REACTIONS OF VALONIA AND OF HALICYSTIS TO COLLOIDS

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It would be foolish to deny that protoplasmic surfaces are usually impermeable to colloids. And it is reasonable to assume that the size of the molecular aggregate plays an important part in this relationship. But it does not seem wise to close one's mind to the possibility that, under certain conditions, healthy protoplasmic membranes may allow the passage of specific colloids in amounts that are ordinarily not detectable by chemical methods. We must not forget that collodion capsules that will not permit the passage of colloids in quantities detectable by the best chemical procedure, can be proved to be semipermeable by serological means. Thus agglutinogens, in tested collodion capsules, when placed in the body cavities of laboratory animals, produce agglutinins (See R. Paltauf).

Along one line of attack, the passage of proteins through the membrane or membranes separating the maternal from the fetal circulation, a considerable literature exists, which has been summarized recently by Ratner, Jackson, and Gruehl (1927). Various investigators have shown that the placenta is permeable to different proteins, such as antitoxins, precipitins, bacteriolysins, etc., in man, guinea pigs, and rabbits, though the placenta is not permeable to such substances in cows, goats, and sheep. Ratner, Jackson, and Gruehl interpret these conflicting results by pointing out that in man and in Rodentia a single cell membrane separates the circulations of mother and fetus, while in Ruminantia there are three cell layers.

Along another line of attack, there is the immense collection of facts connected with allergic manifestations on the skins of different animal species,—facts which can hardly be interpreted unless one

admits that proteins exert specific effects on the membranes of living animal cells.

Admittedly, such phenomena are hard to explain on the basis of our present knowledge, in the face of determinations of diffusion coefficients for proteins such as 0.059 for egg albumen and 0.014 for diphtheria toxin. But the chemistry of living matter is complex; and we are still comparatively ignorant of its fundamental theorems. We know little concerning the chemical rules at the point where solutions pass into colloidal systems, of the effects of mixed colloids, of the behavior of mixtures of electrolytes and non-electrolytes, of dispersing agents, of coagulating agents, of the limitations of the Donnan equilibrium, of previous activity (hysteresis), etc.

In this connection, some observations on *Valonia macrophysa* Kütz and *Halicystis Osterhoutii* Blinks and Blinks, made during February and March, 1932, at the Bermuda Biological Station for Research,[1] may be of interest. These two large-celled coenocytic algae, possessing protoplasmic membranes from 5μ to 8μ thick, are well known because of the extended use that Osterhout and his colleagues have made of them in physiological researches. The permeability of their protoplasmic membranes to crystalloids is probably more accurately known through this work than are the permeabilities of the cells of any other organisms.

These cells were collected in quantity, cleaned carefully, and kept in deep battery jars filled with sea water (pH by Hellige = 8.4), in diffuse light, — dead specimens being removed each day.

Cells used for experiment had been collected at least a week, and were apparently healthy unless otherwise noted. Whether they were actually healthy or not is unknown. Both species are easily disturbed by changed conditions, *Halicystis* being the more delicate. This disturbance can be measured accurately by changes in their electrical properties; but since the apparatus necessary for such measurements was not available to us, all we can say is that the normal death rate, under the laboratory conditions used, was about 1 per 1000 daily for

[1] The writers wish to express their gratitude to Director J. F. G. Wheeler and to Mr. W. Gleeson for many kindnesses, including much time spent in the collection of material; and to Dr. W. J. V. Osterhout and Dr. L. R. Blinks for advice on the care of the algae used.

Valonia and about 1 per 2000 daily for *Halicystis* after the first week subsequent to collection, provided dead cells were removed from the containers at least once a day.

The death of *Valonia* cells manifests itself in three ways. (*a*) In about 2 per cent of the cases, color is wholly lost in about 4 hours, without pseudoplasmolysis. The color due to chlorophyll simply disappears, the chloroplasts meanwhile retaining their shape. After the bleaching occurred, the chloroplasts were usually devoured by a protozoan ($5\mu \times 2\mu$) which could not be stained for study in either gentian violet or hematoxylin. (*b*) In about 8 per cent of the cases, the protoplasm ceases to function properly, the chloroplasts slide together without bleaching immediately, and bare spaces appear where the cell wall is not underlaid with protoplasm. (*c*) The characteristic manner in which about 90 per cent of the *Valonia* cells deteriorate, however, is a sort of pseudoplasmolysis, in which the protoplasm gradually detaches itself from the cell wall, shrinking to about 1/25 of the bulk of the cell in approximately 12 hours. When this pseudoplasmolysis is complete, the cell wall has usually retained its rigidity; but it loses this characteristic within the next 6 hours.

Halicystis cells practically always deteriorate by the second method. They often became flaccid immediately after collection but regained turgidity within 6 hours if they lived.

There was a high death rate for several days after collecting in the case of the *Valonia* cells, due to handling and changed conditions, closely correlated with the size of the cell. After about 4 days, the death rate dropped sharply and remained almost constant.

1. The Reactions of Valonia and of Halicystis to Peptone.—There was apparently some penetration of peptone, as shown by the following experiment.

The peptone used was prepared at the Difco Laboratories (Lot 23368) and was obtained through the courtesy of Mr. H. G. Dunham. It contained 2.72 per cent of ash, 14.92 per cent of nitrogen in the form of peptone (*i.e.*, not precipitated by ammonium sulfate, though part may be amino acids), and the remaining nitrogen, 0.24 per cent, in the form of proteose. The product is of animal origin, but its exact source has not been divulged. Various lots of *Valonia* and of *Halicystis* were allowed to remain indefinitely in solutions of 1 per cent and of 2 per cent of this peptone, dissolved in sea water. The pH of each solution was 7.6, as tested by Hellige apparatus. The Posner biuret test was ++.

Neither *Valonia* nor *Halicystis* appeared to be noticeably affected by this environment. The death rates were only slightly higher than for controls during the 10 day observation periods used. Longer records were impossible because the peptone formed an excellent medium for the growth of a species of bacterium[2] which produced jelly-like cultures of such density that the water always became cloudy and gave off a rank odor by the end of the 10 day period. Such deleterious effects as were observed here were probably due to the bacteria rather than to the peptone, for a similar rise in the death rate was observed when the bacteria were planted in pure sea water, although the bacterial density was here much smaller. At all events, it was observed that both *Valonia* and *Halicystis* were able to withstand this treatment for 10 days, and were often able to live indefinitely when returned to sea water.

Twelve healthy *Halicystis* cells and a like number of *Valonia* cells, or small clusters of medium size, were placed in a 2 per cent solution of peptone in sea water and allowed to remain 6 hours. They were then thoroughly washed in sea water and dried carefully between filter papers. The *Halicystis* cells were apparently unaffected; the *Valonia* cells lost a little of their normal turgidity by this treatment. Six cells of each species were then tested for protein by Posner's modification of the biuret test. The test was made in this manner. The cells were placed in centrifuge tubes, punctured with gold needles, and centrifuged. The clear contents of the vacuoles were used. The results were: *Halicystis* ++, *Valonia* +, controls of each species —. The other six cells were replaced in sea water for viability observations. The *Valonia* cells gradually died, the last one succumbing during the fourth week. At the end of 3 weeks, all the *Halicystis* cells were still healthy.

This experiment was subsequently repeated, using a 1 per cent solution of peptone in sea water and an exposure of 2 hours. The biuret tests were negative for both species. The cells not used for the tests

[2] These bacteria frequently contaminated our dishes when ordinary laboratory finger-bowls were used as containers. No such difficulty was encountered with the use of battery jars. The bacterium thus appears to be highly aerobic. [Dr. Osterhout informs us that Dr. Blinks and he have observed growth of this bacterium in battery jars and in stoppered bottles.]

were returned to sea water and observed for 4 days. All remained alive at the end of that time, though they did not appear to be in perfect condition.

2. The Reactions of Valonia and of Halicystis to Proteose.—There was apparently some penetration of proteose, as shown by the following experiment.

The proteose used was the "bacto-protone" of the Difco Laboratories (Lot 23425), also received from Mr. Dunham. It consists almost entirely of proteoses of animal origin,—there being 12.23 per cent nitrogen in the form of proteose and 2.67 per cent nitrogen in the form of peptone. Various lots of *Halicystis* and of *Valonia* cells were exposed, indefinitely, to 1 per cent and to 2 per cent solutions of this proteose in sea water. The solutions showed a slight cloudiness, and were filtered before use. Each solution had a pH of 7.0, and was ++ to the Posner biuret test. As in the case of the peptone, both species exhibited a high degree of tolerance to the medium. The degree of tolerance to proteose appeared to be somewhat greater than to peptone. This effect may be due to the small amounts of amino acids that were probably present in the peptone and not in the proteose; or, it may be due to the fact that the proteose did not form quite so good a medium for bacterial development. The fact remains that the more resistant cells of both species will tolerate either 1 per cent or 2 per cent proteose in sea water for 10 days, and will live in an apparently healthy condition afterward when returned to sea water.

As in the earlier experiment, twelve healthy *Halicystis* cells and twelve cells, or small clumps, of *Valonia* were exposed for 6 hours to the action of 2 per cent proteose in sea water. They were then washed thoroughly and dried between filter papers. Half of each type were returned to sea water for viability observations, and the vacuole sap of the other half was tested for protein as before. The Posner biuret tests were: *Halicystis* ++, *Valonia* +, controls of each species −.

The six *Valonia* cells replaced in sea water gradually died. At the end of 4 weeks, none remained. The *Halicystis* cells, however, were all apparently healthy at the end of 4 weeks.

A repetition of this experiment with 1 per cent proteose in sea water, and an exposure of 2 hours, gave negative Posner biuret tests. The unused cells were observed for 4 days. The *Halicystis* cells remained normal in appearance. This was also true of the *Valonia* cells, with one exception. The largest cell showed pseudoplasmolysis at the end of 48 hours.

3. The Reactions of Valonia and of Halicystis Cells to Egg Albumen.—No evidence was obtained for the penetration of egg albumen.

Crystallized egg albumen, received through the courtesy of Dr. F. P. Underhill of Yale University, was used in these tests. A solution in sea water was first made. As nearly as could be determined, this was about 1 part egg albumen to 800 parts sea water. This solution was diluted 1:100. The cells were exposed, then, to egg albumen 1 part in 80,000 parts of sea water. This solution gave a strong reaction for albumen in heat tests, Spiegler tests, and Tanret tests. Both the *Valonia* cells and the *Halicystis* cells appeared to be completely tolerant of this solution.

Valonia and *Halicystis* cells exposed to a 0.125 per cent solution of egg albumen in sea water for 2 hours, and then washed thoroughly and dried between filter papers, had their vacuole sap tested for albumen by both the Spiegler and the Tanret methods. All tests were negative. One may say, therefore, that if albumen were present, it was in a lower concentration than 1 part per 200,000.

Observations were made of the unused cells for 4 days. Only one *Valonia* cell died.

4. The Reactions of Valonia and of Halicystis Cells to Diphtheria Toxin.—There was no evidence for the penetration of diphtheria toxin, nor for the production of antitoxin; but *Valonia* cells were killed, while *Halicystis* cells were highly resistant to its action.

The diphtheria toxin used was prepared at the Massachusetts State Antitoxin and Vaccine Laboratory (No. 46, M. L. D. = 0.006). It had a pH of between 8.0 and 8.2. As before, twelve medium sized *Valonia* cells, or clumps, and twelve medium sized *Halicystis* cells, in separate containers, were exposed indefinitely to the action of this product. The strength of the solution used was 1 cc. of toxin to 150 cc. of sea water, — 1 cc. thus containing 1.0 M. L. D. The *Valonia* cells exhibited a very low resistance. They began to deteriorate within 12 hours. All were dead within 30 hours. In contrast, the *Halicystis* cells were highly resistant. They appeared to be entirely normal during 19 days of observation.

Comparable cells of *Valonia* and of *Halicystis* were exposed to the action of diphtheria toxin in sea water solutions containing 1.0 M.L.D., 0.1 M.L.D., and 0.01 M.L.D. per 1 cc., respectively, for 6 hours. After exposure, the cells were washed carefully in sea water, the vacuole sap carefully withdrawn with tuberculin syringes, using separate syringes and separate 26 gauge gold needles for each species. The samples of

sap were placed in vials, stoppered with cellophane-covered corks, and placed in a refrigerator for transportation to Boston for antitoxin determinations.

The sap and protoplasm from these cells, together with similar extracts from untreated cells as controls, were tested for the production of antitoxin by Dr. W. G. Malcolm of the Massachusetts State Antitoxin and Vaccine Laboratory. 0.1 cc. injections were made intradermally on guinea pigs. Marked erythema, with extreme congestion, appeared immediately. In 24 hours, well circumscribed lesions appeared, showing moderate congestion in the outer zone and necrosis at the center. Extracts from untreated cells produced reactions identical with those from cells exposed to the action of diphtheria toxin. Thus it appears that no antitoxin was formed. One cannot conclude that no toxin passed the protoplasmic membrane, however, for tests of cells injected with toxin were also negative for the production of antitoxin.

5. The Reactions of Valonia and of Halicystis Cells to Yeast Nucleic Acid. In this case there was an injurious effect.

The yeast nucleic acid was a concentrated preparation made by Dr. Levene and obtained through the kindness of Dr. Underhill. As in the other experiments, twelve cells, or clumps, of each of the two algae were exposed indefinitely to the action of this material dissolved in sea water. The amount of yeast nucleic acid used was 0.375 gm. per 150 cc. of sea water. To obtain solution, 7.0 cc. of 2 per cent NaOH were required. The solution was then filtered. Pseudoplasmolysis began in the largest *Valonia* cells on the 4th day. The small cells remained normal until after the 10th day. By the 18th day, all the cells had disintegrated. The *Halicystis* cells appeared to be normal on the 10th day, but began to deteriorate immediately thereafter. These cells, also, had disintegrated by the 18th day.

No tests were made for permeability.

6. The Reactions of Valonia and of Halicystis Cells to Edestin.—In these experiments *Valonia* was more resistant than *Halicystis*.

The edestin preparation, a globulin of the hemp seed, was made by Dr. F. P. Underhill. It was used in 1 per cent solution in sea water. The solution was somewhat milky, and was filtered before using. The filtrate gave a precipitate with acetic acid. Again, twelve cells, or clumps, from each species were exposed indefinitely to the action of the solution. The *Valonia* cells proved to be highly resistant to the action of this material. The largest cell showed signs of disturbance

after 2 days and died at the end of 4 days. At the end of 10 days, two more buds showed signs of disturbance. The remainder were apparently normal, though perhaps showing slight signs of disturbance at the end of 18 days. The *Halicystis* cells, on the other hand, went to pieces at the end of 2 days.

No tests were made for permeability.

As a control for these six experiments, twelve *Halicystis* cells and twelve *Valonia* clumps, similar in size to those used previously, were given identical treatment in cleaning and were placed in sea water for viability observations. The type of container was the same as in the other experiments, and the amounts of water identical. The *Halicystis* cells were apparently normal at the end of 18 days. The *Valonia* clumps, containing forty-three cells in all, remained healthy until the 10th day. Between the 10th and the 18th day, three cells died, but the remaining forty cells were still normal at the end of the observation period.

Permeability of Valonia Cells and of Halicystis Cells to Proteose from the Scarlet Runner Bean

Some evidence for penetration was obtained in this case.

The proteose of the scarlet runner bean used was extracted by Mr. Joseph Cianciarulo of the Massachusetts State Antitoxin and Vaccine Laboratory by the method of E. C. Schneider. The preparation contained 2.475 per cent of nitrogen.

This proteose has the power of agglutinating human erythrocytes. Its strength was tested as follows: A, heated to coagulate protein; B, unheated. Various dilutions were made in isotonic salt solution, and 1.0 cc. of each dilution was added to 1.0 cc. of 1 per cent suspension of human erythrocytes also in isotonic salt solution. The suspensions were allowed to stand in 15 cc. centrifuge tubes in a room temperature of 19°C. The readings given below were made at intervals of 3 hours and of 6 hours, respectively.

Dilution.....	1:20		1:200		1:2000		1:20,000		1:40,000		1:80,000	
Reading.....	A	B	A	B	A	B	A	B	A	B	A	B
3 hrs.	+	−	+	+	++	++	+++	+++	+++	+++	0	+++
6 hrs.	++	−	+++	+++	++++	++++	++++	++++	++++	++++	+++	++++

Apparently normal *Valonia* cells (no *Halicystis* available at the time) were transferred to sea water containing 20 per cent bean proteose (pH 7.0). At the end of 4 hours' exposure, the cells, which still appeared to be normal, were washed carefully, the sap withdrawn into clean, gold-needle tuberculin syringes, and tested against human erythrocytes. Equal amounts of sap and of 1 per cent suspension of fresh blood in isotonic salt solution were used. At the end of 3 hours,

there was definite agglutination of the erythrocytes, with clumps adhering to the sides of the tube. There was no agglutination in the control of salt solution plus blood suspension.

This experiment was repeated, using both *Halicystis* and *Valonia* cells. Controls in this case were untreated sap. After 4 hours of exposure, sap from half of the treated cells was tested against human erythrocytes in dilutions by 10 from 1:1 to 1:10,000. No hemagglutination was observed in any of the tubes.

Of the remaining cells of *Halicystis*, half showed distinct signs of deterioration at the end of 18 hours. The *Valonia* cells were still more susceptible to the treatment given. In 11 hours three-fourths of them were dying.

As it was suspected that some of the *Valonia* cells used in the first experiment did not have normal protoplasmic membranes, similar tests were made on three groups of *Valonia* cells showing increasing amounts of protoplasmic deterioration when examined with dissecting binoculars at a magnification of 200 diameters. These cells showed increasing amounts of penetration by the proteose; *i.e.*, they exhibited increased powers of agglutination in direct proportion to the amount of protoplasmic deterioration shown. It was further observed that *Halicystis* cells which showed tiny breaks in the protoplasm under the microscope also gave a plus 4 agglutination test at the end of 6 hours. These tests were made against 1 per cent suspension of fresh blood in isotonic salt solution, using 0.5 cc. of erythrocyte suspension and 0.5 cc. of vacuole sap.

Valonia and *Halicystis* cells showing normal protoplasmic membranes under microscopical examination were then exposed for 4 hours to the action of the 20 per cent bean proteose solution in sea water, washed carefully, the sap withdrawn as before, and tested. The *Valonia* cells tested + at the end of 1 hour, ++ at the end of 2 hours, with sedimentation at the end of 6 hours. The *Halicystis* cells gave precisely the same tests. Controls against salt solution showed no agglutination at the end of 2 hours, but showed sedimentation at the end of 6 hours. Half of the cells remaining at the end of this last test were replaced in sea water and observed for viability. There were no deaths at the end of 3 days.

SUMMARY

From the results of these tests it is clear that both *Halicystis* and *Valonia* have a high degree of tolerance for animal peptone, and a very high degree of tolerance for animal proteose and for egg albumen. The products of bacterial growths fostered by these proteins have a deleterious effect upon both species of algae; but, if it were possible to prevent bacterial growth entirely and at the same time supply proper food, it is probable that *Halicystis* and *Valonia* would show normal growth indefinitely in the presence of these three colloids. This is not true where exposure is made to yeast nucleic acid dissolved in sea water containing 0.00093 gm. per cc. of NaOH. *Valonia* is markedly less tolerant of this medium (perhaps of NaOH rather than the colloid used) than *Halicystis*. Such differential effects, however, reach a high point in the case of the solutions of diphtheria toxin and of edestin. *Halicystis* has a very high tolerance for diphtheria toxin, and *Valonia* a very low tolerance. In the case of edestin, the relationship is reversed. Here *Halicystis* has a very low tolerance, and *Valonia* a very high tolerance. In fact, it may be said that diphtheria toxin has no appreciable effect upon *Halicystis*, and edestin a very slight effect upon *Valonia*; while diphtheria toxin is extremely toxic to *Valonia*, and edestin is extremely toxic to *Halicystis*. We can offer no suggestions, at present, as to the way in which these effects are produced.

It is probable that the very thin protoplasmic layer[3] of these species, which is certainly no thicker than 8μ, is sufficient to obstruct the passage of proteins having large molecules, like egg albumen, with a degree of efficiency that is extraordinary. In the tests we have reported, areas of from 20 sq. cm. to 40 sq. cm. have been submitted to the action of a relatively high concentration of egg albumen for several days without permitting the passage of sufficient amounts to give definable tests either with Spiegler's or with Tanret's method,—presumably less than 1 part in 250,000.

In the tests of the proteins having much smaller molecules (though

[3] Pseudoplasmolized, but turgid, cells of *Valonia* and of *Halicystis*, when subjected to the action of egg albumen in sea water (1:800) for 2 hours, showed the presence of albumen in the cell sap (Spiegler and Tanret). It thus appears that small quantities of albumen pass the cell wall.

the size may not be the explanation), there is some probability that the membranes exhibit a little permeability. The peptone and the proteose of animal origin, or biuret-positive substances[4] derived from them, apparently pass the protoplasmic membranes occasionally in quantities sufficient to give biuret tests. The most probable case of protein passage, however, was that of the proteose of the scarlet runner bean, where specific detection of less than 1 part per 80,000 was possible. In this instance the proteose appeared to pass membranes that were healthy and were functioning normally. But since the cells of the algae had to be destroyed in making the tests, one cannot maintain this point. All one can say is that protein passage was indicated in carefully examined cells of both species, where no breaks in the protoplasmic membrane were discernible, and where samples of the treated cells behaved normally after treatment.

LITERATURE CITED

Osterhout, W. J. V., 1931, Physiological studies of single plant cells, *Biol. Rev.*, **6**, 369.

Paltauf, R., Die Agglutination, in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Microorganismen, Jena, Gustav Fischer, 2nd edition, 1913, **2**, 483.

Ratner, B., Jackson, H. C., and Gruehl, H. L., 1927, Transmission of protein hypersensitiveness from mother to offspring. I. Critique of placental permeability, *J. Immunol.*, **14**, 249.

Schneider, E. C., *J. Biol. Chem.*, 1912, **11**, 47.

[4] The presence of non-protein, biuret-positive substances is not excluded; but the method of preparation of the products used in the experiments is such that the presence of detectable amounts is improbable.