COUNTERACTION OF THE INHIBITING EFFECTS OF VARIOUS SUBSTANCES ON NITELLA.

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I.

INTRODUCTION.

Previous observation[1,2] indicates that cresyl blue enters the vacuole of living cells of *Nitella* and *Valonia* much more rapidly in form of free base (predominating at higher pH values) than in form of salt (predominating at lower pH values), and that upon its entrance part of the free base is at once converted into salt depending on the condition of the sap (*viz.* pH value). Difference in the rate of penetration of these two forms of dye may be attributed to the difference in the extent to which they are taken up by each non-aqueous layer of protoplasm from one aqueous phase and given up to the other aqueous phase.

Any factors affecting the dye or the cell may alter the rate of penetration of dye into the vacuole. For example, the rate is altered by the effect of salt and hydrogen ions on the dye.

As to the control of the rate by the cell, it is uncertain just what part of the cell plays the predominant rôle. Cells of *Nitella* consist of cell wall surrounding a very thin layer of protoplasm enclosing a central vacuole filled with aqueous sap at about pH 5.5. If we neglect the cell wall by assuming that the effect of substances on the cell wall are insufficient in the present case to change the rate of penetration of dye into the vacuole, then we may imagine the penetration of dye as dependent on the diffusion of free base through the protoplasm into the vacuole. The protoplasm may be assumed to consist of an

[1] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561.

[2] Irwin, M., *J. Gen Physiol.*, 1926–27, x, 75.

outer non-aqueous layer in contact with the external medium, a middle aqueous layer, and an inner non-aqueous layer surrounding the vacuole. The surface boundaries at these layers may also play a specific rôle in affecting the rate.

Experiments have been made to determine if alteration in any of these parts of the cell affects the rate of penetration. It was found that a change in the pH value of the sap brought about a change in the rate. For example, a decrease in the rate took place when the pH value of the sap was either increased[3,4] by entrance of ammonia, or decreased[5] by entrance of acetic acid. But these results do not necessarily indicate that the condition in the sap alone controlled the rate, because a simultaneous alteration in the protoplasm might very well be the cause of this decrease.

Evidence that the protoplasm can control the rate of penetration has been given.[5,6] A decrease in the rate takes place when the cells are previously exposed to substances like sodium chloride[6] and phosphate buffer[5] at pH 5.5, etc., which brought about no change in the pH value of the sap.

Such an inhibiting effect was found to be counteracted if the cells were subsequently treated with a substance like magnesium chloride,[6] or if the dye contained a certain concentration of base cations, such as sodium, potassium, or magnesium.

The present paper deals with the counteraction of inhibiting effects (a preliminary report[7] has already been published).

II.

Methods.

A description of methods has been published.[8] Mention may be made, however, of a few points of importance. Cells of *Nitella flexilis* collected in autumn

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[3] McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501.

[4] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 235.

[5] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 54; *J. Gen. Physiol.* 1926–27, x, 271.

[6] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 425.

[7] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 245.

[8] See last reference in Foot-note 5 and also Irwin, M., *J. Gen. Physiol.*, 1925–27, viii, 147, and Foot-notes 2 and 4.

(New York II) were employed. The condition of the cell during the experiment is very difficult to determine exactly. Reversible injury is almost impossible to detect. For this reason it was necessary to carry out experiments on a uniform basis, in order that the condition of the cells might be as uniform as possible. Cells of uniform size, thickness, and turgidity, from the central portion of the plant, were chosen. Cells collected near New York were kept in the laboratory not longer than 4 days after they were collected. Such concentrations of solutions as maintain the cells in as good a condition as possible during the experiments were selected. The criteria for the condition of cells were as follows: (1) turgidity tested by means of touch (as cells become injured they lose turgidity more and more until they collapse after death); (2) the ability of cells to live for several hours in the test solutions while the experiments lasted only a few minutes; (3) the behavior of the cells after experiments: cells were transferred from the test solutions to tap water, and after 1 day the turgidity and mortality of cells were compared with those of the control cells (cells which have not been exposed to any test solutions). Judged by these criteria, the cells referred to in these experiments seemed normal.

Unless otherwise stated the exposure of cells to different solutions other than the dye solution lasted 10 minutes, and such solutions were not stirred. Buffers were made up according to directions given by Clark.[9] For phosphate buffer, a suitable mixture of M/15 Na_2HPO_4 and M/15 KH_2PO_4 was diluted ten times; for borate buffer, a suitable mixture of M/5 boric acid and M/20 borax was diluted ten times. Brilliant cresyl blue (prewar Grübler) was used. If the dye solutions were stirred this was done at the rate of one revolution per minute.

The pH values of the solutions made up with buffer mixtures were determined by means of the hydrogen electrode. Those of other solutions were determined colorimetrically.

In the 7×10^{-5} M dye solution at pH 7.85 made up with borate buffer mixture the proportion of the mixture used was 1 of M/20 borax to 9 of M/5 boric acid and this mixture was diluted ten times with distilled water, while in the dye made up with phosphate buffer mixture the proportion was 8.85 of M/15 secondary to 1.15 of M/15 primary phosphates and the mixture diluted ten times. In 35×10^{-5} M dye solution at pH 6.79 made up with phosphate buffer mixture the proportion used was 4 of M/15 secondary to 6 of the M/15 primary phosphates, and this mixture was diluted ten times with distilled water.

In every case cells were wiped, dipped in distilled water for 3 seconds, and wiped again before they were placed in any solution.

The pH value of the sap was determined colorimetrically using either methyl red or brom cresol purple as an indicator. The pH value of the sap remained unchanged in every experiment.

The concentration of dye in the sap was determined colorimetrically. The end

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[9] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

of the cell was cut, and the sap was gently squeezed out onto a glass slide. The sap was then drawn up into a capillary tube and the color of this tube was matched with that of the capillary tube containing a standard dye solution. The dye measured represents the total amount of dye found in the sap, consisting of uncombined free base and salt, and possibly some dye in combination with constituents of the sap.

The experiments were carried out in an incubator at 25 ± 0.5°C. During the experiment diffused light entered the incubator through small air holes and through a partly open door.

Each figure given in the tables represents an average from 80 to over 100 readings on individual cells and the probable error of the mean is less than 7 per cent of the mean.

III.

Removal of the Inhibiting Effect of Phosphate Buffer Mixture, Phosphoric Acid, and Hydrochloric Acid.

When cells were exposed for 10 minutes to hydrochloric acid, to phosphoric acid, or to phosphate buffer mixture, the pH value of the sap was found to remain unchanged. If such cells were placed in the dye solution made up with a borate buffer mixture at pH 7.85, the rate of penetration as compared to the control was decreased,[10] as shown in Table I.

On comparing the inhibiting effect of these substances it was found, as shown in Table I, that hydrochloric acid brought about the least and phosphate buffer mixture the greatest effect. For example, at both pH 4.2 and at pH 4.8, hydrochloric acid brought about less effect than phosphoric acid, while even at pH 5.5, where practically no effect of hydrochloric acid and only a slight effect of phosphoric acid might be expected, the phosphate buffer mixture brought about considerable inhibiting effect (Table I).

[10] These results confirm those already published by the writer. The percentage decrease given in the experiments described in the present paper, however, is a little higher, but the difference may be considered as due to experimental error or to differences in the cells used, *cf.* Table VI, *J. Gen. Physiol.*, 1926–27, x, 281. Cells in both these cases were collected in New York, and their behavior seemed different from those collected in Cambridge in that they were affected by the phosphoric acid and hydrochloric acid at pH 4.8 to about the same extent as the Cambridge *Nitella* were affected by these acids at pH 4 (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 56 and 57).

TABLE I.

Comparison of the amount of dye in the vacuolar sap of living cells of *Nitella* when cells are first exposed for 10 minutes to one of the solutions given in III, and are then placed in any of the dye solutions given in I. Buffer mixtures are given in Section II in the text.

I		II	III					
External dye solutions			Tap water or control	Phosphate buffer mixture	Hydrochloric acid	Phosphoric acid	Hydrochloric acid	Phosphoric acid
			pH 7.7	*pH 5.5*	*pH 4.8*	*pH 4.8*	*pH 4.2*	*pH 4.3*
When cells are placed for ½ minute in 7×10^{-5} M dye solution at pH 7.85 made up with borate buffer mixture	Dye stirred	Amount of dye in sap, $M \times 10^5$	21.1	9.7	19.2	16.2	15.2	12.8
		Percentage decrease or increase	Standard	54 per cent decrease	9 per cent decrease	23 per cent decrease	28 per cent decrease	39 per cent decrease
	Dye not stirred	Amount of dye in sap, $M \times 10^5$	12.4	5.0	9.5	9.0		
		Percentage decrease or increase	Standard	60 per cent decrease	23 per cent decrease	28 per cent decrease		
When cells are placed for ½ minute in 7×10^{-5} M dye solution at pH 7.85 made up with phosphate buffer mixture	Dye stirred	Amount of dye in sap, $M \times 10^5$	19.7	19.2	20.5	19.7		
		Percentage decrease or increase	Standard	2 per cent decrease	4 per cent increase	No change		
	Dye not stirred	Amount of dye in sap, $M \times 10^5$	11.4	11.7			11.0	12.4
		Percentage decrease or increase	Standard	3 per cent increase			3 per cent decrease	9 per cent increase
When cells are placed for 1 minute in 35×10^{-5} M dye solution at pH 6.79 made up with phosphate buffer mixture	Dye not stirred	Amount of dye in sap, $M \times 10^5$	19.7	15.2				
		Percentage decrease or increase	Standard	23 per cent decrease				

From these results the following conclusion may be drawn.

1. The inhibiting effect of hydrochloric acid seems to be due chiefly to the action of hydrogen ions on the protoplasm since it has been already found by the writer that the chlorides[6] do not seem to bring about any marked inhibiting effect.

2. The greater effect of phosphoric acid may be due either to the specific effect of the anion or to greater penetration of the acid as an undissociated molecule and subsequent dissociation, thereby lowering the pH value of the protoplasm more than is the case with hydrochloric acid. With the lowering of the pH value of the protoplasm the concentration of free base is decreased. We might then expect a decrease in the rate of penetration of dye if we assume that the rate is controlled by the diffusion of free base from the protoplasm into the vacuole.

3. The inhibiting effect produced by phosphate buffer, on the other hand, cannot be attributed primarily to the presence of hydrogen ions and phosphoric anions in the solution, for its inhibiting effect, even when its pH value is 5.5, is greater than that of hydrochloric or phosphoric acid at pH 4.2 (*cf.* Table I). It must be attributed largely to the presence of monovalent base cations, such as sodium and potassium, which bring about an appreciable decrease in the rate of penetration of dye when cells are exposed to them in the same way.

When the dye solution was not stirred the extent of decrease in the rate of penetration of dye was slightly greater than when it was stirred as shown in Table I. It is uncertain whether the greater decrease brought about by lack of stirring is due to the less rapid diffusion of the substances from the cell or to the lowering of the pH value of the solution just outside the cell, as resulting from the accumulation of these substances as they diffuse out of the cell, or due to experimental error.

On the other hand, if cells were exposed to these various solutions and placed in the dye solution made up with phosphate buffer mixture at pH 7.85 the rate of penetration instead of decreasing as before was found to be about the same[7] as that in the case of the cells transferred directly from tap water to the same dye solution. This was the case whether the dye solution was stirred or not (Table I). This absence of the inhibiting effect was not chiefly due to the effect of the phosphate

buffer mixture alone on the protoplasm, because when cells were washed in the phosphate buffer mixture at pH 7.85 for $\frac{1}{2}$ minute (stirred) before they were placed in the dye + borate at pH 7.85, the usual inhibiting effect was observed (Table III).

Unless the cells were first exposed to the inhibiting substances and then placed in the dye solutions there was no marked difference in the behavior of the dye + borate and of the dye + phosphate at pH 7.85. For example, if cells were transferred directly from tap water to these two dye solutions at pH 7.85 the rate of penetration of dye was about the same in dye + borate as in dye + phosphate (see control, Table I).

It would be desirable to know the cause of this difference in behavior. We might assume that the decrease in the rate of dye penetration with the dye + borate at pH 7.85 is due to the decrease in the pH value of the dye solution just outside the protoplasm, which might take place as the dye comes in contact with the substance to which the cells have been exposed before they were placed in the dye and which might adhere to the surface of the protoplasm, and that the decrease does not take place with the dye + phosphate at pH 7.85 because it is sufficiently buffered to keep the pH value constant. In so far as the dye + borate at pH 7.85 and dye + phosphate at pH 7.85 are concerned, this explanation may hold since the latter is more buffered than the former, but when we compare their behavior with that of the dye + phosphate at pH 6.79, we find this interpretation untenable. On this assumption we should expect no decrease in the rate of dye penetration with the dye + phosphate at pH 6.79, because it is even more buffered than the dye + phosphate at pH 7.85, but the experiments show that there is about 23 per cent decrease when cells are exposed, for example, to a phosphate buffer solution at pH 5.5 (*cf.* Table I). Thus we may conclude that the occurrence of a decrease with dye + borate at pH 7.85 and its absence with dye + phosphate at pH 7.85 cannot be attributed to the difference in the changes of pH values of the dye solutions just outside the protoplasm, as a result of the difference in their buffer effects.

A better assumption is that the dye is affected by the cations (sodium and potassium) present in these buffer solutions with which the dye is made up in such a way as to counteract the inhibiting effect of various substances on the protoplasm. According to this theory the

greater the concentration of such cations in the dye the less the inhibiting effect. This assumption is supported by the results which show that the counteraction of the inhibiting effect is proportional to the concentration of base cations such as sodium and potassium in the dye solutions, in the following order: dye + phosphate at pH 7.85 > dye + phosphate at pH 6.79 > dye + borate at pH 7.85 (Table I). If this supposition is correct an addition of the proper concentration of

TABLE II.

Comparison of the amount of dye in the vacuolar sap of living cells of *Nitella* when cells are first exposed for 10 minutes to any one of the solutions (not stirred) stated in III, and are then placed in one of the brilliant cresyl blue solutions (stirred) stated in I for ½ minute. Buffer mixtures are given in Section II in the text.

I	II	III			
External dye solution		Tap water or control	Phosphate buffer mixture	Hydrochloric acid	Phosphoric acid
			pH 5.5	*pH 4.2*	*pH 4.3*
7×10^{-5} M dye solution at pH 7.85 made up with borate buffer mixture containing 0.01 M magnesium chloride	Amount of dye in sap, $M \times 10^5$	22.8	22.8	21.4	22.1
	Percentage decrease or increase	Standard	No change	6 per cent decrease	3 per cent decrease
7×10^{-5} M dye solution at pH 7.85 made up with borate buffer mixture containing 0.02 M sodium chloride	Amount of dye in sap, $M \times 10^5$	24.2	22.4	22.4	22.8
	Percentage decrease or increase	Standard	7 per cent decrease	7 per cent decrease	6 per cent decrease

base cations to the dye + borate at pH 7.85 should cause the dye + borate at pH 7.85 to behave like the dye + phosphate at the same pH value and should counteract the inhibiting effect. The experimental results show that this is precisely what takes place. If cells previously exposed to an inhibiting substance were placed in the dye + borate solution containing sodium chloride the rate of penetration of dye remained the same[7] as that of the control (cells transferred directly from

the tap water to the same dye solution, as shown in Table II). That this counteraction of the inhibiting effect is not due to the effect of the sodium chloride on the borate buffer mixture alone is indicated

TABLE III.

Comparison of the amount of dye in the vacuolar sap of living cells of *Nitella* when cells are first exposed for 10 minutes to one of the various solutions (not stirred) stated in III, are then washed for ½ minute in one of the solutions (stirred) stated in I, and are finally placed for ½ minute in 7×10^{-5} M brilliant cresyl blue solution (stirred) made up with borate buffer mixture at pH 7.85. Buffer mixtures are given in Section II in the text.

I	II	III			
Solution in which cells are washed		Tap water or control	Phosphate buffer mixture	Hydrochloric acid	Phosphoric acid
			pH 5.5	*pH 4.2*	*pH 4.3*
Borate buffer mixture at pH 7.85	Amount of dye in sap, $M \times 10^6$	21.7	9.0	16.6	17.3
	Percentage decrease or increase	Standard	59 per cent decrease	24 per cent decrease	20 per cent decrease
Borate buffer mixture at pH 7.85 containing 0.02 M NaCl	Amount of dye in sap, $M \times 10^6$	22.4	8.6	12.4	9.0
	Percentage decrease or increase	Standard	62 per cent decrease	45 per cent decrease	60 per cent decrease
Borate buffer mixture at pH 7.85 containing 0.01 M $MgCl_2$	Amount of dye in sap, $M \times 10^6$	21.7	23.4	22.1	23.1
	Percentage decrease or increase	Standard	8 per cent increase	2 per cent increase	6 per cent increase
Phosphate buffer mixture at pH 7.85	Amount of dye in sap, $M \times 10^6$	21.0	9.5	16.0	13.0
	Percentage decrease or increase	Standard	55 per cent decrease	24 per cent decrease	38 per cent decrease

by the following experiments. When cells previously exposed were washed for ½ minute in the borate buffer solution at pH 7.85 containing sodium chloride (stirred), before they were placed in the dye + borate solution containing no sodium chloride, the washing did not remove this inhibiting effect but, in fact, the rate of penetration of dye was found to decrease, even more than when the cells had not been washed (Table III).

Not only addition of monovalent base cations but addition of bivalent base cations to the dye + borate solution at pH 7.85 also removed the inhibiting effect (Table II). There was a difference, however, between the behavior of the monovalent and bivalent base cations, in that washing cells with the former just before the cells were placed in the dye increased the inhibiting effect while washing with the latter removed the inhibiting effect (Table III). This removal of the inhibiting effect in this case seems to be due chiefly to the magnesium chloride and only slightly due to the borate buffer mixture since washing the cells for ½ minute in borate buffer mixture at pH 7.85 (stirred) containing no magnesium chloride only partly removed the inhibiting effect of the hydrochloric acid and phosphoric acid and had practically no influence on the inhibiting effect of the phosphate buffer mixture at pH 5.5 (*cf.* Table III).

IV.

Removal of the Inhibiting Effect of Sodium Chloride.

If the assumption is correct that the inhibiting effect of the phosphate buffer mixture is chiefly due to the action of monovalent base cations on the protoplasm, and if it is this effect that is removed under the experimental conditions discussed in Section III, we should expect the inhibiting effect of sodium in sodium chloride to be likewise removed. When the experiments were made to test this point it was found that the inhibiting effect of sodium chloride manifested when the dye was made up with borate buffer solution at pH 7.85 was removed if cells previously exposed to 0.02 M solution of sodium chloride were treated in the following manner.

1. If cells were placed in the dye solution made up with phosphate buffer mixture at pH 7.85 (Table IV). The removal is not due to the

effect of phosphate buffer alone because when cells were washed in this buffer solution containing no dye the inhibiting effect was not removed (Table IV).

TABLE IV.

Comparison of the amount of dye in the vacuole of living cells of *Nitella* when cells are first exposed for 10 minutes to one of the solutions (not stirred) stated in III and are then placed for 1 minute in one of the 7×10^{-5} M brilliant cresyl blue made up with solutions (not stirred) stated in I. Buffer mixtures are given in Section II in the text.

I	II	III			
External dye solution		Tap water or control	NaCl	$MgCl_2$	0.02 M NaCl + 0.01 M $MgCl_2$
			0.02 M	*0.01 M*	
Made up with borate buffer mixture at pH 7.85	Amount of dye in sap, $M \times 10^5$	18.6	7.6	20.7	16.9
	Percentage decrease or increase	Standard	59 per cent decrease	11 per cent increase	9 per cent decrease
Made up with phosphate buffer mixture at pH 7.85	Amount of dye in sap, $M \times 10^5$	19.0	20.0		
	Percentage decrease or increase	Standard	5 per cent increase		
Cells are washed for 1 minute in phosphate buffer mixture at pH 7.85 before they are placed in the dye solution made up with borate buffer mixture at pH 7.85	Amount of dye in sap, $M \times 10^5$	18.7	8.3		
	Percentage decrease or increase	Standard	56 per cent decrease		
Cells are placed in borate buffer mixture at pH 7.85 containing 0.01 M $MgCl_2$	Amount of dye in sap, $M \times 10^5$	19	18.8		
	Percentage decrease or increase	Standard	1 per cent decrease		

2. If cells were placed in dye solution containing sodium chloride[6] or magnesium chloride (Table IV).

3. If cells were washed with bivalent or trivalent cations[6] just before the cells were placed in the dye.

A salt, such as magnesium chloride, may remove the inhibiting effect in absence[6] of dye but phosphate buffer mixture is capable of removing it only in presence of dye (Table IV).

Thus we may conclude that the inhibiting effect of sodium chloride may be removed just as is the case with that of the phosphate buffer mixture.

In addition, it was found that when cells were exposed to a mixture of 0.01 M magnesium chloride and 0.02 M sodium chloride, before they were placed in the dye + borate solution at pH 7.85, the inhibiting effect of sodium was practically removed (Table IV).

As in the case of the other substances discussed in Section III, we may also assume in the case of sodium chloride that the inhibiting effect of sodium on the protoplasm is counteracted by the presence of base cations, such as sodium, potassium, and magnesium, in the dye solution, or by magnesium in absence of dye. It is difficult to state just how this takes place: the base cations above mentioned may act on the dye in such a way that dye is altered so as to be able (1) to penetrate more readily the protoplasm which had been already affected by sodium chloride or (2) to drive the sodium out of the protoplasm or (3) to affect the protoplasm in such a way as to nullify the inhibiting effect of sodium.

Regarding the first assumption the following may be stated. We do not know whether sodium chloride, for example, affects the dye in such a way as to increase its power to penetrate to the extent of counteracting entirely the inhibiting effect of sodium chloride, though experiments[6] have shown that when the dye contains sodium chloride it penetrates more rapidly into the cells and that this increase is a little greater when the cells have been previously exposed to sodium chloride. Spectrophotometric analysis of the dye shows that sodium chloride at 0.1 M does not affect the dye sufficiently for the change to be detected by this method but at higher concentrations it affects the dye in such a way that the absorption curve resembles that of a higher concentration of dye.

As to the second assumption, the experimental results so far obtained are not sufficiently complete to be reported but the writer hopes to be able to present them later.

In relation to the third assumption previous experiments[6] have shown that an inhibiting effect was brought about whether the cells were exposed to the sodium chloride in presence or absence of dye (during this period no dye entered the vacuole) so long as they were subsequently placed in the dye borate containing no sodium chloride at pH 7.85, but this effect was removed if the latter dye solution contained sodium chloride. These results seem to suggest that the dye at such a concentration is incapable of very readily counteracting the inhibiting effect of sodium chloride on the protoplasm but that it is affected by sodium chloride in such a way as to penetrate the protoplasm more readily. A detailed account of these experiments will be subsequently published.

V.

Removal of Inhibiting Effect of Sodium Borate.

In previous sections we have found that the inhibiting effects of phosphate buffer mixture, sodium chloride, hydrochloric acid, and phosphoric acid may be removed. It seems that the counteraction of this inhibiting effect is dependent on the counteraction of the cation effects and that anions do not play an important rôle in this respect. It might be of interest to see if salts and acids with cations common to those already studied, but with different anions, might give some other result. For this purpose sodium borate and boric acid were used.

As shown in Table V, 0.005 M sodium borate (containing 0.01 M sodium), and borate buffer mixture[11] at pH 8.4 (containing 0.0015 M sodium), showed an inhibiting effect while boric acid did not; the cells were first exposed to these solutions and then placed in dye solution at pH 7.85 made up with borate buffer mixture. The inhibiting effect with sodium borate was found to be about 38 per cent, while that of the borate buffer mixture at pH 8.4 was about 22 per cent. The

[11] The borate buffer mixture at pH 8.4 was made up with a proportion of three of M/20 borax to seven of M/5 boric acid and diluted ten times.

TABLE V.

Comparison of the amount of dye in the vacuolar sap of living cells of *Nitella* when they are first exposed for 10 minutes to one of the solutions stated in III, and are then placed for ½ minute in 14×10^{-5} M brilliant cresyl blue solution (stirred) at pH 7.85 as stated in I. Buffer mixtures are given in Section II in the text.

I	II	III				
External dye solution		Tap water or control	Boric acid	Borate buffer mixture	Sodium borate	Sodium chloride
			0.01 M	*pH 8.4*	*0.005 M*	*0.01 M*
Made up with borate buffer mixture	Amount of dye in sap, $M \times 10^5$	41.1	38.7	31.7	25.5	16.9
	Percentage decrease or increase	Standard	6 per cent decrease	23 per cent decrease	38 per cent decrease	59 per cent decrease
Made up with phosphate buffer mixture	Amount of dye in sap, $M \times 10^5$	39.0	42.8	42.4	43.5	41.6
	Percentage decrease or increase	Standard	10 per cent increase	9 per cent increase	12 per cent increase	7 per cent increase
When cells are washed for ½ minute in phosphate buffer mixture at pH 7.85 before they are placed in the dye made up with borate buffer mixture at pH 7.85	Amount of dye in sap, $M \times 10^5$	41.0			29.7	
	Percentage decrease or increase	Standard			28 per cent decrease	

absence of the inhibiting effect with boric acid might be explained on the basis that though it enters the protoplasm as an undissociated molecule it is too weak an acid to lower the pH value of the protoplasm sufficiently to bring about any inhibiting effect, or on the basis of the specificity of the anions.

In view of the fact that boric acid gives no inhibiting effect and that there is greater inhibiting effect the higher the concentration of sodium in the borate solution, we may conclude that the inhibiting effect of the borate buffer mixture is brought about chiefly by the action of sodium on the protoplasm and not by the borate ions. But since sodium borate, and sodium chloride, containing approximately the same concentration of sodium do not bring about an inhibiting effect to the same extent, we may suggest that other ions must also play some part either in counteracting or accelerating the effect of these cations.

The inhibiting effect of sodium borate, just like that of other substances described in Sections III and IV, was found to be removed if cells previously exposed were placed in the dye + phosphate solution at pH 7.85, but merely washing the cells with phosphate buffer solution at pH 7.85 did not remove this effect to any appreciable extent (Table V).

CONCLUSION.

It is assumed that (1) the inhibiting effect of hydrochloric acid is due to the action of hydrogen ions on the protoplasm, (2) the inhibiting effect of phosphoric acid (at the pH value in which hydrogen ions have practically no effect) is due to either a specific effect of the phosphate ions on the protoplasm or due to the entrance of the acid as undissociated molecule and its lowering of the pH value of the protoplasm by its subsequent dissociation, (3) the inhibiting effect of sodium chloride and sodium borate is due to the action of sodium on the protoplasm, (4) the inhibiting effect of phosphate buffer mixture is due primarily to the action of sodium and potassium ions and partly to the action of phosphoric acid on the protoplasm (see Section III).

The inhibiting effect is counteracted when cells previously exposed to these inhibiting substances are placed in the dye solution containing certain concentration of base cations or washed in a salt solution containing bivalent cations. It is not certain whether this removal of the

inhibiting effect in the presence of dye is due primarily to the action of these cations on the dye altering its nature so that it can penetrate the cell more readily or enabling it to nullify the inhibiting effect of these substances on the protoplasm either by displacing them from the protoplasm or by increasing the general permeability of the protoplasm.

It is uncertain as to whether the effect of the ions on the protoplasm is at the surface or in the interior. In case the ions penetrate, this will not necessarily contradict the theory (presented in Section I) stating that the outer layer of protoplasm is non-aqueous.[12] Certain non-aqueous substances absorb ions to a certain extent, and it may very well be that only a very small amount of ions penetrating into the protoplasm is sufficient to bring about an inhibiting effect.

The inhibiting effect discussed here does not indicate that the protoplasm is altered to decrease the rate of penetration of all substances. These experiments apply only to the penetration of brilliant cresyl blue.

SUMMARY.

When living cells of *Nitella* are first exposed to (1) phosphate buffer mixture, or (2) phosphoric acid, or (3) hydrochloric acid, or (4) sodium chloride, or (5) sodium borate, and are then placed in a solution of brilliant cresyl blue made up with a borate buffer mixture at pH 7.85, the rate of penetration of the dye into the vacuole is decreased as compared with the rate in the case of cells transferred directly from tap water to the same dye solution.

When cells exposed to any one of these solutions are placed in the dye solution made up with phosphate buffer solution at pH 7.85, the rate of penetration of dye into the vacuole is the same as the rate in the case of cells transferred from the tap water to the same dye solution.

It is probable that this removal of the inhibiting effect is due primarily to the presence of certain concentration of sodium and potassium ions in the phosphate buffer solution. If a sufficient concentra-

[12] Overton claimed that substances soluble in lipoid penetrated living cells (Overton, E., *Jahrb. wissensch. Bot.*, 1900, xxxiv, 669).

tion of sodium ions is added to the dye made up with a borate buffer mixture the inhibiting effect is removed just as it is in the case of the dye made up with the phosphate buffer mixture.

The inhibiting effect of some of these substances is found to be removed by the dye containing a sufficient concentration of bivalent cations, or by washing the cells with salts of bivalent cations.

The inhibiting effect and its removal are discussed from a theoretical standpoint.