THE EFFECT OF HYPEROSMOTIC AGENTS ON FROG SKIN

by

Thomas J. Franz, B.S.

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(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

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I. INTRODUCTION

One fundamental and necessary property of all living cells is the ability to control their internal environment in the face of a vastly different and constantly changing external environment. Outstanding in this respect is the cell's ionic composition in comparison to the ionic composition of extracellular fluid. Most cells are rich in potassium and low in sodium, although just the opposite condition exists outside the cell. Since these concentration gradients appear to defy known laws, the phenomenon has been the object of much research in the last few decades.

Several alternative theories have been put forth in the past to explain this perplexing problem; the view now generally accepted was originally presented by Dean (3). It is felt that inside the cell there exists a sodium pump, which extrudes most of the sodium that diffuses into the cell, thereby maintaining a low internal sodium concentration. The movement of sodium outward via the pump creates a potential difference across the cell membrane, the inside being relatively electronegative to the outside. This EMF would then act as a driving force for potassium movement and could explain the high intracellular potassium concentration. According to this theory, the potassium ion is at electrochemical equilibrium across the cell membrane, whereas the sodium ion is not. Since it requires energy to maintain an ion out of equilibrium, the term active transport is applied to the process of sodium extrusion.

The proposal that a pump exists inside the cell immediately led to a great deal of research and theoretical consideration as to its nature and operation. Progress has been slow, at least part of the difficulty being due to the technical problems involved in working with single cells. This problem has been partially aided by the discovery that frog skin has the ability to actively transport sodium across itself from anatomical outside to inside. Here one can work easily with a large piece of tissue in which the transporting cells of the epithelium are oriented and pump sodium in one direction only. Basically the epithelial cells are single cells transporting sodium like most other cells, but with the special feature of direction or polarity, which makes them unique and especially valuable to those studying active transport. The frog skin, which is a sheet of these oriented cells, has become a favorite tool of many in the field today because of this special feature of polarity. Since the discovery of active transport in this tissue, other epithelial structures with oriented cells transporting sodium have been found and are also being studied (1, 2, 4, 15).

The discovery of frog skin as a biological tool dates back to the pioneering investigation of du Bois-Reymond (5) who, in 1857, showed that a potential difference exists across living skin. It was not until 1904 that Galeotti (7) showed the potential to be dependent on the presence of sodium salts in the bathing media. He also showed that a unidirectional permeability exists for sodium

in that sodium chloride moved only from the epithelial side inward, but not from the dermal side outward, whereas potassium chloride moved equally well in either direction. This is a property of the living skin only, for the dead skin shows no potential and is equally permeable to sodium in both directions.

In 1935, Ruf (8) showed that the isolated skin, bathed by Ringer's solution on both sides, is able to accumulate chloride in the inside bathing solution. Krogh (13), in 1937, was able to demonstrate that frogs in need of salt take up sodium chloride from the bathing solution, even when the concentration is only 10^{-5} M. A year later, he (14) demonstrated that this process is specific for sodium, for neither potassium nor calcium can be substituted for sodium. The chloride ion, however, can be replaced by either bromide or bicarbonate.

Katzin (10), in 1940, confirmed what Galeotti had shown many years before him, that the movement of sodium through the skin in the inward direction is greater than in the outward direction. Through the use of radioactive sodium he found the influx to be 60 - 300% greater than the outflux.

Ussing (23) also used radioactive sodium and found that it accumulated in the inner bathing solution even though the concentration of sodium outside was 1.64 mM and inside was 115 mM, the influx being usually at least 10 times greater than outflux. Because of the net movement of sodium inward against a concentration

gradient, and likewise against an electrical gradient, he proposed that the sodium ion must be actively transported through the skin. He also proposed that the active transport of sodium is the source of the potential difference which exists across the skin, and that it in turn acts as an attractive force for the chloride ion and thus accounts for the net movement of chloride as observed by Huf. This was the first time an explanation for the skin potential involving the active transport of sodium had been suggested, although the previous work cited above certainly pointed in this direction. Of course, the possibility existed that other ions might also be contributing to the development of the skin potential, but Ussing's data, together with other data, was insufficient to answer this point. In fact, the work of Meyer and Bernfeld (18), published just a few years prior to Ussing's work, stressed the importance of the role of hydrogen ions. Ussing, too, felt that hydrogen ions were important, and proposed that sodium transport might indeed be a forced exchange of sodium for hydrogen, proceeding at a rate sufficient to keep intracellular pH constant.

Although the proposal of Ussing certainly seemed to explain the observations made up to that time, it remained to be shown that his hypothesis was quantitatively correct. In other words, was the sodium ion the only ion tending to create a difference in potential across the skin, and was this potential of sufficient magnitude to account for the net movement of chloride? These were the questions which Ussing set out to answer.

In 1951, Ussing (26) described a technique by which he was able to demonstrate that sodium is the only ion being actively transported through the skin. This is now known as the short-circuit technique and it has been used extensively by many investigators since his original description. Since the net movement of any ion normally depends on the effect of two forces, namely concentration and electrical gradients, no net movement can occur if these gradients are abolished. When the frog skin is mounted as a barrier between two chambers, with Ringer's solution of equal strength on each side, an electrical gradient exists across the skin with the inside being some 20 - 120 mv positive to the outside. Concentration gradients between the solutions are non-existent in this case. Ussing felt that if the electrical gradient could be abolished, the net movement of any ion would then be accountable only on the basis of a third force, namely active transport. The abolition of the normal trans-skin potential was achieved by passing a direct current through the system. Since the passage of current across a resistor, such as the frog skin, results in the development of a potential across that resistor, Ussing was able to nullify the normal trans-skin potential by applying a counter-potential of equal but opposite sign. In this condition only those ions subject to active transport will move, and the number of ions being transported can be determined by measuring the amount of current it takes to maintain the skin potential at zero. For a condition of zero potential to exist across

the skin at a time when a current is flowing across it, it is necessary to have another current of equal strength flowing in the opposite direction. Hence, we see that the ions actively transported through the skin can be viewed as a current flowing through the skin, this current being matched by an equal, imposed, current flowing in the opposite direction. The net result of two currents flowing across the same resistor in opposite directions is, of course, zero, but by measuring the imposed current, the value of the internal current is found automatically. The externally applied current which is just sufficient to drive the skin potential to zero is referred to as the short-circuit current (SCC).

With the technique just described, Ussing was able to measure electrically the amount of Na being actively transported across the skin. On these same skins he simultaneously measured sodium influx with radioactive sodium. When expressed in the same units it turns out that sodium influx very closely approximates the short-circuit current. Since the short-circuit current measures the net amount of ions being transported, the values for sodium outflux must be subtracted from those for sodium influx. When this is done, after measuring these values in separate experiments with radioactive sodium, the agreement between net sodium movement and short-circuit current is almost perfect. Thus Ussing was able to prove that sodium is the only ion being actively transported through frog skin, in agreement with his previous hypothesis.

The second part of Ussing's hypothesis, that the potential difference across the skin could account for net chloride movement, was also quantitatively substantiated a year later (11). In order to establish this, Ussing (24) found it convenient to develop an equation relating the effects of concentration and electrical gradients to ionic movement. For ions moving passively, not subject to active transport, the following equation can be written:

$$\frac{M_{in}}{M_{out}} = \frac{C_o}{C_i} \cdot \frac{f_o}{f_i} \cdot \exp \frac{zF}{RT} (E_o - E_i)$$

where M_{in} is influx; M_{out} outflux; C_i and C_o the concentrations of the ion inside and outside; f_i and f_o the mean activity coefficients for the ion in the two solutions; $E_o - E_i$ the electrical potential difference across the membrane; z the charge on the ion, F Faraday's number, R the gas constant; and T the absolute temperature. By simultaneous measurement of chloride influx and outflux using the radioactive tracers Cl^{36} and Cl^{38}, and direct measurement of the skin potential, Ussing found good agreement with the above equation. Thus he showed that chloride movement in frog skin is strictly passive.

In the study on chloride movement it was noted that there seemed to be an inverse relationship between the spontaneous skin potential and the net chloride influx or chloride permeability. As a general rule, low skin potentials were associated with high chloride permeability and high skin potentials with low chloride permeability. It was

suggested by Ussing that the chloride ion acts as a short-circuit of the potential developed by active sodium transport. In other words, the sodium pump is capable of creating a maximum potential difference across the skin, but this maximum is never attained because the chloride ion is attracted by the potential and diffuses across the membrane to lower it. The magnitude of the potential developed, therefore, depends on the chloride permeability. It thus becomes obvious that if another ion were substituted for chloride, an ion whose permeability approached zero, the maximum pump potential would be realized. The sulphate ion is relatively impermeable in frog skin and was chosen by Koefford-Johnson and Ussing (12) for a study relating the dependence of the frog skin potential to the external sodium and potassium concentrations. In this way, the variable of chloride permeability could be eliminated.

It was found from such a study that the inside of the frog skin behaved as if it were a potassium electrode, and the outside of the skin behaved as if it were a sodium electrode. By this it is meant that the trans-skin potential changes approximately 58 mv for a ten-fold change in concentration of potassium in the inner bathing solution. The same relationship occurs relative to sodium in the outer bathing solution, that is, no change in potential is noted for a change in concentration of potassium in the outer bathing solution or of sodium in the inner bathing solution. It was concluded from these observations that there are two membranes in the frog skin, these probably being the inner and the outer membranes of the cells

of the stratum germinativum, the inner one of which is selectively permeable to potassium but not sodium, and the outer one of which is selectively permeable to sodium but not potassium.

Since the inside of the cell is normally lower in sodium than the outside, sodium will diffuse across the outer membrane into the cell giving rise to a diffusion potential which is predictable by the Nernst equation:

$$E = \frac{RT}{zF} \ln \frac{[Na]_o}{[Na]_c}$$

where R, T, z, and F have their usual meanings, $[Na]_o$ is the sodium concentration in the outer bathing solution and $[Na]_c$ the sodium concentration in the cell. This equation predicts a 58 mv change in potential for a ten-fold change in concentration of sodium in the outer bathing solution, which is what was observed. At the inner membrane a potassium diffusion potential will develop since the intracellular potassium concentration is much higher than that of the inner bathing solution. Again, the magnitude of the potential is given by the Nernst equation,

$$E = \frac{RT}{zF} \ln \frac{[K]_c}{[K]_I}$$

where $[K]_c$ is the potassium concentration in the cell, and $[K]_I$ the potassium concentration in the inner bathing solution. The sum of

the two diffusion potentials, sodium at the outer membrane and potassium at the inner membrane, will give the normal trans-skin potential.

It is obvious that for the frog skin potential and SCC to continue indefinitely, the concentration gradients across the two membranes must be maintained. This is the function of the sodium pump. By extruding sodium ions which enter the cell, it maintains the cellular sodium concentration at a constant low level and thus maintains the sodium gradient across the outer membrane. To account for the maintenance of the potassium gradient across the inner membrane would also require an active pump. Because of the known requirement of the sodium pump for potassium, as shown in frog skin by Huf (9), Koefford-Johnson and Ussing postulated a coupled sodium-potassium pump, one which simultaneously pumped sodium out of, and potassium into, the cell. Thus it can be seen that the development of the normal trans-skin potential can be looked upon in two different ways. Although the potential is the result of active sodium transport, as was originally proposed and demonstrated by Ussing (26), more precisely it is the result of concentration gradients existing within the skin. These gradients are maintained by the coupled sodium-potassium pump.

The model described predicts that the frog skin potential is actually the sum of two diffusion potentials within the skin. This part of the model was tested by Engbaeck and Hoshiko (6) and found to be true. With the use of microelectrodes they were able to

puncture the skin and demonstrate the existence of two definite, spatially separated, potential changes. These two, summed, equal the normal trans-skin potential. The location within the skin of the two steps in potential, roughly corresponds to the sites of the outer and inner membranes of the cells of the stratum germinativum.

With the first part of the model independently verified, it remained for Ussing only to verify the selective permeability properties of the two membranes which he had postulated on the basis of skin potential changes. This was done by studying the changes in volume of the epithelial cells in response to changes in the composition of the two bathing solutions, and was accomplished by direct observation of these cells under the microscope (17). It would be expected that if the outer membrane were permeable to sodium but not to potassium, a change in the sodium concentration of the outer solution should result in a change in the volume of the epithelial cells as a new steady state between cellular and outer bathing solution sodium is established. There would be no volume change expected if the potassium of the outer bathing solution is varied. Just the opposite should be expected with changes in the sodium and potassium concentration of the inner bathing solution, since the inner membrane was predicted to be potassium permeable and sodium impermeable. Good agreement was found between these predictions and actual experimental observation, thus lending more support to the general validity of the model proposed by Koefford-Johnson and Ussing.

The real importance of the model discussed above lies not so much in the fact that it gives a better understanding of the sodium transport system in frog skin, but that it points out a system used by nature which previously had not received attention; a system of two membranes in series, having different permeability characteristics. This concept has had a significant impact on current thinking and has allowed for new theoretical approaches to some of the great mysteries and unsolved problems in the field of biological transport. Examples of these approaches are the recent articles by Ogilvie, McIntosh and Curran (20) and Patlak, Goldstein and Hoffman (22).

This thesis will report on the effect of hyperosmolarity of the outer bathing solution on frog skin permeability. Although the investigation originally began as a study into the possible effect of dimethyl sulfoxide (DMSO) on frog skin permeability, it soon became apparent that the changes observed on addition of DMSO to the outer bathing solution are secondary to the increase in osmolarity and may be produced by other agents added in comparable amounts. These agents will hereafter be referred to as hyperosmotic agents. The changes in frog skin permeability caused by these agents were considerable but there was no obvious explanation for the phenomenon. It appeared both interesting and worthwhile to attempt to elucidate the mechanism of action of the hyperosmotic agents and to determine the site of action within the skin.

II. MATERIAL AND METHODS

All experiments were performed on the non-pigmented abdominal skin of Rana pipiens. The skin is generally mounted as a barrier between two glass or plastic chambers with Ringer's solution of identical composition bathing each side, the composition being 115 mM NaCl, 2 mM KCl, 2 mM Na_2HPO_4, 0.25 mM $CaCl_2$ and 0.05 mM NaH_2PO_4. The pH is adjusted to 7.9 - 8.1. Stirring is accomplished by 1 cm teflon-covered stirring bars which are rotated by externally mounted 600 RPM motors fitted with small permanent magnets.

The skin potential is determined using Beckman calomel sensing electrodes (fiber type no. 241) mounted outside the chambers in small beakers. Electrical contact is made with the chambers by agar bridges, the tips of which are positioned so that they closely approximate the skin surface. The beakers and agar bridges contain Ringer's of the same composition as used in the chambers, thereby avoiding the creation of junction potentials. Agar bridges are prepared by dissolving 3 gm agar in 100 ml Ringer's solution with warming. The agar is then drawn by suction into polyethylene tubing (P.E. 205), allowed to cool, and the filled tubing is cut into appropriate lengths and checked for the presence of air bubbles which would disrupt electrical continuity.

All tracer experiments are performed with the skin "clamped" or "short-circuited" at zero potential; that is, the normal

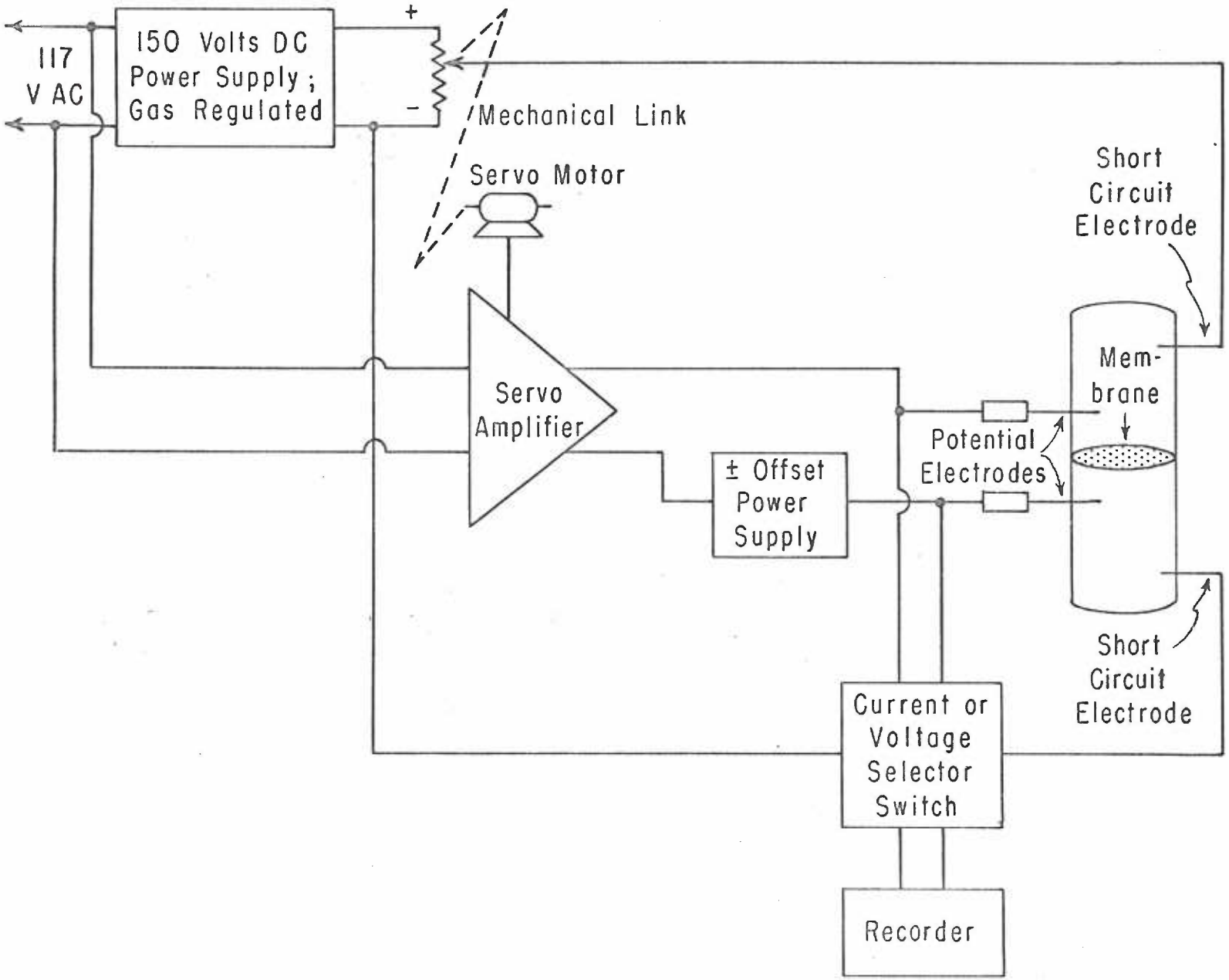
trans-skin potential is driven to and held at zero. This is easily accomplished automatically by a voltage clamp assembly designed and built here. Figure 1 presents a schematic diagram for the voltage clamp assembly. The clamp is a typical null-seeking servo amplifier which responds to the existence of a skin potential by passing sufficient current through the system to negate it. Current enters the chambers via agar bridges similar to those described above but placed as far away from the skin as possible. The other ends of the two current bridges are placed in small vials of Ringer's solution each containing a coil of silver wire which is in turn connected through standard leads to the output side of the voltage clamp. In this way the electrode reactions associated with the passage of current through the system take place outside the chambers in which the skin is mounted and there is little chance for the products of the electrode reactions to alter the experimental conditions. The entire assembly is, for the most part, patterned after that originally described by Ussing and Zerahn (26).

Most experiments consisted in measuring the movement (flux) of a tracer molecule across the skin before and after the addition of a hyperosmotic agent to the outer bathing solution. The general format of such an experiment is as follows. After mounting of the skin, Ringer's solution is added to both sides. Then to the appropriate side there is added a small amount of the radioactive substance to be studied and one hour allowed for equilibration. At the end of one hour

Figure 1

Schematic Diagram for the Voltage Clamp Assembly

Figure 1



SHORT CIRCUIT ASSEMBLY

an aliquot is removed from the opposite side by micropipette or syringe and the same amount of non-labeled Ringer's is added so that the volume of the chamber remains constant. At regular intervals following this (usually thirty minutes) aliquots are removed and replaced with non-labeled Ringer's. Following removal of the fourth sample the hyperosmotic agent is added to the outer bathing solution and sampling is continued for a final three periods.

The radioactivity of the samples is determined on either a Nuclear-Chicago, low background, gas flow counter or a Packard Tri-Carb liquid scintillation spectrometer (3000 series). Samples are prepared for the low background counter by evaporation of the aliquot in shallow, 1.25 inch diameter, stainless steel planchets filled with lens tissue discs to insure an even distribution of the material. Samples are prepared for scintillation counting by addition of the aliquot directly into 10 ml of scintillation fluid contained in special glass vials. The composition of the fluid is 66.7% toluene and 33.3% absolute ethanol, with 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP). The 10 ml of fluid is capable of holding aliquots of up to 0.3 ml Ringer's without separation of phases.

In a large percentage of the experiments the fluxes of two molecular species are determined simultaneously, one being labeled with C^{14}, and the other with H^{3}. The energy spectra of these isotopes are easily separable on the two channels of the liquid

scintillation spectrometer. This type of experiment is extremely valuable in comparing the effect of a hyperosmotic agent on two different molecules since it minimizes the problem of the biological variability between skins.

Although what is actually determined in these experiments is the tracer permeability coefficient, all results are expressed as if the tracer molecules were present at a concentration of 1 mM. This assumes that the movement of a tracer molecule under study is simple diffusion, that is, directly related to concentration. It is also assumed that a 1 mM concentration of the molecule has no effect on the permeability of the membrane itself. Several experiments were actually run with the concentration of the molecular species at 1 mM on both sides of the membrane, and no difference could be seen in the tracer permeability coefficient. Thus, the assumptions appear to be valid up to a concentration of 1 mM.

The various agents used to produce hyperosmolarity of the outer bathing solution were dimethyl sulfoxide (DMSO), erythritol, thiourea and raffinose. All agents were used in amounts equimolar to 2.5% DMSO. This particular level was chosen because DMSO was the first agent to be used, and it was at this level that definite changes in potential and short-circuit current were observed. The osmolality of normal Ringer's solution is 215 mOsm/l, whereas that of 2.5% DMSO in Ringer's is 575 mOsm/l as measured on a Fiske osmometer.

The osmolalities of the other hyperosmotic agents in Ringer's are only slightly different from that of DM30 (±8%).

Several experiments were run to determine the volume of net water flow produced by the presence of the hyperosmotic agent in the outer bathing solution. The apparatus used is shown in Figure 2. The skin is clamped between plastic chambers A and B, a seal being made by a 40 mm diameter O ring. The 11 mm flat face of the rear of the chambers permits stirring by a teflon coated bar actuated by a magnet attached to a small 600 RPM synchronous clock motor mounted outside. A 1/16 inch nylon plate perforated by twenty 4 mm holes and faced with a fine plastic screen is supported by a shoulder in chamber B. This plate permits good diffusion of solution when the skin is standing free during operational periods, yet presents a rigid support for the skin during the time of the reading of volume changes. The leveling bulb is connected to A and permits the imposition of a 7 cm head of water to force the skin against the support plate and thus allows volume changes in chamber B to be made with the skin in a fixed, reproducible position. The readout part of the assembly consists of a capillary outlet from B, a standpipe with bulb and reference point, and a terminal capillary ball joint which receives a modified micropipet-buret assembly with digital readout (A. H. Thomas 2464-U10). Each scale division on the digital readout is equal to 0.2 µl.

After mounting the skin, with the outer surface facing B, the chambers, side arms, buret and leveling bulb are filled with Ringer's

Figure 2

Apparatus Used for the Determination of Net Water Flux

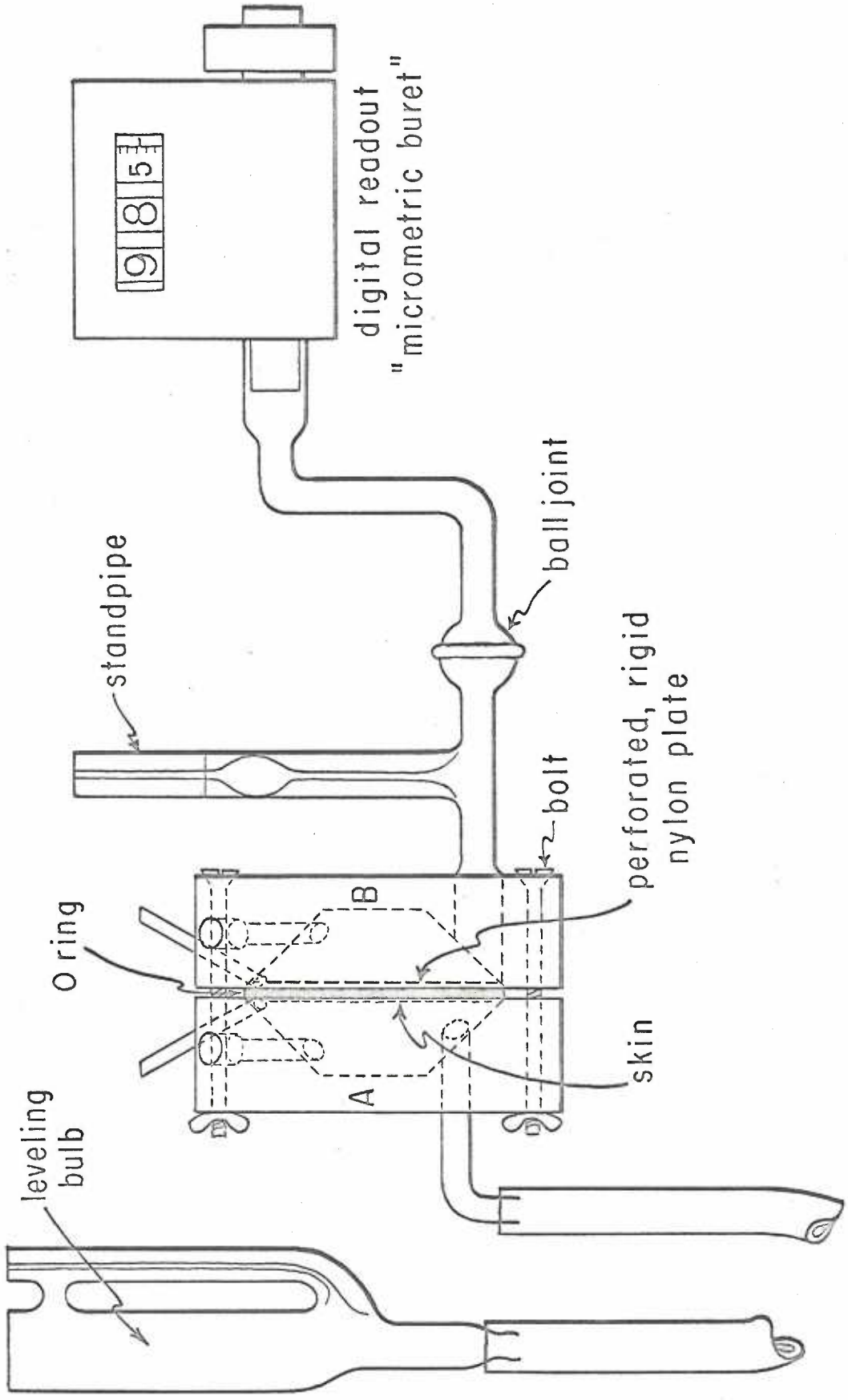

digital readout
"micrometric buret"
9185
standpipe
ball joint
bolt
perforated, rigid
nylon plate
B
O ring
skin
A
leveling
bulb

Figure 2

so that a position corresponding to a 7 cm head in the bulb causes fluid to rise in the standpipe to the reference point in the capillary. The 7 cm head is then withdrawn and fluid in the capillary is lowered into the bulb portion of the standpipe. This puts the fluid level in the bulb and standpipe of the apparatus at the same level as it exists in chambers A and B. The experiments are run in this condition. Only at the time of reading is the 7 cm head imposed and fluid in the capillary returned to the reference point. The sensitivity of this apparatus is ±2 µl. The area of skin used is 7 cm^2, and the volume of each chamber is 4.25 ml.

Several experiments were run to determine the total water content and extracellular water content of the skin in normal and hyperosmotic Ringer's containing radioactive mannitol. In these experiments the skin is equilibrated for one and one-half hours in the appropriate solution. Following this it is carefully blotted on filter paper, put in tared 10 ml volumetric flasks, weighed and dried for two hours at 110° C. Then after cooling in a dessicator, the flask is reweighed. The difference between the two weighings gives the water content of the skin. The size of the extracellular water compartment is determined from the amount of radioactive mannitol in the skin. Following the last weighing, two to three milliliters of 2 N NaOH are added to the flask and the skin is dissolved with warming. After diluting to volume with water, aliquots are taken for radioassay. It is assumed that mannitol

reaches the same concentration in skin water as exists in the bathing solution. Since mannitol is too large to penetrate cell membranes, for example see Page (21), the volume of skin water it equilibrates with is the extracellular space.

III. RESULTS

The presence of 2.5% DMSO in the outer bathing solution always results in an immediate decrease in potential across the skin. The effect on short-circuit current is variable, and is related to the season of the year. Generally an increase in short-circuit current is observed. However, during the winter season when short-circuit currents are low, DMSO causes a decrease in current. Since the normal trans-skin potential is the resultant of two factors, net sodium transport and chloride permeability, it is obvious that a decrease in potential associated with an increase in short-circuit current might result from an increase in chloride permeability. Actual measurements of chloride movement through the skin using the isotope Cl^{36} showed it to be increased some 2-4 times with 2.5% DMSO in the outer bathing solution.

In order to further define the effect of DMSO on skin permeability, it was decided to follow the flux of non-electrolytes as was done for Cl^{-}. This data is presented in Table I with the non-electrolytes listed in decreasing order of permeability, thiourea being the most permeable and sucrose the least permeable. The values given under influx and outflux represent the mean and standard error of the mean of three to eight experiments on each non-electrolyte. The values listed under increase are determined by dividing the means of the treated by the means of the control group. The flux ratio

Table I

Effect of 2.5% DMSO on Frog Skin Permeability.

Non-Electrolyte	Condition	Influx*	Increase	Outflux*	Increase	Influx / Outflux	Difference
Thiourea	Control	7.0 ±1.0	7.2	8.2 ±1.0	5.1	0.9	$P > 0.05$
	Treated	50.7 ±15.8		41.6 ±9.5		1.2	$P > 0.05$
Urea	Control	4.3 ±0.8	16.7	3.2 ±0.7	8.7	1.3	$P > 0.05$
	Treated	71.9 ±14.0		27.7 ±7.0		2.6	$P < 0.05$
Mannitol	Control	0.7 ±0.1	75.0	0.8 ±0.4	11.5	0.9	$P > 0.05$
	Treated	52.5 ±11.9		9.2 ±2.2		5.7	$P < 0.05$
Sucrose	Control	0.3 ±0.1	122.0	0.6 ±0.2	11.3	0.5	$P > 0.05$
	Treated	36.6 ±9.1		6.8 ±1.6		5.4	$P < 0.01$

*μMoles·hr^{-1} x 10^3. Area of skin is 6.3 cm^2.

(influx/outflux) is calculated by dividing either the control or the treated influx by the appropriate outflux.

It can be seen that, for the most part, the control flux ratios of the compounds listed approach one, as is to be expected for passively diffusing molecules under these conditions. No significant difference between influx and outflux exists for any of the four, although the ratio of 0.5 seen with sucrose appears rather low. This is due to the wide variability observed in that particular series of outfluxes. Under the influence of DMSO, grossly different flux values are observed. Although both influx and outflux are greatly increased over the control values with all four non-electrolytes, a much greater increase in influx than outflux is observed with sucrose, mannitol and urea. The end result is a flux ratio significantly different than one. This same tendency is also seen with thiourea, although the difference lacks statistical significance.

The above results indicate that DMSO has a dramatic effect on frog skin permeability. That the results are secondary to hyperosmolarity of the outer bathing solution rather than specific for DMSO is demonstrated in Table II. In these experiments two other compounds, thiourea and erythritol, were added to the outer bathing solution in amounts equimolar to that used in the DMSO experiments. The results are compared with those of DMSO, the DMSO figures having been cited in Table I.

Although there are certainly differences to be noted between the effects of the three hyperosmotic agents, for the most part they

Table II

Effect of Hyperosmotic Agents
in Outer Bathing Solution.

Non-Electrolyte	Hyperosmotic Agent	Influx Increase	Outflux Increase	Influx / Outflux
Thiourea	Erythritol	7.9	10.4	0.8
	DMSO	7.2	5.1	1.2
Urea	Thiourea	28.3	14.1	2.6
	DMSO	16.7	8.7	2.6
Mannitol	Erythritol	19.1	8.5	2.2
	DMSO	75.0	11.5	5.7
Sucrose	Thiourea	141.5	8.4	2.9
	Erythritol	62.4	11.1	2.4
	DMSO	122.0	11.3	5.4

are quite similar in that they all produce large increases in both influx and outflux. The one common feature, a generalized increase in skin permeability to these four non-electrolytes, will be taken to be the result of hyperosmolarity of the outer bathing solution. Differences between the agents will be discussed later.

Although the effects of the agents as reported in Table II are marked, the demonstration that in certain cases a flux ratio significantly greater than one is produced, is of great interest. The rate of passive movement of a molecule through the skin should be the same in either direction, yielding a flux ratio of one. A value significantly different than one means that net movement in one direction is occurring; or in other words the movement of the molecule across the skin in one direction is greater than that in the other direction. This requires the use of some force and the expenditure of work. Since all fluxes have been unitized with respect to concentration a concentration gradient cannot be the origin of the force. Nor can a gradient of potential be considered since we are dealing only with non-electrolytes. Ussing and Anderson (27) have shown that a net flux of water may, by solvent drag, account for a flux ratio other than one. They demonstrated that a bulk flow of water across a membrane, produced by a difference in osmotic pressure, can speed the movement of molecules going in the same direction, and at the same time slow the movement of molecules going in the opposite direction. In the experiments reported above a large osmotic

gradient certainly exists across the skin and a net outward movement of water is therefore to be expected; but this, if solvent drag is to be implicated, should produce a flux ratio (influx/outflux) of less than one, for outflux would be increased over influx by the drag force. Thus, it does not appear that solvent drag can account for these findings, since net water movement is an outflux and net movement of the non-electrolyte is an influx.

The magnitude of the net water outflux in the above experiments is significant. This was measured in separate experiments and the data is presented in Table III. Under normal conditions, when an osmotic gradient does not exist across the skin, there is a small influx of approximately 30 $\mu l \cdot hr^{-1}$ (4.3 $\mu l\ cm^{-2} hr^{-1}$). This is probably associated with the active transport of sodium. However, with the imposition of the osmotic gradient from inside to outside as in the experiments reported above in Tables I and II, the normal influx is reversed and an outflux of greater than 210 $\mu l \cdot hr^{-1}$ is observed. With an outflux of water of this magnitude it is surprising that a net influx of solute could take place at all.

Since the net movement of water is in the wrong direction to explain the observed flux ratios, it is necessary to consider the net movement of the hyperosmotic agent itself. A large gradient exists across the skin for the hyperosmotic agent and the direction for its movement is an influx. If any interaction should occur between the hyperosmotic agent and the tracer material, then as the

Table III

Effect of Hyperosmotic Agents
on Net Water Flow.

Hyperosmotic Agent	Flux ($\mu l \cdot hr^{-1}$)
None	-30*
DMSO	227
Erythritol	210
Thiourea	217
Raffinose	217

*The minus sign indicates an influx.

hyperosmotic agent moves down its large gradient, an increase of tracer influx might be expected. This hypothesis of solute interaction is easily tested since the crucial point in it is that the hyperosmotic agent itself be permeable. If one were to use a non-permeable molecule as the hyperosmotic agent there could be no interaction between the fluxes of hyperosmotic agent and non-electrolyte, and there should be no change in the flux ratio.

The three hyperosmotic agents used to this point are all readily permeable in frog skin as is shown in Table IV. The sucrose permeability coefficient is shown for comparison. Since the permeability of the sucrose molecule in frog skin is very low, any less permeable solute should suffice for a non-permeable hyperosmotic agent. Raffinose, being a larger molecule than sucrose, was chosen and was used in amounts equimolar to 2.5% DMSO. The results of its action on the fluxes of urea and sucrose are seen in Table V.

It can be seen that the effect of raffinose on skin permeability is much less in comparison to DMSO. It is also much less than the changes produced by erythritol and thiourea as shown in Table II. But, as predicted, raffinose does not alter the flux ratio. The ratio is essentially one for both urea and sucrose. The urea outflux appears to be increased more than the influx due to the combination of slight non-significant differences in both the control and treated values. It should be kept in mind that the flux ratio is calculated from the means of the treated values, not the numbers giving the increase over control values as listed in the table.

Table IV

Permeability Coefficients of
Various Solutes in Frog Skin.

Solute	K ($cm \cdot hr^{-1}$)
DMSO	69.0×10^{-4}
Thiourea	11.1×10^{-4}
Urea	6.8×10^{-4}
Erythritol	3.9×10^{-4}
Mannitol	1.1×10^{-4}
Sucrose	0.4×10^{-4}

Table V

Effect of Hyperosmotic Raffinose
in Outer Bathing Solution.

Non-Electrolyte	Hyperosmotic Agent	Influx Increase	Outflux Increase	Influx / Outflux	Difference
Urea	Raffinose	2.1	4.5	0.8	$P > 0.05$
	DMSO	16.7	8.7	2.6	$P < 0.05$
Sucrose	Raffinose	3.5	3.8	1.1	$P > 0.05$
	DMSO	122.0	11.3	5.4	$P < 0.01$

In order to learn more about the nature and site of action of the hyperosmotic agents it is necessary to investigate the changes that are occurring in the skin itself. It must be remembered that the flux experiments reported above do not measure simply the movement of a tracer from one bathing solution across a single membrane to the other bathing solution. The skin is a thick, complex piece of tissue which should more properly be treated as a third compartment as done by Morel (19). From this standpoint every flux can be broken down into two components, the movement into and the movement out of the skin. A simple flux experiment as done above does not enable one to learn anything of the two separate steps involved. However, a study of the labeling of the skin itself from either bathing solution should define these two steps and disclose changes brought about by hyperosmolarity of the outer bathing solution. Since the most pronounced changes in influx are noted with sucrose, this molecule was chosen as the one to study. First, the magnitude of skin labeling by C^{14} sucrose from the inner and outer bathing solutions under normal conditions was determined. Then similar experiments were performed with 2.5% DMSO in the outer bathing solution. The results are presented in Table VI and are expressed in terms of that fraction of the total counts present in the inner or outer bathing solution which is found in the skin after a 1.5 hour equilibration time. This time period is more than enough for a steady state to be reached between the skin and the labeled bathing solution with respect to the concentration of label in the skin.

Table VI

Effect of Hyperosmotic DMSO
on Sucrose-C^{14} Labeling of Skin.

	Control	Treated	Difference
From Outside	3.7×10^{-2}%	12.6×10^{-2}%	$P < 0.01$
From Inside	92.9×10^{-2}%	67.1×10^{-2}%	$P < 0.05$

Table VII

Effect of Hyperosmotic DMSO
on Skin Water Distribution.

	Control*	Treated*	Difference
Total H_2O	120.3 µl	108.7 µl	$P > 0.05$
Extracellular H_2O	55.9 µl	57.5 µl	$P > 0.05$
Intracellular H_2O	64.4 µl	51.2 µl	-

*Area = 5.64 cm^2.

Another aspect of the skin which would be expected to change under the influence of hyperosmolarity is the water content. A certain amount of dehydration and a possible change in the ratio of intracellular to extracellular water might occur. In order to determine the magnitude of changes in water content and distribution produced by outer bathing solution hyperosmolarity a study of total skin water content and extracellular water content was undertaken. The results are to be seen in Table VII. The values for intracellular water were obtained by subtracting the mean values given for extracellular water from the mean values given for total water.

Due to the great variability in the results from the determination of total skin water, the difference between the means of the control and treated groups lacked significance at the 95% confidence level. However, it was significant at the 90% confidence level. This difference is taken to be significant in view of the recent report by Ussing (25) who, under direct microscopic observation, noted a decrease in the thickness of the epithelial cells of frog skin under the influence of outer bathing solution hyperosmolarity. The results cited above lead to the same conclusion, with the decrease in total skin water being due to a loss of water from the cells. No changes occur in the size of the extracellular space.

IV. DISCUSSION

It is quite obvious from the data presented in the previous section that hyperosmolarity of the outer bathing solution produces a marked decrease in the resistance of the skin to the passage of non-electrolytes. However, hyperosmolarity cannot explain all the data since equimolar concentrations of different compounds do not give the same results. Likewise, the small differences in osmolality between the four hyperosmotic solutions (±8%) is not sufficient to account for the observed differences in flux. Similar results were reported by Lindley, Hoshiko and Leb (16) in their study on changes in skin potential and resistance produced by hyperosmotic agents. They found that, although there is a linear relationship between the decrease in skin potential and the concentration of the hyperosmotic agent in the outer bathing solution, different agents at the same concentration do not produce the same decrease in potential. Another similarity between their results and those reported here is that raffinose was found to be the least effective of the hyperosmotic agents.

Of prime importance in trying to elucidate the mechanism of action of the hyperosmotic agents is the localization of the skin structure that is being altered. The work of Koefford-Johnson and Ussing (12) pointed out the existence of at least two membranes in the skin. Other work has led to the conclusion that it is the membrane closest to the outer bathing solution (outer membrane) which offers the

major resistance to movement through the skin. MacRobbie and Ussing (17) demonstrated that the outer membrane is twenty times less permeable to water than is the inner membrane. Also, experiments designed to study the movement of tracers out of the skin, so-called "washout" experiments, have demonstrated that the rate of movement of tracer is always greater to the inner bathing solution than to the outer bathing solution.

If the outer membrane is indeed the rate limiting structure in the skin, then it is likely to be the structure which is altered by hyperosmotic agents. This hypothesis seems to be the most reasonable in light of the skin labeling experiments reported above. When skin labeling was performed with the tracer in the outer bathing solution the addition of a hyperosmotic agent resulted in more than a three-fold increase in the steady state concentration of label found in the skin indicating that the rate of entry of the tracer through the outer membrane into the skin had been increased. When labeling was performed with the tracer in the inner bathing solution the addition of a hyperosmotic agent resulted in a decrease of the steady state concentration of label in the skin. In this case the rate of exit, through the outer membrane, had been increased. Thus the experiments on skin labeling can be easily explained on the basis of increased outer membrane permeability.

The greater labeling of the skins by the inner bathing solution may be due to the presence of the large connective tissue layer on

the inner surface of the skin. The water phase of this layer readily equilibrates with the inner bathing solution. It is difficult to quantitate the effect of this layer but even if the effect is minimal, the greater permeability of the inner membrane would remain as a factor in the labeling of the skin.

It is also possible that the hyperosmotic agents could be affecting the permeability of the inner membrane in addition to that of the outer membrane. In fact, the change in solute permeability could be either a decrease or an increase and not alter the interpretation of the skin labeling data. The critical factor here is the magnitude of the change at the outer membrane. As pointed out by Morel (19), once the steady state has been reached the concentration of a tracer in the skin is determined only by the ratio of the permeabilities of the two limiting membranes and the concentration of tracer in the bathing solution. This may be expressed as:

$$\frac{C_{skin}}{C_{out\ sol}} = \frac{f_{om}}{f_{om} + f_{im}} \quad \text{and} \quad \frac{C_{skin}}{C_{in\ sol}} = \frac{f_{im}}{f_{im} + f_{om}}$$

where C_{skin}, $C_{out\ sol}$, and $C_{in\ sol}$ are the concentrations of tracer in the skin, outer bathing solution and inner bathing solution respectively and f_{om} and f_{im} are the respective fluxes across the outer and inner membranes. From these equations it is more easily seen how the permeability of the inner membrane could vary in either direction and still be compatible with the observed skin labeling results. It all depends on the magnitude of the increase at the outer membrane.

Evidence in favor of the hyperosmotic agents also increasing the permeability of the inner membrane comes from the flux data in Table I. The sucrose influx increased 122 times with hyperosmotic DMSO in the outer bathing solution. The increase in skin labeling of 3.5 fold (Table VI) should result in only a 3.5 fold increase in the influx since flux is directly proportional to concentration. Since the size of the extracellular space did not change under the influence of DMSO (Table VII) the observed 3.5 fold increase in skin labeling actually represents a similar increase in tracer concentration in the skin. Thus, in order to account for a 122 fold increase in sucrose influx, with only a 3.5 fold increase in the concentration of sucrose in the skin, an increase in the permeability of the inner membrane must be postulated. This need not necessarily be a change in the structure of the membrane itself, but may represent the work of an outside force, that same force which produces the flux ratio for sucrose of 5.4. It is not difficult to envision how a hyperosmotic agent could alter the physical characteristics of a membrane when the two are in close contact, but it is not easy to see how this change could occur when separated by other structures including another membrane. Thus it seems likely that hyperosmotic agents in the outer bathing solution might directly alter the physical nature of the outer barrier, but it seems less likely that they could have this same effect on the inner membrane, since their concentration is not the same in the skin as it is in the outer bathing solution. On this basis it seems reasonable to postulate

the inner membrane as the site of action of an unknown force which is producing the high flux ratios.

The exact nature of the force at work in the skin which is causing influx to exceed outflux is not at all clear. That it may somehow be related to the movement through the skin of the hyperosmotic agent itself is suggested by the data in Table V where raffinose is used as the hyperosmotic agent. Raffinose is the only agent used in which the flux ratio remains one and it is the only agent used that does not penetrate the skin. One way to explain these findings is to assume that there is some interaction between the movement of the hyperosmotic agent as it moves through the skin, down its concentration gradient, and the movement of the tracer non-electrolytes, since they both move in the same direction. This interaction may be of a frictional nature as suggested by Ussing and Andersen for solvent drag (27). If that is the case, the greatest interaction should occur with the larger molecules since they offer the largest area for frictional interaction. Since the larger molecules, sucrose and mannitol, showed a greater change in flux ratio than did the smaller molecules, thiourea and urea, the postulated system of interaction must be seriously considered.

However, another explanation may be offered for the greater effect seen with sucrose and mannitol. Trans-skin movement of both of these molecules is confined entirely to the extracellular spaces. The fact that their flux ratios are altered to the greatest extent

may be taken as evidence that the unknown force is at work mostly, or entirely, in the extracellular spaces and that its greatest effect is on that fraction of a molecule's flux which travels by way of the extracellular pathway. Since 100% of the flux of the mannitol and sucrose is through the extracellular route the greatest change in flux ratio should occur with these two molecules. It seems reasonable to assume that as a molecule becomes more permeable in frog skin, that fraction of its flux which is extracellular will become smaller for another pathway is available for its movement, the intracellular route. Although the total amount of a tracer (more permeable than mannitol) going through the extracellular space would be increased, when expressed as the fraction of the total flux, it would decrease. As one studies the flux of the more permeable molecules, a greater amount of flux will be found to go through the intracellular pathway and the fractional flux through the extracellular pathway will decrease even more, in spite of the fact that the absolute amount going through the extracellular pathway is increasing. If these suggestions are true, there should result an inverse relationship between the permeability of a molecule in frog skin and the change in flux ratios produced by the hyperosmotic agents. A review of the data in Table I shows this to be true with the four non-electrolytes used.

Thus, there are at least two hypotheses which can explain why the more permeable molecules are affected the least by the hyperosmotic agents. In fact, there is no reason why both theories may

not be true. This would imply that the interaction is limited to the extracellular spaces, is frictional in nature, and depends on the size and permeability of the non-electrolytes.

From the above discussion, it is also possible to speculate as to why differences exist among the hyperosmotic agents. If interaction in the extracellular spaces is the correct answer, then the important thing in relation to the hyperosmotic agents would be the actual amount going through the extracellular pathway, not the fraction. Thus there should be a direct relationship between the permeability of the hyperosmotic agents and the effect on flux ratios. The data presented in Tables II and V bear out this theory but are not entirely consistent. Certainly raffinose, the least permeable, has the least effect. But thiourea, which is much less permeable than DMSO, seems to be as effective as DMSO. Erythritol seems to fit, in that it is less permeable than thiourea and DMSO and its effect is also less. It is also more permeable than raffinose, and has a greater effect than raffinose. The fact that the data is not entirely consistent with the hypothesis leads one to suspect that all parameters have not been considered. Perhaps, in addition to a frictional interaction, there may also be some chemical interaction. With only the limited number of hyperosmotic agents used this point cannot be explored further.

It is also difficult to speculate on the exact nature of the alteration produced in the outer membrane by the hyperosmotic agents. Since all four agents, including raffinose, increase the permeability

of the outer membrane, the permeability of the hyperosmotic agent itself does not seem to be the key factor here. Two observations would lead one to believe that the effect is of a physical nature. One is the large amount of the hyperosmotic agent required to produce this effect, the tonicity of the bathing solution being more than doubled. The other observation is that the effect is readily reversible upon removal of the agents from the bathing solution. (Data on the reversibility of the hyperosmotic phenomenon are a part of a continuing study and are not presented in this thesis.) Both of these observations make it appear as if the altered membrane permeability is produced "simply" by the presence of the agents in high concentrations. The mechanism may be due to dehydration of the membrane itself or of selective areas of the membrane, a change in the physical nature of water at or in the membrane, or to an alteration in the configuration of lipids or proteins in the membrane itself. The data presented do not allow one to speculate further.

V. SUMMARY AND CONCLUSIONS

This thesis reports on the effect of outer bathing solution hyperosmolarity on the permeability of frog skin to various non-electrolytes. The data show that large increases in skin permeability are produced by hyperosmotic agents, and appear to be explainable on the basis of increased permeability of the outer membrane. The movement of the hyperosmotic agent itself through the skin may also be a factor in the increased permeability since the passage of urea, mannitol and sucrose through the skin is greater in the inward direction than in the outward direction. Because the movement of the hyperosmotic agent through the skin is also in the inward direction, it is suggested that its flux can affect the movement of the tracer non-electrolytes, possibly by frictional interaction. This hypothesis seems even more likely in view of the fact that raffinose, an impermeable hyperosmotic agent, is unable to produce altered flux ratios in contrast to the other hyperosmotic agents. The exact mechanism of action of these agents is not clear at this time, and further investigation is presently underway.

REFERENCES

1. Curran, P. F. and Solomon, A. K. Ion and water fluxes in the Ileum of rats. J. Gen. Physiol., 1957. 41, 143-168.

2. Curran, P. F. and Schwartz, G. F. Sodium, chloride, and water transport by rat colon. J. Gen. Physiol., 1960. 43, 555-571.

3. Dean, R. B. Theories of electrolyte equilibrium in muscle. Biol. Symp., 1941. 3, 331-348.

4. Diamond, J. M. Transport of salt and water in rabbit and guinea pig gall bladder. J. Gen. Physiol., 1964. 48, 1-14.

5. du Bois-Reymond, E. Untersuchungen uber die thierische electrizitat. Berlin: G. Reimer, 1857.

6. Engbaek, L. and Hoshiko, T. Electrical potential gradients through frog skin. Acta. Physiol. Scand., 1957. 39, 348-355.

7. Galeotti, G. Uber die elektromotorischen krafte welche an der oberflache tierischer membrane bei der beruhung nit verschiedenen electrolyten zustande kommen. Zeitschr. Physik. Chem., 1904. 49, 542-562.

8. Huf, E. G. Versuch uber den zusammenhang swischen stoffwechsel, potentialbildung und function der froschaut. Pflug. Arch. ges. Physiol., 1935. 235, 655-673.

9. Huf, E. G. and Wills, J. Influence of some inorganic cations on active salt and water by isolated frog skin. Am. J. Physiol., 1951. 167, 255-260.

10. Katzin, L. I. The use of radioactive tracers in the determination of irreciprocal permeability of biological membranes. Biol. Bull., 1940. 79, 342.

11. Koefford-Johnsen, V., Levi, H., and Ussing, H. H. The mode of passage of chloride ions through the isolated frog skin. Acta. Physiol. Scand., 1952. 25, 150-163.

12. Koefford-Johnsen, V., and Ussing, H. H. The nature of the frog skin potential. Acta. Physiol. Scand., 1958. 42, 298-308.

13. Krogh, A. Osmotic regulation in the frog. Skand. Arch. Physiol., 1937. 76, 60-74.

14. Krogh, A. The active adsorption of ions in some freshwater animals. Zeitschr. Vergleich. Physiol., 1938. 25, 335-350.

15. Leaf, A., Anderson, J., and Page, L. B. Active sodium transport by the isolated toad bladder. J. Gen. Physiol., 1958. 41, 657-668.

16. Lindley, B. D., Hoshiko, T., and Leb, D. E. Effects of D_2O and osmotic gradients on potential and resistance of the isolated frog skin. J. Gen. Physiol., 1964. 47, 773-793.

17. MacRobbie, E. A. C., and Ussing, H. H. Osmotic behavior of the epithelial cells of frog skin. Acta Physiol. Scand., 1961. 53, 348-365.

18. Meyer, K. H., and Bernfeld, P. The potentiometric analysis of membrane structure and its application to living animal membranes. J. Gen. Physiol., 1946. 29, 353-378.

19. Morel, F. In The method of isotopic tracers applied to the study of active ion transport. New York: Pergamon Press, 1959. pp. 155-184.

20. Ogilvie, J. T., McIntosh, J. R., and Curran, P. F. Volume flow in a series membrane system. Biochim. Biophys. Acta, 1963. 66, 441-444.

21. Page, E. J. Cat heart muscle in vitro. J. Gen. Physiol., 1962. 46, 201-213.

22. Patlak, C. S., Goldstein, D. A., and Hoffman, J. F. The flow of solute and solvent across a two-membrane system. J. Theoret. Biol., 1963. 5, 426-442.

23. Ussing, H. H. The use of tracers in the study of active ion transport across animal membranes. Cold Spring Harbor Symp., 1948. 13, 193-200.

24. Ussing, H. H. The distinction by means of tracers between active transport and diffusion. The transfer of iodide across the isolated frog skin. Acta Physiol. Scand., 1949. 19, 43-56.

25. Ussing, H. H. Relationship between osmotic reactions and active sodium transport in the frog skin epithelium. Acta Physiol. Scand., 1965. 63, 141-155.

26. Ussing, H. H. and Zerahn, K. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol. Scand., 1951. 23, 110-127.

27. Ussing, H. H. and Andersen, B. The relation between solvent drag and active transport of ions. In Liebecq, C. (Ed.) Proceedings of the Third International Congress of Biochemistry. New York: Academic Press Inc., 1956. pp. 434-440.

APPENDIX

The following is the data from an actual experiment and will serve to illustrate the method of calculation of all flux experiments.

Thiourea Outflux (70-131)

Time (min)	cpm	x^{45}	x^{46}	Δcpm	$\frac{\mu Moles}{20\ min} \times 10^3$	$\frac{\mu Moles}{hr} \times 10^3$
0	69.9	3,145.5	-	-	-	-
20	94.9	4,270.5	4,365.4	1,219.9	1.35	4.1
40	129.5	5,827.5	5,957.0	1,686.5	1.87	5.6
60	158.8	7,140.0	7,304.8	1,477.3	1.64	4.9
			DMSO Added			
80	222.4	10,008.0	10,230.4	3,084.4	3.42	10.3
100	363.1	16,339.5	16,702.6	6,694.6	7.44	22.3
120	525.8	-	24,186.8	7,847.3	8.72	26.2

The time of the first sample is arbitrarily referred to as time zero, with all the following samples being taken at twenty-minute intervals. The column headed cpm (counts per minute) gives the radioassay of each sample. Since the size of the sample was 0.25 ml, and the total chamber volume was 11.5 ml, the values listed under cpm must be multiplied by 46 in order to obtain the total number of counts in the chamber at the time of sampling. To obtain the net number of counts which have come across the skin in one twenty-minute period, the number of counts which were present in the chamber at the start of that period must be subtracted from

the number of counts present at the end of the period. Although the column under 46 gives the total number of counts present at the end of the period, the column under 45 gives the total number of counts left in the chamber following sampling to be carried into the next period. The number 45 is obtained by dividing the total chamber volume minus the size of the sample (11.5 ml-0.25 ml) by the size of the sample (0.25 ml). Thus, the number listed under 45 for one sample time subtracted from the number under 46 for the next sample time, gives the net counts which came across the skin for the intervening twenty-minute period. This value is listed in the next column, Δcpm. To convert counts into μMoles, the value listed under Δcpm is divided by the specific activity of the opposite bathing solution ($\frac{\text{cpm}}{\mu\text{Mole}}$). In this experiment, the specific activity was 899,575 $\frac{\text{cpm}}{\mu\text{Mole}}$