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SODIUM UPTAKE BY FROG SKIN AND ITS MODIFICATION BY INHIBITORS OF TRANSEPITHELIAL SODIUM TRANSPORT

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SUMMARY

1. The suitability of inulin and mannitol as markers of the diffusional space that exists between the surface of frog skin and the outer barrier to sodium transport has been compared in experiments designed to measure the rapid uptake of sodium.

2. Inulin and mannitol both equilibrate finally with the same space at the outer surface of the frog skin, but the rate at which this equilibration occurs is considerably faster for mannitol.

3. The dependence of the rapid uptake of sodium on the concentration of sodium in the external medium, using mannitol to correct for extracellular sodium, can be described by simple saturation kinetics with an apparent K_m for sodium entry of 24 mM and a maximal rate of uptake of 1·28 μequiv/cm^2.hr.

4. The effect of amiloride is to inhibit sodium uptake, the degree of inhibition depending both on the concentration of sodium in the external medium and on the level of transepithelial transport normally maintained by the skin.

5. Ouabain inhibits sodium uptake when the tissue has been pre-incubated in sodium containing medium. It has no effect on sodium uptake if the pre-incubation takes place in sodium free medium.

6. A short-circuit current and potential difference can be elicited across frog skin in the presence of ouabain, by increasing the concentration of sodium bathing the outside surface. This potential and short-circuit current is abolished by the presence of amiloride.

7. These results provide direct evidence that amiloride acts to stop entry of sodium into the transport compartment and suggest that the

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ouabain inhibition of sodium uptake is mediated primarily through an increase of epithelial sodium concentration.

INTRODUCTION

It is generally accepted that two processes are involved in the active transport of sodium across the frog skin epithelium. First, sodium penetrates into the epithelial cells across the membranes of the outer barrier of the epithelium and then sodium is actively transported from within the cells into the inside solution (Ussing, 1960).

Some effort has been invested in attempts to measure separately these two individual processes. Initially it was thought that a kinetic analysis would provide an estimation of the unidirectional fluxes across each border of the transport compartment (Curran, Herrera & Flanigan, 1963). Subsequent work showed however that the errors involved in curve fitting (Myhill, 1967) and in analysing the part played by connective tissue in delaying isotope fluxes (Hoshiko, Lindley & Edwards, 1964) could be considerable. A more direct technique for measuring sodium entry into the epithelium involves the short-term exposure of the outer surface of frog skin to solutions containing radioactive sodium (Rottuno, Villalonga, Fernandez & Cereijido, 1970; Biber & Curran, 1970). The total radioactive sodium recovered from the skin then consists of two parts, that which has penetrated the epithelium and that which remains adhering to the outer surface of the skin. This second fraction of sodium uptake, which can account for as much as 90% of the total, has been corrected for, using inulin as an extracellular space marker and the residual sodium uptake analysed kinetically (Biber & Curran, 1970). This corrected uptake has then been modified through the use of known inhibitors of sodium transport and conclusions drawn as to which agents act on the outer barrier to sodium transport (Biber, 1971). Unfortunately it has since been shown (Erlij & Smith, 1971) and later confirmed (Biber, Cruz & Curran, 1972) that inulin seriously underestimates the amount of sodium remaining external to the epithelium, making quantitative measurements of sodium entry with this marker virtually meaningless.

The present work expands the initial finding that mannitol provides a better measure of extracellular sodium than does inulin (Erlij & Smith, 1971) and goes on to measure the effects of different inhibitors of sodium transport on the mannitol corrected sodium entry into frog skin epithelium.

METHODS

Isolated skins dissected from the abdomen of the frog (*R. temporaria*) were used Two groups of measurements were carried out: (1) short-term uptake of sodium by the outside surface of the skin; (2) influx of sodium across the skin from outside to

inside. In these cases, and in others where sodium influx was not determined, measurements of potential difference and short-circuit current were also carried out.

The measurement of sodium uptake was performed under open-circuit conditions at room temperature in an apparatus similar to that described originally by Schultz, Curran, Chez & Fuisz (1967). Mounted skins were left for 20 min before determining uptake. During this time they were superfused by a Ringer solution containing: NaCl 115 mM; KCl, 5 mM; $CaCl_2$ 1 mM; Tris-chloride buffer, 3 mM; pH 7·5. Uptake of sodium was determined from a solution containing ^{24}Na, [^{3}H]inulin and [^{14}C]-mannitol. In later experiments [^{3}H]inulin was excluded from this medium. After contact with the isotopes the skins were removed and placed in 0·1 N-HNO_3 for several hours before being counted for ^{24}Na by Cerenkov radiation. [^{3}H]inulin and [^{14}C]mannitol were counted 1 week later, when the ^{24}Na had decayed, on a Packard Tricarb scintillation spectrometer. The amount of sodium that moved into the skin was calculated by subtracting the counts in the extracellular space from the total counts in the skin and then dividing the difference by the specific activity of the loading solution. Most determinations of uptake were made either 30 or 45 sec after placing the isotope in contact with the outside surface of the skin. Some determinations were carried out over longer periods of time up to 10 min after adding the isotopes. Some sodium might be expected to cross the skin during these longer periods of incubation and these measurements will certainly include a large fraction of sodium which has moved beyond the transport compartment. They do, however, provide useful information on the time course of equilibration with extracellular space markers and also give additional support to observations on the blocking effects of drugs. The amount of sodium determined after long intervals may be underestimated, particularly in untreated skins, since some of the sodium may diffuse out of the corium into the filter paper support. In a few experiments we counted the filter paper support and found that the amount of radioactive sodium passing to it after 10 min was only a fraction (about 15%) of the sodium present in the tissue after correcting this value for the outside extracellular space.

Skins treated with ouabain (10^{-4} M) were immersed in sodium free or sodium containing media containing the glycoside for 40 min before being mounted in the uptake chambers. Tris or choline was used to replace sodium as stated in the text. Skins treated with amiloride were allowed a 60 sec contact with the drug in the chambers before addition of isotopes.

Influx of sodium from outside to inside the skin was determined in the usual way using the Ringer solution described above (Ussing, 1949). Potential difference and short-circuit current were measured as described by Ussing & Zerahn (1951).

^{24}Na was obtained as a sterile isotonic solution for injection, with a specific activity approximately equal to 340 μc/mg Na, from The Radiochemical Centre, Amersham, England. [^{3}H]inulin (100–300 mc/m-mole) and [^{14}C]D-mannitol (10–30 mc/m-mole) also came from The Radiochemical Centre. Amiloride hydrochloride was a gift from Merck, Sharp & Dohme Ltd., Hoddesdon, Herts. and ouabain came from BDH Chemicals Ltd., Poole, Dorset. All other reagents were of Analar grade.

RESULTS

Evaluation of extracellular space

Time-dependence of extracellular space determinations. In the frog skin it is the outermost layer of cells in the stratum granulosum which first responds morphologically to imposed changes in electrical gradients (Voûte & Ussing, 1968) and both dyes (Overton, 1904) and lanthanum

(Martinez-Palomo, Erlij & Bracho, 1971) diffuse freely through the stratum corneum until they reach this layer of cells. Both pieces of evidence suggest that sodium has to diffuse some way into the skin before it can reach the outer barrier to transport. An accurate measurement of this diffusional space is essential if it is intended to use uptake data to describe how sodium enters the transport compartment.

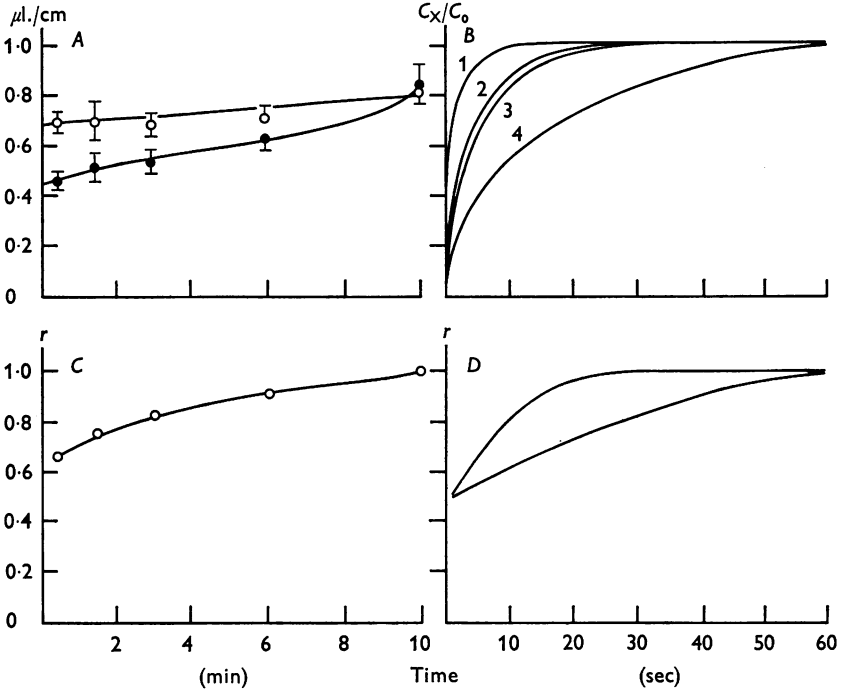

Fig. 1. Dependence of extracellular space determinations on time of incubation.

A, open circles, determinations with mannitol; filled circles, determinations with inulin. Both tracers were measured in each sample of skin. Abscissa: time in min. Ordinate: space in μl./cm^2.

B, calculated values for the degree of equilibration of a space with mannitol (curves 1 and 2) or inulin (curves 3 and 4) which is bounded between the outside solution and a plane either 60 μm (curves 1 and 3) or 100 μm (curves 2 and 4) away from the bulk solution. Abscissa: time in sec. Ordinate: average degree of equilibration of the space at the outer border of the skin calculated from the diffusion equation. Unity represents full equilibration of the space with the bulk solution.

C, average of experimental values for the ratio of inulin to mannitol in individual samples of frog skin. Standard errors were smaller than the radius of points. Ordinate: ratio of inulin to mannitol concentrations.

D, calculated values for the ratio of inulin to mannitol equilibration in the space bounded by planes 60 μm (upper line) or 100 μm (lower line) from the bulk solution.

The results of determinations of the change of extracellular space size with time, using either [^{14}C]mannitol or [^{3}H]inulin as markers in each sample of skin, are illustrated in Fig. 1*A*. Each point is the average of thirty-two determinations. The mannitol space had a value of 690 ± 45 nl./cm^2 ($\pm$S.E.) 30 sec after the addition of labelled solution. This space remained virtually constant with time up to 10 min incubation with this marker. The inulin space, determined after a 30 sec contact, was only 0·65 times the mannitol space. This space increased with time however to become equal to that measured by mannitol 6–10 min after starting the incubation.

The high intercept and negligible slope that fits the mannitol determinations ($y = 655 + 14\,t$), where t is the time in minutes, suggests that this space marker equilibrates rapidly with the extracellular space at the outer surface of the frog skin. Inulin also appears to be a good marker of the same space, but only when the time of contact exceeds 6 min.

The difference in diffusion coefficients in free solution between mannitol and inulin might be responsible for the difference in time needed for the two markers to reach equilibrium. To test this diffusion eq. (42) of Hill (1929) was used, assuming that mannitol and inulin were moving across an unstirred layer of solution adhering to the outer border of the skin and that neither had penetrated the transport compartment for sodium during the time of incubation. The calculations are shown in Fig. 1*B*. Lines labelled 1 and 3 calculate the time course of equilibration in a space bounded between the bulk solution and a plane 60 μm from the outside solution, curves 2 and 4 assume an unstirred layer of 100 μm. An unstirred layer of 60 μm was selected from previous determinations of unstirred layers which sodium has to cross before reaching the outer border of the transport compartment (Kidder, Cereijido & Curran, 1964; Dainty & House, 1966; Aceves & Erlij, 1971). The unstirred layer of 100 μm was selected because it represents a reasonable upper limit for our experiments where conditions were similar to those used in the experiments in which sodium diffusion was measured. The diffusion coefficients for inulin and mannitol in free solution were taken as $2{\cdot}18 \times 10^{-6}$ and $7{\cdot}09 \times 10^{-6}$ cm^2/sec respectively (Pollay, Stevens & Kaplan, 1969). Comparison of Fig. 1*A* with Fig. 1*B* shows that the rate at which mannitol and inulin reach equal distribution within the skin is much slower than that predicted from the diffusion equation. This is more clearly appreciated from comparison of Fig. 1*C* that shows the experimental values for the changing ratio of inulin to mannitol equilibration with time, with Fig. 1*D*, where the ratios of the degree of equilibration of the tracers, as calculated from the solution of the diffusion equation, have been plotted. This presentation has the advantage of normalizing the values as well as allowing a direct comparison to

be made with the results presented in Fig. 1*C*. Again there is a marked discrepancy between the predicted time for inulin equilibration and that found by experiment. Equality of distribution across an unstirred layer 60 μm thick was predicted to take place within 30 sec. Equilibration of both markers should still have been complete within 60 sec, even assuming the outer resistive border to be 100 μm away from the outer solution. In fact equilibration occurred with a time course at least six times greater than this upper calculated value.

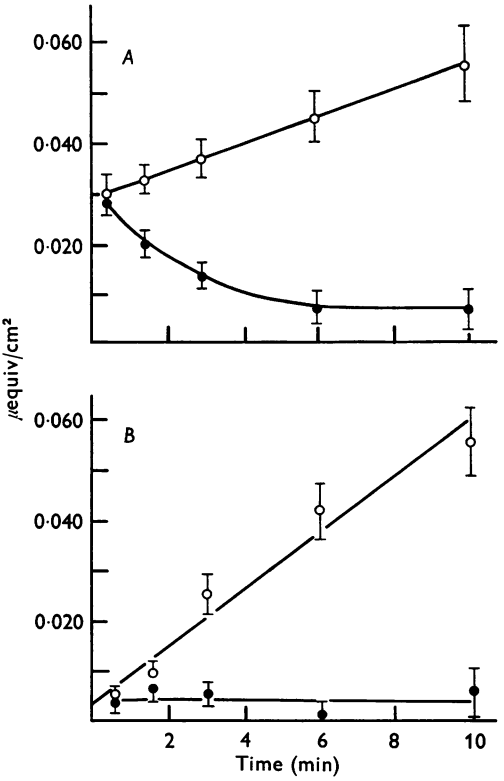

Fig. 2. Time course for sodium uptake using [^{3}H]inulin (*A*) and [^{14}C]mannitol (*B*) to correct for extracellular space. Control skins (open circles) and skins pre-treated with 5×10^{-5} M amiloride (filled circles) were incubated in Ringer containing 115 mM-NaCl. Abscissa: time of exposure to isotope (min). Ordinate: amount of sodium in the skin. Points give mean values ± S.E.

In spite of the fact that several simplifications have been made in arriving at these times for equilibration, it seems unlikely that the differences in diffusion coefficients can be the only factor affecting distribution

of these markers. The movement of inulin in the unstirred layer of the frog skin appears to be hindered out of proportion to its diffusion coefficient, in relation to mannitol.

Effect of space marker on the determination of sodium uptake

Inulin has been used previously to mark the extracellular space, when measuring sodium uptake at the outer surface of frog skin (Biber & Curran, 1970). Its slow distribution in this space, shown above, need not necessarily make it unsuitable for this purpose, provided the distribution of sodium in a similar extracellular phase is equally slow. Experiments were designed to test this point by measuring sodium uptake using both [^{3}H]inulin and [^{14}C]mannitol as markers of extracellular space in the presence and absence of amiloride (5×10^{-5} M), a substance thought to inhibit the movement of sodium across the outer border of the transport compartment. The results are shown in Fig. 2. The following features are of interest. First, the uptake of sodium by control skins, measured after a 30 sec contact with the labelled solution, was seven times larger when inulin rather than mannitol was used to correct for the extracellular space ($31{\cdot}2 \pm 3{\cdot}9$ compared with $4{\cdot}7 \pm 2{\cdot}1$ n-equiv/cm^2). Secondly, this discrepancy ceased to exist when the period of incubation with isotopes was extended to 6 or 10 min. This would be consistent with the known distribution of these markers with time. Now, however, it can be seen that the sodium uptake curve only extrapolates near to the origin when mannitol is used as the space marker (Fig. 2*B*), suggesting that this marker measures quite closely the movement of sodium at the tissue surface before its entry into the transport compartment.

Amiloride inhibits sodium uptake and this effect has been analysed in detail below. Its use here is to show further the unsuitability of inulin as a marker of extracellular space. In amiloride-treated skins the values obtained for sodium uptake using the mannitol correction remain at a low and constant level throughout the experiment. When inulin was used to correct sodium uptake there was an apparent high initial uptake which decreased with time until it became equal to the mannitol corrected value. This incongruous behaviour can again be readily understood if sodium were to diffuse more readily than inulin up to the point where it reaches the outer border of the transport compartment. We conclude from these experiments that the relatively slow movement of inulin into the skin makes it unsuitable for use as a space marker in this tissue.

Sodium uptake by frog skin

The uptake of sodium by the outside surface of the frog skin was next determined as a function of the sodium concentration in the outside solu-

tion. The results obtained are shown in Fig. 3. The data can be analysed as originally suggested by Biber & Curran (1970), who proposed that J_{12}, the uptake across the outer surface of the skin, could be described by the combination of a saturating and a linear component given by

$$J_{12} = \frac{J_m [Na]_0}{K_{Na} + [Na]_0} + \alpha[Na]_0$$

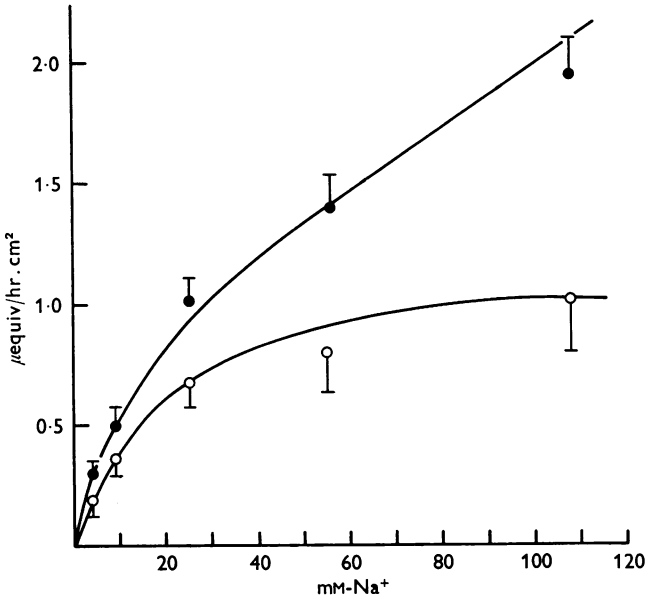

Fig. 3. Dependence of sodium uptake on outside sodium concentration. The amount of sodium taken up by the skin, corrected for the amount of extracellular sodium using either [^{3}H]inulin (filled circles) or [^{14}C]mannitol (open circles), was measured after exposure of the skins to isotope for 45 sec. Choline was used to replace sodium and maintain osmolality. Values give means ± S.E.

in which J_m is the maximal influx for the saturating component, K_{Na} is the apparent Michaelis constant and α is a permeability coefficient. The values for J_m and K_{Na}, $1{\cdot}28 \pm 0{\cdot}13$ μequiv/cm^2.hr and $23{\cdot}8 \pm 4{\cdot}3$ mM respectively, have been calculated using the mannitol corrected data from a plot of J_{12} against $J_{12}/[Na]_0$ as described by Dowd & Riggs (1965). These values have then been used to construct the lower curve in Fig. 3, assuming that the permeability constant α was equal to zero. The agreement between the experimental points and the theoretical curve is good, i.e. there is no requirement on the present evidence to postulate a linear component for sodium uptake in this tissue.

The filled circles in Fig. 3, giving the inulin corrected sodium uptakes,

can also be fitted to a theoretical curve constructed using the same values for J_m and K_{Na} but with α now equal to 0·010 cm/hr. This is less than the value of 0·037 cm/hr reported by Biber & Curran (1970), but it is still significantly different from zero. Its presence in this series of experiments appear artifactual, due to the use of an inappropriate space marker. Evidence presented below however suggests that a small linear component might sometimes exist, even when mannitol is used as space marker. Its presence appears to depend very much on the state of the skin used for experiment.

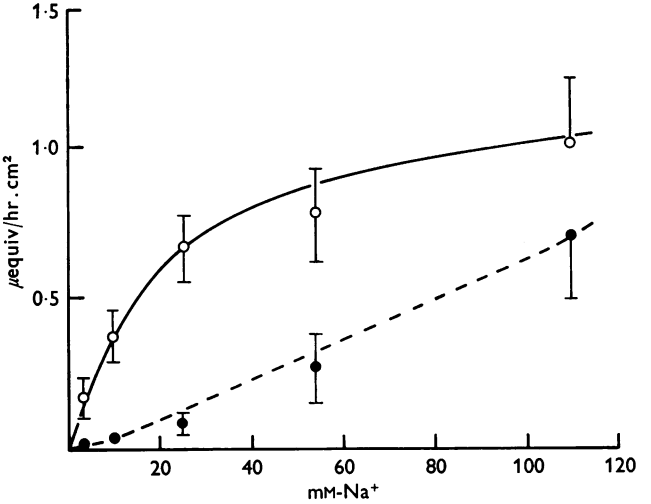

Fig. 4. The effect of amiloride on sodium uptake measured at different external concentrations of sodium. Choline was used to replace sodium. Uptake was measured after a 45 sec exposure of the skin to isotopes. [^{14}C]mannitol was used to correct for extracellular space. Open circles, control uptakes; filled circles, uptake into skins treated with amiloride (5×10^{-5} M). Points give the mean value ± S.E.

Effect of amiloride

There is much circumstantial evidence to suggest that amiloride inhibits some early step in sodium transport across the frog skin (Eigler, Kelter & Renner, 1967; Dörge & Nagel, 1970; Nagel & Dörge, 1970; Salako & Smith, 1970*a*, *b*). The more direct evidence for such an effect however rests on sodium uptake measurements made using inulin as space marker (Biber, 1971). In the present experiments the influx of sodium across the skin was monitored at different times during the year, at the same time as sodium uptake was being measured on skins taken from identical frogs. It was found that frogs could be divided into two types, those taken earlier in the year where the influx of sodium was low (0·70

μequiv/cm^2.hr) and those taken later in the year where the influx of sodium was high (1·40 μequiv/cm^2.hr). Amiloride used at a concentration of 10^{-6} M caused at least 85% inhibition of the short-circuit current of both types of preparation. The concentration of amiloride used in uptake experiments was 5×10^{-5} M. It was assumed that this high concentration of amiloride would produce a maximal effect on sodium uptake.

The effect of amiloride on sodium uptake by skins having a rather low transport capacity for sodium is shown in Fig. 4. Mannitol was used as an extracellular space marker. The uptake of sodium in the presence of amiloride was markedly inhibited provided the outside concentration of sodium did not exceed 25 mM. The inhibition fell to 50% of maximum when 55 mM-NaCl was used to bathe the skin; when the outside bathing solution contained 115 mM-NaCl the amiloride-sensitive uptake became so small as to be statistically insignificant.

Different results were obtained when frog skins having a high influx were used to measure uptake. The uptake of sodium was measured using sixteen pieces of skin, eight control and eight pre-treated with amiloride, paired comparisons being made on the same pieces of tissue. The outside bathing solution contained 115 mM-NaCl. The control uptake, $1{\cdot}49 \pm 0{\cdot}18$ μequiv/cm^2.hr approached the measured influx of sodium (1·4 μequiv/cm^2.hr). No sodium was taken up by amiloride-treated skins in these experiments ($-0{\cdot}10 \pm 0{\cdot}08$ μequiv/cm^2.hr).

This somewhat startling difference in amiloride effect between different types of skin is not so surprising when it is realized that the correction applied for the amount of sodium in the mannitol space can become extremely large in comparison to the total sodium content of the skin. The amount of sodium calculated to be moving across the outer barrier then becomes a small difference between two large quantities, the total amount of sodium taken up by the tissue minus the sodium present in the mannitol space. The problem increases when the concentration of sodium in the bulk solution becomes high and decreases when sodium movements into the transport compartment become larger. For this reason we believe that results obtained using skins showing a high influx probably give a better quantitative measure of the true effect of amiloride than do measurements made when the sodium influx is low, i.e. that the residual uptake measured with high sodium concentrations in amiloride treated skins that had low transport values is the result of inadequate evaluation of extracellular space rather than of movement of sodium into transporting cells.

Effect of ouabain

Ouabain is said to inhibit sodium transport solely by its action on active processes situated at the inner barrier of the frog skin epithelium.

If this were so, and uptake measured movement into the transport compartment only, one would not expect ouabain to have any effect on the short-term uptake of sodium. It is known, however, that lithium can be accumulated within the frog skin to levels which exceed those predicted from its electrochemical distribution (Hansen & Zerahn, 1964; Leblanc & Lemonnier, 1971) and that this cation can also inhibit, apparently competitively, the uptake of sodium by this tissue (Biber & Curran, 1970). It may be then that the movement of lithium and sodium into the transport compartment proceeds through some active transport process which could be sensitive to ouabain. To test if this were so frog skins were pre-incubated for 40 min in sodium free or sodium containing solutions, in the presence and absence of 10^{-4} M ouabain. Radioactive sodium was then added to both and its immediate uptake measured. The sodium concentration used was kept at 10 mM since, as suggested by the amiloride experiments, the use of high sodium concentrations may make it difficult to obtain reliable estimates of initial uptake.

TABLE 1. Effect of ouabain on the short-term uptake of sodium by frog skin. Skins were equilibrated with Tris or Tris containing 10 mM-NaCl for 40 min before measuring uptake. When present, ouabain was allowed to bathe both sides of the skin during equilibration. When amiloride was used, it was added to the outer surface of the skin 60 sec before measuring uptake (final concn. 5×10^{-5} M). All determinations were made using 10 mM-NaCl in contact with the skin for 45 sec. Values give means ± S.E. P^*, significance of difference between ouabain treated and control skins equilibrated in sodium containing medium. P^{**}, significance of difference between skins treated with ouabain in Tris and skins that in addition received amiloride. Degrees of freedom for each P is 12.

	Sodium uptake (μequiv./cm^2.hr)		
	Na-Tris	Tris	Tris-amiloride
Control	0·20 ± 0·01	0·31 ± 0·04	0·08 ± 0·02
Ouabain (10^{-4} M)	0·09 ± 0·01	0·36 ± 0·02	0·10 ± 0·02
	$P^* < 0·001$	$P^{**} < 0·0005$	

Ouabain was found to inhibit sodium uptake provided that the skins had been pre-incubated in the presence of sodium (Table 1). The uptake of sodium by skins pre-treated with ouabain in the absence of sodium was actually higher than that found for control skins, but the difference was not statistically significant. The effect of amiloride was to inhibit sodium uptake, even in skins which had been pre-incubated in the absence of sodium. The amount of inhibition did not depend on the presence of ouabain (Table 1).

The long-term effects of these treatments on sodium uptake is shown in

Fig. 5. In this case the pre-incubation period was shortened to 20 min. The uptake of sodium by skins pre-incubated in sodium free medium increased rapidly during the first 2–4 min and then continued to increase less rapidly over the following 6 min. The presence of ouabain in these skins changed neither the pattern nor the absolute amount of sodium recovered from the skin during the 10 min incubation. Conversely the ouabain inhibition, seen when skins were pre-incubated in the presence of

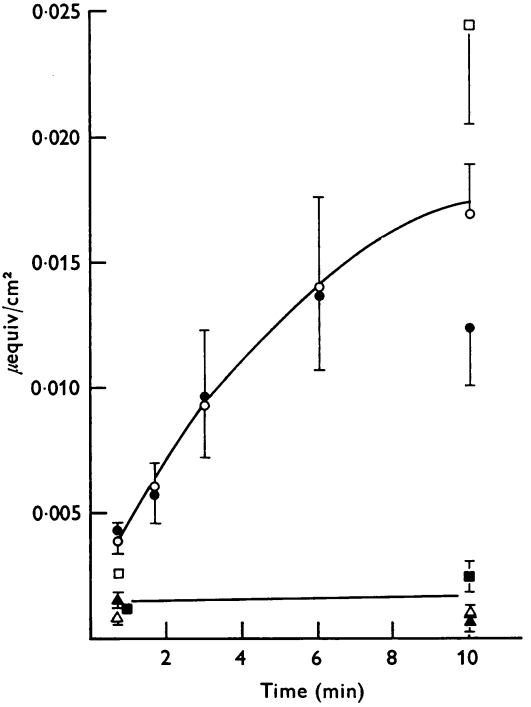

Fig. 5. The effect of ouabain on the long-term uptake of sodium by frog skin. Skins were used in these experiments either pre-incubated in sodium free medium, without ouabain (open circles), with ouabain (filled circles), with amiloride only (filled triangles) or with amiloride plus ouabain (open triangles); or pre-incubated in sodium containing medium, without ouabain (open squares), or with ouabain (filled squares). [^{14}C]mannitol was used to correct for extracellular space. 10 mM-NaCl was present in the radioactive solution Tris being added to maintain osmolality.

sodium continued throughout the experiment. Negligible amounts of sodium were recovered from the skins under these conditions even after 10 min incubation. The control skins incubated with sodium had a high uptake of sodium measured after 10 min incubation, but this was not significantly different from that found in skins pre-incubated in the ab-

sence of sodium. The action of amiloride was just as pronounced after 10 min incubation as after a 45 sec contact with isotope; pre-incubation in sodium free medium did not change this pattern.

If the effect of ouabain is dependent on the intraepithelial concentration of sodium, then pre-treatment of skins in different media should affect the action of this glycoside on both skin potential and short-circuit current. In a preliminary control experiment it was found that 10^{-4} M ouabain, added to the inside chamber of a skin immersed in a solution containing 10 mM-NaCl, reduced both the potential and short-circuit current to values close to zero within 30–45 min. Fig. 6*A* shows one of six experiments where ouabain was added after replacing the solution on both sides of the skin with a sodium free medium. This replacement reduced the potential and short-circuit current to values near to zero. Ouabain caused no further change in these values. Seventy minutes later medium containing 10 mM-NaCl was placed in contact with the outside surface of the skin. The potential increased rapidly to a value equal to 80 % of that recorded during the control period (range in six experiments, 60–100 % of control). The short-circuit current reached a value equal to 30 % of the control level (range in all experiments 30–60 % of the control value). These values decreased to near zero within the following hour.

The short-circuit current recorded with a gradient of sodium concentration across these ouabain poisoned skins probably is not a measurement of active transepithelial sodium movement. Its presence is noted below because it may represent a situation in which sodium behaves in an analogous form to lithium in skin exposed to this cation on the outside. In these experiments it is very likely that the current is carried by lithium penetrating across the outer border of the epithelium while K moves across the inner boundary (Zerahn, 1955; Hansen & Zerahn, 1964).

The next series of experiments were carried out using skins which had been pre-equilibrated in sodium free medium in the presence of 10^{-4} M ouabain. Fig. 6*B* shows one of three experiments where amiloride blocks the increase in potential and short-circuit current, initiated in the skin by placing medium containing 5 mM-NaCl in contact with its outside surface. This inhibition was reversible. Fig. 6*C* shows one of three experiments where a second equilibration of a treated skin in sodium free medium containing ouabain restored the sodium effect in causing a transitory increase in potential and short-circuit current.

Finally, it was shown that it was not necessary to remove sodium completely in the presence of ouabain to show a subsequent increase in potential and short-circuit current. In two experiments, one of which is shown in Fig. 6*D*, skins were poisoned with ouabain in medium containing 5 mM-NaCl. The potential and short-circuit current declined under

these conditions with a time course similar to that seen previously (20–40 min to establish a full block). At this stage the outside solution was replaced by one containing 50 mM-NaCl and ouabain. This solution probuced an increase in potential and short-circuit current similar in character to other responses seen in Fig. 6*A*, *B* and *C*. After washing with medium containing 5 mM-NaCl and ouabain for a further 80 min, a second increase in potential and short-circuit current could again be elicited by

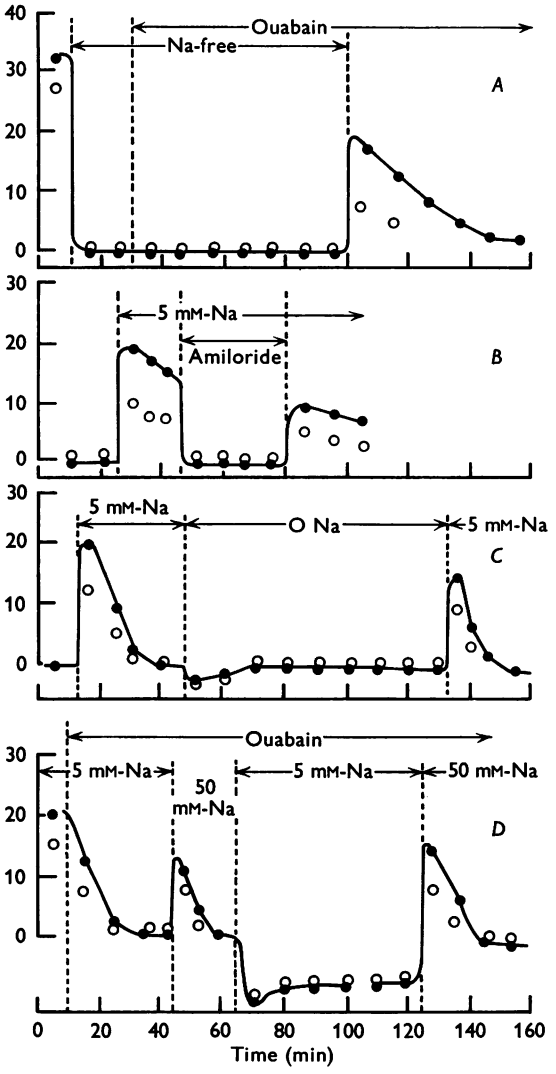

Fig. 6. For legend see facing page.

increasing the outside sodium concentration to 50 mM. These increases in potential and short-circuit current could also be reversibly inhibited by adding amiloride to the outside solution.

DISCUSSION

The present results show that estimation of the volume of extracellular space at the outer border of the frog skin depends on the marker selected for the determination. They also indicate that mannitol provides a closer approximation to the true value of the extracellular space determined after short exposure of the skin to markers.

This last conclusion is based on the assumption that essentially the same space is accessible from the outside to inulin and mannitol during the period of observation in our experiments. The finding that both markers equilibrate with the same volume of solution but at different rates gives some basis for this assumption. The difference in the time course of equilibration must therefore arise because of some structural feature of the extracellular space. Cells in the stratum corneum are known to be permeable to substances that fail to penetrate other cells (Overton, 1904; Martinez-Palomo *et al.* 1971). It is suggested that the 'extracellular space' referred to in the present work will include this layer of cornified cells. This suggestion may provide an explanation for the finding that inulin is retarded out of proportion to its diffusion coefficient in relation to mannitol, since a large and longer molecule will probably move less readily through the 'leaky' cells of the stratum corneum.

Fig. 6. Effect of ouabain (10^{-4} M) on the potential difference (filled circles) and short-circuit current (open circles) of frog skin. Ordinate gives the potential (mV) and the short-circuit current (μA).

A, skin bathed initially in medium containing 10 mM-NaCl. Both solutions were then replaced with sodium free medium. Ouabain was added to the inside chamber and 10 mM-NaCl medium finally added to the outside chamber.

B, this skin had been equilibrated with ouabain in sodium free solution for 80 min when 5 mM-NaCl was added to the outside chamber. Amiloride was then added to the outside solution for 30 min. The inside solution contained ouabain throughout.

C, this skin was equilibrated in sodium free solution containing ouabain and 5 mM-NaCl medium again introduced 90 min later. The inside solution contained ouabain throughout.

D, skin equilibrated in 5 mM-NaCl medium. Ouabain was added at the time indicated by the first interrupted vertical line. 50 mM-NaCl medium was then added to the outside chamber. This solution was later replaced with 5 mM-NaCl medium and 50 mM-NaCl medium again added 60 min later.

The use of mannitol to correct for this space explains two otherwise embarrassing findings. One is the time course of sodium uptake in amiloride treated skins described in this paper. The other is the inulin corrected sodium uptake measured after a 30 sec exposure (0·031 μequiv/cm^2), which is three times higher than the amount of sodium found at equilibrium (0·009 μequiv/cm^2, Aceves & Erlij, 1971). Calculation based on a simple kinetic model of the transport compartment suggests that after a period of 30 sec equilibration between sodium in this compartment and the outside solution would be incomplete. The half-time ($t_{\frac{1}{2}}$) for equilibration of the transport compartment (T) is given by $t_{\frac{1}{2}} = 0{\cdot}693\ T/J_{12}$. When T is taken as 0·009 μequiv/cm^2 and J_{12} as being similar to the rate of transepithelial transport (about 1 μequiv/cm^2.hr) the half time for equilibration becomes equal to 22 sec.

An assessment of the error involved while using an inadequate extracellular space marker can be made if we assume the layer that equilibrates only partially with inulin is only 5 μm thick. The volume of such a layer will be 500 nl./cm^2. This will contain 67·5 n-equiv sodium when in equilibrium with a 115 mM sodium solution. Suppose 35 % of the sodium in this layer is not accounted for in the sodium uptake calculations after a 30 sec exposure of the skin to ^{24}Na. The observed uptake will then exceed the correct value by 2·83 μequiv/cm^2.hr. The depth used in this calculation is close to that of a single layer of cornified cells and the calculated volume is not very different from the present measurements of mannitol space at 30 sec. It is clear, however, that the thickness of the stratum corneum will vary from skin to skin and, although it is likely that the stratum corneum is the site of most of the extracellular space at the outside of the skin, another source of variability could arise from regions in parallel with the transporting cells.

The superiority of mannitol over inulin as a space marker in this tissue has already been remarked upon briefly (Erlij & Smith, 1971) and this has been verified subsequently by Biber *et al.* (1972). These workers could still detect a linear component of sodium uptake, however, even when mannitol was used as a space marker. The discrepancy between their results and ours probably lies in the anatomy or sodium transporting capacity of the skin rather than in any difference in technique used to measure uptake. Though mannitol has been shown to be more useful than inulin, it might still be slightly in error in measuring the extracellular sodium space and this could be more critical with some skins than others. The occasional appearance of a linear component of sodium uptake should therefore be viewed with some caution. It seems better to assume for the present that sodium enters the frog skin by a single process showing saturation kinetics. Neither does it seem necessary to postulate the

existence of an appreciable sodium:sodium exchange across the outer barrier, for the mannitol corrected sodium uptake occurred at a rate similar to that for the transepithelial transport of sodium.

Our results on the effect of amiloride on sodium uptake, taken together with previous findings on the effect of this substance, particularly the observations that it blocks transport only when added to the outside solution and that its effect is observed almost within the time necessary for the drug to reach the outer barrier of the skin (Biber, 1971), constitutes strong evidence that this substance inhibits sodium penetration through the outer border of the transport compartment.

The further finding that amiloride blocks only the saturating component of sodium uptake (Biber, 1971) probably reflects the fact discussed previously that all or a major fraction of the linear component represents movement of sodium into an extracellular space not measured by inulin. The difficulty in detecting the inhibitory effects of amiloride when using high outside concentrations of sodium is in keeping with other findings where neither ouabain (Biber, 1971) nor antidiuretic hormone (Cereijido & Rotunno, 1971) can be shown to affect the uptake at high sodium concentrations though both change sodium uptake at low sodium concentrations. This could be due to problems associated with the correction for extracellular space, problems which make the quantification of an inhibitory response difficult.

The present results show that ouabain only blocks sodium uptake when the skins are equilibrated with the glycoside in the presence of sodium. They also show that sodium uptake in skins equilibrated with ouabain in sodium free solutions proceeds through the amiloride sensitive channel. The increase in potential following addition of sodium to skins treated in this way is also amiloride sensitive and probably associated with the movement of sodium across the outer border of the skin. Two interpretations can be suggested for these findings. Either the inhibitory effect of ouabain is greatly reduced in sodium free solutions or the inhibition is a consequence of sodium accumulation, which can be assumed to occur during the period of equilibration. There is a precedent for believing that the external sodium concentration can affect ouabain binding, the rate of ouabain action on squid giant axon being reduced by removal of external sodium (Baker & Manil, 1968). A similar action in skin, however, seems less likely to explain our experimental findings. After abolishing the potential difference by treatment with ouabain in sodium containing solutions, the potential could be restored by equilibrating the skin with sodium free solutions and then again adding sodium. More conclusive perhaps is the finding that ouabain inhibition of potential and short-circuit current in solutions containing 5 mM-NaCl can be reversed by

changing to a test solution containing 50 mM-NaCl. Treatments that cause a reduction of potential across the epithelium or its cellular membranes are known not to abolish sodium transport (Ussing & Zerahn, 1951; Ussing, 1966; Bricker, Biber & Ussing, 1963). It is therefore proposed that ouabain inhibition of sodium uptake takes place as an indirect consequence of raising the intracellular sodium concentration and not by a direct effect on the outer barrier to sodium transport.

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