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ELECTROPHYSIOLOGICAL STUDIES ON THE HEART OF THE BIVALVE MOLLUSC, *MODIOLUS DEMISSUS*

I. IONIC BASIS OF THE MEMBRANE POTENTIAL

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INTRODUCTION

Intracellular electrical recordings from the isolated ventricles of three bivalves (Irisawa, Shigeto & Otani, 1967; Irisawa, Noma & Ueda, 1968; Ebara, 1969; Wilkens & Greenberg, 1970) have been reported in the literature. The bivalve heart features electrical waveforms similar to those of the vertebrate myocardial pacemaker tissues (Trautwein & Kassebaum, 1961), and behaves functionally as a unitary smooth muscle where impulses are transmitted from cell to cell by way of tight junctions or nexuses. These properties are ideal for extracellular recording by the sucrose-gap technique (Stämpfli, 1954), the method which has been used predominately in the present study.

Recordings from *Modiolus* heart suggest that the pacemaker mechanism is a diffuse property of the entire ventricle. The myogenic pacemaker of bivalves and other molluscs has been discussed in the reviews of Krijgsman & Divaris (1955) and of Hill & Welsh (1966). This paper deals with the ionic basis of the membrane potential in *Modiolus* heart in terms of its characteristic spontaneous nature. Of special significance to this preparation is the low membrane potential due, in part, to chloride and sodium conductances, and the rhythmical contributions of an electrogenic sodium pump. A preliminary account of some of these data has been given previously (Wilkens & Greenberg, 1970).

MATERIALS AND METHODS

The ribbed mussel, *Modiolus demissus*, was collected from salt marshes along the Northern Gulf coast of Florida. This environment subjects the clam to extreme fluctuations in temperature (−5 to 35 °C), salinity (5–35 ‰), and desiccation (Pierce, 1970). Specimens were maintained in salt water aquaria in the laboratory for 1–2 months prior to use. Animals collected during the summer were kept at room temperature (23 °C), while those collected during the winter were stored in a cold room at 10–12 °C. Unless otherwise noted, experiments were performed at room temperature.

Isolation of the bivalve heart has been previously described by Welsh & Taub (1948) and by Greenberg (1965). After the animal had been removed from its shell, the heart was exposed by opening the pericardial chamber. The rectum, which passes

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obliquely through the heart in *Modiolus demissus*, was removed by cutting transversely through the ventricle and rectum, at their anterior and posterior connexions, and teasing the freed rectal segment out of the heart. The ventricle was ligated just medial to the two auricular junctions, and isolated by cutting lateral to the knots.

Although electrical activity was recorded by intracellular and extracellular techniques, most of the experiments reported in this paper have used the extracellular sucrose-gap method (Stämpfli, 1954). This technique is advantageous since it permits continuous perfusion and simultaneous recording of electrical and mechanical responses of the heart to changes in the ionic environment.

Sucrose-gap technique

The single-gap apparatus used in these experiments is illustrated schematically in Fig. 1. The central sucrose chamber lies at the centre of a small Plexiglas wheel. The hole (2·5 mm in diameter) drilled perpendicular to the plane of the wheel forms the actual perfusing chamber. Small holes through the rim of the wheel, and continuing along the length of the spokes, provide the incurrent and excurrent perfusing channels. Two thin rubber membranes, secured on both sides of the wheel by O-rings, are drawn inward by vacuum until they meet midway between the faces of the wheel and enclose the sucrose chamber. A small hole is burned at the centre of the membrane enclosing the sucrose compartment by means of a fine-tip soldering iron.

The two Plexiglas compartments on either side of the gap are mounted in adjustable clamps and pressed tightly against the hub of the sucrose gap, in line with the central compartment. The combination of these three components constitutes a continuous cylindrical perfusion chamber divided into three sections by two thin membranes.

The isolated heart is now drawn through the holes in the two membranes, from left to right, until a small piece enters the depolarizing (right) chamber. This end of the heart is secured, while the greater part of the heart remains in the test chamber and is attached to the tension monitor. The heart now seals off the three compartments and perfusion is begun. The apparatus can be checked for leakage between compartments by perfusing one chamber at a time. The direction of flow of the perfusion fluid is illustrated by arrows in Fig. 1. The tips of two calomel reference electrodes, fitted into each of the side compartments with small O-rings, make electrical contact with the bathing solution as it drains from the chamber; thereby contamination of the perfusate with the saturated KCl in the electrodes is avoided.

The upper middle portion of the left compartment is left open to accommodate one arm of a bellcrank for transmitting tension to a mechano-electric transducer (Grass FT 03). A lightweight spring, interposed between the bellcrank and transducer, allows the heart to beat auxotonically. The transducer was mounted in a micro-manipulator.

The perfusing system is designed to permit rapid switching of solutions with minimal disturbance to the preparation. Flow is maintained by hydrostatic pressure, and controlled independently in each compartment of the apparatus. Care is taken to avoid short-circuiting the sucrose gap through the external plumbing.

The total volume of the test chamber and tubing immediately surrounding the heart is less than 0·1 ml, thereby permitting rapid and complete turnover of the perfusate. Solutions are perfused at a rate of 0·5–1·0 ml/min. A coil of stainless-steel tubing is connected into the flow system and immersed in an ice bath for lowering the tem-

perature of the perfusate. Temperature is monitored by a thermistor embedded in the wall of the test chamber. A more detailed account of the perfusing network has been given elsewhere (Wilkens, 1970).

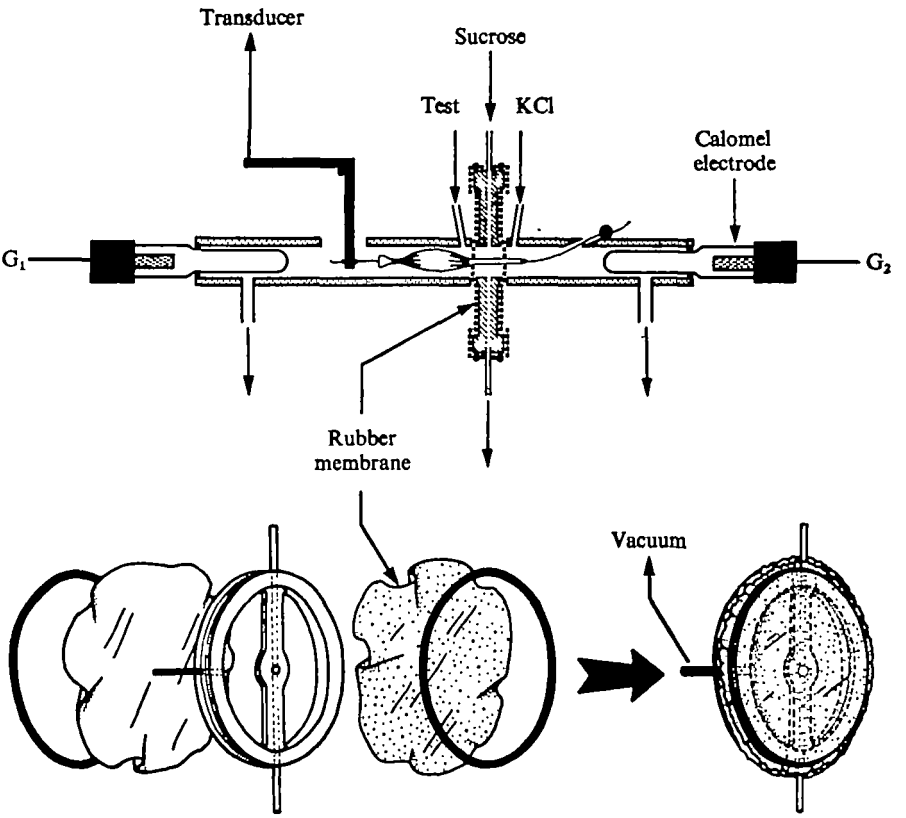


Fig. 1. Cut-away view illustrating construction and operation of sucrose-gap apparatus (top); detailed drawing of the central disk (sucrose chamber) and exploded view showing assembly of rubber membranes and O-rings (bottom). The hole in the hub, in the axis of rotation of the wheel, forms the sucrose compartment. This compartment is separated from the rest of the apparatus by two thin rubber membranes, secured to the outer rim by O-rings, and drawn firmly around the spokes by a vacuum. Two cylindrical Plexiglas tubes, held in adjustable clamps and pressed tightly against the central chamber, form the depolarizing (right) and test (left) compartments. Solutions bathing either end of the heart flow past calomel recording electrodes. One end of the heart is secured with silk thread in the depolarizing chamber. The other end of the heart is connected to a bellcrank inserted into an opening of the test chamber to record tension.

Electrical potentials are led from the calomel reference electrodes into a high-input-impedance cathode follower and d.c. pre-amplifier (Grass Model P6–12). The output of the pre-amplifier is fed directly into one channel of a dual beam oscilloscope (Tektronix Type 565) and into the second channel through a differentiating circuit (time constant, 1·5 msec). A Grass kymograph camera is used to record from the oscilloscope screen. Electrical signals are led simultaneously into the driver amplifier of a two-channel ink-writing polygraph (Grass Model 79). The mechanogram is recorded by the second channel of the polygraph.

The sucrose-gap recording method is illustrated in Fig. 2. Small biphasic potentials and the accompanying mechanical activity are recorded initially. Upon perfusion with isosmotic sucrose (0·72 M), the rise in external resistance of the centre compartment is indicated by an increase in spike amplitude. The increase in external resistance reduces conduction velocity across the gap approximately 50%, as estimated from the temporal separation of the peaks of the biphasic spike. The spike is blocked in the right compartment by perfusion with 0·54 M-KCl resulting in the recording of monophasic action potentials. The resulting depolarization (gap potential) is used as an indication of the actual membrane potential.

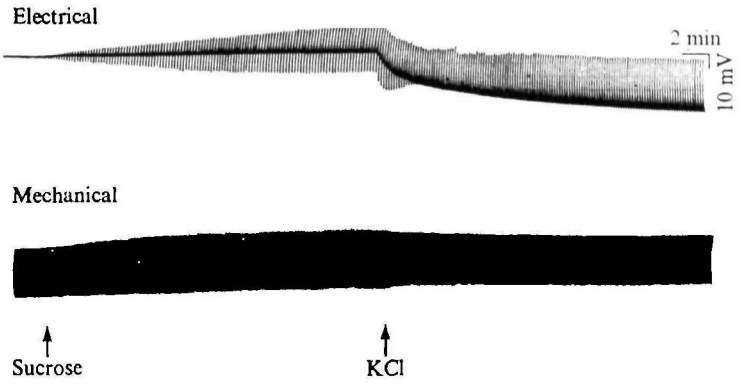


Fig. 2. Electrical (upper) and mechanical (lower) activity recorded by the sucrose-gap method. The amplitude of the biphasic spike increased after switching sucrose into the centre compartment. The spike was blocked in the right compartment by depolarization with KCl (0·54 M), resulting in monophasic action potentials. Membrane potentials were estimated by the difference in base-line potential before and after depolarization with KCl.

The biphasic nature of the spike indicates that the impulse is conducted across the high external resistance of the sucrose gap. The inability of sucrose to block conduction may be caused by the failure of sucrose to replace all of the electrolytes in the extracellular space and by the presence of low-resistance pathways between adjacent cells (Irisawa, Irisawa & Shigeto, 1969).

Microelectrode technique

Intracellular recordings were made with glass pipettes having a resistance of 20–50 MΩ measured in sea water. Microelectrode potentials were recorded with single-ended input into the same amplification and recording system as used with the sucrose-gap. The electrodes were suspended from a Ag–AgCl wire cemented, with silver paint, to a movable coil of 0·001 in. tungsten wire (Woodbury & Brady, 1956). The bathing solution was grounded with a large Ag–AgCl wire.

Solutions

In all experiments the standard perfusing medium was artificial sea water (ASW) prepared from individual salt solutions, each made isosmotic to the sea water in Vineyard Sound, Massachusetts (M.B.L. formula, Marine Biological Laboratory, 1964). The ionic content of ASW, given in millimoles per litre, and of the other experimental salines (e.g. Cl-free SW) are given in Table 1.

Table 1. *Artificial SW and isosmotic test solutions (values for each ion species given in m-moles/l)*

Ion	Art SW	Na-free SW	Ca-free SW	Na- and Ca-free SW	Ca- and Mg-free SW	Na-, Ca- and Mg-free SW	K-free SW	Mg-free SW	Cl-free SW	Li-SW	Ca-free Li SW
Na	463·0	—	478·7	—	540·3	—	472·7	524·8	491·4	—	—
K	9·7	9·7	9·7	9·7	9·7	9·7	—	9·7	10·0	9·7	9·7
Ca	9·9	9·9	—	—	—	—	9·9	9·9	9·8	9·9	—
Mg	51·2	51·2	51·2	51·2	—	—	51·2	—	50·8	51·2	51·2
Li	—	—	—	—	—	—	—	—	—	463·0	478·7
Tris	—	•	—	•	—	•	—	—	—	—	—
Cl	538·6	•	534·5	•	521·3	•	538·6	525·5	—	540·9	536·8
SO_4	27·0	27·0	27·0	27·0	13·2	—	27·0	13·2	65·6	27·0	27·0
HCO_3	2·3	—	2·3	—	2·3	—	2·3	2·3	—	—	—
$C_3H_5O_3$	—	—	—	—	—	—	—	—	491·4	—	—

• The exact chloride and Tris concentrations are unknown since isosmotic Tris Cl was prepared empirically by dissolving 88 g Tris/l H_2O and adding 33 ml conc. HCl.

High-potassium solutions, used in depolarization experiments, were made in multiples of normal sea-water potassium concentration by mixing K-free SW (see Table 1) with either 0·54 M-KCl or 0·44 M-K_2SO_4.

Na-free solutions were prepared by substituting for NaCl either tris(hydroxymethyl) aminomethane (Tris) or LiCl. The method for preparing Tris Cl (85 g Tris/l H_2O + 33 ml conc. HCl) was determined empirically by adjusting the solution to 1040 m-osmoles at pH 7·9.

Propionate was used primarily to replace chloride; in a few experiments sulphate was the major anion. The propionate and sulphate results were indistinguishable. In preparing Cl-free solutions, calcium was added in crystalline form, since $CaSO_4$ is insoluble at concentrations isosmotic to sea water. To eliminate lesser constituents (K, Ca and Mg) a quantity of isosmotic NaCl, equal in volume to that of the deleted solution, was added.

Only large ASW reservoirs were aerated; no attempt was made to oxygenate each test solution. However, no effect was observed in switching from aerated ASW to non-aerated test solutions. Presumably, either a slow rate of perfusion through the external tubing (Medical Grade Silastic) allowed sufficient time for gaseous equilibration, or the heart was insensitive to small differences in oxygen tension.

RESULTS

The isolated ventricle of *M. demissus* produces spontaneous, slow-rising pacemaker waves and action potentials (Fig. 3) arising from a relatively low level of membrane polarization. This potential, as in other spontaneous preparations, cannot be characterized as a resting potential and is measured at the level of maximum diastolic potential (Hutter & Trautwein, 1956).

The amplitudes of spike and membrane potentials are quite variable from one preparation to another. Measured intracellularly from the level of maximum diastolic

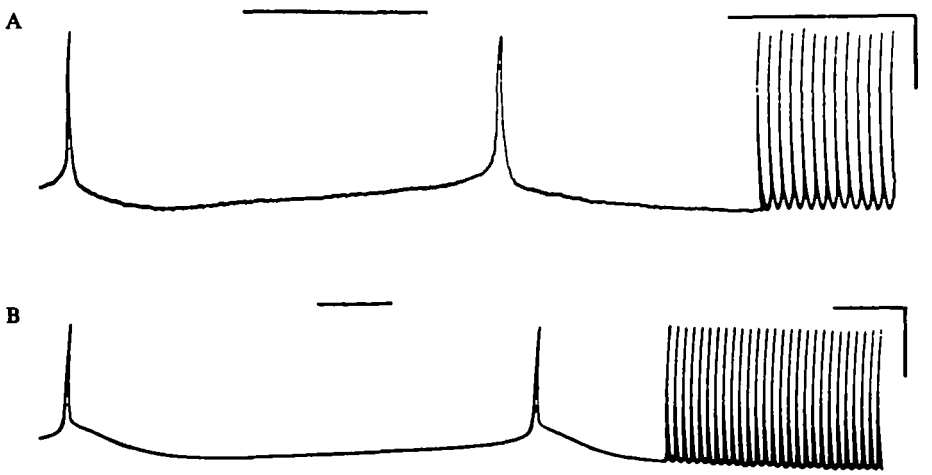


Fig. 3. Electrical activity of *Modiolus* heart by intracellular (A) and extracellular (B) methods. Expanded time-scale portions of each record illustrate the gradual pacemaker depolarization and pre-potential immediately preceding the spike. Vertical bars equal 20 mV. Horizontal bars represent 1 min (right) and 1 sec (left).

potential, spike amplitude ranged from 26 to 56 mV, rarely overshooting zero potential, while values for membrane potentials varied from −42 to −64 mV. Table 2 lists the average value for membrane and action potentials, and compares data obtained by intracellular and extracellular recording techniques. Measurements of the membrane potential by KCl (0·54 M) depolarization in the sucrose gap agreed well with data obtained intracellularly. The extracellular spike amplitude, however, was about 30% less than intracellular values, although the other dimensions of the spike were faithfully recorded by this technique (cf. Fig. 3A, B; see also Wilkens, 1971).

Table 2. *Means and standard deviations for the intracellular (micro-electrode) and extracellular (sucrose gap) recordings of membrane and action potentials*

Method	Membrane potential	Action potential
Intracellular	−55·0 ± 8·2 mV (7)*	46·0 ± 8·4 mV (13)
Extracellular	−53·8 ± 7·2 mV (21)	31·6 ± 5·1 mV (21)

* Parentheses indicate the number of observations in each calculation.

Ionic basis of the membrane potential

Effect of potassium

As in many other excitable cells, the membrane potential of *Modiolus* hearts can be reduced (made less negative) by increasing the external potassium concentration (K_0). In 10–20 mM-K_0 sea water depolarization produces an increase in tone and frequency. At concentrations above 40 mM membrane depolarization blocks the spike and puts the heart into contracture. For these experiments the right compartment (Fig. 1) served as the test chamber; the small amount of tissue in this compartment recovered more rapidly from high concentrations of potassium (2–3 min.).

In the following experiments hearts were depolarized twice with increasing concentrations of potassium – once in KCl and once in K_2SO_4. Following each depolarization sufficient time was allowed for recovery in normal sea water. The data, averaged from three experiments, is shown in Fig. 4. Depolarization varies linearly with log K_0 above 40 mM. In KCl the slope of the line is 45·7 mV per tenfold increase of K_0; in K_2SO_4 it is 49·9 mV per tenfold K_0 increase. The difference between the slopes suggests that the membrane potential is also dependent upon the permeability of the membrane to anions.

Effects of anions on the membrane potential

Although the potential across the cell membrane, in the heart of *Modiolus*, depends primarily on the ratio of intracellular to extracellular potassium, the slopes of the depolarization curves are not indicative of a pure potassium equilibrium potential. The KCl and K_2SO_4 depolarization curves indicate that, in addition to the effect of replacing sodium with potassium, the change in membrane potential reflects the replacement of chloride by sulphate or propionate anions.

To test the effect of anions on the membrane potential the heart was depolarized by K_2SO_4 following replacement of chloride in the normal perfusion fluid with propionate. Isosmotic K_2SO_4 and Cl-free (propionate) SW were mixed to achieve the

desired potassium concentration. In Fig. 5 the heart was first depolarized by KCl in sea water, as in the previous experiment (Fig. 4). The slope for KCl, estimated from the linear portion of the depolarization curve between 40 and 400 mM K_0, is 34 mV with a final membrane depolarization of 46 mV in 540 mM-K_0. The heart was then perfused with Cl-free SW and, after establishing a new equilibrium potential, depolarized with K_2SO_4. The slope appears to have been decreased to 29 mV for a tenfold potassium increase and the membrane depolarization, at 540 mM-K_0, is only 37 mV.

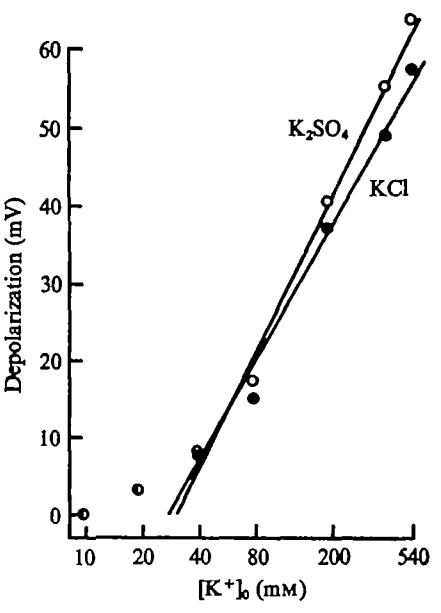


Fig. 4

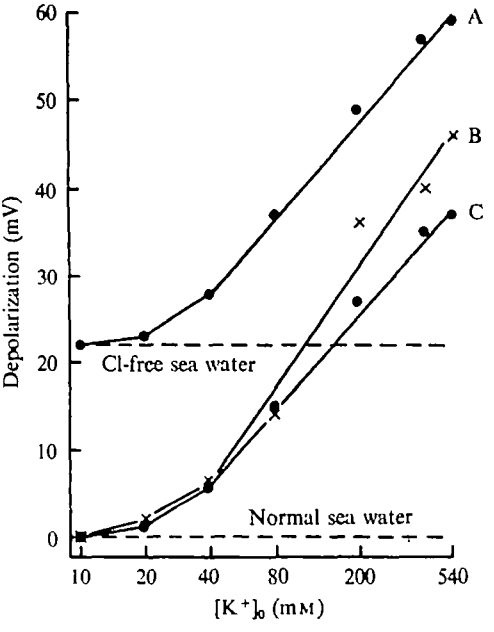


Fig. 5

Fig. 4. Potassium-depolarization curves for KCl and K_2SO_4 in sea water. Membrane depolarization versus the extracellular potassium concentration is plotted on a logarithmic scale. Filled circles are means for KCl; open circles are means for K_2SO_4. The straight lines are least-square calculations based on the data between 40 and 540 mM-K_0. The slope is $45{\cdot}7 \pm 3{\cdot}8$ mV (S.E.) per tenfold increase in K_0 for KCl depolarization; $49{\cdot}9 \pm 4{\cdot}2$ mV (S.E.) per tenfold increase in K_0 for K_2SO_4. Note that the means for KCl and K_2SO_4 at 10, 20 and 40 mM-K_0 are equal.

Fig. 5. Potassium-depolarization curves in normal and Cl-free sea water. Membrane depolarization is plotted versus the extracellular potassium concentration on a logarithmic scale. Depolarization by KCl (crosses) has an estimated slope of 34 mV per tenfold increase in K_0. Depolarization by K_2SO_4 in Cl-free SW (filled circles) has an estimated slope of 29 mV per tenfold increase in K_0. Depolarization, following chloride substitution, is plotted twice; once from the elevated base-line potential of +22 mV in Cl-free SW (curve A), and once from the original potential for comparison with KCl depolarization (curve C). Note the small difference in the magnitude of depolarization below 80 mM-K_0 in KCl and K_2SO_4 solutions (cf. curves B and C).

On the basis of the previous depolarization experiments, in which a gradual chloride replacement increased the K-depolarization slope (Fig. 4), the reverse effect in Cl-free SW seemed questionable. To check the reliability of the experiment the heart was returned to normal sea water and depolarized with 540 mM-K_2SO_4. This procedure depolarized the membrane by 54 mV and the slope of the K-equilibrium potential

was estimated, from this value, to be 42 mV per tenfold increase in K_0. Thus, as in Fig. 4, the slope again appeared to have been increased.

To explain the apparent decrease in slope, it is necessary to consider the effect due to Cl-free SW alone. When propionate or sulphate ions are substituted for chloride, hearts undergo a sustained depolarization of 9–22 mV (Table 3) and maintain this new potential for up to several hours. This response is reversible, as the membrane returns to near the original equilibrium potential in normal sea water.

Table 3. *A summary of the effects of some ions and inhibitors on the membrane potential*

Experiment	Depolarization (mV)	Hyperpolarization (mV)
Na-free SW	—	0–10 (7)
Cl-free SW	9–22 (4)*	—
K-free SW	2–5 (5)	—
Li-SW	6–8 (2)	—
Ouabain (10^{-5} M)	2–4 (4)	—
NaCN (10^{-3} M)	2 (2)	—
Low temperature (ΔC = 15–20)	2–7 (6)	2 (1)

* Parentheses indicate the number of observations.

Curve C must be interpreted in light of the sustained depolarization in Cl-free SW. The same experimental procedure for determining membrane depolarization, i.e. returning the heart to normal sea water between each successive step, was used in these experiments as the heart was returned to Cl-free SW containing potassium at 10 mM between each test. As a result of this procedure, total membrane depolarization was achieved in two steps, by first removing chloride and then by increasing K_0. In the experiment shown in Fig. 5 the heart was depolarized by 22 mV by substituting propionate for chloride and, although the membrane was subsequently depolarized only 37 mV in 540 mM-K_0, the total membrane depolarization, the sum of each step, was 59 mV. This interpretation is illustrated by elevating the potassium depolarization curve to the base-line depolarization level maintained in Cl-free SW (Fig. 5, curve A). The same total depolarization would be predicted by an experimental procedure designed to reduce chloride and increase potassium simultaneously, i.e. resulting in a linear depolarization curve with an estimated slope of 48–50 mV per tenfold increase in K_0 (see Fig. 11). By this interpretation, the slope of the K-depolarization curve would be, as expected (Kuriyama, 1968), increased by reducing the external chloride concentration. The difference in potential between K_2SO_4 depolarization in normal and Cl-free SW, 54 and 59 mV, respectively, is consistent with the fact that 540 mM-K_2SO_4 ASW still contains 214 mM-Cl, or approximately 40% of the original chloride content of sea water (540 mM-KCl is isosmotic to ASW whereas isosmotic K_2SO_4 must be diluted with ASW to have an equal potassium concentration).

The basis of the sustained depolarization in Cl-free SW is not clear. The membrane may be less permeable to chloride than are the membranes of other muscles where chloride is distributed passively (Hodgkin & Horowicz, 1959). Nevertheless, the membrane must be chloride permeable if the potential is sensitive to changes in Cl_0.

Sustained depolarizations have also been reported in visceral smooth muscle by Kuriyama (1968), and by Burnstock & Straub (1958), where replacement of chloride

with sulphate produced a potential decrease of 41 mV per tenfold change in Cl_0. In *Ascaris* muscle the membrane potential depends on the equilibrium potential of chloride to a greater extent than on potassium (del Castillo, de Mello & Morales, 1964).

Experiments in Na-free SW

Between 10 and 40 mM-K_0 the membranc potential departs from the linear relation to log K_0 (Fig. 4). There was no difference, in this range, between K-depolarizations resulting from KCl or K_2SO_4; nor was there a significant difference between depolarizations in normal or Cl-free SW (Fig. 5). This portion of the curve appeared to be independent of the anion.

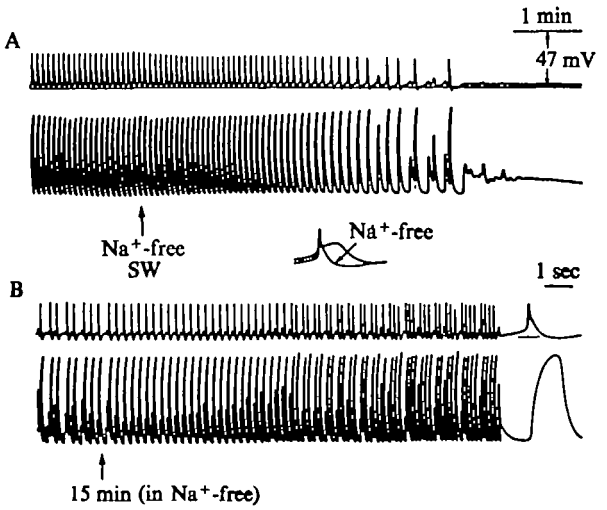


Fig. 6. Response of *Modiolus* heart to Na-free SW. The frequency of spontaneous action potentials (upper trace) and contractions of the heart (lower trace) decrease along with a gradual hyperpolarization of the membrane; the heart becomes quiescent after 4·5 min (A). Partial recovery of spontaneous activity occurs after 15 min in Na-free SW, with the heart becoming progressively more regular (B). The action potential suffers the loss of its plateau in the absence of sodium, cf. spikes in ASW and Na-free SW (Inset).

In the absence of an anion effect, this departure from linearity of the K-depolarization curve is probably due to membrane short-circuiting caused by leakage of sodium ions. Experiments in Na-free SW support this notion. When sodium was replaced by a large impermeant ion, such as Tris, the membrane was hyperpolarized, and the heart stopped beating (Fig. 6). Hearts remained inactive for 10–15 min in Na-free SW, and then recovered spontaneous activity with a characteristic loss of the spike plateau (see inset). The magnitude of hyperpolarization, measured when the membrane potential reached equilibrium (15–20 min), varied predictably with the initial membrane potential of the preparation. Hearts with a less-negative initial membrane potential tended to be hyperpolarized by sodium removal to a greater extent than hearts having a high level of polarization (Fig. 7).

The heart of *Modiolus* is characterized by a low membrane potential and, presumably, by a high interspike sodium conductance which is reduced in Na-free SW. Predictably, therefore, the K-depolarization curve deviates from linearity at a relatively

high level of extracellular potassium (40 mM). As a result, this heart has electrical properties similar to those of other pacemaker tissues, in particular, the pacemaker nodes in vertebrate hearts (Trautwein & Kassebaum, 1961).

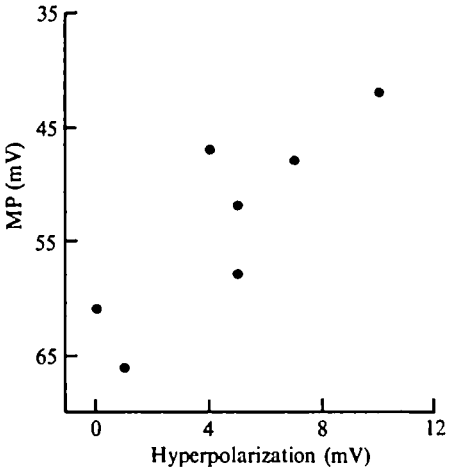


Fig. 7. Membrane hyperpolarization in Na-free sea water. Hyperpolarization (abscissa) versus initial membrane potential (ordinate). Points are scattered but follow a trend in which hearts with a low membrane potential are hyperpolarized to a greater extent than those having higher potentials.

Contribution of an electrogenic sodium pump to the membrane potential

Effects of inhibitors

Modiolus hearts were tested with ouabain and NaCN (Fig. 8). The results of these experiments are listed in Table 3. Depolarization was reversible in both ouabain (Fig. 8B) and, to a lesser extent, in NaCN (Fig. 8C). The actions of both were complete within several minutes, i.e. no additional effects were observed after as much as 20–30 min exposure. Mechanical activity was also sensitive to these substances. An increase in tone resulted from the depolarization in both ouabain and cyanide.

Ouabain is known to inhibit specifically the enzymes regulating active transport of sodium (Schatzmann, 1953). The metabolic inhibiting action of cyanide on oxidative metabolism is indirect. Nevertheless, both inhibit a mechanism contributing to the membrane potential, presumably an electrogenic metabolic pump. The increase in tone can only be explained, from lack of further evidence, if the coupling of electrical and mechanical events has a low threshold (unpublished results), i.e. reflecting the depolarization by a rise in diastolic tone.

Lithium substitution

Membrane depolarization and a rise in tone were also produced by substituting lithium for sodium in artificial sea water (Fig. 8A). In skeletal muscle, lithium substitutes well for sodium in passive ionic fluxes; it does not substitute in the coupled metabolic transport across the cell membrane (Keynes & Swan, 1959). In this muscle, however, the effect of lithium is more complex. Substitution of lithium for sodium depolarizes the heart, while substitution of Tris for sodium results in membrane

hyperpolarization. Both substitutes also reduce the plateau of the action potential (Wilkens, 1971). These differences suggest that, in addition to an effect on the electrogenic pump mechanism, the membrane permeabilities to lithium and sodium are dissimilar.

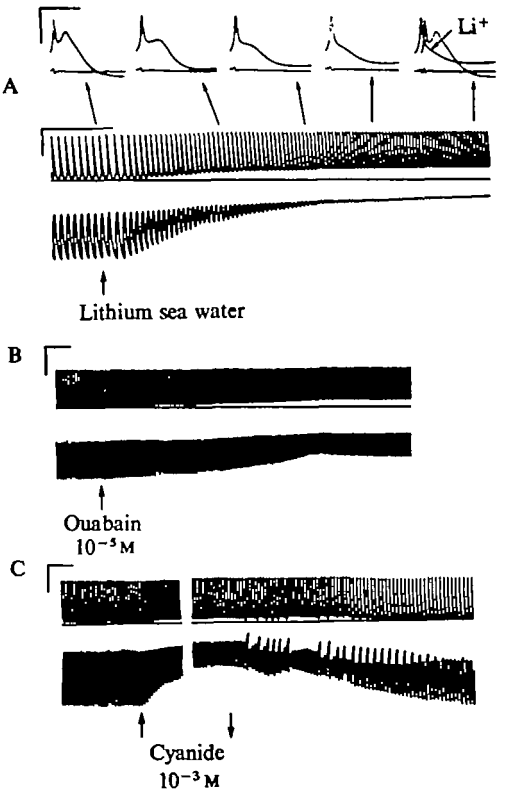


Fig. 8. Depolarizing effects of lithium sea water, ouabain, and cyanide on *Modiolus* hearts. A horizontal line, extending the length of each record, indicates the original membrane potential. The two lower traces in (A), and those in (B) and (C), are polygraph records; electrical above mechanical in each case. In (A), the top set of expanded action potentials (accompanied by their differentiated signals) were photographed by kymograph camera. The last one of the series illustrates the effect of lithium after 6 min, by photographic comparison with a pre-lithium spike. Arrows indicate the position of each spike in the slow-speed polygraph record. Vertical bars are 20 mV. Horizontal bars are 30 sec in (A) and 1 min in (B) and (C) for polygraph records, 1 sec in the kymograph pictures. The record in (C) is shown interrupted by 3 min. Concentrations of ouabain and NaCN are given in molar quantities.

Effects of K-free SW

The membrane also becomes depolarized in K-free SW. Two examples of this response are shown in Fig. 9. The first heart (Fig. 9A) was depolarized 4 mV in K-free SW and, at the end of a 10 min exposure, was subsequently repolarized 6 mV by potassium readmittance, undershooting the original level by 2 mV. After 45 min in K-free SW, another heart (Fig. 9B) was hyperpolarized by return to normal potassium, under-shooting the original level 10 mV and briefly arresting spontaneous activity. Both hearts became irregular in K-free SW and showed a variable increase in frequency. The irregular beat in the first example (see inset) resulted from a spike

with a large plateau and after-hyperpolarization alternating with smaller potentials (Fig. 9A, 3).

These observations are consistent with the idea that extracellular potassium participates in the mechanism of sodium extrusion from the cell and that, in the absence of K_0, sodium enters the cell, decreasing the membrane potential. On this hypothesis, once potassium is returned on the bathing medium, the pump is stimulated by the high internal sodium; the resultant sodium efflux briefly hyperpolarizes the membrane.

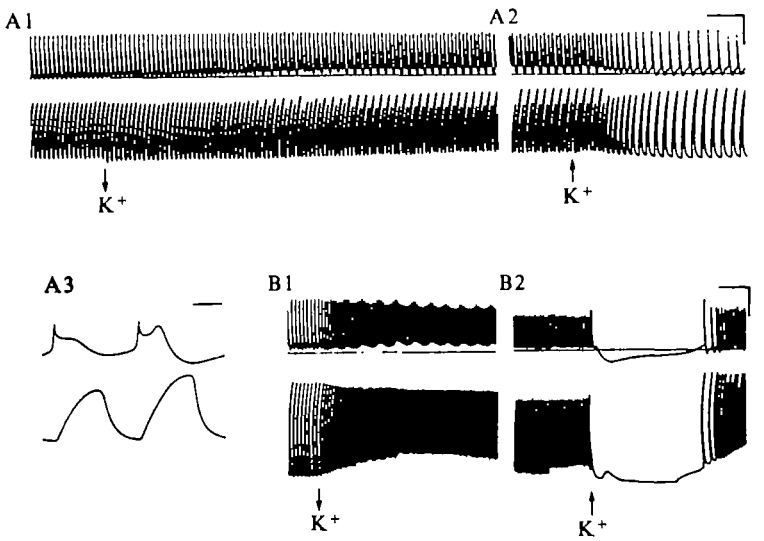


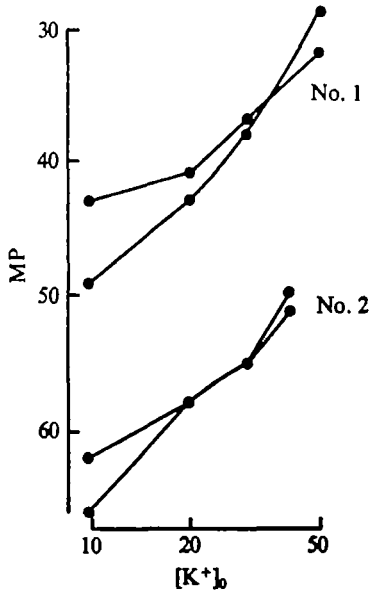
Fig. 9. Effects of K-free sea water. The extent of membrane depolarization is indicated by a line representing the original membrane potential. Arrhythmicity developing in (A) as a result of K-free SW is illustrated in (A 3). Spikes with large plateaus have after-potentials approximating the original membrane potential. However, the extent of depolarization is interpreted as the maximum diastolic potential following the first of the two action potentials in (A 3). Recording has been interrupted 5 min between (A 1, 2). The record in (B) has been interrupted 30 min. Vertical bars are 20 mV. Horizontal bars represent 30 sec (A) and 1 min (B) and 1 sec in the expanded trace (A 3). Electrical activity is shown in the upper traces of each pair; mechanical activity in the lower trace.

Reduced temperatures

The membrane potential in the *Modiolus* heart is sensitive to temperature. A 15–20 °C temperature reduction produced membrane depolarizations of 2–7 mV. This information, by itself, does not lead to the conclusion that the depolarization is due to a metabolism-dependent potential since the potential would be expected to decrease about 3·5 mV by the relation of the equilibrium potential to absolute temperature as predicted by the Nernst equation.

The effect of cooling on the membrane potential was more evident in K-depolarization experiments. In two different preparations the depolarization produced by 20–50 mM excess potassium was greater at room temperature than at 3–4 °C (cf. depolarization values, Fig. 10). In reference to the original membrane potential and the initial cooling depolarization, the depolarization curves at 3–4 °C not only have a reduced slope but also intersect with the depolarization curves at room temperature, the point being that the two curves are more early superimposable toward their linear

ranges (above 40 mM-K_0). Thus, membrane depolarization observed in the cold tends to produce the greatest potential change in the portion of the curves (low K_0) where the effects of a high sodium conductance and offsetting action of the electrogenic, temperature-sensitive pump become important.



	No. 1		No. 2	
K^+	23 °C	4 °C	23 °C	3 °C
20	6	2	8	4
30	11	6	11	7
40			16	11
50	20	11		

Fig. 10. Effect of reduced temperature on potassium depolarization. Membrane depolarization (mV) from increasing concentrations of potassium (m-moles/l) is tabulated in the right half of the figure for two hearts (nos. 1 and 2), each depolarized first at room temperature and then in the cold. These data are plotted in the left half of the figure with the potassium concentration plotted along the abscissa and membrane potential along the ordinate. The lower trace of each pair represents potassium depolarization at room temperature. The upper traces represent potassium depolarization at reduced temperatures and they have been plotted with respect to the initial membrane depolarization in the cold (6 mV for no. 1; 4 mV for no. 2).

DISCUSSION

Ions and the membrane potential

The ionic mechanism underlying the low membrane potential and diastolic pacemaker in *Modiolus* heart appear to be similar, in part, to those in other pacemaker tissues. The basis for the low membrane potential can be better understood by examining the various depolarization curves summarized in Fig. 11. By increasing potassium to 540 mM the membrane was depolarized 46 mV by KCl in sea water with a slope of 34 mV per tenfold increase in K_0; 54 mV by K_2SO_4 in sea water with a slope of 42 mV per tenfold increase in K_0; and 59 mV by K_2SO_4 and Cl-free SW with an estimated slope of 48–50 mV per tenfold increase in K_0. Thus, by replacing chloride with a less rapidly penetrating anion, the increased magnitude of the final depolarization appears to be the result of complementing, and perhaps independent, potentials. This interpretation suggests that 15–30% of the membrane potential in the heart of *Modiolus* is dependent on a chloride equilibrium potential, although the primary membrane permeability is to potassium.

Additional experiments designed to establish the ionic conditions of the new equilibrium (in Cl-free SW) are needed. It would be interesting to pursue the relationship of some organic anions to the membrane potential and Donnan equilibrium. Pierce & Greenberg (1970) have recently demonstrated that the membrane of the heart is permeable to amino acids in dilute sea water and that these anions cross the membrane to maintain osmotic balance. Whether Cl-free SW would alter membrane permeability or whether the transfer of amino acids is sensitive only to a change in osmotic pressure has not been determined.

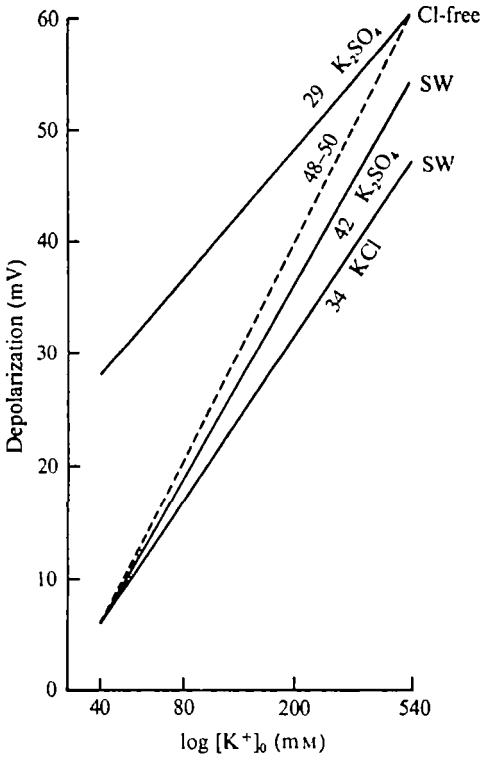


Fig. 11. Summary of potassium-depolarization experiments illustrating the relationship of chloride to membrane potentials. Membrane depolarization versus the extracellular potassium concentration on a logarithmic scale for one experiment. The solid lines have slopes, estimated from the data in Fig. 8 between 40 and 400 mM-K_0, of 34 mV for potassium depolarization by KCl in sea water, 42 mV for K_2SO_4 in sea water, and 29 mV for K_2SO_4 in Cl-free sea water. The latter curve is based on the sustained depolarization in Cl-free sea water. The broken line, with a slope of 48–50 mV per tenfold K_0 increase, is the result which would be predicted by a simultaneous increase in K_0 and decrease in Cl_0.

A second factor contributing to the low membrane potential is a Na-leakage current (I_{Na}), which in low K_0 produces the shift of the observed potential from the linear or more theoretical electrochemical relationship. This sodium current appears to be independent of potassium and chloride conductances, as seen by the superimposability of the curves between 10 and 40 mM-K_0 in Fig. 5. It is possible, however, to increase the membrane potential and shift the curves closer to the K-equilibrium potential by removing Na_0. The linear relation between the membrane potential and log K_0 was

also extended, in *Mytilus* heart, by removing Na_0 (Irisawa *et al.* 1967). In addition, the variability of the membrane potential in *Modiolus* can be attributed to the relative Na-leakage current from one preparation to another. A similar relation between low sodium hyperpolarization and resting potential was observed in Purkinje fibres of sheep with microelectrodes (fig. 1 of Trautwein & Kassebaum, 1961). Thus preparations which exhibit low membrane potentials appear to have a greater sodium permeability.

This continuous sodium current has been interpreted as being the 'driving force' in spontaneous muscles (Hutter & Trautwein, 1956; Noble, 1962). At maximum diastolic potential the membrane permeability to potassium (P_K) is high and the membrane potential is dependent primarily on potassium. As P_K decreases the membrane is depolarized by the contribution of I_{Na} to the overall potential leading, eventually, to the spike threshold. Changing either P_K or I_{Na} could therefore alter the inherent spontaneity of the heart as would be expected, for example, by increasing the inward I_{Na}. A net increase in I_{Na}, resulting from inhibition of the sodium pump by K-free SW, is the apparent explanation for the increased frequency in Fig. 9.

Electrogenic sodium-pump and the membrane potential

The direct effects of metabolic processes on the membrane potentials of muscles and nerves have been the topic of considerable recent research, particularly in the studies on molluscan giant neurones (Kerkut & Thomas, 1965; Carpenter & Alving, 1968; Carpenter, 1970; Moreton, 1969; Thomas, 1969; Gorman & Marmor, 1970). In these systems the exchange mechanism for active transport of sodium and potassium ions across the cell membrane creates electrogenic currents in addition to maintaining the long-term ionic imbalances required for continued regenerative activity. Sodium-pump electrogenesis is normally observed in relation to the membrane currents in the resting state. In *Modiolus*, where the membrane potential is not electrically analogous to a resting potential, the electrogenic pump appears to operate primarily in regenerative processes following each spike.

The evidence for the sodium pump is summarized in Table 3. The membrane potential in *Modiolus* heart is depolarized by 2–8 mV by inhibitors of the enzymes involved in active transport (ouabain and cyanide), by slowing the rates of these enzymatically coupled mechanisms (reduced temperatures), and by removing the substrates necessary for coupling of these transport mechanisms (K-free and Li-free SW).

A further observation concerning the membrane potential, a term used synonomously with the maximum diastolic potential, indicates a direct relationship between the electrical activity in the muscle, a high I_{Na}, and the opposing action of the sodium pump. In arrhythmical preparations (e.g. Fig. 12A), failure to produce a spike also fails to repolarize the membrane to the maximum diastolic potential. By superimposing expanded records of pacemaker depolarizations, with and without a spike, an after-hyperpolarizing phase of the action potential can be identified (Fig. 12B). The membrane potential or maximum diastolic potential is therefore dependent on the magnitude of the after-potential. The amplitude of the slow wave and after-hyperpolarization in this example were 7 and 5 mV, respectively. Action potentials with large plateaus also have a more negative after-hyperpolarization (positive after-

potential) (Figs. 8A, 9A). Evidence that the plateau results from a prolonged sodium current will be presented in the following paper (Wilkens, 1971). Therefore the larger after-hyperpolarizing response appears to represent an increased electrogenic sodium current stimulated by a prolonged influx of sodium during the plateau. Artificial stimulation of the pump, by injecting sodium inside the cell, has been demonstrated in several instances (Kerkut & Thomas, 1965; Koike, Brown & Hagiwara, 1970; Moreton, 1969; Thomas, 1969).

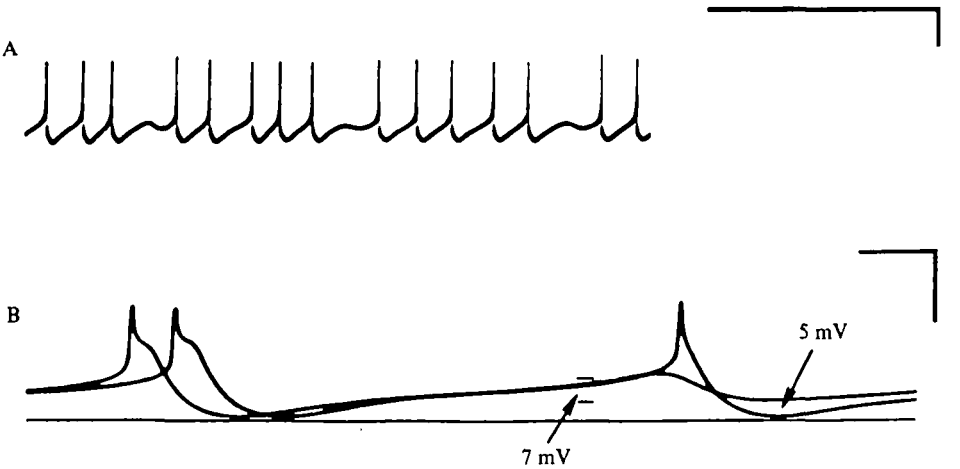


Fig. 12. Illustration of the spike after-potential. Expanded traces of action potentials from an arrhythmic heart, as in (A), are photographically superimposed in (B), showing spike-dependent after-hyperpolarization. Vertical bars equal 20 mV. Horizontal bars equal 1 min in (A), 1 sec in (B).

The relationship between the plateau and after-hyperpolarization was dramatically illustrated (Fig. 8A) by the simultaneous reduction of both of these phases of the action potential in Li-SW. Here, the decrease in magnitude of the after-potential, hence the 8 mV depolarization, reflects the inability of lithium to substitute for sodium in the plateau, the gradual loss of internal sodium and, finally, the inability of lithium to substitute for sodium in the electrogenic pump. The reduction of the after-potential in Li-SW may, on the other hand, represent only the difference between sodium and lithium permeability in the formation of the plateau since the after-potential has already been shown to be dependent on the plateau. Lithium is also, reportedly, unable to generate currents across the membrane in the cultured chick heart (Pappano & Sperelakis, 1969).

Nevertheless, depolarization, or reduced after-hyperpolarization, can be directly attributed to the pump in the case of ouabain treatment, where the effect is present without a simultaneous attenuation of the plateau (Fig. 8B). The plateau, in this slow-speed record, is identifiable as the lower, heavily shaded portion of the electrical trace.

These results, however, do not completely explain the basis for pacemaker rhythmicity in this preparation. One question raised by these experiments concerns the ability of the heart spontaneously to become depolarized in the absence of sodium. The ability to develop action potentials in the absence of sodium, and their dependence on calcium, will be discussed in the following paper (Wilkens, 1971).

The ionic basis for the membrane potential in the heart of *Modiolus demissus*, as the combination of the relative permeabilities of potassium, chloride, and sodium permeabilities, and the operation of a metabolic pump, tentatively explains the wide range in the observed membrane potential. Further study will be necessary to determine the interrelations of these factors quantitatively.

SUMMARY

1. The membrane potential in the heart of *Modiolus demissus* is dependent primarily on the unequal distribution of potassium ions across the cell.

2. The membrane potential is low, however, and the reduced slope of the potassium equilibrium potential is not predictable by the familiar Nernst equation.

3. The membrane undergoes a sustained depolarization in Cl-free SW which reduces the magnitude of subsequent potassium depolarization. Chloride ions contribute, perhaps independently, up to 15–30% of the total potential.

4. The high degree of variability in membrane potential is due, in part, to a sodium leakage current. Membrane hyperpolarization in Na-free SW is greatest in hearts with a low initial potential.

5. An electrogenic sodium pump is described which contributes to the membrane potential in the form of a rhythmical after-hyperpolarization (positive after-potential) following each spike.

6. Inhibition of the pump (reduction of the after-potential) can be achieved using ouabain and cyanide, by substituting lithium for sodium, by removing extracellular potassium and by cooling the heart.

7. The ionic basis of the membrane potential is discussed in relation to the diffuse pacemaker properties of the heart.

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