Determination of Urea Permeability in Red Cells by Minimum Method

A test of the phenomenological equations

R. I. SHA'AFI, G. T. RICH, D. C. MIKULECKY, and A. K. SOLOMON

From the Biophysical Laboratory, Harvard Medical School, Boston, Massachusetts 02115. Dr. Sha'afi's present address is the Department of Physiology, Medical School, The American University of Beirut, Beirut, Lebanon. Please send reprint requests to the Biophysical Laboratory, Harvard Medical School.

ABSTRACT A new method has been developed for measuring the permeability coefficient, ω, of small nonelectrolytes. The method depends upon a mathematical analysis of the time course of cell volume changes in the neighborhood of the minimum volume following addition of a permeating solute to an isosmolar buffer. Coefficients determined by the minimum volume method agree with those obtained using radioactive tracers. ω for urea in human red cells was found to decrease as the volume flow, J, into the cell increased. Such behavior is entirely unexpected for a single uniform rate-limiting barrier on the basis of the linear phenomenological equations derived from irreversible thermodynamics. However, the present findings are consonant with a complex membrane system consisting of a tight barrier on the outer face of the human red cell membrane and a somewhat less restrictive barrier behind it closer to the inner membrane face. A theoretical analysis of such a series model has been made which makes predictions consistent with the experimental findings.

When red cells are placed in a medium containing an isosmolar concentration of impermeant solute, together with a suitable concentration of permeant solute, the cell volume initially shrinks and then returns to its initial volume, after passing through a clearly defined minimum. In 1933 Jacobs (1) had already pointed out that the minimum volume and the time taken to achieve it could be used to determine the permeability coefficient of the permeant solute; the present method is based upon this suggestion. In the shrinking phase water moves out of the red cell because of the osmotic pressure gradient due to the addition of the permeant solute to the medium. At the same time the permeant solute diffuses into the cell down its concentration gradient.

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Since water is moving out of the cell during the shrinking phase, there is a solvent drag effect opposing solute diffusion. The minimum volume is achieved when the volume of solute diffusing inwards is exactly balanced by the volume of water moving outward in response to the remaining osmotic pressure gradient. Subsequently the osmotic pressure gradient reverses its direction, in part because the impermeant solutes are now more concentrated in the cell than in the medium. Water reenters the cell together with the solute so that solvent and solute flux are both in the same direction. The volume change comes to an end when the solute concentration in the cell equals that in the medium and all osmotic gradients disappear.

A great deal of information can be extracted from the time course of the volume change. The permeability coefficient, $\omega$, can be obtained from the minimum volume and the derivative of the rate of volume change at that volume. Although measurements of $\omega$ have been made by this method for a number of solutes, the present study will report results obtained with urea alone since these studies demonstrate certain fundamental limitations of the application of the treatment of Kedem and Katchalsky (2) to solute and solvent fluxes across a red cell membrane.

**THEORETICAL INTRODUCTION**

When the only substance moving across the membrane is solvent and an uncharged solute, Kedem and Katchalsky (2) give the following relations to express the relative fluxes,

$$J_s = -L_p \Delta \pi_i + L_{pd} \Delta \pi_s$$  \hspace{1cm} (1)

$$J_d = -L_{dp} \Delta \pi_i + L_d \Delta \pi_s$$  \hspace{1cm} (2)

$J_s$ is the volume flow per unit area in cm/sec, and flow into the cell is considered to be in the positive direction. The osmotic pressure due to the impermeant solute is denoted as $\Delta \pi_i$, which is defined as $\Delta \pi_i = RT(c_i - c_i^\infty)$; $\Delta \pi_i$ has units of dyne/cm². The solution bathing the external surface of the cell membrane is denoted by the superscript, $o$, and that bathing the internal surface by $\Delta x$. $L_p$ is the hydraulic conductivity of the membrane in cm³/dyne sec. $L_{pd}$ is the cross-coefficient for the volume flow arising from differences in the osmotic pressure of the permeable solute, $\Delta \pi_s$, when there is no difference of hydrostatic pressure (or osmotic pressure produced by impermeant solutes, $\Delta \pi_i = 0$). $L_{dp}$ is the relative diffusional solute mobility per unit hydrostatic (or impermeant solute) pressure difference when there is no osmotic pressure difference ($\Delta \pi_s = 0$). $\Delta \pi_s$ is defined for the permeable solute analogously to the definition of $\Delta \pi_i$. Although $L_p$ is always positive, $L_{pd}$ is negative and has the same units as $L_p$. The Onsager reciprocal relation requires $L_{dp}$ to equal $L_{pd}$ and it was this relationship that we initially desired to test in a biological system. $J_d$ is the diffusional flow and is a measure of the
relative velocity of solute to solvent flux. \(L_d\) is a phenomenological coefficient, related to the permeability coefficient, describing solute movement down its own concentration gradient in the absence of hydrostatic pressure or osmotic pressure differences due to impermeant solutes. Rather than measuring \(L_d\) directly it is much more convenient experimentally to measure \(\omega\) using the following equation derived by Kedem and Katchalsky from the equations above,

\[
J_s = (1 + \frac{L_d \rho}{L_p}) \epsilon \omega J_0 + \omega \Delta \pi_s
\]

(3)

\(\omega\) is related to the phenomenological coefficients by

\[
\omega = (L_d L_p - L_{pd} L_d) \epsilon_s / L_p
\]

(4)

and \(\epsilon_s\) is defined by

\[
\epsilon_s = (\epsilon^* - \epsilon^{\Delta \pi_s}) / \ln (\epsilon^*/\epsilon^{\Delta \pi_s})
\]

(5)

The results of our experiments indicate that \(\omega\) and \(\omega/\epsilon_s\) both depend upon \(J_s\) which is not to be anticipated from equations 1 and 2, if they are applied under the conditions specified by Kedem and Katchalsky to a single uniform membrane.

**GENERAL EXPERIMENTAL METHODS**

Immediately before an experiment human blood was drawn from a healthy male or female donor. Heparin (10,000 units per ml; 4 ml/liter blood) was used to prevent clotting. The blood was diluted in a buffered saline solution to form a suspension of 3% by volume of whole blood. The buffer had the following composition (mM): NaCl, 107; MgCl\(_2\), 0.5; CaCl\(_2\), 1.2; Na\(_2\)HPO\(_4\), 1.7; NaH\(_2\)PO\(_4\), 4.2; KCl, 4.4; Na\(_2\)CO\(_3\), 13.5. The solution was aerated with 5% CO\(_2\)-95% air to pH 7.4. The osmolality as measured with a freezing point osmometer (Fiske Associates, Bethel, Conn. Model 1) was 260–280 milliosmols/liter.

All the experiments have made use of the rapid reaction stop-flow apparatus previously described (3) in which the intensity of 90° scattered light serves as a measure of red cell volume. In all the experiments at least three duplicate runs were made and three control runs; the difference between experimental signal and control was obtained through the use of a computer of average transients (Technical Measurement Corp., North Haven, Conn. CAT 400B) and an IBM 1620 computer according to the general procedure described in detail by Rich et al. (4) for the measurements of \(L_p\) and \(L_{pd}\). A different detailed experimental procedure was required by each of the several sets of measurements. The experimental details are given in the relevant section.

**RESULTS AND DISCUSSION**

In order to study \(\omega\) explicitly, it is first necessary to know how \(L_p\) and \(L_{pd}\) depend upon osmolality since the internal osmolality of the cell changes con-
stantly during the swelling and shrinking process. Rich et al. (4) have previously shown that \( L_p \) is dependent upon the osmolality of the medium but remains independent of the osmolality of the cell interior. As described in the subsequent section, experiments were carried out which showed \( L_{pd} \) to be essentially independent of external osmolality. These were followed by an additional set of experiments which indicated that the determining factor for \( L_p \) was the medium osmolality rather than the osmotic pressure developed across the red cell membrane. That is, the relative permeability of the membrane for a specific solute does not affect \( L_p \). Instead \( L_p \) is controlled by the osmotic pressure as determined by freezing point and is independent of the reflection coefficient of the solute used.

During the course of the experiments it became apparent that it would be desirable to investigate the validity of equations 1–3 in describing coupled flows across the red cell membrane. In order to draw valid conclusions on this point, it was necessary for the experimental studies of \( L_p \) and \( L_{pd} \) to be independent of these equations. Consequently the experimental methods were based on the following definitions (see Katchalsky and Curran [5]) of \( L_p \) and \( L_{pd} \):

\[
L_p = -\left(\frac{J_v}{A\tau_r}\right)_{\Delta \pi_v=0} \quad (6)
\]

\[
L_{pd} = \left(\frac{J_v}{A\tau_r}\right)_{\Delta \pi_v=0} \quad (7)
\]

**Dependence of \( L_{pd} \) on External Osmolality**

In these studies \( L_{pd} \) for urea was measured in human red cells using the zero volume flow principle in the rapid reaction stop-flow apparatus previously described by Sha'afi et al. (3). It is difficult to measure \( J_v \) in absolute terms and so a comparative technique was devised in which the zero time volume flow induced by various concentrations of urea was compared (in relative units) with that induced by various concentrations of glucose, to which the human red cell is impermeable. Suspensions of red cells in the isosmolal salt buffer described in the experimental methods section were mixed in the rapid reaction stop-flow apparatus with hyperosmolal solutions of urea dissolved in the same isosmolal salt buffer. This eliminated any osmotic pressure difference at zero time due to the impermeable species, and thus fulfilled the requirement that \( \Delta \pi_v = 0 \) in equation 7. The time course of cell volume was measured for a 1 sec period, and the zero time volume flow, \( J_{vo} \), was determined by extrapolation as described previously by Rich et al. (4). In the same experiment on the same batch of cells the zero time volume flow, \( (J^u_{vo}) \), was also measured when glucose was used to replace urea. \( L_{pd}^u \) was calculated according to the following equation:

\[
L_{pd}^u = \frac{J^u_{vo} \Delta \pi_{vo} L_{pd}^u}{J^u_{vo} \Delta \pi_{vo}} \quad (8)
\]
in which \( \Delta \pi_{0} \) and \( \Delta \pi''_{0} \) are the osmotic pressure differences at \( t = 0 \) for glucose and urea, respectively. The reflection coefficient, \( \sigma \), is defined (5) as 
\[
\sigma \equiv -L_{pd}/L_{p} \text{ so that } L_{p}^{0} = -L_{p} \text{ for glucose for which } \sigma \text{ is unity in the human red cell. In order to make the computation in equation 8, } L_{p} \text{ must be chosen to correspond to the proper medium osmolality as discussed above (see reference 4). The data in Fig. 1 show the results of two experiments in which } L_{pd} \text{ for urea was studied as a function of medium osmolality. It can be seen that } L_{pd} \text{ changes very little with changes of external osmolality. When these data are combined with those of Rich et al. (4) and the definition above, } \sigma \text{ can also be computed as a function of medium osmolality. The variation of all three coefficients for urea, } L_{p}, \sigma, \text{ and } L_{pd}, \text{ with medium osmolality is compared in Fig. 1. It is clear that the cross-coefficient, } L_{pd}, \text{ is less sensitive to changes in concentration than the straight coefficient, } L_{p}.

![Diagram](image)

**Figure 1.** Dependence of the absolute values of the phenomenological coefficients, \( L_{pd}, L_{p}, \) and \( \sigma \), for urea on medium osmolality of human red cells. The experimental points were determined in the present experiments.

In these experiments the osmolality was varied in the external solution only and the initial internal red cell osmolality was unchanged. As previously described, Rich et al. (4) have shown that \( L_{p} \) is independent of the osmolality of the inner face and we assume that a similar situation obtains for \( L_{pd} \). The initial \( J_{p} \) in the present urea experiments varied by a factor of two as the initial osmolality difference was changed from 180 to 380 milliosmols. In consequence the internal osmolality of the red cell was changing at appreciably different rates under the several experimental conditions. Nonetheless, as Fig. 1 shows, the observed dependence of \( L_{pd} \) on osmolality was relatively
slight, an observation which is consonant with the expectation that \( L_{pd} \) is not strongly, if at all, dependent upon internal osmolality.

Since, as will be shown in a subsequent section, \( \sigma_{\text{urea}} = 0.55 \) it is necessary to know whether \( L_p \) is set by the osmolality or by the osmotic pressure of the medium which differ by a factor of almost two. This specific question was not investigated by Rich et al. (4) who used impermeant solutes in their experiments, so the experiments in the subsequent section were devised to answer this question.

**Does \( L_p \) Depend on Medium Osmolality or Osmotic Pressure?**

The rationale of these experiments was to compare the effect of creatinine and urea on \( L_p \) in human red cells. Rich et al. (4) have previously made use of the fact that creatinine equilibrates rapidly with red cells at 37°C though its half-time for equilibration at room temperature is about 2.5 hr so that \( \sigma_{\text{creatinine}} \) is close to one at room temperature. They have also shown that the presence of creatinine does not affect \( L_p \) in human red cells. Human red cells suspended in isosmolar buffer were divided into two aliquots. A known amount of urea was added to one aliquot to give a final concentration of 0.1 or 0.2 M and an equal amount of creatinine was added to the other aliquot. The two suspensions were incubated at 37°C for 2 hr in order to allow both urea and creatinine to equilibrate with the red cells. Aliquots of the urea suspension were mixed in the stop-flow apparatus with a hyperosmolar solution containing the same amount of urea and the zero time volume flow, \( J_{vo}^u \), was recorded. Likewise \( J_{vo}^c \) was determined on aliquots from the creatinine-treated cells which were mixed with a hyperosmolar solution containing the corresponding amount of creatinine. The solutions were made hyperosmolar by changing the amount of NaCl only. The hydraulic conductivity measured in the presence of urea is determined from equation 6 when \( \Delta \sigma_{\text{urea}} = 0 \) and is denoted by \( L_p^u \), which was compared to \( L_p \) in the presence of creatinine, \( L_p^c \). If \( L_p \) is controlled by medium osmolality, \( L_p^u \) will be equal to \( L_p^c \), whereas if \( L_p \) is determined by the osmotic pressure, \( L_p^u \) will be less than \( L_p^c \) since the reflection coefficient of urea is much less than that for creatinine at room temperature. It is clear from the results of two experiments given in Table I that the two \( L_p \)'s are equal to each other within experimental error. This indicates that \( L_p \) is controlled by the medium osmolality rather than the osmotic pressure.

This observation leads to an interesting conclusion about the mechanism by which the external face of the membrane controls the hydraulic permeability. Rich et al. (4) have suggested that the effect could be mediated by the hydration of the external skin of the membrane. A molecule like urea which can easily permeate equivalent pores of 4.3 A radius is just as effective in controlling \( L_p \) as a molecule like creatinine which cannot. This suggests
that the outer skin of the membrane is impermeable to urea, except for the small population of equivalent pores through which the urea may enter the red cell. In consequence, the controlling effect on the hydraulic permeability appears to be a generalized osmotic shrinkage of a tightly knit gel which forms the outer skin. In turn, this would also lead to a shrinkage of the equivalent pores. Thus passage of water through the equivalent pores or through the gel, or both, would be impeded, causing \( L_p \) to decrease with increased external osmolality in agreement with the experimental observation.

| TABLE I |
| --- |

**COMPARISON OF EFFECT OF CREATININE AND UREA ON \( L_p \)**

| \( \Delta \Phi_{urea}/\Delta \Phi_{creatinine} \) | \( L_p/L_p^* \) |
| --- | --- |

| 0.2 M urea/0.2 M creatinine | 0.2 M urea/0.2 M creatinine |
| --- | --- |
| 0.97 | 0.96 |
| 1.01 | 1.08 |
| 0.94 | 0.83 |
| 0.97 | 0.94 |
| 0.97 | 0.88 |
| 1.27 | 1.01 |
| 1.04 | 0.77 |
| 0.97 | 0.89 |

Average 1.02

SE ± 0.11

| 0.1 M urea/0.1 M creatinine | 0.1 M urea/0.1 M creatinine |
| --- | --- |
| 1.00 | 0.92 |
| 1.23 | 1.07 |
| 0.82 | 0.82 |
| 1.00 | 0.96 |
| 0.88 | 1.91 |
| 1.04 | 1.21 |
| 0.83 | 1.64 |
| 0.99 | 1.03 |

Average 0.97

SE ± 0.13

This general model also fits with the behavior of \( \sigma_{urea} \) as \( L_p \) decreases. Since increases in osmolality are presumed to cause the equivalent pores to shrink, the shrunken membrane becomes able to discriminate more readily between urea and water, thus causing the observed rise in \( \sigma_{urea} \). This would fit with a model in which the urea molecule and the water shared a common pathway.

**Experimental Method for \( \omega \) Determination**

The rapid reaction stop-flow apparatus previously described (3) was used to measure permeability coefficients by the minimum value method. An aliquot
of the standard red cell suspension was rapidly mixed with an equal volume of a solution containing 100 ± 5 milliosmols NaCl/liter plus 750 ± 20 milliosmols/liter of the permeating nonelectrolyte. The scattered light was measured over a 1 or 2 sec interval depending on how rapidly the nonelectrolyte entered the cells. The data were stored in a computer of average transients (CAT), digitized, and subsequently punched out on paper tape. Since the CAT sampled points 200 times at equal time intervals over the whole scan period, data points were obtained at every 5 or 10 msec.

![Figure 2.](image)

**Figure 2.** Variation of the output signal with human red cell hematocrit in the presence and absence of urea.

For every solute studied at least five records were obtained, and subsequently averaged. It was also necessary to carry out control experiments in which the aliquot from the red cell suspension was mixed with a 100 milliosmol NaCl solution brought to the same osmolality as the red cell suspension by the addition of glucose. In this case also at least five records were obtained, averaged, and then subtracted from the record for the permeating solute. This latter procedure compensated for any changes resulting from pH or ionic strength shifts and served to minimize the effects of mechanical noise in the apparatus.

In order to calculate permeability coefficients from the records of scattered light as a function of time (as given by the corrected CAT output) it was necessary to relate the output signal to absolute cell volume. For the calibration of the system, aliquots of the stock red cell suspension were equilibrated
with equal volumes of anisosmolar solutions which contained the same 750 milliosmols/liter of permeating solute but different amounts of NaCl. The output signal was recorded when one aliquot of each suspension was mixed with another aliquot of the same suspension in the stop-flow apparatus. The average control record was subtracted from the records for each calibration suspension. In order to see whether changes in the composition of the solution affected the scattered light, similar experiments were carried out in the absence of red cells over the same concentration range of NaCl with only a negligible effect on the output signal. Thus, for any specific nonelectrolyte concentration in the medium, the signal is a function of the cell volume alone. The cell volume in each calibration suspension was measured by the hemato-crit method described previously (6).

Fig. 2 shows the variation of output signal with cell volume in the presence of salt alone and with salt plus urea. In both cases the relationship is linear. Furthermore the presence of urea does not change the slope of the line, but, because of a refractive index change, merely shifts the line upwards. A single calibration procedure (mean of five runs for each external osmolality) for absolute cell volume served for all the experiments carried out with any single stock red cell suspension.

Theory for Minimum Volume Method

The method of calculation of \( \omega \) from the minimum volume depends upon the definition of \( \omega = (J_s/\Delta \pi_s)_J_s=0 \). In order to make the computation, it is also necessary to make use of equation 1 and the definition of \( \sigma \). In subsequent sections of the paper it will become apparent that equation 1 does not describe the flows across human red cell membranes when \( J_s \) is different from zero. However, it is reasonable to expect the equation to be approximately correct near \( J_s = 0 \), which is the domain of interest since the minimum volume is reached when \( J_s = 0 \). We have first made the assumption that equation 1 may be used near zero volume flow and have applied the zero flow restriction at a later step in the derivation. \( J_s \) may be obtained from equation 1 as follows,

\[
\Delta \pi_s = J_v/L_{pd} - \Delta \pi_v/\sigma
\]

As \( c^2 = n_s/V' \) in which \( n_s \) is the amount of permeant solute in the cell water, whose volume is \( V' \). In the notation of Sha'afi et al. (3) \( V \) is cell volume and \( b \) is the sum of the volume of fixed framework and solute dry weight so that \( V' = V - b \). Using the definitions for \( \Delta \pi_s \) and \( \Delta \pi_v \), that follow equation 1, we may write

\[
n_s = -V'J_v/RTL_{pd} - c^2/V'/\sigma + V'(c^2_s + c^2_v/\sigma)
\]
and

\[ J_s = \frac{1}{A} \left( \frac{dn_s}{dt} \right) = -(ART)^{-1} \left[ \left( \frac{V'}{L_p} \right) \left( \frac{dJ_s}{dt} \right) + \left( \frac{J_s}{L_p} \right) \left( \frac{dV'}{dt} \right) \right] \]

\[ - \left( \frac{V'}{A} \right) \left( \frac{d\Delta \pi_s}{dt} \right) - \left( \frac{c_s^\Delta}{A} \right) \left( \frac{dV'}{dt} \right) + \left( \frac{dV'}{dt} \right) \left( \frac{c_s^\Delta}{A} + \frac{c_s}{A} \right) \]

in which \( A \) is red cell area, considered to remain constant during the experiment (3, 7). \( L_p \) is also considered to remain independent of cell volume.

At the minimum volume, \( J_s = 0 = \left( \frac{dV'}{dt} \right) / A \) and therefore \( d\Delta \pi_s / dt = 0 \)

\[ J_s = -\left( \frac{V'/A}{RTL_p} \right) (dV'/dt)_{\text{min}} \]

(12)

\[ \omega_{\text{min}} = V'_{\text{min}} (dV'/dt)_{\text{min}} / A \]

In these experiments \( A \) was taken as \( 1.67 \times 10^{-4} \) cm\(^2\) and (under isosmolal conditions) \( V'/V = 0.715 \). \( L_p \) was determined from Fig. 2 of reference 4 for the appropriate osmolality as discussed above. \( \pi_i^{\Delta \pi_s} (= RTc_i^{\Delta \pi_s}) \) was determined from calibration curves for each stock red cell suspension in which relative cell volume was measured as a function of medium osmolality. In order to estimate \( (dV'/dt)_{\text{min}} \) the digital computer was used to fit the average data...
curve, after subtraction of control, to an arbitrary sum of two exponentials, using the method of least squares. The computer then calculated the second derivative analytically using the values of the coefficients and exponents in the least squares curve. Fig. 3 shows the results of a typical experiment and the fit obtained by the double exponential. In order to validate the minimum value method, the values of $\omega$ for urea and formamide were compared with the results obtained previously using the continuous flow apparatus and radioactive tracers. The results shown in Table II indicate very good agreement between the two methods.

**Behavior of $\omega$ When $J_s$ Is Nonzero**

The results presented so far indicate good agreement between different methods of measuring $\omega$ when $J_s = 0$. During the course of the minimum value experiments, $J_s$ varies continuously and changes sign as the volume passes through its minimum. Thus the experimental data contain information about the dependence of $\omega$ on $J_s$. The first step to study this dependence explicitly is to compute $\epsilon_s$ as a function of time. This can be done by making use of the definition of $\epsilon_s$ given in equation 5 and substituting for $\epsilon_s$ from equation 10, to obtain

$$\epsilon_s = \frac{-L_p \Delta \pi_i - J_s}{\sigma R T L_p [\ln \sigma L_p \pi_i^0 - \ln (J_s + L_p \Delta \pi_i + \sigma L_p \pi_i^0)]} \quad (15)$$

Equation 15 relates $\epsilon_s$ to the initial conditions, cell volume, and the rate of volume change. Fig. 4 shows the dependence of $\epsilon_s$ on time. It should be pointed out that the derivation of equation 15 involves the use of equation 1, upon which equation 10 is based, under conditions when $J_s$ is nonzero and varies relatively rapidly. The validity of the data in Fig. 4 therefore depends directly on the assumption that equation 1 and the definition of $\epsilon_s$ given in equation 5 are both correct.

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**Table II**

**Comparison of $\omega$ by Minimum Method and Tracer Method**

| Solute      | $\omega$ Minimum method* | $\omega$ Tracer method |
|-------------|---------------------------|-------------------------|
|             | moles/dyne sec $\times 10^{14}$ | moles/dyne sec $\times 10^{14}$ |
| Urea        | 14.1±1.0 (6)              | 16.2±4.5 (7)            |
| Formamide   | 18.1±0.8 (2)              | 20.0±0.6 (2)            |

* The number in parentheses refers to the number of experiments. Errors are SE.
The next step in obtaining an explicit expression for $\omega$ is to substitute equation 9 into equation 3 to obtain:

$$\omega = L_p[1 - (1 - \sigma)\varepsilon_0 J_0]/(J_p + L_p \Delta \pi)$$  \hspace{1cm} (16)

The dependence of $\omega$ on $J_0$ is shown in Fig. 5 in one experiment typical of the three experiments which were analyzed in this way. Contrary to expectation, $\omega$ depends significantly on $J_0$, decreasing from $0.2 \times 10^{-13}$ mole/dyne sec when the volume flow out of the cell is near its maximum to $0.13 \times 10^{-13}$ mole/dyne sec when the volume flow into the cell is $0.6 \times 10^{-6}$ cm/sec. The

![Figure 4. Time course of $\varepsilon_0$ in urea experiment on human red cells.](image)

relationship between $\omega$ and the phenomenological coefficients given in equation 4 (assuming the Onsager relationship) is $\omega = (L_\delta L_p - L_p c)\varepsilon_0/L_p$. Since $\omega$ contains $\varepsilon_0$ explicitly and $\varepsilon_0$ varies continuously throughout the experiment, $\omega/\varepsilon_0$ has been plotted in Fig. 6. This index of cell permeability is much more dependent upon $J_0$ because of the rapid changes in $\varepsilon_0$. The medium osmolality remains constant in these experiments, so that $L_\delta$ should be time-independent, and arguments have already been presented which support the view that $L_\mu$ is independent of internal osmolality. Thus the dependence of $\omega/\varepsilon_0$ on $J_0$ is entirely unexpected.

Since $\omega$ decreases with time, and since the urea concentration is 0.3 M, the possibility exists that the dependence of $\omega$ on $J_0$ may reflect the time course of some chemical action of urea on the membrane. In order to investigate this point, Veatch\textsuperscript{2} carried out two experiments in which THO diffusion was

\textsuperscript{2}Veatch, W. Private communication.
measured by the method of Paganelli and Solomon (8) in the presence and absence of 0.3 M urea. The urea was added 15 min before the beginning of the experiments and the ratios of the THO diffusion in the control to that in the presence of urea were 0.97 and 1.07. This indicates that 0.3 M urea does not cause a generalized deformation affecting human red cell permeability during the 0.5 sec time course of the minimum volume experiments. The THO diffusion experiments were not designed to study the effect of osmolality on solute permeability. Such information would be very interesting but would require experiments especially designed to yield systematic information on the behavior of a number of solutes.

![Graph](image)

**Figure 5.** Dependence of $\omega$ for urea on $J_s$ in human red cells. The line is a least squares line.

The important conclusion that follows from the dependence of $\omega$ on $J_s$ is that equations 1 and 2 do not serve to describe the coupling between solute and solvent flow across the human red cell membrane when urea is the solute. Similar behavior has been observed when other hydrophilic solutes such as acetamide have been studied. Since $\omega$ is a function of $J_s$, equation 3 has no predictive value under our experimental conditions. This finding does not invalidate the use of the minimum volume method to obtain estimates of $\omega$ because the procedure is restricted to data obtained when $J_s$ is at or very near zero. As discussed in the following section, the determination of $\sigma$ by the method of Goldstein and Solomon (9) is also made when $J_s = 0$. $L_p$ was determined by Rich et al. (4) under conditions in which the solute flow was zero. On the other hand, when solute and solvent are moving simultaneously in a biological system, great care must be exercised in the interpretation of
flux measurements because the usual Kedem and Katchalsky treatment described by equations 1 and 2 does not apply even to a system as simple as the human red cell membrane.

There are several possible explanations for the lack of applicability of equations 1 and 2 to a description of coupled flows even across the human red blood cell membrane. The integration required in the derivation of equations 1 and 2 is dependent upon the establishment of steady-state flows. Even though the red cell volume is changing relatively rapidly during these experiments, the steady-state requirement is satisfied under our experimental conditions be-

![Diagram](image)

**Figure 6.** Dependence of $\omega/\tau_s$ for urea on $J_s$ in human red cells.

cause the red cell membrane is only about 100 A thick. The appendix written by one of us (D.C.M.) shows that the half-time required to reach the steady state is about 40 $\mu$sec, whereas a 5 or 10 msec interval elapses between sampling times in the present experiments.

It is much more probable that the difficulty lies with the definition of $\tau_s$, an average concentration within the pores defined by equation 5 in terms of the concentrations of the solutions bathing the membrane on either side but without any reference to $J_s$. Even if the pore had a uniform circular cross-section, it would be hard to imagine that $\tau_s$ could be independent of $J_s$. The simplest considerations would suggest that when volume flow took place from a concentrated solution to a dilute one, the membrane solute concentration would be larger than that when $J_s = 0$; and the converse should hold when
the flow was reversed. Furthermore the concentration profile through the 
pore ought to be distorted as a result of volume flow, so that the average con-
centration ought to depend on $J_\ast$. The appendix shows that such behavior 
is predicted on hydrodynamic grounds. The results following equation A-5 
indicate that a positive slope is to be expected rather than the negative one 
observed in Fig. 5. Moreover the calculated slope is only about 20\% of that 
in Fig. 5 so that $\omega$ for a single uniform very thin membrane would be virtually 
independent of $J_\ast$, in accord with expectations based upon equations 1 and 2 
of Kedem and Katchalsky. Thus the experimental dependence of $\omega$ on $J_\ast$ does 
not result from inadequacies in these equations, but is an indication that the 
red cell membrane does not behave as a thin homogeneous permeability 
barrier.

As already discussed, the human red cell membrane is asymmetric as re-
gards the effect of osmolality on $L_\alpha$, with a rate-limiting barrier present at 
the external face of the cell. Passow (10) has also presented evidence based on 
anion and cation permeation of red cell membrane that strongly suggests the 
presence of at least two separate permeability barriers in the membrane. 
Kedem and Katchalsky (11) have provided a theoretical treatment for a 
series array of pores which can be applied to a system with a tight barrier on 
the outer face and a somewhat less restrictive barrier behind it closer to the 
inner membrane face. In such a model, when $J_\ast$ is directed towards the cell, 
the barrier at the external face will sieve out the solute and the concentration 
within the remainder of the equivalent pore will fall, effectively causing $\omega$ to 
diminish. When the flow is reversed, the solute will accumulate within the 
pore, damned up by the barrier at the exit and hence $\omega$ will be higher than 
when $J_\ast = 0$. Thus the present results which show that $\omega$ decreases as $J_\ast$ in-
creases in the positive direction are consonant with a series membrane model. 
It would be difficult to understand how $\omega$ could reach its maximum value 
when volume flow out of the red cell is at a maximum, if the membrane were 
traversed by uniform pores. The appendix shows that the observed slope in 
Fig. 5 is in quantitative agreement with a series model in which $\omega$ for the thin 
outer barrier is $2 \times 10^{-14}$ moles/dyne sec and that for the less restrictive 
barrier is $6 \times 10^{-14}$ moles/dyne sec. The fact that $\omega/\varepsilon_\ast$ (Fig. 6) depends much 
more sharply on $J_\ast$ than does $\omega$ (Fig. 5) is a reflection of the fact that $\varepsilon_\ast$ is not 
a valid index of membrane concentration when volume flow takes place 
across a series membrane.

Dependence of $\sigma$ on $J_\ast$

There is another instance in which equation 1 does not describe the system 
satisfactorily. Though $\varepsilon_\ast$ does not appear explicitly in equation 1, the concen-
tration gradient of solute across the membrane is the driving force for solute 
movement and deformation of the gradient would be expected to affect the
applicability of the equation. Equation 1 is used to determine the reflection coefficient, $\sigma$, by the method of Goldstein and Solomon (9) under conditions in which $J_r = 0$. $\pi_i$ is kept constant, and $\pi_r$ is varied over a wide range to provide both positive and negative values of $J_{r0}$, the volume flow at $t = 0$. Since the permeant solute is added initially to the external medium only, $\pi_r$

![Figure 7. Dependence of initial rate of volume flow on external osmolality in human red cells in the presence of urea. A linear transformation of the points in experiments 16H and 17H was made to superpose these data on those obtained in experiment 15H.](image)

is known exactly at $t = 0$ without need for analysis of cell solute concentration and $J_{r0}$ is obtained by extrapolation of the points obtained during the first 100 msec. $\sigma = -(\Delta \pi_i/\Delta \pi_r)_{J_r=0}$ and can be obtained by interpolation from a graph similar to that shown in Fig. 7. The validity of this procedure rests upon the assumption that equation 1 is accurate when $J_r = 0$ and that deviations are small when the volume flow approaches zero. Three experiments were carried out with urea in which the experimental points were fitted to a
straight line by the method of least squares, giving the results in Table III. The mean value for $\sigma$ urea is $0.55 \pm 0.02$ which is in satisfactory agreement with the value of $0.62 \pm 0.02$ given by Goldstein and Solomon (9).

When $\Delta \pi_t$ is kept constant equation 1 may be differentiated to give

$$
(dJ_{\pi t}/d\pi_t)_{\Delta \pi_t} = -\Delta \pi_t(dL_p/d\pi_t)_{\Delta \pi_t} + L_{pd} + \Delta \pi_t(dL_{pd}/d\pi_t)_{\Delta \pi_t}
$$

(17)
in which $\Delta \pi_t = \Delta \pi_i + \Delta \pi_t$. If $L_p$ and $L_{pd}$ were independent of medium osmolality the first and third terms would go to zero and $L_{pd}$ could be obtained directly from equation 17 as $(dJ_{\pi t}/d\pi_t)_{\Delta \pi_t}$. Since all the points in Fig. 7 appear to fall close to a straight line, we had initially expected that the first and third terms in equation 17 would indeed be negligible so that the slope of the

| Table III |
|-----------------|-----------------|
| DETERMINATION OF $\sigma$ FOR UREA |

| Experiment | $a_0$ | $a_1$ | $\sigma$ |
|------------|-------|-------|---------|
| 15H        | 2.38  | -0.0058 | 0.53   |
| 16H        | 2.27  | -0.0058 | 0.57   |
| 17H        | 1.96  | -0.0044 | 0.55   |
| Average    |       |        | 0.55 $\pm 0.02$ |

* The equation for the straight line is $(dV_{rel}/dt)_{\pi_t} = a_t + a_1 \pi_t^2$ in which $(dV/dt)_{\pi_t}$ is the initial volume flow in relative amounts and $\pi_t^*$ is the total external osmolality.

The points in Fig. 7 were also fitted by least squares to a second-order polynomial; the coefficient for the term in $(\pi_t^*)^2$ was $3.7 \times 10^{-7}$ and the entire second-order term had a value of $2.1 \times 10^{-1}$. This figure is only about 5% of the value of the linear term of 4.2 and hence the curvature, if any, is not significant. In order to determine the behavior of the curve predicted by equation 17 we have made use of the fact that $L_p$ may be represented empirically as an
exponential function of the inverse of the medium osmolality as shown by Rich et al. (4); $L_{pd}$ may be expressed similarly as shown in Fig. 1. Therefore

$$L_p = A_1 e^{-m_1 \pi_i^0} \quad \text{and} \quad L_{pd} = -A_2 e^{-m_2 \pi_i^0} \quad (19)$$

When advantage is taken of the fact that \((dL_p/d\pi_i^0)_{\Delta \tau} = -[dL_p/d(1/\pi_i^0)] (\pi_i^0)^{-2}\), equation 17 may be transformed into

$$\left(\frac{dJ_v}{d\pi_i^0}\right)_{\Delta \tau} = A_1 m_1 \Delta \pi_i (\pi_i^0)^{-2} e^{-m_1 \pi_i^0} + A_2 m_2 \Delta \pi_i (\pi_i^0)^{-2} e^{-m_2 \pi_i^0} - A_2 n_2 e^{m_2 \pi_i^0} \quad (20)$$

The following are the experimental coefficients:

- \(A_1 = 0.44 \times 10^{-11} \text{ cm}^3/\text{dyne sec} \), \(m_1 = 7.5 \times 10^9 \text{ dynes/cm}^2\),
- \(A_2 = 0.43 \times 10^{-11} \text{ cm}^3/\text{dyne sec} \), and \(m_2 = 2.8 \times 10^6 \text{ dynes/cm}^2\).

Equation 20 predicts that \((dJ_v/d\pi_i^0)_{\Delta \tau}\) will vary from 0.5 to 1.0 when the external osmolality changes from 300 to 600 milliosmols, whereas the entire second-order term in the least squares polynomial fitted to the data in Fig. 7 is only 5% of the linear term. Thus equation 1 leads to predictions which are not satisfied by the experimental data, and hence is not applicable to the measurement of coupled solute and solvent flows across the human red cell membrane. Thus it appears that $\sigma$, just as $\omega$, cannot be determined by using equations 1 and 2 as long as volume flow is taking place in the system. This is a consequence of the series nature of the permeability barrier.

The time course of volume change in similar experiments with permeable solutes has been used by Hempling (12) who determined both $\sigma$ and $\omega$ in ascites tumor cells for a series of homologous glycols. Hempling used the entire time sequence of the experiment and devised an analogue computer solution to the equations in which he treated $\sigma$ and $\omega$ as adjustable constants which could be varied until the computer solution agreed with the experimental time course. Such a procedure involves the assumption that both $\sigma$ and $\omega$ are constants, independent of $J_v$ and that the Kedem and Katchalsky treatment is valid. In the human red cell, these assumptions are not justified and Hempling has not investigated these questions explicitly in the case of the ascites tumor cells. We have also devised a computer program to fit the entire time course of the swelling and shrinking process with single valued constants for $\sigma$ and $\omega$. In agreement with Hempling's observations, a satisfactory fit could be obtained, but the value of $\sigma$ required to obtain these fits differed widely from our independent estimates of $\sigma$ made when $J_v = 0$.

The Kedem and Katchalsky equations discussed here have made an important contribution to our understanding of the physical relations between forces and fluxes in the studies of membrane permeability. The present observations indicate that equations 1–3 may not be used to study coupled
solute and solvent flows across red cell membranes because even this very thin cellular membrane behaves in all respects as a complex structure, which cannot be handled by equations applicable to a homogeneous barrier. Nonetheless, the parameters, \( \sigma, L_p \), and \( \omega \), are illuminating as a formal description of red cell membrane permeability properties provided the measurements are made in the absence of coupled flows. But even these descriptions should be used with caution since all the results in the present study are consonant with the view that the human red cell membrane is an asymmetric and complex permeability barrier, so that the permeability parameters are composites describing properties of more than a single effective barrier.

**APPENDIX**

**D. C. MIKULECKY**

*A Series Membrane Model Obtained from Solving the Local Convection-Diffusion Equation*

It is possible to devise a model to fit our data on the flow dependence of \( \omega \). In this appendix it will be demonstrated that the simplest model consistent with all the data is a series membrane system with a volume-flow-dependent over-all permeability. This nonlinearity arises from the effect of convection (volume flow), which occurs during the swelling and shrinking of the cells, on the membrane concentration profile.

We will proceed by demonstrating that the dependence of \( \omega \) on \( J_o \) cannot be fitted by the solution to the convection-diffusion equation for a single membrane. We will then show that the data can be fit by solving the convection-diffusion equation for two barriers in series. Finally, it will be shown that the use of steady-state equations is justified on the basis of the half-time for the decay of the transient portion of the solution of the nonsteady-state convection-diffusion equation.

**Solution of the Convection-Diffusion Equation for a Single Membrane**

We assume that the local behavior for a slab at some point in the membrane can be described by the equation

\[
J_s = -D(\partial c/\partial x) + c(1 - \sigma^*)J_s
\]

where \( D \) is the local diffusion coefficient and \( \sigma^* \) the local reflection coefficient which is assumed to be independent of \( x \). The continuity relation:

\[
\partial c/\partial t = -\partial J_s/\partial x
\]

combined with the steady-state condition:

\[
\partial c/\partial t = 0
\]
leads to the differential equation,
\[
\frac{\partial^2 \epsilon}{\partial x^2} - u \frac{\partial \epsilon}{\partial x} = 0
\]  \hspace{1cm} (A-2)

in which \( u = J_s(1 - \sigma^*)/D \) and which has the solution,
\[
\epsilon = \langle \epsilon \rangle - \frac{\Delta \epsilon}{2} \frac{\left(2e^{\Delta x} - e^{2\Delta x} - 1\right)}{\left(e^{\Delta x} - 1\right)}
\]  \hspace{1cm} (A-3)

where
\[
\langle \epsilon \rangle = \left(c^0 + \epsilon^{\Delta x}/2 \right) \quad \text{and} \quad \Delta \epsilon = \Delta \pi_s/RT
\]

If we substitute the solution (A-3) into the expression for solute flow (A-1), we obtain
\[
J_s = J_s(1 - \sigma)\langle \epsilon \rangle + J_s \frac{(1 - \sigma)\epsilon^{\Delta x} + 1}{2(e^{\Delta x} - 1)} \Delta \epsilon
\]  \hspace{1cm} (A-4)

in which we assume \( \sigma = \sigma^* \).

By expanding the exponentials and only retaining the terms linear in \( u \) (second-power terms in \( u \) are indeed negligible since \( J_s \) is of the order \( 10^{-8} \) and \((1 - \sigma)\Delta x/D \) is of the order \( 10^9 \)), we obtain
\[
J_s = J_s(1 - \sigma)\langle \epsilon \rangle + [\omega + (1 - \sigma)J_s/RT] \Delta \pi_s
\]
in which
\[
\omega = D/RT\Delta x
\]

The flow-dependent permeability coefficient, \( \omega(J_s) \), is given by:
\[
\omega(J_s) = \omega(0) + (1 - \sigma)J_s/RT
\]  \hspace{1cm} (A-5)

according to which the plot of \( \omega \) vs. \( J_s \) in Fig. 5 would be expected to have a positive slope of \( 2 \times 10^{-11} \) moles/dyne sec. The data in Fig. 5 show a negative slope of \( 1.19 \times 10^{-10} \) moles/dyne sec. Thus the \( J_s \) dependence of \( \omega \) predicted by the theory is too small and its slope has the wrong sign to fit the data.

**Solution of the Convection-Diffusion Equation for a Series System of Two Membranes**

Consider the series membrane system in which a distance, \( l(0 < l < \Delta x) \), separates two regions in the membrane with differing permeability properties. The permeability barrier at the outer face is denoted by the superscript, \( a \), and that near the inner face of the membrane is denoted by the superscript, \( b \). We now seek a two-part solution to equation (A-2), one for each membrane, which satisfies the conditions
\[
u = u^a \quad \epsilon = \epsilon^a(x) \quad 0 \leq x \leq l
\]  \hspace{1cm} (A-6)
\[ u = u^b \quad c = c^b(x) \quad l \leq x \leq \Delta x \]  
(A-7)

\[ c^a(0) = c^a \]  
(A-8)

\[ c^b(\Delta x) = c^a \]  
(A-9)

\[ c^b(l) = c^b(l) \]  
(A-10)

\[ J_s = \text{constant everywhere (steady state)} \]  
(A-11)

The general solution to (A-2) is of the form:

\[ c^a = 
\begin{align*}
1 & + c_1^a \epsilon^{a x} \\
2 & + c_2^a \epsilon^{a x}
\end{align*} \]  
(A-12)

\[ c^b = 
\begin{align*}
1 & + c_1^b \epsilon^{b x} \\
2 & + c_2^b \epsilon^{b x}
\end{align*} \]  
(A-13)

The application of the boundary condition (A-11) leads to the requirement that

\[ D^a u v^a = D^b u v^b \]  

Boundary conditions (A-8) and (A-10) lead to the solutions

\[ c^a = D^b u^b K (1 - \epsilon^{a x}) + c^a \epsilon^{a x} \]  
(A-14)

\[ c^b = D^a u^a K (1 - \epsilon^{b x}) + c^b \epsilon^{b x} \]  
(A-15)

in which

\[ K = \left\{ \frac{\langle \epsilon \rangle (\epsilon^{a x} - \epsilon^{a x}) - \Delta c}{D^b u^b (1 - \epsilon^{a x}) - D^a u^a (1 - \epsilon^{b x})} \right\} \]  
(A-16)

The substitution of (A-14) back into (A-1) yields an expression for the solute flow

\[ J_s = D^a u^a D^b u^b K \]  
(A-17)

If we linearize the exponentials in \( K \) as before we obtain

\[ J_s = D^a D^b \left\{ \frac{\langle \epsilon \rangle (u^b (\Delta x - l) + u^a l) + \Delta c (2 + u^a l - u^b (\Delta x - l))}{D^b l + D^a (\Delta x - l)} \right\} \]  
(A-18)

With the use of the relation \( \omega^i = D^i/RT \delta^i \) where \( \delta^a = l \) and \( \delta^b = \Delta x - l \), the equation can be put in the form:

\[ J_s = \langle \epsilon \rangle (1 - \sigma) J_s + \omega(0) \left\{ 1 + \frac{1}{2RT} \left[ \frac{1 - \sigma^a}{\omega^a} - \frac{1 - \sigma^b}{\omega^b} \right] J_s \right\} \Delta \pi_s \]  
(A-19)

in which we have made use of the expressions derived by Kedem and Katchalsky (11) for a series array

\[ \frac{1}{\omega} = \frac{1}{\omega^a} + \frac{1}{\omega^b} \]  
(A-20)
\[ \sigma = \frac{\sigma^a \omega}{\omega^a} + \frac{\sigma^b \omega}{\omega^b} \]  \hspace{1cm} (A-21)

In the expression for solute flow for a series array (A-19) the flow-dependent permeability is given by

\[ \omega(J_s) = \omega(0) \left\{ 1 + \frac{1}{2RT} \left[ \frac{1 - \sigma^a}{\omega^a} - \frac{1 - \sigma^b}{\omega^b} \right] J_s \right\} \]  \hspace{1cm} (A-22)

In this case the plot of \( \omega \) vs. \( J_s \) may have a negative slope if

\[ \frac{1 - \sigma^b}{\omega^b} > \frac{1 - \sigma^a}{\omega^a} \]

This would be true if barrier \( a \) were a thin membrane with small openings in it in series with a thicker barrier \( b \) with larger equivalent pores. The data can be fitted using the values for \( \sigma^\text{ave} = 0.55 \) and \( \omega \) at \( J_s = 0 \) of \( 0.15 \times 10^{-14} \) mole/dyne sec and expressions (A-20)–(A-22), making the assumption that \( \sigma^b \approx 0 \). The results are that \( \omega^a(0) = 2 \times 10^{-14} \) moles/dyne sec and that \( \omega^b(0) = 6 \times 10^{-14} \) moles/dyne sec. \( \sigma^a \) is found to be approximately 0.67 so that the reflection coefficient for the outer membrane is slightly greater than the composite reflection coefficient measured for the entire series barrier.

The Half-Time for Reaching Steady State in the Presence of Convection and Diffusion

The solution to the nonsteady-state convection-diffusion equation is of the form:

\[ c(x, t) = \sum_{n=1}^{\infty} K_n(x) e^{-D/\Delta x^2} \left[ \frac{\left( \frac{x(1-x)}{D} \Delta x^2 \right)^n}{\pi^n \Delta x^2} \right] \]

For these experiments the first part of the exponential is negligible since

\[ J_s(1 - \sigma)\Delta x/2D = 0.025 \]

Thus at any point in the membrane the half-time for reaching the steady state is

\[ t_{1/2} = \Delta x^2 \ln 2/D \pi^2 = 4 \times 10^{-6} \text{ sec} = 40 \mu\text{sec} \]

DISCUSSION

The series model can account for the dependence of \( \omega \) on \( J_s \) and is consistent with the data previously obtained (4) which show that \( L_p \) varies with the outside osmolarity. The series model has not been used to explain the dependence of \( \sigma \) on \( J_s \) as the number of parameters is too great for the available data. The series model also accounts for dependence of \( \omega/\epsilon_s \) on \( J_s \), since the plot of \( \epsilon_s \) against \( J_s \) in Fig. 8 shows that \( \epsilon_s \) is strongly dependent on \( J_s \). Since \( \epsilon_s \) is much more strongly dependent on \( J_s \) than \( \omega \) is, the graph of \( \omega/\epsilon_s \) vs. \( J_s \) should be hyperbolic as the data in Fig. 6 show.
In the expressions for $J_s$ obtained from the solutions to the convection-diffusion equation we have obtained equations in which $\langle \phi \rangle$ replaces $\bar{\varepsilon}_s$ in the convection term. The difference between the two expressions is small except at the very beginning of the experiment. Nonetheless, the derivation above points out that $\langle \phi \rangle$ is the correct average to be used in the convection term. We should write

$$J_s = \langle \phi \rangle (1 - \sigma) J_s + \omega \Delta \pi_s$$

in place of equation 3. The value of $\bar{\varepsilon}_s$ is in writing expressions linear in $\Delta \pi_s$, rather than $\Delta \mu_s$ by the equation

$$\Delta \mu_s = \Delta \pi_s / \bar{\varepsilon}_s \quad (A-23)$$

The logarithmic average is in no way directly related to the profile in the membrane and has a unique meaning only when it is introduced through (A-23).

The results obtained in this appendix are similar to the rectification of solute and solvent flow predicted by Patlak et al. (13), although the models are somewhat different in detail. In the present case the concentration profile for a two-compartment series membrane is obtained explicitly by the use of an exponential expansion compatible with the low volume flow rates of the order of $10^{-6}$ cm/sec in our experimental system.

A similar approach has been applied to the analysis of desalination membranes by Spiegler and Kedem (14) and Jagur-Grodzinski and Kedem (15).
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