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EFFECTS OF ETHANOL AND RELATED DRUGS ON THE PERMEABILITY OF AMPHIBIAN EPITHELIA

by

THOMAS YORIO

A dissertation submitted to the Graduate Faculty in Pharmacology of the School of Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1975

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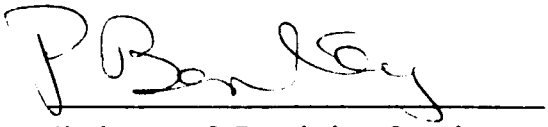
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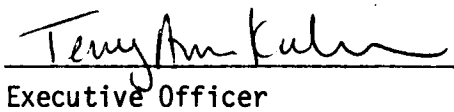
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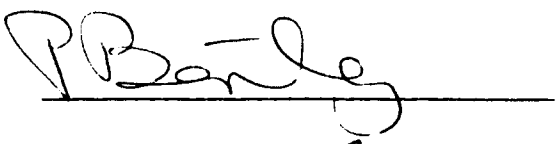
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ABSTRACT

EFFECTS OF ETHANOL AND RELATED DRUGS ON THE PERMEABILITY OF AMPHIBIAN EPITHELIA

by

Thomas Yorio

Adviser: Professor P.J. Bentley

The mechanisms by which ethanol affected the transmural movements of ions and water across three 'model' membrane systems were studied in vitro: using frog's skin, toad urinary bladder and toad lens.

a) Frog skin: Ethanol (3%) decreases the potential difference (p.d.) and short-circuit current (scc) across frog skin. The drug is most effective when present on the outside surface. Unidirectional fluxes of Na and Cl indicate that the drop in scc is due to an inhibition of the Na influx.

Ethanol had no effect on the electrical parameters or Na fluxes, when frog skin was bathed in Cl-free solutions on both sides or the outside alone. Furosemide and thiocyanate also blocked the ethanol response, suggesting anion dependency.

The vasopressin induced stimulation of the scc (natriferic response) and the inhibitory effect of ethanol were found to work independently of each other and different pathways of action are suggested for these drugs.

The intracellular ion content of the isolated epithelium increased

in the presence of ouabain, whereas ethanol had no effect. The O_2 consumption of the isolated frog skin was unaffected by up to 10% ethanol. Acetaldehyde, the metabolite of ethanol, had different effects on the p.d. and scc than that observed for ethanol. A general metabolic action is probably not mediating the ethanol response.

Urea and mannitol in iso-osmotic concentrations to ethanol, did not mimic its effect.

Tritiated-water (THO) fluxes (in the absence of an osmotic gradient) were reduced by 30% in the presence of 3% ethanol. It is suggested that ethanol may impede the flow of water across frog skin by a physicochemical interaction with membrane pores and water molecules. The permeability coefficient (K_{trans}) for ethanol was found to be relatively high as compared to non-permeant solutes; however it was 10-times less than that of water.

b) Toad bladder: Ethanol (9%) decreases the p.d. across the toad bladder when present at the mucosal surface; the scc was unchanged. The electrical resistance decreased indicating a change in ion movements across the bladder. Unidirectional Na and Cl flux measurements showed an increase in the movement of Cl, but no change in Na. The natriferic response to vasopressin was also unaffected by the presence of ethanol. It is suggested that ethanol may be altering the apical tight junctions and be affecting an anion selective pathway.

The hydro-osmotic responses of the toad bladder to vasopressin and cyclic-AMP (which mediates the physiological response), were both decreased

by 3% ethanol indicating an action subsequent to the endogenous formation of the nucleotide. THO fluxes (in the absence of an osmotic gradient) were reduced by 30% in the presence of 3% ethanol. The increase in diffusional water flow in response to vasopressin was similarly reduced. Osmotic water movement across vasopressin-stimulated bladders 'fixed' with glutaraldehyde or N-ethylmaleimide was also decreased by ethanol. However, 3% ethanol had no effect on osmotic water transfer across artificial collodion membranes. Ethanol, therefore, probably interacts with the bladder membrane rather than directly with the molecules of water. The K_{trans} of ethanol and water is increased by vasopressin suggesting that their movement is through similar pathways. It is suggested that ethanol impedes the flow of water across the toad bladder by facilitating a physicochemical interaction between a membrane 'pore' and the water molecules that are passing through it.

c) Toad lens: Hyperosmotic concentrations of NaCl, mannitol, urea and ethanol decreased the p.d. and scc across the amphibian lens in vitro. The solutes were only effective when placed on the anterior side of the lens, which is the site of the lens epithelium.

The p.d. across the anterior side of the lens was decreased by these solutes and the resistance across this side increased. A small decline in the p.d. was observed across the posterior side under these conditions. The decline in the translenticular p.d. and scc was substantially reduced, but not abolished, when Na was excluded from the anterior bathing solution. Absence of Na from the posterior solution also decreased the response.

When the anterior side of the lens was bathed with Na-free Ringer, mannitol produced a much larger increase in resistance than when Na was present.

The lens accumulated Na in the presence of mannitol and ethanol but there was no concomitant decline in K. The extracellular space was unchanged so that the Na was in the cells. The translenticular flux of ^{22}Na in the direction anterior to posterior was increased, while that from the posterior to anterior was not changed significantly.

Lens opacities were observed to occur when the lens was bathed on the posterior surface with mannitol but this was not associated with electrolyte changes.

The osmotic agents appear to influence the electrical activity of the lens by decreasing the activity of the Na-pump in the anterior lens epithelium and increasing its passive permeability to Na.

To Elena for patience, understanding, encouragement and love.

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Introduction:

Man is thought to have first tasted alcohol in the paleolithic era (see for instance Claudian, 1970) and the consumption of this primeval "eau-de-vie" has become a major social and medical problem. Approximately 70% of adult Americans drink and 40% of them are regular drinkers (Mulford, 1964). The main basis for this widespread consumption of ethanol, is its action on the central nervous system. However, ethanol effects not only neural, but hormonal and metabolic processes. Ethanol modifies each system and also alters the complex interactions between various physiological effector and regulatory systems. These processes are ultimately related to the properties of the individual cell types and those processes that are involved in maintaining the functioning of such cells, particularly the permeability of the membrane to solutes and water. The difficulties in disentangling this profusion of intertwined phenomena are further complicated by the diversity of actions that ethanol exhibits on tissues both *in vivo* and *in vitro* particularly in the differences in the range of concentrations used.

Ethanol is classified as a general depressant. Among other compounds in this category are the higher alcohols, the volatile general anesthetics, and the inert gases. As these compounds have been widely investigated, their chemical and physical properties are well known. One property shared by these compounds is that of being somewhat lipid-soluble. The early classical studies of Overton (1901) and Meyer and Hemmi (1935) show that the potencies of the different general depressants *in vivo* correlate

well with their ability to dissolve in a lipid phase. These authors proposed that equal degrees of narcosis are obtained when equal concentrations of the general depressants are present in the lipid phase of the biological system. It has also been proposed that equal degrees of narcosis occur when an equal volume fraction of the cell membrane is occupied by the depressants (Mullins, 1954). Several studies have shown that there is a good correlation between the narcotic activity of the general depressants (which include ethanol and other alcohols) and the free energy of absorption of these compounds to a lipid phase (Fergunson, 1939; Brink and Posternak, 1948; Schneider, 1968).

Proteins have a lipophilic core in which ethanol could also be solubilized, thus changing the molecular characteristics of the protein. In fact, some studies have suggested that there is a good correlation between anesthetic properties and the ability of the depressants to bind to proteins and to alter their molecular structure (Balasubramanian and Wetlaufer, 1966; Schoenborn and Featherstone, 1967; Schoenborn, 1968).

Another property shared by most general depressants, including ethanol, is their ability to increase the stability of microcrystals of hydrates so as to permit them to be formed at temperatures close to physiological temperatures (Miller, 1961; Pauling, 1961). Pauling (1961) suggests that molecules such as ethanol may become part of the hydrogen-bonding framework of lattice structures in cellular water, where they might fill spaces in a crystal framework and thereby have a stabilizing action of Van der Waals-type. This interaction with water molecules imposes a state of rigidity that interferes with normal cell function.

Pauling's hypothesis can be linked with the membrane theory. Miller (1961, 1968) has independently proposed an analogous hypothesis which also assumes a stabilization of a water lattice around proteins and and surface structures. It is interesting that exactly opposite conclusions with respect to the interaction of ethanol with water molecules has been derived from another hypothesis that is based on Ling's theories (1962) concerning the physical state of living matter. According to his theories, narcosis would depend on disordering the protein-stabilized water lattice structure of the cytoplasm, rather than a further increase in stabilization (Fenichel and Herowitz, 1963).

Among attempts to obtain support for the Pauling-Miller hypothesis is a study conducted by Ludmer and Sabelli (1968) which involved experiments with ethanol. It was found that when ordinary water medium was replaced with heavy water containing deuterium, the number of toad sciatic nerves blocked by ethanol at given concentrations was reduced. However, this effect may have resulted from the lower molecular motility distinguishing D_2O from H_2O (Wallgren, 1970) and thus this phenomenon may be related to the antagonistic effect of cooling on ethanol's action. Therefore no clear evidence was obtained for or against the Pauling-Miller hypothesis. There is some evidence which supports the interaction of alcohols with non-polar membrane constituents rather than via hydrogen-bonding in the water lattice. Schneider (1968) calculated the apparent free energies of the binding of methylene groups of alcohols to membrane sites in anesthetic systems. The results suggest that the alcohols were interacting with non-polar groups. However, their experiments did not distinguish between interaction with

lipids or with non-polar moieties of membrane proteins. Schneider (1968) suggests that if proteins are involved they may be subjected to conformational changes when the alcohol is bound. Kwant and co-workers (1969) also obtained evidence that alcohols are bound to hydrophobic sites.

These actions of alcohol on polar and non-polar constituents of membranes may play a prominent role in the mechanism of action of ethanol in the in vivo situation. In addition, as the nature of biological membranes is important to our understanding the functioning of cells and the processes that maintain them, such as water and solute transfer, ethanol may serve as a useful 'pharmacological probe' in helping elucidate the mechanisms of action of these processes.

The fundamental unit of living tissue is the cell. In recent years it has become plain that one cellular component, the membrane, plays a crucial role in almost all cellular activity. Cytoplasmic membrane, the outer envelope surrounding the cell, acts to regulate the internal environment of the cell and to transport substances into and out of it. For many years, theories about biological membranes have been dominated by the concept of the unit membrane and the Davson and Danielli model (1952). According to this concept the membrane consists of a double layer of lipids covered by protein molecules. The lipids are oriented such that the hydrophilic groupings face the exterior combining with the associated protein molecules. Other models have since been proposed which include a mosaic type of structure with a micellar arrangement of lipid components alternating with lipoprotein-protein molecules, and with high molecular polysaccharides in some membrane types. However, the presence of various lipids is still

considered very important. Interaction with lipids thus remains a possible mechanism of ethanol's action in addition to any effects on polar constituents of membrane proteins.

The ability of ethanol to cross the plasma membrane of cells and alter their permeability and excitability is well known. According to Knutsson (1961) von Humboldt in 1797 found that the excitability of frog-nerve muscle preparations was increased by low concentrations of ethanol. Nineteenth century studies demonstrating such changes in excitability include those by Biedermann (1881) and Efron (1885) (cited acc. to Larabee and Posternak, 1952) on motor nerve fibers, and by Blumenthal (1896) on frog muscle. Although ethanol's actions on the central nervous system has been appreciated for several millenia, its mechanism of action on tissues remains obscure. It is, indeed, uncertain whether its ability to alter cell permeability reflects its pharmacological actions in the body, especially since the concentrations that are used in vitro usually far exceed those that are consistent with the survival of an intact animal. It is, however, interesting that many tissues in vitro can withstand exposure to high concentrations of ethanol. Under such conditions the permeability of cells has been shown to be altered. It has thus been found that in frog motor nerves ethanol, in concentrations from 0.1% to 5%, lowers the excitability threshold to direct electrical stimulation (Lucas, 1913; Klensch, 1949). Concentrations of 5 to 15% ethanol transiently increase excitability and then progressively decrease it (Lucas, 1913; Handovosky and Zacharias, 1924; Klensch, 1949). However, these high concentrations, although insulting to the tissue, do

not cause irreversible damage. Thus, the nerve-block induced by a 15% ethanol solution is rapidly reversed by washing with Ringer's solution (Klensch, 1949). Concentrations above 15% rapidly block conduction but even this effect is reversible at concentrations as high as 25 and 40% (Danilewsky and Perichanjanz, 1925; Klensch, 1949).

As the in vivo physiological concentration for survival in man is below 0.5%, these ranges of concentrations of ethanol mentioned above represent in vitro pharmacological doses. However, each tissue appears to have different tolerance levels for ethanol toxicity. It is possible, that under in vivo conditions in man, that some tissues, for instance brain cells, may have a lower tolerance level to ethanol and may thus play a determining role as to the minimum concentration threshold for survival.

Recently, the mechanism of ethanol's actions have been attributed to metabolic effects, such as altering the activity of essential enzyme systems. It has been suggested, that alcohol may affect active transport processes in many tissues by an action of the membrane-bound enzyme Na^+ and K^+ activated ATPase (Israel et al., 1965; Israel et al., 1971; Hegyvary, 1973) and this may reflect the altered electrical behavior that has been observed in nerve and muscle as well as epithelial membranes. The Na^+ - K^+ activated ATPase was first described by Skou (1957). It has properties to be expected of a system actively moving cations across cellular membranes and has been used extensively as a "model" system for biochemical studies on ion transport (Skou, 1965; Albers, 1967; Schwartz, Lindenmayer and Allen, 1975).

Jarnefelt (1961) showed that ethanol inhibits brain Na^+-K^+- ATPase *in vitro*. A number of workers have suggested that interaction of the drug with the enzyme *in vivo* may be partially responsible for the effects of chronic ethanol administration. Israel and co-workers (1965) found partial inhibition by non-lethal concentrations of ethanol in preparations from rat and guinea-pig brain and from eel electroplaque tissue. They found that the effect of ethanol was competitive with potassium, but not with sodium. With rats *in vivo*, injection of potassium chloride counteracted the intoxicating effect of ethanol (Israel-Jacard and Kalant, 1965).

Israel -Jacard and Kalant (1965) also studied re-accumulation of potassium in slices of guinea-pig cerebral cortex depleted of potassium in the preparation phase. The incubation temperature was 37°C and the initial concentration of ethanol was 0.4%. Some ethanol must have been lost during incubation since a gas mixture was bubbled through the incubation medium. This relatively low concentration of ethanol caused approximately 50% inhibition of potassium re-accumulation. In conditions similar to those described by Israel *et al* (1965), Wallgren (1970), was unable to detect an effect of 0.4% ethanol on potassium re-accumulation. In none of the studies just cited was the passive efflux of potassium in the depletion period affected by ethanol. The results of Israel *et al* (1965, 1966) suggest that active cation transport may be a means by way ethanol acts *in vivo*. However, there are some problems to reconcile if ethanol acts in inhibiting active cation transport by an action on the

ATPase. Ethanol has no effect on unstimulated cerebral tissues and in addition ethanol effects ion transport only in selected tissues. Kalant and Israel (1967) in order to explain this response have introduced a concept of a safety factor, which implies that the transport system might become sensitive to inhibiting agents only when it is utilized close to maximal capacity. Such an assumption is compatible with the greater sensitivity of electrically stimulated than of unstimulated tissue that has been found with the administration of ethanol. These authors also suggest that although gross analysis of tissue electrolyte content shows little effect of ethanol, inhibition of active transport might still be important in alcohol-induced depression of nerve function, because only a minute fraction of total sodium might be involved in the very rapid and local ion movements in nerve stimulation. This hypothesis is however difficult to reconcile with the finding that a large proportion of the cerebral potassium and sodium are involved in the response to electrical stimulation (Keesey and Wallgren, 1965; Keesey et al., 1965). Some other observations give further evidence against inhibition of active transport as an important contributor to the depressant action of ethanol. Akera et al (1973) found that despite the development of tolerance to the depressant effects of ethanol on behavior, neither the Na^+-K^+ ATPase activity nor the ^3H-ouabain binding in brain homogenates or microsomal fractions were altered during treatment or withdrawal periods. With cat brain, Knox et al (1972) showed that chronic treatment with ethanol induced small but significant increases on Na^+- K^+-ATPase activity in the

frontal cortex, association cortex and the hippocampus whereas other regions were unaffected. Knutsson and Katz (1967) have also demonstrated increased sodium permeability in muscle fibers exposed to ethanol. Bittar (1966) has reported small and variable effects of ethanol on sodium transport of muscle fibers of crab. Normally electrical stimulation of cerebral cortex slices causes net loss of potassium and an increase in intracellular sodium, evidently as a result of neural activation (Keesey et al., 1965). This response to stimulation has been shown to be blocked by ethanol (Wallgren et al., 1974). Interestingly, the effect is larger on net accumulation of sodium than on potassium loss. In addition, Carmichael and Israel (1975) have also demonstrated a similar effect of ethanol on ion conductance rather than on active transport. If we regard the net changes as the gross sum of a great number of individual action potentials, the results fit in with the findings reviewed above concerning the effect of ethanol on changes in ionic conductances constituting the excitation cycle of isolated axons. These observations support the theories which attribute the depressant action of ethanol to a primary effect on nerve cell membranes rather than the theories which attribute the effect of ethanol to an inhibition of enzyme and metabolic systems. It therefore appears unlikely that if alterations in ion transport occur in vivo as a result of chronic administration of ethanol that it is due to a specific effect on $Na^+ - K^+$ activated ATPase.

As stated earlier, ethanol can interact with the membrane components

of cells by a direct physicochemical interaction. Ethanol molecules, by their tendency to assume particular orientation at lipid-water interfaces (Franks and Ives, 1966), may influence charge distribution and interfere with conformational changes essential for normal membrane functioning.

Progress in our knowledge about the effects of ethanol will depend on the progress in knowledge about membrane structure and function in the living, intact cell. As the physical and chemical properties of ethanol are well known, it can serve as a useful pharmacological tool for such basic investigations on the functionings of epithelial membranes.

EPITHELIAL MEMBRANES

Our understanding of the control of water and ion movements across mammalian epithelia stems from the early observations of Claude Bernard (1859), who recognized that cells were bathed by a specific liquid, 'le milieu interieur', and that its function was to maintain a relatively constant environment within which cellular function can take place. Knowledge into the control of cellular regulation in the mammalian system have been achieved mainly through the investigations of homologous and analogous regulations of other species. The control of water and ion movements in mammals is regulated by the processes of absorption and excretion. This complex system of regulation is under the influence of steroidal hormones (e.g. aldosterone) and the neurohypophysial hormones (e.g. vasopressin). In mammals this regulation is

specifically localized in the kidney, however, in lower vertebrates like the amphibians, the control is more general and operates over the entire surface of the body. At the cellular level however, the operations remain the same.

In order that it may survive, the cell must absorb 'energy-giving materials', substances such as glucose and oxygen, and it must give up products of metabolism, such as carbon dioxide. As indicated earlier, the control over these and other exchanges between the cell and its environment, such as solutes and water, is exercised by the plasma membrane and is controlled by various hormones. The integrity of the epithelial membrane is therefore of major importance for these and other exchanges between the cell and its environment to occur. The study of the mechanisms of transport of solutes and water across epithelial membranes and the processes that may alter their permeability, such as hormones and drugs, will add to our basic understanding of the physiological regulation of cellular function.

As is often the case, the understanding of the more primitive systems can help towards a better understanding of the more highly evolved. It is with this expectation that the present investigation has been directed to the study of the permeability properties of three 'model' membrane systems: the amphibian's skin, bladder and ocular membrane (toad's lens). These epithelial membranes were investigated as to the factors that may modify their permeability with a specific emphasis on the mechanism by which ethanol alters their permeability to solutes and water. As ethanol

has well established chemical and physical properties it may serve as a useful tool in unraveling the complicated mechanisms whereby solutes and water are transferred across these epithelial membranes, as well as offering a possible mechanism of action for its *in vivo* depressant effects.

Amphibian epithelia function in many respects like epithelia from other vertebrates, including man. They show a natural diversity in their roles and functions, and so provide several models for studying the numerous properties and mechanisms of water and ion transport and permeability, along with the changes in these functions that are initiated by the action of hormones and drugs. The morphological simplicity of amphibian skin and urinary bladders, and the ability of these and other membranes, including the toad's lens, to survive for extended periods *in vitro* offer many advantages over the use of mammalian epithelia, especially those that are situated in relatively obscured positions like the kidney. Such tissues when bathed by suitable solutions can stay alive for many hours during which many of their functions are maintained and can be studied under rigidly controlled conditions. One of the most interesting properties of such epithelium is their ability to promote transmural movements (in either direction) of solutes such as Na^+, Cl^-, HCO_3^- and K^+ as well as certain divalent ions, energy substrates and water. These processes may be directly linked to the metabolism of the cell or related to the specific structural features of the membrane which gives it this property.

One method by which physiologists and pharmacologists study the

movements of ions across epithelial membranes is by studying the electrical behavior of the tissues. The amphibian tissues mentioned above offer an excellent system for studying the electrical properties across epithelia membranes.

FROG SKIN

Amphibians have the capacity to absorb sodium from their fresh-water surroundings across their skin. The concentration of sodium in the fresh-water is very low while that in the animals extracellular fluid is high, so that the sodium that is absorbed moves against a high gradient of concentration. When an electrode is placed under the skin and another in the water, a potential difference is measured across the skin, with the inside being positive. Thus the transport of sodium is not only occurring against a concentration gradient but also a potential gradient. In order for this process to occur an expenditure of energy is needed and the process is considered to be an active transport mechanism. The potential across the frog skin is conventionally studied by stretching a piece of isolated skin across a hole separating two lucite chambers. This method was first described by Ussing and Zerahn (1951). Their studies have left little doubt that the cause of the potential is the active transport of sodium. The mechanism by which this occurs is still not yet clear; however, it is generally believed that the sodium ion forms a complex within the skin (possibly a carrier) and, in this unionized form, is rapidly transferred to the inside of the skin and set free as

an ion. Unless a negative ion is transported equally as rapidly, or there is an exchange with a positive ion such as potassium on the other side, the inside of the skin will become positive. Thus the rapid transport of sodium ions causes the inside to become positive up to the point where the potential accelerates chloride ions sufficiently to provide an equal number of sodium and chloride ions passing across the skin in unit time. As the potential builds up to its steady-state value, negative ions will be accelerated more and more while the movement of sodium ions will be decelerated. Based on this, it is reasonable to assume that the potential across the frog skin has been generated by the active transport of sodium, while the movement of chloride and other anions might well be the passive consequence of the movement of sodium. Ussing and Zerahn (1951) devised a very ingenious method for studying this transport. They demonstrated that by applying a counter potential across the skin (in vitro) so as to reduce the spontaneous potential difference to zero, there would be no force driving chloride from one side of the membrane to the other (equal concentrations of solute on both sides of the membrane). The potential would presumably not alter the active transport of sodium, if it was being transported as an un-ionized complex, so that the positive charges brought to the inside of the frog skin by the sodium would be neutralized by electrons provided by this external battery. The current flowing through this external circuit, would be equivalent to the ions carried by the active transport mechanism. Ussing and Zerahn (1951) measured both: the amount of current

that should be passed through the skin to cancel the electrical potential, and the net flux of sodium measured as a difference between the influx and the outflux, using isotopic sodium. They demonstrated that under steady-state conditions there is a larger inward than outward flux of sodium, thus originating a net influx. In addition, the charge moved per unit time by this net flux of sodium was equal to the observed current flowing in the external circuit. In general, this short-circuiting technique has been applied to the study of many actively transporting systems, and useful information has been obtained regarding the actual ion or ions that are being actively transported.

The frog skin consists of a multicellular epithelial sheet, however trans-epithelial transport basically reflects those processes operating across individual cell membranes in this system. Koefoed-Johnsen and Ussing (1958) have proposed a 'two-membrane' hypothesis to explain this phenomena observed in frog skin. This model proposes that sodium first moves across a Na-selective barrier into the cytoplasm of the epithelial cells and then is actively transported across the inner barrier into the inside solution. This treats the entire epithelium as a single giant cell, with an outward and inward facing membrane, both possessing their own permeability and transport properties. The potential across the frog skin can then be theoretically defined in terms of a single layer of epithelial cells with their membranes fused laterally to give a seal preventing significant diffusion of ions between the cells. This can be seen

with the electron microscope as areas of tight junctions of the most apical cells of the epithelial sheet, known as the zona occludens, which would therefore restrict exchanges to a pathway across the cell (see for instance Ussing, Larsen and Erlij, 1974). This potential across the frog skin has been attributed to two diffusion potentials (Koefoed-Johnsen and Ussing, 1952), a sodium-potential governed by the high permeability of the outward facing membrane to sodium compared with that to anions such as chloride, and a potassium-potential due to a high permeability to potassium by comparison to anions. This system is stabilized by the active transport process. If this transport is brought about by the movement of one sodium ion in one direction linked to the movement of one potassium in the other direction, the pump itself would not contribute to the potential and is considered neutral. However, recent data suggests that the coupling ratio between sodium and potassium is not unity (Biber et al., 1972; Candia and Zadunaisky, 1972) and that the pump is electrogenic. However, since the function of the active transport system is merely to maintain the level of sodium at a low value within the cell then the magnitude of the potential across the frog skin would be independent of the active transport of sodium. As long as the concentration of sodium is maintained at this level, the potential would depend primarily on the permeability characteristics of the cell membranes, a high sodium permeability of the outward facing membrane and a high potassium permeability of the inward facing membrane. In fact, the transcellular transport of sodium across the skin is largely independent of the potential, so that

when different skins are compared, large variations in potential may be found but not of sodium transport. In addition, the potential across the skin can be increased by lowering the cell's permeability to anions, e.g. by treating the skin with copper (Lyon, 1974), or replacing the permeant anion in the outside bathing solution with a non-permeant anion (Huf, 1972; Cuthbert et al., 1969). Such increases in potential are not accompanied by parallel changes in the transport of sodium.

As stated earlier, the movement of chloride across frog skin is thought to involve a passive phenomena. Koefoed-Johnsen, Levi and Ussing (1952) established the so-called, 'independence relationship', to determine if an ion moves passively or actively. It states: if an ion is not being actively transported, its fluxes, defined as the number of equivalents crossing unit area of membrane in unit time, will be simply determined by the potential across it, and the concentrations on each side:

$$M_{in} / M_{out} = C_{in} / C_{out} \exp -FE/RT$$

This may also be written

$$M_{in} / M_{out} = \exp(E-E_s)F/RT$$

where E is the actual potential difference across the membrane and E_s is the equilibrium potential appropriate to the particular concentration inside and outside, i.e., $E_s = RT/zF \ln C_{in} / C_{out}$. By varying E, or the values of C_{in} and C_{out} experimentally, and measuring the flux-ratios with isotopes, it is possible to determine whether the migration of a

particular ion is passive or active. Koefoed-Johnsen and co-workers (1952) put this to an experimental test by measuring the potential difference across the isolated frog skin, together with the inward and outward fluxes of chloride (^{36}Cl). The calculated flux-ratios agreed well with those found, so that it could be concluded that the migration of chloride was, indeed, passive. An interesting point brought out by this work was the observation that the higher the potential difference (p.d.) across a given skin, the lower were the fluxes of chloride; as the authors pointed out, if the p.d. was really due to a primary active transport of Na^+, the Cl^- fluxes would represent a partial short-circuit of the p.d.. Thus a high chloride flux would mean that the tendency for the Na^+ and Cl^- ions to separate, on which the p.d. depends, was small, conversely a low chloride flux would lead to a larger separation and a higher p.d..

Recently, however, chloride transport in frog skin under certain conditions, has been found to deviate from this independence relationship. Zadunaisky, Candia and Chiarandini (1963), found an active transport of chloride in the South American frog Leptodactylus ocellatus, the direction of which was in the same direction as the active transfer of sodium ions. They astutely observed that the net fluxes of sodium were greater than the observed short-circuit current (scc) and upon measuring chloride fluxes found a net transport of chloride from outside the frog skin to inside. Jorgensen, Levi and Zerahn (1954) also observed that frogs which have been depleted of chloride actively uptake this anion when it is reinstituted

in the bathing media. Recently, Watlington and Jessee (1974, 1975) have demonstrated active chloride transport in Rana pipien skins bathed in low chloride solutions. Like the South American frog, the chloride transport was in the same direction as the active sodium pathway, and thus would reflect a lower scc than that measured with isotopic sodium.

Another interesting feature of the relationship between transport and the anions present in the solutions bathing the epithelium, is the fact that the rate of sodium transport is markedly dependent on the anion species in the inside bathing solution. The most striking effect found in the skin is that substitution of chloride by sulfate in isotonic solutions leads to a marked depression of transepithelial transport (Cuthbert, Painter and Prince, 1969; Ferreira, 1968; Huf, 1972).

Transepithelial transport of sodium can also be modified by a number of drugs and hormones. One of the most useful pharmacological agents in the study of ion transport across frog skin, as well as toad bladder, is the diuretic amiloride (see for instance Bentley, 1968). This diuretic probably acts on the outside surface of the epithelial membrane by blocking the entry of Na into the cell (Biber, 1971). Apart from exerting its effects from the outside surface only, its speed of action is extremely fast. Direct measurements of Na uptake at the outer border of the epithelium, by tracer methods, shows that this process is blocked when amiloride is added to the outside solution (Biber, 1971; Erlij and Smith, 1973; Moreno et al., 1973; Candia and Reinach, 1975). It has also been shown recently that after chelation

of calcium in the outside solution the inhibiting effects of amiloride on the scc are severely reduced or abolished (Cuthbert and Wong, 1972). An interesting possibility suggested by these findings is that amiloride may combine with calcium and form a ternary complex with the sodium transport sites in the epithelial membrane. This further implies that calcium may play an essential role in the transport of sodium across frog skin.

Another very interesting class of inhibitors of active transport are the cardiac glycosides, typified by ouabain. This agent inhibits the action of an enzyme intimately connected with the active transport of sodium and potassium, namely ATPase, the enzyme that converts adenosine triphosphate to adenosine diphosphate and water. There is more than one ATPase in the cell, however this one is characterized by the fact that it is only active if both Na^+ and K^+ are present in the medium; it is known as the Na-K activated ATPase. Ouabain is thought to inhibit the activity of the enzyme by binding to a phosphorylated complex prior to the potassium binding step and thereby prevents enzyme activation (see for instance Schwartz et al., 1975). This inhibition is reflected as an increase in intracellular sodium concentration and a decrease in potassium, as would be expected if the pump was to become inoperative (Biber, Cruz and Curran, 1972).

The pharmacological use of amiloride and ouabain, has helped in elucidating the mechanism of action of many other drugs on sodium transport processes across frog skin and other epithelial membranes, particularly as their effects are well documented and they both appear to affect different sites of this transport system.

TOAD URINARY BLADDER

In higher animals, such as man, the ability to control the water content of their bodies is an important process for the regulation of normal cellular function. This task is routinely maintained by the kidneys, in which the tubular epithelium of the nephron has the capability of reabsorbing solutes, such as sodium and chloride, without the concomitant reabsorption of water. In the absence of the neurohypophysial hormone vasopressin, under normal hydrated conditions, the epithelium of the ascending limb, distal segment and collecting tubule becomes relatively impermeable to water. This results in a dilute urine being excreted and thus rids the animal of excess water. During extreme periods of water deprivation, vasopressin is secreted from the neurohypophysis into the circulation and modifies the permeability properties of the epithelium in the distal portions of the nephron and water is reabsorbed, so that its content in the animal can be preserved.

Because of the technical problems in trying to work out the mechanisms whereby neurohypophysial hormones modify the permeability properties of renal tubular epithelium of the mammalian kidney, investigators have sought more accessible tissues. The toad's urinary bladder, Bufo marinus, has proven to be an invaluable tool for understanding the mechanisms of water transport and its regulation by vasopressin, as well as adding to our knowledge of other related membrane phenomena.

In the toad, the functions assigned in mammals to the epithelium of the distal portions of the nephron are served as well by the urinary

bladder. The functional properties of this tissue which are of interest to physiologists and pharmacologists are: a) Its ability, selectively and actively, to transport sodium from the urinary (mucosal) surface to the body fluid or serosal surface (Leaf, Anderson and Page, 1958). b) Its responsiveness to antidiuretic hormone (vasopressin) *in vitro*, which is characterized by a stimulation of sodium transport (the natriferic response) (Leaf *et al.*, 1958) and a marked increase in bulk transport of water from mucosal to serosal surfaces in the presence of a transepithelial osmotic gradient (the hydro-osmotic effect) (Bentley, 1958; Sawyer, 1960) as well as increasing the permeability to some non-electrolytes (Leaf and Hays, 1961; Pietras and Wright, 1975). c) Its responsiveness to aldosterone *in vitro*, which is characterized by an increase in trans-epithelial sodium transport (Crabbe, 1961; Porter and Edelman, 1964; Sharp and Leaf, 1964).

Anatomically the toad's urinary bladder is morphologically simpler than the frog's skin. Its fine structure has been described in a detailed microscopic study initially by Keller (1960) and then by Peachy and Rasmussen (1961) and Choi (1963). It consists of a mucosal layer of epithelial cells one layer thick, which can be separated into two major morphological cell types, 'mitochondria-rich' cells and 'granular' cells. These cells rest on a submucosa, which consists mainly of collagen fibers in a network of blood vessels with nerve and muscle bundles. Finally, the serosa consists of a simple layer of squamous cells. However, it is the mucosal cells on the apical side of the bladder that is believed to play a predominant role in transport across this epithelial tissue.

As the bladder is considered to be mainly a single layer of mucosal cells, it represents a simple system to study transmural movements of solutes and water.

The isolated toad bladder transports sodium actively from the luminal or mucosal side to the serosa or blood side. This transfer generates an electrical potential difference, with the serosa being positive. Leaf et al (1958) using the methods of Ussing and Zerahn (1951), examined the electrical properties of the toad bladder in vitro. By placing a piece of bladder (hemi-bladder) between two chambers containing Ringer's solution, the electrical properties across the toad bladder can be measured. Leaf et al (1958) demonstrated, as Ussing and Zerahn (1951) did for frog skin, that the net transport of sodium across the bladder, when measured with labeled sodium, was equivalent to the observed short-circuit current (scc). They also showed that both the scc and the net sodium transport is increased by the neurohypophysial hormone vasopressin. The scc is therefore a good indicator of sodium transport in the toad bladder, and has been used by a number of other investigators for this purpose (Bentley, 1960; Crabbe, 1961; Orloff and Handler, 1962; Green and Matty, 1963; Finn, 1968). It is generally believed that this increase in sodium transport induced by vasopressin results from a complex series of changes, which includes the activation of adenyl cyclase and the formation of cyclic-AMP (Orloff and Handler, 1967) and an increased permeability of the cell membrane at the mucosal surface of the cell

(Civan and Frazier, 1968). This would give rise to an increase in the amount of sodium in the transport compartment derived from the outside solution. However, attempts to obtain evidence in support of this by measuring sodium in the epithelial cells have resulted in contradictory findings. Handler, Preston and Orloff (1972) and MacKnight, Leaf and Civan (1971) have demonstrated an increase in intracellular sodium coming from the outside solution, whereas Lipton and Edelman (1971) could not detect any significant change in ion composition. This latter group of investigators agree that vasopressin stimulates sodium movements across the outer border of the epithelial cell. However, they also suggest that the hormone may have as an additional effect the stimulation of the 'Na-pump' at the serosal side of the epithelium, independently of changes in intracellular sodium. Some evidence in favor of this hypothesis has been obtained by Janáček and Rybová (1967) on frog urinary bladders, who showed an increase in efflux of sodium even when the bladders were equilibrated with mineral oil on the mucosal side and would presumably have no sodium at this surface. In addition, Finn (1968) demonstrated that after loading toad urinary bladder cells with Na, in the presence of amphotericin B, the cells are stimulated by vasopressin to release their load. However, the effect on efflux of sodium by vasopressin is difficult to separate from effects on increasing intracellular sodium, as the increase in efflux may be a consequence of a higher intracellular sodium concentration rather than a stimulation of the pump directly. However, this question still remains to be resolved.

As stated earlier mucosal cells can be separated into two major cell types, the mitochondria-rich and granular cells. The granular cell is the predominant cell type of the toad urinary bladder epithelium and is estimated to represent from about 70% (Danon, Stum and Edelman, 1974) to 83% (Keller, 1963) of those cells forming the luminal surface of the bladder. Therefore, there is a strong possibility that these two diverse cell types may differ with respect to their transport physiology and/or with respect to their response to hormones. Scott and Sapirstein (1974) have developed a technique for separating the two major cell types of the mucosal layer of the toad bladder. They demonstrated that neurohypophysial hormones increase the cyclic-AMP content of mitochondria-rich cells whereas they had no effect on the granular cells. These authors suggest that the mitochondria-rich cells may play a predominant role in the transport of sodium across the toad bladder. However, other investigators (Peachy and Rasmussen, 1961; Dibona, Civan and Leaf, 1969; MacKnight, Leaf and Civan, 1971; Kachadorian, Wade and DiScala, 1975) suggest that it is the luminal membrane of the granular cell whose permeability is dramatically increased in response to the hormone vasopressin. This discrepancy can be reconciled if we consider that the initial response to the hormone affects the mitochondria-rich cell and that either cyclic-AMP or some other chemical messenger via a cell communication process alters the permeability characteristics of the granular cell to bring about the final response. Further investigations as to possible communication processes in toad bladder is needed before

a clear answer as to which cell types are involved in the hormonal response is obtained.

In the presence of an osmotic gradient, the toad urinary bladder also transfers water from its lumen to blood side, and has been helpful in elucidating the mechanisms whereby neurohypophysial hormones regulate this process. An isolated preparation of the amphibian bladder to facilitate the study of this effect has been described by Bentley (1958). A lobe of the bladder is tied to the end of a glass tube, with the mucosal side facing inwards. The bladder is filled with a diluted Ringer solution and placed in a test tube containing aerated Ringers. As water transfer takes place from luminal to blood side it can be measured gravimetrically.

Much evidence has accumulated that vasopressin, which increases the transport of water across this tissue, does so by affecting a permeability barrier located in or near the plasma membrane at the apical surface of the mucosal cells (Civan and Frazier, 1968; Dibona, Civan, and Leaf, 1969; MacKnight, Leaf and Civan, 1971). The transport of water across this tissue is a passive process driven by the osmotic gradient created across the epithelium and proportional to it (Hays and Leaf, 1962). With a dilute mucosal medium very little net transport of water occurs until antidiuretic hormone is added to the serosal side whereupon large net transfers occur. Hays and Leaf (1962) have demonstrated that the cells of the mucosal layer show no change in volume when exposed to a dilute media (hypotonic) until vasopressin is added, whereupon they swell markedly concomitant with the transport of water across this tissue.

It is generally believed that the osmotic flow of water across epithelial membranes, like toad urinary bladder, occurs through aqueous channels (pores) in the cell membrane (Pappenheimer, 1953). The mechanism whereby vasopressin increases this osmotic flow through these channels have followed two schools of thought. One theory known as the 'pore enlargement' hypothesis is based on the observations of Koefoed-Johnsen and Ussing (1953). According to their hypothesis, net movement occurs predominantly by bulk flow in aqueous pores through the membrane rather than by diffusion, and vasopressin increases the net transfer of water by enlarging the radius of individual pores. These authors suggest that neurohypophysial hormones alter the apical membrane from one containing many small pores to one containing fewer larger ones. Little change in area or diffusion permeability would be consistent with large increases in the bulk transfer of water with vasopressin according to this hypothesis of hormonal action. However, Hays and Leaf (1962) have calculated the mean pore radius necessary to accomodate such bulk transfers of water. Their calculations suggest that some 4% of the surface is made up of these pores having a mean pore radius of some 40 Å. Such a size for a channel would be expected to be visible in electron micrographs, however, none have been seen. Additional evidence which is against the pore enlargement hypothesis has been obtained from Maffly, Hays, Lamdin and Leaf (1960) and Leaf and Hays (1962). According to their findings the most striking feature of the effect of vasopressin on the permeability

of the toad bladder to small molecules is its specificity. Only to some molecules, such as uncharged amides and certain alcohols, is the permeability of the toad bladder increased. These compounds like water, penetrate the bladder passively. However, these substances mentioned are capable of hydrogen bonding, and this may play an essential role for the specificity observed in the vasopressin response. Hays, Franki and Soberman (1971), offer additional evidence against the pore enlargement theory. They measured the activation energy for diffusion of water across the toad bladder in the absence and presence of vasopressin. An experimental approach was employed which minimized the effects of unstirred layers and the thick supporting layer of the bladder on the measurements of water diffusion. They found that the activation energy for water in the presence and absence of vasopressin was the same, and also was higher than that expected for the activation energy for the diffusion of water in water. They suggest that their results are consistent with an increase in the number rather than the size of the aqueous channels in the cell membrane. However, further observations are needed before a true picture of the mechanisms of water flow across the toad bladder can be established.

It appears therefore, that neurohypophysial hormones, such as vasopressin, increase water and solute transfer across the mucosal layer of the bladder epithelial cells. It is tempting to postulate the presence of a common receptor site in this tissue which, when vasopressin

binds to it, effects these two processes simultaneously. However, there is good evidence which suggests that the natriferic (sodium transport) and the hydro-osmotic (water transfer) responses to vasopressin are mediated by different receptors. Bentley (1959, 1960) and Edelman and Peterson (1964) have shown that by increasing calcium concentrations, the vasopressin-induced hydro-osmotic response is inhibited whereas the increase in sodium transport is still observed. Peterson and Edelman (1964) further found that the increase in the movement of other solutes that is seen after vasopressin treatment, is not observed in the presence of high calcium concentrations. These authors suggest that there are at least two receptor sites and associated channels involved in the vasopressin action, one for sodium and the other for water and small solutes, such as urea. Further evidence in favor of a two receptor site theory has been obtained through the use of drugs and synthetic hormone analogs. Bourguet and Maetz (1961) have shown that two oxytocin analogs, 8-arginine oxytocin and 8-lysine oxytocin, increase the permeability of the frog skin and bladder to water and sodium in completely different ways. Bentley, Yorio and Fleisher (1975) have shown that the natriferic response of the bladder to vasopressin was unaffected when cadmium was present under conditions that inhibited the hydro-osmotic response, further emphasizing that separate effector mechanisms may be involved for each effect.

The effects of drugs on the actions of neurohypophysial hormones

may lead to helpful information as to the possible mechanisms whereby these hormones effect transport processes and may also lead to information concerning the actual receptor(s) involved.

THE TOAD LENS

Active ionic transport is of major importance to most biological membranes and in the maintenance of normal cellular function. In the crystalline lens these processes also play a major role on the maintenance of a proper ionic composition which may be necessary for the control of a normal degree of hydration and transparency.

Mammalian and amphibian lenses, on which most of the lens membrane studies have been carried out, have a basically similar microanatomy. They are asymmetrical structures, having a single layer of epithelial cells between the capsule (composed of a collagen-like substance) and fibre cell mass on the anterior surface, while the outermost fibre cells on the posterior face makes direct contact with the capsule. The epithelial cell layer on the anterior surface of the lens apparently plays a predominant role in transmural processes. The fibre cells, which make up the major portion of the lens, are closely packed with very little extracellular space between them (Wanko and Gavin, 1961). The contact between adjacent fibre cells and between epithelial and fibre cells is what would one expect for a tight membrane, and represents areas of

tight junctions or occluded zones. It has been suggested (Cohen, 1965) that these junctions might provide low resistance pathways between cells for movements of solutes across the lens.

The crystalline lens of a toad, Bufo marinus, weighs approximately 100 mg and has a diameter of 6 mm. Its fairly large size (as compared to the size of the animal), combined with an ability to survive for extended periods in vitro when bathed by suitable solutions, makes it a convenient system for physiological study.

The similarities between amphibian and mammalian lenses go deeper than a simple morphological similarity. They both have similar ion distributions and resting potentials, and their membranes are more permeable to potassium and chloride ions than to sodium ions (Duncan, 1969; Paterson, 1970; Paterson and Eck, 1971; Brindley, 1956; Sperelakis and Potts, 1959).

The lens is bathed in vivo on each side by two solutions of relatively constant composition, the aqueous and vitreous humor; however, even though they have a similar osmotic composition they are not identical (Toyofuku and Bentley, 1970). The vitreous humor has a slightly lower sodium concentration than the aqueous, but a higher potassium and osmotic concentration. Thus gradients in concentration would appear to be maintained between the two sides of the lens. The aqueous also has a slightly higher osmotic concentration than the serum as also seen in mammals. The intracellular concentration of the toad's lens has the characteristic high potassium and low sodium concentrations compared to the bathing extra-

cellular media (van Heyningen, 1962; Toyofuku and Bentley, 1970).

The problem which faces the lens is that of maintaining this asymmetric distribution of ions. However, there is some controversy as to the relative contributions of the various membranes of the lens in the regulation of this process. There are those who believe that the epithelial cells of the anterior epithelium are alone responsible for actively maintaining the ionic balance of the lens (Kinsey and Reddy, 1965; Bonting, 1965), while others feel that the lens fibre membranes may contribute to this regulation (Rae and Blankenship, 1973; Rae, 1974a, 1974b). Perhaps both membranes play a role in regulating this asymmetric distribution of ions and may contribute to the overall electrical behavior that is seen in the isolated tissue.

The ion transport system in the toad lens is considered to be an asymmetric open system (Candia, 1973), as is the frog skin and toad urinary bladder. However, a superficial look at the lens geometry and ionic gradients may suggest its classification as a closed symmetrical system, similar to the red cell, in which the plasma membrane surrounds the red cell compartment. In fact, some investigators (Harris, 1960; Bonting, 1970) have compared the lens to a giant cell. However, anatomical and functional asymmetric characteristics are present as there exists a layer of epithelial cells only at the anterior surface, and it is therefore considered as an asymmetric composite system. The most important feature of this type of system is that a net transfer between compartments usually occurs and can be easily studied as these compart-

ments are readily accessible.

Perhaps the single most important step in understanding this lens physiology was the procedure developed by Candia, Bentley and Mills (1971) which made available the short-circuit current (scc) technique of Ussing and Zerahn (1951) for the study of transmural movements of ions across the lens. The basic consideration in applying this short-circuit current technique was that regardless of the complexity of the membrane, as the lens is a composite of cellular types, passive unidirectional fluxes should be the same in opposite directions if the electrochemical gradient between the two solutions is zero. In an in vitro system, according to Ussing and Zerahn (1951) this can be accomplished by using solutions of identical composition on both sides bathing the lens, and reducing the spontaneous potential difference developed to zero with the use of an external battery. As stated previously for the frog's skin and toad bladder, the net ionic flux occurring under this short-circuited condition would be the result of an active transport process. The equality between the observed scc and the net flux of the ion in question would further confirm this mode of transport.

Candia, Bentley and Mills (1971) mounted the toad lens in a divided glass chamber, and under these conditions the solutions bathing each surface are separated from each other and an electrical potential difference (p.d.) anterior (aqueous) side positive can be recorded. A

scc can also be measured and one can place radioisotopes on either side and measure their unidirectional fluxes. In addition a microelectrode can be inserted through a small hole in the center of the divided glass chamber, opposite the lens equator, into the central region of the tissue. One can thus, by using this intralenticular electrode as a reference, also measure the electrical parameters across the anterior epithelium and the posterior surface, as well as the entire lens. Candia et al (1971) have found that the transmural p.d. across the toad's lens is about 30mV and the scc about 30 $\mu A/cm^2$, the electrical resistance therefore is approximately 1 $K\Omega\text{-}cm^2$ and the lens is considered to be a 'tight' membrane, similar to the toad bladder and frog skin (see for instance Fromter and Diamond, 1972). Candia et al (1971) also found that there is an active movement of sodium from the posterior to the anterior side of the lens. However, unlike the frog's skin and toad bladder, this active sodium transport only accounts for approximately 30% of the total observed scc. At present the balance of the scc is not clear. According to Candia et al (1971) it does not appear to be potassium, chloride or bicarbonate. An explanation has, though, been offered by Candia (1973) in which he suggests that the additional scc not accounted for by net sodium fluxes can be explained if this additional scc was carried by Na ions moving across the posterior face into the lens and by K moving out across the anterior face. This would provide a net positive charge in the same direction as the active sodium transport, however, no net ion fluxes would be able to detect this. To

support this hypothesis, Candia (1973) measured the uptake of Na^{22} simultaneously with K loss towards both bathing solutions, and found that the lens gains more sodium across the posterior surface than the anterior, whereas more potassium is lost across the anterior than the posterior surface. The difference between these two movements results in a net transfer of positive charges in the posterior to anterior direction. This net transfer of charge was found to be equivalent to the difference in scc observed and that measured as a net Na flux. This finding suggests, that the lens only actively transports sodium (posterior to anterior) and that the scc is at least partly a reflection of this process and would represent a good indicator of ion transport across the lens.

As we have seen, the functional integrity of tissues depends on the concentrations of solutes in the fluids that bathe them as well as the permeability of their respective membranes. Changes in the osmotic concentrations of fluids bathing epithelial membranes may alter their permeability to water and solutes, their metabolism and electrical behavior (Bentley, 1964; Ussing, 1965; Ripoche, Parisi and Bourguet, 1969). The study of such osmotic effects on epithelia are of dual interest, as they not only allow us to predict what may happen *in vivo*, but also furnish information that contributes to our understanding of their normal functioning. The crystalline lens is subject to changes in the osmotic concentration of its bathing solutions, such as may result from evaporation across the cornea (Lazar and Bronson, 1971; Weinstock and Scott, 1967) or during the

administration of osmotic agents that are used to reduce the intraocular pressure (Havener, 1970; Drance, 1970; Becker, Kolker and Krupin, 1968). These observations included increases in osmotic concentration that ranged from about 25 to 300 mOsm/kg H_2O. Increase in the osmotic concentration of the ocular fluids can lead to the formation of reversible cataracts (Fraunfelder and Burns, 1966; Weinstock and Scott, 1967) suggesting that changes in the lens physiology are occurring.

The in vitro amphibian lens offers an excellent system for studying such changes in ion transport systems and lens physiology. By measuring the electrical behavior of the isolated lens, the mechanisms whereby changes in the immediate environment alter the permeability characteristics of the lens membranes, such as increasing the osmotic composition of the bathing fluids or through the administration of drugs, can be investigated.

THE PRESENT INVESTIGATION

The present investigation can be taken as a development of the information that has just been reviewed. The research that is presented has been directed to the study of the permeability properties of three 'model' membrane systems: a) the amphibian's skin, b) the toad's urinary bladder and c) the toad's lens. These epithelial membranes were investigated as to the factors that may alter their permeability properties, particularly to solutes and water, with a major emphasis placed on elucidating the mechanism by which ethanol modifies their permeability

to solutes and water, as well as its effects on the electrical behavior and responsiveness to neurohypophysial hormones. These processes were found to be altered in different ways, which appears to reflect the variety of processes involved in controlling membrane permeability and the different reactions of ethanol with respect to each of these. As ethanol has well established physical and chemical properties it may serve as a useful 'pharmacological probe' for unraveling the complicated mechanisms whereby solutes and water are transferred across epithelial membranes, as well as offering a possible mechanism of action for its in vivo depressant effects.

METHODS

Leopard frogs, Rana pipiens, weighing between 50-150 g, were obtained throughout the year from a biological supplier (Lake Champlain Frog Farm, Alburg, Vermont) and were kept in a container with tap water in the laboratory at 25°C.

Columbian toads, Bufo Marinus, weighing between 150-300 g, were obtained through a commercial supply house (Tarpon Zoo, Tarpon Springs, Florida) and kept on damp peat moss in the laboratory at room temperature.

These animals were maintained under this environment without feeding for a few days prior to their use. They were usually used for experiments within a two week period so that their viability would remain intact. Adult animals of both sexes were used throughout this investigation.

Frog skin preparations:

a) As diaphragms: In vitro preparations of the ventral surface skin were made following double pithing of the frogs. The skin was dissected away from the underlying connective tissue in one continuous sheath and placed in a petri dish containing the appropriate Ringer's solution. For the examination of the effects of the neurohypophysial hormone vasotocin, care was taken to use the skin from the pelvic regions, as this is more susceptible to this hormone's action (Bentley and Main, 1972). In most cases, the dissected sheath was large enough so that two preparations could be prepared from one frog. These pieces of skin were then tied on to the end of a glass tube (open at each end) to form a diaphragm with a surface area

of 3.8 cm^2. The epidermal surface (outside the frog) faced inwards. These preparations were suspended with the dermal surface (inside the frog) immersed in a 50 ml beaker containing 30 ml of Ringer's solution. The other solution, inside the tube, had a volume of 3 ml and consisted of either Ringer's solution or another solution as indicated. This preparation was used to measure the electrical properties across the frog's skin or to determine water flow.

b) In chambers: For the determination of ^{22}Na, ^{24}Na and ^{36}Cl unidirectional flux and simultaneous short-circuit current measurements, frog skins were mounted in Ussing-type lucite chambers with 10 ml of Ringer's solution bathing each side (Figure 1). A parafilm ring was placed on either side of the skin so as to minimize any edge damage. The surface area provided by this divided chamber was 2 cm^2.

The toad urinary bladder preparations:

a) As sacs: This has been described previously (Bentley, 1958, 1960). The bladders were emptied prior to their being used by inserting a blunt instrument into their cloaca. This allowed the bladder to contract and made dissection easier. The animals were then double pithed and the bladder exposed, each lobe was removed separately and tied with silk thread, mucosal (urinary) side facing inwards, onto the end of a Pyrex glass tube. The sac was then filled with either 3 ml (for the measurement of osmotic flow and electrical behavior) or 10 ml of Ringer's solution (for the determination of tritiated water fluxes for diffusional flow). These bladder sac preparations were immersed so that their serosal side was bathed in

20 and 30 ml of Ringer's solution, respectively (Figure 2).

b) In divided chambers: For the determination of unidirectional ion fluxes, using ^{22}Na and ^{36}Cl, bladder halves were mounted in Ussing-type lucite chambers with 15 ml of Ringer's solution bathing each side. A double parafilm ring was placed on either side of the bladder so as to minimize any edge damage. In addition, nylon mesh was stretched across the serosal side of the bladder so as to hold the bladder rigidly in position during stirring. The surface area of the membrane in this divided chamber was 7 cm^2.

Toad lens preparation:

The in vitro preparation of the toad lens was essentially that of Candia, Bentley and Mills (1971). Toads weighing approximately 200 g were used, which provided lenses that weighed between 100 to 200 mg and had a cross-sectional area of about 0.3 cm^2. The toads were immobilized by double pithing, and after cutting out a section of the eyeball, including the cornea and part of the sclera, the lens was carefully dissected out from the ciliary body leaving a thin ring of zonular fibers around its equator which served as a reference for its later mounting in a chamber. After this dissection the lens was placed in a petri dish with the appropriate Ringer's solution. The lens was then picked up by its posterior surface, with the aid of a glass pipette and gentle suction, and was introduced into a divided glass chamber in the manner shown in Figure 3. With practice, lenses could be selected so that they will fit exactly in the

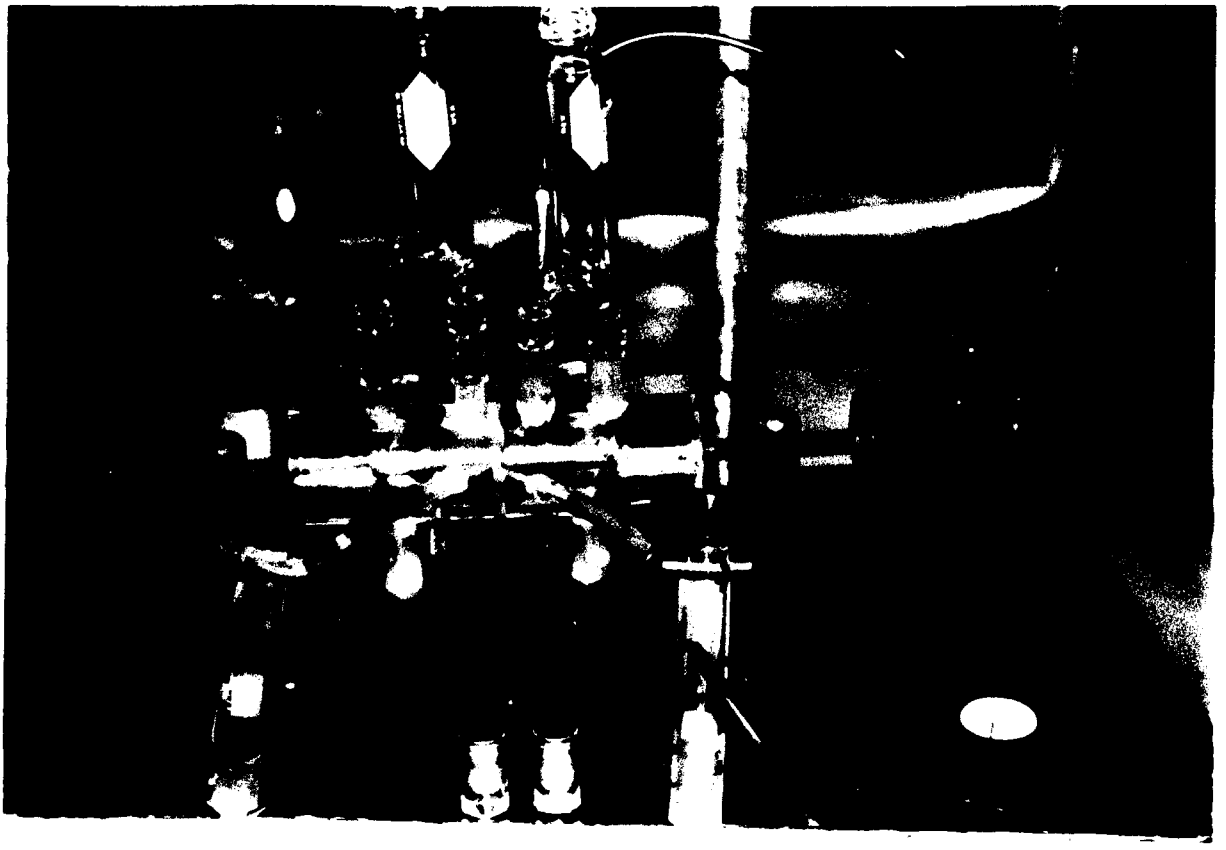

Figure 1. Photograph of the Ussing-type lucite chamber used to mount frog skin and toad urinary bladder for determination of ion fluxes and short-circuit technique.

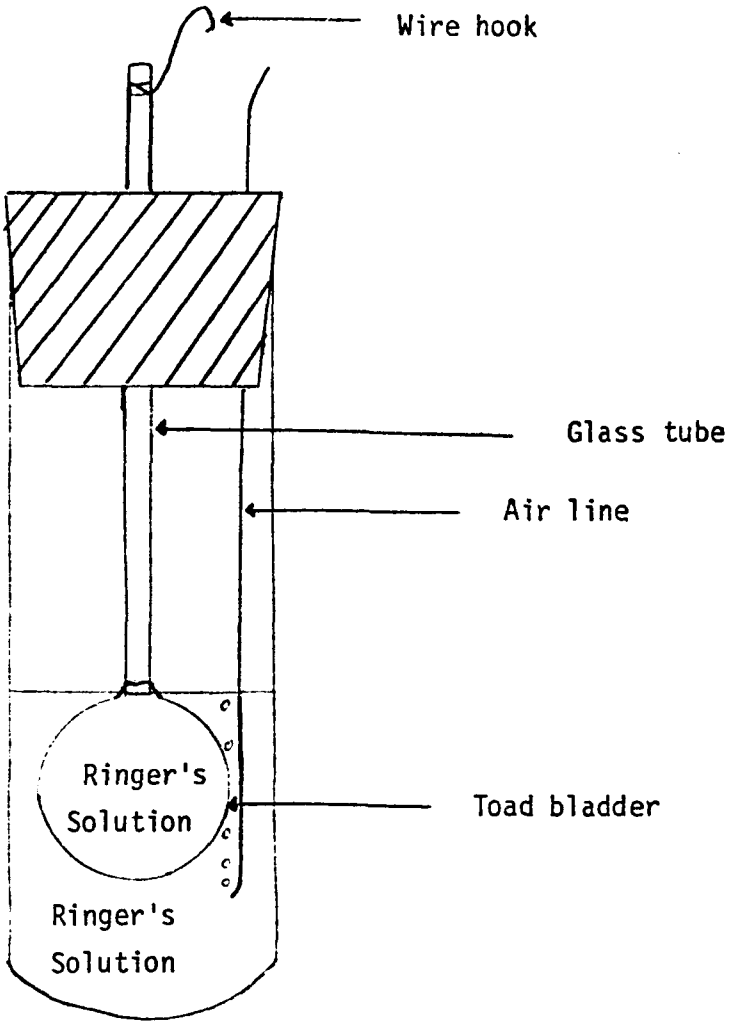

Figure 2. Diagram of bladder preparation used to measure electrical properties and water flow.

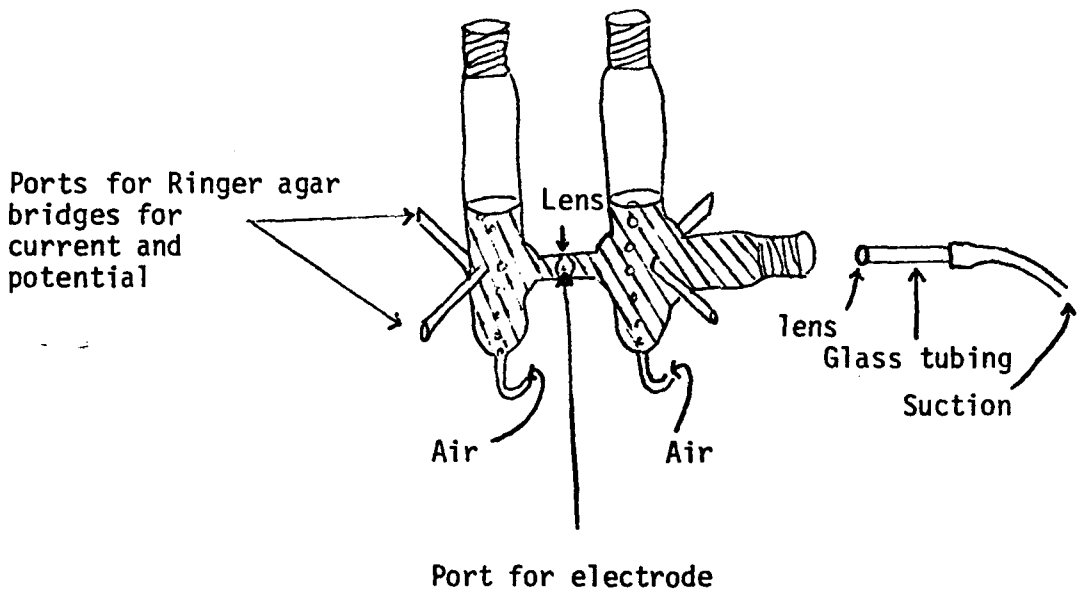

Figure 3. Divided chamber for mounting lens as a membrane with anterior and posterior sides electrically separated.

centerpiece, thereby sealing one compartment from the other.

The glass chamber itself consisted of two compartments joined by a cylindrical centerpiece with a diameter of about 6 mm. The chamber compartments have outlets for two electrical potential and two current electrodes as well as for air bubbling and circulation. The opening at the top of each compartment of the chamber is large enough so that samples may be taken from or drugs added to each compartment separately. In addition the centerpiece of this lens chamber has a small hole (about 0.5 mm diameter) in a place coinciding with the position of the lens equator. A fine glass electrode can be laterally inserted through this hole into the lens so that the electrical properties of the lens can be studied between this electrode and each bathing solution.

Measurement of p.d. and scc:

The electrical potential difference (p.d.) across the frog skin, toad bladder and toad lens, was measured using Ringer-agar or saturated KCl-agar bridges connected through calomel cells to a voltmeter (Keithley, model 200B) or potentiometric recorder. The short-circuit current (scc) was obtained by passing a current, from an external battery connected to a pair of Ag-AgCl cells and KCl-agar bridges (Bentley, 1960) across these tissues so as to bring the spontaneous p.d. to zero. An automatic voltage-clamp with the scc continually recorded on a strip chart recorder was also used, especially to maintain the p.d. at zero in the ion flux experiments. All readings were taken once a steady-state was achieved, this usually occurred within one hour after setting up the preparation. Resistances were also calculated (as p.d./scc) for some experiments.

For the lens, additional measurements were made besides its trans-lenticular electrical behavior. For recording the p.d., scc and resistance across each lens surface, a small glass pipette electrode (filled with saturated KCl, tip diameter, 80μ; electrical resistance about 150 KΩ or less) is inserted through a small hole in the middle of the divided glass chamber, opposite the lens equator, into the central region of the tissue. By using this intralenticular electrode as a reference, the electrical parameters across the anterior epithelium and the posterior surface can be measured. Current across the lens was sent by means of Ringer-agar bridges which were in contact with the solutions bathing the lens and were connected to automatic voltage clamp machines. By using two voltage-clamp recorders and three voltmeters the p.d. between any two electrodes could be voltage-clamped and the scc determined while their p.d.'s with respect to the third electrode can be simultaneously monitored. The inside of the lens is normally negative with respect to either side, and the anterior side is positive with respect to the posterior side (Candia et al., 1971). To short-circuit the p.d. of the anterior side or the trans-lenticular p.d., positive current is sent in the posterior to anterior direction. Because the interior of the lens is negative with respect to either side, when the anterior p.d. is short-circuited, the posterior p.d. is hyper-polarized and vice versa. Current-voltage plots (IV) of the anterior and posterior sides can thus be obtained as shown and described in Figure 4. The voltage response to a current applied for 10 min was stable after 30 seconds and the current-voltage plots were linear. From

Figure 4: Current-voltage plots of the anterior (a) and posterior (p) sides of the isolated toad lens. Points a_0 and p_0 are the open circuit spontaneous potential differences (p.d.) of the anterior and posterior sides. Their difference is the translenticular p.d. with a_0 the anterior side having the larger p.d.. By sending current from the posterior to the anterior direction, the entire lens can be short-circuited (graphed as points a_3, p_3) and the translenticular p.d. would be zero. By further increasing this current the anterior side can be short-circuited (point a_1) while the posterior side is hyper polarized (point p_1). By then sending current in the opposite direction, from anterior to posterior, the posterior side is short-circuited (point p_2) while the anterior side is hyper polarized (point a_2). By this method, pairs of points, lying in the same vertical axis are obtained simultaneously.

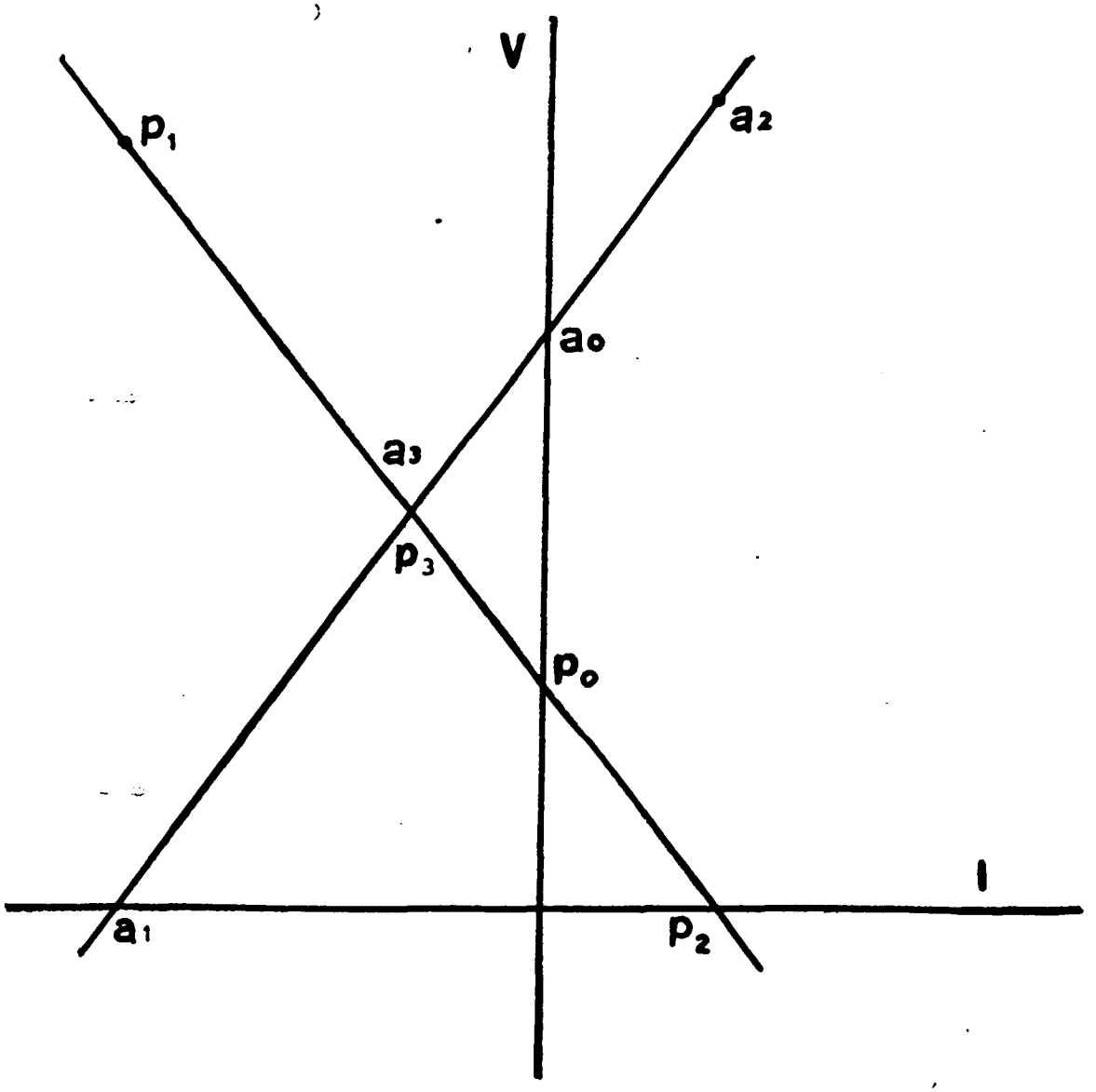

Figure 4 : Current-voltage plots of the anterior (a) and posterior (p) sides of the isolated toad lens. (Current (I), abscissa; Voltage (V), ordinate)

the slope of these lines the resistances across the anterior and posterior surfaces can be calculated.

Measurement of oxygen consumption:

This was determined with an apparatus which utilizes an oxygen electrode to measure changes in oxygen saturation of the bathing medium (Yellow-springs, YSI model 153). The tissues were placed in small chambers which are sealed with the aid of a plunger from which the top of the oxygen electrode emerges (Figure 5). The solutions in this chamber were initially saturated with air. Vigorous mixing was provided by a magnetic stirrer. Changes in oxygen saturation of the fluid in the bath were measured and directly transcribed onto a chart using a recorder (Esterline-Angus). The decline in oxygen saturation was measured between 100 and 90 percent, and was found to be linear over this range. Oxygen consumption was measured on frog skin and toad bladder. For the measurement of oxygen consumption of frog skin two preparations were used: a) Whole skin: Three pieces of frog skin (whole), each with a surface area of 0.78 cm^2 were placed in the oxygen electrode cell containing 5 ml of Ringer's solution saturated with air at 25°C. b) 'Split skin': Sheaths of isolated epithelium, which was prepared from a collagenase-pressure procedure (see later, p. 51), was cut in half so that paired experiments could be run. Each piece, having a surface area of 2 cm^2, was placed in the oxygen electrode chamber containing air-saturated Ringer's solution (5 ml).

For the measurement of oxygen consumption by 'pieces' of toad bladder tissue, 5 ml of fluid was placed in the chamber.

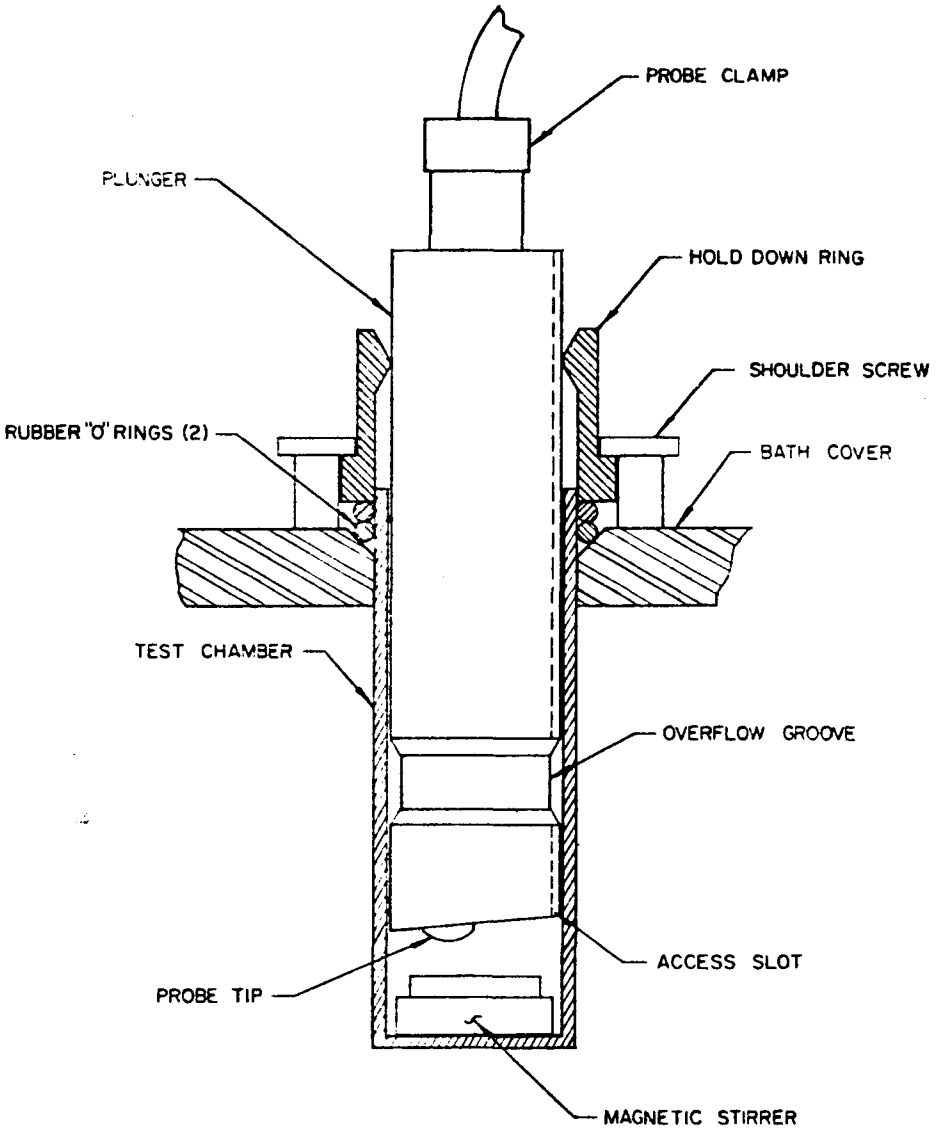

Figure 5. Diagram of the oxygen electrode and sample chamber used to measure oxygen consumption of the frog skin and toad bladder.

All tissues were allowed at least 30 min to equilibrate with the Ringer solution before measurements were begun. Oxygen consumption was then stable for prolonged periods, successive measurements being made for 5 min periods. Oxygen consumption was recorded in tissue preparations immediately before addition of a drug or a change in solution occurred. When choline was used to replace sodium in the Ringer's solution the tissue (frog skin) was rinsed at least three times.

The dry weights of the pieces of skin and bladder were determined by drying them for 24 hours in an oven at 105°C. All volumes of oxygen are expressed as microliters at standard temperature and pressure.

Oxygen consumption was calculated as follows: Ringer's solution was used at 25°C, and at this temperature and pressure contains 5.78 microliters O_2 at STP per milliliter of solution when saturated with air. Therefore if 5 ml of Ringer's solution is being used there is 5 x 5.78 = 28.9 microliters of O_2 in our solution at 100% saturation. To determine the rate of oxygen used, the meter reading at the start and finish of a 5 minute time interval was taken from the recorder.

Start: 97.0%

Finish: 95.3%

So that 97.0 - 95.3 = 1.7% of the oxygen was used up in 5 minutes. Therefore 1.7 of 28.9 = 0.50 microliters of oxygen consumed in 5 minutes. However to normalize the values for the different tissues, this was represented as microliters of oxygen per gram dry weight of the tissue per minute. If the tissue weighed 10 milligrams, the amount of O_2 consumed would be calculated as : 0.50 μl of O_2/5 min x (0.01 g dry weight) = 10 μl of O_2 consumed per gram dry weight per minute.

Separation of frog skin epithelium ('split-skin' preparation):

The following procedure derived from that of Aceves and Erlij, (1971); Rajerison, Montegut, Jard and Morel (1972) and Siegal, Tormay and Candia, (1975) was used.

The ventral surface of large Rana pipiens (about 200 g) was removed following double pithing. The skin was then mounted on the open side of a cylindrical lucite chamber which provided a surface area of 10 cm^2. The frog's skin was secured to the chamber by tying lacing cord around the skin so that the corium (inside the skin) was facing the inside of the chamber. The chamber with the bound skin was immersed in Ringer's solution at 30°C and air was bubbled into the beaker containing the Ringer's solution. The skin chamber had a 60 cm long plastic tube inserted into its closed side so that the bottom portion of the tube just touched the inside surface of the skin. A 20 ml solution of Ringer containing 5 mg collagenase was injected through an outlet into the skin chamber. Following this, air was forced into the chamber, using the same syringe, to maintain a 50 cm high column of solution in the plastic tube attached to the chamber (see Figure 6). In most cases, after 1 to 2 hours, a skin blister was formed. The blister or epithelial layer could be peeled off the skin surface in one continuous piece by cutting around its circumference. This separated epithelium was then cut in half and used for the determination of Na and K content or used for oxygen consumption measurements.

Measurement of intracellular sodium and potassium content:

a) Frog skin isolated epithelium: The sodium and potassium content of the isolated 'split-skin' epithelium was measured by flame photometry

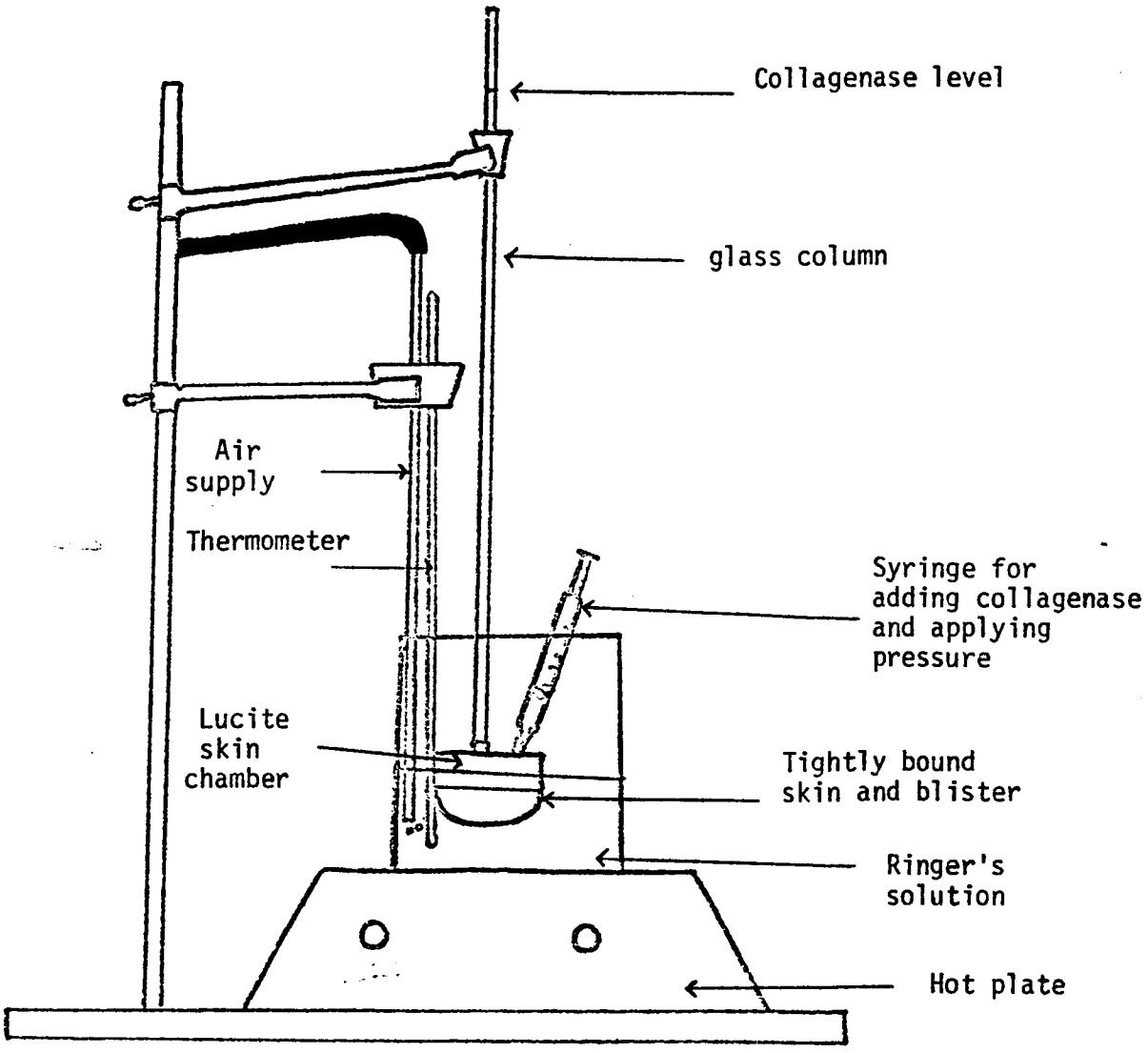

Figure 6: Diagram of apparatus used to prepare isolated 'split skin' epithelium.

(Eppendorf, model 700) by the following method: As large sheaths of isolated epithelium were obtained, paired skin preparations were used and each pair was bathed with Ringers solution containing ^{14}C- inulin (Amersham/Searle) as an extracellular marker. The drug (ethanol, amiloride or ouabain) was then added to the solution bathing one skin of the pair, the other skin served as a control. After the skins were incubated in this media for 1 hr, they were removed and placed in a 2 ml volumetric containing 0.1 N HNO_3 for 24 hours. After this extraction period 1 ml of this solution was used for counting ^{14}C-inulin in a liquid scintillation counter (Packard) for space determination, the other milliliter was used for Na and K measurements in the flame photometer. From this milliliter a 0.1 ml sample was taken and diluted to 10 ml for potassium measurements and 0.2 ml sample was also taken and diluted to 5 ml for sodium determinations. The amounts of Na and K in these solutions were then determined against a standard curve which ranged in values from 100 μeq/liter to 500 μeq/liter for Na and from 50 μeq/liter to 250 μeq/liter for K. At no time did the samples exceed these standard curve ranges. The values obtained were then corrected for the appropriate dilutions and expressed as meq/kg initial wet weight. The final values represent those obtained after the sodium and potassium content in the extracellular space had been subtracted.

b) Toad lens: The sodium and potassium content of the toad lens was also measured by flame photometry after extracting it in 0.1 N nitric acid.

The procedure was essentially that of Toyofuku and Bentley, (1970) and similar to that described for the isolated frog skin. The extracellular space was measured using ^{14}C-inulin or ^{14}C-mannitol. Equilibration was found to occur in 1.5 to 3 hr and the latter time was chosen for these experiments with the toad lens. Paired lenses were used, each pair was bathed with solutions containing the ^{14}C -inulin (or ^{14}C-mannitol) and the osmotic agents were added to the solution bathing one of these. The extracellular space was represented as a percent of the wet weight. The flame photometry procedure was exactly as that described for the frog skin, and the final values are expressed as meq/kg initial wet weight.

Measurement of osmotic water movement:

Water transfer in the presence of an osmotic gradient was measured gravimetrically according to Bentley (1958). Bladder sacs were filled with 1/10 Ringer's solution and suspended in a 20 ml of undiluted Ringer's solution. After a period of 1 hr for equilibration, the bladder was emptied, rinsed and filled again (1/10 Ringer's). It was then weighed complete with the attached rubber bung (see Figure 2) to the nearest 0.1 mg on a Mettler balance. The bladder was out of the Ringer bath for about 30 sec during this procedure. After 30 min the bladder was removed from the bath and reweighed, weight loss was taken as a measure of fluid lost by transfer to the outside solution due to osmosis. The bladder was next emptied, rinsed and refilled to approximately the

same volume and inserted into a tube of fresh Ringer's solution.

When measuring the effects of ethanol on the vasopressin or cyclic-AMP stimulated water flow, ethanol was added during a second period following an initial measurement in the presence of these other agents. Results are shown as weight change in mg per 30 or 60 min.

Preparation of collodion membranes:

Artificial collodion membranes were prepared by pouring liquid collodion (in an ether solution) into test tubes and allowing one minute for partial drying and then emptying the excess content. A thin film of collodion remains adhered to the inside of the test tube. This was blown dry with air and formed a membrane which took the shape of the tube. The membranes thus formed were set up as sacs (similar to bladder sacs) containing 3 ml of deionized water and were immersed in 20 ml of a 240 mM sucrose solution. These artificial membranes were used to measure osmotic water flow in the presence of ethanol.

Glutaraldehyde and NEM fixation procedures.

Glutaraldehyde fixed bladders were prepared according to Eggena (1972). Briefly, the toad bladders were mounted as sacs with 3 ml of 1/10 Ringer's solution added to the mucosal side and 20 ml of Ringer's bathing the serosal side. Ten mU/ml of the neurohypophysial hormone vasopressin was added to the serosal bathing media and 15 min allowed for a response. After this time period the mucosal fluid was removed and re-

placed with a 1% glutaraldehyde solution in 0.05 M cacodylate buffer and resuspended in fresh Ringer's solution. After 5 min the fixative was removed from the bladder and the mucosa was rinsed thoroughly (3 times) with Ringer's solution. These bladders were then refilled with 3 ml of the dilute Ringer's solution and water loss measured gravimetrically as described earlier.

N-ethyl maleimide (NEM) fixed bladders were prepared according to Bentley (1973). The bladders were again set up as sacs containing diluted Ringer's on its mucosal side. These bladders were then exposed to 10 mU/ml vasopressin for 30 min and in the last 5 min of this period 10^{-4}M NEM was placed in the serosal fluid. This agent has been shown in amphibian bladders (Bentley, 1964, 1973) to produce a condition similar to 'fixation' or freezing of the bladder membrane in an osmotically permeable condition following vasopressin treatment. After this 5 min 'fixation' period the bladders were rinsed, each preparation was then refilled with 3 ml of 1/10 Ringer's (on the mucosal side) and placed in 20 ml of Ringer's which bathed its serosal side.

Water transfer was thus measured in the absence of vasopressin but in an osmotically permeable state. Following a control period ethanol was then added to see what effect it had on osmotic water transfer in the bladder under these 'fixed' conditions.

Measurement of unidirectional radioisotope fluxes:

a) Frog skin: Unidirectional fluxes (outside to inside and inside to outside) of sodium and chloride were determined using ^{22}Na, ^{24}Na and ^{36}Cl

as tracers. The skins (whole), mounted in Ussing-type chambers (2 cm^2), were maintained under short-circuited conditions, and at least 30 min was allowed after adding the isotope for equilibration to occur. Samples were then collected from the cold side at 30 min intervals for eight successive periods (4 control plus 4 following drug administration). A stable flow of isotope was an indication of isotope equilibration.

For the determination of tritiated water (THO) and ^{14}C-ethanol flux, the following protocol was used. Whole skins were mounted as diaphragms, as described earlier, but this preparation provided a surface area of 10 cm^2. Vigorous mixing was accomplished with magnetic stirring bars, one of which was suspended on the epidermal side of the skin by a nylon thread attached to a stainless steel fishline spinner. Isotopes (1 μCi/ml) were added to the solution bathing the epidermal surface and samples were taken from the cold side every min for five successive periods. At no time did the activity of the cold side exceed 2% that of the hot solution. The same skin preparation was used when determining drug effects on these fluxes. Following a control series (5 successive flux periods) the hot solution was removed and the chamber and skins were repeatedly washed (5 times). Before the ethanol and the new hot solution were added, (and an additional 5 periods measured) a sample was taken from the cold side to test for residual isotope activity. At all times this was found to be less than 10% above background. Following this drug testing period the skins were washed out again and another control series was run to test for reversibility of

the drug response.

Fluxes were determined from the specific activity of the hot solution. The THO flux measurements are represented as $\mu l/cm^2$ hr or in terms of K_{trans} (the permeability coefficient) as cm/sec. The ^{14}C-ethanol fluxes were used to calculate K_{trans} and are represented as cm/sec.

b) Toad urinary bladder: Unidirectional fluxes (mucosal to serosal and serosal to mucosal) of Na and Cl were determined using ^{22}Na and ^{36}Cl as tracers. The bladders, mounted in Ussing-type chambers (7 cm^2) were maintained under short-circuited conditions, and at least 30 min was allowed after adding the isotope for equilibration to occur. Samples were taken as described above for frog skin.

For the determination of THO and ^{14}C-ethanol fluxes, the same protocol was used as described for the frog skin, however, the bladders were prepared as sacs. Isotopes were added only to the mucosal solution (inside the sac) and samples taken from the cold side every 15 or 30 sec for THO and every min for ^{14}C-ethanol, for five successive periods. Samples were taken at these short intervals so as to keep the level of activity of the cold side below 5% that of the hot solution. The same procedure was then followed as outlined above for measurements in frog skin.

Flux values for sodium and chloride are represented as $\mu eq/cm^2$ hr; for THO fluxes, $\mu l/cm^2 hr$ or as K_{trans} (cm/sec); ^{14}C-ethanol fluxes were used to calculate the permeability coefficient (K_{trans}) and are represented as cm/sec.

c) Toad lens: Unidirectional Na and Cl fluxes were measured according to Candia et al (1971), by adding ^{22}Na or ^{36}Cl, respectively, to one chamber compartment of the divided glass chamber and taking periodic samples from the opposite compartment. In general, different lenses were used to measure each particular ionic flux, however, in some instances paired lenses from the same animal were used to measure fluxes in the two opposite directions. The specific activity of the 'hot side' was kept constant and the activity of the cold side was always 2% or less of that in the hot compartment. For a correct determination of unidirectional fluxes, a steady state between the lens and the hot solution is required. This becomes particularly important in the lens because of its thickness (5-6 mm). When the rate at which isotope appears in the cold side is stable then a steady state has been reached. Therefore, a uniform rate of flow can then be taken as an indication of a steady state. In the lens a steady state was found to be reached after 60 min following the addition of radioisotope. After this one hour equilibration time, samples were taken from the cold side at 30 min intervals for at least 3 hr. A further indication of a steady state was the uniformity of the fluxes over several periods. During these flux measurements the lenses were maintained under short-circuited conditions and only for brief periods, less than 10 sec each and 15 min apart, was this interrupted for measurements of the open circuit potential difference.

All isotopes were counted where appropriate, in a Packard Tri-Carb Liquid Scintillation Spectrometer and a Packard Autogamma Spectrometer.

The bathing solutions: The composition of the solutions used throughout this investigation are presented in Table 1. All solutions were bubbled with air and all solutions were made fresh daily or were made from stock solutions which were previously prepared. The concentration of sodium was checked after preparing the solution by flame photometry, and was always found to be within 3 μeq/liter of the concentration required. In addition, the pH was checked periodically to be certain that the solutions were buffered properly. In ion substitution studies, choline was used to replace sodium; sulfate and gluconate, or nitrate and gluconate was used to replace chloride.

Drugs: The following drugs and hormones were used. Sodium thiocyanate (Eastman Kodak Co., Rochester, N.Y.); ^{14}C-ethanol and ^{14}C-mannitol (Amersham/Searle, Illinois); THO, ^{22}Na, ^{24}Na and ^{36}Cl (New England Nuclear, Boston, Mass.); ethanol and collodion (Mt. Sinai Pharmacy, N.Y.); ouabain, mannitol, N-ethylmaleimide, phospholipase-C and collagenase (Sigma Chemical Co., St. Louis, Mo.); arginine vasotocin and 8-arginine vasopressin (Schwartz/Mann, Orangeburg, N.Y.); furosemide (Hoechst Pharmaceuticals Inc. Sommerville, N.J.); urea (Mallinckrodt Chemical Works, St. Louis, Mo.); cyclic-AMP (Calbiochem, Los Angeles, California); methanol, 2-propanol and butanol (Fischer Scientific, Fairlawn, N.J.); and amiloride (a gift from the Merck Institue for Therapeutic Research, West Point, Pennsylvania).

Table 1. Composition of solutions used in mM concentrations.

Ion	Ringer	Sulfate Ringer	Nitrate Ringer	Choline Ringer	Conway Ringer	Choline Conway
Na^+	115	115	115		104	
K^+	3.35	3.35	3.35	3.35	2.5	3.2
Mg^{++}					1.2	1.2
Ca^{++}	2.7	2.58	2.58	2.7	1.0	1.0
Cl^-	120			120	74.5	81.5
HCO_3^-	4.0	3.35		4.0	25.0	25.0
$H_2PO_4^-$					0.7	0.7
HPO_4^-					2.2	
$Gluconate^=$		2.58	2.58		1.0	1.0
$SO_4^=$		57.0			1.8	1.2
NO_3^-			115			
$Choline^+$				115		104
Glucose	5.0	5.0	5.0		26	26
Sucrose		57				
pH	8.0	8.0	8.0	8.0	8.6	8.6

RESULTS

As this investigation involves the study of three membrane systems, namely, the frog's skin, toad urinary bladder and toad lens, the results that were obtained as to the effects of ethanol and other agents on the transport properties of these membranes will be presented for each system separately.

FROG SKIN

1. Effects of ethanol on oxidative metabolism of the frog skin.

Under *in vivo* conditions the body has a low tolerance to ethanol. It has fatal effects in man when the blood levels reach 500 mg per cent. However, certain tissues *in vitro* can withstand exposure to high concentrations of ethanol. Klensh (1949) for instance found that 25 to 40% ethanol caused a block of electrical stimulation of frog motor nerves. Although these high concentrations were insulting to the tissue there was no irreversible damage as this response was readily reversible following washout of the drug. In order to measure the ability of the frog skin to withstand high concentrations of ethanol, we measured its oxygen consumption in the presence of this drug. Whole skin preparations were initially used for this experiment. Thirty minute exposure to 3% ethanol (650 mM) (on both sides), which is the concentration that had marked effects on the electrical parameters of the frog skin, had no effect on this tissue's oxygen consumption (Table 2). It is possible that an effect on sodium

Table 2. Effects of 3% ethanol on O_2 consumption of isolated 'whole' frog skin

Oxygen consumption $\mu l\ O_2\ g\ dry\ wt^{-1}\ min^{-1}$

	Initial	After 30 min	Wash
Control (10)	11.6 ± 0.7	11.0 ± 0.8	9.9 ± 1.0
3% Ethanol (14)	11.5 ± 0.9	10.8 ± 0.8	10.2 ± 0.8

Results are as means ± S.E.
No. of experiments are in parenthesis.

transport could be obscuring a general metabolic action of ethanol, due for instance to an inhibition of the former and an increase in the latter. Frog skins were thus bathed in Na-free solutions or treated with amiloride to inhibit any sodium transport and remove that fraction of O_2 consumption that is linked to this process. Sodium transport in frog skin accounts for approximately 35% of this tissue's O_2 consumption. As can be seen in Table 3, the O_2 consumption dropped approximately 35% when sodium was removed from or amiloride was added to the bathing solution. When 3% ethanol was added to these pretreated skins, there was no further change in this tissue's oxygen consumption.

To determine at what concentration alcohol had irreversible effects on oxygen consumption further measurements were done using the isolated epithelial (or 'split-skin') preparation. This provides us with an epithelium that is free of connective tissue and should be more sensitive to ethanol's effects than the whole skin preparation. It was found that at a concentration of 30% ethanol (bathing both surfaces of the skin) there was a 90% inhibition of oxygen consumption, whereas, 10% ethanol had no effect on this parameter (Table 4). The concentrations of ethanol that were required to inhibit oxidative metabolism far exceeded those that we observed to alter the permeability of the membrane.

2. Effect of ethanol on the electrical parameters of the frog skin.

When mounted as a flat sheet between two identical Ringer's solutions the frog skin develops an electrical potential difference (p.d.) with the

Table 3. Effects of ethanol on the O_2 consumption of frog skin bathed in Na-free solutions or pretreated with amiloride.

Oxygen consumption $\mu l\ O_2\ g\ dry\ wt^{-1}\ min^{-1}$

	Initial	Na-free	Na-free + ethanol
Control (5)	12.2 ± 1.2	8.2 ± 0.8	7.3 ± 0.9
3% Ethanol (5)	12.7 ± 2.4	7.7 ± 0.9	7.4 ± 0.9

	Initial	Amiloride	Amiloride + ethanol
10^{-4}M amiloride (5)	12.0 ± 0.2	8.4 ± 0.4	7.9 ± 0.3

Results are as means ± S.E.
No. of experiments are in parenthesis.

Table 4. The effects of increasing ethanol concentrations on the oxidative metabolism of isolated 'split-skin' frog epithelium.

	Oxygen consumption $\mu l\ O_2\ g\ dry\ wt^{-1}\ min^{-1}$		
Ethanol conc.	Initial	After 30 min ethanol[a]	p for difference
10% (5)	31.2 ± 4.4	33.8 ± 4.9	N.S.
20% (8)	29.3 ± 3.4	16.4 ± 2.1	< 0.01
30% (8)	28.4 ± 2.9	3.8 ± 0.8	< 0.001

[a]No ethanol was present during the O_2 determinations, so that a change represents an irreversible inhibition.
Results are as means ± S.E.
Number of experiments are in parenthesis.
N.S. not statistically significant ($p > 0.05$)

inside, or corium, positive. This spontaneous p.d. can be brought to zero by the application of an external current, which is called the short-circuit current (scc). Ussing and Zerahn (1951) found that the scc was equivalent to the net active sodium transport from the outside to the inside bathing solutions. The measurement of p.d. and scc across frog skin provides information as to the movements of ions across it, and how these movements can be affected by external forces, such as the administration of drugs.

When ethanol at a concentration of 3% (v/v) was added to the outside solution bathing the frog skin, there was an immediate drop in the p.d. and scc whereas the resistance, calculated as p.d./scc, did not change significantly. This response was readily reversible after removing the drug even after more than 90 min exposure (Figure 7 and Table 5). This effect was also seen with 1% ethanol, although the response was smaller (Table 5). Lower concentrations than this (0.5% ethanol) were without any appreciable effect. Higher concentrations of ethanol (up to 30%) exerted a greater effect which was also reversible following washout of the drug (Table 6). When ethanol was added to the inside bathing solution its response was much slower and smaller in magnitude.

To see if this ethanol response was due to an osmotic effect equimolar concentrations of urea (650mM) and mannitol (650mM) were added to the outside bathing solution. From Table 7 it can be seen that the transmural electrical p.d. decreased in the presence of these osmotic agents, but

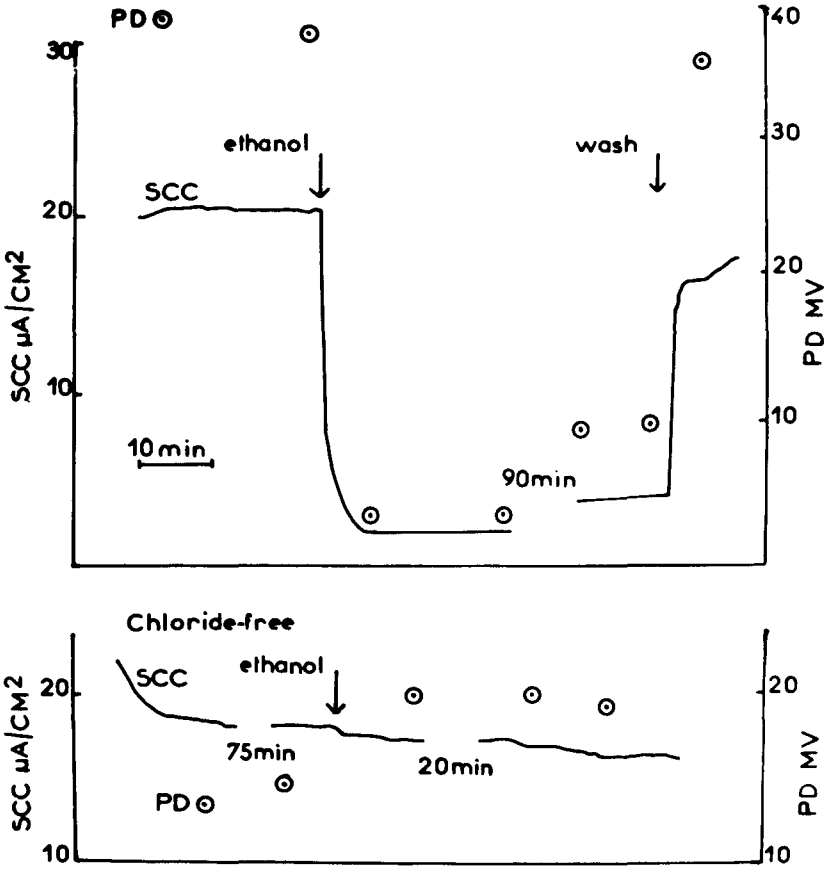

Figure 7. Effect of ethanol on short-circuit current (scc) and potential difference (p.d.) across a preparation of frog skin in vitro. The upper curve shows the response upon the addition of 3% (v/v) ethanol to the outside bathing solution in the presence of chloride. The lower curve shows the response to 3% ethanol (added as above) in Cl-free media.

Table 5. Effect of ethanol on the p.d. (mV) and scc ($\mu A/3.8\ cm^2$) across frog skin *in vitro*.

Treatment		Initial[a]	+ ethanol for 30 min	Washout (ETOH-free) after 30 min
1% ethanol (outside) (11)	p.d.	25 ± 2.9	13 ± 1.7[b]	27 ± 2.3
	scc	68 ± 5.9	44 ± 8.0[c]	76 ± 7.6
3% ethanol (outside) (10)	p.d.	23 ± 2.4	11 ± 1.2[b]	27 ± 3.2
	scc	89 ± 10	46 ± 6.8[c]	106 ± 15
3% ethanol (inside) (8)	p.d.	26 ± 1.4	16 ± 1.5[b]	24 ± 1.4
	scc	83 ± 5.6	66 ± 5.8	84 ± 4.0

[a] This is a steady baseline value measured over a 30 min period.

[b] $p < 0.001$ for differences from initial period.

[c] $p < 0.01$ " " " " "

Results are as means ± S.E.
Number of experiments are in parenthesis.

Table 6. The effects of increasing amounts of ethanol on the electrical properties of frog skin.

	Initial[a]	3% ethanol for 15 min	Washout (ETOH-free) after 30 min	10% ethanol for 15 min	30% ethanol for 15 min	Washout (ETOH-free) after 30 min
p.d.	61 ± 4.7	30 ± 3.0	65 ± 5.4	23 ± 1.6	11 ± 0.7	67 ± 5.3
scc	148 ± 4.0	107 ± 4.0	169 ± 10	84 ±5.0	30 ± 3.4	165 ± 11

[a] p.d. in mV, and scc in $\mu A/3.8\ cm^2$

[b] Ethanol was added to mucosal side only

Results are as means ± S.E. of ten experiments.

Table 7. Effects of urea and mannitol on the electrical p.d. (mV) and scc (μA/3.8 cm^2) across isolated frog skin *in vitro*.

Treatment		Initial[a]	+ solute for 30 min	Washout (urea and mannitol-free) after 30 min
650 mM urea (outside) (8)	p.d.	36 ± 6.0	16 ± 3.0[b]	40 ± 6.8
	scc	102 ± 16	131 ± 22	118 ± 16
650 mM mannitol (outside) (6)	p.d.	44 ± 1.6	15 ± 0.5[c]	43 ± 1.3
	scc	82 ± 4.3	84 ± 4.5	85 ± 5.7

[a]This is a steady baseline value measured over a period of 30 min.

[b]$p < 0.01$ for differences from the initial period.

[c]$p < 0.001$ " " " " " "

Results are as means ± S.E.
Number of experiments are in parenthesis.

there was no change in scc. The effects of hyperosmoticity on frog skin has been well documented (Ussing, 1965, 1966) and similar decreases in p.d. have been described with no concomitant decline in scc reported. These observations suggest that ethanol is not working via an osmotic-type of response on frog skin. In addition, ethanol permeates the frog skin quite readily (see later), so that any osmotic effect would only be transient, as equilibration is rapid.

As described previously, the scc across the frog skin has been shown to be equivalent to the net transport of sodium. However, Cl^- has recently been shown to be also transported actively (Zadunaisky, Candia and Chiarandini, 1963; Kristense 1972; Watlington and Jessee, 1973,1975) and may also play an important role in the active transport process for sodium. To investigate what role Cl played in the ethanol response, frog skins were bathed in chloride-free solutions (SO_4^{-2} Ringer's). When Cl was replaced by SO_4 (osmolality corrected on both sides of the frog skin), there was an increase in the open circuit p.d. and a drop in the scc (Table 8). The drop in scc was not due to an inhibition of an active transport of chloride, (as shown by the flux measurements) indicating that the sodium transport was anionic dependent. Other workers have also demonstrated anion dependent sodium transport in frog skin (Huf, 1972; Ferriera, 1968; Cuthbert, Painter and Prince, 1969).

It was next determined whether the presence of chloride was necessary in the outside or inside bathing solutions. By replacing only the outside

Table 8. Effect of chloride-free (SO_4^{-2}) solutions on the p.d. (mV) and scc ($\mu A/3.8\ cm^2$) across frog skin *in vitro*.

Treatment		Initial[a]	+ Treatment after 30 min	Washout (N-Ringer's both sides) after 30 min
Chloride-free (SO_4-Ringer's)	p.d.	31 ± 4.3	69 ± 4.6[b]	35 ± 4.4
(both sides) (16)	scc	84 ± 6.8	53 ± 6.8[b]	81 ± 6.1
Chloride-free (SO_4-Ringer's)	p.d.	29 ± 3.4	83 ± 6.8[c]	41 ± 7.0
(both sides) (8)	scc	91 ± 10	99 ± 11	110 ± 10
Chloride-free (SO_4-Ringer's)	p.d.	25 ± 3.4	27 ± 3.2	27 ± 2.4
(inside) (11)	scc	85 ± 9.9	41 ± 6.9[b]	73 ± 8.5

[a]This is a steady baseline value measured over a period of 30 min.

[b]$p < 0.01$ for differences from the initial period.

[c]$p < 0.001$ " " " " " "

Results are as means ± S.E. Number of experiments are in parenthesis.

bathing solution with sulfate Ringer's (no Cl) there was an increase in p.d. but no change in scc (Table 8), whereas replacing chloride with sulfate Ringer's on the inside bathing solution reduced the scc. These observations suggest, as confirmed by flux measurements (see later) that the chloride dependent sodium transport depends on the anionic composition of the bathing solution.

When both sides of the frog skin were bathed by chloride-free solutions (SO_4^{-2} Ringer's), 3% ethanol (added to the outside solution) had no significant effect on scc or p.d. (see Figure 7 lower curve and Table 9). These results indicate that the ethanol response is anionic dependent. Further observations indicated that the ethanol response was dependent only on the presence of chloride in the outside bathing solution (Table 9), which is the same side the alcohol is added. This is quite different from our observations that the transepithelial transport of sodium was dependent on the presence of chloride in the inside bathing solution. Therefore, this interaction between chloride and the ethanol response must involve some other mechanism.

To substantiate this observation, that the ethanol effect was dependent on the presence of Cl, additional studies were done on frog skin preparations bathed in solutions containing nitrate substituted for chloride. Under these conditions ethanol also failed to decrease the scc. The scc in nitrate solutions prior to the addition of 3% ethanol was 16 ± 3.0 $\mu A/cm^2$ (10 experiments), and after the addition of the drug it was 15 ± 3.0 $\mu A/cm^2$. Ethanol was also found to be without an effect when NO_3^- was substituted

Table 9. Effect of ethanol on the p.d. (mV) and scc ($\mu A/3.8\ cm^2$) across isolated frog skin bathed in chloride-free (SO_4^{-2}) Ringer's solutions.

Treatment		Initial Cl-free	Cl-free + 3% ethanol (outside) after 30 min	Washout Cl-free after 30 min
Cl-free (both sides) (16)	p.d.	64 ± 3.4	67 ± 3.6	61 ± 3.7
	scc	40 ± 3.3	44 ± 4.0	38 ± 3.6
Cl-free (outside) (8)	p.d.	83 ± 6.8	80 ± 5.8	91 ± 5.5
	scc	99 ± 11	106 ± 14	122 ± 10
				Washout(outside only)after 30 min
Cl-free (inside) (11)	p.d.	24 ± 2.9	13 ± 1.3[a]	26 ± 2.5
	scc	54 ± 7.4	28 ± 3.8[a]	56 ± 4.6

[a] $p < 0.01$ for differences from initial values.
Results are as means ± S.E.
Number of experiments are in parenthesis.

for chloride in the outer solution only.

Certain agents have been useful in helping elucidate the mechanism of chloride transport in many biological membranes. It has been found, for instance, that the Cl^- permeability of many membranes can be reduced by certain anions such as thiocyanate (Epstein, Maetz and De Renzis, 1971; Kristensen, 1972) and furosemide (Burg, Stoner, Cardinal and Green, 1971; Candia, 1973), as well as copper (Lyon, 1974). The effects of ethanol, therefore, were studied on frog skins pretreated with sodium thiocyanate (20 mM in the external bathing solution) and furosemide (10^{-4}M also added to the outside solution). It was found, that ethanol (3% added to the outside solution) did not bring about a decline in the scc as normally seen in the absence of these agents (Table 10). It seems likely that the failure to observe the usual response of the frog skin to ethanol also reflects a lack of available chloride.

Two drugs which have also helped in understanding membrane transport processes have been the diuretic amiloride and the cardiac glycoside ouabain. The mechanism of action of these agents is fairly well known: amiloride blocks sodium entry at the outer barrier of the membrane (Biber, 1971) and ouabain is thought to block sodium transport by an inhibition of the membrane bound enzyme Na-K-activated ATPase, which is believed to be associated with the so-called 'Na-pump' (see for instance Schwartz, Lindenmayer and Allen, 1975). It was therefore interesting to see how their responses were effected by chloride-free solutions. It can be seen in Table 11, that the amiloride (10^{-5}M added to the outside bathing solution) and

Table 10. Effects of ethanol on the p.d. (mV) and scc ($\mu A/3.8\ cm^2$) across frog skin preparations pretreated with sodium thiocyanate and furosemide.

Anion added to outside		Period I[a] Initial	Period II + anion for 30 min	Period III anion + 3% ETOH for 30 min
20 mM Na thiocyanate (8)	p.d.	35 ± 5.4	60 ± 3.8[b]	35 ± 4.4[c]
	scc	54 ± 6.5	68 ± 9.8	63 ± 9.1
10^{-4}M furosemide (9)	p.d.	28 ± 4.1	47 ± 7.9[b]	35 ± 8.8
	scc	81 ± 14	99 ± 15	91 ± 17

[a]This is a steady baseline value measured over a period of 30 min.

[b]$p < 0.01$ for differences from initial values.

[c]$p < 0.01$ " " " Period 2 values.

Results are as means ± S.E.
Number of experiments are in parenthesis.

Table 11. Effects of amiloride and ouabain on the p.d. (mV) and scc (μA/3.8 cm^2) across frog skin preparations bathed in chloride-free (SO_4^{-2}) Ringer's solution.

Treatment		Cl-free both sides	Cl-free + drug after 30 min
10^{-5}M Amiloride (6)	p.d.	65 ± 4.7	4 ± 2.8
	scc	40 ± 2.1	a
			60 min
10^{-4}M Ouabain (6)	p.d.	62 ± 4.3	4 ± 1.9
	scc	43 ± 2.1	a

[a]As the p.d. is so low a true measurement of the scc is not possible.
Results are as means ± S.E.
Number of experiments are in parenthesis.

the ouabain (10^{-4}M added to the inside bathing solution) responses were still observed when Cl^- was absent from the bathing solution.

The ability of ethanol to decrease the p.d. and scc across frog skin *in vitro* seems to be dependent on the presence of chloride. In addition, the observed scc was also found to be depressed in the absence of chloride. It therefore remained to be determined if chloride was involved in a direct or indirect manner. Unidirectional fluxes of sodium and chloride were thus measured.

3. Effect of ethanol on unidirectional sodium and chloride fluxes across frog skin.

Transepithelial sodium and chloride fluxes were measured simultaneously in the same direction (on the same frog skin preparation) using ^{24}Na and ^{36}Cl radioisotopes. Twenty-four experiments were performed: twelve in each direction. Ethanol (3%) decreased the influx of sodium (outside to inside) across the frog skin, but had no effect on the outflux (Table 12). The chloride fluxes in either direction were unaltered (Table 12). The decrease in the sodium influx measured could account for the observed decline in scc when both values are expressed as $\mu A/cm^2$. The decrease in sodium influx was 19.2 ± 2.9 (means ± S.E.) while the drop in scc was 17.0 ± 3.1 $\mu A/cm^2$. Therefore the effect of ethanol on scc appears to be by an inhibition of the sodium influx and that transmural movements of chloride are not contributing to the overall response.

Table 12. Effect of 3% ethanol on unidirectional fluxes across isolated frog skin bathed in NaCl Ringer's solution

$\mu eq/cm^2 hr$

Flux	30 min before ethanol	30 min after ethanol
Sodium:		
influx (outside to inside)	1.9 ± 0.15	1.2 ± 0.13[a]
outflux (inside to outside)	0.4 ± 0.03	0.4 ± 0.02
Chloride:		
influx	0.68 ± 0.09	0.68 ± 0.09
outflux	0.64 ± 0.09	0.69 ± 0.09

[a] $p < 0.01$ for differences from values obtained prior to the ethanol addition.
Results are as means ± S.E.
Represents 12 paired experiments (12 influx; 12 outflux)
The ethanol was added to the external bathing solution.

However, to confirm that ion permeability across the frog skin is unchanged by ethanol in the absence of chloride, unidirectional sodium fluxes were measured using skins bathed in chloride-free solutions. The sodium influx was much less than observed when chloride was present. Under these conditions, ethanol did not alter the sodium fluxes in either direction (Table 13).

These results indicate that chloride must be involved indirectly and the ethanol response is dependent on its presence. This also suggests that sodium transport may be linked to the presence of chloride.

This effect of chloride on the ethanol response was further investigated with a higher ethanol concentration to see if a change in transmural movements of chloride may be detected. The unidirectional fluxes of chloride in both directions were increased in the presence of 9% ethanol (added to the outside solution) (Table 14). In addition, the sodium influx as decreased as observed with the lower ethanol concentration (Table 14). Sodium thiocyanate was also added to these frog skins two hours following the administration of 9% ethanol. As you recall thiocyanate inhibits Cl transport, and it was hoped that this may show up in the flux measurements. It was observed that the unidirectional Cl fluxes decreased in both directions. The influx decreased from 1.16 ± 0.25 $\mu eq/cm^2 hr$ to 0.40 ± 0.11 whereas the outflux decreased from 1.12 ± 0.15 $\mu eq/cm^2 hr$ to 0.46 ± 0.09 (means ± S.E.)

Table 13. Effect of 3% ethanol on unidirectional fluxes across frog skin bathed in chloride-free (SO_4^{-2}) Ringer's.

$\mu eq/cm^2hr$

Sodium flux	30 min before ethanol	30 min after ethanol
Influx	0.96 ± 0.06	0.89 ± 0.07
Outflux	0.30 ± 0.06	0.26 ± 0.04

Results are as means ± S.E. of 14 paired experiments (14 influx; 14 outflux) The ethanol was added to the external bathing solution.

Table 14. Effect of 9% ethanol on unidirectional fluxes of sodium and chloride across frog skin bathed in NaCl Ringer's.

$\mu eq/cm^2 hr$

Flux	30 min before ethanol	30 min after ethanol
Sodium:		
Influx	0.98 ± 0.08	0.49 ± 0.02[a]
Outflux	0.34 ± 0.05	0.32 ± 0.07
Chloride:		
Influx	0.59 ± 0.06	1.16 ± 0.25[b]
Outflux	0.61 ± 0.08	1.12 ± 0.15[b]

[a] $p < 0.001$ for difference from value obtained prior to the ethanol addition.
[b] $p < 0.05$ " " " " " " " " " "

Results are as means ± S.E. of 6 paired experiments (6 influx; 6 outflux)
The ethanol was added to the external bathing solution.

30 min after the addition of 20 mM sodium thiocyanate to the outside solution. This observation confirms the mechanism of action of thiocyanate as well as suggests that the increase in Cl fluxes produced by ethanol is probably due to an increase in its permeability.

4. Effect of ethanol on the vasotocin induced natriferic response in frog skin in vitro.

It has been suggested that ethanol may affect the 'Na-pump' by an inhibition of the Na-K-ATPase (Israel and Mardones, 1971; Hegyvary, 1973). Such an effect could block the natriferic response (increase in scc) to neurohypophysial hormones, such as vasotocin, as the 'Na-pump' at the corium side of the cell is usually rate-limiting to this process. Vasotocin appears to act by increasing the sodium permeability of the cell membrane at the mucosal surface of epithelial cells (Herrera and Curran, 1963; Civan and Frazier, 1968), which is also a site where ethanol could be acting. The effects of ethanol on the vasotocin-induced natriferic response were thus investigated. Vasotocin (AVT) (10 mU/ml) was added to the inside solution bathing the frog skin and after 30 min the scc increased considerably (Table 15). When 3% ethanol was added to these pretreated AVT skins, the p.d. and scc decreased by an amount similar to that when it is added in the absence of AVT; the response was not inhibited. In another series of experiments, ethanol was added prior to the administration of AVT so that the scc declined. In this case the subsequent increase in scc produced by AVT was similar to the increase in the absence of ethanol (Table 15), but the normal baseline scc remained

Table 15. Effect of ethanol on the vasotocin (AVT) induced natriferic response in frog skin.

scc in $\mu A/3.8\ cm^2$

	I Initial	II Vasotocin for 30 min	Difference[a] II vs I	III Ethanol for 15 min	Difference III vs II
AVT (10mU/ml)	107	149	+42 ± 6.6	106	-43 ± 9.9
		Ethanol 15 min		Vasotocin 30 min	
Ethanol (3%)	128	92	-36 ± 5.6	131	+39 ± 4.7

[a]Differences represent increase (+) or decrease (-) in scc from initial control levels.
Differences represented as means ± S.E. of eight paired experiments.

depressed. Vaostocin could not overcome the effect of the ethanol. This suggests that ethanol and AVT are working on different mechanisms in the membrane.

5. Effects of phospholipase C on the ethanol response.

The following experiments were designed so as to help elucidate the nature of the membrane site (s) which ethanol may be affecting. This membrane site can be thought of as a 'drug-receptor', although, a true definition of a receptor involves one in which an endogenous compound interacts with it, rather than some pharmacological agent. However, the term drug-receptor has been generally accepted when explaining a drug-membrane interaction. The isolation and characterization of such receptors is difficult mainly because interactions between drugs and membrane receptors involve, in general, only weak forces which are more difficult to study than the covalent reactions typical of many enzyme-substrate interactions. However, some fundamental questions concerning the nature of such drug-receptors may be obtained by leaving drug receptors intact in the membrane and by studying the effects of degrading enzymes on the pharmacological responsiveness of the tissue. Since the cell membrane consists of proteins and lipids, selective destruction of membrane components by enzymes followed by testing of drug responses might give clues about the nature of receptors and receptor action.

With this in mind, phospholipase C, an enzyme which produces phospholipolysis, was used to help elucidate the nature of the ethanol-membrane

site(s) or which may be termed the 'ethanol-receptor'. Since the electrical potential difference and short-circuit current across the isolated frog skin serves as a good indicator of the ethanol response, the effects of phospholipase C on these parameters was studied by either adding this enzyme prior to or after the addition of ethanol.

When phospholipase C (50 μg/ml) was added to both sides of the frog skin (mounted in Ussing-type chambers) the resting scc increased by about 50% (Table 16) whereas the p.d. did not change consistently. The resistance (p.d./scc) also dropped significantly. To see if the increase in scc was due to an increase in sodium transport, amiloride, which is known to inhibit Na transport, was added to these phospholipase stimulated tissues. As can be seen in Figure 8 the scc dropped to almost zero following the administration of 10^{-4}M amiloride. These results suggest that the increase in scc produced by phospholipase C results from an increase in sodium transport. When ethanol (3% in the outside bathing solution) was added to phospholipase pretreated skins (50 μg/ml both sides) the p.d. and scc decreased by an amount similar to that when it is added in the absence of phospholipase (Table 16); the response was not inhibited. In another series of experiments ethanol was added prior to the administration of phospholipase C so that the scc declined. In this case phospholipase C when added to these ethanol depressed skins increased the scc by an amount similar to that observed in the absence of ethanol (Table 16). This type of interaction is reminiscent of the AVT and ethanol response, that is, phospholipase C and ethanol are probably interacting with

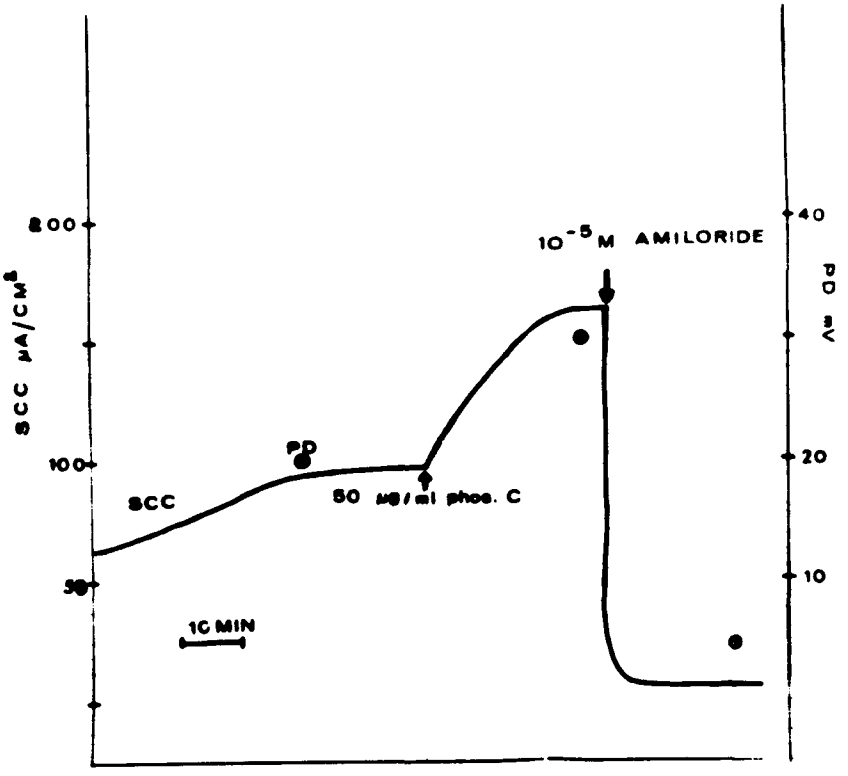

Figure 8. Effects of amiloride (10^{-5}M added to the outside solution) on the phospholipase C (phos. C) (50 μg/ml added to both sides) induced increase in short-circuit current (scc) across the isolated frog skin in vitro.

Table 16. Effect of phospholipase C on the p.d. (mV) and scc ($\mu A/cm^2$) across the isolated frog skin and its interaction with the ethanol response.

		Control	50 µg/ml phospholipase C (both sides) after 30 min	3% ethanol (both sides) after 30 min
(7)	p.d.	16 ± 3.2	21 ± 3.7	10 ± 2.2[c]
	scc	47 ± 5.1	71 ± 7.2[a]	41 ± 5.8[c]
			3% ethanol (outside) after 30 min	50 µg/ml phospholipase C (both sides) after 30 min
(6)	p.d.	28 ± 0.8	8 ± 1.7[b]	12 ± 1.3
	scc	48 ± 3.8	20 ± 3.3[b]	49 ± 6.5[d]

[a] $p < 0.01$ for differences from control value
[b] $p < 0.001$ " " " " "
[c] $p < 0.01$ for differences from phospholipase value
[d] $p < 0.01$ for differences from ethanol value

Results are as means ± S.E.
Number of experiments are in parenthesis.

different components in the membrane. Although ethanol is somewhat lipid soluble it is probably unlikely that the 'ethanol-receptor' contains an accessible phospholipid moiety which is necessary for the ethanol response.

6. Comparative effects of ethanol, amiloride and ouabain on sodium and potassium content of frog skin.

Changes in the intracellular content of ions can be helpful in locating the mechanism of action of drugs on epithelial membranes. Both ouabain and amiloride, like ethanol, inhibit Na transport across frog skin, but they act differently. Ouabain inhibits Na-K-ATPase and has been shown by a number of different methods, which include washout kinetics and tracer studies to increase the intracellular Na 'pool' (Biber, Cruz and Curran, 1972; Candia and Reinach, 1975). Amiloride, on the other hand, inhibits the entrance of Na at the outer barrier of frog skin and toad bladder (Bentley, 1968; Salako and Smith, 1970) and has been shown by some investigators to decrease the intracellular Na 'pool' (Salako and Smith, 1970; Nagel and Dorge, 1970; Biber, 1971). It was therefore interesting to see how ethanol affected the Na 'pool' as compared to these other drugs. For this experiment the isolated epithelium preparation was used (see Methods). It was advantageous to use this preparation as it contains only epithelial cells, with no connective tissues, and thus is more useful when trying to detect changes in intracellular ion content. It was found that ouabain (10^{-4}M) increased the intracellular Na content whereas K decreased (Table 17). Ethanol (3%) like amiloride (10^{-4}M), but in contrast to ouabain, was found to have

Table 17. Effects of ouabain, amiloride and ethanol on the Na and K content of isolated ('split-skin') epithelium.

meq/kg initial wet weight[a]

Treatment		Control	+ Drug[b] after 60 min	p
10^{-4}M Ouabain (9)	Na	51 ± 3.1	82 ± 2.6	< 0.001
	K	60 ± 2.2	32 ± 1.6	< 0.001
10^{-4}M Amiloride	Na	48 ± 5.4	45 ± 7.9	N.S.
	K	63 ± 3.8	60 ± 3.9	N.S.
3% Ethanol	Na	38 ± 6.9	39 ± 4.0	N.S.
	K	58 ± 4.3	59 ± 3.4	N.S.

[a]Final values after extracellular space has been subtracted.
[b]Represents paired skins from the same animal

Results are as means ± S.E.
Number of experiments are in parenthesis.

no significant effect on intracellular Na or K content. The results with amiloride are not consistent with those of some previous investigators and this may reflect differences in the methods used.

7. Effect of ethanol on the permeability of the frog skin to water.

It has been previously shown in another amphibian membrane, the toad urinary bladder, that ethanol and water follow similar pathways (Schucter, Franki and Hays, 1973). Ethanol, also inhibits the movement of water across the toad bladder (see later). It was therefore interesting to see if ethanol had comparable effects on water transfer in frog skin as this may give some clue as to its mechanism of action and may also add to our understanding of how it may affect sodium transfer across this amphibian tissue.

The unidirectional flux of water (measured in the absence of an osmotic gradient) across the frog skin was reduced by nearly 30% in the presence of 3% ethanol (on both sides of the membrane) (Table 18) as seen in the toad bladder. This response was found to be reversible following washout of the ethanol.

8. Permeability of the frog skin to ethanol.

The determination of the permeability of the frog skin to ethanol was important to several aspects of our studies. If ethanol was exerting a classical osmotic-type effect, one would expect that its rate of transfer would be low, especially when compared to more permeant solutes and water. In addition, as ethanol may utilize the same pathways as water, such

Table 18. Effect of ethanol on the diffusion of water across frog skin.

	Water transfer $\mu l/cm^2 hr$		
	Control[a] Period I	+ ethanol Period II	Washout (ETOH-free)
3% Ethanol	308 ± 27	221 ± 19[b]	310 ± 22

[a]Each period is the mean of 5 successive, measured over 5 min.

[b]$p < 0.05$ for differences from the control values.

Results are as means ± S.E. of six experiments.

measurements may suggest possible mechanisms whereby ethanol inhibits the transfer of the latter and could provide information about the nature of the ionic and water pathways in frog skin, as both of these are affected by ethanol.

The permeability of the frog skin to ethanol was found to be 356 ± 36 x 10^{-7} cm/sec (8 exps) (represented as K_{trans}, the permeability coefficient). The permeability coefficient for water was 3111 ± 280 x 10^{-7} cm/sec (6 exps) when measured under identical experimental conditions. In addition the permeability coefficient of ethanol was measured when the frog skin was bathed in chloride-free solutions (both sides), to see if its permeability was altered under conditions which abolished the effect of the drug on Na influx. The K_{trans} for ethanol in chloride-free (sulfate Ringer's) was 336 ± 30 x 10^{-7} cm/sec (8 exps). There was no significant difference in the rate of transfer of ethanol in chloride-free Ringer's as compared to the control.

9. Effects of other aliphatic alcohols on the electrical parameters of the frog skin.

To gain additional insight as to the nature of the 'receptor-site(s)' involved in the ethanol response a series of aliphatic alcohols were used to see if alteration in the structure, that is, changing the length of the carbon chain, will alter the response. Methanol, 2-propanol and n-butanol were studied as to their effects on the electrical p.d. and scc of the isolated frog skin. Equimolar concentrations of these alcohols

compared to ethanol were used so as to normalize the results. Three concentrations were chosen: 22 mM, 220 mM and 650 mM, which are consistent with the concentrations used for ethanol (0.1%, 1%, 3%). As can be seen from Table 19, methanol decreases the p.d. and scc only at a concentration of 650 mM, whereas an effect of ethanol was observed at a concentration of 220 mM (1%). 2-Propanol also decreases the electrical parameters across the frog skin at a 220 mM concentration, however, the magnitude of the response is greater than that observed with ethanol. N-butanol was found to inhibit the p.d. and scc at a concentration as low as 22 mM, which is quite different than that observed for the other alcohols studied. In addition, n-butanol produced irreversible effects when added to the outside solution at a concentration of 650 mM, whereas the other alcohols were found to be reversible. These observations seem to suggest that by increasing the carbon chain length, that is, increase the alcohol's lipid solubility, the effects on the transport processes across frog skin increases. Since the oil/water partition coefficient is a measure of lipid solubility, the higher the coefficient the greater the response. It is therefore possible that lipids may play a prominent role in the ethanol response and make up an essential part of the membrane site(s) in which it interacts.

10. Effects of acetaldehyde on the electrical behavior of the isolated frog skin.

Acetaldehyde is the oxidized product of the metabolism of ethanol. The primary step is the oxidation of ethanol to acetaldehyde by the

Table 19. Effects of other aliphatic alcohols on the p.d. (mV) and scc (μA/3.8 cm^2) across the isolated frog skin.

Alcohol[a]		Initial	+ Alcohol after 30 min
Methanol:			
(6) 22 mM	p.d.	44 ± 3.3	42 ± 3.5
	scc	122 ± 6.7	120 ± 7.2
(6) 220 mM	p.d.	38 ± 3.8	31 ± 3.9
	scc	93 ± 17	88 ± 19
(6) 650 mM	p.d.	43 ± 2.4	30 ± 2.5[b]
	scc	113 ± 12	84 ± 11[b]
2-Propanol:			
(6) 22 mM	p.d.	40 ± 3.7	35 ± 4.1
	scc	121 ± 6.5	109 ± 7.7
(6) 220 mM	p.d.	41 ± 3.7	26 ± 3.9[b]
	scc	118 ± 11	70 ± 18[b]
(6) 650 mM	p.d.	36 ± 4.3	20 ± 1.9[b]
	scc	113 ± 15	53 ± 14[c]
n-Butanol:			
(6) 22 mM	p.d.	42 ± 2.5	30 ± 2.7[b]
	scc	128 ± 5.0	113 ± 5.0[b]
(6) 220 mM	p.d.	35 ± 3.1	18 ± 3.0[c]
	scc	102 ± 17	55 ± 13[c]
(6) 650 mM	p.d.	51 ± 2.5	5 ± 1.7[c]
	scc	122 ± 16	d

[a]The alcohols were added as equimolar concentration to that of ethanol.
[b]$p < 0.05$ for differences from the initial value.
[c]$p < 0.001$ " " " " " "
[d]As the p.d. was so low a true measurement of the scc was not possible.
Results are as means ± S.E. Number of experiments are in parenthesis.

liver enzyme alcohol dehydrogenase, which utilizes NAD as a hydrogen receptor (Wartburg, 1971). Acetaldehyde is eventually converted to acetyl coenzyme A, which is then oxidized through the citric acid cycle or utilized in various anabolic reactions involved in the synthesis of cholesterol, fatty acids and other tissue constituents. Many metabolic changes accompany or follow the metabolism of ethanol. Some changes seem to be a direct consequence of the increased NADH:NAD ratio produced by the oxidation of the ethanol (Lieber, Rubin and DeCarli, 1971). However, most of the oxidation of ethanol occurs in the liver (98%) and peripheral tissues apparently do not metabolize ethanol (Wallgren and Bary, 1970). But to satisfy a curiosity the effects of acetaldehyde on the p.d. and scc across isolated frog skin preparations were investigated to determine if part of the ethanol response was due to its metabolite. There is however a slight problem when working with acetaldehyde, as it is not very stable and can be oxidized quite readily, therefore, it was kept on ice at 0°C up until the time it was added to the chamber. However, once it was added to the bathing media (which is at 25°C) breakdown products would be formed. The results obtained with acetaldehyde were quite varied, which came as no surprise considering the fragile nature of the compound. When a decrease was observed in the scc and p.d., it was found to be slow in onset and irreversible. The concentration that caused this effect was 10 mM when added to the outside or inside bathing solutions. This was much lower than the concentration of ethanol that caused an effect (1% or about 200 mM). However, unlike the ethanol response, acetaldehyde still decreased the scc in skins bathed in chloride-

free media. These results suggest that the ethanol response in frog skin is not due to its metabolic product acetaldehyde, since ethanol's effects were found to be instantaneous in onset, reversible and not obtainable in chloride-free media.

TOAD BLADDER

1. Effects of ethanol on oxidative metabolism of the toad bladder.

Ethanol has fatal effects on man when the blood levels reach about 500 mg/100 ml. However, tissues in vitro appear to be able to withstand much higher concentrations before they succumb. In order to measure the tolerance of the toad's urinary bladder to high concentrations of ethanol we measured its oxygen consumption following 30 min exposure to different concentrations of the drug. As shown in Table 20, this tissue was found to have a remarkable ability to survive exposure to ethanol as the oxygen consumption was not irreversibly abolished until the concentration was 40% (v/v) (about 8M), while at 20% it had no effect. Oxygen consumption was also measured during concurrent exposure to the 20% ethanol, to see if there were transient effects, and it was also unchanged under these conditions. Thus the oxygen consumption in the presence of the ethanol was 98.8 ± 1.2% (6 experiments) of the control level, when the drug was absent. The concentrations of ethanol that were required to inhibit oxidative metabolism far exceeded those that we observed to alter the permeability of the membrane.

Table 20. Effects of ethanol on the oxygen consumption of the toad urinary bladder.

	Oxygen consumption μl O_2/g dry weight		
Ethanol concentration	Initial	after 30 min ethanol[a]	p for difference
40% (5)	16.2 ± 2.5	2.1 ± 1.0	< 0.001
30% (5)	20.3 ± 1.8	11.7 ± 2.1	< 0.05
20% (6)	19.0 ± 3.2	21.6 ± 3.2	N.S.

Results are as means ± S.E. Number of experiments are in parenthesis.

[a]No ethanol was present during the O_2 determinations, so that a change represents an irreversible inhibition. For simultaneous measurements see text.

N.S., not statistically significant ($p > 0.05$)

2. Effects of ethanol on the electrical properties of the toad bladder.

The toad urinary bladder exhibits a transmural electrical potential difference (p.d.), the serosal side being positive. This p.d. can be reduced to zero by the application of an external current, which is called the short-circuit current (scc). In the toad bladder the scc has been shown to be equivalent to the net (active) flux of sodium from the mucosal to the serosal side (Leaf, Anderson and Page, 1958). Neither the p.d. nor the scc were influenced by the presence of ethanol, on either side of the membrane, in concentrations up to 3% (650 mM). However, at a concentration of 9% (1.95 M) on the mucosal or serosal side of the bladder the p.d. dropped but the scc was not changed significantly (Table 21). This effect was not reversible when ethanol was subsequently excluded from the bathing solution. The electrical resistance (calculated as p.d./scc) decreased (Table 21). When 9% ethanol was added to both sides of the toad bladder simultaneously there was a prompt decline in p.d. which approached zero so that the scc could not be measured accurately. The resistance also dropped considerably (Table 21).

The decline in resistance suggests that corresponding changes in ion permeability are occurring. Unidirectional fluxes of Na and Cl were therefore measured in the presence of 9% ethanol, on the mucosal side, (Table 22) to see what ions may be involved in this response. It can be seen that while the unidirectional fluxes of sodium were unchanged those of the Cl were increased considerably. The Cl permeability of many mambranes can be reduced by certain anions such as thiocyanate (Epstein, Maetz

Table 21. Effects of ethanol on the p.d. (mV), scc (μA/100mg wet wt.) and resistance (KΩ-mg) of the toad urinary bladder *in vitro*.

9% Ethanol added		Initial	+ Ethanol after 15 min	Washout after 15 min
Mucosa (20)	p.d.	38 ± 3.6	18 ± 2.5[b]	18 ± 3.5
	scc	211 ± 27	206 ± 26	214 ± 38
	R	22 ± 3.9	9.0 ± 3.9[a]	8.3 ± 2.9
Serosa (25)	p.d.	52 ± 5.4	35 ± 4.5[b]	38 ± 4.8
	scc	218 ± 17	183 ± 19	198 ± 15
	R	27 ± 3.7	21 ± 3.4	21 ± 3.1
Both sides (7)	p.d.	33 ± 5.8	4 ± 0.6[c]	4 ± 0.6
	scc	167 ± 18	d	d
	R	21 ± 4.4	------	------

Results are as means ± S.E.

Number of experiments are in parenthesis.

[a] $p < 0.05$ for differences from initial values
[b] $p < 0.01$ " " " " "
[c] $p < 0.001$ " " " " "
[d] As the p.d. was so low a true measurement of scc is not possible.

Table 22. Effects of 9% ethanol (on the mucosal side) on the unidirectional fluxes of sodium and chloride across the toad urinary bladder.

		Fluxes $\mu eq/cm^2 hr$	
Mucosal to Serosal		Control	+ Ethanol (after 30 min)
Sodium	(5)	0.76 ± 0.13	0.75 ± 0.14
Chloride	(7)	0.06 ± 0.002	0.13 ± 0.02[b]
Serosal to Mucosal			
Sodium	(5)	0.17 ± 0.02	0.18 ± 0.03
Chloride	(8)	0.06 ± 0.005	0.18 ± 0.03[b]

[b] $p < 0.01$ for differences from control period.

Results are as means ± S.E.

Number of experiments are in parenthesis.

and De Renzis, 1973; Kristensen, 1972), furosemide (Burg, Stoner, Cardinal and Green, 1973; Candia, 1973) as well as Cu^{2+} (Lyon, 1974). However, none of these solutes were found to reduce the permeability of the toad bladder to Cl nor did they reduce or prevent the increase in chloride produced by ethanol.

The observed effects of ethanol could be mediated by several mechanisms. Increases in the osmotic concentrations of the solutions bathing the amphibian urinary bladder in vitro has been shown to alter its electrical properties and permeability to ions (Bentley, 1964; Lipton, 1972; Urakabe, Handler and Orloff, 1970; Bentley, Candia, Parisi and Saladino, 1973). Small increases in the osmotic concentrations of the fluids at the serosal side of the bladder decrease the p.d., scc and active Na transport. When 9% ethanol was placed on the serosal side of the bladder only the p.d. was decreased while the scc was unchanged. This response is not comparable to the osmotic type response described previously.

In order to see if the effects of ethanol on the mucosal side of the bladder were consistent with an osmotic effect on the tissue, the actions of an impermeant solute, mannitol, were compared to it. Mannitol, at much lower concentrations (100 mM) than ethanol, decreased the p.d. but did not change the scc when present at the mucosal surface of the bladder. Thus the initial p.d. in 10 experiments was 47 ± 6.1 mV and declined in 15 min to 17 ± 2.3 mV after the addition of mannitol. The scc was 177 ± 22 μA/100 mg tissue before and 141 ± 26 uA/100 mg tissue after the mannitol was added to the mucosal bathing solutions. This effect of mannitol is thus similar to that of

ethanol but, in contrast, the p.d. of mannitol-treated bladders returned to normal after the added solute was removed from the bathing solution (the p.d. after washout returned to 37 ± 5.3 mV). The measurement of unidirectional ion fluxes, in the presence of mannitol, also showed that of Na to be unchanged while Cl was increased (Table 23). The effects of ethanol and mannitol on the mucosal side of the bladder are thus somewhat similar though much higher concentrations of ethanol were needed to produce a response. This may reflect its more permeant character.

3. Effects of ethanol on the permeability of the toad bladder to water; responsiveness to vasopressin.

The toad bladder is permeable to water and this is increased in the presence of neurohypophysial hormones such as vasopressin (Bentley, 1958). It has been shown that this membrane is permeable to ethanol and both molecules may follow similar channels (Schuchter, Franki and Hays, 1973). It is also known that the structure of water may undergo alterations in the presence of alcohols (Franks and Ives, 1966), but the observations of Schuchter et al., (1973) suggest that an interaction could also occur during its passage across the epithelial membrane if both molecular species do follow a similar pathway.

No change in the rate of water movement down an osmotic gradient across the toad bladder could be detected in the presence of ethanol (Table 24). However, the rate of this water movement is small so that

Table 23. Effects of mannitol (on the mucosal side) on the unidirectional fluxes of Na and Cl across the toad urinary bladder.

Fluxes as $\mu eq/cm^2 hr$

Mucosal to Serosal	Control	+100 mM Mannitol (30 min)
Sodium (8)	0.68 ± 0.04	0.69 ± 0.03
Chloride (6)	0.06 ± 0.007	0.18 ± 0.04[b]
Serosal to Mucosal		
Sodium (5)	0.05 ± 0.005	0.07 ± 0.01
Chloride (6)	0.06 ± 0.003	0.17 ± 0.01[b]

[b] $p < 0.01$ for differences from control period.
Results are as means ± S.E.
Number of experiments in parenthesis.

Table 24. Effects of ethanol on osmotic water movement across the toad urinary bladder.

	Water loss as mg/hr/hemi bladder	
Ethanol concentration	Control	Ethanol
3% ethanol, both sides	29 ± 4.4	26 ± 4.6
9% ethanol, both sides	39 ± 6.7	31 ± 4.1

Results are as means ± S.E of 6 experiments at each concentration.

the results are equivocal. The movement of water across the bladder, however, can also be measured with the aid of tritiated-water and this may provide a more sensitive method for detecting an interaction between the water and ethanol. Such measurements are shown in Table 25. It can be seen that the unidirectional flux of water (measured in the absence of an osmotic gradient) was reduced by nearly 30% in the presence of 3% ethanol (on both sides of the membrane).

The osmotic movement of water across the toad urinary bladder in the presence of vasopressin (the hydro-osmotic effect) was reduced by about 70% when 3% ethanol was present on both sides of the membrane (Table 26). The response to vasopressin returned to control levels when the alcohol was subsequently excluded from the bathing solutions. One percent ethanol had no effect on the hydro-osmotic response (Table 26).

While ethanol inhibited the net osmotic water movement across the bladder by about 70% in the presence of vasopressin, the unidirectional water flux, in the absence of the hormone and the osmotic gradient, was only reduced by about 30%. As will be discussed later there are several possible explanations for this apparent inconsistency. These include a difference in the nature of the water channels that may mediate each process or it could reflect the different experimental conditions, especially since unidirectional THO fluxes are measured in the absence of an osmotic gradient. Therefore, unidirectional water fluxes (due to diffusion, with no osmotic gradient present) were also measured in the presence of vasopressin, in order to see if the inhibitory effects of the

Table 25. Effects of ethanol (3% both sides) on the diffusion of water across the toad urinary bladder (mucosa → serosa) in the presence and absence of vasopressin (10 mU/ml).

Water transfer $\mu l/cm^2 hr$

	Control	+ Ethanol		Control	
	[a]Period 1	Period 2	Diff. 1-2	Period 3	Diff. 1-3
Vasopressin absent (5)	549 ± 112	396 ± 89	153 ± 29[b]	490 ± 106	59 ± 32 N.S.
Vasopressin present (6)	1877 ± 259	1204 ± 116	673 ± 114[b]		

[a]Each period is the mean of 5 successive, measured over 2.5 minutes

Results are as means ± S.E.

Number of experiments are in parenthesis.

[b]$p < 0.01$ for differences from the control values.

Table 26. Effects of ethanol on the hydro-osmotic response of the toad bladder to vasopressin (10mU/ml).

	Water transfer mg/30 min		
	Period I vasopressin	Period II vasopressin + ethanol	Period III vasopressin
[a]1% ethanol both sides	1035 ± 81	735 ± 31	736 ± 39
Control	1190 ± 65	1025 ± 95	836 ± 39
3% ethanol both sides	992 ± 113	289 ± 27	708 ± 56
Control	991 ± 45	917 ± 68	725 ± 82

Results are as means ± S.E.

Number of experiments are in parenthesis.

[a]Each group of experiments were performed on 6 paired lobes of the bladder.

ethanol were quantitatively comparable under both types of conditions. As shown in Table 26 the unidirectional flux of water increased about three-fold in the presence of the hormone, and this is comparable to previous observations (Leaf and Hays, 1962; Schuchter et al., 1973). The ethanol clearly reduced the unidirectional flux of water, but the magnitude of the effect, a decline of about 35% was comparable to that observed for the unidirectional flux in the absence of the vasopressin. Thus the water-channels that are apparent under iso-osmotic conditions, as present for the measurement of unidirectional fluxes, exhibit a similar quantitative response to the ethanol in both the presence and absence of vasopressin.

The effect of vasopressin on the amphibian bladder involves a complex series of changes, which include the activation of adenyl cyclase, the formation of cyclic-AMP (Orloff and Handler, 1967) and an increased permeability of the cell membrane at the mucosal surface of the cell (Civan and Frazier, 1968). Thus the ethanol could be inhibiting the rate of water movement by acting at several sites. It was found that the effect of cyclic-AMP in increasing permeability to water was also inhibited by the ethanol (Table 27) suggesting that its action occurs subsequent to the endogenous formation of this nucleotide. It is possible that this may involve the effector mechanism which is thought to be present at the mucosal barrier of the cell.

Vasopressin also increases the scc across the toad bladder. This effect is called the natriferic response and is also mediated by cyclic

Table 27. Effects of 3% ethanol on th hydro-osmotic response of the toad urinary bladder to 2 mM cyclic-AMP.

Water transfer mg/hr/hemi bladder	
Cyclic-AMP	Cyclic-AMP + 3% ethanol
554 ± 53	48 ± 7.3

Results are as means ± S.E. using 6 paired lobes of the toad bladder.

AMP (Bentley, 1960; Orloff and Handler, 1967). However, in contrast to its effects on water movement, the natriferic response was unaffected by the presence of 3% ethanol (Table 28). This suggests that the vasopressin-stimulated natriferic and hydro-osmotic responses are mediated via different mechanisms. Only the latter can be inhibited by ethanol, the sodium channel being unaffected.

In order to explore the possibility that the mucosal hydro-osmotic effector mechanism is inhibited by ethanol, we measured ethanol's effects on the osmotic movement of water across toad bladder preparations which were "fixed" during their exposure to vasopressin. The "fixing" of the bladders in such an osmotically permeable state can be brought about by exposing them to either glutaraldehyde or N-ethylmaleimide (see Methods) while they are under the stimulating influence of vasopressin (Eggena, 1972; Bentley, 1973). Water movement can thus be measured in the absence of the hormone, but with the hydro-osmotic effector mechanism still in its operational condition. The tissues, as judged by their lack of oxygen consumption, appear to be dead under these conditions. Water movement across these "fixed" bladders was also found to be reduced by 3% ethanol (Table 29) suggesting that it is either interacting with the membrane to impede the flow of water or that changes in the properties of the water itself, such as in its structure, are occurring which impedes its movement.

Physicochemically (see for instance Franks and Ives, 1966), it would be expected that the movement of water molecules in alcohol/water mixtures would be greater than in water alone. Alcohol, which is capable of

Table 28. Effects of ethanol (3% v/v on both sides of the bladder) on the natriferic response to vasopressin 10 mU/ml.

scc μA/100 mg wet weight

		I Initial[a]	II + Vasopressin for 15 min	II-I Difference
Control	scc	194 ± 37	490 ± 78	295 ± 50
			+ Vasopressin & Ethanol	
+ 3% Ethanol in II		198 ± 40	455 ± 105	257 ± 71

Results are as means ± S.E. for 7 paired lobes of the bladder.

Table 29. Effects of ethanol (3% v/v both sides) on osmotic water movement across the toad bladder which is 'fixed' in a hydro-osmotically permeable state by exposure to NEM (10^{-3}M) or glutaraldehyde in the presence of vasopressin (10 mU/ml).

	Water transfer mg/30 min/hemi bladder		
'Fixative'	Control	+ Ethanol	p for difference
N-Ethylmaleimide (12)	426 ± 50	208 ± 27	< 0.001
Glutaraldehyde (10)	592 ± 41	329 ± 16	< 0.001

Results are as means ± S.E.

The order of exposure to the ethanol was done in a cross-over manner using half the preparations for each sequence.

Number of experiments are in parenthesis.

hydrogen bonding (Gary-Bobo, Di Polo and Solomon, 1969), may disrupt the water lattice making it less structured. Such an effect on water movement is not consistent with the decline in water movement that we observed. However, in order to investigate this possibility further, we measured the effects of ethanol on osmotic movement of water across artificial collodion membranes (see Methods).

Collodion sacs, containing about 3 ml. of water were bathed in solutions of 240 mM sucrose and the loss of water (from the sac) was measured in the presence and absence of 3% ethanol (added to both sides of the membrane). In the absence of the ethanol the water movement was 10.2 μl cm^{-2} hr^{-1} and this declined to 10.0 μl cm^{-2} hr^{-1} with ethanol present; the mean difference 0.2 $\pm$ 1.7 (5) is not a statistically significant change.

Permeability of the toad urinary bladder to ethanol.

The toad bladder is permeable to ethanol (Leaf and Hays, 1962; Schuchter et al., 1973). The magnitude of this process, under the conditions of our experiments, is relevant to several aspects of our studies. This includes its rate of transfer across the membrane as compared to that of water. This may give an indication as to its possible osmotic effects and in addition, as it may utilize the same pathway as water, suggest its ability to physically interfere with the passage of the latter.

The permeability of the bladder to ethanol was found to be 1314 $\pm$ 217 x 10^{-7} cm/sec (7) (represented as K_{trans}, the permeability coefficient).

The permeability coefficient for water was 5545 ± 1132 x 10^{-7} cm/sec (7) when measured under identical experimental conditions.

If, as has been suggested, ethanol and water follow similar channels across the toad bladder, then it would not be surprising to observe that vasopressin, under the conditions in our experiment, may also increase the movement of the ethanol. The unidirectional flux of ethanol increased by about 35% in the presence of vasopressin (Table 30). This is about 10-times less than the increase in permeability to water that is observed in the presence of this hormone (Table 30). A similar difference has been described previously (Leaf and Hays, 1962; Schucter et al., 1973).

TOAD LENS

1. Under the in vivo situation, the lens is subject to changes in the osmotic concentration of the fluids that bathe its anterior and posterior surfaces. This can occur from the administration of osmotic agents which are used to decrease the intraocular pressure such as during surgery and the treatment of glaucoma. Among the agents used for this purpose is ethanol, however, more effective solutes, such as urea and mannitol, are used more often.

Since the toad lens serves as an excellent in vitro preparation for studying such changes in the osmotic composition of the fluids bathing the lens, the result presented here involves the effects of osmotic

Table 30. Effects of vasopressin (10 mU/ml) on the permeability of the toad bladder to ethanol as compared to water.

K_{trans} in cm/sec

	K_{trans}	p for difference
Ethanol		
control (7)	$1314 \pm 217 \times 10^{-7}$	< 0.01
vasopressin (7)	$2008 \pm 339 \times 10^{-7}$	
Water		
control (5)	$5545 \pm 1132 \times 10^{-7}$	< 0.001
vasopressin (6)	$18959 \pm 2616 \times 10^{-7}$	

agents (which includes ethanol) on the electrical properties across the isolated toad lens.

1. Effects of increased osmotic concentration on the translenticular PD, SCC, and resistance.

When the NaCl concentration in the fluid bathing the anterior side of the lens was increased by 12.5 mM there was a sharp decline in the translenticular p.d. and SCC (Fig. 9). These effects increased as the concentration was raised, were maximal in about 15 min, and returned to normal values when the original Ringer's solution was restored (Fig. 9). To see if this effect was due to an elevation of NaCl concentration alone or reflected an osmotic effect, the experiment was repeated but using mannitol in similar osmotic concentrations. Mannitol produced similar effects as that of NaCl (Table 31 and Fig. 10) suggesting that the effects are osmotic rather than the result of changing gradients of NaCl concentration across the lens membranes. The posterior surface of the lens did not respond in this way to changes in solute levels (Table 31) though a slow decline in SCC cannot be excluded. The electrical resistance across the entire lens did not change significantly with either the administration of NaCl or mannitol (Table 32).

The effects of mannitol were of additional interest, as this is used therapeutically to lower intraocular pressure, reduce cerebral edema, and to promote osmotic diuresis. Ethanol and urea are also known to be effective agents for the former purpose (Peczon, 1965;

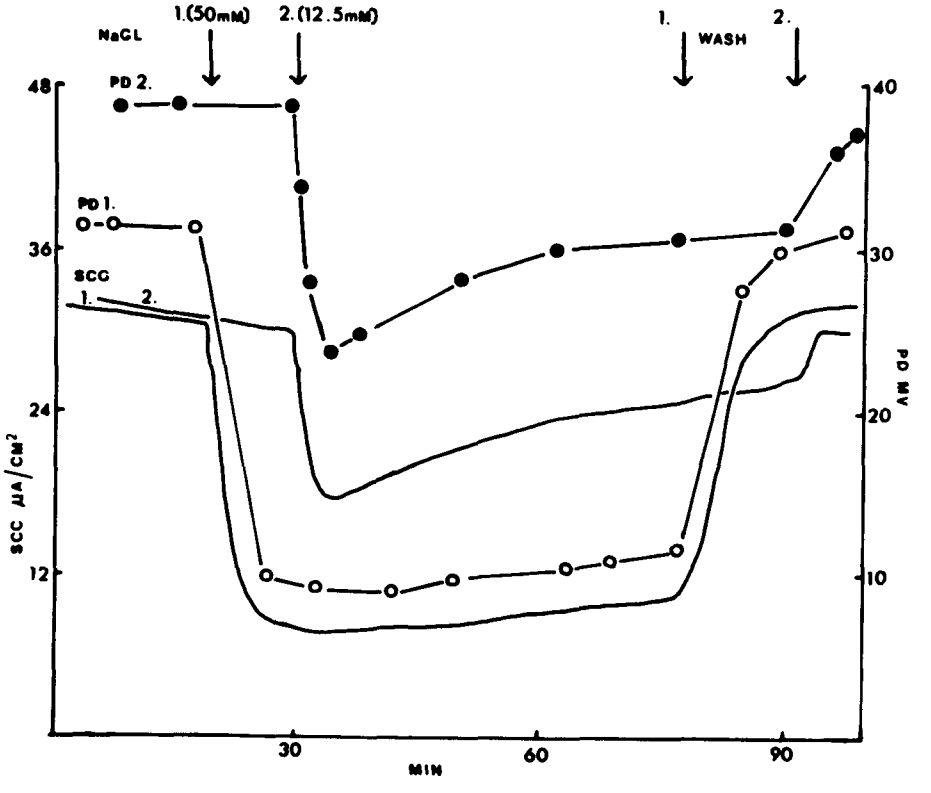

Figure 9. Effects of hyperosmotic NaCl solutions on the translenticular p.d., O—O, and short-circuit current (scc), ——, across the amphibian lens.

Table 31. Effects of hyperosmotic agents on the p.d. (mV) and scc ($\mu A/cm^2$) across the isolated toad lens.

Treatment			Initial[a]	+ Drug after 30 min	Washout Drug-free after 30 min
Drug added to anterior					
50 mM NaCl	(7)	p.d.	22 ± 1.7	4.9 ± 1.2[b]	26 ± 2.7
		scc	25 ± 3.8	5.2 ± 1.5[b]	28 ± 3.9
100 mM Mannitol	(8)	p.d.	26 ± 1.4	8.8 ± 1.6[b]	21 ± 3.6
		scc	34 ± 2.7	12 ± 1.8[b]	28 ± 4.8
200 mM Urea	(7)	p.d.	29 ± 3.4	9.0 ± 2.7[b]	8 ± 3.4
		scc	33 ± 3.4	9.1 ± 2.3[b]	7 ± 3.3
10% Ethanol (about 2M)	(8)	p.d.	27 ± 3.9	18 ± 3.5[c]	20 ± 3.8
		scc	35 ± 4.8	20 ± 3.5[c]	22 ± 3.4
Drug added to posterior					
50 mM NaCl	(7)	p.d.	23 ± 3.0	20 ± 3.1	14 ± 3.0
		scc	29 ± 5.4	23 ± 4.5	14 ± 3.3
100 mM Mannitol	(8)	p.d.	23 ± 2.0	26 ± 2.3	20 ± 1.9
		scc	27 ± 3.3	29 ± 2.3	22 ± 2.1
200 mM Urea	(7)	p.d.	23 ± 1.5	24 ± 2.2	21 ± 2.4
		scc	27 ± 2.9	23 ± 3.2	19 ± 2.7
10% Ethanol	(8)	p.d.	26 ± 2.1	23 ± 2.6	26 ± 2.6
		scc	31 ± 2.1	28 ± 2.4	30 ± 2.5

[a]This represents a baseline value measured over a period of 30 min.

[b]$p < 0.001$ for differences from te initial values .

Results are as means ± S.E.
Number of experiments are in parenthesis.

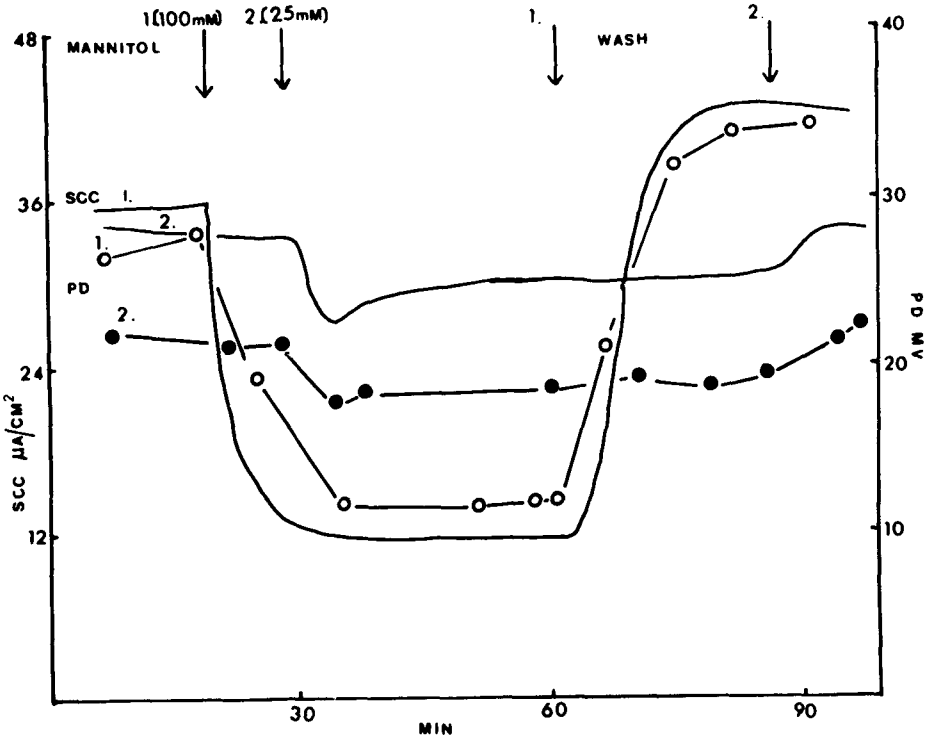

Figure 10. Effects of hyperosmotic mannitol solutions on the translenticular p.d., o—o, and scc, ——, across the amphibian lens.

Table 32. Effects of osmotic agents on the electrical resistance across the amphibian lens

$\Omega - cm^2$

Drug added to ant.	I Initial	II Drug-30 min	III Washout	II-I P
50 mM NaCl (7)	943 ± 75	1019 ± 89	969 ± 71	N.S.
100 mM Mannitol (8)	770 ± 32	707 ± 67	723 ± 34	N.S.
200 mM Urea (7)	933 ± 189	1446 ± 164	1301 ± 209	N.S.
10% Ethanol (8)	794 ± 64	930 ± 98	934 ± 91	N.S.

Results are as means ± S.E.
Number of experiments are in parenthesis.
N.S., not statistically significant.

Houle and Grant, 1967), and their effects on the lens were also tested. Urea was only effective when used at a concentration of 200 mM in the anterior bathing solution (Table 31). Its effects were not reversible following the restoration of normal Ringer's solution. Ethanol had no effect on the electrical parameters of the lens when present at either side in concentrations of 3% (about 650 mM) or even 6% (v/v) but at a concentration of 10% a modest decline in p.d. and SCC were recorded, but only when it was present in the anterior bathing solution (Table 31). Like urea, the ethanol effects were not reversible. The electrical resistance across the entire lens increased with urea, whereas ethanol had no effect on this parameter (Table 32).

2. Effects of osmotic agents on the electrical parameters across the anterior (lens epithelium) and posterior sides of the lens.

The translenticular electrical behavior of the lens is the result of the properties of the limiting membranes at its anterior and posterior surfaces (see Duncan, 1973) and the lens fibers (Rae, 1974a). The anterior is covered by the lens epithelium which probably acts as the limiting barrier at this side. The posterior lacks such an epithelium; its junction is formed by the outer regions of the lens fibers, as well as the capsule which invests the entire lens. The two surfaces, not surprisingly, behave rather differently and when examined in a divided chamber in vitro, the anterior has a higher p.d. than the posterior, the

difference corresponding to the translenticular p.d. (Candia, Bentley and Mills, 1971). It is also possible that the lens fibers contribute to the electrical properties of the lens (Rae, 1974a). However, as the pipet electrode is placed in the central part of the tissue they probably do not influence its electrical asymmetry. When the concentration of the anterior bathing solution was increased with NaCl, mannitol, ethanol, or urea the p.d. across the anterior side of the lens declined sharply (Table 33 and for instance mannitol, Fig. 11) and this was accompanied by a smaller decline across the posterior so that the net effect was to decrease the translenticular p.d. When these same osmotic agents were added to the posterior side there was no effect on the p.d. across either side of the lens.

The electrical resistance of membranes reflects changes in the movements of ions across them. As shown in Table 34, the addition of mannitol to the anterior bathing media resulted in a rise in the electrical resistance across that side and this increase was accompanied by a small decline across the posterior side. Similar results were seen in the presence of NaCl. With the more permeant solutes ethanol and urea, somewhat different effects were observed, as the resistance did not increase until later, after the solute was removed (Table 34). This is not unexpected since these solutes also have irreversible effects on the lens.

Table 33. Effects of hyperosmotic agents on the electrical potential difference (p.d. mV) across the anterior and posterior lens membranes.

'Drug' added to ant.	I Control	II 'Drug'-30 min	III Washout
100 mM NaCl (6)			
Ant. p.d.	58 ± 3.2	30 ± 3.4[a]	65 ± 2.1
Post. p.d.	38 ± 4.2	24 ± 2.5[b]	40 ± 4.4
Overall p.d.	20 ± 1.6	6.0 ± 1.5[a]	25 ± 2.9
200 mM Mannitol (6)			
Ant. p.d.	51 ± 4.7	31 ± 3.4[a]	51 ± 5.3
Post. p.d.	27 ± 3.8	23 ± 2.3	30 ± 3.8
Overall p.d.	24 ± 1.8	8.0 ± 1.5[a]	21 ± 1.8
10% Ethanol (6)			
Ant. p.d.	58 ± 6.9	46 ± 5.4	52 ± 6.6
Post. p.d.	34 ± 6.0	32 ± 4.0	37 ± 4.8
Overall p.d.	25 ± 0.8	14 ± 2.1[a]	15 ± 2.2[a]
400 mM Urea (6)			
Ant. p.d.	61 ± 4.0	24 ± 2.7[a]	30 ± 4.4[a]
Post. p.d.	43 ± 2.5	30 ± 3.6[b]	32 ± 4.4[b]
Overall p.d.	20 ± 3.5	-5.9 ± 0.8[b]	-1.7 ± 0.7[b]

Results are as means ± S.E.
Number of expts. in parenthesis.
[a] $p < 0.01$ for differences from Control
[b] $p < 0.05$ for differences from Control

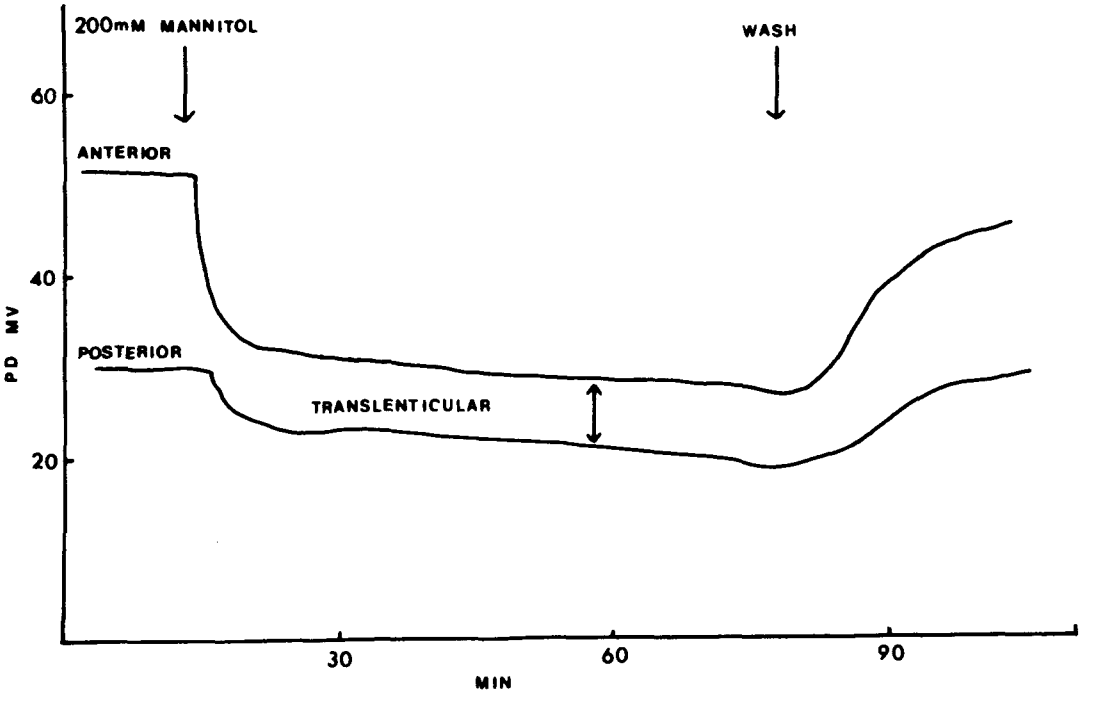

Figure 11. Effects of hyperosmotic mannitol on the p.d. across the anterior epithelium and posterior side of the lens.

Table 34. Effects of hyperosmoticity on the electrical resistance across the anterior and posterior lens membranes.

Resistance Ω-cm^2

Drug added to anterior side

Anterior resistance:		Initial	+ Drug after 30 min	Washout drug-free after 30 min
100 mM NaCl	(4)	401 ± 13	694 ± 52[a]	393 ± 22
200 mM Mannitol	(6)	492 ± 61	711 ± 94[b]	545 ± 47
200 mM Urea	(5)	513 ± 37	538 ± 47	832 ± 47[c]
10% Ethanol (2M)	(6)	537 ± 47	664 ± 89	842 ± 60[c]
Posterior resistance:				
100 mM NaCl	(4)	469 ± 25	369 ± 15[b]	461 ± 13
200 mM Mannitol	(6)	553 ± 45	477 ± 42[b]	574 ± 58
200 mM Urea	(5)	582 ± 4	637 ± 34	519 ± 33
10% Ethanol	(6)	674 ± 75	672 ± 107	719 ± 105

Results are as means ± S.E.
Number of experiments are in parenthesis.
[a] $p < 0.01$ for difference from initial values
[b] $p < 0.05$ for difference from initial values
[c] $p < 0.001$ for difference from initial values

3. The ionic basis for the observed electrical changes with hyperosmotic agents.

The electrical behavior of the lens reflects the movements of ions across its anterior and posterior surfaces and this may involve the passive permeability of the membranes or the activity of ion pumps. Changes in the properties of the interspersed lens fibers could also be contributing.

The effects of the osmotic agents were thus examined when ionic changes were made in the bathing-media. Sodium is known to be actively transported transmurally across the lens, and it also moves passively in either direction. A sodium pump is thought to be located in the anterior lens epithelium which is responsible for the active transport. To see what extent the presence of Na contributed to the effects of these osmotic agents, their actions were examined with Na-free Ringer's solutions (choline substituted for Na) on each side of the lens.

(i) Effects of Na-free Ringer on PD and SCC. When the anterior side of the lens was bathed in Na-free Ringer's solution the PD and SCC declined slightly, probably reflecting some non-specific metabolic inhibition due to the absence of Na (Candia et al., 1971). The addition of mannitol caused a further decline (Fig. 12a), but this was much less than normally observed. The p.d. and SCC returned to normal levels when the Na was restored to the anterior side. Subsequent addition of the mannitol then caused a decline in the SCC such as observed in the earlier

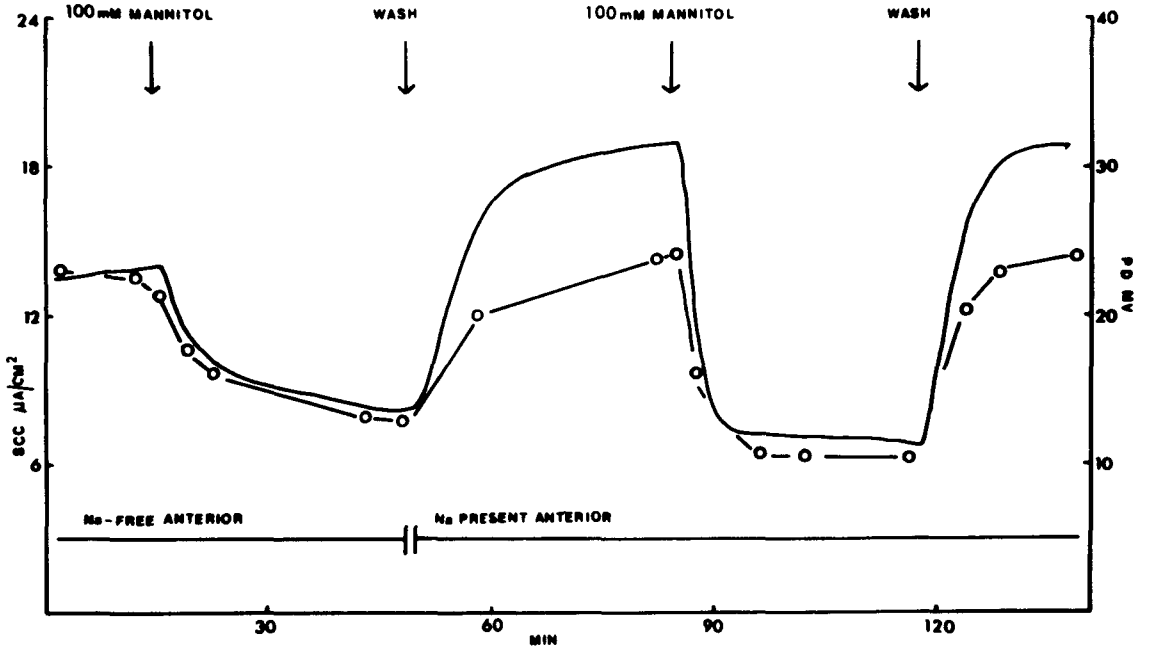

Figure 12. Effects of hyperosmotic mannitol on the translenticular p.d., o—o, and scc, ——.

a) In the presence and absence of sodium at the anterior surface of the lens.

b) In the absence of sodium at the posterior side.

experiments; it was much larger than that observed in the absence of the anterior Na. When the posterior side of the lens was bathed with Na-free Ringer's solution, there was a decline in the p.d. and SCC, which in magnitude (20 to 30%) was consistent with an abolition of the translenticular active Na transport (Candia et al., 1971) (Fig. 12b). Under these conditions mannitol, on the anterior side, still exerted a prominent effect, but this was reduced by an amount similar to that of the initial drop seen when Na was excluded. Thus, the ability of mannitol to decrease the translenticular p.d. and SCC is not completely dependent on an inhibition of active translenticular Na transport, but the evidence suggests that it could be contributing. The effect of hyperosmotic agents may therefore be a dual one.

(ii) Effects of mannitol on electrical resistance in the presence of Na-free Ringer. To further explore the nature of the ionic changes occurring under hyperosmotic conditions, the electrical resistances across the anterior and posterior sides of the lens were examined when each surface was bathed with Na-free Ringer's solutions. The responses to mannitol were measured under these conditions. The presence of Na-free Ringer's solution at the anterior side of the lens resulted in an increased resistance across the anterior surface, but no change at the posterior (Table 35 and Fig. 13). When mannitol was then added to the anterior (Na-free) solution the electrical resistance across the anterior side of the lens nearly doubled; the magnitude

Table 35. Effects of mannitol on the anterior (lens epithelium) and posterior resistances as recorded by a microelectrode in the center of the toad lens in Na-free Ringer's solution.

as $\Omega \cdot cm^2$

	I Initial Na-present	II Na-free	II-I Diff	III + Mannitol	III-II Diff	IV Washout Na-present
	Anterior					
	411 ± 52	583 ± 76	172 ± 39	1082 ± 79	496 ± 62	422 ± 36
			$p < 0.005$		$p < 0.001$	
200 mM Mannitol added to anterior Na-free bathing solution (6)	Posterior					
	573 ± 33	579 ± 28	6 ± 17	448 ± 41	-132 ± 30	546 ± 25
					$p < 0.005$	

The results are as means ± S.E.
Number of experiments in parenthesis.

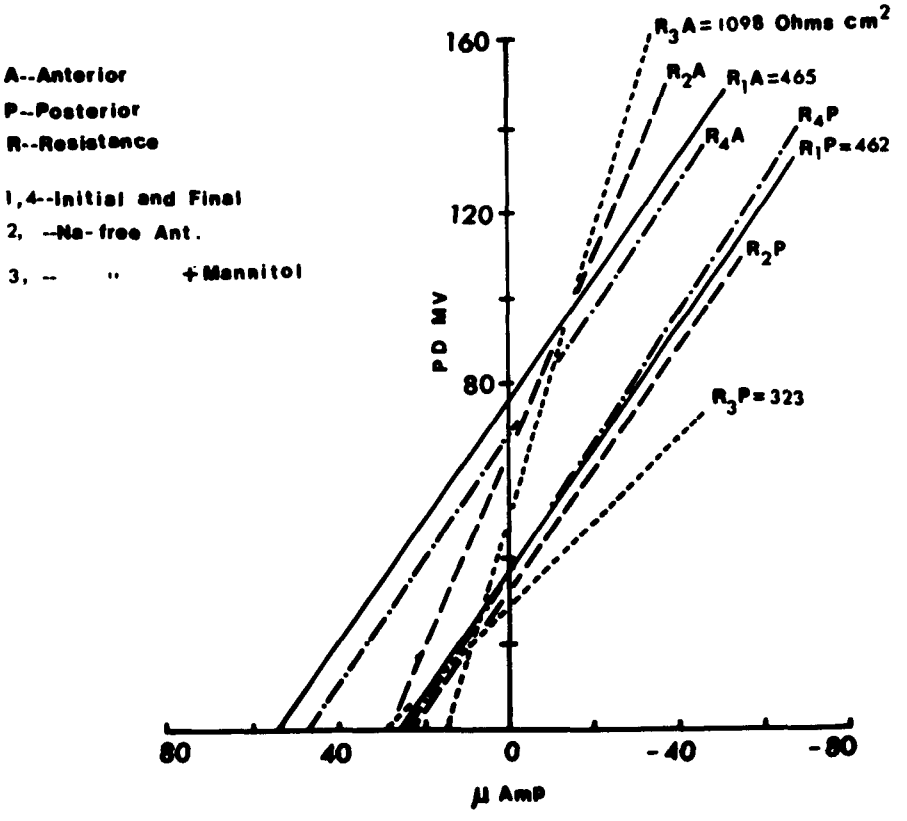

Figure 13. Effects of hyperosmotic mannitol on the electrical resistance across the anterior epithelium and posterior surface of the lens.

The resistances are computed from the slope of the voltage-current relationship (Δp.d./Δscc)

of this response is much greater than that previously observed in the presence of Na (Table 34). Such an increase in resistance would be consistent with an inhibition of ion movements which could involve the active Na pump. An osmotic disruption of the lens fibers could also be contributing to the change if, as suggested by Rae (1974b), they were normally electrically coupled and constituted a lower resistance pathway. There is, however, little evidence for this. The increased magnitude of the rise in resistance in the absence of Na may reflect the absence of an increase in Na backflux which normally accompanies inhibition of the Na pump. Subsequent evidence (see next section) supports this possibility. The resistance at the posterior side of the lens showed an accompanying decline (though much less in magnitude) which may be reflecting the accumulation of Na in the lens (see later).

(iii) Effects on Na accumulation by the lens. The effects of mannitol, ethanol, and urea on the Na and K content of the lens were examined. One lens was incubated in Ringer's solution to which had been added the osmotic agent while the other, paired, lens was used as a control and incubated for a similar time. The presence of mannitol and ethanol resulted in a considerable increase in the accumulation of sodium by the lens but no change in K (Table 36). In contrast urea had no significant effect on the sodium content of the lens and this is consistent (see later) with the observation that the translenticular

Table 36. Effects of osmotic agents on Na and K content of the amphibian lens

m-equiv./kg initial wet weight

Treatment	Control		+ Drug		P for Na Control vs Drug
	Na	K	Na	K	
100 mM Mannitol					
Both sides (6)	20.3 ± 1.4	53.8 ± 1.5	30.1 ± 2.4	56.6 ± 1.9	0.025
Posterior (6)	25.0 ± 1.3	50.6 ± 3.0	27.6 ± 2.1	46.0 ± 2.6	N.S.
10% Ethanol (v/v)					
Both sides (6)	25.2 ± 1.2	70.0 ± 3.4	35.3 ± 1.3	68.3 ± 1.5	0.005
Posterior (6)	33.5 ± 3.7	41.9 ± 4.6	26.8 ± 1.7	41.9 ± 3.0	N.S.
200 mM Urea					
Both sides (6)	19.8 ± 1.1	44.4 ± 1.3	20.0 ± 1.4	40.8 ± 1.6	N.S.

Results are as mean ± S.E.

Number of experiments in parenthesis

Paired lenses were used for this experiment, one as a control for the other.

sodium fluxes were only changed slightly in the presence of this solute. The response to mannitol and ethanol was not seen when the posterior side of the lens alone was exposed to it (in a divided chamber), so that the effect observed can be accounted for by its action at the anterior side (such as previously seen in its effect on the electrical parameters).

The failure of the K content to change, despite the added accumulation of Na, was somewhat surprising as in previous experiments with inhibitors such as ouabain and iodoacetate an accumulation of sodium by the lens was invariably accompanied by a parallel loss of K (Toyofuku and Bentley, 1970). It therefore seemed possible that the Na was accumulating in the extracellular space. Measurements of this, using inulin, failed to demonstrate any change; it was 4.5 ± 0.9% (6) of initial wet weight in the control and 4.3 ± 0.7% after exposure to the mannitol. Therefore, it would thus appear that Na is entering the cells.

To see if changes in water content of the lens were contributing to the osmotic equilibration we measured the weights of lenses after placing them in hyperosmotic solutions. It was found that mannitol and NaCl, but not ethanol or urea produced a 5% loss in weight as compared to the control (Table 37). However, this loss occurred gradually over a prolonged period of time (3 hr) and was not a sufficient decline to balance the osmotic effect produced by these agents. This would suggest that the osmotic balance is not being significantly adjusted by the removal of water from the tissue. Therefore under these

Table 37. Effects of osmotic agents on water loss (as weight loss) from the amphibian lens *in vitro* as % of initial wet weight

Treatment	Control	+ Drug (180 min)	P
50 mM NaCl (5)	100 ± 0.6	95.5 ± 0.8	< 0.01
100 mM Mannitol (5)	98.2 ± 0.4	94.5 ± 0.6	< 0.01
10% Ethanol (5)	98.9 ± 1.0	99.4 ± 0.5	N.S.
200 mM Urea (6)	100.4 ± 1.4	100.0 ± 1.06	N.S.

Results are as means ± S.E.
Number of experiments are in parenthesis.

conditions the influx of sodium would appear to be the predominant mechanism for osmotic adjustment in the lens. In the instance of the more permeant solutes, urea and ethanol, these presumably can enter the cell and contribute to such osmotic balance.

(iv) Effects on translenticular unidirectional Na fluxes. Sodium moves across the lens in either direction, but the unidirectional flux is normally greatest from the posterior to the anterior; a process that is consistent with an active Na transport mechanism. The effects of mannitol, ethanol, and urea on the unidirectional translenticular fluxes of Na^{22} were measured. Both mannitol and ethanol produced a considerable increase in the backflux, anterior to posterior, of Na (Table 38), but no significant change in the forward flux. The experiments were not done on paired lenses, but the changes were of such a magnitude as to suggest that net active translenticular Na transport was abolished. If the change in the backflux indicates an increased permeability to Na, which seems likely, then the failure of the forward flux to increase similarly is somewhat unexpected. If, however, the active Na pump were also inhibited (and the earlier resistance measurements are consistent with this possibility) then the observations can be interpreted satisfactorily. Urea also had similar effects although the magnitude was not as great (Table 38).

Table 38. Effects of osmotic agents on unidirectional Na fluxes across amphibian lens.

		$\mu eq/cm^2 hr$	
Drug added to anterior		Control[a]	60 min after drug
Anterior to Posterior			
100 mM Mannitol	(6)	0.15 ± .04	0.55 ± .08[b]
200 mM Urea	(6)	0.16 ± .04	0.36 ± .06[c]
10% Ethanol	(6)	0.17 ± .04	0.43 ± .04[b]
Posterior to Anterior			
100 mM Mannitol	(6)	0.63 ± .13	0.63 ± .06
200 mM Urea	(6)	0.46 ± .04	0.55 ± .04
10% Ethanol	(6)	0.33 ± .06	0.38 ± .04

Results are as means ± S.E. Number of experiments are in parenthesis.

[a] This represents a stable flux measured 90 min after the addition of the isotope.

[b] $p < 0.01$ for differences from the control values.

[c] $p < 0.05$ " " " " " "

4. The appearance of lens opacities in vitro in the presence of hyperosmotic agents.

Hyperosmotic conditions are known to produce reversible cataracts in vivo. In the present series of experiments using osmotic agents we observed the presence of lens opacities under some conditions. Mannitol had the most pronounced effects and caused clouding of the lens when present at the posterior but not the anterior side, even when little change in electrical behavior or electrolyte content was observed. This clouding was clearly seen within 2 or 3 minutes exposure to the mannitol; it increased with time and extended deeply into the lens tissue. After the removal of the added solute the lens gradually regained its clarity over a period of several hours. When present at the anterior side, it had no effect on opacity despite the prominent effects on p.d., SCC, and Na content. Under iso-osmotic conditions with mannitol present on both sides these opacities were still seen, and thus they apparently do not reflect a translenticular water movement. When lenses were incubated in the presence of 10% ethanol, a slight clouding was observed which appeared to be uniformly distributed over the capsular surface. 50 mM NaCl, or 200 mM urea had no observable effect on the lens transparency. In the present experiments no clear correlation between lens transparency and the electrical properties and changes in Na content of the lens were observed.

5. Measurements of the extracellular space of the isolated toad lens.

The extracellular space of the toad lens was measured as described in the methods section. Two extracellular markers were used, ^{14}C-inulin and ^{14}C-mannitol. It was found that the mannitol equilibrated with the lens space faster than inulin when the lenses were incubated in isotope containing media. However, a common incubation time of three hours was chosen since both markers gave stable space values by this time. The extracellular space was calculated as a per cent of the initial wet weight.

^{14}C-Mannitol consistantly gave slightly higher space values than that for ^{14}C-inulin (Table 39). Since mannitol is a smaller molecule than inulin it may equilibrate with those areas inulin cannot enter.

Additional studies were also done with lenses set up in a divided glass chamber (the same chanber that is used to measure electrical parameters (see Methods) so as to determine what fraction of the extracellular space was measurable from the anterior or posterior surfaces. If the epithelial cells of the anterior surface form tight junctions, this could represent a barrier to the diffusion of large molecules, such as inulin and mannitol. It would thus be possible to measure the space reached from the anterior solution to these tight junctions of the anterior epithelium. By adding the marker to the posterior bathing solution, one may measure the extracellular space across the lens from this solution up to the anterior epithelial tight junctions. This space probably consists of that between the lens fibers. To determine if there was a barrier for diffusion of the marker, fluxes of the inulin were

Table 39. Values of the extracellular space of the isolated lens using inulin and mannitol as markers

Lens incubation time		^{14}C-Inulin	^{14}C-Mannitol
90 min	(6)	3.7 ± 0.5[a]	5.3 ± 0.5
180 min	(6)	4.5 ± 0.9	6.8 ± 1.0

Results are as means ± S.E.
Number of experiments are in parenthesis.
[a]These values are as a percent of the initial wet weight.

measured simultaneously during the incubation period. It was found that the fluxes measured in either direction, were practically zero, as the counts measured on the cold side were only slightly above background. This suggests that there is a barrier, or tight junctions, which may block the diffusion of large molecules, such as inulin and mannitol which were used as extracellular markers in these experiments.

The extracellular space measured by adding inulin to either the anterior or posterior bathing solution gave values of 1.3 ± 0.2 and 3.5 ± 0.3 per cent of initial wet weight, respectively (6 paired experiments). The total space (represented as a sum of the two) was similar to that obtained when whole lens incubations were made (see Table 39). Only 25% of the extracellular space can be measured from the anterior bathing solution so that the anterior epithelium must contain the limiting barrier or tight junctions.

DISCUSSION:

FROG SKIN

The present results demonstrate that ethanol can alter the permeability of a model membrane system, the frog's skin, to ions and water. These effects are seen under a variety of conditions and appear to reflect different mechanisms involved in this tissues transport processes and permeability. The concentrations of ethanol that were effective in eliciting these responses, exceed those which are compatible with human life. As fatal effects in man occur at a concentration of 0.5% (blood level) even the 1% ethanol concentration used, the minimum concentration observed to have an effect on the electrical behavior of the frog skin, was still higher than that consistent with survival *in vivo* in man. The ability of the frog skin to withstand exposure of up to 10% ethanol without any effects on oxidative metabolism suggests that this tissue has a high tolerance to the toxicity of this substance. It is possible, that under *in vivo* conditions (in man), that some tissues, for instance brain cells, may have a lower tolerance level to ethanol and may thus play a determining role as to the minimum concentration threshold for survival. However, as we have demonstrated, the processes of oxidative metabolism of these epithelial cells appears to be intact under our conditions *in vitro*.

Active transport of sodium across the frog skin requires the expenditure of energy. This process accounts for about 35 per cent of the total oxygen consumption of this tissue, as was indicated by the decline

observed when the pieces of frog skin were incubated in Na-free (choline substituted for Na) Ringer's solution or amiloride (an inhibitor of Na transport) was added to NaCl containing media (see for instance Table 3). This drop in oxygen consumption in the absence of sodium transport is in agreement with previous observations with frog skin (Yorio and Bentley, 1973). Therefore, if ethanol was inhibiting sodium transport by an action on Na-K- activated ATPase as has been suggested (Israel, Kalant and Laufer, 1965; Israel, Kalant and LeBlanc, 1966; Sun and Samorajski, 1970; Hegyvary, 1973) a drop in oxygen consumption would have been observed. However, since ethanol effects the transport properties across the frog skin at lower concentrations than that which affected oxygen consumption, a direct effect on the Na-K-ATPase is not a probable mechanism of ethanol's action on this epithelial membrane. Similar conclusions have also been reached concerning ethanol's action in the central nervous system where it decreased the threshold for nerve stimulation (Nikander and Wallgren, 1970; Wallgren et al., 1974; Carmichael and Israel, 1975). Additional support against a general metabolic action is that ethanol's effects on the p.d. and scc across the frog skin were observed to be reversible and could even be blocked under some ionic conditions, and in the presence of anion blocking agents, such as, thiocyanate and furosemide. Also ouabain, which is a known inhibitor of sodium transport by an action on the Na-K-activated ATPase, produces a drop in oxygen consumption as well as retaining its effectiveness regardless of the anion substitutions made. This suggests that the effects of ethanol are not necessarily related to a metabolic action, but may involve a more direct physical interaction with this

epithelial membrane. While metabolic processes ultimately play a prominent role in the movement of molecules across epithelial membranes, they are often difficult to distinguish from those involving interactions with receptors and other membrane components. This makes the present observations potentially interesting as they cannot be related to a prominent metabolic change, though a more subtle such effect cannot be excluded.

Several effects of ethanol on the permeability properties of the frog skin were investigated and have been described.

The ability of ethanol to decrease the p.d. and scc across frog skin in vitro reflects a decline in the influx of sodium. This change is in the same direction as the action of the sodium pump, which is thought to be present at the inner boundary of the cell (Koefoed-Johnsen and Ussing, 1958). The mechanism of the decline of the sodium influx is unknown. It could be reducing the action of the Na-pump indirectly by reducing the entry of sodium across the outer barrier of the cell. In view of the absence of an ouabain-like action, the latter possibility is suggested.

The reduction of Na transport also does not seem to reflect a general metabolic inhibition (for example a cyanide and a DNP -like effect), as O_2 consumption was not significantly changed in the presence of ethanol. More subtle changes, such as an accumulation of metabolites of ethanol cannot however, be excluded. Our observations with acetaldehyde, the metabolite of ethanol, suggest that such an effect is not contributing to the ethanol response, since acetaldehyde, unlike ethanol, had

irreversible effects and the response was still obtained in chloride-free media. In addition, peripheral tissues do not readily metabolize ethanol (Wallgren and Bary, 1970), besides, the speed of onset and reversal of ethanol are difficult to reconcile with such an action.

Ethanol could be exerting an osmotic effect, due to the high concentrations used. However, Ussing (1965), has demonstrated in frog skin that hyperosmolar conditions in the outside bathing solution cause the epithelial cells to shrink. This shrinking is thought to increase pore size and the passive leakage of ions through the skin, including that of Na in the outward direction (Ussing and Windhager, 1964). This leakage represents a shunt for the 'sodium transport battery' and its effect is reflected as a sudden large drop in potential difference. This occurs in spite of a virtually unaltered rate of active sodium transport or short-circuit current. Hyperosmolarity of the outside bathing solution may cause an osmotic shrinkage of the outermost layer of cells and put a strain on the 'tight junctions' or Zona occludens and thereby make them leaky. It is pertinent to this discussion to recall that concentrations of urea or mannitol equimolar to that of ethanol, produced this classical osmotic reaction. However, ethanol was found to decrease both p.d. and scc and did not alter passive sodium movements in the outward direction. In addition, ethanol crosses the frog skin epithelium quite readily, as indicated by its relatively high permeability coefficent compared to other solutes (Table 40) , and would therefore be expected to have much less ability to shrink the cells by an osmotic action than less permeant molecules. Besides, the ethanol response was blocked by removal of chloride, whereas

Table 40. The permeability coefficients (K_{trans}) for electrolytes and non-electrolytes in toad bladder: A comparison to ethanol.

K_{trans} (10^{-7} cm/sec)

SPECIES[a]	K_{trans}	SPECIES	K_{trans}
Inorganic Ions:		Organic molecules:	
Sodium	2.8	Thiourea	13.9
Potassium	26	Glycerol	4.1
Chloride	13	Sucrose	8.9
Sulfate	4.2	Inulin	0.0
Organic Ions:		Water and Alcohols:	
Thiocyanate	8.3	Water	944
Choline	9.1	Methanol	825
Lactate	5.6	Ethanol	575
Urea	26	Butanol	930

[a]These values have been taken from Leaf and Hays, 1962.

osmotic agents still exert their effects in chloride-free solutions (Ussing, 1965). This seems to indicate that ethanol is not having an osmotic effect on frog skin.

An alternative mechanism, which appears more likely, may involve a direct physicochemical interaction with the membrane. Ethanol is a highly polar compound containing a hydroxyl moiety which is capable of hydrogen bonding. This ability of ethanol to hydrogen bond may play an important role in its ability to inhibit the movements of ions across membranes. A similar reversible inhibition of active sodium transport across frog skin has been observed with local anesthetics (undissociated form) (Skou and Zerahn, 1959). There are certain points of resemblance between local anesthetics and ethanol, that is lipid solubility and an ability to penetrate into and change physical properties of lipid containing interfaces (Skou, 1961). It is therefore possible that these physicochemical properties may influence ethanol's action on frog skin. It has been suggested that the polar groups of the outer leaflets of lipids in the membrane form a system of fixed charges which are highly selective for sodium ions (Eisenman, Rudin and Casby, 1957). Cereijido and Rotunno (1968) have proposed that this network of fixed charges, consisting of polar groups, is involved in a possible pathway for sodium ions across frog skin epithelium. This pathway involves the movement of sodium around the cell within the lipid bilayer rather than by penetrating the cell. The presence of the tight-junctions would therefore represent an area which would restrict the movements of ions across it, and represent the rate limiting barrier. Sodium can traverse this barrier whereas potassium cannot. As the sodium pump is thought to be located within the cell membrane facing the intercellular spaces (Farqhar and Palade, 1965) sodium travelling on the outer surface of the cell would be picked up by the Na-pump (having a high affinity for sodium) and can

leave the polar groups, cross a sodium impermeable barrier and reach the intercellular fluid. Ethanol, being somewhat soluble in lipids, may enter such a lipid bilayer and, due to its hydrogen-bonding abilities, form hydrogen bonds with these polar groups. This would then interfere with the movement of sodium ions available for transport. Lipid solubility would therefore be an important factor in reaching the hydrophylic core. This hypothesis is consistent with the observations that by increasing lipid solubility (increasing the chain length of the aliphatic alcohols), as was observed with 2-propanol and n-butanol, the response is increased.

In addition to this tangential movement, sodium may also be transported across the cell through the cytoplasm (Cereijdo and Rotunno, 1968). This transcellular transport can be modified by increasing the concentration of sodium in the outside solution, or by the administration of vasopressin. This hormone increases the permeability of the outside membrane to sodium (Herrera and Curran, 1963) which is reflected as an increase in the short-circuit current.

Our results indicate that ethanol and the homologous neurohypophysial hormone vasotocin are working via different mechanisms as their effects are independent of each other. This result seems to agree with the hypothesis presented above. Our results also indicate that the ethanol response is anionic dependent. There are several possible explanations as to the mechanism of this response. Ethanol's access to the polar groups within the membrane network may require the presence of an anion, particularly Cl. Alternatively, ethanol may enter the lipid matrix via a chloride sensitive channel. Other anions which are larger may not get at the chloride sites and therefore block the alcohol's effects, as our results with sul-

fate and nitrate indicate. In addition, chloride blocking agents, such as thiocyanate and furosemide, also anions, may block access of chloride to its site or may block the chloride channel.

An alternative explanation is that sodium in order to cross this lipid matrix, must do so as a neutral molecule, perhaps as NaCl. Therefore, when chloride is absent from this outside solution or thiocyanate or furosemide are present, the tangential route for sodium transport would not occur. However, the transcellular route would be still operative and would represent the predominant transport path for sodium under these conditions. In fact, Cuthbert, Painter and Prince (1969) have demonstrated that by removing Cl from the outside solution they observed a drop in the transmural movement of sodium. This tangential pathway is therefore one means of interpreting their observation.

Since ethanol may affect only the tangential transport path for Na, its effects would therefore be absent when chloride is removed from the bathing solutions or is blocked by other agents. Substances, such as, vasopressin and possibly phospholipase C, may effect the transcellular pathway and their responses, reflected as an increase in scc, could still occur, even in the presence of ethanol, or under conditions where the ethanol response was blocked. This hypothesis is consistent with the present observations.

These possibilities are only offered as suggestions as to the mechanism of ethanol's effects on sodium transport in the frog skin and more than one such process could be involved in eliciting this response.

It was observed that ouabain, an inhibitor of the Na-K-ATPase, increased the intracellular sodium content of the isolated frog skin epithelium. Amiloride, which blocks sodium entry at the outer boundary of the epithelial cell (Dorge and Nagel, 1970; Ehrlich and Crabbé, 1968; Rick, Dorge and Nagel, 1975), had no effect on the ionic content of this tissue. This effect of amiloride is rather puzzling, as some others have demonstrated decreases in intracellular sodium content (Salako and Smith, 1970; Nagel and Dorge, 1970; Biber, 1971). However, the exact size and localization of the cellular compartment involved in the transepithelial transport of sodium ions by the frog skin is still somewhat controversial (see for instance Ussing, Erlij and Lassen, 1974). Some recent observations suggest that the amount of sodium coming from the outside solution that equilibrates with the cytoplasm is only a small fraction of the total Na in the cell (Aceves and Erlij, 1971; Zylber, Rotunno and Cereijdo, 1975; Morel and Leblanc, 1975). This observation has led to questions concerning the state of the sodium in the frog epithelium, and also whether all the layers of cells participate in the transepithelial process. If a great fraction of the cutaneous intracellular sodium is compartmentalized, either in a bound form or is sequestered in a subcellular compartment or another cell-type, a favorable electrochemical concentration gradient may exist so that its penetration across the outside barrier could be explained without postulating an active step at this border. The observations by Erlij (1971) and Zylber et al. (1975) show that only a small fraction of the total intracellular sodium equilibrates with that in the outside solution so that

its 'transport pool' (involved with transepithelial transport) is small (Erlij, 1971; Zylber et al., 1975; Morel and Leblanc, 1975). This observation has led to the suggestion that perhaps only a single layer of cells, namely the cells in the outer layer of the stratum granulosum, participates in the transport process (Voute and Ussing, 1968; Erlij, 1971; Morel and Leblanc, 1975).

In our experiments, the method imposed measures the total sodium in the epithelium including any that may be present outside the transport compartment. If, as suggested above, the 'transport pool' is just a small fraction of the total sodium measured, any decrease in this small pool, as produced by amiloride, would not be detected as a change in the larger sodium pool measured. Ouabain, on the other hand, increases the total Na of the epithelium presumbably by the accumulation of sodium in subjacent layers. The observations that ethanol, which like amiloride, had no effect on total intracellular sodium content, offers additional support against an ouabain-like action for the mechanism of the ethanol's response. Ethanol may be affecting transepithelial sodium transport by an action on the outer barrier of the frog skin epithelium, which may involve a physicochemical interaction with the membrane components.

It was observed that ethanol decreases the permeability of the frog skin to water by 30 per cent. A similar action was also observed in the toad bladder. The mechanism of action of ethanol on water permeability will be discussed at greater length when considering its effects on the toad urinary bladder (see page 163). However, it seems likely that the same type of mechanism may be responsible for its effects in frog skin.

This may involve a direct physicochemical interaction with the membrane. It has been suggested (Pauling, 1960) for instance, that molecules such as ethanol may become part of the hydrogen-bonding framework of lattice structures in cellular water, where it might fill spaces in a crystal framework and thereby have a stabilizing action of Van-der-Waal's type. This interaction with water molecules imposes a state of rigidity which may interfere with normal cell function. In addition, as water is thought to flow through polar channels in a structured state (Grigera and Cereijdo, 1971) it is possible that ethanol can interact with the walls of the channels or with the water molecules directly to impede its flow. It has also been suggested that in toad bladder ethanol and water flow through similar channels (Schucter, Franki and Hays, 1973) and perhaps this also occurs in frog skin. The results of the present investigation show that the permeability coefficient for ethanol is 10-times less than water, but this could reflect interactions with the membrane channels due to its lipophylic and hydrogen bonding abilities.

It is an observation of some basic significance and interest that the short-circuit current was reduced when chloride was removed from the inside bathing solution. Since this was not attributed to active chloride transport, there may be some step(s) in the transport of sodium that is dependent on the presence of chloride. This may involve a receptor site in which the affinity for sodium is increased in the presence of chloride.

This facilitating influence of external anions on active sodium transport has been previously observed in other epithelial membranes. Smith, Hughes and Huf (1971) compared the permeability of frog skin (Rana pipiens)

to several anions, including chloride, sulfate and nitrate. They concluded that while chloride readily permeates the skin, nitrate and sulfate only diffuse very slowly across the membrane. It was also demonstrated by Huf (1972) that replacement of Cl^- by SO_4^{-2}, on the inside of the skin, invariably led to a decrease in Na^+ influx and net inward sodium flux. The same effect was observed in the present investigation. Huf (1972) has suggested that this decrease in sodium influx may be associated with a decrease in total skin conductance.

Ussing (1965), also noted a rise in scc across the isolated skin of the frog, Rana temporaria, when chloride replaced sulfate in the inside bathing-media. Upon further examination of this phenomena, Voute and Ussing (1968, 1970) observed that the cell layer just beneath the outer cornified cells appears much denser and is shrunk in open and short-circuited skins bathed in osmotically uncompensated (hypo-osmotic) sulfate Ringer instead of chloride Ringer. However, when using such sulfate Ringer another variable, besides changing the anion, is introduced due to its hypo-osmotic concentration. MacRobbie and Ussing (1961), Ussing (1965) and Lindley, Hoshiko and Leb (1964), studied the effect of changes in osmolality of the Ringer's solution when applied to frog skin and observed alterations in the thickness of the skin as well as changes in the open circuit p.d. and scc. Hypotonic solutions bathing the inside of the frog skin cause an increase in the scc and a swelling of the skin, while there is no effect when hypotonic solutions bathe the outside face of the skin alone. When uncompensated sulfate Ringer's solution is used as a substitute for chloride

Ringer's two variables are changed simultaneously and the effect of the hypotonicity can mask the effect of sulfate for chloride substitution. Therefore, in the present investigation care was taken to insure that no other variable capable of influencing the electrical parameters was introduced and all Ringer's solutions were balanced osmotically. However, even under iso-osmotic conditions the substitution of sulfate for chloride may result in cellular shrinkage, as sulfate is a less permeable anion than chloride and thus would create an osmotic gradient across the cell membrane. As changes in cellular volume can reflect changes in short-circuit current and sodium fluxes (MacRobbie and Ussing, 1961; Ussing, 1958, 1965; Lindley et al., 1964) this is a possible mechanism of anion dependent Na transport in frog skin.

Another explanation of this phenomenon has been offered by Watlington and Jessee (1975) using the theoretical model of a neutral 'NaCl pump' that has been proposed by Rehm et al (1974). These authors suggest that the neutral 'NaCl pump' is located on the inner membrane with active transport directed inward. Under short-circuited conditions this neutral pump receives chloride from both inner and outer bathing solutions so that the fraction contributed from either side depends on the relative permeabilities of the respective membranes to chloride. When the chloride conductance of the inner membrane is larger than the outer, no net chloride current, or chloride transepithelial fluxes, would be detected, as most of the chloride supplied to this pump would originate from the inside bathing solution and would just re-cycle from media to cell interior back to media. Therefore,

by removing chloride from the inner solution a decrease in the transepithelial transport of sodium would be observed by reducing the activity of this neutral 'NaCl pump'. Such a process would, however, only make up a small part of the total transmural sodium transport, as there is still a substantial active sodium transport in the presence of Cl-free solutions bathing both sides of the membrane.

Alternatively, chloride may enter the cell from the inside solution making the intracellular environment more electrically negative. Such a change would result in an increase in the electrical gradient for sodium and a subsequent increase in the influx of sodium across the outside barrier. Providing that the sodium pump is not saturated, the extra sodium ions could then be removed across the inner facing membrane. When chloride is removed and substituted with a non-permeant anion, such as sulfate, the cellular interior may become less negative and decreases the electrical gradient for sodium entry.

One problem which arises in interpreting the mechanism whereby Na transport is partially dependent on serosal chloride, is that under these conditions chloride is present in the outside solution and sulfate in the inside. Since chloride is a more permeant anion than sulfate, a diffusional current due to differences in permeability may arise. The direction of this chloride current would be in the same direction as the sodium current, so that the observed scc would be less than that when chloride is present on both sides. However, other results suggest that this is not significant. It was thus demonstrated that with sulfate on both sides, so

that no diffusion gradient exists, the scc still decreases. Direct measurement of the Na fluxes also confirmed this.

At present, there is no conclusive data available as to the pathways by which sodium and chloride travel across frog skin. Both a transcellular pathway (Ussing and Windhager, 1964) and an extracellular pathway, along the cell surface (Cereijido and Rotunno, 1968) has been suggested.

The site of the interactions of anions with sodium transport remains purely speculative and further investigation is needed before the mechanisms involved can be established.

Another interesting observation associated with anion movements across frog skin was noted with the chloride-blocking agents thiocyanate and furosemide. Both of these drugs have been shown to block chloride transport in other epithelia (Epstein, Maetz and De Renzis, 1971; Kristensen, 1972; Burg, Stoner, Cardinal and Green, 1971; Candia, 1973). In the present investigation this was confirmed for frog skin.

When either drug is added to the mucosal bathing solution, the electrical p.d. increases with very little change in scc. Such an effect is an indication of changes in ion permeability, which was further demonstrated to be a reflection of a decrease in the transmural movement of Cl ions. A similar effect on p.d. is reminiscent of replacing chloride by sulfate in the outside bathing solution. However, further confirmation that Cl was being blocked by these drugs was obtained from chloride fluxes. It was thus observed that thiocyanate (in the outside solution only), decreased the fluxes of chloride by 60% in <u>both</u> the outward and inward direction (see page). This is an observation of some basic significance as it

suggests that perhaps a common pathway exists for transmural movements of chloride in frog skin.

TOAD BLADDER

The present investigation demonstrates that ethanol can alter the permeability of the toad's urinary bladder to water and ions. These effects are seen under various conditions and appear to reflect different mechanisms of action on the membrane. The concentrations of ethanol which are effective in such circumstances far exceed those compatible with survival *in vivo*. However, as was demonstrated, the processes of oxidative metabolism in the cells appear to be intact under these conditions *in vitro*. In addition, some of the effects of ethanol on the permeability to water can even be seen in the tissue following its fixation with glutaraldehyde. The responses to the alcohol are thus not necessarily related to a metabolic action and may involve a more direct physical interaction with the membrane. While the latter type of effect undoubtedly plays an important role in the movement of molecules across epithelial membranes they are often difficult to distinguish from those which are controlled metabolically. Since ethanol has also well defined physicochemical properties the present results may provide us with information about the mechanisms by which molecules cross epithelia. In this context these observations on ethanol can be considered in the role of its use as a 'chemical' or 'pharmacological probe'.

The ability of the toad bladder epithelium to withstand exposure to

20 to 30% ethanol without changes in its oxygen consumption, is rather remarkable. In man, a concentration of 0.5% ethanol has fatal effects. It is possible that some tissues, such as the brain, may be far less tolerant to its effects and may thus play a determining role in the ability of an animal to survive. In the present experiments we found that ethanol influenced the permeability of the epithelia even in the absence of any measurable effect on oxidative metabolism. It is thus possible, that the toxic effects *in vivo* are mediated by an action on cell permeability rather than any general inhibition of metabolism. In our experiments these observations on the metabolic actions of ethanol were of importance as they allow a distinction to be made between the possible types of effects that it may exhibit. They confirm that under our experimental conditions the membrane is not drastically compromised metabolically by ethanol and remains viable. Thus the effects we observed may involve a simple physicochemical interaction with the tissue though more subtle metabolic changes cannot be excluded.

Two major effects of ethanol on the amphibian urinary bladder epithelium were described and investigated. a) While the ionic permeability of the membrane was unaffected by exposure to low concentrations of ethanol, 9% (v/v, about 2M) on the mucosal side reduced the transmural p.d. and resistance. There was no change in sodium permeablity but the unidirectional chloride fluxes increased. b) The permeability of the toad bladder to water was decreased in the presence of 3% ethanol and this effect was also apparent in the presence of vasopressin, which stimulates

water movement.

There are several possible mechanisms which could be mediating these effects of ethanol. a) A general metabolic effect could not be demonstrated but more subtle changes such as an accumulation of metabolites of ethanol cannot be excluded. This appears to be unlikely, however, as the peripheral tissues do not readily metabolize alcohol (Wallgren and Bary, 1970) and the response, such as the increase in Cl permeability are very rapid. b) Ethanol can denature proteins, but such effects are usually observed at concentrations above 30%. c) Ethanol may be exerting an osmotic effect which shrinks the tissue, due to the high concentrations used. However, ethanol crosses the bladder epithelium very rapidly, as indicated by its high permeability coefficient and it would therefore be expected to have much less ability to shrink cells by an osmotic action than less permeant molecules. However, relatively high concentrations were present so that such an action, although unlikely, cannot be excluded. d) As distinct from an osmotic shrinkage, the presence of high concentrations of ethanol may be having a non-specific colligative-type of effect on the cell. It is difficult to distinguish this from an osmotic effect since other molecules with which a comparison may be made, for instance urea, may also exert some classical osmotic actions. e) Ethanol is somewhat soluble in lipids. The oil/water partition coefficient is only 1/30, but this could, nevertheless, be contributing to changes in the properties of cell membranes. f) Direct interactions between water, ions and ethanol could also be occurring. Ethanol is a highly polar compound, containing a hydroxyl moiety, which is

capable of hydrogen bonding. This ability of ethanol to hydrogen bond may disrupt the organization of molecules of water in solution and affect their mobility while traversing the membrane. It had been suggested (Gary-Bobo, DiPolo and Solomon, 1969), that hydrogen bonding may play an important role in the movement of non-electrolytes through membranes. The vapor pressure of water in ethanol/water mixtures shows a positive deviation from Raoult's Law (Glasstone and Lewis, 1960), indicating that its mobility is increased. It is also possible that mobility of ions may be changed in such mixtures, though we could detect no changes in ion diffusion potentials of Ringer's solution in the presence of 9% ethanol. g) The ethanol may exert an effect by a direct physicochemical interaction with the membrane. This, apart from its possible solubility in lipids, could involve an interaction with the walls of pores by hydrogen bonding or by a competitive-type of physical obstruction to the free movement of water through the membrane. This may also involve a possible changing of the fluidity of the membrane and thus interfere with normal transmural movements. These are only suggestions as to the mechanisms of ethanol's effects on the bladder and more than one such process could be involved even for each particular effect.

It is an observation of some basic significance and interest that the membrane permeability to chloride ions could be considerably increased by ethanol but that to sodium was unchanged. This suggests that there are specific pathways for anions in this tissue. The decline in resistance is reminiscent of previous observations on the effects of osmotic agents on such membranes. Thus urea, when placed on the mucosal side of

the toad bladder or on the outer surface of the frog skin, has no effect on the scc, but brings about a decline in p.d. and electrical resistance (Ussing, 1965; Bentley, Candia, Parisi and Saladino, 1973; Urakabe, Handler and Orloff, 1970; Civan and Di Bona, 1974). The ionic basis for these effects has not , it seems, been previously described. We found that mannitol, which has a comparable action to ethanol, also increased Cl permeability. The decline in resistance probably reflects an opening of the tight junctions, or zona occludens, at the outside surface of the membrane (Wade, Revel and DiScala, 1973). Solutes enter these fissures and, possibly, by an osmotic effect expand them. This change is reflected, as seen by electron microscopy, by the appearance of bulbous deformations or blisters in the most apical mucosal junctions of the epithelial cells (DiBona and Civan, 1973). Smaller solutes, such as urea, were found to be most effective in eliciting this phenomenon. Ethanol could be having a comparable effect, not necessarily osmotic in nature, but rather due to a structural disruption of lipids or proteins that are present in the zona occludens, and therby make it leaky. It is notable that the effect of ethanol on Cl permeability was, in contrast to other solutes, not reversed when the ethanol was removed from the bathing fluids. More conclusive evidence as to the nature of the morphological effects must await observations with the electron microscope.

Ethanol was found to inhibit the hydro-osmotic reponse to vasopressin by about 70%, but it only reduces the unidirectional water flux, in the presence of this hormone, by about 35%. The reason for this disparity is

unknown. It could, as formerly suggested (Leaf, 1962), reflect a fundamental difference in the processes of water movement by diffusion and down an osmotic gradient. However, there is some doubt about the real existence of two such processes. This question rises from the problems encountered when measuring the diffusion of water across membranes particularly due to the presence of unstirred (or unstirrable) layers (Dainty and House, 1966a,b; Hays and Franki, 1970; Parisi and Piccinni, 1973). It is, however, not unlikely that the membrane may respond differently under each set of conditions; that is in the absence and presence of an osmotic gradient. The latter may result in differences in such processes as cell swelling (Civan and DiBona, 1974) and solute content which could alter its ability to react with the ethanol.

Ethanol decreased the permeability of the bladder epithelium to water. This effect was seen even in the presence of vasopressin and cyclic-AMP which produces a considerable increase in permeability to water. A general metabolic effect would not appear to be involved, as the rate of oxidative metabolism was not changed by such concentrations of ethanol, and the effect was still apparent in metabolically inactive tissues which had been fixed with glutaraldehyde or exposed to NEM. It thus appears likely that some fundamental physicochemical interaction with the membrane, or water, is occurring. As described earlier the mobility of water is increased in alcohol/water mixtures and this is not consistent with the decline in water movement that was observed. In addition, water movement across artificial collodion membranes was not altered by the presence of ethanol in the solutions. It therefore seems

likely that the ethanol is impeding the flow of water across the epithelial membrane itself. The nature of this permeation process is uncertain but it is often conceived of as involving 'pores' or 'channels' which are probably polar in nature. If such pores were of a narrow diameter, the presence of more slowly moving ethanol molecules (our results indicate they move about four-times less rapidly across the bladder than water) could impede the movement of the water molecules. However, the concentration of ethanol is about 70-times less than that of water and yet it still reduces the latter's movement by about 30%. It is thus unlikely that the ethanol molecules, at such relatively low concentrations comparatively, could be impeding the free diffusion of water molecules to the extent observed, even through narrow pores. Alternatively, if some of the ethanol molecules were interacting with the walls of the channels by forming, for instance, hydrogen bonds, it could block this water movement. An interaction with lipids associated with such channels is also possible and could result in changes which could impede the water molecules. Since ethanol contains a lipophilic and hydrophilic moiety, there could be a combination of these two effects. The ethanol molecule could for instance, orient itself with its lipid portion within the pore membrane, leaving its hydrophilic group exposed and free to hydrogen bond with passing water molecules, thereby decreasing their mobility. The movement of ethanol across the bladder was increased by vasopressin but this effect is small when compared to the increase in water movement induced by this hormone. If, as has been suggested (Schuchter et al., 1973), ethanol moves through the same channels as water, this relative

lack of response to the hormone could reflect such binding in the membrane.

TOAD LENS

Hyperosmotic conditions were found to reduce the translenticular p.d. and short-circuit current across the amphibian lens. Mannitol and NaCl were very effective, and the more permeant molecules urea and ethanol less so. The hyperosmotic changes were only effective when they occurred at the anterior epithelial surface of the lens; at the posterior they had no effect.

The translenticular p.d. and scc across the lens probably reflects the permeabilities of the anterior and posterior limiting membranes (Duncan, 1973); the p.d. across the former exceeds the latter, and the difference corresponds to the observed overall p.d.. The scc partly reflects active transmural transport of Na across the lens, and the site of the active transport mechanism appears to be in the lens epithelium (Candia et al., 1971). This process, however, only accounts for about 20 to 30% of the total scc; the origin of the remainder is uncertain, but probably involves differences in the rates of diffusion of ions across each side of the lens (Candia, 1973). The osmotic agents could thus be acting in several ways. Their principal site of action would appear to be the anterior epithelium. Hyperosmotic conditions are known to inhibit active Na transport across other epithelia (Lipton, 1972; Bentley, Candia, Parisi and Saladino, 1973) and such an effect on Na outflux from the lens epithelium could account for part of the response that we observed. This

possibility is supported by the observation that hyperosmoticity increases the electrical resistance across the anterior epithelial lens membrane. However, the scc and p.d. still decline even when active translenticular Na transport is abolished by removing Na from the posterior bathing media. In addition, the response is reduced when Na is absent from the anterior solution. These observations suggested that a second component, apart from active Na transport, is involved and this could be an increased permeability to Na. Measurement of translenticular Na fluxes confirmed that the latter effect was occurring. The lens was also found to accumulate Na at increased rates in the presence of the hyperosmotic agents, and this could be consistent with either an inhibition of the active Na-pump or an increase in Na influx into the lens or, most probably, both. Changes in permeability to other ions could also be contributing,

It is interesting to compare the effects of hyperosmotic agents with those of the polyene antibiotic amphotericin B as both decrease the translenticular p.d. and scc. The mechanism of their effects are, however, different. Amphotericin B has also been shown to increase the Na permeability of the lens (Bentley and Candia, 1975) but, in contrast to hyperosmoticity, it reduces the resistance of the anterior epithelium and increases both unidirectional Na fluxes equally. It does not alter active Na transport.

The hyperosmotic agents were found to primarily influence the properties of the anterior side of the lens, probably the epithelial membrane, but changes at the posterior were also observed. The nature of the

linkage between the two sides is uncertain, but the observed decline in the resistance of the posterior probably reflects an accumulation of Na in the lens which results in an increase in the flux of this ion across the posterior side.

The relative lack of urea on the p.d., scc, resistance and Na fluxes is not surprising as it is a very permeant solute, so that high concentrations are needed to produce osmotic effects similar to NaCl or mannitol. Ethanol can also readily enter cells so that although it has been used as an osmotic agent (Peczon, 1965; Houle and Grant, 1967) to lower intraocular pressure, its effects would also not be expected to be substantial. Neither ethanol or urea produced a measurable reduction of the lens weight. In another amphibian epithelium, the frog skin, ethanol has been shown to reduce active Na transport, and this cannot be accounted for by an osmotic effect (Yorio and Bentley, 1973). It is thus possible that the effects of ethanol and urea on the lens represent a different type of action to that of the other agents.

The present observations in vitro suggest that ionic exchanges in the lens may be influenced in vivo by changes in the osmotic pressure of the aqueous. Hyperosmotic conditions may lead to the accumulation of Na by the lens with a concurrent decline in the p.d.. Such increases in tonicity may occur in vivo during dehydration, hypernatremia and from the accumulation of glucose or urea in the body fluids. The present observations indicate that urea would be relatively innocuous, as compared to the less permeant solutes. Increases in the osmotic concentrations of the body fluids may also occur during the administration of urea, ethanol

or mannitol in order to decrease intraocular pressure. Mannitol, in addition, is gaining increasing popularity for the treatment of cerebral edema and the promotion of osmotic diuresis for the prophylaxis of acute renal failure (Flores, DiBona, Beck and Leaf, 1972). Hyperosmoticity is known to produce reversible cataracts in animals, but the association of this condition with the accumulation of ions in the lens is uncertain. A clouding of the lens under a number of conditions was noted, especially exposure of the posterior surface to mannitol, but alterations in electrical behavior and the accumulation of Na were not invariably associated with such opacities.

SUMMARY

The effects and mechanisms of action of ethanol on ion and water transfer were investigated in three 'model' membranes, the amphibian skin, urinary bladder and lens. Although morphologically different, these epithelia possess similar ion transport processes.

The effects of ethanol on water and ion movements across these three epithelia differ. This may reflect the various processes involved in the transmural movements of ions and water across them. It is, however, interesting that upon careful examination a general mechanism for ethanol's effects may be inferred. It was observed that ethanol, at concentrations that had marked effects on water and ion movements, did not alter the oxygen consumption of the amphibian skin or urinary bladder. This was rather basic to the study as it confirmed that these tissues were not compromised metabolically and had a high tolerance to any toxic effects that may be produced by this compound. It also suggested that a general metabolic action is probably not mediating the response. Ethanol is a highly polar molecule, containing a hydroxyl group capable of hydrogen bonding, and it is also somewhat lipid soluble. The observations that 3% ethanol inhibits the transfer of water by 30% in both the frog skin and toad bladder, and also reduces water movement in the presence of vasopressin, cyclic-AMP or even in 'fixed' bladders, suggests that the physicochemical properties of ethanol may be contributing to its mechanism of action. Specific suggestions as to how this might occur are offered. It may involve the ability of ethanol to enter a lipid phase in the membrane and/or hydrogen bond with molecules of water passing through hydrophilic 'pores', or it could even in-

teract with the walls of the pores themselves.

Ethanol's effects on the electrical behavior of frog skin, urinary bladder and lens were found to be more diverse. These observations may reflect the variety of processes involved in controlling the permeability of the epithelia to ions and the different reactions of ethanol with respect to each of the pathways involved. It was for instance shown that in frog skin, ethanol decreased the influx of sodium, an effect which was anion dependant and reversible. On the other hand, in toad urinary bladder, ethanol had no comparable effect on sodium, but due to an increase in chloride permeability decreased the electrical resistance. This effect was irreversible. In toad lens, ethanol had yet another effect as it increased passive sodium movement but increased the resistance. While these differences in response are quite clear, similar basic mechanisms may nevertheless be involved. The concentrations of ethanol needed to elicit these responses varied in the different tissues; thus 3% ethanol decreased ion movements in frog skin, but about 10% ethanol was needed to increase ion movements in lens and urinary bladder. In frog skin, however, 9% ethanol was also observed to increase chloride permeability. This observation suggests that the higher concentrations of ethanol have a rather similar mechanism of action on all three epithelia and increase their ionic permeability. However, the response depends on the particular epithelium as each possess specific membrane properties and ionic pathways so that different ions will be affected. This increase in ionic permeability may result from the disruption of 'tight junctions', perhaps by altering the conformation of essential membrane components involved in a particular

ionic pathway, or by increasing the membranes fluidity at a particular site(s). As these epithelia are considered to be 'tight', high resistance membranes, such effects could make them 'leaky' and result in increased movements of ions.

It is interesting that the electrical properties of both the toad's lens and urinary bladder were only affected at relatively high ethanol concentrations compared to that observed in frog skin. This may be related to the physiological functions of the membranes. The toad bladder is normally bathed on its mucosal surface (the same side to which the alcohol is added) by a rather obnoxious fluid, the urine. It also seems likely that the aqueous fluid, which bathes the lens of amphibians, may undergo considerable changes in concentration during their normal lives. It is therefore possibly not surprising, to find that high ethanol concentrations are needed to elicit responses. The frog skin is usually only bathed by dilute solutions and thus may exhibit a lower tolerance than the other membranes. In addition, ethanol may act on frog skin in a similar manner to that which has been observed in nerve cells particularly with respect to its effects on Na movements (Walgren et al., 1974). Ethanol, in contrast, had no effect on sodium transfer in the toad urinary bladder. The latter is considered to be a model for sodium transport processes in the mammalian nephron and it is interesting that ethanol has no effect on this tissue either (see Wallgren and Bary, 1970).

The results show that ethanol may provide a useful pharmacological 'probe' for studying the mechanisms involved in transport and permeability phenomena of epithelial membranes. It has been previously suggested that

ethanol has an ouabain-like action, inhibiting Na-K-activated ATPase (Israel, Kalant and Leblanc, 1966; Hegyvary, 1973). Such an effect could account for its depressant effects in man (Kalant and Israel, 1967). The present observations seem to suggest that other mechanisms may be involved. It is proposed that ethanol may affect the movement of ions and water across epithelia, by a direct physicochemical interaction with their membranes rather than by some general metabolic effect or specific enzyme inhibition.

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