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Potentiometric Estimation of Charges in Barnacle Muscle Fibers under Internal Perfusion

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Summary. Single barnacle muscle fibers from *Megabalanus Psittacus* (Darwin) were internally perfused with a number of solutions of K-acetate of concentration C_2 which was increased from 100 to 600 mM maintaining a pH of 7.5. The external solutions at the same pH contained 40 mM $MgAc_2$ and K-acetate of concentration C_1 which was varied from 10 through 60 mM. The tonicity of both internal and external fluids was maintained at 1,000 mosmole with sucrose. The membrane potential E_m (the potential was referred to the external solution as ground) arising across the fiber membrane was measured using those internal and external solutions which maintained the ratio (C_2/C_1) at 10. Similar measurements but now maintaining the ratio (C_2/C_1) at 2, where C_1 was the concentration of Tris-Cl in the internal perfusion fluid and was increased from 100 to 250 mM and C_2 was the concentration of total Cl in the external fluid which contained 40 mM $MgCl_2$ and different amounts of Tris-Cl (120 to 420 mM), were also made using various isotonic solutions at pH 4.0. The measured values of E_m corrected for the liquid junction potentials, on interpolation with the theoretical curves of E_m plotted against log $(1/C_1)$ derived from the theory of membrane potential developed by Teorell and by Meyer and Sievers, gave values of 0.019 and 0.7 M for the density of fixed charges present on the membrane at pH 7.5 (cation selective) and 4.0 (anion selective), respectively. Also, values of 2.3 at pH 7.5 and 1.0 at pH 4.0 were derived for the mobility ratio of cation to anion.

It is well recognized that charges present on the surface of biological membranes influence various aspects of biological behavior. A surface charge model has been used by Gilbert and Ehrenstein (1969) to explain some of the voltage-dependent characteristics of the conductance of the nerve fiber. Other biological phenomena, e.g. muscle contraction, protoplasmic streaming, etc., are likely to be greatly influenced by the nature of the charged membrane surfaces. Consequently, a number of techniques, mostly electrokinetic in nature, have been utilized by a number of investigators (*see* Gilbert, 1971 for a brief review) to estimate the density of charges present in membranes of various biological preparations. Recently,

measurement of electrophoretic mobility of vesicles of fragmented sarcoplasmic reticulum coated onto latex spheres has been used to estimate the charge on the reticulum membrane (Baskin, 1972). In addition, a potentiometric method based on the concepts underlying the theory of membrane potential developed simultaneously by Teorell (1935*a*, *b*, 1953) and Meyer and Sievers (1936) has been used by Meyer and Bernfeld (1945, 1946) to determine the fixed charge density in a number of living and artificial systems. A brief review of this method as applied to artificial membranes has been given by Lakshminarayanaiah (1965, 1969*a*). Since the work of Meyer and Bernfeld (1946), this method has not been applied to any biological preparation because of the complexity of the biological systems and in particular to the inability to control the internal and external ionic environments of the cell membrane.

With the introduction of methods for internal perfusion of the giant axon of the squid (Baker, Hodgkin & Shaw, 1961; Oikawa, Spyropoulos, Tasaki & Teorell, 1961), it is possible to maintain the internal and external concentrations of (1:1) electrolyte at controlled levels (Rojas & Atwater, 1968). This should enable one to use the theory of Teorell, Meyer and Sievers (TMS) to estimate the charge on the membrane of the giant axon of the squid; however, to date no such attempt has been described in the literature.

Recent developments in the laboratory of Professor Rojas in Chile have made it possible to apply the TMS theory to the perfused barnacle muscle fiber. Keynes, Rojas, Taylor and Vergara (1973) have been successful in perfusing the giant barnacle muscle fiber internally with isotonic solutions of K aspartate. Following this, Lakshminarayanaiah and Rojas (1973) were able to manipulate the internal and external environments of the barnacle muscle cell using a variety of salt solutions whose ionic strength was varied over a wide range. The tonicity of the solutions was maintained with sucrose. Under the varied conditions employed, the muscle fibers remained viable in that when they were returned to normal physiological saline they retained their usual contractile characteristics. In view of this remarkable behavior, attempts were made to establish those internal and external ionic conditions that were necessary for the measurement of electrical potentials arising across the cell membrane at rest as a function of (1:1) electrolyte concentration keeping the ratio of its concentrations, internal to external, constant in accordance with the principles of the TMS theory.

It is well established now that the barnacle muscle fiber is selective to cations at pH 7.5 and that as the pH is reduced, it loses its selectivity to cations and becomes selective to anions at a pH of about 4 (Hagiwara,

Gruener, Hayashi, Sakata & Grinnell, 1968; Hagiwara, Toyama & Hayashi, 1971; DiPolo, 1972). Consequently, the required experiments, the results of which are presented in this paper, were performed to estimate the number of anionic and cationic groups present in the fiber membrane at pH 7.5 and 4.0, respectively.

Methods

The barnacle muscle fibers from *Megabalanus Psittacus* (Darwin), available in Chile along the coast of Montemar, were used in this study. The experimental procedures for dissection and internal perfusion of the muscle fibers and for the measurement of membrane potential were similar to those described by Keynes *et al.* (1973).

Solutions

In the preparation of all solutions analar grade salts were used. A 1.0-M stock solution of potassium acetate (KAc) was prepared by neutralizing the exact quantity of KOH to pH 7.5 using nearly 6 M solution of acetic acid. Similarly a 1.0-M stock solution of Tris(hydroxymethyl)aminomethane chloride (Tris-Cl) of pH 4.0 was prepared by neutralizing 100 ml of 2.0 M HCl with Tris base to pH 4.0 and making it up to 200 ml. From these solutions, other solutions both internal and external whose composition and combinations are given in Tables 1 and 2, were made up.

Table 1. Composition of internal and external solutions, pH = 7.5

Solution No.	Internal (mM)			External (mM)			
	KAc (C_2)	Sucrose	Tris-Cl	KAc (C_1)	Sucrose	$MgAc_2$	Tris-Cl
1	100	666	5	10	730	40	5
2	200	560	5	20	710	40	5
3	300	450	5	30	695	40	5
4	400	310	5	40	675	40	5
5	600	50	5	60	640	40	5

The final pH of the solutions was adjusted by adding Tris or HCl.

Table 2. Composition of internal and external solutions, pH = 4.0

Solution No.	Internal (mM)			External (mM)			
	Tris-Cl (C_1)	Sucrose	K-H-Phthalate	Tris-Cl [a]	Sucrose	$MgCl_2$	K-H-Phthalate
1	100	610	10	120	510	40	10
2	150	560	10	220	375	40	10
3	200	480	10	320	205	40	10
4	250	410	10	420	15	40	10

The final pH of the solutions was adjusted by using Tris or H_2SO_4.
[a] C_2, it is the total concentration of chloride (mM).

Tris-Cl (5 mM) and potassium hydrogen phthalate (10 mM) were utilized as buffers to control the pH of solutions used in the estimation of anionic (pH= 7.5) and cationic (pH= 4.0) groups, respectively, present in the barnacle muscle fiber membrane. A sample of 40 mM Mg^{2+} (as acetate or as chloride in solutions of pH 7.5 or 4.0, respectively) was used in the external solutions to maintain the muscle fibers in a physiologically reversible state (Hagiwara *et al.*, 1968, 1971). The osmolarity of all solutions (1000 ± 60 mosmole) was checked on a Mechrolab osmometer. All experiments were performed at room temperature (19 to 21 °C).

Results

In an earlier publication (Lakshminarayanaiah & Rojas, 1973), it was shown that isotonic solutions of KAc when used for internal perfusion of the giant barnacle muscle fibers generated and maintained steady resting potentials whose magnitudes compared well with those existing in the literature (DiPolo, 1972; DiPolo & Latorre, 1972; Keynes *et al.*, 1973). Also it was established that the intracellular concentration of K in the barnacle muscle fiber soaked in normal saline solution (NaCl = 430 mM, KCl = 10 mM, $CaCl_2$ = 20 mM, $MgCl_2$ = 40 mM and Tris-Cl = 5 mM) can not be reduced easily below about 50 mM by internal perfusion of the fiber using isotonic solution of 20 mM KAc. In view of this, it is difficult to decide the lowest concentration of KAc solution to be used inside the fiber to realize a value for the membrane potential which would be steady and reproducible over a period of time. To accomplish this, the muscle fiber must equilibrate easily with the concentration of KAc solution used for its internal perfusion. Consequently, a number of preliminary experiments were carried out using a variety of isotonic solutions of KAc in order to establish the right concentrations, internal and external, required to record steady and reproducible values for the membrane potential. These results showed that the minimum concentration best suited for internal perfusion was 100 mM with respect to K^+ or Cl^- ion at pH 7.5 or 4.0, respectively. The composition of the external solutions was manipulated to give for the ratio (C_2/C_1), internal to external, a value of 10 in the case of KAc solutions at pH 7.5 (Table 1) and a value of 2 (external to internal) in the case of Tris-Cl solutions at pH 4.0 (Table 2). These compositions and combinations of internal and external solutions given in Tables 1 and 2 were found to be most satisfactory in that they generated across the barnacle muscle fiber membrane electrical potentials which were very steady and highly reproducible. Such results of membrane potential measured are given in Tables 3 and 4.

As the electrolyte concentration of the internal perfusion fluid and the total osmolarity of both internal and external solutions are restricted to

Table 3. Membrane potential E_m and liquid junction potential E_L at pH = 7.5

Solution No.	E_m(mV) Measured	E_L(mV) Measured	E_L(mV) Calculated	E_m(mV) Corrected
1	−29.4±0.8 (8)	+3.2	+3.0	−32.5
2	−27.9±0.5 (8)	+4.0	+3.8	−31.8
3	−25.8±0.9 (6)	+5.0	+4.5	−30.6
4	−22.6±0.8 (6)	+5.5	+5.0	−27.9
5	−19.7±0.4 (8)	+6.0	+5.8	−25.6

All potentials are referred to the external solutions as ground. The numbers in parentheses indicate the number of fibers used in the measurements. Values for mV given as ±1 SEM.

Table 4. Membrane potential E_m and the liquid junction potential E_L at pH = 4.0

Solution No.	E_m(mV) Measured	E_L(mV) Measured	E_L(mV) Calculated	E_m(mV) Corrected
1	−14.1±0.5 (7)	+2.8	+2.3	−16.6
2	−11.4±0.3 (9)	+3.0	+2.6	−14.2
3	−10.5±0.3 (7)	+3.5	+2.9	−13.7
4	−10.3±0.2 (9)	+3.5	+3.0	−13.5

All potentials are referred to the external solutions as ground. The numbers in parentheses indicate the number of fibers used in the measurements. Values for mV are given as ±1 SEM.

100 mM (with respect to K^+ or Cl^- ions) and 1,000 mM respectively, there is only limited scope left for the manipulation of outside solutions in order to maintain the values of the ratio (C_2/C_1) constant at significantly divergent levels for at least three different combinations of C_2 and C_1 solutions. For example, it is not possible, with the above restrictions, to prepare even two outside solutions in the case of Tris-Cl (pH = 4.0) so that the ratio (C_2/C_1) is 5. As a consequence, one could only work with the combinations of solutions given in Table 2. However, in the case of KAc solutions (*see* Table 1), it is possible to maintain a value of 2 for the ratio (C_2/C_1). Such solutions were prepared and used in some preliminary experiments to measure the membrane potential E_m. These values of E_m (results not given), although steady and reproducible, were low and changed little at higher concentrations. The highest value measured for $C_2 = 100$ and $C_1 = 50$ mM KAc was about −10 mV and the lowest value was about −4 mV for $C_2 = 600$ and $C_1 = 300$ mM. The relation of these preliminary results derived from very few early experiments (one or two in each case for three combina-

tions of C_2 and C_1 solutions) to those given in Table 3 has been pointed out later in this section.

Other points of interest observed in preliminary studies were that when (i) $C_2 = C_1$, E_m was nearly zero and when (ii) NaCl or KCl electrolyte was used in the place of Tris-Cl (*see* Tables 2 and 4), steady and reproducible values for E_m were not observed. Why this was so is difficult to explain without further investigation.

The measured values of membrane potential (E_m) given in Tables 3 and 4 require corrections for the liquid junction potential E_L which is due to the two liquid junctions, one existing between the tip of the internal capillary electrode filled with 0.5 M KCl (Ag-AgCl) and the internal perfusing solution and the other existing between a similar capillary electrode and the external solution. As the compositions of these solutions are known, E_L can be calculated for the different combinations of internal and external solutions using the Henderson equation (Lakshminarayanaiah, 1969*b*). The values so calculated are given in Tables 3 and 4. Further, E_L can be measured directly by replacing the muscle fiber with an agar-KCl (3.0 M) bridge (Baker, Hodgkin & Meves, 1964) to connect the two half-cells. The results of such measurements are also given in Tables 3 and 4. The average of both measured and calculated values of E_L for various membrane systems are algebraically added to E_m to give the corrected values for E_m.

The values of E_m decrease as the concentration of the cation (Table 3) or anion (Table 4) is increased although the decrease at higher concentrations is not as great when compared to the corresponding decrease at lower concentrations. This is in marked contrast to what is observed in artificial ion-exchange membrane systems where the decrease in the value of E_m at higher concentrations is very large (Hills, Jacobs & Lakshminarayanaiah, 1961*a*; Lakshminarayanaiah, 1966*a*), the decrease being attributed usually to loss in cation or anion, i.e. counter-ion, selectivity arising from the increased leak of co-ions through the membrane. The observed type of behavior in the barnacle muscle fiber (*see* Tables 3 and 4) is in general accord with the TMS theory, according to which the membrane potential is made up of two Donnan potentials at the two membrane-solution interfaces and a diffusion potential existing across the membrane. The net membrane potential E_m is given by (Meyer & Bernfeld, 1945, 1946)

$$E_m = \frac{RT}{F}\left[(1/2)\ln\frac{\sqrt{4C_1^2+X^2}-X}{\sqrt{4C_1^2+X^2}+X}\cdot\frac{\sqrt{4C_2^2+X^2}+X}{\sqrt{4C_2^2+X^2}-X} + U\ln\frac{\sqrt{4C_2^2+X^2}+UX}{\sqrt{4C_1^2+X^2}+UX}\right] \tag{1}$$

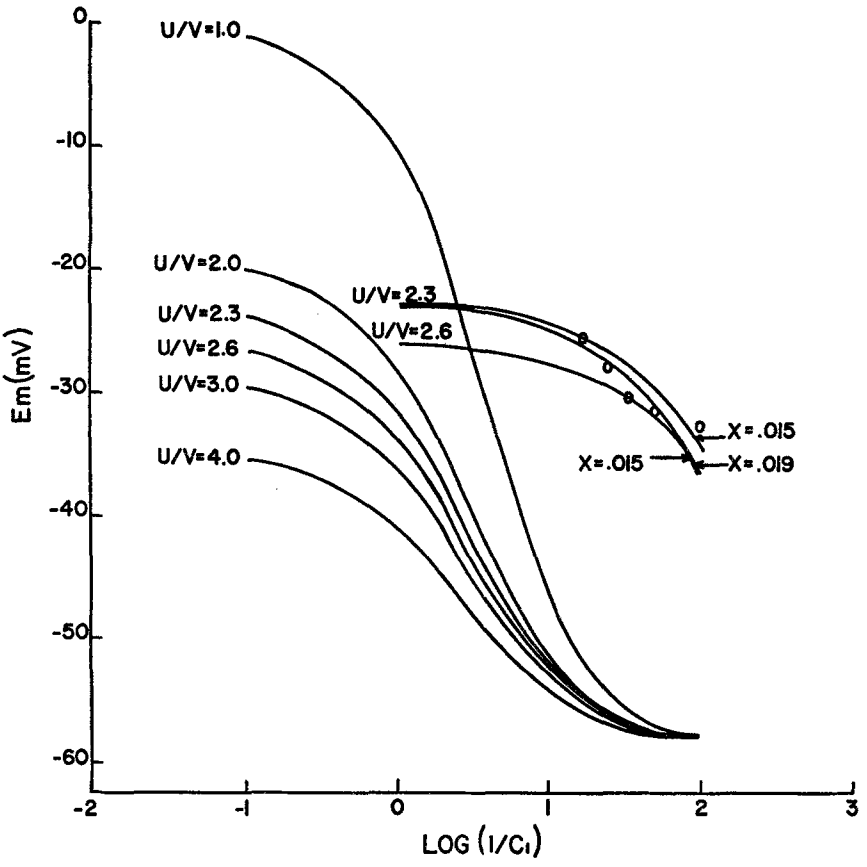

Fig. 1. Estimation of membrane fixed charge density at pH = 7.5 by the potentiometric method due to Teorell, Meyer and Sievers. The smooth curves on the left are theoretical membrane potentials (E_m) for a cation-exchange membrane, (1:1) electrolyte and constant ratio of $(C_2/C_1) = 10$ where C_2 and C_1 are the internal and external solution concentrations, respectively, in moles of K per liter of solution as a function of $\log (1/C_1)$. The curves are for different cation-to-anion mobility ratios (u/v) and fixed charge density value of unity. The experimental E_m values (0) are plotted in the same graph against $\log (1/C_1)$. The shift of the experimental curve coinciding with one of the theoretical curves gave $\log X$ (fixed charge density value) and the coinciding curve gave the value for (u/v). To derive exact values for X and (u/v), theoretical curves (*see* the curves bracketing the experimental points) obtained from approximate values of X and (u/v) are drawn. The values for X and (u/v) so derived are 0.019 M and 2.3, respectively

where X is the concentration of anionic groups in the membrane (cation exchanger), $U = (u - v)/(u + v)$ where u and v are the mobilities of cation and anion, respectively, and R, T and F have their usual meaning. The first and second terms of Eq. (1) represent Donnan and diffusion potentials, respectively. The sign of X in Eq. (1) is changed to make it applicable to an anion exchange membrane.

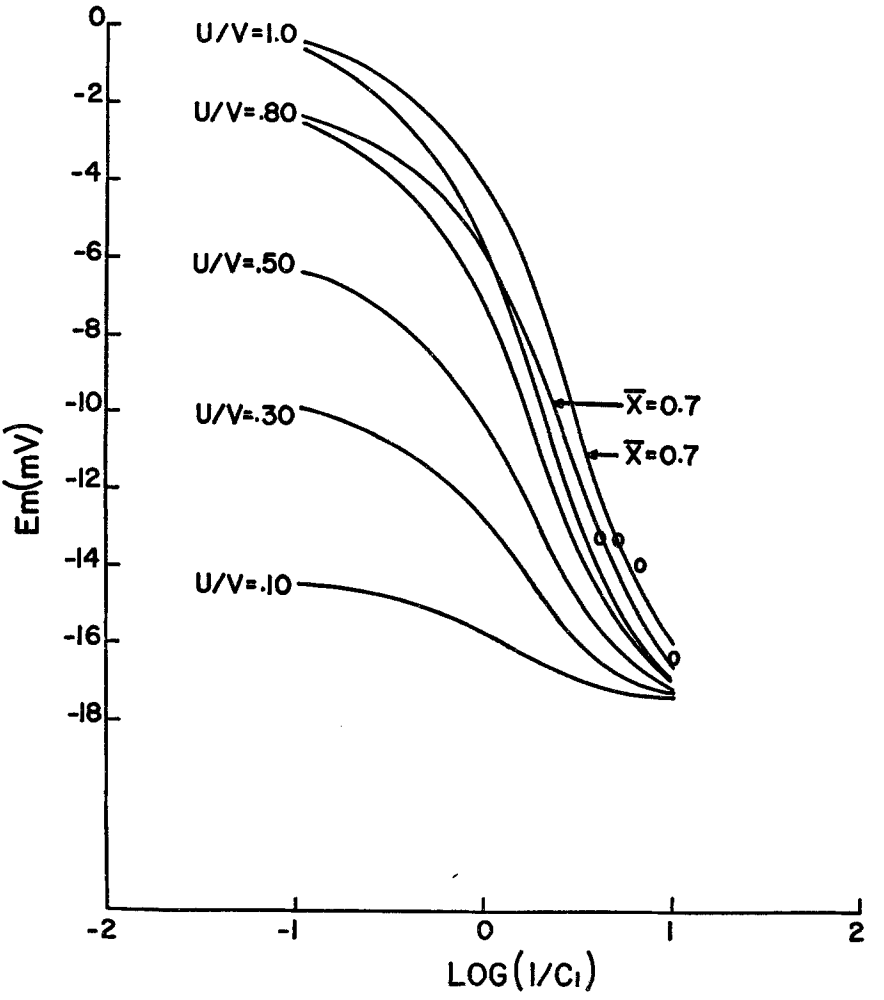

Fig. 2. Estimation of membrane fixed charge density at pH = 4.0 by the potentiometric method due to Teorell, Meyer and Sievers. The smooth curves on the left are theoretical membrane potentials (E_m) for an anion-exchange membrane, (1:1) electrolyte and constant ratio $(C_2/C_1)=2$ where C_2 and C_1 are the external and internal solution concentrations, respectively, of Cl per liter of solution as a function of $\log(1/C_1)$. The curves are for different cation-to-anion mobility ratios (u/v) and fixed charge density value of unity. The experimental E_m values (0) are plotted in the same graph against $\log(1/C_1)$. The shift of the experimental curve coinciding with one of the theoretical curves gave $\log X$ (fixed charge density value) and the coinciding curve gave the value for (u/v). To derive exact values for X and (u/v), theoretical curves (*see* the curves bracketing the experimental points) obtained from approximate values of X and (u/v) are drawn. The values for X and (u/v) so derived are 0.7 M and 1.0, respectively

To use Eq. (1) to estimate a value for X (the density of charge present in the membrane), theoretical values for E_m were calculated for $X=1$ as a function of C_1 keeping the ratio of $(C_2/C_1)=10$, cation exchange membrane

or = 2, anion exchange membrane (note how C_1 and C_2 are labeled in Tables 1 and 2) assigning different values for the mobility ratio (u/v). These calculations were performed on a CDC computer. The values of E_m thus evaluated were plotted as shown in Figs. 1 and 2. The values of E_m measured experimentally (*see* Tables 3 and 4) were plotted in the same graph (Figs. 1 and 2) concerned as a function of log ($1/C_1$). The experimental curve was shifted horizontally and ran parallel, i.e. superimposable, to one of the theoretical curves. The horizontal shift from the coinciding theoretical curve gave the value for log X and the value of (u/v) corresponding to the theoretical curve gave the mobility ratio. The value of X so derived was between 0.015 and 0.019 M and the value of (u/v) was between 2.3 and 2.6 for the cation selectivity of the membrane. The values corresponding to the anion selectivity of the membrane were: X between 0.7 and 0.8 M and (u/v) between 0.8 and 1.0.

To fix these values accurately, theoretical curves were constructed for the estimated values of X and (u/v). In Fig. 1, three curves pertaining to $X = 0.015$ M and $(u/v) = 2.3$, 2.6 and $X = 0.019$ M and $(u/v) = 2.3$ are given bracketing the experimental points. The best fit of experimental points corresponds to values of $X = 0.019$ M and $(u/v) = 2.3$. Similarly, in Fig. 2, two theoretical curves pertaining to $X = 0.7$ M and $(u/v) = 0.8$ and 1.0 enclosing the experimental points are given. In this case, the best fit of experimental points corresponds to $X = 0.7$ M and $(u/v) = 1.0$.

The few preliminary results of measurement of E_m referred to above which were obtained with KAc solutions at pH 7.5 maintaining the ratio (C_2/C_1) at 2, gave also on interpolation with the appropriate theoretical curves (not given) of the type shown in Figs. 1 and 2 values for X and (u/v) which agreed to within 25% of those ($X = 0.019$ M and $(u/v) = 2.3$) derived from exact measurements of E_m (*see* Table 3).

Discussion

There are a number of difficulties, both theoretical and technical, which should be recognized before making an assessment of the applicability of the TMS theory to estimate the number of fixed charges present in a complex biological system such as the barnacle muscle fiber.

The theoretical difficulties which underlie the assumptions upon which Eq. (1) is based, have been discussed at length elsewhere (Hills, Jacobs & Lakshminarayanaiah, 1961*b*). The important points are: (i) the membrane is homogeneous, (ii) the membrane parameters X, u and v do not change with concentration, (iii) ($\Pi\overline{V}/RT$) term, i.e. the swelling pressure and partial

molar volume of electrolyte product, which arises from the consideration of Donnan equilibrium is negligible, (iv) the single ion activity coefficients in the membrane phase are all unity, and (v) transport of water is negligible. Further, there is an additional uncertainty involved in applying Eq. (1) to derive values for the density of fixed groups present in the barnacle muscle membrane. This uncertainty arises from the fact that in the derivation of Eq. (1), the distribution and the diffusion of ions of (1:1) electrolyte only, have been considered. But in the present experiments solutions containing sucrose, Mg^{2+} and other ions in addition to (1:1) electrolyte, have been used. What role these additional species play in determining the values of E_m remain to be assessed.

Assumption (i) stated above is generally made by every investigator although it is commonly recognized that the most homogeneous membrane shows functionally characteristics that are typical of a heterogeneous membrane (Lakshminarayanaiah & Subrahmanyan, 1968). Assumptions (ii) and (iv) have been found to be quite drastic or rather unrealistic (Hills *et al.*, 1961*a*, *b*; Lakshminarayanaiah, 1963; Lakshminarayanaiah & Subrahmanyan, 1964); whereas assumption (iii) has been shown to have little effect on the membrane parameters (Mackie & Meares, 1955; Boyd & Bunzl, 1967). Similarly, the contribution of factor (v) to membrane potential at ordinary concentrations is very small (Lakshminarayanaiah & Subrahmanyan, 1964). Notwithstanding these objections, the TMS theory has been used a great deal in recent years to estimate the fixed charge density in glass membranes (Altug & Hair, 1968; Hersh, 1968; Hersh & Teter, 1972), parchment-supported inorganic precipitate membranes (Siddiqi & Pratrap, 1969; Siddiqi, Lakshminarayanaiah & Saxena, 1970; Beg & Saxena, 1971; Siddiqi, Lakshminarayanaiah & Beg, 1971), thin membranes of Parlodion (Lakshminarayanaiah, 1966*b*) and EIM-doped lipid bilayer membranes (Latorre, Ehrenstein & Lecar, 1972) whose concentration of fixed charge can not be estimated easily by any other means. However, in one of these studies (Lakshminarayanaiah, 1966*b*), it was shown that the TMS theory over-estimated the charge in membranes which had a low concentration of fixed groups and under-estimated the charge in membranes of high fixed charge density. Sollner and Carr (1944) in their very early work on collodion membranes observed similar results.

All of the above considerations should be applicable equally when the TMS theory is applied to estimate the concentration of fixed charges in biological membranes despite their complex structure. The uncertainty referred to above concerning the role of sucrose, Mg^{2+} and other ions in affecting the value of E_m as given by Eq. (1) is not that uncertain. Sucrose

being uncharged should have little effect on the value of E_m. The divalent ion Mg^{2+} or Ca^{2+} (40 mM) could be used in the external solution to maintain the physiologically viable state of the membrane. In the complete absence of either divalent ion, the resting membrane potential could not be measured with confidence as E_m drifted to lower values continuously with time probably due to the deterioration of the muscle fiber. In the present experiments, 40 mM Mg^{2+} ion only was used because its presence did not cause muscle to contract and made the measurements of membrane potential more reliable. In our early work with the barnacle muscle fibers (Lakshminarayanaiah & Rojas, 1973), the dependence of E_m on the concentration of Mg^{2+} (no Ca^{2+} present) in the normal saline solution (*see* p. 148 for composition) was explored using isotonic solution of 200 mM KAc for internal perfusion and Mg^{2+} ions in the concentration range 10 to 60 mM in the outside solution. The resting membrane potential E_m which was about -48 mV when the Mg^{2+} concentration was 60 mM (Lakshminarayanaiah & Rojas, 1973) changed to about -45 mV when the concentration of Mg^{2+} was reduced to 10 mM. In view of this insignificant change in E_m, it is reasonable to state that E_m is not affected significantly by the concentration of Mg^{2+} in the outside solution in the range 10 to 60 mM; but as shown elsewhere it is determined by the concentration of K^+ ions in the case of solutions of pH 7.5 (Hagiwara, Chichibu & Naka, 1964; Lakshminarayanaiah & Rojas, 1973) and by the concentration of Cl^- ions in the case of solutions of pH 4.0 (Hagiwara *et al.*, 1971; E. Rojas, *personal communication*). As the membrane is cation selective in solutions of pH 7.5, the contributions of anions to E_m has been taken care of by the term U in Eq. (1). Similarly, the same term U takes care of the contributions of cations to E_m in the case of solutions of pH 4.0 in which the fiber membrane is anion selective. These considerations thus favor the proposition that Eq. (1) describes adequately the variation of membrane potential E_m with concentration (C_2/C_1); that is, the data given in Tables 3 and 4.

As no data concerning the concentration of charged groups in the barnacle muscle membrane either at pH 7.5 when it is cation selective or at pH 4.0 when it is anion selective exist, it is not possible to assess whether the values derived for X, i.e. 0.019 and 0.7 M, are over- or under-estimated. However, the other information derived by the application of the TMS theory when the fiber membrane is cation selective at pH 7.5, i.e. the mobility ratio $(u/v) = 2.3$, may be checked against similar data derived in our recent publication (Lakshminarayanaiah & Rojas, 1973) in which it is shown that the membrane is permeable equally to Cl^- and Ac^- ions and that $(P_{Cl}/P_K) \approx 0.15$, where P_i is the permeability of the membrane to the

ionic species i and is given by (Hodgkin & Katz, 1949)

$$P_i = \frac{RT}{F} u_i \frac{\beta_i}{d}.$$

(β_i is the distribution or partition coefficient of i between the membrane of thickness d and the surrounding aqueous phase.)

Thus, the mobility ratio is given by

$$\frac{u_{\text{anion}}}{u_{\text{K}}} = \frac{P_{\text{Cl}}\beta_{\text{K}}}{P_{\text{K}}\beta_{\text{anion}}} \approx 0.15 \frac{\beta_{\text{K}}}{\beta_{\text{anion}}} = (v/u).$$

The value of the ratio (u/v) will be 2.3 provided $(\beta_{\text{K}}/\beta_{\text{anion}}) = 2.9$. In view of the presence of negatively charged groups in the membrane (concentration = 0.019 M), the membrane will accumulate cations and repel anions, thereby giving the ratio $(\beta_{\text{cation}}/\beta_{\text{anion}})$, a value definitely greater than unity. In some polymer membranes of high charge density ($X \approx 2.0$ M), this value is found to be about 9 (Hills *et al.*, 1961*a*). The value of 2.9 derived above thus appears reasonable.

In contrast to the information given above, the fiber membrane at pH 4.0 gives a value of 1.0 for the ratio (u/v). This is very perplexing. One would expect that the membrane, because of the presence of cationic groups on it, would be more permeable to anions than to cations and thus make the value of the ratio (u/v) less than unity. As this is not so, the contribution of the second term in Eq. (1), i.e. the potential due to diffusion of ions, to E_m should be considered negligible. The value of E_m is thus determined only by the first term which is due to the Donnan distribution of mobile ions in the membrane phase. This is a consequence of the existence of high density of positive charges (concentration = 0.7 M) present on the membrane. This conclusion is in agreement, although indirect, with the anion permeability and conductance data of Hagiwara *et al.* (1971), who suggested that anion permeation in the barnacle muscle membrane was mediated by membrane charges.

The technical difficulty involves relating the estimated concentration given above to an accepted frame of reference so that the values may be compared with other values derived for different biological systems. In the original papers of Meyer and co-workers, X is expressed as the concentration of fixed groups in equivalents per liter of pore liquid. Although this concentration scale is acceptable for artificial systems whose membranes contain large quantities of interstitial water, it raises some interesting

questions concerning the barnacle muscle fiber membrane and even artificially prepared bimolecular lipid membranes. Does the barnacle muscle fiber membrane in the resting state have pores in a manner similar to those existing in cross-linked polymeric membranes? Where are the charges located in the membrane? Direct answers to these questions can not be given in view of the many unknowns that exist in the present state of our knowledge concerning the structure of biological membranes. However, with the help of a model, one can try to evaluate what the values derived for X mean.

Let us consider the Davson-Danielli model according to which the biological membrane is considered to contain a bimolecular leaflet of lipid with its polar groups oriented toward the two aqueous, intra- and extracellular, phases of the cell. Protein is supposed to exist close to the polar heads of the leaflet (Davson & Danielli, 1943; Robertson, 1960, 1964). Based on the current concepts discussed in the literature regarding the biological membranes, we make the following assumptions. (1) The biological membrane is in a semi-solid semi-fluid (smectic) state; (2) voids between the hydrocarbon chains of the lipid bilayer forming the core of the membrane allow for the freedom of movement of the hydrocarbon chains; (3) liquid associated with the polar groups of the hydrocarbon chains and that which exists in the voids between the hydrocarbon chains are equated to the pore liquid; (4) the dimension of the hydrocarbon chains (bilayer membrane thickness including the polar head groups) is about 35 Å; (5) the double-layer or perhaps the triple-layer thickness extending from the polar head groups of the bilayer membrane is about 10 Å; and (6) the values of X derived from the application of the TMS theory correspond to equivalents per liter of the membrane structure inclusive of interfacial regions.

The total thickness of the membrane which is involved in its electromotive action is about 55 Å because of the existence of two double layers each of 10 Å-thickness on either side of the bilayer membrane. This effective thickness is the minimum value; perhaps it could be even greater. The volume charge density determined for X is 0.019×10^{-3} equiv cm^{-3}. The surface area of 1 cm^3 membrane of 55 Å-thickness is 18.2×10^5 cm^2. So a value of -3.1×10^{12} charges per cm^2 membrane surface is derived considering both the inner and the outer surfaces of the membrane. What the surface charge in one negative charge per N $Å^2$ will be if the effective thickness of the membrane is increased (i.e. surface area decreased) is given in Table 5 along with corresponding values for the positive charge density when the muscle fiber is in solutions of pH 4.0. For purposes of comparision, some values existing in the literature for other biological systems are also

Table 5. Surface charges present in various biological membrane systems

Membrane system	Method used	One negative charge per N, (number of Å^2) (N)	Membrane thickness assigned (Å)	Reference
Fragmented sarcoplasmic reticulum	Electrophoretic	1,000		Baskin (1972)
Muscle fibers, neurons in tissue culture	Application of electric field	2,000		Elul (1967)
Squid axon	Electrophoretic	290 8,000–9,000		Segal (1968); Gilbert (1971) using results of Segal
Lobster axon	Electrophoretic	200 3,500–4,000		Segal (1968); Gilbert (1971) using results of Segal
Squid axon	Conductance changes (curve fitting)	120		Gilbert and Ehrenstein (1969)
Squid axon	Curve fitting	700		Chandler, Hodgkin and Meves (1965)
Squid axon	Membrane potential (curve fitting)	1,600		Rojas and Atwater (1968)
Barnacle muscle fiber	Potentiometric at pH = 7.5 (curve fitting)	3,230 1,460 1,170	55 120 150	present work
		8,730 (according to TMS theory X expressed as mole per liter pore liquid)		
		One positive charge per N	Membrane thickness assumed (Å)	
Barnacle muscle fiber	Potentiometric at pH = 4.0 (curve fitting)	86 40 32	55 120 150	present work
		240 (according to TMS theory X expressed as mole per liter pore liquid)		

given in Table 5. The agreement between different values seems to depend on the nature of assumptions made in the derivation of those values.

In respect to the barnacle muscle fiber, it should be emphasized that the assumptions made above are not divorced from reality. The Davson-Danielli model of the biological membrane, although questioned by some investigators (Maddy & Malcolm, 1965; Korn, 1968), is the one model that has been accepted by a very wide circle of investigators. There is general agreement about assumptions 1 and 2 (Shanes, 1958). Assumption 3 is a consequence of the Davson-Danielli model. Assumption 4 follows from the recent work on various bilayer membranes carried out by Fettiplace, Andrews and Haydon (1971). However, early work on bilayers formed from brain lipids (Mueller, Rudin, Tien & Wescott, 1964) and sheep erythrocyte lipids (Andreoli, Bangham & Tosteson, 1967) indicated membrane thickness in the range of 50 to 130 Å. Assumption 5 is based on the concepts concerning the structure of the electrical double layer (really triple layer) at metal-aqueous solution surfaces (Bockris & Reddy, 1970). Whether this so-called double layer has a multilayered structure in artificial and biological membranes because of the presence of water clusters (Horne, Day, Young & Yu, 1968) is a factor that must be considered in relation to membrane thickness. Further, there is the possibility of overlapping of double layers due to criss-crossing of biopolymeric chains in the neighborhood of membrane surfaces. These possibilities probably would tend to increase the effective membrane thickness. Assumption 6, although realistic, is quite drastic in that it is not in accordance with the original theory. However, if this assumption is completely eliminated, then the thickness of the pore liquid would correspond to the double-layer thickness (10 Å). This would lead to one negative charge per 8,730 $Å^2$ membrane surface in the case of cation selectivity of the membrane and one positive charge per 240 $Å^2$ membrane surface in the case of anion selectivity of the membrane.

The procedure by which the surface charge has been evaluated using the value of volume charge density derived potentiometrically is based on a model which tells where the charges are located and how the liquid around the charges is distributed. Other detailed knowledge pertaining to the physical and chemical structure of the membrane in the barnacle muscle fiber is ignored as it is not required. The other interesting fact emerging from this study is that when the barnacle muscle fiber becomes anion selective at pH 4.0, a 36-fold increase in charge density is noted. This makes one wonder whether the cationic groups in the protein bounding the polar head groups of the phospholipid bilayer become involved in the electromotive action of the membrane. The high density of cationic charges on the

membrane as already pointed out not only determine the distribution of mobile ions in the membrane phase but also, in so doing, control those factors which regulate ion permeation and conductance of the membrane.

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