

THE ROLES OF  
SODIUM TRANSPORT AND ANION PERMEABILITY IN  
GENERATING TRANSEPITHELIAL POTENTIAL  
DIFFERENCES IN LARVAL SALAMANDERS\*

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Recent investigation has shown that the fully aquatic, larval form of the salamander *Ambystoma tigrinum* handles osmotic and ionic regulatory problems as do other fresh water and semiterrestrial animals. Faced with osmotic water entry from a hypotonic environment, it excretes a dilute urine to remain in water balance. Uptake of NaCl from artificial-pond water occurs at the rate of about  $1\mu\text{-equiv. (10 g.)}^{-1}\text{hr.}^{-1}$ . This maintains an ionic steady state by compensating for losses in the urine and by diffusion across the skin and gills (Alvarado, 1962; Alvarado & Kirschner, 1963). Animals in a steady state in pond water ( $[\text{NaCl}] = 1.2 \text{ mM/l.}$ ) have a potential difference across the body surface of 10-20 mV., body fluids positive to the external solution. Nothing is known about the mechanism generating electrical asymmetry in these animals.

Transepithelial potential differences (TEPs) have been studied for many years, and our understanding of the ionic mechanisms underlying them is derived largely from studies on isolated frog skins and toad urinary bladders bathed on both sides by Ringer's solution. Little attention has been paid to the origin of TEPs in intact animals bathed by dilute solutions. Barker-Jørgensen, Levi & Zerahn (1954) showed that when frogs were immersed in 3-5 mM NaCl the TEP was 50-100 mV. body fluids positive to bathing solution. Lower potentials, the polarities of which were variable, were found when the animals were immersed in KCl. Brown (1962) found that the TEP in intact frogs depended on environmental NaCl concentration and that its concentration dependence resembled that of isolated skins. These observations are consistent with a role for active salt transport in generating the TEP, and suggest that the model developed for the *in vitro* preparation (Koefoed-Johnsen & Ussing, 1958; Ussing, 1960) may be applicable to the intact animal in its normal environment. However, some flux data obtained on animals in dilute solutions are hard to reconcile with this model. For example, Krogh (1937) showed that salt-depleted frogs would take up chloride from solutions with a non-penetrating cation ( $\text{K}^+$ ), while the model provides no EMF for anion transfer in the absence of sodium transport. Krogh's animals also absorbed sodium from solutions with non-penetrating anions. Under these conditions the model requires that the TEP approach the EMF of the transport mechanism but that no net ion uptake occur. The observations were confirmed by Barker-Jørgensen *et al.* (1954), who showed that even when NaCl was absorbed the

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movement of neither ion satisfied the flux ratio criterion for diffusion. Hence, chloride appears to be actively transported into the animal from dilute solutions. These data suggest that the ionic basis for electrogenesis in dilute solutions needs further investigation.

Many of these observations have also been made in larval salamanders. The present investigation explores the relationship between ion uptake and electrogenesis in salamanders in dilute solutions. Special attention has been paid to establishing whether active sodium transport is part of the electrogenic mechanism and to the role played by anion absorption.

#### METHODS

The experimental animals were larval salamanders, *Ambystoma tigrinum* captured in local streams. A few experiments were conducted on *A. gracilus*, and no inter-specific differences were noted. Most of the animals weighed between 10 and 50 g. They were stored in porcelain tubs in a lighted room at  $15 \pm 1^\circ$  C. The storage medium was local tap-water.

Experimental animals were removed from the holding tanks and equilibrated with an appropriate experimental solution for a week before measurements were made. Most of the animals were equilibrated with artificial 'pond water' containing 1.2 mM/l. NaCl (Alvarado & Kirschner, 1963). One group was salt-depleted by immersing them in deionized water which was changed daily. This treatment augments active salt transport, and on being returned to pond water these animals absorb NaCl 2-3 times as rapidly as normal (i.e. pond-water-adapted) animals (Alvarado & Kirschner, 1961). Another group of animals was adapted to 40 mM/l. NaCl before being used. This depresses the transport mechanism (unpublished experiments). The animals were used to study changes in TEP under condition of normal, augmented and depressed ion transport. The salt-depleted animals were also used to demonstrate independence of  $\text{Na}^+$  and  $\text{Cl}^-$  transport systems.

Each salamander was anaesthetized with tricaine methane sulphonate (0.1%). The skin and body wall were punctured with a dissecting needle, and a fine polyethylene tube filled with agar-3 M-KCl was inserted into the body cavity. The animal was then transferred to the experimental solution which contained 0.03% tricaine methane sulphonate. A second agar-KCl-filled bridge dipped into the bathing solution. Both bridges were connected through calomel electrodes to a recording potentiometer with an input resistance of 100 k $\Omega$  at balance. Potentiometric precision was about  $\pm 1$  mV. At the end of each experiment electrode asymmetry was measured by connecting the body fluids of the animal through a saturated KCl bridge to the bathing solution. All potentials reported below have been corrected for the small asymmetries noted.

Many experiments were run in which animals were initially placed in distilled water, and the potential differences across the skins were recorded when NaCl was added to the bath. Although these experiments were adequate to demonstrate dependence of the TEP on external salt concentration, they had the disadvantage that both anion and cation concentration varied simultaneously. Since one of the goals of this investigation was to assess the role of each ion in electrogenesis, an alternative procedure was used in all later work. This involved using 5 mM/l.  $\text{K}_2\text{SO}_4$ , rather than distilled water, as the initial solution. Evidence to be presented below shows that both

$K^+$  and  $SO_4^{2-}$  have a low mobility in the skin. The assumption underlying its use is that a non-penetrating salt should have no electrical effect on the animal. This is borne out by the fact that the transepithelial potentials in distilled water and in 5 mM/l.  $K_2SO_4$  fell into the same range, 5–20 mV., body fluids negative to the external solution. By using  $Na_2SO_4$  it was possible to vary the sodium concentration through the range 0.1–10 mM/l. without changing the anion composition or the ionic strength of the medium. The effects of  $(NH_4)_2SO_4$ ,  $Li_2SO_4$ ,  $Ce_2SO_4$  and  $MgSO_4$  were also tested. Chloride concentration was varied through the same range by using KCl. In all experiments the animal was placed in the initial bathing solution and allowed to remain until the TEP was stable. Studies on the dependence of potential on ion concentration began by replacing a measured volume of bathing solution with an equal volume of 5 mM/l.  $Na_2SO_4$  or KCl. When the new TEP was stable it was recorded. This procedure was repeated to give a series of readings through the range 0.1–10 mM/l. with each step increasing the test ion concentration about threefold. Concentrations were measured rather than calculated, because intermittent urination provided an uncontrolled source of ions. Sodium, potassium, and calcium were determined with a direct-reading flame-photometer. Precision of these measurements was about  $\pm 1\%$ . Chloride was determined by silver titration with a Cotlove 'chloridimeter'.

Net fluxes of  $Na^+$ ,  $K^+$  and  $Cl^-$  were measured on animals previously salt-depleted for 1 week. Each animal was placed in a plastic container with a known volume of solution, initially 0.6 mM/l.  $Na_2SO_4$  for one group and 1.2 mM/l. KCl for another. Samples were removed periodically for analysis. After about 24 hr. each animal was transferred to pond water (1.2 mM/l. NaCl) and aliquots were taken for another 24 hr. Changes in concentrations in the bath were estimated by flame-photometry ( $Na^+$ ,  $K^+$ ) or silver titration ( $Cl^-$ ). Net uptake or excretion was calculated from these changes. Chloride determinations were not made on  $Na_2SO_4$  solutions because of sulphate interference.

Sulphate permeability was measured by placing animals in media made from  $Na_2^{35}SO_4$  and measuring disappearance of isotope from the bathing solution. Aliquots were plated on aluminum planchets with a drop of detergent. After drying they were counted with an ultrathin-window GM tube.

The ammonia-excretion experiments were carried out in cylindrical Lucite chambers stirred and aerated by a stream of compressed air. The pH of the bathing solution was adjusted to 6.8 to minimize distillation of ammonia in the air stream. Such losses were shown to be negligible by leading the air through a trap containing boric acid. No ammonia appeared in trap even after 6 hr. of aeration. Ammonia was determined by direct Nesslerization of aliquots removed from the bathing solution. Optical density was measured at 430 m $\mu$ .

All experiments were carried out at a temperature of  $15 \pm 1^\circ C$ .

## RESULTS

### *Dissociation of sodium and chloride transport*

When salamanders are in a steady state in pond water about two-thirds of their salt loss is renal, hence the flux ratio across the body surface must be about three for both ions. Since blood  $[Na^+]$  is about 96 mM/l. a TEP of 137 mV., body fluids

negative, would be necessary to sustain a sodium steady state by diffusion. For chloride a TEP of 137 mV., body fluids positive, would be required. In several hundred measurements on animals in pond water the body fluids have always been positive, but values never exceeded 35 mV., and most were in the range 10–20 mV. Therefore, both ions must be actively transported into the animal under these conditions.

However, these data provide no information on whether the ion movements are coupled through a single mechanism or are independent. To answer this question the following experiment was performed. Four animals were salt-depleted for a week. Two were transferred to 1.2 mM/l. KCl and net movements of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> were measured for 23 hr. The other pair were placed in 0.6 mM/l. Na<sub>2</sub>SO<sub>4</sub> and net transfers of Na<sup>+</sup> and K<sup>+</sup> were measured. At the end of this period all four animals were transferred to pond water and ion movements were followed for another 20.5 hr.

Table 1. *Net movement of ions in salt-depleted salamanders*

(Experimental protocol described in the text. Each value is the mean for two animals. Net absorption is denoted +; net loss -.)

Initial medium	In initial medium (23 hr.): $\mu$ -equiv. taken up per 10 g. body weight			After transfer to pond water (20.5 hr): $\mu$ -equiv. taken up per 10 g. body weight		
	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>
KCl	-7.9	+9.5	+23.6	+37.5	-5.1	+20.5
Na <sub>2</sub> SO <sub>4</sub>	+20.0	-7.3	—	+17.5	+1.6	+37.5

Table 1 shows the total quantity of each ion transferred in each experimental period. It is clear that Cl<sup>-</sup> uptake from KCl occurred. A net loss of sodium occurred during this period. Although unidirectional ion fluxes were not measured here,\* the data show that chloride uptake can occur without simultaneous sodium transport. Uptake of K<sup>+</sup> occurs in some animals but is absent in many. Even when it can be demonstrated (as in Table 1) it is never equal to Cl<sup>-</sup> uptake. When these animals were transferred to normal pond water, influx of Na<sup>+</sup> markedly exceeded that of Cl<sup>-</sup>. There was a net loss of K<sup>+</sup>.

Animals placed in Na<sub>2</sub>SO<sub>4</sub> showed a net uptake of Na<sup>+</sup> while losing some K<sup>+</sup>. Chloride was not measured, but none was present initially and a net loss must have occurred. When this pair was transferred to pond water, Cl<sup>-</sup> uptake was much greater than Na<sup>+</sup> uptake.

Since Na<sup>+</sup> and Cl<sup>-</sup> appear to move independently it is necessary to account for the preservation of electrostatic neutrality. In the case of Na<sup>+</sup> uptake an alternative to anion absorption is exchange with another cation. Fig. 1 shows that animals adapted to pond water excrete ammonia continually in that medium. During the first 2 hr. the untreated animals excreted more ammonia than those with cloacas sutured to prevent urination, but in the period 2.5–7.25 hr. average ammonia efflux was comparable in both groups: 0.52  $\mu$ mole (10 g.)<sup>-1</sup> hr.<sup>-1</sup> for untreated animals; 0.60  $\mu$ mole (10 g.)<sup>-1</sup> hr.<sup>-1</sup> for animals with stitched cloacas. Since the high initial output was seen only in the control animals it probably is due to urinary ammonia.

\* A detailed study of the effects of salt depletion on fluxes of sodium and chloride has been undertaken by R. H. Alvarado. The results will be reported in another publication.

But the sustained efflux in both groups must represent movement across the body surface, and hence may be part of a cation exchange system.

No flux experiments relating to electrostatic neutrality during chloride uptake were conducted.

#### Sodium transport and electrogenesis

When animals were transferred from pond water to a sodium-free solution the normal TEP was replaced by a small one of opposite polarity, usually in the range

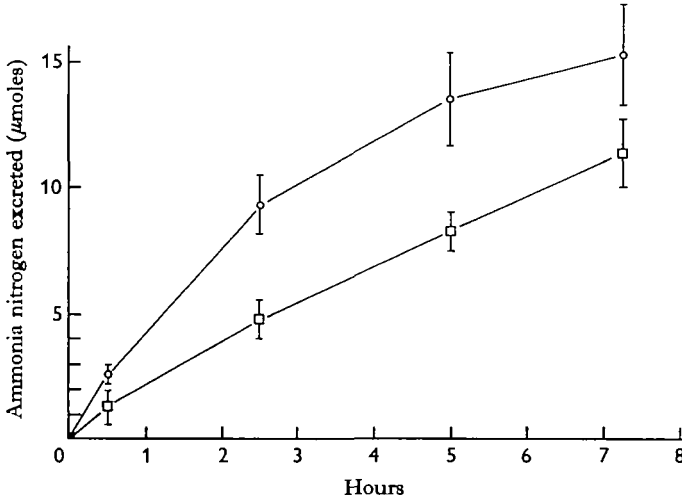


Fig. 1. Ammonia excretion by larval salamanders. Five control animals (O—O) and five animals with stitched cloacas (□—□) were immersed in 5 mM/l.  $K_2SO_4$  containing 1 mM/l.  $Na_2SO_4$ . At the times shown samples of the bathing solutions were removed and analysed for ammonia nitrogen. Each point is the mean of the five determinations. Standard deviations are indicated by the vertical lines.

2–15 mV. body fluids negative. These 'reversed' TEPs have been measured with animals in deionized water, in  $K_2SO_4$  (5 mM/l.), and in KCl (10 mM/l.). This suggests that the normal TEP is dependent on the presence of sodium in the bathing solution. Fig. 2 shows that when  $[NaCl]$  was increased through 4 orders of magnitude the TEP increased non-linearly with concentration to about 20 mV, then decreased at higher concentrations. The same phenomenon is seen in isolated frog skins (Steinbach, 1933; Linderholm, 1951), and in the frog *in vivo* (Brown, 1962). Fig. 2 also shows that in the range 0.1–10 mM/l. the data can be expressed approximately as in equation (1):

$$TEP = a_0 + b \log [NaCl]. \quad (1)$$

Since several functions would describe the data at least as well (a rectangular hyperbola would provide a better fit at lower concentrations), no theoretical significance is attributed to equation (1). However, it generates two easily assessed parameters useful in characterizing the effects of experimental variables. One of these is the TEP at  $[Na^+] = 1$  mM/l. ( $a_0$ ), a concentration approximating that encountered by the animal in nature; the other is the slope of the line ( $b$ ) which expresses the potential change per tenfold concentration change.

Abolition of the TEP in sodium-free solution, and its concentration dependence, both could be explained if the potential were generated by sodium transport. Such a working hypothesis suggests that animals with augmented ion transport should generate high TEPs while those with inactive transport systems should show low TEPs. Three groups of animals were prepared as described under Methods: (1) one group was salt-depleted to stimulate transport; (2) a 'normal' group was adapted to

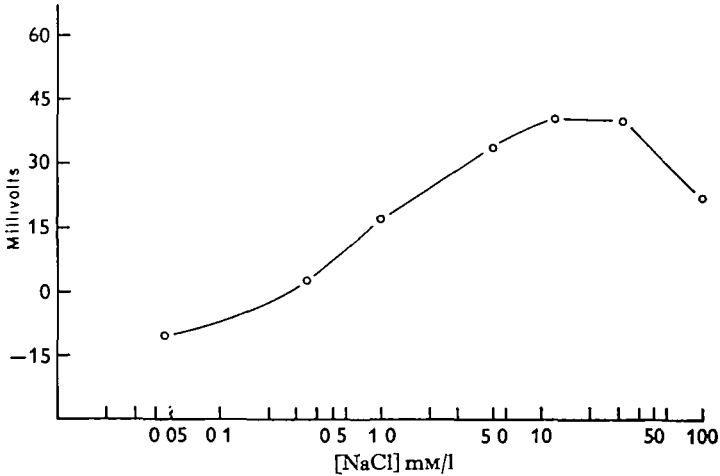


Fig. 2. TEP as a function of [NaCl]. A single larval salamander was anaesthetized and immersed in 50 ml. deionized H<sub>2</sub>O containing 0.02% MS-222. At the beginning of the experiment the animal had lost enough Na<sup>+</sup> to make the initial bath concentration 0.056 mM/l. The concentration was increased in steps by adding NaCl from a stock solution (1M/l.). After each determination of TEP an aliquot of the solution was removed for Na<sup>+</sup> analysis. The sign of the potential is that of the internal electrode.

Table 2. TEP and [Na<sub>2</sub>SO<sub>4</sub>] in animals preadapted to different salinities

Adaptation Medium (mM/l. [Na <sup>+</sup> ])	N*	n†	a <sub>0</sub> (mV.)	b (mV. / log [mM])	s <sub>xy</sub> <sup>2</sup> ‡	s <sub>2</sub> <sup>2</sup> §	s <sub>TEP</sub> <sup>2</sup>
0.0	8	40	21.9	17.4	87.1	0.742	312.3
1.2	12	61	12.9	16.5	61.8	0.512	201.2
40	10	50	3.7	8.1	28.5	0.562	62.1

\* Number of animals used.

† Number of potential measurements made.

‡ Variance of the estimate.

§ Sample variance for concentrations.

|| Sample variance for TEPs.

pond water; (3) transport was depressed in the third group by pre-adaptation to 40 mM/l. NaCl. The relationship expressed in equation (1) was examined, beginning with the animals in K<sub>2</sub>SO<sub>4</sub> (5 mM/l.) as described under Methods. Thus, the anion concentration and total ionic strength of the medium remained constant; only [Na<sup>+</sup>] varied. Measurements on all animals in each group were pooled, and a<sub>0</sub> and b were estimated by the method of least squares. Table 2 summarizes the results, and establishes two facts. First, since b is positive in each case there is a positive correlation between TEP and [Na<sup>+</sup>] in all three groups. Second, animals with increased sodium

uptake generated larger potentials than animals adapted to pond water. The difference between  $a_0$  values is highly significant ( $P < 0.001$ ), although no significance can be ascribed to the apparent differences in slopes ( $P > 0.1$ ). Conversely, animals pre-

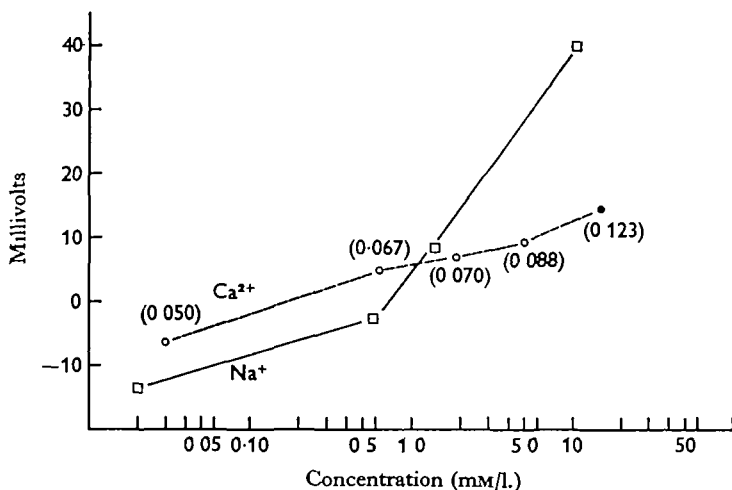


Fig. 3. TEP and  $[Ca^{2+}]$ . A single animal was immersed in 50 ml. 5 mM/l.  $K_2SO_4$ . The TEP was measured at different  $Ca^{2+}$  concentrations (O—O), and after each determination an aliquot of the solution was removed for  $Ca^{2+}$  and  $Na^+$  analyses. At the end of this series fresh  $K_2SO_4$  replaced the last test solution, and the TEP was measured at a series of  $[Na^+]$  (□—□). The numbers in parentheses give the  $[Na^+]$  of the bathing solution for each point in the  $Ca^{2+}$  series.

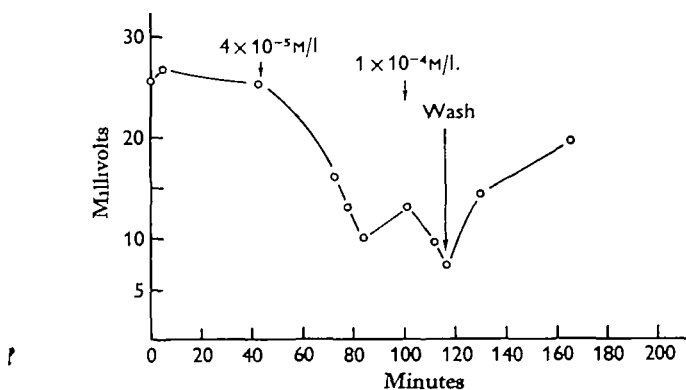


Fig. 4. The effect of dinitrophenol on the TEP. The salamander was immersed in 5 mM/l.  $K_2SO_4$  containing 1 mM/l.  $Na_2SO_4$  about 5 hr. before time zero. The potential had been stable for several hours. At the first arrow dinitrophenol was added to give the concentration shown, and at the second the concentration was increased. The inhibitor was washed out and replaced by fresh bathing solution at the third arrow.

adapted to high salinities show much smaller TEPs over the same concentration range. In this case both  $a_0$  and  $b$  are different ( $P < 0.001$ ) from their values in animals adapted to pond water.

Chemical specificity of the cation requirements was examined by using a series of alkali and alkaline earth metals.  $Li^+$  generated a TEP whose concentration dependence

was similar to that described by equation (1). A small TEP also appeared in the presence of low concentrations (0.1–0.3 mM/l.) of  $\text{Ca}^{2+}$  even in  $\text{Na}^+$ -free solutions, but this potential was little changed by further increasing  $[\text{Ca}^{2+}]$ . Fig. 3 shows the TEPs generated by  $\text{Ca}^{2+}$  and  $\text{Na}^+$  in the same animal, and the differences in magnitude and concentration dependence are apparent. The small increase in TEP at higher  $[\text{Ca}^{2+}]$  was probably due to a gradual rise in  $[\text{Na}^+]$  through the experiment (shown by the figures in parentheses near each experimental point on the calcium curve).  $\text{K}^+$ ,  $\text{Rb}^+$ ,  $\text{Cs}^+$ ,  $\text{Mg}^{2+}$  and  $\text{NH}_4^+$  were also tested; none of them generated a TEP over this concentration range.

Table 3. *TEP and [NaCl] in animals preadapted to different salinities*

Adaption medium (mM/l. $[\text{Na}^+]$ )	<i>N</i>	<i>n</i>	$a_0$ (mV.)	$b$ ( $\frac{\text{mV.}}{\log [\text{mM}]}$ )	$s_{xx}^2$	$S_x^2$	$s_y^2$
0.0	8	35	22.7	19.8	98.9	0.744	391.1
1.2	12	56	14.2	17.7	118.0	0.501	273.7
40	10	50	3.8	11.4	27.9	0.520	95.2

Table 4. *Sulphate uptake by larval Ambystoma tigrinum*

(The animals were placed in 200 ml. of 0.5 mM/l.  $\text{Na}_2\text{SO}_4$ . About 1  $\mu\text{c}$ . of  $^{35}\text{SO}_4$  was added to each container. 1.0 ml. aliquots were removed at the times specified and counted, after drying, with an ultrathin-window GM tube.)

Hours	Counts per min./ml. bathing solution)	
	Pond-adapted*	Salt-depleted†
0	2137	1864
4	2059	1901
9	2142	1986
16	2160	2001
22	2175	1994

\* Mean value for six animals. † Mean value for three animals.

The metabolic inhibitors azide and dinitrophenol abolished the TEP. Fig. 4 shows the time-course of the dinitrophenol effect in one animal, as well as the fact that it acts reversibly. Azide inhibition was irreversible. Strophanthin had no effect on the TEP when added to the bath in concentrations to 10  $\mu\text{g}$ ./ml. or when injected into the animal.

#### *The effect of anions on the TEP*

$\text{NaCl}$  had the same effect on the TEP as  $\text{Na}_2\text{SO}_4$ . This was shown in an experiment similar to that described in Table 2, except that aliquots of the initial  $\text{K}_2\text{SO}_4$  solution were replaced with  $\text{NaCl}$ . Table 3 shows the mean  $a_0$  and  $b$  values for salt-depleted, normal, and salt-loaded animals. In this experiment, too, TEPs were largest for the salt-depleted animals and smallest for the salt-loaded group. Statistical comparison of the parameters for each group in Table 3 with those for the corresponding group in Table 2 shows that there is no significant difference between them. Thus, the magnitude of the potential difference generated at any sodium concentration over this range is independent of the anion used.



These data suggest that there may be little difference between the diffusion mobilities of chloride and sulphate ions in the salamander skin. Sulphate permeability was measured by placing animals in artificial pond water made from  $\text{Na}_2^{35}\text{SO}_4$  and measuring the disappearance of isotope from the bath. Table 4 shows that no measurable uptake occurred; in another experiment a very small uptake ( $0.01-0.02 \mu\text{-equiv. (10 g.)}^{-1} \text{ hr.}^{-1}$ ) was noted. Net sodium uptake by animals under these conditions is in the range  $0.5-1.0 \mu\text{-equiv. (10 g.)}^{-1} \text{ hr.}^{-1}$ . Thus, skin permeability to sulphate is low, and this implies that leak permeability to chloride is also small.

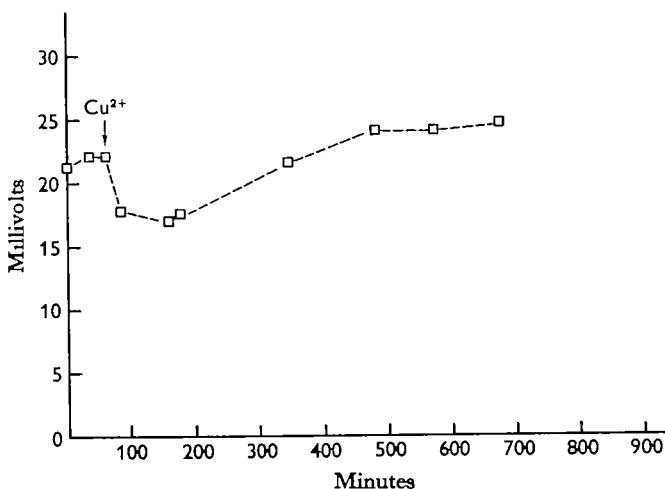


Fig. 5. The effect of copper on the TEP. Two animals were immersed in 5 mM/l.  $\text{K}_2\text{SO}_4$  containing 1 mM/l. NaCl. After 2 hours the TEP was stable and the experiment was begun (time zero). The TEP of the untreated animals was measured for 60 minutes more, then  $\text{CuSO}_4$  was added (arrow) to a final concentration of  $1.25 \times 10^{-6}$  M/l. Measurements were made as shown. Each point is the mean of the two TEPs. TEPs during the control period were nearly the same for both animals. (20.0 and 22.5 mV).

Ussing (1949) has shown that dilute  $\text{Cu}^{2+}$  added to Ringer's solution bathing the outside of the isolated frog skin augments the TEP by decreasing chloride permeability. Fig. 5 shows that low concentrations of cupric ions have little effect on the TEP generated by salamanders in pond water. A transient period of depression occurred as described by Ussing, but even after 15 hr. of exposure the potential difference was within a few millivolts of its initial value. Higher concentrations of the metal only depressed the potential. Lack of copper effect is also consistent with low chloride permeability in the untreated animal under these conditions.

Fig. 6 shows the results of an experiment in which the  $\text{K}_2\text{SO}_4$  solution was replaced by KCl. In the absence of  $\text{Na}^+$  wide variations in  $[\text{Cl}^-]$  had little effect on the TEP. The small change noted was almost certainly a concomitant of an increase in bath  $[\text{Na}^+]$ , probably from urination ( $[\text{Na}^+]$  is shown by the figures in parentheses above the curve). In spite of this the internal electrode never became positive.

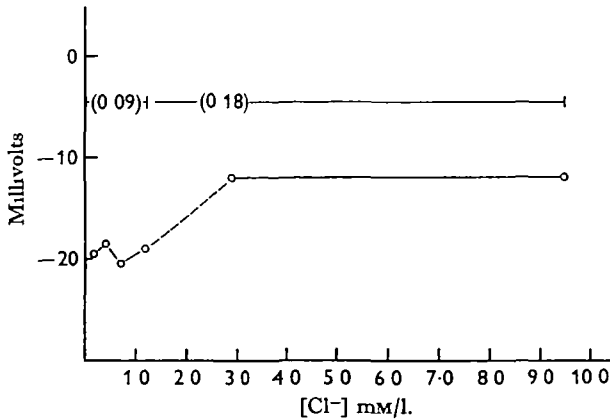


Fig. 6. TEP as a function of  $[Cl^-]$ . Protocol as in Figure 3 except that replacement of  $K_2SO_4$  was made with KCl. After each measurement an aliquot of the solution was removed for  $Cl^-$  and  $Na^+$  analyses. The numbers in parentheses give  $[Na^+]$  over the range included between two vertical lines. The abrupt rise from 0.09 to 0.18 mM/l. probably resulted from urination and is a likely cause of the small increase in TEP shown. The internal electrode remained negative throughout.

#### DISCUSSION

It is clear that when salamanders are in an ionic steady state in pond water both  $Na^+$  and  $Cl^-$  are actively transported inward. In addition, the data in Table 1 show that the transport systems can operate independently of each other. The fact that uptake of each ion in this experiment was not balanced by absorption of a counter ion can only mean that electrostatic neutrality must be maintained by a pair of ion-exchange systems. Fig. 1 shows that these animals excrete ammonia across the body surface. In 3 experiments involving 13 salamanders ammonia excretion was measured for periods of 5–7 hr. The mean efflux was  $0.71 \pm 0.27$  (S.D.)  $\mu\text{mole (10 g.)}^{-1} \text{ hr.}^{-1}$ . Sodium uptake was not measured in these animals but a mean value of  $1.3 \mu\text{mole (10 g.)}^{-1} \text{ hr.}^{-1}$  was reported by Alvarado & Kirschner (1963). Thus, ammonia excretion is somewhat lower than sodium uptake, but would provide electrostatic balance for much of it.  $Na^+/NH_4^+$  exchanges have been reported in the frog (Krogh, 1937), in the goldfish (Maetz & Garcia-Rameau, 1964), and in the crayfish (Shaw, 1960*b*), and ammonia is also excreted by *Necturus maculosus* (Fanelli & Goldstein, 1964). All of these animals have the same ionic regulatory problems as the larval salamanders used in our work, and it appears possible that such an exchange system operates to maintain electrical neutrality during  $Na^+$  uptake in all fresh-water animals. An alternative possibility in  $Na^+/H^+$  exchange, with  $NH_3$  diffusing out of the epithelial cells to maintain a constant cytoplasmic pH.

Our data leave little doubt that active sodium transport plays a central role in electrogenesis under the conditions studied. Of the alkali and alkaline earth metals tested only  $Na^+$  and  $Li^+$  generated a concentration-dependent TEP. The effective concentration range, the size of the TEPs and nature of the relationship, including its maximum at 20–30 mM/l., have also been described in the intact frog (Brown, 1962) and in isolated frog skins (Steinbach, 1933; Linderholm, 1952). In isolated frog skins the same chemical specificity and concentration dependence are characteristic of the sodium-transport mechanism, and provide part of the evidence that it is

involved in generating the TEP (Kirschner, 1955; Ussing & Zerahn, 1951). The correlation between transport rate and TEP, suggested by the concentration dependence, was confirmed by comparing TEPs in salt-depleted (augmented transport), normal, and salt-loaded (depressed transport) animals.

Depression of the potential by metabolic inhibitors may also be a concomitant of transport inhibition. However, these compounds also effect the diffusion permeability of some preparations, for example the isolated frog skin (Nakajima & Hashimoto, 1960; confirmed by unpublished experiments in our laboratory). Such an increase in passive ionic conductance would also tend to depress the potential, and this makes interpretation of inhibitor studies equivocal. However, the results reported here are compatible with participation of sodium transport in electrogenesis.

Lack of effect of strophanthin in the TEP was the only observation apparently inconsistent with a direct role for the sodium pump. Addition of the inhibitor to the external bathing solution may have prevented it from reaching the transport locus, but injection of the compound in quantities sufficient to give blood concentrations of 1–10  $\mu\text{g./ml.}$  were also ineffective. These observations are in contrast with similar studies showing that strophanthin inhibits sodium transport across many epithelia (Nakajima, 1960).

Our experiments also show that salamander skins have a low leak conductance for chloride in dilute solutions.  $\text{Cl}^-$  uptake is thermodynamically active, and can occur independently of simultaneous cation absorption. Fig. 6 shows that  $\text{Cl}^-$  uptake is not electrogenic. These observations suggest that a tightly coupled exchange against another anion must be involved. We have no evidence concerning the identity of the exchangeable anion. A  $\text{Cl}^-/\text{HCO}_3^-$  exchange across the body surface has been described in frogs (Krogh, 1937), crayfish (Shaw, 1960a) and fresh-water teleosts (Maetz & Garcia-Rameau, 1964). Such exchanges are also well known in other epithelia, and the ready availability of  $\text{CO}_2$  makes bicarbonate a probable member of such an exchange system in the salamander.

Thus, the TEP in larval salamanders in dilute saline appears to be generated by active  $\text{Na}^+$  transport with electrostatic neutrality maintained by cation exchange. External anions play no role in electrogenesis. The experiments of Barker-Jørgensen *et al.* (1954) and Krogh (1937) show that this may also be the case in frogs. Yet there is little doubt that isolated frog skins bathed by Ringer's solution have an appreciable diffusion permeability to  $\text{Cl}^-$  and that influx of this ion maintains electrostatic neutrality during  $\text{Na}^+$  transport. Either isolation of the skin or the use of Ringer's solution outside seems to cause a change in leak permeability to chloride. This change is being investigated.

#### SUMMARY

1. Larval salamanders in pond water actively transport both  $\text{Na}^+$  and  $\text{Cl}^-$  inwards. The two fluxes can occur independently, indicating that they are not linked obligatorily through a single mechanism. Ammonia is excreted extrarenally at rates comparable with active  $\text{Na}^+$  influx.

2. A potential difference exists across the body surface of larval salamanders under these conditions. Over the range 0.1–10 mM the transepithelial potential (TEP) varies approximately logarithmically with  $[\text{NaCl}]$ . Only  $\text{Na}^+$  and  $\text{Li}^+$  salts generate

such a TEP, while  $K^+$ ,  $Rb^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ , and  $NH_4^+$  salts have little effect. Azide and dinitrophenol abolish the TEP, while strophanthin has no effect. When active  $Na^+$  uptake is enhanced by salt-depletion, higher TEPs are generated. Conversely, animals with  $Na^+$  transport depressed by salt-loading show significantly smaller TEPs.

3. The magnitude of the TEP is unaffected by replacing  $NaCl$  with  $Na_2SO_4$ . Permeability of the body surface to  $SO_4^{2-}$  is very low, and the similarity in electrical behaviour of the two anions suggests that  $Cl^-$  penetration by diffusion is also small. This surmise is supported by two observations: (1)  $KCl$  generates no TEP over a wide range of concentrations; and (2) cupric ion has no effect on the TEP developed in dilute  $NaCl$  solution.

4. It is suggested that the TEP in salamanders is generated by active inward transport of  $Na^+$ , but that in dilute solutions electrostatic neutrality is maintained by a cation-exchange system involving ammonium ion. Uptake of  $Cl^-$  under these conditions appears to occur by an independent mechanism and is non-electrogenic.

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