J. Membrane Biol. 12, 159–176 (1973)

Urea Transport in the Toad Bladder; Coupling of Urea Flows

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Received 14 July 1972

Summary. Urea transport across amphibian membranes is influenced by interactions with the membrane, the solvent and other solutes. One case of solute interaction, that in which the two species are chemically identical, is investigated here. Because of the effects of hypertonic urea on permeability, the demonstration of interaction required consideration of the ratio r of bidirectional tracer permeabilities. Mucosal-to-serosal ($M \rightarrow S$) and serosal-to-mucosal ($M \leftarrow S$) tracer urea fluxes were determined in paired toad urinary bladders, in the absence and presence of abundant urea. In the control state, r was 1.0. Addition of 0.3 M urea to M increased r, and to S decreased r. These results indicate coupling of abundant and tracer urea flows (isotope interaction), probably occurring in specialized regions. The effects persisted after the addition of antidiuretic hormone, despite the opposing influence of osmotic water flow. Quantitatively different effects of mucosal and serosal hypertonicity, both with and without antidiuretic hormone, are explicable in terms of heterogeneous parallel and series permeability barriers.

Because of the importance of urea in the renal concentrating system, the mechanism of its transport is of considerable interest. Amphibian epithelia are useful model systems for the study of this process and have directed attention to three types of interactions.

First, interaction of urea or its congeners with the membrane, evaluated by tracer permeability in the frog skin and toad bladder, has allowed speculation as to the mechanism of permeation [2, 15, 17, 19]. Second, interaction with water (solvent drag) has been cited as evidence that these compounds share a common pathway with water [2, 17]. A third type of interaction, that of urea with other solute particles, has been observed in the frog skin and toad skin [3, 10, 12, 22, 23].

The findings and interpretations concerning urea-solute interactions have not been consistent. In the toad skin, Ussing and Johansen described net inward movement of sucrose and tracer urea induced by the addition of abundant "cold" urea to the external medium [23]. Because of the non-specificity of the effect they believed direct chemical interaction to be unlikely, and suggested instead a transfer of momentum mediated by the solvent. This "anomalous solvent drag" was attributed to tissue asymmetry such that external hypertonicity would create a circulation of solvent in the cell interspaces, carrying solute molecules inward despite net outward water flow. However, Biber and Curran have described studies of urea and mannitol fluxes in the toad skin [3] in which reversal of the direction of the urea concentration difference resulted in reversal of net mannitol flux. This was considered likely to represent coupling between solute molecules, as suggested also by Franz and Van Bruggen [11] and Franz, Galey and Van Bruggen [10] in studies of the frog skin. More recently, in studies of the effects of hypertonic urea in the toad bladder, Urakabe, Handler and Orloff noted no flux asymmetry with either sucrose or tracer urea [21].

Because chemically different species may utilize different pathways, the significance of their interaction is unclear. Therefore, the study of chemically identical species, which must traverse the same pathways, is of particular interest for the insights it might provide concerning the nature of membranes and mechanisms of permeation. In the present experiments, we investigated interaction of abundant and tracer urea flows in the toad urinary bladder. To facilitate the demonstration of small effects, the experiments were carried out in paired hemibladders which were derived from a single toad and treated identically insofar as possible.

Materials and Methods

I. Biological Membranes – General Methods

Toads (*Bufo marinus*) from the Dominican Republic (National Reagents, Bridgeport, Connecticut) were kept on damp paper without feeding, and sprinkled with tap water daily. After the animals were doubly pithed the bladders were removed and rinsed in 50 ml of sodium Ringer's solution (NaR) (Na 113.5, Cl 116.9, K 4.0, HCO_3 2.4, Ca 1.8 mEquiv/liter, pH 7.5, 222 mOsm/Kg H_2O) three times to remove endogenous antidiuretic hormone. For coupling studies hemibladders were mounted in standard Ussing-Zerahn Lucite chambers of 7.54 cm^2 area, fitted with 3.0 M KCl agar bridges. The transmembrane potential was controlled with a voltage clamp. Except for brief periods for the determination of electrical resistance the membranes were "short-circuited."

When antidiuretic hormone (Pitressin, Parke Davis) (ADH) was used it was added to the serosal bath to give a concentration of some 130 mU/ml.

Studies of volume flow (J_v) were performed in a modified chamber in which one side was sealed except for a calibrated horizontal pipette; stirring was by magnetic rotors.

II. Selection of Paired Tissues

Both hemibladders from a single toad were removed, rinsed, mounted in identical chambers, and bathed in NaR. Following 15 to 30 min of equilibration the open circuit potential was measured. All pairs in which either hemibladder had a value of less than 15.0 mV were discarded. Membranes which were retained were short-circuited, and after an additional 15 to 30 min the electrical resistance was determined from the change in steady-state current induced by rapidly varying the transmembrane potential from -10.0 to $+10.0$ mV. Prior studies have shown that the current-voltage relationship is linear in this range, permitting the use of Ohm's law [6].[1] All pairs in which either membrane had an initial resistance of less than 300 Ω (corresponding to a conductance of 0.442 mmho cm^{-2}) were discarded, except in preliminary experiments. Membranes which satisfied the above criteria were short-circuited and utilized in one of the following protocols.

III. Protocols—Toad Bladder

A. Preliminary Experiments—Effects of Hypertonic Urea. Tracer C-14 urea (New England Nuclear Corporation, Boston, Massachusetts) was added to both mucosal baths *M* or to both serosal baths *S* of a pair. Nonradioactive urea was added to the mucosal bath or to the serosal bath of one hemibladder of each pair to produce a concentration of 0.3 M urea. Following equilibration, samples for measurement of radioactivity were taken at 0, 30 and 60 min.

B. Coupling of Flows—0.3 M Urea. Tracer C-14 urea was added to the mucosal bath of one hemibladder of a pair and to the serosal bath of the other. After 15 min of equilibration, initial, 30- and 60-min samples were taken from each bath. Following this *control* period, nonradioactive urea was added either to both mucosal or to both serosal baths to produce a concentration of 0.3 M urea (*hypertonic* period). After 15 min of re-equilibration, initial, 30- and 60-min samples were again obtained. Following this, ADH was administered. After waiting 10 min for the onset of peak effect, initial, 30- and 60-min samples were again taken, constituting the *ADH* period.

C. Coupling of Flows—Long Control Period. This protocol was similar to that of Section III-B, but with a 120-min *control* period, a 90-min *hypertonic* period, and no *ADH* period.

D. Coupling of Flows—0.1 M Urea. The protocol was identical to that of Section III-B except that the concentration of abundant urea was 0.1 M.

E. Estimation of Reflection Coefficient for Urea. Unpaired membranes mounted in the volume flow chamber were bathed with 0.3 M urea in NaR at one surface [either mucosal (series I) or serosal (series II)] and 0.2 M sucrose in NaR at the other surface. Measurements of volume flow were carried out at 5-min intervals for 15 to 30 min.

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1 *Also* T. Saito, P. D. Lief and A. Essig: Conductance of active and passive pathways in the toad bladder. (*Submitted for publication.*)

Following the addition of ADH, measurements were continued for another 15 to 30 min. The added solute of the serosal bath was then increased to 0.3 M sucrose in series I or 0.45 M urea in series II, and J_v was measured for a final 15 to 30 min.

F. Attempts to Abolish Water Flow. This protocol was identical to that of Section III-B except that in the *hypertonic* period sucrose was added to give a concentration of 0.2 M in the solutions opposite to those containing 0.3 M urea.

IV. Analysis of C-14 Activity

For each flux period 100- to 500-μliter samples from each bath were placed in sealed vials containing 15.0 ml of liquid scintillation fluid [14]. Samples were counted on the day of the experiment whenever possible, or at latest within 96 hr. If not counted promptly, samples were stored in a cool dark place; such samples showed no change in counts for periods of up to four weeks. Samples were counted in a Packard Tri-Carb Liquid Scintillation Counter with an efficiency greater than 95%. Quenching by NaR, 0.3 M urea, 0.2 M sucrose, or ADH was negligible.

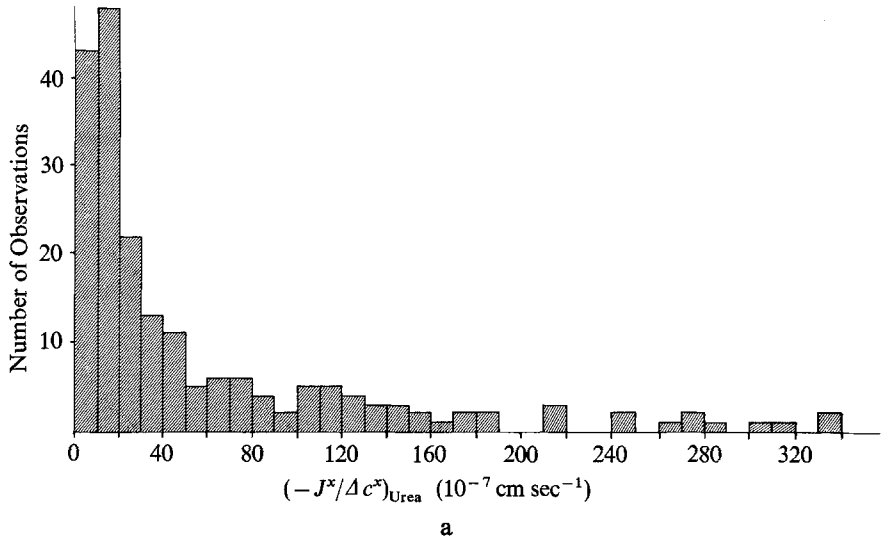
V. Analysis of Data

Tracer permeability was evaluated as $-J^x/\Delta c^x$, where J^x is the tracer flux per unit area per unit time, and Δc^x is the concentration difference of the tracer across the membrane. Placement of radioactivity in the mucosal or serosal solution permitted the determination of $M \rightarrow S$ or $M \leftarrow S$ tracer permeability, denoted $\overrightarrow{J^x/\Delta c^x}$ or $\overleftarrow{J^x/\Delta c^x}$, respectively. For each experimental period the tracer permeability coefficient utilized was the geometric mean of the values of two sequential 30-min determinations. In addition to the initial determination of the resistance of all membranes for purposes of screening, in some cases the conductance was determined systematically near the beginning and end of each period; the mean value was used for further analysis. Statistical analyses were carried out as described by Snedecor and Cochran [20]. Results are presented as the mean $\pm$ the standard error (SE). Means were compared by analysis of variance. Slopes and intercepts were calculated by the method of least squares. Comparison of slopes and intercepts was by analysis of covariance; p values >0.05 were considered insignificant and are not reported.

Results

I. Distribution of Permeability Data

Preliminary inspection of the values for urea permeability ($-J^x/\Delta c^x$) showed a skewed distribution (Fig. 1*a*). Conversion to logarithms produced an apparently more normal (bell-shaped) distribution (Fig. 1*b*), and normal equivalent deviate analysis confirmed this impression [20]. Consequently, all values of ($-J^x/\Delta c^x$) were converted to logarithms for statistical analysis, and all average values cited are geometrical means.



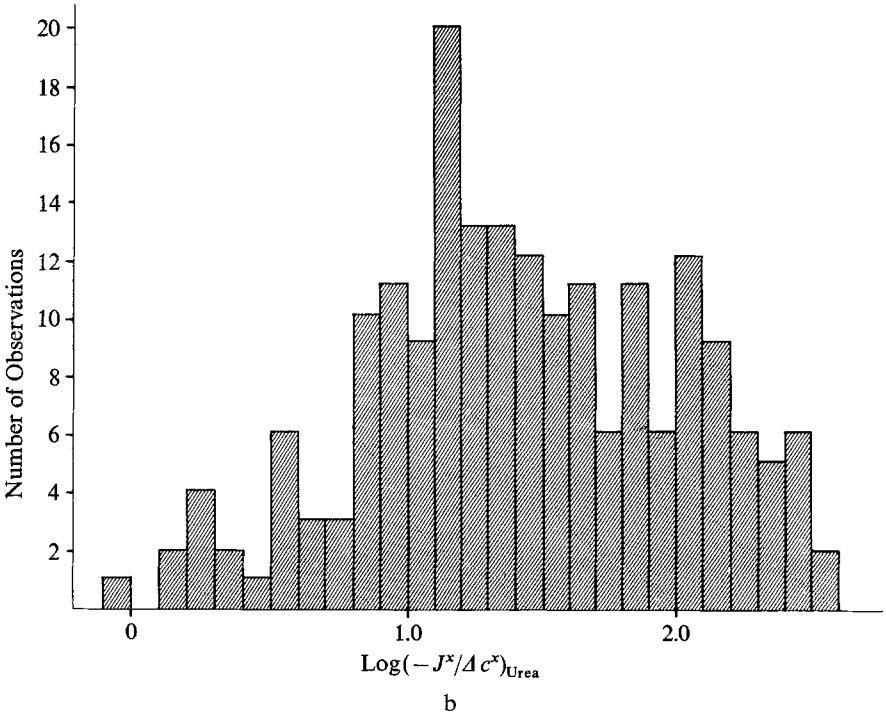


Fig. 1. (*a*) Frequency distribution of tracer urea permeability coefficients. $(-J^x/\Delta c^x)$ is expressed in units of 10^{-7} cm sec^{-1}. Each bar represents the number of observations within the designated interval. The data include both $M \rightarrow S$ and $M \leftarrow S$ measurements. (*b*) Data from Fig. 1*a* after conversion of $(-J^x/\Delta c^x)_{urea}$ to log $(-J^x/\Delta c^x)_{urea}$

II. Preliminary Experiments – Effects of Hypertonic Urea

$M \rightarrow S$ tracer urea permeability was determined in six pairs of hemibladders in which one tissue was treated with 0.3 M urea in either the mucosal or the serosal bath, the other tissue serving as a control. In six identically treated pairs $M \leftarrow S$ permeability was determined. The six hemibladders in which abundant urea was added to M showed larger tracer permeabilities than the paired controls. Conversely, in five of six cases abundant urea in S was associated with lower tracer permeabilities than in the paired controls. Usually the effects on tracer permeability were associated with parallel effects on electrical conductance, but both showed considerable variability in magnitude.

In the face of similar effects of hypertonic urea on $M \rightarrow S$ and $M \leftarrow S$ tracer permeability, but with appreciable variability, the above experiments provided no evidence of interaction between abundant and tracer urea flows.

III. Coupling of Flows – 0.3 M Urea

To investigate the possibility of interaction of urea flows, hemibladders from a single animal were treated identically with abundant urea, one membrane being used for the determination of $M \rightarrow S$ tracer permeability and the other for $M \leftarrow S$ tracer permeability. With coupling to the flow of abundant urea the two permeabilities should be affected differently. To interpret any differences, however, it was necessary first to ascertain the relationship between the two tracer permeabilities in the absence of abundant urea.

A. Control Period. Data from 32 experiments prior to the addition of hypertonic urea are shown in Fig. 2. Over a wide range of values (1.07 to 235×10^{-7} cm sec^{-1}) there was an impressive correlation between the $M \rightarrow S$ and $M \leftarrow S$ tracer permeabilities of paired hemibladders (correlation coefficient $= 0.94$; $p < 0.001$). Since the intercept of the least-squares line differs insignificantly from zero it is justified to calculate a line passing through the origin. The slope (0.995) differs insignificantly from 1.000, indicating equality of the two tracer permeabilities [19], permitting the construction of a ratio $r = \overrightarrow{(J^x/\Delta c^x)}/\overleftarrow{(J^x/\Delta c^x)}$ for each pair of hemibladders. This provides a convenient means of analysis of the data.

In eight experiments the effect of time upon r was investigated. The slope was 0.995 in the first hour, and 0.982 in the second hour. The two values differed insignificantly from each other and from 1.000.

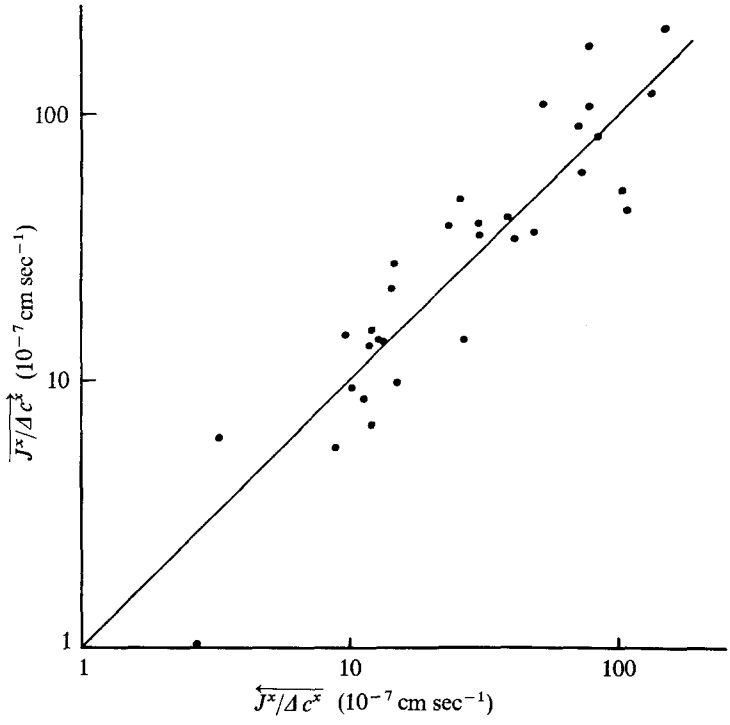


Fig. 2. Relationship of $M \rightarrow S$ and $M \leftarrow S$ tracer urea permeabilities in paired hemibladders. The least-squares line of the data does not differ significantly from the line of equality, $y = 1.000\,x$ (drawn in figure)

B. Mucosal Hypertonicity. In 11 experiments, following the *control* period, nonradioactive urea was added to the mucosal baths of both hemibladders to a concentration of 0.3 M. Increases in the bidirectional tracer permeabilities were noted in 17 of 22 cases. In the 12 cases in which the conductance was systematically evaluated the mean value increased from 0.19 to 2.45 mmho cm^{-2}, an effect described previously by Urakabe *et al.* [21]. Despite these changes, effects on paired hemibladders were similar and the comparison of r in the *control* and *hypertonic* periods permitted the evaluation of coupling. The data are presented in Fig. 3. The (geometric) mean of r increased significantly from 0.832 (mean log $r = -0.080 \pm 0.075$) to 1.22 (mean log $r = 0.087 \pm 0.037$) ($p < 0.001$). These results indicate coupling of flows of abundant and tracer urea.

Following the *hypertonic* period, antidiuretic hormone was added. This further increased the tracer permeabilities, as expected. Moreover, there was a further significant increase in r, to a mean value of 1.82 (mean

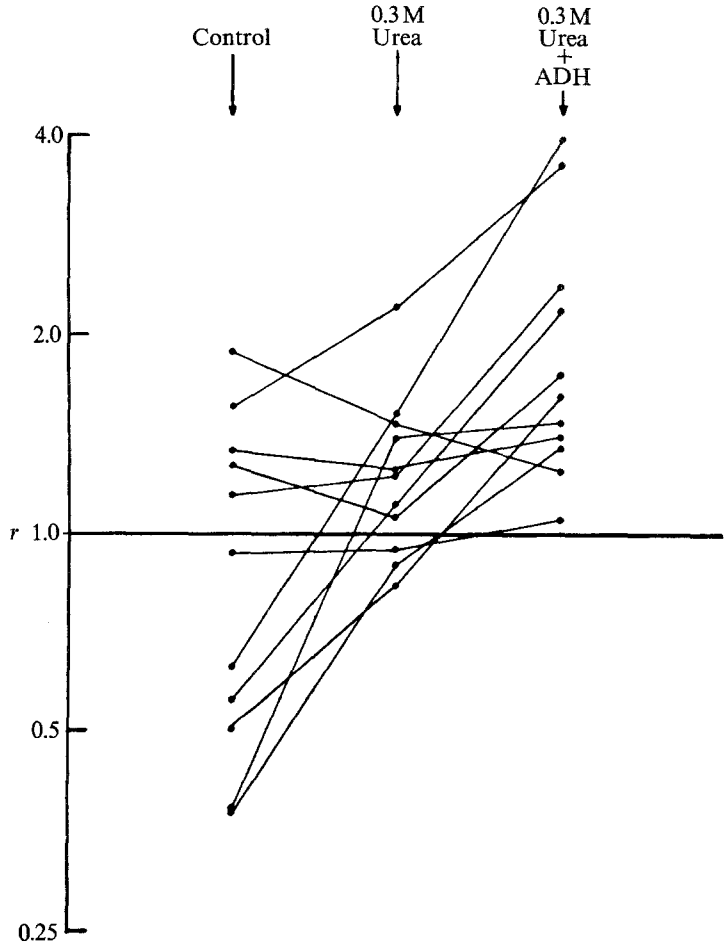


Fig. 3. Effect of mucosal hypertonic urea and ADH on the ratio r of unidirectional tracer urea permeabilities in paired hemibladders. After a 1-hr control period 0.3 M urea was added to both mucosal baths. An hour later, both tissues were treated with ADH

$\log r = 0.261 \pm 0.056$) ($p < 0.001$) in the *ADH* period. Again this is consistent with the coupling of flows.

C. Serosal Hypertonicity. In 12 experiments, following the *control* period, urea was added to the serosal bath to a concentration of 0.3 M. Decreases in the bidirectional tracer permeabilities were noted in 23 of 24 cases; in the 16 cases examined systematically the mean conductance decreased from 0.27 to 0.16 mmho cm^{-2}, again consistent with the data of Urakabe *et al.* [21]. Despite these changes in individual tissues, pairing and the examination

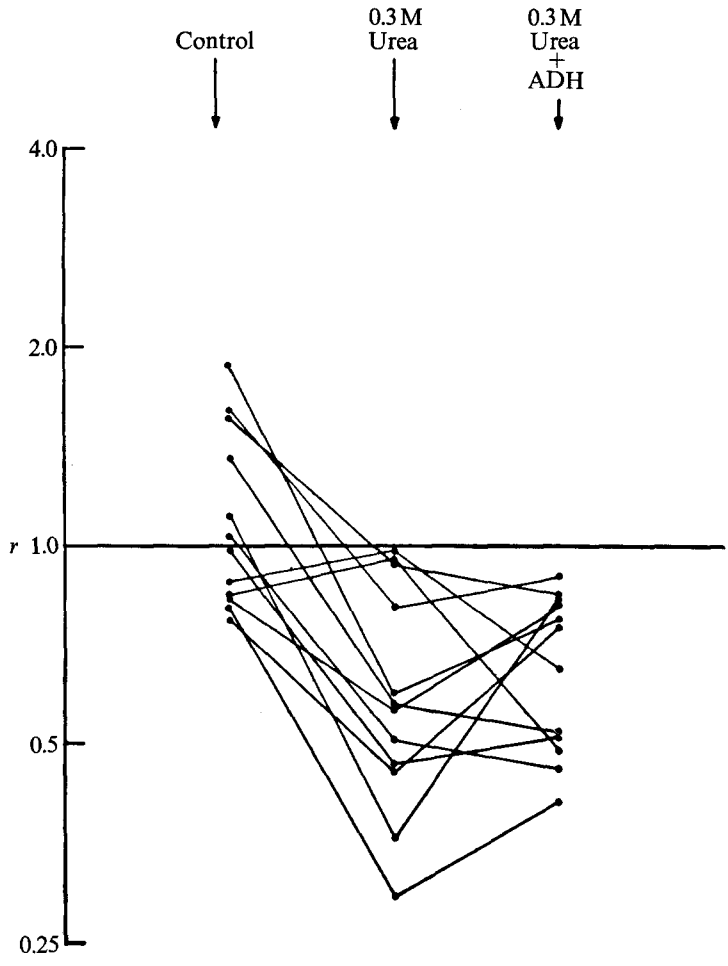


Fig. 4. Effect of serosal hypertonic urea and ADH on the ratio r of unidirectional tracer urea permeabilities in paired hemibladders. After a 1-hr control period 0.3 M urea was added to both serosal baths. An hour later, both tissues were treated with ADH

of r permitted the evaluation of coupling (Fig. 4). Mean r decreased significantly from 1.09 (mean $\log r = 0.037 \pm 0.038$) in the *control* period to 0.585 (mean $\log r = -0.233 \pm 0.049$) in the *hypertonic* period ($p < 0.001$). Again the results were consistent with the coupling of flows of abundant and tracer urea.

As in previous cases, the addition of ADH produced the expected increases in tracer permeabilities. However, the mean value of $r = 0.644$ ($\log r = -0.191 \pm 0.035$) was not significantly different in the *ADH* period.

It should be noted that in the presence of ADH, both with mucosal and with serosal hypertonicity, the effects on r were demonstrated despite the opposing influence of volume flow and presumed solvent drag.[2]

IV. Coupling of Flows – 0.1 M Urea

In contrast to the case with 0.3 M urea, the effects of 0.1 M urea on tracer permeabilities and electrical conductances were less consistent, even for members of a pair. No significant change of r was noted in either the *hypertonic* or *ADH* periods in four experiments with mucosal hypertonicity or in four experiments with serosal hypertonicity. Thus, we were unable to demonstrate coupling at this concentration of urea.

V. Attempts to Abolish Volume Flow

As noted in Section III, effects on r were demonstrated despite the opposing influence of volume flow.[2] To demonstrate the extent of isotope interaction more precisely, an attempt was made to study coupling while abolishing the volume flow resulting from the concentration gradient of urea.

It has been reported that in the toad bladder the reflection coefficient for urea is about 0.67 and that for sucrose is 1.0 [13]. Accordingly, 0.2 M sucrose should approximately balance the osmotic effect of 0.3 M urea. This was tested by exposing the tissues to 0.3 M urea in NaR at either surface, and to 0.2 M sucrose in NaR at the other. Volume flow was reduced from a value of some 200 μliters cm^{-2} hr^{-1} for 0.3 M urea/NaR to undetectable levels (less than 10 μliters cm^{-2} hr^{-1}). When additional solute was later added to the serosal bath the volume flows increased promptly, and in proportion to the new osmotic gradient, indicating the validity of the previous negative observations.

Since 0.2 M sucrose essentially abolished the water flow induced by 0.3 M urea, determinations of inward and outward tracer urea permeabilities

2 It has recently been reported that, in conformity with findings in the red cell [18], the administration of phloretin to the toad bladder produces a virtually complete inhibition of vasopressin-induced urea movement, but has no effect on water flow [9]. Furthermore, upon vigorous stirring apparent solvent drag of acetamide is eliminated. These findings have been interpreted as indicating that urea-like amides and water may traverse independent pathways induced by vasopressin [9]. Be this as it may, with the gentle air-lift mixing techniques employed here the existence of volume flow directed toward the solution of higher urea concentration would interfere with the demonstration of positive coupling of urea flows [17].

were carried out in eight pairs of membranes, utilizing these balanced solutions in the *hypertonic* period. It was apparent that the use of hypertonic solutes in both baths rather than in one produced more variable effects upon unidirectional tracer permeability and conductance. Moreover, effects were more variable within a pair of tissues than had been previously noted for hypertonic urea alone. No consistent effects on r were observed in either the *hypertonic* or *ADH* periods; thus coupling could not be demonstrated under these conditions.

Discussion

With appreciable coupling between abundant and tracer flows a tracer permeability coefficient will fail to quantify the permeability coefficient for net flow. Although the coupling which we have demonstrated is slight, and of no importance in this regard, it is of interest for two reasons: first, the illustration of a technique for the detection of small changes in tissue permeation, and second, the implications as to the mechanism of urea permeation in the toad bladder.

Since it has been felt that urea traverses the toad bladder by an aqueous channel it might have been anticipated that urea-urea interaction would be difficult to demonstrate. In an analysis of data from the literature, Curran, Taylor and Solomon pointed out that at the concentrations employed here, effects of isotope interaction in nonselective membranes would be hidden by the error in estimating unidirectional fluxes [7]. Our preliminary experiments showed that changes in unidirectional tracer permeability, whether spontaneous or induced by hypertonic urea, were far greater than attributable to coupling. We therefore abandoned this approach for one permitting the comparison of bidirectional tracer flows.

Ideally, to minimize nonspecific effects one would simultaneously evaluate both flows in a single membrane. While this is possible [19], it requires the use of N-15 urea and mass spectrometry, which were unavailable to us. Instead, therefore, we used two closely paired membranes exposed to tracer urea at opposite surfaces. Close pairing meant that both hemibladders were from a single toad; that they were handled at the same time; that they were screened according to the same electrical criteria; and that they were treated identically (including exposure to abundant solute and ADH). With careful attention to these details we found an excellent correlation between simultaneously determined bidirectional permeability coefficients in control studies (Fig. 2).

Fig. 5 illustrates how paired tissues can distinguish between effects on permeability and coupling. The upper panel shows the equivalence of control bidirectional tracer permeabilities. The addition of hypertonic solute to the same side of each membrane (center panel) may increase (or diminish) the tracer flows, but in the absence of coupling the effect in each tissue is the same and r remains equal to 1. In contrast, with coupling the two flows are affected differently (lower panel); hence, in spite of changes in both tracer permeabilities, coupling is identified and quantified by a change in the value of r from 1. (It is possible to demonstrate coupling even if the control value of r differs from 1, although less conveniently.)

By this means, in our studies with 0.3 M urea, coupling was shown consistently. Whether solute was added to the mucosal or the serosal bath, with opposite effects on conductance, positive solute-solute interaction was demonstrated.[3] These findings are not explicable in terms of the "anomalous solvent drag" invoked by Ussing and Johansen to explain the interaction between sucrose and urea which they observed in toad skin [23]. While such a model is consistent with the effects of external hypertonicity in their studies it cannot explain the effects of both mucosal (external) and serosal

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3 In principle, it is possible to analyze coupling quantitatively [15]. We write R^x for the "exchange resistance" ($R^x = -\underline{RT}\Delta\rho/J^x$, where ρ is the specific activity and J^x the tracer flow) and R for the phenomenological resistance coefficient for net flow ($R = X/J$, where X is the negative electrochemical potential difference and J the net flow). To the extent that there is coupling of isotope flows R^x will differ from R.

The flux ratio f across an array of identical parallel pathways in the absence of volume flow is given by

$$\underline{RT} \ln f = (R^x/R)\ X. \tag{1}$$

For a nonelectrolyte $X = \underline{RT} \ln (c_M/c_S)$ and

$$\ln f = (R^x/R) \ln (c_M/c_S). \tag{2}$$

Since the flux ratio $f = (c_M/c_S)r$,

$$R^x/R = 1 + \ln r/\ln (c_M/c_S). \tag{3}$$

In practice, several factors prevented the use of this simple expression in this study: (1) in the presence of leak pathways r reflects composite fluxes through parallel selective and nonselective channels; (2) to facilitate the demonstration of coupling c_M and c_S were initially made as different as possible, and hence $\ln(c_M/c_S)$ and $\ln r$ must have changed rapidly during the course of the experiment; (3) the above equation neglects the contribution of coupled water flows.

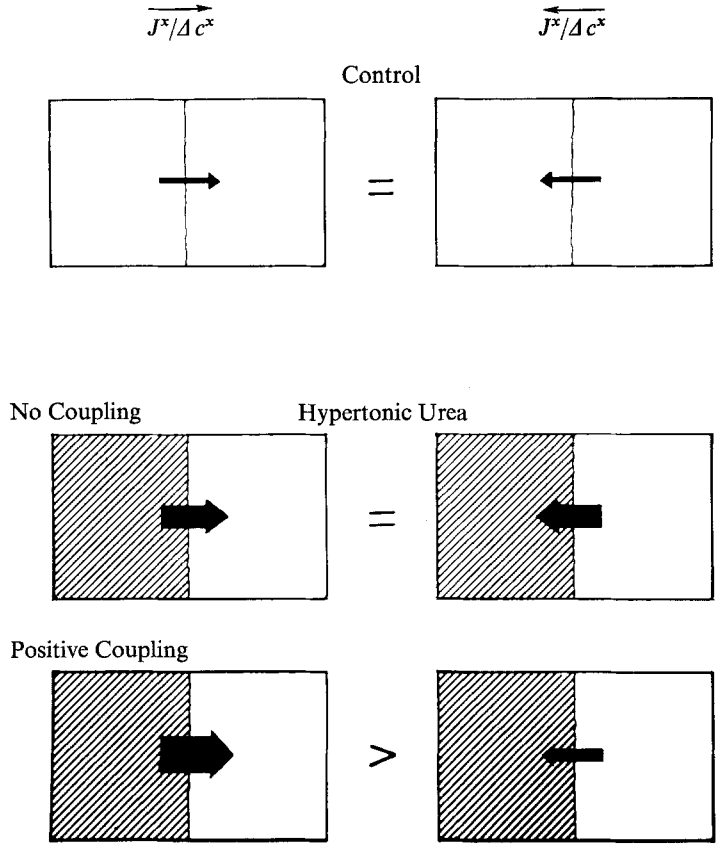

Fig. 5. Two possible results of the addition of hypertonic urea to well-paired hemibladders. As shown in the upper panel, when membranes are well paired, bidirectional permeabilities in the control state are equal. When hypertonic urea is added, the two permeabilities may increase (or decrease) but will remain equal in the absence of coupling (center panel). In the presence of coupling the two permeabilities will be affected differently (lower panel)

(internal) hypertonicity noted in our study and in that of Biber and Curran [3].

Our demonstration of coupling of urea flows differs from the findings of Urakabe *et al.*, who noted no asymmetry of urea tracer fluxes on addition of 240 mM urea to either the mucosal or serosal solutions [21]. Possibly this discrepancy reflects our use of a slightly higher concentration of urea (300 mM); as mentioned, we were unable to demonstrate an effect of 100 mM urea. More important, we believe, is the measurement of ratios of tracer permeability coefficients in paired tissues. It seems un-

likely that we could have demonstrated coupling without this precise technique.

Inspection of Figs. 3 and 4 and the corresponding numerical data shows that, prior to the addition of ADH, the effect of mucosal hypertonicity was less marked than that of serosal hypertonicity. (The pertinent comparison here is between the effect of mucosal hypertonicity on r: $\Delta r/r = (r_{hyp} - r_{cont})/r_{cont} = (1.22 - 0.83)/0.83 = 0.47$; and the effect of serosal hypertonicity on the reciprocal of r: $\Delta(1/r)/(1/r) = (1/r_{hyp} - 1/r_{cont})/(1r_{cont}) = (1/0.585 - 1/1.09)/(1/1.09) = 0.86$.) A possible explanation for this observation may be formulated in terms of two discrete parallel pathways for urea, one selective, the other not. Both the studies of Curran *et al.* [7] and our observations in synthetic membranes (*unpublished observations*) indicate that coupling is unlikely to be demonstrable in a nonselective aqueous channel. Therefore, conditions which increase the relative contribution of a nonselective channel should obscure coupling. Conversely, a relative reduction of the permeability of nonselective pathways should facilitate the demonstration of coupling. In the present studies, the effect of leak was minimized initially by the rejection of membranes of low resistance, and by careful handling of the tissues. After the addition of hypertonic urea to the mucosal medium the electrical resistance fell precipitously, suggesting the opening of large channels. On the other hand, in membranes in which urea was added to the serosal baths the electrical resistance remained at the control level or rose, ruling out the development of large leaks. The inverse relationship between the magnitude of passive leak pathways (as indicated by the electrical conductance) and the apparent magnitude of coupling was striking, and supports the concept that coupling occurs in a selective channel.

Another possible explanation for the greater effect of serosal hypertonicity on r, relating to the composite series character of the membranes, is suggested by the experiments of Urakabe *et al.*, who found that although in the absence of ADH the addition of 240 mM urea to the mucosal solution significantly increased osmotic water flow, the same concentration of urea in the serosal solution had little effect [21]. Since volume flow is in a direction such as to interfere with the demonstration of positive coupling of urea flows these results may well explain the differing effects of mucosal and serosal hypertonicity in the absence of ADH.

It is also noteworthy that, with mucosal hypertonicity, r increased following the administration of ADH. Since this increase was demonstrated despite appreciable transport by way of leak pathways, in which interaction would be insignificant, it is tempting to speculate that ADH may have increased coupling in the specific pathway. Alternatively, of course, it is

possible that ADH influences r solely by increasing the fraction of net flow of urea occurring by way of the specific channel.[4]

If ADH increases coupling in the urea channel it might be expected that r in the *ADH* period with serosal hypertonicity would also be modified and be significantly less than in the corresponding *hypertonic* period without ADH. The failure to demonstrate this effect may possibly again be explained in terms of heterogeneous series barriers, consistent with the observations of Urakabe *et al.* [21] (*see* their Fig. 7). These workers found, in conformity with the earlier conclusions of Bentley [4, 5], that whereas with 240 mM mucosal urea ADH induced only slight enhancement of volume flow ($\Delta < 2$ μliters/min per sac), with 240 mM serosal urea the effect of ADH was large ($\Delta \sim 20$ μliters/min per sac). Since large $M \rightarrow S$ volume flow in the serosal hypertonicity experiments would be expected to increase $M \rightarrow S$ urea flow and to decrease $M \leftarrow S$ urea flow this could well account for the failure to demonstrate a decrease of r following ADH, even if ADH influences coupling in the urea pathway.

In an attempt to determine whether changes in r following ADH are in fact attributable to alteration of urea-urea interaction in the specific channel, we tried to minimize water flow by the use of osmotically balanced solutions, in the hope that the true magnitude of coupling would be thereby revealed.

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4 For transport through parallel arrays of specific "urea" pathways and nonspecific "leak" pathways,

$$1/R = 1/R_u + 1/R_l \tag{4}$$

and

$$1/R^x = 1/R_u^x + 1/R_l^x. \tag{5}$$

Then

$$\begin{aligned} 1/(R^x/R) &= 1/(R_u^x/R) + 1/(R_l^x/R) \\ &= 1/[(R_u^x/R_u)(R_u/R)] + 1/[(R_l^x/R_l)(R_l/R)] \\ &= 1/[(R^x/R)_u(R_u/R)] + 1/[(R^x/R)_l(R_l/R)]. \end{aligned}$$

But we assume $(R^x/R)_l = 1$. Since also $1/(R_l/R) = 1 - 1/(R_u/R)$,

$$1/(R^x/R) = 1/[(R^x/R)_u(R_u/R)] + 1 - 1/(R_u/R) \tag{6}$$

$$= 1 + [1 - (R^x/R)_u]/[(R^x/R)_u(R_u/R)]. \tag{7}$$

Therefore, R^x/R, and thus r, might increase following the administration of ADH as a result of either an increase in $(R^x/R)_u$ [*see* Eq. (6)] or a decrease in (R_u/R). (Note that $1 - (R^x/R)_u$ is negative.) The effect of a decrease in R_u/R would be expected to be more significant with mucosal than with serosal hypertonicity, since in the latter case R_u/R is small even prior to the administration of ADH.

Unfortunately, the addition of solute to both bathing solutions produced inconsistent effects on conductance and urea permeability in the two hemibladders of a pair. While this inconsistency is in itself enough to obscure the demonstration of coupling, there are still other possibilities which might explain our negative results. It is conceivable that positive coupling of sucrose and urea flows, demonstrable in amphibian skins, might have opposed the urea-urea coupling. Alternatively, balanced solutions might have altered the region within the membrane where coupling occurs, thus interfering with the phenomenon. The data are insufficient to distinguish between these possibilities.

In the studies utilizing a lower concentration of urea (0.1 M), coupling could not be demonstrated. We presume that this is the result of dissimilarity of paired tissues and the probable smaller effect on r at a lower concentration of abundant urea, as suggested by the studies of Biber and Curran [3].

Although the difficulty of demonstrating coupling of urea flows might suggest that urea-urea interaction is small, a rough calculation indicates that it is greater than urea-water interaction. Thus, in the ADH periods of our 0.3 M mucosal urea experiments, the mean value of r was 1.82. This value of r was associated with a mean rate of net urea flow of 218×10^{-7} cm sec$^{-1} \times$ 0.3 mole liter$^{-1} = 6.5 \times 10^{-9}$ moles cm^{-2} sec^{-1}. By extrapolation of Leaf and Hays' data on the effect of volume flow on the flux ratio of urea [17], a value of $r = 1.82$ would require water flow of some 270 μliters cm^{-2} hr^{-1} = 4.2×10^{-6} moles cm^{-2} sec^{-1}; i.e., about 600 times the above rate of urea flow. Hence, whether or not urea and water share a common pathway,[2] it would appear that there is a region of the membrane in which urea-urea interaction is appreciably greater than that between urea and water.[5] The most likely explanation would seem to be a region in which the concentration of urea is in excess of that of the bathing solutions, facilitating interaction between urea molecules. This would be consistent with the findings of Galey and Van Bruggen [12]. These workers demonstrated interaction between a variety of solutes in synthetic membranes, with interaction related directly to the concentration and size of the solute particles, and inversely to the radii of the membrane pores.

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5 The above calculation underestimates the discrepancy between the magnitudes of urea-urea interaction and urea-water interaction because it ignores the fact that in the absence of opposing volume flow the mean value of r induced by 0.3 M urea and ADH would presumably have exceeded 1.82.

Biber and Curran have suggested that the degree of mannitol-urea interaction observed in toad skin might be consistent with interaction occurring in aqueous solution, but emphasize that they have neglected effects of solvent flow on solute flux [3].

We thank Dorothy Nadel for skillful technical assistance, Drs. S. R. Caplan, M. M. Civan, and W. R. Galey for careful reading of the manuscript, and Dr. M. Schwartz for valuable aid in the statistical analysis of the data.

This study was supported by grants from the Office of Saline Water (14-30-2156) and the USPHS (HE 13648, HE 00759, HE 05309 and HL 14322 to the Harvard-MIT Program in Health Sciences and Technology). A.E. was a USPHS Career Development Awardee during part of this study.

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