

THE MECHANISM OF VITAL STAINING WITH BASIC DYES.

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That intravital staining with basic dyes occurs more readily in alkaline than in acid solutions has long been known. Among the explanations which have been offered to account for this fact two deserve especial mention. The first is that originally proposed by Overton (1), accepted by Harvey (2), and supported in certain respects by the experiments of Robertson (3) (who, however, on other grounds arrived at a theory diametrically opposed to that of Overton.) According to Overton's theory, cells are freely permeable to the lipoid-soluble dye base but not to the relatively lipoid-insoluble salts of this base with various acids. In acid solutions the dye is present chiefly in the form of salts which enter cells with difficulty, while in alkaline solutions it exists largely in the form of the readily penetrating free base. The difference in staining in the two cases, according to this theory, is due primarily to factors outside of the cell.

The second view, advocated in recent years among others by Bethe (4), Rohde (5), and Irwin (6, 7), depends on the fact that in relatively alkaline solutions proteins combine readily with basic and not with acid dyes and show the reverse behavior in more acid solutions. (In this connection, see also Loeb, (8).) According to this view the state of the dye in the external solution is of less importance than the condition of the proteins within the cell.

If it were possible to vary experimentally and independently of one another the pH inside and outside of a cell it ought to be possible to decide which of these two theories is more in accord with the facts; since if the first is correct, the degree of staining ought to depend largely on the pH of the external solution, while if the second is correct it ought to depend more on that of the cell itself. More specifically, if two cells, one relatively more alkaline internally than the other,

be exposed to two solutions containing the dye at the same pH, then according to the second theory the more alkaline cell should stain more deeply, while according to the first (in its simplest form) the two cells should stain equally.

It is, as a matter of fact, easy to produce, within the range of reactions tolerated by living cells, almost any desired combination of external and internal pH. It is well known that a weak solution of NH_4OH quickly produces intracellular alkalinity while one of NaOH does not. (Bethe (9), Warburg (10), Harvey (3).) Similarly, carbon dioxide produces intracellular acidity far more readily than a mineral acid such as HCl at the same pH (Jacobs (11)). It has also been shown by Jacobs (11, 12) that where carbon dioxide and ammonia are concerned the internal and the external pH may be entirely independent of one another, a cell becoming acid in an alkaline solution and alkaline in an acid solution.

In the following experiments, the pH of the cells and of the surrounding solutions was regulated in this manner by means of NaOH , NH_4OH , CO_2 , and HCl . The dyes employed were neutral red and brilliant cresyl blue, and the living material was: (a) starfish eggs, (b) *Gonionemus* (entire animals), and (c) *Nitella* cells. For the first two types of material the basis of the solutions used was neutralized sea water brought into equilibrium with the air by shaking in a large flask; in the case of *Nitella*, distilled water or boric acid buffer mixture. That the cells were not killed by the treatment they received was shown by the ability of the starfish eggs to undergo cleavage after fertilization, by the movements of locomotion of *Gonionemus*, and by protoplasmic rotation in the case of *Nitella*. Since the results obtained with the three types of cells and with the two dyes agreed in their most essential points it is believed that they represent fairly general conditions.

In general, our results furnish no support for the second theory. If this theory were true, a cell should stain more deeply in a solution of e.g. pH 8.0 produced with NH_4OH , than with NaOH , since in the former case the interior of the cell is made more alkaline while in the latter it is not, or at least is not to the same extent. As a matter of fact, in every case, with all three kinds of material, the staining was less with NH_4OH than with NaOH as will be apparent from the descriptions of several typical experiments.

Starfish eggs were placed in test-tubes which contained neutralized sea water brought to pH 8.0 (determined colorimetrically) with NaOH in one case, and with NH_4OH in the other. In one series of experiments equal amounts of very dilute brilliant cresyl blue were added, and the eggs stained 2 to 8 minutes. Those in NaOH were then found to be more intensely colored than those in NH_4OH . Determination at pH 9.0 gave the same result.

Using neutral red in alkaline solutions, eggs in NaOH were stained red (the acid color), while those in NH_4OH were yellow (alkaline). To compare the amounts of dye taken up by the two lots of eggs, they were very quickly washed and placed in neutralized sea water, and CO_2 was allowed to bubble through, thus producing an acid reaction inside all of the cells. The eggs stained in NaOH were now more deeply red, and evidently had taken up more dye, than those stained in NH_4OH .

The effect of acids on staining was tested as follows: Neutralized sea water was placed in three beakers. The first solution was acidified with HCl; into the second CO_2 was allowed to bubble. Sodium bicarbonate was added to the third, and then into it CO_2 was passed. The three solutions were brought to pH 6.0. With neutral red, starfish eggs stained more intensely in the CO_2 solution (with or without bicarbonate) than in the HCl solution. In some of the experiments a deeper color was obtained in the bicarbonate- CO_2 mixture than in CO_2 alone. The eggs were stained 2 to 7 minutes. Control beakers showed no change in pH at the end of the experiment.

In another series of experiments *Gonionemus* was stained with brilliant cresyl blue for 15 to 30 minutes at pH 8.0. The solutions were prepared as described above and were placed in tall covered glass cylinders. No appreciable alteration in pH occurred in the same time in control cylinders containing animals but no dye. Under these conditions, in the solution containing NaOH the umbrella, manubrium, tentacles, and especially the velum took up the dye freely, while in the NH_4OH solution the staining was very faint. Owing to the constant movements of the animals it was impossible to determine by microscopic examination the exact point of accumulation of the dye, except to be certain that it was in the interior and not on the surface of the body.

Finally, *Nitella* cells 4 to 5 cm. in length were placed in 50 cc. of 0.01 molar buffer solutions which had been brought to a given pH in the one case with NaOH, in the other with NH_4OH . The dye employed was brilliant cresyl blue. After 30 minutes to 26 hours, the ends of several cells were snipped off, the sap was gently expressed and pooled, drawn up into capillary tubes and compared with various concentrations of the dye contained in tubes of the same diameter. This method allows a quantitative determination of the dye in the sap (Irwin (6, 7)).

In thirteen out of fourteen experiments in which quantitative estimates were made, a higher concentration of the dye (in some cases more than ten times as great) was found in the sap vacuoles of the cells stained in NaOH than in those stained in NH_4OH ; in the remaining experiment the concentrations were about equal. In Table I are recorded the results of two typical experiments. It will be noted that while the total amounts of dye taken up in the second case are considerably greater than in the first (due possibly to the fact that the material had been kept for a longer time in the laboratory) the relative concentrations in the NaOH and NH_4OH solutions remain about the same.

TABLE I.

Two typical experiments showing the influence of NaOH and NH_4OH on the pH of the sap of *Nitella*. Cells were stained with brilliant cresyl blue in 0.01 molar solution of boric acid brought to pH 8.0 with either NaOH or NH_4OH . Unstained cells were used for determining the pH of the sap. Time of exposure 1 hr.

Experiment No.	Dye in outside solution.	Dye inside cells in NaOH.	Dye inside cells in NH_4OH .	pH of sap. Cells in NaOH.	pH of sap. Cells in NH_4OH .
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
1	0.0006	0.003	<0.001	5.2	6.6
2	0.0006	0.020	0.006	5.8	6.3

In two experiments *Nitella* cells were placed in the two solutions, and after a certain time some of them were examined and showed, as usual, more intense staining in NaOH. The remaining cells were then transferred from NaOH into NH_4OH , and conversely, from NH_4OH into a NaOH. Subsequent examination showed that the cells transferred to NH_4OH had lost much of their stain, while those transferred to NaOH were now stained more deeply.

It thus appears that the three kinds of organisms employed stain more intensely with certain basic dyes in NaOH than in NH_4OH solutions of the same pH.

That this difference is not due to some specific effect of NH_3 on the partition of dye between fat and water is made probable by the following experiment. Six solutions were prepared of 0.01 molar boric acid

solution and brought to pH 7.1, 7.8, and 8.8 with NaOH in the one case and with NH_4OH in the other. 10 cc. of each solution were shaken with 1 cc. of oil of sweet almonds, using brilliant cresyl blue as the dye. The intensity of staining of the oil was found to increase with increasing pH, but there was no definite difference between tubes containing NaOH and those containing NH_4OH at the same pH.

The experiments so far described, therefore, furnish no support for the second theory. They might also appear to furnish little support for the first theory. If staining depended merely on the proportions of dye-base and dye-salt in the external solution, then, at the same pH, staining should be equally deep regardless of whether alkalinity were produced with NH_4OH or NaOH, or acidity with CO_2 or HCl. This, as has been mentioned, is not the case. With the external pH the same, staining is more intense with NaOH than with NH_4OH . Evidently in the cases mentioned some other factor or factors must produce or at least modify the observed results.

However, certain other observations favor the first theory. For example, when placed in very weak solutions of NaOH or of HCl, starfish eggs undergo no change in reaction for a considerable time, as may be demonstrated by the use of neutral red as an intracellular indicator. Nevertheless, they stain far more rapidly and deeply in the alkaline than in the acid solution. Similarly, in a series of solutions in which the CO_2 tension is the same, the addition of increasing amounts of NaHCO_3 would not, for a time at least, prevent the development of the same degree of intracellular acidity; nevertheless, the presence of bicarbonate in the external solution favors staining as the following experiment shows.

To equal amounts of sea water saturated with CO_2 were added increasing quantities of $\text{M}/2$ NaHCO_3 ; and an equal number of drops of a weak solution of neutral red or brilliant cresyl blue. The pH range obtained was from 5.0 (CO_2 alone) to 6.0. Several specimens of *Gonionemus* were placed in each of the several portions and allowed to remain for 15 to 30 minutes. The intensity of staining was now found to be proportional to the amount of bicarbonate in the solution, *i.e.* it varied directly as the pH of the solution.

It would appear, therefore, that the reaction of the external medium, as opposed to that of the interior of the cell, is a factor of real importance. In certain cases, however, as in experiments cited in the table,

its effects are covered up by a different factor which favors staining when the interior of the cell is less alkaline. As to the nature of this factor, our experiments are not conclusive but it seems probable that it has to do with the retention of the dye rather than with its penetration.

It must constantly be kept in mind that the intensity with which a cell stains is no index of the penetrating power of the dye in question. A dye may penetrate readily and produce little effect, because it finds nothing within the cell with which to combine, and *vice versa*. In the case of basic dyes the accumulation of large amounts of the dye in the cell sap of plant cells has been attributed by Pfeffer (13) to its combination with tannic acid, while Nirenstein (14) has recently shown that the capacity of neutral fats to take up basic dyes is greatly increased by the presence of small amounts of fatty acids. If in the material used in our experiments the *accumulation* of the dye depended on its combination with some acid constituent of the protoplasm, then it is easy to see why the presence of ammonia which penetrates the cell should hinder staining more than that of NaOH which does not, and conversely why, other things being equal, CO₂ should if anything, favor staining as compared with HCl. This is, however, offered merely as a tentative suggestion.

The general result of these experiments is to show that, other things being equal, as demanded by the first theory, alkalinity of the external medium favors, acidity hinders, the staining of cells by basic dyes. On the other hand, alkalinity of the cell itself, in our experiments at least, hinders staining—a result which is directly opposed to the second theory and which neither supports nor opposes the first theory, but which probably indicates the existence of an additional factor concerned in the storage of the dye. This factor may possibly be some substance or substances of acid nature with which the dye forms a salt-like combination.

SUMMARY.

1. Solutions containing NH₄OH and NaOH, and CO₂ and HCl may be used to produce various combinations of extracellular and intracellular reactions in starfish eggs, *Gonionemus*, and *Nitella* cells.

2. Staining by basic dyes is, with a constant intracellular reaction, favored by increased extracellular alkalinity. With a constant extracellular reaction, staining is hindered by increased intracellular alkalinity.

3. These facts are in opposition to the view that staining of cells by basic dyes is chiefly governed by a combination of the dyes with cell proteins. It is more in harmony with the view that the combination is with a substance or substances of acid nature.

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