SOME PHYSICOCHEMICAL PROPERTIES OF DISSOCIATED SPONGE CELLS.

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I. INTRODUCTION.

It has been known since Wilson's discovery (1) that dissociated cells of *Microciona* come together and form aggregates, which by further transformation develop into new sponges. Similar processes were observed in fresh water and in calcareous sponges (2, 3), and in hydroids and alcyonarians (4, 5). Recently one of us (6, 7), in studying the behavior of dissociated cells of *Microciona*, found that the formation of aggregates is due to the ameboid movement of so called archæocytes, that is, unspecialized cells of the sponge mesenchyme, which upon separation creep in various directions and coalesce with other cells of the same species which happen to lie on their route. According to these observations, aggregation is easily affected by changes in the surrounding medium. In pure isotonic solutions of NaCl or KCl the ameboid movement is entirely inhibited, and the addition of at least one of the alkaline earth metals, either Ca or Mg, is necessary to produce the aggregation of cells. The addition of acids or bases to a suspension of cells also causes significant changes in their behavior, inhibiting their movement and changing the adhesive properties of the protoplasm. In mixed suspension, the cells coalesce only with cells of their own species, forming separate aggregates; while in alkaline sea water the *Microciona* aggregates become surrounded by the *Cliona* cells.

The present investigation is an attempt to deal in a quantitative manner with the equilibrium relations involved between the cells of two siliceous sponges, *Microciona prolifera* Ver. and *Cliona celata* Gr., and acid or base.

As a preliminary to the account of the investigation of the sponge cells, we report a titration of 0.00280 molar NaAc in 0.520 molar NaCl solution; this solution served as the medium in experiments with the cells.

II. The Hydrogen Ion Activity in a Solution Containing 0.00280 Mols of NaAc, 0.520 mols of NaCl per Liter, and Various Amounts of HCl and NaOH.

A medium for the titration of the cells of the sponges must answer several requirements. First of all, it must be isotonic with the cells; secondly, it must be of such a nature as to prevent aggregation of the cells (7); and, thirdly, it must have a certain buffer value on the acid side of neutrality (since most of the observations were made in that range) so as to yield reproducible E. M. F. measurements.

After several trials we found that a solution containing 0.00280 mols of NaAc and 0.520 mols of NaCl, and varied amounts of HCl and NaOH, answered practically all of our requirements. It gives fairly reproducible E.M.F. measurements, but its buffer value is not large enough to mask the effect of the acid or base bound by the sponge cells.

Several investigations were made on acetate buffers containing different amounts of NaCl. L. Michaelis and R. Krüger (8) studied the hydrogen ion activity of a 0.02 N mixture of equal amounts of NaAc and HAc in the different salts. They found that in 1 molar NaCl the mixture has a pH of 4.484, the pK′ evidently being equal to 4.484.

L. Michaelis and K. Kakinuma (9) in their contribution to the electrochemical measurements of the activity of ions found that 0.01 molar solutions of equal amounts of NaAc and HAc, containing different amounts of NaCl, have different hydrogen ion activities. A solution containing 0.1 mol of NaCl had a pH equal to 4.607, a 0.5 molar solution a pH of 4.503, and a 1.0 molar solution a pH of 4.448.

G. S. Walpole (10) investigated the pH of a mixture of HAc, NaAc, and NaCl. The concentration of Ac in this system was 0.20 N; a 1:1 mixture gave a pH of 4.58. The measurements were made at 18°C.

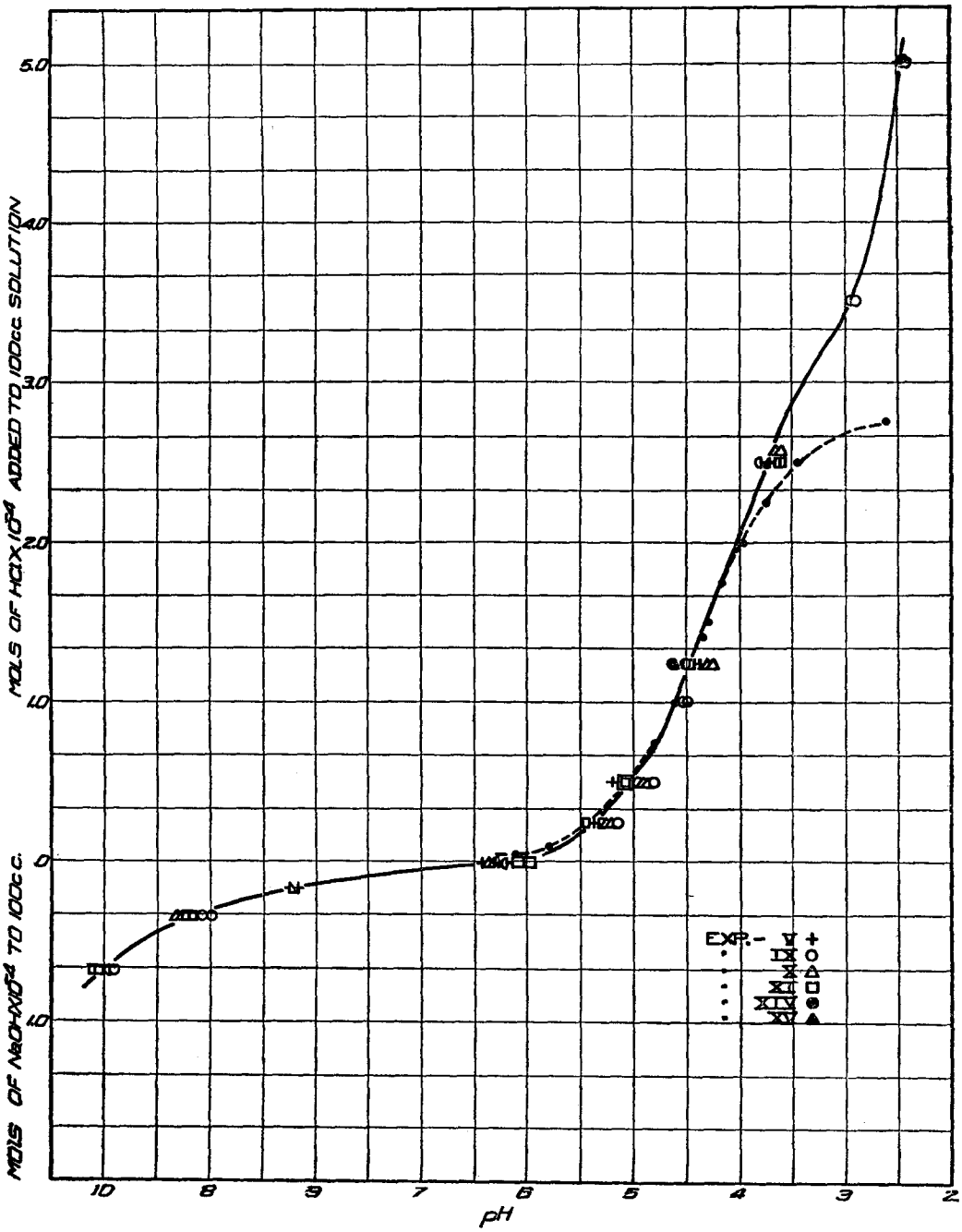

FIG. 1. The hydrogen ion activity in a solution containing 0.00280 mols NaAc, 0.520 mols NaCl per liter, and varied amounts of HCl or NaOH.

J. N. Brönsted (11) recalculated the experimental data obtained by Walpole in terms of fundamental thermodynamic functions.

The results of our investigation are graphically represented in Fig. 1.

All the pH measurements reported were made by means of a Leeds and Northrup potentiometer. The E.M.F. of the hydrogen electrode was measured against a 0.1 N KCl calomel electrode, using a saturated KCl bridge. The pH's reported were recalculated by the equation:

$$\text{pH} = (\text{E.M.F. observed} - \text{E.M.F. calomel})\ 1 \div 0.001983$$

For the E.M.F. of the calomel electrode we used the value given by Lewis and Randall (12). No correction for the diffusion potential was made.

Our experiments were carried out at slightly different temperatures. The effect of temperature on the activity is not a negligible one. We corrected for the influence of temperature by interpolating between experimental points. Fig. 1 represents the titration of our acetate buffer at about 21–22°C.

In this figure the dotted line represents the pH values calculated by the Henderson-Hasselbalch equation:

$$\text{pH} = \text{pK}' + \log [(\text{NaAc}) \div (\text{HAc})]$$

It is evident from Fig. 1 that the equation holds over a considerable range. It fails, however, to describe the experimental data in the range where the amount of NaAc becomes very small. The average pK′ for acetic acid in our system is about 4.37.

III. The Hydrogen Ion Activity in a Suspension of Cells of Microciona prolifera or Cliona celata, in Which the Amount of Acid or Base is Varied, but the Concentration of Cells Is Kept Constant.

In its natural habitat *Microciona* is usually found attached to rocks or shells, frequently occurring on oyster beds. The sulfur sponge, *Cliona*, is a boring sponge; it infests the shells of various pelecypods (both living and dead), and having bored through them, grows farther, reaching an enormous size.

The sponges used in the experiments were collected in the vicinity

of the Woods Hole laboratory. *Microciona* was taken from the mouth of Wareham Bay, and from Waquoit Pond near Falmouth, Massachusetts. *Cliona* was obtained in Squeteague Harbor near North Falmouth, Massachusetts. Both species inhabit shallow waters and normally sustain considerable fluctuations in salinity. In Waquoit Pond and in Wareham River the salinity, according to our observations, varies with each tide from 27 gm. per liter at high tide to 16 at low tide.

Though the salinity in the laboratory tanks at Woods Hole is much higher, varying from 31 to 32, no harmful effect was noticed, and the sponges sustained the new environment very well. As a rule, however, the sponges used for experimentation were not kept longer in the aquarium tanks than 5 days. Experience shows that as a result of prolonged life in the aquarium they undergo physiological and anatomical changes, and become unfit for experimental work.

The following procedure was adopted to obtain a suspension of sponge cells.

(1) Each piece of sponge was washed, all dead portions were cut away, and it was cleaned from all foreign substances, such as small pebbles, sand, mud, or algæ.

(2) The sponge was then placed for 15 minutes in a 0.520 molar NaCl solution, the solution being changed twice.

(3) The material was squeezed through bolting silk No. 20 into a solution containing 0.00280 mols NaAc and 0.520 mols NaCl per liter.

(4) The next procedure consisted in centrifuging and washing twice with a solution of the same concentration of salts as that described in (3).

(5) The suspension of cells thus obtained was transferred to a vessel through which a steady current of CO_2-free air was bubbled. This last process was necessary in order to free the solution from any small amounts of bound or free carbon dioxide which might possibly be present, and also to keep the cells from settling to the bottom of the vessel. By bubbling air through the suspension, it can be kept for 24 hours without sedimentation and aggregation of cells.

The suspension of sponge cells obtained consists of archæocytes, collencytes, pinacocytes, desmacytes, and choanocytes. The percentage composition of the suspension may be given as follows: *Micro-*

ciona; 25.5 per cent archæocytes, 9.9 per cent collencytes, and 64.6 per cent desmacytes, pinacocytes, and choanocytes; *Cliona*; 15.4 per cent archæocytes, 15.0 per cent collencytes, 69.0 per cent desmacytes, pinacocytes, and choanocytes.

During the preparation of the suspension the cells were subjected to rather vigorous mechanical treatment. Part of them might have been cytolyzed. The products of this cytolysis might appear in the watery phase, and might be responsible for the binding of any acid or base added to the system.

TABLE I.

Effect of the Number of Washings on the pH of a Suspension of Cells of Microciona prolifera.

Experiment VI. Washing solution contains 0.520 mols of NaCl, 0.00280 mols of NaAc, and 1.25×10^{-3} mols of HCl per liter. 30 minute intervals between consecutive washings.

No. of washings. (1)	E.M.F. (2)	Temperature. (3)	pH (4)
	volts	*°C.*	
2	0.6230	23.8	4.88
2	0.6218	23.8	4.86
3	0.6274	23.8	4.95
3	0.6272	23.8	4.95
4	0.6251	24.2	4.91
4	0.6247	24.3	4.90
5	0.6235	24.2	4.88
5	0.6233	24.2	4.88
6	0.6205	24.1	4.83
6	0.6206	24.1	4.83
NaCl solution used in this experiment.	0.5952	24.7	4.40
	0.5960	24.7	4.41

In order to determine whether we were dealing in our experiments with the acid- or base-binding property of the cells, or with the effect of some unknown product of cytolysis, we carried out the experiment reported in Table I. In this experiment a suspension, prepared in the way already described, was further washed with a solution containing 0.00280 mols of NaAc and 0.520 mols of NaCl per liter. After each washing the suspension was centrifuged, and the pH of the super-

natant liquid determined. It is evident that the pH of the suspension remains practically constant. If the acid-binding property of this system was dependent upon the product of cytolysis, the pH should gradually have risen until it reached the pH of the washing solution. No such phenomenon was observed. We must conclude, therefore, that in this case we are dealing with a property very closely associated with the living cells.

In our experiment we added acid or base to the suspension of the sponge cells. When any acid or base is added to a system containing

TABLE II.

Effect of Time upon the Establishment of an Equilibrium between the Cells and the Acid Added.

Solution: 0.520 mols NaCl and 0.00280 mols NaAc per liter.

Suspension: 51.9×10^8 cells of *Microciona* suspended in 100 cc. of solution; 1.25×10^{-4} mols HCl added to it.

Time elapsing between the addition of the acid and the E.M.F. measurement. (1)	E.M.F. (2)	Temperature. (3)	pH (4)
min.	*volts*	°C.	
48	0.6219	21.8	4.892
	0.6218	21.8	4.890
63	0.6223	22.0	4.896
	0.6223	22.0	4.896
99	0.6241	22.0	4.919
	0.6240	22.0	4.918
125	0.6249	22.4	4.933
	0.6248	22.4	4.931

basic or acid radicals, a displacement of the equilibrium occurs. The establishment of the new equilibrium takes a certain length of time, depending upon the properties of the system. To test the effect of time on the system containing a rather large amount of acid and a suspension of cells of *Microciona prolifera*, we carried out the experiment reported in Table II. As is seen from the table, the pH values are almost constant. The difference in the pH value of the cell suspen-

sion and the same solution without the cells, in this experiment, is equal to 0.5 of a pH unit, while the difference between the first reading and the last is only 0.04 of a pH unit. There are three possible

TABLE III.

Vitality Tests of the Cells Used in the Titration Experiments.
Microciona prolifera.

Experiment No.	pH of the suspension.	Condition of cells after titration experiments.	
		Immediately.	12 hrs. later.
(1)	(2)	(3)	(4)
VIII	4.56	Normal.	Very small aggregates slightly adhering to glass; many cytolyzed cells.
V	4.82	"	Small globular aggregates adhering to glass; few cytolyzed cells.
III	5.90	"	Normal aggregates adhering to glass; few cytolyzed cells.
V	6.13	"	Normal aggregates adhering to glass.
X	6.36	"	" " " " "
X	6.96	"	" " " " "

TABLE IV.

Vitality Tests of the Cells Used in the Titration Experiments.
Cliona celata.

Experiment No.	pH of the suspension.	Condition of cells after titration experiments.	
		Immediately.	12 hrs. later.
(1)	(2)	(3)	(4)
IX	3.06	Part of cells cytolyzed.	Cells strongly adhering to glass; no aggregation.
IX	3.71	Normal.	" " " " " " "
IX	4.09	"	" " " " " " "
IX	6.59	"	Normal aggregates.
IX	6.70	"	" "
XI	7.34	"	" "

causes of this variation. One of them has already been pointed out; namely, the time factor in the establishment of the new equilibrium. The second factor which must undoubtedly be present in any system containing living material is that of metabolism. The products of

the metabolism might possess acid or basic properties of their own, and might gradually change the pH of the medium. Without knowing the chemical nature of these metabolic products, one cannot determine their influence upon the pH of the suspension. The third factor which may be responsible for this variation is the beginning of cytolysis. It is quite probable that in such acid solution irreversible changes occur in the cells, producing more and more titratable material. This will be seen from the discussion of the vitality tests of the cells treated with acid.

In all subsequent measurements we used 45 minutes for the equilibration time.

The next problem with which an investigator of living matter is confronted, is to determine whether or not his chemical manipulations have caused a permanent injury or death to the object of his experiment. The best test of this, is to examine the cells immediately after the experiment and to observe their behavior when they are brought back to a normal environment.

For this purpose we conducted the following tests in conjunction with the measurements of the pH which resulted from the addition of acid or base to the suspension of sponge cells: the cells used in the experiments were washed with sea water and examined under the microscope to determine whether they were alive or not, then 1 cc. of suspension was added to 9 cc. of sea water, and the mixture left undisturbed for 12 hours. This period is long enough for uninjured cells to coalesce and form globular aggregates which adhere strongly to the glass (7). The cells irreversibly affected by previous treatment are partially cytolyzed and form aggregates of irregular shape. Dead cells form loose sediment not adhering to the glass.

Tables III and IV list the results of the vitality tests carried out on cells taken from the titration experiments. It can easily be seen that the susceptibility of *Microciona prolifera* to acid solutions used in the experiments is much higher than that of *Cliona celata*. Though at the end of Experiments V and VIII the cells of the former remain alive and under the microscope appear to be normal, their ameboid movement is retarded. After 12 hours, instead of forming a few large aggregates as always happens under normal conditions, they coalesce into numerous small groups; many cells at the end of this period be-

come cytolyzed. This occurs when the pH of the supernatant liquid of the suspension is 4.56.

The cells of *Cliona celata* endure much higher acidity remaining uninjured at pH 3.71, though their ameboid movement after such treatment is inhibited.

The difference between the cells of the two sponges can undoubtedly be correlated with the fact that for a given change in pH in fairly acid solutions, *Microciona* binds more acid than *Cliona*. We may suspect therefore that greater chemical changes occur in the first than in the second.

It must be borne in mind that, due to the rough treatment during squeezing, washing, and centrifuging, the cell suspensions may contain a certain amount of cytolyzed material; so the presence of a small number of cytolyzed cells cannot be attributed entirely to the effect of acid solution. Further increase in hydrogen ion activity will certainly cause a complete cytolysis and death of the cells. As can be concluded from the examination of Tables III and IV, the critical concentration probably lies just below pH 4.5 for *Microciona* and about 3.7 for *Cliona*. Above these values, the largest part of the cells examined under the microscope immediately after the titration experiments showed no evidence of injury.

The amount of acid or base bound in titration of a suspension depends upon the concentration of cells in that suspension. Our titration experiments were carried out with suspensions of a definite concentration. We shall express this concentration in terms of the number of cells present in 100 cc. of the suspension. This method of expressing the concentration is not strictly correct. Cells have their own volume; therefore the volume of "free" solution depends upon the number of cells present. Any computation of acid or base bound referred to 100 cc. will deviate from the true value by the volume of cells present in the system. However, we believe that by using rather dilute suspensions of cells we made this error negligibly small.

In determining the number of cells in a given suspension, the following procedure was adopted: 1 cc. of this suspension was diluted one hundred times and shaken well. 1 cc. of this suspension was transferred to a counting cell. A uniform distribution of the cells was secured by the use of a pipette. In about 10 minutes the cells settled

on the bottom and could be counted with a Whipple square micrometer. We counted the cells in ten fields of view taken at random at various parts of the counting cell. From the average number obtained by this procedure the total number of cells in 1.00 cc. of suspension was estimated. The results were usually accurate within 5 per cent.

If the stock suspension was found to differ from the desired concentration, it was diluted to the appropriate extent. After the dilution, the number of cells was checked once more.

In all our experiments we titrated with HCl and NaOH, and for this reason our medium contained a large amount of NaCl. Any addition of small quantities of Cl or Na produced, therefore, a practically negligible change in the concentration of either Na or Cl. Any reaction of the cells will, therefore, be due entirely to the change in the concentration of free acid or base as measured by the hydrogen ion activity or its dependent variable, the hydroxye ion activity. This statement would be accurate if our systems had not contained NaAc. Upon the addition of an acid, however, the concentration of Ac^- decreases proportionally to the acid added. Therefore, in addition to the variables (H^+) and (OH^-) we have the variables (HAc) and (Ac^-). Evidently this second set of variables cannot be neglected. A simple way to test the influence, if any, of the concentration of HAc and Ac^- is to titrate the cells in a solution of 0.520 molar NaCl in the absence of NaAc. Such an experiment is hardly quantitative in a slightly acid solution, but in a medium containing a rather large amount of acid the E.M.F. becomes reliable. Therefore we brought a solution containing 0.520 mols of NaCl per liter to a pH of 4.50 by adding to it a known amount of HCl. Then to the same solution we added about 50×10^8 cells of *Microciona prolifera* and by the addition of HCl brought it to the same pH as the solution of NaCl. It was found necessary to add more acid to the cells than to the NaCl solution in order to make the two solutions isohydrionic. Evidently the amount of acid added to the cells minus the amount of acid added to the NaCl solution is the amount of acid bound by the cells. It was found to be equal to 1.2 ± 0.2 mols HCl $\times 10^{-4}$. If we compare this figure with the one obtained from the titration of the cells of *Microciona prolifera* in the presence of NaAc (Fig. 2) we find a complete agreement. Evidently the (HAc) and (Ac^-) are not the controlling factors in the titration in question.

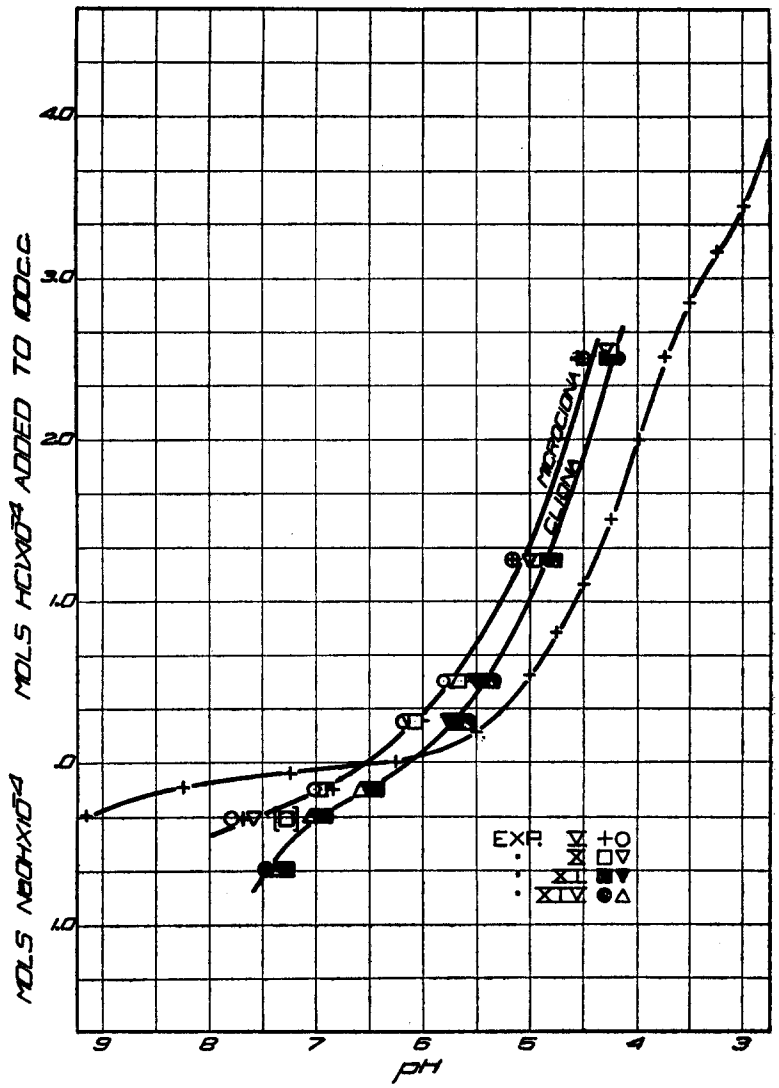

FIG. 2. The hydrogen ion activity in a solution containing NaAc and NaCl, a given number of cells of *Microciona prolifera* or *Cliona celata*, and varied amounts of HCl or NaOH.

Microciona: Experiment V, 52.4 × 10^8 cells; Experiment X, 49.3 × 10^8 cells. *Cliona:* Experiment XI, 57 × 10^8 cells; Experiment XIV, 51.1 × 10^8 cells.

The titration of the suspension was effected by adding to a known amount of cells a given amount of HCl or NaOH. The cells were then centrifuged and the pH determined electrometrically on the supernatant liquid. Two E.M.F. measurements were carried out on each sample. The results of two experiments are given in Fig. 2, together with the titration of the acetate buffer, taken from Fig. 1.

As may be noticed from the titration curve of *Microciona prolifera*, the two experiments disagree slightly with each other in the upper part of the curve. The reason for this disagreement is a difference in concentration of the cells in the two experiments. By drawing a line between the experimental points we can take care of this influence, and the line of titration would represent a titration of suspension having approximately 50.8×10^8 cells per 100 cc. A similar behavior is shown by the cells of *Cliona*, though to a lesser extent.

The titration curves obtained for the sponge cells are only functions related to the acid- or base-binding properties of the cells. The curves will have different shapes in media containing different buffers.

If we subtract at any pH the amount of acid necessary to bring the acetate buffer to that pH from the amount of acid added to the cells to bring them to the same pH, we shall obtain a value characteristic of the suspension—the amount bound by the suspension.[1]

Such a calculation was made for both *Microciona* and *Cliona* for the slightly acid and basic ranges of the titration curve. The results of the estimates are given in Fig. 3. They are probably accurate within about 8 per cent.

The function thus obtained is of fundamental importance for the estimation of the physicochemical properties of cells. Each curve has

[1] This procedure is not strictly correct since the free base or acid is related to the hydrogen ion activity by the equations:

$$(\mathrm{HCl}) = \gamma_1 (a_{H^+})$$

$$(\mathrm{NaOH}) = \gamma_2 K_w \div (a_{H^+})$$

in which the activity coefficients γ_1 and γ_2 vary with the change in concentration of HCl and NaOH.

But, since our system contains a large amount of NaCl, the change in the activity coefficients between the acetate titration curve and the one of the sponge cells is probably small.

two parts; one above the zero point where the sponge behaves as a base, and one below where it behaves as an acid. The zero point,

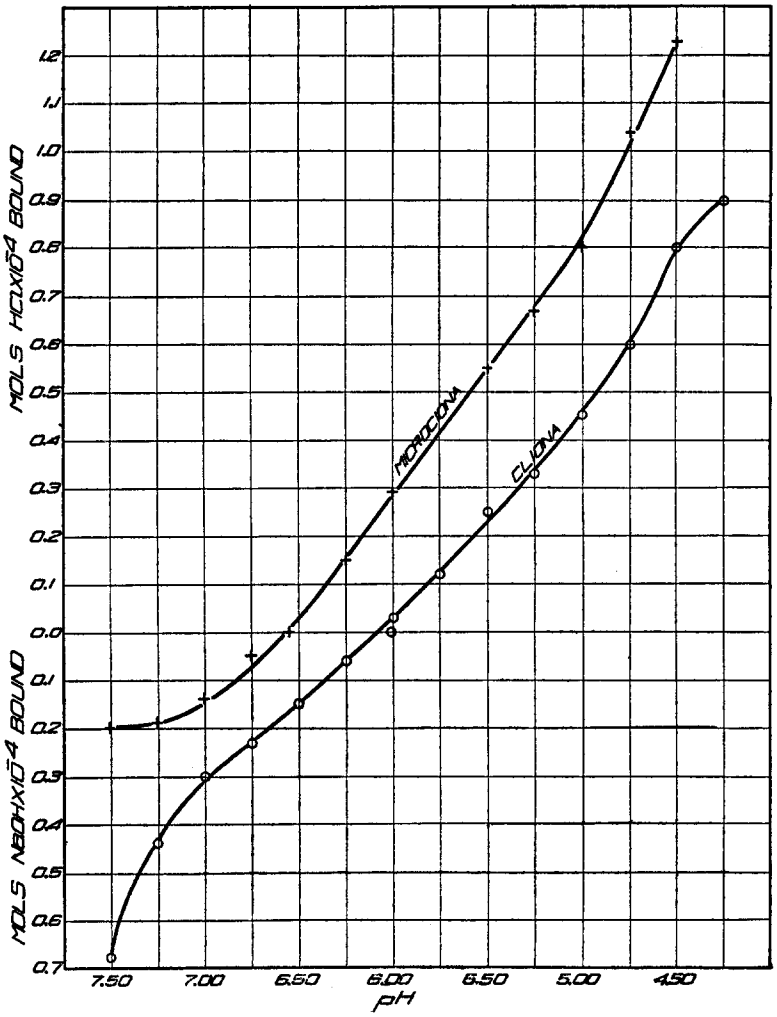

FIG. 3. The acid and base bound by the cells of *Microciona prolifera* and *Cliona celata* at different hydrogen ion activities.

where no base or acid is bound, is of considerable interest for us. It represents the pH of a pure suspension of cells, extracted from the

sponge and washed with isotonic NaCl. These pH values for extracted cells are different for *Microciona* and *Cliona*. The cells of *Cliona* are more acidic than those of *Microciona*. We believe that these pH values may be characteristic of the species concerned, provided the comparison is made at a concentration of about 50–60 $\times$ 10^8 cells.

The hydrogen ion activity of the original suspension for *Microciona prolifera* is equal to a pH value of 6.55 $\pm$ 0.1, and for *Cliona celata* to a pH value of 6.10 $\pm$ 0.1.

Passing to the acid range of the acid- or base-binding curve we observe that *Cliona*, being more acidic than *Microciona*, behaves as a weaker base, and binds less acid than the latter. *Microciona*, being more basic, binds more acid for a given change in pH.

The basic part of the acid- or base-binding curve is even more characteristic for the two species of sponges. While *Microciona prolifera* is almost saturated with the small amount of base at a pH value of 7.50, *Cliona* still has a considerable base-binding capacity at that point. It substantiates once more the conclusion reached upon comparison of the two species in the acid portion of the curve; namely, that *Cliona* behaves as a much stronger acid than *Microciona*.

This conclusion, however, is open to one criticism: the suspensions of the cells of *Microciona* and *Cliona*, though containing an equal number of cells, are composed of cells of different sizes. Therefore, the total surface of the cells is different for *Microciona* and *Cliona*.

If the removal of acid or base from the liquid phase, by the cells, is entirely due to the effect of the active surface, the results reached in this investigation would seem to refer to the surface, but not to the chemical properties of the body of the cells.

It is therefore of considerable interest to provide an experimental evidence to prove that the reagents used penetrated inside the cells.

In the course of the investigation upon the cells of *Cliona* it was found that these cells changed their coloration from yellow to dark brown at a pH ranging from 7.3 to 7.4. Upon microscopic examination of the cells, it was observed that the yellow pigmented granules of the cells were responsible for this change in color.

On treating the cells with absolute ethyl alcohol, this pigment can be extracted, and the same change in color reproduced in a test-tube.

These experiments indicate that the cells of *Cliona* in faintly alka-

line solution are permeable to our reagents. The reaction is not limited to the surface of the cell.

This evidence cannot, however, be extended to the acid range of titration of *Cliona,* nor to *Microciona* suspensions; but, since we have no reasons for believing that an entirely different physicochemical mechanism is involved in these cases, we are inclined to think that the action of our reagents is not limited to the surfaces of the cells.

We may conclude, therefore, that the concentration of cells being equal, the suspensions of cells of *Microciona* and *Cliona* differ from each other in their physicochemical properties, the comparison being made on suspensions of specified composition.

IV. CONCLUSIONS.

1. The activity of the hydrogen ion, in a system containing 0.00280 mols of NaAc, 0.520 mols of NaCl per liter, and varied amounts of HCl or NaOH has been investigated. The average value of pK′ for acetic acid in this system is about 4.37.

2. The effect of the addition of various amounts of HCl and NaOH to a system containing 0.00280 mols of NaAc, 0.520 mols of NaCl, and a known number of cells of either *Microciona prolifera* or *Cliona celata* was then studied. It was found that in weak acid solutions *Microciona* behaves as a stronger base than *Cliona*, the former being practically saturated with base at a pH of 7.5. Similar behavior is shown by suspensions of cells to which no acid or base was added: the cells of *Cliona* are more acidic than the cells of *Microciona.*

3. The microscopic examinations of the cells subjected to the treatment with acid or base indicate that the cells of *Microciona* remain alive down to pH 4.50; the cells of *Cliona* sustain greater acidity,—at pH 3.7 they exhibit no signs of cytolysis. Tests for aggregation of these cells showed that this phenomenon is greatly inhibited even by slightly acid solutions.

4. The conclusion is drawn that the concentration of cells being equal, the suspensions of cells of *Microciona* and *Cliona* differ from each other in their physicochemical properties, the comparison being made on suspensions of specified composition.

BIBLIOGRAPHY.

1. Wilson, H. V., *J. Exp. Zool.*, 1907–08, v, 245.
2. Müller, K., *Arch. Entwcklngsmechn. Organ.*, 1911, xxxii, 397.
3. Huxley, J. S., *Quart. J. Micr. Sc.*, 1920–21, lxv, 293.
4. Wilson, H. V., *J. Exp. Zool.*, 1911, xi, 281.
5. DeMorgan, W., and Drew, G. H., *J. Marine Biol. Assn. U. Kingdom*, 1913–14, x, 440.
6. Galtsoff, P. S., *Biol. Bull.*, 1923, xlv, 153.
7. Galtsoff, P. S., *J. Exp. Zool.*, 1925, xlii, 183, 223.
8. Michaelis, L., and Krüger, R., *Biochem. Z.*, 1921, cxix, 307.
9. Michaelis, L., and Kakinuma, K., *Biochem. Z.*, 1923, cxli, 394.
10. Walpole, G. S., *J. Chem. Soc.*, 1914, cv, 2501.
11. Brönsted, J. N., *J. Chem. Soc.*, 1921, cxix, 574.
12. Lewis, G. N., and Randall, M., Thermodynamics, New York, 1923, 406.