EXIT OF DYE FROM LIVING CELLS OF NITELLA AT DIFFERENT pH VALUES.*

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I.

INTRODUCTION.

The purpose of the present paper is to outline a theory[1] of the penetration of a dye (brilliant cresyl blue) into living cells of *Nitella*,[2] and to examine how far this theory is in harmony with the facts found in studying the exit of the dye from the cell.

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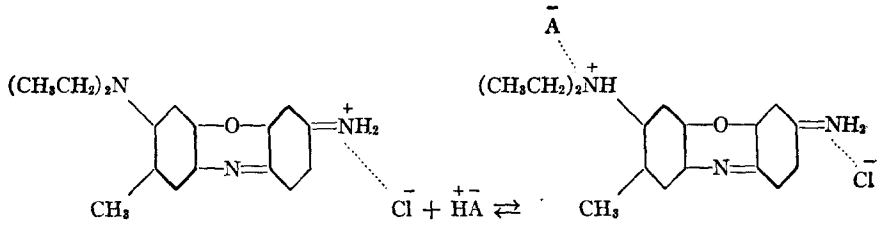
[1] Another theory, previously proposed by the writer (Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 727), regards the rate of penetration and the final equilibrium as dependent primarily on the concentration of the salts of proteins or weak acids, XA, at the surface of the cells, which combine with DS, to form a compound capable of diffusing into the sap. But on further experimentation the writer has concluded that there are many objections to this theory. The most serious objection of all is found in the fact that the rate of penetration seems to be directly proportional to the ratio $\frac{DB}{DS}$ for each particular dye at various pH values, when a comparison of the relative rates at these pH values is made by the writer among several basic dyes having different apparent dissociation constants.

Penetration is regarded by the following writers as dependent on the combining of dye ions with proteins: Bethe, A., *Biochem. Z.*, 1922, cxxvii, 18. Rohde, K., *Arch. ges. Physiol.*, 1920, clxxxii, 114. Pohle, E., *Deutsch. med. Woch.*, 1921, xlvii, 1464. Collander, R., *Jahrb. wissensch. Bot.*, 1921, lx, 354. Mathews, A., *Am. J. Physiol.*, 1898, i, 445.

[2] *Nitella* is a fresh water plant with multinucleate cells up to 4 inches in length, having an outer cell wall, beneath which is a very thin layer of protoplasm surrounding a relatively large central vacuole. The pH value of the sap in the vacuole is about 5.6, and the sap contains about 0.1 M halides in addition to organic acids and protein.

The theory [3,4] states that the dye exists in (at least) two forms, one of which is the "free base"[5] which we may call DB, soluble in chloroform, and another, which we may call DS, little or not at all soluble in chloroform. When the pH value of the solution increases a part of DS is changed to DB (and *vice versa*), so that at each pH value these two forms are in equilibrium with each other (and possibly with a third form, which may be a pseudo base found at still higher pH values). The nature of DB is still an open question. According to the theory of Hantzsch and others[6] both DB and DS may be treated as salts, DB being a quaternary ammonium salt which is capable of undergoing a further salt formation owing to the presence of other basic groups. The following[7] may make this clear by using cresyl blue as an example.



DB: colored blue in water and red in chloroform. Soluble in chloroform and water.

decreasing pH →

increasing pH ←

DS: colored blue in water. Soluble in water but little or not at all in chloroform.

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

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

[5] In former papers (see Foot-notes 3 and 4) this free base was called DOH for convenience but in order to avoid any possibility of confusing DOH with the dye hydrate (which may not be the form we are dealing with), it will be called DB hereafter.

[6] For a discussion of the theory of indicators see Henrich, F., Theories of organic chemistry, translated by John Johnston and Dorothy Hahn, London, 1922.

[7] For the formula see Conn, H. G., Biological stains, Geneva, New York, 1925, 51.

In the case of brilliant cresyl blue, DB and DS have the same color. If the above description of DB and DS is correct DB may be a strongly dissociated salt like DS. On the other hand, DB may be regarded as an undissociated molecule, and DS a strongly dissociated salt. Experiments are being carried out by the writer to determine the behavior of DB in this respect. Dr. Grinnell Jones has kindly determined the change in the conductivity of chloroform with and without the dye. When 100 cc. of pure chloroform were shaken up with 1 liter of M/150 borate buffer solution at pH 9, the specific conductivity of this chloroform was found to be 6×10^{-10}. When the same volume of chloroform was shaken up with 1 liter of M/150 borate buffer solution at pH 9 containing 3.5×10^{-4} M brilliant cresyl blue until there was practically no dye left in the aqueous solution, the specific conductivity of this chloroform was found to be 233×10^{-10} (about forty times greater than that of the chloroform containing no dye). This indicates that some or all of the dye exists in the chloroform in dissociated form.

The behavior of these two forms is very different. Apparently[8] DB can pass through the cell rapidly but DS penetrates extremely slowly or not at all.

Although it is evident that the form of the dye[9] which principally

[8] In connection with this, it may be assumed that DS corresponds with the ions and DB with the undissociated molecules, of a weak base, acid, or salt. In the paper by Hoagland and Davis (Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923–24, vi, 47) it is stated that the time of exposure of the living cells of *Nitella* to solutions containing NO_3 or Br ions, is a matter of days, before a detectable amount is found in the sap even at a favorable external pH value, temperature, and condition of light. The time of exposure, on the other hand, in the case of the penetration of cresyl blue into *Nitella* at a favorable external pH value, and temperature, is a matter of seconds. This fact agrees very well with Osterhout's suggestion (see Foot-note 10) that the undissociated molecules enter the cell, while the ions enter only very slowly or not at all. Furthermore, it agrees with the writer's theory, since the halides are only very slightly soluble in substances like chloroform and benzene, and in this respect the halides correspond with DS of the basic dye.

[9] The following writers state that some basic dyes enter the living cells as a free base: 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. It is stated by Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360, that an acid dye, 2,6,dibromophenol indophenol penetrates *Valonia* only in the form of an undissociated molecule.

penetrates the cell is the one which is soluble in chloroform, the writer does not wish to subscribe without reserve to the lipoid theory in its present form.

Previous experiments[1,2] have shown that when the external concentration is kept constant throughout the experiment, the entrance of the dye is found to follow the equation:

$$\frac{dx}{dt} = k\,(a - x)$$

where a = the concentration of the dye in the sap at equilibrium and x = the concentration of the dye in the sap at the time t, while k = the velocity constant. When the values of x are calculated from this equation they are found to agree very closely with the observed.

This agreement of course does not determine whether the process is governed by diffusion or by chemical reaction.

The temperature coefficient, furthermore, for the rate of penetration between 20°C. and 25°C. is very high (above 4) but this again may not necessarily indicate that the process is controlled by a chemical reaction rather than by diffusion.

Until further knowledge is obtained concerning the temperature coefficient for the diffusion of substances through an artificial system which more or less closely resembles the living cell of *Nitella*, and in which the passage of solute molecules or ions from one solvent phase to another probably does not depend upon forces of the sort usually regarded as "physical" it is not possible to determine whether the rate is governed by simple diffusion or by chemical reaction.

It is quite possible that under some circumstances it is controlled by diffusion and under other circumstances by chemical combination.

Since we are unable at present to decide whether the rate is controlled by diffusion or by chemical reaction let us for the sake of simplicity assume that it is diffusion, since in this case the mechanism is less complicated, and proceed to analyze the data on this basis. After this is done we shall discuss the alternative hypothesis; *i.e.*, that the rate is controlled by chemical reaction.

If we assume that the rate is controlled by diffusion, the mechanism may be explained as indicated by Diagram A (the cell wall being omitted). In this diagram nothing is said regarding combination of

the dye with a cell constituent but this does not indicate that there is no possibility of such a reaction in the protoplasm. It is regarded, for the present, as not affecting the rate; it is therefore omitted in order to simplify the diagram.

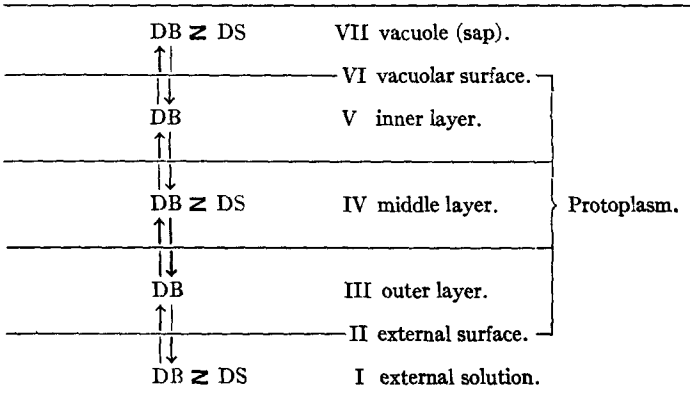

Diagram A. The cell wall is omitted for convenience.

Diffusion is designated by the sign $\rightleftarrows$; equilibrium between DB and DS by the sign ≷. For convenience the process is divided into seven parts. The inner and outer layers (III and V) are hypothetical. The vacuolar surface (VI) represents the protoplasmic surface in immediate contact with the sap, while the external surface (II) represents the protoplasmic surface in contact with the external solution. In I, IV, and VII, DB is in equilibrium with DS and a constant ratio of $\frac{DB}{DS}$ is maintained in each medium as long as the conditions remain unchanged. The concentration of DB in one part is in definite relation with that of DB in any other part of the diagram. Thus, for example, if the concentration of DB in the external solution (I) is changed, successive changes in the concentration of DB in all the parts of the cell take place. It is assumed that DB diffuses through III and V while DS diffuses to such a slight extent as to be negligible in the present case. For penetration the velocity of diffusion of the dye, DB, from I to VII is greater than that from VII to I while for the exit the velocity from VII to I is greater. An equilibrium is established when the velocity of the inward diffusion is equal to the outward

diffusion. At equilibrium the concentration of DB in the vacuole is proportional to the concentration of DB in the external solution depending on the apparent[10] dissociation constant and on the distribution coefficient $C = \frac{\text{DB in the sap}}{\text{DB in the external solution}}$. If C is 1, the concentration of DB in the sap at equilibrium is equal to the concentration of DB in the external solution. If C is lower than 1, the concentration of DB in the sap will be lower than that of DB in the external solution, and *vice versa*. Since there is maintained in the sap a definite ratio of $\frac{DB}{DS}$, the concentration of DS depends on the concentration of DB. Thus the final concentration of the total dye (DB and DS) in the sap at equilibrium will depend on the apparent dissociation constant of the dye $\left(\textit{i.e.}\text{ the ratio of } \frac{DB}{DS}\right)$ in the sap, on the partition coefficient of DB, and on the ratio of $\frac{DB}{DS}$ in the external solution.

According to this scheme it is possible to study the mechanism either of the penetration of the dye into or of the exit from the vacuole, by determining the concentrations of the dye (DB plus DS) in the sap, as long as the color of DB does not differ from that of DS. The rate of penetration will increase and that of exit will decrease when the concentration of DB just outside the external surface (II) is increased. The reverse is the case when the concentration of DB in the sap is increased as, for example, by any change in the medium which changes the apparent dissociation constant of the dye $\left(\textit{i.e.}\text{ the ratio of } \frac{DB}{DS}\right)$, or by a change in the solubility of DB in the sap.

The theory thus outlined accords with the facts previously obtained for the penetration of dye.[3,4] Let us now consider whether it accords with the facts observed in connection with the exit of dye from the cell. We shall proceed upon the assumption that when a stained cell is placed in a solution containing no dye, the dye comes out according to

[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, viii, 131. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255.

the scheme outlined in Diagram A (the outward process VII I from the sap to the external solution). We shall test this assumption by experiments.

II.

Methods.

Living cells of *Nitella* were placed in 8.6×10^{-6} M brilliant cresyl blue at pH 8.2 for 17 minutes, when the concentration of the dye in the sap reached 7.94×10^{-5} M. (The concentration of dye in the sap was determined colorimetrically as described below.) The cells were then removed from the dye solution, gently wiped with a damp cloth, and distributed in solutions at different pH values (pH 5.4 to 8.2) containing no dye. At definite intervals a few cells were removed, and the concentration of the dye in the sap was determined by the colorimetric method as follows: The end of each cell was cut and the sap was gently squeezed out onto a glass slide; the sap was drawn up into a capillary tube the color of which was matched with that of the capillary tube of the same diameter containing a standard dye solution.

In order to avoid experimental error from the presence of the dye in the external solution, only six cells were placed in 200 cc. of solution (without dye) and the solution was constantly stirred and changed every 15 seconds. This method gives the maximum velocity constant for each experiment, *i.e.* there is no further increase in the velocity of the exit of the dye if the frequency of stirring and of changing the solutions is increased.

The concentration of the external dye solution (8.6×10^{-6} M) is chosen because this is sufficiently dilute to avoid error due to the adhering of the dye to the surface of the cell, after the cell is removed and wiped with a damp cloth. The cellulose wall is not stained when cells are placed in this concentration of dye at pH 8.2.

If too high an external dye concentration is used, the exit of the dye from the sap is hindered (even when the cell wall is not stained) when the cells are removed from the dye solution and placed in a solution without dye, though the latter solution is constantly stirred and changed. This decrease in the rate of the exit of the dye is due in all probability to the fact that the dye adhering to the surface of the cell cannot be washed away quickly enough. This complication may

be eliminated by using an external solution which is at least seven times more dilute than the concentration of the dye in the sap which is chosen for the experiments. In order to be absolutely certain that the concentration used avoids this experimental error the experiment was repeated with still lower concentrations but it was found that the result was not altered.

All possible care was taken to have all the cells used at one time as alike as possible, so that the differences in the rates were due chiefly to the experimental conditions and not due to the difference in the condition of the cells before the experiments began. Unless otherwise stated, the *Nitella* used was obtained from Cambridge and the experiments were carried out in early fall when the cells were in excellent condition.

The tests for early stages of injury are very unsatisfactory. The appearance of masses of chlorophyll in the expressed sap, the rapid exit of halides from the intact cell, and the loss of turgidity all indicate advanced stages of injury rather than the first. For this reason it is desirable to control the experiments in some way so that we have a more or less uniform method of detecting the condition of the cell immediately after the experiments. To do this, after each experiment, some of the cells were tested for injury by placing them in distilled water, and for 4 days[11] at intervals of every few hours the percentage mortality was compared with that of the control cells (fresh cells placed in distilled water under same conditions). It was found that the percentage mortality of the cells thus treated was about the same as that of the control cells.

These experiments, like those heretofore described[3,4] by the writer, were carried out in an incubator at 25 $\pm$ 0.5°C., into which diffused light was permitted to enter through small ventilating holes.

The buffer solutions used were M/150 phosphate mixtures. The pH values of these buffer solutions were determined by means of the hydrogen electrode. The dye used was that of Grübler, and was taken from the same stock bottle as the one used in the writer's experiments[3,4] on penetration.

[11] It is not desirable to continue such a test for any longer period since the comparison between the test cells and the control cells becomes more doubtful, in view of the fact that even the control cells do not live indefinitely in the laboratory.

III.

Analysis of the Time Curves.

That lowering of the pH value of the external solution (containing no dye) hastens the exit of the dye from the sap of living cells of *Nitella* is indicated[12] by the curve in Fig. 1. At low pH values (5.4 to 6) the process may be followed until practically all the dye has come out of the sap without causing injury to the cells, but at higher pH values injury or death may occur. The curves given in Fig. 1 represent the process when the cells are not injured.

At higher pH values of the external solutions here employed it is probable that all the dye in the sap would eventually be found to come out of the vacuole if we could continue the experiment long enough and still keep the cell from being injured. The analysis of the time curves therefore is made on the assumption that at the end of the process the concentration of the dye in the sap is zero at all external pH values.

The velocity of diffusion is assumed to be proportional to the difference between the concentration of DB in the sap and that of DB in the external solution. According to the present theory, there is a definite ratio of $\frac{DB}{DS}$ in the sap and in the external solution, so that for mathematical treatment the concentration of DB in both may be replaced, for convenience, by the concentration of the total dye (DB and DS) which we actually measure. Since the concentration of the dye in the external solution is approximately zero, we may in the following equation let a denote the initial concentration of the dye in the sap, x the concentration of the dye that has disappeared from the sap at time t, and k the velocity constant of diffusion. We may then write:

$$\frac{dx}{dt} = k\,(a - x) \quad \text{or} \quad k = \frac{1}{t} \log \frac{a}{a - x}$$

When k is calculated for each time curve it is found to decrease

[12] These results confirm those obtained previously by the writer (Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 223). It may be added here that the writer has chosen to study the exit of the dye by the method presented in this paper first, because other methods offer greater complications.

TABLE I.

Exit of Brilliant Cresyl Blue from Living Cells of Nitella at Varying External pH Values at 25°C.

The process is represented by the equation $\frac{dx}{dt} = k\,(a - x)$, where a is the initial concentration of dye in the sap, $a - x$ is the concentration of dye in the sap at the time t, and k is the velocity constant. The calculation is made with a 20 inch slide rule. $a = 7.94 \times 10^{-5}$ M for all external pH values.

t	pH 5.4			pH 5.7			pH 6.0			pH 6.8			pH 7.5			pH 7.8			pH 8.2		
	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.
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	M × 10^5		M × 10^5	M × 10^5		M × 10^5	M × 10^5		M × 10^5
1	4.83	0.215	4.96	4.83	0.215	5.07	5.59	0.152	5.62	6.21	0.107	6.30									
2	3.05	0.208	3.11	3.28	0.192	3.23	4.00	0.148	3.96	4.83	0.107	5.01	6.14	0.055	6.02	6.56	0.041	6.46	6.9	0.030	7.08
3	2.15	0.189	1.94	2.31	0.179	2.06	2.76	0.153	2.81	4.14	0.094	3.98	5.00	0.067	5.24	5.87	0.043	5.82	6.80	0.023	6.68
5							1.40	0.151	1.40	2.76	0.092	2.51	3.80	0.064	3.97						
7													3.28	0.055	3.02	3.80	0.046	3.84	5.52	0.023	5.31
10																2.59	0.049	2.82	4.49	0.025	4.47
Average.....		0.204			0.195			0.151			0.100			0.060			0.045			0.025	

slightly as the concentration of the dye (DB and DS) in the sap approaches zero (Table I). This decrease in the value of k may be explained on the ground that there is a relative increase in the velocity of the inward process due to an increase in the ratio of DB in the film just outside the external surface (II) to the DB in the outer layer (III) and hence to the DB or to the total dye (DB and DS) in

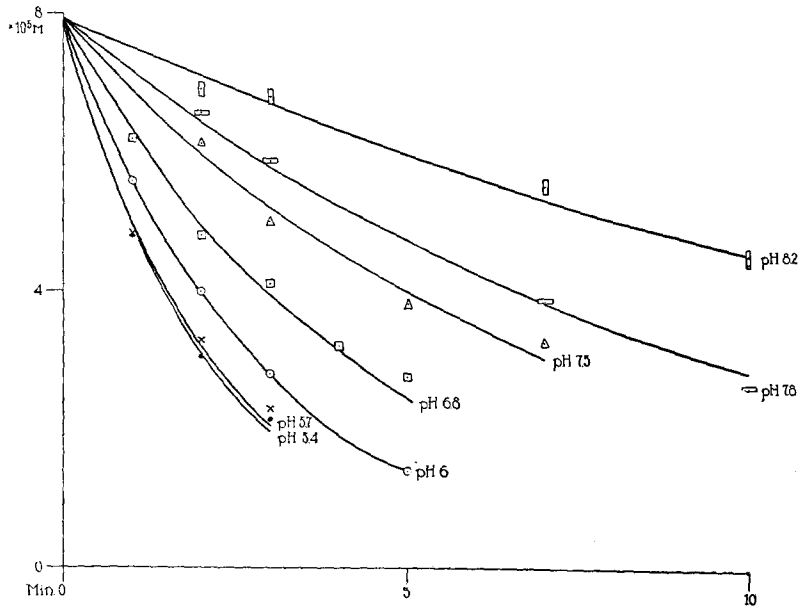

FIG. 1. Time curves showing the exit of brilliant cresyl blue from the living cells of *Nitella* at different external pH values at 25°C., when the initial concentration of the dye in the sap is 7.94×10^{-5} M. The ordinates represent the concentrations of dye in the sap, while the abscissæ represent time. The curves as drawn represent the calculated values of the concentration of the dye in the sap, while the symbols represent the observed values. Each point on every curve is an average of fifty experiments, and the probable error of the mean is less than 8 per cent of the mean.

the sap, since toward the end of the process, where there is a very little dye (DB and DS) left in the sap, the amount of DB in the film just outside the external surface (II) may no longer be a constant fraction of DB in the outer layer (III) and of the total dye in the sap as was the case at the beginning of the process (this will be discussed later on). Since this decrease in the con-

stants is not very great, the average is taken of all the velocity constants at each external pH value. When the values of $a - x$ are calculated for each time curve by using the average value of k, thus obtained, they are found to agree fairly closely with the observed except toward the end of the process, where there is an indication that the calculated values are slightly lower than the observed, as shown under pH 5.4 and 5.7, Table I.

In connection with the analysis of the time curves it may be well to repeat the following in order to avoid misunderstanding. (1) It makes no difference in the form of the time curve whether we measure DB alone or DB + DS in the sap, since DB and DS stand in constant relation as long as the conditions, such as the pH value of the sap, remain unchanged. We actually measure DB plus DS (called the "total dye" for convenience) in the sap and the analysis of the time curves is made by using the concentrations of the total dye. (2) The concentration of the total dye in the sap is affected by the concentration of DB in the other parts of the cell, and in the solution outside the cell. Thus, for example, if the concentration of DB in the outer layer (III in Diagram A) is decreased, the concentration of DB and hence that of the total dye in the sap is decreased.

When the temperature coefficient between 20° and 25°C., for the exit of the dye at pH 5.7 and also at pH 7.8 was determined, Q_{10} was found to be about 4.

IV.

The Relation of the Velocity Constant to the pH Value of the External Solution.

The time curve for each external pH value is found (see Table I) to follow approximately the equation:

$$\frac{dx}{dt} = k\,(a - x)$$

where a denotes the initial concentration of DB in the vacuole minus the concentration of DB in the external solution (which in this case is practically zero), x the amount of DB that has diffused out of the vacuole at the time t, and k the velocity constant. In both cases, DB for convenience is put equal to the total dye which is actually measured.

If the stirring and frequent changing of the external solution kept the concentration of DB equal to zero just outside the external surface of the protoplasm (which is designated by II, the external surface, in Diagram A), we should expect to find the same values of k for all external pH values. But the value of k decreases with an increase in the external pH value, as shown in Fig. 2, and the explanation for

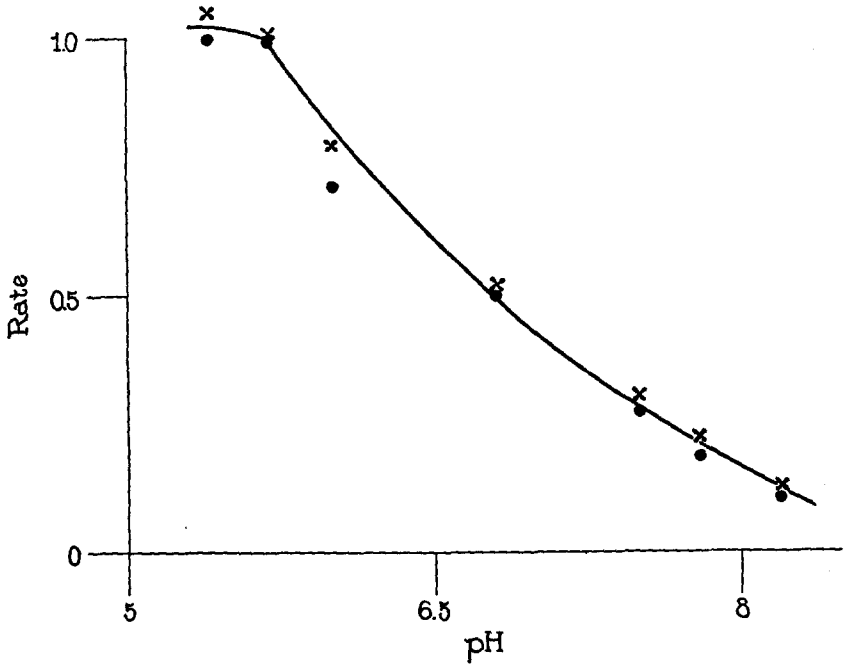

FIG. 2. Curve showing the relation of the external pH values to the rates of the exit of the dye (symbol ×), and also to the velocity constants (symbol ●). The ordinates represent the rates, and also the velocity constants multiplied by 5.2 (for convenience of plotting), and the abscissæ represent the pH values of the external solutions.

this may be that the concentration of DB at the external surface changes as the external pH value changes. Let us assume that just outside the external surface of the protoplasm there is a liquid film more or less protected against the direct effect of stirring by the cellulose wall and that in this film a certain amount of DB (a certain percentage of which is at once transformed to DS) collects as it comes out of the cell; also that the total amount of DB

which comes out is approximately the same in all solutions containing no dye, but the per cent of it which remains in the form of DB depends on the pH value of this film (which is assumed to be approximately the same as that of the external solution since the latter can penetrate freely through the cellulose wall into the film) since a certain amount of DB will change to DS depending on the pH value of the film.

When the cell is removed from the dye, wiped, and placed in a solution in which no dye is present, DB begins to diffuse from the vacuole, the protoplasm, and the film just outside the protoplasmic surface. We may assume that the concentrations in all of these places fall off together, so that when the concentration in the vacuole has fallen to half the value it had at the start, that of the protoplasm and the film will also have fallen to approximately half value. In that case we may regard the falling off in the protoplasm and in the film as following an approximately unimolecular curve (since we have found this to be true of the dye in the vacuole) and consequently the amount of DB in the film will be an approximately constant fraction of that in the sap. If we call the dye in the sap $a - x$ and designate as y the amount of DB in the very thin layer in immediate contact with the outer surface of the protoplasm we may write:

$$y = b\,(a - x)$$

in which b is a constant expressing the amount of DB in the film as a fraction of the amount of DB (which for convenience is put equal to the total dye) in the vacuole throughout the process at any one external pH value.

When the pH value of the external solution changes the value of b will also change, since the per cent of DB in the film will be altered. In order to see how this will affect the rate of exit of the dye from the vacuole, let us first consider the case where there is no effect of y on the velocity constant. Since according to our analysis of the time curves, the dye comes out of the vacuole in a unimolecular fashion, we may write

$$\frac{dx}{dt} = k_1\,(a - x)$$

in which k_1 is the velocity constant of the process when dye is present

on one side of the surface only. This expression gives us the rate of exit of the dye when there is no dye in the film. When dye is present in the film a certain amount diffuses back into the cell. The true rate[13]

[13] Criticism may be made as to this method of mathematical treatment since it involves the consideration of the diffusion of DB through only one very thin surface, when in fact the protoplasm of *Nitella* consists of more than one such layer. Even if we were to treat the entire protoplasmic layer as one surface, the question may be raised as to how far we are justified in considering the protoplasm to be thin enough for such a mathematical treatment. If we consider the diffusion of DB through two surfaces, the vacuolar and the external surfaces (II and VI in Diagram A), one at a time, then we may modify the analysis given in the text in the following manner. The amount diffusing inward through the external surface in unit time when DB is present in the film only $= k_1 y$ (just as described in the text). Let us assume that the amount diffusing inward in unit time through the vacuolar surface when there is no DB in the vacuole is a constant fraction of $k_1 y$ so that we may put this amount equal to $ck_1 y$, in which c is a constant. The amount diffusing outward in unit time through the vacuolar surface when DB is present in the vacuole but not in the protoplasm or in the film (DB fictitiously introduced into the vacuole without getting into the protoplasm) is $k_1 (a - x)$. Hence we take the difference between the amount going outward through the vacuolar surface and the amount passing inward through the vacuolar surface and we have:

$$\frac{dx}{dt} = k_1 (a - x) - k_1 cy$$

put $y = b (a - x)$ in which b is a constant (just as given in the text) then

$$\frac{dx}{dt} = k_1 (a - x) - k_1 bc (a - x)$$

$$\frac{dx}{dt} = (k_1 - k_1 bc) (a - x)$$

or on integration

$$k_1 - k_1 bc = \frac{1}{t} \log \frac{a}{a - x}$$

$$bc = \left(k_1 - \frac{1}{t} \log \frac{a}{a - x} \right) \div k_1$$

Since we are not able to verify the values of the constants b and c experimentally, and since assuming a value for either b or c is very unsatisfactory, we are not able to explain the mechanism any more convincingly than we have done in the text.

Experiments are now in progress to see whether it is possible to determine the

of exit is the resultant of these two processes and may be found by subtracting the amount which would diffuse inward if dye were present on one side only from the amount that would diffuse outward if dye were present on the other side only. Hence we may write

$$\frac{dx}{dt} = k_1 (a - x) - k_1 y$$

Substituting in this equation the value $y = b (a - x)$ we have

$$\frac{dx}{dt} = k_1 (a - x) - k_1 b (a - x)$$

$$\frac{dx}{dt} = (k_1 - k_1 b) (a - x)$$

or, on integration,

$$k_1 - k_1 b = \frac{1}{t} \log \frac{a}{a - x}$$

$$k_1 (1 - b) = \frac{1}{t} \log \frac{a}{a - x}$$

$$k_1 = \left(\frac{1}{t} \log \frac{a}{a - x}\right) \div (1 - b)$$

and

$$b = \left(k_1 - \frac{1}{t} \log \frac{a}{a - x}\right) \div k_1$$

We may put $k_1 (1 - b) = k$; substituting the value $k = \frac{1}{t} \log \frac{a}{a - x}$ we have $b = \frac{k_1 - k}{k_1}$.

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constants experimentally in order that we may know in greater detail what the controlling factor is for the rate of penetration into and that of exit of the dye from the vacuole.

It might be possible that the rate of penetration into and that of the exit of the dye from the vacuole are controlled by the rate of diffusion of DB through only one very thin layer in the cell, (the layer through which the diffusion of DB is the slowest). Whether this is represented by the external surface (II in Diagram A) or by the vacuolar surface (VI) or by some other part of the cell, we are not able to state definitely at present. In all probability under varying conditions the controlling layer varies.

The values of a, x, and t may be obtained experimentally but the values of k_1 and b cannot be obtained in this way. We may, however, assume an approximate value of k_1 and we are justified in doing this since we are interested in relative rather than in absolute values. The analysis of the time curves shows decreasing values of k (see Table I and Section III) with increasing external pH values. When such

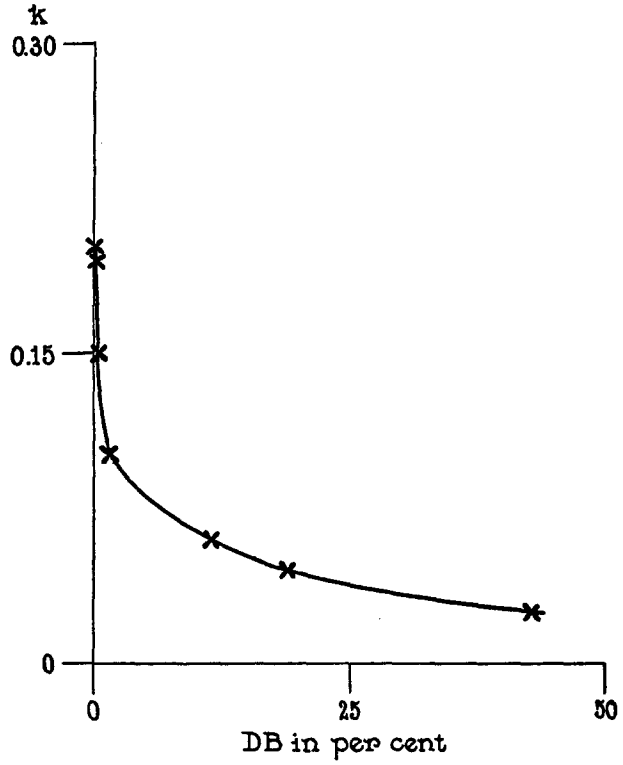

FIG. 3. Curve showing the relation of the velocity constants (k), plotted as ordinates, to the concentrations of free base (DB), in per cent as abscissæ.

values of k are plotted as ordinates and the concentration of DB in the external solution expressed as per cent of the total dye, (obtained from the experiments[4] made by the writer on the distribution of the dye between chloroform and water) are plotted as abscissæ, we obtain the curve shown in Fig. 3. The curve indicates that when DB (and consequently y) equals zero, the value of k is somewhat above

TABLE II.

The Relation of the Dye in the Film Just Outside the Protoplasmic Surface to the Dye in the Vacuole, at Different External pH Values.

The values of b are obtained by the equation $b = (k_1 - \frac{1}{t} \log \frac{a}{a-x}) \div k_1$ where k_1 denotes the velocity constant of the diffusion of the dye into or out of the living cell of *Nitella* where dye is present on one side of the protoplasmic surface only (the value of k_1 is assumed to be 0.37); where a denotes the initial concentration of dye in the vacuole; x the amount of dye that has diffused out of the vacuole at time t; and where b is a constant expressing the concentration of DB in the film as a fraction of the concentration of DB in the vacuole throughout the process at any one external pH value. Knowing the values of b, the values of y are obtained by the equation $y = b\ (a - x)$ where y denotes the DB in the film, $a - x$ the DB in the vacuole. Values of y thus obtained are relative values, since the given observed values of $a - x$ represent the "total dye" (DB + DS). Calculation is made with a 20 inch slide rule.

pH 5.4				pH 5.7				pH 6.0				pH 6.8			
$a-x$ obs.	k	b	y when $b = 0.45$	$a-x$ obs.	k	b	y when $b = 0.47$	$a-x$ obs.	k	b	y when $b = 0.59$	$a-x$ obs.	k	b	y when $b = 0.73$
M × 10^5			M × 10^5	M × 10^5			M × 10^5	M × 10^5			M × 10^5	M × 10^5			M × 10^5
4.83	0.215	0.42	2.17	4.83	0.215	0.42	2.27	5.59	0.152	0.59	3.30	6.21	0.107	0.71	4.55
3.05	0.208	0.44	1.37	3.28	0.192	0.48	1.54	4.00	0.148	0.60	2.36	4.83	0.107	0.71	3.53
2.15	0.189	0.49	0.97	2.31	0.179	0.52	1.09	2.76	0.153	0.59	1.63	4.14	0.094	0.75	3.02
								1.40	0.151	0.59	0.83	2.76	0.092	0.75	2.02
Average....		0.45				0.47				0.59				0.73	

pH 7.5				pH 7.8				pH 8.2			
$a-x$ obs.	k	b	y when $b = 0.84$	$a-x$ obs.	k	b	y when $b = 0.88$	$a-x$ obs.	k	b	y when $b = 0.93$
M × 10^5			M × 10^5	M × 10^5			M × 10^5	M × 10^5			M × 10^5
6.14	0.055	0.85	5.16	6.56	0.041	0.89	5.78	6.90	0.030	0.92	6.42
5.00	0.067	0.82	4.20	5.87	0.043	0.89	5.16	6.80	0.023	0.94	6.32
3.80	0.064	0.83	3.19	3.80	0.046	0.88	3.35	5.52	0.023	0.94	5.14
3.28	0.055	0.85	2.76	2.59	0.049	0.87	2.27	4.49	0.025	0.93	4.18
Average.....		0.84				0.88				0.93	

0.3. Extrapolation has been attempted by various methods but with such a curve it is very difficult to obtain any reliable result. We may assume, however, that we are not too far from the true value if we take the maximum value of k to be 0.37.

If we solve for the values of b in the above equation, we find that they remain fairly constant for each external pH value, but they increase with an increase in the external pH value as shown in Table II. It may be stated here that the values of b are the same whether $a - x$ represents the "total dye" or DB.

Knowing the values of b and $a - x$, we may calculate the values of y by means of the equation: $y = b\ (a - x)$ for any value of $a - x$ as shown in Table II.

In calculating the values of y, the observed values of $a - x$ (Table II) representing the "total dye" in the sap are used for convenience. Since we are interested primarily in the relative values of y, such values will give us the desired information. It is needless to state that if the values of DB in the sap were used instead of those of the total dye (DB plus DS), the values of y would be considerably lower than those given in Table II, but the ratio of one value of y to another would remain unchanged.

At each external pH value the values of y are found to increase with increase in the value of $a - x$. If we take a fixed value of $a - x$ and compare the values of y at different pH values, we find that the value of y increases with an increase in the external pH value.

In order to bring out clearly the effect of y on the velocity constant of exit we may return to the equation on page 90

$$k = k_1 - k_1 b$$

and substitute the value $b = \frac{y}{a - x}$ (see page 88). We then have

$$k = k_1 - \frac{k_1 y}{a - x}$$

It may be added here that the values of k are the same whether $a - x$ represents the "total dye" or DB.

Let us now consider the relation of y to the per cent[14] of DB in

[14] The discussion of the apparent dissociation constant is given in detail in the paper referred to in Foot-note 3.

the film (as determined by the pH of the external solution). We shall take for convenience the values of y where $a - x$ is 4.5×10^{-5} at different external pH values and take the percentage of DB as calculated from the distribution of DB between chloroform and water at different pH values of the external solution.

Let us first see if the values of y at different pH values are proportional to the values in per cent of DB obtained from the dissociation[3] curve of the dye (which gives the DB in per cent calculated from the data obtained by the experiments on the distribution of the dye between chloroform and water). If we take for convenience the value 4.5×10^{-5} M for $a - x$ and find the value of y at pH 7.8 at which pH value 20 per cent of the dye is in the form of DB (according to the dissociation curve) we are able to calculate the values of DB in per cent on the basis of the values of y at other pH values (since we know the values of y for this fixed value of $a - x$), by the following equation.

$$\frac{y_1}{y_2} = \frac{m_1}{m_2}$$

when y_1 = the value of y at pH 7.8 = 4×10^{-5} M.
y_2 = the value of y at another pH value, say pH 7.5 = 3.8×10^{-5} M.
m_1 = 20 per cent.
m_2 = the DB in per cent at pH 7.5.
By substituting we get

$$\frac{4 \times 10^{-5}}{3.8 \times 10^{-5}} = \frac{20}{m_2}$$

$$m_2 = 19 \text{ per cent}$$

Where the values of DB are thus obtained for different pH values, they are found to be higher than the values of DB of the dissociation curve.

Since the values of y do not appear to be directly proportional to the values of DB in the dissociation curve, we may look for another relationship. If we plot the values of $\frac{\text{per cent DB in external film}}{y}$ (at varying pH values of the external solution) against the per cent of DB in the film (z), we get a line which is fairly straight, as shown

in Fig. 4. This indicates a relation corresponding to Langmuir's[15] equation for adsorption,

$$y = \frac{m\,n\,z}{1 + zm}$$

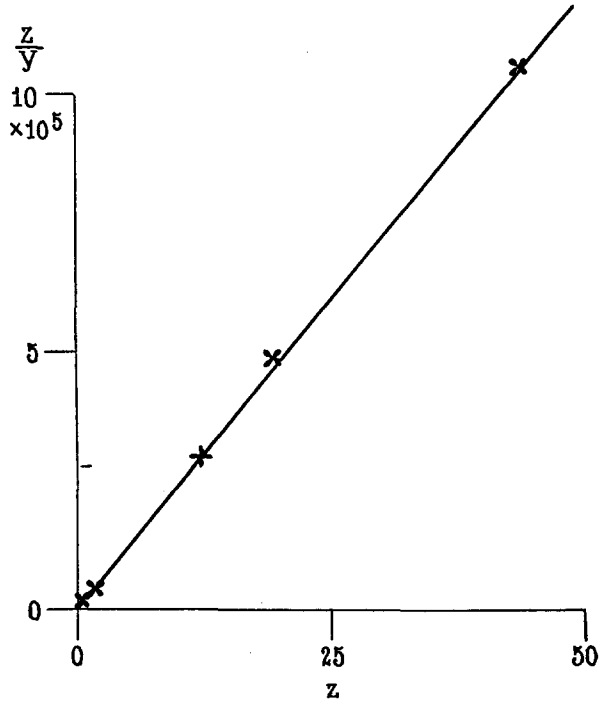

FIG. 4. Graph showing that Langmuir's equation $y = \frac{mnz}{1 + mz}$ (in which z = DB) may be applied to the process of the exit of the dye since the graph is a straight line. Ordinates represent $\frac{z}{y}$, and the abscissæ represent z.

where y is the substance adsorbed by a fixed concentration of an adsorbent, z is the concentration of the solution at equilibrium, and m and n are constants.

This might be regarded as indicating that the velocity constant (k) of the exit of the dye depends on the value of y which represents the

[15] Langmuir, I., *J. Am. Chem. Soc.*, 1918, xl, 1368.

amount of DB adsorbed by the protoplasmic surface from the film of external solution just outside the surface. But the applicability of this equation does not necessarily mean that we have to do with adsorption. For example, as Hitchcock[16] has pointed out, a similar relation applies if we have to do with a reversible chemical reaction where one of the reactants has a constant value.

It may be objected that if y represents the amount of DB adsorbed at the surface it will not be a constant fraction of $a - x$ during the entire process of exit of the dye but will be relatively greater during the latter part of the process. From the results of calculations which neglect this factor it is evident that it is not one of sufficient importance to effect any material change in the calculations here given.

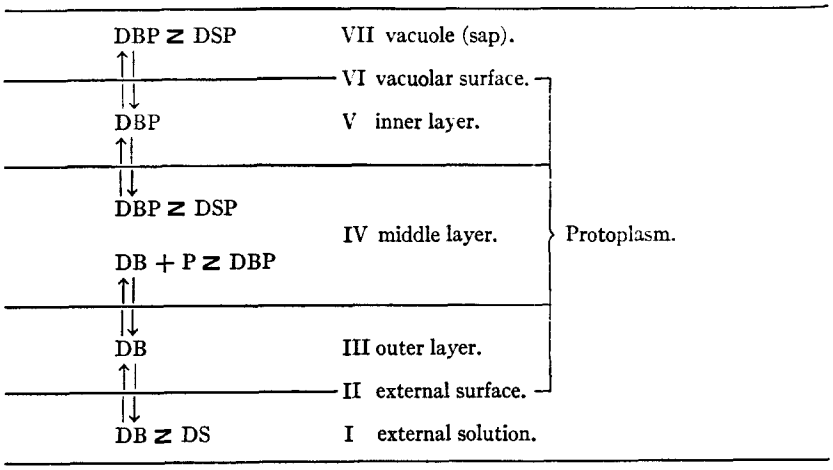

Diagram B. The cell wall is omitted for convenience.

If the surface forces constrain the molecules of DB so that they are not free to diffuse we meet with a difficulty. This difficulty would disappear if a constant fraction of the molecules is so constrained since that would merely lead us to divide the values of y by a constant factor.

The writer does not wish to lay any emphasis upon the fact that adsorption might possibly explain the relations observed but prefers to give the formula as a purely empirical one leaving the interpretation to future research.

[16] Hitchcock, D. I., *J. Gen. Physiol.*, 1925–26, viii, 61.

The preceding discussion of diffusion applies whether DB is in the form of undissociated molecules or ions.

Let us now consider the hypothesis that the rate of penetration and likewise of exit of dye from the vacuole is controlled by a chemical combination between DB and a constituent of the protoplasm. For this purpose we may modify Diagram A to conform to Diagram B or C.

Equilibrium between the two forms of dye is designated by the sign ⩵ and diffusion by arrows ⇄. The entire mechanism represents a reversible process. Let us first take up Diagram B. DB can pass through III but DS cannot. As DB enters IV, it reacts with P, a protoplasmic constituent to form DBP. This form of dye compound, DBP, enters into equilibrium (according to the apparent dissociation constant) with another form of dye compound, DSP, which may represent a tautomere, or a complex compound. The ratio of $\frac{DB}{DS}$ and that of $\frac{DBP}{DSP}$ in IV depends on conditions in the protoplasm (pH value, solubility, etc.). DB, DS, and DSP are unable to pass through V, while DBP can pass through V but not through III. When DBP enters the vacuole it establishes an equilibrium with DSP, the ratio of $\frac{DBP}{DSP}$ being dependent on conditions in the sap, so that as long as the latter remain unchanged this ratio remains constant. The concentration of DBP in the sap is dependent on its concentration in the protoplasm, and on the concentrations of DB in all the parts described in the diagram. Thus, if the concentration of DB in III diminishes by its exit from III to I, then DB in IV decreases by its exit from IV to III, thus resulting in a decrease in DBP which in turn causes a corresponding amount of DBP to diffuse out from VII to IV.

We may consider a cell of *Nitella* in a solution as representing a heterogeneous system consisting of at least three phases: (1) the external solution, (2) the protoplasmic layer, and (3) the sap in the vacuole. If we venture to suppose that the protoplasm has non-aqueous layers at its outer and vacuolar surfaces, we shall consider the system to be composed of at least five phases.

The relation of the reaction DB + P ⩵ DBP in the protoplasm to the DB in the external solution may be made clearer if we consider the

hydrolysis[17] of an ester in hydrochloric acid when the ester is distributed between HCl and benzene. As fast as the ester is hydrolyzed in hydrochloric acid, more ester passes in from the benzene. The rate of hydrolysis is controlled by the distribution coefficient, C, of ester between hydrochloric acid and benzene, since the lower the value of C the less ester diffuses from benzene to the hydrochloric acid in a given time. The equation for this process resembles that for a unimolecular reaction in a homogeneous system. The only difference is that this equation contains the correction for the partition coefficient C.

Thus in the case of the reaction DB + P $\rightleftarrows$ DBP, the rate may be assumed to be dependent on the amount of DB that passes into the protoplasm. If the concentration of DB in the external solution is raised, more DB will enter the protoplasm in a given time, and this will increase the rate of reaction. Exit of the dye from the protoplasm may also be explained on this basis. If there is no DB outside the cell, DB will come out of the protoplasm, and with the decrease in the concentration of DB in the protoplasm, the reaction DBP $\rightarrow$ DB + P will proceed faster; DB thus formed will continue to come out until there is no DBP in the protoplasm. But if the DB which comes out is not at once removed, a certain amount will diffuse back into the protoplasm, so that in a given time the decrease in the concentration of DB in the protoplasm will be less than when there is no DB outside. This will correspondingly retard the process DBP $\rightarrow$ DB + P, and hence diminish the rate of exit of DB.

So far as the relation of the reaction DB + P $\rightarrow$ DBP in the protoplasm to the DBP in the sap is concerned, the same explanation will hold. With an increase in the concentration of DBP in the protoplasm more DBP will diffuse into the sap. If DBP in the protoplasm decreases, on the other hand, DBP will tend to come out of the vacuole into the protoplasm. The rate of the reaction will depend on the concentration of DBP in the protoplasm. If for example the concentration of DBP in the sap is increased, causing a decrease in the amount of DBP diffusing out of the protoplasm into the vacuole, the concentration of DBP in the protoplasm will increase. This increase will retard the reaction DB + P $\rightarrow$ DBP. Thus the rate of reaction DB + P $\rightarrow$ DBP

[17] Goldschmidt, H., and Messerschmidt, A., *Z. physik. Chem.*, 1899, xxxi, 235.

is controlled by the concentration of DB and DBP in the protoplasm. Increase in DB will hasten the reaction DB + P → DBP, while increase in DBP will retard it. The concentration of DB in the protoplasm depends on the amount of DB that enters or goes out of the protoplasm at a given time, and hence on the velocity of diffusion of DB through II or III in the diagram. The concentration of DBP in the protoplasm depends on the amount of DBP that goes out of the protoplasm into the vacuole, and the amount of DBP that enters the protoplasm from the vacuole, at a given time, and hence the rate of diffusion of DBP through V or VI in the diagram, B. It must be added here that the concentrations of DB and DBP are obviously interdependent.

In view of the fact that the time curve for the hydrolysis of ester in hydrochloric acid, as described above, follows an equation similar to that of an irreversible unimolecular reaction in a homogeneous system, it is not surprising that we find in the case of penetration of dye into *Nitella* the unimolecular time curves for a homogeneous system.

Thus the analysis of the time curve of the exit of the dye may be made in this case by the use of the same equation as in the case of diffusion

$$\frac{dx}{dt} = k_1 (a - x) - (k_1 y)$$

where a denotes the concentration of DB at the start in the protoplasm, $a - x$ the amount left combined with protoplasm at time t, and y the amount of DB in the film of liquid just outside the external protoplasmic surface. The presence of DB in the film will cause some DB to diffuse back into the protoplasm, and thereby retard the decrease of DB in the protoplasm. This retards the rate of the reaction DBP → DB + P and hence it retards the exit of the dye from the vacuole.

What we actually measure is the concentration of the total dye (DBP and DSP) in the sap, and the value of $a - x$ is taken from the amount of the total dye in the sap at equilibrium. This method is justified since we are interested primarily in the relative values, and since we assume that the amount of the dye in the sap has a definite ratio to that of the dye in the protoplasm.

Another method of explanation is the following, as described in Diagram C.

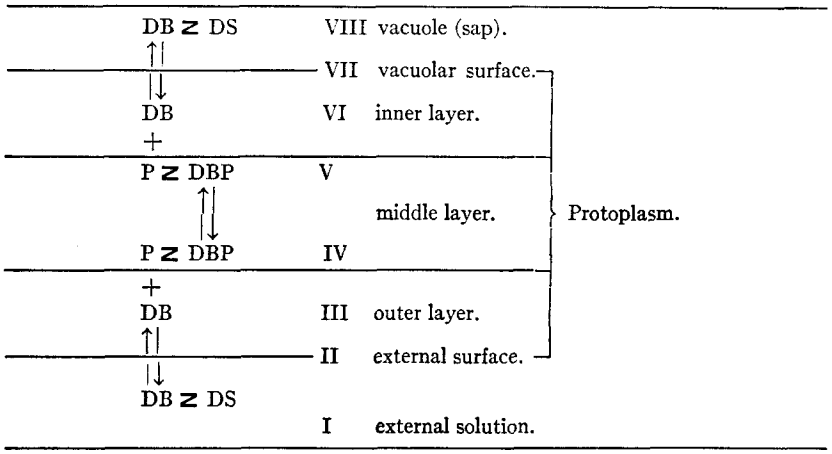

Diagram C. The cell wall is omitted for convenience.

DB can pass through III and VI. As DB enters at IV (the boundary between the outer layer and the middle layer) it combines with P of the protoplasm to form a complex compound DBP. DBP now diffuses from IV to V (the boundary between the middle layer and the inner layer) and DB is given off at V to VI. DB now diffuses into VIII. In I, II, VII, and VIII DB is in equilibrium with DS. The rate of DB + P → DBP is controlled by the concentration of DB and DBP in the protoplasm, and the concentrations of these substances are dependent on the amount diffusing in and out of the protoplasm in a given time. It is hardly necessary to undertake a detailed description of this diagram, as it closely resembles Diagram B. The only important differences are that the nature of the dye in the vacuole is not changed in this case, and that the reaction of DB + P → DBP in the protoplasm takes place at the boundaries IV and V. The latter may bring in complications to such an extent that we may have no justification for using an equation for a homogeneous reaction. Since so little is known in regard to this, the investigation of this question will be left to the future.

The experimental results thus far obtained do not show conclusively which one of the theories represents the mechanism. It may be possible that though there are reactions taking place between the dye and the protoplasmic constituents, the final result in both entrance of dye into and exit from the vacuole is dependent on the diffusion (see Section I) of the dye (see Foot-note 13).

A rough analogy to the passage of dye may be found in the case of entrance and exit of water into and from a reservoir, where the rate of inflow and outflow of the water depends on the conditions at the entrance and exit, and not on the conditions in the body of water between these two points.

V.

Rate of Exit When the pH Value of the Sap Is Changed.

The following experiments were carried out to determine if the theory thus proposed is supported by the observations on changes in the rate of the exit of the dye when the pH value of the sap is varied.

One lot of cells was placed in M/150 borate buffer solution at pH 8.5 containing 8.6×10^{-5} M cresyl blue and 0.005 M NH_4Cl; at the end of 5 minutes there was 8.6×10^{-5} M dye in the sap. The cells were then removed, wiped with a damp cloth, and placed in an M/150 phosphate buffer solution at pH 6.5 containing no dye. After 2 minutes, the concentration of the dye in the sap was found to be 2.6×10^{-5} M.

A second lot of cells was placed in 0.005 M NH_4Cl at pH 8.5 M/150 borate buffers: at the end of 5 minutes the pH value of the sap had increased from pH 5.6 to 6.9. The pH value of the sap remained at 6.9 when such cells were placed in a buffer solution at pH 6.5 for 2 minutes.

A third lot of cells was placed in M/150 borate buffer solution at pH 8.5 containing 8.6×10^{-5} M cresyl blue: at the end of 45 seconds there was 8.6×10^{-5} M dye in the sap. The cells were now removed, wiped with a damp cloth, and placed in an M/150 phosphate buffer solution at pH 6.5 containing no dye. After 2 minutes the concentration of the dye in the sap was determined and was found to be 5.9×10^{-5} M.

In all cases the experiments were carried out at 25 ± 0.5°C., and the solutions were constantly stirred and changed.

From these experiments it may be concluded that the rate of the exit of the dye from the cell sap is increased by presence of NH_3 in the sap which increases the pH value of the sap. Whether this increase in the rate is due to the increase in the pH value of the sap, or to the possible presence of NH_3 and consequent increase in pH value in the protoplasmic layer, or to NH_3 adhering to the cell surface, the writer is at present unable to determine.

The above observation is in agreement with the theory since the increase in the pH value would increase the concentration of DB in the sap and hence increase the concentration gradient, but in view of the fact that no appreciable changes in the pH value of the sap may be brought about without an injury to the cells, such a conclusion must necessarily be made with reserve. Furthermore, the fact that the rate of penetration is decreased,[18] while that of the exit of the dye is increased, when NH_3 enters the sap, does not prove that the dye enters the cell only in the form DB. As already suggested by the writer, in case DS enters,[19] the rate of penetration[20] may very well be decreased by the competition between the DS and the aqueous NH_3 for the substances (*viz.* salt of proteins or weak acid) in the protoplasm which may take place as NH_3 and DS enter the cell. The presence of aqueous NH_3 then would hasten the exit of DS from the cell, if the affinity of NH_3 for the cell substance is greater than that of DS.

SUMMARY.

Experiments on the exit of brilliant cresyl blue from the living cells of *Nitella*, in solutions of varying external pH values containing no dye, confirm the theory that the relation of the dye in the sap to that in the external solution depends on the fact that the dye exists in two forms, one of which (DB) can pass through the protoplasm while the other (DS) passes only slightly. DB increases (by transformation of DS to DB) with an increase in the pH value, and is soluble in substances like chloroform and benzene. DS increases with decrease in pH value and is insoluble (or nearly so) in chloroform and benzene.

The rate of exit of the dye increases as the external pH value decreases. This may be explained on the ground that DB as it comes out of the cell is partly changed to DS, the amount transformed increasing as the pH value decreases.

The rate of exit of the dye is increased when the pH value of the sap is increased by penetration of NH_3.

[18] McCutcheon and Lucke (see Foot-note 9) believe that the decrease in the rate of penetration of a basic dye into *Nitella* with an increase in the pH value of the cell sap is a direct disproof of the theory that the dye combines with a protein in the cell.

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

[20] When the pH value of the sap is decreased by an entrance of acetic acid the rate of penetration of dye is either increased or decreased, depending on the condition (probably of the protoplasm) of the cell.