# MECHANISM OF THE ACCUMULATION OF DYE IN NITELLA ON THE BASIS OF THE ENTRANCE OF THE DYE AS UNDISSOCIATED MOLECULES.

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## I.

## INTRODUCTION.

The mechanism of the accumulation of the basic dye, brilliant cresyl blue, in the sap of *Nitella* has been discussed by the writer[1] on the basis of experiments made with different concentrations of the dye at one pH value. More recently a preliminary report[2] was made by the writer on experiments[3] with one concentration of the dye at different pH values. A fuller account of these experiments is given in the present paper. In order to understand the mechanism it is necessary to analyze the data for (1) the rate of penetration of the dye into the cell sap, and for (2) the final equilibrium.

## II.

## *Methods.*

The living cells[4] of *Nitella* were placed at 25°C. ± 0.5° in $2 \times 10^{-5}$ M dye solutions (brilliant cresyl blue) at different pH values, from pH

[1] Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.

[2] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 251.

[3] Experiments such as these were made by the writer on *Nitella* found in Woods Hole, Massachusetts, with brilliant cresyl blue made by the National Aniline Chemical Company, but at that time the dissociation constant of the dye was not known, so that the analysis of the data was not complete. Since it is no longer possible to obtain the *Nitella* at Woods Hole, the experiments were repeated with *Nitella* found at Cambridge, and with the dye made by Grübler, the dissociation constant of which was found by the writer as will be described later. Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 727.

[4] For details of technique see the writer's paper referred to in Foot-note 1. The *Nitella* used was obtained from Cambridge, Massachusetts.

6.1 to pH 9.3 (M/150 phosphates or borates as buffers).[5] The concentration of the dye was kept constant throughout each experiment. At definite intervals the cells were removed from the solutions. The end of each cell was then cut and the sap was squeezed out upon a glass slide. The sap was then drawn up into a capillary tube, the color of which was matched with that of the capillary tube containing the standard dye solution.

III.

*Analysis of the Rate of Penetration.*

When the concentrations of the dye in the sap were thus determined at definite intervals, it was found that the greater the pH value of the external solution, the higher was the rate of penetration. The maximum was reached at about pH 9.3, where further increase in pH value of the external solution brought about no appreciable increase in the rate of accumulation. We may assume that the dye behaves as a weak base and that the dye ions cannot enter but that the dye penetrates only in the form of undissociated molecules of the free base which for convenience will be referred to as DOH. In that case, the rate of penetration at the start should be proportional to the concentration of DOH in the external solution. With a constant concentration of the dye the concentration of DOH will depend on the pH value. If this assumption is correct, we can calculate the concentration of DOH, expressed as per cent of the total dye present from the rates of penetration into *Nitella*, and this should agree with the values obtained by other methods such as that of determining the distribution[6] of

[5] The readings made at pH 6.1 are rather uncertain because at this pH value the cellulose wall becomes deeply stained, and it is difficult to avoid contamination of the sap from the dye adhering to the cell wall at the cut end. The readings made with pH values lower than this cannot be used since the lower the pH value the more rapid is the staining of the cellulose wall and the greater the chance of contamination.

[6] Pure chloroform was added to $1.4 \times 10^{-5}$ M dye solution previously saturated with chloroform at different pH values (M/150 phosphates or borates) at 24°C. ± 1°, and the determination of the amount of dye taken up by chloroform at equilibrium was made colorimetrically. The color of the dye in water is blue at the pH values used, but in chloroform it is pink (when the dye comes out again into water it is blue). In order to make the colorimetric determinations accurate,

the dye between chloroform and water, which was employed by the writer.

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it was necessary to use different volumes of chloroform and the dye solutions at different pH values, so that at equilibrium the concentration of the dye will be reduced to about 0.000007 M. Such mixtures were shaken vigorously in a separatory funnel, and after equilibrium was established the chloroform was allowed to separate and was then drawn off. The aqueous solution was then collected in a test-tube, and tightly stoppered at once. Extreme care must be taken to avoid the slightest evaporation of the chloroform in the funnel or in the test-tube, or else the aqueous solution will at once become more concentrated by taking up the dye left by the evaporated chloroform. The color of the tube containing the aqueous solution was matched with that of tubes of the same diameter containing standard dye solutions. Since the volume of the aqueous solution, the volume of the chloroform, and the concentration of the dye in the aqueous solution at start and at equilibrium were known, the concentration of dye in the chloroform at equilibrium could be readily calculated. When this was done, it was found that the relative amount (distribution coefficient) of dye taken up by the chloroform increased with increase in the pH value of the aqueous solution, until a maximum was reached at about pH 9.3, when further increase in the pH value brought about no appreciable increase in the taking up of the dye. This was not due to the saturation of the dye in the chloroform, because more dye was taken up on raising the concentration of the dye in the aqueous solution. At this pH value it may be assumed that 88 per cent of the dye in the aqueous solution is in the form of undissociated DOH. On this basis, it is possible to calculate the value of the constant at the pH value where 100 per cent of the dye is undissociated DOH, by the equation $\frac{C_1}{C_2\,(1-\alpha)} = K$, in which $C_1$ is the concentration of undissociated DOH in chloroform, $C_2$ is the concentration of the total dye in water, $K$ is the constant, and $\alpha$ is the molar fraction of the dye dissociated. By substituting 0.88 for $\alpha$ and the observed values of $C_1$ and $C_2$ at pH 9.3, the value of $K$ was determined where all of the dye in the aqueous solution was in the form of undissociated DOH, and was found to be 780.

There seems to be no association of the dye in the chloroform because dilution of the aqueous dye solution does not change the value of the constant.

The degree of dissociation of the dye may now be calculated at different pH values by using the above equation.

The percentage of undissociated DOH calculated in this manner for various pH values of the aqueous solution from pH 5.3 to 9.3 is shown by the symbol $\times$ in Fig. 1. From this curve the dissociation constant of DOH is determined graphically to be $10^{-5.6}$. *Cf.* Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922, 44, 46, in which pOH is substituted for pH and OH for H.

This method was checked by another in which the chloroform containing the dye was removed from the aqueous solution and placed in 2 cc. of water after

When the rates[7] (the reciprocal of time taken for the concentration of the dye in the sap to reach $3.45 \times 10^{-5}$ M) for different pH values of the external dye solutions are calculated, it is found that a maximum is reached at about pH 9.3, as already stated. This is regarded as indicating that the percentage of undissociated DOH has nearly reached its maximum value. We assume[8] that this is 88 per cent of the total dye and the per cent of undissociated DOH at different pH values is calculated on this basis by assuming that the rate of penetration is directly proportional to the concentration of DOH. When such values are plotted against the external pH values, the curve agrees closely with that obtained by the experiments on the distribution of the dye between chloroform and water, as shown by the symbols $\times$ and $\bigcirc$ in Fig. 1. The theoretical curve, calculated from the dissociation constant of the dye,[9] $K = 10^{-5.6}$ follows these two curves,

which the chloroform was driven off by a current of air. After complete evaporation of the chloroform the solution was diluted to a point at which a good colorimetric determination could be made. The results thus obtained agreed closely with those described above.

Distribution of the dye between benzene and water was determined at different pH values. The constant, $K$, of the partition coefficient was found to be lower than for chloroform so that for very high pH values it was more satisfactory but for pH values below 8 it was so unsatisfactory that the results obtained by this method were not seriously considered. The dissociation constant was found to be about $10^{-5.2}$.

[7] The rates taken with 0.000014 M dye in the sap gave the same type of curve. The rates were taken at a low concentration to avoid the possibility of error from having the pH values of the sap affected by the dye. The results seem to indicate that so long as the concentration of the dye in the sap does not go above 0.0000345 M such errors as the above described are avoided. If we compare the amounts of dye taken up by the sap at different pH values near the start of the experiment (at 3 minutes) we get the same type of curve.

[8] This value was chosen as producing the best agreement among the curves shown in Fig. 1.

[9] The equation used for the calculation of $\alpha$, the fraction of the dye dissociated is:

$$\alpha = \frac{1}{1 + \frac{OH}{K}}$$

in which $K$ is $10^{-5.6}$. In a previous paper (Foot-note 1) this was stated as $K = 10^{-6.4}$. Since, however, the dissociation constant could not be accurately determined at that time, the calculations were not published in detail. This has been remedied by improved technique.

as shown by the curve as drawn in Fig. 1 until about pH 7, below which the chloroform curve becomes lower than the calculated (unfortunately this difference is not well marked in the figure because the scale

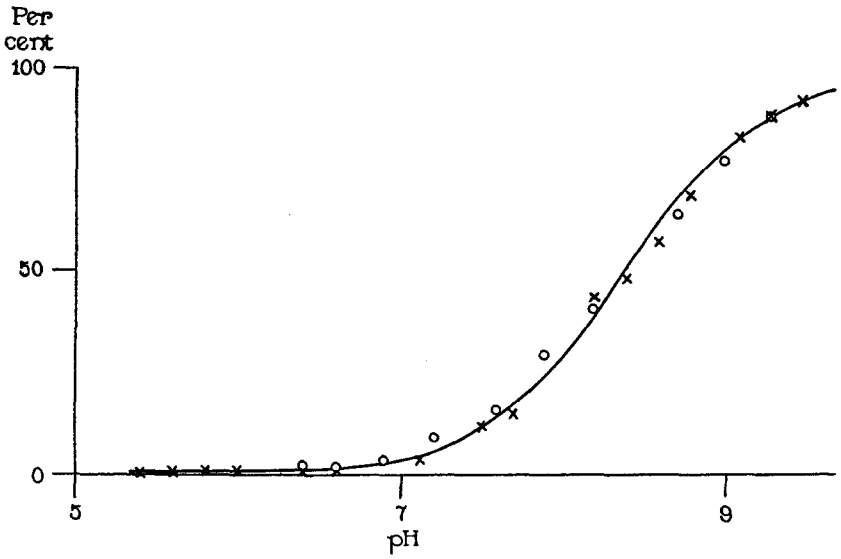


FIG. 1. Curve showing the percentage of undissociated molecules of brilliant cresyl blue at different pH values. The ordinates represent the percentage and the abscissæ represent the pH values. Symbol ○ represents the data from rates of penetration of the dye into *Nitella*. Symbol × represents the data from the distribution of the dye between chloroform and water. The curve as drawn represents the calculation made from the equation, $\alpha = \frac{1}{1+\frac{OH'}{K}}$, when $K = 10^{-5.6}$.

for plotting is very small at these pH values). This difference may be due either to experimental errors or to the dissociation of a second salt-forming group in the dye molecule. If we put $K_1 = 10^{-5.6}$ and $K_2 = 10^{-8}$ the theoretical[10] curve agrees more closely with the chloro-

[10] The equation used for the calculation of $\rho$, the fraction of the dye undissociated is $\rho = \frac{1}{1+\frac{K_1}{OH}+\frac{K_1 K_2}{(OH)^2}}$ in which $K^1 = 10^{-5.6}$, and $K_2 = 10^{-8}$.

form curve, but since the chances for experimental errors are rather great at these lower pH values, the writer does not wish to make a definite statement as to the nature of this difference until an opportunity presents itself to determine the dissociation constants more accurately.

These results seem to indicate that the dye enters only in the form of DOH, and that the rate of penetration is directly proportional to the concentration of DOH in the external solution, provided the conditions in the cell are kept constant. Changes in the condition of the cell sap, for example, can alter the rates of penetration, as has already been shown,[11] though none of the experiments are very reliable since the cells might have been injured with the changes in the pH values of the sap. The sap of *Nitella* is buffered, according to Hoagland and Davis,[12] so that, in all probability, the presence of $3.45 \times 10^{-5}$ M dye in the sap brings about no change in the pH value of the sap. Even if the pH values are increased by the presence of this amount of dye in the sap, the relative rates will not change so long as the pH value of the sap is changed to the same extent for all external pH values.

[11] McCutcheon and Lucke, and later the writer, have found that an increase in the pH value of the sap brought about a decrease in the rate of penetration (cells may be injured). Recently the writer has found that when acetic acid penetrates the living cells of *Nitella* until the pH of the sap is changed from pH 5.6 to 5, an increase in the rate of penetration of the dye takes place. This experiment is unreliable because there is formed a white precipitate in the sap (in all probability the protein in the sap has reached its isoelectric point), and the cells may be injured. The fact that after a brief exposure to $NH_3$ the rate may be decreased before the pH value of the sap is increased may be due to the fact that the $NH_3$ is at that time confined to the protoplasm and the outer portion of the sap, where it could affect the rate by locally raising the pH value without, however, affecting the pH value of the sap as a whole when squeezed out on the slide, or due to the fact that in correspondence with the conditions in the sap, there is present $NH_3$ (without the change in the pH value) in the parts of the cell other than the vacuole (McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501. Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 235.)

[12] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629.

## IV.

### *Analysis of the Equilibrium.*

The experiments described in Section II may be regarded as indicating that the dye enters the cell only in the form of DOH. It is desirable to inquire whether the analysis of the equilibria will support this interpretation.

At pH 6.4, 6.6, and 6.9, the absorption of dye reaches an equilibrium, but at higher pH values of the external dye solutions the cells die before the equilibrium was attained. The equilibrium values thus obtained increase as the pH values of the external solutions rise.

If the dye penetrates as DOH we shall expect that at equilibrium the internal and external concentrations of DOH will be the same. Thus when the external dye solution is $2 \times 10^{-5}$ M and the external pH is 6.9, the concentration of DOH in the external solution may be taken as 3.16 per cent of 0.00002 M (since according to the theoretical curve, 3.16 per cent of the dye is in the form of DOH at pH 6.9). Hence we have $6.31 \times 10^{-7}$ M DOH in the external solution and in the sap at equilibrium. In the sap the pH value may be taken as 5.6, at which value DOH forms 0.16 per cent of the total dye (according to the theoretical curve). Hence when DOH enters the sap it must dissociate, forming a sufficient number of ions to constitute 99.84 per cent of the total dye inside. If we assume that these ions cannot escape from the cell vacuole, then the total dye, $x$, inside will be

$$x = \frac{100}{0.16} \times 0.000000631 \text{ M} = 0.000395 \text{ M}$$

whereas we actually find 0.00014 M by observation.[13] Table I shows the corresponding values calculated for the pH values 6.4 and 6.6.

At all of the pH values the calculated values are higher than the observed.

If the above assumption is correct, then it should be possible to treat in the same manner the previous determinations[1] of the concentrations of the dye in the sap at equilibrium with different concentrations of external dye solutions at one pH value. When such

[13] *Cf.* Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255.

TABLE I.

From the percentage dissociation of brilliant cresyl blue calculated when $K = 10^{-5.6}$ the values of the total dye ($D^+$ ions and DOH) in the sap of *Nitella* at equilibrium are calculated at different pH values of the external dye solutions at 25°C. The concentration of the external dye solution is 2 M $\times 10^{-5}$, and the pH value of the sap is 5.6, at which pH value 0.16 per cent of the total concentration of the dye is undissociated. The calculations for Tables I and II were made with a 20 inch slide rule.

| pH of the external dye solution. | Concentration of undissociated molecules in the external solution. | Concentration of undissociated molecules in the sap and in the external solution. | Observed values of the total concentration of the dye in the sap. | Calculated values of the total concentration of the dye in the sap. |
|---|---|---|---|---|
| | *per cent* | M $\times 10^{-5}$ | M $\times 10^{-5}$ | M $\times 10^{-5}$ |
| 6.4 | 1 | 0.03 | 6 | 12.5 |
| 6.6 | 1.6 | 0.036 | 9 | 20.0 |
| 6.9 | 3.16 | 0.076 | 14 | 39.5 |

TABLE II.

From the percentage dissociation of brilliant cresyl blue calculated when $K = 10^{-5.6}$ the values of the total dye ($D^+$ ions and DOH) in the sap of *Nitella* are calculated for different concentrations of external dye solutions at 25°C. The pH of the sap is 5.6, at which pH 0.16 per cent of the total concentration of the dye is undissociated. At the pH value of the external dye solution (pH 6.9) 3.16 per cent of the dye is undissociated.

| Concentration of external dye solution. | Observed values of total concentration of dye in the sap. | Calculated values of the total concentration of dye in the sap. |
|---|---|---|
| M $\times 10^{-5}$ | M $\times 10^{-5}$ | M $\times 10^{-5}$ |
| 0.40 | 2.8 | 7.9 |
| 0.65 | 4.4 | 12.8 |
| 1.00 | 6.9 | 19.8 |
| 1.30 | 9.0 | 25.7 |
| 1.5 | 11.0 | 29.6 |
| 1.7 | 12.4 | 33.5 |
| 2.0 | 14.1 | 39.5 |
| 2.6 | 27.5 | 51.3 |
| 3.1 | 32.0 | 61.2 |
| 4.1 | 46.5 | 81.0 |

calculations are made, it is found, as shown in Table II, that the values of the total dye in the sap are higher than the observed. The fact that the observed values are lower than the calculated cannot be

wholly due to the increase in the pH value of the sap brought about by the presence of the dye in the sap, because, if this were the case, the extent of the lowering of the concentration of the dye in the sap should increase proportionally as the concentration of the dye in the sap is increased, but this does not seem to be the case. Such a lowering may be due to the fact that the dye is not so soluble in the sap as it is in the external solution or that the dissociation constant of the dye is not the same in the sap as it is in the external solution. Unfortunately there is not sufficient quantity of sap available to determine this point.

When the concentration of the dye has reached about 0.00014 M in the sap, the disagreement between the observed values and the calculated becomes less. This may be due to the occurrence of secondary changes in the cell, which increase the final concentration of the dye in the sap, as already suggested[1] by the writer.

If the values of the dye in the sap are calculated on the assumption that there are two dissociation constants, by using the values for the undissociated DOH calculated from the equation[10] already described, the discrepancy between the calculated values and the observed values of the dye in the sap is still greater.

Furthermore, if a correction is made for the ionic strength of the sap (about 0.1 M, comprising NaCl and KCl in about equal proportions) the discrepancy becomes still greater.

Let us now see if the values calculated on the basis of the Donnan equilibrium which is based on the entrance of ions will not agree with the observed. According to the Donnan equilibrium the relation $\frac{H^+ \text{ inside}}{H^+ \text{ outside}} = \frac{D^+ \text{ inside}}{D^+ \text{ outside}}$ must hold if the dye behaves as a monoacid base. When the values of $D^+$ ions inside are calculated on this basis, it is found that the calculated values are higher than the observed to the same extent as found in the case of the values calculated on the basis of the entrance of the dye as undissociated molecules of DOH when $K = 10^{-5.6}$

If the dye behaves as a diacid base with $K_1$ equal to $K_2$, the relation $\frac{D^+ \text{ inside}}{D^+ \text{ outside}} = \frac{(H^+)^2 \text{ inside}}{(H^+)^2 \text{ outside}}$, in which case the discrepancy between the observed and the calculated values is still greater.

In case $K_1$ is not equal to $K_2$ the calculation of the dye in the sap is somewhat complicated.

Whether the dye behaves as a monoacid or diacid base it would not be possible to distinguish from an analysis of the conditions in the sap at equilibrium if the dye enters the cell as undissociated dye base or as ions. We cannot assume that $\frac{H^+ \text{ inside}}{H^+ \text{ outside}} = \frac{D^+ \text{ inside}}{D^+ \text{ outside}}$ unless $H^+$ ions are diffusible through the protoplasm, but such is not the case with *Nitella*. The Donnan equilibrium requires that all the diffusible cations should stand in the same relation (inside to outside) as the H ions, but this is not the case. Furthermore the relation of Cl ions should be the reverse of that of the cations, but as a matter of fact the contrary is true. It therefore does not seem probable that the results can be explained on the basis of the Donnan equilibrium. Moreover, in all probability the ions do not enter, as pointed out by Osterhout and Dorcas,[13] because, if the rate of penetration increases with increase in the outside concentration of undissociated molecules the conditions at equilibrium cannot be due to the Donnan effect unless the undissociated molecules penetrate much more rapidly than the ions.

## V.

## DISCUSSION.

The above analysis seems to indicate that the rate of penetration of the dye into living cells of *Nitella* is proportional to the concentration[14] of undissociated molecules of the dye in the external solution, provided the conditions in the cell remain the same for all the external pH values. Since the temperature coefficient between 20° and 25°C.

[14] The following writers have assumed that a basic dye enters a living cell in the form of undissociated molecules, Overton, E., *Jahrb. wissensch. Bot.*, 1900, xliii, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1. McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501. Brooks, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 265. Referring to her experiments on the penetration of 2, 6-dibromophenol-indophenol into *Valonia*, Brooks states that "the amount of dye in the sap at equilibrium is proportional to the amount of undissociated dye in the external solution." Without further details, the writer is unable to determine whether in this case the dye enters as undissociated molecules or not.

is about 4.8, the rate cannot be dependent on the simple diffusion of DOH into the cell vacuole. The process may be complicated by a chemical combination of the dye in the protoplasm, or in the membranes, which might be a slower process than diffusion. This idea is supported by the following observation. In the experiments of Osterhout and Dorcas[13] on the penetration of $CO_2$ into living cells of *Valonia*, the temperature coefficient is very low (that of diffusion), while in the case of the writer's experiments on the penetration of brilliant cresyl blue into *Valonia* the temperature coefficient is very high (that of a chemical reaction). This leads the writer to believe that $CO_2$ enters the cell vacuole without combining with protoplasmic constituents, while the dye enters into combination. It may be possible that the dye enters by diffusion complicated by some other factors which are unknown to us at present.

In either case, it might well happen that the time curve of penetration of the dye into the cell vacuole would follow the equation for a unimolecular reaction as described.[1]

The treatment of the time curves made in the writer's previous papers on the penetration of the dye into the living cells of *Nitella* will hold on the basis of the present theory.

As to the conditions at equilibrium, the analysis seems to indicate that the final concentration of the dye is governed by the concentration of DOH in the external solution and by the percentage of dissociation of DOH in the sap (provided there are no complications due to other factors), as previously suggested by Osterhout and Dorcas[13] in discussing the penetration of $CO_2$ into *Valonia*.

In case there is a combination of the dye with a constituent, XA, of the sap, according to the equation $DOH + XA \rightleftharpoons DA + XOH$, the total dye in the sap would be composed of DOH, $D^+$ ions, and D*A* (all of the same color), and the calculations would have to be made accordingly. If D*A* were slightly soluble or slightly ionized, the concentration of $D^+$ ions and of DOH would remain the same as if DA were not present, unless the solubility or the pH values are changed by the presence of DA.

It may be added that all that has been said regarding DOH would apply equally well to a tautomer of the dye which acts similarly to DOH.

The solubility of the dye in the sap is also an important factor to be considered in relation to the penetration. It is of interest to mention here that methyl red, even at pH 8 or 9, where the dye is practically in the form of undissociated DOH and can be readily absorbed by the chloroform, cannot enter the cell sap, and it may be that this is due to the fact that methyl red is not very soluble in the sap. Or, it may be possible that there is a specificity in the behavior of the cell toward the undissociated molecules. That not all undissociated molecules enter may be still further shown by the fact that the acid dyes, such as thymol blue, brom thymol blue, phenol red, brom cresol purple, at pH 5.5, where the greater percentage of the total dye is in the form of undissociated HD molecules, do not enter the cell. These indicators are not very soluble in aqueous solution and in chloroform, so that this may be interpreted as being due to the still greater lack of solubility of the dye in the sap and in a lipoid, but a dye, such as acid fuchsin, which is readily soluble in water and slightly soluble in chloroform does not enter the cell. Further investigation is now being undertaken, and in the near future the writer hopes to throw some light on this problem.

The assumption that the ions do not enter appreciably is still further supported by the experiments on other basic dyes, crystal violet, malachite green (nitrate), and tetramethyl diaminophenoxazonium nitrate, neutral red, and methylene blue,[15] the rate of penetration of which depends chiefly on the concentration of undissociated DOH molecules.

[15] The writer is indebted to Dr. W. A. Jacobs and Dr. M. Heidelberger of this Institute for their kindness in supplying her with the first three dyes in highly purified form.

The methylene blue was purified by repeated recrystallization and extraction with chloroform. This dye does not appreciably enter the living cells of *Nitella* and *Valonia* at pH 5.4. In case the ions enter they enter so slowly that it is difficult to determine whether or not the presence of the dye in the sap is due to the contamination of the sap from the stained cell wall, or to an injury. This result is contrary to the results obtained by Brooks[14] on the penetration of the dye into *Valonia*. Methylene blue is not a good dye to use for this purpose because it is very difficult to separate it in pure form from other dyes which behave as weaker bases and which are mixed with it in great quantity, so that we cannot tell at higher pH values whether the dye which enters is methylene blue or other dyes which are not so strongly dissociated.

The mechanism of the penetration of the dye into living cells of *Nitella* represents by no means a simple process, and though the results tend to confirm more and more the assumption discussed in this paper, the writer disclaims any intention of attempting a complete explanation at present.

## SUMMARY.

The rate of penetration of brilliant cresyl blue into the living cells of *Nitella* indicates that the dye enters only in the form of the undissociated molecule. At equilibrium the total concentration of the dye in the sap is proportional to the concentration of the free base in the outside solution.

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