Effects of D_2O and Osmotic

Gradients on Potential and Resistance

of the Isolated Frog Skin

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ABSTRACT Exposure of the outside surface of isolated frog skin (*R. pipiens* and *R. catesbeiana*) to sulfate solution made up with D_2O decreased skin potential and resistance. Exposure of the inside surface to D_2O solution decreased the potential slightly but increased the resistance. The changes were linearly related to the D_2O concentration. Since D_2O acts like a hyperosmotic solution, the skin potential and resistance were studied upon exposure to solution made hyperosmotic by addition of sucrose, mannitol, acetamide, urea, thiourea, Na_2SO_4, or K_2SO_4. Skin potential and resistance decreased when the outside solution was made hyperosmotic. The changes depended upon the concentration and the nature of the solute. Thiourea and urea solutions were the most effective. Treatment of the inside surface gave relatively small decreases in potential; the resistance either increased or remained unchanged. These effects appeared to depend upon the direction of the osmotic gradient across the skin rather than upon the value of the osmolarity compared to normal body fluids. Experiments with a series of six polyhydric alcohols from methanol to mannitol and the polysaccharides, sucrose and raffinose, showed adonitol with 5 carbons to decrease the potential the most. Smaller and larger compounds of this set gave lesser effects. As yet no consistent explanation of the effects is forthcoming, but their demonstration calls for caution in the indiscriminate use of solutes such as mannitol or sucrose "to make up the osmolality" and in the neglect of urea because "it penetrates freely."

INTRODUCTION

In 1939 T. C. Barnes (1) reported experiments in which he replaced the inside and outside solutions bathing frog skin with D_2O Ringer's (chloride). He observed large decreases in potential. In our initial experiments with D_2O effects on frog skin the magnitude of the depression depended upon which side of the skin was exposed to D_2O: outside exposure gave the largest depression, inside exposure gave the least depression, and simultaneous exposure

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of both sides gave an intermediate depression. In view of the suggestion of S. C. Brooks (2) that D_2O is "hyperosmotic" to H_2O, we have studied the effects of osmotic gradients and of D_2O on the electrical behavior of frog skin. Previously, Motokawa (3) had presented data on the effects on the frog skin potential of NaCl solutions made hypertonic with addition of some non-electrolytes. MacRobbie and Ussing (4) and Whittembury (5) have reported on volume changes of the epithelial cells in response to osmotic gradients.

METHODS

All experiments were performed on pieces of abdominal, thigh, or calf skin from winter and spring bullfrogs, *Rana catesbeiana* (obtained from Lemberger's, Oshkosh, Wisconsin); in some cases identical experiments were performed on skin from large leopard frogs (*Rana pipiens*, also from Lemberger's). The skins (2 cm^2 area) were mounted in lucite chambers of the Ussing-Zerahn (6) type. Potentials were monitored through 3 M KCl-agar bridges with Philbrick P-2 differential operational amplifiers, and recorded on a speedomax G strip chart recorder. Skin resistance was estimated by passing a small constant current pulse (5 to 8 $\mu a/cm^2$) through the skin by way of $MgSO_4$-agar bridges and recording the potential deflection produced. Skins with initial potential difference below 70 mv in our regular sulfate solution were rejected. For part of the experiments including the D_2O replacements, the composition of our regular solution was 115 mEq/liter Na_2SO_4, 5 mEq/liter K_2HPO_4, pH 8.2. For other experiments, as noted below, the basic electrolyte composition was 60 mEq/liter Na, 5 mM/liter tris, pH 8.2 (the anion was sulfate in all cases). Skin potentials remained stable in the absence of added calcium. Special solutions as noted were also used. Osmolalities are given in terms of added non-electrolyte; the actual osmolalities are greater because of the electrolyte content. The sodium activities of the solutions were checked with a sodium-sensitive glass electrode (Beckman 78178V), and the osmolalities with a Fiske osmometer. All solutions at a given nominal osmolality within any single experiment had the same measured osmolality within 3 per cent. The solutions were bubbled with washed, compressed air. D_2O solutions were prepared by drying down a suitable volume of the regular solution (in a double boiler or in a flash evaporator, then in a drying oven) and taking up the residue in D_2O.

Reagents used and suppliers are: D_2O (Abbott, Bio-Rad); methanol; ethanol (Gold Shield); ethylene glycol (Eastman 133); acetamide, urea, and thiourea (Fisher certified); mannitol, glucose, K_2SO_4, $Na_2SO_4 \cdot 10H_2O$, and sucrose ("Baker analyzed"); glycerol (General Biochemicals); raffinose (Mann Research Laboratories); tris(hydroxymethyl)aminomethane (Sigma 7-9).

Changes in potential are reported as the difference between the steady-state value during the treatment and that immediately preceding the treatment. If no steady-state was reached, the potential reading was taken at a specified time after the change of solutions. Resistance measurements were made at a time corresponding to the potential value used.

In many cases a split plot experimental design was used (7). In these experiments several pieces of skin from the same frog were used.

The method of relating potential changes and resistance changes needs some justification. If we take the equivalent circuit proposed by Ussing and Zerahn (6) for the frog skin (Fig. 1),

$$V = \frac{R_s}{R_i + R_s} E \tag{1}$$

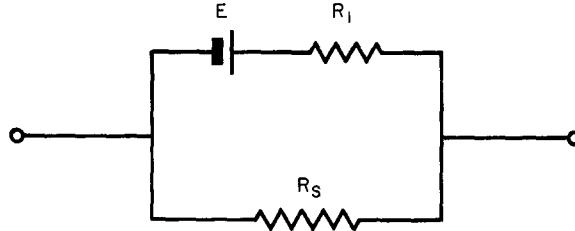when no current is drawn from the skin. V is the skin potential and E is the EMF of the skin.

FIGURE 1. Frog skin equivalent circuit of Ussing and Zerahn. E is the skin EMF, R_s is the resistance of the shunt pathway, and R_i is the internal resistance associated with the skin EMF.

If an external source of current is present, the potential deflection divided by the current gives the estimated resistance of the skin.

$$R = \frac{R_i R_s}{R_i + R_s}$$

If one then measures the skin potential and the skin resistance under control (subscript o) and experimental (subscript exp) cases,

$$\Delta V = V_{exp} - V_o = E\left[\left(\frac{R_s}{R_i + R_s}\right)_{exp} - \left(\frac{R_s}{R_i + R_s}\right)_o\right]$$

$$\Delta R = R_{exp} - R_o = \left(\frac{R_i R_s}{R_i + R_s}\right)_{exp} - \left(\frac{R_i R_s}{R_i + R_s}\right)_o$$

(assuming the EMF to be unchanged)
Then,

$$\frac{\Delta V}{V_o} = \frac{(R_s)_{exp}}{(R_s)_o} \frac{(R_i + R_s)_o}{(R_i + R_s)_{exp}} - 1$$

$$\frac{\Delta R}{R_o} = \frac{(R_i R_s)_{exp}}{(R_i R_s)_o} \frac{(R_i + R_s)_o}{(R_i + R_s)_{exp}} - 1$$

If the shunt resistance R_s changes without the internal resistance R_i changing,

$$\frac{\Delta V}{V_o} = \frac{\Delta R}{R_o}$$

Thus, if the experimental solutions increased leakage paths through the skin, a plot of $\Delta V/V_o$ vs. $\Delta R/R_o$ is a straight line of unit slope if the changes in potential are due to changed leakage resistance. This is the type of plot used to present resistance measurements.

RESULTS

1. *D_2O Replacements*

Fig. 2 shows a typical experiment following replacement of the control outside bathing solution (115 mEq/liter Na, from here on given as 115 Na)
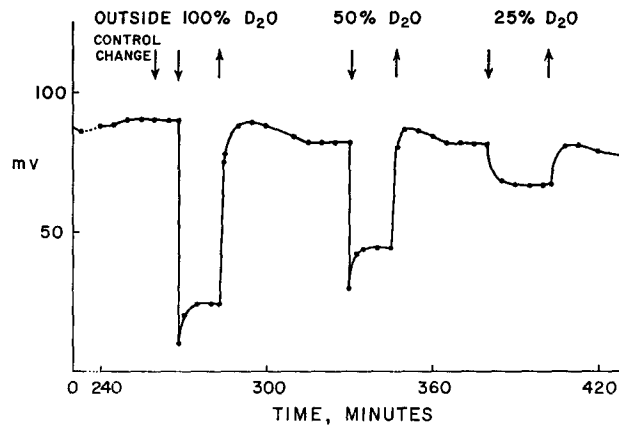

FIGURE 2. Typical experiment showing effect on bullfrog skin potential of replacement of outside bathing solution with D_2O solution. Redrawn from the original strip chart recording.

with D_2O solutions. The potential fell and reached a minimum within half a minute. The new potential, after a slight initial rise, was quite stable for long periods of time (as long as 1 hour), and the skin recovered fully and rapidly upon return to H_2O solutions. It can be seen that the response appears to be roughly proportional to the D_2O concentration. Replacement of the inside solution with D_2O solutions gave similar, but much smaller decreases in potential.

Fig. 3 shows a plot of the relation between the change in potential (*i.e.*, difference in steady-state potentials, see Methods) and the deuterium concentration. The lower solid line shows that the response to outside replacement was approximately a linear function of the deuterium concentration with a mean change of −68 mv at 100 per cent D_2O outside (sixty values represented). The dotted lines indicate the 99 per cent confidence limits for the regression line (constrained to pass through the origin). The upper solid line shows the response to inside replacement. A mean change of −27

mv occurred at 100 per cent D_2O. Again, the dotted lines are the 99 per cent confidence limits (thirty-four values represented). One point (labeled "both sides") shows that replacement of both bathing solutions with 100 per cent D_2O gave an intermediate change of −51 mv (six values), which was significantly different from both other groups.
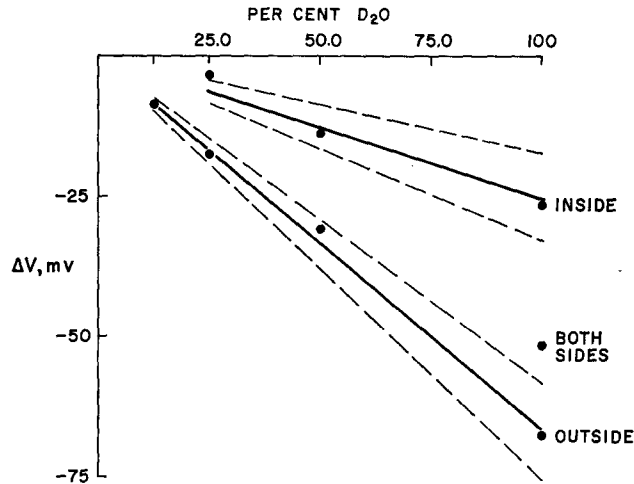

FIGURE 3. Relation between change in frog skin potential and D_2O concentration in the solution bathing the inside and the outside surfaces. The solid lines are the regression lines, the broken lines indicate the 99 per cent confidence limits for the slope. All lines were constrained to pass through the origin. The residual variance was assumed proportional to the concentration of D_2O. The single point shows the potential decrease seen when both bathing solutions contained 100 per cent D_2O. All solutions contained 115 mEq/liter Na, 5 mEq/liter K with sulfate as the anion and were buffered with phosphate to pH 8.2.

2. Osmotic Gradients in "Regular" Solution

A number of different solutes were first used in order to gain some idea of the general effects of hyperosmotic solutions. In these experiments the control condition was regular sulfate solution (115 Na, 5 K) on both sides of the skin. The nominal osmolality of this solution is 180 mOsm/liter; the measured value is about 150 mOsm/liter. The experimental solutions were made hyperosmotic to varying degrees by adding solute to the solution. Except when the added solute was Na_2SO_4 or K_2SO_4 the sodium activities in the experimental solutions were equal to that in the control solution. Hyperosmotic solutions gave large decreases in potential similar to those with D_2O. In Fig. 4 are shown regression lines for the changes in potential obtained upon exposure of the outside surface to solutions containing sucrose, acetamide, mannitol, urea, thiourea, sodium sulfate, or potassium sulfate. Especially noteworthy,

since the skin potential at the outer border is usually considered to be a sodium diffusion potential, is the fact that Na_2SO_4 also decreased the potential.

A second set of experiments in this group was designed to allow comparison of D_2O solution and some hyperosmotic solutions (acetamide, thiourea, and mannitol) on the outside surface within the same frogs. Concentrations used were 70, 140, and 210 millimolal (in sulfate solution) and 25, 50, and 75 per cent for the D_2O. In addition, resistance measurements were made on this set. Fig. 5 shows the plot of $\Delta V/V_o$ vs. $\Delta R/R_o$ for these four sets of solutions.
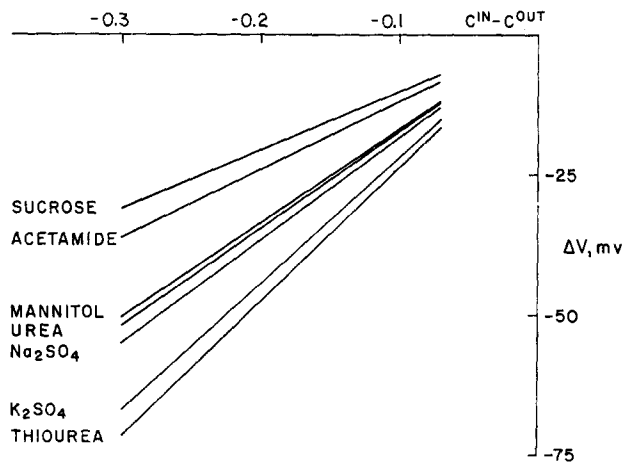

FIGURE 4. Relation between change in frog skin potential and concentration of osmotic agent bathing the outside surface (in Osm/liter). Regression lines are shown for the agents listed in the figure and were constrained to pass through the origin. Points have been omitted for the sake of clarity.

It can be seen that the large potential decreases were accompanied by large resistance decreases. Similar experiments with some of the other solutes mentioned in Methods and with leopard frogs gave qualitatively similar results.

The above experiments with osmotic gradients depended on raising the tonicity of the outside solution by added solute. Further experiments of modified types were undertaken to distinguish between the effects of raised solute concentration and those of the gradient *per se*. One set of experiments was done with mannitol, acetamide, thiourea, and D_2O in order to investigate the relative effects of replacing the inside solution only, the outside only, or both sides. After a control level was established in regular sulfate solution, either the outside or the inside solution was replaced with a solution containing 210 millimolal non-electrolyte (total milliosmolality about 360) or with 100 per cent D_2O solution. Following a period of 20 to 25 minutes, the solution on the other side of the skin was made identical to that on the first ex-

perimental side. Following this treatment of both sides, the first experimental side was returned to the control solution. Thus, the experiment gave "inside, outside, both sides" replacement data, with random assignment of the first side to be treated. The means (seven frogs) of the potential measurements are given in Fig.6, and the means of the resistance measurements are given in Fig. 7. Comparison of the mean skin potentials by Keuls' sequential range test (reference 7, p. 253) using the error calculated for acetamide and D_2O
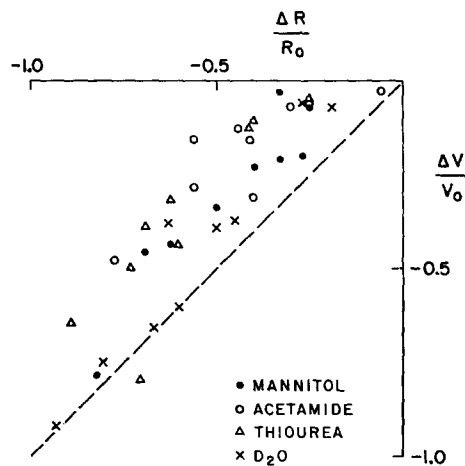

FIGURE 5. Relation between potential and resistance changes when the outside surface was exposed to D_2O solutions and to solutions made hyperosmotic with added acetamide, thiourea, and mannitol. $\Delta R/R_o$ is the resistance change divided by the control resistance and $\Delta V/V_o$ is the potential change divided by the control potential. Three frogs were used in this experiment. See Methods for explanation of this way of presenting resistance data. The broken line is the 45° line of equality which would be followed if the relative potential changes were directly proportional to the relative resistance changes. Further details of this experiment are given in Results.

on the one hand and for thiourea and mannitol on the other showed that treatment of the outside surface with all agents gave a significant ($p < 0.05$) decrease below both the control and recovery potentials. In addition, the outside treatments with acetamide and D_2O gave significant decreases below either inside or both sides treatments. Thiourea, mannitol, and D_2O when present on the inside surface or at both surfaces simultaneously gave significant decreases below their respective control levels.

Comparison of the mean skin resistances (using the error calculated for each agent) showed that for acetamide and thiourea the resistances with outside treatment were significantly ($p < 0.05$) lower than those under all other conditions. For acetamide the resistance with inside treatment was also significantly higher than those under all other conditions. For mannitol

the resistance with outside treatment was significantly lower than those under all other conditions except initial control; the resistance with inside treatment was significantly higher than that under control conditions, and the recovery resistance was also significantly higher than the initial control resistance. For D_2O the resistances under inside and both sides treatment were significantly higher than that with outside treatment. The resistance with inside treatment was also significantly higher than that for either the control or
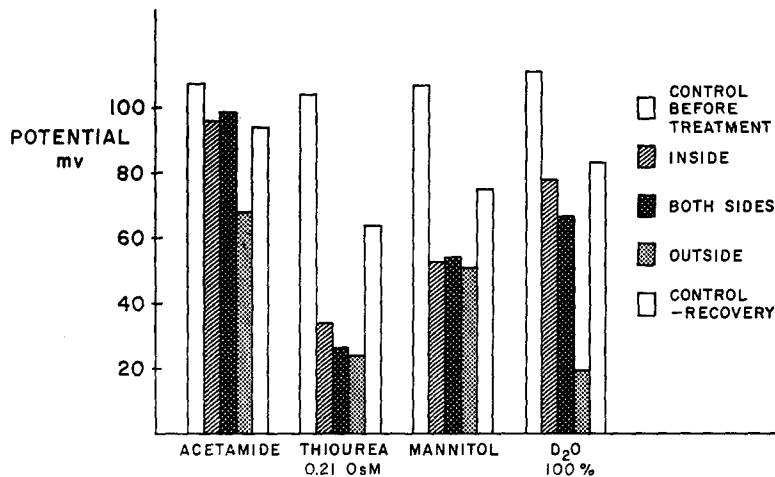

FIGURE 6. Frog skin potentials before, during, and after exposure of the skin surfaces to 100 per cent D_2O solution and to solutions made hyperosmotic with added (0.21 molal) acetamide, thiourea, and mannitol. The experiment was analyzed in two parts because of differences in residual error and carryover effect for the agents. For mannitol and thiourea, the standard error of the difference between means of "positions" for the same solute was 7.31 mv with 47 degrees of freedom. For acetamide and D_2O the standard error of the difference between means of positions for the same solution was 6.49 mv with 48 degrees of freedom.

recovery periods. The appropriate standard errors are given in the legend to Fig. 7.

3. Osmotic Gradients in Modified Solutions

Another set of experiments was done in order to determine the effect of hypotonic as well as hypertonic solutions on the outside surface. The outside surface was exposed to a dilute solution (20 Na 5K) in which mannitol or thiourea was added in concentrations of 0, 50, 100, 150, 200, 250, and 300 millimolal (total milliosmolality 35 to 335). The 150 millimolal mannitol and thiourea solutions were used as controls and the outside solution was changed to the other concentrations. The inside solution was regular sulfate solution (150 mOsm/liter). In these experiments the direction of the osmotic

gradient varies. However, the control condition is one with no nominal osmotic gradient (but a small actual gradient; measured values were 185 mOsm/liter outside and 150 mOsm/liter inside). The assignment order of the solution concentrations was random. The data are summarized in Table I. When the outside solution was made hyperosmotic, the usual large decreases in potential occurred. When the outside solution was made hypoosmotic, there were only very slight decreases in potential. On the other hand, the resist-
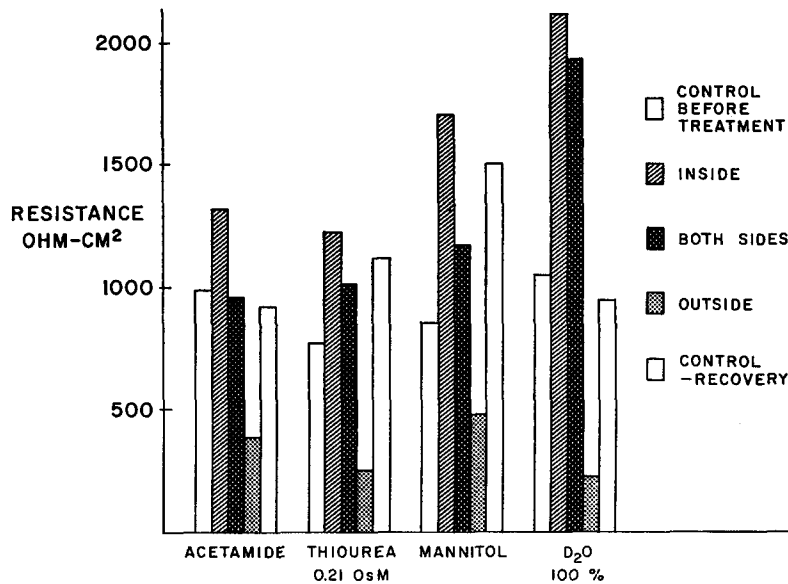

FIGURE 7. Frog skin resistances before, during, and after exposure of skin surfaces to 100 per cent D_2O solution and to solutions made hyperosmotic with added (0.21 molal) acetamide, thiourea, and mannitol. The experiment was analyzed in four parts (that is, four randomized blocks). The standard errors of the difference between means of positions for the same solution were: acetamide, 95.7 ohm-cm²; thiourea, 142.9 ohm-cm²; mannitol, 175.5 ohm-cm²; D_2O, 311.4 ohm-cm² (each with 24 degrees of freedom).

ance decreased when the outside was hyperosmotic, but increased (thiourea) or did not change significantly (mannitol) when the outside was hypoosmotic.

In a similar experiment the outside surface was exposed to dilute sulfate solution (20 Na, 5 K, phosphate buffer) in which acetamide was dissolved (in concentrations of 80, 160, 240, 320, and 400 millimolal) or to a dilute D_2O solution. The D_2O solution contained 20 Na, 5 K (phosphate buffer) dissolved in 20, 40, 60, 80, and 100 per cent D_2O. The outside control solution was the dilute solution. The inside solution was regular sulfate solution at all times. Thus, the osmotic gradient was established in both directions during these experiments, although the changes in solute concentration were always made at the outside surface. In the control condition the outside solution was hypoosmotic (35 mOsm/liter) compared to the inside solution (150 mOsm/

liter). The acetamide solutions in concentrations up to 240 millimolal caused very slight increases in potential, but at higher concentrations, small decreases in potential. The D_2O solution caused decreases in potential which were linear with the D_2O concentration as before, but the regression line did not go through the origin. Rather, it intersected the concentration axis at about 20 per cent D_2O. In other words, changing the outside solution from the control (20 Na, 5 K) to 20 per cent D_2O solution containing 20 Na, 5 K gave a negligible change in potential.

TABLE I

EFFECTS ON BULLFROG SKIN POTENTIAL AND
RESISTANCE OF EXPOSURE OF OUTSIDE SURFACE TO THIOUREA
AND MANNITOL IN DILUTE SOLUTION

Solute concentration (millimolal)	Thiourea		Mannitol	
	ΔV	$\dfrac{\Delta R}{R_0}$	ΔV	$\dfrac{\Delta R}{R_0}$
	mv		mv	
	(Hypoosmotic)			
0	−2.2±1.8	+0.26±0.08	−0.4±1.7	+0.09±0.09
50	−3.6±2.0	+0.18±0.08	+0.4±0.7	−0.06±0.03
100	−1.6±1.6	+0.15±0.06	−4.0±2.9	+0.04±0.07
	(Hyperosmotic)			
200	−6.8±1.6	−0.10±0.03	−7.0±1.8	−0.06±0.02
250	−21.4±5.8	−0.24±0.07	−11.6±2.1	−0.31±0.18
300	−43.0±3.4	−0.41±0.04	−23.6±6.7	−0.25±0.07

All figures are given as means ± SEM (five experiments).

The experiments (Figs. 4 to 7 and Table I) collectively show that the potential difference across the isolated frog skin is reduced when the solution bathing the outside is made hyperosmotic relative to the inside. The magnitude of the depression of the skin potential depends not only upon the concentration but also upon the nature of the solute. Thus, urea has a more pronounced effect than sucrose. Furthermore, a hyperosmotic solution has less effect at the inside of the skin than at the outside. When the outside solution was hyperosmotic, skin resistance decreased. However, when the outside solution was hypoosmotic, the resistance increased or remained unchanged. These effects appeared to depend upon the direction of the osmotic gradient rather than upon the osmolality of the solutions relative to that of the normal frog body fluids.

4. *Osmotic Gradients with Members of a Homologous Series*

In order to investigate the effect of molecular size on the depression of the potential, a graded series of polyhydric alcohols and two sugars were studied.

The experiments were carried out with a sulfate solution of reduced electrolyte content (60 Na, 5 K, tris-pH 8, 90 mOsм/liter). Solute was added to increase the concentration by 70, 140, or 210 millimolal. Thus the effects are those of elevated solute concentration on the side indicated. Fig. 8 reports part of an experiment of prolonged duration. The ordinate gives the change in potential from the control value and the abscissa gives the time in minutes. In this case raffinose, glycerol, and adonitol (concentration of 140 millimolal in sulfate solution) were used. It can be seen that the potential fell rapidly at first, then slowly throughout the period. Recovery was good, even after 100 minutes.
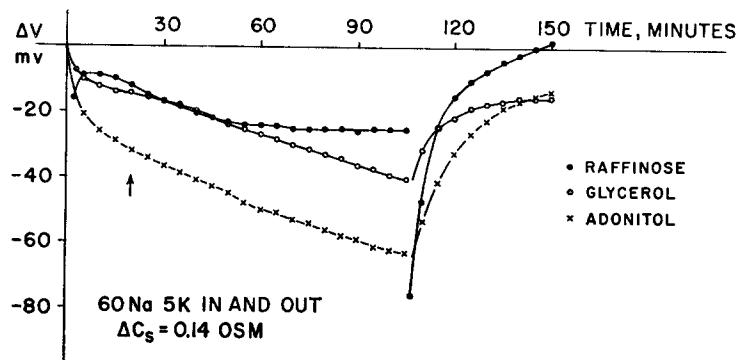

FIGURE 8. Typical experiment showing change in skin potential during prolonged exposure of outside surface to sulfate solutions made hyperosmotic with added (140 millimolal) raffinose, glycerol, and adonitol. The arrow is at 20 minutes, which is the duration of exposure used in the experiments summarized in Fig. 9. Redrawn from original strip chart recording.

In Table II the means from three such experiments are given during exposure and after return to the control solution.

A large transient decrease in potential was seen when the raffinose was washed out in this experiment. Similar decreases were observed at times when sucrose was washed out. Since the recording equipment printed at 30 second intervals, it could not be expected to register faithfully the earliest and largest changes which probably occurred. No experiments were performed to study the conditions for occurrence of the transient.

In order to allow more extensive comparisons, most of our experiments were done using 20 minute treatment periods. The order of effectiveness of the solutes is relatively unchanged measured at 20 instead of 60 minutes (Table II).

The effects of a series of polyhydric alcohols and two sugars on bullfrog skin potential are summarized in Fig. 9. The ordinate represents change from the control potential, usually a decrease. The abscissa represents the difference in osmolality between the inside and outside solutions, produced by added

solute. Thus, the left-hand side represents hyperosmotic outside solutions and the right-hand side hyperosmotic inside solutions. The points are mean values at each concentration (except 0.07 osmolal inside) for each solute. The regression lines were calculated from data at three concentrations with six frogs for the outside experiment and five frogs for the inside experiment. Since the experiments were carried out in a solution of reduced concentration, it might be of interest to indicate that the applied solutions were approximately isoosmotic with frog plasma at the point 0.12 Osm. The data have been sub-

TABLE II

EFFECT ON FROG SKIN POTENTIAL OF
PROLONGED EXPOSURE OF OUTSIDE AND INSIDE
SURFACES TO HYPEROSMOTIC SOLUTIONS

See Fig. 8 for typical experiment showing time course of potential changes
during exposure of outside surface.

Solute (140 millimolal)	Skin potential				
	Time of exposure			Recovery	
	Control	20 min.	60–65 min.	20 min.	35–40 min.
	mv	*mv*	*mv*	*mv*	*mv*
	Outer surface				
Glycerol	99	86	74	81	83
Adonitol	86	49	32	67	72
Raffinose	89	78	62	77	82
	Inside surface				
Glycerol	85	85	83	86	86
Adonitol	98	79	61	81	85
Raffinose	85	72	56	63	75

jected to an extended analysis of variance; *viz.*, regression in terms of orthogonal polynomials. For the outside replacements, all agents gave regression lines with slopes significantly different from zero. Only in the case of adonitol was there a significant deviation from linearity. The regression lines depart significantly from a common slope ($p < 0.05$). The following slope differences were significant ($p < 0.05$): adonitol (A)-glycerol (G), erythritol (E)-G, mannitol (M)-G, sucrose (S)-G; A-raffinose (R), E-R, M-R, S-R; A-S.

For the inside replacements, all solutes except glycerol gave slopes significantly different from zero. In no case was the curvature significant. Significant slope differences were M-G, A-G, S-G, E-G. Table III gives the means of the skin potentials before treatment and after recovery.

Thus, a wide variety of hyperosmotic solutions produce large potential decreases when applied to the outside of the frog skin, and the decrease varies directly with the difference in osmolality. The same substances produce slight decreases (again proportional to the difference in osmolality) when applied

to the inside. In the experiments in 60 Na, 5 K solution only in the case of prolonged treatment with raffinose did the potential decrease produced by a given agent on the inside compare in magnitude with that produced by the same agent on the outside (see also Fig. 6).
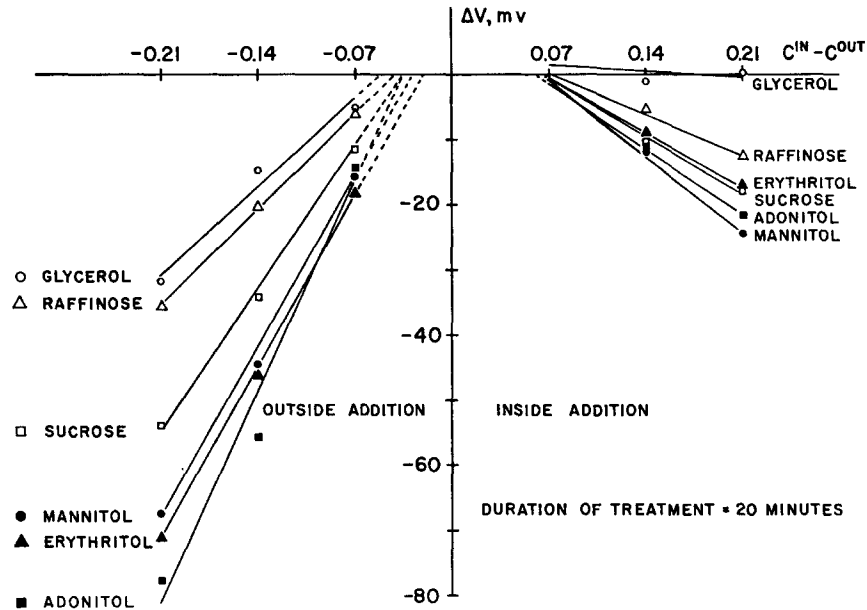

Figure 9. Relation between potential change and concentration of osmotic agent added to the outside and the inside bathing solutions. Four polyhydric alcohols and two sugars were used at three concentrations. On the left of the figure are shown regression lines calculated for the changes observed upon exposure of the outside surface and on the right are shown the corresponding lines for the exposure of the inside surface. The broken line is the extrapolated regression line drawn to show the intercept. Points for the mean values at each concentration (except 0.07 osmolal inside) for all agents are shown. The standard error of the difference between two slopes (for replacements on the same side) was 0.407 mv/0.01 molal for the outside replacements (60 degrees of freedom) and 0.387 mv/0.01 molal for the inside replacements (48 degrees of freedom). The standard error of the difference between means for two solutes at the same concentration on the outside surface was 4.75 mv (with between 25 and 60 degrees of freedom); on the inside surface, 4.34 mv (with between 20 and 48 degrees of freedom).

The skin resistance decreased when the outside was made hyperosmotic to the inside solution. The skin resistance increased when the inside solution was made hyperosmotic to the outside solution, whether by adding solute to the inside or by removing solute from the outside.

Fig. 10 shows the relationship between resistance change and potential change for the compounds shown in Fig. 9. Solid circles represent outside hyperosmolality, each point being the mean of six experiments at a given

TABLE III

RECOVERY OF SKIN POTENTIAL AFTER
EXPOSURE OF INSIDE AND OF OUTSIDE SURFACES
TO POLYHYDRIC ALCOHOLS AND SUGARS

	Skin potential			
	Outside treated		Inside treated	
Agent	Control	Recovery	Control	Recovery
	mv	*mv*	*mv*	*mv*
Glycerol	97.5±3.6	95.8±4.8	93.0±4.2	93.1±5.5
Erythritol	98.1±3.6	93.3±3.5	98.0±6.3	84.0±5.0
Adonitol	96.3±3.7	88.7±6.7	91.0±8.5	76.8±8.3
Mannitol	103.8±4.4	95.3±6.5	92.7±4.8	83.0±7.3
Sucrose	96.8±5.2	91.0±4.8	98.1±3.5	82.1±5.7
Raffinose	102.0±4.0	98.3±3.8	92.3±5.5	81.8±9.0

concentration of a given solute. Crosses represent inside hyperosmolality, each point, the mean of five experiments. The data were also plotted separately for each solute to check for spurious correlation introduced by the composite plot. Any given solute produced a similar plot. It can be seen that
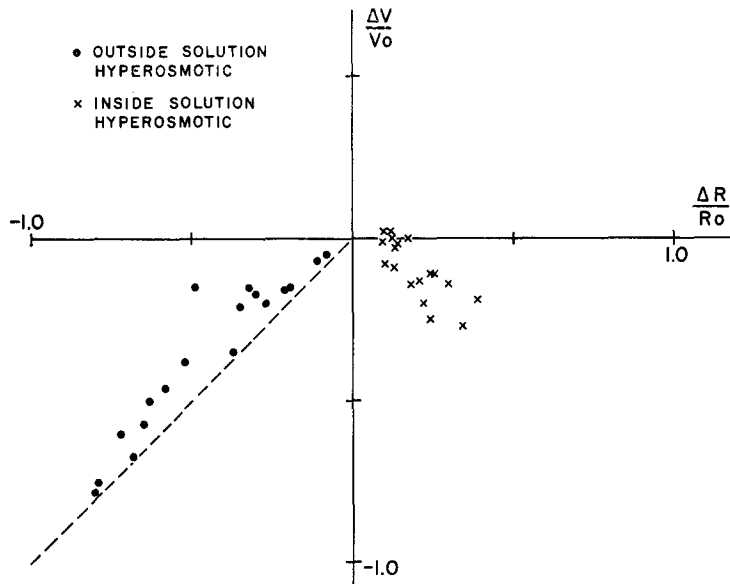

FIGURE 10. Relation between potential change and resistance change observed upon exposure to sulfate solution made hyperosmotic with polyalcohols and sugars. The data were obtained in the same experiment shown in Fig. 9. The solid points show results from exposure of the outside surface and the crosses, those from exposure of the inside surface. The broken line is the equality line. See text for explanation.

outside hyperosmolality was accompanied by resistance decreases proportional to the potential decrease, whereas inside hyperosmolality produced resistance increases.

Experiments in which both inside and outside surfaces were exposed simultaneously to solutions 210 millimolal in the non-electrolyte were also carried out. In adonitol, mannitol, sucrose, and raffinose, the rate of decline in potential persisted for the duration of the experiment (1 hour). After an initial fall, the potential of the skin in the presence of erythritol rose again slightly in the second half-hour. In glycerol the potential initially decreased, then returned to the control level. At 20 minutes, all potentials were higher than those seen with replacements at the same concentration of only the outside solution. In all except sucrose and raffinose the resistance decreased initially, and then rose steadily after 15 minutes, exceeding the control level for erythritol and adonitol. In sucrose and raffinose the resistance rose throughout the experimental period. Thus, except for an initial period with some of the agents, resistance and potential changes were dissociated.

The effects of simultaneous exposure of both surfaces are to be contrasted with the findings during prolonged treatment of only the outside or only the inside surface with hyperosmotic solutions. In the latter cases the situation was always qualitatively the same as for the 20 minute values reported above. As mentioned previously, the change did tend to become larger (*e.g.*, greater decrease in potential) with time, and did not level off or diminish during the treatment.

DISCUSSION

It is evident that the effects of D₂O on skin potential and resistance are similar to those of hyperosmotic solutions. Thus, the decreases in potential and the changes in resistance in D₂O display the same large size and asymmetry (inside compared to outside) as with hyperosmotic solutions. In view of the observations that D₂O produced osmosis across inanimate membranes (8, 9), the red cell membrane (2), and leopard frog skin (Lindley and Hoshiko, unpublished), it seems reasonable to suppose that a large part of the effects of D₂O on frog skin is "osmotic" in origin. We have been unable to obtain consistent, reliable, and quantitative values for water flow across bullfrog skin and therefore cannot present direct evidence for a direct correlation between depression of skin potential and osmotic flow. Preliminary experiments on the effect of exposure of both sides of the isolated bullfrog skin to the same D₂O solution (*i.e.*, no osmotic difference across the skin) indicated that the short-circuit current may be depressed more than might be expected from the lower conductivity of D₂O solution. This is consistent with the finding (Fig. 7) that the resistance often increased when D₂O solution was present on both sides. We must therefore leave open the possibility of other mecha-

nisms behind the D_2O effect in addition to an osmotic one. However, the experiments reported here raise questions which extend beyond the original aim of comparison of D_2O and hyperosmotic solutions into more general aspects of the problem of osmotic gradients.

Fig. 11 gives an alternative presentation of data (see Fig. 9) from potential measurements upon exposure of the outside surface, as compared to molecular size. The bars are identified by the number of carbons in the compound used. The two bars in lighter hatching are from a different experiment. The
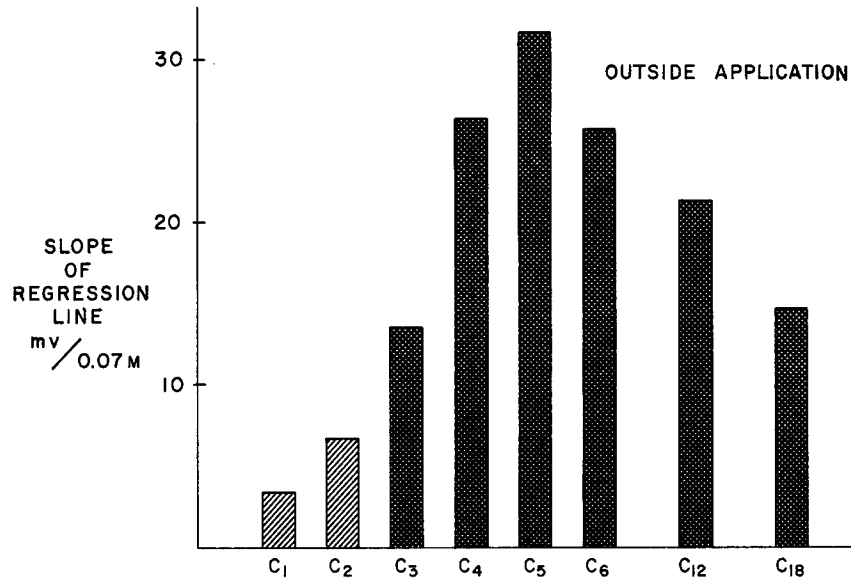

FIGURE 11.   Relation between the skin potential depression effect (of polyhydric alcohols and sugars in the outside bathing solution) and molecular size. The slopes of the regression lines in the left side of Fig. 9 are given as bars and are identified by the number of carbon atoms in the compound represented. The lightly hatched bars are from a different experiment. The standard error of the difference between slopes is 2.85 mv/0.07 M (60 degrees of freedom) for the solutes from the experiment shown in Fig. 9.

compounds are methanol, ethylene glycol, glycerol, erythritol, adonitol, mannitol, sucrose, and raffinose. The ordinate represents the relative effectiveness in depressing the potential as indicated by the slope of the regression line. It appears that a maximum occurs in the 4–6 carbon range, and that the effect falls off both with smaller compounds and with larger compounds. Other substances which we have investigated indicate similar relationships, although the maximum is displaced—perhaps because of different solubility characteristics of different classes of compounds. The order of effectiveness in depressing the potential is thus quite different from the order of increasing reflection coefficients (see Whittembury (5)).

A major shortcoming of the experiments reported above in addition to the lack of information on water flow is the fact that many of the measurements do not represent steady-state values. Under many of the conditions and within the times employed, the bullfrog skin does not appear to reach a steady-state. However, the experiments of prolonged duration would seem to indicate that no errors in qualitative conclusions arise from the use of short term experiments. There are some interesting rapid transients observed when the solutions are changed; these have also been ignored in the present report.

Ussing (10), in a discussion of unpublished observations, mentioned finding high resistance when the inside surface was bathed in hypertonic sucrose solutions. When he made the inside solution hypotonic, there was a decrease in resistance. He suggested that these findings might be explained on the basis of altered potassium permeability of the inside border. However, as described in Results, potential and resistance changes can be dissociated.

Huf (11) has reported "rectification" of water flow in frog skin (*R. esculenta*). That is, the rate of osmosis produced by a given gradient depends on the direction of the gradient. The basis for directed osmosis would seem to have something in common with the asymmetry of the effects of hyperosmotic solutions on the frog skin potential.

Motokawa (3) reported that skin potential in chloride Ringer's decreased when urea, glucose, or sucrose was added to the outside bathing solution. The above order was the order of decreasing effectiveness for depressing skin potential when full strength Ringer's was used to dissolve the solutes. However when dilute Ringer's was used, the order of effectiveness was reversed. Motokawa believed that his results could be explained in terms of competitive adsorption of NaCl *versus* the other solutes as the first step in the generation of the skin potential.

The ultimate explanation may draw upon the findings of MacRobbie and Ussing (4). They reported that only 21 μ of the 58 μ thick epidermis is osmotically active. They suggest that the outermost cornified layers may not be osmotically active. Since the effects we have observed are not in a steady-state, it is possible that a slow sieving is occurring at the outer border of the cornified layer. Although normally this layer does not contribute to the skin potential, it is the seat of some type of potential (12) and could possibly generate some of the effects seen upon treatment of the outside surface. Another possible barrier is the lining of the lymph space.

There are thus a number of mechanisms by which the decrease in potential caused by hyperosmotic solutions could be brought about (assuming a Koefoed-Johnsen-Ussing model).

1. Change in ionic gradients
     a. Change in cell volume without loss of intracellular ions.

  b. Alteration in intracellular ionic activities on a non-proportional basis, as
  by water-binding by the added solute.

  c. Alteration in active transport leading to a shift of the intracellular Na-K
  ratio, as by enzymatic inhibition.

2. Change in membrane selectivity

  a. Dehydration of pores.

  b. Blockage of pores on a selective basis (see Motokawa's explanation).

  c. Elevation of intracellular ion concentrations leading to altered transference
  numbers.

3. Shunting

  a. Cell shrinkage leading to intercellular leakage pathways.

4. Superposition of "IR drops"

  a. Streaming potential (13, 14).

  b. Entrainment of ion movements by the solute species.

The condition for an alteration of type 1*a* can be investigated. Suppose as an expression for the skin potential the sum of two membrane potentials in series, each given by an expression such as that suggested by Staverman (15) on the basis of irreversible thermodynamics:

$$V = \frac{RT}{F} \sum \frac{t_{i,\text{out}}}{z_i} \ln \frac{a_i^{\text{outside}}}{a_i^{\text{cell}}} + \frac{RT}{F} \sum \frac{t_{i,\text{in}}}{z_i} \ln \frac{a_i^{\text{cell}}}{a_i^{\text{inside}}}$$

If the volume of the cell compartment is changed without net changes in the amounts of the various ions present, and without changes in the transference numbers t_i,

$$\Delta V = \frac{RT}{F} \ln \frac{(a_i)_2}{(a_i)_1} \left[-\sum \frac{t_{i,\text{out}}}{z_i} + \sum \frac{t_{i,\text{in}}}{z_i} \right]$$

since the activities of all ions will change proportionally (but see possibility 1*b*).

Thus if the sums of transference numbers of ions of like valence are the same at both the inner and the outer borders, $\Delta V = 0$. Such would be the case if the only permeating species are Na and K. With a finite anion permeability, different at the two borders, $\Delta V \neq 0$ and may be of either sign. For anion transference numbers larger at the inner border than at the outer border, $\Delta V < 0$ (the observed case). Condition 1*a* thus appears to be possible. Conditions 1*b* and 1*c* lead to potential changes in an obvious fashion.

The presence of asymmetry in the potential change occasioned by inside replacement *versus* outside replacement would be explained in group 1*a* and 1*b* models by a difference in the degree of alteration of cell volume due to different reflection coefficients at the two borders or different accessibility of the two borders to the solute (*e.g.*, very slow movement of the solute into the corium). The peculiar order of effectiveness of the various solutes could be explained by the interaction of solute mobilities (high for low molecular weight) and reflection coefficients (low for low molecular weight). In group 1*c* models the asymmetry would be explained purely on the basis of penetrability of the substances. The order of effectiveness of the solutes then presents some difficulty.

Models involving group 2 conditions do not appear to be prevalent in the literature. The formulation of such models would in essence be changes in the transference numbers produced by the hyperosmotic solutions. The increased transference number of co-ions in ion exchange membranes as the ambient electrolyte concentration is raised (mechanism 2c) has been discussed by Helfferich (16). Mullins (17) has discussed the role of hydration in ionic selectivity.

Shunting (mechanism 3) has been discussed above in connection with the formulation of our method for presenting the relation between resistance changes and potential changes. Ussing (18) has reported that both skin resistance and potential decreased when urea was added to the outside bathing solution. He found increased sulfate and sodium permeability and suggested that shunting may be the mechanism. However, increased resistance with inside hyperosmolality makes a simple explanation based on shunting unlikely in this case.

The interpretation of resistance changes presents formidable difficulties, which cannot be overcome with the limited types of measurements made in the present experiments. It is to be noted that resistance measurements in tissues performing active transport do not pertain simply to the passive permeability characteristics of the membranes involved. Rather, the active transport of ions increases the conductance of a membrane (decreases the resistance). Thus alterations in resistance may be either changes in passive permeability or the result of changes in active transport (see also (19)).

At this time no single explanation for the various effects of osmotic gradients on skin potential appears to be sufficient. It is probable that the phenomena we have observed represent the summation of a number of types of effects, at least one of which is determined by the direction of the osmotic gradient and not by the side of the skin on which the foreign solute is present (see the experiments with dilute solution outside and regular solution inside). A further factor which must be held in mind in working with the frog skin is the possibility that the skin glands contribute to observed effects.

We should like to emphasize what we regard as the salient features of the effects demonstrated in these experiments:

1. Large magnitude of potential and resistance changes.
2. General asymmetry of potential and resistance changes.
3. Linear relation between concentration gradient and potential change for a given direction of the gradient.
4. Peculiar order of effectiveness (with respect to molecular size).
5. Variable time course (extremely rapid in some cases, very slow in others) and general reversibility.

These observations may prove to be of direct functional significance, as for example with respect to the role of urea in renal concentrating mechanisms. On the other hand, they may prove to be simply artifacts of extreme experimental conditions. In the latter case we may still expect the extension of these

studies and the elucidation of the mechanisms involved to be pertinent to the understanding of the functional behavior of epithelial membranes.

The major purpose of this paper has been to point out the need to clarify the nature of the effects of the osmolality of the bathing solutions on the frog skin potential. It is our feeling that such clarification is essential before one indiscriminately uses a solute such as sucrose or mannitol to make up the osmolality or ignores the presence of a solute such as urea or acetamide because it "penetrates freely anyway."

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