

Hyperosmolarity and the Net Transport of Nonelectrolytes in Frog Skin

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ABSTRACT The permeability of frog skin to a series of nonelectrolytes (thiourea, urea, mannitol, and sucrose) under the influence of 2.5 times normal osmolarity in the outer bathing solution has been investigated. Although the flux of the tracer nonelectrolytes across the skin in either direction is greatly increased by hyperosmolarity, the influx is found to be increased to a significantly greater extent than the outflux. Flux ratios as high as 3:1 can be observed. The net inward movement of the nonelectrolyte proceeds in spite of a sizeable bulk flow of water in the opposite direction. Possible driving forces for this phenomenon are discussed.

INTRODUCTION

Several investigators have studied the effects of hyperosmotic solutions on frog skin. Lindley, Hoshiko, and Leb (1) have shown that if the outer bathing solution is made hyperosmolar, skin potential and resistance invariably fall, these changes being dependent upon the nature and concentration of the solute. Identical effects were not observed with hyperosmotic solutions bathing the inner surface. In this case, there was a lesser drop in skin potential and skin resistance remained the same or even increased. Ussing and Windhager (2) found a large decrease in skin resistance when the osmolarity of the outer bathing solution was doubled by the addition of urea to Na_2SO_4 Ringer's solution. These authors demonstrated this decrease in resistance to be due to an increase in the passive flux of sodium and sulfate ions through the skin via the "shunt pathway."

During the course of our studies on the possible mechanism of action of dimethyl sulfoxide (DMSO), an agent reported to have high solvent, penetrant, and analgesic properties, we observed changes in skin conductance and potential similar to those reported above. We also observed some unusual effects on the passive movement of certain solutes. These effects were shown to be due to an induced hyperosmolarity and apparently were not due to any unusual structural characteristics of the DMSO. The following studies report

the findings in detail and also record the effects of several other hyperosmotic agents.¹

MATERIALS AND METHODS

In the present studies, abdominal skins of *Rana pipiens* were used as described by Franz and Van Bruggen (3), the area of skin enclosed by the glass chambers being 6.3 cm². The Ringer's containing Na⁺ 117, K⁺ 2, Cl⁻ 117, Ca⁺⁺ 0.25 μ eq/ml was lightly buffered with phosphate and adjusted to pH 7.9–8.1. The osmolarity of the Ringer's has been determined to be 215 milliosmols per liter (mOsm) on an Advanced Osmometer (Model No. 66-31KS, Advanced Instruments, Inc., Newton Highlands, Mass.).

In the flux studies, the mounted skin was bathed with Ringer's on both sides. To an appropriate side, tracer was added and the skin allowed to equilibrate for 30–45 min before sampling was begun. Aliquots were then taken to establish the base radioactivity of both bathing solutions. Three 30 min control flux periods were evaluated by sampling prior to the imposition of hyperosmolarity. The liquid solute DMSO was added to the outer bathing solution directly by micropipette and three more 30 min samples were taken. The effects of the other solutes were studied by replacing the outer bathing solution with Ringer's made hyperosmotic by the addition of the indicated solute. Unless otherwise indicated, all agents were added in an equimolar concentration to that of 2.5% DMSO, the concentration used in our initial studies. This 2.5% DMSO in Ringer's was determined to be 575 mOsm.

A separate series of flux measurements was made by adding both the tracer and the hyperosmotic agent to the outer bathing solution at the start, allowing 1 hr for equilibration, then taking six samples at 20-min intervals. By means of this second method it was possible to obtain a new steady state with respect to the tracer flux. When the first method was used, a steady state was not obtained following addition of the hyperosmotic agent.

In many of the flux studies both bathing solutions were made 1 mM with nonisotopic solute. In others, only tracer was added to a single side. In either case, the flux measurements reported are those measured at or calculated to the flux of a 1 mM solution. No significant difference in the fluxes was observed by the two methods of measurement. All isotopes were obtained from New England Nuclear Corporation, Boston, Mass.

In uptake studies, mounted skins were equilibrated with tracer on the appropriate side for 1 hr. The bathing solution from the opposite (nonlabeled) side was replaced with fresh Ringer's several times during the equilibration to maintain a low concentration of tracer on that side. The skin was then removed, quickly rinsed, blotted, and a 5.6 cm² center section cut with a modified cork-borer tool. This skin was then dissolved in warm N NaOH in a small volumetric flask. Aliquots of this solution were assayed for radioactivity.

In the washout studies, the skin was equilibrated with the tracer substance on the

¹ Hyperosmotic agent will be used to refer to the solute added to the outer bathing solution to make the solution hyperosmolar.

inside for 90 min while mounted in the usual chamber assembly. The bathing solutions were removed, the skin and chambers quickly rinsed with Ringer's, and the chambers then refilled with fresh nonlabeled Ringer's. At 2.5-min intervals the bathing solution was removed in toto by means of an aspirator-trap assembly. The entire solution was removed to maintain the concentration of tracer in the recipient bathing solution near zero. The solutions were subsequently radioassayed.

The total water content and the extracellular water content of skins in normal or hyperosmotic Ringer's containing tracer mannitol were determined in a series of

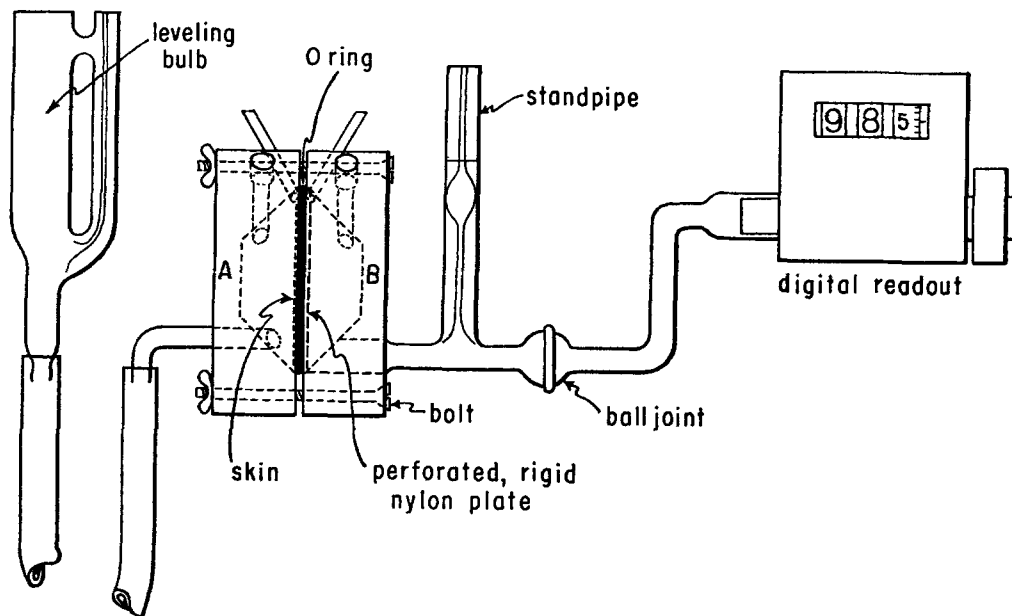


FIGURE 1. Schematic drawing of the apparatus used for the rapid measurement of net water movements, either inward in the control condition or outward as the result of the addition of hyperosmotic agent to the outer bathing solution. For description, see text.

experiments. The skins were equilibrated for 90 min with the tracer on both sides and then removed, rinsed, blotted, and a standard section removed as above. The sections were placed in tared volumetric flasks, weighed, and dried for 2 hr at 100–110°C. After desiccation the flasks were reweighed and the water content calculated. The volume of the extracellular water space was determined after radioassay of the amount of tracer mannitol in the skin following solution of the skin in *N* NaOH. It was assumed that mannitol reaches the same concentration in the skin as exists in the bathing solution.

Measurements of net water fluxes were made with the apparatus shown in Fig. 1. 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 allows for stirring by a Teflon-coated bar actuated by a magnet attached to a small 600 RPM

synchronous clock motor which is mounted outside. Such mounting eliminates heat transfer from the water to the chamber. A $\frac{1}{16}$ 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 (Arthur H. Thomas, Co., Philadelphia, Pa., Manostat Digi-Pet 2464-U10). Each scale division on the digital readout is equal to 0.2 μ l.

After mounting the skin with the outer surface toward *B*, the chambers, side arms buret, and leveling bulb are filled with Ringer's 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 $\pm 2 \mu$ l. The area of skin used is 7 cm², and the volume of each chamber is 4.25 ml. The apparatus should be maintained at a uniform temperature during an experiment. All water measurements were done in a 21°C room controlled to $\pm 1^\circ$ C.

To measure net water influx, skins are mounted in the apparatus and allowed to equilibrate with normal Ringer's for 30–60 min with the chambers open to the atmosphere and the skin not "clamped." Equilibration is necessary because immediately after mounting, skins show a large and highly variable net water influx. After the chambers are sealed with the potential and short circuit current bridges, water influx should be measured within an hour.

The water influx of some skins remains constant for prolonged periods but the water influx of others drops off to very low levels after the 1 hr period. That the oxygen supply of the closed system might be the sole cause of the decrease in influx has been considered, but preliminary studies do not bear this out.

RESULTS

Effect of Hyperosmolarity on Nonelectrolyte Permeability

The addition of DMSO, at a level of 2.5%, to the outer bathing solution results in changes in skin potential and short circuit current (SCC). Skin potential invariably falls but the response of the SCC is entirely unpredictable and often biphasic. Except during the winter season, when SCC is very low, the response is generally an immediate increase in current, which will then slowly decline. During the winter season the SCC always falls. Since changes

in electrical characteristics can often be explained on the basis of alterations in skin permeability, this point was investigated.

First to be studied were changes in the permeability of the skin to four nonelectrolytes following the addition of DMSO to the outer bathing solution. In these experiments, after an initial period of equilibration, three 30 min periods of flux were measured before, and three 30 min periods of flux after the addition of DMSO. The rate of movement of the tracer species through the skin during the control periods was constant indicating that the skin had reached a steady state with respect to the tracer. However, after the addition

TABLE I
EFFECT OF 2.5% DMSO ON FROG SKIN PERMEABILITY

Nonelectrolyte	Condition	Influx*	Outflux*	Influx		Difference
				Outflux		
Thiourea	Control	7.0±1.0	8.2±1.0	0.9		<i>p</i> > 0.05
	Treated	50.7±15.8	41.6±9.5	1.2		<i>p</i> > 0.05
Urea	Control	4.3±0.8	3.2±0.7	1.3		<i>p</i> > 0.05
	Treated	71.9±14.0	27.7±7.0	2.6		<i>p</i> < 0.05
Mannitol	Control	0.7±0.1	0.8±0.4	0.9		<i>p</i> > 0.05
	Treated	52.5±11.9	9.2±2.2	5.7		<i>p</i> < 0.05
Sucrose	Control	0.3±0.1	0.6±0.2	0.5		<i>p</i> > 0.05
	Treated	36.6±9.1	6.8±1.6	5.4		<i>p</i> < 0.01

* $\mu\text{moles} \cdot \text{hr}^{-1} \times 10^3$ (mean \pm SEM)

of DMSO there was an immediate and continuous increase in the rate of appearance of the tracer and a new steady state was not reached by the end of the third sampling period. The results of these experiments are presented in Table I. For simplicity of presentation a mean has been taken of the three treated periods in the same manner as the control periods. Although this number has no absolute meaning by itself, it is useful as a means of comparing the effect of DMSO on the four tracer species.

It can be seen from the table that the addition of DMSO markedly alters the permeability of the skin. The unidirectional fluxes, both in and out, of the four nonelectrolytes have been greatly increased. However, a comparison of the influx and outflux values for a given nonelectrolyte in the treated condition reveals that a difference exists. For three of the four nonelectrolytes the influx, under the influence of DMSO, is larger than the corresponding outflux. A convenient way of expressing this is as a flux ratio (influx/outflux). The flux ratios for urea, mannitol, and sucrose in treated skins are much

greater than 1.0. The flux ratio for thiourea is statistically not different from 1.0.

Molecules moving passively across the skin, subject only to the forces of diffusion, should have a flux ratio of 1.0. In other words, the rate of movement across the skin in one direction is the same as the rate in the opposite direction. This is to be expected since, if the same pathway is used, all the forces encountered by a molecule moving through the skin in the inward direction should similarly be encountered as it moves in the outward direction. It can be seen from Table I that the flux ratios of control skins are not statistically different from 1.0, suggesting that their movement is entirely passive. The

TABLE II
EFFECT OF HYPEROSMOTIC AGENTS IN OUTER BATHING SOLUTION

Nonelectrolyte	Hyperosmotic agent	Influx*	Outflux*	Influx	
				Outflux	Difference
Thiourea	Erythritol	66.2±12.4	102.4±18.8	0.6	<i>p</i> > 0.05
	DMSO	50.7±15.8	41.6±9.5	1.2	<i>p</i> > 0.05
Urea	Thiourea	96.3±7.3	36.6±10.7	2.6	<i>p</i> < 0.05
	DMSO	71.9±14.0	27.7±7.0	2.6	<i>p</i> < 0.05
Mannitol	Erythritol	45.2±12.3	28.1±5.0	1.6	<i>p</i> < 0.05
	DMSO	52.5±11.9	9.2±2.2	5.7	<i>p</i> < 0.05
Sucrose	Thiourea	46.7±3.1	16.1±0.5	2.9	<i>p</i> < 0.05
	Erythritol	31.2±7.3	12.7±1.7	2.5	<i>p</i> < 0.05
	DMSO	36.6±9.1	6.8±1.6	5.4	<i>p</i> < 0.01

* $\mu\text{moles} \cdot \text{hr}^{-1} \times 10^3$ (mean \pm SEM)

fact that three of the flux ratios of treated skins are greater than 1.0 is evidence that some additional force, other than diffusion, is operative.

These results indicate that DMSO has a dramatic effect on frog skin. That the increase in both permeability and flux ratios is due to hyperosmolarity of the outer solution and not to DMSO per se is demonstrated by the following experiments in which erythritol and thiourea were added to the outer solution in amounts equimolar to DMSO. Data comparing the effects of thiourea and erythritol with the effect of DMSO are presented in Table II, the DMSO data being the same as that in Table I. Only the values from treated skins are presented in the table since the control values did not differ appreciably from those seen in Table I. It is apparent that the three hyperosmotic agents cause increased skin permeability to tracer thiourea, urea, mannitol, and sucrose and are also able to produce flux ratios greater than 1.0 for urea, mannitol, and sucrose. As was the case with DMSO, erythritol did not produce a flux ratio greater than one for tracer thiourea.

Due to the fact that the previous two tables report nonsteady-state data, a serious question can be raised as to their significance. It may well be that the rates of change of the influx and outflux after the imposition of hyperosmolarity have different time courses. Indeed, it is possible that a flux ratio of 1.0 may be obtained once a new steady state has been reached. To rule out this possibility, a series of flux measurements was made under steady-state conditions. In these experiments, the hyperosmotic agent was added immediately upon mounting the skin. Following a 1 hr equilibration period, six 20 min samples were taken. Table III reports the results of three different hyperosmotic agents on the mannitol flux, the only tracer that was checked by this

TABLE III
EFFECT OF HYPEROSMOTIC AGENTS ON THE MANNITOL FLUX

Hyperosmotic agent	Influx*	Outflux*	Influx	Difference
			Outflux	
Erythritol‡	53.7±6.4	24.4±2.1	2.2	<i>p</i> < 0.05
	76.5±4.7	41.2±3.1	1.9	<i>p</i> < 0.05
Thiourea	126.0±16.1	44.1±14.5	2.9	<i>p</i> < 0.05
DMSO	55.7±11.2	19.8±0.6	2.8	<i>p</i> < 0.05

* $\mu\text{moles}\cdot\text{hr}^{-1} \times 10^3$ (mean \pm SEM)

‡ Results obtained on two successive shipments of frogs from the same supplier. The figures obtained from the two groups show little overlap of values. The values from the two groups may be averaged without loss of significance in the change in flux ratios.

method. When done in this way the flux value obtained is essentially constant throughout all six periods indicating that a steady state had been reached after 1 hr and that flux ratios greater than 1.0 can be maintained under steady-state conditions.

The flux ratios reported in Table III are not too different from those of Table II, with the possible exception of the ones for DMSO, which suggests that either method of determination of fluxes (steady-state vs. nonsteady-state) is valid. Whether the differences seen with DMSO are a result of the different methods used or some other variable is not known at this time.

The demonstration that flux ratios greater than 1.0 may be produced by hyperosmolarity is most significant. This same phenomenon has also been reported recently by Ussing (4). The increase in flux ratio means that the influx is greater than the outflux or that net movement of the nonelectrolyte is occurring in the absence of a concentration gradient. What force can account for such a movement?

A net water movement through the frog skin may, by frictional interaction (solvent drag), alter the rate of movement of a solute as shown by the studies

of Andersen and Ussing (5). This phenomenon of solvent drag may cause a flux ratio different from 1.0 but its net effect will be in the direction of the net water movement. In our experiments, a net water outflux is to be expected because of the osmotic gradient created by hyperosmolarity of the outer solution. A net outflux of water might well increase solute outfluxes, but this should result in flux ratios less than 1.0, not greater as found.

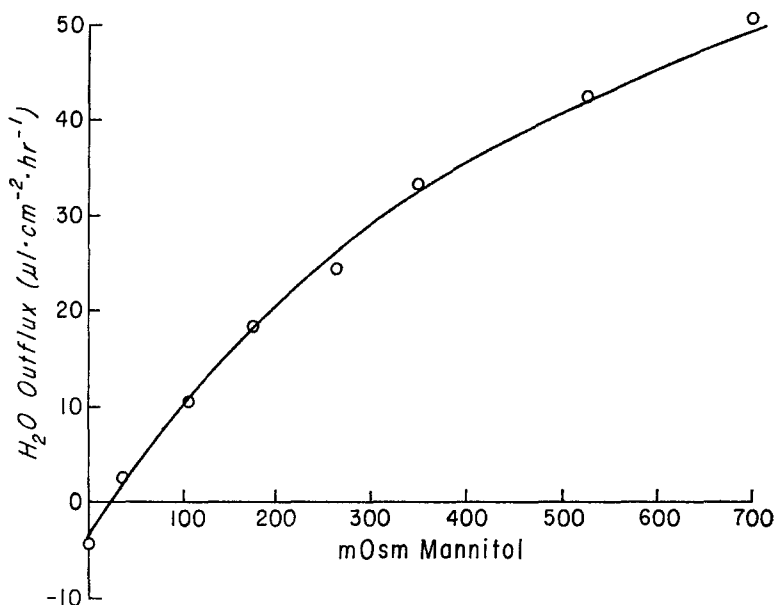


FIGURE 2. Net water outflux across frog skin produced by the addition of mannitol to the Ringer's of the outer bathing solution. The value of $-4 \mu\text{l}$, at 0 milliosmol mannitol, represents a net influx of water in the absence of any osmotic gradient across the skin.

Effect of Hyperosmolarity on Water Movement

In order to rule out solvent drag as a possible cause for the asymmetric solute fluxes, the direction and magnitude of water movement across the skin were measured. This was done by using the apparatus described earlier. The average net water influx in the absence of an osmotic gradient was found to approximate $4 \mu\text{l} \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$ with a range of 0–12 μl . Since the mechanism(s) of the net water influx are not known, it is not possible to explain the wide range of values observed. However, we feel that the major causes of variability are inherent in the skin itself. The mean value of 4 μl is similar to figures given by Huf (6), House (7), and Schoffeniels and Tercafs (8).

When DMSO, erythritol, thiourea, mannitol, or raffinose are added to the outer Ringer's solution at levels of 35–700 mOsm, the net water influx is

reversed and a net outflux is produced. Fig. 2 shows this outflux as a function of mannitol concentration. Attention is directed to the value of some $33 \mu\text{l} \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$ for the outflux when the outer solution contained 350 mOsm mannitol, the concentration approximately equivalent to that used in the solute flux studies reported in Table II (350 mOsm mannitol + Ringer's = 575 mOsm). Thus, as expected, a sizeable net water outflux is produced by the hyperosmotic outer bathing solution, and solvent drag is excluded as a mechanism for the observed net solute influx.

The data presented for the water fluxes resulting from mannitol-induced hyperosmolarity do not differ significantly from those produced by the other agents cited (range $30\text{--}33 \mu\text{l} \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$) with the exception of DMSO which was $17 \mu\text{l} \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$. The latter agent, having a higher permeability for frog skin, has a lower effective osmotic force.

It can be seen from Fig. 2 that the curve is nonlinear over the entire range studied. These results appeared to be in conflict with the data of House (7) who found an apparent linear response for the range -175 to $+175$ mOsm. This author (9) has, however, recently described a statistical analysis of the earlier data and now reports that the response is indeed nonlinear. Thus the frog skin exhibits rectification of water flow in response to osmotic gradients when bathed by chloride Ringer's.

A comparison of the magnitude of water movement for a given osmotic gradient reveals a substantial difference between the data presented by House and ours. Calculation of the hydraulic conductivity coefficient (L_p) for our preparations in the osmotic range of 0–100 milliosmols mannitol, yields the value of $16.5 \times 10^{-7} \text{ cm} \cdot \text{sec}^{-1} \cdot \text{atm}^{-1}$. This coefficient is about fourfold larger than that of 3.9×10^{-7} reported by House. Species variability and/or seasonal effects may account for the apparent differences.

Effect of Hyperosmolarity on Skin Compartments

In an attempt to define the mechanism by which hyperosmolarity influences skin permeability, the individual processes of solute movement into the skin and out of the skin were investigated. For proper interpretation of this data it became necessary to know the volume of skin water and its compartmentation, as well as the changes produced in these values under the influence of hyperosmolarity.

On a purely anatomic basis, it is possible to divide skin water into intracellular and extracellular compartments. The volume of extracellular water can be estimated by use of solutes such as mannitol, sucrose, and inulin, which do not penetrate cell membranes. Because there is evidence that inner bathing solution sodium is unable to penetrate the transporting cells (10), it was expected that sodium might also give a good estimate of the extracellular space. In these studies tracer mannitol, sucrose, and inulin were present on both

sides of the skin, whereas tracer sodium was added only to the inner bathing solution. Although the time course of equilibration was not determined, it is known that skin labeling is complete well within the 90-min period used in these studies.

Table IV presents data on the distribution of skin water. All determinations of total water, within each of the two groups, were pooled to give the one mean value reported. A significant difference exists between the total water present in the two groups studied with group II having some 20 % less water. It is possible that seasonal variations are responsible for the difference since the data of the first group were obtained from fall skins and the data of the second group from winter skins. In spite of the difference in total water between

TABLE IV
DISTRIBUTION OF SKIN WATER ($\mu\text{l}\cdot\text{cm}^{-2}$)

	Group I				Group II		
	ECW	ECW/TW	TW		ECW	ECW/TW	TW
Mannitol (8)	13.6±0.7	0.53		(23)	11.0±0.5	0.52	
Sucrose (14)	13.9±0.5	0.54	25.8±0.6	(11)	13.9±0.8	0.66	21.0±1.0
Inulin	—	—		(6)	10.5±0.8	0.50	
Sodium (5)	14.4±1.4	0.56			—	—	

ECW, extracellular water \pm standard error; TW, total skin water \pm standard error. Numbers in parentheses represent number of skins.

groups I and II, extracellular water seems to be a constant fraction (0.52–0.53) of the total water. The apparent discrepancy of the sucrose value of 0.66 is unexplained at this time. It seems unlikely that sucrose with its larger molecular size could equilibrate with a greater extracellular space than that available to mannitol.

It is interesting to note that the volume of water available to Na^+ (from the inner solution only) closely approximates the extracellular space. This is consistent with the concept of a Na^+ -impermeable barrier as proposed by Koefoed-Johnsen and Ussing (10); that is, sodium from the inner bathing solution cannot enter the transporting cells of the epidermis. It might be argued that the value obtained with tracer sodium is an underestimate of the true space actually available to sodium, since it is theoretically impossible for a tracer to attain equilibrium with a space when the label is present solely on one side. An exception to this is the case of an impermeable structure bounding the space on the side opposite the label. Since this is not the situation, that is, Na^+ outflux > 0 in frog skin, there must be an error in the value obtained with tracer Na^+ . However, it has been shown by Winn et al. (11) that about 99 % of the skin resistance to sodium movement is located in the epidermis

so that the error in underestimating the true space available to Na^+ from the inner bathing solution is only about 1 %.

The effect of 2.5 % DMSO on the water content of skin is reported in Table V. As is to be expected, the presence of hyperosmolarity in the outer bathing solution causes a loss of water from the skin. In group I the water loss represents 18 % of the total skin water, whereas in group II the loss is only 10 %. Again, group I represents fall skins and group II winter skins. It should be mentioned that the data of Table V represent steady-state values since it has been determined that all water loss takes place within the first 20 min. There appears to be little change in the size of the extracellular space as determined with mannitol. The apparent loss of extracellular water (ECW) in group I under the action of DMSO is unexplained at this time. At least there is no increase in ECW following DMSO, which would lead one to suspect that the cell membrane is now permeable to mannitol.

TABLE V
EFFECT OF 2.5% DMSO ON SKIN WATER ($\mu\text{l}\cdot\text{cm}^{-2}$)

		ECW	TW
Group I	Norm (8)	13.6±0.7	26.2±0.9
	DMSO (7)	11.4±0.8	21.5±0.9
Group II	Norm (6)	10.0±0.3	21.5±1.5
	DMSO (7)	10.3±0.4	19.4±0.6

Effect of Hyperosmolarity on Solute Movement into and out of Frog Skin

Presentation of trans-skin fluxes such as those of Tables I, II, and III are of greatest use as a survey or a general approach. These measurements inadvertently focus attention on the skin as if it were a single membrane. Realizing that the skin is a complex structure, but being unable to define the contribution of each structural component to a trans-skin flux, we can as a minimum, study two parts of the over-all process, that is, movement into and out of the skin.

Movement of solute into the skin was followed by measurement of the uptake of sucrose- ^{14}C in control skins and in others in the presence of 2.5 % outer bathing DMSO. Uptakes from both the inner and outer bathing solutions were followed and experiments were of sufficient length so that the tracer in the bathing solution reached a steady state with the skin. Table VI presents the data. The results are expressed as the fraction total skin cpm/total solution cpm with the volume of the inner and outer bathing solutions being identical.

For a proper interpretation of the data presented three things must be kept in mind. First, 75–85 % of the skin is dermis (11, 12). In addition, since sucrose

remains entirely extracellular and the comparative size of the epidermal extracellular space must be small, the greatest part of the tracer sucrose in the skin will be found in the dermis. Secondly, the dermis freely communicates with the inner bathing solution but is separated from the outer bathing solution by the relatively impermeable epidermis (11). Whether it is the entire epidermis or only a part of it which is rate limiting makes little difference at this point. The third consideration relates to the magnitude of sucrose labeling of the skin. It must be remembered that in the steady state this is dependent on both the rate of entrance into and the rate of exit from the skin.

Because of the above, it is possible to view the skin in the following manner. It consists entirely of dermis bounded on the outside by a rate-limiting "membrane," the epidermis, but with no limiting structure between it and the inner bathing solution. This model applies only to molecules confined to the extra-

TABLE VI
EFFECT OF 2.5% DMSO ON SUCROSE-¹⁴C LABELING OF SKIN

	Control*	Treated*	Difference
From outside	3.7×10^{-4}	12.6×10^{-4}	$p < 0.01$
From inside	92.9×10^{-4}	67.1×10^{-4}	$p < 0.05$

* Total skin cpm/total solution cpm.

cellular space. There is no need to use the two membrane model which is commonly used with respect to Na⁺ and K⁺ (10).

The data of Table VI appear to follow the model nicely. The control values demonstrate the resistivity of the epidermis in restricting the movement of sucrose from the outer bathing solution into the dermis. Labeling from the outside is only $\frac{1}{25}$ of that from the inside. Following the imposition of hyperosmolarity the permeability of the rate-limiting membrane is increased. Because of its asymmetric location in the skin the result is increased skin labeling from the outside (increased rate of entrance) and decreased skin labeling from the inside (increased rate of exit). Movement of the tracer in the dermis and at the dermal-inner bathing solution interface is assumed to remain constant. This seems justified in the view of the "washout" data to be presented.

The second part of these experiments involved following the loss of tracer from prelabeled skins, washouts. Because of the extreme impermeability of the outside of the skin relative to the inside, it has not been possible to obtain meaningful data from the outer bathing solution as yet.

During the labeling period, the tracer was present in only the inner bathing solution and hyperosmolarity was present during both labeling and washout, in the case of the treated skins. The washout of mannitol to the inside for control and treated skins is shown in Fig. 3, each point representing the mean

of four values. It can be seen that the rate of washout is linear after 10 min and that the results from the two groups can be represented by lines with nearly identical slopes. The DMSO slope is slightly greater. This is explainable on the basis of the increased rate of loss through the outer barrier. The fact

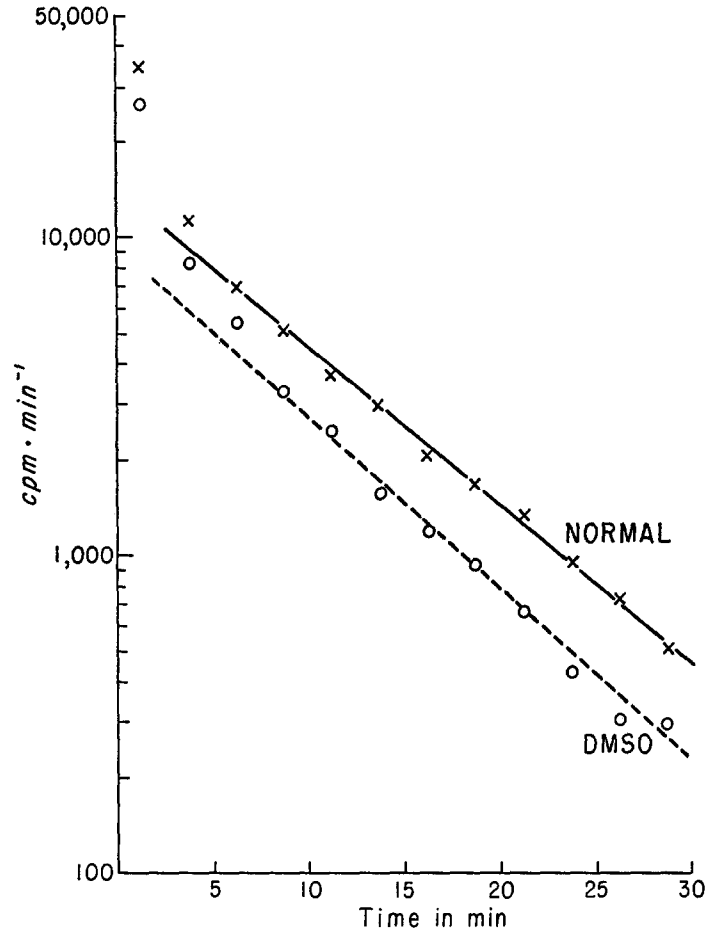


FIGURE 3. Time course of washout of tracer mannitol to the inner bathing solution in control and DMSO treated skin.

that the two slopes are so nearly identical indicates that DMSO is not affecting the permeability of the dermis. The lower position of the line for treated skins demonstrates that these skins initially contained less tracer mannitol in agreement with the findings of the sucrose experiment listed in Table VI in which there was a decreased labeling from the inside in the presence of hyperosmolarity.

The nonlinearity of the curves in the first 10 min requires some comment.

Commonly, this type of curve leads to the assumption of a two compartment system. However, this same type of curve is obtained for the case of diffusion out of a "plate" or "slab," e.g., see Jost (13). A slab may be defined as a homogeneous phase having the same resistance to diffusion throughout. Although the movement of tracer out of a slab would normally proceed in both directions, the same equation is applicable and the same type of curve is obtained when one face of the slab is bounded by an impermeable membrane as would seem to be the case in frog skin. The dermal connective tissue may be looked upon as a slab and the rate-limiting barrier of the epidermis as an impermeable membrane. On this basis, then, the shape of the washout curve is exactly as would be expected and there is no need to postulate the presence of additional compartments in the skin for sucrose and mannitol. This data together with the data of Table V, confirm the fact that mannitol and sucrose behave as if they were confined entirely to the extracellular space, even under hyperosmotic conditions, and lend support to the model presented earlier. This will be an important consideration in proposing a mechanism of action for outer bathing solution hyperosmolarity.

DISCUSSION

Although it has been shown previously that hyperosmolarity of the outer bathing solution can markedly alter frog skin permeability, the present apparent active transport (flux ratio > 1.0) of nonelectrolytes is an unexpected finding. Ussing (4) reported a similar phenomenon in which SO_4^{4-} and sucrose moved asymmetrically across skins of *Rana temporaria* under the influence of hyperosmolarity of the outer bathing solution.

Because of the diverse molecular structures that show asymmetric movement with hyperosmolarity, it is easier to conceive of a common mechanism underlying this transport rather than individual mechanisms for each solute or ionic species. Before a mechanism can be proposed to account for the observed net transport, it is necessary to understand the driving force involved. Under the conditions of our experiments there appear to be at least three processes, each of which has a net or directional component to it, which might supply the energy for the asymmetric fluxes. These three processes are bulk flow of water, active transport of sodium, and the movement of the hyperosmotic agent down its concentration gradient.

As mentioned previously the net water outflux reported is in the wrong direction to account for a net influx of solute. This would appear to rule out a simple solvent drag mechanism as the force responsible for the net solute influx. It does not, however, preclude a more complex mechanism by which water movement might serve as the driving force. However, in the absence of definitive information on localized movements of water within the skin itself, it does not appear profitable to pursue this point at this time.

That the net solute influx might in some way be coupled to net sodium transport must be considered because of the relationship demonstrated between sugars, amino acids, and sodium transport in other epithelial structures (14, 15).

Ussing (4) has shown a relationship between the asymmetric solute movement and active sodium transport on the basis of the following observations: (a) a linear relationship between net solute movement and the capacity to generate a short circuit current, (b) a linear relationship between the log of the sucrose flux ratio and the open circuit potential, (c) a loss of net sucrose movement after stoppage of sodium transport by 2 mM cyanide treatment, and (d) a loss of net sucrose movement after removal of sodium from the bathing solution.

Although the observations of Ussing suggest some relationship between asymmetric solute movement and sodium transport, the relationship may not be as direct as it seems. This is borne out by the fact that when large changes in SCC are brought about by cyanide (10^{-4} M) or ouabain (10^{-4} M) there is little change in the asymmetric fluxes.²

The lack of stoichiometry between the net solute flux and the net sodium flux suggests that the latter is not the direct driving force for the former. This does not preclude the possibility that a functioning sodium pump is an absolute requirement for net solute movement.

Further evidence against the pump being directly coupled to the process of solute movement is the fact that the solutes affected to the greatest extent are the extracellular molecules sucrose and mannitol. Even under hyperosmotic conditions these two molecules do not enter the cells of the frog skin. Since active sodium transport is generally considered to be a function of cell membranes, it seems unlikely that the two processes could interact.

A third source of energy for net solute movement may come from the movement of the hyperosmotic agent itself down its concentration gradient, e.g., 350 mM out vs. 0 in. It has been shown in free solution that the movement of one solute down a concentration gradient can cause the net movement of a second solute even though no concentration gradient exists for the second solute (16). This phenomenon, probably the result of frictional interaction between the two diffusing solutes may be occurring in the skin system; that is, the movement of the hyperosmotic agent (DMSO, thiourea, erythritol) down its concentration gradient will interact with the tracer species in the skin to increase its inward flux. Certainly the direction is correct. The net movement of the hyperosmotic agent is inward as is the net tracer solute movement. Although the magnitude of solute interactions is small in free solution, some special structural features in the skin may promote a greater interaction.

As a consequence of the above considerations it is to be expected that a

² Franz, T. J., and J. T. Van Bruggen. Data to be published.

nonpermeable hyperosmotic agent should not produce an alteration in the flux ratio. This is amply borne out by our previous studies with raffinose (17), which is essentially impermeable in frog skin. Although raffinose was capable of producing an increase in the bidirectional fluxes of urea and sucrose across the skin, a flux ratio of 1.0 was maintained. These studies suggest that there are two mechanisms by which hyperosmolarity may alter the permeability of the skin. The first is simply related to the concentration of a given solute in the outer bathing solution since so many different solutes, at least qualitatively, have similar effects in that they increase skin permeability. The second effect, the production of a flux ratio greater than 1.0, would seem to be related to the permeability of the hyperosmotic agent itself.

On the basis of the present studies it appears that the force required for the observed asymmetric fluxes cannot be precisely defined. The raffinose experiments suggest that it may be the movement of the hyperosmotic agent down its concentration gradient. However, the potential role of active sodium transport cannot be ruled out. In the absence of precise knowledge of all the forces involved it is not possible at this time to describe a mechanism by which the asymmetric fluxes are produced.

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