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Sodium Influx at the Outer Surface of Frog Skin

Evaluation of Different Extracellular Markers

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Summary. The unidirectional sodium influx across the outside surface of the frog skin epithelium was measured. The method was identical to the one described by Biber and Curran [4] except that mannitol instead of inulin was used as the indicator for the amount of tracer containing test solution remaining on the surface of the skin after blotting. The space of distribution for ^{3}H-mannitol is about twice as large as the corresponding space for inulin after 32-sec exposure to the tracer. At a sodium concentration of 6.7 mM the sodium influx determined with inulin as marker for the test solution is 0.146 μEquiv $hr^{-1}cm^{-2}$ larger than the influx measured in the same preparation with mannitol. This difference increases in proportion to the sodium concentration and can be ascribed to diffusion of sodium into a space which is accessible to mannitol but not to inulin during a 30-sec interval. Hence, nearly two-thirds of the previously described linear component of the sodium influx proceeds, as was suspected previously, into a compartment which is not directly connected to net sodium transport across the skin. The nature of the remaining one-third of this linear compartment is not clear but previous experiments suggest that it is also not involved in net sodium transport.

The frog skin can transport sodium from outside to inside against up to 10,000-fold concentration gradients. The initial step in this process, the sodium influx across the outer surface of the skin epithelium, is of considerable interest especially since recent experiments indicate that this uptake of sodium may not proceed by simple diffusion alone. Observations made by Biber and Curran [4] suggest that the unidirectional sodium influx across the outer border of the epithelium is made up of two components, a diffusion component and a saturating one which is competitively inhibited by lithium ions. In addition, this sodium uptake is decreased in the presence of ouabain or under anaerobic conditions [2]. Rotunno, Villalonga, Fernandez and Cereijido [9] used an entirely different method for measuring the sodium influx across the outer border of the skin and also came to the conclusion that the penetration of sodium into the epithelium does not

occur by simple diffusion. Zerahn [10] concluded from his experiments that active transport is involved in sodium uptake by the skin since he found that the sodium pool determined by the method of Andersen and Zerahn [1] has already undergone active transport.

Several lines of evidence emerged from previous measurements of the unidirectional influx of sodium to suggest that the linear component of the process is proceeding into a compartment that is not concerned with net Na transport across the skin [2, 4]. We mentioned among other possibilities that this compartment may be located in "extracellular" spaces accessible from the outside bathing solution. However, the nature of this compartment was by no means clear.

Our earlier studies used inulin as an indicator for the amount of tracer containing test solution which remains on the outside surface of the skin after blotting. However, recent measurement of spaces of distribution at the serosal side of the isolated epithelium of the frog skin showed that the spaces determined with ^{3}H-mannitol were consistently larger than the spaces measured by ^{14}C-inulin although both spaces were essentially constant during exposure times ranging from 15 to 60 sec [3]. These findings suggested that at the serosal side of the cells, mannitol (and sodium) may enter spaces that are not readily accessible to inulin. If a similar effect occurred at the outside surface of the tissue, we might be able to locate at least part of the linear component of the sodium influx in a space which is accessible to mannitol but not to inulin. The present experiments were carried out to examine this possibility and they indicate that indeed about two-thirds of the linear component can be attributed to a space that equilibrates within 30 sec with ^{3}H-mannitol but not with ^{14}C-inulin.

Materials and Methods

The chambers and the methods for measuring the unidirectional influx across the outside of the frog skin epithelium were practically identical with those described previously [4]. Circular pieces of the abdominal skin of *Rana pipens* were mounted between two chambers and bathed on both sides with identical solutions (exposed area 0.475 cm^2) at an ambient temperature of 22 °C. The skins were kept under short-circuit conditions by an automatic clamping device and the short-circuit current (SCC) was recorded continuously. After equilibration, the solution bathing the outside surface of the skin was withdrawn, and was replaced by a test solution which was identical except for the addition of ^{3}H-mannitol (20 μC/ml) and/or ^{14}C-inulin (3 μC/ml) alone or in combination with ^{24}Na (2 to 3 μC/ml) or with ^{22}Na (1 to 3 μC/ml). After an appropriate exposure time to the tracers (10 sec to 60 min) the skin was removed from the test solution and in rapid succession blotted, punched out and dropped into 2 ml of 0.1 N nitric acid. After 2-hr extraction, aliquots of the eluate were used for determination of radioactivity by liquid scintillation photometry.

The spaces for ^{3}H-mannitol, ^{14}C-inulin and radioactive Na were calculated by dividing the total tissue counts by the counts obtained per µliter of test solution. The counting efficiency of the tissue samples and of the test solution were identical. Calculations of the sodium influxes (Figs. 5 and 6) were made using exposure times of 15, 24 and 32 sec at a Na concentration of 6 mM and of 24 and 32 sec at a Na concentration of 115 mM since shorter exposure times were found to give somewhat less accurate data. Standard errors (SE) are used throughout this study. The regression lines are calculated by the least squares method.

Special attention was paid to the stirring of the bathing solutions during the measurements since large changes in stirring rate caused different rates of distribution of the tracer. In each frog, we aimed to obtain an equal number of points at each particular time of exposure to the test solution. The measurements at different exposure times were alternated. The Ringer's solution was composed of 115 mM NaCl, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$. In some experiments, all but 6 mM NaCl of the Ringer's solution was replaced by choline chloride.

Results

Two series of experiments were carried out to determine the relationship between inulin and mannitol spaces. For this purpose, only ^{3}H-mannitol and ^{14}C-inulin were present in the test solution and the pieces of skin were exposed to the tracers for different lengths of time. In the first experiments, 102 measurements were made in 10 frogs with exposure times ranging from 10 to 240 sec; results are summarized in Fig. 1. The space for both these markers increases with time but appears to approach a final value at times between 120 and 240 sec. Initially, there is a significant difference between the mannitol and inulin spaces as indicated more clearly in Fig. 2 where the averages of the differences obtained in each preparation are plotted against the exposure time. At 240 sec the spaces do not differ significantly.

In a second series of experiments, 26 measurements were carried out in 3 frogs in order to follow the change in the inulin and mannitol spaces over much longer exposure times. The results are summarized in Table 1. The spaces given by inulin and mannitol do not differ significantly when measured at times ranging from 240 sec to 60 min in contrast to the situation at the shorter time periods. There is a slow but significant increase with time in both spaces. The slope of the regression line calculated from the individual values is approximately 3.5×10^{-5} µliters sec^{-1}. Since this rate of increase in space is very small, it can be disregarded over short periods of time. For example, a mannitol space of 0.24 µliter will only increase by approximately 0.4% in 30 sec.

In the remaining experiments presented in this study, the rate at which the bathing solutions were stirred was increased. Using 146 pieces of skin from 13 frogs, we measured the mannitol spaces after 10.2-, 15.8-, 24.2- and

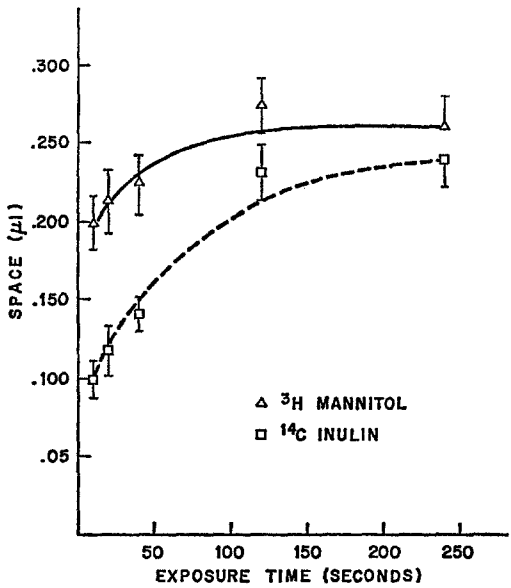

Fig. 1. Spaces for ^{3}H-mannitol (△) and for ^{14}C-inulin (□) observed 10.2, 20.2, 40.5, 119.8 and 240.3 sec after injection of the tracer containing solution into the outside chamber. Numbers of observations of different times were 22, 21, 21, 20 and 18, respectively. Na concentration, 115 mM. The difference between the curves suggests that D_{mannitol} is about 2 to 3 times greater than D_{inulin}

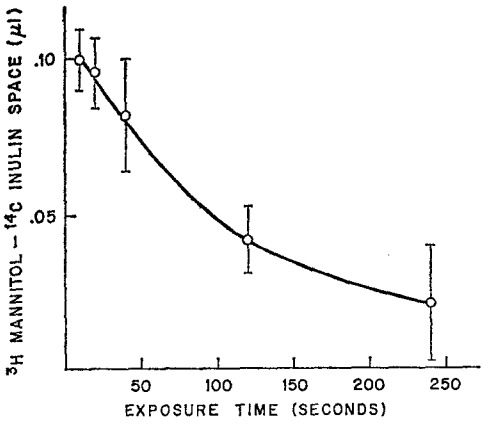

Fig. 2. Averages computed from difference between ^{3}H-mannitol and ^{14}C-inulin space observed in each preparation. Exposure times and numbers of observations identical with those on Fig. 1. Na concentration, 115 mM

Table 1. Simultaneous determination of ^{3}H mannitol and ^{14}C inulin spaces at exposure times from 4 to 60 min

Exposure time (ΔT) (sec)	^{3}H-mannitol space (μliters)	^{14}C-inulin space (μliters)	No. of observations
240.0±0.1	0.246±0.021	0.251±0.021	11
899.8±0.1	0.287±0.015	0.318±0.015	9
3600.0±0.1	0.372±0.035	0.379±0.017	6

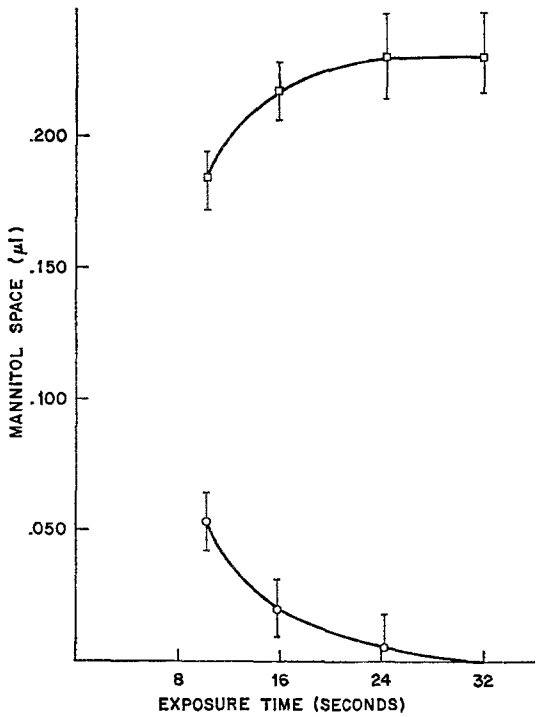

Fig. 3. *Top*: ^{3}H-mannitol space 10.2, 15.8, 24.2 and 31.8 sec after exposure of the outside surface of the skin to the tracer. Numbers of observations were 34, 37, 35 and 40, respectively. Na concentration, 115 mM. *Bottom*: Averages calculated from the difference between the average space obtained in each frog at 32 sec and the individual spaces measured in the same frog at shorter time. Na concentration, 115 mM

31.8-sec exposure to the tracer solution. The results shown on the top of Fig. 3, indicate that the mannitol space increases at first and then levels off after 24 sec.

A comparison of the curves obtained for mannitol spaces in Figs. 1 and 3 shows that the increased stirring rate causes an appreciably faster equilibration of the tracer. The value of the mannitol space obtained at such a high stirring rate after 32 sec was not significantly different from the values

obtained at a low stirring rate after 240 sec (Table 1 and Fig. 1). Similar increased stirring rates were maintained for the experiments depicted in Figs. 4, 5 and 6. The points shown on the bottom of Fig. 3 were obtained by subtracting all the individual values of the mannitol space measured at short exposure times from the average of the values obtained at 32 sec using the skin from the same frog. The data obtained from different preparations were then pooled and the SE calculated. It becomes clear from this plot that the mannitol spaces observed at 10.2 sec are significantly smaller (0.053 ± 0.011 µliter in 34 measurements) than at 31.8 sec. On the other hand, measurements of the time course of change in potential following addition of Na to the outside solution [2, 7] indicate that Na reaches a barrier across which a potential difference is generated in times less than 1 sec. The reason for the difference in behavior of mannitol and Na is unknown, but the effect suggests that Na has an effective diffusion coefficient in some region approximately 10 times larger than that of mannitol. If we make the reasonable assumption that the barrier is also the one across which Na influx occurs, the difference in diffusion has some interesting consequences for the calculation of Na influxes at short exposure times. For instance, a sodium influx calculated from an exposure time of 10 sec will overestimate the amount of sodium isotope in the tissue by the amount of tracer contained in 0.053 µliter of test solution. Hence for the calculation of uptake of sodium tracer by the tissue the differences shown on the bottom of Fig. 3 were added to the spaces found in the individual measurements made at shorter exposure times [1].

The uptake of radioactive sodium which was obtained with a sodium concentration of 6 mM in both (outside and inside) bathing solutions is shown on Fig. 4. The uptake is linear with time and the intercept of the regression line is very close to zero. Additionally, 102 measurements were made in 11 frogs to obtain a corresponding plot with a sodium concentration of 115 mM on both sides of the skin. The rate of tracer uptake was about 4.3 times as high but otherwise the plot was very similar to the one shown in Fig. 4.

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1 Such a procedure does not affect significantly the influxes calculated from experiments with an exposure time of 24 and, of course, 32 sec nor does it change appreciably the Na uptake obtained at shorter exposure times for a Na concentration of 6 mM (Fig. 4). On the other hand, the influxes measured at shorter exposure times are lowered to a variable degree when measured at a Na concentration of 115 mM. Curves similar to the ones depicted in Fig. 3 can be obtained with inulin. However, a corresponding correction does not affect the results reported previously [4] since the fluxes were obtained after 30-sec exposure time and since the uptake curve (Fig. 3 of ref. [4]) was measured at a Na concentration of 6 mM.

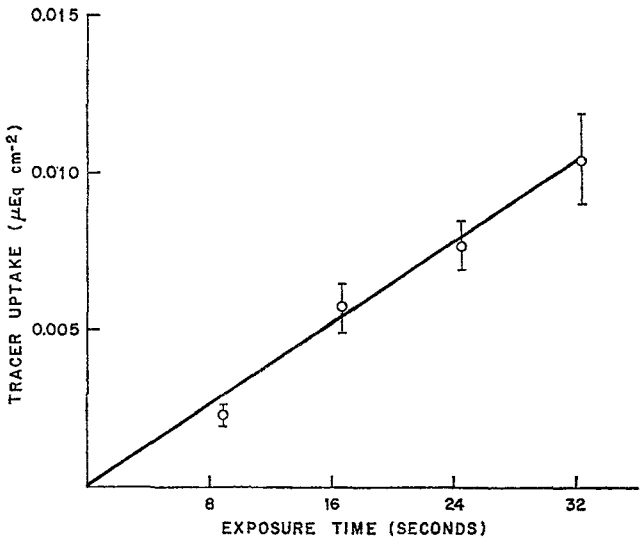

Fig. 4. Uptake of radioactive sodium. 25 measurements obtained in 3 frogs. Exposure times 8.9, 16.6, 24.4 and 32.2 sec. Numbers of observations 3, 7, 6 and 9, respectively, since only in one frog all four exposure times were tested. In the other 2 frogs only the three longer exposure times were employed. Na concentration, 6.7 mM. Regression line calculated from all individual values (25 pairs) 0.00032 ($\pm$0.00007) x +0.00007 ($\pm$0.00178), r=0.673

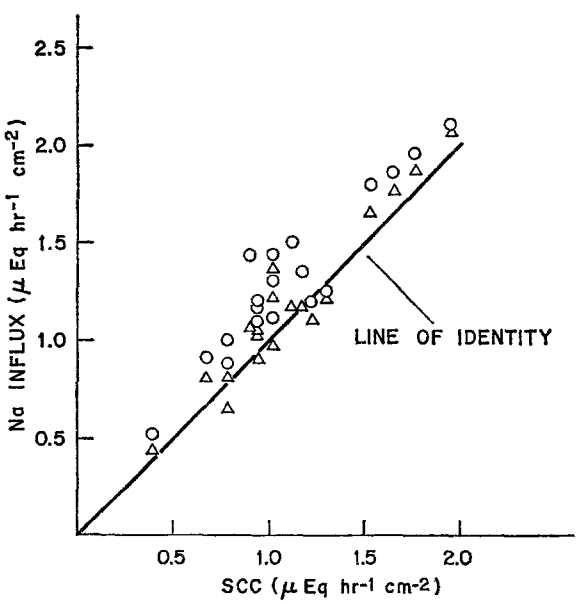

Fig. 5. Sodium influx and short-circuit current (SCC) at a Na concentration of 6.7 mM. Values corrected for inulin space (o) or for mannitol space (Δ). For details, *see* text

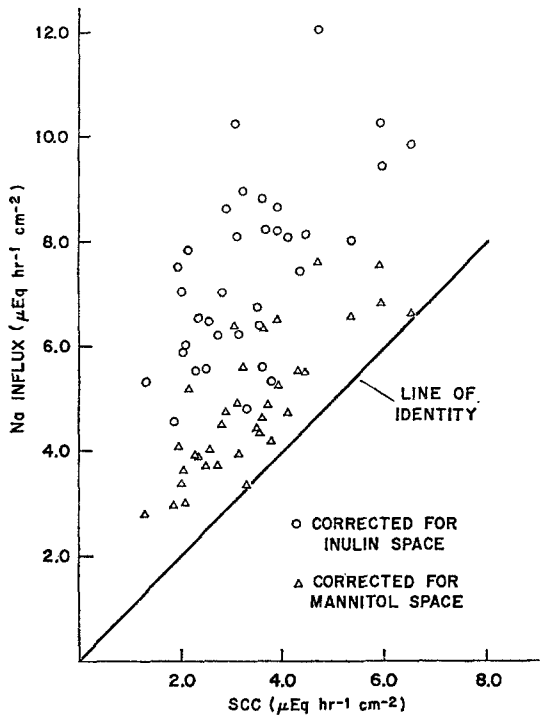

Fig. 6. Sodium influx and short-circuit current (SCC) at a Na concentration of 114.5 mM. 35 measurements carried out in 7 frogs. For details, *see* text

Nineteen sodium influx determinations were made in 2 frogs at a sodium concentration of 6 mM using simultaneously ^{3}H-mannitol and ^{14}C-inulin as markers for trapped test solution. The results of these experiments are shown in Fig. 5. The influxes calculated by subtracting the sodium isotope located in the inulin space were consistently higher than those obtained from the same preparation using the mannitol space. The average of the 19 individual differences between inulin- and mannitol-corrected sodium influx was 0.146 ± 0.022 μEquiv hr^{-1} cm^{-2}. Furthermore, the sodium influx is proportional to the SCC measured in the individual preparations. The data suggest that at a Na concentration of 6 mM the backflux of Na across the outside surface of the skin is negligible under steady state conditions. For each set of data, the slope of the regression line is not significantly different from one.

Corresponding data were obtained at a sodium concentration of 115 mM and are shown in Fig. 6. Again, the mannitol-corrected sodium influxes were always lower than the inulin-corrected ones calculated from the same piece of tissue. The slopes of the regression lines computed for the two

sets of data were not significantly different from one. However, the intercept of the regression line was much higher for the inulin-corrected influxes (4.50 ± 0.69 μEquiv hr^{-1} cm^{-2}) than for the mannitol-corrected values (1.94 ± 0.39 μEquiv hr^{-1} cm^{-2}). The average of the 35 individual differences between inulin- and mannitol-corrected sodium influxes was 2.60 ± 0.14 μEquiv hr^{-1} cm^{-2}.

Discussion

The data in Figs. 1 and 2 indicate that the spaces of distribution for ^{3}H-mannitol at the outside surface of the frog skin can be as much as twice as large as the corresponding ^{14}C-inulin spaces. In all the experiments carried out at a high stirring rate the mannitol spaces averaged 0.231 ± 0.015 μliter (40 measurements) after an exposure time of 31.8 sec whereas the corresponding space for inulin was only 0.138 ± 0.013 μliter (29 measurements). However, this difference between the two spaces decreases as the exposure time is prolonged and they become essentially identical after 4 min. The initial discrepancy can be simply a consequence of pronounced difference in rates of diffusion in these two compounds. The exact location of these spaces is difficult to establish. The fact that the mannitol and inulin spaces are practically identical for exposure times from 4 to 60 min suggests that both substances distribute in the same space. Since the spaces increase very slowly after 4 min and since the size of the spaces constitutes only a small fraction (approximately 20%) of the total cell volume of the epithelium, it might be reasonable to assume that both tracers enter the cells very slowly if at all. Alternatively, both mannitol and inulin might enter certain cell types of the epithelium quite rapidly. Recently, Martinez-Palomo, Erlij, and Bracho [8] have found that lanthanum penetrates fairly readily into the cells of the cornified layer of the skin. Thus, it is possible that mannitol and inulin (and sodium) also enter this cell layer and that it accounts for most of the "extracellular" space. In fact, one could regard the cornified cell layer of the epithelium as functional extracellular space. It is perhaps of interest to note in this regard that the space measured by mannitol after blotting the tissue is equivalent to a layer 5 to 6 μ thick. The thickness of the cornified layer of the skin is approximately of this magnitude.

Regardless of the exact anatomical location of these "spaces", the different behavior of the two markers at short time intervals does influence the evaluation of sodium influxes. From previous measurements using inulin-corrected values, we suggested [4] that the total influx was a com-

bination of a saturating component and a linear one. The linear component was characterized by a term $\alpha\ [Na]_0$ in which α is a permeability coefficient and $[Na]_0$ is sodium concentration in the outside solution in μEquiv/ml; the value of α obtained was 0.037 cm hr^{-1}. The present results indicate that at least a portion of this linear component of influx can be accounted for by entry of sodium into a "space" which has not equilibrated with inulin during the short observation period (30 sec). On the basis of the studies with mannitol and inulin, it appears that this portion of sodium influx can be identified to a reasonable approximation with the difference between mannitol- and inulin-corrected influxes. At a sodium concentration of 6.7 mM, this difference is 0.146 μEquiv hr^{-1} cm^{-2} and at a concentration of 114.5 mM it is 2.56 μEquiv hr^{-1} cm^{-2}. The difference between inulin- and mannitol-corrected influxes is thus almost exactly proportional to sodium concentration and would have the character of a linear component of the total influx. If the previous data [4] are corrected for this difference between inulin and mannitol (assuming a direct proportionality to sodium concentration as indicated above) a corrected value of α of 0.015 cm hr^{-1} is obtained.

It should be pointed out, however, that the size of the linear component may vary considerably from frog to frog depending on the condition of the epithelium. This is not surprising in view of the fact that shedding of the stratum corneum of the amphibian epithelium may lead to changes in sodium conductance [5, 6]. Hence, it is possible that the frogs used for collection of our earlier data [4] may have had a larger linear component than the frogs tested in this study. The corrected value for α could, therefore, be even less than 0.015 cm hr^{-1}.

A second estimate of a new value of α can be obtained from the data in Fig. 6. If we assume, as previously suggested, that the saturating component of influx is proportional to net sodium flux (or SCC), a value of α can be calculated directly from the y-intercepts of regression lines for the data shown in Fig. 6. The inulin-corrected data have an intercept of 4.50 μEquiv hr^{-1} cm^2 corresponding to $\alpha = 0.039$ cm hr^{-1}; the mannitol-corrected data have an intercept of 1.94 μEquiv hr^{-1} cm^{-2} yielding $\alpha = 0.017$ cm hr^{-1}. These results suggest that approximately 60% of the linear component of sodium influx previously reported [4] can be accounted for by movement of sodium into a "space" in excess of inulin during the 30-sec flux measurement. However, after correction for this effect a linear component of influx still remains. Part of this residual influx may represent movement of sodium through shunt-pathways. An evaluation of the shunt-path by Mandel and Curran (*personal communication*) indicates that

under these conditions about 0.5 μEquiv hr^{-1} cm^{-2} proceed through this pathway which would not be connected with net Na transport (and therefore with the saturating component). If we subtract the Na flux attributed to the shunt-path from the value of the intercept calculated for the mannitol-corrected influx values shown on Fig. 6 we obtain a value for α of 0.013 cm hr^{-1}. Hence, even after this correction a linear component of influx appears to remain. The nature of this flux is unknown at present. It could be caused by entry of sodium into an "extracellular" space in excess of mannitol during the influx measurement or into a space not accessible to mannitol. Finally, the linear component of influx could be caused by entry of sodium into the transporting cells. Since more than one of these possibilities may apply, a clear-cut determination of the nature of this component by techniques presently available seems rather difficult.

Note Added in Proof: Erlij and Smith have reported in abstract (D. Erlij and M. W. Smith, 1971, *J. Physiol.* **218**:33P) a study of Na uptake across the outer surface of frog skin using mannitol as an "extracellular" marker. They report no remaining linear component, i.e. $\alpha = 0$. However, their experimental conditions appear to differ appreciably from ours so that meaningful comparison of results is difficult at present.

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