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POTASSIUM REACCUMULATION BY ISOLATED FROG EPIDERMIS *

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Summary

The involvement of potassium in transepithelial sodium transport was tested by studying net potassium reuptake by potassium-depleted frog skin epidermis. Normal potassium content in half-strength Ringer's (0.244 μequiv/mg dry weight) fell 43% after 16 h in K-free medium at 5°C. Reaccumulation, against an electrochemical potential gradient, to 83% of the initial tissue potassium content occurred following incubation for 4 h at 22°C in K-containing medium. Sodium was required in the solution bathing the inside, but not the outside surface of the skin, for net potassium reaccumulation. Ouabain caused an additional potassium loss from potassium-depleted epidermis, but did not have the same effect on potassium-depleted isolated cells. Procaine, lithium and caffeine completely inhibited, antidiuretic hormone and cyclic AMP may partially inhibit and amiloride had no effect on potassium reaccumulation. In many cases decreases in sodium and water content were found to occur even in the absence of net potassium reaccumulation. The results suggest (1) potassium is actively transported into the epidermis, (2) this transport is not rigidly coupled to sodium extrusion or water loss, (3) potassium uptake is not rigidly coupled to transepithelial sodium transport, or only a small fraction is involved, (4) potassium diffusion is restricted in the extracellular space.

Introduction

The involvement of potassium in transepithelial sodium transport by frog skin was first demonstrated by Huf and Wills [1,2]. They showed that sodium

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transport was drastically reduced when potassium-free bathing solutions were used and that an Na-K exchange seemed to be involved. A role for potassium in transepithelial sodium transport in frog skin was postulated in the model of Koefoed-Johnsen and Ussing [3]. Implied in the original model was the notion applied to the red cell by Tosteson and Hoffman [4] that the Na/K active transport mechanism regulated cell volume. Since then red cell volume regulation has been shown to be more complex and not necessarily linked to the ouabain inhibitable Na/K active transport system [5]. In frog skin evidence on the role of potassium in cell volume regulation has not been available primarily because the corium in intact frog skin presented a large potassium pool unrelated to transepithelial Na transport. Development of large sheets of isolated epidermis [6] has now made possible a reexamination of the role of potassium in transepithelial sodium transport and in the maintenance of cell volume.

Many attempts have been made to measure potassium fluxes across the basolateral border and to establish correlation with transepithelial sodium transport. Such studies involved analysis of tracer fluxes which were complicated by the presence of the corium [7,8]. Biber et al. [9] studied fast uptake of ^{42}K from the solution bathing the inside surface of the epidermis of the frog skin which had been separated from the corium. However, these isotope movements may involve only isotope exchange and could be unrelated to net movements. We have chosen to study net potassium uptake by the isolated epidermis by measuring net changes in cellular ionic content.

Net loss and reaccumulation of cell potassium has been demonstrated in Ehrlich ascites tumor cells [10], rat and guinea pig renal cortical slices [11,12] and in rat renal medullary slices [13]. Loss of tissue potassium can be induced by incubation in potassium-free medium at reduced temperature in these tissues. Subsequent reincubation at room temperature in the presence of potassium allows net reaccumulation of potassium. The present study employs similar methods to explore the following three issues. What is the relationship between cell sodium and potassium, and is active transport of potassium involved? What is the relationship between potassium uptake and transepithelial sodium transport? How is potassium involved in the regulation of cell volume?

Methods

Leopard frogs, *Rana pipiens* (northern variety) Mogul-Ed (Oshkosh, Wisc.) and Bay Biological (Port Credit, Ontario, Canada) were doubly pithed and abdominal skins were dissected. Large sheets of epidermis were isolated by the method of Hoshiko and Parsons [6]. Briefly, the method is as follows. The inside or corium surface was lightly scored with a scalpel in a grid-like pattern with successive strokes 2—3 mm apart. This inside surface was then exposed to half-strength sulfate Ringer's (Na, 55.0; K, 5.0; Ca, 0.5; SO_4, 33.0; Tris, 5.0 mM/l; titrated to pH 7.8 with H_2SO_4) containing 80 U/ml of trypsin (Worthington), for a period of 45 min in winter frogs and 30 min in summer frogs. The trypsin solution was then discarded and the epidermis was teased free of the corium.

In order to study the potassium depletion process the isolated epidermis was divided along the axis of symmetry of the animal. One-half was immediately

analyzed to determine pre-incubation sodium, potassium, and water contents, and, in some cases, extracellular space. The other half was subjected to the depletion procedure and was then similarly analyzed. The isolated epidermis was depleted of its normal content of potassium by incubation in 30 ml of a potassium-free medium (Na, 60; Ca, 0.5; SO_4, 33; Tris 5 mM/l; pH 7.8) for 16 h at 5° C. The epidermis was spread out in the bottom of the beaker so as to prevent curling and to retain orientation of the inside and outside surfaces. Compressed air saturated with water was used to bubble the solutions gently so as not to disturb the position of the tissue.

Reaccumulation of tissue potassium was studied by first depleting both halves of an isolated epidermis as described above. At the end of the depletion period one-half was immediately analyzed to establish the ionic content before reaccumulation was allowed to occur. The other half was incubated for 4 h at 22° C in a beaker containing 30 ml of a solution identical to that used for depletion, but having 1.0 mM potassium substituted for 1.0 mM sodium.

To study the effect of sodium and drugs on reaccumulation the epidermis was sandwiched between nylon mesh and mounted in an Ussing chamber [14]. The mesh protected the tissue against rupture by temporary hydrostatic pressure gradients. Petroleum jelly ensured a waterproof seal at the edges while avoiding edge pressure. Each half chamber, with its attached reservoir, contained 5 ml of sulfate Ringer's and was equipped with current and potential bridges.

In drug experiments the epidermis from a single frog was divided into three parts. One part was analyzed for ion and water contents after each of the following procedures: depletion, the normal reaccumulation in a beaker, and after reaccumulation in chambers in the presence of the agent.

The electrical potential difference across an epidermis mounted in the Ussing chamber was recorded on a strip chart recorder. Calomel electrodes were connected to the chambers through bridges of 3% agar in 3 M KCl. No corrections were made for junction potentials which were checked before each experiment and found to be less than 1 mV.

All chemicals were reagent grade and solutions were made with glass distilled water. Pharmacological and biochemical agents and their sources were as follows: Amiloride HCl, from Merck, Sharp and Dohme Research Lab; ouabain, from Nutritional Biochemical Company; procaine base, from Matheson, Coleman and Bell; caffeine hydrate from Mann Research Laboratories Incorporated; 2-deoxy-D-glucose (glucose-free) and dibutyryl cyclic adenosine 3′-5′ monophosphate (dibutyryl cyclic AMP) from Calbiochem; Pitressin (antidiuretic hormone) from Parke Davis Company. Tritiated mannitol (>3 Ci/mM) was obtained from New England Nuclear Corporation in 1 : 1 water/ethanol.

Isolated cell experiments

Isolated epithelial cells were prepared from sheets of isolated epidermis following depletion by a procedure adapted from that of Swallow and Sayers [15]. The potassium-depleted epidermis from three frogs was placed in 30 ml of a trypsin dispersion medium at 22° C and stirred (400—500 rev./min) with a glass paddle for 2 h. This medium consisted of 250 mg of lyophilized trypsin (Worthington) per 100 ml of the K-free depletion medium. All glassware which

came in contact with the cell suspension was siliconized. The cell suspension was divided among four conical-bottom tubes and centrifuged (1000 rev./min) for 10 min. One tube, to which 25 μCi of tritiated mannitol had been added prior to centrifugation, was analyzed immediately. The remaining three aliquots were resuspended in appropriate experimental media for 4 h. The resuspension media contained 50 mg per 100 ml lima bean trypsin inhibitor (Worthington) and 500 mg per 100 ml bovine serum albumin (Sigma), in order to keep the cells dispersed. For analysis the cells were spun down, resuspended in a small volume of distilled water, transferred to a tared Vycor crucible and analyzed as described below. Tritiated mannitol was added to the experimental cell suspension before centrifugation. Light microscopy verified that these procedures yielded mainly single cells and aggregates of a few cells.

Analysis

Following incubation the epidermis was blotted with moist filter paper, placed in a tared Vycor crucible and immediately weighed. In the case of chamber mounted epidermis the exposed area was blotted, punched out with a cork borer and weighed. Following overnight drying at 110° C the crucible and epidermis were reweighed to determine tissue dry weight. 2 ml of 0.1 M HNO_3 was added and the crucible gently shaken for 2 h. In some cases an aliquot of the extract was taken to determine the mannitol space. The remaining extract was dried overnight on a hot plate at a surface temperature of 120° C. The residue was resuspended in lithium diluent, and sodium and potassium were determined by flame photometry using the lithium internal standard method. Bathing media Na and K were routinely analyzed.

Counting samples were assayed in a Packard Tri-Carb 3000 liquid scintillation counter using a commercial scintillant (Scintisol or Insta-gel). Quench rates were determined by internal standardization and tissue sample efficiencies were determined to be 2 to 15%.

Data reduction

Derived quantities were determined according to the following equations:

$$ECS = N \cdot Z \cdot \frac{T(\text{cpm})}{M(\text{cpm})} \cdot \frac{M'(\text{cpm})}{T'(\text{cpm})}$$

$$I = TC/D$$

$$Y = (TC - ECS \cdot C)/D$$

$$H_2O = (W - D)/W$$

where ECS is the extracellular space volume, N is the correction factor required because only part of the tissue sample is used in the ECS determination, Z is the volume of the aliquot of the bathing medium, T(cpm) and M(cpm) denote the counts per min in the tissue and the bathing medium samples, respectively. Primes indicate the increment in these values after addition of the internal standard. I and Y are the tissue contents per mg of dry weight, the latter having an extracellular space correction. TC is the total tissue content for a given ion, C is the concentration of the ion in the bathing medium, D denotes the dry weight

in mg of the tissue, H_2O is the tissue water content and W the wet tissue weight in mg.

Student's t-test was used for two sample comparisons. For multiple comparisons analysis of variance and Tukey's test [16] were employed. Linear correlation coefficients were determined by a least squares fit and their significance evaluated by an iterative method [17].

Microelectrode experiments

Microelectrodes were pulled from capillary tubing (Pyrex 7740). They were filled by boiling in methanol at reduced pressure after which they were transferred first to distilled water then to 3 M KCl. Microelectrodes with resistances of 25—45 MΩ were used. The epidermis was mounted horizontally in an open-topped chamber, inside up, supported from below by a nylon mesh. Both sides of the tissue were bathed by K-free medium. The microelectrode was advanced from the inside to the outside of the epidermis using a micro-drive unit. Reference electrodes were present on both sides of the epidermis and short-circuiting could be accomplished through a separate pair of electrodes. Microelectrode position was observed through a binocular microscope until contact with the epidermis was achieved. Thereafter position was determined by the calibrated drive unit. The microelectrode was advanced in steps of 2—10 μm.

Results

As shown in Fig. 1, 16 h incubation in potassium-free depletion medium at 5°C causes a loss of tissue potassium to a level that is 57% of that found in fresh, isolated epidermis. This loss of potassium is accompanied by an increase in tissue sodium and water content.

Fig. 1 also indicates that tissue potassium content is restored to 83% of the fresh epidermis value when a potassium-depleted epidermis is reincubated for 4 h at 22°C in reaccumulation medium which contains 1 mM/l potassium. There is a concurrent fall in tissue sodium and water content. If potassium is

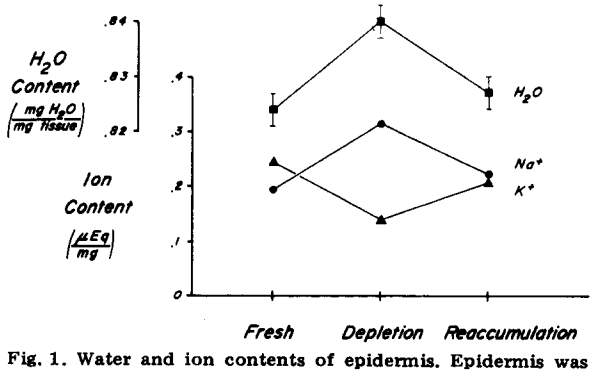


Fig. 1. Water and ion contents of epidermis. Epidermis was isolated and immediately analyzed to give "fresh" values (n = 21). "Depleted" epidermis was incubated for 16 h in K-free medium at 5°C (n = 138). "Reaccumulation" values were obtained from epidermis following the above treatment and subsequent incubation for 4 h at 22°C in medium containing 1 mM/l K (n = 124). Ion contents are given as μequiv/mg dry tissue weight. Water content is given as mg H_2O/mg wet tissue weight. Error bars indicate one S.E.M; if missing, the error was smaller than the size of the symbol.

distributed uniformly throughout the cells of the epidermis [18] and within each cell, then the data indicates that reaccumulation proceeds against a concentration gradient of at least 25 : 1. For reaccumulation against such an apparent concentration gradient to be passive, the electrical potential difference would have to be at least 84 mV, cell negative. Despite recent reports of large potential differences across the mucosal border of frog skin [19,20] we have not observed comparable potentials in potassium-depleted epidermis.

Microelectrode experiments were performed on K^+-depleted, isolated epidermis under open circuit conditions. Measurements were accepted if the offset potential had changed less than 1 mV, the microelectrode impedance had changed less than 1 MΩ during the puncture and if there was an abrupt change in potential which did not quickly decay. Typically, potentials were stable for at least 2 min. Table I gives the results of seven successful punctures in three pieces of isolated epidermis. The mean cellular potential recorded was −16.7 mV. Short-circuiting the epidermis did not lead to a detectable change in cellular potentials probably due to the low trans-epithelial potentials (mean 5 mV when mounted for micropuncture). Even allowing for the considerable scatter the potentials do not approach the calculated potassium equilibrium potential.

Taken together with the calculated tissue concentration of potassium and the known concentration of potassium in the bathing medium, the data from the microelectrode experiments suggests that potassium reaccumulation occurs against an apparent electrochemical potential gradient of 67 mV (84—17 mV). Therefore, the observed potassium reaccumulation would seem to be an active process. Potassium reaccumulation seems to occur with potassium at the inside surface only (ΔK = +0.010 μequiv/mg, n = 2) but not at the outside surface only (ΔK = −0.55 μequiv/mg, n = 3). Thus the K reaccumulated probably comes from the inside bathing solution exclusively.

Energy requirements

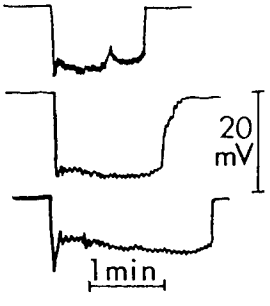
Since all of the experiments described above were carried out with no exo-

TABLE I

MICROELECTRODE DATA

The table gives the intracellular potential (mV) 1 min after puncture with the microelectrode. Some typical tracings are shown along with calibration bars: Horizontal, 60 s, vertical, 20 mV.

Puncture	Cell potential (mV)	Typical records
1	− 7.6	
2	− 7.3	
3	−13.7	
4	−31.4	
5	−11.9	
6	−10.6	
7	−34.7	



genous substrate, it was apparent that reaccumulation must be fueled by a stored energy supply. Endogenous glycogen falls during overnight incubation of frog skin and glucose will then stimulate short circuit current [21]. This raised the question of whether reaccumulation could be stimulated by the addition of substrate. The effect of glucose on potassium reaccumulation is shown in Fig. 2A. Although significant reaccumulation of tissue potassium occurred in both the control and the glucose-treated epidermis there were no significant differences in any quantity measured between these two cases. Atypically, water content did not fall during the reaccumulation period in this experiment. This may be due to the fact that post-depletion water contents were abnormally low. Since the same conditions existed for reaccumulation with and without glucose, this should not invalidate the comparison. Thus, in contrast to the stimulation of short-circuit current in substrate-depleted skins caused by glucose, glucose does not augment potassium reaccumulation.

The metabolic inhibitor 2-deoxy-D-glucose was used to further explore the

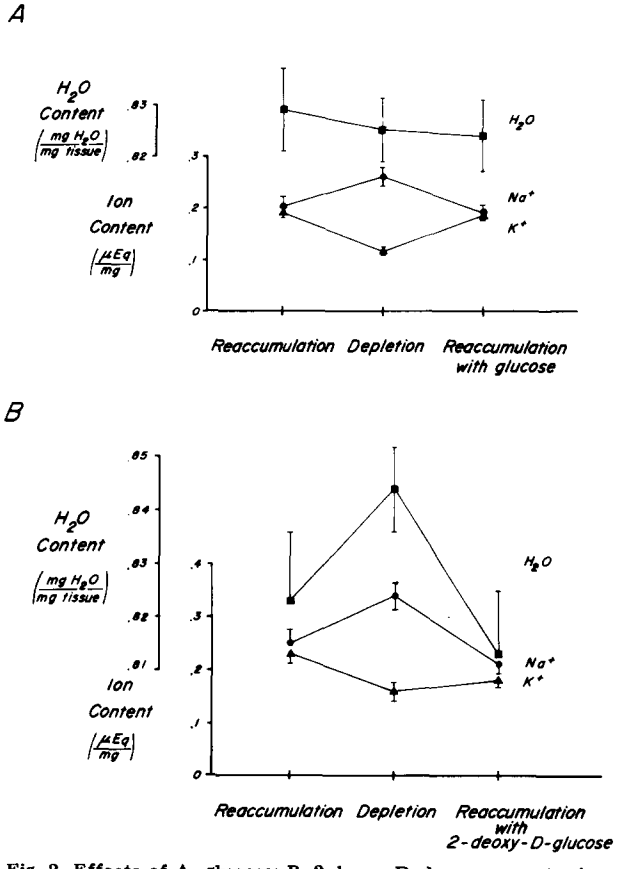


Fig. 2. Effects of A, glucose; B, 2-deoxy-D-glucose on potassium reaccumulation. The figures indicate the change in tissue content of sodium, potassium and water. "Depletion" and "Reaccumulation" values are determined as for Fig. 1. The points at the right of each figure indicate the tissue contents following incubation as for normal reaccumulation with either (A) 10 mM/l glucose, (n = 12), or (B) 10 mM/l 2-deoxy-D-glucose (n = 12) added to the medium.

energy requirements of reaccumulation. 2-deoxy-D-glucose (10 mM/l) was applied to both sides of the epidermis in Ussing chambers. Fig. 2B indicates that potassium uptake is abolished by this drug. However, extrusion of tissue sodium and water are seen despite inhibition of potassium reaccumulation ($P < 0.001$).

Sodium dependence

The dependence of the reaccumulation process on sodium was explored in a series of experiments in which pieces of epidermis were mounted on Ussing chambers. Sodium-free reaccumulation medium, in which Tris was substituted mol for mol for sodium, bathed the outside only, the inside only, neither side or both sides. The sodium concentration of the bathing medium on the sodium-free side measured at the end of the reaccumulation period was less than 2.5 mequiv/l, except for one case in which the "inside" sodium concentration rose to 4.5 mequiv/l. Excluding data from this skin does not alter the results.

Table II shows the unexpected result that although potassium reaccumulation is not as great in the absence of sodium bathing the outside surface of the skin, there is no detectable reaccumulation of potassium in the absence of sodium at the inside surface. When sodium was present at neither surface the potassium content actually decreased during the reaccumulation period. Since net reaccumulation of potassium reflects an imbalance between influx and outflux either an increased outflux or a decreased influx could account for diminished reaccumulation. Other experiments presented below show that the potassium uptake mechanism appears to be extremely effective in retaining potassium that has leaked into the extracellular space. Thus, it would seem that decreased reaccumulation in the absence of "inside" sodium is primarily due to an effect on the active potassium uptake mechanism.

The final intracellular sodium concentration in the pieces of epidermis with no sodium outside was actually lower than the final sodium concentration of pieces of epidermis with no sodium "inside". Therefore, the poorer potassium

TABLE II

SODIUM DEPENDENCE OF POTASSIUM REACCUMULATION

The values indicate the change in tissue ion and water content when potassium-depleted epidermis was exposed to sodium-free medium at the surfaces indicated. The standard error of the mean and significance levels are indicated. N.S., not significant; n, number of pieces of epidermis.

	Change in sodium content (μequiv/mg dry wt.)	Change in potassium content (μequiv/mg dry wt.)	Change in water content (mg H_2O/mg tissue)
Sodium both sides $n = 10$	−0.123 ± 0.023 $P < 0.005$	+0.083 ± 0.020 $P < 0.005$	−1.8 ± 0.4 $P < 0.001$
No sodium outside $n = 8$	−0.179 ± 0.028 $P < 0.005$	+0.042 ± 0.014 $P < 0.01$	−0.8 ± 1.2 N.S.
No sodium inside $n = 10$	−0.115 ± 0.059 N.S.	−0.017 ± 0.011 N.S.	−0.9 ± 0.6 N.S.
Sodium neither side $n = 8$	−0.240 ± 0.031 $P < 0.005$	−0.047 ± 0.008 $P < 0.001$	+2.4 ± 1.0 N.S.

reaccumulation in the latter case cannot be attributed to a lack of cellular sodium available for exchange with potassium.

The water content of epidermis after the reaccumulation period is not significantly lower than after depletion if sodium is not present in one of the bathing media. When sodium is present at both surfaces there is a loss of tissue water during the reaccumulation ($P < 0.001$) as was seen when reaccumulation was allowed to occur in beakers. With no sodium in either solution there is an actual increase in water content ($P < 0.025$). This may reflect a net uptake of Tris, which was not analyzed.

Drug effects

A number of compounds with known effects on sodium transport were used to test for correlation between sodium transport and potassium reaccumulation. The modifiers may be grouped according to their effects on short circuit current. The inhibitors ouabain, procaine and amiloride will be discussed first, followed by two substances which have equivocal effects on short-circuit current under the conditions used; amiloride at the inside surface and lithium at the outside surface. Finally the stimulators antidiuretic hormone, dibutyryl cyclic AMP, and caffeine will be considered.

Unless otherwise stated all of the experiments on modifiers were performed by treatment of an epidermis mounted in the Ussing chamber and the control piece incubated in a beaker during the reaccumulation period. In control experiments no difference was detected between tissue contents after reaccumulation in chambers ($n = 10$) or beakers ($n = 124$). The probabilities that the mean contents are not different are: sodium ($P > 0.3$), potassium ($P > 0.3$) and water ($P > 0.1$).

Table III shows the changes in tissue ion and water uptake of K^+-depleted epidermis in response to the sodium transport modifiers. In testing for an oua-

TABLE III

CHANGE IN TISSUE CONTENTS IN RESPONSE TO SODIUM TRANSPORT MODIFIERS

The values in the table represent the difference between the change in tissue content following exposure to the modifier and the change in tissue content following normal reaccumulation. It, thus, represents the excess gain or loss attributable to the action of the modifier. Significance levels are indicated as follows: $P < 0.05$ *, $P < 0.01$ **.

Modifier	No. of skins	$\Delta K_{experimental} - \Delta K_{control}$ (μequiv/mg)	$\Delta Na_{experimental} - \Delta Na_{control}$ (μequiv/mg)	$\Delta H_2O_{experimental} - \Delta H_2O_{control}$ (mg H_2O/mg tissue)
Ouabain	6	−0.171 **	+0.182 **	+0.014
Procaine	12	−0.154 **	+0.041	−0.006
Amiloride: outside	10	+0.009	−0.077 *	−0.016
Amiloride: inside	11	+0.009	−0.028	−0.001
Amiloride: both sides	8	−0.010	−0.098 **	−0.021 **
Lithium: outside	10	−0.080 **	−0.092 **	−0.034
Antidiuretic hormone:				
chambers	10	−0.037 **	−0.023	−0.017 **
beakers	7	+0.010	+0.018	+0.014
Cyclic-AMP	4	−0.035	−0.002	−0.014
Caffeine	6	−0.063 **	−0.001	−0.011

bain effect on reaccumulation, pieces of epidermis were exposed to 0.1 mM/l ouabain in beakers. Ouabain induces a greatly accelerated loss of potassium, despite the fact that the bathing medium contains 1 mM/l potassium. There was a concommitant rise in sodium content with ouabain treatment, but the sum of sodium and potassium contents was not significantly altered. While the mean water content increased significantly in the ouabain-treated skin, an increase of marginal significance was also seen in the water content during normal reaccumulation in this experiment.

In contrast, 10 mM/l procaine applied to both sides of the epidermis caused a significant loss of tissue potassium with no difference in sodium or water content from the values found in controls allowed to reaccumulate potassium normally.

Amiloride had no effect on potassium reaccumulation. At a concentration of 0.1 mM/l at either the outside only, the inside only or both sides, there was no detectable difference in potassium reaccumulation. When amiloride had access to the outside surface, tissue sodium and water content decreased, but these quantities did not change when amiloride had access to the inside only.

Lithium, substituted for sodium at the outside surface, induced a loss of sodium, total cation and water content which was consistent with the losses seen when Tris substituted for sodium at that border. However, lithium completely blocked potassium reaccumulation in contrast to Tris substitution which allowed a significant reaccumulation of tissue potassium.

Both antidiuretic hormone (pitressin, Parke Davis 100 U/l, inside only) and dibutyryl cyclic AMP (0.1 mM/l, both sides) caused a decrease in potassium reaccumulation, but not to the levels of the depleted epidermis. As a check, an experiment was performed to determine the effects of antidiuretic hormone in beakers on potassium reaccumulation. Under these conditions there was no effect on any quantity determined. Since no difference has been observed between tissue contents following potassium reaccumulation in beakers or chambers under control conditions, this effect may be unique to the antidiuretic hormone-treated epidermis, which probably has an increased water permeability.

In contrast to the lack of effect exerted by antidiuretic hormone and dibutyryl cyclic AMP, caffeine (10 mM/l, both sides), which is thought to stimulate sodium transport by inhibiting phosphodiesterase [22], completely blocks the normal reaccumulation of tissue potassium. Caffeine does not appear to affect sodium extrusion or to inhibit water loss.

Isolated cell experiments

Pieces of epidermis that are left in potassium-free Ringer's at room temperature for 4 h following the depletion procedure do not lose additional potassium in significant amounts (Table IV, column 1). The potassium retention was unexpected in view of the fact that ouabain-treated, potassium-depleted epidermis loses additional potassium at a higher rate than that found during normal depletion. There appeared to be two possible explanations for these results. First, ouabain could increase the permeability of the epithelial cells to sodium and potassium. Second, the epithelial cells may be very efficient at retaining the potassium that is lost into the restricted extracellular space and unstirred layers

TABLE IV

ISOLATED CELL EXPERIMENTS

Mean tissue potassium content (μequiv/mg dry wt.) ± S.E.M. are given for pieces of isolated epidermis following the incubation period indicated. *n*, number of pieces of epidermis. Depletion, 16 h, 5°C, K^+-free medium. Reaccumulation, 4 h, 22°C, 1 mM/l K^+, following depletion. Reaccumulation with ouabain, same as reaccumulation with 10^{-4} M/l ouabain. Continued depletion, 4 h, 22°C, K^+-free medium, following depletion.

	Isolated epidermis (μequiv/mg dry wt.)	Isolated cells (μequiv/mg dry wt.)
Depletion	0.108 ± 0.003 *n* = 17	0.052 ± 0.009 *n* = 6
Reaccumulation	0.197 ± 0.007 *n* = 6	0.098 ± 0.010 *n* = 6
Reaccumulation with ouabain	0.026 ± 0.008 *n* = 6	0.041 ± 0.003 *n* = 6
Continued depletion	0.112 ± 0.007 *n* = 11	0.034 ± 0.005 *n* = 6

adjacent to the skin. If this were so, then a ouabain-treated skin would lose potassium and gain sodium since its pump would be blocked. The untreated epidermis would be able to retain potassium even in potassium-free solutions.

In order to test these two possibilities experiments were performed on isolated cells. This preparation effectively eliminates the diffusion restrictions of the normal extracellular space (unstirred layers remain). Aliquots of potassium-depleted cells were suspended in reaccumulation medium, in reaccumulation medium with 0.1 mM/l ouabain and in depletion medium. The fourth aliquot was analyzed at the beginning of the reaccumulation period (i.e., immediately after depletion and isolation). Table IV, column 2 gives the results. The first point to note is that cells incubated for 4 h in reaccumulation medium contain significantly more potassium per mg of dry weight in the intracellular compartment than do those that were assayed after depletion. This indicates that the isolated cells are able to reaccumulate potassium as does the isolated epidermis.

If ouabain alters cellular permeabilities then the ouabain-treated cells should have a much lower potassium content than either the potassium-depleted cells or those which were exposed to potassium-free solutions during the reaccumulation period. However, the figure clearly shows that the potassium content of the ouabain-treated cells is not significantly different from either of these. In fact, the mean is actually higher than that for the cells which were depleted for an additional 4 h. All three of these potassium contents are significantly lower ($P < 0.01$) than that of the cells reincubated in normal reaccumulation medium. In general, the potassium content of the isolated cells is much lower than in depleted epidermis. Although these lower contents (expressed per mg dry weight) may be due to problems with the isolated cell preparation such as cellular debris or highly permeable, disrupted cellular junctions they are not unexpected in view of the low potassium content of intact epidermis treated with ouabain. The mean tissue potassium content of intact, potassium-depleted epidermis after reincubation in reaccumulation medium with ouabain was 0.026

μequiv/mg dry weight. If potassium loss is retarded by the restricted extracellular space then it would not be surprising that isolated cells exposed to a potassium-free medium would have a potassium content approaching the value after ouabain.

A number of studies have shown that ouabain either causes no change in passive ion fluxes or actually reduces ionic permeability [23—26]. In favor of a restricted extracellular space, many reports have indicated that inaccurate results may be obtained due to recycling of potassium within the tissue [25,27, 28].

Thus, it is reasonable to expect that potassium may be recycled to a significant extent by the cells of the epidermis. These results indicate that extreme caution must be exercised in interpreting the results of radiopotassium flux studies in intact epidermis. In particular, quantitative estimates of the coupling coefficient between K^+ uptake and sodium transport must be carefully evaluated. In addition, experiments showing continued maintenance of sodium transport in the absence of serosal potassium cannot be used to discount the presence of a Na-K pump at the serosal border.

Discussion

Cellular potassium and the inside bathing solution

It has been shown that potassium can be taken up against a large concentration gradient, probably by active transport. Potassium reaccumulation requires sodium at the inside surface but not at the outside surface of the epidermis. Hurlbut [29] has shown that the desheathed frog-nerve preparation requires sodium in the solution bathing the nerve in order for net potassium uptake to occur. Both frog nerve and epidermis are thought to possess a sodium-potassium ATPase responsible for transport. In both cases the observed sodium requirement is at the surface from which potassium is normally pumped. Though a sodium requirement at the inside border is not postulated by the Koefoed-Johnsen and Ussing [3] or any other model, it has been previously suggested. Rabito et al. [30] reported that the composition of the inside bathing solution drastically affects the electrical parameters in frog skin and concluded that lack of sodium in the inside bathing solution leads to an inhibition of sodium transport across the tissue. Hoshiko and Parsons [31] have also observed that the absence of sodium at the inside surface of isolated frog epidermis abolishes short-circuit current.

The results of the present study and those just cited point to a vital role for sodium at the inside border of the skin. This ion is required for the maintenance of cellular contents and transepithelial transport. However the mechanism for this requirement is not at all clear.

Role of K^+ in transepithelial Na^+ transport

The effects on potassium reaccumulation of agents known to modify sodium transport can be determined with reference to Table V. No increase in potassium reaccumulation above control levels can be seen with the transport stimulators glucose (see Results), antidiuretic hormone, or dibutyryl cyclic AMP. Caffeine actually inhibited the reaccumulation process. Conversely, the sodium

TABLE V

CHANGE IN CELL CONTENTS IN RESPONSE TO TRANSPORT MODIFIERS

The change in the mean ionic and water contents are given for both the control conditions and experimental conditions for each experiment (one drug effect = one experiment). All ion contents are given as μequiv/mg dry weight, while water contents are given as mg water per mg wet tissue weight, times 100.

	Controls				Experimental			
Modifier	ΔNa	ΔK	ΔCation	ΔH_2O	ΔNa	ΔK	ΔCation	ΔH_2O
Glucose	−0.069	+0.077	+0.008	+0.4	−0.084	+0.070	−0.014	−0.1
2-Deoxy-D-glucose	−0.096	+0.055	−0.041	−2.1	−0.116	+0.009	−0.107	−3.4
Ouabain	−0.068	+0.089	+0.019	+3.9	+0.116	−0.082	+0.030	+5.2
Amiloride:								
outside	−0.105	+0.048	−0.057	−1.8	−0.182	+0.055	−0.125	−3.4
inside	−0.128	+0.038	−0.090	−3.0	−0.156	+0.047	−0.109	−3.1
both sides	−0.106	+0.086	−0.020	−1.4	−0.204	+0.076	−0.128	−3.6
Procaine	−0.144	+0.072	−0.072	−1.9	−0.103	−0.082	−0.021	−2.5
Antidiuretic hormone:								
chambers	−0.143	+0.057	−0.086	−2.6	−0.165	+0.020	−0.145	−4.3
beakers	−0.064	+0.055	−0.009	−0.5	−0.046	+0.065	+0.019	+0.9
Cyclic AMP	−0.067	+0.082	+0.015	+1.0	−0.069	+0.047	−0.022	−1.3
Caffeine	−0.062	+0.051	−0.011	−2.1	−0.063	−0.012	−0.051	−3.3

transport inhibitor amiloride exposed to the outside or both sides of the tissue did not inhibit potassium reaccumulation. It is clear that the modifiers tested do not have the same effect on sodium transport and potassium reaccumulation.

It is tempting to conclude that potassium uptake and sodium extrusion across the serosal border are not coupled. Such a conclusion, however, would be an oversimplification. The fraction of total tissue ions involved in the transport process is not a well defined quantity.

A number of lines of evidence indicate that the entire epidermis may be involved in transport. The microelectrode investigations of Ussing and Windhager [32] suggested intercellular junctions offering low resistance to the passage of ions. Farquahar and Palade [33] demonstrated the existence of intercellular junctions anatomically. Based on measurements of the low frequency electrical impedance Smith [34] concluded that all cells are electrically coupled. Recent investigations employing microelectrodes [35] and electron microprobe analysis [36] are in accord with these views. Even Voute and Ussing [37] whose first reacting cell layer data is frequently quoted by those who believe that transport is accomplished by only a fraction of the cells, recognized that their evidence is not conclusive on this point and so state in their paper.

Numerous studies have been aimed at determining the size of the "sodium transport compartment". Most of these investigations have been performed using tracer kinetics or steady-state tracer equilibration. As techniques and preparations have been refined the data has indicated ever smaller transport compartments. Earlier studies found sodium transport compartments of about 0.1 $\mu equiv/cm^2$ [38,39], while later studies have found the same compartment to

be about one order of magnitude lower [40,41]. Such findings led Cereijido and Rotunno [42] to propose that the sodium transport compartment is not intracellular at all, but represents sodium bound to sites on the surface of the cell membrane. Their hypothesis does not postulate an active involvement of potassium in transport.

Since the size of the transport compartment is not known, the failure to observe a correlation between the effects of transport modifiers on potassium reaccumulation may mean (1) potassium is not involved in sodium transport, or alternatively, (2) the amount of potasssium is too small to be detected by analysis of the whole epidermis. Compartmentalization is a problem in studies of ionic contents because there is most certainly a division of cellular ions among various cellular constituents as well as among different cell types [43,44]. Electron microprobe analysis [18] suggests that potassium concentration increases as one progresses from the outside to the inside of the skin from 100 to 150 mequiv/l cell water. In isolated frog epidermis, Zylber et al. [45] have shown that 96% of tissue potassium exchanges with a single time constant, the remainder being in a much faster compartment. If the 4% of cell potassium in the fast compartment is associated with a small sodium transport compartment, then sodium-potassium exchange might go undetected in our investigations. However, if this fast compartment represents extracellular potassium, as the authors suggest, and the large potassium compartment is involved in transport, then our methods could detect a significant correlation between effects on sodium transport and potassium reaccumulation. Therefore, either potassium uptake is not associated with sodium transport or only a small compartment of the epithelium is involved in that transport. Such a compartment would have to be smaller than the detectable content difference measurable in these experiments, about 0.040 μequiv/mg dry weight, or about 15% of the content of fresh skins equilibrated with half-strength sulfate Ringer's.

Zerahn [46] has proposed that potassium may be expelled when lithium is accumulated by cells of the skin. In previous studies, tissue analysis has not yielded sufficiently consistent results to determine whether potassium loss had occurred [46,47]. Comparison of Table II and III shows that when lithium replaces sodium at the outside surface potassium reaccumulation does not occur. Tris substitution for sodium does not block reaccumulation. Lithium seems to have been taken up at the expense of potassium as Zerahn proposed. It should be noted that the effects of lithium are extremely complex. Potential and impedance oscillations lasting hours may be induced by lithium [48]. Lithium has been reported to decrease [49] or cause no change in normal short-circuit current [50].

It is of interest to note that no agent was capable of stimulating potassium reaccumulation. In studies of toad urinary bladder Robinson and Macknight [51] have found that although they could depress ^{42}K uptake with amiloride, antidiuretic hormone had no effect. Biber et al. [9] however, did find that antidiuretic hormone stimulated fast uptake of ^{42}K across the serosal border of frog skin.

Cell volume regulation

Water content increases when the isolated epidermis is incubated at 5°C

during potassium depletion and then decreases again when rewarmed during reaccumulation. It is striking that these water content changes and parallel changes in sodium content are found whether or not potassium reaccumulation has occurred. Ouabain appears to be a notable exception to this rule. A test for linear correlation of the mean values of sodium, potassium and total cation contents with the mean water contents was performed. For the experimental reaccumulation data there is a high degree of correlation between the change in total cation content and water content ($r = 0.833$, $P < 0.002$). There is no correlation between the degree of potassium reaccumulation and the change in water content ($r = 0.346$, $P > 0.297$). It would seem reasonable to assume that the water content is governed by the sodium content of the tissue, and indeed a strong correlation is evident ($r = 0.904$, $P < 0.0001$).

Such findings are not consistent with the Koefoed-Johnsen and Ussing model which postulates that sodium transport, potassium uptake, and by inference, cell volume are all regulated by the same coupled sodium-potassium pump. The present data may be interpreted to mean that cell volume can be maintained independently of net potassium uptake, suggesting that these two processes involve different transport mechanisms.

In the preceding paragraph the assumption that tissue water content reflects cell volume was tacitly made. Based on a mean extracellular space of 1.4 μl/mg dry weight (mannitol space measured in this study) a simple calculation from the data of Fig. 1 shows that in order for such a change to be entirely extracellular the volume of that compartment would have to change nearly 50%. It seems unlikely that such large changes would occur without a concommitant change in cell volume.

The increase in water content with ouabain treatment may represent an exception to the rule. In this case there was a large, significant ($P < 0.01$) increase in water content, (Table V). There was also a marginally siginificant ($P < 0.05$) increase in water content after normal potassium reaccumulation in this particular experiment, causing the results to be somewhat equivocal.

Huf et al. [52] have suggested that transepithelial sodium transport may be a separate process from maintenance of electrolyte concentrations (and thereby cell volume) in cells of the frog skin. They showed that sodium transport could be stimulated or depressed independent of detectable changes in whole skin sodium and potassium content. Recent computer simulation of this model demonstrates that such independent effects can occur, and stresses the need for studies on isolated epidermis [53].

Sodium and volume regulation independent of Na-K exchange has been reported in other tissues. Slices of guinea pig renal cortex can be depleted of potassium by incubation at 0.6°C in potassium-free solution [12]. When rewarmed without potassium, sodium and chloride were expelled along with water. When potassium was added it was taken up in exchange for sodium. The sodium extrusion could be inhibited by ethacrynic acid while Na-K exchange was ouabain inhibitable. Macknight et al. [54] have not found a relationship between cell volume and either cell potassium content or transepithelial sodium transport in toad bladder. In frog epidermal cells tracer flux studies have revealed additive inhibition of sodium extrusion by ouabain and ethacrynic acid [45]. In view of these studies and the present data we suggest that two

pump systems are operative, one functioning in sodium-potassium exchange and a second that can expel sodium independent of potassium uptake, thus regulating cell volume.

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