Involvement of the Endocrine Dorsal Bodies and the Central Nervous System in the Physiology of Reproduction in *Helisoma duryi* (Mollusca : Pulmonata)

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ABSTRACT

Helisoma duryi is a freshwater pulmonate snail common to many small bodies of water throughout North America. This snail is easily maintained in the laboratory and is capable of reproducing throughout the year. *H. duryi* normally cross-fertilize and after mating, they deposit gelatinous egg masses upon underwater substrata. Egg production in hermaphrodite snails is a complex process which involves oocyte development, ovulation of mature oocytes, fertilization, egg and egg mass formation, and finally oviposition. The albumen gland, an exocrine female accessory sex gland, plays an integral part in the egglaying process as it synthesizes a nutrient-rich perivitelline fluid which is secreted around individual eggs as they move along the reproductive tract. The perivitelline fluid is composed mainly of polysaccharides and proteins and serves as the main nutrient source for the developing embryos. This thesis investigates the endocrine and neurosecretory factors which regulate the synthetic and secretory activities of the albumen gland in *H. duryi*.

In freshwater pulmonates, there are two known gonadotropic centers thought to control reproduction, the non-nervous, endocrine dorsal bodies, and the neurosecretory caudodorsal cells in the cerebral ganglia. The endocrine dorsal bodies stimulate oocyte maturation and the synthetic activity of the albumen gland, whereas the caudodorsal cells stimulate ovulation and also regulate the activity of the albumen gland. Although many putative substances of endocrine or nervous origin have been proposed to influence reproductive activities, few have been chemically identified and shown to have specific physiologic effects on reproductive functions.

Albumen gland polysaccharide synthesis can be monitored *in vitro* by incubating albumen gland explants with a radiolabelled monosaccharide such as ^{14}C-glucose and measuring the total radiolabelled polysaccharides. Since the albumen gland is a major target organ for an endocrine factor from the dorsal bodies, the measurement of polysaccharide synthesis was used as a bioassay to monitor the activity of dorsal body extracts in order to purify and characterize the bioactive substance(s). Extracts of dorsal body tissue and dorsal body-conditioned culture medium were separated by high-performance liquid chromatography and the resultant fractions were tested with radioimmunoassay or bioassay. The material from the dorsal bodies is not proteinaceaous, and displayed characteristics of an ecdysteroid-related molecule. In addition, another bioactive substance from the dorsal bodies was detected which differs in hydrophobicity. A partial purification of these substances was achieved.

A 66 kDa glycoprotein was identified in crude albumen gland extracts. The *in vitro* release of this protein, and others, was enhanced in the presence of an acidic extract of the brain. The stimulatory factor from the brain was partially characterized as a basic peptide of less than 10 kDa. This neuropeptide stimulated the production of the second messenger cyclic AMP in the albumen gland, which in turn evoked the release of secretory material. The relationship between the secretory and synthetic activity of the albumen gland is discussed with respect to the endocrine and neurosecretory factors which influence reproduction in pulmonate molluscs.

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INTRODUCTION

The Mollusca constitutes a large diverse phylum, and second to the Arthropoda, it includes the most abundant species in the animal kingdom. Over 100,000 living species are distributed among seven classes: Monoplacophora, Aplacophora, Polyplacophora, Scaphopoda, Bivalvia, Gastropoda, and Cephalopoda (Barnes, 1980). Among these classes, the Gastropoda have colonized nearly every major habitat, and consequently, they have the largest number of species and show the greatest diversity within the phylum. A major factor contributing to the evolutionary success of gastropods, and of the Mollusca as a whole, is the diversity of their reproductive systems (for reviews see Tompa, 1984; Saleuddin, 1998b). Reproductive processes such as mating, gametogenesis, fertilization, egglaying, and embryonic development have been described in some detail, but the physiological mechanisms controlling some of these processes remain largely unknown.

The freshwater snail *Helisoma duryi* (Weatherby) is a pulmonate gastropod belonging to the order Basommatophora, and family Planorbidae. The distribution of various freshwater snails in the United States and Canada has been documented by Baker (1928, 1945) and Clarke (1981). Members of the genus *Helisoma* are found in small lakes, streams, and ponds throughout North America. They inhabit the littoral zone and are mostly found at a depth of less than a meter. *H. duryi* is found in the waters of southeastern United States, while its close relative *H. trivolvis* (Say) occupies a more northern habitat ranging from northeastern United States and eastern Canada, and westward to Alberta (Clarke, 1981). Planorbid snails

from the genera *Biomphalaria*, *Bulinus*, and *Australorbis* are the intermediate hosts for the trematode genus *Schistosoma* (Cheng, 1973). In Africa, South America, and parts of Asia, parasitic infection by *Schistosoma spp.* debilitates millions of people. Over the past 30 years, considerable effort has been directed toward understanding the internal factors regulating reproduction in freshwater snails as a means of controlling snail populations, and therefore, limiting the spread of schistosomiasis. It is thought that specific molluscicides can be created that interfere with the snails' reproductive system or its ability to regulate key reproductive processes such as the synthesis or release of hormones.

Organization of the Basommatophoran Reproductive System

The reproductive system of the basommatophoran pulmonates is complex. All basommatophorans are simultaneous hermaphrodites, however, in some of the primitive species, a short male-phase precedes the hermaphroditic phase (Duncan, 1975). The reproductive system consists of a hermaphroditic gonad, and the male and female reproductive tracts with their associated accessory sex organs (ASOs). A single ovotestis at the posterior end of the snail produces both male and female gametes. The gametes are periodically released and pass through a narrow hermaphroditic duct (spermoviduct) that has small lobular seminal vesicles located along it. Further anterior, this duct joins the male and female portions of the reproductive tract where the gametes are transported via their separate tracts. The junction of the hermaphroditic duct and the male and female tract is called the carrefour. A small sac-like structure, the fertilization pocket, is sometimes associated with

the carrefour. The female tract which consists of the albumen gland, oviduct, uterus, muciparous gland, oothecal gland, and bursa copulatrix is a glandular structure (Fig. 1). Its main function is to synthesize and secrete material for egg and egg mass formation, and to receive foreign sperm from a copulating partner and transport it to the site of fertilization (Abdel-Malek, 1954; de Jong-Brink, 1969; Plesch *et al.*, 1971). The principal function of the male tract which consists of seminal vesicles, sperm duct, prostate gland, vas deferens, and penis complex is to provide nutrition to the spermtozoa and to transport them to a mating partner.

Ovotestis

In the Basommatophora, the gonad is believed to be of mesodermal origin whereas the rest of the reproductive tract (male and female) and its associated ASOs arise from ectoderm (Runham, 1983). The gonad is surrounded by a thin layer of connective tissue and composed of very few muscle fibers (Plesch *et al.*, 1971). Within the ovotestis is a variable number of acini, bounded by a basal lamina and a layer of connective tissue. Both male and female gametes are found in the acini. The gametes, Sertoli cells, and follicle cells develop from a germinal epithelium which is confined to the upper portion of the acini (Joosse and Reitz, 1969). The developing oocytes migrate to the bottom of the acini and become surrounded by follicle cells. The developing sperm cells and Sertoli cells also migrate to the bottom of the acini and pass over the female cells. The spermatozoa project into the lumen of the acinus, whereas the oocytes are located peripherally (de Jong-Brink *et al.*, 1977).

Fig. 1 A diagramatic representation of the reproductive system of *Helisoma duryi* showing the formation of the egg mass (from Saleuddin *et al.*, 1990).

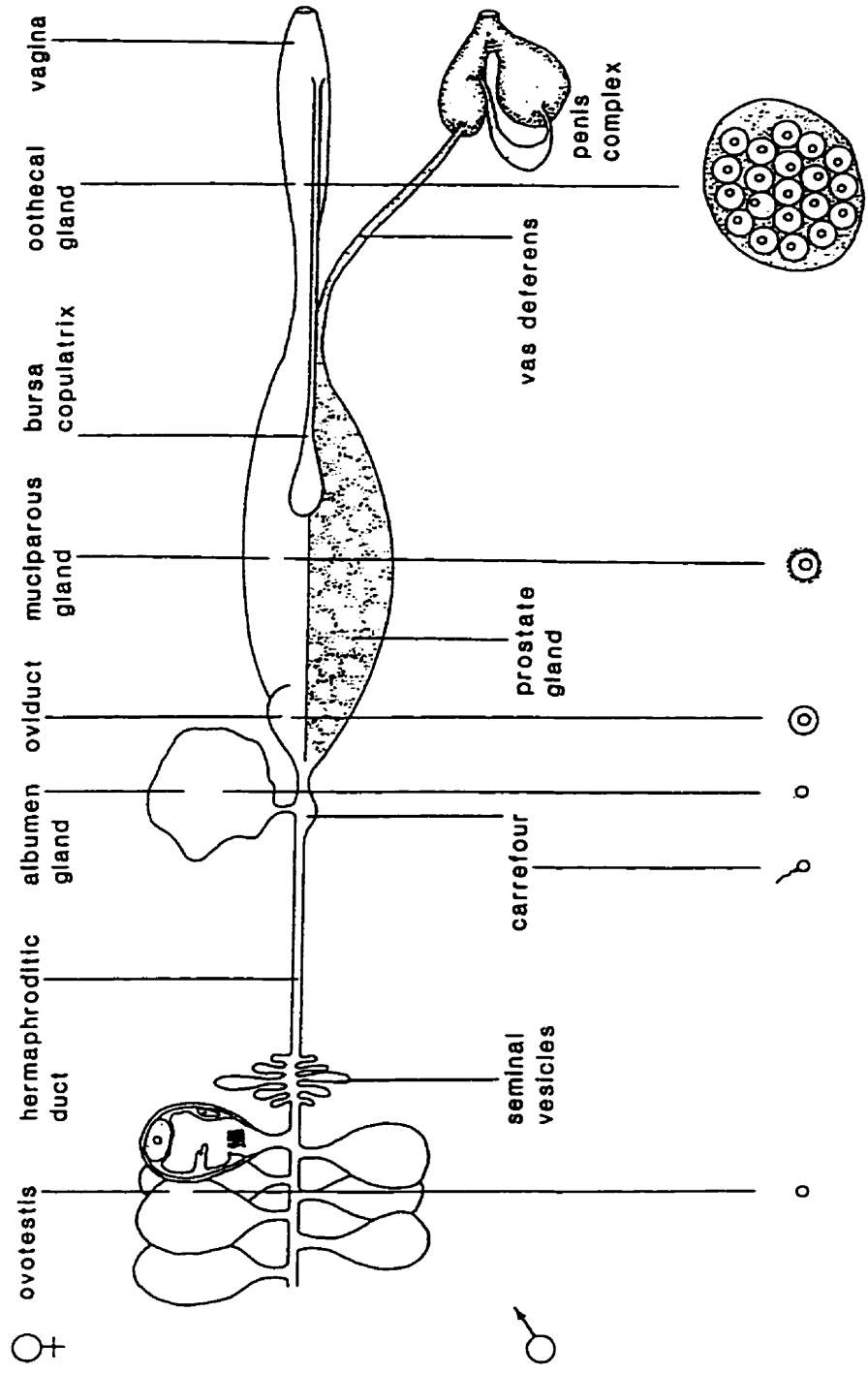

vagina
penis complex
oothecal gland
vas deferens
bursa copulatrix
mucilparous gland
prostate gland
oviduct
albumen gland
carrefour
hermaphroditic duct
seminal vesicles
ovotestis

Fig. 1

Spermatogenesis

The developing male sex cells arise from the germinal epithelium and are connected to the Sertoli cells initially through desmosome-like junctions and later by gap junctions which synchronize cell division (deJong-Brink *et al.*, 1977). The Sertoli cells are thought to provide nutrition to the spermatogenic cells and produce paracrine factors involved in spermiation (deJong-Brink *et al.*, 1981). Spermatogonia undergo mitotic divisions and develop into spermatocytes. The spermatocytes undergo meiosis and differentiate into spermatids near the rim of the vitellogenic area (see below) (Geraerts and Joosse, 1984). Excess cytoplasmic material from the acinus is phagocytosed by the Sertoli cells after spermiation, and the Sertoli cells gradually degenerate. Molluscs exhibit a great diversity of spermatozoan form even within particular orders, but among the Basommatophora sperm morphology is relatively uniform (Duncan, 1975; Maxwell, 1983).

Oogenesis

Primordial cells of the germinal epithelium differentiate to become primary oogonia and secondary oogonia. The oogonia then pass to the basal region of the acinus, the vitellogenic area, where they become surrounded apically by follicle cells (deJong-Brink *et al.*, 1983). In the freshwater snail *Lymnaea stagnalis*, oocyte differentiation and maturation can be morphologically characterized into 4 stages: (1) a premeiotic phase (formation of a germinal vesicle); (2) oocyte enlargement and accumulation of RNA in the cytoplasm; (3) formation of yolk granules (vitellogenesis); (4) oocyte maturation (polar body formation) (Ubbels, 1968; de Jong-Brink *et al.*, 1982).

Follicle Cells

The follicle cells are attached to the oocytes by septate junctions (Khan and Saleuddin, 1983) and gradually cover the portion of the oocyte protruding into the acinus. Later on in development, a follicular cavity appears and spreads laterally between the oocyte and follicle cell (deJong-Brink *et al.*, 1976). The follicle cells of pulmonates have characteristics of protein synthesizing cells, and have been suggested to synthesize yolk proteins (Rigby, 1979). However, their function with respect to vitellogenesis is unclear. The follicle cells have also been suggested to be involved in the formation of the follicular cavity, a structural feature thought to be necessary for ovulation to occur (de Jong-Brink *et al.*, 1976). In *H. duryi*, the follicle cells appear more synthetically active in egg-laying snails than in non egg-layers, and the cell junctions between follicle cells and oocytes are more numerous in egg-layers (Saleuddin *et al.*, 1980).

Vitellogenesis

Unlike the oocytes of cephalopods, those of basommatophoran snails contain little yolk, and can be characterized as oligolecithal. In *L. stagnalis*, the yolk has been characterized into two types: proteinaceous yolk and fatty yolk (Raven, 1975). Proteinaceous yolk is contained within membrane-bound granules and consists of muco-glycoproteins, phosolipids, ferritin, and basic proteins, whereas fatty yolk is composed mainly of neutral lipids (Ubbels, 1968). Proteinaceous yolk is believed to be derived from both autosynthetic processes within the oocyte (de Jong-Brink *et al.*, 1976) and heterosynthetic processes (Saleuddin *et al.*, 1980; Bottke *et al.*, 1988). In the freshwater snails, the follicle

cells are few (4-6 cells/oocyte in *H. duryi*) (Khan and Saleuddin, 1983), and do not appear to produce yolk proteins (Bottke and Tiedke, 1988). Furthermore, there is no correlation between the activities of the oocyte and follicle cells during oocyte development and the appearance of pinocytotic profiles in the oocyte. Ultrastructural studies of pulmonate oocytes indicate the presence of abundant RER, active Golgi complexes, and numerous yolk granules which coincide with the development of the oocyte (Saleuddin *et al.*, 1980). Therefore, the endogenous synthesis of proteinaceous material by the oocyte is believed to be the major mechanism of yolk formation in the Basommatophora (Geraerts and Joosse, 1984).

In pulmonates, the iron storage protein ferritin is the only identified extragonadal protein known to be taken up pinocytotically by the oocyte (Bottke *et al.*, 1982; Saleuddin *et al.*, 1980). Its site of synthesis in *H. duryi* is thought to be within the mantle pore cells (Miksys and Saleuddin, 1986, 1987a,b), but in *L. stagnalis* and *Planorbarius corneus*, the digestive gland is proposed to synthesize ferritin (Bottke *et al.*, 1988). Although ferritin synthesis is regulated by iron levels, there is evidence suggesting the pinocytotic uptake of ferritin from the hemolymph into the oocytes is under endocrine control (Miksys, 1987). Thus, ferritin could be considered as a vitellogenin. In *P. corneus*, two forms of ferritin have been isolated, one from the general visceral mass (without gonad), and one from the yolk granules of oocytes (Bottke and Sinha, 1979; Bottke, 1982). The somatic ferritin is composed of multiple homopolymeric subunits of 19 kDa. It has a native molecular mass of 500 kDa and is widely distributed in snail tissue. The yolk ferritin is a slightly larger molecule comprised of 24 kDa subunits. It has a native molecular mass of 560 kDa and is found only

in the oocytes. Both somatic and yolk ferritins are synthesized by the digestive gland and secreted into the hemolymph where they are taken up by their respective tissues (Bottke *et al.*, 1982). The somatic ferritin differs in both structure and immunoreactivity from the yolk ferritin and they are suggested to have different functions (Bottke, 1985, 1986; Bottke and Crichton, 1984). With the exception of ferritin, there is little information regarding the biochemical composition of other pulmonate yolk proteins.

Hermaphroditic Duct

The male and female gametes are released at different times into the narrow hermaphroditic duct which is continuous with the main collecting duct arising from the ovotestis. They are transported by ciliary movement to the glandular portion of their respective tracts (de Jong-Brink, 1969). The ovulation of mature oocytes into the hermaphroditic duct is likely accomplished by the amoeboid movement of the oocytes from their follicle cells (Saleuddin and Khan, 1981; Saleuddin *et al.*, 1983a). It is suggested that microfilaments present in the oocytes are induced by a neurally-derived ovulation-stimulating hormone, which allows the oocyte to become motile. The middle to distal portion of the hermaphroditic duct is enlarged by globular lateral processes of the seminal vesicles which may have a role in sperm storage. Glandular cells lining the hermaphroditic duct serve to resorb old sperm (Duncan, 1975).

Carrefour

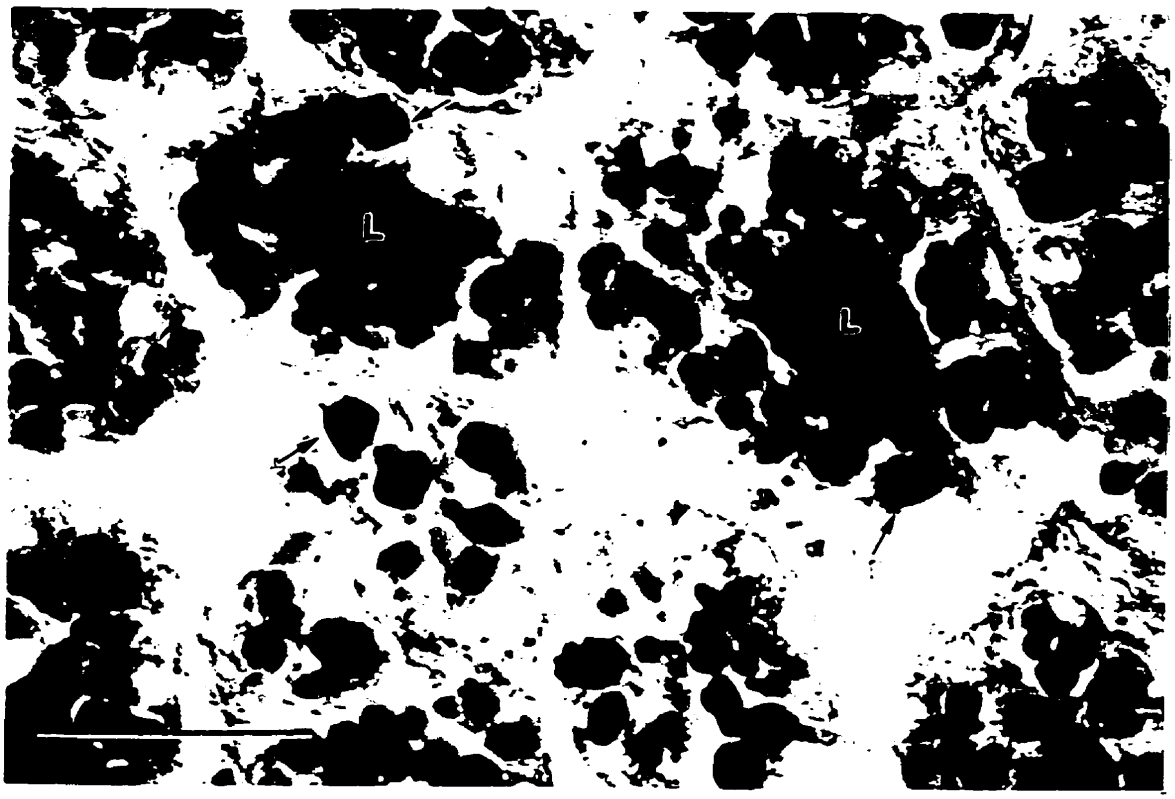
The carrefour is a structure located at the junction of the hermaphroditic duct and the male and female tracts. The albumen gland duct also opens into the carrefour at this point. There are sensory nerve endings, cilia, and muscle that participate in the detection of the zygote so that it may receive secretions from the albumen gland (Plesch *et al.*, 1971; Brisson and Collin, 1980). In some species, fertilization is thought to occur in the anterior portion of the hermaphroditic duct rather than the carrefour itself (Duncan, 1975).

The Female Tract and its Associated Accessory Sex Organs

Albumen Gland

The albumen gland is an exocrine female accessory sex organ composed of many compound tubules and normally has a yellowish appearance in *H. duryi*. It has a central duct lined with a ciliated epithelium which opens at the carrefour. The tubules are lined with a single layer of large (~40-60 μm) cuboidal to pyramidal secretory cells, occasionally interdispersed with some ciliated (centroacinar) cells, and moderate amounts of secretory material in the lumen of the tubules (Abdel-Malek, 1954). Ultrastructurally, the secretory cells have prominent Golgi, numerous ribosomes, and an extensive array of ER cisternae (Nieland and Goudsmit, 1969; Cousin *et al.*, 1995). Small granules packaged by the Golgi coalesce and form large (up to 10 μm) periodic acid-Schiff (PAS)-positive secretory vesicles that occupy the majority of the cytoplasm (Fig. 2). Within these vesicles are numerous small (20 nm) particles consisting of the polysaccharide galactogen, which is the major constituent

Fig. 2 A light micrograph of a cross section through the albumen gland of *Helisoma duryi*. The gland is composed of many closely-associated tubules containing a single layer of cuboidal secretory cells surrounding a central lumen. Note the large secretory globules (arrows) stained positively with the periodic acid-Schiff (PAS) reaction within the cytoplasm, and the considerable amount of PAS-positive material in the lumen (L) of the tubules. Scale bar, 50 μm.


L
L

produced by the albumen gland (Nieland and Goudsmit, 1969; Goudsmit and Ashwell, 1965). Other major components of the secretory vesicles have been shown histochemically to contain protein, protease inhibitors, and lectins (deJong-Brink, 1969; Okatore *et al.*, 1982). The secretory cells of the albumen gland are innervated by a neuronal plexus, which may be involved in regulating the release of secretory material (deJong-Brink and Goldschmeding, 1983). The contents of the secretory vesicles are released into the lumen by exocytosis, and are likely carried by the action of ciliated cells to the main collecting duct (Duncan, 1975).

The secretory fluid produced by the albumen gland and which surrounds the zygote is referred to as the perivitelline fluid (PVF). The amount of PVF per egg is constant, suggesting the albumen gland releases equal-sized drops around each egg as it enters the carrefour (Plesch *et al.*, 1971). The PVF consisits of galactogen, glycogen, proteins, glycoproteins, glucose, glucosamine, free amino acids and various salts (Morrill *et al.*, 1964; deJong-Brink, 1973; Wijsman and van Wijck-Batenburg, 1987).

Galactogen is the best studied component of the PVF. It is a highly branched polysaccharide composed of D- or D-L-galactose units linked glycosidically β-(1→3) and β-(1→6) (Goudsmit *et al.*, 1989; Stangier *et al.*, 1995), and its concentration is 30-40% (dry weight) in the albumen glands (Livingstone and de Zwaan, 1983). In *Helix pomatia*, the average molecular weight of galactogen is 4×10^6 and in *L. stagnalis* it is 2.2×10^6 (see Geraerts and Joosse, 1984). The albumen gland can utilize both glucose and galactose as distal precursors for the synthesis of galactogen. The enzyme UDP-galactose 4-epimerase converts UDP-glucose to UDP-galactose, which in turn is enzymatically converted to

galactogen by galactogen synthase (Goudsmit and Ashwell, 1965). The PVF serves as a source of nutrients for the developing embryos, which catabolize the majority of galactogen before they emerge as young snails from the egg mass (Goudsmit, 1976).

Uterus, Oothecal Gland, Muciparous Gland, Bursa Copulatrix

The coated eggs pass from the region of the carrefour through a short ciliated oviduct and a glandular uterus, where an outer membrane is formed around the egg from the coagulation of the PVF (deJong-Brink, 1969). The uterus has mucus-secreting cells and opens into the muciparous gland. This gland possesses columnar cells which secrete a jelly-like mucopolysaccharide matrix in which the eggs are embedded (Plesch *et al.*, 1971). The eggs are then bounded by the ootheca or egg capsule which is secreted by the oothecal gland. The walls of this gland are folded and consist of columnar and ciliated cells which produce mainly acid mucopolysaccharides.

The oothecal gland leads to a muscular vagina, which is composed of scattered secretory cells lining its inner ciliated epithelium (Abdel-Malek, 1954). The egg mass is extruded through the vagina and out the female genital opening. A thin gelatinous layer is deposited on the surface of the egg mass by secretory cells located around the female gonopore, and serves to stick the egg mass to a substrate (Plesch *et al.*, 1971).

The bursa copulatix or gametolytic gland is a sac-like organ attached by a ciliated duct to the vagina near the genital opening. It is lined with tall columnar cells, goblet cells, and secretory cells (Abdel-Malek, 1954). Unpackaged eggs and the majority of unused foreign sperm after copulation are hydrolyzed by this gland (Geraerts and Joosse, 1984).

The Male Tract and Associated Accessory Organs

Arising from the hermaphroditic duct is the sperm duct, consisting of several secretory cell types which secrete lipoproteinaceous material (Plesch *et al.*, 1971). The sperm duct enters the prostate gland, which consists of a series of follicles enclosed within a common envelope. These follicles are lined with large cuboidal secretory cells opening into a series of collecting ducts, which are connected by a common duct to the sperm duct (Duncan, 1975). The prostatic secretion consists of lipo-, phospho-, muco-, and glyco-, proteins. The prostatic fluid serves for transport, nutrition, and activation of the sperm (Geraerts and Joosse, 1984), and modulates egg laying (Lucarz, 1991). The region of the sperm duct after leaving the prostate is sometimes called the vas deferens. This duct is muscular and lined with a ciliated epithelium, and opens into the verge (penis) sac. Various neuronal substances have recently been identified which modulate vas deferens motility, and are believed to influence semen transfer during copulation (van Golen *et al.*, 1995a; Li *et al.*, 1995).

Penial Complex

Sperm is transferred to a copulating partner by the intromittent, muscular penis. In most Basommatophora, the penis (verge) is enclosed within a sheath (verge sac) and is everted through the action of hydrostatic pressure and penis retractor/protractor muscles (Geraerts and Joosse, 1984). The penis muscle and copulatory behavior appear to be under neuronal control (Li *et al.*, 1994; van Golen *et al.*, 1996). Associated with the verge sac is the preputium, a structure composed of longitudinal muscle layers. The preputium likely functions as a hold-fast during copulation in the genus *Helisoma* (Abdel-Malek, 1952).

Egg Mass and Embryonic Development

The egg masses of planorbid snails are generally disc-shaped with a variable number of eggs per egg mass. In *H. duryi*, an adult snail will deposit an egg mass that contains between 10-30 eggs. Development proceeds through morula, blastula, gastrula, trochophore, and veliger entirely within the egg mass (Raven, 1975). The egg mass protects against predators, bacterial infections, osmotic stress, and mechanical forces of waves (Geraerts and Joosse, 1984). In *H. trivolvis*, the duration of embryonic development is approximately 9 days at 26.5°C (Goldberg and Kater, 1989). The young hatch as crawling juvenile snails and immediately begin grazing. Environmental factors such as photoperiod, temperature, water quality, and availability of food are known to affect egg mass production (see Geraerts and Joosse, 1984). Favourable environmental stimuli are translated into an internal signal which initiates the synthesis/release of reproductive hormones (Joosse, 1984).

HORMONES AND RECEPTORS

Hormones are chemical substances that can be produced by a single cell, or small groups of cells, or a defined organ. These substances are typically secreted into the body fluid in one part of an organism, and transported to another tissue or organ, or distributed throughout the body (Gorbman and Davey, 1991). Hormones serve as chemical messengers conveying information to their respective target cells to regulate a vast array of cellular processes.

In addition to defined, non-nervous endocrine organs, (for example, the vertebrate

pancreas), many animals have specialized nerve cells referred to as neurosecretory cells, which are sources of hormones. Neurosecretory cells often contain proteins with sulfhydryl groups and thus have an affinity for certain histochemical stains such as paraldehyde fuchsin, chrom-alum hematoxylin, and alcian blue/alcian yellow (Berlind, 1977; Wendelaar-Bonga, 1970). Ultrastructurally, neurosecretory cells contain electron-dense, membrane-bound secretory vesicles ranging from 100-300 nm in diameter (Berlind, 1977; Maddrell and Nordmann, 1979). They can be distinguished from conventional nerve cells which contain smaller (60-100 nm in diameter) membrane-bound granules. These neurosecretory granules are transported along the axon to concentrations of nerve terminals referred to as neurohemal organs, then exocytotically released into the body fluids. This is in contrast to the mechanism by which another class of chemical messengers, the neurotransmitters, are known to operate. These substances, typically consisting of catecholamines,indoleamines, or amino acids are synthesized and released by specialized nerve cells or neurons, which send long processes or axons that contact other cells some distance away. In response to an external (environmental) or internal signal, the neuron sends action potentials along its axon to stimulate the release of a chemical neurotransmitter. Specialized cell junctions between the axon and its target cell called chemical synapses help to deliver the neurotransmitter rapidly (within milliseconds), as opposed to hormone-mediated effects which are relatively long-lasting (minutes to days). Chemicals that act as hormones regulating a specific function may possess other non-endocrine functions and can act as neurotransmitters and vice versa. Thus, the distinction between hormone and neurotransmitter action has become somewhat blurred.

The concentrations of hormones and the specific effects they elicit on their target cells are sensed by specialized proteins called receptors. Most hormones can be placed into one of four general categories : (1) peptides and proteins, (2) amines, (3) prostaglandins, and (4) steroids. These chemical messengers can be divided into two main groups based on their mode of action. In general, steroids and other lipophilic molecules are able to penetrate the cell membrane and each interacts with an intracellular receptor to control activities such as transcription. Proteins, peptides, and amines stimulate (or inhibit) a specific response by binding to receptors located on the plasma membrane. Regardless of whether the receptor is located on the plasma membrane or inside the cell, they will bind their respective ligands with high affinity and specificity. The presence of an appropriate receptor at the target cell therefore provides a molecular mechanism through which the hormone exerts its biologic effect.

Receptors located on the plasma membrane must be able to transduce the hormonal signal or 'first messenger' into specific intracellular changes. Activation of the receptor by an extracellular agonist in many instances causes the formation of soluble transduction molecules known as 'second messengers' that initiate a cascade of intracellular events, ultimately leading to an appropriate biologic effect. These cascades of signalling events are referred to as signal transduction pathways.

The plasma membrane receptors comprise a diverse group of signalling proteins but share some common features such as the presence of a number of hydrophobic membrane-spanning domains that serve to anchor the receptor at the cell surface. In general, the

extracellular sequences participate in ligand binding whereas the intracellular sequences are associated with an effector protein (G-protein) or have direct catalytic (enzymatic) activity. Receptors for structurally and functionally different hormones can be very similar, particularly with respect to its intracellular domains. Thus, despite the enormous array of extracellullar signals presented to target cells, there is a conservation of basic signalling mechanisms that are used repetitively by a large number of different receptors (Ji *et al.*, 1995; Mayo, 1997).

There are three general classes of cell surface receptors which are distinguished by their mechanism of signal transduction. The first class of receptors are those linked to ion channels, often referred to as ligand-gated ion channels. These receptors typically possess four transmembrane domains (Stroud *et al.*, 1990). Binding of the ligand, typically a neurotransmitter, causes a rapid opening of ion channels that are selectively permeable to specific ions. This alters the membrane potential of the cell to stimulate or inhibit neurotransmission. Examples of ligand-gated ion channels are nicotinic acetylcholine, ionotropic glutamate, and GABA receptors. The second class of plasma membrane receptor is the catalytic type which has intrinsic enzymatic activity as part of their structure. This class of receptor has a single transmembrane domain, and when activated, phosphorylates intracellular target proteins, as well as the receptor itself (Cadena and Gill, 1992). The insulin, epidermal growth factor, and insulin-like growth factor receptors are examples of catalytic receptor proteins. The third type of cell surface receptor, and the one which will be discussed in this thesis, is the G-protein-coupled receptor. It generally has seven transmembrane-spanning domains and is linked to intracellular effector proteins called GTP-binding proteins

(G-proteins). Binding of the ligand, typically a protein, peptide, or neurotransmitter, initiates a series of reactions that generates a soluble second messenger such as cyclic adenosine monophosphate (cAMP), calcium or inositol phospholipids (Strader *et al.*, 1994). Since it is now widely accepted that numerous signal transduction pathways are capable of communicating (crosstalk) with one another to regulate distinct cellular processes (Houslay, 1991), investigations in this thesis will be limited to aspects of intracellular signalling through the production of cAMP.

cAMP and Hormone Action

Studies on the mechanism by which hormones regulate glycogenolysis by altering the balance between active and inactive forms of glycogen phosphorylase lead to the discovery of a heat-stable factor synthesized from ATP. The amount of this factor was dramatically increased in liver homogenates treated with either glucagon or catecholamines. This soluble, heat-stable factor was later chemically identified as cyclic-3', 5'-adenosine monophophate (cAMP) (reviewed by Sutherland *et al.*, 1960). Cyclic AMP was the first second messenger to be discovered and provided a system by which the concept of signal transduction cascades were first realized. With the advent of more refined biochemical techniques to assay for cAMP, it became apparent that cAMP regulated an enormous array of biochemical and cellular processes in a variety of cells from diverse organisms. The various components of the cAMP signalling pathway following cell surface binding of an extracellular ligand to a receptor are briefly described below.

G-Proteins

Guanosine 5'-triphosphate or GTP-binding proteins (G-proteins) comprise a family of heterotrimeric glycoproteins situated on the plasma membrane. They are coupling proteins that regulate the activity of numerous intracellular effectors. The G-proteins consist of 3 polypeptide subunits : an alpha (α) subunit (36-52 kDa) which binds and hydrolyses GTP and contains intrinsic GTPase activity; a beta (β) subunit (35-36 kDa); and a gamma (γ) subunit (8-10 kDa)(Neer, 1995). The β and γ subunits exist as functional dimers and only dissociate when they are denatured. When GDP is bound, the α-subunit associates with $\beta\gamma$ to form an inactive heterotrimer which is coupled to an appropriate receptor. After a chemical signal stimulates the receptor, it changes conformation leading to a decrease in affinity for GDP and an increase in affinity for GTP by the α subunit. After GTP is bound, the α-subunit dissociates from the $\beta\gamma$, and both free α and $\beta\gamma$ subunits are able to interact with other intracellular target molecules. The α subunit contains intrinsic GTPase activity and the free subunits remain active until GTP is hydrolyzed back to GDP. Once GTP is converted to GDP, the α and $\beta\gamma$ subunits reassociate, and return to the receptor where it awaits another signal (see Neer, 1995; Rens-Domiano and Hamm, 1995).

There are four main classes of α subunits, grouped according to amino acid sequences, and each class having various members : (1) the α_s class subunits stimulate adenylate cyclase and regulate calcium channels ; (2) the α_i class subunits inhibit adenylate cyclase, regulate potassium and calcium channels, and activate cyclic guanine monophosphate (cGMP) phosphodiesterase; (3) the α_q class subunits activate phopholipase C; and (4) the α_{12}

class subunits regulate sodium/potassium exchange. The α subunits show the greatest diversity and number (over sixteen different G-α subunits have so far been identified in mammals). Recent protein crystalization studies combined with molecular cloning have revealed specific domains that are involved in GTP binding and hydrolysis, βγ subunit association, and receptor recognition (Neer, 1995; Neer and Smith, 1996).

Unlike the numerous α subunits, the genes for only five β subunits and eleven γ subunits have been identified. The γ subunits are more different from one another than the β subunits (Birnbaumer *et al.*, 1991; Hildebrandt, 1997). The βγ complex can interact with receptors, α subunits, adenylate cyclase, phospholipase C, and calmodulin.

Adenylate Cyclase

At present, nine isoforms of mammalian adenylate cyclase have been identified by molecular cloning (Sunahara *et al.*, 1996). The adenylate cyclases are complex regulatory enzymes containing approximately 1080-1248 amino acid residues and show a remarkable resemblance to certain membrane transporters such as the P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (Cooper *et al.*, 1995). Molecular cloning and hydropathicity studies predict adenylate cyclase to have two hydrophobic regions (M_1 and M_2) which span the membrane six times. The two hydrophobic domains are connected by two cytoplasmic domains (C_1 and C_2). The two cytosolic regions are thought to bind ATP, whereas the hydrophobic domains are implicated to be the site for activation by forskolin, a diterpene activator of adenylate cyclase (Tang and Gilman, 1991; Cooper *et al.*, 1995). The different isotypes of adenylate cyclase are impinged upon by multiple regulatory factors (G_s,

G_i, $\beta\gamma$, calcium, calmodulin, forskolin, protein kinases) and are often expressed in discrete anatomical regions, especially in vertebrate nervous tissue (Mons and Cooper, 1995).

cAMP

Cyclic AMP has been identified in practically all tissues of multicellular organisms. It has also been detected in body fluids of vertebrates such as cerebrospinal fluid, plasma, and gastric juice but so far no function has been ascribed to its presence there (Butcher *et al.*, 1972). In the absence of extracellular stimulation, cAMP content in most cells remain relatively constant (~1 μM). However, upon stimulation, cAMP concentration often undergoes dramatic increases (over fifty-fold), depending on the system being studied (Sutherland, 1972). The time course of cAMP production can also vary considerably from tissue to tissue, but in general, the levels of cAMP increase within seconds to minutes after ligand binding.

Cyclic AMP has four main actions once it is activated : (1) it alters the permeability of the cell membrane, allowing for the selective passage of ions into and out of the cell ; (2) it alters intracellular activity by increasing or decreasing the degree of protein phosphorylation; (3) it can alter protein synthesis by interaction with elements of the cell nucleus; (4) it can cause release of calcium (itself a second messenger) from intracellular stores. In certain cell types, an uneven distribution of intracellular cAMP has been reported. This compartmentalization of cAMP may have profound effects on the spatial-temporal aspects of its signalling (Hempel *et al.*, 1996).

cAMP-Dependent Protein Kinase

The primary intracellular effector regulated by cAMP is the cAMP-dependent protein kinase or protein kinase A (PKA). Protein kinase A catalyzes the transfer of the γ phosphoryl group of ATP to hydroxyl groups of serine and threonine residues on proteins. Protein kinase A is a tetramer composed of two heterodimers, each containing regulatory (50 kDa) and catalytic (42 kDa) subunits (Taylor, 1993). Cyclic AMP binds to the regulatory subunits causing them to dissociate from the catalytic subunits. The released catalytic subunits become activated, and in turn, phosphorylate specific target proteins. The catalytic subunits of PKA (Cα and Cβ) are similar, whereas the regulatory subunits have two functional forms, the type I (RIα, RIβ) and type II (RIIα, RIIβ) isoforms, each encoded by a unique gene (Doskeland *et al.*, 1993). The major difference between RI and RII is the RII subunits are autophosphorylated by the catalytic subunits and are membrane-associated, whereas the RI subunits are not phosphorylated and are mainly cytosolic (Hoppe, 1985). Thus, the intracellular location of PKA plays an important role in defining its activity. The regulatory subunits have two types of binding sites to their catalytic units, type α (fast-dissociating site) and type β (slow-dissociating site). The rate at which the regulatory subunits dissociate from their catalytic subunits is an important control mechanism, determining the rate of initiation of the signal. The α forms have a widespread distribution, whereas the the β-forms display a more restricted pattern of expression (Brandon *et al.*, 1997).

Membrane-permeable cAMP analogues such as dibutryl cAMP and 8-bromo- cAMP are thought to activate the catalytic subunits of PKA by first binding to certain regions of the

regulatory subunits, which initiates the release of activated catalytic subunits (Francis and Corbin, 1994). Various analogues that are selective for either the fast- or slow-sites on the regulatory subunits are now available, and combinations of these site-selective analogues may provide an indication of which regulatory subunit is mediating the biologic effect being examined.

Cyclic Nucleotide Phosphodiesterases

The intracellular concentration of cAMP is regulated by the rate of synthesis (via adenylate cyclase) and the rate of degradation of cAMP. The two major mechanisms that regulate cAMP removal in cells is the degradation by cyclic nucleotide phosphodiesterases and export through the plasma membrane. About 30 isoforms of cyclic nucleotide phosphodiesterases have been identified, each possessing specific affinity for cAMP and cGMP (Manganiello *et al.*, 1995). Both particulate and cytosolic forms of phosphodiesterase exist, which can be targeted to specific intracellular structures. The ability of the cell to regulate its phosphodiesterase activity provides a way to alter the magnitude and duration of the cAMP-mediated response. The cAMP phosphodiesterases hydrolyze cAMP to 5'- AMP which is generally inactive. Methyl xanthine derivatives such as theophylline and 3-isobutyl-1-methylxanthine are conventional phosphodiesterase inhibitors used in most systems to prolong cAMP action in the cell. They are thought to function as competitive inhibitors and also antagonize adenosine receptors (Beavo, 1995). In some eukaryotic cells, cAMP is actively extruded through the cell membrane. For example, in slime molds, secreted cAMP serves as a chemotactic signal for amoeba to congregate into a muticellular colony

(Devreotes, 1983). The function of this efflux in other systems is uncertain, other than to reduce the intracellular concentration of cAMP (Barber and Butcher, 1983).

The differential regulation and spatial distribution of the multiple forms of adenylate cyclase, cyclic nucleotide phosphodiesterases and PKA allow for specific intracellular targeting. In addition, calcium, G-protein $\beta\gamma$ subunits, and other intracellular enzymes such as protein kinase C (PKC) impinge on various components of the cAMP pathway permitting cross-talk between different signalling systems, thus allowing for continuous adjustments to the sensitivity at which the system can respond to extracellular signals (Houslay and Milligan, 1997).

Criteria Used to Determine if a Cellular Response is Mediated by cAMP

In order to demonstrate that a hormone-receptor system uses cAMP as a signalling molecule, there are several criteria that need to be satisfied (Sutherland and Robison, 1966): (1) application of agonist should stimulate cAMP accumulation in cells, (2) the agonist should stimulate adenylate cyclase in membrane preparations, (3) cAMP levels should be potentiated by inhibitors of cAMP phosphodiesterase, (4) biologic actions should be mimicked by cAMP analogues. This list of criteria has been modified and extended in recent years with the introduction of new and specific cAMP agonists and antagonists (Doskeland *et al.*, 1991), but the basic criteria listed by Sutherland and Robison (1966) are still valid and serve as useful guidelines.

Organization of the Central Nervous System of *Helisoma duryi*

The CNS of *Helisoma duryi* is representative of the general organization of the pulmonate nervous system. It includes 5 paired ganglia; buccal, cerebral, pedal, pleural, parietal, and a single visceral ganglion (Fig. 3). The microscopic anatomy of the CNS of *H. tenue* has been described in detail by Simpson *et al.* (1966) and Simpson (1969). The endocrine centers that control reproduction in pulmonates are located in the cerebral ganglia. Various neuroendocrine and CNS-associated endocrine centers have been reported to directly influence the gonads, accessory reproductive organs, or to modulate reproductive processes (see Geraerts *et al.*, 1988; Joosse, 1988; Saleuddin *et al.*, 1994). Endocrine centers in the cerebral ganglia which have been examined in detail are the neurosecretory growth-stimulating mediodorsal cells (light green cells in *L. stagnalis*), the lateral lobes, the caudodorsal cells (CDCs), and the CNS-associated endocrine dorsal bodies (DBs).

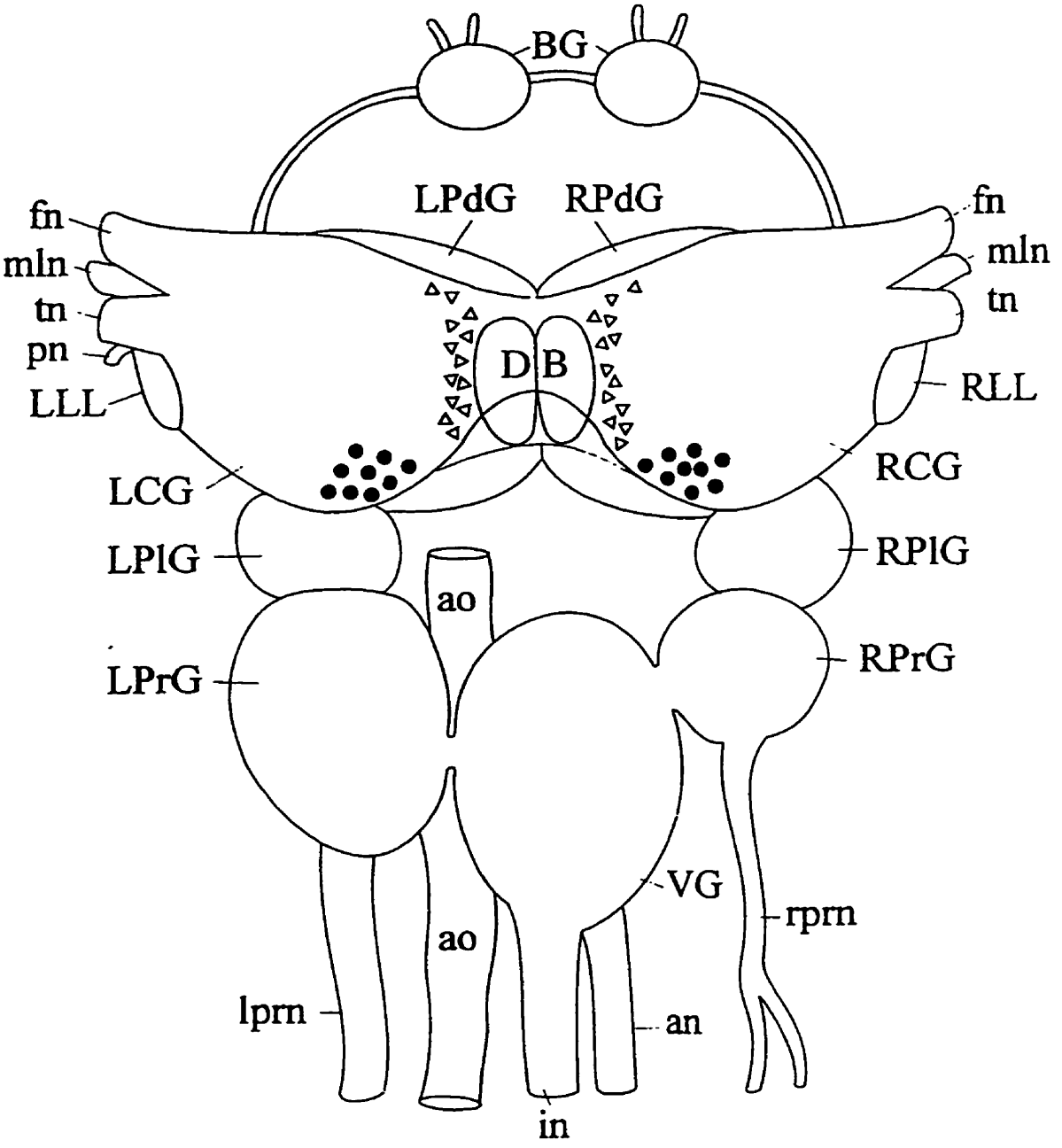
Mediodorsal Cells

The mediodorsal cells (MDCs) of *H. duryi* are located in a single cluster of approximately 30 cells at the mid-dorsal region in each cerebral ganglion. The MDCs lie adjacent on either side of the cerebral commissure and DBs (Khan *et al.*, 1992). The MDCs and their axons stain intensely with paraldehyde fuchsin, and release their secretory products at the periphery of the median lip nerve (labial nerve). In *H. duryi*, the MDCs are thought to be involved in regulating shell and body growth (Saleuddin and Kunigelis, 1984; Kunigelis and Saleuddin, 1985). Khan *et al.* (1992) demonstrated the MDCs of *H. duryi* contain insulin-like immunoreactivity and are capable of secreting insulin-like peptides (ILPs) *in vitro*.

Fig. 3 A diagram of the central nervous system of *Helisoma duryi*. The position of some of the neurosecretory cells in the cerebral ganglia and the endocrine dorsal bodies is shown.

DB	dorsal bodies
●	caudodorsal cells
△	mediodorsal cells
BG	buccal ganglia
LPdG, RPdG	left and right pedal ganglion
LLL, RLL	left and right lateral lobe
LCG, RCG	left and right cerebral ganglion
LPlG, RPlG	left and right pleural ganglion
LPrG, RPrG	left and right parietal ganglion
VG	visceral ganglion
fn	frontal lateral nerve
mln	median lip nerve
tn	tentacular nerve
pn	penis nerve
ao	aorta
lprn	parietal nerve
in	intestinal nerve
an	anal nerve

Fig. 3


BG
LPdG
RPdG
fn
mln
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LLL
D
B
fn
mln
tn
RLL
RCG
LCG
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RPlG
ao
RPrG
LPrG
VG
rprn
ao
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in

The hemolymph concentration of ILPs increased in shell-regenerating animals and, mammalian insulin stimulated *in vitro* protein synthesis in the mantle (Saleuddin *et al.*, 1992), suggesting insulin-related molecules produced by the MDCs are involved in growth.

In *L. stagnalis*, the neurosecretory light green cells (LGCs) are homologous to the MDCs of *H. duryi*. The LGCs are large (~90 μm in diameter) cells located in a medio- and latero-dorsal group of cells in each cerebral ganglion. The LGCs are thought to produce a growth hormone since removal of the LGCs retarded body growth, and reimplantation of cerebral ganglia containing LGCs restored growth (Geraerts, 1976a). Mobilization of glycogen stores, shell formation and stimulation of cell multiplication in the soft body parts are some of the functions attributed to the LGCs (Geraerts, 1976a, 1992). Immunohistochemical studies have demonstrated that the LGCs, their axons, and the median lip nerve (neurohemal area) react with an anti-mammalian insulin antibody (Schot, 1981, Ebberink *et al.*, 1987). In order to identify the peptides produced by the LGCs, mRNA was extracted from these cells, then cDNAs were constructed and used to screen a CNS-specific cDNA library. Light green cell-specific clones revealed that the encoded precursor molecule had a similar structure to vertebrate insulin, and it was named molluscan insulin-related peptide (MIP) (Smit *et al.*, 1988). In the CNS, MIP is expressed in the LGCs and in the canopy cells of the lateral lobe. Biochemical and molecular studies have shown that MIP belongs to a multigene family of which 4 members have been identified. Each of the MIPs is thought to regulate distinct aspects of growth and metabolism in *L. stagnalis* (van Minnen *et al.* 1989b; Geraerts *et al.*, 1992). A putative MIP-related receptor has also been cloned,

which possesses some of the typical features of an insulin receptor protein (Roovers *et al.*, 1995).

Lateral lobes

The lateral lobes (LLs) are small ganglia that project laterally from of each cerebral ganglion and are thought to be neuroendocrine coordinating centers involved in the control of growth and reproduction (Geraerts, 1976b; Roubos *et al.*, 1980). Extirpation of the LLs stimulated growth (Geraerts, 1976b) and reduced egg-laying activity (Roubos *et al.*, 1980), suggesting the LLs inhibit the growth hormone-producing center (LGCs) and stimulate the reproductive centers (DBs and CDCs). In *L. stagnalis*, the LLs contain two types of neurosecretory cells, a canopy cell (CC) and a droplet cell (DC) (Geraerts, 1976a), whereas in *H. duryi*, two lateral lobe cells, lateral lobe cell 1 (LLC_1) and lateral lobe cell 2 (LLC_2), and a CC are present (Saleuddin *et al.*, 1996). Both of these snails also have a structure associated with the LLs called a follicle gland (FG). Axons from the left and right canopy cells cross through the cerebral commissure to the contralateral ganglion and run closely to the axons from the opposite LGCs (Benjamin *et al.*, 1976). In the cerebral commissure, the LGC axons show intricate branching patterns as they project varicosities that run closely to the CDCs and DBs. Saleuddin and Ashton (1996) have shown direct innervation of the DBs by LLC_1, LLC_2, and the CC, from only the right lateral lobe neurosecretory cells. The MIP-immunoreactive canopy cell is a specialized LGC that is believed to transmit regulatory signals to the LGCs and the female reproductive centers (Geraerts *et al.*, 1992). The neurosecretory activity of the LLs in *L. stagnalis* is thought to be influenced by photoperiodic

input (van Minnen and Reichfelt, 1980). In *H. duryi*, neurites from the optic nerve form synapse and synapse-like contacts with the neurosecretory cells of the LLs (Saleuddin and Ashton 1996), and the activity of these cells changes under different photoperiodic conditions (A.S.M. Saleuddin, unpublished observations).

Caudodorsal Cells

The caudodorsal cells (CDCs) are neurosecretory cells located in the caudo-dorsal part of each cerebral ganglion and were first described in *L. stagnalis* by Joosse (1964). Cells homologous to the CDCs have been found in the cerebral ganglia from all basommatophorans studied thus far (Boer *et al.*, 1977; Roubos and van de Ven, 1987; van Minnen *et al.*, 1992). In the opisthobranch *Aplysia californica*, functionally identical neurosecretory cells called the bag cells (BCs) are located in the abdominal ganglion (Geraerts *et al.*, 1988). The CDCs and BCs have been the subject of intense endocrinological, cellular, and molecular studies for over 30 years, and consequently there are numerous reviews reflecting the physiology and biochemistry of these cells (Joosse and Geraerts, 1983; Geraerts *et al.*, 1988; Nagle *et al.*, 1989a, 1989b; Conn and Kaczmarek, 1989; Arch and Berry, 1989). The CDC somata are located in two clusters in each cerebral ganglion. In *L. stagnalis*, the left ganglion contains a group of 20-40 cells, and the right ganglion contains 50-100 cells (Joosse 1964; Boer 1965; Wendelaar-Bonga 1970, 1971). Individual CDC perikarya can attain a size of up to 90 µm in diameter. In *H. duryi*, the CDCs occur as clusters in the left ($\sim$ 40 cells) and right ($\sim$30 cells) cerebral ganglia and are approximately 40-50 µm in diameter (Khan *et al.* 1990b, Saleuddin *et al.*, 1990). All the CDCs have axons that emerge from their cell bodies and run

along the anterior portion of the cerebral ganglia, forming an area where they lie close together called the loop area. From the loop area, the axons project in the peripheral layer of the ipsilateral portion of the cerebral commissure, which is the neurohemal area of the CDCs (Wendelaar-Bonga, 1971). The neurohemal area is intimately associated with the hemolymph through an extensive network of blood spaces in the perineurium (Bekius, 1972). This area is morphologically separated from the inner compartment of the of the cerebral commissure by a glial cell sheath. Some of the ventral CDCs also project axons that cross through the cerebral commissure contacting other CDCs in the contralateral ganglion (de Vlieger *et al.*, 1980). These crossing axons give rise to an extensive network of diffuse collaterals which ramify within the inner portion of the cerebral commissure (Schmidt and Roubos 1989; Schmidt *et al.*, 1989). The collaterals end blindly and release their products into the intercellular spaces and are thought to communicate with various other cells within the CNS in a paracrine fashion. In *H. duryi*, many CDCs also project axons which pass through the subesophageal ganglia and may innervate peripheral and visceral organs (Saleuddin *et al.*, 1990).

The CDCs are known to produce a neurohormone called caudodorsal cell hormone (CDCH), also called ovulation hormone, which stimulates ovulation and oviposition (Geraerts and Bohlken, 1976), and controls various behavioral aspects of the egg-laying process (see Geraerts *et al.*, 1988). The egg-laying cycle in *L. stagnalis* occurs every 1-3 days and during this time the CDCs exhibit three states of electrical excitability (Kits, 1980). The CDCs are usually electrically quiescent (resting state). However, prior to egg-laying the cells display

a marked increase in electrical activity. During the active state (~1 hr), all the CDCs show synchronous spiking activity (the afterdischarge), resulting in an increase in exocytotic release from the axon terminals (Burna and Roubos,1985), and a concomitant rise in the CDCH titre in the hemolymph (Joosse, 1986). The CDCs within one group make cell contacts with each other through gap junctions and synchronization between groups of cells is brought about by crossing axons within the cerebral commissure which contact the contralateral group of CDCs (Schmidt and Roubos, 1987). A lack of spontaneous electrical activity by the CDCs (the inhibited state) follows, and persists for several hours. The ovotestis becomes refractory during this phase and egg mass production ensues. The CDCs then resume their resting state again until the next bout of egg-laying (Joosse and Geraerts, 1983).

Egg-laying in *L. stagnalis* and *A. californica* is an example of a neurohormone-induced stereotyped behavior (Geraerts *et al.*, 1988). It lasts for several hours and involves a number of internal physiological events (ovulation, egg mass formation, oviposition), which are coincident with a series of fixed overt behaviors. In *L. stagnalis*, the CDC system can be activated by a combination of external factors such as abundant food, clean oxygenated water, and long-day photoperiods (Bohlken and Joosse, 1986; ter Maat *et al.*, 1983; Joosse, 1984). At the cellular level, the ventral CDCs receive nervous input on the cell perikarya and along their axons (see Geraerts *et al.*, 1988). However, the exact mechanism by which the CDCs are activated *in vivo* is unclear.

The primary structure of the CDCH in *L. stagnalis* was determined as an amidated peptide containing 36 amino acid residues having a molecular mass of 4477 Da and an

isoelectric point of 9.3 (Ebberink *et al.*, 1985). The CDCH shares approximately 44% homology at the amino acid level to the egg laying hormone (ELH) of *A. californica*. The BCs of *A. californica* and the CDCs of *L. stagnalis* synthesize and secrete their products according to the cellular paradigm of regulated protein secretion (Sossin *et al.*, 1989; Perone *et al.*, 1997). The CDCH precursor (preproCDCH) is a polypeptide of 259 amino acid residues and is predicted to have a 34 amino acid signal sequence, five potential dibasic cleavage sites, and six potential tribasic cleavage sites (Vreugdenhil *et al.*, 1985, 1988). After the signal peptide is cleaved off in the membrane of the RER, the prohormone passes into the Golgi apparatus where it is post-translationally processed, packaged into secretory granules, then sorted for export or retained for degradation (Geraerts *et al.*, 1988; Sossin *et al.*, 1989). If all the potential processing sites are utilized, the precursor would yield 12 peptides. However, recent studies have indicated that not all processing sites are used *in vivo* and about 10 peptides are derived from the CDCH precursor (van Heumen and Roubos, 1991; van Heumen *et al.*, 1992).

Several other CDC products have also been isolated and sequenced : a 14-residue peptide called calfluxin (CaFl), which stimulates the influx of calcium into mitochondria of the albumen gland secretory cells (Dictus *et al.* 1987b, 1988; Dictus and Ebberink, 1988); a set of autoexcitatory peptides named alpha (α)-caudodorsal cell peptide and three beta (β)-caudodorsal cell peptides (Vreugedenhil *et al.*, 1988; Brussaard *et al.*, 1990); and a 44-residue peptide sharing high amino-terminal homology to CDCH (now called CDCH-II), has recently been isolated but its function remains unknown (Li *et al.*, 1992). Molecular studies

have shown that these peptides are encoded by genes that belong to a small multigene family (Geraerts *et al.*, 1988; Vreugdenhil *et al.*, 1988; Nagle *et al.*, 1989b). In *L. stagnalis*, cDNA cloning has shown there are two genes encoding the CDC peptides, the CDCH-I and CDCH-II genes. The CDCH-I gene codes for the precursor containing the 36- residue CDCH (CDCH-I), CaFl, and the α- and β- caudodorsal cell peptides, whereas the CDCH-II gene codes for the 44-residue CDCH-II.

The CDCH gene family is also expressed in a variety of neural and non-neural tissues. CDCH-related transcripts are present in the CDCs as well as other neurons in the CNS and in peripheral neurons innervating the accessory sex organs (van Minnen *et al.*, 1989a). The oothecal gland, muciparous gland, and oviduct have neuronal processes terminating on their secretory cells, suggesting the secretory activities of these cells are regulated by the nervous system. Caudodorsal cell hormone-immunoreactive neurons and their processes have been detected in male accessory sex glands such as the prostate and spermduct. In addition, CDCH-like immunoreactive fluid is present in the lumen of the male tract, indicating CDCH-related peptides might be transferred to a partner during copulation (van Minnen *et al.*, 1989a).

Endocrine Structures Associated With the CNS in Molluscs

The gastropod molluscs possess distinct endocrine cells that are associated with the central nervous system. Lever (1958) observed a cluster of cell bodies on the dorsal surface of the cerebral commissure from the freshwater snail *L. stagnalis* and referred to these

structures as dorsal bodies (DBs). Investigations of other molluscs confirmed the presence of CNS-associated endocrine structures analogous to the pulmonate DBs (see Saleuddin *et al.*, 1994). In the prosobranchs and opisthobranchs, they are called the juxtaganglionar organs (JOs) and in the cephalopods they are called the optic glands (OGs) (Fig. 4). These CNS-associated endocrine structures are thought to produce a hormone(s) involved in the regulation of female reproductive activity (see Joosse, 1988; Saleuddin *et al.*, 1994, Saleuddin, 1998a). The location, cellular organization, and the control of the DBs, JOs and OGs as well as current information about their secretory products will be discussed below.

Dorsal Bodies

Basommatophora

The dorsal body cells of the Basommatophora occur as two yellowish-white masses located on the dorsal surface of the cerebral commissure. The DBs are spatially separated by the cerebral commissure in the genera *Ancylus* and *Lymnaea*, but in the genera *Helisoma*, *Planorbarius*, and *Austrolorbis*, they lie close together or are fused (Boer *et al.*, 1968; Simpson *et al.*, 1966; Simpson, 1969). The embryonic origin of the DBs is not firmly established. Boer *et al.* (1968) have suggested that the DBs are of mesodermal origin because the DB cells are not separated from the mesodermally-derived myoblasts and fibroblasts by a clearly delineated basement membrane. However, Saleuddin *et al.* (1997) have suggested that the DBs may be of ectodermal origin based on the presence of DBCs within the cerebral ganglia of *Siphonaria pectinata*. Ultrastructurally, Golgi bodies were

rarely present in basommatophoran DBCs and granular ER was sparse. The large mitochondria with distinct intramitochondrial granules and lysosome-like bodies were the DBCs most conspicuous features described by these authors.

In *H. duryi*, the DBC perikarya are generally 10-15 μm in diameter and are grouped together as 6-12 cells forming a lobule which is separated from neighboring lobules by collagen fibers, muscle and connective tissue, and pore cells (Saleuddin *et al.*, 1989). There are two distinct zones in the DBs, the cortex, containing the cell body region, and the medulla, containing the cell processes with their secretory granules. The DB cell processes project into the medulla where they branch into finer processes that interdigitate with other DBCs (Khan *et al.* 1990). These short cytoplasmic processes contain moderately electron-dense, membrane-bound granules about 70-90 nm in diameter, which are released by exocytosis (Boer *et al.*, 1968; Saleuddin *et al.*, 1989; Saleuddin *et al.*, 1997). The exocytotic release of these granules increases dramatically in first-mated snails compared to virgins (Saleuddin *et al.*, 1989). The contents and function of the DBC granules are unknown.

In the Basommatophora, the DBs were not originally believed to be innervated by neurons from the CNS (see Joosse, 1988). However, recent detailed electron microscopic studies of the DBs of *H. duryi* reveal that the DBs are indeed innervated (Saleuddin and Ashton, 1996). Three large neurosecretory cells, a canopy cell and two lateral lobe cells, from the right lateral lobe project their axons to the DBs. The neurites from the the canopy cell innervate the the cell bodies, whereas the neurites from the lateral lobe cells innervate the DB cell processes. An additional level of complexity about the regulatory control of the DBs

is the observation that the optic nerve forms synapses with one lateral lobe cell and synapse-like contacts with the other lateral lobe cell (Saleuddin and Ashton, 1996).

Stylommatophora

In the terrestrial pulmonates, the DBs are less discrete structures than those seen in freshwater snails. The DBs consist of groups of cells dispersed within the thick connective tissue sheath that surrounds the cerebral ganglia (Saleuddin *et al.*,1991; Ohtake and Takeda, 1994; Takeda and Ohtake, 1994b). Surrounding the DBCs are glycogen-rich support cells, mucous cells and calcium cells. Examination of the fine structure of the DBCs shows that they have many ellipsoidal lipid droplets, secretory granules, well-developed Golgi complexes, SER, and numerous large mitochondria with electron-dense inclusions (Nolte, 1983; Saleuddin *et al.*, 1991; Takeda and Ohtake, 1994a). Similar morphological findings were reported for the DBs of the slugs *Limax maximus* (Van Minnen and Sokolove, 1984) and *Arion rufus* (Ezzughayyar and Wattez, 1989). In *Achatina fulica*, the presence of cilia in the DBCs were reported but their function is unknown (Takeda and Ohtake, 1994b). A diurnal rhythm of DB activity has been reported for field-collected *H. aspersa* (Mounzih *et al.*, 1988). Based upon ultrastructural observations, it appeared the synthetic activity of the DBCs increased during the scotophase and release of secretory material occurred in the photophase.

Functions of the Pulmonate Dorsal Bodies

The physiological role of the DBs has been established through classical endocrinological ablation and replacement experiments. In *L. stagnalis*, removal of the DBs reduced the proportion of mature oocytes in the ovotestis, and prevented the development

of the female accessory sex organs and their cellular differentiation in juvenile snails (Geraerts and Joosse, 1975; Geraerts and Algera, 1976). In *H. duryi*, ablation of the DBs from mated snails drastically reduced egg-laying and the proportion of mature oocytes in the ovotestis (Schollen and Saleuddin, 1986). The *in vitro* synthetic activity of the albumen gland from DB-ablated snails was significantly reduced as was the wet weight of the female accessory sex organs (Miksys and Saleuddin, 1987). Spermatogenesis and the male accessory sex organs remain unaffected by DB-ablation as in *L. stagnalis*, hence, the effect of the DBs on reproduction appear to be female-specific. Similar findings have been reached in the terrestrial pulmonates *Agriolimax reticulatus* (Wijdenes and Runham, 1976), *Arion rufus* (Ezzughayyar and Wattez, 1989), and *Helix aspersa* (Griffond and Vincent, 1985). In *H. aspersa*, coculture of juvenile ovotestis with DBs from reproducing adults stimulated the uptake of [^{14}C]leucine and [^{3}H]fucose (Barre *et al.*, 1990). In *H. duryi* and *H. aspersa*, crude extracts of cerebral commissure and cerebral ganglia (including DBs) generated amoeboid movement of mature oocytes from the enclosing follicle cells *in vitro* (Saleuddin and Khan, 1981; Saleuddin *et al.*, 1983a), indicating a possible role in ovulation. Dorsal body extract was reported to increase the activity of adenylate cyclase in the follicle cells of *L. stagnalis*. The physiological significance of this activation remains uncertain (deJong-Brink *et al.*, 1986).

The DBs of *H. aspersa* receive innervation from the growth-stimulating neurosecretory cerebral green cells (Vincent *et al.*,1984; Wijdenes *et al.*,1987). These cells are suggested to exert an inhibitory effect on DB activity, since long-term *in vitro* incubation of whole CNS with gonad inhibits the development of mature oocytes. In *H. aspersa*, the

DBs also receive innervation from FMRFamide-immunoreactive axons from unidentified cells within the CNS (Griffond *et al.*,1990). These authors report that FMRFamide inhibits the uptake of [^{3}H]-methionine in DBCs (Griffond and Mounzih, 1989). The methionine is thought to be involved in the synthesis of Met-enkephalin which is immunohistochemically detectable in DBCs of *H. aspersa* (Marchand and Dubois, 1986; Marchand *et al.*, 1991).

Juxtaganglionar Organs

In the prosobranchs *Gibbula umbilicalis*, *G. pennanti*, *G. cineraria*, *Monodonta lineata*, and *Haliotis rufescens*, the juxtaganglionar organs (JOs) are light yellow structures found in both sexes along the posterior portion of the the cerebral commissure (Herbert, 1982; Clare, 1987; Miller *et al.*, 1973). The JOs lie within the perineurium and are composed of glandular cells interspersed with connective tissue, much like the arrangement of the DBs in stylommatophoran pulmonates. The JOs are penetrated by a hemolymph space lined with basal lamina. At the ultrastructural level, numerous electron-dense secretory granules (100-200 nm in diameter) and many mitochondria are present. The amount of ER and the cellular activity of the JO cells varies, depending on seasonal reproductive cycles (Herbert, 1982; Clare, 1987). Some cells bear cilia which project into the hemolymph spaces between the cells as seen in the pulmonate *A. fulica* (Takeda and Ohtake, 1994a). Cytoplasmic processes arising from the JO cells interdigitate with one another and granule release is thought to occur by exocytosis. In the abalone *H. rufecens*, the juxtaganglionic cells are concentrated at the base of the cerebral-pleural connective, but are also spread over the bases of the tentacular and optic nerves (Miller *et al.*, 1973). During the breeding season, the JOs are rich

in mitochondria, lipid, and some large electron-dense vesicles.

The JOs of the opisthobranch *Aplysia juliana* are more condensed than the DBs of terrestrial pulmonates but less compact than the DBs of freshwater snails (Switzer-Dunlap, 1987). Ultrastructurally, the cells of the opisthobranch JOs are similar to prosobranch JOs and the pulmonate DBs. They share similar nuclear morphology, numerous mitochondria, secretory granules (80-90 nm), limited RER, and abundant lipid droplets. In *A. juliana*, the JO cells are ciliated and appear to project into the matrix surrounding other JO cells. The JOs of opisthobranchs and prosobranchs appear to be poorly innervated but well vascularized. Unfortunately, there have not been any physiological studies done on the JOs, and only circumstantial evidence exists regarding their role in reproduction.

Optic Glands

The optic glands (OGs) of cephalopods contain only one cell type called the stellate cell. These cells are presumed to be of ectodermal origin (Bonichon, 1967). The stellate cells are characterized by the presence of abundant, tubular mitochondria and free ribosomes, scarce RER, electron-dense granules, and round nuclei with prominent nucleoli (Bjorkman, 1963; Bonichon, 1967). Stellate cell processes project and release their secretions near blood capillaries (Nishioka *et al.*, 1970). In *Octopus vulgaris*, the OGs become a bright orange colour as the animals become sexually mature and the ablation of OGs from maturing octopus causes regression of the gonads (Wells *et al.*, 1975). The OGs are under nervous inhibitory control by the the brain. Precocious sexual development can be induced by severing the optic nerve or the optic tract connecting the OGs to the CNS, thereby stimulating the activity of

the OGs. Activation of the OGs can also be induced by removal of the optic lobe or the subpendiculate lobe of the CNS (Wells and Wells, 1977). Nerve fibers immunoreactive to the neuropeptide FMRFamide have been localized within the optic tract and innervating the OGs (LeGall *et al.* 1988), and it is possible FMRFamide-related peptides (FaRPs) might be involved in regulating the activity of the OGs as was reported for the DBs in *H. aspersa* (Griffond and Mounzih, 1989).

The optic gland hormone (OGH) appears to be neither sex- nor species-specific (Wells and Wells, 1975; Richard, 1970). The OGH regulates yolk protein synthesis by the follicle cells and stimulates the synthetic activity of both male and female accessory sex organs (Wells and Wells, 1972; O'Dor and Wells, 1973, 1975). In vitellogenic female *O. vulgaris*, the OGH inhibits somatic protein synthesis and increases free amino acid levels in the hemolymph. The excess free amino acids are thought to be taken up by the follicle cells during vitellogenesis (O'Dor and Wells, 1978). Optic gland extracts and hemolymph from maturing animals stimulates follicle cell mitosis in the cuttlefish *Sepia officinalis*, suggesting a role for OGH in gonadal cell proliferation and differentiation (Koueta *et al.*, 1992).

Chemical Nature of the DBH and OGH

The chemical nature of the DBH remains a matter of controversy. In *L. stagnalis*, biological activty of crude DB tissue extracts was shown to be protease-sensitive and associated with a protein having a molecular mass of about 30 kDa, indicating the DBH was probably a large polypeptide (Ebberink *et al.*, 1983). However, further purification attempts were unsuccessful as biological activity was lost or greatly diminished. In the slug *L.*

maximus, a factor from cerebral ganglia extracts (including DBs) stimulated *in vitro* galactogen synthesis from albumen glands (vanMinnen *et al.*, 1983). This factor was presumed to be of DB origin and was called galactogen-synthesis stimulating factor (GAL-SF). Galactogen-synthesis stimulating factor was partially characterized as heat- and protease-sensitive, and not extractable with mild acid or base (vanMinnen and Sokolove, 1984). Gel filtration chromatography estimated GAL-SF to have a molecular mass between 4-7 kDa. The DBH of *H. aspersa* is suggested to be a peptide based upon the presence of a well-developed Golgi, RER, elementary granules and increased exocytosis frequency when the animals are egg-laying (Griffond and Vincent, 1985; Mounzih *et al.*, 1988).

The DBs of pulmonates also have features reminiscent of steroid-synthesizing cells (Saleuddin *et al.*, 1989; Saleuddin *et al.*, 1991; Saleuddin *et al.*, 1997). Nolte *et al.* (1986) have detected the *in vitro* release of the steroid hormone ecdysone from the DBs of *H. pomatia*. Steroid biosynthetic enzymes are also present within the DB tissue (Krusch *et al.*, 1979), and a novel cytochrome P-450 gene whose metabolic function remains unknown has recently been cloned in *L. stagnalis* (Theunissen *et al.*, 1992). This gene is expressed solely in the DBs and may be involved in the synthesis of the DBH.

At present, there have been no biochemical or physiological studies on the JOs. The nature of the cephalopod OGH is still unknown. In *S. officinalis*, the OGH was partially characterized as a heat-labile, trypsin-sensitive molecule of molecular mass (4-5 kDa), suggesting that it is probably a polypetide (Koueta *et al.*, 1992). In contrast, ultrastructural investigations by Froesch (1979) have suggested that the OGs may produce a steroidal

substance. In *O. vulgaris* and *Loligo pealii*, a substance from the OGs was partially characterized which had physico-chemical characteristics of an ecdysteroid-like molecule (R. O'Dor personal communication).

Ecdysteroids

Ecdysteroids comprise a group of polyhydroxylated steroid hormones that were initially discovered as the molting hormones of insects and crustaceans. Ecdysone (E), the first molecule identified from this family of steroid hormones, was successfully isolated from 500 kg of *Bombyx mori* pupae by Butenandt and Karlson in 1954. As with all steroids, E has the usual four ring nucleus, but unlike the vertebrate steroids it retains the full side chain of cholesterol (Thompson and Lusby, 1989). Some characteristic features of the E molecule are a *cis*-fused A/B ring, a keto group in the B ring conjugated to a double bond, a 7-ene-6-one chromophore, and a 14α-OH (Fig. 4). The steroid nucleus of E is rather rigid, whereas the side chain is flexible depending on its environment. This is thought to be important with respect to the interaction of ecdysteroids with their receptors and other macromolecules (binding proteins). At least 61 different 'zooecdysteroids' and over 100 'phytoecdysteroids' have been identified (Rees, 1989).

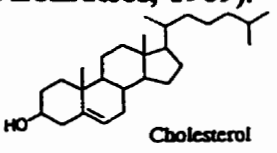
Biosynthesis of Ecdysone

In larval insects, the source of ecdysteroids is the prothoracic glands, and in crustaceans it is the Y-organ (Redfern, 1989). In adult female insects, the ovarian follicle cells synthesize ecdysteroids (Goltzene *et al.*, 1978), whereas in male insects, the testis are thought to produce ecdysteroids (Loeb *et al.*, 1982). Despite the numerous ecdysteroid

molecules that have been isolated, the complete biosynthetic pathway of E is not known, even in insects. A brief description of what is known about the synthesis of ecdysteroids in insects is given, since this is the best characterized system. As with many steroid hormones, E is synthesized from a cholesterol precursor. Unlike the vertebrates, arthropods cannot synthesize cholesterol *de novo*, consequently, it must be derived from their diet. The steroid nucleus is first modified by the introduction of a second double bond into the B ring and then cholesterol is converted to 7-dehydrocholesterol. The A/B *cis*-ring, the keto group of the B ring, and the hydroxyl at C14 are introduced by a series of reactions that are not fully understood, often called the 'black box' reactions. The enzymes participating in E synthesis are microsomal and mitochondrial located enzymes of the cytochrome P-450 superfamily (Kappler *et al.*, 1989). An intermediate steroid, 5β-ketodiol (2, 22, 25-trideoxyecdysone) is produced, which undergoes three consecutive hydroxylations at C25, C22, and C2. Alternative biosynthetic routes appear to exist between 7-dehydrocholesterol and 5β-ketodiol, and 5β-ketodiol and E, depending on the species of insect examined. This suggests there are multiple pathways of E synthesis. The products and specific enzymes involved in many of these alternative pathways are just beginning to be examined (see Greneisen, 1994).

Ecdysteroids are immediately released into the hemolymph after they are synthesized, and only small amounts can be detected in ecdysiosynthetic tissues. Therefore, release of ecdysteroids reflects their synthesis (Redfern, 1989). The hormone is transported through the hemolymph to peripheral tissues where it undergoes hydroxylation at C20 to produce 20-hydroxyecdysone (20E). This is the major circulating ecdysteroid in the majority of

Fig.4 The structural formulae of selected ecdysteroids which have been isolated from invertebrates (modified from Rees, 1989).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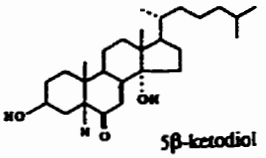
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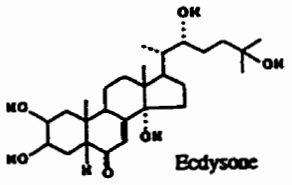
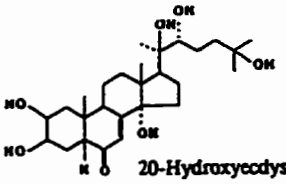
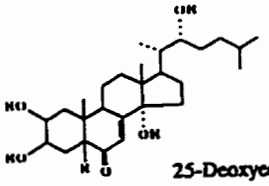
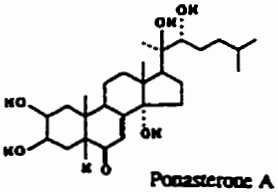
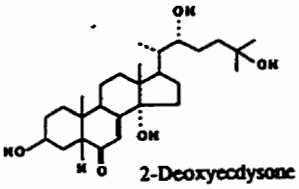
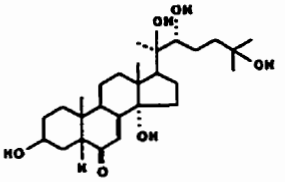

Ecdysone

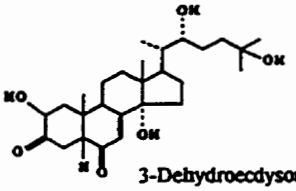

20-Hydroxyecdysone


25-Deoxyecdysone


Ponasterone A


2-Deoxyecdysone


2-Deoxy-20-Hydroxyecdysone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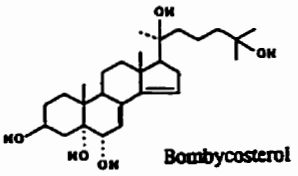
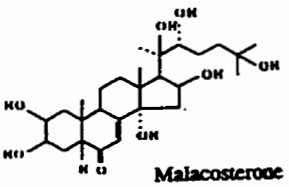
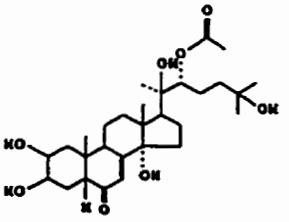

Bombycosterol


Malacosterone


20-Hydroxyecdysone 22-Acetate

arthropods, and is generally the most potent ecdysteroid in bioassays. Ecdysteroids can circulate in either free or bound (carrier proteins) form, but it is thought the free form represents the active hormone.

Metabolism of Ecdysteroids

Ecdysteroids can be metabolized intracellularly by a variety of tissues, and converted to either biologically active or inactive forms. Conjugation of ecdysteroid molecules to phosphate esters, glucosides, long chain fatty acids, or esters of acetic acid render the ecdysteroid molecule inactive (Lafont and Connat, 1989). These ecdysteroid conjugates can be stored in appreciable quantities in the gut or can be readily excreted. Therefore, the ecdysteroid titer can be regulated by the rate of synthesis, metabolism and excretion of hormone.

Mode of Action

Ecdysteroids, like their vertebrate steroid counterparts, enter their target cells and travel to the nucleus where they bind to their receptor. The activated ecdysteroid/receptor complex then interacts with specific sites on DNA to induce the synthesis of new RNA transcripts. The ecdysteroid receptor has recently been identified by molecular cloning (Koelle *et al.*, 1991), and biochemical purification (Luo *et al.*, 1991) and belongs to the steroid hormone receptor superfamily (Segraves, 1991). However, the cloned ecdysteroid receptor is incapable of high affinity DNA binding or transcriptional activation on its own (Koelle *et al.*, 1991). The activity of the receptor is dependent upon its association with another member of the steroid receptor superfamily called ultraspiracle (USP). Together,

they form a functional heterodimer which bind ecdysteroids with high affinity (Yau *et al.*, 1993).

Presence and Function of Ecdysteroids in Non-Arthropod Invertebrates

In arthropods, ecdysteroids serve as a multifunctional hormonal system capable of eliciting specific responses from their target tissues in a temporally-specific manner (Steel and Vafopoulou, 1989). Ecdysteroids are not only involved in regulating growth and development in larval arthropods, but also play an important role in the regulation of reproduction in the adults (see Hagedorn, 1989). Ecdysteroids have also been biochemically identified in a number of non-arthropod invertebrate phyla (Cnidaria, Platyhelminthes, Nematoda, Nemertea, Annelida, Mollusca), and their proposed activity in these groups has attracted the attention not only of ecdysteroid specialists, but of comparative endocrinologists as a whole.

Cnidaria

The cnidarians represent one of the most primitive metazoan phyla. Ecdysteroids and ecdysteroid-related molecules have been isolated from some of these animals. In the soft coral *Lobophytum pauciflorum*, an ecdysteroid-related molecule called lobosterol was isolated by Tursch *et al.* (1976). Stuaro *et al.* (1982) isolated large quantities of 20E from the anthozoan *Gerardi savaglia*, while Searle and Molinski (1995) isolated a novel ecdysteroid named 4-dehydroecdysterone from *Parazoanthus* sp. The cellular origin and function of these ecdysteroids are unknown.

Platyhelminthes

In the Platyhelminthes, 20E stimulates growth and asexual reproduction of the larval cestode *Mesocestoides corti* (Kowalski and Thorson, 1976). Ecdysteroids, both free and conjugated forms, have been detected by HPLC/RIA in other cestodes such as *Moniezia expansa* (Mendis *et al.*, 1984), *Hymenolepis diminuta* (Mercer *et al.*, 1987a), and *Echinococcus granulosus* (Rees and Mercer, 1986). Concentrations of ecdysteroids appear to be higher at the anterior portions of the worms and vary according to development. Ecdysteroids have also been detected in cultures of *H. diminuta* and are believed to be excreted by the worms (Mercer *et al.*, 1987b).

In trematodes, ecdysteroids have been demonstrated by RIA/ GC/MS in *Schistosoma mansoni* and *Fasciola hepatica* (Foster *et al.*, 1992). In *S. mansoni*, the concentrations of E and 20E fluctuate with respect to specific developmental stages and sexual maturation, implicating possible functions in growth and reproductive processes (Nirde *et al.*, 1983, 1984a). Basch *et al.* (1986) detected ecdysteroid-like immunoreactivity near the opening of the vitelline duct, suggesting ecdysteroids may be involved in egg production. DeJong-Brink *et al.* (1989) found ecdysteroid immunoreactive material in sporocysts and cercaria of *Trichobilharzia ocellata.*

Nematoda

Ecdysteroid and ecdysteroid conjugates have been found in a variety of nematodes and some possible functions of ecdysteroids in molting and reproduction have been reported. In *Nematospiroides dubius* and *Ascaris suum*, ecdysteroids are capable of stimulating molting

after the worms are cultured in the presence of the hormone (Flemming, 1985a; Dennis, 1976). Whole body titers of ecdysteroids appear to be correlated with ecdysis in larval *A. suum* (Flemming, 1985b), suggesting ecdysteroids may be involved in regulating molting as in insects. In *Dirofilaria immitis*, ecdysteroids are involved in reinitiating meiosis during the pachytene stage (Delves *et al.*, 1986), indicating a role for ecdysteroids in oocyte development. In addition, adult *Brugia pahangi* release microfilaria when maintained with ecdysone *in vitro* (Mercer *et al.*, 1990), and ecdysone increases egg laying activity in *Nippostrongylus brasiliensis* (Goudy-Perriere, 1992), providing further evidence that ecdysteroids modulate reproductive processes in nematodes. In the human filarial worm *Onchocerca vovolvus*, genes encoding nuclear hormone receptor molecules similar to the ecdysteroid receptor have been isolated and are expressed maximally during embryonic development (Yates *et al.*, 1995).

Nemertea

In female *Carcinonemertes errans*, ecdysteroids were detected in tissue extracts and increases in ecdysteroid levels were associated with reproductive development (Okazaki *et al.*, 1988). Synder *et al.* (1992) detected significant changes in ecdysteroid concentrations between reproducing and non-reproducing females of *Paranemertes peregrina* and *Pantinonemertes californiensis*. In addition, ecdysteroid-immunoreactive material is present in the egg string (Synder *et al.*, 1992), and application of synthetic 20E promotes hatching and metamorphosis in *C. errans* (Okazaki *et al.*, 1988).

Annelida

Similar functions for ecdysteroids (molting and reproduction) have been reported to occur in the annelids. In the leech *Hirudo medicinalis*, high concentrations of ecdysteroids have been detected during the shedding of the old cuticle and synthesis of the new one (Sauber *et al.*, 1983; Porchet *et al.*, 1984). Injection of radiolabelled ecdysteroids demonstrated that *H. medicinalis* is capable of conjugating and metabolizing ecdysteroids (Garcia *et al.*, 1989). In addition, the concentration and metabolism of ecdysteroids undergo changes similar to the situation observed during insect embryonic development (Welter *et al.*, 1986). Ecdysone accelerated gametogenesis in both male and female reproductive organs of *Nephelopsis obscura*, while decreasing glycogen, lipid, and triglyceride levels (Kalarani *et al.*, 1995). In the polychaete *Perinereis cultifera*, high titers of ecdysteroids are detected in the oocytes near the end of vitellogenesis (Porchet *et al.*, 1984). These observations suggest that ecdysteroids might play important roles in reproduction and metabolism in annelids.

Although ecdysteroids have been identified in the Cnidaria, Platyhelminthes, Nematoda, Nermertea and Annelida, and there is an increasing body of evidence to suggest that ecdysteroids may function to regulate important physiological processes, an endogenous source (tissue) of ecdysteroids has yet to be demonstrated in these groups.

Mollusca

The occurrence of ecdysteroids in pulmonates has been reported for the stylommatophorans *H. aspersa*, *H. pomatia*, *Cepaea nemoralis* (Whitehead and Sellheyer, 1982; Romer, 1979; Nolte *et al.*, 1986; Garcia *et al.*, 1986), and the basommatophorans

Biomphalaria glabrata and *L. stagnalis* (Whitehead and Sellheyer, 1982; Nolte *et al.*, 1986; deJong-Brink *et al.*, 1989). These authors used combinations of RIA, HPLC, and GC/MS to demonstrate that E and 20E are the principle ecdysteroids present in pulmonates.

Nolte *et al.* (1986) demonstrated that the endocrine DBs of the terrestrial snail *H. pomatia* are capable of secreting E *in vitro*. This was the first study that identified a putative ecdysiosynthetic tissue in a non-arthropod invertebrate. If the DBs synthesized ecdysteroids, then some of the known physiological effects exerted by the DBs may be attributable to E or a related molecule such as 20E. In *H. aspersa*, 20E stimulates the *in vitro* synthesis of polysaccharides from the albumen gland, a known target organ of the dorsal body hormone (Bride *et al.*, 1991). In *C. nemoralis*, tritiated E is converted into labelled 20E and other metabolites *in vivo*, suggesting that snails metabolize E and possess a 20-hydroxylase activity (Garcia *et al.*, 1986). Together, these observations indicated that ecdysteroids such as 20E might be involved in controlling the activity of the albumen gland, and perhaps other reproductive processes in pulmonates.

In all non-arthropod invertebrates, and even in some primitive arthropods, the source of ecdysteroids (endogenous or exogenous) has been the subject of controversy, particularly since ecdysteroids are present in many plant species upon which these animals feed. Cholesterol labelling experiments, either by the injection of radioactive cholesterol, or *in vitro* incubation, have not proven successful (see Franke and Kauser, 1989). This is not surprising considering the large cholesterol pool in most invertebrates, the small quantities of ecdysteroids detected, and the poor incorporation rates of labelled cholesterol even in well

characterized insect systems. In pulmonate snails, exogenously derived ecdysteroids may be ingested as part of the diet, however, deJong-Brink *et al.* (1989) did not detect an increase in ecdysteroid levels between fed and non-fed snails. Furthermore, a steroid-synthesizing enzyme system is present in the DBs of *H. pomatia* (Krusch *et al.*, 1979), and a novel cytochrome P-450 gene is uniquely expressed in the DBs of *L. stagnalis* (Theunissen *et al.*, 1992). Taken together, the evidence suggests that the pulmonate DBs appear to be capable of synthesizing steroids or ecdysteroid-related molecules, and these molecules may serve as hormones regulating reproductive activities.

THESIS RATIONALE

The endocrine DBs of pulmonate snails have been shown to play an important role in influencing reproduction (Joosse, 1988; Saleuddin *et al.*, 1994; Saleuddin, 1998a). Oocyte maturation, stimulation of growth/differentiation and the synthetic activity of the female ASOs are among the reproductive processes controlled by the DBs. Endocrine structures functionally similar to the pulmonate DBs occur in other gastropods (the JOs) and in the cephalopods (the OGs). Since the first studies describing the hormonal function of the DBs (Geraerts and Algera, 1975, 1976), the characterization and isolation of the DBH has generated great interest from molluscan endocrinologists. However, the pharmacological characterization of the DBH has been controversial. On one hand, it has been described to be a peptide (Ebberink *et al.*, 1983; van Minnen and Sokolove, 1984; Vincent *et al.*, 1984), and on the other, it has been described as being steroidal in nature (Nolte, 1983; Nolte *et al.*,

1986; Miksys and Saleuddin, 1988).

The initial objective of this thesis was to characterize the nature of the DBH, that is, determine whether it is a peptide or steroidal in nature, and then attempt to isolate the hormone for definitive chemical identification. To accomplish this, a suitable bioassay was required to monitor the activity of DB extracts during purification. In the Basommatophora, yolk protein synthesis occurs primarily via autosynthetic mechanisms (Geraerts and Joosse, 1984). Saleuddin *et al.* (1980) reported that oocytes from over-wintering, non-egglaying *H. trivolvis* were less synthetically active than those oocytes taken from summer, reproducing *H. trivolvis* or laboratory-reared *H. duryi*. This indicated that the oocyte itself probably contributed a significant proportion of proteinaceous yolk as it developed. In addition, injection of DB extracts from egg laying snails into over-wintering non-egglaying snails induced egg production in the recipients. In *H. aspersa*, ovotestes from juvenile snails cultured in the presence of DBs, showed increased levels of protein synthesis (Barre *et al.*, 1990). These observations suggest a possible involvement of the DBs in regulating the synthetic activity of the ovotestis. The first part of this thesis examines the feasibility of using the ovotestis and albumen gland, two major target organs for the DBH, as an *in vitro* bioassay system to test the influence of the DBs on protein and polysaccharide synthesis respectively.

In 1986, Nolte and coworkers reported that the DBs of the terrestrial snail *H. pomatia* were capable of secreting the steroid hormone ecdysone *in vitro*. This was the first study that identified a specific molecule secreted by the DBs. At approximately the same time, Garcia *et al.* (1986) demonstrated the pulmonate snail *C. nemoralis* converted E to 20E, suggesting

that snails are capable of metabolizing E in a manner similar to arthropods. A few years later, Miksys and Saleuddin (1988) partially characterized biological activity of DB tissue extracts of *H. duryi* as being a methanol-extractable, heat-labile, and protease-insensitive molecule, and suggested the DBH is steroidal in nature. The second part of this thesis re-examines some of the work by Nolte *et al.* (1986), specifically, whether the DBs of the freshwater snail *H. duryi* are capable of producing ecdysteroids, and whether they have effects on reproduction in *H. duryi*.

In addition to the polysaccharide galactogen, another major secretory product produced by the albumen gland is protein (Wijsman and van Wijck-Batenburg, 1987). In *L. stagnalis*, protein synthesis by the albumen gland increased as the animals begin laying eggs (Dictus and de Jong-Brink, 1987a), and in *B. glabrata*, albumen gland protein synthesis and secretion is markedly higher in egg laying snails compared to non-egg layers (Crews and Yoshino, 1991). In addition to the synthesis of secretory material, the control of secretion is also a vital function of the albumen gland, as it must release an appropriate quantitiy of PVF to surround each egg. The third part of this thesis investigates some of the secretory protein components of the albumen gland, and the partial biochemical characterization of a neurally-derived factor mediating protein release in the albumen gland.

Despite the numerous reports of various endocrine and neuroendocrine substances involved in the regulation of PVF synthesis and release, little is known about the mode of action of these substances on the albumen gland. In *H. pomatia*, cAMP analogues stimulated galactogen synthesis by the albumen gland, suggesting cAMP is involved in the regulating the

synthetic activity of the albumen gland (Goudsmit and Ram, 1982). However, there was no evidence of an endogenous substance which stimulated cAMP production in the albumen gland. In *L. stagnalis*, the neuropeptide calfluxin is thought to stimulate the influx of calcium into albumen gland cells, and mobilize calcium from intracellular stores (Dictus *et al.*, 1987b, 1988; Dictus and Ebberink, 1988). In these studies, the increase in intracellular calcium was detected ultracytochemically as calcium deposits on mitochondria, but the ultimate physiologic response as a result of this apparent increase in intracellular calcium is unknown. The last portion of this thesis investigated the effect of nervous system extracts on the *in vitro* secretion of protein in the albumen gland. The intracellular signal transduction pathway participating in the secretion of protein in the presence a stimulatory factor from the brain was characterized.

The results from this thesis provide further information about the physiology of the DBs and the nature of the DBH of *H. duryi*. A partial purification of the DBH is achieved, which should clarify some of the controversy concerning its biochemical properties. A neurosecretory factor from the brain is demonstrated to induce the secretion of perivitelline fluid from the albumen gland via the cAMP pathway. Examination of the endocrine and neurosecretory factors affecting the synthetic activity and secretion of albumen gland polysaccharides and proteins elucidate possible mechanisms of regulation, and the relationship between these two processes. The information gathered in this thesis contributes new information and insight regarding the role of CNS-associated endocrine structures in the egglaying process of pulmonate molluscs, in particular, the regulation of the synthetic activity

of the albumen gland and the chemical nature of the DBH. The regulation of perivitelline fluid synthesis and its precise release are critical elements contributing to egg mass production and are indispensable to the survival of pulmonate embryos.

Chapter I : Effect of Endocrine Factors on the Synthetic Activity of the Ovotestis and Albumen Gland

SUMMARY

The ovotestis or the albumen gland from the freshwater snail *Helisoma duryi* were co-incubated with the central nervous system or dorsal bodies to determine if putative endocrine factors influenced gonadal protein synthesis and polysaccharide synthesis respectively. The results indicated neither the brain nor dorsal bodies affected general protein synthesis in the ovotestis under long term *in vitro* incubation. Some differences in electrophoretically separated proteins were detected between the ovotestes from mated snails compared to virgins. In contrast, the dorsal bodies had a stimulatory effect on albumen gland polysaccharide synthesis, whereas the brain (including dorsal bodies) did not. Both dorsal body tissue extracts and dorsal body-preconditioned medium were able to stimulate polysaccharide synthesis, and the release of secretory products in a dose-dependent manner. It is concluded that the measurement of general gonadal protein synthesis in *Helisoma duryi* is not a suitable bioassay to examine the effects of gonadotropic endocrine factors, despite previous reports claiming endocrine factors stimulated ovotestis protein synthesis in pulmonates. The albumen gland responds to the dorsal bodies *in vitro* by increasing glandular polysaccharide production, thus demonstrating its potential as a bioassay to examine the role of reproductive hormones in freshwater snails.

INTRODUCTION

The dorsal body hormone (DBH) of pulmonate basommatophoran molluscs influences several aspects of female reproduction . In *Lymnaea stagnalis* and *Helisoma duryi*, the DBH stimulates oocyte maturation (Geraerts and Joosse, 1975; Schollen and Saleuddin, 1986), and the synthetic activity of the albumen gland (Veldhuizen and Cuperus, 1976; Wijdenes *et al.*, 1983; Miksys and Saleuddin, 1985, 1988). The presence of the DBs is also necessary for the growth and differentiation of the female reproductive tract and its associated accessory sex organs (Geraerts and Joosse, 1975; Geraerts and Algera, 1976; Miksys and Saleuddin, 1987b). Despite its importance in regulating key reproductive events in pulmonates, there is a paucity of information regarding the chemical characterization of the DBH. Studies on the precise nature of the DBH (peptide or steroid) have been reported but these data remain controversial (see Saleuddin *et al.*, 1994; Saleuddin, 1998a).

A principal target organ of the DBH in pulmonates is the ovotestis, where it stimulates vitellogenesis (Geraerts and Joosse, 1975; Saleuddin *et al.*, 1980; Schollen and Saleuddin, 1986). The synthesis of yolk proteins in gastropods is thought to occur primarily via autosynthetic mechanisms (de Jong-Brink and Geraerts, 1982). Saleuddin *et al.* (1980) reported the oocytes of reproducing *H. duryi* and *H. trivolvis* showed ultrastructural features of cells that were actively engaging in protein synthesis compared to the oocytes from non-egglaying snails. In *Planorbarius corneus*, vitellogenic oocytes incorporate labelled amino acids into yolk platelets, whereas the follicle cells did not appear to be involved in yolk protein synthesis or transport (Bottke and Tiedtke, 1988). In *H. aspersa*, incubation with either the

brain or DBs stimulated gonadal protein synthesis (Barre *et al.*, 1990). These observations suggest that endogenous yolk protein synthesis might be regulated by factors from the central nervous system (CNS) or DBs.

In both the Basommatophora and Stylommatophora, it has been demonstrated that DBH acts on the albumen gland to stimulate polysaccharide synthesis (Veldhuijzen and Cuperus, 1976; Wijdenes *et al.*, 1986; Miksys and Saleuddin, 1985, 1988; van Minnen *et al.*, 1983; van Minnen and Sokolove, 1984). Galactogen, the main polysaccharide synthesized by the albumen gland, is secreted around the eggs as part of the perivitelline fluid (PVF), and provides an important source of nutrients during development (Goudsmit, 1976). The current study examines the feasiblity of using the *in vitro* culture of the ovotestis and albumen gland from *H. duryi* as bioassays to study the effects of the DBH on gonadal protein and albumen gland polysaccharide synthesis.

MATERIALS AND METHODS

Animals

A laboratory stock of reproducing *H. duryi* were maintained under 16L : 8D photoperiod and reared in 2-liter plastic containers with dechlorinated tap water at room temperature. Snails were fed a diet of boiled lettuce and fish pellets every 2-3 days, and the water was changed at least once per week. A colony of virgin snails was established by collecting juvenile snails 3-4 weeks after hatching and placing them individually in 200 ml plastic cups. These virgin animals were maintained under the same conditions as the stock

colony until they reached adult size (10-12 mm shell diameter). A colony of reproducing snails with low egglaying activity was used as recipients for bioassay experiments. These animals were maintained in continuous darkness (DD) by raising them in plastic containers which were placed in a light-tight wooden incubator. The animals were exposed to light for several minutes a week during water changes and feeding. This exposure to light did not appear to have significant effect on egglaying activity (Miksys and Saleuddin, 1985).

In Vitro Culture of Ovotestis

The CNS, DBs, or subesophageal ganglia from reproducing snails were dissected out in snail saline (51.3 mM NaCl, 1.7mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.0 mM Hepes, pH 7.3 120 mOsm/kg H_2O) containing an antibiotic-antimycotic mixture of 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone (GIBCO-BRL Life Sciences Inc., Burlington, Ont.), and cocultured individually with pieces of ovotestes from DD snails. Both tissues were maintained for 48 h in Falcon 96-well culture plates (Becton-Dickinson, USA) containing 200 µl Medium 199 (pH 7.3, 140 mOsm H_2O) with antibiotics and 10 µCi/ml ^{3}H-amino acid mixture (Amersham Life Sciences, Oakville, Ont.). All dissections and tissue culture were performed in a laminar flow hood. After the incubation, the tissues were thoroughly rinsed in snail saline containing non-radioactive amino acids, then homogenized in 50 µl of deionized water in a glass-on-glass microgrinder (Jencons-Hertz, England). Duplicate aliquots of 20 µl were spotted onto 2 cm squares of Whatman #42 filter paper, allowed to dry, and processed for liquid scintillation counting according to Mans and Novelli (1961). The filter squares were placed in 20 ml scintillation vials containing 10 ml of

scintillation cocktail (Beta-Max, ICN, Quebec), and the radioactive protein quantified in a Packard Minmaxi Tricarb 4000 scintillation counter (United Technologies, USA). After counting, the papers were washed in acetone and dried. The protein content of the spotted sample was determined by the method of Bramhall *et al.* (1968) using BSA as a standard. Data are expressed as dpm/µg protein.

Mating Experiment

To determine the effect of a first mating on gonadal protein synthesis, virgin snails were paired and allowed to mate. At approximately 6 h intervals following a first mating, the ovotestis were removed and incubated for 6 h in Medium 199 containing tritiated amino acid mixture. The tissues were processed for further characterization of amino acids incorporated into proteins as described above.

Electrophoresis

The ovotestes from either virgin or reproducing snails were placed in a 1.5 ml microtube and homogenized in 100 µl of 50 mM Tris-HCl (pH 7.2, containing 1mM PMSF, 2 mM $MgCl_2$) using a motor-driven polypropylene teflon pestle. The homogenate was centrifuged at 20,000 x g for 20 min and the supernatant used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein determinations were performed using a commercial protein assay kit (Bio-Rad, USA) with BSA as a standard. Approximately 10 µg total ovotestis protein was loaded for each sample and separated on a 10% mini-gel (Bio-Rad Life Sci., USA) according to Laemmli (1968). Separated proteins were stained with 0.1% Coomassie Brilliant Blue R-250.

In Vitro Culture of Albumen Glands

Albumen glands were dissected free from surrounding tissue and rinsed thoroughly in antibiotic saline. The glands were cut into pieces with a scalpel, randomized, then incubated in sterile Falcon 96-well culture plates with 200 µl Medium 199 containing antibiotics and 1 µCi/ml UDP-^{14}C-glucose (Amersham, Oakville, Ont.). Albumen glands were either cocultured with the CNS, DBs, subesophageal ganglia (SG), extracts of DB tissue, or DB-conditioned culture medium for 48 h (N= 4-6 glands for each experiment). After the incubation period, the glands were rinsed thoroughly in saline containing non-radioactive glucose, carefully blotted on filter paper, then placed into pre-weighed 1.5 ml microtubes and their wet weight recorded using a Mettler AE 163 microbalance (Mettler Instruments, Switzerland). The tissues were homogenized in 150 µl of ice-cold 10% trichloroacetic acid (TCA) with a motor-driven polypropylene pestle, and centrifuged at 10,000 x g for 10 min. After the precipitated proteins were removed, 60 µl of the supernatant was spotted onto 2 cm squares of Whatman #42 filter paper and allowed to dry. To determine the release of newly synthesized polysaccharides, 100 µl of the surrounding medium was placed in a tube, and treated with an equal volume of 20% TCA. After proteinaceaous material was removed, a 100 µl aliquot was spotted onto filter squares. Total polysaccharide synthesis was determined according to Thomas *et al.* (1968). Briefly, the filter papers were washed twice in ice-cold 66% ethanol (20 min each), and then for 5 min in acetone and dried. The dried filter papers were placed in 20 ml scintillation vials with 10 ml of Beta-Max cocktail (ICN, Quebec), and radioactivity measured by liquid scintillation

counting.

Preparation of Test Material

For coculture experiments, whole CNS, DBs, or SG were dissected out from reproducing snails and washed well in antibiotic saline before being placed with a piece of albumen gland. DB tissue was homogenized in 100% methanol using a glass-on-glass microgrinder. Homogenates were centrifuged at 10,000 x g, and the supernatant evaporated to dryness, then resuspended in sterile saline. Preconditioned DB medium was prepared by maintaining 10 DBs/well in Falcon 96-well plates containing either modified L-15, pH 7.3 130 mOsm/l (Wong *et al.*, 1981) or Medium 199 (pH 7.3, 130-140 mOsm/ kg H_2O) for 24-30 h. After incubation, aliquots of the medium were collected and used directly in the bioassay.

All statistical calculations were done using one-way ANOVA ($p<0.05$) where variances permitted, or the non-parametric Kruskal-Wallis test. Where means from ANOVA or Kruskal Wallis procedures were found to be different, multiple sample comparisons with controls were determined using the Dunnett or the Dunn Test ($Q_{\alpha=0.05,\,k}$).

RESULTS

In Vitro Protein Synthesis by the Ovotestis

Pieces of ovotestis were incubated for 24 h, 48 h, and 72 h in Medium 199 containing a tritiated amino acid mixture to examine the time course of protein synthesis (Fig. 1). Protein synthesis by ovotestes removed from DD snails displayed a gradual increase in protein synthesis between 24 h to 72 h *in vitro*. No detectable release of protein into the surrounding

culture medium could be detected for any of the incubation periods.

The CNS, DBs, or SG were cocultured with pieces of ovotestes and the incorporation of labelled amino acids into protein was determined after 48 h. Neither the CNS, DBs, nor SG had any effect on ovotestis protein synthesis compared to controls (Fig. 2).

Mating is a known stimulator of egg production in virgin *H. duryi*. The effect of mating on ovotestis protein synthesis was investigated every 6 h after a first mating for a 48 h period. Surprisingly, general gonadal protein synthesis showed a steady decline in synthetic activity between 0-12 h post-mating (Fig. 3). The synthetic activity of the ovotestes from virgin snails was 481.42±84.53 dpm/µg protein and that of 12 h post-mating snails was 347.52 ± 67.24 dpm/µg protein. The activity of the ovotestis exhibited some minor fluctuations during the next 36 h, but there was no statistically significant difference between any of the time periods. However, the trend appeared as though the actively reproducing snails had a slightly lower basal rate of ovotestis protein synthesis than virgin snails.

Electrophoretic Separation of Ovotestis Extract

Extracts of ovotestis proteins from virgin and mated snails separated by SDS-PAGE revealed many bands as might be expected from crude extracts (Fig. 4). The examination of the Coomassie Blue stained gel showed there were two protein bands (~48 kDa and >97 kDa) which were stained more intensely in mated snails as compared to virgins (Fig. 4, lane 2).

In Vitro Polysaccharide Synthesis by the Albumen Gland

The time course of *in vitro* polysaccharide synthesis by albumen gland explants between 24-72 h is shown in Fig. 5. Synthesis was linear over 72 hours, and the release of newly synthesized polysaccharides into the medium increased linearly during this period. An incubation time of 48 h was chosen for subsequent experiments since synthesis is linear at this point, and release of radiolabelled products into the medium is readily detectable.

Coculture of Albumen Glands With CNS, DBs, or SG

The CNS, DBs, or SG were cocultured with pieces of albumen glands and the *in vitro* synthesis and release of polysaccharides was determined after 48 h. Coculture with DBs induced a significant increase in polysaccharide synthesis after a 48 h period (Fig. 6). In addition, a significant increase in the accumulation of radiolabelled polysaccharides in the culture medium was detected. In contrast, neither the CNS nor SG significantly increased the synthesis or release of albumen gland polysaccharides.

Effect of DB-Conditioned Media and DB Tissue Extracts on Polysaccharide Synthesis

Dorsal bodies were maintained in either Medium 199 or L-15 (10 DBs/100 μl) for 24-30 h, and aliquots of DB-conditioned medium were tested for their ability to stimulate albumen gland polysaccharide synthesis. Preconditioned medium stimulated polysaccharide synthesis and release in a dose-dependent fashion (Fig. 7). Modest stimulation of synthesis occurred at approximately 0.5 animal equivalents (ae), and maximal stimulatory activity was induced at 1.0 ae. In contrast, the accumulation of released polysaccharides increased significantly at a dose of 0.5 ae, and was maximal between 1.0-2.0 ae (Fig. 7).

Extracts of DB tissue were also tested to determine if they possessed any stimulatory activity on the albumen gland explants. Dorsal body tissue extracts stimulated albumen gland polysaccharide synthesis and release in a concentration-dependent manner (Fig. 8). The threshold for stimulation of polysaccharide synthesis occurred at a dose of 0.5 ae, and was maximal between 1.0-2.0 ae. A significant increase in the accumulation of secreted polysaccharides was observed at a dose of 0.5 ae, and reached a maximum between 1.0-2.0 ae.

DISCUSSION

Ovotestis Protein Synthesis

The DBs of pulmonate snails are known to regulate important gonadotropic events such as the stimulation of vitellogenesis (see Joosse, 1988; Saleuddin *et al.*, 1994). Yolk protein synthesis is thought to be mainly autosynthetic, since there are relatively few endocytotic profiles seen on the surface of mature oocytes, whereas the cytoplasm of the maturing oocyte displays characteristics of active protein synthesis (Saleuddin *et al.*, 1980; de Jong-Brink and Geraerts, 1982). In this study, coculture of *H. duryi* ovotestis with either the CNS, DBs, or SG from reproducing snails had no discernable effect on gonadal protein synthesis. This is in contrast to the situation in the terrestrial snail *H. aspersa*, where the incubation of the ovotestis with the brain or DBs was claimed to increase protein synthesis (Barre *et al.*, 1990). However, the study of Barre *et al.* (1990) did not actually quantify protein synthesis, only total amino acid uptake into tissues.

In *H. duryi*, mating is a prerequisite for the production of viable eggs from virgin

snails (Saleuddin *et al.*, 1983b). Virgin ovotestes contain an abundance of immature oocytes compared to the ovotestes from reproducing animals (Schollen and Saleuddin, 1986). Mating is believed to induce the production of a 'matedness factor', activating the gonadotropic centers (neurosecretory caudodorsal cells and endocrine DBs), which in turn, act upon the ovotestis to stimulate ovulation and subsequent oocyte development. When virgin snails were paired for a first mating and the ovotestis removed at 6 h intervals thereafter, there was no increase in the synthesis of gonadal proteins even after 48 h. In fact, protein synthesis showed a decrease during the first 12 h, and then maintained a relatively steady level for the duration of the experiment. This decrease in synthetic activity following mating may result from the ovulation of radiolabelled oocytes out of the ovotestis into the hermaphroditic duct, which decreases the total amount of detectable radiolabelled protein in the ovotestis.

The ovotestes of pulmonates contain many heterogeneous cell types associated with both male and female gamete production (see Geraerts and Joosse, 1984). The concurrent development of the male gametes probably contributes a substantial proportion of non-specific (non-oocyte) labelling to the total activity which could mask increases in endocrine-stimulated synthesis of a specific protein(s). Electrophoretic analysis of extracts of ovotestes proteins from virgin and reproducing snails showed a very complex protein profile, and two proteins were identified which appeared to be more abundant in mated snails. However, general protein synthesis levels are not different in glands cocultured with either brain or DBs. These findings are supported by the study of Crews and Yoshino (1991) who were unable to detect an increase in general ovotestis protein synthesis between parasitized (non-

reproducing) and non-parasitized (reproducing) *B. glabrata*. Therefore, the measurement of non-specific protein synthesis is not a suitable assay system to study the effects of endocrine factors on oocyte development in the Basommatophora. Perhaps incubating ovotestis with either DBs or brain and a high specific activity isotope (eg. ^{35}S-methionine), followed by autoradiographic analysis might reveal a specific ovotestis protein which is regulated by endocrine factors.

Albumen Gland Polysaccharide Synthesis and Release

The time course of polysaccharide synthesis is linear over a 72 h period under the current incubation conditions. A time of 48 h was chosen since this is when the effect of the DBs on albumen gland polysaccharide synthesis and release is optimal. This agrees with a previous study which also used a 48 h incubation period to assay the effect of endocrine factors on albumen gland synthetic activity (Miksys and Saleuddin, 1988).

The incubation of albumen gland explants with various (neuro)endocrine tissues demonstrates that only the DBs are capable of stimulating polysaccharide synthesis and release under long term culture. Although the CNS contain intact DBs, significant stimulation of polysaccharide synthesis could not be induced in cocultures, suggesting the brain might exert some inhibitory influence on the DBs. In both the Basommatophora and Stylommatophora, the DBs are innervated by axons originating from various neurons within the CNS (Saleuddin and Ashton, 1996; Saleuddin *et al.*, 1997; Griffond and Mounzih, 1991). In *H. aspersa*, surgically isolating the DBs from the rest of the CNS (denervation), increased the synthetic activity of the DBs (Vincent *et al.*, 1984). The DBCs of *H. aspersa* are known

to be innervated by FMRFamide-immunoreactive varicosities (Gifffond and Mounzih, 1990), and the activity of the DBs is inhibited by the neuropeptide FMRFamide (Griffond and Mounzih, 1989). Together, these observations suggest that in pulmonate molluscs, the brain exerts an inhibitory control on the synthesis and/or release of DBH.

Culture medium preconditioned with DBs for 24-30 h or extracts of DB tissue is capable of stimulating polysaccharide synthesis and release in a dose-dependent manner. Maximum stimulation of synthesis occurred at a dose of 1.0-2.0 ae. In albumen gland explants from snails raised under DD conditions, biosynthesized material is secreted into the lumen of the ducts at a constant rate of cellular release, accumulating in the ducts with incubation time, and gradually extruded through the cut ends of the ducts into the surrounding medium. The relative amounts detected in the tissue and medium would depend on the gland's rate of synthesis, the rate of cellular release, and culture time. These data show that the DBs increase the rate of glandular polysaccharide synthesis, and possibly enhance the release of newly-synthesized products into the lumen of the albumen gland ducts or its extrusion from the lumen into the surrounding medium. Therefore, the measurement of *in vitro* polysaccharide synthesis and release from albumen gland explants shows promise as a bioassay to detect DBH activity.

Fig. 1 Time course of *in vitro* protein synthesis by the ovotestis of *Helisoma duryi*. Pieces of ovotestis were incubated with ^{3}[H]-amino acid mixture in Medium 199 (10 μCi/ml) for the indicated times, then quantified for incorporation of labelled amino acids into TCA-precipitable protein. Each point represents the mean ● SE of 4-6 samples.

Fig. 1

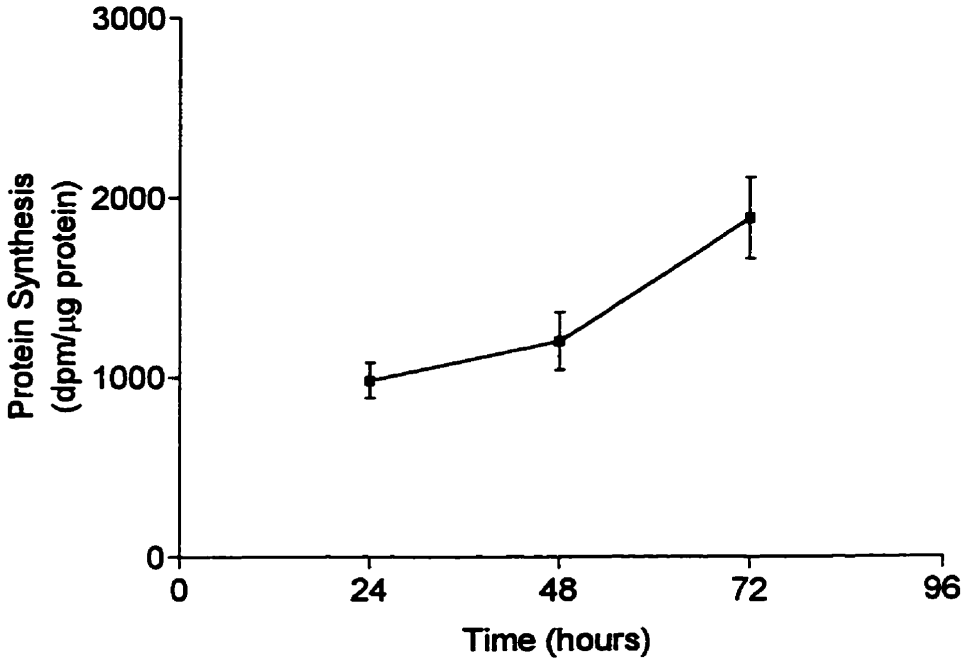

3000
2000
1000
0
Protein Synthesis
(dpm/µg protein)
0
24
48
72
96
Time (hours)

Fig. 2 Effect of various endocrine tissues on ovotestis protein synthesis. Pieces of ovotestis were cocultured with the CNS or brain (BR), subesophageal ganglia (SG), or dorsal bodies (DBs) for 48 h in Medium 199 containing labelled amino acid mixture. Bars represent the mean ± SE of 4-6 samples. Note that there was no significant effect of the various tissues on general ovotestis protein synthesis.

Fig. 2

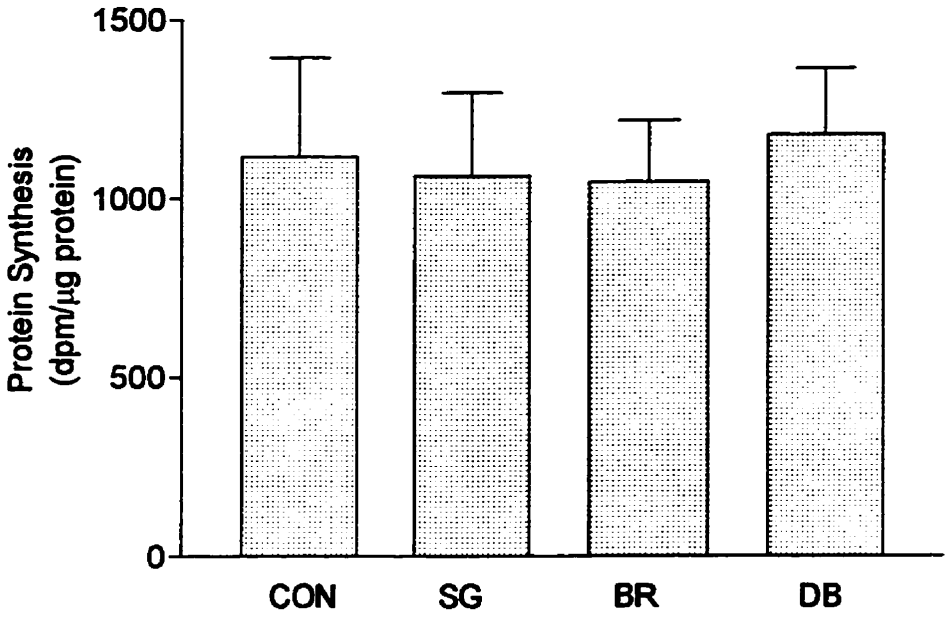


Fig. 3 Effect of a first mating on *in vitro* protein synthesis by the ovotestis. Virgin snails were raised in isolation until they reached approximately 10-12 mm shell diameter, then paired for a single mating. The ovotestes were removed from first mated snails at 6 h intervals following copulation and maintained in Medium 199 containing tritiated amino acid mixture for 6 h. Each point represents the mean ● SE of 4 samples. Note a general decline in protein synthesis was observed between 0 h to 12 h post-mating.

Fig. 3

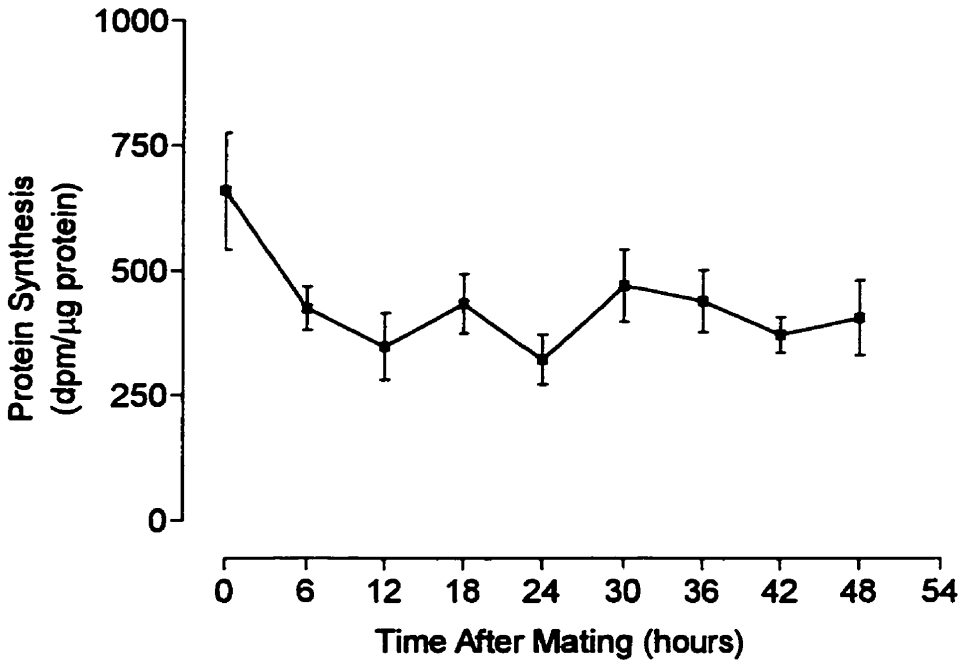

1000
750
500
250
0
Protein Synthesis
(dpm/µg protein)
0
6
12
18
24
30
36
42
48
54
Time After Mating (hours)

Fig. 4 Electrophoretic separation of soluble ovotestis protein from virgin and mated *Helisoma duryi*. Extracts from virgin (lane 1) and mated (lane 2) snails were separated on a 10% T, 3% C gel by SDS-PAGE. Each lane contained approximately the same concentration of protein (~10 μg). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (0.1%), and then destained. The molecular weight markers are represented in the far left lane. Note the presence of two protein bands (arrows), one of approximately 48 kDa, and the other (>97 kDa), which were stained more intensely in mated animals compared to virgin snails.

4

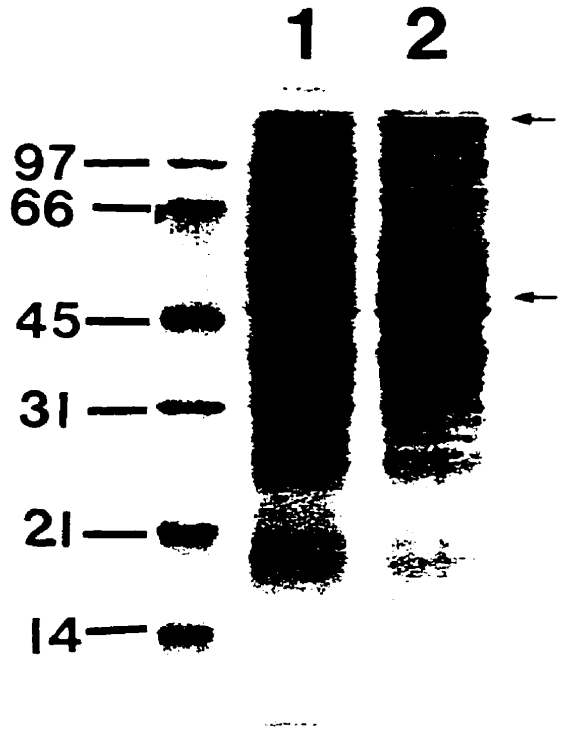


Fig. 5 Time course of *in vitro* synthesis and release of newly labelled polysaccharides from the albumen gland of *Helisoma duryi*. Albumen glands from snails raised in continuous darkness were incubated for various times in Medium 199 containing ^{14}[C]- glucose (1 μCi /ml). Total polysaccharide synthesis and release was determined in pieces of tissue and culture medium after TCA extraction and ethanol precipitation. Each point represents the mean ± SE of three separate experiments.

Fig. 5

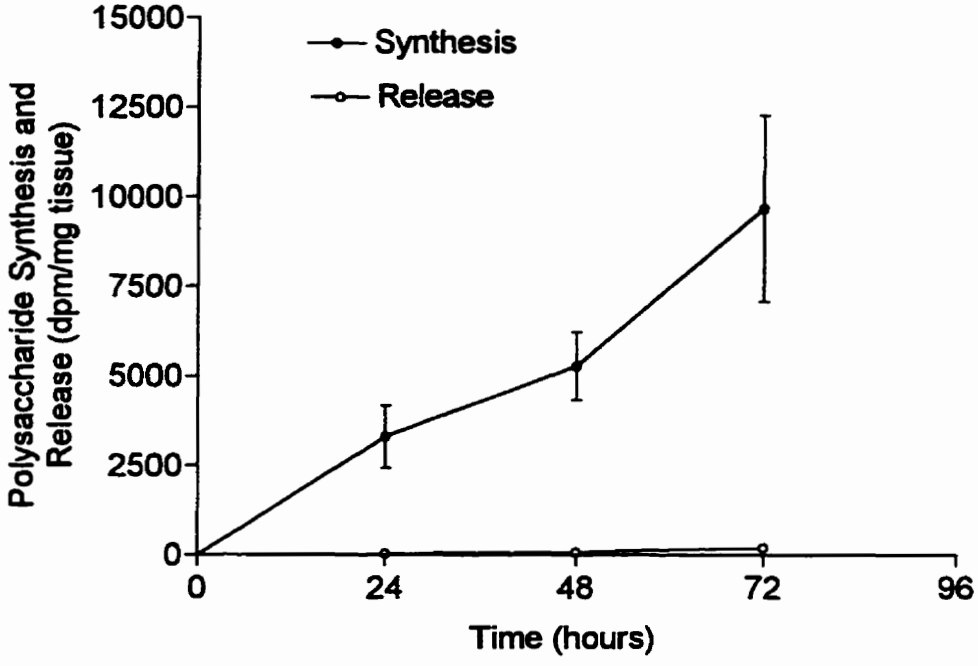

Synthesis
Release
Polysaccharide Synthesis and Release (dpm/mg tissue)
15000
12500
10000
7500
5000
2500
0
0
24
48
72
96
Time (hours)

Fig. 6 Effect of various endocrine and neurosecretory tissues on albumen gland polysaccharide synthesis and release. Albumen glands were cocultured with brain (BR), subesophageal ganglia (SG), or dorsal bodies (DBs) for 48 h in Medium 199 containing radiolabelled glucose. Each bar represents the mean ± SE of five separate experiments. Note that the DBs are the only tissue capable of significantly stimulating both polysaccharide synthesis (**Kruskal-Wallis, $p=0.029$, Dunn Test) and release (* Kruskal-Wallis, $p<0.05$, Dunn Test).

Fig. 6

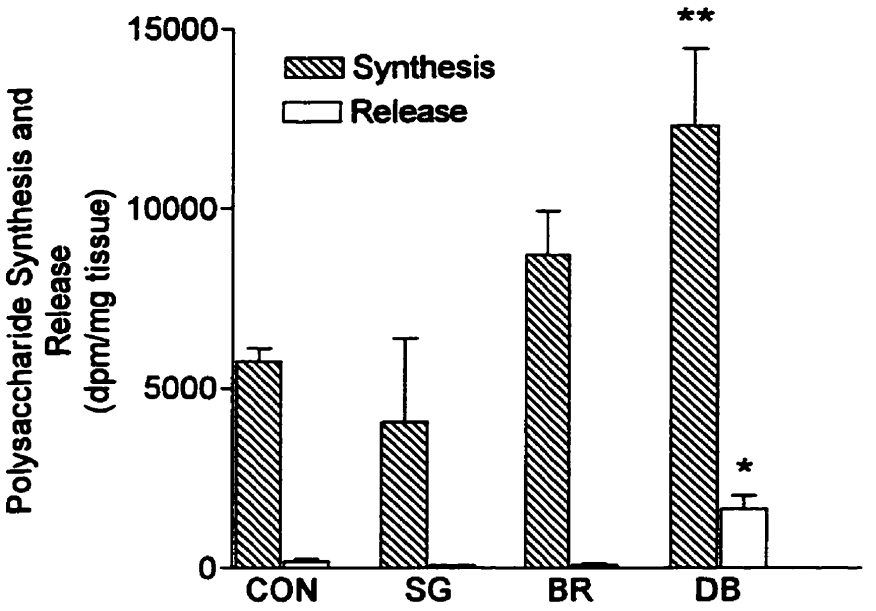

Polysaccharide Synthesis and Release (dpm/mg tissue)
15000
10000
5000
0
Synthesis
Release
**
*
CON
SG
BR
DB

Fig. 7 Effect of DB tissue extracts on albumen gland synthetic activity and release of labelled polysacharides. Dorsal body tissue was homogenized and extracted in methanol, then tested for bioactivity at various concentrations. Each bar represents the mean ± SE of four separate experiments. Note significant stimulation of polysaccharide synthesis at a dose of 1.0 and 2.0 animal equivalents (ae) (**ANOVA $p<0.05$, Dunnett Test) and significant accumulation of labelled polysaccharides in the culture medium at a dose of 0.5-2.0 ae (*Kruskal-Wallis $p<0.05$, Dunn Test).

Fig. 7

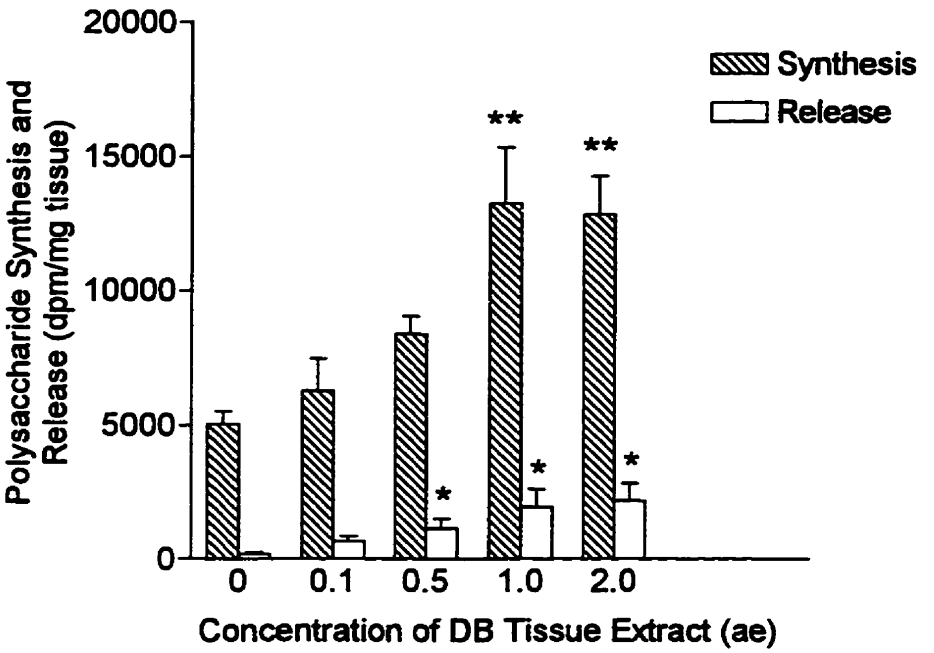
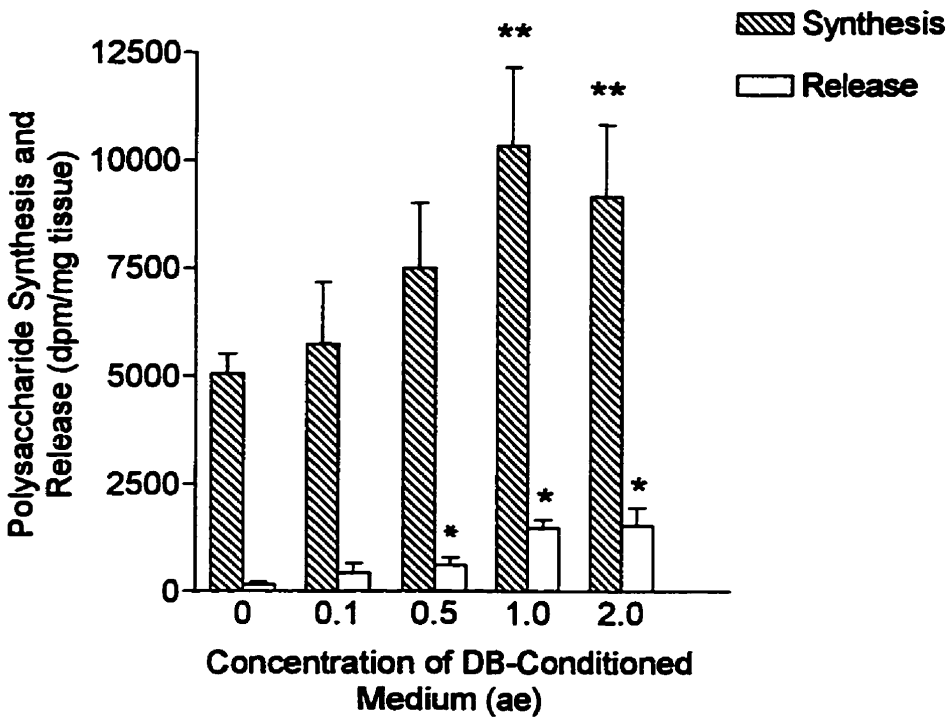

Polysaccharide Synthesis and Release (dpm/mg tissue)
20000
15000
10000
5000
0
Synthesis
Release
**
**
*
*
*
0
0.1
0.5
1.0
2.0
Concentration of DB Tissue Extract (ae)

Fig. 8 Effect of DB-conditioned medium on albumen gland polysaccharide synthesis and release of labelled polysaccharides. DBs were incubated in Medium 199 for 24-30 h, and aliquots of DB-conditioned medium were directly tested for bioactivity. Bars represent the means ● SE of five experiments. Significant stimulation of polysaccharide synthesis is notable at a dose of 1.0 and 2.0 ae (**ANOVA $p<0.04$, Dunnett Test), and significant release of labelled material is noted at 0.5- 2.0 ae (*Kruskal-Wallis $p<0.05$, Dunn Test).

Fig. 8


Synthesis
Release
Polysaccharide Synthesis and Release (dpm/mg tissue)
12500
10000
7500
5000
2500
0
**
**
*
*
*
0
0.1
0.5
1.0
2.0
Concentration of DB-Conditioned Medium (ae)

Chapter II : Partial Characterization of the Dorsal Body Hormone

SUMMARY

Both dorsal body tissue extracts and dorsal body-conditioned medium are known to stimulate *in vitro* polysaccharide synthesis in albumen gland explants of *Helisoma duryi*. A partial pharmacological characterization of tissue extracts and conditioned media was performed by subjecting extracts to heat and proteolytic enzymes. The extracts from dorsal body tissue and conditioned media were passed through solid-phase extraction cartridges, then eluted with increasing concentrations of methanol (20%, 70%, and 100%), and the various eluates tested for their ability to stimulate albumen gland polysaccharide synthesis. The results showed that both tissue and media extracts are heat- and protease-resistant, suggesting the bioactive material from the dorsal bodies is not a polypeptide. Both dorsal body tissue extracts and conditioned medium contained hydrophobic material (eluted in 100% methanol) which stimulated albumen gland synthetic activity. In addition, the conditioned medium also contained a more polar substance (eluted in 70% methanol) which also possessed bioactivity.

INTRODUCTION

The dorsal bodies (DBs) of pulmonate snails display features of both protein- and steroid- synthesizing tissues. The presence of membrane-bound granules and well-developed rough endoplasmic reticulum (RER), indicate the DBs produce proteinaceous material, while the abundance of lipid droplets, tubular mitochondria with dense inclusions, and smooth endoplasmic reticulum (SER), suggest the DBCs also have steroidogenic capacity (see Saleuddin *et al.*, 1994; Saleuddin, 1998a). Isolation and elucidation of the precise chemical structure of the DBH has been extremely difficult, and only fragmented evidence from different animals is present in the literature. In the freshwater snail *Lymnaea stagnalis*, the active material from DB tissue extracts was suggested to be a proteinaceous molecule of approximately 30 kDa (Ebberink *et al.*, 1983). In the terrestrial snail *Limax maximus*, the synthetic activity of the albumen gland was stimulated by a peptidergic factor called galactogen-synthesis stimulating factor (GAL-SF), presumed to be of DB origin (van Minnen *et al.*, 1983; van Minnen and Sokolove, 1984). A similar factor was demonstrated to be present in whole CNS or cerebral ganglia extracts of *Helix pomatia* (Goudsmit and Ram, 1982). However, the precise cellular source of DBH activity in the Stylommatophora was uncertain since whole ganglia were used for extraction.

In *Helisoma duryi* and *Helix aspersa*, the disappearance of lipid droplets from the DBCs coincides with the onset of reproductive activities (Saleuddin *et al.*, 1989; Griffond and Vincent, 1985), suggesting that lipids are being mobilized, perhaps for the production of lipophilic substances. Miksys and Saleuddin (1988) demonstrated that a polysaccharide

synthesis-stimulating factor could be extracted from DB tissue with either saline or methanol, but not under acidic or basic conditions. The active material was heat-sensitive, but resistant to proteolytic digestion, suggesting it is not a peptide. Nolte (1983) proposed the DBs synthesized a steroidal product based upon ultrastructural and biochemical studies. The presence of steroid-synthesizing enzymes in the DB tissue of *H. pomatia* (Krusch *et al.*, 1979), and of a novel cytochrome P-450 gene expressed solely in the DBs of *L. stagnalis* (Theunissen *et al.*, 1992), indicates the DBs have the synthetic capacity to produce steroids or steroid-related molecules.

It was previously shown that the albumen gland of *H. duryi* responds to both crude DB tissue extracts (Miksys and Saleuddin, 1985, 1988), and to culture medium preconditioned with DBs (Chapter 1, this thesis) by increasing glandular polysaccharide synthesis and release. The present study examined the effect of different pharmacological treatments of DB tissue extracts and DB-conditioned medium, and its effect on the synthesis and release of albumen gland polysaccharides. The active material from DB tissue and DB-conditioned media were separated according to hydrophobicity with Sep-Pak C_{18} cartridges in an effort to further purify DBH activity for future chromatographic separation and characterization.

MATERIALS AND METHODS

Bioassay

The *in vitro* incubation of albumen glands from snails maintained under DD conditions

is described in a Chapter I of this thesis. All test material was resuspended in sterile antibiotic snail saline and added to albumen gland explants in 20 µl aliquots. After a 48 h incubation, total polysaccharide synthesis and release of newly-synthesized material into the medium was quantified as previously described.

Preparation of DB Tissue Extracts

Dorsal body tissue from reproducing *H. duryi* were carefully dissected free from surrounding nervous tissue, homogenized in methanol, then centrifuged at 10,000 x g (10 min). The pellet was reextracted with methanol once more, centrifuged, and the resultant supernatants were pooled. The extract was evaporated to dryness using a Speed-Vac (Savant, USA), and the residue resuspended to its appropriate concentration in sterile saline. Some of these dried DB extracts were resuspended in HPLC-grade water and applied to a Sep-Pak Light C_{18} cartridge (Waters, Bedford, Ma), previously conditioned with methanol, and then rinsed with water. The material not adsorbed to the cartridge was washed with water, and the material bound to the cartridge was eluted with successive 2 ml washes of 20% methanol, then 70% methanol, and finally with 100% methanol. The respective methanol eluates were dried, and tested at a dose of 4-5 ae.

Preparation of DB-Conditioned Medium

Dorsal bodies were maintained for 24-30 h in culture medium as described in the previous chapter. For Sep-Pak C_{18} studies, DB-conditioned medium was collected and applied directly to a preconditioned cartridge. The bound material was eluted with successive methanol rinses as described above, and tested at 4.0-5.0 ae.

Heat and Protease Treatment of DB Tissue Extracts and DB-Conditioned Medium

Extracts of both DB tissue and DB-conditioned medium were subjected to heat treatment by aliquoting aqueous extracts in 1.5 ml polypropylene tubes and placing them in boiling water for approximately 15 min. After heating, the extracts were cooled, briefly centrifuged, and placed on ice until required. Aqueous extracts of DB tissue and DB-conditioned medium were subjected to proteolytic digestion using protease-coated agarose beads (Pronase F, 100 μg/ml; Sigma) for 2 h at 37°C with continuous agitation. The insoluble protease was pelleted by centrifugation at 5,000 x g for 10 min, and portions of the supernatant were used directly for the bioassay.

Analysis of data was performed using one-way ANOVA or the Kruskal-Wallis procedure ($p<0.05$), followed by the Dunnett Test or the Dunn Test ($Q_{\alpha=0.05, k}$) for assessing differences among multiple samples compared to controls. For each experiment, sample sizes were between 4-6.

RESULTS

Effect of Heat and Protease Treatment

The effect of heat and protease treatment on the polysaccharide synthesis-stimulating activity of DB tissue is shown in Fig. 1. Neither treatment diminished the activity of DB tissue extracts on polysaccharide synthesis or release compared to the untreated control. A similar result was obtained when DB-conditioned medium was subjected to the same treatments (Fig. 2).

Sep-Pak C_{18} Fractionation of DB Tissue and DB-Conditioned Medium

DB tissue extracts were fractionated from Sep-Pak C_{18} cartridges with successive 20, 70, and 100% methanol rinses. The 100% methanol eluate contained significant polysaccharide synthesis stimulating activity, as well as increasing the release of labelled material into the medium (Fig. 3). No activity could be detected in the 20% and 70% methanol washes (Fig. 3) or the flow-through material (data not shown). Fractionation of DB-conditioned medium showed significant stimulation of both polysaccharide synthesis and release activity in the 70% and 100% methanol eluates (Fig. 4), although the 100% methanol fraction was slightly more effective in stimulating synthesis and release than the 70% eluate.

DISCUSSION

The purification of the DBH of pulmonates has been attempted by several molluscan endocrinology labs. A preliminary report by Ebberink *et al.* (1983) suggested that the DBH of *L. stagnalis* is a protein of approximately 30 kDa with an isoelectric point of about 4.0. However, the bioassay used to detect DBH activity was the induction of ovulation, an assay supposedly specific for the caudodorsal cell hormone (CDCH) (Dogterom *et al.*, 1983; Ebberink *et al.*, 1985). The studies on the chemical nature of the DBH of terrestrial snails are also unclear, since the cellular source of the active material is unknown (van Minnen and Sokolove, 1984; Goudsmit and Ram, 1982). The isolation of the DBs from the Stylommatophora is complicated by the scattered location of the DBCs throughout the heavy connective tissue sheath surrounding the CNS (Marchand and Dubois, 1986). In this regard,

the DBs of planorbid snails such as *H. duryi* are more suitable for the isolation of DB tissue, since they are located as a discrete mass above the cerebral commissure.

Crude extracts of DB tissue or DB-conditioned medium were capable of stimulating *in vitro* polysaccharide synthesis from albumen gland explants (Chapter 1, this thesis). The stimulatory activity of the DB extracts was unaffected after it was subjected to heat treatment or to proteolytic enzymes, suggesting that the factor from the DBs is not a peptide. These results support the study by Miksys and Saleuddin (1988) who found biological activity of DB tissue extracts from *H. duryi* as being protease-insensitive, however, their study determined the DB preparation was heat-sensitive, as opposed to this study which shows that DB tissue extracts and DB-conditioned medium are heat-stable. The reason for this discrepancy is unclear.

The fractionation of DB tissue with Sep-Pak C_{18} cartridges revealed stimulatory activity resided solely in the 100% methanol eluate, indicating the factor within the DB tissue is relatively hydrophobic. Fractionation of DB-conditioned media showed two fractions (70% and 100%) which contained significant biological activity. Both fractions were approximately equal in potency, although the 100% methanol eluate contained slightly greater stimulatory activity. The distribution of biological activity from the DB-conditioned medium is difficult to explain. The stimulatory activity of the 100% methanol fraction from DB tissue could represent a less polar precursor of the bioactive substance detected in the 70% methanol fraction of the DB-conditioned medium. This hydrophobic precursor might be modified (hydroxylated), and then secreted by the DBs into the culture medium during *in*

vitro incubation. The active material detected in the 100% methanol fraction of DB-conditioned medium may represent a non-polar substance secreted into the medium or possibly liberated from DBCs through cut portions of the perikarya or cell processes during the isolation from the CNS. Alternatively, there may be two separate substances produced by the DBs which stimulate the albumen gland and possess different polarities. Further chromatographic separation and testing of the fractions is necessary for the characterization of DB secretions.

Fig. 1 The effect of protease and heat treatment of DB tissue extracts on albumen gland synthesis and accumulation of radiolabelled polysaccharides in the culture medium. Dried methanol extracts of DB tissue were resuspended in sterile snail saline and incubated with proteolytic enzyme (100 µg/ml Pronase F beaded agarose) for 2 h at 37°C. The insoluble enzyme suspension was pelleted by centrifugation, and the supernatant was tested for bioactivity. Dorsal body tissue extracts were also subjected to heat treatment by boiling extracts for 15 min. Bars represent the means ±SE of four separate experiments. Note that neither protease nor heat treatment significantly reduced the stimulatory activity of DB tissue extracts on albumen gland polysaccharide synthesis (**ANOVA $p<0.02$, Dunnett Test) and release (*Kruskal-Wallis $p<0.05$, Dunn Test) T, DB tissue extract; T+PR, DB tissue extract treated with protease; T+HE, DB tissue extract subjected to heat.

Fig. 1

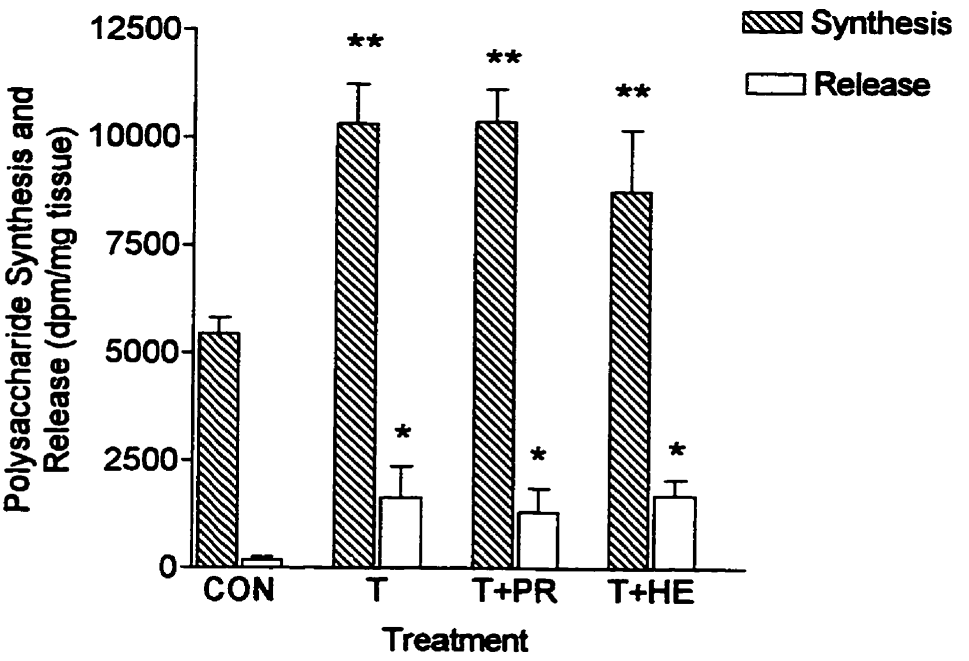

Synthesis
Release
Polysaccharide Synthesis and Release (dpm/mg tissue)
12500
10000
7500
5000
2500
0
**
**
**
*
*
*
CON
T
T+PR
T+HE
Treatment

Fig. 2 The effect of protease or heat treatment of DB-conditioned medium on albumen gland synthesis and release of radiolabelled polysaccharides. Dorsal body-conditioned medium was incubated with protease (Pronase F beaded agarose 100 μg/ml) for 2 h at 37°C. The insoluble enzyme suspension was pelleted by centrifugation, and the supernatant tested for bioactivity. Conditioned medium was also subjected to heat treatment by boiling for 15 min, then tested for bioactivity. Bars represent the means ±SE of five experiments. Note that neither protease nor heat treatment significantly diminished the stimulatory activity of DB-conditioned medium on albumen gland polysaccharide synthesis (**ANOVA $p<0.04$, Dunnett Test) and release (*Kruskal-Wallis $p<0.05$, Dunn Test). M, DB-conditioned medium; M+PR, DB-conditioned medium treated with protease; M+HE, DB-conditioned medium subjected to heat.

Fig. 2

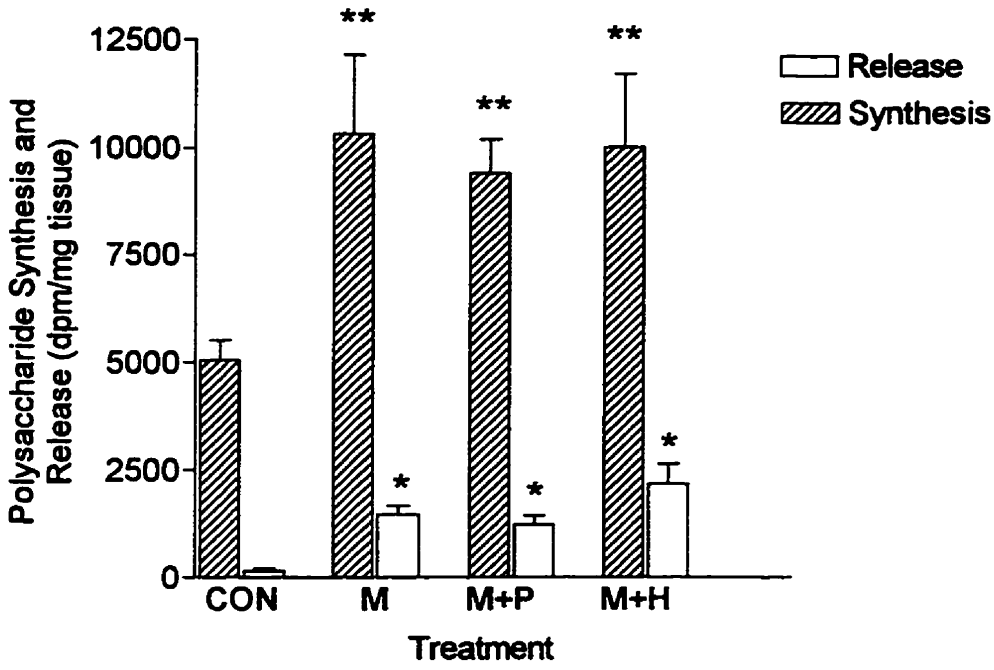

Polysaccharide Synthesis and Release (dpm/mg tissue)
12500
10000
7500
5000
2500
0
**
**
**
*
*
*
CON
M
M+P
M+H
Treatment
Release
Synthesis

Fig. 3 Sep-Pak C_{18} fractionation of DB tissue extracts. DB tissue extracts were passed through a pre-conditioned Sep-Pak C_{18} cartridge, then eluted with successive rinses of 20%, 70%, and finally 100% methanol. Each eluate was dried, resuspended in sterile snail saline, then tested for bioactivity. Bars reperesent the means ± SE of four experiments. Note that only the 100% methanol eluate stimulated polysaccharide synthesis (**Kruskal-Wallis $p=0.0374$) and release (* Kruskal-Wallis $p<0.05$).

Fig. 3

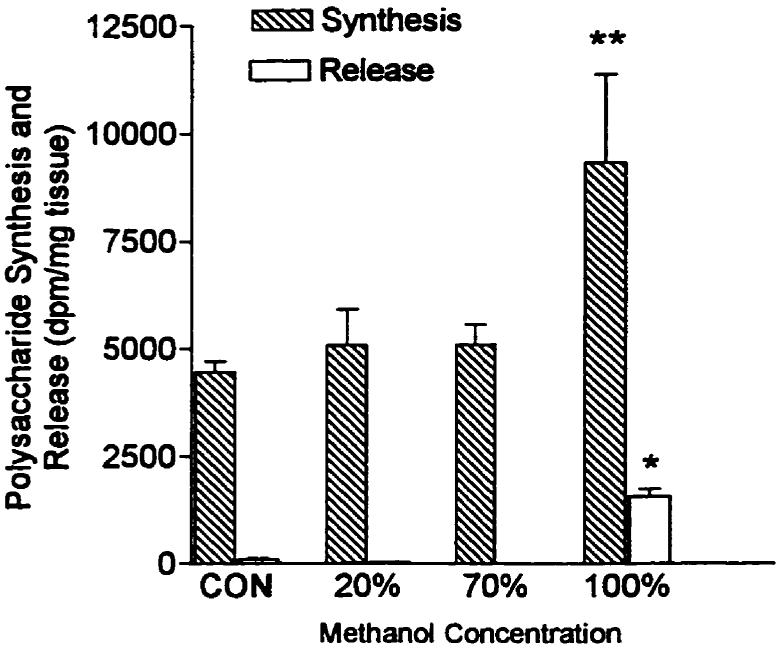
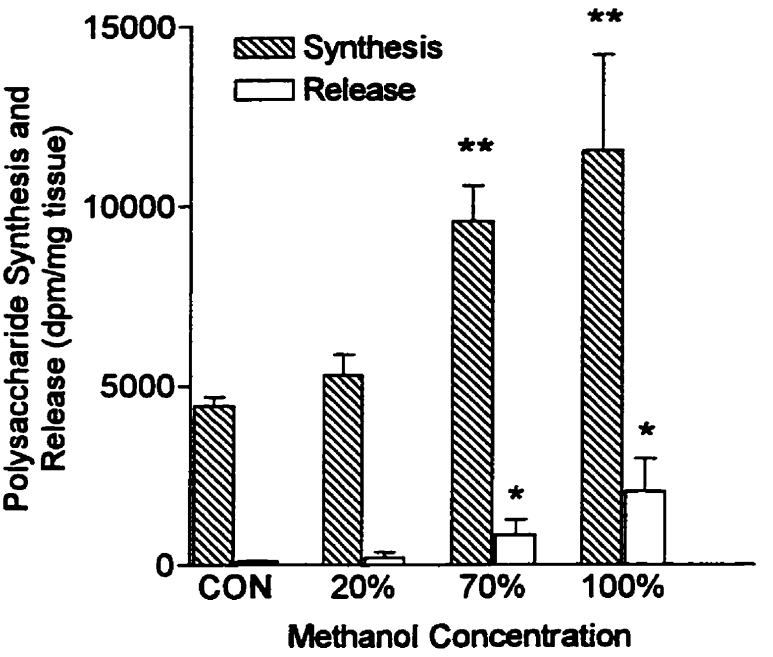

Synthesis
Release
12500
10000
7500
5000
2500
0
Polysaccharide Synthesis and Release (dpm/mg tissue)
**
*
CON
20%
70%
100%
Methanol Concentration

Fig. 4 Sep-Pak C_{18} fractionation of DB-conditioned medium. Dorsal body conditioned medium was passed through a preconditioned Sep-Pak C_{18} cartridge, then eluted with successive washes of 20%, 70%, and finally 100% methanol. Each eluate was dried, resuspended in saline, and tested for bioactivity. Bars represent the means ±SE of five experiments. Note that both the 70% and 100% methanol eluates stimulated polysaccharide synthesis (**Kruskal-Wallis $p<0.05$, Dunn Test) and release (*Kruskal-Wallis $p<0.05$, Dunn Test).

Fig. 4


15000
10000
5000
0
Synthesis
Release
**
**
*
*
CON
20%
70%
100%
Methanol Concentration
Polysaccharide Synthesis and Release (dpm/mg tissue)

Chapter III : Secretion of Ecdysteroids by the Dorsal Bodies and Effect of Synthetic Ecdysteroid on Reproductive Functions

SUMMARY

The endocrine dorsal bodies of the freshwater snail *Helisoma duryi* were maintained *in vitro*, and following incubation, the culture medium was collected and tested for the presence of ecdysteroids. Radioimmunoassay of the culture medium demonstrated the presence of ecdysteroid-like immunoreactivity, suggesting the dorsal bodies are capable of secreting ecdysteroids *in vitro*. To test for possible physiological functions of ecdysteroids in *Helisoma duryi*, 20-hydroxyecdysone (a potent ecdysteroid in arthropods) was injected into non-egg laying virgin snails. Injections of ecdysteroid induced low egg laying activity and the maturation of oocytes in the ovotestis. Incubation of albumen glands with ecdysteroid stimulated polysaccharide synthesis. Identification of released ecdysteroids by HPLC/RIA revealed a number of immunoreactive fractions which were tested for bioactivity. The results are discussed in relation to the possible function of ecdysteroids in pulmonate snails.

INTRODUCTION

In 1986, Nolte and coworkers reported the detection of ecdysteroid-like immunoreactive material in the culture medium from which dorsal bodies (DBs) of the terrestrial snail *Helix pomatia* had been maintained (Nolte *et al.*, 1986). The identity of the DB secretory material from culture media was determined by high-performance liquid chromatography (HPLC) and a specific radioimmunoassay (RIA) for ecdysteroids, and demonstrated that the DBs of *H. pomatia* were capable of secreting ecdysone (E) *in vitro*. This study represented the first demonstration of a putative ecdysteroid-secreting endocrine organ in a non-arthropod invertebrate. The authors proposed that pulmonate DBs produced E, and that ecdysteroid-related molecules might be involved in regulating the reproductive events attributed to the DBs. Bride *et al.* (1991) subsequently found that 20E (the more biologically potent ecdysteroid in insects), stimulated *in vitro* polysaccharide synthesis in the albumen glands of *H. aspersa*, providing further evidence that ecdysteroids influenced reproduction in snails. However, no further studies in the Stylommatophora were conducted to determine if ecdysteroids were functioning as authentic hormones.

In the freshwater snail *Helisoma duryi*, the active material from DB extracts and DB-conditioned medium was reported to be a methanol-extractable, protease-resistant substance, suggesting it is not proteinaceous (Miksys and Saleuddin, 1988; see Chapter 2, this thesis). The present study attempts to determine if the DBs of *H. duryi* are capable of synthesizing ecdysteroids, and whether these molecules exert specific physiological effects on reproductive processes. Initial experiments were conducted to determine the physiological effect of 20E

on two major reproductive processes, oocyte maturation and albumen gland polysaccharide synthesis. Chromatographic studies using HPLC were undertaken to separate and purify the previously determined bioactive DB secretions from the 70% and 100% methanol eluates from Sep-Pak C_{18} cartridges. The Sep-Pak C_{18} eluates and HPLC fractions were then tested for ecdysteroid-like immunoreactivity (by RIA) and biological activity (stimulation of polysaccharide synthesis) in an attempt to characterize the substance(s) produced by the DBs of *H. duryi*.

Materials and Methods

Ecdysteroid Measurements

Ten DBs or ovotestes from reproducing *H. duryi* were maintained in either Medium 199 or modified L-15 as described previously (Chapter 1, this thesis). After 24-30 h, the DB- or ovotestis-conditioned medium was removed, and evaporated to dryness. The dried residue was resuspended in borate buffer and quantified for ecdysteroids by RIA according to Steel *et al.* (1982). The polyclonal antiseum (H3) raised in rabbit demonstrates a 4:1 affinity for E to 20E (Gilbert *et al.*, 1977). The standard curve was generated using 20E, therefore all results are expressed as pg 20E eqts. Some DB culture media were also passed through a preconditioned Sep-Pak C_{18} solid-phase extraction cartridge (Waters, Bedford, MA) and eluted with successive 2 ml washes of 20%, 70%, and 100% methanol (Watson *et al.*, 1982; Lafont *et al.*, 1982). Each methanolic eluate was then measured for ecdysteroids using RIA. Hemolymph ecdysteroid concentrations from reproducing snails or from snails following a

first mating were quantified by RIA. The excess water surrounding the opening of the shell was absorbed with facial tissue and the shell was gently crushed. A known volume of hemolymph was removed with a glass micropipettor (Drummond Scientific Co., Broomall, PA) and four to five volumes of methanol were added to the hemolmyph to precipitate large proteins and extract ecdysteroids. The sample was centrifuged at 15,000 x g (10 min), and the pellet resuspended by sonification in methanol, then centrifuged once more. The resultant supernatants were evaporated to dryness, and assayed for ecdysteroids as described above.

Injection of 20E into Virgin Snails

Virgin snails were raised to adult size (10-12 mm shell diameter), placed in four groups (8 snails/group), and received injections of 10, 25, and 100 ng 20E in 2 µl of saline. Controls received injections of saline alone. A small hole was made with a dental drill (Emesco Dental Co., NY) in the ventral surface of the the shell near the vicinity of the digestive gland, and test solutions were injected with a Hamilton syringe. After the injections, the hole was sealed with dental wax. Injections were performed daily over a period of three weeks, and the total number of eggs and egg capsules laid by each snail was recorded. At the end of the experiment, the animals were sacrificed and the ovotestis dissected free from the digestive gland, and placed in Hutchison's fixative overnight (Hutchison, 1953). Tissues were dehydrated in ethanol, embedded in Paraplast Plus (Oxford Labware, St. Louis, MO), and serially sectioned at 8 µm. Sections were stained with the Prussian Blue reaction for iron-containing proteins, then counterstained with safranin (Humason, 1979). Iron-stainable material in mature oocytes serves as a histological marker in aiding to score oocyte

maturation. The number of oocytes per ovotestis was determined by counting the total number of oocytes in every tenth section. Oocytes ranged in size from 10-120 μm and were arbitrarily separated into two size classes according to Schollen and Saleuddin (1986). The oocytes were measured at their largest diameter and followed serially so as not to count the same oocyte twice. Oocytes < 50 μm were classified as immature oocytes, and those ≥ 50 μm were classified as mature. The values obtained were expressed as a percentage of the oocytes measured, and the arcsine transformed data was analyzed using ANOVA. Multiple comparison *a posteriori* tests were performed using the Tukey Test ($p<0.05$).

In Vitro Culture of Albumen Glands With 20E

Albumen glands were dissected from DD snails as described previously and incubated in 200 μl Medium 199 containing antibiotics and 1 μCi/ml ^{14}C-glucose. A stock solution of 10^{-3} M 20E was dissolved in ethanol and serial dilutions were made in snail saline. A concentration range between 10^{-12} to 10^{-7} M was applied to glands in a volume of 20 μl, and the synthesis and release of polysaccharides was determined as described previously (see Chapter 1, this thesis).

HPLC/RIA/Bioassay

The HPLC studies were performed with a Waters Chromatography System consisting of a U6K injector, a 600E System Controller, and a 486 Tunable Absorbance Detector (set at 242 nm). The HPLC separation was done on the Sep-Pak C_{18} 70% methanol eluate of DB-conditioned media, previously shown to contain ecdysteroid-like immunoreactivity and polysaccharide synthesis stimulating activity. The eluate was evaporated to dryness, then

resuspended in 12% acetonitrile (ACN). A 200 µl aliquot was injected into a Nova Pak C_{18} column (3.9 x 150 mm, 4µm) and the mixture separated using a linear gradient from 12-32% ACN over 60 min, then 32%-62% ACN in 10 min. The flow rate was 1 ml/min. The fractions (1 ml) were collected into 1.5 ml polypropylene tubes by hand. Each fraction was measured for the presence of ecdysteroids by RIA. For the bioassays, every 6-7 fractions were pooled, evaporated to dryness, and resuspended in 100 µl sterile saline. A dose of 4-5 ae was used on the albumen gland bioassay. For the HPLC separation of the Sep-Pak 100% methanol eluate from DB-conditioned medium, a different gradient scheme was used. Dried extracts were resuspended in 20% ACN, and separated with a Nova Pak C_{18} column using a linear gradient from 20-100% ACN in 40 min. The flow rate and collected fraction volume were the same as described above. Every 5 fractions were pooled, then dried and assayed for bioactivity. Statistical calculations were performed with ANOVA, or the non-parametric Kruskal-Wallis procedure ($p<0.05$), and when found significant was followed by the Dunnett Test or the Dunn Test ($Q_{\alpha=0.05,\,k}$) repectively.

RESULTS

Ecdysteroid Secretion by the DBs

The culture medium in which the DBs were maintained was analyzed for the presence of ecdysteroids using RIA. After 24-30 h incubation, approximately 100 pg 20E eqts could be detected from 10 DBs (Table 1). Dorsal body tissue, either before or after incubation, was extracted in methanol and analyzed for the presence of ecdysteroid-like immunoreactivity. Ecdysteroid-immunoreactive material could not be detected within DB tissue either before

or after incubation. The ovotestis was also incubated for the same duration as the DBs, however, ecdysteroid-like immunoreactivity could not be detected in the culture medium by RIA (Table 1). Since the DBs appeared to be capable of synthesizing ecdysteroids, synthetic 20E, the more biologically potent ecdysteroid in arthropods, was initially tested for its effect on oocyte maturation and albumen gland synthetic activity.

Effect of 20E Injections on Egg Production and Oocyte Maturation

Daily injections of 10, 25, 50 ng 20E into virgin *H. duryi* over a three week period stimulated egg laying activity (Table 2). The number of snails responding to ecdysteroid injection was clearly greater than saline injected controls. Both egg and egg capsule (containing empty eggs) production were significantly increased. The histological examination of the ovotestes from virgin controls showed an abundance of immature oocytes (Fig. 1). Injections of 20E appeared to increase the proportion of mature oocytes within the ovotestis compared to saline injected controls. Significant stimulation of oocyte maturation occurred at a concentration of 10 ng 20E and increased in a dose-dependent manner. Light microscropic examination of the ovotestes from snails injected with 20E (Fig. 2b) showed that there was a noticeable increase in iron-stainable material in the oocytes compared to controls (Fig. 2a).

Effect of 20E on Albumen Gland Polysaccharide Synthesis

The effect of 20E (10^{-12} to 10^{-7} M) on the *in vitro* synthesis of polysaccharides in albumen gland explants was examined after 48 h incubation. This ecdysteroid showed a biphasic effect on albumen gland polysaccharide synthesis (Fig. 3). Incorporation of

radiolabelled glucose into polysaccharides increased slightly at a concentration of 10^{-12} M, and significantly between 10^{-11} to 10^{-6} M. However, the release of newly synthesized polysaccharides could not be detected in the culture medium. Preliminary experiments using ecdysone (E) or the vertebrate steroid hormone β-estradiol (10^{-7} M) showed they did not have stimulatory effects on albumen gland polysaccharide synthesis (data not shown).

Effect of Mating on Polysaccharide Synthesis and Hemolymph Ecdysteroid Levels

Virgin snails were raised to adult size, then paired for a single mating. Albumen gland polysaccharide synthesis and hemolymph ecdysteroid concentrations were measured every six hours following mating. The *in vitro* synthetic activity of the albumen gland remained at low levels for the first 18 h following mating (Fig. 4). Between 18-24 h post-mating, the synthetic activity of the albumen gland increased dramatically, and continued to increase until 30 h after mating. Albumen gland polysaccharide synthesis exhibited cyclical changes in activity showing peaks at 30 h and 48 h post-mating, and troughs at 36 h and 60 h post-mating. The times at which the synthetic activity of the albumen gland increased was coincident with bouts of egg laying. The corresponding hemolymph ecdysteroid profile is also shown (Fig. 4). However, it did not appear to undergo any significant changes in concentration following mating.

Fractionation of DB-Conditioned Medium

Since 20E was capable of inducing oocyte maturation and stimulating albumen gland polysaccharide synthesis, attempts were made to further purify ecdysteroid-like material from DB secretions. The DB-conditioned medium was fractionated using a Sep-Pak C_{18} cartridge

and the methanol eluates (20%, 70%, 100%) assayed for ecdysteroids using RIA. All ecdysteroid-like immunoreactivity was localized in the 70% methanol eluate, whereas the 20% and 100% methanol rinses did not contain any ecdysteroid-like immunoreactivity (Fig.5).

HPLC/RIA Analysis of DB Media

The HPLC separation of the Sep-Pak 70% methanol fraction of DB-conditioned medium revealed only trace amounts of UV-absorbing material (Fig. 6). The RIA analysis of this eluate revealed a number of ecdysteroid-immunoreactive fractions, several of which had the same retention times as the authentic ecdysteroid standards. The fraction coeluting with 25dE contained the most ecdysteroid-like immunoreactivity, followed by E, 20E22Ac, 20E, and Pon A (Fig. 7). A few unidentified fractions which are slightly more polar, and some which are less polar than E, also contained low amounts of ecdysteroid-like immunoreactivity. Pooled fractions from another HPLC run were then tested for their ability to stimulate polysaccharide synthesis in albumen gland explants. The activity profile showed modest polysaccharide synthesis stimulating activity in pooled fractions 1-6 and 54-60, and statistically significant activity in pooled fractions 33-39 (Fig. 8). Pooled fractions 1-6 did not correspond to any of the known ecdysteroid standards available, and did not possess ecdysteroid-like immunoreactivity. The only ecdysteroid standards eluting between fractions 33-39 and 54-60 are 2dE and 25dE respectively.

The HPLC separation of the 100% methanol eluate (not ecdysteroid-immunoreactive) from DB-conditioned medium revealed a prominent UV-absorbing peak near the end of the gradient (Fig. 9). The pooled fractions of this HPLC separation were tested for their ability

to stimulate albumen gland polysaccharide synthesis. Fractions 31-35 and 36-40 eluted with high concentrations of acetonitrile and possessed significant biological activity compared to controls incubated with saline alone (Fig. 10).

DISCUSSION

The presence and structural identification of ecdysteroids has been demonstrated in all the major protostomian invertebrate phyla (see Walgreave and Verhaert, 1988; Franke and Kauser, 1989). Although there is no doubt regarding the presence of these steroids in the invertebrates, definitive evidence of their hormonal function exists only in the arthropods. In the Platyhelminthes, Nemertines, Nematodes, and Annelids, circumstantial evidence indicates a role for ecdysteroids in development and reproduction, however, a putative ecdysiosynthetic tissue has never been identified in any of these groups (Franke and Kauser, 1989; Lafont, 1991). Since ecdysteroids are often consumed from the plants upon which some of these animals feed, the endogenous production of ecydsteroids by non-arthropod invertebrates has been questioned.

In molluscs, Romer favoured the notion that ecdysteroids were of endogenous origin (Romer, 1979). De Jong-Brink *et al.* (1989) demonstrated that fed *L. stagnalis* contained less ecdysteroid-like immunoreactivity in the hemolymph and tissues than starved snails, suggesting the ecdysteroids were not exogenously derived. The results of this study demonstrate that the DBs of the freshwater snail *H. duryi* secrete ecdysteroids *in vitro* and support the work of Nolte *et al.* (1986) in *Helix*. In addition, we did not detect the presence

of ecdysteroid-like material in the food (lettuce) of *H. duryi* using the current RIA system. The ovotestis, another potential steroid-synthesizing tissue did not secrete detectable ecdysteroid-like material. Fractionation of DB-conditioned medium with Sep-Pak C_{18} cartridge demonstrates all ecdysteroid-like immunoreactivity elutes with the 70% methanol rinse. This is consistent with the polaritites of most ecdysteroid molecules (Lafont *et al.*, 1982; Watson *et al.*, 1982). It is proposed that the DBs of *H. duryi* are capable of synthesizing ecdysteroids based upon the observation that ecdysteroid-like immunoreactivity could not be detected in DB tissue either before or after incubation, yet significant quantities were detected in the culture medium. Since ecdysteroids are released as soon as they are synthesized (Redfern, 1989), the net increase in ecdysteroid-like immunoreactivity suggests that synthesis has occurred.

In *H. duryi*, virgin snails reared in isolation, lay markedly fewer egg masses than mated snails (Saleuddin *et al.*, 1983b). Mating is necessary for the production of viable eggs and egg laying. The virgin ovotestis is known to contain a majority of immature oocytes (~ 70% of total oocytes), whereas in mated snails the proportion of immature to mature oocytes is approximately equal (Schollen and Saleuddin, 1986). The injection of 20E into virgin *H. duryi* appeared to stimulate egg production. However, the number of eggs laid by the injected snails was much lower compared to reproducing snails, and the percentage of mature oocytes within the ovotestis still remained less than the immature oocytes (see Schollen and Saleuddin, 1986). This indicates that 20E is only partially effective in inducing oocyte maturation and egg laying. Thus, another factor such as CDCH (Mukai and Saleuddin, 1989)

or a 'matedness factor'(Kunigelis and Saleuddin, 1986) is probably required for normal egg laying activity. The histological examination of the ovotestes from ecdysteroid-injected animals showed an increase in the proportion of mature oocytes relative to controls, indicating 20E is involved in the maturation of the oocytes. The mechanism by which 20E exerts its effect is unclear, perhaps involving the synthesis or uptake of yolk precursors. In the Diptera and Crustacea, ecdysteroids are known to stimulate yolk protein synthesis (Dhadialla and Raikhal, 1994; Wilder *et al.*, 1991; Okumura *et al.*, 1992). Since a specific yolk protein whose synthesis is regulated by hormones has not been isolated in any mollusc, investigations on the regulation of yolk protein synthesis remain stalled.

The iron storage protein ferritin is known to be pinocytotically taken up by pulmonate oocytes (Saleuddin *et al.*, 1980; Bottke and Sinha, 1979), and it is suggested the uptake of the exogenous ferritin is controlled by the DBs (Miksys and Saleuddin, 1987a, b). The Prussian Blue iron-staining of the ovotestes from animals injected with 20E, displayed more intense staining of the oocyte cytoplasm than oocytes from saline injected snails, indicating that 20E might influence the uptake of iron-containing proteins (ferritin). The observation that 20E injections also increased egg caspule production indicates it also has effects on those female accessory sex glands which participate in egg capsule formation (eg. oothecal gland).

In pulmonate molluscs, the albumen gland is a known target organ for the DBH (see Joosse, 1988; Saleuddin *et al.*, 1994; Saleuddin, 1998a). Dorsal body-conditioned culture medium contained a heat-stable, protease-resistant factor which is capable of stimulating polysaccharide synthesis in the albumen gland of *H. duryi* (Chapter 2, this thesis), and this

DB-conditioned medium also contained ecdysteroid-like immunoreactivity. The *in vitro* culture of albumen glands with synthetic 20E showed that this ecdysteroid is able to induce significant increases in polysaccharide synthesis. The concentrations of 20E which stimulated synthesis appeared to be in the physiological range of ecdysteroid-like immunoreactivity determined in the hemolymph (10^{-10} to 10^{-8} M). These results are in agreement with those of Bride *et al.* (1991) who demonstated 20E stimulated the *in vitro* synthesis of polysaccharides in the albumen gland of the terrestrial snail *H. aspersa.*

The observation of a change in titer of a particular chemical substance concomitant or preceding a specific physiological event is a key criterion to demonstrate the substance is functioning in a classical hormonal fashion. In *H. duryi*, mating is known to activate the two gonadotropic centers, the CDCs and DBs, thereby stimulating the release of their products into the hemolymph (Mukai and Saleuddin, 1989; Saleuddin *et al.*, 1989; Khan *et al.*, 1990a, b). A marked increase in albumen gland polysaccharide synthesis was observed between 18-24 h following a first mating, and peaked at about 30 h post-mating. It is during this period of time that the first clutch of eggs was laid. The albumen gland synthetic activity showed peaks and troughs of activity over the next 36 h. The periods of increasing synthetic activity was correlated with bouts of egg laying. After snails are mated, the albumen gland appeared to maintain an elevated level of synthetic activity which was approximately two to three-fold higher than virgins.

The hemolymph ecdysteroid concentration from first-mated snails was measured by RIA and showed only small fluctuations in ecdysteroid titer. There were no significant

changes in hemolymph ecdysteroid levels up to 66 h post-mating, although there were general correlations of some minor increases in hemolymph ecdysteroid concentration with albumen gland activity. One possible explanation is the DBs may secrete an unusual ecdysteroid(s) into the hemolymph, which is biologically active on the albumen gland, but is not recognized by the current antiserum.

The HPLC/RIA analysis of DB-conditioned medium shows that 25dE and E are the predominant ecdysteroids secreted by the DBs of *H. duryi*. This is in contrast to the results of Nolte *et al.* (1986) who reported the DBs of the terrestrial snail *H. pomatia* secreted primarily E. In this respect, the secretory products of *H. duryi* DBs resemble those of the decapod crustaceans, where 25dE is also a major secretory product of the Y-organs (Lachaise *et al.*, 1989; Pis *et al.*, 1995). This ecdysteroid is then converted to Pon A by peripheral tissues, and along with 20E, are the major circulating ecdysteroids in the hemolymph of several crustacean species (Lachaise *et al.*, 1989).

The bioactivity of pooled HPLC fractions show there are two fractions (1-6 and 54-60) which possess modest polysaccharide-synthesis stimulating activity, whereas pooled fractions 33-39 contained significant bioactivity. The only ecdysteroid standard which elutes between fractions 33-39 is 2dE (~37 min). In this study, the only ecdysteroid tested extensively was 20E. Based on HPLC/RIA data, some other possible candidates that await further testing are E, 25dE, or PonA. Biosynthetic studies by Garcia *et al.* (1995) found that terrestrial snails did not appear to produce the typical insect ecdysteroids (E, 20E) via the same pathways. They proposed that snails might use an alternative ecdysteroid biosynthetic

pathway, similar to that observed in primitive arthropods to produce unusual ecdysteroid molecules. Bombycosterol is an example of a unique ecdysteroid-related molecule isolated from *Bombyx mori* ovaries (Fujimoto *et al.*, 1985). Such novel ecdysteroid-related molecules may have similar polarity to the typical ecdysteroid molecules (for example, E, 20E), but react poorly with current ecdysteroid antisera. In our study, weak ecdysteroid-like immunoreactivity could be detected in some fractions that showed modest activity in the bioassay. Therefore, it is possible *H. duryi* DBs might produce an unusual ecdysteroid-like molecule which is not recognized using the current antiserum. Analysis of other ecdysteroid species present in DB-conditioned media and in the hemolymph of *H. duryi* by HPLC/differential RIA (the use of antisera with differing affinities toward various moieties of the ecdysteroid molecule), and biosynthesis studies using radiolabelled precursors should provide useful information regarding the type of ecdysteroid molecule(s) to focus attention upon in pulmonates.

The HPLC separation and bioactivity profile of the 100% methanol eluate (not ecdysteroid-immunoreactive) from the solid-phase extraction cartridge revealed significant stimulatory activity in two pooled fractions eluting near the end of the acetonitrile gradient, suggesting that the material from these fractions is hydrophobic in nature. At present, the chemical identity of the substance(s) within these bioactive fractions is unknown.

Table 1 Secretion of ecdysteroid-like immunoreactive material from the DBs of *Helisoma duryi*. Groups of ten DBs or ovotestes were maintained for 24-30 h in Medium 199. After the incubation period, the culture medium was collected and measured for ecdysteroids using RIA (antiserum H3). Dorsal body tissue either before or after incubation was extracted in methanol, then measured for ecdysteroids. Note that ecdysteroids could be not be detected in DB tissue either before or after the incubation period. However, significant quantities of ecdysteroid-like immunoreactive material is detected in the culture medium from which the DBs had been incubated. The ovotestis did not secrete detectable amounts of ecdysteroid into the surrounding culture medium. nd-not detectable.

Table 1

Organ Extracted or Cultured	pg 20E eqts	(N)
DB Tissue (before culture)	nd	(5)
DB Culture Medium (after 24 h)	100.00 ± 7.07	(9)
DB Tissue (after 24 h culture)	nd	(9)
Ovotestis Culture Medium (after 24 h)	nd	(3)

Table 2 The effect of 20-hydroxyecdysone (20E) injections into virgin *Helisoma duryi* on egglaying activity. Virgin snails were divided into four groups (8 snails per group), each receiving injections of 10, 25, and 100 ng 20E (in 2 µl of saline) every day for three weeks. Control animals received saline alone. The cumulative number of egg masses and egg capsules produced at the end of the experiment was recorded for each group. Note that 20E stimulated egg mass production in a dose-dependent fashion (10-100 ng) compared to controls.

Table 2

Injections	No. of snails responding to 20E injections	Total no. of egg masses laid	Total no. of empty egg capsules laid
Control (saline)	1/8	1	1
10ng 20E	6/8	12	3
25ng 20E	6/8	21	11
100ng 20E	8/8	32	6

Fig. 1 The effect on ecdysteroid injection in virgin *Helisoma duryi* on oocyte maturation. The ovotestis were removed from 20E injected snails, fixed in Hutchison's solution, then processed for light microscopy. Serial 8 μm thick sections were cut and stained for iron-containing proteins using the Prussian Blue reaction. Oocytes < 50 μm in diameter were scored as immature and those ≥ 50 μm in diameter were scored as mature. The data is represented as the % of mature oocytes relative to immature oocytes. Note that ecdysteroid injections cause an increase in the percentage of mature oocytes compared to saline injected controls (N=6 for each group). *Statistically different from control (ANOVA $p<0.002$, TukeyTest).

Fig. 1

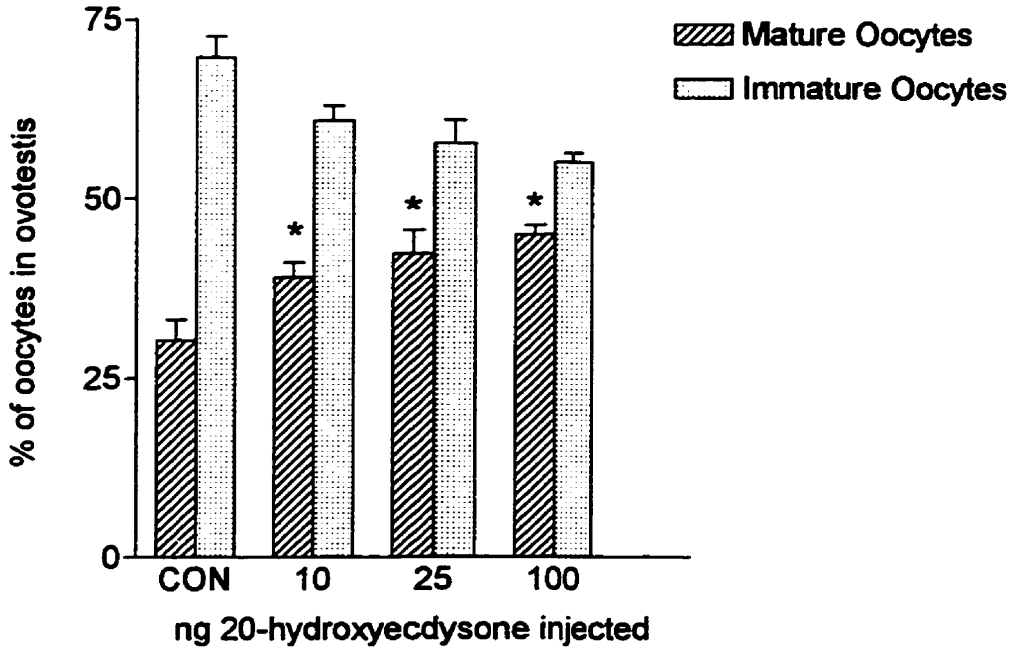

75
50
25
0
% of oocytes in ovotestis
Mature Oocytes
Immature Oocytes
*
*
*
CON
10
25
100
ng 20-hydroxyecdysone injected

Fig. 2 Light micrograph of the ovotestis from a virgin snail injected with either saline (2a) or 100 ng 20-hydroxyecdysone (2b). Sections were stained with the Prussian Blue Reaction for iron-containing proteins (green-blue) and counterstained with safranin (red-brown). Note the increase in number of mature oocytes (>50 μm) in the ovotestis from the ecdysteroid injected animal compared to the control. ac-acinus. Scale bar, 100 μm.


2a
ac

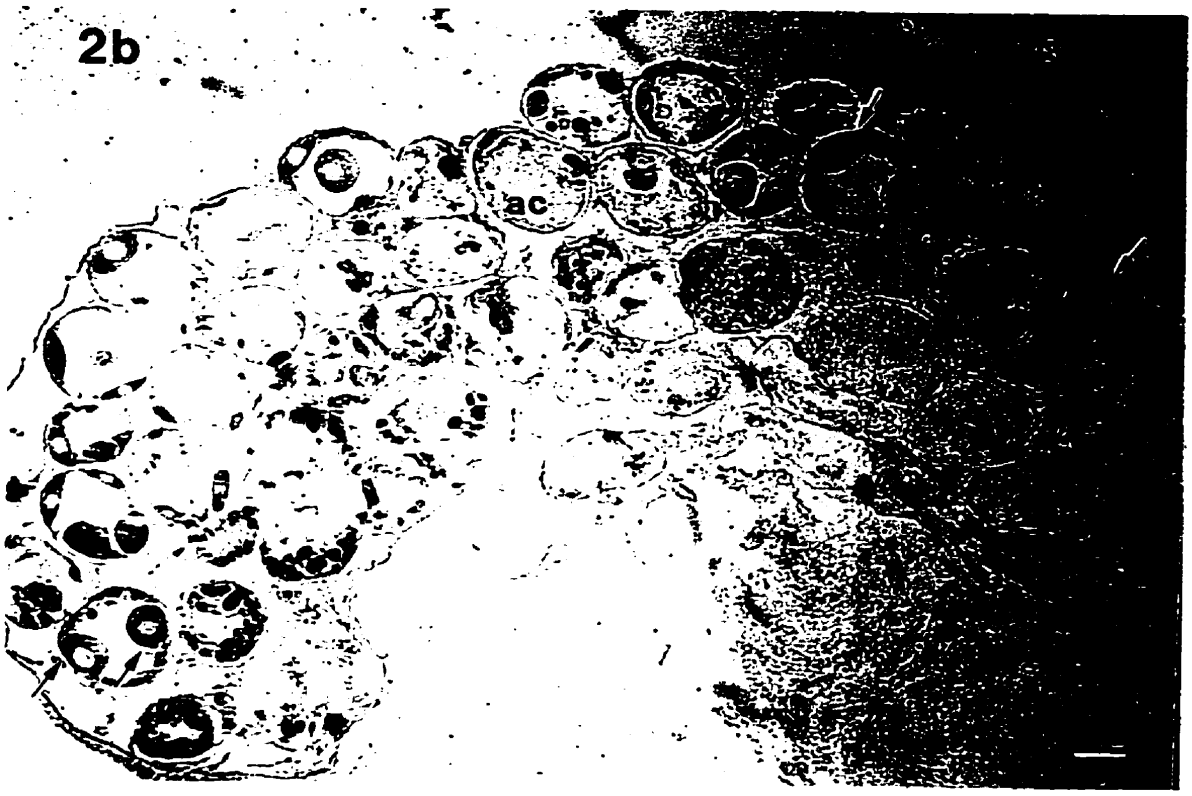

2b
ac

Fig. 3 The effect of 20E on albumen gland synthetic activity. Albumen glands were incubated for 48 h with 20E (10^{-12} to 10^{-7} M) in Medium 199 containing radiolabelled glucose (1 μCi/ml). Total polysaccharides synthesized by the tissues and released into the culture medium was quantified after TCA extraction and ethanol precipitation. Bars represent the means ± SE of 10-20 samples. Note significant stimulation of polysaccharide synthesis between 10^{-11} M to 10^{-7} M 20E (*Kruskal Wallis $p<0.05$, Dunn Test).

Fig. 3

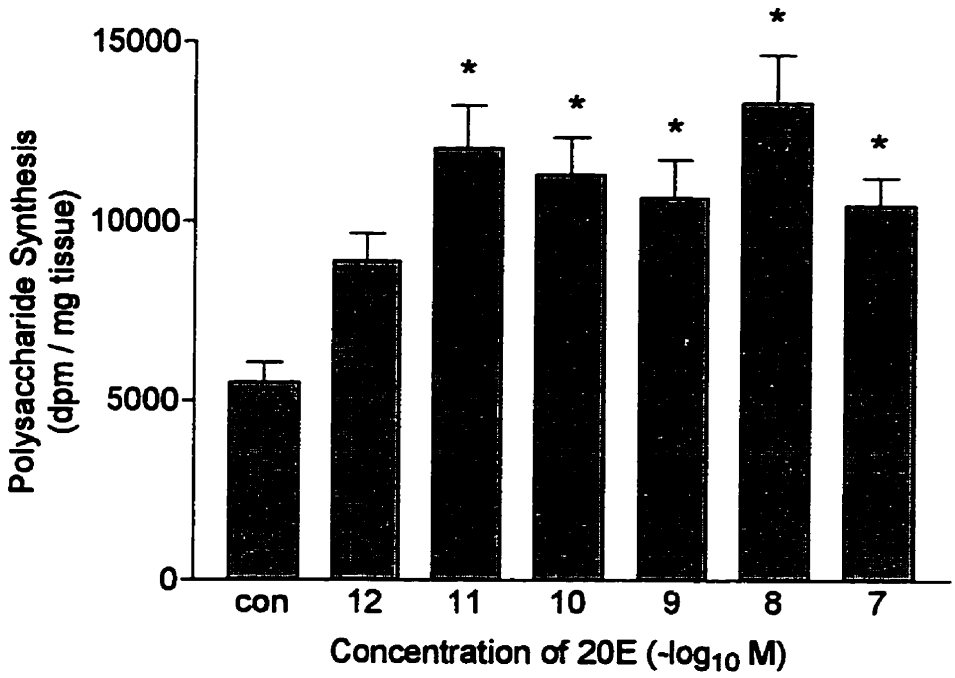

15000
10000
5000
0
Polysaccharide Synthesis
(dpm / mg tissue)
*
*
*
*
*
con
12
11
10
9
8
7
Concentration of 20E (-log10 M)

Fig. 4 The effect of mating on albumen gland polysaccharide synthesis and hemolymph ecdysteroid concentration. Virgin snails were raised in isolation to a size of 10-12 mm shell diameter, then paired for a single mating. After copulation, the *in vitro* synthetic activity of the albumen gland was determined every six hours. Hemolymph ecdysteroid levels were also quantified by RIA. Note a dramatic increase in synthetic activity of the albumen gland between 18-30 h post-mating. The horizontal bars represent approximate time frame of oviposition. The hemolymph ecdysteroid concentration does not show statistically significant changes after a first mating. Each point for the determination of polysaccharide synthesis and hemolymph ecdysteroid concentration represents the mean ± SE of 4-6 samples, and mean ± SE of 4-12 samples respectively.

Fig. 4

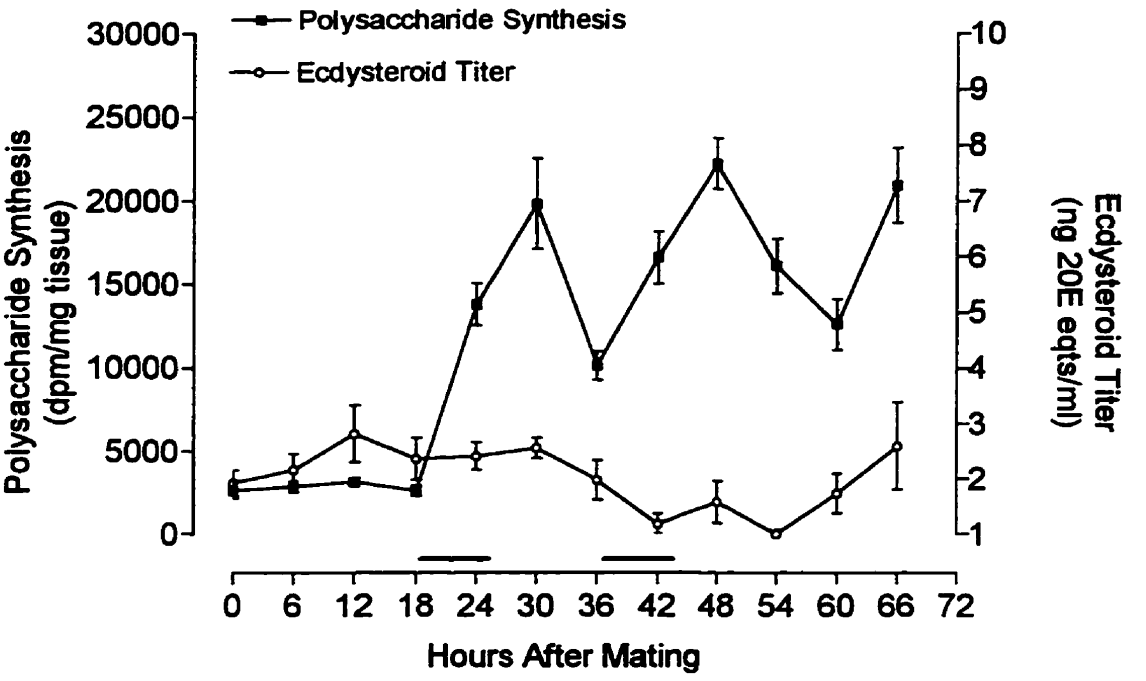

Polysaccharide Synthesis
Ecdysteroid Titer
Polysaccharide Synthesis (dpm/mg tissue)
Ecdysteroid Titer (ng 20E eqts/ml)
30000
25000
20000
15000
10000
5000
0
10
9
8
7
6
5
4
3
2
1
0 6 12 18 24 30 36 42 48 54 60 66 72
Hours After Mating

Fig. 5 Fractionation of dorsal body-conditioned medium using Sep-Pak C_{18} solid-phase extraction cartridges, and subsequent detection of eluates for ecdysteroids. Approximately 20 DBs were incubated in Medium 199 for 24 h. After incubation, the medium was loaded directly onto a preconditioned cartridge and eluted with successive washes containing 20%, 70%, and 100% methanol. The individual eluates were dried down and analyzed for ecdysteroids using RIA. Note that only the 70% methanol eluate contained ecdysteroid-like immunoreactivity.

Fig. 5

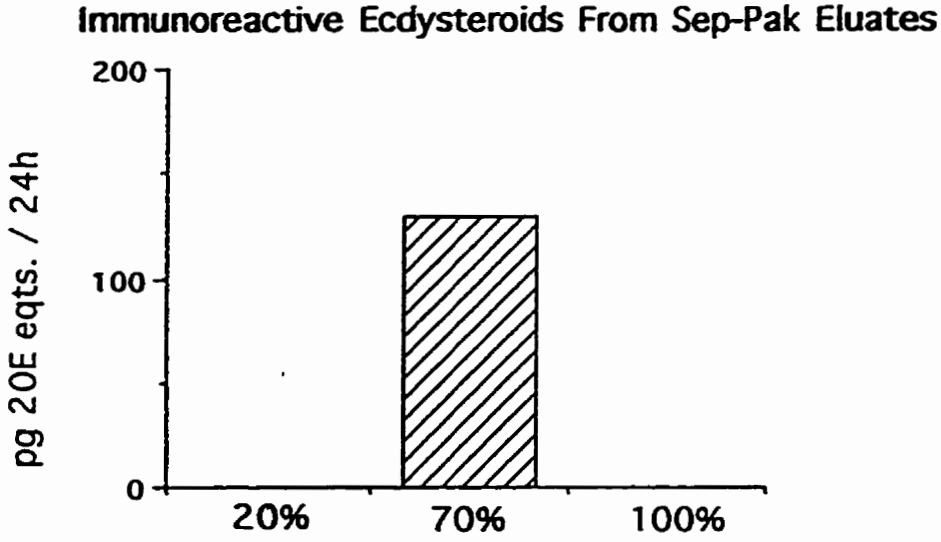

Immunoreactive Ecdysteroids From Sep-Pak Eluates
pg 20E eqts. / 24h
200
100
0
20%
70%
100%

Fig. 6 HPLC chromatogram of DB-conditioned medium after Sep-Pak C_{18} fractionation. Approximately 200 DBs were incubated in culture medium for 24 h and the medium passed through a Sep-Pak C_{18} cartridge. After salts and unretained material were washed from the cartridge with water, the adsorbed material was eluted with 70% methanol. The eluate was dried, resuspended in 12 % acetonitrile (ACN), and a portion was injected into the HPLC column (Nova Pak C_{18} , 3.9 x 150 mm, 4 μm). The extract was separated using a linear gradient from 12-32% ACN over 60 min, then a linear gradient from 32-62% ACN in 10 min. The flow rate was 1 ml/min, and fractions (1 ml) were collected into 1.5 ml polypropylene tubes. The vertical axis represents the absorbance units (AU) at 242 nm, and the horizontal axis represents the duration of the gradient.

Fig. 6

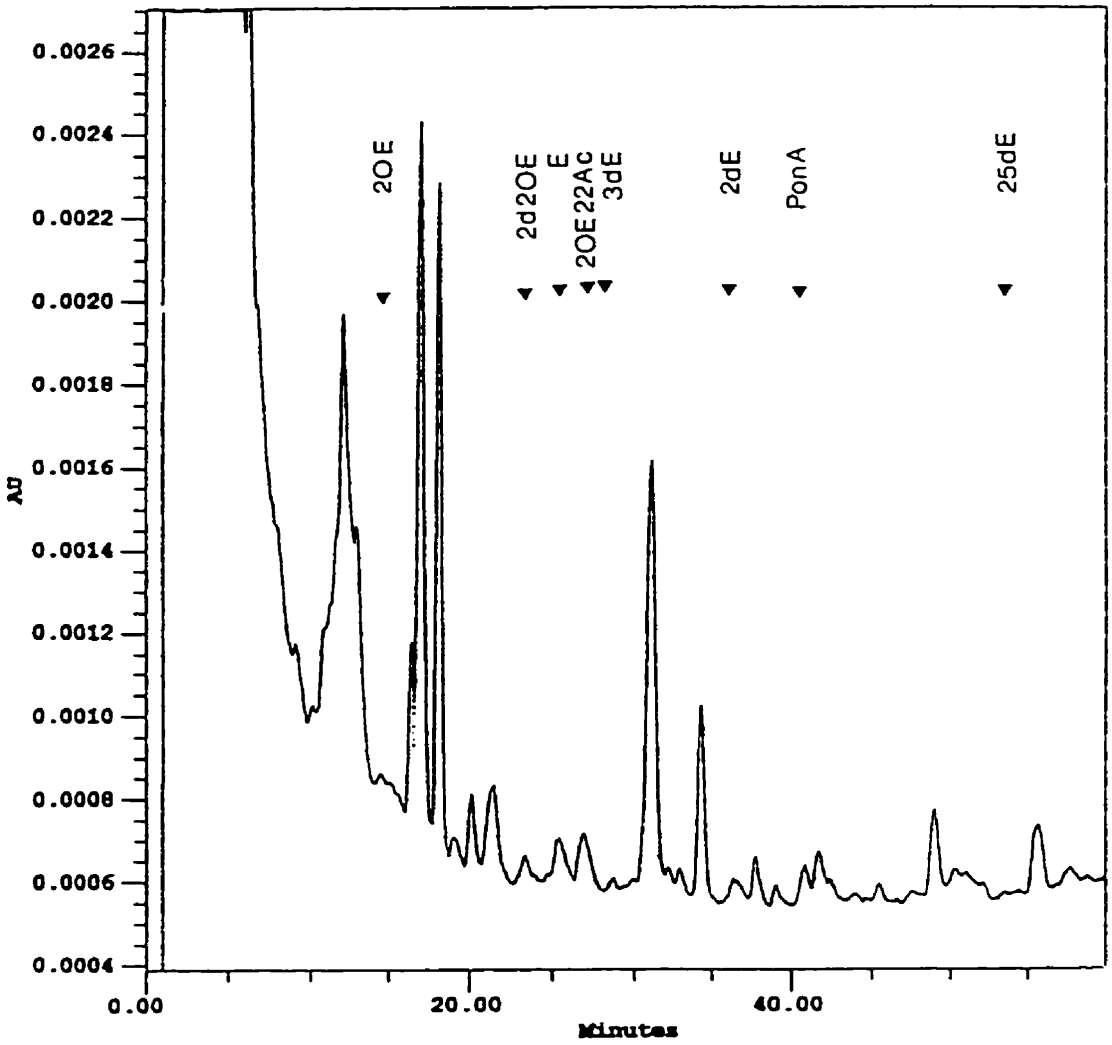

0.0026
0.0024
0.0022
0.0020
0.0018
0.0016
0.0014
0.0012
0.0010
0.0008
0.0006
0.0004
AU
0.00
20.00
40.00
Minutes
2OE
2d2OE
E
2OE22Ac
3dE
2dE
PonA
25dE

Fig. 7 Ecdysteroid quantification of HPLC-separated fractions from DB-conditioned medium. The HPLC separation was performed as described in Fig. 6, and each of the the fractions analyzed for ecdysteroids using RIA. The fractions coeluting with 25dE and E contained the highest ecdysteroid-like immunoreactivity. The data were not corrected for cross-reactivity to the H3 antiserum. Arrows represent the retention times of the authentic ecdysteroid standards. 20E, 20-hydroxyecdysone; E, ecdysone; 20E22Ac, 20-hydroxyecdysone 22 acetate; 3dE, 3-dehydroecdysone; 2dE, 2-dehydroecdysone; Pon A, ponasterone A; 25dE, 25-deoxyecdysone.

Fig. 7

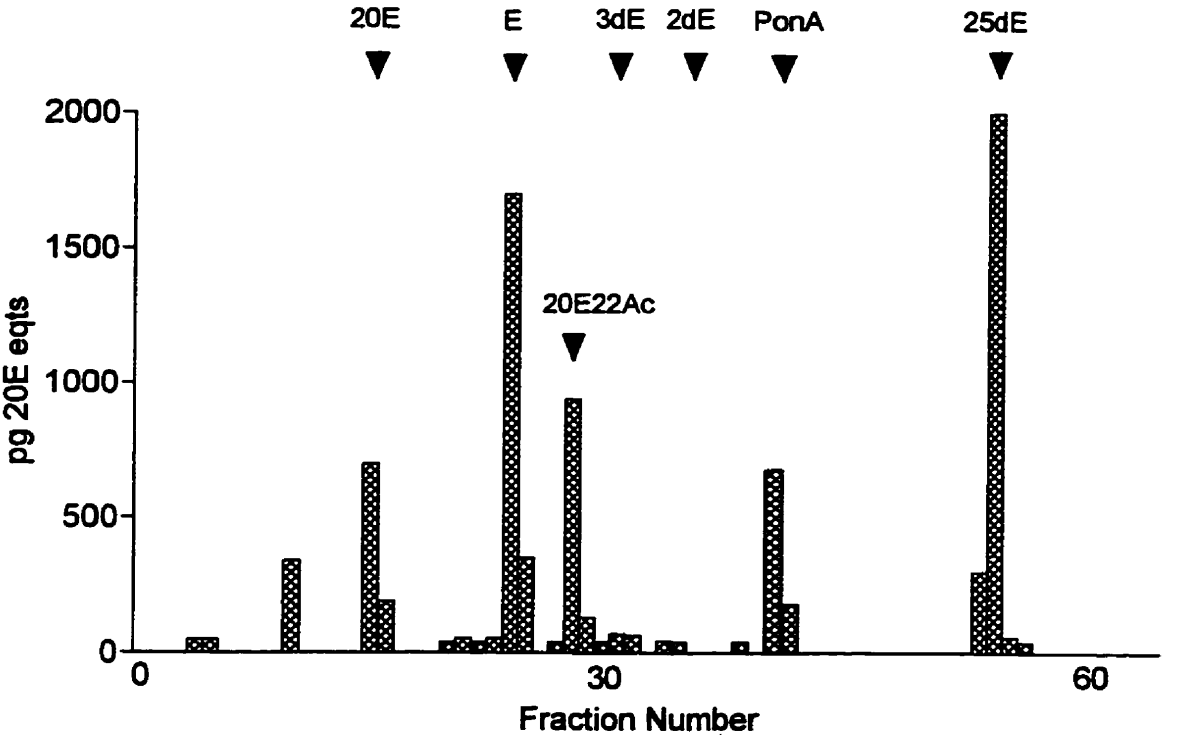

20E
E
3dE
2dE
PonA
25dE
20E22Ac
2000
1500
1000
500
0
pg 20E eqts
0
30
60
Fraction Number

Fig. 8 The effect of HPLC-separated fractions on albumen gland synthetic activity. Dorsal body-conditioned medium was separated by HPLC as described in Fig. 6a. Six to seven fractions were pooled and dried down. The residue was resuspended in sterile saline and tested for bioactivity at a dose of 4-5 ae. Sample 1 (fractions 1-6); sample 2 (fractions 7-12); sample 3 (fractions 13-18); sample 4 (fractions 19-25); sample 5 (fractions 26-32); sample 6 (fractions 33-39); sample 7 (fractions 40-46); sample 8 (fractions 47-53); sample 9 (fractions 54-60); sample 10 (fractions 61-70). Bars represent the means ± SE of 4-6 samples. Note significant stimulation of albumen gland polysaccharide synthesis in sample 6 (fractions 33-39)(*ANOVA p=0.0016, Dunnett Test).

Fig. 8

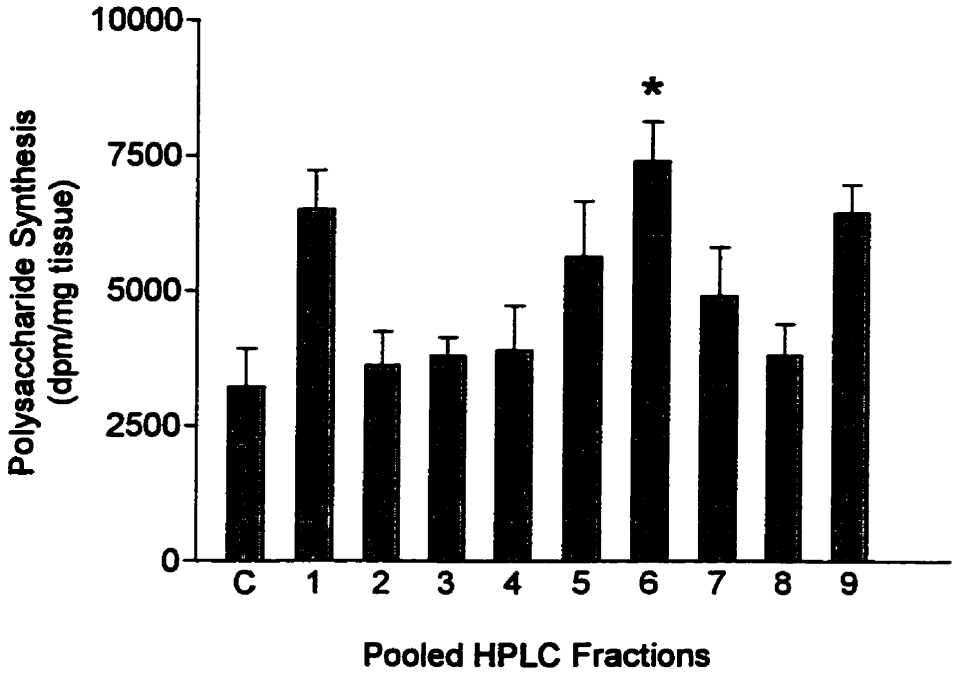

10000
7500
5000
2500
0
Polysaccharide Synthesis
(dpm/mg tissue)
*
C
1
2
3
4
5
6
7
8
9
Pooled HPLC Fractions

Fig. 9 HPLC chromatogram of DB-conditioned medium eluted from a Sep-Pak C_{18} cartridge with 100 % methanol. Approximately 200 DBs were incubated in culture medium for 24 h. The conditioned medium was passed through a preconditioned Sep-Pak C_{18} cartridge. Ecdysteroid-like immunoreactive material was eluted with 70 % methanol and set aside. The remainder of the hydrophobic material retained by the cartridge was eluted with 100 % methanol, then evaporated to dryness. The residue was resuspended in 20 % acetonitrile (ACN) and injected into the HPLC column (Nova Pak C_{18} 3.9 x 150 mm, 4 μm). The extract was separated using a linear gradient from 20%-100% ACN in 40 min. The vertical axis represents the absorbance units (AU) at 242 nm, and the horizontal axis represents the duration of the gradient.

Fig. 9

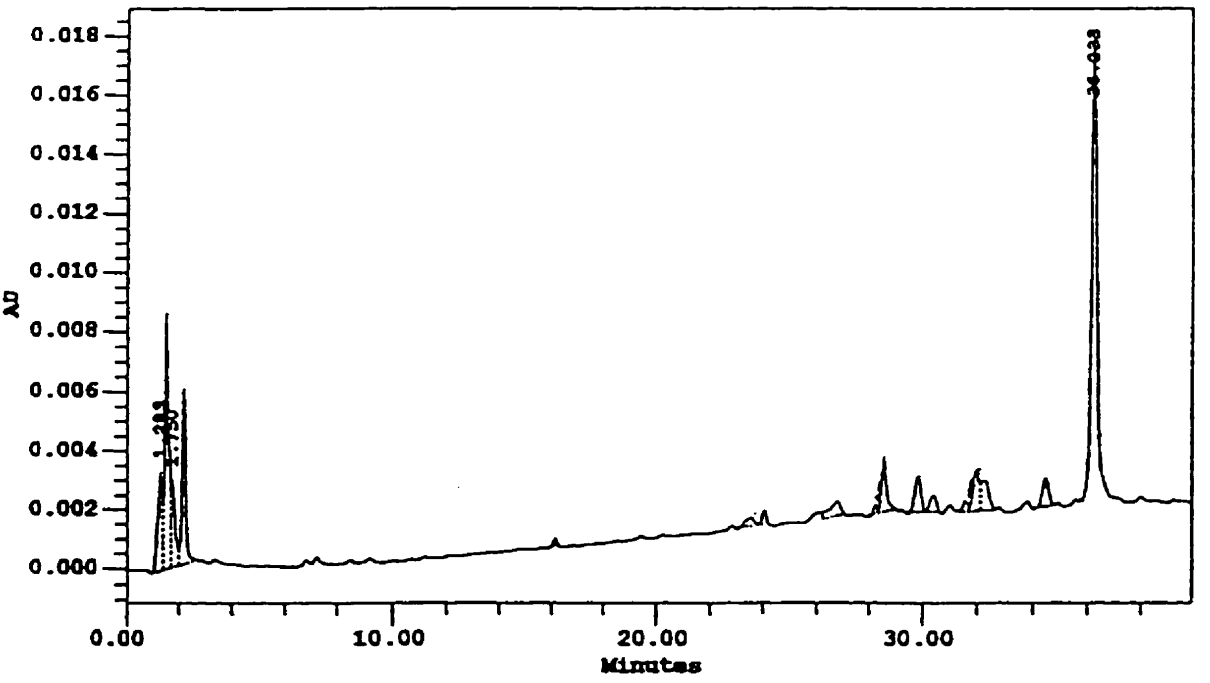
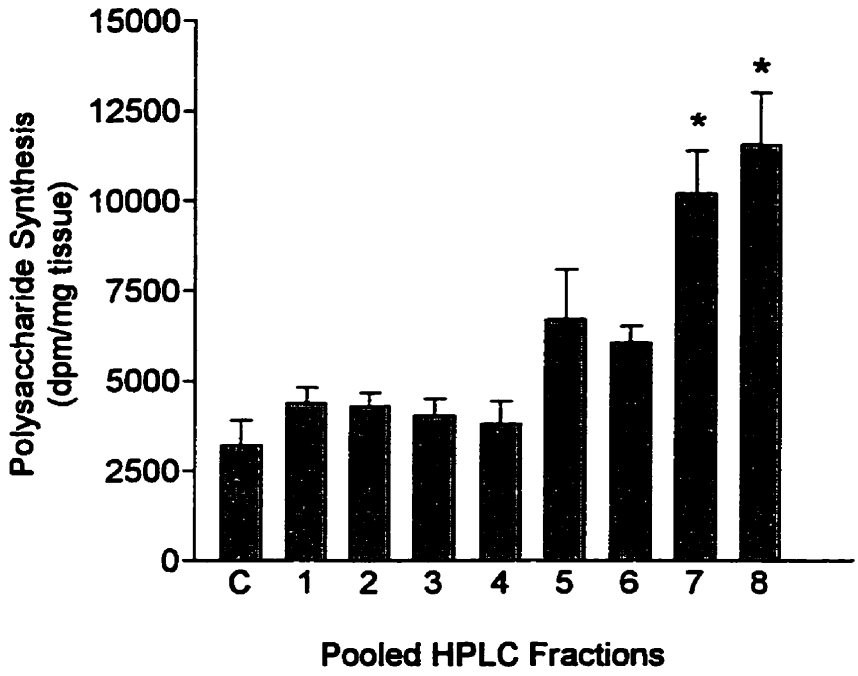

0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
AU
1.203
1.750
34.033
0.00
10.00
20.00
30.00
Minutes

Fig. 10 Activity profile of the hydrophobic material from DB-conditioned medium separated by HPLC. The 100% methanol eluate from DB-conditioned medium was separated by HPLC as described above. Every 5 fractions were pooled, then evaporated to dryness. The fractions were tested for bioactivity at a dose of 4-5 ae. Bars represent the means ± SE of 4-6 samples. Sample 1 (fractions 1-5); sample 2 (fractions 6-10); sample 3 (fractions 11-15); sample 4 (fractions 16-20); sample 5 (fractions 21-25); sample 6 (fractions 26-30); sample 7 (fractions 31-35); sample 8 (fractions 36-40). Note that samples 7 and 8 significantly stimulated albumen gland polysaccharide synthesis (* Kruskal-Wallis $p<0.007$, Dunn Test).

Fig. 10


15000
12500
10000
7500
5000
2500
0
Polysaccharide Synthesis
(dpm/mg tissue)
C
1
2
3
4
5
6
7
8
*
*
Pooled HPLC Fractions

Chapter IV: Identification of Albumen Gland Proteins and the Effect of Brain Extracts on Their Release

SUMMARY

A 66 kDa glycoprotein was identified in crude albumen gland extracts from the pulmonate snail *Helisoma duryi*. This glycoprotein is the major protein constituent of the albumen gland secretory cells and is also secreted by glands maintained *in vitro*. The albumen gland glycoprotein appeared to be metabolized by developing embryos and likely serves an important role in providing nutrition and/or protection to the embryos. Protein release from the albumen gland can be enhanced by addition of a brain extract. The albumen glands dissected from mated snails were more sensitive to brain extract than those of virgin animals. There appears to be two factors from the brain that stimulate the secretion of albumen. One factor has been partially characterized as a basic peptide having a molecular weight less than 10 kDa, and the other is the biogenic amine dopamine.

INTRODUCTION

The production, transport, and packaging of eggs is a complex process in gastropod molluscs and is known to involve the endocrine system (Geraerts and Joosse, 1984; Saleuddin *et al.*, 1990). Oocyte maturation, as well as the differentiation and synthetic activity of the female accessory sex organs is governed by the endocrine dorsal bodies (DBs) in pulmonate snails (see Joosse, 1988; Saleuddin *et al.*, 1994). The release of mature oocytes (ovulation) from the ovotestis is stimulated by the peptidergic egg-laying hormone (ELH) in the opisthobranch *Aplysia californica* and by the caudodorsal cell hormone (CDCH) in the pulmonate *Lymnaea stagnalis* (Geraerts *et al.*, 1988). In freshwater pulmonates, the ovulated oocytes travel along the hermaphroditic duct and are fertilized near the carrefour, a glandular outpocketing of the hermaphroditic duct. As the eggs move along the female tract, they receive secretions from various female accessory sex organs and are assembled as an egg mass to be oviposited.

Oocytes in the Basommatophora are quite small (~100 μM in diameter) and contain little yolk protein (deJong-Brink *et al.*, 1983; Geraerts and Joosse, 1984). The embryos receive most of their nutrition from the perivitelline fluid (PVF) which surrounds the individual eggs. The PVF is composed mainly of the polysaccharide galactogen and various proteins, which are synthesized and secreted by the albumen gland, a compound tubular exocrine female accessory sex gland (Okatore *et al.*,1982; Wijsman and van Wijck-Batenburg, 1987). The secretion of PVF into the carrefour must be synchronized with the arrival of eggs, and thus, the precise control of secretion is necessary. Ultrastructural studies indicate

that a neuronal plexus is present in the albumen gland (Nieland and Goudsmit, 1969; deJong-Brink and Goldschmeding, 1983), and it has been suggested that a nervous mechanism is responsible for controlling the release of PVF (deJong-Brink *et al.*, 1982). In the freshwater snail *Helisoma duryi*, significant *in vitro* release of radiolabelled polysaccharides was detected from albumen glands following long term incubation with the central nervous system (CNS) or dorsal body (DB) extracts (Miksys and Saleuddin, 1985; this thesis), suggesting a factor from the brain or DBs promotes the release of the newly synthesized polysaccharides into the surrounding medium.

Current bioassays that monitor the *in vitro* synthetic activity of the albumen gland are time-consuming (only 1-2 assays can be performed per week), expensive (costly isotopes, scintillation equipment, and culture supplies), and are subject to considerable variability, depending on the physiological status of the albumen gland. A unique 66 kDa glycoprotein from the albumen gland of *H. duryi* has been recently identified, and a sensitive, rapid bioassay for release of albumen gland proteins and polysaccharides developed (Morishita *et al.*, 1998). This bioassay has been modified and used to determine the effects and distribution of activity from various regions of the CNS of *H. duryi* on albumen gland protein secretion. A partial characterization of a stimulatory factor from the CNS was achieved. Several neuroactive agents known to be present in pulmonate nervous tissue were also tested for potential secretion-promoting activity.

MATERIALS AND METHODS

Animals

Laboratory stocks of reproducing *H. duryi* were reared in 4-liter plastic containers (15-20 snails/container) in dechlorinated tap water containing 0.025% artificial sea water (Instant Ocean, Ohio) and maintained under 16L:8D photoperiod. The snails were fed a diet of boiled lettuce and fish pellets every 2-3 days and the water was changed at least once a week. Adult animals (10-12 mm shell diameter) were taken from the general population and individually placed in plastic cups 2-3 weeks prior to experimentation to monitor egg-laying rates of the isolated snails. Albumen glands were dissected from those snails that had not laid egg masses within the last 24 hours.

Bioassay

Albumen glands were dissected free from surrounding tissue and cut into halves, then washed in several changes in *Helisoma* saline (51.3 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.0 mM Hepes, pH 7.3, 120 mOsm/kg H_2O). Individual tissue pieces were placed in each well of a 96-well culture plate (Becton-Dickinson and Co., Lincoln Park, NJ) containing 100 µl saline. All the external saline was removed and replaced with 100 µl of fresh saline every 20 min. The test compounds were dissolved in saline to their final concentrations, then applied to the glands at 60 min. The test extracts were removed at 80 min, and the release of protein was monitored for another 60 min. The collected saline was centrifuged at 2,000 x g for 1 min to remove any debris, and 80 µl of the supernatant was added directly into a disposable 1.5 ml polystyrene cuvette containing 420 µl Triton X-100

(0.012% v/v). Protein content of the released material was determined by adding 125 µl of commercial Bio-Rad dye reagent concentrate (Bio-Rad Life Sciences , Mississauga, Ont.) and measuring the OD_{590} after 10-20 min. Bovine serum albumen was used as the standard. At the end of the experiment, the albumen gland wet weight was recorded and the data normalized with this value.

Electrophoresis

Albumen glands were dissected out in snail saline and washed in several changes of saline to remove cellular debris and hemolymph. The glands were homogenized in ice-cold 50 mM Tris-HCl (pH 7.2, containing 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF), then centrifuged at 20,000 x g for 20 min. The pellet was discarded and the extract was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoretic separation was carried out on 9% slab gels using the Bio-Rad mini-gel apparatus (Bio-Rad Life Sciences, Inc., Mississauga, Ont.) according to Laemmli (1970). Following electrophoresis, the gels were stained overnight with 0.2% Coomassie Brilliant Blue R-250 (CBB) in 50% methanol, 10% acetic acid. Excess stain was removed with 50% methanol, 10% acetic acid and the gels stored in distilled water so that they could be photographed or dried onto filter paper for further analysis. For the demonstration of carbohydrate moieties on proteins, the gels were stained using the Periodic Acid-Schiff (PAS) technique according to Van-Seuningen and Davril (1992). Protein released from glands stimulated with brain extract was collected, concentrated with a Speed-Vac (Savant, France), and mixed with SDS-PAGE sample buffer, then separated as described above.

Preparation of CNS Extracts

Buccal ganglia (BG), cerebral ganglia (CG), subesophageal ganglia (SG) or dorsal bodies (DBs) from *H. duryi* were dissected out under ice-cold saline and immediately frozen on an aluminum block that had been previously cooled with dry ice. The frozen tissues were pooled and stored at -80^{0}C until required for extraction. Extraction of stimulatory material from the nervous tissue was performed by boiling the tissue in 0.1 M acetic acid for 3 min to destroy endogenous proteases. The preparation was rapidly cooled, then homogenized with a motor-driven pestle in a 1.5 ml polypropylene microtube (Kontes, NJ) held on ice. The homogenate was centrifuged at 20,000 x g (5 min), and the pellet was reextracted in a small volume of acetic acid and centrifuged once again. The supernatants were pooled and two volumes of acetone (0^{0}C) were added (final concentration 66% v/v) to the acid extract and held at approximately 0^{0}C for 20 min. This concentration of acetone is known to precipitate most proteins above 10 kDa in an aqueous solution (Scopes, 1988). The precipitated protein was pelleted at 20,000 x g (20 min), and the supernatant was evaporated to dryness. For the bioassay, the dried brain extract was resuspended just prior to use in 5 µl of 0.01 M acetic acid and an appropriate volume of saline. In some cases, the DBs were homogenized in methanol, centrifuged at 20,000 x g, and the supernatant evaporated to dryness. Dorsal bodies were also maintained in Medium 199 (pH 7.3, 140 mOsm) for 48 h to allow for secretory material to be released and accumulate in the culture medium. After the incubation period, the DBs were removed and the culture medium concentrated with a Speed-Vac. The concentrate was loaded onto a preconditioned Sep-Pak C_8 solid-phase extraction cartridge.

Salts and unretained material were eluted with water and the adsorbed material was eluted with 100% methanol. The methanol eluate was dried, resuspended in saline, and then tested for bioactivity.

Protease Treatment of CNS Extracts

Dried brain extracts were resuspended in saline to a concentration of 1 brain equivalent (be) and incubated with protease-beaded agarose (1 mg/ml of Pronase F, Sigma Chemical Co., St. Louis, Mo.) in 1.5 ml microtubes at room temperature for 4-5 hours on a Vibrax shaker (Janke and Kunkel, Germany). After the incubation period, proteolytic enzymes were pelleted by centrifugation at 10,000 x g (5 min), and the saline extracts tested for activity. Control CNS extracts were not subjected to protease treatment or were incubated with heat-inactivated enzyme, then tested for activity.

Membrane Ultrafiltration of CNS Extracts

For determination of the molecular weight of the stimulatory factor from the CNS extract, a molecular weight cut-off membrane filter of 3 kDa was used (Microcon MWCO-3, Amicon, Mississauga, Ont.). Brain tissue was boiled in acid and large proteins were acetone-precipitated as described earlier. The extract was partially reduced in volume, then loaded into the ultrafiltration unit and centrifuged at 12,000 x g until 10-20 µl of retentate remained. The filtrate and the retentate were evaporated to dryness and resuspended in saline prior to bioassay.

Sep-Pak C_8 Separation of CNS Extract

The brain extracts after acetone precipitation were reduced in volume to 100-200 µl

and loaded onto a Sep-Pak C_8 solid phase extraction cartridge that had been previously primed with acetonitrile (ACN), then washed with 0.1 M acetic acid. The acid extract was passed through the cartridge and then washed with 0.1% trifluoroacetic acid (TFA). Substances not adsorbed to the cartridge were eluted with 0.1% TFA which was designated as the flow-through (FT). The retained material was eluted with two washes of 60% ACN/0.1%TFA (2 ml), then with two washes of 100% ACN/0.1% TFA (2 ml). The three separate eluates were dried and tested for activity as described above.

Ion Exchange Separation of CNS Extracts

Dried brain extracts after acetone precipitation were resuspended in 3 ml of 10 mM ammonium acetate (pH 5.0) containing 20% ACN. The extract was passed through two different ion exchange cartridges connected in series, a Sep-Pak CM cartridge and a Sep-Pak QMA cartridge (Bennett, 1986). The CM unit is a strong cation exchanger and will bind basic peptides, whereas the QMA unit is an anion exchanger and will bind acidic peptides. Neutral peptides will not bind to either cartridge and are washed through with an additional 5 ml of buffer (neutral pool). The cartridges were then disconnected and each washed with buffer containing 1 M NaCl. The CM wash was designated the basic pool, and the QMA wash was designated the acidic pool. The high salt eluates were reduced to approximately half their volume and loaded onto a Sep-Pak C_8 cartridge. The salts and unretained material were washed away with 0.1% TFA and the retained material eluted with 60% ACN/0.1% TFA. The fractions were tested at a dose of 2.0 and 4.0 be.

Statistical analysis of the data were performed with the paired t-test or the Wilcoxin paired sample test ($p<0.05$).

RESULTS

SDS-PAGE Analysis of Albumen Gland Proteins

When a crude extract of albumen gland was separated by SDS-PAGE, a protein band of approximately 66 kDa was stained intensely (Fig. 1a, lane 2). This protein appeared to be the most abundant protein in soluble albumen gland extracts and is likely a glycoprotein as it also stained positively with the PAS technique (Fig. 1a, lane 4). When freshly dissected albumen glands were maintained in saline, they released a number of proteins into the surrounding medium (Fig. 1a, lane 3). A similar protein profile was detected whether an intact gland or pieces of the gland are used (Morishita *et al.*, 1998). The most abundant protein in the releasate was the 66 kDa glycoprotein. Proteins extracted from freshly oviposited egg masses were electrophoretically separated and the major constituent protein was again the 66 kDa glycoprotein (Fig. 1b, lane 2, 3). The embryonic development of *Helisoma trivolvis* requires about 12 days at 26.5 °C from the time of oviposition to the emergence of the juvenile snails, and is synchronous among eggs within individual egg masses (Goldberg and Kater, 1989). Approximately mid-way through embryonic development (~6-7 days), the staining of the 66 kDa protein was noticeably reduced (Fig. 1b, lane 4), and just prior to hatching, the 66 kDa protein was barely detectable (Fig. 1b, lane 5).

To determine if an endogenous factor from the brain is capable of evoking protein

release from the albumen gland, an acid extract prepared from *H. duryi* CNS was applied to the gland. The basal release of the 66 kDa glycoprotein from the albumen gland is shown during the first 60 min in Fig. 2. The application of one brain equivalent (1 be) at 60 min markedly increased the release of this protein over the next 20 min. Following the removal of the extract at 80 min, protein release returned to basal levels. Although other minor proteins were released by the albumen gland upon stimulation, the 66 kDa glycoprotein was clearly the most abundant.

Since the assay of protein release by SDS-PAGE is unsuitable for the analysis of a large number of samples, a more rapid and convenient method was required to measure *in vitro* protein release by the albumen gland before and after application of test material. This involved the use of a commercial protein dye reagent from Bio-Rad based on the method of Bradford (1976). The method was slightly modified by adding the protein sample (80 µl) directly to a cuvette containing 420 µl Triton X-100 (0.012%), then adding 125 µl dye reagent concentrate and mixing thoroughly with the pipettor. The inclusion of low concentrations of Triton X-100 has been shown to increase the sensitivity and linearity of the original Bradford assay (Friedenauer and Berlet, 1989; Loffler and Kunze, 1989). Therefore, this protocol increases the sensitivity of the assay while minimizing the use of supplies as well as saving time.

Effect of CNS Extract on Protein Secretion

When freshly dissected albumen glands are maintained in saline, they will release protein from their ducts into the surrounding medium. The time course of a typical rate of

in vitro protein release from albumen gland explants is shown in Fig. 3. Protein release was initially around 1.4 µg protein/mg tissue/20 min, and gradually decreased over the next 60-80 minutes until protein levels were nearly undetectable using the current protein detection system. A time of 60 min was chosen as the point at which the test extracts were applied.

The application of 1 be evoked significant release of protein from albumen glands *in vitro* (Fig. 4). The release of protein persisted at approximately the same rate as long as the stimulus (brain extract) was present in the surrounding medium (60 min). After removal of the stimulus, the rate of protein release declined rapidly until it attained levels comparable to that seen before brain extract application. Addition of a single dose of brain extract for 20 min evoked release of protein that was similar in terms of magnitude compared to glands that had been exposed to stimulus for longer than 20 min (data not shown), thus all subsequent experiments used only a single dose of test material. The basal level of protein release 20 min prior to the addition of test extract was compared to the level of protein release 20 min after the addition of test extract.

The dose-response curve after brain extract application revealed the threshold for stimulation of protein release from the albumen gland appeared to occur at approximately 0.25 be and was maximal at about 1.0 be (Fig. 5). The protein content of the brain extract was negligible and did not interfere with total protein measurements from albumen gland releasates.

When a brain extract was applied to virgin albumen glands, there was no significant increase in protein secretion compared to basal levels (Fig. 6). The same extract when applied

to albumen glands from mated snails was capable of inducing significant protein release. The location of protein secretion stimulatory factor in the CNS was assessed by surgically separating the CG, SG, BG, and DBs, and processing the tissues as described above. Significant stimulatory acitivty was contained within the CG and SG (Fig. 7). Both ganglia seemed to be equally effective in stimulating protein release from the albumen gland. The BG and DBs did not contain any acid-extractable stimulatory activity. Likewise, a methanol extract of the DBs or application of DB-conditioned medium did not evoke protein release from the albumen gland (Fig. 7).

Partial Characterization of the Stimulatory Factor From the CNS

Since the stimulatory factor from the CNS retained biological actvity after being extracted in boiling 0.1 M acetic acid and subjected to acetone treatment, it suggested that the stimulatory factor is relatively heat-stable and probably not a large protein. A further pharmacological characterization of the stimulatory factor from *H. duryi* CNS was performed by treating extracts with proteolytic enzyme. Incubation of brain extract with protease abolished its stimulatory effect on the albumen gland, indicating the brain factor is likely a peptide (Fig. 8).

The acetone treatment of the acid extract indicated that the brain factor is probably a peptide less than 10 kDa. The use of a disposable ultrafiltration devices with a specified molecular weight cut-off membrane (MWCO 3 kDa) provides a method to separate the stimulatory activity originating from the brain based upon relative molecular weight. This experiment showed that the stimulatory peptide from the CNS is present in both the filtrate

(molecules less than 3 kDa), and the retentate (molecules above 3 kDa). The filtrate displays slightly higher activity but is not significantly different compared to the activity of the retentate (Fig. 9). The ultrafiltration procedure was unable to clearly define a molecular weight region for the brain peptide since molecules greater than 3 kDa can sometimes pass though the membrane and conversely, molecules less than 3 kDa can sometimes be retained.

The crude CNS extract was also fractionated on a Sep-Pak C_8 cartridge to in an attempt to partially purify the CNS factor for future peptide purification studies using HPLC. The acid extract was loaded into a Sep-Pak C_8 cartridge, then eluted with 0.1% TFA (flow-through), 60% ACN/0.1% TFA and finally with 100% ACN/0.1% TFA. The only fraction to possess stimulatory activity on the albumen gland was the 60% ACN/0.1% TFA eluate (Fig. 10). Significant stimulation of protein release was elicited by a dose of 1.0 be.

The Sep-Pak solid phase extraction cartridges were also used to determine the relative ionic nature of the peptide of interest. The eluate from the Sep-Pak CM cartridge (basic pool), the Sep-Pak QMA cartridge (acidic pool), and the flow-through (neutral pool) were assayed at 2.0 and 4.0 be for their ability to induce protein release from the albumen gland (Fig. 11). Neither the acidic nor the neutral peptide pool contained stimulatory activity. In contrast, the basic pool was capable of stimulating significant protein release from the albumen gland *in vitro* at concentrations of 2.0 be and 4.0 be (Fig. 10).

Effect of Some Neuropeptides and Biogenic Amines on Albumen Gland Protein Secretion

Electron microscopic studies have indicated that the albumen gland of *H.duryi* is innervated by peptidergic and aminergic varicosities (A.S.M. Saleuddin, unpublished

observations). Therefore, some neuropeptides and biogenic amines known to be involved in pulmonate reproductive functions were tested for bioactivity. The neuropeptides caudodorsal cell hormone (CDCH) and calfluxin from *L. stagnalis*, the molluscan neuropeptide FMRFamide, and the biogenic amines serotonin, and dopamine were tested at 0.1 μM and 1.0 μM for their ability to stimulate protein secretion from albumen glands (Table 1). The CDCH, calfluxin, FMRFamide, and serotonin were all ineffective, whereas dopamine at a concentration of 1 μM evoked a significant release of protein (1.7-fold increase). At a higher concentration (10.0 μM), dopamine stimulated protein release approximately 4-fold over control levels. No effect on protein secretion was seen at a dose of 0.1 μM dopamine.

DISCUSSION

The albumen gland of freshwater pulmonate snails serves an important role in the reproductive physiology of these animals. The secreted PVF coats individual eggs as they pass through the carrefour, the site at which the albumen gland releases its products (deJong-Brink, 1969). The coated eggs then pass along the female portion of the reproductive tract to receive further secretions from other female accessory sex glands. The eggs are packaged together forming a circular egg mass, then oviposited upon a suitable substrate.

In addition to the polysaccharide galactogen, a quantitatively important constituent of the PVF is protein. In *L. stagnalis*, protein accounts for nearly 50% of the dry weight of the freshly laid eggs (Wijsman and van Wijck-Batenburg, 1987). A crude soluble extract from *H. duryi* albumen gland reveals the gland is rich in proteinaceous material. The major

component of the soluble extract is a protein of approximately 66 kDa as determined by SDS-PAGE. This protein appears to be glycoslylated as it also stains positvely with the PAS reaction. When freshly dissected albumen glands are placed *in vitro* they secrete a number of different proteins, the major constituent of the secreted material being the 66 kDa glycoprotein. This protein is also the major protein present in extracts of freshly laid egg masses. During embryogenesis, this glycoprotein is slowly metabolized, and prior to hatching, it is noticeably reduced in quantity. This consumption of the albumen gland glycoprotein by developing embryos of *H. duryi* parallels the enzymatic breakdown of the polysaccharide galactogen by embryos of *L. stagnalis* (Goudsmit, 1976). The precise function of the 66 kDa glycoprotein is unknown, but it likely serves as a nutritive source for the embryos along with galactogen.

In the sea hares *Aplysia kurodai* and *Dolabella auricularia*, antibacterial proteins have been isolated and characterized from egg mass and albumen gland extracts (Kamiya *et al.*, 1984; Kisugi *et al.*, 1989). These glycoproteins in opisthobranch molluscs are similar in native molecular weight and subunit composition to the 66 kDa glycoprotein from *H. duryi* albumen gland (Morishita *et al.*, 1998). Since many basommatophoran planorbid snails dwell in shallow muddy ponds and slow-flowing streams, the albumen gland glycoprotein may also function to protect the egg mass from bacterial infection in stagnant waters. In another planorbid snail, *Biomphalaria glabrata*, the synthesis of an albumen gland protein having a similar subunit composition to the *H. duryi* glycoprotein was significantly reduced in schistosome-infected (non-reproducing) snails as compared to uninfected (reproducing) snails

(Crews and Yoshino, 1991). This lends further support to the importance of this protein in reproduction.

The secretion of PVF around the eggs appears to be a regulated process as each oocyte must receive an equal-sized drop of PVF around it as it passes through the carrefour (deJong-Brink, 1969). Since the albumen gland and the reproductive tract receive innervation from the CNS, it has been suggested that secretion of PVF is controlled by a nervous or neurohormonal mechanism (deJong-Brink and Goldschmeding, 1983). An acidic extract from *H. duryi* CNS stimulates the *in vitro* secretion of proteins from the albumen gland. Electrophoretic analysis of the proteins released before and after stimulation demonstrates the major component of the secreted proteins is the 66 kDa glycoprotein. If the stimulus (CNS extract) is present for a prolonged time, the magnitude of the response is not enhanced, but protein release remains elevated until the stimulus is removed. A dose of 1 be is the most effective concentration in stimulating protein secretion from the albumen gland.

In virgin *Helisoma duryi*, the secretory cells of the albumen gland contain an abundance of secretory material (Miksys and Saleuddin, 1985), and at least morphologically, they appear to be capable of releasing their contents given an appropriate stimulus. However, treatment of virgin glands with brain extract failed to evoke significant release of proteins, indicating the action of some other factor (DBH, matedness factor ?) may be required for the brain extract to exert its effect.

The general distribution of the stimulatory activity in the CNS was determined by testing extracts of CG, SG, BG and DBs on the albumen gland *in vitro*. The only nervous

tissues to possess stimulatory activity are the CG and SG. The exact cellular origin of the stimulatory factor within these ganglia is not known or whether the stimulatory factor from the CG is identical to that from the SG. The BG extract does not stimulate protein release from the albumen glands. The BG contains mainly small neuropeptides (FMRFamide-related peptides, myomodulins, small cardioactive peptides, buccalins) and biogenic amines (serotonin, dopamine, acetylcholine) which are involved in the muscular control of feeding in snails (Weiss *et al.*, 1992).

The DBs have been reported to stimulate the synthesis of polysaccharides in the albumen glands of freshwater pulmonates (Wijdenes *et al.*, 1983; Miksys and Saleuddin, 1985, 1988; this thesis). An acidic or methanol extract prepared from DB tissue is ineffective in stimulating protein release, suggesting that soluble material from DB tissue is ineffective in evoking the rapid release of proteins from the albumen gland. However, under long term incubation conditions (~48 h), significant release of newly synthesized polysaccharides was observed with DB extracts (this thesis). Miksys and Saleuddin (1988) also reported that polysaccharide synthesis-promoting activity from DB tissue was not stable under acidic conditions, however, biological activity could be extracted with methanol. Culture medium in which the DBs were previously maintained was capable of stimulating albumen gland polysaccharide synthesis and release under long term culture conditions (this thesis), but was not able to promote short term protein release. Taken together, these results indicate the DBH exerts its effect primarily by influencing the long term synthetic activity of the albumen gland, and has little direct effect on the short term release of its secretory products.

These findings are consistent with the mechanism by which the albumen gland is believed to operate. The DBs have been proposed to maintain a certain level of albumen gland synthetic activity in reproducing snails over a relatively long period, whereas a nervous or neurosecretory factor stimulates PVF release only when eggs are present in the carrefour. The mechanism by which the approaching eggs are detected in the carrefour and how this information is conveyed to elicit release of the PVF secretion-promoting peptide is not clear. In *Bulinus truncatus*, sensory recepto-secretory neurons are present in the carrefour region (Brisson and Collin, 1980), and could transmit information to albumen gland secretory cells which are innervated by both peptidergic and aminergic axons from the CNS (Nieland and Goudsmit, 1969; Brisson, 1983; A.S.M. Saleuddin, unpublished observations).

In order to determine the nature of the stimulatory factor from *H. duryi* CNS extracts, the nervous tissue was subjected to proteolytic digestion and then tested for activity. Treatment with protease abolished its stimulatory activity, implying that it is a proteinaceous substance. This result, coupled with the observation that the brain extract retained activity after boiling in acid and was not precipitated with acetone treatment, suggests that the CNS factor is probably a peptide less than 10 kDa. Separation of the brain peptide with Amicon ultrafiltration membranes (MWCO-3) results in activity being distributed in both the filtrate and retentate.

The purification and characterization of the stimulatory peptide from the CNS is an important step towards the understanding of its role in the reproduction of *H. duryi*. To this end, a partial purification was achieved using Sep-Pak reverse-phase and ion-exchange

cartridges. Brain extracts were fractionated using a Sep-Pak C_8 cartridge and eluted with consecutive washes of 0.1% TFA, 60% ACN/0.1% TFA, and 100%ACN/0.1% TFA. The only fraction possessing stimulatory activity was the 60% ACN/0.1% TFA eluate. This is consistent with the retention properties of most peptides from solid-phase extraction cartridges (Herraiz and Casal, 1995). The separation of the CNS extract by ion exchange cartridges showed only the basic pool was capable of stimulating protein release from the albumen gland, suggesting that the CNS peptide is rich in basic amino acid residues.

Wijdenes *et al.* (1983) have suggested that there are two gonadotropic hormones, the DBH and the CDCH that act on the albumen gland. Crude extracts of the DBs or the cerebral commissure (presumed to contain CDCH) from *L. stagnalis* stimulated *in vitro* polysaccharide synthesis. Since DBH has not been chemically isolated, the unequivocal effect of DBH on the biosynthetic activity of the albumen gland has not been demonstrated. Likewise, it has always been assumed that CDCH stimulates the synthetic activity of the albumen gland based on the activity of cerebral commissure extracts, the neurohemal area of the CDCs. Although the primary structure of CDCH has been known for some time (Ebberink *et al.*, 1985), synthetic CDCH has never been shown to stimulate albumen gland synthetic activity. Another neuropeptide named calfluxin has been reported to influence intracellular calcium levels in albumen gland secretory cells of *L. stagnalis* (Dictus and Ebberink, 1987b; Dictus *et al.*, 1988). Calfluxin is synthesized by the CDCs as part of the CDCH precursor protein, and is then processed, and transported to the cerebral commissure to be released (van Heumen *et al.*, 1992). Calfluxin was isolated from commissure extracts

by virtue of its ability to stimulate the influx of extracellular calcium into the mitochondria of the albumen gland. Although a physiological function as a result of calcium entry into albumen gland secretory cells has not been described, it was postulated that it may be important for either stimulating the synthesis and/or the release of PVF (Dictus *et al.*, 1988). Experiments to examine the role of calcium in protein secretion from *H. duryi* albumen glands using calcium-free medium, calcium channel blockers, ionophores, and intracellular calcium mediators are now in progress.

These data indicate that a peptide from the CNS stimulates protein release when applied to albumen glands *in vitro*. Several peptides and amines present in molluscan nervous tissue were tested as potential candidates to determine if they were capable of eliciting protein secretion. The two neuropeptides reported to stimulate the albumen gland of *L. stagnalis*, CDCH and calfluxin, were ineffective in stimulating protein secretion from the albumen gland of *H. duryi*. DeJong-Brink *et al.* (1982) reported that the albumen gland of *L. stagnalis* is innervated by fibers containing FMRFamide, a neuropeptide originally isolated as a cardioaccelerator from the clam *Macrocallista nimbosa* (Price and Greenberg, 1977). However, the application of FMRFamide to *H .duryi* albumen glands did not evoke protein release.

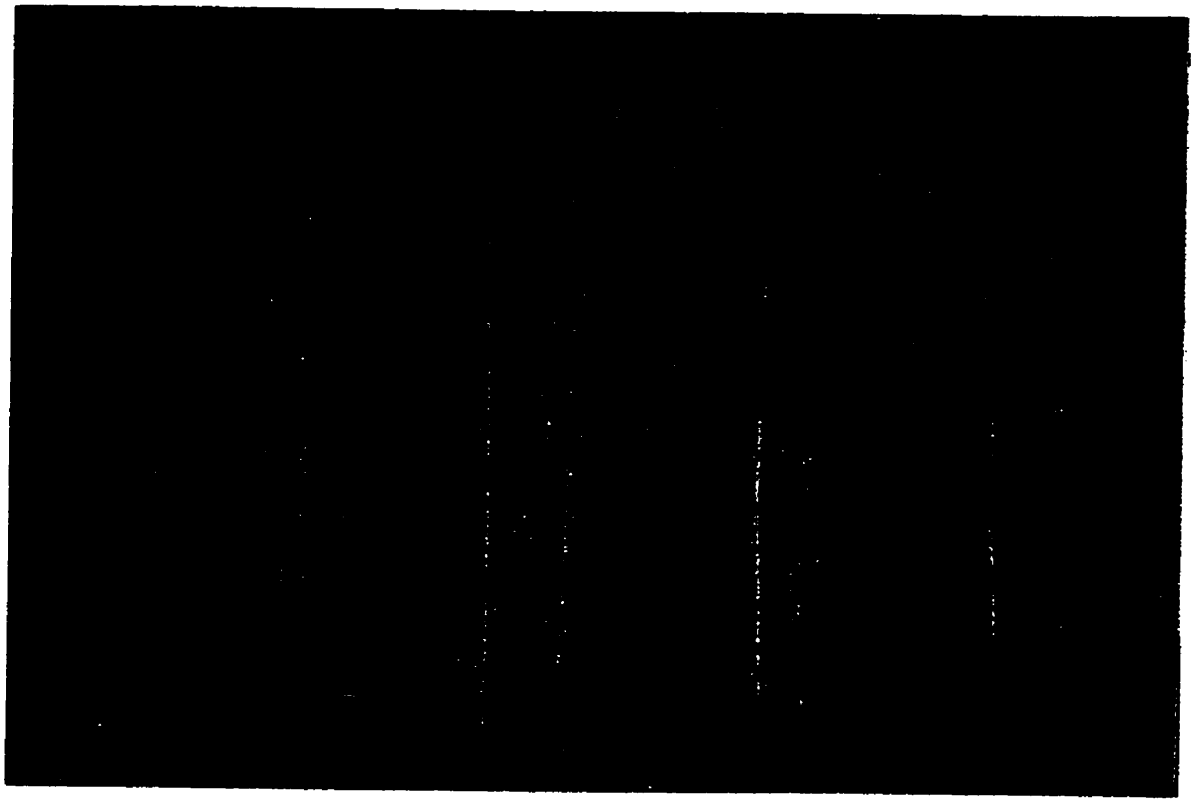
The biogenic amine serotonin or 5-hydroxytyptamine influences reproductive processes in a number of molluscs. In bivalves, serotonin induces oocyte maturation and stimulates spawning (Hirai *et al.*, 1988), whereas in pulmonates, serotonin is suggested to modulate egg production (Manger *et al.*, 1996) and sexual behavior (Adamo and Chase,

1991). Application of either 0.1 or 1.0 μM serotonin to *H.duryi* albumen glands did not stimulate protein secretion. The other neurotransmitter tested, 3-hydroxytyramine or dopamine, was capable of evoking protein secretion from the albumen gland of *H. duryi*. The response of the albumen gland to dopamine appears to be a concentration-dependent effect. A 1.7-fold increase in protein secretion occurred at a dose of 1.0 μM, and at 10.0 μM dopamine, a 4-fold increase was observed. In the related planorbid snail *B. truncatus*, the albumen gland and carrefour region, along with other areas of the reproductive tract, were found to be innervated by intrinsic and extrinsic catecholaminergic varicosities (Hartwig *et al.*, 1980; Brisson and Collin, 1980). These authors proposed that sensory inputs, perhaps from approaching eggs, are detected by the intrinsic system and convey information to the extrinsic system, which in turn, regulates the secretory or muscular activity of the reproductive tract. Since pulmonate nervous tissue is known to contain dopamine (Croll and Chiasson, 1990; Werkman *et al.*, 1990a), the treatment of the brain extract with protease should not have affected dopamine, and thus stimulation of protein secretion should have been observed. However, dopamine is easily oxidized and is light-sensitive, and perhaps the harsh extraction procedure prior to protease treatment might have oxidized the CNS dopamine, thereby rendering it inactive. It is also possible that dopamine may exert an indirect effect on the albumen gland by stimulating the release of a peptide factor from neurosecretory terminals contacting the albumen gland. The finding that dopamine was also capable of stimulating protein secretion adds another dimension of complexity regarding the mechanisms controlling the secretory activity of the pulmonate albumen gland. The results from this study contribute

important information about neurosecretory control of albumen gland protein secretion in pulmonate snails, and provides a foundation to begin isolation of the stimulatory peptide from the CNS of *H. duryi*.

Fig. 1a SDS-PAGE separation of soluble albumen gland extract of *H. duryi*. Crude protein extracts of albumen gland (10 µg per lane) were separated on a 9% T, 3% C gel, and stained with Coomassie Brilliant Blue R-250 (CBB). Lanes 2 and 4 represent crude albumen gland extracts stained with CBB and the PAS-reaction respectively. Lanes 3 and 5 represent secreted material from albumen glands maintained *in vitro*, then stained with CBB and PAS-reaction respectively. Note the presence of a prominent 66 kDa protein component in both tissue extracts and secreted material. This 66 kDa protein along with a high molecular weight protein stained positively with the PAS technique. The heavily-stained PAS-positive material at the top of the gel represents polysaccharide (galactogen) which does not enter the pores of the gel. Lane 1, molecular weight markers. DF-dye front.

Fig. 1b SDS-PAGE separation of egg mass proteins during development. Note the 66kDa protein was the most abundant component in the freshly laid egg mass (lane 2). Approximately mid-way through development (6-7 days post-oviposition), the relative amount of the 66 kDa protein appeared to decrease (lane 4), and just prior to hatching the 66 kDa protein was considerably reduced (lane 5). Each lane contained approximately equal amounts of total protein. Lane 1, molecular weight markers. DF-dye front.



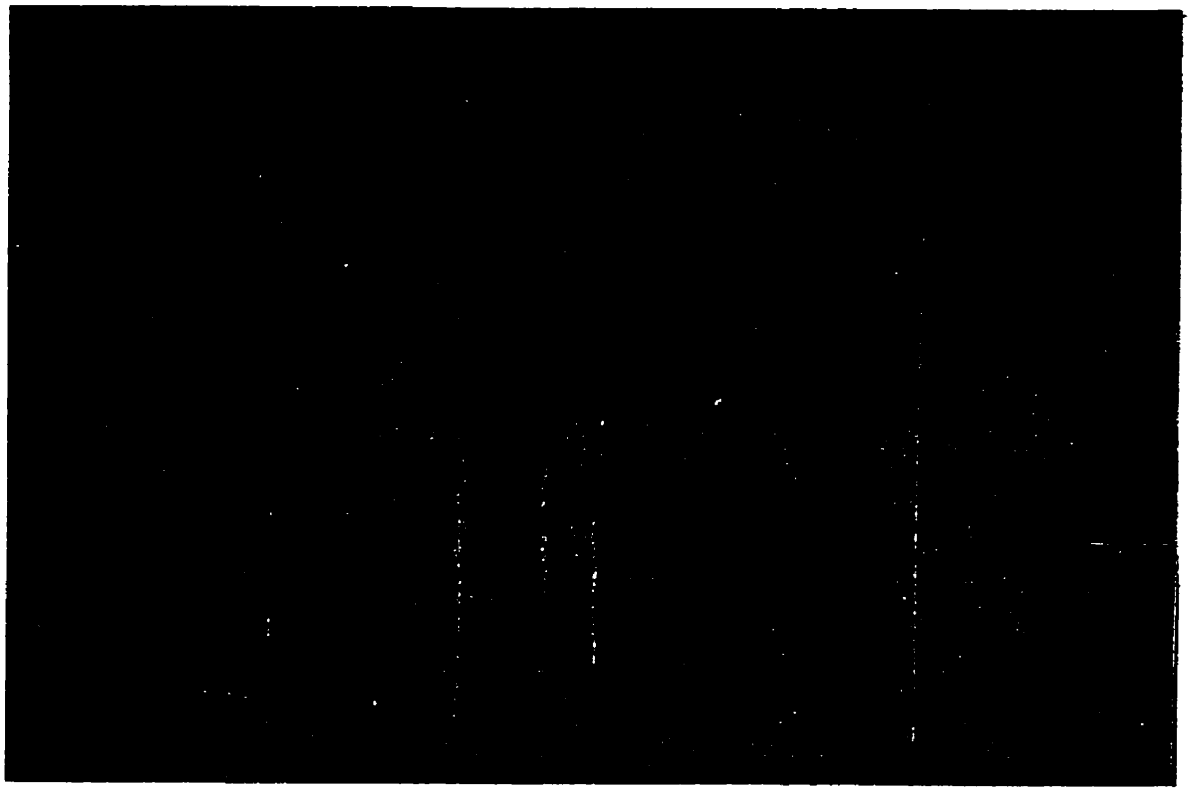


Fig. 2 Effect of a CNS extract on the *in vitro* release of albumen gland proteins. Basal protein secretion was monitored for 60 min using SDS-PAGE (lanes 1 to 3). One brain equivalent (1 be) was added at lane 4 for 20 min, then replaced with normal saline from lanes 5 to 8. Lane 9 represents the brain extract alone, and lane 10 represents the molecular weight markers. Note the marked increase in the release of albumen gland proteins in the presence of a brain extract, in particular, the prominent 66 kDa protein.

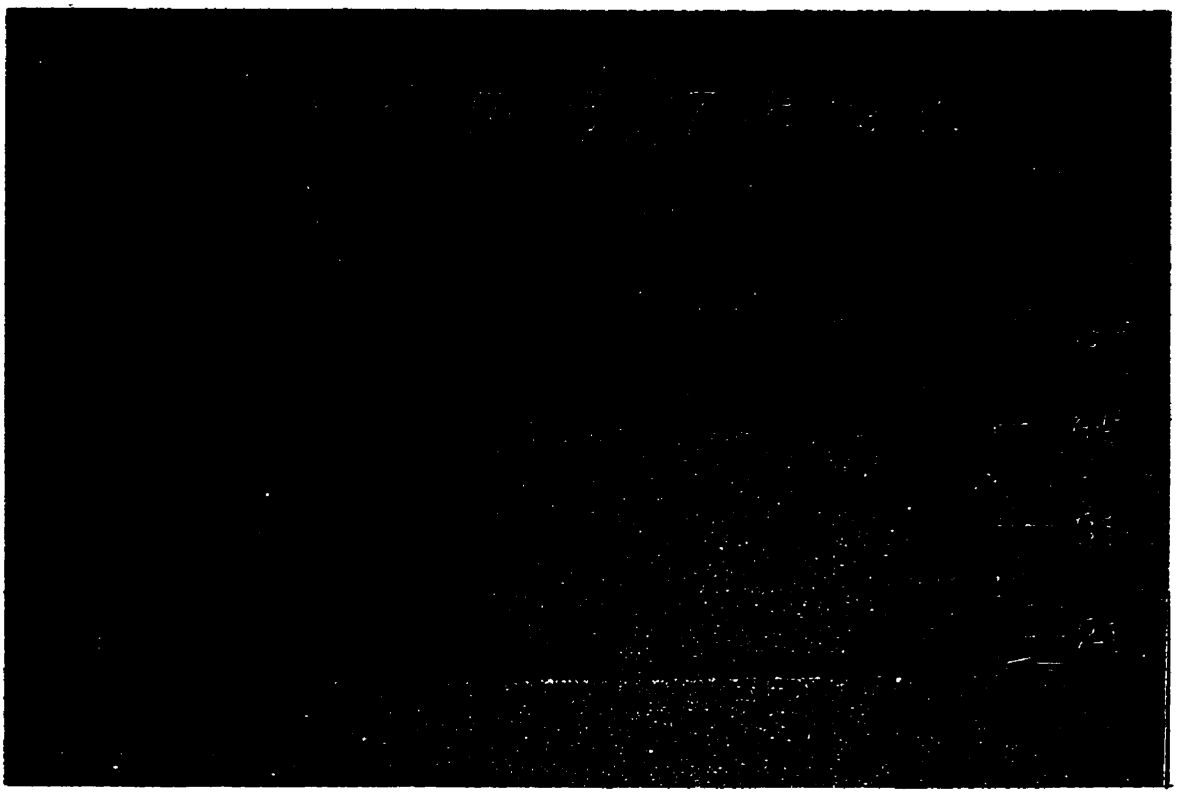


Fig. 3 Time course of *in vitro* protein secretion from *H. duryi* albumen gland explants. Individual tissue pieces were incubated in 100 µl saline in 96-well plates and the saline was replaced every 20 min with fresh saline. An 80 µl aliquot from each sample was removed for protein determinations and the wet weight of each tissue was recorded at the end of the experiment. Each point represents the mean ± SE of 6 samples.

Fig. 3

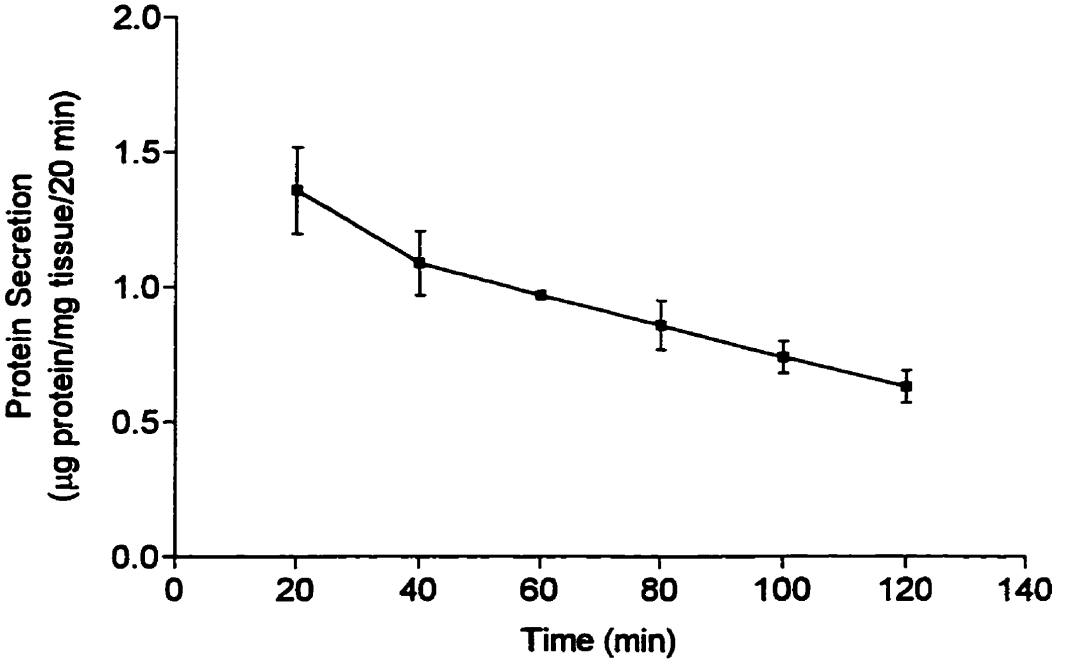

Protein Secretion
(µg protein/mg tissue/20 min)
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
140
Time (min)

Fig. 4 The effect of brain extract on *in vitro* protein secretion from the albumen gland. Basal protein secretion was measured for the first 60 min, then the gland was challenged with 1.0 be (as described in the methods) at 60 min. The extract was removed 60 min later and protein secretion was measured for another 60 min. An rapid increase in the release of albumen gland proteins was seen after brain extract was applied. In the presence of brain extract (indicated by the solid horizontal line), protein secretion was maintained significantly higher than basal levels. After removal of the stimulus, albumen gland protein secretion rapidly returned to basal levels. Each point represents the mean ± SE of 6 samples.

Fig. 4

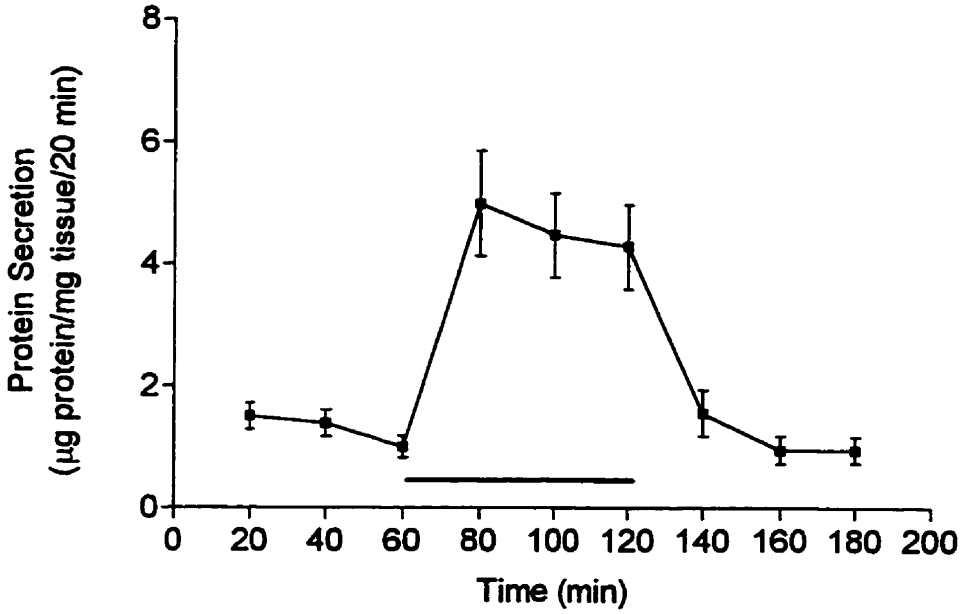

Protein Secretion
(µg protein/mg tissue/20 min)
8
6
4
2
0
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Fig. 5 Dose-response curve for the stimulation of protein secretion by brain extract. Brain extracts were tested at concentrations of 0.1, 0.25, 0.5, 1.0, and 2.0 brain equivalents (be). The basal protein levels 20 min before extract application were compared to the protein levels 20 min after extract application and quantitated as the change in protein secretion (Δ μg protein). Each point represents the mean ● SE of 5-8 samples.

Fig. 5

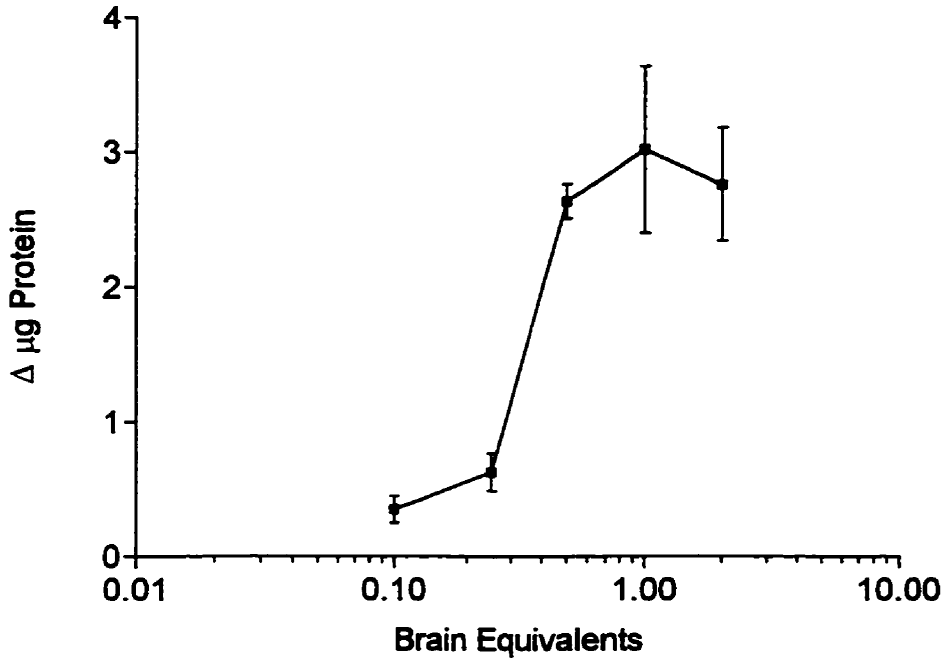


Fig. 6 The effect of brain extract from a mated snail on albumen gland protein secretion from virgin and mated animals. Brain extract (1.0 be) was applied to pieces of virgin or mated albumen glands (positive control) and the amount of proteins secreted into the surrounding medium in the presence of the stimulus was quantitated. Note that brain extract did not significantly stimulate the release of proteins from the virgin albumen gland (VIR), whereas in the glands from mated snails (MAT), it evoked significant release of secretory material (*Wilcoxin Test, p=0.0156)

Fig. 6

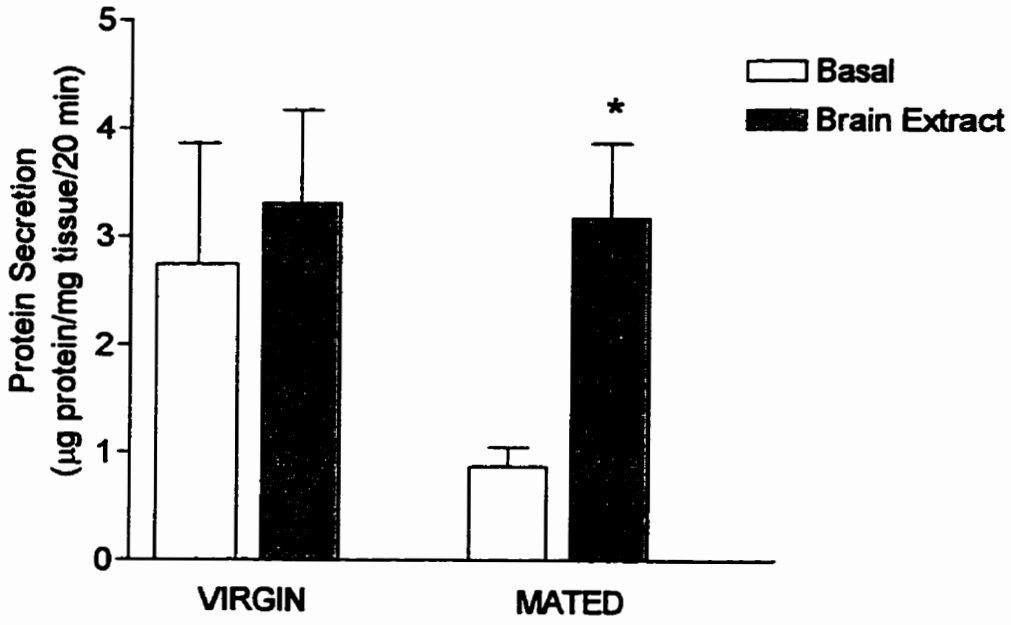

Protein Secretion
(µg protein/mg tissue/20 min)
5
4
3
2
1
0
*
Basal
Brain Extract
VIRGIN
MATED

Fig. 7 The activity of various portions of the CNS and DBs on albumen gland protein secretion. Buccal ganglia (BG), cerebral ganglia (CG), subesophageal ganglia (SG), or DBs were extracted in acid and tested for bioactivity as described in the Methods. In some experiments, the DBs were extracted in methanol (meth), or incubated in culture medium (med) for 48 h, then tested for bioactivity. The CG and SG are the only tissues that evoke protein secretion from the albumen gland. Bars represent the means ± SE of 6-10 samples. (*Wilcoxin Test, $p<0.008$ for CG and SG).

Fig. 7

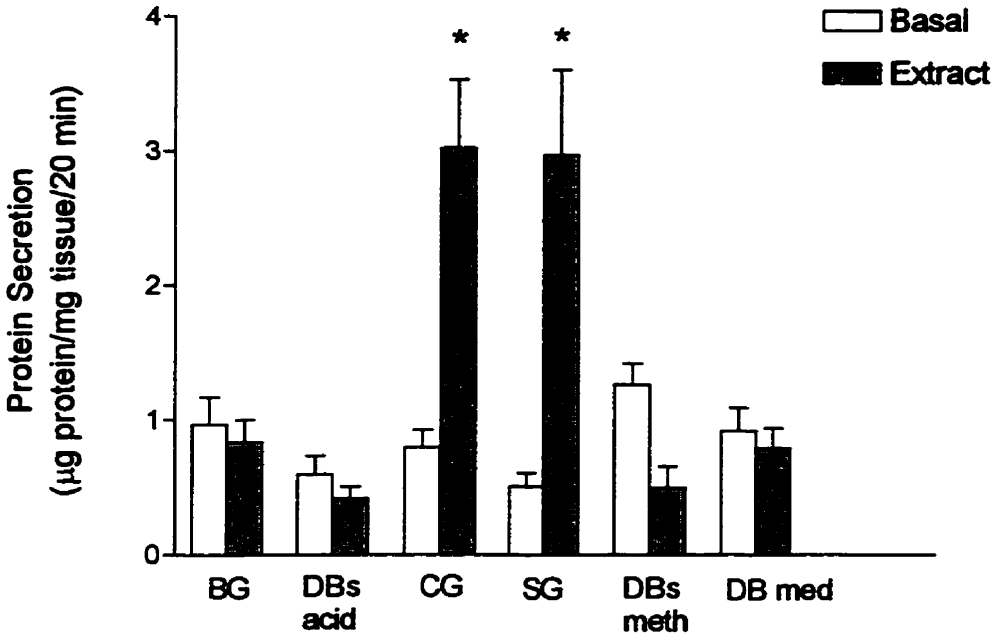

Protein Secretion
(µg protein/mg tissue/20 min)
4
3
2
1
0
*
*
Basal
Extract
BG
DBs acid
CG
SG
DBs meth
DB med

Fig. 8 The effect of protease on the activity of brain extracts. Brain extracts (1.0 be) were incubated in snail saline containing Pronase F-beaded agarose (1 mg/ml) for 4-5 h. Following protease treatment, the insoluble enzymes were pelleted by centrifugation and the supernatant tested directly for activity. Note the treatment with proteolytic enzyme (PR+BR) destroyed the stimulatory activity of the brain extract. Control extracts received no enzyme (BR) or previously heat-inactivated enzyme (BR+INAC). Bars represent the means ● SE of 13 samples for protease and control treatments, and 10 samples for the heat-inactivated enzyme controls (*Wilcoxin Test $p<0.002$ for BR and BR+INAC).

Fig. 8

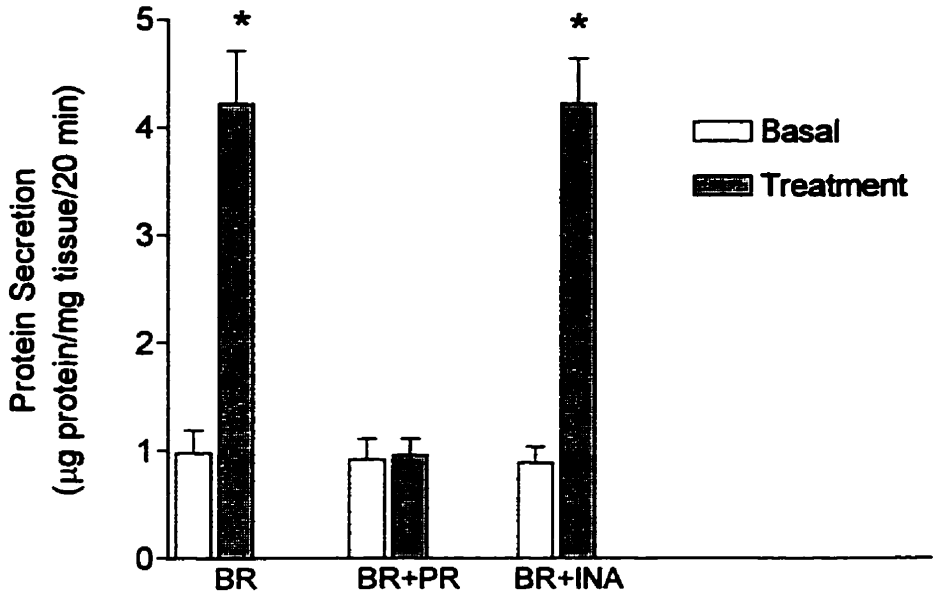

Protein Secretion
(µg protein/mg tissue/20 min)
5
4
3
2
1
0
*
*
Basal
Treatment
BR
BR+PR
BR+INA

Fig. 9 Ultrafiltration of *H. duryi* brain extract through an Amicon ultrafiltration membrane unit. Brain extracts were loaded into an Amicon MWCO-3 centrifugal unit and processed according to manufacturers instructions. Both the filtrate (molecules less than 3 kDa) and the retentate (molecules larger than 3 kDa) were tested at 2.0 brain equivalents. Note both the filtrate and retentate have strong stimulatory activity. Bars represent the means ± SE of 13 samples for the retentate and 8 samples for the filtrate (*Wilcoxin Test, $p<0.008$ for filtrate and retentate).

Fig. 9

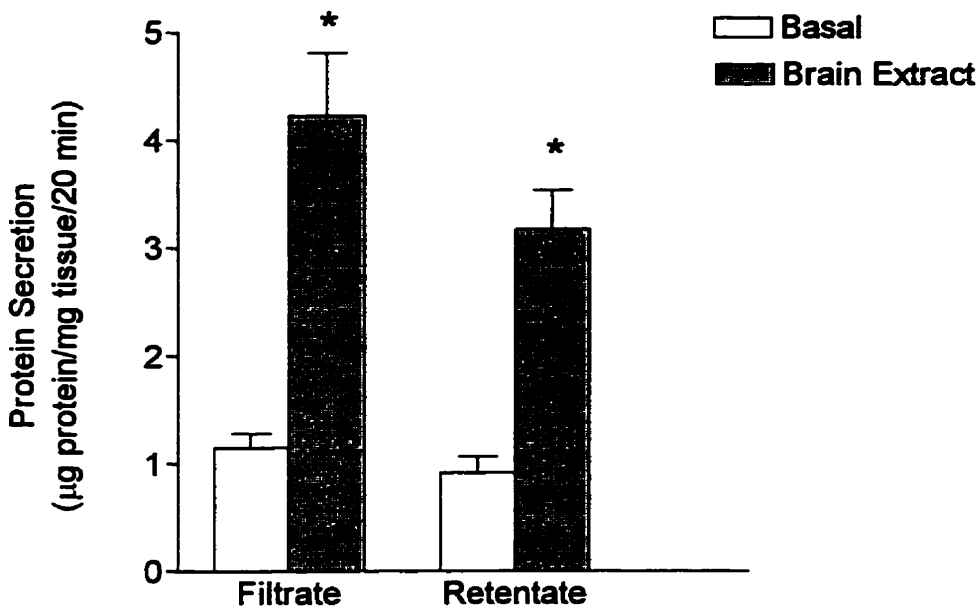

Basal
Brain Extract
Protein Secretion
(μg protein/mg tissue/20 min)
0
1
2
3
4
5
*
*
Filtrate
Retentate

Fig. 10 Elution of stimulatory activity of brain extracts from Sep-Pak C_8 cartridges. Brain extracts were loaded onto a preconditioned cartridge and unretained material or flow-through (FT) was washed with 0.1% TFA. Adsorbed compounds were eluted with 60% ACN/0.1% TFA, then with 100% ACN/0.1% TFA. The individual eluates were dried, resuspended in saline and tested for activity at 1.0 be. The 60% ACN/0.1% TFA eluate was the only fraction that stimulated protein secretion from the albumen gland. Bars represent the means ± SE of 6 samples (*Wilcoxin Test p=0.0313).

Fig. 10

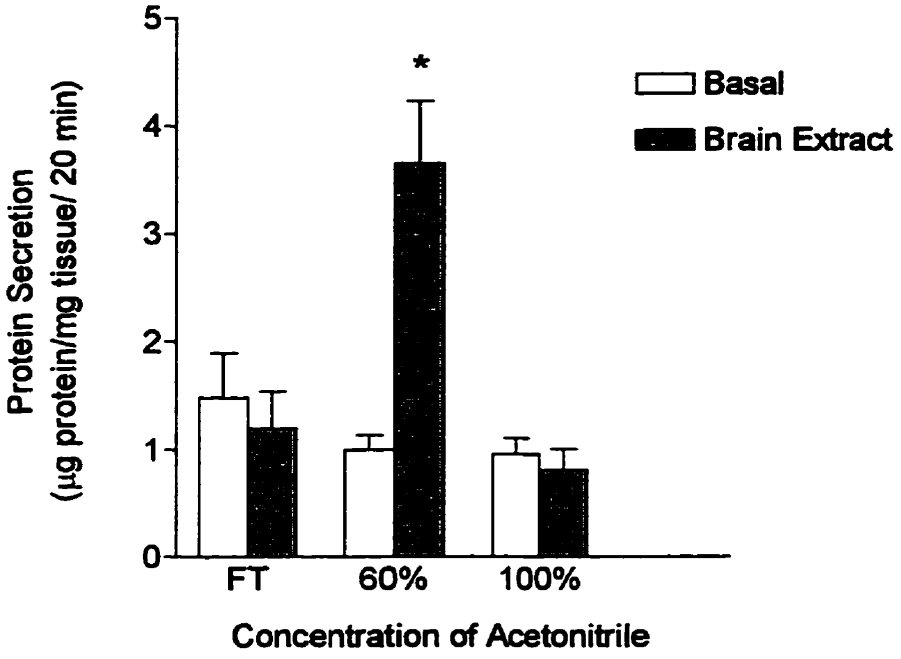

Protein Secretion
(µg protein/mg tissue/ 20 min)
5
4
3
2
1
0
*
Basal
Brain Extract
FT
60%
100%
Concentration of Acetonitrile

Fig. 11 Ionic nature of the albumen secretion-promoting peptide from the brain of *H. duryi*. Brain extracts were passed through a Sep-Pak CM cartridge and a Sep-Pak QMA cartridge connected in series and washed with 50 mM sodium acetate buffer containing 20% acetonitrile. The material that passed through both cartridges was termed the neutral pool. The cartridges were then disconnected and each eluted with buffer containing 1 M NaCl. The CM eluate was designated as the basic pool whereas the QMA eluate was called the acidic pool. Samples were desalted with a Sep-Pak C_8 cartridge as previously described and tested at 2.0 and 4.0 brain equivalents (be). Note the basic pool (CM eluate) was the only fraction to have stimulatory activity. Bars represent the means ± SE of 4-6 samples (*Wilcoxin Test, $p<0.04$ for 2.0 and 4.0 be).

Fig. 11

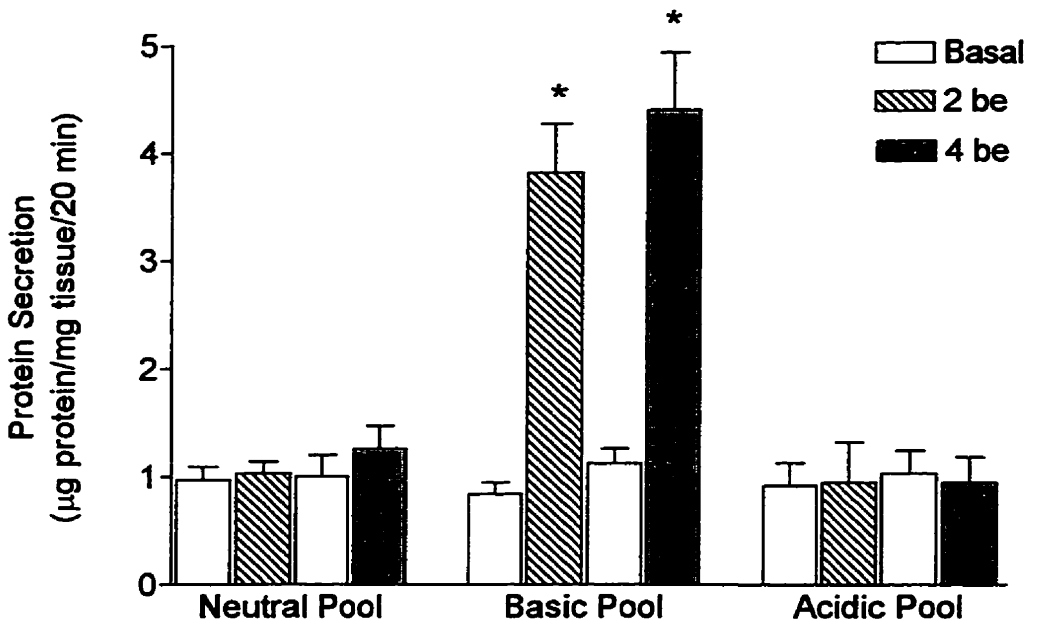

Protein Secretion
(µg protein/mg tissue/20 min)
5
4
3
2
1
0
*
*
Basal
2 be
4 be
Neutral Pool
Basic Pool
Acidic Pool

Table 1 The effect of some bioactive peptides and biogenic amines on protein secretion in the albumen gland of *Helisoma duryi*. Albumen glands were treated with 0.1 μM or 1.0 μM CDCH and calfluxin from *Lymnaea stagnalis*, FMRFamide, serotonin or dopamine (0.1, 1.0, 10.0 μM). The total amount of protein secreted during the 20 min application of neuroactive agents (treated) was compared to the total amount of protein secreted during the 20 min immediately prior to the application of the agents (basal). N = 5-6 for all agents. Dopamine evoked significant protein release from albumen glands (*p=0.0042 for 1.0 μM and **p=0.0112 for 10.0 μM, paired t-test). All other agents tested were ineffective in stimulating protein secretion.

Agent	Concentration	Protein Secretion (μg protein / mg wet weight / 20 min)		Ratio
		Basal	Treated	
CDCH	0.1 μM	0.98 ± 0.24	1.18 ± 0.24	1.20
	1.0 μM	1.10 ± 0.57	1.35 ± 0.23	1.23
Calfluxin	0.1 μM	1.30 ± 0.18	1.20 ± 0.22	0.92
	1.0 μM	1.10 ± 0.24	0.98 ± 0.26	0.89
FMRFa	0.1 μM	1.26 ± 0.41	1.00 ± 0.24	0.79
	1.0 μM	1.49 ± 0.13	1.28 ± 0.14	0.86
Serotonin	0.1 μM	1.17 ± 0.32	1.17 ± 0.45	1.00
	1.0 μM	1.62 ± 0.29	1.46 ± 0.24	0.90
Dopamine	0.1 μM	1.61 ± 0.75	1.30 ± 0.52	0.81
	1.0 μM	0.93 ± 0.20	1.66 ± 0.29	*1.78
	10.0 μM	0.87 ± 0.23	3.61 ± 0.74	**4.15

Chapter V: Brain Extract Mediates Protein Secretion in the Albumen Gland Through the cAMP Signal Transduction Pathway

SUMMARY

Application of an acidic extract of the central nervous system to the albumen gland in the freshwater snail *Helisoma duryi* stimulated the production of the second messenger cAMP. The factor from the brain was heat-stable and sensitive to protease digestion indicating it is a polypeptide. The adenylate cyclase activator forskolin elevated albumen gland cAMP levels and evoked *in vitro* protein secretion in a concentration-dependent fashion. In addition, membrane-permeable analogues of cAMP and an inhibitor of cAMP phosphodiesterase were capable of promoting the release of proteins from the albumen gland. Thus, a majority of the essential criteria outlined by Sutherland and Robison (1966) to demonstrate cAMP signalling are fulfilled. These results demonstrate that protein secretion by the albumen gland in the presence of a peptide messenger from the brain is mediated, in part, through the adenylate cyclase/cAMP signal transduction pathway.

INTRODUCTION

The albumen gland of freshwater pulmonate snails is an exocrine female accessory sex gland which synthesizes and secretes proteins and polysaccharides (Okatore *et al.*, 1982; Wijsman and van Wijck-Batenburg, 1987). These compounds are secreted into the glandular carrefour as a viscous fluid referred to as perivitelline fluid (PVF). The PVF coats individual eggs as they enter the carrefour, and provides nourishment for the developing embryos (de Jong-Brink *et al.*, 1983).

The synthesis and release of PVF are believed to be under hormonal control from the endocrine dorsal bodies (DBs) and neuropeptides from the cerebral ganglia (Wijdenes *et al.*, 1983; deJong-Brink and Goldschmeding, 1983; Miksys and Saleuddin, 1985, 1988; Morishita *et al.*, 1998). Despite the considerable body of evidence regarding the effect of various endocrine and neurosecretory substances on the pulmonate albumen gland, there has been no direct evidence of a chemically identified substance mediating a specific physiological response such as synthesis or secretion (see Joosse and Geraerts, 1983; Joosse, 1988; Saleuddin *et al.*, 1994). As a consequence, there are few studies examining the intracellular messengers which mediate the activity of the albumen gland (Goudsmit and Ram, 1982; Dictus *et al.*, 1987b, 1988; Dictus and Ebberink, 1988). Recently, we have identified a 66 kDa glycoprotein from the albumen gland of the freshwater pulmonate snail *Helisoma duryi*, and demonstrated that a central nervous system (CNS) peptide evokes the *in vitro* release of this glycoprotein (Morishita *et al.*, 1998; this thesis). Forskolin and cAMP analogues mimic the effect of brain extract on the albumen gland, suggesting that the adenylate cyclase/cAMP

signal transduction pathway is involved in the exocytotic release of secretory material (Morishita *et al.*, 1998).

In many eukaryotic cells, cAMP serves as an important intracellular signalling molecule in the regulated secretory pathway. In gastropod molluscs, the neuroendocrine bag cells (BCs) of *Aplysia californica* and the caudodorsal cells (CDCs) of *Lymnaea stagnalis* release egglaying neuropeptides when treated with cAMP analogues, phosphodiesterase inhibitors, or forskolin (see Geraerts *et al.*, 1988). The activity of adenylate cyclase and the influx of calcium also increases as these neurosecretory cells begin releasing their peptides, indicating that secretion of reproductive hormones is mediated, at least in part, by cAMP. Neurotransmission and memory (Jarrard *et al.*, 1993; Haydon *et al.*, 1991; Berry, 1996; Kaang *et al.*, 1993), visceral muscle contraction (Ishikawa *et al.*, 1981;Gies, 1986; Hooper *et al.*, 1994), and cardiac activity (Painter, 1982; Lloyd *et al.*, 1985; Reich *et al.*, 1997) are other examples in gastropods in which cAMP functions as a second messenger and the cAMP-mediated responses have been examined in detail.

This study extends upon the observations of Morishita *et al.* (1998) by examining the influence of a stimulatory factor from the CNS of *H. duryi* on cAMP production in the albumen gland. In addition, various agents which increase cAMP levels as well as those that mimic the biological actions of cAMP were tested on albumen glands *in vitro*. The cAMP-stimulating activity from the CNS is abolished after digestion with protease, suggesting that the CNS factor is peptidergic. The adenylate cyclase activator forskolin, increased intracellular cAMP production and potentiated cAMP levels when applied together with a

CNS extract. Forskolin, cAMP analogues or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) were all capable of stimulating *in vitro* protein secretion by the albumen gland. It is suggested that a CNS peptide(s) stimulates the exocytotic release of proteins by activating the cAMP signal transduction pathway in the albumen gland of *H. duryi*.

MATERIALS AND METHODS

Animals

Laboratory stocks of reproducing *H. duryi* were maintained as previously described in this thesis. Animals used for the bioassay and cAMP studies were individually isolated for 1-2 weeks prior to experimentation so that egg laying rates could be monitored. Albumen glands were dissected from those snails that had not laid eggs within the last 24h. Virgin snails were raised as isolates soon after hatching and allowed to grow to adult size, then used for experiments.

Bioassay

The *in vitro* release of proteins by albumen gland explants as described in Morishita *et al.*, (1998) and as modified in this thesis was used to test the effects of forskolin, cAMP analogues [N^6, 2'-O-dibutyryl cAMP (dibutyryl cAMP), 8-bromoadenosine cAMP (8-bromo cAMP), and 8-(4-chlorophenylthio) cAMP (8-cpt cAMP)], and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). Forskolin and IBMX were prepared as stock solutions in DMSO and diluted to their final concentrations in snail saline. All reagents were

purchased from Sigma (St. Louis, Mo., USA) unless mentioned otherwise.

Preparation of CNS Extract

The *H. duryi* CNS extract was prepared as described in the previous chapter. Briefly, brains were dissected out from reproducing snails and immediately frozen on dry ice. Batches of 20-30 brains were boiled in 0.1 M acetic acid, homogenized, then centrifuged. Two volumes of acetone were added to the supernatant and the mixture was centrifuged to pellet large proteins. The resultant supernatant was evaporated to dryness, resuspended in snail saline, and tested for bioactivity. Some extracts were treated with protease-beaded agarose (1 mg/ml Pronase F), then tested for their ability to stimulate cAMP in albumen glands.

cAMP Measurements

Albumen glands were dissected free from surrounding tissue and rinsed in snail saline several times. The glands were then halved, one serving as a control and the other receiving brain extract. The glands were washed in several saline rinses, then maintained in saline for about 60 min prior to the start of the assay. To initiate the assay, control albumen glands were placed individually in Falcon 96-well tissue plates with 100 µl saline containing 0.1 mM IBMX, while their corresponding halves were placed in 100 µl saline containing 0.1 mM IBMX with 1 brain equivalent (be). The reaction was terminated by immersing the tissue directly into liquid nitrogen and storing the tissue in 1.5 ml Eppendorf tubes (Brinkmann, USA) at -80^{0}C until required. The tissues were then homogenized in 6% PCA to precipitate proteins and to extract cAMP, and the homogenate centrifuged at 3,000 x g for 10 min. The pellet was resuspended and homogenized in a small volume of 6% PCA and centrifuged

again. The precipitated protein in the pellet was redissolved in 0.1 M NaOH and the protein content was measured using a commercial protein assay kit (Bio-Rad, Mississauga, Ont.) with BSA as a standard. The supernatants were pooled, neutralized with 30% KOH to approximately pH 3.0-4.0, then centrifuged at 5,000 x g (10 min). The supernatant was then transferred to a fresh tube and evaporated to dryness. The dried extract was resuspended in 100 µl of deionized water and an appropriate volume was removed for cAMP determination using a commercial ^{125}I-cAMP radioimmunoassay (RIA) system (Amersham, Oakville, Ont.). The data are expressed as fmol cAMP/µg protein.

Statistical analysis of paired data were performed with either the paired t-test or the Wilcoxin paired sample test ($p<0.05$). Multiple sample tests were done with ANOVA or the Kruskal-Wallis procedure ($p<0.05$), then *a posteriori* multiple sample comparison tests were performed with either the Dunnett Test or the Dunn Test ($Q_{\alpha=0.05, k}$).

RESULTS

Effect of Forskolin, cAMP Analogues, and IBMX on Albumen Gland Protein Secretion

The adenylate cyclase activator forskolin was tested at concentrations of 1, 10, and 100 µM. A 1 µM dose of forskolin was ineffective in stimulating release of protein from the albumen gland, but at a concentration of 10 µM, a 5-fold increase in protein secretion was seen (Fig. 1). At 100 µM forskolin, a large increase (8-fold) in protein secretion was elicited.

The cAMP analogues dibutyryl cAMP, 8-bromo cAMP, and 8-cpt cAMP were tested for activity at concentrations of 0.1 mM or 1 mM. All the analogues were ineffective in

stimulating protein release at a concentration 0.1 mM (Fig. 2). At 1 mM concentration, 8-bromo cAMP or 8-cpt cAMP were capable of stimulating a significant increase in protein release over basal levels. Dibutyryl-cAMP caused only a small increase (not statistically significant) in protein secretion and was the least effective analogue. The analogue 8-bromo cAMP appeared to be the most potent agent, and 8-cpt cAMP was slightly less effective. When combinations of the cAMP derivatives were tested, their effects on protein secretion were synergistic. Analogues 8-bromo cAMP+8-cpt cAMP were the most potent combination in stimulating protein secretion, followed by 8-bromo cAMP+dibutyrl cAMP, and 8-cpt cAMP+dibutyrl cAMP (Fig. 3).

The phosphodiesterase inhibitor IBMX was tested at concentrations of 0.1, 1, and 10 mM on the albumen gland bioassay. All concentrations of IBMX used stimulated a significant increase in protein release (Fig. 4). However, the response to the inhibitor did not appear to be dose-dependent, since a 1 mM concentration did not induce a significant increase in protein release compared to a 0.1 mM dose.

cAMP Content of Albumen Glands From Virgin and Mated Snails

The basal cAMP levels in the absence of phoshodiesterase inhibitor were measured from freshly dissected albumen glands from virgin snails and random mating snails. Albumen gland cAMP was quantified using RIA. In mated animals, basal cAMP levels were approximately three-fold greater compared to the basal cAMP levels in glands from virgin snails (Fig. 5).

Stimulation of Albumen Gland cAMP Production By CNS Extract

The time course of cAMP accumulation was examined in *H. duryi* albumen glands at 2, 5, 10, and 20 min after application of a CNS extract. The basal level of cAMP in control albumen glands increased relatively slowly over 20 min in the presence of IBMX. Following application of 1 be to the albumen glands, there was a rapid elevation of cAMP which reached a maximal level (5-fold over controls) after 5 min (Fig. 6). The increase in albumen gland cAMP appeared to be a transient occurrence as the levels of cAMP declined after 5 min, then maintained a relatively steady concentration (2-3-fold over controls) from about 10 to 20 min after application of brain extract. Since maximal stimulation occurred at approximately 5 min, this incubation time was chosen for subsequent experiments.

The adenylate cyclase activator, forskolin, was also capable of stimulating *in vitro* cAMP production by albumen glands. At a dose of 0.1 μM, forskolin had no effect on cAMP production and at 1.0 μM, a small increase in cAMP levels was detected. A concentration of 10 μM forskolin elevated cAMP levels about 5-fold over basal levels (Fig. 7), which was similar to its effect on protein release (Fig. 1). When brain extract (1 be) and forskolin (10 μM) were applied together, cAMP production was enhanced compared to the effect of either compound used by itself (Fig. 8).

In order to determine the nature of the factor from the CNS which stimulated albumen gland cAMP production, a CNS extract was subjected to proteolytic digestion and then tested for its ability to increase intracellular cAMP. After treatment with protease, the stimulatory effect of the CNS extract was completely abolished, suggesting the factor from the brain is

likely a polypeptide (Fig. 9).

DISCUSSION

In pulmonate molluscs, there is little information regarding the production of second messengers by the gonad or the accessory reproductive glands in response to extracellular peptide messengers. Neuropeptides such as CDCH (Ebberink *et al.*, 1985), calfluxin (Dictus *et al.*, 1987), small cardioactive peptides (SCPs)(A.S.M. Saleuddin, unpublished observations), FMRFamide-related peptides (deJong-Brink *et al.*, 1983; van Golen *et al.*, 1995b), myomodulins (Li *et al.*, 1994; van Golen *et al.*, 1996), APGWamide (van Kesteren *et al.*, 1995c), and conopressin (van Kesteren *et al.*, 1995a, b) are reported to be involved in modulating muscle contraction in reproductive tissues. However, there are few studies which have examined the generation of second messengers in reproductive tissues and the specific physiological responses which they mediate. In *L. stagnalis*, a crude saline extract of DBs stimulates adenylate cyclase in the follicle cells of the ovotestis, but the physiological significance of this activation is unknown (deJong-Brink *et al.*, 1986). In *Helix pomatia*, an unidentified factor from the CNS called 'galactogenin' stimulates galactogen (polysacccharide) synthesis by the albumen glands (Goudsmit and Ram, 1982). The cAMP analogue 8-bromo cAMP mimics the effect of galactogenin. In *L. stagnalis*, a neuropeptide called calfluxin was isolated from the neurosecretory CDCs (Dictus and Ebberink, 1988). Calfluxin was reported to stimulate the influx of extracellular calcium and mobilize calcium from intracellular reserves, but the ultimate cellular response it mediates is unknown.

In *H. duryi*, sexually immature juveniles can be reared in isolation as virgins until they attain adult size (10-12 mm shell diameter). This population of snails does not produce viable eggs even though they are simultaneous hermaphrodites. Mating is thus a prerequisite for egg production in these animals (Saleuddin *et al.*, 1983b). Thus, distinct reproductive populations (mated vs virgin) can be easily established in the laboratory. Striking differences in egg production (Saleuddin *et al.*, 1983b), albumen gland polysaccharide synthesis (Miksys and Saleuddin, 1985; this thesis), dorsal body activity (Saleuddin *et al.*, 1989; Khan *et al.*, 1990a), and synthetic activity of the neurosecretory CDCs (Mukai and Saleuddin, 1989; Khan *et al.*, 1990b) have been demonstrated between virgin and mated *H. duryi*.

The basal cAMP levels were compared from albumen glands removed from both virgin and randomly mated snails and assayed for cAMP. Basal albumen gland cAMP levels were approximately three-fold higher in mated snails than those of virgin animals. The *in vitro* synthetic activity of albumen glands from reproducing snails is also markedly higher as compared to albumen glands from virgins (Miksys and Saleuddin, 1985; this thesis). Therefore, in reproducing *H. duryi*, both basal cAMP production and polysaccharide synthesis by the albumen gland are significantly elevated as compared to non-reproducing snails. Goudsmit and Ram (1982) reported that cAMP analogues enhanced galactogen synthesis in the albumen gland of *H. pomatia*, suggesting that cAMP is involved in regulating glandular synthetic activity. However, Dictus *et al.* (1988) stated that they could not detect any changes in cAMP production during different stages of the egg laying cycle of *L. stagnalis*, and argued the principle signalling mechanism used by the albumen gland is via calcium and

turnover of inositol phosholipids. In the present study, albumen glands from reproducing *H. duryi* have significantly higher (3-fold) basal cAMP levels as compared to glands from virgin snails. This increased cAMP production by albumen glands of reproducing snails might function to maintain an elevated level of synthetic activity in the albumen gland which is required for increased egg production.

This study demonstrates that in the presence of an acidic extract of the CNS, the albumen gland responds by increasing the production of intracellular cAMP. The increase in cAMP in the presence of a CNS extract appeared to be phasic, since the concentration of cAMP increased rapidly, then peaked within 5 min to levels which were about five-fold over controls. Cyclic AMP levels then declined over the next five minutes to approximately two-fold above untreated controls and remained at this level for the duration of the experiment. Transient increases in cAMP in some systems may indicate a loss of intracellular cAMP. This may be due to incomplete inhibition of cAMP phoshodiesterase or extrusion of cAMP from cells (Houslay, 1991). In another pulmonate snail, *Helix aspersa*, transient increases in cardiac tissue cAMP in response to small cardioactive peptide B (SCP_B) were reported even in the presence of two phosphodiesterase inhibitors (Reich *et al.*, 1997), and efflux of cAMP from the whole CNS of *L. stagnalis* has been reported (Werkman *et al.*, 1990b). In the present work, the medium surrounding the albumen gland was not measured for cAMP, therefore it is possible that some of the cAMP could have been extruded into the saline, particularly since the albumen glands were cut into halves. Alternatively, the cAMP phosphodiesterase activity of the albumen gland was not completely inhibited by IBMX.

The rapid increase in cAMP and its subsequent decrease can also be explained with respect to the secretory physiology of the albumen gland. The amount of PVF required to coat one egg in response to a single dose of brain extract, represents a relatively small fraction of the secretable pool. However, following ovulation, many oocytes (15-30) are released from the ovotestis, and are fertlized in the region of the carrefour. Each egg must receive a precisely equal amount of PVF (deJong-Brink, 1969). Therefore, prolonged activation of the secretory machinery would be unnecessary. An efficient way to lower intracellular cAMP levels rapidly would be to have the cell increase its phosphodiesterase activity shortly after receptor activation, ensuring the termination of the signal, and thereby maintaining sufficient secretory reserves for the rest of the egg clutch.

The diterpene forskolin has been used extensively to demonstrate the specific activation of adenylate cyclase in many eukaryotic systems (Laurenza *et al.*, 1989). A slight increase in cAMP was observed using forskolin at 1 μM, and a significant elevation of cAMP was achieved at a dose of 10 μM (five-fold). When brain extract and forskolin were added simultaneously to albumen glands *in vitro*, a potentiating effect on cAMP production was observed. In many systems, it is well known that forskolin can act together with hormones to potentiate adenylate cyclase activity or cAMP production (Seamon and Daly, 1986). This is achieved as forskolin bypasses the receptor by directly activating the catalytic subunit of the enzyme (Seamon and Daly, 1983). Forskolin was also capable of stimulating the release of newly-synthesized proteins and polysaccharides from albumen glands which had been previously incubated in radiolabelled amino acids or galactose (see Appendix).

Treatment of *H. duryi* CNS extract with protease abolished its ability to elevate albumen gland cAMP, indicating that the factor originating from the CNS is likely peptidergic. This result was also confirmed using the albumen gland protein secretion bioassay (Chapter 4, this thesis). These results suggest that a peptide from the CNS stimulates the secretion of proteins from the albumen gland. This brain peptide activates the adenylate cyclase/cAMP system of the gland and likely promotes the exocytotic release of secretory material.

Calcium and cAMP are widely recognized as the major regulatory agents mediating exocytosis in a wide variety of cells from different sources. In vertebrates, exocytotic release of secretory material by exocrine glands is, in part, mediated by the second messenger cAMP. Examples of such systems include the secretion of enzyme and fluids from rat exocrine pancreas (Schafer *et al.*, 1994), and amylase release from parotid acinar cells (Takuma and Ichida, 1994). In arthropods, cAMP analogues and crustacean hyperglycemic hormone stimulates the release of amylase from crayfish hepatopancreas (Keller and Sedlmeier, 1988), and biogenic amines elevate cAMP levels in insect salivary glands (Ali and Orchard, 1993, 1996). In molluscs, cAMP analogues and dopamine stimulate amylase release from dispersed digestive gland cells of scallop (Giard *et al.*, 1995). In the pulmonate snails, various neurotransmitters and peptides are capable of stimulating adenylate cyclase in salivary glands (Ferretti *et al.*, 1993,1996), and cAMP analogues are able to stimulate galactogen synthesis in the albumen gland (Goudsmit and Ram, 1982). Therefore, in both vertebrate and invertebrate exocrine glands, ligand-activated cAMP production is an important component

of the the signal transduction cascade promoting the exocytotic release or synthesis of secretory material.

Adenylate cyclase activity of albumen gland membrane preparations can also be stimulated by a brain extract or forskolin, as well as aluminum fluoride and a slowly hydrolyzable GTP analogue (GTPγS) (S. Mukai, K. Almeida, S. Saleuddin, unpublished observations). In addition, the presence of the stimulatory α-subunit of the G-protein (G_s) in albumen gland membrane preparations provides further evidence that the receptor for the CNS peptide in the albumen gland is a G-protein-coupled receptor that activates adenylate cyclase (Morishita *et al.*, 1998).

The ability of the membrane-permeable cAMP analogues, dibutyryl cAMP, 8-bromo cAMP, and 8-cpt AMP to induce protein secretion, indicates the release of secretory material from the albumen gland secretory cells involves the cAMP-dependent protein kinase or protein kinase A (PKA). Cyclic AMP analogues are thought to activate PKA by binding to its regulatory subunit and causing the release of the catalytic subunit which then phosphorylates various intracellular target proteins (Walsh and Van Patten, 1994). The analogues 8-bromo cAMP and 8-cpt cAMP evoke the largest increase in protein secretion, whereas dibutyryl cAMP induces only a small increase in release. Combinations of the three cAMP analogues produce synergistic effects. The analogues 8-bromo cAMP + 8-cpt cAMP produced the largest response, followed by dibutyrl cAMP + 8-bromo cAMP, and dibutyrl cAMP + 8-cpt cAMP. The N^6 derivatives of cAMP are believed to bind to the α site (fast-dissociating site) with respect to type I (RI) and type II (RII) regulatory subunits, whereas

the 8-thio- and 8-bromo- cAMP derivatives bind to the β site (slow-dissociating site) on the RII subunit (Francis and Corbin, 1994). Since the 8-thio and 8-bromo analogues appeared to be more effective than the N^6 cAMP derivative, this suggests that the RIIβ isoform of PKA might be involved in mediating protein secretion. In addition, pre-incubation of albumen glands with the PKA antagonist R_p-cAMP attenuated protein secretion when challenged with a brain extract, supporting the notion that PKA activation mediates protein release (Morishita *et al.*, 1998). Activation of PKA will undoubtedly phosphorylate a number of different proteins, some of which may be involved in the exocytotic release of secretory material (Takuma and Ichida, 1994). The identification of target proteins phosphorylated by PKA in response to the CNS peptide, and their role in the exocytotic release or synthesis of secretory material in the albumen gland merits further investigation.

Methylxanthine derivatives such as theophylline or IBMX are commonly used to prolong the action of cAMP in a variety of systems by inhibiting cyclic nucleotide phosphodiesterases. The inhibitor IBMX is able to enhance protein secretion from the albumen gland of *H. duryi* when applied at 0.1, 0.5, or 1 mM concentration. Therefore, the accumulation of cAMP caused by the inhibition of cyclic nucleotide phosphodiesterases, activates the secretory machinery of the albumen gland, culminating in the release of protein. However, even at high concentrations of phosphodiesterase inhibitor (1 mM), only a modest increase in protein secretion is achieved.

At present, the neuropeptide calfluxin is the only chemically identified molecule in molluscs which has a specific cellular effect on the albumen gland (Dictus *et al.*, 1987b,

1988). These authors suggest that elevation of intracellular calcium is achieved through the activation of PKC and the inositol phosphate pathway rather than by cAMP. They based their conclusions on the stimulatory effect of phorbol ester and lithium-induced inhibition of calfluxin action. Dictus *et al.* (1988) concluded that extracellular calcium was not required for calfluxin to exert its effect, but it appeared to be necessary to achieve a full response.

In vertebrate exocrine glands, both cAMP and IP_3 production can be stimulated by a single agonist depending on its concentration (Trimble *et al.*, 1986; Kimura *et al.*, 1986; O'Sullivan and Jamieson, 1992). It is possible a similar situation may occur in *H. duryi*, whereby cAMP and calcium are both involved in mediating PVF secretion in the presence of a stimulatory peptide from the CNS. In addition, the effect of the biogenic amine dopamine (another known stimulator of albumen gland protein secretion) on cAMP production or on other second messengers has yet to be determined. More detailed dose-response studies using a purified brain peptide, combined with cAMP, IP_3, calcium and concurrent protein/polysaccharide secretion measurements are required to elucidate the participation of these signal transduction pathways in regulating PVF secretion. The determination of cAMP in the albumen gland in the presence of brain extract may provide a parallel detection method, along with bioassays, for the isolation of this particular peptide from *H. duryi*. This approach has been used successfully to isolate neurally-derived diuretic peptides from a number of insect species where conventional bioassay methods showed too much variability or proved technically cumbersome (Morgan and Mordue, 1985; Kay *et al.*, 1991a, 1991b; Furuya *et al.*, 1995; Spittaels *et al.*, 1996).

Fig. 1 Effect of the adenylate cyclase activator forskolin on albumen gland protein secretion. The saline (100 µl) surrounding the albumen gland was collected every 20 min for a period of 140 min. Basal secretion (open bars) represents the amount of protein released between 40-60 min. Forskolin (1, 10, or 100 µM final concentration) was added between 60-80 min (filled bars), and the saline measured for protein that was secreted by the albumen gland. Note that forskolin stimulated albumen gland protein secretion 5-fold at a dose of 10 µM (*Wilcoxin Test p=0.002), and up to 8-fold at a dose of 100 µM (**Wilcoxin Test p=0.0078). Bars represent the means ± SE of 6-10 samples.

Fig. 1

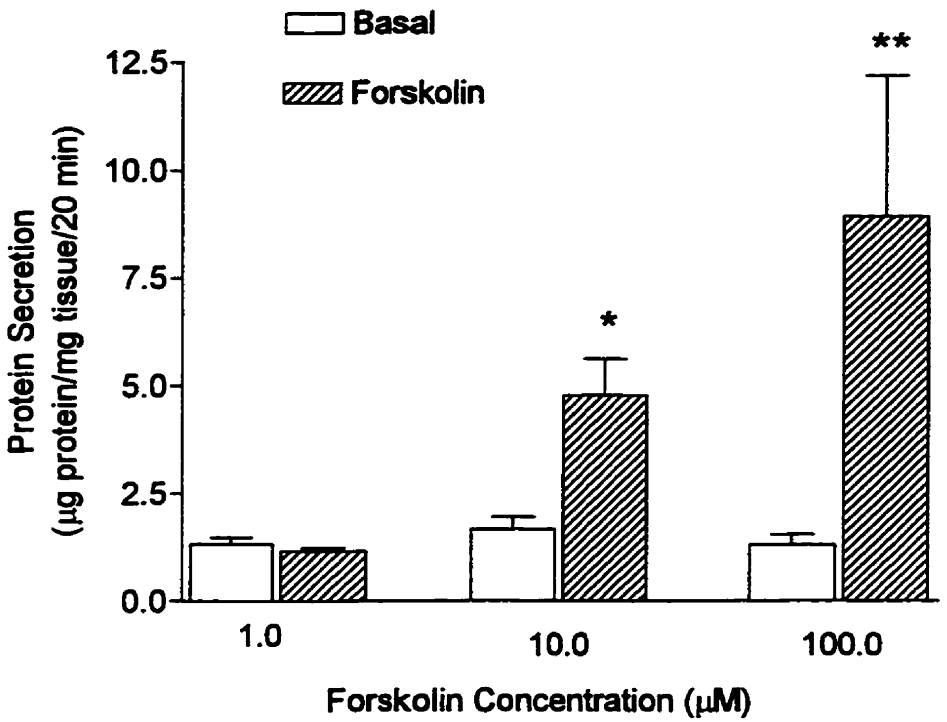

Basal
Forskolin
12.5
10.0
7.5
5.0
2.5
0.0
Protein Secretion
(µg protein/mg tissue/20 min)
*
**
1.0
10.0
100.0
Forskolin Concentration (µM)

Fig.2 Effect of cAMP analogues on albumen gland protein secretion. The cAMP analogues dibutyryl cAMP (db-cAMP), 8-bromo-cAMP (8-br-cAMP), and 8-4-chlorophenylthio-cAMP (8-cpt-cAMP) were tested at either 0.1 mM or 1.0 mM (final concentration). All analogues were ineffective in stimulating protein secretion when used at 0.1 mM. Dibutyryl cAMP (1.0 mM) did not significantly stimulate protein release ($p=0.0777$), whereas 1.0 mM 8-br-cAMP or 8-cpt-cAMP (*Wilcoxin Test, $p<0.03$) induced a 3-fold and 2-fold increase in secretion respectively. Bars represent the means ± SE of 5-8 samples. Basal secretion (open bars); cAMP analogues (filled bars).

Fig. 2

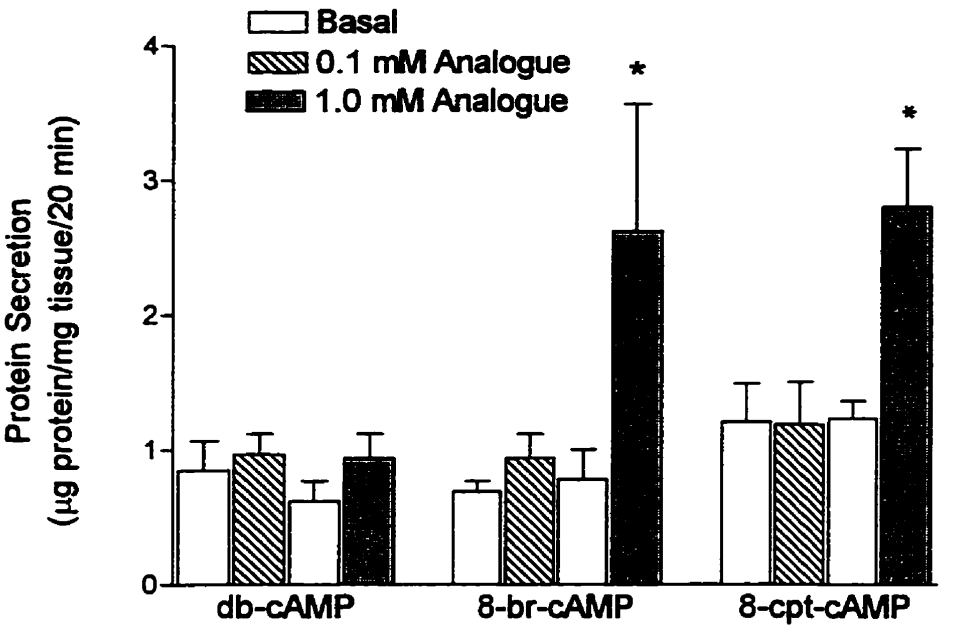

Basal
0.1 mM Analogue
1.0 mM Analogue
Protein Secretion
(µg protein/mg tissue/20 min)
0
1
2
3
4
*
*
db-cAMP
8-br-cAMP
8-cpt-cAMP

Fig.3 Synergistic effect of cAMP analogues on albumen gland protein secretion. Three combinations of cAMP analogues were tested at 1.0 mM (final concentration) : dibutyryl cAMP and 8-bromo cAMP (db+8br); dibutyryl cAMP and 8-4-chloropenylthio-cAMP (db+8cpt); 8-bromo cAMP and 8-4-chlorophenylthio-cAMP (8-br+8cpt). Note all combinations of cAMP analogues tested strongly stimulated protein secretion. The combination of 8-br+8cpt was the most potent (14-fold increase), followed by db+8cpt (6-fold), and db+br (5-fold). Bars represent the means ± SE of 4 samples (*Mann-Whitney Test, $p<0.03$ for all analogue combinations). Basal secretion (open bars); combination of cAMP analogues (filled bars).

Fig. 3

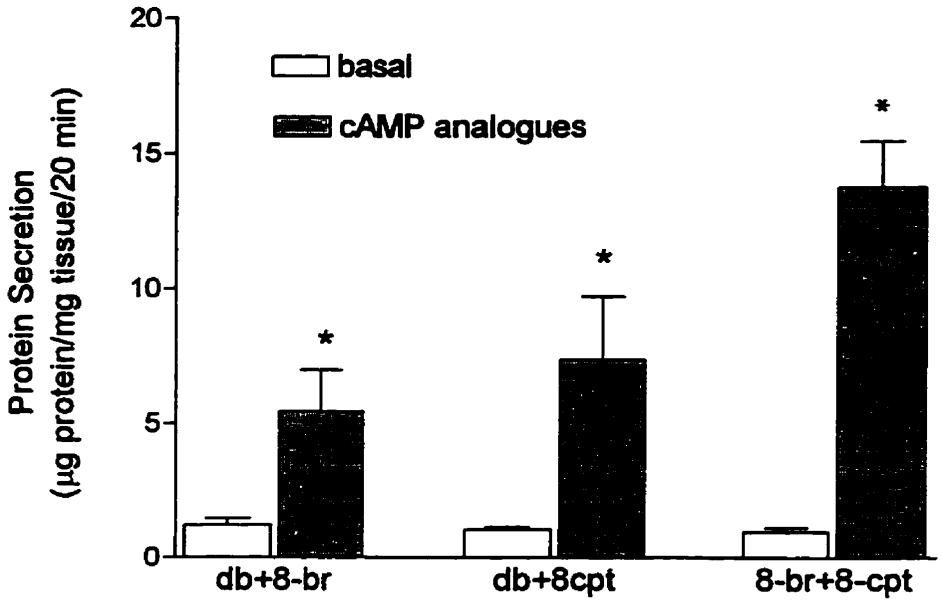

Protein Secretion
(µg protein/mg tissue/20 min)
20
15
10
5
0
basal
cAMP analogues
*
*
*
db+8-br
db+8cpt
8-br+8-cpt

Fig 4 Effect of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) on albumen gland protein secretion. The inhibitor IBMX was tested at 0.1, 0.5, or 1.0 mM (final concentration). Note IBMX was capable of stimulating protein release at all concentrations tested. Bars represent means ± SE of 4-6 samples (paired t-test, $p<0.05$ for all concentrations of IBMX). Basal secretion (open bars); IBMX (closed bars).

Fig. 4

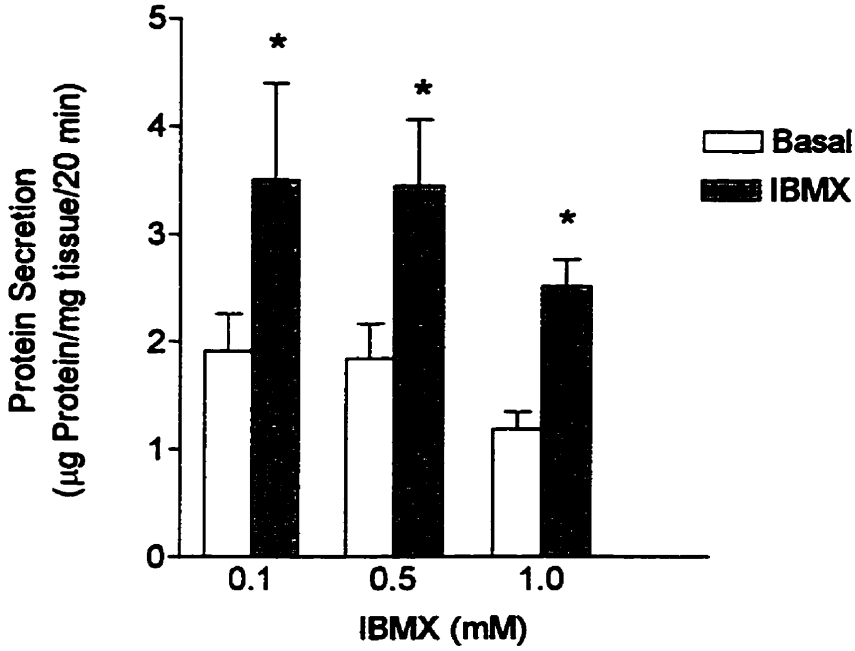

Protein Secretion
(µg Protein/mg tissue/20 min)
5
4
3
2
1
0
*
*
*
0.1
0.5
1.0
IBMX (mM)
Basal
IBMX

Fig. 5 Basal albumen gland cAMP levels in virgin and mated snails. Albumen glands were surgically removed from virgin and reproducing snails, and immediately placed in ice-cold 6% PCA and homogenized. The homogenate was centrifuged, and the protein content of the pellet was determined. The supernatant was measured for cAMP using a commercial RIA kit. The data are expressed as fmol/μg protein. Bars represent the means ± SE of 4 samples. Note the basal levels of cAMP are significantly higher in mated compared to virgin snails (*t-test, p=0.022).

Fig. 5

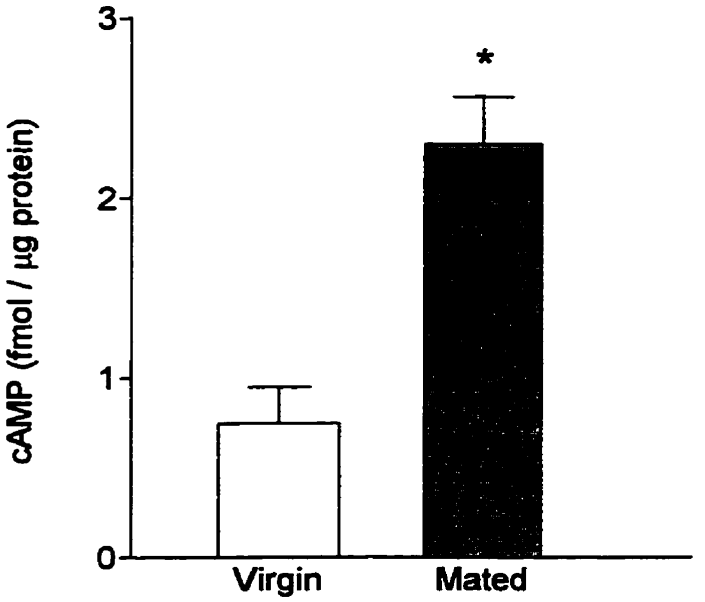


Fig. 6 Time course of cAMP production in albumen glands in the presence of brain extract. Albumen glands were halved, one serving as a control and the other as recipient of brain extract (1be). All incubations were performed in the presence of 0.1 mM IBMX in the saline. Application of brain extract stimulated cAMP production after 2 min, and peaked at approximately 5 min (5-fold). The level of cAMP in the albumen gland was maintained at 2-fold higher concentrations for the duration of the experiment. Data are expressed as fmol/μg protein. Each point represents the mean ± SE of 3-5 samples.

Fig. 6

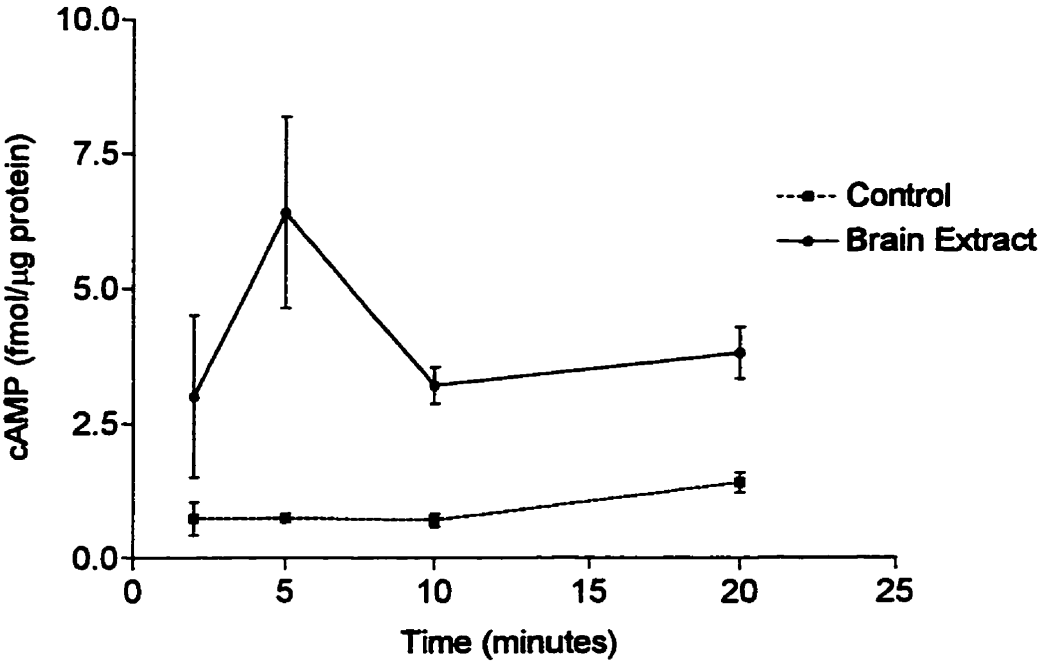

10.0
7.5
5.0
2.5
0.0
cAMP (fmol/µg protein)
0
5
10
15
20
25
Time (minutes)
Control
Brain Extract

Fig. 7 Effect of the adenylate cyclase activator forskolin on albumen gland cAMP production. Albumen glands were halved, one serving as a control, the other receiving 0.1, 1.0, or 10.0 μM forskolin (final concentrations). All incubations were performed in the presence of 0.1 mM IBMX for 5 min. Data are expressed as fmol cAMP/μg protein. Bars represent the means ± SE of 5-6 samples. Significant elevation of cAMP was observed at a dose of 10 μM (*Wilcoxin Test p=0.0313).

Fig. 7

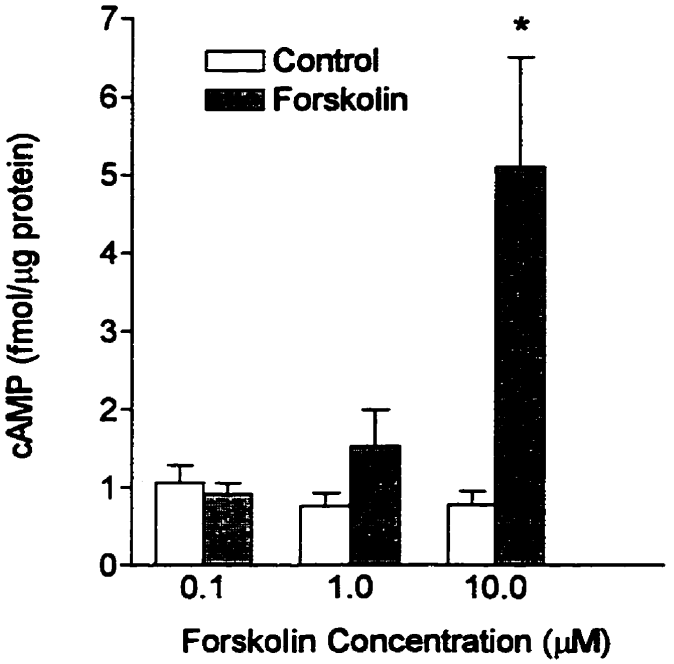

7
6
5
4
3
2
1
0
cAMP (fmol/µg protein)
Control
Forskolin
*
0.1
1.0
10.0
Forskolin Concentration (µM)

Fig. 8 Potentiating effect of forskolin on brain extract-stimulated cAMP production in the albumen gland. Albumen glands were dissected into four equal-sized pieces, one piece served as a control, a second piece was treated with 10.0 μM forskolin, the third piece was treated with brain extract (1 be), and the fourth piece was treated with both forskolin (10 μM) + brain extract (1 be). All incubations were performed in the presence of 0.1 mM IBMX for 5 min. Data are expressed as fmol cAMP/μg protein. Bars represent the means ● SE of 5-6 samples. Note that brain extract+forskolin enhanced cAMP production compared to brain extract or forskolin alone.

Fig. 8

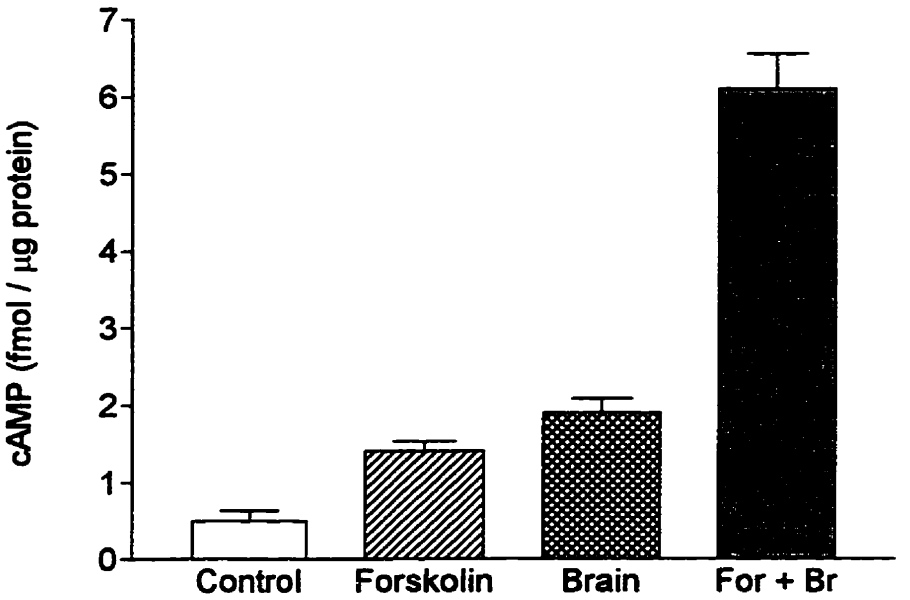
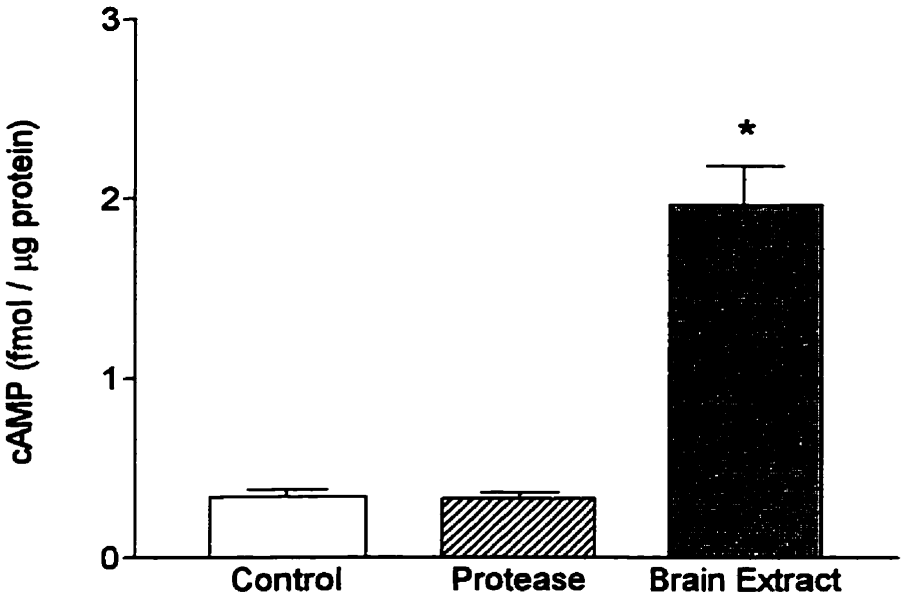

cAMP (fmol / µg protein)
0
1
2
3
4
5
6
7
Control
Forskolin
Brain
For + Br

Fig. 9 Effect of brain extract treated with protease on cAMP production in the albumen gland. Albumen glands were cut into three equal-sized pieces, one served as a control, another was treated with brain extract (1 be), and the last piece was treated with brain extract which had been previously subjected to proteolytic enzymes. All incubations were done in the presence of 0.1 mM IBMX for 5 min. Data are expressed as fmol/μg protein. Bars represent the means ● SE of 5-6 samples. Note that protease treatment of brain extract abolished its ability to stimulate cAMP production (*Kruskal-Wallis p=0.0142)

Fig. 9



GENERAL DISCUSSION

Hormones regulate many physiological processes in metazoan animals such as growth, reproduction, and osmoregulation. However, in comparison to the vertebrates, most of the invertebrates possess only few glandular endocrine organs for which a definitive function has been demonstrated (Highnam and Hill, 1977). It is only in the arthropods and molluscs that endocrine glands have been morphologically described and their functions identified. Examples of some endocrine organs in insects that control various aspects of growth and reproduction are the ectodermally-derived prothoracic glands and corpora allata. In molluscs, the only non-nervous endocrine organs known to control specific physiological functions are the dorsal bodies of pulmonates, and the optic glands of cephalopods (see Saleuddin *et al.*, 1994; Saleuddin, 1998a). In contrast to the few characterized endocrine glands present in invertebrates, neurosecretory cells are found in great numbers and types in many invertebrates, including molluscs. Many invertebrates use neurosecretory products (peptides and biogenic amines) as chemical messengers to control physiological functions such as muscle contraction, growth, reproduction, and osmoregulation. Indeed, several invertebrate models have contributed important information toward the studies of neurosecretion and comparative endocrinology in general (for references see Laufer and Downer, 1988; Davey *et al.*, 1994; Nassel, 1996).

The molluscan neuroendocrine system is exemplary of the diversity seen among the invertebrates. In particular, many molluscan neurosecretory cells are large and unipolar, readily identifiable, and located at the periphery of the ganglia (Joosse, 1979). Anatomically

distinct neurohemal organs such as the corpus cardiacum of insects or the sinus gland of crustaceans are not present in molluscs. Instead, most molluscs release their neurosecretory products at the peripheries of nerves, connectives or commissures (Joosse and Gerearts, 1983). Bioactive substances may be released from neurosecretory cells into the general circulation, or in some cases, released in or near target tissues located some distance from the perikarya (Joosse, 1979).

Studies on the hormonal control of reproduction in molluscs have been confined mainly to the gastropods. This is probably due to the fact that this group of molluscs can be maintained in the laboratory relatively easily, and their nervous systems and neurosecretory cells have been described in some detail (Joosse, 1988; Bulloch and Ridgeway, 1995). At present, the best characterized system for studying the hormonal regulation of reproduction in molluscs is the egg-laying hormone producing bag cells of *Aplysia californica*. These neurosecretory cells are present in large numbers (~ 400 cells/cluster) with their axon terminals situated in an anatomically distinct location (Arch and Berry, 1989; Conn and Kazcmarek, 1989). Since these cells are polyploid, they produce a proportionately large amount of mRNA making them an ideal system in which to study the cellular and molecular aspects of neuropeptide synthesis and secretion.

The endocrine control of reproduction in basommatophoran pulmonates has been studied extensively in two snails, *Lymnaea stagnalis* and *Helisoma duryi*. There are two main gonadotropic centres, the endocrine dorsal bodies and the neurosecretory caudodorsal cells, which regulate various aspects of reproduction. The dorsal bodies stimulate vitellogenesis

in addition to the development and synthetic activity of the female accessory sex glands (see Joosse, 1988; Saleuddin *et al.*, 1994). The caudodorsal cells are known to stimulate ovulation, overt behaviors associated with oviposition, and the activity of the albumen gland (see Geraerts *et al.*, 1988). One of the main target organs for the dorsal body hormone is the ovotestis, where it is believed to stimulate vitellogenesis. During vitellogenesis, nutrient reserves in the form of yolk accumulate in the oocyte cytoplasm, resulting in an increase in the size of the oocyte (Ubbels, 1968). In oviparous invertebrates such as annelids and arthropods, the major proteinaceous component of yolk is referred to as vitellin. Vitellins are generally high molecular weight glycolipoproteins derived from special serum proteins, vitellogenins, which are synthesized extragonadally. These vitellogenins are secreted into the circulation and are subsequently taken up by maturing oocytes (Gilbert, 1997). In most pulmonates, yolk protein is thought to be synthesized within the oocyte (Ubbels, 1968; deJong-Brink *et al.*, 1983), although the iron storage protein ferritin has been reported to be taken up during vitellogenesis and is an extragonadal component of yolk (Bottke and Sinha, 1979; Miksys, 1987).

The synthesis and uptake of vitellogenins is under hormonal control in most egg-laying animals. In snails, the only identified yolk component is ferritin, but its synthesis is regulated by iron reserves rather than hormones and it constitutes only a small fraction of proteins in the mature oocyte (Miksys, 1987). In cephalopods, general protein synthesis by the follicle cells (the site of yolk protein synthesis) is stimulated by the optic gland hormone (Wells and Wells, 1977). In *Helix aspersa*, gonadal protein synthesis is increased in the presence of

endocrine factors from the brain or dorsal bodies (Barre *et al.*, 1990), or with high doses of methionine-enkephalin, somatostatin or insulin (Monnier and Bride, 1995). However, in both these examples a specific protein which was regulated by the endocrine system was not identified. It is apparent from the experiments in this thesis involving the *in vitro* incubation of the ovotestis of *Helisoma duryi* that endocrine factors from the brain or dorsal bodies do not stimulate general protein synthesis levels, although two unknown proteins are present in higher amounts in the ovotestis of mated snails compared to virgins. Injection of the steroid hormone 20-hydroxyecdysone into virgin animals stimulated oocyte maturation, as evidenced by the increase in the number of mature oocytes. It is possible that ecdysteroid treatment somehow exerts a permissive effect on the gonad, allowing for the uptake of exogenous yolk precursors (ferritin) from the hemolymph. The yolk ferritin has been speculated to be involved in the production of iron-containing enzymes such as cytochromes, and in the mineralization of the radular teeth to allow the juveniles to immediately start grazing (Miksys and Saleuddin, 1986). Due to the presence of many heterogeneous cell types in the pulmonate ovotestis, it is likely that a more specific bioassay is necessary to test for the involvement of endocrine factors on yolk protein synthesis. This would require the isolation and characterization of a specific yolk protein from mature oocytes. However, since most basommatophoran oocytes are small, and cannot be easily dissected from the acini, harvesting an appreciable amount of starting material for protein purification is a daunting task.

The pulmonate digestive gland (hepatopancreas) has been implicated as a potential extragonadal source of yolk protein precursors (Bottke *et al.*, 1988; Barre *et al.*, 1991).

However, there is so far no evidence of hormones affecting protein synthesis in the digestive gland. In *Helisoma duryi*, the involvement of the endocrine system on digestive gland protein synthesis has not been assessed due to the massive amounts of proteolytic enzymes liberated *in vitro* which drastically alters the pH of the incubation medium (unpublished observations). Clearly, modifications to current tissue culture techniques for example, the use of dissociated cells and/or inclusion of various protease inhibitors, are required before a long term incubation of the digestive gland is ever achieved. A more feasible approach to determine the role of the endocrine system in yolk protein regulation would be to isolate specific proteins from mature oocytes of dioecious gastropods such as prosobranchs, or to isolate yolk proteins from the oocytes of a cephalopod such as the squid *Sepioteuthis lessoniana*. This squid has been recently bred and maintained in the laboratory for several generations and appears to be ideal for endocrinological studies since it grows large and rapidly attains sexual maturity (Lee *et al.*, 1994). If specific yolk proteins can be isolated, antibodies can be raised against the native protein or its subunits, and its presence can be detected in various tissues with sensitive immunoassays.

Unfortunately, the present literature regarding the endocrine regulation of the pulmonate ovotestis is fragmentary and remains inconclusive. On the other hand, the endocrine regulation of perivitelline fluid synthesis and its release by the basommatophoran albumen gland is more clearly understood. In both *Lymnaea stagnalis* and *Helisoma duryi*, polysaccharide synthesis in the albumen gland is stimulated by the dorsal body hormone and a neuropeptide from the cerebral ganglia, most likely caudodorsal cell hormone or calfluxin

(Wijdenes *et al.*, 1983; Dictus *et al.*, 1987b; Miksys and Saleuddin, 1988). Ironically, in *Lymnaea stagnalis*, both caudodorsal cell hormone and calfluxin have been chemically identified and synthetically manufactured, but their specific physiological effects on the albumen gland have never been shown.

Although many efforts have been undertaken to isolate and identify the dorsal body hormone of pulmonates, there has been little evidence in terms of a definitive chemical characterization. Both a steroidal and proteinaceous nature for the dorsal body hormone have been proposed, and it is apparent the dorsal body cells possess the cellular machinery to manufacture both these products (see Saleuddin *et al.*, 1994; Saleuddin, 1998a).

Dorsal bodies from mated animals are more synthetically active compared to those from virgins (Saleuddin *et al.*, 1989; Khan *et al.*, 1990a). Immediately after mating, dramatic changes in the cellular machinery occur in the dorsal body cells. Among these changes is the rapid disappearance of the majority of the large cytoplasmic lipid droplets. It is speculated this mobilization of lipid reserves is required for the production and release of a lipophilic compound into the hemolymph. In addition to the disappearance of lipid inclusions, the dorsal body cells from mated snails also begin releasing small (70-90 nm) moderately electron-dense granules, whose contents and function remain unknown (Boer *et al.*, 1968; Saleuddin *et al.*, 1989). In insects, ultrastructural and biochemical studies on the ecdysteroidogenic prothoracic glands have demonstrated these endocrine glands also produce and secrete a number of different proteins, some of which fluctuate with the ecdysteroid titer during development (Rybczynski and Gilbert, 1994; Hanton *et al.*, 1993; Richter and

Baumann, 1997). The pulmonate dorsal bodies appear to possess morphologically similar secretory granules to insect prothoracic glands, and in insects these granules have also been demonstrated to be exocytotically released (Hanton *et al.*, 1993; Watson *et al.*, 1996). The contents of these granules have been speculated to contain a carrier protein for steroidal secretions. Such transport proteins are known to exist in insect hemolymph (Whitehead, 1989) and also in vertebrate blood (Hammond, 1995), and may regulate hormonal access to, or recognition at target tissues.

The results from this work indicate the secretory material(s) from the dorsal bodies of *Helisoma duryi* is not a peptide, and support the study of Nolte *et al.* (1986) which suggest the pulmonate DBs produce a steroidal product, possibly an ecdysteroid. Furthermore, synthetic 20-hydroxyecdysone stimulates polysaccharide synthesis in the albumen glands of both *Helix aspersa* (Bride *et al.*, 1991) and *Helisoma duryi* (this thesis), and induces egg laying, suggesting a reproductive function for ecdysteroids in pulmonates. Both dorsal body tissue extracts and dorsal body-conditioned medium proved to be effective in stimulating *in vitro* polysaccharide synthesis in albumen gland explants. Based upon elution from solid phase extraction cartridges, the dorsal body tissue extracts contained a hydrophobic substance, whereas the dorsal body-conditioned medium possessed two bioactive fractions with slightly different polarities. One bioactive eluate from the conditioned medium contained a substance with similar hydrophobic properties to the bioactive material from dorsal body tissue, while the more polar fraction was demonstrated to possess ecdysteroid-like immunoreactivity. Based on the observed stimulatory activity of synthetic ecdysteroid on

Helisoma duryi and *Helix aspersa* albumen glands *in vitro*, and its effect on oocyte maturation in *Helisoma*, it was postulated that some of the bioactivity may be attributed to an ecdysteroid-related moleule secreted by the dorsal bodies. Thus, attempts were made to isolate the active material from dorsal-body conditioned medium using HPLC separation, RIA detection, and bioassay. Further HPLC separation of conditioned media revealed immunoreactive fractions coeluting with several ecdysteroid standards. When pooled fractions were tested for bioactivity, an increase in polysaccharide synthesis-stimulating activity was detected, however, the bioactivity was spread about in a several fractions and the release of newly synthesized secretory material in the medium was not evident.

The observations of other investigators clearly demonstrate that ecdysteroids are present in molluscs, as well as all the major non-arthropod invertebrate phyla. Moreover, in those groups that have been examined in some detail (Nematoda, Annelida, and Mollusca), ecdysteroids appear to have physiological functions (see Introduction). Although it was shown the dorsal bodies of *Helisoma duryi* were capable of secreting ecdysteroids *in vitro*, and ecdysteroid immunoreactive material could be detected in the hemolymph, significant changes in hemolymph ecdysteroid concentration were not apparent between virgin and mated snails. Garcia *et al.* (1995) postulated that if gastropods are capable of synthesizing ecdysteroids, they probably employ a different biosynthetic pathway than observed in insects. The identification of the types of ecdysteroid molecules circulating in the hemolymph is may be necessary before further bioassays are conducted. Experiments to determine if a humoral reproductive factor is present in the hemolymph were not succcesful. Long term incubation

of albumen glands from virgin animals with even small quantities of hemolymph from reproducing snails had a toxic effect on organ cultures. This phenomenom has been reported for another freshwater snail, *Biomphalaria glabrata*, and is probably due to the oxidation of hemoglobin or the interaction of another unidentified component of snail hemolymph (Hansen, 1976). From the biochemical and physiological studies conducted in this thesis, it can be concluded that if the dorsal body hormone of *Helisoma duryi* is an ecdysteroid, it is not likely 20-hydroxyecdysone but could be an unusual ecdysteroid-related molecule whose biochemical detection and physiological effect(s) have eluded current assay methods. Metabolic labelling studies by Garcia *et al.* (1986) have demonstrated that pulmonate snails are capable of forming novel ecdysteroid-related molecules such as 16β,20-dihydroxyecdysone (malacosterone) or unusual conjugates (20-hydroxyecdysone 22-acetate). It is unknown if these metabolites are biologically active. The observation of an additional bioactive hydrophobic substance (distinct from ecdysteroids), which could be extracted from DB tissue and DB-conditioned media, and the presence of a novel cytochrome P450 gene expressed solely in the dorsal bodies (Theunissen *et al.*, 1992) suggest further characterization of this substance should prove informative.

The examination of albumen gland synthetic activity is a useful method to determine the relative activity of glands from animals under different reproductive conditions (mated vs virgin) but may not be sensitive enough to detect more subtle changes in albumen gland activity. As the albumen gland secretory cells normally contain a certain amount of secretory material, the responsiveness of the albumen gland to stimulatory substances can sometimes

vary depending on how 'full' the cells are. Preliminary observations on SDS-PAGE separated albumen gland proteins revealed distinct protein bands which were present in higher amounts in reproducing snails compared to virgins, suggesting reproductive status may alter the synthesis or expression of various proteins in the albumen gland involved in the synthesis or release of secretory products. Since the dorsal body hormone of *Helisoma duryi* is suggested to be a lipophilic molecule(s), and these substances generally exert their effects by altering processes such as gene expression (Reichel and Jacob, 1993), the effect of the dorsal bodies on gene expression in virgin albumen glands may provide a novel assay for detecting dorsal body hormone activity. This type of molecular screening can be accomplished by differential display polymerase chain reaction (DD-PCR) and has been successfully used to isolate and identify many bioactive substances regulating development in coelenterates (Takahashi *et al.*, 1997).

The partial characterization of a peptidergic factor from the brain of *H. duryi* which acts upon the albumen gland to elevate intracellular cAMP levels, ultimately evoking the secretion of perivitelline fluid is also a noteworthy contribution. It provides the first demonstration in molluscs of a neurosecretory factor from the brain mediating a specific physiological event in an accessory reproductive gland through a known signal transduction cascade. Two reproductive neuropeptides from *Lymnaea stagnalis*, the caudodorsal cell hormone and calfluxin are ineffective in stimulating protein secretion from the albumen gland of *Helisoma duryi*. Also, the neuroactive agents FMRFamide, YGGFMRFamide, small cardioactive peptide B, and serotonin were without stimulatory effect (Morishita *et al.*, 1998),

whereas the biogenic amine dopamine was capable of inducing protein secretion (this thesis). At present, it is not known whether dopamine acts directly on the albumen gland secretory cells to promote secretion, or indirectly by triggering the release of peptide from neurosecretory terminals around the cells. Clearly, more work is needed in this area.

Based on the results from this study, and together with previous observations in the Basommatophora, the following scheme regarding the endocrine and neurosecretory control of reproduction in *H. duryi* is proposed (see Fig. 4). Some of the interpretations and speculations will undoubtedly prove incorrect in the long term, especially in areas where the data are fragmented, but it should provide a reasonable framework for future studies.

In virgin snails, the albumen gland secretory cells are packed full of secretory material since these animals are not laying eggs (Miksys and Saleuddin, 1985; this thesis). Therefore, a large increase in synthetic activity is not required to maintain sufficient reserves of secretory material, hence glands from these animals display low synthetic activity. In contrast, the basal synthetic activity of glands from mated animals is significantly greater due to the demands of egg mass production. Copulation of virgin animals serves to activate the two known reproductive centers, the dorsal bodies and the caudodorsal cells. The mechanism by which these gonadotropic centers are activated may be through a nervous connection or perhaps via the production of a humoral 'matedness' factor (Kunigelis and Saleuddin, 1986).

The caudodorsal cells release an ovulation hormone (CDCH) which acts upon the ovotestis to induce the release of mature oocytes into the hermaphroditic duct. After fertilization, the eggs arrive at the carrefour where they are detected by intrinsic sensory nerve

endings (Brisson and Collin, 1980) which transmit a nervous or chemical (dopamine?) signal to the albumen gland. Does dopamine directly stimulate the release of secretory material from the gland by binding to, and activating specific receptors on the albumen gland? If so, what is the second messenger pathway(s) involved? Another possibility is that the ovulated oocytes triggers the release of the CNS peptide from axon terminals innervating the albumen gland. The brain peptide would then bind to, and activate, a G-protein-coupled receptor on the albumen gland membrane, activating intracellular signal transduction pathways linked to the production of cAMP, and possibly other second messengers. The increase in intracellular second messenger concentration induces the exocytotic release of secretory globules containing nutrient-rich perivitelline fluid for the egg. Since synthesis and secretion are closely coupled in most endocrine and exocrine cells (Harper, 1988; Peterson, 1992), the release of perivitelline fluid triggers increased synthesis within the albumen gland. This period of increased synthesis (~6 h) generally coincides with bouts of egglaying and is reflected by the peaks of synthetic activity in the albumen gland after mating.

In first mated snails, polysaccharide synthesis does not increase until 18-24 hours post-mating, even though the dorsal bodies are actively engaged in secretion well before this (Saleuddin *et al.*, 1989; Khan *et al.*, 1990a). It is speculated that the dorsal bodies exert control over albumen gland activity at various levels in both virgin and mated snails. In first mated snails, the release of the dorsal body hormone may provide the initial stimulus to activate or 'prime' the virgin albumen gland to respond to a peptide from the brain, which in turn, evokes the release of secretory material. The dorsal body hormone might accomplish

this via a nuclear mechanism, for example, by acting on specific mRNA synthesis to increase the number of receptors to a peptide messenger from the brain. This may explain the attenuated response of the 'virgin' gland to brain extract as compared to the response from a 'mated' gland . Alternatively, the dorsal body hormone may act further downstream of receptors by altering the amount of cyclic nucleotide-dependent protein kinases or the amount of substrate available to them, thus tailoring the physiological response of the albumen gland to extracellular stimulatory or inhibitory signals.

In addition to controlling specific functions such as albumen gland synthetic activity and vitellogenesis, the dorsal bodies also have long term effects in stimulating the growth and cellular differentiation of the female accessory sex organs. In randomly mated snails, the dorsal bodies are proposed to have a long term effect on the albumen gland by sustaining a high basal rate of synthesis within the gland which is required for egg mass production. Support for this stems from the observation that ablation of the dorsal bodies reduced the synthetic activity of the albumen gland to levels comparable to glands from virgin animals (Miksys and Saleuddin, 1985), and caused a reduction in female accessory gland wet weight (Miksys and Saleuddin, 1987b). Furthermore, the basal intracellular levels of cAMP were also significantly higher (2-3 fold) in mated animals as compared to virgins (this thesis). The increased basal concentrations of cAMP in mated snails might function to maintain high albumen gland synthetic activity in reproductively active animals. It is well known that protein kinase A is able to phosphorylate transcription factors which can then interact with specific DNA sequences to activate cell-specific mRNA for activities such as protein synthesis

(Karin and Smeal, 1992; Sassone-Corsi, 1994). In the future, it should be interesting to determine the effect of dorsal body ablation on basal albumen gland cAMP levels, and what effect long term alteration of cAMP concentration has on its synthetic activity, or its ability to secrete perivitelline fluid in response to the brain peptide. The partial characterization of the dorsal body hormone of *Helisoma duryi* as a lipophilic substance, its observed pleiotropic functions in various pulmonates, and its proposed mode of action presented in this thesis is reminiscent of the mechanism by which the vertebrate reproductive steroids, and the insect juvenile hormones are thought to regulate reproduction (Wyatt and Davey, 1996).

The development of very sensitive myotropic bioassays, immunoassays, and significant improvements in analytical equipment (HPLC, protein sequencers, mass spectrometry) during the last 10-15 years has allowed investigators to isolate and identify an unprecedented number of novel bioactive substances (mainly peptides) from molluscs, some of which have been implicated to regulate reproductive functions. This has resulted in a situation referred to as 'reverse endocrinology'in which the structures of many different compounds are known, but not their functions. A careful scrutiny of the literature reveals there are only a few chemically identified substances in molluscs for which there exists substantial physiological evidence for a role in reproduction. For example, the egg laying peptides from *Aplysia* and *Lymnaea* and the FMRFamide-related peptides in pulmonates. The great majority of molluscan peptides have been isolated using myotropic bioassays. Molluscan muscle and invertebrate muscle in general, appears to be regulated by a multitude of neurally-derived compounds, and is therefore a relatively non-specific bioassay. Since one can only detect bioactivity for which

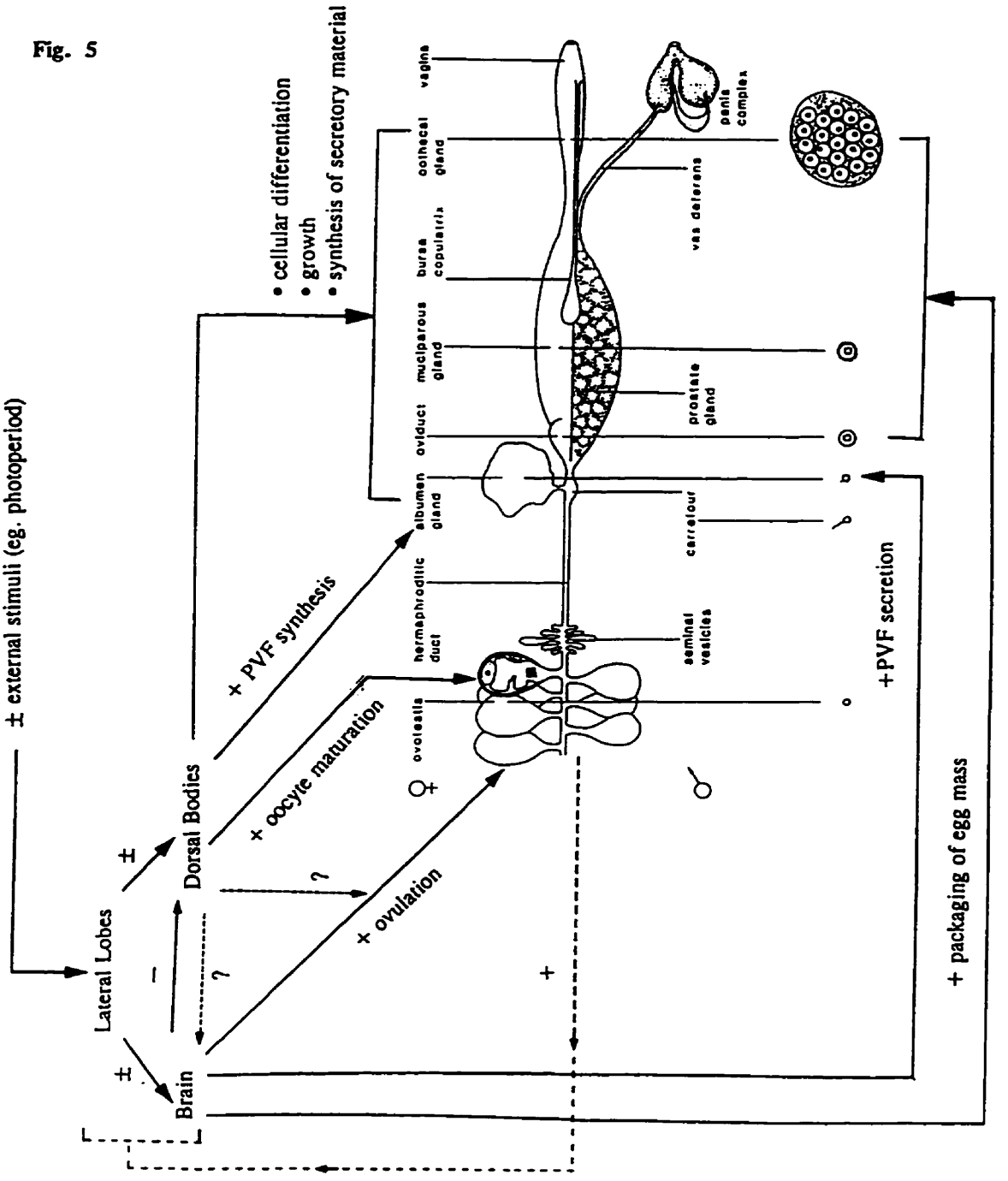
one is assaying for, this may or may not be the primary function of these peptides. Other than their ability to alter muscle contraction, little else is known regarding their synthesis or even if they are released under physiological conditions. Thus, the challenge facing molluscan reproductive physiologists will be to develop sensitive bioassays having greater specificity, especially concerning those processes presumed to be under hormonal control. Moreover, anaesthetization, surgical procedures, and post-operative survival for most molluscs, particularly small aquatic species requires further improvement before a better understanding of hormonal control mechanisms are achieved.

Although this thesis examines a relatively small area of molluscan reproductive physiology, it nonetheless provides important information regarding some of the physico-chemical properties of the dorsal body hormone in *Helisoma duryi* and its role in reproduction. In addition, a foundation is established with respect to the neurosecretory control of perivitelline fluid secretion and its mediation through an intracellular signal transduction pathway. Finally, this thesis points out important observations on some of the pitfalls and technical difficulties encountered when studying molluscan reproductive endocrinology, some of the gaps in our current knowledge, and suggests possible approaches for future research.

Fig. 5 A model showing some aspects of the endocrine and neurosecretory control of reproduction in *Helisoma duryi*. The model incorporates some features of what is known in a related snail *Lymnaea stagnalis*. External stimuli such as photoperiod, temperature, water quality, availability of food and mating stimuli can be received by the lateral lobes or the brain which transmit information to the reproductive centres (CDCs or DBs), which in turn stimulate (or inhibit) the synthesis/release of neuro(endocrine) substances. The brain and DBs are probably capable of influencing each others' activities. The DBH stimulates oocyte maturation in the ovotestis, possibly by affecting the uptake of yolk precursors (ferritin?). The gonad is believed to exert a positive feedback on the reproductive centres. The DBs also exert long term effects during reproductive activity by stimulating the growth and differentiation of the female accessory sex organs.

A factor from the brain (CDCH) or DBs induces ovulation of ripe oocytes from the ovotestis into the hermaphroditic duct. The DBs likely manufacture a steroidal product(s) which stimulates polysaccharide synthesis in the albumen gland. As the oocytes arrive in the carrefour region they receive a coat of perivitelline fluid. A peptide from the brain or the biogenic amine dopamine evokes the release of proteins and polysaccharides from the albumen gland. This secretion of perivitelline fluid is mediated, at least in part, by the cAMP signal transduction pathway. Neurosecretory factors from the brain are also involved in egg mass formation by influencing the secretory and muscular activities of the female accessory sex organs, and to coordinate egg laying behavior.

Fig. 5


± external stimuli (eg. photoperiod)
Lateral Lobes
Brain
Dorsal Bodies
±
±
–
?
?
+
+ PVF synthesis
× oocyte maturation
× ovulation
• cellular differentiation
• growth
• synthesis of secretory material
vagina
oothecal gland
bursa copulatrix
mucoparous gland
oviduct
albumen gland
hermaphroditic duct
ovotestis
vas deferens
penis complex
prostate gland
carrefour
seminal vesicles
+PVF secretion
+ packaging of egg mass

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APPENDIX: Simultaneous Measurement of Radiolabelled Proteins and Polysaccharides in Albumen Gland Releasates

INTRODUCTION

The principal secretory product of the albumen gland of pulmonate snails is called perivitelline fluid (PVF). Galactogen, a high molecular weight molecule composed of β(1-3), and β(1-6)-linked galactose residues, and glycogen, represent the main polysaccharides in the PVF of pulmonates (Goudsmit, 1976), whereas a 66 kDa glycoprotein, is the most abundant proteinaceous component in the PVF of *Helisoma duryi* (Morishita *et al.*, 1998; this thesis). We have recently developed a rapid bioassay to measure the release of these two major components of PVF in the presence of a brain peptide (Morishita *et al.*, 1998).
This brain peptide stimulates the production of cAMP, which in turn induces the secretion of PVF. Forskolin, a potent activator of adenylate cyclase, increases PVF secretion by about 8-fold, and stimulates intracellular cAMP in the albumen gland (Chapter 5, this thesis).

In the freshwater snail *Biomphalaria glabrata*, the *in vitro* synthesis and release of PVF varies according to the reproductive state of the animal (Crews and Yoshino, 1989, 1991). Furthermore, endocrine factors are known to regulate the synthesis and release of albumen gland polysaccharides and proteins in *H. duryi* (see Saleuddin *et al.*, 1994, Saleuddin, 1998a; this thesis). The recent identification of a unique 66 kDa glycoprotein that is released by the albumen gland in the presence of brain extract, forskolin or cAMP analogues, prompted the notion this glycoprotein could be labelled with a radioactive

monosaccharide for secretion studies. In *H. duryi*, knowledge of the specific carbohydrate moieties of the 66 kDa glycoprotein and the nature of their covalent linkage to its protein is presently unknown. In the keyhole limpet *Megathura crenulata*, the monosaccharides galactosamine, galactose, and fucose were determined to be the major carbohydrate components from the glycoprotein of the egg vitelline envelope (Heller and Raftery, 1976). In the pulmonate snail *Lymnaea stagnalis*, the albumen gland was found to possess a highly active N-acetylgalactosaminyl transferase which was suggested to be involved in the synthesis of glycoproteins (Mulder *et al.*, 1995). Based on these biochemical studies, the monosaccharide galactose was chosen as a precursor for glycoconjugate labelling. In addition, radiolabelled galactose can serve as a precursor for the biosynthesis of the polysaccharide galactogen (Stangier *et al.*, 1995), therefore it should be possible to label both glycoproteins and polysaccharides in the same sample. Since forskolin was the most potent stimulator of PVF secretion we have tested so far, it was the compound of choice to determine if newly synthesized albumen gland secretory products are capable of being released under short term *in vitro* conditions.

MATERIALS AND METHODS

Radiolabelling of Albumen Gland Proteins and Polysaccharides

Albumen glands from egg-laying *H. duryi* were dissected free of surrounding tissue in sterile antibiotic saline and cut longitudinally into two equal-sized halves. The tissue pieces were rinsed thoroughly in saline and incubated for 48 h in 200 µl Medium 199 containing

either 0.5 μCi/ml ^{14}C-amino acid mixture or 1.0 μCi/ml^{14} C-galactose (both radioisotopes were purchased from Amersham Life Sciences, Oakville, Ont.). After the incubation, the tissues were rinsed in several changes of saline and then placed individually in Falcon 96-well tissue culture plates with 100 μl saline. The surrounding saline from galactose-labelled glands was removed every 20 min and transferred to 1.5 ml polypropylene microtubes. The well was quickly replaced with another 100 μl of fresh saline. An equal volume of 25% trichloroacetic acid (TCA) was added to the collected saline from each time point, and allowed to stand overnight at 4^{0}C. Precipitated proteins were centrifuged at 20, 000 x g (20 min), and the supernatant placed in a separate tube. The pellet was washed with 12.5% TCA, centrifuged, and the supernatants were pooled. The TCA from the pellet was removed with a mixture of ethanol:ether (1:1), centrifuged again, and the supernatant discarded. The residual organic solvent was dried under a stream of N_2 gas. The pellet was redissolved in 50 μl 0.1 M NaOH, and 1 ml of aqueous counting scintillant (ACS, Amersham) was added to the tubes. The radioactive proteins were quantified by liquid scintillation counting. Two volumes of 100% ethanol was added to the TCA supernatants and allowed to stand overnight at 4^{0}C. The labelled polysaccharides were pelleted by centrifugation, and the pellet washed with 66% ethanol, and centrifuged once more. The supernatants were discarded and the pellet was dried. The pellet was resuspended in 50 μl of distilled water, mixed with 1 ml ACS, and counted. Basal protein and polysaccharide secretion was measured for the first 60 min, then forkolin (100 μM, final concentration) was immediately added for 20 min and its effect on secretion was monitored for another 60 min. A similar protocol to the one

described above was followed for determining the release of radiolabelled proteins from albumen glands incubated in ^{14}C-amino acids. Results are expressed as dpm protein or polysaccharide released/mg tissue weight/20 min.

RESULTS AND DISCUSSION

Initially, albumen glands were radiolabelled with an amino acid mixture. The basal release of radiolabelled protein from albumen glands incubated with amino acids shows a linear decrease during the first 60 min of culture (Fig. 1). Application of forskolin (100 μM), a potent adenylate cyclase activator, induces a three-fold increase in release of labelled protein into the medium. Although forskolin was applied for only 20 min, a strong stimulatory effect persists for at least another 40 min. This occurrence was also observed when non-radioactive protein release was determined after forskolin stimulation (data not shown). The magnitude of release of radiolabelled proteins is about half of that seen when measuring conventional protein release due to the dilution of the isotope with amino acids in the culture medium.

Sugar nucleotides such as UDP-galactose and UDP-N-acetylgalactosamine are the immediate precursors for polysaccharides (galactogen) and glycoproteins respectively. However, since these molecules cannot be readily taken up by cells or tissues in culture, the metabolic labelling of oligosaccharides is typically accomplished with radiolabelled monosaccharides (Varki, 1989,1994). When albumen glands are labelled with galactose, and then treated with forskolin for 20 min, a three to four-fold increase in release of radiolabelled secretory products is observed (Fig. 2). Even after forkolin is removed, there is an

augmentation of release for the next 20 min. Thereafter, the levels of secretory material begin to decline and approach their basal rates. The concentration of forskolin (100 μM) used in these studies is pharmacological, which might account for the prolonged release after it is removed. It is also possible another signal transduction pathway is activated with or by cAMP, which serves to enhance the release of secretory material.

The time courses for the release of labelled proteins and polysaccharides are indistinguishable, suggesting that these secretory products are co-released (Fig. 2). This supports the morphological studies of the pulmonate albumen gland which indicate both protein and polysaccharide components coalesce to form large (1-10 μM) secretory vesicles (Nieland and Goudsmit, 1969; Cousin *et al.*, 1995). Light microscopic studies of forskolin-treated albumen glands show the secretory vesicles are released from the cytoplasm of the glandular cells into the lumen of the ducts, and finally extruded into the surrounding medium (Morishita *et al.*, 1998). These observations suggest that radiolabelled galactose should serve as a valuable precursor in future studies to examine the effect of endocrine factors on both glycoprotein and polysaccharide synthesis/release in a single piece of albumen gland.

Fig. 1 Release of radiolabelled protein from albumen gland pieces following forskolin stimulation. Pieces of albumen glands were maintained in Medium 199 containing 0.5 μCi/ml ^{14}C- amino acid mixture for 48 h. After the incubation, the glands were washed thoroughly in normal saline and the release of radiolabelled TCA-precipitable proteins into the surrounding medium was quantified every 20 min by liquid scintillation counting. Basal release of labelled proteins was determined for 60 min, then forskolin (100 μM) was added between 60-80 min. The medium surrounding the tissue was replaced with normal saline from 80-140 min. Note that in the presence of forskolin (solid horizontal bar) a marked increase in the release of radiolabelled proteins was observed. Each point represents the mean ± SE of 6 samples.

Fig. 1

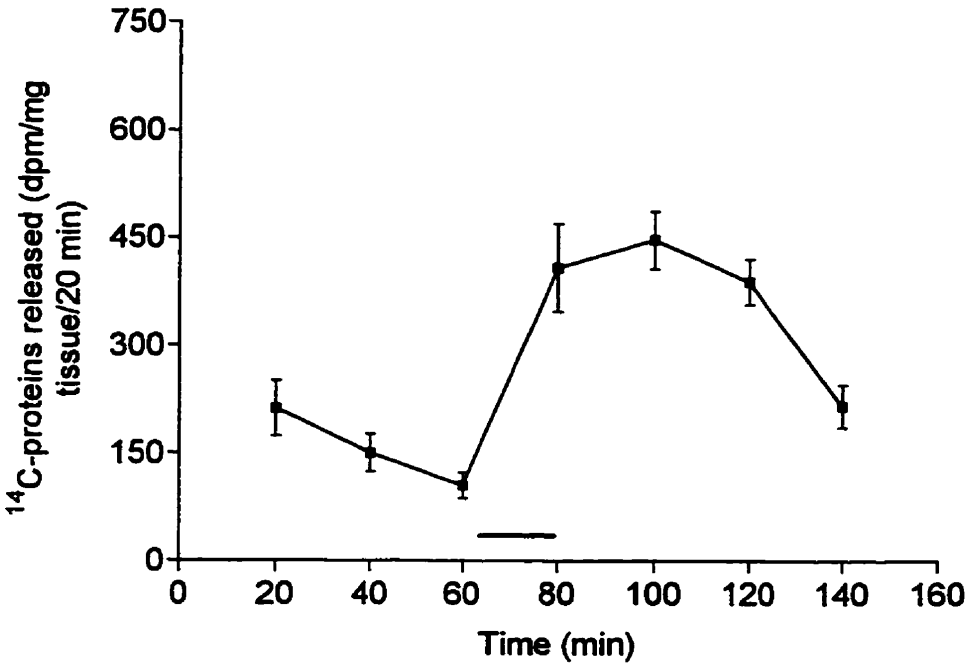
750
600
450
300
150
0
^{14}C-proteins released (dpm/mg tissue/20 min)
0
20
40
60
80
100
120
140
160
Time (min)

Fig. 2 Co-release of radiolabelled glycoproteins and polysaccharides from albumen gland pieces. Albumen glands were maintained in Medium 199 containing 1 μCi/ml ^{14}C-galactose for 48 h. After the incubation, the glands were washed thoroughly in normal saline and the release of radiolabelled glycoproteins and polysaccharides into the medium determined every 20 min. Radiolabelled glycoproteins were TCA-precipitated, centrifuged, and the pellet quantified by liquid scintillation counting. To the resultant TCA supernatant were added two volumes of 100 % ethanol, and the precipitated polysaccharides were quantified as mentioned above. Note that forskolin (100 μM) evokes a significant increase in release of both labelled glycoproteins and polysaccharides, and that the time courses of release of secretory products were identical. The solid horizontal bar denotes the time at which the glands were treated with forskolin. Each point represents the mean ± SE of 6 samples.

Fig. 2

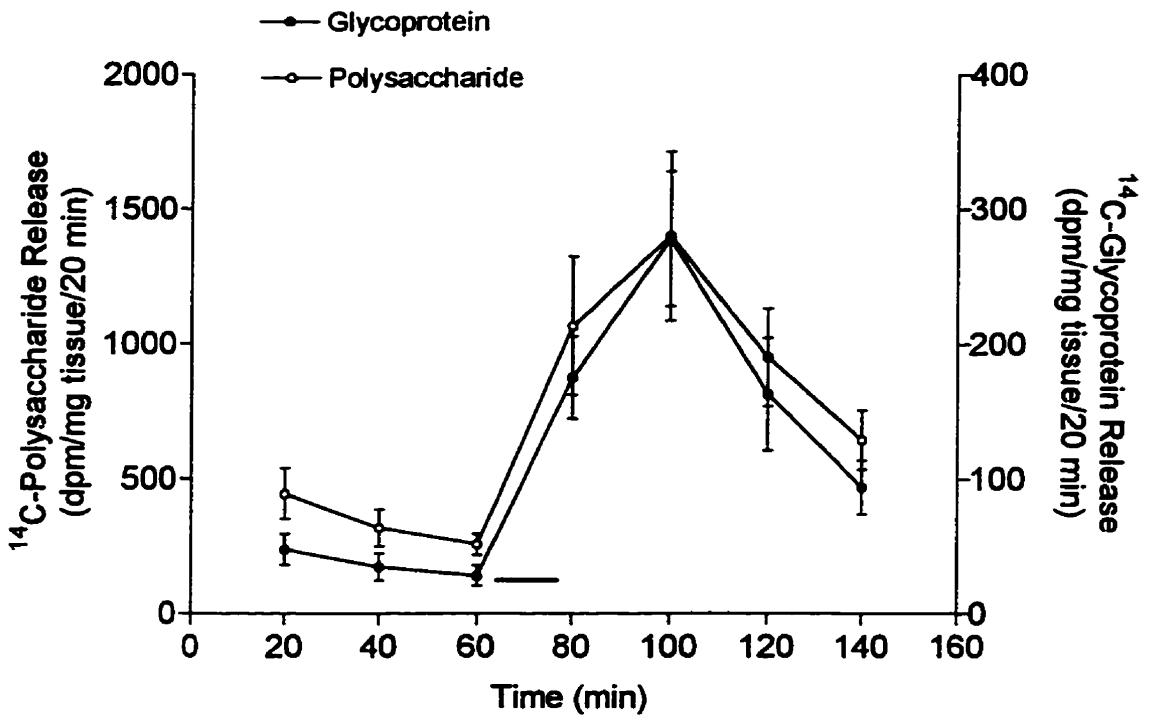
Glycoprotein
Polysaccharide
2000
1500
1000
500
0
400
300
200
100
0
0
20
40
60
80
100
120
140
160
Time (min)
14C-Polysaccharide Release (dpm/mg tissue/20 min)
14C-Glycoprotein Release (dpm/mg tissue/20 min)

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IMAGE EVALUATION
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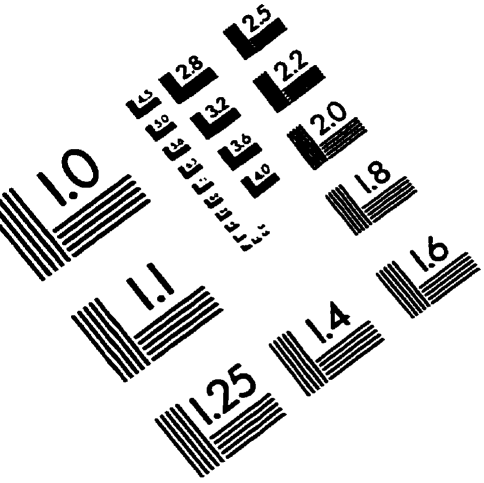

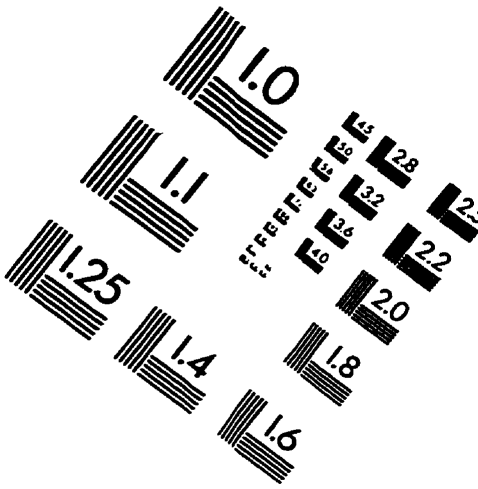

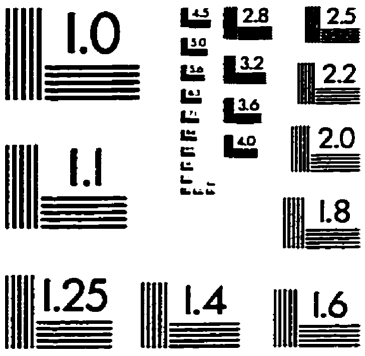

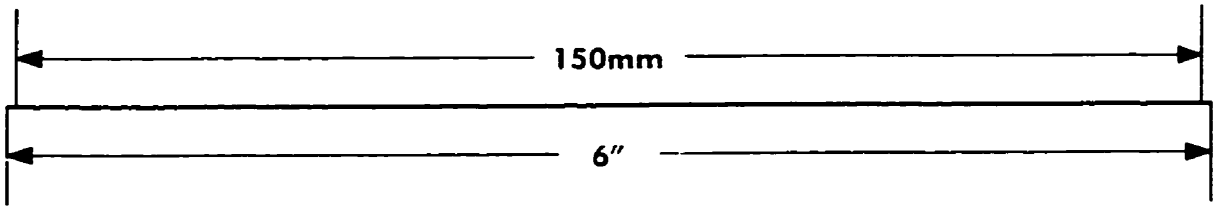

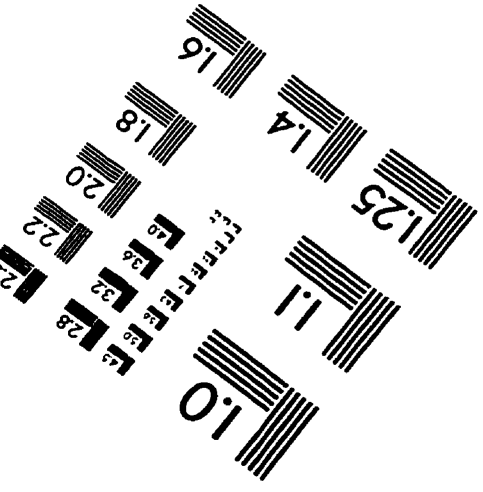

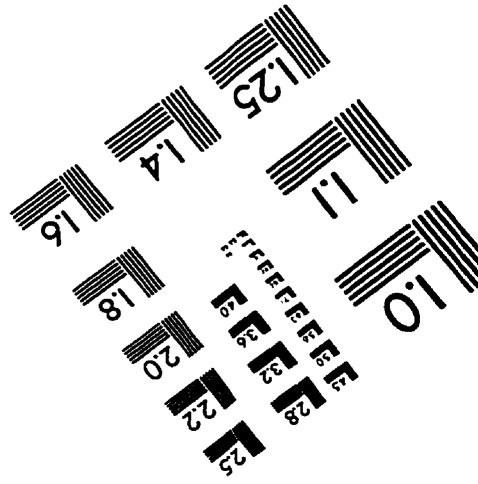

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