IONIC MECHANISMS
INVOLVED IN THE SECRETION OF SWEAT.

by

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" Veil after veil will be lifted – but there must be veil after veil behind ".

Sir Edwin Arnold.

CONTENTS.

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SUMMARY.

Previous EPXMA studies on sweat glands from the horse and the human demonstrated that a fall in the intracellular concentration of K^+ occurs in response to thermal stimulation. There is no similar information on the ionic changes that occur in the sweat glands of the cow, the sheep and the goat, during thermal stress. Moreover, the patterns of sweat output exhibited by the cow, sheep and goat differ to those of the horse and the human. As a first objective sweat glands from the cow, sheep and goat were examined by electron probe x-ray microanalysis (EPXMA) to determine whether the different patterns of sweat output exhibited by these animals, could be explained by changes in the glandular intracellular concentrations of sodium, potassium and chlorine.

This study demonstrated, that upon thermal stimulation, there was a significant increase in the intracellular concentration of sodium in the secretory cells of the cow, and trends for chlorine to increase and potassium to decrease. In general the changes were qualitatively the same as those reported in the horse and human after thermal stimulation, and suggests that the cow has a similar mechanism of sweat production. No ionic changes were detected in the secretory fundus of the sheep and the goat indicating that sweat output in these animals is a slow continuous process and that the pattern of sweat output is due to the expulsion of preformed sweat by myoepithelial contraction.

Furthermore, the results of the EPXMA studies in the horse and the human contrasted with the findings of electrophysiological studies which failed to detect a K^+ efflux from sweat glands upon stimulation. A second objective therefore, was to determine by radiotracer methods if a K^+ efflux occurs in sweat glands in response to stimulation.

Radioisotopic studies demonstrated that a K^+ efflux occurs in the isolated human sweat gland in response to agonist-induced stimulation, and that the nature of the K^+ loss differs between cholinergic, α and β adrenergic agonists. The K^+ efflux in human sweat glands in response to ACh was resolved into

two phases; a transient first phase which persisted under Ca^{2+}-free conditions and a second more sustained phase dependent on extracellular Ca^{2+}. The initial phase has been attributed to a release of Ca^{2+} from bound intracellular stores and the sustained phase to an influx of Ca^{2+} into the cell.

The response to α-adrenergic agents consisted of a single phase which was sensitive to the presence of extracellular Ca^{2+}. In contrast β-adrenergic stimulation caused a slowly increasing rate of K^+ efflux, which was independent of increased intracellular Ca^{2+}.

The increase in K^+ permeability in response to ACh was further investigated using Na^+-free and Cl^--free conditions. The results of the experiments performed under Na^+-free conditions demonstrated that the first phase of the ACh-induced K^+ efflux was abolished when $NMDG^+$ was used as a Na^+ substituent while the second phase persisted. This result suggests that the ACh-induced release of bound intracellular calcium is dependent on the presence of extracellular Na^+. However, when Li^+ was used as a Na^+ replacement both phases of the response persisted. Lithium is known to support proton extrusion to a limited extent, via a Na^+-H^+ exchanger, and therefore the results of this study are consistent wth a role for the activation of Na^+-H^+ exchange in normal stimulus-secretion coupling. The experiments involving Na^+-free conditions also demonstrated that consistent responses to ACh could not be evoked in bicarbonate-free media and suggest that the human sweat gland has a requirement for the presence of bicarbonate.

The results of the Cl^- replacement experiments show that in contrast to the simian sweat gland, the ACh-induced efflux of K^+ from the isolated human sweat gland is not reduced using Cl^--free conditions. Under these conditions ACh evoked a biphasic increase in the rate of K^+ efflux. It appears that the presence of extracellular Cl^- is not a prerequisite for the ACh-evoked K^+ efflux from human sweat glands.

The results of the experiments in this thesis would suggest that no single model of the mechanisms involved in sweat production can be applied in detail to the sweat glands of different species.

CHAPTER 1.

SWEAT GLANDS.

INTRODUCTION.

Sweat glands, tubular exocrine skin glands by definition, are characteristic of mammals although they are not present in all species. They are amongst the smallest exocrine glands, typically averaging 6-8 mm in length and 40-50μm in diameter in the human. The sweat gland consists of a fundus and a duct (see figure 1.1). The fundus, which ranges in shape from a simple sac, as in some species of cattle, to the tight coil of the horse and man, is composed of a secretory epithelium surrounded by a layer of myoepithelium, a well developed basement membrane and, outermost, a fibrocyte sheath (Jenkinson,1973). The duct, which leads from the fundus to the exterior, is generally convoluted or straight and is lined with a cubical epithelium. Jenkinson (1990), following a number of studies (Bligh, 1967; Jenkinson, 1973; Montgomery, Jenkinson, Elder, Czarnecki & MacKie, 1984), has proposed descriptions of the sweat glands on the anatomical basis of whether they are associated with a hair follicle (epitrichial), or not (atrichial). The sweat glands of the general body surface of man and those of the footpads of rodents, cats and dogs are examples of the latter, and are often referred to in the literature as eccrine, whilst the sweat glands of most other mammals are epitrichial, often called apocrine in the literature.

FINE STRUCTURE.

In most mammalian sweat glands the luminal cells of the unstimulated fundus are usually all of the one type, although flattened in cattle and cuboidal in the horse and sheep. The human atrichial gland is an exception, where two secretory cell types can be identified; dark (granular) and clear (non granular), cells which occur in approximately equal numbers. The granular cells border the luminal surface of the secretory tubule and occur either as a cuboid cell which rests on the clear cells or are wedged shaped with cytoplasmic

processes extending down towards the basement membrane between two clear cells. The clear cells rest either directly on the basement membrane or on the myoepithelial cells. The basolateral membranes of the clear cells form extensive interdigitations and intercellular canaliculi are often observed between them. All the secretory cells are joined to their neighbours by tight junctions at the apical surface.

The duct of the epitrichial sweat gland has two readily distinguishable regions; an intrafollicular region and that within the dermis. The lumen of the duct within the intrafollicular region is formed from either a single cell connected to itself or a ring of two or three cells, which are lined with microvilli (Montgomery, Jenkinson & Elder, 1982a & b). The dermal region of the duct consists of two layers of epithelium surrounded by a fibrocyte sheath. The atrichial sweat gland has two equivalent zones to the intrafollicular and dermal regions of the epitrichial gland. The intradermal duct in man, has a coiled segment which consists of an inner ring of columnar cells surrounded by one or two layers of flat basal cells with an outer fibrocyte sheath.

In some species, such as the cow and sheep, the myoepithelium forms a complete layer, whereas in others such as the horse and man, the secretory cells penetrate to the basal lamina between the myoepithelial cells forming a network.

FUNCTION OF SWEAT.

Mammals which are devoid of sweat glands generally avoid extremes of temperature by moving to more acceptable environments e.g. diving by the cetacea, burrowing by rodentia and wallowing by the hippopotamus (Luck & Wright, 1964; Schmidt-Nielsen, 1964). In contrast, the profuse sweating response to exercise and thermal stimulation in the horse and human has led to the belief that sweat glands are basically thermoregulatory organs. There is a marked variation in the efficiency of these glands in the regulation of body temperature (Ingram, 1967; Hulbert & Rose, 1972; Bligh, 1967). In contrast to

the horse and the human, the glands of the pig do not respond to thermal stimulation (Ingram, 1967) and sweating in sheep has only a minor role in the maintenance of body temperature, which is regulated largely by panting (Bligh, 1967). It is apparent therefore, that sweat glands are not necessarily efficient thermoregulatory organs in many species and those in specialised regions such as the footpads, palms and eyelids which have a low output are unlikely to have such a function (Jenkinson, 1986).

Sweat glands have been shown to be situated not only in specialised regions but throughout the skin of the general body surface. The above evidence has led to the view that they have a number of possible functions which can be regarded overall as protective (Jenkinson, 1973). It has been suggested that they may 1). protect against extremes of temperature by acting as thermoregulatory organs, 2). reduce frictional damage in specialised regions such as the eyelids and palms, 3). prevent accumulation of waste products, 4). inhibit bacterial action by producing sweat, which may be effective as an anti-bacterial agent, and 5). act as a fluid phase to aid the flow of sebum across the skin. It seems, that the principal purpose of a sweat gland is not necessarily the same in all species or even between body regions and apart from those in primates, equidae and some bovidae, they play only a minor role in the control of body temperature. It is therefore, probable that individual sweat glands may have more than one function (Jenkinson, 1973).

CONTROL OF SWEATING.

The major centre for the control of sweating is in the hypothalamus (Sato, 1977a; Jenkinson, 1986; Quinton, 1987). However, an increase in temperature of the hypothalamic region does not appear to be the only determinant of sweat output. Other evidence, such as a reduction in thermoregulatory sweating brought about by mental stress, suggests that the hypothalamus acts as a coordinating centre for inhibitory inputs from other areas in the brain (Quinton, 1987).

Evidence that an intact sympathetic nerve supply to sweat glands is necessary for heat-induced sweating (Jenkinson, 1973; Thaysen, 1978) has led to the conclusion that sweat glands are basically under the control of the sympathetic nervous system (Lovatt Evans & Smith, 1956; Findlay & Robertshaw, 1965; Robertshaw & Taylor, 1969; Jenkinson, 1973; Jenkinson, Montgomery & Elder, 1978, Jenkinson, 1986). It has also been established that sweating occurs in the glands of all species studied to date in response to adrenaline and noradrenaline (see Jenkinson, 1986). In addition, circulating catecholamines have been shown to augment exercise-induced sweating in the equidae (Robertshaw, 1977).

The role of acetylcholine and parasympathomimetic agents in sweating is obscure, since they have a stimulatory action in only a few species, including the human, the dog, some primates, equidae and the rodent and feline footpads (Aoki & Wada, 1951; Lovatt Evans & Smith, 1956; Robertshaw & Taylor, 1969; Sakurai & Montagna, 1964; Jenkinson, 1973; 1986). The sweat glands of the cow, sheep, goat and pig do not respond to injection of either acetylcholine or similar agents (Findlay & Robertshaw, 1965; Kimura & Aoki, 1962; Robertshaw, 1968; Jenkinson, 1969). Even though the central control of sweating is mediated through the sympathetic nervous system, physiological sweating results from the release of acetylcholine from post ganglionic terminals and subsequent binding to muscarinic receptors on the glands (Sato, 1977a; Thaysen, 1978; Quinton, 1987). Sweating, in most of the species which respond to cholinergic agents, can be blocked by atropine (Randall & Kimura, 1955; Sato, 1973; Sato, 1977a; Sato & Sato, 1981a) excepting the equidae where heat-induced sweating is unaffected (Robertshaw, 1977). This suggests that a muscarinic cholinergic response is not involved in the sudomotor mechanism of these animals.

The situation is further complicated by the finding that in some of the sweat glands studied, there is not a direct nerve supply to the gland (Jenkinson, Sengupta & Blackburn, 1966; Jenkinson & Blackburn, 1967; Bell &

Montagna, 1972; Jenkinson *et al.*, 1978). Only the sweat glands of man, the horse, some primates and the footpads of rodents, cats and dogs have nerves in close association with the glands (Jenkinson & Blackburn, 1967; Jenkinson, 1970; Jenkinson *et al.*, 1978). It has been postulated (Jenkinson *et al.*, 1978) that the sudorific control of sweating is essentially similar in all mammals. These authors propose that the sudorific mechanism involves the action of sympathetic adrenergic and cholinergic nerves on cutaneous blood vessels, causing a local transfer of a local transmitter, most likely catecholamine, which would elicit sweating. In species where the blood vessels are distant from the gland (e.g. the cow) acetylcholine would have no direct effect, whereas those with blood vessels in close proximity to or within the fibrocyte sheath (eg man and the horse), acetylcholine, in addition to catecholamine, would elicit sweating.

PATTERNS OF SWEATING.

In those species of mammals which respond to thermal stimulation, three general patterns of evaporative loss have been demonstrated using ventilated capsules placed on the skin surface (figures 1.2 & 1.3)(McLean, 1963; Bligh, 1967; Allen & Bligh, 1969; Robertshaw & Taylor, 1969; Jenkinson, 1972; Johnson, Maloiy & Bligh, 1972). Cattle, an example of the first type show a stepwise increase in evaporative loss to a plateau level which can be maintained for more than five hours (McLean, 1963). In the second type, exhibited by sheep and goats, there is a discrete decremental pattern of intermittent sweat discharges to a state of fatigue, which is reached after 90-120 minutes (Bligh,1967; Robertshaw,1968; Jenkinson, *et al.*,1979). Sheep and other ungulates in fact depend more on panting for temperature regulation (Finch, 1972; Jenkinson, 1972). The third type of pattern, found in man and the horse, shows a smooth onset to a plateau which is then sustained, although after 4 hours there is often a decline in activity (Allen & Bligh,1969; Robertshaw & Taylor,1969; Montgomery, Jenkinson & Elder, 1982b).

Combining anatomical and physiological data on the sweat gland, Bligh (1967) proposed a hypothesis to explain the three different patterns. That of the cow he attributed to the effect of a rapidly increasing secretory rate superimposed upon myoepithelial contractions. In the sheep and goat the pattern was considered to be phasic myoepithelial contractions expelling a secretion which was produced continuously in a slow uncontrolled manner. Jenkinson & Robertshaw (1971), however, demonstrated that thermal stress does stimulate sweat formation in the goat. They concluded that the subsequent fatigue was due to the rate of sweat formation being insufficient to provide enough secretion to maintain output in the presence of relatively strong myoepithelial contractions. The third pattern, displayed by the horse and man was believed (Bligh, 1967) to result from secretory activity with little or no myoepithelial action.

ROLE OF MYOEPITHELIUM.

The role of the myoepithelium in sweat output is not well defined. Observations of glands present in the wing of the bat demonstrated that repetitive myoepithelial contractions can occur upon stimulation and that glandular contents could be expelled by forceful contraction of the gland wall (Murphy, 1960; Cortese & Nicholl, 1970). Tucker, (1968) using histological sections of glands from the palmar region of the Koala (*Phascolarctos cinerus*) concluded that the myoepithelium was involved in the output of sweat from the glands. Nicolaidis & Sivadjian, (1972) proposed that the pattern of sweat output they observed on the human forehead, was similarly due to repetitive myoepithelial contractions. In addition, heat and exercise have been shown to cause a reduction in glandular volume in the cow, sheep and goat (Hayman & Nay, 1958; Findlay & Jenkinson, 1964; Jenkinson & Robertshaw, 1971). These results support the view that the myoepithelium contributes to the expulsion of sweat and supports Bligh's (1967) hypothesis.

However, in contrast, ultrastructural evidence of sustained myoepithelial contraction, upon thermal stimulation, in all of these species, casts doubt on

the involvement of repetitive myoepithelial action in the expulsion of sweat (Jenkinson, Montgomery & Elder, 1979; Montgomery, Jenkinson & Elder, 1982a; Montgomery, Jenkinson & Elder, 1982b; Montgomery *et al.*,1984). Pharmacological studies performed by Sato and colleagues (1977b, 1979 & 1980) using sweat glands from the palmar region of the monkey and human epitrichial glands, demonstrated that while myoepithelial cells do contract in response to cholinergic stimuli they do not respond to other stimulatory agents of sweat secretion such as alpha or beta-adrenergic compounds. This series of experiments also showed that there was no pulsatile contraction during continued exposure to acetylcholine and that myoepithelial contraction did not push out any appreciable amount of sweat *in vitro.* These observations led to the conclusion that the primary function of the myoepithelium surrounding the secretory portion of the gland is the provision of structural support for the tubular wall during sweat formation.

MODES OF SECRETION.

Comparative studies of sweat gland ultrastructure in man, the horse and ungulates before and after thermal stimulation (Jenkinson *et al.*, 1979; Montgomery *et al.*, 1982a; Montgomery *et al.*, 1982b; Montgomery *et al.*, 1984) revealed marked species variations in the appearance of luminal epithelial cells. These studies showed, that Schiefferdecker's (1917) original criteria, namely, that eccrine glands release their secretion through the luminal membrane of the secretory cell without rupture and loss of cytoplasm and that apocrine glands exhibit both eccrine secretion and necrobiotic discharge is no longer valid. In spite of the differences between the species, the mode of sweat formation in the cow, the sheep and the goat (Jenkinson *et al.*, 1979; Montgomery *et al.*,1982a) and the horse and man (Montgomery *et al.*, 1982b; Montgomery *et al.*, 1984), which exhibit different patterns of output, is basically the same. Sweat in all species studied to date, is formed from 1) the products of cell death and 2) secretion, which involves exocytosis, fluid transport (including ions) and, in some species a microapocrine process.

The different patterns of evaporative loss therefore, can not be explained on the basis of the general mode of secretion. As neither myoepithelial involvement nor varying modes of secretion can fully explain the different patterns of sweating it would seem therefore, that they are likely to be reflected by variations in the mechanism of fluid and ionic transport and hence by the intracellular ionic composition of the fundus and ductal cells of the different species.

The concept of an active secretory process during sweating in cattle, sheep, goats, man and the horse is supported by evidence from studies of the ionic composition of sweat, where the electrolyte composition of surface sweat has been shown to vary widely amongst species (Schultz, 1969; Johnson, 1970; Jenkinson & Robertshaw, 1971; Jenkinson & Mabon, 1973; Sato, 1977a; Kerr, Munro & Snow, 1980). These variations in the electrolyte composition, however, are unlikely to be solely a reflection of differences in composition of fluid secreted by the fundus or of fluid reabsorption occuring in the ductal portion of the gland. The involvement of an active secretory process is illustrated in cattle by a reduced Na/K ratio in surface skin washings upon thermal stimulation (Johnson, 1970; Jenkinson & Mabon, 1973) and in other species by morphological evidence of secretory cell involvement (Jenkinson et.al., 1979; Montgomery *et al.*, 1982b; Montgomery *et al.*, 1984). Evidence from the monkey (Sato, 1977a) demonstrated changes in Na and K concentration between the secretory coil and the proximal duct and emphasised the importance of ductal reabsorption in this species.

Investigations using x-ray microanalysis have examined the intracellular ionic compositions in cells of the fundus and duct of human and rat footpad atrichial glands (McWilliams, Montgomery, Elder, Jenkinson & Wilson, 1987, 1988; Wilson, Elder, Sutton, Jenkinson, Cockburn, McWilliams & Bovell, 1988a) and the epitrichial glands of the horse (Wilson, Elder, Jenkinson & McWilliams, 1988b) upon thermal activation and most recently isolated monkey palm glands (Saga & Sato, 1989). These studies demonstrated that

significant changes occur in the intracellular electrolyte levels of the sweat gland cells after thermally and secretagogue-induced activation. The finding of a significantly elevated level of intracellular Na in the active fundus of the horse, human and rat footpad accords with the findings from other exocrine glands during secretory activity (Izutsu & Johnson, 1986) and provides evidence of a secretory response to thermal stimulation in these species.

Studies using lanthanum, an extracellular tracer, demonstrated that it could not pass the zonula occludentes and enter the lumina of cattle, sheep and horse sweat glands (Jenkinson, Nimmo, Jackson, McQueen, Elder, Mackay & Montgomery, 1983). This finding further suggests that fluid transport in the sweat glands of the above species is largely due to ionic mechanisms, while the situation in man however is unclear.

Missing from the above studies however are data on the mechanisms underlying the sweating patterns showing a stepwise increase (cattle) or the rapidly fatiguing phasic output type of the sheep and goat.

IONIC SECRETORY MECHANISMS IN EXOCRINE GLANDS.

Fluid secretion in exocrine glands is regulated by alterations in membrane permeability to osmotically important electrolytes or by alterations in the rates of active transport of these electrolytes. The current model for fluid secretion in mammalian exocrine glands is based on the hypothesis that chloride ions are moved from the interstitium to the lumen by secondary-active transport. This generates a transepithelial potential difference (lumen negative), thereby providing a driving force for the movement of Na^+ and consequently water by osmosis (Frizzell, Field & Schultz, 1979; Petersen & Maruyama, 1984).

The features of this model were first described in the shark rectal gland (Silva, Stoff, Field, Fine, Forrest & Epstein, 1977; Epstein, Silva & Stoff, 1983; Greger & Schlatter, 1984). Silva *et al.*, (1977), using perfused, isolated glands, demonstrated that the intracellular concentration of chloride was 2-4 times higher, than that predicted by the Nernst equation, from cells in which the

potential difference across the basolateral membrane was simultaneously recorded. The authors calculated from their results that there was an approximate 36mV electrochemical gradient opposing the movement of chloride into the cell. These results suggested that chloride was being transported into the cell against an electrochemical gradient and accumulating at a concentration above that expected for passive distribution.

Chloride transport in isolated shark rectal gland has been shown to be highly dependent on the sodium concentration of the perfusing solution and similarly, the transport of sodium depends on the presence of chloride in the perfusate (Silva *et al.*, 1977; Epstein *et al.*, 1983; Greger and Schlatter, 1984). Sodium ions enter the cell 'down' an electrochemical gradient generated by a Na^+/K^+ pump on the basolateral membrane, inhibition of the pump, by the glycoside ouabain, blocked the accumulation of chloride within the rectal gland secretory cells (Silva *et al.*, 1977; Epstein *et al.*, 1983; Greger & Schlatter, 1984). The discovery that the accumulation of chloride was linked to both external Na^+ concentration and the maintenance of a Na^+ gradient, suggested that chloride movement was tightly coupled to the inward movement of Na^+. Strong evidence in support of a Na^+/Cl^- co-transport mechanism came from studies of membrane vesicles prepared from the shark rectal gland, which further demonstrated the interdependence of Na^+ and Cl^- for their uptake into vesicles (Epstein *et al.*, 1983). An inhibitory effect of furosemide (a loop diuretic) on the transport of Na^+ and Cl^- into the cells has also been described (Epstein *et al.*, 1983).

Further investigations of the shark rectal gland demonstrated that the Na/Cl co-transport system required the presence of potassium ions (Hannafin, Kinne-Saffran, Friedman & Kinne, 1983). Using rubidium, as a marker for potassium movement, these authors demonstrated that rubidium uptake was higher in the presence of NaCl than when the NaCl was replaced with either lithium chloride or choline chloride. The rubidium uptake was also shown to be inhibited by the loop diuretic bumetanide (Hannafin *et al.*, 1983) which

added support to the postulate of an $Na^+/K^+/Cl^-$ co-transporter. Evidence that the $Na^+/K^+/Cl^-$ co-transporter, in the shark rectal gland, moved ions in the ratio of 1Na :1K :2Cl, was provided by studies on the energetic efficiency of the process (Epstein *et al.*, 1983) and electrophysiological studies (Greger & Schlatter, 1984). The latter authors demonstrated that cellular Cl^- activity in shark rectal gland cells fell transiently upon reduction of the K^+ concentration in the perfusate, and that this fall in cellular Cl^- was accompanied by a decrease in the driving force for Cl^- exit across the apical membrane. The loss of Cl^- from the cells has been shown to depolarise the cells, reducing the driving force for further Cl^- exit, while loss of K^+ caused a hyperpolarisation, which maintained the driving force for continued Cl^- efflux via the luminal membrane (Greger & Schlatter, 1984).

A similar movement of Cl^- into cells coupled to Na^+ entry by means of either an $Na^+/K^+/2Cl^-$ (Petersen & Maruyama, 1984; Exley, Fuller & Gallacher, 1986) or an Na^+/Cl^- (Singh, 1988) co-transporter mechanism has been proposed in mammalian exocrine glands (figure 1.4). The sodium ions enter the cell 'down' an electrochemical gradient, generated by a Na^+/K^+ pump on the basolateral membrane driven by adenosine tri phosphate (ATP) (Bundgaard, Møller & Poulsen, 1977; Bundgaard, Møller & Poulsen, 1981; Quinton & Tormey, 1976).

In general the effect of stimulation in exocrine glands is to increase the permeability of the membrane to one or more of the ions which have an equilibrium potential different from the resting membrane potential, resulting in a secretory membrane potential change (Petersen, 1976). One consequence of this stimulation is for Cl^- ions to exit passively down a favourable electrochemical gradient, through an apical membrane channel in to the lumen (Marty, Tan & Trautmann, 1984; Findlay & Petersen, 1985; Evans & Marty, 1986). Therefore, factors that regulate the permeability of the apical membrane to Cl^-, such as increased concentrations of intracellular Ca^{2+}, which

activate apical Cl^--channels (figure 1.4), control the rate of transepithelial secretion (Welsh, 1987; Wakui & Petersen, 1989).

Electrophysiological studies on the rat parotid gland demonstrated a brief hyperpolarisation in response to muscarinic and adrenergic stimuli, which the authors reasoned was due to an increase in K^+ permeability (Petersen & Pedersen, 1974). This stimulant-evoked K^+ release is due to the activation of K^+ channels by calcium (figure 1.4)(Petersen & Maruyama, 1984; see review by Petersen & Gallacher, 1988). The K^+ channels are activated by both an increase in the concentration of intracellular free calcium ($[Ca^{2+}]_i$), and by a decrease in membrane potential, which open the channels allowing K^+ to leave down an electrochemical gradient, thereby hyperpolarising the cell and permitting sustained secretion. The existence of these K^+ channels which are sensitive to the internal free $[Ca^{2+}]_i$ within the cell has been demonstrated only in the last few years by the patch clamp method (see review by Petersen & Gallacher, 1988). The calcium-activated K^+ and Cl^- channels are the keys to the understanding of the Ca^{2+} dependence of fluid secretion as well as the stimulant-evoked K^+ release and membrane hyperpolarisation.

In secretory cells, such as those of the salivary glands and exocrine pancreas, in which the K^+ channels are under dual control of the membrane potential and $[Ca^{2+}]_i$ (Maruyama, Gallacher & Petersen, 1983; Maruyama, Petersen, Flanagan & Pearson, 1983) an increase in $[Ca^{2+}]_i$ will increase the open state probability of the channel, which consequently will increase the membrane potential and so in turn reduce the probability of the K^+ channel opening. Thus the efflux of K^+ has a negative feedback control.

A general scheme for receptor control of permeability in mammalian exocrine glands, as related to ion permeability has been proposed by Putney (1978) where the occupation of receptors on acinar cell membranes by agonists (muscarinic, α adrenergic and peptidergic) triggers a breakdown of phosphatidylinositol bis-phosphate, following cleavage of which, calcium is released from binding sites and the intracellular calcium activates ionic channels.

The increase in intracellular Ca^{2+} concentration in response to muscarinic stimulation appears to consist of two phases; a first phase being due to the release of bound intracellular calcium and a second phase due to the influx of Ca^{2+}. The release of Ca^{2+} from intracellular stores has been attributed to the action of inositol 1,4,5 trisphosphate (1,4,5 IP_3) (Streb, Irvine, Berridge & Schulz, 1983; Aub, McKinney & Putney, 1982; Aub & Putney, 1987; Petersen & Gallacher, 1988). The second more sustained phase is thought to require the influx of Ca^{2+} into the cells (Putney, 1976; 1977; 1979; Marier, Putney & van de Walle, 1978). Evidence from microinjection studies performed on sea urchin oocytes (Irvine & Moor, 1986) suggests that such a calcium influx is mediated via inositol 1,3,4,5,tetrakisphosphate (1,3,4,5 IP_4). Secretagoues, such as ACh, which act via an increase intracellular calcium, increase the rate of hydrolysis of a membrane phospholipid (Hokin & Hokin, 1954; Michell, 1975) inositol 4,5 bis-phosphate (PIP_2). Both 1,4,5 IP_3 and 1,3,4,5 IP_4 are water soluble by products of the hydrolysis of PIP_2 which is formed from phosphatidyl inositol (Ptd Ins) present in the cell membrane.

PIP_2 is split by phospholipase C to form diacylglycerol (DAG) and 1,4,5 IP_3. The DAG is then phosphorylated to form phosphatidic acid (PA), while the inositol phosphates are dephosphorylated and then re-coupled with PA to form Ptd Ins once again. Agonists accelerate this cycle by activating phosphoinositidase C, a membrane enzyme which may be coupled to receptors. The phosphorylated product, PIP_2, is hydrolysed to produce 1,4,5 IP_3 which has been shown to be very effective in releasing calcium from intracellular stores (Berridge, Dawson, Downes, Heslop & Irvine, 1983; Putney, Burgess, Halenda, McKinney & Rubin, 1983). 1,4,5 IP_3 is rapidly dephosphorylated within the cell to form inactive di & mono-phosphoinositol before being reincorporated into Ptd Ins in the membrane. 1,4,5 IP_3 can be metabolised by another pathway in addition to the one already described. In the alternative pathway a kinase catalyses phosphorylation of 1,4,5 IP_3 to 1,3,4,5 IP_4. The enzyme which catalyses dephosphorylation of 1,4,5 IP_3 also

catalyses dephosphorylation of 1,3,4,5 IP_4 to inositol 1,3,4 trisphosphate which is further dephosphorylated eventually to inositol.

While the release of intracellular Ca^{2+} is mediated by 1,4,5 IP_3 and Ca^{2+} influx by 1,3,4,5 IP_4 the mechanism by which 1,4,5 IP_3 and 1,3,4,5 IP_4 act has yet to be fully described. It has been shown that separate internal application of these compounds fails to elicit a release of Ca^{2+} similar to the effect of acetylcholine in lacrimal glands (Morris, Gallacher, Irvine & Petersen, 1987; Changya, Gallacher, Irvine & Petersen, 1989) or of sperm in fertilisation in sea urchin oocytes (Irvine & Moor, 1986) Dual application of these two substances is therefore required to increase intracellular calcium in order to obtain a sustained increase in Ca^{2+}-activated K^+-efflux similar to that elicited by acetylcholine (Changya *et al.*, 1989).

The stimulant-evoked K^+ loss has been well described in salivary and pancreatic acinar cells (Burgen, 1956; Petersen, 1970, 1971; Putney, 1976, 1978; Singh, 1984, 1988; Petersen & Singh, 1985). Following release of K^+ from stimulated salivary glands, there is a reuptake of K^+. It has been postulated, that in the steady secretory state, K^+ recirculates via pump and cotransporter (Petersen & Maruyama, 1984). Ouabain can block the reuptake of K^+ in salivary glands and is thought to act on the ATP-driven Na^+/K^+ pump, on the basolateral membrane. K^+ reuptake is also abolished when the Cl^- component of the perfusion fluid is replaced by either nitrate or sulphate (Petersen, 1970) and is also sensitive to reductions in external Na^+ concentrations ($[Na^+]_o$) (Petersen, 1970). The fact that secretion is markedly reduced under these conditions adds support to the postulated existence in exocrine glands of a $Na^+/K^+/2Cl^-$ cotransporter to move these ions into the cell. The ability of ouabain to block the K^+ reuptake further supports the suggested presence of a $Na^+/K^+/2Cl^-$ cotransporter, since uptake through the cotransporter will be dependent on the presence of the sodium gradient.

IONIC MECHANISMS IN THE SWEAT GLAND.

The current understanding of the mechanisms involved in sweat secretion is outlined in figure 1.5.

Sato & Sato, (1981a) demonstrated that sweating occurred in isolated simian sweat glands in response to cholinergic (muscarinic) and α and β adrenergic agents which acted through different receptors. The latter were less effective. Sweat production, induced by ACh and α-adrenergic stimulation, was shown to be wholly dependent on the presence of extracellular calcium (Sato & Sato, 1981a). In contrast, sweating induced by β-adrenergic stimulation was unaffected by removal of external calcium (Sato & Sato, 1981b) and appeared to be induced via increased levels of cyclic adenosine monophosphate (cAMP) within the secretory cells (Sato & Sato, 1981c).

In common with salivary glands, there is an ATP - driven Na^+/K^+ pump on the basolateral membrane of sweat glands (Quinton & Tormey, 1976) and sweat secretion, both *in vivo* (Schultz, 1969) and *in vitro* (Quinton, 1981), is inhibited when ouabain is bound to it. This indicates that secretory fluid transport in sweat glands is dependent on Na^+/K^+ ATPase activity. The abolition of sweat secretion in the simian palm gland by the removal of extracellular Na^+ or Cl^-, or the presence of bumetanide, strongly suggests the existence of a $Na^+/K^+/2Cl^-$ cotransporter in sweat glands (Sato & Sato, 1987) similar to that found in salivary glands (Petersen & Maruyama, 1984). Although furosemide, another loop diuretic, which is thought to act on the $Na^+/K^+/2Cl^-$ cotransporter, has been shown to block secretion in the rat submanibular gland (Martinez & Cassity, 1983) it does not affect secretion from human sweat glands (Quinton, 1981). This suggests that either the blocking capabilities of furosemide on a $Na^+/K^+/2Cl^-$ cotransporter is gland specific or that such a cotransporter does not exist in human sweat glands.

Similar to findings described in the shark rectal gland (Silva *et al.*, 1977; Greger & Schlatter, 1984), a negative electrical potential difference develops

across the luminal membrane, of sweat glands, in response to stimulation (Sato, 1973; 1986; Quinton, 1981). Sato (1986) further demonstrated that cholinergic stimulation of monkey palm glands evoked a biphasic change in the potential difference across the basolateral membrane; an initial depolarisation followed by repolarisation to near the resting potential difference.

However, in spite of the many similar properties that exist between sweat glands, other exocrine glands and other epithelial tissues, electrophysiological studies have failed to reveal a K^+ efflux in sweat glands in response to stimulation (Sato, 1973; 1986). By contrast, EPXMA studies have reported that a K^+ efflux accompanies stimulation in different sweat glands (McWilliams *et al.*, 1987;1988; Wilson *et al.*, 1988a; 1988b; Saga & Sato, 1989), which could account for the repolarisation reported by Sato (1986). Further evidence in support of a role for a K^+ efflux in the maintenance of sweat secretion, comes from the studies of Sato & Sato (1987), who inhibited sweat secretion by blockade of the K^+ channels with Ba^{2+}.

OBJECTIVES.

As discussed earlier Jenkinson and his co-workers proposed that the mode of sweat secretion is the same in all species. This conclusion was based on ultrastructural studies of glands, before and after thermal stimulation, in species with (man; Montgomery *et al.*, 1984; and horse; Montgomery *et al.*, 1982b) and without (cow, sheep & goat; Jenkinson *et al.*, 1979) direct innervation. The different patterns of sweat output from these species could therefore not be explained on the basis of the general mode of secretion.

EPXMA studies on the sweat glands of the horse (Wilson *et al.*, 1988 b) and human (Wilson *et al.*, 1988 a) demonstrated that in common with other exocrine glands, changes in the intracellular electrolyte content of the cells occurred upon thermal stimulation. The authors concluded that the patterns seen in both the human and the horse, of increasing secretory rate, was a

reflection of changes in intracellular elemental concentration in the epithelial cells of both types of sweat glands.

As a first objective, a similar investigation using EPXMA was undertaken to examine the changes in electrolyte levels that occurred in the cells of the sweat glands from the cow, sheep and goat, which exhibit different patterns of output.

The methodology involved in EPXMA is still rapidly developing and it is therefore essential to have a sound knowledge of the techniques involved in the preparation of samples and their analysis. A brief account of these techniques is given in chapter 2.

EPXMA studies on the horse (Wilson *et al.*, 1988 b), human (Wilson *et al.*, 1988 a) and monkey palm gland (Saga & Sato, 1989) all demonstrated a drop in the intracellular concentration of potassium. These results are in contrast to the findings of electrophysiological studies which failed to establish an efflux of K^+ from the cells of the monkey palm gland (Sato, 1973, 1986). A second objective was therefore to investigate the anomaly between EPXMA and electrophysiological results in sweat glands, using as a different approach, radioisotopic tracers; the choice of isotope and the methodology are outlined in Chapter 4.

The development of an improved method for the isolation of large numbers of sweat glands from strips of human skin (Lee, Jones & Kealey, 1984) in conjunction with a frequent supply of human skin from the nearby university teaching hospital, offered an ideal opportunity to investigate, in man, the anomaly between EPXMA and electrophysiological results.

FIGURE LEGENDS CHAPTER 1.

Fig 1.1. Shows a generalised sweat gland structure illustrating the fundus and the duct. The fundus is comprised of a secretory epithelium (SE) surrounded by a myoepithelium (MyoE) and a fibrocyte sheath (F).

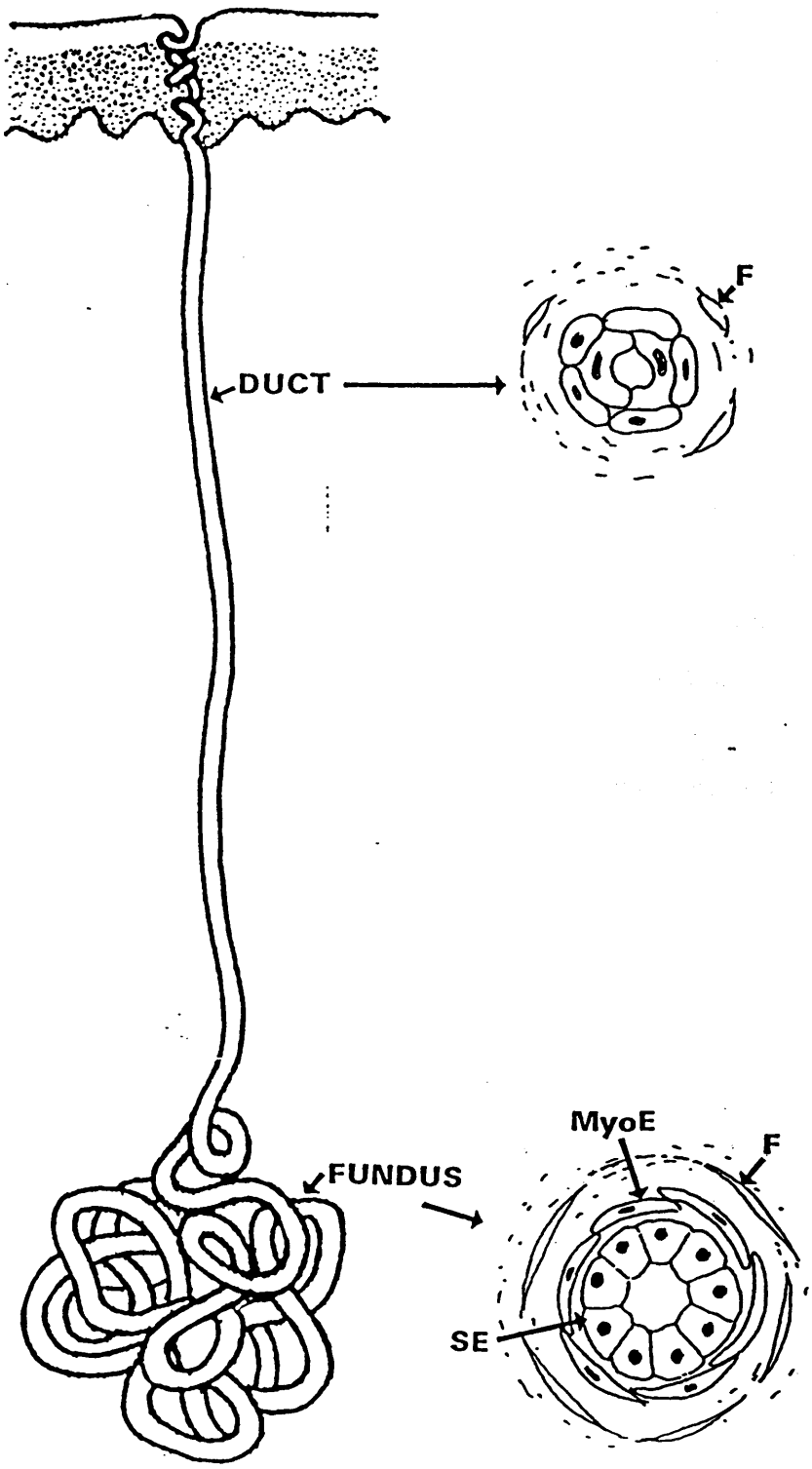

DUCT
F
FUNDUS
MyoE
F
SE

Fig. 1.2. A ventilated capsule (V.C) on the skin of a cow. Equivalent metered airflows pass through the capsule and the room, and the humidity of the air in the two tubes is measured by wet bulbs (WB). The difference in humidity between the two airways gives a measure of evaporative loss from the animal. At high enviromental temperatures the contribution of water from transport through the skin is relatively low and total evaporative loss approximates to sweat output.

1
2

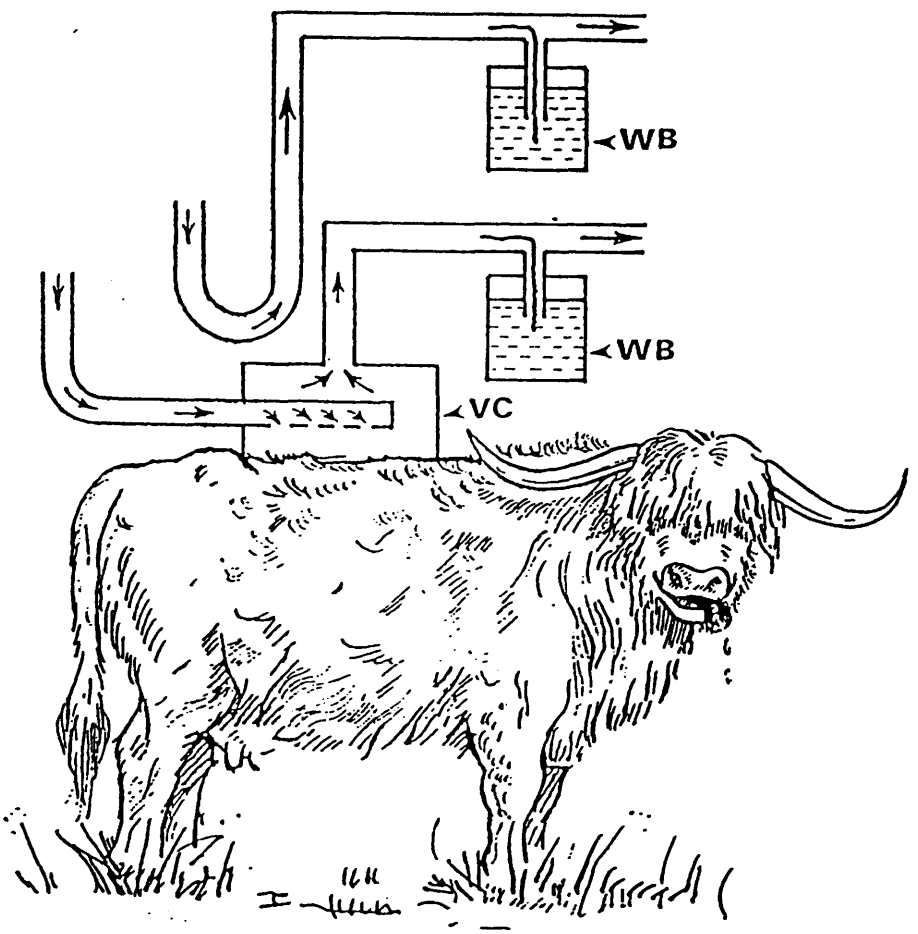
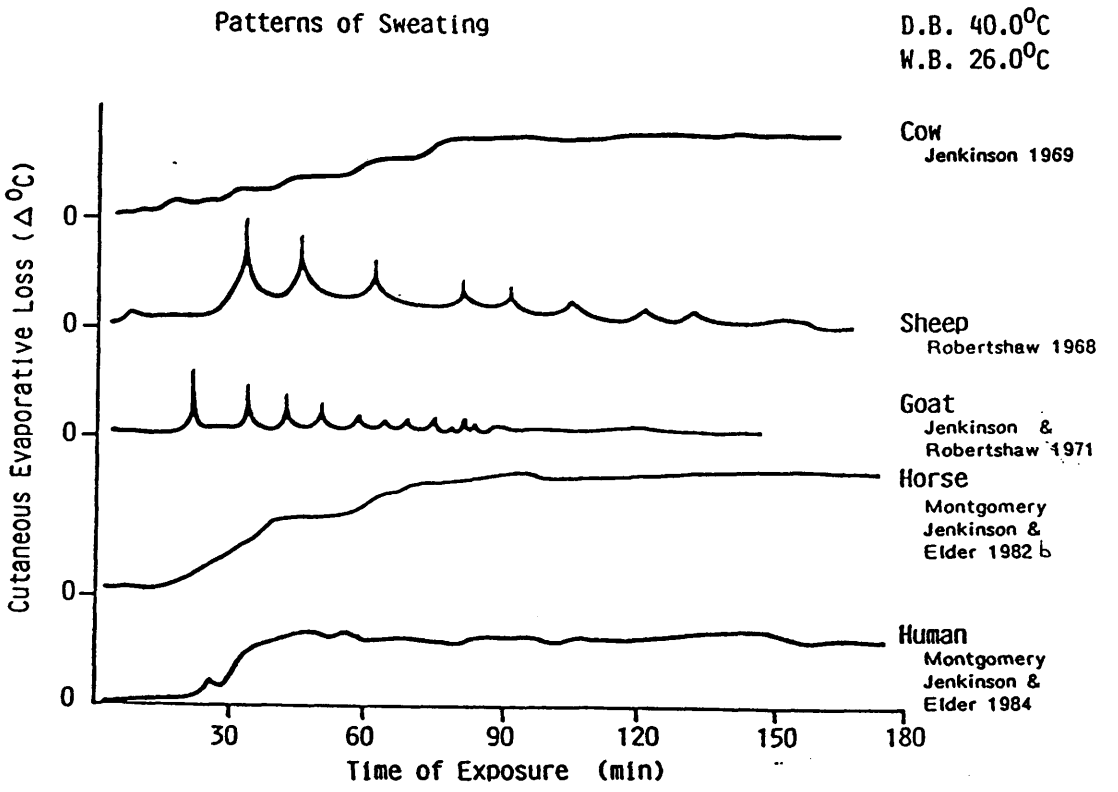


Fig. 1.3. The patterns of cutaneous evaporative moisture loss against time for five different species at high environmental temperatures.


Patterns of Sweating
D.B. 40.0°C
W.B. 26.0°C
Cutaneous Evaporative Loss (Δ °C)
0
0
0
0
0
Cow
Jenkinson 1969
Sheep
Robertshaw 1968
Goat
Jenkinson & Robertshaw 1971
Horse
Montgomery Jenkinson & Elder 1982 b
Human
Montgomery Jenkinson & Elder 1984
30
60
90
120
150
180
Time of Exposure (min)

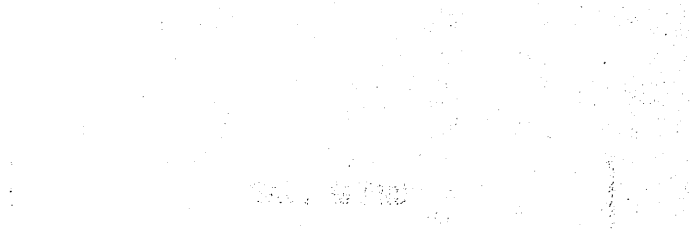
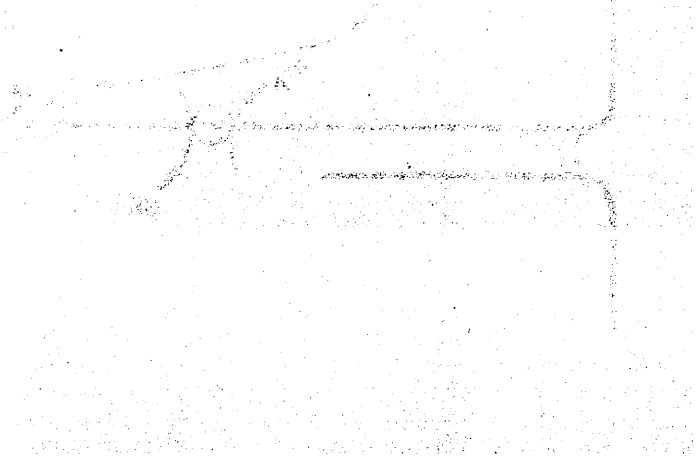


Fig. 1.4. Shows a diagram outlining the ionic mechanisms involved in secretion from salivary glands in response to cholinergic stimulation. Further details are given in the text (see pages 11-18).



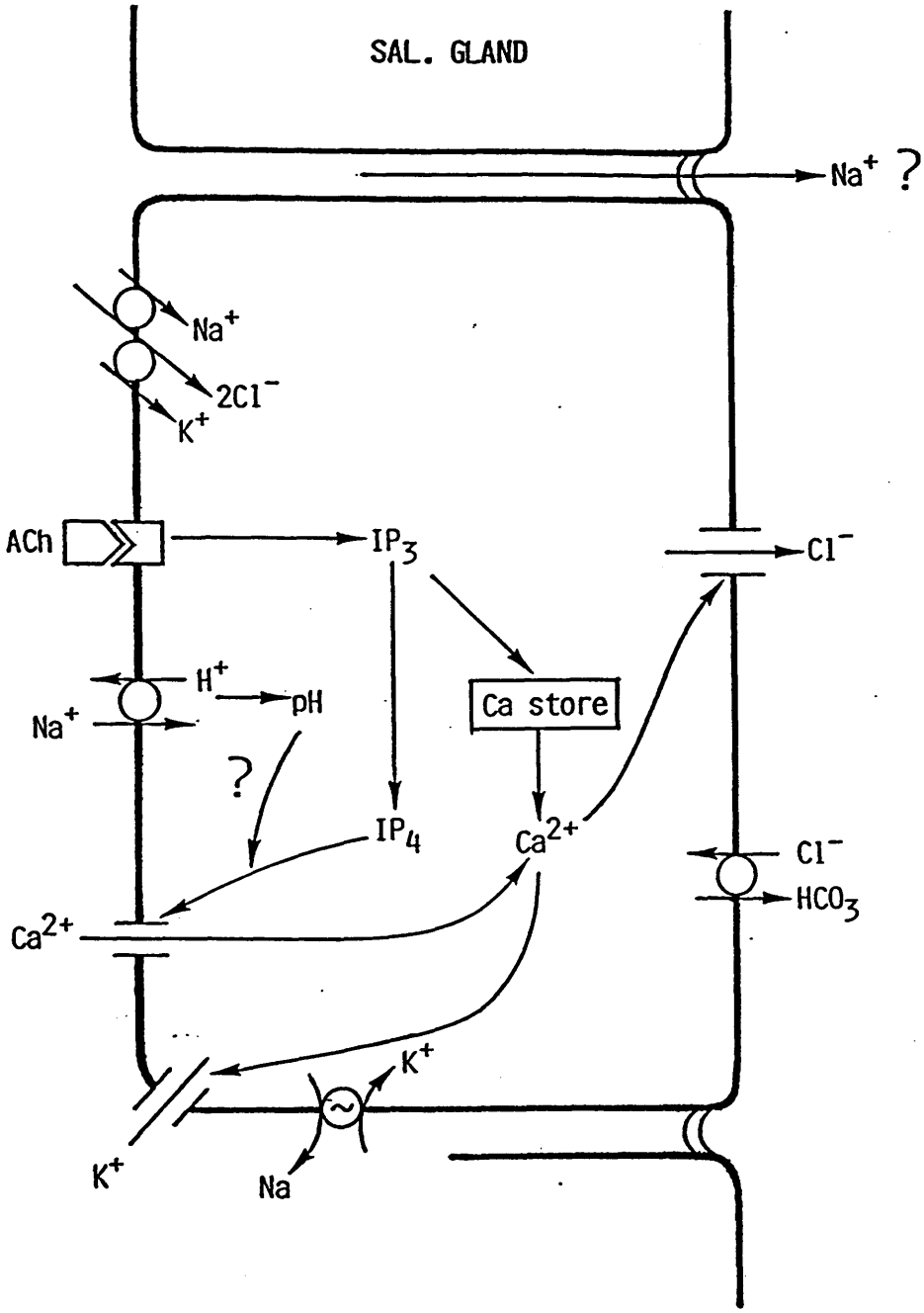
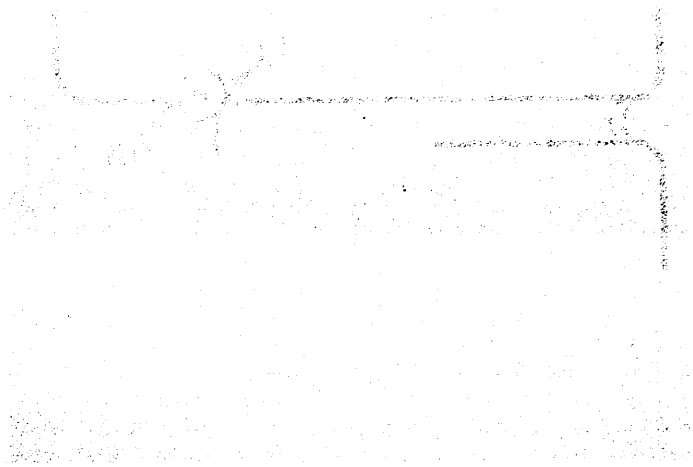
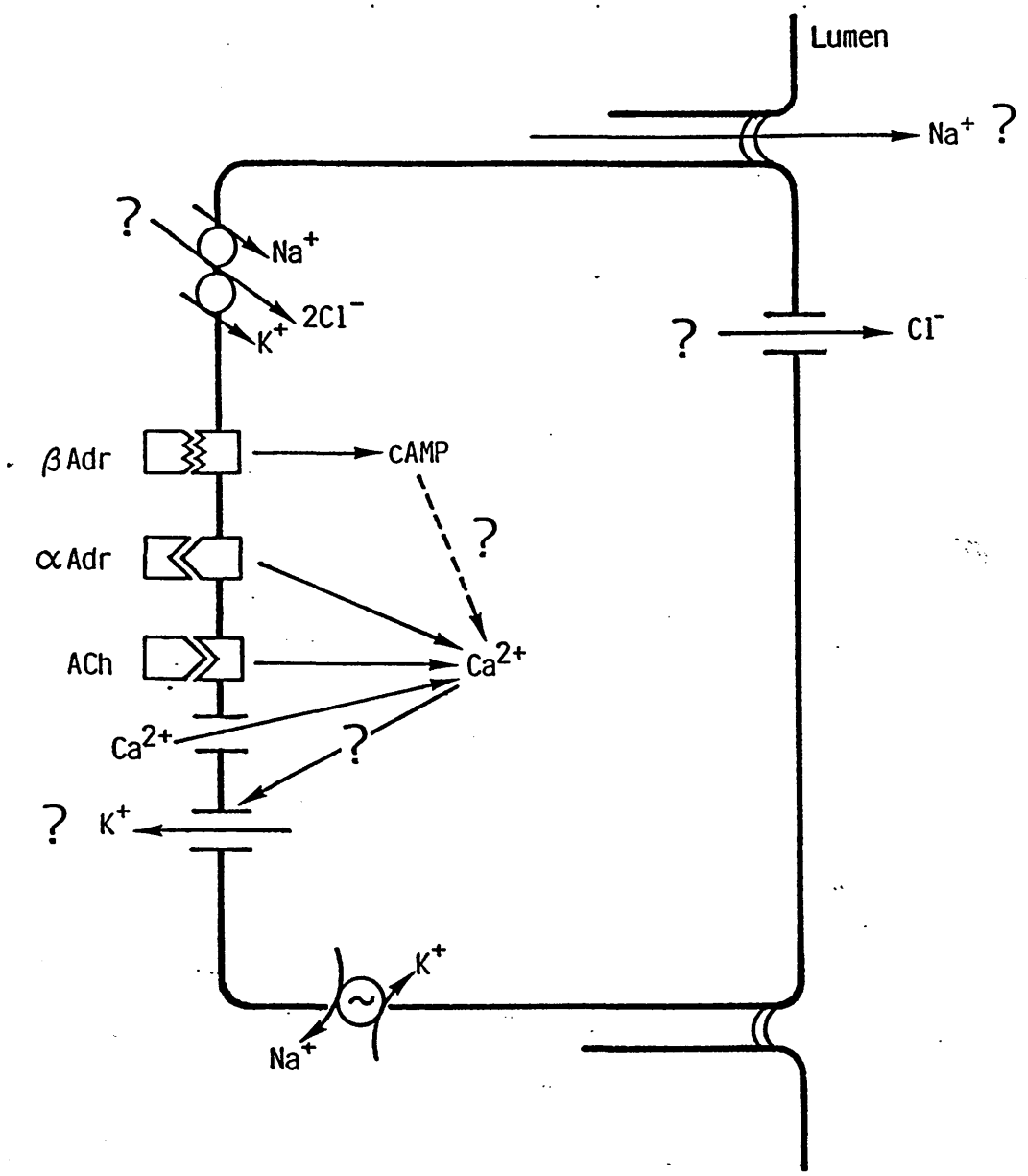

SAL. GLAND
Na^+ ?
Na^+
$2Cl^-$
K^+
ACh
IP_3
Cl^-
H^+
pH
Na^+
Ca store
?
IP_4
Ca^{2+}
Cl^-
HCO_3
Ca^{2+}
K^+
K^+
Na

Fig. 1.5. A diagramatic representation of the ionic mechanisms thought to be involved in the secretion of sweat in response to to catecholaminergic and cholinergic agonists. Further details are given in the text (see pages 18 & 19).




Lumen
Na+ ?
?
Na+
2Cl−
K+
?
Cl−
βAdr
cAMP
?
αAdr
ACh
Ca2+
Ca2+
?
?
K+
K+
Na+

CHAPTER 2.

SPECIMEN PREPARATION AND THE PHYSICAL BASIS OF ELECTRON PROBE X-RAY MICROANALYSIS (EPXMA)

INTRODUCTION.

Electron probe x-ray microanalysis (EPXMA) is an analytical technique in which x-radiation is produced by the interaction of a focussed beam of accelerated electrons with a specimen. A proportion of the emitted x-rays have energies characteristic of the elements in the specimen, thus permitting their identification. The quantities of the identified elements can be calculated by measurement of the number of x-rays with energies characteristic of the elements.

Two important stages are involved ;

1) Specimen preparation (see below), and

2) Specimen analysis (see below)

Successful application of EPXMA requires the careful use of the appropriate methodology in both of the above stages.

SPECIMEN PREPARATION.

Specimen preparation is the most difficult and critical step in biological microprobe analysis (Coleman 1975, Warner & Lechene 1979). The main aim is to ensure that the processing procedures preserve the specimen as near as possible to its *in vivo* state, so that elemental distribution can be quantitated with a high degree of spatial resolution.

In EPXMA, the major demand of any preparative technique is the prevention of ion displacement from or within the cell. Therefore, it is of great importance to avoid any procedure which fails to meet this condition. For this reason the commonly used methods of chemical fixation, dehydration and embedding in preparing material for transmission electron microscopy (TEM), which lead to changes in cell structure with extraction of many of the soluble

materials and redistribution of ions and other mobile components are generally unacceptable.

To overcome these problems alternative methods have been studied and implemented, whereby the specimen is 'fixed' by rapid freezing. This process has been termed cryofixation.

CRYOFIXATION (RAPID FREEZING)

The basis of cryofixation is the rapid conversion of water from a liquid to solid state. The transition between these two phases is a complex series of events which is influenced by cooling rate, the presence of solutes and pressure.

When pure water is cooled to 273 K at normobaric pressure it does not freeze immediately, because at this temperature the small ice crystals which form, are unstable due to their energetically unfavourable surface to volume ratio (Rasmussen, 1982). However, as the temperature continues to drop, the crystals become more stable and act as foci for general growth. Such freezing is termed homogeneous nucleation. The subsequent rate of ice crystal growth is temperature dependent and maximises at 260 K. As the temperature decreases still further, the growth rate declines until 140 K is reached. At and below this temperature, known as the recrystallisation point, ice crystals no longer grow. Thus, ice crystals will form and grow at all temperatures between the freezing point and the recrystallisation point and hence crystallisation can only be totally avoided if this range of temperatures is traversed sufficiently rapidly. If cooling is fast enough no ice crystal structures will form, giving a glass like state to the frozen water molecules (vitrification).

Vitrification of pure water is predicted to occur when it is cooled at a rate in excess of 10^6 K s^{-1} (Fletcher, 1971) and has been achieved with extremely small samples (<1μm diameter) (Brüggeller & Mayer, 1980; Dubochet & McDowall, 1981; Mayer & Bruggeller, 1982). However,the behaviour of biological specimens is not equivalent to pure water due to the presence of dissolved material and physical barriers to diffusion. This has led to the

prediction of 'vitrification' in biological specimens at cooling rates between 2 x 10^6 K s^{-1} (Riehle, 1968; Franks, 1978) and 10^4 K s^{-1} (Moor, 1971).

The quality of cryopreservation for EPXMA may be judged by the extent of ice crystal damage in the specimen. The larger the specimen the greater the latent heat which must be removed as quickly as possible from it. As biological tissues have poor thermal conductivity, it follows that the size of the material being cryofixed must be kept small for good preservation. Vitrification has been achieved in rat liver preparations (McDowall, Chang, Freeman, Lepault, Walter & Dubochet, 1983). It is possible with cooling rates of approximately I x 10^6 K s^{-1} (Moor, 197I; Bald 1983) to obtain specimens which have an outer layer approximately 10μm thick, where the ice crystal diameter is below the resolution of the electron microscope. Within this area there is little structural disruption or elemental redistribution (Elder, Gray, Jardine, Chapman & Biddlecombe, 1982).

The physical properties of each of the cooling media (cryogens) are of great importance in determining their cooling rates. There has been a number of publications reviewing the physical properties of cooling media and their use with different rapid freezing methods (Sitte, 1979; Costello, 1980; Elder *et al.*, 1982; Escaig, 1982; Plattner & Bachmann, 1982; Sitte, 1984). These reviews have concluded that: 1) the coolant should have a high thermal conductivity with high heat capacity per volume. 2) the coolants used should have a very low temperature, since large temperature gradients facilitate heat diffusion. 3) the formation of an insulating gas layer (Leidenfrost phenomenon) should be avoided when the specimen comes in contact with the cryogen, by employing coolant which has a large temperature difference between its freezing and boiling points.

Consideration of thermophysical properties indicated that liquid nitrogen could be the most efficient coolant if certain criteria can be satisfied (Bald,1985). These are 1) that excessive vapour formation is avoided by operating at a pressure/temperature combination above the critical point, 2) entry velocity into the cryogen is high enough to allow the heat transfer

coefficient to be proportional to velocity and 3) that cooling is completed during the plunge whilst the specimen is moving rather than in the stationary phase. Unfortunately, the reduction of excessive vapour formation has proved the most difficult and expensive to overcome and so although nitrogen when liquefied (a temperature of 77 K) it is only suitable for use as a primary cryogen, and so is used to cool the cryogenic agents.

The most commonly used cryogenic fluids include Freon 13 (Gupta, Hall & Moreton, 1977; Barnard, 1982) sub-cooled Freon 22 (Somlyo, Shuman & Somlyo, 1977), Propane (Rick, Dörge, Gehring, Bauer & Thurau, 1979; Elder *et al.*, 1982), ethane (Silvester, Marchese-Ragona & Johnson, 1982), nitrogen slush (Appleton, 1974; Seveus, 1978) and propane containing 1-25% iso-pentane (Jehl, Bauer, Dörge & Rick, 1981; Ward & Murray, 1987). The cooling rates obtained using these fluids have been measured (Costello & Corless, 1978; Schwabe & Terracio, 1980; Elder *et al.*,1982; Silvester *et al.*,1982; Ryan, Purse, Robinson & Wood, 1987) and ranked as follows; Ethane > Propane > Freon 22 > Freon 12 > Nitrogen slush > liquid nitrogen.

Bald (1984) assessed the properties of various liquid coolants and concluded that at atmospheric pressure ethane or propane are the most efficient depending upon the specimen geometry and nature of the tissue holder and this has been confirmed by Ryan *et al.* (1987).

Mathematical modelling to determine the optimum rate of freezing has shown that the speed of propulsion of the specimen into and through a liquid cryogen is an important factor (Elder *et al.*,1982; Robards & Crosby, 1983). As the specimen "sees" only the temperature of the layer of cryogen in direct contact with its surface any warming of this layer due to the finite thermal diffusivity of the cryogen can be partially compensated by propelling the specimen through it. Experiments with increased rates of propulsion demonstrated that cooling rates also increased up to propulsion velocities of 10 m s^{-1} (Handley, Alexander & Chien, 1981., Elder *et al.*,1982; Robards & Sleytr, 1985, Sitte, Edelmann & Neumann, 1987). Sitte *et al.* (1987a)

concludes that for most purposes the best cooling rates are obtained up to entry velocities of 3 m s^{-1}.

Although the hydrocarbon and fluorocarbon refrigerants give faster cooling than nitrogen slush, they remain on the surface of the specimen when it is maintained at temperatures below the melting point of the coolant used. This may adversely affect sectioning properties of the block by causing the block face to be 'wet' (Robards & Sleytr, 1985) or may, if fluorocarbons are used, give contaminating peaks if the specimen is subsequently used for EPXMA (Gupta, 1979). The now well documented damage to the ozone layer in the earth's atmosphere by fluorocarbons should preclude their use in the future.

Propane and ethane have clear safety problems associated with their use (Bald, 1984; Ryan & Liddicoat, 1987, Sitte *et al.*,1987b). They can form explosive gas mixtures with oxygen, which condenses at 93 K, unless stringent safety measures are applied. Aspects of safety considerations have been discussed by a number of workers (Elder *et al*, 1982; Robards & Sleytr, 1985; Silvester *et al.*, 1982; Ryan & Liddicoat,1987, Sitte *et al.*, 1987b). The freezing points of ethane and propane are several degrees higher than that of liquid nitrogen and consequently may freeze during cooling. This problem may be overcome by the addition of 1-25% iso-pentane (Bell,1956; Jehl *et al.*,1981; Ward & Murray, 1987) without seriously affecting the cooling rate.

Liquid cryogens can be used in a variety of ways but are all bound by the constraint that their heat transfer characteristics (and hence the cooling rates) are strongly dependent on the temperature difference between the specimen and the coolant plus the size of the sample. In general, the specimen is either plunged into a coolant (Rebuhn,1972; Echlin & Moreton, 1976; Bald & Robards, 1978; Costello & Corless, 1978) or held stationary as the coolant is brought up over the specimen (Barnard, 1982). To minimise the possible adverse effects of using cryogens (poor rates of freezing) a number of different approaches have been used (see Review by Elder & Bovell, 1988):- A) Spray freezing:- An elegant method of cryofixation,

where single cells in suspension are sprayed into a liquid cryogen (Bachmann & Schmitt, 1971; Plattner & Bachmann, 1982, Plattner, Schmitt-Fumian & Bachmann, 1973) which has limited use in cryofixing samples for EPXMA. B) Propane Jets:- To avoid the problem of the coolant temperature rising through the heat of the specimen, Müller, Meister & Moor (1980) described a method of jet freezing using propane whereby the specimen was kept static and a coolant at constant temperature was projected over it. However, this technique requires larger quantities of cryogen and it has additional safety requirements. C) High pressure freezing:- When water is subjected to a pressure of about 2.1×10^8 Pa, its freezing point is lowered to about 183K. This depression of the freezing point reduces the rate of cooling required to minimise the formation of ice crystals. The principle was first suggested in detail by Riehle (1968) and now allows specimens up to 600μm to be frozen without the formation of ice crystal segregation compartments (Moor, 1987). However, the availability of the equipment has as yet limited its applications in this field. D) Metal surface freezing (Slamming):- In this process tissues up to 1 mm^3 can be 'slammed' into a block of very pure metal e.g. silver (Van Harreveld & Crowell, 1964; Christensen, 1971 Van Harreveld, Trutbach & Steiner, 1974; Bald, 1983) or copper (Dempsey & Bullivant, 1976 a & b; Heuser, Reese, Dennis, Jan, Jan & Evans,1979; Escaig, 1982; Bald, 1983; Sitte, 1979) which have been precooled by liquid nitrogen (Van Harreveld & Crowell, 1964; Dempsey & Bullivant, 1976 a & b; Bald, 1983) or liquid helium (Heuser *et al*, 1979; Escaig, 1982). This method relies on the fact that the solid surfaces of these metals have a higher thermal conductivity and diffusivity than a liquid interface and that their heat transfer characteristics are improved with lower temperatures.

To achieve ideal cooling the specimen must be brought into rapid (but $<5m\ s^{-1}$)(Sitte *et al.*,1987a) close contact with the metal block which undoubtedly results in some degree of surface distortion of the deeper tissues (Seveus, 1978; Sitte,1987a).Using this method excellent cryopreservation to a depth of 25μm has been achieved by Escaig (1982).

TECHNIQUES EMPLOYED AFTER CRYOFIXATION.

Freeze-drying and resin embedding.

The technique of freeze-drying in combination with embedding the dried material in resin was developed as an alternative to chemical fixation and dehydration with organic solvents. It is a method of dehydration in which the partial pressure of water vapour is reduced by means of vacuum or a dry gas environment to promote the sublimation of ice from a frozen specimen. Sublimation occurs in this temperature range as the partial pressure of water at the surface of the specimen is less than the saturation vapour pressure of ice. This method is extremely popular because redistribution of elements induced by fixatives and other solvents in conventional TEM and freeze-substitution procedures is eliminated and so the technique is highly suitable for EPXMA (Ingram & Ingram, 1984; Kuijpers, van Nooy, de Pont & Stols, 1984; Roos & Barnard, 1985; Elder *et al.*,1985; Zierold, 1985; Edelmann, 1986; McWilliams *et al.*, 1987; Bovell, Elder, Jenkinson & Wilson, 1987; Bovell, Jenkinson, Elder & Wilson, 1988a; Wilson *et al.*, 1988 a & b).

The saturation vapour pressure of ice is proportional to the temperature (Robards & Sleytr, 1985) and freeze-drying therefore occurs in the temperature range 193-173 K, when the partial pressure of water at the surface of a specimen is less than the vapour pressure of the ice at that temperature. This requires a compromise between keeping the specimen below the recrystallisation temperature and having a high enough temperature to allow freeze-drying. The recrystallisation temperature for ice in biological systems is variously estimated at 213K (Appleton,1974:) and 203 K (Christensen,1971; Dempsey & Bullivant,1976a & b). At 173 K the saturation vapour pressure in the specimen is low (10^{-5} Torr for pure ice) and drying takes place at an exceedingly slow rate. Water released from the specimen during the drying process must be continuously removed to keep the partial pressure of the water vapour low. Control of the partial pressure of water in the freeze-dryer can be achieved by performing the process in a vacuum below the desired water vapour pressure. Accuracy in the prediction of drying

rates is limited by uncertainty in the estimation of the partial pressure in either a vacuum or a dry gas. Robards (1974) found that between 10-50% of the residual molecules in the vacuum were water. The best means of controlling residual water vapour in the vacuum is to make use of an anticontaminator cold-trap at liquid nitrogen temperature. This relies on the fact that the saturation vapour pressure of ice at the temperature of liquid nitrogen (77 K) is 10^{-24} torr and that a large metal surface at this temperature placed close to the specimen (~2 mm) (Moor, 1969; 1987; Ingram & Ingram, 1984) will readily attract any remaining water molecules. The importance of including a 'cold-trap' cannot be underestimated. The 'cold-trap' can be supplemented by the use of a physical absorbent, such as an activated molecular sieve (aluminium silicate) or a chemical absorbent (P_2O_5).

The technique of freeze-drying relies on the ice being sublimed at the surface of the specimen. As this occurs an increasing layer of dried specimen presents a resistance to further freeze-drying. As drying progresses both heat transfer and mass transfer (of the water molecules) must occur through the dried area. The water in the cell is not homogeneous as is water in a dilute solution and so exhibits different physicochemical properties (Edelmann, 1986). This is often referred to as "ordered" water and is closely associated with surfaces of intracellular membranes or macromolecules (Negendank, 1986). The consequence of this is that during the drying process the frozen bulk water of the cell is more easily removed than the 'bound' water. As only a few tens of microns of the surface layer of most specimens have been well cryofixed, it is best to freeze-dry this area at a temperature (193 K) which gives little risk of recrystallisation. Once freeze-drying of this area has been achieved, drying of the internal areas, which are more likely to have ice crystal damage, can be accomplished at a much higher temperature (213 K).

Infiltration should be performed under dry gas conditions, to minimise possible specimen rehydration. Numerous workers have developed techniques which allow infiltration to be performed while the specimen is still in the drying chamber (Ingram & Ingram, 1975, Edelmann, 1978; Pfaller, 1979; Elder *et al.*, 1986). Spurr (1969) found that an increase in polymerisation

temperature from 258 K - 293 K produced poorer structural preservation. Edelmann (1978), also obtained improved structural preservation when polymerisation was performed at 313 K compared with 343 K. Now with the development of low temperature embedding resins (Armbruster, Carlemalm, Chiovetti, Garavito, Hobot, Kellenberger & Villiger, 1982; Carlemalm *et al.*, 1982) these problems can be minimised. Sectioning may be done on any suitable ultramicrotome. To avoid any possible elemental displacement during sectioning it is important to use only 'dry' cutting procedures.

In microanalytical studies, it is important to ensure that the benefit of trapping diffusible specimen components by good cryofixation is not lost by poor freeze-drying or infiltration procedures (Ingram & Ingram, 1975). Freeze-drying for x-ray microanalysis has been evaluated by Ingram & Ingram (1980); Edelmann, (1986); Elder, Bovell, Pediani, Wilson, McWilliams & Jenkinson, (1988b). Good morphology, as judged by light microscopy, is preserved if drying is completed before the tissues are warmer than 203 K and with vacuums greater than 10^{-3} Torr, where the rate of transport of water through the tissue becomes the rate determining step (Boyde & Wood, 1969; Ingram & Ingram, 1975). Suitable media for infiltration and embedding should be hydrophobic with a low viscosity and they should contain little or none of the elements to be measured in the specimen (McWilliams Ph.D Thesis, University of Glasgow, 1987) which would otherwise interfere with the results obtained by EPXMA. Controversy exists as to whether and how far ions maybe redistributed during resin infiltration. Some studies have shown that after freeze-drying and embedding ionic redistribution occurs (Roos & Barnard, 1985; Wroblewski & Wroblewski, 1986). However, studies using cryoquenched droplets of different 'standard' solutions, which were infiltrated and embedded 'back to back', gave evidence of little or no ionic redistribution (Elder *et al.*, 1988b).

Although the method has its own particular artefacts (Rebuhn, 1972; Robards & Sletyr, 1985), it has been successfully applied to ion localisation by EPXMA (Chandler, 1977; Ingram *et al.*, 1975; Edelmann, 1978 ; Elder *et al.*,

1985; McWilliams *et al.*, 1987; Wilson *et al.*, 1988 a & b; Bovell *et al*, 1987; Bovell *et al*, 1988a).

PHYSICAL BASIS OF EPXMA.

The elemental content in sub-cellular volumes of sections can be localised or measured by biological EPXMA. The technique is based on the interaction of the focussed beam of electrons in the electron microscope with the atoms composing the area of the section being irradiated by the beam. The requirements for EPXMA are 1) a means of focussing the electron beam, 2) a means of detecting the emitted x-rays, and 3) the capability to visualise the specimen, (Moreton, 1981). The technique can therefore be performed using either scanning, transmission or scanning-transmission electron microscopes. In this thesis only features relevant to EPXMA in the TEM are discussed.

PRODUCTION OF X-RAYS.

The emission of x-rays is straightforward in principle. High energy incident electrons may strike on one or more of the bound, inner shell electrons of the material, displacing them from their orbits or energy levels. This is called inelastic scattering and generates two types of x-rays (figure 2.1): 1) characteristic, and 2) background.

CHARACTERISTIC X-RAYS.

An atom consists of a nucleus (positively charged) surrounded by "shells" of orbiting electrons. These shells are designated 'K', 'L' & 'M' etc. moving away from the nucleus. The binding energy of the orbiting electrons increases with increasing distance away from the nucleus.

The interaction of a high energy incident electron with an inner shell electron results in ejection of the bound electron from the atom. The atom is

then energetically unstable (or ionised) with a vacancy in the innershell, an event which may last approximately 10 ns (Borowitz & Beiser, 1971).

The resting state is restored when the vacancy is filled by an electron dropping from a higher energy orbital within the same atom. The excess energy, which exists between the starting and finishing 'states', is usually emitted as an x-ray photon. The energy of the x-ray photon is the difference in potential energy between the two orbitals involved in the transition, and is characteristic of that orbital transition for each element. The x-rays produced are named according to the shell where the original vacancy occurred. If the original vacancy was created in the K shell (innermost shell) the resultant x-rays are designated 'K' x-rays. Since the transition to fill the vacancy can occur from either the 'L' or the 'M' shells, and since these shells have different energy states, the 'K' x-rays are subdivided with the subscript α, β etc. to describe the origin of the transition (e.g. α; transition from ' L' shell).

The intensity of the emitted x-rays, which is a measure of the number of atoms of that element present in the irradiated volume, varies and in biological EPXMA the 'K' line is the most easily detected and is used to identify and quantify elements.

Only some inner shell ionisations result in the radiative emission of x-rays. The energy released during some transitions may be imparted to eject an electron from an outer orbit (Auger electron). The probability of a radiative transition (i.e. characteristic x-ray emission) is called the fluorescent yield (W). The fluorescent yield is strongly dependent on the atomic number of the element. The intensity of characteristic x-ray emission depends on the product of the fluorescent yield (W) and ionisation cross-section (Q). 'Q' is a function of the energy of the incident electron (E_o) and the critical ionisation energy (E_c) of the particular shell of the element concerned.

$$Q = \frac{7.92 \times 10^{-14}}{E_o E_c} \times \ln (E_o) \qquad \text{(Roomans, 1980)}$$

where E_o is the initial energy of the incident electron (keV), E_c is the critical ionisation energy (KeV). Overall, x-ray production (Ia) is therefore a product of 'W' and 'Q'.

$$Ia = W\,(N/A)\,Q\,\sigma\,dz \qquad \text{(Russ, 1975).}$$

Where W= fluorescent yield, N= avogadro's number, A= atomic weight, σ= density, dz= thickness of specimen and Q= ionisation cross section.

Background x-rays (also described as Continuum x-rays, Bremsstrahlung radiation and white radiation).

These are produced from inelastically scattered incident electrons. When an incident electron passes close to the electrostatic field surrounding the nucleus of an atom, the electron undergoes a deceleration and change in direction with a resultant loss of energy which can be from 0-100% of its original energy. This loss of energy is released as x-ray photons. The intensity of the background x-rays is inversely proportional to the accelerating voltage and is dependent on the total atomic number of the irradiated volume thus giving a measure of the local specimen mass thickness. When the average atomic number of the irradiated volume is known, the Bremsstrahlung is thus a function of section thickness.

Spatial Resolution.

Spatial resolution in bulk samples (i.e. specimens of thickness greater than the range of the incident electrons) depends upon the volume through which electrons will diffuse in a given sample or more precisely by that part of the range where electron energy is still above the x-ray excitation threshold. This in turn depends upon the accelerating voltage and the density of the specimen. Due to differences in critical excitation potential of different elements the volume of excitation will be different for every element at any particular accelerating voltage. As electrons diffuse through a sample they lose energy, and diffusion paths may be long, particularly in biological specimens. The shape of the volume of excitation varies with the average atomic number of the sample. In samples, with low atomic number such as

biological material the volume is pear shaped. The irradiated volume or beam broadening (b) can be calculated (Goldstein, Costley, Lorimer & Reed, 1977):

$$b \alpha 6.25 \times 10^2 (Z/E_0) (\rho/A)^{1/2} (t)^{3/2}$$

where A = atomic weight, Z = atomic number, ρ = density,

E_0 = accelerating voltage and t = thickness.

For most biological specimens the mean Z and density are so low that beam broadening can be ignored (Morgan, 1985). Thus in thin sections, of biological material, spatial resolution can be regarded as primarily dependent on the diameter of the incident beam (Goldstein *et al.*, 1977), particularly at high accelerating voltages.

Detection of x-rays.

There are 2 methods of collecting and counting x-rays leaving an irradiated specimen: 1). Wavelength-dispersive spectrometry (WDS); 2). energy-dispersive spectrometry (EDS). EDS nowadays has a much more widespread use in biology than the WDS (Russ, 1978; Morgan, 1985).

A major limitation of WDS is that the spectrometer is only able to detect a single band of x-rays (i.e. from a single element) leaving the specimen. This represents a major constraint in biological microanalysis where it is frequently necessary to perform multi-element analyses and in which the organic matrix is sensitive to radiation damage. The EDS, however, allows simultaneous display of all mid-energy (e.g. 1-20 KeV) x-rays collected during a single analytical period.

The EDS detector is essentially a wafer thin silicon crystal diffused with electron-donating lithium and maintained under vacuum and at liquid nitrogen temperature. Cryogenic cooling of the crystal is essential to reduce the mobility of the lithium and to reduce the electrical noise in the preamplifier. The silicon is coated at the ends with a thin (20 nm) contact layer of gold and is normally isolated from the microscope by a Beryllium window (~8 microns thick) which protects the crystal from deposition of extraneous microscope contamination, and absorbs back-scattered electrons. The silicon crystal acts

as a semi-conductor and converts the incident x-rays to an electrical signal which can be processed by a multi-channel analyser and computer. The major advantages and disadvantages of an EDS detector, compared to the WDS, have been previously described (Chandler, 1977). The major advantages are summarised as follows; 1) it can be placed closer to the specimen so that the specimen subtends a larger solid angle to the detector. This allows the system to detect a greater number of x-rays thereby reducing specimen damage by enabling lower probe currents to be used. 2) it simultaneously detects all characteristic x-rays and the background; 3) it is more sensitive than the WDS detector. Its major disadvantage is that the resolution between spectra (e.g. peak overlap in the detection of $K_{k\beta}$ and $Ca_{k\alpha}$) is much less accurate when using a WDS detection system. The EDS system is also disadvantaged by the presence of the Beryllium window and the gold coating which attenuates soft x-rays from low atomic number elements (e.g. Na). More recently new 'windowless' detectors have been developed and used, however, these tend to be expensive and are only suitable for electron microscopes with ultra clean vacuum systems. The EPXMA work in this thesis was performed using a Kevex (U.S.A) windowed EDS detector for x-ray detection with a Link Systems 290 microanalysis system.

Methods of Quantification.

A wide range of quantitative procedures are available for correcting measured x-ray intensities to yield the relative or absolute concentrations of elements in biological specimens. Corrections for atomic number effects (Z), x-ray absorption (A) and for x-ray fluorescence (F) (ZAF correction factors) are mandatory for bulk specimens. By contrast, quantification procedures for "analytically thin" specimens are relatively straightforward because they are all based on the "thin film" definition that the absorption of generated x-rays within thin sections is slight or negligible. Consequently, in thin specimens a measured characteristic x-ray intensity is always proportional to the amount of the element present per unit area.

The prevalent schemes for the deduction of the elemental concentration utilise either a combination of characteristic and continuum x-ray data (continuum-normalisation (C-N) method) (Hall, 1971; 1979; Hall & Gupta, 1982; 1984) or characteristic alone (C-A) (Dörge *et al.*, 1978; Rick, Dörge, Gehring, Bauer & Thurau, 1979; Rick, Dörge & Thurau, 1982; Hall & Gupta, 1982).

Continuum-Normalisation.

The peak to background (P/B) ratio of an element is related to its concentration in the analysed area of the specimen. As the continuum intensity is also dependent on the total composition of the analysed area of the sample, the P/B ratio is also influenced by the concentration of other elements in the sample. The method commonly used for quantitation in thin biological specimens is the continuum' method (Hall, 1971; Hall, Anderson & Appleton, 1973). The 'mass fraction' or elemental mass per unit tissue mass of an element (Cx), can be calculated as follows

$$Cx = Rx\ Ax\ (Nx / \Sigma NZ^2)_{st}\ Z^2/A_{sp}$$

where

Cx = mass fraction of element x,

Rx = $\frac{(\text{characteristic counts element x / continuum count})_{specimen}}{(\text{characteristic counts element x / continuum counts})_{standard}}$

Ax = atomic weight of element x

$(Nx/\Sigma NZ^2)_{st}$= the number of atoms of element x divided by the total of the number of atoms multiplied by the square of the atomic number in the standard.

Z^2/A_{sp} = a matrix factor to account for all the elements in the section including those light elements which do not produce characteristic x-rays (see Hall, 1971). According to the approximate theory of Kramers (1923), continuum intensity per unit mass thickness is proportional locally to Z^2/A).

In calculating values for background radiation it must be considered that this not only emmanates from the specimen but also from additional components such as the specimen mounts, supporting film and specimen holder. This extraneous component has to be subtracted from the total Bremsstrahlung radiation to obtain a precise determination of continuum.

The C-N method has a number of advantages:- 1) the results are unaffected by variations in section thickness and density neither of which can be accurately determined, 2) standards used need only be of known composition and thin in comparison to the specimen. The method is particularly suitable for the quantitative analysis of sections of freeze-dried material.

The major disadvantages lie in the assumption (discussed below) made in the equation $Cx = Rx\ Ax\ (Nx/\Sigma NZ^2)_{st}\ Z^2/A_{sp}$ and in the accuracy of measurement of the background. Firstly, the equation used to calculate the total mass of the section from the spectrum, is initially based on Kramer's (1923) approximate assumption. However, although a more accurate theory for background production now exists, the modified Bethe-Heitler equation (Chapman, Gray, Robertson, Nicholson, 1983; Nicholson & Chapman, 1983), it has been shown experimentally that the above expression is quite adequate for the range of compositions encountered in biology (Shuman, Somlyo and Somlyo,1976) calculated that the approximation for the background, is proportional to ΣNZ^2, and is adequate at least up to $Z = 21$ i.e. the elements of biological interest (Hall & Gupta, 1984). Secondly, the matrix correction Z^2/A is only an approximation for the elements not measured. Hall (1971) calculated the correction factor for the major elements not measured in a typical matrix of biological material (i.e. C,N & O) to be 3.28. Fortunately the variations in Z^2/A for different biological matrices are small (Hall, 1979) except for water (important in analysing frozen-hydrated sections), or such as mineralised tissues.

A series of important factors which can affect quantitation by the C-N method are contamination, mass loss and x-ray contributions from extraneous sources.

Contamination can result in the deposition of hydrocarbons on the surface of the specimen from the oil and grease vapours that circulate even in good vacuum systems. The effect is to reduce the quality of the image and absorb a proportion of the low energy x-rays emerging from the specimen. Contamination can also make a significant contribution to the apparent mass of the section and decrease the P/B ratio and the mass fractions of the characteristic elements. However, contamination can be limited, and is unlikely to be present in a 'clean' microscope system using an efficient anti-contaminator (Nicholson, 1981).

A focussed electron beam can remove anything between 10-90% of the mass of an irradiated volume of an organic specimen (Bahr, Johnson & Zeitler, 1965; Hall & Gupta, 1974) thus potentially introducing an intolerable error into any quantitative determination involving mass fractions. It has been reported that organic material is lost from an irradiated specimen both as an immediate and rapid consequence of the formation of radiation products (Stenn & Bahr,1970; Hall & Gupta, 1974). Mass loss normally found in organic matrices in biological EPXMA is 20-40% (Hall *et al.*, 1973; Hall, 1979) under most operating conditions. Bahr *et al.* (1965) reported that mass loss occurs at electron doses below those normally used in EPXMA. Calculations performed by Hall (1979) demonstrated that, for biological material, mass loss would occur at a dose of approximately 1.8×10^{-9} C/ μm^2, which is exceeded in almost all instances of practical EPXMA. However, beam induced mass loss can be significantly reduced by cooling the specimen to low temperatures (Hall & Gupta, 1974), although Egerton (1980) calculated that resistance to mass loss was improved by no more than a factor of 100 using low temperatures compared with room temperature analysis.

It is evident that errors in peak estimation will be reduced by lowering or eliminating extraneous x-ray contributions. There is a number of steps which

can be taken to reduce these contributions:- 1) by careful collimation of the electron beam, 2) by limiting the amount of solid material in close proximity to the specimen and constructing them from low atomic number materials such as aluminium (Nicholson, Gray, Chapman & Robertson, 1982) producing x-rays which can be measured and subtracted as described below.

To reduce the residual extraneous contribution from the specimen rod and immediate surroundings two different approaches have been adopted. The first is to coat all the surroundings with a heavy layer of C or Be (Panessa, Warren, Hren, Zadunaisky & Kundrath, 1978; Russ, 1977; Sauberman, Echlin, Peters & Beeuwkes 1981). However, although the use of C or Be may mask the instrumental peaks a considerable fraction of the measured Bremsstrahlung may be generated in such low atomic number elements (Nicholson & Chapman, 1983) which cannot be calculated.

The second approach (Nicholson & Dempster,1980) and Nicholson & Chapman (1983), is to construct remaining components from a material which will produce a characteristic peak which, with reference to a pure standard, would allow its contribution to be deducted from the specimen background. It should be noted that, to avoid a peak overlap, the material chosen should not produce its characteristic peak in a region of interest in the spectrum (Hall, 1971).

Characteristic.

A significant alternative to the continuum method, this is normally carried out using a peripheral standard which is cut and analysed at the same time as the specimen (Hall & Gupta, 1982; Dörge *et al.*,1978; Rick *et al.*,1979; Rick *et al.*, 1982). Using this method elemental contributions are derived solely from the characteristic x-ray intensity.

The calculation of concentrations is achieved by dividing the intensity (I) of the characteristic radiation in the specimen by the intensity of the characteristic radiation in the standard multiplied by the concentration (C) of the element of interest in the standard:-

$$C_{specimen} = (I_{specimen} / I_{standard}) \times C_{standard}$$

However, this technique is based on two assumptions which must be taken into consideration:- 1) that section thickness is uniform and equal to that of the standard in the areas used for analysis (Hall & Gupta, 1982). This also assumes that there are no section irregularities such as discontinuous chipping or cutting during cryosectioning and that no differential compression occurs during sectioning or manipulation of the sections; 2) that during sectioning, there is no differential shrinkage or swelling of the specimen or the standard (Hall & Gupta, 1982).

The great advantage of this technique is that it makes no use of the background radiation and so no errors are introduced into this measurement.

Figure Legends Chapter 2.

Fig. 2.1. Represents the generation of x-rays as produced in the electron microscope.

The right hand side of the diagram illustrates the generation of characteristic x-rays. An electron in the beam (1) impinges on an atom (2) which knocks out an electron from an inner atomic orbit. The incident (3) and the ionised (4) electrons leave the atom, and (5) an electron from an outer orbit "drops" into the empty orbit. The loss of energy in this transition is emitted as characteristic x-ray quantum.

The left hand side of the diagram illustrates the generation of x-ray continuum. An incident electron (A) is decelerated (B) resulting in the emission of a quantum (C) in the x-ray continuum.

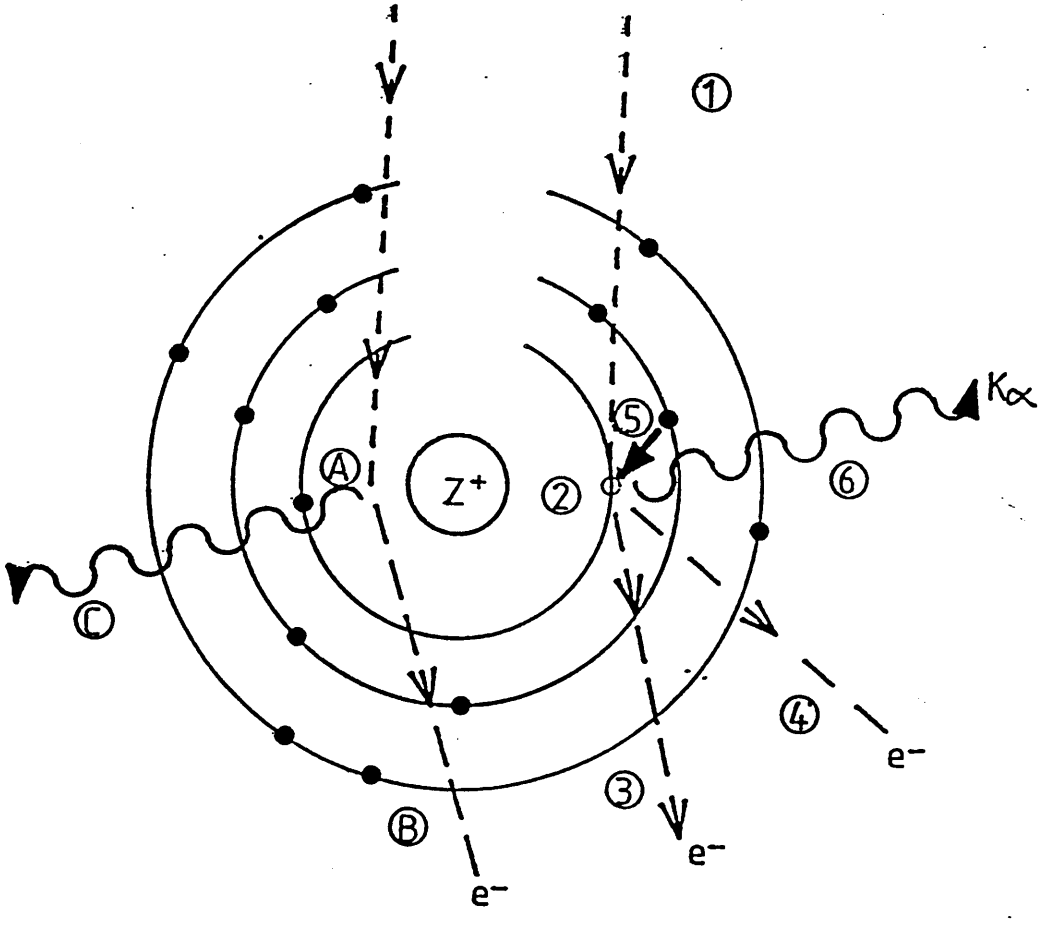
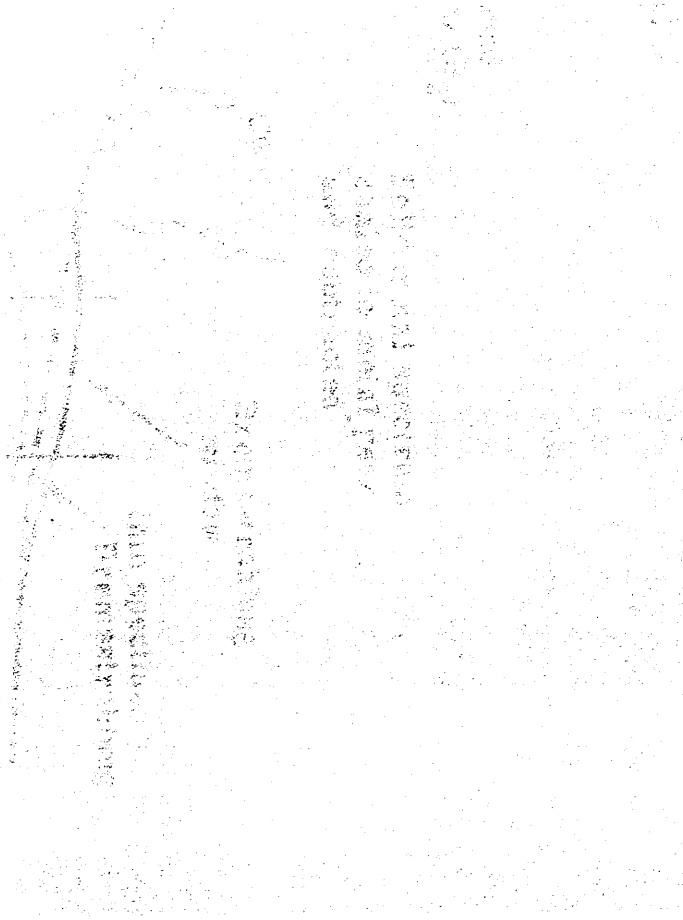
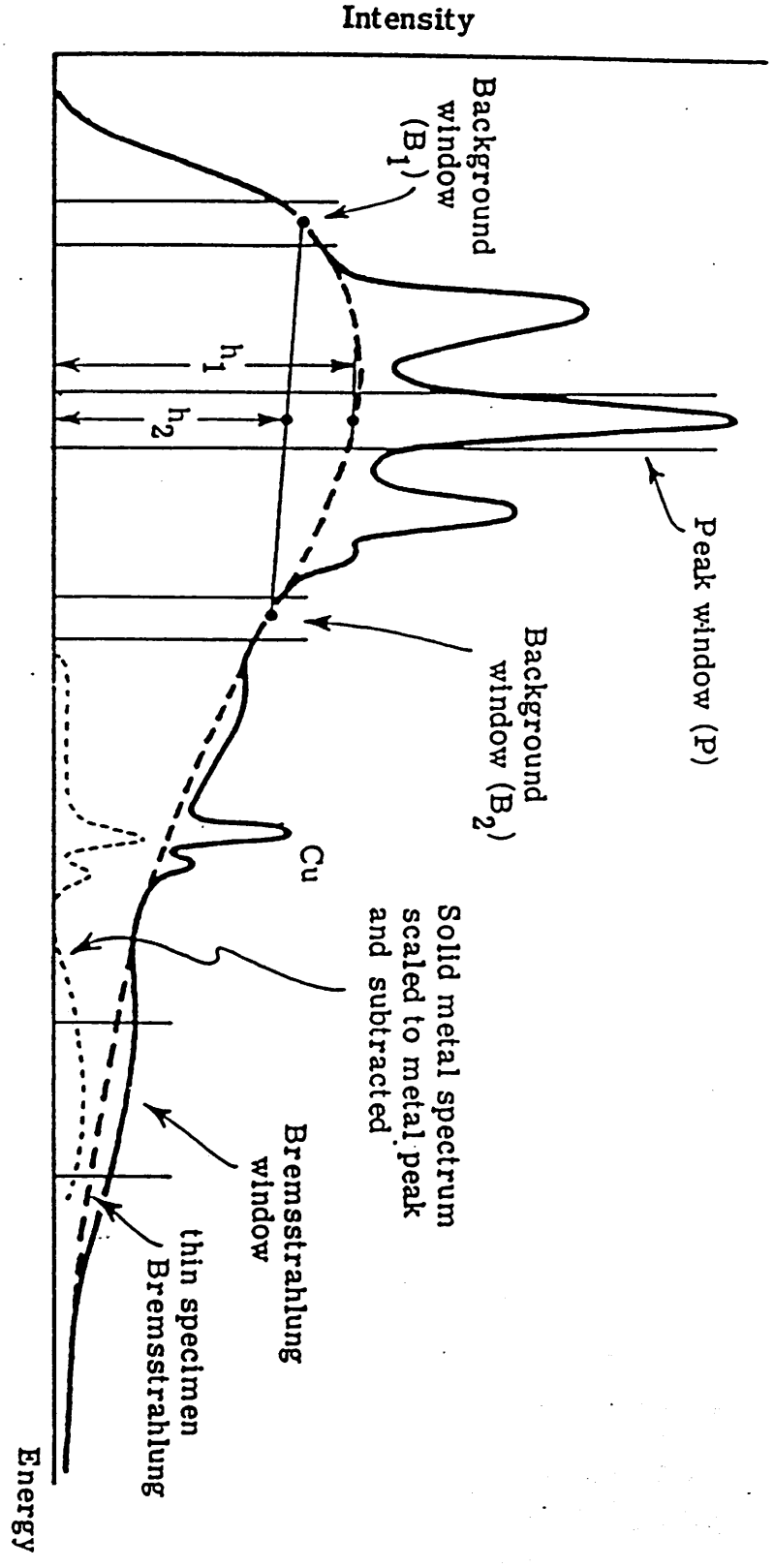

①
Kα
⑤
Ⓐ
Z+
②
⑥
Ⓒ
④
e⁻
③
Ⓑ
e⁻
e⁻

Fig. 2.2. Methods for calculating characteristic peak counts and correcting spectra for instrumental background / Bremsstrahlung after Nicholson & Dempster (1980).





Peak = $P - \frac{1}{2}(B_1 + B_2) \times k$

Correction factor, $k = h_1/h_2$

CHAPTER 3.

EPXMA OF THE INTRACELLULAR ELEMENTS IN THE SWEAT GLANDS OF THE COW, THE SHEEP AND THE GOAT, BEFORE, DURING AND AFTER THERMAL STIMULATION.

INTRODUCTION.

Different patterns of evaporative moisture loss from the skin surface have been demonstrated, amongst species which sweat in response to thermal stress, using ventilated capsules (figure 1.2) (McLean, 1963; Bligh, 1967; Allen & Bligh, 1969; Montgomery *et al.*,1982 a,b;1984). Three general patterns have been described (See Chapter 1). The smooth onset of sweating in the horse and human, contrasts with the stepwise pattern characteristic of the cow and the discrete discharges and rapid fatigue exhibited by sheep and goats. Bligh (1967) proposed a tentative explanation of these patterns on the basis of secretory rate and the role of the myoepithelium. In the horse and human, an example of the first type, he proposed that the pattern was due to increasing secretory rate with little or no myoepithelial involvement. The second pattern, exhibited by the cow, was interpreted as being due to an increasing secretory rate superimposed upon myoepithelial contractions and the third, exhibited by the sheep and goat, was proposed to be due to the rate of sweat expulsion exceeding that of production.

Although observations of the walls of glands present in the wing of the bat (Murphy,1960; Cortese & Nicoll, 1970), and of the sweat output from the human forehead (Nicolaidis & Sivadjian, 1972), support Bligh's (1967) hypothesis that myoepithelial contractions can occur upon stimulation, ultrastructural studies in the human, cow, sheep and goat (Jenkinson *et al.*, 1979; Montgomery *et al.*,1982; 1984) and pharmacological studies of the simian palm gland (Sato, 1977b, 1980; Sato, Nishiyama & Kobayashi, 1979) suggest that the myoepithelium acts as a support tissue, rather than being responsible for the expulsion of sweat from the gland. The variations in the modes of sweat output are therefore, more likely to be a result of species differences in the rate of sweat formation rather than of myoepithelial activity.

Previous studies (Jenkinson *et al.*, 1979; Montgomery *et al.*,1982 a,b; 1984) have shown that the mode of sweat formation is similar in the horse, human, cow and the sheep and goat, involving fluid transport, cell death and exocytosis of vesicles. It would, therefore, appear from ultrastructural studies (Jenkinson *et al.*, 1979; Montgomery *et al.*,1982 a,b; 1984), that the different patterns of sweat output can not be fully accounted for by cell death and loss of vesicles which were similar in all the species examined. The most likely explanation for the different patterns is variations in the electrolyte content of the fluid produced by the glands.

Results from electron probe x-ray microanalytical studies of horse and human sweat glands have demonstrated that, in common with other exocrine glands, the secretory cells lose potassium in response to thermal stimulation (Elder *et al.* , 1985; Wilson *et al.*, 1988a; Wilson *et al.*, 1988b). The studies of Elder *et al.* (1985) and Wilson *et al.* (1988 a & b) provide evidence that the active transport mechanisms found in the sweat glands are similar to those previously described in salivary and pancreatic acinar cells (Burgen, 1956; Schneyer, Young & Schneyer, 1972; Parod & Putney, 1978a & b; Izutsu & Johnson, 1986). The horse and human studies of Wilson *et al.* (1988 a & b) suggest that active transport mechanisms are modifying the primary fluid, secreted in the fundus, as it passes along the duct. These changes in the electrolyte content of the duct cells support the findings of comparative studies which demonstrated that primary secretion and the sweat that appears on the skin surface differ in their electrolyte content (Schultz, 1969; Quinton, 1981; Bijman, 1987). Other studies have noted that electrolyte concentrations found in surface secretions, change as sweating progresses (Johnson, 1970; Jenkinson & Mabon, 1973; Kerr, Munro & Snow, 1983). Therefore, if the increased rate of fluid output in response to thermal stimulation, is due to the involvement of active transport processes in the cow sheep and goat sweat glands, then these processes are likely to be reflected by altered intracellular ionic compositions of the fundus and ductal cells.

The glands of the sheep and goat, which respond poorly to heat stress, would be expected to show little intracellular elemental change upon activity

compared with those in the cow, in which changes comparable to those detected in the horse (Elder *et al.* 1985; Wilson *et al.*, 1988b), rat footpad (McWilliams *et al.* 1988) and man (McWilliams *et al.*, 1987; Wilson *et al.*,1988a) would be predicted. However, it is not known if the patterns of sweat output of the cow, sheep and goat reflect modulation of the mechanisms of sweat production so experiments were performed on the cow, sheep and goat to test this hypothesis.

MATERIALS & METHODS.

Procedure.

Seven 11 month old Ayrshire oxen, four adult castrated male Dorset Horn x Finnish Landrace sheep and four adult castrated British Saanen goats, were placed in a climatic chamber at a controlled environment of 15°C Dry Bulb (DB)/16°C Wet Bulb (WB) for a period of 3-4 hours. Cutaneous evaporative moisture loss was monitored continuously throughout, using the ventilated capsule technique (see figure 1.3) (McLean, 1963).

Skin samples (4 mm diameter) were removed, without anaesthesia, by biopsy using the high speed punch technique of Findlay and Jenkinson (1964) 1) prior to entry into the climatic chamber, and 2) before removal of the animals from the chamber 3-4 hours later. The patterns of output characteristic of each of the five species occur synchronously on both sides of the thorax (Findlay & Robertshaw, 1965; Allen & Bligh, 1969). Hence while sweating was monitored on a shaved area of skin overlying the eighth and ninth ribs, on the upper aspect of the body, towards the thoracic spinous process, skin samples were taken at known stages of output.

The following day the experiment was repeated in an experimental environment of 40°C DB/ 26°C WB designed to induce sweating. Skin samples were taken prior to entry into the chamber, at the onset of sweating and 2 hours later.

Sample preparation.

Each sample was sliced perpendicularly to the skin surface, into four strips (1mm wide) which were rapidly cryoquenched in liquid propane, containing 20-25% iso-pentane, at 87 K by the method of Elder *et al.* (1982) and stored in liquid nitrogen.

All specimens were freeze-dried for three days at 195 K under a vacuum of 1 x 10^{-6} torr in the presence of freshly activated molecular sieve (type 3A, BDH Chemicals Ltd.). After drying, the specimens were slowly warmed to room temperature (10^{o} hr^{-1}) and infiltrated with a degassed, non polar resin, whilst under vacuum. The tissue samples were then removed from the freeze-dryer and placed in fresh resin for 48 hours before polymerisation in fresh resin at 60°C. Sections from the polymerised blocks were cut dry using a diamond knife at one micron and stained with 1% Toluidine Blue in distilled water, for light microscopical identification of suitable gland profiles. Once suitable gland profiles had been located, unstained sections, approximately 150 nm thick, were mounted on formvar coated single hole titanium mounts, coated with a 20 nm layer of carbon (Temcarb 500 coating unit, EMScope Ltd, U.K.) and used for microanalysis.

EPXMA.

Sections from each animal group were examined in a modified TEM adapted for EPXMA (Nicholson *et al.*, 1982). Gross morphological features of the glands, such as, secretory cells, myoepithelium and nuclei were identifiable in spite of the lack of contrast and decreased resolution in the unfixed and unstained sections. The titanium mounts, with specimen, were loaded into the microscope using a side entry specimen rod, which was then tilted by 30^{o} from the horizontal towards the detector. The spectrometer was then placed to within 31 mm of the specimen to maximise the collection of x-rays from the sample.

Analyses were performed for one hundred seconds at room temperature with an electron beam accelerating voltage of 80 KeV, using a Link Systems 290 EDS system (Link Systems PLC, U.K.) incorporating a Kevex x-ray

detector (Kevex Corp, U.S.A.). The probe diameter was approximately 200 nm. Beam current was approximately 0.4 nA as measured by a Keithley electrometer (Keithley Electronics, UK.) connected to a collection plate within the microscope column (Nicholson, 1981).

The continuum radiation recorded from the sections includes a contribution from the resin (McWilliams Ph.D. Thesis) and so the calculated mass fractions do not equate directly with those obtained from freeze-dried sections (Izutsu & Johnson, 1986). As tissue water is replaced by the resin (Ingram & Ingram, 1983; 1984; Meyer, Schmidt & Zierold, 1985) the mass of the specimen will approximate to the mass of hydrated sections.

Thirty spectra were recorded from the secretory epithelia of each profile. All spectra were analysed using the continuum normalisation procedure (Hall, 1971) and standardised as described by Hagler, Lopez, Lundswick & Buja (1983) using amino plastic resin impregnated with appropriate salts as standards (Roos & Barnard, 1984). The chloride content of the resin (McWilliams Ph. D. Thesis, Glasgow, 1987), calculated from analyses of thin sections prepared from pure resin, was automatically subtracted, from the acquired data, by quantification routines for the computer. The data were examined by analysis of variance and resulting differences were analysed using students paired 't' test.

RESULTS.

Patterns of evaporative moisture loss.

Placing the animals in a warm environment caused measureable increase in cutaneous evaporative moisture loss in contrast to the control environment, where cutaneous moisture loss was not detectable. The patterns of this loss, recorded from each of the animals used (figures 3.1 & 3.2), were similar to those previously described (Bligh, 1967; Allen & Bligh, 1969; Jenkinson *et al.*, 1979; Montgomery *et al.*,1982 a,b; 1984), and the underlying mechanism of sweat production, could be regarded as representative of each species.

EPXMA.

Each set of thirty spectra collected were regarded as individual homogeneous populations and the means for each group were calculated (Tables 3.1 & 3.2). Representative spectra from the fundus of each species are shown in figure 3.3. The intracellular elemental concentrations of sodium, potassium and chlorine for each animal in the experimental groups are shown in Appendix 1a & b.

1) The fundus.

The results obtained for the fundus of each animal are given in Table 3.1 and shown in Figure 3.4.

In the fundus of the cow, the concentration of sodium rose significantly ($p<0.02$) at the onset of sweating but in spite of continued sweating had returned to the pre-stimulation level two hours later. There were no significant alterations in the cellular concentrations of potassium or chlorine in response to the thermal stimulus, although the pattern exhibited by the chlorine values resembled that for sodium.

In the sheep and goat no statistically significant changes were detected in the concentrations of sodium, potassium or chlorine in secretory cells during thermal stimulation although again the cellular concentrations of chlorine, like those of the cow, were elevated at the onset of sweating.

2) The duct.

The results obtained in the analyses of the ductal epithelia of each animal are given in Table 3.2 and shown in Figure 3.5.

The only significant change ($p<0.02$) in the mean elemental values (sodium, potassium and chlorine) in the ductal epithelia of the cow, sheep and goat, in response to thermal stimulation was a fall in the chlorine concentration in the cells of the sheep at onset (Fig. 3.4) . In both the cow and the sheep intracellular sodium values fell at the onset of sweating, however, this fall was not significant.

DISCUSSION.

The finding, in this study, of a secretion-related increase in the intracellular sodium content, of the secretory cells in the bovine fundus, is consistent with previous reports from other exocrine glands (Burgen, 1956; Schneyer, Young & Schneyer, 1972; Izutsu & Johnson, 1986). The increase (127%) in sodium content of the secretory cells during activity compares with the findings in the rat plantar gland (138%, McWilliams *et al.*, 1988) but is higher than the values found in the human (74%, Wilson *et al.*, 1988b) and the equine sweat gland (48%, Wilson *et al.*, 1988a).

The increase in intracellular Na provides evidence of a secretory response to thermal stimulation in cattle and supports the hypothesis (Bligh,1967) that active secretion contributes to the pattern of sweating in the cow. Despite maintained sweat output, the transient nature of the rise in intracellular sodium and hence transepithelial sodium flux, suggests that the bovine secretory cells can compensate for large excursions of intracellular sodium concentrations during sweating.

Loss of potassium from active secretory cells has been well documented in a number of exocrine glands (Burgen, 1956; Petersen, 1970; Putney, 1976; Singh, 1984; Izutsu & Johnson, 1986). Failure to detect a significant change in intracellular potassium, in either the fundus or duct of the cow, is not necessarily indicative of lack of involvement of this element in bovine sweat formation since a change in the intracellular concentration of an element can be detected only when efflux exceeds influx. The net flux of this element must have increased in the fundus since its concentration on the skin surface of sweating cattle is significantly elevated (Johnson,1970; Jenkinson & Mabon, 1973).

In many exocrine glands the influx of sodium into the cell also moves chloride in via a Na/K/2Cl cotransporter (Welsh, 1987; Petersen & Maruyama, 1984). Results of this study, in common with other studies, have demonstrated an increase in intracellular chloride during activity (Sasaki, Nakagaki, Mori & Imai, 1983; McWilliams *et al.*, 1988; Wilson *et al.*, 1988a; 1988b). Although, it

was not possible to detect a significant change in intracellular chlorine, the output of this element is also known to increase on the skin surface during sweat formation by cattle (Jenkinson & Mabon, 1973).

The absence of significant changes in the intracellular concentrations of Na, K and Cl in the fundus of either the sheep or goat indicates either that secretory rate was not influenced by thermal stimulation or that the influx and efflux rates of all three elements rose in parallel. The latter seems much less likely, and therefore to that extent the results support the hypothesis of slow continuous secretion (Bligh, 1967). However, in view of preliminary evidence of altered Na/K ATPase distribution and activity in the secretory cells of the sheep on thermal stimulation (Elder *et al.*, 1987), the possibility of a small increase in secretory output in response to heat, as concluded by Jenkinson and Robertshaw (1971), cannot be excluded.

Unlike the ducts of the cow and goat which exhibited no significant alterations in the values for the intracellular electrolyte elements there was a significant fall in the concentration of Cl in the sheep on thermal stimulation. The reason for this fall, which contrasts with the rise in Cl concentration in the duct of the horse (Elder *et al.*, 1985) and man (Wilson *et al.*, 1988b) and which was not found in the goat, is unknown. The evidence from this study of intracellular elemental composition does not provide any clear evidence on the role of the duct in ungulate sweat formation. However, the fall in the level of Cl^- would suggest that some active transport mechanism is activated in the sheep ductal cells, upon thermal stimulation, and adds further support to Jenkinson and Robertshaw's (1971) conclusion that there is a small increase in sweat output in response to heat.

It has been described (Thaysen, 1978) that the responsiveness of glands from one particular animal and from the same skin location, can vary considerably and may be the reason for the variation (as measured by the standard error) in concentrations of the elements within each group of animals. It is also possible that biological variation between animals may also account for the variation. In both sets of conditions it is extremely likely that the

glands will not all be at the same state of activity and will therefore have different elemental concentrations. Also to detect a change in the intracellular concentration of an element using EPXMA, the rate of influx or efflux must exceed the other. However, these aspects can be overcome using radiotracer flux experiments which have successfully been used in other exocrine glands (Putney, 1976; 1977; 1978; Parod & Putney, 1978a; Aub *et al.*, 1982; Singh, 1984). Tracer flux measurements can often detect transient changes in permeability that do not result in a net concentration change. Likewise sustained but small changes in permeability not distinguishable by electrophysiological measurements, can often be discerned by following the kinetics of tracer exchange.

The use of isolated glands, which can now be obtained (Lee, Jones & Kealey, 1984) in combination with tracer flux measurements offers an opportunity to examine elemental fluxes in sweat glands.

Although Bligh's hypothesis on the patterns of sweating was based on secretory activity and myoepithelial involvement the results of this study demonstrate that secretory activity contributes to the patterns of sweat secretion and suggests that in the cow the myoepithelium does not play a major role in sweat output. However, the results in the sheep and goat sweat glands neither prove or disprove myoepithelial involvement in sweat expulsion but they confirm low secretory activity. Therefore, the role of myoepithelium in sweat expulsion has still to be clarified but because of its minor role in sweat output, will not be examined in any further detail in this thesis.

TABLE 3.1.

Intracellular elemental concentrations of Na, K and Cl in the funduses of cow, sheep and goat sweat glands, before, at the onset of, and during the sustained phase of evaporative moisture loss. Refer to Appendix 1a.

ANIMAL.

COW.	'n'	Na	K	Cl
Before	5	11 ± 4	146 ± 9	40 ± 7
Onset	5	25 ± 5	157 ± 27	58 ± 9
Plateau	5	10 ± 2	145 ± 7	41 ± 7
SHEEP.				
Before	3	7 ± 3	167 ± 13	38 ± 11
Onset	3	7 ± 2	158 ± 17	50 ± 12
Plateau	3	10 ± 5	130 ± 44	37 ± 13
GOAT.				
Before	3	9 ± 3	142 ± 15	39 ± 4
Onset	3	13 ± 9	104 ± 18	62 ± 14
Plateau	3	13 ± 7	95 ± 14	52 ± 6

Values are the mean ± s.e.m and the units are mMoles/Kg dry wt. 'n' represents the number of animals used from each group. Chlorine values are corrected for the chlorine content of the resin.

TABLE 3.2.

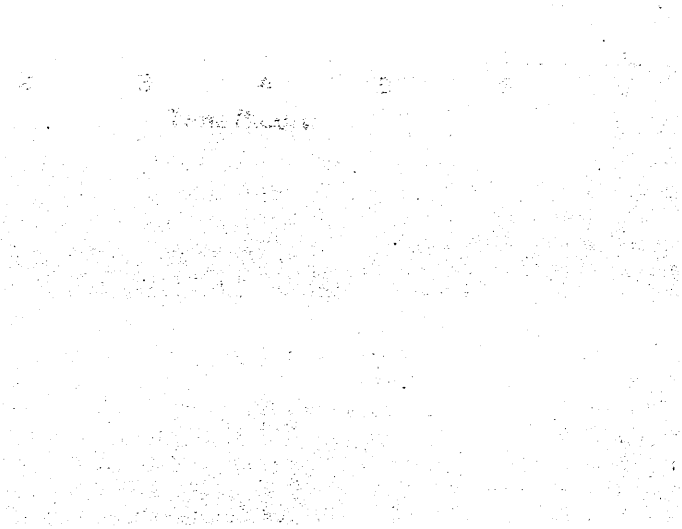
Intracellular elemental concentrations of Na, K and Cl in the ducts of cow, sheep and goat sweat glands, before, at the onset of, and during the sustained phase of evaporative moisture loss. Refer to Appendix 1b.

ANIMAL.

COW.	'n'	Na	K	Cl
Before	5	27 ± 10	116 ± 16	58 ± 11
Onset	5	16 ± 4	107 ± 17	56 ± 9
Plateau	5	26 ± 8	110 ± 14	56 ± 11
SHEEP.				
Before	3	18 ± 6	131 ± 10	69 ± 9
Onset	3	11 ± 3	117 ± 15	50 ± 5
Plateau	3	17 ± 5	131 ± 14	82 ± 17
GOAT.				
Before	3	10 ± 4	141 ± 21	54 ± 10
Onset	3	15 ± 7	116 ± 22	59 ± 6
Plateau	3	12 ± 11	116 ± 11	38 ± 17

Values are the mean ± s.e.m and the units are mMoles/Kg dry wt. 'n' represents the number of animals used for each group. Chlorine values are corrected for the chlorine content of the resin.

Fig 3.1. The patterns of cutaneous evaporative moisture loss from four oxen in an environment of 40°C DB / 26°C WB. Samples of skin were removed from the contralateral side of the animal at the times indicated by the arrows.



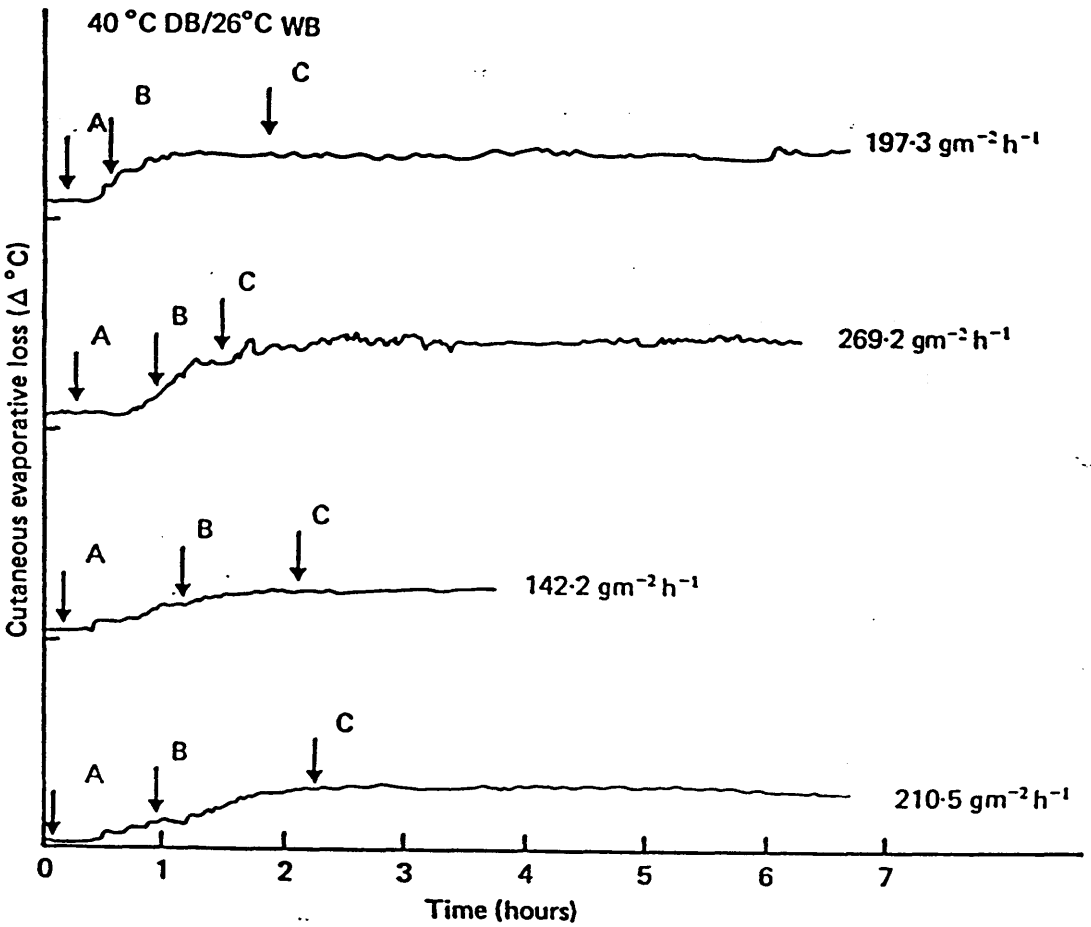
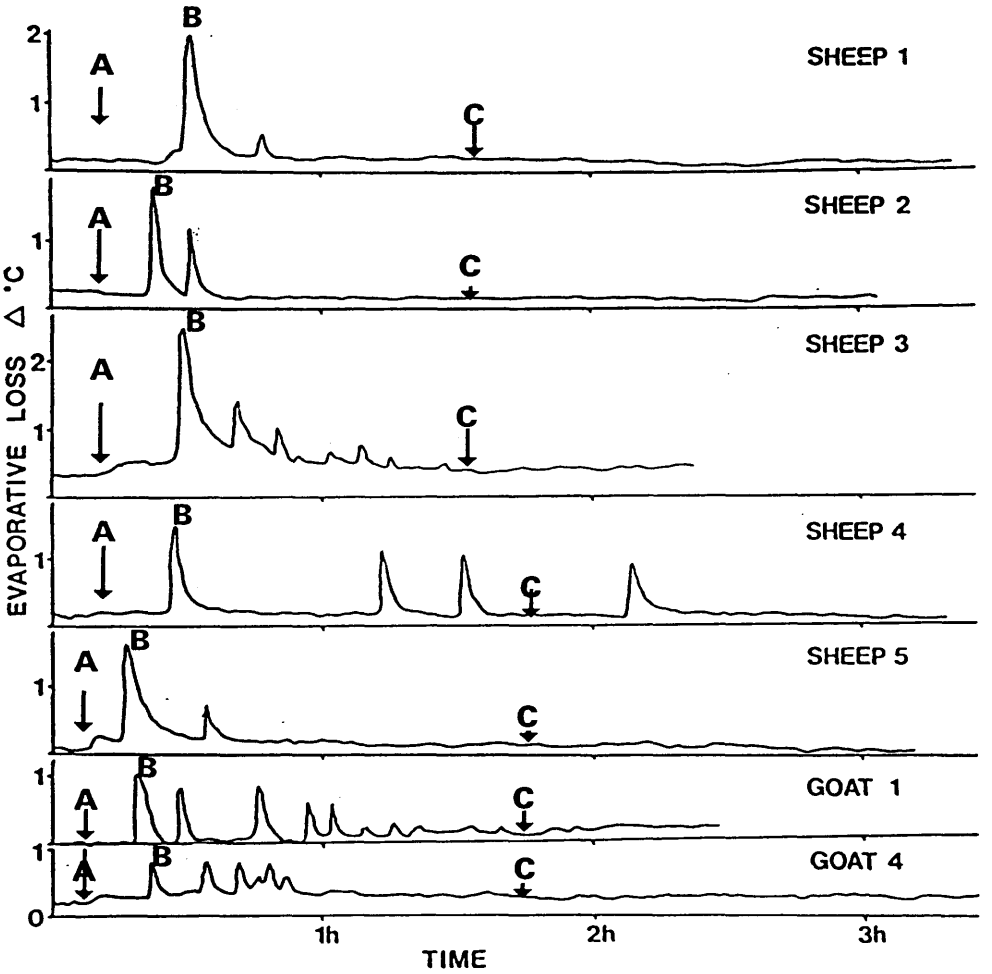

40 °C DB/26°C WB
A
B
C
197·3 gm⁻² h⁻¹
269·2 gm⁻² h⁻¹
142·2 gm⁻² h⁻¹
210·5 gm⁻² h⁻¹
Cutaneous evaporative loss (Δ °C)
0
1
2
3
4
5
6
7
Time (hours)

Fig. 3.2. The patterns of cutaneous evaporative moisture loss from 5 sheep and 2 goats in an environment of 40°C DB / 26°C WB. Skin samples were taken from the contralateral side at the times indicated by the arrows.


SHEEP 1
SHEEP 2
SHEEP 3
SHEEP 4
SHEEP 5
GOAT 1
GOAT 4
A
B
C
EVAPORATIVE LOSS Δ °C
TIME
1h
2h
3h

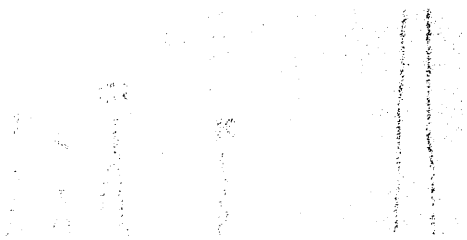
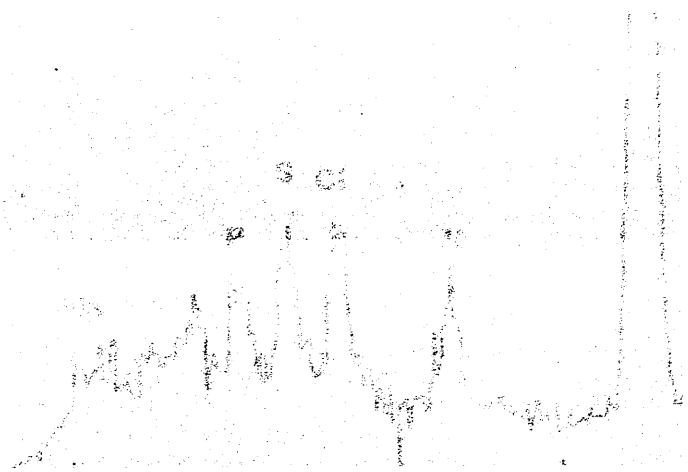


Fig. 3.3 Shows a typical spectrum from the secretory cells present in the fundus of the cow (A), sheep (B) and goat (C), as recorded by the EDS used in this study. Characteristic peaks (Na, P, Cl, S, K & Ti) and the background on which they are superimposed are illustrated.



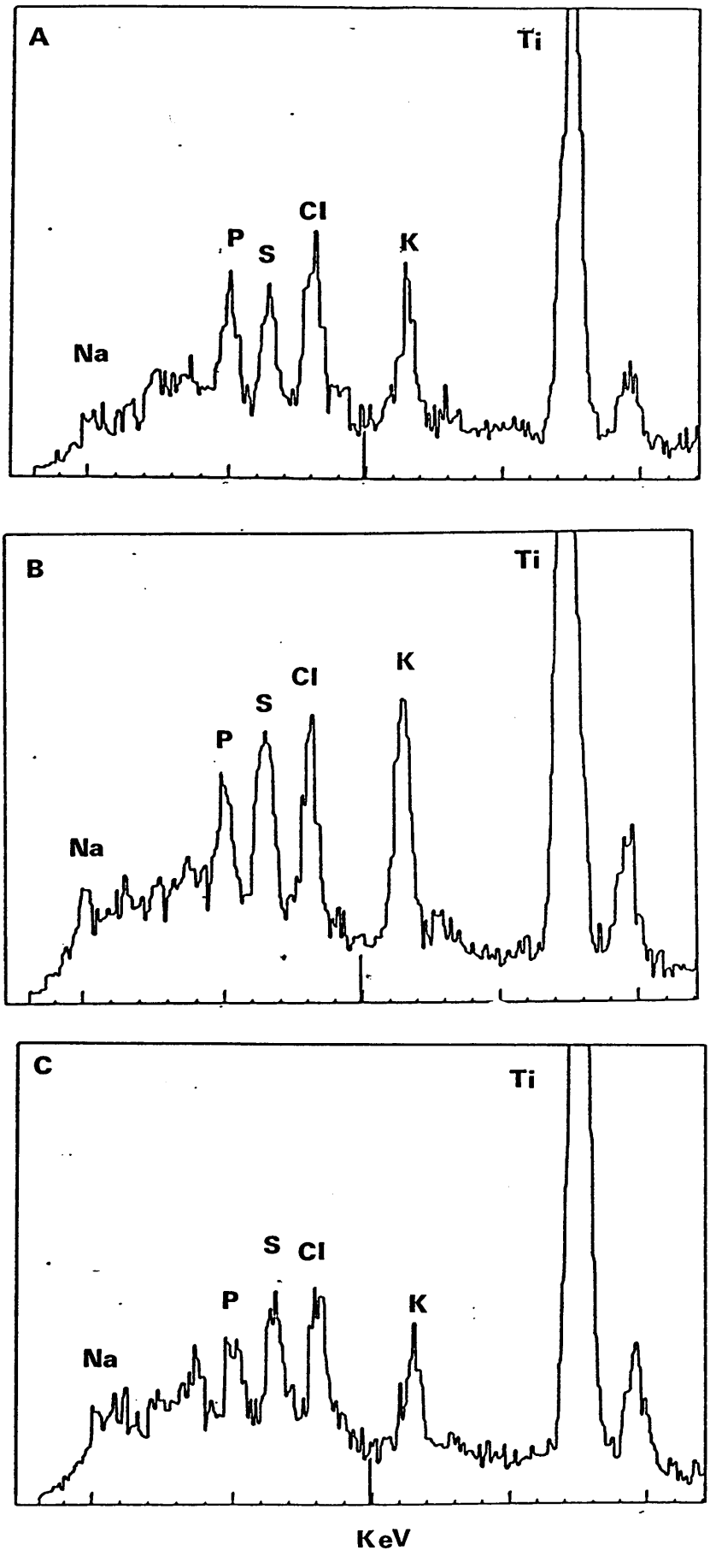

A
Ti
Cl
P
S
K
Na
B
Ti
Cl
K
S
P
Na
C
Ti
S
Cl
P
K
Na
COUNTS / CHANNEL
KeV

Fig. 3.4. The mean concentrations of Na, K & Cl (± standard error) in the secretory cells of the fundus of cow, sheep and goat sweat glands before (B), at the onset (O) of and after two hours of continuous sweating (C). "n" refers to the number of animals. ** = $P<0.02$.

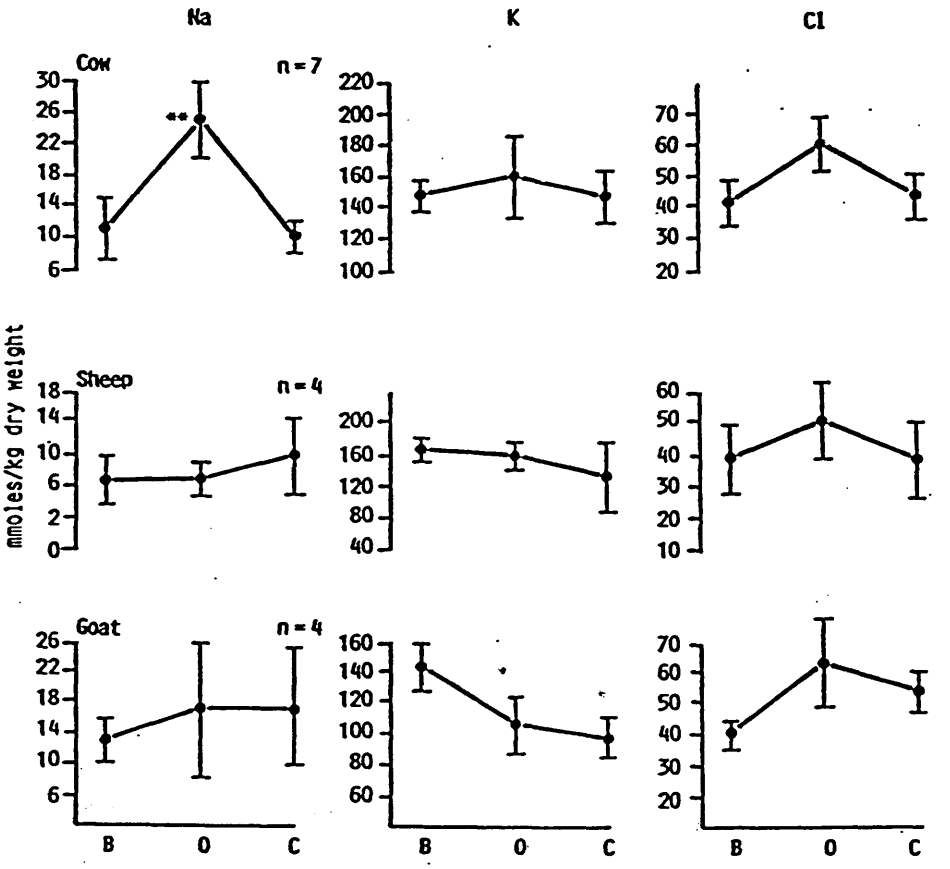
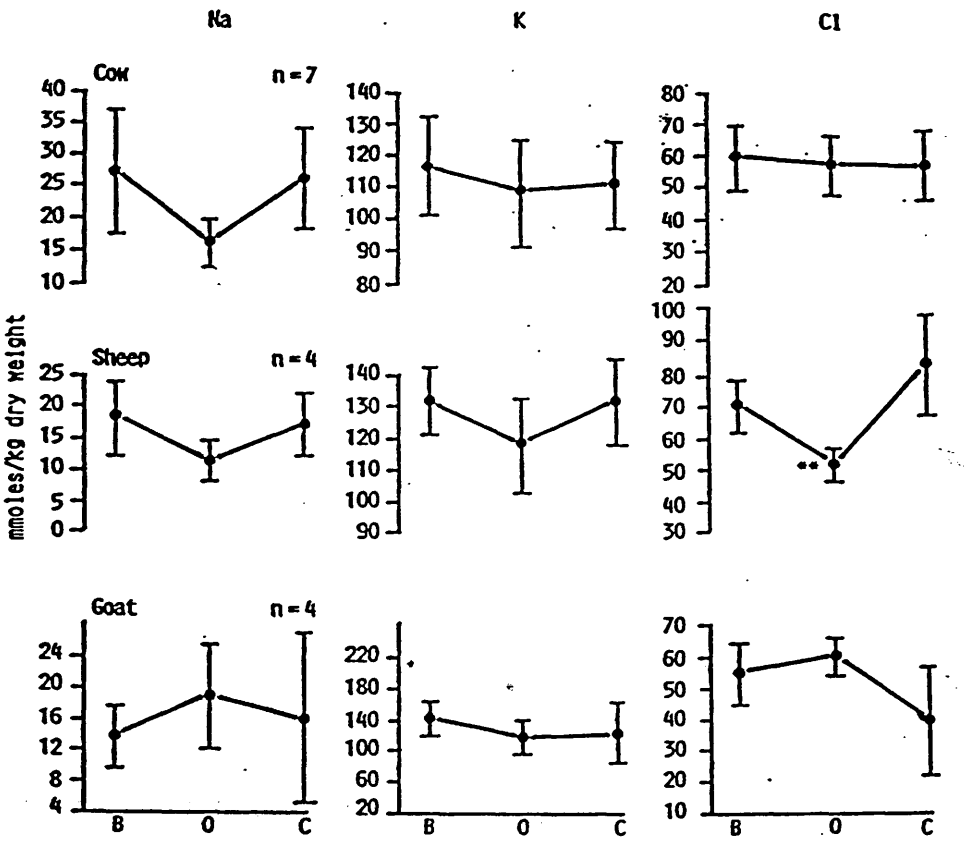

Na
K
Cl
Cow
n = 7
Sheep
n = 4
Goat
n = 4
**
mmoles/kg dry weight
B
O
C

Fig. 3.5. The mean concentrations of Na, K & Cl (± standard error) in the epithelial cells of the duct of cow, sheep and goat sweat glands before (B), at the onset (O) of and after two hours of continuous sweating (C). "n" refers to the number of animals. ** = $P<0.02$.


Na
K
Cl
Cow
n = 7
Sheep
n = 4
Goat
n = 4
mmoles/kg dry weight
**
B
O
C

CHAPTER 4.

THE BASIS OF RADIOISOTOPIC FLUX STUDIES.

INTRODUCTION.

The main aim of the isotope experiments was to examine the ionic fluxes associated with the secretory process in exocrine glands. One of the most versatile ways of investigating these phenomena is to use a radioactive form of the element under investigation. A small quantity of radioactive tracer, is mixed with a much larger amount of its non-radioactive counterpart, and the behaviour of the two in the experimental system is then assumed to be identical. As the tracer can be detected by its radioactivity, it provides information about the non-radioactive form.

Radioisotopes are characterised by the presence of more or fewer neutrons than are present in the nuclei of the naturally occurring stable isotope of the element. Such nuclei are unstable, and they tend in time to change into stable configurations by various processes known collectively as radioactive decay. The primary radioactive decay process is always either the emission of a charged particle (α, β^+ & β^-) or the capture by the nucleus of an orbital electron. These captures are often accompanied by electromagnetic radiation in the form of γ-rays or x-rays.

Quantities of most ordinary materials are determined by weight or size. With radioactive substances, however, the weights of the radioisotopes involved are usually extremely small, and the radionuclides themselves cannot easily be isolated from the stable isotopes of the same element. Consequently, determination by weight is not usually possible and so special methods have to be used. In practice radioisotopes are nearly always specified on the basis of their 'activity' which is the disintegration rate of the radioactive source. Activity can be quoted in Becquerels (Bq; 1 Becquerel = 1 disintegration per second) or Curies (Ci; 3.7×10^{10} Bq) (and their respective derived units e.g. KiloBq, MegaBq, GigaBq; milliCurie, microCurie, nanoCurie etc). Activity is not constant with time but decays at a rate which is

characteristic of the radionuclides present. The time required for the activity of a radioisotope to decay to half its initial value is termed its 'half-life'. The half-lives of known radioisotopes vary from fractions of a microsecond to thousands of years. Therefore, activity is only true at a specified time, known as the 'Reference Date'.

EXPERIMENTAL PROTOCOL.

The isotope used in this study was selected on the basis of previous studies which demonstrated that a K^+ efflux accompanies secretion in exocrine glands (Burgen, 1956; Petersen, 1970). This efflux has been successfully examined by isotope flux experiments using potassium-42 (^{42}K) as a tracer for potassium transport (Putney, 1976).

There are however several disadvantages to the use of ^{42}K or ^{43}K for the study potassium fluxes, particularly their short half-lives (12.4 & 22.4 hours respectively). Therefore any experiment which, from beginning to end, lasts for more than the 12.4 or 22.4 hours, would require correction for specific activity. Moreover ^{42}K & ^{43}K are expensive and repetitive experiments require substantial amounts of isotope. Both ^{42}K & ^{43}K are not pure β emitters, they also emit a proportion of γ rays and thus have gamma coefficients (1.4 & 5.6). The higher the gamma coefficient the more hazardous they are to work with and the more important aspects of safety become (see appendix 2).

Fluxes of K^+ ions across biological membranes can also be assessed using Rubidium-86 ($^{86}Rb^+$). Rubidium belongs to the alkali metal series of elements and rubidium ions behave in many aspects physicochemically and biologically like potassium. $^{86}Rb^+$ has a half life of 18.7 days, has a lower gamma coefficient (0.5), is much less expensive and has thus been used quite extensively as a marker for potassium transport in exocrine cells (Putney, 1976; 1977; 1978; Parod & Putney, 1978; Marier, Putney & Van de Walle, 1978; Singh, 1984). However, although it has been shown that K^+ channels of salivary glands have an extremely low pure rubidium conductance (Gallacher, Maruyama & Petersen, 1984), $^{86}Rb^+$ is a reliable indicator of potassium

transport when the [Rb^+/ K^+] ratio in the cytoplasm is low (Gallacher *et al.*, 1984).

Figures 4.1 and 4.2 illustrate the superfusion apparatus.

Samples were transferred to a flow cell (figure 4.2) (volume 120µl), (Apollo Biomedical Engineering, U.K.), where they were continuously superfused with a bicarbonate-buffered physiological saline, gassed with 95% O_2 & 5% CO_2, at 2mls min^{-1} using a peristaltic pump (LKB Produkter, Sweden.)(figure 4.1). They were maintained at 37°C by immersing the flow chamber in a water bath and by using superfusion media warmed to 37°C, as it passes through coils of tubing submerged in the water bath. Thermocouple measurements established that the media had reached 37°C upon entering the flow chamber.

Loading with rubidium.

Samples were loaded with $^{86}Rb^+$ by continuous loop superfusion for 1 hour with bicarbonate-buffered physiological saline containing 0.1-0.3µCi ml^{-1} of $^{86}RbCl$ (Amersham Int. PLC., U.K.). The specific activity of the solutions used varied between 385 and 20 Ci per mol, depending on the number of days after the reference date. The total RbCl concentration of the solutions also varied accordingly from 0.97-3.99 µmol l^{-1} (see Table 4.1). Perspex was use to shield against the β emissions of $^{86}Rb^+$, while lead was used as a shield against emitted gamma rays.

After the loading of the glands with $^{86}Rb^+$, superfusion was continued with unlabelled medium. The remains of the loading solution was 'washed' out of the system for 2 minutes prior to collection of sequential samples every 30 seconds. Effluent samples were collected into vials automatically using a 'Redirac' fraction collector (LKB Produkter, Sweden). At the end of the experiment glands were recovered from the flow chamber and dissolved in 2ml of concentrated nitric acid.

Data Analysis.

Each effluent sample and aliquots of the dissolved tissue were mixed with 5 mls of scintillation fluid and the amount of radioactivity in each sample was determined by liquid scintillation counting (see Appendix) on a Tri-Carb 300 counter (Packard Ltd, U.K.). Aliquots of the superfusion medium taken before and after each experiment, were used to determine background radiation. The instrument was programmed for the automatic subtraction of background radiation and 'windows' were set to detect the energy values of $^{86}Rb^{+}$ thereby reducing the component of electrically generated noise from the instrument.

The sum of the activity present in all the samples of effluent plus the tissue's residual activity gave a measure of the $^{86}Rb^{+}$ content present in the samples just prior to collection.

First-order rate coefficients for $^{86}Rb^{+}$ efflux were calculated from these data (Putney,1976) as follows:

$$kn = \Delta \ln\% \, n \, / \, \Delta tn$$

where kn is the rate coefficient over the 'n' th interval, $\Delta \ln\%$ n is the change in the natural logarithm of the percent radioactivity remaining in the tissue over that particular interval and Δtn is the length of time of each interval. A computer programme (written by Dr S.M. Wilson for use on a BBC Master computer) based on this formula, enabled automatic calculation of the rate constant for each interval and the percentage activity remaining. The programme also calculated the disintegrations per minute (DPM's) for each sample. A sample result sheet is shown in Appendix 4.

It was possible to calculate the amount of Rubidium taken up by each sample since the counting efficiency of the counter (see Appendix 4) and the DPM's for each sample were known.

See Appendix 5 for details of disposing of radioactive waste.

Table 4.1.

The table shows, the weight (in μg ml^{-1}) and the molarity (mmol l^{-1}) of Rb^+ in each batch of isotope used. The concentration of Rb^+ (μmol l^{-1}) in the 'loading' solution, prepared from each batch of isotope, is given in the last column.

Rb batch	μg ml^{-1} Rb	mmol l^{-1} Rb	μmol l^{-1} [Rb]
1	221	2.60	0.97
2	392	4.61	1.71
3	399	4.69	1.74
4	465	5.47	2.03
5	496	5.83	2.16
6	855	10.06	3.74
7	912	10.73	3.99

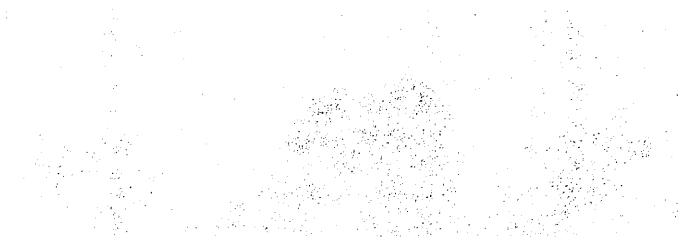


Figure 4.1. The photograph shows the superfusion apparatus involved in the radioisotopic experiments. Isolated sweat glands were harvested into the flow chamber (FC) and then superfused at 37°C by immersing the chamber into a water bath (WB). The glands were superfused at a rate of 2ml min^{-1} using a peristaltic pump (PP). Effluent samples were collected automatically every 30 seconds, using a 'Redirac' fraction collector (RFC).

Figure 4.2. The photograph shows the internal sections of the flow chamber.

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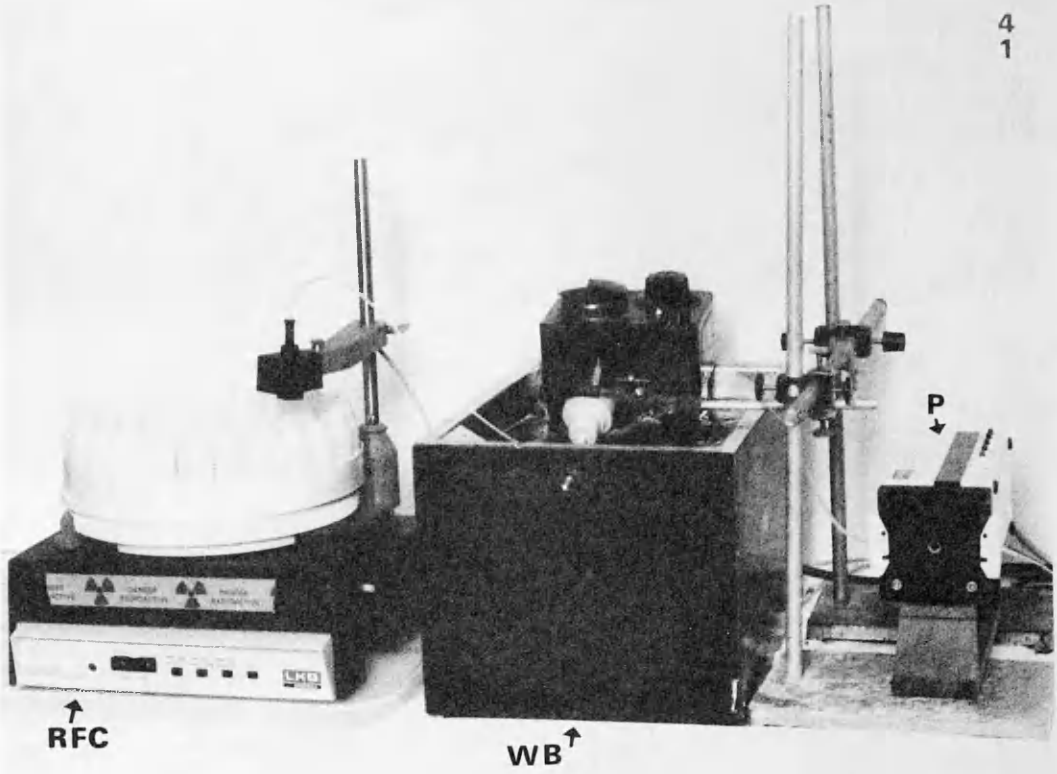
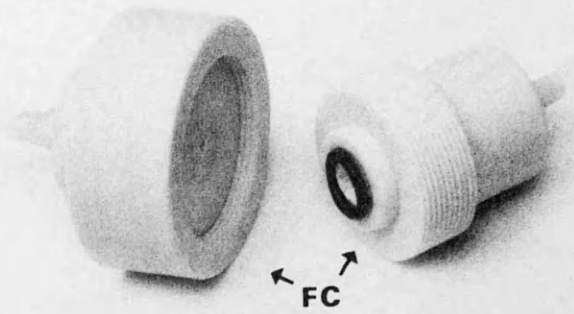


42



CHAPTER 5.

RADIOISOTOPIC FLUX STUDIES ON THE ISOLATED HUMAN SWEAT GLAND AND THE EFFECTS OF CHOLINERGIC AND α & β-ADRENERGIC AGONISTS.

Introduction.

Fluid secretion in mammalian exocrine glands (as described in chapter one) relies on the secondary active transport of Cl^- from the interstitium to the lumen. One of the major ionic movements of this mechanism is the loss of K^+ from the cell which causes a repolarisation, and maintains the driving force for secretion. The increase in K^+ permeability of the cells in response to stimulation has been well documented in salivary glands and can be induced by nerve stimulation (Burgen, 1956) or evoked by direct stimulation with acetylcholine or adrenaline (Petersen, 1970; Batzri, Selinger & Schramm, 1971). Douglas & Poisner, (1963) demonstrated that salivary secretion induced by acetylcholine and adrenaline was dependent on the presence of calcium ions. Further studies investigating secretagogue-induced K^+-efflux from salivary gland cells provided evidence that the loss of K^+ was a consequence of the activation of a K^+ channel (Petersen & Pedersen, 1974; Petersen, 1976) which was dependent on the presence of calcium ions (Schramm & Selinger, 1975).

Putney (1976), using radiotracers (^{42}K & ^{86}Rb) as markers for potassium transport, demonstrated that the release of K^+ (^{86}Rb), from the parotid gland, in response to cholinergic and adrenergic agonists was biphasic with an initial transient phase which persisted under calcium free conditions, and a subsequent sustained phase, dependent upon extracellular calcium. Such a biphasic response occurs in salivary and lacrimal glands, and the pancreas (Parod & Putney, 1978a, b; Putney, Leslie & Marier, 1978; Singh, 1984) and is believed to be mediated via an increase in the concentration of intracellular free calcium.

Secretion from sweat glands, in common with other exocrine glands, has been shown to be responsive to cholinergic (muscarinic) and adrenergic

agonists (Sato & Sato, 1981a). The maximal sweating rate was highest after stimulation by acetylcholine, was lower with the beta-adrenergic agent isoprenaline and was least with the alpha agonist phenylephrine. The removal of Ca^{2+} from the bathing medium prior to the addition of ACh, or phenylephrine terminated secretion within 5 minutes of stimulation (Sato & Sato, 1981b). The effects of isoprenaline, however, were unaffected by the removal of Ca^{2+} from the bathing medium.

Because of the similarities between sweat glands and other exocrine organs with regard to the cholinergic and adrenergic mechanisms of action and the pivotal role of calcium, it has been proposed that sweat secretion is another example of secondary active Cl^- transport (Sato & Sato, 1987). However, electrophysiological studies of sweat glands failed to reveal a secretagogue-induced increase in potassium permeability (Sato, 1978, 1986). This is in direct contrast to the findings of x-ray microanalytical studies, which have shown that a fall in the cytoplasmic potassium concentration accompanies human sweat secretion in-vivo (Wilson *et al.*, 1988a; Wilson *et al.*, 1988b; McWilliams *et al.*, 1988). Furthermore, studies undertaken using K^+ channel blockers have shown that blockade of the K^+ channels inhibits sweat secretion (Sato & Sato, 1987). This would strongly suggest that K^+ efflux is a vital feature of sweat secretion.

Experiments were therefore performed using the radiotracer technique described by Putney (1976), as means of investigating secretagogue-induced loss of potassium from isolated human sweat glands and the role of calcium in that loss.

In the interim, Saga, Sato & Sato (1988) have recently reported that there is a K^+ efflux from the simian palm gland in response to activation by secretagogues.

MATERIALS AND METHODS.

Gland Isolation.

Strips of skin (0.5cm x 5.0cm) were obtained from the trunk of patients undergoing general surgery. Informed consent was granted by the patients and the procedure had the approval of the local medial ethics committee.

Immediately upon removal, the skin strips were immersed in isolation medium (see Appendix 6) and kept on ice throughout the entire isolation procedures (30-90 minutes).

A homogenate of the skin was prepared by repeated mechanical shearing with sharp scissors and sweat glands were isolated, as described by Lee, Jones and Kealey (1984). Glands were found free-floating and easily identifiable and those with minimal connective and adipose tissue (Fig 5.1) were selected for experimentation. The viability of each batch of glands was checked by phase contrast microscopy and the Trypan Blue exclusion test (see Appendix 7). Isolated glands were also prepared for light and electron microscopical examination (see Appendix 8).

Batches of 30-40 glands were transferred to the flow cell and continuously superfused (2ml min^{-1}) at 37°C with a bicarbonate buffered physiological salt solution (control superfusate; see Appendix 6) gassed with 95% O_2 & 5% CO_2. The glands were loaded with $^{86}Rb^{+}$ as described in Chapter 4.

Experimental Procedures.

After loading with $^{86}Rb^{+}$, the glands were superfused with either control or Ca^{2+}-free superfusates. Effluent samples were collected every 30 seconds and the activity present in each sample and in the residual tissue samples, was determined by liquid scintillation counting (as described in Appendix 3).

The responses of the glands to acetylcholine and adrenaline were studied and the effects of stimulating adrenergic receptor subclasses present on the glands, using phenylephrine, an α-adrenoceptor agonist, and isoprenaline, a β–adrenoceptor agonist, were also investigated. The responses to each

secretagogue were quantified by subtracting the basal efflux rate from the peak response.

All drugs were administered, dissolved in the superfusate, from a stock solution (10^{-2}M) at a final concentration of 10^{-5}M.

Low Ca^{2+} solutions were prepared by omitting Ca^{2+} and adding ethylene glycol-bis-(aminoethylether) tetra acetic acid (E.G.T.A. 0.1 mmol l^{-1}). This gave nominally Ca^{2+}-free solutions with an estimated concentration of calcium, $[Ca^{2+}]_i$ of 0.02 µmol l^{-1} (Miller & Smith, 1984).

RESULTS.

Viability.

Viable sweat glands, with normal morphology as judged by, light, phase contrast and electron microscopy and the ability of the glands to exclude trypan blue were obtained. Figures 5.2, 5.3 and 5.4 show cross sections of secretory coil and figure 5.5 coiled duct, from a freshly isolated human sweat gland. All the cell types present appear to be in a good state of preservation. Open canaliculi are present in the secretory coil, while in both the duct and the secretory coil the intercellular spaces are dilated.

Figures 5.6 and 5.7 are electron micrographs showing the morphology of sweat glands after undergoing superfusion and experimental procedures. Inspite of continued stimulation by ACh, the cells within the glands still appear to be in a good state of preservation.

Rubidium Uptake.

The amount of rubidium which accumulated in the cells of each batch of glands (20-1560 pmoles) was linearly ($r=0.82$, $P<0.001$) related to the RbCl concentration of the superfusing solution, which in turn varied with the specific activity of each batch of $^{86}Rb^{+}$.

Basal Washout.

The graph presented in figure 5.8 shows the glandular $^{86}Rb^+$ content of a single batch of glands plotted on a natural logarithmic scale against superfusion time. Initially the glands contained just over 60 pmoles of Rb^+ which declined with time. The near linearity of the slope of the line reflects the efflux of Rb^+ from the glands and suggests that this process is exponential.

Rate constants calculated from 9 such experiments, plotted against superfusion time, are shown in figure 5.9. In five of these experiments glands were superfused with control solution (●). Equivalent data from 4 experiments obtained during superfusion under Ca^{2+}-free conditions are also shown in figure 5.9. (■). In both sets of experiments the efflux rate constants initially fell but, after 4-5 minutes, reached a level of efflux which was maintained. A comparison of the mean efflux rate constants calculated over this maintained period demonstrated that $^{86}Rb^+$ efflux was significantly lower under Ca^{2+} free conditions (Table 5.1; $P<0.05$, Student's t-test).

In subsequent experiments drugs were always administered during the maintained phase of the basal efflux to enable clear identification of their effects on the rate of $^{86}Rb^+$ efflux from the glands.

Effects of acetylcholine.

Fig. 5.10. shows the effect of adding acetylcholine (10^{-5} M) upon the efflux rate of $^{86}Rb^+$ from the glands. Eleven batches of glands were initially superfused with control superfusate, to six of which acetylcholine was added. All diagrams have been constructed to show the addition of drugs at the time they reached the sweat glands.

Acetylcholine caused a biphasic response which consisted of a rapid and immediate increase in efflux rate, to a peak within about 1 minute (Table 5.2) (Fig 5.10), followed by a rapid decline to a more slowly declining phase.

When the glands were superfused under Ca^{2+}-free conditions the efflux rate constant again rose promptly in response to acetylcholine (Fig. 5.11), but the efflux rate returned to control values within 3 minutes and there was no

slowly declining phase. When $CaCl_2$ (2.56 mmol l^{-1}) was then added to this solution, in the continued presence of acetylcholine, the rate of $^{86}Rb^+$ efflux again rose substantially to a level which was sustained (Table 5.3.) (Fig 5.11). The addition of calcium thus restored the more slowly declining second phase of the response. In the absence of acetylcholine the readmission of calcium caused only a slight increase in the rate of efflux which was not statistically significant.

Effects of adrenergic agonists.

Under control superfusate conditions, the response to adrenaline at 10^{-5} M, consisted of a slight, but prolonged rise in the rate of $^{86}Rb^+$ efflux. Increasing the concentration of adrenaline to 5 x 10^{-5}M potentiated the increase in the rate of efflux (Fig. 5.12A). Under calcium free conditions the effects of adrenaline (5 x 10^{-5} M) were abolished but the addition of calcium to the superfusate, in the continued presence of adrenaline, caused an increase in the rate of efflux (Table 5.3.) (Fig. 5.12B).

Phenylephrine (10^{-5} M), an a-adrenoceptor agonist, caused a sustained increase in the rate of $^{86}Rb^+$ efflux (0.015 ± 0.003 min^{-1}) (Table 5.2.) (Fig. 5.13). This effect was slightly smaller than that observed after an equimolar dose of adrenaline, but was otherwise indistinguishable. Because of the similarity between the results obtained using adrenaline and phenylephrine under control conditions, the effect of removing calcium on the response to phenylephrine was assumed to be similar and was therefore not investigated.

The β-adrenergic agent isoprenaline (10^{-5} M) had no immediate effect, but after a delay of 3-4 minutes, caused a slow, increase in $^{86}Rb^+$ efflux rate (Fig. 5.14). This response to isoprenaline was consistent in all the preparations tested and, in one experiment, was observed to continue increasing the rate of efflux over a total period of 12 minutes. The response to isoprenaline was not affected by the removal of calcium from the superfusate (Fig. 5.15).

DISCUSSION.

The skin strips used in this study were removed from patients with no obvious skin complaints or disease, and who were not receiving any drugs (which might otherwise affect glandular function), apart from anaesthetics. Anaesthetics are known to affect sweat gland function (McWilliams, *et al.*, 1987) but, because of the length of time involved in the isolation, loading and superfusion procedures, it is likely that any effects of anaesthetics would have diminished or disappeared.

By recording the age, sex and the area of the body from where each skin sample was removed, it was apparent that striking differences existed in the size of sweat glands isolated from; 1) different areas of the body; 2) similar areas from men and women, and 3) from patients of different ages. The largest glands were found in skin samples from young people particularly young males. These subjective observations correlate with Sato and Sato's (1983) findings that sweat gland size and output was larger in young males rather than in older subjects. The differences in sweat gland size did not appear to affect the physiological responsiveness of glands to secretagogues.

The morphology of the isolated sweat glands as seen in figures 5.2-5.5 demonstrates that in spite of the mechanical disruption the skin undergoes during the isolation procedures, the cellular architecture of the sweat glands remains intact. Lee *et al.*, (1984) proposed that the reason the sweat glands remained intact throughout the rapid mechanical shearing process was due to an inherent line of weakness between the connective tissue sheath surrounding the gland and the encompassing collagen fibres. Observations from this study suggest that the proposed line of weakness was not uniform throughout the range of skin samples received. It proved to be far more difficult to isolate sweat glands in skin samples removed from areas with a dense collagen fibre network or from older patients.

Electron microscopic examination of the isolated sweat glands demonstrated that the glands appeared to be active, as judged by the morphological criteria of dilated basal infoldings and intercellular canaliculi

(Montgomery *et al.*, 1984). It is not surprising to find the glands in an active state for two reasons: 1) The shearing process would mechanically stimulate any nerves present in the skin samples to release some neurotransmitter, thereby activating the glands; 2) Anaesthetics, which are known to activate sweat glands, may still be present in the sample and would require some time to be washed out of the glands. Although the finding in this study of 'active' sweat glands contrasts to those glands isolated by Lee *et al.*, (1984) which appeared quiescent, the sweat glands, which those authors 'maintained' by short term culture methods, also displayed characteristics of activation (H.Y.Elder personal communication).

The fact that sweat glands, which had undergone both superfusion and acetylcholine-induced activation, still displayed an intact internal morphology (Fig. 5.6 & 5.7), further validates the results of this study.

Sweat glands, in common with other exocrine glands, are not composed exclusively of secretory cells but consist of both a secretory fundus and a reabsorptive duct. However, since EPXMA studies demonstrated a significant decrease in K^+ concentration in the stimulated secretory fundus but not in the duct of the activated human sweat gland (Wilson *et al.*, 1988a), the results are attributed to changes in the secretory cells.

Although it is often used as a marker for potassium (K^+) transport, discrepancies between the behaviour of $^{86}Rb^+$ and of K^+ have been reported in several exocrine organs (Putney, 1976; Parod & Putney, 1978; Findlay, 1984; Gallacher, Maruyama & Petersen, 1984). Gallacher *et al.*, (1984) reported that the Rb^+ conductance of large K^+ channels in exocrine acini was extremely low and did not carry current through the channel. Gallacher *et al.*, (1984), further suggested that the permeabilities for Rb^+ and K^+ may be about the same, but the rate constant for passing through the barriers in the channel could be much lower for Rb^+ than K+, while the reverse is true with regard to the equilibrium constants for binding sites within the channel. While $^{86}Rb^+$ may have a high affinity for

channel sites, if it is present in low concentrations, there will be strong competition for the sites from the higher concentrations of K^+ present in the solutions and so more K^+ will pass through the channel. The mobility of Rb^+ may therefore be much better in low concentrations. In this study the $^{86}Rb^+/K^+$ ratio was estimated by flame photometry, to be 0.00003 and assumed to be in a low enough concentration to give meaningful results. The secretagogue-induced changes of $^{86}Rb^+$ are therefore interpreted as indicative of changes in the rate of potassium efflux.

It would appear from the lower $^{86}Rb^+$ output in calcium-free solution that the presence of extracellular calcium is a requirement for the maintenance of potassium permeability in the unstimulated human sweat gland.

The biphasic K^+ ($^{86}Rb^+$) efflux in response to acetylcholine was similar to that found in other exocrine glands (Putney, 1976; Parod & Putney, 1978; Putney, Leslie & Marier, 1978) and is thought to be mediated by an increase in the intracellular Ca^{2+} concentration. The initial transient component of the ACh-evoked response was attributed to the mobilisation of bound Ca^{2+} from intracellular stores (Putney, 1976; Aub *et al.*, 1982; Aub & Putney, 1987).

It now seems that the response of the human sweat gland to acetylcholine requires both the release of intracellular calcium and a calcium influx. Sato & Sato (1988) showed that cholinergic agents increase intracellular calcium in secretory cells isolated from simian sweat glands but concluded that this increase was dependent upon extracellular Ca^{2+}. This implies that mobilisation of intracellular Ca^{2+}, in response to acetylcholine does not occur, which is in direct contrast to this study and to the findings in other exocrine glands (Putney, 1976; 1977; 1978; Haddas, Landis & Putney, 1979; Arkle , Gillespie & Greenwell, 1988). However, subsequent studies of the simian sweat gland (Saga, Sato & Sato, 1988) suggest that the cholinergically-evoked response in these glands is mediated through an initial mobilisation of bound intracellular Ca^{2+}.

Although acetylcholine is the major secretagogue in human sweat glands, glands from other species have been shown to respond to adrenaline (Sato, 1973,1977a;Jenkinson *et al.*, 1978). In the isolated human sweat gland the K^+ efflux in response to adrenaline and the α-adrenergic agonist, phenylephrine are essentially the same. It would appear therefore that adrenaline-induced sweating is elicited via α-adrenoceptors.

The adrenaline-induced K^+ efflux in contrast to the cholinergic response, consisted of a single phase dependent on extracellular calcium. This indicates that adrenaline and phenylephrine increase the level of intracellular calcium by an influx of calcium ions rather than calcium mobilisation from bound stores. In salivary glands, adrenaline mobilises bound calcium via α-receptors (Putney, 1976, 1977) this would suggest there is a functional difference between sweat glands and salivary glands.

In the monkey palm gland isoprenaline is known to induce sweat secretion at a rate approximately 1/2 the maximal rate induced by acetylcholine (Sato & Sato, 1981b). From the onset of isoprenaline-induced sweating <u>in vitro,</u> it takes approximately 5 minutes for the sweating to reach maximal output. Isoprenaline caused a slow progressive increase in the rate of $^{86}Rb^+$ efflux, in the isolated human gland which after 7 minutes, was still rising. The similarity between the rising rate of secretion and the increasing rate of K^+ efflux supports the view that K^+ efflux maintains the secretory process.

The removal of external calcium had no effect on the isoprenaline induced efflux of $^{86}Rb^+$ from the sweat glands. This is in contrast to salivary glands where isoprenaline fails to cause a K^+ efflux but rather induces a K^+ uptake (Katoh, Nakasato, Nishiyama & Sakai, 1983). In sweat glands it would seem therefore, that the mechanism of action of isoprenaline, does not depend on intracellular calcium, or require the presence of extracellular calcium (Sato & Sato, 1988). Sato & Sato (1981c) demonstrated that isoprenaline elevated the level of cyclic adenosine monophosphate (cAMP) within the cell and that the accumulation of cAMP was not inhibited by the removal of extracellular

calcium, whereas, acetylcholine, adrenaline and phenylephrine all failed to raise cAMP levels. This is strong evidence that the action of isoprenaline is mediated via cAMP.

The results from the present study show that in common with other exocrine glands there is a secretagogue-induced K^+ efflux from human sweat glands.

TABLE 5.1.

Rate constants for the basal rate of $^{86}Rb^{+}$ efflux during superfusion with calcium-containing and calcium-free solutions.

	$^{86}Rb^{+}$ efflux rate constant (min^{-1}).	
Control washout.	.076 ± .002	n = 5
Ca^{2+}-free washout.	.055 ± .001	n = 4 *

Data are presented as means ± s.e. and *n* values refer to the number of experiments. * $p < 0.05$.

TABLE 5.2.

Rate constants in response to stimulation by differing secretagogues. Responses were quantified by subtracting the rate of basal efflux from the peak response.

	$^{86}Rb^{+}$ efflux rate constant (min^{-1}).	
Secretagogue		
ACh (10^{-5}M)	.100 ± .020	n = 6
Adr (10^{-5}M)	.032 ± .005	n = 3
Adr (5×10^{-5}M)	.039 ± .012	n = 3
PE (10^{-5}M)	.015 ± .003	n = 3

Data are presented as means ± s.e. and *n* values refer to the number of experiments.

TABLE 5.3.

Rate constants in response to stimulation by differing secretagogues under Ca^{2+}-free conditions to which Ca^{2+} was subsequently added. Responses were quantified by subtracting the rate of basal efflux from the peak response.

$^{86}Rb^{+}$ efflux rate constant (min^{-1}).

Secretagogue	Ca^{2+} independent	Ca^{2+} dependent	
ACh (10^{-5}M)	.141 ± .015	.052 ± .008	n = 6
Adr (5×10^{-5}M)	.006 ± .002	.037 ± .012	n = 4

Data are presented as means ± s.e. and *n* values refer to the number of experiments.

Fig. 5.1. Shows a light micrograph of an isolated human sweat gland photographed under differential interference contrast microscopy. The secretory cells can be distinguished by their granular appearance while the cells of the reabsorptive duct do not have these cells. See appendix 9 for key to figures.

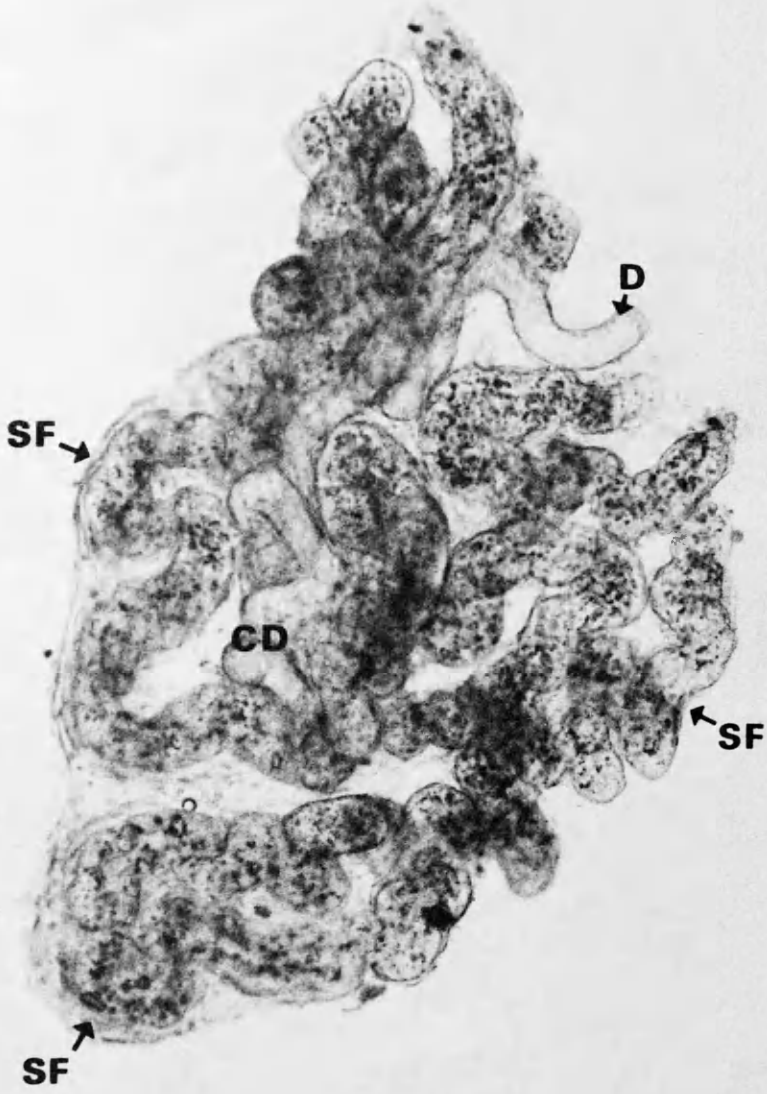
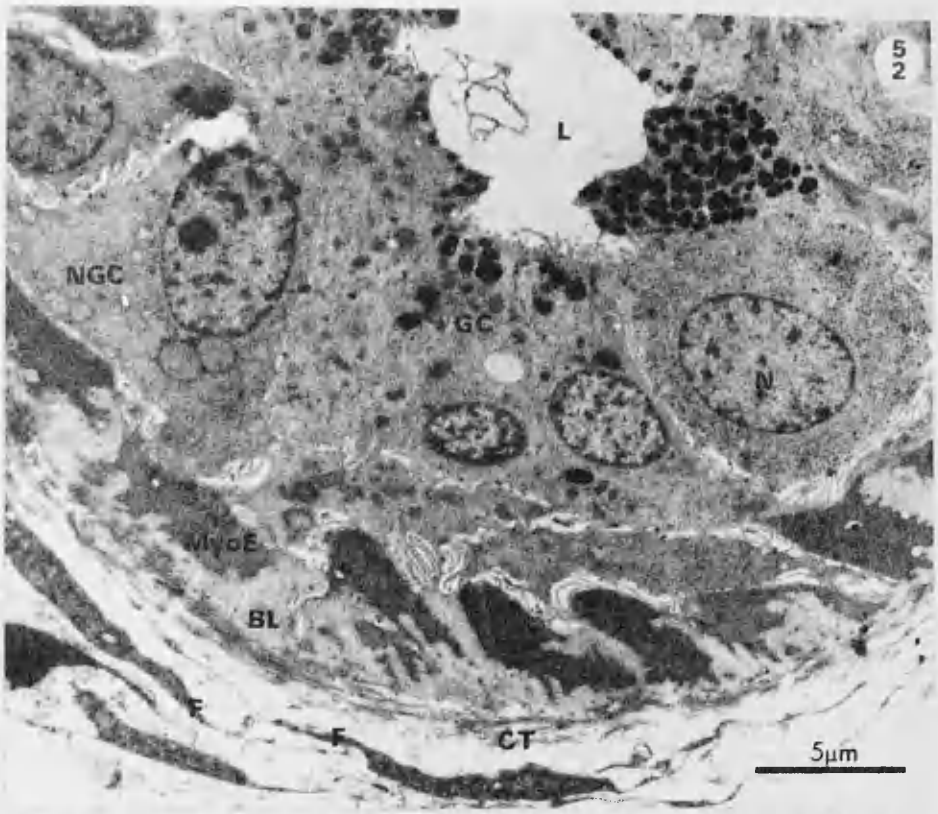

D
SF
CD
SF
SF
100μm

Fig. 5.2. An electron micrograph of the secretory fundus of a freshly isolated human sweat gland, illustrating the granular (GC) and non granular (NGC) cells, as well as the myoepithelial cells, the surrounding connective tissue and the fibrocyte sheath.

Fig. 5.3. A higher power view of the basal portion of a non granular cell illustrating the complex basal infoldings (BI), processes of which rest on the basal lamina (BL).


52
L
NGC
GC
N
MyoE
BL
F
F
CT
5µm

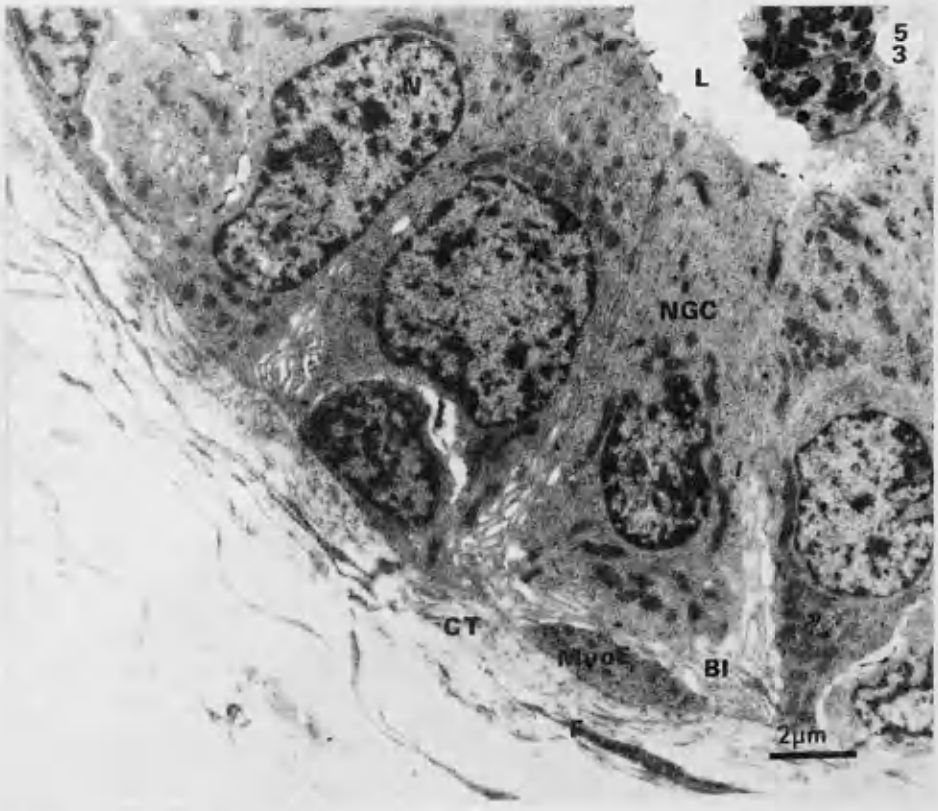
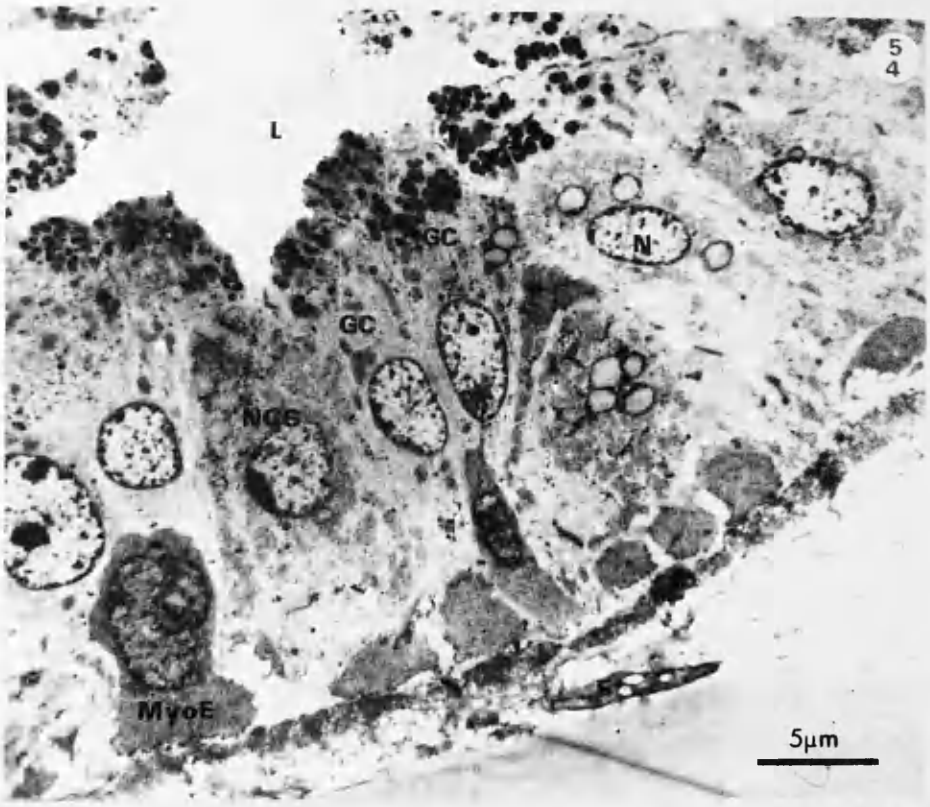

53
N
L
NGC
CT
MyoE
BI
2µm

Fig. 5.4. Numerous granular cells with granules lying apposed to the lumen (L) of the gland. Myoepithelial cells appear shorter and more puckered, indicative of tension. The basal infoldings, are wider than in the previous micrograph indicating slight activity within the gland.

Fig. 5.5. The duct of a freshly isolated human sweat gland. The wall is approximately 2 layers thick and is composed of basal cells (BC) and luminal cells (LC). Luminal and basal cells are interconnected by complex interdigitations.


5
4
L
GC
N
GC
NGS
MyoE
5µm

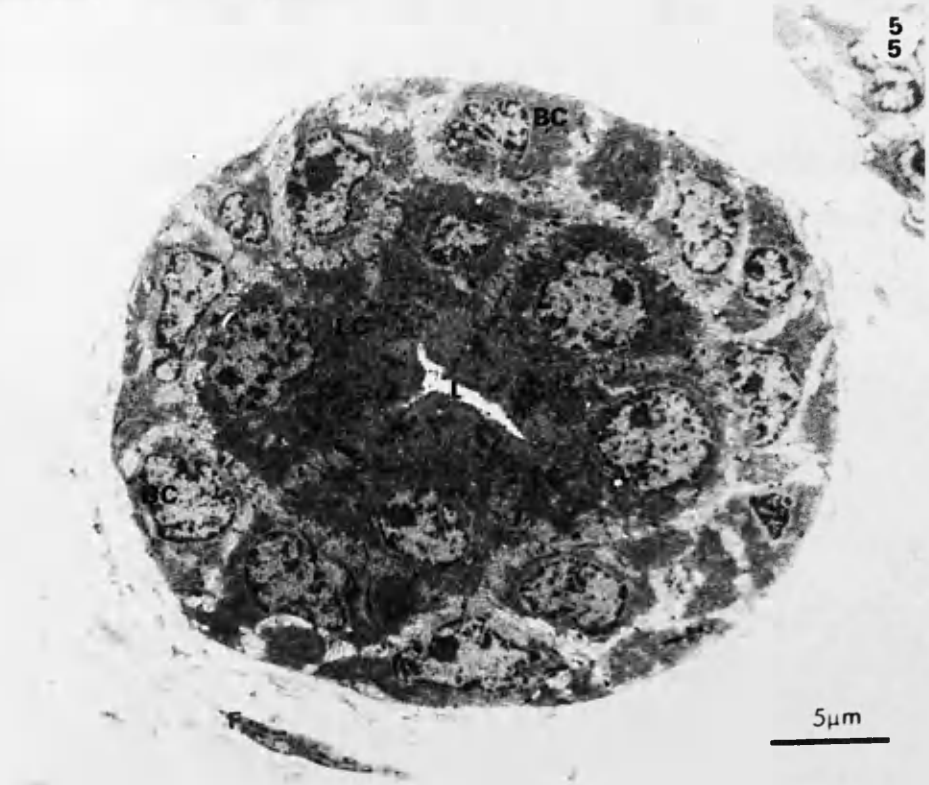
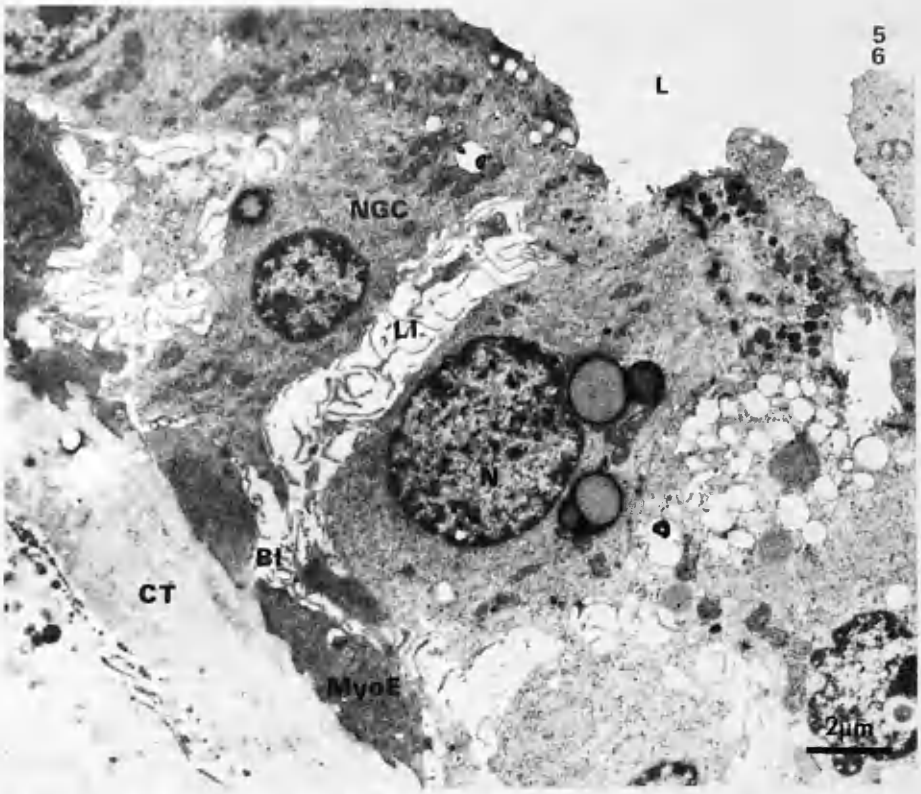

5
5
BC
LC
L
BC
5µm

Fig. 5.6. The appearance of the fundus after superfusion and stimulation procedures. Some granular cells exhibit evidence of granule depletion and the dilatation of the basal infoldings between granular and non granular cells is striking, indicative of activity.

Fig. 5.7. A higher power view of the fundus after superfusion and stimulation procedures. Non granular cells show mitochondrial damage with wide lateral interdigitations. (Key to figures in Appendix 9, page 172).


5
6
L
NGC
LI
N
BI
CT
MyoE
2µm

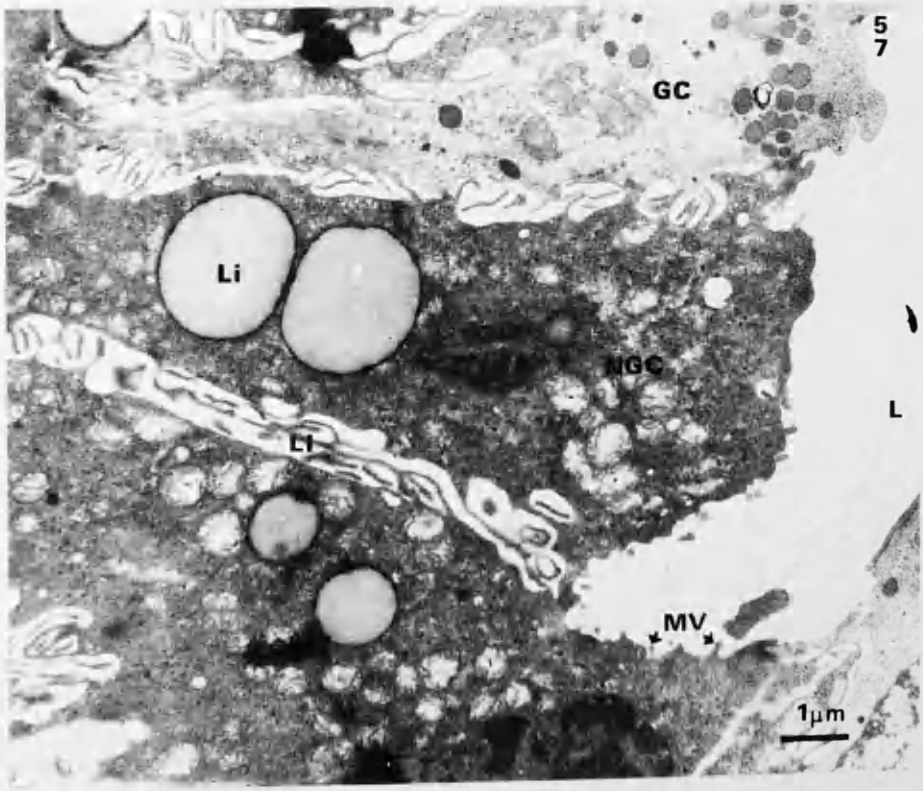
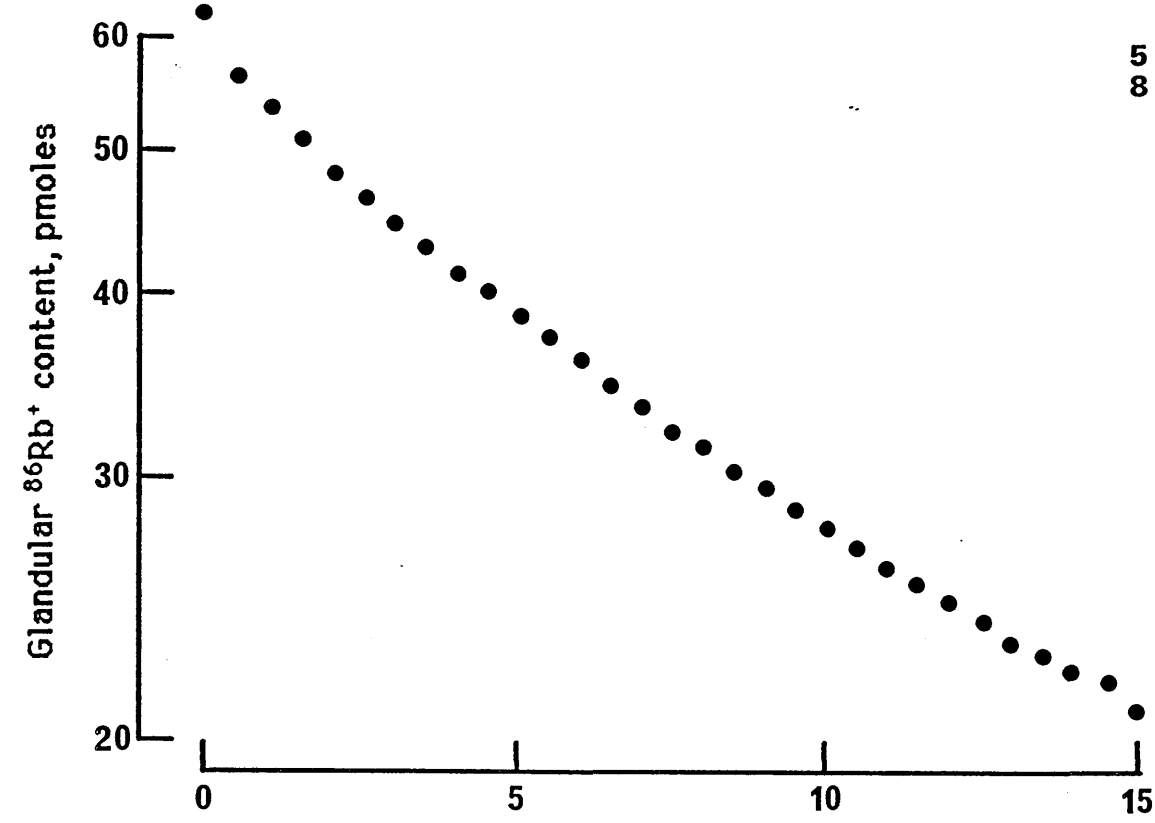

5
7
GC
Li
NGC
L
LI
MV
1µm

Fig. 5.8. The declining $^{86}Rb^{+}$ content of a single batch of glands, plotted on a natural logarithmic scale, as a function of superfusion time.

Fig. 5.9. Shows the calculated rate constants (mean ± s.e.) plotted against superfusion time. The circles (•) show data obtained during superfusion with control saline (n=5). Equivalent data obtained under Ca^{2+}-free conditions are represented by the squares (■, n=4). The insert shows a histogram comparing the efflux rate constants between control (open column) and Ca^{2+}-free (hatched column) conditions; * $P < 0.05$.


Glandular $^{86}Rb^{+}$ content, pmoles
60
50
40
30
20
0
5
10
15
5
8

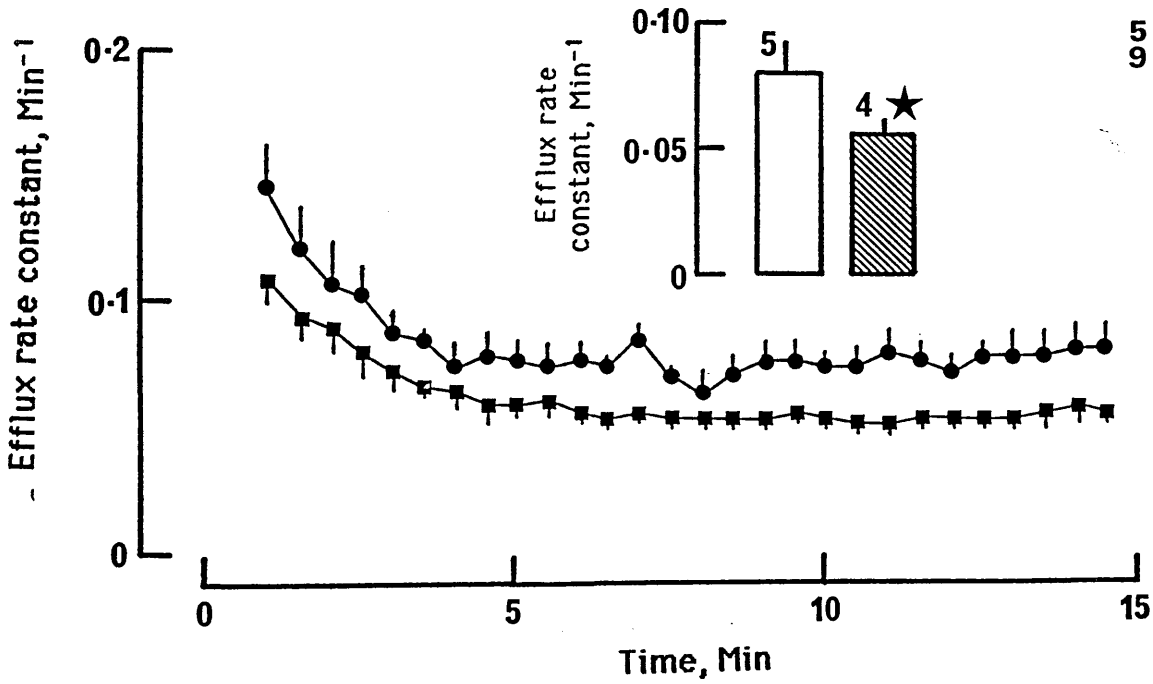

Efflux rate constant, Min^{-1}
0·2
0·1
0
0
5
10
15
Time, Min
Efflux rate constant, Min^{-1}
0·10
0·05
0
5
4
5
9

Fig. 5.10. The effects of ACh under control conditions, upon $^{86}Rb^{+}$ efflux. The figure shows data (mean ± s.e.) from a total of eleven separate experiments initially superfused with control saline to which ACh was added to five batches (■).

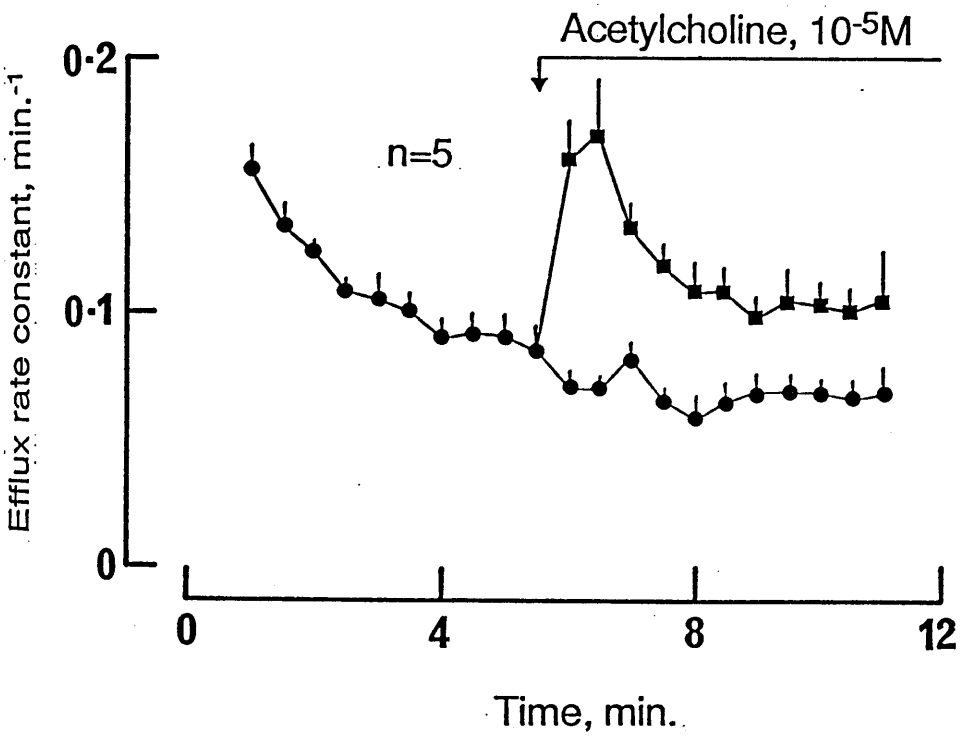

Acetylcholine, 10^{-5}M
n=5
0·2
0·1
0
Efflux rate constant, min.$^{-1}$
0
4
8
12
Time, min.

Fig. 5.11. The effects of ACh and then $CaCl_2$ on batches of glands (n=6) initially superfused with Ca^{2+}-free solution.

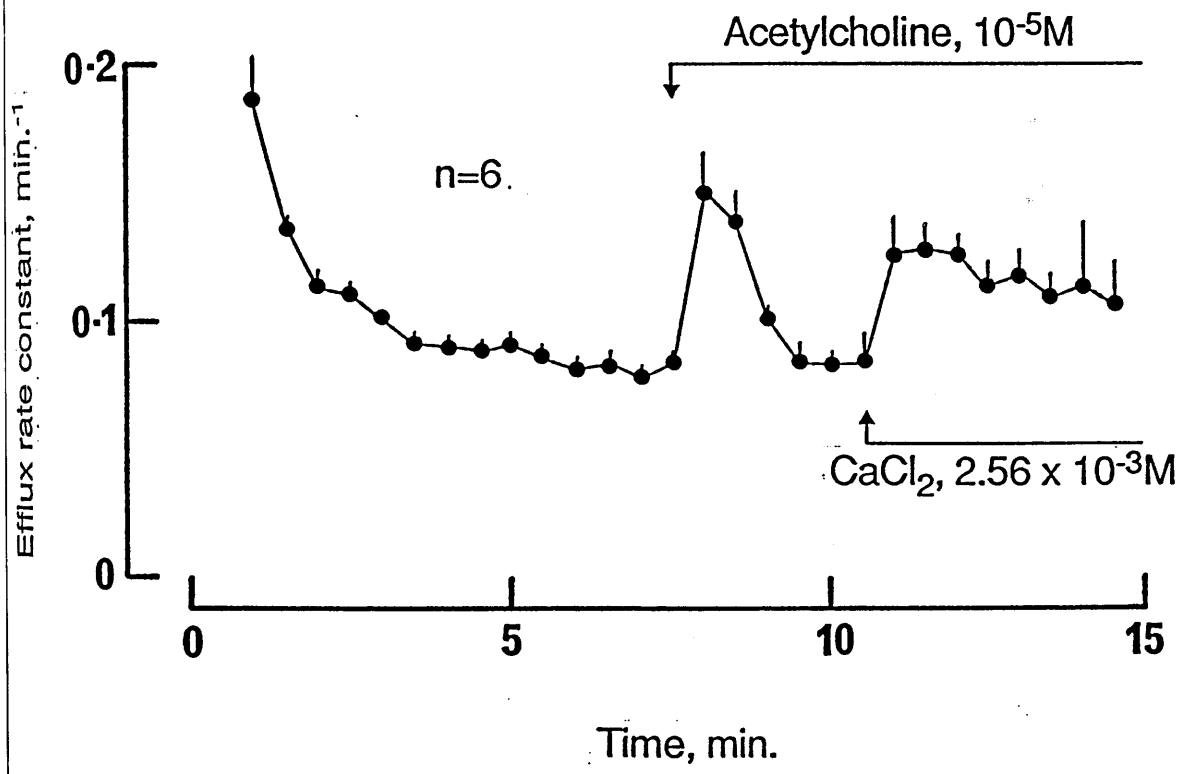

Acetylcholine, 10^{-5}M
n=6.
$CaCl_2$, 2.56 x 10^{-3}M
Efflux rate constant, min.$^{-1}$
0·2
0·1
0
0
5
10
15
Time, min.

Fig. 5.12. Response of the sweat gland to adrenaline(5×10^{-5}M): A: under control conditions (n=3); B: in Ca^{2+}-free solution to which Ca^{2+} was subsequently added (n=3).

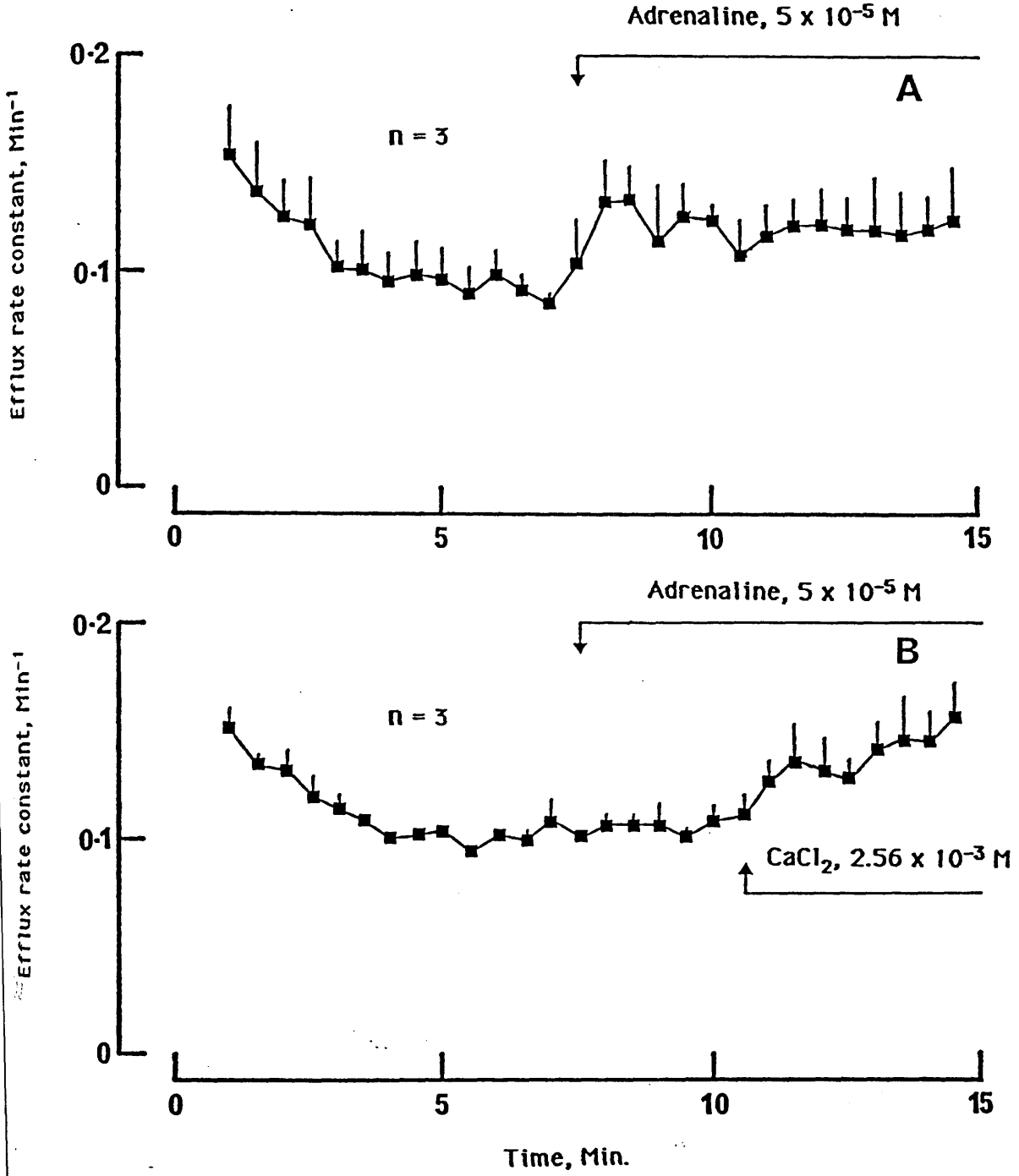

Adrenaline, 5 x 10⁻⁵ M
A
n = 3
0·2
0·1
0
Efflux rate constant, Min⁻¹
0
5
10
15
Adrenaline, 5 x 10⁻⁵ M
B
n = 3
CaCl₂, 2.56 x 10⁻³ M
Time, Min.

Fig. 5.13. The effect of the α-adrenergic agent phenylephrine, under control conditions, on the rate of $^{86}Rb^{+}$ efflux.

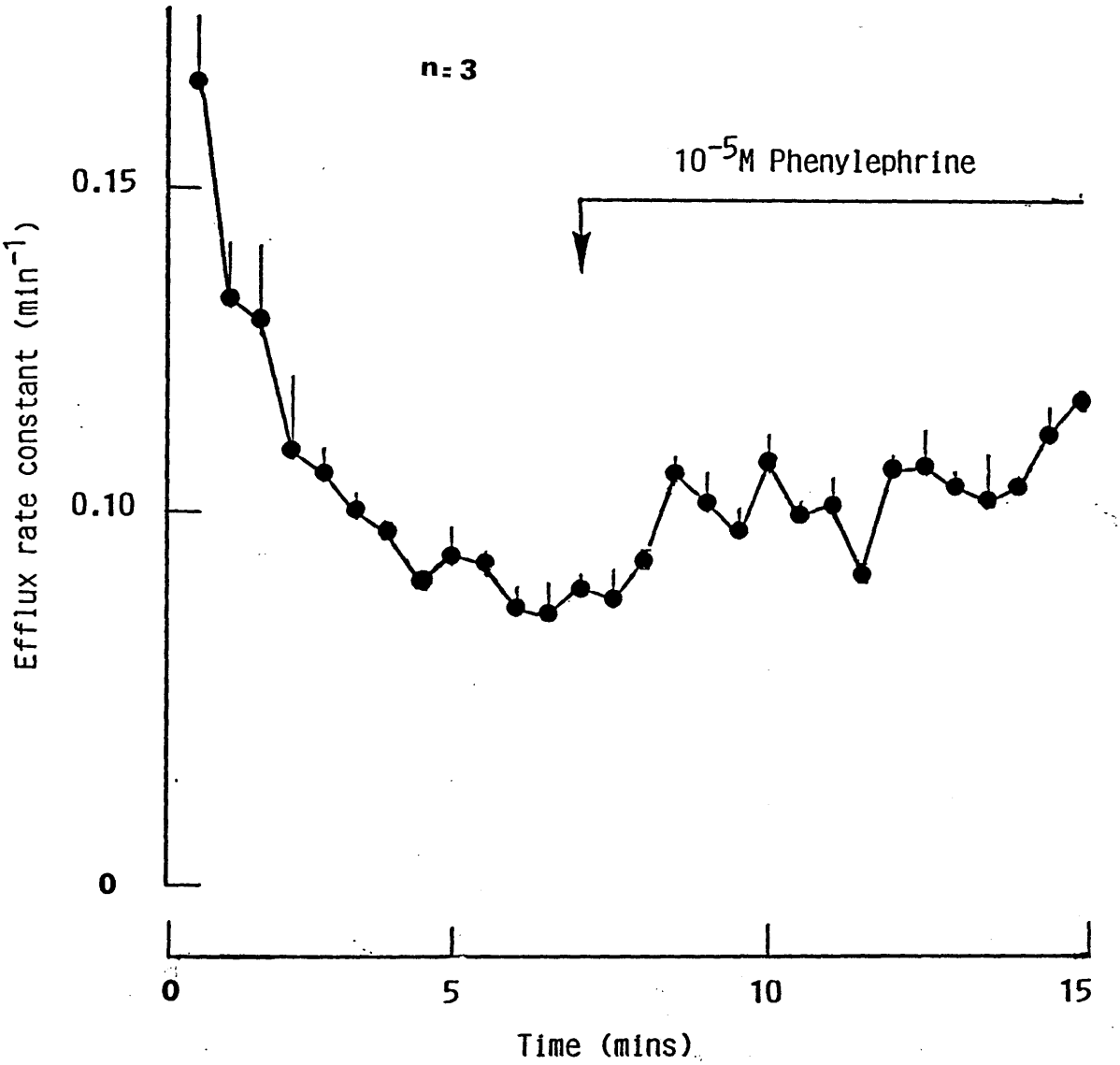

n=3
10^{-5}M Phenylephrine
0.15
0.10
0
Efflux rate constant (min^{-1})
0
5
10
15
Time (mins)

Fig. 5.14. The effects of isoprenaline, the β-adrenergic agonist, on $^{86}Rb^{+}$ efflux under control conditions (n=3).

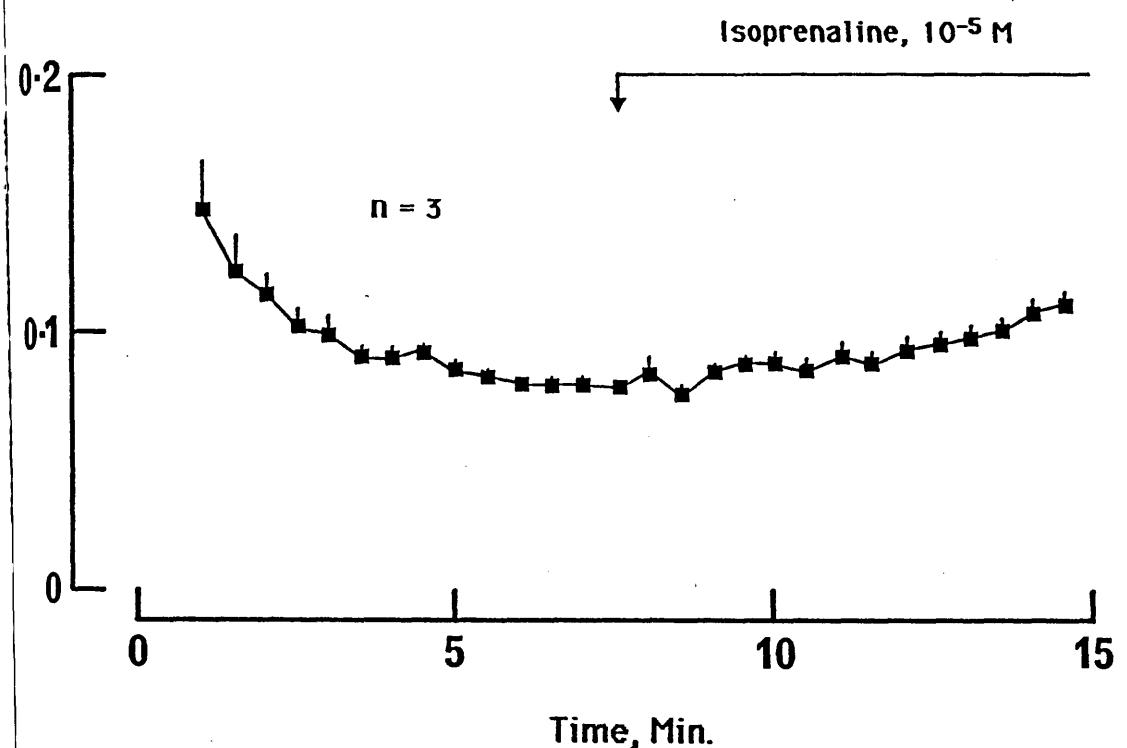
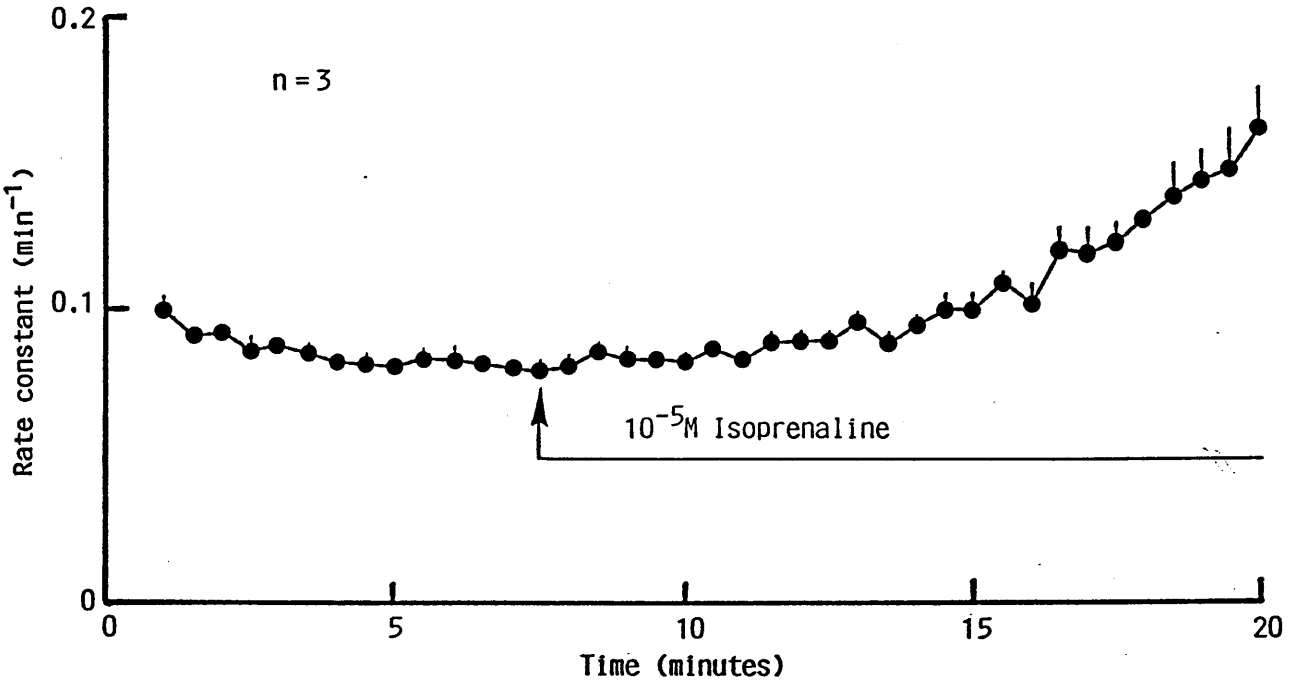

Isoprenaline, 10^{-5} M
0·2
0·1
0
n = 3
0
5
10
15
Time, Min.

Fig. 5.15. The effects of isoprenaline under Ca^{2+}-free conditions (n=3).


0.2
0.1
0
n = 3
Rate constant (min^{-1})
10^{-5}M Isoprenaline
0
5
10
15
20
Time (minutes)

CHAPTER 6.

THE EFFECTS OF REMOVING EXTRACELLULAR Na^+ OR Cl^- ON THE ACh-INDUCED EFFLUX OF $^{86}RB^+$ FROM ISOLATED HUMAN SWEAT GLANDS.

INTRODUCTION.

The results from the previous chapter have now established that in common with other exocrine glands, there is a K^+ loss which accompanies secretagogue-induced activation in the isolated human sweat gland. In particular, the major secretagogue in promoting secretion from human sweat glands, acetylcholine, caused a biphasic change in the rate of efflux similar to that previously described in salivary glands (Putney, 1976, Parod & Putney, 1978 a,b; Marier *et al.*, 1978). Furthermore, the studies described in the previous chapter resolved the acetylcholine-evoked loss of K^+ from isolated human glands into two phases similar to that described in other exocrine glands (Putney, 1976; Parod & Putney, 1978 a,b; Marier *et al.*, 1978; Aub *et al.*, 1982). The response consists of an initial transient phase independent of extracellular calcium, which has been attributed to the release of bound intracellular calcium, and a second sustained phase dependent on the presence of extracellular calcium.

Patch clamp studies on mouse submandibular gland acinar cells (Gallacher & Morris, 1987), have suggested that the acetylcholine-evoked calcium-influx, which supports the sustained efflux of K^+ from the acinar cells, is dependent on the presence of extracellular Na^+. The conclusions of the patch clamp studies of Gallacher and Morris (1987) contrast with the results of $^{86}Rb^+$ efflux studies performed on the rat submandibular gland (Bovell *et al.*, 1989c). The rubidium efflux studies demonstrated that acetylcholine can still evoke an increase in K^+ efflux under Na^+-free conditions.

Although it has been demonstrated in the monkey palm gland that removal of extracellular Na^+ inhibits sweat secretion (Sato & Sato, 1987) and the cholinergically-induced loss of K^+ (Saga *et al.*, 1988), there is no

information on the effects of removing extracellular Na^+ on either of the phases of the acetylcholine response in the human sweat gland.

In common with salivary gland secretion (Case, Hunter, Novak & Young, 1984; Martinez & Cassity, 1985; Young, Cook, Evans & Pirani, 1987), sweat secretion *in vitro* in the simian palm gland, is abolished when external Cl^- is removed from perfusion fluids (Sato & Sato, 1987) and the acetylcholine-induced efflux of K^+ is inhibited (Saga *et al.*, 1988). As yet there is no information on the effects of removing extracellular Cl^- on the acetylcholine-induced biphasic response of either salivary glands or sweat glands. Therefore, the effects of removing extracellular Na^+ and Cl^- on the two phases of the acetylcholine-evoked K^+ ($^{86}Rb^+$) efflux from the isolated human sweat gland were investigated.

Materials and Methods.

The procedures used for isolating glands, loading with radioactive tracer, superfusion and the calculation of efflux rate constants have been described in the preceding chapters.

To examine the effects of removing either extracellular sodium or chloride on the two phases of the acetylcholine-evoked response, the following basic protocol was employed: glands were superfused with calcium-free superfusate containing 0.1mM E.G.T.A. to which acetylcholine (10^{-5} mmol l^{-1}) and calcium (2.56 mmol l^{-1}) were subsequently added. During experimental procedures the sodium content of the superfusate was replaced with either N-methyl-D-glucammonium ($NMDG^+$) or lithium (Li^+), or alternatively the chloride content was replaced with gluconate or nitrate.

Sodium-free solutions.

$NMDG^+$ or Li^+ were used as sodium substituents in solutions in which the $NaHCO_3$ content of the solution had been isosmotically replaced with 4-(2-hydroxyethyl)-1-piperazine sulphonic acid (HEPES) buffer (see Appendix 6). All substrates were added to the solutions as free acids and the pH was

adjusted to 7.4 with either hydrochloric acid or lithium hydroxide. All HEPES buffered solutions were gassed with 100% O_2.

Bicarbonate buffered, Na^+-free solutions were prepared in the following way: NMDG+ solution was prepared as before (see Appendix 6) except that 115 mmol/l of NMDG was added to the solution and the pH was adjusted to 6.9 - 7.1 with HCl. A further 25 mmol l^{-1} NMDG base was then added and the resulting alkaline (pH > 13) solution brought to final volume and gassed with 95 % O_2 / 5 % CO_2. After 1-2 hours gassing with the 95 % O_2 / 5 % CO_2 mixture the pH of the solution was 7.3-7.4.

Chloride-free solutions.

Chloride-free solutions were prepared by replacing the chloride content of the superfusate with either gluconate or nitrate (see Appendix 6). These solutions contained 25 mmol l^{-1} $NaHCO_3$ and were gassed with 95 % O_2 / 5 % CO_2. The pH was adjusted to 7.3-7.4.

During experiments involving chloride-free solutions the subsequent addition of calcium to the superfusate was achieved using either 2.56 mmol l^{-1} calcium gluconate or calcium bromide.

RESULTS.

Basal efflux rates.

Table 6.1 shows the basal efflux rate constants during superfusion with HEPES-buffered and bicarbonate (HCO_3^-) buffered solutions. The basal rate of efflux during superfusion with HEPES-buffered Na-containing superfusate was significantly ($p<0.01$) lower than during control conditions (Na^+-containing HCO_3^--buffered solution). The replacement of Na^+ by Li^+ and $NMDG^+$ in HEPES-buffered superfusates, significantly ($p<0.05$) elevated the rate of efflux when compared with Na^+-containing HEPES-buffered solutions.

During superfusion with $NMDG^+$ or Li^+ replacement for Na^+, in HCO_3^- buffered solutions, neither $NMDG^+$ or Li^+ replacement caused a significant increase in the basal efflux rate.

Responses to ACh under HEPES buffered Na-free conditions.

Figure 6.1 illustrates the responses of 5 batches of glands initially superfused with Ca^{2+}-free solution, in which Na^+ was replaced by $NMDG^+$ and to which acetylcholine and Ca^{2+} were subsequently added. Under these conditions, the first phase of the ACh-evoked response was inhibited. The subsequent addition of Ca^{2+} caused a significant ($p<0.002$) increase in the rate of $^{86}Rb^+$ efflux which was sustained.

Figure 6.2 shows the responses to the addition of ACh and subsequently Ca^{2+}, from a total of six experiments, using Li^+ as a Na^+ substituent. The results demonstrate that the responses to ACh and Ca^{2+} in each experiment were extremely inconsistent.

Responses to ACh under HEPES buffered Na-containing conditions.

The responses to ACh from a total of seven experiments, superfused with HEPES-buffered Na^+-containing solutions, are shown in figure 6.3. Evidence of a biphasic increase in the K^+ permeability was seen in only two experiments while the response in the remaining five experiments was extremely variable with no consistent response being observed.

Effects of acetylcholine in Na-free, bicarbonate-buffered solutions.

Figure 6.4 shows the mean efflux rate constant for five experiments using bicarbonate (HCO_3^-)-buffered $NMDG^+$-containing superfusate. In the presence of $NMDG^+$, the initial response to ACh was again inhibited. The subsequent addition of Ca^{2+} to the superfusate increased the rate of efflux to a level, which was sustained.

Acetylcholine evoked increased rates of efflux, when glands were superfused with calcium-free solutions containing lithium . Addition of Ca^{2+} to

the superfusate, in the continued presence of ACh, caused a further increase in the rate of $^{86}Rb^+$ efflux (figure 6.5.). The changes in efflux rates evoked by ACh when added to the Li^+-containing superfusate were not significantly different from those observed under control (Na^+ containing, HCO_3^- buffered) conditions.

The effects on the acetylcholine response of readmitting Na to the superfusate.

The effects, on the acetylcholine response, of readmitting 72 mmol l^{-1} Na to an initially Na-free superfusate ($NMDG^+$-containing and HCO^{3-}-buffered solution) are shown in figure 6.6. In the presence of $NMDG^+$ acetylcholine failed to increase the rate of efflux. The subsequent addition of sodium caused a transient increase in the rate of efflux. When 2.56 mmol l^{-1} $CaCl_2$ was added to the superfusate, in the continued presence of acetylcholine, the rate of efflux increased to a more sustained level.

ACh-evoked response under calcium and chloride-free conditions.

The responses to ACh during superfusion with chloride-free solutions, which contained no calcium are presented in figure 6.7. In the gluconate-containing solution ACh evoked an increased rate of efflux which reached a peak within 1 minute and then declined to a maintained level above the basal rate. The subsequent addition of calcium to the superfusate in the continued presence of acetylcholine, caused a further increase in the rate of efflux. The initial transient change in the rate of efflux (i.e. Peak response - Basal rate) using gluconate replacement was not significantly different from the change in efflux observed under chloride containing solutions. However, the overall response of the glands was augmented.

Acetylcholine-evoked an essentially similar response when nitrate instead of gluconate was used as a replacement for the chloride content of the superfusate (Fig 6.7).

DISCUSSION.

The initial experiments on the effects of removing extracellular Na^+ on either or both phases of the acetylcholine-evoked response in human sweat glands, were conducted using superfusates in which Na^+ had been replaced by either the large impermeant anion $NMDG^+$ or Li^+. As the bicarbonate salts of $NMDG^+$ or Li^+ are not commercially available, it was necessary to replace the bicarbonate with HEPES buffer. The removal of extracellular bicarbonate has been shown to have no apparent effect on cholinergically-induced sweat secretion from isolated simian palm glands (Sato & Sato, 1987). Similarly, results from a previous study investigating $^{86}Rb^+$ efflux from the rat submandibular gland have also shown that replacement of HCO_3^- with HEPES buffer did not affect the basal rate of efflux from the gland (Bovell *et al.*, 1989c). However, in contrast to the latter findings the basal rate of K^+ efflux from the isolated human sweat gland was significantly ($p<0.02$) lower in Na^+-containing HEPES buffered solutions than under control conditions ($NaHCO_3^-$-containing solutions). Both Li^+ and $NMDG^+$ caused significantly increased ($p<0.05$) basal efflux rates compared to Na^+-containing HEPES-buffered conditions (Table 1.), similar to that described in the rat submandibular gland (Bovell *et al.*, 1989c).

In the isolated human sweat gland, replacement of sodium with $NMDG^+$-HEPES inhibited the initial transient calcium-independent phase of the response, while the second calcium-dependent phase was restored by the addition of calcium to the superfusate. This result strongly suggests that, in the human sweat gland the acetylcholine-evoked mobilisation of intracellular calcium is inhibited by the replacement of extracellular Na^+. This is in contrast to patch clamp studies of mouse submandibular gland, where Gallacher and Morris (1987) concluded that the influx of calcium which maintains the sustained efflux of K^+ from the gland, was dependent on the presence of extracellular Na^+. Using Li^+ as a Na^+ substituent, the effects of Na^+ removal on the rate of K^+ efflux from sweat glands were further investigated.

Experiments with Li^+-containing HEPES-buffered superfusates demonstrated that acetylcholine and calcium could not consistently evoke biphasic increases in K^+ permeability. The results from both the $NMDG^+$ and Li^+ replacement experiments were in contrast to similar experiments performed in the rat submandibular gland (Bovell *et al.*, 1989c), where consistent results to acetylcholine could be obtained in both $NMDG^+$ and Li^+-containing solutions. In order to verify that the inconsistent results obtained using Li^+ substitution were caused by lithium ions, control experiments with HEPES-buffered solutions containing Na^+ were performed. However, HEPES-buffered superfusates containing Na^+ also failed to elicit consistent results in response to acetylcholine.

Repeat experiments were therefore undertaken using Na^+-free solutions buffered with HCO_3^- / CO_2. The results of these experiments confirmed that consistent results could be achieved in HCO_3^--containing solutions and that the initial transient phase of the acetylcholine-induced K^+ efflux is inhibited by the replacement of extracellular Na^+ with $NMDG^+$. The variation in responsiveness to acetylcholine in HCO_3^--free solutions, a feature not noted in the rat salivary gland (Bovell *et al.*, 1989c), would suggest that the dependence of basal K^+ efflux on the presence of bicarbonate, is a physiological feature of sweat glands. Exposure to $NMDG^+$-containing solutions has been shown to decrease pH_i and reduce responsiveness to acetylcholine, in both mouse submandibular and rat parotid salivary glands (Arkle *et al.*, 1988). The removal of extracellular HCO_3^- has been shown to lower pH_i (Steward, Seo & Case. 1989) and could account for the variability in the response of the sweat glands to acetylcholine. The results of $NMDG^+$ replacement would strongly suggest that in the human sweat gland, the presence of extracellular Na^+ is a prerequisite for the mobilisation of bound intracellular calcium stores. This is in direct contrast to the conclusion of Gallacher and Morris (1987) and to the finding in rat salivary gland (Bovell *et al.*, 1989c).

Under chloride-free conditions acetylcholine evoked a biphasic response, with both the Cl- substituents giving virtually identical results. The fact that the initial transient increase in K^+ efflux did not return to control levels suggests that intracellular calcium levels were augmented sustaining K^+ efflux. When calcium was added to the superfusate there was a further increase in efflux rate constant. Although the increase in efflux rate constant (peak-basal), was not significantly different from control values, the fact that the change occurred from an increased level of efflux suggests an augmentation of the overall response. The finding that K^+ efflux persists under Cl^--free conditions is in contrast to the findings in the simian gland (Saga *et al.*, 1988) where removal of extracellular Cl^- abolished K^+ efflux. However, the results are similar to findings in the rat lacrimal gland where removal of Cl^- failed to affect the acetylcholine-induced K^+ efflux (Singh, 1988).

There is no evidence in this study that calcium entry into the human sweat gland is inhibited by removal of extracellular Na+, which is in contrast to findings in rat salivary glands (Gallacher & Morris, 1987). The reason for this difference and the apparent necessity for the presence of bicarbonate in human sweat glands is unclear and requires further investigation.

TABLE 6.1.

Rate constants for the basal rate of $^{86}Rb^+$ efflux during superfusion with Bicarbonate and HEPES-buffered solutions.

	$^{86}Rb^+$ efflux rate constant (min^{-1}).	
Principal cation	HCO_3^--buffered	HEPES-buffered
Na^+	0.080 ± 0.003 (6)	0.065 ± 0.005 (7)*
Li^+	0.080 ± 0.005 (5)	0.086 ± 0.008 (6)†
$NMDG^+$	0.081 ± 0.006 (5)	0.092 ± 0.009 (5)†

*$p<0.01$ with respect to bicarbonate containing conditions. † $p<0.05$ with respect to appropriate sodium-containing solution. Number of experiments given in parenthesis.

Chapter 6. FIGURE LEGENDS.

Fig. 6.1. The response of 5 batches of glands during superfusion with calcium-free, HEPES-buffered, $NMDG^+$-containing solution to which acetylcholine (10^{-5} mol l^{-1}) and calcium (2.56 mmol l^{-1}) were subsequently added.

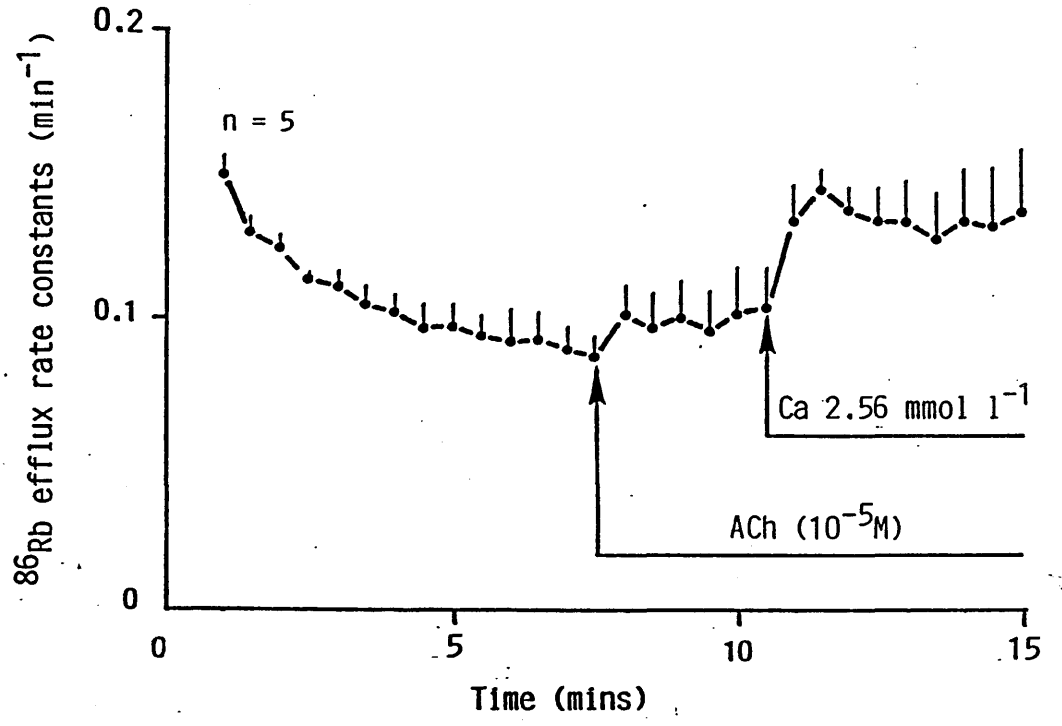
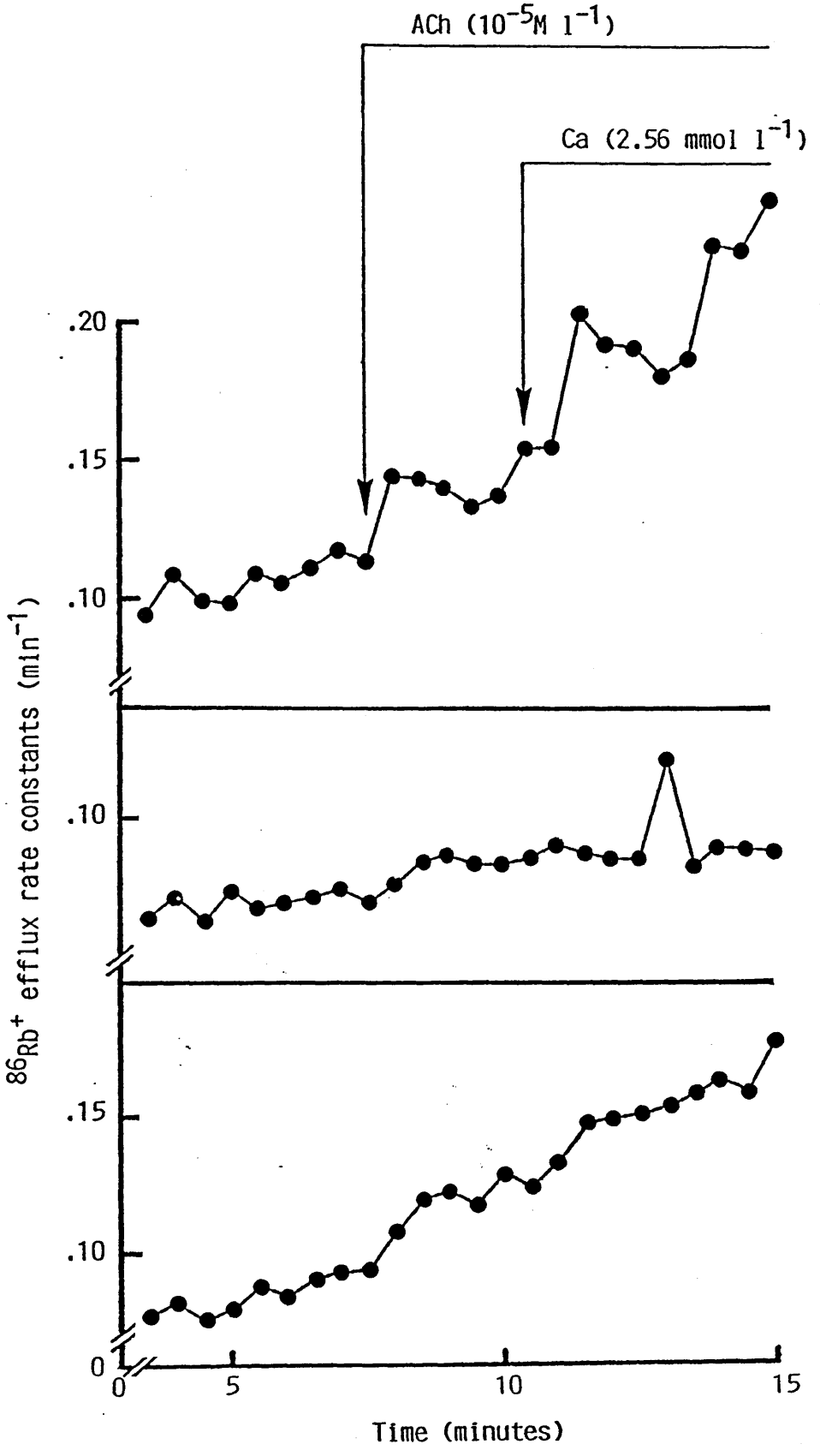

0.2
0.1
0
n = 5
Ca 2.56 mmol l^{-1}
ACh (10^{-5}M)
0
5
10
15
Time (mins)
^{86}Rb efflux rate constants (min^{-1})

Fig. 6.2 A & B. Increases in the rate of potassium ($^{86}Rb^{+}$) efflux evoked by acetylcholine and calcium during superfusion with a HEPES buffered solution in which extracellular sodium had been entirely replaced with Li^{+}. Each panel shows data from a single experiment, a total of six such experiments were performed.


ACh (10^{-5}M l^{-1})
Ca (2.56 mmol l^{-1})
.20
.15
.10
.10
.15
.10
0
0
5
10
15
$^{86}Rb^{+}$ efflux rate constants (min^{-1})
Time (minutes)

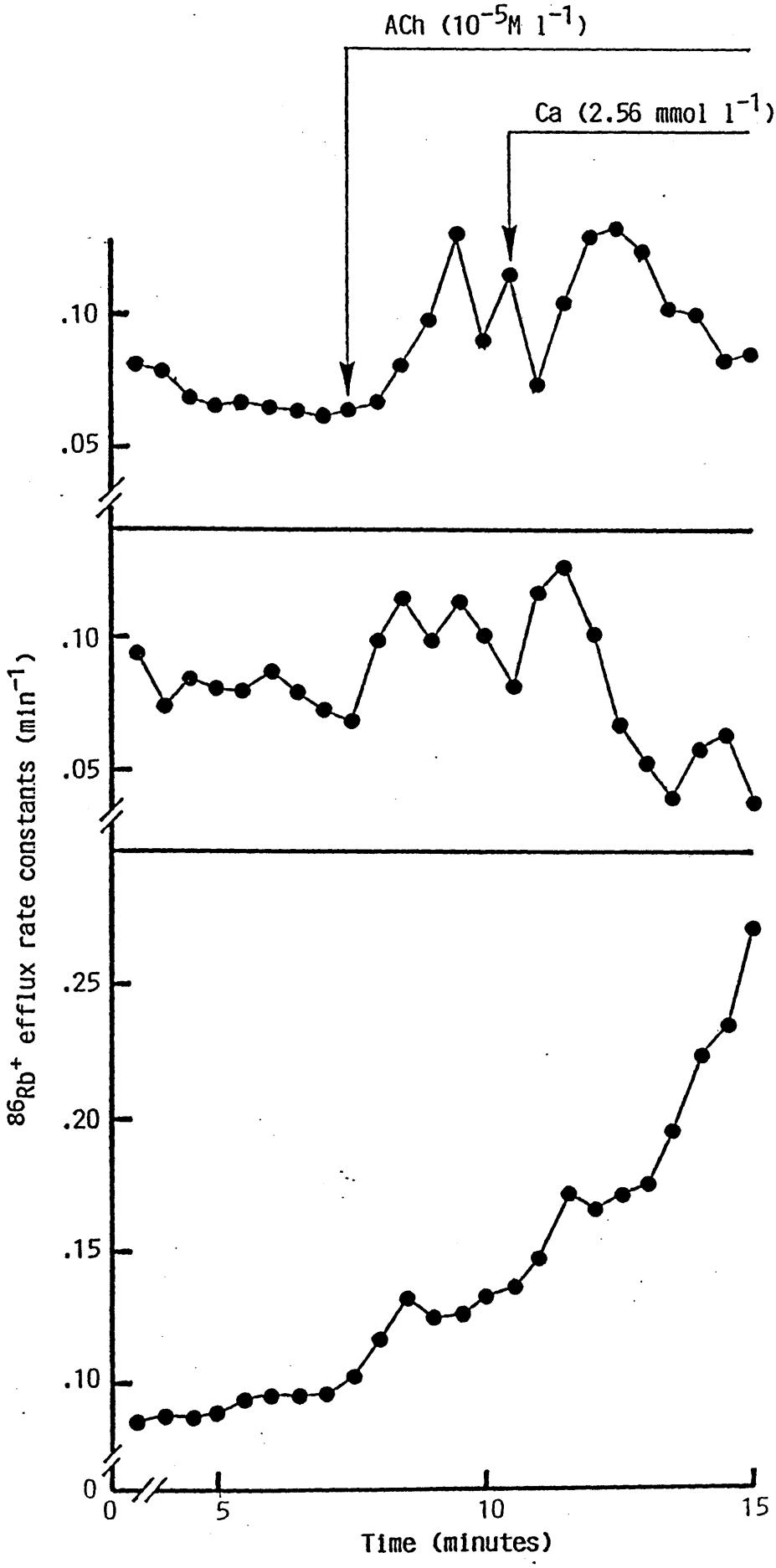
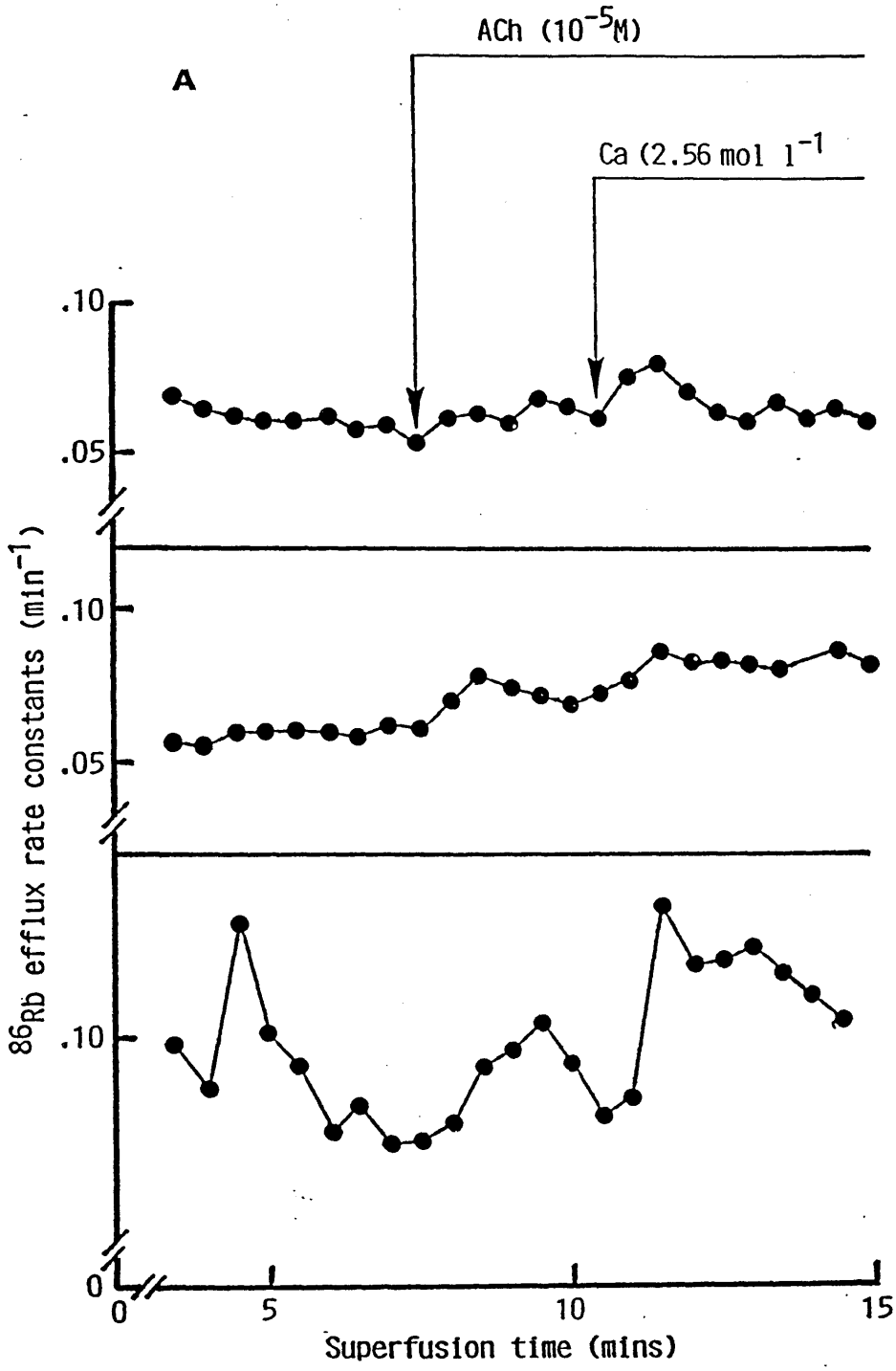

ACh (10^{-5}M l^{-1})
Ca (2.56 mmol l^{-1})
.10
.05
.10
.05
.25
.20
.15
.10
0
0
5
10
15
Time (minutes)
$^{86}Rb^{+}$ efflux rate constants (min^{-1})

Fig. 6.3 A & B. Effects of acetylcholine and calcium upon the efflux of $^{86}Rb^{+}$ during superfusion with Na-containing, HEPES-buffered solution. The figure shows data from a total of seven experiments.


ACh (10^{-5}M)
A
Ca (2.56 mol l^{-1}
.10
.05
.10
.05
.10
0
0
5
10
15
^{86}Rb efflux rate constants (min^{-1})
Superfusion time (mins)

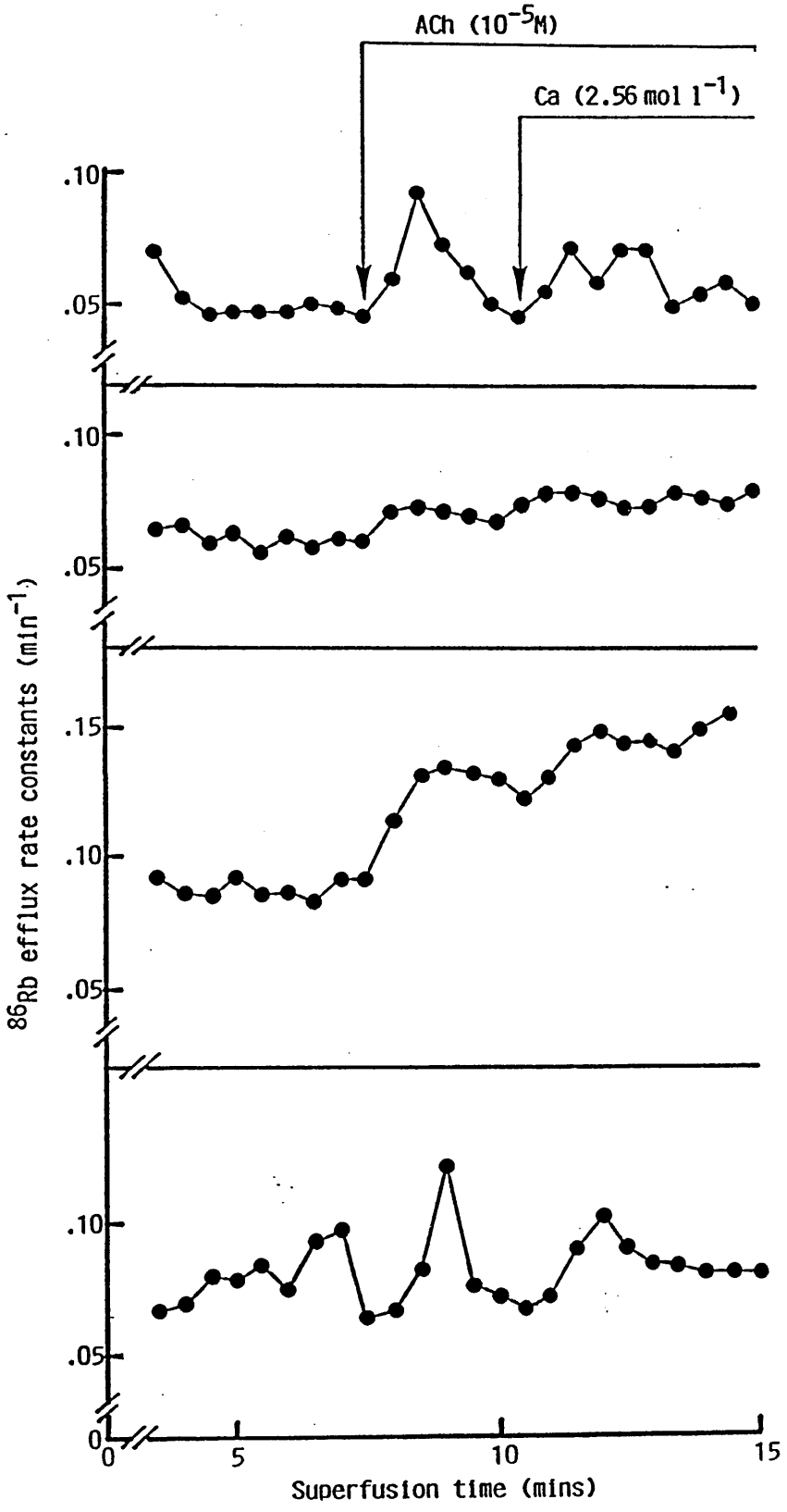

ACh (10^{-5}M)
Ca (2.56 mol l^{-1})
.10
.05
.10
.05
.15
.10
.05
.10
.05
0
0
5
10
15
Superfusion time (mins)
^{86}Rb efflux rate constants (min^{-1})

Fig. 6.4. Calcium-dependent and calcium-independent responses to acetylcholine during superfusion with sodium-free, HCO_3^--buffered, $NMDG^+$-containing solutions (n=5).

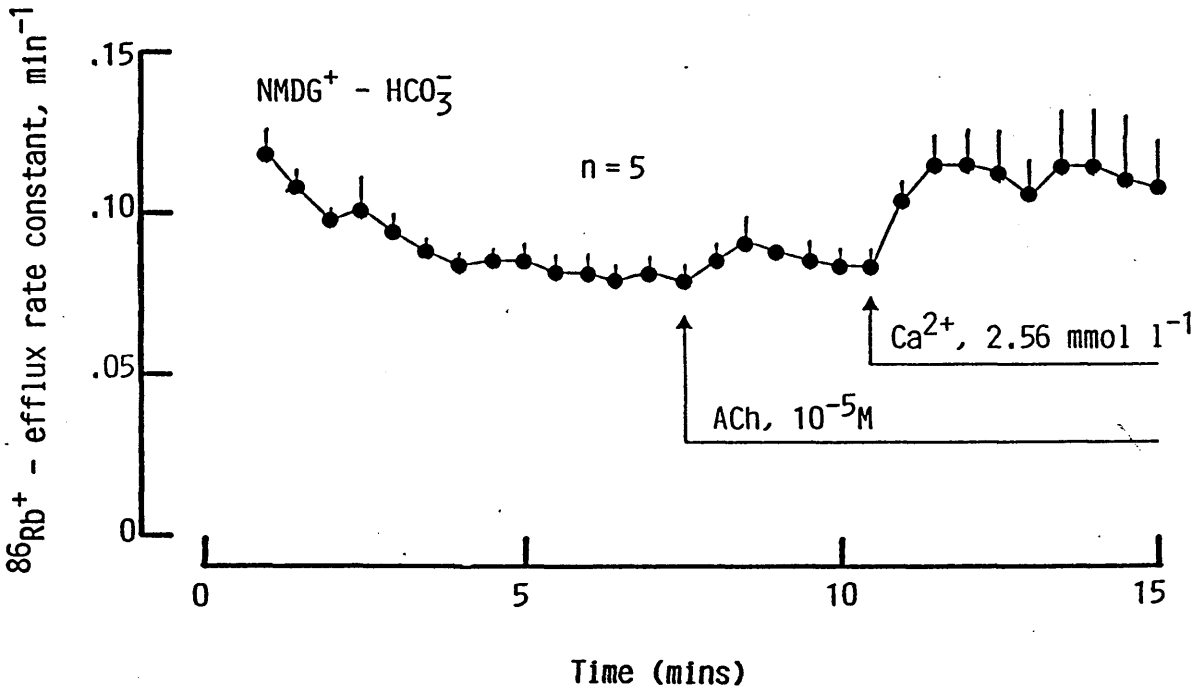

$^{86}Rb^+$ - efflux rate constant, min^{-1}
.15
.10
.05
0
$NMDG^+ - HCO_3^-$
n = 5
Ca^{2+}, 2.56 mmol l^{-1}
ACh, 10^{-5}M
0
5
10
15
Time (mins)

Fig. 6.5. The response of six batches of glands to acetylcholine and calcium, while being superfused with a Na^+-free, HCO_3^--buffered, Li^+-containing solution.

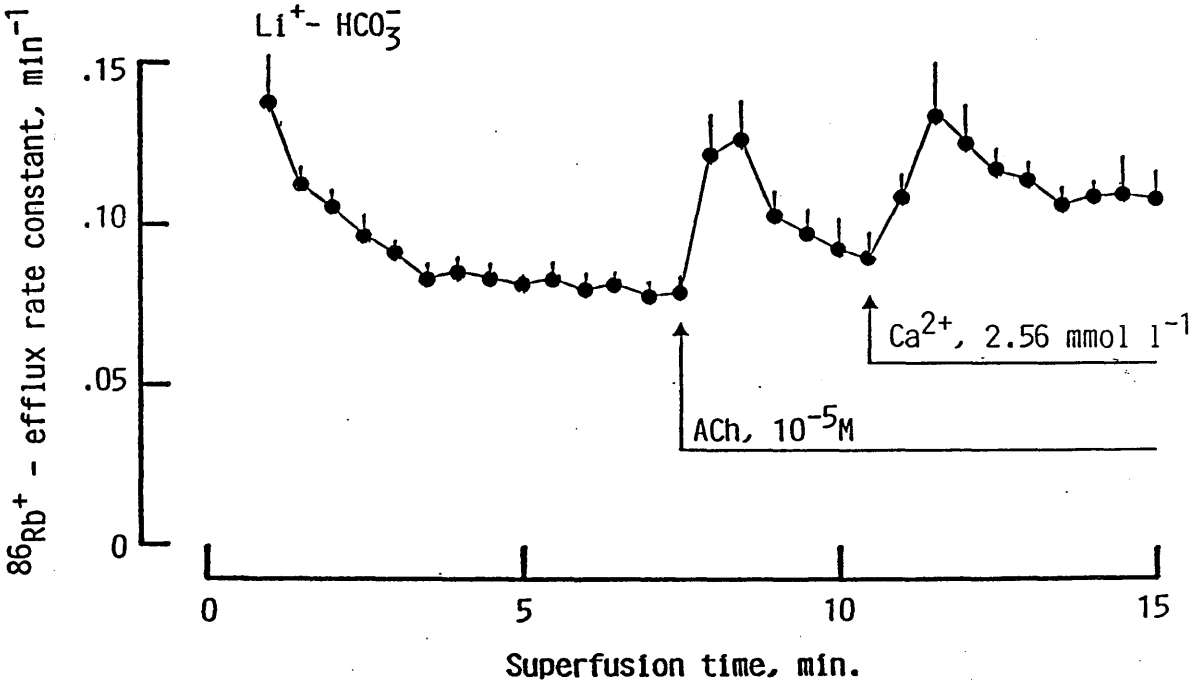

$Li^+ - HCO_3^-$
.15
.10
.05
0
$^{86}Rb^+$ - efflux rate constant, min^{-1}
Ca^{2+}, 2.56 mmol l^{-1}
ACh, 10^{-5}M
0
5
10
15
Superfusion time, min.

Fig. 6.6. The figure shows the response to acetylcholine in $NMDG^+$ solution containing bicarbonate, to which 72 mmol l^{-1} sodium and 2.56 mmol l^{-1} calcium were subsequently added (n=3).

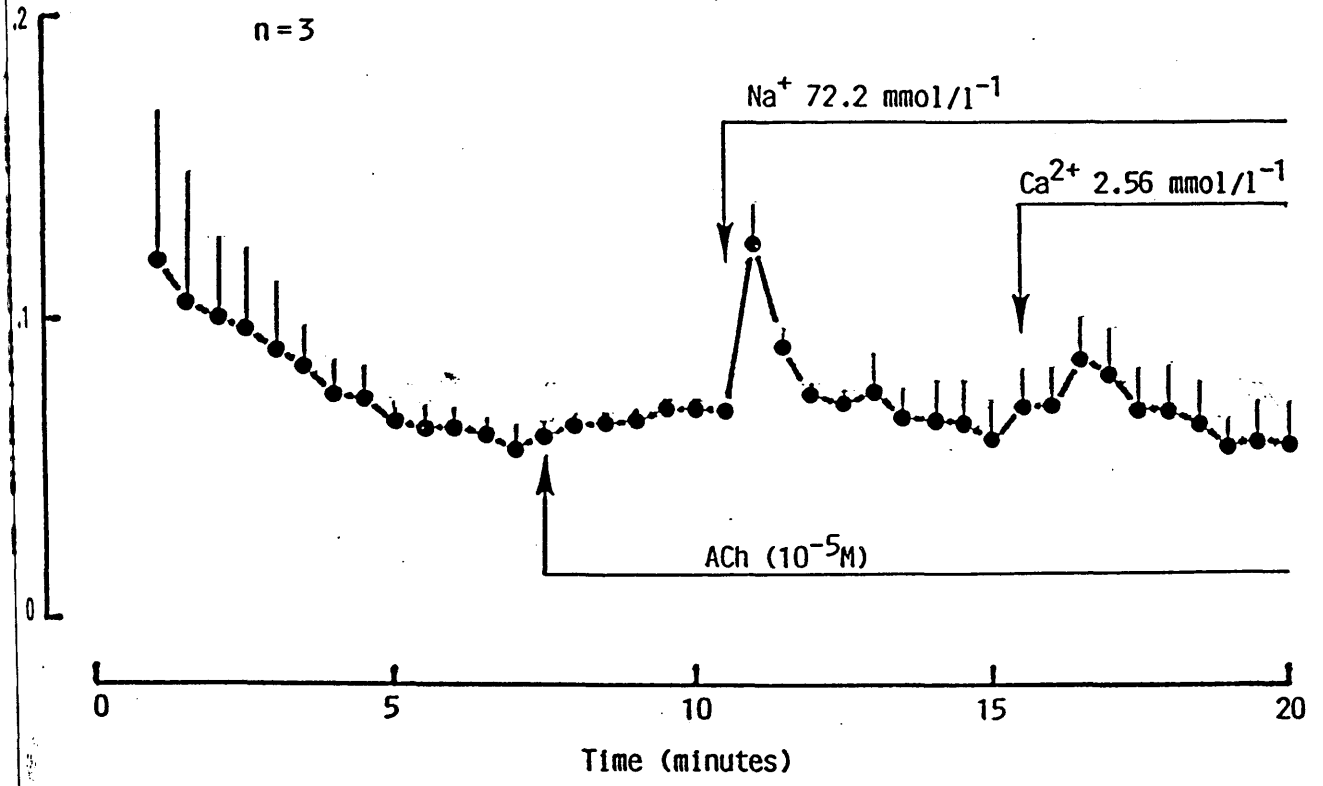
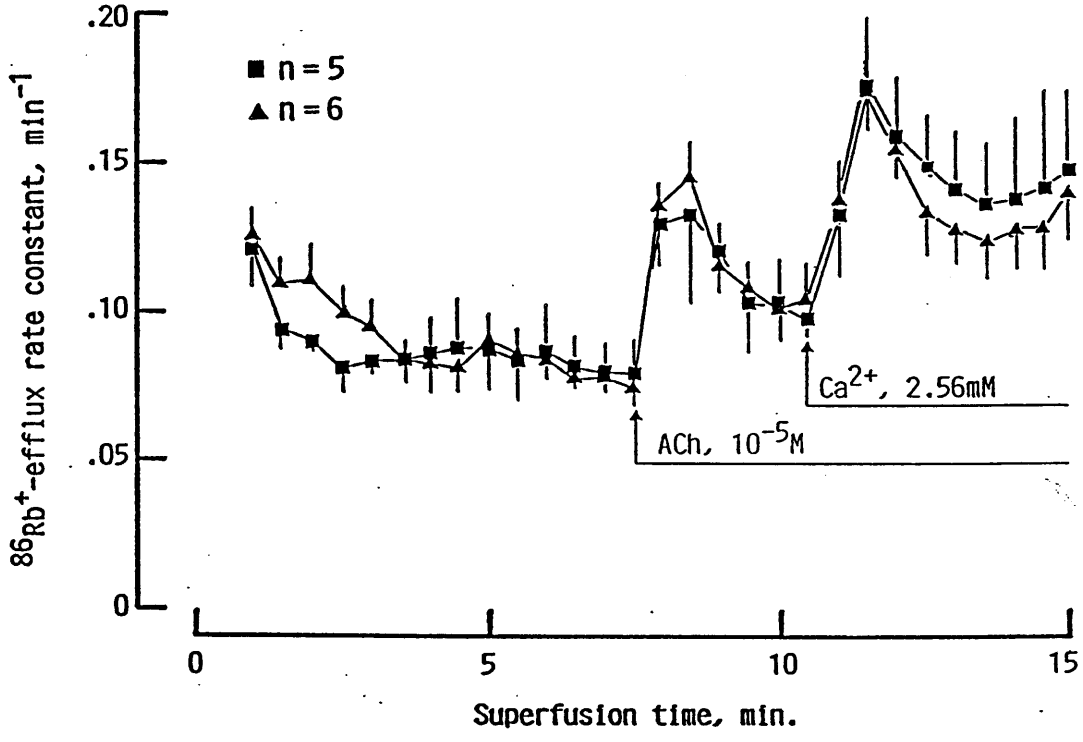

.2
.1
0
n = 3
Na+ 72.2 mmol/l-1
Ca2+ 2.56 mmol/l-1
ACh (10-5M)
0
5
10
15
20
Time (minutes)

Fig. 6.7. The response of sweat glands to superfusion with chloride-free solutions (gluconate replacement (■)(n=5), or nitrate replacement (♦)(n=6)) to which acetylcholine and calcium were subsequently added.


.20
.15
.10
.05
0
■ n = 5
▲ n = 6
$^{86}Rb^{+}$-efflux rate constant, min^{-1}
Ca^{2+}, 2.56mM
ACh, 10^{-5}M
0
5
10
15
Superfusion time, min.

CHAPTER 7.

GENERAL DISCUSSION.

The results obtained in this study clearly demonstrate that a K^+-efflux occurs in the isolated human sweat gland, in response to secretagogues (Bovell, Elder, Jenkinson & Wilson, 1988b; 1989a) and are in contrast to electrophysiological findings in the simian palm gland (Sato, 1973, 1986). The demonstration by radioisotopic methods of a K^+-efflux in sweat glands, agree with the findings of EPXMA studies, on sweat glands in the rat footpad (McWilliams *et al.*, 1988), the horse (Wilson *et al.*, 1988b), the human (Wilson *et al.*, 1988a) and, most recently, the simian palm (Saga & Sato, 1989).

Both cholinergic (muscarinic) and adrenergic agonists (α & β), have now been shown to increase the rate of K^+ ($^{86}Rb^+$) efflux from pre-loaded, isolated human sweat glands (Chapter 5)(Bovell *et al.*, 1988b; 1989a). The results in this study further demonstrate that the K^+ efflux in response to each agonist is generated via differing mechanisms. The response to acetylcholine, the major secretagogue of the human sweat gland, has been resolved into two components (Chapter 5), an initial transient phase, which persists in the absence of extracellular calcium and a second more sustained phase, which is dependent on extracellular calcium. A two phase response to ACh, such as that seen in this study, which is similar to that seen in salivary glands, is attributed to an increase in intracellular calcium ($[Ca^{2+}]_i$) (Putney, 1976; Parod & Putney, 1978 a & b; Putney *et al.*, 1978). The increase in $[Ca^{2+}]_i$ is thought to be mediated initially, via a release of bound calcium ions from an intracellular store (Putney, 1976; Aub *et al.*, 1982). Further evidence in support of this hypothesis comes from the finding in pancreatic (Gillespie, Greenwell & Scratcherd, 1988), and salivary acini (Aub & Putney, 1987; Arkle *et al.*, 1988) that ACh elevates $[Ca^{2+}]_i$ in the absence of extracellular calcium, as determined by fluorescent ratio imaging

Changes in the levels of calcium within the cells of the sweat glands used in this study were not quantified by EPXMA for two major reasons. Firstly, the level of calcium thought necessary to activate the K^+ channels, 50 nmol l^{-1} -

200 nmol l^{-1} (Petersen, 1984), is vastly below the level of detection available (~1 mmol l^{-1}) in bulk freeze-dried resin embedded sections. Secondly, since EPXMA measures total concentrations of elements, it is not possible to discriminate between 'free' and 'bound' states. Therefore any release of calcium from internal stores may not change the overall concentration within the cell and any release would remain undetected. Consequently, attempts were made to investigate ACh-induced increases in $[Ca^{2+}]_i$ by the fluorescent ratio imaging technique, in intact isolated human sweat glands. However these proved unsuccessful as the intensity of light generated by the fluorescent dye 'Indo' was completely masked by autofluorescence from the glands (Bovell, Elder & Wilson, unpublished observations). The autofluorescence appeared to originate in granules (thought to be lipofuscin granules) within the secretory cells and could not be quenched or inhibited. Sato & Sato (1988) did not have this problem with their study, using 'quin-2', on dispersed secretory cells from simian palm glands, to determine the relationship between changes in cytosolic calcium and sweat secretion. These authors did not detect any increase in cytosolic calcium in response to ACh when extracellular calcium was absent and concluded that any increase in $[Ca^{2+}]_i$ was due to an influx of calcium. This result contrasts with the findings in salivary (Aub & Putney, 1987; Arkle *et al.*, 1988) and pancreatic acini (Gillespie *et al.*, 1988) and maybe due to the ability of 'quin-2' to bind calcium (Grynkiewicz, Poenie & Tsien, 1985). Further studies to detect changes in calcium in dispersed human secretory cells, using different fluorescent probes (e.g. Fura-2), may resolve the apparent difference existing between the simian gland and the human gland, in the changes of $[Ca^{2+}]_i$ upon ACh-induced activation.

Although there is still some doubt as to the site of the intracellular stores of calcium, which are released in response to ACh, IP_3 is believed to activate a release of calcium from vesicular non-mitochondrial intracellular stores thought to be the endoplasmic reticulum (Petersen & Gallacher, 1988). IP_3 binding receptors have recently been identified on the endoplasmic reticulum

in rat cerebellar Purkinje neurons (Ross, Meldoles, Milner, Satoh, Supattapone & Snyder, 1989). Putney (1986) has suggested that both calcium stores and the calcium influx pathway are functionally coupled such that the calcium released from the E.R. will then activate the influx of calcium into the cells. It is known that the drug 'Ryanodine' blocks the release of calcium from the sarcoplasmic reticulum in muscles, but there is no information on the effects of this drug on calcium release from epithelial cells. It would therefore be interesting to examine the effects of 'Ryanodine' on the release of calcium from intracellular stores in sweat gland cells and from this it may be possible to resolve the question as to whether the release and influx mechanisms of calcium, are functionally coupled.

In this study it is assumed that the initial transient component of the response to acetylcholine reflects the IP_3-mediated release of a limited, store of intracellular calcium. The results obtained from sweat gland cells, suggests that the mobilisation of bound intracellular calcium, rather than an influx, of calcium, is dependent on the presence of extracellular Na^+. This result contrasts with findings of a patch clamp study performed on the mouse submandibular gland (Gallacher & Morris, 1987), where calcium influx was inhibited by the removal of extracellular sodium. Arkle *et al.*, (1988) observed that the mobilisation of calcium in salivary acini was inhibited during exposure to sodium-free solutions which contained the large impermeant cation $NMDG^+$. These authors attributed this result to a direct action of $NMDG^+$, rather than a consequence of removing sodium. However, the inhibitory effect demonstrated in chapter six of this study, was clearly a consequence of removing the sodium, as responsiveness to ACh was restored when the glands were exposed to 72.2 mmol l^{-1} Na^+, in the continued presence of equimolar $NMDG^+$.

It is not immediately apparent how removal of extracellular sodium may inhibit calcium release, particularly as the hydrolysis of PIP_2 is not sodium-dependent (Jones & Michell, 1976). However, the hydrophobic products of PIP_2 breakdown, diacylglycerols, are biologically active and allosterically

modulate the activity of protein kinase C (Kishimoto, Takai, Mori, Kikkawa & Nishizuka, 1980). As this enzyme regulates the activity of the Na^+/H^+ countertransport system, secretagogues which mobilise calcium stores may also influence pH_i by increasing the rate of proton extrusion (Dufresne, Bastie, Vaysse, Creach, Hollende & Ribet, 1985; Stoltoff, McMillian, Cantley, Cragoe & Talamo, 1989).

A similar phenomenon has been described in platelets by Siffert and Akkermann (1987), where cellular responses are mediated via an increased intracellular calcium concentration, and secretory activity is accompanied by an elevation of pH_i due to increased Na^+-H^+ exchange. In platelets, the mobilisation of calcium in response to activation is abolished when Na^+-H^+ exchange is inhibited by pharmacological means (Siffert & Akkermann, 1987). These authors propose that cytoplasmic alkalinisation is an essential component of stimulus-secretion coupling. The mobilisation of bound intracellular calcium by the action of IP_3, may be modulated by changes in pH_i where acidification reduces the affinity of IP_3 for the stores, resulting in calcium not being released, and alkalosis augmenting calcium release (Brass & Joseph, 1985). The data in this study suggest that such a cytoplasmic alkalinisation is a requirement for the mobilisation of calcium stores in the human sweat gland. The interactions between calcium and pH_i are complex (Busa & Nuccitelli, 1984) especially when internal sodium has been changed (Mullins & Requena, 1987) and therefore the consequences of altering these parameters are hard to predict.

The results from experiments described in chapter six of this study suggest that a similar mechanism to that proposed for the platelets, may operate in the isolated human sweat gland whereby replacement of Na^+ with $NMDG^+$-containing solutions would prevent an Na^+- H^+ countertransport system from regulating internal pH (Bovell, Elder, Jenkinson & Wilson, 1989b). The series of lithium replacement experiments, where calcium mobilisation proceeds normally, support the hypothesis that Na^+- H^+ exchange is involved in the release of intracellular calcium. Li^+ has been shown to support the extrusion

of H^+ to a limited extent, in barnacle muscle fibres (Boron, McCormick & Roos, 1981), renal microvilli (Kinsella & Aronson, 1981) and sheep heart purkinje fibres (Ellis & McLeod, 1985) but not in skeletal muscle (Aickin & Thomas, 1977). In the rat submandibular gland Li^+ appears to augment K^+ efflux (Bovell *et al.*, 1989c) while in the parotid gland 10 mmol l^{-1} lithium did not affect the cholinergically evoked efflux of $^{86}Rb^+$ (Downes & Stone 1986). In the mouse pancreas, however, the acetylcholine-evoked K^+ release is reversibly abolished when Li^+ is used as a replacement for Na^+ (Singh, 1984; Petersen & Singh, 1985).

An alternative explanation of the results obtained with $NMDG^+$ replacement, could be that the initial phase of potassium efflux occurs via a $Na^+/2Cl^-/K^+$ co-transport system, as has been postulated for the murine pancreas (Singh, 1984; Petersen & Singh, 1985). The chloride requirement for secretion in sweat glands (Sato & Sato, 1987) appears to be essentially similar to that observed in other exocrine glands (Case, Conigrave, Hunter, Novak, Thomson & Young, 1981; Case, Conigrave, Favaloro, Novak, Thomson & Young, 1982; Case, Hunter, Novak, & Young, 1984; Martinez & Cassity, 1985). In the mandibular glands of the rat (Martinez & Cassity, 1985) and the rabbit (Case et al., 1981; 1982; 1984), replacement of external chloride with impermeant anions does not completely abolish secretion. In simian sweat glands replacement of extracellular chloride by nitrate or isethionate does not completely inhibit sweat secretion (Sato & Sato, 1987). As one of the major events involved in the secretory mechanism is a K^+ efflux, in response to the loss of Cl^- from the cell, which maintains the driving force for secretion, one would expect that when Cl^- is reduced then the K^+ efflux would also be reduced. Saga, Sato & Sato (1988) describe such a result in simian palm glands, where replacement of extracellular chloride with nitrate substantially reduced the cholinergically-induced K^+ efflux. A similar result has also been described in the mouse pancreas using nitrate replacement, where K^+ ($^{86}Rb^+$) efflux was inhibited (Singh, 1984; Petersen & Singh, 1985). However, as described in chapter six, the replacement of Cl^- with either NO_3^- or Gluconate caused no such inhibition of K^+ ($^{86}Rb^+$) efflux from the isolated

human sweat gland (Bovell, Elder, Jenkinson & Wilson, 1989d). Under these conditions ACh evoked a biphasic increase in the rate of K^+ efflux. Similarly, removal of chloride did not affect the acetylcholine-induced K^+ efflux from rat lacrimal glands (Singh, 1988). Therefore, potassium efflux in the human sweat gland does not appear to be mediated by such a $Na^+/K^+/2Cl^-$ co-transporter system.

The results of this study, which show that the ACh-induced efflux of K^+ in the human sweat gland is not reduced under Cl^--free conditions and those of Saga *et al.*, (1988), who under similar conditions found that the K^+ efflux from the simian gland was not totally abolished, suggest that some other mechanism is inducing a K^+ efflux. The removal of all extracellular Cl^- has been shown to partially inhibit secretion in salivary glands and under these conditions the saliva that is produced is rich in HCO_3^- (Young *et al.*, 1987). The continued secretion of saliva under Cl^--free conditions demonstrated that the movement of negatively charged HCO_3^- from the cell into the lumen of the gland can support secretion (Young *et al.*, 1987). Although the removal of Cl^- has been shown to inhibit ACh-induced secretion from simian sweat glands (Sato & Sato, 1987), there is no information as to the concentration of HCO_3^- in the lumen of the glands under these conditions. The reason for a K^+ efflux in both the human and the simian gland, may therefore be in response to the movement of HCO_3^- from the cells, which would otherwise tend to depolarise the cell thereby reducing the driving force for secretion.

The lack of inhibition of the ACh-induced K^+ efflux from human sweat glands under Cl^--free conditions, compared with the reduction seen in the simian gland over a similar time period, may be a species difference between the two glands. A role for HCO_3^- is further emphasised by the finding that the removal of extracellular HCO_3^- gave inconsistent results when human sweat glands were stimulated with ACh, while removal of HCO_3^- did not appear to affect sweat secretion in the simian gland (Sato & Sato, 1987). Further investigations are therefore necessary in the human sweat gland to determine: 1) the concentration of HCO_3^- present in the lumen of the glands under Cl^--free

conditions, and 2) whether ACh can still evoke a K^+ efflux after a longer period of superfusion with a Cl^--free solution.

Glands of those species which do not show a response to ACh and which display different patterns of sweat output were also examined, using the technique of EPXMA (Chapter 3). An advantage of using EPXMA is that the elemental concentrations within the sample can be measured with a high degree of spatial resolution and changes in the levels of elements which occur, can be quantified. Although the use of freeze-dried resin embedded material, as in this study, restricts analyses to intracellular areas, reliable measurements can be obtained from these areas (Elder *et al.*, 1988b). Analyses of both intracellular and extracellular compartments are possible if frozen-hydrated sections are used instead of freeze-dried material, however, frozen-hydrated sections are technically more difficult to obtain, and to examine using the electron microscope. Although it was possible to prepare frozen-hydrated sections and to store them satisfactorily, it was not possible to analyse them. This was because the technology involved in transferring the frozen-hydrated sections to the electron microscope had not advanced sufficiently throughout the duration of this project to make regular, reproducible analyses possible (Bovell unpublished).

The finding by EPXMA, of an increase in the concentration of sodium in the secretory cells of activated cow sweat glands (Bovell, Elder, Jenkinson & Wilson, 1987; 1988a), concurs with the findings in other sweat glands (Elder *et al.*, 1985; McWilliams *et al.*, 1988; Wilson *et al.*, 1988 a & b; Saga & Sato, 1989) and the results obtained from other exocrine glands (Izutsu & Johnson, 1986). The finding of an increased level of Na^+ at the onset of sweating and a trend for Cl^- to similarly follow suit in the cow, suggests that the secretory epithelia belongs to the group of those Cl^- secreting epithelia which involve, at least, a Na/Cl cotransporter. In the cow there was a trend for chloride to increase upon activation and this result differs from that found in the monkey palm gland, where a fall in the concentration of chloride, upon activation, was reported (Saga & Sato, 1989). However, these results may not be inconsistent. There is currently no information as to how a cotransporter

system may account for the results in the cow, but two possible explanations exist. Firstly, the cotransporter may only function upon stimulation, accumulating both Na^+ and Cl^- in the secretory cells. A second, and alternative explanation may be that the cotransporter functions all the time and on activation the rate of transport is accelerated to a rate of influx initially greater than the rate of efflux of both ions. The latter explanation seems more likely as evidence from the shark rectal gland (Silva *et al.*, 1977; Hannafin *et al.*, 1983) suggests that the cotransporter mechanism functions continuously maintaining Cl^- above its equilibrium potential.

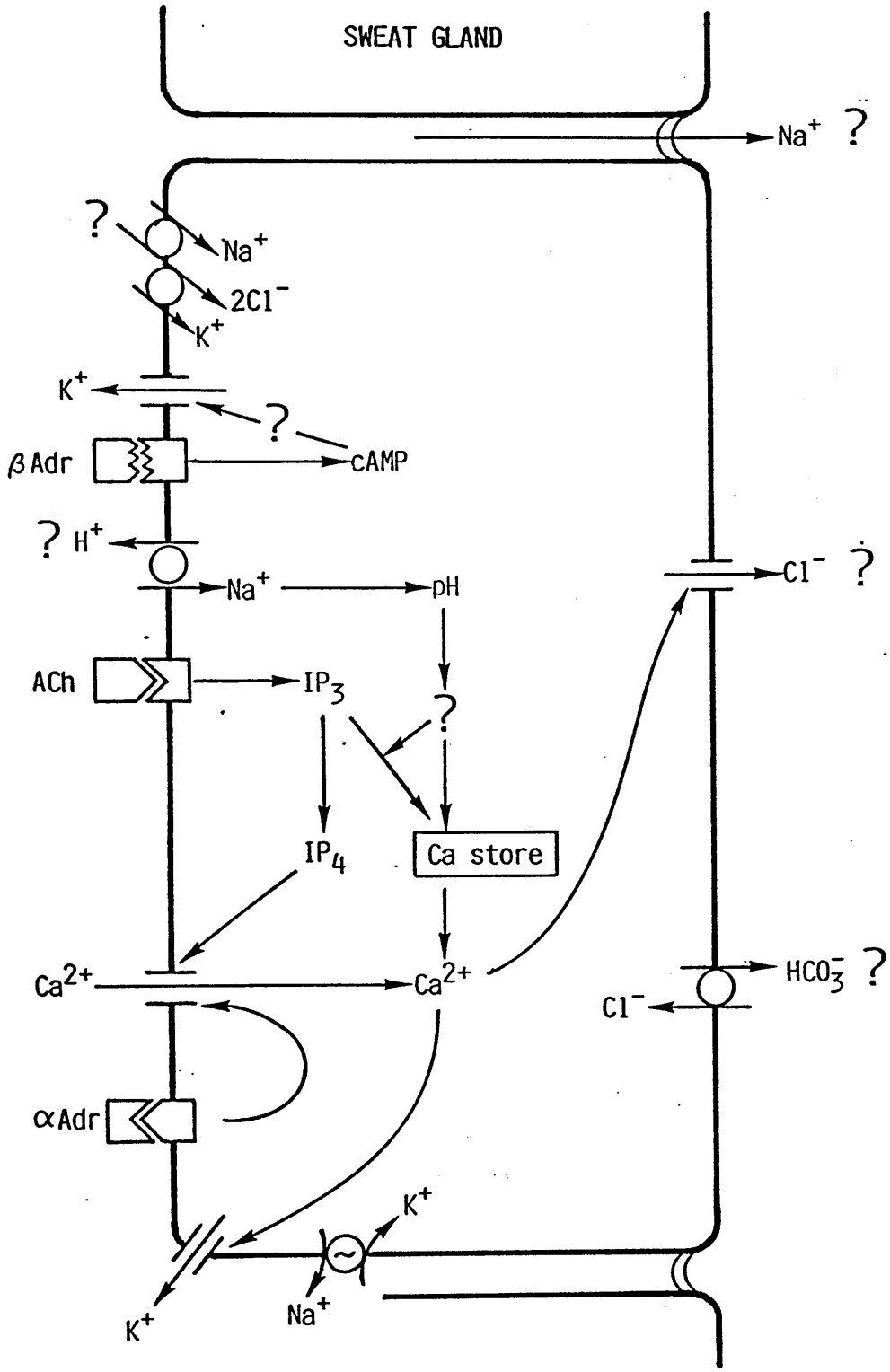
The finding that the levels of both Na^+ and Cl^- in the cow, return to control values during prolonged sweating would suggest that after an initial phase in which influx exceeds efflux, the cells achieve a new equilibrium where net ion fluxes, in the continued presence of stimulation, are again in balance though presumably at an increased rate.

It would appear from the EPXMA studies that active transport processes account for the increasing secretory rate in the cow but the results can not explain the 'step' like increases in sweat output. The results obtained from the secretory fundus of the sheep and goat sweat glands failed to show any evidence of active transport processes. The output of secretion in the sheep and goat, may be continuous and of such a low level that there are no overall changes in the intracellular elemental concentrations of the secretory fundus, when these animals are subjected to thermal stress. This would add support to Bligh's (1967) thesis that sweat production in these animals is a slow continuous process and that the pattern of evaporative loss is due to myoepithelial contractions expelling the sweat. The significant decrease in the level of intracellular Cl^- in the ductal cells of the sheep, contrasts with the findings of Wilson *et al.* (1988 a & b), who reported an increase in Cl^- in ductal cells of the human and the horse during thermal stimulation, and requires further investigation with larger numbers of animals.

Thaysen (1978) proposed that the responsiveness of individual glands upon initial activation, can vary substantially. These varying states of activity may be a possible explanation for the lack of significant changes in the level of both K^+ and Cl^- in the fundus of the cow and also the lack of change in the secretory cells of both the sheep and goat. Those glands removed for analyses may not all have been at the same stage of activation. In addition, the preparation of samples for EPXMA relies on the tissue being instantaneously frozen thus arresting sweat gland function at possibly differing states of activation. The use of larger numbers of isolated sweat glands from each of these species, would help to overcome the possibly varying states of activation. Isolated glands could either be stimulated by direct application of secretagogues, such as adrenaline and then immediately cryofixed for EPXMA, or used for radioisotopic flux studies. Further investigations by these techniques could determine if a K^+ efflux occurs in the cow, sheep and goat in response to secretagogues. The pattern of sweat output exhibited by the sheep and goat may be due to a lack of a K^+ efflux in response to stimulation, which would fail to maintain the driving force for secretion. This failure to maintain secretion may therefore account for the state of apparent fatigue exhibited by the sweat glands of these animals after prolonged thermal stimulation.

Fig. 7.1.

A detailed diagram of the mechanisms of action of cholinergic and adrenergic (α & β) agonists on the secretory cells in the fundus of the human sweat gland.


SWEAT GLAND
Na+ ?
?
Na+
2Cl−
K+
K+
?
βAdr
cAMP
? H+
Na+
pH
Cl− ?
ACh
IP3
?
IP4
Ca store
Ca2+
Ca2+
HCO3− ?
Cl−
αAdr
K+
K+
Na+

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Appendix 1 (a).

Original data for Na, K and Cl in the funduses of cow, sheep and goat sweat glands, before, at the onset of, and during the sustained phase of evaporative moisture loss. Data are given as percentage absolute mass fractions ± s.d. 'n' represents the number of spectra obtained from each animal. Chlorine values have been corrected for the chlorine content of the resin.

FUNDUS.

ANIMAL.

COW.	'n'	Na	K	Cl
			BEFORE.	
1	30	.021 ± .012	.492 ± .120	.268 ± .100
2	30	.009 ± .012	.730 ± .101	.214 ± .059
3	30	.001 ± .005	.536 ± .111	.200 ± .057
4	30	.003 ± .004	.589 ± .201	.120 ±. 090
5	30	.050 ± .020	.524 ± .125	.101 ± .054
6	30	.032 ± .020	.453 ± .114	.095 ± .046
7	30	.057 ± .044	.656 ± .211	.219 ± .086
			ONSET.	
1	30	.032 ± .030	.390 ± .078	.171 ± .067
2	30	.081 ± .050	1.09 ± .898	.352 ± .090
3	30	.032 ± .016	.354 ± .058	.158 ± .062
4	30	.024 ± .019	.747 ± .272	.173 ± .088
5	30	.100 ± .062	.814 ± .226	.145 ± .050
6	30	.070 ± .043	.454 ± .142	.293 ± .098
7	30	.058 ± .024	.450 ± .116	.151 ± .040
			PLATEAU	

			PLATEAU	
1	30	.015 ± .003	.687 ± .266	.242 ± .094
2	30	.018 ± .016	.658 ± .128	.184 ± .059
3	30	.032 ± .014	.821 ± .106	.197 ± .031
4	30	.018 ± .016	.284 ± .140	.141 ± .100
5	30	.013 ± .007	.409 ± .055	.072 ± .035
6	30	.014 ± .007	.507 ± .109	.089 ± .045
7	30	.051 ± .024	.591 ± .113	.087 ± .034

ANIMAL.

SHEEP.	'n'	Na	K	Cl
			BEFORE	
1	30	.0016 ± .005	.697 ± .150	.080 ± .040
2	30	.0123 ± .010	.732 ± .100	.231 ± .080
3	30	.0120 ± .012	.671 ± .056	.058 ± .033
4	30	.0390 ± .020	.504 ± .073	.164 ± .041
			ONSET.	
1	30	.020 ± .020	.422 ± .194	.140 ± .039
2	30	.018 ± .014	.687 ± .097	.300 ± .231
3	30	.020 ± .014	.722 ± .100	.121 ± .025
4	30	.005 ± .005	.636 ± .098	.145 ± .042
			PLATEAU	
1	30	.006 ± .005	.404 ± .075	.250 ± .082
2	30	.022 ± .014	.363 ± .056	.161 ± .050
3	30	.008 ± .008	.853 ± .194	.055 ± .028
4	30	.052 ± .032	.313 ± .119	.052 ± .069

ANIMAL.

GOAT.	'n'	Na	K	Cl
			BEFORE.	
1	30	0	.541 ± .066	.107 ± .042
2	30	.026 ± .014	.432 ± .100	.151 ± .065
3	30	.020 ± .012	.991 ± .112	.135 ± .060
4	30	.027 ± .020	.529 ± .079	.123 ± .040
			ONSET.	
1	30	.015 ± .011	.489 ± .100	.119 ± .035
2	30	.070 ± .025	.265 ± .052	.281 ± .087
3	30	.007 ± .015	.975 ± .180	.250 ± .046
			PLATEAU	
1	30	.009 ± .014	.343 ± .072	.176 ± .029
2	30	.017 ± .013	.371 ± .065	.219 ± .050
3	30	.081 ± .035	.252 ± .085	.212 ± .062
4	30	.011 ± .011	.521 ± .125	.123 ± .045

Appendix 1 (b).

Original data for Na, K and Cl in the ducts of cow, sheep and goat sweat glands, before, at the onset of, and during the sustained phase of evaporative moisture loss. Data are given as percentage mass fractions ± s.d. 'n' represents the number of spectra obtained from each animal. Chlorine values have been corrected for the chlorine content of the resin.

DUCT.

ANIMAL.

COW.	'n'	Na	K	Cl
			BEFORE.	
1	30	.057 ± .012	.624 ± .150	.233 ± .060
2	30	.052 ± .017	.352 ± .043	.107 ± .043
3	30	.019 ± .017	.495 ± .104	.139 ± .040
4	30	.007 ± .006	.229 ± .041	.201 ± .051
5	30	.171 ± .045	.493 ± .100	.226 ± .065
6	30	.111 ± .033	.678 ± .179	.403 ± .115
7	30	.023 ± .016	.297 ± .075	.135 ± .041
			ONSET.	
1	30	.086 ± .042	.558 ± .075	.313 ± .180
2	30	.020 ± .017	.274 ± .100	.220 ± .080
3	30	.031 ± .034	.269 ± .053	.131 ± .046
4	30	.014 ± .011	.287 ± .065	.249 ± .048
5	30	.046 ± .021	.318 ± .120	.209 ± .150
6	30	.033 ± .019	.515 ± .065	.042 ± .032
7	30	.031 ± .019	.720 ± .152	.227 ± .089
			PLATEAU	
1	30	.163 ± .076	.206 ± .050	.074 ± .038
2	30	.004 ± .010	.556 ± .114	.166 ± .127
3	30	.038 ± .018	.574 ± .060	.188 ± .056

3	30	.038 ± .018	.574 ± .060	.188 ± .056
4	30	.032 ± .020	.594 ± .089	.235 ± .046
5	30	.051 ± .018	.375 ± .078	.412 ± .110
6	30	.066 ± .019	.382 ± .087	.161 ± .047
7	30	.064 ± .017	.331 ± .077	.153 ± .049

ANIMAL.

SHEEP.	'n'	Na	K	Cl
			BEFORE.	
1	30	.054 ± .020	.541 ± .052	.265 ± .054
2	30	.063 ± .022	.615 ± .140	.256 ± .070
3	30	.050 ± .030	.426 ± .070	.305 ± .098
4	30	0	.470 ± .044	.156 ± .041
			ONSET.	
1	30	.025 ± .012	.339 ± .100	.191 ± .125
2	30	.020 ± .018	.473 ± .070	.177 ± .056
3	30	.043 ± .022	.401 ± .074	.205 ± .078
4	30	.010 ± .010	.609 ± .048	.129 ± .033
			PLATEAU	
1	30	.050 ± .029	.448 ± .074	.236 ± .069
2	30	.043 ± .023	.483 ± .067	.228 ± .068
3	30	.006 ± .014	.449 ± .042	.164 ± .050
4	30	.056 ± .015	.671 ± .093	.342 ± .169

ANIMAL.

GOAT.	'n'	Na	K	Cl
			BEFORE.	
1	30	.003 ± .009	.631 ± .134	.138 ± .068
2	30	.040 ± .020	.718 ± .214	.288 ± .126
3	30	.009 ± .005	.518 ± .078	.139 ± .038

4	30	.038 ± .028	.340 ± .042	.204 ± .048
			ONSET.	
1	30	.067 ± .028	.283 ± .058	.235 ± .057
2	30	.010 ± .009	.562 ± .066	.168 ± .059
3	30	.028 ± .014	.513 ± .080	.212 ± .040
	PLATEAU			
1	30	.003 ± .007	.575 ± .073	.065 ± .046
2	30	0	.583 ± .083	.124 ± .061
3	30	.080 ± .055	.200 ± .056	.210 ± .104

APPENDIX 2.

Safety.

Provided that proper precautions are observed, radioactive materials present less of a hazard than other laboratory substances. Safety, in the use of radioactive materials comes by limiting the exposure of the user to the ionising radiations to acceptable levels. Where the quantities of radioactivity in use are small, adequate safeguarding can be obtained by working at a large enough distance away from the source. The radiation dose received is proportional to the number of particles of photons incident on unit area. This means that if we double the distance (d) between the irradiated area and the point source of emissions the radiation dose received is proportional to $1/d^2$. This is known as the inverse square law.

There is however, a maximum distance (arm length) to which it is possible to work conveniently with a vial containing isotope. Therefore, it is necessary to reduce exposure by placing a 'shield' between the source and the operator. Commonly used materials for shielding include glass, perspex, lead and cement.

The type of shielding used depends on the source of emissions. For the purposes of this thesis, precautions were taken to minimise exposure to β & γ emissions. Beta particle emissions, which have an effective range of a few metres in air can be stopped by glass or perspex. The thickness ($t\beta$) of shielding required to absorb beta emissions from a radioactive source can be calculated using the formula:

$t\beta = R\beta$ x density of shielding material.

where $R\beta = E\beta/2.$ gm.cm^2

$R\beta \equiv$ the range of emissions,

$E\beta \equiv$ the energy of the source (MeV).

It is important to note that pure β emitters should be shielded by low atomic number materials. As beta particles pass through matter they may cause the emission of a photon of electromagnetic radiation (Bremsstrahlung). High atomic number materials are much more efficient at generating this Bremsstrahlung. It is important in this context to remember that any isotope which has a component of γ emissions will require lead shielding for protection of the user. Therefore, to avoid Bremsstrahlung production from beta emissions passing through lead shielding, the lead can be placed behind the primary perspex shielding. This effectively reduces the amount of beta emissions capable of producing Bremsstrahlung when from the high atomic number lead.

Another safety consideration is the possible exposure to radiation via contamination. It is essential to minimise contamination and restrict the area within which it can occur. All dispensing of radioisotopic materials as well as being carried out behind shielding, is done over a drip tray lined with paper towelling.

APPENDIX 3.

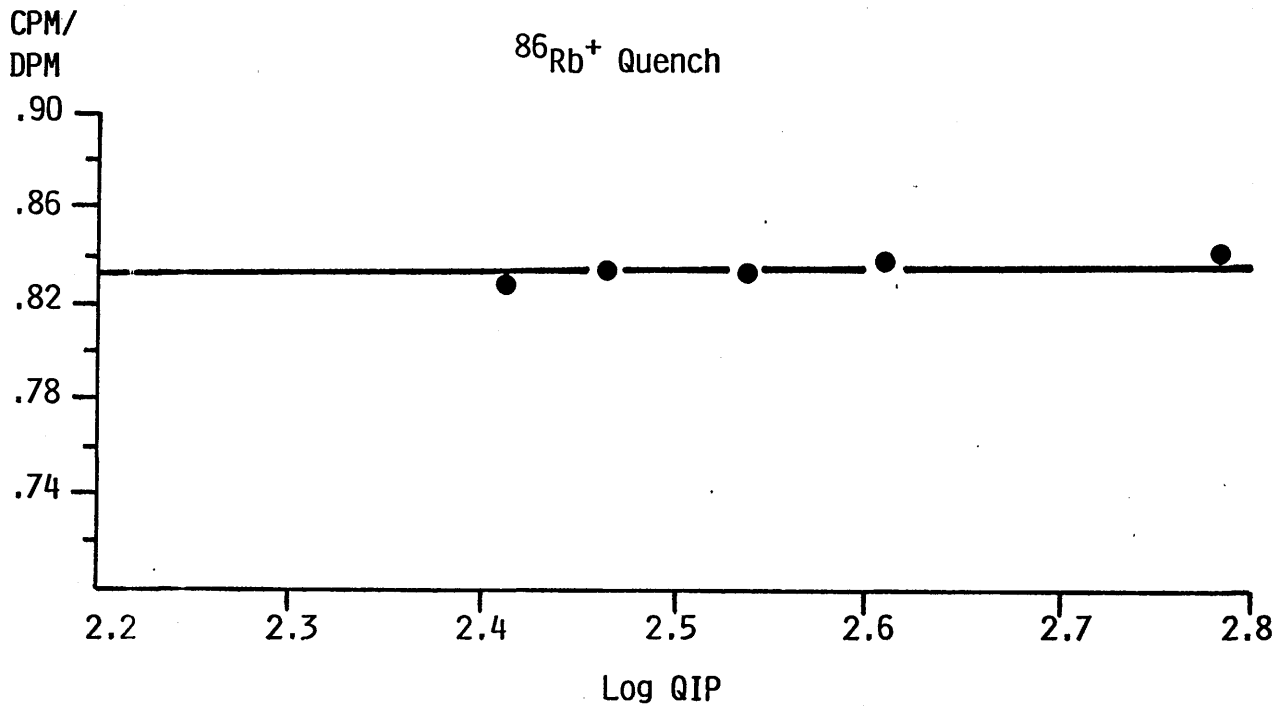
Liquid scintillation counting.

Three basic instruments are used for the quantitation of radioactive materials: gas filled chamber (Geiger-Müller type), the semiconductor detector and the scintillation counter.

For measurements of many isotopes the scintillation counter has the greatest sensitivity, and its introduction has made possible the accurate measurement of very small amounts of a wide range of radioactive materials. In this type of instrument, energy from emitted radiation is absorbed by a fluorescent material (scintillator or fluor) and re-emitted as light photons; which are detected by a photomultiplier tube and converted to electrical energy (pulse voltage) for quantification. In liquid scintillator counting the radioactive material is brought into close relationship with a scintillator usually by dissolving both in a suitable solvent. This procedure is particularily suitable for the quantitative measurement of radiations which have limited penetrating power, such as α or β particles.

The number of counts recorded by a liquid scintillation counter is always less than the number of β-particles emitted in the sample. This discrepancy is usually expressed in terms of counting efficiency, which is the percentage (or fraction) of decaying nuclei actually counted. Counting efficiency depends on a number of factors the most important being quenching. Quenching is due to the reduction of pulse voltage by material present in the sample or within the scintillant mixture reducing the number of photons of light detected.

The counting efficiency of each counter can be determined by reference to a set of quenched standards which consist of vials containing a known amount of radioactivity together with graded quantities of a quenching agent. They thus provide a series of standards whose disintegration rates are known and whose differences in counting efficiency are caused by the quenching agent. The counting efficiency of the counter used for the experiments in this thesis was approximately 80% (see accompanying graph).


CPM/
DPM
$^{86}Rb^{+}$ Quench
.90
.86
.82
.78
.74
2.2
2.3
2.4
2.5
2.6
2.7
2.8
Log QIP

APPENDIX 4.

A sample printout of the results obtained using Dr S. M. Wilson's computer program to calculate efflux rate constants. The program also calculates the amount of $^{86}Rb^{+}$ (pmoles) present in the sample at the begining of the experiment.

SWEAT GL LI-HCO3 22/3/89

Total Counts in tissue = 53190.405 DPM

= 23.960 nCi

Specific activity = 0.079 nCi/pmole

Total tissue Rb content = 304.508 pmoles

n	CPM	QIP	% Eff.	DPM	Fraction	pmole	k
1	4627	1201	83	5511	0.896	272.952	-
2	3595	1149	83	4274	0.816	248.481	-0.188
3	2252	1171	83	2664	0.766	233.228	-0.127
4	1992	1130	83	2352	0.722	219.760	-0.119
5	1714	1153	83	2019	0.684	208.201	-0.108
6	1488	1154	83	1748	0.651	198.193	-0.099
7	1203	1147	83	1406	0.624	190.141	-0.083
8	1177	1151	83	1375	0.599	182.267	-0.085
9	1101	1146	83	1284	0.574	174.916	-0.082
10	1113	1135	83	1298	0.550	167.482	-0.087
11	1068	1155	83	1244	0.527	160.356	-0.087
12	904	1172	83	1047	0.507	154.357	-0.076
13	924	1162	83	1071	0.487	148.220	-0.081
14	860	1087	83	995	0.468	142.523	-0.078
15	806	1148	83	930	0.451	137.196	-0.078
16	992	1141	83	1153	0.429	130.593	-0.099
17	979	1106	83	1137	0.407	124.078	-0.102
18	852	1116	83	985	0.389	118.436	-0.093
19	896	1080	83	1038	0.369	112.491	-0.103
20	1038	1074	83	1208	0.347	105.572	-0.127
21	733	1137	83	842	0.331	100.747	-0.094
22	752	1149	83	865	0.315	95.790	-0.101
23	834	1083	83	964	0.296	90.272	-0.119
24	726	1105	83	834	0.281	85.494	-0.109
25	681	1103	83	780	0.266	81.025	-0.107
26	623	1125	83	711	0.253	76.955	-0.103
27	564	1132	83	640	0.241	73.289	-0.098
28	613	1139	83	699	0.228	69.287	-0.112
29	517	1099	83	583	0.217	65.944	-0.099
30	507	1174	83	571	0.206	62.670	-0.102

APPENDIX 5.

Disposal of radioactive waste.

The radioactive waste was disposed in accordance with the regulations within the University of Glasgow. The $^{86}Rb^{+}$ 'loading' solutions were stored in plastic containers behind perspex and lead until at least three half-lives had elapsed. Effluent/Scintillator mixtures and their vials were stored in large plastic drums. Both the stored loading solutions and the experimental samples were then delivered to the University's Radiation Services for final disposal.

APPENDIX 6.

PHYSIOLOGICAL SALT SOLUTIONS.

The solutions used for isolating, loading and superfusing the sweat glands in Chapters 5 & 6 are detailed in the following section.

1). Isolation medium:

Solution A.

NaCl.	(1.03M).	=	58.44g		
KCl.	(48mM)	=	3.578g		
KH_2PO_4	(12mM)	=	1.633g		
Distilled water		=	To 1 litre		

Solution B.

HEPES	(250mM)	=	59.575g		
$NaHCO_3$	(200mM)	=	16.8g		
$MgSO_4 7H_2O$	(12mM)	=	2.96g		
Distilled water		=	To 1 litre		

Working solution.

Solution A		=	50ml	or	100ml
Solution B		=	50ml	or	100ml
Distilled water		=	300ml	or	600ml
Glucose		=	0.99g	or	1.98g

Gas solution with 95% O_2/5%CO_2

Molar $CaCl_2$		=	0.65ml	or	1.3ml

Adjust to pH 7.4 with NaOH

Distilled water		=	to 500ml	or	to 1000ml

Gas solution with 95% O_2 / 5%CO_2

Taken from Lee, Jones & Kealey (1984).

2). Normal (modified Krebs / Henseleit solution).

Solution A.

NaCl.	(1.03M).	=	60.193g
KCl	(47mM)	=	3.504g
$MgCl_26H_2O$	(11.3mM)	=	2.297g
Distilled water		=	To 1 litre

Solution B.

$NaHCO_3$	(250mM)	=	21.003g
NaH_2PO_4	(115mM)	=	21.003g
Distilled water		=	To 1 litre

Working solution.

Solution A		=	50ml	or	100ml
Solution B		=	50ml	or	100ml
Distilled water		=	300ml	or	600ml
Glucose		=	0.275g	or	0.55g
Na Pyruvate		=	0.269g	or	0.539g
Na_2 Fumarate		=	0.216g	or	0.432g
Na Glutamate		=	0.414g	or	0.828g

Gas solution with 95 % O_2 / 5 % CO_2

Molar $CaCl_2$		=	1.28ml	or	2.56ml
Distilled water		=	To 500ml	or	To 1000ml

Gas solution with 95 % O_2 / 5 % CO_2

Adjust pH to 7.4

3). Sodium-free (NMDG replacement).

1Molar Pyruvic Acid.

Pyruvic Acid		=	3.467ml
Distilled water		=	To 50ml

Aliquot into 5ml and freeze.

Working solution.

N-Methyl-D-glucamine	(144mM)	=	28.19g
KCl	(3.55mM)	=	0.265g
KH_2PO_4	(1.15mM)	=	0.157g
$MgCl_2$	(1.13mM)	=	0.230g
HEPES	(25mM)	=	5.958g
D-Glucose	(2.8mM)	=	0.556g
E.G.T.A.	(0.1mM)	=	0.038g

The following are free acids

Pyruvic acid	(4.9mM)	=	4.9ml
Fumaric acid	(4.9mM)	=	0.721g
Glutamic acid	(2.7mM)	=	0.313g
Distilled water		=	800ml

Adjust pH to 7.4 with normal HCl (~110ml)

Add distilled water		=	To 1000ml

Gas solution with 100 % O_2

4). Sodium-free (Lithium replacement).

LiCl	(108mM)	=	4.578g
KCl	(3.55mM)	=	0.265g
KH_2PO_4	(1.15mM)	=	0.157g
1M $MgCl_2$	(1.13mM)	=	1.13ml
HEPES	(25mM)	=	5.958g

D-Glucose	(7.8mM)	=	0.556g
E.G.T.A.	(0.1mM)	=	0.038g

The following are free acids

Pyruvic acid	(4.9mM)	=	4.9ml
Fumaric acid	(4.9mM)	=	0.721g
Glutamic acid	(2.7mM)	=	0.313g

Distilled water = 800ml

Check pH.

Adjust to pH 7.4 with 1M LiOH (~23-24ml)

Add distilled water = To 1000ml

Gas solution with 100 % O_2.

5). HCO_3^- buffered sodium-containing ($NMDG^+$ replacement) solution.

Working solution.

KCl	(3.55mM)	=	0.265g
KH_2PO_4	(1.15mM)	=	0.157g
$MgCl_2$	(1.13mM)	=	1.13 ml 1M stock solution
D-Glucose	(2.8mM)	=	0.556g

Add the following free acids

Pyruvic acid	(4.9mM)	=	4.9ml 1M stock solution
Fumaric acid	(4.9mM)	=	0.721g
Glutamic acid	(2.7mM)	=	0.313g

Distilled water		=	800ml
E.G.T.A.	(0.1mM)	=	0.038g
N-Methyl-D-glucamine	(119.4mM)	=	23.307g

Adjust to pH 7.0 with normal HCl

Add

N-Methyl-D-glucamine	(25mM)	=	4.880g

Gas solution with 5 % CO_2 & 95 % O_2 until pH is near to 7.4

6). HCO_3^- -buffered sodium-free (Li^+ replacement) solution.

LiCl	103mM	=	4.366g
KCl	3.55mM	=	0.265g
KH_2PO_4	1.15mM	=	0.157g
D-Glucose	2.8mM	=	0.556g
Pyruvic acid	4.9mM	=	4.9ml stock solution
Fumaric acid	2.7mM	=	0.313g
Glutamic acid	4.9mM	=	0.721g
EGTA	0.1mM	=	0.038g

Add to 800mls of distilled water and adjust the pH to 7.0 with LiOH.

Add LiOH	25mM	=	1.049g

Gas with 95 % O_2 / 5 % CO_2 and then add

$MgCl_2$	1.13mM	=	1.13ml stock solution

7). Chloride-free (Nitrate replacement).

Solution B.

$NaHCO_3$	(250mM)	=	21.003g
NaH_2PO_4	(115mM)	=	21.003g
Distilled water		=	To 1 litre

Solution E.

$NaNO_3$.	(1.03M).	=	60.193g
KNO_3.	(47mM)	=	3.504g
$MgSO_4$	(11.3mM)	=	2.297g
Distilled water		=	To 1 litre

Working solution.

Solution B	=	50ml	or	100ml
Solution E	=	50ml	or	100ml

Distilled water		=	300ml	or	600ml
D-Glucose	(2.8mM)	=	0.275g	or	0.55g
Na Pyruvate		=	0.269g	or	0.539g
Na_2 Fumarate		=	0.216g	or	0.432g
Na Glutamate		=	0.414g	or	0.828g
E.G.T.A.	(0.1mM)	=	0.038g		
Distilled water		=	To 500ml	or	To 1000ml

Gas solution with 95% O_2/5%CO_2

Adjust to pH 7.4

8). Chloride-free (Gluconate replacement).

Na-Gluconate	(103mM)	=	22.464g
K-Gluconate	(4.7mM)	=	1.101g
Mg-Gluconate	(1.13mM)	=	0.234g
$NaHCO_3$	(25mM)	=	2.10g
NaH_2PO_4	(1.15mM)	=	0.138g
Na Pyruvate		=	0.539g
Na_2 Fumarate		=	0.432g
Na Glutamate		=	0.828g
D-Glucose	(2.8mM)	=	0.55g
E.G.T.A.	(0.1mM)	=	0.038g
Distilled water		=	To 1000ml

Gas solution with 95% O_2 / 5%CO_2

Adjust to pH 7.4

APPENDIX 7.

Trypan Blue. The solution for the Trypan blue exclusion test was prepared by dissolving 0.1g of the dye Trypan Blue, in 100ml of phosphate buffered saline. A drop of the Trypan Blue solution was placed on a microscope slide and some sweat glands were added to the solution. A coverslip was placed on top and the preparation examined under phase contrast microscopy.

APPENDIX 8.

TRANSMISSION ELECTRON MICROSCOPY.

The isolated sweat glands were fixed and processed using the following solutions and method.

FIXATIVES.

The fixative solutions are from those of Sabatini, Bensch & Barrnett (1963).

PRIMARY FIXATIVE.

1M. Sodium Cacodylate	=	10ml
Distilled Water	=	70ml
25% Glutaraldehyde	=	8ml
Sucrose	=	1.7g
1M Calcium Chloride	=	0.1ml

Adjust pH to 7.2, bring to 100ml with distilled water.

BUFFER WASH.

1M. Sodium Cacodylate	=	10ml
Distilled Water	=	80ml
Sucrose	=	5.9g

Adjust pH to 7.2, bring to 100ml with distilled water.

POST FIXATIVE.

1M. Sodium Cacodylate	=	10ml
Distilled Water	=	50ml
Sucrose	=	5.1g

Adjust pH to 7.2, bring to 100ml with distilled water.

Buffer = 3 parts

4% Osmium Tetroxide = 1part

METHOD.

Isolated glands were processed as follows:

1). Primary fixative = 3 hours

2). Buffer wash = 3 x 20 minutes

3). Post fixative = 2 hours

4). Buffer wash = Rinse

5). Dehydrate slowly using graded alcohols or acetone.

6). Propylene oxide as intermediate solvent

7). Propylene oxide / Araldite = overnight

8). Fresh unpolymerised resin for 3-4 days

9). Embed in beem capsules at 60°C

STAINING.

1µm sections were mounted on glass microscope slides and stained

Toluidine Blue/ Pyronin Y. (Ito & Winchester, 1963).

Sodium Borate = 0.8g

Distilled Water = 100ml

Dissolve, then add in order

Toluidine Blue = 0.8g

Pyronin Y = 0.2g

Dissolve, then filter into a stock bottle.

Sections were stained at room temperature, washed in running water, air dried and the mounted with synthetic mounting media.

ULTRATHIN SECTIONS.

Ultrathin sections, cut using a diamond knife, were mounted on copper grids then stained with 2% uranyl acetate in 50% ethanol (Stempak & Ward, 1964) followed by Reynolds Lead citrate (Reynolds, 1963).

MICROSCOPY.

Stained 1μm sections for light microscopy were examined using a Zeiss GFL standard microscope. Ultrathin sections for electron microscopy were examined using a Zeiss 109 electron micrscope.

Appendix 9.

Key to figures.

B. C.	=	BASAL CELL
B. I.	=	BASAL INFOLDINGS
B. L.	=	BASAL LAMINA
C. D.	=	COILED DUCT
C. T.	=	CONNECTIVE TISSUE
D.	=	DUCT
F.	=	FIBROCYTE
G. C.	=	GRANULAR CELL
L.	=	LUMEN
Li.	=	LIPID
L.I.	=	LATERAL INTERDIGITATIONS
L. C.	=	LUMINAL CELL
MV.	=	MICROVILLI
MyoE	=	MYOEPITHELIUM
N.	=	NUCLEUS
NG. C.	=	NON-GRANULAR CELL
S. F.	=	SECRETORY CELL
F. C.	=	FLOW CELL
P.	=	PUMP
W. B.	=	WATER BATH
R. F. C.	=	'REDIRAC' FRACTION COLLECTOR

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