NONSTEADY-STATE THREE COMPARTMENT TRACER KINETICS

II. Sodium Flux Transients in the Toad Urinary Bladder in Response to Short Circuit

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ABSTRACT The theoretical approach presented in the previous paper provides an analytical method for determining the unidirectional, nonsteady-state fluxes in a three compartment system. Based on this a study was made of the sodium flux transients in the toad urinary bladder. A transient time-dependent state was generated by suddenly short-circuiting a bladder previously maintained in an open-circuited steady state. The sequence of experiments suggested by the theory provided the data required for the analysis.The results of these tracer experiments were consistent with the complex non-three compartmental structure of this tissue. As a result both of the inadequacy of the three compartment model in representing the tissue and of certain experimental difficulties, attempts at a quantitative solution were not entirely successful. Useful information was nevertheless obtained through a careful use of this model, and a qualitative analysis implied that the sodium influxes into the tissue at both of its surfaces are sensitive to changes in electrical potential while both effluxes are insensitive to this change. This suggests that both of the effluxes result from active processes while both influxes are associated with passive processes. The net transepithelial transport of sodium would then necessarily result from a more complex polarization than that proposed by Koefoed-Johnsen and Ussing.

INTRODUCTION

Although tracer techniques have commonly been used to investigate ionic fluxes across epithelial structures such as the isolated urinary bladder of the toad, with but one exception these have dealt exclusively with steady states. Even in this exceptional case, Frazier and Hammer (1963) could make only a partial analysis of some of the sodium tracer fluxes in the toad bladder because of the attendant difficulties. This situation is regrettable because the transient response of a system is often more revealing of underlying mechanisms than its steady-state response.

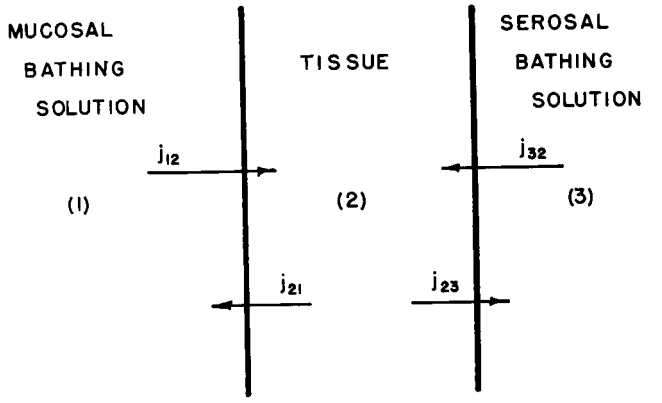


FIGURE 1 The three compartment model.

An in-series three compartment model is the simplest that can be used to represent such isolated tissues and their bathing media (Fig. 1). Although this model is only an approximate representation of the tissue and the experimental apparatus, it is also the most complex model for which a general time-dependent solution has been devised (Schwartz and Snell, 1968; hereafter denoted as (I)). In (I) we demonstrated that for a system described by this model, a particular set of sequential nonsteady-state experiments can serve to determine the four unidirectional fluxes of a traced substance across the boundaries of the central compartment as well as the size of that compartment. The time-dependent state that we examined was the result of a sudden change in some condition prevailing during an initial steady state. The required experiments were described, and the related equations were developed and solved to yield the fluxes and the central compartment size as functions of time.

In the present paper we present the results of an investigation utilizing this theory to determine the response of the sodium ion fluxes in the isolated urinary bladder of the toad, *Bufo marinus*, to the sudden short circuit of a previously open-circuited bladder. We will demonstrate that even though the tracer results are consistent with the complex histology of this tissue, useful information about the transient period can nevertheless be obtained from the simple three compartment model. The notation used follows that employed in (I).

METHODS

Animals

The toads were obtained commercially and stored on damp sand in plastic trays at 12–15°C. They could be kept thus without feeding for as long as two months with no apparent deterioration.

Solutions

The bathing solutions contained 115 mM NaCl, 2.5 mM $KHCO_3$, 1.5 mM $CaCl_2$, and 10 mM dextrose—a total of 249.5 milliosmols/liter. Reagent grade chemicals were used. All solutions

were air equilibrated prior to use after which the pH was 8.3–8.5. Dextrose was added just before each experiment to slow the decline in sodium transport occurring in the absence of substrate (Maffly and Edelman, 1963). Aldosterone (3.47×10^{-8} M, Ciba Pharmaceutical Co., Summit, N. J.) was added in several of the longer experiments because an exogenous supply of this hormone stimulates sodium transport across bladders deprived of their endogenous supply (Edelman, Bogoroch, and Porter, 1963; Sharp and Leaf, 1964).

^{24}Na obtained as $^{24}NaCO_3$ from the Western N. Y. Nuclear Research Center, Buffalo, N. Y., was titrated to a pH of 5–7 with 0.300 N HCl. ^{22}Na was supplied by Iso/Serve Inc. of Cambridge, Mass. as carrier free NaCl solution.

5 ml of Ringer's solution containing either 1 mc of ^{22}Na or 2–3 mc of ^{24}Na were prepared from fresh, aerated, stock solutions as required during an experiment. The pH of the radioactive Ringer's solution was 8 as measured with pH paper. Concentrations in the "hot" and "cold" solutions were identical to within 1%. We further required that there be no change in the short-circuit current or the membrane potential upon changing from one solution to the other.

Apparatus[1]

1. Bladder Chamber and Accessories. A small contained volume and a large ex-

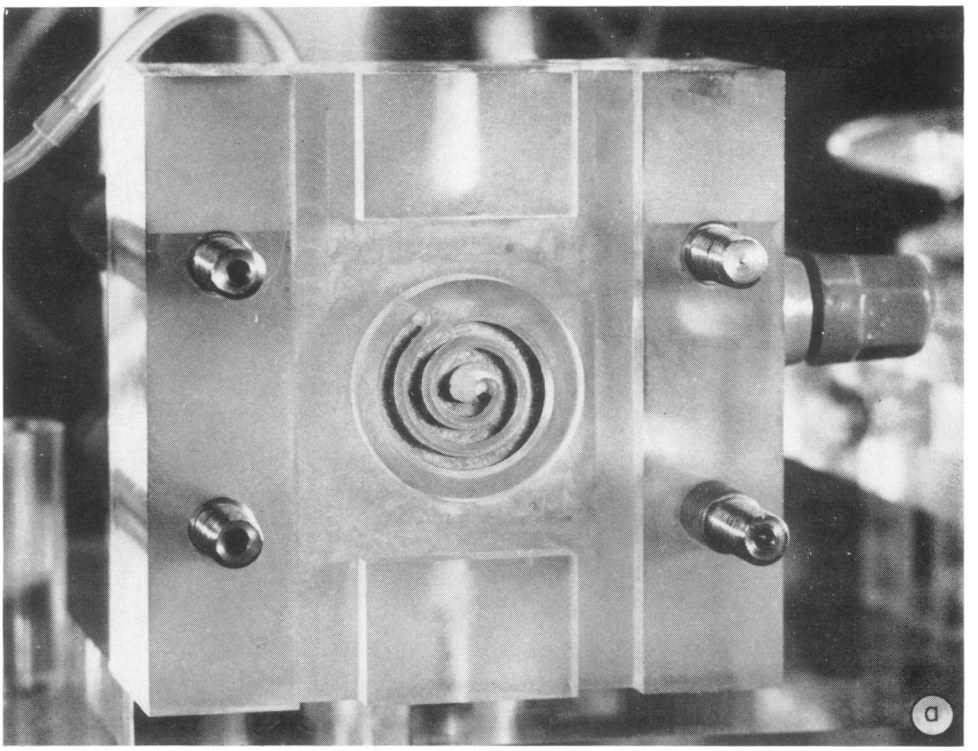


FIGURE 2*a* The face of one half-chamber. The spiral groove, its entrance and exit ports, and the ends of the four bolts used to fasten the chamber halves together can be seen.

[1] A more detailed description of the apparatus and the experimental procedure can be found elsewhere (Schwartz, 1966).

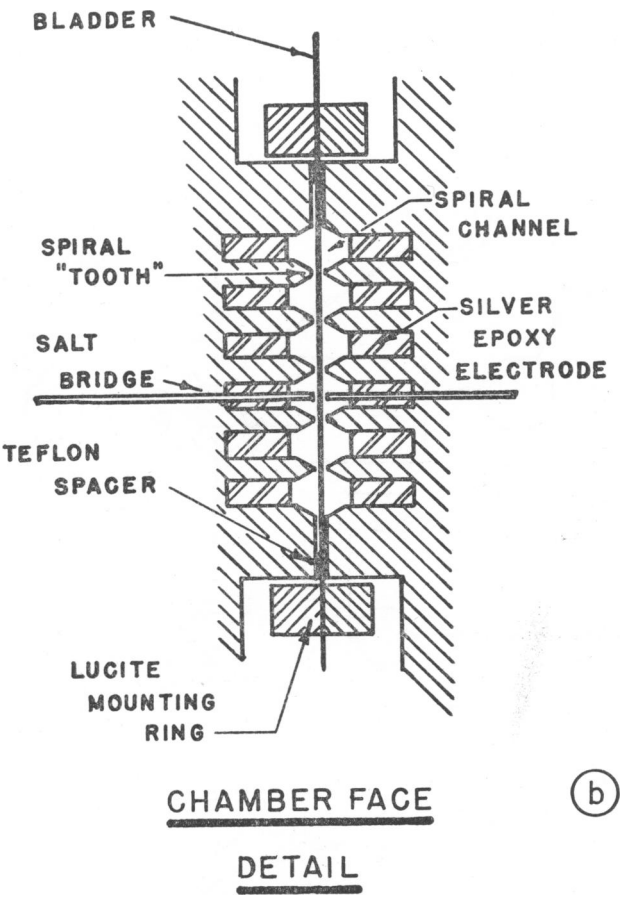


FIGURE 2*b* A cross-section of the assembled chamber with a membrane in place.

posed tissue area were achieved by machining matching, shallow, spiral grooves in two Lucite blocks (Fig. 2). The membrane was clamped between these half-chambers with four fitted bolts. Entrance and exit ports were provided at the ends of each groove. The tooth-like groove shape was designed to support the tissue without damaging it. Teflon spacers, 1×10^{-2} inches thick, prevented pressure by the "teeth." Further support was provided by nylon nets, and in some experiments a high wet-strength lens-tissue (Fisher Scientific Co., Pittsburgh, Pa., #11-995) was interposed between the "teeth" and the bladder (Fig. 5). The contained volume of each channel was 0.36 cc without the spacer, and 0.47 cc with it. The exposed tissue area was 4.45 cm^2.

The bottom of each groove was filled with silver epoxy, machined flat, and a thin layer of silver chloride was deposited to provide current electrodes. A thin polyethylene tube was sealed into a hole in the bottom of each channel to provide salt bridges leading to saturated KCl, calomel electrodes for monitoring the membrane potential. The tubes were terminated close to the membrane, and were filled with Ringer's solution thickened into a paste by the addition of 4–5% by weight of sodiumcarboxymethylcellulose (Hercules Powder Co., Wilmington, Del., type CMC-7HS). KCl diffusion in the region of the membrane was thus avoided. The bathing solutions were flowed past both membrane surfaces at 6 ml/min. Bubble

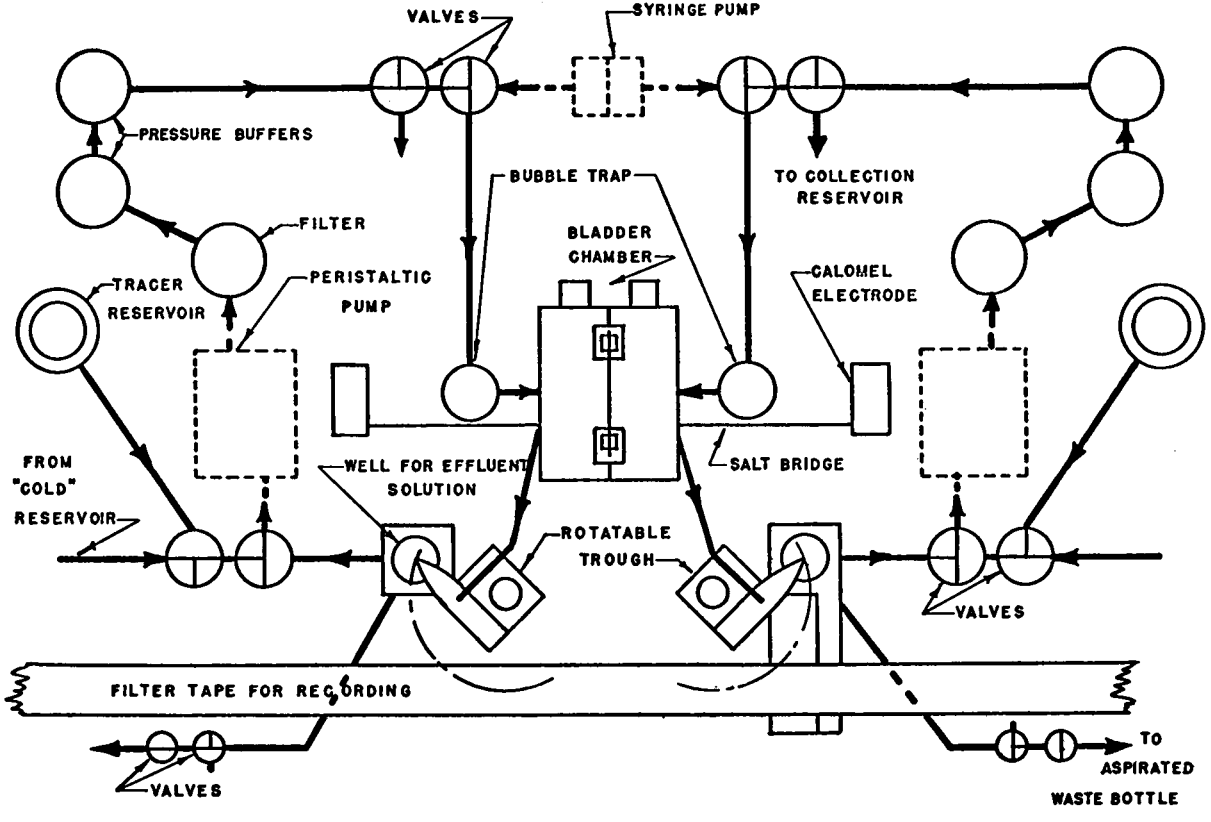


FIGURE 3 A schematic of the apparatus. Each trough could be turned to arrange for the collection of the appropriate effluent solution on the moving filter tape. The left bathing solution is shown being drawn from the "cold" reservoir and aspirated into waste bottles after passing through the chamber. On the right side the same, small volume of solution is shown repeatedly circulated. The system is symmetric and the components of only one side are labeled.

traps with fittings for either a water manometer or a pressure transducer preceded the chamber (Fig. 3). The pressure differential across the tissue was checked periodically during each experiment. This differential had to be maintained well below 1 cm of water to avoid damaging the tissue.

2. Pumps and Accessories. A syringe pump was used in all experiments in which the tissue, having been previously loaded with tracer, was washed on both sides with tracer-free solution. A peristaltic pump was used for all other pumping. Two in-series air buffers directly following the pump on both sides of the system eliminated pressure pulses. In-line filters preceded the buffers to prevent clogging, and all solutions were filtered prior to use.

Either pump could be chosen at will by appropriate valve settings. With the peristaltic pump in use, the solution source could be the reservoir containing "cold" solution or that containing "hot" solution. The effluent could be discarded or circulated. With either pump, solution discarded from either side of the system could be sent to waste or collected either on the moving strip of filter tape—by rotating the appropriate trough into position—or in vials placed under the troughs. A bypass enabled the peristaltic pump to be cleared of tracer without interrupting an experiment. The volume of each side of the system with the valves set to circulate the solution was 3.7 cc.

3. Tape Drive. A constant speed drive moved a strip of 1 inch wide, highly absorbent chromatography tape (Whatman, type ET31) at 2.9 ft/min. A relay-operated pen marked the tape at 10 sec intervals prior to wetting. The wet tape was dried in-line in a stream of warm air and automatically rolled on a reel.

4. Voltage Clamp. The voltage clamp described by Menninger, Snell, and Spangler (1960) and modified as shown in Fig. 4, was used in conjunction with a two channel recorder utilizing low level preamplifiers (Sanborn Co., Cambridge, Mass., Models 150 and 150–1500). The lower operational amplifier clamped its voltage electrode to the potential E_2, which was adjusted at the start of each experiment to correct for unequal diffusion or electrode potentials. The upper amplifier served as a unity gain voltage measuring device in the potential measuring mode. In the voltage clamp mode, it held its electrode at ground potential, thereby short-circuiting the tissue. The output current was then the short-circuit current. Both amplifiers were type UPA-2 (George A. Philbrick Researches, Inc., Boston, Mass.). The response time of the clamp, recorder, and chamber connected as in an actual experiment was always negligible compared with the minimum sampling time of 10 sec.

5. Counting Apparatus. Automatic, 100 sample, well-type scintillation counters (Packard Instrument Co., Inc., Downers Grove, Ill.), and a similar manual instrument were used.

Tissue Preparation

Experiments were conducted in all seasons of the year. The toad was pithed and washed to remove skin gland secretions. A ventral incision was made, both lobes of the bladder were dissected free, tied off near the cloaca, and removed from the animal. One lobe was opened and mounted on a Lucite ring equipped with stainless steel pins (Fig. 5). The tissue was gently stretched to a uniform thickness and the rings were assembled.

The apparatus was flushed of all air bubbles. The mounted tissue was placed in the chamber, which was then closed and bolted. Ringer's solution was gently flushed through to remove all air. Caution was required to avoid irreversible damage to the bladder due to pressure pulses. Both sides of the bladder were then flushed with fresh solution during an equilibration

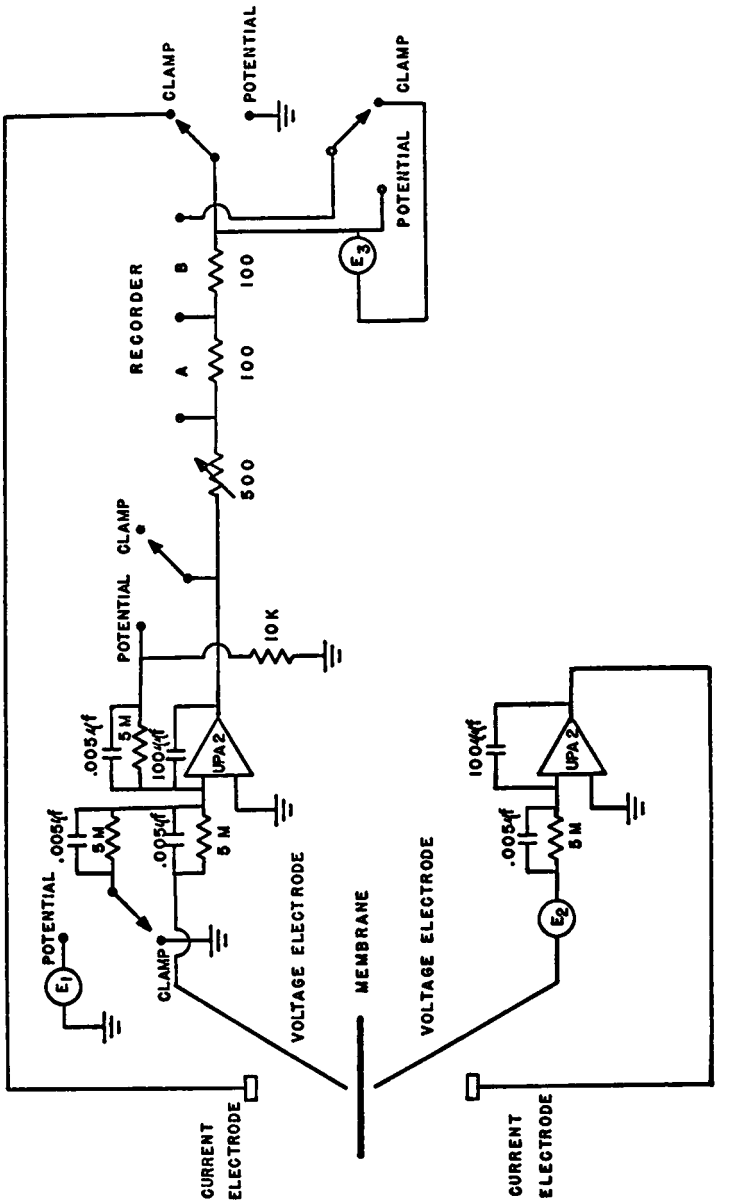
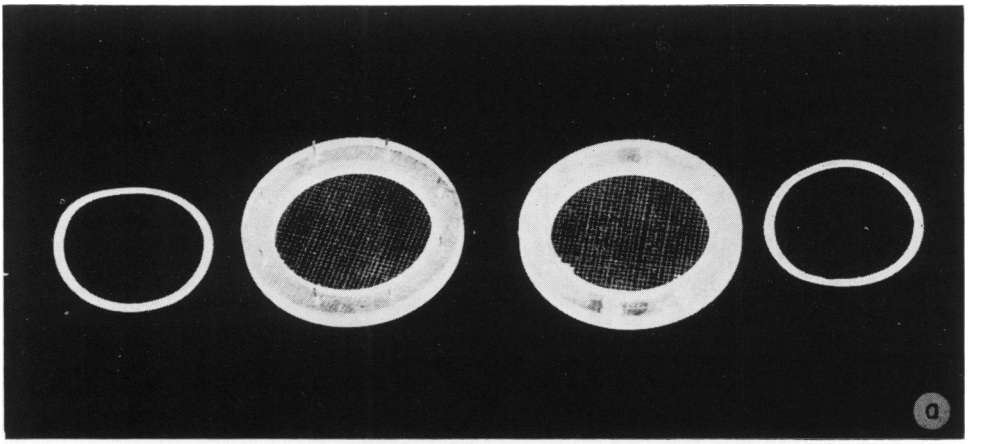


FIGURE 4 The voltage clamp. The magnitudes of E_1 and E_3 were adjusted by varying calibrated, ten-turn, potentiometers. E_1 served to shift the zero of the potential recording scale. E_3 provided zero suppression in channel B of the recorder in the clamped mode. The 500 Ω potentiometer was used for calibration when recording potentials. Switching between the two operating modes was accomplished with a single switch.



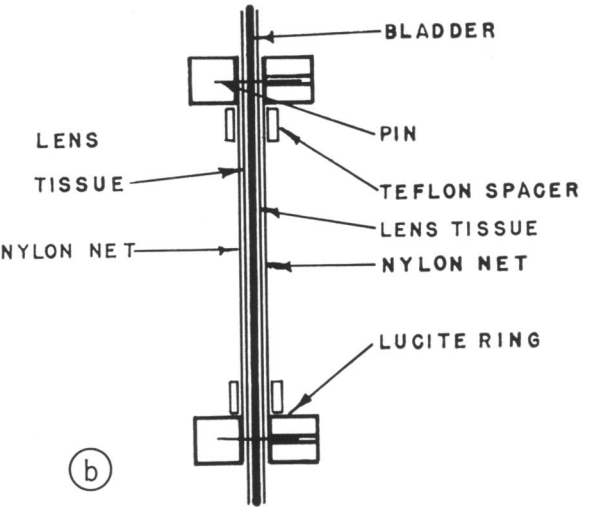


FIGURE 5 Assembly to support the bladder. (*a*) Pins held the bladder in place during the mounting process, and nylon nets provided support without diffusion delay. In long experiments, lens tissue (*b*) was helpful in extending the life of the tissue. The nets were woven into a $\frac{1}{32}$ inch mesh out of 2.5×10^{-4} inch diameter monofilaments (E. I. du Pont de Nemours & Co., Wilmington, Del.), and were cemented to the rings.

period of 2–3 hr until a steady state was reached. Bladders yielding potentials less than 20 mv at this point were rejected.

Experimental Procedure

Each experiment was performed with a single piece of tissue and was composed of several sequential runs corresponding to those outlined in (I). In two experiments the entire set of runs was completed. In most instances only selected runs were performed. Compartment 1 of (I) now represents the solution bathing the mucosal surface of the bladder and compartment

3 represents that bathing the serosal surface. A step change in the conditions prevailing during an initial steady state was produced by suddenly short-circuiting the initially open-circuited bladder at an appropriate moment during the run. A continuous recording of either the membrane potential or the short-circuit current was made depending on whether the tissue was open- or short-circuited. Periods of approximately 40 min were allowed between runs to permit an open-circuited steady state to be reached.

Data were continuously collected on the filter paper tape during each of Runs A through D. A portion of the initial open-circuited steady state and the transient state following the abrupt short circuit were included. During Runs B and D the collection of data was either delayed until both sides of the chamber had been rinsed with "cold" Ringer's solution for 5–6 min, or note was made on the tape when this time had elapsed. This point corresponds to $t = 0$ of (I), and is similarly denoted in this paper. Effects due to residual tracer activity in the compartment from which the tissue had been preloaded were thus isolated and minimized.

Two other runs—D′ and E—were made on two occasions to acquire additional information. The initial conditions during both were identical to those of Runs C and D. Thus the mucosal bath initially contained high tracer activity during both. In both runs the serosal side was continuously flushed with "cold" Ringer's solution and its effluent was collected on tape for a 5 min period during the initial, open-circuited, steady state. This established additional independent determinations of the open-circuit mucosal to serosal tracer flux. In Run D′ the mucosal compartment was flushed with "cold" Ringer's solution after the initial period, and a continuous collection of the effluent from the serosal side was made in vials in 60 sec samples. The bladder was maintained in an open-circuited steady state during this entire run. During Run E the tissue was removed from the apparatus while still mounted on the rings immediately after the initial period. That portion of bladder exposed in the chamber was cut out, rinsed in "cold" Ringer's solution for several seconds, dried at 100°C for 36 hr in a tared flask, and then weighed. It was then dissolved in concentrated HNO_3 and an aliquot was counted. This run thus corresponds to Run E of (I) which was designed to determine $S_2^*(0)$.

Samples of the "hot" preloading solutions were used as standards. The dried tapes were cut into strips corresponding to successive 10 sec intervals. Each strip was tamped into a test tube. These were interspersed with other tubes containing standards or blanks and all were then counted. A portion of each liquid sample obtained during Run D′ was counted in the same manner. Each sample was counted at least twice to reveal machine errors. Uniform counting times were chosen to yield standard deviations generally less than 2%. Only approximately 5% could be achieved during the terminal portions of Runs B, D, and D′.

^{24}Na was used during Runs A and B. ^{22}Na was used in all other runs in any experiment in which two tracers were required. The ^{24}Na samples were counted immediately. ^{22}Na samples were counted after a delay of at least 1 week to permit the decay of traces of ^{24}Na not washed out in the course of experimental routine.

Calculations

Most of the calculations were made using a digital computer. The method of least squares was used to fit a line to a graph of the background counts against total elapsed counting time in the automatic counters. This line was then used to subtract the proper background count from each sample and standard. A linear regression was again used to fit a line to a plot of the logarithm of the corrected standard counts against total elapsed counting time. This line was used to calculate the tracer fluxes from the relationship

$$\text{Tracer flux} = \frac{(\text{Sample} - \text{Background}) \times (\text{Sodium content of standard})}{(\text{Standard} - \text{Background}) \times \Delta t}$$

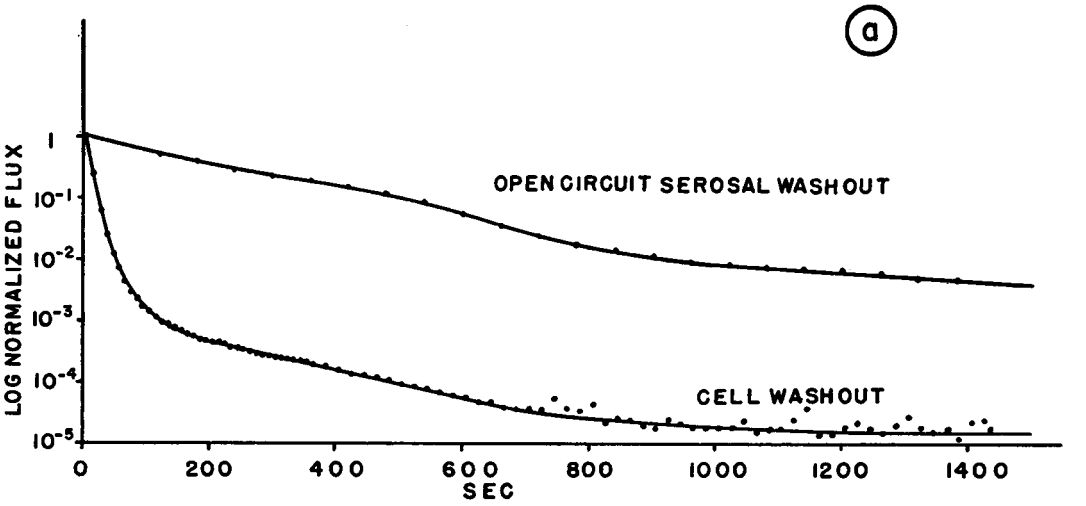


FIGURE 6*a* Comparative washout characteristics. Data were normalized to unity. The data for the open-circuit serosal washout are those of Run D′ of Experiment 21.

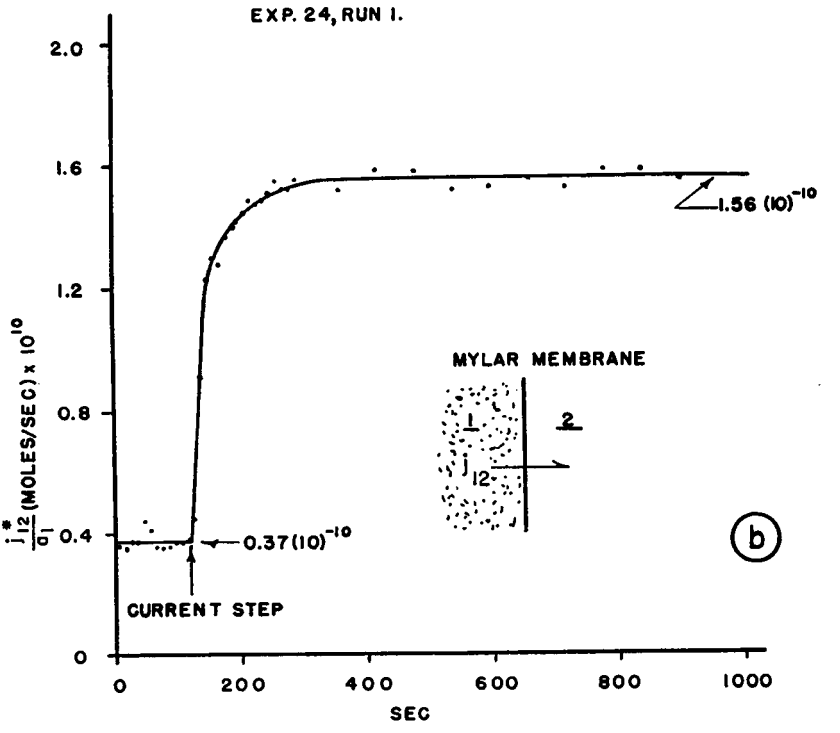


FIGURE 6*b* Flux of ^{24}Na across a thin Mylar membrane in response to a 200 μa current step. The membrane was supported on nylon nets and lens tissue.

where Δt was the time interval over which the sample had been collected. The separate results following from the repeated counting of each single sample were weighted by their standard deviations, averaged, and the standard deviation of the resulting tracer flux was calculated.

The equations used for calculations based on the three compartment model can be found in (I).

Apparatus Characteristics

In order to evaluate the results one must know the characteristic behavior of the apparatus itself under time-varying conditions. For this purpose the washout pattern of the chamber and the response of the apparatus to a step change in the flux at the membrane surface were determined. A thin (2.5×10^{-4} inches) Mylar membrane was mounted in the chamber in precisely the same configuration as a bladder. The Mylar had been made permeable to ions by treatment with hot, concentrated NaOH. Compartment 1 was filled with circulated Ringer's solu-

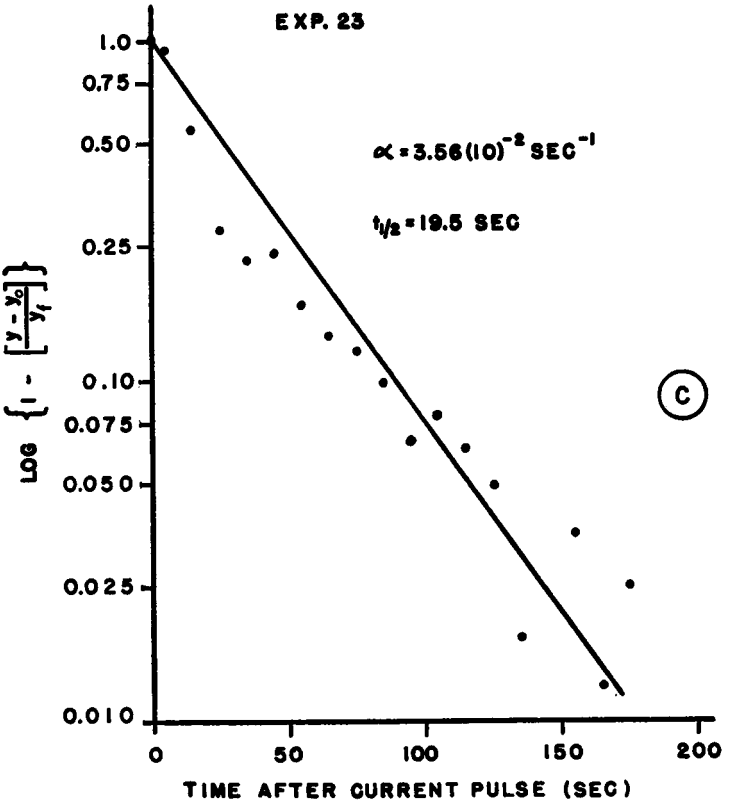


FIGURE 6*c* The exponential behavior of the flux in Fig. 6*b*. Time zero corresponds to the moment of application of the current step. Data were plotted according to the relation

$$-\alpha t = \ln\left(\frac{y_\infty - y}{y_\infty - y_0}\right)$$

where y_0 and y_∞ are the initial and final magnitudes of the flux. The line was drawn by eye.

tion containing ^{24}Na while compartment 3 was flushed with "cold" Ringer's solution. The flow rate was the same as in the bladder experiments. The chamber washout pattern was then studied by suddenly flushing out compartment 1 while collecting its effluent on tape. 5 min after the start of washout the activity in this effluent was only $\frac{1}{3600}$th of its initial value (Fig. 6*a*). This confirmed the effectiveness of the flushing period preceding $t = 0$ during Runs B, D, and D'. The chamber washout pattern is seen to be distinctly different from the washout of a bladder.

A step change in the flux at the Mylar surface was produced by suddenly imposing an electrical current through the Mylar from compartment 1 to compartment 3 under initial conditions similar to those above. The effluent from compartment 3 was continuously collected during this process. The apparatus response is shown in Figs. 6*b* and 6*c*. The half time of rise, which gives an indication of the time resolution of the apparatus, was 19.5 sec. Flow rate variations of as much as 15% had no detectable effect. Flow variations during bladder experiments were less than this. The above information regarding the response of the apparatus to a transient was later used to calculate appropriate corrections to the bladder data.

RESULTS, INTERPRETATION, AND DISCUSSION

The Initial and Final Steady States

The steady-state tracer fluxes and the short-circuit current are compared in Table I. The tracer flux from the tissue into the serosal bathing medium during Run C, normalized by the specific activity in the mucosal compartment of origin, is ξ_{23} ((I), equations 7). The corresponding normalized tracer flux into the mucosal bath during Run A is ξ_{21}. The net mucosal to serosal steady-state tracer flux is given by $\xi_{23} - \xi_{21}$. This quantity also gives the net sodium flux during a steady state. The short-circuit current in Table I is the average of the final currents during Runs A and C. An "average bladder" calculated from the data of columns 2–6 was treated on a par with bladders for which complete information was available in the calculation of the last two columns.

TABLE I

THE SHORT-CIRCUIT CURRENT AND THE NET SODIUM FLUXES DURING THE INITIAL OPEN-CIRCUITED AND FINAL SHORT-CIRCUITED STEADY STATES

Experiment	Open circuit		Short circuit		I_{sc}	Net flux/I_{sc}	
	ξ_{21}	ξ_{23}	ξ_{21}	ξ_{23}		Open	Short
10	—	5.80	—	8.79	7.98	—	—
14	7.41	—	5.00	—	19.06	—	—
19	8.12	25.5	5.11	36.6	27.61	0.63	1.14
20	8.01	—	6.23	—	26.32	—	—
21	10.3	21.2	7.12	31.9	23.73	0.46	1.04
Average Bladder	8.46	17.5	5.87	25.8	21.92	0.41	0.91

All units are 10^{-10} moles/sec.

Changes could and in fact, did occur in the tissue during the successive runs that constituted a single experiment. The variations of $\xi_{23}(0)$ and the short-circuit current from run to run during typical experiments are shown in Table II and Fig. 7. They were sufficiently small in the steady-state regions to permit reasonable calculations to be made. Thus the data of Table I may be interpreted to show an essential equality between the net short-circuited sodium flux and the short-circuit current. This equality was previously demonstrated for both the frog skin and the toad bladder (Ussing and Zerahn, 1951; Koefoed-Johnsen, Levi, and Ussing, 1952; Leaf, Anderson, and Page, 1958). It is also evident from Table I that the net, open-circuited, sodium flux was approximately one-half the short-circuit current. This relationship was previously suggested by the results of a tracer experiment with a single isolated frog skin which were incomplete in that only the tracer flux from the outside to the inside was measured (Ussing and Zerahn, 1951).

TABLE II

$\xi_{23}(0)$ DURING DIFFERENT RUNS OF THE SAME EXPERIMENT

Experiment	$\xi_{23}(0)$ in 10^{-10} moles/sec			Mean value	Deviation from the mean		
	Run C	Run D′	Run E		Run C	Run D′	Run E
19	25.46	—	29.91	27.69	−8.1%	—	+8.1%
21	21.17	22.38	24.15	22.57	−6.2%	−0.8%	+7.0%

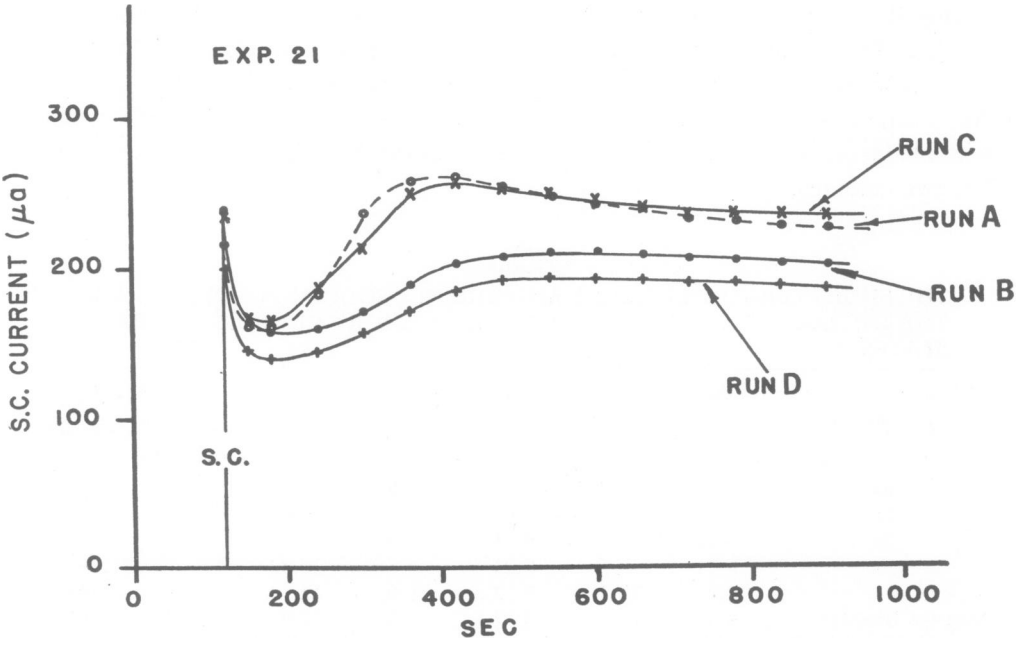


FIGURE 7 Short-circuit current during Runs A through D of Experiment 21.

The Nonsteady State

Results typical of Runs A through D′ are presented in Figs. 8–10. The particular set presented was obtained from a single experiment on a single bladder. All points during the initial open-circuited period were averaged to calculate the values of $\xi_{21}(0)$ and $\xi_{23}(0)$. The values of $\xi_{21}(f)$ and $\xi_{23}(f)$ were similarly calculated from points occurring after the corresponding tracer flux had reached its final steady state. The

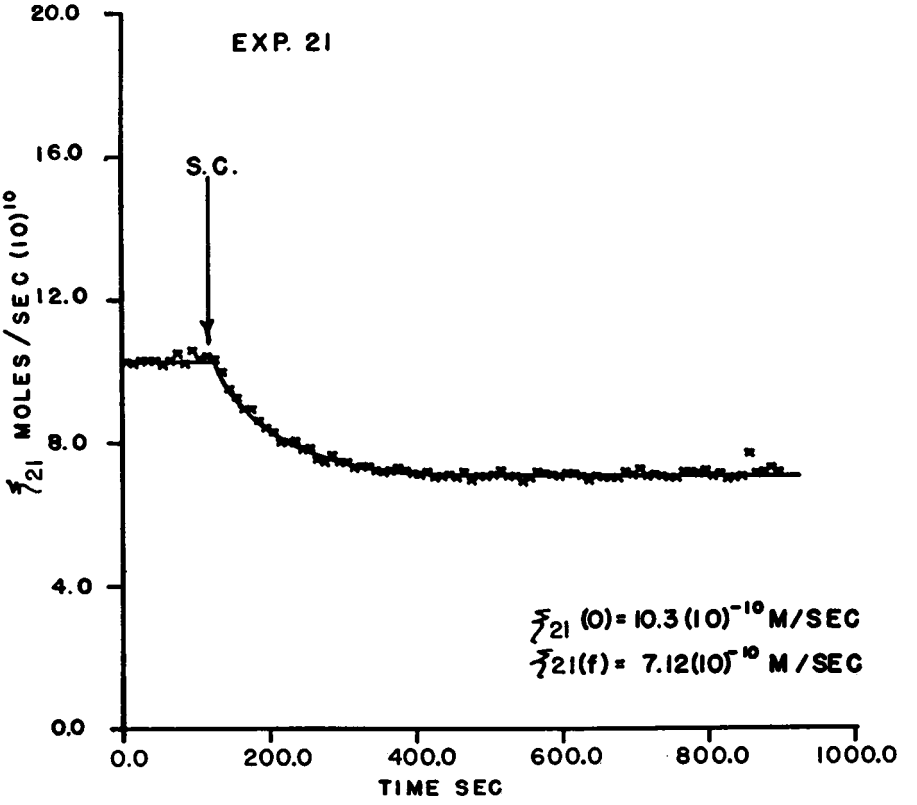


FIGURE 8 Run A. The membrane potentials directly before and after the short-circuited period were 54.5 and 61.0 mv, respectively.

data in Fig. 10 were plotted semilogarithmically as suggested by the development in (I) (equations 8*b*, 8*d*, 33*d*, and 33*e*). The tracer flux from the tissue into the serosal bath during Run D, normalized by the specific activity in the mucosal medium during the preloading period, is denoted by η_{23} ((I), equations 7). The corresponding normalized tracer flux into the mucosal bath during Run B is η_{21}.

Calculated corrections to these data that take the apparatus delays into account produced no profound changes in the experimental curves (Fig. 11). These corrections were made according to a procedure developed by Schwartz (1966) which is more general than that suggested by Burch (1891). The corrections were significant

only in regions of rapid change where they merely accentuated the rapidity. We therefore do not concern ourselves with them any further.

The results of both Runs A and C (Figs. 8 and 9) show a striking response to short circuit. In both runs there was an abrupt change of slope directly following short circuit. The increase in ξ_{23} and the decrease in ξ_{21} with both reaching new steady-state levels are as expected if these variables are sensitive to electrical potential changes. The more rapid response of ξ_{21} suggests that the potential difference

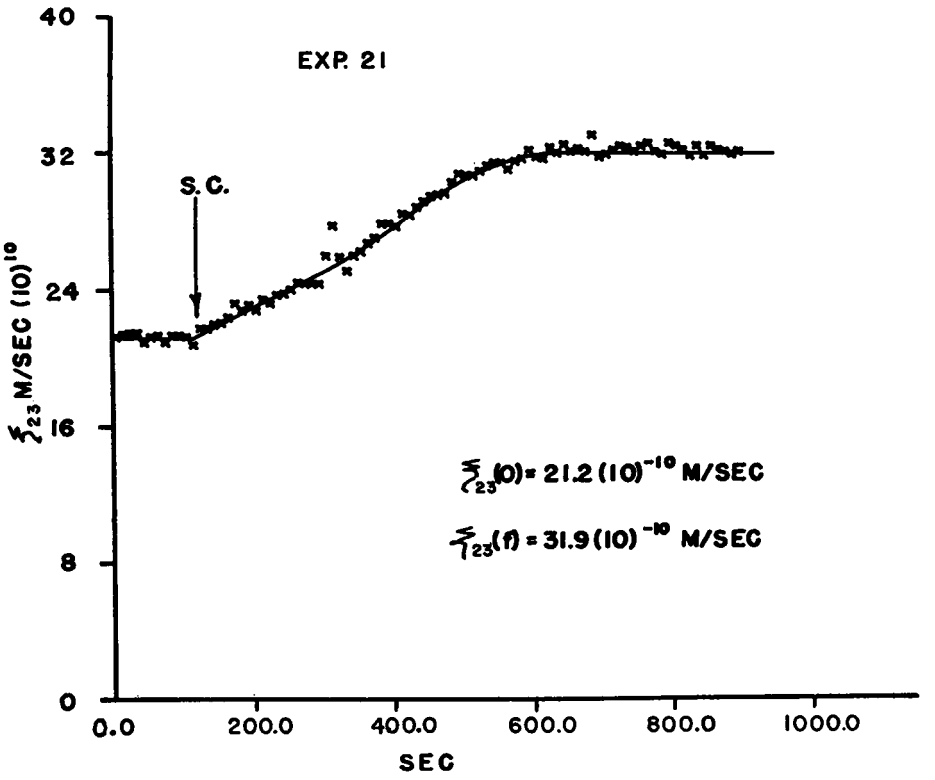


FIGURE 9 Run C. The membrane potentials directly before and after the short-circuited period were 52.5 and 61.0 mv, respectively.

abolished by short-circuiting the bladder occurred across a compartment located closer to its mucosal than to its serosal surface. Since this potential difference is known to be associated with the active transport of sodium, it is thus implied that the active compartment is near the mucosal side and is separated from the serosal side by a passive compartment. The active compartment may be identified with the layer of epithelial cells on the mucosal surface, and the passive compartment with the serosal connective tissue. This agrees with the conclusion drawn by Frazier and Leaf (1963) from other evidence, and is consistent with the known structure (Peachey and Rasmussen, 1961; Choi, 1963).

No striking response to short circuit such as that discussed above was present during Runs B, D, and D′ (Fig. 10). Indeed, comparison of the results of Runs D and D′ makes it clear that the structure of these curves and the act of short-circuiting do not have any simple correlation. These two particular runs were performed successively under conditions which were identical in all but one respect: during Run D the bladder was short-circuited at the indicated moment while it was maintained in open circuit throughout Run D′. In spite of this the results of the two runs

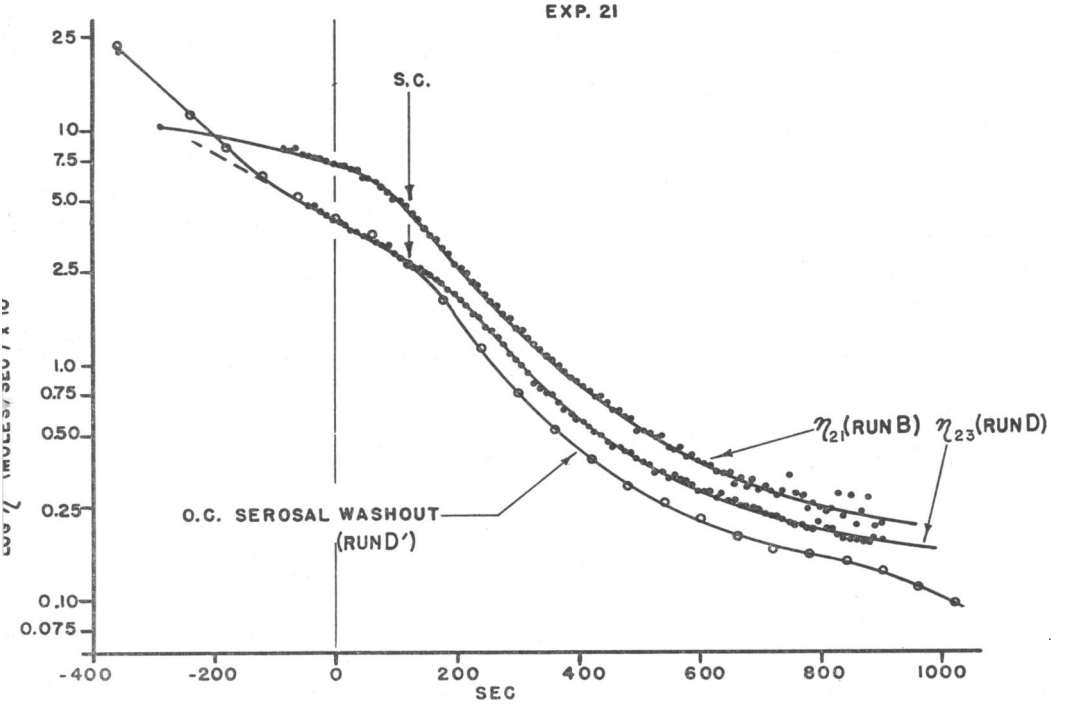


FIGURE 10 Runs B (η_{21}), D (η_{23}), and D′ (open-circuited serosal washout). The membrane potentials directly before and after the short-circuited period during Run B were 55.0 and 57.5 mv, respectively. The corresponding values during Run D were 45.5 and 53.5 mv. The potential varied between 41.5 and 46.5 mv during Run D′. No correlation was found between this variation and the structure of the curve. The first point on each curve corresponds to the start of washout; and that for Run D can be seen to lie quite close to that for Run D′.

resembled each other rather closely. Thus the main cause of the structure of these curves cannot be the sudden short circuit. To investigate this question more closely the difference (Run D − Run D′) was plotted linearly (Fig. 12). This difference would, in the absence of other more subtle causes, be entirely due to the effects of short circuit, and it is at first tempting to conclude that the positive peak following that event in Fig. 12 might truly represent such an effect. But further examination argues against such a conclusion. This positive peak is of approximately the same magnitude as an earlier negative peak (drawn dotted) which occurred during the

initial open-circuited period. The earlier peak could only have been caused by some spontaneous variation in the state of the bladder itself between runs since the initial states of these two runs were identical in all other respects. Thus the later peak may also be the result of biological variability. This conclusion is strengthened by comparison of Figs. 9 and 12. Although these two figures correspond to different tracer conditions in the mucosal bath, the observable in both cases was the reaction to short circuit of the efflux of tracer from the tissue into the serosal bath. In Fig. 9 where

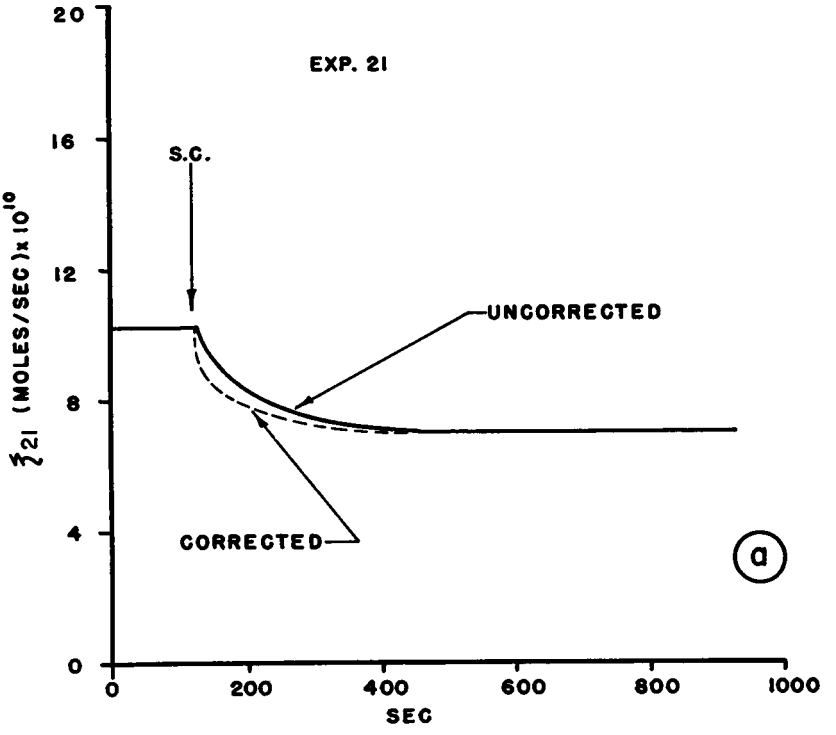


FIGURE 11*a* Corrections for delay in the apparatus. Run A.

the response was unambiguous it was approximately 30 times as large as the maximum response seen in Fig. 12. It is therefore doubtful that one can interpret Fig. 12 as depicting the effects of short circuit. Indeed, in the light of all of the above evidence it is more reasonable to conclude that the tracer fluxes of Runs B and D were not significantly influenced by short circuit. It is of interest to note that Frazier and Hammer (1963) similarly found these tracer fluxes to be insensitive to the total replacement of the mucosal sodium by choline.

An understanding of the structure of the curves in Fig. 10 must therefore be sought in the anatomy of the tissue. Run D′ would have yielded a straight line if the tissue-bathing solution system could truly have been represented by a three com-

partment model ((I), equation 33*e*). Since the actual curve is decidedly nonlinear, a more complex structure is implied. Certain of its aspects can be deduced from the tracer data.

The early phase of η_{21} has a downward facing concavity. In that run the tissue was loaded with tracer from a "hot" serosal bath which was then flushed with "cold" solution during the period preceding $t = 0$. Data collection was from the mucosal side. A washout delay such as would be caused by an interposed compartment on the serosal side is therefore suggested by this feature of η_{21}. This compartment would

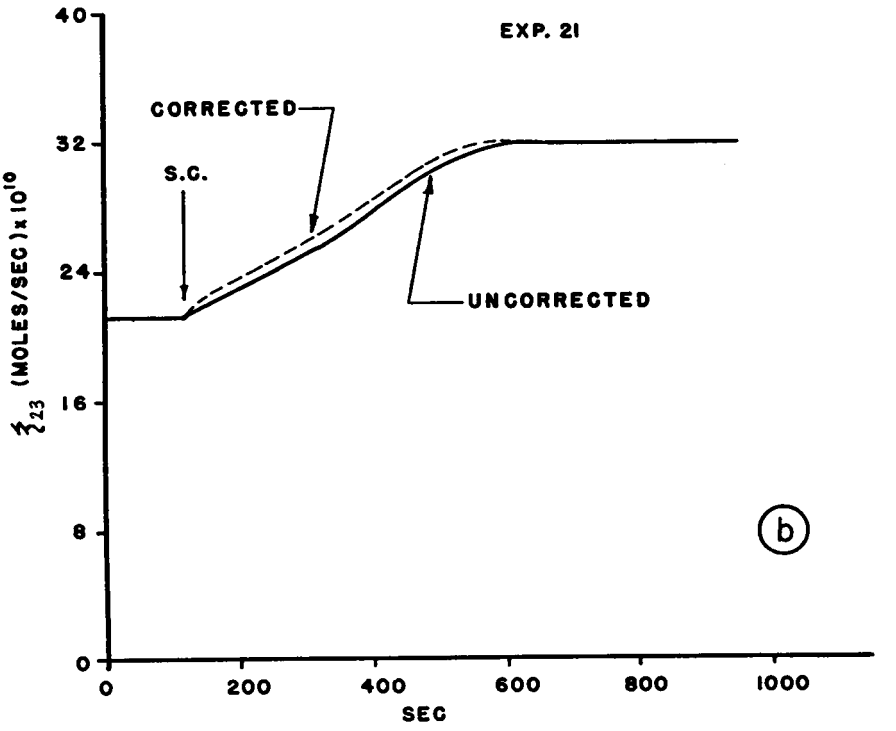


FIGURE 11*b* Corrections for delay in the apparatus. Run C.

have to exchange fairly rapidly with its bathing medium, and is probably to be identified with the serosal layer of connective tissue. The plots for both η_{23} and Run D′ display an upward-facing concavity during this same period. In those runs, loading was from the mucosal side while data were collected from the serosal side. A small, direct path between the mucosal surface and the serosal connective tissue would account for this concavity. It would permit the tracer specific activity in the connective tissue to decrease rapidly when the initially "hot" mucosal bathing solution is suddenly changed to one containing no tracer, thus producing a rapid initial drop in the serosal tracer efflux. Such a "leak" might occur through small damaged epithelial areas, through one of the less numerous of the several cell types in the

epithelial layer (Peachey and Rasmussen, 1961; Choi, 1963), or through regions permeable to sodium between the epithelial cells. A proposal of the latter type was made by Ussing (1963) and Ussing and Windhager (1964) for the isolated skin of *Rana temporaria*. Civan, Kedem, and Leaf (1966), who utilized a different technique, also have found evidence for parallel sodium pathways in the urinary bladder of the toad, *Bufo marinus*.

While, as we have indicated, the contrasting behavior of η_{21} and η_{23} during the early washout phase implies a polar structure containing compartments in series,

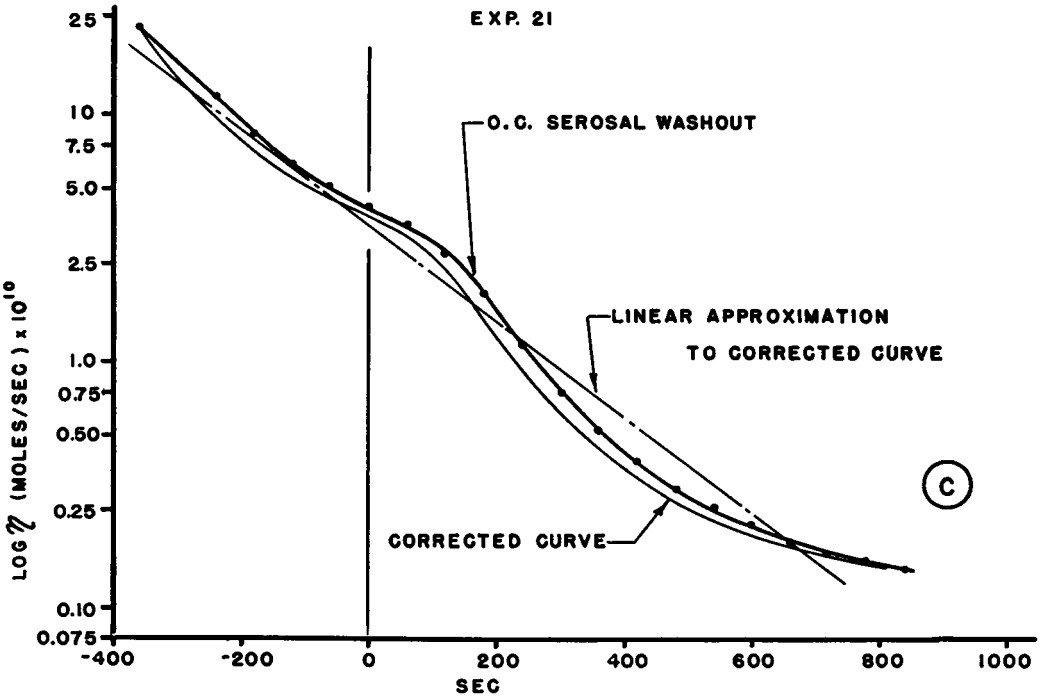


FIGURE 11*c* Corrections for delay in the apparatus. Run D'. The linear approximation was calculated by the method of least squares. It yielded a half time of washout of 2.58 min.

the main features of curve structure following time zero can be explained by a fast and a slow compartment in parallel. Washout experiments with short-circuited skins of *Rana pipiens* and *Rana catesbiana* have also revealed a late, slow phase which has been interpreted as evidence for a slow, parallel compartment (Cereijido, Herrera, Flanigan, and Curran, 1964; Hoshiko, Lindley, and Edwards, 1964). However Hoshiko and his colleagues felt this to be an artifact arising from the clamped fringes of their preparations. The half time of washout of their artifactual compartment was 30–60 min. The half time of washout of the late compartment in the present work is typically much smaller—9.7 min in the experiment presented

here. Furthermore, a toad bladder is much thinner than a bullfrog skin, and each bladder was very tigthly clamped during our experiments. It is thus unlikely that the slow compartment reported here is due to such an artifact. While the tracer data do not provide any way to identify these parallel compartments anatomically, it is tempting to relate them to the different cell types in the epithelium.

These tracer studies thus reveal a compartmental structure consistent with the known histology and at least as complex as that shown in Fig. 13. A three compart-

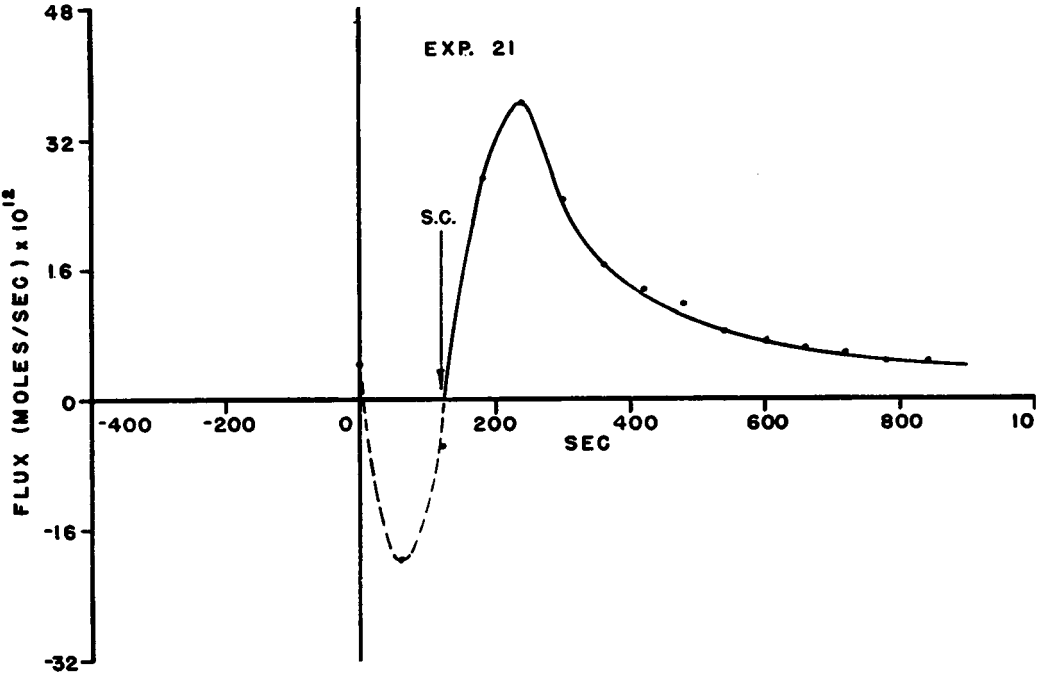


FIGURE 12 Apparent changes in serosal tracer washout fluxes produced by short-circuiting. This curve was obtained by subtracting the data of Run D′ from those of Run D. The dotted portion refers to the open-circuited period after time zero.

ment model is not adequate for a description of this system. Indeed, attempts at a quantitative solution based on nonsteady-state three compartment tracer kinetics (I) and the measured value of $S_2^*(0)$ were not entirely successful. The complexity of this analysis made it impossible to pinpoint any single cause for the problems which developed. While the inadequacies of the model and the consequent impossibility of measuring the required unique washout slope appearing in the theory ((I), equation 32) were of great importance, the biological changes in the tissue between runs and the fact that measurement of $S_2^*(0)$ is subject to uncertainty and dependent on technique were certainly also influential. Nevertheless, calculations utilizing estimates of $S_2(0)$ made from data in the literature confirmed the qualitative

conclusions regarding the sodium fluxes which are presented in the following discussion (Schwartz, 1966).

In spite of these difficulties certain valuable qualitative interpretations can be made with the aid of a three compartment model if it is used with discretion. The linear approximation to the washout curve for Run D′ (Fig. 11*c*) shows that the single exponential, steady-state washout, characteristic of the model does, in a gross way, apply to the tissue. However it is even more significant that the initial transient response of the tissue to short circuit should be independent of the sizes and arrangement of its internal compartments. This follows from the fact that the new electrical forces act instantly while the forces originating in concentration gradients take time

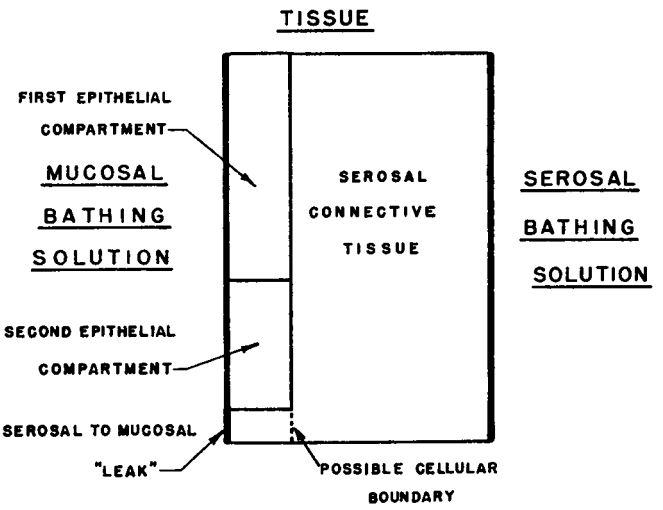


FIGURE 13 A compartmental model suggested by the data.

to change. The three compartment model can therefore be used to obtain a good qualitative understanding of events closely following the suddenly applied short circuit and as a rough approximation thereafter.

Bearing this in mind we can examine the significance of a striking feature of these data: the contrast between the dramatic sensitivity to short circuit of ξ_{21} and ξ_{23}, and the insensitivity of η_{21} and η_{23} (Figs. 8–10). The abrupt short circuit did not produce discontinuities in any of the observed tracer fluxes. It follows that j_{21} and j_{23} were also continuous at short circuit since j_{21} is proportional to both ξ_{21} and η_{21} while j_{23} is proportional to ξ_{23} and η_{23} ((I), equations 4 and 7). However, the short circuit did discontinuously alter the slopes of both ξ_{21} and ξ_{23}. That of ξ_{21} became negative while that of ξ_{23} became positive. An assumption that this can be explained by appropriate changes in dj_{21}/dt and dj_{23}/dt leads to a contradiction because the slopes of ln η_{21} and ln η_{23} remained unaltered ((I), equations 8*b* and 8*d*). The changed slopes of ξ_{21} and ξ_{23} can therefore only reflect abrupt changes in either j_{12} or j_{32}, or

in both. In the absence of changes in j_{21} and j_{23} the undisturbed, exponential decrease of η_{21} and η_{23} must reflect an invariant rate of dilution of the tissue tracer activity by the influx of "cold" sodium into the bladder from the bathing compartments. Hence the sum, $j_{12} + j_{32}$, which represents the net influx of "cold" sodium, must remain constant at short circuit even if the individual terms have changed. This can be seen quite clearly by inspecting equations 8*b* and 8*d* of (I), while recalling that S_2 can, of course, not change discontinuously. We have thus found, so far, that the data imply that j_{21}, j_{23}, and the sum, $j_{12} + j_{32}$, did not immediately respond to short circuit even though its individual terms did.

To account for the increase in ξ_{23} and the decrease in ξ_{21} seen in Figs. 8 and 9 we conclude that j_{12} must have increased while j_{32} decreased by the same amount in response to short circuit. This appears reasonable since, during Run A, there would then have been a sharp decrease in the influx of tracer into the tissue from the "hot" serosal compartment, and a sharp rise in the influx of "cold" sodium from the mucosal bath at short circuit. The result would be a decreased specific activity in the tissue and a consequent decrease in ξ_{21}. During Run C, short-circuiting would then have resulted in an increased tracer flux into the tissue from the "hot" mucosal bath, and a decrease in the influx of "cold" sodium on the serosal side. This would produce an increased tracer activity in the tissue, and a consequent rise in ξ_{23}. While we have directly measured only tracer effluxes from the tissue in these experiments, other workers have measured one of the influxes. Candia (1965) and Candia and Zadunaisky (1965, personal communication) reported a sharp, sustained rise in the flux of tracer sodium into isolated bullfrog skin from the outer bathing medium upon short circuit of this tissue. This result is in agreement with our conclusion regarding the analogous flux on the mucosal side of the bladder—j_{12}.

It thus appears that the influxes into the tissue, j_{12} and j_{32}, are sensitive to the change in electrical potential while the effluxes, j_{21} and j_{23} are not. The two sets of fluxes thus seem to be governed by different force-flux relationships with the implication that they are the result of different mechanisms. The difference in sensitivity to short circuit suggests that the influxes are governed by passive processes, while the effluxes are the result of active processes involving mechanisms which are insensitive to electrical forces. The directions of change in the influxes are precisely those expected of the passive fluxes of a cation in response to the elimination of this electrical potential difference. The existence of active processes functioning to extrude intracellular sodium has been suggested for most cells.

It thus appears that the sodium "pump" in the cells of the epithelial layer of the toad bladder extrudes intracellular sodium along both their serosal and mucosal aspects. Supporting indirect evidence for this is found in the reported localization of ATPase activity in the luminal, basal, and lateral surfaces of the epithelial cells of the bladder (Novikoff, Essner, Goldfischer, and Heus, 1962; Bartoszewicz and Barnett, 1964). While there has been some debate as to whether the localized ATPase is the one involved in sodium transport (Tormey, 1966), it is felt that the presence

of the active transport ATPase at these sites is implied (Kaye and Pappas, 1965; Farquhar and Palade, 1966). We would therefore suggest that the mechanism of net mucosal to serosal sodium transport is more complex than that originally proposed for the frog skin by Koefoed-Johnsen and Ussing (1958), modified by Ussing (1963) and Ussing and Windhager (1964), and generally assumed to apply in an analogous manner to the toad bladder. According to that model the "pump" is located only along the inward (serosal) side, and this feature was basic to an understanding of the observed net transtissue sodium transport. According to our evidence this degree of polarization due to "pump" location is not present, and it would be completely lacking if the density of distribution of "pump" sites about the cellular periphery should prove to be uniform. Unfortunately the nature of this distribution density is not known beyond the fact that it is not discretely localized at the serosal surface. Further work will be required to investigate this question. But it is interesting to note that, should the density of "pump" sites indeed prove to be basically uniform around the cellular periphery, it would then be essential that the apical aspects of the epithelial cells be much more permeable to sodium than their lateral and basal aspects for a net mucosal to serosal transfer of sodium to take place. This follows from the fact that occluded zonules apparently seal these cells together along their apical surfaces (Peachey and Rasmussen, 1961; Choi, 1963). Thus a permeability difference previously suggested to exist in both the frog skin and the toad bladder by electrophysiological evidence (Koefoed-Johnsen and Ussing, 1958; Leaf, 1964; Snell and Chowdhury, 1965) may be the basic polar feature required for the purpose of active transtissue sodium transport.

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