THE EFFECT OF ACETATE BUFFER MIXTURES, ACETIC ACID, AND SODIUM ACETATE, ON THE PROTOPLASM, AS INFLUENCING THE RATE OF PENETRATION OF CRESYL BLUE INTO THE VACUOLE OF NITELLA.

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An adequate theory of the penetration[1,2,3] of cresyl blue into the vacuole of living cells of the fresh water plant *Nitella* may be stated as follows. The dye, in form of free base, which predominates at a high pH value, diffuses in and out of the vacuole very freely, while the dye in form of salt, which predominates at a low pH value, diffuses so slowly that its rate of penetration and that of the exit may be neglected in experiments of the present type. Upon the entrance of free base into the vacuole it is partly converted at once into the salt (the extent depending on the condition of the sap, *i.e.* the pH value). The concentration of free base inside the vacuole is always proportional to that of the free base in the external solution.

We may picture the entrance of dye into the vacuole as a reversible process[2] depending primarily on the diffusion of free base through a very thin layer of protoplasm which lies between the cell wall and an inner central vacuole filled with aqueous sap (at about pH 5.5 and containing about 0.1 M halides). The consideration of cell wall may be neglected since the substances in this case do not seem to affect it enough to change the rate of penetration of dye into the vacuole.

[1] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561; *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 251.

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

[3] The following investigators have stated that basic dyes in form of free base penetrated living cells: Overton, E., *Jahrb. wissensch. Bot.*, 1900, xxxiv, 669; Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507; Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1.

The protoplasm may therefore be treated here as if it were in direct contact with the external aqueous solution. The protoplasm proper[2] may be assumed to consist of (1) an outer non-aqueous layer[4] in contact with the external solution, (2) a middle aqueous layer, (3) an inner non-aqueous layer surrounding the central vacuole, and (4) their respective surface boundaries.

Thus the penetration of basic or acid dye into the vacuole may depend (1) on the ease with which each non-aqueous phase takes up the dye from one aqueous phase and gives it up to the other aqueous phase, (2) on the condition of the surface boundaries at the outer and inner non-aqueous layers, and (3) on the ratio of free base or free acid to salt in the aqueous phases in case the solubility of free base or free acid in non-aqueous layers differs from that of the salts. Thus there are to be considered two partition coefficients of a dye between non-aqueous (behaving like a lipoid) and aqueous phases at each protoplasmic boundary (external and vacuolar). It is uncertain as to whether the rate of penetration of dye is controlled by both boundaries or by one only. This theory serves to explain why many basic dyes enter the vacuole more readily in form of free base than salt, while some basic or acid dyes even in form of free base or free acid, as well as some other substances in dissociated or undissociated forms do not readily enter the vacuole of living cells. A detailed account will be subsequently published.

An alteration in any of these parts may change the rate of penetration if the rate is controlled by that particular part of the cell.

It may be possible therefore to locate the factors controlling the rate by changing the conditions in the various ways outside and inside the cell.

It was shown[1,2,5] previously that by altering the external solution (*viz.*, the pH value), it was possible to change the rate of penetration of dye.

An alteration in the protoplasm by various substances was previously found to decrease the rate of penetration of cresyl blue.

Experiments have been made[6,7] showing that decrease in the rate of penetration of dye occurred when the pH value of the sap was raised by the entrance of ammonia. But such experiments do not necessarily prove that the decrease in the rate of penetration of dye is caused by the change in the pH value of the sap, since they do not show that the rate is not controlled by a change in the protoplasm. In fact it was found[7] that a decrease in the rate of penetration of dye took place even before a measurable rise in the pH value of the sap occurred. This decrease

[4] Overton claimed that dyes soluble in lipoid penetrated the living cells (*cf.* Foot-note 3).

[5] Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 427; 1925–27, viii, 147.

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

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

in the rate may therefore be due to the increase in the pH value of the protoplasm, or to some other effect of ammonia or ammonium salt on the protoplasm (either at the surface or in the interior). Since there is no way of settling this experimentally, it is not possible to draw any final conclusion as to the cause of the decrease in the penetration of dye. Thus these experiments do not seem to prove or disprove the theory presented.

The supposition that the factor controlling the rate of penetration of dye might be located in the protoplasm under certain conditions is supported by the following experimental results. When cells are exposed to either (1) sodium chloride[8] or (2) phosphate buffer solution,[9] and are then placed in brilliant cresyl blue solution, the rate of penetration of dye into the vacuole is decreased, without any change in the sap. This decrease is found to be caused by the action on the protoplasm (1) of sodium present in sodium chloride, and (2) of monovalent base cations and phosphoric acid present in the phosphate buffer mixture.

If cells are exposed to an acetate buffer mixture,[9] in which the pH value of the sap is lowered, a decrease in the rate of penetration of dye takes place. If this decrease were actually caused by the lowering of the pH value of the sap, then the present theory of penetration of dye would have to be revised. But if this decrease is brought about by the action of acetate buffer mixture on the protoplasm, rather than by the changes in the pH value of the sap, such a result would not necessarily discredit the theory. In order to determine this point it is necessary to compare the behavior of the respective constituents of the acetate buffer mixture, namely acetic acid and sodium acetate, and to show if hydrogen ions, at the pH value used, play any part. The data and discussion of these experiments are reported in the present paper.

II.

Method.

The cells employed were collected near New York, but not in the same place as those used in previous experiments. Owing to the mild winter and sheltered

[8] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 425; *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 54.

[9] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 271.

locality, the cells were still in the same condition as they were in the early fall though these experiments were made at the beginning of winter. For convenience we shall call these *Nitella* New York II, as contrasted with the New York *Nitella* employed for the experiments described in some of the previous publications. So long as a series of comparative experiments was made within a few days with the same lot of cells it was possible to obtain consistent results.

Since the methods of choosing the best type of cells, of detecting the condition of the cells during the experiment, and of colorimetric determination of the dye in the sap have been described in detail in the writer's previous publications,[2,5,7] they will be omitted here. Great care was taken to keep the cells from injury during the experiments. The experiments were carried out in an incubator at 25 ± 0.5°C., and the dye used was prewar Grübler's brilliant cresyl blue. In all cases the buffer mixtures used were made up from Clark's[10] table; M/15 phosphate buffer mixture was diluted ten times, and acetate buffer mixture was diluted thirty times.

The dye solution at pH 6.79 was made up with M/15 phosphate buffer mixture at pH 6.64 diluted ten times, and at pH 7.85 was made up with either M/15 phosphate buffer mixture at pH 7.7 or borate buffer mixture (M/5 boric acid and M/20 borax) at pH 7.36, diluted ten times.

The pH values of the solutions of buffer mixtures were determined by means of the hydrogen electrode; the pH values of acetic acid, sodium acetate, and hydrochloric acid were determined colorimetrically. The pH values of the sap were determined by use of methyl red.

In all cases the cells were wiped, dipped in distilled water for 5 seconds, and again wiped before they were placed in the dye solution. The cells were always wiped before they were placed in any solution.

Each experiment represents an average of over 100 readings on individual cells. The probable error is less than ± 6 per cent of the mean.

III.

When the Dye Solution was Made up with Phosphate Buffer at pH 6.79.

It was necessary first of all to repeat some of the experiments previously made,[9] since the cells employed here were not of the same lot as those used before.

When cells were placed (without stirring) for 10 minutes in phosphate or acetate buffer solution at pH 5.5 and then placed in brilliant cresyl blue solution made up with phosphate buffer mixture at pH 6.79, there was 30 per cent decrease in the rate of dye penetration with acetate and 23 per cent decrease with phosphate (Table I).

[10] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, pp. 81–83.

These results confirm those previously published,[9] except that the percentage decrease is lower; in former experiments the acetate gave 47 per cent decrease and the phosphate 44 per cent decrease. This difference in the percentage decrease with these two groups of cells may be due to difference in the locality as well as in the season of the year.

When cells were exposed to acetate buffer mixture at pH 5.5 for 10 minutes, the pH value of the sap decreased from pH 5.5 (normal) to pH 5.0, while phosphate buffer mixture brought about no change in the pH value of the sap.

TABLE I.

Comparison of the amount of dye found in the vacuole of living cells of *Nitella* when cells were first exposed for 10 minutes either to an acetate or phosphate buffer solution at pH 5.5 and were then placed for 1 minute in 34.5×10^{-5} M brilliant cresyl blue at pH 6.79 made up with phosphate buffer mixture. The solutions were not stirred. The buffer mixtures are described in Section II in the text.

External solution to which cells were exposed.....	Tap water or control	Phosphate buffer at pH 5.5	Acetate buffer at pH 5.5
Amount of dye in sap, $M \times 10^6$.....	19.7	15.2	13.8
Percentage decrease or increase.....	Standard	23 per cent decrease	30 per cent decrease
pH value of the sap before the cells are placed in the dye solution.....	5.5	5.5	5.0

Since the inhibiting effect is brought about irrespective of the changes in the pH value of the sap, we may conclude as before[9] that the factor controlling the rate of dye penetration may not lie in the vacuole, in this case, but that it lies in the protoplasm (either at the surface or the interior).

IV.

When the Dye Solution is Made up with Borate Buffer Mixture at pH 7.85.

When uninjured cells of *Nitella* were placed in various solutions and after 10 minutes the pH value of the sap was determined, the results

TABLE II.

Comparison of the amount of dye found in the vacuole of living *Nitella* when cells were first exposed for 10 minutes to one of the various solutions given below and were then placed for ½ minute in 13.8×10^{-5} M brilliant cresyl blue solution at pH 7.85 made up with diluted borate or phosphate buffer mixture. The pH values of the sap after cells were exposed for 10 minutes to these various solutions are given below.

Buffer mixtures are described in Section II in the text.

			Tap water or control	Phosphate buffer mixture	Acetate buffer mixture	Acetate buffer mixture	Acetic acid	Acetic acid	M/150 sodium acetate	Hydrochloric acid	Hydrochloric acid
				pH 5.5	*pH 5.5*	*pH 4.8*	*pH 4.8*	*pH 4*	*pH 7.1*	*pH 4.8*	*pH 4.2*
Dye solution made up with borate buffer mixture	Dye solution stirred	Amount of dye in sap, $M \times 10^6$	41.4	19.3	15.5	10.7	37.6	11.4	18.5	37.7	30.0
		Percentage decrease or increase	Standard	53 per cent decrease	63 per cent decrease	74 per cent decrease	9 per cent decrease	72 per cent decrease	55 per cent decrease	9 per cent decrease	28 per cent decrease
Dye solution made up with phosphate buffer mixture	Dye solution stirred	Amount of dye in sap, $M \times 10^6$	40.7	38.7	19.0	15.9		20.0	36.6		43.7
		Percentage decrease or increase	Standard	5 per cent decrease	53 per cent decrease	61 per cent decrease		51 per cent decrease	10 per cent decrease		8 per cent increase
	Dye solution not stirred	Amount of dye in sap, $M \times 10^6$	22.4	22.8	12.1						
		Percentage decrease or increase	Standard	2 per cent increase	46 per cent decrease						
pH values of the sap			5.5	5.5	5.0	4.8	5.2	4.9	5.5	5.5	5.5

given in Table II were obtained. In phosphate buffer mixture, and M/150 sodium acetate, the pH value of the sap remained unchanged, while in acetate buffer mixture and in acetic acid the pH value of the sap decreased. In either acetate buffer mixture or acetic acid the lower the external pH value, the greater the decrease in the pH value of the sap but the extent of decrease was greater in acetate buffer mixture at pH 4.8 than in acetic acid at the same pH value. This difference may be due to the difference in the supply of acetic acid. With acetate buffer mixture more acid is formed in the external solution during the experiment, replacing the acid which has diffused into the cell, while with acetic acid this does not take place so that in a given period more acetic acid enters the vacuole from the former than from the latter.

When the cells thus exposed to various solutions were placed in the solution of brilliant cresyl blue made up in borate buffer mixture at pH 7.85, the following results shown in Table II were obtained.

With cells previously exposed to sodium acetate, the rate of penetration of dye was decreased considerably without any change in the pH value of the sap. This decrease seems to be a result of the action of sodium ions on the protoplasm since acetic acid at this pH value has no inhibiting effect.

When cells previously exposed to hydrochloric acid at pH 4.8, were placed in the dye solution, the rate of penetration did not decrease appreciably, as shown in Table II, but at a lower pH value, 4.2, about 30 per cent decrease was brought about. Since previous experiments indicated that the inhibiting effect of various other chlorides was not due to the chloride ions, this effect of hydrochloric acid may be due to the hydrogen ions and not to the chloride ions.

With cells previously exposed to acetic acid the rate of penetration of dye was found to decrease with the lowering of the pH value of the external solution and with the lowering of the pH value of the sap as shown in Table II. For the reason already discussed, the inhibiting effect of acetic acid at pH 4.8 is not due to the hydrogen ions in the external solution but at pH 4 it is partly due to the hydrogen ions and partly to the acetic acid in the external solution. The latter may have a specific effect on the protoplasm or may enter as undissociated molecules and dissociate in the protoplasm thereby

lowering the pH value of the protoplasm. Though the extent of decrease in the rate of penetration of dye corresponds with the lowering of the pH value of the sap, it does not necessarily signify that this decrease is brought about by the lowering of the pH value of the sap. In view of the fact that hydrogen ions and base cations may have an inhibiting effect without changing the pH value of the sap, we may suppose that in the case of acetic acid the decrease in the rate of penetration of dye is brought about by the inhibiting effect of acetic acid on the protoplasm.

With cells previously exposed to an acetate buffer mixture it is found (Table II) that the rate of penetration of dye decreases with the lowering of the pH value of the external solution and that of the sap. In this case the effect of hydrogen ions in the external solution cannot be the chief cause, because at these external pH values there is practically no effect of the hydrogen ions on the protoplasm. Since the acetate buffer mixture is made up of sodium acetate and acetic acid, both of which are found to have an inhibiting effect, the decrease in the rate of penetration must therefore be due to the effect of sodium acetate and acetic acid on the protoplasm.

The fact that at the external pH value of 4.8 the acetic acid has practically no inhibiting effect while the acetate buffer mixture has a considerable inhibiting effect (about 70 per cent) may be explained as due to two factors: (1) the presence of sodium in the acetate buffer mixture which is found to have an inhibiting effect on the protoplasm, and (2) the greater supply of acetic acid in the acetate buffer mixture.

V.

When the Dye Solution is Made Up with Phosphate Buffer Mixture at pH 7.85.

In the writer's previous publication[11] it was shown that when cells were exposed to phosphate buffer mixture at pH 5.5 or to phosphoric acid or to hydrochloric acid at pH 4.2 in which the pH value of the sap remained unchanged, and were then placed in the dye solution made up with phosphate buffer mixture at pH 7.85, the inhibiting effect of these solutions on the protoplasm was entirely masked. It

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

is of interest to see if such a counteraction of the inhibiting effect of acetate buffer mixtures and that of acetic acid in which the pH value of the sap is decreased will likewise take place if cells previously exposed to these solutions are placed in such a dye solution.

Living cells were therefore exposed for 10 minutes to various solutions and were then placed in brilliant cresyl blue solution at pH 7.85 made up with phosphate buffer mixture (Table II). The inhibiting effect described in Section IV was almost completely removed in the case of the phosphate buffer mixture at pH 5.5 and sodium acetate, but only slightly diminished in the case of the acetate buffer mixture and acetic acid, as shown in Table II.

That the inhibiting effect on the protoplasm is only very slightly removed in the case of acetate buffer mixtures and acetic acid may be due to the fact that the acetic acid in the sap serves as a reservoir which enables the inhibiting effect to persist for a time even when the cells are placed in the dye solution made up with phosphate buffer mixture at pH 7.85, while there is no inhibiting substance in store to act as a reservoir with phosphate buffer mixture and with sodium acetate, since these substances do not collect in the vacuole.

The decrease in the rate of penetration of dye in the case of the cells exposed to acetate buffer mixture is not due to the lowering of the pH value of the external solution immediately surrounding the cell, resulting from a diffusion of acetic acid from the cell after the cells are transferred from the acetate buffer mixture into the dye solution, since the rate of penetration of dye is decreased to about the same extent whether the external dye solution is stirred or not (Table II).

VI.

CONCLUSION.

The experiments show that the inhibiting effect of sodium acetate is due to the action of sodium and acetic acid on the protoplasm. Just as in the case of phosphate buffer mixture, this inhibiting effect may be removed when the dye solution is made up with a salt solution containing a certain concentration of base cations.

The inhibiting effect of acetic acid at pH 4.8 is due either to the specific effect of acetic acid on the protoplasm or due to the entrance of

undissociated molecules of acetic acid and their subsequent dissociation in the protoplasm thereby lowering the pH value of the protoplasm. With the lowering of the pH value the concentration of the dye in form of free base decreases in the protoplasm. If, therefore, we assume that the rate of penetration of dye in this case is controlled by the diffusion of dye in form of free base from protoplasm into the vacuole and that the inhibiting effect of acetic acid on the protoplasm exceeds the accelerating effect on the sap in the vacuole, we would expect a decrease in the rate of penetration to occur. Since both a non-aqueous substance and the vacuole take up the dye more readily in form of free base than in form of salt, we may assume that the vacuole is surrounded by a non-aqueous layer ("inner layer of protoplasm" in Section I). The rate of penetration of dye therefore may depend on the amount of free base taken up by this non-aqueous layer from the aqueous layer of protoplasm ("middle layer" in Section I) and given up to the sap in the vacuole, in a given time. If any part of the protoplasm is affected in such a way as to change the absorption and giving up of free base by this non-aqueous layer, the rate of penetration of free base into the vacuole will be accordingly altered.

For reasons already stated in Section III, the inhibiting effect of acetic acid at pH 4 may be considered to be due partly to the hydrogen ions in the external solution.

The inhibiting effect of acetic acid and of the acetate buffer mixture is not completely counteracted in presence of the dye made up with a diluted phosphate buffer mixture at pH 7.85. This may be due to the presence of acetic acid in the vacuole, which acts as a reservoir so that the inhibiting effect on the protoplasm persists until this is used up.

We may conclude that in all these cases the inhibiting effect is brought about by the action of these substances on the protoplasm. Under these conditions therefore the factor controlling the rate of penetration of dye into the vacuole is located in the protoplasm and not in the vacuole.

These experiments do not contradict the theory of penetration of basic dye given in this paper (Section I), although in some cases the rate of penetration of dye appears to decrease with the lowering of the pH value of the sap.

SUMMARY.

When living cells of *Nitella* are exposed to a solution of sodium acetate and are then placed in a solution of brilliant cresyl blue made up with a borate buffer mixture at pH 7.85, a decrease in the rate of penetration of dye is found, without any change in the pH value of the sap. It is assumed that this inhibiting effect is caused by the action of sodium on the protoplasm.

This effect is not manifest if the dye solution is made up with phosphate buffer mixture at pH 7.85. It is assumed that this is due to the presence of a greater concentration of base cations in the phosphate buffer mixture.

In the case of cells previously exposed to solutions of acetic acid the rate of penetration of dye decreases with the lowering of the pH value of the sap. This inhibiting effect is assumed to be due chiefly to the action of acetic acid on the protoplasm, provided the pH value of the external acetic acid is not so low as to involve an inhibiting effect on the protoplasm by hydrogen ions as well. It is assumed that the acetic acid either has a specific effect on the protoplasm or enters as undissociated molecules and by subsequent dissociation lowers the pH value of the protoplasm.

With acetate buffer mixture the inhibiting effect is due to the action of sodium and acetic acid on the protoplasm.

The inhibiting effect of acetic acid and acetate buffer mixture is manifested whether the dye solution is made up with borate or phosphate buffer mixture at pH 7.85. It is assumed that acetic acid in the vacuole serves as a reservoir so that during the experiment the inhibiting effect still persists.