CERTAIN EFFECTS OF SALTS ON THE PENETRATION OF BRILLIANT CRESYL BLUE INTO NITELLA.

By MARIAN IRWIN.

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

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I.

INTRODUCTION.

The effect of salts on the rate of penetration of dye into living cells has been studied by various investigators[1] by comparing the rate in dye solution containing salt with that in dye solution containing no salt, but it is not possible to determine from their results whether the salt acts on the protoplasm or on the dye or on both.

The writer therefore has suggested a method[2,3] by which we are able to separate the effect of salt on the protoplasm from that on the dye. This is done as follows: (1) Cells are placed for a given time in a salt solution, after which they are placed in dye solution containing no salt. The rate of penetration of dye in the case of such cells is compared with that of the control (cells which have not been previously exposed to the salt solution). This gives the effect of salt on the protoplasm since it has previously been shown[2,3] that there is no error due to salt adhering to the surface of the cell wall (which cannot be removed by wiping and washing). It might be thought that an error could arise from the fact that salt might diffuse out from the cell when removed from the salt solution and placed in the dye solution

[1] *Cf.* Endler, J., *Biochem. Z.*, 1912, xlii, 440; xlv, 359. Szücs, J., *Sitzengsber. k. Akad. Wissensch., math.-naturw. Cl., Wien*, 1910, cxix, 1. These papers contain references to earlier literature. Lack of space prevents a detailed comparison of their results with those of the writer, but it may be said that in general they found that in some cases the presence of salts in the dye solution accelerated and in other cases inhibited the penetration of dye.

[2] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 54.

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

containing no salt, and enough of this might collect in the solution just outside the protoplasm to affect the results. This, however, has been shown[2,3] not to take place. (2) Cells previously exposed to water or salt solution are placed in dye solution containing the salt, and the rate of penetration in these cells is compared with that of the control (cells previously exposed to distilled water and then placed in dye solution containing no salt).

Preliminary accounts of such experiments have been given in the writer's previous papers.[2,3] These experiments are of interest because they are helpful in locating the factor controlling the rate of penetration of dye under various experimental conditions, which is one of the important problems in permeability.

The theory underlying the mechanism of the penetration of basic dye into living cells has been presented[4] by the writer and need not be discussed in detail here. But it may be stated that we assume that we are concerned only with the diffusion of the dye in the form of free base (for convenience called DB) into the vacuole, since the salt (called DS for convenience) does not enter the cell very readily. As DB enters[5] the vacuole, some of it changes to DS, and more DB enters until there is in the vacuole a definite ratio of DB/DS depending on the condition of the sap, *e.g.* the pH value and the salt content. At equilibrium, DB[6] in the vacuole is either equal to or proportional to DB in the external solution, depending on the solubility, etc.

II.

Methods.

Since the method of determining the amount of dye in the vacuole and the general experimental precautions and procedure have been repeatedly described in the writer's previous publications, a detailed account will not be given here.

The determination of the amount of dye in the sap was made colorimetrically. The dye, brilliant cresyl blue, was dissolved in M/150 borate buffer at pH 7.7 unless

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

[5] The same may be said of DB in the protoplasm.

[6] What we actually measure is the total dye (DB and DS) but since the color of DB and DS is the same, and since there is a definite ratio of DB/DS, we are justified in using this method.

otherwise stated. The pH values of the solutions were determined by means of the hydrogen electrode. The experiments were carried out in an incubator at 25 ± 0.5°C.

Only living cells (collected in Cambridge unless otherwise stated) free from foreign growths, such as diatoms on the surface, were used and great care was taken to obtain uniform cells and to keep them all under the same conditions before experimentation. The cells were invariably wiped and washed in distilled water for 5 seconds, and again wiped, before they were placed in any solution.

Each experiment represents an average of about 60 readings on individual cells, and the probable error of the mean is less than 7 per cent of the mean.

In carrying out a series of comparative experiments, approximately the same number of readings was made for each experiment each day, and an average was taken of all the readings of each experiment after 60 readings were made. There may be a great source of error if comparative experiments are not made at the same time and with uniform cells.

III.

Salts with Monovalent Cations.

The experiments[7] (see Table I) with 0.013 M NaCl solution show that the rate of penetration of dye (as compared with that of control cells transferred from distilled water to the dye solution) decreases greatly when cells, after exposure to the salt solution for 10 minutes,

[7] The experiments were carried out as follows: Cells were divided into four groups:

(*a*) Cells were placed for 10 minutes in distilled water (pH 5.4), after which they were wiped and placed in 0.00014 M brilliant cresyl blue solution at pH 7.7 (M/150 borate buffer mixture). After 2 minutes the concentration of the dye in the sap was determined. This experiment represents the control, and this rate of penetration is used as a standard of comparison.

(*b*) Cells were exposed for 10 minutes in 0.013 M NaCl solution (made up in the distilled water), after which they were wiped and washed for 5 seconds in distilled water. Then they were again wiped and placed in the same dye solution as group *a*. After 2 minutes the concentration of dye in the sap was determined.

(*c*) Cells were placed in distilled water for 10 minutes, after which they were wiped, and placed in 0.00014 M dye solution containing 0.013 M NaCl at pH 7.7 (M/150 borate buffer mixture). After 2 minutes the concentration of dye in the sap was determined.

(*d*) Cells were exposed for 10 minutes in 0.013 M NaCl solution, after which they were wiped, and washed for 5 seconds in distilled water. Then they were again wiped and placed in the same dye solution as group *c*. After 2 minutes the concentration of dye in the sap was determined.

TABLE I.

Penetration of Brilliant Cresyl Blue into the Vacuole of Living Cells of Nitella in Presence of NaCl at Different Concentrations.

I. Concentration of external NaCl solution.

II. Concentration of dye in the vacuole when cells were placed in NaCl solution (Column I) for 10 minutes and then placed in 0.00014 M dye solution at pH 7.7 (borate).

III. Concentration of dye in the vacuole, when cells were placed in distilled water for 10 minutes and then in 0.00014 M dye solution at pH 7.7 (borate) containing the same concentration of salt as in I.

IV. Cells were placed for 10 minutes in the salt solution stated in I, and then placed in the same dye solution as in III.

As a basis of comparison the concentration of dye in the vacuole was taken when cells were placed in distilled water for 10 minutes and then placed in the same dye solution as in II. This concentration was 25.0×10^{-5} M.

In all cases the concentration of dye in the vacuole was determined after 2 minutes exposure to the dye solution.

I	Concentration of dye in the vacuole		
	II	III	IV
M	M × 10^5	M × 10^5	M × 10^5
0.0500	7.9	30.1	33.0
0.0250	7.2	29.3	32.2
0.0125	6.9	29.5	31.4
0.0063	7.6	29.3	30.5
0.0016	15.5	23.8	23.7
0.00063	22.8	24.5	24.0

TABLE II.

Concentration of brilliant cresyl blue in the vacuole of living cells of *Nitella* when cells previously exposed to tap water or for different lengths of time to distilled water or to 0.05 M or 0.01 M NaCl solution are placed in 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture) for 2 minutes.

In tap water at pH 7.0	In distilled water pH 5.4		In NaCl solution				
	1/2 min.	10 min.	5 sec.	1/2 min.	2 min.	5 min.	10 min.
M × 10^5	M × 10^5	M × 10^5	M × 10^5	M × 10^5	M × 10^5	M × 10^5	M × 10^5
22.4	21.4	22.1	24.2	5.2	5.9	4.5	5.2

are subsequently placed in the 0.00014 M dye solution made up with borate buffer mixture at pH 7.7 for 2 minutes. Cells previously exposed either to distilled water or to 0.013 M NaCl for 10 minutes and then placed for 2 minutes in the same concentration of dye, at the same pH value, containing 0.013 M NaCl solution, show a slight increase in the rate of penetration of dye (Table I).

These experiments were repeated with different concentrations of salt between 0.05 M and 0.0006 M (Table I). Both the inhibiting and accelerating effects of NaCl remain about the same between 0.05 M

TABLE III.

Experiments Showing That the Effect of NaCl on the Protoplasm Causing a Decrease in the Rate of Penetration of Brilliant Cresyl Blue into the Vacuole of Nitella (Collected in New York) Is Not Readily Reversible.

I. Represents the control experiment. Cells placed for 10 minutes in distilled water and then placed in the dye solution.

II. Cells were exposed for 2 minutes to 0.0016 M NaCl solution after which they were placed in the dye solution.

III. Cells were exposed for 2 minutes to 0.0016 M NaCl after which they were washed in a large volume of distilled water for 1 hour, and then placed in the dye solution.

In every case the cells were placed for 1 minute in 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture).

	I	II	III
	M × 10^5	M × 10^5	M × 10^5
Amount of dye in sap.....	31.8	18.9	20.0

and 0.006 M, but below the latter concentration both effects diminish as the concentration decreases until at 0.0006 M solution there is practically no more effect.

This inhibiting effect of NaCl may be brought about in $\frac{1}{2}$ minute at 0.01 M or above (Table II). This effect is not removed by transferring the cells after 2 minutes from the salt solution to distilled water and leaving them for 1 hour, as shown in Table III.

When the experiments were repeated with KCl, KNO_3, LiCl, Na citrate, and Na_2SO_4 at 0.01 M solution the same results were obtained as is shown in Table IV.

TABLE IV.

Penetration of Brilliant Cresyl Blue into the Vacuole of Living Cells of Nitella in the Presence of Salts.

I. Cells were exposed for 10 minutes to 0.01 M salt solution after which they were placed in the 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture).

II. Cells were exposed for 10 minutes to distilled water after which they were placed in the 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture) containing 0.01 M salt.

III. Cells were exposed for 10 minutes to 0.01 M salt solution after which they were placed in the same dye solution as in II.

As a basis of comparison the concentration of dye in the vacuole was taken when the cells were placed in distilled water for 10 minutes and then transferred to the same dye solution as in I. This concentration was 24.2×10^{-5}M.

In all cases the cells were placed in the dye solution for 2 minutes.

0.01 M external salt solution.	Concentration of dye in the vacuole		
	I	II	III
NaCl	6.9	28.1	31.0
LiCl	7.9	27.8	32.5
KCl	8.3	29.0	31.3
KNO_3	7.3	27.5	30.0
Na_2SO_4	5.8	29.5	32.2
Na citrate	5.5		
$CaCl_2$	26.0	26.2	26.5
$MgCl_2$	26.2	25.9	27.0
$MgSO_4$	25.8	26.5	26.8
$LaCl_3$	29.2		
$La(NO_3)_3$	28.3		

IV.

Salts with Bivalent and Trivalent Cations.

When the experiments[8] were carried out using salts with bivalent and trivalent cations, namely,[9] $MgCl_2$, $CaCl_2$, $MgSO_4$, $LaCl_3$, and

[8] These experiments were carried out by dividing the cells into four groups in the same manner as with NaCl, as described in Foot-note 7, which should be consulted for the details of the experiments.

[9] $LaCl_3$ and $La(NO_3)_3$ reduced the pH value of the solution considerably so that the experiments were carried out with lower concentrations of the salt and the same result was obtained.

$La(NO_3)_3$, it was found that there is a very slight accelerating effect in the case of cells previously exposed to the salt solution and then placed in the dye solution containing either salt or no salt, as well as in the case of cells directly transferred from distilled water to the dye solution containing salt (Table IV).

TABLE V.

Experiments Showing That the Effect of NaCl on the Protoplasm Causing a Decrease in the Rate of Penetration of Brilliant Cresyl Blue into the Vacuole of Living Cells of (New York) Nitella May Be Removed by Salts with Bivalent and Trivalent Cations.

The cells were left for 5 minutes in each salt solution, and then placed in 0.00014 M dye solution at pH 7.7 (M/150 borate buffer mixture) for 1 minute.

The cells were exposed to the various solutions in the order given. The cells kept in distilled water for 5 minutes and then transferred to the dye solution for 1 minute had 28×10^{-5} M dye in the vacuole.

The cells exposed to 0.001 M NaCl for 5 minutes and then transferred to the dye solution had 15.6×10^{-5} M dye in the vacuole.

Salt solution	0.001 M NaCl, then salt solution, then dye	Salt solution, then 0.001 M NaCl, then dye
	M × 10^5	M × 10^5
0.01 M $MgCl_2$	29.5	16.1
0.01 M $MgSO_4$	30.0	16.6
0.001 M $MgSO_4$	29.2	15.0
0.001 M $LaCl_3$	28.4	17.0

In considering the results obtained with all the salts given above, we may conclude that the decreasing effect[10] of the salts with monovalent cations is due to the effect of the cations and not to that of the anions, while the bivalent and trivalent cations have no inhibiting effect on the protoplasm.

[10] For results with certain concentrations of dye and salts (including a decrease in penetration of dye in dye solution containing either 0.05 M $CaCl_2$ or $MgCl_2$ at higher pH value). See Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, No. 4.

V.

The Inhibiting Effect of NaCl May Be Removed by Salts with Bivalent and Trivalent Cations.

The question now arises whether the inhibiting effect of NaCl may be entirely or partially removed by other salts. To test this question the following experiments were made.

Cells were exposed to 0.001 M NaCl solution for 5 minutes after which they were washed in 0.01 M $MgCl_2$ for 5 minutes, and then transferred to the dye solution. The inhibiting effect of NaCl was found to have been completely removed[11] since the rate of penetration of dye was the same as that of the control, as shown in Table V. This inhibiting effect, however, was not removed when cells were first exposed to the $MgCl_2$ solution and subsequently treated with NaCl and then placed in the dye solution (Table V)

The same result is obtained (Table V) when $LaCl_3$ and $MgSO_4$ are used instead of $MgCl_2$.

No inhibiting effect of NaCl is produced if cells are exposed to 0.02 M NaCl containing 0.01 M $MgCl_2$ and then placed in the dye solution. In some cases the inhibiting effect of NaCl is removed in dye made up with phosphate buffer mixture, or with borate buffer mixture at high pH value in which borax predominates, or at lower pH value containing NaCl or $MgCl_2$.

Theoretical Considerations.

The experimental results described above appear to involve two factors, (1) effect of salt on the dye, (2) action of salt on the protoplasm (either at the surface or in the interior).

A salt (with monovalent, bivalent, or trivalent cations) may affect the dye in three ways, (*a*) increase the dissociation of the dye, (*b*) decrease the solubility of the dye, (*c*) have some other (possibly specific) effect on the dye.

In order to study the effect of salt on the dye experiments were made on the distribution of the dye between chloroform and the 0.00014 M

[11] The inhibiting effect brought about by 0.01 M NaCl may also be removed by phosphate buffer mixture containing 0.00014 M dye, while it cannot be removed by the buffer solution containing no dye.

dye solution (borate buffer mixture) at pH 7.7. These show that the addition of 0.01 M NaCl or 0.01 M $MgCl_2$ to the dye solution does not increase the amount of dye taken up by the chloroform as would be the case if the dye were being salted out but on the contrary decreases it, which may indicate that the solubility of the dye (at such concentrations of the dye and the salts as are here employed) is not affected as much as the dissociation constant of the dye. The salt appears to increase the dissociation of the dye which would cause less dye to enter the chloroform.

The question arises whether we can apply these results directly to *Nitella*. In view of the fact that the rate of penetration of the dye at various pH values was previously found[4] by the writer to correspond with the amount of dye absorbed by the chloroform from aqueous solution at these pH values, we might expect a similar correspondence to exist when cells are placed in the dye solution containing the salt, but the experiments show the contrary. Less dye is taken up by the chloroform while more dye is taken up by the cells when the aqueous dye solution contains salt. This may indicate that the effect of change in solubility exceeds that of the shift in dissociation constant, in the case of *Nitella,* since the partition coefficient of DB between the cell vacuole and the external solution is small, probably less than 1. Hence a slight change in the solubility of DB in the external solution may bring about a great change in the partition coefficient, while the partition of DB between chloroform and M/150 buffer solution is found to be exceedingly large so that a slight change in the solubility may have no appreciable effect on the partition coefficient. But it does not seem possibe to explain these results on *Nitella* entirely on the basis of solubility because the salting out effect of a salt with monovalent cation, such as NaCl, would probably be less than that of a salt with bivalent cation, such as $MgCl_2$, so that at the same concentrations one might expect less accelerating effect with NaCl than with $MgCl_2$, but the experiments show that NaCl brings about a greater effect than $MgCl_2$ (which has very little effect) which indicates that the situation may be complicated by additional factors such as the specific effect of cations on the dye and on the protoplasm.

That the accelerating effect in dye plus NaCl is due to the direct

action of the salt on the dye in the external solution and not on the protoplasm is shown by the experiment where the cells are exposed to the salt solution and then placed in the dye solution containing no salt, in which case there is a decrease in the rate of penetration of dye instead of an increase. This decrease cannot be due to the entrance of salt into the vacuole since the halide content remains unchanged. It must therefore be due to the presence of the salt in the protoplasm (either at the surface or in the interior). We may then assume that the salt acts on the protoplasm in such a way that the rate of penetration of dye from the external solution into the protoplasm is decreased (it might, for example, decrease the solubility of dye in the protoplasm or increase the viscosity of the protoplasm) and if the penetration of dye into the outer surface of the protoplasm is slower than the diffusion of the dye from the protoplasm into the vacuole we may expect a decrease in the amount of dye in the vacuole.

A striking fact is that this inhibiting effect not only disappears but is replaced by an accelerating effect when cells thus exposed to the salt solution are placed in the dye solution containing salt. The accelerating effect is a little greater in this case than when cells are first exposed to distilled water and then placed in the same dye solution. It has been shown by experiments that at the concentration of NaCl employed the effect of the salt on the protoplasm is immediate so that irrespective of whether the cells have been formerly exposed to the salt or not the condition of the protoplasm may be practically the same very soon after the cells are placed in the dye solution containing the salt.

If this is true we may assume that the reason the accelerating effect is a little greater in the case of the cells previously exposed to the salt is because there is a greater accumulation of salt in the very thin film of liquid between the protoplasm and the cell wall and that this acts on the dye.

It would seem that this accelerating effect is due to a change in the nature of the dye (produced by the salt) which makes the dye penetrate more rapidly. It may not be necessary to suppose that the combination of salt plus dye affects the protoplasm so as to increase

penetration because cells exposed[12] to such a combination behave subsequently like those exposed to salt without dye (showing a decrease in the rate of penetration when placed in the dye). We may therefore suggest that the effect of salt on the protoplasm is not affected by presence of dye. Further experiments are necessary, however, to determine this conclusively.

In striking contrast to the behavior of NaCl is that of salts with bivalent and trivalent cations. When cells are exposed to a solution of $MgCl_2$ and then placed in the dye solution there is no inhibiting effect such as is produced by previous exposure to NaCl. On the contrary, there is apparently an accelerating effect but this is so small that it may possibly be due to experimental error.

There is another difference[13] between these salts. The inhibiting effect of NaCl may be reversed by adding $MgCl_2$ to the solution containing NaCl or by washing the cells with a solution of $MgCl_2$ after they have been exposed to the solution of NaCl.

SUMMARY.

The effect of various substances on living cells may be advantageously studied by exposing them to such substances and observing their subsequent behavior in solutions of a basic dye, brilliant cresyl blue.

The rate of penetration of the basic dye, brilliant cresyl blue, is

[12] From the time curves it is evident that the same amount of accelerating effect is produced whether the cells are placed in 0.02 M NaCl solution at pH 7.7 (borate buffer mixture) containing 14×10^{-5} M or 1.7×10^{-6} M dye. An inhibiting effect is produced when cells are exposed for 3 minutes to 0.02 M NaCl solution at pH 7.7 (borate buffer mixture) containing either 1.7×10^{-6} M dye or no dye, and then placed in 14×10^{-5} M dye solution at the same pH value for 1 minute.

[13] These differences may result from the fact that each salt affects the protoplasm or the dye in more than one way so that the net result may differ because with one salt one effect is of primary importance while with another salt a different effect predominates. One salt may affect diffusion from the external solution into the protoplasm a great deal while another may have more effect on the diffusion from the protoplasm into the vacuole and the location of these effects may change with alterations in concentration. The same may be said of differences in viscosity, solubility, etc.

decreased when cells are exposed to salts with monovalent cations before they are placed in the dye solution (made up with borate buffer mixture). This inhibiting effect is assumed to be due to the effect of the salts on the protoplasm.

This effect is not readily reversible when cells are transferred to distilled water, but it is removed by salts with bivalent or trivalent cations. In some cases it disappears in dye made up with phosphate buffer mixture, or with borate buffer mixture at the pH value in which the borax predominates, and in the case of NaCl it disappears in dye containing NaCl.

No inhibiting effect is seen when cells are exposed to NaCl solution containing $MgCl_2$ before they are placed in the dye solution.

The rate of penetration of dye is not decreased when cells are previously exposed to salts with bivalent and trivalent cations.

The rate is slightly increased when cells are placed in the dye solution containing a salt with monovalent cation and probably with bivalent or trivalent cations. In the case of the bivalent and trivalent salts the increase is so slight that it may be negligible.