Further Observations on Asymmetrical Solute Movement across Membranes

T. J. FRANZ, WILLIAM R. GALEY, and J. T. VAN BRUGGEN

From the Department of Biochemistry, University of Oregon Medical School, Portland, Oregon 97201. Captain Franz's present address is General Delivery, Letterman General Hospital, San Francisco, California 94129. Address communications regarding this work to J. T. Van Bruggen, Department of Biochemistry, University of Oregon Medical School, Portland, Oregon 97201

ABSTRACT The permeability of frog skin under the influence of urea hyperosmolarity has been studied. Flux ratio asymmetry has been demonstrated again for tracer mannitol. The inhibitors DNP, CN^- , and ouabain have been used to eliminate active sodium transport and it was found that urea hyperosmolarity produces asymmetrical mannitol fluxes on frog skins having no short-circuit current. These findings suggest that flux ratio asymmetry is due to solute interaction and is unrelated to sodium transport. Studies with a synthetic membrane show clearly that bulk flow of fluid can produce a "solvent drag" effect and change flux ratios. When bulk flow is blocked and solute gradients allowed their full expression, then solute interaction "solute drag" is easily demonstrable in a synthetic system.

INTRODUCTION

It has been observed recently that, in frog skin, flux ratios (influx/outflux) greater than one may be produced for certain solutes by the presence of a hyperosmotic outer bathing solution (1-3). This implies a net inward transport for the tracer solute in the absence of a concentration gradient, in spite of the fact that a sizeable osmotic flow of water is proceeding in the opposite direction. The mechanism involved is as yet unknown. However, one might assume that this phenomenon is somehow coupled to active sodium transport because of the known dependence of intestinal sugar and amino acid transport on the sodium pump (4, 5). Indeed, Ussing (1) has stated that in frog skin the net, or asymmetrical, movement of sucrose may be abolished by complete removal of Na from the bathing solutions or by the addition of 2 mM cyanide, both methods causing cessation of active sodium transport.

An alternative explanation has been proposed by Franz and Van Bruggen (3). We have shown that the asymmetrical solute fluxes are also dependent upon the nature of the hyperosmotic agent. When a relatively nonpermeable

molecule such as raffinose is used as the hyperosmotic agent, flux ratios of one are observed. On the basis of this we suggested that an interaction between the hyperosmotic agent (as it moved down its concentration gradient) and the appropriate solute could account for the observed flux ratios. However, the possible role of sodium transport in this system as suggested by Ussing could not at that time be ruled out on the basis of our existing data. The present paper deals with studies on a variety of metabolic inhibitors and documents the essential independence of asymmetrical solute movement and sodium transport. Additional studies on synthetic membranes confirm the conclusion arrived at with frog skin, that an interaction between the hyperosmotic agent and other solutes may cause flux ratios to exceed one. Taken together, the studies illustrate the presence of a potent physical force which may explain asymmetrical solute movement.

MATERIALS AND METHODS

The abdominal skins of fall and winter *Rana pipiens* were mounted in small plastic chamber assemblies, each side of which had a volume of 1.6 ml. The area of skin exposed was 1.5 cm² and the two chamber assemblies available permitted simultaneous influx and outflux studies of tracer mannitol to be run on a single skin. For subsequent analysis, the solutions of the acceptor side were removed *in toto* by a suction device and fresh, nonlabeled solution added as replacement. Tracer movement is expressed in terms of the permeability coefficient P (cm hr⁻¹) which is the tracer flux divided by the concentration gradient.

The regular Ringer solution used contained Na⁺ 117, K⁺ 2, Cl⁻ 117, Ca⁺⁺ 0.25 meq/liter, was lightly buffered with phosphate, and adjusted to pH 7.9–8.0. The osmolarity of this solution was 220 milliosmols/liter. To this Ringer's solution, urea was added to a concentration of 220 mM producing a total osmolarity of 440 milliosmols/liter. Osmolarities were determined by freezing point depression on an Advanced Osmometer (model 66-31KS, Advanced Instruments, Inc., Newton Highlands, Mass.).

In all experiments, unless otherwise stated, the mounted skins were determined to have normal potentials and short-circuit currents, (SCC), before flux measurements were made. Urea-Ringer's was used as the outer bathing solution and regular Ringer's as the inner bathing solution. Tracer mannitol was added and the system allowed to equilibrate for some 30 min before beginning the sampling periods. Samples were taken at 15 min intervals over a 4 hr period. In the following figures only the 30 min points are plotted to simplify the presentation.

Inulin-¹⁴C (carboxy) and sucrose-¹⁴C (UL) were obtained from The New England Nuclear Corp., Boston, Mass. The molecular weight of the inulin was stated by the supplier to be 5000–5500. Mannitol-1-¹⁴C was obtained from Nuclear Research Chemicals Inc., Orlando, Fla. Water solutions of all tracers were purged with ¹²CO₂ in order to remove volatile impurities. Paper chromatography confirmed the class or group identity of the tracers.

In outflux studies, the "outside" urea-Ringer solution was renewed each 15 min by

the sampling procedure which introduced fresh urea-Ringer's. The "inside" labeled Ringer was replaced with fresh solution after 2 hr. In influx studies, the urea-Ringer was renewed after 2 hr. After this time the solution had decreased from its initial 440 to about 410 milliosmols/liter.

In experiments in which net volume changes were measured, the apparatus previously described (3) was used. In the studies on nonbiological membranes in which volume changes were held at zero, the length of the U tube leveling system was extended or a Hg-H₂O pressure system was used so that hydrostatic heads could be imposed.

FIGURE 1. Effect of urea hyperosmolarity on the permeability of frog skin to mannitol. The curves are drawn freehand between mean values and the vertical lines represent ± 1 SE. The numbers between the curves represent flux ratios (influx/outflux). At 1 hr urea-Ringer's (220 mM urea) was used to replace the outer bathing solution. Number of experiments was five each.

In the studies on synthetic membranes, the DiaFlo UM-3 membrane was used. This anionic, synthetic, hydrated polymer ultrafilter, which is thermally and dimensionally stable, is available from the Amicon Corp. (Cambridge, Mass.). The membrane is asymmetric, consisting of the polymer complex, approximately 75 μ thick, coated on a very porous backing, approximately 125 μ thick. The coated side is shiny when compared to the backing side and the terms shiny and dull are used in the manuscript to describe the sides of the membrane. We have determined that the UM-3 membrane used here had the following characteristics: L_p 1.8×10^{-10} cm³ dyne⁻¹ sec⁻¹; σ sucrose 0.02; P sucrose 20×10^{-3} cm hr⁻¹; and, P inulin 5×10^{-3} cm hr⁻¹.

RESULTS

Studies with Frog Skin

In order to establish the presence or reveal the absence of a direct coupling between active sodium transport and net solute movement induced by hyperosmolarity, a variety of methods was used to inhibit sodium transport. Urea was chosen as the hyperosmotic agent and mannitol-¹⁴C influx and outflux

values were determined (2, 3). The agents used to inhibit sodium transport were added to the test systems after urea had produced an asymmetrical flux of the extracellular solute, mannitol. The response of the system to urea hyperosmolarity alone, without the addition of inhibitor, can be seen in Fig. 1.

In these experiments the skin was bathed with normal Ringer's on both sides for 1 hr. During this interval the flux ratio is seen to approximate unity. In other studies, mannitol flux ratios of one or less were maintained for many hours when Ringer's bathed both sides of the skin.

Urea-Ringer's was introduced after 1 hr. The outflux is seen to increase linearly throughout the experiment while the increase in influx is seen to be

FIGURE 2. The effect of urea hyperosmolarity induced 30 min prior to zero time and continued through the experiment. The curves are drawn freehand between mean values. The vertical lines represent ± 1 SE. The numbers between the lines represent flux ratios (influx/outflux). Number of experiments was eight each.

less regular. The effect of urea is immediate, for by one-half hour, a flux ratio of 4.9 was obtained. A maximal value of 5.6 was found 1.5 hr after urea treatment. However, this value slowly declined over the period of observation. It is to be noted that the large flux ratios were retained in spite of the increase in skin permeability. These data are presented in part to show the rapidity of response to urea hyperosmolarity. There is a marked variation in the response of different skins after adding urea. Partly for this reason, the base period of subsequent figures is seen to vary somewhat in different groups.

Fig. 2 illustrates in another way the effect of urea hyperosmolarity on frog skin. In all the following frog skin experiments, the skin had a one-half hour equilibration period with urea-Ringer's and the curves of Fig. 2 represent the base line of comparison for the subsequent experiments. It is clear that in the absence of other treatment, the frog skin bathed with urea will show a continued increase in both mannitol influx and outflux P values throughout the experiment, and that deviation from the general shape of these curves will

represent the effect of an added factor. The lower flux ratios and generally lower P values of this group of skins are indicative of the variation seen from skin to skin as mentioned above.

The first method of interference with sodium transport consisted of the use of 1 mM 2,4-dinitrophenol (DNP). This agent was used to uncouple oxidative phosphorylation and thus deprive the sodium transport system of a sustaining supply of "high energy" phosphate. The results of this study are shown in Fig. 3. It is clear that DNP has a profound effect on the transport

FIGURE 3. The effect of 2,4-dinitrophenol (DNP) on urea hyperosmolarity. The curves are drawn freehand between mean values. The vertical lines represent ± 1 se. The numbers between the lines represent flux ratios (influx/outflux). The dotted line approximates the SCC before and also after the introduction of DNP. The values on the ordinate may be read as $\mu\text{a}/\text{cm}^2$ for the SCC. Number of experiments was five each.

process for the SCC begins to decrease within a few minutes of the addition of the agent and in one-half hour has decreased to about 25% of its starting value. While sodium transport had been grossly deranged, the mannitol influx showed only a minor initial change and then showed a general increase for several hours similar to that seen in the control Fig. 2. The flux ratio of greater than one shown for the system prior to the addition of the DNP did not change significantly during the period when the SCC was changing so drastically indicating a lack of coupling between sodium transport and the urea-induced asymmetric flux of mannitol. The flux ratio did subsequently decrease some but this was also seen in the control.

The second chemical inhibitor of transport used was 2 mM NaCN. Cyanide combines with cytochrome oxidase to prevent its transfer of electrons in the respiratory chain thus preventing high energy phosphate formation. Again, the large decline in SCC was not followed by a significant change in the asym-

metrical flux ratio.¹ In keeping with the observations seen with the other inhibitors, we are forced to conclude that there does not appear to be a coupling of sodium transport and/or intermediary metabolism with asymmetrical solute fluxes in the frog skin system. These results appear to differ from those of Ussing (1) who stated that 2 mM CN^- abolished the asymmetrical sucrose flux produced by urea.

The third method involved the use of 1 mM ouabain to inhibit sodium transport. Ouabain is assumed to exert its chief action on an ATPase system that is

FIGURE 4. The effect of cyanide (CN^-) on urea hyperosmolarity. The lines are drawn freehand between mean values and the vertical lines represent ± 1 SE. The numbers between the lines represent flux ratios (influx/outflux). The dotted line approximates the SCC before and also after the introduction of CN^- . The values on the ordinate may be read as $\mu\text{a}/\text{cm}^2$ for the SCC. Number of experiments was eight each.

related to the transport mechanism. As shown in Fig. 4, ouabain caused a decrease in SCC similar to that caused by DNP and CN^- . During the decrease in SCC and after the current was essentially zero, flux ratios greater than one were maintained. This agent has a somewhat different effect on the mannitol fluxes. Instead of the gradual increase seen in Figs. 2 to 4, the fluxes remained constant and the corresponding flux ratios remained constant as well, in contrast to the usual decreases seen in fluxes and their ratios.

In another series of experiments, not being reported in detail, the inhibitors DNP, CN^- , and ouabain were added to mounted skins and the SCC monitored. When the SCC had fallen to essentially zero and the sodium pump could be considered to be inoperative, urea hypertonicity was imposed upon

¹Note Added in Proof Addition of the NaCN to the lightly buffered Ringer solution results in a recently noted increase in pH. When buffered NaCN is added, the decrease in SCC is obtained as before, and flux ratios greater than 1.0 are maintained but the increase in P values is less.

the system. With few exceptions, the systems demonstrated subsequent asymmetric mannitol fluxes and ratios of 2 to 6 were obtained. In most cases the influx and outflux values obtained at equivalent time periods were well within the range of values of the fluxes shown in Figs. 3 to 5.

The question then arises as to the nature of the force operative in the asymmetry of fluxes. As we previously suggested (2, 3), the movement of hypertonic solute down its concentration gradient and its interaction with another solute appear to answer the need for the driving force required for asymmetry.

FIGURE 5. The effect of ouabain on urea hyperosmolarity. The lines are drawn freehand between mean values and the vertical lines represent ± 1 SE. The numbers between the lines represent flux ratios (influx/outflux). The dotted line approximates the SCC before and also after the introduction of ouabain. The values on the ordinate may be read as $\mu\text{a}/\text{cm}^2$ for the SCC. Number of experiments was five each.

Studies with Nonbiological Membranes

The studies described above indicate that asymmetrical solute fluxes induced by hyperosmolarity are not dependent upon active sodium transport per se or on the continuation of metabolic processes sensitive to DNP, CN^- , or ouabain. The studies, however, do not eliminate the possibility that changes in a living, dynamic system such as frog skin may in some unknown way be responsible for the observed phenomena. In other words, the presence of uncontrollable variations inherent in biological systems makes it difficult to preclude their participation in the production of the asymmetrical fluxes reported here. The use of a nonbiological membrane circumvents this problem. If asymmetrical flux ratios can be demonstrated in systems containing structurally stable, synthetic membranes, then a strong argument can be made for physical forces as the causative agents of the flux asymmetry. To this end we have studied a variety of synthetic membranes with 0.3 M sucrose as the "hy-

perosmotic agent” and tracer amounts of inulin- ^{14}C as the solute species whose flow is modified.

Studies with the DiaFlo UM-3 membrane mounted in place of frog skin in the apparatus previously described (3) are shown in Table I. In series 1 and 2, inulin fluxes were determined in the absence of hydrostatic or osmotic gradients.

As is to be expected, with identical Ringer's or Ringer's-sucrose solutions bathing both sides of the membrane, flux ratios not differing from unity were observed. When 0.3 M sucrose was included in the Ringer on one side (series 3) and $115 \mu\text{l hr}^{-1} \text{cm}^{-2}$ osmotic flow into this hyperosmotic solution allowed to proceed, the flux in the direction of flow increased while that in the direction opposite to the flow decreased resulting in a flux ratio of 0.2.² This flux ratio of less than one demonstrates the phenomenon of solvent drag as described by Andersen and Ussing (6) and it would at first glance appear that flux ratios greater than one cannot be produced in the UM-3 membrane. However, it must be noted that bulk flow of water through a “wide-pore” membrane such as the UM-3 will have a much greater effect on solute fluxes than would a comparable flow across a “narrow-pore” membrane such as frog skin. This is shown quite clearly by an equation derived by Kedem and Katchalsky (7) and rewritten as:

$$J_s = P\Delta C_s + \bar{C}_s(1 - \sigma)J_v$$

where P is the permeability coefficient; ΔC_s the concentration gradient across the membrane; $\bar{C}_s$ is the mean concentration in the membrane; σ is the reflection coefficient; J_v the volume flow; and, J_s the solute flux across the membrane.

The first term of the equation represents the contribution of diffusion while the second shows the effect of solvent flow (solvent drag) on the transmembrane flux of solute. In the case of frog skin, since σ (mannitol) closely approximates one the second term will be very nearly zero. Thus in frog skin, bulk flow of solvent contributes little to the flux of large intercellular molecules such as mannitol. However, with the wide-pore UM-3 membrane where σ is small, the contribution of solvent flow to J_s becomes appreciable. As a result, the presence of a force such as solute drag may well be masked, and in order to demonstrate it, the solvent drag effect must be blocked.

In series 4, hydrostatic heads sufficient to reduce net volume flow to essentially zero were imposed on the system cited in series 3. In this condition the

²The flux ratio for the synthetic membrane studies is defined as:

$$\frac{\text{Flux of the tracer out of the hyperosmotic solution}}{\text{Flux of tracer into the hyperosmotic solution}}$$

Flux of tracer into the hyperosmotic solution

Thus, a flux ratio greater or less than one has the same meaning as did ratios for frog skin where the influx represented movement out of the hyperosmotic solution.

flux in the direction out of the hyperosmotic solution increased while that in the opposite direction decreased. These flux values yield a flux ratio of 13.7 which is much greater than 1.0. Since in this experiment there was no net volume flow, the only obvious force which could cause a net flux across the membrane is the gradient created by the diffusible hyperosmotic agent. Thus,

TABLE I
INULIN FLUX AND FLUX RATIOS WITH DIAFLO UM-3 MEMBRANES

Se- ries	No. of samples	Bathing solution	Net volume flow	Tracer*	Flux†	Ratio§	Conclu- sion
1	20	Ringer's on both sides	None	D→S	5.9±0.6	1.3	=1.0
	12		None	S→D	4.5±0.3		
2	16	0.3 M sucrose-Ringer's on both sides	None	D→S	4.0±0.4	1.0	=1.0
	14		None	S→D	4.0±0.5		
3	18	0.3 M sucrose-Ringer's on D side	Osmotic flow allowed	D→S	1.6±0.2	0.2	<1.0
	15			S→D	8.9±0.5		
4	12	0.3 M sucrose-Ringer's on D side	Osmotic flow blocked by hydrostatic head	D→S	23.3±0.8	13.7	>1.0
	21			S→D	1.7±0.2		
5¶	8	0.3 M sucrose-water on on S side	Osmotic flow blocked by hydrostatic head	S→D	23.0±1.3	12.1	>1.0
	8			D→S	1.9±0.2		
6¶	12	Water on both sides	None	S→D	3.6±0.2	1.1	=1.0
	12		None	D→S	3.2±0.3		

* The symbols S → D refer to the movement of tracer inulin from the solution bathing the shiny surface to the solution bathing the dull surface.

† Flux as $\mu\text{mole cm}^{-2} \text{ hr}^{-1} \times 10^3$ with the concentration of tracer assumed to be 1 μM .

§ ratio = $\frac{\text{flux of tracer out of the hyperosmotic solution.}}{\text{flux of tracer into the hyperosmotic solution}}$

|| As indicated by "t" test of difference from 1.0 with a confidence level of 0.95 or better.

¶ A UM-3 membrane similar to those in 1-4 above but with a different lot number.

a flux ratio of 13.7 illustrates in a striking way the significance of solute interactions, for the mere blocking of solvent flow allowed the flux ratio to increase from 0.2 to 13.0.

That the flux ratio much greater than one is not due to instrumental error or our inability to hold the volume flow to precisely zero is validated by experiments not reported in detail in which a hydrostatic head was applied to a similar membrane (but with a different lot number) to produce a flow of $\sim 24 \mu\text{l hr}^{-1} \text{ cm}^{-2}$ which resulted in a flux ratio of ~ 12 . Since it required this large a volume flow to produce a flux ratio of ~ 12 , it is unlikely that the result shown in series 4 is due to our inability to maintain the volume flow at exactly

zero, for our measurements of the net volume change were equal to or less than $2 \mu\text{l cm}^{-2} \text{ hr}^{-1}$.

It was considered possible that the asymmetrical nature of the UM-3 membrane might be responsible for the asymmetrical flux cited in series 4. To eliminate this possibility, a UM-3 membrane was reversed, the hyperosmotic solution was added to the "S" side, and osmotic flow was blocked as before. This condition (series 5) is identical to that of series 4, with the membrane turned 180 degrees. It is clear that flux ratios greater than one are obtained and that the fluxes themselves are of the same magnitude as before. It is acknowledged that an additional experimental detail was altered, for, water and water-sucrose solutions were used in series 5 and 6 in place of Ringer's and sucrose-Ringer's solutions. The validity of this approach is attested to by the similarity of the flux values under the two conditions. The data also suggest that the electrolyte composition of the bathing solutions does not play a significant role in the observed phenomena.

Preliminary studies similar to those reported above have been conducted on a variety of other membranes. Details of the final experiments will be reported in a subsequent paper. It is clear, however, that the structure of the membrane is closely related to the magnitude of the flux asymmetry. With dialysis tubing and some cellophane preparations, it is difficult to obtain ratios significantly greater than 1.0 with the limited number of solute pairs used to date. Flux ratios near 2.0 have been obtained on an uncoated cellophane membrane (Crown Zellerbach Lustro-Roll, Crown Zellerbach Corp., Portland, Ore.).

DISCUSSION

The evidence presented in this and in the preceding papers allows the formulation of certain conclusions: (*a*) flux ratio asymmetry opposite to that caused by bulk solvent flow can be demonstrated when a membrane separates solutions of unequal solute composition; (*b*) the membrane must be permeable to the solute of the more concentrated solution (hyperosmolar) to permit the flux asymmetry; (*c*) the asymmetry demonstrable with certain solutes and the frog skin system is not directly related to sodium transport or to metabolic processes supportive of it; (*d*) the flux asymmetry is not restricted to biological systems but is also demonstrable with synthetic membranes using suitable solute pairs; and, (*e*) the magnitude of the asymmetry appears to be a function of the characteristics of the membrane and/or of the solute pairs used.

These conclusions appear to support our original suggestion that net movement of one solute may result from its interaction with a second solute moving down a concentration gradient. Dunlop (8) has shown that such interaction can occur in free solution in the presence of an initial concentration gradient for only one of the components. In such systems, the cross-term coefficients

will have nonzero values. Our systems differ from those of Dunlop in that a membrane structure has been interposed between solutions of unequal concentration and the interaction we are discussing is expressed in terms of transmembrane fluxes. It is quite likely that the presence of the membrane introduces special conditions not present in the work with free solutions and it is also possible that the membrane itself becomes a component in the system of interaction.

Although it is recognized that the eventual description of the mechanisms of the interaction and the precise quantitation of the forces involved may well be a formidable task, it is, however, possible to speculate on mechanisms for the solute interaction. The interaction forces are possibly frictional in nature and result from collisions between solute species or interaction between the fields of force surrounding the solute. In a free, homogeneous solution the kinetic energy of interaction would be expected to dissipate in all directions and not lead to a net movement of solute. When a concentration gradient is introduced, a directional force is available which may result in a net movement of the tracer solute. In this instance the tracer species can be visualized as being swept along with the flow of a solute.

With the introduction of a membrane into the system, new possibilities for interaction occur. Consider only a simple system in which the hyperosmolar solute and the tracer species may both cross the membrane barrier by way of water-filled spaces. At the entrance to and in the interstices of the membrane the movements of the solutes are likely to be more restricted and the directional component imparted to the tracer by the hyperosmotic agent enhanced. It is possible to conceive of conditions associated with biological systems in which solute drag would play a significant role in solute transport across membranes. Indeed, this mechanism may be operative in certain transport systems in which the movement of solute has been loosely termed "active" or in systems in which transport of solute requires the presence of other solutes.

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