

# The Kinetics of Sodium Transport in the Toad Bladder

## I. *Determination of the transport pool*

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**ABSTRACT** A compartmental model of toad bladder sodium content has been developed, whereby it is possible to measure the four unidirectional fluxes across the opposite faces of the transport compartment, as well as the amount of sodium in the compartment.  $^{24}\text{Na}$  is added to the mucosal medium of a short-circuited bladder mounted between halves of a chamber in which the fluid is stirred by rotating impellers. After a steady state is reached, nonradioactive medium is flushed through both sides of the chamber, collected, and counted. The data from each chamber are fitted to sums of exponentials and interpreted in terms of conventional compartmental analysis. Three exponentials are required, with half-times of 0.2, 2.2, and 14.0 min. It is shown that the first of these represents chamber washout, the second the transport pool, and the third a tissue compartment which is not involved in active sodium transport and which does not communicate with the transport pool. The second compartment contains 10.5  $\mu\text{Eq}$  of sodium per 100 mg dry weight, an amount equal to approximately 30 % of total tissue sodium. The results also indicate, as expected from electrophysiological data, that the mucosal-facing side of the transport compartment is over 10 times as permeable to sodium as the serosal, or pump, side.

The isolated urinary bladder of the toad, like other oriented multicellular membranes, carries out the net transport of sodium against an electrochemical potential gradient. It has been assumed that sodium must cross at least two barriers in order to traverse the bladder, one at the mucosal, the other at the serosal, boundary of the cells. In actual fact, satisfactory evidence for the presence of such a simple model has been difficult to obtain. Furthermore, there are at least four cell types which make up the epithelial layer of the bladder (Choi, 1963; Peachey and Rasmussen, 1961). Quantitative considerations make it likely that the most numerous of these, the granular layer, carries

out the transport function, but there are, at present, no clear data on this. In addition, there are cells in the subepithelial layer, and although it seems clear from electrophysiological data (Frazier, 1962) that these cells do not play a significant role in production of the transepithelial potential difference, their role in the kinetics of sodium transport has not been defined; since they must contain some sodium it is possible that tracer kinetics may include a pool of sodium from this layer. Finally there is evidence that there must be a shunt, or parallel, pathway for sodium as well as a transcellular pathway (Civan et al., 1966).

It would be of considerable interest to be able to determine that fraction of tissue sodium involved in the transport process, the so-called "transport pool;" however, this has proven to be a difficult problem. Herrera (1965, 1966) was able to measure a sodium pool by a method which involved the abrupt addition of  $^{24}\text{Na}$  to the mucosal medium, and the monitoring of tracer appearance rate in the serosal solution. The resulting equations could be solved so as to yield values for unidirectional influxes and effluxes at both mucosal and serosal borders, as well as the pool size. This method also yielded evidence that the toad bladder sodium pool seemed to be represented by a single compartment. However, the nature of the method of calculation made it unlikely that more than a single exponential could be observed, a problem also present in the studies by Curran et al. in frog skin (1963).

Crabbé and DeWeer (1965, 1969) made measurements of a sodium pool in the toad bladder, but their calculations involved the implicit assumption of a single sodium compartment, and it was impossible to test adequately whether this pool, in fact, represented the transport pool.

Evaluation of flux rates and, in some cases, pools, has also been made by utilizing tracer washout from preloaded tissues (Frazier and Hammer, 1963; Hoshiko and Ussing, 1960; Leaf, 1966; Schwartz and Snell, 1968; Zerahn, 1969; Vanatta and Bryant, 1970). However, Leaf (1966) and Zerahn (1969) have argued that the sodium transport pool is not measurable. On the other hand, preliminary results from this laboratory (Finn, 1969) suggested that determination of a transport pool, and of the fluxes and rate coefficients at either side of the transporting compartment, could in fact be made in the toad bladder.

Thus, the purpose of these experiments was to evaluate the kinetics of sodium transport in the toad bladder by observing the rates of washout of tracer from preloaded bladders into both mucosal and serosal media. It was necessary to devise a technique which would allow the measurement and discrimination of fairly rapid events since Hoshiko and Ussing (1960) and Crabbé and DeWeer (1965) had shown that the half-time of buildup of tracer to a steady state following addition of tracer to the mucosal medium was of the order of 2 min. Once it could be established that consistent results could be obtained,

it was expected that the data might serve to establish a kinetic model of the transport system.

#### MATERIALS AND METHODS

The toads used in these studies were *Bufo marinus*, from Colombia, South America. They were obtained commercially from the Pet Farm, Miami, Florida, and kept on moist San-i-cel (Paxton Processing Co., Inc., Paxton, Ill.) prior to use. They were pithed and the bladders removed and placed in Ringer solution of the following composition, in millimoles per liter: NaCl 109, KCl 2.5, CaCl<sub>2</sub> 1, NaHCO<sub>3</sub>, 2.4, glucose 5.5. Solutions were gassed with room air and had a pH of 7.8.

Bladders are mounted in the chamber in the following manner: a Lucite ring, with an outer diameter of 2.5 cm, is placed on a Lucite block (Fig. 1). A piece of nylon stocking is stretched over the ring, and held in place on the block with a rubber band.

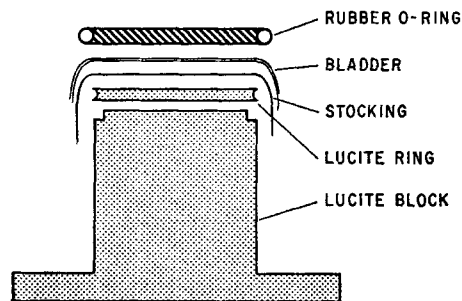


FIGURE 1. Assembly for mounting bladder. A piece of nylon stocking is stretched tightly over the Lucite ring, and held down with a rubber band. The bladder is placed gently over the stocking, and the rubber O-ring is snapped in place. Excess bladder is then cut off.

The bladder is carefully placed over the stocking, and a rubber O-ring is then snapped in place over the assembly. The excess stocking and bladder are then trimmed off, and the assembly is placed in Ringer solution. Another similar stocking and O-ring assembly is prepared, but without a bladder. The twin assembly is then mounted between halves of a Lucite chamber.

The chamber (Fig. 2) is fitted with entrance and exit ports, and silver-silver chloride pellets<sup>1</sup> are used to measure PD. The current electrodes consist of loops of silver-silver chloride wire placed around the base of the chamber, as shown in the figure. The bladders were short-circuited throughout these experiments, and the short-circuit current (SCC) was continuously recorded.

Within each half-chamber is a teflon disc with impeller blades carved on the surfaces. Within each disc are small bar magnets, and the impellers are rotated at approximately 500 rpm by using a pair of external horseshoe magnets attached to a rotary motor. The fluid volume contained in each chamber, with the teflon disc in place, is 2.24 ml.

After mounting the bladder-stocking assembly, Ringer solution is pumped through each chamber at a rate of 7.8 ml/min, using a separate Harvard infusion-withdrawal

<sup>1</sup>The electrodes are prepared by a method similar to that described by C. G. Phipps and G. G. Lucchina, U. S. Patent No. 3,137,291, June 1964.

pump for each chamber. The fluid flowing from the chambers is collected separately. For these studies, no bladder was used unless the open circuit PD was 20 mv or greater.

After the achievement of a constant short-circuit current, radioactive sodium was introduced into the mucosal chamber by a third Harvard syringe pump. The tracer was placed in Ringer solution with a final sodium concentration equal to that of the control Ringer; a tracer concentration of approximately 0.2 mCi/ml was used. An aliquot of the loading solution was collected and subsequently counted. Tracer was allowed to remain in the chamber for at least 45 min, during which time nonradioactive Ringer was continuously pumped through the serosal chamber. This period of time is adequate for achievement of a steady state for tissue radioactivity, since the rate of appearance of tracer on the serosal side reaches a constant value by 8–9 min. It may be shown that tissue radioactivity is constant when this criterion is met.<sup>2</sup>

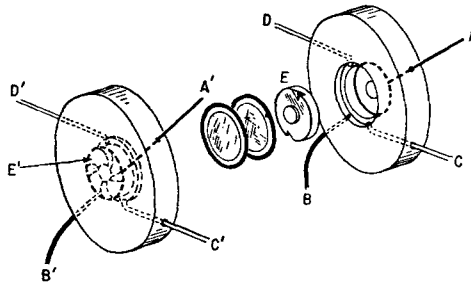


FIGURE 2. Washout chambers. The double O-ring assembly, one of which contains the bladder, is in the center. The chambers are held together by screws and mounted between two large horseshoe magnets. *A, A'*, current-passing electrodes, leading to silver-silver chloride wire loops at the base of each chamber. *B, B'*, PD electrodes. *C, C'*, fluid entry ports. *D, D'*, fluid exit ports. *E, E'*, teflon discs, each of which has several small bar magnets inside. On the back surface of each disc (shown in *E'*) there is a series of impeller blades. On the bladder-facing surface (shown in *E*) two smaller blades are carved.

<sup>2</sup> The rate equation describing tracer appearance in the serosal solution is

$$\frac{dP_s}{dt} = k_{sT} P_T - k_{Ts} P_s$$

where *P* is tracer activity in cpm, *k* is the rate constant in min<sup>-1</sup>, *T* is tissue, and *S* is serosal medium. The subscript convention, as described later, is *k<sub>ij</sub>* = rate constant for flow into compartment *i* from compartment *j*. Since *P<sub>s</sub>* is approximately zero with nonradioactive Ringer flowing through the medium at all times, the equation reduces to:

$$\frac{dP_s}{dt} = k_{sT} P_T.$$

Thus, since it is assumed that *k<sub>sT</sub>* is constant, the condition for tissue radioactivity to be constant with time is that the rate of appearance of tracer in the serosal medium, *dP<sub>s</sub>/dt*, be constant. This is strictly true, of course, only for compartments which communicate with the serosal bathing medium; however, as shown below, we have no evidence for the existence of any noncommunicating compartment(s).

After the achievement of this steady state of tracer in the tissue, the tracer was washed out of the chambers by introducing nonradioactive solution into both sides at a rate of 38.2 ml/min per chamber (nominal pump rate). This rate of flow was continued for 1 min. At that time, the rate of flow was abruptly reduced to 7.6 ml/min per chamber, and all effluent was collected into test tubes mounted in a fraction collector. The fluid was collected from each side for 30 sec periods for 30 min, and subsequently the test tubes were placed in an automatic gamma-counter and counted. (Because of limitations imposed by the number of samples and the problems of decay, all samples from the first 30 collection periods, and alternate samples from the last 30 periods, were counted.) Samples were counted long enough to give at least 10,000 counts, and all count rates were subsequently corrected for decay.

At the completion of one or more experiments on a bladder, the assembly was removed and the exposed portion of the bladder punched out with a cork borer, dried to constant weight, and the dry weight obtained.

#### THEORETICAL CONSIDERATIONS

A compartmental model for the analysis of the data has been developed. A lower bound on the number of compartments required was found by fitting the data simultaneously with linear combinations of exponentials (Coddington and Levinson, 1955; Hearon, 1963). Thus

$$\text{Mucosal efflux} = A_{M1}e^{-\alpha_1 t} + A_{M2}e^{-\alpha_2 t} + \dots + A_{Mn}e^{-\alpha_n t} \quad (1 a)$$

$$\text{Serosal efflux} = A_{S1}e^{-\alpha_1 t} + A_{S2}e^{-\alpha_2 t} + \dots + A_{Sn}e^{-\alpha_n t}. \quad (1 b)$$

When this fitting procedure was carried out it was found that two exponentials were never adequate, three were adequate in most studies, and four were needed in a few of the studies.<sup>3</sup> (See Results.) The role of the fourth exponential will be dealt with later. Thus a minimum of three compartments in the model was required.

#### *Role of the Chambers*

Since the chambers contribute a "compartment" their washout characteristics must be evaluated. The effluent (in counts per minute) washed out from  $t - \Delta t$  to  $t$  is given by

$$\text{cpm}(t) = \frac{f}{v} P_M \Delta t \quad (2)$$

where  $f$  is the flow rate in milliliters per minute,  $P_M$  is the total activity in the chamber in counts per minute, and  $v$  is the volume of the chamber in milli-

<sup>3</sup> All the data fitting and model computations were done with the computer program called "SIMULATION ANALYSIS AND MODELING," ("SAAM") developed by M. Berman at the National Institutes of Health (Berman et al., 1962).

liters. The data are normalized by dividing by the length of collection,  $\Delta t$ . The radioactivity in the chamber is nearly constant for small  $\Delta t$ , and its value at time  $t - \frac{\Delta t}{2}$  is approximated, using equation (2), by the radioactivity of the effluent at time  $t$ . Thus

$$\frac{\text{cpm}}{\text{min}}(t) = k_{OM} P_M \left( t - \frac{\Delta t}{2} \right) \quad (3)$$

where  $k_{OM} = \frac{f}{v}$ .

In order to evaluate the mixing characteristics of the chamber, washouts were performed with either Parafilm or Saran wrap in place of the bladder. The equation describing the radioactivity in the chamber in this case is

$$\frac{dP_M}{dt} = -\frac{f}{v} P_M = -k_{OM} P_M \quad (4)$$

with solution

$$P_M = P_M(0)e^{-k_{OM}t} \quad (5)$$

As shown in Table I, the observed decay rates for chamber washout at different flows compare well with those predicted. The data for each experiment were well-fitted by a single exponential over at least five orders of magnitude, indicating that the chamber is well-mixed and may be considered to be a single compartment.

From the results in Table I, it may be calculated that during 1 min of washout at 38.2 ml/min (such as is used at the beginning of a bladder experiment), the amount of tracer in the chamber falls to approximately  $0.37 \times 10^{-7}$

TABLE I  
CHAMBER KINETICS

Flow rate	$k_{OM}$ or $k_{OS}$ , $\text{min}^{-1}$	
	Theoretical	Observed
<i>ml/min</i>		
7.64	3.42	3.57 (3)
15.3	6.84	6.81 (2)
38.2	17.08	17.51 (3)

Theoretical decay constants are calculated as flow rate: volume of chamber, as described in the text. The observed constants (number of experiments in parentheses) are the computer-calculated values from the best fit to a single exponential; washouts from mucosal and serosal chambers yield identical values.

times its initial steady-state value. The reason for doing the rapid wash is to be able to neglect the backflux of labeled sodium from the chambers into the tissue in analyzing the data. The model presented assumes that backflux can be neglected. The validity of this assumption has been checked computationally and is discussed below.

### Three-Compartment Model

Since the chamber itself accounts for one of the exponentials in equation 1, the minimum number of tissue compartments is two. A simple configuration which neglects backflux and has two tissue compartments is given schematically in Fig. 3, where the circles labeled 1 and 2 represent the two compartments in the tissue and the circles labeled M and S represent the chambers on the mucosal and serosal sides of the bladder respectively.

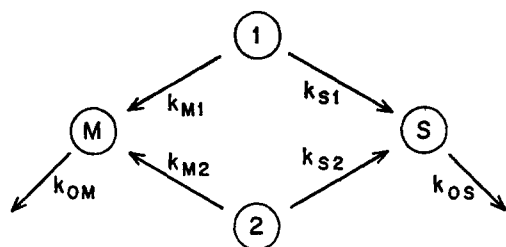


FIGURE 3. A simple three-compartment model. See text.

After achievement of the steady state, tracer washout begins. The equations for the radioactivity in the tissue compartments during washout (neglecting backflux) and assuming that the volumes of the compartments remain constant, are

$$\frac{dP_1}{dt} = - (k_{M1} + k_{S1})P_1 \quad (6 a)$$

$$\frac{dP_2}{dt} = - (k_{M2} + k_{S2})P_2 \quad (6 b)$$

with solutions:

$$P_1(t) = P_1(0)e^{-(k_{M1}+k_{S1})t} \quad (7 a)$$

$$P_2(t) = P_2(0)e^{-(k_{M2}+k_{S2})t} \quad (7 b)$$

where  $P_i(0) = P_i$  at time  $t = 0$ . In particular, at time  $t_1$  when the chamber wash rate is reduced,

$$P_1(t_1) = P_1(0)e^{-(k_{M1}+k_{S1})t_1} \quad (8 a)$$

$$P_2(t_1) = P_2(0)e^{-(k_{M2}+k_{S2})t_1} \quad (8 b)$$

The equation describing the radioactivity in the mucosal chamber is:

$$\frac{dP_M}{dt} = -k_{OM}P_M + k_{M1}P_1 + k_{M2}P_2. \quad (9)$$

Using equations (7) this becomes

$$\frac{dP_M}{dt} = -k_{OM}P_M + k_{M1}P_1(0)e^{-(k_{M1}+k_{S1})t} + k_{M2}P_2(0)e^{-(k_{M2}+k_{S2})t}. \quad (10)$$

The solution for this equation with initial condition  $P_M(t_1)$  given is

$$P_M(t) = \frac{k_{M1}P_1(0)}{k_{OM} - (k_{M1} + k_{S1})} e^{-(k_{M1}+k_{S1})t} + \frac{k_{M2}P_2(0)}{k_{OM} - (k_{M2} + k_{S2})} e^{-(k_{M2}+k_{S2})t} + \left[ P_M(t_1) - \frac{k_{M1}P_1(0)e^{-(k_{M1}+k_{S1})t_1}}{k_{OM} - (k_{M1} + k_{S1})} - \frac{k_{M2}P_2(0)e^{-(k_{M2}+k_{S2})t_1}}{k_{OM} - (k_{M2} + k_{S2})} \right] e^{k_{OM}t_1} e^{-k_{OM}t}. \quad (11)$$

Similarly, the equation satisfied by the radioactivity in the serosal chamber is

$$P_S(t) = \frac{k_{S1}P_1(0)}{k_{OS} - (k_{M1} + k_{S1})} e^{-(k_{M1}+k_{S1})t} + \frac{k_{S2}P_2(0)}{k_{OS} - (k_{M2} + k_{S2})} e^{-(k_{M2}+k_{S2})t} + \left[ P_S(t_1) - \frac{k_{S1}P_1(0)e^{-(k_{M1}+k_{S1})t_1}}{k_{OS} - (k_{M1} + k_{S1})} - \frac{k_{S2}P_2(0)e^{-(k_{M2}+k_{S2})t_1}}{k_{OS} - (k_{M2} + k_{S2})} \right] e^{k_{OS}t_1} e^{-k_{OS}t}. \quad (12)$$

The equations for the serosal and mucosal effluent data are obtained by multiplying both sides of equations 11 and 12 by  $k_{OM}$  and  $k_{OS}$ , respectively and using equation 3. The data on each side can be fitted simultaneously as linear combinations of the same three exponentials, constraining one of the exponential decay rates to be  $\frac{f}{v}$  which is known:

$$\text{Mucosal effluent} = \frac{dP_M}{dt} = -k_{OM}P_M = D_1e^{-d_1t} + D_2e^{-d_2t} + D_3e^{-d_3t} \quad (13 a)$$

$$\text{Serosal effluent} = \frac{dP_S}{dt} = -k_{OS}P_S = \tilde{D}_1e^{-d_1t} + \tilde{D}_2e^{-d_2t} + \tilde{D}_3e^{-d_3t}. \quad (13 b)$$

When the best (in the least squares sense) values for  $D_1, D_2, D_3, \tilde{D}_1, \tilde{D}_2, \tilde{D}_3, d_1, d_2,$  and  $d_3$  are found, the following identifications can be made:

$$d_1 = k_{OM}$$

$$d_2 = k_{M1} + k_{S1}$$

$$d_3 = k_{M2} + k_{S2}$$



$$D_2 = \frac{k_{OM} k_{M1} P_1(0)}{k_{OM} - (k_{M1} + k_{S1})}$$

$$\tilde{D}_2 = \frac{k_{OS} k_{S1} P_1(0)}{k_{OS} - (k_{M1} + k_{S1})}$$

$$D_3 = \frac{k_{OM} k_{M2} P_2(0)}{k_{OM} - (k_{M2} + k_{S2})}$$

$$\tilde{D}_3 = \frac{k_{OS} k_{S2} P_2(0)}{k_{OS} - (k_{M2} + k_{S2})}$$

Since  $k_{OM} = k_{OS}$ , these equations can be solved for  $k_{M1}$ ,  $k_{M2}$ ,  $k_{S1}$ ,  $k_{S2}$ , and the initial pools,  $P_1(0)$  and  $P_2(0)$ .

#### *Validation of the Model*

The model presented is a simple one that permits easy calculation of the parameters from a straightforward fit of the data to three exponentials. In order to validate the model, SAAM was used to simulate it, i.e. to solve the differential equations starting at time 0, at which time the rapid wash is begun, and taking into account the change in wash rate that occurs at 1 min. When the parameters of the model are obtained in this manner, initial pool sizes  $P_1(0)$  and  $P_2(0)$  are additional unknowns to be estimated, while the initial radioactivity in the chambers is known (see Methods). The results obtained for the parameters in both of these ways were the same, as would have been anticipated since the same model was involved. The principal reason for simulating the model and solving the associated differential equations was to study possible changes in the model. Thus, the following configurations were considered: two including connections between the tissue compartments (series and parallel configurations were studied separately) and one including backflux from the chambers into the tissue compartments. In no case was the "fit," as measured by the sum of squared deviations between the predicted and observed values, improved. The additional unknowns (i.e.,  $k_{12}$  and  $k_{21}$ ) gave too much freedom to the parameters and they could not be determined with precision. Several attempts were then made to fix the values of  $k_{12}$  and  $k_{21}$  over a 100-fold range, rather than to estimate them. No matter what combinations of values were chosen for either the series or the parallel model, the fit was never better than when  $k_{12} = k_{21} = 0$ .

In a few cases, an additional early fast component could be observed. This component could not be well-determined numerically but the computed parameters for compartments 1 and 2 (above) were identical whether the data from these experiments were fit to three or four exponentials. The possible significance of this compartment will be discussed.

RESULTS

A. Data Analysis

Figs. 4 and 5 show the results of a typical washout experiment on a bladder. The data are plotted semilogarithmically against time, and it is clear from

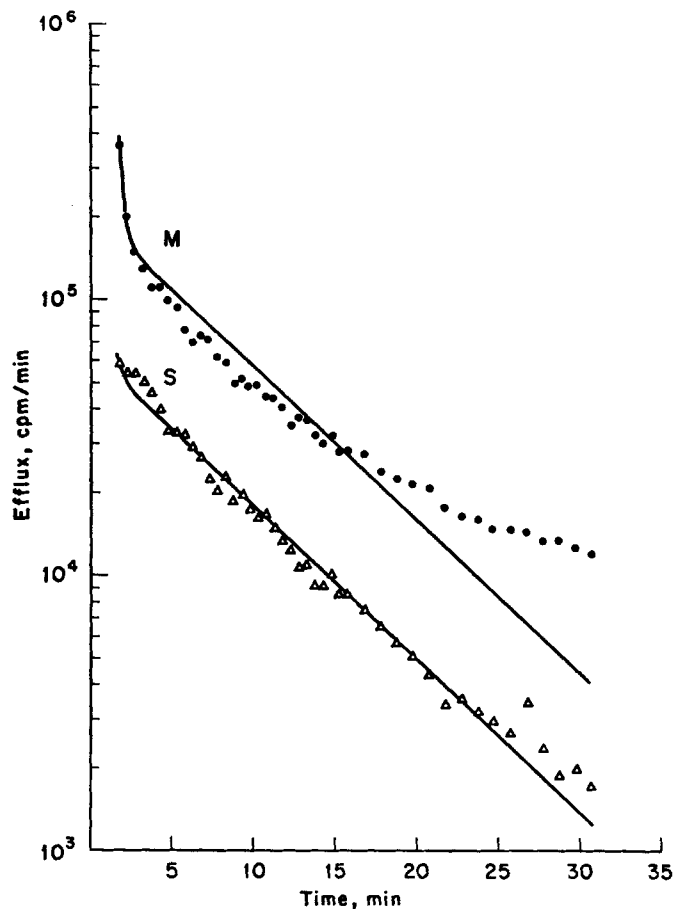


FIGURE 4. Two-exponential fit. The data are fit by the computer to two exponentials. *M* (solid circles), mucosal, and *S* (open triangles), serosal points. It should be noted that there is a systematic deviation of both sets of points from the calculated best fit (the solid lines). This is more marked in the data from the mucosal chamber.

inspection that at least two exponentials are required to describe the data adequately. The experiment shown has been fitted by the computer to both two (Fig. 4) and three (Fig. 5) exponentials. Not only is the sum of squared deviations considerably reduced by fitting with three exponentials, but also the latter gives a better qualitative fit, as seen by the lack of systematic devi-

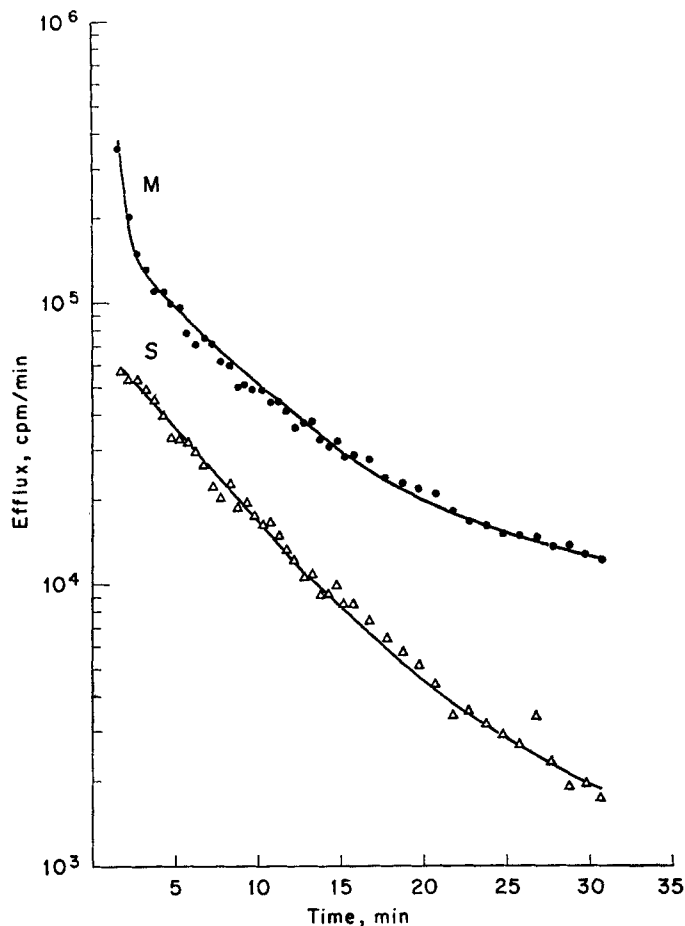


FIGURE 5. Three-exponential fit. The same data as in Fig. 6 are plotted but the calculated lines (the solid lines) represent the best least squares fit to three exponentials, as described in the text. Note the lack of systematic deviation.

ations between the observed and predicted values. On the other hand, fitting the data to four exponentials generally did not improve the quality of the fit, although the sum of squared deviations decreased somewhat. Also, the computed values for the additional pair of coefficients and decay rate could not be determined with precision.

When all experiments were fitted to three exponentials, the value for the fastest exponential constant was  $3.36 \pm 0.42 \text{ min}^{-1}$ . This value is not different from that found for the chamber itself, as previously described. Hence, attempts to determine the appropriate kinetic model for the tissue include the assumption that this first component is due only to the chamber.

Thus as stated above, there appear to be, in general, two exponential terms

due to sodium washout from the toad bladder itself. The slower of these components has a decay rate of  $0.050 \pm 0.004 \text{ min}^{-1}$ , or a mean half-time of 14.0 min. This value is quite close to that described by Leaf (1966), and in the latter's experiments, drastic inhibition of sodium transport did not affect this rate. Thus it seemed that the sodium pool involved in transport could not be reasonably attributed to such a component.

In order to evaluate this possibility further, two experiments were performed in which sodium cyanide was added to the serosal medium for the second washout, after a control washout had been run. In two additional experiments the bladder potential was clamped at a value of 250 mv, serosa positive. In neither of these types of experiments was there a change in the slow component of the washout curves. In addition, neither vasopressin nor changes in sodium concentration (Finn, 1971) affected the parameters involving this slow compartment, while there were significant changes involving the faster of the two tissue components, again suggesting that the slow component did not represent the toad bladder transport pool.

In addition to the portion of bladder exposed to the media, there is a rim of the tissue which is compressed between the plastic ring and the rubber O-ring. Since this tissue might, in fact, represent the slow component seen in the washouts, the following experiments were done. The portion of tissue exposed to the circulating media was cut out carefully, and a piece of Saran wrap was mounted on one side of the remaining rim of tissue. Tracer was then added to the medium facing the tissue, and a washout was performed as previously described, with the exception, of course, that samples were collected from one chamber only. Under these circumstances, as shown in Fig. 6, the data were satisfactorily fitted to only two exponentials, one of

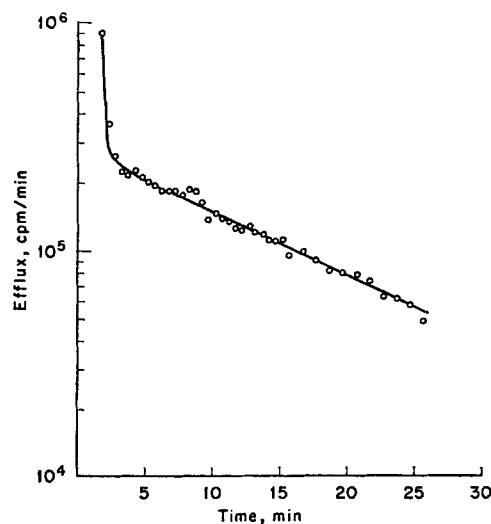


FIGURE 6. Rim washout. In this case, the portion of the bladder exposed to the chambers has been carefully cut away, and a piece of Saran wrap placed between the O-ring assemblies. The data (open circles) are fit to two exponentials (—).

which was equal to the chamber washout, the other of which was approximately equal to the slow component of the intact tissue washouts. In three experiments, the half-times of the slow component were 12.5, 13.0, and 11.1 min. For these reasons, the slow component has been tentatively ascribed, at least in part, to the rim of tissue which is compressed along the margins of the chamber. This will be discussed further below.

The mean decay rate for the second, or fast tissue component, was  $0.319 \pm 0.024 \text{ min}^{-1}$ , corresponding to a half-time of 2.17 min. From the computer output, the values for  $k_{M1}$ ,  $k_{S1}$ , and  $P_1(0)$  can be obtained as described in the section on theory. In order to obtain the unidirectional fluxes and the sodium content of the pool, the following calculations are necessary. Conventions and definitions are as follows:

$k_{ij}$ = rate constant for sodium movement into compartment $i$ from compartment $j$ , $\text{min}^{-1}$	$p_i^*$ = specific activity in compartment $i$ , $\text{cpm}/\mu\text{Eq}$
$P_i$ = total radioactivity in compartment $i$ , $\text{cpm}$	$J_{ij}$ = flux into compartment $i$ from compartment $j$ , $\mu\text{Eq}/\text{min}$
	$A_i$ = total sodium in compartment $i$ , $\mu\text{Eq}$ .

After the addition of isotope to the mucosal compartment and before washout begins, the change of tracer with time in the tissue compartment of interest will be as follows:

$$dP_1/dt = k_{1M}P_M + k_{1S}P_S - P_1(k_{M1} + k_{S1}). \quad (14)$$

After allowing sufficient time for the steady state for tracer to be reached, and taking  $P_S$  to be negligible (nonradioactive Ringer continuously flowing through the serosal chamber), equation (14) becomes, after rearranging,

$$k_{1M}P_M = P_1(k_{M1} + k_{S1}) \quad (15)$$

Furthermore, since

$$J_{ij} = k_{ij}A_j \quad (16)$$

and

$$p_i^* = P_i/A_i \quad (17)$$

equation (15) becomes:

$$J_{1M}p_M^* = P_1(k_{M1} + k_{S1}). \quad (18)$$

Following achievement of the steady state, tracer washout begins. During washout, the model satisfies the equations previously discussed. Assuming that  $P_1(t)$  is a continuous function, and, in particular, is continuous at time

$t = 0$ , and again, that tracer flux from mucosal compartment to tissue is negligible during washout, equation (18) may be solved for  $J_{1M}$  at  $t = 0$ , since  $p_M^*$  is known and  $P_1(0)$  and  $(k_{M1} + k_{S1})$  can be calculated from the washout data.

We now make the following assumptions: (a) a steady state for total sodium exists, (b) the short-circuit current equals the net sodium flux (in separate experiments this was shown to be true for the present system), (c) for the purpose of testing this model, the net sodium flux across compartment 1 is equal to the net sodium flux across the bladder. Therefore,

$$J_{\text{net}} = J_{SM} - J_{MS} = J_{1M} - J_{M1} = J_{S1} - J_{1S} \quad (19)$$

Thus  $J_{M1}$  may be determined as

$$J_{M1} = J_{1M} - J_{\text{net}} \quad (20)$$

From equations (16) and (20),  $A_1$  may be obtained, and from this value and equation (16),  $J_{S1}$  may be obtained. Finally, knowing  $J_{\text{net}}$  and  $J_{S1}$ ,  $J_{1S}$  may be obtained from equation (19).

### B. Calculated Results

The results of the calculations based on the model discussed are shown in Table II,<sup>4</sup> for the faster of the two tissue components. Pool sizes and fluxes are expressed relative to the dry weight of the portion of the bladder exposed to the media. This reference was used because of the great variability in the degree that a bladder may be stretched when it is mounted, although there is still, as seen in the table, considerable variation in pool sizes.

Table III shows the same calculations obtained from the slower of the two tissue compartments. It is immediately obvious that this compartment cannot alone be the transport pool. First of all, the backfluxes from the serosal medium to the pool have relatively large negative values due to the fact that the net flux is considerably larger than the calculated unidirectional efflux,  $J_{S2}$ . Second, the fluxes and rate coefficients are far smaller than those calculated for the faster compartment. Third, as previously noted, there is no effect on this pool of cyanide or of an adverse electrical potential gradient; finally, a compartment with similar kinetics seems to be present even after removal of the central portion of tissue. It is realized that this compartment will, in general, have reached only about 90% of the tracer steady-state values (half-time 14 min, minimum loading period 45 min). Thus the calculations in Table III will differ slightly from the true values for this pool; the

<sup>4</sup> The data in Tables II and III were obtained in experiments used as controls for some of the vasopressin experiments detailed in the accompanying paper (Finn, 1971).

TABLE II  
KINETIC PARAMETERS: FAST TISSUE POOL

	$J_{1M}$	$J_{M1}$	$J_{S1}$	$J_{1S}$	Pool	$k_{M1}$	$k_{S1}$
	2.76	2.34	0.620	0.196	11.36	0.206	0.055
	0.83	0.73	0.181	0.075	5.62	0.129	0.032
	2.79	2.63	0.212	0.054	6.92	0.380	0.031
	3.86	3.72	0.151	0.010	11.62	0.320	0.013
	5.83	5.56	0.330	0.064	11.80	0.471	0.028
	1.74	1.63	0.187	0.071	9.60	0.169	0.019
	5.83	5.72	0.134	0.027	16.35	0.350	0.008
Mean	3.37	3.19	0.259	0.071	10.47	0.289	0.027
±	±	±	±	±	±	±	±
SEM	0.71	0.72	0.065	0.023	1.34	0.047	0.005

Symbols are as described in text.

Fluxes are expressed in milliequivalents·100 mg dry weight<sup>-1</sup>·min<sup>-1</sup>, pools in milliequivalents·100 mg dry weight<sup>-1</sup>, and rate coefficients in min<sup>-1</sup>.

TABLE III  
KINETIC PARAMETERS: SLOW TISSUE POOL

	$J_{2M}$	$J_{M2}$	$J_{S2}$	$J_{2S}$	Pool	$k_{M2}$	$k_{S2}$
	0.453	0.033	0.0086	-0.411	0.85	0.039	0.0102
	0.270	0.170	0.0279	-0.071	7.02	0.024	0.0040
	0.322	0.164	0.0036	-0.154	2.98	0.055	0.0012
	1.182	1.041	0.0223	-0.119	18.59	0.056	0.0012
	1.438	1.172	0.0440	-0.222	13.32	0.088	0.0033
	0.175	0.059	0.0015	-0.115	14.75	0.004	0.0001
	0.585	0.478	0.0464	-0.061	14.48	0.033	0.0032
Mean	0.632	0.521	0.0220	-0.165	10.28	0.043	0.0033
±	±	±	±	±	±	±	±
SEM	0.184	0.168	0.0063	0.046	2.53	0.010	0.0013

Data are expressed as in Table II.

conclusions would not be altered by these minimal deviations. It should be added, furthermore, that since compartment 2 cannot be identified with the transport pool, the assumption that it carries out net transport of sodium is probably not valid. When the data obtained from the washout curves are used, we obtain, as shown above,  $J_{2M}$  and the rate constants,  $k_{M2}$  and  $k_{S2}$ . If we now assume that there is no net flux across this compartment (i.e.,  $J_{2M} = J_{M2}$ , and  $J_{2S} = J_{S2}$ ), we can recalculate the size of this pool. Such calculations yield a pool size of  $18.24 \pm 4.65 \mu\text{Eq} \cdot 100 \text{ mg dry weight}^{-1}$ , a value which is probably closer to the true size of this compartment.

A critical measurement may be made, which should yield conclusive evidence as to which of these two pools is involved in transepithelial transport, if indeed either of them is. It is possible to calculate the transepithelial mucosa-

to-serosa flux ( $J_{SM}$ ) from the four unidirectional fluxes. Since the former may also be measured independently just prior to the start of a washout experiment, by serial collections of effluent from the serosal side (while isotope is still present in the mucosal chamber), these two measurements may be compared. Agreement would indicate that the model almost certainly represents the same compartment involved in transepithelial sodium transport. The derivation (after Ussing and Zerahn [1951]), follows:

From equations (17) and (18),

$$J_{1M}p_m^* = p_1^*(J_{M1} + J_{S1}). \quad (21)$$

During the loading period, we may write the following:

$$dP_s/dt = (k_{s1}P_1 - k_{1s}P_s) + (k_{s2}P_2 - k_{2s}P_s). \quad (22)$$

We may, as before, take the total tracer in the serosal compartment as negligible (since nonradioactive ringer is flowing through that chamber continually); furthermore, in order to test the hypothesis being proposed here, we may ignore the contribution of the slow compartment (it is, in any event, less than 10% of that from the fast compartment, as shown in Tables II and III). Thus, from equations (17) and (22),

$$dP_s/dt = J_{s1}p_1^*. \quad (23)$$

The transepithelial flux,  $J_{SM}$ , is measured as the rate of appearance of tracer in the serosal chamber (in the steady state) divided by the specific activity of the loading solution in the mucosal chamber, i.e.,

$$J_{SM} = \frac{dP_s/dt}{p_m^*}. \quad (24)$$

From equations (23) and (24)

$$J_{SM} = J_{s1}p_1^*/p_m^*. \quad (25)$$

Solving equations (21) and (25) for  $J_{SM}$  yields

$$J_{SM} = \frac{J_{s1}J_{1M}}{J_{M1} + J_{S1}}. \quad (26)$$

In order to test whether the measured and calculated fluxes are equal, five additional experiments were done (since in the above experiments, the transepithelial flux was not routinely determined). Table IV shows the remarkable agreement between the two independent measurements. If one uses the slow compartment for the calculated  $J_{SM}$ , a value of approximately one-tenth of the measured  $J_{SM}$  is obtained.



TABLE IV  
COMPARISON OF MEASURED AND CALCULATED  $J_{SM}$

	$J_{1M}$	$J_{M1}$	$J_{S1}$	$J_{SM}$	
				Calculated	Measured
	1.11	0.66	0.543	0.501	0.491
	3.45	3.29	0.219	0.215	0.277
	3.88	3.65	0.408	0.390	0.384
	5.81	5.43	0.402	0.400	0.382
	1.31	1.13	0.291	0.267	0.283
Mean	3.11	2.83	0.373	0.355	0.363
±	±	±	±	±	±
SEM	0.87	0.87	0.055	0.051	0.039

The calculated fluxes are obtained as follows:

$$J_{SM} = \frac{J_{S1} J_{1M}}{J_{M1} + J_{S1}}$$

The measured fluxes are determined by collecting timed samples from the serosal chamber after tracer has been present in the mucosal chamber for at least 45 min, and are determined as follows.

$$J_{SM} = \frac{dP_s/dt}{p_M^*}$$

Thus, of the two pools readily identified in the bladder, one seems to be related to transbladder sodium transport. In order to provide still more evidence that such a pool is the biologically significant "transport pool," one needs to show that it responds to biological perturbations. The following paper (Finn, 1971) indicates that such is the case.

As previously mentioned, there appears to be, in a few studies, a third tissue compartment. This compartment is a very rapid one, with a mean half-time of 32 sec. The presence of such a pool was suggested by the fact that in those studies, the first few mucosal data points fell above the calculated best fit for the model heretofore presented. The addition of an extra compartment, which empties only into the mucosal chamber, completely accounts for these points. Calculations were performed on other models, in which this extra compartment was allowed to communicate with both chambers, but the data did not support these models. In order to evaluate this further, four experiments were performed in which the flow rate through the chambers for the first 5 min after the rapid wash was 15.3 instead of 7.6 ml/min. In those experiments, effluent was collected at 12 sec intervals for this 5 min period. In only one of the four experiments was there evidence of a fourth exponential; again, the best fit was obtained when the extra com-

partment was allowed to communicate only with the mucosal chamber. In the other three experiments only three exponentials were needed, and in all four experiments the pools, fluxes, and rate coefficients were not different from those reported above. Finally, an experiment was performed in which the flow rate through the chamber was continued at the same rate as that used for the rapid wash, namely 38.2 ml/min, for the first 5 min, and effluent was collected at 6 sec intervals during this period. In this experiment, there was no evidence of a fourth exponential component.

Thus, the presence of a third tissue component is questionable; at any rate, since such a pool is so small and so inconsistent, further speculation is unwarranted at this time. As mentioned previously, however, whether or not this compartment is included in the curve-fitting procedure, the calculated values for the fluxes and sizes of the other compartments are not affected. Thus, for all studies indicated above, the calculated values are for the two-compartment tissue model previously discussed.

#### DISCUSSION

The experiments described above indicate that the kinetics of transbladder sodium transport in the toad bladder may be described by a simple three-compartment system, with a single transport pool interposed between mucosal and serosal media. It must be mentioned, of course, that a model with more compartments would also satisfy the data, as indicated above; this is one of the limitations of a compartmental analysis. On the other hand, we have been guided by the principle that the simplest model consonant with all the data should be chosen; this led us to the system described. If, furthermore, the parameters obtained can be shown to have biological significance, and if they respond to biological perturbations in a meaningful way, then the simple model will have been shown to be a reasonable representation of the tissue. The results of the present experiments must be viewed in this way.

As shown above, there are two tissue compartments. For reasons discussed in part already, the slower of these compartments cannot be ascribed to the transport pool. In the first place, the pool does not respond to a number of biological perturbations, including the application of inhibitors or of an adverse potential gradient. Second, it appears to be present after removal of the central portion of the tissue, so that it may represent a nonfunctioning edge of the preparation. Most critical are the calculations of the transepithelial flux from the unidirectional fluxes, as shown for compartment 1 in Table IV. The fact that these two independent measurements agree strongly suggests that the model is correct, and that the assumptions made in deriving equations 19 and 20 are valid. Similar calculations for compartment 2 yield a value that is only one-tenth of the value measured directly. Although we have tentatively ascribed the source of this pool to the rim of tissue around

the periphery, such cannot be definitive. What we can say is that compartment 2 is not the transport pool.

The rim studies showed that only a single tissue component is present under those circumstances, but this is not the same as saying that if we could study only the central portion we would also see a single component, with a half-time equal to that of the fast tissue pool. Thus the slow component of the washout of intact tissue may well include a portion of the bladder sodium not located in the rim; it would then have to be assigned parameters which are not distinguishable from those characteristic of the rim. Again, it is a limitation of compartmental analysis that compartments with similar kinetics cannot be distinguished. Such a component may also include the remainder of the intracellular sodium not involved in the transport pool. Although there is no way at present to locate this pool adequately, it is clear that it is not the transport pool; it seems equally clear that the fast tissue pool, compartment 1, does represent that pool.

Others have utilized tracer techniques to measure the size and kinetics of the toad bladder transport pool (Frazier and Hammer, 1963; Herrera 1965; Leaf, 1966; Schwartz and Snell, 1968); in general, their conclusions have been limited by the fact that they measured only the slow component of the transport system. In the experiments described by Frazier and Hammer (1963) and by Leaf (1966), for example, the time which elapsed between the start of the elution of tracer and the first collection was at least 6–10 min, so that any fast component, such as that described here, would have been overlooked. Indeed, the half-time observed by Leaf was about 10 min, essentially equivalent to the slow component seen in the present studies. In the studies of Herrera, as previously noted, the technique did not allow separation into more than a single component, and it is of interest that the pool size was not varied by any of the perturbations reported. With the use of a similar technique Hoshiko and Ussing (1960) observed a mean buildup half-time of 3.7 min, a value reasonably close to that seen here for the transport pool, and similar to that reported by Frazier, Dempsey, and Leaf (1962). Similarly, Crabbé and DeWeer (1965) estimated a half-time of the sodium pool of about 2 min by an entirely different method. Recently, Vanatta and Bryant (1970) have attempted to analyze sodium kinetics by a method similar in principle to that employed here. However, they used bladders whose viability is open to question, and they did not attempt to fit the data simultaneously, as in equation (1); thus their conclusion that there are eight compartments, none of which is open to both chambers, gives no indication that any of them represents the transport pool.

Recent studies of sodium transport in frog skin have been interpreted as evidence for a new transport model in that system (Cerejido et al., 1967, 1968; Rotunno et al., 1970). The authors have identified a fraction of sodium

which they designate a transport compartment (and whose magnitude is about one-fifth of that calculated here), but unfortunately they offer no evidence that this compartment is in fact involved in transepithelial transport, nor that it varies with biological perturbations. On the other hand, we have chosen first to define the pool kinetically, second, to show that it is involved in transepithelial transport, and third, to test the kinetic model by altering the biological conditions. In the only studies in which an attempt was made at a direct comparison of transport pools between frog skin and toad bladder, Hoshiko and Ussing (1960) found that the toad bladder pool was about twice that of frog skin, when expressed per unit area. It is clear that the frog skin is a far more complex tissue, and there is no particular reason to expect that the Cereijido model should apply to a cell layer that is one cell thick.

Another possible reason for the small size of the pool in the Cereijido studies is the fact that all experiments were performed in open-circuit conditions. Under such conditions in toad bladder, the sodium pool is, as one might expect, considerably smaller than in short circuit, since mucosal entry is inhibited and mucosal exit is enhanced.<sup>5</sup>

One must also question the significance of the influx reported by Rotunno et al., since, again, no evidence is provided that this influx is into a compartment involved in transport across the skin. On the other hand, in the present studies, we show clearly that the fluxes measured are into and out of the same pool responsible for transepithelial transport. Thus it is difficult to compare their values in frog skin with those reported here for toad bladder.

In summary, then, previous techniques have yielded different results as to the kinetics, and it has been difficult to measure toad bladder transport parameters and pools which respond to experimental perturbations in consistent and meaningful ways. It appears, however, that with the technique used here, it is possible to separate and to measure these several components.

As shown in Table II, the fluxes and rate constant at the mucosal border of the transport cells are several times higher than those at the serosal surface. This is in agreement with the data of others (Gatzy and Clarkson, 1965; Leb et al., 1965) that the mucosal surface is relatively more permeable to sodium ion than is the serosal surface. The lower flux rates at the serosal side may imply that the pump mechanism, which is presumably located at this surface of the transporting cells, is the rate-controlling step for transbladder transport. However, as is shown in the accompanying paper (Finn, 1971) the rate coefficient for the pump appears to be inversely related to the size of the pool, suggesting the presence of a saturating system. More evidence is needed as to the role of sodium entry at the mucosal border as a controlling step in transbladder transport.

<sup>5</sup> A. L. Finn. Unpublished observations.

The mean size of the pool in these studies is  $10.5 \mu\text{Eq}/100 \text{ mg}$  dry weight. When the wet weight:dry weight ratio of 4.2 is taken from previous studies (Finn, 1968), this yields a pool size of  $2.5 \mu\text{Eq}/100 \text{ mg}$  wet weight, or some 30% of total tissue sodium.

From previous data using inulin as an extracellular marker (Finn, 1968) simple calculations reveal that of total tissue sodium, 62% is intracellular. If we assume that the transport pool measured in the present experiments is in cells, then this pool represents 30:62, or 48% of intracellular sodium in the control preparation.

The size of the transport pool reported here is larger than that reported by others (Frazier et al., 1962; Herrera, 1965). The main reason for this appears to be that the pool evaluated previously was the slower component of the washouts; as noted above, this pool is probably not related to transport. Thus, for example, the experiments of Herrera yielded a control value of  $5.1 \mu\text{Eq}/100 \text{ mg}$  dry weight, a value approximately one-half of that found here.

Frazier et al. (1962) calculated a pool size that was considerably smaller than that obtained here, but those experiments were performed on bladders exposed to a sodium-free serosal medium. One may, in fact, calculate from their data that the pool contained some 52% of total tissue sodium. Under the circumstances of their experiments, total sodium falls by about 80% (Essig and Leaf, 1963). Since the bulk of the remaining sodium in these experiments must have been in the epithelial cells, this calculated fraction is quite similar to that observed here. It should be pointed out, of course, that a measurement of total tracer content done in the manner of those studies will give a falsely low value, since considerable time is spent in washing off the tracer, cutting the tissue down, and blotting it, especially since the half-time of washout of this pool is approximately 2 min.

Finally, it must be added that in the present experiments, no unequivocal evidence has been found of an additional sodium pool which might be an intercellular pathway for transport. Such a component almost certainly exists (Civan et al., 1966), but its role in transepithelial sodium transport in this tissue is not known. It is, of course, possible that such a pool has rate coefficients similar to those found in the pool described above, so that it could not be determined with the experimental approach used here. The fast component seen in some of the studies may represent such a component; this seems unlikely, however, since no evidence for washout of this pool to the serosal side could be detected, suggesting that it could not be a transepithelial shunt. It seems likely that the shunt pathway could not be detected either because of its small magnitude, or because it is similar to one of the other components of the washout curves and hence cannot be distinguished.

It was noted earlier that the rate constant for the first, or fastest, component

of the washout curves represented the chamber washout. One may also calculate that any component due to simple diffusion through the tissue would probably occur during the 1 min of rapid wash and hence would be missed. By utilizing the solution to the diffusion equation for diffusion through a plane sheet (Jacobs, 1967), it may be shown that diffusion from the cells to the serosal medium would occur with a half-time of about 3 sec (assuming a diffusion constant for sodium of one-half its value in water, and a distance of 60  $\mu$  (Choi, 1963) from serosal cells to serosal medium). The value for the half-time for diffusion into the mucosal medium is about 0.3 sec (since the diffusion distance is so much smaller on that side). Since all sodium in the connective tissue layer may well be in diffusion equilibrium with the media, it is not surprising that a substantial amount of total tissue sodium is not accounted for by washout experiments.

Finally, one must ask whether the pool being measured here is, in fact, the transport pool, or whether it represents a moiety of sodium already subjected to transport. One answer to that question lies in the data presented in Table IV. It is clear that the two transepithelial fluxes of sodium include that fraction which is "pumped;" in fact, the difference between these two fluxes is the active component of sodium transport, and is equal to the short-circuit current. Now if the compartment we refer to as the transport pool were in fact already transported, then no equality between the calculated and measured transepithelial fluxes would be expected. The rather remarkable agreement between these two moieties gives strong support to the contention that this pool is "awaiting transport," as it were, and that the fluxes and rate constants are measures of the parameters of this particular pool. Additional substantiation of the model, however, requires knowledge of its behavior in response to biological perturbations. Some of these are shown in the accompanying paper, and they add further weight to the conclusion as to the identity of this tissue sodium compartment with the transport pool in the toad bladder.

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