The Effect of Ca and Antidiuretic Hormone on Na Transport across Frog Skin

II. *Sites and mechanisms of action*

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ABSTRACT A method has been developed for determining unidirectional Na fluxes across the two faces of the transporting cells in the frog skin. The method has been used to investigate the location of the sites at which Ca and antidiuretic hormone act to alter the rate of active Na transport across the skin. The results have indicated that the primary effect of both agents is on the Na permeability of the outward facing membrane of the cells. Ca decreases and the hormone increases permeability of this barrier. Neither agent appears to have a direct effect on the active transport system itself assuming that it is located at the inner membrane of the cells. The rate of active Na transport is altered as a result of changes in the size of the Na pool in the cells which occur because of changes in the rate of Na entry through the outer membrane. Thus, the results indicate that the Na permeability of the outer membrane plays an important role in controlling the rate of net active Na transport across the skin.

The previous paper in this series (1) described effects of Ca and antidiuretic hormone (ADH) on net Na transport across isolated frog skin. The results of these studies led to the suggestion that both agents acted on the outward facing membrane of the transporting cells of the skin. The observed effects could be explained qualitatively in terms of changes in the Na permeability of the outer membrane without postulating a direct effect on the active Na transporting system. The present experiments were designed to test this hypothesis by obtaining more specific information regarding the sites and mechanisms of action of Ca and ADH.

THEORETICAL CONSIDERATIONS

A modification of the methods previously described by Schoffeniels (2) and Hoshiko and Ussing (3) has been used in an attempt to estimate some of the parameters describing the Na transport system and to determine the changes brought about by Ca and ADH. The method is based on the kinetic analysis outlined below and depends on measurement of the rate at which the flux of radioactive Na across the skin builds up to a steady value, plus an estimation of the amount of radioactive Na in the transporting cells when the flux has reached a steady value. The model of the skin used for kinetic description is shown schematically in Fig. 1. This model requires only that the skin present two significant barriers to Na movement arranged in series. At the outset, no specific assumptions need be made concerning the nature of these barriers. The particular parameters of this model which are of interest in attempting to evaluate the effects of Ca and ADH are the Na permeability of the outward facing membrane, the rate constant k_{23} (assumed to represent primarily active

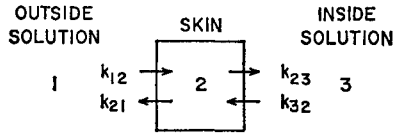

FIGURE 1. Model of frog skin used for kinetic analysis. The k_{ij} are rate constants for undirectional Na movements. Compartment 2 need not represent the entire skin, but only that portion of the skin between the two major barriers to Na movement.

Na transport in the model of Koefoed-Johnsen and Ussing (4)), and the pool of Na within the skin compartment.

When Na^{24} is added to the outside bathing solution, the change with time of the amount of Na^{24} in compartment 2 (taken to represent the transporting cells) and in the inside solution will be given by the following equations:

$$\frac{dP_2}{dt} = -(k_{21} + k_{23})S_2p_2^* + k_{12}S_1p_1^* + k_{32}S_3p_3^* \quad (1)$$

$$\frac{dP_3}{dt} = -k_{32}S_3p_3^* + k_{23}S_2p_2^* \quad (2)$$

in which P_i = total radioactivity in compartment i (CPM)
S_i = total Na in compartment i (μeq)
p_i^* = specific activity in compartment i (CPM/μeq)
k_{ij} = rate constant for Na movement from compartment i to j ($hr.^{-1}$).

The values of the k_{ij} are dependent on the volumes of the compartments. For example, $k_{12}V_1[Na]_c = \Phi_{12}$, in which Φ_{12} is the unidirectional flux from

compartment 1 to 2 and V_1 is the volume of compartment 1. In using Equations 1 and 2, we shall assume that the volumes (V_i) and amounts of Na (S_i) in each compartment remain constant throughout a given experiment. For the conditions used in the present experiments, these equations may be simplified since p_1^* remains constant and since $p_3^* \cong 0$ throughout the experimental period. Using these assumptions and the relationship $p_i^* = P_i/S_i$, Equation 1 becomes

$$\frac{dP_2}{dt} + \lambda P_2 = k_{12}P_1 \tag{3}$$

in which $\lambda = k_{21} + k_{23}$. Taking as initial conditions $P_2 = 0$ at $t = 0$ and assuming that $k_{12}P_1$ is constant, Equation 3 has the solution

$$P_2 = \frac{k_{12}P_1}{\lambda}(1 - e^{-\lambda t}) = P_{2\infty}(1 - e^{-\lambda t}) \tag{4}$$

in which $P_{2\infty}$ is the steady state value of P_2 reached as t becomes large.

Using the above assumptions, Equation 2 becomes

$$\left[\frac{dP_3}{dt}\right] = k_{23}P_2 = k_{23}P_{2\infty}(1 - e^{-\lambda t}) \tag{5}$$

in which Equation 4 has also been used. As t becomes large, Equation 5 reduces to

$$\left[\frac{dP_3}{dt}\right]_\infty = k_{23}P_{2\infty} \tag{6}$$

in which $(dP_3/dt)_\infty$ is the steady state rate of tracer appearance in compartment 3. Inserting this relationship into Equation 5, rearranging, and taking the natural logarithm of both sides, we obtain

$$\ln\left[1 - \frac{(dP_3/dt)}{(dP_3/dt)_\infty}\right] = -\lambda t \tag{7}$$

The quantity on the left hand side can be determined directly by measuring the rate of tracer appearance in the inside solution and a plot of this quantity against time should give a straight line having a slope equal to $-\lambda$. From Equation 4, we find that

$$k_{12} = \frac{P_{2\infty}\lambda}{P_1} \tag{8}$$

Thus, k_{12} can be determined if the value of $P_{2\infty}$ is known. This quantity can

be estimated by the means described in the Methods section. From Equation 6,

$$k_{23} = \frac{(dP_3/dt)_\infty}{P_{2\infty}} \tag{9}$$

and therefore, k_{21} can be calculated from the values of λ and k_{23}.

If the skin is in a steady state, the net flux of Na across the skin must be equal to the net flux across each of the two barriers in the series arrangement. Thus, we may write

$$\Phi_n = \Phi_{12(n)} = k_{12}S_1 - k_{21}S_2 \tag{10}$$

in which Φ_n is net Na transport across the skin, measured by short-circuit current and $\Phi_{12(n)}$ is the net Na transport from compartment 1 to compartment 2. Equation 10 can be solved for S_2.

$$S_2 = \frac{k_{12}S_1 - \Phi_n}{k_{21}} \tag{11}$$

Since all quantities on the right hand side are either determined directly or calculated as described above, S_2, the Na pool in compartment 2, can be calculated.

The calculations made up to this point do not rest on any particular model of the Na transporting system, but on the basis of certain assumptions, calculation of additional parameters is possible. Assuming, in accordance with the model of Koefoed-Johnsen and Ussing (4), that Na diffuses passively across the outer membrane, and also that under conditions of short-circuiting, there is no electrical potential difference between compartments 1 and 2, the movement of Na may be described in terms of a single permeability coefficient.[1] Equation 10 may be then rewritten as

$$\Phi_n = P^o_{Na}\{[Na]_o - [Na]_c\} \tag{12}$$

in which P^o_{Na} is Na permeability of the outer membrane and $[Na]_o$ and $[Na]_c$ are Na concentrations in the outside solution and in the cell compartment respectively. Comparison of Equations 10 and 12, keeping in mind the definitions of k_{12} and S_1, indicates that P^o_{Na} is related to k_{12} as follows:—

$$P^o_{Na} = V_1k_{12} \tag{13}$$

in which V_1 is the volume of compartment 1, the outside solution. Equation 12 may then be solved for $[Na]_c$.

$$[Na]_c = \frac{P^o_{Na}[Na]_o - \Phi_n}{P^o_{Na}} \tag{14}$$

[1] In terms of rate coefficients, these assumptions require that $k_{12}V_1 = k_{21}V_2$.

Since both the size of the Na pool in compartment 2 and the Na concentration in the pool can be estimated, the volume of this compartment may be calculated directly. Assuming that the surface area of the compartment is equal to the geometrical area (A) of the skin exposed in the chamber, it also is possible to calculate the thickness (x) of compartment 2

$$x = \frac{S_1}{A[\mathrm{Na}]_c} \tag{15}$$

Thus, by measuring the rate at which the flux of Na^{24} builds up to a steady value, the amount of Na^{24} in the skin, and the net Na transport across the skin, the parameters which are necessary to analyze the effects of Ca and ADH in more detail can be estimated.

METHODS

The skin of *Rana pipiens* was mounted between two small lucite chambers which were equipped with two sets of electrodes as previously described (1, 5). The area of skin exposed was 3.14 cm^2, and the volume of each bathing solution was 5.0 ml. The experiments were carried out in pairs using symmetrical pieces of skin from the same frog; one piece always served as a control and the other was treated with either Ca or ADH. The skins were mounted in the Ringer's solution described in the preceding paper (90 mM NaCl, 22.6 mM choline chloride, 2.4 mM $NaHCO_3$, and 2 mM KCl, but no Ca) and were allowed to equilibrate for at least 1 hour. They were then short-circuited and the short-circuit current was taken as a measure of the net Na transport (5, 6).

After the short-circuit current had reached a steady value, one of the paired skins was treated with either Ca (11.3 mM added to the outside solution as previously described (6)) or ADH (0.2 U/ml of pitressin, Parke, Davis Co., added to the inside solution). After 15 to 20 minutes, approximately 25 microcuries of Na^{24} were added to the outside solution of both skins and sampling of the inside solution was begun at once. Ten samples were taken at either 1 or 1.5 minute intervals followed by several samples at 5 or 10 minute intervals. The duration of the experiments was 45 to 50 minutes, a time sufficient for the tracer flux to reach a steady state. Samples of 0.5 ml were withdrawn from the inside solution, pipetted directly into tubes, and counted in a well-type scintillation counter. Immediately after sampling, the fluid volume in the chamber was restored by adding inactive Ringer's solution. Near the end of the experiment, a sample was withdrawn from the outside solution, diluted, and counted. All counting rates were corrected for decay. At the end of the experiment, the skin was removed from the chamber and blotted with filter paper. The portion of the skin not exposed to the bathing solutions was then cut away and the exposed portion was counted directly in the scintillation counter to determine the amount of Na^{24} present. In this series of experiments, the total amount of Na^{24} present in the skin at the end of the experiment was taken as the value of $P_{2\infty}$. The Na concentrations of the solutions used were determined by flame photometry after each experiment.

Estimation of parameters of the Na transport system using these methods is based

on several assumptions. We have assumed that the quantity $P_{2\infty}$ is given by the total amount of Na^{24} in the skin when the tracer flux has reached a steady value. Some of this Na^{24} must be present in the extracellular spaces of the skin and possibly in cells other than those involved in the process of active Na transport, particularly those cells in the epithelium which are in the process of being cornified. MacRobbie and Ussing (7) found that these cells do not respond to changes in the osmolarity of the outside solution suggesting that they are relatively impermeable to water. It seems reasonable to assume that they would also be relatively impermeable to Na and would not lead to an important error in estimation of $P_{2\infty}$. Thus, the major error in $P_{2\infty}$ probably arises from the contribution of Na^{24} in the extracellular space available from the outside solution. However, some Na^{24} may be lost from the skin during the process of blotting and cutting away unexposed parts of the skin, a process requiring approximately 2 to 3 minutes, and this would tend to cancel the error caused by the extracellular space. Further, estimation of this outer extracellular space in separate experiments using C^{14}-inulin has indicated that it is relatively small (0.31 ± 0.03^2 μl/cm^2 area) and is not altered by either Ca or ADH. Thus, the errors involved in determination of $P_{2\infty}$ should be of similar magnitude in control and treated skins, and they should not cause any alteration in the basic conclusions concerning the changes caused by Ca and ADH.

In using Equations 12 and 14 to calculate the Na concentration in the transporting compartment, we have had to assume that there is no electrical potential difference across the outer barrier when the skin is short-circuited. Measurements in both frog skin (8) and toad bladder (9) using microelectrodes have indicated that this potential difference may be quite small and it therefore seems reasonable to neglect it. However, estimation of the size of the Na pool does not require knowledge of this potential difference since any contribution of the potential difference will be included in the rate coefficients k_{12}, k_{21}, and k_{23} which are used to estimate S_2. The existence of a potential difference would alter the estimated values of $[Na]_c$ and would have to be taken into account in calculation of the permeability coefficient of the outer membrane. Although the value of P^o_{Na} reported here is determined directly by k_{12} only and may not necessarily represent the true permeability coefficient of the membrane, we shall refer to it as a permeability coefficient for convenience of discussion.

These considerations indicate that the methods used in this study should give reasonably reliable results for the purpose of evaluating the effects of Ca and ADH on the Na transport system since this evaluation depends on determining changes in parameters. On the other hand, they also indicate that appreciable errors are possible, and the absolute values of the parameters obtained cannot be considered exact until methods have been devised for checking them more directly.

RESULTS AND DISCUSSION

The Experimental Method

The results of a typical experiment are shown in Fig. 2 in which $\log \{1 - [(dP_3/dt)/(dP_3/dt)_\infty]\}$ is plotted against time. As predicted by

[2] Standard error of the mean.

Equation 7, a straight line can be drawn through the experimental points. However, the best straight line through the points does not pass through the point 1.0 at $t = 0$ although Equation 7 predicts that it should do so. Such an effect could be explained on the basis of a delay in Na^{24} movement caused by an extracellular space located between compartments 2 and 3 (the transporting system and the inside solution). Assuming that the transporting system is located in the epithelium of the skin, a delay might be expected due to the rather thick layer of connective tissue located between the epithelium and the inside solution.

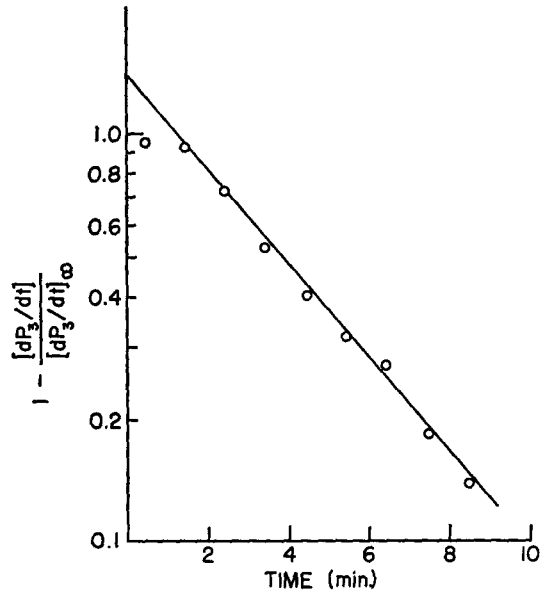

FIGURE 2. Results of a control experiment showing the approach of tracer flux to a steady value. The data are plotted in the manner suggested by Equation 7. The line has been drawn by eye, neglecting the first point.

As shown in Appendix A (Equation A-6), such an extracellular space would give rise to the experimentally observed results, but would not alter the calculation of parameters of the skin. As described in the Appendix, a rate constant for Na movement through this extracellular space can be calculated from values of the intercept at $t = 0$ and λ, the slope of the line. The average value obtained for all experiments in this series was 0.75 $min.^{-1}$, which is in good agreement with the value of 0.86 $min.^{-1}$ reported by Hoshiko and Ussing (3) and obtained by direct measurement of Na washout from this space. In no experiment was there evidence of more than one exponential after a time of about 1 to 1.5 minutes. Thus, the build-up of tracer flux to a steady value follows the predictions based on the system shown in Fig. 1, with the

exception just noted, and this does not require any alteration in the method of calculation. The results shown in Fig. 2 are in good agreement with those previously reported by Hoshiko and Ussing (3) for toad bladder and the skin of *Rana temporaria*. In all experiments, the unidirectional Na influx across the skin, computed from $[dP_3/dt]_\infty$ was greater than the short-circuit current by 5 to 15 per cent as expected if the current is equal to net Na flux (5). The observed changes in net Na flux with Ca and ADH were due primarily to changes in Na influx as previously observed (5, 6).

Analysis of the effects of Ca and ADH on the skin is based on the assumption that separate experiments on two pieces of skin from the same frog will give comparable results. Table I shows values of some of the parameters calculated as described in the theoretical section for two experiments in which both pieces of skin were kept under control conditions. The parameters

TABLE I

COMPARISON OF RESULTS ON PAIRED SKINS*

	λ	Φ_n'	$P_{Na}^{o\prime}$	k_{23}	S_2'	$[Na]_c$	x
	hr.$^{-1}$	*μeq/hr. cm*^{2}	*cm/hr.* × 10^2	*hr.*$^{-1}$	*μeq/cm*^{3}	*mM*	*μ*
Skin A							
1	18.2	1.41	3.5	12.6	0.20	34	58
2	19.0	1.38	3.6	11.9	0.17	38	46
Skin B							
1	20.0	1.31	4.1	9.6	0.15	39	39
2	21.7	1.68	4.9	10.5	0.18	41	44

* Primed quantities are expressed per unit area of skin.

given are the ones of primary interest in interpreting the effects of Ca and ADH. Although calculation of Na concentration in the skin compartment, $[Na]_c$, and the thickness of this compartment, x, rest on assumptions which have yet to be verified, they are included in the table since they may give some information concerning the validity of the methods used. The interpretation of experimental results does not, however, rest on the values of these two parameters. The data given in Table I indicate that the method yields reproducible results within the limits of the rather appreciable experimental errors involved. In the second experiment given in the table, net Na flux differed by more than 20 per cent in the two pieces of skin and some of the differences in parameters may be ascribed to this. All experiments testing the effects of Ca and ADH were carried out on pairs of skins in which the initial net Na flux under control conditions differed by less than 20 per cent. Thus, the results in Table I give some indication of the variability between pieces of skin from the same frog.

The Effects of Ca and ADH

The effects of Ca and ADH on Na transport were each tested in 13 experiments. The averaged results of these two groups of experiments are given in Tables II and III. The results have been analyzed statistically as paired data so that each piece of experimental skin has been compared with a control skin taken from the same frog. Addition of Ca to the outside solution

TABLE II
EFFECT OF Ca ON FROG SKIN

	λ	Φ_n'	$P^{o\prime}_{Na}$	k_{23}	S_2'	$[Na]_c$	π
	hr.$^{-1}$	*μeq/hr. cm*^{2}	*cm/hr* × 10^2	*hr.*$^{-1}$	*μeq/cm*^{2}	*mM*	*μ*
Control	18.0	1.35	3.2	9.4	0.20	50	41
Ca (11.3 mM)	16.0	0.76	1.4	11.7	0.10	35	31
Mean difference*	−2.0	−0.59	−1.8	2.3	−0.10	−15	−10
±SE	±0.8	±0.08	±0.2	±1.0	±0.02	±2	±3
p	<0.05	<0.01	<0.01	<0.05	<0.01	<0.01	<0.01

* Calcium-treated − control.

TABLE III
EFFECT OF ADH ON FROG SKIN

	λ	Φ_n'	$P^{o\prime}_{Na}$	k_{23}	S_2'	$[Na]_c$	π
	hr.$^{-1}$	*μeq/hr. cm*^{2}	*cm/hr* × 10^2	*hr.*$^{-1}$	*μeq/cm*^{2}	*mM*	*μ*
Control	16.4	1.40	2.7	10.7	0.17	39	44
ADH (0.2 U/ml)	19.5	1.84	4.5	9.7	0.24	51	47
Mean difference*	3.1	0.44	1.8	−1.0	0.07	12	3
±SE	±1.3	±0.77	±0.2	±0.8	±0.02	±3	±5
p	<0.05	<0.01	<0.01	<0.2	<0.01	<0.01	<0.8

* ADH-treated − control.

caused a decrease in net Na transport. There were significant decreases in the Na permeability of the outer membrane and in the Na pool in the cells. The Na concentration in this compartment decreased proportionately less than did the Na pool. This is apparently the result of decrease in thickness (or volume) of the compartment after treatment with Ca. The increase in k_{23} observed upon addition of Ca is probably statistically significant ($p < 0.05$). Addition of ADH to the inside solution caused the expected increase in net Na transport and also resulted in statistically significant ($p < 0.01$) increases in Na permeability of the outer membrane, the size of the Na

pool, and the Na concentration in the transporting compartment. The changes observed in x and k_{23} were not significant.

On the basis of the results given in Tables II and III and calculation of other rate coefficients as indicated in the theoretical section, a model of the frog skin in terms of Na fluxes can be constructed for the different experimental conditions. The results are shown in Fig. 3 and serve to illustrate the changes in unidirectional Na fluxes at the two sides of the skin caused by Ca and ADH. As would be expected from the above results, Ca decreased the unidirectional Na fluxes across the outer membrane, as well as the net flux, while ADH caused an increase in both unidirectional and in net Na fluxes across this membrane. There were no significant changes in Na flux from the inside solution toward the cell compartment, but the flux from cell toward inside solution, assumed to represent the active transport step, is increased in the presence of ADH and is decreased by Ca.

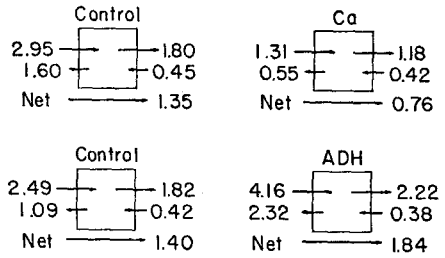

FIGURE 3. Average values for calculated unidirectional Na fluxes across the two barriers to Na movement in the frog skin. Fluxes are given as μeq Na/hr. cm^2. The average net Na fluxes across the skin, determined by short-circuit current, are included.

Curran and Gill (6) and Herrera and Curran (1) have suggested that the effects on Na transport of Ca (added to the outside solution) and of ADH could be explained in terms of changes in Na permeability of the outer membrane. The results presented in Tables II and III and in Fig. 3 indicate that this hypothesis is essentially correct. Ca decreases the Na permeability of the outer membrane while ADH increases permeability. As a result of these changes the rate at which Na enters the transporting compartment from the outside solution must be altered. This should lead to a reduction in the Na pool of the transporting system when Ca is added and an increase in the pool when ADH is added. The rate of active Na transport (across the inner membrane) would then be altered as a result of these changes in pool size.

There is no evidence that the effect of either agent can be explained in terms of a direct alteration in the active Na transporting system assuming that it is located at the inner membrane. As shown in Tables II and III, the rate coefficient k_{23} is larger in Ca-treated skins than in controls and is smaller

in ADH-treated skins than in the corresponding controls. However, Ca causes a decrease in net Na transport and to explain this in terms of a direct effect on the transport system would require that k_{23} be reduced rather than increased. Similarly, if ADH acted by direct effect on the active transport system, it would cause an increase in k_{23} rather than the slight decrease which was actually observed. Thus, the changes in the unidirectional Na flux from the cells toward the inside solution caused by Ca and ADH must be ascribed to the changes in the Na pool of the cells and not to changes in the system which brings about Na movement across this membrane. Since the properties of the inner membrane are not altered, the changes in size of the Na pool, and, hence, in the rate of Na transport, must be the direct result of the observed changes in the outer membrane. Frazier, Dempsey, and Leaf (10) have suggested a similar mechanism to explain the effect of ADH on Na transport across the isolated toad bladder and have recently shown that ADH does increase the Na permeability of the mucosal (or outer) membrane of the cells and the Na pool in this tissue. Skou and Zerahn (11) have suggested that local anesthetics also increase Na transport across frog skin by increasing the Na permeability of the outer membrane.

The quantitative changes in the parameters of the skin are in good agreement with those expected on the basis of the observed changes in net Na transport. In the case of Ca treatment, Na transport decreased by 44 per cent and the permeability of the outer membrane by 56 per cent. ADH caused an increase of 31 per cent in Na transport and of 67 per cent in permeability. In both cases, the relative change in Na permeability was greater than that in transport. This is, however, expected on the basis of changes in the Na concentration in the cellular compartment. Addition of Ca causes a decrease in $[Na]_c$ and this will increase the driving force for Na movement from outside solution into the cells. Thus, the change in net Na flux across this membrane should be relatively less than the change in Na permeability. Similarly, ADH causes an increase in $[Na]_c$ which will decrease the driving force for Na movement across the outer membrane and the increase in flux should be relatively less than the increase in permeability. The exact relationships between permeability coefficients and driving forces cannot be evaluated without knowledge of the electrical potential difference across the outer membrane, even though it is likely to be rather small (9). However, the results which have been presented do appear to fit into a self-consistent pattern.

The Na Transport System

In addition to showing that the primary effects of Ca and ADH are on the outer barrier of the skin, the present experiments offer additional information concerning the Na transport system itself. They lead to estimates of unidi-

rectional Na fluxes across the two barriers in the skin, of the distance between these barriers, and of the Na concentration within the transporting compartment. The value of 45 mM (mean of all control experiments) for Na concentration in the cells may appear rather high, but it is in reasonable agreement with results on other epithelial membranes which actively transport Na. Thus, Whittembury, Sugino, and Solomon (12) reported a value of 37 mM for *Necturus* kidney cells and Leaf (13) has given a value of 55 mM for cells in toad bladder.

The present results appear to be consistent with the hypothesis that the Na transport system is located in the epithelial layer. The non-zero intercept shown in Fig. 2 would be expected if the transport system were in this layer since it implies the existence of an appreciable extracellular space between the transporting system and the inside solution. Further, the figure of 45 μ obtained for the thickness of the cell layer is much less than the total thickness of the skin (180 to 250 μ measured in 6 experiments) and is very nearly equal to the thickness of the epithelium. The thickness of this layer appears to be approximately 40 to 60 μ (7, 14, 15). However, Koefoed-Johnsen and Ussing (2) have suggested that the stratum germinativum is the site of active Na transport and this layer has a thickness of about one-half the total epithelium or 20 to 30 μ. The present thickness estimate is probably maximal since we have assumed that the surface area of the transporting cells is equal to the geometrical surface area of the skin exposed in the chamber. The true surface area may easily be greater than this and such an effect would reduce the estimate of thickness (Equation 15). It is also possible that the actual thickness of the layer serving as the "transporting compartment" is greater than that of the stratum germinativum and this might be an indication that the outer layer of the epithelium is, in fact, part of the outward facing barrier to Na movement.

The present methods do not have sufficient resolution to establish the location of the outer barrier, but they have shown that the permeability of this barrier plays an important role in controlling the rate of net Na transport across the skin as previously suggested (1, 6). This is illustrated clearly in Fig. 4 in which the rate of net Na transport across the skin is plotted against Na permeability of the outer membrane. Each point represents the result of a single experiment, and since the primary effects of Ca and ADH are on P^o_{Na}, all the data have been included in a single plot. There is a strong correlation between rate of net transport and permeability of the outer membrane, but the relationship is not linear over the entire range of observation; there appears to be a tendency toward saturation at higher values of the permeability.

Such a relationship could be predicted on the basis of the model of the skin suggested by Koefoed-Johnsen and Ussing (4). Assuming that Na diffuses

passively across the outer membrane and is actively transported across the inner membrane, the following relationship between net Na flux and permeability of the outer membrane may be derived (Appendix B)

$$\Phi_n = \frac{P^o_{Na}(V_2k_{23} - P^i_{Na})[Na]_o}{P^o_{Na} + V_2k_{23}} \quad (16)$$

in which V_2 is volume of compartment 2 and P^i_{Na} is permeability of the inner membrane. If V_2k_{23} and P^i_{Na} may be regarded as constants, a reasonable assumption on the basis of the data presented in Tables II and III and Fig. 3, a relationship between Φ_n and P^o_{Na} of the type shown in Fig. 4 would be

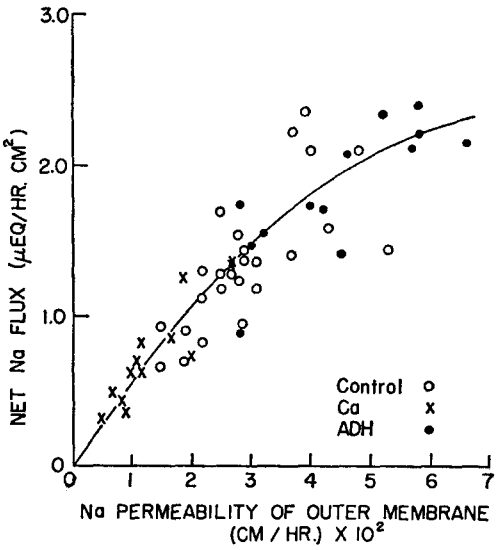

FIGURE 4. Relationship between net Na flux across the skin and Na permeability of the outer membrane. Each point represents a single experiment. The line has been drawn by eye.

expected. Under these conditions, net Na transport should increase with increasing permeability of the outer membrane until $P^o_{Na} \gg V_2k_{23}$, when it should become independent of P^o_{Na}.

The data obtained also make possible an estimate of the relationship between rate of active transport (taken as equal to net Na flux) and the Na pool of the transporting cells, as shown in Fig. 5. For this purpose, the results of all control experiments have been pooled and only average values for the three experimental conditions are shown. Over the range available there appears to be a linear relationship between rate of transport and amount of Na present in the cells, suggesting that the transport system is not saturated. This is a reflection of the fact that there is no appreciable variation of k_{23}

over the range of the present experiments. It is more usual to consider the relationship between flux and concentration; this has not been done in the present case since calculation of $[Na]_c$ is based on the assumption that there is no potential difference across the outer barrier. However, if the Na transport system were saturated under control conditions, ADH could not cause an increase in Na transport by merely increasing the Na permeability of the outer barrier.

This observation that the Na-transporting system itself is not saturated at the normal cellular levels of Na when the skin is bathed in solutions of approximately 100 mM Na, plus the finding that the rate of net Na movement is influenced strongly by the Na permeability of the outer membrane, requires some consideration. The rate of net Na transport across the skin saturates at outside Na concentrations of about 20 mM (16, 17) but on the

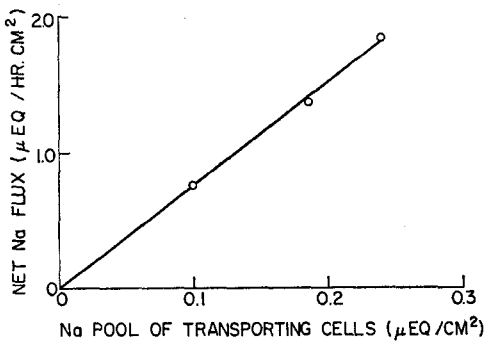

FIGURE 5. Relationship between net Na flux across the skin and size of the Na pool in the transporting cells. The three points are average values for each set of conditions used in these experiments, control, Ca-treated, and ADH-treated.

basis of the present results, this cannot be ascribed to saturation of the carrier system itself and appears to represent a property of the outward facing membrane. If the Na flux across this membrane saturates with increasing Na concentration, we must consider the possibility that Na entry into the transporting cell may involve more than simple passive diffusion. However, until further information regarding the nature of Na movement across this membrane becomes available, a definite conclusion cannot be made. It is possible that Na crosses the outer membrane, at least in part, by a mechanism such as facilitated or carrier-mediated diffusion (18) which could exhibit saturation effects. Alternatively, Na may diffuse passively and the permeability of the membrane may be altered by changes in Na concentration of the outside solution. Such an explanation would require that the permeability of the membrane increase with decreasing Na concentration. The observation that in sulfate Ringer's solution the outer membrane behaves as a nearly

perfect Na electrode (4) indicates that at least a part of the Na movement across this membrane must take place by free diffusion.

On the other hand, assuming that all the Na passes through the epithelial cells, a value for Na permeability of the inner membrane of 0.46×10^{-2} cm/hr. may be calculated from the values of Φ_{32} given in Fig. 3 and the Na concentration of the inside solution. This is less than P^o_{Na} by a factor of only 6. If this value relates to Na ions moving freely through this membrane, it seems unlikely that the membrane would behave as though it were impermeable to Na, but evidence from electrical measurements indicates that it does (4). However, this membrane of the cells would appear impermeable to free Na if Na outflux took place between the cells or if Na crossed the inner membrane from inside solution to cells in non-ionic form, perhaps by a mechanism of exchange diffusion. Thus, the present results suggest that further examination of Na movement across the two barriers in the skin might yield additional information concerning the over-all mechanism of transport.

APPENDIX A

Derivation of Equations Accounting for the Effect of Extracellular Space at the Inside of the Skin

We shall assume that in the system shown in Fig. 1, compartments 2 and 3 are separated by an extracellular space forming an extra compartment designated X. The movement of tracer in these compartments may be described by the following equations:

$$\frac{dP_X}{dt} = k_{2X}P_2 - k_{X3}P_X \tag{A-1}$$

$$\frac{dP_3}{dt} = k_{X3}P_X \tag{A-2}$$

in which k_{X3} is the rate constant for Na movement from compartment X to compartment 3. We have assumed that $k_{X2} \ll k_{X3}$; this is a reasonable assumption since there appears to be relatively little Na movement across the inner membrane in this direction (5). k_{2X} is equivalent to k_{23} in Equation 2 in that it represents transfer of Na from cell toward inside solution. In the text, we shall continue to designate this coefficient as k_{23}. From Equation 4 of the text,

$$P_2 = P_{2\infty}(1 - e^{-\lambda t})$$

in which $\lambda = k_{21} + k_{2X}$. Using this value, Equation A-1 becomes

$$\frac{dP_X}{dt} + k_{X3}P_X = k_{2X}P_{2\infty}(1 - e^{-\lambda t}) \tag{A-3}$$

For the initial conditions $P_X = 0$ at $t = 0$, Equation A-3 has the solution

$$P_X = k_{2X}P_{2\infty}\left[\frac{1}{k_{X3}} - \frac{1}{k_{X3} - \lambda}e^{-\lambda t} + \frac{\lambda}{k_{X3}(k_{X3} - \lambda)}e^{-k_{X3}t}\right] \tag{A-4}$$

Using this expression for P_X and the identity $k_{2X}P_{2\infty} = (dP_3/dt)_\infty$ for the steady state tracer flux, Equation A-2 becomes, after rearrangement

$$1 - \frac{(dP_3/dt)}{(dP_3/dt)_\infty} = \frac{k_{X3}}{k_{X3} - \lambda}e^{-\lambda t} - \frac{\lambda}{k_{X3} - \lambda}e^{-k_{X3}t} \tag{A-5}$$

At longer times, assuming that $k_{X3} > \lambda$, Equation A-5 may be written as

$$\ln\left[1 - \frac{(dP_3/dt)}{(dP_3/dt)_\infty}\right] = -\lambda t + \ln\frac{k_{X3}}{k_{X3} - \lambda} \tag{A-6}$$

which will give a result of the form shown in Fig. 2 when plotted semilogarithmically. According to Equation A-6, the line through the experimental points in this figure should have an intercept at $t = 0$ equal $\ln [k_{X3}/(k_{X3} - \lambda)]$. Since λ is known from the slope of the line, the value of k_{X3} can be calculated directly from the intercept. It should be noted that the presence of this extracellular space does not alter any of the calculations which have been described in the text since λ is determined from exactly the same relationship described for the simpler three compartment system (Equation 7 of the text).

APPENDIX B

Derivation of Equation 16

The model system of Koefoed-Johnsen and Ussing (4) suggests that Na diffuses passively across the outer membrane of the skin. The net flux of Na across this barrier, assuming that the potential difference may be neglected under conditions of short-circuiting, would be given by Equation B-1

$$\Phi_n = P^o_{Na}\{[Na]_o - [Na]_c\} \tag{B-1}$$

The net flux across the inner membrane, which must be equal to the net flux across the outer membrane would be, in our notation

$$\Phi_n = V_2k_{23}[Na]_c - P^i_{Na}[Na]_o \tag{B-2}$$

in which V_2 is the volume of the cellular compartment and P^i_{Na} is the permeability coefficient of the inner membrane for Na. In Equation B-2, we have used the condition, valid for our experimental conditions, that Na concentrations in the inner and outer solutions are equal. Solving these two equations simultaneously by elimination of $[Na]_c$ yields

$$\Phi_n = \frac{P^o_{Na}(V_2k_{23} - P^i_{Na})[Na]_o}{P^o_{Na} + V_2k_{23}} \tag{B-3}$$

which illustrates the dependence of net flux on permeability of the outer membrane if the other quantities in the equation remain constant.

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