Liquid and Solid-State Cl⁻-Sensitive Microelectrodes

Characteristics and Application to Intracellular Cl⁻ Activity in Balanus Photoreceptor

JAMES H. SAUNDERS and H. MACK BROWN

From the Department of Physiology, University of Utah College of Medicine, Salt Lake City, Utah 84108

ABSTRACT   When intracellular chloride activity (a_{Cl}^i) was monitored with chloride-sensitive liquid ion exchanger (CLIX) microelectrodes in *Balanus* photoreceptors, replacement of extracellular chloride (Cl_0) by methanesulfonate or glutamate was followed by a rapid but incomplete loss of a_{Cl}^i. When propionate was used as the extracellular anion substitute, CLIX electrodes detected an apparent gain in a_{Cl}^i, while a newly designed Ag-AgCl wire-in glass microelectrode showed a loss of a_{Cl}^i under the same conditions. This discrepancy in Cl⁻ washout when propionate replaced Cl_0 is explained by the differences in selectivity of CLIX and Ag-AgCl electrodes for native intracellular anions and for the extracellular anion substitute which also replaces Cl_i and interferes in the determination of a_{Cl}^i. Both electrodes indicate that $E_{Cl} \approx E_m$ when the cells are bathed in normal barnacle saline, and both electrodes showed the rate of Cl washout $(\tau \approx 5$ min$)$ to be independent of Cl_i when Cl_0 was replaced by glutamate. Details of Ag-AgCl microelectrode construction are presented. These electrodes were tested and found to be insensitive to the organic anion substitutes used in this study. Selectivity data of CLIX electrodes for several anions of biological interest are described.

INTRODUCTION

Using chloride liquid ion exchanger (CLIX) microelectrodes similar to those described by Orme (1969) and Walker (1971), Brown showed (1976) that chloride was passively distributed in the dark-adapted barnacle photoreceptor, i.e. he reported a range of values for the internal chloride activity (a_{Cl}^i) and he noted that the calculated E_{Cl} was generally within 1-2 mV of the measured resting potential. A previous study (Brown et al., 1970) showed that the dark-adapted photoreceptor membrane potential was relatively insensitive to changes in external chloride activity (a_{Cl}^0) when chloride was replaced by methanesulfonate. Methanesulfonate was chosen for that study (Brown et al., 1970) because it was one of the least permeant anion substitutes studied in muscle fibers of another barnacle, *Balanus nubilus* (Hagiwara et al., 1971). A recent study of

cation permeability ratios in *Balanus* photoreceptor was justified by the observation that chloride is passively distributed in this preparation (Brown and Saunders, 1977).

The studies cited above raise several questions concerning anion permeation in *Balanus* photoreceptor. First, one cannot decide whether the "electrical silence" of Cl^- is due to a relatively low or a comparatively high membrane permeability to anions. Second, since CLIX electrodes are liable to interference by unknown native intracellular anions and to a number of extracellular anion substitutes, an evaluation of these electrodes for interference from naturally occurring or experimentally used anions is necessary to allow a choice of anion substitutes to which CLIX electrodes are relatively insensitive. Third, another probe was needed to independently measure a_{Cl}^i to verify the passive distribution of Cl^-. The Ag-AgCl electrode described in Materials and Methods was chosen because it could be easily fabricated and was expected to have different and fewer interferents than the ion exchanger. This electrode provided another measure of the completeness of Cl^- washout after substitution of an "impermeant anion" for the extracellular chloride.

Both CLIX and Ag-AgCl microelectrodes were used to make static and dynamic measurements of a_{Cl}^i in the dark-adapted photoreceptor. Static a_{Cl}^i determined by either electrode type after equilibration in normal barnacle saline, was comparable to that reported by Brown in that the calculated Cl^- equilibrium potential agreed well with the measured membrane potential. Dynamic measurements of a_{Cl}^i during chloride washout and determinations of the final a_{Cl}^i after washout with the two different electrode types were not always in agreement. The terms "apparent chloride activity" or "apparent change in chloride activity" were used to indicate serious deviations from the expected observation, for example when CLIX electrodes indicated a 38-fold increase in a_{Cl}^i during substitution of Cl_0 with iodide.

A preliminary account of the work has appeared (Saunders and Brown, 1975).

MATERIALS AND METHODS

Preparation

Barnacles (*Balanus eburneus*) were obtained from Marine Biological Laboratory, Woods Hole, Mass. The lateral ocellus was removed and dissected as described by Brown and co-workers (1970). The preparation was superfused with normal barnacle saline (NBS) which contained (mM): 462 NaCl; 8 KCl; 12 $MgCl_2$; and 20 $CaCl_2$, buffered to pH 7.6 with 10 mM Tris (hydroxymethyl) aminomethane.

Recording

Preliminary experiments in this study were conducted with a conventional recording system, i.e. E_m and the potential of the intracellular chloride electrode (V_{Cl}^i) were measured against a common 3 M KCl-filled reference electrode. This technique was satisfactory for measurements of a_{Cl}^i when the cell was bathed in NBS, but changes in the extracellular saline composition resulted in some change in the liquid junction potential of the extracellular reference electrode. In many experiments this potential change was persistent, producing uncertainty in the values of E_m and V_{Cl}^i from which the internal chloride activity (a_{Cl}^i) was calculated (cf. Brown, 1976). To avoid the

influence of extracellular saline changes on the reference electrode and on the calculation of a_{Cl}^i an "inside-out" recording system was used in the experiments reported here except where noted (see Fig. 7).

In the inside-out method the membrane potential was recorded between an intracellular reference electrode and an extracellular potential-sensing electrode. This procedure provided an intracellular reference for the Cl⁻-sensing electrode, thereby reducing the change in liquid junction potentials that invariably occurred when an extracellular reference electrode was used and when a test anion was substituted for Cl_0. Changes in junction potential after replacement of Cl_0 by the test anion could produce some error in measurement of the membrane potential under these conditions, but this would not influence the determination of a_{Cl}^i since there is no E_m term to consider in the determination of a_{Cl}^i (see Eq. [1] in Brown, 1976). Microelectrodes for membrane potential recording were filled with 3 M KCl which we found gave results the same as those obtained with K acetate-filled electrodes. The intracellular reference electrode was maintained at virtual ground potential by an operational amplifier in current-to-voltage configuration. Suitable high-input impedance amplifiers were used for recording E_m and V_{Cl} (Brown, 1976).

The results from experiments with conventional and inside-out techniques were comparable, provided the artifacts produced by anion substitution in the conventional recording system were taken into consideration and when the chloride-sensitive electrode was calibrated against the same reference electrode used in the biological experiment.

Chloride Electrodes

Chloride-sensitive microelectrodes were either the liquid ion exchanger (CLIX) or solid-state Ag-AgCl type, calibrated in advance of cell penetration (see Fig. 3 A). The chloride activity of the NBS (a_{Cl}^0) was determined from the calibration curve. When a cell was penetrated with both the ion-sensitive and reference electrodes, the cell was illuminated at intervals to determine that the chloride-sensitive electrode recorded no light-induced membrane potential change, verifying that both had impaled the same cell. After at least 15 min of dark adaptation, the potentials of both electrodes reached steady-state values and the intracellular chloride activity was calculated from

$$a_{Cl}^i = a_{Cl}^0 \cdot 10^{(V_{Cl}^i - V_{Cl}^0)/S},\tag{1}$$

where V_{Cl}^i was the potential measured between the intracellular chloride-sensitive electrode and the intracellular reference, V_{Cl}^0 was the potential between the same electrodes in the extracellular NBS, a_{Cl}^0 was the chloride activity of NBS determined during electrode calibration, S is the slope, dV/da_x of the electrode obtained from the calibration curve.

Changes in electrode tip potential are difficultt to assess. Results were considered acceptable only when the reference potential in the saline bath was the same before and after penetration of the cell. A change of tip potential while in the cell would contribute equivalently to V_{Cl}^i whether the reference electrode was intra- or extracellular. Broken, 3 M KCl-filled reference electrodes have been shown to be preferable to other types and show only small changes in liquid junction potential, which are in good agreement with those predicted from the Henderson-Planck equation (Hagiwara et al., 1971).

In general, the biological experiments were designed so that after impalement, cells were allowed to reach a dark-adapted steady state in NBS with $E_{Cl} \approx E_m$. The preparation was then superfused with one of the chloride-substituted salines until a new steady-state (final) a_{Cl}^i was attained. The preparation was then superfused with NBS, returning chloride to the intracellular space and bringing E_{Cl} back to agreement with

E_m. After this, a second chloride-substituted saline was used to wash out chloride for a second time; this was followed by the original anion substitute. Some experiments were done with only partially substituted salines and several experiments fell short of the ideal, with only one or two of the washout cycles completed before impalement was lost. The time constant for change of extracellular saline in the bath was 1 min.

Solutions

The composition of normal barnacle saline is given above. Chloride-free barnacle salines were prepared by taking the bases of the metals and of Tris (hydroxymethyl) aminomethane in aqueous solution and titrating to pH 7.6 with methanesulfonic acid, or by more conventional means using the monobasic salts of glutamic or propionic acid. Cation concentration, pH, and osmolarity (Advanced Digimatic Osmometer, model 3D, Advanced Instruments, Inc., Needham Heights, Mass.) were the same in NBS and chloride-free salines.

Several types of solutions were used to evaluate the selectivity of CLIX microelectrodes, including: (a) two-component mixed solutions of salts with a common cation (Na^+ or K^+). These solutions were made to contain chloride and one interfering anion at constant ionic strength, e.g., $[Cl^-] + [A^-] = 100$ mM ("reciprocal dilutions"); (b) separate serial dilutions of chloride and interfering anion salts, e.g., 10^0, 10^{-1} and 10^{-2} M KCl to be compared to the same concentrations of $KHCO_3$ ("pure solutions"); (c) solutions containing various concentrations of chloride, but all containing the same amount of interfering anion ("constant interferent"); (d) solutions containing various amounts of interferent, but all containing the same amount of chloride ("constant primary"); and (e) solutions containing the same amount of KCl with pH varied by adding small amounts of solid KOH or glutamic acid. Table I gives the composition of representative solutions.

Sodium isethionate and sodium propionate were obtained from K & K, Inc., (Irvine, Calif.). Sodium propionate supplied by Pfaltz & Bauer (Stamford, Conn.) was found to contain substantial halide contaminant, precipitated by $AgNO_3$. Data obtained with this chemical are not reported. Propionic and L-aspartic acids were obtained from Sigma Chemical Co. (St. Louis, Mo.). D-L-Glutamic acid was obtained from Aldrich Chemical Co. (San Leandro, Calif.), while the Na, K, Mg, and Ca monobasic salts were obtained from City Chemical Corp. (New York). Methanesulfonic acid was obtained from Eastman Kodak Corp. (Rochester, N. Y.). All inorganic chemicals were reagent grade.

Electrode Construction

CLIX microelectrodes were prepared with Corning chloride exchanger (no. 477315 Corning Medical, Medfield, Mass.) by a method similar to that described by Walker (1971). The electrodes routinely prepared in this laboratory are filled from the back, rather than by immersion of the micropipette tip in the exchanger. These electrodes were judged acceptable for use when they showed an initial calibration slope of at least −54 mV per decade increase in Cl^- activity. Virtually all CLIX electrodes prepared in this manner were acceptable. All electrodes were calibrated in a shielded cage by use of high-input impedance unity gain amplifiers with broken KCl reference electrodes. Experiments were acceptable only when the ion-sensitive electrodes were recalibrated after use, again showing slopes comparable to the original calibration slopes.

Ag-AgCl microelectrodes were prepared by starting with a 10-cm length of clean 0.127-mm 99.9% silver wire. This was immersed for 8–10 mm in a solution of 10% NaCN-5% NaOH and etched under microscope observation to a fine point by passing an anodal current of 60–80 mA (Neild and Thomas, 1973). If the wire produced by this method was not smoothly tapered, dilution of the etching solution (25–50%) with distilled water generally remedied this condition. After etching, the wire was rinsed in distilled water

and the tip (5 mm) of the wire was then immersed in a bath of 100 mM HCl and chlorided by passing an anodal current of 5 μA for 1 min.

Micropipettes were prepared by using Pyrex (Corning No. 7740) tubing (1.3–1.4 mm OD, 1.0 mm nominal ID) drawn in a vertical puller (Narishige PE-2) to a tip diameter of approximately 1 micrometer. The tip of the pipette was then immersed in 50% aqueous glycerol for about 2 s to allow the solution to fill the pipette to a depth of approximately 100 micrometers from the tip.

The sharpened, chlorided wire was then carefully inserted in the pipette under microscope observation until the tip of the wire extended 75–95 micrometers beyond the glycerol meniscus, i.e. 5–25 micrometers behind the tip of the pipette (see Fig. 1 B). The best results were obtained when the chlorided end of the wire was centered in the pipette tip and not in contact with the wall. This ensured that the glycerol was not drawn up between the wire and the glass, and made it easier to form the wax seal. With the pipette still on the microscope stage, the butt of the pipette was gently heated by holding a match

TABLE I
COMPOSITION OF REPRESENTATIVE TEST SOLUTIONS

	[Cl⁻]	[HCO₃⁻]	a_{Cl}^o	γCl
	mM	*mM*	*mM*	
A, *Mixed solutions at constant ionic strength* $\mu =$	100	0	77.0	
100 mM (reciprocal dilutions)	75	25	57.8	
	50	50	38.5	
	25	75	19.3	
	10	90	7.7	
	1	99	0.77	
B, *Mixed solutions (constant interferent)*	1,000	10	604	0.604
	500	10	324	0.647
	100	10	76.2	0.762
	50	10	40.2	0.803
	10	10	8.7	0.867
	5	10	4.4	0.882
	1	10	0.9	0.897

above it, and the wire was tacked to the glass with a small amount of sealing wax (melting point ~150°C) with care taken to leave the lumen patent. A sliver of wax was then dropped into the pipette and the whole assembly moved to a microforge where it was placed tip down in the center of a 3-mm, three-turn coil of resistance wire. The coil was gently heated and the electrode observed under the microscope (×400) until the wax sliver melted. The electrode tip was then raised and lowered through the coil until the molten wax rested on the glycerol where it was allowed to cool, forming a rigid, fixed seal about 50 micrometers from the pipette tip (some of the aqueous glycerol evaporated during heating).

The assembly was completed by sealing the butt with more wax, and the glycerol was displaced from the dead space by immersing the electrode tip in a KCl solution for a few minutes. This technique is very reliable. Since the entire process is conducted under the microscope, few defective electrodes reach the calibration steps. The entire process can be completed in 10–15 min, which is a substantial saving in time compared to either the Neild-Thomas technique (1973) or that described by Kerkut and Meech (1966), both of which require many hours for completion. The wax seal defines a fixed dead space, while the chlorsilane barrier described by Neild and Thomas was unreliable in our experience;

the barrier either formed at the tip or failed, allowing the dead space to increase enormously.

These electrodes characteristically showed slopes of 56–58 mV/decade and the time constant of the voltage change after a decade change in chloride concentration was approximately 1 min (Fig. 1 A). Selectivity coefficients for silver halide electrodes have been reported in the literature, notably for halide pairs (Bishop and Dhaneshwar, 1963; Janz and Ives, 1968). In all other cases where the solubility constant products ($K_{SP}s$) are known, selectivity coefficients for Ag-AgCl electrodes may be estimated from the ratio of the solubility product of AgCl to that of the silver salt of the interfering anion (Buck, 1968; Covington, 1969; Lakshminarayanaiah, 1976). A search of the literature has failed to yield any data on solubility properties of silver glutamate, silver propionate, or silver methanesulfonate. Evidence that these anions are not potent interferents for the Ag-AgCl microelectrode was obtained in this study by two different methods: (a) addition of $AgNO_3$ to Na-salts of these organic acids produced no precipitation; and (b) addition of up to 500 mM Na glutamate, Na propionate, or $NaMeSO_3$ to a 10-mM solution of NaCl produced no change in electrode potential, and hence no detectable interference by these anions.

RESULTS

Selectivity of Ion Exchanger Microelectrodes

Some selectivity coefficients for CLIX microelectrodes have been reported previously (Orme, 1969; Walker, 1971). The reported selectivities may not be appropriate for the present study, since the selectivity coefficient (K_{ij}) might be expected to show some dependence on pH, ionic strength, or the microconfiguration of the electrode. The selectivity of chloride-sensitive exchanger electrodes has been reported to be relatively good, approaching 20:1 for chloride vs. bicarbonate (K_{ij} = 0.05; Walker, 1971). Since bicarbonate is a known intracellular anion which could be an important interferent in chloride washout experiments and since the selectivity for chloride vs. bicarbonate has been shown to be greater than for Cl⁻ vs. other proven or probable intracellular anions, a detailed study of chloride-bicarbonate selectivity should give an estimate of the best possible performance of liquid ion exchanger microelectrodes.

EFFECT OF pH   Mixed solutions of bicarbonate and chloride show variation in pH which could vary the selectivity coefficient of the exchanger. To assess the influence of pH on the CLIX microelectrode, samples of three stock solutions (100, 200, and 500 mM KCl) were adjusted to different pHs by adding small amounts of solid KOH or glutamic acid, giving three families of constant chloride solutions ranging from pH 3.5 to pH 10. The results of one of these studies are shown in Fig. 2. The electrode potential was independent of pH between 4.0 and 10.0. At low pH, there was some deviation from the expected value. The range of pH for the solutions used to investigate chloride-bicarbonate selectivity is indicated by the arrows above the curves in Fig. 2. Within this range there was no substantial interference due to hydroxyl ions, as might be expected from the $K_{ij}s$ determined for this ion pair in this and similar exchangers (Moody and Thomas, 1971; Orme, 1969).

SELECTIVITY AT CONSTANT IONIC STRENGTH   Mixed solutions at constant ionic strength were chosen for the evaluation of electrode selectivity. Tests
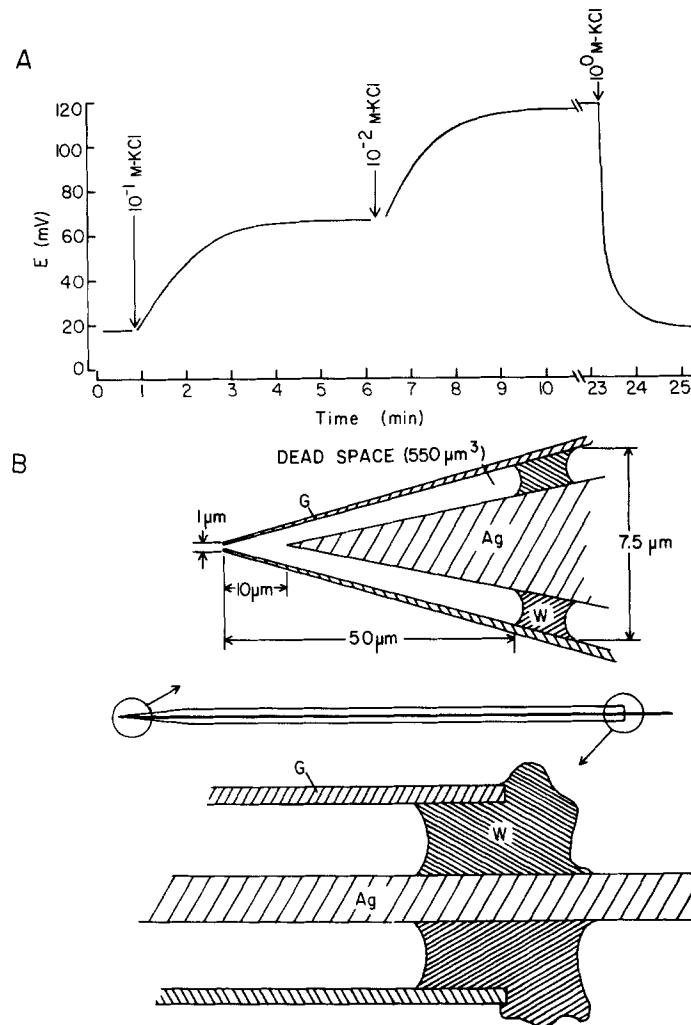
FIGURE 1. A, Typical time course of voltage changes in an Ag-AgCl electrode after decade changes in chloride concentration. Arrows mark time at which electrode was immersed in the indicated solutions. This is a chart recording of the electrode potential made by using a unity gain electrometer amplifier. Four concentration changes in each of three electrodes ($n = 12$) gave a time constant of 69.6 ± 28.4 s ($\bar{y}$ ± SD). B, Scheme of the Ag-AgCl electrode. Details of the tip and butt construction are shown. Dimensions given are averages for a number of electrodes: G = glass pipette, Ag = Ag-AgCl wire, W = wax seals.

were run in 100 or 500 mM solutions. In the case of [Cl⁻] + [HCO₃⁻] = 100 mM, the pH varied between 8 and 10, on the flat portion of the curve in Fig. 2. The dependency of electrode potential on chloride activity is shown in Fig. 3 A for four different electrodes. Triangles represent KCl solutions and circles Cl-HCO₃ mixtures. The mean and standard deviation of the difference between

the potential measured in any given calibrating or test solution and that potential measured in 100 mM KCl is shown. The selectivity coefficient (K_{ij}) was calculated from

$$K_{ij} = \frac{a_{Cl}^i \cdot 10^{(E-E_0)/S} - a_{Cl}^0}{a_{HCO_3}^0},\tag{2}$$

where all the terms have the same meaning as in Eq. (1), except that here a_{Cl}^0 and $a_{HCO_3}^0$ are the calculated activities of chloride and bicarbonate in the test solution and a_{Cl}^i is the activity of chloride (published values) in the electrode-filling solution. Point by point calculation of K_{ij} may yield a range of values for the selectivity coefficient. This calculation can be avoided by rearrangement of Eq. (2) to the form

$$10^{(E-E_0)/S} = a_{Cl}^0 \cdot \frac{1 - K_{ij}}{a_{Cl}^i} + \frac{MK_{ij}}{a_{Cl}^i},\tag{3}$$

where M is the total (geometric mean) anion activity in the test solution; the
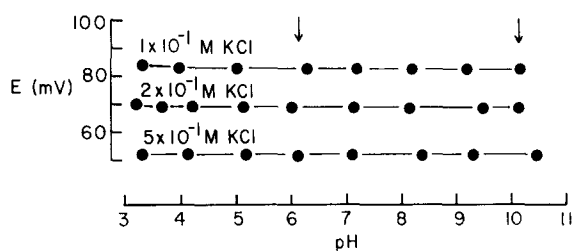

FIGURE 2.   Effect of pH on electrode potential in 100, 200, and 500 mM KCl. This electrode read slightly more positive values in 100 and 200 mM solutions below pH 4. All solutions used to determine electrode selectivity for bicarbonate vs. chloride ranged between pH 6.1 and pH 10.1 as indicated by arrows above the curves.

molal activity coefficients have been used because they are the readily available published values. The error introduced by the use of molal activity coefficients is quite small at the ionic strengths used in these experiments. In plotting data in the slope-intercept form of Eq. (3), we have adjusted the value of M to account for the changes in activity coefficients as chloride is replaced by another anion. This has been done by taking a weighted average of the mean molal activity coefficients of the two salts in the test solution.

The K_{ij} obtained from Eq. (3) can be expressed as

$$K_{ij} = \frac{y}{bM + y},\tag{4}$$

where b is the slope of the line described by Eq. (3) and y is the intercept. The estimated regression for these points and the values of y and b were computed (Li, 1957). In practice, M was taken as the activity of chloride in pure solution at the appropriate ionic strength. Correlation coefficients of the data points to the regression line were generally 0.95 or better and were 0.98 in 70% of the cases. The relation between the antilog of voltage change and a_{Cl}^i (Eq. [3]) is
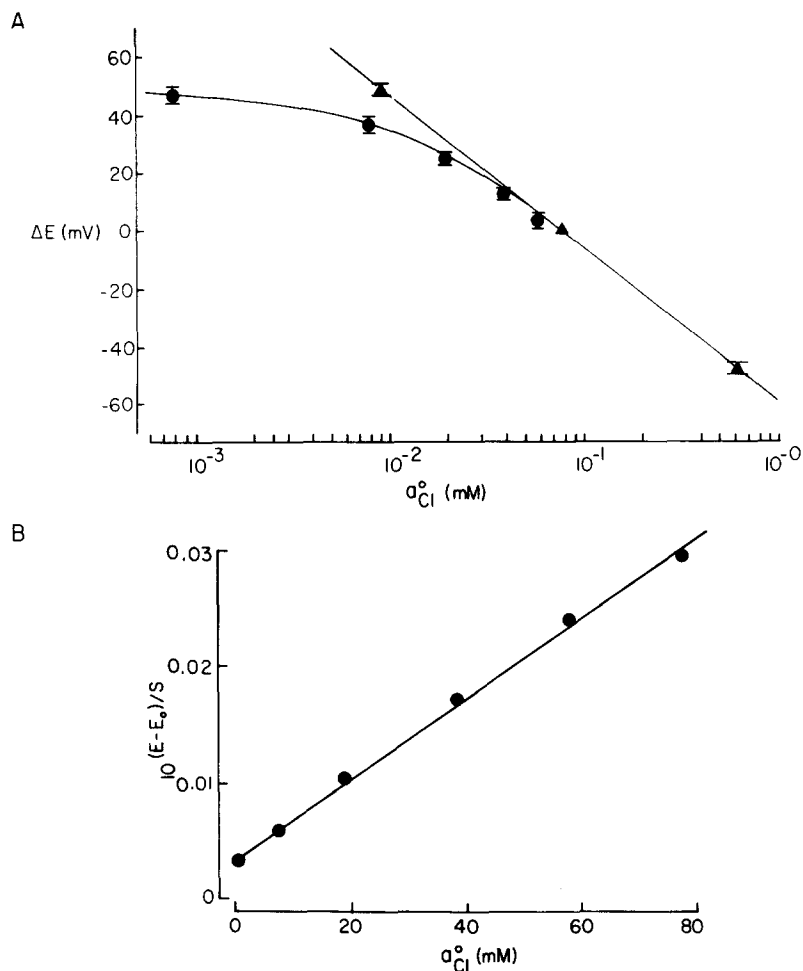
FIGURE 3. A, Triangles show an electrode calibration curve in solutions containing 10, 100, and 1,000 mM KCl. Solid circles represent electrode potential change from the potential measured in 100 mM KCl ($\bar{y}$ ± SD) as a function of a^0_{Cl} in chloride-bicarbonate mixtures at constant ionic strength (μ = 100 mM) for four different electrodes. The curved line is drawn to satisfy Eq. (2) with K_{ij} = 0.12 (Table IV, column 1). B, Antilog of electrode potential as a function of a^0_{Cl} (graphic representation of Eq. [4]). The line was drawn from a least-squares regression analysis; correlation coefficient for the points to the regression line was 0.999. The K_{ij} given from this relation was 0.10 for this electrode.

plotted in Fig. 3 B for one electrode. The values of y and b were determined by regression analysis and K_{ij} was 0.10, i.e. the selectivity of the electrode for bicarbonate with respect to that for chloride. Selectivity coefficients determined by this method for several different electrodes are given in Table II for a variety of biologically occurring anions and common anion substitutes. CLIX electrodes are clearly better I⁻ and Br⁻ electrodes than Cl⁻ electrodes and

discriminate Cl^- best in F^-, aspartate, or glutamate mixtures; no clear effect of different ionic strengths was evident. Table III shows the mean and standard deviation of $K_{ij}s$ determined at ionic strength of 100 and 500 mM and compares the results of this study with previously reported values.

Since other workers have used different methods or have not specified their method of determining $K_{ij}s$, we used several additional treatments to determine to what extent $K_{ij}s$ obtained by various methods would differ. Details of the following treatments have been presented by Moody and Thomas (1971). These

TABLE II

SELECTIVITY COEFFICIENTS ($K_{ij}s$) DETERMINED FOR CHLORIDE-SENSITIVE LIQUID ION EXCHANGER MICROELECTRODES IN TWO-COMPONENT MIXED SOLUTIONS AT CONSTANT IONIC STRENGTH

				Interferent species						
μ	HCO_3^-	1	2	I^-	Br^-	F^-	3	4	5	6
mM										
100	0.13	0.22	0.89	26	3.4	0.09	0.32	0.022	0.086	0.21
	0.12	0.13	0.85	13	3.0	0.07	0.27	0.015	0.01	0.26
	0.12	0.30	0.78	11	3.4	0.09			0.089	0.31
	0.11	0.27	0.79	6.4	2.7	0.08	0.29	0.015		0.23
		0.35	0.77	94	4.2					0.25
										0.27
500	0.16	0.24		19		0.13	0.40	0.04		0.23
	0.15	0.18	0.82	11	3.0	0.14	0.39	0.05		0.26
	0.13	0.17	0.83		2.9	0.11				0.26
	0.18			5.4	2.8	0.09	0.38	0.05		0.20
		0.43	0.80		3.4					
				18						

1, isethionate⁻; 2, propionate⁻; 3, acetate⁻; 4, monobasic aspartate; 5, monobasic glutamate; 6, methanesulfonate⁻. Data are arranged so that selectivities are given at 100 and 500 mM ionic strength for the same electrodes in some cases, with data for any given electrode on corresponding lines. In addition, isethionate, propionate, iodide, and bromide were tested in one population of electrodes; fluoride, acetate, and aspartate in another; and the remaining anions in individual populations where they were the only anions tested.

methods involve either separate solutions of primary and interfering ions or mixed solutions with a range of ionic strengths.

SEPARATE SOLUTION METHOD   This involves calibration of the electrode with decade dilutions of primary ion. In addition, the electrodes were "calibrated" with the same dilutions of the interfering ion. The ion activities were calculated from published activity coefficients (Gregor et al., 1963; Parsons, 1959; Robinson and Stokes, 1970) and the electrode potential was plotted as a function of primary or interfering ion activity. Two different treatments were used to calculate selectivity coefficients from the data.

A, The ratio of primary and interfering anion activities which produce the same electrode potential is determined from:

$$E_i = E_0 + S \log a_i, \quad \text{and} \tag{5}$$

$$E_j = E_0 + S \log K_{ij} a_j; \quad \text{since} \quad E_i = E_j \tag{6}$$

$$K_{ij} = \frac{a_i}{a_j}. \tag{7}$$

Selectivity coefficients were calculated by this method ($E_i = E_j = 100$ mV) for four electrodes where the primary and interfering anions were chloride and bicarbonate, and results are shown in Table IV.

TABLE III

COMPARISON OF ESTIMATED SELECTIVITY COEFFICIENTS (K_{ij}s) OF CHLORIDE-SENSITIVE LIQUID ION EXCHANGER MICROELECTRODES AND MACROELECTRODES

	Microelectrodes				Commercial macroelectrodes	
	Present study*‡		Walker‡		Corning‖	
Interfering anions	$\mu = 100$	$\mu = 500$	$\mu = 100, 1,000$	Orme§	476131	Orion¶ 92-17
Iodide	30±36 (5)	13±6 (4)			15	17 (4.4-26.7)
Bromide	3.3±0.6 (5)	3.0±0.3 (4)			2.5	1.6 (1.72-3.73)
Fluoride	0.08±0.01 (4)	0.12±0.02 (4)			0.2	0.1
Bicarbonate	0.12±0.01 (4)	0.16±0.02 (4)	0.05	0.15		0.19
Acetate	0.29±0.03 (3)	0.39±0.01 (3)		0.26	0.21	0.32
Propionate	0.82±0.05 (5)	0.82±0.02 (3)	0.5			
Methanesulfonate	0.26±0.03 (6)	0.24±0.03 (4)				
Isethionate	0.25±0.08 (5)	0.26±0.12 (4)	0.2			
H-Glutamate	0.077±0.018 (3)					
H-Aspartate	0.017±0.004 (3)	0.047±0.006 (3)				

* $\bar{y}$ ± SD, (n) = number of observations, mixed solutions at constant ionic strength.
‡ Corning exchanger no. 477315.
§ Orion exchanger no. 92-17-02.
‖ Corning Bulletin, Electrode no. 476131.
¶ Srinivasan and Rechnitz, 1969.

TABLE IV

COMPARISON OF K_{ij}s (HCO₃⁻ vs. Cl⁻) DETERMINED BY VARIOUS METHODS

Electrode no.	Mixed solutions $\mu=100$	Separate solutions				Varying [Cl⁻] constant [HCO₃⁻]			Varying [HCO₃⁻] constant [Cl⁻]			$\bar{y}$±SD
		$E_i=E_j$	$a_i=a_j$									
			10	100	1,000	10	25	50	10	25	50	
1	0.13	0.15	0.17	0.19	0.20	0.11	0.10	0.09	0.09	0.14	0.12	0.14±0.04
2	0.12	0.09	0.14	0.12	0.11							0.12±0.02
3	0.12	0.10	0.18	0.14	0.11	0.15	0.12	0.07	0.13	0.11	0.10	0.12±0.03
4	0.11	0.08	0.17	0.12	0.09	0.13	0.12	0.09	0.10	0.10	0.10	0.11±0.02
$\bar{y}$±SD	0.12±0.01	0.11±0.03	0.17±0.02	0.14±0.03	0.13±0.05	0.13±0.02	0.11±0.01	0.08±0.01	0.11±0.02	0.12±0.02	0.11±0.01	0.12±0.03

B, The potentials at which the primary and interfering ion activities are equal in the two solutions can also be used to calculate the K_{ij}. From Eq. (5) and (6), when $a_i = a_j$,

$$K_{ij} = 10^{(E_j - E_i)/S}. \tag{8}$$

The selectivity coefficients of the same four electrodes were evaluated by this treatment at $a_i = a_j = 10$, 100, and 1,000 mM. The results were somewhat more variable in this case, as shown in Table IV.

MIXED SOLUTIONS AT VARYING IONIC STRENGTH   The third method of estimating electrode selectivities involves the use of mixed solutions where: (a) the interfering ion activity is varied in the presence of a constant background of primary ion; or (b) the primary ion activity is varied while the interferent background remains constant. By plotting electrode potential as a function of primary or interfering ion activity, both methods show smooth curves with distinct right and left hand asymptotes. The two asymptotes may be extended to intersect, and at the point of intersection, K_{ij} is given by Eq. 7. These methods were used to estimate K_{ij} in the same four electrodes described above. The composition of a representative test series is given in Table I, while the calculated K_{ij}s are given in Table IV. The selectivity coefficients calculated from these data are in good agreement with those obtained by the other methods.

Measurement of External Chloride Activity

Some workers in this field apparently base measurements of internal chloride activity on calculated values of a_{Cl}^0. We have empirical evidence that this yields high values, of a_{Cl}^0, at least for *Balanus*, *Aplysia*, or *Helix* saline solutions. Thus, calculations of a_{Cl}^i based on these high values of a_{Cl}^0 are also elevated. A chloride activity for barnacle saline can be obtained by multiplying the reported activity coefficient for 0.5 M NaCl by the sum of the chloride salts. In the case of normal barnacle saline, the activity coefficient would be 0.685 (Parsons, 1959; Robinson and Stokes, 1970). Since there is a total of 535 mM chloride, one would estimate that the total chloride activity would be approximately 366 mM. The activity of chloride can also be calculated from the extended Debye-Huckel equation or one of the modified forms of that equation, which yields a slightly lower value. No method agrees well with the values measured by either the CLIX or the Ag-AgCl electrode. Both consistently give values of 315–325 mM chloride activity in NBS. This corresponds to a γ_{Cl} of approximately 0.6.

An empirical approach to the problem is shown in Fig. 4. The closed and open circles represent chloride activity as a function of chloride concentration calculated from published activity coefficients for NaCl and $CaCl_2$. An Ag-AgCl wire was calibrated in NaCl, and a reading was taken in 500 mM NaCl and then in mixed solutions containing 500 mM NaCl and increasing amounts of $CaCl_2$. This gives a series of readings in solutions of increasing ionic strength. The data are plotted (▲) to show the measured chloride activity as a function of the chloride concentration in the solutions. Adding up to 50 mM $CaCl_2$ had little effect on measured Cl^- activity. Addition of 100 mM $CaCl_2$ did increase the activity from 330 to 380 mM, whereas the calculated value would be approximately 465 mM.

Determination of a_{Cl}^i with the Liquid Ion Exchanger Microelectrode

Brown (1976) has previously reported that E_m is generally in good agreement with E_{Cl} in *Balanus* photoreceptors with CLIX electrodes. The same observation was made in the present study; if a disparity was observed, E_{Cl} was generally more negative than E_m. In the present investigation, a_{Cl}^i was monitored with CLIX electrodes during chloride washout after extracellular chloride substitu-

tion by methanesulfonate, glutamate, propionate, or iodide. In a few experiments, chloride was only partially replaced by the test anion for several practical reasons. For example, iodide damages the Ag-AgCl reference in pH electrodes so that titration with HI is impractical, while a Tris-HCl buffered iodide saline can be quickly checked and adjusted to proper pH. Membrane potential and a_{Cl}^i were measured before washout in all cells in which a_{Cl}^i was monitored by CLIX electrodes. In 12 cells, E_m was -45.1 ± 3.8 mV, a_{Cl}^i was 59.3 ± 10.1 mM, and the calculated E_{Cl} was -44.1 ± 4.6 mV ($\bar{y} \pm$ SD). A paired comparisons t-test indicated that E_m and E_{Cl} were not different.

METHANESULFONATE AS ANION SUBSTITUTE. Six cells were studied with ion exchanger electrodes after full chloride substitution by methanesulfonate. Internal chloride activity generally decreased rapidly during the first 20–30 min
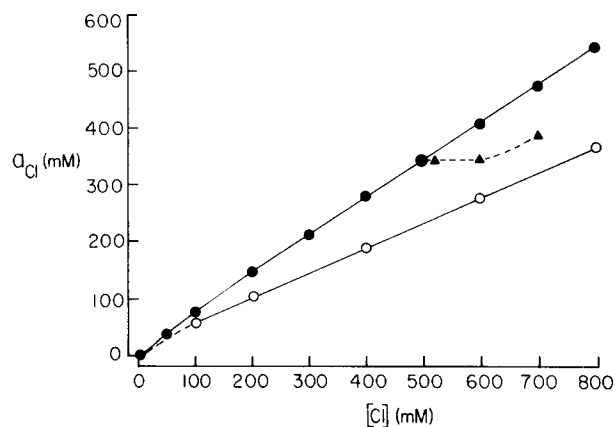

FIGURE 4. Effect of Ca^{2+} on chloride activity in mixed solution. Chloride activity is plotted as a function of chloride concentration for pure solutions of NaCl (●) and $CaCl_2$ (○). Dotted line (▲) shows observed activity obtained from an Ag-AgCl electrode in mixed solutions of NaCl and $CaCl_2$ when 10, 50, and 100 mM $CaCl_2$ were added to 500 mM NaCl.

in chloride-free barnacle saline, and in some cases a_{Cl}^i continued to decrease very slowly for as long as the experiment was continued (up to 3 h). Even after this amount of time, there remained an appreciable a_{Cl}^i. Results from a representative cell are shown in Fig. 5. The initial a_{Cl}^i was 50.3 mM. The membrane potential was -46 mV and E_{Cl} was -47 mV. After replacement of the extracellular chloride by methanesulfonate, a_{Cl}^i decreased to 33.6 mM within 10 min, and over the next 10 min a_{Cl}^i slowly decreased to 29.4 mM. The change in a_{Cl}^i was 20.9 mM which represents a 42% reduction in a_{Cl}^i. The time constant for this change was 4.8 min. In the six cells in which Cl^- was fully replaced by $MeSO_3^-$ the time constant for chloride washout was 5.0 ± 1.7 min ($\bar{y} \pm$ SD). The initial a_{Cl}^i before washout with methanesulfonate was 57.3 ± 9.9 mM; the final a_{Cl}^i in these six cells was 28.3 ± 8.9 mM. In five cells Cl_0 was replaced by glutamate in washout runs preceded and followed by runs in which methanesulfonate replaced Cl_0. In these five cells there was a small increase (3–

4 mM) in the final a^i_{Cl} between the first and third runs. The time constant for washout was somewhat less for the third run than for the first in some cells. Since this was not a consistent feature of all experiments, the time constants from multiple runs on the same cell were averaged.

GLUTAMATE AS ANION SUBSTITUTE   Glutamate replaced extracellular chloride in 12 cells studied with ion exchanger microelectrodes. The time constant of chloride washout was 4.1 ± 1.7 min. Extracellular chloride was fully replaced by glutamate in only nine of these cells. In these nine cells the initial a^i_{Cl} was
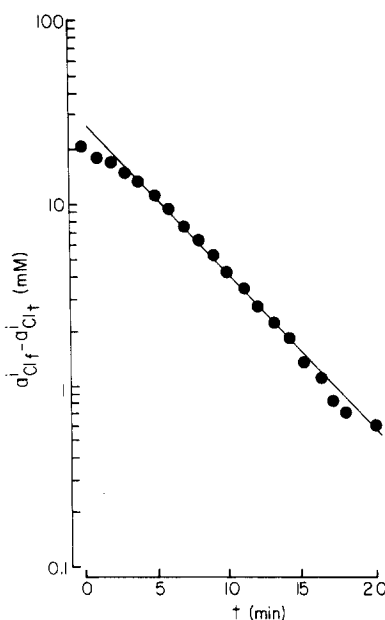

FIGURE 5. Time course of change in internal chloride activity measured with liquid ion exchanger microelectrode after replacement of extracellular chloride by methanesulfonate at $t = 0$ min. The time constant of chloride washout was 4.8 min. Deviation of the initial points from the line was a characteristic of these experiments and is attributed to mixing of test and control solutions in the bath; abscissa is time in minutes after washout began, ordinate is change in intracellular chloride activity in mM.

58.7 ± 9.6 mM; after washout the final a^i_{Cl} was 30.0 ± 7.2 mM. For the five cells in which both methanesulfonate and glutamate were used to wash out chloride, the final a^i_{Cl}s were 31.3 ± 5.7 mM and 30.4 ± 7.1 mM, respectively. These were judged not to be different. However, the time constant was always less when glutamate was used as the anion substitute.

PROPIONATE AS ANION SUBSTITUTE   Extracellular propionate fully replaced chloride in three cells; a^i_{Cl} did not diminish as expected. Two cells showed an apparent gain in a^i_{Cl} (from 65.6 to 88.4 mM and from 61 to 68 mM), whereas the other showed an apparent loss of a^i_{Cl} (from 49.8 to 42.5 mM).

When chloride was replaced by glutamate in these same three cells, a^i_{Cl} diminished with a time constant of 3.5 ± 0.85 min. These data are a subset of

the data reported for glutamate above, and are represented here to show (a) that the electrode was responsive to changes in a_{Cl}^i, and (b) that chloride washed out in the expected manner when glutamate was the extracellular anion substitute.

IODIDE AS ANION SUBSTITUTE Iodide was used to replace part of the extracellular chloride in three experiments. In each case, the apparent a_{Cl}^i increased and far exceeded the original a_{Cl}^0, as shown in Fig. 6. The substitution was well tolerated, but the return to control a_{Cl}^i was very prolonged, requiring approximately 2 h. Apparent time constants were not calculated. Fig. 6 A shows (top trace) the potential change of the CLIX electrode after substitution of methanesulfonate for Cl_0; the bottom trace shows that the calculated a_{Cl}^i decreased from 43 to 12 mM. Partial replacement of Cl_0 by iodide (Fig. 6 B) was followed by a large negative potential change of the CLIX electrodes which translated to an apparent increase in a_{Cl}^i from 37 mM to 1.432 M. This apparent increase provides evidence that I^- entered the cell during chloride washout and, since I^- is a potent interferent, the electrode measured a large but spurious a_{Cl}^i.

Determination of a_{Cl}^i with the Ag-AgCl Microelectrodes

The calculated dead space of the Ag-AgCl electrode represented in Fig. 1 is approximately 0.1% of the cytoplasmic volume of the lateral photoreceptor cell of *Balanus eburneus* as determined by Krebs and Schaten (1976). Due to the recessed-tip construction of the Ag-AgCl microelectrode, this electrode has a time constant of about 1 min which is considerably longer than that of the ion exchanger electrodes. As with the liquid ion exchanger microelectrode, the internal chloride activity measured with the Ag-AgCl electrode varies from cell to cell, but the calculated chloride equilibrium potential is generally within a few millivolts of the measured resting membrane potential. Internal chloride activity was monitored with Ag-AgCl microelectrodes during chloride washout after extracellular substitution of glutamate or propionate for chloride. Some very early experiments in which chloride was replaced by methanesulfonate gave similar results but were technically inferior and are not reported here. Iodide was not used as an anion substitute since a small amount of I^- (as low as 0.1 mM) is sufficient to irreversibly damage these electrodes.

Fig. 7 shows membrane potential (upper trace) and V_{Cl} (lower trace) determined from an Ag-AgCl electrode in a short experiment (about 1 h). The essential features of all such experiments are illustrated here. This particular record was made with the conventional recording system, by use of an extracellular reference electrode for both potential and chloride-measuring electrodes. After impalement (at A), the top tracing shows membrane potential and receptor potentials in response to 1-s flashes of white light presented every 10 s. At B, the chloride probe was turned on and within 2 min reached a steady-state potential of 48 mV. This was V_{Cl}^0 and corresponded to $a_{Cl}^0 = 325$ mM. Long-lasting membrane depolarizations were recorded at C and D in response to illumination of the preparation during placement of the chloride-sensing electrode before the second impalement. At E the second electrode impaled the photoreceptor. There was an increase in V_{Cl}^i (corresponding to a_{Cl}^i)
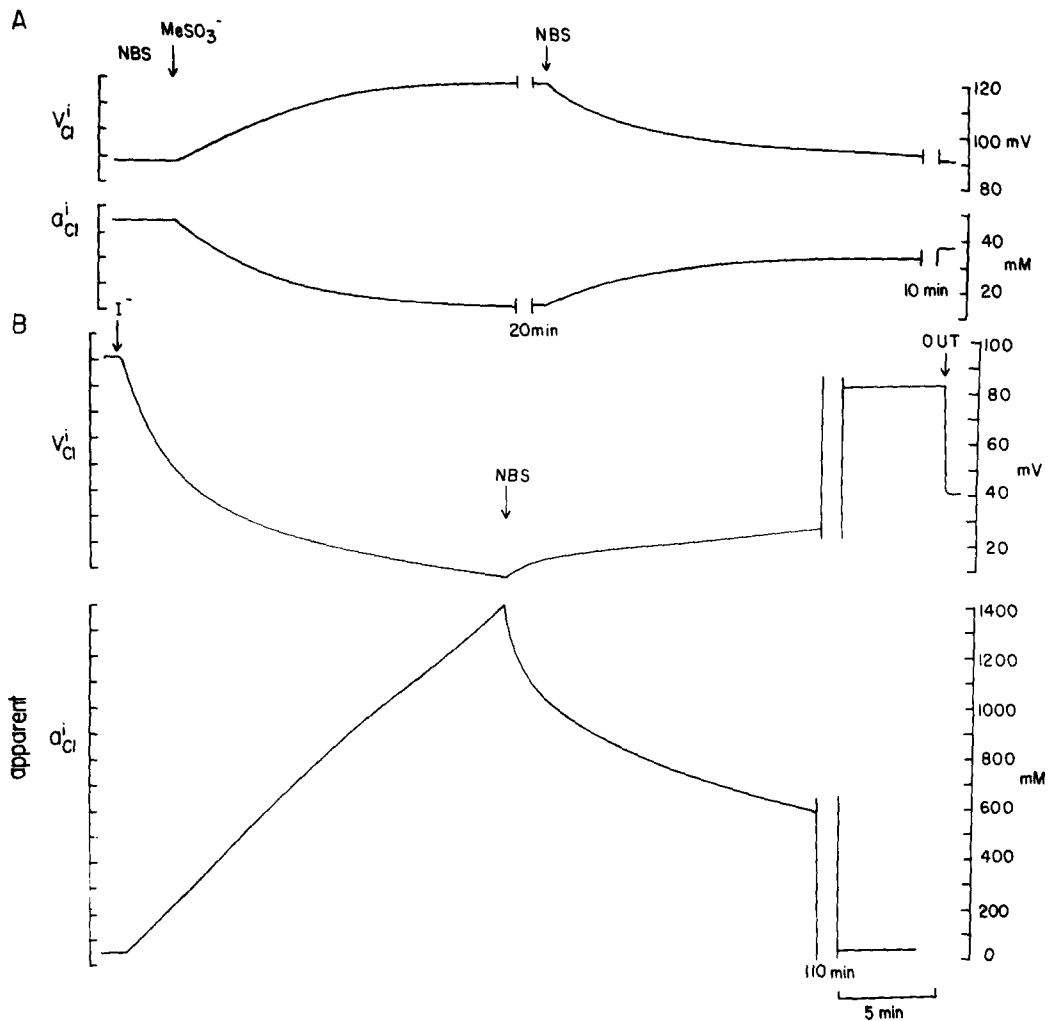
FIGURE 6. Comparison of recordings from an intracellular CLIX electrode when MeSO$_3$ (A) and I$^-$ (B) are substituted for Cl$^-$. Apparent intracellular chloride activity (a_{Cl}^i) is shown below the voltage trace. In panel A, Cl$_0$ was fully replaced by MeSO$_3$ and V$_{Cl}^i$ increased, while a_{Cl}^i decreased from 43 to 12 mM (electrode slope is negative). In panel B, V$_{Cl}^i$ decreased rapidly after partial (86%) substitution of I$^-$ and Cl$_0$, while a_{Cl}^i appeared to increase from 37 mM to 1.432 M due to interference as I$^-$ entered the cell. Recovery was prolonged but complete. CLIX was removed from cell after experiment and recorded $V_{Cl_{final}}^0 = 41$ mV $= V_{Cl_{initial}}^0$.

which represents the equilibration of the electrode dead space with the cell interior. Note that membrane potential recorded in the upper trace was diminished after the second impalement. F and F' are responses to illumination recorded from both electrodes. The potential change was the same for both electrodes. At G and G' both electrodes were simultaneously withdrawn. The

KCl microelectrode returned to the original reference level and the Ag-AgCl microelectrode returned to the original V_{Cl}^0 (48 mV). The electrode was recalibrated, giving the same -57 mV/decade slope as in the original calibration. For this experiment a_{Cl}^i was calculated from

$$a_{Cl}^i = a_{Cl}^0 \cdot 10^{(V_{Cl}^i - E_m - V_{Cl}^0)/S},\tag{9}$$

where all terms are the same as in Eq. (1); V_{Cl}^i is the voltage of the Ag-AgCl electrode in the cell. In this cell E_m was -39 mV, a_{Cl}^i was 70 mM, and E_{Cl} was -39 mV. Membrane potential and a_{Cl}^i were measured with Ag-AgCl electrodes in many cells; there was generally good agreement between E_m and E_{Cl}.
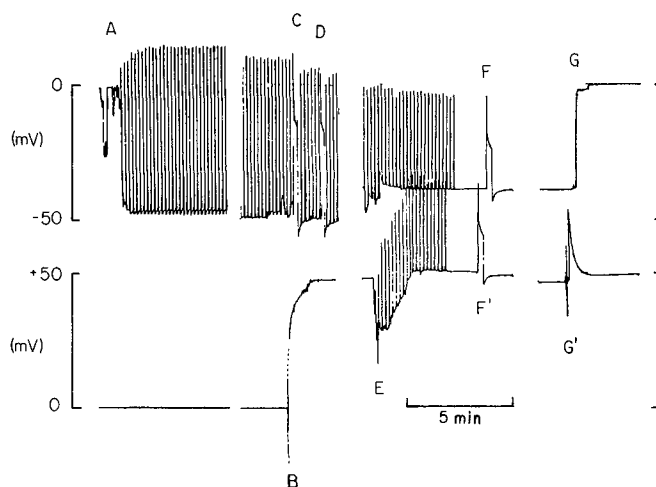

FIGURE 7. Simultaneous recording of membrane potential from a conventional KCl electrode (top trace) and potential of a chloride-sensitive Ag-AgCl microelectrode (bottom trace) using a common extracellular reference electrode (broken KCl-filled pipette). The top tracing is offset about 23 s by the chart recorder. See text for description.

However, in long experiments electrode performance can change slightly (slope, or value of V_{Cl}^0) and this can introduce some uncertainty in the determination of a_{Cl}^i and E_{Cl}. For example, a change in electrode slope from -58 to -55 mV/decade introduces a 10% uncertainty in a_{Cl}^i and a 5% uncertainty in E_{Cl}. On the other hand, the same change in electrode slope produces negligible error in the determination of the time constant of chloride washout, i.e. less than 1%.

GLUTAMATE AS ANION SUBSTITUTE   Glutamate was used to partially or totally replace Cl_0 in eight cells. The time constant of chloride washout was 5.2 ± 2.4 min ($\bar{y}$ ± SD). The final level of a_{Cl}^i was less than 20 mM in more than 50% of the cells investigated with Ag-AgCl electrodes when glutamate was used to wash out Cl⁻. For example, in one cell where E_m and E_{Cl} were -30 and

-30.8 mV before washout, the initial a_{Cl}^i was 95.5 mM and final a_{Cl}^i was 16.5 mM after washout.

PROPIONATE AS ANION SUBSTITUTE   Extracellular chloride was fully replaced by propionate in three cells. The time constant of chloride loss was 10.8 $\pm$ 3.06 min. Since the Ag-AgCl electrode is insensitive to propionate, this relatively long time constant cannot be attributed to electrode interference. The time constant of chloride washout after glutamate substitution in these same three cells (also included as part of the larger set above) was 5.6 $\pm$ 3.3 min.

Estimation of Net Chloride Flux during Washout

The net chloride flux can be calculated from data similar to those shown in Fig. 5. Chloride efflux was calculated for a single washout run in which a_{Cl}^i was monitored with an Ag-AgCl microelectrode during washout in glutamate saline. Initial a_{Cl}^i was 95.5 mM, decreasing 79 mM to a final a_{Cl}^i of 16.5 after 38 min. The time constant for this change was 8.5 min, from which the chloride loss during the 1st s was calculated to be 1.2×10^{-4} M.

Estimates of the surface area of a *Balanus* photoreceptor can be obtained from electrophysiological or recent morphometric data. Brown et al. (1970) published values for the apparent membrane capacitance based on the time course of membrane potential changes in response to rectangular changes of current passed across the membrane. They obtained a value of 180 $\mu F/cm^2$ calculated from the surface area of a sphere 100-μm in diameter. If one assumed a more conventional value for the specific membrane capacitance (1 $\mu F/cm^2$), the surface area would be 1.5×10^{-2} cm². This value is in close agreement with recent morphometric data (Krebs and Schaten, 1976) which indicate that the surface area of the rhabdomeres of a single *Balanus* photoreceptor cell is approximately 2.5×10^{-2} cm². Krebs and Schaten (1976) also report that the plasma membrane of a single photoreceptor has an area of 4×10^{-3} cm². The volume of a single photoreceptor cell was found to be 4×10^{-7} cm³ or 4×10^{-10} liter (Krebs and Schaten, 1976).

Chloride flux can be estimated from the rate of chloride washout and the estimates of photoreceptor surface area and volume. If one assumes that chloride moves out through plasma and rhabdomeric membrane, the peak (during the 1st s) net chloride efflux is approximately 3.0 pM cm^{-2} s^{-1}. If the same values are used from $t = 0$ to $t = \tau$, the net Cl$^-$ flux would be 1.6 pM cm^{-2} s^{-1}. If only the plasma membrane were involved, the peak and τ fluxes would be 19 and 9.8 pM cm^{-2} s^{-1}, respectively. These estimates are based on the assumption that chloride is lost uniformly from the entire volume of the cell and that the activity coefficient is unity.

DISCUSSION

This study describes measurements of a_{Cl}^i in *Balanus* photoreceptor with two different chloride-sensitive probes. Both probes provided similar steady-state measurements of a_{Cl}^i in dark-adapted photoreceptors, insofar as both electrodes showed that the chloride distribution was generally in equilibrium with the membrane potential ($E_{Cl} \approx E_m$); however, values of a_{Cl}^i and the time constant of chloride washout can be dependent on several factors, as described below.

Electrode Stability

Some authors have argued that Ag-AgCl electrodes might be reduced by such intracellular moieties as sulfhydryl groups (Tasaki and Singer, 1968; Neild and Thomas, 1974). If this were so, the electrode should not recalibrate after use. In some experiments we observed this to be true, but this did not occur with any higher frequency with the Ag-AgCl electrode than with the CLIX electrode and seemed to be due to plugging of the electrode tip. The present experiments were acceptable only when there was good agreement between pre- and postexperimental calibration of the chloride-sensitive electrodes.

Choosing a Value for a_{Cl}^0

Determinations of a_{Cl}^i based on calculated values of a_{Cl}^0 can be particularly misleading when the external saline contains even a small quantity of divalent cation. Whether a_{Cl}^0 is estimated by taking the product of Cl_0 and some published activity coefficient or more rigorously calculated from the Debye-Huckel equation, a_{Cl}^0 for normal barnacle saline will be overestimated by 10–15%, probably due to the "Bjerrum effect" (Lewis and Randall, 1961). This would imply that in mixed solutions at relatively high concentration, the divalent cations would associate with anions to a greater extent than the univalents so that the a_{Cl} would be lower than that predicted by Debye-Huckel. The effect of using any calculated value of a_{Cl}^0 is to overestimate a_{Cl}^i. This error may be less serious than that resulting from poor electrode selectivity; if both factors are not considered, a_{Cl}^i could be significantly overestimated.

Electrode Selectivity and Errors in the Measurement of the Initial a_{Cl}^i

Electrode selectivity is the central problem in interpreting the biological data in this study. Few, if any, ion-selective probes are "specific." The consequence of this nonspecificity is that interfering ions will consistently cause overestimation of primary ion activity, as shown by Eq. 2. The Ag-AgCl microelectrode is liable to interference by sulfide, thiocyanate, cyanide, iodide, and bromide, among others, as indicated by any table of the electrochemical series. The most probable naturally occurring interferents in marine biological preparations are iodide and bromide. The CLIX electrode is also more selective for iodide or bromide than chloride, and in addition is sensitive to many more anions which do not affect the Ag-AgCl probe. The CLIX probe used in this study was as sensitive to propionate as it was to chloride. Bicarbonate, acetate, isethionate, fluoride, and methanesulfonate can also interfere significantly with determinations of a_{Cl}^i made by the liquid ion exchanger, but not with the Ag-AgCl probe. Aspartate and glutamate do not appreciably interfere with determinations made by the liquid ion exchanger, and have no effect on the Ag-AgCl electrode.

If intracellular chloride had been overestimated in the resting cell, then the corrected a_{Cl}^i would give $E_m > E_{Cl}$, suggesting the existence of an outward-directed "chloride pump." In fact, when there was an appreciable difference between E_m and E_{Cl} in the present study, E_m was greater than E_{Cl}. This discrepancy probably was due to depolarization of the photoreceptor membrane due to injury occurring in the double impalement of the cell. Ascher et al. (1976) have recently reported that chloride moves slowly in response to changes in the

membrane potential of *Aplysia* neurons. Our preliminary experiments in *Balanus* photoreceptors were qualitatively similar, so it seems reasonable to conclude that when $E_m > E_{Cl}$, the discrepancy is due to injury and depolarization of the photoreceptor, and failure to allow adequate time for re-equilibration.

Electrode Selectivity and the Measurement of the Final a^i_{Cl}

Measurement of a^i_{Cl} during and after washout magnifies all the problems considered above, as demonstrated by the differences between data obtained with the CLIX and Ag-AgCl microelectrodes. While the time constants of chloride washout in glutamate are essentially the same, the extent to which chloride appears to wash out depends on the probe. The lowest a^i_{Cl} from a CLIX-monitored experiment was 18 mM after washout in glutamate saline. Four of eight Ag-AgCl experiments yielded a final a^i_{Cl} less than 20 mM whereas only one of nine CLIX experiments had a comparable final a^i_{Cl}. These results are consistent with interference by native intracellular anions in determination of the "final a^i_{Cl}."

If any chloride remains after washout, the Ag-AgCl electrode should provide the best estimate of the final a^i_{Cl}. The most likely biologically occurring interferents for this electrode are bromide and iodide. Since these anions are more permeant than chloride (Brown and Saunders, 1977), they should be lost during washout unless they are present as contaminants in the extracellular saline. We discovered that halides are used in the production of propionates and may remain as contaminants in some manufacturers' products. Some halide contaminant ion could explain the longer washout time constants observed when propionate replaced Cl_0 and Ag-AgCl electrodes were used to monitor a^i_{Cl}; however, we detected no interference by propionate when we tested Ag-AgCl microelectrodes with the pure chemical.

If chloride washout were complete, neither electrode could show this. From theoretical considerations the limit of sensitivity of the Ag-AgCl electrode should be approximately 1.25×10^{-5} M chloride (pure solutions). Empirically, sensitivity is limited to about 10^{-4} M. We observe "final" a^i_{Cl}s approximately two orders of magnitude greater than this. In the absence of Cl^-, a variety of half-cell reactions could interfere in the determination of the final a^i_{Cl} after washout, for example:

$$Ag_2O + H_2O + 2e^- = 2Ag + 2OH^-,$$
$$Ag_2CO_3 + 2e^- = 2Ag + CO_3^=.$$
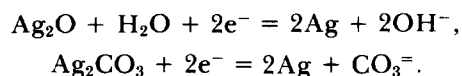
The electrode potential is undefined in these cases in the sense that the electrode has not been calibrated for these reactants and, more seriously, the types and quantitites of interferent anions are unknown.

Evidence that the photoreceptor is permeable to anions other than Cl^- is provided by the substantial apparent increase in a^i_{Cl} shown by CLIX electrodes when Cl_0 was replaced by iodide. In some cases a more complete interpretation of the biological data can be obtained by comparison of the data obtained by using two probes with different selectivities. For example, the decrease in a^i_{Cl} measured by Ag-AgCl electrodes when propionate replaced Cl_0 showed that

Cl^- washed out of the cell, while the apparent increase in a_{Cl}^i shown by CLIX electrodes under the same conditions suggests that propionate replaces intracellular chloride at the same time. CLIX electrodes cannot distinguish between propionate and Cl^- (see Tables II and III), while Ag-AgCl electrodes are insensitive to propionate.

The selectivity coefficients which we have determined for the CLIX microelectrode are generally in agreement with previously reported values for electrodes made with this type of exchanger, but selectivity coefficients vary from electrode to electrode and are also dependent upon the technique of evaluation as shown in Tables II and IV. Notably, the selectivity coefficients which we determined for bicarbonate vs. chloride are in better agreement with Orme's data (1969) on the Orion exchanger than with Walker's data (1971) for the same brand of exchanger (Corning) that we used. The selectivity coefficients for propionate and isethionate agree quite well with Walker's reported values.

Comparisons with Previous Studies

In the literature, different techniques have sometimes provided different estimates of a_{Cl}^i. In squid axons, Ag-AgCl electrodes have typically given higher estimates of a_{Cl}^i (Mauro, 1953; Keynes, 1963) than other methods (Steinbach, 1941). The same observation has recently been made in *Aplysia* giant neurons (Owen et al., 1975). In some cases, apparently similar techniques have led to different results in the same preparation (Kerkut and Meech, 1966; Neild and Thomas, 1974).

In a previous study (Brown, 1976) the mean a_{Cl}^i obtained in nine cells was 65 mM. In the present study, a_{Cl}^i was measured with two different probes. Internal chloride was 70.9 ± 10.9 mM obtained from four determinations with the Ag-AgCl microelectrode and 59.3 ± 10.1 mM ($\bar{y} \pm SD, n = 12$ when measured with the CLIX electrode. On the average, the Ag-AgCl electrode measured a higher a_{Cl}^i than the CLIX electrode. However, the difference between E_{Cl} and E_m for the two populations of cells was considered to be negligible: $E_m = -39.4 \pm 7.1$ mV and $E_{Cl} = -38.0 \pm 5.1$ mV in the four Ag-AgCl determinations and in the CLIX experiments $E_m = -45.1 \pm 3.8$ mV and $E_{Cl} = -44.1 \pm 4.5$ mV ($n = 12$).

The net chloride flux during washout was calculated to be between 2 and 20 pM cm^{-2} s^{-1} depending upon the assumptions made for the area of surface membrane involved. There are no data on other preparations in which direct comparison can be made since different techniques or different procedures were used. Nevertheless, surprisingly good agreement of chloride flux was found for *Balanus* photoreceptor, *Aplysia* neurons, and frog skeletal muscle. The data of Russell and Brown (1972) on *Aplysia* neuron are perhaps most germane since they also used liquid ion-exchanger electrodes. They loaded cells with chloride by cooling or exposure to increased extracellular potassium. Their reported chloride efflux upon return to normal saline or upon rewarming ranged from 1 to 37 pM cm^{-2} s^{-1}. Adrian (1961) measured chloride efflux from frog sartorius muscle using tracer techniques and obtained a value of 50 pM cm^{-2} s^{-1}. A calculated value of 330 pM cm^{-2} s^{-1} was obtained by Hodgkin and

Horowicz (1959) on the basis of membrane potential changes that occurred after rapid changes in external potassium.

Conclusions

Both types of electrode show that chloride is distributed in equilibrium with the resting potential of the dark-adapted photoreceptor membrane. When the cell is bathed in normal barnacle saline and has a normal complement of internal chloride, intracellular interferents should be relatively unimportant in the initial determination of a^i_{Cl}. From Table III, this would be so if the major intracellular interferents included bicarbonate, isethionate, glutamate, and aspartate. If chloride is not actually distributed in equilibrium with the membrane potential, then both electrodes must be in error, the Ag-AgCl electrode overestimating a^i_{Cl} due to an intracellular interferent halide (probably bromide, since these electrodes are irreversibly damaged in small quantitites of iodide), while the exchanger coincidentally overestimates a^i_{Cl} to the same extent due to interference by bromide, bicarbonate, propionate, isethionate, etc. This seems unlikely, not only because of the coincidence required, but also because of the great difference in bromide-chloride selectivity; the two electrodes cannot read the same a^i_{Cl} if both chloride and bromide are present in roughly equivalent quantities. The presence of millimolar quantities of bromide in *Aplysia* has been reported to cause substantial discrepancy between readings of a^i_{Cl} with the two electrode types (Owen et al., 1975).

We conclude that both electrodes provide accurate estimates of a^i_{Cl} when the cell is bathed in normal barnacle saline and contains the normal amount of chloride, that the absolute value of a^i_{Cl} depends on the level of E_m and that chloride is passively distributed. Chloride also appears to be quite permeant, moving with a time constant of about 5 min in response to changes in the chemical gradient. Changes in membrane potential produced by increasing extracellular potassium or by illumination resulted in an increase in a^i_{Cl} with the chloride equilibrium potential moving toward the new membrane potential. The quantitative description of these observations will be the subject of a later paper.

Since we have found that Ag-AgCl electrodes are insensitive to propionate, it is probable that the rather large difference in washout τs between glutamate and propionate (5 min and 11 min, respectively) represents true differences due to some effect of the substituted anion on membrane permeability to Cl⁻. Thus, methanesulfonate (observed with CLIX electrodes) and propionate (observed with Ag-AgCl electrodes) could interfere directly with Cl⁻ washout. Such differences have been observed in radiochloride flux studies of frog skeletal muscle by Adrian (1961) and Moore (1969).

The possibility that KCl is lost from the cell and that the cell shrinks during chloride washout cannot be excluded on the basis of the present experiments. The fact that the extracellular anion substitute also replaces internal chloride suggests that this is an unlikely alternative. Thus it is clear from the present experiments that Cl⁻ washes out of the *Balanus* photoreceptor quite rapidly upon anion substitution and that the characteristics of washout can be strongly influenced by the kind of probe used and selection of the anion substitute.

REFERENCES

ADRIAN, R. H. 1961. Internal chloride concentration and chloride efflux of frog muscle. *J. Physiol. (Lond.).* **156:**623–632.

ASCHER, P., D. KUNZE, and T. O. NEILD. 1976. Chloride distribution in *Aplysia* neurones. *J. Physiol. (Lond.).* **256:**441–464.

BISHOP, E., and R. G. DHANESHWAR. 1963. Silver and halide-ion responsive electrodes. *Analyst.* **88:**424–432.

BROWN, H. M. 1976. Intracellular Na⁺, K⁺, and Cl⁻ activities in *Balanus* photoreceptors. *J. Gen. Physiol.* **68:**231–296.

BROWN, H. M., S. HAGIWARA, H. KOIKE, and R. W. MEECH. 1970. Membrane properties of a barnacle photoreceptor examined by the voltage clamp technique. *J. Physiol. (Lond.)* **208:**385–413.

BROWN, H. M., and J. H. SAUNDERS. 1977 Cation permeability and conductance sequence in *Balanus* photoreceptor. *J. Gen. Physiol.* **70:**531–543.

BUCK, R. P. 1968. Theory of potential distribution and response of solid state membrane electrodes. *Anal. Chem.* **40:**1432–1439.

COVINGTON, A. 1969. Heterogeneous membrane electrodes. *In* Ion-Selective Electrodes. R. A. Durst, editor. National Bureau of Standards Special Publication 314. p. 89–106.

GREGOR, H. P., M. ROTHENBERG, and N. FINE. 1963. Molal activity coefficients of methane- and ethanesulfonic acids and their salts. *J. Phys. Chem.* **67:**1110–1112.

HAGIWARA, S., K. TOYAMA, and H. HAYASHI. 1971. Mechanisms of anion and cation permeations in the resting membrane of a barnacle muscle fiber. *J. Gen. Physiol.* **57:**408–434.

HODGKIN, A. L., and P. HOROWICZ. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibers. *J. Physiol. (Lond.).* **148:**127–160.

JANZ, G. J., and D. J. G. IVES. 1968. Silver, silver chloride electrodes. *Ann. N.Y. Acad. Sci.* **148:**210–221.

KERKUT, G. A., and R. W. MEECH. 1966. The internal chloride concentration of H and D cells in the snail brain. *Comp. Biochem. Physiol.* **19:**819–832.

KEYNES, R. D. 1963. Chloride in the giant squid axon. *J. Physiol. (Lond.)* **169:**690–705.

KREBS, W., and B. SCHATEN. 1976. The lateral photoreceptor of the barnacle *Balanus eburneus.* Quantitative morphology and fine structure. *Cell Tissue Res.* **168:**193–207.

LAKSHMINARAYANAIAH, N. 1976. Membrane Electrodes. Academic Press, Inc., New York.

LEWIS, G. N., and M. RANDALL. 1961. Theories of electrolyte solutions. *In* Thermodynamics 2nd edition. K. S. Pitzer and L. Brewer, revisors. McGraw-Hill Book Company, New York, Ch. 23. 332–348.

LI, J. C. R. 1957. Introduction to Statistical Inference. Edwards Bros., Ann Arbor, Mich.

MAURO, A. 1953. The electrochemical potential difference of the chloride ion in the giant squid axon sea water system. *Biol. Bull. (Woods Hole).* **105:**378.

Moody, G. J., and J. D. R. Thomas. 1971. Selective Ion Sensitive Electrodes. Merrow Publishing Co., Ltd., Watford, England.

Moore, L. E. 1969. Anion permeability of frog skeletal muscle. *J. Gen. Physiol.* **54:**33–52.

Neild, T. O., and R. C. Thomas. 1973. New design for a chloride-sensitive microelectrode. *J. Physiol. Proc. Physiol. Soc.* **232:**7P–8P.

Neild, T. O., and R. C. Thomas. 1974. Intracellular chloride activity and the effects of acetylcholine in snail neurones. *J. Physiol. (Lond.).* **242:**453–470.

Orme, F. W. 1969. Liquid ion-exchanger microelectrodes. *In* Glass Microelectrodes. N. Lavallée, O. F. Schanne, and N. C. Hébert, editors. John Wiley & Sons, Inc., New York. 376–395.

Owen, J. D., H. M. Brown, and J. H. Saunders. 1975. Chloride activity in the giant cell of *Aplysia. Biophys. J.* **15**(2, Pt. 2):45a. (Abstr.).

Parsons, R. 1959. Handbook of Electrochemical Constants. Butterworths Scientific Publications, London.

Robinson, R. A., and R. H. Stokes. 1970. Electrolyte Solutions. 2nd edition. Butterworth & Co. (Publishers) Ltd., London

Russell, J. M., and A. M. Brown. 1972. Active transport of chloride by the giant neuron of the *Aplysia* abdominal ganglion. *J. Gen. Physiol.* **60:**499–518.

Saunders, J. H., and H. M. Brown. 1975. Determination of intracellular chloride in *Balanus eburneus* photoreceptor. *Biophys. J.* **15**(2, Pt. 2):323a. (Abstr.).

Srinivasan, K., and G. A. Rechnitz. 1969. Selectivity studies of liquid membrane ion-selective electrode. *Anal. Chem.* **41:**1203–1208.

Steinbach, H. B. 1941. Chloride in the giant axons of the squid. *J. Cell. Comp. Physiol.* **17:**57–64.

Tasaki, I., and I. Singer. 1968. Some problems involved in electric measurements of biological systems. *Ann. N. Y. Acad. Sci.* **148:**36–53.

Walker, J. L., Jr. 1971. Ion specific liquid ion exchanger microelectrodes. *Anal. Chem.* **43:**89A–91A.