THE PENETRATION OF DYES AS INFLUENCED BY HYDROGEN ION CONCENTRATION.

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The purpose of this investigation is to analyze the mechanism by which dyes are taken up by the cell under the influence of different concentrations of hydrogen ion.

I.

Methods.

The technique has been described in a previous paper.[1] The plant employed was *Nitella*, whose very long, multinucleate cells contain a large, central vacuole filled with sap which is surrounded by a delicate layer of protoplasm. If the end of the cell is cut off, and gentle pressure applied, the clear sap flows out, free from protoplasm or chlorophyll granules. If the cell has been previously placed in a dye which has penetrated through the layer of protoplasm into the cell the concentration of the dye in the sap may be measured in a very simple manner. A drop of the sap is taken up into a capillary tube and the color is then compared with the colors of other tubes, of the same diameter, containing different concentrations of the same dye.

The dye used is brilliant cresyl blue (from the National Aniline and Chemical Company).[2] As it was not completely soluble in water the stock solution was made up by placing 2 gm. of dye in 2 liters of distilled water, dissolving as much as possible and filtering off the residue. Although the actual concentration was not known, this is

[1] Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 223.

[2] It is often impossible to compare measurements made with the same dye obtained from different manufacturers, since there is apt to be too much variation in the composition.

of no consequence, since in these experiments only relative concentrations were important. This stock solution was called 0.1 per cent and the other percentages mentioned in the paper are to be understood as representing corresponding dilutions of this stock solution.

All the experiments were made at 20 ± 0.1°C. Every determination made for the amount of dye present in the sap, is an average of forty experiments.

To make up buffer solutions, the standard buffer mixtures were diluted to $\frac{m}{100}$ (this did not appreciably change the pH value in any case). From pH 3 to pH 5 phthalates were employed; from pH 5.6 to pH 8, phosphates; from pH 8 to pH 9, borates. Check experiments showed that at the same pH value phthalates had the same effect as phosphates, and phosphates the same as borates, so that their effect on the cells must be due to hydrogen ion concentration, and other differences in chemical composition have little influence.

II.

Absorption of Dye by the Cellulose Wall.

In studying the penetration of a dye into the vacuole of *Nitella*, both the cellulose wall and the protoplasmic layer must be considered, since the dye must diffuse through these two layers before it reaches the vacuole.

The protoplasmic layer is so very thin that it is not possible to determine accurately to what extent it stains with the dye.

At first sight it might seem that the rate of penetration of the dye into the vacuole might depend on the amount of absorption of the dye by the cell wall so that the more the dye is taken up by the cell wall, the less it enters the vacuole. It was necessary, therefore, to carry out experiments to determine whether this is the case.

The results of some of these experiments are shown in Table I. It is evident that at low pH values (pH 5.6) the cellulose wall takes up the stain rapidly, while the sap acquires it very slowly. Between pH 8 and 9 the cell wall stains very slightly, while the sap takes up the stain rapidly. It is therefore evident that if there is any competition for the stain between the sap and the cell wall it would be most effective at the lower pH values. It is evident, however, from

Experiments 1 and 2 that at pH 5.6 the cell wall ceases after a few minutes to take up the stain and the competition must therefore be confined to the first few minutes of the experiment. As all of the experiments at lower pH values (pH 6.6 and 7) were long (lasting for several hours) this competition, if it really exists, is a negligible factor.

TABLE I.

Relation between the Absorption of the Dye by Cellulose Wall and by Cell Sap.

Experiment No.	pH	Time of exposure.	Concentration of dye in outside solution.	Concentration of dye in vacuole.	Color of cellulose wall.
			per cent	*per cent*	
1	5.6	40 min.	0.004	0	Sky-blue.*
2	5.6	24 hrs.	0.004	0.0005	" *
3	6.6	22 "	0.002	0.0017	Medium blue.*
4	7.0	14 "	0.002	0.007	" " *
5	7.7	12 "	0.002	0.06	Pale blue.*
6	5.6	40 min.	0.002	0	" "
7	8.0	50 "	0.002	0.04	No stain.
8	9.0	50 "	0.002	0.096	" "

* Signifies that the color of the cellulose wall was deeper than that of the outside solution.

III.

Penetration of Dye without Injury.

To study the penetration of the dye into the vacuole the cells were placed in an aqueous solution of cresyl blue (0.002 per cent) at pH 6.6 and 7.

At these pH values, the dye was taken up until an equilibrium was reached (Fig. 1 and Table II) beyond which no accumulation of dye occurred. In Fig. 1, the observed values are represented by symbols, and the curves show the values calculated from the equation representing reaction of the first order, $K = \frac{1}{t} \log \frac{a}{a - x}$, in which a = the amount of dye present in the cell sap at the end of the reaction when equilibrium is reached; x = the amount of dye present

in the cell sap at the time t; $a - x$ = the additional amount of dye which must accumulate in the sap before equilibrium is reached, and K = the velocity constant. For pH 6.6, a = 0.0017 per cent, and K = 0.00182, while for pH 7, a = 0.007 per cent, and K = 0.00186 (Table II). It is evident that the observed values agree with the calculated quite closely.

The fact that the process behaves as a reaction of the first order might suggest that it is due merely to diffusion since diffusion experi-

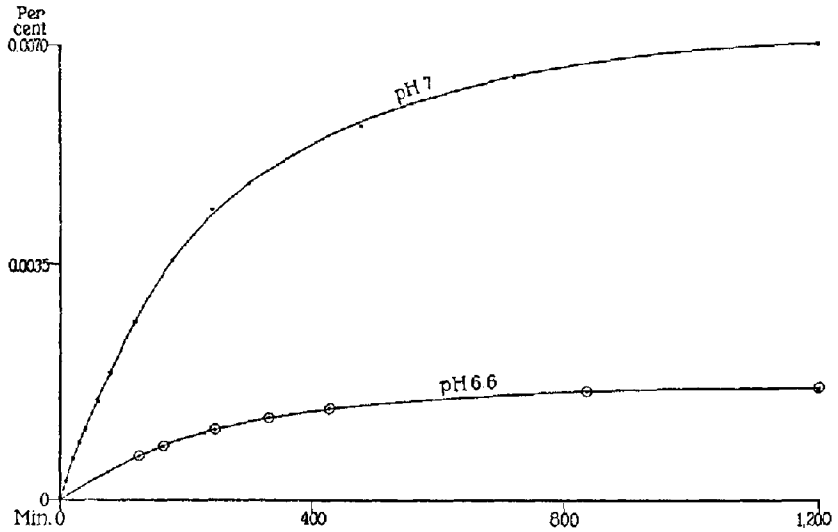

FIG. 1. Curves showing the penetration of 0.002 per cent cresyl blue solution into living cells of *Nitella* at pH 6.6 and pH 7. The concentrations of dye (in per cent) in the cell sap are plotted as the ordinates, and the time in minutes as the abscissæ. The smooth curves show the calculated values, while the symbols represent the observed values.

ments are commonly arranged in such a manner that the process behaves as a reaction of the first order. We find, however, that the temperature coefficient is high (in the neighborhood of 3),[3] which indicates that we are dealing with a chemical reaction which is slower than the process of diffusion and which therefore determines the temperature coefficient.

[3] Results on the temperature coefficient will appear in a later paper.

It is evident that the final equilibrium is higher at pH 7 than at pH 6.6. In order to explain this we may assume that the dye in the vacuole is combined with a protein[4] which acts somewhat like gelatin, as described by Loeb,[5] in that the increase of pH from 6.6 to 7.0 causes an increase in the amount of active or ionized protein which can combine with the dye (and a corresponding decrease of inactive

TABLE II.

Penetration of 0.002 Per Cent Solution of Cresyl Blue into Living Cells of Nitella at 20°C. at pH 6.6 and pH 7.

pH 7 (a = 0.007 per cent)				pH 6.6 (a = 0.0017 per cent)			
t observed.	X	$K = \frac{1}{t} \log \frac{a}{a-x}$	t calculated for K=0.00186	t observed.	X	$K = \frac{1}{t} \log \frac{a}{a-x}$	t calculated for K = 0.00182
min.	*per cent*		*min.*	*min.*	*per cent*		*min.*
10	0.0003	0.00190	10.3	120	0.0007	0.00193	126
20	0.00065	0.00211	22.8	180	0.00086	0.00170	167
30	0.0009	0.00198	32.2	240	0.0011	0.00188	247
40	0.0011	0.00185	40.1	360	0.00128	0.00168	332
60	0.00155	0.00181	58.5	480	0.00142	0.00163	428
80	0.002	0.00182	79.0	720	0.00165	0.00212	835
120	0.0028	0.00184	120.0	1,200	0.0017		
180	0.0038	0.00188	178.0	2,000	0.0017		
240	0.0046	0.00193	250.0	Average.....		0.00182	
300	0.005	0.00182	294.0				
480	0.0059	0.00167	435.0				
720	0.0066	0.00172	690.0				
1,200	0.007						
2,000	0.007						
Average.....		0.00186					

[4] The word protein is used in this paper as a convenient expression to signify either protein or any other amphoteric electrolyte, which acts like protein in combining with dyes. *Cf.* Foot-note 5. It is quite possible that there are several proteins or other amphoteric electrolytes in the cell which combine with the dye.

It might be thought that the dye when combined with the protein would have a different color and that in consequence the apparent concentration would differ from the concentration as measured. It was found, however, that on mixing dye with cell sap the change of color was the same as on mixing with an equal amount of water. It may therefore be assumed that this source of error is negligible.

[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

protein which cannot combine with the dye). This ionized protein must be negatively charged because the dye with which it combines is basic and its colored ion is positively charged.

As the amount of active protein increases, more dye is able to combine. The process may be represented as follows:

$$I \rightarrow A$$

$$A + D = AD$$

where I = inactive protein, A = active protein, and D = dye (cation).

If we assume that the transformation of I to A takes place rapidly (as compared with the second reaction) we need, in our explanation of the time curve, to consider only the latter, $A + D = AD$. Since the amount of D is constant (because it is present in great excess) we may regard the reaction as monomolecular, just as it is in the case of the inversion of cane-sugar in the presence of great excess of water.

The concentration of the dye in the external solution remains constant during the reaction because it is present in great excess. The question arises whether this is also the case inside the cell where the reaction $A + D = AD$ takes place. Since the temperature coefficient of the whole process is high it would appear that the chemical reaction is slower than the process of diffiusion (if the latter were slower we should find a low temperature coefficient). Under these circumstances we may assume that the concentration of D would not diminish as the reaction proceeded, and if it increased the amount of increase could not be very great during the period of the experiment which is employed in our calculations and could in no case go above 0.002 per cent (the concentration in the external solution). It therefore appears that we cannot go far astray in assuming it to be constant.

IV.

Penetration of Dye Accompanied by Injury.

When we apply this explanation to the curves at pH values higher than 7.0 (Fig. 2), we meet with a complication owing to the fact that at these higher pH values some injury occurs after a time (as indicated by the fact that chlorophyll granules appear in the sap when it is squeezed out of the cell). This seems to be due to the

accumulation of the dye in the vacuole and to the effect of the buffer solutions. For example, the death of the cells takes place in about 5 hours, when they are placed in the buffer solution[6] at pH 9, while it takes place in about 4 hours in the solution of dye at pH 9, when the concentration of the dye in the sap reaches about 0.2 per cent, as shown in Fig. 3. As a result of this injury, the cell dies before an equilibrium is reached. If the injury goes far enough the accumulation of the dye stops and the dye begins to diffuse out of the cell very rapidly.

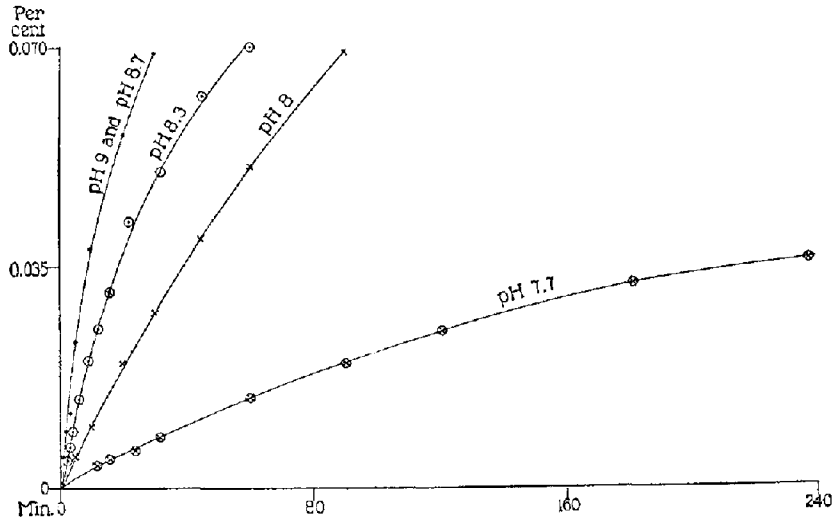

FIG. 2. Curves showing the penetration of 0.002 per cent cresyl blue solution into living cells of *Nitella*, at pH 7.7, 8, 8.3, 8.7, and 9. The concentrations (in per cent) of the dye in the cell sap are plotted as the ordinates, and the time in minutes as the abscissæ.

In dealing with the curves for pH values above 7.0 I have disregarded that part of the curve which is affected by this injurious action. In order to be on the safe side I have disregarded these portions which lie above 0.04 per cent.

If the reaction $A + D = AD$ behaves as one of the first order (since D is practically constant), its velocity constant will remain the same no matter how much the initial concentration of A varies.

[6] The effect of the buffer solutions in the cells will be discussed later in this paper.

It will therefore be the same for all values of pH (if the chemical nature of A does not change with change of pH). We find that this is true for pH 6.6 and 7.0 where the equilibria are known, and we assume that it is true for the higher values where the equilibria cannot be precisely determined (owing to the onset of injury).[7] We therefore assume that the value of K is in all cases 0.00184 (average of 0.00182 and 0.00186) and calculate the equilibria at each pH on

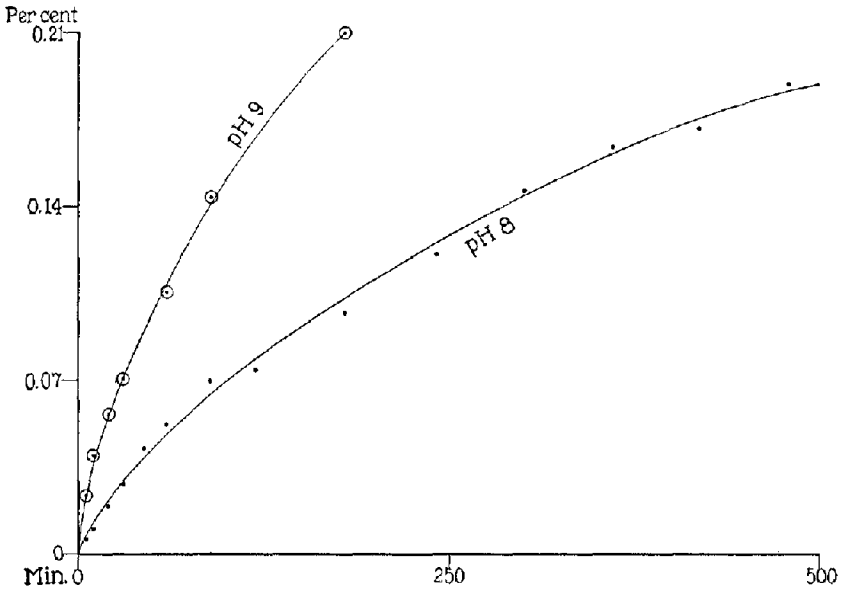

FIG. 3. Curves showing the penetration of 0.002 per cent cresyl blue solution at pH 8 and pH 9, into living cells of *Nitella* until the death of the cells takes place. The concentrations (in per cent) of the dye in the cell sap are plotted as the ordinates, and the time in minutes as the abscissæ.

this basis. When this is done we find that the values of K are approximately constant[8] at 0.00184 throughout each curve.

[7] It might be possible to fit these curves with a formula depending on consecutive reactions, but since injury seems to be involved and we are therefore dealing with variables which are not directly measured, it seems unnecessary to carry out such calculations.

[8] Above 0.04 per cent concentration of the dye in cell sap a decrease in the velocity constants takes place, which is in all probability due primarily to the injury.

On comparing the equilibria obtained at different pH values, it is found that the equilibrium increases with increase[9] in pH, as shown in Fig. 4, Curve E, where the equilibria are plotted as the ordinates, and pH values as the abscissæ.

If it is possible for the chemical nature of the active protein to differ at different pH values, and if this should be true in the present instance, the same velocity constants may not be maintained for

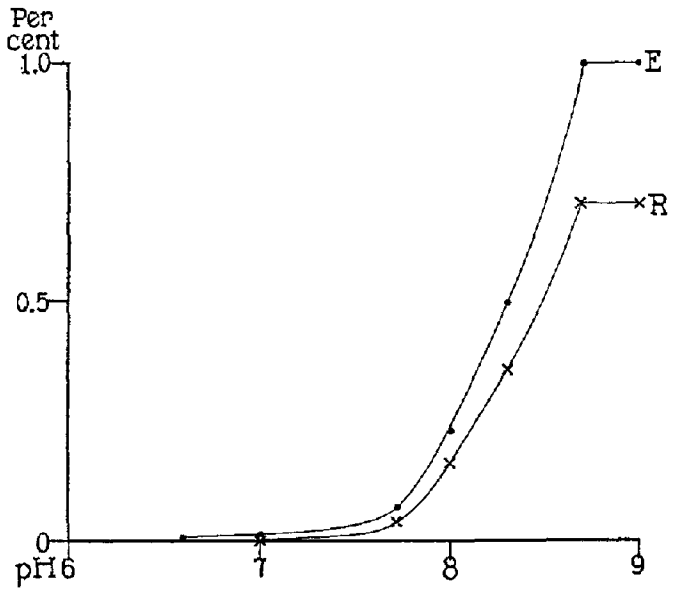

FIG. 4. Curves showing the penetration of 0.002 per cent cresyl blue solution at different pH values into living cells of *Nitella*. In Curve E, the equilibria are plotted as the ordinates, and pH values as abscissæ. In Curve R, the ordinates represent rates, (reciprocals of the times required to bring the concentration of the dye in cell sap to 0.006 per cent).

[9] The rate of penetration of dye was found for each pH by obtaining the reciprocal of the time it took the dye to reach the concentration of 0.006 per cent in the cell sap. This rate increases with the increase in pH as shown in Curve R, Fig. 4.

This seems to be true for all the basic dyes employed. Experiments with acid dyes show that they do not penetrate even at low pH values (pH 4.0); experiments at lower pH values are not feasible owing to the rapid injury which occurs. It seems probable that pH 4 does not lie far enough below the isoelectric point of the protoplasm to provide active protein which can combine with the acid dye.

all pH values; in such a case our assumption might have to be modified, but this may not alter the main conclusion that the equilibrium rises with the increase in pH value.

V.

Presence of Active Protein in the Cell.

The active protein might influence the equilibrium reached by the dye in two ways. In the first place, an increase in the amount of active protein in the vacuole would enable more dye to combine. If this explanation is correct, there should be an increase in the pH of the cell sap when the cells are placed in a solution at higher pH than that of the cell sap. The pH of the sap[10] of cells growing in tap water (the pH value of which is about 6.5) is about 5.6. If the cells are left in a buffer solution at pH 9 for 5 hours, the pH of the sap increases gradually from pH 5.6 to 6.5, when the death of the cells takes place. After the cells have been in the solution for a little less than an hour, a great deal of chlorophyll appears in the sap when the sap is pressed out of the cell (which indicates an injury in many instances). There is no appreciable change in the pH of the sap before this phenomenon appears. The same is true in the case of the cells placed in pH 8 except that here the change in the pH from 5.6 to 6.4 takes place in about 9 hours instead of 5 hours. When the cells are placed in the buffer solutions (without dye) at pH 8 and 9, there is no marked change in the pH of the sap after 10 minutes. Cells placed for this length of time in 0.002 per cent cresyl blue solution would show a concentration in the sap of 0.04 per cent at pH 9, and 0.012 per cent at pH 8. These experiments show that there is a considerable difference in the amount of dye combined at pH 8 and 9, and yet there is no appreciable change in the pH of the sap. With the present method, however, it is not possible to detect a change in pH of the sap less than 0.1 pH so that a difference of less than 0.1 pH must bring about the above difference in the amount of dye combined at pH 8 and 9.

[10] To determine the pH value, sap was drawn into a capillary tube to a certain height and blown out on to a slide; nine parts of sap were mixed with one part of of a very dilute solution of brom-cresol purple which was measured in the same way. The color was then compared with a mixture of nine parts of buffer solution and one part of the same indicator.

We may therefore conclude that if the increase in the active protein in the vacuole is responsible for the increase in equilibrium, a small increase in the pH of the cell sap suffices to bring about a considerable change in the amount of active protein.

It is quite possible, however, that this is not a fair comparison since the increase in the pH of the sap with the same external pH may be greater when the dye is present in the external solution. The cell sap contains organic acids, and if the dye cation penetrates in combination with hydroxyl ion (as some investigators believe), the organic acids will be neutralized and the pH value increased by the penetration of the dye. (In that case the amount of dye in combination with the organic acid will be constant at equilibrium and will thus form a constant fraction of the total concentration of dye at equilibrium.)

It is, of course, possible that the sap near the surface of the vacuole may undergo a much more rapid change in pH and that this may affect the result.

Since this explanation assumes the presence of protein in the sap some experiments were made on this point. The presence of protein in the vacuole is shown by the fact that it contains protein globules which float about in it, and dissolved protein is shown by the fact that the sap gives the xanthoprotein reaction.

When the cell is killed, some of the amphoteric electrolytes appear to diffuse out; the protein globules, however, remain intact and these globules can be stained by placing the dead cell in the dye. It is found that these globules stain more rapidly at high pH than at low pH. This agrees with the experiments on the penetration of dyes into the cells.

In the second place, it is of course, possible that the increase in the amount of dye taken up is due to changes in the protoplasmic layer. Since this is very thin it might undergo rapid changes in pH with a consequent increase in active or ionized protein. If the protoplasmic layer takes up the dye and the dye is actively driven into the vacuole it is quite conceivable that the rate and the final equilibrium of this process would depend on the amount of active protein present. Such a process would involve the expenditure of energy derived from the physicochemical activity of the cell.

VI.

DISCUSSION OF LITERATURE.

Some investigators[11] suggest that the basic dye penetrates more rapidly at high pH because the dye cation can enter only when combined with OH, and that more of this compound, which will be called DOH, is formed at the higher pH values. If this were the case it could affect only the rate of penetration (in the case of *Nitella*) since the final equilibrium would depend only on the amount of substance combining with the dye. If it affected the rate it would change our assumption that the velocity constants are the same at each pH but it would not affect our conclusion that the final equilibrium increases with increase of pH.

If the dye did not combine with something in the cell the concentration inside could not rise above that of DOH. If the dye combines with something inside the cell this combining substance will determine the final equilibrium.

Harvey maintained that the dye accumulated because the dye was unable to diffuse out from the sap, because the sap was acid, which decreased the amount of DOH. If this is true there should not be a diffusion of dye from the cell sap when the stained cells are placed in a buffer solution of low pH. But experiments[1] show that the dye diffuses out much more readily at low pH than at high pH, so that neither the penetration nor the accumulation can be dependent primarily on the amount of DOH.

The solubility of basic dye in fatty substances is known to increase in alkali.[12] If the plasma membrane were lipoid, as some investigators suppose, the basic dye could diffuse out of the cell more readily at high pH than at low pH, but my experiments show that the dye comes out more readily when the pH value of the external solution is low.

Pfeffer[13] states that a basic dye forms an insoluble compound with

[11] Harvey, E. N., *J. Exp. Zool.*, 1911, x, 508.

[12] Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1.

[13] Pfeffer (Pfeffer, W., *Untersuch. Bot. Inst. Tub.*, 1886, ii, 179) supposes that in some cases the dye forms soluble compounds in the sap which are unable to diffuse out. Various substances such as protein, phloroglucin, etc., have been supposed to combine with the dye in some cases.

tannic acid in the sap of *Spirogyra*, for which reason accumulation of the dye takes place. If this compound is the primary factor in the process, the equilibrium should be the same at all pH values, provided the tannic acid is not neutralized by anything except DOH, as would be the case if the cell is impermeable to alkali. If the cell is permeable to alkali it will diffuse in and compete with the DOH for the tannic acid, in which case the dye equilibrium will be decreased as the external pH value increases.

MacArthur[14] showed that planarians took up more basic dye at high pH than at low pH. He stated that he was unable to determine how much of this effect was due to the influence of hydrogen ion concentration on dissociation or rate of diffusion of the dyes, and how much to the alteration of "membranes" and "deeper tissues."

Bethe[15] has drawn an analogy between the vital staining and staining of protein with dyes at different hydrogen ion concentrations. In both cases, there was a greater staining with a basic dye at high pH, and with an acid dye at low pH.

Redfern[16] states that different equilibria were reached at different concentrations, when discs of vegetable tissue were placed in the solutions of basic dye. She concluded that the process of staining was due to adsorption. Since her method differs greatly from mine, I shall be obliged to defer the comparisons to the future.

A theory[11,15,17] of the penetration of dye into the cells must be reserved for the future when greater knowledge of the facts may solve some of the complications. I feel, however, that the final equilibrium is determined by the amount of active protein present. Whether this is dependent primarily on the protein in the cell sap, or

[14] MacArthur, J. W., *Am. J. Physiol.*, 1921, lvii, 350.

[15] Bethe, A., *Biochem. Z.*, 1922, cxxvii, 18. For other recent papers on the influence of pH on vital staining see Rohde, K., *Arch. ges. Physiol.*, 1920, clxxxii, 114. Pohle, E., *Deutsch. med. Woch.*, 1921, xlvii, 1464. Collander, R., *Jahrb. wissensch. Bot.*, 1921, lx, 354.

[16] Redfern, G. M., *Ann. Bot.*, 1922, xxxvi, 511.

[17] For reviews of the literature on the penetration of dyes, see Bayliss, W. M., Principles of general physiology, New York, London, Bombay, Calcutta, and Madras, 1915. Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic and Berlin, 1914. Overton, E., *Jahrb. wissensch. Bot.*, 1900, xxxiv, 669. Schulemann, W., *Biochem. Z.*, 1917, lxxx, 1.

in the protoplasm (either at the surface or throughout the protoplasm), must remain undecided for the present. If it is dependent on the protein in the cell sap, a small change in the pH must bring about a considerable change in the amount of active protein. If, on the other hand, it is dependent on the protein in the protoplasmic layer (the pH of which is assumed to change rapidly), the dye must be driven into the cell sap from the protoplasm by the energy supplied by the physicochemical processes in the cell.

VII.

SUMMARY.

When cells of *Nitella* are placed in buffer solutions at pH 9, there is a very slow and gradual increase in the pH of the sap from pH 5.6 to 6.4 (when death of the cells takes place). If the living cells are placed in 0.002 per cent dye solutions of brilliant cresyl blue at different pH values (from pH 6.6 to pH 9), it is found that the rate of penetration of the dye, and the final equilibrium attained, increases with increase in pH value, which can be attributed to an increase in the active protein (or other amphoteric electrolyte) in the cell which can combine with the dye.