HORMONAL CONTROL OF ACTIVE SODIUM TRANSPORT IN ANURAN MEMBRANES

by

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THESIS

PRESENTED FOR

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RESEARCH CAREER.

I graduated in Zoology from the University of Durham with a B.Sc. (Hons.). The research work recorded in this thesis was carried out during the nine terms between September 1962 and August 1965, during which time I held a research Studentship from the Department of Scientific and Industrial Research, at the Gatty Marine Laboratory of the University of St. Andrews.

DECLARATION.

I hereby declare that the work recorded in this Thesis has been carried out by myself, and that it is of my own composition. I further declare that it has not been submitted in any previous application for a Higher Degree.

Signed

SUPERVISOR'S CERTIFICATE.

I certify that Margaret Ann Foster has fulfilled the conditions laid down in the regulations for a degree of M.Sc. under the Ordinances of the University Court of the University of St. Andrews and that she is accordingly qualified to submit this Thesis for the degree of Master of Science.

Signed .....

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I would like to thank the staff of the departments of Zoology and Pharmacology of The University of St. Andrews at the Gatty Marine Laboratory for their tolerance and guidance during the time that I spent with them, and in particular my supervisor Dr. (now Professor) A.J.Matty for his patience and forbearance during my many periods of difficulty.

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CHAPTER ONE.

Introduction.

The majority of Anuran Amphibia spend large parts of their lives in fresh water and it is, therefore, necessary for them to maintain a much higher internal osmotic pressure and ionic concentration than they find in the external medium. It was found over a hundred years ago that an electrical potential difference can be recorded across the frog skin (Du Bois - Raymond, 1843) and more recent work has shown that this potential difference is due to the active transport of sodium ions into the body against an electrochemical gradient (Ussing, 1949). It has also been shown that, although many Anura produce large volumes of urine, the salt concentration of this is very low due to the active uptake of sodium during storage. This resorption does not take place in the kidney tubule, as in mammals, indeed as was pointed out by Crane (1927) there is no loop of Henlé present in the frog kidney, but the same effect is achieved by resorption of salt across the bladder wall. The bladder is very large in size, enabling considerable volumes of urine to be stored and hence the urine is able to remain there for a greater length of time. (The bladder, when distended, can occupy up to two thirds of the body cavity in the common toad.)

Both the frog skin and the toad bladder are very hardy tissues and also they are large enough to be manipulated easily. Hence they provide a much easier material for the investigation of the process of active transport of sodium than does the kidney tubule which is very small and consequently needs extremely refined techniques, or other tissues which are often too delicate to be studied in vitro. There is also the advantage of using a thin sheet of undamaged cells (except at the outer edges which can be excluded from the area under experiment) hence overcoming one of the major difficulties of working with tissue slices.

Studies of a mechanism like the sodium pump and the action of hormones on it are greatly facilitated by the use of in vitro preparations rather than attempting in vivo studies. In an isolated preparation one is able to eliminate many of the factors which otherwise confuse the issue in question This is particularly important when studying the effects of hormones and drugs at the cellular level. The hormone can be applied as closely as possible, often directly onto the surface of the cell where it has its action. Hence the exact concentration of the hormone reaching the receptor cells is known, which it never is when injected into the entire body, also the possibility of other effects of the

hormone or drug interfering with the system under study are eliminated. Hence, for example, the direct effects of A.C.T.H. may be observed without having to take into account the variation in the blood adrenocortico-steroid level.

Ussing (1949) first demonstrated that the electrochemical potential difference across the frog skinwas due to the active uptake of sodium ions rather than, as had been suggested previously, to the active transport of chloride ions. He demonstrated this using double labelling techniques measuring the simultaneous flux rates of ^{24}Na and ^{22}Na or of ^{36}Cl and ^{38}Cl across the isolated membrane. He then fitted the results of his experiments into the following equation which describes the flux rates of ions when they are moving passively across a membrane in response to an electrochemical gradient:-

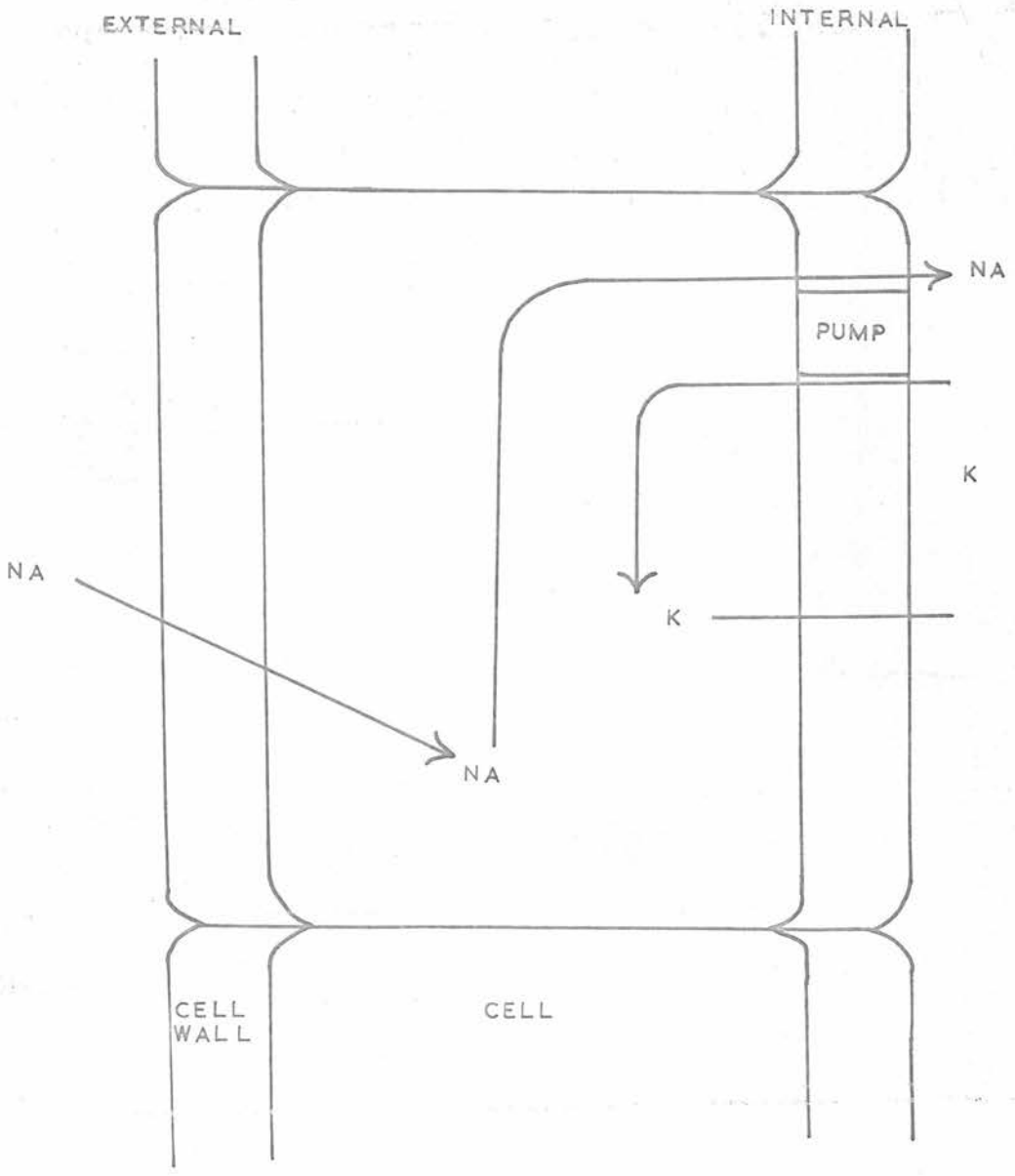
$$\frac{K_i}{K_e} = \frac{C_e}{C_i} \log_e \frac{zFE}{RT}$$

(K_i is the rate of influx of the ion species and K_e is the rate of efflux, C_e is the external concentration of the ion and C_i is the internal concentration, z is the valency of the ion, F. is Faraday's constant, E. is the potential difference across the membrane, R is the gas constant and T is the temperature in degrees absolute).

He found that the figures for chloride movement would complete the equation whereas those for the sodium fluxes would not. The movement of chloride ions, therefore was likely to be passive caused by the imbalance of the ions due to the selective permeability of the cell membrane.

Koefoed Johnsen and Ussing (1958) suggested a model system to account for the position of the sodium pump and the relative permeabilities of the various cell membranes involved in the processes. This model is shown diagrammatically in Fig. I : 1. Their conclusions from the existing data were that the pump was probably located in a single cell layer, on the inner (serosal) surface of the cells. The outer cell membrane would be freely permeable to the passive movement of sodium ions (possibly carrier-mediated) but would be impermeable to potassium ions, whereas the inner cell membrane would be readily permeable to the passive movement of potassium but would be impermeable to any passive movement of sodium ions. The sodium/potassium pump would be located in this inner cell membrane and there would have to be some means of preventing the back-flow of sodium ions round the outside of the cells through the intercellular space system. This model is still

FIG. 1:1 MODEL OF TRANSPORTING MEMBRANE



accepted by most authorities as substantially accurate although some slight modifications have had to be made. Studies by Biber et al (1966) and Cereijido and Rotunno (1967) have demonstrated that the sodium in the frog skin is in at least two compartments, only one of which is available to the active transport process. This was demonstrated by the loading of the tissue with ^{22}Na, when it was found that only about one third of the sodium in the cells is readily exchangeable. This helps to explain a major fault in the Koefoed Johnsen-Ussing model. Most of the experiments on frog skin have been carried out with a high mucosal sodium concentration and under these conditions the external sodium concentration is higher than that in the cells, hence the sodium would diffuse into the cells down a concentration gradient. Under normal circumstance however, the external concentration of sodium is very low and it seems unlikely that the sodium in the cells is at an even lower concentration. Hence there would have to be some facilitation of the entry of sodium ions across the serosal surface of the transporting membrane. If however, only about one third of the sodium in the cells is exchangeable this reduces the effective concentration of the sodium pool to such a level

that it is not necessary to postulate any active mechanism on the mucosal surface of the membrane.

The passage of sodium ions through the outer membrane of the model, although passive, is apparently not completely free since, when the concentration of sodium in the external medium is increased beyond a certain level, the rate of diffusion into the cell remains constant. It has been demonstrated (Frazier et al, 1962) that the rate of sodium movement across the outer membrane can be fitted into the Mechaelis-Menton equation:-

$$\text{Rate} = R_{max} \frac{C}{C + K}$$

where R_{max} is the maximum rate of movement of the ion and C is the concentration of the ion in the external medium. K is a constant. This equation typically describes the rate of action of an enzyme on its substrate but it can be extended to apply to any process which reaches a saturation level in the same manner.

The fact that the sodium permeability of the outer membrane shows saturation kinetics indicates that the sodium ions are passing through the membrane attached to a "carrier" system rather than as free ions. The carrier becomes saturated at high external sodium concentrations. There is,

however, another possible explanation of this restricted movement of the ions. If the ions had to attach to certain sites on the outside of the membrane before they were able to pass through it, even if they passed through completely freely after that attachment, the system would also show saturation kinetics. In this case also the passage of the sodium ions could not be called completely free.

The sodium pump, located on the inner side of the model, is controlled by three factors. These are first the activity of the pump itself governed by the rate at which the enzymes etc. which make up the pump can go through their cycle, and also governed by the energy available from the metabolism of the cell. The second controlling factor is the size of the sodium pool in this cell, which in turn is controlled by the rate at which sodium ions can enter the outer membrane, and the third factor is the potassium available in the inner medium (plasma or artificial medium) to be exchanged with the sodium. In point of fact the serosal potassium level rarely exerts a controlling effect on the pump because of the high permeability of the serosal membrane to the back-flux of potassium ions. It should be noted, however, that Curran and Cereijido (1965), measuring the potassium fluxes into

the skin, have found that there is no correlation between the rate of potassium uptake and sodium transport. This would imply that the sodium pump is not a simple Na/K pump as postulated in the Koefoed Johnsen-Ussing model.

In the whole animal it is possible for hormones to act upon the sodium pump through all of these controlling factors. By isolating the membrane and studying it in vitro, however, it is possible to eliminate the third factor by maintaining the membrane in an artificial medium with a constant, and sufficient,quantity of potassium ions on the inner (serosal) surface of the pump to ensure that this is not a rate-limiting factor. Hence, if a hormone or drug can be shown to affect the sodium pump it must be working on the first or second of the factors listed. As will be seen later (Chapter 5) the different hormones which have been shown to influence the sodium pump do so in different ways, using both of these remaining factors.

Due to the different sites of action of the hormones which have been shown to control the active transport of sodium across the amphibian skin and bladder, studies of the effects of these hormones have proved helpful in investigations of the mechanisms involved in all the phases

of the transport process. For example the work of Leaf and others on the effects of vasopressin on the toad bladder has greatly increased the knowledge of the structure of cell membranes and that of Crabbé with aldosterone has helped formulate ideas on links between the active transport processes, hormone action and the production of messenger RNA in the cells of the membranes. For these reasons it was considered that if other hormones could be found which assisted in the control of the active sodium transport processes in either the frog skin or the toad bladder, and particularly if such other hormones had different sites of action at the cell level to those already investigated, a study of their effects would help to elucidate even further the mechanism of the active transport process and the physiology and structure of the cell membranes involved. The work reported in this thesis was undertaken to discover which, if any, of the known hormones which had not as yet been applied to the membranes, would influence the processes under investigation, and subsequently to make a closer study of the actions of any of these hormones which proved to have such effects.

CHAPTER II.

THE STRUCTURE OF THE MEMBRANES AND THE SITE OF ACTIVE TRANSPORT.

Several attempts have been made, using both physiological and histological techniques, to isolate the site of active transport in the bladder and skin of Amphibia, but so far there has been little success.

The skin of the frog is a relatively thick membrane made up of about four layers which are histologically distinct. The outermost of these layers is the epidermis, which is divided from the stratum spongiosum by a basement membrane. It is in the stratum spongiosum that most of the glands are located. A thin, densely-staining layer divides the stratum spongiosum from the stratum compactum and below this lies the tela subcutanea (Fig. II : 1).

The epidermis is subdivided into several layers (Fig. II : 2). The outermost of these is the stratum corneum consisting of one or, in some species, two rows of flattened, elongated cells which are only loosely attached to the rest of the skin. These cells are heavily cornified, as their name implies and, other than keratin fibres, they contain only occasional traces of nuclear and mitochondrial fragments.

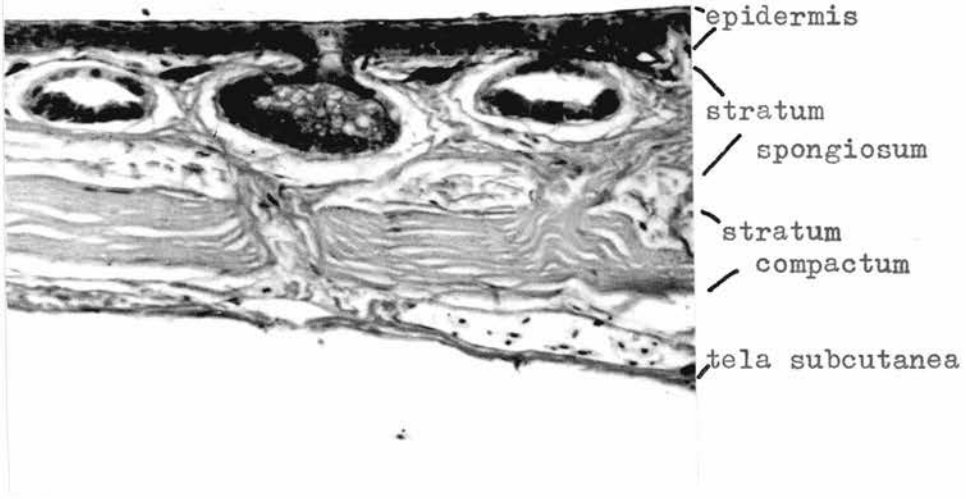


Figure II : 1

Vertical section through ventral skin of the frog stained with Azan to show general arrangement of tissues. Magnification approximately 120.

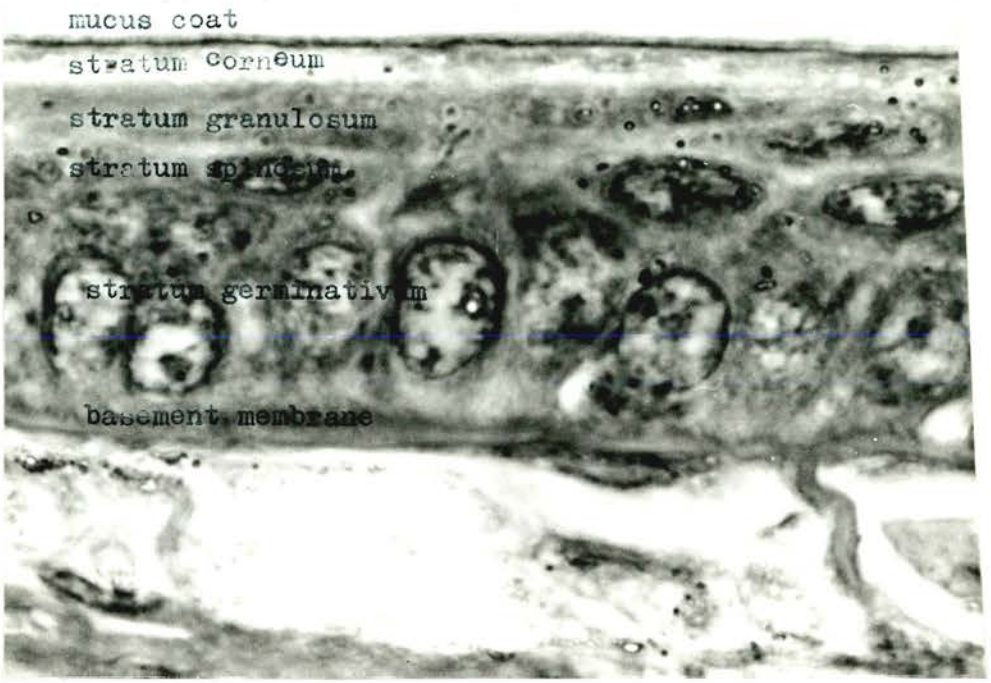


Figure II : 2

Section through epidermis of frog skin.

Stained with Azan. Magnification approximately 600.

There are no signs of golgi vesicles or ribonuclear particles. Occasional mucus droplets may be present (Parakkal and Matoltsy, 1964). Below this layer are the strata granulosum and compactum which consist of several rows of cuboidal or polyhedral cells. These contain flattened nuclei, golgi vesicles and occasional mitochondria. This layer, unlike the stratum corneum, gives the appearance of being alive (Parakkal and Matoltsy, 1964). The final layer, the stratum germinativum, is a single row of columnar cells which are attached to a basement membrane, the presence of which was first demonstrated by Ottoson et al (1952).

The stratum spongiosum consists of a loose network of collagen fibres along with blood vessels, melanocytes and the skin glands. The stratum compactum is formed from a series of regularly-arranged bundles of collagen fibres and the tela subcutanea is a thin cell layer at the base of the dermis. This latter may be the wall of lymphatic sacs (Farquhar and Palade, 1965).

Several layers have been suggested as possible sites for the active transport process. The Ussing model (see Chapter 1) has led various people to conclude that all the

functions take place in a single cell layer but more recent evidence has shown that this is unlikely, and that Ussing's Na-permeable membrane is probably a separate cell-layer from that containing the sodium pump mechanism.

In 1964 Farquhar and Palade, in an electron microscopic study of the frog skin, found that the outermost edges of the cells of the stratum corneum are connected by zonules occludens (Fig. II : 3) while in 1965 they found that such occluding areas do not exist between the cells of the stratum germinativum. These zonules occludens are areas where the outer leaflets of the cell membrane come together and fuse into a single band some 30 to 40 A in thickness. The length of the fusion is between 0.1 and 0.3μ. Below these areas the intercellular space system forms a continuous network between the cells of between 200 and 300 A in thickness. The cells of the stratum corneum are connected below the occluding zonules by a series of modified desmosomes (Fig. II : 4) and the cells in the lower strata by normal desmosomes and maculae adherens. Composite desmosomes are found between the cells of the stratum corneum and stratum granulosum linking the cells across a large and irregular intercellular space (Fig. II : 4). The zonules occludens are found between the cells of the

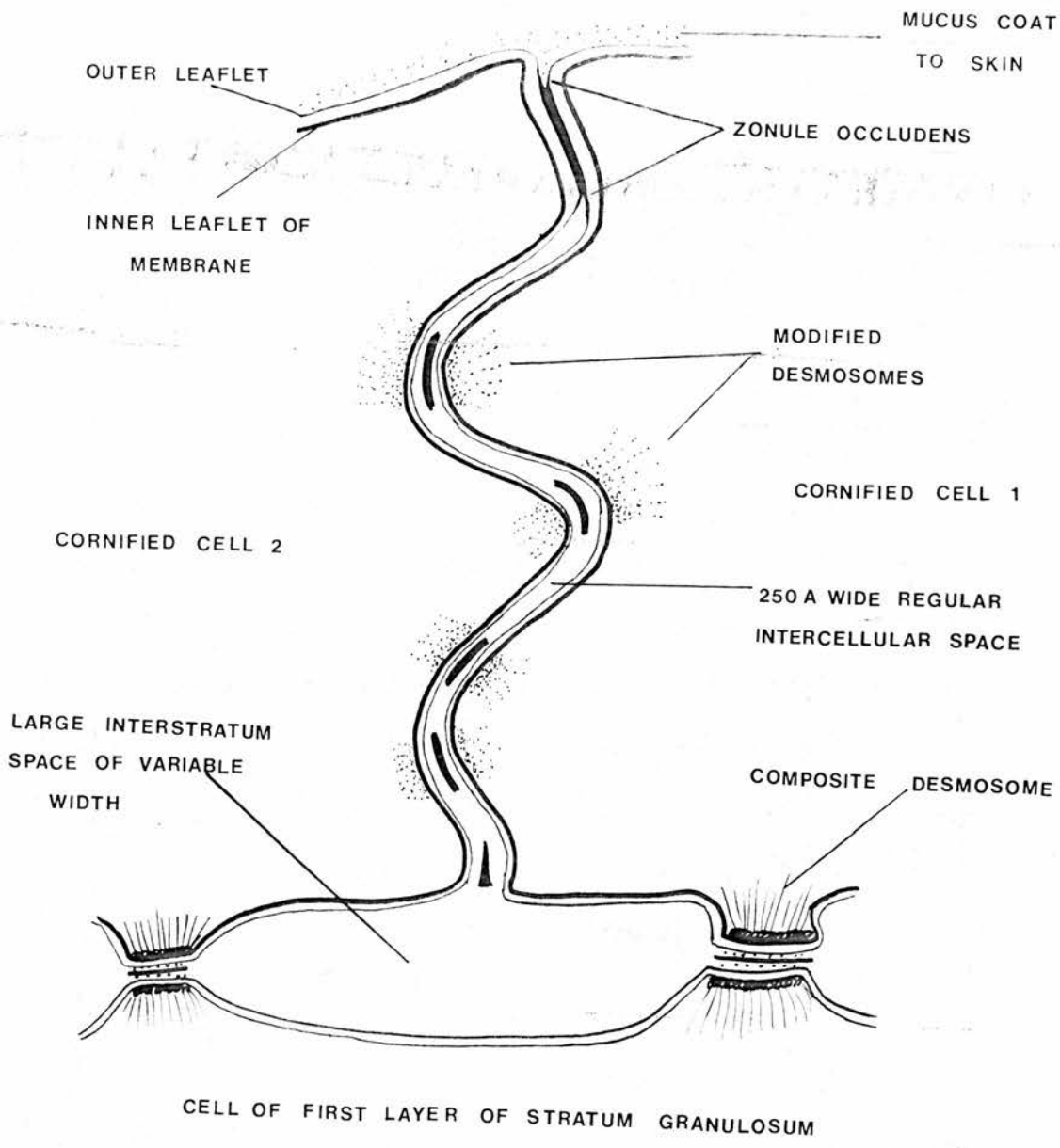


Figure II : 3

Junction between two cells of the stratum corneum and a cell of the stratum granulosum of frog skin. Magnification approximately 45,000.

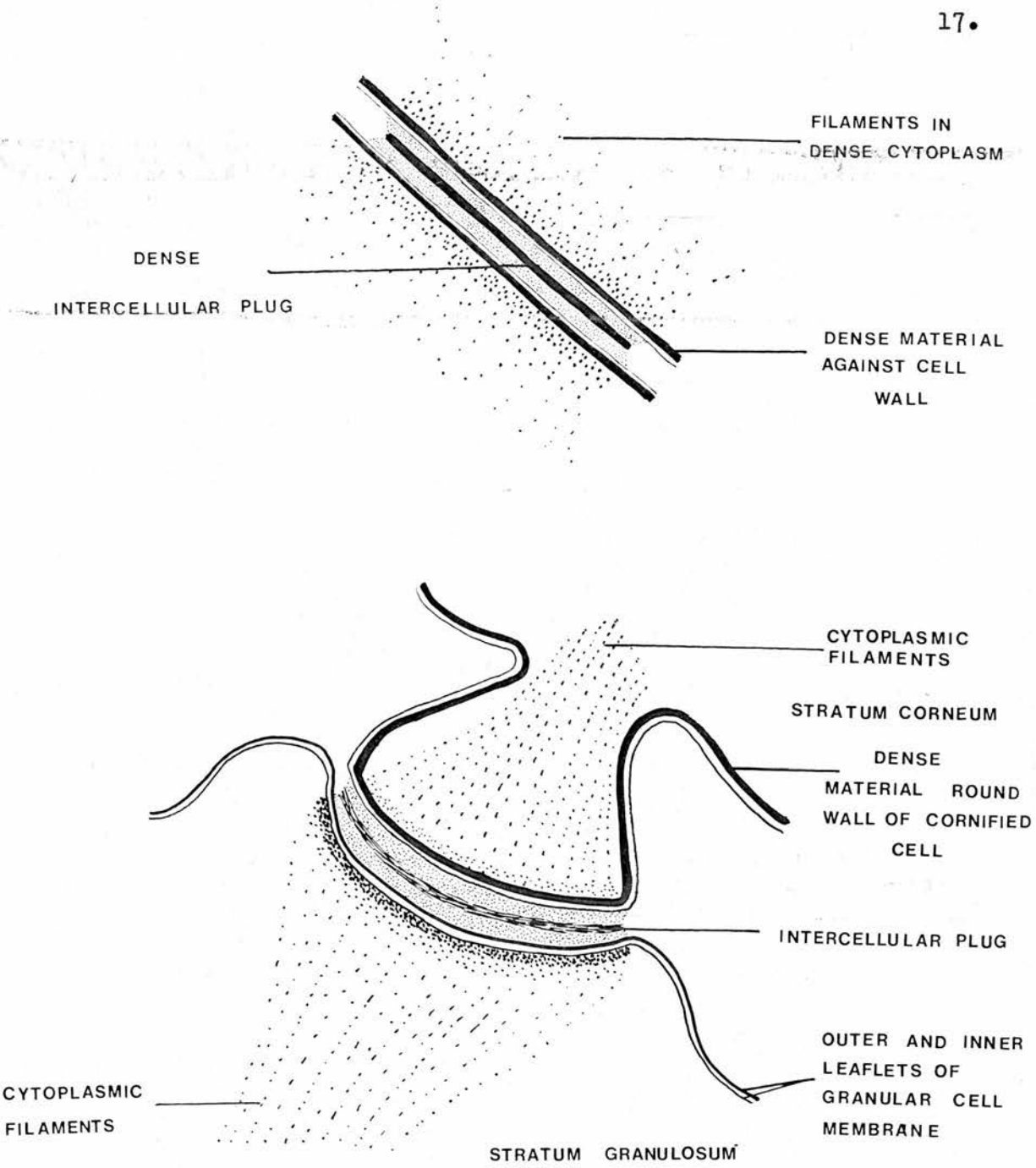


Figure II : 4 Structural details from frog skin epidermis.

Above: Modified desmosome from lateral wall of cornified cells. The plate of intercellular material is more dense than normal and the bundles are less distinct. There is an aggregation of dense cytoplasmic material immediately behind the inner leaflet of the cell wall. This is continuous round the cell.

Below: Composite desmosome between cells of strata corneum and granulosum. Normal desmosome structure modified by presence of layer of dense cytoplasm surrounding cornified cell. Magnification approximately 200,000.

outer cell row of the stratum corneum, between the cells of the lower cell row of this layer where one is present, and also between the outermost cells of the stratum granulosum. Thus the epidermis is divided into several compartments. There is some evidence that these occluding areas are impermeable. MacRobbie and Ussing (1961) found that if hypotonic solutions are placed on the outside of the skin there is no swelling of the epidermis whereas if they are placed on the inside the epidermis swells markedly. This also adds support to the statement of Farquhar and Palade that there is no continuous layer below the occluding layer in the stratum granulosum.

These findings point strongly to the outer layers of the epidermis as the sodium permeable/potassium impermeable layer of the Ussing model which is supported by the work of Koefoed-Johnsen (quoted by Ussing and Windhager, 1964) using ^{42}K to examine the permeability of the various layers of the epithelium. They do not, unfortunately, give much help towards locating the site of the sodium pump. It is possible, however, that the pump is more generally spread than was previously thought. It appears likely that after the sodium ions have entered the outer cells of the skin there are only two possible sites for them to be pumped

out into the body. These are (1) the inward-facing side of the outer cell layer of the stratum granulosum, or (2) the surface of all the cells, or of certain types of cells in the layers below those connected by the zonules occludens. If the first of these was the site of the pump it would be expected that at least some of the reports on the potential profile of the frog skin would have indicated a single potential step across the outer layers of the epidermis. Since there have been no such reports it is unlikely that this is the site. As far as (2) is concerned, only the cells of the epidermis need to be considered since the basement membrane forms a structural barrier between the cells of the epidermis and those of the dermis. Hence there will be no direct exchange of ions between these cells except via the intercellular space system. The basement membrane, although long considered to be a barrier to ion movement across the epidermis/dermis junction, is, in fact, unlikely to act in this capacity. Farquhar and Palade have shown, in membranes other than the frog skin, that similar structures are freely permeable to the passage of both sodium and potassium ions. If, as is likely, this is also the case

with the basement membrane below the epidermis of the frog skin, then it would not form a barrier to the movement of the sodium ions from the intercellular space system in the epidermis to the same system in the dermis, even though its presence precludes the possibility of cellular junctions existing between these two layers. However all the cells in the epidermis are connected by desmosomes and maculae adherens and hence it may be possible to have a completely free interchange between all the cells in this system. This means that there is a continuous sodium pool for the active transport mechanism.

If this mechanism is located in all the walls of the cells of the strata granuosum (except the outer side of the outer layer), spinosum and germinativum this would give the advantage of a very large surface area. Having been pumped into the intercellular space system the ions would then be free to move through the permeable basement membrane and the dermis into the animal. Although, once again, this is not supported by examinations of the potential profile it is not in such direct conflict with these measurements as the first site would be. Also there is much difficulty in

recording the potential steps precisely due to the impossibility of knowing whether the electrodes are inter- or intracellular. If all the cells of the epidermis were actively transporting sodium ions into the extracellular space system this would be even more important.

There is some evidence in favour of this view of the site of the sodium pump from biochemical examination of the frog skin. Fig. II : 5 is a diagram taken from a section of the frog skin stained for ATP-ase using the method of Padykula and Herman (1955). The ATP-ase, which has been shown by various workers to be closely connected with the active transport mechanism (Skou, 1960), is seen to be located round the edges of the cells of the lower layers of the epidermis. It is not in the cell membrane itself but slightly separated from it by an area apparently free of the enzyme. There is little or no ATP-ase present in the basement membrane or in the ends of the cells of the stratum germinativum which abut on to this membrane. It is possible that the pump is located in the outer edge of the cells where the ATP-ase is present. Another feature of this technique is the demonstration of cells in the epidermis which are extremely rich in ATP-ase. These are goblet-shaped cells but, unlike most cells of that type, they are

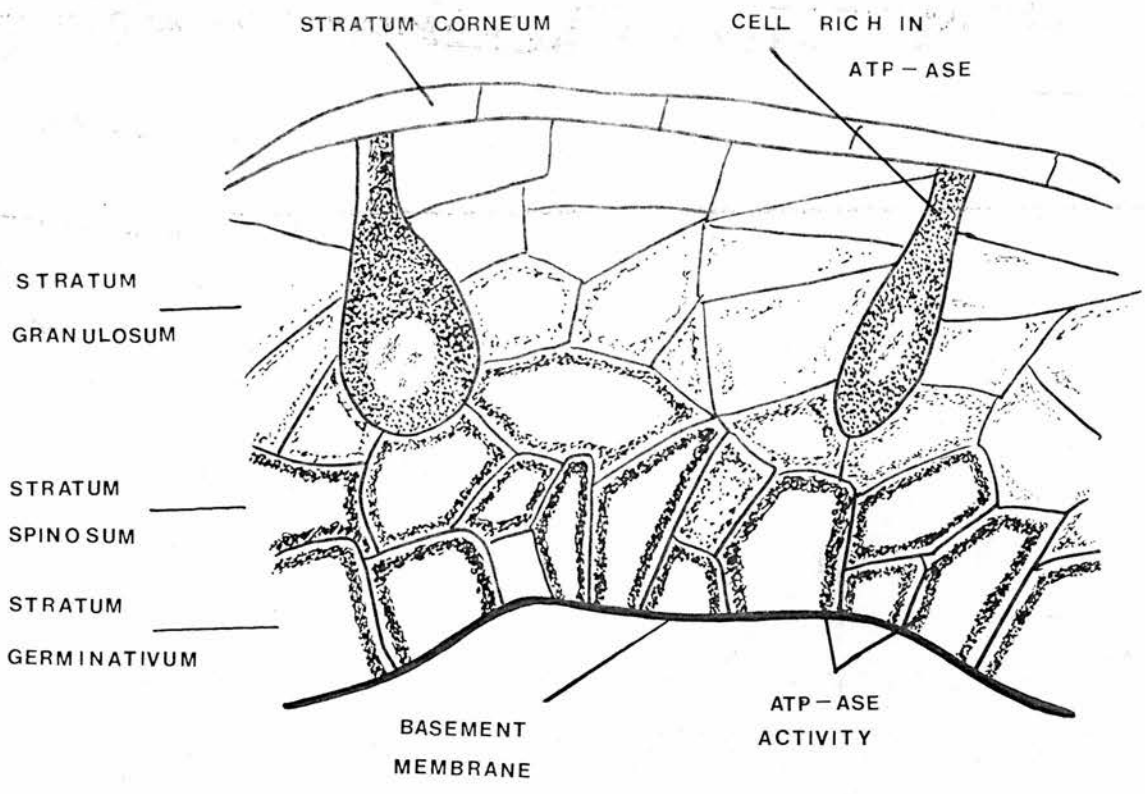


Figure II : 5

Diagram of epidermis of frog showing localisation of Adenosine triphosphatase activity. Drawing from section stained by the method of Padykula and Herman (1955). ATP-ase activity round edges of lower layers of cells (strata spinosum and germinativum) and in 'ATP-ase rich cells'. No concentration of enzyme in basement membrane. Magnification approximately 800.

not PAS-positive and therefore are not producing mucopolysaccharides. They were not demonstrable using normal staining techniques and their function is unknown.

Various alternative theories as to the site of active transport have, however, been suggested. Cholinesterases are of great importance to the active transport of sodium across the frog skin, as had been shown by the work of Kirschner (1953) involving the use of cholinesterase inhibitors, and Koblick (1958) demonstrated that these enzymes were concentrated in the tela subcutanea. Hence he assumed that this layer played an important part in the active transport process. This argument was strengthened by Sheer and Mumbach (1960) who measured the potential difference across the membrane at various levels and found that the frog skin had a two-step potential profile, one step being at the epithelium and the other at the level of the tela subcutanea. However Franz and van Bruggen (1964) found that removing the tela subcutanea had no effect on the short-circuit current and this layer could not, therefore, be the site of the sodium pump. Farquhar and Palade (1965) reported that the tela subcutanea does not form a continuous layer, in that the intercellular spaces are open inwards, hence there would be nothing to prevent the backflow of any ions which were actively transported into the animal.

In another attempt to locate the site, Engbaek and Hoshiko (1957) made more refined measurements of the potential profile of the skin and found that there were two potential steps at the base of the epidermis, which they placed at the level of the stratum germinativum. This finding was later confirmed by Whittenbury (1964). Otteson et al (1953) and Voute (1963) suggested that the basement membrane at the base of the stratum germinativum is the only continuous membrane in the skin. Such a barrier would be necessary to prevent the backflow of sodium ions and if this did represent the barrier then presumably the pump would be located in it since it would be impossible for it to be permeable to the passive movement of sodium ions in one direction only. These findings lent very strong support to the hypothesis that either the stratum germinativum or the basement membrane was the layer in which the pump was located. As has been stated already, however, the basement membrane is very likely to be readily permeable to the passage of water and small ions and possibly even small organic molecules. Hence it would not form the type of barrier which would be necessary at this site.

From this it would appear that the first of these suggested sites is the most likely, that is, that the outer cell layers represent the Na-permeable/K-impermeable membrane of the

Ussing model, whereas the sodium/potassium pump is most probably located in the cell walls of all the cells in the epidermis below the occluding zonules.

The toad bladder has a much simpler histological structure than the frog skin, being composed of a mucosal and a serosal epithelium between which runs a submucosa of varying thickness. The submucosa consists of a network of collagen bundles embedded in a matrix which also contains capillaries and venules, nerve fibres and bundles of smooth muscle cells. The serosal cells form a thin layer of simple squamous epithelium. The cells of this layer contain many vesicles and show evidence of pinacytosis at both surfaces (Fig. II : 6). According to the study by Choi (1963), there are no desmosomes between the serosal epithelial cells in Bufo marinus. Some of the serosal cells also bear villi (Fig. II : 7). The serosal epithelium lies on a thin, apparently complete basement membrane.

The mucosal epithelium also sits on a basement membrane and its cells also support large numbers of microvilli. There are three main types of cells present in this epithelium. The most numerous cell-type is the 'epithelial cell'. These cells contain a densely granular cytoplasm and their mitochondria are small and few in number (Fig. II : 8). The second most numerous type of cell is the 'mitochondria-rich cell'. These

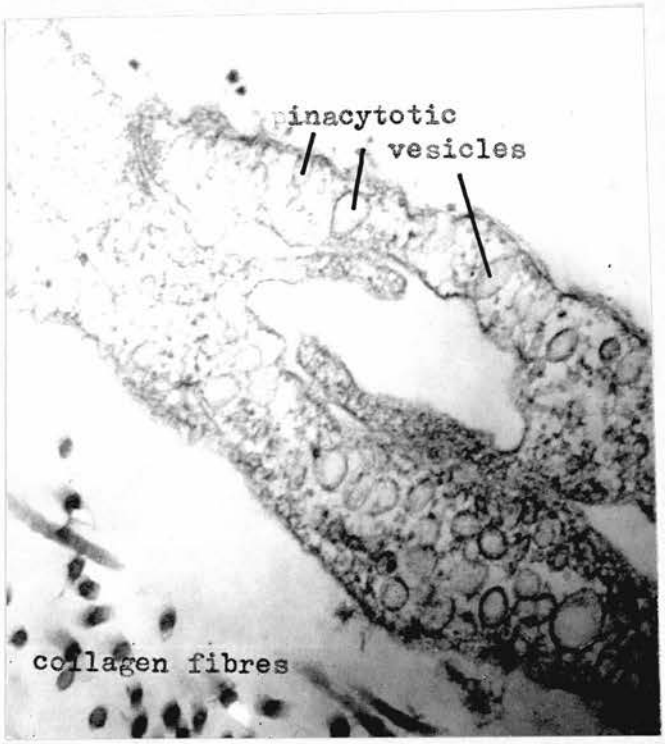


Figure II : 6

Electron micrograph of part of a cell of serosal epithelium of toad bladder.
Magnification 26,000.

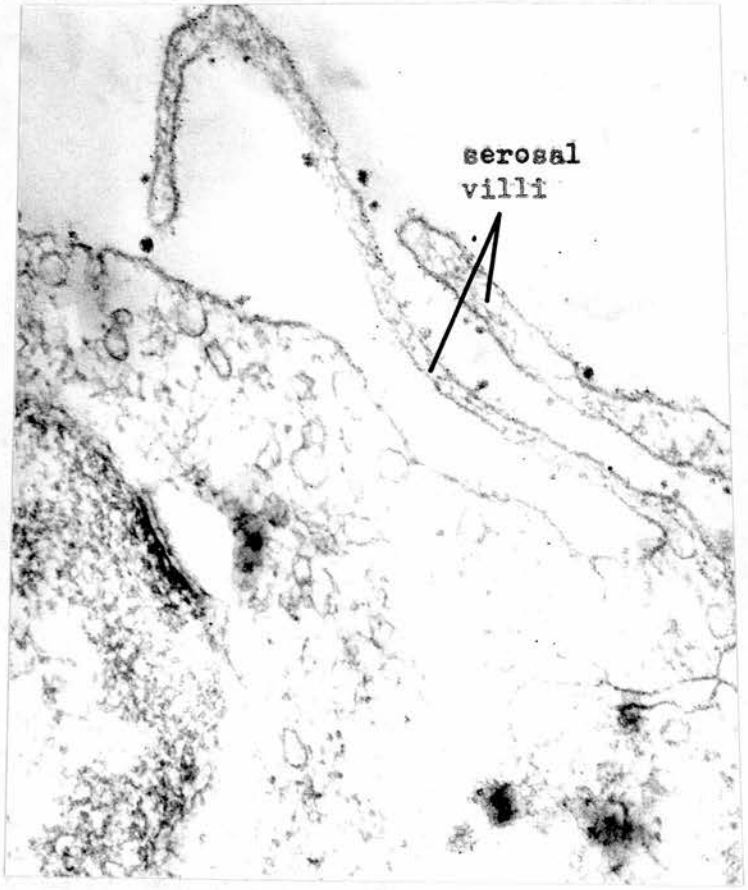


Figure II : 7

Electron micrograph of part of serosal epithelium cell of toad bladder showing villi. Magnification 24,000.

have a somewhat more clear cytoplasm than the epithelial cells, and contain very large numbers of large mitochondria. These organelles are not arranged in any obvious alignment (unlike those of the cells of the kidney tubule which are placed along folds of the cell membrane - a common feature in cells which contain active pumps), but are placed at random in the cell cytoplasm, often concentrated at the side of the cell away from the mucosal surface (Fig. II : 9). The other major type of cell in this epithelium is the 'goblet cell' (Fig. II : 10). These are large cells, the interior of which is almost completely filled with mucus droplets. They are strongly PAS-positive (Keller, 1963). According to Keller these three cell types appear in the ratios - 83% ordinary epithelial cells to 11% mitochondria rich cells to 6% goblet cells. The mitochondria-rich cells, however, account for only about 1% of the total surface area of the mucosal side of the membrane since they tend to be conical in shape with the narrow end of the cone at the mucosal surface. More detailed descriptions have been made of the various cell-types by Choi (1963) and Peachey and Rasmussen (1961).

The site of the sodium pump is as uncertain in the toad bladder as it is in the frog skin. Leaf and his co-workers, however, have suggested that the pump is likely to be located

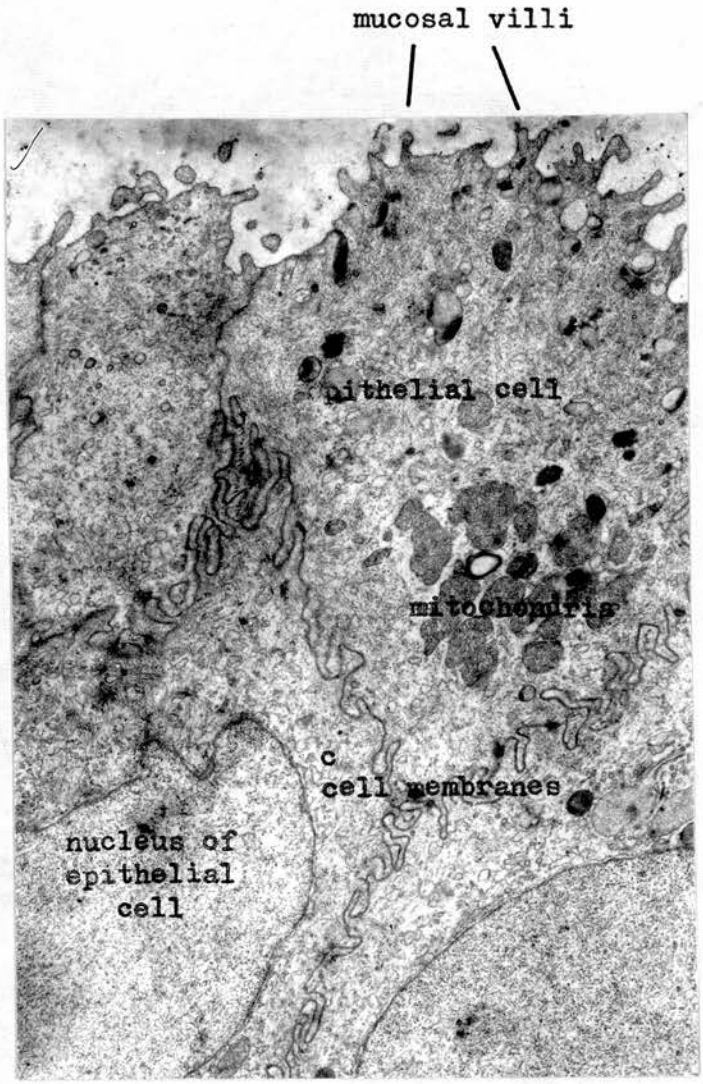


Figure II : 8

Electron micrograph of cells of mucosal surface of toad bladder. Magnification 18,000.

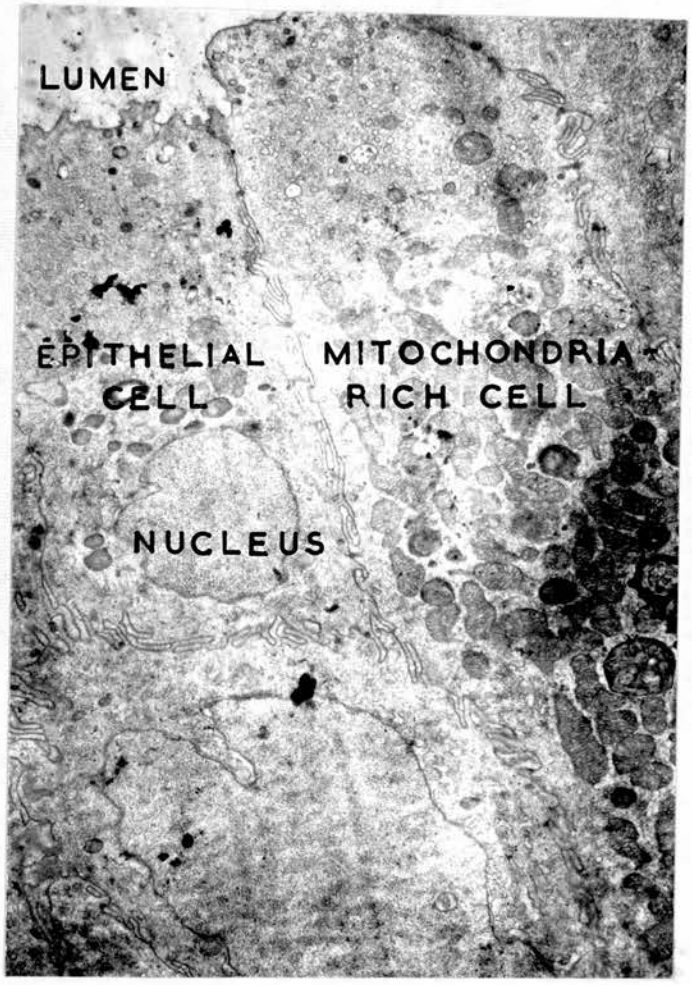


Figure II : 9

Electron micrograph of cells of mucosal side of toad bladder. Magnification 12,000.

in the basal region of the cells on the mucosal surface of the membrane. There is some support for this view from various histochemical studies which have been made of the toad bladder. It has been found (Matty and Guinness, 1964) that most of the enzymes connected with energy supply are located on the mucosal side, and specifically Keller (1963) has reported that there are membrane-associated ATP-ases in the basal cell membranes of the mucosal cells. He has also demonstrated that there is, as might be expected, a concentration of dehydrogenase activity in the mitochondria-rich cells. Occluding areas, similar to those described for frog skin, have been observed between the outer edges of the cells of the mucosal epithelium, hence it is possible for this to be the Na permeable/K impermeable layer of the bladder, and in such a situation, since this membrane is so much simpler than the frog skin, it would seem likely that the pump itself would be located along the parts of the walls of these same cells which are adjacent to the extracellular space system.

Although it has been suggested that the transport mechanism is located in the serosal cells this would seem less likely since it has been shown that there are no desmosomes or maculae adherens between the cells of the serosal epithelium as there

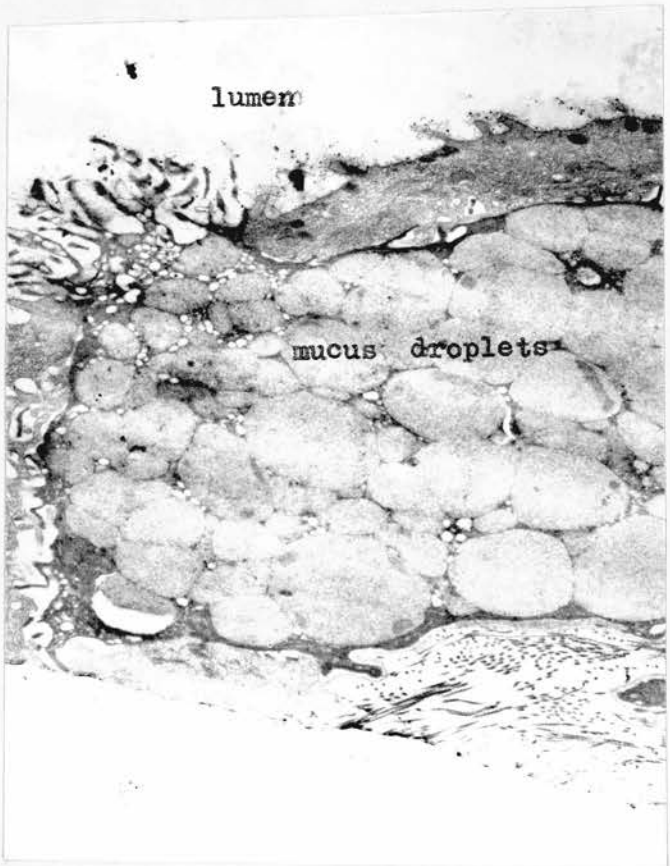


Figure II : 10

Electron micrograph of goblet cell of toad bladder. Magnification 6,000.

are between those of the mucosal epithelium. Hence if the pump was located on the serosal side of the bladder there would be no free passage of ions among the cells to balance the sodium pools in the cells, whereas this could take place in the cells on the opposite side of the membrane. Also there is little in the way of enzyme activity on the serosal surface of the membrane, even of those enzymes which would be necessary to provide the energy for the active transport process.

Among the cells in the mucosal epithelium it would seem at first glance that the mitochondria-rich cells are the ones most likely to be the site of the sodium pump since not only are they morphologically similar to other transporting cells in, for example, the kidney tubule, in that they possess numerous mitochondria but also they possess the highest concentration of the various enzymes which may be associated with the pump mechanism. However Hoshiko and Ussing (1960) have shown that the pump could not be located in these cells alone since the 'active transport pool' of sodium in the membrane is too large to be contained in only 10% of the cells. From the location of the ATP-ases it would appear likely that the pump is located along the internal surfaces of all, or nearly all the mucosal epithelium cells.

It will be apparent from these descriptions that the two membranes under consideration present very much the same picture when it comes to an attempt to locate the sodium pump geographically. It would seem that the membranes, although histologically very different, have various points of structure in common. Both have occluding zonules at the outer border of the mucosal cell layer, effectively separating the intercellular space system from the external medium. It is not known definitely whether the occluding areas are impermeable to salt but there is reason to believe that they are impermeable to water, hence it would only be possible for molecules and ions of a larger size to pass through attached to a carrier. Working on the assumption that there is no such carrier present, especially likely since these 'plugs' are outside the cells, this means that the outermost surface of the outer cell layer in each case is likely to be the outer surface of the Ussing model. The inner surface, where the pump itself is placed, is more difficult to locate. There is some evidence, from the distribution of ATP-ases, that in both membranes it may be the walls of all the cells beneath the occluding layer down to the next basement (the entire epidermis in the skin and the mucosal epithelium in the bladder). There are

several questions, however, which these hypotheses leave unanswered. Of these the two most important are concerned with the role of the mitochondria-rich cells of the bladder since it is difficult to see what function these cells serve if they are not the site of the pump, and also an explanation is needed for the fairly consistent demonstration of a two-step potential profile in the frog skin with the steps at each side of the stratum germinativum. Unfortunately there is not sufficient data at this time to make even a tentative hypothesis regarding these questions.

I would like to thank the Hon.F.E.Guinness for her help with the electron microscopy and for allowing me to use some of her electron micrographs.

CHAPTER THREE

MATERIALS AND EXPERIMENTAL METHODS

(i) Animals.

The animals used in this study were *Rana temporaria* and *Bufo bufo*. The *R. temporaria* were all obtained from a commercial source (L. Haigh & Son, Surrey), as were some of the *B. bufo*. Other toads were collected locally around St. Andrews. No animals were used until they had been in the laboratory for at least two days. The toads were kept indoors in large tanks floored with peat or gravel and containing dishes of water. During the course of some experiments animals were kept at a constant temperature of 12°C, but it was found that this made no apparent difference to the basal rate of sodium transport across the membranes, so, for the most part, the animals were kept at ambient temperature. The frogs were housed in large aquaria in which there was either a floor of gravel and a large dish of water or else the aquarium was tilted and water and gravel put on the floor. All the animals were cleaned out once a week and given fresh water each day. The photoperiod was of natural length for the time of year.

The animals were generally fed on blowfly larvae, but this diet was occasionally supplemented with a mixture of minced liver and raw egg which was force fed to them through a syringe.

This was particularly necessary with some of the locally collected animals which appeared to ingest the maggots quite readily, but then passed them straight through the gut and egested them with no sign of digestion having taken place. The reason for this was not known, but it did not appear to be universal, and was never met in the frogs.

No diseased animals were used in these experiments. The two diseases most frequently found in the animals were the bacterial infection known as 'red-leg' and a parasitic infection of the bladder by platyhelminths. Any animals suffering from either of these were discarded.

It was found that there was quite a considerable seasonal variation in the potential across the bladder and skin of the toads, and to a lesser extent, across the frog's skin. During the months immediately after spawning, the potential was very low in both sexes. The majority of the sodium movement experiments were performed, therefore, between July and January.

The animals were killed by stunning and double pithing, and were pinned out prior to dissection. In the case of the frog a sheet of skin was removed from the belly region of the animal sufficient in size to be used across two chambers. In the toads either a sheet of skin from the belly region was removed, or the animal was opened and the bladder removed by

holding up each lobe separately whilst freeing it from the mesentery and then cutting it free completely at the cloacal end. The tissue was placed in a petri dish containing physiological saline solution as soon as it had been removed. The skins were mounted directly, but in the case of the bladder the two lobes were separated and each one opened out before mounting to give a relatively flat sheet of tissue.

(ii) The hormones.

Most of the hormones used in this study were obtained from commercial sources.

The thyrotrophic hormone (T.S.H.) preparation Ambinon 'B' (Organon Laboratories Ltd.) was obtained as a lyophilised powder and used at a concentration of 0.3 U.S.P. units per ml. of bathing solution on frog skin and toad skin and bladder. Another preparation of T.S.H., obtained from Parke Davies Ltd. was used on frog skin at concentrations of 0.15, 0.10, 0.05 and 0.015 U.S.P. units per ml. of bathing fluid. Thytropar, the Armour preparation of bovine T.S.H., was used on frog skin at concentrations of 0.15, 0.005 and 0.0002 U.S.P. units per ml. of bathing solution.

Preparations of follicle-stimulating hormone (F.S.H.) were obtained from Armour Ltd. (F.S.H.-F) and the National Institute of Health (N.I.H.), Bethesda, U.S.A. (batch no. NIH-FSH-S1). The Armour product, which was porcine in origin, was applied to

frog skin at concentrations of 400, 100, 10 and 1 milliunit per ml. of saline. The preparation of ovine F.S.H. supplied by N.I.H. was used on frog skin at concentrations of 0.05 mg. and 0.025 mg. per ml. of bathing solution, and on toad bladder at a concentration of 0.025 mg. per ml. of bathing fluid. According to the data sheet supplied with the preparation, the hormone had been assayed against the Armour product and was equivalent to 2.7 units F.S.H. per mg. lyophilised powder.

The preparations of luteinising hormone used were also supplied by Armour (P.L.H.) and by N.I.H. Bethesda (batch of ovine L.H. no. NIH-LH-S7). The Armour preparation was applied to frog skin at concentrations of 0.25 and 0.01 milliunits per ml. of bathing fluid. The N.I.H. preparation was applied to frog skin at concentrations of 0.05 and 0.025 mg. per ml. of saline and to toad bladder at a concentration of 0.025 mg. per ml. saline. This latter preparation had a potency of 0.42 U.S.P. units per mg. of lyophilised preparation.

Two preparations of adrenocorticotrophic hormone (A.C.T.H.) were used. Actar-Gel was obtained from Armour Ltd. and applied to frog skin at a concentration of 400 milliunits per ml. of bathing fluid. The high purity preparation ACTH-Al, obtained from Organon, was applied to frog skin at a concentration of 0.05 mg. per ml. of bathing fluid. It was not possible to obtain

a figure for the activity of this latter preparation for purposes of comparison with the former, since ACTH-Al is a fraction of A.C.T.H. which does not show all the properties of the entire substance.

The N.I.H. preparation, NIH-GH-B7, of bovine growth hormone (somatotrophic hormone or S.T.H.) was applied to frog skin at a concentration of 0.05 mg. per ml. of bathing fluid. This substance assayed at 0.88 U.S.P. units per mg. lyophilised preparation. Since this hormone is minimally soluble between pH 6 and 8 in distilled water and physiological saline, it was necessary to first suspend the lyophilised powder in a very small quantity of physiological saline and then add a small amount of 0.1 normal sodium hydroxide solution (about 0.05 ml. per mg.hormone). This does not impair the biological activity of the preparation, and control runs showed that such an amount of sodium hydroxide has no measurable effect on the transport of sodium ions across the membranes used in this study. Two preparations of high S.T.H. activity were obtained from Ferring (Sweden). These were Prolactin and Somacton. The S.T.H. activity of the ovine preparation Prolactin was approximately three times as high as that of the porcine preparation Somacton, when compared on a weight basis. Somacton was used on frog skin at a concentration of 0.05 I.U.S.T.H. activity per ml. of bathing fluid. The Prolactin was applied to

frog skin at a concentration of 1 International Unit Prolactin per ml. of saline, and to toad bladder at a concentration of 0.5 I.U. prolactin per ml. bathing fluid. The amount of the Prolactin preparation which contained 1 I.U. of prolactin also contained approximately 0.1 units of S.T.H. activity.

Since almost all of the preparations of the anterior pituitary hormones were contaminated with small amounts of posterior pituitary hormones, it was necessary to perform control experiments with the contaminant level of posterior pituitary hormone for each anterior pituitary preparation. The increase in sodium transport brought about by the vasopressin at the contaminant levels was compared with the increase caused by the hormone preparation, and thus the actual effect of the anterior pituitary preparation was found. The preparations of posterior pituitary hormones used to measure the contamination effects were Pitressin, a vasopressin preparation by Parke Davies, and a preparation of lysine vasopressin, given by Professor Heller of Bristol University. These were used at the level relevant to the hormone under study, the lysine vasopressin being used to measure the possible contaminant effects of hormones of porcine origin since these would be contaminated with lysine rather than arginine vasopressin (Kleeman and Cutler, 1963; Sawyer, 1961).

Among the other hormones used in this study were thyroxine supplied by Light and Son Ltd. This was applied to frog skin, toad skin and toad bladder at concentrations of 10^{-5}M per litre of saline. The preparation used was a sodium salt of tetra-iodothyronine and was relatively insoluble in water or physiological saline. It was, therefore, first dissolved in a small amount of 0.1 normal sodium hydroxide and subsequently added to the saline solution in which it remained in solution.

Oestradiol sulphate and oestrone sulphate were obtained from Organon Ltd. Both of these preparations were considerably more soluble in water than the parent steroid, and were used for that reason. The oestradiol sulphate was used on frog skin at a concentration of 0.08 mg. pure substance per ml. bathing fluid, and on toad bladder at a concentration of 0.03 mg. per ml. The oestrone sulphate was applied to frog skin at a concentration of 0.015 and 0.03 mg. of pure substance per ml. saline.

Angiotensin, obtained as the preparation Hypertensin, from Ciba Ltd., was applied to frog skin at a concentration of 0.015 mg. per ml. bathing solution. Hydrocortisone, obtained from Ciba Ltd., was applied to frog skin at a concentration of 0.06 mg. per ml. bathing fluid, and glucagon, from Parke Davies, was used on frog skin at a concentration of 0.015 mg. per ml. bathing saline.

All hormone preparations were applied to the membranes in solution in the physiological saline. The hormone/saline preparations were made up fresh for each experiment.

(iii) Physiological saline solutions.

Experiments were performed with several amphibian "Ringer" solutions to determine that which was best for the study in hand. It was found that the salines of Matty and Green (1963), Boyle and Conway (1941), Forster (1948) and Harris (1953) (see Lockwood, 1961) were all capable of keeping the tissues alive and in such a condition that they could transport sodium.

A modification was used of the saline described by Boyle and Conway in their experiments on frog muscle. This contained 4.24 gm. NaCl/l, 0.148 gm. KCl/l, 0.2 gm. $CaCl_2$/l (the original contained 0.4 gm. Ca gluconate/l rather than $CaCl_2$), 0.146 gm. $MgSO_4$/l, 0.092 gm. Na_2SO_4/l, 2.1 gm. $NaHCO_3$/l, 0.356 gm. Na_2HPO_4/l, 0.068 gm. KH_2PO_4/l and 4.648 gm. glucose/l. This solution was equilibrated with a mixture of 95% O_2 and 5% CO_2 for one hour. The pH of the solution, before bubbling, was about 7.8. Since, during the course of the study, it was necessary to aerate the tissue with air through a Hi-flo pump, this method of aeration was used with this solution after the initial equilibration.

The saline used by Forster contained 5.3 gm. NaCl/l, 0.19 gm. KCl/l, 0.22 gm. $CaCl_2$/l, 0.2 gm. $MgCl_2$/l, 1.26 gm. $NaHCO_2$/l,

0.07 gm. NaH_2PO_4/l and 0.54 gm. glucose/l. The pH of this before bubbling was also 7.8.

Harris's saline consisted of 5.26 gm. NaCl/l, 0.22 gm. KCl/l, 0.22 gm. $CaCl_2$/l, 0.12 gm. $MgSO_4$/l, 2.52 gm. $NaHCO_3$/l, 0.33 gm. Na_2HPO_4/l, and this had a pH of 8.0 before aeration.

The "Ringer" used by Matty and Green contained 6.5 gm. NaCl/l, 0.2 gm. KCl/l, 0.2 gm. $CaCl_2$/l, 0.1 gm. $MgSO_4$/l, 1.5 gm. $NaHCO_3$/l, 0.75 gm. glucose/l. The pH was 8.2.

100 ml. of each of these salines was made up and the pH adjusted to 7.2 in each case with hydrochloric acid. Before adjusting the pH it was noticed that the salines of Boyle and Conway and Harris had precipitated slightly, but the solutions cleared when the pH was adjusted. These were then aerated with air via a Hi-flo pump. Forty minutes later the Harris saline had precipitated again. This saline was then found to have a pH of 8.2. Hydrochloric acid was added until the precipitate redissolved (pH 6.9) and the pH was then brought back to 7.25 with NaOH. Aeration was then continued but 25 minutes later it was found to be milky again with a pH of 8.25. Ninety minutes after the start of aeration the Boyle and Conway saline was found to have turned milky and had a pH of 8.2. This was adjusted until the precipitate disappeared, when the pH was 6.8. After adjusting the pH to 7.2 aeration was re-started and the solution precipitated again after one hour. Three hours after the commencement of

aeration both the Matty and Green and the Forster salines were still clear, the Matty and Green saline having a pH of 8.4 and the Forster saline with a pH of 8.6.

If the solution were aerated with a mixture of 95% O_2 and 5% CO_2 Harris's and Forster's salines precipitated. When 100 ml. samples of fresh saline were left overnight without aeration it was found that Harris's and Boyle and Conway's salines precipitated during the night whilst those of Matty and Green and Forster remained clear, Matty and Green's having a pH of 8.4 and that of Forster having a pH of 8.9. From these data it was decided to continue with the saline used by Matty and Green (1963) whilst undertaking a similar study.

This saline was used throughout the work at a pH of 7.8 (that used by Matty and Green). The choice of pH was, within limits, arbitrary, since there appeared to be little effect, by pH, on the sodium transport across any of the tissues used, between 7.0 and about 8.6, beyond these limits there was a sharp decrease in active transport.

The saline was normally made up from concentrated stock solutions and distilled water, the pH being adjusted immediately before the commencement of the experiment. The saline was normally stored overnight before use but was never used after being stored for more than four days. It was always kept at 2°C, the amount to be used being allowed to return to room temperature immediately

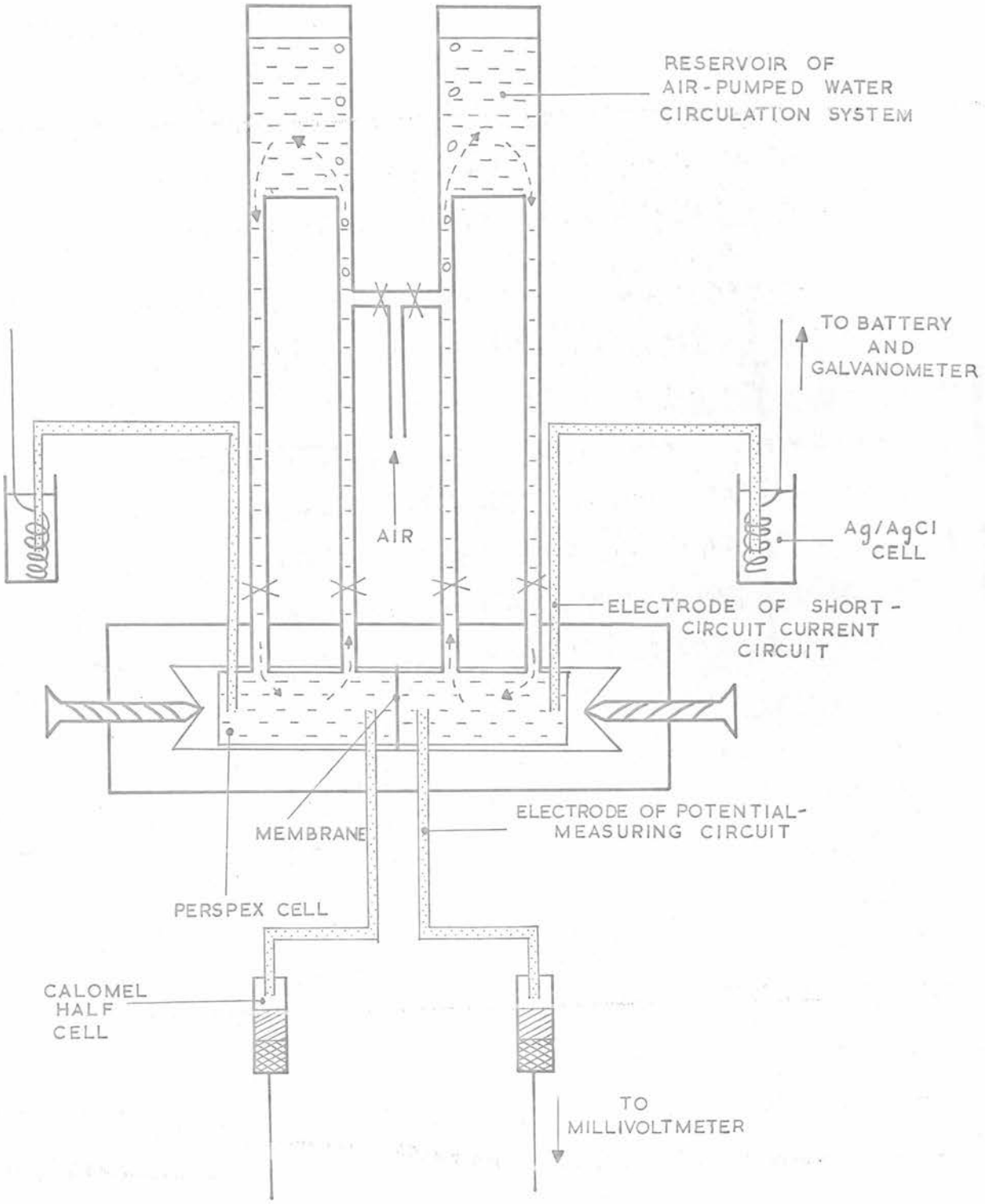
before the beginning of each experiment, and any excess was discarded at the end.

During the majority of the experiments the saline was aerated with air throughout the complete period but this did not appear to have any effect upon the membrane. It did, however, ensure circulation of the hormones etc. within the system.

(iv) Apparatus for the measurement of sodium transport.

The apparatus for the measurement of sodium transport across the membranes was similar to that used by Ussing and Zerahn (1951). It consisted, basically, of two perspex chambers between which the membrane was mounted (Fig. III : 1). These chambers were filled with physiological saline solutions. A polythene tube filled with saturated KCl/agar was inserted through the wall of the chamber to within 1 mm. of the centre of the membrane on each side. These tubes acted as current bridges between the chambers and the calomel half-cells of the circuit used for the measurement of the electro-chemical potential difference across the membrane. This potential was measured on the millivolt range of a pH meter. The two pH meters used during the course of the work were a Radiometer pH Meter 4 and a Pye Dynacap to which an extension meter had been attached, enabling the first twenty millivolts of the range to be read to an accuracy of 0.1 mV. The extension meter was an Eel microammeter. The calomel half-cells

FIG. III: 1 APPARATUS A

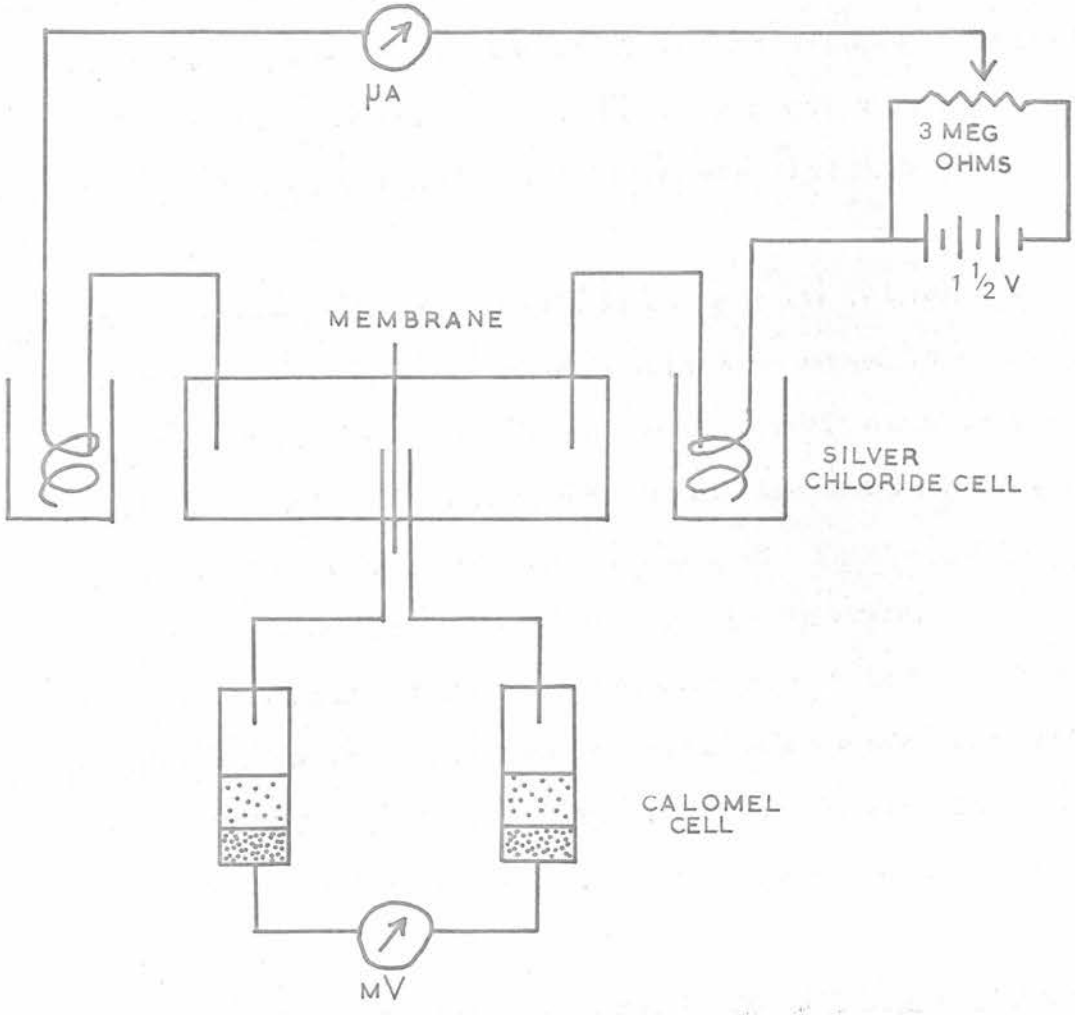


were glass tubes into the bottoms of which were sealed half-inch pieces of platinum wire. The wire to the potentiometer was attached to the outside end of the platinum wire, the end inside the tube being immersed in mercury. Above the mercury was a layer of calomel paste, consisting of equal parts of mercury, mercurous chloride and potassium chloride, ground to a homogeneous paste with a little saturated KCl. Above this was a saturated solution of potassium chloride into which the agar bridges were dipped.

At the end of the chamber furthest from the membrane two more saturated KCl/agar bridges were inserted through the chamber wall. These linked the chambers to the silver/silver chloride cells of the short-circuit current, through which the external E.M.F. was applied. The voltage for this was supplied by a 45 volt battery and adjusted by a 3 meg-ohm potential divider. The silver/silver chloride cells were composed of a spiral of chlorided silver wire immersed in a saturated solution of potassium chloride into which was dipped the agar bridge. The short-circuit current was read from an Eel microammeter. For a diagram of the circuit see Fig. III:2.

Three types of chamber were used during the course of this study. The first two of these were similar to those used by Green (1964). In Apparatus A the perspex cells were enclosed

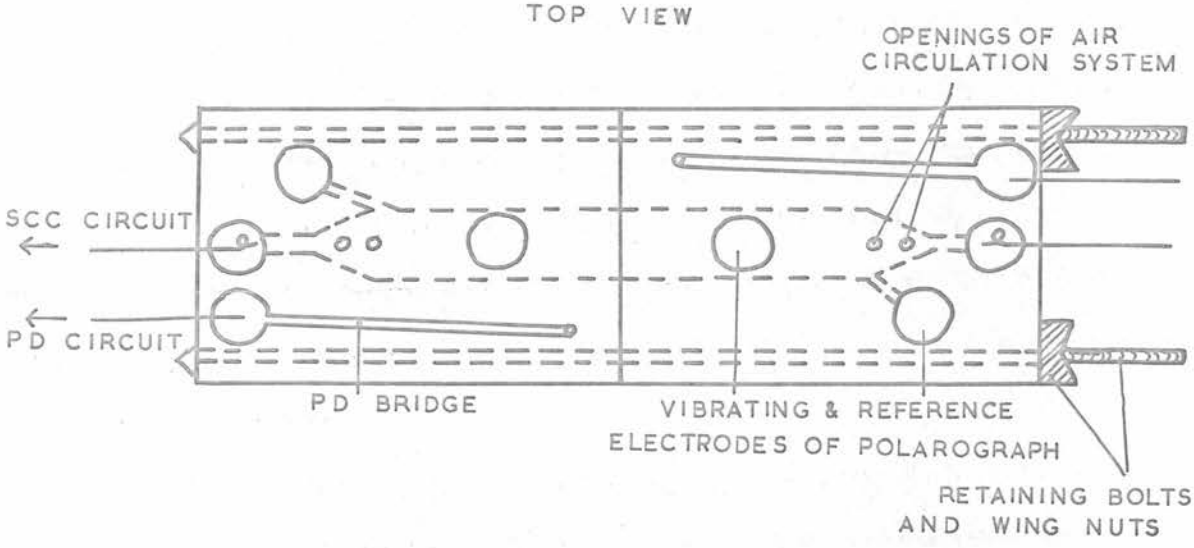
FIG. III :2 CIRCUIT DIAGRAM

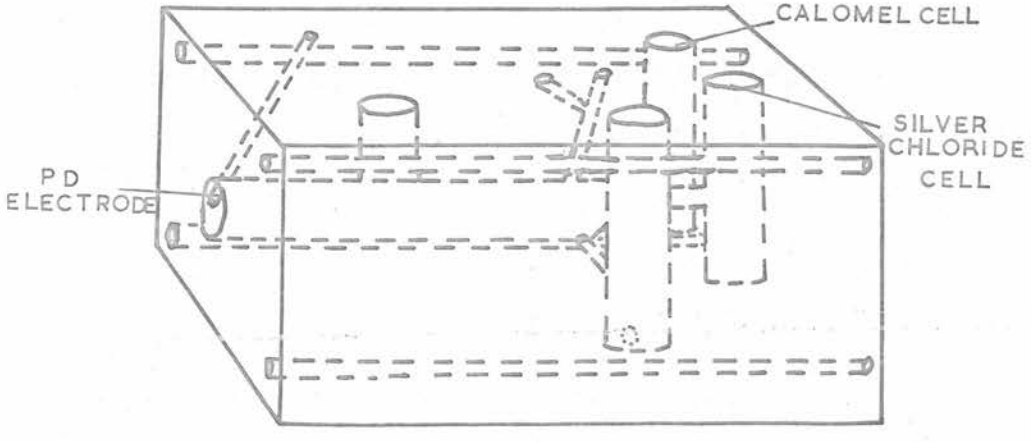


within a wooden trough through the ends of which ran two brass screws which fitted into conical grooves in the cells. By tightening these screws the cells were drawn together forming a water-tight seal about the membrane. An aerator system was fixed to the chamber which also served as a circulatory system, joining the cells to an outside reservoir of liquid; the chambers, tubes and reservoir held a total volume of 30 ml. of saline. Polythene tubes connected the cells to the glass reservoirs as shown in Fig. III:1. The air supply opened into the side of one of these tubes, thus causing the saline to be drawn round the entire system. The area of the open end of the chamber was 0.5542 sq. cm.

The second type of apparatus is shown in Fig. III:3, in which the silver/silver chloride cells and the calomel half-cells were incorporated into the same block from which the chambers were machined. In this case there was provision made for the introduction of vibrating platinum electrodes and reference electrodes for the measurement of oxygen concentrations within the chambers. These, however, were never used. The chambers were clamped together by means of four screws which ran the full length of the two halves and were tightened together by means of wing nuts. This apparatus was found to have several disadvantages, the major one being that any necessary tilting during the mounting of the membrane tended to

FIG. III:3 APPARATUS B





SIDE VIEW OF ONE HALF

upset the calomel cells. Also the agar bridges, being injected into small tubes drilled in the perspex, were difficult to keep in good condition, tending to be forced into the chambers by hydrostatic pressure. The area of the open end of the chamber was 0.5608 sq. cms. and the volume of each side was 1.5 ml.

Apparatus C (Fig. III:4) was designed to allow several experiments to be run simultaneously. The apparatus was much simplified, consisting of a single perspex cell across the end of which the membrane was secured by means of a perspex gasket held in place by four nylon screws. The whole was immersed in a bath of saline which served as the outer chamber. The area of the open end of the chambers was 0.4418 sq. cms. The volume of saline held in the chamber was 1 ml. The block was immersed in a bath of 30 mls. of saline. The agar bridges were inserted through the top of the chamber and in the bath at the relevant distance. Glass tubes were used to make these bridges, rather than polythene, since it was necessary to use something which would hold its own position, unsupported by the walls of the chambers, as took place in the other types of apparatus. Using this apparatus it was possible to run several experiments at the same time, using the same circuits, since the bridges could be lowered into the appropriate chamber to take a reading, and afterwards moved on to another chamber. This, however, had the disadvantage of not giving a continuous recording of the potential.

FIG. III:4 APPARATUS C

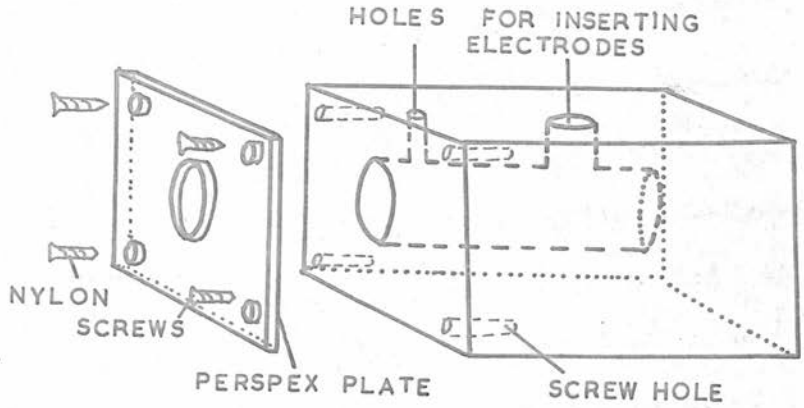
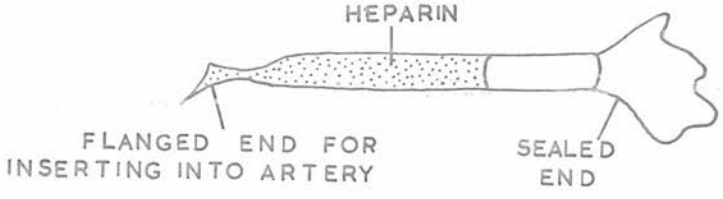


FIG. III:5 ARTERY CANNULA



When it was necessary to obtain a continuous recording of the potential difference, a Cambridge 4-pen recorder was incorporated into the circuits with apparatus A or B. This also recorded the short-circuit current when the potential was adjusted to zero.

(v) Technique for measurement of sodium transport.

After sacrificing the animal the tissue was removed and placed in a petri dish of physiological saline. The two halves of the bladder were separated and opened up, thus all three tissues could be used as flat plates. When apparatus A was to be used the membrane was draped across the open end of one of the chambers, the other chamber was held against it, the unit placed in the trough and the screw tightened. In the case of apparatus B and C the membrane was layed across the face of the block, over the open end of the chamber and the other block or, in apparatus C, the gasket, was pressed against the membrane and the screws tightened. The chambers were filled with liquid, care being taken to prevent any great excess of hydrostatic pressure at either side which would cause the membrane to stretch, this being particularly necessary in the case of the toad bladder which was very delicate. When using apparatus C it was necessary to partly fill the chamber before immersing it in the bath of saline which served as the other cell. The chamber was then filled completely. The saline

was inserted into the chambers of apparatus B and C by means of a hypodermic syringe to which was attached a polythene tube.

The membranes were left in physiological saline to stabilise since it was found that the potential difference fluctuated immediately after mounting the membrane. The toad bladder was left for one hour and the toad and frog skins were left for one and a half hours. The bathing fluid was changed twice during this equilibration period, the second time being about ten minutes before the first reading was taken.

When apparatus A and B were in use the potential difference across the membrane was continuously monitored and the short-circuit current was measured at intervals of either ten or fifteen minutes. When the pen recorder was not in use the potential difference was noted immediately prior to measuring the short-circuit current. To obtain a measure of the short-circuit current the potential difference was adjusted to zero using the potential divider in the external circuit and the amount of current necessary to do this was read from the microammeter. When apparatus C was in use the chamber and bath were raised to the electrodes and the readings taken in exactly the same way as with apparatus A and B when the recorder was not in use.

For the first hour after the equilibration period the readings were taken when the membrane was immersed in saline which did not contain any hormone. This enabled a basal value

to be obtained for the sodium transport across each membrane used. After this hour the solution was removed from both sides of the membrane and replaced by fresh saline. The saline now applied to one or both sides of the membrane contained a solution of hormone of the appropriate concentration. The readings were continued for at least one hour, and in many cases considerably longer, after the application of the hormone. In some cases the hormone solution was removed after one or two hours and the membrane washed for at least thirty minutes. A second control period was then recorded and an application of the same or a different hormone made at the end of the control hour.

(vi) Interpretation of Results.

Ussing and Zerahn (1951) performed experiments upon frog skin during which they "short-circuited" the membrane. In this condition the potential difference across the skin is reduced to zero and therefore, since the saline has effectively the same composition on both sides of the membrane, there will be no net transfer of passive ions from one side to the other. Those ions, however, which are actively transported, will continue their unidirectional movement across the skin. This will cause

a current to run through the short circuit which will be equivalent to the sum of the active transport of charged particles through the membrane.

In a series of experiments during which the flux of sodium ions (measured isotopically) across the frog skin was measured simultaneously with the short-circuit current, Ussing and Zerahn showed that the net flux of sodium ions (i.e. the influx minus the outflux) in the short-circuited skin was equal to the current measured in the short circuit when both were expressed in the same units, e.g. coulombs/hour. This relationship was shown to hold true when the electrical potential was increased, e.g. by application of vasopressin to the membrane, or when it was reduced by higher CO_2 tensions.

Similar experiments were performed on the toad bladder by Leaf, Anderson and Page (1958) and on the bladder and skin of Bufo bufo by Green and Matty (1963). In these membranes, also, the short-circuit current was found to be equivalent to the net active transport of sodium ions. Because these experiments are so well authenticated it was considered unnecessary to repeat the tracer experiments needed for proof, and throughout this study the relationship between short-circuit current and active transport has been accepted.

Most of the results of the experiments performed during this study have been expressed in micro-equivalents of sodium

actively transported per square centimeter of membrane per minute. These figures were calculated from the fact that a movement of 0.01036267 equivalents of a monovalent ion across a membrane in one second gives rise to a current of one milliamp. Hence:

$$\mu \text{ eq Na/min./sq.cm.} = \frac{60 \times 10.36267 \times 10^{-6} \times \mu \text{ amps/sec.}}{\text{Area of membrane in sq. cms.}}$$

(vii) Assay of Pressor Activity.

Some of the preparations used, particularly those of the anterior pituitary hormones, were found to be contaminated with unknown amounts of posterior pituitary hormones. Since, as has already been discussed, vasopressin and its analogues have a very marked effect on the sodium transport across frog skin and toad bladder, it was necessary to find out what proportion of the total effect of the preparation was due to the contaminants. This was done by assaying the anterior pituitary hormones for their pressor activity and then doing control experiments with a saline containing the equivalent amount of vasopressin and subtracting the control results from the experimental results.

The technique for assaying the pressor activity of the preparations was Dekanski's modification (1952) of the procedure

of Landgrebe et al (1946). Rats of about 200 to 300 gms. body weight were anaesthetised by an intraperitoneal injection of 25 per cent w/v solution of urethane, at a dose level of 0.5 ml. per 100 gms. body weight. Occasionally it was necessary to give slightly more anaesthetic, but this was done with care since the rats proved very susceptible to an overdose of urethane. The rat was normally under surgical anaesthesia within ten minutes.

It was found that the external jugular vein was the easiest to use for the venous cannulation, since it was large and easily accessible. Care had to be taken, however, because the vessel was so close to the heart that any mistake could lead to death by exsanguination in a very short time. The cannula used for this purpose was a very fine piece of polythene tubing. A size 20 syringe needle just fitted into this cannula.

The next step was the cannulation of the trachea. This was done because it was found that animals left uncannulated had a tendency to choke themselves to death with their saliva. The carotid artery was then cannulated as close to the heart as possible. The cannula used in this case was once again of polythene, but slightly larger in diameter than that used for the venous cannula. This was pulled at one end to fit into the carotid, and it was filled with heparin solution. The other end was sealed until the animal was coupled to the manometer (see Fig. III:5). During the cannulation of the carotid artery it

was found necessary to take great care to avoid handling, cutting, or including in the ligature, the vagus nerve, since this had deleterious effects on the breathing ability of the animal.

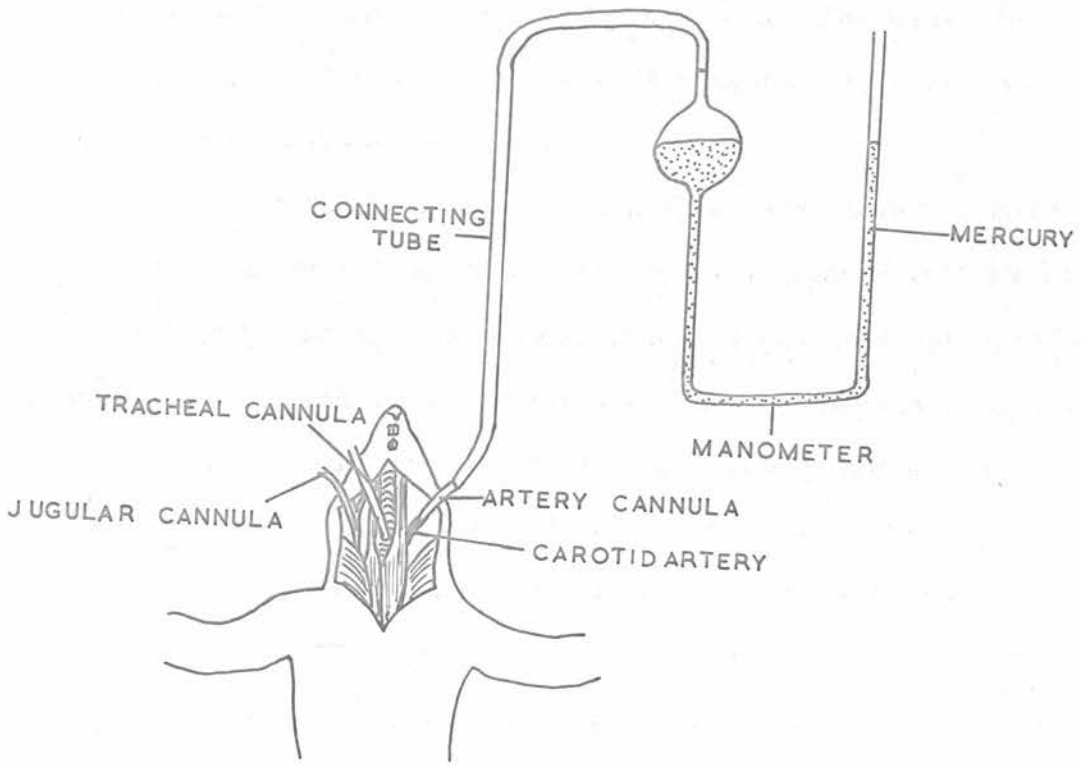
The manometer was the standard Condon manometer (see Fig. III:6) filled with mercury. A fine rubber tube was used to connect the manometer to the cannula. This was of such a size as to fix directly on to the cannula. The tubes connecting the mercury of the manometer with the cannula were filled with 0.9 per cent saline solution.

Before attachment of the animal to the Condon manometer, it was given an i/v injection of 500 i.u. heparin per 100 gm. body weight, through the venous cannula, and left for a minute to allow the heparin to circulate. The cannula was attached by cutting off the sealed end and immediately inserting it into the tubber connecting tube. During this process the cannula was closed with an artery clip to prevent blood loss.

Immediately after attachment the rat was given an i/v injection of dibenylene (0.1 mg. per 100 gm. body weight of rat, obtained from Smith, Kleine and French) to block the pressor effect of adrenalin. The animal was then left for about five minutes until the blood pressure had settled. Immediately the base line was level the assay was started.

The actual assay followed the standard pattern, using

FIG. III:6 CANNULAE AND MANOMETER



two doses of vasopressin of known presser activity and two doses of the unknown substance. The doses of unknown activity were then varied until they gave the same response as the known doses. From this could be calculated the presser activity (in terms of presser units per unit of hormone) of the hormone preparations. It was assessed that the pressor activity of the unknown was entirely due to vasopressin since no presser effects have been recorded for pure preparations of anterior pituitary hormones.

CHAPTER 4.

RESULTS

1. Anterior Pituitary Hormones.

a) Thyrotropin.

The first extract of the anterior pituitary gland applied to the membranes in this study was the relatively crude Organon preparation of thyrotropin (thyroid-stimulating hormone or T.S.H.) called Ambinon 'B'. A concentration of 300 mU/ml. serosal bathing fluid was found to significantly increase both the potential difference (P.D.) and the short-circuit current (S.C.C.) across the skin and bladder of the toad and the frog skin (see Fig. IV:1). The effect of the preparation on frog skin reaches its peak much faster than on either of the other membranes. The amount of sodium transported per square centimeter of membranes is approximately doubled within ten minutes of the addition of the hormone and then slowly decreases, still showing a 60% increase over the basal level 1½ hours after application of the preparation. The potential difference follows this very closely, both in time course and extent of increase, having a basal level for the P.D. of 15.5 mV and reaching 30 mV ten minutes after addition of the preparation. After 1½ hours incubation in the hormone solution the P.D. has dropped to 25.3 mV. The extent of the

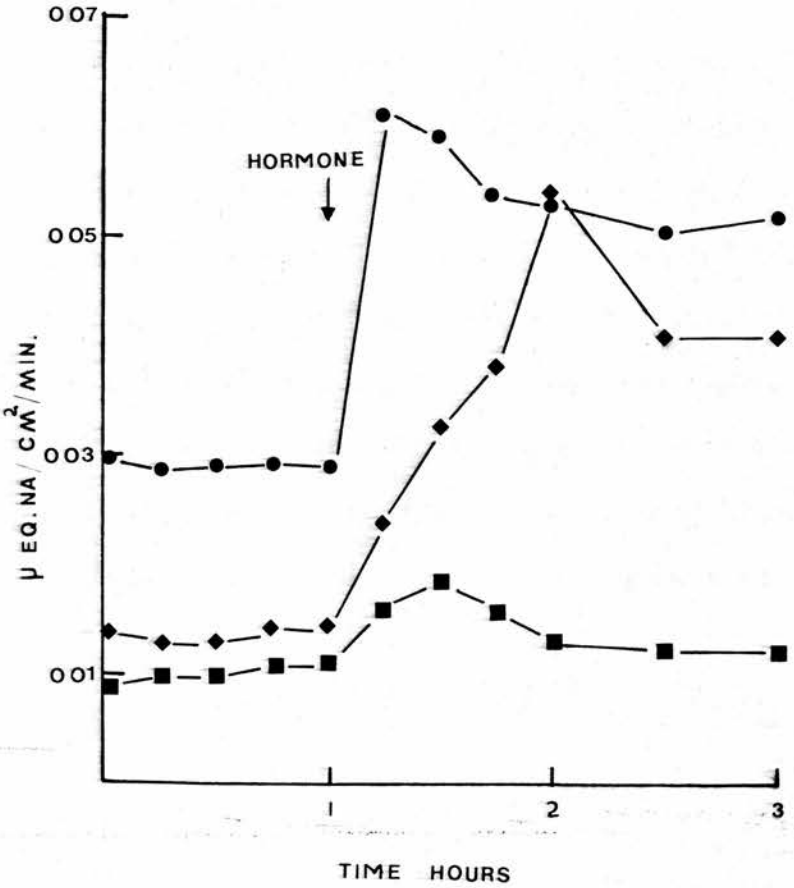


Figure IV:1 Graph to show effect of Ambinon"B" on net sodium flux across toad bladder (■), toad skin (◆) and frog skin (●).

effect shown by toad skin is greater than that of the frog skin, but the time course of action is slower. The level of sodium transport rises from approximately 0.014 µeq. Na/sq.cm./min. in the control period to 0.054 µeq./sq.cm./min. one hour after addition of the hormone to the serosal bathing fluid. After 1½ hours incubation the level is still much higher than the control level. In this case the effect on the P.D. does not exactly follow that on the S.C.C. The P.D. rises from a control value of 15 mV to a level of 27 mV one hour after the addition of the hormone, but then continues to rise slightly to 29 mV after 90 min. incubation. The toad bladder shows less response and a quicker return to approximately control values than either of the other membranes. From a control value of 0.01 µeq./sq.cm./min. there is rise to a peak of 0.0183 µeq./sq.cm./min. 30 mins. after the addition of the hormone which, after 60 mins. incubation, has fallen to 0.0134 µeq./sq.cm./min. This continues falling, but less sharply until it reaches the control figures about 2½ to 3 hours after the start of incubation. Once again the P.D. very closely follows both time course and extent of the action shown by the net active sodium flux.

A second preparation of T.S.H., obtained from Parke Davies Ltd. was applied to frog skin. This preparation was not so heavily contaminated with gonadotrophic hormones as was the Ambinon 'B' but it had not been assayed for other possible

contaminants before application to the frog skin. The preparation was applied to the serosal surface of the membrane at a series of dose levels (see Fig. IV:2). The higher concentrations gave rise to much the same order of total increase, the only apparent difference being the time taken to reach the peak level. At a concentration of 150 mUnits/ml. bathing fluid the highest level was reached 25 mins. after application of the hormone, at 100 mU/ml. it was reached 40 mins. after application and at 50 mU/ml. the level of sodium flux was still rising 2 hours after application. At a concentration of 15 mU/ml. bathing fluid there was little or no demonstrable effect. It is surprising that there should be such a sharp decrease in response to a relatively small change in concentration of the hormone. The preparation, however, was only applied to three membranes at this dose level and, since one of these membranes showed a marked drop in the S.C.C. throughout the course of the experiment, the average figures may not indicate the true action. The individual results of the membranes used in this experiment are shown in Fig. IV:3.

A third preparation of T.S.H. of much greater purity than either of the others, was applied at various dose levels to frog skin. This was the Armour product Thytropar. When applied to the serosal surface of the skin at a level of 0.2 and 5 mU/ml. bathing fluid there was very little response in

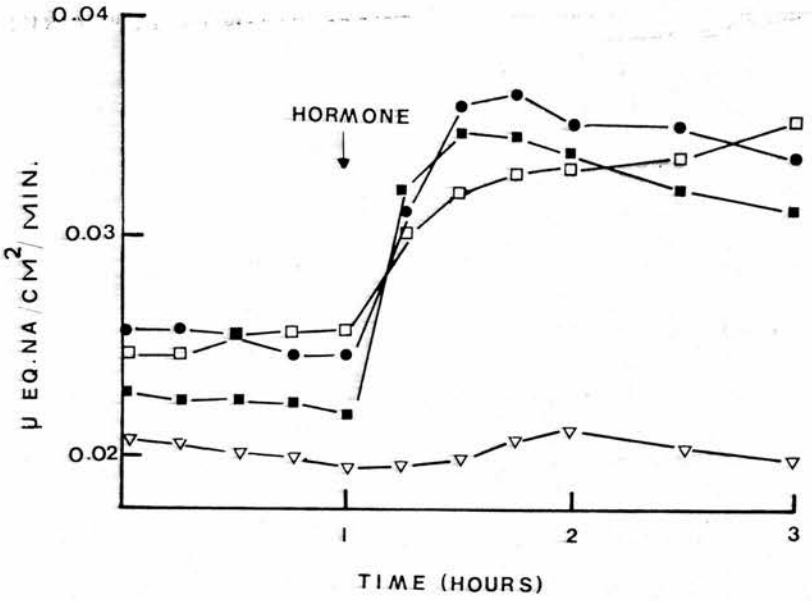


Figure IV:2 Effect of Parke Davies T.S.H. on frog skin (■) dose level 150mU/ml. (av. of 6), (●) does level 100mU/ml. (av. of 5), (□) dose level 50mU/ml. (av. of 5), (▽) dose level 15 mU/ml. (av. of 3).

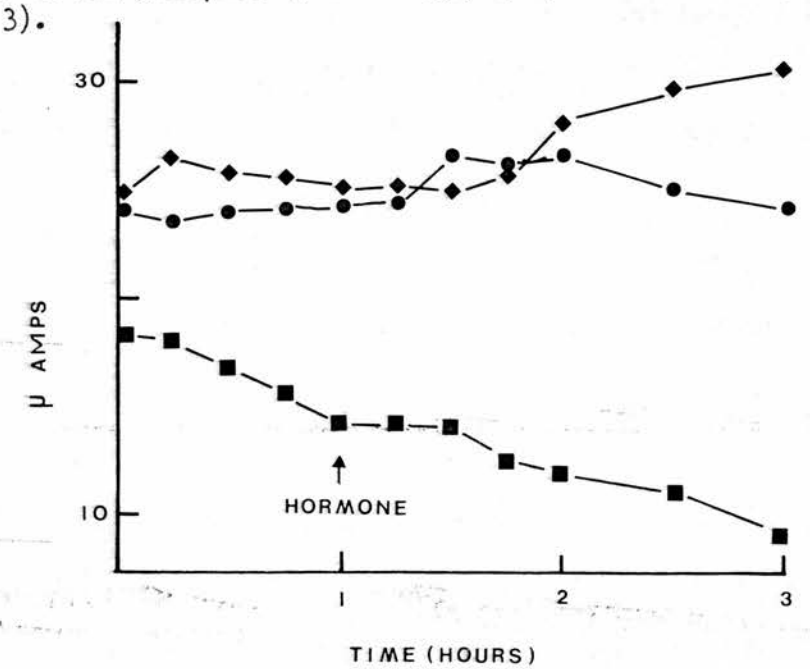


Figure IV:3 Effect of Parke Davies T.S.H. on individual membranes at a dose level of 15mU/ml. (●) 18/3/63, (■) 18/3/63, (◆) 25/3/63. Sodium flux given as μamps.

the net flux of sodium across the membrane (see Fig. IV:4). When applied at a concentration of 150 mU/ml. bathing fluid, however, it caused an increase in sodium flux, from a control level of 0.0154 µeq.Na/sq.cm./min. to 0.0197 µeq. Na/sq.cm./min. after 40 mins. incubation. The rate remained at round about this level for a further hour, and was not measured after that time. The electrical potential across the membrane showed a similar increase to that of the net sodium flux.

It will be noticed that the response to the application of these hormone preparations of both net sodium flux (measured as short-circuit current) and of electrical potential difference across the membranes is very similar to the response shown by these and other membranes to vasopressin (compare Figs. IV:1, IV:2 and IV:4 with Leaf, 1960, Fig. 6. For action of pituitary extract on frog skin P.D. see Furhmann and Ussing, 1951). Because of this similarity it was decided to find out if there was any contamination of the anterior pituitary hormone preparations by posterior pituitary hormones at such a level as to have any effect on the results obtained.

From Armour Ltd. information was received that the batch of Thytropar used in these experiments (Lot X6008) was contaminated at a level of 0.01 to 0.02 U.S.P. pressor units per unit of T.S.H. (In this context I would like to thank

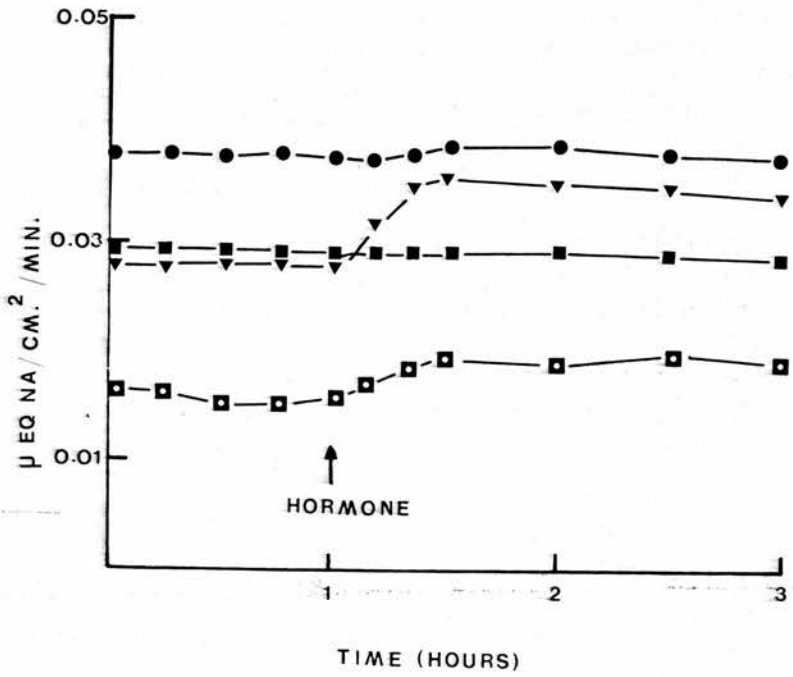


Figure IV:4 Effect of Armour Thytropar on frog skin.
(■) dose level 150 mU/ml. (average of 6)
(●) dose level 5 mU/ml. (average of 5)
(■) dose level 0.2 mU/ml. (average of 6)
(▼) Pitressin at dose level of 3 mU/ml. (average of 4).

Mr. D. Purdie, manager of Armour Medical Services Department for his help). Assuming that the higher of these two figures is in fact the correct one, the level of pressor activity would be 3 mU/ml. bathing fluid at the highest Thytropar concentrations used (i.e. 150 mU/ml.). For the purposes of this study the pressor activity was assumed to be entirely due to vasopressin and its analogues and control runs to assess the effect of the contaminants, were performed with Parke Davies Pitressin if the original hormone preparation was of bovine or ovine origin, or with lysine vasopressin (supplied by Professor H. Heller) if the preparation was of procine origin.

The effect of 3 mU vasopressin/ml. bathing fluid was assessed on the frog skin (see Fig. IV:4) and it was found that whereas 150 mU Thytropar/ml. bathing fluid had given an increase from 0.0154 to 0.0197 µeq. Na/sq.cm./min., i.e. of 28% this dose of vasopressin gave, in an average of four animals, a rise (from a higher base level - cause unknown) from 0.0278 to 0.0362 µeq. Na/sq. cm. /min. i.e. 30%. Thus it can be seen that a contamination of 0.02 U.S.P. units vasopressin per unit T.S.H. will fully account for the increase in net sodium flux across the frog skin when treated with Thytropar.

Since both the other T.S.H. preparations were known to be of a lower standard of purity than Thytropar, and gave response curves similar to that obtained with the latter product,

it was assumed that the effect in the case of all three of these preparations was due to contamination by posterior pituitary hormones. Rat blood pressure assays showed a significant amount of pressor action in both the Organon and the Parke Davies preparations, but the exact level was not ascertained.

b) Gonadotrophic Hormones.

The follicle-stimulating hormone and luteinizing hormone used in this study were obtained from two sources - Armour Ltd. (Armour F.S.H. and Armour P.L.H.) and from the National Institute of Health (N.I.H. - F.S.H. and N.I.H. - L.H.)

Armour F.S.H. was applied to frog skin at a series of concentrations, namely 10 mU/ml, 100 mU/ml. and 400 mU/ml. bathing fluid (see Fig. IV: 5). At a concentration of 10 mU/ml. the preparation caused an increase of 17% in the net sodium flux within 20 mins. of application. At 100 mU/ml. the increase was 30% after the same time and at 400 mU/ml. there was a peak increase of 34% 25 mins. after addition of the hormone preparation. The electrical potential was increased by an equivalent amount in all cases. Information received from Armour put the contamination level of pressor activity in this preparation of 0.02 U.S.P. units per unit of F.S.H. This would mean that at a dose level of 10 mU F.S.H./ml. bathing fluid a dose of 0.2 mU/ml. of vasopressin was being administered to the

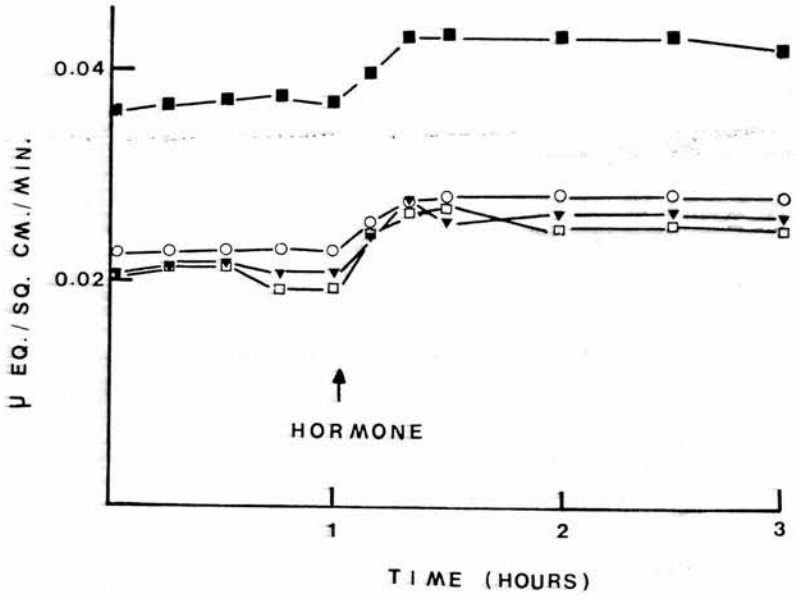


Figure IV:5 Effect of Armour F.S.H. on frog skin sodium fluxes at various doses. (■) dose level 10mU/ml. (▼) dose level 100 mU/ml. (□) dose level 400 mU/ml. (○) 0.2 mU lycine vasopressin /ml.

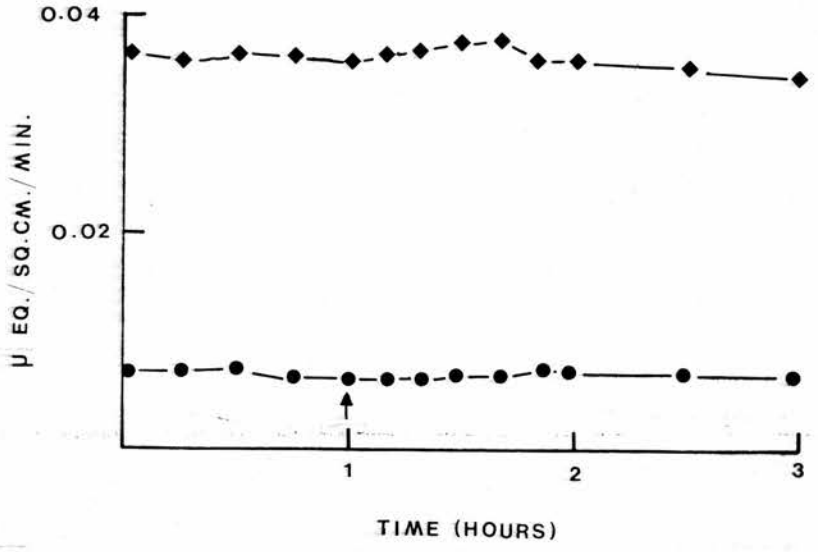


Figure IV:6 Effect of N.I.H.-F.S.H. on sodium fluxes.
(◆) dose level 125 mU/ml. on frog skin (average of 6)
(●) dose level 62.5 mU/ml. on toad bladder (average of 4).

membrane. Accordingly an assay of the effect of this dosage of vasopressin on the frog skin was made using lysine vasopressin since the F.S.H. was prepared from porcine material. It was found (see Fig. IV:5) that an increase of 19% in 30 mins. resulted from the application, the difference between that and the effect shown by the F.S.H. being insignificant. Hence, it was surmised that the effect of the F.S.H. preparation was due entirely to contamination by posterior pituitary hormones.

To establish this further the preparation of N.I.H.- F.S.H. was applied to frog skin at concentrations of 125 mU/ml. There was no change in potential difference across the membrane during incubation with this preparation, but there was a slight rise (3.5%) in net Na flux (see Fig. IV:6). Very slight traces of posterior pituitary hormones would be necessary to cause this increase.

A concentration of 62.5 mU F.S.H./ml. serosal bathing fluid was also applied to toad bladder (Fig. IV:6) but once again there was no significant effect. This preparation is extremely pure and gave no sign of an increase in blood pressure when injected into a rat. It appears therefore that F.S.H. has no intrinsic effect on the permeability of frog skin and toad bladder to sodium ions.

The picture obtained by application of luteinizing hormone to the membranes was very similar to that for F.S.H.

When the Armour preparation was applied to frog skin at a concentration of 10 mU/ml. bathing fluid, an increase in sodium flux of 48% was observed after 30 minutes (see Fig. IV:7). The amount of vasopressin contamination in this preparation was not known but when the much purer N.I.H.- L.H. was assayed on frog skin and toad bladder at a dose level of 0.025 mg/ml. bathing fluid, (it was not possible to compare the amount used with the unit measurement of the Armour preparation), there was no significant effect (Fig. IV:7). From these data it was deduced that luteinizing hormone had no effect on this parameter of action of the membranes.

c) Adrenocorticotrophic hormones.

The Armour preparation Actar-gel was applied to frog skin at a concentration of 400 mU A.C.T.H./ml. serosal bathing fluid. This application resulted in an increase of 15.5% in net sodium flux, accompanied by a similar increase in potential difference across the membrane (see Fig. IV:8). Unfortunately no information was available as to the amount of the vasopressin contamination of this preparation but the appearance of the response curve after application was very similar to that obtained in all the experiments cited so far. The peak of activity was reached 30 minutes after introduction of the hormone and from this point the activity gradually

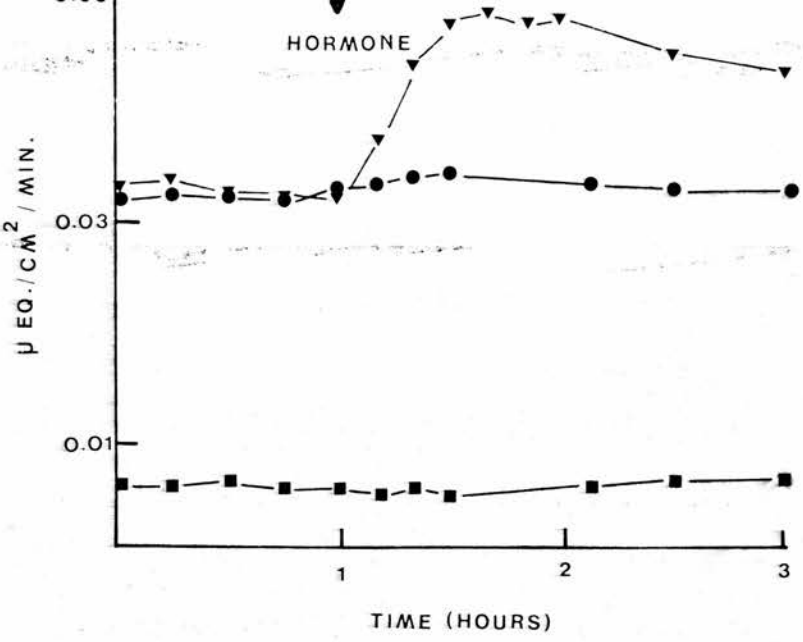


Figure IV:7 Effect of Armour P.L.H. and N.I.H.-L.H. on sodium flux. (▼) dose level 10mU Armour P.L.H./ml. on frog skin, (●) dose level 0.025 mg N.I.H.-L.H./ml. on frog skin, (■) dose level 0.025 mg N.I.H.-L.H./ml. on toad bladder (all averages of 6).

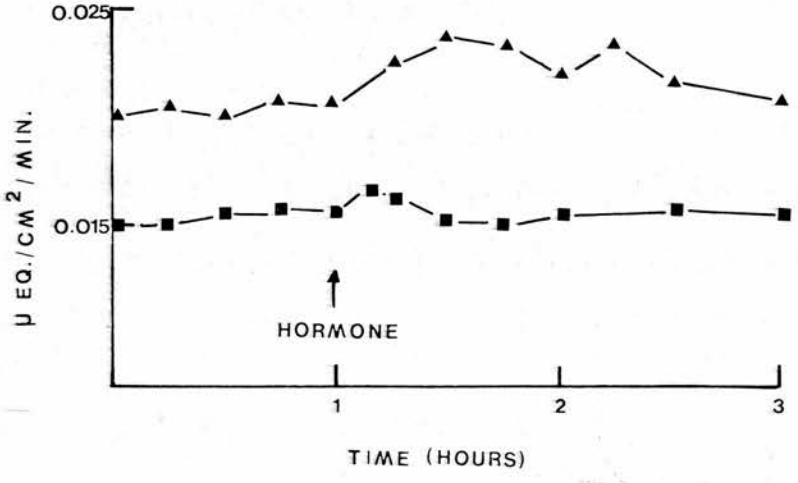


Figure IV:8 Effect of Actar-Gel at a concentration of 400 mU/ml. (▲) and A.C.T.H.-Al at a concentration of 0.05 mg/ml. (■) on sodium flux across frog skin.

fell back towards the control level, reaching it after approximately 2 hours incubation.

An A.C.T.H. preparation of mucher higher purity was obtained from Organon Ltd. This was known as A.C.T.H.-Al. According to the manufacturers this is only a fraction of A.C.T.H. and shows a somewhat different type of activity to that of other fractions in that it has extremely little adreno-cortical growth effect but a very high steroid output-stimulating action. When applied to the serosal surface of frog skin at a concentration of 0.05 mg./ml. bathing fluid there was little other than a slight peak (an increase of about 3%) in short-circuit current ten minutes after application. This had regained the control value within 20 minutes after application (see Fig. IV:8).

Although no definite assumptions can be made regarding the activity shown by the Actar-gel without further information on pressor contamination, the lack of effect of A.C.T.H.-Al makes it appear unlikely that A.C.T.H. has any intrinsic effect on sodium transport across the frog skin. It must be remembered, however, that the A.C.T.H.-Al is only one fraction of the substance, and hence no great reliability can be placed on this assumption.

d) Growth hormone and Prolactin.

A preparation of growth hormone (G.H.) obtained from N.I.H. was applied to frog skin at a concentration of 50 mU/ml. serosal bathing fluid. An increase of 30% was observed in the short-circuit current after incubation in the hormone for ten minutes. The effect persisted with very little fall-off for a further hour. Although this preparation was reported to be of a very high level of purity, it was thought that the effect may be due to contamination, and, since no figures were available for the pressor level in this preparation, it was subjected to a rat blood pressure assay. The hormone was found to give rise to an increase in the blood pressure of the rat, and this assay showed a level of contamination equivalent to 10 mU vasopressin per unit G.H. Further tests were made on the frog skin adding vasopressin to a concentration of 0.05 mU/ml. in the serosal bathing fluid (this is the amount which would be present at the dose level of G.H. used.) Fig. IV:9 shows the results of this. Although the G.H. had increased the short-circuit current by 30%, the vasopressin at contamination level only caused an increase of 20% after the same time interval. This would suggest that the G.H. had an effect in its own right, amounting to an increase of 10% over the control value. It should be noted,

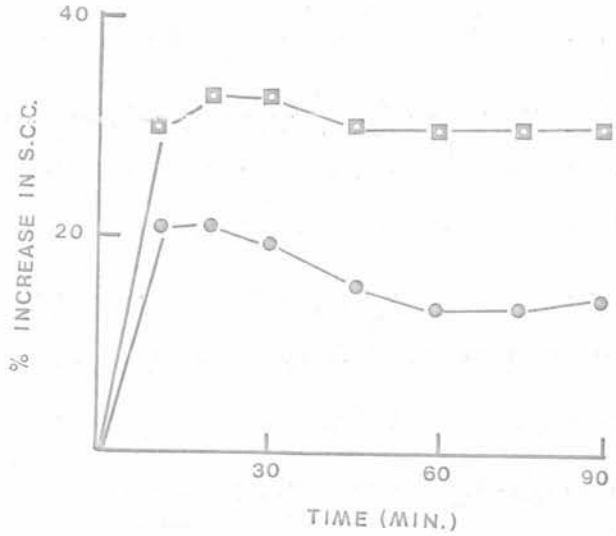


Figure IV:9 Graph to show % increase in short circuit current across frog skin after addition of 0.05 units G.H./ml. (■) and vasopressin at contamination level of 10mU/unit G.H.(●).

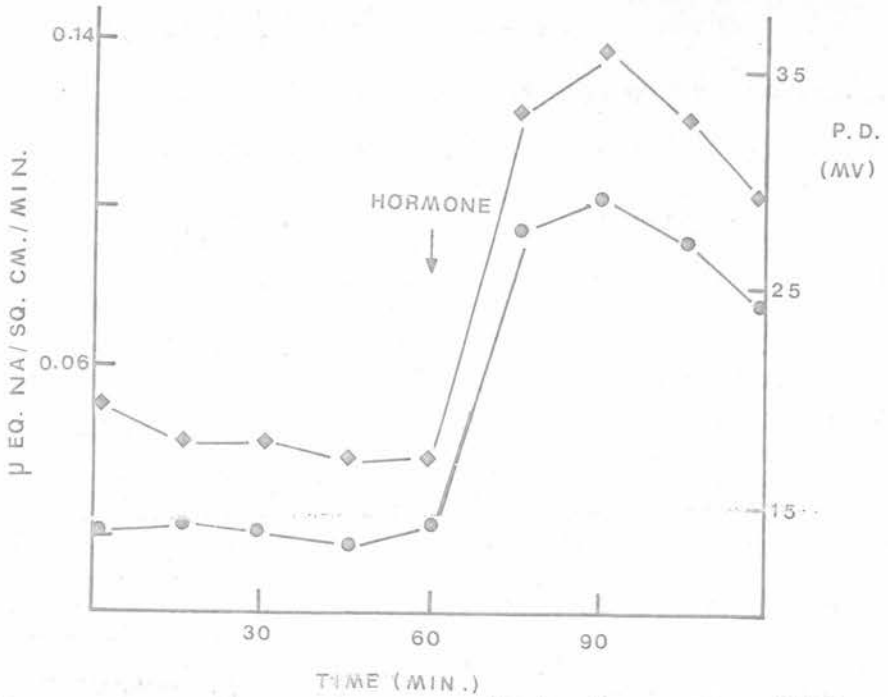


Figure IV:10 Graph to show P.D. (◆) and Na flux (●) across bladder on addition of 0.5 units Prolaction/ml. Average of 6.

however, that vasopressin was not the only hormone with which this preparation was contaminated. Also present was a very high level of prolactin activity, amounting to 0.5 I.U. Prolactin/unit G.H.

The preparations Somacton and Prolactin obtained from Ferring AB, were found, in each case, to contain both prolactin and growth hormone. The first of these to be applied to the membranes was Prolactin. Treatment of frog skin at a level of 1 unit Prolactin/ml. serosal bathing fluid, and of toad bladder at a level of 0.5 units Prolactin/ml. serosal bathing fluid both resulted in a marked increase in the potential difference and net Na flux across the membraines (see Figs. IV:10 and IV:11). In both cases the potential difference showed an increase proportional to the increase in short-circuit current, and the maximum effect occurred 30 minutes after the application of the hormone, giving a response curve very similar to that shown by vasopressin.

Inquiries to the manufacturers of this preparation yielded the information that the Prolactin was contaminated quite heavily with posterior pituitary hormones, vasopressin having been assayed, in one batch, at a level of 24 mU vasopressin/ unit Prolactin. An assay on the frog skin showed that a contamination level of less than half of this (10 mU vasopressin /unit prolactin) was sufficient to account for all the increase

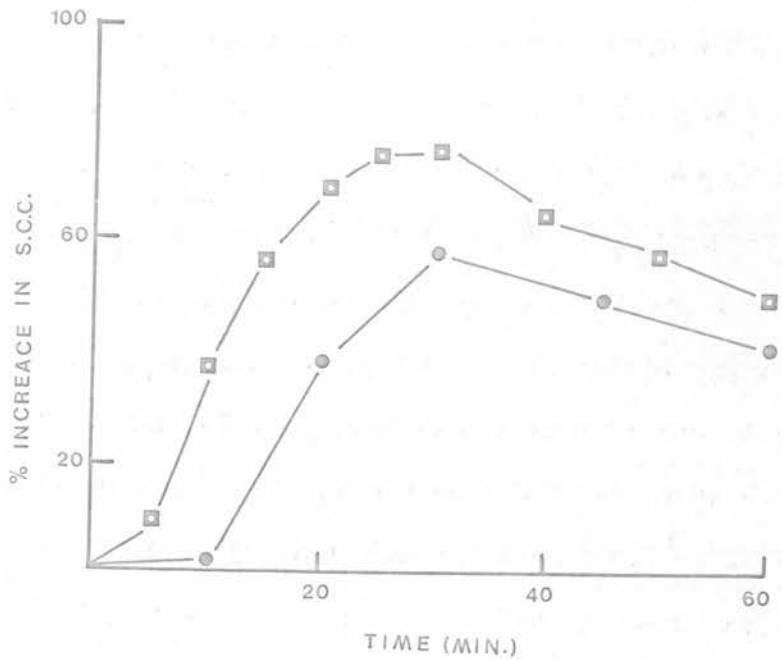


Figure IV : 11

Graph to show percentage increase in short circuit current of frog skin after addition of 1 unit Prolactin/ml. bathing solution (■) and 10 mU vasopressin/ml. of bathing solution (●). Averages of 7.

arising from the application of the Prolactin (see Fig. IV:11). A rat blood pressure assay of the preparation confirmed a level of 5 to 10 mU. vasopressin/unit prolactin (Fig. IV:12).

According to the manufacturers the preparation Somacton is made by a process of separation from an oxy-cellulose filtrate of material extracted from isolated anterior pituitary lobes. It is, thus, extremely unlikely that there would be any of the small-chain polypeptide hormones of the posterior pituitary present. This was confirmed by application of the preparation to the frog skin at a level of 50 mU/ml. serosal bathing fluid (Fig. IV:13) where no increase in electrical potential or short circuit current was observed within an hour of application. As well as demonstrating the purity of the preparation, the absence of stimulation suggested that neither porcine growth hormone nor prolactin have any effect on the parameter of action measured on the frog skin.

2. Oestrogens.

Oestrone sulphate and oestradiol sulphate were used in this study, these compounds being more soluble in water than the natural steroids, and hence capable of being applied to the membranes at a higher concentration.

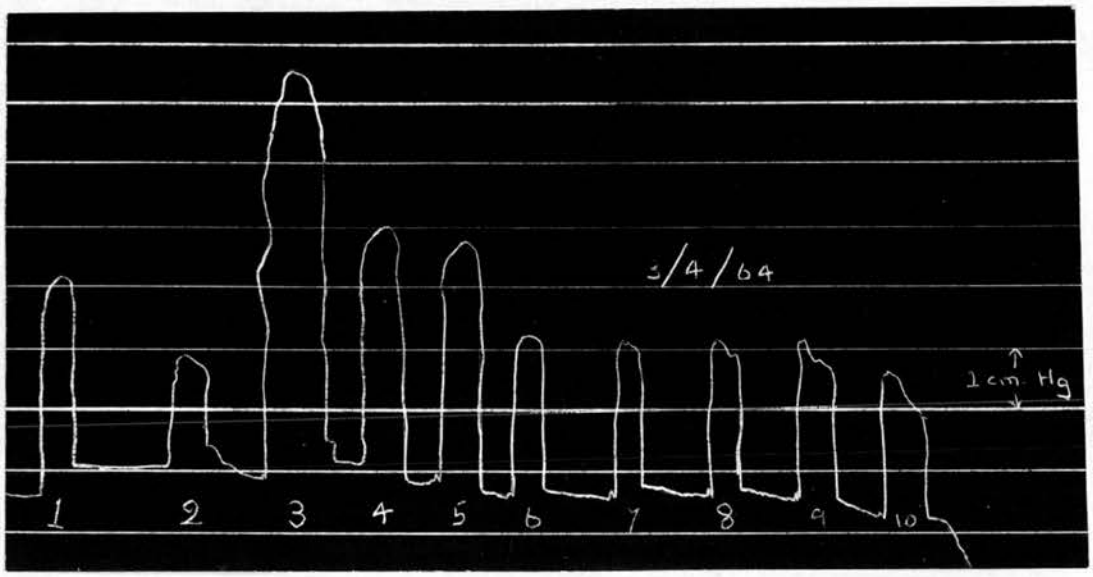


Figure IV : 12

Assay of pressor activity in Ferring Prolactin.

1. 10 mU Vasopressin. 2. 5 mU Vasopressin. 3. 4 units Prolactin. 4. 2 units Prolactin. 5. 10 mU Vasopressin. 6. 2 units Prolacting. 7. 10 mU Vasopressin. 8. 10 mU Vasopressin. 9. 2 units Prolactin. 10. 2 units Prolactin.

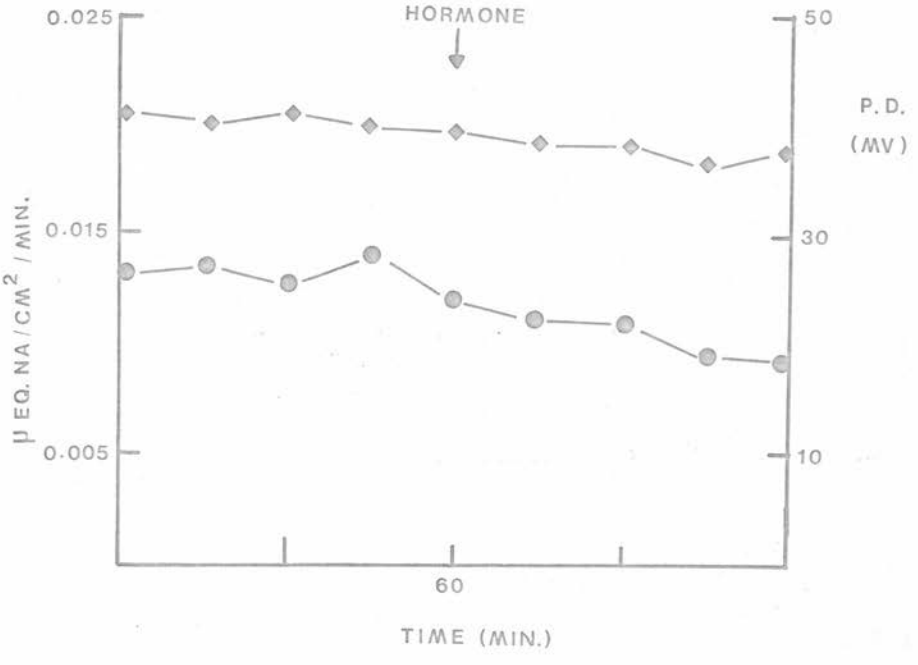


Figure IV : 13

Graph to show effect of addition of 50 mU Somacton/ml. serosal bathing fluid on net Na flux (◆) and P.D. (●) of frog skin. Average of 6.

Frog skin was treated with oestrone sulphate at two concentration levels, namely 0.017 and 0.034 mg. hormone/ml. serosal bathing fluid, (see Fig. IV:14). Neither concentration appeared to have any effect on either electrical potential difference across the membrane, or on the active transport of sodium.

Oestradiol sulphate, which usually shows a greater biological potency than oestrone, was applied to the frog skin at a concentration of 0.0085 mg/ml. and to the toad bladder at a concentration of 0.034 mg. hormone/ml. serosal bathing fluid. As will be seen in Fig. IV:15 neither membrane showed any effect from the addition of this hormone to the serosal medium on either P.D. or net sodium flux.

3. Thyroxine.

Green and Matty (1963) reported that thyroxine (T_4), at a concentration of 10^{-6}M, caused a 50% increase in active sodium transport across the bladder and skin of Bufo bufo, within one hour of application of the hormone. They also showed an effect of thyroxine on water movement across the toad bladder (Green and Matty, 1962) where it was found that a concentration of 10^{-6}M T_4 resulted in an increase of 20µl/ sq. cm./hr. and at 10^{-5}M T_4 there was an increase of over

Figure IV : 14 Graph to show effect of addition of oestrone sulphate at a concentration of 0.017 mg. and 0.034 mg. on active Na transport (◘ and ◆ respectively) and P.D. (■ and ● respectively) across frog skin. Average of 6.

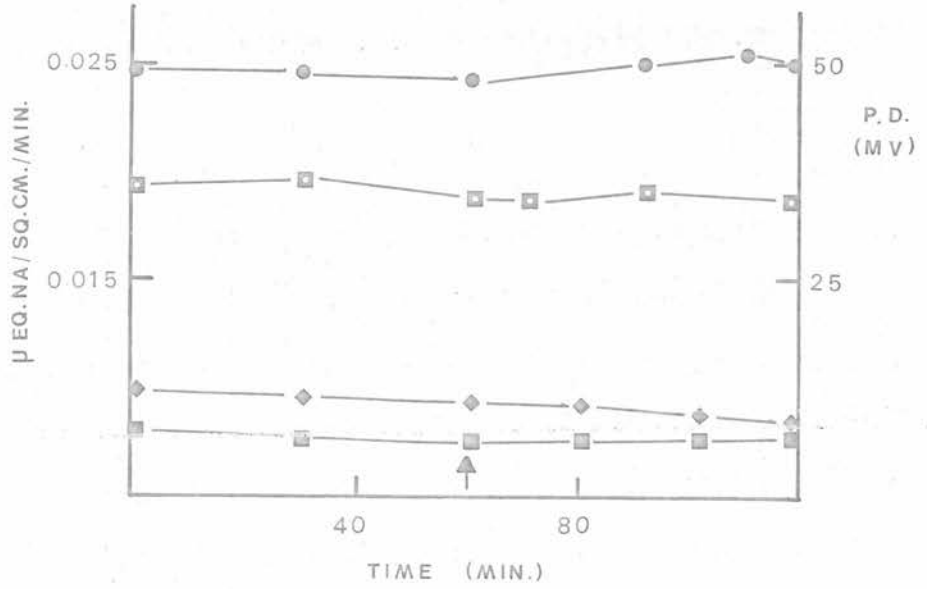


Figure IV : 15 Graph to show effect on P.D. and net Na flux of addition of oestradiol sulphate to toad bladder at a concentration of 0.034 mg./ml. (■ and ◆ respectively) and frog skin at a concentration of 0.0085 mg./ml. (● and ◘ respectively). Average of 9.

30µl/sq. cm./hr. It was decided, therefore, to investigate the effects of the application of thyroxine at a concentration of 10^{-5}M on active sodium transport across isolated frog skin. As will be seen in Fig. IV:16 the potential difference dropped throughout the experiment but the net sodium flux remained constant. In Green and Matty's experiments the potential difference had remained constant but the net flux of sodium had increased. It was difficult to say whether or not the drop in potential difference in my study masked an increase in net sodium flux, i.e. if the fall in P.D. would normally have been echoed by a fall in short-circuit current, but the hormone had held the net flux up at a constant level, this would, in effect, have been an increase in net flux over the control level.

Thyroxine was applied to the toad skin and bladder at a concentration of 10^{-5}M and in neither case was there any apparent effect on the active sodium transport across the membrane (see Fig. IV:17). In these experiments the electrical potential difference did not show any reduction throughout the experiment. In the series on the toad skin the P.D. maintained a level of 10.5 mV throughout the entire length of the experiment, but the average of the results from the toad bladders showed a regular rise in P.D. from 9.5 mV to 13.5 mV in two hours. This rise, however, is seen in the

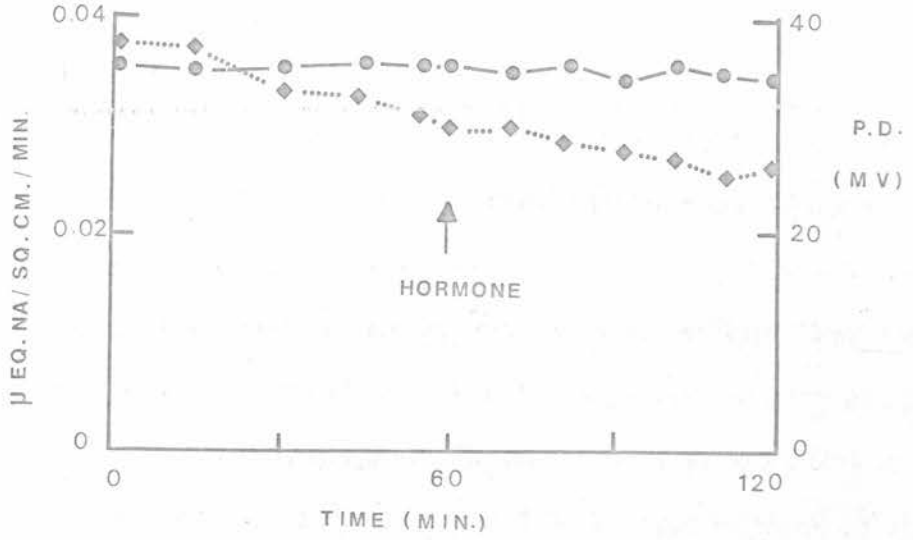


Figure IV : 16 Graph to show effect of addition of 10^{-5}M T_4 on net Na flux (●) and P.D. (◆) across frog skin. Average of 6.

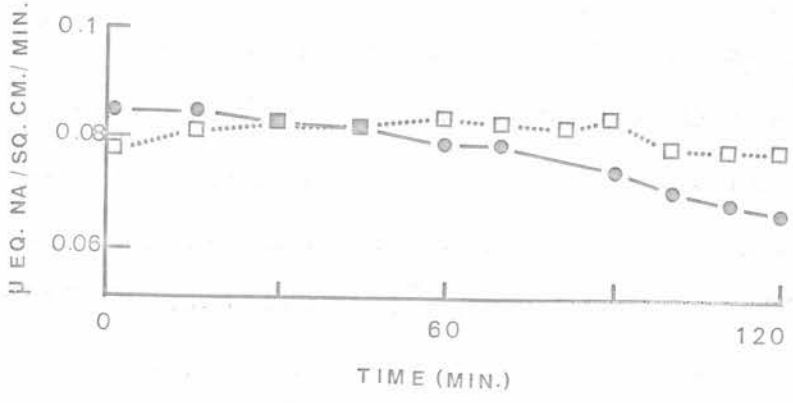


Figure IV : 17 Graph to show effect of addition of 10^{-5}M T_4 to toad bladder (□) and skin (●). Average of 6.

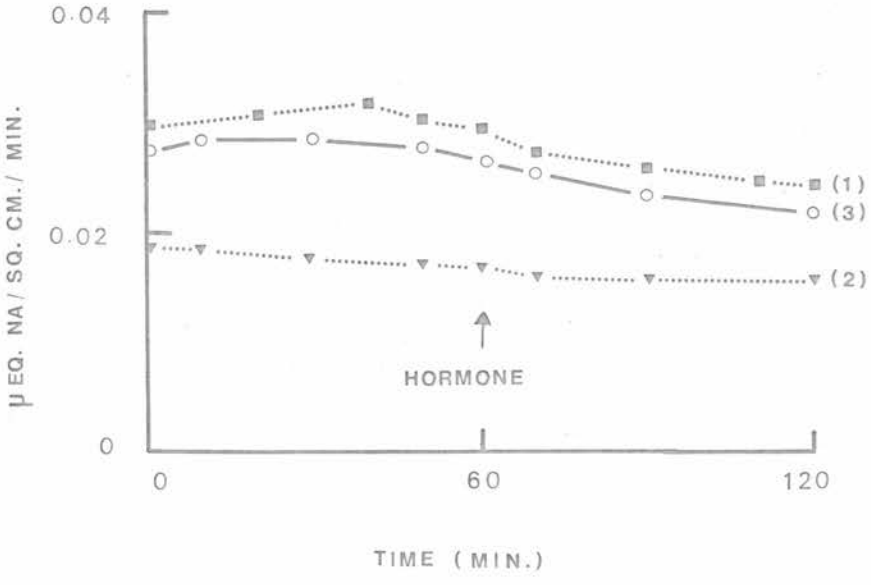


Figure IV : 18

Graph to show effect of addition of (1) hydrocortisone at a concentration of 0.067 mg./ml; (2) angiotensin at a concentration of 0.017 mg./ml. and (3) glucagon at a concentration of 0.017 mg./ml. on active Na transport across frog skin. Average of 6 animals.

initial control period as well as in the time after the application of the hormone.

4. Other hormones.

Three other hormones, which have been shown to have effects on membrane permeability, were assayed on the frog skin.

Hydrocortisone was applied to the serosal bathing fluid at a concentration of 0.067 mg/ml; angiotensin was applied at a concentration of 0.017 mg/ml. and glucagon was used at the same level as the angiotensin. In none of these cases did the addition of the hormone to the serosal fluid give rise to any effect on either the electrical potential difference or on the short-circuit current (see Fig. IV:18).

CHAPTER FIVE.

Discussion and Conclusions.

As will have been seen already, this work failed to bring to light any hormones, other than those already known, which influenced the rate of active transport of sodium ions across either the skin of the frog or the skin and bladder of the toad. In consequence it was not possible to proceed to the second part of the project, namely an investigation of the effects, at the cellular level of other controlling hormones and an examination of the new light they threw on the mechanism of active sodium transport.

This being so it is impossible to discuss the results in relation to the physiology of the membranes. In this discussion, therefore, I will give a brief survey of the literature concerning the permeability effects of the hormones I used in this study, and various others, to see in what way the various hormones exert their effects and also if any general statements can be made at this time on hormonal control of membrane permeability.

Hormones of the Adenohypophysis.

The anterior pituitary gland produces several hormones including thyroid-stimulating hormone (T.S.H.) follicle-stimulating hormone (F.S.H.) luteinizing hormone (L.H.)

growth hormone (G.H. or somatotrophin), prolactin and the adrenocorticotrophic hormone (A.C.T.H.) The majority of these are primarily concerned with the regulation of the secretions of other endocrine glands throughout the body, on which they act as part of a feed-back system - the adenohypophysis, for example, producing A.C.T.H. which controls the secretion of adrenocortical steroids while the amount of these hormones in the blood stimulates an increase or decrease in the rate of production of A.C.T.H. All the hormones produced by the anterior pituitary are proteins with molecular weights varying from 4,500 (porcine A.C.T.H.) to over 100,000 (porcine L.H.).

Thyroid-stimulating Hormone.

Investigations have been made on some of the peripheral effects of T.S.H., e.g. Girolamo et al (1961) found that T.S.H. had no effect on serum free fatty acid concentration in rabbits, although Engel and White (1960) had indicated the reverse, deducing that although pituitary hormones as a whole are not essential for free fatty acid mobilization, they may be required for the optimal response. It has also been demonstrated that T.S.H. enhances uptake of I^{131}-labelled thyroxine into the abdominal muscle of the mouse (Tonoue, Suzuki and Yamamoto, 1963).

The majority of work upon the effects of T.S.H. on permeability however, has been done on its main target tissue, the thyroid gland. This includes investigations on amino acid accumulation, the results from which are rather equivocal, e.g. Debons and Pittman (1962) found that T.S.H. caused an increase in uptake of α-aminoisobutyric acid into bovine and canine thyroid slices, but not into muscle and liver, whereas Raghupathy et al (1964) showed that although the unstimulated thyroid gland does concentrate AIB, application of T.S.H. does not enhance this effect in the sheep thyroid. The evidence in favour of a stimulatory effect of T.S.H. on the uptake of sugars into the mammalian thyroid is less confused. Tarui and Nonaka (1963) showed that T.S.H. increased the uptake of d-xylose and l-arabinose into bovine thyroid, and Irie and Slingerland (1963) showed that uptake of glucose into rat thyroid was enhanced by this hormone. However, this might indicate a general metabolic effect of T.S.H. rather than a specific action on the cellular permeability since it has been found that thyrotropin does not cause any increase in the accumulation of the biologically inactive optical isomers of xylose and arabinose, i.e. l-xylose and d-arabinose (Tarui et al, 1963).

Although work with T.S.H. has centred mainly on accumulation of organic compounds, there have been some investigations on the effects of thyrotropin on the permeability of the thyroid gland, and in particular the follicular membrane, to inorganic anions and cations. Lewitus, Guttman and Anbar (1962) found that there was an increased accumulation of perchlorate and fluoroborate ions into the rat thyroid gland after injection of the animal with T.S.H. The hormone appears to have an effect on accumulation of water in thyroid slices although Debons and Pittman (1962) indicated that it did not enhance the extracellular/intracellular water ratio. Bakke et al (1957) demonstrated that if T.S.H. was added to the medium bathing bovine thyroid slices, these increased rapidly in weight, the effect being sensitive enough for use as a standard assay procedure for T.S.H. activity. Although they gave histoquantitative evidence to show that the weight increase was due to swelling of the follicles, presumably by uptake of water from the external medium, they did not investigate any possible change in ionic concentration of the medium or the follicular contents. Soloman (1961) found that administration of T.S.H. to young chicks resulted in an increase in the in vitro accumulation of both

water and electrolytes, particularly sodium, in the thyroid follicles. Gorbman and Ueda (1963) found that injection of T.S.H. into Rana pipiens prior to sacrifice resulted in a lowering of the electrical potential and resistance across the follicular membrane in the isolated thyroid gland. They did not, however, investigate whether the potential was due to a flux of sodium, or the passage of any other charged particle through the follicular membrane or an active iodide pump, although they did mention the latter as a possible explanation.

In this connection it might be mentioned that a non-specific halide pump (such as may exist in the cells of the endostyle of ammocoete larvae; Morris, 1966 pers. comm.) or a sodium pump which drags in halide ions to balance the electrical potential gradient across the membrane would account for the accumulation of iodide in the cells of the thyroid gland providing that the iodine is bound after entering the cell whilst the other ions are free to diffuse out again. It is interesting to note here the findings of Pastan and Katzen (1967) who showed that T.S.H. causes activation of the enzyme adenyl cyclase in thyroid homogenates and that the T.S.H. binding site is either near or on the adenyl cyclase molecule in the plasma membrane. As will be seen in the discussion of the action of vasopressin in the membrane, adenyl cyclase plays a part, possibly indirect,

in the control of the active sodium pump.

The interactions between T.S.H. and its target tissue are very complex and cover a wide range of activities e.g. the hormone increases the accumulation and incorporation of iodide by the gland, controls its glucose uptake and hence its metabolism (Irie and Slingerland, 1963; Field et al, 1963) and even acts as a growth hormone for the thyroid gland, having effects on the nuclei and on the cytoplasmic R.N.A. (Tepperman and Tepperman, 1960), as well as affecting the permeability of the gland to organic and inorganic substances and stimulating the synthesis and release of thyroxine. There are, however, few indications that the hormone plays any major role in the body other than on the target organ. It is not surprising, therefore, that application of T.S.H. to the amphibian skin and bladder did not give rise to any detectable increase or decrease in the active transport of sodium across the membranes, although most preparations showed an effect due to vasopressin contaminants (Matty and Foster, 1963). It may be that thyrotropin is capable of increasing the permeability of certain tissues to thyroxine itself, as in the case of the gut. Although this was not examined in the amphibian membranes, it seems unlikely that

there would be any enhancement of uptake of thyroxine completely across either the skin or the bladder since it would have little physiological value. A mechanism for increasing the permeability of the individual cell membranes to T_4 and its active analogues would have greater physiological significance.

Gonadotropic Hormones.

The gonadotropic hormones F.S.H. and L.H. have received very little attention from the point of view of their effects on the physiology of systems other than their target organ, and there have been very few reports of work concerning their effects on the permeability of ovarian tissue. Ellis (1961) showed that L.H. caused a substantial increase in the accumulation of radio-iodinated serum albumen by the immature rat ovary, while F.S.H., although showing the same effect, had a considerably lower potency. L.H. has also been shown to increase the rate of glucose uptake by isolated whole ovaries of the young rat. There is a proportionally greater amount of lactic acid produced by the tissue after this treatment which indicates that the effect is by increasing the rate of metabolism of the tissue rather than being a simple increase of the permeability to glucose or by stimulating the active uptake of glucose. F.S.H. shows a similar effect on this tissue but

its activity is only about 4% of that of L.H. (Armstrong, Kilpatrick and Greep, 1963).

The gonadotropic hormones have also been shown to enhance the uptake of amino acids by the isolated rat ovary. Ahren and Kostyo (1963) showed that F.S.H. stimulates the rate of uptake of α-aminoisobutyric acid into the isolated prepubertal rat ovary if it is injected into the animal before removal of the organ. There was, however, no effect if the hormone was added to the incubation medium. Injection of L.H. did not produce a similar response. Since these hormones show few, if any, general effects in the body it was not surprising that they failed to increase the net sodium transport across the anuran bladder or skin.

Adrenocorticotrophic hormone.

During the last few years various workers have shown that A.C.T.H. affects the permeability of its primary target organ, the adrenal cortex or inter-renal body, and also of other tissues, to a variety of substances. The effect may be either an intrinsic one of the hormone itself, that is it exerts the effect directly, or an indirect effect due to the stimulation of production of the steroid hormones of the adrenal cortex, these latter exerting the direct influence on permeability. In the former of these categories lie the findings of Sharma et al (1963) who showed that A.C.T.H. inhibited the active

transport of ascorbic acid in slices of adrenal cortex tissue, but not in brain slices. Other workers have demonstrated direct effects of A.C.T.H. on the permeability of cells and tissues. For example Eichhorn et al (1960) showed that A.C.T.H. increases the permeability of cells of the adrenal gland to D-xylose and Verner, Blackard and Engel (1962) reported a stimulation in uptake and utilization of glucose by adipose tissue after treatment with adrenocorticotrophin.

Few reports have appeared of effects of A.C.T.H. on the permeability of cells or membranes to inorganic ions, other than that of Notter (1962) who indicated that A.C.T.H. increases the accumulation of radio-iodine into the human thyroid gland in adrenalectomised patients, and the demonstration by Beigelman and Hollander (1964) that A.C.T.H. does not alter the resting potential across adipose tissue cell membranes. In this connection it should be noted that there may be some significance in the findings by Taunton et al (1967) that A.C.T.H. stimulates the activity of the enzyme adenyl cyclase in the plasma membranes in adrenal gland homogenates. As will be discussed later, stimulation of this enzyme may be the cause of the stimulation of the sodium pump by vasopressin.

Removal of the anterior pituitary of Rana pipiens is followed by an increase in the passive permeability of the skin to sodium ions, as indicated by an increase in the loss of Na^{22}, and a decrease in the active sodium pump, shown by a drop in the resting potential and short circuit current across the skin. The effect can be reduced by treatment of the animals with A.C.T.H. (Myers, Bishop and Scheer, 1961). It is likely that this effect is, at least in part, a secondary one due to the action of A.C.T.H. on the inter-renal body causing an increase in the production of aldosterone, since it was shown by these authors that treatment with aldosterone has a similar effect to treatment with A.C.T.H. and it is well known that aldosterone, administered to toad skin and bladder in vitro, stimulates the active transport of sodium across these membranes (Crabbé, 1961 a and b, 1962, 1963; Porter and Edelman, 1964). As A.C.T.H., applied in vitro to amphibian membranes produced no apparent stimulation of sodium transport, it is likely that all the effect reported by Myers, Bishop and Scheer was caused by stimulation of production of the adrenocorticosteroids. This may also be the case in the work reported by Bentley and Follett (1962) who found that A.C.T.H. decreased the rate of sodium loss in Lampetra fluviatilis. The effect was also found after injection with aldosterone,

while injection of the aldosterone blocking agent SC11927 increased the rate of sodium loss.

From these reports it appears that A.C.T.H. has very little effect on ionic and water balance in its own right. It is, however, of great importance to ionic regulation due to its action on the adrenal gland.

Growth Hormone (Somatotrophin).

Noall et al (1957) first demonstrated that growth hormone increased the cellular uptake of amino acids by injecting the non-metabolisable amino acid α-aminoisobutyric acid (AIB) into rats and then administering growth hormone. They showed that the hormone caused an increase in the intracellular accumulation of AIB within two hours. Since that time much evidence has been put forward to suggest that amino acid transport into muscle is one of the major sites of action of growth hormone (Brande and Knobil, 1962; Knobil and Hotchkiss, 1964). It apparently has little or no effect on the rate of uptake of amino acids by the mammalian kidney or intestine (Marsh et al, 1962) although this may not be conclusive since these workers used a preparation of bovine growth hormone which is now known to have only a slight effect even on the mammalian diaphragm. Ovine growth hormone is the most potent in its effect on the uptake of amino acids.

Few studies have been made of the effect of growth hormone on the movement of ions or water. Corvilain and Abramow (1962) showed that treatment with growth hormone caused an increase in the serum phosphate level which apparently was the result of a direct effect of the hormone on the tubular reabsorption of this ion. Another direct effect of growth hormone on the mammalian kidney is that demonstrated by Lockett and Roberts (1963) who found that if isolated cat kidneys were treated with the hormone there was a reduction in the rate of excretion of water and ions. It was suggested that the site of action of the hormone was probably the proximal end of the kidney tubule. This finding was confirmed on rats by Lees et al (1964) who found that although the hormone caused a retention of water and sodium and potassium ions, there was no change in the urine Na/K ratios. They also found that growth hormone antagonised the effect of aldosterone in the rat kidney. There have been no reports of work on the effects of growth hormone on ionic regulation in amphibia. There is some difficulty in separating growth hormone from prolactin and in the present survey the hormones were applied together to the membranes. The preparation had no significant effect in its own right.

Prolactin.

This hormone has received very little attention from

the point of view of any possible effects on cellular permeability, except in the fishes where it has been shown to increase the ability of hypophysectomised animals to live in fresh water. Burden (1956) showed that the killifish Fundulus, although euryhaline under normal circumstances, was unable to live in fresh water after hypophysectomy. Since then various workers have demonstrated that regular injections of mammalian prolactin enable this and other fishes to survive in media of very low salinity even after removal of the adenohypophysis. For example, Potts and Evans (1966) found that in fresh water the normal rate constant for sodium efflux from Fundulus heteroclitus was 0.050. After hypophysectomy, however, this rate increased to 0.192. Since the rate constant for the influx of sodium was not greatly increased, the animals were losing sodium at a rate of 14% of the total body sodium per hour. These animals died within 24 hours of the operation. A similar batch of fish survived indefinitely when injected with 20u of prolactin every two days. Ovine prolactin is believed to be more effective in this respect than hormone from any other source. It has been suggested that the hormone works by increasing the thickness of the layer of mucus over the gill surface (Pickford, Pang and Sawyer, 1966). This

would increase the thickness of the unstirred layer, in which the concentration of sodium could build up and the animal would thus loose less by diffusion through the gills. It seems unlikely however, that this alone would be sufficient and it is possible that the hormone exerts a direct effect on the cells of the gill. Prolactin has also been shown to cause a transient diuresis, accompanied by natriuresis in rats which was followed by a long-lasting antidiuresis accompanied by the retention of sodium and potassium (Lockett, 1965).

A substance similar to prolactin has been localised in the anterior pituitary of teleosts and if it is true that the lactogenic hormone of mammals has developed from a hormone which appears to play a major part in ionic regulation in fishes, it may be rewarding to examine the effects of prolactin on water and ionic permeability of mammary tissue.

Although produced by the same gland (but not the same cells) and being of the same chemical nature (i.e. protein) the hormones of the anterior pituitary work in quite different ways on a variety of tissues. Those hormones which have a definite target tissue appear to have very few peripheral effects, and often little is known of their mode of action on their target tissue, this being particularly true of

F.S.H. and L.H. A.C.T.H. is an exception in that it appears to have effects on several tissues other than the adrenal cortex. All these hormones may control their target tissues at least in part by varying the permeability of the cell membranes to various substances, this being especially so for T.S.H.

Growth hormone has a more general regulatory function over the whole body, and once again a major part of its effect may be on the regulation of uptake of substances, particularly amino acids, by cells. Prolactin is somewhat strange because, although in mammals it has a definite effect on a target tissue, in fishes where this tissue is not present, it plays a completely different role in the body. The actual effects of the hormone are difficult to evaluate because the work has been done on the whole animal rather than on isolated tissues. It is thus not possible to say whether the prolactin acts directly on the gill cells or whether it stimulates the production of some other hormone.

Melanocyte-Stimulating Hormone.

M.S.H. is a polypeptide produced by the pars intermedia of the pituitary gland and acts mainly, as its name implies, on the chromatophores of the skin. Novales (1959) postulated that the hormone worked by increasing the permeability of the chromatophores to water and sodium ions. The effect of

the hormone on sodium and water flux across the isolated skin of Rana temporaria was investigated by Tercafs (1963) who found that the rate of flux of water down an osmotic gradient was unchanged after application of M.S.H. to the serosal surface of the skin, but the electrical potential difference across the membrane was increased. This latter effect was possibly due to an increase in the passive permeability of the membrane to sodium ions.

M.S.H. is found in two forms α - M.S.H. and β - M.S.H. Both of these forms bear marked similarities to A.C.T.H. in molecular construction, although this is particularly true of α - M.S.H. which has the same arrangement of amino-acids as the first part of the A.C.T.H. molecule. A.C.T.H. shows a marked melanocyte-stimulating effect. This being so it is curious that A.C.T.H. does not have the same effect on the electrical activity of the skin of Rana temporaria as that shown by M.S.H.

Hormones of the Neurohypophysis.

The neurohypophysis secretes a number of closely related octopeptide amides which are very similar in structure, each having a cyclic pentapeptide with a tripeptide amide side-chain. Arginine vasopressin is typical of the group and has the structure

$$\overline{\text{CyS - Tyr - Phe - Glu}(NH_2) - \text{Asp}(NH_2) - \text{CyS}} - \text{Pro - Arg - Gly}(NH_2)$$

These hormones have been known for many years to regulate water balance in vertebrates (for a review on the subject see Heller, 1945), and more recently they have been shown to have very marked effects on the movement of ions, particularly sodium, across cellular and multicellular membranes.

In the lamprey Bentley and Follett (1962) found that the neurohypophyseal hormones increased the net loss of sodium from the animal, and that oxytocin was more active in this respect than was vasopressin. It seems strange that the animals were losing salt at all if they were well adapted to the medium in which they were living, but since they were caught on their way up-river from the sea it may be that they were not completely adapted. Although both teleost and elasmobranch pituitaries have been shown to produce a substance which has an antidiuretic action in tetrapods, this substance (or substances) has not been shown to produce any effect on water or electrolyte balance in fishes (Potts and Parry, 1964). Indeed there are very few reports of any neurohypophyseal hormone producing positive effects in fishes. This is in keeping with the lack of effect in teleosts of most other hormones which help in the maintenance of salt or water balance in the tetrapods. Even in the lung - fish Protopterus, it has been shown that the characteristic antidiuretic action of these hormones is either not present, as

with 8- isoleucine oxytocin, or is reversed, e.g. arginine vasotocin increases the rates of glomerular filtration, water excretion and sodium excretion (Sawyer, 1966).

The urodeles Necturus and Ambystoma both show increased retention of water after injection of antidiuretic hormone (A.D.H.) although the site of action of the hormone is unknown (Potts and Parry, 1964). Bentley and Heller (1964) showed that A.D.H. increased sodium transport and water movement across the isolated skin of the newt Triturus and the same authors (1965) showed that arginine vasotocin, when injected into intact fire salamanders brought about an increase in weight of up to 14% which was mainly caused by an increased rate of water absorption from the urine in the bladder, since water transfer across the skin was not increased. Cofré and Crabbé (1967) demonstrated that treatment with A.D.H. could increase the active sodium transport pool and the short current across the colon of Bufo marinus. Bentley (1962) found that neurohypophyseal hormones had no effect on either sodium transport of water flux across tortoise large intestine or bladder. In the mammals Aulsebrook (1961) found that Pitressin caused an increase in the net transfer of sodium across the rat colon, although Green and Matty (1966) were unable to repeat this or demonstrate a similar effect on the intestine of the mouse, the toad or the fish

Cottus. Grantham and Burgh (1966) showed that vasopressin and cyclic-AMP increased net water absorption along an osmotic gradient and also diffusional permeability of water in isolated collecting tubules of the mammalian kidney. A great deal of other work has been done on the effects of the hormones of the posterior pituitary in the mammals.

The effects of vasopressin and its analogues on movement of salts and water in the anuran amphibia have been very widely studied. Both Leaf (1955) and Bentley (1958) have demonstrated that vasopressin increases the net flux of water across the toad bladder. There have been several mechanisms suggested for this action on water permeability. Capraro and Bernini (1952) suggested the presence of an active water-transporting system which was stimulated by the hormone, presumably through some metabolic process. This accounted for their results which showed that the hormone increased the net flux of water but caused only a slight increase in the rate of diffusion. Capraro and Grampani (1956) suggested that the movement of water was controlled by electro-osmosis and that the hormone stimulated this process. Sawyer (1951) showed that although neurohypophyseal hormones increased water uptake across frog skin, they could not stimulate an action if the frog was bathed in a hypertonic medium, hence the flow of water is likely

to be passive. Koefoed Johnsen and Ussing (1953) also considered that the movement of water across frog skin was passive. They found however, that the net flux of water was greater than would be expected by calculation from normal diffusion equations. This lead them to propose that the water actually moved through water-filled pores across the membrane, and that the effect of vasopressin was to increase the diameter of these pores. Hays and Leaf (1962) suggested that the pores through the membrane offer a high resistance to the penetration of water, and the water in them is in a structured state. After application of vasopressin the aqueous channels are enlarged so that the core of water they contain possesses the physical properties of ordinary bulk water. In this state the membrane would have a higher permeability to water and net flow along an osmotic gradient would be faster. Rasmussen, Schwartz, Schoessler and Hochster (1960), having shown that the permeability change caused by vasopressin was not a direct effect on oxidative metabolism even though it is decreased by metabolic inhibitors such as 2-4 DNP and cyanide, proposed that the hormone became bound to the membrane by a disulphide-sulphydryl bond and caused a structural reorganisation of the membrane in a physical rather than in a metabolic manner.

It has also been established that the neurohypophyseal hormones increase the rate of sodium transport across the frog

skin and toad bladder. Furhman and Ussing (1951) found that the potential difference across the skin of the frog was increased when an extract of the mammalian posterior pituitary gland was applied to the serosal surface of the membrane. In the same year Ussing and Zerahn, using both isotopic and electrical techniques showed that this increase in electrical potential was due to an enhancement of the rate of active transport of sodium across the membrane. Leaf (1960) and Leaf, Anderson and Page (1958) confirmed that vasopressin caused an increase in the active uptake of sodium ions by the toad bladder, and also found that there was a decreased resistance to the movement of this ion across the membrane. Ussing (1960) suggested that the major effect of the vasopressin was on the outer border of the cell, where it increased the passive intake of sodium, which in turn stimulated the sodium pump, rather than directly on the pump itself. Frazier and Hammer (1963) supported this theory by showing that, after a bladder has been loaded with radioactive sodium, the rate of efflux from the mucosal surface is increased in the presence of vasopressin, whilst loss from the serosal surface shows no significant change. It is surprising that they were able to make such a clear demonstration of this phenomenon, since one would expect a greatly increased loss from the serosal surface of the bladder in the presence of vasopressin whether its effect on the sodium pump

was direct or indirect also, although there is an increase in the sodium pool, much of this is being taken up by the pump, so there should be no great increase in the passive efflux of sodium from the cell. Civan and Frazier (1968) also found that vasopressin increases the net sodium transport across the toad bladder by increasing the mobility of sodium ions within the tissue rather than by directly influencing the sodium pump. They showed this by impaling cells of the mucosal epithelium with glass electrodes and measuring the D.C. resistance to current pulses passed through the bladder. They found that the apical permeability barrier of the mucosal cells contributed 54% of the initial total transmembrane resistance but this increased to 98% following treatment with vasopressin. Hence the vasopressin increased ionic mobility selectively across this apical permeability barrier of the transporting cells.

Although both water and sodium entry through the mucosal surface of the skin and bladder is thought to be passive, and both are increased by the addition of posterior pituitary hormones to the bathing medium, it has been demonstrated that these effects are independent of each other. Bourquet and Maetz (1961) showed that both these parameters of action of the membrane are affected by oxytocin and its two analogues arginine vasotocin and lysine vasotocin. They also demonstrated, however, that whilst the permeability of the membrane to water is affected to an equal

extent by equal doses of all three analogues, the permeability to sodium ions is increased to a different extent by each hormone. This can be interpreted as showing that vasopressin acts not on one site on the mucosal membrane, but has two targets, possibly in the same epithelial cell. Anderson and Tomlinson (1965) found that by varying the calcium concentration of the medium bathing toad bladders the response to the addition of neurohyphyseal octapeptides could be varied. However this was only the case with water transport, there is no apparent inhibition of the sodium transport in response to octapeptides in the presence or absence of calcium ions. This supports the evidence that the action of the hormone on the two parameters is independent. Recently Elliot (1968) studied the action of various natural and synthetic peptides after injection into Bufo melanostrictus. He found that the two effects were completely separate but also by studying the structure of the various peptides he surmised that the hydrosmotic effect is correlated with an arginine in position 8 whereas the natriferic effect is associated with an isoleucine in position 3 of the octapeptide molecule.

Orloff and Handler (1961, 1962) demonstrated that cyclic adenosine 3',5',-phosphate (cyclic AMP) had a very similar effect to vasopressin on both water movement and the electrical charges in toad bladder. They suggested, because of this, that

the hormone exerts its effect both on this tissue and on the kidney by stimulation of the enzyme adenyl cyclase which produced AMP from adenosine triphosphate. Since theophyline prevents the conversion of cyclic AMP to inactive 5' AMP, they also studied the effects of this compound and found that it too mimicked the effect of vasopressin. It is not known whether the hormone increases the activity or the production of the enzyme, but it is apparently the enzyme which is the rate-limiting factor in the production of cyclic AMP since addition of ATP does not have any effect on either the rate of water movement or the electrical potential across the toad bladder. It would be of interest to apply vasopressin and ATP to the membrane at the same time and see if the increase in net flux of sodium across the bladder was any greater than that which would normally be expected from that dose of hormone.

Hormones of the Thyroid Gland.

The iodinated amino acid hormones of the thyroid gland have been shown to exert numerous effects on the permeability of tissues, cells and sub-cellular particles. There are so many reports, covering such a wide field that it seems possible that membranes (cellular, mitochondrial etc.) are one of the primary loci of action of this group of hormones.

Although effects of T_4 on glucose uptake by some membranes e.g. rat diaphragm, are well authenticated there is some debate as

to the extent of action of the hormone on sugar uptake into other tissues. For example Althausen and Stockholm (1938) reported that an extract of thyroid gland increased the absorption of sugars by the small intestine of both the normal and thyroidectomised rat. Bronk and Parsons (1964) and Levin and Smyth (1963), although both agreeing that an increase in the amount of thyroid hormones brought about an increase in the absorption of sugars into the mucosa, found that the effect was considerably smaller than that reported by the earlier workers. In the amphibia it has been found that thyroxine at very low concentrations can increase glucose absorption across the gut of Rana esculenta (Gellhorn and Northup, 1963). Other work on mammalian gut has shown that treatment with thyroxine brought about an increase in the net movement of calcium and phosphate ions across the rat gut, although Noble and Matty (1967) were unable to confirm these findings.

A considerable body of information on the action of the thyroid hormones and sub-cellular particles has accumulated in recent years. Some of the effects are definitely caused by a change in permeability in response to application of the hormones. For example Greif and Alfano (1964) showed that there is a marked acceleration in the rate of swelling of isolated dogfish liver mitochondria when subjected to treatment with the thyroidal hormones. Other aspects of mitochondrial function e.g. uptake and incorporation of amino acids (Freeman, Roodyn and

Tata, 1963) and respiration (Tata et al, 1963) are also enhanced by treatment with thyroxine and tri-iodothyronine.

There are several indications that thyroxine or its analogues have some control over ionic permeability of cells and possibly over osmotic and ionic regulation in general. Menozzi and Gatto (1961) showed that after treatment of epididymal adipose tissue with thyroxine there was a marked increase in the sodium content of the cells while Stephan, Jahn and Réville (1964) reported a greater loss of sodium and water through the kidney of hypothyroid rats than in normal animals. Effects of thyroid hormones on osmotic and ionic regulation are perhaps most marked in teleost fishes. Gasterosteus aculeatus showed markedly diminished euryhalinity after treatment with thyroid hormones, the level of chloride in the blood increases and the fish are no longer able to tolerate salinities in which control animals can live, (Kock and Heuts, 1942). The correlation between salinity of the medium and thyroid function has been discussed by Dodd and Matty (1963) who commented that marine teleosts are able to survive for a longer time in fresh water after injection with thyroxine than is normal for stenohaline fishes.

Various people have examined the effect of thyroid hormones on the permeability of amphibian membranes to ions and other substances. The earliest of these were Embden and Adler who in 1922, found that when an extract of the thyroid gland was added

to the saline bathing isolated frog skin, there was an increase in the flow of water through the membrane. Saito (1930)(quoted by Gellhorn and Northup, 1933) showed that thyroid extract increases the permeability of frog skin to dyestuffs.

Recently a considerable body of information has been amassed on the effects of thyroxine and some of its analogues on active sodium transport and metabolism of amphibian skin and bladder. Green and Matty have reported that thyroxine, at a range of concentrations, increases the short circuit current without influencing the electrical potential difference across the skin and bladder of Bufo bufo i.e. it increases the activity of the sodium pump and at the same time enhances the passive permeability of the membranes to sodium and other ions (Green, 1964; Green and Matty, 1963; Matty and Green, 1962, 1964). Active transport of sodium was increased by 50% when thyroxine was applied to the serosal bathing fluid at a concentration of 10^{-6}M whilst at 10^{-5}M oxygen consumption of the bladder was doubled within 40 minutes (Matty and Green, 1963; Green and Matty, 1962, 1964). However Thornburn and Matty (1964) found that thyroxine, at the same concentration, only caused an increase of 23% in the level of respiration of the bladder of B. bufo when measured by Warburg manometry rather than polarographically. Marusic and Torretti (1964) reported

that in vitro application of thyroxine to the bladder of B. spinolosus caused an increase in active sodium transport.

There has, however, been some difference of opinion about these results. Marusic et al (1966) found that when T_4 was administered in vivo it failed to either increase water permeability and metabolic rate of subsequently isolated bladders or alter the overall water balance of intact toads. Taylor and Barker (1965) were unable to obtain any increase in oxygen consumption of the skin or bladder of B. marinus, B. americanus and B. bufo after treatment with thyroxine. Even tissues from tadpoles of Rana catesbeiana, which are known to respond to thyroxine injected in vivo did not respond when exposed to the hormone in vitro. The same workers measured the short-circuit current across ventral skin sheets from Rana pipiens, Rana catesbeiana, Bufo americanus and Bufo bufo and bladder from B. marinus, B. americanus and B. bufo and were unable to detect any increase in the active sodium transport across these membranes after treatment with thyroxine, although all the tissues responded to the addition of vasopressin to the bathing medium. They also showed that treatment with thyroxine and its analogues failed to increase the rate of water movement across isolated bladder and skin of various anura including Bufo bufo (Taylor and Barker, 1967).

It has been shown (Figs. 4: 16 and 4: 17) that thyroxine at a concentration within the range of those used by Matty and Green has no apparent effect of sodium transport across the skin of Rana temporaria, nor was it possible to repeat the stimulation of short-circuit current across the bladder or skin of Bufo bufo. Conditions, in these experiments were as close as possible to those used by Matty and Green. A saline solution having a similar composition was used and the hormone was of the same batch, dissolved in the corresponding amount of caustic soda. The apparatus used was, for most of the experiments, identical to that used by Matty and Green and for some of the experiments was, in fact, the actual apparatus which they had used. Animals were kept in the same rooms, under the same conditions. During some of these experiments, after incubation of the membrane in thyroxine solution for an hour, the thyroxine saline was removed and the membrane was first washed and then treated with vasopressin. It was invariably found that the membrane responded normally to the vasopressin treatment. Since the experiments were so very similar it is difficult to explain the discrepancy in the results so obtained.

Parathyroid Hormone.

The parathyroid gland produces a polypeptide of molecular weight about 9000 which is sometimes called "parathormone"

or P.T.H. It has been known for many years that this substance plays a major role in the maintenance of the normal level of blood calcium, although the mechanisms whereby it does this are still in debate. At one time it was thought that the effect on calcium might be a secondary one, the main action of the hormone being on the renal reabsorption of phosphate (Albright and Reifenstein, 1948) but more recent investigations have shown that the hormone has a direct effect on the bone calcium. P.T.H. has been shown, however, to have some direct control over movement of phosphate ions in, for example, mitochondria where the hormone stimulates the uptake of inorganic phosphate and the dependent movement of magnesium ions (Sallis, DeLuca and Rasmussen, 1963). Borle and his co-workers (1963) have shown that P.T.H. also increases the flux of phosphate ions across the rat duodenum.

Very few studies have been made of the effects of this hormone on movement of ions other than calcium and phosphate. The influence of the hormone on water movement in amphibia was studied by Rasmussen et al (1963) who showed that P.T.H. had no effect on the movement of water across toad bladder in the presence of an osmotic gradient.

Hormones of the Adrenal Medulla.

The adrenal medulla produces the catacholamines epinephrine

and norepinephrine, which, although closely linked to the autonomic nervous system, are of definite endocrine nature. Both of these hormones are known to affect permeability of certain tissues. Aulsebrook (1965) found that epinephrine and norepinephrine stimulated the absorption of glucose and sodium from the small intestine, Jenkinson and Morton (1967) found that nor-adrenaline (norepinephrine) increased both inward and outward fluxes of potassium in smooth muscle, although it has no effect on the exchange of chloride or sodium uptake. Sutherland and Rall (1960) reported that the catacholamines increased the transmembrane potentials in cardiac muscle of mammals. Davoren and Sutherland (1963) showed that epinephrine stimulates the activity of the enzyme adenyl cyclase which catalyses the conversion of ATP into cyclic-AMP. Orloff and Handler (1963) suggested that the neurohypophyseal hormones also work by stimulation of this enzyme and if this is the case it would be expected that epinephrine and vasopressin would have a similar effect on a membrane such as the frog skin. Epinephrine has been applied to this membrane (Barker Jorgensen, 1947; Ussing and Zerahn, 1951) where it was found to increase the loss of salt to the outside. It has been shown, however, that this effect is due to a stimulation of the secretion of chloride to the outside which drags the sodium passively with it (Koefoed-Johnsen, Ussing and Zerahn, 1952). The hormone has been reported to have no effect on the passage of

ions across the toad bladder, (Leaf, Anderson and Page, 1958) other than causing an inexplicable transient drop in the short-circuit current. Rasmussen, Schwartz, Young and Marc-Aurele (1963) found that the hormone had no effect on water movement across the toad bladder in the presence of an osmotic gradient. Since this hormone does not mimic the effect of vasopressin on these membranes it casts some doubt on the theory that vasopressin controls sodium permeability by its effects on adenyl cyclase, although this would seem to be well authenticated.

Hormones of the Adrenal Cortex.

The steroid hormones of the adrenal cortex of mammals (the interrenal tissue of lower vertebrates have, for some time, been known to exert a considerable influence on salt balance in animals. During the past decade a considerable amount of work has been published on the effect of these and other steroids (including artificial ones) on the permeability of cells, organs and organisms to sodium and other small ions. Investigations have been made on representatives of most of the major groups of vertebrates, for example, Lockett and Roberts, (1963) found that perfusion of isolated cat kidney with aldosterone caused a retention of water, sodium and potassium due to an action of the hormone on the proximal nephron. Also in the mammals Spach and Streeten (1964) showed that when the blood of the dog is incubated in

aldosterone the sodium exchange between the red cells and the plasma is retarded. The effects of these hormones, particularly aldosterone, have also been investigated in the reptiles. Bentley (1962) showed that d-aldosterone, applied in vitro to the bladder of Testudo graeca, causes an increase in the short-circuit current, but it has no such effect on the intestine. It is interesting to note, however, that neither hydrocortisone, 0-α-fluorohydrocortisone nor d, l-aldosterone had any consistent effect on sodium or potassium loss from the stenohaline wrasse (Thalassoma dupperey), the euryhaline mullet (Mugil cephales) or the euryhaline Tilapia mossambica (Edelman, Young and Harris, 1960). From this it would appear that like many other hormones the corticosteroids do not play such a prominent role in ionic regulation in fishes as they do in most other groups of vertebrates. However in the lamprey, Lampetra fluviatilis Bentley and Follett (1962) have shown that aldosterone causes a marked decrease in the rate of sodium loss from the whole animal.

A small amount of work has been done on the urodele Amphibia e.g. Alvarado and Kirschner (1964) showed that aldosterone increases the sodium influx across the body wall of the larval Ambystoma tigrinum. Ferreri et al (1967) and Socino and Ferreri (1965) have shown that both aldosterone and cortisol increased sodium retention in excised muscles of the newt Triturus cristatus,

aldosterone being the more active in this respect. The majority of studies on the Amphibia, however, have been made on the Anura.

In 1956 Taubenhaus and his co-workers demonstrated that when sacs of skin from the hind legs of frogs were treated with certain steroids there was a greater change in the osmotic pressure of the internal solution than was found in control skin sacs. They found that deoxycorticosterone acetate (DOCA), fluorohydrocortisone and hydrocortisone caused a marked increase, estriol caused an increase only when applied at very high concentrations and testosterone had no detectable effect. In the same year Levinsky reported that DOC had no effect on sodium transport across toad bladder, although later Sharp and Leaf (1964) showed that this compound did cause a small increase in sodium transport as did Pasqualini and Riseau (1952). Maetz, Jard and Morel (1958) were unable to demonstrate any effect of aldosterone, applied in vitro, on sodium transport across frog skin, but found an increase in short-circuit current, measured in vitro after the whole animal had been injected with the hormone. This confirmed the work of Huf and Wills (1953) who were unable to demonstrate an increase in sodium transport after addition of adrenal cortex extract to the bathing medium. A similar study was made by Bishop, Mumbach and Sheer (1961) showing that destruction of the interrenal tissue of frogs in vivo lead to a decrease in active sodium transport across

the skin but they found that administration of adrenal steroid extract in vitro to some extent reversed this effect. Further work on the frog skin showed that 2-methyl-9-α-fluorohydrocortisone and hydrocortisone sodium succinate increases the rate and duration of sodium transport (McAfee and Locke, 1961) and that aldosterone increases Na uptake whilst SC8109 (an inhibitor of aldosterone) decreases the short-circuit current (Penny, McAfee and Locke, 1961). Imamura and Suski (1962) reported that de-oxycorticosterone gluconate, cortisol and corticosterone at concentrations of 3×10^{-6}M elicit equal rises in short-circuit current across frog skin.

During the past five years the bladder of the toad (usually B. marinus) has received more attention than any other amphibian membrane in respect of its response to aldosterone. Other than the work of Levinsky already referred to, the first reports to be published on the effects of steroids on sodium transport across toad bladder were those of Crabbé (1961a, 1961b) who showed that aldosterone causes an increase in the active transport of sodium across B. marinus bladder whether applied to the membrane in vitro or in vivo. When applied in vitro the hormone was found to cause a stimulation after about one hour, at concentration levels of 2.0 to 25 x 10^{-7}M/l. This was confirmed by Sharp and Leaf (1963) who showed that the action of aldosterone was

antagonised by progesterone and spirolactone as well as by ouabain. They also found that if aldosterone was applied and then removed after five minutes treatment the tissue responded as if the hormone was still present and the effect followed the normal time course. Porter and Edelman (1964), showed that aldosterone affected the transport of sodium across toad bladder if it was applied to the mucosal surface, although a greater dose was required than that necessary when applied to the serosal surface.

These reports authenticated the action of aldosterone on this parameter of cellular permeability and were followed by work on the mode of action of the hormone. Crabbé (1963) suggested that aldosterone stimulates active sodium transport at least in part by increasing the passive permeability of the epithelial cells of the toad bladder to sodium. This causes an increase in the size of the sodium 'pool' and means that the amount of sodium available to the pump is increased. It is interesting to note in this connection that Leaf (1960) suggested that vasopressin works in a similar manner. It was suggested by Edelman, Bogoroch and Porter (1963) that aldosterone induces the synthesis of enzymes which couple metabolism to active transport. This was based on the finding that the hormone was selectively localised in the nuclei of the epithelial cells and that actinomycin D and puromycin block

the action of aldosterone without affecting that of vasopressin. Porter and Edelman (1964) also showed that the action of aldosterone is dependent on an adequate supply of metabolic substrate. By the use of antibiotics such as actinomycin D and puromycin, which inhibit steps in protein synthesis, Crabbé and de Weer (1964) concluded that aldosterone could be stimulating the synthesis of some fraction of nuclear RNA which migrates to the cytoplasm and induces the ribosomes to synthesise more 'carrier' for the sodium uptake. The first part of this suggestion was to some extent affirmed by the report of Porter, Bogoroch and Edelman (1964) that aldosterone increased the rate of incorporation of radioactive uridine into epithelial cell RNA and that this effect preceded the effect of the hormone on sodium transport.

From this it appears that the primary effect of aldosterone on the toad bladder is on the passive permeability of the mucosal border of the epithelial cells. This passive influx is mediated through a carrier system and the amount of carrier present appears to be its limiting factor. The aldosterone increased the amount of carrier available, hence increasing the intake of sodium into the cell. Since the size of the sodium pool is the rate limiting factor for the sodium pump, the active transport of sodium is increased accordingly.

Although this mechanism is basically very similar to that

suggested for vasopressin (Crabbé and de Weer, 1965) it would appear unlikely that the hormones use the same site. The onset of action of aldosterone begins much later than that for vasopressin, an unlikely event if both hormones were affecting the same part of the same system, also the two hormones complement each other's action if applied together to the membrane, the total effect being the sum of the effects which would be expected from each hormone. It seems likely that whilst the aldosterone affects the synthesis of the carrier thus causing an increase in the total amount of carrier present, the vasopressin could act by increasing the efficiency of the carrier system itself.

Sex Steroid Hormones.

There have been very few reports of the effects of the sex steroids on the permeability of vertebrate cells or membranes to monovalent cations, and it is possible that some of the actions attributed to this group of hormones are, in fact, due to secondary effects with the steroids affecting the production of other hormones, e.g. from the pituitary gland which in turn could alter the rate of secretion of the adrenal cortex etc. One example of this may be the sex difference in the short-circuit current across the small intestine of the mouse reported by Matty (1964). He found that the female had a higher potential and short-circuit current than the male, although it is not clear whether this is due to an increase in

the net flux of sodium across the gut or to the movement of some other ion. Injection of oestradiol-17 into the male mouse increased the short-circuit current across the intestine, measured in vitro, but ovariectomy of the female did not cause any reduction. It is quite possible that this is entirely due to a secondary action.

Bitman et al (1959) showed that oestrogens and progesterone can affect the intracellular K/Na ratio in rabbit endometrium, oestrogen causing an increase in the amount of sodium present whilst progesterone has the reverse effect. Gimeno, Gimeno and Webb (1963), working on cellular membrane potentials of isolated rat atrium, found that oestradiol, testosterone and progesterone all increased the duration and area of the action potential, and decreased the rate of depolarisation of the membrane, although none of the hormones had any effect on the resting potential. The experiments were performed in vitro using a near-physiological dose of the hormones (10^{-5}M) in each case and apparently show a real and direct effect of the hormones at least on the passive permeability of the cell membranes.

Reports of studies using the sex steroids on amphibian membranes are scarce. Francis and Gatty (1938) using Rana catesbiana reported a difference in P.D. across the skin of male and female frogs, but this was denied by Katzin (1940) and there have been no subsequent reports of such differences. Taubenhaus,

Fritz and Morton (1956) measured the effects of various steroids on frog skin by filling hind leg sacs with a Ringer solution of known composition, measuring the osmotic pressure after treatment with a hormone and comparing the figures with those from the control sacs. They found that oestriol had a slight effect, but only at a very high dose level, and that testosterone had no effect at all. Schoffeniels and Baillien (1960) found that iodinated N-methyl-pyridine-2-aldoxine (2-PAM) increases the electrical potential and net sodium flux across the skin of Rana temporaria except during the immediate post-reproductive period. During this time, however, if the female was injected with oestradiol and the male with testosterone, the 2-PAM effect was the same as that throughout the rest of the year. It has been found, however, that testosterone has no effect when added to the frog skin in vitro and it is possible that the action of this hormone on the skin after 2-PAM treatment is not a direct effect on permeability characteristics (Schoffeniels, 1964 pers. comm.). None of the sex steroids used in this study showed any effects in their own right.

Pancreatic Hormones.

The pancreas produces two distinct hormones, insulin and glucagon. The effect of the first of these has been known for some time and the hormone itself was one of the first to be separated. It is a low molecular weight protein consisting of a disulphide-linked ring with two polypeptide side-chains.

Insulin alters the permeability characteristics of various types of cells. The majority of the work with this hormone has been performed on mammals, particularly in relation to the accumulation of glucose and amino acids. For example insulin has been shown to increase the uptake, into diaphragm muscle, of sugars, α-aminoisobutyric acid (Krahl, 1961), of various amino acids (Wool, 1964) and also to regulate the sodium uptake in this membrane (Crease, D'Silva and Northover, 1958). Studies have been made which indicate that insulin increases the permeability of muscle to ions and affects the potential difference across the muscle membrane (Zierler, 1957) although this has been disputed (Kernan, 1961). In 1963 Beigelman and Hollander demonstrated that the resting electrical potential of rat epididymal adipose tissue was increased by insulin in either the presence or the absence of glucose, hence showing that the effect of insulin on the sodium pump was independent of any possible effect on a glucose-transporting mechanism.

There have been several reports of studies of the action of insulin on amphibian tissues. The earliest of these (Rehm, Schumann and Heinz, 1961) demonstrated that insulin causes an increase in the potential difference and short-circuit current across frog gastric mucosa. This, however, is due to an effect of the hormone on the chloride pump across

the membrane rather than on a sodium-transporting mechanism. Bower and Grodsky (1963) found that the application of insulin to the bladder of Bufo marinus caused an increase in the uptake of glucose by the membrane. Also in 1963, Rasmussen et al reported that application of insulin to B. marinus bladder had no effect on net water flux across the membrane, a finding which has been confirmed by Leaf (reported by Herrera, 1965).

Insulin has also been shown to affect the transport of sodium across amphibian skin and bladder. Herrera, Whittembury and Planchard (1963) reported that glucagon-free insulin at a concentration of 4 units/ml brought about an increase in the short-circuit current across frog skin (Rana pipiens) after the sodium transport had been inhibited by high concentrations of calcium or magnesium ions in the bathing medium. These experiments have little physiological significance since the membranes would never be subjected to such high concentrations (22.4 mM/l) of either calcium or magnesium in normal conditions. Also the concentration of hormone needed to bring about the relatively small change in sodium flux was several orders of magnitude larger than the normal physiological concentration. If, however, any reliance can be placed on these results, it should be noted that the bathing solutions did not contain any glucose - once again

indicating that the effect on sodium transport is independent of any effect of permeability of the membrane to glucose.

The effects of insulin on sodium transport across the bladder of Bufo marinus have been investigated under more normal conditions (Leaf, in Goodfriend and Kilpatrick, 1963; Herrera, 1965). Herrera has shown that a concentration of 2.6×10^{-5}M insulin gives rise to an increase of 54% in the short-circuit current across B. marinus bladder, and he has also demonstrated that this effect is due to an action of the hormone on the sodium pump itself rather than on the passive permeability of the cell membrane. This latter finding is of interest since it has been suggested that vasopressin and insulin might have similar effects on the membrane, possibly through the disulphide-sulphhydril linkage since the hormones have the S-S group in common (Carlin and Heckter, 1962). It has been shown, however, that vasopressin acts on the sodium-transporting mechanism of the toad bladder by increasing the passive permeability of the mucosal cells to sodium ions (Leaf, 1960). Insulin, apparently, does not have any effect on the passive permeability of the cell, either directly or through some stage of the associated metabolism, but stimulates the pump itself. Under these conditions the sodium pool would decrease and the passive permeability of the cell membrane would be the rate-limiting factor rather than the activity of the pump.

Glucagon, the second hormone produced by the Islets of Langerhans of the pancreas, is also a low molecular weight protein, containing 29 amino acids. This hormone plays a part in controlling the release of glucose by hydrolysis of glycogen from the liver, although it appears to have no effect on the permeability of tissues to glucose. Aulsebrook (1965) found that neither dopamine nor glucagon had any effect on absorption of glucose or sodium by the small intestine of the rat, and although Randle (1958) found that a glucagon preparation increased the uptake of glucose by the isolated rat diaphragm he considered that this was due to contamination of the hormone with insulin. It has been thought that glucagon may show a slight effect on potassium mobilization (Ellis and Beckett, 1963).

Tissue Hormones.

Very little work has been done on the permeability effects of those hormones which, being produced in tissue diffused throughout the body rather than in discrete organs, may be known as tissue hormones. These hormones include serotonin (SHT), histamine, bradykinin and angiotensin (hypertensin).

Villegas (1963) reported that histamine at a concentration of 2.10^{-5}M, when applied to the gastric mucosa of the frog in vitro, caused an increase of nearly 100% in the permeability coefficient of the tissue for potassium. It also increased the permeability to water. Serotonin has been shown to

increase the rate of transport of calcium ions into muscle cell of the rat uterus, acetylcholine and epinephrine showed a similar effect to S.H.T. (Woolley and Gommi, 1963). Rasmussen et al (1963) applied numerous hormones to isolated toad bladder and measured their effect on the net flux of water down an osmotic gradient. He found that neither angiotensin, bradykinin nor serotonin increased this rate of flux. Angiotensin has been found to increase the action of vasopressin on sodium transport in amphibia, (Coviello and Crabbé, 1965), and has also been shown to cause a depression of tubular resorption rates of salt in mammals (Leyssac, 1965).

General Discussion.

Substances can move across cellular boundaries in diverse ways and several things can happen to a substance once it has entered a cell. Because of this diversity there are various ways in which a hormone can alter the amount of a substance entering or leaving a cell or tissue. It is possible for a hormone to alter the net flux of a substance without changing the permeability characteristics of the membrane. An example of this is the action of L.H. on the rat ovary. This hormone increases the total influx of glucose into the tissue. However, it also causes an increase in the production of lactic acid which indicates that the actual effect of the hormone is to increase the rate of metabolism

of glucose. This results in a decrease in the passive loss of the sugar from the cell without altering the rate of influx. Since the net influx is the rate of influx minus the rate of efflux, the net influx of glucose will be increased by addition of L.H. to the tissue.

A hormone may have a direct effect on the cell membrane itself. If it increases the passive permeability of the membrane in both directions there will be no change in the net flux across the barrier unless some other factor is present. This can be illustrated by reference to the action of vasopressin on the permeability of the toad bladder to water. The hormone increases the passive permeability of the membrane but if the saline on both sides of the bladder is of the same concentration there is no detectable effect on net-water flux after addition of the hormone - the increase in rate of efflux is equal to the increase in rate of influx. If, however, there is an osmotic gradient across the membrane, in other words, the rate constant of influx did not equal the rate constant of efflux before addition of the hormone, the stimulation increases the difference between the rates of water flux and hence net flux is greatly increased.

A third way in which a hormone can influence the rates of movement of substances is by acting on a passive carrier system, or any other system which allows a limited passive

permeability in either direction. The influx of sodium into the frog skin appears to be an example of such a system, since the rate of entry of sodium ions through the mucosal surface of the cell is limited by the amount of carrier available, according to one theory, or by the number of attachment sites according to another. If there was an increase in the rate of passive efflux of the ion then the net flux would remain the same but if the efflux rate remained the same there would be an increase in net influx into the cell. A hormone would also affect the rate of efflux from the cell by increasing the passive permeability without increasing the carrier-mediated intake, and this situation would lead to an increase in the net efflux of the substance from the cell.

A fourth site of hormone action would be directly on an active transport system, as for example the action of insulin on the transport system for glucose in mammalian muscle cells. In such a case stimulation of the active transport system without a complementary increase in the passive permeability of the cell to the substance being pumped, would lead to a decrease in the intracellular pool of that substance. This in turn would decrease the passive efflux of the substance from the cell, and once again net flux would be regulated by the rate of influx.

Hormones may work by one of these systems, or by several at one time, for example, vasopressin directly increases the passive influx of sodium into toad bladder cells and indirectly increases the rate of active transport from the cells. In consequence it is not possible to make any general statements on the action of hormones in cell membranes from which the effects of other hormones at the cellular level would be predicted.

Although the present study has not discovered any previously unknown effect of hormones on the permeability of anuran membranes to sodium ions it has shown that no general principles can be established and also that the number of hormones in the amphibian which control salt and water balance is very limited.

SUMMARY.

1) The structure of the frog skin and toad bladder are discussed in relation to the site of the Na permeable/K impermeable layer of the Koefoed Johnsen/Ussing model, and to the site of the pump for the active transport of sodium ions.

2) A wide range of hormones and their contaminants, where present, were assayed for their possible effects on the active transport of sodium ions across the anuran membranes using the short circuit current technique of Ussing and Zerahan (1951). These hormones included those of the anterior pituitary (with posterior pituitary contaminants), oestrogens, thyroxine, hydrocortisone, angiotensin and glucagon.

3) None of these hormones either directly or indirectly affected the rate of active transport of sodium when applied in vitro to the skin of the frog or skin and bladder of the toad.

4) The effects are discussed of these and various other hormones on permeability of vertebrate cells and membranes to ions, water and small organic molecules.

5) It was found impossible at this stage to draw any general conclusions on the effects of hormones on permeability since each one appeared to control a different parameter of the action of the cells.

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APPENDIX.

Measurement of water movement across toad bladder.

Bentley (1958) described a gravimetric method for measurement of water movement across the toad bladder in the presence of an osmotic gradient. This was done by separating the two lobes of the bladder, maintaining them intact, and tying each half bladder onto a flanged glass tube. The bladder is then filled with a saline medium, or a different concentration of the same one. The bladder and tube are then weighed repeatedly, the difference in weight representing the movement of water into or out of the membrane sac. Using this method Bentley found that he could repeat the weighing process sufficiently accurately to enable measurements of hormonal effects to be made, both on Bufo marinus and on Bufo bufo. An attempt was made, during the course of this work, to apply this technique to assess the effects of the various hormones on water movement across the bladder of the toad Bufo bufo.

Bladders were removed from adult B. bufo of both sexes. These were placed in a saline solution and the two lobes separated. Each lobe was attached to a flanged glass tube $\frac{1}{4}$ inches in diameter with sewing cotton. The sac was filled with 1 ml saline of approximately the same osmotic pressure as the toad plasma

and then immersed, to the level of the collar above the ligature, in a boiling tube containing saline at a lower concentration under aeration through a fine polythene tube. The tube was held in place in the boiling tube by a rubber stopper. The bladders were left undisturbed for an hour to equilibrate, after which the saline was removed and replaced by fresh saline at the same concentrations. The bladder was then removed from the boiling tube, blotted and weighed. It was replaced in the saline and re-weighed at 15 minute intervals.

The area of the bladder was obtained by draining the sac after the final weighing and then re-weighing to find the weight of saline it contained. It was assumed that 1 gm. of saline would have a volume of 1 c.c. and from this the area could be assessed since:-

$$\text{area} = 4 \sqrt[3]{\frac{\text{volume}}{4/3\ \pi}}^{2}$$

$$\text{or area} = 12.57 \left(\frac{\text{volume}}{4.197}\right)^{2/3}$$

Although theoretically this technique should yield the necessary results it was found in practice, the weights were very variable. This variability was attributable to the varying thickness of the water layer remaining over the outer surface of the bladder sac and around the collar

above the ligature after blotting. The variability in the weighing was so great that it could easily have masked any hormone action which caused an increase of less than 100% in the net flux of water.

Several methods were used to try to eliminate this error.

1) No liquid was allowed to touch the glass tube and the level of liquid in the boiling tube was kept such that the collar above the cotton ligature was not immersed in the saline, also the cotton was cut off as short as possible. This meant that there was no water film on the glass or adhering to long lengths of cotton, also that there was no possibility of drops of water becoming entangled in the collar. These precautions helped to decrease the variability but by so small a part that it was obvious that the major factor involved was the water layer over the surface of the bladder sac.

2) A standardised technique was developed for draining the bladders. They were brought out of the saline and wiped four times round the top of the boiling tube before weighing. The weight differences in a typical set of experiments using this procedure is shown in Table 1.

3) In case water had seeped up into the collar the bladders were drained as above and then the collar was blotted by lightly dabbing with paper tissue. A typical set of results for this modification are given in Table 2.

4) If the entire bladder was blotted it was found that the variability was as great (Table 3).

5) In many of the experiments it was found that the rate of water across the bladder dropped during the first hour. However if the internal solution was changed or the same solution taken up in a syringe and then replaced (pseudo-change) the water loss rate increased again. This is possibly due to the lack of stirring within the bladder sac itself (Table 4).

From this it will be seen that the inherent error in the method was sufficiently great to make it impracticable as a method for assessing hormone effect on water movement. Bentley (pers. comm.) suggested that leaving the bladder in the saline for a greater length of time would reduce the error, since the true difference in weight between weighings would be greater and hence the difference caused by the inherent variability would be less. Unfortunately the variability was still too great to allow use of the technique (Table 5).

From this it was inferred that the possibilities of error inherent in the technique are too great to allow any reliance on results obtained. In consequence these studies were not pursued.

Table 1.

Weight difference in four bladders after a period of 1 hour, when treated as in condition 2)in the text.

Time(min)	Difference between weight at beginning and end of period(mg			
	Bladder (1)	Bladder (2)	Bladder (3)	Bladder (4)
0 - 15	12.9	27.5	20.0	22.4
15 - 30	19.0	41.4	14.6	27.6
30 - 45	13.0	25.5	12.1	12.8
45 - 60	16.0	13.5	17.3	19.4

Table 2.

Weight differences in four bladders over a period of 1 hour, when treated as in condition 3) in the text.

Time(min)	Difference between weights at beginning and end of period(m			
	Bladder (1)	Bladder (2)	Bladder (3)	Bladder (4)
0 - 15.	17.0	5.4	4.2	3.7
15 - 30	17.4	5.6	7.3	5.7
30 - 45	10.5	4.1	4.6	3.3
45 - 60	12.2	2.0	5.2	5.2

Table 3.

Weight differences in four bladders over a period of 1 hour, when treated as in condition 4) in the text.

Time(min)	Difference between weights at beginning and end of period (mg)			
	Bladder (1)	Bladder (2)	Bladder (3)	Bladder (4)
0 - 15	14.4	12.1	6.1	4.0
15 - 30	7.2	8.9	4.1	4.8
30 - 45	9.2	6.6	3.2	3.4
45 - 60	8.0	6.5	2.2	4.2

Table 4.

Weight differences in four bladders after a period of 1 hour, when treated as in condition 5) of the text.

Time(min)	Difference between weight at beginning and of period (mg)			
	Bladder (1)	Bladder (2)	Bladder (3)	Bladder (4)
0 - 15	6.9	14.6	2.1	4.4
15 - 30	4.0	15.6	3.6	9.4
30 - 45	2.9	10.5	1.7	15.6
45 - 60	3.7	3.2	1.8	1.6
60 - 65	inside soln.changed.		inside solution pseudochanged.	
65 - 80	5.5	34.9	8.2	5.4
80 - 95	7.2	7.6	6.4	6.6
95 -110	2.9	12.4	1.3	4.2

Table 5.

Weight difference in four bladders over a period of 3 hours when treated as per Bentley's suggestions.

Time(min)	Difference between weights at beginning and end of periods(mg)			
	Bladder (1)	Bladder (2)	Bladder (3)	Bladder (4)
0 - 60	153.7	120.0	22.7	87.4
60 -120	143.7	259.4	20.8	98.6
120-180	75.3	157.2	18.1	85.0