

## THE REACTIONS OF HALICYSTIS AND OF VALONIA TO INJECTIONS OF CERTAIN PROTEINS

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During the months of February and March, 1932, the writers injected several hundred specimens of *Halicystis Osterhoutii* Blinks and Blinks, and of *Valonia macrophysa* Kütz with solutions of certain proteins, with the dual purpose of learning the tolerance of these algae to the presence of proteins in the vacuole and of determining whether antibodies are formed.<sup>1</sup>

After numerous trials, it was found that the following procedure was essential to success in making the injections. Micro pipettes were drawn out, having an end with a diameter of not over 0.02 cm., passing to a diameter of 0.1 cm. at a distance of about 0.6 cm. from the end, and then rapidly enlarging to 0.2 cm. The pipettes were fitted with rubber bulbs, and were inserted quickly to this point of enlargement. Both the cell wall and the protoplasmic membrane were thus penetrated, leaving the end of the pipette in the midst of the vacuole something over half a centimeter beyond the cell wall. With a little practice, this could be done without allowing the liquid from the vacuole to escape around the sides of the pipette or into the pipette,—the latter point being determinable by the constancy of the height of the liquid. After the insertion of the point of the pipette in this manner, it could be withdrawn slightly and the pressure on the rubber bulb increased sufficiently for a definite amount of liquid to be delivered, the displaced liquid of the vacuole escaping around the walls of the pipette. When this procedure was followed with due care, the pipette could be withdrawn without the admission of air or the further escape of liquid from the vacuole. It is impossible to say, of course, just how much of the injected liquid took a straight course from the end of the pipette and escaped during the manipulation; but from tests using dyes, the amount is believed to be negligible. It is also believed that the amount of liquid lost after the return of the cells of the algae to sea water was very small.

<sup>1</sup>This work was done at the Bermuda Biological Station for Research. We wish to express our thanks to the Director, Dr. J. F. G. Wheeler, and to Mr. W. Gleeson for their many courtesies, and for their aid in obtaining material.

The cells remained turgid if properly operated upon; and within 5 minutes after the operation, it took distinct pressure with the fingers to force the cell sap out. Within 24 hours, the wound was completely healed, microscopical examination showing a discernible wound callus of the material of the cell wall underlaid by protoplasm.

The pipettes used were calibrated to deliver 0.02 cc., 0.01 cc., and 0.005 cc. at 20°C. This calibration, sufficiently accurate for the purpose, was done by delivering 0.01 cc. or 0.02 cc. of aqueous gentian violet from a standardized pipette on a dry glass slide, and then calibrating the micro pipettes by the amount delivered that would make a drop of the same size.

The solutions used on *Halicystis* were made in sea water, since the liquid in the vacuoles of this species has about the same salt concentration as sea water. The solutions used on *Valonia* were made in "Valonia artificial sap," as determined by Osterhout (1931). This artificial sap consisted of NaCl 2.632 gm., KCl 18.65 gm., and CaCl<sub>2</sub> 0.0944 gm. made up to 500 cc. with distilled water. Controls of both *Halicystis* and of *Valonia* were made by injecting with sea water and with artificial sap, respectively.

Somewhat more than 100 cells of each species were injected, not counting those manifestly injured, before the injection technique was perfected to a point where less than 10 per cent of the controls of *Halicystis* died during an observation period of 1 week. The work reported is concerned only with what was done subsequently.<sup>2</sup>

The cells used varied in volume from 0.25 cc. to 4.0 cc.; but 80 per cent of them had volumes between 0.8 cc. and 1.8 cc., the cells of *Halicystis* averaging a little larger than those of *Valonia*. The quantity of liquid injected into the cells having volumes varying from 2.0 cc. to 4.0 cc. was 0.02 cc., thus giving percentages within the vacuoles ranging from 0.5 to 1.0. The remaining cells ordinarily were given injections of 0.02 cc., 0.01 cc., and 0.005 cc., according to size. The solutions injected, therefore, usually formed between 0.7 per cent and 1.1 per cent of the contents of the vacuole. Occasionally, injections of double these amounts were given.

Solutions of 1.0 per cent Difco "bacto-peptone," which had 2.72 per cent ash, 14.92 per cent animal peptone nitrogen, and 0.24 per cent animal proteose nitrogen—a preparation obtained through the kindness of Mr. H. G. Dunham of the Difco Laboratories—were given to both types of cells. About 50 cells of each species were operated upon. No effect was detectable over a period of observation of 10 days, though slightly more *Valonia* died than with the controls.

Similar injections with Difco "bacto-protone" having 2.18 per cent ash, 12.23 per cent animal proteose nitrogen, and 2.67 per cent animal

<sup>2</sup> The one exception to this statement is the experiment with injections of proteose.

peptone nitrogen, induced different reactions. The *Halicystis* cells were affected but little. About 25 per cent died within 10 days, as compared with 15 per cent in the controls made at the same time; but the remainder appeared to be in perfect condition at the end of an observation period of 2 weeks. The injected *Valonia* cells, on the other hand, all died within 2 days; while 80 per cent of the controls lived. This effect is in marked contrast to the behavior of *Valonia* when exposed to the action of Difco "protone" on the outside of the protoplasmic membrane, the cells being very tolerant to the action of the protease under these conditions.

Three sets of *Valonia* were injected with solutions containing 1 part to 800 (Series A), 1 part to 8,000 (Series B), and 1 part to 80,000 (Series C) of crystallized egg albumen. Injections of artificial sap (Series D) were used as controls. The casualties are shown in the table.

Series	No. injected	No. dying on designated day after injection														No. alive
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
A	57	2	6	21	10	0	0	0	2	1	0	0	0	1	0	14
B	60	2	4	10	11	10	7	5	2	0						9
C	55	2	3	7	9	5	3	0	5	1						20
D	55	2	4	6	8	4	2	1	2	1	0	0	0	1	0	24

Obviously, *Valonia* is adversely affected by the rough treatment experienced during the injections. The death rate is high. But there appear to be significant differences in the trends of the mortality curves and in the percentage of cells remaining alive during the period of observation in the four series. In Series C (1:80,000 egg albumen) and Series D (control) the results are sufficiently similar so that one may consider them to be random samples of a single population. Traumatic shock is probably the primary cause of death. In Series A (1:800 egg albumen) and Series B (1:8,000 egg albumen) there is an additional cause of death. Presumably this cause is the toxicity of egg albumen in the higher concentrations. It is certain that a relatively high percentage of egg albumen was present in the cell sap, for several tests of dead cells showed that a minimum of 0.5 cc. would show albumen by the Spiegler test and that a minimum of 1.0 cc.

would show albumen by the heat test. It should also be noted that, though in each series about the same number of individuals died on the 1st and the 2nd days, during the next 3 days the number of deaths was thirty-one for Series *A*, thirty-one for Series *B*, twenty-one for Series *C*, and eighteen for Series *D*. A death rate of this type, with a lag of a few days followed by a rapid rise, is the characteristic effect of vegetable albumens such as ricin when injected subcutaneously into laboratory animals. The cells of Series *A* and *B* that remained alive until the end of the observation period may have been more tolerant to the foreign protein, but it is somewhat more probable that they actually retained smaller amounts of the injected material.

The nine cells of Series *B* remaining alive on the 10th day after injection were given a precipitin test in order to find out whether antibodies had been formed. The sap plus protoplasm was syringed out with gold-neededled tuberculin syringes, centrifuged for 15 minutes, and the supernatant liquid layered carefully on egg albumen in "artificial sap" 1:800. No trace of a precipitin ring was formed.

The fourteen remaining cells of Series *A* and the 20 remaining cells of Series *C* were reinjected with egg albumen 1 part to 800, for the purpose of determining any possible development of increased tolerance—with or without the production of antibodies, or of hypersensitiveness. The cells of Series *A* were thus reinjected 14 days after the first injection, while those of Series *C* were reinjected 9 days after the first injection. The casualties are shown in the table below, all the records except those of the first few days being taken by Mr. W. Gleeson.

Series	No. injected	No. dying on designated day after second injection																							No. alive
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
<i>A</i>	14	2	0	0	1	1	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	7
<i>B</i>	20	2	1	3	1	0	1	2	2	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	4

Unfortunately, the controls surviving from the first injection were discarded by mistake, and therefore were not available for comparison. Despite this deficiency, it is clear that the records show nothing resembling anaphylaxis after the second injection, and no markedly

increased power of resistance. There is a somewhat higher proportion of survivors in Series *A*, which had previously had the more concentrated dosage of 1:800 albumen, than there is in Series *C*, which had had only a dosage of 1:80,000 albumen. There is also the further suggestion, in the death rate frequencies involved, that traumatic shock alone accounts for practically all the deaths, since there is not the sharp rise in mortality after a short "incubation" period that characterizes the effect of vegetable albumens on animals and that is found in our own first injections where concentrated solutions were used. It would be necessary to deal with a much larger population before one could attach much significance to these indications, however, both because sampling errors are highly probable and because the survivors of the first injection may have been subject to selection.

The sap and protoplasm from the surviving cells in Series *A* and *C* were shipped from Bermuda to Boston in sealed tubes and tested for precipitin reactions by Dr. W. G. Malcolm of the Massachusetts State Antitoxin and Vaccine Laboratory. Dry egg albumen (Merck) 1 part in 100 parts of physiological salt solution was used as antigen. The sap plus protoplasm centrifuged was used in physiological salt solution dilutions by powers of 2 from 1:1 to 1:512. The tubes were incubated 5 hours at 37°C. There were no precipitin reactions shown either here or in controls of untreated cells.

Eighty-five cells of *Halicystis* were injected with crystallized egg albumen 1 part to 800 parts sea water on March 14. After 24 hours, the sap from six cells was used for an albumen test. The Spiegler test was plus 3. 10 days after injection, the sap and protoplasm from eight cells, centrifuged, were used for a precipitin test with egg albumen in sea water 1:1 as antigen. A plus 1 ring was obtained which remained visible for 60 minutes and then faded; but as the upper layer of sap and protoplasm was slightly cloudy, we are not inclined to conclude that there was antibody formation. Of the 71 injected cells remaining, one died after 48 hours. There were no other casualties during a 34 day observation period. The sap and protoplasm of these cells were shipped to Boston and tested for precipitins by Dr. Malcolm, as described above. The test was negative. A small number of control injections were made on *Halicystis*. Out of twenty cells injected with sea water, one died.

It seems reasonable to conclude from these experiments that *Halicystis* shows a high resistance to traumatic shock and a virtually complete tolerance to egg albumen in the concentration of 1:800 when amounts are injected forming between 0.7 per cent and 1.1 per cent (occasionally 2.0 per cent) of the contents of the vacuole. There is no indication, except possibly in one test, that antibodies are formed as a reaction. It appears probable that practically all the albumen is retained within the tonoplast and does not come in contact with the protoplasm in sufficient quantities to cause disturbance; though it is possible that minute quantities may be broken down and utilized as food, or even that minute quantities, as albumen molecules, may egress through the membrane.

In contrast to *Halicystis*, *Valonia* exhibits a low resistance to traumatic shock. It also shows an apparent tolerance to egg albumen in the concentration 1:80,000. In the higher concentrations of albumen, 1:8,000 and 1:800, however, there is evidence of the toxicity of the injected material which manifests itself by a rapid rise in the death rate on the 3rd, 4th, and 5th days after injection. When the survivors of the first injection are again injected with albumen in the concentration 1:800, there is some indication that the toxic effects are less marked.

*Halicystis* having been observed to have a very high tolerance and *Valonia* to have a very low tolerance to diphtheria toxin when exposed to its presence in sea water, a number of injections were made with sea water (for *Halicystis*) and "artificial sap" (for *Valonia*) containing 1.0 M. L. D. per cc. Of *Halicystis*, twenty-four cells were injected (Series *E*); of *Valonia*, twelve cells were injected (Series *F*). The casualties were as follows:

Series	No. injected	No. dying on designated day after injection										No. alive
		1	2	3	4	5	6	7	8	9	10	
<i>E</i>	24	0	0	20	2	0	0	0	0	0	0	2
<i>F</i>	12	0	0	12	—	—	—	—	—	—	—	—

The figures in this table show, in a marked degree, the delay in the fatal action of diphtheria toxin which is so characteristic of its effect upon susceptible animals.

It is evident that something occurred here that was far more definite and striking than our usual experience with injected cells. *Valonia* is susceptible to a rather high death rate from traumatic shock, as shown by our other records. But it was the common experience to have about 5 per cent die from acute shock during the first 24 hours, and then to have a rise in the death rate between the 3rd and 5th day, at which times the death rates ranged from 10 to 40 per cent. In this case, however, sudden death overtook the entire population on the 3rd day.

A similar situation is evident in the case of *Halicystis*. *Halicystis* is highly resistant to traumatic shock, as witness the 71 cells injected with egg albumen, of which only one died in 34 days. In contrast, twenty out of twenty-four *Halicystis* cells died suddenly on the 3rd day after injection, and two more were found to be dead on the morning of the 4th day. It is true that two of these injected cells remained alive at the end of the 10th day (when discarded); but one cannot be certain that they were exposed to the same conditions. It is possible that they were tolerant to the diphtheria toxin, but it appears to be more probable that the injected material was lost.

There was not sufficient *Halicystis* material available to make a more extended test of the effect of this preparation. It was possible, however, to make a second test on *Valonia*. Accordingly, 92 cells of *Valonia* were injected with diphtheria toxin dissolved in "artificial sap" in the same proportion as before; *i.e.*, 1 cc. of solution contained 1 M. L. D. of toxin. The results for the first 10 days were as follows:

No. injected	No. dying on designated day after injection										No. alive
	1	2	3	4	5	6	7	8	9	10	
92	1	3	5	3	45	0	7	3	0	3	22

From the 11th to the 16th days, inclusive, there were five casualties,—one each day with the exception of the 12th day. Subsequently there was but one casualty from the 17th to the 40th day.

Here, again, there was an extraordinary rate of death during the first few days, culminating in the death of half the population on the 5th day. The death rate peak's coming on the 5th day instead of the 3rd day can possibly be ascribed to the fact that the air temperature

was several degrees lower than it was when the first series of *Valonia* was injected.

There were sixteen cells remaining alive at the end of 40 days of observation. It is, of course, possible that these cells received no toxin; but we are not inclined to accept this explanation, (a) because they were the last cells injected, and the technique used was the best we were able to devise, and (b) because all the cells, including those which finally survived, gave definite evidence of "sickness" between the 4th and the 8th days. It is more probable that the surviving cells received smaller doses,—that is to say, sublethal doses of the toxin. It is known that the cells dying on the 5th day received quantities of toxin detectable by chemical means, for extracted sap gave definite biuret reactions, something that uninjected cells never do.

The sap and protoplasm from these sixteen cells—and also sap and protoplasm from *Valonia* (two injections) and *Halicystis* (one injection) injected with egg albumen—were brought to Boston and used—after centrifuging—by Dr. Malcolm of the Massachusetts State Antitoxin and Vaccine Laboratory for intradermal injections into guinea pigs. The untreated saps of *Halicystis* and of *Valonia* were used as controls. 0.1 cc. injections were made. There was immediate reaction. A marked erythema appeared which had the appearance of extreme congestion. After 24 hours, a lesion was observable with moderate to severe edema. The lesions were well circumscribed, with moderate congestion of the outer zone, and necrosis at the center. All the tests, including controls, were similar. Thus, while one can say that the sap from the vacuoles of *Halicystis* and from *Valonia* produces marked necrotic lesions when injected under the skin of normal guinea pigs,—reactions that can hardly be attributed to the amount of salts contained,—there is no evidence that diphtheria antitoxin was produced as a reaction to the injections of diphtheria toxin. One cannot determine from these tests, in fact, whether or not the surviving *Valonia* cells injected with diphtheria toxin did, in actual truth, receive it.

#### SUMMARY

It is shown (1) that *Valonia* and *Halicystis* cells exhibit varying degrees of tolerance to injections of animal peptone, animal proteose,



crystallized egg albumen, and diphtheria toxin; (2) that *Valonia* cells display decreased tolerance to egg albumen in increasing dosages, although *Halicystis* is completely tolerant of the highest dosage used; (3) that the mortality curves of *Valonia* injected with egg albumen and of both *Valonia* and *Halicystis* injected with diphtheria toxin show the delayed effect characteristic of laboratory mammals when treated similarly; (4) that *Valonia* cells injected twice with egg albumen exhibit no change in susceptibility to its effects; and (5) that neither species of algae gives evidence of having formed antibodies against the antigens used.