INTERNAL VERSUS EXTERNAL TOXICITY IN VALONIA.

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(Accepted for publication, June 5, 1928.)

Our bioelectric measurements indicate that in *Nitella* and *Valonia* the protoplasm consists of layers, 1 of which the inner is more sensitive to the action of chloroform than the outer (when both are in contact with the same electrolytes at the same concentration). 2

It is a matter of considerable interest to determine the relative sensitivity of these layers by applying a toxic substance to each one separately. The experiments here described were made for this purpose. The cells were divided into two groups. Those of group A were allowed to stand in sea water containing a small amount of the toxic agent so that only the outer layer (X) was directly exposed. In group B sap containing the toxic agent was injected into the cells. 3 Here only the inner layer (Y) was directly exposed. It seems probable that both X and Y are relatively impermeable to the toxic agent here employed (MnCl₂), at least as long as the protoplasm is not severely injured, and if this is the case we may expect death to occur more rapidly when the poison is applied to the more sensitive layer. 4

The experiments were carried out in Bermuda on cells of *Valonia macrophysa* (containing from 0.3 to 1.2 cc. of sap) at about 20-25°C.

It was necessary to find a toxic agent which could be measured with a fair degree of accuracy even in very small amounts. Such organic reagents as chloroform or formaldehyde were ruled out because of analytical difficulties. After a number of trials in which Ni++, Cd++, Cu++, Fe++, Cr++, and Mn++ were investigated our choice fell on the last. From the analytical point of view Mn is excellent, since

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1 It is assumed that there is an inner layer Y and an outer layer X both of which are probably non-aqueous: between them there is an aqueous layer W.


4 But if the protoplasm were freely permeable to the toxic agent it would make little difference whether it is injected into the cell or applied from the outside.
it can be determined to 0.01 mg. with good accuracy. It has, however, several valences (2, 3, 4, 6, and 7). In our experiments it was employed in the form of the bivalent salt, MnCl₂. This may be considered as the salt of a fairly strong base and a strong acid since it undergoes no appreciable hydrolysis. A possible objection is the uncertainty in regard to oxidation. In the sea water the chance of oxidation must be very small. On the other hand we know very little about the oxidation conditions within the vacuole of the cell. We do know, however, that the normal oxidation potentials of the electrodes

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\begin{align*}
\text{Mn}^{IV} & \text{Mn}^{II} + \text{Pt} + 15 \times \text{H}_2\text{SO}_4 & \text{Mn}^{III} & \text{Mn}^{II} + \text{Pt} + 15 \times \text{H}_2\text{SO}_4
\end{align*}
\]

are strongly positive: according to Grube and Huberich over 1.5 plus referred to the hydrogen electrode taken as zero. This indicates such a strong tendency of the higher valence compound to be reduced that it does not seem likely that the cell can oxidize MnCl₂.

It was hoped at the start that we might be able to determine the maximum concentration of Mn⁺⁺ within the vacuole which the cell would tolerate indefinitely. This was not realized. A considerable number of cells were found which fulfilled the conditions required, but on analysis they were found to contain so little Mn that the results were very inaccurate. In 17 out of 20 cases where the cells lived for a long time not more than a trace was found. For a similar reason we were unable to determine very accurately the maximum concentration of MnCl₂ in the sea water that the cell would tolerate indefinitely. The plan was therefore adopted of injecting the cells in group A with sap containing MnCl₂, and allowing them to stand in sea water until dead. Analyses were then made of the cell contents. In this way data were obtained giving the length of life of cells containing 0.004 to 0.185 per cent of MnCl₂ in their sap. In group B the cells (stabbed with a capillary but not injected with MnCl₂) were simply placed in sea water containing MnCl₂ and allowed to remain until dead.

**Technique.**

In a previous paper we have stated that occasionally cells which had been impaled on a capillary and then shaken from the capillary into sea water, healed

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6 "Indefinitely" is here used in a limited sense. We know that cells will live for periods of months in sea water in the laboratory, without showing any change in the protoplasm except a slight fading of the color. In this work "indefinitely" simply indicates a period of a month or more during which there is no more change in the appearance than would be displayed by a cell not exposed to Mn.

7 The sense in which this word is employed is explained on page 215.

up, becoming as turgid as unpunctured cells and continuing to live. This observation formed the basis of our present work. It was found that the proportion of punctured cells which could be made to heal could be very greatly increased by careful handling. Glass capillaries were used, and these were attached to a Luer hypodermic syringe of 2 cc. capacity. The capillaries were considerably finer than those used by us in making the measurements of potential difference. The glass at the tip was extremely thin, and the point was broken off at an angle to facilitate the piercing of the relatively tough cellulose wall. During injection the syringe was filled with sap containing a definite concentration of MnCl₂. The cell was then grasped firmly between the thumb and forefinger and quickly impaled on the capillary and at the same time a slight pressure was applied to the plunger of the syringe. As soon as the cell was in place on the capillary it was relinquished by the fingers, and then a considerable pressure was applied to the piston. At this stage, those cells which showed any sign of leakage around the capillary were rejected. This was done because any severe leak indicated that an undue displacement of the natural sap by artificial sap plus MnCl₂ had probably occurred, and also because when a cell leaked on puncturing there was usually some scouring and tearing of the protoplasm at the site of the leak. This was probably due to the sudden rush of sap out through the minute annular space. Cells so damaged usually failed to heal. The passage of sap + MnCl₂ into the cell could be observed owing to the difference of refractive index of the solutions. It was thus possible to control in a very rough way the amount injected. It is obvious that at the moment of injection the pressure in the vacuole must have been above normal. Nevertheless in only a very few cases (excepting those cells which leaked at the puncture) was there any evidence of rupture of the cell wall.

FIG. 1.

FIGS. 1 and 2. Methods of inserting a hollow steel needle into the cell.
Further it is of interest to note that those cells which showed any sign of such rupture were invariably large, containing 0.75 cc. or more. The actual transfer of the solution from the syringe to the cell took only a few seconds. While the pressure was still maintained the cell was brushed quickly from the capillary into a large volume of sea water.

This method of injection yielded the greatest number of cells which healed. Deflation of the cell was completely avoided. A second method which was tried and abandoned involved the use of a very fine hollow steel needle. This was ground off at the point at a steep angle. The cell was brought to the needle and forced up on it rather slowly. As Fig. 1 shows there was at the start a passage through which sap could escape as indicated by the arrow. This passage was closed as soon as the impalement was complete (Fig. 2). By applying a slight pressure to the piston the loss of sap was made up by the injected solution. The advantage was that no excess of pressure had to be applied to the interior of the cell, and that after practice it was possible to determine the amount of entering solution quite accurately by controlling the speed at which the hole was closed up. This plan, however, involved some deflation of the cell which is often fatal. If the cell wall is creased or folded the protoplasm is likely to become detached at this point, and this disturbance spreads until death occurs. When the cell is transferred from the capillary to the sea water after injection by the first method described, it is obvious that the moment it leaves the tip there must be a fall in the internal pressure by the escape of liquid through the orifice. Undoubtedly the time of outflow would be very short, both because the internal pressure would be high, and the orifice (which may be regarded as a very short capillary of relatively great diameter) would offer very small resistance to the outflow. Hence we should anticipate a great acceleration of the liquid at the orifice but we should not expect any great scouring of the protoplasm at the circumference for owing to the size of the opening the greatest acceleration would be opposite the center of the orifice, decreasing outward to the circumference. This is in marked contrast to the case previously discussed, where the flow was through a tiny annular orifice. Here owing to the smallness of the opening the greatest acceleration would be opposite the edges. It is probable that most of the material which escaped from the cell when the pressure fell was natural sap; for the injected sap plus MnCl₂ had a greater density and would tend to fall to the bottom of the cell away from the site of the puncture. This could actually be observed in certain preliminary experiments where the pink Co ion was injected. The ideal condition would have been to have released the pressure on the piston at the moment the cell left the capillary. This, however, could not be realized in practice and in consequence the exterior of the cell was subjected to a rain of the toxic solution at this time. However, by holding the cell just above the surface of a large beaker of sea water the length of this exposure was cut down and after injection the cell was removed from the beaker at once and washed in a stream of sea water. This served to rock and rotate the cell, and helped to bring about a thorough mixing of the sap and the slightly heavier toxic solution which was injected. The cell was then
placed in a 50 cc. crystallizing dish containing fresh sea water. This was kept covered to prevent evaporation. Each cell was preserved in a separate crystallizing dish to avoid the effect of exosmosis from other cells.

It was recognized that before healing was complete a certain amount of MnCl₂ might escape from the cell by diffusion through the orifice. In order to minimize any effect that this might have on the exterior of the cell, the sea water was changed a few hours after injection and then daily until the cell was healed.

To apply the toxic solution to the exterior the cells were placed in sea water containing varying concentrations of MnCl₂, 1 cell to a dish. Weighed amounts of MnCl₂·2H₂O were added to sea water, but owing to the uncertain water content, the solutions so made up were analyzed for manganese.

It is plain that when the solution is injected into the cell we must consider not only the action of the toxic solution of the layer Y but also the effect of the puncture on the protoplasm. An attempt was made to eliminate the effect of stabbing by trying to determine the percentage of cells dying as the result of the stab alone but this was abandoned in favor of the plan of impaling all cells. All the cells of group B before being placed in the sea water containing MnCl₂ were injected with artificial sap containing no Mn. The possibility of the diffusion of MnCl₂ through the orifice into such cells must be considered. We might have permitted each cell to heal in sea water before placing it in the toxic sea water. But it was felt that this would give an advantage to these cells in comparison with those of group A, which were exposed to the Mn from the moment of injection.

Our experiments were carried out in two series, the following routine being observed. A few cells were injected with sap containing MnCl₂, washed, and placed in separate vessels of sea water, then the syringe was washed out with distilled water and finally with artificial sap. Then the same number of cells were injected with artificial sap, washed, and transferred to separate vessels of sea water containing MnCl₂. Finally a smaller but proportional number of cells were injected with the sap containing no MnCl₂, and these were placed, after washing, in sea water to serve as controls. This process was repeated, until the required number of cells for the series had been obtained. By proceeding in this way we made certain that the cells in each group were injected under exactly comparable conditions.

It was not possible to control, except very roughly, the concentration of MnCl₂ applied to the interior of the cell. A small measure of control could be exercised by varying the concentration of the MnCl₂ in the injected sap. In practice two solutions were used, containing respectively about 0.05 N and 0.25 N MnCl₂. These concentrations represented a compromise. When a solution 0.01 N was injected into 25 cells 18 of them lived for more than 2 weeks without sign of injury. At the end of this period the experiment was terminated. On analysis only 6

9 The needles were of course very fragile and when one broke during the stabbing the cell was rejected.
TOXICITY IN VALONIA

cells showed more than a trace of MnCl₂ present, while in two cases no Mn was detected. On the other hand, when a solution containing 0.1 N MnCl₂ was injected into a group of 9 cells, 8 of them failed to heal and within 48 hours the protoplasm had detached itself from the cellulose wall.

During the first 2 days after injection all the cells were examined carefully for signs of healing. In this respect the behavior of the control cells was of great interest. Many of these showed definite signs of recovery in 6 hours, and in nearly all cases healing was complete (as far as the eye could detect) within 24 to 48 hours. The visible signs of healing are the deposit of a black material in the puncture and the regaining of full turgidity. Most of these cells continued to live indefinitely. A group of such healed cells compared with a group of unpunctured cells kept under similar conditions failed to show an appreciably higher mortality in 20 days. The effect of impalement is apparently shown at once, and if impaled cells heal they will survive almost as well as unpunctured cells. This conclusion is in line with our previous experiments on potential difference in which cells were impaled and left on capillaries in sea water for periods up to a month, without suffering any apparent injury.

We believe that our procedure eliminated the effect of the impalement. All cells which persistently remained soft were rejected, and all cells which showed complete or even partial healing were kept (by partial healing we mean that a few cells showed the deposition of the black material in the puncture but died before they had completely recovered their turgidity).

It was observed that in the presence of Mn a series of changes took place with considerable regularity. Sometime after the sealing of the puncture, the protoplasm showed long thin areas of a lighter color. These widened, and became still lighter or even colorless. This process continued until the protoplasm was without color except for very small dark irregular patches. During these changes the cell retained its usual turgidity. At length it softened abruptly. At this stage the protoplasm either detached itself spontaneously from the cellulose wall or the detachment occurred when the cell was gently rolled between the fingers. These changes took place in both group A and group B when the concentration of the MnCl₂ was high. At lower concentrations the appearance of the lighter areas was either very much delayed or if they appeared early they spread comparatively slowly. In these cases the protoplasm usually detached itself spontaneously and the "colorless" and "soft" stages were absent. Variations of these main types of behavior were also observed. It was therefore necessary to select a stage in the protoplasmic changes to serve as a reference point in comparing the length of

10 About 7 per cent failed to heal. These remained soft and within 24 to 72 hours showed a definite detachment of the protoplasm from the cellulose wall. In many of these cases the protoplasmic structure was greatly altered, and in others on gentle rolling between thumb and forefinger the protoplasmic layer broke up.
life of the cells. Only two points seemed suitable, namely: the first appearance of the light streaks, and the detachment of the protoplasm. The first of these was less desirable for it was not always possible to distinguish slight irregularities present at the start from the incipient streakiness. The detachment of the protoplasm was therefore selected as our reference point. There was no detectable diffusion of Mn$^{++}$ or SO$_7^-$ into the cell (and no diffusion of Mn$^{++}$ from the cell) at any stage preceding the detachment of the protoplasm. A few hours after this had occurred, however, sufficient diffusion of these ions across the membrane had taken place to be readily detected. It would therefore seem as though this is the point at which the protoplasm becomes freely permeable. In the discussion which follows we shall designate cells which have reached this point as "dead."\textsuperscript{11}

**Analyses.**

It was not, of course, possible to tell the exact moment when the protoplasm became freely permeable in cases where its detachment was spontaneous and hence in cells of group A it was necessary to take into account MnCl$_2$ which might have passed out into the sea water by diffusion. In making the analyses, therefore, the sea water was tested for Mn with the delicate reagent potassium periodate. When Mn was detected, care was taken to extract all the sap from the cell. This was done by piercing it with a fairly fine capillary pipette and by applying considerable suction. The external pressure caused the cell to collapse completely, and drove not only the sap but also the detached protoplasm into the pipette. The sap was blown from the pipette into a small glass-stoppered bottle and weighed. A certain amount of inaccuracy was therefore introduced into the analysis since the weight would be too great by the weight of the protoplasm. On the other hand since some of the Mn had passed out into the sea water the weight of sap from this cause was too little. We cannot accurately estimate these errors which tend to compensate each other, nevertheless it does not seem that they could have affected the final results very seriously. After the sap had been weighed it was added to the sea water in which the cell had stood and the solution was evaporated with 0.5 cc. of concentrated HNO$_3$, to oxidize the protoplasm, and 0.5 cc. of concentrated H$_2$SO$_4$, to change all the chlorides to sulfates. As an extra precaution, because of the large amount of chloride present, a second evaporation with H$_2$SO$_4$ was performed. The Mn was then oxidized according to the well known bismuthate method, and compared with standards containing known amounts of Mn.\textsuperscript{12} In the group B analyses, where no appreciable amounts of Mn were detected in the sea water bathing the cells, the sea water was not included for the analysis, and since it was not necessary to empty the cell com-

\textsuperscript{11} See also page 210.

\textsuperscript{12} For details of the analysis see Standard methods for the examination of water and sewage, American Public Health Association, Boston, 5th edition, 51.
pletely, sap free from protoplasm was obtained for analysis. We have not been able to detect in these two sets of group B results any systematic variation comparable with the natural variation of the cells themselves.

**DISCUSSION.**

The effect of the treatment is shown in Fig. 3. For group A (Mn applied externally) the figures were obtained by averaging the length of life of all the cells (usually 18) at each concentration. For group B (Mn applied internally) the length of life of each cell was plotted and a curve drawn free-hand without attempting anything more than a rough fit. The scattering of the points at low concentrations is to be expected since in general the application of dilute solutions of toxic substances gives very irregular results. (In general the controls lived more than a month after being stabbed.)

It is obvious that the cells of group A lived about twice as long as
those of group B. The difference might have been a little greater if
the amount of Mn available for combination with the protoplasm had
been the same in both cases; but where the Mn was applied externally
at the same concentration as in the sap the external volume of solution
was greater (10 to 15 cc. for each cell). Hence if the protoplasm com-
bined with most of the Mn there would be more available in the latter
case but it is not probable that more than a small fraction of the Mn
was taken up by the protoplasm.\textsuperscript{13}

Earlier in this paper we spoke of the possibility of the diffusion of
MnCl\textsubscript{2} from the sea water into the cell before healing was complete.
This might tend to reduce the average life of the cells by subjecting
\(Y\) as well as \(X\) to the action of the toxic agent. This would tend to
bring the curves closer together. Hence any error involved would
not invalidate the conclusion that the injection of the MnCl\textsubscript{2} pro-
duces death more quickly than application to the exterior.

Hydrolysis\textsuperscript{14} of MnCl\textsubscript{2} might affect the results by increasing the
acidity but our tests indicate that this effect is negligible.

If we take the curves as drawn we see that, for example, when
0.0003 \(\text{M}\) MnCl\textsubscript{2} is applied internally the cells live about 12 days; to
shorten the life of the cell to the same extent by an external application
would require about 0.0045 \(\text{M}\), a concentration more than 10 times as
great.

The simplest interpretation might be to regard the inner layer,
\(Y\), as more sensitive than the outer, \(X\), and to suppose that as soon as
the inner layer is sufficiently altered to become permeable to MnCl\textsubscript{2}
the latter diffuses through \(Y\) and attacks \(X\). An attack from the out-
side would be slower because of the greater resistance of \(X\).

An alternative assumption might be that \(X\) is permeable to the
toxic agent and \(Y\) is not, and that the layer which is permeable is not

\textsuperscript{13} It is of course possible that some Mn may combine with the cell wall in such
a way as to be removed from the sphere of action but it does not seem probable
that this is a factor of importance, especially as each cell was placed in a sepa-
rate dish containing 10 to 15 cc. of solution.

\textsuperscript{14} The hydrolysis of MnCl\textsubscript{2} appears to have been measured only once. Thus
Kullgren (\textit{Z. physik. Chem.}, 1913, lxxxv, 473; see Landolt-Börnstein, Physikalisch-
chemische Tabellen, Berlin, 5th edition, 1923, ii, 1170) found by the sugar inver-
sion method that a 0.25 \(\text{M}\) MnCl\textsubscript{2} solution at 100°C. was hydrolyzed only 0.0017
per cent.
injured by the MnCl₂ which diffuses through it so that when the toxic solution is injected Y will be attacked at once, but X will not be injured until after the alteration of Y.₁⁵ When the toxic solution is applied to the outside of the cell Y will be subject to attack only after the toxic substance has diffused through X.

Even if there are differences of permeability it is probable that we also have differences of sensitivity as well to deal with. The assumption that X and Y are unlike is in harmony with our previous work on potential differences across the protoplasm of Valonia₁⁶,₁⁷ and with experiments on the marine alga Griffithsia₁⁸ and with the investigations of de Vries₁⁹ in so far as they may be interpreted to mean that the inner and outer surfaces do not act alike. But the statement of Höber²⁰ that the inner and outer surfaces of the protoplasm may be unlike seems to apply rather to a difference between the inner and outer surfaces of X.

It is probably not worth while to attempt to compare our results with those of Chambers and his coworkers who have in some cases found much less toxicity when a substance is injected into Amoeba than when applied to the outside,²¹ since in these cases the toxic substance was not injected into a pre-existing vacuole as in the case of Valonia where the mechanism involved may be quite different.

Throughout this part of the discussion the destruction of either layer is considered to be equivalent to death.


₁₇ Experimental evidence seems to indicate that X is actually permeable to certain ions, while Y may not be.

₁₈ Osterhout, W. J. V., Science, 1913, xxxviii, 408.


SUMMARY.

When MnCl₂ is injected into the cells of *Valonia macrophysa* they live only about half as long as when the same concentration is applied to the exterior of the cell. This is due to toxic action and not to the mechanical disturbance accompanying the injection (since all cells were stabbed in the same manner by the capillary).

A variety of explanations are suggested, all of which involve a difference between the inner and outer layers of the protoplasm.