STUDIES ON PENETRATION OF DYES WITH GLASS ELECTRODE

IV. Penetration of Brilliant Cresyl Blue into Nitella flexilis

By MARIAN IRWIN

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

(Accepted for publication, June 19, 1930)

I

INTRODUCTION

Although basic dyes play a special rôle in biology, because many of them are readily taken up by living cells, the form in which they penetrate has never been satisfactorily determined.

A basic dye is usually obtained in the form of the salt some of which may be transformed in aqueous solution to free base (the amount depending on the "apparent" dissociation constant of the dye and the pH value), and in the range of pH values generally employed with living cells (approximately between pH 5 and 9.5) both forms of dye must be considered. In this range methylene blue, for example, exists only as a salt. It is very strongly basic[1] so that even at pH 9.5 no free base is formed. Lauth's violet, on the other hand, is less basic, with a basic dissociation constant[1] of 1.9×10^{-3}.

Since the nature of the transformation of the dye salt into the free base is still in dispute[2] the structure of the salt and free base of the dye will not be discussed.

Furthermore, the dissociation constant of cresyl blue has not been determined by such methods as the oxidation-reduction potential or the glass electrode, so that the basicity of the dye is not accurately known.

It is, however, possible to determine approximately the relative ratio of the dye in form of free base/salt by shaking the chloroform with the dye solution and de-

---

[1] Clark, W. M., Cohen, B. C., and Gibbs, H. D., *Hygienic Laboratory Bulletin*, 1928, No. 151, 202–206.

[2] Henrich, F., Theories of organic chemistry, translated by John Johnson and Dorothy Hahn, London, 1922.

termining colorimetrically the "apparent" partition coefficient $k_{ap} = \frac{C_1}{C_2}$, where C_1 is the concentration of free base of the dye in chloroform and C_2 the concentration of the total dye (free base in equilibrium with the salt) in the aqueous solution. Such a determination was previously made[3] and it was found that the values of the k_{ap} increased with rise in the pH values as if the basic dissociation constant of the dye were about $10^{-5.6}$. But owing to the extreme solubility of the free base in chloroform and to the progressive alteration in the color of the dye in the aqueous solution from about pH 10 upward, it was not possible to determine the value of the true partition coefficient $k_{tr} = \frac{C_3}{C_4}$ where C_3 represents the concentration of free base of the dye in chloroform and C_4 the same in the aqueous solution. The pH value at which the dye existed almost entirely in form of free base was assumed and the value of k_{tr} was accordingly calculated. The dissociation constant was then calculated on basis of this assumption so that it may prove to be different when the determination is made with a more reliable method. But the significance of this observation does not lie in the accurate determination of the dissociation constant but in the proof that the relative increase in the values of k_{ap}, showing roughly the relative ratio of free base/salt, more or less corresponds to the relative increase in the rate of penetration of the dye into *Nitella* as the pH value of the aqueous dye solution is raised.

It has been suggested that basic dyes penetrate living cells in the form of free base[4] and not in the form of salt,[5] but this, despite many attempts, has never been really proven. The rate of penetration of a basic dye has been found to increase with a rise in the pH value of the external dye solution. This observation might be interpreted as showing penetration in the form of free base since the concentration of this form of dye increases as the external pH value rises. But it might be held to indicate that the dye penetrates as positive ions since with a rise in the external pH value an increase of the negative charges in the cell surface might occur which might result in greater penetration of the positive dye ions.

---

[3] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, **23**, 251; *J. Gen. Physiol.*, 1925–26, **9**, 561.

[4] The following authors assume that basic dyes penetrate as free base. Overton, E., *Jahrb. wissensch. Bot.*, 1900, **43**, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, **10**, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, **4**, 1. McCutcheon, M., and Lucké, B., *J. Gen. Physiol.*, 1923–24, **6**, 501. Irwin, M., *J. Gen. Physiol.*, 1925–26, **9**, 561.

[5] The following authors account for the penetration of basic dyes through combining of dye cations with protoplasmic constituents. Bethe, A., *Biochem. Z.*, 1922, **137**, 18. Rohde, K., *Arch. ges. Physiol.*, 1920, **182**, 114. Pohle, E., *Deutsch. med. Wochn.*, 1921, **47**, 1464. Collander, R., *Jahrb. wissensch. Bot.*, 1921, **60**, 354.

If the dye penetrates as free base and combines[6] with the sap to form salt which cannot readily escape from the vacuole, an increase in the pH value of the sap might be expected to decrease the rate of penetration. When the pH value of the sap of *Nitella* was increased by entrance of ammonia, the rate of penetration of cresyl blue was decreased[7] from which it was concluded[7] that the dye penetrated as free base. If such a result were indicative of the penetration of the dye as free base we should expect that in decreasing[8] the pH value of the sap an increase in the rate of penetration of cresyl blue would take place. But on lowering the pH value of the sap, through the entrance of acetic acid, a decrease[8] in the rate of penetration was observed, instead of an increase. This decrease may be due to an inhibitory effect of the acid on the protoplasm irrespective of the changes in the sap since various[9] other substances produce an inhibiting effect without penetrating the vacuole.

It is therefore desirable to find a satisfactory way of distinguishing between the penetration of the free base and that of the dye salt. This can be done by observing the effect of the dye on the pH value of the sap. The hydrogen electrode cannot be utilized, owing to the poisoning of the electrode by the dyes and to their oxidation-reduction potential, and the glass electrode is therefore employed. Since this requires the use of about 0.4 cc. of sap it is necessary to choose large cells which yield sap in sufficient amount for analysis. This can easily be collected from the vacuoles of cells of *Nitella*. Its behavior toward dyes is roughly comparable to that of such cells as *Paramecium*, *Spirogyra*, *Elodea*, and *Valonia* so that by studying the mechanism of dye penetration with *Nitella* we may gain some insight into the behavior of other cells as well.

In the present paper therefore determinations of the pH values of the sap of *Nitella* by the glass electrode[10, 11] are used as a means of studying the form of dye penetrating the vacuoles of living cells.

---

[6] E. N. Harvey was the first to suggest that the free base was converted to the salt in the sap. See reference under footnote 4.

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

[8] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24**, 54; *J. Gen. Physiol.*, 1926–27, **10**, 271; 1927–28, **11**, 111.

[9] See footnote 8, and also Irwin, M., *J. Gen. Physiol.*, 1926–27, **10**, 425.

[10] Through the kindness of Dr. D. A. MacInnes and of Dr. M. Dole a glass electrode apparatus was devised with which it was possible to measure a very small amount of liquid. Through their cooperation it was possible to determine whether this method was applicable to the studies of the penetration of cresyl blue into *Nitella* cells, as mentioned in their article (MacInnes, D. A., and Dole, M., *J. Gen. Physiol.*, 1928–29, **12**, 805).

[11] Applicability of the glass electrode methods to the measurement of the sap, mentioned under footnote 9, was described separately by Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 132. Preliminary report of the present paper was published, *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 991.

II

Methods

Solutions Employed.—All buffer solutions were made up according to Clark's[12] standards and diluted ten times. The pH value of these solutions was checked by means of the glass electrode. The dyes were in all cases dissolved in these dilute solutions.

Condition of the Dye Used.—The cresyl blue was purchased as a salt. The brilliant cresyl blue (called cresyl blue in the text) was manufactured by Grübler previous to 1914, and appears to contain (in the range of pH values between pH 5.5 and pH 9.2) chiefly cresyl blue in form of salt or free base. It also contains another dye, *X*, but in the solutions employed the amount is so small in comparison to the cresyl blue (free base or salt) that *X* may be neglected. The difference in behavior between the cresyl blue and *X* is well marked so that it can be readily distinguished. Cresyl blue is absorbed as free base by the chloroform and appears orange. This orange dye can be entirely removed from the chloroform on shaking the chloroform with the artificial sap of *Nitella* (0.1 N KCl at pH 5.5) in which it appears blue. On shaking the cresyl blue solution with chloroform, the dye is absorbed much more from pH 9.2 than from pH 5.5, showing that the free base of cresyl blue is much more soluble in chloroform than the dye salt, and that the ratio of free base/salt of cresyl blue increases with the rise in the pH value. The dye *X*, on the other hand, appears pinkish red in chloroform. The amount of *X* absorbed by the chloroform is so much less than that of the free base of cresyl blue that it becomes visible only when the experimental condition is favorable (*i.e.*, at a high concentration and low pH value). For example, on shaking 0.07 per cent cresyl blue solution at pH 5.5 with the chloroform, the dye appears red in chloroform. On shaking with artificial sap, the red dye is not removed from the chloroform; in fact only a trace of the blue dye appears in the sap, which may very well be a trace of the cresyl blue extracted from the chloroform. On shaking 0.005 per cent cresyl blue solution at pH 9.2 or at pH 5.5, or 0.07 per cent cresyl blue solution at pH 9.2, or 0.025 per cent cresyl blue solution at pH 6.8 with chloroform, the dye absorbed appears orange in chloroform. But when this chloroform is shaken with the sap, until the orange dye is completely removed from the chloroform, the red dye becomes visible in the chloroform, and is not extractable by the sap from the chloroform. This red dye is absorbed by the chloroform to about the same extent from pH 9.2 or from pH 5.5 cresyl blue solution, but the amount absorbed increases with the rise in the concentration of aqueous cresyl blue solution. The amount of *X* absorbed is so small, however, that the absorption causes only a very slight decrease in the concentration of the total dye solution

[12] Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928.

even when the chloroform is shaken with 0.07 per cent cresyl blue solution at pH 5.5. These experiments are being continued and their results will be reported in later publications.

Spectrophotometric measurements of these solutions of cresyl blue show that they give absorption curves characteristic of cresyl blue.

The free base of cresyl blue was obtained as follows: 0.005 per cent cresyl blue solution at pH 9.2 was shaken with chloroform. The chloroform was then carefully freed from the aqueous solution and immediately placed in a pyrex glass vial and caused to evaporate rapidly by bubbling air through the solution. The freshly collected sap was then placed in this glass vial, and the concentration of the dye was determined colorimetrically by comparison with the standard dye solutions of known concentrations. There was a small amount of the dye residue which was not readily soluble in the sap, and which was therefore discarded. The pH value of the sap containing the dissolved dye was at once determined by the glass electrode.

To ascertain whether this dye was contaminated by other alkaline substances absorbed by the chloroform from the buffer solution which might alter the pH value of the sap the following experiments were made. The chloroform was shaken with the buffer solution at pH 9.2 containing no dye and was evaporated in the vial as before. The freshly extracted sap was placed in the vial and the pH value was immediately determined by the glass electrode. Since the pH value of the sap collected in this vial was the same as that of the sap collected in a control vial (which had not come in contact with the chloroform) it is apparent that the chloroform does not absorb any alkaline substance from the buffer solution in the absence of dye which would alter the pH value of the sap. Whatever alteration occurs in the pH value of the sap in presence of the dye must therefore be due to the dye alone.

It may be asked whether this dye is actually free base of cresyl blue. It cannot be a pseudo base, since it is very soluble in water and is readily reversible, forming a salt at lower pH values (appearing blue), while the pseudo base is not readily soluble in water, in which it appears colorless, and is irreversible. On shaking this dye solution (made up in buffer mixtures) with chloroform, the behavior is found to be very much like that of the original cresyl blue solution. The spectrophotometric determination shows that the absorption curve of the dye taken up by chloroform is identical with that of brilliant cresyl blue. It would therefore appear that the dye represents primarily the free base of brilliant cresyl blue. A detailed account of these results will be subsequently published.

Condition of Cells before Experiments.—As previously described,[13] single cells were cut from the central portion of the plant and kept about 20 hours in pans of

[13] Regarding the care of cells and detection of injury see Irwin, M., *J. Gen. Physiol.*, 1925–28, **8**, 147; 1926–27, **10**, 271, 927; 1927–28, **11**, 123; 1928–29, **12**, 147.

tap water at about 20°C. in a well ventilated room in diffused light. The cells which have suffered severe injury at the time of cutting die over night and those which may be slightly injured may recover so as to be suitable for use. The effect of cutting and of the subsequent treatment of the cells as described in connection with electrical measurements[14] is not manifested in the same way in the determination of penetration of dyes into the vacuole, so that it is not necessary, for example, to keep each cell in a separate test-tube for a day or more before an experiment. The precaution, however, was always taken of placing only a few cells in a large pan of water so as to minimize any possible effect of the sap diffusing from the dead cells to the living cells.

Experiments on Penetration.—Cells were placed in solutions with one end (only a very small portion, just enough to avoid contamination) exposed to the air in a moist chamber, at the temperature at which the glass electrode measurement was subsequently made. The cells were kept in the dye solution about 15 minutes. Leaving one end of the cell out of the solution for this period in a moist chamber was not injurious since cells kept in this way in tap water containing no dye even after 8 hours showed no signs of injury.

Extraction of the Sap.—The cells were removed from the solution, rinsed rapidly in distilled water and wiped gently first with a soft dampened cheese cloth and then with the dry cloth. Then each cell was cut open by a pair of scissors (the cut being made at the end which projected out of the solution) and the sap was allowed to flow out into a pyrex glass vial (small enough to prevent evaporation). By thus extracting the sap from the unstained portion of the cell, it was possible to avoid contamination of the sap by dye diffusing out of the cell wall at the time of extraction. Two successive extractions of sap were made from the vacuole of each living cell. The first extraction, called for convenience "sap," was made without pressing on the cell wall, while the second extraction called "sap mixture" was made by squeezing the cell wall vigorously so as to press the protoplasm into the vacuolar sap. The pH value of the sap was found to be about 0.6 pH lower than that of the sap mixture. It took about 15 minutes for one person to collect the sap and the corresponding sap mixture sufficient for analysis. For each reading by the glass electrode method it required about 0.4 cc. of sap collected from the vacuoles of 40 or more cells. The extraction was made at the same temperature as the temperature at which the electrode measurement was made.

Standardization of the Glass Electrode and Method of Measurement.—The apparatus used is identical with the one described by MacInnes and Dole[15] but the method of standardization of the glass electrode and the calculation of the pH value of the unknown solution used was different. The standard solution of the M/20 potassium hydrogen phthalate was made up to pH 3.97 and its pH value was determined by means of the hydrogen electrode. This solution was made up every

[14] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929–30, **13**, 467.
[15] For reference see footnote 10.

2 weeks to keep the pH value constant. Due to the very low temperature coefficient of this solution the alteration in the pH value with varying temperature is negligible in the range in which the experiments were carried out (between 20° and 23°C.).

Before each series of experiments the temperature was noted, and the glass electrode was standardized by determining the potential of the standard phthalate solution[16] at pH 3.97. Then the pH value of the standard M/15 phosphate buffer solution at pH 6.647 (the pH value of which was checked by the hydrogen electrode) was determined by means of the glass electrode to ascertain the behavior of the electrode; in no case did the reading vary more than ±0.03 pH. The potential of the unknown solution was then determined, and the pH value of the unknown solution was calculated for the given temperature according to the usual formula[17]

$$pH_m = pH_n + \frac{E_m - E_n}{\frac{RT}{F}}$$

where pH_m denotes the pH value of the unknown solution and pH_n that of the standard, while E_m represents the potential of the unknown solution and E_n that of the standard (the value of $\frac{RT}{F}$ at different temperatures is given by Clark[11]).

The temperature during the day did not usually vary more than ±0.5°C. but in case a variation greater than ±0.5°C. occurred, the electrode was re-standardized. From day to day the temperature varied between 20° and 23°C., but this variation did not bring about errors, since the glass electrode was always standardized and a temperature correction was made accordingly. After each series of experiments the electrode was again standardized with the phthalate and the phosphate buffer to see whether the electrode remained unaltered during the experiments. In many cases where the test solution is much less buffered than the standard phosphate solution, it may be desirable to check the glass electrode before and after a series of experiments with a standard solution which is buffered to about the same extent as the test solution. This was done in the case of the *Nitella* sap, by using M/150 phosphate buffer solution at pH 5.5, or the control sap, whenever there was a possibility of the effect of the sap in presence of the dye on the electrode in a series of experiments. In the case of all the experiments presented in the text the electrode remained unchanged through each series of experiments, in that there was no variation of more than ±0.05 pH value with these standard solutions.

---

[16] For review and bibliography on the glass electrode methods see Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928.

[17] The equation is taken from the one described by Kerridge, P. T., *Biochem. J.*, 1925, **19**, 611.

Three electrodes were employed for the experiments described in the text. In no case did the pH value of the standard phosphate buffer solutions determined by these electrodes vary more than ±0.03 pH.

Method of Washing the Glass Electrode when Successive Determinations Are to be Made.—When the glass electrode is not in use, it is kept in 0.1 N HCl. When two measurements with different samples are to be made in rapid succession washing with distilled water (without washing with the acid) is adequate if the test solution is such that it does not measurably affect the electrode or the reading, or if the solution is sufficiently buffered, but if the test solution does affect the electrode and if it is not sufficiently buffered, the alteration will result in a wrong pH value. An example of such cases may be cited as follows. The pH value of a strongly buffered solution, such as standard phosphate buffer at pH 6.647, in presence or absence of basic dye, or that of the distilled water containing no dye, may be measured repeatedly without showing any effect of the test solution on the electrode whether the electrode is washed after each measurement with 0.1 N HCl and then with distilled water or only with distilled water. But if the distilled water contains, for example, 0.07 per cent cresyl blue in form of salt or free base, an effect on the electrode is manifested by an increase in the pH value after each successive measurement if the electrode is washed only with distilled water each time. But such an effect is not manifested if the electrode is first washed with acid each time before applying distilled water.

The freshly extracted sap is more buffered than the distilled water but not sufficiently so to enable us to omit the washing with acid each time, even though the electrode is always washed with the test sap before each measurement. If the electrode is not washed with the acid there is a small but progressive increase in the observed pH value of sap containing cresyl blue (in form of salt or in form of free base) with each successive drop of the same sample of sap taken for each one of the five measurements, where the maximum increase is a little over 0.1 pH. The observed pH value of the same sap remains constant throughout such a series of experiments when the electrode is washed with 0.1 N HCl and then with distilled water followed by final washing with test solution after each measurement. There is no danger of the acid diffusing out of the electrode which has been washed with acid into the sap, since the pH value of the sap containing no dye is found to be the same whether the electrode is first washed with the acid or not.

To avoid the errors arising from this source, the following procedure was adopted in the experiments described in the text. Before using the electrode it is removed from 0.1 N HCl, wiped with filter paper, washed in distilled water, and wiped again. Then the electrode is washed quickly in the solution, the potential of which is to be determined. After measurement, the electrode is washed in distilled water, wiped, and placed in 0.1 N HCl until the effect of the test solution on the electrode is completely removed. When the electrode is not in use, it is always kept in the 0.1 N HCl. The temperature of the solutions used to wash the electrode is kept within ±0.5°C. of the temperature of the test solution.

Time Curve for Glass Electrode Measurement.—It is always necessary to continue measurement for about 5 or 10 minutes to see if there is any change in the potential with time. Such a change may occur from the effect of the solution on the electrode or from the change in the solution proper. The results show that there is no alteration in the pH value of the buffer solution at pH 6.65 within 5 minutes, nor in the pH value of the control sap. There is a tendency for the pH value of the sap containing the dye to rise with time, but the maximum rise in 5 minutes is only 0.05 pH, which is within an experimentl error. The two measurements made within 2 minutes agree within ±0.03 pH; the measurements given in the text represent the average of two readings made, (*a*) one-half minute after the electrode has first come in contact with the test solution, and (*b*) one minute and a half later.

The pH Value of the Control Sap or the Sap Mixture. If the pH value of the control sap changed rapidly after extraction, it would interfere with such experiments as are described in the text, but fortunately there was no alteration in the pH value of the sap nor of the sap mixture even on standing in a pyrex glass vial (stoppered) 2 hours after extraction. Even on keeping the sap in the refrigerator for 20 hours the pH value of the sap was found to increase only 0.2 pH.

Furthermore there was practically no alteration in the pH value of the sap nor of the sap mixture during the 15 minutes required for extraction. This test was made colorimetrically,[18] since by this method only a small capillary tube of sap was sufficient so that it was possible to complete the first determination only 3 minutes after the extraction had begun. The indicator method was found to be adequate since the pH value of the sap determined by this method agreed closely enough (within ±0.1 pH) with the pH value determined by the glass electrode.

From day to day there was some variation in the pH value of the control sap, the maximum variation being about ±0.2 pH. Owing to this an alteration in the pH value of the sap less than 0.2 pH should not be taken seriously. Though the pH value of the sap did not alter on standing for several hours, the measurement with the glass electrode was always made immediately after the sap was extracted.

Each reading given in the text is an average of 10 to 15 experiments employing altogether 400 cells or more.

III

Nitella flexilis (*A*)

The Sap.—The average sap of the control cells (taken from tap water) is at about pH 5.36 (Table I).

When the cresyl blue salt at a concentration in the neighborhood of 0.07 per cent is dissolved in the sap *in vitro* the pH value of the sap

[18] For method see Irwin, M., *J. Gen. Physiol.*, 1925–26, **9**, 240, Section III.

is found to decrease by about 0.07 pH, which is too small an alteration to be considered seriously (Table I).

When about 0.07 per cent free base [19] of cresyl blue is dissolved in the sap *in vitro*, the pH value is found to increase by about 0.55 (Table I).

TABLE I

pH Value of the Sap of Living Cells of Nitella flexilis (A)

In presence of cresyl blue. Average temperature 21°C. Electrode I was used. Unless otherwise stated, penetration experiments were carried out with one end of the cell outside the solution in moist chamber, to avoid contamination of the sap. The duration of the experiments was about 15 minutes.

	Conc. of dye in sap in per cent	pH of sap	Increase in pH of sap over the control sap	Conc. of dye in sap containing protoplasm in per cent	pH of sap containing protoplasm	Increase in pH of sap containing protoplasm over the control sap containing protoplasm
Control cells in tap water	0	5.36		0	5.97	
Dye salt added *in vitro*	0.069	5.29				
Free base of dye added *in vitro*	0.071	5.91	0.55	0.070	6.48	0.51
Penetration from 0.002 per cent dye at pH 9.2	0.073	5.86	0.50	0.065	6.45	0.48
Penetration from 0.025 per cent dye at pH 6.8	0.071	5.87	0.51	0.074	6.37	0.40
Penetration from 0.004 per cent dye at pH 9.2	0.18	6.66	1.30			
Free base added *in vitro*	0.16	6.50	1.24			
Penetration from 0.002 per cent dye at pH 9.2, when cells are completely immersed	0.070	5.88	0.52			
After 8 hours in tap water containing no dye	0	5.40		0	6.00	
After 1 hour in buffer solution at pH 9.2, containing no dye	0	5.37		0	5.94	

These results prove that the two forms of the dye are distinguishable by determining the pH value with the glass electrode, the dye salt having very little effect on the sap, the free base of the dye distinctly raising the pH value of the sap. If therefore the same amount of dye penetrating the vacuole raises the pH value to about the same extent

[19] For method see Section II in the text.

as the free base of the dye, we may conclude that the dye penetrates as free base and not as salt. The following results show this to be the case.

When cells are placed[19] for about 15 minutes in 0.002 per cent dye solution at pH 9.2 until about 0.07 per cent dye has accumulated in the vacuole, the pH value of the sap is found to increase about 0.5 over that of the control sap (Table I). This increase[20] agrees closely with the increase obtained by dissolving 0.07 per cent free base of the dye in the sap (Table I), thus indicating that the dye penetrates chiefly as free base and not as salt.

Previous experiments[21] have shown that at pH 9.2 cresyl blue exists predominantly as free base so that it would not be surprising if the dye penetrated as free base from this solution. But at a lower pH value, where the dye salt predominates, does the dye enter as salt or as free base? To test this point the dye was allowed to penetrate for about 15 minutes from 0.025 per cent cresyl blue solution at pH 6.8 (in which there was a preponderance of the dye salt).[21] As shown in Table I, 0.07 per cent dye penetrating from this solution has brought about approximately the same increase (about 0.51 pH) in the pH value as 0.07 per cent dye penetrating from the solution at pH 9.2. These results prove that irrespective of the ratio of the free base to salt of the dye in the external solution, the dye penetrates as free base and not as salt.

The dye was furthermore allowed to penetrate to a still higher concentration (0.18 per cent) from 0.004 per cent cresyl blue solution at pH 9.2 in about 15 minutes, to see if further increase in the pH value of the sap would occur with a higher concentration of the dye in the sap. An increase[20] of about 1.30 pH over the control was found to take place, which agreed closely with the rise in the pH value by dissolving 0.16 per cent free base of cresyl blue in the sap *in vitro* (Table I).

The pH value of the sap in the presence of a lower concentration of

[20] These results prove that the alteration in the rate of entrance and exit of the dye formerly reported to occur in the cell after penetration of dye are not due to the production of acid in the sap to the extent of lowering the pH value of the sap below the normal. Irwin, M., *J. Gen. Physiol.*, 1925–28, **8**, 147; *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24**, 247.

[21] For reference see footnote 3.

cresyl blue (below 0.07 per cent) was determined but owing to the variation in the pH value of the control sap (to the extent of 0.2 pH) it is not desirable to draw conclusions from the lower concentration of the dye in the sap which brought about this increase in the pH value of the sap.

Owing to the fact that these experiments were carried out when a portion of the cell was exposed to the air in a moist chamber (to avoid contamination of the sap from the stained cell wall at the time of extraction) the question may be raised whether the increase in the pH value of the sap may not be caused by the penetration of the alkaline buffer as a result of injury to the cells under these experimental conditions. This "partial immersion" method, however, is proved to cause no injury in the duration of these experiments, as shown by the following experiments.

When the experiments were repeated by completely immersing the cells in 0.002 per cent dye at pH 9.2 for a little less than 15 minutes until 0.07 per cent dye had collected in the sap, the pH value of the sap was found to increase about 0.52 pH, which agrees closely with the rise brought about when cells were only partly immersed in the solution as previously stated (Table I). In this dye solution at pH 9.2 so little staining of the cell wall occurs (with this length of exposure) that no question of errors arising from contamination of the cell sap from the stained cell wall enters in the case of the cells completely immersed in this solution. If the partial immersion method causes injury, thereby hastening the penetration of the alkaline borate buffer, the rise in the pH value of the sap may be expected to be greater when this method is used than the rise when the cells are completely immersed. Since the rise in the pH value is about the same in both cases, we may conclude that the alteration in the pH value of the sap is not due to injury caused by the partial immersion method.

Furthermore the pH value of the cells which were partially immersed in this way in tap water for 8 hours or in the buffer solution at pH 9.2 (containing no dye) for 1 hour was the same as that of the control sap, thus showing that the partial immersion method does not injure the cells (for the duration of exposure described in the text) enough to affect the pH value of the sap (Table I).

Sap Containing Protoplasm.—The most striking fact is that after the

sap has been allowed to flow out without pressure, if the cell wall is squeezed so as to press the protoplasm into the sap, the pH value of this mixture is found to be about 0.6 pH higher than the sap proper (Table I). This may be chiefly due to the presence of the protoplasm, the pH value of which may be higher than that of the sap. Another possible explanation is that the fluid[22] which may exist in the space between the protoplasmic layer and the cell wall may also be squeezed into the sap and may help to raise the pH value of the sap.

In presence of 0.07 per cent cresyl blue free base the pH value of this mixture is increased about 0.51 over that of the control mixture (Table I). The cresyl blue penetrating from the dye solution at pH 9.2 or at pH 6.8 also raises the pH value of sap containing protoplasm by about 0.4 to 0.5 pH (Table I), thus showing that the dye penetrates chiefly as free base into this mixture. Undoubtedly not all the protoplasm can be squeezed into the sap and the protoplasmic layer is very thin so that in the mixture the sap preponderates over the protoplasm. For this reason it would be difficult to conclude from these results whether the dye penetrates chiefly as free base into the protoplasm as it does into the vacuole.

IV

Nitella flexilis (*B*)

To determine if the penetration of cresyl blue as free base will occur with another sample of *Nitella* obtained in a different locality, the experiments were repeated with cells collected elsewhere which are distinguished for convenience from *Nitella* (*A*) by the term *Nitella* (*B*).

The Sap.—The average pH value of the sap of *Nitella* (*B*) is pH 5.6 (Table II), which is about 0.2 pH higher than the pH value of the sap of *Nitella* (*A*).

On dissolving the 0.086 per cent free base[19] of cresyl blue in this sap *in vitro* the pH value of the sap is found to increase about 0.37 pH (Table II). This increase is about 0.2 pH less than is the case with *Nitella* (*A*). This may mean that the sap of *Nitella* (*B*) is more buffered than the sap of *Nitella* (*A*) (Tables I and II). On

[22] Irwin, M., *J. Gen. Physiol.*, 1926–27, **10**, 75.

adding the dye salt to the sap there is practically no alteration in the pH value of the sap. When about 0.075 per cent dye penetrates the vacuole from the cresyl blue solution at pH 9.2 or 6.8, there is an increase in the pH value of 0.4 or 0.45 pH over that of the control sap (Table II), which agrees closely with the rise in the pH value when the free base of the dye is dissolved in the sap *in vitro*. In this case

TABLE II

pH Values of the Sap of Living Cells of Nitella flexilis (B)

In presence of cresyl blue. Average temperature at 22°C. Electrode I was used unless otherwise stated. The penetration experiments lasted about 15 minutes (with precautions to avoid contamination of the sap).

	Conc. of dye in sap in per cent	pH of sap	Increase in pH of sap over the control sap	Conc. of dye containing protoplasm in per cent	pH of sap containing protoplasm	Increase in pH of sap containing protoplasm over the control sap containing protoplasm
Control	0	5.60		0	6.11	
Free base of dye added *in vitro*	0.086	5.97	0.37	0.077	6.43	0.32
Dye salt added *in vitro*	0.080	5.55		0.085	6.07	
Penetration from 0.002 per cent dye at pH 9.2	0.076	6.00	0.40	0.065	6.35	0.24
Penetration from 0.025 per cent dye at pH 6.8	0.077	6.05	0.45	0.069	6.36	0.25
Penetration from 0.01 per cent dye in tap water	0.076	5.96	0.38	0.072	6.37	0.26
Cells placed for ½ hour in buffer solution at pH 9.2 after cells had been in 0.002 per cent dye at pH 9.2 for about 15 minutes	0.075	5.95	0.35	0.070	6.31	0.20
Penetration from 0.002 per cent dye at pH 9.2 Electrode II	0.079	5.98	0.38	0.070	6.39	0.28
Penetration from 0.002 per cent dye at pH 9.2 Electrode III	0.074	5.95	0.35	0.068	6.36	0.25

the increase in the pH value is a little less (about 0.1 to 0.2 pH) than that of the *Nitella* (*A*).

Thus the results on *Nitella* (*B*) confirm those on *Nitella* (*A*), showing that in both cases the dye penetrates the vacuole chiefly as free base.

The Sap Containing Protoplasm.—The pH value of the mixture of

the sap and the protoplasm extracted as before behaved very much like the mixture of *Nitella* (*A*) in that the pH value of the mixture is raised when the free base of the dye is dissolved in the mixture *in vitro* (Table II). On examining the pH values of the mixture after penetration of the dye they are found to be 0.1 to 0.2 pH lower than the corresponding pure sap containing approximately the same amount of dye (Table II). Such results may signify that some dye salt[23] penetrates into the protoplasm or that the pH value of the protoplasm is decreased when the dye penetrates as a result of production of acid by the protoplasm.

Though the results definitely show the penetration of cresyl blue into the sap to be chiefly as free base there is still one more uncertainty to be removed and this is in relation to the possibility of the rise in the pH value of the sap taking place as a result of penetration of an alkaline substance other than the dye owing to the injury caused by the penetration of the dye. Such a possibility is imminent owing to previous[20] results showing changes in the rate of penetration from the normal as a result of penetration of over 0.006 per cent dye. Since the concentration of cresyl blue in the vacuole in the present case is high (about 0.07 per cent), it is necessary to prove that the increase in the pH value of the sap is not due to the penetration of an alkaline substance other than the dye into those cells the condition of which is altered from the normal by penetration of the dye.

Since in all the experiments the length of exposure and the concentration of the dye which has penetrated into the sap are approximately equal, if the increase in the pH value of the sap were due to the penetration of an alkaline substance other than the dye, there should be a greater increase in the pH value of the sap when cells are exposed to the dye solution at pH 9.2 than at pH 6.8 or in tap water at pH 7.7. But this increase is found to be about constant irrespective of the external pH values and the external alkaline buffer concentrations, provided the concentration of dye in the sap is approximately the same (Table II). These results therefore point to the fact that whatever

[23] Experiments on penetration of dye from methylene blue, described in the subsequent paper, show that such a lowering is not due to the penetration of the dye salt, so that in all probability the same is true with the cresyl blue. Irwin, M., *J. Gen. Physiol.*, 1930–31, **14**, 19.

secondary changes that might occur in the cell they do not admit an alkaline substance other than the dye into the vacuoles of these cells.

Another proof is as follows. If the increase of 0.4 pH in the sap over the control sap is not due to the penetration of alkaline buffer as a result of secondary changes, but is due to the penetration of about 0.08 per cent cresyl blue free base from the dye solution at pH 9.2, then by transferring such cells from the dye solution into the buffer solution at the same pH value containing no dye there should be no progressive increase in the pH value of the sap. Such is found to be the case experimentally, as shown in Table II, when even after $\frac{1}{2}$ hour exposure of these cells to the buffer solution at pH 9.2 there is no further increase in the pH value of the sap. But a further increase is found to occur if more dye penetrates the vacuole.

These experiments were made with one electrode (I) only: afterward check experiments were made with several electrodes, which gave about the same results as Electrode I. The results from two other electrodes are recorded in Table II.

The spectrophotometric measurements show that the dye which has penetrated from the cresyl blue solution into the sap or into the sap mixture is identical with the external dye solution as well as the cresyl blue free base dissolved in the sap *in vitro* in that the absorption curves are characteristic of cresyl blue.

V

CONCLUSION

From these results we may conclude that the cresyl blue penetrates chiefly as free base and upon coming in contact with the sap at the vacuolar surface it is at once partly converted into the dye salt. At equilibrium the concentration of free base in the sap is proportional to that in the external solution. Thus the rate of penetration of the dye in form of free base and the concentration of the total dye (free base and salt) in the sap at equilibrium are dependent on the dissociation constant of the dye. For this reason both the rate of penetration and the concentration of the dye in the sap at equilibrium are increased with a rise in the external pH value (which increases the concentration

of free base of the dye) or with a decrease in the pH value of the sap (which increases the conversion of the free base to the dye salt).

It is possible that some of the dye may be converted to something different from the dye salt after the dye has penetrated the vacuole. Previous experiments[20] have shown that as the concentration of the dye in the sap through penetration reached a certain point the rate of penetration progressively increased while that of the exit decreased from that of the normal. Such an alteration in the rate is not due to the excess production of acid in the sap as shown by the preceding results. It may be caused by some kind of change in the dye as the dye penetrates the sap. Such a dye may be either another tautomer of cresyl blue, or a compound formed between the cresyl blue and the sap, or another dye. To affect the rate, as already stated, the reaction producing this dye must be less reversible than that producing cresyl blue salt or this dye must have a lower rate of exit from the vacuole into the external solution than cresyl blue salt.

These results cannot be due to exchange of dye cations for hydrogen ions, as this would involve a lower rate of penetration since the surfaces are non-aqueous and the concentration of hydrogen ions in the sap is too low. This will be discussed in subsequent papers.

SUMMARY

Glass electrode measurements of the pH value of the sap of *Nitella* show that cresyl blue in form of free base penetrates the vacuoles and raises pH value of the sap to about the same degree as the free base of the dye added to the sap *in vitro*, while the dye salt dissolved in the sap does not alter its pH value. It is proved conclusively that the increase in the pH value of the sap is due only to the presence of the dye and not to some other alkaline substance.

Spectrophotometric measurements show that the dye which penetrates the vacuole is chiefly cresyl blue.

When the protoplasm is squeezed into the sap, the pH value of the sap is higher than that of the pure sap. Such a mixture behaves very much like the sap in respect to the dye.