SPECTROPHOTOMETRIC STUDIES OF PENETRATION.

IV. Penetration of Trimethyl Thionin into Nitella and Valonia from Methylene Blue.

By MARIAN IRWIN.

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

(Accepted for publication, May 15, 1928.)

I.

INTRODUCTION.

Methylene blue has been generally regarded as a vital stain, but recent experiments[1,2] have shown that it is not necessarily methylene blue that stains living cells. Using very large cells (*Valonia macrophysa*) yielding enough sap for spectrophotometric analysis, it was found that the dye in the sap of the vacuole is mostly, if not entirely, azure B, which is present as an impurity[3] in the external solution of methylene blue. Methylene blue was not found in the sap even in reduced form.

In order to discover whether these results have general validity it became necessary to perform similar experiments on the fresh water plant *Nitella*[4] and the marine alga *Valonia;* the results of this comparison are given in the present paper.

[1] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 425.

[2] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 927.

[3] The following investigators have stated the presence of methylene azures in methylene blue. Scott, R. E., and French, R. W., *Milit. Surg.*, 1924, lv, 1. Conn, H. J., Biological stains, 1925, published by the Commission on Standardization of Biological Stains. Haynes, R., *Stain Technol.*, 1927, ii, 8. MacNeal, W. J., *J. Infect. Dis.*, 1925, xxxvi, 538. Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905, xiii, 358.

[4] Preliminary reports have been published (*cf.* foot-notes 1 and 2, and Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927–28, xxv, 563).

II.

Methods.

1. Conditions of the Experiments.—The experiments were made with *Nitella flexilis* and *Valonia macrophysa*. The dye used for *Nitella* was dissolved in phosphate or borate buffer solutions at pH 5.5 or 9.2 (made up according to the standards given by Clark,[5] and diluted ten times). The pH values of the buffer solutions were checked by means of the hydrogen electrode. The dye used for *Valonia* was dissolved in sea water at pH 9.5 (0.8 cc. of 0.2 N NaOH added to 100 cc. of Bermuda sea water). The pH value was determined as previously described.[6] The concentration of dye in the sap was determined[7] by matching the color of the tube containing the sap with that of the tube containing a known concentration of dye. The experiments were carried out at 25 ± 0.5°C. Care was taken not to expose cells to direct sunlight. The solutions were changed every hour.

2. Criteria for the Condition of the Cells.—Irreversible injury is very often followed by collapse of the cell and disintegration of the chlorophyll bodies, but the loss of turgidity if not too far advanced is often found to be reversible. Reversible injury cannot be satisfactorily determined. Turgidity seems to be the best criterion, but the outcome will vary with the judgment and experience of the observer. If we employ this criterion and find that cells die in the solutions within 24 hours, are we justified in thinking that the cells are not injured when placed in such solutions for only 1 hour? Here again the outcome will vary with the personal judgment of the experimenter, though this is one of the methods used for controlling the experiments.

The least reliable method of detecting injury is to compare the mortality of the test cells (which have been transferred after the experiment from the dye solution to the normal medium) with the mortality of the control cells. If such cells begin to die very shortly after they are transferred we may conclude that in all probability severe irreversible injury occurred before the cells were removed from the dye solution, but if after a longer period they begin to die more rapidly than the control cells we are in no position to decide whether they were injured in the original solution or died as a result of the subsequent toxic action of the dye which had not been washed away (from the cell surface or the interior). In case these cells after transference are found to live just as well as the control cells, we are in no position to say that the cells had not been reversibly injured before transference since they may very well have been injured and recovered after transference.

---

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

[6] *Cf.* foot-note 2.

[7] Test-tubes were employed for *Valonia* and capillary tubes for *Nitella*.

On the basis of the first two criteria the results presented in this paper involve no injury, unless otherwise stated.

3. Method of Collecting the Sap for Spectrophotometric Analysis.—Owing to the fact that the volume of sap collected from the vacuole of each cell of *Nitella* is very small there is danger of contamination of the sap from the stained cell wall at the time of extraction of the sap from the vacuole. Contamination may occur whether the sap is extracted by puncturing the cell wall with a capillary tube and drawing the sap into it or by cutting one end of the cell and allowing the sap to flow out into a glass container. To avoid contamination the cells were placed with only about $\frac{3}{4}$ of their length in the solution, as will be described later. About six cells were placed in each dish and were removed after a few minutes. It took about 1 hour to extract sufficient sap (1 cc.) for spectrophotometric analysis.

With *Valonia* the cells were completely immersed in the dye solution. After they were taken out of the solution, the superfluous dye was removed as much as possible in the following manner. The cells were wiped with cheese cloth dampened with the sea water in which they are normally kept, dipped in the sea water, wiped again with the dampened cloth, and finally wiped with a dry cloth. Even after all this washing and wiping, the dye still comes out onto the cloth when cells are wiped. The sap was extracted from the vacuole by puncturing the cell wall with a sharp capillary end of a glass tube into which the sap was drawn up. It took about 10 minutes to extract sufficient sap for analysis. Medium sized cells were chosen (the sap from each cell would have a volume of about 0.4 cc.). Unless otherwise stated the amount of dye penetrating from solutions at pH 9.5 into the vacuoles of such cells was sufficient to make the error resulting from contamination of the sap from cell wall at the time of extraction relatively negligible.[8]

The vial containing the extracted sap of *Nitella* or *Valonia* was shaken very gently several times to mix the contents. The sap was drawn up into a pipette and placed in the cell for immediate spectrophotometric analysis unless otherwise stated. The analysis itself took about $\frac{1}{2}$ hour. Measurements at critical wave-lengths (between 645 mμ to 670 mμ) were determined twice, once before the regular series of measurements were made and a second time when the usual series were measured from 550 mμ to 690 mμ (proceeding from the lower to the higher wave-lengths). In no case were the readings found to differ which showed that the light had not affected the dye during the measurement.

4. Accuracy of the Measurements.—The concentration of the dye and the thickness of the layer of solution were adjusted as much as possible to ensure accurate measurements. The thickness of the layers employed for each curve is described in Table I.

The solvent for dye employed was (1) either the sap of *Nitella* containing about 0.11 M halides (mostly potassium chloride) at pH 5.5, or (2) diluted buffer solution (described in the text), or (3) sap of *Valonia* containing 0.6 M halides (mostly potassium chloride) at pH 5.8, or (4) sea water (0.58 M halides).

---

[8] *Cf.* foot-note 2 (p. 929).

As already stated in a previous publication[2] the shape of the absorption curve differs with varying concentrations of dye and of salt present in the solvent. An increase in the concentration of either one tends to accentuate the secondary absorption maximum and obscure the primary, without disturbing actually the position of these maxima in relation to the wave-lengths. For example, irrespective of the concentration the primary absorption maximum of trimethyl thionin or azure B is at about 650 mμ while the secondary is at about 600 mμ, though with an increase in concentration the primary absorption maximum tends to become less distinct, and the secondary more accentuated.

For identification of dyes the primary absorption maximum is more important than the secondary and if dilution of the dye accentuated the former, we might at first sight suppose that the greater the dilution the greater the accuracy *ad*

TABLE I.

Showing the Thickness of Layers of Solution in Centimeters Used for Spectrophotometric Analysis. Nature of the Solvents is Described in the Text.

Figures	Symbols	Thickness of layer
		cm.
1	All	1.0
2	×, ▲, and ●	1.0
	□	0.6
	○	0.2
3	▲	1.0
	●, ■, ×, and ○	0.3
4	All	1.0

infinitum. But this is not found to be the case. Beyond a certain dilution the measurement becomes very inaccurate on account of the distortion of the spectra.

The average eye is relatively insensitive in the spectral region of the absorption maxima of methylene blue and azure B or trimethyl thionin (between 645 and 670 mμ), and particular care was taken to avoid the distortion of spectra which might have occurred through minor errors in measurements carried out on solutions which were too lightly or too strongly colored for optimum results. The collaboration of experienced technicians in the spectrophotometric analysis of dyes was obtained to insure reliable results.

5. *Acknowledgments.*—Unless otherwise stated, the measurements were made by the use of the Hilger wave-length spectrometer with a Nutting photometer or Bausch and Lomb (improved model) spectro-

photometer by W. C. Holmes[9] of the Bureau of Chemistry, U. S. Department of Agriculture, Washington, D. C. I wish to express my deep appreciation to Mr. Holmes for his collaboration and to the Color Laboratory for its hospitality.

At the request of Mr. W. C. Holmes, a few measurements were repeated with a König-Martens spectrophotometer by Dr. K. S. Gibson of the Bureau of Standards, for whose collaboration as well as for the hospitality of the Bureau of Standards I wish to express my gratitude.

I also desire to thank the Marine Biological Laboratory at Woods Hole for the use of facilities during last summer.

I am greatly indebted to Mr. Holmes, Dr. B. Cohen, and to Sergeant French for samples of dyes. The samples are designated throughout the paper by the name of the donor placed in parentheses. The samples furnished by Dr. Cohen were Samples G and F of Dr. Mansfield Clark.[10]

III.

Spectrophotometric Analysis.

A. Experiments on Nitella flexilis.

(*a*) *Collected in New York.*—The experimental procedure was as follows: It was first of all necessary to determine whether the dye was stable in solution and to find out what penetrates the cell. This led to an investigation of errors due to contamination of the sap during extraction in proportion to the rate of penetration which in turn led to a study of the effects of injury. Consideration was then given to the important question whether azure B penetrates as such or is produced from methylene blue after the latter has entered the cell.

The results tend to indicate that azure B penetrates very much more rapidly than methylene blue.

[9] Cooperating expert in the field of spectroscopy of dyes for International Critical Constants.

[10] Clark, W. M., Cohen, B., and Gibbs, H. D., Hygienic Laboratory Bulletin No. 151, 1928, p. 174.

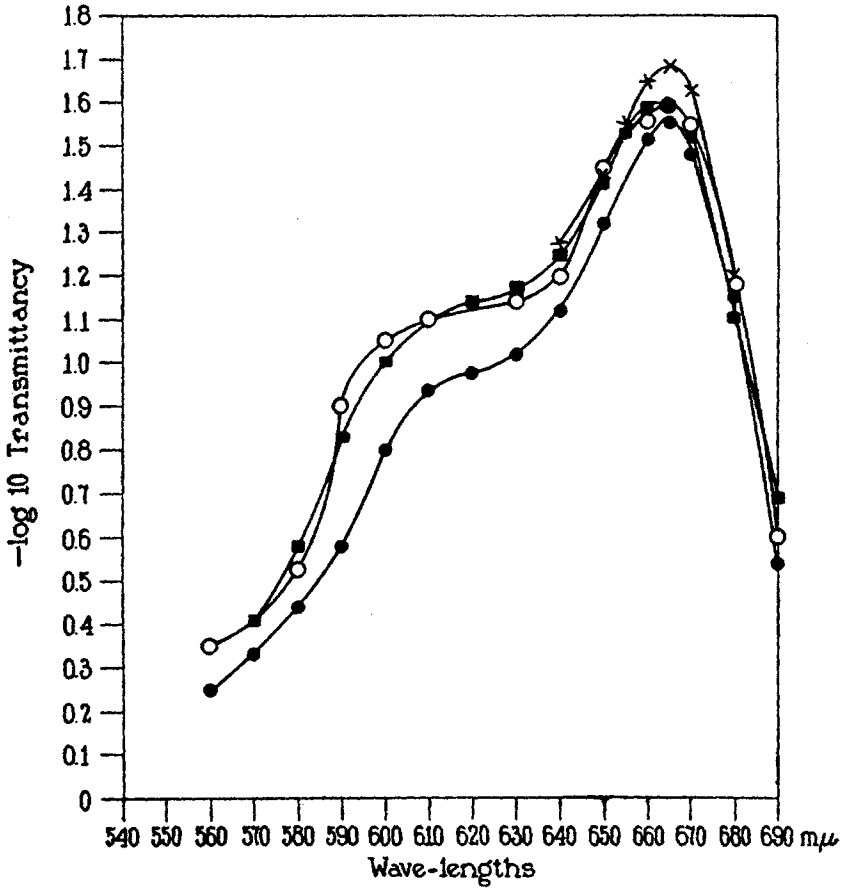

FIG. 1. Absorption curves showing that the sample of methylene blue in solution remains unchanged and that there may be contamination of the sap from cell wall at the time of extraction of sap from the vacuole of *Nitella flexilis* (New York) placed in methylene blue solution (French). Analysis made about 20 hours after extracting the sap or dissolving the sample. Symbol ● represents methylene blue dissolved in borate buffer solution at pH 9.2 or at pH 5.5. Symbol × represents the dye extracted by distilled water from the cell wall of uninjured cells stained in the methylene blue solution at pH 5.5 before the dye has penetrated into the vacuole. Symbol ■ represents the dye extracted from vacuoles of cells immersed completely in methylene blue solution at pH 9.2 where contamination may play a predominant rôle. Symbol ○ represents the dye extracted from vacuoles of cells placed in methylene blue solution at pH 5.5, where contamination occurred without any penetration. The heights of these curves have no significance other than attempts to select a condition as favorable for accurate measurement as possible under these circumstances.

1. Stability of the dye solution: Methylene blue solution (French) either at pH 9.2 or at pH 5.5 gives an absorption curve characteristic of methylene blue with absorption maximum at 664 mμ (Fig. 1, symbol ●) whether it is examined immediately after dissolving or 3 hours later.

2. Nature of the dye entering the vacuole when error from contamination was not avoided: Analysis was made of the sap extracted from the cells which had been completely immersed for a few minutes in 0.01 per cent methylene blue solution (French) at pH 9.2. The dye in the sap gave an absorption curve characteristic of a mixture of methylene blue and azure B with a primary absorption maximum at about 661 mμ (Fig. 1, symbol ■). Owing, however, to a severe contamination of the sap from the stained cell wall at the time of expressing the sap this result cannot be taken as conclusive evidence that the methylene blue present in the sap is a result of penetration rather than of contamination. Let us now consider the question of contamination.

3. Contamination of the sap during the process of extraction:

(*a*) In the solution just mentioned a very deep staining of the cell wall occurs before penetration of dye into the vacuole takes place. Dye extracted from the cell wall of such cells consisted chiefly of methylene blue with a primary absorption maximum at 664 mμ (Fig. 1, symbol ×). This is important since the cell wall can give off this dye to the sap during the process of extraction.

Cells were placed in the 0.01 per cent methylene blue solution (French) at pH 5.5 or at pH 9.2 and were removed after the cell wall had become heavily stained but before any penetration into the vacuole took place. Such cells were removed from the dye solution, wiped, and were then placed in a small volume of distilled water until sufficient dye for analysis was extracted from the cell wall. Several extractions were analyzed and they were found to give the same results. Similar results were obtained when the extraction from the cell wall was made after the sap and the protoplasm had been removed.

(*b*) Sap known to owe most of its color to contamination at the time of extraction showed an absorption curve characteristic of methylene blue with an absorption maximum at 664 mμ (Fig. 1, symbol ○).

Cells were placed in a solution of methylene blue (French) at pH 5.5 until the cell wall was deeply stained. The end of the cell was cut and a drop of sap was

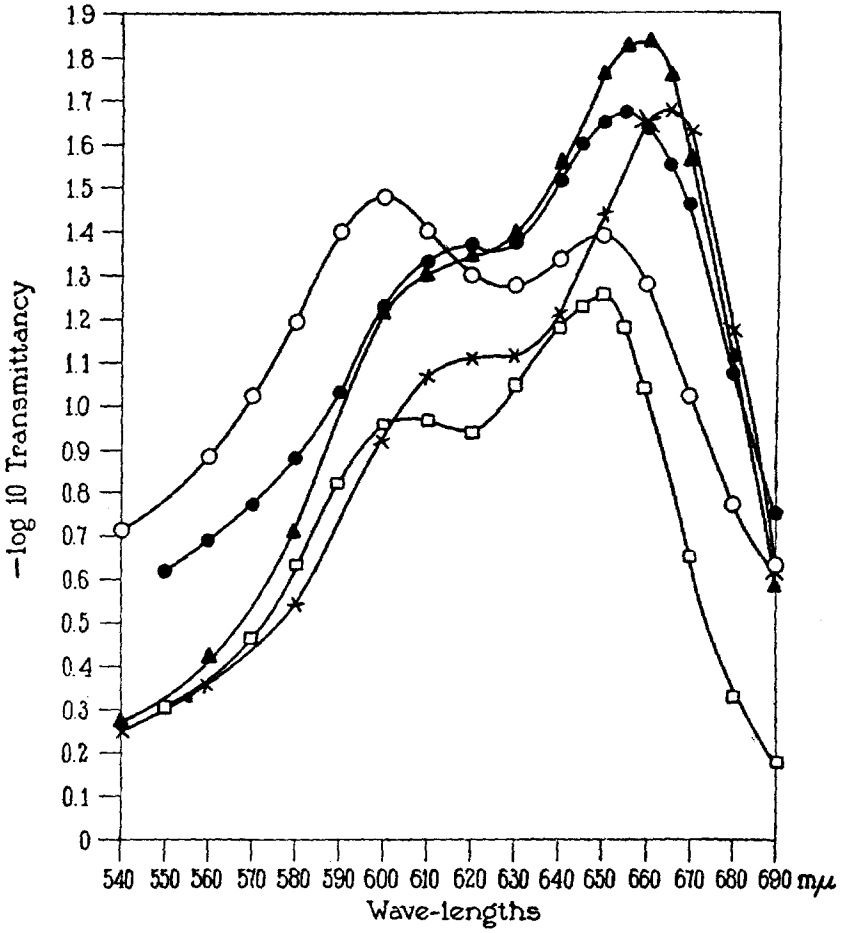

FIG. 2. Absorption curves showing the nature of the dye collected from the vacuole of *Nitella* (New York) placed in solutions of methylene blue (French) or azure B (Holmes), analysis being made about 20 hours after extracting or dissolving the sample. Symbol ● represents the dye collected from the vacuole of living cells after several minutes exposure to methylene blue solution at pH 9.2, where contamination was eliminated by allowing part of the cell to project out of the solution as described in the text. Symbol × represents methylene blue (French), at about the same concentration as the dye represented by symbol ●, dissolved in freshly extracted sap and analyzed after a period equal to the duration of penetration experiment and subsequent measurement. Symbol ▲ represents the dye collected in the vacuole of cells which have lost some turgidity as the result of injury during exposure to methylene blue solution at pH 9.2 where contamination was likewise eliminated. The dye in the sap was diluted with freshly extracted sap containing no dye since it was too concentrated for measurement. Symbol □ represents the pure sample of azure B (Holmes) dissolved in borate buffer solution at pH 9.2. Symbol ○ represents the dye collected from the vacuole of living cells after a few minutes exposure to the azure B solution at pH 9.2, where contamination was eliminated. The heights of these curves have no significance other than an attempt to adjust the concentration and thickness of the layer of solution so as to obtain as accurate a measurement as possible under these circumstances.

allowed to fall onto a glass slide. The drop of sap appeared colorless except for a very dark blue spot in the center which was caused by the dye diffusing from the cell wall contaminating the sap. The contamination occurred when the hanging drop of sap came in contact with the stained cell wall before it separated from the cut end and flowed out upon the surface of the glass slide.

These results show that the error may arise from contamination of the sap from the stained cell wall at the time of extraction. It was therefore necessary to avoid this contamination.

4. Nature of the dye entering the vacuole of uninjured cells when contamination was eliminated: To avoid contamination only about $\frac{3}{4}$ of the length of the cell was immersed in the methylene blue solution (French) and the remaining $\frac{1}{4}$ was allowed to rest on the edge of the dish. The latter was kept moist by wet absorbent cotton which, however, was separated from the dye solution by a short segment of the cell. Cells were thus placed in 0.01 per cent dye at pH 9.2 for a few minutes. The dye in the sap of such cells was found to give an absorption curve characteristic of chiefly azure B and a smaller percentage of methylene blue with a primary absorption maximum at 655 mμ (Fig. 2, symbol •). When the sap thus extracted was exposed to the air in an alkaline solution the color of the dye in the sap did not deepen, thus showing that there was no methylene blue in reduced form in the sap.

5. Nature of the dye penetrating the vacuole of injured cells: Since reversible injury is difficult to detect, we may inquire whether the azure B found in the vacuole as described under (4) is a demethylation product of methylene blue as associated with injury. If this were the case, the more injury the more azure B would be formed. To test this point experiments were carried out with cells which began to lose their turgidity during the experiment. It was found that methylene blue penetrated the vacuole more rapidly when cells began to lose their turgidity, which is shown by the fact that the dye from the vacuole then has a primary absorption maximum at 659 mμ (Fig. 2, symbol ▲).

Obviously azure B penetrates much more rapidly than methylene blue unless injury has occurred. But it is possible that this penetration is only apparent and that after all it is methylene blue which penetrates and is transformed to azure B after it has entered. We must now consider this question.

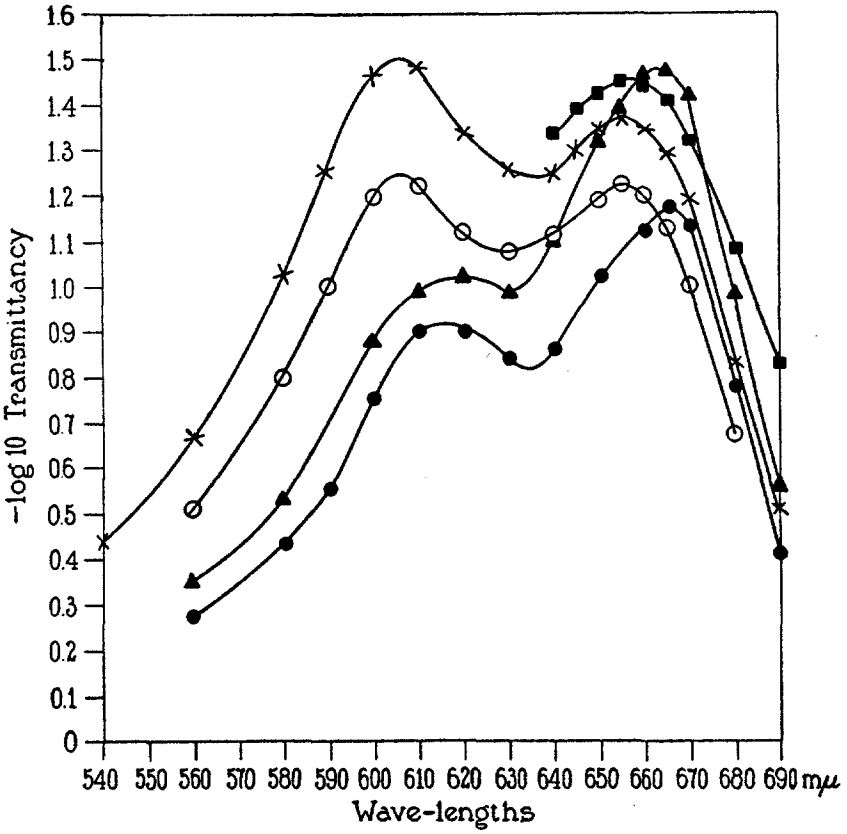

FIG. 3. Absorption curves showing the results obtained with *Nitella* (collected in Massachusetts) proving that the results are identical whether analysis is made immediately or 20 hours after extraction. Symbol ● represents the sample (Merck's medicinal) dissolved in freshly extracted sap and analyzed 4 hours later. Symbol ■ represents the dye collected from the vacuole of living cells placed in methylene blue (Merck's medicinal) at pH 9.2, when analysis was made immediately after extraction. Symbol ▲ represents methylene blue (Merck's medicinal) dissolved in buffer solution at pH 9.2 and analyzed 4 hours later. Symbol × represents the dye extracted from the vacuole of living cells placed in methylene blue (French) at pH 9.2, and analyzed immediately after extraction. Symbol ○ represents the dye extracted from the vacuole of living cells placed in methylene blue (French) at pH 9.2 and analyzed 20 hours after extraction. The heights of these curves have no significance other than an attempt to make measurement in the region where accuracy is possible under these circumstances. Contamination was avoided in all cases.

6. Is the azure B in the sap produced from methylene blue which has entered the cell? To throw some light upon this question methylene blue (French) was dissolved in freshly extracted sap (at about the same concentration as that found in the vacuole in penetration experiments) and allowed to stand for about the same length of time as was the case with the penetration experiment followed by the measurement. The dye in the sap was found to give an absorption curve characteristic of methylene blue with a primary absorption maximum at 664 mμ (Fig. 2, symbol $\times$). This indicates that the sap is not able by itself to change methylene blue to azure B to any noticeable extent during the time of experiment.

(*b*) *Collected in Massachusetts.*—Owing to the fact that the extraction of sap from the vacuole of cells of *Nitella* required the cooperation of several technicians it was necessary in the experiments just described to extract the sap in New York City while the measurements were made by Mr. Holmes in Washington. Although these measurements were made about 20 hours after extraction of the sap, the validity of these analyses is shown by the fact that later, when it was possible to make in Woods Hole extractions followed immediately by measurements the dye in the sap (extracted from cells collected in Massachusetts, care being taken to avoid contamination) gave the same absorption curve as before whether it was measured immediately after extraction or 20 hours later. The primary absorption maximum was at 655 mμ (Fig. 3, symbols $\times$ and $\bigcirc$). This primary absorption maximum closely resembles that obtained with New York *Nitella* (Fig. 2, symbol $\bullet$).

The same result was obtained when the experiments were repeated with methylene blue (Merck's medicinal) (Fig. 3, symbols $\bullet$, $\blacksquare$, and $\blacktriangle$).

Experiments with pure azure B. Although the sap has been shown to be incapable of demethylating methylene blue to azure B is the protoplasm able to do so? Since Holmes[11] has found that demethylation of azure B to azure A takes place more readily than demethylation of methylene blue to azure B, if we find azure B to penetrate the vacuole without demethylation, from pure azure B solution, we may be justi-

[11] Holmes. W. C., to be published later.

fied in concluding that it is unlikely that the protoplasm is capable of changing methylene blue to azure B. Dye from cells placed in azure B solution (Fig. 2, symbol □) gives an absorption curve characteristic of azure B with a primary absorption maximum at 650 mμ (Fig. 2, symbol ○), thus showing that in all probability neither the protoplasm nor the sap is capable of demethylating azure B (contamination was avoided).

B. Experiments on Valonia macrophysa.

Dye from uninjured cells left for about 1½ hours in methylene blue (French) at pH 9.5 gave an absorption curve characteristic of azure B with an absorption maximum at 650 mμ (Fig. 4, symbol ●), while the external solution consisted chiefly of methylene blue with an absorption maximum at about 664 mμ (Fig. 4, symbol □).

If the conclusion is correct that the presence of azure B in the vacuole of cells placed in methylene blue solution is due to the more rapid penetration of azure B from the outside solution (which contains azure B as impurity) we should expect an increase in the rate of penetration on the addition of more azure B to methylene blue. This was found to be the case experimentally. Enough dye for analysis collected in half an hour in the vacuoles of cells (at pH 9.5) in 0.04 per cent methylene blue solution (Merck's medicinal) to which about 25 per cent of the same concentration of azure B was added, whereas during the same period not sufficient dye for analysis penetrated from the same sample of methylene blue solution to which no addition of azure B was made. Spectrophotometric analysis of the first solution showed a mixture of methylene blue and azure B with a primary absorption maximum at 660 mμ (Fig. 4, symbol ○) while the dye in the vacuole was chiefly azure B with a primary absorption maximum at 650 mμ (Fig. 4, symbol ▲).

When cells are placed in pure azure B solution (Holmes) with a primary absorption maximum at 650 mμ sufficient dye for analysis enters in a very few minutes and this dye is found to be azure B with a primary absorption maximum at 650 mμ (Fig. 4, symbol △) which indicates that neither the protoplasm nor the sap is capable of changing azure B.

All these measurements were made immediately after extraction of the sap. When some of the measurements in connection with *Valonia* were repeated by Dr. Gibson with a König-Martens spectrophotometer the results confirmed those previously obtained. Whether Samples F and G of Clark, or the sample of French is used, the dye which

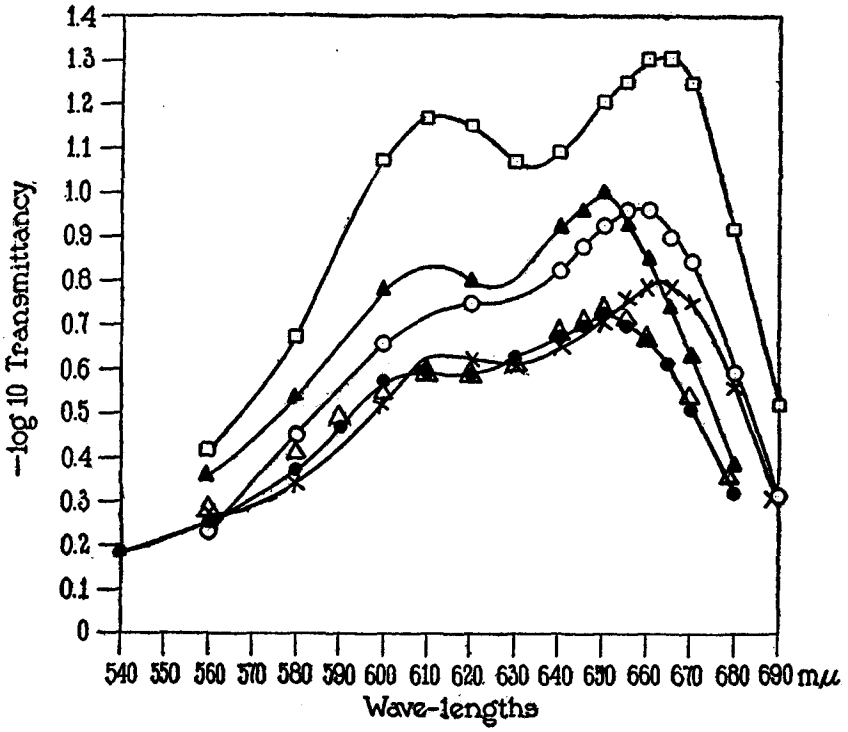

FIG. 4. Absorption curves showing the penetration of dye into *Valonia* from a mixture of azure B and methylene blue in comparison with the dye from methylene blue solution containing a trace of azure B as impurity. Symbol □ represents methylene blue (French) dissolved in sea water at pH 9.5 and analyzed 4 hours later. Symbol ● represents the dye which has penetrated from this solution in 1½ hours when analysis was made immediately after extraction. Symbol × represents methylene blue (French) dissolved in freshly extracted sap and analyzed 4 hours later. Symbol ○ represents a mixture of about 75 per cent of methylene blue and 25 per cent of azure B at pH 9.2. Symbol ▲ represents the dye which has penetrated from this solution in ½ hour when analysis was made immediately after extraction. Symbol △ represents the dye which has penetrated from pure azure B solution in less than ½ hour.

collects in the vacuoles of uninjured cells of *Valonia macrophysa* placed in methylene blue solution at pH 9.5 gives an absorption curve characteristic of dye chiefly consisting of azure B. With the sample of French, this absorption maximum is at 649 mμ while with Clark's samples it is at about 653 mμ.[12] The absorption maximum of azure B is at 647 mμ and of methylene blue at 664 mμ.

The difference in the absorption maximum between 650 mμ (from French's samples at pH 9.5) and 653 mμ (from Clark's samples at pH 9.5) is due to the fact that there is practically no methylene blue in the former while there is some in the latter. Methylene blue is detectable in the latter case because the rate of penetration of azure B is slower and the concentrations of dye found in the sap after 2 hours are much lower (at about 5.9×10^{-6} M and 7.9×10^{-6} M). It is difficult to determine whether this methylene blue in the sap is due to contamination of the sap from the stained cell wall at the time of extraction of the sap or to penetration. One way of determining this point would be to continue the experiments at pH 9.5 until the concentration of dye in the sap increased considerably and see if the presence of methylene blue would become masked by the increase of azure B in the sap. But this is inadequate on account of the danger of an added complication arising from possible injury.

The experiments previously[2] carried out with French's sample showed that at pH 9.5 with concentrations of dye up to about 1.6×10^{-5} M, unless the experiments were done with great care, a small amount of methylene blue was found with azure B in the sap giving an absorption maximum at about 653 mμ. When there was more dye in the sap the methylene blue was masked by azure B, thereby giving an absorption maximum at 650 mμ which was identical with that of pure azure B. This indicates that at lower concentrations of dye in the sap there is a considerable possibility of error arising from contamination.

[12] Experiments were continued up to 3½ hours when one reading gave an absorption maximum at 653 mμ and the other at 656 mμ. At such a slow penetration it is impossible to determine whether the presence of methylene blue (together with azure B) in the sap is due to very slight injury, contamination, or very slow penetration (much slower than the penetration of azure B). The results of such experiments must therefore be considered doubtful.

These results do not disagree with the theory presented in the text since they show that at higher external pH value the dye in the vacuole of uninjured cells consists chiefly of azure B while the external solution contains chiefly methylene blue with a trace of azure B as impurity. This indicates that azure B in form of free base possibly penetrates more rapidly than methylene blue.

IV.

DISCUSSION.

These results on *Valonia* and *Nitella* confirm those previously obtained with *Valonia*.[1,2] The fact that by eliminating errors from contamination the primary absorption maximum in the case of dye penetrating from methylene blue solution at about pH 9.5 is at 650 mμ with *Valonia* while it is at 655 mμ with *Nitella* may be due to differences in the condition of the cells brought about during the experiments or to the normal compositions of the two types of cells or to the difference in the external media. Though there is this difference, the fact still remains that at about pH 9.5 both take up azure B from methylene blue solution containing a small amount of azure B as impurity. At this external pH value methylene blue is detected in the sap (1) when cells are injured or (2) when the contamination of the sap from the stained cell wall at the time of extraction occurs. The errors arising from these two sources are frequent so that experiments must be carried out with a great deal of care.

Has this azure B actually penetrated as such from the external solution or has methylene blue entered and become demethylated to form azure B? Holmes[13,14] has shown that a progressive demethyla-

[13] Since Holmes has found that demethylation of methylene blue to azure B at pH 11 does not occur instantly but only after standing for a long period this increase in the rate of penetration of azure B with a rise in the external pH value to pH 10.9 where at maximum the solution has stood for only 1 hour is not due to the increase in the concentration of azure B resulting from demethylation of methylene blue in presence of greater akalinity.

[14] Transformation of methylene blue to methylene azure in presence of alkalinity has been found by others. Bernthsen, A., *Ann. Chem.*, 1885, ccxxx, 137. Kehrmann, F., *Ber. chem. Ges.*, 1906, xxxix, 1403. Baudisch, O., and Unna, P. G., *Dermat. Woch.*, 1919, lxviii, 4.

tion of methylene blue to azure B and then to azure A does not take place readily at a pH value below 10. Since the pH value of the sap is at about pH 5.8 and since the work of others[15] indicates the pH value of the protoplasm in general to be considerably below 10, we may conclude that methylene blue cannot be demethylated by the protoplasm nor by the sap at their normal pH values unless they contain a substance capable of bringing about demethylation at a pH value below 10. Such substances must be absent from the sap for freshly extracted sap is incapable of demethylating methylene blue (at the concentration of the blue dye actually penetrating the vacuole) even during a period longer than that required for the penetration and its subsequent measurement in my experiments.

An additional reason for doubting that the protoplasm and the sap of these cells can demethylate methylene blue is that azure B is found to penetrate from pure azure B solution into the vacuole as such without undergoing demethylation to azure A. Since Holmes[11] has found that demethylation of methylene blue to azure B is brought about less readily than that of azure B to azure A, it is unlikely that the system which is incapable[16,17] of changing azure B could transform methylene blue.

The azure B is not formed from methylene blue as a result of the change in the protoplasm or in the sap caused by an injury, because so long as cells are not injured azure B collects in the vacuole while as soon as it becomes injured methylene blue begins to penetrate freely.

The spectrophotometric measurement of the dye in the vacuolar sap of *Valonia macrophysa* has been recently repeated by M. M. Brooks,[18] who concludes that the azure B present in the vacuole in my experiments is due to the transformation of methylene blue by the

[15] A review of work on the pH values of the protoplasm is given by Chambers, R., *J. Gen. Physiol.*, 1926–27, x, 739.

[16] This statement, however, must be made with reservation, since it has been found that in dogs the methyl group in position seven of the xanthine ring is most readily removed while in rabbits the opposite is the case (Krüger, M., and Schmidt, J., *Ber. chem. Ges.*, 1899, xxxii, 2677; *Z. physiol. Chem.*, 1902, xxxvi, 1).

[17] When methylene blue is injected intravenously azure B is found in the urine and feces (Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905, xiii, 358).

[18] Brooks, M. M., *University California Publications, Zoology*, 1927, xxxi, 90.

sap after extraction. She bases this conclusion on the assumption that my measurements were made after a lapse of time sufficient for the transportation of the extracted sap from New York to Washington. In view of the fact that my measurements, even when completed within 1 hour after extraction, gave absorption curves characteristic of azure B there is no basis for her conclusion.

If we suppose that azure B penetrates as such, diffusing through the protoplasm from an external solution containing mostly methylene blue, with only a small percentage of azure B, we may conclude that azure B penetrates the vacuole much more rapidly than methylene blue. This conclusion is supported by experiments showing that azure B collects in the vacuole much more rapidly from pure azure B than from methylene blue solution. The behavior of azure B is similar to that of many other basic dyes which are capable of forming free base and salt, since it is found to penetrate the vacuole much more readily as free base than as salt (the higher the pH value of the external solution the more rapid is the rate of penetration). This increases with rising pH value of the external solution for both azure B and methylene blue.[19,20,21]

These results are in agreement with the theory[22] that the rate of entrance of the dye is higher the more rapidly it enters the non-aqueous layers of the protoplasm and passes from them into the

[19] The following investigators have found an increase in the rate of penetration of dye with an increase in the external pH value of methylene blue solution. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. MacArthur, J. W., *Am. J. Physiol.*, 1921, lvii, 350.

[20] My statement showing that the blue dye penetrated the vacuole of living cells from methylene blue solution more rapidly at pH 9.5 than at pH 5.5 (Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, foot-note 15, p. 572; 1926–27, x, 927) was contrary to the first conclusion drawn by Brooks, M. M. (*Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 265; *Am. J. Physiol.*, 1926, lxxvi, 360), but my conclusion was confirmed by her in her later publication (Brooks, M. M., *University California Publications, Zoology*, 1927, xxxi, 90).

[21] Baudisch and Unna have found that methylene azure enters chloroform and appears red (see foot-note 14). Kehrmann (14) has stated that methylene azure enters ether, chloroform, and benzene in form of a base and not in form of a salt.

[22] Irwin, M., *J. Gen. Physiol.*, 1927–28, xi, 112; *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 127.

vacuolar sap. The entrance of the dye therefore depends on the concentration gradient and on the partition coefficient between the non-aqueous layers of the protoplasm and the aqueous phases.

In the present case the cell behaves as if the rate is controlled by only three phases (1) external solution, (2) one non-aqueous layer, and (3) aqueous sap in the vacuole. Since this will form the subject of the next paper it will not be discussed at length here.

On basis of this theory we may expect a very slow penetration of both azure B (in form of salt) and methylene blue, which might be detected if the dye penetrating the vacuole from methylene blue solution at pH 5.5 could be measured, but unfortunately the penetration is too slow for accurate analysis.

If methylene blue penetrates, its presence might be detected if there were little or no penetration of azure B. This might be ensured by placing cells in a sample of methylene blue exceptionally free from azure B but in this case the penetration of the blue dye is so slow that we are unable to obtain data and errors may result from injury or contamination.

Although the experiments show that unless cells are injured, azure B collects in the vacuole much more rapidly than methylene blue from methylene blue solution at a higher pH value, they do not tell us anything about the penetration of methylene blue into the protoplasm (which forms a layer so thin that no experiments suitable for our present purpose can be made on it directly). They merely point out the danger of drawing any conclusion as to the permeability of cells or the oxidation reduction potential of cells based on penetration of a blue dye from methylene blue solution without satisfactory determinations of the nature of the dye inside and outside the cell.

SUMMARY.

Spectrophotometric measurements show that it is chiefly the trimethyl thionin that is present in the sap extracted from the vacuoles of uninjured cells of *Nitella* or *Valonia* which have been placed in methylene blue solution at a little above pH 9. Whether these measurements were made immediately or several hours later the same results were obtained. Methylene blue is detected in the sap

(1) when the cells are injured or (2) when the contamination of the sap from the stained cell wall occurs at the time of extraction.

The sap is found to be incapable of demethylating methylene blue dissolved in it even on standing for several hours.

It is somewhat uncertain as to whether the trimethyl thionin penetrated as such from the external methylene blue solution which generally contains this dye as impurity (in too small concentration for detection by spectrophotometer but detectable by extraction with chloroform), or whether it has formed from methylene blue in the protoplasm. The evidences described in the text tend to favor the former explanation.

Theory is discussed on basis of more rapid penetration of trimethyl thionin (in form of free base) than of methylene blue, or of trimethyl thionin in form of salt.