

THE PENETRATION OF BASIC DYE INTO NITELLA AND  
VALONIA IN THE PRESENCE OF CERTAIN ACIDS,  
BUFFER MIXTURES, AND SALTS.

By MARIAN IRWIN.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, October 7, 1926.)

I.

INTRODUCTION.

It has been shown that the pH value of the cell sap plays<sup>1</sup> an important rôle in the accumulation of the basic dye, brilliant cresyl blue, in the living cell of *Nitella*, and in view of this it is important to study the changes in the rate of penetration produced by varying the pH value of the sap. Experiments of this sort, made by McCutcheon and Lucke,<sup>2</sup> and by the writer,<sup>3</sup> showed that the penetration of ammonia increases the pH value of the sap and decreases the rate of penetration of the dye.

The present paper deals with experiments on the rate of penetration of the dye in presence of acids and buffer mixtures. These experiments are of interest in connection with the hypothesis<sup>4</sup> that brilliant cresyl blue exists in aqueous solution in two forms, called for convenience DB and DS. DB, the form which predominates at higher pH values, represents a free base while DS exists predominantly at lower pH values and is a dissociated salt. A normal living cell of *Nitella* is assumed to be chiefly permeable to DB and only very slightly permeable to DS. The present problem is to find the nature of the factors controlling the penetration of DB.

<sup>1</sup> Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, 561.

<sup>2</sup> McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923-24, vi, 501.

<sup>3</sup> Irwin, M., *J. Gen. Physiol.*, 1925-26, viii, 147.

<sup>4</sup> Irwin, M., *J. Gen. Physiol.*, 1926-27, x, 75.

## II.

*Methods.*

Only general methods will be given here: special methods for each set of experiments will be described in connection with the results.

The experiments were carried out in an incubator at 25°C.  $\pm$  0.5° having air holes through which diffused light entered.

Only living cells were used. In order to obtain cells in the same condition for experimentation uniformity as to length, thickness, and external appearance was attended to. In the case of *Nitella flexilis* the cells used were taken from the central portion of the plant, midway between the tip and the root. Seasonal changes bring about differences in the permeability of the cells, so that a series of comparative experiments were made on the same lot of cells collected within a short period (near New York in spring unless otherwise stated).

A control experiment was always carried out by removing cells directly from tap water and placing them in the same dye solution as in the case of the test experiment (in which the cells were given some special treatment before being placed in the dye). The rate of penetration obtained from the control experiment was used as a standard of comparison in order to determine the change in the rate of penetration of the dye caused by varying the media in which the cells were placed previous to exposure to the dye solution.

Every determination given represents an average of over 60 experiments and the probable error of the mean is in all cases less than 7 per cent of the mean.

In the case of *Valonia macrophysa* (collected in Bermuda), the procedure was as follows: The clusters of cells were pulled apart and the individual cells were allowed to stand in pans of sea water (which was changed daily) for over 2 weeks in the laboratory (exposed during the day to diffused light). During this period the cell wall at the point of detachment thickened somewhat. This precaution was taken to diminish irregularity in the rate of penetration and the susceptibility of cells to injury upon exposure to solutions.

Small cells (each having a volume of about 0.1 cc.) with one point of detachment, and having practically no attached cells, were chosen. Care was taken to remove adhering organisms or deposits from the surface of the cell.

The detection of an early stage of reversible injury is a very difficult matter, especially with *Valonia*. In the case of *Nitella* an increase in the rate of accumulation of the dye may serve as an indication of a preliminary stage of an injury under certain conditions but this does not seem to be markedly evident in *Valonia*. The exit of halides from the vacuole of *Nitella* or the entrance<sup>5,6</sup> of SO<sub>4</sub> into *Valonia* seems to indicate advanced stages of injury.

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<sup>5</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1925-26, viii, 131.

Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, ix, 255.

<sup>6</sup> Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360.

Cells in good condition are turgid: as they become injured the turgidity diminishes. An experienced experimenter can, to a certain extent, predict the degree of resistance of the cells of *Nitella* and *Valonia* to experimental treatment by the turgidity as ascertained by touching them. This method was used by the writer as a rough guide to the condition of the cells but it does not serve to tell whether injury is reversible. The criterion of irreversibility of injury employed by the writer was as follows: cells which had been exposed to experimental conditions were replaced in the normal medium (*Nitella* in tap water, *Valonia* in sea water), and at intervals during 2 days the rate of mortality was compared with that of the control cells (without exposure to experimental conditions). The criterion of death for *Nitella* was a complete and permanent loss of turgidity, and for *Valonia* either a complete collapse of the cell or disarrangement of chlorophyll and its appearance in the vacuole, so that the greater part of the cell surface appeared colorless. Another test of the condition of the cells is to observe the length of time it takes for them to die in the experimental solutions.

It is not possible, however, to determine experimentally whether the cell was injured at the time of experiment, unless the injury happened to be irreversible. By using these tests an attempt was made to keep the cells uniform during the experiments.

The dye used was made by Grüber and was dissolved in buffer solutions (M/150) in the case of *Nitella* and in sea water in the case of *Valonia*. The pH values of the solutions determined colorimetrically were checked as much as possible by means of the hydrogen electrode. Solutions were not stirred unless otherwise stated.

The determination of the concentration of the dye in the sap was made colorimetrically. With *Nitella*, the cell was gently wiped and was cut at one end, so that the sap could be squeezed out onto a glass slide. With *Valonia* the surface of the cell was punctured with a sharp capillary tube and the sap was drawn up from the vacuole into the tube, from which it was pushed out onto a glass slide. In both cases the sap was drawn up into capillary tubes and the color was matched with capillary tubes of the same diameter containing standard dye solutions.

To determine the pH value of the sap a definite volume was taken by filling a tube for 2 inches with the sap. Indicator solution was drawn up into another tube for a distance of  $\frac{1}{8}$  of an inch. The contents of both these tubes were pushed out onto a glass slide and thoroughly mixed. This mixture was then drawn up into a capillary tube and the color matched with that of the capillary tube containing a mixture of standard buffer solution at a known pH value and the same amount of the indicator (the mixture was prepared in the same manner as in the case of the sap). Care was taken to have the least possible contamination of the sap by CO<sub>2</sub> from the breath of the experimenter, as well as to prevent escape of CO<sub>2</sub> into the air, as far as possible.

The color of the indicators changed on standing in a buffer solution containing artificial *Valonia* sap, and also on standing in the natural expressed sap, but the color of the indicators did not change during the time required to determine the pH value of the sap.

The salts in the sap of *Nitella* (about 0.1 M halides) do not seem to affect the indicator seriously, but those in the sap of *Valonia* (about 0.6 M halides) have a very definite effect. In view of the fact that we know so very little about the salt error in general, and possible specific effects of individual salts on these indicators, it will be necessary to study this question carefully before absolute pH values of the sap of *Valonia* can be given.

Another possible source of error in the case of *Valonia* is that the sap is so little buffered that an addition of indicator solution may bring about a change in the pH value of the sap; careful experiments must therefore be made to avoid this error. On the other hand, the sap of the *Nitella* used by the writer is buffered so that this source of error may be negligible. Since only approximate and relative values are desired the pH values of the sap of *Valonia* and *Nitella* given in this paper represent values without a correction for salt error, determined by means of one concentration of indicator dissolved in distilled water of pH 5.8 (approximately the pH value of the sap), or indicator dissolved in alcohol (methyl red). For one series of changes in the pH values only one indicator is used. For example, when experiments were made by exposing cells to a solution of  $\text{NH}_4\text{Cl}$ , brom-cresol purple was used: in the case of cells placed in acid solutions methyl red was used. Brom-cresol green was used to check the values obtained with methyl red, but in view of the fact that the color above pH 5.2 was not satisfactorily matched, only a very rough estimation of the pH value of the normal sap could be made by this indicator. Each indicator is taken from the same stock solution for each series of experiments.

### III.

#### *The Decrease in the Rate of Penetration of Dye When the pH Value of the Sap Is Lowered by Entrance of Acetic Acid.*

The cells were divided into four lots. One lot was placed in an acetate buffer mixture at pH 5.1, and at the end of 10 minutes the pH value of the sap was compared colorimetrically with that of the normal cell sap. It was found to have decreased<sup>7</sup> from pH 5.5 (normal) to pH 4.9.

<sup>7</sup> It may be added here that these experiments show that acetic acid enters the vacuole rather easily from an acetate buffer mixture and decreases the pH value of the sap until the internal pH value is less than the external. This agrees with the results obtained by many investigators showing that weak acids enter the living cells. The writer's experiments also show that the pH value of the sap may be raised again when acetic acid is allowed to come out of the vacuole by placing the cells in a solution containing no acetic acid (the more alkaline the external pH value, the more rapid is the rate of exit of acetic acid from the vacuole).

The second lot of cells was placed in the acetate buffer mixture at pH 5.1, and after 10 minutes they were removed, wiped, rinsed for 5 seconds in phosphate buffer mixture at pH 6.6, wiped, and placed in fresh phosphate buffer mixture at pH 6.6. After 1 minute the cells were removed, and the pH of the sap was determined. It was found to be pH 5.2, which is 0.3 pH lower than that of the normal cell sap.

TABLE I.

Comparison of the amount of brilliant cresyl blue in the vacuole when the living cells of *Nitella* are placed for 1 minute in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) after previous exposure to M/150 acetate buffer solution at pH 5.1 for different lengths of time. The rate of penetration of dye in the case of cells directly transferred from the tap water to the dye solution is used as the standard of comparison.

External solutions.		In tap water at pH 7.7.	In acetate buffer solution 5 sec.	In acetate buffer solution 1 min.	In acetate buffer solution 10 min.
When dye solution is not stirred or changed.	Amount of dye in sap.....	M 0.000073	M 0.000069	M 0.000069	M 0.000037
	Percentage decrease on basis of 0.000073 as standard.....		5 per cent	5 per cent	50 per cent
When dye solution is stirred and changed every 5 sec.	Amount of dye in sap.....	M 0.00012			M 0.000056
	Percentage decrease on basis of 0.00012 as standard....				47 per cent

The third lot of cells was first exposed to the acetate buffer solution at pH 5.1 for 10 minutes, after which they were removed, wiped, washed for 5 seconds in phosphate buffer mixture at pH 6.6, again wiped, and placed in the 0.00035 M dye solution at pH 6.6 (phosphate buffer mixture). After 1 minute they were removed from the dye solution and the concentration of the dye in the sap was determined colorimetrically, and was found to be 0.000037 M.

The fourth lot of cells was taken directly from the tap water (at pH 7.7) and placed in the same dye solution as the third lot of cells. At the end of 1 minute the concentration of the dye in the sap was found to be 0.000073 M.

Cells thus treated did not live so well as the control cells when replaced in tap water so that in all probability they were more or less injured, but during the experiment the actual appearance of the cells, in respect to chlorophyll arrangement and turgidity, seemed about the same as that of control cells except that the sap appeared slightly murky. Cells kept continuously in the acetate buffer solution began to die in about 3 hours, so that after an exposure of 10 minutes there may have been a very slight injury.

Thus these experiments show that the decrease in the pH value of the sap brought about by acetic acid may be associated with a decrease<sup>8,9</sup> in the rate of penetration of dye amounting to about 50 per cent, as shown in Table I.

This fact is of particular interest in connection with the theory<sup>1,4</sup> that the dye is chiefly in the form of free base (for convenience called DB), at high pH values, and that this alone can penetrate the proto-

<sup>8</sup> This decrease in the rate of penetration of dye is not due to the lowering of pH value of the external solution immediately surrounding the cell wall as a result of diffusion of acetic acid from the vacuole, because when the experiment is repeated by stirring the external solution, the relative amount of decrease in the rate is about the same as when the external solution is not stirred, as shown in Table I. Furthermore, this decrease is not caused by the adhering of acetic acid to the surface of cell wall in such a manner that it cannot be removed by washing and wiping before the cells are placed in the dye solution, because when the cells are placed in the dye solution, after they have been dipped in the acetate buffer solution only for 5 seconds or for 1 minute instead of 10 minutes, during which exposure the pH value of the sap remains normal, there is no decrease in the rate of penetration of dye, as shown in Table I.

<sup>9</sup> This result is contrary to the result obtained with Cambridge *Nitella* previously described (Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, 566, Foot-note 11) where an increase in the rate of penetration took place, but the extent of this increase was not so great (about 25 per cent). Since this work on Cambridge *Nitella* was done in midwinter, the experiments were repeated with the cells obtained in the summer, and it was found that in the majority of cases a decrease took place (about 25 per cent), which is less than the decrease in the case of New York *Nitella*. Such a difference in the behavior of cells may be due to the difference in the condition of the protoplasm.

plasm and enter the vacuole, and that the extent of accumulation of the total dye is dependent on the extent of change of this form, DB, on entering the vacuole into another form, DS, which cannot pass through the protoplasm. In that case we might expect the rate of penetration into the vacuole to be increased when the pH value of the sap is decreased, since with this decrease in the pH value of the sap the ratio of DB/DS decreases in the sap so that as DB enters the vacuole more of it will change to DS, thus causing more DB to enter. But since the experimental results give evidence to the contrary it is evident that the factor which controls the rate of penetration of dye into the vacuole cannot be wholly dependent on the condition of the sap. Under the present experimental conditions the rate of penetration of the dye must be controlled primarily by the effect of the acetate buffer on some other part of the cell. A series of experiments was therefore undertaken to determine the cause of this decrease in the rate of penetration of dye into the vacuole.

## IV.

*Can the Decrease in the Rate of Penetration of the Dye be Produced without Change in the pH Value of the Sap?*

If the theory<sup>4</sup> outlined in Sections I and III were correct we might assume that the decrease in the rate of penetration of dye associated with a decrease in the pH value is due to a change either at the surface or inside the protoplasm caused by the acetate buffer mixture. In that case we might very well expect a decrease in the rate of penetration when the cells are exposed<sup>10</sup> to the solution only long enough for the protoplasmic surface or the interior of the protoplasm to be affected before a change in the pH value of the sap occurs. Unfortunately it is not possible to use the acetate buffer solution for this purpose since the pH value of the sap changes after a very few

<sup>10</sup> The detailed description of the method of experimentation will be omitted hereafter since it is given in Section III. It may be repeated here that in all cases the cells were washed for about 5 seconds in a buffer solution at the same pH value as that of the dye solution before they were placed in the dye solution and the cells were invariably wiped before they were placed in any solution. Cells were exposed for 1 minute in the solution of dye, 0.00035 M made up with phosphate buffer mixture at pH 6.6 unless otherwise stated.

minutes exposure of the cells to the solution, even at the highest possible pH value (pH 5.4, M/150 acetate buffer mixture). For this reason it was necessary to expose the cells to a phosphate buffer solution at pH 5.4 for 10 minutes, in which the pH value of the sap remained unchanged, and to compare the rate of penetration of dye in the case of cells thus treated with the rate in the case of cells previously exposed for the same length of time in an acetate buffer solution at pH 5.4 where the pH value of the sap decreased from 5.5 (normal) to 5.0. As shown in Table II, there is about the same

TABLE II.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella*, when the cells are placed in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) after a previous exposure of the cells for 10 minutes either to the M/150 acetate buffer solution (when the pH of the sap is decreased) or to the M/150 phosphate buffer solution (when the pH value of the sap is not decreased).

External solutions.	In tap water at pH 7.7.	In acetate buffer solution at pH 5.4.	In phosphate buffer solution at pH 5.4.
	M	M	M
Amount of dye in sap.....	0.000073	0.000039	0.000041
Percentage decrease on basis of 0.000073 as standard.....		47 per cent	44 per cent

amount of decrease<sup>11</sup> in the rate of penetration whether the pH value of the sap is lowered or remains normal. The mortality of the cells thus treated is lower than that of the cells exposed to the acetate buffer mixture.

<sup>11</sup> Since there is about the same amount of decrease in the rate of penetration of dye whether the pH value of the sap is decreased or not, such a decrease cannot be due primarily to the decreasing of the pH value of the film<sup>4</sup> of liquid between the protoplasmic surface and the cell wall as result of diffusion of acetic acid from the vacuole into the film. This film is the only part of the external system which affects penetration since it alone determines the number of dye molecules striking the protoplasmic surface. The condition of the external solution may be regarded as of importance only in so far as it affects this film.



## v.

*Is the Decrease in the Rate of Penetration of the Dye Due to the Effect of H Ions on the Surface or to Their Penetration (as Ions) or to the Entrance of Acids in Undissociated Form?*

The decrease in the rate just described was about the same whether the pH value of the sap was lowered or not, and this suggests that the decrease in the rate might be due to the direct action of H ions on the surface or their penetration as ions when the pH value of the external solution changed from pH 7.7 (tap water) to pH 5.4 (buffer solutions). If this assumption were correct we might expect the rate of penetration to be about the same whether the cells were previously exposed to tap water, to phosphate, or to borate buffer solutions at pH 7.7 providing equal numbers of hydrogen or hydroxyl ions enter in each case.

In order to test this the rates of penetration of dye were compared among the three groups of cells previously placed<sup>10</sup> for 10 minutes (1) in tap water (control), (2) in phosphate buffer solution, and (3) in borate buffer solution, all at pH 7.7, and it was found (as shown in Table III) that with phosphate buffer solution there was about 30 per cent less dye in the vacuole than in the case of the control, and with borate buffer about 13 per cent less dye (which may not be significant since the probable error of the mean is rather high).

The experiments were extended to higher pH values, pH 8.1 and 7.3, and it was found, as shown in Table III, that the rate of penetration of dye is again lower in the case of cells previously exposed<sup>10</sup> to the phosphate buffer solution than that in the case of cells exposed to the borate solution. Such a difference in behavior between the borate and the phosphate buffer mixtures cannot be due to the effect of H or OH ions as such on the cell, since the pH value is the same in both these solutions.

Other experiments are therefore needed to determine just what causes this difference.

This difference between the phosphates and the borates, as affecting the rate of penetration of dye, is not due to the difference in the effect<sup>12</sup>

<sup>12</sup> It is not possible, unfortunately, to determine if there is an effect of acetate buffer mixture on the dye, since it is impossible to determine the penetration of dye at a pH value lower than pH 6.2 in the case of *Nitella*.

of these buffer mixtures directly on the dye, as is proved by the fact that when the cells are transferred<sup>10</sup> directly from the tap water to

TABLE III.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella*, when cells were previously exposed to M/150 borate and phosphate buffer solutions at different pH values for 10 minutes after which they were placed for 1 minute in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture).

External solutions.	In tap water at pH 7.7.	In phosphate buffer solution at pH 7.7.	In borate buffer solution at pH 7.7.	In phosphate buffer solution at pH 8.1.	In borate buffer solution at pH 8.1.
	M	M	M	M	M
Amount of dye in sap.....	0.000079	0.000055	0.000069	0.000059	0.000079
Percentage decrease on basis of 0.000079 as standard.....		30 per cent	13 per cent	26 per cent	0 per cent

External solutions.	In phosphate buffer solution at pH 7.3.	In borate buffer solution at pH 7.3.	In phosphate buffer solution at pH 6.6.	In borate buffer solution at pH 8.7.
	M	M	M	M
Amount of dye in sap.....	0.000052	0.000069	0.000048	0.000079
Percentage decrease on basis of 0.000079 as standard.....	35 per cent	13 per cent	39 per cent	0 per cent

TABLE IV.

Comparison of the amount of brilliant cresyl blue in the sap after 1 minute in 0.00017 M dye solutions at pH 7.7 made up with different buffer mixtures (M/150).

External dye solutions.	Borate buffer mixture.	Phosphate (ordinary) $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$ .	Phosphate (lacking K) $\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$ .
	M	M	M
Amount of dye in sap.....	0.00041	0.00035	0.00040

the dye solution at pH 7.7 made up (1) with borate buffer mixture and (2) with phosphate buffer mixture, the rate of penetration of the dye is the same, as shown in Table IV.

This difference furthermore is not due to a specific action of the K in the phosphate buffer mixture since the experiments were repeated<sup>10</sup> with the solution made up with  $\text{Na}_2\text{HPO}_4$  containing  $\text{NaH}_2\text{PO}_4$  instead of  $\text{KH}_2\text{PO}_4$ , at pH 5.4 and 7.7 and the same result was obtained, as shown in Table V.

TABLE V.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella* after 1 minute exposure to 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) following a 10 minute exposure to the M/150 phosphate buffer mixtures consisting of  $\text{Na}_2\text{HPO}_4$  and either  $\text{KH}_2\text{PO}_4$  or  $\text{NaH}_2\text{PO}_4$ .

External solutions.	In tap water at pH 7.7.	In $\text{Na}_2\text{HPO}_4$ + $\text{KH}_2\text{PO}_4$ at pH 7.7.	In $\text{Na}_2\text{HPO}_4$ + $\text{NaH}_2\text{PO}_4$ at pH 7.7.	In $\text{Na}_2\text{HPO}_4$ + $\text{KH}_2\text{PO}_4$ at pH 5.4.	In $\text{Na}_2\text{HPO}_4$ + $\text{NaH}_2\text{PO}_4$ at pH 5.4.
	M	M	M	M	M
Amount of dye in sap.....	0.000084	0.000055	0.000059	0.000048	0.000048
Percentage decrease with 0.000084 as standard.....		33 per cent	29 per cent	42 per cent	42 per cent

TABLE VI.

Comparison of amount of brilliant cresyl blue in the vacuole when cells of *Nitella* (autumn) are placed in 0.00004 M dye solution (stirred) at pH 7.7 (M/150 borate buffer) for  $\frac{1}{2}$  minute, after they have been exposed for 10 minutes to various solutions.

External solutions.	Tap water pH 7.7.	Boric acid pH 4.8.	Hydrochloric acid pH 4.8.	Phosphoric acid pH 4.8.	Phosphate buffer pH 5.4.
	M	M	M	M	M
Dye in sap.....	0.000072	0.000076	0.000069	0.000058	0.000042
Percentage decrease on basis of 0.000072 as standard.....		5 per cent increase (?)	5 per cent	20 per cent	42 per cent

The inhibiting effect of phosphate buffer mixtures is greater the lower the pH value, as shown in Table III. It may be that this is due to the greater amount of phosphoric acid present in the buffer mixture, if we assume that as a weak acid it penetrates the protoplasm as undissociated molecules and dissociates after entering and

lowers the pH value of the protoplasm, so that when cells are subsequently placed in a dye solution there will be less DB (since DB changes to DS more at a low pH value) in the protoplasm than in the case of the control cells which are transferred directly from tap water to the dye solution. The rate of penetration of DB from the protoplasm to the vacuole will therefore be less than in the case of the control cells.

This assumption<sup>13</sup> is partly supported by the following result. When the cells<sup>13</sup> are exposed for 10 minutes to three separate solutions, (1) hydrochloric acid, (2) boric acid, (3) phosphoric acid, all at pH 4.8, and then placed in the dye solution<sup>13</sup> (borate) for  $\frac{1}{2}$  minute, the rate of penetration of dye (as compared with that of the control cells, which are transferred directly from tap water to the same dye solution) in the case of hydrochloric acid and boric acid is about the same as that of the control. This indicates that H ions do not affect<sup>13</sup> the cell and that if boric acid enters the cell as undissociated molecules it does not afterward dissociate sufficiently to lower the pH value to any appreciable degree. Phosphoric acid behaves differently in that the rate of penetration of the dye in the case of the cells exposed previously to this acid is found to be about 20 per cent lower than that of the control, which indicates that phosphoric acid enters the

<sup>13</sup> There are several other possible explanations, for example:

(1) On the basis that phosphoric acid enters more rapidly than boric. We are unable to prove this experimentally, for which reason the explanation described in the text is used instead.

(2) On the basis that a weak acid enters the protoplasm as undissociated molecule and by dissociating lowers the pH value of the protoplasm and that when such cells are removed from the buffer solution to the dye solution, the weak acid diffuses out from the protoplasm into the film of liquid between the protoplasmic surface and the cell wall, and lowers the pH value of the film thereby decreasing the ratio of DB/DS in the film. This will explain the difference between boric acid and phosphoric acid, in that boric acid does not change the pH value of the film since it is too weak an acid, while phosphoric acid is sufficiently strong to bring about this change. But this assumption is not so satisfactory as the one described in the text when we consider the fact that there is an inhibiting effect on the rate of penetration of dye even with cells previously exposed to a phosphate buffer solution at pH 8.1 and then placed in dye solution at much lower pH value (pH 6.6). In such a dye solution one would expect further entrance of phosphoric acid into the cell, rather than exit of the acid from the protoplasm to the exterior of the cell. These cells were collected in autumn.

The dye solution was stirred. At a lower pH value both phosphoric acid and hydrochloric acid have an inhibiting effect which is greater in the case of the former.

protoplasm and then dissociates sufficiently to lower the pH value or else that it has a specific effect on the surface (Table VI).

The cause of the decrease brought about by the phosphate buffer mixture may be threefold, (1) due to undissociated phosphoric acid, (2) due to the Na and K salts present in the buffer mixture, and (3) due possibly to certain anions.

It may be of interest to add here the following. When cells (collected in Cambridge) are exposed for 10 minutes to solutions at different concentrations (0.05 M to 0.006 M) of NaCl, LiCl, KCl, Na<sub>2</sub>SO<sub>4</sub>, and NaNO<sub>3</sub> made up in distilled water, after which they are washed in distilled water for 5 seconds, wiped, and are placed in 0.00014 M dye solution at pH 7.7 (borate buffer mixture) for 1 minute, the rate of penetration is considerably decreased as compared with the control. If cells are placed directly for 1 minute (without such treatment) in 0.00014 M dye solution at pH 7.7 (borate buffer mixture) containing any one of these salts, the rate is found to be slightly higher than in the case of cells placed in dye solution containing no salt.

Solutions of MgCl<sub>2</sub>, MgSO<sub>4</sub>, CaCl<sub>2</sub>, LaCl<sub>3</sub>, and LaNO<sub>3</sub> all behave alike, in that when cells are exposed to these solutions for 10 minutes and then transferred to the dye solution, the rate of penetration of dye is about the same as the control. When cells are placed without such treatment in dye solutions containing any one of these salts (LaCl<sub>3</sub> omitted), the rate is found to be somewhat higher than that of the control.

Thus there is evidence for the inhibiting effect<sup>14</sup> of the salts with

<sup>14</sup>The experiments described in the text (see Table III) show that the borate buffer mixtures have no inhibiting effect on the rate of penetration of dye. In view of the fact that the borate buffer mixtures at higher pH values contain a considerable amount of Na, there is an apparent discrepancy between the results obtained in this case and those in the case of NaCl solutions in which there is a considerable inhibiting effect due to the presence of Na (this discrepancy was mentioned in the writer's previous paper (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, No. 1)). This, however, may be due to the fact that in the case of cells previously exposed to the borate buffer mixtures the dye was made up with phosphate buffer mixtures which seem to diminish the inhibiting effect of Na, while in the case of cells previously exposed to NaCl solutions the dye used was made up with borate buffer mixture which does not seem to have this effect. The following experiments may make this clear. When cells previously exposed to (1) 0.01 M NaCl and (2) to 0.005 M sodium borate solutions for 10 minutes were washed for 5 seconds in distilled water, wiped, and placed in 0.00014 M dye solution at pH 7.7 (borate buffer mixture), there is a considerable inhibiting effect

monovalent base cations which is not easily reversible, since the effect may be brought about by placing the cells in 0.01 M NaCl solution for 5 minutes, but this effect does not disappear after the cells have been transferred to distilled water and left for over an hour. This effect, however, may be readily removed if cells are placed in a solution of salt with bivalent or trivalent cations, such as  $MgCl_2$  and  $LaCl_3$  at certain concentrations.

Further experiments are being carried out on this subject by the writer.

## VI.

### *Experiments on Valonia.*

The experiments have been repeated with *Valonia macrophysa* but the results described below are approximate and show only relative values, owing to the fact that the pH value of the sap cannot be accurately determined without special experiments and that the sea water not only shifts the dissociation constant considerably, but seems to change the nature of the dye, especially at lower pH values.

*A. The Effect of Aqueous Ammonia (Free and Combined).*—Since the method of determining the change in the pH value after placing cells in solutions has been described in detail in Section II, it will be omitted here. When cells of *Valonia* were placed for 1 hour in sea water containing 0.003 M  $NH_4Cl$  solution, the pH of the sap increased from 6 (normal) to 6.6 (determined colorimetrically by using brom-cresol purple). When such cells were replaced in sea water and left for 1 hour the pH value decreased from 6.6 to 6.1.

One group of cells was placed in sea water containing 0.00035 M dye, a second group in sea water containing 0.003 M  $NH_4Cl$  and

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which is slightly greater with (1) than with (2). If such cells (1) and (2) are placed in 0.00014 M dye solution at pH 7.7 (phosphate buffer solution) they show no inhibiting effect at all.

At a higher concentration of NaCl (0.05 M) this inhibiting effect is not removed in 0.00014 M dye made up with phosphate buffer mixture at pH 7.7.

The inhibiting effect of previous treatment with the phosphate buffer mixture at pH 5.4 (Table II) is increased in 0.00014 M dye solution at pH 7.7 made up with borate buffer mixture.

0.00035 M dye. A third group of cells was first exposed for 1 hour to sea water containing 0.003 M  $\text{NH}_4\text{Cl}$  and then transferred to the dye solution used in the case of Group 1. After 1 hour there was a decided decrease in the rate of penetration of dye in the case of cells placed in the dye solution containing  $\text{NH}_4\text{Cl}$  (Group 2) and also in the case of cells previously exposed to  $\text{NH}_4\text{Cl}$  solution (Group 3), as compared with the control (Group 1). These results show that the presence of ammonia in the cell brings about a decrease in the rate of penetration of dye. Whether this decrease is entirely due to the increase in the pH value of the sap in the presence of ammonia or due partly to the former and partly to the presence of ammonia in the protoplasm (at the surface or the interior), it is not possible to determine. These results confirm those obtained with *Nitella*<sup>3</sup> (see Section I).

*B. Effect of Acetic Acid and HCl at pH 5.9.*—Let us first see if the same results may be obtained as with *Nitella* when the pH value of the sap is decreased by entrance of acetic acid. When cells were placed in sea water containing acetic acid at pH 5.9, the pH value of the sap decreased in 1 hour from 5.5 (normal) to 4.8 (methyl<sup>15</sup> red used as an indicator). The pH value of the sap thus decreased was found to be raised to the normal when such cells were placed in sea water for 20 minutes. When cells whose pH value had been thus decreased were placed for 20 minutes in sea water containing 0.00035 M dye, the amount of dye in the sap was less<sup>16</sup> than in the sap of cells transferred directly from the sea water to the same dye solution (control). These experiments show that there is a decrease in the rate of penetration of dye when the pH value of the sap is decreased

<sup>15</sup> Difference between the determination of the pH value of the sap made with brom-cresol purple and with methyl red lies in the fact that the effect of salt on the indicator is not corrected. The explanation of the use of the indicators is described in Section I.

<sup>16</sup> Brooks exposed cells of *Valonia macrophysa* to sea water (1) containing  $\text{NH}_4\text{Cl}$  until the pH value of the sap increased, and (2) containing  $\text{CO}_2$  until the pH value of the sap decreased, after which they were placed in sea water containing 2, 6, dibromophenol indophenol, and found that the rate of penetration of dye decreased with (1) and increased with (2). She interprets these results on the basis that the rate of penetration of dye is affected by the change in the pH value of the external solution surrounding the cell as a result of diffusion of (1)  $\text{NH}_4\text{Cl}$  and (2)  $\text{CO}_2$  from the vacuoles. (See Foot-note 6.)

by entrance of acetic acid, which agrees with the results discussed in Section III on *Nitella*.

Let us now see if a decrease can be brought about without a change in the pH value of the sap. Cells were exposed for 1 hour to sea water containing HCl at pH 5.9, after which they were placed in 0.00035 M dye for 20 minutes. The rate of penetration in this case was found to be less than the control but the extent of decrease in the rate is not so great as it was in the case of cells exposed to acetic acid.

When cells are placed for 1 hour in sea water containing 0.0007 M dye at pH 5.9, (1) containing acetic acid and (2) containing HCl, the rate of penetration was found to be higher with acetic acid than with HCl.

C. *Effect of Sea Water at pH 6.5 Containing either Acetic Acid or HCl (No Change in the pH Value of the Sap)*.—The question now arises as to what will happen if we put cells in sea water containing acetic acid at a pH value at which there is no decrease in the pH value of the sap. Cells were placed in sea water at pH 6.5 containing acetic acid for 1 hour after which they were transferred to sea water containing 0.00035 M dye for 20 minutes: the pH value of the sap remained normal. When the rate of penetration of dye in the case of the cells thus treated was compared with that of the control (cells directly removed from the sea water and placed in the same dye solution), it was found to be the same. In the case of the cells previously exposed to sea water containing HCl at pH 6.5 the rate of penetration of dye was also found to be the same.

Cells placed in 0.00017 M dye in sea water at pH 6.5 containing (1) acetic acid and (2) HCl, showed no difference in the rates.

Thus these experiments show that in the case of *Valonia* also the rate of penetration of dye may be retarded when (1) the pH value of the sap is decreased in presence of acetic acid, and (2) the pH value of the sap is increased in presence of  $\text{NH}_3$ , when cells are exposed to these solutions before they are placed in the dye solutions.

#### SUMMARY.

When living cells of *Nitella* are exposed to an acetate buffer solution until the pH value of the sap is decreased and subsequently placed in a solution of brilliant cresyl blue, the rate of penetration of dye into the vacuole is found to decrease in the majority of cases,



and increase in other cases, as compared with the control cells which are transferred to the dye solution directly from tap water. This decrease in the rate is not due to the lowering of the pH value of the solution just outside the cell wall, as a result of diffusion of acetic acid from the cell when cells are removed from the buffer solution and placed in the dye solution, because the relative amount of decrease (as compared with the control) is the same whether the external solution is stirred or not.

Such a decrease in the rate may be brought about without a change in the pH value of the sap if the cells are placed in the dye solution after exposure to a phosphate buffer solution in which the pH value of the sap remains normal. The rate of penetration of dye is then found to decrease. The extent of this decrease is the greater the lower the pH value of the solution.

It is found that hydrochloric acid and boric acid have no effect while phosphoric acid has an inhibiting effect at pH 4.8 on stirring.

Experiments with neutral salt solutions indicate that a direct effect on the cell (decreasing penetration) is due to monovalent base cations, while there is no such effect directly on the dye.

It is assumed that the effect of the phosphate and acetate buffer solutions on the cell, decreasing the rate of penetration, is due (1) to the penetration of these acids into the protoplasm as undissociated molecules, which dissociate upon entrance and lower the pH value of the protoplasm or to their action on the surface of the protoplasm, (2) to the effect of base cations on the protoplasm (either at the surface or in the interior), and (3) possibly to the effect of certain anions. In this case the action of the buffer solution is not due to its hydrogen ions.

In the case of living cells of *Valonia* under the same experimental conditions as *Nitella* it is found that the rate of penetration of dye decreases when the pH value of the sap increases in presence of  $\text{NH}_3$ , and also when the pH value of the sap is decreased in the presence of acetic acid. Such a decrease may be brought about even when the cells are previously exposed to sea water containing HCl, in which the pH value of the sap remains normal.

The writer wishes to thank Miss Helen McNamara for her faithful assistance in carrying out the experiments.