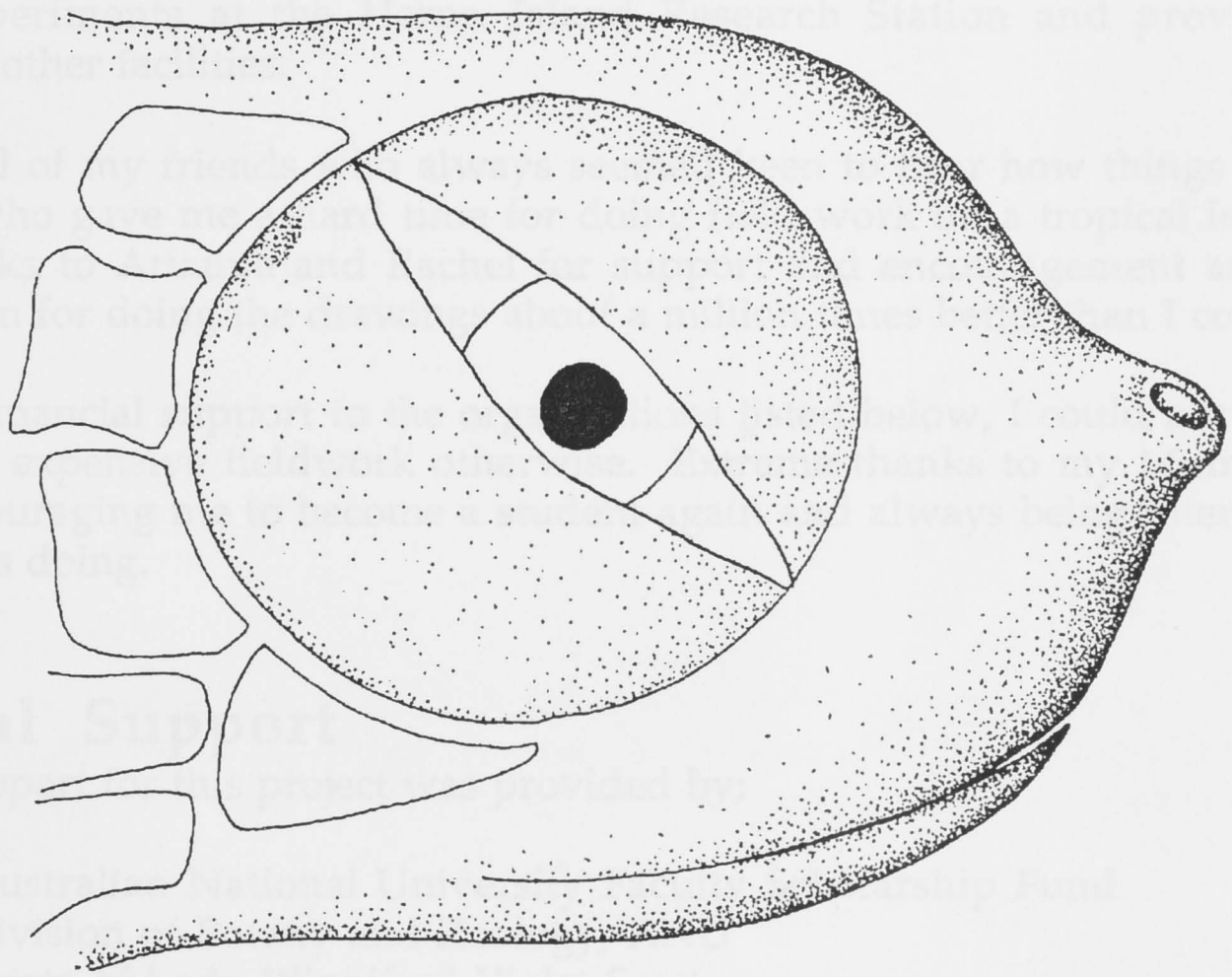
Regulation of salt gland function in the green sea turtle, *Chelonia mydas*.



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Richard David Reina
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Prior to their commencement, experiments conducted in this thesis were examined and approved by The Australian National University Animal Experimentation Ethics Committee and The Great Barrier Reef Marine Parks Authority (GBRMPA). Permit numbers were;

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Declaration

I declare that the research presented in this thesis is my own work and has not been submitted towards any other degree. I have conducted all experiments myself and the work of field assistants has been limited to collection and care of study animals.

Abstract

Regulation of salt gland function in the hatchling green sea turtle *Chelonia mydas* was investigated to test a model proposed for its control. The salt gland is an efficient means of extra-renal salt excretion used by marine turtles to remove excess salt while minimising associated water loss. The mechanisms by which marine turtles regulate the function of the salt gland have not previously been investigated and are poorly known. Possible sites of control were identified and experiments were conducted which examined these possible sites of control. Experimental goals were; 1) to quantify the salt gland response to salt-loading and measure a number of blood parameters in association with secretory activity, 2) to determine the response of the salt gland to exogenous application of possible modifying chemicals *in vivo*, 3) to examine gland histology, reconstruct serial sections and measure morphometry of gland components, 4) to quantify changes in capillary blood flow in the salt gland following salt-loading and chemical application *in vivo*, and 5) to measure the oxygen consumption of salt gland slices *in vitro* following removal from salt loaded and control animals, as well as measure the effect of application of possible secretory modifiers *in vitro*.

The salt gland responds rapidly to injection of a salt load greater than 400 - 600 μmol NaCl.100g BW^{-1} to produce tears containing approximately 800 - 900 $mmol.l^{-1}$ sodium and chloride, with the rate of sodium removal about 800 μmol Na.100g $BW^{-1}.hr^{-1}$. Plasma osmolarity and sodium concentration both rose following salt loading, but fell as secretory activity by the salt gland continued.

Exogenous application of chemicals was able to influence the activity of the salt gland. Adrenalin and methacholine (a cholinergic agonist) inhibited salt gland activity, while atropine (a cholinergic antagonist) stimulated salt gland activity when applied simultaneously with a sub-threshold salt load. The peptide arginine vasotocin (AVT) has a transient inhibitory effect on salt gland secretion, while atrial natriuretic peptide, vasoactive intestinal peptide, neuropeptide Y, and molsidomine (a nitric oxide donor) did not influence secretion under the conditions of the experiments. Immunohistochemical technique confirmed earlier histochemical evidence of adrenergic innervation in salt gland.

Histological technique showed that salt gland tissue had the characteristic arrangement of secretory cells forming tubules which drained into a central canal. Three dimensional reconstruction of serial sections revealed a branched arrangement of central canals draining into a main collection duct. Morphometric analysis of an active and inactive gland showed that blood vessel volume was twenty times greater in the active gland, with a slight increase in central canal volume, while tubule lumen volume did not differ.

Blood flow through salt gland capillaries was measured using coloured microspheres injected into the systemic circulation. Blood flow through salt gland capillaries was about 180 times greater in active glands than in inactive glands, indicating that circulation was a site of regulation. Exogenous atropine increased blood flow although tears were not produced, and adrenalin and methacholine decreased capillary blood flow concomitant with inhibition of tear production.

The oxygen consumption of salt gland slices *in vitro* was measured as an indicator of energy-dependent ion transport. Glands were taken from animals which had been salt-loaded as well as those which were not, but there was no significant difference in oxygen consumption, with both about 35 μmol O_2.g wet weight^{-1}.hr^{-1}. Addition of methacholine or adrenalin to the incubation medium did not change the respiration rate of salt gland tissue, but did influence the respiration of cardiac tissue in the same preparation. It was calculated that the measured rate of oxygen consumption was sufficient to fuel observed secretion rates *in vivo*. It did not appear that secretory cells were a site of direct regulation of salt gland activity under the experimental conditions.

A revised secretory model is presented which identifies blood circulation within the salt gland as being the primary site of control of gland activity. This control is exerted by adrenergic and cholinergic nerves to regulate the supply of blood, oxygen and metabolic substrates to the secretory tubules and so indirectly control the ion transport activity of secretory cells. Two alternative secretory models are also given which rely on the conduct of further experiments to clarify. These differ in that the presence of cholinergic nerves is questioned and it is suggested that the influence of methacholine on gland activity is through an indirect and unrelated action. Future directions of research are suggested which will increase understanding of salt gland function and further test the models presented.

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CHAPTER 1
General Introduction

1.1 OSMOREGULATION IN THE MARINE ENVIRONMENT

Life in the marine environment presents a considerable osmoregulatory challenge to vertebrate animals. The concentration of sodium chloride and other salts in the internal fluids of nearly all vertebrates is much lower than that found in seawater, so that there is a tendency for ions to enter the body and water to be lost to the environment. Ions which enter the body in excess of requirements must be removed, and potential water loss associated with excretion of excess salt is of major physiological significance. Water loss associated with ionoregulation and across the integument means that the ocean can be a desiccating environment and so water must be conserved by marine vertebrates by utilising mechanisms for concentrated salt excretion.

Marine mammals possess a kidney capable of producing urine in which the salt concentration exceeds that of seawater (Schmidt-Nielsen, 1990). Consequently, for any given volume of seawater that enters the body, the salt acquired can be excreted in an equal or lesser volume of water and no net water loss occurs. However, other classes of marine vertebrate such as sea turtles do not have a kidney with the mammalian concentrating ability (Dantzler, 1976; Evans, 1979; Willoughby and Peaker, 1979). In the absence of some extrarenal osmoregulatory mechanism, the ionic concentration of body fluids would increase and exceed physiological tolerance, ultimately leading to death.

Various secretory glands have been developed by vertebrates to excrete concentrated salt solutions and overcome the problem of water conservation. Teleost fish possess chloride transporting proteins on the gill epithelium (Mayer-Gostan *et al.*, 1987; Staurnes, 1993), elasmobranchs have a rectal salt-secreting gland (Silva *et al.*, 1977; Stoff *et al.*, 1979), marine reptiles (sea snakes, sea turtles, marine iguanas and salt water crocodiles) utilise a variety of cephalic glands (Schmidt-Nielsen and Fänge, 1958; Dunson and Dunson, 1974; Taplin and Grigg, 1981) and marine birds (such as the albatross and gull) possess paired nasal glands (Schmidt-Nielsen *et al.*, 1958). Although there are a number of structural, physiological and biochemical differences between the gland types, they all share the common

feature of elaborating a solution of sodium, chloride and sometimes potassium which has a concentration greater than that of seawater. With the exception of the teleost gill, these are collectively referred to as "salt glands". Thus, through concentrated salt gland secretions large quantities of salt can be removed with little water loss, which could not be achieved through the kidney of these vertebrates alone. Considerable research has been undertaken into the function of elasmobranch and avian salt glands, however, the nature of most reptilian salt glands remains relatively unexplored.

1.2 EVOLUTIONARY RELATIONSHIPS OF ANIMALS POSSESSING SALT GLANDS

Salt glands are possessed by distantly related vertebrate groups. It seems likely that the reptilian ancestor of modern birds possessed a nasal salt gland because salt glands are not found in any other location in birds, unless multiple, convergent evolution of the gland occurred. The location of the gland among living reptilian families may be orbital, lingual, sublingual, nasal or premaxillary (Figure 1.1). The evolutionary history of reptilian salt glands is unclear because of the variety of cranial locations in which they are found. Marine turtles (chelonians), sea snakes and crocodilians must have independently evolved different non-nasal salt glands which enabled them to osmoregulate in a marine environment. Nasal salt glands may be unsuitable for a marine existence because the external nares must be closed when diving, hence preventing secretion of fluid through the nostrils. However, secretions from orbital glands can be flushed away with water when diving and oral glands can be flushed with water, as the glottis prevents water passage into the digestive system. Terrestrial lizards cannot flush glands with water, so salt glands which empty into the nasal cavity are most suitable because secretions can be easily expelled by exhaling. These differences in gland location strongly suggest that avian and reptilian salt glands are not homologous structures but have an analogous function. The nasal salt glands of birds show many structural similarities to the reptilian cranial salt glands, despite differences in embryological origin (Schmidt-Nielsen, 1960). The rectal gland of elasmobranchs shows many similarities to avian and reptilian salt glands in the secretory role that it plays. It is important to be aware that physiological and biochemical mechanisms operating in the avian and elasmobranch salt gland may bear little resemblance to those yet to be elucidated in the reptilian salt gland because of likely differences in evolutionary development of the glands. However,

important clues may be gained from a comparative examination of the mechanisms operating for elaborating concentrated salt solutions.

1.3 PHYLOGENETIC SIGNIFICANCE OF THE CHELONIAN SALT GLAND

Chelonians possess skull morphological characters not found in any other living reptile, bird or mammal (Goin *et al.*, 1978). The Cotylosauria ("Stem reptiles") from the Upper Carboniferous period 310 million years ago also possessed these characteristics and it is thought that the turtles probably represent the most ancient and continuous link with these reptilian ancestors (Bellairs and Attridge, 1975). From the Cotylosauria arose all reptiles, birds and mammals, both living and extinct, but in these other animals the characteristic skull morphological features have been lost. The sea turtles are thus members of an order that is in some ways most likely to reflect the ancestral state of all living reptiles and birds. In comparison to other vertebrates, the salt glands of sea turtles have received very little attention since their discovery and they may reveal information regarding early vertebrate osmoregulatory mechanisms.

1.4 THE GREEN SEA TURTLE, *CHELONIA MYDAS*

The green sea turtle, *Chelonia mydas* is a species of turtle which lives in tropical and subtropical seas throughout the world with large populations around the coast of Queensland, Australia (Limpus and Reed, 1985). The turtles lay eggs on coastal and island beaches, but otherwise spend their entire lives at sea, migrating between feeding and breeding areas from time to time. Hatchlings are primarily carnivorous but adults are herbivorous; the preferred diet changes sometime during the juvenile years but the exact age is unknown (Bjorndal, 1985). The green turtle is a suitable subject for investigation of reptilian salt glands, as they possess functional salt glands at hatching, always remain in a marine environment (unlike the estuarine turtle and salt water crocodile) and exist in reasonably large populations in Australian waters. Access to adult turtles as experimental animals is restricted, as they are protected in Australia, difficult to catch and difficult to handle because of their large size. However, the hatchling turtles are more suitable as experimental subjects because they hatch on land and are easily collected. Mortality rate of hatchling turtles is extremely high, primarily due to predation in waters close to shore (Bustard, 1970), so the impact on populations of collection of hatchings is much less than collection of adults. Thus for both practical and conservation reasons, investigations into the

function and regulation of salt gland of *Chelonia mydas* in this thesis used hatchlings as the experimental subject.

The mechanisms of control of chelonian salt glands are not known, but comparative information provides a useful background from which to consider likely possibilities worthy of investigation. Structural features, nervous and endocrine influences are important indicators of possible mechanisms of regulation. Comparative data on these features as well as information known about sea turtle salt glands are important to form hypotheses about regulation of the chelonian salt gland. The rest of this review will describe the information currently known about activity, structure and modifiers of the salt gland so that a model may be proposed for experimental investigation of regulation of the hatchling green turtle salt gland.

1.5 COMPARATIVE SALT GLAND ACTIVITY

1.5.1 Reptiles

The salt gland of sea turtles was first identified by Schmidt-Nielsen & Fänge (1958) as a lachrymal gland located in the orbit behind each eye with a main secretory duct opening onto the posterior corner of the eyelid (Figure 1.1a). Secretion can be experimentally initiated in animals with an injected sodium chloride load. Increase in plasma sodium concentration appears to be the critical factor in initiating secretion, as increase in plasma osmolarity alone by sucrose injection is not effective (Marshall and Cooper, 1988), nor is injection of potassium chloride. The osmotic concentration of tears is approximately twice that of seawater and tears are composed almost entirely of sodium and chloride with a small amount of potassium present. The rate of tear production ranges from about 6 to a maximum of 5600 $\mu l.100g^{-1}.hr^{-1}$ in adults and hatchlings respectively (Prange and Greenwald, 1980; Marshall and Cooper, 1988).

A net water gain can be achieved from ingestion of seawater, as the salt contained in any given volume can be excreted by the salt gland in a lesser volume of fluid. This net water gain can be used to satisfy physiological water requirements such as the removal of nitrogenous wastes via the urine. Unfed green (*Chelonia mydas)* and loggerhead (*Caretta caretta*) turtle hatchlings have been observed to gain weight over several days through drinking seawater in response to dehydration (Bennett *et al.*, 1986; Marshall and Cooper, 1988).

The salt glands of different reptiles produce secretions which vary in osmotic concentration and the major salts produced. This difference in ionic composition of secretions is probably largely due to differences in dietary and environmental ionic composition as well as access to fresh water. The ratio of sodium to potassium in secretions from some herbivorous lizards approaches 1:100 (Minnich, 1979), whereas the ratio in sea turtles is approximately 30:1 (Marshall and Cooper, 1988) reflecting the ratio of these ions encountered in the food and the environment. Other reptiles demonstrate abilities to concentrate different ions between that of the herbivorous lizards and the marine turtles although the concentration of the primary ions excreted is always higher than in the plasma.

There are several species of entirely marine snakes, the most studied of which are probably the yellow banded (*Laticauda semifasciata*) and yellow bellied (*Pelamis platurus*) sea snakes. There was an initial confusion as to the identity of the salt glands of these snakes, but it subsequently became apparent that they possessed modified lingual or sublingual poison or salivary glands which served this function (Figure 1.1c). The salt secreting ability of sea snakes is not great, ranging from about 30 to 165 μmol $Na.100g^{-1}.hr^{-1}$ (Dunson and Dunson, 1974) at a concentration of 400 to 900 $mmol.l^{-1}$ NaCl.

A salt secreting gland in crocodiles was first positively identified in the estuarine crocodile *Crocodilus porosus* (Taplin and Grigg, 1981). It is a modified lingual salivary gland with many pores which open onto the surface of the tongue (Figure 1.1d). This permits animals to survive along the salinity gradients found in the estuarine and tidal rivers where many crocodiles live, as well as enabling water conservation during the dry season.

1.5.2 Birds

Although the nasal gland of marine birds had been described earlier, it was not understood to have a salt secreting function until secretions from the double crested cormorant, (*Phalocrocorax auritus*) were analysed by Schmidt-Nielsen, Jörgensen and Osaki (1958). They found a secreted solution containing about 520 $mmol.l^{-1}$ sodium with an approximately equal amount of chloride present. It was subsequently shown that marine birds could be stimulated to secrete by stomach loading or injection with

seawater and were capable of secreting the salt from large seawater loads. The black-backed gull (*Larus marinus*) secreted a load of 10% of its total bodyweight in about 3 hours, commencing minutes after loading and at rates from 600 to 2200 $\mu l.100g^{-1}.hr^{-1}$ containing up to 900 $mmol.l^{-1}$ sodium (Schmidt-Nielsen, 1960). The concentration of sodium in the secretion of other marine birds ranges from 500 to 1100 $mmol.l^{-1}$ at rates from 168 to 1362 $\mu l.100g^{-1}.hr^{-1}$ (Skadhauge, 1981). In those instances where both sodium concentration and rate of fluid production were measured, total sodium secretion rates ranged from 122 to 1067 $\mu mol\ Na.100g^{-1}.hr^{-1}$. It was subsequently found that non-marine birds which had marine ancestors (such as the Pekin duck) possessed an atrophied gland which could be stimulated to hypertrophy and become fully functional if animals were given 1 to 3% salt water to drink (Ellis *et al.*, 1963; Schmidt-Nielsen and Kim, 1964). The process of fully adapting to salt takes approximately two weeks, during which time changes in gland structure occur with an increase in gland size. The significant morphological and biochemical changes which take place include differentiation of cell types, formation of a branched duct arrangement and specialisation of secretory cell membranes (Ellis *et al.*, 1963; Ernst and Ellis, 1969; Ernst and Mills, 1977).

Secretion by the avian salt gland is initiated by an increase in plasma sodium as well as an increase in total plasma osmolarity by sucrose infusion (Schmidt-Nielsen *et al.*, 1958; Hanwell *et al.*, 1972) and is detected by osmoreceptors in the heart or great blood vessels. The relationship between salt gland secretion, extracellular fluid tonicity and extracellular fluid volume is complex and has been extensively reviewed (Simon, 1982; Gerstberger and Gray, 1993).

1.5.3 Elasmobranchs

The rectal gland of elasmobranchs responds to an increase in the volume of the extracellular fluid to produce a secretion of approximately 500 $mmol.l^{-1}$ sodium chloride which is isosmotic with plasma but does not contain the high concentration of urea (approximately 300 mM) found in the plasma (Silva *et al.*, 1990). The absence of urea permits sodium and chloride to comprise almost all of the osmotic pressure of the solution and thereby exceed the concentration of these ions found in the plasma. Secretion rates have been measured at 190 $\mu l.100g^{-1}.hr^{-1}$ with a rate of sodium removal of approximately 100 $\mu mol\ Na.100g^{-1}.hr^{-1}$ from the spiny dogfish, *Squalus acanthias* (Burger and Hess, 1960).

1.6 SALT GLAND STRUCTURE

1.6.1 Macroscopic structure

The salt glands of birds and reptiles show a large number of common structural features at both macroscopic and microscopic level. All have an arrangement of blind ended secretory tubules emptying into a series of progressively larger ducts (central canals) which then drain into the main collecting duct and to the external environment. Avian salt glands are situated in or around the orbit (Figure 1.2) and empty into the nasal cavity and out the external nares or along the internal nares to the tip of the beak (Peaker and Linzell, 1975). Reptilian salt glands empty into the nasal cavity (lizards), posterior corner of the eye (chelonians), or mouth (sea snakes and crocodiles). There appear to be different cell types present along the duct network (Schmidt-Nielsen and Fänge, 1958; Marshall, 1989), possibly contributing to the concentration of secretions. Unlike birds and turtles, the salt glands of lizards and sea snakes are not lobular, but they do retain the organisation of many branched secretory tubules which drain into collecting ducts and then an excretory duct (Dunson *et al.*, 1971; Minnich, 1979; Barnitt and Goertmiller, 1985). Crocodiles possess salt glands which show the same basic structural features as those in birds and turtles, but about 30 to 40 separate, small multilobular glands open onto the surface of the tongue (Taplin and Grigg, 1981; Franklin and Grigg, 1993).

1.6.2 Microscopic structure and cellular histochemistry.

The microscopic structure of avian and reptilian salt glands also show many similarities. The histochemical and ultrastructural features of the avian gland have been extensively reviewed by Gerstberger and Gray (1993) and will be briefly described here. In the individual secretory tubules, principal secretory cells are arranged radially around a central lumen, with peripheral cells at the blind ends (Ernst and Ellis, 1969). Peripheral cells are smaller, undifferentiated and do not appear to contribute significantly to the secretory process. Avian principal secretory cells show extensively folded basal and lateral cell membranes, which interdigitate and greatly increase the surface area of the cell. These extensive foldings possess many mitochondria and form many intracellular and extracellular compartments, features present only in birds adapted to salt water. The apical surface of the cells form the walls of the tubule lumen and many small microvilli are apparent. The lateral surfaces of principal cells form junctions at the lumen which appear to be "leaky" (Ellis *et al.*, 1977) and so form an extensive

intercellular channel which may permit fluid movement between cells and into the tubule lumen.

Histochemical staining of principal secretory cells indicates a high phospholipid, succinic dehydrogenase and cytochrome oxidase content, as well as a high level of Na-K-ATPase activity (Ellis *et al.*, 1963; Ernst and Ellis, 1969), suggestive of a major ion transport function. In contrast, peripheral cells are small, without folded membranes, possess few mitochondria and have low Na-K-ATPase activity. As salt adaption takes place, these cells divide and differentiate to form the principal cells as tubules increase in length and diameter.

The central canals and main collection ducts have multi-layered epithelia composed of small flat cells and large cylindrical cells with folded lateral surfaces, apical microvilli and other features which suggest that the ducts may also have a transport role in concentrating primary secretions from the secretory tubules (Marshall *et al.*, 1987).

The salt glands of all reptiles so far examined have a microstructure very similar to the avian salt gland (Figure 1.3). Principal secretory cells are found along the length of secretory tubules and show extensive folding of the lateral membrane, although the basal membrane remains relatively unfolded (Abel and Ellis, 1966; Dunson *et al.*, 1971; Lemire *et al.*, 1972; Taplin and Grigg, 1981; Franklin and Grigg, 1993). Many mitochondria are present and the level of Na-K-ATPase and oxidative enzyme activity is very high (Abel and Ellis, 1966; Lemire *et al.*, 1972). The infolding of lateral membranes differs slightly from the avian gland in that the space between cells is greater and interdigitation between neighbouring cells is not as extensive (Peaker and Linzell, 1975; Marshall, 1989). Unlike the avian gland, the intercellular space formed between adjacent lateral membranes of cells is filled with a mucopolysaccharide (Ellis and Abel, 1964). Apical and lateral membrane junctions join the cells in a manner similar to the avian gland. Small peripheral cells are found at the blind tubule ends which are similar in appearance to avian peripheral cells. The duct system appears stratified in the central canals, secondary and main ducts with the presence of wide intercellular spaces between large mucocytes (Marshall, 1989). It has been proposed that this arrangement serves an ion transport function in concentrating the glandular secretion as it travels down the ducts (Marshall *et al.*, 1987). The estuarine diamond-back terrapin *Malaclemys terrapin*

shows similar cellular arrangement although salt secreting ability is substantially less than the marine turtle (Dunson, 1970; Cowan, 1971).

Although the rectal gland of elasmobranchs has a more simple structure it nevertheless has many of the characteristic features described. The rectal gland of the dogfish *Squalus acanthias* has a single central canal which runs the length of the gland, with numerous secretory tubules arranged radially around and emptying into it. Secretory cells show folding of the basal and lateral membranes and have intercellular junctions similar to those in the avian gland (Bulger, 1965; Stoff *et al.*, 1979). The elasmobranch, avian and chelonian salt glands appear to show a clear example of convergent anatomy with independent evolution of similar salt-secreting structures.

1.7 ENDOCRINE MODIFIERS OF SALT GLAND ACTIVITY

A number of compounds have been reported to have some activity in control of salt glands in various animal species but have rarely been investigated in reptiles, particularly the chelonians. Comparative data may provide useful clues about possible control mechanisms operating in *Chelonia mydas*. Extensive discussion of the role of hormones on salt gland function can be found in reviews by Gerstberger and Gray (1993) and Peaker and Linzell (1975). A summary of hormone effects and the experimental conditions employed is presented in Table 1.1.

1.7.1 Steroid hormones

Early investigations into the role of hormones in salt gland function primarily focussed on aldosterone, corticosterone and the effect of adrenalectomy on the domestic duck (Holmes *et al.*, 1961; Phillips *et al.*, 1961; Holmes *et al.*, 1963). It is very difficult to identify the specific actions of the adrenal hormones on any particular gland without measuring and understanding the broader role they play, as they influence many physiological systems. Later experiments demonstrated that the effect of adrenalectomy on salt gland function was short-lived, with normal function resuming in seven days, provided animals were force-fed and kept well hydrated. Other evidence showed that the action of corticosteroids on the avian salt gland actually resulted from interference with normal cardiac function and prevented the increase in blood flow that the gland required for secretory activity (Butler, 1984; Butler, 1987). This led to the conclusion that there was no direct influence of steroids on salt gland activity, but through their numerous other actions, they played a permissive role

necessary for maintenance of normal function. Certainly the steroid influence on protein synthesis would be expected to play a major role in the adaptive process during hypertrophy of the avian salt gland (Peaker and Linzell, 1975).

The action of steroid hormones on reptilian salt glands is likely to be similar. Holmes and McBean (1964) investigated the action of corticosterone on salt gland activity in hatchling *C. mydas* by administering amphenone B to chemically simulate adrenalectomy. Following amphenone B treatment, the salt gland response to a salt-load was diminished and replacement therapy with corticosterone restored the response to control levels. However, there was no enhancement of the secretory response by corticosterone in the absence of amphenone B treatment, and secretion following a salt-load still occurred despite injection with amphenone B, at about 50% of control rates. Effects on blood flow or cardiac function were not measured. In any case, no direct effect on salt gland activity had been demonstrated and so it is likely that steroids in the turtle play a permissive role much the same as in the bird. It would be interesting to repeat the study of Holmes and McBean with normal animals, as the hatchlings that they used were extremely small for their age. They weighed only 50 to 60 g at six months of age compared with a normal weight of one to two kilograms at eight months (Wood and Wood, 1993). Clearly some factor must have retarded their development and may have been associated with abnormal adrenocortical function.

The action of steroids on salt glands of desert dwelling lizards is probably more complex, as there appears to be some involvement with the ability of these animals to regulate the cation and anion ratios in secretion. Adrenalectomy resulted in an elevated sodium concentration in salt gland secretions of the desert iguana, *Dipsosaurus dorsalis*, which was reversed by addition of aldosterone (Templeton *et al.*, 1972). Aldosterone in intact animals reduced the sodium concentration, but potassium concentration was unaffected. Mineralocorticoids were not capable of inducing salt gland secretion directly. It is possible that aldosterone acts to reduce sodium excretion by the salt gland so that potassium is preferentially secreted, although there is currently no evidence to support this suggestion. However, such a function would be consistent with the anti-natriuretic action of aldosterone on the kidney.

In the animals examined, steroid hormones apparently have a role in the long term regulation of salt glands to prepare them for activity, but they are not involved in direct stimulation or inhibition of secretion, with the possible exception of aldosterone in the desert iguana.

1.7.2 Arginine vasotocin

Arginine vasotocin (AVT) is a highly conserved peptide found in many vertebrate groups with a variety of actions on osmoregulatory organs, particularly the kidney. AVT is the only hormone reported to have initiated avian salt gland secretion in the absence of a salt-load (Peaker, 1971) although the secretion was slow, with an osmotic concentration lower than that normally following a salt-load. There seems to be a complex interaction with renal parameters affecting homeostasis which may well lead to changes in salt gland activity via indirect means such as changing plasma sodium concentration. AVT receptors are not detectable in the salt gland itself (Keil *et al.*, 1990) and high levels of AVT can be detected in the blood both when the gland is active and inactive (Mohring *et al.*, 1980; Gerstberger and Gray, 1993), so it appears unlikely that stimulation reported by Peaker (1971) was a direct effect.

AVT influences renal activity in reptiles also (Butler, 1972), but its effect on salt gland activity has not been investigated. An increase in circulating AVT levels is seen during nesting of sea turtles (Figler *et al.*, 1989) and is believed to stimulate egg laying (Owens and Morris, 1985). Tear samples collected from nesting *Chelonia mydas* during the period of egg laying showed that tear ionic concentrations were much lower than normal and were similar to plasma levels (Cooper, unpublished observations). The tears with a low ionic concentration produced by many nesting females may be a side effect of the high circulating levels of AVT, in a similar manner to that seen by Peaker (1971) in the goose. However, no studies have investigated this observation or determined whether there is a direct effect of AVT on salt gland function of reptiles.

1.7.3 Atrial natriuretic peptide

Atrial natriuretic peptide (ANP or ANF) acts on a number of vertebrate osmoregulatory organs where it generally causes increased diuresis and natriuresis (Oshima *et al.*, 1984; Keil *et al.*, 1990; Solomon *et al.*, 1992; Gunning *et al.*, 1993; Uva *et al.*, 1993). The peptide has been detected immunohistochemically in avian salt gland (Lange *et al.*, 1989). Infusion of

ANP briefly enhances the response of secreting glands to a salt-load in ducks (Schütz and Gerstberger, 1990) by binding to receptors distributed throughout the secretory tissue. Circulating levels of ANP increase significantly during hypertonic saline infusion (Gray, 1994). However, despite these circumstantial findings, the precise role played by ANP remains unclear.

Infusion of ANP will initiate chloride secretion by the dogfish rectal salt gland (Solomon *et al.*, 1985; Solomon *et al.*, 1992; Gunning *et al.*, 1993), in which it has been shown to stimulate the release of vasoactive intestinal peptide (VIP) from neural stores within the gland to increase gland activity (Silva *et al.*, 1987).

No studies have been conducted to investigate the role or activity of ANP in reptiles.

1.8 NEURAL MODIFIERS OF SALT GLAND ACTIVITY

The rapid activation of the avian and reptilian salt glands following a salt-load (Schmidt-Nielsen *et al.*, 1958; Marshall and Cooper, 1988) suggests neural initiation of secretion. Exocrine glands are commonly under neural control, with antagonistic sympathetic (adrenergic) and parasympathetic (cholinergic) innervation serving to inhibit and stimulate the secretory response respectively (Eckert *et al.*, 1988; Withers, 1992). In addition, a number of neuropeptides have been discovered which are manufactured or stored in peptidergic nerve terminals for release in response to neural stimulation to affect the target tissue. There may be a co-release of classical neurotransmitters such as adrenalin and acetylcholine with these neuropeptides to finely regulate organ function. A summary of neural influences on salt gland function is presented in Table 1.2.

1.8.1 Sympathetic and parasympathetic influence on the salt gland

The demonstration that injection of acetylcholine or methacholine (a cholinergic agonist) stimulated salt gland secretion in the herring gull *Larus argentatus* (Fänge *et al.*, 1958a) and the finding that anaesthetics suppress secretory activity indicated the importance of nervous control (Schmidt-Nielsen, 1960). As a consequence of these findings, extensive investigations were carried out into the type of innervation present in the avian salt gland. Histochemical studies showed the presence of adrenergic and cholinergic innervation associated with both secretory tubules and vascular elements

of salt glands in birds which had been acclimated to salt water (Ellis *et al.*, 1963; Ash *et al.*, 1969; Haase and Fourman, 1970; Peaker and Linzell, 1975). Another study found that acetylcholine led to vasodilation and adrenalin caused vasoconstriction of blood vessels supplying the avian salt gland (Fänge *et al.*, 1963).

The anterior branch of the seventh cranial nerve is considered to be the secretory nerve in birds (Ash *et al.*, 1969; Cottle and Pearce, 1970) carrying mainly parasympathetic innervation, a feature characteristic of exocrine glands. Cholinergic innervation was examined using histochemical techniques for detecting the presence of acetylcholinesterase. This revealed stained elements which ran between the columns of secreting cells and also along the walls of the ducts, as well as elements in the interlobular, intralobular, peritubular connective tissue and endothelial cells of glandular capillaries (Ellis *et al.*, 1963; Ash *et al.*, 1966; Ash *et al.*, 1969). These features are absent or greatly diminished in birds not adapted to salt water. Sympathetic adrenergic innervation was also revealed (Haase and Fourman, 1970) with a pattern of adrenergic nerve distribution which closely matched that of cholinergic fibres. In the goose most adrenergic fibres were present in the walls of blood vessels, suggesting a major role in the regulation of circulation (Peaker and Linzell, 1975). Other fibres passed along the secretory tubules, although it is unclear what role this innervation plays.

The first account of salt glands in a marine turtle by Schmidt-Nielsen and Fänge (1958) reported that salt gland secretion could be initiated by injection of 10 mg.kg bodyweight^{-1}* methacholine. Methacholine also stimulates salt gland function (Dunson, 1970) and increased oxygen consumption of dissociated salt gland cells *in vitro* (Shuttleworth and Thompson, 1987) of the euryhaline diamondback terrapin, *Malaclemys terrapin*. Methacholine will also stimulate salt gland secretion in the marine iguana, *Amblyrhynchus cristatus* (Schmidt-Nielsen and Fänge, 1958) and estuarine crocodile, *Crocodilus porosus* (Taylor *et al.*, 1995). These studies suggested that there might be a role for cholinergic innervation in control of the sea turtle salt gland although this has received relatively little attention, with the only study conducted being that of Abel and Ellis (1966). Using histochemical localisation of butyrylcholinesterase, they identified cholinergic nerve fibres which ramified through the peritubular connective tissue. Butyrylcholinesterase is a catabolic enzyme which will degrade both butyrylcholine and acetylcholine, but it is generally found associated with

* Here and subsequently, "bodyweight" properly refers to "body mass."

acetylcholinergic neurons, particularly in the lower vertebrates (Cooper *et al.*, 1991). Histochemical localisation of monoamine oxidase indicated the presence of putative adrenergic fibres which appeared to be restricted to the perilobular connective tissue. Abel and Ellis (1966) suggested that cholinergic innervation affected the interlobular arteries to influence blood flow to the gland, but the role of adrenergic innervation was unclear. However, cholinergic and adrenergic receptors have not yet been demonstrated in the gland, nor has there been immunohistochemical evidence. A recent study examining innervation of the lachrymal gland in the estuarine diamondback terrapin, *Malaclemys terrapin* failed to show any presence of cholinergic innervation using immunohistochemical techniques (Belfry and Cowan, 1995). Adrenergic innervation was shown in association with blood vessels in the gland. There is some question as to the precise function of the lachrymal gland in *Malaclemys* (Cowan, 1981; Cowan, 1990) with the possibility that it performs other activities not related to osmoregulation. It is possible therefore that its structure and control differ from the salt glands of marine turtles.

It has been widely assumed that the neural control of secretion in marine turtles was similar to that of marine birds, with cholinergic innervation initiating and maintaining gland secretion. However, the report by Schmidt-Nielsen and Fänge (1958) remains the only instance where cholinergic stimulation has been investigated in a strictly marine turtle. The role of adrenergic stimulation on salt gland activity has never been investigated in marine turtles. Thus the precise nature of neural influences on salt gland function in turtles is unclear and requires further investigation.

1.8.2 Nitric oxide influence

Some exocrine glands such as mammalian salivary glands are under the control of nitric oxide-containing nerves (Edwards and Garrett, 1993; Rand and Li, 1995). Nitric oxide (NO) is formed in the nerves by neuronal nitric oxide synthase and is generally associated with a relaxation of blood vessel musculature resulting in vasodilation and increased blood flow (Edwards and Garrett, 1993; Umans and Levi, 1995). A recent study reported histological evidence for the presence of non-adrenergic, non-cholinergic (NANC) innervation in the avian salt gland which was proposed to be nitric oxide innervation. The nerve fibres appeared to be close to secretory tubules and arterioles which suggested that nitric oxide innervation may be

involved in regulation of the secretory response of the salt gland (Hübschle *et al.*, 1995). It remains to be seen whether nitrergic nerves exert significant or minor control over avian salt gland function. Any potential role of NO has not been determined in regulating any reptilian salt gland and should be examined.

1.8.3 Vasoactive intestinal peptide

Vasoactive Intestinal Peptide (VIP) appears to play an important role in the secretory function of a number of exocrine glands. VIP has been identified immunohistochemically in the dogfish rectal gland (Holmgren and Nilsson, 1983; Chipkin *et al.*, 1988), as well as salivary glands in the cat, rat, dog and human (Shimizu and Taira, 1979; Lundberg *et al.*, 1980; Uddman *et al.*, 1980; Johansson and Lundberg, 1981). VIP is co-released from nerve terminals and acts cooperatively with acetylcholine to regulate blood flow and secretion in cat salivary gland (Lundberg *et al.*, 1981a; Lundberg *et al.*, 1981b). With the many functional similarities of salt glands to other exocrine glands, it is not surprising that several workers (Lowy *et al.*, 1987; Gerstberger, 1988; Gerstberger *et al.*, 1988) subsequently identified a stimulatory role of VIP in the avian salt gland. Current evidence suggests that VIP has a vasodilatory effect in the avian salt gland, analogous to that in the cat salivary gland, although the precise mechanism of action is not yet established (Gerstberger and Gray, 1993). In addition, experiments conducted on preparations of dissociated salt gland cells showed that VIP stimulated chloride secretion via an increased phosphorylation of cyclic AMP (Torchia *et al.*, 1992).

The role of VIP in the elasmobranch rectal gland has been examined both immunohistochemically and physiologically. VIP immunoreactive fibres surround the circumference of the gland and penetrate the connective tissue between secretory tubules (Chipkin *et al.*, 1988), probably acting to increase blood flow. In the rectal gland perfused *in situ*, addition of VIP leads to increased blood flow, secretory rate, chloride concentration of secretion and an activation of adenylate cyclase to raise levels of cAMP (Stoff *et al.*, 1979; Stoff *et al.*, 1988).

The only reported investigation of VIP activity in salt gland function of reptiles has been in the estuarine crocodile *Crocodylus porosus* where it was shown to stimulate lingual salt gland secretion (Taylor, 1992; Taylor *et al.*, 1995). VIP-like immunoreactivity has been shown in the lachrymal gland

of *Malaclemys terrapin* next to the basement membrane of the secretory cells (Belfry and Cowan, 1995).

VIP appears to have a generally stimulatory role and is a possible candidate for involvement in control of the sea turtle salt gland but has never been examined.

1.8.4 Neuropeptide Y

Neuropeptide Y (NPY) has been reported to inhibit chloride secretion in the dogfish (Silva *et al.*, 1993) by inhibiting the stimulatory effect of VIP in the rectal gland. NPY has a vasoconstrictive role in the gastrointestinal tract of mammals (Friel *et al.*, 1986; Wang *et al.*, 1987) where it also inhibits chloride secretion. The direct influence of NPY on salt secretion has not been reported in any other animal, although NPY-like immunoreactivity has been shown in the lachrymal gland of the diamondback terrapin (Belfry and Cowan, 1995).

1.9 MECHANISM OF IONIC TRANSPORT

Regulatory mechanisms may operate to control the activity of the secretory cells of the salt gland, so it is useful to understand the process by which a concentrated salt solution is elaborated by the gland. The proposed cellular events which take place to secrete salt by salt gland cells are described below, and potential sites of regulation considered.

Considerable research has investigated the ionic transport events that take place in salt glands in order to produce a hypertonic solution. This work has been restricted to avian and elasmobranch salt glands and has been conducted *in vitro* due to the difficulties in measuring intracellular activity in the living animal. Initial work generally involved the use of perfused salt gland slices but later techniques allowed culture of dissociated salt gland cells into monolayer sheets with an apical and basal surface. This experimentally simulated the serosal (blood or internal) side and the mucosal (or tubule lumen) side of the secretory cell and permitted measurement of the chemical and electrical properties of both sides simultaneously.

Two models have been suggested to explain the process of concentrated salt secretion by the salt gland which differ in the identity of the primary secretory cells although the process of fluid elaboration is similar in both.

These models have been constructed on experimental evidence gained from study of the avian and elasmobranch salt glands, but it is reasonable that they are likely to apply to the chelonian salt gland as well. The first model draws on a number of earlier theories based on gland histology and biochemical evidence from cell culture studies. The second model proposes a similar means of ionic transport but a different location of concentrating the secretion.

The first model drew on early histochemical and biochemical evidence indicating that the cells forming the secretory tubules concentrate fluid by an energy-dependent ion transport process (Lowy *et al.*, 1989). This formed the basis of a model proposed by Gerstberger and Gray (1993) to combine both active and passive transport mechanisms (Figure 1.4). Essentially the same model is proposed for the elasmobranch rectal gland (Silva *et al.*, 1990). Sodium, potassium and chloride are pumped from the extracellular space into the secretory cell against a concentration gradient by a Na-K-2Cl cotransporter on the basal membrane. Energy for this process is provided by exchange of potassium into the cell for sodium out of the cell by a Na-K-ATPase. Potassium then passively leaves the cell through basal potassium channels. Chloride leaves the cell through channels on the apical surface which have been shown to be homologous to cystic fibrosis transmembrane conductance regulator protein which permit passive chloride movement (Ernst *et al.*, 1994). Movement of chloride into the tubule lumen creates an electrochemical gradient which draws sodium down the lateral spaces between secretory cells and through the leaky junctions at the apical contact point between neighbouring cells. A concentrated solution is formed in the tubule lumen because salt is transported in one direction only and limited passive entry of water from the blood occurs because the membrane is relatively impermeable to water. This energy-dependent ionic transport process is proposed to create a highly concentrated solution at the secretory tubule which does not undergo any subsequent modification in its passage down the collecting ducts. It should be pointed out however, that several assumptions must be made when constructing this model from data gathered using cell culture techniques. It must be assumed that cellular resistance does not change, as the recording of ionic currents under short circuit conditions only measures voltage and current. Any change in resistance will affect the relationship of V=IR but resistance is not generally measured. It is possible that the conductance of other channels or ionic gates is changed by experimental manipulation to change resistance

although this has not been demonstrated. Additionally, it must be assumed that expression of cell transport proteins is normal in cell cultures where innervation and vascularisation are absent. Cell morphology is certainly different (Hootman and Ernst, 1980; Lowy *et al.*, 1985), but it is not clear whether membrane structure remains normal.

The second model has been proposed on the basis of data from birds and turtles which suggests that secretory cells produce a solution which is not highly concentrated but is isosmotic or slightly hyperosmotic with plasma and subsequently concentrated in the duct system of the gland (Marshall, 1986; Marshall *et al.*, 1987; Marshall, 1989; Marshall and Saddlier, 1989). It is proposed that the primary secretion is concentrated by the addition of salt or removal of water across the epithelia of the duct, through an unspecified energy-dependent process. So although the mechanism by which salts are transported are similar in both models, the location and identity of the cells doing the osmoregulatory work in concentrating the secretion is different.

There are currently insufficient experimental data to say beyond doubt which of the two proposed models is operating in the salt gland; indeed, there may be a combination of both. It is possible that the duct system plays a role in further concentrating a hyperosmotic primary secretion produced by secretory cells. In either case, salt gland secretion must incorporate a highly effective transport process for concentration of salt against a large osmotic gradient. An understanding of the processes operating to control salt gland secretion may provide evidence to support one or the other model of ionic transport, perhaps by showing in which region of the gland control is exerted thereby indicating which regions of the gland (tubules or ducts) are regulated.

1.10 THE SECRETORY MODEL

Control of salt gland secretion may involve regulation of blood supply or circulation within the gland, ionic transport processes within the gland cells, or possible water resorption from a primary secretion. The secretory response requires blood flow to the salt gland to supply ions for transport by secretory cells, and cellular transport mechanisms for movement of ions into secretory tubules. When salt levels are returned to normal, salt gland secretion is not required and so must be inhibited or inactivated by some means. Inhibitory modifiers may act to suppress either blood circulation or

cellular transport activity, or these processes may be suppressed in the absence of stimulatory modifiers.

There are three potential sites at which the secretory process can be controlled (Figure 1.5). 1) Vasodilation and vasoconstriction can regulate the circulation of blood and ions through and within the salt gland, 2) the activity of transport proteins can control the movement of ions through the secretory cell, and 3) the secondary removal of water from a primary secretion by cells lining the secretory ducts may be modified. When attempting to identify the role of possible modifiers of salt gland function, the putative modifier should meet at least one of several criteria. It should be demonstrated to have an effect on salt gland activity *in vivo*; be shown to be present in or around the gland or have receptors in those areas; to influence cellular activity *in vitro*; or to cause changes in blood circulation to and through the gland. If a single neural or hormonal factor demonstrates all these attributes, it is extremely likely that it plays an important role in regulation of salt gland function. A failure to meet one or more of these criteria does not necessarily mean that a substance is not involved, as its role in the model may be restricted to only one of the control sites mentioned or experimental design and execution may not be appropriate.

1.11 THESIS ORGANISATION

In order to investigate the function of the green turtle salt gland and the modifiers operating to control the secretion of the gland, experiments were conducted to test their role in the proposed secretory model. The response of the salt gland to salt-loading *in vivo* was measured and quantified. Accompanying changes in blood osmotic parameters were also measured following a salt challenge. These results are described in Chapter 2. The ability of a number of potential modifying substances to alter the secretory response of the salt gland was examined by quantifying any changes to the secretion by the gland in comparison to the normal response to salt-loading previously determined. In addition, immunohistochemical technique was employed to identify adrenergic nerves in the salt gland. These results are described in Chapter 3. Salt gland histology, vasculature and changes in blood circulation within the salt gland caused by salt-loading, adrenalin and methacholine administration are presented in Chapter 4. The influence of salt-loading and addition of adrenalin and methacholine on the rate of oxygen consumption of salt gland tissue *in vitro* is presented in Chapter 5.

The synthesis and general implications of the results are discussed in Chapter 6, with the proposed secretory model reviewed in light of experimental results.

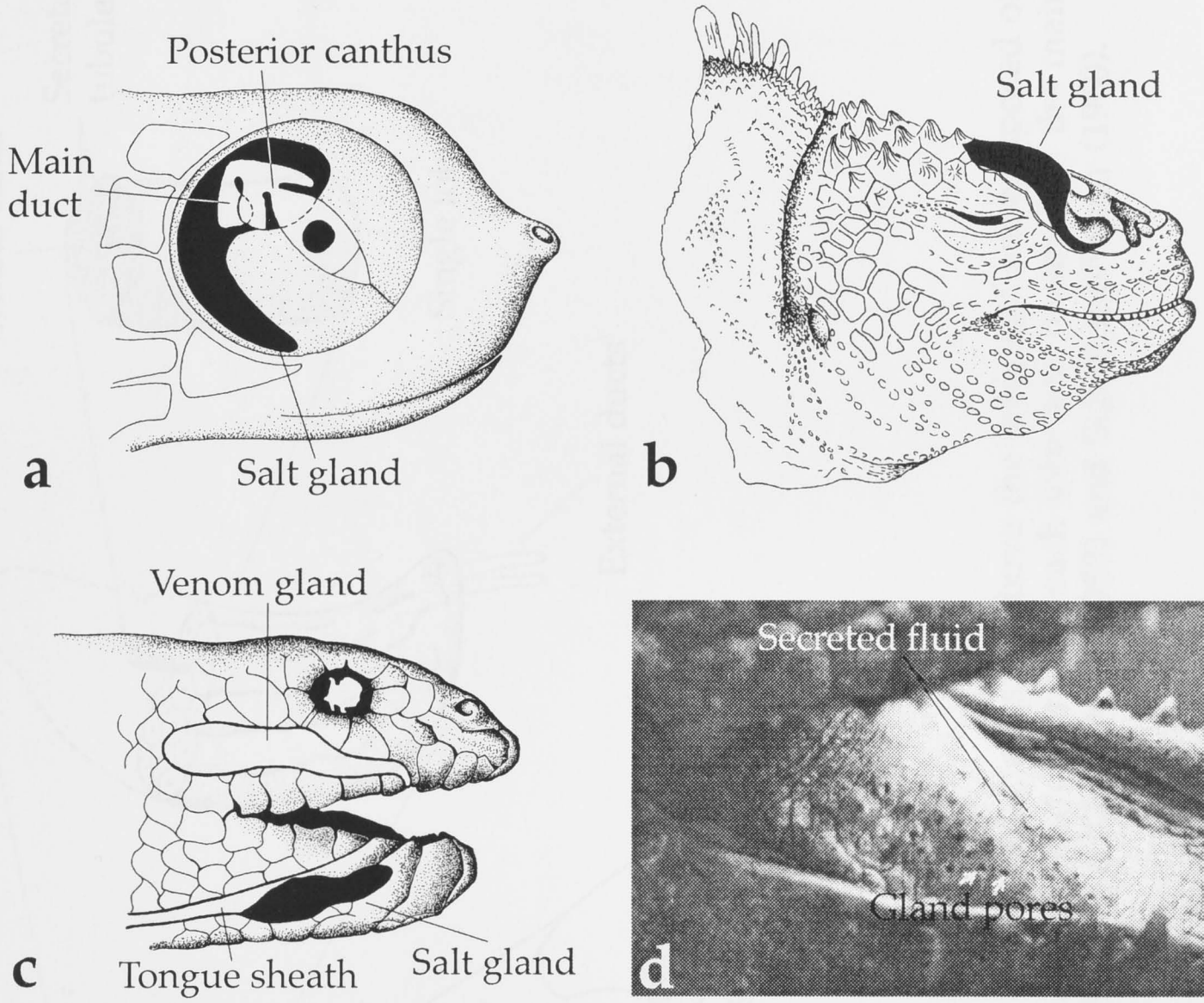


Figure 1.1
Location of salt-secreting glands in different reptiles.
a) Sea turtles have a lachrymal salt gland located behind each eye which empties onto the surface of the eyeball. Redrawn from Marshall and Saddlier (1989). Chelonia mydas
b) The Galápagos iguana possesses a nasal salt gland which extends from the nasal region to above the eye and drains into the nasal cavity. Redrawn from Dunson (1976). Amblyrhynchus cristatus
c) The sublingual salt glands of sea snakes (Pelamis spp.) are located under or around the tongue sheath of the lower jaw. Redrawn from Dunson and Dunson (1974).
d) Lingual salt glands of crocodiles are located inside the tongue and open through pores on its upper surface. Droplets of secreted fluid can be seen on the surface of the tongue. From Taplin *et al.* (1982). Crocodylus porosus

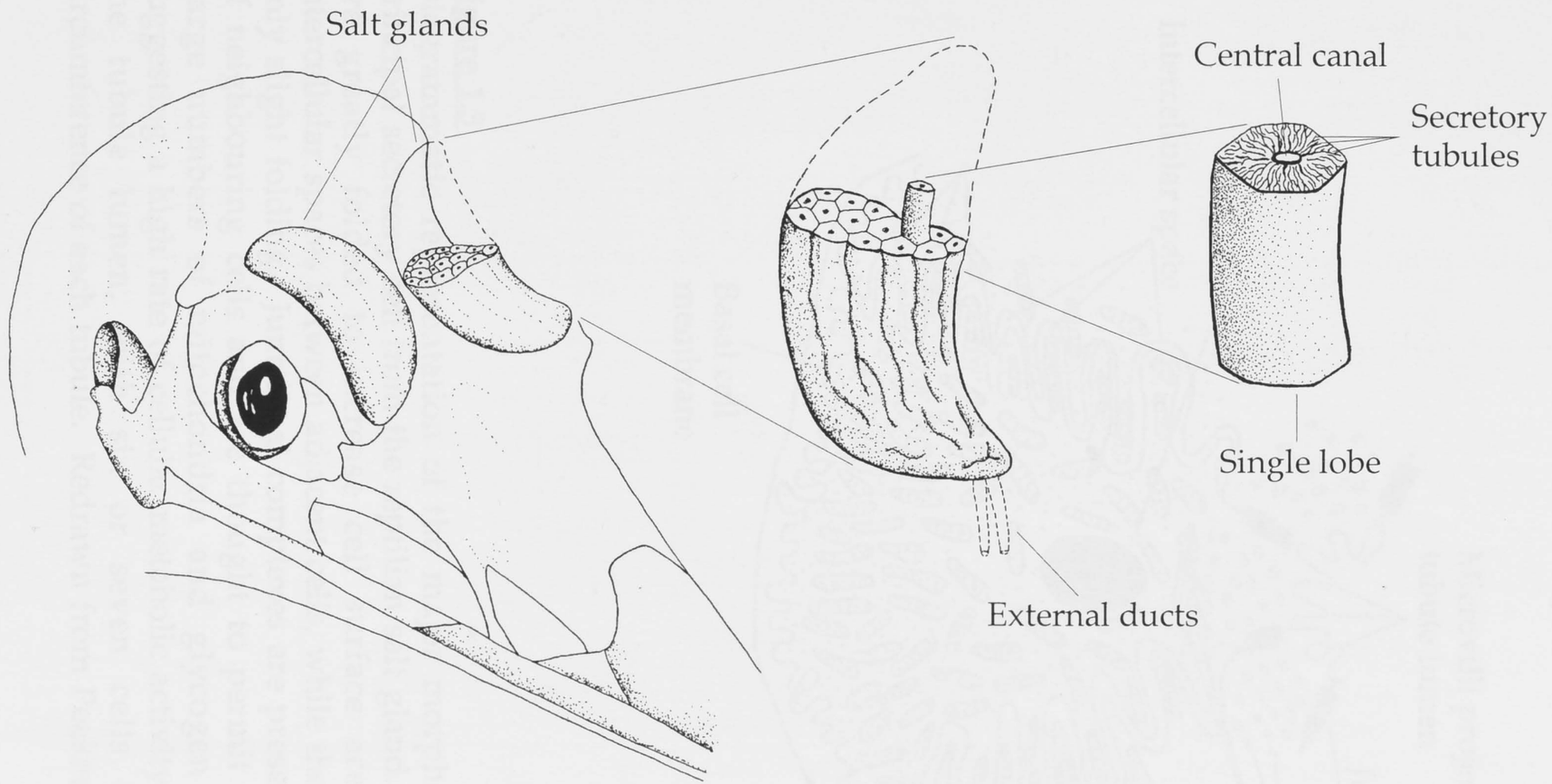


Figure 1.2
Location and gross morphology of the avian salt gland. The paired glands lie above the orbit and are composed of numerous lobes of secretory tubules. The secreted fluid flows down the central canal of each lobe and drains into the main collecting ducts which lead to the external or internal nares. Modified from Fänge *et al.* (1958) and Schmidt-Nielsen (1960).

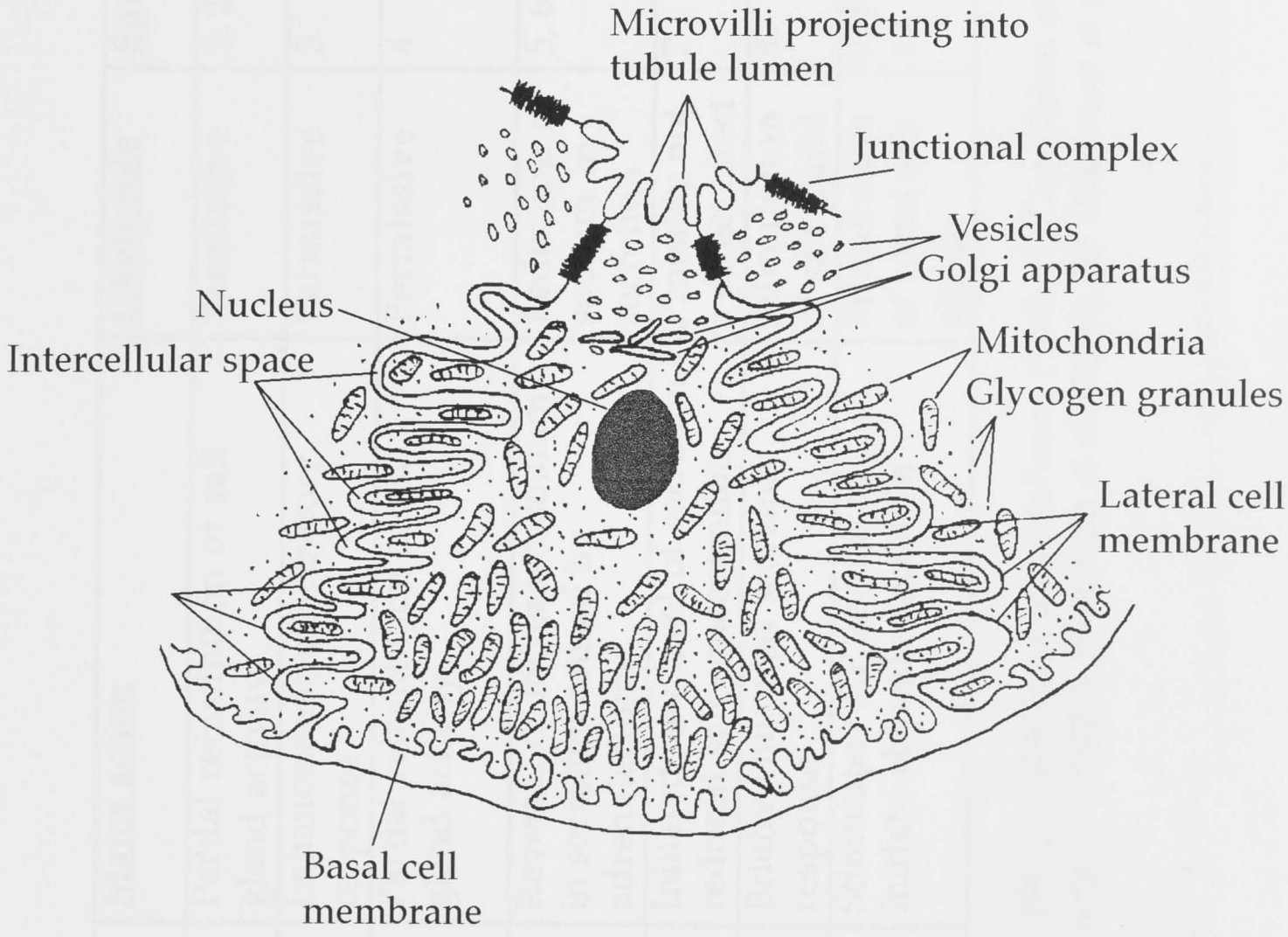


Figure 1.3
Diagrammatic representation of the major morphological features of the principal secretory cell from the reptilian salt gland. The lateral membranes are greatly folded to increase cell surface area and form extensive intercellular spaces between adjacent cells, while the basal membrane shows only slight folding. Junctional complexes are present at the apical junction of neighbouring cells and are thought to permit passive ion movement. Large numbers of mitochondria and glycogen granules are present, suggesting a high rate of cellular metabolic activity. Microvilli project into the tubule lumen, with six or seven cells typically forming the circumference of each tubule. Redrawn from Peaker and Linzell (1975).

Table 1.1
Possible endocrine influences on salt gland function.

Hormone	Animal	Experimental technique	Major action	Likely role	Source
Corticosteroid hormones	Salt adapted duck	Adrenalectomy/ replacement therapy	Partial reinstitution of salt gland activity	Permissive	1, 2
		Administered with salt-load	Enhancement of secretory response	Permissive	3
	Green turtle	Chemical adrenalectomy/ replacement therapy	Partial reinstitution of salt gland activity	Permissive	4
	Desert iguana	Adrenalectomy/ replacement therapy	Reversal of increased sodium in secretion caused by adrenalectomy	Reduction of sodium in secretion	5, 6
AVT	Salt adapted goose	Intact animal	Initiated low level of secretion, reduced salt concentration	Probably not a direct effect	7
ANF	Salt adapted duck	Administered with salt-load	Briefly enhanced secretory response	Stimulation of salt gland	8
	Dogfish	Intact animal, perfused rectal gland, dissociated cells	Stimulates VIP release to initiate chloride secretion	Stimulation of rectal salt gland	9, 10, 11, 12

Sources:
1 (Holmes *et al.*, 1961), 2 (Holmes *et al.*, 1963), 3 (Phillips *et al.*, 1961), 4 (Holmes and McBean, 1964), 5 (Templeton *et al.*, 1972), 6 (Shoemaker *et al.*, 1972), 7 (Peaker, 1971), 8 (Schütz and Gerstberger, 1990), 9 (Solomon *et al.*, 1985), 10 (Solomon *et al.*, 1992), 11 (Gunning *et al.*, 1993), 12 (Silva *et al.*, 1987)

Table 1.2
Possible neural and neurohormone influences on salt gland function.

Neurohormone/ Neurotransmitter	Animal	Experimental technique	Major action	Likely role	Source
Acetylcholine Methacholine	Herring gull	Intact animal, anaesthetised animal	Stimulates salt secretion, causes vasodilation	Stimulation of salt gland	1, 2
Acetylcholine Methacholine	Estuarine crocodile	Intact animal	Stimulates salt secretion	Stimulation of salt gland	16
Methacholine	Loggerhead turtle	Intact animal	Stimulates salt secretion	Stimulation of salt gland	3
	Marine iguana	Intact animal	Stimulates salt secretion	Stimulation of salt gland	3
	Non salt adapted duck	Salt gland slices	Increased cellular oxygen consumption	Stimulation of salt gland	4
	Estuarine terrapin kept in fresh water	Salt gland slices	Increased cellular oxygen consumption	Stimulation of salt gland	4
	Estuarine terrapin	Intact animal	Stimulated low level of salt secretion	Stimulation of salt gland	5
	Salt adapted duck	Dissociated cells	Increased cellular oxygen consumption, increase in sodium pump activity	Stimulation of salt gland	6, 7
Adrenalin	Herring gull	Anaesthetised animal	Reduction of salt gland blood flow	Inhibition of salt gland	8

Table 1.2 (cont.)
Possible neural and neurohormone influences on salt gland function.

Neurohormone/ Neurotransmitter	Animal	Experimental technique	Major action	Likely role	Source
VIP	Salt adapted duck	Dissociated salt gland cells	Stimulation of Cl^- secretion via increased phosphorylation of cAMP	Direct effect on salt gland cells	9
	Salt adapted duck	Intact animal at threshold salt conditions	Stimulation of secretion, increased blood flow	Stimulation of salt gland	10, 11
	Estuarine crocodile	Intact animal	Stimulation of secretion	Stimulation of salt gland	12
	Dogfish	Rectal gland slices	Increase in blood flow, secretory rate, Cl^- concentration and cAMP levels	Stimulation of rectal salt gland	13, 14
NPY	Dogfish	Perfused gland, isolated tubules, cultured cells	Inhibited chloride secretion stimulated by VIP	Inhibition of rectal salt gland	15

Sources:
1 (Fänge *et al.*, 1958b), 2 (Fänge *et al.*, 1963), 3 (Schmidt-Nielsen and Fänge, 1958), 4 (Shuttleworth and Thompson, 1987), 5 (Dunson, 1970), 6 (Hootman and Ernst, 1982), 7 (Hootman and Ernst, 1981), 8 (Fänge *et al.*, 1963), 9 (Torchia *et al.*, 1992), 10 (Hammel *et al.*, 1980), 11 (Gerstberger, 1988), 12 (Taylor, 1992), 13 (Stoff *et al.*, 1979), 14 (Stoff *et al.*, 1988), 15 (Silva *et al.*, 1993), 16 (Taylor *et al.*, 1995)

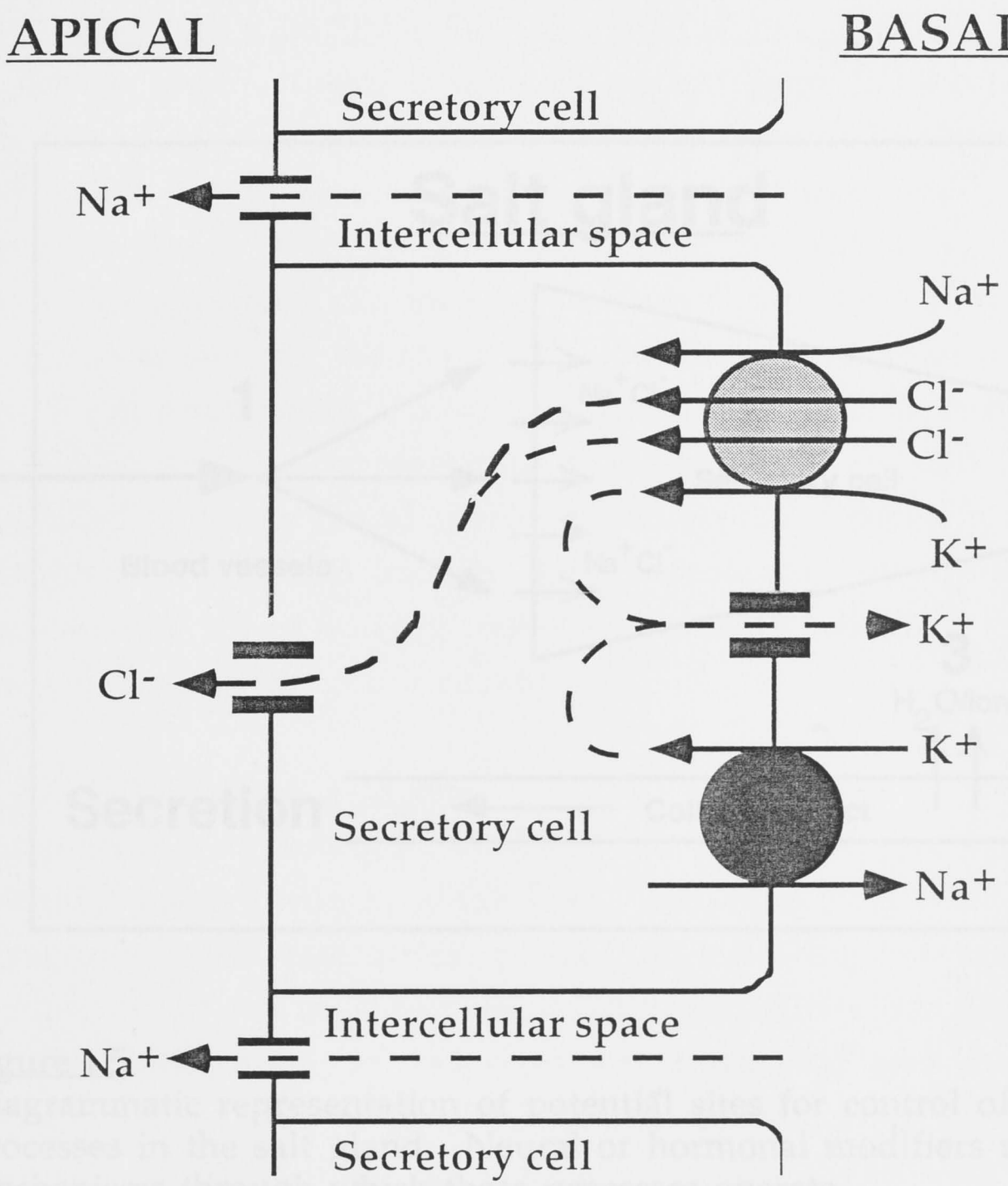


Figure 1.4
Proposed model for ionic transport by principal cells of secretory tubules. Chloride enters the cell via an energy-dependent basal Na-K-2Cl cotransport protein, sodium is pumped out of the cell in exchange for potassium by a Na-K-ATPase and potassium leaves the cell through passive channels. Chloride leaves the cell through the apical (luminal) membrane, creating an electrochemical gradient which draws sodium through the intercellular spaces and leaky cell junctions. Solid arrows indicate active ion pumping, dashed arrows indicate movement of ions due to concentration or electrical gradients. Adapted from Gerstberger & Gray (1993) p 174.

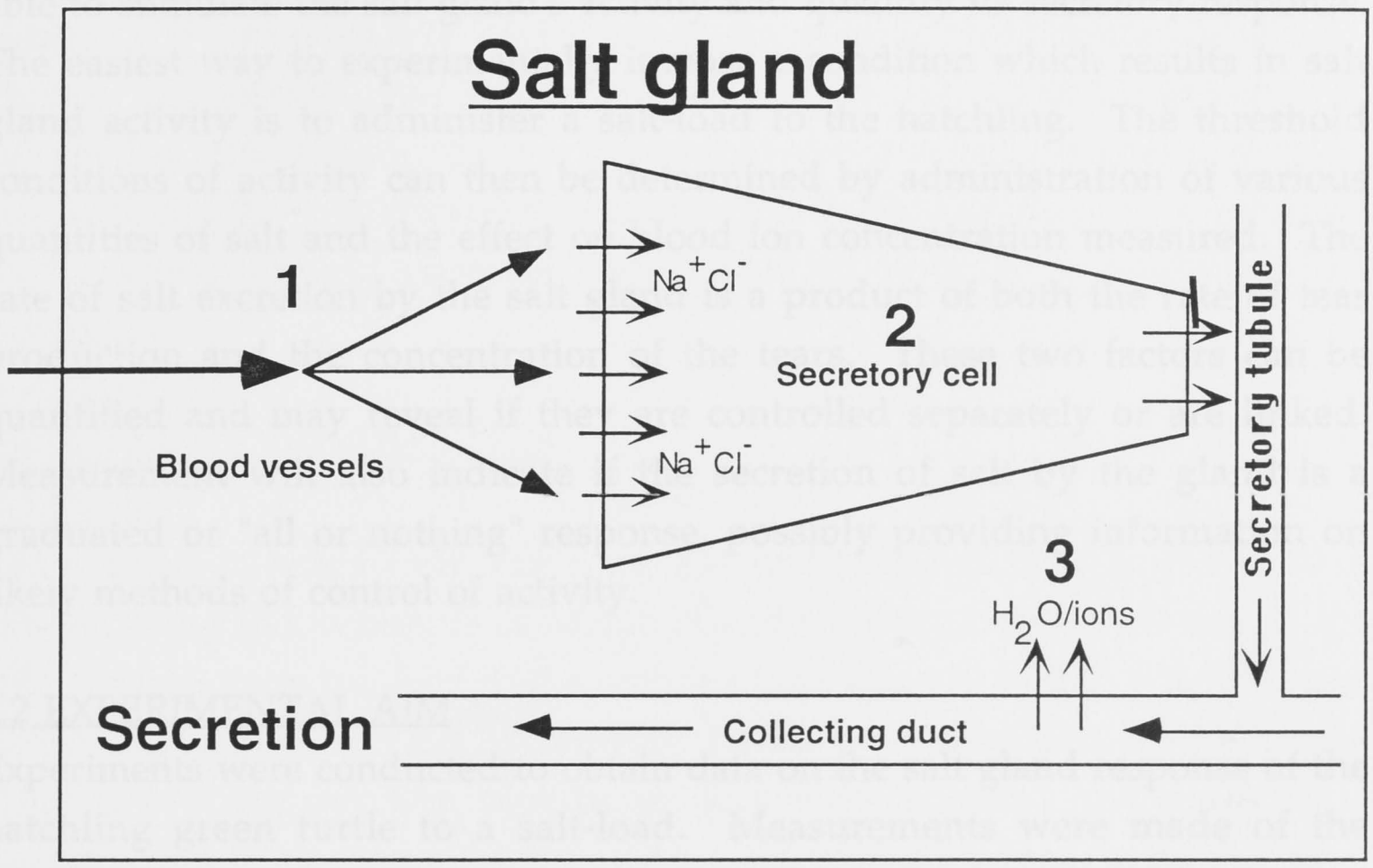


Figure 1.5
Diagrammatic representation of potential sites for control of the secretory processes in the salt gland. Neural or hormonal modifiers may affect the mechanisms through which these processes operate.
1) Constriction and relaxation of muscles surrounding blood vessels influence the circulation of blood and hence supply of ions and nutrients to the salt gland secretory cells.
2) Ion transport activity may be controlled to regulate the movement of ions through the secretory cell from the blood to the tubule lumen.
3) Control of possible extraction of water from the primary secreted fluid will affect the final concentration and rate of fluid production.

CHAPTER 2
Characteristics Of Salt Gland Function

INTRODUCTION

2.1 SECRETORY RESPONSE OF THE HATCHLING TURTLE SALT GLAND

In order to test the proposed secretory model (Figure 1.5) it is necessary to be able to stimulate the salt gland's activity and quantify its secretory response. The easiest way to experimentally impose a condition which results in salt gland activity is to administer a salt-load to the hatchling. The threshold conditions of activity can then be determined by administration of various quantities of salt and the effect on blood ion concentration measured. The rate of salt excretion by the salt gland is a product of both the rate of tear production and the concentration of the tears. These two factors can be quantified and may reveal if they are controlled separately or are linked. Measurement will also indicate if the secretion of salt by the gland is a graduated or "all or nothing" response, possibly providing information on likely methods of control of activity.

2.2 EXPERIMENTAL AIM

Experiments were conducted to obtain data on the salt gland response of the hatchling green turtle to a salt-load. Measurements were made of the concentration and rate of tear production following injection of a known quantity of salt and the threshold of salt gland activity was determined. The pattern of the secretory response was quantified and the relationship between rate of tear production and tear concentration was investigated. This also provided data for comparison with subsequent experimental treatments (Chapter 3) to quantify the influence of possible modifiers of secretion. Changes in blood ionic composition and packed red blood cell volume (hematocrit) were measured to investigate what effect salt-loading and salt gland activity had on these blood parameters and to quantify the movement of sodium into and out of the blood.

METHODS

2.3 ANIMALS

Hatchling turtles (approximately 25 to 30 g body mass) were collected from Heron Island, located at 151^0 55' E. 23^0 26' S on the Great Barrier Reef, Australia as they emerged from the nest and made their way to the sea. Following collection they were maintained in fibreglass aquaria 1.7 m x 0.7 m, filled to a depth of approximately 15 cm with continuously flowing sea water. The temperature of the seawater was approximately 28 ± 1^{0}C. Feeding commenced after about five days and thereafter hatchlings were fed on shelled raw shrimp daily. Animals were marked on the shell with liquid paper for identification. Following experiments they were returned to the aquarium and observed for a week or more then released to the ocean.

2.4 COLLECTION OF SAMPLES

2.4.1 Tears

Tears were collected from the right eye of hatchlings in a five microlitre pipette using the technique of Marshall and Cooper (1988). One end of the micro-pipette was touched to the corner of the eye where the secretory duct of the salt gland emerges. By carefully holding the pipette in position, tears were drawn up into the pipette as they were secreted and before any evaporation. The rate of secretion was calculated by measuring the time taken to fill the pipette and calculated in microlitres per minute. Collected tears (5 µl) were absorbed onto paper osmometer discs and measured immediately for total osmolality (Wescor Vapour Pressure Osmometer 5500). Discs were then sealed in Eppendorf tubes for subsequent analysis for sodium and potassium concentration by atomic absorption spectrophotometry (Varian Techtron) and chloride concentration by ion chromatography (Dionex QIC analyser).

2.4.2 Blood

Blood samples of approximately 0.3 ml were collected from the cervical sinus of hatchlings in heparinised 1.0 ml insulin syringes (Terumo, 27G x 1/2). The cervical sinus is a paired, blood filled cavity lying on either side of the vertebrae of the neck and is a suitable location for sampling venous blood (Owens and Ruiz, 1980; Bennett, 1986). Plasma was separated by centrifugation and frozen immediately at -70^{o}C for subsequent analysis of ionic composition (techniques as for tears).

2.5 EXPERIMENTS

2.5.1 Threshold salt-load for salt gland activation

The threshold salt-load required for initiation of salt gland secretion was determined by injecting various salt-loads into the thoracic cavity of hatchlings and determining the proportion of animals which produced tears within 30 minutes of injection. Salt-loads injected were 0, 200, 400 and 600 μmol NaCl.100g BW^{-1} in a volume of 18 ml.kg BW^{-1}. If secretion had not commenced within 30 minutes of salt-loading, a blood sample was taken from the cervical sinus for measurement of plasma sodium concentration. If animals produced tears within 30 minutes of salt-loading, a blood sample was obtained at the time tears first appeared. Blood samples were immediately centrifuged and plasma frozen for analysis of sodium concentration. The plasma sodium *concentration* was calculated for each of the salt-loads administered and *total actual* plasma sodium (μmol.100g BW^{-1}) was calculated, assuming that plasma volume comprises about 4.4% of total body mass (Thorson, 1968). This permitted a comparison of the amount of sodium injected per 100g BW with the amount detected in the plasma per 100g BW. The change in plasma sodium as a proportion of the total injected sodium could then be determined.

2.5.2. Response of salt gland to salt-loading

Tear production rate and tear concentration in response to salt-loading were determined to provide a comparison for results obtained under subsequent experimental conditions. Hatchlings were injected with sodium chloride solution (2700 μmol NaCl.100g BW^{-1}) into the thoracic cavity. Control animals had isotonic sodium chloride (PBS) solution (NaH_2PO_4 20 $mmol.l^{-1}$, NaCl 154 $mmol.l^{-1}$, pH 7.2) injected into the thoracic cavity (270 μmol NaCl.100g BW^{-1}). For both groups, the time in minutes from injection to commencement of any salt gland secretion was measured and samples of tears were collected every five minutes from tear initiation over an eighty minute period. Tear production ($\mu l.min^{-1}$) and ionic concentration ($mmol.l^{-1}$) were determined to permit calculation of the total rate of sodium removal (tear production *X* ionic concentration).

2.5.3. Blood parameters following salt-loading

A time-course study was conducted to measure changes in hematocrit, plasma osmolarity and plasma sodium concentration following salt-loading. These parameters were measured in animals injected with a salt-load of 2700 μmol NaCl.100g BW^{-1}, as well as those which received an

injection of PBS (270 μmol NaCl.100g BW^{-1}), or were untreated. Blood samples were obtained from the cervical sinus of some animals immediately after salt-loading, then several samples were obtained from other animals during the next 70 minutes of the secretory response. Significant changes which occurred following treatment or over the course of the experiment were determined.

2.6 STATISTICAL ANALYSIS

The Mann-Whitney rank test (Sokal and Rohlf, 1981) was employed to determine any significant differences in hematocrit, plasma osmolality or plasma sodium concentration. Significance was assumed if $p<0.05$. All results are shown as the mean ± 1 standard error.

RESULTS

2.7 THRESHOLD OF SALT GLAND ACTIVITY

The threshold salt-load required to initiate salt gland tear production was between 400 and 600 µmol NaCl.100g BW^{-1}. At 400 µmol NaCl.100g BW^{-1}, one of four animals commenced secreting within 30 minutes of salt-loading. At 600 µmol NaCl.100g BW^{-1} all animals produced tears within 30 minutes of salt-loading (Table 2.1) and a significant increase in plasma sodium of 18.6% was measured. Not all of the injected sodium was detected in the plasma, as injection of 600 µmol.100g BW^{-1} of sodium chloride only resulted in a rise in plasma sodium of 112 µmol.100g BW^{-1}.

2.8 SALT GLAND RESPONSE TO SALT-LOADING

Since 400 - 600 µmol NaCl.100g BW^{-1} was the threshold for secretion, subsequent experiments used 2700 µmol NaCl.100g BW^{-1} for initiating secretion and to ensure that sufficient sodium had been introduced to result in a sustained secretory response. Isotonic saline (270 µmol NaCl.100g BW^{-1}) was used as a volumetric control. The delay between injection of salt-load and start of secretion averaged 12 minutes (n = 9) with a range from 5 to 20 minutes. Upon initiation of secretion, tears reached maximal osmotic concentration within two to seven minutes (Figure 2.1) with an average osmotic concentration of 1758 ± 10 mosmol.kg^{-1}. Osmotic concentration remained high for the period of the experiment. Average sodium, chloride and potassium concentrations were 816 ± 15, 887 ± 21 and 18 ± 0.5 mmol.l^{-1} respectively (n = 9). Chloride concentration was greater than the sum of sodium and potassium ($p<0.0001$). Sodium and chloride accounted for approximately 97% of the total osmotic concentration of salt gland secretions.

The rate of tear production (Figure 2.2) reached maximum in about fifteen minutes. Secretory rate is shown for the right gland only, although glands on both sides of the head usually secreted simultaneously. The total rate of sodium removal is the product of tear concentration and flow rate, and is shown for the right salt gland (Figure 2.3). The maximal secretion rate averaged approximately 415 µmol Na.100g $BW^{-1}.hr^{-1}$ or 830 µmol Na.100g $BW^{-1}.hr^{-1}$ if we assume that right and left glands secrete at the same rate and concentration. This was determined by calculating the average of the highest rate of all hatchlings. The maximum secretory rate measured from a single salt gland was 593 µmol Na.100g $BW^{-1}.hr^{-1}$.

2.9 BLOOD PARAMETERS FOLLOWING SALT-LOADING

Hematocrit of animals injected with PBS (25.7 ± 1.7%, n = 4) was not significantly different from that of untreated animals (26.8 ± 1.6%, n = 15) or hematocrit immediately following salt-loading (24.56 ± 2.73%, n = 4). Hematocrit of salt-loaded animals then rose rapidly and remained elevated over the next 80 minutes (Figure 2.4). Hematocrit was approximately 29% when secretion commenced.

Plasma osmolality of untreated animals (371 ± 6 mosmol.kg^{-1}, n = 15) was not significantly different from osmolality of animals injected with PBS (352 ± 7 mosmol.kg^{-1}, n = 4). Plasma osmolality increased rapidly following salt-loading (Figure 2.5) and was significantly higher than control values for the next 70 minutes. Plasma sodium concentration following salt-loading was also significantly higher than concentrations measured in control animals (Figure 2.6), showing both a greater relative increase and a more rapid increase than total osmolality.

DISCUSSION

Injection of salt will initiate secretion from the salt gland of *Chelonia mydas*. A salt-load of between 400 and 600 µmol NaCl.100g BW^{-1} (equivalent to ingestion of approximately 0.2 to 0.3 ml seawater for a 30 g hatchling) is sufficient to stimulate secretion. A previous study (Nicolson and Lutz, 1989) reported a similar threshold salt-load required to initiate secretion in turtles of 17 to 40 kg body weight, suggesting that the threshold is independent of body mass. Injection of 600 µmol NaCl.100g BW^{-1} led to a rise in plasma sodium of 112.4 µmol NaCl.100g BW^{-1}. Approximately 19% of the injected salt was detected in the plasma, the remainder presumably entering intracellular and interstitial spaces. Therefore at least some of the injected salt-load reaches the salt gland in the blood and is available for uptake and secretion by the secretory cells.

The secretory response of hatchlings to a salt-load of 2700 µmol NaCl.100g BW^{-1} was similar to that previously reported (Marshall and Cooper, 1988) although rate and concentration of secretion differed slightly. The secretory response was very rapid, with tears sometimes visible within five minutes after salt-loading. The secreted tears contained approximately 1.7 times the sodium chloride concentration of seawater and over 35 times the sodium chloride concentration of urine from normally hydrated animals (Prange and Greenwald, 1980). Sodium, potassium and chloride comprised 97.0% of the total osmotic pressure, with magnesium, bicarbonate and urea accounting for the balance in adult turtles (Nicolson and Lutz, 1989). The sodium concentration of tears remains relatively constant during periods of secretion and there is only minor fluctuation in tear production rate while the gland is active. This suggests that the salt gland shows an "all or nothing" response and so is either fully active or fully inactive. This may indicate a relatively simple regulation of activity with the gland operating at maximal rate when active and ceasing activity completely when secretion is not required.

If it is assumed that both glands secrete at the same rate, approximately 30% of the injected salt-load was removed in the first hour of secretion. It has been reported that the rate and concentration of secretion from right and left glands varies (Marshall and Cooper, 1988; Nicolson and Lutz, 1989) but it is assumed that variation is equally likely in either gland and balances out over the period of measurement. At the measured rate of tear secretion the entire salt-load injected could be removed in an average of less than four

hours, although it was not determined if animals secreted continuously or intermittently until the excess salt was excreted. However, at the observed rate of plasma sodium decrease, plasma sodium in salt-loaded animals would take only two hours to reach control levels. It is not clear why this time differs to that calculated from the salt gland secretory rate, unless sodium is being removed from the plasma into some other compartment simultaneous with removal and secretion by the salt gland. It may be that salt-loading initially overcomes the ability of the salt gland to reduce plasma sodium quickly enough, so some sodium leaves the blood and enters interstitial or intracellular spaces. This could slowly re-enter the blood at a rate which the salt gland could remove. The movement of sodium from interstitial fluid back into the plasma may also require plasma sodium to fall to a certain level to provide a sufficient driving force for diffusion to occur. Marshall and Cooper (1988) showed that plasma osmotic pressure had returned to control levels within 18 hours of salt-loading. There may be an interaction between the salt gland and the kidney, with the salt gland initially removing sodium through concentrated tears to achieve a net water gain. If there is a subsequent, slow re-entry of sodium into the blood this may be slowly removed by the kidney, utilising some of the water gained through salt gland activity. Thus the concentrated secretory activity of the salt gland could provide the extra water required for a more dilute salt excretion by the kidney, thereby avoiding dehydration.

The rate of sodium removal from both salt glands of salt-loaded animals averaged 814 ± 3.7 μmol Na.100g $BW^{-1}.hr^{-1}$, a rate considerably higher than 117 μmol Na.100g $BW^{-1}.hr^{-1}$ found in an earlier study (Holmes and McBean, 1964). The lower rate measured may have resulted from the animals used in that study which were very small for their age (Chapter 1). This abnormality in growth may have affected the rate of secretion by their salt glands. The measured rate of sodium secretion by hatchling turtles in this study was much higher than that observed in other reptiles, with the exception of the report of Marshall and Cooper (1988) also on hatchling turtles (Table 2.2).

If the salt gland is capable of secreting at the measured rate continuously , up to 12 mls of seawater could be drunk by a 30 g hatchling per day. About half of this water would remain as freshwater after secretion of the salt, to be available for other physiological requirements. Although it is unlikely that

this amount of seawater would be intentionally drunk, it illustrates the efficiency of this extra-renal route of salt removal.

The changes in plasma osmolality following salt-loading showed that injected salt entered the blood very quickly. In the time between injecting the salt-load and taking a blood sample from the cervical sinus (about two minutes), plasma osmolality rose from 371 to 394 $mosm.kg^{-1}$ and plasma sodium from 137 to 188 $mmol.l^{-1}$. Plasma sodium concentration at threshold conditions in the experiment described earlier was 162.8 $mmol.l^{-1}$ and so had been exceeded in the first minute or so following salt-loading. At the commencement of secretion, plasma sodium concentration had only increased slightly further to 190 $mmol.l^{-1}$, indicating that plasma sodium sufficient to stimulate the secretory response was reached nearly 10 minutes before secretion actually commenced. It is unclear why the rise in osmolality was smaller and occurred more slowly than the rise in plasma sodium. There may be movement of some other osmotically active ion out of the blood which counteracts the osmotic change caused by entry of sodium following the salt-load, thus reducing the change in plasma total osmolality.

The time difference between plasma sodium reaching threshold and the commencement of secretion may reflect the time taken for the detection of increased sodium by receptors and stimulus of the secretion pathway. Alternatively, the mechanism which detects elevated sodium concentration may not be responding to plasma sodium but rather the concentration of sodium in some other body compartment which rises more slowly. This seems possible given that less than 20% of injected sodium is detected in the plasma. There are four reasons why this may be the case. 1) The study of Kooistra and Evans (1976) showed that there was a part of the exchangeable sodium pool with a much higher concentration of sodium than in the plasma. They presumed this to be the salt gland itself, but it may be that turtles have some other unknown space into which excess sodium can be directed. 2) Such a space could store the excess injected sodium not seen in the plasma of salt-loaded animals and may be the site at which elevated sodium is detected to initiate secretion. 3) If the space is larger in larger animals it will take longer to become filled to the point where salt gland activity is initiated. The delay between salt-loading and secretion is much greater in larger animals (Schmidt-Nielsen and Fänge, 1958; Nicolson and Lutz, 1989) but is unlikely to be the result of a higher threshold, as

evidenced by the similarity of threshold salt-load in hatchling and sub-adult animals. 4) Movement of sodium into a storage space would also permit the rapid removal of sodium from the blood to be stored and slowly re-released as proposed earlier and so explain the difference in the rate of sodium removal by the gland and sodium concentration in the blood.

Hematocrit of salt-loaded animals rose following salt-loading to a level above that of untreated animals and was significantly higher for the duration of the experiment. Three possible explanations can be presented for understanding the rise in hematocrit. Firstly, there is a movement of water out of the blood which is driven by an osmotic gradient caused by salt movement into the interstitial spaces. Secondly, there may also be a release of stored red blood cells (RBC) from the spleen in response to changing salt levels. Thirdly, entry of salt into RBCs causes them to swell as water follows the osmotic gradient, so that there is a change in RBC volume rather than cell numbers. It seems unlikely that the change in hematocrit has a significant role to play in the secretory process, rather it is a secondary effect of salt-loading with its own physiological implications but it probably does not directly influence the secretion of salt itself.

Comparing maximal salt secretory rate of hatchlings with larger turtles indicates a semi-logarithmic relationship between secretory rate and body mass (Table 2.3 and Figure 2.7). The power function is -0.265 and so although the threshold for secretion is independent of body mass, the rate of secretion is proportionally greater in small animals than large ones. This may be associated with the more rapid growth rate of hatchlings (Frazer and Ladner, 1986; Wood and Wood, 1993) resulting in a proportionally higher intake of food and seawater requiring greater capacity to remove salt. So while a range of secretory rates have been reported, it is likely that these are simply size-related. A scaling of sodium efflux was also observed in the saltwater crocodile, *Crocodylus porosus* (Taplin, 1984; Grigg *et al.*, 1986), with hatchling crocodiles having a greater efflux of sodium and water than larger animals. When collecting published data to calculate the relationship between secretory rate and body mass, two sources were excluded. These were data from Holmes and McBean (1964) and Kooistra and Evans (1976). In the first case, body mass of hatchlings used was abnormal as explained earlier and hence it is not appropriate to use the generated data for an analysis which relies upon normal growth patterns. In the second case, the

methodology employed meant that it is not possible to accurately calculate a rate of secretion per unit time.

In summary, the hatchling green turtle has a highly active salt-secreting gland. Following a salt-load, hatchlings were easily capable of secreting 1 mmol NaCl.100g $BW^{-1}.hr^{-1}$ at the maximum rates observed. This rate of secretion is sufficient to permit a hatchling to drink up to 12 ml of seawater each day with about half that amount retained as freshwater following salt removal. Activity of the gland appears to be an "all or nothing" response, with tear production rate and concentration changing little during periods of secretion. The observed rate of salt secretion was much higher than that found in other reptiles and elasmobranchs, and similar to the capacity of marine birds such as the black-backed gull. However, the allometric relationship between body mass and secretory rate probably accounts for at least some of this difference, as adult animals were usually used in other studies. Nevertheless the high secretory rate is striking when it is considered that the ectothermic hatchling turtle has a rate similar to the endothermic gull. The measurement of secretory parameters under conditions of salt-loading permits the quantitative comparison of experimental treatments on the basis of tear production rate, tear concentration and total rate of sodium removal by the salt gland. The influence of potential controlling modifiers on these parameters are investigated in Chapter 3.

Table 2.1
Percentage of animals secreting and plasma sodium (± 1 std error) 30 minutes after salt-loading. P values of significant differences in plasma sodium compared to control are shown. n = number of animals sampled, n.s. = not significant.

Salt-load (μmol.100g BW^{-1})	**% of animals secreting after 30 minutes**	**Plasma Sodium (mmol.l^{-1})**	**Plasma Sodium (μmol.100g BW^{-1})**
0 (n = 15)	0	137.2 ± 1.04	603.0 ± 4.6
200 (n = 4)	0	148.4 ± 1.08	652.2 ± 4.7
400 (n = 4)	25	155.6 ± 1.02	683.8 ± 4.5
600 (n = 4)	100	162.8 ± 1.10	715.4 ± 4.8 $P<0.05$
2700 (n = 9)	100	191.9 ± 2.8	843.3 ± 12.3 $P<0.01$

Table 2.2
Salt secreting abilities of different reptiles.

Animal	Gland location	Concentration of secretion	Major ions secreted	Rate of secretion	Source
Green turtle *Chelonia mydas*	Orbital (lachrymal)	1600 - 2000 mosm.kg^{-1}	Na^+, Cl^-	100-900 µmol Na. 100g^{-1}.hr^{-1}	1, 2, 3, 4, 5
False iguana *Ctenosauria pectinata*	Nasal	330 - 1300 mosm.kg^{-1}	K^+, Cl^-, lesser amounts of Na^+	1 µmol Na.100g^{-1}.hr^{-1} 10 µmol K.100g^{-1}.hr^{-1}	6, 7
Chuckwalla *Sauromalus obesus*	Nasal	660 - 1350 mosm.kg^{-1}	K^+, Cl^-, lesser amounts of Na^+	3.2 µmol Na.100g^{-1}.hr^{-1} 31.1 µmol K.100g^{-1}.hr^{-1}	6, 7
Yellow banded sea snake *Laticauda semifasiculata*	Sublingual	1150 mosm.kg^{-1}	Na^+, Cl^-	68 µmol Na.100g^{-1}.hr^{-1}	8
Yellow bellied sea snake *Pelamis platurus*	Sublingual	1200 mosm.kg^{-1}	Na^+, Cl^-	142 µmol Na.100g^{-1}. hr^{-1}	8
Estuarine crocodile *Crocodilus porosus*	Lingual	770 - 1160 mosm.kg^{-1}	Na^+, Cl^-	45 µmol Na.100g^{-1}.hr^{-1}	9

Sources:
1 (Holmes and McBean, 1964), 2 (Kooistra and Evans, 1976), 3 (Prange and Greenwald, 1980), 4 (Marshall and Cooper, 1988), 5 (Nicolson and Lutz, 1989), 6 (Peaker and Linzell, 1975), 7 (Dunson, 1976), 8 (Dunson and Dunson, 1974), 9 (Taplin *et al.*, 1985)

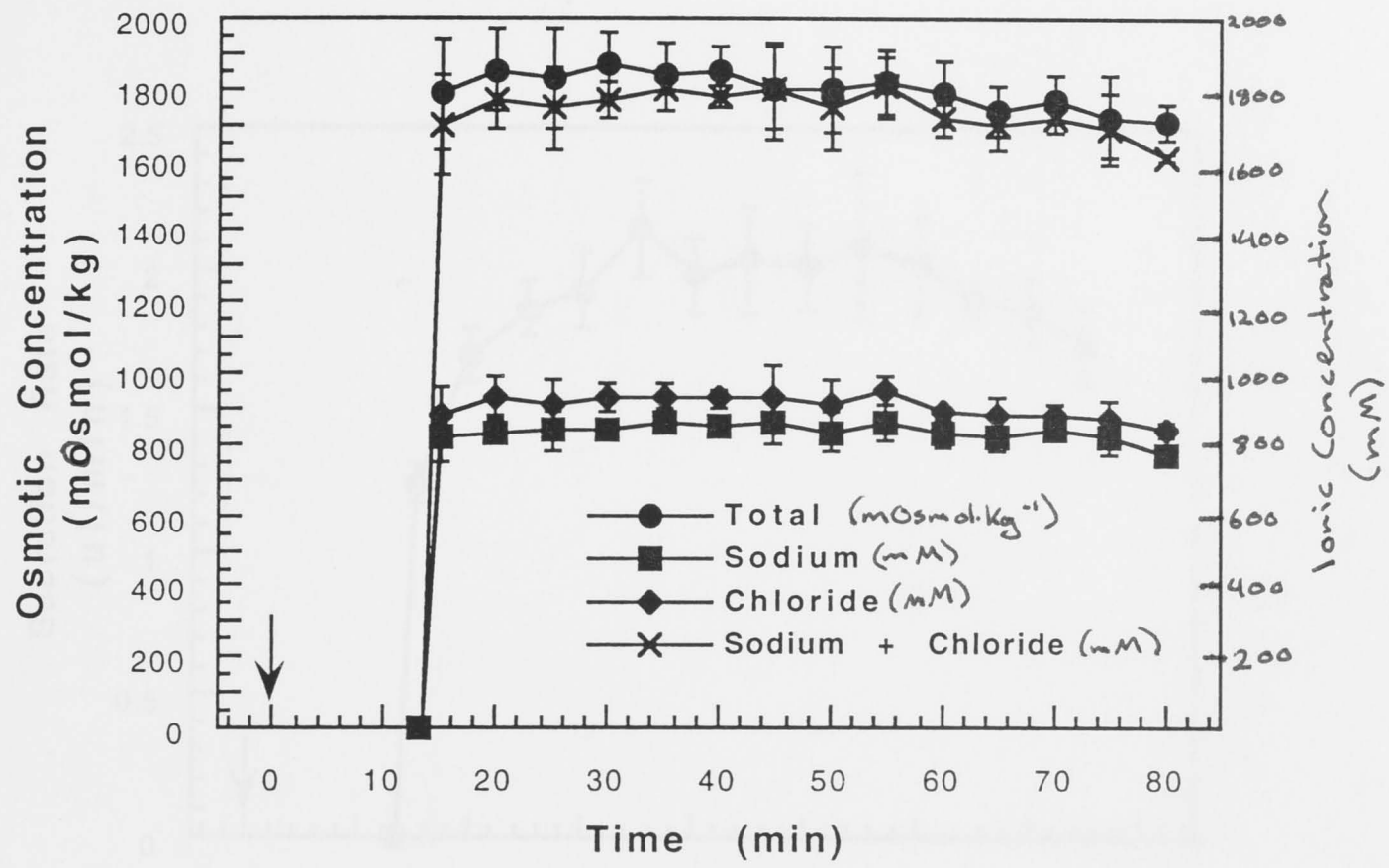


Figure 2.1

Ionic composition (mosmol.kg^{-1}) of salt gland secretions from hatchlings loaded with 2700 µmol NaCl.100g BW^{-1}. The salt-load was injected at 0 minutes as indicated by the arrow (n = 9). The time at which secretion commenced has been standardised by using the average time of commencement. Points are then shown as the mean of 'commencement time' plus 5 minutes, 10 minutes and so on. In this way, the average ~~time~~ concentration of secretions can be shown over time, regardless of the actual time of commencement of secretion.

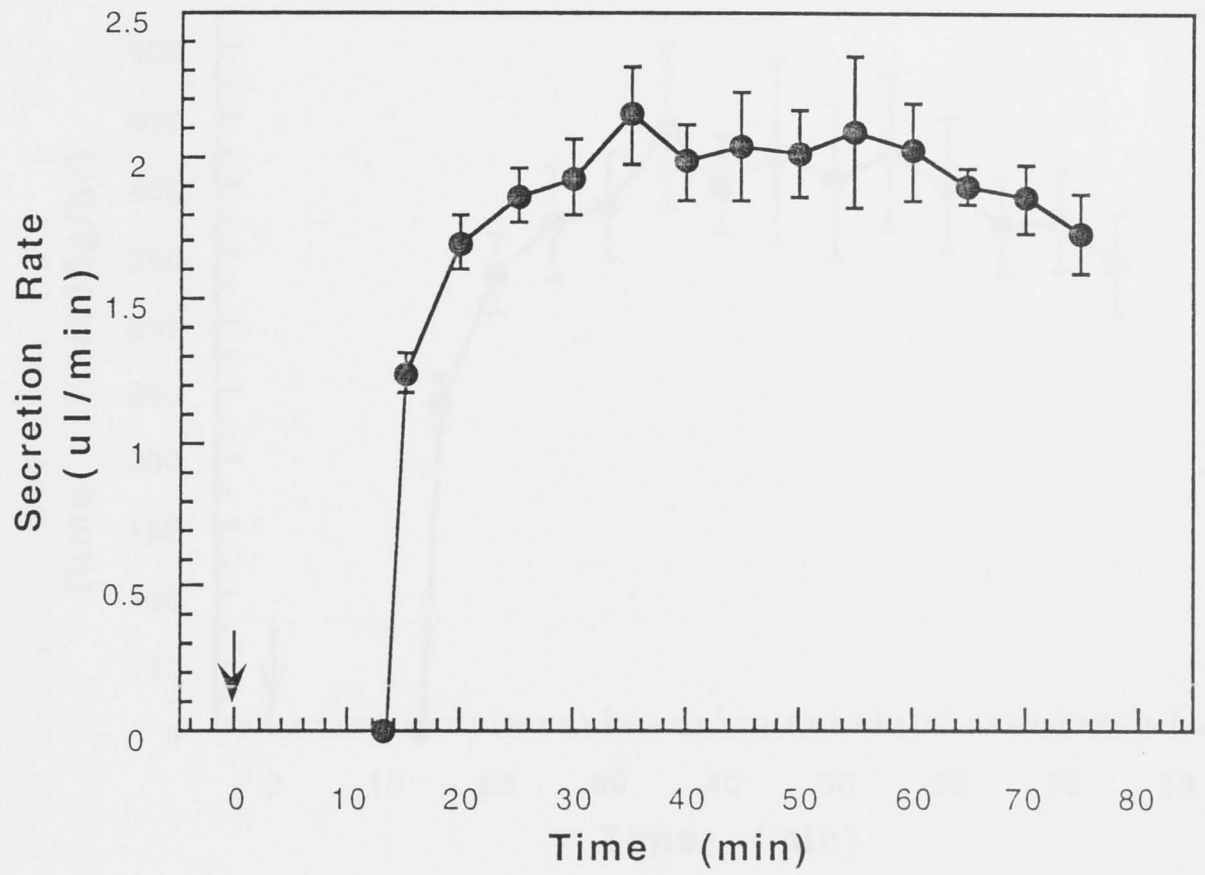


Figure 2.2
Rate ($\mu l.min^{-1}$) of salt gland secretions from hatchlings loaded with 2700 μmol NaCl.100g BW^{-1}. The salt-load was injected at 0 minutes as indicated by the arrow (n = 9).

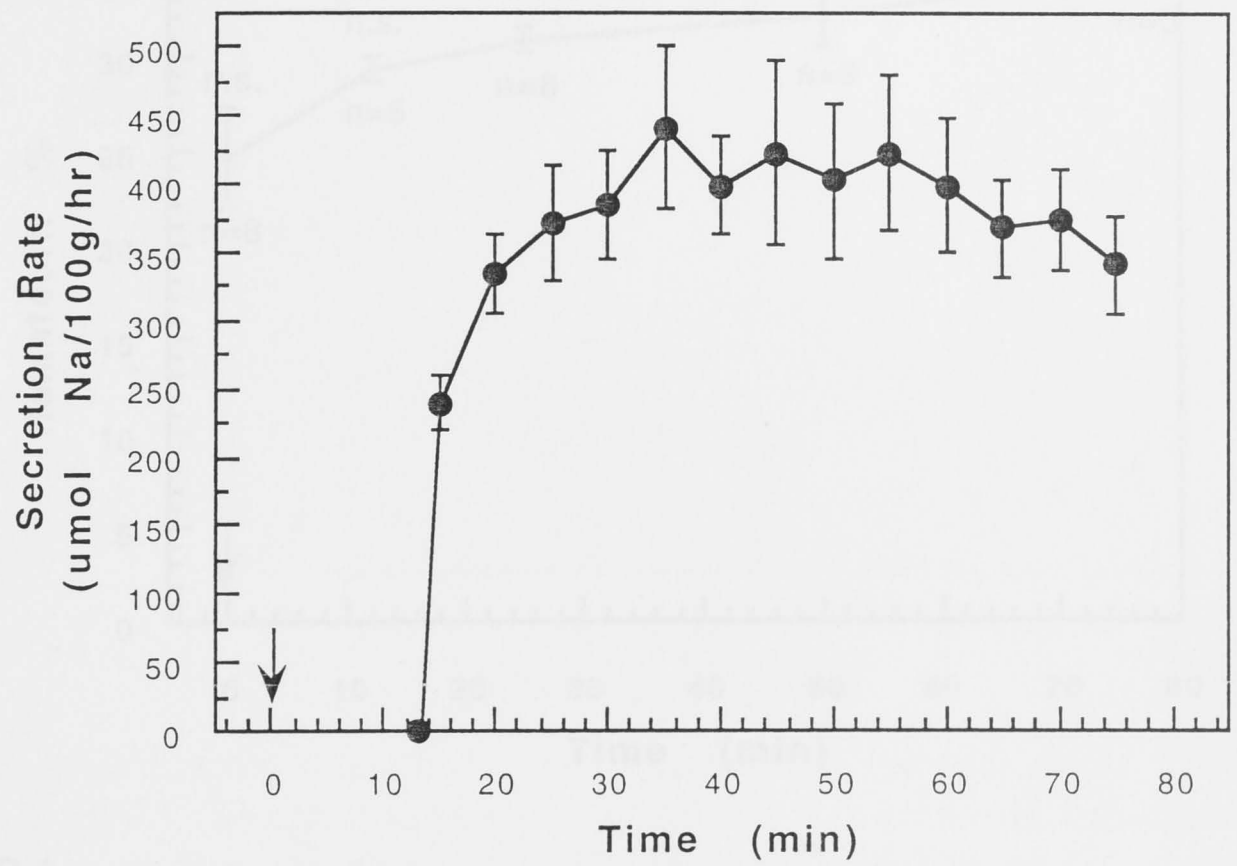


Figure 2.3
Total rate of salt gland sodium removal (μmol.100g $BW^{-1}.hr^{-1}$) by hatchlings loaded with 2700 μmol NaCl.100g BW^{-1}. The salt-load was injected at 0 minutes as indicated by the arrow (n = 9).

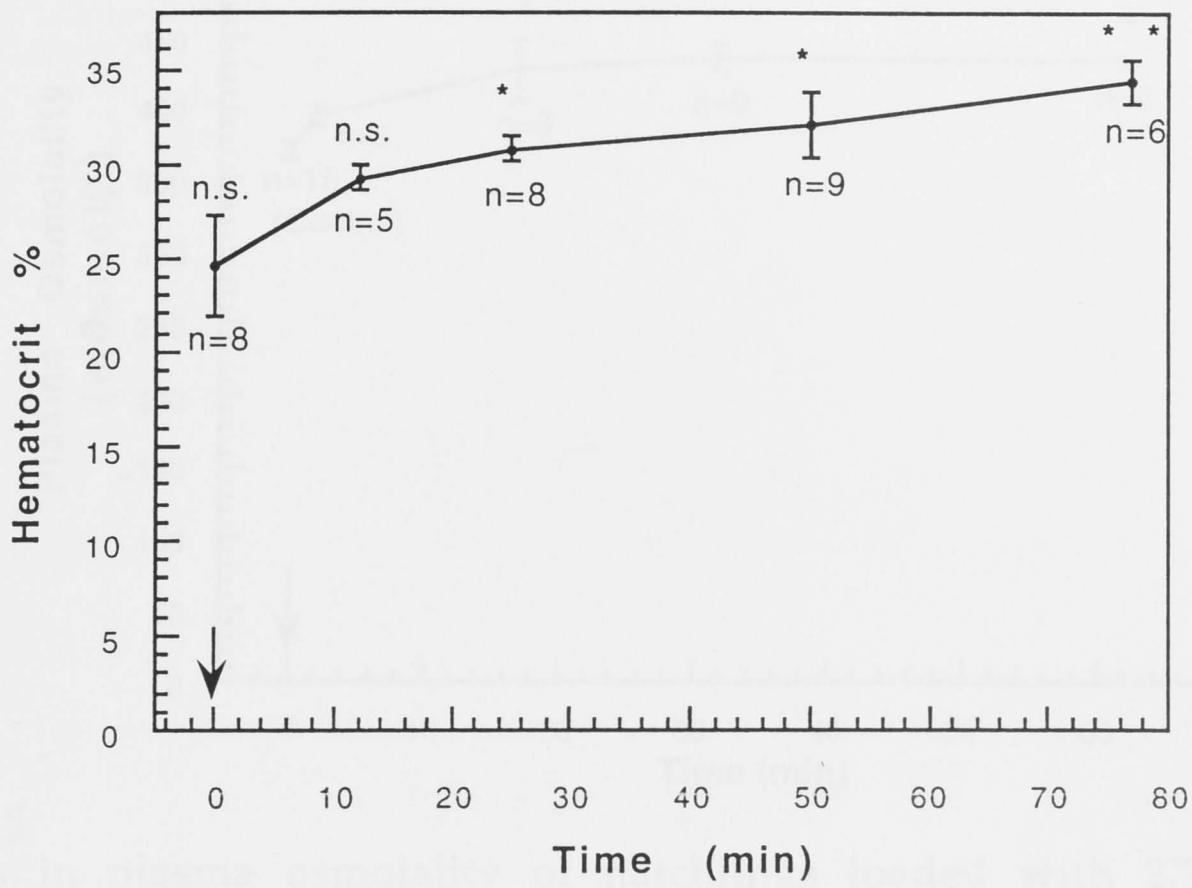


Figure 2.4
Changes in percentage hematocrit of hatchlings loaded with 2700 µmol NaCl.100g BW^{-1}. The salt-load was injected at 0 minutes as indicated by the arrow and secretion commenced at 12 minutes. Significant differences from the control hematocrit of 25.7% are indicated at each data point. n.s. = not significant, * = $p<0.05$, ** = $p<0.01$, n = number of animals.

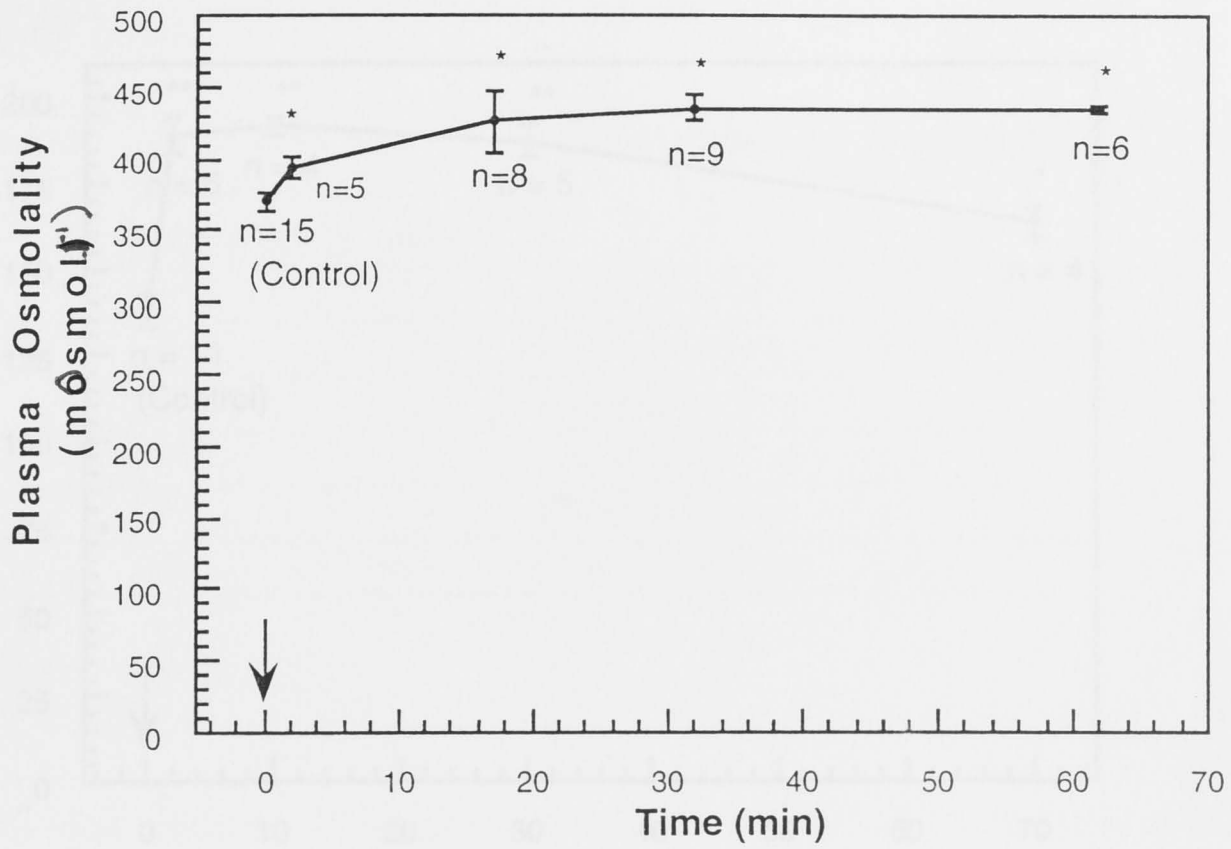


Figure 2.5
Changes in plasma osmolality of hatchlings loaded with 2700 µmol NaCl.100g BW^{-1}. The salt-load was injected at 0 minutes as indicated by the arrow and secretion commenced at 12 minutes. Significant differences from control are indicated at each data point. n.s. = not significant, * = $p<0.05$, ** = $p<0.01$, n = number of animals.

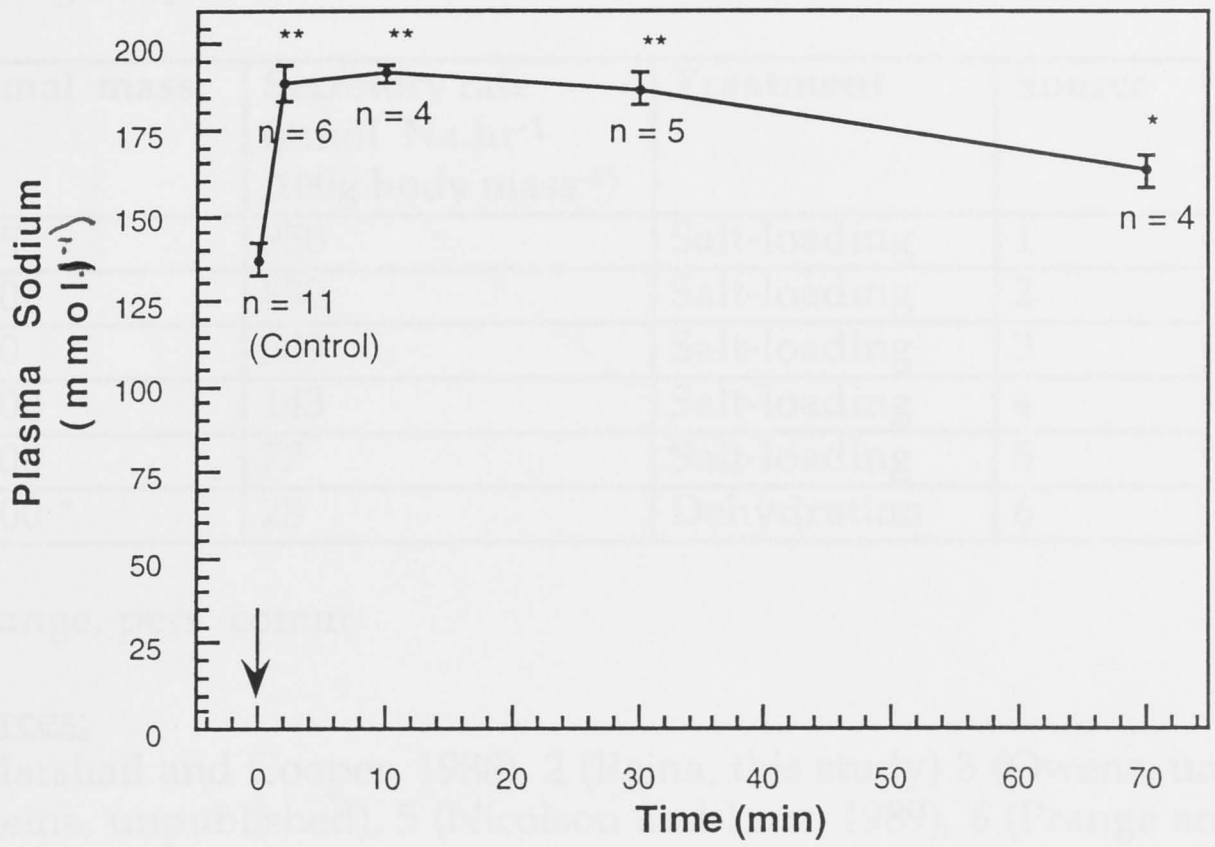


Figure 2.6
Changes in plasma sodium of hatchlings loaded with 2700 µmol NaCl.100g BW^{-1}. The salt-load was injected at 0 minutes as indicated by the arrow and secretion commenced at 12 minutes. Significant differences from control are indicated at each data point. n.s. = not significant, * = $p<0.05$, ** = $p<0.01$, n = number of animals.

Table 2.3
Summary of salt gland secretory rate and treatment of green turtles of varying body mass.

Animal mass (kg)	**Secretory rate (μmol Na.hr^{-1} .100g body mass^{-1})**	**Treatment**	**Source**
0.025	950	Salt-loading	1
0.030	830	Salt-loading	2
1.220	509	Salt-loading	3
12.500	143	Salt-loading	4
15.500	77	Salt-loading	5
50.000 *	28	Dehydration	6

* Prange, pers. comm.

Sources:
1 (Marshall and Cooper, 1988), 2 (Reina, this study) 3 (Owens, unpublished), 4 (Reina, unpublished), 5 (Nicolson and Lutz, 1989), 6 (Prange and Greenwald, 1980)

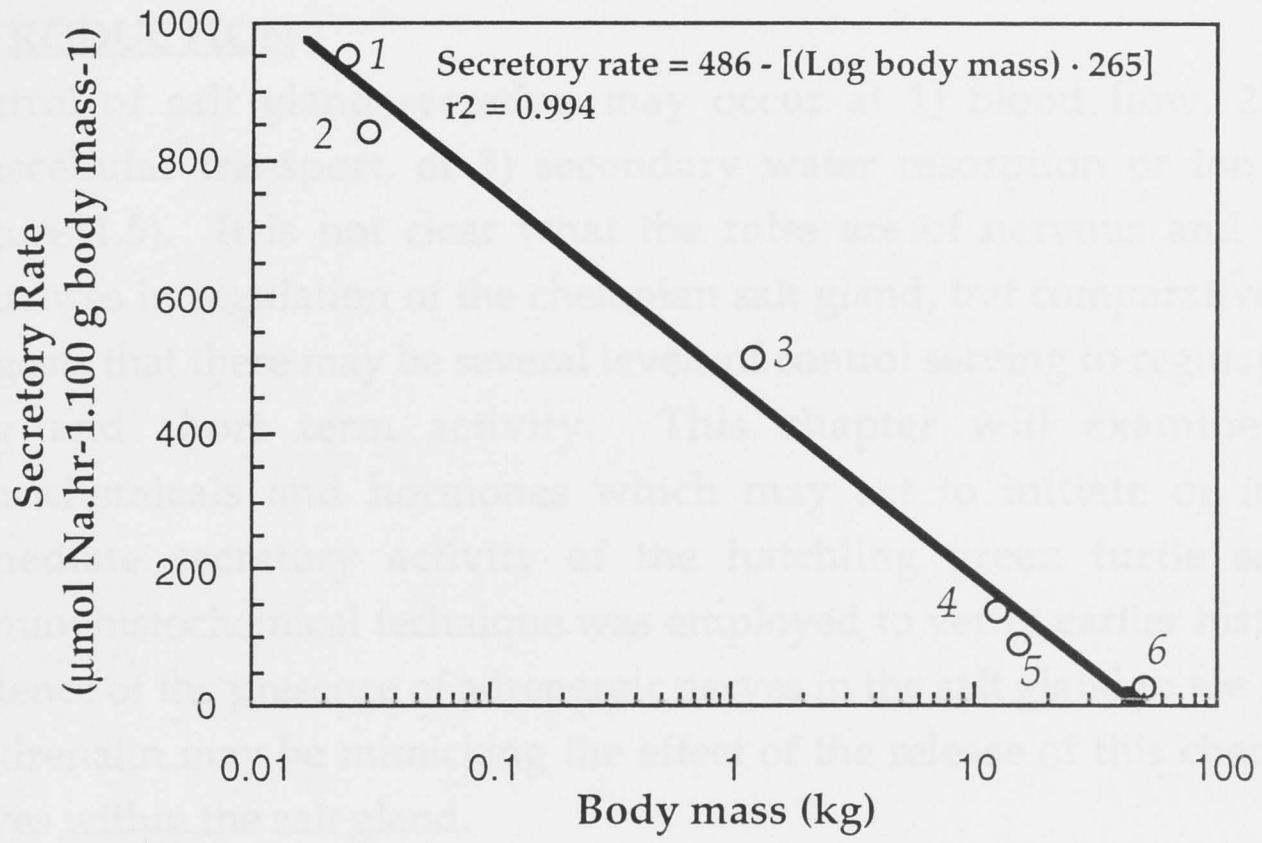


Figure 2.7
Secretory rate (µmol $Na.hr^{-1}.100g$ body $mass^{-1}$) plotted against body mass on a logarithmic scale. Numbers next to data points indicate the source as shown in Table 2.3.

CHAPTER 3
Innervation and Influence of Possible Modifiers Of Salt Gland Activity

INTRODUCTION

Control of salt gland secretion may occur at 1) blood flow, 2) primary transcellular transport, or 3) secondary water resorption or ion transport (Figure 1.5). It is not clear what the roles are of nervous and endocrine influences in regulation of the chelonian salt gland, but comparative evidence suggests that there may be several levels of control serving to regulate both the long and short term activity. This chapter will examine possible neurochemicals and hormones which may act to initiate or inhibit the immediate secretory activity of the hatchling green turtle salt gland. Immunohistochemical technique was employed to verify earlier histochemical evidence of the presence of adrenergic nerves in the salt gland to see if injection of adrenalin may be mimicking the effect of the release of this chemical from nerves within the salt gland.

3.1 CHEMICALS TO BE EXAMINED

Cholinergic, aminergic and peptidergic control of a variety of salt glands has been demonstrated (Tables 1.1 and 1.2). Non-adrenergic, non-cholinergic (NANC) nerves are a novel group of nervous cells which are known to influence the duck salt gland (Hübschle and Gerstberger, 1994; Hübschle *et al.*, 1995). Adrenergic and muscarinic cholinergic receptors have been identified in avian salt gland tissue and it is proposed that adrenergic and cholinergic agonists and antagonists influence secretory cell ion transport through these receptors (Stewart and Sen, 1981; Hootman and Ernst, 1982; Snider *et al.*, 1986; Lowy *et al.*, 1989). It is thought that the intracellular environment is influenced by receptors to regulate the activity of ion transport proteins and so control the movement of ions from the blood to a primary secreted fluid in the secretory tubule lumen.

This study examined several neurochemicals and hormones for an effect on the salt gland *in vivo*. General circulation injection was used, as identifying and cannulating blood vessels supplying the salt gland in the intact animal was difficult. The evidence points to the likely possibility that acetylcholine and adrenalin play important roles in controlling secretion of the avian salt gland so the effect of these on the chelonian salt gland was examined. Atropine is a

competitor for muscarinic cholinergic receptors and has been used in a number of studies to examine the role of acetylcholine in exocrine glands. It has been shown to inhibit secretion in the avian salt gland (Kaul *et al.*, 1983; Lowy *et al.*, 1987) and cat submandibular gland (Lundberg *et al.*, 1981a). The effect of atropine on reptilian salt glands has never been reported

Comparative studies have revealed salt gland regulatory activity for vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) in the avian salt gland and elasmobranch rectal gland (Hammel *et al.*, 1980; Torchia *et al.*, 1992; Silva *et al.*, 1993; Taylor *et al.*, 1995) but their activity has not been examined in the sea turtle. Experiments were conducted to measure the influence on salt secretion from the hatchling sea turtle of application of these neuropeptides in *vivo.*

Nitric oxide (NO) has recently been implicated in control of the avian salt gland (Hübschle and Gerstberger, 1994; Hübschle *et al.*, 1995). Molsidomine is a nitric oxide agonist which is used *in vivo* as an NO donor (Reden, 1990) to influence blood vessel musculature to regulate blood flow. If it does influence secretion this suggests that NO containing nerves may be present which affect the blood vessels supplying the gland. Its ability to influence secretion and hence indicate if nitrergic innervation is involved has never been examined in the sea turtle salt gland.

Arginine vasotocin (AVT) and atrial natriuretic peptide (ANP) are potential endocrine neuromodifiers of salt gland activity and were examined for any short term effect on the secretory response.

3.2 INNERVATION

Abel and Ellis (1966) inferred cholinergic innervation in the salt gland of *Chelonia mydas* by using histochemical techniques to detect the presence of cholinesterase as described in Section 1.6.1. They also histochemically identified the presence of monoamine oxidase, presuming the presence of adrenergic nerves. However, monoamine oxidase is an enzyme with a number of substrates including dopamine, tyramine and tryptamine as well as adrenalin (Cooper *et al.*, 1991). Abel and Ellis proposed that the enzyme was associated with adrenergic nerves in the salt gland, relying on circumstantial evidence from the histochemical technique employed. Belfry and Cowan (1995) identified adrenergic nerves but not cholinergic nerves in the estuarine turtle, despite reports that methacholine stimulated salt gland secretion from this animal (Dunson, 1970). Reptilian antibodies to cholinergic nerves have not

been produced and those raised in mammals do not appear to bind to salt gland tissue (Prof. M. Schemann, personal communication).

A re-examination of the proposed adrenergic innervation in the green sea turtle salt gland is justified, as more specific techniques have become available since the study of Abel and Ellis (1966). By using immunohistochemical technique, more definitive identification of nerve types can be made. It is not the intention to describe proposed adrenergic innervation in detail as was done in the report of Abel and Ellis, but to confirm the identity of the nerves that they describe. Confirmation of the presence of adrenergic nerves within the salt gland will clarify whether adrenalin exerts an influence on salt gland activity through a localised release from neural stores, or is carried through the vascular system to the salt gland from some remote site.

3.3 EXPERIMENTAL AIM

Experiments were conducted to identify possible controlling modifiers by testing the hypotheses that methacholine, adrenalin, atropine, molsidomine, VIP, AVT, ANP and NPY affect the activity of the salt gland *in vivo*. These substances were examined for their ability to influence the salt gland in several ways; 1) to initiate secretory activity, 2) to increase secretion from an active gland, and 3) to suppress secretion from an active salt gland. The response of the salt gland to these chemicals was quantified and compared with that observed in the absence of drug treatment described in Chapter 2. Significant changes in secretory activity following application of chemicals implied that the chemical affected one or more of the three control points to regulate activity. The presence of adrenergic nerves in the gland was examined using immunohistochemical techniques.

METHODS

3.4 ANIMALS

Animals used were as described in Section 2.3.

3.5 COLLECTION OF SAMPLES

Samples were collected as described in Section 2.4.

3.6 EXPERIMENTS

The chemicals affecting salt gland activity were examined for their ability to either stimulate or inhibit secretory activity of the salt gland. Chemicals were injected into the thoracic cavity of hatchlings under one or more of four experimental conditions.

1) In the absence of any salt-load to determine if salt gland secretion could be initiated by the chemical alone.
2) Simultaneously with a salt-load of 2700 µmol NaCl.100g BW^{-1} to determine if the start of tear secretion effected by the salt-load was accelerated or delayed by the chemical.
3) 20 minutes after secretion had been initiated by a salt-load of 2700 µmol NaCl.100g BW^{-1} to determine if secretory parameters were affected by the chemical.
4) Simultaneously with a sub-threshold salt-load to determine if secretion could be initiated by the chemical in these circumstances. Subthreshold salt-load was calculated on the basis of results of experiment 2.5.1 described in Chapter 2.

A summary of experimental chemicals, dosages and conditions is presented in Table 3.1.

3.6.1 Control

Control animals received an injection of phosphate buffered saline (PBS, 0.9% NaCl) in a volume of 2 ml.kg BW^{-1} into the thoracic cavity under all the experimental conditions described for drugs injected.

3.6.2 Chemicals

Where exogenous application of acetylcholine is necessary, methacholine is preferred because it is more resistant to degradation by cholinesterases within the body (Cooper *et al.*, 1991) and so has a greater opportunity to affect the organ of interest. Methacholine (acetyl-β-methyl choline chloride, Sigma), adrenalin (adrenalin bitartrate, Sigma), atropine (Sigma), molsidomine

(Corvaton®, Cassella-Riedel Pharma), VIP (chicken, Sigma), AVT (Arg^8-vasotocin acetate salt, Sigma), ANP (chicken, Auspep) or NPY (human, Auspep) were dissolved in PBS and injected in a volume of 2 ml.kg BW^{-1} at a concentration appropriate to deliver the dosage rates listed in Table 3.1.

An experiment was conducted to determine if atropine could reverse any influence of methacholine in the presence of a salt-load. Animals were injected with a salt-load of 2700 µmol NaCl.100g BW^{-1} simultaneously with methacholine 5 mg.kg BW^{-1}. Atropine 10 mg.kg BW^{-1} was injected into the thoracic cavity 10 minutes later and secretory parameters measured. Control animals received methacholine with a salt-load followed by injection of PBS instead of atropine.

3.6.3 Doses and source of peptides employed

The doses of chemicals used were estimated on the basis of comparative data of AVT dose (Butler, 1972) because the other peptides have never been examined in sea turtles. Other reports have utilised infusion techniques, but this was very difficult in the hatchling turtle, so a single injection was given in the thoracic cavity. A range of doses for most substances was examined within permit restrictions on the number of experimental animals which could be used. The peptides employed were selected from the types available to resemble the amino acid sequence most likely to be found in the green sea turtle. Chicken VIP is identical to alligator VIP (Blomqvist *et al.*, 1992; Wang, 1993) which was presumed to be the most similar to chelonian VIP. Reptilian ANP has not been sequenced, so chicken ANP was selected as most likely to be suitable (Miyata *et al.*, 1988). AVT has been found to have the same amino acid sequence in all non-mammalian vertebrates (Perks, 1987). Human NPY has the same sequence as alligator NPY (Wang, 1993) and so was chosen because chelonian NPY has not been sequenced.

3.7 IMMUNOHISTOCHEMICAL TECHNIQUE

Immunohistochemical technique permits specific and highly resolved identification of many substances in experimental tissue. The principle of the technique relies on antibodies which specifically recognise certain substances such as enzymes, peptides, neurotransmitters or many other chemicals. Details regarding manufacture of antibodies and experimental technique are presented elsewhere (Peters and Coons, 1976). In order to visualise the primary antibody bound to tissue, a secondary antibody is used which recognises part of the primary antibody and is linked to a label which fluoresces or is a substrate of a

chemical reaction which results in a visible product. By utilising immunohistochemical technique, the location of substances can be identified within experimental tissues.

Immunohistochemical technique was used to attempt identification of adrenergic nerves in the salt gland. A primary mouse antibody to tyrosine hydroxylase (Incstar) was employed to identify the presence of this enzyme which is involved in the synthesis of adrenalin and is used to localise adrenergic nerves in the peripheral nervous system (Marfurt and Ellis, 1993; Wrobel and Kujat, 1993). Salt glands were collected from hatchling turtles following decapitation and were fixed in 4% paraformaldehyde in PBS (pH 6.8 - 7.2). Tissue was mounted in Tissue Tek, frozen in a cryostat (Cryocut E) at -20^{0}C and 14 µm sections cut. Sections were placed on slides coated with poly L-lysine and allowed to stand at room temperature for five minutes before incubating for five minutes in 0.2% hydrogen peroxide, followed by three washes of five minutes in PBS with 0.3% Triton-X (PBT). Non specific antibody binding was blocked by incubation with PBT with 5% normal goat serum (PBT + N) at room temperature for two hours. Sections were then incubated with the primary antibody diluted 1:200, 1:500 or 1:1000 in PBT + N at 4^{0}C for 16 hours, followed by three washes of five minutes each in PBT then two washes of 30 minutes each in PBT. Secondary antibody (sheep anti-mouse conjugated to horseradish peroxidase, Serotec) was diluted 1:100 in PBT + N and applied to the tissue for incubation for three hours followed by three washes of five minutes in PBT. A 2:1 ratio of PBT/diaminobenzidine (DAB) solution (1 mg/ml) was prepared, with 8 µl nickel chloride/ml DAB and 10 µl/ml 0.2% hydrogen peroxide. PBT/DAB was applied to the tissue for 10 minutes, then the reaction was stopped by washing slides several times with PBT then three washes of five minutes with PBS. Tissues were dehydrated in ethanol solutions from 50% to 100% then cleared in methyl salicylate overnight and mounted in Fastmount with Colourfast (Histo-labs, Australia). Tissue sections were examined under light microscopy with the presence of immunoreactivity to the primary antibody detected by dark staining and presumed to indicate identification of adrenergic nerves. Rabbit adrenal tissue was used as a positive control, with negative controls having incubation with the primary or secondary antibody omitted from the procedure.

3.8 STATISTICAL ANALYSIS

Differences in secretory parameters among salt gland treatments were determined using the non-parametric Mann-Whitney rank test, with

significance assumed if $p<0.05$. The data for each time point in identical experiments were grouped and the time from the commencement of secretion was standardised. This was because drug treatments were administered 20 minutes after the start of tear production, rather than a certain time from injection of the salt-load, to allow for individual differences in the delay between salt-loading and start of visible gland activity. All results are shown as the mean ± 1 standard error.

RESULTS

3.9 CONTROL

Injection of PBS after secretion had been initiated by a salt-load did not affect the rate of sodium secretion by the salt gland (Figure 3.1).

3.10 INFLUENCE OF CHEMICALS ON SALT GLAND SECRETION

3.10.1 Methacholine

Methacholine had no measurable effect on salt gland secretion in the absence of a salt-load but salt gland secretion stimulated by a salt-load ceased immediately following injection of methacholine at a dose of 10 mg.kg BW^{-1} (Figure 3.2). Tears stopped flowing from the gland onto the eye within 2 minutes of methacholine administration, while injection of an equal volume of PBS had no effect. When methacholine was injected into hatchlings at the same time as a salt-load, the onset of secretion was delayed in an apparently dose dependent manner (Figure 3.3). The small change in delay between dosages of 5 and 10 mg.kg BW^{-1} suggests that maximal inhibition was reached somewhere between these dosages. When secretion commenced there was a dose dependent time delay for secretion to reach control levels (Table 3.3). Animals receiving a dose of 1 mg.kg BW^{-1} methacholine secreted at control rates almost immediately, but higher doses resulted in a depression of total sodium secretion. At a dose of 5 mg.kg BW^{-1}, secretion commenced at approximately 67% of control, rising to 100% after 23 minutes. At a dose of 10 mg.kg^{-1}, secretion commenced at approximately 62% of control and took 48 minutes to reach 100% of control rates.

3.10.2 Adrenalin

Adrenalin did not measurably influence salt gland activity in the absence of a salt-load. Secretion initiated by a salt-load ceased within 2 minutes following injection of adrenalin at doses of 25 µg.kg BW^{-1} (n = 5), 100 µg.kg BW^{-1} (n = 5), 500 µg.kg BW^{-1} (n = 5) and 2 mg.kg BW^{-1} (n = 2). When adrenalin was administered simultaneously with a salt-load, there was a dose dependent delay in the onset of secretion (Figure 3.4). When secretion commenced following inhibition by adrenalin it took about 10 minutes to return to the control rate, this period did not change with increasing dose (Figure 3.4 & Table 3.4). Following initiation of secretion, rates first were 80% of control (25 and 100 µg.kg BW^{-1}) or 50% of control (500 µg.kg BW^{-1}).

3.10.3 Atropine

In the absence of a salt-load, atropine at a dose of 10 mg.kg BW^{-1} led to tear formation in three of four hatchlings, although tears did not appear until an average of 50 minutes after injection (Figure 3.5). The secretory rate was low and tears ceased after about 15 minutes. Tears did not appear at doses below 10 $mg.kg^{-1}$. When atropine was injected simultaneously with a subthreshold salt-load of 200 µmol NaCl.100g BW^{-1}, tears appeared in five of seven hatchlings (Figure 3.5). Tears were formed an average of ten minutes (± 2 min) after injection and secretion continued for about 15 minutes.

Atropine reversed the inhibition of salt gland secretion caused by methacholine. Methacholine 5 $mg.kg^{-1}$ was injected at the same time as a salt-load, inhibiting the onset of secretion. After 10 minutes, animals received either atropine 10 $mg.kg^{-1}$ or received PBS, with the atropine treated animal producing tears an average of 7 minutes later (range 5 to 10 minutes). Animals which received PBS instead of atropine remained inhibited and did not secrete (Figure 3.6).

3.10.4 Molsidomine

Molsidomine did not initiate salt secretion in the absence of a salt-load at any of the doses injected up to 50 $mg.kg^{-1}$ (n = 8).

3.10.5 VIP

VIP did not initiate secretion in the absence of a salt-load (n = 11) and had no significant effect on salt gland secretion under any of the other experimental conditions (Figure 3.7).

3.10.6 AVT

AVT did not initiate secretion in the absence of a salt-load (n = 11). Injection of AVT 30 $ng.kg^{-1}$ produced a transient reduction in sodium secretion by the active salt gland (Figure 3.8). This occurred immediately after AVT administration and lasted approximately ten minutes, after which secretion returned to control values. There was a significant reduction in tear osmotic concentration and rate of tear production fell but the change was not significant. Total sodium secretion is a product of both osmotic concentration and fluid secretion rate, so the effect of AVT on sodium removal is greater than that seen in either of these factors alone. There did not appear to be any dose dependence of this inhibition, as the reduction in secretory rate caused by a 300 $ng.kg^{-1}$ dose of AVT was not significantly different from that of 30 $ng.kg^{-1}$.

3.10.7 ANP

ANP did not initiate secretion in the absence of a salt-load (n =11) and had no measurable effect on salt gland secretion under any of the other experimental conditions (Figure 3.9).

3.10.8 NPY

NPY did not initiate secretion in the absence of a salt-load (n = 7) and had no measurable effect on salt gland secretion under any of the experimental conditions (Figure 3.10).

A summary of the effects of chemicals injected is shown in Table 3.2.

3.11 ADRENERGIC INNERVATION

Immunoreactivity to tyrosine hydroxylase (assumed to represent adrenergic nerves) was observed in the salt glands and the rabbit adrenal tissue (positive control), but not the negative control. A positive reaction was observed when the primary antibody was diluted 1:200 and 1:500, but the reaction was weak at a dilution of 1:1000. Nerves were observed around the edges of the gland (Figure 3.11a) where they branched several times in towards the centre of the gland. Other nerves extended into the lobes of the gland (Figure 3.11b) but it could not be determined if they were innervating the secretory tubules themselves or passing through the lobe. It was not possible to tell if nerves were associated with any particular element of the glandular structure.

DISCUSSION

The results demonstrate that the secretory response of the chelonian salt gland can be modified *in vivo* by the application of some exogenous chemicals. The hypotheses that methacholine, adrenalin, atropine and AVT influenced the rate of sodium secretion under certain experimental conditions were supported by the experimental data. The hypotheses regarding the ability of molsidomine, VIP, ANP and NPY to affect the secretory response of the salt gland were not supported. Immunohistochemical technique confirmed the presence of proposed adrenergic nerves within the salt gland.

The inhibitory action of methacholine was both immediate and sustained, with tear production ceasing within two minutes of methacholine injection at doses as low as 1 mg.kg BW^{-1}. Its action may have been even more rapid than this, as tears observed may have been formed in the gland prior to injection and taken a short time to travel down the ducts to the surface of the eye. In another investigation on cholinergic stimulation in the crocodile it had been found that methacholine had an inhibitory action at higher doses and a stimulatory effect at lower doses (Grigg, personal communication). However, in this study doses of methacholine down to 100 $ng.kg^{-1}$ did not stimulate the gland, indicating that there was not a reversal of action in this range. When methacholine was administered at doses of 5 and 10 mg.kg BW^{-1} after a salt-load, secretion commenced at a rate less than the control rate and took some time to reach control levels. It is not apparent from the data how the inhibition of secretory activity was achieved, but it resulted in the complete abolition of tear production for some time. Histochemical evidence indicated the presence of cholinergic nerves within the salt gland (Abel and Ellis, 1966), so it is presumed that methacholine mimics the effect of these nerves. However in the absence of definitive immunohistochemical evidence, the possibility remains that cholinergic nerves are absent and that methacholine is influencing gland activity through some unrelated or pharmacological mechanism.

The inhibitory action of methacholine is in contrast to the earlier report of Schmidt-Nielsen and Fänge (1958), where they found that methacholine initiated the secretory response. The experimental history of the animal injected in that study was unclear and it is possible that it had been salt-loaded in a previous experiment or had become dehydrated during transport from the site of capture (Schmidt-Nielsen, personal communication). If this was the case, secretion observed may have been spontaneous or as a result of prior salt-loading and not related to the methacholine injection. The delay between salt-

loading and salt gland secretion is quite lengthy in large animals (Nicolson and Lutz, 1989) and it would be expected that initiation of secretion could take several hours in an adult animal. When one considers the period of inhibition by methacholine measured in hatchlings (30 to 50 minutes) this is considerably less than the delay between salt loading and tear production of about 70 to 100 minutes reported by Nicholson and Lutz (1989) in juvenile turtles. It is not known what delay occurred between injection of methacholine and appearance of tears in the study of Schmidt-Nielsen and Fänge (Schmidt-Nielsen, personal communication), but it is possible that the inhibitory influence of methacholine had diminished during the period between an earlier salt-load and the secretion observed.

Application of exogenous atropine had a stimulatory effect on salt gland secretion, which was slight in the absence of a salt-load but which led to secretion when administered with a sub-threshold salt-load. There was a long delay between injection of atropine and the appearance of tears (about 50 minutes) when no salt-load was given. The mechanism by which atropine stimulated secretion is unclear. Atropine may act either directly by affecting salt gland secretory cells or vasculature, or indirectly by influencing cardiac function or some other factor which affected the salt gland.

The long delay between atropine injection and the appearance of tears and the brief secretory response indicate that the effect of atropine under the experimental conditions was not characteristic of the normal secretory response to a salt-load. It is possible that a greater dose of atropine may have been sufficient, but this was not attempted for two reasons. 1) Systemic injection of a large dose of a cholinergic antagonist may severely interfere with cardiac function of the study animal. 2) Any effect of atropine observed at high doses may be pharmacological rather than physiological, hence providing misleading information.

Atropine stimulated tear production when administered with a subthreshold salt-load, suggesting that elevated plasma sodium may be a prerequisite of normal salt gland secretion. However, the secretion evoked by atropine and a subthreshold salt-load was only transient. Perhaps after a short time plasma salt was reduced by the salt gland or some other means to a level not sufficient for secretion to continue. It is feasible that plasma salt must be above a certain threshold for the secretory cells to remove it from the blood and so produce tears, but from the data it can not be determined if this is the case.

The reversal of methacholine inhibition of the salt gland by atropine suggests that there may be chronic, cholinergic down-regulation of the gland when secretion is not required, which is transiently counteracted by the injection of exogenous atropine . Thus in the case of tear production following atropine injection, the atropine had not stimulated the gland *per se*, but rather had temporarily prevented methacholine from inhibiting the gland activity. In the context of gland control *in vivo*, the cessation of inhibition by cholinergic nerves may permit the gland to become active.

Adrenalin also exerts an inhibitory influence on salt gland activity in hatchlings, but is more potent than methacholine. A dose of 25 μg.kg BW^{-1} adrenalin inhibited the commencement of secretion by 20 minutes, while a dose of 1 mg.kg BW^{-1} methacholine was needed to cause the same delay in secretory response. On recovery from inhibition, animals secreted at control rates after about 10 minutes. The time for recovery once secretion commenced did not change with increasing dose of adrenalin, although the time taken for secretion to start was lengthened. It is not possible to determine the location of the inhibitory activity of adrenalin from the experiments conducted.

The detection of adrenergic nerves by immunohistochemical technique confirms the localisation of nerves proposed to be adrenergic following detection of monoamine oxidase by histochemical methods (Abel and Ellis, 1966). It was not possible to determine if nerve fibres were associated with blood vessels or other elements of the gland structure, but they could be seen around the periphery of the gland as well as penetrating into the secretory lobes. The identification of these adrenergic nerves supports the proposition that the injection of exogenous adrenalin into hatchling turtles to influence the salt gland mimics the release of adrenalin from nerve fibres within the gland itself.

The nitric oxide donor molsidomine did not stimulate salt gland activity in the absence of a salt-load. This may be because there are no receptors sensitive to nitric oxide in or around the salt gland or the specificity of NO donor may be a critical factor. Molsidomine is a commonly used NO donor with vasoregulatory action, but most studies are carried out on mammalian systems. Reptilian nitrergic innervation may require different NO donors. Examination of a wider range of donors may reveal if this is the case.

VIP has a major stimulatory role in avian and elasmobranch groups so it was surprising that it was unable to influence the secretory activity of the turtle salt gland at the doses used. The fact that the peptide could not be infused into the blood vessels supplying the salt gland may have been an important factor. The chicken VIP used may not have been suitable for use in the sea turtle if there is a difference in amino acid sequence. However, a larger dose may have produced some effect.

ANP and NPY did not appear to affect turtle salt gland activity under the experimental conditions, suggesting that control mechanisms differ between vertebrate groups possessing salt glands. AVT had a transient inhibitory influence on salt gland activity, but only when administered after initiation of salt gland secretion by a salt-load. This was mediated primarily through a reduction in tear ionic concentration. AVT did not reduce the concentration of secretion when administered simultaneously with a salt-load, probably because its influence had ceased in the intervening period from salt injection to tear formation. The reduction of tear ionic concentration is consistent with the observation that dilute tears are produced by nesting female turtles at a time when circulating AVT concentrations are very high (P. Cooper, personal communication). It is unclear whether AVT has a direct influence on the salt gland itself, as its impact on total sodium removal by the gland is relatively slight.

One of the limitations of the experimental technique employed in this study is that exogenous chemicals are introduced into the systemic circulation. It is not possible to inject directly into the blood vessels supplying that salt gland of the turtle hatchling, as epidermal scales lie over the area and the blood vessels are very small. The possibility exists that no effect on salt gland activity was seen for some chemicals because they were degraded rapidly or for some reason did not reach the salt gland. Application of higher doses than those employed may reveal if this was the case, but a limited number of doses could be examined. However, the results of AVT administration shows that peptide at least was able to exert an influence on the salt gland. Thus some confidence may be held that the other injected peptides at similar doses were also potentially able to influence the gland and that absence of effect was a real result and not solely a limitation of the experimental technique. Until the amino acid sequences for the peptides examined are determined in turtles, there remains a possibility that peptides were not recognised by receptors for the endogenous peptide due to differences in sequence.

Examination of the comparative data shows that the antagonistic actions of adrenergic and cholinergic agonists found in other exocrine glands and the avian salt gland were not found in the green turtle salt gland. There is not a consistency of cholinergic action among reptilian salt glands, with a stimulatory role found in the estuarine crocodile and marine iguana but an inhibitory action in the hatchling green turtle. Within experimental limitations, the peptidergic influences exerted on salt gland secretion in other animals and the nitrergic influence seen on the avian gland are not seen in the chelonian gland. There may be a role for peptidergic control in the salt gland despite the absence of evidence from the experiments conducted. It is possible that an osmoregulatory function for a known peptide not tested exists in the chelonian salt gland, or there may be novel peptides involved not yet identified.

In summary, secretion from the salt gland of the hatchling green turtle is inhibited by the neurotransmitters methacholine and adrenalin. Atropine has a slight stimulatory influence on the gland and reverses the inhibition of methacholine. AVT transiently reduces the secretion from the gland but the other peptides examined did not show any measurable effect under the experimental conditions used. It is not clear from the data how these modifiers exerted their influence, whether by changing blood flow, secretory cell activity or other means. It is unusual that both cholinergic and adrenergic inhibition of the gland occur, as acetylcholine and adrenalin generally have antagonistic roles in controlling activity of other exocrine glands. Adrenergic nerves were demonstrated by immunohistochemical means, confirming an earlier report which inferred their presence histochemically and indicating that adrenalin probably affects the gland through release from adrenergic nerves in the gland. Production of suitable antibodies will allow collection of immunohistochemical evidence to determine if cholinergic nerves are present. Identification of potential neural modifiers of salt gland activity permits an investigation into the separate processes which may operate to regulate the salt gland, these are described in the chapters following.

Table 3.1

Summary of chemicals and doses used under different experimental conditions. All doses are per kg body mass. "No salt-load" refers to injection of chemical in the absence of any salt-load. "With salt-load" refers to injection of chemical simultaneously with a salt-load of 2700 µmol NaCl.100g BW^{-1}. "After salt-load" refers to injection of chemical 20 minutes after initiation of salt gland secretion by a salt-load of 2700 µmol NaCl.100g BW^{-1}. "With subthreshold salt-load" refers to injection of chemical simultaneously with a salt-load of 200 µmol NaCl.100g BW^{-1}. A blank box indicates that the chemical was not tested under that particular condition.

Chemical	No salt-load	With salt-load	After salt-load	With subthreshold salt-load
Methacholine.kg BW^{-1}	100 ng, 1 µg, 10 µg, 100 µg, 1 mg, 10 mg	1 mg, 5 mg, 10 mg	2 mg, 10 mg	
Adrenalin.kg BW^{-1}	10 mg, 100 mg	25 µg, 100 µg, 500 µg, 2 mg, 10 mg	25 µg, 100 µg, 500 µg, 10 mg	10 mg
Atropine.kg BW^{-1}	10 µg, 100 µg, 1 mg, 10 mg			10 mg
Molsidomine.kg BW^{-1}	100 ng, 10 µg, 100 µg, 1 mg, 10 mg, 50 mg			
VIP.kg BW^{-1}	3.5 ng, 10 ng, 30 ng, 60 ng, 300 ng	30 ng	30 ng	30 ng
AVT.kg BW^{-1}	30 ng	30 ng	30 ng, 300 ng	
ANP.kg BW^{-1}	30 ng, 60 ng	30 ng	30 ng	
NPY.kg BW^{-1}	30 ng	30 ng	30 ng	

Table 3.2
Summary of effects of chemicals injected on salt gland activity under different experimental conditions. "0" indicates that the chemical had no measurable effect, "—" indicates that there was an inhibitory effect , "+" indicates that there was a stimulatory effect. "No salt-load" refers to injection of chemical in the absence of any salt-load. "With salt-load" refers to injection of chemical simultaneously with a salt-load of 2700 µmol NaCl.100g BW^{-1}. "After salt-load" refers to injection of chemical 20 minutes after initiation of salt gland secretion by a salt-load of 2700 µmol NaCl.100g BW^{-1}. "With subthreshold salt-load" refers to injection of chemical simultaneously with a salt-load of 200 µmol NaCl.100g BW^{-1}. A blank box indicates that the chemical was not tested under that particular condition.

Chemical	No salt-load	With salt-load	After salt-load	With subthreshold salt-load
Methacholine	0	—	—	
Adrenalin	0	—	—	0
Atropine	+?			+
Molsidomine	0			
VIP	0	0	0	0
AVT	0	0	—	
ANP	0	0	0	
NPY	0	0	0	

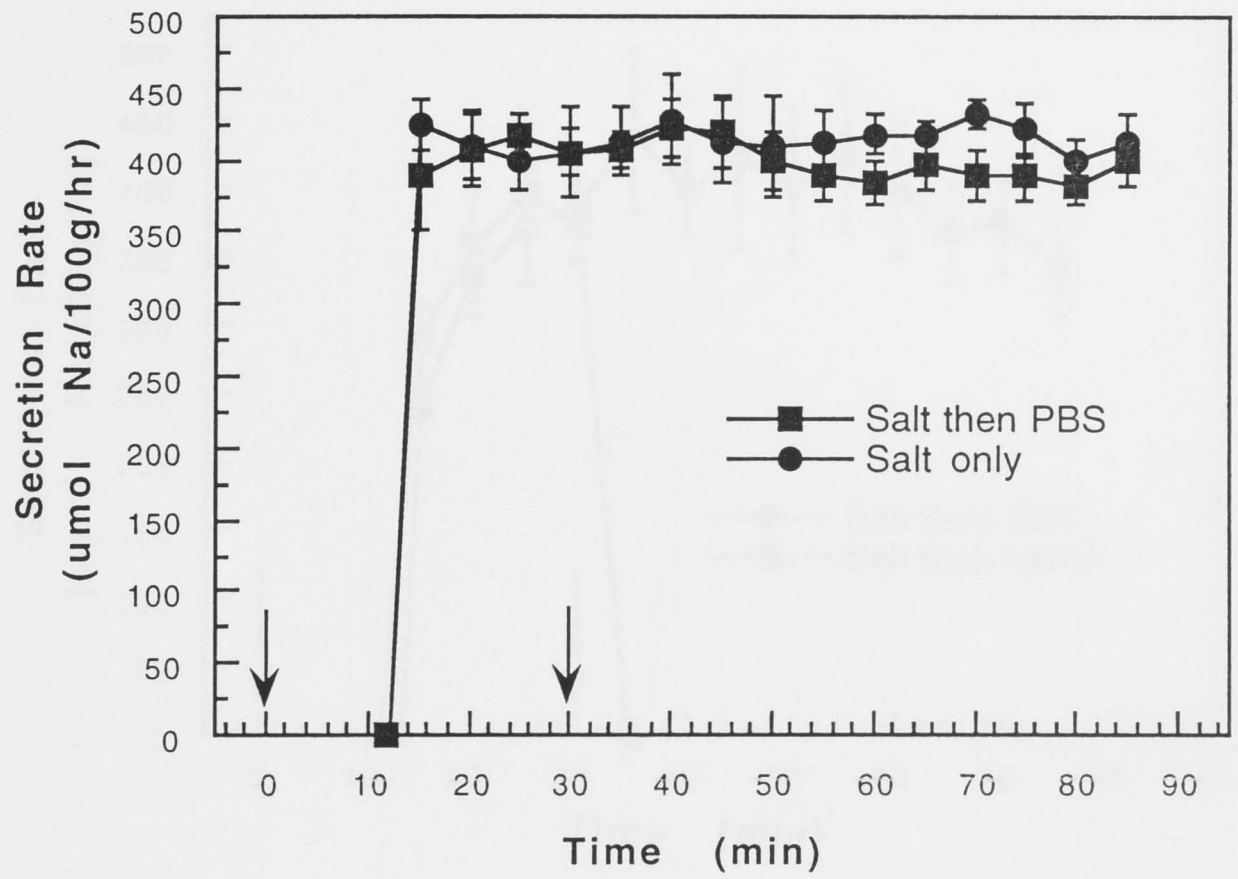


Figure 3.1
Effect of injection of phosphate buffered saline (PBS) on sodium secretion rate initiated by salt-loading. Average secretion rate is shown ± 1 standard error. The first arrow indicates the time of injection of all animals with a salt-load of 2700 μmol NaCl.100g BW^{-1}. At the second arrow, experimental animals received an injection of 2 $ml.kg^{-1}$ PBS (n = 6), while control animals were untreated (n = 6). There were no significant differences in secretion rate following the PBS injection.

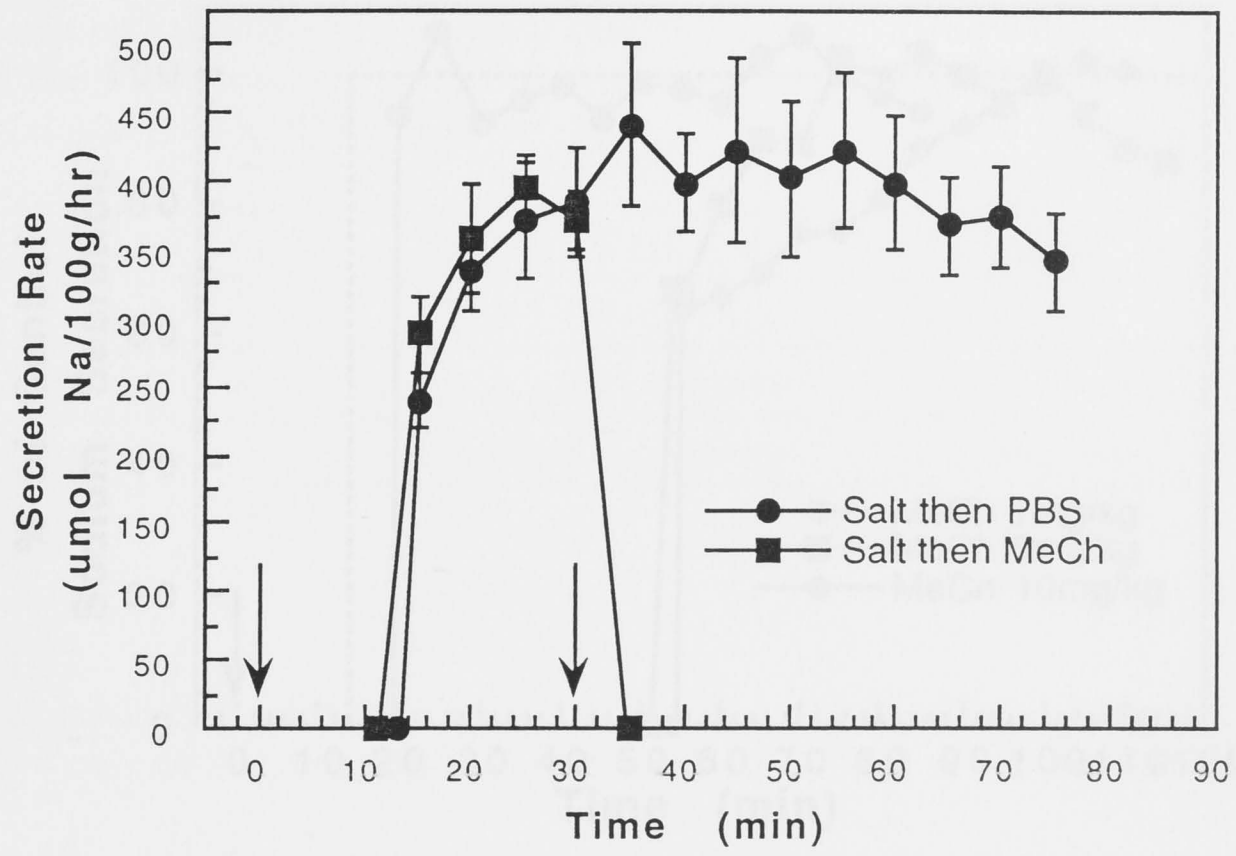


Figure 3.2
Effect of injection of 10 mg.kg BW^{-1} methacholine (MeCh) on sodium secretion rate initiated by salt-loading. Average secretion rate is shown ± 1 standard error. The first arrow indicates the time of injection of all animals with a salt-load of 2700 µmol NaCl.100g BW^{-1}. At the second arrow, experimental animals received an injection of 10 mg.kg BW^{-1} methacholine (n = 6), while control animals received an equal volume of PBS (n = 6). Secretion was abolished in about two minutes in treated animals, but continued in controls. The same inhibition was seen at doses of 1 and 5 mg.kg BW^{-1} methacholine.

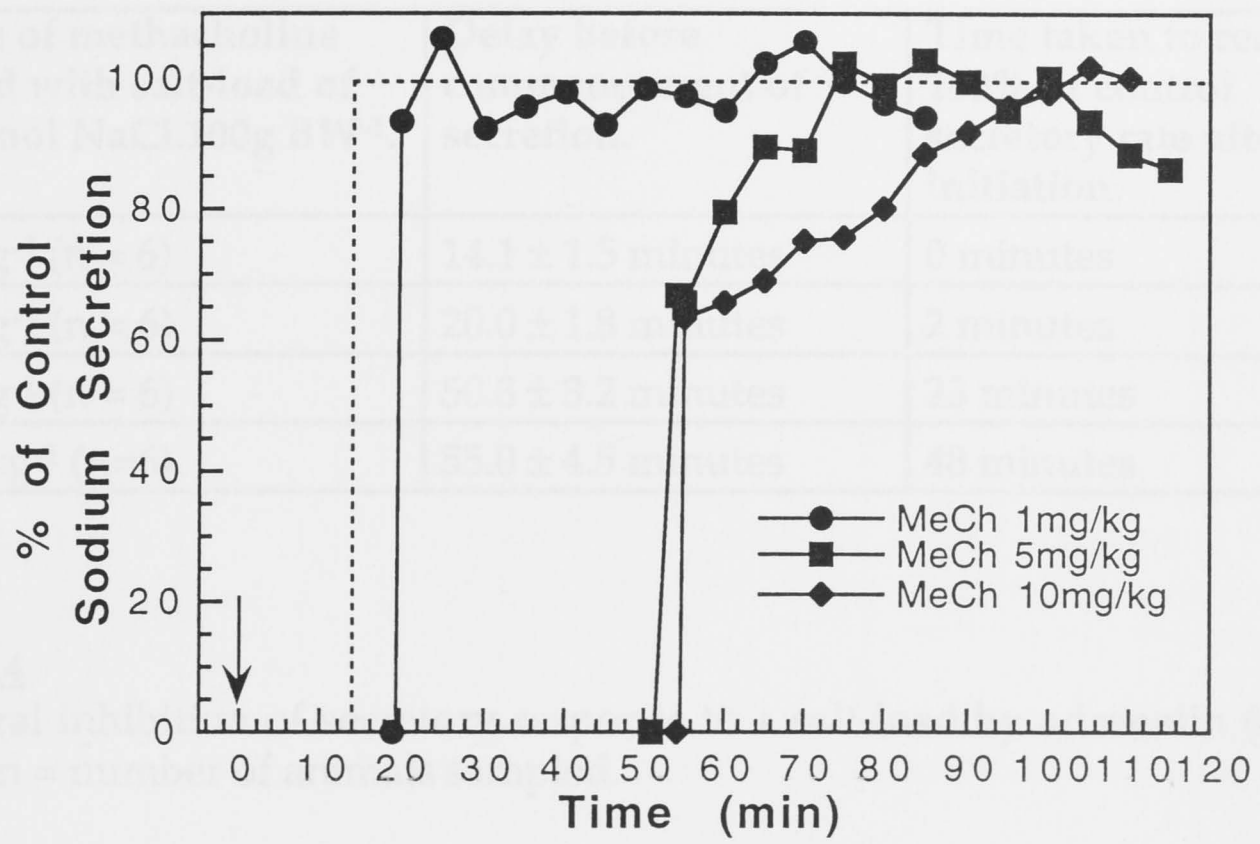


Figure 3.3
Effect on sodium secretion of methacholine injection of 1 mg.kg BW^{-1} (n = 6), 5 mg.kg BW^{-1} (n = 6), 10 mg.kg BW^{-1} (n = 6) when injected simultaneously with a salt-load of 2700 µmol NaCl.100g BW^{-1}. The dashed line indicates the rate from animals which received a salt-load only (control). Secretion was measured in µmol $Na.100g^{-1}.hr^{-1}$ and calculated as a percentage of secretion of control (n = 9). No error bars are shown because only two values were compared at each time point - the average control secretory rate and the average experimental secretory rate.

Table 3.3
Temporal inhibition by methacholine of secretory response to a salt-load (± 1 std error). n = number of animals sampled.

Dosage of methacholine injected with salt-load of 2700 µmol NaCl.100g BW^{-1}.	**Delay before commencement of secretion.**	**Time taken to reach 100% of control secretory rate after initiation.**
0 $mg.kg^{-1}$ (n = 6)	14.1 ± 1.5 minutes	0 minutes
1 $mg.kg^{-1}$ (n = 6)	20.0 ± 1.8 minutes	2 minutes
5 $mg.kg^{-1}$ (n = 6)	50.8 ± 3.2 minutes	23 minutes
10 $mg.kg^{-1}$ (n=6)	55.0 ± 4.5 minutes	48 minutes

Table 3.4
Temporal inhibition of secretory response to a salt-load by adrenalin (± 1 std error). n = number of animals sampled.

Dosage of adrenalin injected with salt-load of 2700 µmol NaCl.100g BW^{-1}	**Delay before commencement of secretion**	**Time taken to reach control secretory rate after initiation**
0 $mg.kg^{-1}$ (n = 6)	14.1 ± 1.5 minutes	0 minutes
25 $\mu g.kg^{-1}$ (n = 5)	20.0 ± 2.2 minutes	8 minutes
100 $\mu g.kg^{-1}$ (n = 5)	34.0 ± 1.8 minutes	10 minutes
500 $\mu g.kg^{-1}$ (n = 5)	80.0 ± 4.6 minutes	8 minutes

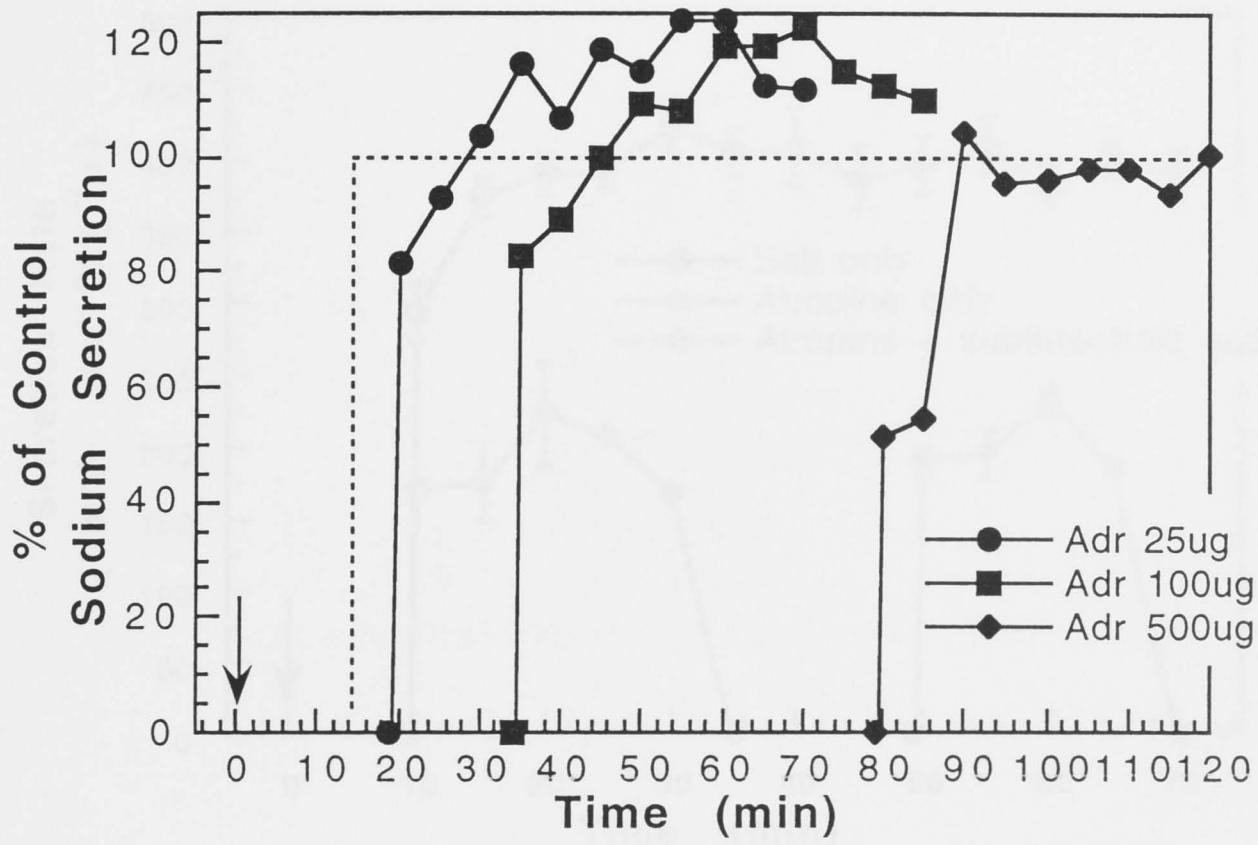


Figure 3.4
Effect on sodium secretion of adrenalin injection of 25 µg.kg BW^{-1} (n = 5), 100 µg.kg BW^{-1} (n = 5) and 500 µg.kg BW^{-1} (n = 5) when injected simultaneously with a salt-load of 2700 µmol NaCl.100g BW^{-1}. The arrow indicates the time at which the salt-load and adrenalin were injected. The dashed line indicates the rate from animals which received a salt-load only (control). Secretion was measured in µmol $Na.100g^{-1}{\cdot}hr^{-1}$ and calculated as a percentage of secretion of control (n = 9). No error bars are shown because only two values were compared at each time point - the average control secretory rate and the average experimental secretory rate.

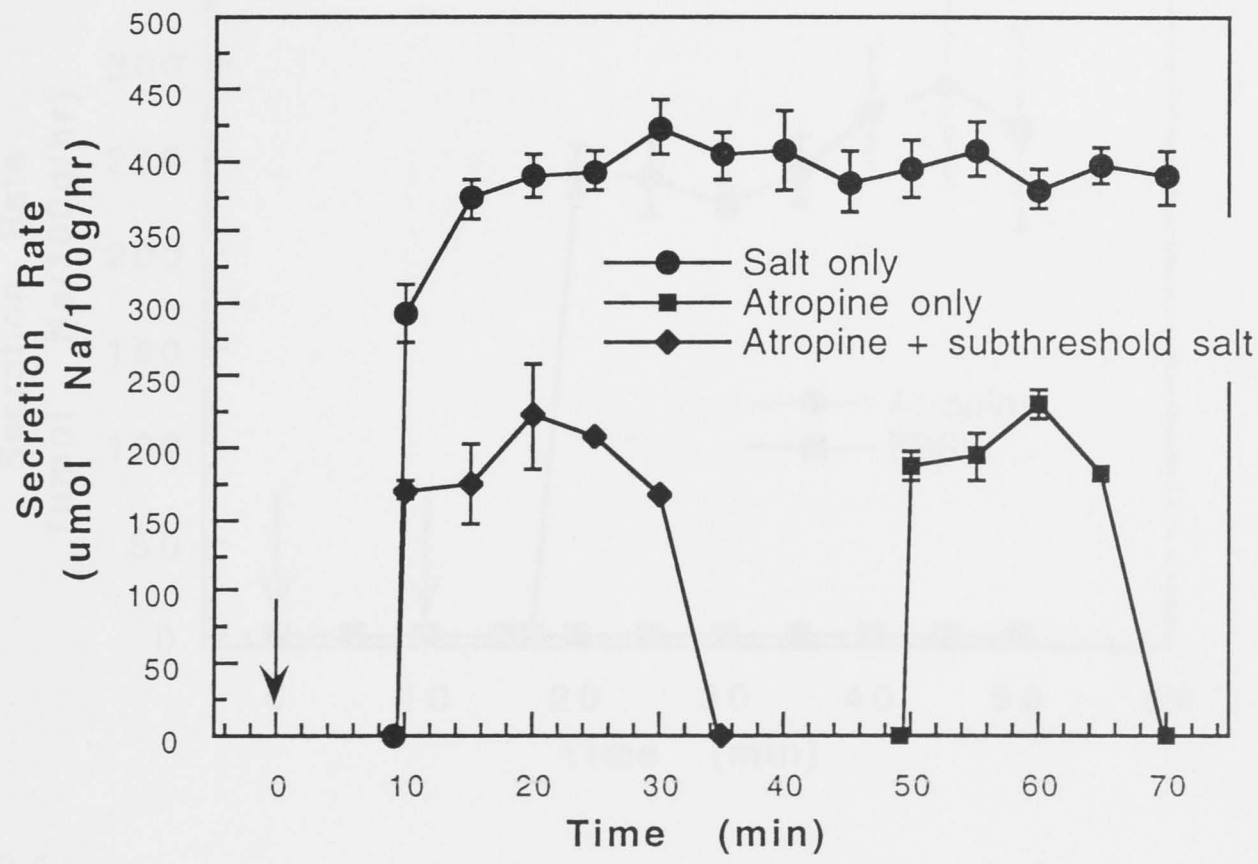


Figure 3.5

Influence of atropine on secretion by the salt gland. Average secretion rate is shown ± 1 standard error. The arrow indicates time at which animals received an injection of salt only (2700 µmol NaCl.100g BW^{-1}, n = 9), atropine 10 mg.kg BW^{-1} only (n = 4), or atropine 10 mg.kg BW^{-1} with a subthreshold salt-load of 200 µmol NaCl.100g BW^{-1} (n = 7). 1 of 4 animals injected with atropine did not secrete and 2 of 7 injected with atropine and a subthreshold salt-load did not secrete.

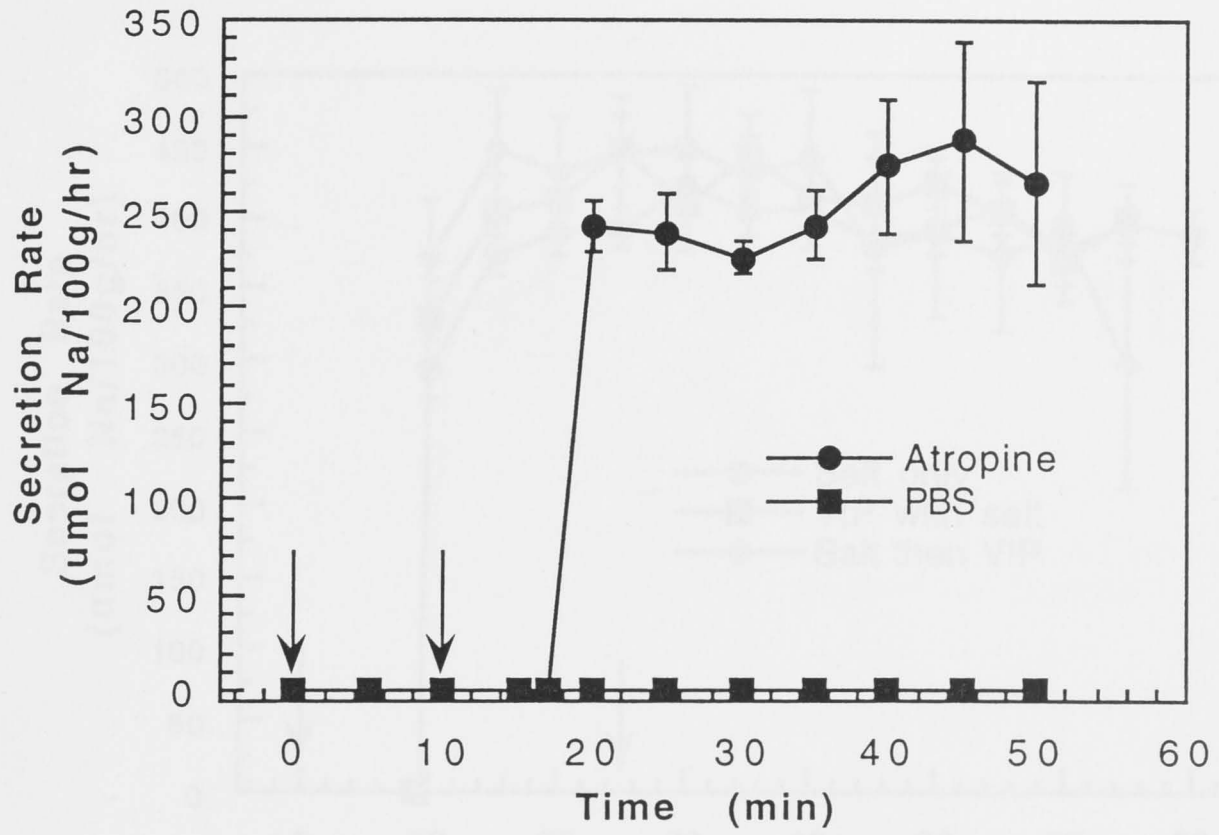


Figure 3.6
Atropine reversal of methacholine inhibition of salt secretion. Average secretion rate is shown ± 1 standard error. The first arrow indicates time of injection of a salt-load of 2700 µmol NaCl.100g BW^{-1} simultaneous with methacholine 5 mg.kg BW^{-1}. The second arrow indicates the time at which animals were injected with either atropine 10 mg.kg BW^{-1} (n = 4) or the same volume of PBS (n = 4). Animals which received atropine began secreting an average of seven minutes after atropine injection, while those which received PBS did not secrete for the duration of the experiment.

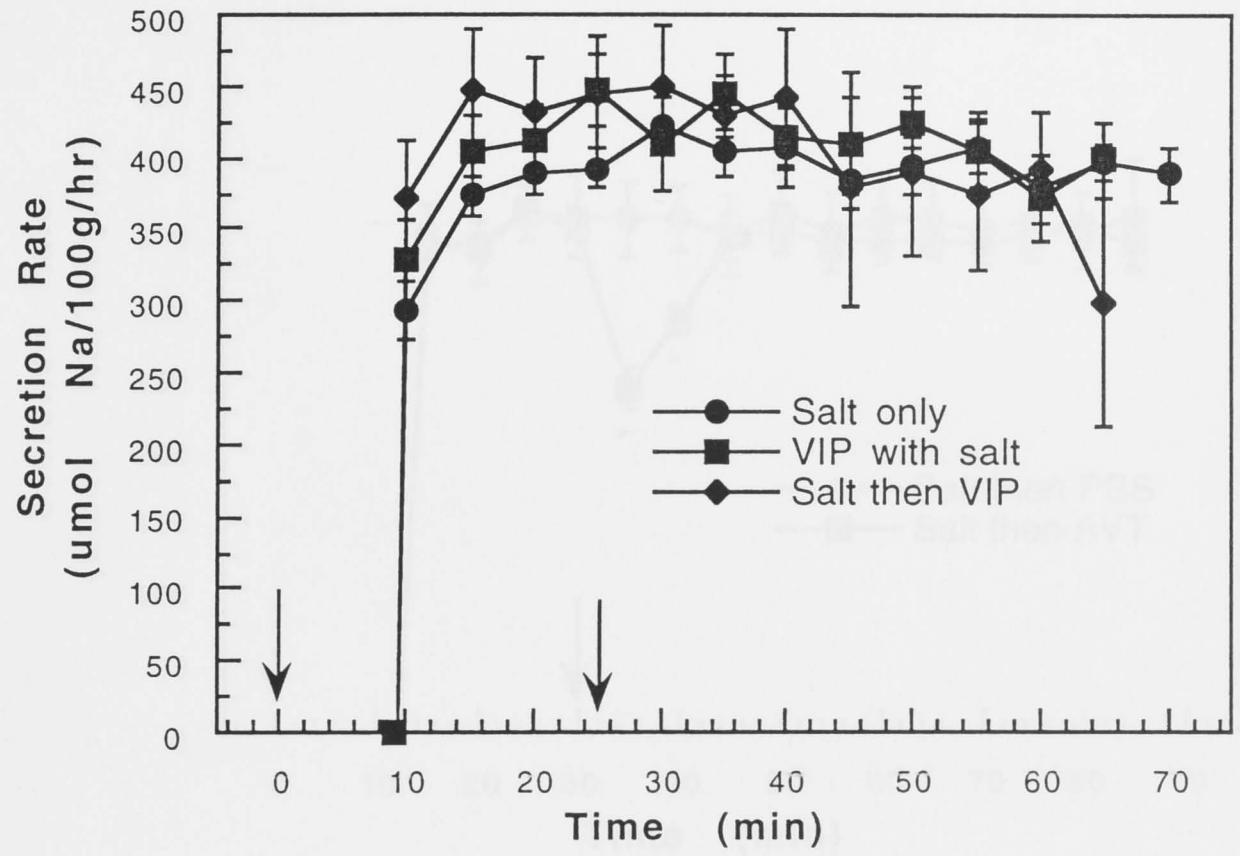


Figure 3.7
Effect of VIP injection (30 ng.kg BW^{-1}) on secretory rate of salt gland. Average secretion rate is shown ± 1 standard error. The first arrow indicates when all animals were injected with a salt-load of 2700 µmol NaCl.100g BW^{-1}. Some animals also received VIP with the salt-load(n = 11), while others received VIP at the time indicated by the second arrow (n = 9). There were no significant differences in secretory rate of animals treated with VIP compared to those receiving a salt-load alone (n = 9).

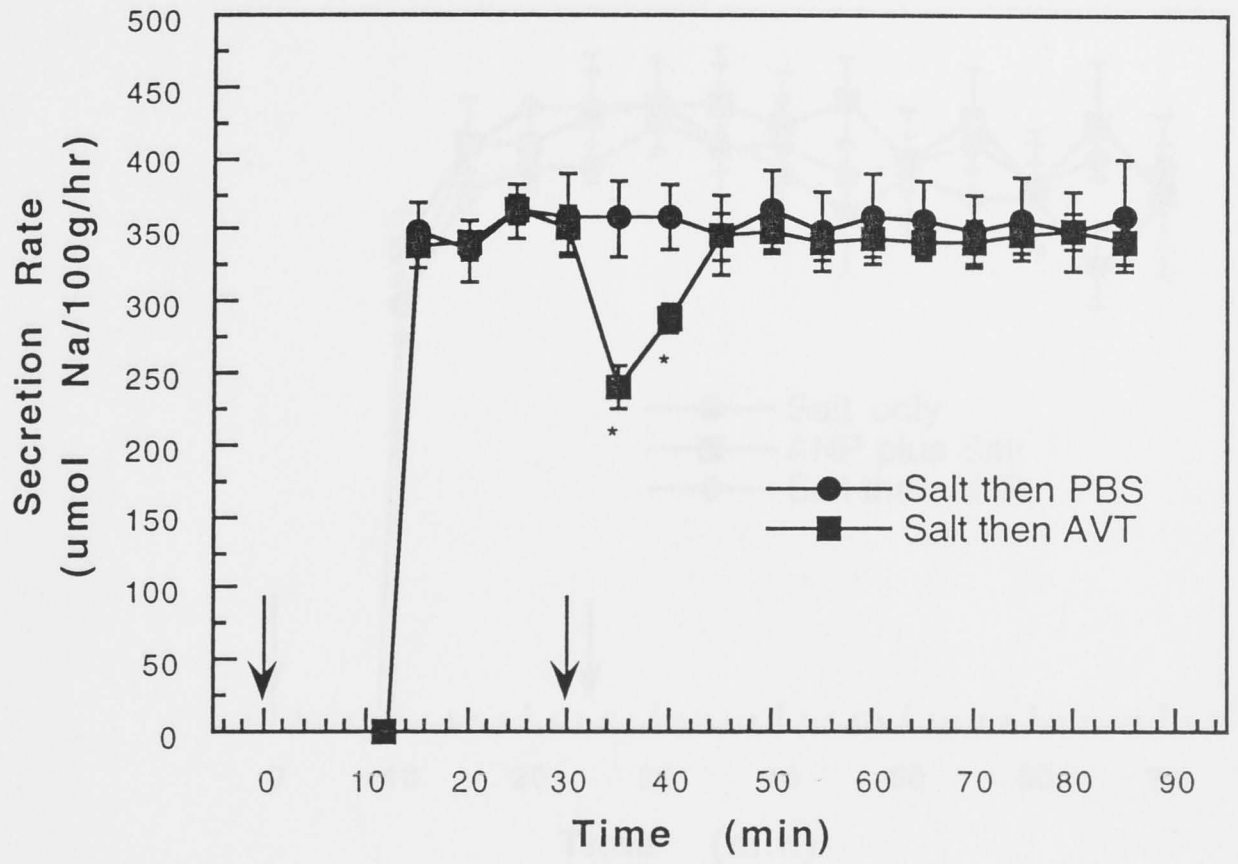


Figure 3.8
Effect of AVT injection (30 ng.kg BW^{-1}) on secretory rate of salt gland. Average secretion rate is shown ± 1 standard error. The first arrow indicates when all animals were injected with a salt-load of 2700 µmol NaCl.100g BW^{-1}. The second arrow indicates when AVT was injected in some animals (n = 9), while control animals received PBS (n = 9). Asterisks indicate significant differences in secretory rate, $p<0.05$.

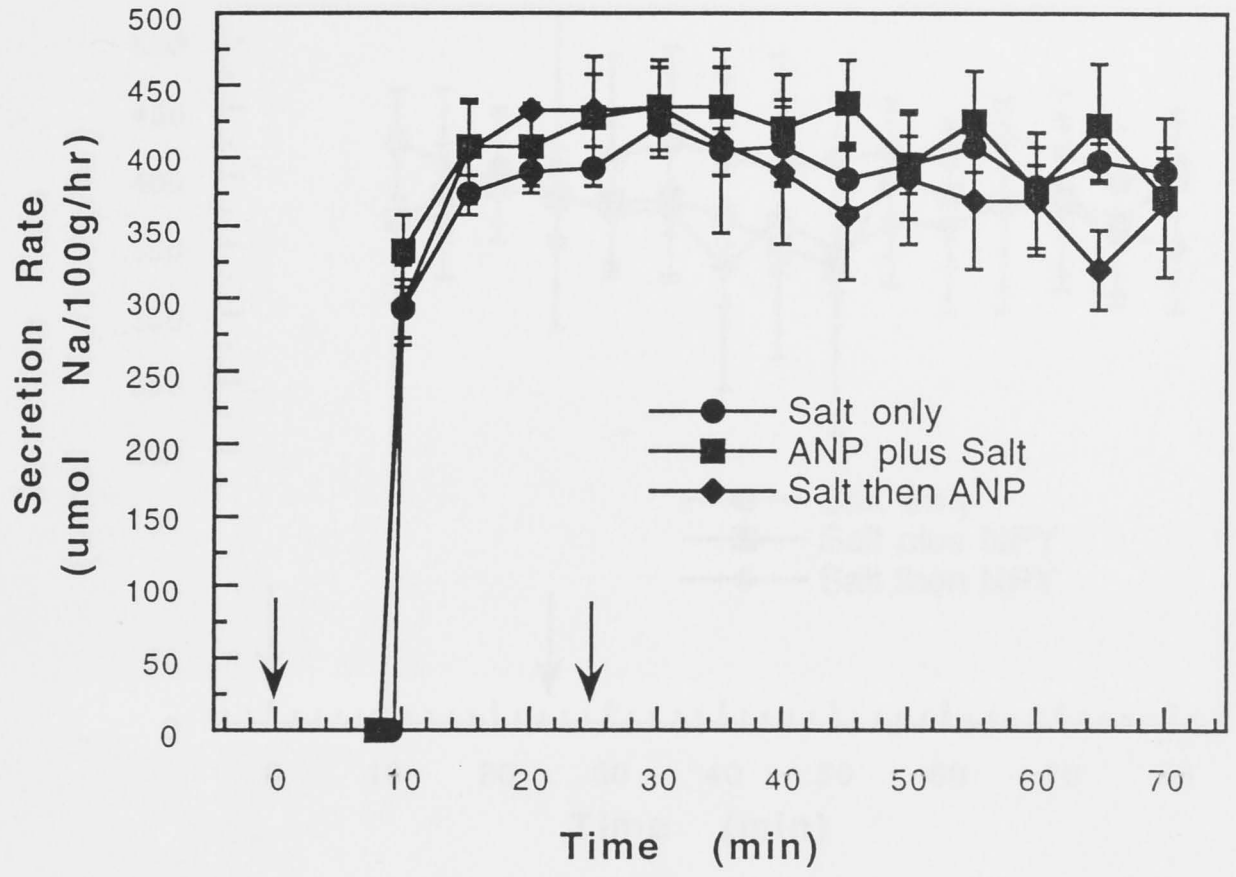


Figure 3.9

Effect of ANP injection (30 ng.kg BW^{-1}) on secretory rate of salt gland. Average secretion rate is shown ± 1 standard error. The first arrow indicates when all animals were injected with a salt-load of 2700 µmol NaCl.100g BW^{-1}. Some animals also received ANP with the salt-load(n = 9), while others received ANP at the time indicated by the second arrow (n = 9). There were no significant differences in secretory rate of animals treated with ANP compared to those receiving a salt-load alone (n = 9).

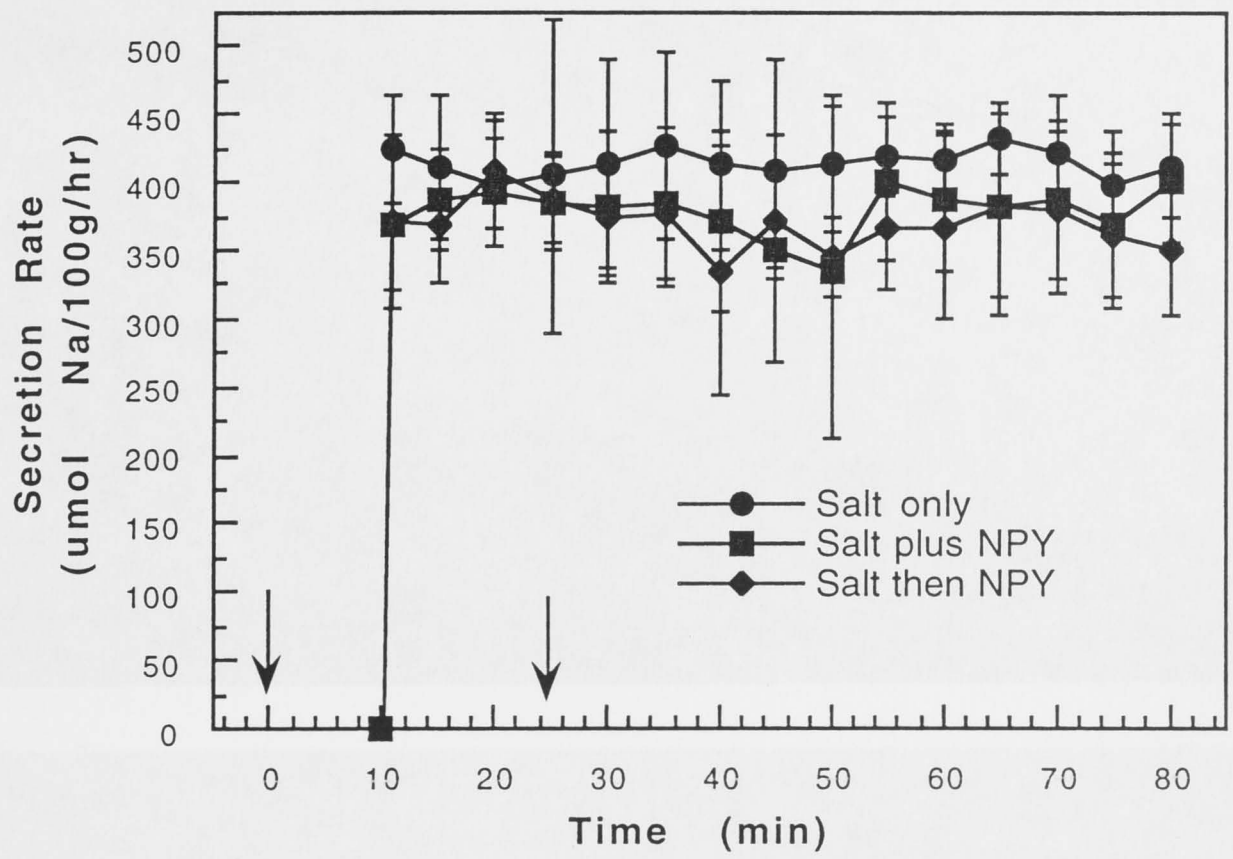
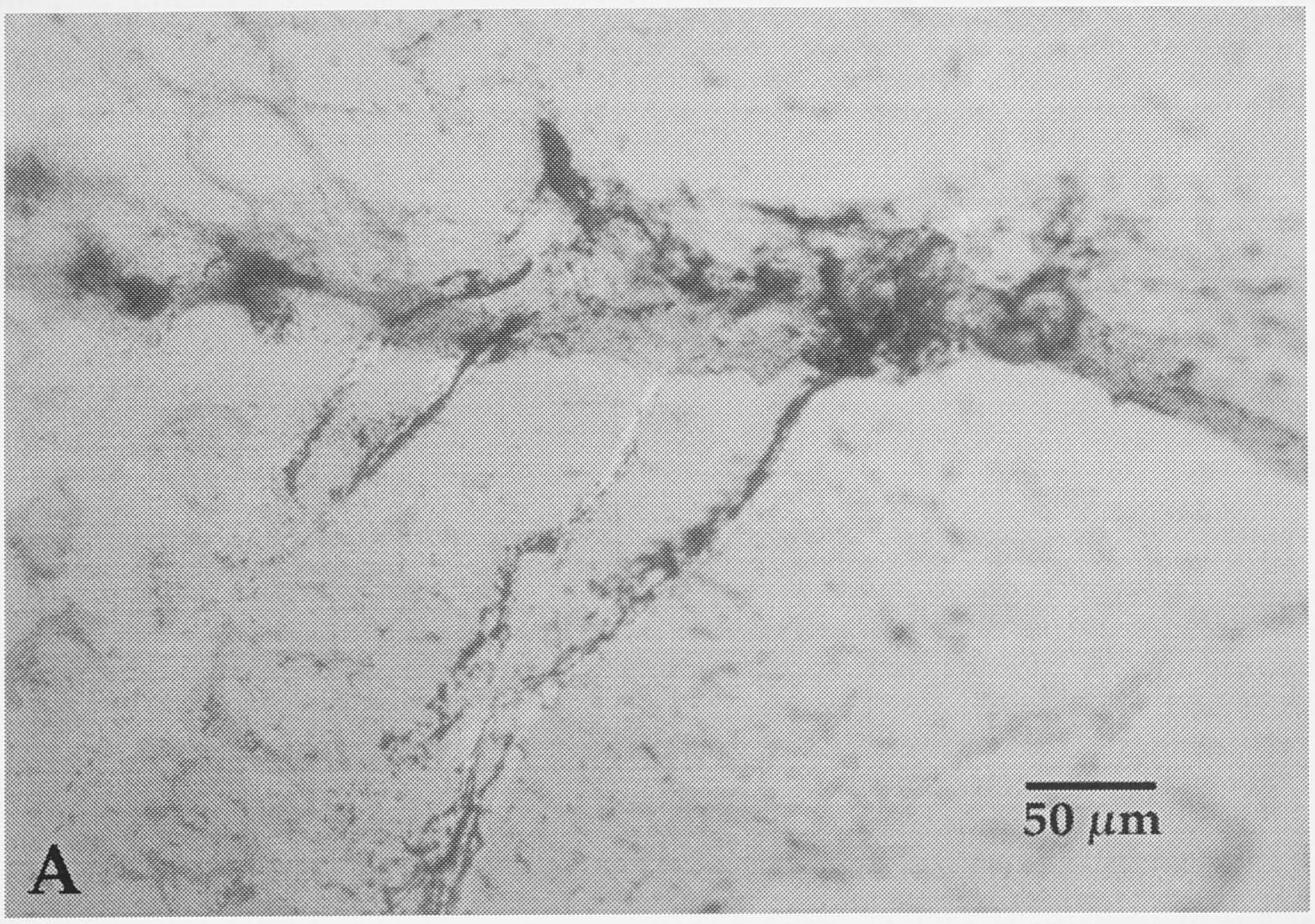


Figure 3.10

Effect of NPY injection (30 ng.kg BW^{-1}) on secretory rate of salt gland. Average secretion rate is shown ± 1 standard error. The first arrow indicates when all animals were injected with a salt-load of 2700 µmol NaCl.100g BW^{-1}. Some animals also received NPY with the salt-load(n = 5), while others received NPY at the time indicated by the second arrow (n = 5). There were no significant differences in secretory rate of animals treated with NPY compared to those receiving a salt-load alone (n = 9).



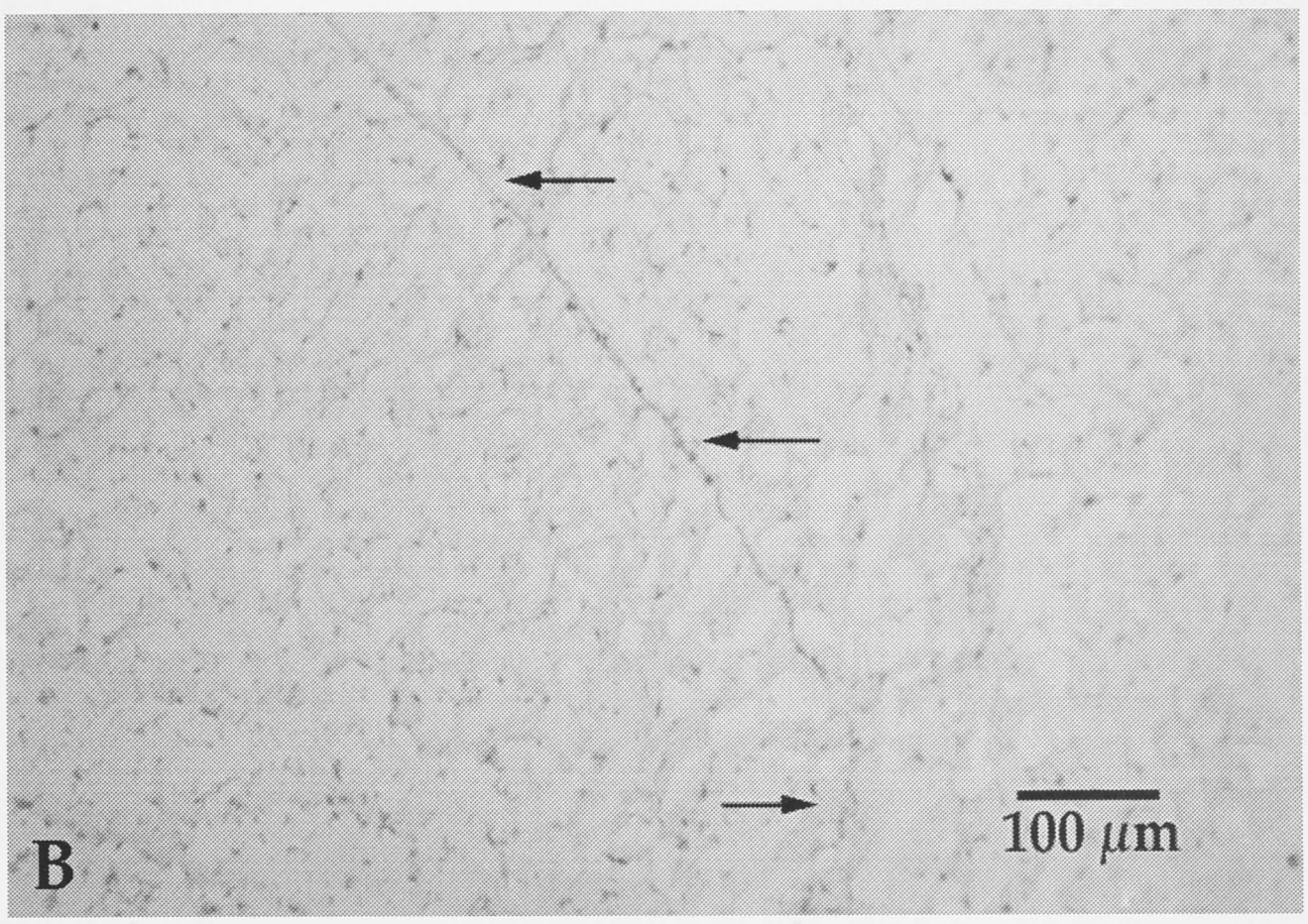


Figure 3.11
A) Immunoreactivity to tyrosine hydroxylase was observed at the periphery of salt gland sections, with putative adrenergic nerve fibres branching towards the interior of the section. Dilution of primary antibody was 1:200.
B) Other putative adrenergic nerve fibres indicated by the arrows ran through the secretory lobes of the salt gland sections, but it could not be seen if they terminated within the lobes. Dilution of primary antibody was 1:500.

CHAPTER 4
Salt Gland Histology and Blood Flow

INTRODUCTION

To produce a concentrated secretion, the salt gland must be supplied with blood from which to extract salt. Blood supply to the gland was identified as a possible site of regulation of gland function (Figure 1.5) and may be controlled to increase or decrease the availability of salt and other metabolic substrates which limit the secretory activity of the gland. This chapter investigates whether there are alterations in structure or vasculature and blood circulation which accompany changes in the activity of the hatchling green turtle gland. Any alterations which occur imply that the flow of blood in the gland is regulated, so the ability of possible modifiers to effect such changes was examined. Investigation of structure and vasculature was conducted using histological and stereological techniques, while the circulation of blood through the salt gland was measured using a technique employing coloured microspheres injected *in vivo*.

4.1 HISTOLOGY

The histology of the sea turtle salt gland has not been extensively examined, with the only comprehensive report being that of Abel and Ellis (1966). The gland structure is essentially similar to that of the avian salt gland, with a large number of secretory tubules formed by a circular arrangement of principal secretory cells. The secretory tubules drain into central canals in each lobule of the gland and these in turn form a large collecting duct. Abel and Ellis (1966) reported that like the avian salt gland, secretory tubules of the green turtle salt gland were surrounded by a network of capillaries which contacted the basal (outer) membrane of the secretory cells, similar to the arrangement in the avian salt gland. However, the salt glands examined were not active when removed, so the histological report made was of salt glands which were not secreting salt. There may be important structural changes which occur when the salt gland is active, particularly involving vasculature around the secretory tubules. The supply of blood may change with gland activity, with blood vessels associated with secretory tubules and central canals changing diameter or carrying more red blood cells. A comparison of gland structure under conditions of activity and inactivity may reveal if changes such as these do occur. The structure of salt glands from hatchling green turtles was examined and stereological methods were

used to quantify any changes associated with secretory activity. New, computer three-dimensional reconstruction techniques were used to reveal the internal arrangement of central canals and collecting duct in the salt gland. Stereological and reconstructive techniques are based upon analysis of serial sections of the tissue being examined (Weibel, 1980), so the salt gland tissue was cut serially and used for all techniques.

4.2 THE IMPORTANCE OF SALT GLAND BLOOD SUPPLY

Salt glands in birds, crocodiles and turtles are highly vascularised, with capillary networks encircling secretory tubules (Schmidt-Nielsen, 1960; Abel and Ellis, 1966; Hossler and Olson, 1990; Franklin and Grigg, 1993) as well as excretory ducts (Marshall *et al.*, 1987). Each secretory tubule is surrounded by six or seven capillaries which are in close contact with the basal membranes of principal secretory cells (Figure 4.1). Blood appears to flow in the opposite direction to the movement of secretory fluid down the tubule (Fänge *et al.*, 1958a), but it has not been shown that this arrangement is important in the process of moving ions from the blood to the tubule lumen. The high degree of vascularisation indicates that an adequate supply of blood is essential to the process by which salt glands produce concentrated secretions. Studies of the avian salt gland demonstrated that salt leaves the blood in its passage through the gland, with sodium concentration in blood exiting the gland 15 to 20% lower than in blood entering (Hanwell *et al.*, 1971; Kaul *et al.*, 1983). Clearly a reduction in the blood supply to the gland will restrict the availability of salt for uptake by secretory cells and so may be a means of controlling salt gland activity. Oxygen supply to the secretory cells and the gland as a whole will also be affected by changes in the blood flowing through the gland. The availability of oxygen for aerobic processes is a regulating factor in the metabolic activity of certain animals (Withers, 1992) and the possibility exists that the secretory cells of the salt gland are also regulated by the available blood-borne oxygen.

It is important to make the distinction between whole organ blood flow and circulatory changes which regulate the amount of blood flowing through the capillaries which contact the secretory tubules. An early investigation into the vasculature of the herring gull salt gland (Fänge *et al.*, 1958b) showed that blood can enter and flow through the gland and rejoin the main artery distal to the gland. If there is a specific constriction of the arterioles which branch from the artery to supply the capillaries surrounding the secretory tubules, then blood may pass through the gland but not come into contact

with secretory cells. So when blood flows through the salt gland, unless the circulatory route is such that the blood is available to the secretory cells then salt and other substrates are not available to the secretory tubules (Figure 4.2). In the context of regulation of blood supply to the secretory cells, it can only be said with certainty that regulation occurs if there is a change in the *capillary* blood flow, as the secretory cells are in contact with these vessels. Measurements of changes in capillary blood flow with different states of gland activity provide evidence of circulatory changes within the gland but not necessarily that blood flow through the gland as a whole has changed. Therefore it is necessary to employ experimental techniques which measure blood flow through capillaries rather than arteries or arterioles.

Blood flow through the chelonian salt gland has never been measured, so it is not known if there are differences associated with secretory activity. Experiments were conducted to test the hypothesis that there are circulatory differences between active and inactive salt glands.

The drugs methacholine and adrenalin inhibited secretion and atropine stimulated secretion by the chelonian salt gland *in vivo* (Chapter 3) but it was not possible in those experiments to identify how this was achieved. Experiments were conducted to measure salt gland circulation *in vivo* following administration of these chemicals to test the hypothesis that they were changing the circulation of blood through the gland. If this was the case, it implied that at least a portion of their effects on salt gland secretion were achieved in this way.

4.3 MEASUREMENT OF BLOOD FLOW

Several techniques may be used to measure blood flow *in vivo*, such as laser-doppler flowmetry (Gerstberger, 1991), marker diffusion (Hanwell *et al.*, 1971) and several types of microsphere techniques (Prinzen and Glenny, 1994). However, limitations imposed by small size and epidermal scales of the study animal meant that a modification of one employing coloured microspheres was the most suitable. The microspheres technique involves injection of small plastic beads into the systemic circulation which are trapped by capillaries in the organs as blood flows throughout the body. Because spheres are found in the organ itself, it can be conclusively shown that blood carrying the spheres had passed through capillaries of that organ. The number of spheres trapped is proportional to the rate of blood flow (Kowallik *et al.*, 1991) and the assumption is made that changes in the

number of spheres trapped in a tissue (concentration of spheres per gram of tissue) is a direct measure of changes in the rate of blood flow through the capillaries of that tissue. Also it is assumed that spheres are equally likely to be trapped in all tissues, proportional to their share of the total systemic blood flow, provided that capillaries are of similar diameter. In this study, the number of spheres trapped in the salt gland and other tissues as a measure of capillary blood flow was compared under the experimental conditions described above.

4.4 EXPERIMENTAL AIM

The aims of the experiments were; 1) to employ histological techniques to investigate the structural arrangement of the salt gland including three-dimensional reconstruction of duct arrangement, 2) to use stereological methods to measure whether the volume of blood vessels or other gland components associated with secretory tubules changes with salt gland activity, and 3) to use the coloured microsphere technique to quantitatively test the hypothesis that blood circulation in the active hatchling turtle salt gland is different than that in the inactive salt gland. In addition, the hypotheses were tested that methacholine, adrenalin and atropine all affect blood circulation through the hatchling turtle salt gland. Cardiac and pectoral muscle tissue samples were also examined to provide comparative information on capillary blood flow in other tissues under the experimental conditions employed. The aim was to determine whether the site of blood flow regulation proposed in the secretory model was indeed influenced by modifiers of salt gland activity.

METHODS

4.5 ANIMALS

Animals used were as described in Section 2.3.

4.6 HISTOLOGICAL TECHNIQUE

Many techniques may be employed for examining the histology of tissues and are generally selected on the basis of the information that is being sought. Thin sections of tissue are useful for showing microscopic structure of tissues when stained appropriately to reveal different tissue types. Tissues are fixed following removal from the animal and may be stained and examined some time later. Paraformaldehyde or Bouin's fixative preserve cellular structure and allow tissues to be subsequently embedded in resin or paraffin wax for cutting into thin sections (Pearse, 1985). The thickness of sections varies with the embedding medium, 7 to 14 µm wax sections and 1 to 3 µm resin sections are typically taken. Selection of the appropriate stain will differentially colour tissues and so reveal the presence of connective tissue, blood cells, secretory cells etc. Wax embedding and resin embedding histological techniques were employed in this study to examine the fine structure of hatchling turtle salt glands.

4.6.1 Collection of tissue samples

Two methods were used to collect salt glands. 1) Animals were killed by decapitation and the salt glands quickly removed and placed in Bouin's fixative or 4% paraformaldehyde in PBS (pH 6.8 - 7.2), or 2) animals were cooled in the refrigerator for about 15 minutes before placing in a -70^{0}C freezer for about 30 minutes to kill them. Animals were then thawed sufficiently to permit removal of salt glands, which were placed in Bouin's fixative and embedded for sectioning in wax. Glands were collected from animals stimulated to secrete by injection of a salt-load of 2700 µmol NaCl.100g BW^{-1}, as well as from animals which were untreated and so not secreting.

4.6.2 Wax embedding

Histological technique was used to identify visible differences in salt gland structure from animals which were producing tears as well as those which were not. In order to initiate secretion, animals were injected with a salt-load of 2700 µmol NaCl.100g BW^{-1} and the salt glands were removed 15 minutes after the appearance of tears. Glands were collected both from decapitated and frozen animals. Glands were fixed in Bouin's fixative for

subsequent wax embedding in a Shandon® Hypercentre and cutting into serial sections. Embedding protocol is detailed in Appendix 2. Following embedding, tissue was removed and attached to a small wooden block for slicing. Sections of 7µm thickness were cut on a Sorvall® JB-4 Microtome (Dupont Instruments) slicer and were attached to a gelatine-coated glass microscope slide. Slides were then placed on a slide heating rack at 65°C for a minimum of 2 hours to fix the sections.

4.6.3 Resin embedding

Salt glands were collected from decapitated animals both with active and inactive glands as described above. Glands were fixed in 4% paraformaldehyde in PBS (pH 6.8 - 7.2) prior to embedding in resin and sectioning. Tissue was dehydrated in ethanol for five minutes at 20%, 40%, 50%, 75% and 90% and overnight at 100%, followed by three washes in 100% ethanol for one hour. Infiltration with London White resin was done at room temperature three times for one hour each, before curing at 60°C for 24 hours in a nitrogen atmosphere. Sections of 1 µm thickness were cut on an ultramicrotome (Reichert) and mounted on glass slides.

4.6.4 Staining of tissue sections

4.6.4.1 Wax embedded sections.

Salt gland tissue sections which had been wax embedded were stained using the Periodic acid-Schiff (PAS) method counterstained with haematoxylin and fast green (recipes in Appendix 1). Staining protocol is detailed in Appendix 2. Mounted sections were examined under light microscopy.

4.6.4.2 Resin embedded sections.

Slides were placed on a slide heater at 80°C and stained for 20 seconds with 0.05% toluidine blue in PBS. Stain was then rinsed off the slide with distilled water.

4.7 STEREOLOGICAL TECHNIQUE

Stereological technique can be employed to permit morphometric measurements of internal structures of biological specimens (Weibel, 1980). Volume and surface area measurements of various structures within a tissue can be made by applying the technique to serial sections of the tissue. By measuring two-dimensional area of a particular feature in adjacent sections, volume and surface area calculations can be made on the basis of two dimensional area or perimeter measurements, section thickness and

distance between sections. Although the technique is very time-consuming, it permits accurate morphometric quantification of internal structures not possible using conventional microscopic technique.

4.7.1 Three-dimensional reconstruction

Stained, wax embedded serial sections were used as described in Section 4.6 above. Images of sections were obtained using a video camera (Sony CCD) mounted on a light microscope (Zeiss Axioscope). The images were captured and stored using the computer program Adobe Photoshop on a Macintosh computer. Images were converted from colour to greyscale to reduce computer file size and saved in TIFF format. Every third section was captured in this way, with about 60 sections collected for the reconstruction. Sections were aligned on a Silicon Graphics workstation so that ducts could be accurately tracked through the gland. Using the program "Imod" (Kremer *et al.*, 1996) ducts in each section were traced and a closed contour formed around them. The program then reconstructed the internal duct structure by joining overlapping contours in adjacent sections and rendering them in three-dimensional form.

4.7.2 Morphometric analysis of gland components

Analysis was conducted on sections of glands taken from two frozen hatchlings, one which was salt loaded and one which was not. Six lobes from each gland were examined, with the lobes followed through the gland until they entered the main collecting duct or became indistinguishable from other lobes. Every third section was captured on computer using the video technique described above. "Imod" was used to display a computer image of the section and individual blood vessels, central canals, lobes and tubule lumens within the lobe were outlined and contoured. The area of closed contours was calculated in square microns and thus volume was determined from section thickness. Not all contours could be followed from one section to the next, so the sum volume was calculated from each individual section and was not interpolated between sections. The total surface area and volume of the different lobular structures from about 400 tissue sections comprised of over 2300 object contours was calculated in this way. The size of lobes varied, so the volume of lobular structures was calculated and expressed as a percentage of the total lobe volume. The average values for lobes from an active gland were compared with the average values of lobes from an inactive gland.

MICROSPHERE TECHNIQUE

4.8.1 Injection of microspheres

Microspheres used were coloured and made from cross-linked polystyrene-divinylbenzene (Ultrasphere™, E-Z Trac, Los Angeles). These spheres are resistant to alkaline digestion and are colour coded to permit identification of different sizes. In order to be securely trapped by capillaries, spheres should be about 50% larger than the dimensions of the red blood cell (Thorarensen, personal communication). Green turtle RBCs are elliptical, approximately 12 µm by 17 µm (Frair, 1977), so 15 µm and 25 µm diameter spheres were selected as the most appropriate sizes. Spheres were added to PBS until a total of 300,000 spheres of both sizes were contained in a volume of 100 µl PBS. The number of spheres to be injected was calculated from estimates of cardiac output, salt gland blood flow and salt gland mass according to an equation described by Prinzen and Glenny (1994). The suspension was vortexed before injection to ensure even distribution of spheres and to prevent clumping. 100 µl of suspension containing 300,000 spheres was injected into the systemic blood of subject animals by cardiac puncture using a heparinised insulin syringe (Terumo, 27G x 1/2). The needle was inserted to a depth of about 8 mm in the joint between scutes immediately above the external yolk sac. A slight amount of negative pressure was applied to the needle to draw blood to determine whether the needle penetrated the heart, then the suspension containing the spheres was steadily injected over 2 seconds. The initial withdrawal of blood ensured that the spheres were injected into the ventricular space and not into the muscle of the heart wall. Five minutes after sphere injection the animal was killed by decapitation for immediate removal of tissue samples. A time period of five minutes between injection and decapitation was based on estimates of heart rate and blood volume of hatchlings. Decapitation rather than freezing was used to kill the animals because it was necessary to ensure an immediate stop in blood flow after five minutes. The salt glands, pectoral muscle and cardiac muscle were removed, with tissues always collected in the same order. Blood adhering to the surface of the sample was blotted off, tissues were weighed and placed into individual Eppendorf tubes for microsphere extraction. Left and right salt glands from each animal were treated separately in case the order of their removal influenced microsphere concentration.

4.8.2 Extraction of microspheres

Microspheres were extracted from tissue samples using a modification of the method recommended by the supplier (Ultrasphere™ Extraction Protocol 91001.4). Ultrasphere™ tissue/blood digest reagent 1 (0.5 ml) was added to each sample. In addition, 2000 reference 25 µm spheres in 100 µl PBS were added to each sample so that extraction efficiency could be measured. Tissue samples were pushed or shaken to the bottom of each Eppendorf tube and the cap closed. Tubes were placed in an oven at 85°C for at least 2 hours for tissue digestion to occur and then allowed to cool to room temperature before vortex mixing. Any tissue samples that did not form a uniform suspension were returned to the oven for a further hour to complete digestion. Following digestion, Ultrasphere™ tissue/blood digest reagent 2 (1 ml) was added to the suspension and vortexed. Tubes were centrifuged at 6500 RPM for 30 minutes so that microspheres formed a small pellet in the bottom of the tube. The supernatant was removed with a Pasteur pipette to a level slightly above the pellet and discarded. 1.5 ml of Ultrasphere™ microsphere counting reagent was added to the tube and the pellet resuspended by vortex mixing. If the pellet did not completely resuspend, tubes were returned to the oven for 30 minutes before vortexing again. This process was repeated until all sediment aggregates had disappeared. Tubes were centrifuged at 6500 RPM for 15 minutes and the supernatant removed to a level slightly above the pellet. The tube was briefly centrifuged again to spin down any liquid adhering to the sides so that liquid volume could be accurately determined using a Gilson pipette. Liquid volume was recorded and the liquid retained for counting of microspheres. The liquid volume was important for calculating total number of microspheres after taking a subsample for counting of spheres.

4.8.3 Counting technique

The sampling and counting technique was validated by preparing solutions of 200 each of 15 µm and 25 µm microspheres in a total volume of 100 µl PBS. Five subsamples of 20 µl each were taken and the number of spheres of each colour counted under a microscope. Three separate 100 µl standards were used and variations between 20 µl subsamples were compared to determine the accuracy of subsampling. It was found that number of spheres varied by less than ± 5% in subsamples so this subsampling technique was employed for counting spheres in experimental samples.

A 20 µl subsample was taken from experimental samples following vortexing of the Eppendorf tube to disperse microspheres evenly throughout the solution. The number of spheres of each colour was counted and recorded and the number of spheres per µl was calculated. The total number of spheres was then determined by multiplying the number of microspheres per µl by the total volume of solution measured previously with a Gilson pipette . Extraction efficiency was determined by dividing the total number of reference spheres in the solution by the 2000 that had been added to the original tissue sample. The number of spheres in individual samples was corrected for extraction efficiency by dividing by the number of spheres by the proportion of reference spheres counted. For example, if 1700 of the 2000 reference spheres were present in the sample, the extraction efficiency was 85% and 15% of the reference spheres had been lost during the extraction process. It was assumed that 15% of the 15 and 25 µm spheres had also been lost and so the number in the sample was divided by 85% to correct accordingly. The total number of each sphere size in the original tissue sample was calculated by this method. The number of 15 µm and 25 µm spheres was multiplied by the sample tissue weight to determine the number of spheres per gram of tissue.

4.8.4 Experiments

Experiments were conducted to measure blood flow in the presence and absence of a salt-load. Salt-loaded animals were injected with 2700 µmol NaCl.100g BW^{-1} while control animals were injected with an equal volume of PBS (270 µmol NaCl.100g BW^{-1}). Fifteen minutes after salt gland secretions had first appeared in salt-loaded animals, both salt-loaded and control animals were injected with spheres by cardiac puncture and tissues collected as described above.

Other experiments were conducted to investigate if the inhibitory influence of methacholine and adrenalin and the stimulatory influence of atropine on salt gland secretion was produced by changing blood flow. Two groups of animals received a salt-load of 2700 µmol NaCl.100g BW^{-1} followed ten minutes after the first appearance of tears by injection of adrenalin (500 µg.kg BW^{-1}) or methacholine (10 mg.kg BW^{-1}) into the thoracic cavity. The reason a salt-load was administered prior to injection of these chemicals was to replicate the conditions under which their inhibitory effects were found in Chapter 3. Microspheres were injected 5 minutes later and tissue samples collected as above. A third group of animals received an injection of

atropine (10 mg.kg BW^{-1}) in the absence of a salt-load, with spheres injected 15 minutes later and tissue samples collected.

4.8.5 Statistical analysis

The Mann-Whitney rank test was used to determine if there were statistical differences in microsphere numbers between salt-loaded and non salt-loaded animals. The same test was applied to determine statistical differences among groups of turtles receiving treatment and controls. In all cases, significance was assumed if $p<0.05$. All results are shown as the mean $\pm$ 1 standard error.

RESULTS

4.9 HISTOLOGY

4.9.1 Wax sections

Wax sections of the hatchling turtle salt gland show the major features of secretory tubules forming numerous lobes within the gland (Figure 4.3). Connective tissue surrounds the gland and is visible between secretory lobes, with occasional blood vessels apparent between lobes. The main collecting duct of the gland was seen as sections approached the middle of the gland and the central canal of each lobe was usually seen. Secretory tubules were approximately hexagonal in cross section but the lumen of the secretory tubule was not always visible, probably due to slight compression of the tissue as sections were cut. Blood vessels were seen occasionally associated with secretory tubules in both active and inactive glands which had been collected by decapitation of the animal. The blood vessels observed were small and generally lay in the junction between adjacent secretory tubules so that they were in contact with the secretory cells of both. Similar blood vessels were also seen around the central canal of some lobes. It was apparent that the number of blood vessels in an active gland collected by chilling and then freezing the animal (Figure 4.4) was much greater than in an inactive gland collected in the same way. It seemed likely that this was a more suitable collection technique for preserving the blood within the gland during and following its removal, so sections from these glands were used for morphometric analysis of blood vessel volume.

4.9.2 Resin sections

Resin-embedded sections revealed the same gross morphological arrangement of salt gland lobes separated by connective tissue (Figure 4.5a). More detail was seen of the secretory tubules and the radial arrangement of secretory cells was clear when tubules were cut in transverse section and the lumen of some tubules could be seen (Figure 4.5b). The orientation of secretory tubules varies through the gland, so that in a single section they were cut variously transversely or longitudinally or some orientation in between. However, tubules in close proximity to each other were sectioned in generally similar planes. Intercellular spaces created by the extensively folded lateral cell membranes of secretory cells were seen, with about six to ten cells usually forming the circumference of the tubule (Figure 4.6a & b). Cell nuclei could be seen, but other intracellular structures could not be resolved. The structure and type of cells forming the central canal of each lobe was different to the secretory tubules, with cells forming layers around

the canal lumen. The diameter of the central canal lumen was much greater than the lumen of secretory tubules (Figure 4.6c). Large mucocytes were present in the cells around the central canal and intercellular spaces were visible between cells.

4.10 STEREOLOGY

4.10.1 Three-dimensional reconstruction

Reconstruction of serial sections shows that the canals from secretory lobes join and drain into a common collecting duct. The collecting duct is quite large and drains via a duct which folds underneath it and runs anteriorly to the posterior canthus on the surface of the eye (Figure 4.7a & b). The collecting duct is situated at the posterior end of the gland and canals run from lobes anterior to it to drain primarily into its anterior end. An anterior view of the ducts (Figure 4.7c) shows that they run from the lateral regions of the gland as well and so form a network which converges from a wider lateral and dorsal area into a relatively narrow collecting duct. A small number of ducts enter the dorsal surface of the collecting duct directly (Figure 4.7d), presumably from lobes positioned directly above it. Some run from the posterior end of the gland and join other ducts at the anterior part of the collecting duct. The reconstructed region had a volume of 2.9×10^8 μm^3 and the surface area was 11.4×10^6 μm^2. The main collecting duct (coloured red) comprised 72% of volume and 39% of total surface area.

4.10.2 Morphometric measurement of gland components

The percentage of total lobe volume constituted by central canals, blood vessels and tubule lumens from one active and one inactive gland is shown in Table 4.1. It was assumed that the volume of lobes did not change with activity, supported by the finding that active and inactive glands did not differ significantly in weight (see Section 4.10.3 below). Secretory tubules constitute the vast majority of lobular tissue, accounting for over 99% of the lobular volume. The percentage volume of central canals was slightly higher in lobes of the active gland than the inactive gland although they still comprised only a fraction of a percent of total lobe volume. The percentage volume of blood vessels in sections of the active gland was about 20 times greater than in the inactive gland, increasing from 0.034% to 0.699%. The volume occupied by tubule lumens did not change with activity and was very low in both cases. It is probable that many lumens were not visible because their diameter is small and they would be easily compressed and

closed by sectioning. A statistical comparison of the volumes was not conducted because of the small sample size.

4.10.3 Salt gland weight and appearance

There was no significant difference in weight of active (n = 12) and inactive (n = 12) salt glands, averaging 41 ± 1 mg. When the overlying tissue was dissected free but prior to removal of the gland itself from the animal, active salt glands had a pink colour while inactive glands were pale. There was no significant difference in microsphere concentration between left and right salt glands taken from the same animal under any of the experimental conditions. The average microsphere concentration of the two glands was used in statistical analysis.

4.11 MICROSPHERE MEASUREMENTS OF BLOOD FLOW

4.11.1 Microsphere recovery

Microsphere extraction efficiency as determined by recovery of reference spheres averaged 84.8 ± 0.19% (n = 112), ranging from 80.7% to 89.0%. There was no significant difference in extraction efficiency of 15 μm and 25 μm spheres.

Significantly more 25 μm diameter spheres were caught in tissues than 15 μm diameter spheres ($p < 0.0001$), with the number of 15 μm spheres only 50.04 ± 2.6% of the number of 25 μm spheres (n = 112). The ratio of 15 μm to 25 μm microspheres found was not significantly different in any of the tissues or any of the experimental groups. As there was no significant difference found with microsphere size, only the data from 25 μm spheres are shown in the graphs, the data from 15 μm spheres is included in Table 4.2.

4.11.2 Effect of salt-loading

All experimental animals were producing tears when sacrificed but tears were not produced by control animals which were injected with PBS. Salt-loading significantly increased the number of microspheres present in the salt gland tissue compared to control animals injected with PBS (Figure 4.8). There was no significant difference in the number of spheres found in cardiac or pectoral muscle tissue of salt-loaded animals compared to controls.

4.11.3 Effect of methacholine

All animals produced tears after salt-loading, but stopped secreting within two minutes of methacholine injection (10 mg.kg BW^{-1}). The concentration of spheres per gram wet weight caught in salt gland and cardiac tissue compared to salt-loaded animals were also significantly reduced following methacholine treatment (Figure 4.9). Although the concentration of spheres was lower in the salt glands of methacholine treated animals, these animals had a significantly higher concentration of spheres in the salt gland than those which did not receive a salt-load at all ($p<0.0001$). The concentration of spheres trapped in pectoral muscle slightly decreased but the change was not significant.

4.11.4 Effect of adrenalin

All animals secreted after salt-loading but tear production ceased within two minutes after injection of adrenalin and did not resume prior to sacrifice. Adrenalin (500 $\mu g.kg\,BW^{-1}$) administered after a salt-load significantly reduced the number of spheres caught in the salt gland compared to animals which received a salt-load only (Figure 4.9). Adrenalin also significantly increased the number of spheres found in the cardiac tissue and there was an increase in the number of spheres found in pectoral muscle although this was not significant. There were significantly more ($p<0.0001$) spheres caught in the salt gland after salt-loading followed by adrenalin injection than in animals which did not receive a salt-load.

4.11.5 Effect of atropine

No animals were secreting following injection of atropine (10 mg.kg BW^{-1}), but there was a significant increase in the number of spheres in the salt gland compared to control animals (Figure 4.8). There was a significant increase in the number of spheres found in cardiac tissue and a slight but not significant decrease in the number of spheres in pectoral muscle compared to controls. There were significantly fewer ($p<0.05$) spheres in the salt gland following atropine treatment than in the salt gland of animals which received a salt-load.

The concentration of microspheres trapped in tissues under different experimental conditions is summarised in Table 4.2.

DISCUSSION

The gross histology of the hatchling green turtle salt gland is the same as that described for turtles of an unspecified age (Abel and Ellis, 1966) and similar to that of the avian salt gland (Ellis *et al.*, 1963) so will be only briefly discussed. The gland is composed of numerous lobes of secretory tubules which are round in transverse section, with the tubule lumen occasionally visible. The lumen runs along the secretory tubule, before draining into the central canal. Intercellular spaces between secretory cells are clearly visible in thin resin sections, with about six or seven cells forming the circumference of the tubule. Secretory cells comprise the majority of the glandular tissue, with only a small proportion of the gland composed of connective tissue, blood vessels or cells forming central canals or the main collecting duct to the posterior canthus. Three-dimensional reconstruction shows that the main collecting duct is located at the posterior side of the gland and empties through the posterior canthus via a duct which folds under it and runs anteriorly. Thus the movement of secreted fluid in the salt gland is generally in a latero-posterior direction. If the suggestion that blood flows opposite to the direction of fluid movement is correct then blood generally moves in an anterior direction. Blood vessels and blood cells are sometimes seen in the connective tissue surrounding the gland as well as in the connective tissue between lobes. The cells forming the central canal of each lobe were arranged differently to those forming the secretory tubules. The canals and collecting duct were much larger in diameter and did not have the characteristic radial arrangement of cells, with a more stratified appearance. Larger cells were present around the ducts, identified by Marshall (1989) as mucocytes. There were intercellular spaces visible between the cells of the ducts, these have been proposed to play a role in concentrating secreted fluid as it passes down the ducts (Marshall and Saddlier, 1989).

Morphometric analysis of structures within secretory lobes showed structural differences between an active and an inactive salt gland. While the secretory tubules occupied the vast majority of lobular tissue, the volume occupied by central canals, blood vessels and tubule lumens was also measurable. The volume occupied by tubule lumens did not change with gland secretory activity, while the volume of central canals increased slightly in active glands. However, the volume of blood vessels was about 20 times greater in the active gland than in the inactive gland, with large numbers of vessels seen around the secretory tubules. The apparent greater

volume of central canals in the active gland may be a reflection of greater secreted fluid volume passing through these canals and resulting in their dilation. However, the diameter of central canals is much larger than blood vessels or tubule lumens and this introduces a possible source of measurement error as they were not present in all sections. Measurement of a small number of large volume canals is inherently more prone to error than measurement of a large number of small volume vessels. The greater volume of blood vessels found in the active salt gland suggests that capillaries were dilated in active glands to increase the blood supply to secretory tubules. The association of blood vessels with secretory tubules in the hatchling turtle matches that described for the avian salt gland, with the secretory tubule surrounded by capillaries in the herring gull. The increase in capillary blood supply with secretory activity has not previously been detected by histological or stereological means. The proposed secretory model (Figure 1.5) suggested that salt gland blood flow was a site of regulation, the morphometric evidence indicates that this may be the case.

It is interesting that the gross differences in vasculature of active and inactive glands were more apparent in frozen glands than glands collected from decapitated animals. This is probably because freezing the animals keeps the blood cells within the gland and they remain there when it is removed from the animal. In comparison, perhaps blood drains from the gland when the animal is decapitated and so the differences in small blood vessels associated with secretory tubules in active and inactive glands is disguised. However, the differences were clear in the microsphere entrapment experiments, so while blood cells may drain out, the microspheres remain securely lodged, probably because they are larger and do not deform in shape. However, the 15 μm microspheres were close to the largest dimension of the red blood cells, so it is possible that some of them may have drained out of capillaries with the blood cells, explaining why they were found at a lower concentration than the 25 μm spheres which would not drain.

Quantification by the microsphere technique of the morphometric blood vessel volume differences with gland activity shows changes in the circulation of blood through salt gland capillaries with secretory activity. In considering microsphere entrapment in different tissues, the assumption is made that sphere concentration and rate of capillary blood flow are very closely related. There is experimental evidence to support this assumption,

which has been reviewed and shown elsewhere (Bassingthwaite *et al.*, 1987; Prinzen and Glenny, 1994). Thus in the discussion of results, a doubling of microsphere concentration is presumed to indicate a change in the rate of blood flow of similar magnitude. Changes in sphere concentration may not represent changes in total blood *flow* through the salt gland, due to the possibility of specific constriction or relaxation of arterioles branching from the main blood vessels passing through the salt gland, as described earlier. However, changes in microsphere concentration do show differences in the *distribution* of blood, with blood flow rate through capillaries inferred from this.

Hatchlings were not restrained during the period between microsphere injection and tissue collection, but little movement was observed. Thus it would be expected that pectoral muscle would be in a relatively inactive state with a small requirement for blood flow. In comparison, the concentration of spheres found in inactive salt glands was less than 2% of that found in pectoral muscle, indicating that blood flow through capillaries of inactive glands is very low indeed. This suggests that the salt gland is in a very quiescent state when salt secretion is not required, presumably with a very low need for blood transported nutrients. However, following administration of a salt-load, circulation through the hatchling turtle salt gland changes. Nearly 200 times more microspheres were trapped in the capillaries of active glands than inactive glands. The primary secreting cells of secretory tubules in active glands were then in close contact with blood-filled capillaries, thereby permitting uptake of salts from the blood. Circulation within the gland differs with gland activity and capillary blood flow appears to increase concomitant with secretory activity. Circulatory changes following salt-loading do not appear to occur in all organs, as the number of spheres trapped in cardiac and pectoral muscle did not significantly change.

Thus the experimental evidence clearly shows that the circulation or pattern of blood flow through the hatchling turtle salt gland changes as the gland becomes active and initiates secretion. Presumably the change in flow pattern results from a neural or endocrine modification of the local circulation. The possible site of regulation at blood vessels identified in the proposed secretory model is then confirmed as one means by which the activity of the salt gland may be controlled.

Salt gland weights did not differ significantly between active and inactive states. Morphometric measurements show that blood vessel volume in an active gland is less than one percent of total volume, so it is not surprising that a difference in mass could not be detected. It is also possible that the volume of blood within the gland is similar in both inactive and active states, but its distribution changes from being contained within a small number of large diameter blood vessels to being distributed into a much larger number of capillaries.

My experiments did not determine exactly when circulatory changes in the salt gland occurred, whether before, at the same time, or after initiation of tear production. However, given the need for increased availability of salt for secretion by the gland, it seems probable that the change occurred before tears were observed. Comparative data supports this suggestion, as vasodilation was detected before secretion in the herring gull salt gland (Thesleff and Schmidt-Nielsen, 1962).

Methacholine was identified in Chapter 3 as having an inhibitory influence on the activity of the salt gland *in vivo*. Experiments showed that this chemical is capable of modifying the circulation of blood through the salt gland. Methacholine rapidly inhibited secretion from the salt gland when injected after a salt-load, and the concentration of microspheres in the salt gland following a salt-load was significantly reduced after injection of the drug when compared with animals which received a salt-load alone. This infers a reduction in capillary blood flow through the gland following methacholine treatment. Thus it would appear that methacholine influenced salt gland circulation to reduce capillary blood flow as measured by entrapment of microspheres and this explained at least part of its ability to inhibit gland secretory activity.

While the concentration of spheres caught in the salt gland following methacholine treatment was reduced to about half that of salt-loaded animals, it was still significantly higher than that found in animals which had not received a salt-load. Secretions were not produced in methacholine-treated animals, despite capillary blood flow being above control levels and a salt-load having been administered, indicating that the requirements for tear production were not met. Perhaps capillary blood flow needs to be at or near some maximum in order for the secretory process to operate.

However, interpretation of the circulatory changes seen in salt glands following methacholine treatment is complicated by the possibility that methacholine reduced the cardiac output of blood to the systemic circulation through a decrease in heart stroke rate and stroke volume (Withers, 1992). The observed reduction of about 50% in sphere concentration in the musculature of the salt gland, heart muscle and the pectoral muscle would be consistent with this cholinergic action. It is possible also that methacholine influences the flow of blood at some rate-limiting step, (including cardiac output) prior to the salt gland in a way which is not related to regulation of gland activity. This may be due to a pharmacological effect or mimicking the effect of cholinergic nerves unrelated to the salt gland. So while administration of methacholine to salt-loaded animals certainly results in a reduced microsphere concentration in the salt gland, it is not completely clear whether this is due to a localised effect on salt gland circulation, a general reduction in systemic blood flow or an unrelated effect on blood flow at some rate limiting step supplying the gland.

The concentration of spheres found in the salt gland was about eighty times higher in animals treated with atropine than in those which did not receive any treatment. Thus in the absence of a salt-load, some circulatory change occurred which permitted entry of microspheres into the salt gland capillaries where they became trapped. This would occur through an antagonism of whatever cholinergic influences were involved in reducing blood flow following administration of methacholine as discussed above. Part of this was probably due to an increase in total systemic circulation through an increase in cardiac output. Some capillaries must be open in the inactive gland to trap the small number of spheres found and an increase in heart output may be capable of pushing blood through these at a faster rate to increase the number of spheres trapped per unit time. However, the eighty fold increase in sphere concentration suggests that there may have been also a circulatory change either in the gland or at some rate-limiting step to result in the huge increase in sphere entrapment. It would be necessary to confirm the presence and exact location of cholinergic nerves to determine which of these was the case. Atropine antagonises the effects of acetylcholine, so the change in blood circulation that it caused was not due to an intrinsic action on the salt gland but rather a reversal of any cholinergic action. Thus while there was an antagonism by atropine of cholinergic inhibition of salt gland blood flow which resulted in an increased concentration of spheres trapped in capillaries it is not possible to

determine at precisely which point in the circulatory system it occurred. It may have been due to an antagonism of cholinergic nerves in the gland, a change in cardiac output, an effect on blood flow at a rate limiting step supplying the gland, or a combination of these.

Adrenalin significantly reduced the concentration of microspheres trapped in salt gland tissue following a salt-load when compared to animals which received a salt-load alone. Microsphere concentration in the gland was reduced by 95% but it was still significantly higher than that in control animals with inactive glands. Sympathetic stimulation of cardiac tissue by adrenalin is known to increase cardiac stroke rate, stroke volume and total cardiac output (Withers, 1992). Adrenalin significantly increased the blood flow through the cardiac musculature, suggesting that if an increase in cardiac output did occur then the extra work done by the heart was associated with an increase in blood supply to the heart muscle, thereby resulting in more spheres being trapped. While cardiac microsphere concentration approximately doubled, salt gland microsphere concentration changed in the opposite direction and so was presumably independent of cardiac output increase. The reduction in salt gland blood flow clearly shows a specific vasoconstrictory response in vessels supplying the gland regardless of any increase in systemic blood flow that adrenalin may have caused. Adrenalin appears to be capable of shutting down the microcirculatory system involved in active gland blood flow. Although immunohistochemical identification of adrenergic nerves could not resolve whether fibres innervated blood vessels directly (Chapter 3), it seems that this is the case. The specific vasoconstriction in the salt gland indicates this, and adrenergic innervation has been seen surrounding salt gland arterioles in the duck (Peaker and Linzell, 1975), with a similar effect on salt gland circulation (Fänge *et al.*, 1963).

Similar observations of increased blood flow during secretory activity have been reported in studies of the avian salt gland, where blood flow to the gland increases by about 15 to 20 fold during periods of activity (Hanwell *et al.*, 1971; Kaul *et al.*, 1983; Gerstberger *et al.*, 1988; Gerstberger, 1991). Several techniques have been used in these studies, the results of which are summarised in Table 4.3. Estimates of blood flow from microsphere data have been supported by alternative methods. Thus there is support for the accuracy of the microspheres technique in salt gland studies, as it measured blood flow changes similar to those seen using other techniques. While the

magnitude of change measured in the chelonian salt gland is greater than that observed in the avian salt gland, this is probably due to a lower basal rate of blood flow in the inactive chelonian gland rather than a greatly higher rate of blood flow in the active state. The effects of methacholine and atropine in the chelonian salt gland are opposite to the effects observed in the avian gland, where the chemicals increase and reduce blood flow respectively (Fänge *et al.*, 1963; Kaul *et al.*, 1983). However, in both the turtle and the bird, these chemicals had effects on salt gland circulation consistent with their overall influence on salt gland secretion. The inferred decrease in salt gland capillary blood flow stimulated by adrenalin in the turtle is similar to the effect in the bird (Fänge *et al.*, 1963).

In the context of the secretory model, the results indicate that blood circulation is a site of salt gland regulation. Reduction of capillary blood flow via vasoconstriction deprives the secretory tubules of salt and other metabolic substrates, the reverse is true when vasodilation occurs. It is not possible to determine from the technique employed in this study whether vasoconstriction and vasorelaxation occurred in capillaries within the gland or in a few major blood vessels supplying the gland. However, it is certain that the circulatory changes which took place affected the entrapment of microspheres in salt gland capillaries, and from this we can infer that the supply of blood to the secretory tubules was modified. Changes in circulation following salt-loading are very clear, with the salt gland being the only tissue examined in which a change was detected. Methacholine appears to exert its influence on gland activity by reducing capillary blood flow, with atropine demonstrating the reverse effect. The conclusions to be drawn from methacholine data are weakened somewhat by the possibility that cholinergic influences on cardiac activity may reduce blood flow to all organs or that it exerted an unrelated effect on a rate-limiting step of blood supply to the gland. The effect on salt gland circulation of atropine injection was the reverse of methacholine, with an increase in microsphere concentration. However, the data on adrenergic influence is clear, because capillary blood flow through the salt gland decreased while blood flow through cardiac muscle increased presumably as cardiac output increased and adrenergic nerves are known to lie within salt gland tissue.

In summary, the hatchling green turtle salt gland shows the characteristic cellular arrangement of the chelonian salt gland described in detail by Abel

and Ellis (1966). The three dimensional, branched arrangement of central canals draining into a main collecting duct is shown for the first time using computer-aided stereological technique. Morphometric data suggests that the circulation of blood through salt gland capillaries is increased in active glands. While the small sample size prevents statistical demonstration of significant differences, the results of blood vessel volume measurements support measurements of microsphere entrapment in active and inactive glands. Exogenous modifiers of salt secretion from the gland appear to exert at least part of their influence by affecting the flow of blood through salt gland capillaries, either directly or indirectly.

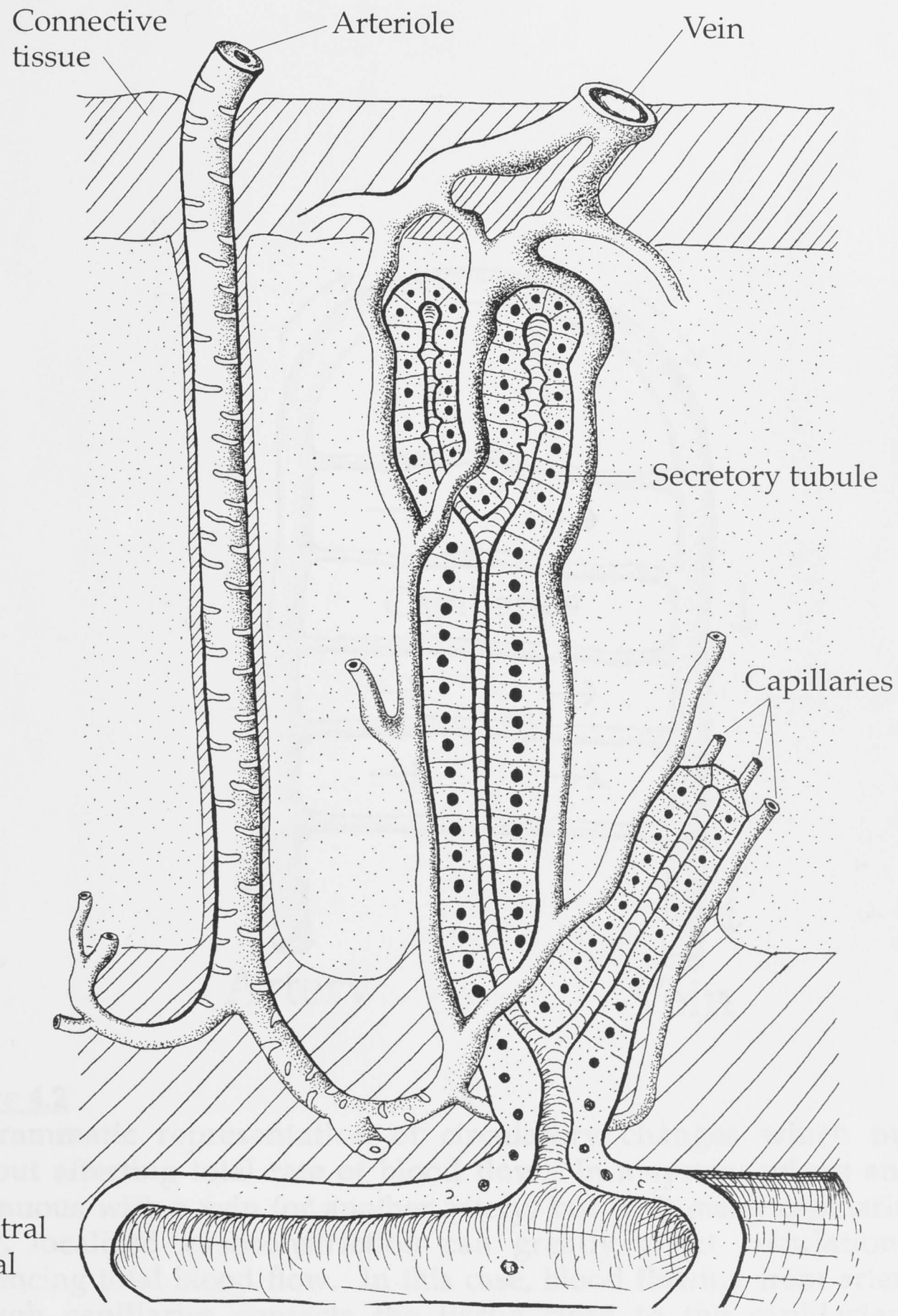


Figure 4.1
Diagram of the close association between blood vessels and the secretory tubules of the avian salt gland. Arterial blood flows towards the centre of the gland and then through capillaries surrounding the secretory tubules towards the exterior of the gland. The direction of blood flow is opposite to the flow of fluid down the tubule to the central canal. Blood vessels and secretory tubules are packed tightly together and occupy the area indicated by stippling in the drawing. The arrangement in the chelonian salt gland is similar. Redrawn from Fänge *et al.* (1958).

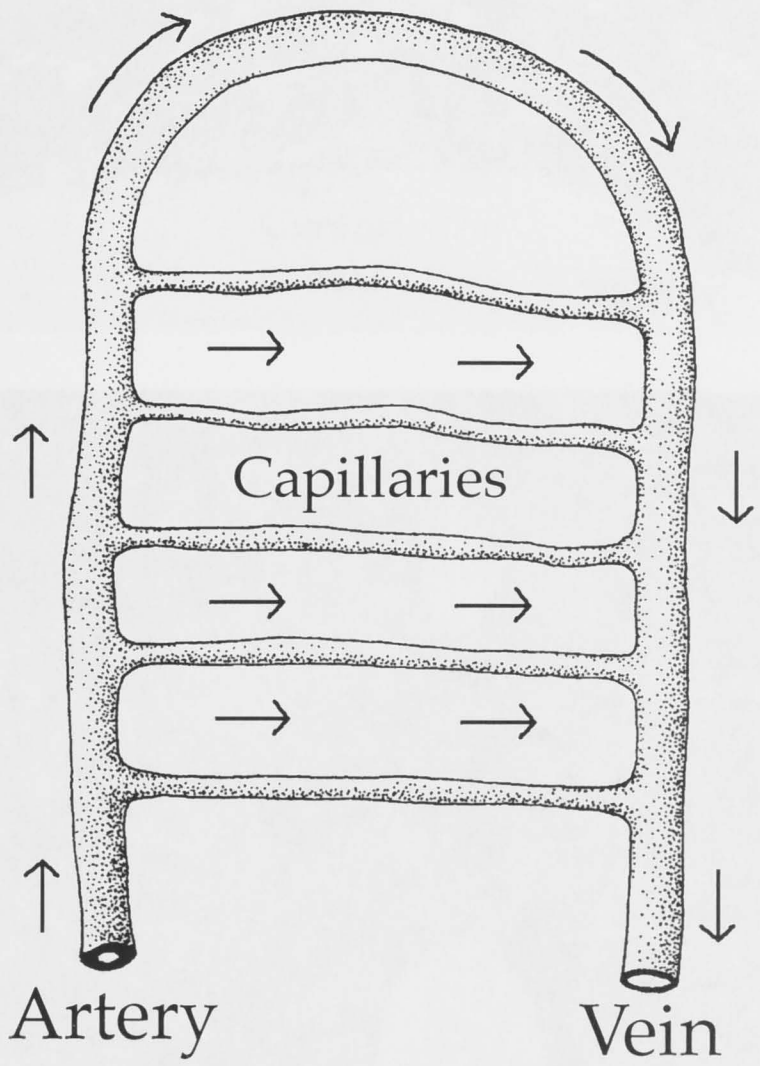
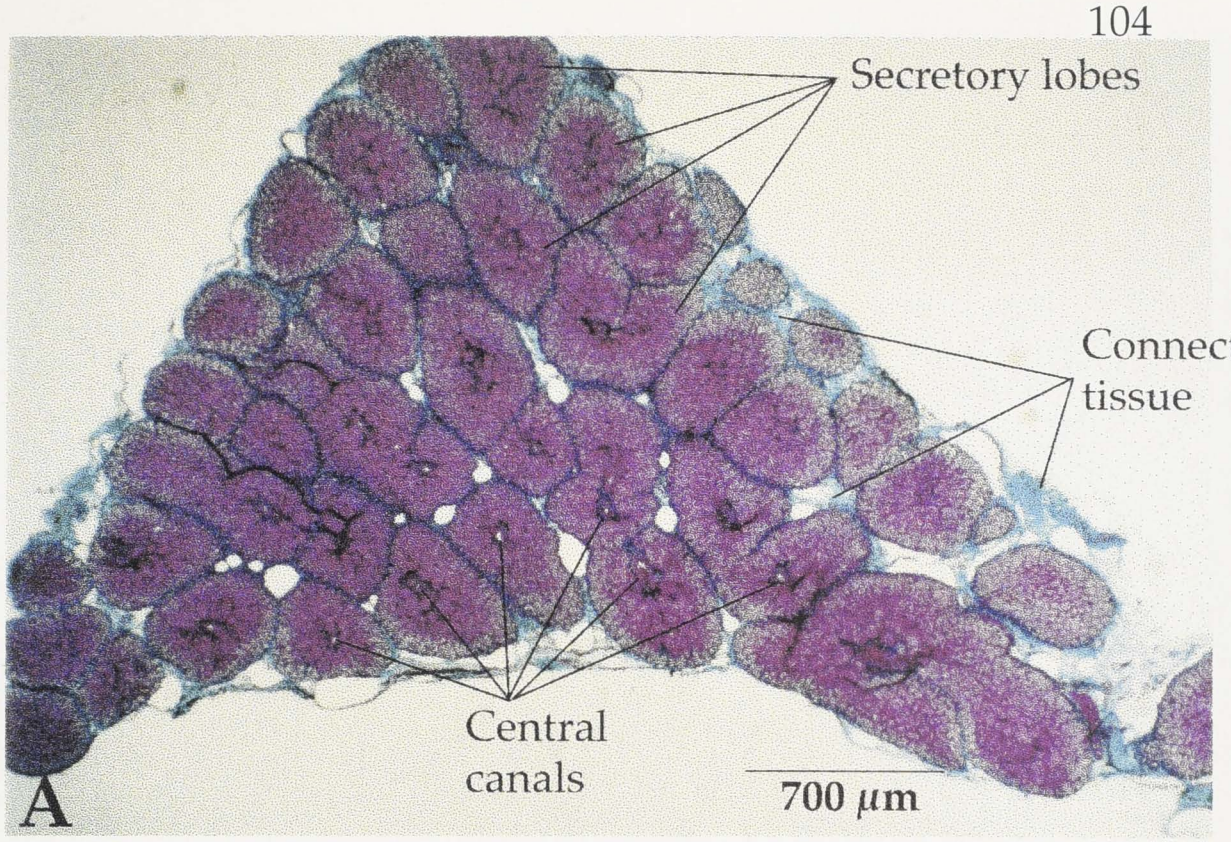


Figure 4.2
Diagrammatic representation of circulatory changes which may occur without affecting total rate of blood flow. In a system where an artery is continuous with a vein (or another artery) but with many capillaries linking them, localised vasoconstriction can greatly affect circulation without influencing total blood flow. In this case, blood flowing from artery to vein through capillaries contacts the tissue close to the capillaries. If the capillaries constrict and prevent blood flowing through them, then the blood is shunted around and enters the vein directly. Thus the amount of blood entering and leaving the tissue is unchanged but its distribution is greatly different.



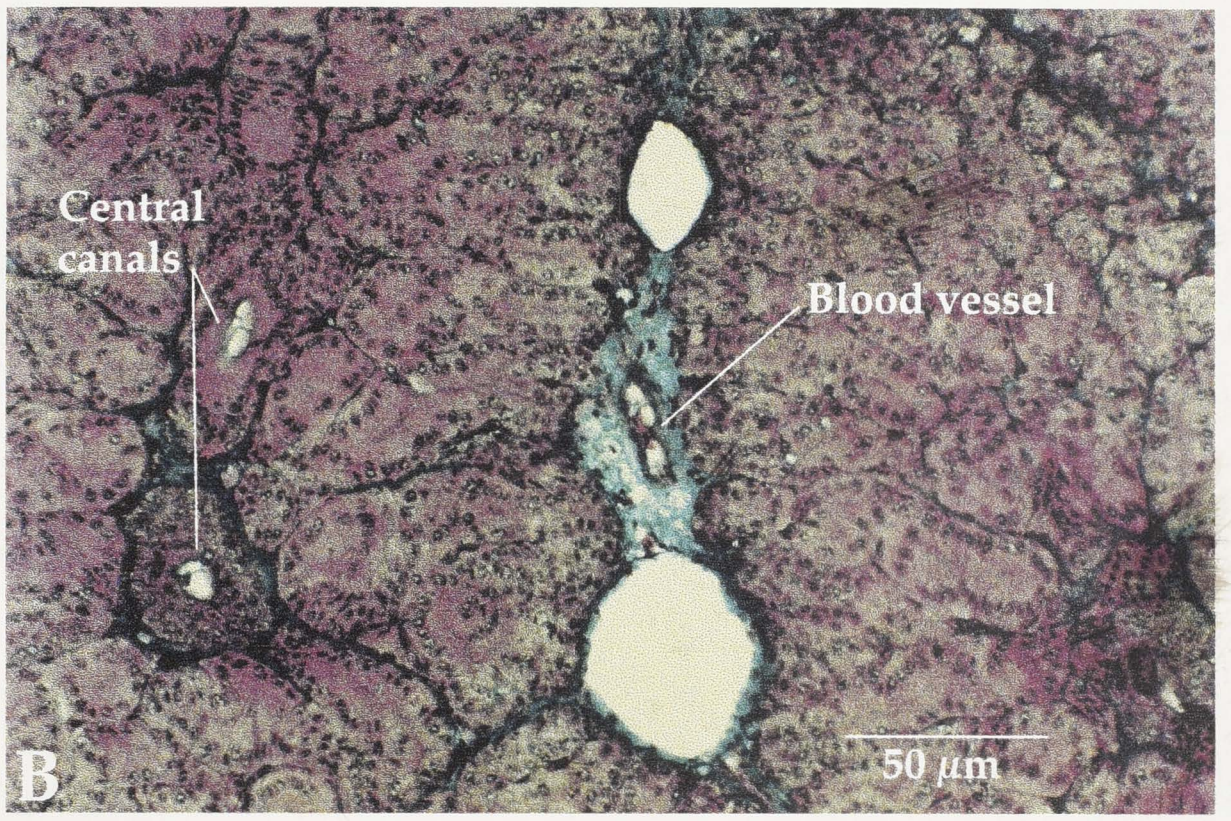
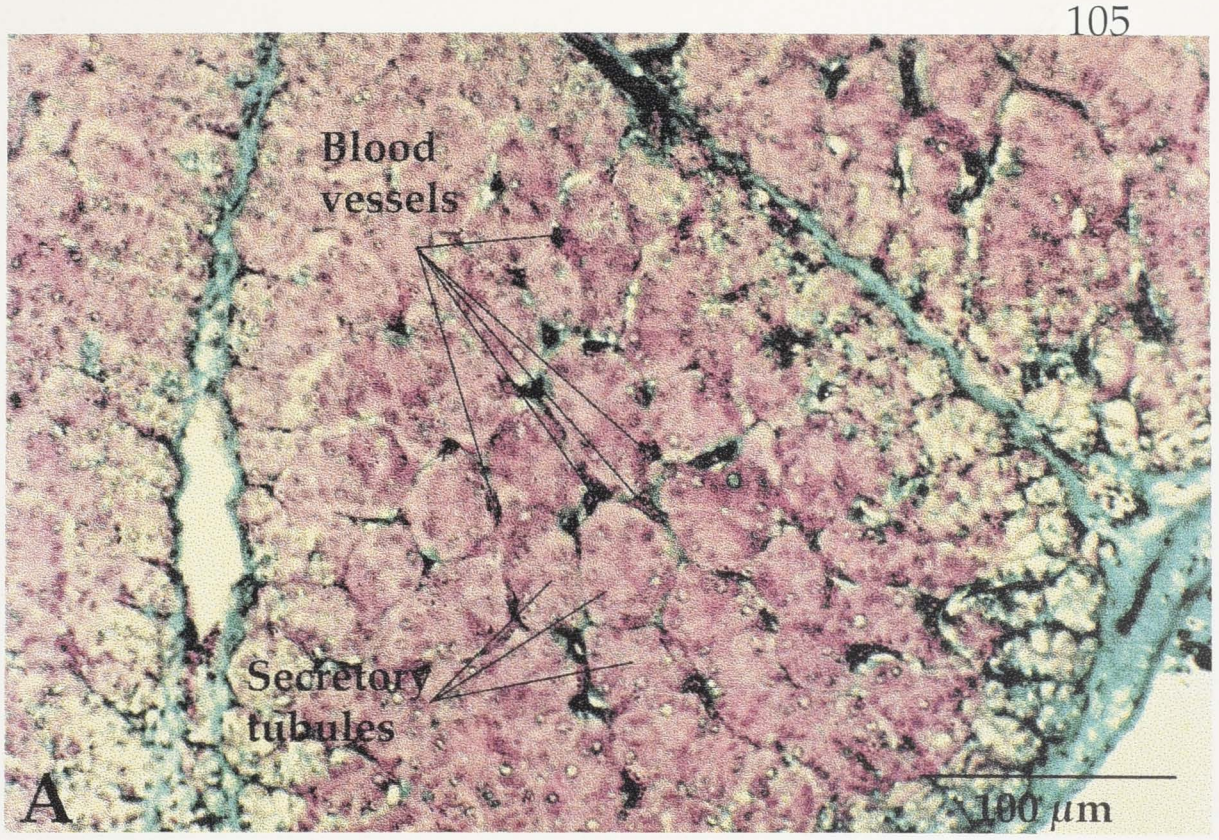


Figure 4.3
Stained wax sections of 7 μm thickness from inactive salt gland.
A) Longitudinal section of the salt gland reveals numerous secretory lobes surrounded by connective tissue and with a central canal often visible. Each lobe is made up of many individual secretory tubules packed tightly together. The large spaces between some of the lobes are artefacts of the slicing technique.
B) Secretory tubules are composed of secretory cells, the nuclei of which are seen surrounding the tubule lumen when cut in transverse section. Blood vessels pass through the connective tissue between lobes.



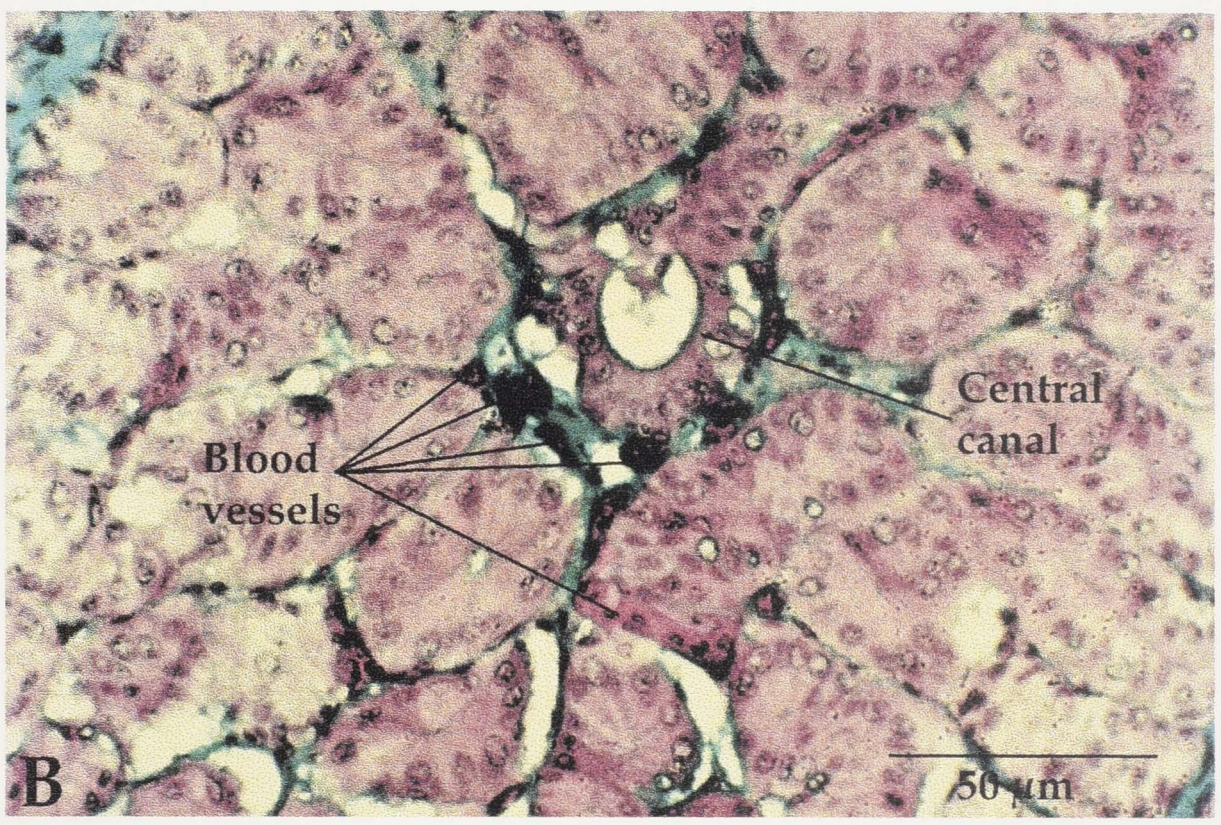
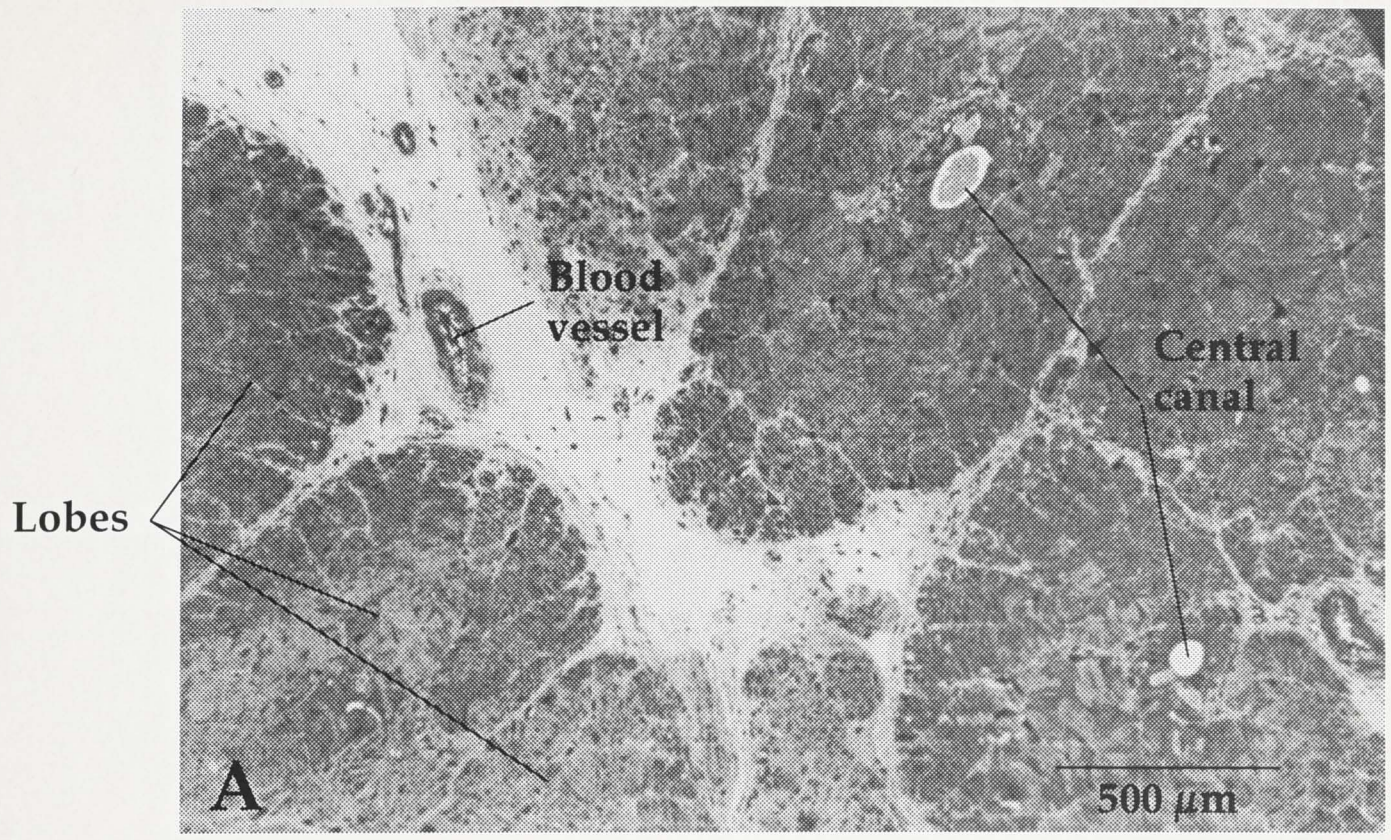


Figure 4.4
Stained wax sections of 7 μm thickness from an active salt gland taken from a frozen animal.
A) Blood cells are seen around secretory tubules, often lying in the "corners" at the junction between adjacent tubules.
B) Small blood vessels surround the central canal of each lobe, lying in the connective tissue.



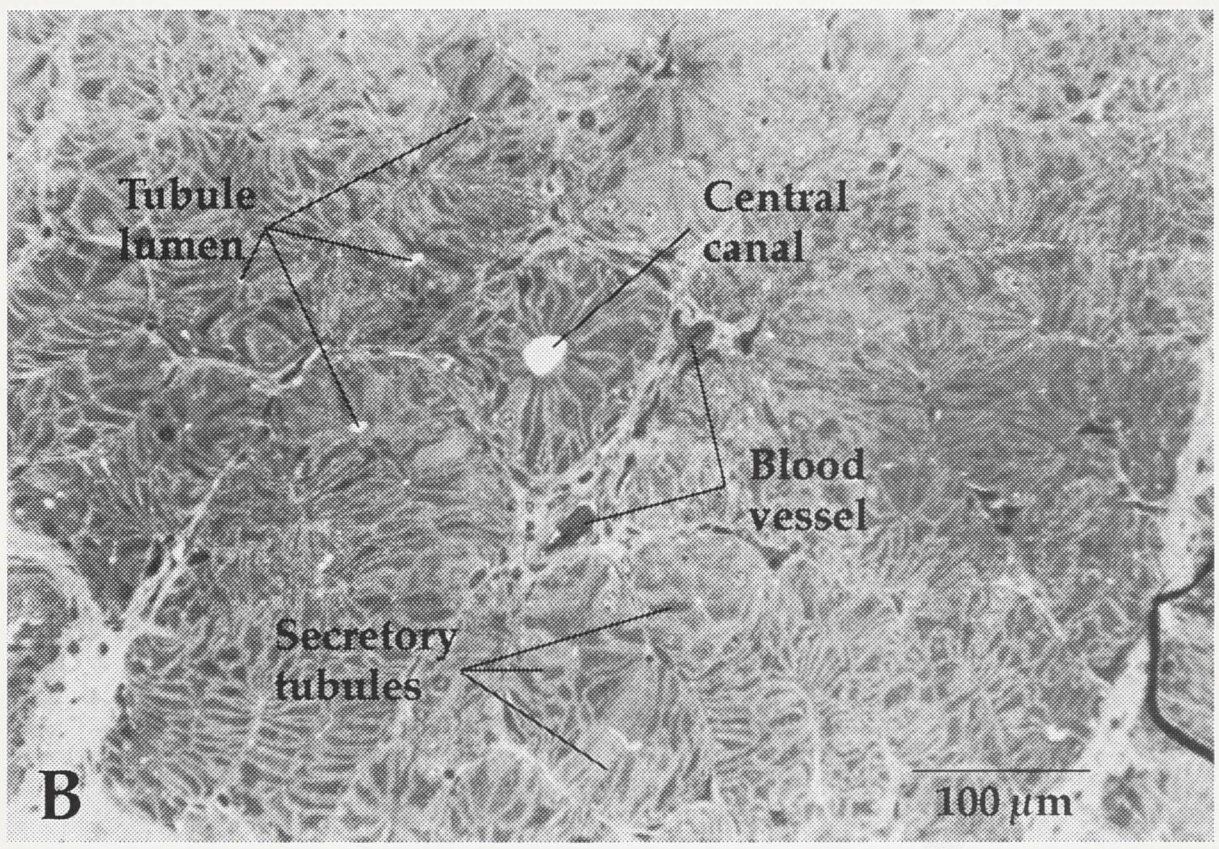
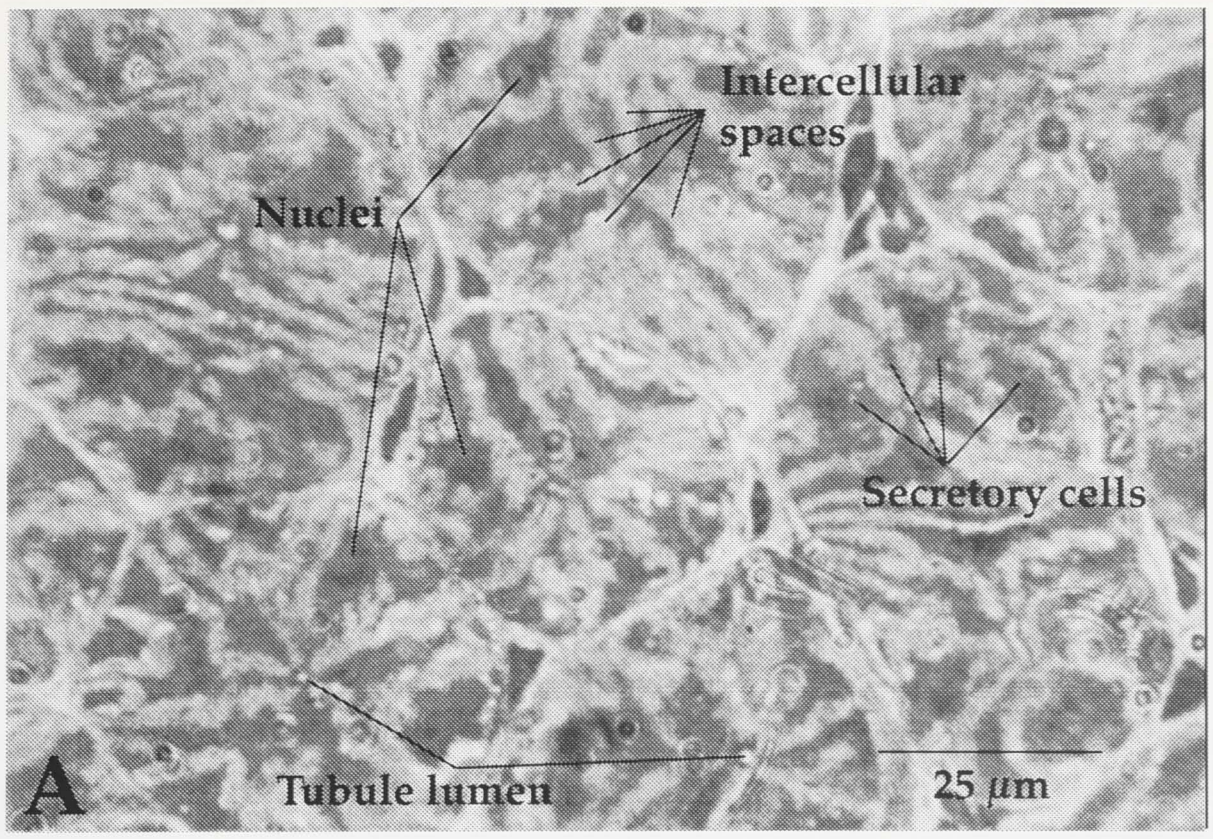
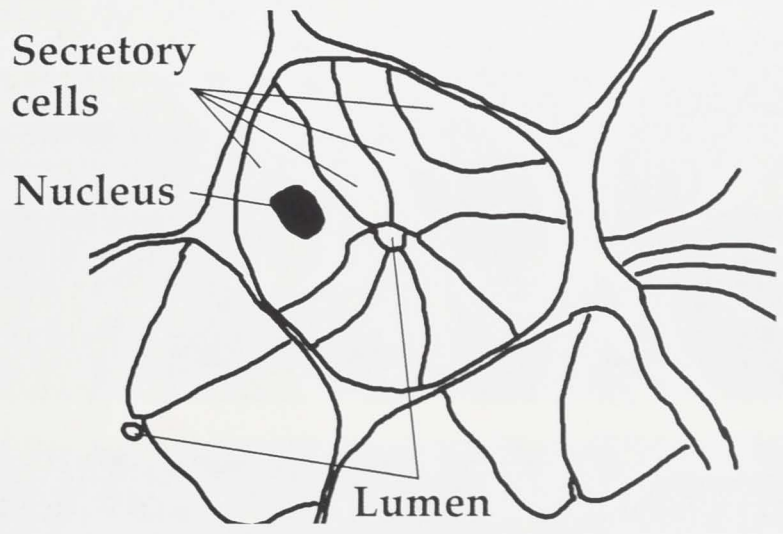


Figure 4.5
Salt gland sections of 1 μm thickness embedded in London White resin.
A) Gross morphology of the salt gland showing secretory lobes, each with a central canal and connective tissue separating them.
B) Secretory tubules are tightly packed and are roughly circular, the tubule lumen can sometimes be seen. The lumen of the central canal is much larger and has a different cellular arrangement.

Figure 4.6 (opposite)
Salt gland sections of 1 μm thickness embedded in London White resin.
A) Transverse section of salt gland showing radial arrangement of secretory cells to form secretory tubules. Approximately six to ten cells form the circumference of the tubule, with the intercellular spaces seen between them. The nuclei of cells were visible, but other intracellular structures were not apparent.
B) Interpretive drawing for A above. The secretory tubule in the centre of the micrograph is redrawn in approximately the same position to illustrate its major features more clearly. The tubule is composed of about 8 cells separated by intercellular spaces and surrounding a central lumen. Other tubules are packed closely together and have the same arrangement, although the plane of section through the tubule sometimes affects the observed radial arrangement of the secretory cells.
C) Transverse section of salt gland through the central canal showing epithelial and mucous producing cells. The diameter of the canal is much greater than that of the secretory tubule lumen and the cellular arrangement is different. Intercellular spaces are visible between cells and blood vessels can be seen around the canal. A layer of basal cells surrounds the outer circumference of the canal.


Intercellular spaces
Nuclei
Secretory cells
A
Tubule lumen
25 μm


Secretory cells
Nucleus
B
Lumen

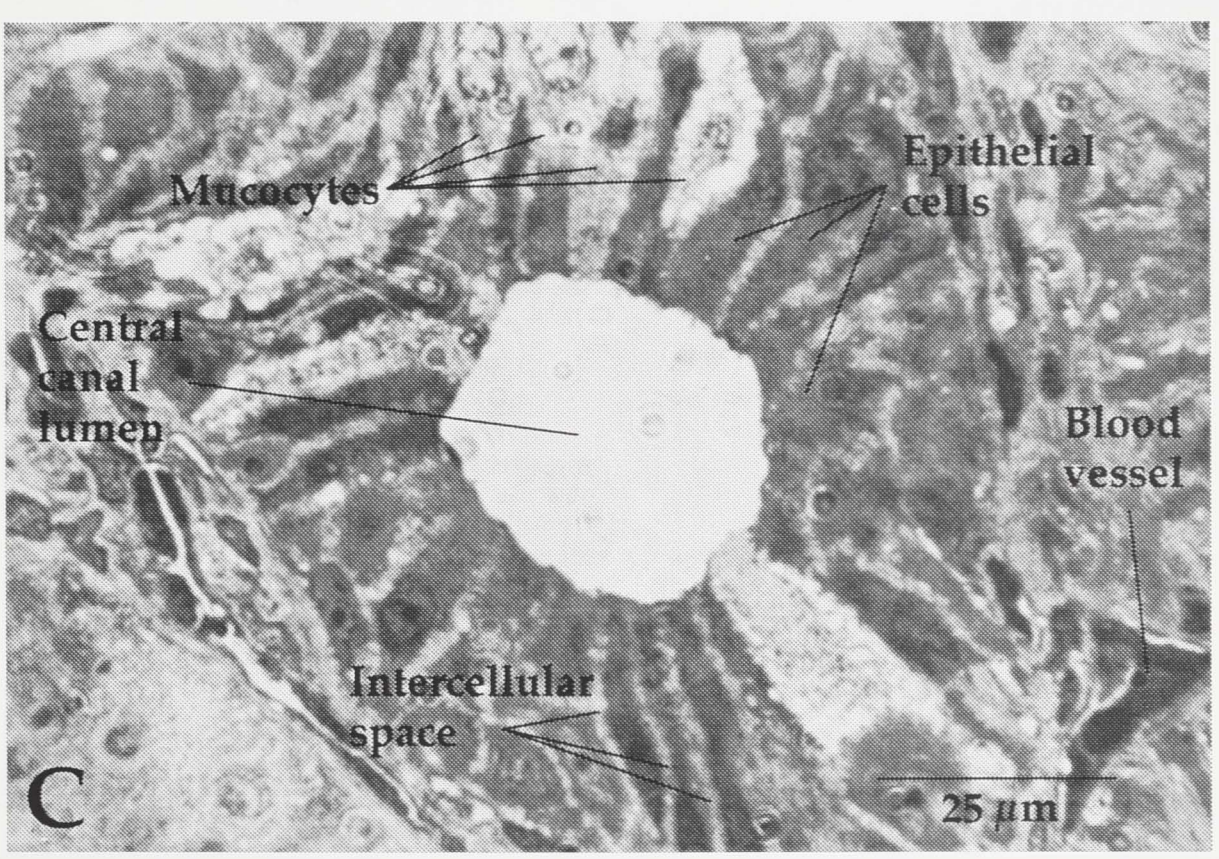
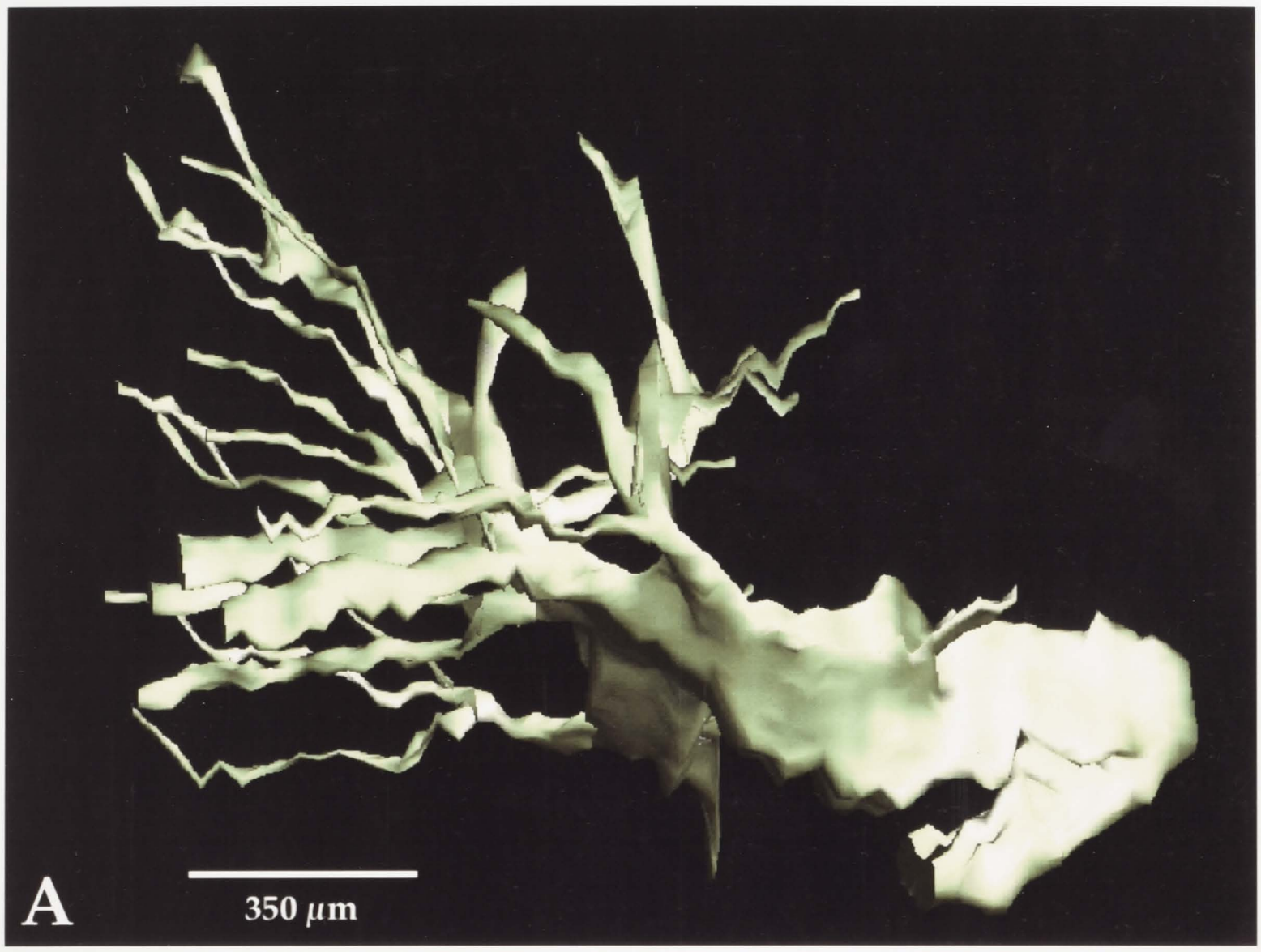
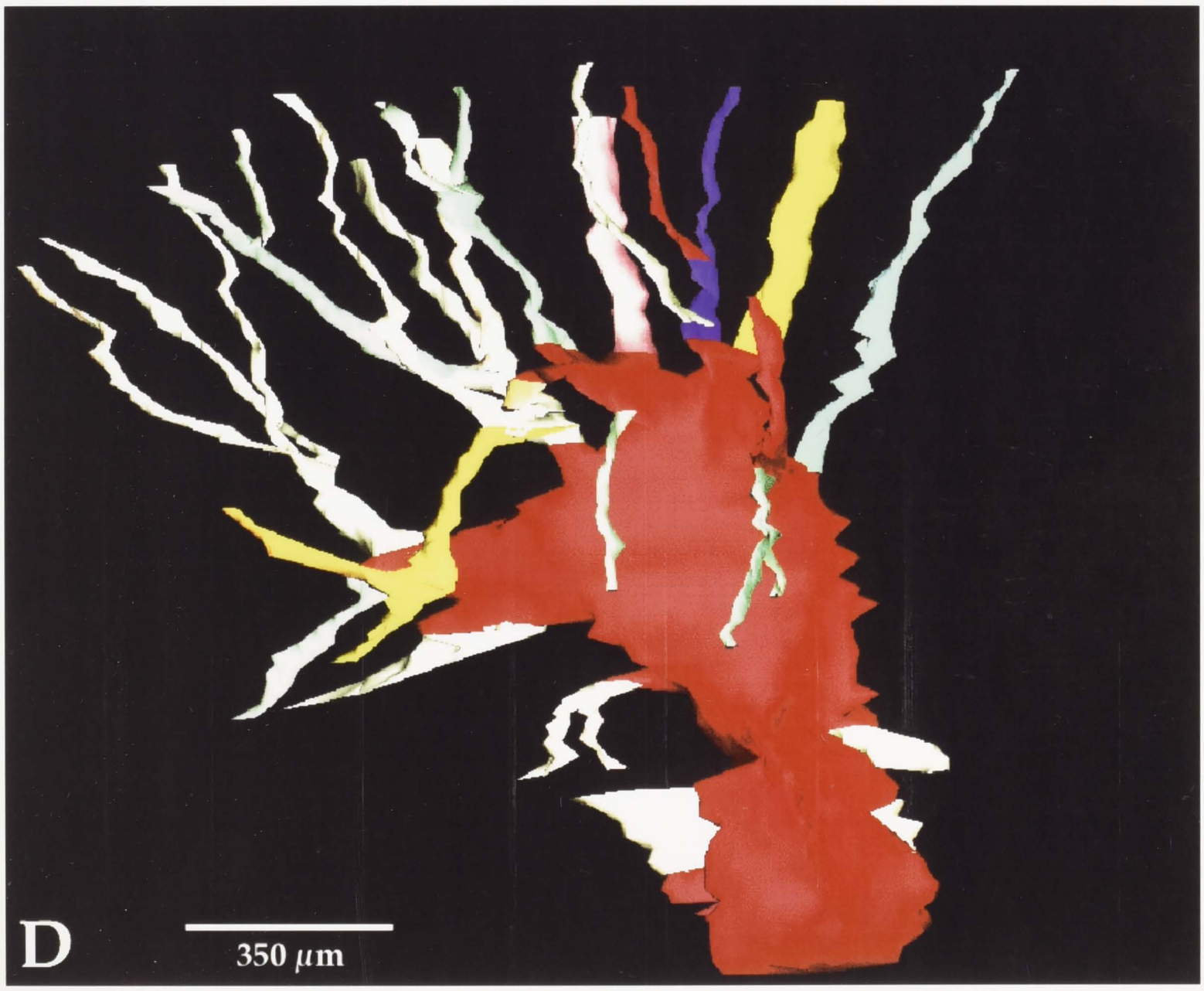

Mucocytes
Epithelial cells
Central canal lumen
Blood vessel
Intercellular space
C
25 μm

Figure 4.7 (opposite and following page)
Three dimensional computer reconstruction of the branched arrangement of ducts in an inactive salt gland. Central canals from individual lobes join and enter the main collecting duct. From this a duct leaves the gland to drain from the posterior canthus onto the surface of the eye.
A) Arrangement of ducts shown in side view. The main collecting duct is at the posterior side of the gland, with the anterior surface of the gland and the anterior of the head to the left of the illustration. The gland extends laterally also, with ducts generally running in a latero-posterior direction.
B) Side view with individual ducts in different colours to show connections more clearly.
C) Anterior view shows that ducts converge from a wider lateral and dorsal area into a relatively narrow entrance to the main collecting duct.
D) Dorsal view shows that a few ducts drain directly into the main collecting duct from lobes above.


A
350 μm


B
350 μm


C
350 μm


D
350 μm

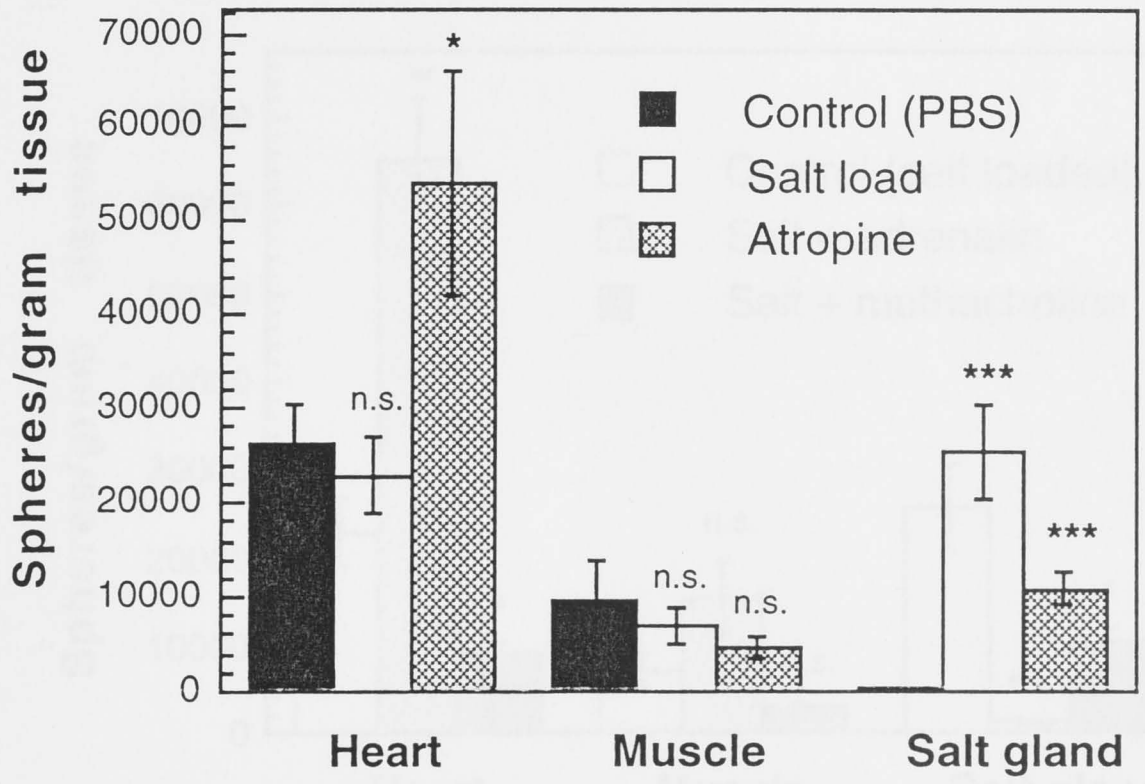


Figure 4.8
Number of 25 µm diameter spheres per gram wet weight of tissue in heart, pectoral muscle and salt gland of animals which received either PBS (n = 6), a salt-load of 2700 µmol NaCl.100g BW^{-1} (n = 6), or atropine 10 mg.kg BW^{-1} (n = 5). Asterisks indicate significant differences in microsphere number when compared with control, * = $p<.05$, *** = $p<.0001$, n.s. = not significant.

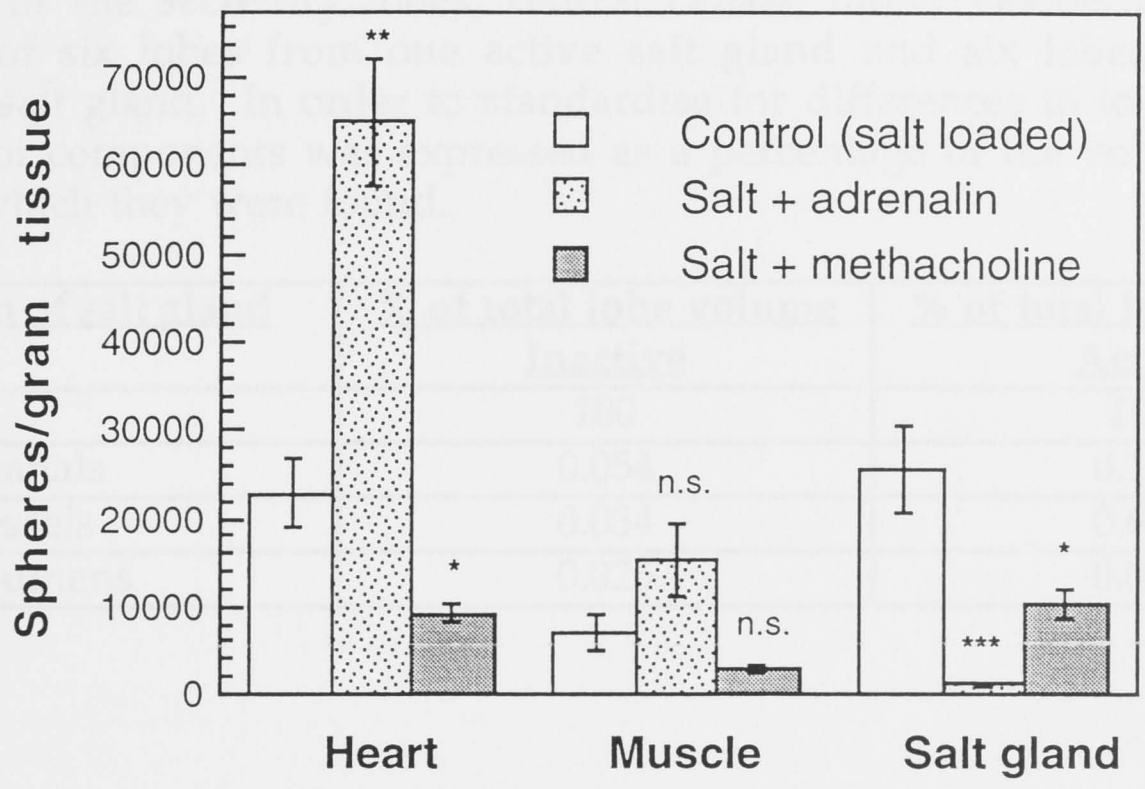


Figure 4.9
Number of 25 µm diameter spheres per gram wet weight of tissue in heart, muscle and salt gland of animals which received either a salt-load of 2700 µmol NaCl.100g BW^{-1} (n = 6), a salt-load of 2700 µmol NaCl.100g BW^{-1} followed by adrenalin 500 µg.kg BW^{-1} (n = 5), or a salt-load of 2700 µmol NaCl.100g BW^{-1} followed by methacholine 10 mg.kg BW^{-1} (n = 5). Asterisks indicate significant differences in microsphere number when compared with control, * = $p<.05$, ** = $p<.001$, *** = $p<.0001$, n.s. = not significant.

Table 4.1
Percentage volumes of salt gland components. Computer-aided stereological technique was used to measure the volume in salt gland serial sections of the secretory lobes, central canals, blood vessels and tubule lumens of six lobes from one active salt gland and six lobes from one inactive salt gland. In order to standardise for differences in lobe size, the volume of components was expressed as a percentage of the volume of the lobe in which they were found.

Region of salt gland	% of total lobe volume Inactive	% of total lobe volume Active
Lobe	100	100
Central canals	0.054	0.125
Blood vessels	0.034	0.699
Tubule lumens	0.025	0.024

Table 4.2
Summary of microsphere concentrations found in tissue samples under different experimental conditions. Mean concentration of 25 μm and 15 μm spheres are shown per gram wet weight of tissue ± 1 std error. n = number of animals sampled. Two salt glands were collected from each animal and the average microsphere concentration used in calculations.

Treatment	Tissue	25 μm spheres.g tissue^{-1}	15 μm spheres.g tissue^{-1}
Control (n = 6)	Salt gland	141 ± 38	38 ± 19
	Cardiac muscle	26318 ± 4254	15569 ± 2396
	Pectoral muscle	9713 ± 4255	3037 ± 1670
Salt-loaded (n = 6)	Salt gland	25430 ±4918	13867 ± 2647
	Cardiac muscle	22858 ±3969	16173 ± 2630
	Pectoral muscle	6970 ± 1908	4153 ± 1848
Methacholine (n = 5) plus salt load	Salt gland	10303 ±1792	4453 ± 854
	Cardiac muscle	9201 ± 932	4548 ± 488
	Pectoral muscle	2698 ± 416	1091 ± 246
Atropine (n = 5)	Salt gland	10916 ± 1756	7619 ± 1386
	Cardiac muscle	53897 ± 12042	32372 ± 7873
	Pectoral muscle	4508 ± 1112	1800 ± 367
Adrenalin (n = 5)	Salt gland	1103 ± 129	462 ± 81
plus salt load	Cardiac muscle	64956 ± 7173	32372 ± 7873
	Pectoral muscle	15275 ± 3992	7919 ± 1993

Table 4.3
Comparison of salt gland blood flow measurements as measured by different techniques. There is close agreement between reports of avian salt gland blood flow, with similar values obtained using different experimental techniques.

Technique	**Animal**	**Blood flow (ml.min^{-1}.g tissue^{-1})**	**Source**
Sapirstein/Fegler [a]	Salt adapted goose	0.8 ± 0.2 Control 11.6 ± 1.3 Secreting	1
Microsphere [b]	Salt adapted duck	0.9 ± 0.1 Control 12.5 ± 2.5 Secreting	2
Microsphere Laser-Doppler flowmetry [c]	Salt adapted duck	1.1 ± 0.2 Control 15.2 ± 1.3 Secreting 6.0 ± 1.0 VIP treated VIP treated 170% increase over control	3
Microsphere [b]	Salt adapted duck	1.3 ± 0.2 Control 15.1 ± 1.4 Secreting	4
Microsphere [e]	Hatchling green turtle	Secreting 180 times greater than control	This study

[a] The Sapirstein/Fegler technique combines measurements of diffusion of an indicator from the blood into tissues proportional with their share of cardiac output, with a determination of cardiac output by a thermodilution method.

[b] Microspheres were radioactively labelled and injected into the left ventricle, with radioactivity in the salt glands measured. By ongoing collection of a reference arterial blood sample, flow rate per unit time could be calculated.

[c] Laser-Doppler technique measures movement of red blood cells (flux) in salt gland tissue by a laser probe positioned against the salt gland surface. It is assumed that superficial blood flow is representative of blood flow throughout the entire gland. Only relative flow rates may be measured, it is not possible to calculate blood flow per unit time. Increases in flux are not necessarily linear with increases in flow rate, hence VIP stimulated a 600% increase in blood flow when measured by radioactive microspheres technique, but a 170% increase in flux was measured by the laser-Doppler flowmetry method.

[e] Coloured microspheres were injected into the systemic circulation, with numbers trapped in the salt gland counted. An ongoing arterial blood sample could not be collected so blood flow per unit time could not be calculated.

Sources: (1) (Hanwell *et al.*, 1971), (2) (Kaul *et al.*, 1983), (3) (Gerstberger *et al.*, 1988), (4) (Gerstberger, 1991)

CHAPTER 5
Oxygen Consumption Of Salt Gland Tissue

INTRODUCTION

The salt gland concentrates salt by removing sodium chloride from the blood and producing a final secretion which is five to six times the blood concentration. There are several reasons why this is almost certainly an energy-dependent process of active ion transport. Although the tubular and vascular arrangement of the chelonian and avian salt gland allows blood and secreted fluid to flow in opposite directions (Fänge *et al.*, 1958b; Abel and Ellis, 1966), a passive mechanism such as this is not sufficient to concentrate ions to the levels measured in secretions. Secretory cells of the salt gland are packed with mitochondria and glycogen granules (Abel and Ellis, 1966), indicating a high degree of oxidative metabolic activity. The secretory cells also have a high level of Na-K-ATPase activity (Ellis *et al.*, 1963; Ernst and Ellis, 1969), again indicating substantial energy requirement. Other enzymes which are present in principal secretory cells but not the undifferentiated peripheral cells include phosphatases, succinic dehydrogenase and cytochrome oxidase (Abel and Ellis, 1966; Ernst, 1986). These features are clearly indicative of active ion transport fuelled by aerobic metabolism within the secretory cell. Thus a measurement of the aerobic metabolic rate, or oxygen consumption of the secretory cells of the salt gland may reflect the rate of ion transport activity. Similarly, changes in the rate of oxygen consumption imply changes in the rate of energy-dependent ion transport.

A secretory model has been proposed by Gerstberger and Gray (1993) modified from that of Lowy *et al.* (1989) (Figure 1.4) and another model is proposed by Marshall (1987) to explain how active ion transport occurs in the avian salt gland. The chelonian salt gland shows sufficient structural and biochemical similarity to suggest that these models probably apply to ionic transport in the sea turtle salt gland as well. The histological and histochemical evidence on which the avian secretory models are based is largely also present in the chelonian salt gland, further supporting this suggestion. The first model describes a number of steps to move sodium, chloride and potassium across the principal secretory cell membrane with the result that sodium and chloride are highly concentrated in the secretory tubule lumen. This is consistent with the high enzymic activity and large numbers of mitochondria in the principal secretory cells. The second model suggests that the secretion from the secretory tubules is isosmotic and

subsequently concentrated by cells lining the collecting ducts. The histological evidence described above tends not to support this model, as cells lining the collecting ducts do not have the features described above which indicate a high degree of ion transport activity. If they were doing the majority of ion transport they should be very active indeed, particularly as they constitute a small percentage of the total number of cells in the salt gland. Whichever model is accepted, a key feature of both is that they rely on transport against a concentration gradient by an energy-dependent process which consumes oxygen.

The secretory model (Figure 1.5) indicates that control of ionic transport can be at the level of primary salt production. An indirect method for examining if this is true is to measure the oxygen consumption of tissue slices. The rate of respiration by isolated tissue slices can be measured *in vitro*, and changes in oxygen consumption detected. However, in order for measurement of respiration rate to reveal the activity of salt gland tissue, three assumptions must be made. First, the tissue slices must maintain their activity *in vitro* in the absence of innervation and blood flow. Second, the oxygen consumption associated with ionic transport must comprise a significant proportion of total oxygen consumption by the gland so that differences in states of activity can be detected. Third, an increase in respiratory rate of tissue must be coupled to an increase in the transport of ions and vice versa. In this chapter the rate of oxygen consumption of salt gland slices from hatchling turtles was measured *in vitro*, following removal from animals which were actively secreting and also from those which were not. The hypothesis was tested that salt glands taken from animals which were secreting would consume oxygen at a faster rate than inactive salt glands because of an increased rate of energy-dependent ionic transport. The ability of modifiers of gland activity to change the oxygen consumption of salt gland tissue was examined to determine if their effects were exerted directly on the activity of secretory cells.

5.1 IONIC TRANSPORT AND OXYGEN CONSUMPTION

Provided that the assumptions made regarding the relationship between oxygen consumption and ionic transport activity are correct, it may be possible to detect whether the secretory cells change in respiratory rate with the overall secretory activity of the gland. If the secretory cells themselves can exist in states of high and low rates of ion transport, this suggests that they may be a target for regulatory modifiers of gland activity as a whole.

Studies have shown that oxygen consumption of salt gland tissue from other animals can be measured (Borut and Schmidt-Nielsen, 1963; Shuttleworth and Thompson, 1987; Silva *et al.*, 1993), so this technique is suitable for examining possible changes in oxygen consumption with ion transport by the gland.

5.2 MODIFIERS OF OXYGEN CONSUMPTION

Methacholine and adrenalin both inhibit secretion from the salt gland *in vivo* (Chapter 3) but it was not possible to determine what aspect of salt gland function they were affecting. By examining their ability to influence respiration rate of salt glands, the possibility was investigated that they were changing the activity of secretory cells directly. The hypothesis was that methacholine reduces the respiration rate of salt gland tissue as a result of inhibiting energy-dependent ion transport. Similarly, it was hypothesised that adrenalin reduces the respiration rate of salt gland tissue for the same reason. In the context of the secretory model, this attempted to identify if the secretory cells of the tubules or the cells lining the ducts are targets for regulation of the activity of the salt gland as a whole.

5.3 EXPERIMENTAL AIM

The aim of the experiments was to determine oxygen consumption of salt gland tissue *in vitro* as a measure of energy-dependent ionic transport using the oxygen electrode recording technique. Respiration rate of active and inactive glands was measured to identify any differences associated with secretory activity of the gland. Respiration rate of tissue following administration of methacholine and adrenalin was measured to discover if at least part of their inhibitory action occurred through inhibition of ionic transport by secretory cells.

METHODS

5.4 ANIMALS

Animals used were as described in Section 2.3.

5.5 COLLECTION OF TISSUE SAMPLES

Animals were killed by decapitation and salt glands immediately removed and placed in turtle Ringer's solution bubbled with carbogen gas (95% O_2, 5% CO_2). Turtle Ringer's was the same as that used for the estuarine turtle, *Malaclemys terrapin* (Shuttleworth and Thompson, 1987) and contained (in mmol.l^{-1}); NaCl 90, KCl 3, $CaCl_2$ 2, $MgSO_4$ 1, $NaHCO_3$ 10, Na_2SO_4 10, urea 10, glucose 5.5, NaH_2PO_4 0.5, Na_2HPO_4 0.5, adjusted to pH 7.2 with NaOH while being bubbled with carbogen. Following pH adjustment, total sodium was approximately 150 mmol.l^{-1}. Salt glands were mounted on a cutting block with cyanoacrylate glue (Selleys Supaglue) in a bathing medium as above. Prior to slicing, some salt glands were killed by freezing at -70°C for 30 minutes or placing them in 100% methanol for 15 minutes. Tissue slices of 200 µm thickness were cut with a vibrating slicer (Campden Instruments) and transferred to rest on wire mesh above a small stirrer in the oxygen electrode incubation chamber (Figure 5.1). The Ringer's incubation medium was bubbled with carbogen and a water jacket maintained the medium at 28°C. Chambers were sealed immediately after the carbogen bubbler was removed and oxygen concentration measured over the course of the experiment. At the conclusion of the experiment tissue slices were blotted dry and weighed. Sodium dithionite was added to the incubation medium to scavenge any remaining oxygen to verify that some oxygen remained in the medium and that the recording electrodes were still measuring falling oxygen concentration.

5.6 MEASUREMENT OF OXYGEN CONSUMPTION

Three recording electrodes were used simultaneously, each measuring oxygen concentration of a different incubation chamber. Recording electrodes were of the Clark polarographic type (Rank Brothers Instruments, Cambridge U.K.), with a platinum cathode in the incubation medium, a silver anode isolated from the medium and with a KCl bridge linking the two (Figure 5.1). A small magnetic stirrer circulated the incubation medium during the experiment. A polarising voltage of 0.6 volts was applied across the electrodes so that a current passed between the poles when oxygen was present in the incubation medium, with oxygen reduction at the cathode and silver oxidation at the anode. The current flowing at the voltage applied was

linearly proportional to the concentration of dissolved oxygen. Changes in this current were converted to a potential difference and captured by computer through a MacLab (AD Instruments). The Chart v3.4 (AD Instruments) program was run on a Macintosh SE 30 computer to record, store, convert and analyse data. Changes in incubation medium oxygen concentration were observed as changes in the voltage displayed on the computer. The computer recorded voltage for the duration of the experiment, so that the time course of voltage changes could be seen. This permitted calculation of the rate of oxygen consumption over time.

5.7 EXPERIMENTS CONDUCTED

The oxygen consumption rate of salt gland tissue was measured both from glands taken from animals which had been stimulated to secrete with a salt-load of 2700 µmol NaCl.100 g BW^{-1}, and from glands taken from animals which were injected with an equal volume of PBS and not secreting. Any passive oxygen absorption by the tissue was determined by measuring the oxygen consumption rate of the tissue killed by freezing or methanol.

To determine whether methacholine or adrenalin directly affected oxygen consumption of salt gland tissue, the tissue was incubated with and without these chemicals in the incubation medium. Once oxygen consumption rate had stabilised for approximately 20 minutes, methacholine or adrenalin was added to experimental incubation chambers, while control tissues received PBS. Twenty five µl of methacholine (5 $mg.ml^{-1}$ methacholine in PBS) was added for a final concentration in the incubation medium of 2.6×10^{-4} M while 25 µl of adrenalin (250 $\mu g.ml^{-1}$ adrenalin in PBS) was added for a final concentration of 1.4×10^{-5} M. The dose of methacholine administered was similar to that used by Shuttleworth and Thompson (1987) of 5×10^{-4} M when measuring oxygen consumption of the salt gland from the estuarine terrapin. The dose of adrenalin was lower than that of methacholine and was selected on the basis of the relative effective doses of these chemicals *in vivo*. In order to act as a positive control, cardiac tissue from experimental animals was removed and sliced as described above. The cardiac slices were exposed to the same concentrations of methacholine and adrenalin and the effect on oxygen consumption rate measured. A typical experiment was conducted with salt gland tissue in one chamber, salt gland or cardiac tissue from the same animal in another chamber, and no tissue in the third chamber. One tissue received the injected chemical and the second received

PBS except when it contained cardiac tissue, in which case it also received the injected chemical.

5.8 CALCULATION OF OXYGEN CONSUMPTION RATE

The incubation medium was presumed to be saturated with oxygen when the voltage observed on the computer reached a maximum and remained at that level while the solution was still being bubbled with carbogen. The medium was presumed to be completely anoxic following addition of sodium dithionite when the voltage observed on the computer reached a minimum value. The total oxygen content of the turtle Ringer's was measured by saturating the solution using carbogen gas and then measuring oxygen partial pressure with a Radiometer ABL505 blood gas analyser. After correcting for temperature and salinity, total oxygen content of the incubation medium was calculated to be 1.017 $\mu mol.ml^{-1}$. The difference between maximum and minimum voltage (after addition of sodium dithionite) for each experiment then represented a 100% fall in oxygen concentration of the medium. Thus for each experiment the measured voltage fall as a proportion of the total could be expressed as a fall in micromoles of oxygen consumed over the period of the experiment. Oxygen consumption rate per unit time could then be calculated and expressed in micromoles of oxygen consumed per gram of tissue per hour. For example, if the maximum voltage reading of an incubation chamber was 75 mV and the minimum was 25 mV, then a decrease from 75 mV to 50 mV represented a 50% fall in oxygen concentration, a decrease of 12.5 mV represented a 25% fall and so on. This was converted to the same percentage fall from the oxygen concentration of 1.017 $\mu mole.ml^{-1}$ in a volume of 2.5 ml and the time period over which the fall occurred was determined. This could then be expressed as a fall in oxygen concentration in $\mu mole.min^{-1}$ and calculated per gram of tissue in the incubation chamber. Typically about 40 - 60% of the total oxygen in the incubation medium was consumed by salt gland tissue during the course of the experiment.

When measuring fall in recorded voltage, the highest value was taken after an initial equilibration of about 10 minutes. The minimum value was taken immediately prior to addition of sodium dithionite. The rate of fall from control chambers which did not contain tissue or contained dead tissue was calculated in the same way. The control rate of oxygen decrease was deducted from that of experimental chambers when determining oxygen

consumption, as it was presumed to indicate passive uptake by tissue as well as spontaneous loss or leakage of oxygen from the incubation chambers.

In order to identify whether addition of PBS, methacholine or adrenalin changed the rate of oxygen consumption of either salt gland or cardiac tissue, or of the control chamber, the rate was measured before and after chemical addition. The rate was calculated by determining the voltage fall in the five minutes before and five minutes after chemical addition and converting to oxygen consumption as described above.

5.9 STATISTICAL ANALYSIS

The Mann-Whitney rank test was used to determine any significant difference in the rates of oxygen consumption by active and inactive salt glands. A paired t-test was employed to determine any significant difference in oxygen consumption before and after chemical addition. In all cases, significance was assumed if $p<0.05$. All results are shown as the mean $\pm$ 1 standard error.

RESULTS

5.10 OXYGEN CONSUMPTION OF ACTIVE AND INACTIVE SALT GLAND TISSUE

There was no significant difference in oxygen consumption rates of salt gland slices from animals which had been salt-loaded compared to those which had not. After allowing for the rate of oxygen loss from control chambers which did not contain tissue (n = 19), salt-loaded glands consumed oxygen at 33.938 ± 2.902 μmol O_2.g wet weight^{-1}.hr^{-1} (n = 7), while non salt-loaded glands consumed oxygen at 39.975 ± 2.518 μmol O_2.g wet weight^{-1}.hr^{-1} (n = 7). The rate of oxygen loss from chambers containing salt gland tissue killed by freezing (n = 3) or methanol (n = 2) was not significantly different from the empty control chambers. Sample voltage traces from an empty control chamber, tissue from salt-loaded (active) and non salt-loaded (inactive) animals indicating the fall of oxygen concentration in the medium are shown in Figure 5.2a, b & c.

5.11 INFLUENCE OF METHACHOLINE AND ADRENALIN ON OXYGEN CONSUMPTION

Methacholine did not significantly influence the rate of oxygen consumption of salt gland tissue, either from salt-loaded or non salt-loaded animals (Figure 5.3). However, the rate of oxygen consumption by cardiac tissue was significantly reduced from 52.98 ± 15.72 to 13.32 ±10.2 μmol O_2.g wet weight^{-1}.hr^{-1} following methacholine addition to the incubation medium (Figures 5.2d and 5.3). Consumption rate was reduced for approximately five minutes before returning to the rate seen prior to the addition of methacholine.

The addition of adrenalin also did not change the oxygen consumption rate of salt gland tissue (Figure 5.4), but it transiently increased consumption by cardiac tissue slices from 43.20 ± 11.52 to 270.00 ± 10.20 μmol O_2.g wet weight^{-1}.hr^{-1} (Figures 5.2e and 5.4).

DISCUSSION

There was no observed difference in the rate of oxygen consumption of slices from salt glands whether removed from salt-loaded or control hatchlings. Therefore under the conditions and assumptions of these experiments, the hypothesis that ion transport activity measured by oxygen consumption differs with gland activity is not supported. On the strength of this result, it would be unlikely that the secretory cells (both of the tubules and those proposed to line the ducts) of the salt gland are a target for regulation as suggested in the secretory model. The inability of methacholine and adrenalin to alter the rate of salt gland tissue respiration means that the hypotheses relating to a reduction in secretory cell respiration by these chemicals are also not supported under these experimental conditions. Thus the results indicate that the energy-dependent ion transport of secretory cells proceeds with the same oxygen requirement irrespective of the overall state of activity of the gland and independent of the influence of modifiers of gland activity. However, a major limitation of the *in vitro* experimental technique is that the tissue being examined is isolated from any controlling factors present *in vivo*.

Measurements of oxygen consumption *in vitro* make the assumption that the tissue respires normally and responds to modifiers normally. However, removal of the salt gland obviously isolates it from neural and circulatory influences and these may be important for maintenance of the gland in a state of activity or inactivity. When removed from these influences, an active gland may become inactive, or the reverse may be true. If this is the case, it would not be possible to distinguish between different states of activity by measuring oxygen consumption, as the tissue will assume one or the other state following removal from the animal. Detection of a difference in oxygen consumption of slices from active and inactive glands would tend to refute this problem, but this was not observed in this study, and no other published studies have measured respiration of the gland in different states of activity. Furthermore, it is not currently possible to measure oxygen consumption and ion transport of slices simultaneously, so it is impossible to be certain that ion transport is actually taking place in the *in vitro* preparation. Results will be discussed in the light of these limitations.

The rate of oxygen consumption measured in glands removed from salt-loaded and non salt-loaded animals was approximately 35 μmol O_2.g wet weight^{-1}.hr^{-1}. In order to determine what proportion of the total respiration

rate is due to the requirements of ion transport in the active gland, the oxygen needed to fuel this work must be calculated. In a salt-loaded animal which is secreting, the metabolic cost to the secretory cells of transporting ions is calculated from the equation;

$$\text{Work (Joules.mole}^{-1}) = RT \ln C_{bl}/C_{medium} \quad \text{(Withers, 1992)}$$

where R is the gas constant, T is the absolute temperature and C_{bl} and C_{medium} are the blood and medium (secretion) ion concentrations. Substituting the appropriate values ($R = 8.3143$ J.mole^{-1}.K^{-1}, $T = 301^0$ Kelvin, $C_{bl} = 190$ mmol.l^{-1}, $C_{medium} = 816$ mmol.l^{-1}), the energy requirement is 3646 J.mole^{-1} salt transported. The amount of salt transported by a single salt gland of 40 mg mass in a 30 g hatchling is 125 µmol NaCl.hr^{-1}, thus the work done is 0.456 Joules.hr^{-1}. Assuming that energy is provided by carbohydrate metabolism with a production of 21.4 kilojoules per litre of oxygen, then the oxygen required is 525 µl O_2.hr^{-1}.g^{-1}, or 22.06 µmol O_2.hr^{-1}.g salt gland weight^{-1}. The measured respiratory rate is capable of meeting the calculated ion transport energy requirement provided the efficiency of transport is at least about 60%. The remaining component of the observed respiration must be for the support of cellular metabolic activity not related to osmoregulation. It seems reasonable that the salt gland would have a high proportion of its oxygen consumption used for osmoregulation, as suggested by the large numbers of mitochondria and glycogen granules seen in principal secretory cells (Abel and Ellis, 1966). The result suggests that the secretory cells of the salt gland of the hatchling turtle remain in the same state of activity regardless of whether the gland is secreting or not. However, given the limitations of the experimental technique discussed earlier, it is entirely possible that the salt gland tissue assumed either an active or inactive state following removal from the animal and isolation from any controlling modifiers. Two cases can be considered, with either the inactive salt gland slices in the absence of *in vivo* inhibition increasing their activity to match that of the active glands, or the reverse occurring in the absence of *in vivo* stimulation. In the first case, the rate of oxygen consumption measured and therefore the ionic transport activity inferred is the rate which occurs in salt gland tissue which is actively transporting salt. The calculated oxygen requirement for active transport is less than the measured rate, indicating that secretory activity could have been taking place. In the second case considered, the rate of oxygen consumption measured and therefore the ionic transport activity

inferred is the rate of basal metabolism by the salt gland when it is not producing secretions.

A further possibility presents itself, that the activity of the salt gland secretory cells is limited by the available oxygen concentration. The oxygen supply to the salt gland may be tightly controlled *in vivo* by regulating blood supply so that the activity of ion transporting cells is reduced or increased as required. Following removal from the animal and incubation in an oxygen-saturated environment, the secretory cells may consume oxygen as rapidly as possible and consequently transport ions at their maximum rate, regardless of their experimental history. The fact that the measured respiration rate exceeds the calculated minimum requirement for secretion indicates that this could certainly occur.

Measurements of oxygen consumption from the hatchling turtle salt gland are similar to those made from other animals (Table 5.1). The fact that the rates measured from avian salt glands were close to those measured from turtles and the shark suggests that the avian glands were not in an active state. This is suggested because active avian salt glands would be expected to have a respiration rate much higher than those of turtles and the shark simply because of the comparative metabolic rate of ectothermic and endothermic animals. In addition, salt-adapted birds have a greater secretory rate per unit mass of salt gland tissue than reptiles or sharks. It is not possible to determine if there were comparative differences between active and inactive salt glands as this was the only study which measured respiration under both conditions.

Methacholine and adrenalin both inhibit the secretory activity of the salt gland *in vivo* but had no measurable effect on the oxygen consumption rate of salt gland tissue *in vitro* under the experimental conditions. The respiration rate of heart muscle responded to injection of methacholine and adrenalin into the incubation medium, indicating that the technique was working. This would suggest that the inhibitory influence on salt gland secretion of both these modifiers is not achieved via a direct effect on the secretory cells to change their transport activity. However, in view of the possible effects of removal of the gland from the study animal discussed earlier, it cannot be ruled out that these chemicals may exert an influence on secretory cell ion transport through some intermediate factor which is absent in the *in vitro* preparation. In contrast, methacholine increases the

respiration rate of avian salt gland tissue by 50 to 80% (Borut and Schmidt-Nielsen, 1963; Stewart *et al.*, 1979; Hootman and Ernst, 1982) presumably exerting at least part of its stimulatory influence on the avian gland in this way. The effect of methacholine on the estuarine turtle lachrymal gland and shark rectal gland is similar to the bird (Shuttleworth and Thompson, 1987; Silva *et al.*, 1993). However, as mentioned in the general introduction the salt glands in these animals are not homologous structures, so control mechanisms may be different. It does not seem likely that this is due to differences in technique, as the experimental methods are similar, and observed respiration rates in the absence of drug treatment are also similar. Again it is not possible to distinguish whether this is due to methacholine really having no influence on ion transport at all, or some result of removing the gland from the study animal which prevents its effect being observable in the hatchling green turtle. It is not possible to make any comparisons on the effect of adrenalin, as its effect on oxygen consumption in other animals has not been reported.

One of the assumptions made in measuring oxygen consumption as an indicator of ionic transport is that respiration and transport activity are directly linked. However, if respiration and ion transport can be coupled and uncoupled, it is possible for the two to be dissociated. In this instance where it is suggested that the secretory cells consume oxygen at a rate dependent on its availability, respiration may have increased *in vitro* but if it is uncoupled from transport, ion movement will not change. In the context of the gland *in vivo*, a change in blood flow and hence oxygen supply may be associated with a concomitant coupling of respiration and ion transport in the secretory cells by a modifier of activity. Thus, *in vivo* there would not be a futile increase in respiration which does not result in any greater work done by the gland. In the absence of the modifier the process uncouples but again there is no futile respiration because the uncoupling is associated with a reduction in blood flow and hence oxygen availability to the secretory cells. The result of such a control system is that *in vitro* the coupling or uncoupling described above will not be detected by any change in respiratory rate. Although the assumption of a direct link between oxygen consumption and ion transport is fundamental to respiration experiments, the possibility that the link can be uncoupled cannot be ruled out in the absence of a simultaneous measurement of both.

A consideration of the measured oxygen consumption rate may reveal information about the different models of ionic secretion proposed by Gerstberger and Gray (1993) and Marshall (1987) if we assume that these models apply to both the turtle and avian salt glands. The first model relies on energy-dependent ion transport by the principal secretory cells of secretory tubules, while the second relies on the majority of ion transport work being done by the cells which line the central canals and collecting ducts. The rate at which these cells would have to transport ions and consume oxygen differs, as they are not present in the salt gland in equal numbers. The area of lobular tissue comprised of principal secretory cells is approximately 150 times the area of central canals and ducts around which the secretory cells proposed by Marshall lie (Marshall *et al.*, 1987). The same amount of osmoregulatory work has to be done in both models, but in that proposed by Marshall the membrane area over which this transport can occur is very much smaller. The size of secretory cells in the tubules and the cells lining the ducts is similar (Marshall and Saddlier, 1989) so it is reasonable that the relative number of these cells is proportional to the relative surface areas. Thus if cells surrounding ducts and central canals are responsible for producing the concentrated secretion they must work and consume oxygen at a rate about 150 times that of principle secretory cells proposed in Gerstberger and Gray's model. From the measured rate of the whole gland, the oxygen consumption rate of the cells around collecting ducts would be approximately 4400 μmol O_2.g wet weight^{-1}.hr^{-1}. This assumes that the salt is concentrated from the blood to the secreted fluid by a factor of six times, with the secretory cells of the lobules performing one sixth of the total work by producing an initial isosmotic solution and all non-osmoregulatory cells in the gland have a comparatively low rate of oxygen consumption. So while the total oxygen consumption of the gland is the same in both models, there is a difference in the relative activity of cell types. The model of Gerstberger and Gray relies on a large number of cells which comprise the majority of the salt gland tissue working at a certain rate, while the model of Marshall relies on cells which comprise a small fraction of the salt gland working at a greatly higher rate. Thus the measured rate of total oxygen consumption by the gland tends to support the model of ionic transport of Gerstberger and Gray, as the model of Marshall requires an extremely high rate of oxygen consumption by cells surrounding collecting ducts, particularly for tissue from an ectothermic animal. Theoretically Marshall's model could occur, but it seems more probable that a distribution

of osmoregulatory work between a large number of cells (with each cell consequently working at a lower rate) would be most likely.

In view of the potential limitations of the experimental technique used in these experiments, the question arises of what may be a more suitable method to employ in the future. In order to determine that ion transport is actually taking place, a modification of cell culture techniques already developed (Lowy *et al.*, 1989) will be useful. By modifying the technique using a cultured cell monolayer to simultaneously measure ion concentration and oxygen consumption on both the basal and apical sides of the monolayer it will be possible to detect the transport of ions and the oxygen consumption associated with it. Thus any question about the relationship between respiration and energy-dependent ion transport can be resolved. In addition, modifiers of gland activity can be examined more closely to determine if they influence ion transport without affecting oxygen consumption, or vice versa by coupling or uncoupling the link. However, the question of overcoming any effect of isolating the salt gland from modifiers *in vitro* is more problematical. This would require development of some technique which can measure localised respiration of the gland *in vivo*, preferably simultaneously with measurement of other parameters such as blood flow and secretory rate. One alternative method this may be achieved is to measure oxygen and salt concentration of blood in the main vessels entering the salt gland and comparing this to the oxygen and salt concentration of blood leaving the salt gland. Thus the data to be gained from conventional measurements of oxygen consumption *in vitro* using the oxygen electrode technique have limitations in relating measurements and future experiments may be more informative if cultured cell monolayers or other techniques are used.

In summary, the hypotheses proposed were not supported by data within experimental limitations. The oxygen consumption of tissue slices from active and inactive glands did not differ and modifiers of gland secretory activity *in vivo* did not change the rate of oxygen consumption *in vitro*. It is possible that this occurred because secretory cells respired at maximum rate in the presence of a high oxygen concentration and inhibitors of gland activity do not affect secretory cells. It is also possible that the link between respiration and ion transport can be uncoupled and that the secretory modifiers acted on this link rather than oxygen consumption *per se*.

However, the proposed secretory model (Figure 1.5) is not directly supported in identifying the secretory cells of the tubules and the ducts as a target of regulators of gland activity.

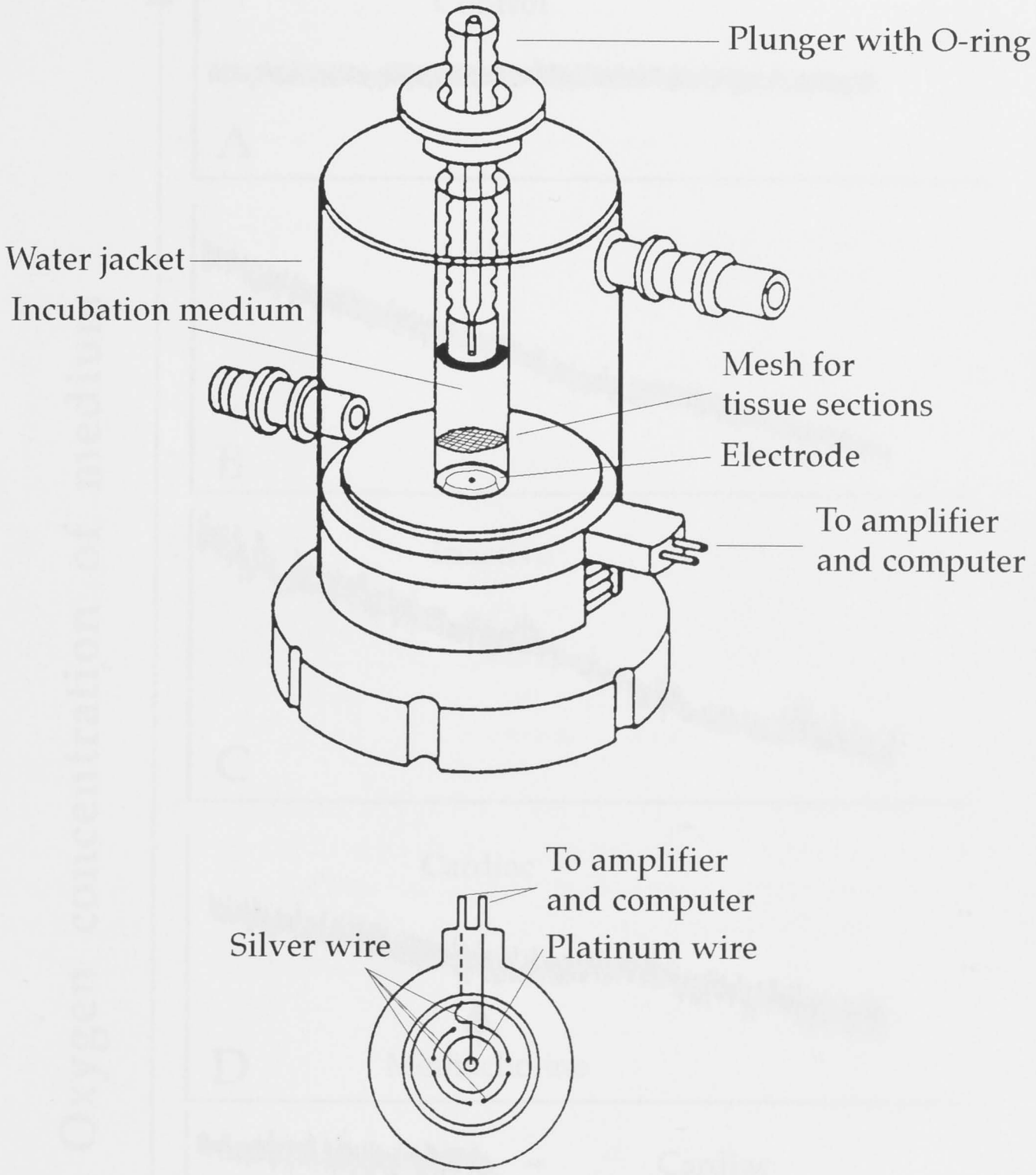


Figure 5.1
Incubation chamber and oxygen electrode for recording of oxygen consumption by salt gland tissue slices. The incubation medium is saturated with oxygen and the chamber sealed with the plunger after tissue slices are placed on the mesh. The water jacket maintains the incubation medium at a constant temperature. A stirrer lies between the electrode and the mesh but for clarity this is not shown. A KCl bridge joins the platinum and silver wires in the diagram of the electrode. The current flowing between the silver anode and platinum cathode of the oxygen electrode is proportional to the oxygen concentration in the incubation medium. As the oxygen concentration falls in the incubation medium, the current measured by the electrode also falls and this is converted to a potential voltage and recorded by computer.

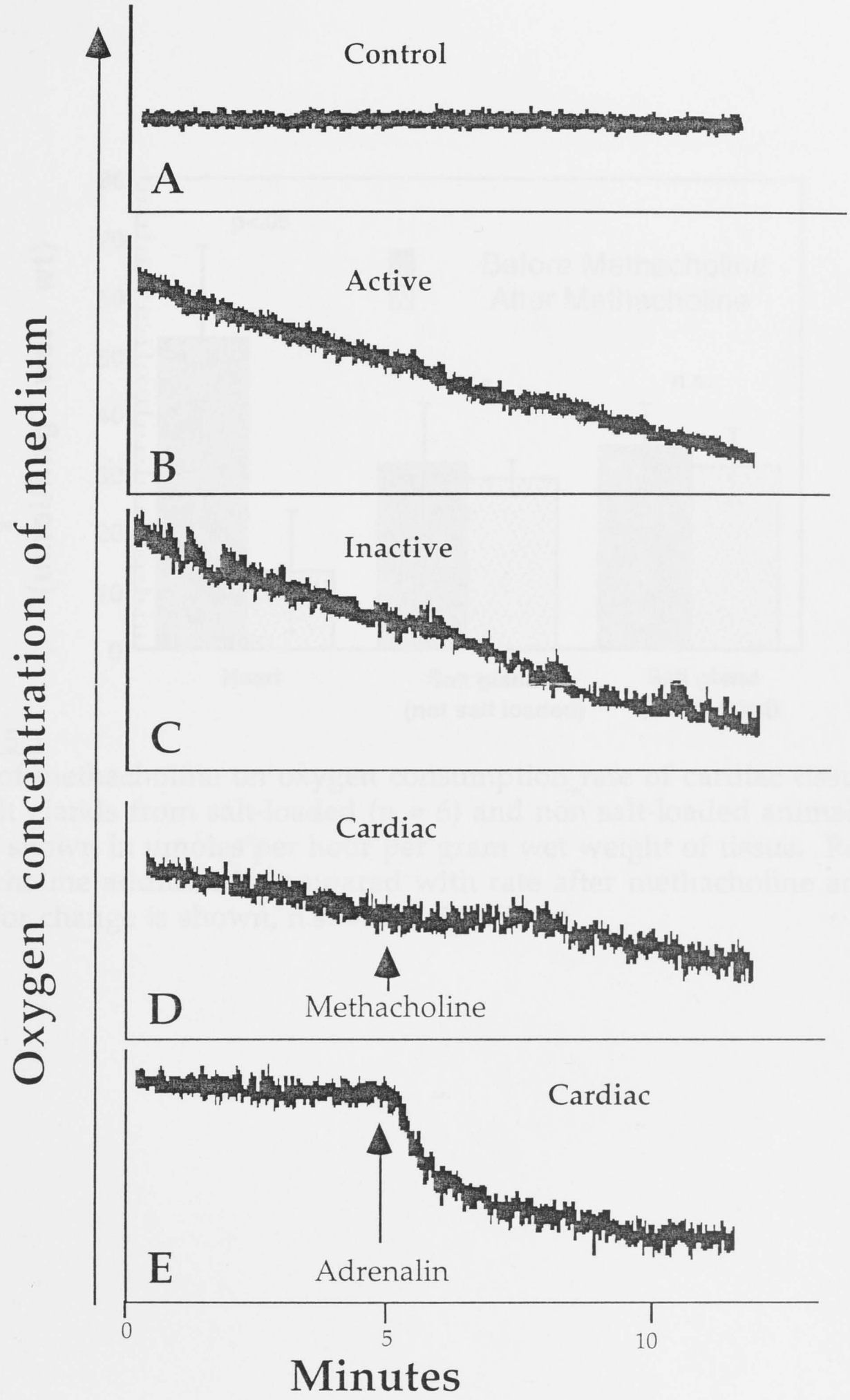


Figure 5.2

Sample voltage traces indicating the oxygen consumption of different tissues. A fall in the voltage trace indicates a fall in the oxygen concentration of the incubation medium and hence the rate of consumption by the tissue can be calculated.

A) Empty control chamber.
B) Salt loaded (active) salt gland.
C) Non salt loaded (inactive) salt gland.
D) Cardiac tissue treated with methacholine 2.6×10^{-4}M.
E) Cardiac tissue treated with adrenalin 1.4×10^{-5} M.

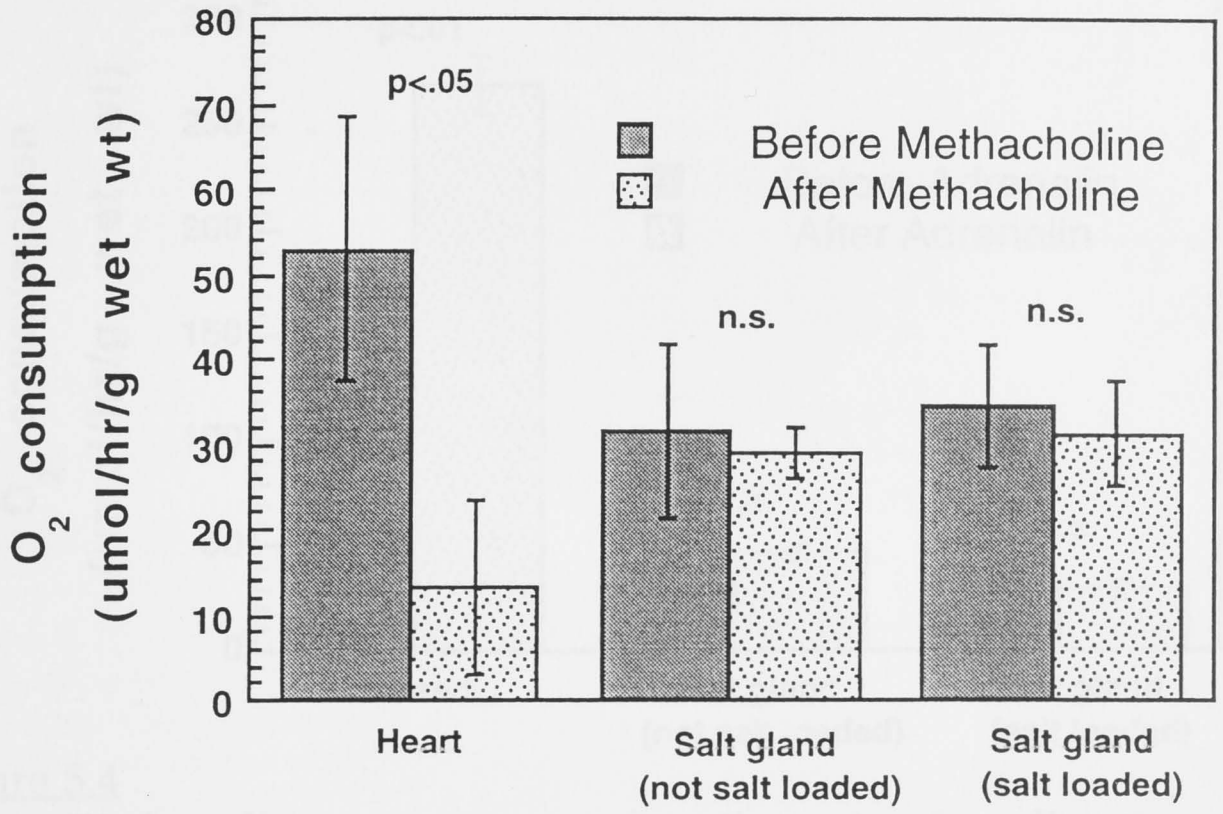


Figure 5.3
Effect of methacholine on oxygen consumption rate of cardiac tissue (n = 3) and salt glands from salt-loaded (n = 6) and non salt-loaded animals (n = 4). Rate is shown in μmoles per hour per gram wet weight of tissue. Rate before methacholine addition is compared with rate after methacholine addition, p value for change is shown, n.s. = not significant.

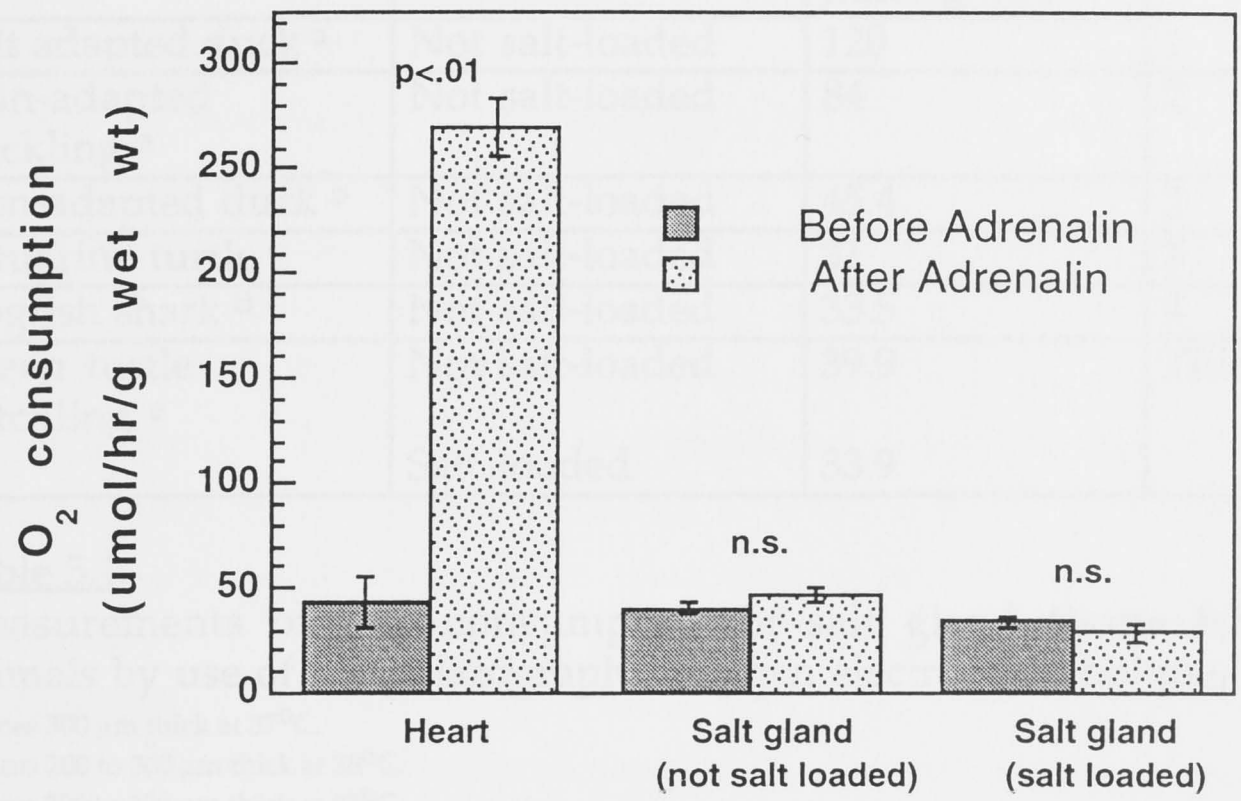


Figure 5.4
Effect of adrenalin on oxygen consumption rate of cardiac tissue (n = 3) and salt glands from salt-loaded (n = 12) and non salt-loaded animals (n = 8). Rate is shown in μmoles per minute per gram wet weight of tissue. Rate before adrenalin addition is compared with rate after adrenalin addition, p value for change is shown, n.s. = not significant.

Animal	Treatment	Oxygen consumption $\mu mol.g^{-1}.hr^{-1}$	Source
Salt adapted duck [a]	Not salt-loaded	120	1
Non-adapted duckling [a]	Not salt-loaded	84	2
Non-adapted duck [b]	Not salt-loaded	45.4	3
Estuarine turtle [c]	Not salt-loaded	31	3
Dogfish shark [d]	Not salt-loaded	33.5	4
Green turtle hatchling [e]	Not salt-loaded Salt-loaded	39.9 33.9	This study

Table 5.1
Measurements oxygen consumption of salt gland tissue from different animals by use of the polarographic oxygen electrode technique.

[a] Slices 300 µm thick at 37°C.
[b] Slices 200 to 300 µm thick at 38°C.
[c] Slices 200 to 300 µm thick at 23°C.
[d] Separated tubules at 25°C.
[e] Slices 200 µm thick at 28°C.

Sources: (1) (Stewart *et al.*, 1979), (2) (Lingham *et al.*, 1980), (3) (Shuttleworth and Thompson, 1987), (4) (Silva *et al.*, 1993)

CHAPTER 6
General Discussion

6.1 INTRODUCTION

The aims of this study were to investigate the control of salt gland secretion in the hatchling green sea turtle, *Chelonia mydas* and to test a model proposed to explain its regulation. Experiments were conducted to investigate the ability of various modifiers to influence activity of the gland under a number of experimental conditions, as well as measurements of blood flow and oxygen consumption. In addition, histological, immunohistochemical and morphometric evidence was collected to test the model. In this chapter the results of these experiments are summarised and the implications of these results on the regulation of salt gland secretion are discussed. Some general implications of comparative extra-renal osmoregulatory control are considered and future directions of research suggested. The secretory model presented in Chapter 1 is re-examined in light of experimental data, and revised to reflect the results obtained. A model is proposed based on the results obtained in this study which explains regulation of hatchling turtle salt gland activity through adrenergic and cholinergic nerves. Blood flow through the salt gland is identified as an important regulatory site which controls the supply of oxygen and salt to secretory cells. Two alternative models are also proposed, based on speculation about possible alternatives which require further experiments to test. It is suggested that the secretory cells themselves may also be a site of neural control, which was not apparent under the conditions of the experiments.

6.2 SUMMARY OF RESULTS

6.2.1 Characteristics of salt gland function

The lachrymal salt gland of hatchling green sea turtles produced tears of concentrated sodium chloride about 10 minutes after a salt-load was administered. The threshold salt-load was 400 - 600 µmol NaCl.100g BW^{-1}. Tear concentration changed little during periods of secretion and contained 800 - 900 $mmol.l^{-1}$ sodium and chloride, with the rate of sodium removal apparently largely dependent on the rate of tear production as this showed more variation. Sodium removal rate averaged 814 ± 3.7 µmol Na.100g $BW^{-1}.hr^{-1}$, assuming both salt glands secreted at approximately the same rate. With only slight variations in the concentration and rate of production, the salt gland appeared to respond in an "all or nothing" manner, with secretory rate

remaining high during periods of activity. Both plasma osmolarity and plasma sodium concentration increased following salt-loading, although less than 20% of the injected sodium was detectable in the plasma during the first hour. These parameters then fell as secretion of salt through the gland progressed. It was estimated that removal of a salt-load of 2700 μmol NaCl.100g BW^{-1} would take approximately four hours. Hematocrit changes were observed following salt-loading, but it seems unlikely that they influenced the secretion of salt.

6.2.2 Influence of possible modifiers of salt gland activity

A number of possible modifiers of salt gland activity were examined for their ability to influence the secretory response of the salt gland. Tear concentration and rate of tear production following administration of various chemicals were compared with those found in their absence. Methacholine and adrenalin completely abolished the secretory response of the salt gland when they were administered after secretion had been initiated by a salt-load. The duration of inhibition increased with increasing dose. When administered simultaneously with a salt-load, both delayed the onset of secretion in a dose dependent manner. Atropine stimulated a brief secretory response by the salt gland when administered with a sub-threshold salt-load although the rate of secretion was lower than normal. The peptide AVT transiently reduced secretion from the gland when administered after secretion had been initiated by a salt-load. Molsidomine (an NO donor), ANP, VIP and NPY did not influence the salt gland under the conditions of the experiments. The data showed that activity of the salt gland could be modified by application of exogenous chemicals. It was inferred that exogenous application of these chemicals was mimicking their release *in vivo*. Immunohistochemical technique verified histochemical identification of adrenergic nerves from an earlier study, indicating that the influence of adrenalin *in vivo* is probably through release from neural stores within the gland rather than from some remote site. However, the presence of cholinergic nerves is not unequivocal, as circumstantial histochemical evidence exists but immunohistochemical evidence does not.

6.2.3 Salt gland histology, morphometry and blood flow

The histology of the salt gland from hatchling green turtles showed the characteristic arrangement of secretory tubules and lobes as seen in salt glands from other animals. Tubules were composed of radially arranged secretory cells with a central lumen which drained into a central canal in each lobe. Three dimensional reconstruction of serial sections showed a branching arrangement of central canals in lobes entering the main collecting duct at the

posterior of the gland. Secretory tubule vasculature differed between active and inactive glands when measured using morphometric technique. The percentage of total lobe volume occupied by blood vessels as indicated by the presence of red blood cells was about 20 times greater in an active gland than in an inactive gland. The volume of tubule lumens did not differ with gland activity and central canal volume was slightly higher in the active gland. The difference in blood vessel volume with gland secretory activity provided supporting evidence for the identification of regulation of blood circulation proposed in the secretory model. This evidence was quantified by measuring the circulation of blood through the salt gland using coloured microspheres which are trapped in the capillaries. The concentration of spheres trapped per gram of tissue was compared between salt glands under different experimental conditions as well as cardiac and pectoral muscle. Capillary blood flow was inferred from microsphere concentration and so circulatory changes in the tissues detected. Salt gland circulation changed significantly in active salt glands following salt-loading compared to inactive glands, with capillary blood flow much higher in active glands. Capillary blood flow following salt-loading was unchanged in cardiac and pectoral muscle compared to control animals. The administration of methacholine to salt-loaded animals resulted in a circulatory change to reduce salt gland capillary blood flow compared to salt-loaded animals which did not receive methacholine. However, pectoral and cardiac muscle circulatory changes also occurred which suggested that the change in the salt gland following methacholine injection may have been due to a reduction in systemic blood flow. It was also suggested that exogenous methacholine was having an unrelated or pharmacological influence on a rate-determining step of blood supply to the gland. Adrenalin also influenced salt gland circulation to reduce capillary blood flow when administered after a salt-load, while it increased capillary blood flow in cardiac and pectoral muscle. Atropine was administered in the absence of a salt-load and changed salt gland blood circulation to greatly increase capillary blood flow when compared to control animals. This suggested that atropine was either abolishing a tonic, localised cholinergic inhibition of blood flow in the gland, or was increasing blood flow at the rate-determining step. The proposed secretory model identified salt gland circulation as being a possible site of control and this was supported by the experimental data. The influences on local circulation of the chemicals examined were consistent with their action on salt gland secretion, indicating that at least part of their influence was exerted in this way.

6.2.4 Oxygen consumption of salt gland tissue

The polarographic electrode technique was used to measure the oxygen consumption of salt gland slices as an indicator of ionic transport activity. There was no difference in the oxygen consumption of salt glands taken from animals which were actively secreting following a salt-load compared to those which were not secreting. The measured oxygen consumption rate of about 35 μmol.g wet weight^{-1}.hr^{-1} was sufficient to meet the calculated minimum energy requirements for ionic transport *in vivo*. Addition of methacholine or adrenalin to the incubation medium did not change the oxygen consumption of salt gland but did influence the respiration rate of cardiac tissue. The data suggested that the inhibitory action of these chemicals was not mediated through a change in the ion transport activity of secretory cells, unless this was not apparent in the *in vitro* preparation. This may be the case if modifiers affect ion transport through intermediate factors only present *in vivo*, or if the link between respiration and ion transport can be coupled and uncoupled.

6.3 RESTING BLOOD FLOW AND ACCESS OF HORMONES OR INJECTED CHEMICALS

The blood flow of the inactive salt gland is very low. Endogenous or injected chemicals may not reach the gland itself when it is not secreting because they rely on transport via the blood to their site of activity. This infers that the stimulation of the salt gland is not via the systemic release of hormones or other chemicals but is through a localised release from nerves at their site of activity. Thus exogenous compounds with a stimulatory influence may not show any effect in activating an inactive gland when they are injected because they cannot reach it. This may explain why VIP did not initiate secretion. However, chemicals were also injected while the gland was active and consequently blood flow was high but they did not influence the rate or concentration of secretion. In the avian salt gland, injection of stimulatory chemicals when the salt gland is active will increase the rate of secretion (Gerstberger *et al.*, 1988), a result not seen in this study. It is possible though that when active the hatchling turtle salt gland is secreting at the maximal rate and cannot be stimulated further. Perhaps the application of atropine to stimulate a sub-threshold salt gland blood flow followed by the application of the chemical of interest will induce a suitable condition for a stimulatory effect to be seen.

6.4 SUBTHRESHOLD BLOOD FLOW

Capillary blood flow through the salt gland inferred from microsphere entrapment can be elevated significantly above control levels without tears

being produced. As was seen with the injection of atropine *in vivo*, a simultaneous subthreshold salt-load was necessary for the secretory response. However, atropine was shown to significantly increase microsphere entrapment in the absence of a salt-load. It was presumed that this was due to atropine blocking cholinergic inhibition of blood flow, but the microsphere entrapment was less than that measured following a salt-load. This would suggest that there was an atropine-resistant vasoconstriction within the gland, most likely those vessels controlled by adrenergic nerves. So even though capillary blood flow was elevated, tears were not produced. The addition of salt may further stimulate whatever mechanism increases blood flow to result in tear formation. The presence of elevated plasma sodium is probably also necessary for the production of tears. Secretion may not occur if sodium is not above a certain concentration for transport, regardless of capillary blood flow. Blood pressure within salt gland capillaries may be important in order to provide a force to drive fluid into or between secretory cells through a hydrostatic mechanism. It would be interesting to experimentally manipulate these parameters to determine their importance in the blood flow/secretion relationship.

6.5 BLOOD FLOW AND OXYGEN CONSUMPTION

Morphometric examination showed that the active salt gland had a greater capillary volume than the inactive salt gland, when tissues were collected under appropriate conditions. The differences in circulation between salt glands in these two states was quantified in experiments using coloured microspheres. When measured by this method, the circulation of blood through salt gland capillaries when the gland is inactive was extremely low in comparison to other tissues. The massive increase which follows salt-loading is associated with secretory activity of the gland and serves to deliver salt and oxygen to the secretory cells. Measurements were made of oxygen consumption of salt glands taken from animals which were secreting as well as those which were not, but no significant difference was detected. The assumption was made that oxygen consumption indicated the rate of energy-dependent ion transport, so it was inferred that ion transport occurred at the same rate *in vitro* regardless of whether the gland was active *in vivo* or not. The possibility exists that respiration and ion transport can be uncoupled so that while the measured oxygen consumption rate is sufficient to support the observed ion transport *in vivo* ion transport does not actually occur. However, for the purposes of a consideration of whether measured respiration was from an active gland or an inactive gland, we can consider the gland to be *potentially*

active if respiration is sufficient to meet the requirements of ion transport. In the consideration of blood supply and activity, a potentially active gland will have the same requirements as an actually active gland, so the two can be considered together, and are commonly referred to as being "active" in this section of the discussion So when considering the experimental data the question arises as to whether the rate of respiration measured was that of active glands actually (or potentially) transporting ions, or inactive glands carrying on non-osmoregulatory metabolism. A consideration of the observed changes in salt gland capillary blood flow detected using microspheres following salt-loading clarifies this question.

The rate of blood flow required to supply the salt gland with the oxygen necessary to fuel the measured rate of oxygen consumption can be estimated and from this I can infer capillary blood flow rate in active and inactive glands. If I assume that the respiration rate measured was that of an active salt gland, then I can estimate the blood flow required to support it and compare this with the results of microsphere entrapment experiments. It is possible to make some estimates of capillary blood flow rate through the turtle salt gland based on assumptions about oxygen carrying capacity and efficiency of extraction. Assuming that blood carries about 20% oxygen (Eckert *et al.*, 1988) or about 9 $\mu mol.ml^{-1}$, then the salt gland needs around 64 $\mu l.g$ wet $weight^{-1}.min^{-1}$ of blood to supply the measured respiration rate of 35 $\mu mol.g$ wet $weight^{-1}.hr^{-1}$ (0.58 $\mu mol.g$ wet $weight^{-1}.min^{-1}$). The efficiency of oxygen extraction by the salt gland tissue is likely to be about 25% (Peaker and Linzell, 1975), so the requirement for blood flow is therefore 256 $\mu l.g$ wet $weight^{-1}.min^{-1}$. This is considerably less than the blood flow measured in the Pekin duck of 12.5 $ml.g$ wet $weight^{-1}.min^{-1}$ (Kaul *et al.*, 1983) but is offset by differences in the blood volume and relative salt gland mass in these animals. The salt glands are proportionally much larger in the hatchling turtle, approximately 0.26% of body mass compared to 0.047% body mass (Kaul *et al.*, 1983) in the salt adapted duck. Assuming a blood volume in the turtles of about 15% of body mass (Thorson, 1968) the salt glands of hatchling turtles require 5.7% of the total blood volume.g wet $weight^{-1}.min^{-1}$ calculated on the basis of the blood required to supply the measured oxygen consumption above. In comparison, the active salt glands of the duck require approximately 3.6% of the total blood volume.g wet $weight^{-1}.min^{-1}$ calculated from the rate of blood flow, body mass and salt gland mass measured by Kaul *et al.* (1983) with the total blood volume being a similar proportion of body mass to the turtle. Thus although the volume of blood flow per gram of salt gland mass is lower in the turtle than the bird, the

proportion of the total blood volume and the secretory rate per unit body mass is very similar. The net result is that the salt glands of the turtle have a lower rate of blood flow per gram of salt gland mass fuelling a lower rate of oxygen-consuming ion transport. However, the rate of secretion as a percentage of *body mass* is similar in the two animals, because in the turtle the glands are proportionally larger, requiring the same percentage of total blood per unit time, although they are less active in terms of output per unit *salt gland mass*. Thus the blood flow requirements calculated from oxygen consumption of the turtle salt gland are consistent with observations of blood flow in the active salt gland of the duck.

To return to the question of whether the rate of oxygen consumption measured in Chapter 5 was that of active glands, it appears that this is indeed the case, as the proportion of total blood required to support that rate *in vivo* is the same as that measured in active glands of the duck. The difference in capillary blood flow in active and inactive glands inferred from microsphere entrapment was approximately 180 fold. Thus if the rate of blood flow estimated above was from oxygen consumption of an inactive gland, the rate of blood flow in an active gland should also be 180 fold greater, or over 1000% of total blood volume.g wet weight^{-1}.min^{-1}. The salt glands of a 30 g hatchling turtle weigh approximately 80 mg and would therefore receive over 80% of the total body blood volume through their capillaries each minute. This is equal to 6 mls per minute, or greater than the combined weight of the salt glands every second. Such an enormous rate of blood flow is extremely unlikely, further supporting the conclusion that the oxygen consumption rate measured and blood flow calculated were of active salt glands from the hatchling turtle. Furthermore, this indicates that the rates of oxygen consumption measured of avian tissue are not truly indicative of active salt glands as they do not match the rate expected from the flow of blood per gram of salt gland tissue. In order for this to be the case, the efficiency of oxygen extraction from the blood by the avian salt gland would be extremely low, in the order of 2%. The measurements of avian salt gland respiration were taken from birds which had not been salt-loaded and so presumably were not secreting, so it is not unexpected that the rates observed were of inactive glands. Indeed, Borut and Schmidt-Nielsen (1963) measured respiration of salt gland slices from the herring gull which was not salt-loaded and concluded that the rate they detected *in vitro* was not capable of supplying the energy required for the secretion rates observed following salt-loading *in vivo*. Following from this there must be an intrinsic difference in the regulation of avian and chelonian salt gland activity so that

turtle salt glands become active (or potentially active) in terms of respiration rate *in vitro*, while avian salt glands remain inactive *in vitro*. Methacholine increased respiration rate of avian salt gland tissue in experiments, but only by about 80% (Borut and Schmidt-Nielsen, 1963; Hootman and Ernst, 1982) and this change is not great enough to require the rate of blood flow measured in the avian salt gland *in vivo*. Thus regardless of whether the hatchling turtle salt glands were actually transporting ions or not, the measured rate of oxygen consumption and the inferred blood flow was not that of inactive glands and was sufficient to supply the requirements of active glands. It was not possible to tell if the glands were transporting ions, but they were theoretically capable of doing so if one accepts that respiration and ion transport can be uncoupled. The assumption of respiration experiments of ion transporting tissues is that uncoupling does not occur, but it remains a possibility.

6.6 OXYGEN AS A SECRETORY MODIFIER

The results of oxygen consumption and blood flow experiments discussed above suggest an interesting possibility of secretory cell regulation. While oxygen consumption experiments certainly isolate the gland from endogenous inhibitory modifiers such as nerves, they also put the salt gland tissue in an oxygen-saturated environment. Some animals are oxygen conformers (Withers, 1992) with their metabolic activity modified by the available oxygen concentration. It is intriguing to speculate that a similar situation may occur in the salt gland of the green turtle hatchling. When respiration is measured *in vitro* it may be that the absence of any oxygen limitation allows the tissue to carry on metabolic activity at its maximum rate, potentially including ion transport. We know that the supply of blood to the secretory cells is regulated, so in this way the supply of oxygen is regulated as well. The inactive salt gland has a very low capillary blood flow compared to pectoral muscle, so the availability of oxygen to cells of the inactive gland is also very low. However, following salt-loading, the circulation changes to greatly increase capillary blood flow and hence the supply of oxygen and salt. Perhaps in the presence of increased oxygen availability the secretory cells are stimulated to respire more quickly and consequently transport the salt which is also available. Such a system of control can be regulated entirely by changes in blood circulation, and is consistent with the suggestion that modifiers do not change the respiration of salt gland tissue in the presence of an oxygen saturated environment unless respiration and transport are uncoupled.

6.7 PEPTIDERGIC CONTROL

There is clear evidence of peptidergic innervation and regulation of the elasmobranch rectal gland (Stoff *et al.*, 1979; Chipkin *et al.*, 1988) and this animal would be considered an earlier evolutionary development than the turtle. It is therefore surprising that peptidergic mechanisms of salt gland regulation were not detected in the turtle. However, the rectal gland of the elasmobranch and the lachrymal gland of the turtle have obviously evolved independently, so it cannot be assumed that their control mechanisms will be the same. Nevertheless the failure to detect peptidergic control of the chelonian salt gland does not preclude its presence, as experiments may not be using the right peptides, or not under the right conditions. It has been mentioned also that low blood flow in the inactive gland may prevent exogenous peptides from reaching their target.

6.8 A SECRETORY MODEL BASED ON THE RESULTS OF THIS STUDY

The secretory model presented in the General Introduction (Figure 1.5) needs revision now that the components of salt gland activity are better understood and to incorporate the experimental data obtained. The effect on the secretory model of modifiers examined is presented in Table 6.1. A model is presented which explains salt gland regulation through the activity of nerves. This is for two reasons, 1) the response of the salt gland to salt and other active chemicals is extremely rapid and 2) the results of microsphere experiments show that the blood flow through the inactive gland is extremely low so that hormones or other substances released into the blood will have great difficulty in reaching the salt gland itself. This second point suggests very strongly that any substances affecting the blood flow or cellular activity of the salt gland are locally released from nerve endings in or close to the gland. The model also identifies that blood supply to the gland and its secretory cells is a target of regulatory modifiers. This is based on the clear evidence that the active gland has a greater capillary blood flow than the inactive gland and that blood flow can be modified experimentally. The model shows a system of control through dual inhibitory innervation by adrenergic and cholinergic nerves. The influence of AVT on salt gland secretion is not included in this or the alternative models as it is necessary to determine precisely where AVT is binding, to know whether it has a direct or indirect effect.

6.8.1 Model 1 - Inhibitory cholinergic and adrenergic innervation

Exogenous methacholine and adrenalin both inhibit secretion by the salt gland and reduce blood flow through salt gland capillaries. In the model (Figure 6.1)

it is assumed that adrenalin and methacholine are mimicking the action of adrenergic and cholinergic nerves in salt gland tissue. Thus, both sympathetic and parasympathetic nerves inhibit the activity of the salt gland, rather than having the antagonistic effects typically associated with exocrine glands and seen in the avian salt gland. In the model, elevated sodium is detected by the central receptor which inhibits both adrenergic and cholinergic nerves in the salt gland. These nerves act to restrict blood flow to the gland and so when they are inhibited the supply of salt and oxygen to secretory cells by the blood is increased and secretion results. The model suggests that the salt gland is subject to a system of inhibitory control, with activity resulting when the inhibition is absent rather than becoming active following stimulation from an inactive state. It is possible also that cholinergic nerves influence the activity of the secretory cells directly, as reported in comparative studies (Borut and Schmidt-Nielsen, 1963; Stewart *et al.*, 1979; Hootman and Ernst, 1982), although in this case the influence is inhibitory rather than stimulatory as in those reports. Respiration of salt gland tissue did not change following methacholine administration in this study, but if methacholine acted to uncouple respiration and ion transport then its inhibitory influence would not be detected by this means. Although the underlying assumption in all experiments which measure oxygen consumption as an indicator of ion transport is that this does not occur, an experimental setup which measures respiration and ion transport simultaneously is the only way to be absolutely sure. It is necessary to identify the precise location of cholinergic nerves within the gland to determine if they contact secretory cells and so could affect them in this way.

Possibly the salt gland in hatchling turtles is active most of the time and temporarily inhibited from time to time by inhibitory nerves, rather than normally being inactive and then being switched on from time to time by stimulatory nerves. Hatchling turtles may be secreting while immersed and this has not been detected, but hatchlings removed from the water only occasionally secrete spontaneously (personal observation). The results of adrenalin administration suggest that this may be a result of being removed from the water and handled. The salt water crocodile, *Crocodilus porosus* will not secrete salt from the lingual salt glands unless it is immersed and unrestrained (Taylor *et al.*, 1995), indicating that emersion probably causes a stress response. A similar situation may occur in green turtle hatchlings with the release of adrenalin which inhibits the salt gland as a result of stress following removal from the water. This inhibition could be over-ridden by injection of a salt-load to produce the secretory response observed. It would be

interesting to determine if adrenalin also has an inhibitory influence on salt gland secretion in the crocodile.

A consideration of the salt threshold required to initiate salt gland secretion *in vivo* indicates that it is likely that the gland in hatchings is secreting a large proportion of the time. It has been calculated that the exchangeable sodium pool of hatchling green turtles is 7300 μmol.100g BW^{-1} (Kooistra and Evans, 1976), so the threshold sodium influx represents between 5.5% and 8.2% of this. An increase of 5% of the exchangeable sodium pool would occur through ingestion of only 200μl of seawater by a 25 g hatchling, irrespective of any additional salt ingested in the food. Hatchlings have been reported to drink 2-8.5 $ml.100g^{-1}.day^{-1}$ of seawater (Marshall and Cooper, 1988) and ingest 700 μmol $NaCl.100g^{-1}.day^{-1}$ in shrimp (Jones *et al.*, 1995) when maintained under conditions similar to those in this study. A rapid growth rate is important for small turtles to reduce the risk of predation and they must be able to secrete salt at a rate which permits maximum growth. At the rate of secretion measured in Chapter 2, the salt gland could secrete the salt from about 30 $ml.100g^{-1}.day^{-1}$ of seawater. So there is evidence that when hatchlings are freely swimming, feeding and drinking they ingest a large amount of salt as well as an unknown quantity which must diffuse into the body from the environment, and that the salt gland is capable of removing this quantity. Total sodium influx then represents a large percentage of the hatchling salt gland total secretory capacity. In order to remove this, the gland would have to operate a large proportion of the time to reduce internal sodium concentration, and would be inhibited from time to time when sodium falls sufficiently. In this situation, a system of control which is inhibitory rather than stimulatory is more likely to occur.

The question remains as to why both adrenergic and cholinergic elements would be present in the gland, both apparently having the same effect on gland activity. The effect of adrenalin on secretion and blood flow is in agreement with other reports (Fänge *et al.*, 1963; Peaker and Linzell, 1975), while the action of methacholine in hatchling turtles in this study is different to other animals (Fänge *et al.*, 1958a; Schmidt-Nielsen and Fänge, 1958; Taylor *et al.*, 1995). Some possible reasons for this difference have been discussed in Chapter 3, but information on sodium intake and growth rate of hatchling turtles suggests an alternative. The possibility exists that there are ontogenetic changes occurring in the control mechanisms operating in turtles of different ages. The small size and rapid growth rate of hatchling turtles means that they eat proportionally

more food and ingest proportionally more seawater than adult turtles. As turtles get larger their growth rate slows (Bjorndal and Bolten, 1988) and their energy requirements are proportionally much smaller and met by a herbivorous rather than an omnivorous diet (Bjorndal, 1980). Through a reduction in feeding and drinking requirements, their total sodium intake is probably greatly reduced. In addition, scaling of the body mass to surface area ratio means that passive entry of salt into the body is reduced as size increases. Although the secretory rate of the salt gland is allometrically related to body mass it is likely that the salt gland changes from being usually active in hatchlings to being intermittently active in adults to maintain salt balance, as is the case in marine birds. If so, a system of control which is both inhibitory and stimulatory in adults would be suitable. When salt secretion is required, the gland is stimulated by some modifier and when secretion is no longer required it is inhibited by some other modifier. In this case, the role of acetylcholine or adrenalin in the salt gland may switch from being inhibitory to being stimulatory. If acetylcholine switches to having a stimulatory effect, this would be consistent with the sympathetic/parasympathetic model of control of the avian salt gland and other exocrine glands. There is currently no experimental evidence to support this proposition but it is interesting to speculate that this may occur. The problem in obtaining adult turtles makes this difficult to examine experimentally. However, it may be that the model of antagonistic roles for cholinergic and adrenergic control of salt gland secretion in the bird simply does not apply to the chelonian salt gland. Both systems in the turtle may have an inhibitory function and stimulation of the gland occurs through an absence of inhibition or some other mechanism. One of the nerve types might exert immediate but short-lived inhibition to stop secretion after it is initiated, while the other exerts a more long term inhibitory influence during periods when secretion is not required. I am not aware of other exocrine glands controlled in this way and although such a dual inhibitory system does not match current dogma concerning regulation of secretory tissues, it is consistent with the experimental data in this thesis.

6.9 ALTERNATIVE MODELS

Two alternative models are presented which are based on the experimental data obtained but also incorporate data collected in other studies. They are also dependent on the conduct of further experiments to clarify areas of uncertainty, primarily regarding the presence of cholinergic innervation and the effect of exogenous cholinergic agonists.

6.9.1 Model 2 - Inhibitory adrenergic innervation

The innervation of the green turtle salt gland has not been unequivocally determined; although immunohistochemical technique in this study has demonstrated the presence of adrenergic nerves, the same has not been reported for cholinergic innervation. There is circumstantial evidence through the histological studies of Abel and Ellis (1966) but until a suitable antibody for the green turtle salt gland is tested, the immunohistochemical evidence is absent. Commercially available mammalian choline-acetyl transferase (ChAT) antibodies do not bind to the salt gland of the estuarine turtle (Belfry and Cowan, 1995) even though cholinergic agonists stimulate the gland both *in vivo* and *in vitro*. Mammalian ChAT antibodies do not bind to the salt gland of the duck either (Prof. M. Schemann, personal communication) so it seems that antibodies may need to be raised directly from the green turtle to definitively determine if cholinergic nerves are present in the salt glands of these animals. The second model (Figure 6.2) presents regulation of secretion whereby secretory activity of the hatchling green turtle salt gland is controlled solely by adrenergic regulation of salt gland blood supply.

Exogenous methacholine and adrenalin both inhibit the activity of the hatchling green turtle salt gland, at least part of this effect is achieved by influencing the circulation of blood so that salt gland capillaries are not filled. If the histochemical evidence of cholinergic nerves is not accepted, it follows that the influence of methacholine and atropine on the salt gland is not mimicking cholinergic nerves and so occurs for some other reason. Methacholine is known to reduce cardiac stroke rate and output (Withers, 1992) and so will reduce blood flow rate to some extent. If the changes measured in salt gland circulation following methacholine administration are due solely to this reduction, then an indirect, inhibitory effect will be seen. Cholinergic influence on the heart induces bradycardia and is involved in the diving response (Schmidt-Nielsen, 1990). If the injected methacholine stimulates a diving response, bradycardia (with consequent reduction in cardiac output) as well as a peripheral vasoconstriction will result. This may explain the inhibitory effect seen. Alternatively, if methacholine exerts an inhibitory effect on the rate-determining step of blood flow (possibly including cardiac output), then salt gland activity will also be reduced. The second model presented therefore explains the cholinergic effect seen as being due to a secondary or pharmacological response and so the regulation of the salt gland is through the action of inhibitory adrenergic nerves alone. Like the first model, this also suggests the salt gland is subject to a system of inhibitory control. A solely

inhibitory system of control was discussed with regard to the first model above, the same arguments apply here.

6.9.2 Model 3 - Inhibitory adrenergic and stimulatory cholinergic innervation

The third model (Figure 6.3) describes a system of control where the histochemical evidence for the presence of cholinergic nerves is accepted but the role of the cholinergic nerves is stimulatory. There is an antagonistic role of adrenergic and cholinergic innervation which is not apparent in experiments conducted because of the effect of exogenous methacholine described in the second model. Elevated sodium is detected by the central receptor which inhibits adrenergic nerves and stimulates cholinergic nerves. These act to restrict and increase blood flow respectively so that salt and oxygen delivery to the salt gland is controlled. In experiments conducted, methacholine caused an inhibition of secretion, this is explained by the inhibitory effect on cardiac output or on a blood flow determining step so that blood flow to the gland is reduced below threshold. The site of action of methacholine cannot be the same as the site of cholinergic innervation because of the opposite effects and it must be a rate-determining step because of the action of atropine. Atropine antagonises the cholinergic influence and increases overall salt gland blood flow but at the site of cholinergic nerves it must inhibit blood flow. By having the opposite effect to methacholine at the rate-determining step, an increase in blood flow can overcome the inhibition at the site of cholinergic nerves so that blood flow through the gland as a whole increases. In the reverse case, cholinergic nerves stimulate blood flow but because methacholine inhibits flow at the rate-determining step the total salt gland blood flow is reduced. This may provide an explanation as to why the administration of methacholine in this study differed from that in the study of Schmidt-Nielsen and Fänge (1958). It seems highly possible that the experimental animal used was dehydrated or had received an earlier salt load (Schmidt-Nielsen, personal communication), both of which are likely to result in a subthreshold blood flow. If this pre-existing stimulation is sufficient to overcome the inhibitory action of methacholine on the rate-determining step of blood flow, then the methacholine can reach the salt gland tissue. There it can mimic the action of cholinergic nerves to increase the local blood flow through capillaries and exert any possible effect on ion transport by secretory cells. Another site of cholinergic nerves may be at the secretory cells themselves, where they act to couple the processes of respiration and ion transport. Thus ion transport activity can be increased without a detectable change in oxygen consumption of secretory cells. The result is that cholinergic nerves increase salt gland blood

flow, or couple respiration and ion transport, or both. It is necessary to identify the exact location of nerves to discriminate between these two possibilities.

An antagonistic sympathetic/parasympathetic system of control is the classical scheme for an exocrine gland and matches that proposed for the avian salt gland. In this model it is proposed that the inhibitory effects of exogenous methacholine disguise the stimulatory influence of cholinergic nerves.

6.10 EARLY REPTILIAN EXTRA-RENAL OSMOREGULATION

It was mentioned in the general introduction that skull morphology identified turtles as possibly being the closest living descendants of the stem reptiles. Whilst it is difficult be confident about physiological comparisons, it seems that the control of extra-renal osmoregulation in turtles is a "simple" system, perhaps what might be expected in a primitive reptilian ancestor. It is interesting to speculate that salt gland regulation in the green turtle is similar to that of the stem reptiles and that subsequent development of extrarenal osmoregulation by more advanced lizards, snakes, crocodiles and birds relies on more complex regulatory systems.

6.11 FURTHER EXPERIMENTS

Future research should clarify the secretory models presented here. The immunohistochemical experiments required to do this have been described in the discussion of the models to identify which elements of the gland, if any, are contacted by cholinergic nerves. Other experiments have been suggested and described in the discussion of the components of salt gland activity, they are briefly summarised below; 1) It will be useful to further examine possible peptidergic influences on salt gland activity, both *in vivo* and *in vitro* under different experimental conditions. 2) Cannulating the blood vessels supplying the salt gland will permit localised, exogenous application of proposed modifiers of gland activity. Stimulating subthreshold blood flow prior to addition will resolve the question of injected chemicals reaching the gland. 3) Measurements of blood circulation through the salt gland *in situ* will provide quantitative measurements to support the inferences made based on oxygen consumption and microsphere experiments discussed above. 4) An experimental preparation which measures both respiration and ion transport activity of the gland simultaneously will conclusively demonstrate whether these two aspects of gland function can be uncoupled. 5) Experiments similar to those in this study should be conducted on green turtles of various ages, to

determine if regulation of salt gland activity changes with age and associated changes in diet.

6.12 SUMMARY

The experimental evidence presented demonstrates that salt secreting activity of the salt gland of the hatchling green turtle is regulated. Secretion commences following administration of a salt-load above a threshold and the mechanisms by which this initiation and suppression of secretion were effected were examined experimentally. A model was presented based on the experimental evidence which identifies control of blood circulation in the gland by adrenergic and cholinergic nerves as being the means by which gland activity is regulated. Two other models were suggested as alternatives which require further experiments to clarify. These differed in that the presence of cholinergic nerves is not accepted in one, and in the other the inhibitory effect of exogenous methacholine disguises the true stimulatory influence of cholinergic nerves. Measurement of oxygen consumption of the salt gland *in vitro* indicated that respiration rate of secretory cells is not modified by the exogenous application of adrenalin or a cholinergic agonist. Calculations of the oxygen requirement and blood flow through the gland show that it appears to assume a state of activity following removal from the animal, possibly due to increased oxygen availability. However, it is possible that the link between respiration and ion transport can be coupled and uncoupled so that oxygen consumption does not change following chemical administration but ionic transport is affected. It is necessary to devise a technique which simultaneously measures oxygen consumption and ion transport to determine if this is the case. Peptidergic mechanisms of control were not found, although they may yet be identified in subsequent studies. Possible reasons for different patterns of innervation of adrenergic and cholinergic nerves in the models were discussed, with the suggestion that ontogenetic changes may occur as turtles grow. The simple, neural control mechanisms proposed to operate in the hatchling turtle may reflect a similar state in extra-renal osmoregulation of early reptiles. Future experiments are suggested which will make it possible to refine and clarify the secretory models.

Table 6.1
Summary of the effects of chemicals examined on salt gland secretion and the proposed control points identified in the secretory model. "+" indicates that the chemical had a stimulatory effect, "—" indicates that the chemical had an inhibitory effect, and "0" indicates that the chemical had no measurable effect under the conditions of the experiment. A blank box indicates that the chemical was not examined in that particular experiment. Salt gland secretion was measured as the total rate of sodium removal in tears produced by the gland. Circulation changes were detected as changes in the flow of blood through capillaries of the gland. The activity of secretory tubule cells and duct cells was measured by the rate of oxygen consumption as an indicator of energy-dependent ion transport activity.

Chemical	Salt gland secretion *in vivo*	Circulation (Site 1)	Secretory tubule cells (Site 2)	Duct cells (Site 3)
Salt	+	+	0	0
Methacholine	—	—	0	0
Adrenalin	—	—	0	0
Atropine	+	+		
Molsidomine	0			
AVT	—			
ANP	0			
VIP	0			
NPY	0			

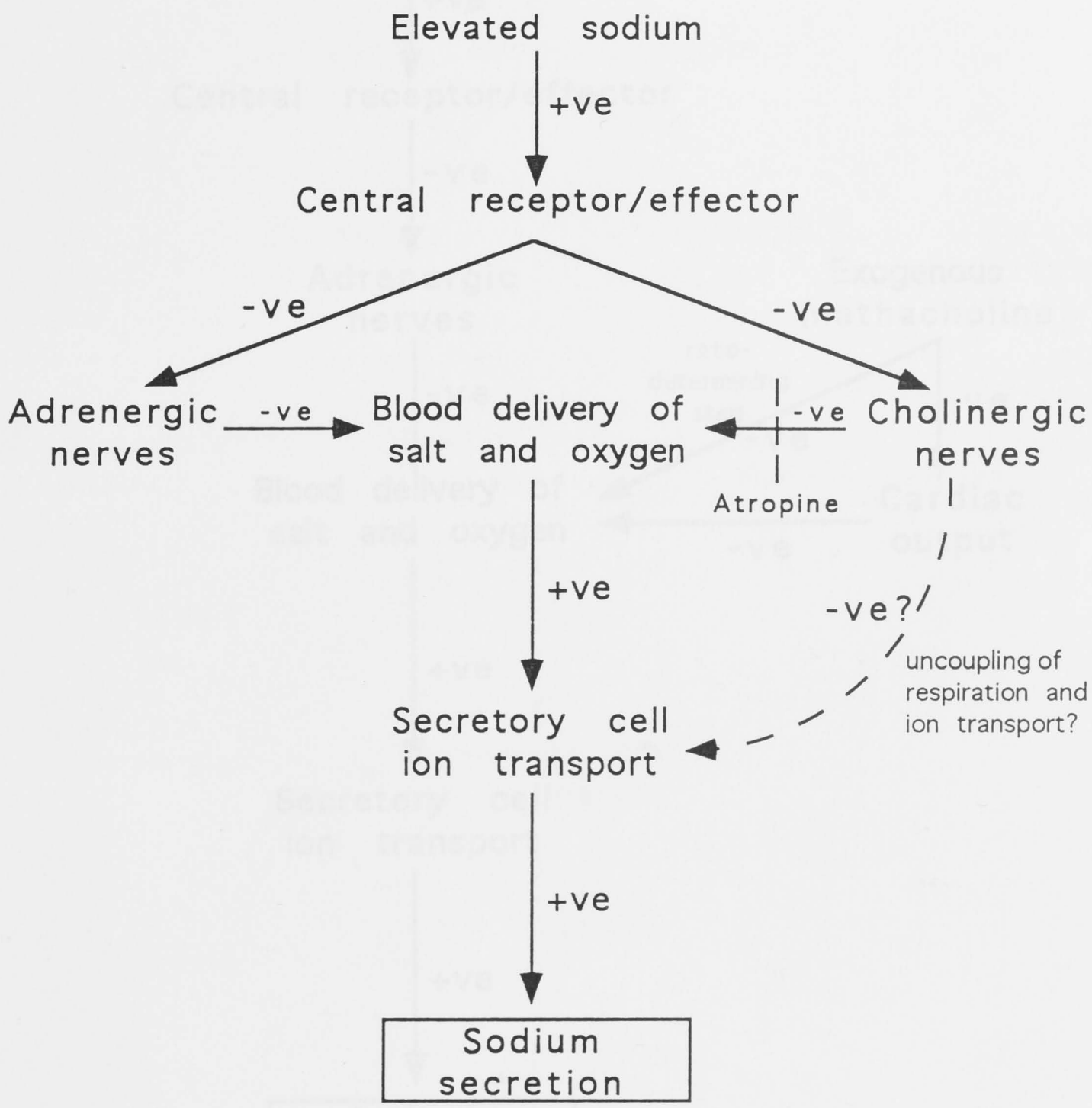


Figure 6.1
Model of salt gland regulation in the hatchling green turtle though inhibitory action of adrenergic and cholinergic nerves. Elevated sodium is detected by a central receptor which inhibits adrenergic and cholinergic nerves in the salt gland. These nerves act to restrict the flow of blood to the salt gland secretory tubules, either by affecting arterioles supplying the gland or capillaries in the gland. Thus through inhibition of the nerves, the delivery of salt and oxygen to secretory cells is increased and salt secretion by the gland results. Cholinergic nerves may also affect secretory cells directly if they act to uncouple respiration and ion transport so that although oxygen consumption is unaffected, the transport of salt is inhibited.

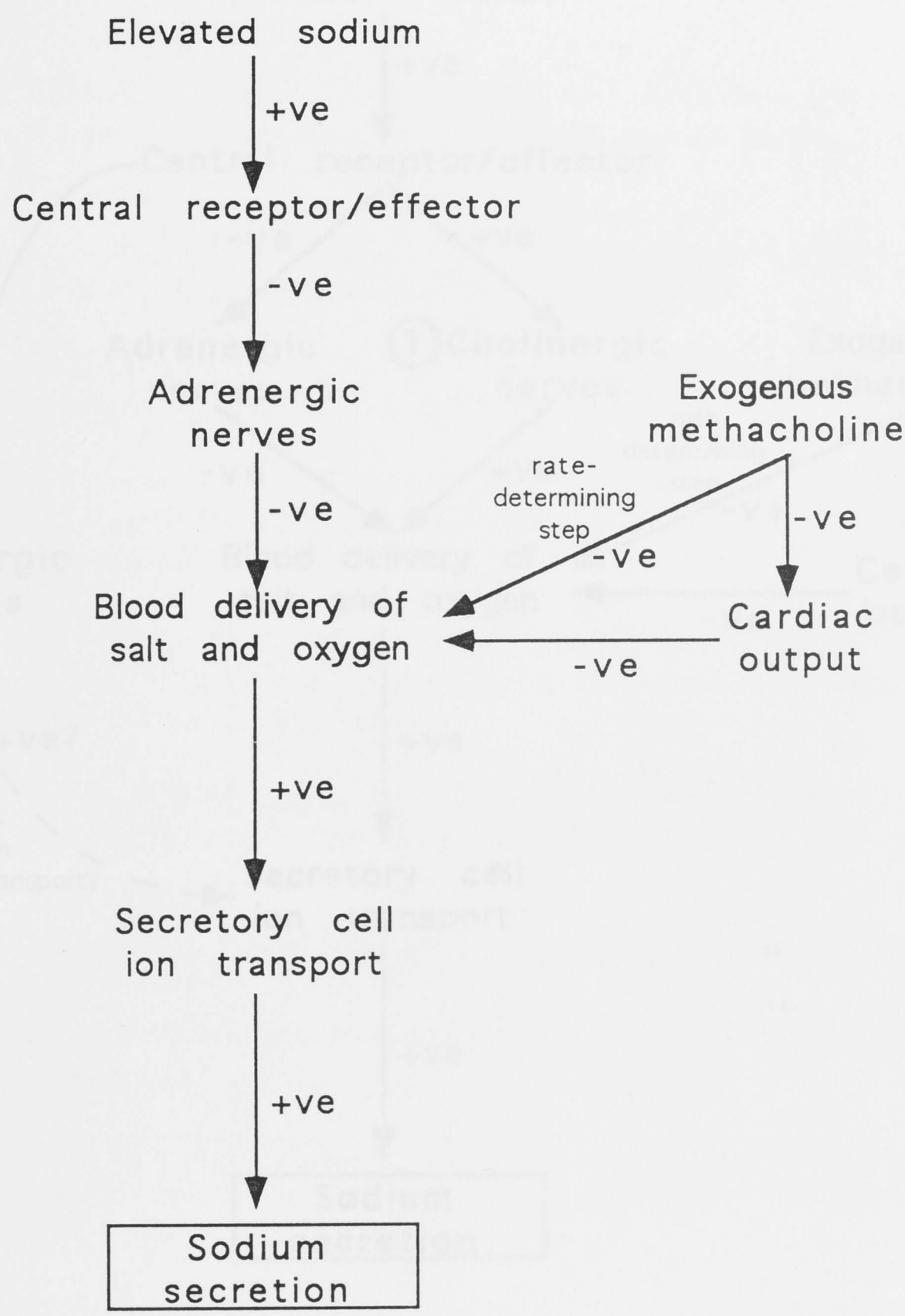


Figure 6.2
Model of salt gland regulation based on adrenergic innervation of the gland. Elevated sodium is detected by a central receptor which inhibits the activity of adrenergic nerves in the salt gland which normally restrict blood flow. In this way the delivery of salt and oxygen to secretory cells is controlled. In the absence of cholinergic nerves, it is proposed that the effect of exogenous methacholine acts to reduce salt gland blood flow through a secondary or pharmacological means. Methacholine will reduce cardiac stroke rate and output and may result in a 'dive response' resulting in peripheral vasoconstriction. Alternatively, there may be an effect on blood flow at some rate-determining step through a pharmacological rather than physiological action. It must affect a rate-determining step because atropine causes the reverse effect. The result is to restrict blood flow so that salt secretion by the gland is inhibited.

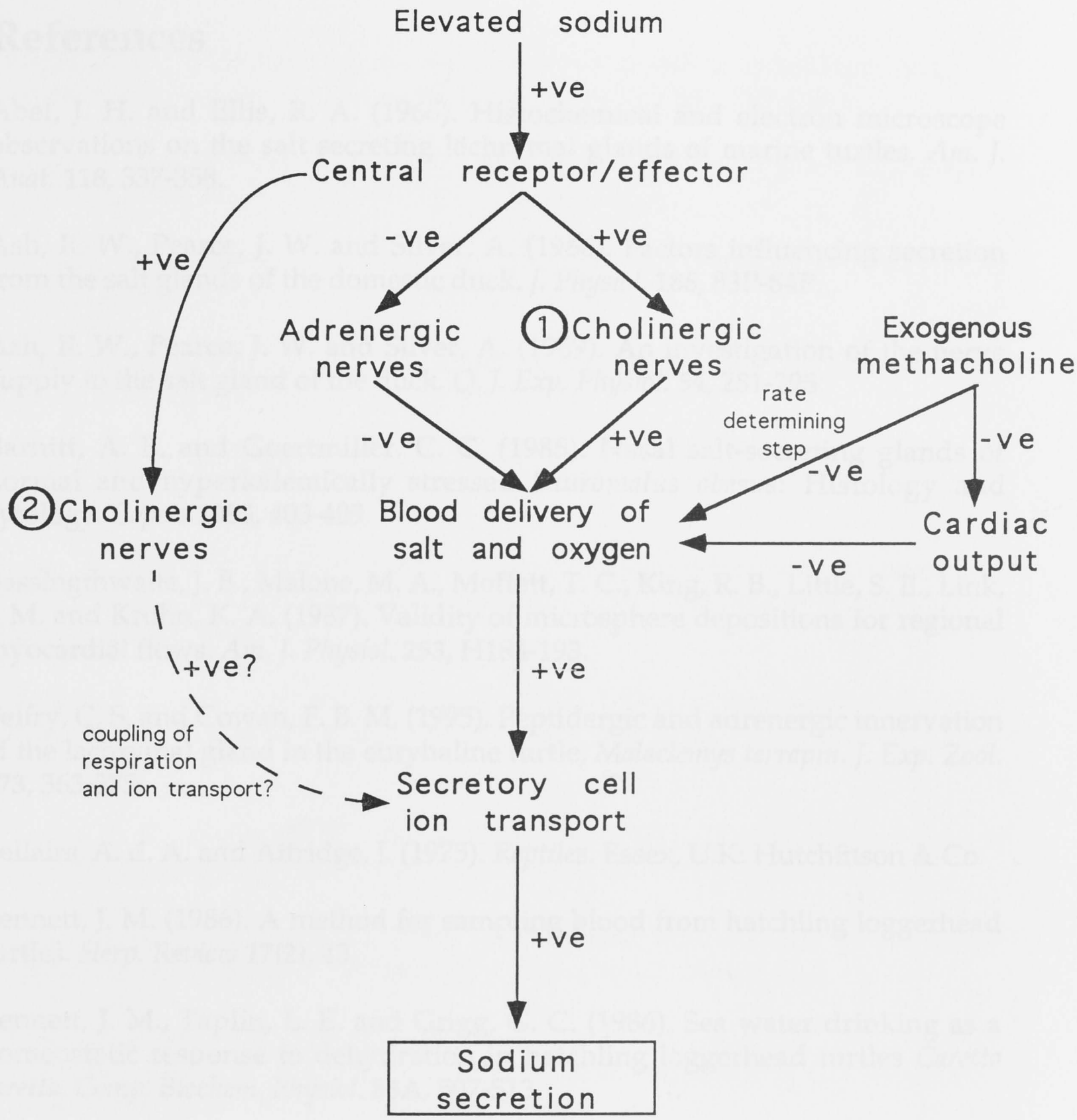


Figure 6.3
Model of salt gland regulation through inhibitory adrenergic and stimulatory cholinergic innervation. A central receptor detects elevated sodium concentration and has an inhibitory influence on adrenergic nerves and a stimulatory influence on cholinergic nerves. These nerves inhibit and stimulate salt gland blood flow respectively to control the supply of salt and oxygen to the secretory cells so that sodium secretion is regulated. There may also be an stimulatory effect on cholinergic nerves by the receptor/effector to couple respiration and ion transport of secretory cells so that salt secretion occurs without an increase in oxygen consumption. However, exogenous methacholine affects a rate-determining blood flow point to inhibit salt gland activity in the same way as described in the second model. Thus the stimulatory influence of cholinergic nerves is disguised by the inhibitory influence of the injected cholinergic agonist. It is necessary to identify precisely where cholinergic nerves terminate in order to distinguish between points 1 and 2.

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APPENDIX 1.
Recipes

ACID ALCOHOL
Dilute 700 ml 100% ethanol with 300 ml distilled H_2O.
Take 1 ml of concentrated HCl (36%) and dilute with 35 ml distilled H_2O to give 1% HCl solution.
Add 10 ml 1% HCl to 990 ml 70% ethanol.

1% ALCIAN BLUE pH 1.0
Add 10 g alcian blue powder to 1000 ml 0.1N HCl.
Shake until dissolved.

1% PERIODIC ACID
Add 10 g periodic acid to 1000 ml distilled H_2O.
Shake until dissolved.

SCOTT'S TAPWATER SUBSTITUTE
Add 2 g sodium bicarbonate or potassium bicarbonate and 20 g magnesium sulphate to 1000 ml distilled H_2O.
Shake until dissolved.

MAYER'S ACID HAEMALUM
Dissolve 50 g ammonia potassium sulphate in 700 ml distilled H_2O by heating. Add 5 g haematoxylin and dissolve, followed by 1 g sodium iodate.
Add 300 ml glycerine or glycerol, 20 ml glacial acetic acid and mix thoroughly.

SCHIFF'S REAGENT
Dissolve 5 g basic fuschin in 1000 ml boiling distilled water in a stoppered 2 litre flask.
Shake for 5 minutes.
Cool to exactly 50°C, filter and add 100 ml of 1N HCl to the filtrate.
Cool further to 25°C and add 5 g sodium or potassium metabisulphite.
Store for 18-24 hours in the dark, add 20 g activated charcoal and shake for 1 minute.
Remove charcoal by filtration and store in the dark at 0-4°C.

FAST GREEN 0.5%
Dissolve 10 g fast green in 990 ml distilled water, then add 10 ml glacial acetic acid.

APPENDIX 2.
Embedding and Staining

WAX EMBEDDING

1) 70% Ethanol	5 minutes
2) 70% Ethanol	5 minutes
3) 70% Propanol	15 minutes
4) 70% Propanol	15 minutes
5) 90% Propanol	15 minutes
6) 90% Propanol	15 minutes
7) 95% Propanol	15 minutes
8) 95% Propanol	15 minutes
9) 100% Propanol	30 minutes
10) 100% Propanol	30 minutes
11) Wax @ 60°C under vacuum	30 minutes
12) Wax @ 60°C under vacuum	60 minutes

STAINING OF WAX EMBEDDED SECTIONS

1) Wash in histolene.	7 mins
2) Rinse in 100% ethanol	20 secs
3) Rinse in 75% ethanol	20 secs
4) Rinse in 50% ethanol	20 secs
5) Rinse in distilled H_2O	20 secs
6) Second rinse in distilled H_2O	20 secs
7) Oxidise in 1% periodic acid	9 mins
8) Rinse in distilled H_2O	30 secs
9) Schiff's reagent	20 mins
10) Rinse in running H_2O	30 secs
11) 1% Alcian Blue pH 1.0	20 mins
12) Rinse in running H_2O	2 mins

Counterstain with haematoxylin

13) Mayer's acid haemalum	6 mins
14) Rinse in running H_2O	30 secs
15) 1% acid alcohol	5 secs
16) Rinse in distilled H_2O	30 secs
17) Scott's tapwater substitute	2 mins
18) Rinse in distilled H_2O	30 secs

Counterstain with fast green

19) Fast green	5 mins
20) 50% ethanol	5 mins
21) 100% ethanol	5 mins
22) 100% ethanol	5 mins

23) Mount with Colourfast and cover with coverslip.