

THE IONIC RELATIONS OF *ARTEMIA SALINA* (L.)

II. FLUXES OF SODIUM, CHLORIDE AND WATER

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INTRODUCTION

The ability of *Artemia salina*, the brine shrimp, to maintain haemolymph ion concentrations which are very much smaller than those in the external solution (Croghan, 1958*a*) might suggest that this animal has a very low permeability to ions. However, it has been found that the tracer fluxes of sodium and chloride are extremely rapid (Croghan, 1958*b*; Thuet, Motais & Maetz, 1968). The values of both sodium and chloride efflux fall considerably in distilled water; this effect, and the rapidity of the fluxes, has led to the postulation that both these ionic species cross the gill epithelium by an exchange diffusion process, as described by Ussing (1947).

A similar situation occurs in certain euryhaline teleosts (Motais, Garcia Romeu & Maetz, 1966). The value of the tracer efflux of sodium in *Platichthys*, the flounder, was estimated as $3600 \text{ pmole cm.}^{-2} \text{ sec.}^{-1}$, an extremely large value when compared with those observed in other tissues. This efflux dropped considerably in distilled water, again suggesting that exchange diffusion was occurring.

The present paper describes experiments on both sodium and chloride efflux in *Artemia*, and the effect on these fluxes of rapid changes in the composition of the external medium. The results are considered in relation to the electrical measurements reported in the previous paper (Smith, 1969); it is concluded that exchange diffusion of chloride is most probably present, but that exchange diffusion of sodium is unlikely to occur. Measurements of water fluxes are also reported.

THEORETICAL SECTION

Definition of the ionic permeability coefficient

The description of the movement of ions across membranes is intrinsically more complex than the description of non-electrolyte movement, for ions are subject to gradients not only of concentration, but also of electrical potential. The characterization of ion movement by a permeability coefficient has to rely upon a specific model for the movement of that ion through the membrane, involving somewhat arbitrary assumptions (Dainty, 1962). The simplest satisfactory approach is that due to Goldman (1943), for a homogeneous membrane, which assumes that the gradient of electrical potential is constant through the membrane. Using this theory, it is possible to predict the way in which ion fluxes depend on differences of concentration and electrical

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potential across the membrane. For a membrane separating two phases, the expression for the net flux of ionic species i from phase 1 to phase 2, \mathcal{J}_i (mole cm.⁻² sec.⁻¹) is

$$\mathcal{J}_i = P_i(z_i F V_{12}/RT) \frac{C_{i2} - C_{i1} \exp(z_i F V_{12}/RT)}{1 - \exp(z_i F V_{12}/RT)}, \quad (1)$$

where P_i (cm. sec.⁻¹) is the permeability coefficient of ion i , V_{12} is the electrical potential of phase 1 with respect to that of phase 2, C_{i1} and C_{i2} (mole cm.⁻³) are the concentrations of species i in phases 1 and 2 respectively, and z_i , F , R and T have their usual meanings. Phase 1 will be identified with the inside, or haemolymph, phase, and phase 2 with the external solution; \mathcal{J}_i thus represents the net efflux of ion i .

In the derivation of this equation, it is assumed that the movement of an ion through the membrane is independent of the movement of all other species (thus excluding solvent-drag effects, for example), and also independent of the movement of other ions of the same species (thus excluding exchange diffusion and single-file diffusion). It is further assumed that active transport does not occur. These conditions can be summarized by stipulating that the ions move passively and independently. Use of equation (1), therefore, is valid only when there is evidence that ion movement is passive and independent.

In this paper equation (1) is used to determine a value for P_i in a given set of experimental conditions. The equation is then used to predict the value of ionic flux under other conditions, assuming that P_i remains constant.

It is usually the tracer flux of an ionic species which is actually measured. If the internal and external specific activities of ion i are s_{i1} and s_{i2} (Ci mole⁻¹) respectively, the net tracer efflux \mathcal{J}_i^* (Ci cm.⁻² sec.⁻¹) is given by

$$\mathcal{J}_i^* = P_i(z_i F V_{12}/RT) \frac{s_{i2} C_{i2} - s_{i1} C_{i1} \exp(z_i F V_{12}/RT)}{1 - \exp(z_i F V_{12}/RT)}. \quad (2)$$

The unidirectional efflux of ion i , here denoted by \mathcal{J}_{i1} (mole cm.⁻² sec.⁻¹) is defined by

$$\mathcal{J}_i^* = s_{i1} \mathcal{J}_{i1}, \quad (3)$$

when $s_{i2} = 0$. Substituting (3) into (2) and setting s_{i2} equal to zero gives

$$\mathcal{J}_{i1} = P_i(z_i F V_{12}/RT) \frac{C_{i1} \exp(z_i F V_{12}/RT)}{\exp(z_i F V_{12}/RT) - 1}. \quad (4)$$

Note that equation (4) predicts correctly that \mathcal{J}_{i1} is always positive, no matter what the sign of z_i or V_{12} . An equation for unidirectional influx can be derived similarly.

Ion flux and electrical resistance

It was shown by Hodgkin (1951) that if an ion is in equilibrium across a membrane, the relation between its partial conductance g_i and unidirectional flux \mathcal{J}_{i1} is

$$g_i = \frac{z_i^2 F^2}{RT} \mathcal{J}_{i1}. \quad (5)$$

(g_i is measured in units of mho. cm.⁻².) The validity of equation (5) is subject to the restriction that ion movement be passive and independent. However, in the derivation of the equation no arbitrary assumptions concerning the nature of the electrical

potential gradient are necessary, nor is it assumed that the membrane is homogeneous. For an ion in equilibrium across a membrane unidirectional influx and efflux are equal in magnitude, and either could be used in equation (5).

For application to the present situation equation (5) was modified to give the partial resistance of ion i (reciprocal of the partial conductance and measured in $\Omega\text{cm.}^2$). Both sides of the equation were then multiplied by the epithelial area A , so that the partial resistance r_i of the whole epithelium, predicted from a measurement of ion flux, was given by

$$\frac{1}{r_i} = \frac{z_i^2 F^2}{RT} j_{i1} A. \quad (6)$$

MATERIALS AND METHODS

Animals from the sea-water culture described previously (Smith, 1969) were used. Experiments were confined to adult animals acclimatized to sea water, and were performed at room temperature (20–25° C). The compositions of the experimental solutions were also given in the previous paper. All isotopes and labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks.; animals were loaded with the tracers in 20 ml. radioactive sea water.

Sodium efflux

The isotope ^{22}Na , obtained as the chloride at high specific activity, was used for the measurement of sodium efflux. Single animals were loaded overnight using a solution at a specific activity of 20 $\mu\text{Ci m-mole}^{-1}$, 10 $\mu\text{Ci ml.}^{-1}$. It was shown by Croghan (1958*b*) that all the internal sodium is exchanged within 12 hr.; these animals were therefore completely loaded.

Two methods of measuring efflux were used. In the first of these the radioactivity remaining in the animal was measured. An animal was transferred from the loading medium to a small Perspex cell, similar to that used for the electrical measurements (Smith, 1969), but with a barrier of nylon gauze to prevent the animal from being carried away by the flow of solution. It was not necessary to immobilize the animal. The volume of the cell was about 0.2 ml., and inactive solution flowed through at a rate of about 0.7 ml. min.⁻¹. Immediately beneath the cell was a scintillation detector connected to a scaler with a programme and print-out unit (IDL 1700 and 2007 with Addo-X printer). This system was set to record the number of counts accumulated every 400 sec.; print-out required 20 sec., and so the activity remaining in the animal was recorded every 420 sec. The efficiency of the counting system was determined by counting samples of the loading medium in a cell where the geometry and γ -ray scattering characteristics were closely similar to those of the cell used for efflux measurements. Knowing the sodium concentration of the loading medium it was then possible to relate a given counting rate to a specific quantity of radioactive sodium.

A graph of logarithm of activity remaining in the animal was plotted against time, and the rate constant k_{Na} for efflux of sodium determined from the gradient. The unidirectional efflux $j_{\text{Na}1}$ (mole cm.⁻² sec.⁻¹) was then calculated from the equation

$$k_{\text{Na}} = \frac{j_{\text{Na}1} A}{Q_{\text{Na}}} \quad (7)$$

where Q_{Na} (mole) is the sodium content of the animal, and A the area (cm.^2) through which the flux takes place. The value of A was taken as 0.03 cm.^2 , the area of the gills (Smith, 1969) through which most of the exchange of ions takes place (Croghan, 1958*b*); the value of Q_{Na} was calculated from the counting rate at zero time (determined by extrapolation).

In one set of experiments sodium efflux and electrical resistance were measured simultaneously. The procedure was as described previously for electrical measurements (Smith, 1969), but the animal was loaded with radioactive sodium before the experiment. Loss of sodium was then followed as described above, and the total unidirectional efflux of sodium $\mathcal{J}_{\text{Na}1}A$ was calculated from equation (7). The sodium partial resistance r_{Na} was then calculated from equation (6); it has been found previously (Smith, 1969) that the distribution of sodium between the blood and the external medium is close to equilibrium, in sea water, and so the use of this equation is justified.

The above method for measuring efflux allowed an experiment to be continued for a long time, but was not sufficiently sensitive for the observation of the rapid changes in efflux which occurred when the composition of the external solution was changed. In order to make such measurements a second method was used, where the activity lost from the animal was determined directly. The efflux solution was collected every minute, and the radioactivity in each sample determined. The activity lost from the animal in 1 min. was small, and it was necessary to use a low-background Geiger counting system (Nuclear Chicago 4312) for these experiments. The collected solution samples were dried down on planchettes for counting. It was necessary to make corrections for the absorption of β -particles, which differed in the deposits left by the different solutions; the appropriate correction factors were determined by counting standard samples. At the end of the experiment, the activity remaining in the animal was determined, using the same counting system. The activity present in the animal during each 1 min. period was then obtained by adding back the efflux counts; for each efflux period the activity lost was divided by the total activity present to give the rate constant for efflux.

Chloride efflux

The isotope ^{36}Cl was obtained as 2 N-HCl solution at a high specific activity. This solution was neutralized with NaOH and made up to 20 ml. solution with the composition of artificial sea water, and a specific activity of $50 \mu\text{Ci m-mole}^{-1}$, $25 \mu\text{Ci ml.}^{-1}$. Single animals were loaded overnight in this solution.

This isotope emits β -particles only, of low energy (0.7 MeV); these were not detectable by the scintillation counter used for sodium efflux, and so it was not practicable to perform long-term chloride efflux experiments. Short-term experiments were carried out using the second method described for sodium. NaOH solution was added to the planchettes before drying down, to prevent loss of radioactive chloride as HCl gas. Values for the unidirectional efflux of chloride were calculated from equation (7).

Drinking rate

Drinking rate can be determined from the rate of uptake by the whole animal of a molecule to which the external surface is impermeable. A convenient molecule for this

purpose is polyvinylpyrrolidone (PVP) labelled with ^{131}I ; it has been shown by Evans (1968) that PVP labelled with ^{128}I is a suitable compound for measuring drinking rates in fish.

Before use the compound was dialysed against sea water to remove any free iodide; a negligible quantity was in fact found to be present. A group of animals was loaded in a solution with a specific activity of $10 \mu\text{Ci ml.}^{-1}$. Single animals were removed at intervals of 5–10 min., rinsed for 3 min. in a continuous flow of sea water, and their radioactivity was determined by means of a well-crystal scintillation counter. The activity in samples of the loading medium was also determined. Dissection of the gut from loaded animals showed that by far the larger part of the activity was in this organ, so that the volume of solution swallowed was easily calculated, on the assumption that no excretion had occurred. The finding of Croghan (1958*c*) that phenol red remained in the gut for periods as long as 72 hr. indicates that this assumption is likely to hold during the time over which the experiment was carried out ($2\frac{1}{2}$ hr.).

Diffusional water influx

The rate of exchange of water was measured in *Artemia* using tritiated water, THO. A group of animals was placed in a solution at a specific activity of $10 \mu\text{Ci ml.}^{-1}$. At intervals of 10–20 min. an animal was removed, rinsed for 30 sec. in a continuous flow of distilled water, blotted gently on paper tissue and placed in 5 ml. distilled water in a closed polythene container. After 48 hr., when it was assumed that all activity in the animal had been lost to the external solution, 1 ml. solution was withdrawn and added to 14 ml. liquid scintillator (Bray, 1960) for counting. Counting was carried out using a Packard Tricarb Liquid Scintillation Spectrometer, model 3314. The activity in samples of the loading medium was also determined.

In order to determine the fraction of water in the animal which had exchanged it was also necessary to measure the total water content of each animal. The usual method of carrying out such a determination would be to measure the difference between the fresh and dry weights of each animal. However, measurement of the fresh weight was inaccurate, owing to difficulties in removing all external water without puncturing the cuticle; an indirect method of determining water content was therefore used. The total sodium content of each animal was determined by analysis of the solution remaining in the polythene containers after the sample had been withdrawn for counting; a Unicam SP900 flame photometer was used for sodium analysis. The relation between sodium content and water content was then determined by measuring the sodium content and radioactivity in animals loaded to completion with THO. This relation was expressed as the sodium concentration in total body water.

Thus the fraction of water which had exchanged, b_w , was determined. This was related to the unidirectional water influx \mathcal{J}_{w2} by the equation

$$\ln(1-b_w) = -\frac{\mathcal{J}_{w2}A}{Q_w}t, \quad (8)$$

where Q_w is the water content of the animal, and t the length of time for which the animal was loaded.

Results are given as mean \pm standard error of mean (number of observations).

RESULTS

Sodium efflux in sea water

A representative graph of logarithm of internal activity against time is plotted in Fig. 1; this was obtained from an experiment in which sodium efflux was measured by the first method. The graph shows clearly that sodium exchange can be explained by a single-compartment model. The mean value of the sodium efflux rate-constant k_{Na} in sea water, obtained from experiments using both methods, was $0.0115 \pm 0.0005 \text{ min.}^{-1}$

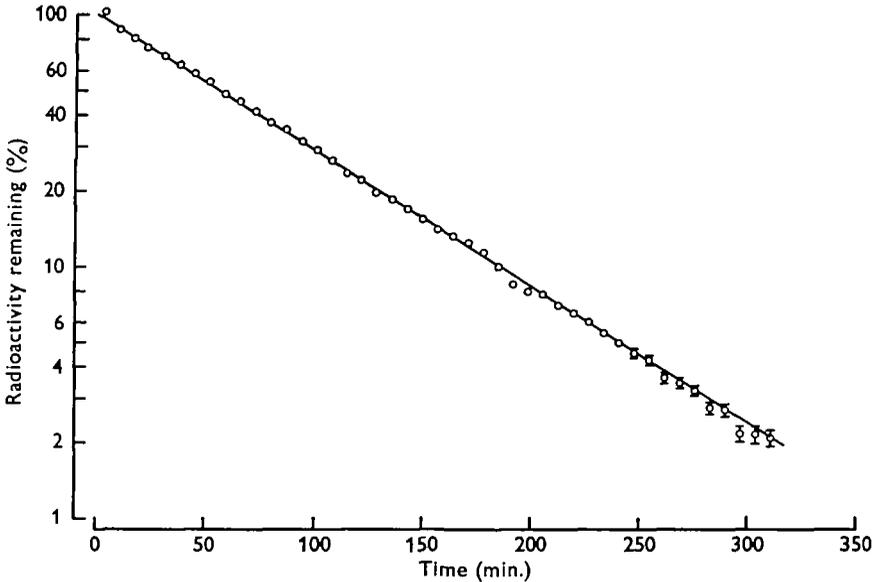


Fig. 1. Sodium efflux, in sea water. In this experiment the first method of measurement was used; the rate-constant k_{Na} is 0.0124 min.^{-1} . Error bars are \pm standard deviation.

(48). In experiments where the sodium content of the animal was also determined the value of the unidirectional sodium efflux for the whole animal, $\mathcal{J}_{Na1}A$, was $215 \pm 25 \text{ pmole sec.}^{-1}$ (10); this corresponds to a value for \mathcal{J}_{Na1} of $7200 \text{ pmole cm.}^{-2} \text{ sec.}^{-1}$ ($\text{pmole} = 10^{-12} \text{ mole}$). Taking the epithelial p.d. V_{12} as $+23.4 \text{ mV}$. (Smith, 1969), the value for the sodium permeability P_{Na} , calculated from equation (4), was $2.8 \times 10^{-5} \text{ cm. sec.}^{-1}$.

These results are in agreement with those of Croghan (1958*b*) and Thuet *et al.* (1968), and confirm the extremely high values of sodium flux in *Artemia*.

Sodium efflux and electrical resistance

In the group of experiments where the measured epithelial resistance was compared with that calculated from the sodium flux, the value of the measured resistance was $1100 \pm 180 \Omega$ (10). The sodium partial resistance r_{Na} , calculated from the sodium flux using equation (6), was $1320 \pm 100 \Omega$ (10). The mean difference between the two was $230 \pm 170 \Omega$ (10), which is not significantly different from zero, indicating that the observed resistance can be accounted for by sodium movement alone.

Chloride efflux in sea water

It was shown by Croghan (1958*b*) that efflux of bromide could be explained by the presence of a single internal compartment, and this was confirmed for chloride by Thuet *et al.* (1968). In the present experiments no evidence for the existence of more than a single internal chloride compartment was found in experiments which lasted for a period of 1 hr.

The mean value of the chloride efflux rate-constant in sea water was $0.0145 \pm 0.0023 \text{ min.}^{-1}$ (11). Assuming that the animals used in these experiments were the same size as those used in the sodium efflux experiments this corresponds to a flux of $220 \text{ pmole sec.}^{-1}$ for the whole animal, or $7400 \text{ pmole cm.}^{-2} \text{ sec.}^{-1}$. It is not possible to calculate a diffusional chloride permeability directly from these results, for there is evidence (considered below) that a large part of the chloride flux is not diffusional in character.

This value of chloride efflux is similar to the flux of bromide measured by Croghan (1958*b*), and is not significantly different from the value of the sodium efflux. In contrast, Thuet *et al.* (1968) found that the efflux of chloride was about twice as rapid as that of sodium; apparently there were differences between the population of animals used in the present work and that used by Thuet *et al.* (1968).

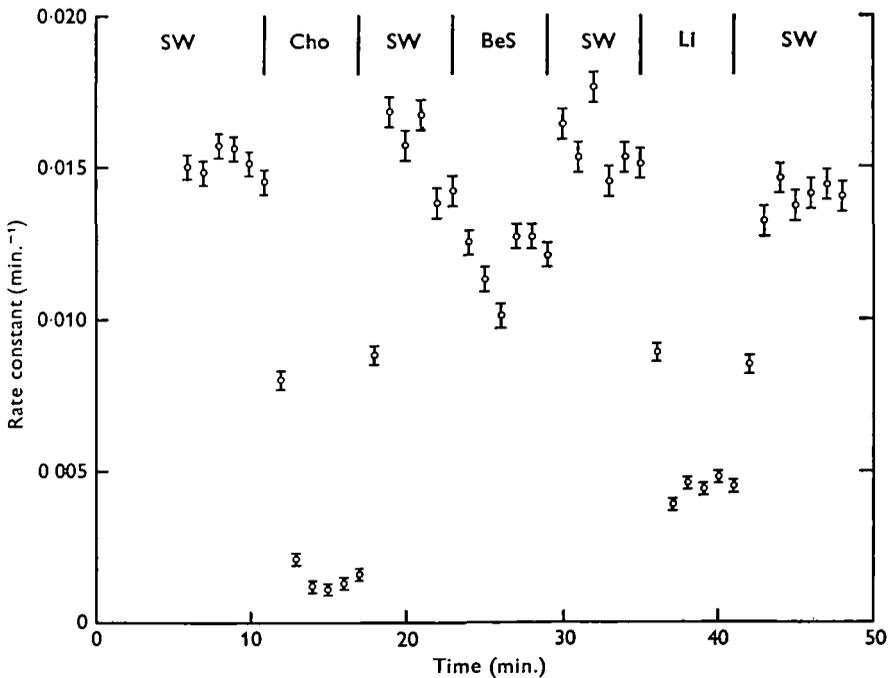


Fig. 2. Sodium efflux in different solutions. In this experiment the second method of measurement was used. Li, lithium SW; Cho, choline SW; BeS, benzenesulphonate SW. Error bars are \pm standard deviation.

Sodium efflux in different solutions

The effect of rapid changes in the composition of the external solution on sodium efflux was observed, using the second method of efflux measurement. A graph of efflux rate-constant against time, obtained in one of these experiments, is shown in Fig. 2. There is no evidence for a lag of change of efflux behind change of solution, as the observed rate of change can be explained by the rate of solution mixing in the efflux cell. The results of similar experiments on several animals are summarized in Table 1.

Table 1. *Changes of sodium efflux in various solutions, as ratios of the values in sea water*

Solution	Efflux ratio
Lithium SW	0.38 ± 0.03 (14)
Choline SW	0.25 ± 0.06 (8)
Benzenesulphonate SW	0.92 ± 0.06 (7)

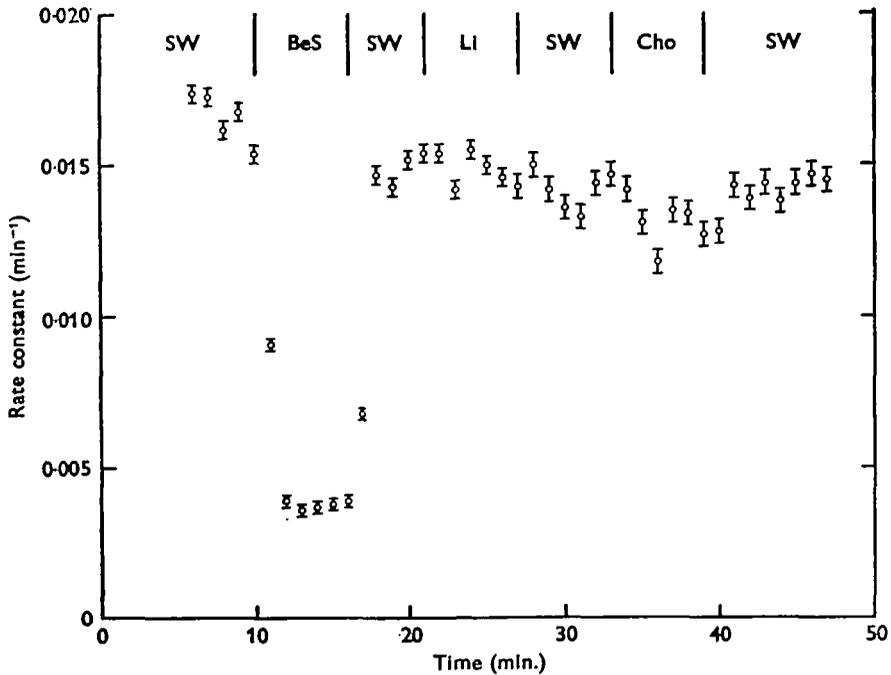


Fig. 3. Chloride efflux in different solutions. Li, lithium SW; Cho, choline SW; BeS, benzenesulphonate SW. Error bars are \pm standard deviation.

In benzenesulphonate sea water (SW) sodium efflux remains at the same level as in normal SW. In lithium SW there is a marked fall in efflux, and in choline SW an even greater drop is observed; a pronounced 'sodium-free' effect is therefore present, which since the solutions are nominally isosmolar cannot be due to an osmotic effect.

Chloride efflux in different solutions

The effect on chloride efflux of rapid changes in the composition of the external solution was also observed. A representative graph of efflux rate-constant against time is shown in Fig. 3, and the results of several similar experiments are summarized in Table 2.

Table 2. *Changes of chloride efflux in various solutions, as ratios of the values in sea water*

Solution	Efflux ratio
Lithium SW	0.91 ± 0.05 (6)
Choline SW	0.92 ± 0.04 (6)
Benzenesulphonate SW	0.30 ± 0.04 (8)

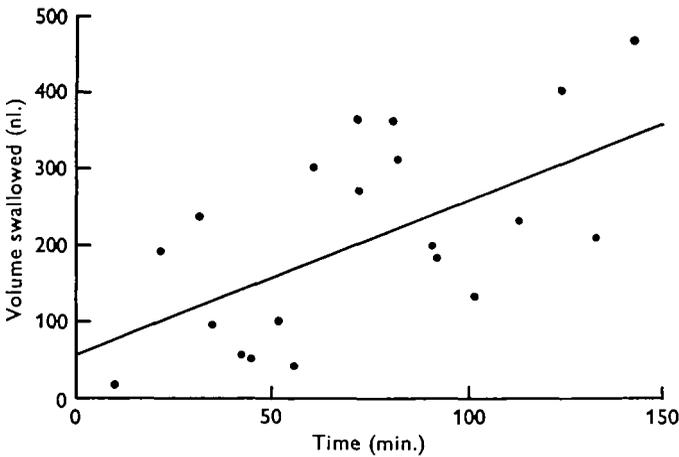


Fig. 4. Drinking rate. The regression of volume v (nl.) on time t (min.) is $v = 2.14 t + 55$.

Chloride efflux is not significantly affected by substitution of lithium SW or choline SW. In benzenesulphonate SW there is a marked fall in efflux, indicating a 'chloride-free' effect.

Drinking rate

The volume of external solution swallowed is plotted against time in Fig. 4; it can be seen that there is considerable scatter in the results. The regression of volume v (nl., 10^{-9} l.) on time t (min.) is given by

$$v = 2.14 t + 55.$$

The gradient is significantly different from zero at the 1% level. The presence of a significant uptake at zero time may be due to adsorption of radioactivity on to the cuticle of the animal.

In order to be able to express the drinking rate as % body weight hr.^{-1} , the weight of two groups of ten animals was determined approximately. The mean weight was found to be 6.3 mg. The drinking rate ($2.1 \text{ nl. min.}^{-1}$, or 36 pl. sec.^{-1}) therefore

corresponds to 2.0% body weight hr.^{-1} , or 20 ml. $\text{kg.}^{-1} \text{hr.}^{-1}$, rather smaller than the rate of 30 ml. $\text{kg.}^{-1} \text{hr.}^{-1}$ observed by Thuet *et al.* (1968).

Diffusional water influx

The value of the sodium concentration in total body water was found to be $140 \pm 6 \text{ mM}$ (11), in agreement with that found by Croghan (1958*a*). Using this value it was possible to calculate the water content of an animal from the sodium content.

A graph of $\ln(1-b_w)$ is plotted against time in Fig. 5 (b_w is the fraction of the total body water which has exchanged at time t). An approximate straight-line relationship is apparent. For each animal the total THO influx $\mathcal{J}_{w2}A$ (pl. sec.^{-1}) was calculated according to equation (8). The mean value was $242 \pm 14 \text{ pl. sec.}^{-1}$ (30), of which 36 pl. sec.^{-1} represents swallowing of the medium (see above). The influx across the gills was therefore 206 pl. sec.^{-1} , or 6900 $\text{pl. cm.}^{-2} \text{sec.}^{-1}$; this corresponds to a diffusional water permeability coefficient P_d of $6.9 \times 10^{-6} \text{ cm. sec.}^{-1}$.

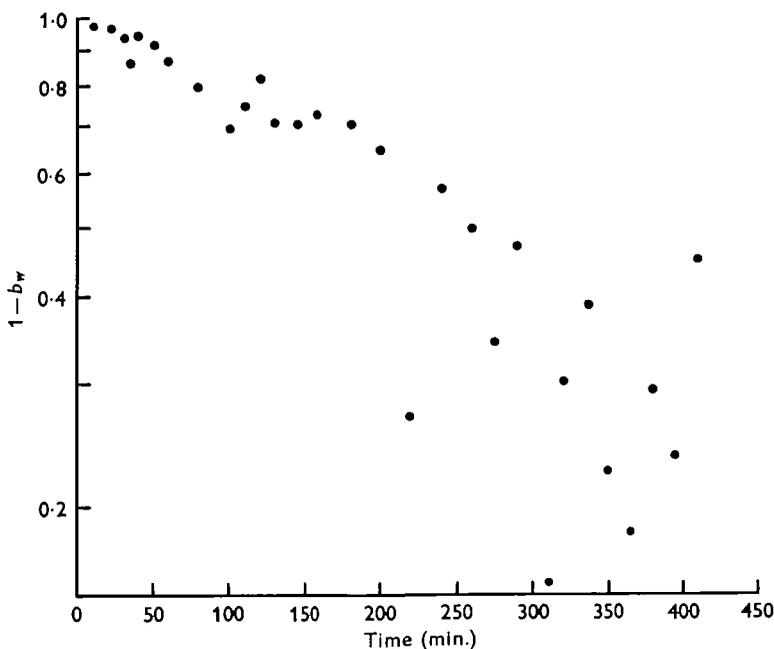


Fig. 5. Tritiated water influx. b_w is the fraction of total body water which has exchanged at time t .

DISCUSSION

Nature of the ion fluxes across the gill epithelium

The constant-field equation [equation (1)] has been used here to define ionic permeability. This equation was also applied to the study of ion fluxes in whole animals by House (1963) and by Potts & Parry (1964). While it is acknowledged that this theory is by no means strictly applicable to a structure as complex as the gill epithelium, it is the best approximation available in the absence of any detailed knowledge of the properties of the epithelium.

For sodium the evidence is strong that ion movement is passive and that the ions move independently. The equality of measured resistance and that calculated from the sodium flux on the assumption of passive, independent movement of ions, taken together with the large transport number of this ion (Smith, 1969), leaves little doubt that sodium movement is diffusive in character. Calculation of a sodium permeability coefficient from equation (4) is therefore justified.

In the case of chloride it was concluded in the preceding paper (Smith, 1969) that active transport occurs; there is also evidence for the presence of exchange diffusion (see below). It is therefore not possible to calculate the chloride permeability coefficient

Table 3. *Summary of changes in epithelial properties in various solutions.*

Solution	Sodium efflux	Chloride efflux	P.d. (mV.)	Conductance
Lithium SW	0.38	0.91	+ 1	0.53
Choline SW	0.25	0.92	- 36	0.30
Benzenesulphonate SW	0.92	0.30	- 3	0.77

Flux and conductance values are given as ratios of the values in sea water; values of p.d. are given as differences from the values in sea water.

directly from equation (4). However, from the electrical measurements reported in the preceding paper, it was calculated that the ratio of the diffusional permeability of chloride to that of sodium was 0.11; thus from the sodium permeability of 2.8×10^{-5} cm. sec.⁻¹, it can be calculated that the chloride permeability is 3.1×10^{-6} cm. sec.⁻¹.

The effects of changes in the composition of the external medium on the ion fluxes, p.d. and conductance are summarized in Table 3. The values of the electrical parameters are taken from the previous paper. Three groups of animals were used in the experiments from which the results of Table 3 are taken; one group for the electrical measurements, and one group each for the sodium and chloride fluxes.

It has already been concluded that sodium efflux is diffusional in character; the results given in Table 3 are in agreement with this. In lithium SW the decrease in sodium efflux corresponds to a decrease of similar magnitude in conductance. While the origin of the decrease remains unexplained, the approximate equality of the effects on efflux and conductance indicates that the effect is upon some diffusional process. In choline SW the blood potential becomes negative with respect to that of the external solution; this is expected to produce a decrease in the efflux of the positively-charged sodium ion, and thus a decrease also in conductance. Both of these effects are observed. From the constant-field theory [equation (4)] it is possible to predict the ratio of the effluxes in choline SW and normal SW, for the observed p.d. change, assuming the internal ion concentrations to remain unchanged; the value obtained is 0.51. The observed ratio of 0.25 is considerably smaller than this, and it therefore appears that choline, as well as lithium, reduces the diffusional permeability coefficient of sodium. Benzenesulphonate SW has little effect on the p.d., and so small effects only are expected on efflux and conductance, as in fact was observed.

The magnitude of the chloride efflux is similar to that of sodium. It was concluded from the electrical measurements that the diffusional permeability coefficient of chloride was much smaller than that of sodium, and it therefore appears that the greater part of the efflux must be due either to active transport by a neutral ion pump,

or to exchange diffusion, as described by Ussing (1947); fluxes mediated by either of these two mechanisms would not be detected by electrical measurements. The results given in Table 3 support this conclusion. In choline SW, where the p.d. becomes negative, and increased efflux of the negative chloride ion would be expected, if the efflux were diffusional in character; no change was observed. Furthermore, in benzenesulphonate SW, with little change in p.d., chloride efflux decreased to 30% of its value in normal SW; it is unlikely that this could be explained by a decrease in chloride permeability, because the conductance is not greatly affected. The proportions of the chloride efflux occurring by the different mechanisms are considered further below.

Variation of ion flux with external concentration

The behaviour of the efflux of both sodium and chloride in different external solutions, as observed in the present experiments, is very similar to that found by Thuet *et al.* (1968) under the same experimental conditions. However, Thuet *et al.* concluded that exchange diffusion of both sodium and chloride was present; in particular, it was found that the variation of unidirectional sodium efflux with external sodium concentration could be described by an equation of the Michaelis-Menten type, as would be expected for a carrier-mediated process. The equation used was

$$\Delta \bar{J}_{\text{Na}1} = \Delta \bar{J}_{\text{Na}1 \text{ max.}} \frac{C_{\text{Na}2}}{C_{\text{Na}2} + K_m}$$

$\Delta \bar{J}_{\text{Na}1}$ is the increase in unidirectional sodium efflux over that observed in distilled water, and $\Delta \bar{J}_{\text{Na}1 \text{ max.}}$ the maximum value of this increase (i.e. that observed at high external sodium concentration). K_m is the 'affinity' of the hypothetical carrier molecule—the external concentration at which a half-maximal increase in sodium efflux is observed.

The conclusions of Thuet *et al.* do not agree with those of the present work. It was therefore of interest to see whether the variation of sodium efflux with external concentration, as found experimentally, would be predicted if sodium movement were purely diffusional. Upon dilution of the external medium the electrical potential of the blood decreases (Smith, 1969); this would be expected to cause a decrease in sodium efflux if sodium movement were simply diffusional. It is possible to calculate the magnitude of this decrease from the Goldman (constant-field) theory, if the values of p.d. between the blood and external medium are known. In the Appendix the variation of efflux with external concentration is calculated, on the basis of two different assumptions for the values of p.d. The first is that the p.d. is equal to the sodium equilibrium potential [equation (9)]; this assumption was found to hold when the external medium was sea water (Smith, 1969), but is unlikely to be correct as the external concentration is decreased below that of the haemolymph, when increasingly large negative potential differences are predicted. The variation of efflux with external concentration predicted by this model [equation (12)] is shown in Fig. 6A. The second, and more realistic, assumption for the values of p.d. [equation (14)] involves the permeability coefficients of both sodium and chloride, and uses the permeability ratio calculated in the preceding paper. The variation of efflux predicted on this basis [equation (16)] is shown in Fig. 6B.

It can be seen that both graphs have a shape similar to that given by the Michaelis-

Menten equation [cf. Fig. 6 with Fig. 11 of Thuet *et al.* (1968)]. It is therefore concluded that a flux-concentration curve of this type does not necessarily constitute evidence for the presence of a carrier-mediated process. The observed variation of sodium efflux with external concentration in *Artemia* can be explained if sodium movement is simply diffusional.

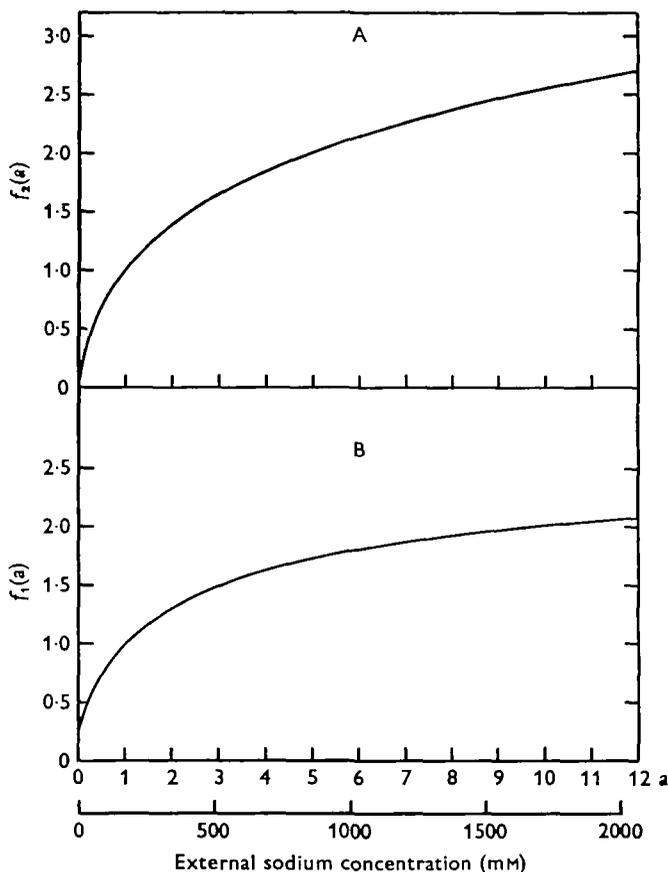


Fig. 6. Theoretical graphs of sodium efflux against external concentration. The ordinate of both graphs is linearly related to the unidirectional efflux \mathcal{J}_{Na} by the equation

$$\mathcal{J}_{Na1} = P_{Na} C_{Na1} f(a).$$

The functions $f_1(a)$ and $f_2(a)$ are defined by equations (13) and (17) of the Appendix.

The theoretical approach described here is more satisfactory than that employed by Thuet *et al.* in the respect that it has only one parameter which can be varied to fit the experimental results, namely the sodium permeability coefficient. The Michaelis-Menten equation has two, the maximum flux increment $\Delta \mathcal{J}_{Na1 \max}$, and the 'affinity' K_m of the hypothetical carrier molecule.

The external concentration $C_{\frac{1}{2}}$ at which half-maximal increase in sodium efflux occurs (the K_m of Thuet *et al.*) can be calculated from equation (16) [equation (12) is not asymptotic at high external concentrations, and therefore $C_{\frac{1}{2}}$ cannot be calculated from this equation]. The value obtained for $C_{\frac{1}{2}}$ is 430 mM—considerably in excess of

the value of 65 mM found experimentally by Thuet *et al.* However, experimentally little increase in efflux was found above an external concentration of 500 mM Na; if this is taken as the concentration at which maximal efflux occurs, the value obtained for $C_{\frac{1}{2}}$ is 125 mM, which although in better agreement with the experimental value, is still approximately twice as large. The reason for this discrepancy is not clear.

The value of $C_{\frac{1}{2}}$ predicted by the present approach is determined solely by the haemolymph concentration, if the ratio of sodium and chloride permeabilities remains unchanged. Haemolymph concentration varies little with the salinity to which the animal is acclimatized, and so the finding of Thuet *et al.* that $C_{\frac{1}{2}}$ did not vary with the salinity of acclimation is predicted by the present approach.

It is also possible from equation (16) to calculate the reduction in sodium efflux expected when distilled water is substituted for sea water. The predicted ratio of the two fluxes is 0.18. Thuet *et al.* observed values of 0.23 in fresh water and 0.28 in fresh water made isotonic to sea water by the addition of mannitol; these values are in moderately good agreement with theory.

In considering the variation of efflux with external concentration, it is necessary to distinguish between 'rapid-transfer' experiments, where no acclimation to the new concentration occurs, and experiments in which the animal is allowed to acclimatize to the new external medium. The two types of experiment give quite different results, both in *Artemia* (Thuet *et al.* 1968) and *Platichthys* (Motais, Garcia Romeu & Maetz, 1966). Thus the experiments of Croghan (1958*b*), in which *Artemia* was allowed to acclimatize to the new concentration, are not comparable to those discussed here.

The considerations here have been confined to sodium efflux in *Artemia*. It is evident that similar doubts must be raised about the conclusion of Motais *et al.* (1966) that sodium exchange diffusion is present in certain euryhaline teleosts.

This approach can be extended to predict the variation of influx with external concentration. If sodium is in electrochemical equilibrium across the epithelium, influx and efflux will be equal, and Fig. 6A will also give the variation of influx with external concentration. If the more realistic assumption for the p.d., which takes into account both sodium and chloride permeability [equation (14)], is used, a different curve is obtained; however, this has the same general shape as Fig. 6A. There are many papers which describe measurements of the half-maximal influx of ions, using as a basis the Michaelis-Menten equation. Most of these studies were performed at low external concentrations. Under such conditions, the sodium equilibrium potential would be extremely large, and unlikely to correspond at all closely to the actual p.d.; equation (9) for the p.d. would therefore not apply. Furthermore, equation (14) for the p.d. predicts only a slow variation of p.d. with external concentration under these conditions, and so an approximately linear variation of influx with external concentration would be predicted. The validity of these studies is therefore unaffected by the considerations described here. Nevertheless, it must be emphasized that measurements of ion flux without parallel p.d. measurements can lead to fallacious conclusions.

Partition of chloride efflux

From the observed rate of swallowing of the external medium maximum values for the rate of absorption of ions from the gut into the blood can be calculated, assuming

That all the ingested ions are absorbed. This calculation gives the following values, per animal:

Sodium absorption from the gut: 17 pmole sec.⁻¹.

Chloride absorption from the gut: 20 pmole sec.⁻¹.

These values are an order of magnitude smaller than the fluxes across the gills, confirming that by far the greater part of the ion flux takes place across the gill epithelium.

It is possible to calculate, from the observed values for influx, the rate at which outward active transport of chloride must occur. The net passive influx across the gills can be calculated from the constant-field theory [equation (1)], using the chloride permeability coefficient of 3.1×10^{-8} cm. sec.⁻¹; this gives a value of 70 pmole sec.⁻¹ for a single animal. The value for active transport is the sum of the net influx across the gills and the influx across the gut, i.e. approximately 90 pmole sec.⁻¹. Of the observed value for efflux, 90 pmole sec.⁻¹ must be due to active transport; it can be calculated from the constant-field equation that the diffusional chloride efflux contributes 10 pmole sec.⁻¹, so that the total chloride efflux accounted for is 100 pmole sec.⁻¹. The observed value of efflux is 220 pmole sec.⁻¹, of which 120 pmole sec.⁻¹ must therefore be due to exchange diffusion. Thus in benzenesulphonate SW, the efflux is expected to fall to $100/220 = 0.45$ of its value in sea water. The observed value (Table 2) is 0.30, which in view of the assumptions made is not too different from this.

Water fluxes

The observed value of the drinking rate allows a value for the hydraulic conductivity of the epithelium, L_p , to be calculated. The water influx across the gut, assuming that all the ingested water is absorbed, is 36 pl. sec.⁻¹. This influx must equal the osmotic loss of water. L_p (cm. sec.⁻¹. atm.⁻¹) can then be calculated using the equation (e.g. Dainty, 1963):

$$j_v = L_p(\Delta P - \sigma RT\Delta C_s)$$

where j_v (cm.³ cm.⁻² sec.⁻¹) is the volume flow, which is negligibly different from the water flow j_w , ΔP is the mechanical pressure difference, σ the reflexion coefficient for the particular solute-membrane combination and ΔC_s the difference in solute concentrations. Any mechanical pressure difference across the gill epithelium is much smaller than the large osmotic pressure difference, and ΔP can be neglected. If the gill epithelium behaves as an ideal semi-permeable membrane to the solute, which is largely sodium chloride, the reflexion coefficient σ is equal to unity; in the absence of evidence to the contrary, it has been assumed that this is so. Taking the values of internal and external concentrations as 1.2 and 3.2% NaCl respectively (Croghan, 1958a), and assuming the solutions to be ideal, the value obtained for L_p is 7.1×10^{-8} cm. sec.⁻¹ atm.⁻¹. (In this calculation, it has also been assumed that the osmotic water flow takes place only across the gill epithelium, with area 0.03 cm.².) This compares with values (in the same units) of 2.4×10^{-7} in frog skin (Dainty & House, 1966); 8.5×10^{-7} in frog stomach (Durbin, Frank & Solomon, 1956); and 4.7×10^{-8} in teleost gall bladder (Diamond, 1962). The value in *Artemia* is rather smaller than these; thus osmotic loss of water in high external salinities is reduced.

The rate of diffusional exchange of water, measured here using tritiated water, is in

close agreement with the rate measured by Ussing (in Krogh, 1939) using deuterated water. The value of the diffusional water permeability P_a (6.9×10^{-6} cm. sec.⁻¹) is considerably smaller than that of the sodium permeability P_{Na} (2.8×10^{-5} cm. sec.⁻¹). This is a most unusual situation; it seems unlikely that it would occur if sodium and water traversed the epithelium through the same pathway, and it therefore appears necessary to postulate separate pathways for the two chemical species. The value of P_a (using the same units) is 6.5×10^{-5} in frog skin (Dainty & House, 1966); 4.8×10^{-5} in frog stomach (Durbin *et al.* 1956); and 8.3×10^{-5} in teleost gall bladder (Diamond, 1962). Again the value in *Artemia* is somewhat lower than these, confirming that the gill epithelium is more 'tight' to water than are other epithelial tissues.

The osmotic and diffusional water permeabilities can be compared after multiplication of L_p by the factor RT/\bar{V}_w , \bar{V}_w being the partial molar volume of water (18 cm.³ mole⁻¹). The expression $L_p RT/\bar{V}_w$ is often called the osmotic permeability coefficient, P_{os} , for water. The calculation gives a value for P_{os} of 9.6×10^{-5} cm. sec.⁻¹—some 14 times larger than that for P_a . Such a discrepancy is often observed in epithelial tissues, and has been commonly attributed to the presence of pores in the cell membranes. This interpretation has been criticized by Dainty & House (1966) on the grounds that the value of P_a has been underestimated because the presence of 'unstirred layers' has not been taken into account. The present determination of P_a in the gill epithelium of *Artemia* is also open to the criticism that the experiments were not performed under conditions of zero volume flow, as is necessary for a true estimate of the value of P_a (Dainty & House, 1966). Furthermore the assumptions necessary in the calculations of the values of the two parameters have introduced uncertainties, and it would therefore be unwise to attach too much importance to the observed size of the discrepancy, although it seems likely that a true disparity is present.

SUMMARY

1. The effects of different external media on the sodium and chloride efflux in *Artemia salina*, the brine shrimp, have been observed, using animals acclimatized to sea water. In sea water, both sodium and chloride fluxes across the epithelium are approximately 7,000 pmole cm.⁻² sec.⁻¹.

2. Sodium efflux drops markedly in sodium-free media, and chloride efflux falls in chloride-free media; the two effects are independent, and are not due to changes in external osmolarity.

3. The decreases in sodium efflux can be explained by changes in electrical potential difference and diffusional permeability; exchange diffusion of sodium does not occur.

4. Approximately 70% of the chloride efflux is due to exchange diffusion, and most of the remainder is due to active transport.

5. It is shown that graphs of ion efflux against external concentration which can be fitted by a Michaelis-Menten equation do not constitute evidence for the presence of exchange diffusion; graphs of similar shape can be obtained if the flux is simply diffusional.

6. The drinking rate, determined from the rate of uptake of ¹³¹I-polyvinylpyrrolidone, is 36 pl. sec.⁻¹, or 2.0% body weight hr.⁻¹.

7. The diffusional influx of water is 240 pl. sec.⁻¹.

APPENDIX

Dependence of unidirectional sodium efflux upon external concentration

The object is to calculate the way in which the tracer efflux of sodium depends upon its external concentration. It is assumed that the constant-field theory holds true.

The tracer efflux \mathcal{J}_{Na}^* (Ci cm.⁻² sec.⁻¹) is the measured quantity. This is given by equation (2) of the Theoretical Section. The epithelial p.d. V_{12} is first equated to the sodium equilibrium potential:

$$V_{12} = \frac{RT}{F} \ln \frac{C_{Na2}}{C_{Na1}}. \tag{9}$$

Equation (9) is substituted into equation (2), and the external specific activity s_{Na2} set equal to zero. This gives, after rearrangement:

$$\mathcal{J}_{Na}^* = s_{Na1} P_{Na} \frac{C_{Na1} C_{Na2}}{C_{Na2} - C_{Na1}} \ln \frac{C_{Na2}}{C_{Na1}}. \tag{10}$$

For rapid changes in the external sodium concentration C_{Na2} , the internal sodium concentration C_{Na1} can be considered constant. Writing $a = C_{Na2}/C_{Na1}$, equation (10) becomes

$$\mathcal{J}_{Na}^* = s_{Na1} P_{Na} C_{Na1} \frac{a}{a-1} \ln a. \tag{11}$$

Substituting equation (3) into equation (11) gives

$$\mathcal{J}_{Na1} = P_{Na} C_{Na1} \frac{a}{a-1} \ln a. \tag{12}$$

In equation (12), only the parameter a varies with the external concentration, and a graph of the function

$$f_1(a) = \frac{a}{a-1} \ln a, \tag{13}$$

will give the shape of the graph of efflux against external concentration. A graph of $f_1(a)$ is plotted in Fig. 6A. Note that this function is not asymptotic at large values of a , increasing beyond all bounds as a tends to infinity. As a tends to zero, $f_1(a)$ also tends to zero; this theory therefore predicts that the efflux in distilled water will be zero.

A more realistic equation for V_{12} is (Smith, 1969)

$$V_{12} = \frac{RT}{F} \ln \left[\frac{C_{Na2} + \beta C_{Cl1}}{C_{Na1} + \beta C_{Cl2}} \right], \tag{14}$$

where β is the ratio of chloride permeability to sodium permeability, and has the value 0.11. In order to simplify the algebra, equation (14) can be approximated to

$$V_{12} = \frac{RT}{F} \ln \left[\frac{C_{Na2} + \beta C_{Na1}}{C_{Na1} + \beta C_{Na2}} \right]; \tag{15}$$

here the concentrations of sodium and chloride have been equated in both the internal and external solutions. Equation (15) can then be substituted in equation (2) to give,

after setting s_{Na2} equal to zero, substituting for J_{Na}^* from equation (3), and re-arranging

$$J_{Na1} = P_{Na} C_{Na1} \frac{1}{1-\beta} \frac{a+\beta}{a-1} \ln \left[\frac{a+\beta}{1+\beta a} \right], \quad (16)$$

the parameter a having been defined above. Again, only the parameter a varies with the external concentration, and a graph of the function

$$f_2(a) = \frac{1}{1-\beta} \frac{a+\beta}{a-1} \ln \left[\frac{a+\beta}{1+\beta a} \right], \quad (17)$$

will give the shape of the graph of efflux against external concentration. A graph of $f_2(a)$ is plotted in Fig. 6B, the value of β having been taken as 0.1. Note that this function, unlike $f_1(a)$, is asymptotic at large values of a , and has a finite value at $a = 0$.

The value of $f_2(a)$ at $a = 0$ is 0.26, and that at $a = \infty$ is 2.56. Thus a half-maximal increase in efflux is equivalent to a value for $f_2(a)$ of $(2.56 + 0.26)/2$, or 1.41; this corresponds to a value for a (determined from Fig. 6B) of 2.50. The internal sodium concentration C_{Na1} is 173 mM (Smith, 1969), so that this value of a represents an external concentration of 430 mM. If, however, the value of external sodium concentration at which maximal efflux occurs is taken as 500 mM (see Discussion), which corresponds to a value for a of 2.89 and for $f_2(a)$ of 1.48, half-maximal increase in efflux is equivalent to $f_2(a) = (1.48 + 0.26)/2 = 0.87$, corresponding to a value for a of 0.72, and an external sodium concentration of 125 mM.

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