and teleosts must be considerably larger since the surface is greatly convoluted at this time (Wallace and Dumont, 1969). However, in *Xenopus*, where uptake rates of VTG remain fairly constant over the major part of vitellogenic growth, use of the same formula to calculate surface area appears to normalize data obtained from follicles over similar size ranges (Wallace and Dumont, 1969) suggesting that this

term reflects a physiological surface.

Figure 6.2 shows that the larger groups of follicles from the single ovary measuring 1.8 to 2.3mm in diameter, sequestered similar amounts of VTG per unit follicle surface, suggesting that on reaching approximately 1.8mm the maximal rate of VTG uptake per mm^2 follicle surface is attained. The data from the seasonal study on VTG uptake however, suggested otherwise and indicated that the relative rate of VTG sequestration continues to increase until the follicles approach their maximum size. It is likely that the latter study which included many individuals, will have provided the more reliable result. However, more detailed analyses would need to be conducted to discount the observation from experiment 1, for it may have some bearing on achieving synchrony of follicle size that occurs during the early phase of vitellogenic development.

In *Xenopus* as follicles approach ovulation VTG uptake is reduced significantly; follicles between 1.2-1.3mm incorporate as little as 5% of that sequestered by the smaller vitellogenic follicles (0.8-1.1mm; Keem *et al.*, 1979). The limited data presented here for rainbow trout follicles approaching ovulation suggests that a similar reduction in VTG sequestration (both total and relative) occurs before VTG uptake stops and ovulation is initiated. Both groups of follicles that appeared by superficial observations, to be approaching ovulation in the third sampling period of the population study (mean follicle diameters of 3.9 and 3.7mm) sequestered VTG at half the rate compared to the remaining follicle groups at this time (which measured 3.0-3.4mm in diameter).

The results from this chapter clearly show that both the total and relative rates of VTG sequestration increase with increasing size of the vitellogenic follicle. These observations, in conjunction with the increasing levels of blood VTG during vitellogenic development explain, at least in part, how the enormous rates of increase in follicle size (increasing in volume by 2-4 times, Sumpter pers. comm.) are maintained during the later stages of exogenous vitellogenesis when the follicles surface area (area for VTG sequestration) becomes much reduced in relation to the follicle volume in comparison to the smaller vitellogenic follicles.

CHAPTER SEVEN

THE HORMONAL CONTROL OF VITELLOGENIN UPTAKE

7.1 Introduction

The series of interrelated stages comprising the ovarian development in the rainbow trout, described in detail in Chapter 1, require precise co-ordination and control to produce eggs at the optimum time of the year for the subsequent survival of the offspring. Although most salmonids rely on the seasonally changing of cycle of daylength to time their annual cycle of reproduction (Bromage and Cumaranatunga, 1987) it is the endocrine system that directly controls the different phases of ovarian development and maturation. The hormonal mechanisms controlling the female reproductive cycle in fish have been the subject of much research in recent years, due partly to a general awareness of the value of comparative studies in solving fundamental physiological problems and partly to the fact that purified fish hormones have become more readily available. Many hormones have been implicated in both the initiation and regulation of follicle development and principally they involve secretions from the hypothallamus, pituitary gland and ovaries. Of all the series of physiologically integrated events invoked by artificially administered hormones in teleosts, the selective uptake of VTG by developing follicles has received the least study.

In the salmonids the large number of follicles develop simultaneously (ie. they show synchronous development), and because each require the same hormonal signals this may mean that these signals have to be much amplified than in animals that show an asynchrony in follicle development; this may account for the very much higher circulating levels of hormones found in this group than, for example, in the mammals. The trout, therefore offers an attractive model for studying the control of reproduction. However, the number and nature of hormones involved in the regulatory mechanisms of VTG incorporation have not been resolved in salmonids, nor indeed in any fish, teleost or otherwise.

Ascribing specific functions to naturally circulating hormones and determining the effects of hormonal treatments *in vivo* are often difficult because their involvement may be either direct, which in the case of VTG sequestration may be an affect on follicular receptivity and competence, or indirect, their actions being relayed by other endocrine tissues. Furthermore in salmonids the large numbers of different hormones which undergo pronounced fluctuations during vitellogenesis and ovulation make it difficult to discriminate essentially dominant factors from modulating ones.

This chapter investigates the range and nature of hormones involved during exogenous vitellogenesis in the regulation of VTG uptake into trout follicles. Using the established culture procedures in the previous two chapters, 7 hormones were tested including the hypophyseal products, gonadotropin (GtH) and growth hormone (GtH), the thyroid hormones thyroxine (T4) and tri iodothyronine (T3), the steroids 17ß-oestradiol and testosterone, and also insulin. These hormones were chosen for study from the array of hormones implicated in VTG sequestration because: firstly, evidence in the literature from a number of previous studies (in teleosts and other oviparous vertebrates) have suggested their involvement in VTG uptake, and/or secondly, where known, their concentrations in the serum become elevated during the vitellogenic development of the ovary and thirdly, it was possible to obtain preparations of these hormones. The following introductory passages summarise the known functions of the major hormones in teleostean reproduction, in particular those selected for investigation; they consider the seasonal cycles, where known, and in particular discuss suggested involvements of these hormones in VTG uptake.

7.1.2 Pituitary hormones

One of the pituitary glands primary products, GtH, has been consistently considered to be one of the major endocrine mediators in the control of reproduction influencing both vitellogenesis and maturation (Campbell and Idler, 1976): GtH induces oestrogen production by the ovary which in turn is responsible for the stimulation of hepatic VTG production (Crim and Idler, 1978; Fostier *et al.*, 1979; Yaron and Barton, 1980). GtH also acts on the ovarian follicle both to stimulate pinocytotic uptake of VTG (Campbell, 1978; Upadhyay *et al.*, 1978; Campbell and Idler, 1980; Abraham *et al.*, 1984) and subsequently to induce the synthesis of maturation stimulating hormones, the progestagens (reviewed in Nagahama, 1987).

Gonadotropin secretion is stimulated by gonadotropin releasing hormone (Gn-RH), produced in the hypothalamus in response to various sensory inputs, which include environmental signals, (particularly photoperiod in the salmonids) which in turn is modulated by steroid negative feedback (Goos, 1978; see Figure 7.1).

Controversy exists in the literature, however, regarding the numbers and nature of GtH's in fish. One hypothesis maintains that a single GtH, rich in

Figure 7.1 The hormonal control of follicle development in the rainbow trout (solid lines indicate known pathways of action and broken lines suggested ones; modified from Bromage and Cumaranatunga, 1988)

carbohydrate, is responsible for inducing both maturation and ovulation (Burzawa-Gerard, 1974) and the second hypothesis suggests that 2 distinct GtH's exist, each with separate functions; one inducing VTG uptake and the second inducing ovulation (Campbell and Idler, 1976; Ng *et al.*, 1980).

The first concept of a single GtH is supported by the findings that a single glycoprotein, absorbed on the affinity gel Concanavalin-A has been isolated from the pituitary of the carp (Burzawa-Gerard, 1971), chinook salmon (Donaldson *et al.*, 1972) and sturgeon (Burzawa-Gerard, 1982). This preparation stimulates the rate of VTG incorporation by follicles in a number of teleosts (Burzawa-Gerard, 1974; Sundararaj *et al.*, 1982; Le Menn and Burzawa-Gerard, 1985) and has a capacity to induce steroid production by the gonad (Ng *et al.*, 1980). This GtH was shown by Ng and co-workers to induce the production of 17ß-oestradiol during the vitellogenic phase of gonad development and 17α,20ß-dihydroxy progesterone shortly preceeding ovulation.

The second hypothesis, suggesting that 2 forms of GtH exist, was put forward by Idler and his co-workers from work on the winter flounder (*Pseudopleuronectes americanus*), American plaice and chum salmon. The types of pituitary GtH's are characterized on the basis of their affinities on a concanavalin A-Sepharose column. The first fraction known as Con AI is low in carbohydrate (and hence not retained by the column) and is said to stimulate incorporation of VTG into vitellogenic follicles (Campbell and Idler, 1976; Ng *et al.*, 1980). The second fraction, known as Con AII, in contrast to the Con AI is rich in carbohydrate and is bound by the column of lectins. This Con AII GtH is primarily involved with the stimulation of steroid secretion by the ovary, final maturation of the follicle and ovulation. These authors emphasise that the Con AI is a distinct GtH and not a partially glycosylated form from the carbohydrate-rich Con AII GtH. They further demonstrated a non-interference of the salmon Con AI hormone (so called vitellogenic GtH) in the RIA for Con A II GtH (so called 'maturational' GtH) reflecting a dissimilarity between the antigenic sites of the 2 molecules (Ng and Idler, 1978). When compared with gonadotropin of higher vertebrates the Con AII GtH bears a resemblance to luteinising hormone in having a steroidogenic and maturational role and the carbohydrate-poor, Con AI GtH a superficial resemblance to follicle stimulating hormone (FSH) in that both stimulate follicle growth. Difficulties with the '2 GtH' concept from Idler and his co-workers were met however, in studies on hypophysectomised winter flounder (animals dissected

of their pituitary gland and therefore of any endogenous gonadotropin production), where salmon carbohydrate rich GtH stimulated the formation of yolky follicles (Ng *et al.*, 1980). These authors suggested that the follicles had been primed by Con Al-GtH in the recipient flounders prior to hypophysectomy.

Recent attempts to resolve the controversy surrounding the number and function of teleost GtH(s) have complicated the issue further. Kawa uchi *et al.*, 1986) isolated 2 separate GtHs in the chum salmon and both were found to exist in carbohydrate-rich and carbohydrate poor forms. Similarly in the goldfish, Van der Kraak and co-workers (1987) isolated 2 GtH(s), both existing in carbohydrate rich and carbohydrate poor forms. Both of the goldfish GtH forms were steriodogenic, however, they showed marked differences in biological potency with differences in degrees of glycosylation. As yet it is unclear whether the different steroidogenic potencies are due to alterations in receptor-binding affinity and/or differences in clearance from the blood. To date the 'vitellogenic' activity of these gonadotropins has not been assessed. Clearly the multiplicity of forms and/or isohormones of fish GtHs is a complicated problem and it seems likely that discrepancies in the numbers and functions of GtH's in teleosts may arise, at least in part, from different purification procedures and through the use of heterologous bioassays.

Radioimmunoassays for the measurement of plasma levels of salmonid GtH have been developed in various laboratories. However, the uncertainty in number, biological properties and chemical structure of GtH in salmonid pituitaries make it difficult to know exactly what many of the radioimmunoassays measure. Even so, all the salmonid GtH RIA's developed to date, although they use different hormone preparations and different antibodies, appear to measure similar material(s) in the blood (Crim *et al.*, 1973; 1975; Breton *et al.*, 1976; Tan, 1976; Billard and Breton, 1978).

Annual seasonal cycles for GtH have been published for a variety of salmonid species: brown trout (*Salmo trutta*) (Crim *et al.*, 1975, Billard *et al.*, 1978; Campbell and Idler, 1978), rainbow trout (Billard and Breton, 1978; Bromage *et al.*, 1982a,b; Scott and Sumpter, 1983), brook trout (*Salvalinus fontinalis*) (Crim *et al.*, 1973), Atlantic salmon (Crim *et al.*, 1975; Crim and Evans, 1978; Dodd *et al.*, 1978) chinook salmon (*Oncorhynchus tshawytscha*) (Crim *et al.*, 1973) and sockeye salmon (*0.nerka*, Crim *et al.*, 1973). In these studies there is wide agreement relating to the presence of high GtH values at and

around ovulation, (in the rainbow trout these serum levels may reach 25 $ng.ml^{-1}$ or more), but in most of the studies throughout the major part of the vitellogenic growth phase of ovarian development they remain constantly below $1ng.ml^{-1}$ often being non-detectable (Sumpter and Scott, 1987). These seasonal patterns of GtH, showing a peak in plasma concentrations at around ovulation, clearly demonstrate that all of the radioimmunoassays currently being used depend at least in part, on the antigenicity of the Con AII, maturational GtH fraction (Breton *et al.*, 1976; Peter, 1981, Sumpter *et al.*, 1984). It should be stressed that the majority of studies following the seasonal cycles of GtH have used heterologous assays. Using a homologous rainbow trout assay developed by Breton *et al.*, (1976) a number of studies have consistently demonstrated 2 phases of increased levels of blood gonadotropin during the ovarian cycle of the rainbow trout (Bromage *et al.*, 1982a,b; Whitehead *et al.*, 1983). The second and largest peak in immunoreactive gonadotropin, coincided with the final maturation of the follicle and ovulation (Billard *et al.*, 1978; Bromage *et al.*, 1982a,b; Zohar *et al.*, 1982) similar to that seen using the heterologous assays, however, there was also a second much smaller increase occurring early in the cycle around the time at which exogenous vitellogenesis begins (Figure 7.2). This early increase in GtH is probably involved in the initiation of vitellogenic ovarian growth (Bromage and Cumaranatunga, 1988): GtH is known to stimulate oestrogen secretion by the ovary and in turn VTG synthesis and release by the liver. It is unclear therefore why this initial increase in plasma GtH is not observed in the pre-ovulatory development of rainbow trout and other salmonids in other studies, but it may be due to the heterologous assays employed. An alternative explanation is that 2 GtHs do exist in the rainbow trout and both were employed in raising antibodies during the development of Breton's assay. Subsequently when used to measure seasonal changes in serum GtH this assay would detect both the 'vitellogenic' GtH (corresponding to the initial peak at the onset of exogenous vitellogenesis) and the 'maturational' GtH at ovulation. However, this is purely speculation and as yet the discrepancies in pre-ovulatory GtH levels using the different assays remains unclear. Zohar *et al.* (1982) suggested that GtH secretion in trout is pulsatile (as is the case in mammals), particularly during early gametogenesis, and this could account for differences in pre-ovulatory levels. In contrast, however, recent studies by Sumpter and Scott (1987) suggest that no episoidic release of GtH occurs throughout the reproductive cycle of the rainbow trout.

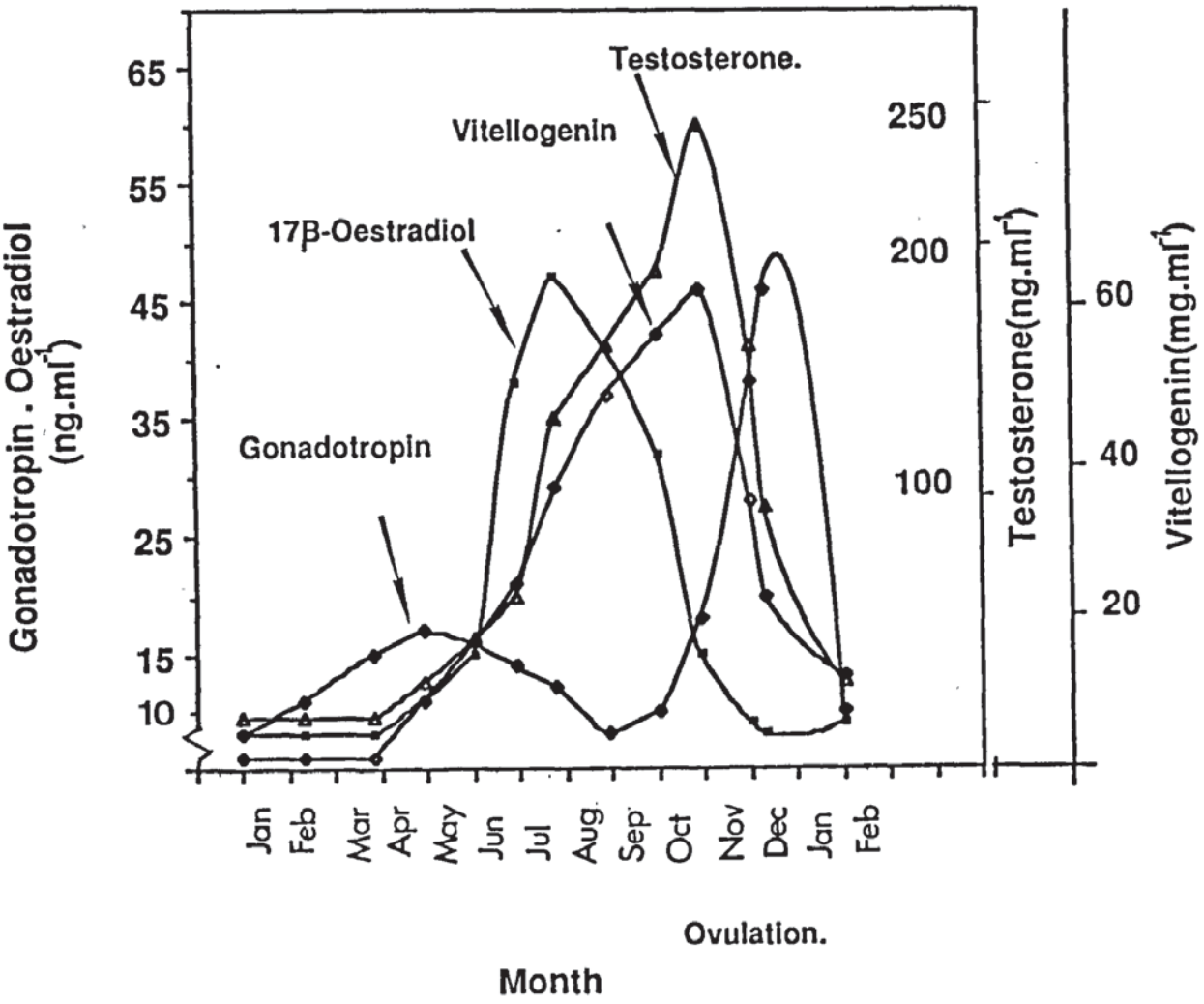


Figure7.2. Composite graph showing the changes in serum concentration of vitellogenin and a number of hormones associated with gonadal maturation in the female rainbow trout. The vertical lines denote the first day of the indicated months. (Taken from Bromage and Cumaranatunga, in press).

The control of VTG uptake by the developing follicles remains unclear. Although there is evidence that the maturational (glycosylated) GtH can stimulate the uptake of VTG by growing follicles in salmonids (Breton and Derrien Gerard, 1983; Wiegand and Idler, 1983), no measurable changes in serum GtH occur during the period of most active vitellogenesis. It is possible, however, that high serum levels are not necessary for VTG incorporation, as they appear to be for final follicle maturation. Alternatively a different 'vitellogenic' hormone may be active at this time. Idler (1987) recently reported that serum levels of carbohydrate-poor (Con AI) GtH paralleled the seasonal pattern for serum VTG in land-locked Atlantic salmon, and that the levels were very much higher than those found for carbohydrate-rich GtH during exogenous vitellogenesis; this supports their view that carbohydrate-poor GtH has the greater control on VTG sequestration. The lack of agreement between the different studies reviewed here highlights the present unsatisfactory understanding of GtH(s) in teleost pituitaries, and more specifically in salmonids. Clearly it constitutes an area of high priority for research. The following experiments investigated the action of a CHO-rich gonadotropin on VTG sequestration by vitellogenic trout follicles.

The role of hypophyseal hormones other than GtH(s) in teleost reproduction is even more questionable (Fontaine, 1976). Recent studies on the goldfish have suggested that the release of growth hormone from the pituitary is, at least in part, regulated by a common releasing factor (GnRH) to that of GtH (Marchant *et al.*, 1987). If this is the case then the secretion of both hormones could be concomitant and therefore growth hormone may have a role in controlling teleost reproduction. Singh *et al.*, (1987) working on hypophysectomized mummichogs, showed that salmon growth hormone had an effect on gonadal steroidogenesis and directly stimulated oestrogen production by the developing ovary. Presently, however, there are no data available on circulating levels of growth hormone and therefore it is not possible to assess whether they alter during the reproductive cycle. Studies on teleost fish have been limited by the lack of purified fish growth hormone and to date there is no evidence for a direct involvement of growth hormone in VTG uptake. However, the recent purification of the first fish (salmon) growth hormone (Kawa uchi *et al.*, 1986) and the subsequent cloning and expression of the salmon growth hormone gene should now allow more rapid progress in this area, and provided the opportunity to investigate its action(s), if any, on VTG sequestration in this study.

Other pituitary hormones may exhert direct or indirect effects in reproduction in the salmonids, these include corticotropin, thyrotropin (TSH) and prolactin. Almost nothing is known about the direct action of corticotropin in fish reproduction however, it stimulates the production of adrenocorticosteroids which have frequently been implicated in reproduction of teleosts, especially migratory salmonids. These steroids are discussed later in this text. Thyrotropin (TSH) has an intrinsic gonadotropic activity in certain teleost species (Fontaine and Burzawa Gerard, 1977). Yaron *et al.* (1982) showed that bovine TSH had double the potency of a relatively crude salmon GtH preparation in stimulating 17ß-oestradiol production in *Saratheroden aure i.s.* Mammalian TSH has also been reported to have marginal steroidogenic effects on cultured teleost follicles (Iwamatsu, 1978; Hirose, 1980). These studies involving TSH must, however, be interpreted with care because contamination with only trace amounts of GtH may be enough to elicit a threshold response (Sage and Bromage, 1970). Furthermore, the homology between GtH and TSH (Fontaine *et al.*, 1976) could explain some 'gonadotropic actions' exhibited by TSH, especially when heterologous TSH's are used in studies on fish. Little is known about the action of the pituitary product, prolactin, in fish reproduction. Most of our knowledge of prolactin action in fish is based on osmoregulation studies in euryhaline species where prolactin is often recognised as the 'freshwater adapting hormone', favouring ion retention and water exclusion (Bern, 1985). A recent report, however, suggested that purified salmon prolactin (Kawa uchi *et al.*, 1987) has a steroidogenic action, stimulating testosterone production by the testes of hypophysectomised *Fundulus* (Singh *et al.*, 1987). Unfortunately TSH and corticotropin have not been purified from any fish to date and prolactin from fish is in very short supply. Because of the problems of availability of these hormones and of those incurred when using pituitary products from different vertebrate groups mentioned above, the effects of these 3 pituitary hormones on VTG sequestration by developing follicles were not examined in this study.

7.1.3 Thyroid hormones

In teleosts, as in elasmobranchs, the likely involvement of thyroid hormones in reproduction has long been recognised, though their precise roles have yet to be established (reviewed by: Sage, 1973, Eales, 1979; Nagahama, 1983). Extensive unpublished studies by Dodd and Sumpter (communication

through JP Sumpter) have shown that in the lesser spotted dogfish thyroidectomy, if done before VTG uptake begins, prevents subsequent VTG uptake, but if performed after VTG sequestration has started does not prevent VTG uptake, suggesting that tri-iodothyronine (T_3) and thyroxine (T_4) somehow initiate VTG sequestration in the elasmobranch. Evidence for an involvement of thyroid hormones in reproduction in teleosts derives mainly from the close relationship that exists between thyroid and reproductive cycles (Bromage and Sage, 1968; Sage and Bromage, 1970; Ichikawa *et al.*, 1974; Osbourne and Sympsom, 1978; Cyr *et al.*, 1987). Circannual cycles of T_3 and T_4 in the rainbow trout (Osborn *et al.*, 1978; Leatherhead and Sunstegard, 1980, 1981; Cyr *et al.*, 1987) suggest a strong interrelationship between the annual variations and sex steroids and indicate a role for thyroid hormones in early ovarian development (see Figures 7.2 and 7.3). Cyr and Eales (1986) demonstrated that thyroid hormones, especially T_3, acted upon ovarian follicles of rainbow trout *in vitro* to amplify GtH action with regard to oestrogen secretion. Further involvement of T_3 in the ovarian cycle was indicated when T_3 restored the ovarian response to exogenous pituitary extracts in sturgeons subjected to captivity stress or experimental cooling (Detlaff and Davydova, 1974, 1979). There is some evidence that T_4 enhances the ovarian uptake of VTG (Lewis and Dodd, 1974; Hurlburt, 1979), however, a more recent study by Ng and co-workers (1984) reported that elevations of endogenous T_3 and T_4 levels and treatment with thiourea had no effect on yolk incorporation.

In conclusion although the thyroid axis is clearly involved in fish reproduction it is unclear whether the thyroid hormones play a permissive or synergistic role in gonadal growth or whether they have a direct role in stimulating protein incorporation by the developing ovary. The direct effects of T_3 and T_4 on VTG sequestration were therefore examined in this study.

7.1.4 Steroids

During the early part of exogenous vitellogenesis there are increases in serum levels of the steroids, 17ß-oestradiol, oestrone and teleosterone (Figure 7.2; Elliott *et al.*, 1984). 17ß-oestradiol is one of the most important steroids

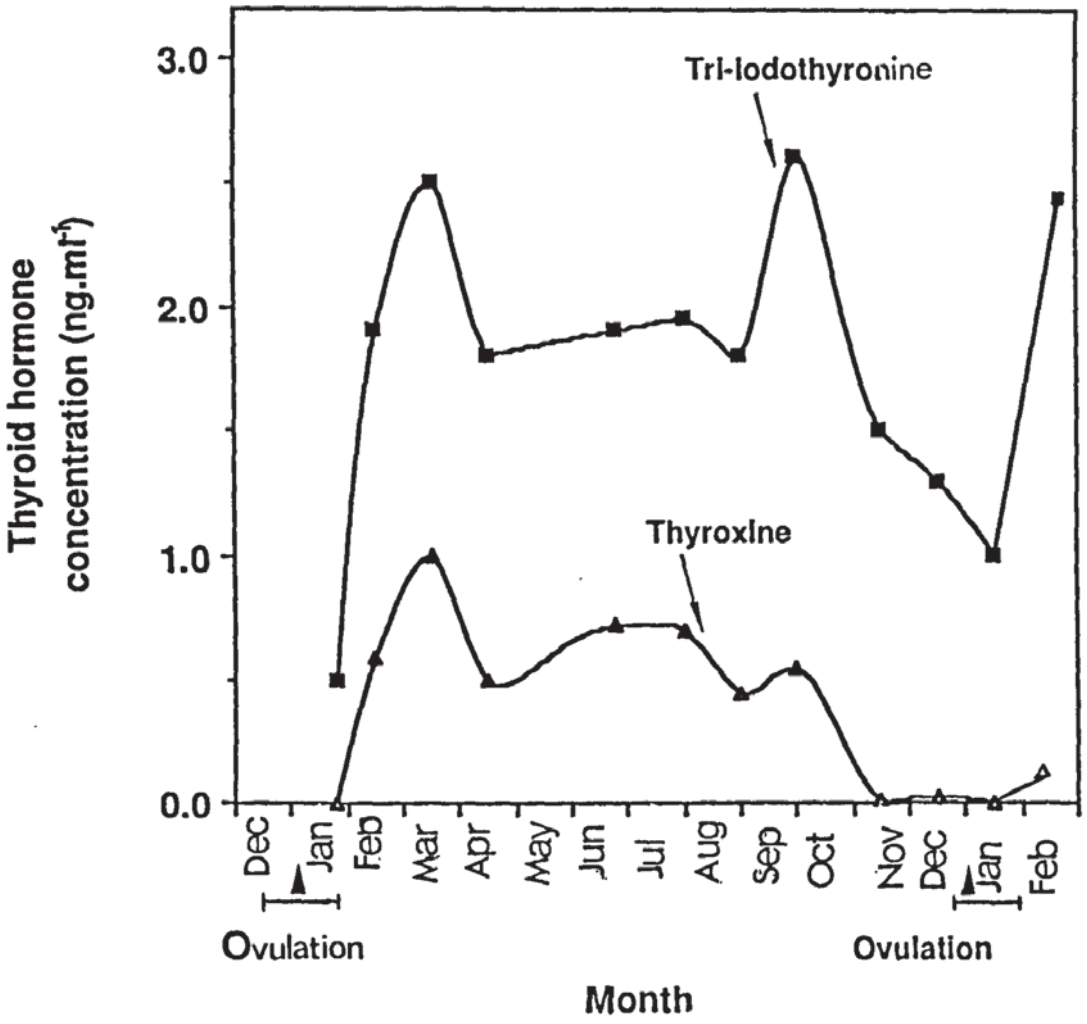


Figure 7.3 Graph showing changes in the serum of the thyroid normones, thyroxine(T4) and tri-iodothyronine(T3), associated with gonadal maturation in the female rainbow trout.The vertical lines denote the first day of the indicated months. (Taken from Cyr *et al.*, in press).

involved in the control of vitellogenesis. In non-mammalian oviparous vertebrates this hormone, produced in teleosts by the ovary in response to GtH, has a universal stimulatory action on the hepatic production of VTG (lamprey (Pickering, 1976), hagfish (Yu *et al.*, 1981), elasmobranch (Craik, 1978b), teleosts (Elliott *et al.*, 1984), amphibians (Wallace and Bergink, 1974), reptiles (Callard and Banks, 1970; Ho *et al.*, 1981) and birds (Heald and Machlan, 1964)). In addition to inducing the synthesis of VTG, 17ß-oestradiol may have other roles related to vitellogenesis. In the rainbow trout, for example, it increased plasma levels of very low density lipoprotein, which carries triglycerides into the developing follicles (Skinner and Rogie, 1978).

In vitro studies on the amago salmon provide evidence that 17ß-oestradiol is produced in granulosa cells of the follicle from substrates, including testosterone provided by the theca (Kagawa *et al.*, 1983; Young *et al.*, 1983). Little is known about the transport of metabolites between the theca and granulosa; it has been suggested they may simply diffuse across the basement membrane or be actively transported (Wingfield and Grim, 1977). In the rainbow trout blood levels of 17ß-oestradiol start to increase at the onset of exogenous vitellogenesis and peak during the period of most active vitellogenesis, generally the autumn (Figure 7.2, Whitehead *et al.*, 1978a,b; Bromage *et al.*, 1982a,b; Fostier *et al.*, 1978, 1983; Scott *et al.*, 1980; Scott and Sumpter, 1983; Elliott *et al.*, 1984). Subsequently, levels of oestradiol fall to reach basal levels approximately one month prior to ovulation (Whitehead *et al.*, 1978a; Fostier *et al.*, 1978, 1983; van Bohemen and Lambert, 1981; Elliott *et al.*, 1984). Vitellogenin levels in the blood similarly increase with the rising concentration of 17ß-oestradiol, however, despite the fall of 17ß-oestradiol before ovulation, VTG levels continue to increase, reaching their highest values at the time of ovulation (Figure 7.2) and only return to basal levels a month or so after spawning (Bromage and Cumaranatunga, 1988). Studies conducted on the rainbow trout to date suggest that oestradiol does not stimulate VTG incorporation into the follicle (Campbell and Idler, 1976; Cumaranatunga, 1985). Indeed experiments conducted by Cumaranatunga showed that pharmacological doses of 17ß-oestradiol can inhibit VTG uptake and cause follicular atresia. These investigations were performed *in vivo* however, and one cannot determine whether the effects were directly attributable to the hormone's action on the sequestration process; a feedback-inhibition of endogenous gonadotropin secretion

may lead to atresia (Fostier *et al.*, 1983). The *in vitro* culture methods developed in Chapters 5 and 6, allowed the direct involvement(s), if any, of 17ß-oestradiol to be investigated.

Oestrone shows a similar pattern of production to 17ß-oestradiol during exogenous vitellogenesis, serum levels reaching a peak during the period of most active vitellogenesis (Whitehead *et al.*, 1978a,b; van Bohemen *et al.*, 1982). However, oestrone is far less potent than 17ß-oestradiol in stimulating hepatic VTG production (Elliott, Bromage and Sumpter, 1980, unpublished) and its importance in this process remains unclear. van Bohemen and Lambert (1981) and van Bohemen *et al.*, (1982) suggested that oestrone primes the liver for subsequent 17ß-oestradiol induction. It is possible however, that oestrone levels merely reflect the biosynthesis precursors and products during the production of 17ß-oestradiol by the ovary. The effects of oestrone on VTG uptake were not investigated in this study and although it appears unlikely that this oestrogen is involved in the direct stimulation of VTG sequestration this hormone has yet to be studied in an *in vitro* culture system.

The functional role of testosterone in female teleost reproduction is less clear than that of most other circulating steroids. Testosterone levels in the serum start to increase in the trout at approximately the same time as the oestrogens (Figure 7.2) and can reach concentrations of greater than 300 ng. ml^{-1}, peaking about a week before ovulation and well after the peak in 17ß-oestradiol. These levels are considerably higher than those found in mature males (Scott *et al.*, 1980). Testosterone is a precursor of other sex steroids and therefore may have an important regulatory role in dictating the amount of 17ß-oestradiol secreted into the circulation for the stimulation of VTG production. If so this process would clearly affect the availability of VTG for sequestration and subsequently the amount incorporated by the developing follicles. However, as suggested by Bromage and Cumaranatunga, (1987) the very high levels of testosterone reached in female fish would imply that this hormone plays a more definite role in ovarian maturation. There is evidence that testosterone is involved in the development of atresia in higher vertebrates (Magoffin and Erickson, 1981). Studies by Cumaranatunga *et al.* (1985) on female trout showed that treatment with testosterone during exogenous vitellogenesis produces atresia. However, as with the treatments with 17ß- oestradiol, mentioned earlier , it is not possible to

differentiate specific effects of the steroid administered, in this case testosterone, from a general increase in the level of steroidal negative feedback on gonadotropin secretion (Fostier *et al.*, 1983). Reductions in levels of GtH might in turn decrease VTG uptake by the developing follicles, resulting in atresia (Bromage and Cumaranatunga, 1988). Although oestrogen levels fall to basal after a month or so before spawning testosterone levels remain above basal for 2 months after ovulation and Bromage and Cumaranatunga (1988) have speculated that testosterone may be involved in controlling some of the changes that occur in the ovary after ovulation in preparation for the next cycle. Clearly the few studies conducted to date on the direct involvements of testosterone in vitellogenesis have not provided any conclusive answers and a definite function of the high plasma levels has yet to ascribed. The uncertainty of testosterone's function during exogenous vitellogenesis and the parallel profiles of serum testosterone and VTG during this phase of reproductive development prompted us to investigate the effects of this steroid on VTG sequestration in this study.

Approximately 1-2 weeks before ovulation the ovary synthesises maturation-inducing steroids, the progestagens (Nagahama *et al.*, 1985). Several progestogens are produced and secreted into the blood, these include: 17αhydroxyprogesterone (17αOH-P); 17αhydroxy-20ß-dihydroxyprogesterone (17α20ß-P); 17αhydroxy-20αprogesterone (17α20α-P); deoxycortisone, and 11α-hydroxyprogesterone.17α 20ßOH-P in particular has been identified as the maturation-inducing steroid in the rainbow trout (Scott and Baynes, 1982; Scott *et al.*, 1982; Goetz, 1983) and may reach concentrations of 500 $ng.ml^{-1}$ at ovulation. The very high levels at ovulation, and injections of this hormone, lead to germinal vesicle breakdown (Goetz, 1983, Figure 7.2). It has been suggested that the extremely rapid rise of 17α20ßOH-P during the few days prior to ovulation, thought to be triggered by gonadotropic hormone stimulation, is made possible, at least in part, by the presence of high titres of serum testosterone and a change in the steroid biosynthetic pathway in the ovary. Both Young *et al.*, (1983) and Nagahama *et al.*, (1984) working on the follicles of amago salmon *in vitro* have shown that the decrease in plasma 17ß-oestradiol towards ovulation and the concurrent increase in 17α20ßOH-P result from a progressive loss of aromatase and increased amounts of 20ß hydroxysteroid dehydrogenase, the key enzymes mediating 17ß-oestradiol and 17α20ß OH-P production from testosterone, respectively. How the biosynthetic pathway is switched from

oestrogen to progestagen production is controlled, however, is not yet known. The progestagens clearly play an integral role in follicle maturation, however, they are not present in the serum of females during the major part of exogenous vitellogenesis and cannot have a controlling influence on VTG sequestration during this period, therefore they were not investigated in this study.

Sexual maturation in several species of salmonids is accompanied by a hyperplasia of the interrenal tissue (Robertson and Wexler, 1959; Hayl and Carpenter, 1972; McBride and van Overbeeke, 1969) and an elevation of the levels of circulating corticosteroids, especially cortisol (Home and Roberts, 1959; Pickering and Christie, 1981). In the sockeye salmon, the increase in interrenal activity is characterized by increases in both the secretion rate and metabolic clearance rate of cortisol (Donaldson and Fagerlund, 1968). A single report by Sundararaj and Goswami (1977) suggested that adrenal corticosteroids were involved in VTG sequestration in the catfish *Heteropnuestes fossilis.* In the salmonids, however, it is suggested that changes in levels of circulating corticosteroids are primarily related to the demands of spawning and the reduction or cessation of feeding during the later stages of vitellogenic development (Pickering and Christie, 1981), i.e. caused by the stress imposed by reproduction. Indeed the extent of hyperplasia of the interrenal tissue varies considerably between individuals of the same species and is much less pronounced in hatchery reared rainbow trout than wild migrating salmonids (Robertson and Wexler, 1959). The catabolic effects of corticosteroids in wild salmon, particularly during the later stages of migration, would facilitate the mobilization of energy from body reserves or tissues for increasing gonadal mass and helping migration (Sower *et al.*, 1984). Relating cortisol levels to gonadal growth is difficult, for it is produced in response to stressful stimuli and therefore becomes raised in the blood when handling the animals during sampling. The role of cortisol during teleost reproduction is probably an indirect one, stimulating changes in general fish metabolism and not direct one stimulating gonadal growth. From the evidence provided in salmonids, the corticosteroids do not appear to be major hormones in mediating VTG sequestration by the ovary and therefore were not examined further in these experiments.

7.1.5 Other hormones

Very little is known about other hormones in relation to reproduction in fish. In mammals, however, during the last decade it has become apparent that insulin and insulin-like growth factors are regulators of ovarian function, as well as the GtH's, FSH and LH. Potential mechanisms (precise involvements have yet to be established) underlying the 'gonadotropic' activity of insulin in mammals include; direct effects on steroidogenic enzymes, modulation of FSH and LH receptor numbers or synergism with FSH and LH (Poretsky and Kalin, 1987).

The available data in amphibians, far exceeding that in teleosts, indicates that blood insulin levels follow a seasonal cycle paralelling ovarian development (Schlaghcke and Blum, 1981). *In vitro* studies indicate that insulin can stimulate the re-initiation of meiosis in *Rana pipiens*(Lessman and Marshall, 1984) and is capable of stimulating VTG uptake into vitellogenic follicles of *Xenopus laevis* (Wallace, 1983). Together these findings in the amphibian suggest that insulin may play a direct role in follicle development, as well as a supportive one, in the lower vertebrates generally.

Blood insulin levels in fish also fluctuate according to their life cycles and in salmonids plasma insulin levels may reach 30 $ng.ml^{-1}$ (Plisetskaya *et al.*, 1986).

In teleosts however, insulin plays a role in amino acid metabolism, as well as in the homeostasis of glucose (although its role here is less important than in mammals (Thorpe and Ince, 1977; Ottlengli *et al.*, 1982; Huth and Rapport, 1982)). In the sea bass, *Dicentrarchus labrax* plasma insulin levels show daily variations which are dependent on both the feeding times and composition of the diets (Gutierrez *et al.*, 1984). Furthermore, Huth and Rapport (1982) have demonstrated that the synthesis of insulin in carp islets *in vitro* is clearly influenced by temperature. Clearly the multiple functions of insulin in metabolism in fish make it difficult to relate levels in the blood to reproduction and it is not known whether this hormone plays a direct role in stimulating follicle growth. The finding in *Xenopus* that insulin was capable of stimulating VTG uptake and the availability of purified salmon insulin prompted an investigation on insulins action of VTG uptake in the rainbow trout conducted here.

The involvement of hormones in fish vitellogenesis, especially VTG uptake, is clearly complex and in many areas poorly understood. To determine the direct effect of hormones on VTG uptake one has to isolate their actions. This is

most easily accomplished using an *in vitro* system. Two experiments were conducted in this chapter to investigate the hormonal control of VTG uptake using the chosen hormones: a highly purified CHO-rich (maturational) GtH from chum salmon, chinook salmon growth hormone, a pituitary extract (PE) obtained from a female rainbow trout undergoing exogenous vitellogenesis, thyroxine (T_4), tri-iodothyronine (T_3), 17ß-oestradiol and testosterone. The first experiment examined the influence of the various hormones and pituitary preparation on VTG sequestration in isolated vitellogenic follicles. The second study determined the dose-responses to GtH and insulin on both VTG uptake and ovarian steroidogenesis.

7.2 Materials and methods

The first section in these materials and methods describes the hormonal preparations, including their origins, used in this study. The succeeding sections explain the protocols for the experiments.

7.2.1 Hormone preparations

Growth hormone (GH)

Chinook salmon GH was a gift to J Sumpter from Dr. P. Le Bail Rennes, France. It was supplied at a concentration of 1 $\mu g.ml^{-1}$ in sterile phosphate buffered saline. Although this preparation was the most purified available at the time, recent reports suggested that it may have been contaminated with a little gonadotropin.

Gonadotropin (GtH)

Chum salmon GtH was purified by Paul Copeland (Brunel University; coded D2-GtH). The preparation was the carbohydrate-rich, maturational GtH. It was supplied at a concentration of 5 $\mu g.ml^{-1}$ in sterile phosphate buffered saline.

Pituitary extract (PE)

The pituitary gland of Fish I, from when the first group of vitellogenic follicles for culture was obtained, was carefully dissected out and thoroughly washed in physiological saline. The gland was then homogenised in 1 ml of culture medium in a glass homogenizer and the extract centrifuged at 1500g for 10 minutes. The supernatant was withdrawn and used in the subsequent experiments.

Thyroxine (T4) and tri-iodothyronine (T3)

These thyroid hormones were purchased from Sigma Chemical Company and after weighing made up to a concentration of 5 $\mu g.ml^{-1}$ in 0.1M sodium hydroxide (T_3 and T_4 dissolve more easily in basic pH's than at neutrality).

17ß-Oestradiol and testosterone

17ß-oestradiol and testosterone were supplied in crystalline forms by Sigma Chemical Company. Both steroids were dissolved in solutions of culture medium and ethanol (in an equal ratio), at an initial concentration of 10 mg. ml^{-1}.

Insulin

The teleost insulin was from chum salmon, purified by Dr E Plisetskaya (Plisetskaya *et al.*, 1986) and given to J Sumpter. The preparation was supplied at a concentration of 5 $\mu g.ml^{-1}$ in 0.1% hydrochloric acid (it is more soluble in dilute acid).

All the purified hormones were serially diluted in culture medium to produce stock solutions containing $100ng.ml^{-1}$.

7.2.2 Experiment 1: The effects of several different hormones on vitellogenin uptake in cultured follicles

Separate groups of 20 follicles from 2 vitellogenic fish were cultured with 13 ± 0.5 mg ^{3}H.VTG ml^{-1} for 18 hours. Follicles from Fish I (3.2 ±0.1mm) were cultured with the following hormones: carbohydrate-rich GtH, GH, insulin, T_3, T_4 or a pituitary extract. The PE was obtained from the female donating the follicles for culture and added at a concentration of 0.16% of the total soluble extract per ml of culture media; this dilution was based on the fact that a pituitary gland from a maturing female fish contains approximately 50 000 ng carbohydrate-rich GtH (Sumpter and Scott, 1987), and thus the dose of 0.16% (v/v) of the extract added per 1 ml media should have produced a dose of about 100ng GtH ml^{-1} (it should be realized, however, that this extract would contain all other pituitary hormones). Follicles from Fish II (4.0 ±0.15mm) were cultured with GtH, insulin, testosterone or 17ß-oestradiol. All purified hormones were added so that their final concentration was 100 ng ml^{-1}. Follicles cultured under the same conditions but without any hormone were used as controls. At the end of the cultures viable follicles were washed, counted for radioactivity and the rates of VTG sequestration determined. The rates of uptake in each follicle were compared using a Multiple Comparison of the Means test (Gabriel, 1978; see Chapter 2).

7.2.3 Experiment 2: Dose-responses of gonadotropin and insulin on both vitellogenin sequestration and ovarian steroidogenesis

Vitellogenic follicles (4.0 ± 0.15mm) were incubated in a medium containing 13 mg VTG.ml^{-1} with a range of GtH or insulin doses between

0.1-1000 ng.ml^{1} for 18 hours. Control groups were cultured without any hormone. The abilities of the hormones to stimulate VTG uptake were determined by measuring VTG sequestration in the normal way, and were examined on the basis of both hormone weight and molar concentrations. In parallel the steriodogenic potencies of these hormones were assessed by measuring testosterone levels in the culture media. For each hormone dose the levels of testosterone were assayed at the beginning and end of the culture period.

7.2.3.1 Radioimmunoassay for testosterone

The assay technique used for testosterone measurements was developed by Whitehead (1979) and Elliott (1982).

7.2.3.1.1 Materials

(a) Assay buffer

$Na_2H\ PO_4$ 8.88g, $NaH_2\ PO_4$ 5.82g, sodium chloride 4.5g sodium azide 0.03g, and gelatin 0.5g.

All the constituents were dissolved in 'Analar' water and made up to a final volume of 500ml.

(b) Ethyl acetate

(c) 1,2,6,7-^{3}H testosterone (Amersham International plc): 80 curie mM^{-1}) Stock solution 2.22 x 10^{7} dpm, working sol ution 20 000 dpm 100 µl^{-1}.

100 µl of the stock solution (made up in absolute ethanol) was dried down under a flow of pure nitrogen and then reconstituted with 10 ml of the assay buffer.

(d) Antiserum

Antiserum was supplied as freeze dried pellets (Steranti research, St. Albans, Herts). The dry pellet was dissolved in 1ml of assay buffer and frozen in 100 µl aliquots until required. When required each aliquot was thawed and made up to 10 ml with the assay buffer.

(e) Standard

A standard stock solution of 1000 pg 100 μl^{-1} was prepared in ethanol and subsequently a working solution made up by diluting 100 µl of the standard in 1 ml of ethanol. The working solution contained 100 µg 100 μl^{-1}. Using the working solution a series of dilutions, ranging from 4 to 500 pg was prepared in assay buffer.

(f) Dextran-coated "separex" charcoal tablets (Steranti research)

A single tablet was dissolved in 50ml of assay buffer and thoroughly mixed to produce a homogenous suspension. Once prepared this solution was maintained at 4°C.

7.2.3.1.2 Method

(a) 50 µl of each culture medium, before and after culture procedures, were pipetted into separate polypropylene tubes (Hughes and Hughes Ltd, Romford, England).

(b) To each tube 2ml of ethyl acetate was added and the tube tightly stoppered.

(c) After 1 hour on a rotary mixer the tubes were centrifuged at 1500 rpm for 10 minutes and 50 µl aliquots of the supernatant solution transferred to rimless soda assay tubes for assaying.

(d) A series of dilutions of the standard hormone were made, ranging between 0-1000 pg.100μl^{-1}. Each dilution was made in duplicate and added to similar soda assay tubes.

(e) Extracts and standards were dried in a vacuum oven at 30°C; and the samples subsequently cooled in a refrigerator at 4°C.

(f) 100 µl of buffered antiserum was added to each of the sample and standard tubes, vortexed, for approximately 10 seconds and allowed to incubate on ice (at 4°C) for 30 seconds.

(g) 100 µl of the working solution of labelled testosterone was added to each of the tubes, vortexed for approximately 10 seconds and placed in the refrigerator (at 4°C) overnight.

(h) 0.5ml of the dextran-coated charcoal suspension was added to each tube, vortexed and incubated for 10 mins at 4°C.

(i) The mixtures were centrifuged at 2000rpm at 4°C and 0.4ml of the total 0.7ml supernatant pipetted into scintillation vials containing 10ml of Optiphase 'safe' scintillant.

(j) 100µl of the working solution of testosterone was added in duplicate to vials containing 10 mls of scintillation fluid to obtain the total number of counts added to each of the assay tubes.

(k) All vials were counted for 5 minutes.

7.2.3.1.3 Calculations

(a) Counts obtained for 100 µl of labelled hormone (total counts) = T (background counts (10ml scintillation fluid only were zero)).

$$\text{Corrected counts obtained for standards} = \text{counts} \times \frac{0.7}{0.4} \times \frac{\text{Total supernatant}}{\text{Fraction of supernatant counted}} = S$$

$$\text{Corrected counts obtained for unknowns} = \text{counts} \times \frac{0.7}{0.4} = U$$

(b) Therefore % bound for standards and unknowns = $\frac{S \text{ or } U}{T} \times 100$

(c) The percentage bound for each standard was plotted against the concentration of testosterone in each standard to obtain a 'standard curve' (see Figure 7.6)

(d) The concentration of steroid, in pg, present in each of the unknown or control samples was read off the standard curve using the percentage bound calculation formula.

(e) The amount of testosterone calculated in 50µl of culture medium was multiplied by 20 to give the amount present in 1ml of medium

(f) The intra-assay coefficient of variation, representing the difference between the calculated levels of known, equal standards placed at the beginning and end of the tubes to be analysed was approximately 5%.

(g) Cross reaction data for the testosterone antiserum, provided by Elliott (1982) are given in Appendix III.

7.3 Results

7.3.1 Experiment 1

The rates of VTG uptake in the presence of the various purified hormones are shown in Figure 7.4. Control follicles, receiving no hormone treatments, sequestered VTG at a rate of 23.74 ± 2.26 and 16.32 ± 2 $ng.mm^2$ hr^{-1} in Fishes 1 and 2 respectively. Analyses of variance each group of follicles showed there were significant differences in the rates of VTG uptake between the treated groups ($P < 0.001$). Multiple comparisons of the Means using 95% confidence units showed that GtH and insulin in both groups, and PE in group 1, significantly increased VTG sequestration. VTG incorporation was stimulated in the follicles of group 1 treated with GtH, insulin and PE by 34%, 27% and 22%, respectively. In Fish II GtH and insulin stimulated uptake by 24% and 20% respectively. Using the Multiple Comparison of the Means test T_3 treated follicles sequestered significantly less VTG than the control group. The remaining hormones showed no significant stimulatory or inhibitory action on VTG uptake.

7.3.2 Experiment 2

Vitellogenin uptake was stimulated in a dose-dependent manner with both GtH and insulin, but this uptake was only significantly elevated at doses of 100 $ng.ml^{-1}$ and above for both hormones ($P < 0.05$ Multiple Comparisons of the Means Test). GtH at doses between 0.1-1000ng gave uptake rates of VTG between 17.1-21.4$ng.mm^{-2}$ hr^{-1}., respectively, corresponding to percentage increases in sequestration above the controls (follicles receiving no hormones) between 4.5-30.8, respectively (Figure 7.5). Insulin stimulated VTG uptake at doses between 1-1000 $ng.ml^{-1}$, producing uptake rates between 16.9-20.6 $ng.mm^{-1}$ hr^{-1} respectively, which corresponded to percentage increases in sequestration above the control group of follicles between 3.5-26, respectively (Figure 7.5).

Figure 7.6 illustrates the standard curve from the testosterone RIA from which levels in the culture medium were determined. The mean value of testosterone in the culture medium containing the follicles at the onset of the incubations for the GtH- and insulin-treated groups was 1.7 ± 0.4 $ng.ml^{-1}$. GtH clearly stimulated testosterone production in a dose-dependent manner (Figure 7.7a) at doses of GtH between 1-1000 $ng.ml^{-1}$ testosterone production increased from 2 to 17 $ng.ml^{-1}$, respectively. At the maximum GtH concentration (1 $\mu g.ml^{-1}$) each follicle was producing approximately 77$pg.ml^{-1}$ hr^{-1}. Unlike

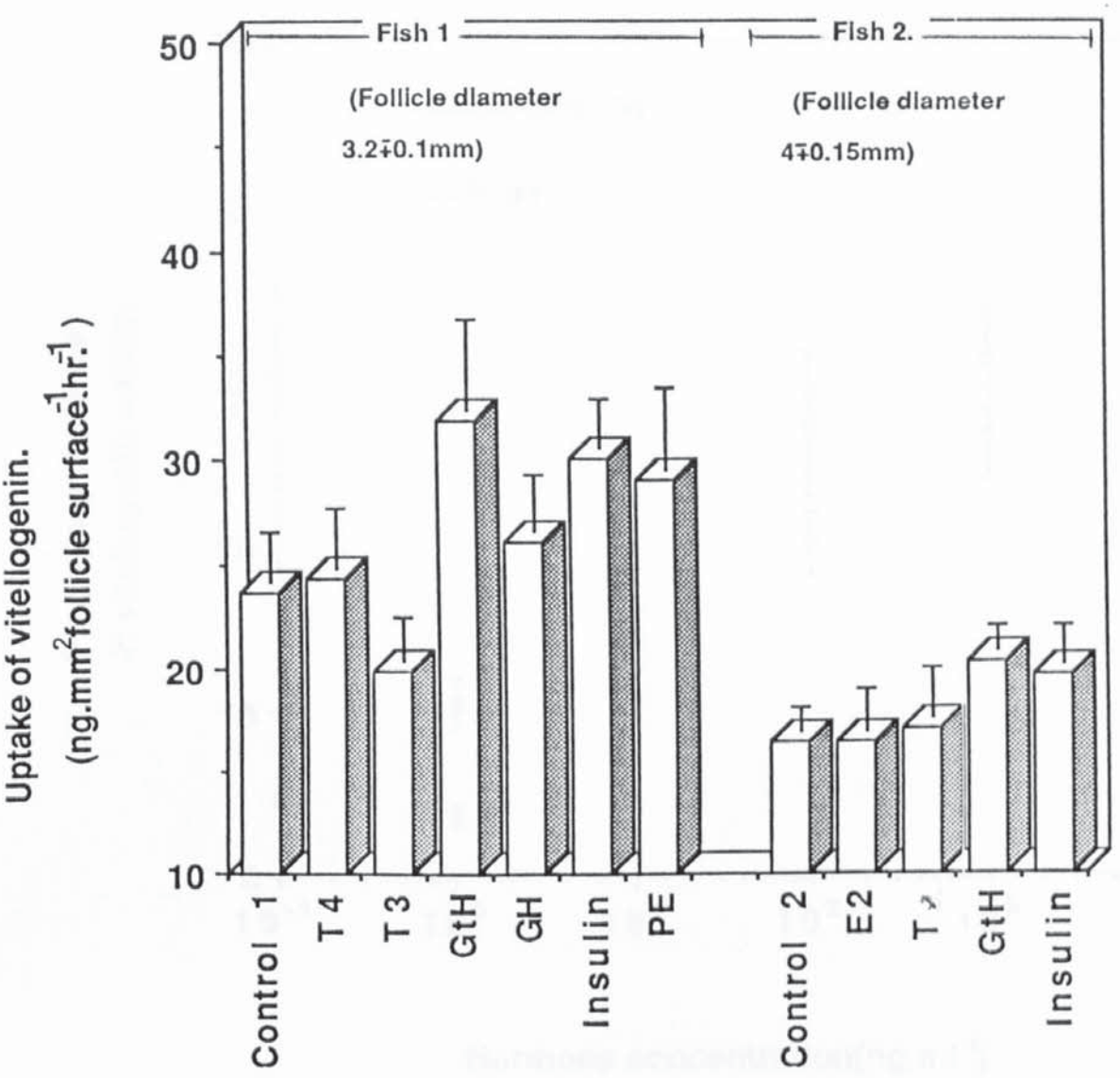


Figure 7.4. Rates of vitellogenin uptake in cultured vitellogenic follicles in the presence of several hormones. Each rate of sequestration represents the mean from 10 follicles. Vertical bars denote the standard deviations.

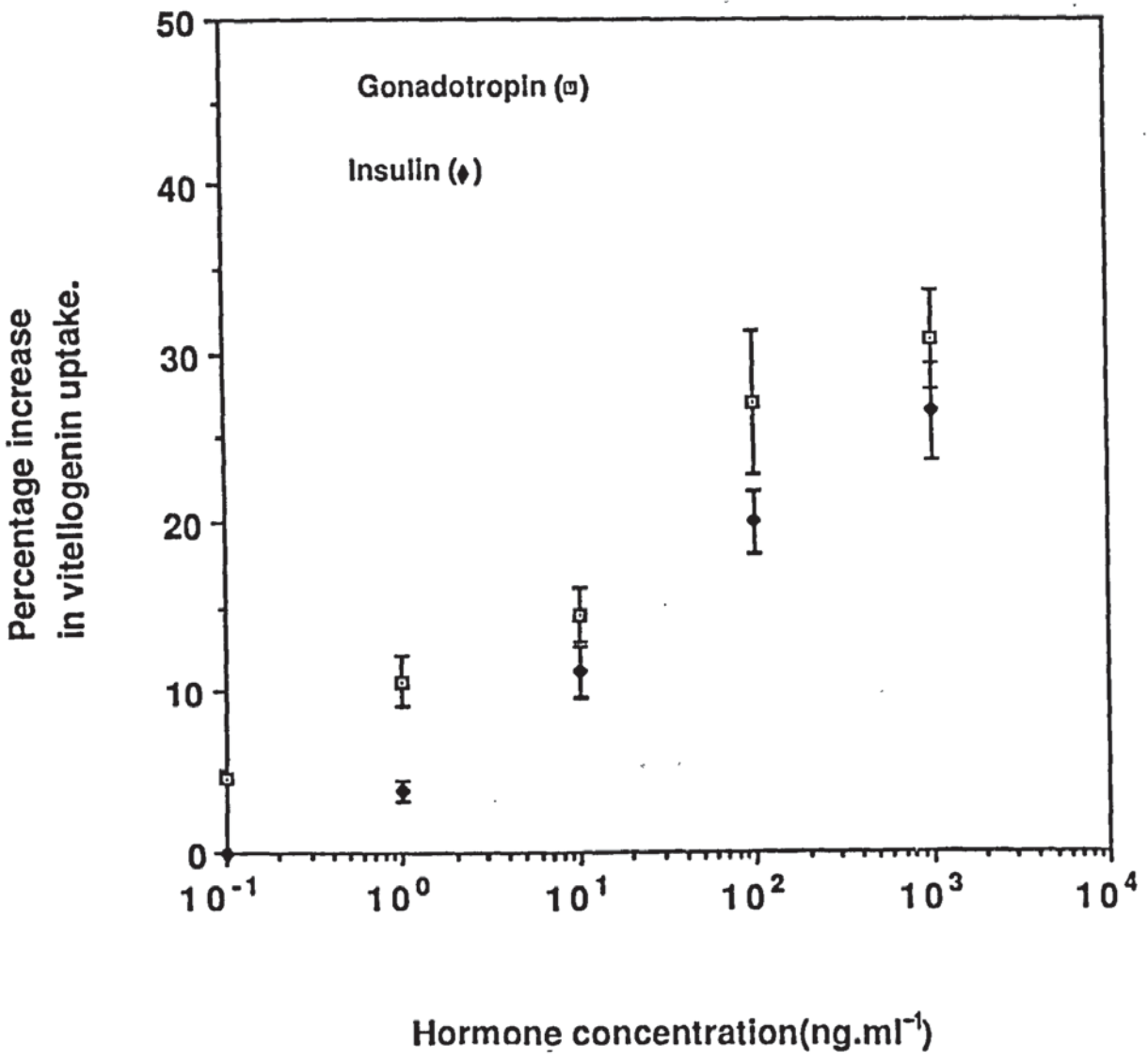


Figure 7.5. Dose-response relationships of gonadotropin and insulin on vitellogenin uptake in cultured vitellogenic follicles. Each point represents the mean uptake from 10 follicles. Vertical bars denote standard deviations.

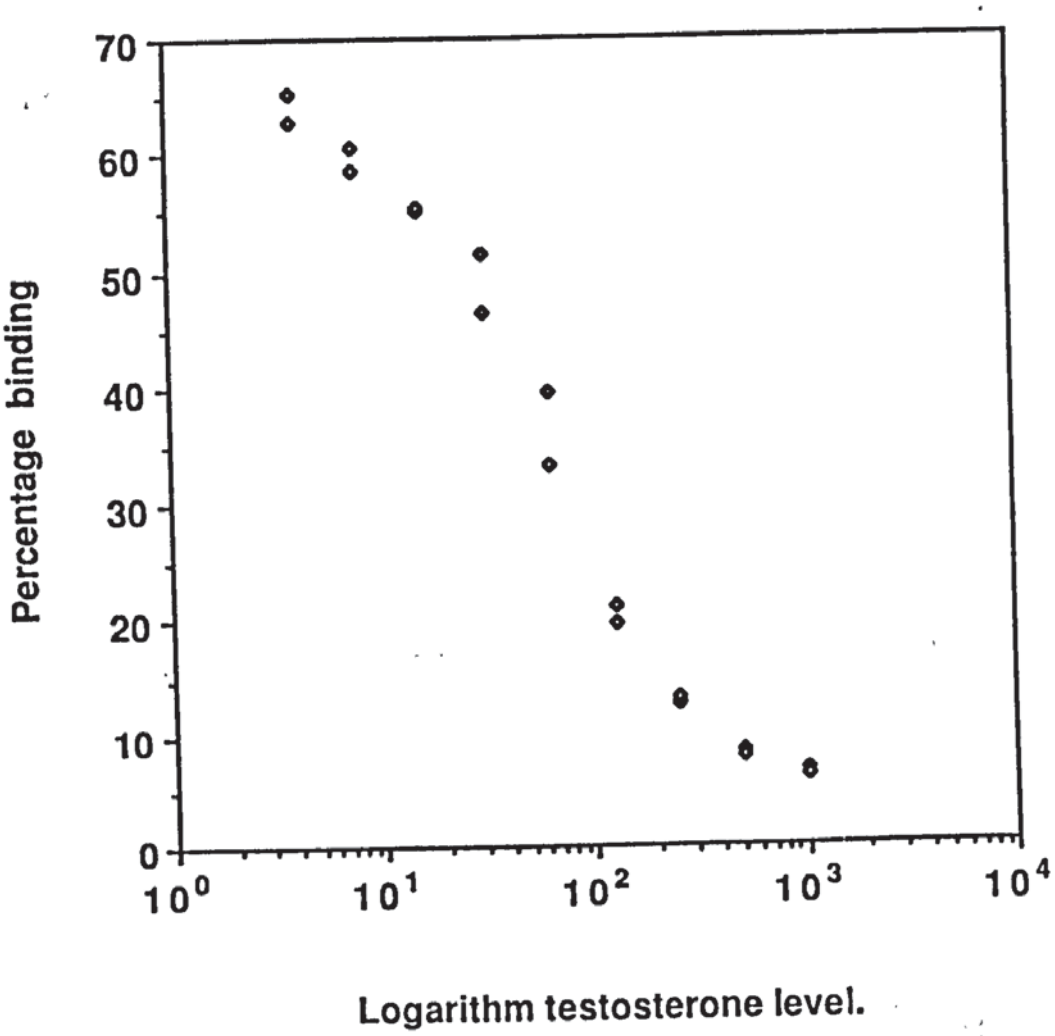


Figure 7.6. Standard curve for the testosterone radioimmunoassay.

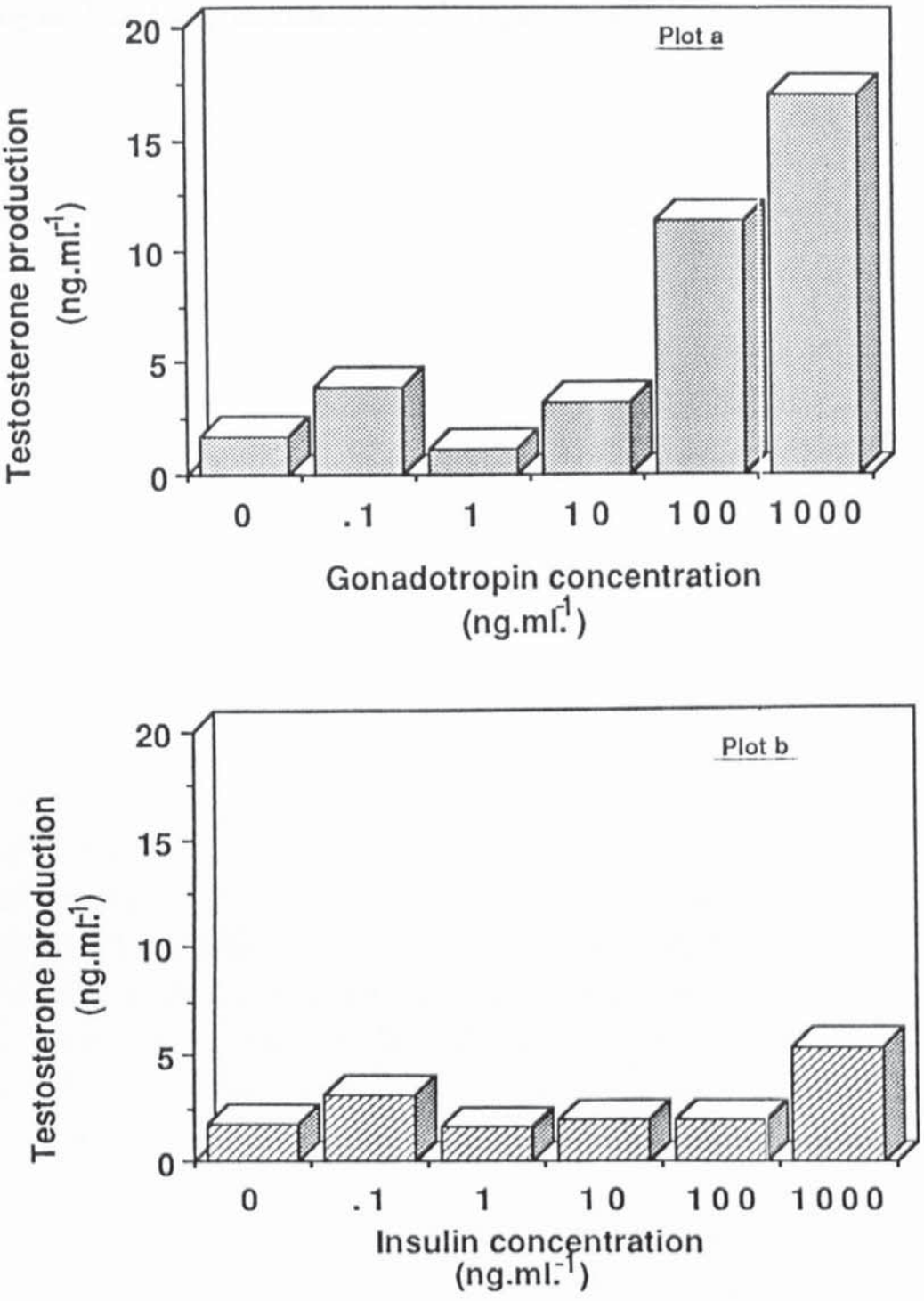


Figure 7.7. Dose-response relationships for gonadotropin (a) and insulin(b) on the production of testosterone by cultured vitellogenic follicles. The rates of testosterone production are expressed in ng.ml^{-1}. from10 follicles in18hours.

GtH, insulin had no steriodogenic activity, (stimulation of testosterone production), at doses up to and including 100 $ng.ml^{-1}$. At a concentration of 1 $\mu g.ml^{-1}$ insulin caused a slightly enhanced rate of testosterone production (from about $2ng.ml^{-1}$ to 5.4ng $testosterone.ml^{-1}$; Figure 7.7b).

7.4 Discussion

The rainbow trout pituitary extract and the chum salmon carbohydrate-rich GtH both significantly increased VTG uptake, thus demonstrating a direct involvement of teleost pituitary product(s) in VTG sequestration. A vitellogenic action for 'maturational' GtH in the rainbow trout has also been described by Breton and Derrien-Guimand (1983), who showed that a highly purified Con A-II GtH stimulated an increase in the rate of radiolabelled VTG incorporation by follicles maintained in a perfusion system. Similar actions were also reported on the silver eel, *Anguilla anguilla in vivo* after injections of carp Con A II GtH (Burzawa-Gerard, 1982). Together these studies and the data provided here suggest that the carbohydrate-rich GtH mediates VTG sequestration in vitellogenic follicles as well as having a role in maturation and ovulation.

Analysis of the culture medium from the follicles treated with rainbow trout PE in a chum salmon GtH assay (Sumpter, 1985) showed that it contained more than 2.5 times the concentration of carbohydrate-rich GtH than that in the follicle cultures containing purified chum salmon GtH, however, the stimulatory effect on VTG uptake of PE was only 65% of that achieved using the purified GtH. It is difficult to interpret the potencies of the salmon and trout GtH's on VTG sequestration however, because of the heterologous assays employed; this study used a chum salmon GtH to stimulate rainbow trout follicles and a chum salmon GtH assay to measure GtH levels in the trout PE. Care has to be taken when using heterologous asssays, even as in this study between species within the same family, because heterologous hormones may have differences in stability, binding affinity or other properties to those normally found in the biological system effected. Although salmon hormones are frequently used in studies on trout and other non salmonid species, often dictated by the limited availability of purified fish preparations, we do not know how similar the hormones are either between species or within species.

The validity of the physiological role of carbohydrate-rich GtH *in vivo* in controlling VTG uptake could be questioned because of the high doses applied in this study *in vitro*. As mentioned previously, in the natural reproductive cycle, during the period of most active vitellogenic growth plasma GtH concentrations are generally below $1ng.ml^{-1}$ whereas in this study doses of $100ng\ ml^{-1}$ had to be employed before significant effects were obtained. However, it is difficult to compare a dose of $100ng.GtH.ml^{-1}$ presented *in vitro* to isolated follicles, with a

much lower level found *in vivo* during the period of maximal plasma steroid levels and VTG uptake. Furthermore, other authors have found that GtH doses of greater than 100 $ng.ml^{-1}$ are necessary to obtain significant steroidogenesis from cultured salmonid follicles (Kagawa *et al.*, 1982; Young *et al.*, 1983) and even a dose of $100ng.ml^{-1}$ does not produce a maximal response (reviewed by Nagahama, 1985). The dose of $100ng.ml^{-1}$ was chosen to investigate VTG uptake because it was expected to be high enough to ensure a good penetration through the follicular tissues during static conditions of incubation. It is likely that *in vivo* effective dosages of GtH are somewhat lower.

The involvement of carbohydrate-poor GtHs in VTG uptake in rainbow trout follicles remains unclear; the present data does not support the findings of Idler and co-workers, discussed in the introduction to this chapter, who have suggested that carbohydrate poor GtHs control the sequestration of VTG, because we found a carbohydrate-rich GtH was effective.

The *in vitro* production of testosterone by follicles with maturational GtH showed a clear dose-response, the output increasing to over 4 times that of untreated groups at a dosage of 1µg GtH ml^{-1}. At the maximal rate of stimulation each follicle produced approximately 1.4ng testosterone over the 18 hour incubation period. Similar studies conducted on follicles from the amago salmon, using the less purified SG-G100 gonadotropin preparation (Donaldson *et al.*, 1972), produced similar amounts of testosterone (Kagawa *et al.*, 1982; Young *et al.*, 1983; Nagahama *et al.*, 1984). However, it is difficult to make direct comparisons of steroid output by ovarian follicles between species, because the latter authors clearly showed that production in response to SG-G100 changes markedly during the pre-ovulatory period. These researchers also demonstrated that the rate of steroid production in response to SG-G100 was strongly correlated with the plasma steroid titres of the recipient fish. The rainbow trout follicles used in this study to examine the effects of GtH on both VTG uptake and testosterone were close to ovulation (as indicated by their large size), and it is likely that the steroidogenic responses seen to GtH are at or close to their maximum, because it is at this time in the natural reproductive cycle when testosterone levels are highest in the blood (Scott *et al.*, 1980; Sumpter and Scott, 1983).

Given the current state of knowledge, together with the findings of the present work, it is suggested that in the rainbow trout there is a unique GtH

(which may possess different degrees of glycosylation and consequently different potencies) controlling many diverse aspects of gonadal function, ranging from VTG sequestration to steroidogenesis. From the data in Chapters 5 and 6, however, it can be seen that VTG uptake can occur at rates comparable to those observed *in vivo*(Chapters 3 and 4), in the absence of gonadotropin(s). Furthermore even when GtH was present at 1000ng.ml^{-1} in the medium in this study uptake was increased only by 34%. These observations suggest that GtH may not necessarily have a strong, driving influence on VTG incorporation throughout the vitellogenic cycle, as frequently implied, but may function to 'fine tune' the endocytotic processes which contribute to follicle growth. However, although GtH does not appear to have a strong influence on the rate of VTG uptake once the process of sequestration has been started, it may initiate the process (in follicles of approximately 1mm in diameter) and thus play an all-important role; this has yet to be investigated.

Insulin's somewhat surprising stimulation of VTG sequestration has also been observed in *Xenopus* follicles (Wallace, 1983). Furthermore recent studies by Opresko and Wiley (1987b) on *Xenopus* showed that insulin increased VTG sequestration by between 100-500%, depending on the experiment. Although this is a considerably greater effect than observed in this study on the rainbow trout these authors used insulin at a dose of 5 µg.ml^{-1}, 5 times as much as the highest dose used here, and this may account for the differences. In this study on the rainbow trout insulin was not as potent in stimulating VTG sequestration as GtH. Many studies on hormones express their potencies on a molar basis, for a single hormone molecule binds and subsequently triggers the action of a single specific hormone receptor. If this approach is adopted in this study salmon GtH, having a molecular weight of almost 5 times that of salmon insulin (31 000 Daltons versus 6100 Daltons, respectively) is approximately 30-40 times more potent than insulin in stimulating VTG sequestration.

Little is known about how insulin and GtH mediate their stimulation of VTG sequestration in the trout or indeed if they occur, in any other fish, however, recent detailed studies on *Xenopus* by Opresko and Wiley (1987b) have given a insight into how actions of these hormones may be conducted. Both insulin and human chorionic gonadotropin (hCG) increased the rate of VTG uptake in *Xenopus* follicles (although it should be stressed that the effect of hCG could only be achieved *in vivo*) similar to that seen for insulin and GtH in this study on the

rainbow trout and Opresko and Wiley (1987b) suggested that these hormones do so through different mechanisms. By treating maximally hCG stimulated follicles with insulin these authors showed that the rate of VTG sequestration increased further showing that the effect of the different hormones on uptake was additive, and therefore suggesting that they did not share the same rate limiting step in the uptake pathway. Both hormones increased the rate of fluid phase endocytosis, but the magnitude of stimulation was not sufficient to account for the observed increase in VTG uptake. Using kinetic and steady state analyses these authors found that the stimulation of VTG uptake was not due to an increase in surface VTG receptors but due to an increased rate of internalization of the VTG receptor. They suggested 4 possible ways by which the internalization rate of the receptor could be increased; firstly, by increasing the number of coated pits at the cell surface, secondly by increasing the rate at which coated pits are internalized, thirdly increasing the affinity of the VTG receptor for coated pits (or other rate limiting component in the endocytotic pathway) or fourthly by increasing the probability of a receptor encountering a coated pit (eg. increasing lateral mobility of the receptor in the surface membrane). With regards to these suggestions human chorionic gonadotropin has been shown to increase the follicle surface area and therefore increase the number of coated pits (Wallace and Dumont, 1968). In their detailed studies Opresko and Wiley further showed that insulin not only increased the number of coated pits but also increased the affinity of the VTG receptor for its ligand.

Insulin and gonadotropin are thought to interact at different sites in the follicle; insulin interacts directly with the vitelline membrane (Maller and Koontz, 1980) whereas the action of GtH appears to be mediated by the surrounding granulosa cells which are in communication with the oocyte via gap-junctions (Browne *et al.*, 1979). In conclusion from the studies on *Xenopus* it appears that 2 distinct, independent mechanisms could thus exist, both of which increase the rate of VTG uptake.

Insulin had a mild steroidogenic action in inducing testosterone production but only at the highest dose adopted (1 $\mu g.ml^{-1}$) and even then the effect was small, being less than 30% of the stimulatory effect on steroidogenesis observed with the same dose of GtH.

Insulin's stimulatory action on VTG sequestration in amphibians (Wallace, 1983; Opresko and Wiley, 1987b) and here in the trout on both VTG

uptake and ovarian steroidogenesis and its involvement in amino acid metabolism and glucose homeostasis in both fish and amphibians, suggests that in both these vertebrate classes, where large numbers of eggs are ovulated during a brief natural spawning, insulin probably plays diverse functions in mobilizing and aiding energetic and endocrine resources focussed around and upon follicle growth and completion of the spawning act. Clarifying the role of insulin in teleost reproductive physiology requires further work, especially with respect to plasma concentrations and its seasonal fluctuations.

An involvement for thyroid hormones in teleost reproduction has frequently been suggested, based mainly on the circumstantial evidence of changes in thyroid activity throughout the reproductive cycle (see references in introduction), but because many other changes are occurring at the same time it has been impossible to ascribe 'vitellogenic' actions to thyroid hormones. The data presented here demonstrated that T_3 and T_4 were unable to directly stimulate VTG sequestration, even at concentrations 25-50 times that found in trout plasma during ovarian recrudescence (Cyr *et al.*, 1987). Similarly T_4 had no effect on VTG sequestration in the winter flounder *in vivo* (Ng *et al.*, 1980).

The slight inhibitory action on VTG uptake shown by T_3 in this study is probably an artifact of the experimental design, rather than a natural phenomenon; the T_3 was made up in 0.1M NaOH and even upon dilution to obtain the appropriate concentration for culture (100 $ng.ml^{-1}$) the pH of the medium was altered, rising slightly above pH 8.0 (as indicated by the colourimetric properties of the phenol-red in the leibovitz L15 culture medium). Thus the slight (but significant) decrease in rate of VTG uptake observed may have been due to this pH change of the medium. In the salmonids thyroid hormones appear to exert constant stimulatory actions on somatic growth (Donaldson *et al.*, 1979) and have direct actions on the follicle to amplify the steroidogenic action of GtH (Cyr and Eales, 1986) but from this study they do not appear to be directly involved in the endocytosis of VTG. However, the effects of T_3 and T_4 need further study before it will be unequivocally possible to define the role, if any, in regulating VTG uptake.

As with the thyroid hormones, chinook salmon GH did not have a significant stimulatory action on VTG uptake. Slight elevations in the rate of VTG

uptake into follicles treated with the GH were, however, observed. It is possible that this 'effect' is due, at least in part, to the contamination of the preparation with GtH because the complete separation of these pituitary products is difficult due to some similarities in their chemical composition. Growth hormone is known to exert stimulatory actions on the somatic growth in fish and is therefore likely to participate indirectly in ovarian growth; possibly its actions on non-ovarian tissue have to be reduced in order that available energy can be channelled into ovarian growth during the reproductive cycle, rather than into somatic growth.

The ovarian steroids tested, 17ß-oestradiol and testosterone, failed to alter the rate of VTG uptake. Studies conducted *in vivo* by Campbell and Idler (1976) and by Cumaranatunga *et al.*, (1985) similarly demonstrated that 17ß-oestradiol was unable to enhance VTG sequestration. Cumaranatunga and co-workers further showed that pharmological doses of 17ß-oestradiol inhibited VTG uptake, possibly acting through a negative feedback action on GtH production. Testosterone's function in the reproductive cycle of rainbow trout remains unclear; it does not appear to enhance or inhibit VTG sequestration at the dose adopted, which was within the normal physiological range.

Hormonal regulation of sexual reproduction is an ubiquitous phenomenon in the animal kingdom and the reproductive physiology of the lower vertebrates, especially the bony fishes which are the ancestral vertebrates, is of great interest to comparative endocrinologists. Using the culture system developed for trout follicles only GtH and insulin, of the hormones tested, directly stimulated VTG sequestration. However, it should be stressed that these are not necessarily the only hormones involved in VTG uptake by vitellogenic follicles and other hormones especially the hypophyseal products, prolactin and TSH (discussed previously), and peptides such as insulin-like growth factors (see Chapter 8) need to be researched. Furthermore *in vivo* the situation is likely to be more complex. By using combinations of hormones *in vitro*, it should be possible to determine any synergistic, antagonistic or passive actions on VTG uptake, and in turn generate a more complete picture on the control of ovarian recrudescence. If combinations of hormones need to act on the follicle in the correct sequence for maximal ovarian growth, then the hormonal signal which has been received *in vivo* by the ovary before the beginning of an *in vitro* experiment may be decisive on the further responses to different exogenous factors. Therefore in future studies information on the accurate hormonal status before and at the time of sampling could help in

the interpretation of *in vitro* data.

CHAPTER EIGHT

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDY

The initial *in vivo* studies on $^{32}P.^{3}H$-VTG uptake clearly demonstrated, for the first time in teleosts, that VTG was processed in the ovary to the yolk proteins lipovitellin and phosvitin, in a similar fashion to that occurring in other oviparous vertebrates. These yolk proteins were subsequently found to be stable throughout the periods of experimental study (up to 4 weeks) thus providing a convenient feature for subsequent VTG uptake studies. However, it should be stressed that this study provided the 'basic model' for VTG's processing within the oocyte, and that lipovitellin and phosvitin are not necessarily the only derivatives of VTG; other yolk proteins such as the 'ß' component shown to be immunologically related to VTG in the rainbow trout (Campbell and Idler, 1980; see Chapter 3) may enter the follicle as a component of the VTG complex. A further important consideration is that this study considered VTG as though it existed in a single form, whereas in both birds and amphibians there are multiple VTGs, coded for by distinct genes. As salmonid fish are considered to be tetraploid, it seems likely that they could possess 2 or 4 VTG's; they certainly have two quite distinct types of many hormones (melanophore stimulating hormone, GtH, endorphin and growth hormone) so are quite likely to possess multiple VTG's. Clearly this could lead to a complex array of proteins within the ovary, all derived from VTG(s). Detailed structural studies on both VTG and yolk proteins clearly requires analytical techniques with greater powers of resolution than gel-filtration alone. For example gel filtration (which separates molecules on the basis of their size) in combination with ion-exchange chromatography (which separates molecules on the basis of their surface charge) would provide greater resolution. Ion-exchange chromatography allows similarly sized proteins with subtle differences in charge to be separated and therefore could provide a useful method for determining the number of VTG's, lipovitellins and phosvitins which are present in the rainbow trout. The use of other separative techniques such as high pressure liquid chromatography (possibly reverse-phase columns working by hydrophobicity would be the initial method of choice), which provides a method that is both fast and has great resolving powers would further allow components of the protein complexes to be determined. Together data of this kind could help explain the conflicting results obtained in different studies of teleosts regarding the size and number of different VTGs and components of the VTG complex.

Further *in vivo* studies showed that VTG was sequestered far more rapidly than BSA suggesting that VTG is incorporated into vitellogenic follicles by receptor-mediated endocytosis. It is important to realize however, that due to the small numbers of fish involved (n=3) this study was qualitative rather than

quantitative and although the investigation successfully demonstrated that uptake rates were different for the two different proteins, accurate assessments of the relative sequestration rates between VTG and non-vitellogenic proteins would require greater numbers of both fish and proteins to be used. It would also be informative to examine the uptake of proteins of a similar size to VTG, such as ferritin (440 000 Daltons) and thyroglobulin (669 000 Daltons), to determine whether uptake is in anyway affected by steric considerations. Also by investigating the uptake of a number of other homologous i.e. naturally occurring blood proteins , it should be possible to more accurately quantify 'true' selectivity of protein sequestration. Furthermore by 'loading' vitellogenic females with very large amounts of labelled non-vitellogenic proteins (preferably tens of microcuries) it should be possible to get sufficient counts in the developing oocytes (perhaps a few thousand) to accurately monitor and indeed quantify the pathways followed within the oocyte of non-vitellogenic proteins, to determine what proportion, if any, of these sequestered proteins remain 'stable' in the oocyte and what fraction undergoes proteolysis and/or secretion from the oocyte. This type of experiment, using a series of homologous serum proteins, would give a further idea of what proportion of the total yolk reserves in trout are derived from blood proteins other than VTG.

An interesting observation arising from the studies reported in this thesis relates to follicle synchrony during vitellogenic development of the ovary. Both Chapters 4 and 6 document an asynchrony of follicle development during early vitellogenic development of the ovary but subsequently, after the follicles reached about 2.5mm in diameter, they showed a homogeneity in size which persisted throughout the remainder of vitellogenic growth until ovulation. As yet it is still unclear how this synchrony is achieved. This parallels the situation amongst other teleosts where generally there has been little research on the dynamics of vitellogenic growth of the ovary and many questions remain to be answered. For example: What factor determines when a follicle begins VTG sequestration? Is the timing controlled by the oocyte itself or externally by a signal from the blood? The observation of an initial follicle asynchrony during vitellogenic development would suggest the former because a blood signal would be received by all follicles simultaneously and subsequently stimulate the appropriate follicles concomitantly. Such questions relating to vitellogenic follicle growth could be answered, or at least more fully understood, with a more detailed study on the vitellogenic cycle of the rainbow trout. By sampling a single population of maturing females at intervals during ovarian development and measuring ovarian growth rates in parallel with

blood VTG levels, and the numbers and sizes of follicles at different stages of vitellogenic development, it should be possible to determine: growth rates of the different sized vitellogenic follicles, assess exactly when follicles begin to sequester VTG (in relation to their size and the time in the spawning cycle) and more precisely determine at what stage follicle synchrony is achieved during exogenous vitellogenesis.

As mentioned briefly in Chapters 1 and 5 *in vivo* studies are, at times, prone to difficulties of interpretation, whereas *in vitro* studies can allow the direct effect(s) of individual parameters to be investigated under constant controlled conditions. *In vitro* studies using the culture system developed here for trout follicles clearly provide an important approach for future research and where many questions relating to vitellogenic growth of the ovary could be answered. However, although the results presented in Chapters 5, 6 and 7 showed that the culture system developed provided favourable conditions, it appeared that these conditions were not optimal. This was indicated by the slower rate of processing of VTG *in vitro* compared to that seen *in vivo* (Chapters 3 and 4). Furthermore, although VTG uptake rates paralleled that measured *in vivo*, simple calculations based on the known growth rate of the ovary during the reproductive cycle suggest that the rate of uptake of VTG must reach approximately 1.5 $\mu g.mm.^{-2}hr.^{-1}$ in order for the follicles to grow at the rate they do. It is unclear exactly which parameter(s) are limiting both uptake and processing of VTG *in vitro*, and to a lesser extent uptake *in vivo*, but it may in part be linked to the stress imposed on the female fish prior to removal of the ovary and isolation of follicles. Indeed recent studies by Sumpter and Carragher (pers. comm.) indicate that stress strongly suppresses many elements of the reproductive cycle, including blood gonadotropin and sex steroid levels and possibly the rate of ovarian growth. Clearly further research is required to clarify these problems.

Possibly one of the most important findings of the present work was that CHO-rich (maturational) GtH was capable of stimulating VTG sequestration. The stimulation of VTG uptake by the gonadotropin known to mediate follicle maturation in the rainbow trout (CHO-rich fraction) favours the idea of a single gonadotropin controlling both follicle growth and maturation, processes which are controlled separately by luteinizing hormone and follicle stimulating hormone, respectively, in mammals. However, the doses required to stimulate the uptake of VTG were far above the levels of these hormones that occur in the blood of female fish during the major part of exogenous vitellogenesis and even at a dose of 100ngml^{-1} VTG uptake was only

stimulated by 34%; together these observations illustrate that the precise hormonal elements controlling VTG uptake are still unclear. To help resolve the present controversy surrounding the numbers and functions of gonadotropins present in teleosts the culture system developed here seems ideal, for its offers an oppo-tunity to examine the biological activities (in relation to VTG uptake and ovarian steroidogenesis) of the various purified GtH preparations available (see Chapter 7). In such an experiment the potencies of the different gonadotropin preparations could be assessed in parallel and direct comparisons made between them.

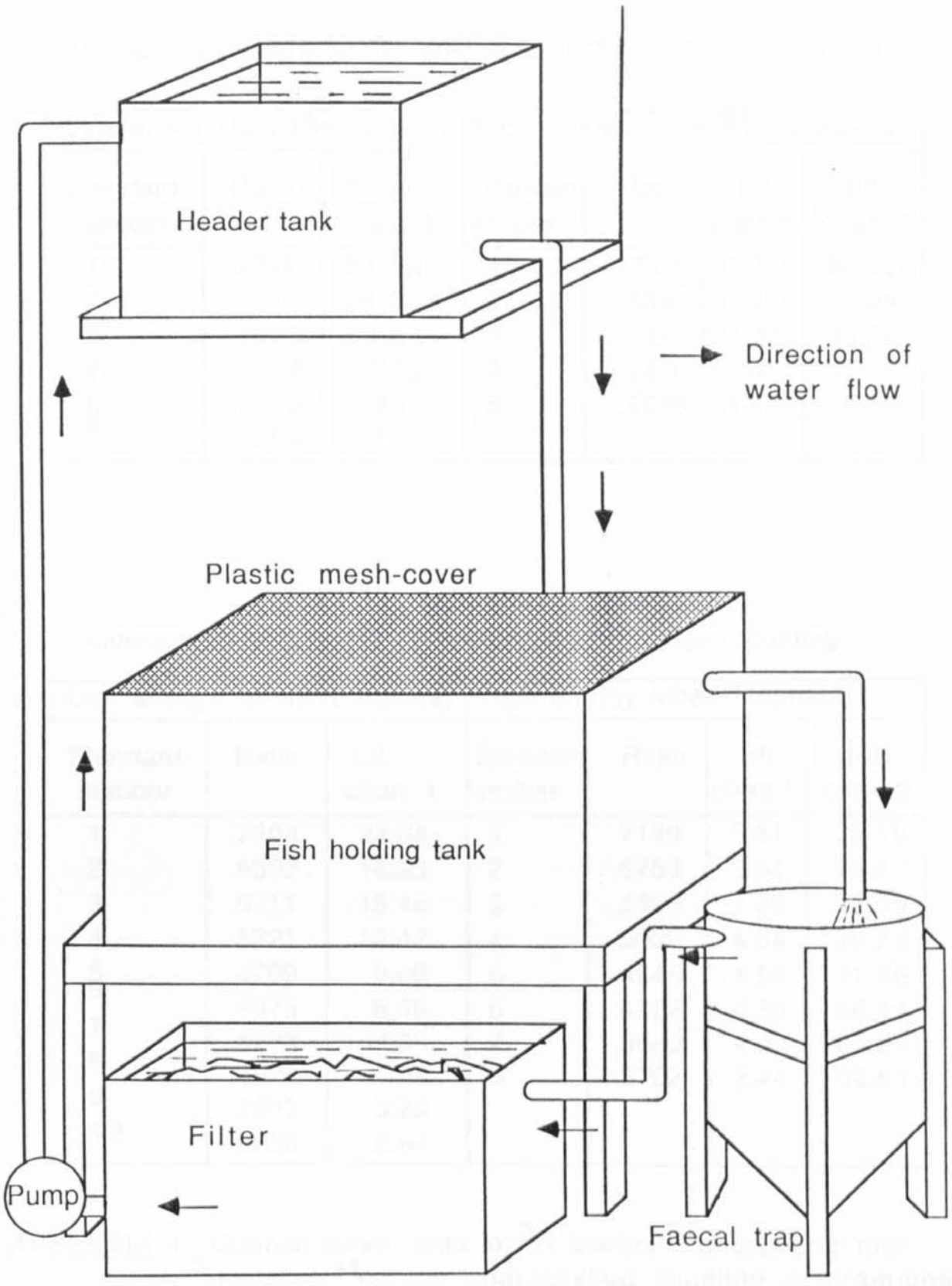
Insulin was also shown to stimulate VTG uptake but was not as potent as GtH. Recently much attention on cell growth in the mammalian field has focused on insulin-like growth factors, which are usually small molecular weight proteins. There are many types of insulin-like growth factors (GFs), the better known of which include epidermal growth factor and fibroblast growth factor. These growth factors are located within specific tissues and have been found to have growth promoting activities far above that induced by insulin. Presently very little is known about what controls the quite phenomenal growth of the oocyte. One distinct possibility is that it, or more likely the surrounding follicle cells (which have been demonstrated to secrete growth factors in mammals), synthesise and secrete growth factors which regulate oocyte growth. If so it is possible that the stimulation of VTG uptake by insulin in this study is brought about by insulin's resemblence to the follicles own insulin-like growth promoters, if, that is , they occur. Cultures of vitellogenic follicles in the presence of insulin-like growth factors (of mammalian origin because fish have not been purified yet) would not only give an idea of whether they play a role in VTG uptake in fish but also, if so, whether gonadotropins really do have a direct driving influence on VTG sequestration, which on assessment of the available literature appears unlikely, though it is often postulated.

The availability of an *in vitro* culture system for trout follicles also allows questions relating to how VTG uptake is initially stimulated and controlled to be realistically investigated. Results in this thesis have shown that once initiated (when follicles reach approximately 0.9mm in diameter) VTG sequestration and processing can occur in culture without any hormone supplements; that is without GtH(s), insulin, or any other factor. However, follicles below 0.9mm did not sequester VTG in culture. Thus it appears that at a certain follicle size VTG sequestration is triggered, after which it continues until ovulation. By culturing pre-vitellogenic follicles just prior to the onset of exogenous vitellogenesis with various stimulantes such as gonadotropins and insulin-like growth factors it might

be possible to determine whether an outside stimulus 'switches on' the uptake process of VTG or whether it is controlled from within the oocyte ; for example, if the follicle has to reach a certain stage of development or critical follicle size be before VTG uptake can occur. If stimulants such as gonadotropin were found to initiate the uptake of VTG it would be of interest to know whether this effect was mediated by the generation of surface receptors for VTG or by opening channels between the follicular layers thus allowing VTG access to the oocyte surface.

As in most fields of cell biology, much has yet to be determined. However, with the development of an *in vitro* culture system for trout follicles studies relating to follicle growth and development in fish should progress rapidly.

APPENDICES



Appendix I Recirculating water-system for holding fish in the laboratory.

Quench curve data for ^{3}H leucine and ^{32}phosphorus counting.

Low energy label(^{3}H leucine)			High energy label(^{32}phosphorus)			
Standard number	Ratio	Eff. chan. 1.	Standard number	Ratio	Eff. chan.1.	Eff. chan.2.
1	.3394	31.38	1	.4073	1.74	50.23
2	.3319	24.38	2	.3399	1.48	49.56
3	.2875	20.52	3	.2948	1.33	49.92
4	.2234	13.55	4	.2376	1.41	47.92
5	.1512	7.30	5	.1615	1.13	45.35
6	.1269	5.70				

Quench curve data fof ^{3}H leucine and ^{14}carbon counting.

Low energy label(^{3}H leucine)			High energy label(^{14}carbon)			
Standard number	Ratio	Eff. chan. 1.	Standard number	Ratio	Eff. chan.1.	Eff. chan.2.
1	.7303	22.64	1	.7199	5.61	75.59
2	.6302	18.23	2	.6753	5.54	76.07
3	.5711	15.45	3	.5593	4.86	73.69
4	.5221	12.47	4	.5029	4.54	70.79
5	.4769	8.00	5	.4845	4.59	71.26
6	.4373	6.98	6	.4322	4.36	68.41
7	.4283	7.20	7	.3680	4.03	65.51
8	.3023	4.04	8	.2702	3.44	62.81
9	.2899	3.29				
10	.2639	2.64				

APPENDIX II. Quench curve data for ^{3}H leucine/^{32}phosphorus and ^{3}H leucine/^{14}carbon dual-labelled counting programmes.

Steroid	Percent cross reactivity
Testosterone	Taken as 100%
11-Ketotestosterone	34.5
Dehydroepiandosterone	20.0
17B-Oestradiol	5.8
Oestrone	2.9
Cortisol	<0.5
17β-Hydroxy-20B-Dihydroprogesterone	1.66
17β-Hydroxy-progesterone	0.5
Pregnenolone	3.3
Non-specific	1.8

Appendix III. Cross reaction data for testosterone antiserum with eight other steroids.
(From Duston and Bromage 1987)

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