STUDIES ON PENETRATION OF DYES WITH GLASS ELECTRODE

V. Why Does Azure B Penetrate More Readily Than Methylene Blue or Crystal Violet?

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I

The results[1] from measurements made with the glass electrode and spectrophotometer show that brilliant cresyl blue penetrates as free base into the vacuoles of living cells of *Nitella*. The present paper describes similar experiments with azure B, methylene blue, and crystal violet.

Since the methods for the condition of cells, extraction of the sap, penetration experiments, extraction of free base of dyes, making up of dye solutions, and measurements by means of the glass electrode have been published[1] in detail, they are omitted here. The dyes were obtained as salts. The azure B (in pure form) was made by W. C. Holmes of the Color Laboratory, Washington, D. C., methylene blue (medicinal) by Merck and Co., crystal violet by Grübler (obtained before 1914). These dyes affected the electrode slightly more than cresyl blue (causing the pH value of the sap containing the dye to increase between 0.1 and 0.2 pH value after the fifth measurement (when the electrode was not washed with acid), but these effects were entirely eliminated by washing the electrode with acid as already described.[1] Just as with cresyl blue, the readings did not vary within 5 minutes after the electrode was brought in contact with any one of these dyes.

[1] Irwin, M., *J. Gen. Physiol.*, 1930–31, **14**, 1.

II

Azure B or Trimethyl Thionin

A. Nitella (A)

The Sap.[1]—When about 0.07 per cent azure B salt is dissolved in the sap *in vitro* the pH value remains practically the same as that of the control (Table I), but when the same concentration of the dye in the form of free[1] base is dissolved in the sap *in vitro* the pH value is found to be about 1 pH above that of the control sap (Table I). Owing to this difference it is possible to find out which form enters the vacuoles of living cells of *Nitella* by determining the pH value of the sap after penetration.

When cells are placed in 0.01 per cent azure B solution at pH 9.2 for about 15 minutes until 0.07 per cent dye has penetrated the vacuole, the pH value of the sap is found to increase about 1 pH above that of the control sap (Table I).

Since azure B which has penetrated the vacuole increases the pH value of the sap to about the same extent as the same concentration of the dye in form of free base added to the sap *in vitro*, while the dye salt dissolved in the sap *in vitro* does not alter the pH value of the sap, it would seem that the azure B penetrates chiefly as free base and not as salt.

But the experimental results supporting this conclusion are not so numerous as with cresyl blue.[1] There is a greater possibility of injury to cells exposed to azure B than to cresyl blue. Since the rate of penetration at pH 9.2 is much slower with azure B than with cresyl blue, it was necessary to use a higher concentration[2] of azure B in order to maintain a comparable length of exposure to both these solutions. Cells were dead in this azure B solution after 3 hours' exposure while these cells in the cresyl blue solution were not dead until after 6 hours' exposure. Since injury hastens the entrance into the vacuole of substances whose penetration under normal conditions is negligible it is

[2] A lower concentration of azure B at pH 9.2 (causing penetration of 0.03 per cent dye in 15 minutes) was employed in which a rise in the pH value of the sap was found to be at about 0.5 pH over that of the control, and in which cells lived somewhat longer than in the solution described in the text. But even in this case, the results were not so conclusive as with cresyl blue.

all the more necessary to ascertain whether the cells were injured to such an extent that alkaline substances other than the azure B penetrated. Unfortunately it was not possible to determine this point so

TABLE I

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

In presence of azure B and methylene blue. Average temperature 22.5°C. Duration of experiments was about 15 minutes with azure B and about 80 minutes with methylene blue (precaution was taken to avoid contamination of the sap). Electrode I was used unless otherwise stated.

	Conc. of dye in sap in per cent	pH of sap	Increase in pH of sap over the control sap	Conc. of dye in sap containing protoplasm in per cent	pH of sap containing protoplasm	Increase in pH of sap containing protoplasm over the control sap containing protoplasm
Nitella A, azure B						
Control	0	5.25		0	6.09	
Dye salt added *in vitro*	0.07	5.27	0.02	0.070	6.14	0.06
Free base of dye added *in vitro*	0.73	6.37	1.10	0.080	7.20	1.06
Penetration from 0.01 per cent dye at pH 9.2	0.075	6.39	1.14	0.070	6.73	0.64
Nitella B, azure B						
Control	0	5.52			6.00	
Penetration from 0.01 per cent dye at pH 9.2	0.08	6.52	1.0	0.080	6.50	0.5
Penetration from 0.01 per cent dye at pH 9.2. Electrode II	0.08	6.45	0.93	0.075	6.43	0.43
Nitella A, methylene blue						
Control	0	5.30		0	6.10	
Dye salt added *in vitro*	0.07	5.28		0.075	6.17	0.07
*Free base added *in vitro*	0.067	6.31	1.01	0.065	6.99	0.89
Penetration from 0.04 per cent dye at pH 9.2	0.07	6.43	1.13	0.073	6.78	0.61

* This free base is azure B extracted from methylene blue solution at pH 9.2.

satisfactorily as with cresyl blue. Owing to the heavy staining of the cell wall when cells were placed in the azure B solution (at all pH values) the dye at once began to diffuse into the vacuoles from the

cell wall when cells were transferred from the dye solution at pH 9.2, for example, into the buffer solution at the same pH value containing no dye so that it was not possible to prove whether or not the pH value of the sap would continue to increase without further penetration of the dye. Furthermore it was not possible to determine the penetration of azure B from tap water in the given period without employing a very high concentration of dye (too high for experiments without danger of causing injury to cells).

Thus the proof of the penetration of the dye in form of free base was less conclusive than with cresyl blue, but it seems probable that the azure B also penetrates as free base.

Sap Mixture[1] (*Sap Containing Protoplasm*).—Addition of 0.07 per cent dye salt or the free base to the mixture of sap and protoplasm produces much the same effect as on sap in that the dye salt brings about practically no alteration in the pH value of the mixture while the free base of the dye increases the pH value about 1 pH over that of the control mixture (Table I). But when the azure B penetrates until 0.07 per cent dye has collected in the vacuole, the increase in the pH value of this mixture is found to be only about 0.6 pH instead of 1 pH as was the case with the sap. Judging from the results obtained with penetration of dye from methylene blue (described in following section) we may conclude that this difference may be due to the production of acid by the protoplasm as a result of penetration of dye, and not to the penetration of azure B salt into the protoplasm. It is not due to the greater buffer action of the protoplasm since the pH value is increased to about the same extent when the azure B free base is added *in vitro* to the sap or to the mixture of the sap and the protoplasm. As the protoplasm consists of only a very thin layer it is not possible to obtain it free from the sap so as to study its behavior.

B. Nitella (*B*)

Approximately the same results were obtained when the experiments were repeated with *Nitella*[1] (*B*) (Table I).

On employing Electrode II, the same results were obtained (Table I).

Spectrophotometric measurements show that the dye which has penetrated the vacuole is like the free base dissolved in the sap *in vitro*, and the external dye solution employed in giving absorption

curves characteristic of azure B with primary absorption maxima at about 650 mμ.

III

Methylene Blue or Tetramethyl Thionin

Nitella (A)

The Sap.[1]—The pH value of the sap after 0.07 per cent dye had penetrated from 0.04 per cent methylene blue solution at pH 9.2 in 80 minutes was found to be about 1 pH higher than the control (Table I), which agreed closely with the rise in the pH value brought about by dissolving the 0.07 per cent free base of azure B in the sap (Table I). The methylene blue chloride when dissolved in the sap brought about practically no change in the pH value of the sap (Table I).

There was a striking similarity in the behavior of the dye absorbed by the vacuole and by the chloroform. When chloroform was shaken up with the methylene blue solution at pH 9.2, the dye appeared red in the chloroform. After evaporation of the chloroform, the dye residue was dissolved in sufficient sap to make the concentration 0.07 per cent. The pH value of this sap was found to be again about 1 pH higher than that of the control sap (Table I).

On determining by means of the spectrophotometric measurements the nature of the dye which has penetrated the vacuole from this methylene blue solution until 0.07 per cent has collected in the sap, the absorption curves showed that the dye consisted more of azure B than methylene blue with primary absorption maxima at 653 to 655 mμ. The dye absorbed by the chloroform was also found to be chiefly azure B with a primary absorption maximum at 650 mμ.

These results therefore confirm those obtained previously by spectrophotometric analysis[3,4] and support the conclusion previously made that the azure B free base present as impurity in methylene blue solution penetrates the vacuole so much more rapidly than methylene blue salt that the dye which collects in the vacuole consists chiefly of azure B and not methylene blue.

[3] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24**, 425; 1927–28, **25**, 563; *J. Gen. Physiol.*, 1928–29, **12**, 147, 407.

[4] Experiments on spectrophotometric measurements were repeated by Brooks, who found methylene blue to penetrate instead of azure B. Brooks, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 290. Protoplasma, 1929, **7**, 46.

As soon as cells are injured, however, the methylene blue penetrates more rapidly so as to shift the absorption curve of the dye in the sap from that of a mixture containing more azure B than methylene blue to that of a mixture containing more methylene blue than azure B. Since in the present instance where there is 0.07 per cent penetration of dye from methylene blue solution at pH 9.2 it is the azure B free base that still penetrates predominantly, the cells thus exposed cannot be injured to the extent of permitting the dye salts to penetrate freely although the cells are dead in the methylene blue solution within three hours' exposure.

Owing to this toxicity of methylene blue solution and to the necessity of longer exposure of these cells to the solution, the results are not so convincing as those obtained on cresyl blue, but the evidence is in favor of the conclusion that predominantly azure B in form of free base penetrates the vacuoles unless cells are injured.

"*Sap Mixture*"[1] (*Sap Containing Protoplasm*).—The same results as in the case of the sap were obtained when the methylene blue or the dye extracted by the chloroform from the methylene blue solution was dissolved in the sap mixture *in vitro* (Table I). But on penetration of the dye from the methylene blue solution at pH 9.2 into the vacuoles the pH value of the sap mixture was raised only 0.6 pH over that of the control sap mixture instead of 1 pH increase, as was the case of the sap. This difference is not due to the greater penetration of methylene blue into the protoplasm than into the sap, because the dye in the sap mixture gives the same absorption curve as the dye in the sap, with a primary absorption maximum at 653 to 655 mμ (characteristic of a mixture of azure B and methylene blue with preponderance of the former). In all probability it is due to the production of acid by the protoplasm as a result of the dye penetration.

IV

Crystal Violet

Nitella (A)

Crystal violet is more toxic than other dyes employed here and the rate of penetration at any pH value into the cells of *Nitella* is not sufficiently rapid to cause an adequate penetration of the dye before there is a danger of injury to cells. In 0.001 per cent dye solution, for ex-

ample, the cells are dead after 1 hour, while the penetration after 15 minutes is too small to show whether the dye increases the pH value. The rate of penetration is approximately the same at pH 9.2 as at pH 5.5. As the dye penetrates, a violet precipitate appears in the sap with very little of the dissolved dye. The penetration of the dye is in all probability mostly due to injury. Crystal violet is a basic dye and it is soluble in chloroform so that according to Overton's theory it should penetrate the cells rather rapidly. Since the penetration is found to be slow, it is of interest to find out more about the behavior of this dye toward the sap of *Nitella in vitro* as a basis for an explanation of why it does not penetrate the vacuole readily.

When 0.07 per cent crystal violet chloride was dissolved in the freshly extracted sap, the pH value was not higher than that of the control sap (about pH 5.3).

The dye was absorbed by chloroform from aqueous solution at pH 5.5 and pH 9.2 in about the same amount. The stained chloroform was freed from the aqueous solution, allowed to evaporate, and the colored residue was dissolved in the sap. This did not raise the pH value of the sap above that of the control sap (whether the dye thus dissolved was obtained by shaking the chloroform with crystal violet solution at pH 5.5 or pH 9.2).

The behavior of crystal violet in sap is therefore different from that of the free base of cresyl blue or of azure B in that with crystal violet there is no difference between the dye salt and the dye absorbed by the chloroform from the solution at pH 9.2, while with cresyl blue or azure B the dye salt does not alter the pH value of the sap while the dye absorbed by the chloroform from the dye solution at pH 9.2 increases it.

Spectrophotometric determination shows that the absorption curves characteristic of crystal violet are obtained with the dye in buffer solutions at pH 9.2 or at pH 5.5, or with the dye which was absorbed by the chloroform from these solutions.

V

Theoretical Considerations

Do these facts help us to understand why azure B enters the vacuoles of living cells of *Nitella* rapidly while methylene blue and crystal violet penetrate very slowly?

The results show that in aqueous solution azure B exists chiefly in two forms, the dye salt and free base, while methylene blue and crystal violet exist chiefly in one form. They also show that azure B penetrates chiefly as free base and not as salt. Since the free base of azure B predominates over the salt at higher pH values, the rate of penetration of the dye increases as the external pH value rises. The increase of free base over salt depends on the "apparent dissociation constant" of the dye.

As soon as the dye in form of free base passes through the protoplasm and comes in contact with the sap in the vacuole, the greater portion of it is converted into the dye salt. The extent of this conversion is dependent on the basicity of the dye, and on the constituents of the sap (such as the hydrogen ion, organic salts, and protein). With *Nitella* sap, the chief factor controlling the conversion is the pH value of the sap. Owing to the low pH value of the sap and to the apparent dissociation constant of azure B, the free base of the dye is converted to the salt to such an extent that only a very small proportion of the dye exists in the sap as free base. At equilibrium the concentration of free base of the azure B in the sap is proportional to that of the free base of the dye in the external solution, so that the higher the concentration of the free base outside, the higher is the concentration of the total dye (free base and salt) in the sap. This explains why at equilibrium the concentration of the total dye in the sap is found to be higher when the pH value of the external solution is raised or the concentration of the dye is increased.

Why is it that the free base of the dye penetrates more rapidly than the dye salt? In these cases the living cells of *Nitella* behave as if the rate of penetration of the dye into the vacuoles is controlled by three phases only, that is, the non-aqueous layer lying between the external aqueous phase and the internal aqueous sap in the vacuole. The rate of penetration is partly controlled by the partition coefficients of the dye at these two phase boundaries. Azure B free base, for example, diffuses into the non-aqueous layer readily because the partition coefficient K_e of this form of dye between the non-aqueous layer and the external solution is high. But no matter how high the K_e is, the dye cannot diffuse into the vacuole unless the partition coefficient K_i of the free base between the non-aqueous layer and the sap is

low or the free base is largely converted by the sap into another form, the K_i of which is low. With azure B, the free base is predominantly converted to the salt, the K_i of which is low, so as to promote a rapid penetration and accumulation of the dye in the sap. The rate of penetration of dye will be greatly reduced by increasing the pH value of the sap and so causing less transformation of free base to the salt.

The exit of the dye may also be accounted for on the same theoretical basis which explains why the rate of exit is hastened with a decrease in the external pH value.

Methylene blue does not penetrate the vacuole readily because it exists as salt even at a high pH value such as pH 9.2, and the partition coefficient K_e of the dye salt is low.

The reason crystal violet does not penetrate the vacuoles readily, though the partition coefficient K_e is high, is because the partition coefficient K_i of the dye is also high. The results indicate that crystal violet exists only in one form in the range of pH values available for living cells of *Nitella* (between pH 5 and pH 9.2), so that there is no such transformation of free base into the salt as shown by azure B. It is uncertain whether the crystal violet exists as salt or as free base. From the structure it appears to be a strongly basic dye, but owing to its solubility in lipoid it may be a very weak base. In case it is a weak base, it is too weak to increase the pH value even of the distilled water.

Owing to the penetration being dependent (at least in part) on more than one partition coefficient, the theory was called the "multiple partition coefficient theory."[5] It is uncertain yet whether this non-aqueous layer is lipoid in nature but its behavior is very much like that of lipoid. This theory is not identical with Overton's even if the non-aqueous layer proves to be a lipoid because Overton considered only one partition coefficient, K_e, between the non-aqueous layer and the external solution. His theory is tenable in so far as those dyes which are not soluble in lipoid do not readily penetrate the vacuoles of these living cells, but it fails to explain why those dyes which are soluble in a lipoid do not readily penetrate. In the case of crystal violet, for example, it becomes explainable only when the second par-

[5] For the theory of multiple partition coefficients see Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927–28, **25**, 127; *J. Gen. Physiol.*, 1927–28, **11**, 111; 1928–29, **12**, 147, 407.

tition coefficient, K_i, is considered. Furthermore, the accumulation of the dye in the vacuole cannot be explained on the basis of his theory.

Such instances show the simplest type of penetration. In more complicated cases we must consider other partition coefficients. In view of the fact that a living cell of *Nitella* consists of a heterogeneous system composed of an outer non-aqueous layer (in contact with the external solution), the middle aqueous layer of the protoplasm, the inner, non-aqueous layer (in contact with the sap in the vacuole), and the aqueous sap, the rate of penetration may be controlled by two or more of the four partition coefficients. The discussion of these cases will be deferred to a later publication.

SUMMARY

Glass electrode measurements of the pH value of the sap of cells of *Nitella* show that azure B in the form of free base penetrates the vacuoles and raises the pH value of the sap to about the same degree as the free base of the dye added to the sap *in vitro*, but the dye salt dissolved in the sap does not alter the pH value of the sap. It is concluded that the dye penetrates the vacuoles chiefly in the form of free base and not as salt.

The dye from methylene blue solution containing azure B free base as impurity penetrates and accumulates in the vacuole. This dye must be azure B in the form of free base, since it raises the pH value of the sap to about the same extent as the free base of azure B dissolved in the sap *in vitro*. The dye absorbed by the chloroform from methylene blue solution behaves like the dye penetrating the vacuole. These results confirm those of spectrophotometric analysis previously published.

Crystal violet exists only in one form between pH 5 and pH 9.2, and does not alter the pH value of the sap at the concentrations used. It does not penetrate readily unless cells are injured.

A theory of "multiple partition coefficients" is described which explains the mechanism of the behavior of living cells to these dyes.

When the protoplasm is squeezed into the sap, the pH value of the mixture is higher than that of the pure sap. The behavior of such a mixture to the dye is very much like that of the sap except that with azure B and methylene blue the rise in the pH value of such a mixture

is not so pronounced as with sap when the dye penetrates into the vacuoles.

Spectrophotometric measurements show that the dye which penetrates from methylene blue solution has a primary absorption maximum at 653 to 655 mμ (i.e., is a mixture of azure B and methylene blue, with preponderance of azure B) whether we take the sap alone or the sap plus protoplasm.

These results confirm those previously obtained with spectrophotometric measurements.