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THE IONIC REQUIREMENTS OF TRANSEPITHELIAL POTENTIALS IN *HYDRA*

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The epithelial layers of hydra maintain an electrical potential across the body wall; the enteron is electrically positive with respect to the bathing medium (Josephson and Macklin, 1967). Negative-going action potentials are superimposed on the transepithelial resting potential. These action potentials are called contraction pulses (CP's) because they precede and are probably causally related to contraction of the hydra body column, a shortening due to contraction of muscular elements in epitheliomuscular cells of the ectoderm (Passano and McCullough, 1964; Josephson, 1967). The CP's appear both spontaneously and in response to electrical stimulation. They propagate in the column ectoderm at a velocity of 4 to 5 cm/sec (Kass-Simon and Passano, 1969; Josephson, 1967). There are nerve cells in the ectoderm but their influence on the initiation and propagation of CP's is unknown at present.

The transepithelial potentials of hydra are of interest for several reasons. Hydra live in fresh water. Osmotic experiments and chemical analysis indicate that the cells of hydra are hyperosomotic to the medium and are permeable to water (Lilly, 1955; Steinbach, 1963; Koblick and Yu-Tu, 1967); therefore, there should be a net influx of water. However, hydra have no known organs or organelles for extruding water. It has been suggested that the resting potential of hydra reflects activity of ion transport mechanisms used in osmoregulation (Macklin, 1967). Evidence supporting this is given below. Further, the CP system in hydra may be an example of an epithelial conducting system of which several are known in Cnidaria (Mackie, 1965; Mackie and Passano, 1968). Epithelial conducting systems are likely precursors of nerve cells and an understanding of the physiology of epithelial conduction should give insight into the early evolution of nervous systems. Epithelial conduction has been recently demonstrated in a larval amphibian (Roberts, 1969) suggesting that excitable epithelia may be widespread in the animal kingdom and possibly a significant component in the control of behavior in higher animals as well as in Cnidaria.

In the previous study we used electrical techniques to examine properties of the epithelial layers of hydra (Josephson and Macklin, 1969). The body wall acts as a linear resistance of approximately 5 Kohms-cm^2 to low frequency currents with a density less than about 4 μA/cm^2. With stronger transverse current the column resistance is nonlinear, the nonlinearity having both initial and delayed components. Impedance analysis using A. C. current indicates that the body wall can be represented as an electrical network with a minimum of three time constants. The amplitude and frequency of CP's are unaltered by imposed current, and there is no significant change in column impedance during CP's. These features of the CP are consistent with the hypothesis that the CP-generating membrane forms but a small fraction of the total body wall impedance.

TABLE I

Experimental solutions used. All concentrations are in mM/1.

Solution name	Na^+	K^+	$Tris^+$	Ca^+	Mg^{++}	Cl^-	HCO_3^-	SO_4^-	EDTA	Sucrose	$CH_3SO_3^-$
Culture Solution	1.68			1.5		3.0	1.2		0.12		
Normal	1.5			1.5		3.0	1.5				
Na^+free, K^+		1.5		1.5		3.0	1.5				
Na^+free, $Tris^+$			1.5	1.5		3.0		0.6		0.9	
Ca^{++}free, Mg^{++}	1.5				1.5	3.0	1.5				
Ca^{++}free, Sucrose	1.5						1.5			4.5	
Ca^{++}free, $EDTA^-$	1.5								0.48	5.52	
Cl^-free, $CH_3SO_3^-$	1.5			1.5			1.5				3.0
HCO_3^-free, $CH_3SO_3^-$	1.5			1.5		3.0					1.5
$Cl^-+HCO_3^-$free, SO_4^-	1.5		1.61	1.5				2.89			
$Cl^-+HCO_3^-$free, $CH_3SO_3^-$	1.5			1.5							4.5

Having characterized the electrical properties of the body wall, we next investigated the role played by each of the ions found in the hydra culture medium. Ham, Fitzgerald and Eakin (1956) found that hydra grew well in a medium containing only $CaCl_2$ and Na_2EDTA. Loomis and Lenhoff (1956) used a medium containing $CaCl_2$, Na_2HCO_3 and Na_2EDTA. In each case additional ions are supplied by the food, which is, in most laboratory cultures, newly hatched *Artemia*. Since the Loomis and Lenhoff medium is most commonly used, the effects of sodium, calcium, chloride, and bicarbonate ions and of osmotic pressure on the generation of the resting potential and CP's were studied and are reported here.

MATERIALS AND METHODS

All of the experiments were performed on *Hydra oligactus* raised as described in Josephson and Macklin (1969). The animals were starved for 24 hours before being used and all experiments were conducted at room temperature (20 to 24° C).

A number of solutions varying in ionic composition was used in our study. The composition of these is listed in Table I. Each of the test solutions was prepared so that it had a calculated osmolarity of 7.5 mosmol, the same osmolarity as the culture solution. The standard for comparison in the experiments was termed "normal solution." This solution differs from the culture solution primarily in the absence of EDTA. EDTA is included in the culture medium to remove heavy metal ions which can be toxic in small concentrations (Loomis and Lenhoff, 1956). Generally the effect of each solution on electrical activity in an animal was obtained (a) when the animal was bathed by the test solution while its gut was perfused with normal solutions, or (b) while the gut was perfused by test solution with the animal bathed in normal solution. The choice of normal solutions as the usual gut perfusate is somewhat arbitrary. The mouth of hydra is normally kept closed so the enteron fluid is separated from and is probably of a composition different than the bathing medium. However, hydra sometimes opens its mouth for extended periods and contact with bathing medium is seemingly not deleterious to the endodermal cells. Also osmotic and ionic gradients across the body wall are minimized by using normal solution as the internal perfusate.

All of the solutions were prepared in distilled water with reagent grade chemicals. When methane sulfonate ($CH_3SO_3^-$) was substituted for the chloride or

bicarbonate anions, stock solutions were prepared by titrating methanesulfonic acid with sodium hydroxide to a pH of 7.4 ± 0.2. To obtain the proper pH for the calcium free EDTA solution a mixture of Na_4EDTA and Na_2H_2EDTA was used. When tris base was used, it was titrated with sulfuric acid for pH control.

Other than for these three cases there was no control over pH of the test solutions. However, the pH measured for all solutions used fell between 7.1 and 7.7.

In all experiments a hydra was mounted on the glass holder previously described (Josephson and Macklin, 1969) and shown schematically in Figure 1. The experimental dish holding the external medium had two compartments—a large chamber to permit the animal to be mounted easily on the holder and a small perfusion chamber in which the actual measurements were done. At the start of each experiment the dish was filled with normal solution and the animal was placed on the holder in the large chamber. The holder was then moved horizontally from the large chamber into the perfusion chamber, which had a capacity of 10 ml, and a partition was inserted to isolate the perfusion chamber from the large compartment.

The exchange of fluid in the test chamber was studied by measuring the time constant for the appearance and disappearance of a dye. The dye concentration in the test chamber was monitored by the transmission of light measured with a photocell. The time constant for fluid exchange was about 10 seconds. Assuming good mixing and a time constant of 10 seconds approximately 99.9% of the fluid is exchanged in 70 seconds. To be conservative, external perfusion was continued for 150 seconds each time a solution change was made.

The gut of the test hydra was perfused in these experiments by passing a fine pipet through the holder into the gut. This pipet (P_i in Fig. 1) was connected to a perfusion pump which supplied fluid at a flow rate of 0.7 to 3.5 μl/min. The lower flow rate was only used when flow out of the gut of the animal up into the cup of the holder was retarded due to debris (cells and mucus) collecting in the narrow annulus between the perfusion pipet and the holder. When the flow from the exit was impeded in this way the animals would swell if the inlet flow was not reduced. To maintain a constant pressure head in the gut of the hydra, fluid was removed from the holder cup by a suction pipet, S_i. The gut capacity was estimated to be one microliter from measurements of the outside dimensions of hydra on the test holder. Therefore the turnover of the internal fluid was considerably less than the external fluid. To increase the exchange rate of fluid the internal perfusion tube was placed close to the bottom of the gut so that the perfused fluid rose through the animal as a column. However, because of the slow rate of internal perfusion, only qualitative results for these experiments are reported.

The electrical potential across the body wall was recorded with a high impedance D.C. amplifier and salt bridge electrodes consisting of chlorided silver wires in glass pipets filled with 2 M KCl-Agar.

The transverse impedance of the body wall was determined by measuring the voltage change to imposed sinusoidal current. The current intensity was 0.1 or 0.2 μA and the frequency 1 or 5 Hz. To currents of this amplitude and frequency the body wall acts as a purely resistive element (Josephson and Macklin, 1969). The internal current electrode replaced the internal perfusion pipet so

these animals were not internally perfused. After each test with a set of different solutions the impedance of the apparatus alone was determined by removing the animal without disturbing the current and voltage electrodes and again determining the ratio between voltage and imposed current in each of the solutions used. The apparatus impedance varied slightly between the different test solutions because of their different conductivities. The impedance of the apparatus for a given solution was subtracted from that measured with the animal in place to get the impedance of the body column alone.

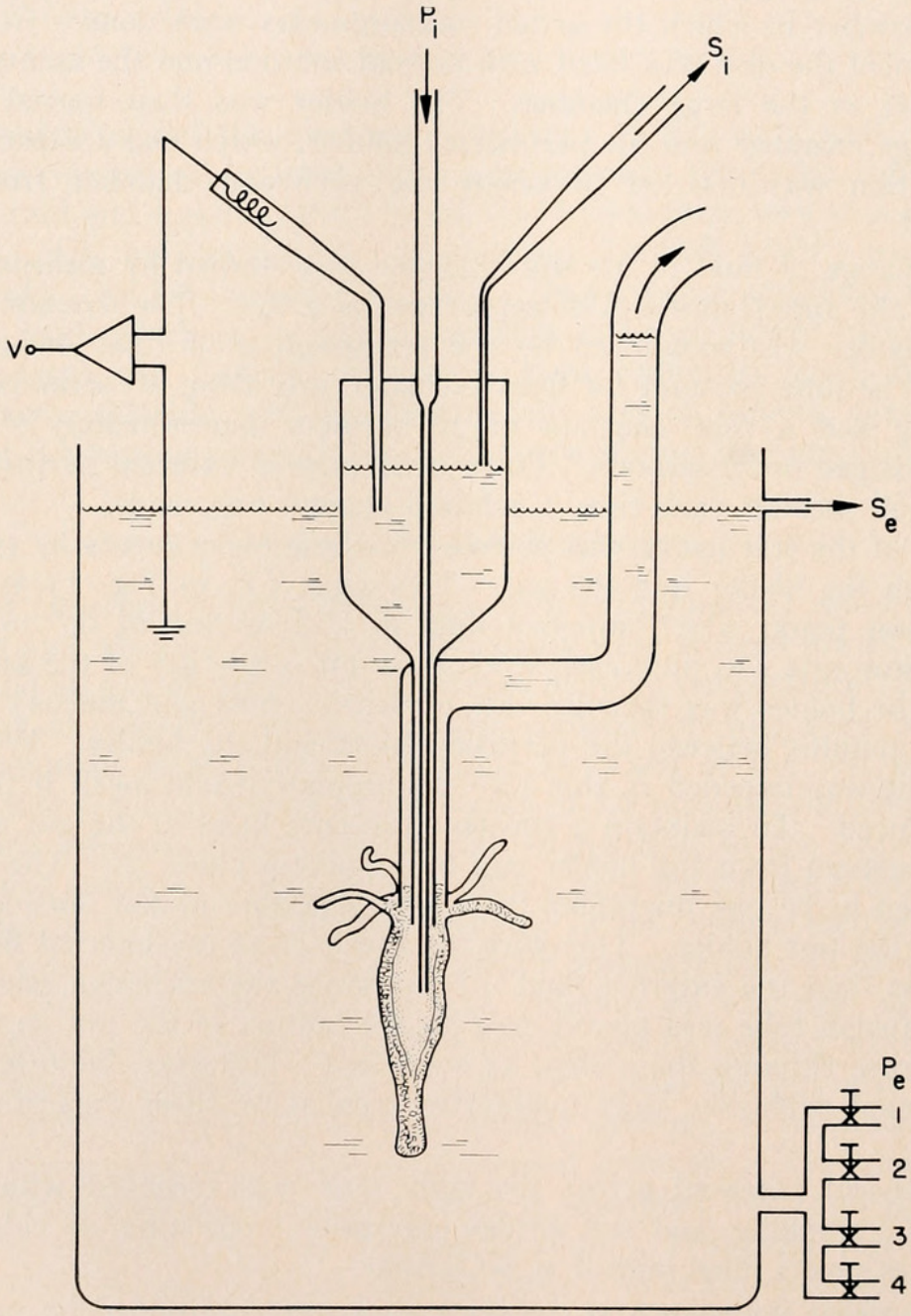

FIGURE 1. The experimental apparatus. An animal is shown on the glass holder held on by suction applied to the unmarked tube. The animal was internally perfused through the pipet P_i and the perfusate was removed *via* S_i. The external fluid was perfused through one of the four inlets, P_e, and removed *via* S_e. The transepithelial potential was measured between the cup of the holder and the bathing solution.

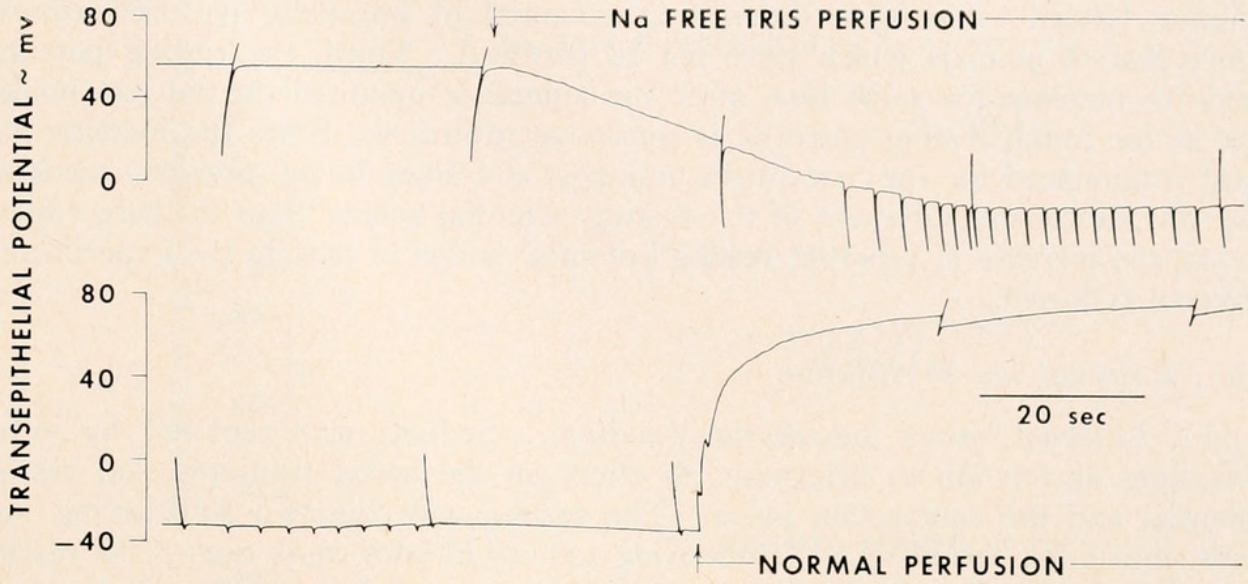

FIGURE 2. Effect of Na-free tris solution on the outside of the animal. The electrical records in this and the following figures were recorded with a curvilinear penwriter. The record begins (top) prior to external perfusion with Na-free tris. The record is broken before perfusion was complete and begins again (bottom) one minutee prior to perfusion with normal solution. Positive going pulses are stimulus artifacts which are separated by 30 sec. Note the decrease in CP magnitude during perfusion and oscillations during the readmission of normal solution. This was the second ten minute period of exposure to Na-free tris for this animal.

When testing different solutions, systematic errors were minimized by changing solutions in random sequences with the use of a random number table. For each solution change several readings were taken. Preliminary tests established that the potentials became stable less than 5 minutes following a solution change. Accordingly 5 to 10 minute test periods were selected for different experimental solutions. For a 5 minute test period, the resting potential was measured at 3, 4 and 5 minutes after the beginning of the solution change. For a 10 minute test period, measurements were made at 6, 7, 8, 9 and 10 minutes. The resting potential for the test period was then taken as the average of the measured values. For any test sequence all test periods were of the same length and the external perfusion during solution changes always lasted 150 seconds. Each test sequence was repeated several times for each animal. The average values of the potential obtained in each test period were themselves averaged. The data reported are a result of the analysis of data from several animals for each separate experiment.

RESULTS

The transepithelial resting potential reported in this paper for the test animals are noticeably higher than the values previously reported (Josephson and Macklin, 1967, 1969). We can attribute this increase to several factors. All previous experiments were done with culture solution (Table I) on the outside of the animal, whereas the standard for comparison in this study was the normal solution (Table I) which did not contain EDTA. A preliminary test indicated that the resting potential is greater when the animal is bathed in normal solution than when it is bathed in culture solution. Second, the perfusion of the gut with normal

solution tended to increase the resting potential of internally perfused animals above that of animals which were not so perfused. Third, the resting potential tends to increase for some time after the animal is mounted on the test holder, and so the initial resting potential is somewhat arbitrary. Since the increase was most pronounced for the first fifteen minutes, and since in the previous work we had started the measurement of the resting potential sooner than in these experiments, the increase in reported resting potential is due in part to the experimental protocol followed.

(A). *External ion substitution*

(1). *External cation substitution—sodium.* Sodium was replaced by either potassium or tris ion to determine its effect on the hydra transepithelial resting potential and the contraction pulse. The results with the two substituting ions were similar but not identical. Removing sodium in both cases caused the resting potential to fall. For three animals the average potential in normal solution was 67 mv (range 61 to 75) and in sodium-free potassium solution it was 1.1 mv (range −4.4 to 9.1). Similarily for three other animals the average resting potential in normal solution was 63 mv (range 52 to 77) and in sodium free tris solution it was −19 mv (range −26 to −10). These resting potentials were measured for the last five minutes of ten minute test periods as described above; two to five test sequences were done with each animal. The results show that maintenance of the potential requires sodium in the external medium and neither potassium nor tris will substitute.

The changes in the resting potential at the onset of perfusion with sodium-free solution and at the readmission of solution containing sodium are not symmetrical; the latter is much more abrupt (Figs. 2, 3 and 4). This suggests that the relation

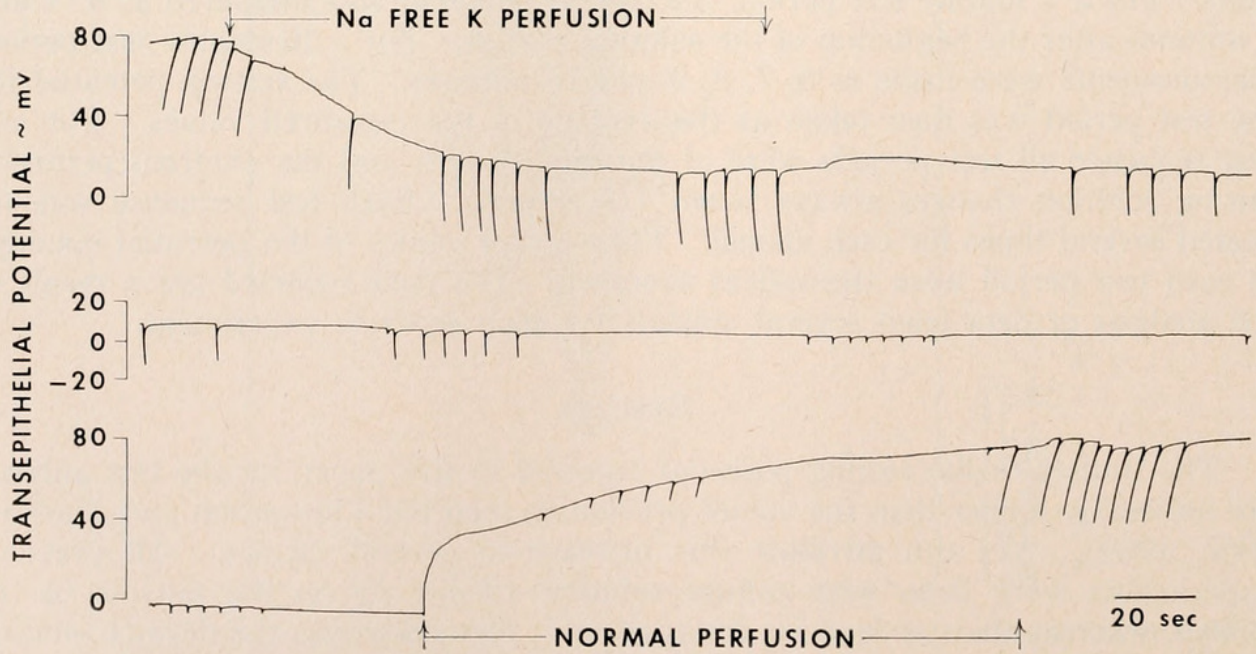

FIGURE 3. The effect of external Na-free-K perfusion. The complete record for the first ten minute period of exposure to Na-free-K for this animal is shown. Note the two step recovery of resting potential following readmission of normal solution.

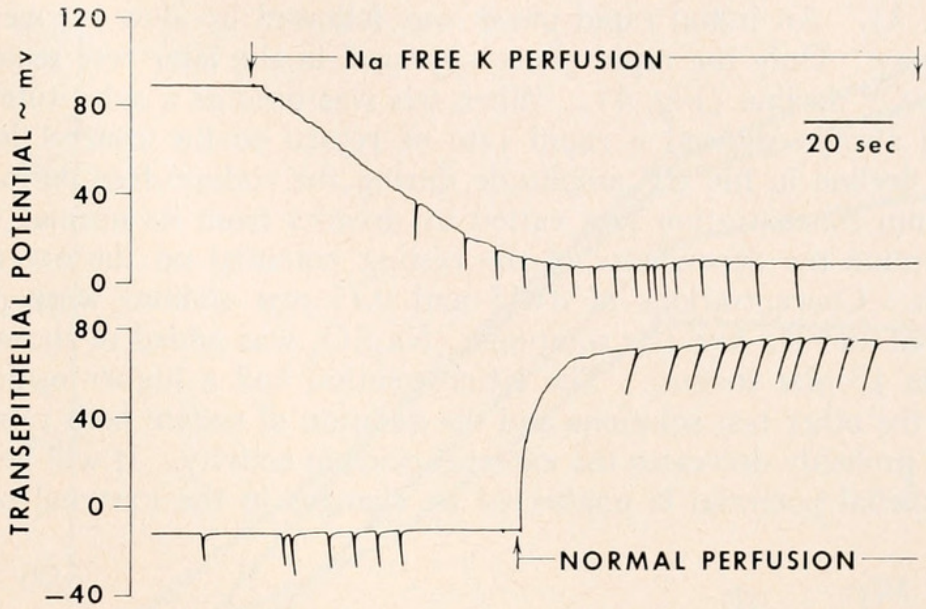

FIGURE 4. The effect of external Na-free-K perfusion. This is a portion of the record for the fourth ten minute exposure to Na-free-K for the same animal as Figure 3. Note constancy of CP size and the one step recovery of the resting potential.

between sodium concentration and the resting potential is nonlinear, and that an appreciable resting potential is present at relatively low sodium concentrations. Since the solution exchange during perfusion is approximately exponential it takes considerable time to reduce the sodium concentration to low levels when perfusing with sodium-free solutions, while the sodium concentration rises steeply when solution containing sodium is readmitted. The resting potential records frequently showed two or three oscillations when sodium was readmitted to the external medium (Fig. 2). We do not know whether these oscillations are inherent properties of the mechanism generating the resting potential or if they are due to sudden surges of sodium rich solution passing the animal in the turbulent inflow, although the former seems more likely.

The CP amplitude often falls during the period when the animal is in a sodium-free external medium (Figs. 2 and 3). The time course of CP decline was examined by stimulating the column electrically to produce CP's at regular intervals. The stimulating electrode was a suction electrode on the basal disc (see Josephson, 1967). The stimuli were 1 to 3 msec current pulses somewhat above CP threshold. Examples of results from these experiments are shown in Figures 2 and 5. The decline of the amplitude of both evoked and spontaneous CP's was most marked during the initial exposures with sodium free solution; in later exposures the CP size showed little or no changes throughout the period in sodium-free solution (Fig. 5 and compare Figs. 3 and 4). The CP generating system seemingly adapts to changes in the external environment. Even when their amplitude has decreased greatly, CP's return to normal size within a few minutes after sodium is reintroduced in the external medium (Figs. 3 and 5).

The resting potential did not show the same type of adaptation during repeated test sequences as did the CP's. However, when the sodium was replaced by potassium, it was noted that if the CP's declined in sodium-free solution then the resting potential return contained two distinct phases when the sodium was read-

mitted (Fig. 3). An initial rapid phase was followed by slow recovery lasting a minute or more. Only the rapid phase appeared in the later test sequences when there was no CP decline (Fig. 4). When tris was used as a substitute for sodium the potential always showed a rapid rate of return to the control level even if there was a decline in the CP amplitude during the sodium-free period (Fig. 2).

The sodium concentration was varied by decades from its normal value of 1.5 mM to determine the dependence of the resting potential on the external sodium concentration. Concentrations of 0.015 and 0.15 mM sodium were prepared by mixing normal and Na-free-tris solutions. Na_2SO_4 was added to the normal solution to obtain 15 mM sodium. The latter solution had a higher osmotic concentration than the other test solutions and the addition of sulfate both raises the ionic strength and probably decreases the external calcium activity. It will be shown that the transepithelial potential is unaffected by changes in the external osmotic con-

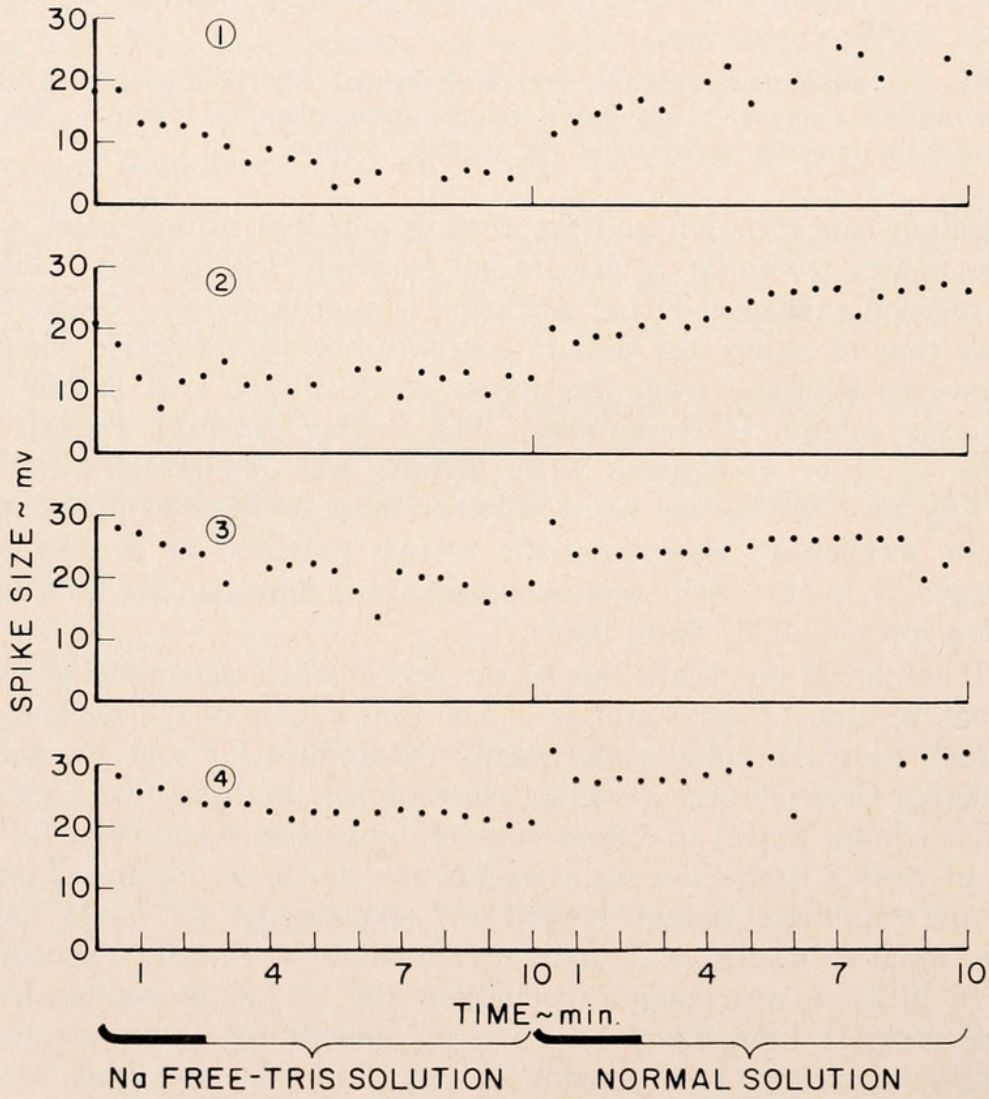

FIGURE 5. Pattern of CP adaptation to external Na-free perfusion. Every 30 seconds a CP was evoked by a stimulus and its magnitude measured. Note the decline and recovery of the CP's in the first fluid exchange sequence and the gradual adaptation with each subsequent exchange. External perfusion occurred during the first 2.5 minutes of each 10 minute period. Occasional missing points are due to increases in the CP threshold. Each time the CP system failed to respond the stimulus intensity was increased slightly until the stimuli were again effective.

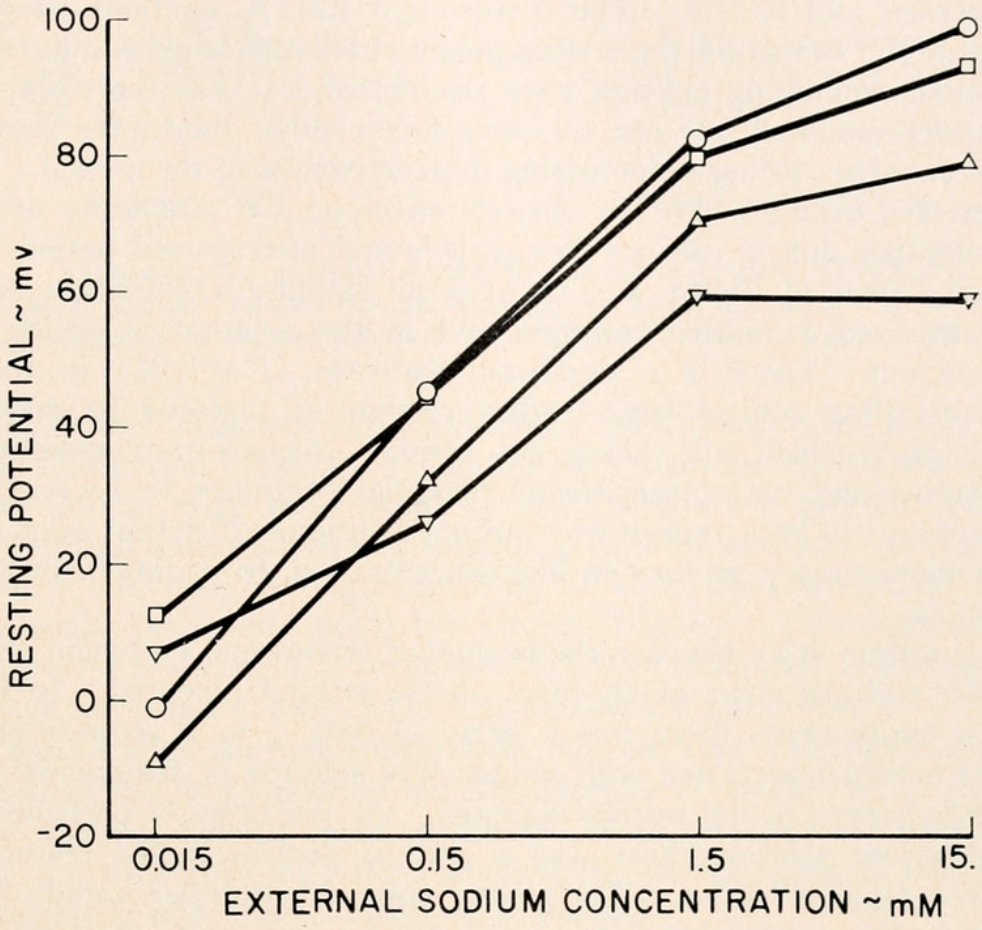

FIGURE 6. Dependence of transepithelial resting potential on external sodium concentration. Records for four animals are shown. Each point is the average of four measurements taken in random order.

centration in this range and that sulfate can replace both Cl^- and HCO_3^- in the bathing solution with no significant change in the transepithelial potential. Although the effects of small changes in external calcium concentration and of ionic strength have not been investigated it seems likely that the changes in transepithelial potential seen with this solution are due solely to the increased sodium concentration.

The resting potential increased monotonically with sodium concentration (Fig. 6). The rise is initially logarithmic but shows evidence of saturating at higher concentrations. The average slope for the initial logarithmic phase (0.015 to 1.5 mM sodium) is 35 mv per decade change in sodium concentration, rather less than the 58 mv per decade expected if the resting potential were entirely attributable to a sodium diffusion potential. This and the saturation at high external sodium concentration suggests that the resting potential is largely the result of an inwardly directed sodium transport system. Externally applied 10^{-5} M and 5×10^{-4} M ouabain was without obvious effect on the resting potential or CP's. No other inhibitors have been tried.

(2.) *External cation substitution—calcium.* Calcium is the only cation besides sodium reported as being necessary for normal growth in the bathing solution of hydra (Ham *et al.*, 1956; Loomis and Lenhoff, 1956). To determine the effects of external calcium we replaced it in the bathing solution sequentially with mag-

nesium, sucrose, and EDTA. The sucrose and EDTA solutions were obviously detrimental to the animal for the resting potential fell and failed to return to normal when solutions containing calcium were readmitted. It was therefore not appropriate to order randomly the test solutions used; rather they were presented in a sequence ordered according to increasing destructiveness to the animal. In no case did calcium-free medium have an obvious effect on CP amplitude or frequency. Resting potentials during calcium free periods and interspersed periods in normal solution are shown in Figure 7. There is no significant difference between the potential measured in normal solution and in the solution in which magesium replaced calcium. There is a significant decrease ($P < 0.01$) in the potential from the preceding control period when calcium is replaced by sucrose or by EDTA. EDTA, which will chelate any residual calcium or magnesium, is especially damaging and the transepithelial potential continues to decline even after EDTA solution has been replaced by normal solution. External calcium or magnesium seems necessary, at least in low concentration, to maintain the integrity of the animal.

There is a time delay between the beginning of external perfusion with calcium free solution and the onset of an effect on the resting potential. In the sodium substitution experiments there was a delay of only 1 to 3 seconds between the initiation of external perfusion with sodium free solution or readmission of normal solution and changes in the resting potential. At the onset of perfusion with calcium free sucrose solution there was a gradual decline in the resting potential rather than a rapid reduction. The resting potential decreases rapidly but after a delay of 15 to 20 seconds when calcium free EDTA was used. In both cases there are changes in the resting potential 1 to 3 seconds after readmission of calcium.

When normal solution is admitted following EDTA perfusion, the resting

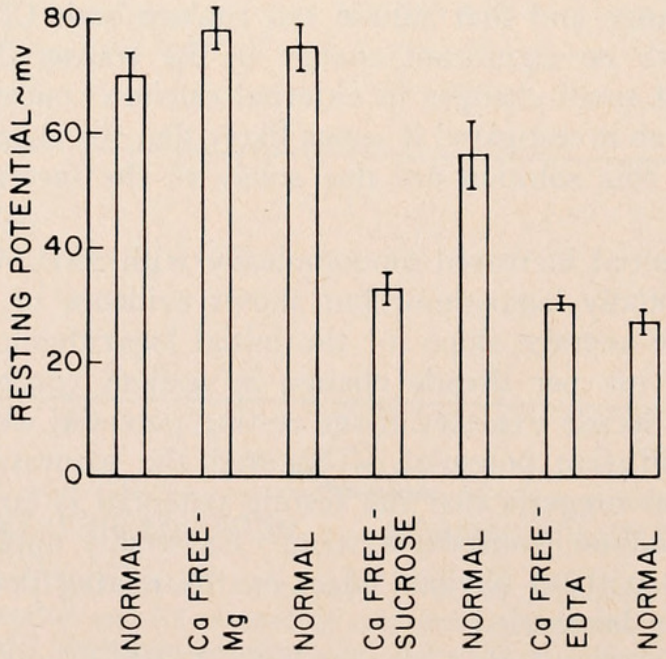

FIGURE 7. Dependence of transepithelial resting potential on presence of calcium and various calcium replacements. Data points were taken in the order plotted. Test periods were each 10 minutes long and only one test sequence was done with each animal. Each bar shows mean ± SE for 6 animals.

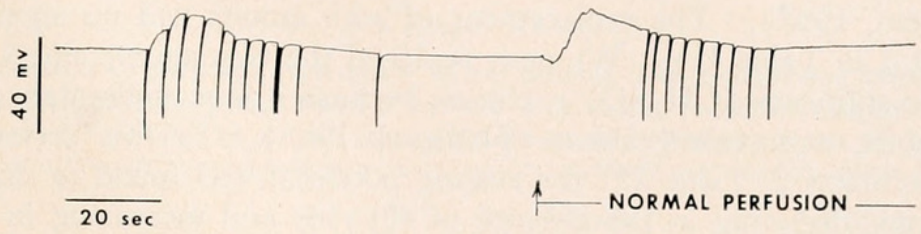

FIGURE 8. Transepithelial potential during CP bursts in Ca-free EDTA and immediately after admission of normal solution to the external perfusion chamber. Note the transient accompanying admission of normal solution. The internal perfusate was normal solution.

potential transiently increases and then drops back toward the level reached in the calcium free period (Fig. 8). The transient increase sometimes had oscillations on its leading edge reminiscent of those seen when sodium is readmitted following perfusion with sodium free solution. In most solutions the resting potential is stable or decreases during a burst of CP's; in EDTA solution the resting potential typically increased during CP bursts (Fig. 8). The significance of the increase in potential during CP firing in EDTA solution and following the readmission of normal solution after EDTA perfusion are yet obscure.

These results with calcium substitution indicate that external calcium is not directly involved in CP production but that calcium or magnesium is necessary in the bathing medium for the maintenance of the transepithelial resting potential. Judging by the time course of the response, the participation of calcium in the resting potential is less direct than that of sodium.

(3.) *External anion substitution.* The anions in the usual hydra culture solution are chloride and bicarbonate. The effect of these was first tested by replacing them both with sulfate which is generally assumed to be less permeant than chloride

TABLE II

Effect of external anion substitution on resting potential. For experiment 1, 8 animals were tested for 6 or 7 test sequences each, and for experiment 2, 6 animals were tested for 5 test sequences each. Each test solution was used for a 5 minute period. Normal solution was perfused interally during all tests. The difference between the measured values is not statistically significant in experiment 1 ($P > 0.1$). The values obtained in $Cl^- + CH_3SO_3^-$ and $CH_3SO_3^-$ are not significantly different ($P > 0.1$), all of the other values are significantly different from one another ($P < 0.01$, except $P < 0.05$ for pair $Cl^- + HCO_3^-/HCO_3^- + CH_3SO_3^-$). The difference in resting potential in normal solution ($Cl^- + HCO_3^-$) in the two experiments is indicative of the variations seen in experiments conducted with different groups of animals.

Anions present	Resting potentials ± SE mv
Experiment 1	
$Cl^- + HCO_3^-$	71 ± 2
$SO_4^=$	67 ± 4
Experiement 2	
$Cl^- + HCO_3^-$	56 ± 3
$Cl^- + CH_3SO_3^-$	73 ± 3
$HCO_3^- + CH_3SO_3^-$	49 ± 2
$CH_3SO_3^-$	69 ± 2

(see *e.g.*, Stein, 1967). The replacement of both anions had no significant effect (Experiment 1 in Table II). We next replaced the two anions singly and jointly with methane sulfonate ($CH_3SO_3^-$), chosen because it is a moderately large, seemingly innocuous, monovalent anion (Milligan, 1965). In this series of experiments (Experiment 2, Table II) the resting potential was found to depend on the external anions, declining in the absence of chloride and increasing in the absence of bicarbonate.

(4.) *Change in impedance during ionic substitution.* In the first set of experiments the impedance and resting potentials were measured for 5 animals in normal solution immediately before perfusing with sodium or calcium free solution (tris or sucrose substitution). The impedance and transepithelial potential were then measured after 10 minutes in the ion substitution solution and then again 3 minutes after the beginning of external perfusion with the normal solution. In these experiments (A and B in Fig. 9) there was a significant change in potential but no statistically significant change in impedance due to the ion substitution. In a second set of experiments (C in Fig. 9) the column impedance and resting potential were compared for five animals when the animals were bathed with chloride-free methane sulfonate and with bicarbonate-free methane sulfonate, the solutions which gave the greatest difference in resting potential in Table II. In these experiments the test periods were five minutes long and each animal was alternately bathed in the two solution 3, 4 or 9 times; the series being ended when the animal pulled off the holder or pulled the current electrode through the body wall during column contraction. There was again no significant impedance change although

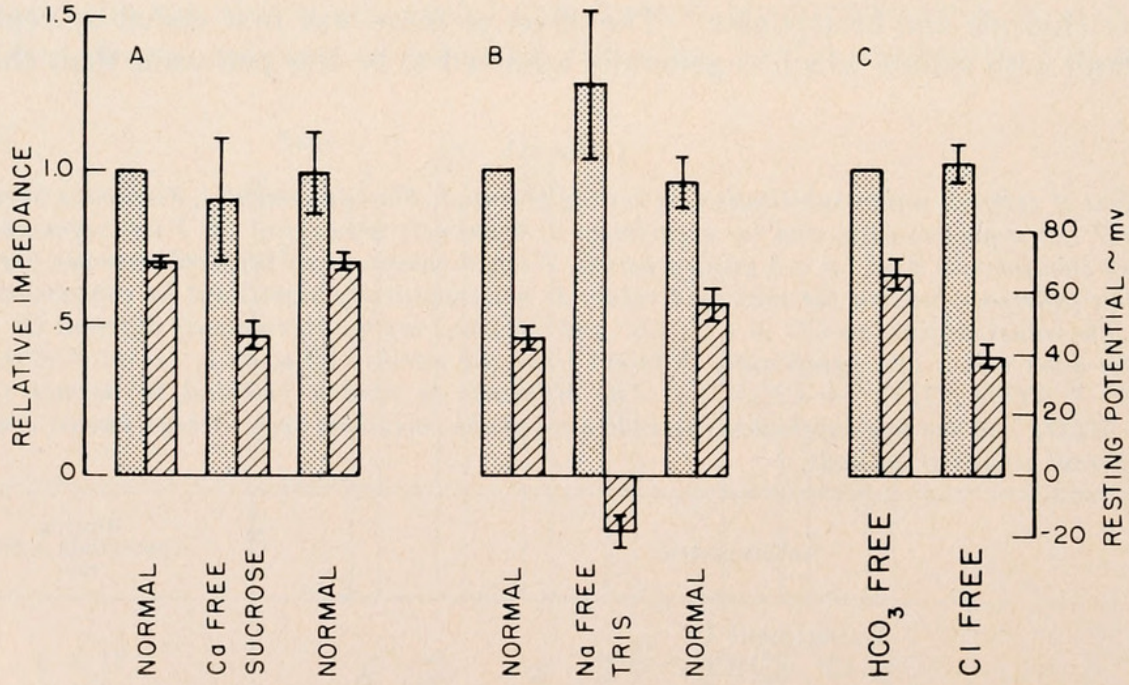

FIGURE 9. Effect of external ion substitution on body wall impedance. The left bar of each pair is the column impedance and the right bar is the mean resting potential. The impedance is shown relative to the first solution of each series. Means ± S.E. are plotted for five different animals in A, B and C. In all three cases the resting potential differs significantly in the two solutions ($P < 0.01$) but the impedances do not ($P > 0.1$). Test currents used were: A—0.2 μAmp at 1 Hertz, B—0.1 μAmp at 1 Hertz, C—0.2 μAmp at 5 Hertz. No internal perfusion was used in these tests.

TABLE III

Effect of anions on body wall impedance for one animal. This animal was tested for 9 test sequences; each test period lasted 5 minutes. The resting potential is significantly different ($P < 0.01$) in the two solutions whereas the impedance is not ($P > 0.1$).

Solution	HCO_3^- free, $CH_3SO_3^-$	Cl^- free, $CH_3SO_3^-$
Anions present	$Cl^- + CH_3SO_3^-$	$HCO_3^- + CH_3SO_3^-$
Potentials ± SE~mv	76 ± 2	43 ± 1
Impedance ± SE~Kohms	117 ± 7	124 ± 8
Current ± SE~μAmp	0.67 ± 0.04	0.36 ± 0.03

the resting transepithelial potential differed markedly in the two test solutions. Table III gives the results from the single animal from which the most replicate measurements were obtained in the anion substitution series.

If a hydra has its mouth closed and the resting potential is constant there is no net current flow across the body wall. Active current due to ion transport mechanisms and gradients is balanced by passive current due to the voltage gradient. The active current component can be estimated by the ratio of transepithelial potential and transverse impedance. For hydra this ratio is a good estimate of short circuit current, the current measured when the transepithelial potential is held at zero (Table III of Josephson and Macklin, 1969; in this table the resting potential should be 22 ± 2 mv). The short circuit current calculated in this way for one experiment is shown in Table III. Chloride enhances and bicarbonate reduces transepithelial current.

(B.) *Internal ion substitution*

For the internal ion substitution experiments, two perfusion pipets were used which were driven by the same syringe pump. The animal was first perfused internally with normal solution and then the pipet used was replaced by one delivering the ion replacement solution. The animal was perfused internally with the test solution for 20 minutes and then again perfused internally with normal solution. Perfusion pipets were changed in 20 to 30 seconds.

Most internal ion substitutions were without obvious effect. There was no noticeable change in resting potential or CP's when the gut was perfused with sodium free solution (tris or potassium substitution), with solutions in which chloride and bicarbonate were replaced singly or together with sulfate or with solutions in which calcium had been replaced by sucrose. For periods of up to 2 hours the gut was perfused with normal solution which is potassium free without any effect on the resting potential or CP's, indicating that these potentials do not depend on potassium in the enteron fluid. When EDTA was used as a calcium substitute in the internal perfusate, the resting potential slowly decreased by 30 to 40 mV but recovered essentially to the pretest level within three minutes after normal solution was introduced into the enteron. In this case the effect of EDTA solution is not irreversibly destructive as it is on the outside of the animal.

Of greater interest was the effect of EDTA on the CP's. The CP is normally a monophasic, negative going spike superimposed on the positive resting potential. During internal perfusion with EDTA solution the CP magnitude gradually decreases and in many cases CP's became biphasic or of reversed polarity (Fig. 10).

CP recovery is rapid when normal solution is readmitted just as with the resting potential. The effects of EDTA perfusion were variable and with some animals the CP's became small but remained negative. One reasonable explanation for the variability in CP response to EDTA perfusion is that only a very small amount of divalent cation is required at the CP generating locus, an amount so small that it is but ineffectively removed by EDTA chelation in the face of diffusion from surrounding tissue.

(C.) *Osmotic pressure-effects*

Earlier it was hypothesized that the resting potential of hydra is the result of ion accumulating mechanisms used in osmoregulation (Macklin, 1967; Josephson and Macklin, 1969). One might therefore expect the size of the resting potential to reflect the osmotic stress faced by the animal. This was examined by determining the effects on transepithelial potentials of bathing media which were hyperosmotic to the normal medium. Increasing the osmotic concentration of the bathing medium somewhat above that of normal solution reduces the osmotic gradient between the tissues of the animal and its environment. In the first set of experiments (short term tests) the osmotic gradient was altered for 10 minute periods; in the second set of experiments (long term tests) the animals were kept in a solution with elevated osmotic concentration for two weeks before testing.

In the first short term tests the osmotic concentration of the bathing medium was varied by adding sucrose to normal solution (7.5 mosmol) to give calculated osmotic concentrations up to 67.5 mosmol. Animals did not fare well when exposed for 10 minute periods to these solutions presented in random order; the rest-

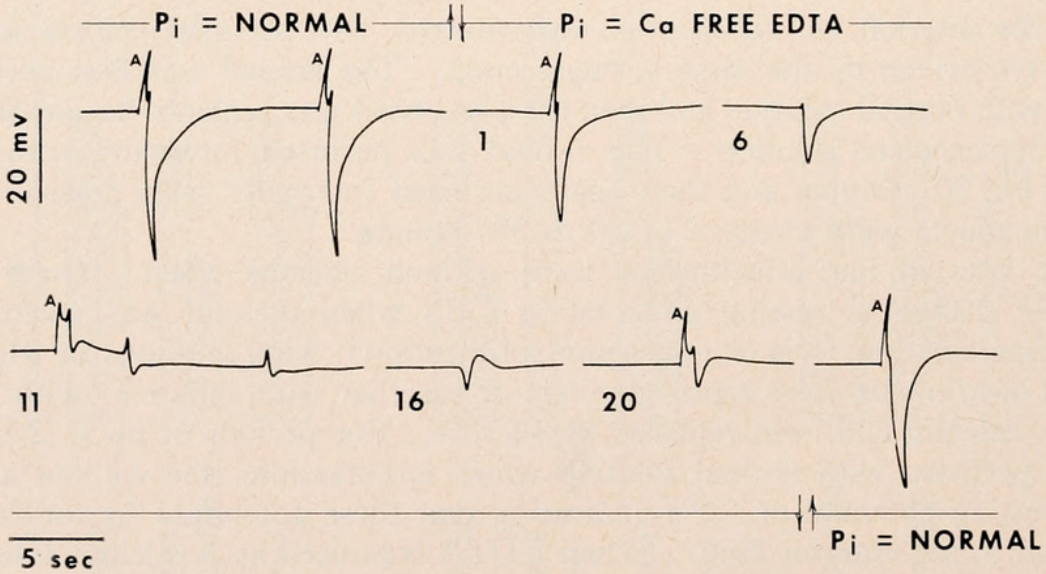

FIGURE 10. The effect on CP's of internal perfusion with Ca-free-EDTA. The animal was bathed in normal solution. Here the penwriter was capacitor-coupled so the records do not show the resting potential. The numbers below the record segments indicate the time in minutes since the onset of perfusion with Ca-free solution. The last CP was recorded 10 seconds after the perfusion pipet delivering Ca-free solution was replaced by one delivering normal solution. Some of the CP's shown were triggered by electrical stimuli, the rest were spontaneous. The stimulus artifacts preceding the triggered CP's are the upward deflections marked "A." Note that in the middle of the perfusion period the CP's were biphasic or principally positive. The CP shape seen at 11 minutes continued until 14 minutes.

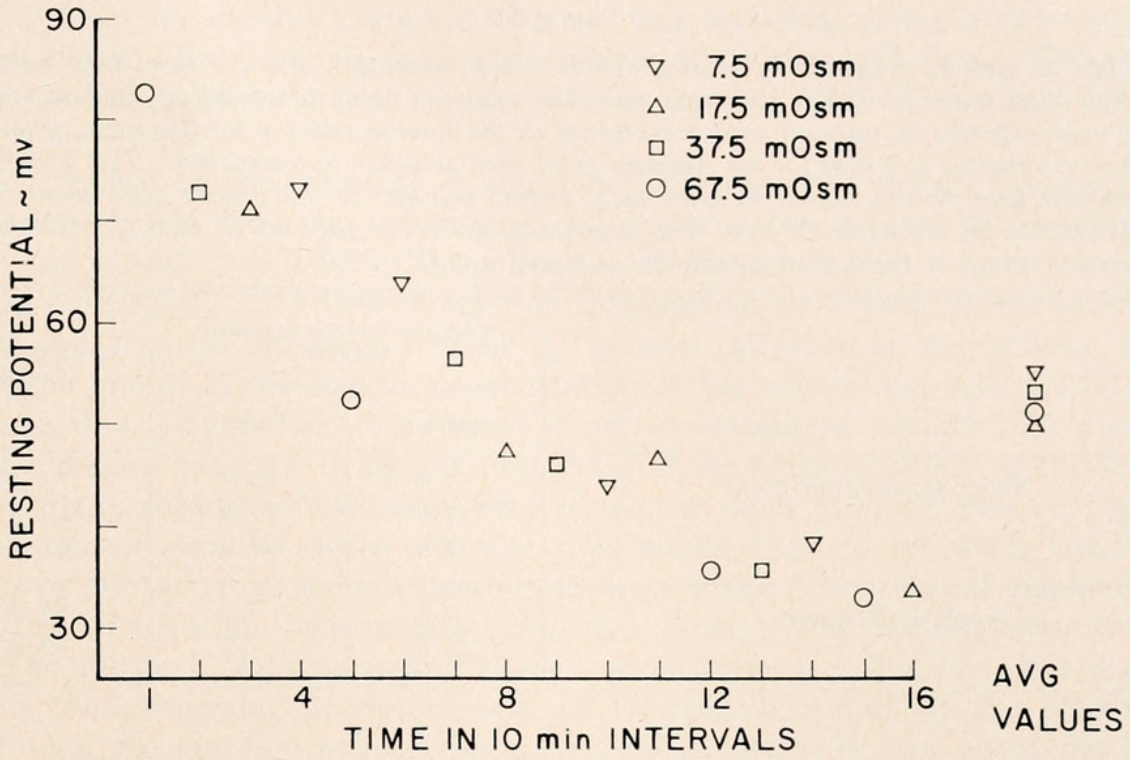

FIGURE 11. Destructive effect of high osmotic pressure on the resting potential of one animal. The resting potential continually fell when solutions of the indicated osmotic pressure were presented in random order for ten minute periods. Internal perfusate was normal solution which has a calculated osmotic pressure of 7.5 mosmole.

ing potential fell throughout the test series (Fig. 11). Toward the end of the series the animals were visibly moribund. Although not totally satisfactory because of the irreversible changes in the animals, these experiments did suggest that the resting potential was not greatly different during brief periods in solutions of differing osmotic concentration (see average values, Fig. 11). The test was then repeated omitting the 67.5 mosmol medium. There was no obvious degeneration when the 67.5 mosmol solution was omitted and reasonable resting potentials were maintained throughout the test series (Table IV). There was a small but not significant difference between the resting potentials in the different solutions. This result was not anticipated and it led to examination of the effects of long term exposure to a medium, with an elevated osmolarity.

For the long term test, twenty hydra were placed in each of two finger bowls, one bowl contained culture solution and the second bowl contained culture solution plus 10 mM/l NaCl. NaCl was used to increase the osmotic pressure rather than sucrose to avoid bacterial growth. The two cultures were fed normally and maintained in the two media for two weeks. During this time both cultures reproduced asexually at essentially the same rate—the population in culture solution increased from 20 to 28 animals and in culture solution plus 10 mM NaCl the population increased from 20 to 30. At the end of the two week period the animals were placed in randomly numbered vials. The vials were arranged in pairs, one containing an animal raised in normal solution and the other with an animal raised in the solution with an elevated osmotic strength. The sorting was done by an assistant who did not reveal the rearing solution of the animals to the authors until after they were tested.

TABLE IV

Effect of osmotic pressure on transepithelial resting potential. For the short term experiment, 6 animals were tested for 2 test sequences each, the solutions being presented in random order. For the two week experiment, animals were maintained in the rearing solution for two weeks prior to testing, then the resting potential for ten animals from each solution was measured. Test periods of 10 minutes were used for the short term experiment and 15 minutes for the 2 week experiment. The differences between the means in the short term experiment are not significant ($P > 0.1$) while the difference between means in the 2 week experiment is significant ($P < 0.01$).

	Solution osmotic pressure mosmol		Resting potential ± SE mv
	Rearing	Testing	
Short term experiment	7.5	7.5	72 ± 3
	7.5	17.5	69 ± 3
	7.5	37.5	67 ± 3
2 week experiment	7.5	7.5	65 ± 4
	27.5	7.5	49 ± 4

The animals were each placed on the test holder with normal solution on the outside and perfusing the gut. The recorded resting potential for each animal was determined as the average of readings taken every minute for the last 5 of the 15 minute test period. Measurements were made on ten animals from each culture, and the animals then identified. The animals grown for two weeks in the higher osmotic strength solution had a significantly lower resting potential than those grown in regular culture solution (Table IV). Thus animals accustomed to living in a higher osmotic concentration, and therefore a reduced osmotic gradient, have a lower resting potential. Hydra do not adapt to short term changes in osmotic gradient but there is long term adaptation.

DISCUSSION

Of the ions examined sodium is most directly involved in the transepithelial potential. The monotonic increase in the resting potential with increasing concentration of external sodium and the polarity of the potential suggest that at least part of the resting potential if not all of it results from inward movement of sodium —a transfer of sodium from the medium to the animal's tissue. The tissue sodium concentration in hydra is approximately 20 mM which is ten times the concentration in the usual bathing solution (Steinbach, 1963). A net inward movement of sodium must therefore involve an active transport mechanism. The slope of the potential versus external sodium concentration curve (Fig. 6) is less than the Nernst potential of 58 mv per decade and there is apparent saturation at high sodium concentration; both of these findings support the conclusion that sodium movement involves a transport system rather than solely passive diffusion. Although inwardly directed sodium transport appears to contribute to a potential across the epithelium and an inward current flow in animals whose transepithelial potential is clamped at zero (Josephson and Macklin, 1969), the active transport itself need not be electrogenic. For example, the transport mechanism might ex-

change sodium for another cation at one cell face and thus create a concentration gradient down which sodium passively diffuses at another cell face. This is the explanation originally offered for potentials across frog skin by Koefoed-Johnsen and Ussing (1958).

The rapidity of the potential change when the external sodium concentration is altered indicates that the ectodermal cell layer is the source of the potential change and indeed that it is the outer surface of the ectodermal cells at which the potential originates. It follows from this that sodium movement at the outer cell face involves charge separation, either by passive diffusion or the activity of an electrogenic pump. The sodium concentration in the ectodermal cells would have to be less than 1.5 mM for there to be a net movement of sodium into them by passive diffusion from the usual environment. If the transepithelial potential were due entirely to passive sodium movement across the outer cell face then the internal sodium concentration in the ectodermal cells would have to be about 0.015 mM judging by the external sodium concentration at which the potential changes sign in the tris substitution experiments (Fig. 6). Such a low sodium concentration is hard to reconcile with measured tissue concentrations. This line of argument supports the alternative possibility—*i.e.* an electrogenic pump at the ectodermal surface—but the question can not be settled until more is known about the intracellular sodium concentration and details of the potential profile across the body wall.

Although the resting potential declines when there is neither calcium nor magnesium in the bathing medium, the requirement for a divalent cation is less direct than for sodium as shown by the delay between the removal of calcium and the onset of the potential decline. Part of the decline in Ca^{++} free solutions may be due to cell damage. However there is little impedance change while the potential declines, indicating that the epithelia and the cells of which they are composed are remaining intact.

We think it particularly significant that the CP's become smaller and sometimes of reversed polarity when calcium is removed from the enteron by EDTA perfusion. Contraction pulses are probably generated by the basal surface of the ectodermal cells (Josephson and Macklin, 1969). The results of internal perfusion with EDTA suggest the following model for the genesis of CP's: They result from calcium moving inward across the basal membranes of the ectodermal cells, moving from the extracellular space on the mesogleal side into the ectodermal cells. The movement follows an increase in the calcium permeability of the basal membranes and the driving force is a gradient in calcium concentration from the extracellular space to the cell interior. When the enteron is perfused with EDTA the extracellular calcium concentration is lowered, reducing the gradient and the CP amplitude. During long perfusion periods the extracellular calcium concentration can be reduced sufficiently that the gradient is reversed; calcium now leaves the cells when the calcium permeability increases and the CP polarity is reversed. We are suggesting that CP's are calcium spikes, similar to those described for crayfish muscle fibers (Fatt and Ginsborg, 1959) and for barnacle muscle fibers (Hagiwara, Chichibu and Naka, 1964). Although CP's decline during initial test periods in Na free solution they do not do so in later periods (Fig. 5). Thus external sodium may influence CP amplitude, but its exact role is obscure.

Contraction pulses precede and are probably causally related to contraction of the ectodermal musculature. There is ample evidence in higher animals that the activity of muscle protein is controlled through the ambient calcium concentraction (Ebashi, Endo and Ohtsuki, 1969). It may be calcium influx during CP's which initiates contraction of hydra muscle fibers.

The transepithelial potential depends to some extent on the anionic composition of the bathing medium as is shown by the change in the potential when methane sulfonate replaces chloride or bicarbonate. The fact that both chloride and bicarbonate in the external medium can be replaced by sulfate with no significant change in the potential suggests that anion pumps, if they exist, are not major contributors to the transepithelial potential. It seems useful to see if the anion substitution results can be interpreted on the basis of passive anion movements acting as shunts for potentials developed by cation transport. The effectiveness of an anion species in shunting the transepithelial potential will vary with the conductance of the anion across the potential-generating structure. The potential changes in the methane sulfonate substitution experiments require the ionic conductances at the existing concentrations to decrease in the following order: $HCO_3^- > CH_3SO_3^- > Cl^-$. There is the difficulty that substitution among these ions was not reflected in a change in the column impedance. A possible explanation for this is that the barrier across which the potential is generated and at which ion shunting is effective makes up only a small part of the total transverse impedance, the remainder of the epithelia forming a large, passive impedance which is unaffected by short term changes of the external solution. This explanation is similar to the one which we proposed to account for the observation that there is little change in the transverse column impedance during CP's (Josephson and Macklin, 1969). Then we proposed that the CP generating membrane contributed only a small fraction of the total transverse impedance; now we are suggesting that the resting potential source also makes up but a small part of the transverse impedance.

In sum we have found that external sodium concentration is directly related to the transepithelial resting potential in hydra indicating a sodium transport mechanism. The effect of anions is as yet uncertain. Removing external divalent cations causes physiological deterioration, whereas removal of them from the gut with EDTA causes the CP's to change their shape. The latter result suggests that divalent cations are involved in CP production on the endoderm side of the CP generating membrane.

These experimental results support the following model for the maintenance of volume and osmotic equilibrium by hydra (see also Marshall, 1969). Sodium is transported from the outer medium to the gut, possibly in several steps. Anions passively follow the sodium. Because of the osmotic gradient, water enters cells of the epithelia from the outer bathing medium. In the animal, water movement is coupled to the movement of salt, either directly or through the creation of osmotic gradients, so that it too moves from the tissue to the gut. In this way water entering the animal is transported to the gut. The gut contents, which are hyperosmotic to the outer medium (R. Prusch and D. Benos, personal communication, Department of Biology, Case Western Reserve University; Marshall, 1969), are excreted by bulk flow to the environment, presumably through the mouth. This hypothesis is weakened by the observation that if an animal is transected, the cut

surfaces heal over. A regenerate without either a mouth or a basal disc has a closed gut cavity but is still able to osmoregulate (Macklin, unpublished). However, the regenerate continues to contract spontaneously and may thus force fluid through adventitious openings in the body wall. The suggested mechanism by which hydra maintain both ionic and volume regulation is the same as the one recently described for another fresh water coelenterate, *Craspedacusta sowerbyi* medusae, by Hazelwood, Potts and Fleming (1970). Their conclusions derive from measurements of radioactive sodium and water transport rates and concentrations, whereas our conclusions are based primarily on electrophysiological measurements.

The ability of hydra to adapt to changes in osmotic pressure over a two week period but not within a ten minute period indicates that the effectiveness of the transport system can be slowly modified in response to environmental requirements, possibly by the synthesis of new carriers.

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Summary

(1) The resting potential across the body wall of hydra varies monotonically with the external sodium concentration.

(2) Replacing bicarbonate and chloride, the normal external anions, with methane sulfonate changes the resting potential but not the transverse column impedance. If the anions are acting as passive shunts, the barrier across which the potential is developed must form but a small portion of the total transverse impedance.

(3) Changing the concentration of ions in the gut of hydra was generally without effect, but removal of calcium with EDTA caused the contraction pulses to become reduced or reversed in sign suggesting that these are calcium spikes.

(4) The resting potential changes in response to long term but not short term changes in the osmotic stress faced by the animal. It is proposed that the resting potential results from a sodium transport mechanism which is involved in osmotic regulation.

LITERATURE CITED

Ebashi, S. M., M. Endo and I. Ohtsuki, 1969. Control of muscle contraction. *Quart. Rev. Biophys.*, **2**: 351–384.

Fatt, P., and B. L. Ginsborg, 1959. The ionic requirements for the production of action potential in crustacean muscle fibres. *J. Physiol.*, **142**: 516–543.

Hagiwara, S., S. Chichibu and K.-I. Naka, 1964. The effects of various ions on resting and spike potentials of barnacle muscle fibres. *J. Gen. Physiol.*, **48**: 163–179.

Ham, R. G., D. C. Fitzgerald, Jr., and R. E. Eakin, 1956. Effects of lithium ion on regeneration of hydra in a chemically defined environment. *J. Exp. Zool.*, **133**: 559–572.

Hazelwood, D. H., W. T. W. Potts and W. R. Fleming, 1970. Further studies on the sodium and water metabolism of the fresh-water medusa, *Craspedacusta sowerbyi*. *Z. Vergl. Physiol.*, **67**: 186–191.

JOSEPHSON, R. K., 1967. Conduction and contraction in the column of hydra. *J. Exp. Biol.*, **47**: 179–190.

JOSEPHSON, R. K., AND M. MACKLIN, 1967. Transepithelial potentials in hydra. *Science*, **156**: 1629–1631.

JOSEPHSON, R. K., AND M. MACKLIN, 1969. Electrical properties of the body wall of *Hydra*. *J. Gen. Physiol.*, **53**: 638–665.

KASS-SIMON, G., AND L. M. PASSANO, 1969. *Hydra* conduction pathways. *Amer. Zool.*, **9**: 1113–1114.

KOBLICK, D. C., AND L. YU-TU, 1967. The osmotic behavior of digestive cells of *Chlorohydra viridissima*. *J. Exp. Zool.*, **166**: 325–330.

KOEFOED-JOHNSEN, V., AND H. H. USSING, 1958. Nature of the frog skin potential. *Acta Physiol. Scand.*, **42**: 298–308.

LILLY, S. J., 1955. Osmoregulation and ionic regulation in *Hydra*. *J. Exp. Biol.*, **32**: 423–439.

LOOMIS, W. F., AND H. M. LENHOFF, 1956. Growth and sexual differentiation of hydra in mass culture. *J. Exp. Zool.*, **132**: 555–573.

MACKIE, G. O., 1965. Conduction in the nerve-free epithelia of siphonophores. *Amer. Zool.*, **5**: 439–453.

MACKIE, G. O., AND L. M. PASSANO, 1968. Epithelial conduction in hydromedusae. *J. Gen. Physiol.*, **52**: 600–621.

MACKLIN, M., 1967. Osmotic regulation in hydra: Sodium and calcium localization and the source of the electrical potential. *J. Cell. Physiol.*, **70**: 191–196.

MARSHALL, P. T., 1969. Towards an understanding of the osmo-regulation mechanism of *Hydra*. *School Sci. Rev.*, **51**: 857–861.

MILLIGAN, J. V., 1965. The time course of the loss and recovery of contracture ability in frog striated muscle following exposure to Ca-free solutions. *J. Gen. Physiol.*, **48**: 841–858.

PASSANO, L. M., AND C. B. MCCULLOUGH, 1964. Co-ordinating systems and behavior in *Hydra*. I. Pacemaker system of the periodic contractions. *J. Exp. Biol.*, **41**: 643–664.

ROBERTS, A., 1969. Conducted impulses in the skin of young tadpoles. *Nature*, **222**: 1265–1266.

STEIN, W. D., 1967. *The Movement of Molecules Across Cell Membranes*. Academic Press, New York, 351 pp.

STEINBACH, H. B., 1963. Sodium, potassium and chloride in selected hydroids. *Biol. Bull.*, **124**: 322–336.