SPECTROPHOTOMETRIC STUDIES OF PENETRATION.

V. RESEMBLANCES BETWEEN THE LIVING CELL AND AN ARTIFICIAL SYSTEM IN ABSORBING METHYLENE BLUE AND TRIMETHYL THIONINE.

BY MARIAN IRWIN.

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

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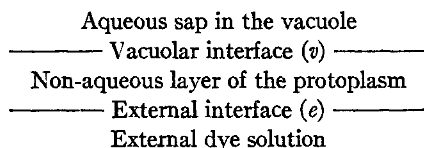
I.

INTRODUCTION.

This paper deals with an attempt to imitate the action of the living cell in respect to penetration of dyes.

The protoplasm of a living cell probably consists of two non-aqueous layers[1] (one in contact with the external medium and the other with the vacuolar sap), which are separated by an aqueous middle layer. But in setting up an artificial system we may confine ourselves to the simple case where the rate of diffusion appears to be controlled by three phases, as if a living cell consisted of only one non-aqueous layer lying between the external solution and the aqueous sap of the vacuole, as shown by the following diagram:



[1] We are unable to say which layer plays the more important rôle in regulating the rate of penetration into the vacuole. The controlling factor may change with alterations in the condition of the cells brought about by experiments. It seems possible that the external non-aqueous layer is more polar than the vacuolar layer, in that the ions penetrate the former more rapidly than the latter. In the case cited in the text the vacuolar non-aqueous layer may be responsible for the control of the rate. But in any case it seems probable that the net result may be treated as if the rate were controlled by a system containing only one non-aqueous layer.

In such a system the diffusion of a dye will be greatly affected by the partition coefficients:

$$K_e = \frac{\text{Concentration of dye in the non-aqueous layer}}{\text{Concentration of dye in the external solution}}$$

$$K_v = \frac{\text{Concentration of dye in the non-aqueous layer}}{\text{Concentration of dye in the aqueous sap}}$$

The higher K_e is, the more rapid is the rate of diffusion from the external solution through non-aqueous layer. On the other hand, the lower K_v is, the more rapid is the rate of diffusion through the non-aqueous layer. The values of K_e and K_v and concentrations of dye in the aqueous solutions will determine the diffusion gradient in the non-aqueous layer.

In the case of methylene blue, diffusion into the vacuole from the external solution is very slow because K_e is so small that though K_v is low there is very little dye in the non-aqueous layer[2] to diffuse into the sap. When a dye exists in two forms, with free base predominating at higher pH values and salt predominating at lower pH values, it is necessary to consider the partition coefficient of each form of dye at each interface. In the case of azure B the partition coefficient of the dye in the form of free base,[3] K_{eb}, is so high that its diffusion through

[2] According to Overton's theory penetration depends on the solubility of the dye in the lipoid layer of the living cell, which involves only one partition coefficient, K_e. This is sufficient to account for the lack of penetration of dyes which are insoluble in lipoid and for the rapid penetration of some of the dyes soluble in lipoid, but does not explain why a dye like crystal violet, which is very soluble in lipoid, does not readily penetrate the cell. On the basis of the theory presented in the text the slow penetration of crystal violet into the cell is explainable; though the dye penetrates the non-aqueous layer it does not enter the vacuole rapidly on account of K_v being so high.

[3] Though the dye in form of free base is often considered to be undissociated, this is an open question. The free base may be dissociated just as much as the dye in form of salt. We must therefore leave the question of the extent of dissociation of the "free base" for future solution by organic chemists, while we may conclude with more certainty that the "salt" is dissociated. This "free base" must be distinguished from the "pseudo base" which exists at still higher pH values. "Free base" may possibly be represented by an anhydro-base, or by a structure like the salt except that the halide is replaced by the hydroxyl group. (Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 927 and 928, foot-note 3.)

non-aqueous layer from the external solution is rapid. But even if K_{vb} (the partition coefficient of the same form of dye at the vacuolar interface) is high, the dye readily diffuses through the non-aqueous layer into the vacuole because it is transformed to salt as soon as it comes in contact with the sap (on account of the low pH value of the sap) and this greatly increases the diffusion gradient. Since the partition coefficient of the dye in the form of salt at the vacuolar interface, K_{vs}, is low, its backward diffusion from the vacuole through non-aqueous layer is very slow. The dye salt readily collects in the vacuole. The same type of behavior occurs if a dye enters into combination with the sap to form a very slightly soluble compound.

This view would explain why azure B penetrates more rapidly than methylene blue into the vacuole of a living cell, as described in previous papers,[4-6] and would lead us to predict that the same thing would happen with an artificial system consisting of (1) the same dye solutions as those employed for the living cells, (2) chloroform to represent the non-aqueous layer of the protoplasm, and (3) sap freshly extracted from the vacuoles of living cells or artificial sap. Since the non-aqueous part of the protoplasm is but crudely represented by chloroform, the system may bear only a qualitative resemblance to the living cell.

If this theory[7] be correct we shall expect that when mixtures of dyes are employed the order of penetration will be the same in the artificial system as in the living cell.

II.

Methylene Blue.

A. Comparison of the Artificial System with Valonia.

Previous analyses[5,6] have shown that the vacuole of *Valonia* takes up azure B from a solution of methylene blue in sea water at pH 9.5. In order to compare this with the artificial system two types of

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

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

[6] Irwin, M., *J. Gen. Physiol.*, 1928–29, xii, 147.

[7] This theory has been successfully tested in the case of many dyes (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 127; 1928, xxvi, 125).

experiments were made. (1) One experiment is to show the absorptive power of the chloroform in relation to the aqueous dye solution (representing the external dye solution used for living cells). This is done by first shaking the chloroform with the aqueous dye solution, allowing the chloroform to evaporate, and then dissolving the dye residue in the sap or in distilled water. The dye thus obtained is called for convenience "the dye obtained from chloroform by evaporation."

(2) The second experiment is to determine how readily the chloroform is able to give up the dye to the sap. This is done by first shaking the chloroform with the aqueous dye solution and subsequently extracting the dye from the chloroform by shaking it up with the sap.

If chloroform adequately represents the non-aqueous layer of a living cell we may by studying the behavior of the chloroform[8] toward the dye in these two aqueous phases learn something of the behavior of the non-aqueous layer of the cell in relation to phases inside and outside the cell.

The experiments were therefore carried out as follows.

200 cc. of solution of methylene blue (French) dissolved in sea water at pH 9.5 or at pH 5.5 was shaken up with 50 cc. of pure chloroform in a separatory funnel; the chloroform was then removed from the funnel and if it contained even a trace of water in the form of droplets the latter was removed by decanting the chloroform repeatedly from one beaker to another: the drops of water then adhered to the wall so that eventually chloroform free from drops of water could be obtained by this method. The chloroform was then divided into two equal parts: one portion was allowed to evaporate and the dye residue was dissolved in freshly extracted sap of *Valonia* (the sap contained about 0.6 M halides and had a pH value of about 5.8) or in distilled water. The second portion was shaken with sap until a concentration sufficient for spectrophotometric analysis was obtained. The sap was separated from the chloroform by pipetting it off (there was not sufficient chloroform in the sap to affect the absorption spectra of the dye in the sap).

The sample of methylene blue (French) gave an absorption curve with primary absorption maximum at 664 $m\mu$ when dissolved in sea water at pH 9.5 or pH 5.5 (Fig. 1, symbol □), which is characteristic of a dye solution consisting chiefly of methylene blue.

[8] Though the non-aqueous layer is represented by the chloroform, it does not signify that it resembles chloroform in chemical composition. It is uncertain as to whether the non-aqueous layers are "lipoid," but their behavior suggests it. The question of the character of the non-aqueous layers must therefore be left undecided for the present.

With this sample of methylene blue in sea water at pH 9.5, the dye (1) obtained from chloroform by evaporation, and (2) extracted from the chloroform by shaking with sap, gave an absorption curve characteristic of a dye consisting chiefly of azure B (with primary absorption

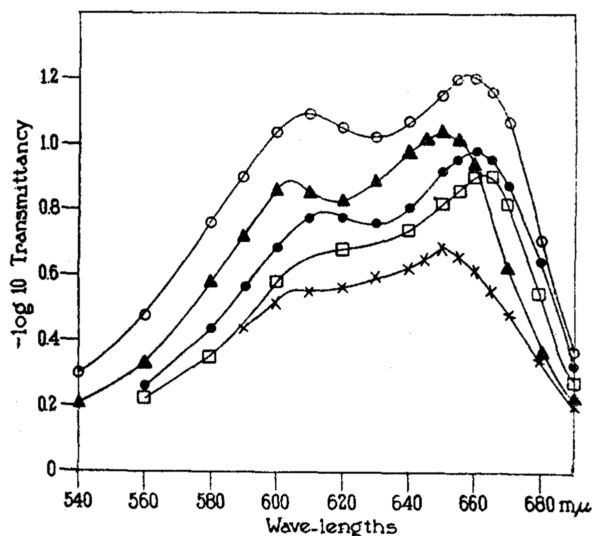


Fig. 1. Absorption curves obtained in experiments on methylene blue (French). (Comparison with *Valonia*.) Symbol □ represents the methylene blue dissolved in sea water at pH 5.5 or pH 9.5. Symbol ▲ represents the dye taken up by chloroform from methylene blue in sea water at pH 9.5, then freed from chloroform by evaporation, and finally dissolved in distilled water. Symbol × represents the dye extracted by sap of *Valonia* from chloroform which had previously been shaken with methylene blue in sea water at pH 9.5. Symbol ○ represents the dye taken up by chloroform from methylene blue in sea water at pH 5.5, then freed from chloroform by evaporation, and finally dissolved in distilled water. Symbol ● represents the dye extracted by the sap of *Valonia* from chloroform which had previously been shaken with methylene blue in sea water at pH 5.5. The measurements were made on a layer 1 cm. thick.

maximum at 650 mμ; Fig. 1, symbols ▲ and ×), which readily enters the chloroform because at pH 9.5 it is largely in the form of free base; it readily comes out into sap because the acidity of the sap changes it to dye salt which has a low value of K_v. The reddish violet of the dye in chloroform may indicate the absorption chiefly of azure B in the

form of free base and a smaller amount of azure B in form of salt and methylene blue. With the solution at pH 5.5 the dye obtained from chloroform by evaporation gave a primary absorption maximum at 658 mμ (Fig. 1, symbol ○) and the dye extracted by the sap from the chloroform gave a primary absorption maximum at 660 mμ (Fig. 1, symbol ●), indicating that in both cases there was a mixture of azure B and methylene blue (the former containing slightly less methylene blue than the latter). The color of the dye in chloroform was blue, thus showing that if azure B was absorbed it was in form of salt. The amount of azure B in form of salt and of methylene blue absorbed is less than in the case of azure B in form of free base.

The result obtained at pH 9.5 is in complete agreement with that obtained with living cells of *Valonia* (uninjured). Unfortunately it is not possible to compare the result with that of the living cell in solutions at pH 5.5 since in the latter case penetration is so slow that analysis is not possible at present. But if the present theory be correct we might expect a mixture of these two dyes at pH 5.5 to penetrate the cell but only more slowly than azure B from the solution at pH 9.5.

B. Comparison of Nitella flexilis with the Artificial System.

Previous experiments[9] have shown that from methylene blue solution (French or Merck's medicinal) at pH 9.2 azure B is readily taken up by the vacuole of living cells of *Nitella*. For comparison with the behavior of an artificial system the following experiments were carried out.

The sample of methylene blue (Merck's medicinal) dissolved in buffer solution at pH 9.2 or pH 5.5 gave an absorption curve characteristic of methylene blue (Fig. 2, symbol ■).

The same technique as described under *A* was employed (1) for absorption of dye by chloroform from this sample of methylene blue dissolved in diluted buffer solution at pH 9.2 and pH 5.5, and (2) for the extraction of dye from the chloroform by the artificial sap of *Nitella*. It was not possible to use the real sap

[9] In 1926–27 preliminary statements of these results on *Nitella* were made on page 426 of the paper referred to in foot-note 4, and on page 945 of the paper referred to in foot-note 5. In 1928 these results in detail were reported in the paper referred to in foot-note 6, and in *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 563.

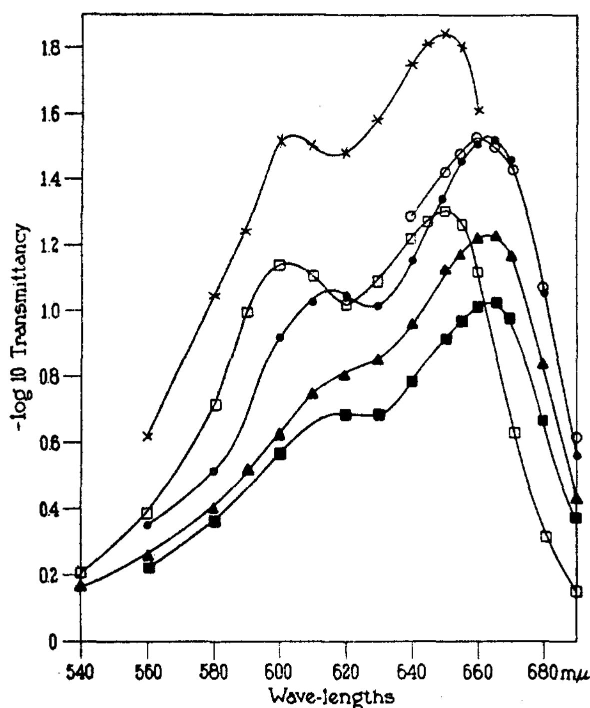


FIG. 2. Absorption curves obtained in experiments on methylene blue solution (Merck's medicinal). (Comparison with *Nitella*.) Symbol ■ represents methylene blue solution at pH 9.2 or at pH 5.5. Symbol □ represents the dye absorbed by chloroform from methylene blue solution at pH 9.2, freed from chloroform by evaporation, and then dissolved in artificial sap of *Nitella*. Symbol × represents the dye extracted by artificial sap of *Nitella* from chloroform which has absorbed the dye from methylene blue solution at pH 9.2. Symbol ○ represents the dye absorbed by chloroform from methylene blue solution at pH 5.5. The chloroform was allowed to evaporate and the dye residue was dissolved in artificial sap of *Nitella*. Symbol ● represents the dye extracted by artificial sap of *Nitella* from chloroform previously shaken with methylene blue solution at pH 5.5. Symbol ▲ represents the dye extracted with distilled water at pH 5.5 from chloroform previously shaken with methylene blue solution at pH 5.5. The curves with the symbols ●, ■, × refer to measurements made with a layer 1 cm. thick, the others to measurements made with a layer 0.3 cm. thick.

of *Nitella* because the protein coagulates on shaking. The artificial sap was therefore made up with 0.11 M KCl dissolved in a solution of M/150 phosphate buffer at pH 5.5. All measurements described in the text were made immediately after extractions.

With the solution at pH 9.2 the dye obtained from chloroform by evaporation or by extraction with the sap gave an absorption curve characteristic of a dye containing chiefly azure B with a primary ab-

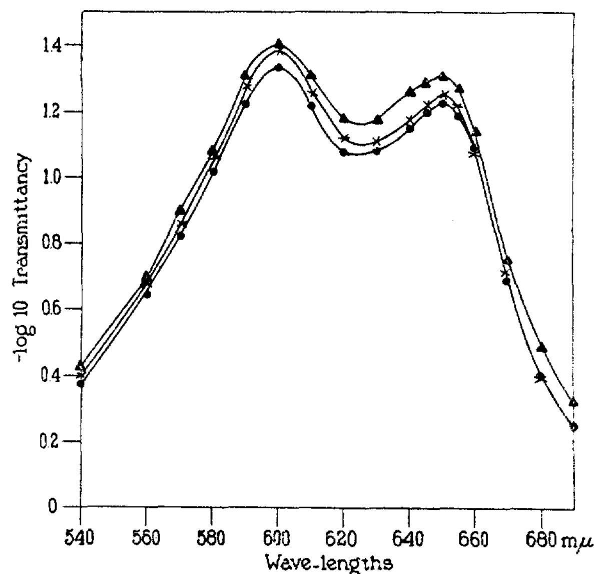


FIG. 3. Absorption curves obtained in experiments on azure B or trimethyl thionine (Holmes) in sea water. (Comparison with *Valonia.*) Symbol × represents the azure B dissolved in sea water at pH 9.5 or pH 5.5. Symbol ● represents the dye extracted by sap of *Valonia* from chloroform shaken with azure B in sea water at pH 9.5; symbol ▲ the same at pH 5.5. The measurements were made on a layer 0.6 cm. thick.

sorption maximum at 650 mμ (Fig. 2, symbol □ and ×). The color of the dye in chloroform was violet red thus indicating that azure B in the form of free base was absorbed. But with the solution at pH 5.5 the dye obtained from chloroform by evaporation gave a primary absorption maximum at 660 mμ (Fig. 2, symbol ○); the dye extracted by the sap from the chloroform gave a primary absorption maximum at 663 mμ (Fig. 2, symbol ●), indicating that a mixture containing chiefly

methylene blue with some azure B was absorbed by the chloroform, but mostly methylene blue was extracted from the chloroform by the sap. The color of the dye in chloroform was blue, thus showing that some azure B in form of salt or methylene blue was absorbed.

When the experiments were repeated at pH 9.2 with methylene blue (French) the same result was obtained as with Merck's medicinal.

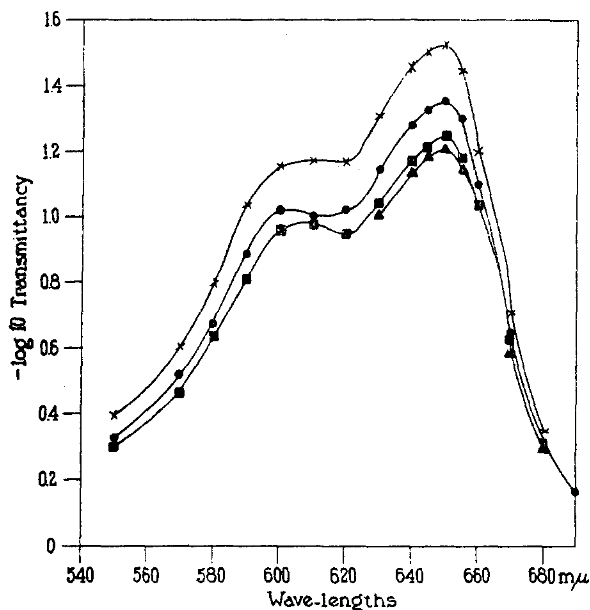


FIG. 4. Absorption curves obtained from experiments on azure B (Holmes) dissolved in buffer solutions. (Comparison with *Nitella*.) Symbol ■ represents the dye dissolved in buffer solution at pH 9.2 or at pH 5.5. Symbol × represents the dye absorbed by chloroform from azure B solution at pH 9.2. The dye was freed from chloroform by evaporation and dissolved in distilled water. Symbol ● represents the dye extracted by artificial sap of *Nitella* from chloroform shaken with azure B solution at pH 9.2; symbol ▲ the same at pH 5.5. The measurements were made on a layer 0.6 cm. thick.

The results obtained with methylene blue at a pH value a little above 9 were in general agreement with the experiments on living cells (uninjured). At pH 5.5, the comparison is not possible since the penetration is so slow that there is not sufficient dye collected for analysis in the sap before there was a possibility of injury to the cell.

III.

Azure B or Trimethyl Thionine.

Using the method described in section II experiments were repeated with azure B for comparison of the artificial system with *Valonia* (Fig. 3) and with *Nitella* (Fig. 4). In both cases the dye absorbed by chloroform and set free by evaporation or extracted by the sap from chloroform gave the absorption curve of a dye consisting chiefly of azure B with a primary absorption maximum at 650 mμ (Fig. 3, symbols ● and ▲ and Fig. 4, symbols ×, ●, and ▲), which is identical with the dye solutions in which chloroform was shaken up (Fig. 3, symbol × and Fig. 4, symbol ■). These results are in exact agreement with those obtained with uninjured cells of *Valonia* and of *Nitella*.

IV.

CONCLUSION.

The rate of diffusion[10] through the non-aqueous layer of the protoplasm depends largely on the partition coefficients mentioned above. Since these cannot be determined we have employed an artificial system in which chloroform is used in place of the non-aqueous layer of the protoplasm. The partition coefficients may be roughly determined by shaking up the aqueous solutions with chloroform and analyzing with the spectrophotometer (which is necessary with methylene blue because we are dealing with mixtures). This will show what dyes may be expected to pass through the protoplasm into the vacuole in case it behaves like the artificial system.

From these results we may conclude that the artificial system and the living cell act almost alike toward methylene blue[11] and azure B, which supports the notion of non-aqueous layers in the protoplasm.

There is a close resemblance between *Valonia* and the artificial system in their behavior toward these dyes at pH 9.5.

[10] *Cf.* Northrop, J. H., *J. Gen. Physiol.*, 1928–29, xii, 435.

[11] Methylene blue solution is generally found to contain azure B as impurity but in too small a concentration to affect the primary absorption maximum which is at about 665 mμ. It is, however, possible to determine roughly by extraction with chloroform the relative amount of azure B present in methylene blue solutions

In the case of *Nitella*, on the other hand, with methylene blue solution at pH 9.2 the sap in the artificial system takes up relatively more azure B (absorption maximum at 650 mμ) than the vacuole of the living cell (655 mμ). But both take up azure B much more rapidly than methylene blue.

A comparison cannot be made between the behavior of the artificial system and that of the living cell at pH 5.5 since in the latter case there arises a question of injury to cells before enough dye is collected in the sap for analysis.

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since azure B in form of free base is absorbed by chloroform from methylene blue solution much more rapidly than methylene blue itself. Azure B may therefore be readily extracted from methylene blue solution at about pH 9. The dye thus extracted is freed from chloroform by evaporation and is dissolved in various aqueous solutions. The dye thus extracted contains various proportions of azure B and methylene blue giving primary absorption maxima from 650 mμ (that of the pure azure B) to 662 mμ (nearly that of purest available methylene blue), depending on the amount of azure B in proportion to methylene blue present in the methylene blue solution from which the extraction was made.

Another method of detection is by observing the color of the dye in chloroform since azure B in form of free base appears violet red, while methylene blue appears blue. But this method is inadequate if the extracted dye is a mixture or if the concentration is low where color matching becomes difficult.

At about pH 9 we find that the more azure B a methylene blue solution contains the more rapid is the rate of penetration of the blue dye into the vacuole of a living cell as well as into the artificial "vacuole."

If the penetration is very slow there are possibilities of errors arising from injury and contamination as already stated (Irwin, M., *J. Gen. Physiol.*, 1928–29, xii, 147) so that the sample "French" was chosen, which had a higher rate of penetration than any other samples (it contained more azure B than others). With *Valonia*, even with the sample "French," the rate of penetration was so slow that it could not very well have been lowered without rendering the results doubtful on account of the possibilities of error already stated. For this reason conclusive results were not possible from experiments with other samples which showed a slower rate of penetration. But with *Nitella* the penetration was more rapid with each of the samples employed so that it was possible to employ a greater variety of samples.

The analogy between chloroform and the living cell is only a rough one. The resemblance, for example, exists in so far as both systems (living and artificial) take up azure B much more rapidly than methylene blue but we need not suppose that the dyes absorbed by both systems will give exactly identical absorption curves in every instance.

These resemblances both in *Valonia* and in *Nitella* become less as injury increases on account of the increase in penetration of methylene blue into the vacuoles of injured cells.