ON THE NATURE OF THE DYE PENETRATING THE VACUOLE OF VALONIA FROM SOLUTIONS OF METHYLENE BLUE.

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I.

INTRODUCTION.

Experimental results[1] favor the theory that a basic dye penetrates a living cell very rapidly in the form of free base (which predominates at a high pH value) but so slowly in the form of salt (which predominates at low pH values) that its penetration is comparatively negligible. If this theory were correct, we should not expect a dye like methylene blue, which is very strongly basic,[2] to enter a living cell, since at the range of pH values generally available for living cells this dye exists in the form of salts. Yet methylene blue is widely known as one of the most commonly used vital stains. What is the explanation for the discrepancy between the theory presented and the observed facts? Does this indicate that the theory is inadequate, or does it mean that the dye which penetrates is not methylene blue but a less basic lower homologue, such as azure B or trimethyl thionine,[3] which is found in methylene blue solutions

[1] 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. MacArthur, J. W., *Am. J. Physiol.*, 1921, lvii, 350. Irwin, M., *J. Gen. Physiol.*, 1925–27, viii, 147; 1925–26, ix, 561; 1926–27, x, 75.

[2] For discussion of the apparent dissociation constant of methylene blue *cf.* Clark, W. M., and his collaborators (Clark, W. M., Cohen, B., and Gibbs, H. D., *Pub. Health Rep., U. S. P. H., No. 23*, 1925, 1131).

[3] The apparent dissociation constant of azure B has not been determined, but we have the following reason to assume that it is a weaker base than methylene blue. In general it is found that a substance whose amino groups are completely substituted with alkyl radicles, such as tetramethyl ammonium hydroxide,

especially at a higher pH value and which is capable of existing partly in form of free base? It has been stated by some investigators[4] that lower homologues are found in samples of methylene blue, and others[5] have found that in presence of air and with an alkaline reaction methylene blue in aqueous solution is partly converted to methylene

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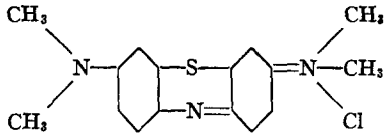
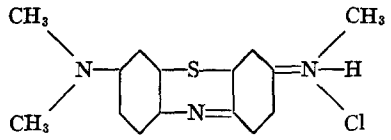
is a strong base; while a substance whose amino groups are only partially substituted by alkyl radicles, such as trimethyl ammonium hydroxide is a weaker base. Since amino groups of methylene blue or tetramethyl thionine are completely substituted with alkyl radicles, while those of azure B or trimethyl thionine are only partially substituted by alkyl radicles, as shown below, it would seem reasonable to suppose that methylene blue behaves like a strong base while azure B behaves like a weaker base.

Methylene blue or tetramethyl thionine.



Azure B or trimethyl thionine.



Difference between the chemical structure of the dye in form of free base and that of the dye in form of salt must be left undecided until further studies are made. It is uncertain as to whether such a difference between free base and salt as represented in the previous publication (Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 76) is correct. It may very well be that the salt is represented by the structure given above and that the free base is represented by a structure in which the Cl is replaced by the OH, or it is an anhydro-base.

[4] 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. A delicate and reliable method for the detection of the presence of trimethyl thionine in methylene blue has been devised by Holmes and will appear in an early issue of *Stain Technology*. By means of this method it was found that the purest samples of methylene blue available for testing invariably contained small proportions of trimethyl thionine.

[5] 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.

azure, which was found[6] to enter chloroform and to appear red. Furthermore, Kehrmann[7] has stated that methylene azure, which is a mixture of trimethyl thionine (azure B) and asymmetrical dimethyl thionine (azure A), enters substances like ether, chloroform, and benzene in form of a base and not in form of a salt, while methylene blue is not soluble in ether.

In view of the fact that the dye from methylene blue solution does not enter[8] the living cells except when the pH value of the solution is high, we may have a good reason for suspecting that the dye capable of entering a living cell is not methylene blue (in the form of a salt), but a less basic homologue (in the form of a free base), just as in the case of absorption by a substance like ether (already discussed). In this case the theory first presented would prove adequate.

One way to test this question is to study by spectrophotometric analysis the nature of the dye inside and outside the living cell. Heretofore this has not been attempted. The writer therefore proposes to give in the present paper[9] a series of spectrophotometric analyses of the dye penetrating from solutions of methylene blue into the vacuole of the living cell of marine alga *Valonia macrophysa*.

II.

Penetration of Dye into Valonia from a Solution of Methylene Blue.

Details of technique will be omitted here since they have previously been given by the writer.[10] Mention may, however, be made of several points of importance. Medium sized cells were chosen to avoid errors caused either by injury or by contamination of the sap from the stained cell wall. If too large a cell was employed, it took so long for the dye to collect in the vacuole that injury often occurred before there was a sufficient quantity of dye in the sap for spectrophotometric analysis. If too small a cell was used, the dye derived from the stained cell wall when the cell was punctured by a capillary tube for the purpose of collecting the sap exceeded the concentration of dye in the vacuole, which had

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[6] *Cf.* Baudisch, O., and Unna, P. G., Foot-note 5.

[7] *Cf.* Kehrmann, Foot-note 5.

[8] Harvey, E. N., MacArthur, J. W., and Irwin, M., see Foot-note 1.

[9] A preliminary report of these analyses has been made by the writer (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 425).

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

penetrated from the external dye solution before the cell was removed from the solution and punctured.

It is desirable to possess a sound basis for judging the condition of cells during the experiment. It is not a difficult matter to determine an irreversible injury, but a reversible injury is almost impossible to detect. It was therefore necessary to have a more or less arbitrary basis for judging if the cells were injured. Cells during experiment were considered to be uninjured (1) if they continued to live after they were kept in the test solution for several hours beyond the time required for experiment, (2) if they were found to be living a day or so after they had been transferred from the test solution to normal sea water, (3) if the turgidity of the cell, as detected by touch, remained the same as that of control cells (as the cells become injured they lose their turgidity).

The pH values of the sea water employed were about pH 5.5, 9.5, and 10.9. The pH value of the Bermuda sea water in which the dye was dissolved was altered and determined in the following manner. To sea water, hydrochloric acid was added until the color of the test-tube containing the sea water and brom cresol purple matched that of the standard phosphate buffer solution at pH 6 containing the same concentration of the indicator and 0.6 M sodium chloride, which roughly corresponds to the halide concentration of Bermuda[11] sea water. To sea water sodium hydroxide was added until the color of the test-tube containing the sea water and cresol phthalein matched that of the test-tube containing standard borate buffer solution at pH 9.7 or at pH 11.2, containing the same concentration of the indicator and 0.6 M sodium chloride. Owing to the slight difference in the pH value of different samples of Bermuda sea water the volume of hydrochloric acid and sodium hydroxide added varied slightly, but to 100 cc. of sea water on an average was added 1.08 cc. of 0.2 M hydrochloric acid (for pH 5.5) or 0.8 cc. of 0.2 M sodium hydroxide (for pH 9.5) or 0.45 cc. of 0.5 M sodium hydroxide (for pH 10.9).

Since the addition of sodium chloride alters the pH values of the buffer solutions, the pH values of the standard phosphate and borate buffer solutions containing 0.6 M sodium chloride were determined by means of the hydrogen electrode, and they were found to be pH 5.5 (phosphate), pH 9.5 (borate), and pH 10.9 (borate). These pH values represent only approximate pH values of the given sea water, because the colorimetric determination is not accurate for the following reason. Since in the standard buffer solutions the halide content of the sea water is represented only by sodium chloride, and since some salts are known to change the color of the indicators more than the others, the pH value of the standard buffer solution containing 0.6 M sodium chloride and that of the sea water may not be exactly the same even though the color of the test-tube containing the one matches that of the test-tube containing the other.

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[11] Bermuda sea water contains about 0.58 M halides (Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924–25, vii, 637).

Furthermore, above pH 10.3, the magnesium in the sea water is precipitated, which in some cases interferes with colorimetric determination, and no indicators are very sensitive in this range, so that at pH 10.9 the error may not be small.

But no effort was made to avoid such sources of error since only the relative pH values were desired for these experiments. These pH values therefore are sufficiently accurate to serve the purpose in the present case, though they are approximate values.

Methylene blue is partially salted out in sea water so that two kinds of solutions were used; (1) a dye solution in which all the precipitate was allowed to remain, (2) a dye solution from which the precipitate was in greater part removed by filtering. In both cases the concentration of the dye before and after the experiment was found to remain unchanged and approximately the same results were obtained.

The following samples of methylene blue were employed, which according to the writer's knowledge represent some of the purest available.

(1) $C_{16}H_{18}N_35Cl + 1H_2O$ sent by Dr. Benda from Germany.

(2) $C_{16}H_{18}N_35Cl + 3H_2O$ sent by Dr. Benda from Germany.

(3) Sample F and Sample G, given by Dr. W. M. Clark and Dr. B. Cohen of the Hygienic Laboratory, Washington, D. C.

(4) Bleu de methylen pour Bacteriologie, Microbiologie, Physiologie, Produit, given by Dr. R. H. French of the Color Laboratory, United States Department of Agriculture, Washington, D. C.

(5) Merck's medicinal.

Owing to the difference in the solubility of different samples in sea water, different concentrations[12] varying from 0.01 to 0.04 per cent were employed.

In determining the concentration of dye in the sap the following method was used. Sap was collected by puncturing the cell (previously removed from the dye solution and wiped) by means of a sharp glass capillary tube, and by drawing up the sap from the vacuole. About 2 cc. of sap was then placed in a small test-tube and the color of this test-tube was matched with that of a test-tube of the same diameter containing a known concentration of methylene blue.

Merck's medicinal was used as the standard solution for all the samples employed because this was the only sample available in sufficient quantity to make up a series of standard solutions at different concentrations. When the concentration of dye in the sap was below 0.00004 per cent, the color appeared more greenish than the standard so that it was difficult to match the color. Furthermore, above 0.0003 per cent the color of the test-tube containing the sap appeared more purplish than that of the standard so that it again became difficult to match.

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[12] Other concentrations were used as check experiments. In all cases, if any dye entered the vacuole of uninjured cells more entered from the external solution at pH 9.5 than at 5.5, provided the experimental errors described in the text are absent.

Experiments were carried out at 25° ± 0.5°C. in an incubator with air holes through which diffused light was allowed to enter.

When living cells of *Valonia* were placed in methylene blue dissolved in sea water at these two pH values it was found that at about pH 5.5 practically no dye penetrated the vacuole, while at about pH 9.5 more entered. For example, with Merck's medicinal methylene blue, after 1 hour, at pH 9.5, the concentration of dye in the sap was about 0.00006 per cent, while at pH 5.5 it was too dilute for determination. When other samples of methylene blue, already described, were used, it was found that with some samples more dye entered than from the Merck's medicinal, while from others less entered the vacuole. But in all cases the rate of penetration of the dye into the vacuole was higher with the external dye solution at pH 9.5 than at pH 5.5. But with the samples in which the penetration was extremely slow the amount of dye found in the vacuole was so small even after several hours of exposure that unless extreme care was taken there were possibilities of experimental error arising from (1) contamination of the sap from dye in the cell wall at the time of puncturing exceeding the actual penetration of dye into the vacuole before puncturing; (2) inability to match the color of the test-tubes accurately; (3) more rapid penetration of dye due to a slight and reversible injury which cannot be detected, and which may occur if experiments are extended for several hours or if the cells at the start are not in excellent condition (the dye enters more rapidly as the cells become injured).

These sources of error might in some cases cause the rate of penetration of dye to appear the same at pH 5.5 and at pH 9.5. Furthermore, since at pH 5.5 the cells become injured more rapidly than at pH 9.5, in some cases where the injury occurred to the extent of a very slight loss of turgidity the rate of penetration at pH 5.5 was found to be higher than that at pH 9.5.

Experiments have been made with cells which have been kept in stoppered glass bottles containing some sea water for several months, as well as with cells which have been kept in a pan of sea water for several weeks. In both cases it was found that so long as the cells were not injured, the dye entered more rapidly at pH 9.5 than at pH 5.5, though in the case of the cells which have been kept in the laboratory for several months, as described above (cells appeared

less green than more recently collected cells), the dye entered more rapidly than in the case of the cells which have been kept only for a few weeks.

When the sap collected from the uninjured cells which had been exposed to the dye solution was oxidized by shaking and exposing to air with an alkaline reaction, no increase in coloration took place so that we may conclude that there was no dye in reduced form present in the sap.

When the pH value of the sap was determined after the living cells of *Valonia* had been exposed to sea water at pH 5.5, 9.5, and 10.9 for 4 hours, no change in the pH value of the sap occurred if the cells were not injured.

III.

Spectrophotometric Analysis.

The nature of the dye in the external solution and in the vacuolar sap of uninjured and injured cells was tested by means of spectrophotometric determinations. The measurements were made at the Color Laboratory in Washington, D. C., by W. C. Holmes, of whose collaboration the writer desires to express her appreciation.

The instrument employed was a Hilger wave-length spectrometer, equipped with a Nutting photometer. Either 1 or 2 cm. layers of solution were examined, depending on the concentration of dye in the solutions and the quantities of solution available. The measurements were carried out over the spectral range between 540 and 690 mμ. The concentration of dye was adjusted, insofar as was possible, to afford maximum visual sensitivity at and near the absorption maximum of the dye in dilute aqueous solution. All recorded values in this restricted region are averages of a considerable number of measurements.

A brief statement of spectroscopic criteria is advisable at this point. The absorption maximum of methylene blue in dilute aqueous solution is approximately 665 mμ. The corresponding maximum of trimethyl thionine is approximately 650 mμ. Although the average visual sensitivity in this region of the spectrum is relatively inferior it is readily possible to locate absorption maxima (with favorable dye concentrations) within a possible variation of about $\pm$1mμ.

The determination of the approximate absorption maximum, accordingly, differentiates the two dyes with absolute certainty. It affords, moreover, a reliable, if somewhat rough, criterion of the relative proportions of the dyes in question when both are present. Owing to the relatively limited spectral interval between their bands the band of a mixture of the dyes does not inhibit the individual maxima of its two component bands, but, rather, a single composite maximum of which the location varies with dye proportions. The absorption maximum of a mixture containing 66 per cent of methylene blue and 33 per cent of trimethyl thionine, for example, falls at approximately 660 mμ, while that of a mixture of 33 per cent of methylene blue and 66 per cent of trimethyl thionine falls at approximately 655 mμ.

It may be noted that the employment of suitable spectrophotometric ratios would afford a more exact definition of relative dye proportions. The basic data requisite for this procedure, however, were not available when the present investigation was begun and it was felt that the mere determination of the approximate absorption maxima of solutions would afford ample evidence of their character for present purposes.

Both methylene blue and trimethyl thionine exhibit secondary absorption in the general spectral region near 600 mμ. Both dyes are held to exist in aqueous solutions in a state of tautomerism between two dye forms.[13] In the present investigation considerable variations were noted in the apparent tautomeric equilibria between dye forms. These arise primarily from variations in dye concentration and are also influenced by other factors. A discussion of these phenomena is unnecessary in this paper. It is sufficient to note that their occurrence does not modify in any appreciable degree the relative absorption of the dyes at different wave-lengths within the critical spectral region between 650 and 665 mμ, or invalidate conclusions derived from variations in absorption within that region.

When such analyses were made some very interesting facts were obtained, as follows:

I. A sample of methylene blue,[14] dissolved in (1) Bermuda sea

[13] Holmes, W. C., *Ind. and Eng. Chem.*, 1924, xvi, 35; *Stain Technol.*, 1926, i, 17.

[14] Several samples were employed (see Section II in text).

water, (2) sap of *Valonia macrophysa*, and (3) artificial sap,[15] gave absorption maxima[16] characteristic of methylene blue, about 665 mμ (see Table I and Fig. 1, Curves *A*, *B*, and *C*).

II. The dye allowed to diffuse out of the cell wall (which had been previously stained by placing the living cells for a few minutes in sea water containing methylene blue), into artificial sap of *Valonia* gave

TABLE I.

Solutions	Primary absorption maximum
	mμ
Methylene blue dissolved in sea water	665
Methylene blue dissolved in the sap of *Valonia*	665
Methylene blue dissolved in artificial sap of *Valonia*	665
Dye which has diffused out of the cell wall into artificial sap, after the cell wall of living cells of *Valonia* has been stained in methylene blue (dissolved in sea water)	665
Dye found in sap from the vacuole of injured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 9.5	663
Dye found in sap from the vacuole of injured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 5.5	665
Trimethyl thionine dissolved in sap of *Valonia*	650
Dye found in the vacuole of uninjured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 9.5	650
Dye absorbed by chloroform from methylene blue dissolved in sea water at pH 9.5: This was freed from chloroform by absorbing it in distilled water	650
Dye absorbed by chloroform from methylene blue dissolved in sea water at pH 5.5: This was freed from chloroform by absorbing it in distilled water	655
Methylene blue dissolved in distilled water	665
Dye absorbed by chloroform from methylene blue dissolved in M/150 buffer mixtures at pH 5.5 or at pH 9.5: This was freed from chloroform by absorbing it in distilled water	650

the absorption maximum of 665 mμ characteristic of methylene blue (Table I and Fig. 1, Curve *D*).

[15] The pH value of the sap is about 5.8. The sap contains about 0.6 M halides (*cf.* Osterhout, W. J. V., and Dorcas, M. J., Foot-note 11).

[16] The absorption curve thus obtained resembles that of a higher concentration of methylene blue dissolved in distilled water. This may be due to the effect of salt on the dye, as suggested by Dr. W. C. Holmes.

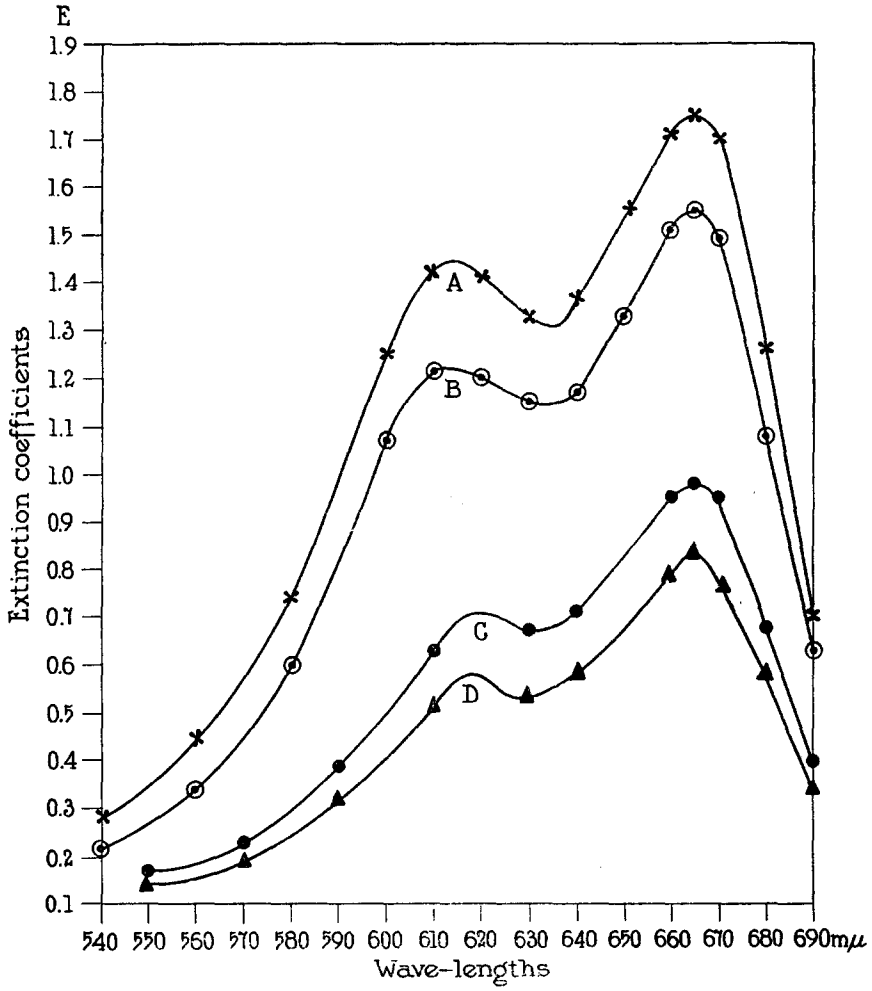

FIG. 1. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curve *A* represents the methylene blue dissolved in sea water, Curve *B* in the sap of *Valonia*, Curve *C* in the artificial sap of *Valonia*, Curve *D* the dye that has diffused from the cell wall into artificial sap after the cell wall of living cells of *Valonia* has been stained in methylene blue dissolved in sea water.

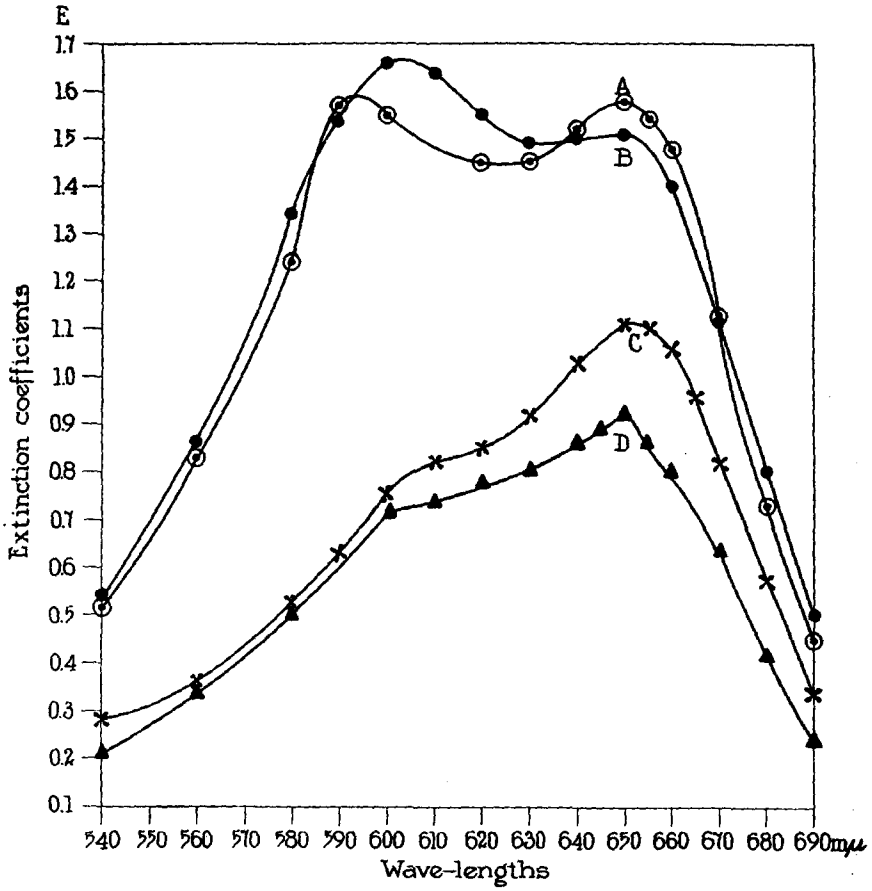

FIG. 2. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves *A* and *C* (symbols ⊙ and ×) represent the dye which was found in the vacuolar sap of uninjured cells of *Valonia* after the cells have been stained for 1 hour in one sample of methylene blue dissolved in sea water at pH 9.5 (Curve *A*) and in another purer sample at pH 10.9 (Curve *C*). Curves *B* and *D* represent the sample of trimethyl thionine or azure B dissolved in sap of *Valonia* at two dilutions, corresponding approximately to two dilutions represented by Curves *A* and *C* respectively. Curve *B* corresponds with Curve *A* and Curve *C* with Curve *D*.

III. Two concentrations of trimethyl thionine or azure B (sent by W. C. Holmes) dissolved in the sap of *Valonia* (Table I, Fig. 2, Curves *B* and *D*) gave absorption maxima of 650 mμ. This sample is obtained by the oxidation of methylene blue. Holmes suggests that though it is shown to be a fairly pure product it is possible that it contains small proportions of both methylene blue and asymmetrical dimethyl thionine.

IV. The dye in the sap collected from the vacuole of uninjured cells after an exposure of 1 hour to sea water saturated with two samples of methylene blue[17] (1) 0.04 per cent at pH 9.5 (Curve *A*, Fig. 2), and (2) 0.01 per cent at pH 10.9 (Curve *C*, Fig. 2). In Curve *A* the absorption maximum is 650 mμ which shows that the dye is chiefly trimethyl thionine and it gives no visible evidence of the presence of methylene blue. The absorption maximum of Curve *C* is about 652 mμ which shows that there is a trace of methylene blue, though the dye is chiefly trimethyl thionine. The presence of a trace of methylene blue in all probability is due to the contamination of the sap from the stained cell wall at the time the cell was punctured to collect the sap. Such a contamination plays an important part whenever the concentration of the dye in the sap is relatively small.

V. The dye collected from the vacuole of injured cells (slightly soft) after 12 hours' exposure to sea water saturated with methylene blue, either at (1) pH 5.5, or (2) at pH 9.5 gave an absorption maximum of 665 mμ (methylene blue) for (1) and 663 mμ (methylene blue and a little azure B) for (2) (Table I, and Fig. 3, Curves *A* and *B*). Since in both cases the dye in the sap collected from the vacuole was diluted with the sap collected from the vacuole of unexposed living cells, the heights of the curves which vary with dilution (the higher curve corresponding to the higher concentration) given in Fig. 3 do not show true relative concentrations of the dye found in the vacuole.

The azure B found by spectrophotometric analysis in the sap collected from the vacuole of uninjured cells of *Valonia* is not due to the transformation of methylene blue into azure B after methylene blue

[17] Samples employed are specified in the text in Section II. Owing to the fact that the purpose of these experiments is not to determine the purity of these samples, the sample used for each result is not specified.

has penetrated from the external dye solution into the vacuole because not enough conversion takes place during 1 to 3 hours in the methylene blue dissolved in the sap of *Valonia* to be detected by this method.

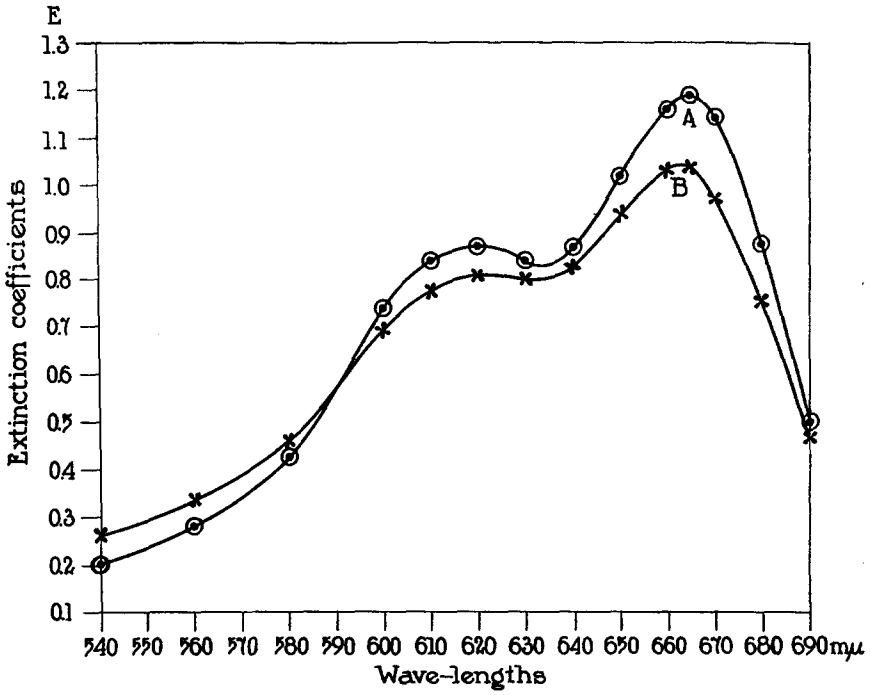

FIG. 3. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves representing the dye in the sap collected from the vacuole of injured cells of *Valonia* and diluted with sap after the cells have been stained in the methylene blue dissolved in sea water, Curve *A* at pH 5.5 and Curve *B* at pH 9.5.

IV.

Absorption of Dye by Chloroform from Methylene Blue Solution.

In view of the fact that a similarity was found between *Valonia* and chloroform in their behavior toward other basic[18] dyes, in that

[18] When living cells of *Valonia* were placed in different basic dyes, Lauth's violet, neutral red, and brilliant cresyl blue, it was found that the higher the pH values of the dye (*viz.* between pH 5 and pH 8), the more rapidly the dye

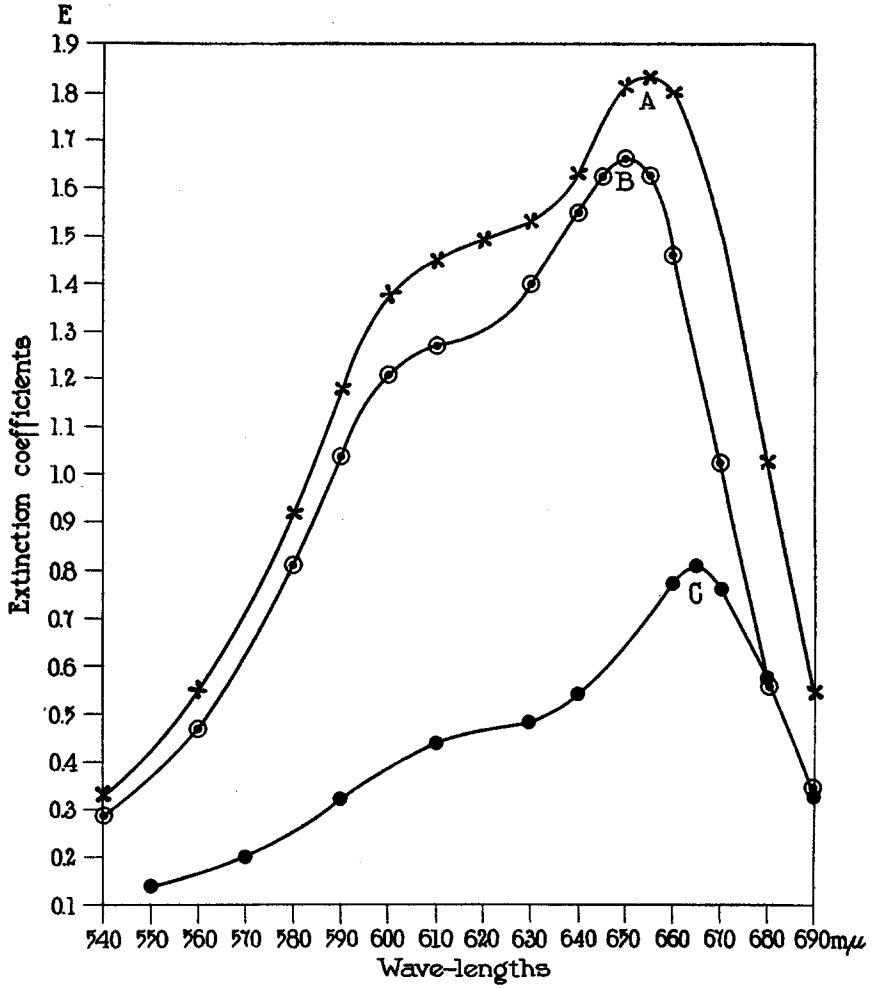

FIG. 4. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves *A* and *B* represent the dye which was absorbed by chloroform from methylene blue dissolved in sea water, Curve *A* at pH 5.5 and Curve *B* at pH 9. In both cases the dye was freed from chloroform by its subsequent absorption in distilled water. Curve *C* represents methylene blue dissolved in distilled water.

both take up the dye in the form of free base much more readily than in the form of salt, it seemed possible that chloroform, like *Valonia*, takes up chiefly trimethyl thionine or azure B from a solution of methylene blue, which may be determined by means of spectrophotometric analysis and by the determination of the partition coefficient of the dye between chloroform and water.

Heretofore no spectrophotometric analysis of the dye absorbed by chloroform from methylene blue has been made. This was accordingly done in the case of the dye absorbed by chloroform from a solution of methylene blue (made up in sea water). From chloroform thus stained, dye was freed by subsequent absorption in distilled water. When the sea water was at pH 9.5, the dye absorbed by chloroform gave the absorption maximum 650 mμ, characteristic of azure B (Table I, and Fig. 4, Curve *B*), and at pH 5.5 an absorption maximum of 655 mμ (which showed that there was a small amount of methylene blue in addition to azure B, Table I, and Fig. 4, Curve *A*). The methylene blue dissolved in distilled water gave an absorption maximum of 665 mμ (Table I, Fig. 4, Curve *C*).

The dye absorbed by chloroform from aqueous methylene blue solution (made up with M/150 buffer mixtures) was found to be azure B, both at pH 5.5 and at pH 9.5 (Table I).

These analyses show that in the presence of sea water at pH 5.5 azure B and a small amount of methylene blue are absorbed by chloro-

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entered the vacuole. Relative rate of penetration differed with various basic dyes. Such differences in the rates corresponded roughly with the differences in the degree of absorption of these dyes by chloroform at different pH values of the sea water, and in the basicity of the dyes. When the amount of brilliant cresyl blue absorbed by chloroform or by *Valonia* is plotted against the external pH values, an S-shaped curve is obtained in both cases.

In view of the fact that in presence of sea water a basic dye in form of salt, as well as in form of free base, enters the chloroform, it is difficult to obtain with any accuracy the distribution coefficient of the dye only in form of free base between chloroform and sea water. This complication, however, was absent in the case of the basic dyes dissolved in M/150 buffer solutions, so that it was possible to make a comparison on a quantitative basis between the absorption of dye in form of free base by the vacuole of *Nitella* and by chloroform (Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561).

form, while from sea water at pH 9.5 and from dilute buffer solutions at both pH values chiefly azure B is absorbed.

As to the experiments on the distribution of the dye between methylene blue dissolved in sea water and chloroform the following results were obtained.

When 3 cc. of chloroform were shaken up with 10 cc. of 0.002 per cent methylene blue dissolved in sea water at pH 9.5 and pH 5.5, it was found that the apparent partition coefficient of the dye between chloroform and sea water at pH 9.5 was about 2 (*i.e.* more concentrated in chloroform), while at pH 5.5 it was about 1.3. These might be called the apparent partition coefficients, since they merely represent the ratio of the concentration of the dye in the chloroform to that of the dye in sea water, without taking into account whether the dye is azure B, methylene blue, or a mixture of both.

The color of the dye in the chloroform when absorbed from methylene blue in sea water at pH 9.5 is reddish purple and from methylene blue in sea water at pH 5.5 is blue. Since the dry salt of methylene blue and that of azure B dissolved in chloroform is blue, the dye in the chloroform absorbed from methylene blue in sea water at pH 5.5 may possibly represent a mixture of methylene blue and azure B, in form of salt, while the dye in chloroform absorbed from methylene blue in sea water at pH 9.5 is azure B chiefly in form of free base. These experiments are insufficient to show whether or not these dyes in form of salt enter chloroform as undissociated molecules which may possibly be formed to a certain extent in presence of so high a concentration of salt such as sodium chloride in the sea water.

Just as in the case of the vacuole of uninjured cells of *Valonia* the dye which is taken up by chloroform from methylene blue in sea water at pH 9.5 is chiefly azure B in form of free base and not methylene blue.

Whether methylene blue is capable of penetrating the vacuole from methylene blue in sea water at pH 5.5 cannot be determined, since the dye does not penetrate in sufficient quantity for spectrophotometric analysis. Since the concentration of undissociated molecules of methylene blue in form of salt possibly present in sea water is not determined, this result neither proves nor disproves the theory that the undissociated molecules enter the vacuole of uninjured cells more rapidly than the ions.

It is not certain as to whether methylene blue enters the chloroform in constant amount at all pH values or only at lower pH values. Further experiments are necessary to determine this point.

V.

Penetration of Azure B into the Vacuole of Valonia and into Chloroform.

If our supposition is correct that azure B is a weaker base than methylene blue, and capable of existing in form of free base at higher pH values, then, according to the theory presented in Section I, a pure sample of azure B should penetrate into the vacuole of *Valonia* and into chloroform more when the pH value of the sea water is higher.

Living cells of *Valonia* were therefore placed for $\frac{1}{2}$ hour in 0.04 per cent azure B (1) made by Holmes, (2) extracted by chloroform from methylene blue solution (made up with borate buffer at pH 9.5). With both samples approximately the same results were obtained, in that at pH 9.5 the rate of penetration was much higher (about 0.001 per cent dye in sap) than at pH 5.5 (dye in sap was too dilute for accurate determination).

When 3 cc. of chloroform were shaken up with 10 cc. of the azure B dissolved in sea water at pH 9.5 and at pH 5.5, the partition coefficient of the azure B (made by Holmes) between chloroform and sea water at pH 9.5 was 14.9 and at pH 5.5 was 1.8 (*i.e.* the dye was more soluble in chloroform than in sea water).

The color of the dye in chloroform when sea water was at pH 9.5 was reddish purple, while it was blue at pH 5.5.

Since the dry salt of azure B dissolved in chloroform appears blue, the azure B taken up by chloroform from sea water at pH 5.5 may be in form of salt, and at pH 9.5 in form of free base (which is reddish purple).

VI.

DISCUSSION.

From these results we may conclude that the vacuole of uninjured cells of *Valonia macrophysa* takes up chiefly trimethyl thionine (azure B) from the solution of methylene blue which contains so little azure B (as impurity) that it cannot be detected by the spectro-

photometer. As soon as cells are injured methylene blue enters. The writer's results and conclusion are contrary to those obtained by M. M. Brooks,[19] who states that the vacuole of uninjured cells of *Valonia macrophysa* takes up dye from methylene blue solution with the same speed at all pH values (from pH 5 to 9), from which she concludes (without analysis of the dye in the vacuole) that methylene blue (in form of salt) enters the vacuole of uninjured cells.

The writer's experiments show that the penetration of dye into the vacuole of uninjured cells from a solution of methylene blue does not discredit the theory that the basic dye enters the vacuole chiefly in the form of free base, since the dye which penetrates is found to be chiefly a lower homologue of methylene blue, azure B, which is less basic and capable of existing in part in the form of free base at higher pH values. Azure B behaves like all other basic dyes in that its relative rate of penetration depends on the amount of dye in form of free base present, which corresponds with the pH value of the external solution (the higher the pH value the more dye is in form of free base and the more rapid is the rate of penetration). That this difference in the rate of penetration at varying pH values is not due primarily to the effect of different pH values on the protoplasm is shown by the fact that the relative rates of penetration at a given series of pH values differ with different basic dyes.

The writer's previous statement that the vacuole of living cells, such as that of *Valonia*, behaves very much like chloroform toward basic dyes, in that they both take up the dye in the form of free base, is still further supported by the fact that they both take up primarily azure B from methylene blue solution at higher pH value.

Undoubtedly the penetration of a basic dye depends chiefly on two factors under such experimental conditions, (1) on the apparent dissociation constant of the dye, (2) on the partition coefficient of the dye between the vacuolar sap and the external solution, and in some cases on that of the dye between the vacuolar sap and the protoplasm. In case there is a combination of dye with some constituent of the sap, this factor must be brought into consideration. With chloroform also penetration depends on the dissociation constant and the partition coefficient.

[19] Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360.

There is a similarity between chloroform and the vacuole of uninjured cells of *Valonia* in that they are both capable of taking up azure B and some other basic dyes in form of free base, but in certain cases chloroform and *Valonia* are found not to behave alike. For example, some acid dyes are slightly soluble in chloroform but they do not penetrate the vacuole of uninjured cells of *Valonia*. However such an analogy is not complete since the ability of the dye to collect in the vacuole may not only depend on the ability of the protoplasmic layer (between protoplasm and external solution, or between protoplasm and the vacuole) to absorb the dye but also on its power to give up the dye. Experiments are being done with this consideration in view.

The fact that azure B instead of methylene blue is found in the vacuole is not proof that methylene blue does not enter the protoplasm. It might enter the protoplasm though it does not penetrate into the vacuole. One way to arrive at a definite conclusion is to determine the nature of the dye inside the protoplasm, after the dye has been allowed to penetrate the cell in uninjured condition; but with the protoplasmic layer of *Valonia* this cannot be accomplished since it cannot be removed for examination without contamination or injury. Furthermore there is no way of determining whether or not methylene blue enters protoplasm and is converted to azure B or trimethyl thionine.

These experiments show the danger of drawing conclusions as to permeability or as to oxidation-reduction potentials from the experiments on the penetration of dye from a solution of methylene blue into living cells, unless we know the nature of the dye both in the external solution and inside the cells.

These experiments were repeated with *Nitella* and gave approximately the same results.

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

SUMMARY.

When uninjured cells of *Valonia* are placed in methylene blue dissolved in sea water it is found, after 1 to 3 hours, that at pH 5.5

practically no dye penetrates, while at pH 9.5 more enters the vacuole. As the cells become injured more dye enters at pH 5.5, as well as at pH 9.5.

No dye in reduced form is found in the sap of uninjured cells exposed from 1 to 3 hours to methylene blue in sea water at both pH values.

When uninjured cells are placed in azure B solution, the rate of penetration of dye into the vacuole is found to increase with the rise in the pH value of the external dye solution.

The partition coefficient of the dye between chloroform and sea water is higher at pH 9.5 than at pH 5.5 with both methylene blue and azure B. The color of the dye in chloroform absorbed from methylene blue or from azure B in sea water at pH 5.5 is blue, while it is reddish purple when absorbed from methylene blue and azure B at pH 9.5. Dry salt of methylene blue and azure B dissolved in chloroform appears blue.

It is shown that chiefly azure B in form of free base is absorbed by chloroform from methylene blue or azure B dissolved in sea water at pH 9.5, but possibly a mixture of methylene blue and azure B in form of salt is absorbed from methylene blue at pH 5.5, and azure B in form of salt is absorbed from azure B in sea water at pH 5.5.

Spectrophotometric analysis of the dye shows the following facts.

1. The dye which is absorbed by the cell wall from methylene blue solution is found to be chiefly methylene blue.

2. The dye which has penetrated from methylene blue solution into the vacuole of uninjured cells is found to be azure B or trimethyl thionine, a small amount of which may be present in a solution of methylene blue especially at a high pH value.

3. The dye which has penetrated from methylene blue solution into the vacuole of injured cells is either methylene blue or a mixture of methylene blue and azure B.

4. The dye which is absorbed by chloroform from methylene blue dissolved in sea water is also found to be azure B, when the pH value of the sea water is at 9.5, but it consists of azure B and to a less extent of methylene blue when the pH value is at 5.5.

5. Methylene blue employed for these experiments, when dissolved

in sea water, in sap of *Valonia*, or in artificial sap, gives absorption maxima characteristic of methylene blue.

Azure B found in the sap collected from the vacuole cannot be due to the transformation of methylene blue into this dye after methylene blue has penetrated into the vacuole from the external solution because no such transformation detectable by this method is found to take place within 3 hours after dissolving methylene blue in the sap of *Valonia*.

These experiments indicate that the penetration of dye into the vacuole from methylene blue solution represents a diffusion of azure B in the form of free base. This result agrees with the theory that a basic dye penetrates the vacuole of living cells chiefly in the form of free base and only very slightly in the form of salt. But as soon as the cells are injured the methylene blue (in form of salt) enters the vacuole.

It is suggested that these experiments do not show that methylene blue does not enter the protoplasm, but they point out the danger of basing any theoretical conclusion as to permeability on oxidation-reduction potential of living cells from experiments made or the penetration of dye from methylene blue solution into the vacuole, without determining the nature of the dye inside and outside the cell.