AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS.

I. THEORETICAL.*

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(Accepted for publication, July 26, 1926.)

The attempts to explain a part of the behavior of living cells or tissues on the assumption that they act as simple ampholytes, while fairly satisfactory and suggestive, must be considered only as a first suggestion. Those who employ this concept admit that living cells are more complicated systems than simple proteins, so that clear-cut results from studying them from such a point of view are not to be expected. Certain recent researches cannot, however, be easily correlated on the basis of mere lack of "clear-cutness." For example, Robbins (1), from the staining reactions and water absorption of potato tuber, has shown that it acts as an ampholyte with an isoelectric point at a pH of about 6. However, Cohn, Gross, and Johnson (2) have found that the typical potato protein, tuberin, obtained by acid precipitation of potato juice, has an isoelectric point at a pH of about 4.

Winslow, Falk, and Caulfield (3) have studied the electrophoretic behavior of the organism *Bacillus cereus* over a wide pH range, and while, in the main, the curve obtained may be explained on the basis of a simple Donnan equilibrium, there is a comparatively wide pH range through which such an explanation cannot hold.

Certain unicellular organisms seem to show little tendency to retain either acid or basic dye, through a comparatively wide pH range, in place of through only a narrow range as might be expected to be characteristic of the isoelectric behavior of a simple ampholyte (4). Other organisms show no point where combination with one

* Contributions from the Gates Chemical Laboratory, California Institute of Technology, No. 115.

or the other type of dye does not seem to take place to a considerable extent.

It is the purpose of this paper to examine the probable behavior of a system of two amphoteric substances between which mutual combination may take place under proper conditions, and to show that, by employing the considerations involved, it is much easier to explain much of the physical and chemical behavior of living tissues than it is by using the concept of a simple ampholyte. In the two following papers experimental evidence is adduced, from a study of certain simple systems of two ampholytes, in support of this idea. It may, however, be pointed out that living cells are by no means as simple as this above concept would seem to indicate. Its justification lies in the fact that, by sacrificing only very little of the simplicity of treatment which suffices for consideration of simple ampholytes, one gains greatly in comprehensiveness.

Concept of a Conjugate Protein.

Consider an aqueous solution of two amphoteric substances, HAOH with ionization constants K_a and K_b and an isoelectric point at a hydrogen ion concentration I, and HBOH with corresponding constants K'_a and K'_b and an isoelectric point at I'. Suppose I is larger, *i.e.* more acid, than I'.

In solutions whose hydrogen ion concentration is appreciably greater than I both components will act as bases and tend to retain acids. When the hydrogen ion concentration is appreciably less than I' both components will act as acids and tend to retain bases. In solutions whose hydrogen ion concentration lies between I and I', however, one component will act as a base and the other as an acid, and there will be a tendency toward mutual combination, resulting in a decreased retention of acid or basic reagent. This pH range, from I to I', is the range of mutual combination; and the stability of the system, so far as its behavior as a distinct individual is concerned, depends among other things on the magnitude of this range. This might explain, for example, why certain conjugated proteins, such as lecithoproteins, though they are thought to exist, have not been definitely isolated, while others such as nucleoproteins or phosphoproteins can be easily obtained.

Apparent Isoelectric Behavior.

In a system of two ampholytes we have the following equilibria:

$$\overset{+}{H} + AO\overset{-}{H} \rightleftarrows HAOH \rightleftarrows H\overset{+}{A} + \overset{-}{OH} \text{ with constants } K_a, K_b, \text{ and } I;$$

and

$$\overset{+}{H} + BO\overset{-}{H} \rightleftarrows HBOH \rightleftarrows H\overset{+}{B} + \overset{-}{OH} \text{ with constants } K'_a, K'_b, \text{ and } I'.$$

If HAOH is the more strongly acidic, there will be an isoelectric point at the pH at which

$$(AO\overset{-}{H}) + (BO\overset{-}{H}) = (H\overset{+}{A}) + (H\overset{+}{B})$$

The value of the hydrogen ion concentration corresponding to this point is obtained by expressing the above quantities in terms of $(\overset{+}{H})$ and the various constants and solving. We obtain

$$(\overset{+}{H}) = \sqrt{K_w \frac{K_a\,(HAOH) + K'_a\,(HBOH)}{K_b\,(HAOH) + K'_b\,(HBOH)}} \qquad (1)$$

The isoelectric point is not exactly the point of maximum mutual combination. The latter point may be expected to be governed by the condition

$$(AO\overset{-}{H}) = (H\overset{+}{B})$$

and will occur at a hydrogen ion concentration obtained from the following expression:

$$(\overset{+}{H}) = \sqrt{K_w \frac{K_a\,(HAOH)}{K'_b\,(HBOH)}}$$

The two points, though not identical, will lie very close together; and the simple expression may be used for calculating the isoelectric point of the system.

Electrophoretic Behavior.

From the amphoteric equilibria given above we can, by applying the mass-action law and differentiating with respect to the logarithm

of the hydroxide ion concentration, obtain the following four equations:

$$\frac{d(\mathrm{A\bar{O}H})}{d\ ln(\mathrm{\bar{O}H})} = \frac{K_a\ (\mathrm{HAOH})\ (\mathrm{\bar{O}H})}{K_w}. \tag{2}$$

$$\frac{d(\mathrm{\overset{+}{H}A})}{d\ ln(\mathrm{\bar{O}H})} = -\frac{K_b\ (\mathrm{HAOH})}{(\mathrm{\bar{O}H})}. \tag{3}$$

$$\frac{d(\mathrm{B\bar{O}H})}{d\ ln(\mathrm{\bar{O}H})} = \frac{K'_a\ (\mathrm{HBOH})\ (\mathrm{OH})}{K_w}. \tag{4}$$

$$\frac{d(\mathrm{\overset{+}{H}B})}{d\ ln(\mathrm{\bar{O}H})} = -\frac{K'_b\ (\mathrm{HBOH})}{(\mathrm{\bar{O}H})}. \tag{5}$$

from which

$$\frac{d(\mathrm{A\bar{O}H})}{-\ d(\mathrm{\overset{+}{H}B})} = \frac{K_a\ (\mathrm{HAOH})\ (\mathrm{\bar{O}H})^2}{K'_b\ (\mathrm{HBOH})\ K_w}$$

or, since $$(\mathrm{\bar{O}H}) = K_w/(\mathrm{\overset{+}{H}}),$$

$$d(\mathrm{A\bar{O}H}) = -\frac{K_a\ (\mathrm{HAOH}\ (\mathrm{\bar{O}H})}{K'_b\ (\mathrm{HBOH})\ (\mathrm{\overset{+}{H}})}\ d(\mathrm{\overset{+}{H}B})$$

Either an increase in $(\mathrm{A\bar{O}H})$ or a decrease in $(\mathrm{\overset{+}{H}B})$ will increase the resultant negative charge on the micella, and thus increase the velocity toward the anode in a constant electric field. At the isoelectric point, as the hydroxide ion concentration is increased, $\frac{d(\mathrm{A\bar{O}H})}{d\ ln(\mathrm{\bar{O}H})}$ is of the same order of magnitude as $\frac{-\ d(\mathrm{\overset{+}{H}B})}{d\ ln(\mathrm{\bar{O}H})}$ but the former increases with increasing alkalinity while the latter decreases (equations (3) and (4)). Through the pH range I to I', the value of $-d(\mathrm{\overset{+}{H}A})$

is small compared to that of $d(\overline{AOH})$, and $d(\overline{BOH})$ is small compared to $-d(\overset{+}{HB})$. As a result there will be a rather rapid increase in negative charge through a certain range, passing through the isoelectric point, until nearly all the HAOH is ionized to $\overline{AOH}$, which ionization takes place increasingly rapidly as $(\overline{OH})$ is increased. When such a condition is reached, the only possible significant increase in negative charge before the point I' is reached is from $-d(\overset{+}{HB})$, which has less and less effect on the magnitude of the charge as $(\overset{+}{HB})$ becomes smaller, *i.e.* as $(\overline{OH})$ is increased (equation (4)). This means that through a certain pH range the negative charge will remain nearly constant. When I' is reached, however, we have $\frac{-d(\overset{+}{HB})}{d(\overline{BOH})} = 1$, and from this point on $\frac{d(\overline{BOH})}{d\,ln(\overline{OH})}$ is the predominant factor. Its value increases with $(\overline{OH})$, and the negative charge again begins to increase more rapidly, and continues until the HBOH is completely ionized. From this point on, since we are now on the alkaline side of the isoelectric points of both components and they are thus both in the same ionic state, we may expect the curve to be the same as would be predicted by the application of the Donnan equilibrium to a simple ampholyte.

A similar condition would prevail on the acid side of the isoelectric point of the system as the hydrogen ion concentration is increased.

DISCUSSION.

It may be well to cite some of the observations which originally led to the more definite formulation of the concept of a mixed amphoteric system.

In connection with certain bacteriological problems the staining reactions of a large number of organisms have been studied by the author (4). Certain of the typical curves are given in Fig. 1.[1] Ab-

[1] Bacterial cells furnish a very satisfactory material to study. The individual cell as a system can be easily observed, thin smears can be obtained fairly free from débris, and equilibrium can be quickly reached. We found little difference in results between buffering for several minutes and for as long as 150 hours. Gern-

scissæ are pH values and ordinates are arbitrary functions of the intensity of retained color. Values for the latter were obtained by repeated comparison of slides under the microscope, and are there-

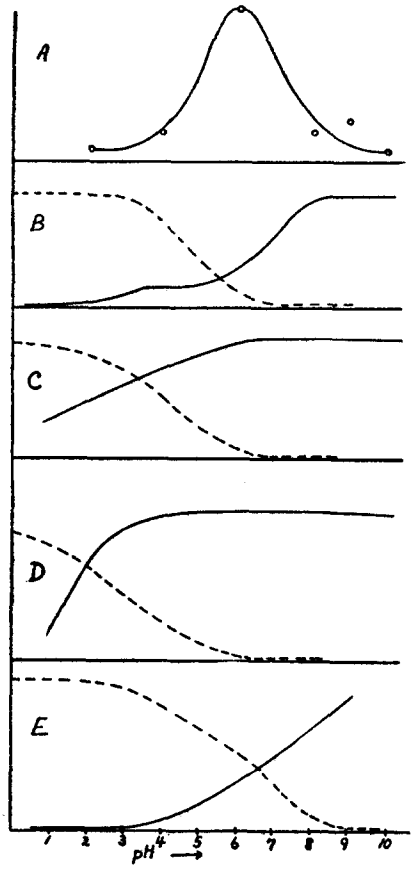

FIG. 1. For explanation see text. Broken lines represent behavior toward acid fuchsin, unbroken lines (except Curve A) behavior toward gentian violet.

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gross, along the same line (6), states that hide powder in small quantities, unhardened by formaldehyde, reaches complete reversible equilibrium with acid solutions in 2 minutes. Our final technique consisted in preparing thin smears on microscope slides, staining with "carbol gentian violet" or "carbol acid fuchsin" solution, "fixing" by treatment with buffer solution, and finally decolorizing with acetone. Slides of any series were then repeatedly compared under a microscope.

fore only qualitative. They are, however, comparative and the curves represent the behavior of the organism. The intensity plotting has been conservative and quantitative methods would, we feel sure, merely accentuate the contrast between the greater and smaller dye retention.

In Curve A of this figure the buffer ratios for the organism *Bacillus coli*, obtained by Falk and Shaughnessy (5) are plotted just above the color curve for the same organism (Curve B).[2] The results are suggestive, showing that the buffering power of this organism shows itself over a wide pH range with a maximum near the isoelectric point of the system as determined by minimum dye retention.

The curves in this figure are typical of classes of bacteria. A fair number of organisms have their point of least color retention around a pH of about 3 (Curve D), another large class has a corresponding point around a pH of 5 to 6 (Curve B), and there are a few intermediate (Curve C).

Other types of cells were studied, such as red blood cells from both human blood (Curve E) and sheep blood. In the case of the former the smears were made from whole blood and the isoelectric point of such a system lies a little below a pH of 7. The sheep cells used were washed cells and showed a corresponding point at a pH very near 7.

Such a system as *Bacillus coli* (Curve B) exhibits a range through which little dye is retained—either acid or basic—suggesting a considerable stability of the system as a chemical individual, while the system *Bacillus dysenteriæ* Shiga (Curve C), even at its isoelectric point, still retains fairly strongly both acid and basic dyes, showing that there is still a fair concentration of both cation and anion in the system, and that either actual combination between these two is limited, or that the combination is comparatively unstable.

The condition of maximum combination between the two components of a system of two ampholytes need not mean a condition of much combination. When $(\overset{-}{AOH}) = (\overset{+}{HB})$, though this is the optimum condition for combination, the system may act as a majority

[2] In plotting Curve A the pH range given in the table by Falk and Shaughnessy is plotted at its lowest value, thus where they give the range 6–7, it is plotted as 6.

of ionogens and remain largely in ionic form. On the other hand it may act in a manner analogous to such salts as lead acetate, mercury salts, etc., and the two ions may almost entirely combine. The extent of this combination will determine the behavior toward anions and cations. In systems in which the two components are largely combined at the isoelectric point we may have behavior simulating a simple ampholyte in that there will be a pH range through which no appreciable combination with added cation or anion takes place.

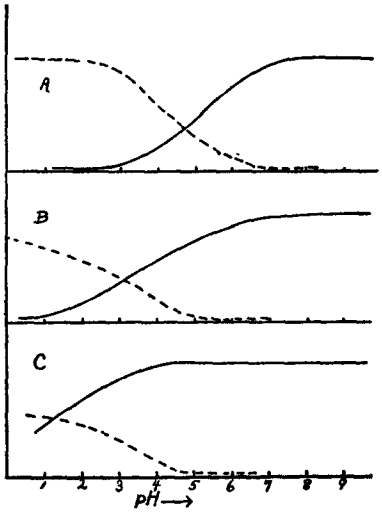

FIG. 2. Effect of oxidation on the behavior of *Bacillus typhosus* toward acid fuchsin (broken line) and gentian violet (unbroken line). Curve A—original organism, Curve B treated with N/50 iodine, Curve C treated with N/50 potassium dichromate.

On the other hand, we may have systems in which even at the isoelectric point there is still a fair concentration of both $(\overline{AOH})$ and $(\overset{+}{HB})$ and thus there will be no point at which added anions and cations will not be appreciably bound.

There are at least two ways in which the isoelectric point of a mixed system or of a simple ampholyte may be changed. The system may be transformed into a new mixed system either by altering the relative amounts of the components or by adding another component,

or the acid or basic properties may be altered by oxidation or reduction.

An example of the first effect is reported by Gerngross (7) who found that the electrophoretic isoelectric point of gelatin was changed from a pH of 4.75 to 4.3 by treatment with formaldehyde.

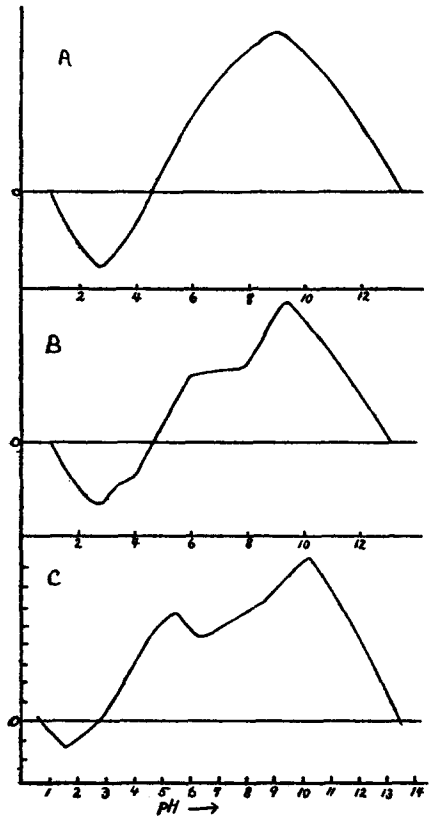

FIG. 3.

Change in isoelectric point by oxidation has been studied by the author (4), and certain results are shown in Fig. 2. The magnitude of the change depends on the degree of oxidation. A mild oxidizing agent such as iodine (Curve B) renders the system more acidic than it was originally (Curve A), but such an oxidizing agent as potassium

bichromate produces a distinctly greater shift in the same direction (Curve C).[3]

While reducing agents do not seem to have any effect on the original organism, the effect produced by oxidation has been repeatedly reversed by treatment with stannous chloride. This behavior is, of course, analogous to the behavior of practically all substances in the effect of oxidation or reduction on acidic strength.

An interesting application of the concept of a mixed system is the electrophoretic behavior of the organism *Bacillus cereus* as worked out by Winslow, Falk, and Caulfield (3) and by Winslow and Shaughnessy (8). Their results are roughly represented by Curve C of Fig. 3. Abscissæ are pH values and ordinates are migration velocities in an electric field. These are measured toward the anode on that portion of the curve above the zero line and toward the cathode below this line. Curves A and B are both theoretical. Curve A represents the theoretical electrophoretic behavior of a micella composed of a single ampholyte to which the Donnan equilibrium applies. Curve B represents the theoretical electrophoretic behavior of a system of two ampholytes as worked out above. The intersection of the curves with the zero line represents the isopotential point.

In all the cases mentioned above the concept of a mixed system of ampholytes, so simple as to contain only two components, offers a much more obvious explanation of the experimental facts than the concept of a simple ampholyte.

SUMMARY.

The amphoteric behavior of a system of two amphoteric components is theoretically examined; and this is shown to correspond more nearly with certain of the physical and chemical behaviors of living tissues than does the concept of a simple ampholyte.

[3] Oxidizing agents were incorporated in the buffer solutions in N/50 concentrations.[1] A goodly number of oxidizing agents were studied, and, in general, they could be arranged in a series on the basis of the magnitude of their effect on the organisms, which series was roughly the same as arrangement on the basis of oxidizing potential. The increase in acid properties upon treatment with an oxidizing agent, illustrated in Fig. 2, was noted in all organisms studied.

It is a pleasure to acknowledge the criticism and suggestions of Dr. A. L. Raymond in the preparation of the manuscript of this and the three following papers.

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