DISSIMILARITY OF INNER AND OUTER PROTOPLASMIC SURFACES IN VALONIA. III

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The dissimilarity of the inner and outer surfaces of the protoplasm of Valonia macrophysa is strikingly demonstrated by the large p.d. across the protoplasm observed when the solution applied to the external surface has the same composition as the vacuolar sap. While in some of the earlier experiments the external solution was natural Valonia sap, in the later measurements with improved technic it was artificial sap made up according to the analyses of L. M. Van der Pyl. The use of artificial sap may be open to criticism on the ground that it possibly lacks some substance which, although present in natural sap only in traces, may still be supposed to exert a considerable effect on the p.d. (Such effects have been reported for artificial systems: thus, Beutner finds that the addition of 4 mg. of pilocarpine to 100 cc. of physiological salt solution causes a decrease of 57 mv. in the p.d. between this solution and a layer of nitrobenzol containing oleic acid.) Accordingly, it seemed worth while to carry out a few additional measurements using natural sap when our supply of Valonia permitted the sacrifice of a few hundred cubic centimeters of cells to provide a sufficiently large sample.

1 Similar evidence for the asymmetry of protoplasm has been found in Nitella (Osterhout, W. J. V., and Harris, E. S., J. Gen. Physiol., 1927–28, 11, 391), in Halicystis (Blinks, L. R., J. Gen. Physiol., 1929–30, 13, 223), and possibly in muscle (Osterhout, W. J. V., Biol. Rev., 1931, 6, 390).
5 Beutner, R., J. Pharmacol., 1927, 31, 305.
The cells available for this purpose were taken from a lot which had been kept at the (Bermuda) laboratory for more than a year; they had been stored out of doors, but shaded from direct sunlight, in large glass bottles containing more than ten times their volume of sea water; the sea water had been changed occasionally. Under these conditions the cells had grown to more than twice their volume when collected. They appeared healthy, although they were lighter green in color than recently collected cells, and were somewhat more sensitive to rough handling. 5 months later, practically all the unused cells from this lot were still in good condition. Several samples of sap were extracted at different times in order to provide fresh material for the p.d. measurements. After these measurements had been completed, L. L. Burgess (of this laboratory) kindly determined the concentrations of KCl and NaCl in the combined samples.

<table>
<thead>
<tr>
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<th>Moi per liter</th>
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<tr>
<td></td>
<td>KCl</td>
</tr>
<tr>
<td>This sample of natural sap</td>
<td>0.542</td>
</tr>
<tr>
<td>Proportions used in making artificial sap</td>
<td>0.517</td>
</tr>
</tbody>
</table>

A considerable variation is to be expected in the composition of different samples of sap, since, as Jacques and Osterhout have pointed out, the K + Na ratio of the sap changes during the growth of the cell, and is easily affected by external conditions (e.g., by handling the cells, by changing the sea water, or by illumination). Comparison of measurements in which fresh sap was used with others in which the sap had been kept from 1 to 4 days after extraction showed no differences which could be attributed to changes in the sap on standing. The impaled cells used in the measurements of p.d. were taken from a more recently collected lot (collected November 14, 1930, measured January 21–February 18, 1931) which had been stored out of doors as described above, with change of sea water at least once a week.

Since the limited supply of natural sap did not permit the use of a flowing contact for applying solutions to the cell (as in earlier measurements with artificial sap), a less wasteful method was adopted in which the cell was completely immersed in a small volume of solution. For this procedure it proved convenient to impale the cell from above while supporting it on a glass ring as shown in Fig. 1. Advantages of this form of support are that it adds very little to the depth of solution needed to cover the cell and that it is easily rinsed, thus reducing the danger of carrying over sea water into the natural sap when the external solution is changed. (The rinsing consisted in dipping the cell rapidly several times in a second beaker of natural or artificial sap before placing it in the sample to be used in the p.d.

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The external and internal solutions were connected with balanced calomel electrodes in the same manner as described in an earlier report. The E.M.F. of the system was measured by a compensation method using a simple type of potentiometer and a Compton quadrant electrometer. It proved most convenient to use this combination as a deflection potentiometer: i.e., the principal part of the unknown P.D. was balanced by a known P.D. from the potentiometer while the remaining small portion of the unknown P.D. was estimated from the electrometer deflection. The electrometer was adjusted to give a deflection of 5 to 6 mm. per millivolt with the scale 4 feet from the mirror. The distance measured in practice, however, was the sum of the deflections to right and to left when the sign of the charge applied to the insulated quadrants was reversed (by means of a reversing switch in the electrometer circuit); since this total deflection amounted to 10 to 12 mm. per millivolt, the P.D. could easily be read to 0.1 millivolt. Since the time required for full deflection was somewhat less than 20 seconds, as many as

7 Osterhout, Damon, and Jacques, p. 195.
8 Blinks, L. R., J. Gen. Physiol., 1930–31, 14, 139; also unpublished experiments.
3 readings per minute were possible provided that the p.d. did not change so rapidly as to require a new setting of the potentiometer.

The conclusions reported in this paper are based on 28 measurements of p.d. using 13 impaled cells. Of these measurements, 12 were with natural sap, using 7 different cells, and 16 were with artificial sap, using 9 cells. The average duration of exposure to sap in each experiment was 90 minutes. The measurements were carried out at room temperature, which varied between 14° and 21°C., but which in most cases was between 16° and 18°C.

**Fig. 2.** Time curves showing changes in p.d. across *Valonia* protoplasm when the external solution is changed from sea water (shaded circles) to *Valonia* sap (open circles). The interior of the cell is positive in the external circuit with respect to both sea water and sap applied externally; i.e., positive current tends to flow from the capillary through the measuring instrument to the solution bathing the outside of the cell. Curve A₁ represents a measurement using natural *Valonia* sap, Curve A₂ a later measurement on the same cell using artificial sap. Curves B₁ and B₂ represent similar measurements with natural sap on a second cell. To prevent confusion, the graphs are separated by a vertical and horizontal displacement.

Comparison of the p.d.-time curves with natural sap and with artificial sap fails to disclose any effects which can be ascribed to differences between the two solutions. Any such effects are evidently much smaller than the variations among individual cells measured with the same solution, or than changes in a single cell measured at different
times with the same solution. This is illustrated by the P.D.-time curves in Fig. 2, where Curve A1, representing a measurement using natural sap, has the same general shape as Curve A2, representing a later measurement on the same cell using artificial sap. Differences between these two curves are closely paralleled by differences between Curves B1 and B2, both of which represent measurements with natural sap on a second cell. Reasonably good agreement is found also in the values of the first maximum in the curves with natural and artificial sap, summarized in the following table:

<table>
<thead>
<tr>
<th></th>
<th>Sea water</th>
<th>Valonia sap</th>
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<tbody>
<tr>
<td></td>
<td>mV</td>
<td>mV</td>
</tr>
<tr>
<td>Experiments with natural sap (12 measurements on 7 cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extremes</td>
<td>4.1-13.0</td>
<td>42.4-74.9</td>
</tr>
<tr>
<td>Mean</td>
<td>7.6</td>
<td>61.3</td>
</tr>
<tr>
<td>Average deviation from mean</td>
<td>2.2</td>
<td>6.5</td>
</tr>
<tr>
<td>Experiments with artificial sap (16 measurements on 9 cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extremes</td>
<td>4.6-13.3</td>
<td>42.6-81.0</td>
</tr>
<tr>
<td>Mean</td>
<td>8.2</td>
<td>63.0</td>
</tr>
<tr>
<td>Average deviation from mean</td>
<td>2.1</td>
<td>7.6</td>
</tr>
</tbody>
</table>

In some cases, later maxima in the P.D.-time curves were considerably higher than the first maxima reported in the above table. The highest P.D. observed was 100.5 mV. (with artificial sap).

Comparison of the values for P.D. with sap in this report and in earlier reports shows that our recent values are much higher than the values formerly reported as usual (25-35 mV.) and more like the value (82 mV.) formerly reported as unusually high. Since it is known that the K + Na ratio in *Valonia* sap varies greatly among healthy cells depending on various factors, especially on the amount of illumination which the cells have received, it is not surprising that the P.D. produced by potassium-rich solutions should vary. The cells used in the earlier experiments had been kept in the laboratory, and hence had been much less strongly illuminated than the cells used in the recent experiments, which had been stored out of doors exposed to light from the sky, although shaded from direct sunlight. The effect of illumination on the P.D. requires further study.
In an earlier paper it was reported that prolonged exposure to KCl solutions produced irreversible (or very slowly reversible) changes in the protoplasm, which were made evident by changes in the shape of the P.D.-time curve when the cell was measured for a second time with the same solution; i.e., the P.D. failed to rise to a second maximum. To explain this alteration, it was suggested that a part of the KCl which had entered the protoplasm during exposure to sap was not leached out again when the cell was returned to sea water, and that the change in behavior of the cell when measured for a second time was due merely to this increase in the concentration of KCl in the protoplasm.

In the recent experiments it has been found that in most cases if the cells are allowed to stand in sea water for 24 hours between experiments their behavior when measured with sap for a second or third time is quite similar to their behavior in the original measurement. Indeed, instead of failing to rise to a second maximum, the P.D. sometimes actually rises higher than in the first measurement (compare Curves A1 and A2 in Fig. 2). We must conclude, therefore, that with these cells the penetration of KCl into the protoplasm is reversible, and that on standing in sea water the concentration of KCl in the protoplasm returns to approximately its original value. The irreversible change observed in the earlier experiments was apparently due to some more complex type of alteration of the protoplasm. Presumably the cells used in these earlier experiments were in a less vigorous state, corresponding to their lower P.D. with sap outside, and hence were less able to resist alteration. In all cases, irreversible changes in the protoplasm must be expected if the experiment is continued too long, since, as Osterhout has shown, intact Valonia cells live but a short time (as a rule less than a week) if kept immersed in natural or artificial Valonia sap.

An earlier report included a discussion of the sort of (reversible) alteration which might be expected to cause the typical fluctuations in P.D. across the protoplasm of a Valonia cell immersed in Valonia sap. It was concluded that the decrease in P.D. from the initial maximum value followed by a rise to a second maximum can be explained on the simple assumption that this alteration consists merely in an increase in the concentration of KCl in the main body of the pro-

toplasm. In a number of the time curves obtained in recent experiments, however, the P.D. fluctuated in a somewhat more complicated manner as shown in Fig. 2. Here we find the P.D. passing rapidly through two maxima in the first 15 to 20 minutes, then falling to a second minimum and rising slowly to a third maximum. It is interesting to see whether this more complicated behavior can be explained from the same simple assumptions.

![Diagram](image)

**Fig. 3.** Hypothetical diagram illustrating the theory of protoplasmic layers. The length of the arrows indicates the relative magnitudes of the P.D.'s assumed to exist across the inner and outer surface layers. The direction of the arrows is that in which positive current tends to flow. The resultant P.D. (the value observed) is shown by a feathered arrow.

The large P.D. which is observed with the system, sap/protoplasm/sap, led to the conclusion that the protoplasm itself is unsymmetrical; i.e., that its external and internal surface layers are different. In their electrical behavior and in certain other respects these surface layers act like non-aqueous films immiscible with water. As a working hypothesis, the protoplasm is regarded (Fig. 3) as made up of an outer, non-aqueous layer, $X$, the aqueous main body of the protoplasm, $W$, and the inner layer, $Y$.

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and an inner non-aqueous layer, $Y$, different from $X$. The observed P.D. is then the algebraic sum of the E.M.F. across $X$ between the external solution and $W$, plus the E.M.F. across $Y$ between $W$ and the vacuolar sap. For the case of a cell immersed in sea water it is assumed that these E.M.F.'s have opposite signs, $W$ being positive in an external circuit to both the vacuolar sap and the external sea water, and that both E.M.F.'s are large as compared with their algebraic sum. These assumptions are based on the changes in P.D. observed when a cell is killed by the addition of a toxic non-electrolyte to the external sea water under such conditions that $X$ may be supposed to have been destroyed before enough of the toxic substance has diffused through $W$ to produce much injury at $Y$.

In Fig. 4, $X-X$ is a hypothetical curve representing the sort of changes which we may expect in the P.D. across the outer surface of the protoplasm, $X$, when sap replaces sea water as the external solution, if we assume that the only change in the protoplasm is an increase in the concentration of KCl in the aqueous layer, $W$. (We need not consider here the mechanism by which K and Cl are transferred through the non-aqueous surface layers, $X$ and $Y$, whether as ions, undissociated KCl, undissociated KOH and HCl, or in some other form.) The first rapid rise in P.D. caused by changing from sea water to sap is followed by a fall in P.D. as the concentration of KCl at the inner surface of $X$ increases, approaching that at the external surface. Since we may suppose that the concentration of KCl in the main body of the protoplasm, $W$, is initially very low, the penetration of a small amount of KCl into $W$ will at first cause a great decrease in the ratio $\frac{\text{KCl outside}}{\text{KCl in } W}$. As a result, we may expect that the P.D. will decrease at first at a correspondingly rapid rate, but that this rate will fall off as the concentration of KCl in $W$ increases.

Meanwhile, KCl diffusing across $W$ will reach the inner surface of the protoplasm and will produce a similar decrease in the P.D. there. As at the outer surface, this decrease will presumably be very rapid at first, but the rate will then fall off. (The concentration of KCl in $W$ does not necessarily increase at a uniform rate: the entrance of KCl

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from the external solution may be partially offset by diffusion from \( W \) into the vacuole; furthermore, an increase in the concentration of salts in the protoplasm may alter its permeability.) The curve Y-Y

![Graph showing electrical potential differences](image-url)

**Fig. 4.** Curves in which the observed p.d. across *Valonia* protoplasm in contact with *Valonia* sap (Curve A) is represented as the difference between oppositely directed E.M.F.'s at the outer (X-X) and inner protoplasmic surfaces (Y-Y). Differences between the ordinates of Curves X-X and Y-Y are equal to the corresponding ordinates of Curve A directly above. Curve A represents an actual experiment, Curves X-X and Y-Y are hypothetical.
in Fig. 4 shows these hypothetical changes in the p.d. across the inner surface layer of the protoplasm between W and the vacuolar sap. The p.d.'s plotted in X-X and Y-Y have opposite signs as in Fig. 3. The observed p.d. is then represented by differences between Curves X-X and Y-Y. This difference is represented in Curve A (above) the ordinates of which are equal to the difference between the corresponding ordinates of Curves X-X and Y-Y. Curve A, which obviously has the same general shape as the p.d.-time curves in Fig. 2, represents an actual measurement with artificial sap, the observed values being indicated by the ordinates which have been drawn.

It should be emphasized that while Curve A represents an actual experiment, Curves X-X and Y-Y are purely imaginary and may equally well be drawn in other ways. They are intended to show merely that in order to explain the fluctuations in p.d. with sap, we need not assume that the protoplasm suffers any further alteration than an increase in the concentration of KCl. If this explanation is correct, it is apparent from Fig. 4 that the values of the p.d. across the surface layers of Valonia protoplasm must be very large. The actual values are of course unknown; this is shown in Fig. 4 by interrupting the scale of ordinates toward the base by a dotted line, to indicate that below this point the ordinate extends for an indefinite distance.

SUMMARY

Evidence that the inner and outer protoplasmic surfaces in Valonia are unlike is found in the high p.d. across the protoplasm when the external solution has the same composition as the vacuolar sap. Earlier experiments with artificial sap have been repeated, using natural as well as artificial sap. Good agreement between the data with the natural and the artificial solution was found both in the magnitude of the p.d.'s observed and in the shape of the p.d.-time curves. The p.d.'s, however, were considerably higher than the values formerly reported as usual, while the cells proved much less liable to alteration produced by exposure to sap. It is suggested that the cells used in the recent experiments were in a more vigorous condition, perhaps as a result of exposure to stronger illumination.
The interpretation of the shape of the P.D.-time curves, proposed in an earlier report, and based on the theory of protoplasmic layers, is further discussed. It is assumed that the fluctuations in P.D. are due to an increase in the concentration of K in the main body of the protoplasm.