Liquid Junction and Membrane Potentials of the Squid Giant Axon

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ABSTRACT The potential differences across the squid giant axon membrane, as measured with a series of microcapillary electrodes filled with concentrations of KCl from 0.03 to 3.0 M or sea water, are consistent with a constant membrane potential and the liquid junction potentials calculated by the Henderson equation. The best value for the mobility of an organic univalent ion, such as isethionate, leads to a probably low, but not impossible, axoplasm specific resistance of 1.2 times sea water and to a liquid junction correction of 4 mv. for microelectrodes filled with 3 M KCl.

The errors caused by the assumptions of proportional mixing, unity activity coefficients, and a negligible internal fixed charge cannot be estimated but the results suggest that the cumulative effect of them may not be serious.

Since direct measurements of the electrical potential differences between the cytoplasm and the external solution of many cells have been found possible, these data have become increasingly important in the investigation of the properties and mechanisms of cell membranes. As a consequence, it has become correspondingly important to understand the limitations of the methods.

For some cells, such as marine eggs (Tyler, Monroy, Kao, and Grundfest (1956)), it was difficult even to penetrate the membrane and be certain of it, but for most the appearance of a potential difference has been accepted as an adequate criterion. The next question was that of injury and survival. Large plant cells (Osterhout, Damon, and Jacques (1927)) seemed to be able to form a rather durable and satisfactory seal around quite a large capillary. The long, large squid axon permitted measurements at points well beyond the range of the electrical disturbance caused by the entry of a large internal electrode (Curtis and Cole (1940)), but the injury spread steadily and less obvious effects have been suspected. So many cells have tolerated

Moore and Cole (1955) made a preliminary report. The opinions stated are those of the authors and are not necessarily the opinions of the Navy Department.

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penetration by electrodes with diameters of the order of 1 micron so well as to arouse considerable curiosity about the underlying phenomena.

Metallic microelectrodes (Grundfest, Sengstaken, Oettinger, and Gurry (1950)), insulated except at the tip, have been useful in the investigation of transient phenomena, such as excitation, for which the polarization impedance or any uncertainty of the electrode reaction at the metal-cytoplasm interface has not been important. A large majority of steady state or resting potential measurements have been made with electrolyte-filled glass capillaries which act as a salt bridge between a large reversible electrode and the cytoplasm at the tip. The large capillaries inserted into *Valonia* vacuoles and *Loligo* axons have been filled either with the external solution or with an approximation to the internal electrolyte composition as has also been done with the microcapillary electrodes (Ling and Gerard (1949)). Later (Nastuk and Hodgkin (1950)), as has now become almost universal practice, the microelectrodes were filled with 3 M KCl in order to decrease their electrical resistance and the accompanying electrical difficulties and to approximate the time-honored saturated KCl bridge. There has been no certain evidence of injury by the diffusion from the concentrated solution into the interior of the cells and although large and erratic potentials have been found, careful techniques (Adrian (1956)) have produced a high proportion of similar electrodes—particularly those of comparatively low resistance (Moore and Cole (1960)).

The only obvious components of the various cell potential measurements that should not be attributed to the membrane are the liquid junction potentials to be expected between the electrode systems and the external medium on one hand and the cytoplasm of the cell on the other hand. These extramembrane potentials were of particular interest when it first seemed probable that the squid action potential might overshoot the resting potential but they were estimated (Curtis and Cole (1942)) to be too small to prevent the conclusion that there was an overshoot. Although more complete analyses (Koechlin (1955)) and conductances of axoplasm have become available, the isethionate ion conductance is not known and the range of conductance values is considerable, so that there has not been any very good basis to improve the early liquid junction potential estimates. On the other hand, resting and action potentials are approaching so close to those predicted by analyses for potassium and sodium potentials, respectively, as to indicate a need for careful consideration of all factors.

The usual measurement of a cell potential is the change of the potential difference between two stable reversible electrodes, one fixed in the external solution and the other connected to the solution in a microelectrode, as the tip of the microelectrode is either inserted from the external medium through the cell membrane and into the cytoplasm of the cell or as it is withdrawn. This measurement is then, with obvious reservations, equivalent to the

potential difference between two identical reversible electrodes connected to two identical microelectrodes, with the tip of one in the external medium and the tip of the other in the cytoplasm. The potential difference of this array must then include the liquid junction potential between the electrolyte within the microneedle and the medium, the membrane potential, and the liquid junction potential between the cytoplasm and the same electrolyte in the same microelectrode as is outside. The outside liquid junction potential may be calculated reasonably well, but a calculation for the inside junction is no better than the measured and assumed properties of the cytoplasm and is without an indication of its possible error.

For this reason, when it became practical to do so, measurements were made across a single squid axon membrane with a series of similar microelectrodes filled with KCl solutions at concentrations ranging from 0.03 to 3.0 M or with sea water. On the necessary assumption that the membrane potential is independent of the microelectrode electrolyte concentrations, measurements with two concentrations suffice to determine this potential and the relative conductance of a single ion species of the axoplasm. The use of additional concentrations can determine more ion conductances or, more usefully, improve the confidence in the reliability of the membrane potential and single ion conductance values. This conductance with the values used for the other ions then gives an axoplasm conductance for comparison with the direct experimental values.

EXPERIMENTS

The experiments were made in conjunction with those of the preceding paper (Moore and Cole (1960)) and with identical techniques. Mantle preparations with the axons *in situ* were found to give satisfactorily constant and reproducible measurements and were used throughout. The observed potentials were low—both for this reason and because all measurements were made during the 1954 midsummer slump in squid viability. The microelectrodes were drawn and filled as described but several, filled with 0.03, 0.1, 0.3, 0.5, 3.0 M KCl or sea water, were prepared for each experiment. The measured potentials for each composition were constant, reproducible, and independent of sequence to within about 2 mv. and a measurement at 3.0 M KCl was usually made before and after each of the others. The temperatures were those of the tap sea water and in the neighborhood of 20°C. The data on four axons were satisfactory and complete enough for analysis.

Calculations

The well known Henderson (1907) liquid junction equation was used for all calculations and a few of these values were satisfactorily checked by solutions of the transcendental Planck (1890) equation. Ionic conductances were

calculated from the appropriate electrolyte conductances and transference numbers and used as suggested by Lewis and Sargent (1909) instead of the products of mobility and concentration. The liquid junction potential is then

$$E_{12} = \frac{RT}{F} \frac{(U_1 - V_1) - (U_2 - V_2)}{(U_1 + V_1) - (U_2 + V_2)} \log \frac{U_1 + V_1}{U_2 + V_2}, \quad (1)$$

where U, V are the cation and anion conductances for the two sides of the junction designated by the subscripts.

The assumed sea water composition approximated that at Woods Hole with a salinity of 32/00 and a specific resistance of 22.7 ohm cm. at 18°C. The axoplasm was assumed to have the K, Na, and Cl concentrations given for fresh axoplasm (Steinbach and Spiegelman (1943); Steinbach (1941)) and the mobilities appropriate for such a simple aqueous solution as has been well demonstrated for K^+ in axoplasm (Hodgkin and Keynes (1953)). The remaining anion, primarily isethionate or a precursor, was assumed to be entirely monovalent.

The following values were used for the cation and anion conductances, U, V, in millimhos per centimeter at 18°C.:

	KCl					
SW	0.03 M	0.1 M	0.3 M	0.5 M	3 M	Axoplasm
U 16.3	1.734	5.5	15.55	25.0	133.2	22.7
V 27.8	1.805	5.7	16.25	26.1	139.8	12.7–16.7

The external junction potentials between sea water and the various concentrations of KCl were calculated first and added to the observed potentials to give the sum of membrane and internal junction potentials. The axoplasm junction potentials for each concentration of KCl and for sea water were computed and plotted as functions of the axoplasm anion conductance. An overlay of the experimental values corrected for the external junction potential was translated to obtain an anion conductance and the corresponding membrane potential that gave the best agreement between experimental and calculated potentials.

RESULTS

Twenty-two measurements on four axons have been analyzed but two low terminal measurements with 3 M KCl and the values found immediately preceding them were inconsistent and are omitted. The remaining results are shown in Fig. 1 as a function of the microelectrode KCl concentration and include values in which sea water was used. After the first correction for

sea water–KCl junction potentials the best values found for the anion conductances were 12.7, 13.5, 15.7, and 16.7 m mho/cm. The corresponding membrane potentials obtained with these values were 60.4, 59.6, 60, and 58.4 mv. and are shown in Fig. 1. The maximum difference between the observed and calculated potentials was 2 mv. In particular the calculated value of the sea water–3 M KCl junction was 1.0 mv. and the 3 M KCl–axoplasm values were 3.7, 3.5, 3.0, 2.6 mv.

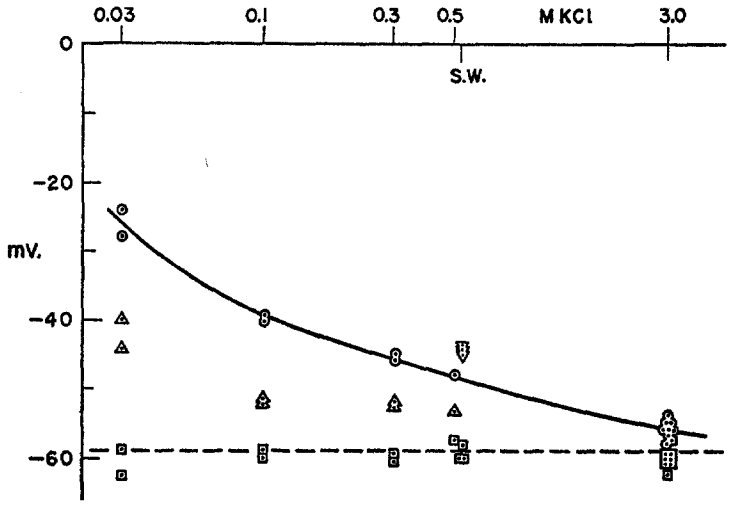

FIGURE 1. The potential differences, ordinate, across squid axon membranes as measured with microelectrodes filled with KCl (circles) at concentrations given as abscissae, or with sea water (S. W. inverted triangles). The solid curve indicates an average change of the observed potentials with the KCl concentrations. The observed potentials as corrected for the external sea water–KCl junction are shown as triangles. The membrane potentials obtained by the second correction for the KCl–axoplasm or SW–axoplasm junction are shown as squares. The dotted line is the average of the estimated individual axon resting potentials.

For the average $V = 14.6$ m mho/cm. the axoplasm specific resistance is about 1.2 times that of sea water. Subtraction of a chloride conductance of 2.3 m mho/cm. leaves the assumed remaining univalent anion with a mobility of about 0.4 that of K^+.

DISCUSSION

The obvious aim of this and much other biological work is to measure the electrical potential difference of a cell membrane between the different aqueous phases outside and inside the cell as a guide to the structure and mechanism of the membrane. In such work a number is sought and obtained

in the hope and belief that it is, at the very least, a useful piece of information. On the other hand, at this point it is more likely than not that Guggenheim (1929) will be quoted—"The electric potential difference between two points in different media can never be measured and has not yet been defined in terms of physical realities: it is therefore a conception which has no physical significance." This statement has not gone unchallenged (Bates (1954, p. 39)) but it has been quite consistently upheld since its appearance (Guggenheim (1950, p. 332); Harned and Owen (1958, p. 440)).

It is most unlikely that attempts to measure membrane potentials and to assign a physical significance to them will not continue for a while longer. Nor is there any real prospect that a completely rigorous meaning for the results can be found in the near future. Consequently it is important that the imperfections of both rigor and expediency be isolated, identified, and to whatever extent possible understood.

The theoretical development stems from chemical thermodynamics as modified by the concept of the activity. This concept was subsequently shown to have a considerable physical reality in terms of ionic electrostatic energies (Debye and Hückel (1923)) and the first approximation has been only improved by the more detailed particulate theory (Kirkwood and Poirier (1954)).

In the first place it seems clear that the full force of Guggenheim's emphatic statement is not directed at the present situation in which the two media are not different because the solvent in each case is water (Guggenheim (1929); (1950, p. 335)). Then the measurable potential difference, E_{12}, between identical half-cells is

$$E_{12} = -\sum_{1}^{n} \frac{RT}{N_i F} \int_1^2 t_i \, d \log a_i \tag{2}$$

in terms of transference numbers, t_i and activities, a_i. This expression also has a satisfying and firm base in irreversible thermodynamics (Harned and Owen (1958, p. 38)) and is a very useful intuitive guide. Expressed in terms of concentrations, m_i, and activity coefficients, γ_i, $a_i = m_i \gamma_i$,

$$E_{12} = -\sum_{1}^{n} \frac{RT}{N_i F} \int_1^2 t_i \, d \log m_i - \sum_{1}^{n} \frac{RT}{N_i F} \int_1^2 t_i \, d \log \gamma_i, \tag{3}$$

the difficulty is explicit. No matter what their other shortcomings, calculations of the liquid junction potentials—such as by Planck and Henderson—are frankly concerned only with the first summation and so assume each $\gamma_i = 1$, identically. The argument then proceeds that the second summation cannot be evaluated because only the activity coefficients of compounds, $\gamma_+ \cdot \gamma_-$, are experimentally available, whereas the individual ion coefficients, γ_+ and γ_-, are not—except only that by definition they both approach

unity at infinite dilution. However, for dilute solutions of strong electrolytes, γ_+, γ_- as calculated by the electrostatic contribution to the free energy, are equal and the mean activity coefficients $\gamma = \sqrt{\gamma_+ \cdot \gamma_-} = \gamma_\pm$ are in generally good agreement with experiment. Under such conditions, the second integral is definable in terms of physical realities and liquid junction potentials have been calculated on this basis (MacInnes (1939)). Calculations with $\gamma_+ = \gamma_-$ have, however, been carried to concentration ranges in which the electrostatic theory is not adequate and are consequently based on an arbitrary assumption. That this may not be critical is suggested by calculations showing that so long as $\gamma = \sqrt{\gamma_+ \cdot \gamma_-}$ the junction potential was not much affected by the choices of γ_+ and γ_- (Harned (1926)). Although this approach is limited because the necessary data cannot be obtained and the necessary theory is not available, it can be helpful when it may be possible to estimate that the second summation is within the present biological needs of perhaps 2 mv.

The ideal theory would be for a realizable liquid junction including ion atmosphere and such other factors as necessary at high concentrations. Although this should be possible in principle, it can be expected to be very difficult in view of the shortcomings of the present formulations and impractical for high concentrations until they are better understood. But unless a more immediate and practical approach is found, it may be that this ideal will be needed to provide a further physical reality for the thermodynamic concepts.

Since it is apparent that the second summation of equation (3) is inadequate by an amount that cannot now always be evaluated, this error will have to be ignored in the hope that, for present biological purposes at least, it will not be significant. But the first summation itself is not without difficulties. The classical solutions of Planck and Henderson with their respective assumptions of constrained diffusion and proportional mixing are the best known and, along with numerous modifications, are the most widely used. Here again, in the absence of a less restricted theory, it is difficult to estimate the errors caused by the simplifying and inappropriate assumptions. However, the fact that these two theories are identical for three simple types of liquid junction may be taken as an indication that the effects of the assumptions may not be radically different. This is, however, no indication that either or both are necessarily correct and it can only be said that this term of equation (3) also involves unknown errors. With no internal basis to judge the adequacy of the theories it is the more necessary that they be considered in terms of their ability to represent experiment.

The experimental results with liquid junctions are not completely satisfactory because the measured potentials depend upon the particular technique used to form and maintain the junction and, for some, upon the time after formation. Without specific reference to the voluminous detailed literature

(MacInnes, (1939); Bates (1954); Harned and Owen (1958, chapter 10)), an opinion may be offered that in general the more reproducible and constant potentials usually agree to within 2 mv. and often to within 1 mv. Such experiments seem to be paralleled by various theoretical values that again, in a general way, have a similar agreement not only among themselves but also with the results of experiment. Furthermore, in those cases so far considered for a liquid junction potential that can be predicted from experimental values obtained entirely from electrolytic cells without transport and entirely sound theoretically, the values so predicted again are found to be within 1 or 2 mv. of those obtained both by liquid junction theories and experiments.

It has been suggested that the presence of the fixed charges to be expected in a gel, such as that in the squid axoplasm (Chambers and Kao (1951)), will introduce Donnan considerations (Overbeck (1956)) into the internal liquid junction potential calculation. Since no estimate of such a charge density is available and there is no evidence as yet that it should be considered for the squid axon, this complication has been ignored without investigation.

There are now two points to be kept in mind. The first is that an uncertainty of 1 mv. is quite unacceptable for many electrochemical purposes, and particularly so for relatively simple electrolytic systems. The second is that although such an uncertainty is not yet of crucial importance in the biological systems here considered there is no basis to do more than to hope that the errors for these more complicated living systems will not be considerably increased. It would, of course, be preferable to have whatever experimental evidence that it may be possible to obtain.

If, for example, the cell membrane were replaced by an electrode known to be reversible only to potassium, sodium, or chloride ions, a value for the difference of the two junction potentials would come from each measurement and the appropriate activities. The first two electrodes may be technically possible as artificial membranes or amalgams and the third, although apparently simple, may not be feasible with Ag–AgCl electrodes until the properties of such an electrode in axoplasm are proven simpler than is now suspected. Even in the absence of acceptable values for the activities, any differences between the potentials observed with the natural and such artificial membranes can only be an indication of the imperfection of the natural membrane. However, in the absence of some such experiments, the situation is reversed and calculations of the junction potentials lead to the conclusions that the axon membrane is a rather good potassium electrode at rest and almost as good a sodium electrode at the height of activity!

It is necessary to question the appropriateness first of the use of the fresh axoplasm analyses to arrive at liquid junction corrections consistent with measurements on equilibrated axons having a lowered resting potential and then of the application of these corrections back to measurements on fresh axons (Moore and Cole (1960)). It seems possible that the present measurements correspond to an axon state lying somewhere between that for the

fresh and for the 3 hour analyses and that even the fresh axoplasm analyses may not be appropriate for the earliest potential measurements in the preceding paper. However, the axoplasm cation conductance, U, is so dominated by that of K^+ that the partial replacement of K^+ by Na^+ between the two analyses does not make an important difference. Although the axoplasm Cl^- concentration change is considerable, it does not enter except into the interpretation of the value for the anion conductance, V, found to best fit the data. What may be of more significance is that the lowest anion conductance corresponds to the highest membrane potential and *vice versa*.

With the data then available, the over-all liquid junction corrections were estimated by Curtis and Cole (1942) to be 10 mv. for KCl isosmotic with sea water and by Hodgkin and Katz (1949) to be 14 mv. for sea water. Fig. 1 shows that both are in good agreement with the present conclusions.

The average value of the axoplasm resistivity of about 1.2 times that of sea water is clearly less than the ratios of 1.5 to 9 with an average of 4 obtained by Curtis and Cole (1938). It lies at the lower end of the range 1.15 to 1.9 with an average of 1.4 given by Cole and Hodgkin (1939) and is significantly less than 1.95 as determined by Schmitt (1955). An unpublished investigation by Freygang also gave about 1.95. Evidence has been found for the existence of a thin poorly conductive layer close to the membrane (Cole and Marmont, unpublished); Hodgkin, Huxley, and Katz (1952)) which would reduce the ratio of 4 obtained by transverse impedance (Cole and Curtis (1950)). This layer would not have an immediately obvious effect on the value given by Schmitt and would have no effect on the other results.

If, as now seems likely, the axoplasm has a specific resistance somewhat higher than the value used to produce the best estimates of the liquid junction correction consistent with the measurements, the difference may well be the cumulative effect of the assumptions of proportional mixing in the Henderson equation, unity activity coefficients, negligible internal fixed charge, and perhaps others. Since no quantitative evidence has been produced to show that all errors would be sufficiently small or that any would tend to cancel, it is hardly to be expected that so roundabout a procedure could produce as good a value for the specific resistance as a more direct measurement.

Consequently, it is believed that the use of such an effective conductance, which is adequate for a series of potential measurements, is more apt to give a better correction for each than is the use of the correct conductance for any. It is also quite obvious that even more drastic changes of the parameters used in the equation will not produce changes in the 3 M KCl liquid junction correction of about 4 mv. that are apt to be significant for present biological purposes.

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REFERENCES

ADRIAN, R. H., *J. Physiol.*, 1956, **133**, 631.

BATES, R. G., Electrometric pH Determinations, New York, John Wiley & Sons, Inc., 1954, chapter 3.

CHAMBERS, R., and KAO, C., *Biol. Bull.*, 1951, **101**, 206.

COLE, K. S., and CURTIS, H. J., Medical Physics. II, (O. Glasser, editor), Chicago, Year Book Publishers, Inc., 1950, 82.

COLE, K. S., and HODGKIN, A. L., *J. Gen. Physiol.*, 1939, **22**, 671.

CURTIS, H. J., AND COLE, K. S., *J. Gen. Physiol.*, 1938, **21**, 757.

CURTIS, H. J., AND COLE, K. S., *J. Cell. and Comp. Physiol.*, 1940, **15**, 147.

CURTIS, H. J., and COLE, K. S., *J. Cell. and Comp. Physiol.*, 1942, **19**, 135.

DEBYE, P., and HÜCKEL, E., *Physik. Z.*, 1923, **24**, 185.

GRUNDFEST, H., SENGSTAKEN, R. W., OETTINGER, W. H., and GURRY, R. W., *Rev. Scient. Instr.*, 1950, **21**, 360.

GUGGENHEIM, E. A., *J. Physic. Chem.*, 1929, **33**, 842.

GUGGENHEIM, E. A., Thermodynamics, Amsterdam, North Holland Publishing Co., 1950.

HARNED, H. S., *J. Physic. Chem.*, 1926, **30**, 433.

HARNED, H. S., and OWEN, B. B., The Physical Chemistry of Electrolytic Solutions, New York, Reinhold Publishing Corp., 3rd edition, 1958.

HENDERSON, P., *Z. physik. Chem.*, 1907, **59**, 118.

HODGKIN, A. L., HUXLEY, A. F., and KATZ, B., *J. Physiol.*, 1952, **116**, 424.

HODGKIN, A. L., and KATZ, B., *J. Physiol.*, 1949, **108**, 37.

HODGKIN, A. L., and KEYNES, R. D., *J. Physiol.*, 1953, **119**, 513.

KIRKWOOD, J. G. and POIRIER, J. C., *J. Physic. Chem.*, 1954, **58**, 591.

KOECHLIN, B. A., *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 511.

LEWIS, G. N., and SARGENT, L. W., *J. Am. Chem. Soc.*, 1909, **31**, 363.

LING, G. and GERARD, R. W., *J. Cell. and Comp. Physiol.*, 1949, **34**, 383.

MACINNES, D. A., Principles of Electrochemistry, New York, Reinhold Publishing Corp., 1939, chapter 13.

MOORE, J. W., and COLE, K. S., *Fed. Proc.*, 1955, **14**, 103.

MOORE, J. W., and COLE, K. S., *J. Gen. Physiol.*, 1960, **43**, 961.

NASTUK, W. L., and HODGKIN, A. L., *J. Cell. and Comp. Physiol.*, 1950, **35**, 39.

OSTERHOUT, W. J. V., DAMON, E. B., and JACQUES, A. G., *J. Gen. Physiol.*, 1927, **11**, 193.

OVERBEEK, J. TH. G., Progress in Biophysics, (S. A. V. Butler, editor) New York, Pergamon Press, 1956, **6**, chapter 3.

PLANCK, M., *Ann. Physik u. Chem.*, 1890, **40**, 561.

SCHMITT, O. H., Electrochemistry in Biology and Medicine, (T. Shedlovsky, editor), New York, John Wiley & Sons, Inc., 1955, 91.

STEINBACH, H. B., *J. Cell. and Comp. Physiol.*, 1941, **17**, 57.

STEINBACH, H. B., and SPIEGELMAN, S., *J. Cell. and Comp. Physiol.*, 1943, **22**, 187.

TYLER, A., MONROY, A., KAO, C. Y., and GRUNDFEST, H., *Biol. Bull.*, 1956, **111**, 157.