STUDIES ON THE EFFECTS OF N-ETHYLMALEIMIDE (NEM) IN THE URINARY BLADDER OF BUFO MARINUS

D. Phil. Thesis

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This thesis sets out a characterisation of the effects of the sulphydryl agent N-ethylmaleimide (NEM) on water flow across the urinary bladder of the toad, Bufo marinus, and compares the effects of NEM with those of vasopressin (VP). This has been done using functional, biochemical, and ultrastructural studies of bladders treated with NEM or VP.

The introduction reviews the action of VP in the toad bladder, and summarises the previous experiments with NEM in this tissue. The results show that NEM causes a specific, irreversible, increase in transepithelial osmotic water flow, but inhibits sodium transport. The increase in water flow does not appear to be mediated by cAMP, or changes of pH or calcium ion concentration, but can be inhibited by low pH, raised calcium, or disruption of the cytoskeleton or cellular metabolism. NEM-induced water flow is additive with that induced by a sub-maximal, but not a maximal, VP dose. NEM treatment leaves the overall epithelial structure intact, while causing changes consistent with increased water flow through the granular cells. Membrane fusion profiles, similar to those seen after VP stimulation, are visible at the apical plasma membrane, and particle aggregates morphologically identical to those believed to mediate the hydrosometric response to VP can be visualised by freeze-fracture electron microscopy.

These results suggest that NEM induces a specific increase in transepithelial water flow by activating, via one or more sulphydryl reactions, the cellular apparatus stimulated by VP. This activation must occur distal to cAMP production, but proximal to cytoskeletal involvement. NEM may prove valuable in understanding the action of vasopressin, by providing an agonist that acts later in the pathway than others currently available. The implications of the experiments reported here, and suggestions for further work, are discussed.
This work is dedicated to my parents, whose teaching and enthusiasm for the natural world has been an encouragement and inspiration for me as long as I can remember, and whose support, both financial and practical, throughout my education has been of inestimable value.
Acknowledgements

First and foremost in this section must come my supervisor, Dr. Ann Taylor, without whose wisdom and knowledge this work could never have been done. She not only provided the lab space and facilities, but her advice and practical experience were invaluable in coming to understand the field, and in the design of the experiments. In this context Dr. Mirilee Pearl, with her enthusiasm and interest was also most useful, particularly in the biochemical and electron-microscopic fields. Barbara Barber provided a great deal of practical advice and experience, and by keeping the lab running so smoothly made life much easier. Bob Pinches gave valuable help in the fluorescence studies. These four also deserve special thanks for their friendship, which made the lab such a pleasant place to work, and encouragement, which kept things moving when they threatened to get bogged down.

I would also like to acknowledge the technical staff in the department: in particular Ingrid White, for her help with all aspects of electron microscopy, from sectioning to the final micrographs, Lawrance Waters, for his help and advice on photographic work, and Barbara Coles, for her help in the instrument room.

My college, Exeter, and the University, have provided financial support for this work, particularly the part spent in Dr Bourguet's lab at Saclay, for which I am very grateful. I wish to thank Dr. Bourguet and all those working in his lab for their support in what was probably the most intense and exciting three weeks of this period.
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INTRODUCTION

Brunn (1921) first discovered that anurans (frogs and toads) injected with neurohypophyseal extracts would take up water through their skin. This was the first demonstration that the water permeability of an anuran epithelium was under hormonal control. The other body system which plays a major role in the regulation of water balance in amphibia is the urinary bladder. Steen (1929) first observed that frogs can resorb water from the urine in the bladder when dehydrated, or after being placed in hypertonic salt solutions. He pointed out that the bladder could act as a source of water when it was not available to be absorbed across the skin. Ewer (1952) showed that water resorption from the toad urinary bladder could be induced by the injection of neurohypophyseal extracts.

It is now generally accepted that the urinary bladder of anurans is a functional analogue of the collecting tubule of the mammalian kidney (MacKnight et al., 1980), permitting the resorption of water from the urine under the control of the neurohypophyseal hormone, antidiuretic hormone (ADH). ADH, released from the posterior pituitary in response to the osmotic status of the animal, causes an increase in the water permeability of the bladder epithelium, hence allowing the passive flow of water down an osmotic gradient from the hypotonic urine into the bloodstream. The mechanism by which this occurs is discussed in detail below.

The toad bladder is most responsive to the amphibian antidiuretic hormone, arginine vasotocin (Eggena et al., 1968). However, water permeability is also increased in response to the mammalian equivalents, arginine vasopressin (Bentley, 1958) and, to a lesser extent, oxytocin (Eggena et al., 1968). The easier availability of arginine vasopressin has resulted in this hormone being used in most studies of ADH action in the toad bladder. For clarity, vasopressin (VP) will be used throughout the rest of this thesis as a generic term covering all antidiuretic hormones.

Early studies of VP function were carried out in intact animals, but experimental manipulations, and subsequent interpretation of the results, is much easier in isolated tissues. The isolated toad urinary bladder was first used by Leaf et al. (1958) in studies of
transepithelial sodium transport, while Bentley (1958) developed a simple gravimetric technique for the measurement of transepithelial osmotic water flow in response to VP using this preparation. The large size and responsiveness of the urinary bladder of the South American toad, Bufo marinus, make it particularly well suited to studies of the actions of vasopressin.

In seeking to elucidate the mechanisms by which VP controls the water permeability of the tissue, its functional effects have been characterised, and biochemical and ultrastructural changes associated with its action have been extensively studied (DeSousa, 1984; MacKnight et al., 1980). Treatments that inhibit or enhance the response to VP have been used in an attempt to elucidate some of the cellular mechanisms involved. Similarly, agents able to mimic part or all of the hormonal response have been investigated.

The sulphydryl reagent N-ethylmaleimide (NEM) has been found to induce an increase in transepithelial water flow under certain circumstances (Rasmussen et al., 1960). This thesis describes a detailed characterisation of the activation of the water permeability pathway in toad urinary bladder by NEM, involving functional, biochemical, and morphological studies. As a foundation for this, the introduction contains a review of what is known of the morphology of the toad bladder and its functional characteristics, with particular emphasis on the cellular mechanisms underlying the increase in water permeability brought about by VP.

**Morphology of the Toad Urinary Bladder**

The urinary bladder of the toad is a large, highly distensible, bilobed organ, which can occupy half the abdominal cavity, and contain up to 200 ml. of urine (Bentley, 1966; Leaf, 1960; Macknight et al., 1980). The bladder has a relatively simple structure (Leaf et al., 1958; Peachey & Rasmussen, 1961; Choi, 1963; DiBona et al., 1969a). It consists of a pseudo-stratified epithelium on the mucosal (luminal) surface, with a basement membrane, supported on a submucosa made up of bundles of collagen and smooth muscle. Small blood vessels and nerves run through this supporting network. The whole structure is covered with a simple squamous mesothelium on the serosal surface.
In a classical electron microscopical study, Choi (1963) described the four cell types that make up the epithelium. Three of these, the granular, mitochondria-rich and goblet cells, are exposed at the mucosal surface, while the fourth type, the basal cells, lie on the basement membrane, partly supporting the other cell types. It has been shown that all the cells which make up the mucosal face of the epithelium also abut the basement membrane, so the epithelium is functionally a single layer of cells (DiBona et al., 1969a).

The epithelial cells are joined together near their luminal borders by classical tripartite junctions (Claude & Goodenough, 1973; Farquhar & Palade, 1963). These are made up of a zonula occludens, consisting of up to eight strands of membrane fusion, a zonula adherens, in which the membranes are closely apposed, but not fused, and a band of desmosomes. The combined effect of these components is to provide a junction that is highly impermeable to both water and solutes, and which will hold the cells together even in severely distended bladders (Peachey & Rasmussen, 1961). There is a very high electrical resistance across the epithelium. On the basis of these characteristics, the toad bladder epithelium is classified as a tight epithelium (MacKnight et al., 1980).

Below the junctional complex, the cells are held together by occasional desmosomes, which link all classes of cells to each other. Gap junctions have also been seen, but these only link granular cells with basal cells (Wade, 1978).

Granular Cells

The majority of the epithelium is made up of granular cells. In Bufo marinus of Dominican origin, these cells constitute 80% of the total epithelial cell population, and cover over 95% of the luminal surface of the bladder (MacKnight et al., 1980). These cells have been described as funnel-shaped (DiBona et al., 1969a), with a large area facing the bladder lumen, and a relatively small region applied to the basement membrane.

When the apical surface of the epithelium is viewed by scanning electron microscopy, the granular cells appear as large, polygonal cells, which form a pavement (Davis et al., 1974;
Macknight et al., 1980). In the resting state, unstimulated by VP, these cells show a pattern of anastomosing ridges covering the entire cell surface. The junctions between cells are marked by two parallel, closely apposed ridges. Occasionally, short microvilli rise above the mean level of the ridges, particularly at the junctions (Davis et al., 1974). In transverse section, the ridges give the appearance of stubby microvilli (Choi, 1963), although sections cut close to the plane of the apical surface show their ridge-like character (Peachey & Rasmussen, 1961). After stimulation with VP, the microvilli change from a ridge-like to a finger-like arrangement (Davis et al., 1974; Dratwa et al., 1979). There is a prominent PAS-positive (periodic acid schiff-positive) glycocalyx on the apical surface of these cells (Peachey & Rasmussen, 1961; Choi, 1963).

Below the apical junctions, the basolateral membranes of these cells are heavily folded, being tightly interdigitated with those of adjacent cells in unstimulated tissue. If the tissue is stimulated with VP in the presence of an osmotic gradient the interspaces become dilated (Carasso et al., 1962), as water flows out of the cells and along the interspaces (Croker & Tisher, 1972).

Membrane-limited granules, for which this cell type is named, are found in the apical cytoplasm. Typically, granules are present both in the immediate subplasmalemmal region (which is occupied by a dense terminal web made up of microfilaments), and in the subjacent cytoplasm (Peachey & Rasmussen, 1961). The granules are discoid, PAS positive, and are rendered electron-dense by treatment with osmium tetroxide (Choi, 1963; Wade et al., 1975). Their contents appears to consist primarily of glycoprotein precursors of the glycocalyx (Masur et al., 1986). Their membrane composition differs from that of the plasma membrane, both in lipid and protein content: they are low in free cholesterol, and the pattern of membrane proteins does not in general match that of the plasma membrane (Masur et al., 1986). The granule membrane is highly impermeable to water (Masur & Verkman, 1988).

Lucent, tubular vesicles are also found, often in clusters, in the apical cytoplasm. In freeze-fracture electron microscopic views, some of these can be seen to have aggregates of membrane particles in their walls (Humbert et al., 1977; Wade, 1978), as discussed in detail below. The origin of these tubules is not clear: it has been suggested that they may represent elements of a tubulo-cisternal reticular system (Mollgard & Rostgaard, 1981a), as described in
other transporting epithelia (Mollgard & Rostgaard, 1981b). Certainly, the granular cells contain extensive rough endoplasmic reticulum (E.R.), and smaller amounts of smooth E.R. These reticular components may be connected to one another, and to the nuclear membrane (Peachey & Rasmussen, 1961). There is a well developed supra-nuclear Golgi apparatus in these cells, and the tubular vesicles may be derived from this. The tubules may be derived directly from the Golgi, or they may be built up by the coalescence of smaller, Golgi-derived vesicles (Franki et al., 1986; Hays, 1983).

The nuclei generally lie close to the base of the granular cells. There are frequent mitochondria: in thin section these appear as round or elongated profiles, which occasionally branch (Peachey & Rasmussen, 1961), but they may really form a tubular network (DiBona, 1981). Multilamellar and multivesicular bodies are also found in these cells (Peachey & Rasmussen, 1961).

Networks of actin filaments have been identified in the microvilli and terminal web region of the cytoplasm using heavy meromyosin decoration (Pearl & Taylor, 1983). Intermediate filaments are also common, often running in bundles beneath the apical network of actin filaments (Pearl & Taylor, 1983).

Microtubules occur singly, rather than in bundles, and they show no preferred orientation (Reaven et al., 1978). Reaven et al. (1978) found that microtubules were sometimes associated with organelles, but such associations did not occur significantly more often than expected in a random distribution. Microtubules were significantly less common in the microfilament-rich region immediately underlying the apical plasma membrane than in the rest of the cytoplasm, and microtubules were never seen to contact the plasma membrane (Reaven et al., 1978). However, Muller et al. (1980), working with highly stretched bladders, showed that microtubules do occasionally appear to run up to the plasma membrane, as well as to other membranous elements.

As discussed below, the granular cells are responsible for active transepithelial sodium transport, and also represent the site of the increases in water, sodium and urea permeability induced by vasopressin.
Mitochondria Rich Cells

The mitochondria-rich (MR) cells make up about 15% of the epithelial cells, but only 3% of the apical surface. They are flask-shaped cells, with a large body from which a narrow neck projects up to the lumen (Choi, 1963). The neck contains numerous small, lucent, tubular vesicles, occasional multivesicular bodies, conspicuous microtubules, but few other organelles. The main body of the cell contains the nucleus, and large numbers of mitochondria. There is little endoplasmic reticulum, and the Golgi apparatus tends to be fragmented (Peachey & Rasmussen, 1961).

Recent evidence indicates that MR cells are the site of transepithelial Cl⁻ movement in frog skin (Foskett & Ussing, 1986). In the Columbian sub-species of Bufo marinus, where MR cells make up 35% of the epithelium, they have been implicated in proton secretion (Rosen et al., 1974; Ludens & Fanestil, 1972). The MR cells of Dominican Bufo marinus are assumed to have similar functional characteristics.

Goblet Cells

The third type of cell which reaches the luminal face of the bladder is the goblet cell. Goblet cells constitute 6% of the epithelial cells (MacKnight et al., 1980). They possess prominent microvilli at their apical surface. The lower part of the cell contains the nucleus, small amounts of endoplasmic reticulum, and mitochondria, while the main part is occupied by large mucin granules, which are PAS positive (Choi, 1963).

Basal Cells

The basal cells lie on the basement membrane, to which they are attached by hemidesmosomes (DiBona et al, 1969a). Organelles are scarce within these cells (Choi, 1963), but there are often many microfilaments and intermediate filaments (Kraehenbuhl et al., 1979). Little is known of the
function of these cells, but it has been suggested that they are young, undifferentiated cells (Choi, 1963), or that they may play a role in cell-cell communication (Wade, 1978).

Functional Characteristics of the Toad Urinary Bladder and Their Modification by Vasopressin

Transepithelial Sodium and Urea Movement

There is a transepithelial potential difference of 10-110 mV, serosa positive, across the bladder of Bufo marinus of Dominican origin (Leaf, 1960; MacKnight et al., 1980). This potential difference is associated with the active transport of sodium from the mucosal to the serosal surface of the bladder: sodium appears to be the only ion actively transported across the epithelium in significant quantities (Leaf et al., 1958). The granular cells are believed to be the sites of transepithelial sodium transport (Bobryiki et al., 1978; Rick et al., 1978). Sodium enters the cells passively across the apical plasma membrane through amiloride-blockable channels, and is pumped out across the baso-lateral plasma membrane by a ouabain-sensitive, adenosine triphosphate (ATP) dependent Na⁺/K⁺ pump (Na/K ATPase) (MacKnight et al., 1980).

Vasopressin stimulation causes a transient increase in sodium transport (Leaf et al., 1958). This response is mediated by adenosine 3',5'-cyclic monophosphate (cAMP) (Orloff & Handler, 1962). The weight of evidence suggests that the increase in sodium transport depends on the activation of a population of sodium channels that exist preformed in the apical plasma membrane (Li et al., 1982; Sariban-Sohraby & Benos, 1986).

The unstimulated toad bladder epithelium is not very permeable to urea, but after vasopressin stimulation there is an increase in urea permeability of up to 40 fold (Maffly et al., 1960; Leaf, 1960). Permeability to certain other small amides, such as acetamide, also increases (Leaf & Hays, 1962). Urea movement across the tissue is a passive process, and the barrier is at, or near, the mucosal surface (Maffly et al., 1960; Leaf & Hays, 1962). Little is known about the mechanism by which urea crosses the membrane; a carrier system, inhibited by
phloretin, has been proposed (Levine et al., 1973).

**Basal Transepithelial Water Movement**

In the unstimulated bladder, there is almost no water flow across the bladder, even in the presence of a steep osmotic gradient across the tissue (Bentley, 1958). The apical plasma membrane, in contrast to the basolateral membrane, is virtually impermeable to water: a hypotonic mucosal bath, in the absence of VP, does not cause swelling of the epithelial cells, while a hypotonic serosal bath causes swelling of all cell types (DiBona et al., 1969b). Thus it is the apical membrane, together with the tight junctions discussed above, which provides a barrier to transepithelial water flow in the unstimulated bladder (DiBona et al., 1969b).

**Vasopressin Stimulation of Transepithelial Water Movement**

If the toad bladder is stimulated with vasopressin in the presence of an osmotic gradient (mucosa hypotonic), water flow across the tissue increases up to 50-fold (Bentley, 1958). Studies of accumulation of tritiated water, used as a tracer, showed that the site of increased permeation was the apical plasma membrane (Leaf, 1960; Hays & Leaf, 1962). This finding was later confirmed and localised to the granular cells by studies of cell swelling in the presence of vasopressin and an osmotic gradient (DiBona et al., 1969b). Prominent vacuoles are often seen in the granular cell cytoplasm after VP stimulation (Carasso et al., 1962). There is some uncertainty as to whether these vacuoles represent infoldings of the basolateral membrane (Strange & Spring, 1987), or whether they are truly intracellular structures (Kirk, 1988). As mentioned above, following vasopressin stimulation the interspaces between the basal parts of the cells become dilated, as water flows out of the cells and along the interspaces (Peachey & Rasmussen, 1961; DiBona, 1969b).
Cellular Events Underlying the VP-Induced Increase in Apical Plasma Membrane Water Permeability

Development of the Pore Theory: Biophysical Evidence for Water Channels

Hays and Leaf (1962) demonstrated that vasopressin causes an increase in both osmotic (Pf) and diffusional (Pd) water permeability in the toad bladder. However, in their experiments Pd was only about doubled, while the osmotic flux increased about 40 fold; the difference between the two suggested that the increased water flow was not simply due to increased diffusion of water across the rate-limiting apical membrane. These findings paralleled those of Koefoed-Johnsen & Ussing (1953) in frog skin, who had proposed that neurohypophyseal hormones caused the opening, or widening, of pores, allowing bulk flow of water across the tissue. This explanation was adopted for the toad bladder (Hays & Leaf, 1962).

Vasopressin was also shown to increase the permeability of the tissue to urea and sodium (Leaf, 1960; Leaf et al., 1958), and it was suggested that a single mechanism could explain the increase in permeability to all three molecules, with vasopressin causing an increase in pore size from 8Å to about 40Å (Hays & Leaf, 1962). However, there were problems for this theory. The tissue showed a highly selective permeability: Leaf (1960) had found a marked increase in permeability to urea, but not to thiourea, a very similar molecule. There was also no significant increase in permeability to other small ions and non-electrolytes which would be expected to pass through a pore of this size (Leaf & Hays, 1962). Faced with similar results from studies in frog skin, Andersen & Ussing (1957) had proposed a dual-barrier hypothesis. They postulated a permeability barrier opened by hormonal stimulation in series with another, size-specific, barrier that is highly permeable to small molecules but prevents larger molecules passing, even when the pores in the hormone-sensitive barrier are open.
Hays & Franki (1970), in experiments in which the fluid bathing the tissue was stirred as vigorously as possible, found that there was a large (at least ten fold) increase in $P_d$ following VP stimulation, but that this had been masked in earlier experiments by the effects of unstirred layers. By removing much of the apparent discrepancy between $P_f$ and $P_d$, this finding undermined the principle evidence for the pore theory of vasopressin action. Hays & Franki (1970) proposed that the effect of VP might be to open large numbers of small pores, or might even simply increase diffusion of water across the lipid part of the membrane (Hays, 1968; Hays & Franki, 1970; Hays et al., 1971).

Pietras & Wright (1974) reported that VP caused an increase in the permeability of the toad bladder to lipophilic solutes. They interpreted this as being due to an increase in membrane fluidity, and proposed that the increase in water permeability seen after vasopressin stimulation might be due to selective increases in the fluidity of parts of the apical plasma membrane (Pietras & Wright, 1975). While testing this idea, Finkelstein (1976a) reported that changes in fluidity of artificial lipid bilayers could alter their permeability to water and to a variety of non-electrolytes, but that such changes occurred to a similar extent for all the molecules tested, and the ratio between the permeabilities of different substances remained constant. This was not the case with molecules crossing the toad bladder (Pietras & Wright, 1975), where the increase in the permeability to water, urea, and acetamide after hormonal stimulation was much greater than that seen for a range of other compounds. On the basis of these findings, Finkelstein (1976b) concluded that the increase in water permeability was not due to an increase in membrane fluidity, but must involve the creation of specific pores, or channels.

Gluck & al-Awqati (1980) provided further indirect evidence for the existence of aqueous pores when they showed that VP increased the proton conductance of the apical plasma membrane: this is consistent with the presence of a continuous line of water molecules across the membrane, as would be found in a pore, where protons can effectively be transferred along the chain by a series of bond rearrangements, but not with single water molecules diffusing through a lipid environment.
The finding that transepithelial movement of water, sodium, and urea could each be inhibited independently (by methohexital (Levine et al., 1976) or cytoskeleton disruptive drugs (Taylor et al., 1973), amiloride (Bentley, 1968), and phloretin (Levine et al., 1973), respectively) provided evidence that there were separate routes of permeation across the apical plasma membrane for each of these molecules. These findings effectively refuted the dual barrier model, and necessitated the postulation of channels selective for each type of molecule. Hays (1968) had suggested that such specific water channels would need to have a radius close to 2 Å, in order to permit the passage of water molecules while excluding other small compounds, such as urea.

More recent measurements of Pf and Pd have shown there to be a marked increase in the Pf:Pd ratio in the presence of vasopressin even when allowance is made for unstirred layers. Parisi & Bourguet (1983), working with frog bladders, found that the ratio was close to 1 in unstimulated tissue, suggesting that water movement under these conditions might be due almost entirely to free diffusion across the membrane. When VP was added, the ratio increased to greater than 9, implying that free diffusion across the membrane could no longer be the predominant mechanism. Levine et al. (1984), in similar studies using toad bladders, found a value for Pf/Pd of about 17 after VP stimulation. If the discrepancy between Pf and Pd is not due to bulk flow through wide pores, as suggested by Koefoed-Johnsen & Ussing (1953), another explanation is needed. Levitt (1974), on theoretical grounds, and Rosenberg & Finkelstein (1980), based on experiments with gramicidin channels, had concluded that for single-file movement of molecules through a channel, the Pf:Pd ratio should equal the number of molecules within the channels at any one time. Taken together, the findings described above suggest that the VP-induced increase in water permeability may be due to the appearance of long, narrow water channels in the apical plasma membrane, through which water molecules move by single file diffusion.
The VP-Induced Increase in Water Permeability is Mediated by cAMP

Vasopressin is only active at the serosal surface of the bladder, yet the increase in water permeability occurs at the mucosal face (Hays & Leaf, 1962; Leaf et al., 1958). How does VP bring about the creation of channels in the apical plasma membrane?

Orloff & Handler (1961) first proposed that the effects of VP might be mediated by a rise in intracellular cyclic adenosine monophosphate (cAMP) levels. They found that exogenous cAMP could mimic the effects of the hormone, as could theophylline (an inhibitor of phosphodiesterase, the enzyme that breaks down cyclic nucleotides). More recently forskolin, which activates adenylate cyclase directly, has also been shown to mimic the effects of VP in the toad bladder (DeSousa & Grosso, 1983).

Vasopressin stimulation causes a rise in cellular cAMP content, as measured in epithelial cells isolated after stimulation (Omachi et al., 1974; Eggena et al., 1975). Intracellular cAMP levels reach a peak within 3 minutes of maximal hormonal stimulation (Sapirstein & Scott, 1973), and the rise in cAMP occurs ahead of the increases in both sodium transport (Sapirstein & Scott, 1973), and water permeability (Chevalier et al., 1983). Thus cAMP fulfills Sutherland's criteria for a second messenger, and it is now generally accepted that this nucleotide mediates the vasopressin response (DeSousa, 1984).

Vasopressin binds to specific receptors (defined as \( V_2 \) by Michell et al., 1979) located in the basolateral membrane of the epithelial cells. Binding studies have shown that only a relatively low occupancy is required to produce a near-maximal stimulation of cAMP production (Handler & Orloff, 1981). It is believed that the VP receptors activate adenylate cyclase via a 3 part membrane protein complex involving a GTP-binding regulatory protein (Ausiello et al., 1987; Handler & Orloff, 1981). This arrangement has now been identified in a large number of systems where cAMP acts as a second messenger (Alberts et al., 1983).

A rise in the cAMP level activates cytosolic type II cAMP dependent kinase (Schlondorff & Franki, 1980). It is widely presumed that an alteration in the phosphorylation pattern within the cells represents the next step in the vasopressin pathway (Handler & Orloff, 1981), although
specific phospho-proteins have not yet been identified. In vitro studies have suggested that cAMP-dependent protein kinase may also phosphorylate an actin binding protein, and hence modulate cytoskeletal function (Ausiello & Hartwig, 1985). Recently, four proteins (m. wt. 15.5, 17, 28, and 34 KD) have been found in the apical plasma membrane whose phosphorylation state changes in response to VP stimulation of the toad bladder (Konieczkowski & Rudolph, 1985). These proteins have not as yet been characterised, nor has any role in the response been determined for them. However recent attempts to isolate the channels from apical membranes have produced proteins of similar molecular weights (Harris et al., 1987).

**Cytosolic Calcium May Play a Modulatory Role**

Recently, there has been speculation that calcium may act as an intracellular messenger for vasopressin action in the toad bladder (Hardy, 1978; Taylor, 1981; Levine et al., 1981). However, studies with fluorescent calcium indicators in isolated toad bladder epithelial cells have shown no increase in cytosolic calcium following VP stimulation (Burch & Halushka, 1984; Taylor et al., 1984, 1985), and whereas Burch & Halushka (1984) reported a reduction in free calcium ion concentration after VP stimulation, Taylor et al. (1984, 1985) found no such reduction, if precautions were taken to avoid artifacts.

Functional experiments, involving manoeuvres designed to raise cytosolic calcium concentration, indicate that increases in intracellular calcium inhibit the water permeability response to vasopressin in the toad bladder (Taylor, 1975; Taylor et al., 1979). Inhibitors of calmodulin activity also inhibit the hydrosmotic response to VP (Levine et al., 1981; Grosso et al., 1982). Although the weight of evidence suggests that the cytosolic calcium level modulates the vasopressin response, the precise roles played by calcium and calmodulin are not clear. Vasopressin might act via cAMP-dependent phosphorylation events to decrease the calcium sensitivity of cellular processes: a similar change in calcium sensitivity has been described for smooth muscle (Conti & Adelstein, 1980). In this way calcium could modulate the hydrosmotic response in the absence of any apparent change in calcium levels (Taylor & Windhager, 1985).
**Vasopressin Induces Exocytosis and Endocytosis**

It has become clear that both exocytosis and endocytosis are increased at the apical surface of the granular cells after VP stimulation of the toad bladder. Masur et al. (1971) reported that stimulation of the bladder with vasopressin or cAMP caused endocytosis of horseradish peroxidase (HRP) from the mucosal medium; HRP was taken up into tubules and vesicles, and later appeared in lysosomes and multivesicular bodies. Masur et al. (1972) later showed that granules lying in the sub-apical cytoplasm could be induced to fuse with the apical plasma membrane by the addition of VP. Combining these findings, they suggested that insertion of granule membrane (or components of the granules themselves) into the apical plasma membrane might underlie the increased permeability of the apical plasma membrane, and that regulation of permeability might depend on the balance between ADH-induced exocytosis and endocytosis (Masur et al., 1972).

Gronowicz et al. (1980), using morphometric methods, found that granule exocytosis correlated well with the onset of VP-induced osmotic water flow (the hydrosmotic response). There was an initial net increase in apical plasma membrane area, followed by a return to the starting level. The increase in apical membrane area after VP stimulation has been confirmed in membrane capacitance studies (Palmer & Lorenzen, 1983; Stetson et al., 1982).

**Vasopressin Induces the Appearance of Particle Aggregates**

The use of freeze-fracture electron microscopy led to the discovery, first in frog bladder (Chevalier et al., 1974) and then in toad bladder (Kachadorian et al., 1975), that VP or cAMP stimulation causes the appearance of organised arrays of membrane particles, known as particle aggregates, in the p fracture face of the apical plasma membrane of the granular cells. A strong correlation between the percentage area of aggregates in the apical plasma membrane and magnitude of transepithelial water flow has been demonstrated, both with changing concentrations of agonists (Kachadorian et al., 1977a), and temporally during the onset of the
hormonal response (Kachadorian et al., 1978). Water flow and aggregate frequency can be modified in parallel by a variety of treatments. Cold slows the onset of both the hydrosomatic response and aggregate appearance (Bourguet, 1966; Chevalier et al., 1983; Kachadorian et al., 1979a), while colchicine (Kachadorian et al., 1976) and methohexital (Kachadorian et al., 1977b) inhibit both. Hydrazine, which enhances the hydrosomatic response, also increases the number of aggregates (Levine et al., 1980). All these are treatments that modify the hydrosomatic response without affecting urea or sodium flux; conversely, phloretin, which specifically inhibits urea permeability, does not affect the aggregation response (Kachadorian et al., 1977b). This close correlation between transepithelial water flow and the presence of particle aggregates has led to the view that the aggregates represent the site of water channels in the apical plasma membrane.

**Origin of Particle Aggregates**

How do these aggregates arise? They do not appear to form by association of particles already present in the apical plasma membrane, since there are no particles of the correct size in the resting membrane, and the pattern of particles is not altered by VP, except for the appearance of the aggregates themselves (Bourguet et al., 1981). The rapidity of their appearance (they can be detected in the apical plasma membrane within one minute of VP stimulation (Chevalier et al., 1979)) precludes de novo synthesis, suggesting that they must be stored. Humbert et al. (1977) first described the presence of tubular vesicles containing aggregates in the cytoplasm of the granular cells. Occasional fortuitous freeze-fracture views have shown such tubular vesicles fused to the apical plasma membrane after VP stimulation, with particle aggregates lying across the junction of the tubule wall with the plasma membrane (Muller et al., 1980). At early time points in the response aggregates are often seen closely associated with fusion sites (Muller et al., 1980). These findings have led to the view that the aggregates exist preformed in the walls of tubular vesicles, and are inserted into the apical plasma membrane by exocytic fusion of the tubules with the plasma membrane (Humbert et al., 1977; Wade, 1978; Muller et al., 1980).
Wade (1978, 1980) first proposed the "membrane shuttle hypothesis". He suggested that aggregate-containing tubular vesicles (named aggrephores by Muller & Kachadorian (1984)) continuously move up to and fuse with the surface, thus delivering aggregates to the plasma membrane, while endocytosis subsequently removes them. This proposal is very similar to that of Masur and colleagues (Masur et al., 1972; Gronowicz et al., 1980) discussed above; however, the absence of aggregates in the membrane of the granules (Humbert et al., 1977), together with the very low permeability of the granule membrane (Masur & Verkman, 1988), makes it unlikely that the granules themselves are directly involved in the increase in water permeability. Recent experiments combining HRP labelling with the freeze-fracture technique have demonstrated that some endocytic vacuoles contain particle aggregates within their walls (Coleman et al., 1987), confirming the theory that they are retrieved endocytically. Shi & Verkman (1989) and Harris et al. (1989) have isolated endocytic vesicles, and shown that they have a very high water permeability, implying that they have functional aggregates within their walls. It is not yet clear how much reprocessing occurs before the particle aggregates can be re-used, if they are indeed recycled at all.

The balance between VP-induced exocytosis and endocytosis is affected by the transepithelial osmotic gradient. In the absence of an osmotic gradient, and hence transepithelial water flow, there appears to be much less endocytic activity at the apical plasma membrane than is observed when water flow occurs (Masur et al., 1984, Muller & Kachadorian, 1984). Under these conditions the increase in water permeability is greater than that seen in bladders stimulated in the presence of a transepithelial osmotic gradient (Ellis et al., 1980). The increased permeability is associated with greater numbers both of aggregates on the cell surface (Ellis et al., 1980), and aggrephores fused to the plasma membrane (Muller & Kachadorian, 1984). Conversely, studies using cationised ferritin, which increases endocytosis, have shown reduced water flow in response to hormonal stimulation (Beauwens et al., 1986). These studies provide support for the theory that water permeability is determined by a dynamic balance between exocytosis and endocytosis.
Problems With Aggregates as Sites of Water Flow

It has proved possible under certain circumstances to dissociate the number of aggregates present in the apical plasma membrane from the observed water flow. As this correlation is the major evidence that the aggregates represent the site of water flow, such conditions need consideration.

Low pH Can Dissociate Water Flow and Aggregate Numbers

The hydrosmotic response to vasopressin or cAMP can be strongly and rapidly inhibited if the pH of the serosal medium is reduced below pH 7 (Brem et al., 1985). Lowering the pH rapidly can lead to a dramatic fall in transepithelial water flow within three minutes; however, if the tissue is fixed at this time, aggregates can still be seen in the membrane at levels unchanged from those observed under conditions of maximal water flow (Parisi & Bourguet, 1984). If the pH is then raised again, the water flow rapidly resumes. Thus water permeability can be switched on and off by pH changes, despite the continued presence of aggregates in the plasma membrane. Bourguet et al. (1981) suggest that pH changes may act directly at the level of the aggregates, switching them between open and closed states.

Is There a Second Barrier to Water Flow?

Aggregate numbers also seem to become dissociated from water flow after about 30 minutes of continuous hormonal stimulation: aggregate numbers remain maximal, but there is an "intrinsic inhibition" of the water flow, which declines to about half its peak value (Muller et al., 1980). This may be due to a reduction in the permeability of the apical plasma membrane, for example by closure of some of the channels. It has also been interpreted as implying the development of a second barrier to water flow (Kachadorian et al., 1985). The evidence for such a barrier is, however, still equivocal (Levine & Jacoby, 1987).
**Aggregates May be a Recovery Form**

A further question about the role of the particle aggregates is raised by evidence from rat collecting tubules that equivalent particle clusters are associated with a clathrin coat on the cytoplasmic face of the membrane (Brown & Orci, 1983): this could imply that the aggregates are a recovery form, rather than sites of active water movement.

**The Role of the Cytoskeleton**

**Evidence for Involvement of Microtubules**

Microtubules appear to play a part in the development of the hydrosmotic response to VP (Taylor et al., 1973, 1975; Kachadorian et al., 1979b). Colchicine, which disrupts microtubules (Borisy & Taylor, 1967), inhibits the initiation of the hydrosmotic response to vasopressin or exogenous cAMP in the toad bladder (Taylor et al., 1973, 1975). Detailed studies have shown that this effect is likely to be due to disruption of microtubules (Reaven et al., 1978; Taylor et al., 1978; Wilson & Taylor, 1978), rather than to changes in membrane fluidity (Wunderlich et al., 1973; Kachadorian et al., 1979b) or nucleoside transport (Taylor et al., 1973, 1978). A cautionary note is provided by the findings of Burch & Halushka (1982), who obtained evidence that colchicine might be inhibiting the VP response, at least in part, via the production of prostaglandins, and the subsequent inhibition of adenylate cyclase. However, nocodazole, a rapidly acting microtubule-disruptive drug (De Brabander et al., 1981), also inhibits the hydrosmotic response to VP (Lees & Taylor, 1982; Brady et al., 1981), as does taxol, which binds specifically to assembled microtubules (Manfredi et al., 1982).

Further support for the view that microtubules play a role in the VP response has been obtained in morphological studies using colchicine, which has been shown to inhibit the VP-dependent addition of new membrane (Gronowicz et al., 1980; Palmer & Lorenzen, 1983), and the appearance of both particle aggregates and vesicle fusion sites on the apical plasma membrane after VP stimulation (Kachadorian et al., 1979b; Muller et al., 1980). In conjunction, these
experiments have provided indirect evidence that microtubules are necessary for the normal fusion of aggrephores with the apical plasma membrane during the onset of the hydrosomatic response to vasopressin (Kachadorian et al., 1979b; Muller et al., 1980).

Ultrastructural studies showed that the microtubule content of the granular cells increases slightly but significantly when the toad bladder is stimulated by VP (Reaven et al., 1978). Experiments using high hydrostatic pressure to depolymerise microtubules (Coluccio et al., 1983) suggest that it may be the polymerisation of microtubules, rather than merely the presence of an intact microtubule network, that is crucial to the onset of the hydrosomatic response. It is possible that VP initiates a rapid restructuring of the cytoskeleton in association with the onset of exocytosis, as has been described in cytotoxic leukocytes (Kupfer et al., 1985), where the new microtubules direct vesicles towards the cell surface. The concept of rapid remodelling of the cytoskeleton is in line with recent studies on microtubule turnover, which suggest that the microtubule network within a cell is in a state of dynamic instability, and rapidly adapts to new requirements (Mitchison & Kirschner, 1984; Schulze & Kirschner, 1986).

One possible role for microtubules in the development of the hydrosomatic response is in the movement of aggrephores up to the apical surface of the cells (Taylor et al., 1973; Kachadorian et al., 1979b). Such a role for microtubule-directed organelle movement is suggested by the ability of vanadate (DeSousa & Grosso, 1979; Beauwens et al., 1981) and EHNA (erythro-9-(3-(2-hydroxynonyl))adenine) (Marples & Taylor, unpublished observations) to inhibit the vasopressin response. Both of these substances have been shown to inhibit ATPase activity of ciliary dynein (Gibbons et al., 1978; Bouchard et al., 1981). They have also been implicated as inhibitors of at least one other putative microtubule motor, as has NEM (Koonce et al., 1987).

**Evidence for the Involvement of Microfilaments**

As described above, microfilaments are present in large numbers in the sub-apical cytoplasm of the granular cells. Cytochalasin B (CB), which disrupts microfilaments (Wessels et al., 1971), inhibits both the increase in transepithelial water flow, and also the
appearance of particle aggregates in the apical plasma membrane (Kachadorian et al., 1979b). CB does not appear to affect the number of vesicles that fuse with the apical membrane following VP stimulation (Muller et al., 1980). Accordingly, it has been suggested that microfilaments are involved at a step distal to the fusion of aggregaphores with the plasma membrane, and may mediate the translocation of aggregates from the aggregaphores onto the apical surface (Muller et al., 1980). However, Palmer & Lorenzen (1983) found that CB inhibits the increase in membrane capacitance (a measure of membrane area) seen after VP stimulation, suggesting that the drug is inhibiting the insertion of new membrane.

The effects of colchicine and cytochalasin B on the onset of the VP response have been shown to be additive (Kachadorian et al., 1979b; Parisi et al., 1985). However, colchicine has no effect on the maintenance (Kachadorian et al., 1979b; Brady et al., 1981) or offset (Parisi et al., 1985) of the hydrosomotic response, while CB has been shown to inhibit both its maintenance (Kachadorian et al., 1979b; Parisi et al., 1985) and its offset (Masters & Fanestil, 1979), as well as its onset. These findings, together with the morphological and functional evidence described above, suggest that microtubules and microfilaments are involved in separate, sequential stages of the hydrosomotic response. The available evidence is consistent with a role for microtubules in the translocation (Taylor et al., 1973, 1978; Kachadorian et al., 1979b) and/or reorientation (Sasaki et al., 1984) of aggregaphores, so that they can fuse with the apical plasma membrane (Muller et al., 1980), and for microfilaments in the movement of aggregates onto the surface, and also in their subsequent removal (Kachadorian et al., 1979b). Microfilaments may have an additional role in the organisation and restructuring of the granular cell cytoplasm and microvilli (DiBona, 1983; Parisi et al., 1985; Pearl & Taylor, 1985).

**Metabolic Dependence of the Hydrosomotic Response**

Bentley (1958) first reported that metabolic inhibitors markedly reduced the hydrosomotic response to VP in the toad bladder. Handler et al. (1966) subsequently showed that such agents also inhibited the response to exogenous cAMP, so the effect is not due merely to
inhibition of cAMP production. The effect of inhibitors of glycolysis can be abolished with pyruvate, supporting the theory that they act via inhibition of ATP synthesis (Handler et al., 1966). A metabolic dependence of the initiation of the VP response is consistent with a cAMP-dependent phosphorylation step, and also with the involvement of energy-dependent cytoskeletal transport and/or remodelling processes.

**Offset of the Response**

Removal of VP from the tissue results in a rapid decline in transepithelial water flow. This is closely correlated with the disappearance of particle aggregates from the apical plasma membrane (Kachadorian et al., 1978, Chevalier et al., 1983). The offset of the response is also accompanied by an increase in endocytic activity (Harris et al., 1986): some of these endocytic vesicles contain aggregates, as discussed above (Coleman et al., 1987). The offset of the VP response appears to be an energy-dependent process (Masters & Fanestil, 1979).

**Summary of Cellular Mechanisms Underlying the Hydrosmotic Response to Vasopressin**

The available evidence fits with a model of the mechanism of vasopressin action, as illustrated in figure 1. According to this model, VP binds to $V_2$ receptors in the basolateral membrane of the granular cells, activating adenylate cyclase and causing a rise in intracellular cAMP levels. Through unknown intermediate steps, this leads to activation of the microtubule network and translocation of aggregphores towards the apical plasma membrane. Subsequent to their fusion with the membrane, the particle aggregates in their walls are moved onto the apical cell surface by microfilaments. These aggregates are believed to be sites of specific water channels. When VP is removed, the aggregates are recovered from the plasma membrane by endocytosis, and the water permeability of the epithelium returns to its low resting level.
Figure 1: A Model of Vasopressin Action
Previous Studies With NEM in VP-Sensitive Anuran Epithelia

Rasmussen et al. (1960) first used NEM to explore the mechanism of action of VP in the toad urinary bladder. Working on the theory that a sulfhydryl-disulphide interchange reaction between VP and its receptors was central to the action of the hormone, they investigated the effects of sulfhydryl reagents, both alone and in conjunction with VP. Their main finding was that NEM, and a number of mercurial compounds, inhibited the hydrosmotic response to VP when applied to either face of the bladder.

In the course of these studies, Rasmussen et al. (1960) noted that NEM could cause an increase in transepithelial water flow when applied to the mucosal surface of the tissue at a concentration of 0.1mM. The effect of NEM was irreversible. Moreover, if NEM was applied to a bladder already stimulated with vasopressin, it appeared to "fix" the bladder in its state of increased permeability even after the removal of vasopressin. However they showed that water flow declined substantially after such "fixation" if the pH of the bath was lowered to 6.3. Rasmussen et al. (1960) further noted that, while prolonged exposure to 0.1mM mucosal NEM caused increased water flow, 1mM NEM had little or no effect when applied to the mucosal surface; however, short (2 minute) applications of 1mM NEM had an effect similar to the continued presence of 0.1mM NEM.

Orloff & Handler (1962) subsequently found that 1mM serosal NEM inhibited the hydrosmotic responses evoked by both VP and cAMP, and also inhibited the short circuit current in the toad bladder. They concluded that the effects of NEM were probably nonspecific. Bentley (1964), working in frog bladders, obtained similar results.

Bentley (1973) confirmed that NEM, applied to the serosal surface in low doses (0.01-0.1mM) for 5 minutes could "fix" an established vasopressin response. This capacity was used by Chevalier et al. (1981) in frog bladder and skin to study the structure of the membrane particle aggregates in the absence of glutaraldehyde fixation. In the course of their experiments, Chevalier et al. (1981) noted that 0.1mM mucosal NEM, applied at the peak of the response to oxytocin, sometimes caused a further increase in water flow. However, they did not pursue this finding.
Objectives

Previous studies of NEM in the toad bladder have concentrated on its ability to inhibit or to "fix" the hydrosmotic response to VP when applied to the serosal surface of the bladder. The initial report (Rasmussen et al., 1960) that NEM can stimulate transepithelial water flow in its own right when applied to the mucosal surface has not been followed up. It was therefore of interest to examine this effect of NEM in some detail, particularly in the light of evidence from other systems that NEM can activate specific membrane-related processes (e.g. Kaplan et al., 1985; Carter & Martin, 1969).

Characterisation of the functional and morphological changes brought about by NEM treatment may contribute to our understanding of how the tissue responds to natural agonists. This thesis sets out the results of such a characterisation, and discusses how this may help to illuminate the mechanisms involved in the action of vasopressin.
METHODS

Materials

Toads, *Bufo marinus* of Dominican origin, were obtained from National Reagents, Bridgeport, USA. They were kept without food, but with free access to running water.

Vasopressin, NEM, maleimide, succinimide, sodium iodoacetate, cytochalasin D, native and cationised ferritin, dithiothreitol, and quinidine sulphate were supplied by Sigma Chemical Co. Ltd. Cytochalasin B came from Aldrich Chemical Co. Ltd., ionomycin from Calbiochem, nocodazole from Janssen Pharmaceuticals, and EHNA (erythro-9-(3-(2-hydroxynonyl))adenine) from Welcome Research Laboratories. Standard laboratory reagents were obtained from BDH Chemicals Ltd. or Sigma.

Isolation of Bladders

Toads were killed by double pithing. The abdominal cavity was opened on the ventral surface with a longitudinal incision as two layers; skin and peritoneum. Lateral incisions were then made to allow reflection of the peritoneum, and the bladder was freed from the mesenteries supporting it along its lateral border.

For gravimetric or cell isolation experiments, sutures were placed under each hemibladder, and a small incision was made at the base. A glass cannula was inserted through this, and the hemibladder sac tied to it using the sutures. Two such ties were made for each hemibladder. The hemibladder was then freed from its remaining attachment to the cloaca, and washed, on both surfaces, three times.

For volumetric studies, the bladder was filled through the cloaca, and then the two hemibladders were tied off with a suture, to form two balloons, which were freed from the cloaca. Four ties were attached around the perimeter of each balloon, and the balloon was lifted carefully on to the bottom part of the chamber. An incision was then made along the balloon,
which could then be opened out flat across the chamber, and held in place with the ties. The top half of the chamber was then clamped in place.

For short-circuit current experiments, the hemibladders were removed using a curved incision, which made it possible to lay the tissue out as a flat loosely-stretched diaphragm across a spiked ring, which was then mounted in the chamber.

**Solutions**

All experiments involving water flow or sodium transport measurements, except those involving EHNA, were performed using a bicarbonate Ringer's solution of the following composition (in meq/l):

\[
\begin{align*}
\text{Na}^+ & \quad 114 \\
\text{K}^+ & \quad 3.4 \\
\text{Ca}^{++} & \quad 1.78 \\
\text{Cl}^- & \quad 117 \\
\text{HCO}_3^- & \quad 2.4
\end{align*}
\]

This solution has an osmolality of 220 mOsm./Kg.H_2O, and a pH of 8.2 when bubbled with air.

During experiments in which the effect of changing the serosal pH was investigated, the serosal bath was bubbled with a 1\% CO_2/99\% O_2 mixture to give a pH of 7.1, or a 5\% CO_2/95\% O_2 mixture to give a pH of 6.5.

Because of the insolubility of EHNA at pH 8.2, experiments in which it was used were carried out using a Heps-buffered Ringer's solution of the following composition (in mEq/l):

\[
\begin{align*}
\text{NaCl} & \quad 111 \\
\text{NaHCO}_3 & \quad 2.4 \\
\text{KCl} & \quad 3.4 \\
\text{KH}_2\text{PO}_4 & \quad 0.5 \\
\text{Glucose} & \quad 6.0 \\
\text{Hepes} & \quad 10.0 \\
\text{MgSO}_4 & \quad 1.2 \\
\text{CaCl}_2 & \quad 0.89
\end{align*}
\]

pH 7.4, osmolality 240 mOsm./Kg. H_2O.
Gravimetric Measurement of Water Flow

Water flow was measured gravimetrically using the method of Bentley (1958). The apparatus is shown in figure 2. Hemibladder sacs were isolated as described above. The sacs were normally filled with 5 ml. of a 1/5 strength Ringer's solution, but in experiments involving 0.3mM quinidine distilled water was used for consistency with previous experiments. The sacs were suspended in a 50 ml. bath of full-strength aerated Ringer’s solution. Care was taken to ensure that the fluid levels inside and outside the sacs were the same, thus avoiding any hydrostatic pressure gradient across the tissue. Every 10 minutes, the bladders were carefully blotted dry on the outside, and weighed on a Mettler H20T analytical balance to an accuracy of ± 0.1mg. Water flow out of the sac could then be calculated, in mg/min., by subtracting successive weights.

Before any experimental procedures were carried out, the bladders were weighed three times over a period of twenty minutes to establish a basal flow rate, and to allow any endogenous vasopressin to be cleared from the tissue. At the end of this period, the water flow was generally less than 1mg/min. Flow rates higher than this normally indicated a leak in the bladder. This was repaired if possible; if not, that pair of bladders was discarded.

In general, bladders from 2, 3, or 4 toads were used at one time: this yielded 4, 6, or 8 sacs. One of each pair of these sacs underwent the experimental manipulations, while the other acted as its control. Thus in most cases the experimental and control hemibladders were paired: this reduced variation between experimental and control measurements, and facilitated statistical analysis of the data.

Application of Hormone and other Compounds

In experiments in which hemibladders were stimulated with vasopressin, a small volume (50-100μl) of a stock solution of the hormone (2 - 10 U/ml.) was added to the solution bathing the serosal surface of the tissue at the required time.
Figure 2. Bentley gravimetric method for measurement of transepithelial water flow in toad bladders.
NEM and its analogues were usually applied to the mucosal surface of the bladder. They were made up at the chosen concentration in 1/5 strength Ringer's solution. At the required time, each bladder was weighed, then each was emptied and refilled with the appropriate solution; finally, each bladder was reweighed to provide a new baseline figure. This whole procedure took ten minutes, half before, and half after, the exchange of fluid. No data on weight loss were available for this time interval, and for clarity this period has been omitted from graphs and statistics.

When NEM was applied to the serosal surface of the tissue, it was dissolved at the desired concentration in full-strength Ringer's solution and, at the required time, bladders were transferred to new baths of the NEM-containing Ringer's solution. The paired hemibladders were also transferred to fresh baths, to control for any effect of the transfer to fresh bathing solution.

Ionomycin, nocodazole, dithiothreitol, and cytochalasins B and D were made up as concentrated stock solutions in dimethyl sulfoxide (DMSO). A small aliquot of the stock solution was then added to the serosal bath of the experimental hemibladders to give the required final concentration, as shown in the table below. An equal amount of DMSO was added to the solution bathing the paired hemibladders to control for any effect of the DMSO itself. In no case did the final DMSO concentration exceed 0.2%: at this level, DMSO has been found to have no detectable effect on the water permeability of the bladders (Taylor, pers. comm.).

Quinidine and EHNA were made up at the required concentration in Ringer's solution, as described above for NEM. Because it is essentially insoluble at pH 8.3, EHNA was dissolved in a HEPES buffered Ringer's solution with the pH adjusted to 7.1. The entire experiment was carried out using this solution.

Ferritin (native or cationised) was applied in the mucosal bath, as described above for NEM. Because ferritin was supplied in a solution containing 0.15M NaCl, it was made up in 1/6 strength Ringer's solution, to give a solution with a final osmolality close to that of the 1/5 strength Ringer's solution used in the majority of the other experiments. In studies with ferritin, experimental hemibladders were filled with a solution containing cationised ferritin, while the paired controls contained native ferritin. NEM was applied to the serosal surface of the tissue in these experiments, to avoid any possible interaction with the ferritin.

Iodoacetate was applied to both surfaces of the bladder using the methods described for NEM.
Table 1: Drugs and Chemicals Used

<table>
<thead>
<tr>
<th>Drug</th>
<th>Stock (mM)</th>
<th>Volume Added</th>
<th>Final Conc.</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasopressin</td>
<td>2 U/ml.</td>
<td>50 μl</td>
<td>20 U/ml.</td>
<td>S1</td>
</tr>
<tr>
<td></td>
<td>20 U/ml.</td>
<td>100 μl</td>
<td>200 U/ml.</td>
<td>S1</td>
</tr>
<tr>
<td>NEM</td>
<td>0.01 mM</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>S2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maleimide</td>
<td>0.1 mM</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinimide</td>
<td>0.1 mM</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ionomycin</td>
<td>1 mM</td>
<td>100 μl</td>
<td>2 μM</td>
<td>S3</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>10 mg/ml.</td>
<td>50 μl</td>
<td>10 μg/ml.</td>
<td>S3</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>10 mM</td>
<td>100 μl</td>
<td>20 μM</td>
<td>S3</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>1 mM</td>
<td>50 μl</td>
<td>1 μM</td>
<td>S3</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1 M</td>
<td>50 μl</td>
<td>1 mM</td>
<td>S3</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.3 mM</td>
<td>S2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>S2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHNA</td>
<td>1 mM</td>
<td>S2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.5 mg/ml.</td>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>1 mM</td>
<td>M &amp; S2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key for methods:  
S1: concentrated aqueous stock added to serosal bath  
S2: dissolved at required conc. in serosal bath  
S3: concentrated stock in DMSO added to serosal bath  
M: applied to the mucosal surface

Monitoring of the Osmotic Gradient

Preliminary studies with NEM showed that it could affect the osmotic gradient across the tissue, particularly when used at a dose of 1mM for two hours. Because of this, experiments were generally limited to one hour, and the osmolality of the fluid bathing the mucosal surface was monitored. In most experiments with NEM, 30 μl samples were taken after each weighing in order to monitor, and if necessary correct for, any diminution of the osmotic gradient. Samples
were taken at the end of all experiments. The osmolality of the mucosal fluid was measured with a Wescor 5100c vapour pressure osmometer. Experiments were discarded if the osmolality of the mucosal fluid filling any hemibladder increased by more than 75 mOsm/Kg. overall, or if the discrepancy between the two halves of a pair was greater than 50 mOsm/Kg. When samples had been taken only at the end of an experiment, the results were discarded if there was a difference of more than 15 mOsm/Kg H2O between the final osmolalities of the mucosal solutions of the two hemibladders.

Where the discrepancy between the osmolalities of the mucosal fluids of any pair of hemibladders within an experimental series varied by between 15 and 50 mOsm/Kg., the measured water flow for each hemibladder was adjusted by multiplying the measured flow by the original gradient, and dividing by the measured gradient at that time point. This correction was applied to about 1/3 of the experiments in which NEM was used.

**Volumetric Measurement of Water Flow**

During experiments in which bladders were being prepared for freeze-fracture, water flow was measured using the volumetric method of Bourguet and Jard (1964), illustrated in figure 3. In this technique, a mucosal bath of fixed volume is kept full by the automatic injection of new fluid as water flows across the tissue to the serosal bath. The amount injected is monitored electronically, and stored digitally every minute, using an Apple II computer. The data are also fed to a pen recorder which provides a continuous trace: the pen returns to baseline at the beginning of each minute and the rate of water flow is thus represented as the height of a series of spikes. An example of such a trace is shown in figure 4.

The volumetric method has a number of disadvantages. Although it is straightforward to add drugs to the serosal bath, addition to the mucosal bath involves substantial disassembly and re-assembly of the equipment, which causes a break in the recording, and inevitably endangers the bladder tissue. It also effectively precludes the taking of samples to monitor osmolality of the mucosal fluid during the experiment. The system is also very sensitive to any bubbles in the sealed part of the system.
Figure 3. Apparatus for volumetric measurement of transepithelial water flow, as described by Bourguet & Jard (1964). Bladder tissue, supported by nylon net, is mounted in paired perspex chambers of 1.6 cm$^2$ cross-sectional area. The serosal bath, which is uppermost, is bubbled with air. The lower, mucosal, chamber, is filled through a hole in its side, and then firmly plugged with a rubber bung. Passing through this bung are two tubes. One of these is an outflow tube, which is clamped off during the experiment, while the other leads to a detector, which senses any loss of fluid, and replaces it. The detector is at a higher level than the chamber: this provides a hydrostatic pressure of about 10 cm. of water that ensures that the mucosal chamber is kept full (as fluid flows out of the detector into the chamber), and also holds the bladder tissue against the net, preventing it from sagging. When water flows out of the detector, the level drops in the tube leading up to the contact pin, and the contact is broken, switching on the drive for a nanolitre syringe. R: Ringer's solution. R/20: 1/20 strength Ringer's solution.
Figure 4. This is a sample trace from the pen recorder used in the volumetric method.
Protein Measurements

Solutions

Reagent A
2% Na$_2$CO$_3$ 0.01% CuSO$_4$ 0.02% K$_2$Na Tartrate

Each component was dissolved separately, and then mixed with rapid stirring. The solution was made up to volume, and stored at 4°C.

Reagent B

1 part of stock 2N Folin and Ciocalteau Phenol Reagent mixed with 1 part of distilled water. This was made up fresh before use.

Measurement Procedure

Protein content of a sample was measured by a modification of the method of Lowry et al. (1951). Trichloroacetic acid was added to give a final concentration of 5%, and the protein was allowed to precipitate for 3-18 hours, and then centrifuged at 4°C in a Burkard Koolspin uP at 15,000g for 10 minutes. The supernatent was discarded, and the pellet was dissolved in 0.1M NaOH. Duplicate 200μl aliquots of this solution were taken for the assay.

Standards were set up as follows:

| Protein stock (μl): | 0 | 5 | 10 | 10 | 15 | 15* | 25 | 50 | 100 | (200) |
|---------------------|--|--|--|--|--|--|--|--|--|--|---|
| 0.1M NaOH (μl):     | 200| 195| 190| 190| 185| 185| 175| 150| 100| (0)  |

The protein stock was a 1mg/ml solution of bovine serum albumin in 0.1M NaOH. The highest concentration standard was only used when a high concentration was expected in the sample tubes, as it was found to depart from the linearity generally shown by the lower concentrations.

One ml of Reagent A was added to each tube, which was then vortexed briefly. The tubes
were then allowed to equilibrate for 10 minutes before addition of 100μl of Reagent B. The tubes were again vortexed, and allowed to stabilise for 30-120 minutes before their optical density was read at 750nm in a Cecil CE272 spectrophotometer. A curve was constructed using the standards, and a best fit line was derived by linear regression analysis. From this the experimental values were calculated.

**Short Circuit Current Experiments**

Sodium transport was measured in quarter-bladders using the short-circuit current (SCC) method of Ussing & Zerahn (1951). The apparatus used is shown in figure 5. Each hemibladder was mounted as a diaphragm in a pair of divided lucite chambers. The two sides of each chamber were filled simultaneously with 5ml of bicarbonate Ringer's solution, and the quarter-bladders were allowed to stabilise for 2 to 3 hours while the SCC and transepithelial potential difference (PD) were monitored. NEM, made up at 0.1mM in Ringer's solution, was applied to the mucosal surface in one chamber at the appropriate time point by changing the bath (the Ringer's solution of the serosal bath was changed simultaneously). The Ringer's solution bathing the tissue in the other chamber, which acted as a paired control, was also replaced with fresh solution.

The SCC and PD were recorded at 5 minute intervals for 30 minutes. Vasopressin (20 mU/ml) was subsequently applied by adding it (10μl of 10 U/ml stock solution) to the serosal reservoirs of both experimental and control quarter bladders. The SCC was then measured at 2 minute intervals for 20 minutes, and then at 5 minute intervals for a further 40 minutes. The PD was not measured during vasopressin stimulation.

In order to compensate for the marked differences in the absolute values of the SCC across different pairs of quarter-bladders, the results for each quarter-bladder at a time t were expressed as a fraction of the SCC at time 0 (the end of the equilibration period), i.e. SCC_t/SCC_0 (Taylor et al., 1978). Quarter-bladders were compared with their paired controls, and the results analysed by paired t test.
Figure 5. Measurement of transepithelial SCC and PD. Bladder tissue was mounted as a diaphragm between two half chambers, each connected to an upper reservoir by two channels. Air was bubbled into one of these: this both aerated the Ringer's solution, and also kept it circulating, to ensure rapid mixing of drugs added to the reservoir. There were separate circuits for the measurement of potential difference (PD) and application of short circuit current (SCC) across the bladder. Connection to the chambers was via agar bridges (3% agar in 0.11 M KCl): two such bridges ran into the wall of each chamber. One lead from each half chamber was connected to a calomel electrode, itself connected to a voltmeter (Keithley 614 electrometer). When measurements of the potential difference across the bladder were made, the current flowing through the other circuit was interrupted briefly. The second lead ran to a silver/silver chloride electrode. A set of batteries provided a potential difference that could be set to oppose that generated by the tissue: the magnitude of the applied current when the potential across the bladder was brought to 0, the short-circuit current, was measured on the ammeter (Keithley 160B multimeter).
cAMP Assay Procedure

Cellular cAMP content was measured using a cAMP binding protein assay kit supplied by Amersham.

Solutions and Reagents

All solutions were made up in a Tris buffer containing 50 mM Tris/HCl, 4 mM EDTA (ethylenediaminetetraacetic acid)(pH 7.5). The buffer itself also contained 1 mM IBMX (iso-butyl methyl xanthine). EDTA chelates divalent cations, while IBMX inhibits phosphodiesterase: both were used to minimise the breakdown of cAMP. Tritiated cAMP was 18 fmolar. Cold cAMP standard was 3.2 pmolar.

Procedure

Hemibladders were isolated as sacs from two toads as described above, except that, after pithing, the vascular system was perfused with Ringer's solution through an aortic cannula for 10-15 minutes before removal of the bladder, to clear as much blood as possible from the bladder tissue and thus minimise contamination of the isolated epithelial cells. Water flow was measured gravimetrically, as described above, with experimental hemibladders being stimulated with either 0.1 mM mucosal NEM or 20 mU/ml serosal vasopressin. The protocol was arranged so that there were never more than two hemibladders to be weighed at any time point, and that the hemibladders reached the end of the functional part of the experiment one at a time, allowing rapid processing of individual tissues.

After each hemibladder sac received its final weighing, it was immediately emptied, and cut off the cannula. The hemibladder was cut in half and laid out flat on an ice-cold glass plate. The epithelial cells were then scraped off with a smooth-edged double coverslip, and put in an Eppendorf tube with 500 μl of Tris buffer containing EDTA and IBMX. The Eppendorf tube
was then plunged into a dry ice/alcohol mixture to freeze the cells. This whole procedure, from emptying the bladder to freezing the cells, took less than one minute.

When cells from all hemibladders had been removed and frozen, they were boiled for 2 minutes. They were then frozen, boiled, and frozen again. This procedure was designed to disrupt the cells, thus releasing intracellular cAMP, and to denature the proteins, thus inactivating enzymes. To test for cAMP breakdown during this treatment, and to monitor cAMP recovery, samples of tritiated cAMP underwent the same procedure; recovery averaged 95 + 4% (n=6).

After thawing, the tubes were centrifuged twice at 12000g for 2 minutes. Sometimes coagulated protein formed a pellet on the surface of the fluid; when this occurred, aliquots for the assay were carefully drawn from the central portion of the tube. Duplicate or triplicate 50 µl samples were assayed in parallel.

In the assay, 50 µl of buffer, 50 µl of tritiated cAMP, and 100 µl of binding protein were added to 50 µl of the sample in each tube. Blanks (lacking tritiated cAMP) and standards (containing unlabelled cAMP at various concentrations) were also run in each experiment. After mixing, tubes were left to equilibrate for 2 hours at 4°C. 100 µl of a suspension of activated charcoal (to absorb any cAMP not bound to the binding protein) was added to each tube, and then the tubes were centrifuged for 5 minutes at 12000g to pellet the charcoal. 200 µl samples were taken from the supernatant, containing the binding protein and bound cAMP, and added to scintillation fluid. After 1 hour, to allow decay of chemiluminescence, the samples were counted in a Searle Isocap 300 scintillation counter.

In order to determine the protein content of the original cell samples, 600 µl of 10% trichloroacetic acid (TCA) was added to the Eppendorf tubes in which the cells had been placed, to precipitate any protein which had remained dissolved in the buffer. After at least three hours, the tubes were centrifuged at 12000g for 10 minutes to pellet all the protein, and the supernatant was discarded. The pellet was homogenised in 1 ml. of 0.1 M NaOH, and assayed for protein by the method of Lowry, as described above.

In the cAMP binding assay, the amount of cAMP in the original sample is reciprocally related to the number of counts measured: the binding protein binds a constant amount of cAMP, and the concentration of unlabelled cAMP is calculated by how much of the known quantity of
tritiated cAMP is displaced from the binding protein. Thus if there are equal quantities of "hot" and "cold" cAMP, the radioactivity associated with the binding protein will be only half what is measured in the absence of "cold" cAMP.

To calculate the cAMP level in the sample, the number of counts in the blanks (which lacked tritiated cAMP, and hence were a measure of background radiation) was subtracted from all the sample counts. These were then divided into the value for the 0 standard (which had "hot", but no "cold" cAMP), to give a value of $C_0/C_x$. This was then plotted against cAMP concentration for the standards. A linear regression line was then calculated, and the values for the unknown samples could be found from this. The results were expressed as pMol of cAMP per mg of protein for each sample. Experimental and control data came from paired hemibladders, so paired t-tests were used to analyse differences in cAMP levels.

**Measurement of Intracellular pH and Calcium Levels**

Intracellular calcium and pH levels were measured in isolated epithelial cells, using the fluorescent indicators Fura-2 and Quene, respectively. Both of these can be loaded into cells in the form of an ester: once inside the cell they are hydrolysed to membrane-impermeant free acids.

**Low Calcium Method for Cell Isolation**

**Solutions**

Cell isolation was carried out in the Hepes-buffered Ringer's solution described above, except that 0.05% bovine serum albumen was included, and CaCl$_2$ was excluded. The serosal bathing fluid also contained 0.5mM EGTA, to remove any trace of calcium.
Isolation procedure

Cells were isolated using a modification of the low calcium method of Sapirstein & Scott (1973). The bladders were dissected out as sacs, filled with Ca\(^{++}\)-free Ringer's solution, and suspended in aerated baths of EGTA-containing Ringer's solution. This treatment weakens the junctions between the epithelial cells. After 20 minutes, the bladders were massaged to release the epithelial cells from the serosa. The mucosal fluid, containing the cells, was collected, and centrifuged at 1000g for 2 minutes, to pellet the cells. The cells were then washed twice in the same buffer, before being pooled and resuspended in 10ml of calcium-containing Hepes buffer. The concentration of cells was measured using a haemocytometer, and the suspension diluted to give a final concentration of 5 x 10\(^5\) cells/ml. The cells were then incubated in calcium-containing medium for 30 minutes, to allow recovery from the low calcium treatment. The fluorescent indicator was then added to the bath as a concentrated stock dissolved in DMSO, and allowed to load into the cells for 60 minutes. Cells were washed (by centrifugation and resuspension) to remove extracellular indicator. Each aliquot of cells was washed again immediately before use.

Calcium measurement: Fura 2

Fura \(2^*\) was added from a 1mM stock to give a final concentration of 1\(\mu\)M. At the beginning and end of the loading period, an aliquot of cells was scanned with excitation light in the range 240-490nm, with the emitted fluorescence being measured at 510nm. The initial scan showed a peak at 380nm, representing the activity of the ester, which is calcium insensitive. After 60 minutes, the peak had moved to 340 nm, which is the absorption maximum for the free acid with calcium bound.

During a run, cells were loaded into a cuvette, and placed in the fluorimeter (Applied Photophysics). The excitation was switched manually between 340 and 380nm at intervals of approximately 15 seconds. The ratio between the signals at 340 and 380 nm provides a measure of the calcium level that is independent of the concentration of the indicator (Gryniewicz et
al, 1985). Emission was monitored continuously at 510 nm. The fluorescence was monitored for several minutes, before NEM was added from an 0.1 M aqueous stock, to give a final concentration of 0.1 mM. The mixture was carefully stirred, and the effects monitored for 20 minutes, with the cuvette being stirred twice during this period, to avoid settling of the cells. A control run, during which all manipulations were performed, was carried out on unloaded cells, to provide a measure of baseline autofluorescence: this was then subtracted from the values measured during the experimental runs.

In order to calibrate the trace produced the following procedure was used. At the end of the run, EGTA in Tris buffer was added, to give a final concentration of 5 mM EGTA, and 10 mM Tris. This removed free calcium from the bath, and quenched the signal from any dye that was free in the bath: the 340:380 nm ratio under these conditions was defined as $R_q$. The Tris raised the pH to about 8.5. The cells were then lysed with Triton X-100 (0.05%), to release the dye into the bath, and give a calcium-free signal: the 340:380 nm ratio under these conditions was defined as $R_{	ext{min}}$. Finally, 10 mM calcium was added to saturate both the EGTA and the Fura-2, to give a maximal calcium-bound signal: the 340:380 nm ratio under these conditions was defined as $R_{	ext{max}}$. Note that $R_{	ext{min}}$ and $R_{	ext{max}}$ have been calculated directly for each run, as the use of ratios defined beforehand in free solutions, as described by Grynkiewicz et al. (1985), has been found unsatisfactory in this tissue (Taylor & Pinches, pers. comm.). The $K_d$ for calcium binding to Fura-2 in free solution had previously been calculated in this lab to be 158 nM in a solution designed to mimic amphibian intracellular fluid. This information, together with the ratio between the total calcium-free and calcium-bound signals at 380 nm, $C_f/C_b$, is enough to calculate the cytosolic free calcium ion concentration for a given 340:380 ratio $R$:

$$[\text{Ca}^{++}] = K_d (R - R_{\text{min}})/(R_{\text{max}} - R) (C_f/C_b)$$

(Gryniewicz et al., 1985).

In order to correct for leaked dye, a correction factor was calculated:

$$CF = 1 - (R - R_q)/(R - R_{\text{min}})$$
Intracellular pH: Quene

Quene was added from a 5mM stock to give a final concentration of 5μM. At the beginning and end of the loading period, an aliquot of cells was scanned with an excitation light of 390nm: the emitted fluorescence being measured in the range 430-630nm. The initial scan showed a peak at 520nm, representing the activity of the ester. After 60 minutes, the peak had moved to 540 nm, which is the emission maximum for the free acid.

During experimental runs, the dye was excited at 390nm, and the fluorescence monitored continuously at 540nm. The magnitude of this fluorescence provided a measure of the pH. As with Fura 2, a control run was carried out before the experimental ones to correct for autofluorescence, and the cells were stirred to avoid sedimentation.

At the end of the run, the cells were lysed with 0.05% Triton X-100, in a solution containing 0.5mM EGTA and 0.5mM EDTA, to remove calcium and magnesium, which affect the indicator. The bath was then acidified to about pH 6.8 with 2mM HCl, and then gradually alkanilised with 4 aliquots of 2mM Tris. At each step, the pH was measured with a pH electrode, and the fluorescence signal measured. Thus a calibration curve could be constructed to analyse the data.

Preparation for Thin-Section Electron Microscopy

Fixation and Embedding

Solutions

The functional part of these experiments was carried out using the bicarbonate-buffered Ringer's solution described above. All solutions used during preparation for electron microscopy were made up with deionised distilled water, to avoid any contamination.

The fixative contained 50 mM sodium cacodylate and 1.25% glutaraldehyde. It had a pH of 7.4, and an osmolality of 230-235 mOsm/Kg. Washes were performed using 0.1M sodium cacodylate buffer (pH 7.4, osmolality 220 mOsm/Kg.). Osmium tetroxide was made up at a
concentration of 1% in 0.1M sodium cacodylate buffer. Uranyl acetate was made up in 0.1M acetic acid, and stored, protected from light, at 4°C. The solution was filtered before use.

The embedding resin was made up as follows:

EPON 812 8.2 ml.
DDSA (dodecenyl succinic anhydride) 18.4 ml.
Araldite Cy 212 5.6 ml.
DMP 30 (2,4,6 tri(dimethylamino-methyl) phenol) 0.6 ml.

Components were added in this order, with constant stirring. All bubbles were allowed to escape before use. Resin could be stored for short periods at -18°C in a sealed container to avoid hydration.

Reynolds lead citrate solution (Reynolds, 1963) was made by dissolving half a pellet of NaOH in 12 ml. of cooled, boiled deionised distilled water, and adding 50 mg. of lead citrate. This was shaken for two minutes, after which it was centrifuged to remove any precipitate. The solution was stored at 4°C, sealed from air, and inspected before use for any precipitate. If any was observed, the solution was discarded.

**Experimental procedure**

Hemibladders were isolated as described above, and stimulated with 0.1mM NEM. Paired controls were unstimulated. Water flow was measured gravimetrically during the functional part of the experiment to provide information about the permeability of the bladders at the time at which they were fixed.

After 20 or 40 minutes stimulation, the hemibladder was weighed, emptied, and filled with 5 ml. of fixative. After 30 seconds, it was immersed in a bath of fixative for 2 hours. (Prefixation of the mucosal surface was performed in order to minimise any distortion of the epithelium due to removal of water from the cells through their more permeable serosal surface (Reaven et al., 1978; Croker & Tisher, 1971). Any risk of this was further minimised by the use of a fixative that was essentially isotonic (Dratwa et al., 1979).)
The following procedure was used for embedding of tissue; all manoeuvres being carried out inside a fume hood:

1) At the end of the fixation period, the apical portion of each hemibladder was cut off and divided into four pieces.

2) These were then washed in 3 changes of buffer.

3) The tissue pieces were postfixed in osmium tetroxide for exactly 30 minutes.

4) They were then washed in 3 changes of deionized distilled water, before being en bloc stained overnight at 4°C in 1% uranyl acetate.

5) After 3 washes in deionized distilled water, the pieces were dehydrated in a graded series of alcohols and propylene oxide as follows:

   5 minutes in 70% ethanol
   5 minutes in 95% ethanol
   3 x 7 minutes in dehydrated 100% ethanol
   3 x 7 minutes in propylene oxide

6) The pieces were finally transferred to a 1:1 mixture of propylene oxide and resin, and put on a rotator for at least two hours. The tubes were loosely sealed with parafilm, to allow gradual evaporation of the propylene oxide.

7) The tissue pieces were put in fresh tubes of pure resin, and left overnight on the rotator in tightly sealed tubes.

8) The central portion of each piece was cut out and embedded in a resin-filled mould. Thus four blocks were prepared from each original hemibladder.

9) The resin was cured at 70°C for 48 hours.
Sectioning and Staining

Silver to gold sections were cut on an LKB Ultratome III ultramicrotome, using glass or diamond knives. Some sections were examined unstained, but the majority were stained for 5 minutes with a filtered saturated aqueous solution of uranyl acetate, and for 5 minutes with Reynold's lead citrate (Reynolds, 1963).

Sections were examined on a Jeol 100CX electron microscope at 60 KV (for unstained sections) or 80 KV (for stained sections).

Morphometric Analysis

Morphometric analysis was carried out using the point counting stereological technique described by Weibel (1969). Hemibladders was stimulated with 0.1mM NEM for 20 or 40 minutes, while paired controls were unstimulated. Only the controls of the 40 minute group were used in this study, as unpaired analysis was to be used to compare the three groups (control, 20 minutes NEM, and 40 minutes NEM). Tissue was prepared and sectioned as described above. Once sections had been cut and stained, they were coded, and all procedures apart from data analysis were carried out without knowledge of the status of the tissue.

At the microscope, a random grid square was selected, with the requirements that the whole width of the epithelium should be visible, crossing the full width of the square, and that the technical quality of the tissue should be adequate. Two such squares were selected for each of the three hemibladders in each group, and all the epithelium within each square was photographed. This resulted in approximately 20 micrographs of each bladder. Prints were made at a 13,000 fold magnification, and a point count made using a 13.5 mm lattice. All points within the epithelium (i.e. lying between the apical plasma membrane and the basement membrane) were allocated to one of seven categories, as discussed in Chapter 6.
Horseradish Peroxidase Labelling

Solutions

Diaminobenzidine (DAB) solutions contained 5mg of DAB dissolved in 10 ml. of 0.05M Tris HCl buffer (pH 7.6). DAB/hydrogen peroxide buffer also contained 0.1 ml of 1% hydrogen peroxide. These solutions were made up fresh immediately before use.

Procedure

Horseradish peroxidase (HRP) was used in studies of endocytosis. HRP at a concentration of 3mg/ml. was included in the mucosal fluid of the hemibladders during the functional part of the experiment, and then the tissue was fixed as described above. After the washes in cacodylate buffer (stage 2 above), the tissue pieces were preincubated with diamino benzidine (DAB) for 30 minutes. They then spent 30 minutes in Tris buffer containing both DAB and hydrogen peroxide. This caused the formation of a brown reaction product at the sites where HRP was present.

After 3 washes in cacodylate buffer, the tissue was postfixed with osmium tetroxide, dehydrated and embedded as described above. In order to maximise the visibility of the peroxidase reaction product the tissue was not en bloc stained with uranyl acetate, nor were the sections stained prior to examination in the electron microscope.

Negative Staining

To look for membrane fragments in the mucosal fluid from toad bladders incubated with NEM, the following protocol was used. A formvar film was made by dropping a 2% solution of formvar in dichloroethane onto a bath of water. Electron microscope grids were
then laid on the flat part of the film, so that the grids formed a rectangular array. The whole array was then picked up on a piece of parafilm. After drying, the grids were carbon coated for 30 seconds.

A drop of mucosal fluid was placed on a grid for 30 seconds. It was then washed off and replaced with filtered saturated aqueous uranyl acetate: again, this was left on for 30 seconds. The grid was then washed with deionized distilled water, and allowed to dry before examination in the electron microscope.

**Freeze-Fracture Electron Microscopy**

These experiments were performed under the guidance of Dr. Bourguet in his laboratories at Saclay, and with the help of Renée Gobin. Hemibladders were isolated, and water flow was followed by the volumetric technique described above. At the required time, a fixative of 2% glutaraldehyde in 0.1M sodium cacodylate (pH 7.4) was applied, first to the mucosal face, then to the serosal side. The tissue was fixed for at least 20 minutes.

After 3 washes with Ringer's solution, the chamber was opened, and the bladder was carefully cut from the central portion of the chamber, to avoid any tissue damaged by being clamped in the chamber. After cryoprotection in 30% (v/v) glycerol/Ringer's solution, small discs of tissue were stamped out and placed between small copper plates. They were then rapidly frozen in liquid nitrogen-cooled freon 22, and stored in liquid nitrogen.

The samples were fractured and etched in a Balzers 301 cryopump freeze etch unit, and coated with a carbon/platinum film. The tissue was dissolved away from this replica for 24 hours in bleach, and the replica was mounted on a copper grid for examination in a Philips EM 300 electron microscope.
FUNCTIONAL CHARACTERISTICS OF THE NEM RESPONSE

Effects on Transepithelial Water Flow

Studies were first carried out to determine the basic characteristics of the increase in osmotic water flow across the toad bladder brought about by treatment with mucosal or serosal NEM. Transepithelial water flow, from mucosa to serosa, was measured gravimetrically in isolated hemibladders by the method of Bentley (1958), as described in the Methods chapter.

0.1 mM Mucosal NEM induces an increase in water flow

Figure 6 shows the mean water flow plotted against time for a series of paired experiments (n=4) in which 0.1 mM NEM was applied to the mucosal surface of the experimental bladders (this concentration was chosen on the basis of Rasmussen's previous results (Rasmussen et al., 1960)). This treatment induced an increase in water flow across the tissue: in unstimulated control bladders transepithelial water flow averaged 0.5 ± 0.04 mg/min, while water flow across the NEM-treated bladders averaged 10 ± 2 mg/min, calculated over the 60 minute period of exposure to NEM.

Figure 7 shows the mean results of 29 unpaired experiments with 0.1 mM mucosal NEM (these data were collated from the controls of later experiments in which inhibition of the NEM response was studied). For comparison, the results are also shown of twelve bladders stimulated with vasopressin, and 18 untreated control bladders. In the NEM-treated bladders, water flow averaged 18 ± 1 mg/min over 60 minutes and reached maximal levels in about 30 minutes, while the water flow induced by VP stimulation averaged 36 ± 5 mg/min and reached peak levels at around 20 minutes. Basal water flow in unstimulated bladders was 0.9 ± 0.5 mg/min.

Several differences between the responses to VP and mucosal NEM are apparent. The onset of the response to application of NEM is more gradual, and the maximal level of water flow is lower.
Figure 6. Mean water flow for a preliminary series of 4 experiments with 0.1 mM mucosal NEM, showing a slow but substantial increase in transepithelial water flow.

Figure 7. This illustrates the time course of water flow induced by 0.1mM mucosal NEM (n=29) or 20mU/ml. serosal VP (n=12), compared to unstimulated controls (n=18). This data is unpaired.
Furthermore, the response to vasopressin climbs rapidly to a peak, and then declines significantly; in contrast, after NEM the water flow rises slowly towards a plateau. In general, this pattern was followed in the response of individual bladders to NEM, but in some cases, particularly when an unusually high maximal water flow was achieved, a peak similar to that seen with vasopressin was observed. An example of this type of response is shown in Figure 8.

**Dose Dependence**

To investigate the dose dependence of the increase in water flow induced by NEM, the effects of 10μM and 1mM mucosal NEM were determined. In a series of 5 paired experiments on bladders treated with 10μM NEM, transepithelial water flow averaged 2.5 ± 1.1 mg/min over a one hour period, compared to control flows of 1.6 ± 0.4 mg/min (n.s.). Although in each pair the experimental hemibladder lost water faster than the untreated control, water flow across the NEM-treated tissues was not statistically significantly greater than that across the controls during this period. However, over two hours, water flow in the NEM treated bladders averaged 2.7 ± 0.4 mg/min, compared to 1.6 ± 0.1 mg/min in the controls (n=5, p<0.01). Thus a concentration of 10μM appears to be close to the threshold at which mucosal NEM can induce transepithelial water flow.

In a series of 12 experiments, 1mM mucosal NEM caused water flow that averaged 7.7 ± 1 mg/min over 60 minutes; water flow in the paired controls averaged 0.82 ± 0.1 mg/min. As shown in Figure 9, in these experiments NEM-induced water flow reached a maximum of 8.6 ± 1.6 mg/min in twenty to thirty minutes.

Figure 10 shows the biphasic dose-dependence of NEM-induced transepithelial water flow. There is little increase in water flow at a dose of 10μM NEM. 1mM NEM induces a smaller increase in water flow than 0.1mM NEM, however maximal levels are reached slightly more quickly.
NEM-Induced Water Flow: Unusually Large Response

Figure 8. Occasionally, NEM induced a particularly large response, as shown here. Note that in this case there is a peak and decline in water flow similar to that seen during VP stimulation. The time course is also more like that of VP, climbing rapidly to a peak at 20 minutes stimulation.

Figure 9. This illustrates the increase in water flow brought about by stimulation with 1mM mucosal NEM.
Figure 10. Dose dependence of the water flow response to mucosal NEM. Maximal flows are seen after stimulation with 0.1mM NEM.
Reversibility

In order to determine whether the increase in water permeability brought about by mucosal NEM was reversible, hemibladders were stimulated with 0.1mM NEM on their mucosal surface for 20 minutes; the NEM was then washed out, and the water flow monitored for a further 40 minutes. Paired control hemibladders were stimulated with NEM throughout the whole 60 minute period. As shown in figure 11, water flow in the experimental hemibladders was not merely maintained at the level achieved before the removal of NEM, but continued to rise in line with that in the control tissues which were still exposed to NEM. Thus the course of the response was unaffected by the removal of NEM after 20 minutes stimulation.

Figure 12 illustrates the results of a 5 minute application of either 0.1mM or 1mM NEM to the mucosal surface of the bladder. These experiments were done without paired controls, to conserve animals. Although 5 minutes exposure to 0.1mM NEM produced an increase in water flow, this was substantially smaller than seen with continuous treatment. Thus water flow averaged only $4.6 \pm 0.9$ mg/min over a 60 minute period ($n=6$, $p<0.001$ compared to 29 unpaired hemibladders stimulated for 60 minutes with 0.1mM NEM, in which mean flow was $18 \pm 1$ mg/min). Water flow following 5 minutes exposure to 0.1mM NEM reached levels within the first ten minutes not significantly different from the maximum attained: thus there was both a smaller increase in water flow, and a less prolonged response, than in the continued presence of 0.1mM NEM. Conversely, 5 minutes exposure to 1mM mucosal NEM caused a rise in water flow averaging $7.1 \pm 1.5$ mg/min ($n=6$), peaking at around 20 minutes: this response was not significantly different from that observed in the 12 experiments in the continued presence of 1mM NEM, described above.

Thus the increase in water flow induced by treatment with mucosal NEM is essentially irreversible. Stimulation for 5 minutes with 0.1mM NEM caused only a small response, but the effects of 5 minutes treatment with 1mM NEM, or 20 minutes treatment with 0.1mM NEM, were indistinguishable from the effects observed in the continued presence of that dose of NEM.
Figure 11. Comparison of 20 minutes stimulation with 0.1mM NEM with continuous stimulation. Washout after 20 minutes has no effect on the development of the response.

Figure 12. Stimulation of hemibladders for 5 minutes with 1mM or 0.1mM mucosal NEM. 1mM NEM produces a response identical to the produced by its continued presence, but 0.1mM NEM produces a much smaller response, which may not increase after removal of the NEM.
Development of the Response in the Absence of an Osmotic Gradient

To study whether NEM could induce an increase in permeability in the absence of a transepithelial osmotic gradient, NEM, dissolved in full-strength Ringer's solution, was applied to the mucosal surface of the experimental hemibladders, while the controls received NEM dissolved in 1/5 strength Ringer's solution. Water flow was monitored for thirty minutes, after which all hemibladders were refilled with NEM-containing 1/5 Ringer's solution, and water flow was monitored for a further 30 minutes. The results are illustrated in Figure 13, which shows that water flow out of the experimental hemibladders reached levels greater than that seen in paired controls even during the first time period after the osmotic gradient was imposed. Water flow in the final 30 minutes of the experiment was 26.7 ± 2.5 mg/min in the bladders without a transepithelial osmotic gradient in the early part of the experiment, compared to 19.7 ± 1.0 mg/min in the controls. This is a difference of 35 ± 8% (n=4, p<0.05). Thus NEM can induce an increase in water permeability in the absence of a transepithelial osmotic gradient; indeed, this increase appears to be greater than that developed in the presence of a gradient.

Response to 0.1mM Serosal NEM

In a series of 6 experiments, 0.1mM NEM was also found to cause an increase in transepithelial water flow when applied to the serosal surface of the bladder. As shown in Figure 14, the mean flow induced by serosal NEM, 0.1mM, over 60 minutes was 14 ± 0.8 mg/min, compared to that in unstimulated paired controls of 1.1 ± 0.3 mg/min. The response to 0.1mM serosal NEM was similar to that seen with 0.1mM mucosal NEM, although the development of the response was considerably slower; water flow did not reach maximal levels in these experiments until after 50 minutes exposure to NEM.
Figure 13. Effect of NEM on water permeability in the absence of an osmotic gradient. The rapid rise in water flow when a gradient was applied across the tissue suggests that an increase in permeability had already occurred.

Figure 14. Application of 0.1 mM NEM to the serosal surface of the tissue also produced an increase in water flow, as shown in this figure. Water flow increases more slowly than seen after mucosal application of 0.1 mM NEM.


**Effects of NEM on Transepithelial Sodium Transport**

To investigate the effect of mucosal NEM on transepithelial net sodium transport, its effect on the basal short circuit current (SCC), and also on the vasopressin-stimulated SCC, was examined. The results are shown in Figure 15. Mucosal NEM, 0.1mM, caused a rapid and substantial decline in the basal SCC, which was inhibited by 24 ± 5% (n=6, p<0.01) relative to paired controls within the first 5 minutes; after 30 minutes, the inhibition had reached 54 ± 6% (n=6, p<0.001). Subsequent addition of vasopressin caused a rise in SCC, but this response was strongly inhibited in the NEM treated bladders. At the peak of the natriferic response to VP, the SCC in the NEM treated bladders was inhibited by 69 ± 7% (n=6, p<0.001).

If the ratio of the SCCs in the NEM-treated and control bladders was plotted against time, there was a smooth decline, reflecting an increasing inhibition of the SCC by NEM. Addition of VP to both bladders caused no break in this decline, which reached a constant level at about 70% inhibition. This implies that the response to vasopressin was reduced in proportion to the inhibition of the basal short circuit current.

**Summary**

NEM, applied to the mucosal surface of the toad bladder, can cause an increase in transepithelial water flow qualitatively similar to that induced by vasopressin. The increase in flow induced by NEM is slower and smaller than that seen after vasopressin. Unlike the hormonal response, the effect of NEM is not reversed by removal of the drug. The response to NEM has a biphasic dose dependence, with a maximum response at 0.1mM. At this dose it induces an increase in water flow when applied to either the mucosal or serosal surface of the bladder.

In contrast to the similarity of its effect on water flow to that of vasopressin, NEM strongly inhibits basal sodium transport across the tissue, and also reduces the natriferic response to VP.
Figure 15. This illustrates the effect of 0.1 mM NEM on the SCC. There is a rapid and substantial decline in basal SCC, and the response to VP is also strongly inhibited.
CELLULAR EFFECTS OF NEM

In order to elucidate the mechanism by which NEM induces an increase in transepithelial water flow experiments were performed to determine whether it is acting as a sulphydryl reagent, and whether it causes changes in cellular cAMP content, cytosolic calcium ion concentration, or intracellular pH: these have all been suggested as possible mediators or modulators of the vasopressin hydrosmatic response (Orloff & Handler, 1961; Hardy, 1978; Parisi et al., 1983). Finally, studies were performed to determine whether NEM causes shedding of membrane from the apical region of the cells, as described with cultured cells (Belkin & Hardy, 1961).

**Is NEM Acting as a Sulphydryl Reagent?**

To seek an answer to this question, experiments were carried out with maleimide, succinimide, and phenyl arsine oxide (PAO). Maleimide is a compound closely related to NEM which also has the ability to react with sulfhydryl groups. Succinimide is structurally identical with maleimide except for the absence of the double bond in the ring which give maleimide and NEM their reactivity with sulfhydryl groups (Webb, 1966). PAO is an unrelated sulphydryl reagent (Kaplan et al., 1985). Dithiothreitol, which can reduce disulphide linkages in an exchange reaction (Cleland, 1964), was tested to see if it could reverse the increase in permeability induced by NEM.

Maleimide, applied to the mucosal surface of the bladder at a dose of 0.1 mM, caused a significant increase in water flow. Bladders treated with maleimide averaged a flow of 5.5 ± 1.5 mg/min over 60 minutes, compared with untreated controls which had a mean flow of 1.1 ± 0.1 mg/min (p<0.05, n=7). The time course of the response is shown in Figure 16, which shows that the peak flow of just over 9 mg/min was not achieved until 50 minutes after stimulation: this is rather slower, and less than half the maximum magnitude seen with NEM. Nevertheless, the two responses are qualitatively very similar.

In a series of 3 experiments in which the effect of succinimide was investigated, there was no
Figure 16. Mucosal maleimide (0.1 mM) induces an increase in transepithelial water flow qualitatively similar to that seen with NEM, but the response is smaller and develops more slowly.

Figure 17. Dithiothreitol (1 mM) has little effect on NEM-induced water flow.
indication that it could cause an increase in transepithelial water flow: indeed, water flow was lower during the experimental period that during the preliminary period in which basal water flow was measured, although it was slightly, but not significantly, higher than in the control bladders.

PAO, although it is a sulfhydryl reagent, caused no significant increase in water flow: over a 60 minute period after mucosal application of 1mM PAO water flow averaged 1.2 ± 0.3 mg/min, while flow across paired control hemibladders averaged 1.0 ± 0.6 mg/min (n=4).

In a series of 6 experiments, 1mM dithiothreitol (DTT) was applied to the bladders 20 minutes after stimulation with NEM, to examine its ability to reverse the response. The results showed that water flow across DTT-treated bladders was reduced by 17 ± 6% (n=6, p<0.05): however, although this result is statistically significant, its biological significance is questionable, as in 3 of the experiments the water flow across the bladders that would later receive DTT was already markedly lower than their paired controls during the initial period of stimulation with NEM: as shown in Figure 17, there is almost no increase in the separation of the lines representing DTT-treated and control groups during the presence of the DTT. If the data is normalised to compensate for this, the apparent inhibition is reduced to 6 ± 5% (p>0.3). Thus it appears that DTT, at this dose, is unable to counteract the effects of NEM on water flow.

Investigation of Possible Intracellular Signals

Effect of NEM on Intracellular cAMP Levels

The vasopressin response is mediated by a rise in intracellular cAMP levels, as discussed in the introduction. Experiments were performed to determine whether NEM also causes a rise in intracellular cAMP levels. Hemibladders were stimulated for 20 minutes with VP or NEM, while paired controls received no stimulation. The epithelial cells were isolated, and cellular cAMP was extracted and assayed, as described in the Methods chapter. In 5 experiments, 20 minutes exposure to 20 mU/ml. vasopressin caused a rise in cAMP content of the epithelial
Figure 18. Vasopressin produces a substantial increase in cytosolic cAMP levels, while NEM produces a small decrease, relative to unstimulated controls.
cells from $11.6 \pm 2.1$ pmol./mg. of protein to $29.9 \pm 3.7$ pmol./mg.: this is an increase of $175 \pm 33\% \ (p<0.01)$. In contrast, NEM caused a drop from $11.0 \pm 1.8$ pmol./mg. to $8.2 \pm 0.8$ pmol./mg., a reduction of $22 \pm 6\% \ (n=5, \ p<0.02)$. These results are illustrated in Figure 18.

**Effect of NEM on Cytosolic Calcium Levels**

As cytosolic calcium ion concentration appears to modulate the response to VP, as discussed above, it was of interest to investigate the effects of NEM on ionised calcium levels. Intracellular free calcium ion concentration was measured in isolated epithelial cells using the fluorescent dye, Fura-2, as described in the Methods chapter.

Addition of 0.1mM NEM had no dramatic effect on free calcium ion concentration over a period of ten minutes. Over 20 minutes, there was a slow increase of the signal at 340 nm, and a decrease of the signal at 380 nm, consistent with an increase in calcium ion concentration. However, interpretation of the traces was complicated by leakage of dye into the bathing medium.

As described in the Methods chapter, the amount of leaked dye was determined using EGTA at the end of the run. Depending on whether this correction is applied to data throughout the run, or only to the final time points, the change in free calcium ion concentration can be estimated at either $32 \pm 5\%$ or $-17 \pm 1\%$ (data not shown): it is likely that the true value lies between these limits. Such small changes cannot be readily interpreted in the light of such uncertainty.

**Effect of NEM on Intracellular pH**

As discussed in the introduction, changes in pH have profound effects on the response to VP. It has been suggested that pH may modulate the response to VP, and indeed VP has been shown to cause a change in intracellular pH (Parisi et al., 1983). Intracellular pH was measured using the pH-sensitive dye, Quene. In a series of four experiments, 20 minutes exposure to 0.1 mM NEM caused no change in the intracellular pH from its baseline value of $7.45 \pm 0.03$ (data not shown).
NEM Induced Mucosal Protein Release

Measurement of Protein in the Mucosal Fluid

It has been reported that NEM can induce blebbing and vesiculation of the plasma membrane of cultured cells (Belkin & Hardy, 1962). In the light of this, the protein content of the mucosal fluid was measured after experiments. The mucosal fluid was collected, pooling the fluid from hemibladders which had undergone the same treatment, and protein was precipitated using trichloroacetic acid, and assayed by the method of Lowry, as described in the methods section. Protein yields are expressed as mg. of protein/hemibladder (mg/hb). Control bladders which underwent no drug treatment yielded 0.015 ± 0.002 mg/hb (n=41), although the normal range included values as high as 0.04 mg/hb. After 60 minutes vasopressin stimulation in the presence of a gradient this increased only very slightly to 0.031 ± 0.003 (n=54, p<0.001). In the absence of a gradient, vasopressin caused no increase in protein release (0.014 ± 0.004 mg/hb; n=7).

NEM, applied to the mucosal surface at 0.1mM in the presence of a transepithelial osmotic gradient, caused protein yields of 0.069 ± 0.003 mg/hb (n=34), more than a four-fold increase relative to untreated hemibladders, and more than two-fold greater than vasopressin stimulated ones. Application of NEM to the serosal surface had a similar effect, leading to 0.065 ± 0.004 mg/hb (n=6). Mucosal NEM applied simultaneously with vasopressin, which caused a high rate of water flow, led to the release of 0.12 ± 0.009 mg/hb (n=8). Conversely NEM stimulation of tissue in a bath at pH 6.5, a treatment which strongly inhibits water flow, yielded only 0.035 ± 0.005 mg/hb (n=4), suggesting that NEM-induced protein yield is dependent on transepithelial water flow.

An increase in the protein found in the mucosal fluid was also found when quinidine, 1mM, was applied to the serosal surface of the bladders after they had been stimulated with vasopressin: the yield was 0.078 ± 0.012 mg/hb. This effect required the presence of vasopressin, quinidine, and an osmotic gradient; the omission of any of which reduced protein
release to essentially basal levels.

**Negative-stain studies of the mucosal fluid**

To try and visualise any membranous elements or vesicles that might be present in the mucosal fluid at the end of the experiments described above, negatively stained samples, prepared as described in the Methods section, were examined in the electron microscope. Occasionally, membrane fragments were seen that appeared to be part of the plasma membrane: these were quite large sheets, that appeared to have microvilli still present in them. A large number of smaller fragments, which often appeared to form tubular or irregular vacuolar structures, were also seen. An analysis of the diameter of 91 of the tubular elements showed that the modal size was in the range 70-80 nm. This is somewhat smaller than the diameter of 120 nm reported for the tubular vesicles in this tissue (Muller et al., 1980). Occasionally, what appeared to be the remains of whole cells were seen on these grids: these were very infrequent, and may have represented no more than normal shedding of dead cells from the epithelium.

**Summary**

These experiments indicate that NEM probably produces its effects on water permeability via one or more sulphhydryl reactions. The response to NEM does not appear to be mediated by changes in cellular cAMP content or cellular pH; nor is there clear evidence for a change in calcium levels within the cells.

The appearance of protein in the fluid bathing the mucosal surface after NEM stimulation may reflect vesiculation of the apical plasma membrane, and may be dependent on transepithelial water flow.
STUDIES WITH MODIFIERS OF THE VP RESPONSE

In order to characterise the hydrosmotic response to NEM further, a comparison of the effects of a number of factors known to affect the VP response on NEM-induced and VP-induced water flow was made.

**Effect of Low pH**

As discussed in the introduction, the response to VP in the toad bladder is inhibited at low serosal pH. In order to investigate whether low pH has a similar effect on NEM-induced water flow, experiments were performed in which the serosal bath of the experimental hemibladder was acidified to pH 6.5 or 7.1 by bubbling with CO₂, as described in the Methods chapter. The control hemibladder was in a bath bubbled with air, at pH 8.2. Paired experimental and control hemibladders were stimulated with VP (20mU/ml) or mucosal or serosal NEM (0.1mM). The effect of lowering the pH of the serosal bath after establishment of the response was also determined.

The water flow response to VP at pH 6.5, calculated over a 60 minute period, was 6.4 ± 0.7 mg/min, compared to 35.8 ± 2.8 mg/min in paired control hemibladders at pH 8.2, an inhibition of 74 ± 1% (n=6, p<0.001). The response to mucosal 0.1mM NEM was affected similarly: water flow at pH 6.5 averaged 1.9 ± 0.6 mg/min over 60 minutes, compared to a flow of 16.1 ± 2.9 mg/min in controls at pH 8.2, an inhibition of 85 ± 5% (n=6, p<0.001). Likewise, the response to serosal 0.1mM NEM at pH 6.5 averaged 1.9 ± 0.3 mg/min, compared to 16.6 ± 1.2 mg/min in controls at pH 8.2, a reduction of 90 ± 3% (n=6, p<0.001).

Acidification can also inhibit an established water flow response. In experiments in which bladders were stimulated with VP for twenty minutes at pH 8.2 and then moved to new baths at pH 6.5, the mean flow over the next 40 minutes was 10.1 ± 1.0 mg/min; mean water flow across paired hemibladders which remained at pH 8.2 was 37.4 ± 1.7 mg/min, an inhibition by low pH of 74 ± 1% (n=6, p<0.001). Water flow across hemibladders stimulated with 0.1mM mucosal NEM at pH 8.2 averaged 18.7 ± 2.3 mg/min., while hemibladders moved to baths at pH
Figure 19a. Effect of serosal acidification on an established response to VP.

Figure 19b. Effect of serosal acidification on an established response to NEM.
Figure 20. There is no evidence that the response to NEM can be initiated at pH 6.5: the increase in water flow seen when the pH is raised follows the same time course as seen in the absence of preincubation with NEM at pH 6.5.
6.5 after 20 minutes stimulation at pH 8.2 showed a mean water flow of 6.5 ± 0.5 mg/min. This represents an inhibition of 63 ± 4 % (n=6, p<0.001). The time courses of these inhibitions is shown in Figures 19a and 19b. It is clear that lowering the pH does not merely prevent any further increase in NEM-induced water flow, but actually causes a reduction in flow.

In similar experiments using serosal NEM, the change to pH 6.5 was made after 30 minutes stimulation, because of the slower development of this response. Transepithelial water flow over the next 30 minutes was reduced from 21.8 ± 1.8 mg/min to 9.1 ± 1.0 mg/min, an inhibition of 59 ± 2% (n=6, P<0.001).

When preincubated at an intermediate pH of 7.1, the response of bladders to 0.1mM mucosal NEM was inhibited by 53 ± 4% (n=5, p<0.01), relative to the controls incubated at pH 8.2. In a series of 6 experiments in which the pH was lowered to 7.1 after 30 minutes exposure to 0.1mM mucosal NEM at pH 8.2 there was an inhibition of 17 ± 4% (n=6, p<0.01) relative to controls incubated at pH 8.2 throughout.

In an attempt to elucidate how low pH is acting, experiments were carried out in which bladders were exposed to 0.1mM mucosal NEM for 20 minutes at pH 6.5, before being moved to baths at pH 8.2. The response was then monitored over a 40 minute period. The results are illustrated in Figure 20, which shows that the response takes 30 - 40 minutes from the time the pH is changed to 8.2 to reach its peak. This is closely comparable to the time taken for the development of the response when NEM is applied to tissue initially at pH 8.2.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>20mU/ml VP</th>
<th>0.1mM mucosal NEM</th>
<th>0.1mM serosal NEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>60' at pH 6.5</td>
<td>74 ± 1%</td>
<td>85 ± 5%</td>
<td>90 ± 3%</td>
</tr>
<tr>
<td>20' at pH 8.2, 40' at pH 6.5</td>
<td>74 ± 1%</td>
<td>63 ± 4%</td>
<td>---</td>
</tr>
<tr>
<td>30' at pH 8.2, 30' at pH 6.5</td>
<td>---</td>
<td>---</td>
<td>59 ± 2%</td>
</tr>
<tr>
<td>60' at pH 7.1</td>
<td>---</td>
<td>53 ± 4%</td>
<td>---</td>
</tr>
<tr>
<td>30' at pH 8.2, 30' at pH 7.1</td>
<td>---</td>
<td>17 ± 4%</td>
<td>---</td>
</tr>
</tbody>
</table>
Effect of Drugs Altering Cytosolic Calcium

Drugs which are believed to raise cytosolic free calcium levels, such as quinidine (Fuchs et al., 1968) and ionophores such as ionomycin, are known to inhibit the hydromotic response to vasopressin (Taylor et al., 1987). The effects of these drugs on responses to NEM and VP was compared.

When bladders were preincubated for 30 minutes with 0.3mM quinidine sulphate in the serosal bath, the response to NEM was inhibited by $82 \pm 6\%$ ($n=5$, $p<0.001$). This dose of quinidine inhibited the response to vasopressin by $69 \pm 7\%$ ($n=6$, $p<0.001$). Quinidine (1mM) also inhibited the response when applied 20 minutes after NEM or VP; as illustrated in Figure 21 it inhibited the NEM response by $35 \pm 6\%$ ($n=4, p<0.05$), and the response to VP by $49 \pm 6\%$ ($n=3$, $p<0.02$), calculated as the mean reduction in water flow over 40 minutes exposure. Note that quinidine actually causes a diminution in water flow when applied after the onset of the response.

Serosal ionomycin (2μM) had a different effect on NEM-induced water flow, as can be seen in Figure 22. Ionomycin slowed the onset of the response to NEM, but did not significantly reduce the maximal rate of water flow. Ionomycin has been found to slow the response to VP similarly, and also to reduce the peak flow (Taylor & Barber, unpublished observations).

Effect of Metabolic blockade: Iodoacetate

Iodoacetate has been shown to inhibit the hydromotic response to vasopressin or cAMP, probably by blocking glycolysis (Handler et al., 1966). To investigate the metabolic dependence of the response to NEM, experiments were carried out in which bladders were preincubated for 30 minutes with 1mM iodoacetate on both surfaces of the tissue, and then treated with 0.1mM NEM. The results are shown in Figure 23. Water flow in the iodoacetate-treated bladders was inhibited by $69 \pm 2\%$ ($n=4$, $p<0.001$). Thus the NEM response appears to show a marked metabolic dependence.
Figure 21a. 1mM Quinidine markedly inhibits an established VP response.

Figure 21b. 1mM Quinidine also inhibits an established NEM response. Note that water flow across bladders actually falls following application of quinidine.
Figure 22. Ionomycin delays the increase in water flow induced by NEM, but has no significant effect on the final maximum flow.

Figure 23. Iodoacetate causes a profound and prolonged inhibition of NEM-induced water flow.
Effect of Drugs Affecting the Cytoskeleton

In order to investigate whether the cytoskeleton plays a part in the response to NEM, experiments were carried out with (a) cytochalasins B and D, which prevent elongation of actin microfilaments, and hence disrupt the microfilament network (Brown & Spudich, 1979), (b) nocodazole, which binds specifically to tubulin (DeBrabandier et al., 1981) and causes depolymerisation of microtubules, and (c) EHNA (erythro-9-(3-(2-hydroxypropyl) adenine), an ATP analog that is known to inhibit dynein (Bouchard et al., 1981), the ATPase that drives (microtubule-based) ciliary movement.

Cytochalasins B and D

One hemibladder of each pair was preincubated with cytochalasin for 20 minutes, after which both were stimulated with NEM. The inhibition was calculated using the mean water flow over the first 30 minutes after stimulation with 0.1mM NEM. Cytochalasin B (20|μM) produced an inhibition of 22 ± 5% (n=5, p<0.02), while cytochalasin D (1|μM) reduced the response by 28 ± 7% (n=6, P<0.02), as shown in figure 24. In both cases, the inhibition during the final 30 minutes of the experiment was smaller. Under these conditions, neither CB nor CD caused a significant increase in the osmolality of the mucosal fluid.

In order to determine the effect of CD on the maintenance phase of the NEM response, 1|μM CD was applied to the serosal surface of the tissue 20 minutes after stimulation with 0.1mM mucosal NEM. There was no significant difference in water flow across CD-treated and control hemibladders (n=6, p>0.6) (figure 25a). Because this result was at odds with reports of the effect of CB on the maintenance phase of the VP response (Kachadorian et al., 1979b; Pearl & Taylor, 1983), similar experiments were performed to determine the effect of CD on the maintenance of the VP response. The results are illustrated in figure 25b. These experiments also showed no significant difference between CD-treated and control hemibladders (n=6, p>0.2).
Effect of Cytochalasin B on NEM-Induced Water Flow

Figure 24a. Cytochalasin B causes a moderate inhibition of the response to NEM.

Effect of Cytochalasin D on NEM-Induced Water Flow

Figure 24b. Cytochalasin D produces a very similar pattern to that seen with CB.
Figure 25a. Cytochalasin D, applied 20 minutes after NEM, had no effect on the development of the response.

Figure 25b. Cytochalasin D also had no effect on the response to VP when applied 20 minutes after stimulation.
**Nocodazole**

After a 30 minute preincubation with 33µM nocodazole, the response of hemibladders to NEM stimulation was reduced by 50 ± 5% (n=6, p<0.001) over the first 30 minutes relative to paired controls not treated with nocodazole. The inhibition was lower during the second 30 minutes of the experiment. As shown in Figure 26, both groups reached the highest levels of water flow at 40 minutes, but the control bladders showed a greater subsequent decline.

**EHNA**

As shown in Figure 27, the pattern of inhibition of NEM-induced water flow caused by 60 minutes preincubation with 1mM EHNA was very similar to that seen with nocodazole: over the first 30 minutes after exposure to NEM water flow was inhibited by 43 ± 10% (n=6, p<0.02). Maximum water flow in these experiments occurred at 30 minutes, after which the EHNA treated bladders showed almost constant flow, while there was a marked decline in the flow across the control bladders.

**Effect of Cationised Ferritin**

The presence of cationised ferritin in the mucosal bathing solution has been reported to inhibit the hydrosomotic response to VP, and to increase endocytosis (Beauwens et al.,1986). Experiments were carried out to confirm this, and to determine if the response to NEM was similarly affected. The results are illustrated in figure 28. In a series of 5 experiments, cationised ferritin was found to inhibit VP-induced water flow (calculated over the first 30 minutes after hormonal stimulation) by 27 ± 8% (p<0.05) relative to controls containing native ferritin. In 5 experiments in which bladders were stimulated with serosal NEM (to avoid any direct interaction of the reagents), water flow was inhibited by 52 ± 9% (p<0.01) over the first 30 minutes of stimulation.
Figure 26. Nocodazole appears both to slow the onset of the response to NEM, and also to reduce the maximum flow seen.

Figure 27. EHNA also reduces both the rate and magnitude of the hydrosomotic response to NEM.
Figure 28a. Cationised ferritin both slows the onset of the response to VP, and reduces the peak flow reached.

Figure 28b. The effect of cationised ferritin on the response to NEM is very similar to, although even more marked than, the effect on VP.
Additivity of the Responses to Vasopressin and NEM

To test the additivity of the hydrosmotic responses to VP and NEM, 0.1 mM NEM was applied to the mucosal surface of the tissue simultaneously with the addition of VP to the serosal bath. VP was used at two concentrations: a maximal or near maximal dose of 20mU/ml, and 2mU/ml, which was a submaximal dose, but still produced a substantial increase in water flow. In these experiments, the controls received VP, but no NEM. The responses produced are illustrated in Figure 29. In a series of 7 experiments, NEM had no significant effect on the response to a maximal dose of vasopressin. However, it did cause a significant increase in water flow over the first 30 minutes when used in conjunction with a submaximal dose (2mU/ml) of vasopressin. Under these conditions NEM enhanced the response to VP by 26 ± 8% (n=8, p<0.05).

Summary

These results demonstrate that drugs that disrupt the cytoskeleton or metabolism within the cells inhibit the increase in transepithelial water flow induced by NEM, as do agents that raise the cytosolic free calcium concentration. Lowering the serosal pH also markedly inhibited the development of the response. The effects of the various agents on NEM-induced water flow closely mirror their effects on the hydrosmotic response to VP. The induction of water flow by VP and NEM is additive only when a sub-maximal dose of VP is used.
Figure 29a. When a maximal dose of VP is used, the addition of 0.1mM mucosal NEM has no effect on the water flow induced.

Figure 29b. When a submaximal dose of VP is used, addition of 0.1mM mucosal NEM causes a significant increase in transepithelial water flow.
ULTRASTRUCTURAL STUDIES USING THIN-SECTION ELECTRON MICROSCOPY

In order to investigate the effects of NEM on the morphology of the epithelium and its constituent cells, studies were carried out in which hemibladders were treated with 0.1mM mucosal NEM for either 20 or 40 minutes, and then fixed, embedded, and sectioned for electron microscopy as described in the Methods chapter. Paired control hemibladders, untreated with NEM, were processed for microscopy at the same time.

The appearance of the unstimulated epithelium is shown in figures 30 and 31. The low power micrographs in figure 30 show the full thickness of the tissue: the epithelium together with its underlying connective tissue, which contains smooth muscle and bundles of collagen. Figure 30b also shows a small blood vessel. All the epithelial cells in figure 30a are granular cells, but figure 30b shows a goblet cell and a mitochondria-rich (MR) cell lying next to each other. Figure 31a shows a higher power view of a granular cell. The tight junction with the adjacent cell, and the tightly interdigitated basolateral membranes, are well illustrated in this view. The microvilli, cut in cross-section, appear finger-like in most cases. Within the cytoplasm there are copious granules, and a number of mitochondria, set in a meshwork of microfilaments. There is also a multivesicular body: such structures are often seen in the apical cytoplasm of these cells, as are multilamellar bodies, such as that illustrated in figure 31b. In this figure, which shows the apical portion of another granular cell, the plane of section is at a more acute angle to the plane of the epithelium, and thus reveals the branching, ridgelike nature of the microvilli better than figure 31a.

Qualitative Assessment of Morphological Changes Induced by NEM

After 20 minutes treatment with NEM, the basic organisation of the epithelium remained intact, as shown in figure 32. The tight junctions between cells were normal in appearance, and the interspaces were dilated, consistent with the occurrence of transepithelial water flow.
Figure 30. Sections through unstimulated toad bladders. Micrograph (a) shows a section made up entirely of granular cells, backed by a serosa of collagen bundles. Micrograph (b) shows a goblet cell (arrow) and a MR cell (arrowhead) lying next to each other. A blood vessel can be seen running through the serosa. x 4,000.
Figure 31. Sections through unstimulated toad bladders. Micrograph (a) shows a higher power view of a granular cell (x 14,000). Note the tightly interdigitated junction between the cells (arrow), a multivesicular body (*), and the prominent granules under the apical plasma membrane. The section in (b) is oblique to the surface of the bladder, revealing the anastamosing, ridge like nature of the microvilli. There is a prominent multilamellar body (*) in the cytoplasm. x 43,000.
The most obvious change brought about by NEM was the appearance of vacuoles within the cytoplasm of most of the granular epithelial cells. These were large, multiple, membrane-limited structures, which could be found in the cytoplasm of all parts of the cell, but were often close to the apical surface. Examples of such vacuoles are shown in more detail in figure 33. Other cellular organelles, including the mitochondria, which are often regarded as a sensitive guide to the state of the cell, appeared to be unaffected by NEM treatment; however, the cytoplasm of the granular cells often appeared swollen. Microtubules and microfilaments both appeared to be unaffected following exposure to NEM. Occasionally, a granular cell would be seen which did not appear to have been affected in any way by the NEM: examples can be seen in figures 32 and 36.

The microvilli in figure 33, but not those in figure 32, appear rather distorted. Such an appearance was not uncommon, but was by no means universal. It is not easy from these views to determine whether the microvilli have been transformed from a ridgelike to a fingerlike form by the NEM treatment or not. However, figure 34a shows a fortuitous section which grazes through the top of a granular cell, and reveals that the microvilli remain in a pattern of anastamosing ridges. Figure 34b consists of a stereo pair of views of a thicker section, taken at higher power. These also suggest that the microvilli are in the form of ridges. These findings imply that NEM does not cause the microvillar transformation seen after VP stimulation.

After 40 minutes treatment with NEM, the epithelium looked very similar, except that the vacuoles in the granular cells had become enlarged (figure 35a), and had apparently started to fuse together (figure 35b). The granular cells seen in these figures are markedly swollen, possibly due to the formation of the large vacuoles; however the cytoplasm, and in particular the intracellular organelles, have an essentially normal appearance.

The other cell types in the epithelium appeared essentially normal after NEM treatment: a MR cell can be seen in figure 35a, which shows little change from its unstimulated state, apart from some flattening of the microvilli. Similarly, goblet cells showed no obvious changes. Basal cells appeared to show increased pinocytotic activity: small saccular membrane structures surrounded by blebs, having the appearance of rosettes, were often seen in the cytoplasm of the basal cells. An example is shown in figure 37. Similar structures were sometimes seen in granular cells.
Figure 32. Section through a bladder stimulated for 20 minutes with 0.1 mM NEM. Note that the tight junctions (arrows) are intact, but the interdigitations of the basolateral membranes have opened up. There are vacuoles (*) in the cytoplasm of one granular cell, while the other seems unaffected. x 9,000.

Figure 33. Higher magnification view of membrane-limited vacuoles in a granular cell. The cytoplasm has been somewhat diluted, but mitochondria and granules retain their normal morphology. x 18,000.
Figure 34. Micrograph (a) shows a section passing obliquely through the apical part of a granular cell. The microvilli can be seen to form anastamosing ridges, even after 20 minutes treatment with NEM. x 14,000. Figure 5b consists of a stereo pair of micrographs which illustrate the ridgelike nature of the microvilli. x 42,000.
Figure 35. Sections through bladders treated with NEM for 40 minutes. Micrograph (a) shows that the vacuoles have grown larger than at 20 minutes, but morphology is otherwise intact. Note that the MR cell looks virtually unchanged by NEM treatment. x 10,000. Micrograph (b) shows a large vacuole that appears to consist of two fused vacuoles. x 8,000.
Figure 36. Even after 40 minutes stimulation with NEM, this granular cell appears unaffected, unlike its neighbours. x 8,000.

Figure 37. Saccular "rosettes" (arrowed) apparently derived from endoplasmic reticulum, were often seen in the cytoplasm of basal cells. x 33,000.
Membrane Fusion Profiles Induced by NEM

Careful examination of the apical plasma membrane of the granular cells showed the presence of membrane profiles which appeared to represent sites of fusion of intracellular vesicles with the plasma membrane. Such sites were relatively infrequent, and quantitation was not attempted. Profiles could be divided morphologically into two types. The first type consisted of long, narrow, parallel-sided structures, with approximately the dimensions reported for aggrephores (Muller et al., 1980); two examples of this are shown in the stereo pairs in figure 38. The second type, illustrated in the stereo pairs in figure 39, consisted of more classical omega-shaped figures. That the latter may reflect granule fusions is suggested by the occasional section showing such a fusion with electron-dense material issuing from it (Figure 40). Figure 41 shows an example of each type of fusion in close proximity.

Occasionally, particularly after 40 minutes treatment with NEM, bizarre saccular membrane structures could be seen in which the plasma membrane appeared to form large blebs protruding into the mucosal solution, as shown in figure 42a. These may be sites at which the vacuoles have fused with the plasma membrane and everted. However, similar structures were also (very rarely) seen in control bladders (Figure 42b), and have been noted before (Taylor, pers. comm.): it may be that they reflect normal turnover of the cell membrane.

Morphometric Analysis Of NEM-Induced Changes in Intracellular Components

In order to obtain a more detailed picture of the changes in intracellular components caused by NEM treatment, a quantitative study, using a point-counting stereological analysis was performed, as described in the methods chapter. Three treatment regimes were analysed: 20 minutes exposure to 0.1mM mucosal NEM, 40 minutes exposure, and untreated controls. Three hemibladders were used in each group, with approximately 20 micrographs from each hemibladder. A 13.5mm lattice was laid over the micrographs, and the structure underlying each
Figure 38. These two stereo pairs show tubular structures fused to the apical plasma membrane of granular cells. x 67,000.
Figure 39. These two stereo pairs show omega-shaped profiles at the apical plasma membrane of granular cells. x 45,000.
Figure 40. The contents of a granule can be seen escaping into the mucosal space of the bladder. x 79,000.

Figure 41. This micrograph shows a tubular (arrow) and an omega-shaped (*) fusion in close proximity. x 68,000.
Figure 42. Blebbing or exfoliation of the apical plasma membrane (a) was sometimes seen in NEM-treated bladders. × 29,000. Similar structures were also occasionally seen in control bladders (b). × 55,000.
lattice point, if it lay between the apical plasma membrane and the basement membrane, was allocated to one of seven categories, as shown in table 3. Counts from the micrographs for each bladder were summed, and the mean and standard errors for each category were calculated using the results from the three hemibladders that had undergone each experimental procedure. These data are listed in table 3, and presented graphically in figure 43.

These results show that vacuoles, defined as membrane-limited spaces larger than 250nm, increased from about 2% of the epithelial volume to 13% at 20 minutes, and 18% after 40 minutes treatment with NEM. This was almost exactly matched by a 15% decline in the area occupied by cytoplasm from 62.1% to 50% at 20 minutes, and 47.5% after 40 minutes. The only other category to show any consistent change following NEM treatment was the area of the interspaces between the cells, which increased from 1.1% to 2.6% of the epithelial area, consistent with the occurrence of transepithelial water flow; however, this difference was not statistically significant. If the data are recalculated excluding all points within vacuoles (which appear to be swelling the epithelium), the data shown in table 4 and figure 44 are obtained. After 40 minutes treatment with NEM the increase in the interspaces is approximately 3 fold: this is now significant at the 2% level. There is also a small, statistically insignificant, increase in the volume of the endoplasmic reticulum category (which also includes small lucent vesicles, etc.).

Studies Using Horseradish Peroxidase

When the toad bladder is stimulated with vasopressin, there is a marked increase in endocytosis at the apical surface of the granular cells (Masur et al. 1971). In an attempt to determine whether NEM produced a similar effect, horseradish peroxidase (HRP) was used as a fluid phase marker. The method was a modification of that of Masur et al. (Pers. Comm.), as described in the Methods chapter.

After 20 minutes stimulation with NEM, HRP-labelled profiles were occasionally seen within the cytoplasm, but were rare (data not shown). After 40 minutes stimulation there were considerably more cytoplasmic profiles labelled with HRP. Examples are shown in figure 45.
Table 3. Percentage of epithelium occupied by each category following NEM stimulation.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Controls</th>
<th>20' NEM</th>
<th>40' NEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>S.E.</td>
<td>mean</td>
</tr>
<tr>
<td>Vacuoles</td>
<td>2.11</td>
<td>0.81</td>
<td>13.09</td>
</tr>
<tr>
<td>Nucleus</td>
<td>18.28</td>
<td>3.13</td>
<td>15.83</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>62.1</td>
<td>2.62</td>
<td>50.62</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>3.31</td>
<td>0.49</td>
<td>4.91</td>
</tr>
<tr>
<td>Granules</td>
<td>1.56</td>
<td>0.69</td>
<td>2.62</td>
</tr>
<tr>
<td>Interspaces</td>
<td>1.12</td>
<td>0.28</td>
<td>1.74</td>
</tr>
<tr>
<td>E.R. etc.</td>
<td>11.49</td>
<td>0.72</td>
<td>11.15</td>
</tr>
</tbody>
</table>

Table 4. Percentages excluding vacuoles.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Controls</th>
<th>20' NEM</th>
<th>40' NEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>S.E.</td>
<td>mean</td>
</tr>
<tr>
<td>Nucleus</td>
<td>18.63</td>
<td>3.07</td>
<td>18.19</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>63.49</td>
<td>3.21</td>
<td>58.31</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>3.39</td>
<td>0.51</td>
<td>5.64</td>
</tr>
<tr>
<td>Granules</td>
<td>1.59</td>
<td>0.69</td>
<td>3.04</td>
</tr>
<tr>
<td>Interspaces</td>
<td>1.14</td>
<td>0.28</td>
<td>1.99</td>
</tr>
<tr>
<td>E.R. etc.</td>
<td>11.73</td>
<td>0.76</td>
<td>12.80</td>
</tr>
</tbody>
</table>
Figure 43. Percentage of epithelial area occupied by each category, in control bladders, and after 20 or 40 minutes of NEM stimulation.
Figure 44. Percentage of epithelial area excluding the vacuoles.
Both tubular and spherical profiles were seen, and these often occurred in clusters. It is noteworthy that HRP was not seen within the large vacuoles. Although formal quantitation was not carried out, there appeared to be substantially fewer HRP-labelled profiles after 40 minutes stimulation with NEM than after an equal period of stimulation with VP.

Epithelial folds containing trapped HRP revealed the presence of membrane fusion events, as illustrated in figure 46, which shows both a tubular and a circular profile. It is essentially impossible to decide from such views whether such profiles represent exocytic or endocytic activity.

HRP-labelled profiles were also seen in the cytoplasm of mitochondria-rich cells, as shown in figure 47. These did not appear to be caused by NEM stimulation, as they were also seen in unstimulated controls (not shown).

**Summary**

The results presented here demonstrate that 0.1 mM NEM causes a number of changes, including the appearance of vacuoles within the granular cells, and the opening of the intercellular spaces. These changes are similar to those seen after VP stimulation. However, NEM does not cause significant disruption of overall epithelial morphology.

Significantly, stimulation of the tissue with NEM leads to the appearance of fusion profiles at the apical plasma membrane. These may be of tubular or spherical forms, presumed to reflect the fusion of aggrephores and granules, respectively. NEM also induces uptake of HRP, indicating that it (directly or indirectly) stimulates endocytosis.
Figure 45. Following NEM stimulation in the presence of mucosal HRP, both tubular and spherical HRP-labelled profiles were seen in the cytoplasm of granular cells. These sections are unstained. x 54,000-59,000.
Figure 46. HRP trapped in a fold in the epithelium, outlining the surface. Here, both tubular (*) and omega-shaped (+) profiles can be seen. x 54,000.

Figure 47. HRP labelling in an MR cell. Similar appearances were seen in control and NEM-treated bladders. x 44,000.
FREEZE-FRACTURE ELECTRON MICROSCOPY

As described in the introduction, the only way to visualise the membrane particle aggregates believed to mediate the increase in water permeability of the apical plasma membrane caused by vasopressin is by freeze-fracture electron microscopy. This technique was applied to tissues that had been exposed to mucosal NEM, to see whether NEM induces the appearance of particle aggregates in the apical plasma membrane. In combined functional and morphological studies, water flow was monitored using the volumetric method of Bourguet & Jard (1964) before the tissue was fixed for fracturing, as described in the Methods chapter.

Particle Aggregates are present after exposure to NEM

In unstimulated control bladders, aggregates were not seen in fractures of the mucosal surface (25 cells from 3 experiments). Figure 48 shows the appearance of the P-fracture face of the apical membrane of a granular cell from an unstimulated tissue. The surface appears flat and smooth, with occasional microvilli. There are randomly scattered membrane particles, but no clusters or aggregations of such particles. This micrograph also shows an intercellular junction.

When the fracture plane passed through the sub-apical cytoplasm of the granular cells, a large number of membrane-limited structures were visualised. Amongst these, aggregate-containing tubular vesicles were sometimes found, containing the characteristic banded pattern of particle aggregates, as shown in figure 49.

When NEM was applied to the mucosal surface for 20 (n=2) or 40 (n=4) minutes before the cells were fixed, particle aggregates were seen on the P fracture face. The aggregates were often in clusters, as shown in figure 50a, and often associated with membrane fusion events (figure 50b). These aggregates were morphologically indistinguishable from those which have been demonstrated after vasopressin stimulation (Chevalier et al., 1974, Kachadorian et al., 1975). Figure 51 displays a peculiar patch of membrane which is virtually bare of particles apart from the presence of some aggregates. This appearance was seen on only one occasion, and its significance is not clear.
Figure 48. This micrograph shows the p fracture-face of the apical plasma membrane of an unstimulated granular cell. An intercellular junction runs across the bottom left. No aggregates are present. x 31,000.

Figure 49. The fracture plane passes through the cytoplasm, revealing 3 aggrephore p faces (*), and 2 e faces (+). x 52,000.
Figure 50. Following stimulation with NEM, particle aggregates could be seen in the p fracture face. These often occurred in clusters (a, arrow) (x 61,000), and were often associated with fusion sites (b, *). x 90,000.
On the E fracture face, the aggregates leave a pattern of parallel grooves (Kachadorian et al., 1975), as seen in figure 52. Occasionally, careful examination of both the P and E fracture faces would reveal complementary views. Two such examples are presented in figure 53.

**Relationship Between Transepithelial Water Flow and Aggregate Area**

The strongest evidence for the role of the aggregates as the sites of water movement across the apical plasma membrane after vasopressin stimulation is the close correlation between the rate of water flow, and the percentage of the apical plasma membrane occupied by particle aggregates (Kachadorian et al., 1975;1977b). To investigate whether a similar relation holds for the aggregates that appear in response to NEM, the correlation between water flow at the time of fixation and the percentage of the apical plasma membrane occupied by aggregates was calculated for 11 experiments in which tissues were stimulated for 20 or 40 minutes with 0.1 mM NEM before fixation. A graph of these results is shown in figure 54. There is a strong correlation ($r=0.94$, $p<0.01$) between these values.

In two experiments in which 0.1 mM NEM was applied to the mucosal surface for 20 minutes before fixation, a mean peak water flow of $9.5 \pm 3.5$ ml/min. was recorded. In a sample of 15 cells taken from the two experiments, aggregates were found to occupy $0.07 \pm 0.04$ % of the apical surface area. After 40 minutes exposure to NEM, the mean peak water flow in a series of four experiments was $19.25 \pm 4.1$ ml/min. This was associated with aggregates which occupied $0.19 \pm 0.08$ % of the apical surface area, in 38 cells drawn from the four experimental bladders.

Of a total of 70 cells measured from NEM-treated bladders, 19 had aggregates on them. The incidence in individual experiments varied from 18 to 77 % of the cells. There was some tendency for bladders showing large numbers of aggregates to have a high percentage of active cells, but this correlation was not statistically significant with the limited number of experiments available. It is also important to note that not all of the surface of a cell could be seen, so it is possible that there could have been aggregates on a part of the cell that had not been photographed. This is made more likely by the fact that the aggregates occur in clusters, with large areas of the surface of even highly active cells being clear.
Figure 51. This micrograph shows an area of plasma membrane virtually devoid of particles apart from a cluster of aggregates. This appearance was only seen once. x 63,000.

Figure 52. This micrograph shows the fracture face of the apical plasma membrane of a granular cell. Arrays of parallel grooves can be seen. x 106,000.
Figure 53. Micrographs (a) and (c) show p fracture faces containing particle aggregates, while (b) and (d) show the complementary e faces, with arrays of parallel grooves matching the aggregates seen on the p face. x 76,000, x 87,000.
Figure 54. Correlation between water flow and the percentage of the surface area covered by aggregates. There are three points at the origin, giving a total $n$ of 11. The correlation $r=0.94$, $p<0.01$. 

Correlation Between Water Flow and Aggregate Area
The highest percentage of the granular cell apical plasma membrane occupied by aggregates was 0.38%. This is about 1/3 1/2 of the values reported for vasopressin (Kachadorian et al., 1977a, 1977b), which correlates well with the relative peak water flows of the two responses, as described above.

**Effect of NEM in the Absence of an Osmotic Gradient**

In a single experiment, 0.1 mM NEM was applied to the mucosal surface of the bladder for 40 minutes, in the absence of a transepithelial osmotic gradient. After fixation, freeze-fracture showed the presence of aggregates, which covered 0.17% of the apical surface in a series of 13 cells. This value is very close to that reported above for the series of experiments in which NEM was applied for 40 minutes in the presence of an osmotic gradient. This suggests that the appearance of the aggregates is not an artifact of water flow through the tissue.

**Effect of Removal of NEM**

In the light of the functional irreversibility of the response to NEM, experiments were carried out in which 0.1 mM NEM was applied to the mucosal surface of the bladder for twenty minutes, and then washed out. Water flow was followed for a further twenty minutes before the tissues were fixed. In two experiments, the mean peak water flow prior to fixation was 12.5 ± 3.5 ml/min., and aggregates occupied 0.19 ± 0.03% of the apical surface area in 17 measured cells. Neither of these values was significantly different from those obtained when NEM was present continuously throughout a forty minute period.

**Effect of NEM at pH 6.5**

The functional experiments described above demonstrated that the onset of the response to NEM was inhibited at low pH. In an attempt to clarify further whether this was due to inhibition of aggregate insertion or aggregate function, two experiments were carried out in which
bladders were exposed to 0.1mM NEM at pH 6.5, and then fixed. In these experiments, transepithelial water flow did not rise above the basal level. Freeze-fracture showed a few aggregates in only one of the experimental tissues: the mean aggregate area was 0.02 ± 0.02 % of the granular cell apical plasma membrane area. This value was not significantly different from that of the controls, in which no NEM was applied. This suggests that low pH can inhibit the response to NEM at some stage prior to the insertion of the aggregates. This does not exclude an additional effect of pH at a later stage.

Summary

Exposure to 0.1mM mucosal NEM induces the appearance of particle aggregates in the P fracture face of the apical plasma membrane of the granular cells. Such aggregates are morphologically indistinguishable from those seen after VP stimulation, and similarly often occur in clusters, and in association with membrane fusion profiles. The area of these aggregates is closely correlated with the observed rate of transepithelial water flow induced by NEM.

The appearance of aggregates on the plasma membrane was not affected by the absence of an osmotic gradient, or by the removal of NEM after 20 minutes stimulation. However, virtually no aggregates were seen if the serosal bath was maintained at pH 6.5 during NEM stimulation.
DISCUSSION

The results presented above demonstrate that NEM induces an increase in transepithelial water flow, but inhibits net sodium transport, across the toad urinary bladder. The increase in water flow induced by NEM is accompanied by the appearance of particle aggregates in the apical plasma membrane, as seen by freeze fracture electron microscopy; fusion profiles can also be demonstrated in thin sections. These findings are consistent with the view that the increase in water flow is brought about via cellular mechanisms also activated following stimulation of the bladder by vasopressin. This discussion will seek to elucidate what can be determined about the mode and site of action of NEM in this tissue by comparing the effects of NEM and VP. Possible intracellular signals mediating the increase in water permeability will be discussed, as will the effects of NEM on exocytosis and endocytosis. Finally, there will be a discussion of how the findings reported here may contribute to our understanding of the natural hormonal response.

As a background to this discussion, it is useful to consider the actions of NEM that have been reported in other tissues, as this may cast light on its action in the toad urinary bladder.

**Possible Modes of Action of NEM: Evidence from Other Systems**

N-Ethyl maleimide (figure 55) has generally been regarded as reacting specifically with sulphydryl (SH) groups (Webb, 1966; Trudinger, 1969), although other reactions have been reported at very high concentrations (Smyth et al., 1960). This specificity of action, combined with its good penetration into cells, makes NEM useful in studies of the role of SH groups in cellular function (Webb, 1966). However, although some SH groups in cellular proteins react rapidly, others are resistant to reaction with NEM (Webb, 1966). Thus there is some selectivity of action, particularly at low concentrations (Webb, 1966).
Figure 55. N-Ethylmaleimide. Reaction with sulphydryl reagents occurs by fission of the double bond in the ring.
Effect of NEM on Membrane Transport Processes

NEM has been shown to have effects on a number of ion transport processes. The first of these to be demonstrated was inhibition of Na\(^+\)/K\(^+\) transport. The Na\(^+\)/K\(^+\) ATPase was shown to have three NEM binding sites, which reacted at different rates (Skou, 1963). In addition to an inhibition of Na\(^+\)/K\(^+\) exchange, NEM also unmasks the ability of the pump to act as a transphosphorylation enzyme (Fahn et al., 1966). More recently, NEM has been shown to inhibit non-mitochondrial proton pumps, including those in chromaffin granules (Cidon & Nelson, 1986), and coated vesicles and lysosomes (Ohkuma et al., 1982; Stone et al., 1983). In contrast, NEM stimulates KCl cotransport across the erythrocyte membrane (Lauf, 1983; Logue et al., 1983) and Ehrlich ascites tumour cells (Kramhoft et al., 1986). NEM has also been shown to cause release of calcium from sarcoplasmic reticulum vesicles, probably by opening specific channels (Bindoli & Fleischer, 1983).

In addition to its effects on ion transporters, NEM has been shown to inhibit the transport of glucose into erythrocytes (Dawson & Widdas, 1963), and catecholamines into storage granules (Bashford et al., 1976). Carlin & Hechter (1962) examined the effect of NEM on the action of insulin, another SH-containing hormone, in the rat diaphragm. They found a variable degree of inhibition of the actions of the hormone, but 0.5mM NEM produced changes in xylose distribution similar to those seen after insulin stimulation. Carter and Martin (1969) found that NEM had a biphasic effect on glucose handling by rat adipocytes: while 50uM NEM inhibited both basal and insulin-stimulated transport, 20uM NEM had an insulin-like stimulatory effect. NEM has also been shown to inhibit a GTPase linked to adenosine receptors in rat brain (Fredholm et al., 1985): as discussed above, a receptor/G protein complex mediates the VP response in the toad bladder.
Disruption of Intracellular Motility

The acto-myosin system (Karlsson & Lindberg, 1985), ciliary dynein, and some microtubule-associated transport ATPases such as kinesin and cytoplasmic dyneins (Porter et al., 1987; Paschal & Vallee, 1987) can be inhibited by NEM. Kinesin is much more resistant to the effects of NEM than are dynein-like proteins such as MAP 1c (Paschal & Vallee, 1987). The ATPase believed to be responsible for slow axonal contraction and microtubule-associated gelation-contraction can also be inhibited by NEM (Gao & Weisenberg, 1988).

Effects on Cellular Membranes

Belkin & Hardy (1961) reported that NEM could cause the formation of blebs in the plasma membrane of cultured cells. Later studies showed that this was an energy- and ion-dependent process, possibly caused by increased permeability of the plasma membrane to water (Scott, 1976; Scott & Maercklein, 1979). Such blebs, when detached, seemed to contain little except plasma membrane, and a small amount of soluble cytosolic material. Jacob & Jandl (1962) showed that NEM could destabilise the membrane of erythrocytes, measured as an increased rate of haemolysis. In a more detailed study, Chasis & Mobandas (1986) showed that low doses of NEM (0.1 mM) stabilised the membrane for a period, before destabilising it.

Kaplan et al. (1985) found that NEM, at doses around 1 mM, could induce the fusion of receptor-containing vesicles with the plasma membrane of rabbit macrophages. In contrast, Jackson et al. (1985) found that 5 mM NEM could prevent calcium-induced exocytosis in sea urchin oocytes, while lower doses raised the calcium level required to trigger exocytosis. Another block of the exocytotic pathway was found by Weideman et al. (1989), who showed that NEM binds to a fusion protein involved in Golgi activity, preventing fusion of transport vesicles with the Golgi lamellae, and hence movement of proteins through the Golgi apparatus. This protein has since been shown also to have a role in the cellular management of endocytosed membrane (Diaz et al., 1989). Thus NEM can apparently affect both the plasma membrane and the flow of membrane through the organelles of the cell.
Effects of NEM in the Toad Urinary Bladder

Is NEM Acting as a Sulphydryl Reagent?

In order to determine whether NEM is acting as a SH reagent in the toad bladder, experiments were carried out using maleimide and succinimide. Maleimide, which shares the ability of NEM to participate in reactions with SH groups, was found to induce an increase in transepithelial water flow. On the other hand, succinimide, which lacks the double bond that reacts with SH groups, had no effect on water flow. An unrelated SH reagent, the arsenical compound phenylarsine oxide, failed to induce an increase in water permeability. However, another unrelated SH reagent, methyl mercuric bromide, has been reported to increase water permeability of the toad bladder (Rasmussen et al., 1960).

Taken together, these results suggest that the increase in transepithelial water flow observed after NEM stimulation is likely to be due to a specific reaction, or reactions, with NEM acting as a SH reagent.

Comparison of the Effects of NEM and VP

The increase in water permeability seen after stimulation of the bladder with mucosal 0.1 mM NEM occurs somewhat more slowly, and reaches a lower maximum, than that seen after VP stimulation. When the toad bladder is stimulated with VP, hormone-receptor binding causes adenylate cyclase activation, which provides a rapid amplification of the signal and presumably very efficient activation of subsequent stages. Initiation of the response to NEM appears to occur at some stage after this cascade, as there is no increase in intracellular cAMP levels, and this in itself is probably sufficient to explain the relatively slow onset of the response to NEM.

The rate of water flow observed after stimulation with 0.1 mM NEM generally rises to reach a plateau at about 1/3 to 1/2 of the peak flow seen after VP stimulation. However, it is close to the level typically reached after the peak and decline in water flow normally seen with the hormone,
as illustrated in figure 7. This VP-induced peak may represent an "overshoot" phenomenon, as could occur if water flow itself led to an inhibition of water flow. The slower onset of the response to NEM may give time for opposing processes to reach equilibrium, avoiding such an overshoot. A peak and decline is sometimes seen when there has been a rapid onset of water flow in response to NEM, as illustrated in figure 8, supporting this supposition.

In contrast to its effect on water permeability, NEM differs fundamentally from VP in its effect on transepithelial sodium transport. Mucosal NEM (0.1mM) causes a substantial inhibition of both basal and VP-stimulated SCC. This implies that NEM is neither producing non-specific pathways across the epithelium, nor simply acting at the VP receptor sites as a partial agonist. The inhibition of the SCC by NEM may reflect a direct effect of NEM on the sodium channels at the apical plasma membrane: this would be consistent with the finding of a proportional inhibition of the VP-induced increase in SCC. Alternatively, NEM may be inhibiting the Na/K pump in the basolateral membrane, as described in other tissues (Skou, 1963).

**Dose-dependence**

The biphasic dose-dependence of the response to NEM may reflect two opposing actions of NEM on water movement, one stimulatory and one inhibitory. Previous studies have shown that 1mM NEM, applied to the serosal surface of the tissue can produce a profound inhibition of the response to vasopressin (Rasmussen et al., 1960; Bentley, 1973). The nature of this inhibition is not yet clear, but presumably at 1mM, an inhibitory effect of NEM is starting to outweigh its stimulatory effect. As discussed above, at this dose NEM is known to interfere with cytoskeletal function and membrane flow in other systems: either or both of these effects could inhibit the induction of water flow.

**Effect of Inhibitors**

In order to determine what cellular systems are involved in the response to NEM, experiments were performed under conditions known to inhibit VP-induced water flow.
**Effect of low serosal pH**

At a serosal bath pH of 6.5, the response to both mucosal and serosal NEM, like that to serosal VP, was profoundly inhibited. Bladders stimulated with NEM at low pH (6.5), and then moved to baths at pH 8.2 showed a response closely comparable in time course to bladders which had had no preincubation with NEM at low pH, implying that the low pH is preventing the initiation of the response to NEM rather than just keeping the channels closed once they have been added to the surface (in which case a prompt increase in water flow would have been seen as soon as the pH was changed). Because the response to NEM is not mediated by cAMP, this block must be distal to the effects of adenylate cyclase.

Lowering the pH of the serosal bath after increased water flow had become established caused a substantial decline in water flow across both VP- and NEM-stimulated bladders. Such inhibition of the response to VP is not surprising: low pH inhibits hormone-receptor binding (Gulyassy & Edelman, 1965), and adenylate cyclase activity (Bochaert et al., 1972), as well as causing closure of the channels (Bourguet et al., 1981), and leading to removal of the aggregates from the apical plasma membrane, even in the continued presence of exogenous cAMP (Parisi & Bourguet, 1984). The inhibition of the response to NEM may reflect reduced insertion of new water channels into the plasma membrane (as implied by the results discussed above in connection with the onset of the response), closure of the channels, increased removal of channels, or some combination of the above. Preliminary freeze-fracture studies performed in an attempt to determine the number of aggregates remaining on the plasma membrane after a reduction in pH unfortunately produced ambiguous results. Further functional and freeze-fracture studies will be necessary to determine the site(s) at which the response to NEM is being inhibited by low pH.

**Effect of drugs altering cytosolic calcium**

Drugs which raise cytosolic free calcium levels are known to inhibit the response to VP. Quinidine, which may prevent sequestration of calcium in the endoplasmic reticulum (Fuchs et
al., 1968), was found to inhibit both the onset and the maintenance of the responses to VP and to NEM by a similar degree. Ionomycin, a calcium ionophore, appeared to slow the onset of the response to NEM, without significantly reducing the maximum water flow observed. The reason for the differences between the effects of these two drugs is not clear. It may be that ionomycin produces a smaller increase in cytosolic calcium concentration, or it may be that quinidine has actions in the toad bladder other than those due to the rise in calcium. However, these results imply that a raised cytosolic calcium concentration can inhibit the response to NEM, although further work would be needed to determine the mechanism of this inhibition. Raised cytosolic calcium levels may disrupt the cytoskeleton, or may modify enzymatic activity via a calmodulin-like calcium-binding protein.

**Effect of metabolic inhibition**

The hydrososmotic response to VP can be inhibited by metabolic blockade (Handler et al., 1966). Iodoacetate, which inhibits the VP response by blocking glycolysis (Handler et al., 1966), inhibited the response to NEM severely, suggesting that induction of water flow by NEM is an energy-requiring process. This finding implies that the increase in water flow is unlikely to be a non-specific phenomenon, or due merely to a fusogenic effect of NEM causing the passive release of aggregates from aggrephores onto the apical plasma membrane.

**Effect of drugs affecting the cytoskeleton**

As discussed in the introduction, both microtubules and microfilaments appear to play a role in the hydrososmotic response to VP. Experiments with nocodazole, a rapidly acting microtubule disruptive drug (DeBrabandier et al., 1981), and EHNA, an ATP analogue which is known to block the activity of several microtubule-associated transport proteins (Bouchard et al., 1981; Koonce et al., 1987), were performed to determine whether microtubules are involved in the response to NEM. Both agents produced a substantial inhibition of the onset of the response, and maximal water flow was significantly reduced. Interpretation of the effects of the inhibitors
in the later period of the response is difficult to interpret, as control hemibladders in this series of experiments showed a large, rapid response to NEM, with a subsequent decline in water flow that brought their flow back towards the level measured in the drug-treated hemibladders. The results are consistent with the involvement of microtubules in the initiation of the response, but are equivocal about their involvement in its maintenance. This is closely comparable with the results obtained in experiments on the VP response, as discussed in the introduction.

As discussed above, cytochalasin B (CB), which disrupts microfilaments, inhibited both development and maintenance of the VP response by 30-50% (Pearl & Taylor, 1983; Kachadorian et al., 1979b). Preincubation with dihydrocytochalasin B, the action of which is more specific for microfilament disruption, produced a similar, but slightly smaller inhibition (Pearl & Taylor, 1983). Preliminary results with cytochalasin D (CD), a related compound highly specific for its action on microfilaments (Schliwa, 1982) suggested that this had a still smaller inhibitory effect (Pearl, unpublished results).

Experiments were carried out to determine the effects of both CB and CD on the response to mucosal NEM. Preincubation with either CB or CD produced a small inhibition of the response: this was comparable to the inhibition of the VP response by CD. The calculated inhibition reflects both a small decrease in the maximum flow obtained, and a slowing of the onset of the response. This is consistent with involvement of microfilaments in the development of the response.

When CD was applied after 20 minutes stimulation with NEM, no effect was seen. Because this is not consistent with the results reported above for the effect of CB on the response to VP, experiments were carried out in which CD was applied 20 minutes after VP stimulation: these also showed no effect of CD. These results imply either that microfilaments are not involved in the maintenance of the response to either VP or NEM, or that CD does not disrupt microfilaments as much as CB does. Previous results, using CB, may reflect an action of this less specific agent unrelated to its ability to disrupt microfilaments. This may also underlie the larger inhibition of the onset of the VP response seen with CB.
Additivity

Experiments in which NEM and VP were applied simultaneously to opposite faces of the bladder showed that NEM could cause an increase in flow (relative to controls stimulated only with VP) when a submaximal (2mU/ml), but not a maximal (20mU/ml), dose of VP was used. The flow induced by even 2mU/ml VP alone was greater than that normally seen after NEM stimulation. These results strongly suggest that NEM induces water flow via a specific pathway, at least part of which is also used in induction of water flow by VP: when the pathway is saturated by VP, NEM can cause no further effect, but when there is spare capacity, NEM can make a contribution, and cause increased water flow.

Effects of NEM on Tissue Morphology

Electron microscopic studies were performed to determine the effect of NEM on tissue architecture. At a dose of 0.1mM, NEM produced very little disruption of the overall epithelial organisation. There were, however, changes in the granular cells comparable to those seen after VP stimulation in the presence of a transepithelial osmotic gradient, consistent with the passage of water through the cells. These included the opening of the interspaces between the epithelial cells and the appearance of prominent membrane-limited vacuoles within the cytoplasm. Similar vacuoles have been reported after VP stimulation, but those seen in the studies reported here probably represent an exaggerated form, possibly because NEM induces them to fuse together. The significance of these vacuoles will be discussed further below.

The appearance of fusion profiles at the apical plasma membrane is a part of the responses to both VP and NEM. In both cases two populations of profiles can be observed: rounded omega-shaped figures, which probably represent granule fusions, as evidenced by the occasional visualisation of electron-dense contents issuing from such fusion profiles, and parallel-sided figures believed to represent the fusion of aggrephores with the plasma membrane. As described in the introduction, the fusion of aggrephores with the plasma membrane is a late stage in the development of the hydrososmotic response to VP, and the finding of
such profiles after NEM stimulation would be expected if NEM is inducing water flow via the same mechanism as VP.

The appearance in the apical plasma membrane after NEM treatment of particle aggregates morphologically indistinguishable from those seen after VP stimulation is strong evidence for coincidence of at least the final stage of the mechanisms by which they induce water flow. As with VP, the area of aggregates observed after NEM stimulation was closely correlated with water flow, and the ratio was similar to that seen following VP stimulation. This provides further support for the view that the aggregates are closely associated with water flow, and may represent the sites of water channels.

The studies using HRP suggest that there is an increase in endocytosis following NEM treatment, just as there is after stimulation with VP. Both parallel-sided and circular profiles were seen after NEM, suggesting that aggrephores were being retrieved, and that there were also other endocytic processes occurring. The frequent appearance of HRP-labelled profiles in clusters suggests that endocytosis may occur at active sites in the plasma membrane, rather than randomly all over it. Although not formally quantitated, there appeared to be fewer HRP profiles after NEM- than after VP-stimulation of the tissue. This may reflect the lower water flow induced by NEM if endocytosis is stimulated by transepithelial water flow, or it may be that NEM is partially inhibiting the induction of endocytosis. Alternatively, VP may induce endocytosis by some more direct means. It would be desirable in the future to perform double label experiments using HRP and ruthenium red to exclude the remote possibility that all the profiles observed were still surface connected.

The evidence presented in chapter 6 suggests that NEM does not induce a transformation of the granular cell microvilli from a ridge-like to finger-like form, unlike VP. The significance of the microvillus transformation observed during the VP response is not clear; neither is its mechanism. However, it is clear from the results with NEM that transformation is not an essential part, or consequence, of the induction of water flow.

The occasional appearance of membrane blebs at the apical surface after NEM stimulation is rarely paralleled in the VP response. The shedding of such blebs may explain the protein release into the mucosal fluid observed after NEM treatment. Such blebs may reflect fusogenic effects of NEM on membranes, or disturbance of the normal cytoskeletal support for the membrane.
Formation of the blebs may be an exaggeration of a normal process of tissue shedding, or may be due to a toxic effect of NEM. Loss of substantial amounts of aggregate-bearing membrane would reduce the water permeability of the apical plasma membrane: this may be one cause of the reduced water flow observed with high doses of NEM. This effect of NEM may, by providing a relatively pure apical plasma membrane preparation, represent a possible way of isolating water channels in the future.

Investigation of Possible Intracellular Signals

It is known that the response to VP is mediated by a rise in intracellular cAMP concentration. However, the results reported here show that NEM does not cause a similar rise, and hence the response to NEM cannot be mediated by cAMP. There is similarly no evidence that a change in pH is important in the response to NEM. The possible role of cytosolic calcium is more problematic: as discussed above, interpretation of the results obtained is virtually impossible, but there does not appear to be any significant change in the period immediately following stimulation with NEM. The inhibitory effects of drugs that raise the cytosolic calcium concentration on the hydrosmotic responses to both VP and NEM make it unlikely that a rise in cytosolic calcium ion mediates the response to NEM. Indeed, it is possible that an NEM-induced rise in calcium levels could be one mechanism explaining both the inhibitory effects of high doses of NEM on the response to VP, and the biphasic dose-dependence of NEM-induced water flow.

Where is NEM acting?

The results presented above place limits on the site(s) at which NEM may be acting to produce an increase in transepithelial water flow. The similarities of the effects of inhibitors and the changes of morphology seen after stimulation with VP or NEM, together with the additivity of their action when a submaximal, but not a maximal, dose of VP is used, are consistent with the view that NEM is activating at least part of the cellular apparatus utilised in the hormonal response. Because NEM does not cause a rise in intracellular cAMP concentration, it must be acting at a
site distal to adenylate cyclase. The presence of fusion profiles at the apical plasma membrane, and the appearance of particle aggregates on it, suggest that by this stage the pathways are common.

Drugs which disrupt the cytoskeleton inhibit the increase in water flow induced by NEM, suggesting that the response is dependent on cytoskeletal action, implying a site of action at, or before, the involvement of such elements in the VP response. The involvement of cytoskeleton-mediated transport processes in the response to NEM is also consistent with the metabolic dependence of the response. However, metabolic dependence would also be expected if NEM acted by stimulating a protein kinase, as occurs in the VP response. This is perhaps a more likely site of action, as it could provide a more coordinated activation of the cellular apparatus than would be expected if NEM acted, for example, by stimulating cytoskeleton-mediated transport proteins. Simple activation of cytoskeletal transport is unlikely to be the whole answer, as it would be unlikely to provoke fusion of the aggrephores with the plasma membrane, even if it made more aggrephores available. However, NEM may also play a direct fusogenic role which would overcome this problem.

**Irreversibility**

Removal of vasopressin leads to a rapid return to the low basal water permeability of the toad bladder. In contrast, removal of NEM seems to have little effect. Three theories may be considered to account for this irreversibility. These are:

1) Fixation of the aggregates in the apical membrane: either by actually fixing them to the membrane, or by making their retrieval impossible, for example by making them too rigid to be taken up into vesicles.

2) Inhibition of their recovery: either a general block of endocytosis, or a block of a specific retrieval system: for example, by blocking localisation of aggregates into coated pits for recovery. If their endocytosis became a hit and miss affair recovery would be greatly slowed: they only occupy a very small part of the apical plasma membrane, so most endocytosis would be unproductive. Indeed they might be actively excluded from coated pits. One mechanism that may be important in recovery is the cytoskeleton; microfilaments have been implicated in the offset of
the response (Masters & Fanestil, 1979).

3) NEM might cause an irreversible activation of the mechanisms leading to insertion, for example by binding irreversibly to an enzyme such as a protein kinase. If this were the case, it would be acting as a fixative of the response, rather than the aggregates. Retrieval of the aggregates, and their recycling if it happens, would continue, but removal of the agonist would not lead to cessation of insertion.

Experiments in which NEM has been used as a fixative of the VP response have tended to depend on theory 1. However, short exposures to NEM can lead to the development of a response that continues to increase even after removal of the NEM, as described above. This is not really consistent with theories 1 or 2, although it cannot exclude them as additional mechanisms. On the other hand, Bentley (1973) found that if NEM was applied for 5 min (0.1mM, serosal), and then washed out before the addition of VP, the response to the hormone developed normally, but the decline on washout was markedly reduced (but not eliminated). This treatment did not increase basal water flow (Bentley, 1973). This might suggest an effect on the retrieval mechanism, supporting theory 2, as it could not be fixing the cellular apparatus in the "on" state induced by VP. However, the experiments reported above suggest that even this exposure might have been sufficient to cause some activation of water flow, which might be sufficient to maintain the response once it had been initiated by VP.

In order to investigate this further, experiments were performed using cationised ferritin. Application of cationised ferritin (CF) to the mucosal surface of the bladder inhibited the hydrosmotic response to VP, probably by increasing endocytosis, and hence removing some of the aggregates from the plasma membrane (Beauwens et al., 1986). If NEM was fixing aggregates in the plasma membrane or blocking endocytosis, CF would not be expected to produce any inhibition of NEM-induced water flow. However, CF produced an even larger inhibition of the response to NEM than that to VP. This may reflect the slower onset of the NEM response: the same rate of removal of aggregates by CF-mediated endocytosis would remove a greater proportion of those whose insertion had been caused by NEM. These experiments suggest that NEM does not cause irrevocable fixation of the aggregates in the membrane, or substantially impair their recovery. The morphological studies with HRP also appear to contradict any block of endocytosis. These results are all consistent with theory 3: that
NEM irreversibly activates the insertion mechanism; however, insertion can be inhibited by low pH, cytoskeletal disruption, and a raised cytosolic calcium concentration.

**How can Studies With NEM Help Our Understanding of VP Action?**

If we conclude that NEM induces transepithelial water flow by activating part of the cellular apparatus that is involved in the hydrosmotic response to VP, then we can use the results of NEM experiments, particularly when they differ from equivalent experiments with VP, to further our understanding of the action of the hormone in this tissue.

The principal difference between the functional responses to NEM and VP is the irreversibility of the response to NEM. As discussed above, this is probably due to irreversible activation of some part of the pathway, between adenylate cyclase and the involvement of microtubules. This simplifies interpretation of experiments involving changes of pH: because of the multitude of its effects on hormonal action, it is difficult to decide at what sites it is acting. However, the ability of low pH to delay the onset of the response to NEM implies that there must be a block of the activation pathway that lies distal to the site of action of NEM, and prevents insertion of the channels into the plasma membrane. It is interesting that in this context the relative slowness of the response to NEM is an advantage: the initiation of the response is much slower than the change of state of the channels observed by Parisi & Bourguet (1984). The interpretation of the effects of low pH on an established NEM response remains uncertain: the decline in water flow may reflect the inactivation of channels, or their removal from the surface (with continued insertion prevented by the block deduced above). A more detailed freeze-fracture study will be needed to resolve this issue, by determining the number of aggregates present in the membrane when water flow has declined.

Morphological studies threw up a number of questions. The most notable change after NEM treatment was the appearance of large membrane-limited vacuoles. Similar vacuoles are also seen after stimulation with VP, but are generally smaller. The studies with NEM have shown that these vacuoles can lie in all parts of the cell, including the apical cytoplasm, above the cell nuclei. This makes it unlikely that they are merely invaginations of the basolateral membrane, as has been suggested by Strange & Spring (1987). On the other hand, studies with HRP have shown that
these vacuoles do not become filled with HRP, implying that they are not formed by fusion of endocytic vesicles from the apical plasma membrane. Morphometric analysis of the cellular compartments showed no decline in the volume of the endoplasmic reticulum and other small vesicular structures (the "ER" fraction) within the cells, implying that there is not wholesale fusion of these elements to form the vacuoles. The origin of the vacuoles therefore remains unclear. The membrane of which they are made may come from the basolateral membrane (which could be retrieved endocytically after the interdigitations between cells had opened), as suggested by Kirk (1988). This would be consistent with the occasional finding of HRP in vacuoles within cells where HRP had got into the spaces around the basolateral surface. Alternatively, they may exist, in a collapsed form, in the unstimulated tissue. They would then have been counted as part of the "ER" fraction of unstimulated tissues during the morphometric study. Their loss from this compartment could have been concealed if the other "ER" elements also swell to some extent, as might be expected during periods of transcellular water flow, thus maintaining their percentage of epithelial volume.

The cause, and any possible role for such vacuoles remain unclear. They may result from a dramatic increase in membrane flow as water flow through the cell induces increased endocytosis, and this increase may overwhelm the cells' capacity to process and redirect the membrane: if NEM disrupts this processing it would result in an exaggeration of the vacuoles, as is observed. Once such vacuoles exist, they may provide a pathway through which water molecules can diffuse more freely than they can through the cytoplasm: thus the majority of water flow may actually pass through the vacuoles, rather than through the cytosol. Alternatively, it is possible that they play a role similar to the vacuoles in protozoans, which are actively involved in the control of intracellular osmolality: this could explain the remarkable preservation of the morphology of other organelles. However, there is no evidence at present for any such active element to their role.

Morphological studies also revealed that NEM did not cause transformation of the microvilli, demonstrating that this is not an essential part of the hydrosmotic response. The significance of the transformation is unclear; although it will increase the surface area of the cells, this will only be significant for water movement if more aggregates are inserted. It has been suggested that the transformation may be caused by exocytic events breaking up the ridges (Pearl, pers.
NEM probably induces fewer such fusions, and hence this effect may be less marked.

Morphologically, some granular cells showed no sign of having responded to NEM stimulation. Such heterogeneity of response has been reported in VP-stimulated tissues (Kachadorian et al., 1989). Such cells must have a defect in a late part of the pathway, rather than in the receptor system for VP.

The freeze-fracture studies demonstrated that NEM-induced water flow was correlated with the appearance of membrane particle aggregates, and that the ratio was close to that seen after VP stimulation. This is further confirmation of the association between aggregates and water flow.

**Where From Here?**

The precise site of action of NEM has not been defined. As it appears to act by irreversibly activating something, presumably by binding covalently to it, it may be possible to use labelled NEM to isolate the protein it is activating. The protein itself may be of interest, as will its role in the response. Such isolation will probably require the use of differences in binding at different NEM concentrations to determine which of the proteins to which it binds are likely to be significant in the induction of water flow. This will necessitate more detailed study of the dose-dependence of the response.

The cellular components involved in the induction of the hydrosmotic response are poorly understood distal to the production of cAMP, and studies with NEM may help elucidate this. By providing activation at a different site, it may prove very useful in studying the site at which inhibitors act, as exogenous cAMP has done in the past. This advantage will be greater when its precise site of action has been defined.

Further experiments in which the effects of pH on the response to NEM are examined, particularly in association with freeze-fracture studies, would be useful in determining the effects of pH in this system.

Further morphological studies will be needed to clarify the origin of the vacuoles, as discussed above. In particular, studies with serosal HRP might be helpful. Definitive studies of endocytosis also need to be carried out: this will necessitate the use of a double-labelling...
technique, for instance HRP plus ruthenium red, to determine which of the HRP-labelled profiles are truly intracellular. Such studies, including experiments without a transepithelial gradient, may be of value in determining whether endocytosis is driven by water flow, or directly by some part of the apparatus activated by NEM and VP.

NEM has advantages over VP as a means of stimulating the tissue in some experiments. On occasions, a slow onset of water flow is an asset, as is the ability to stimulate the tissue from either surface. As discussed above, NEM acts at a late step on the pathway, so the site at which inhibitors act can be more precisely defined. Finally, NEM produces an irreversible activation of the cellular mechanism involved in the insertion of the aggregates.

**Summary**

The results presented here are consistent with the view that NEM induces a specific increase in transepithelial osmotic water flow by activating the later part of the cellular apparatus stimulated by VP. This activation must occur distal to the generation of cAMP, but proximal to the involvement of microtubules and microfilaments. It is possible that NEM binds irreversibly to a protein kinase or similar catalytic protein, “fixing” it in an active state. Further experiments with NEM may prove valuable in understanding the action of the natural hormone, by providing an agonist that acts later in the pathway than others (such as cAMP) currently available.
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