

## THE INFLUENCE OF AMMONIUM SALTS ON CELL REACTION.

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The recognition of the importance of the true reaction or hydrogen ion concentration of the blood and body fluids of organisms, as opposed to their titratable acidity, has been responsible in recent years for many important advances in physiology and for a large and rapidly growing body of literature. So great has been the interest in the determination, made possible by the development of the indicator and the electrometric methods, of the pH, as such, in the case of body fluids, sea water, culture media, etc., that the fact has rather generally been overlooked that the pH of the medium to which a cell is exposed bears no necessary relation to the pH produced by that medium within the cell itself.

This is particularly true in the case of the most important of all of the various buffer systems found in organisms; namely, the carbonic acid-bicarbonate system. The author<sup>1,2</sup> has shown that the pH produced within a cell by a medium containing both CO<sub>2</sub> and bicarbonate can by no means be predicted from the pH of the medium as measured with indicators or electrometrically. Thus, cells composing the flowers of *Symphytum peregrinum*, which contain a natural indicator sensitive to weak acids, when exposed to the slightly alkaline solution resulting from the saturation of  $m/2$  NaHCO<sub>3</sub> with CO<sub>2</sub>, give visible evidence of a rise in intracellular acidity which is almost as rapid and striking as in the case of a strongly acid saturated solution of CO<sub>2</sub> in distilled water, and far more rapid than in the case of solutions of HCl whose hydrogen ion concentration is still greater.

<sup>1</sup> Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457.

<sup>2</sup> Jacobs, M. H., *Biol. Bull.*, 1922, xlii, 14.

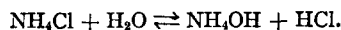
Likewise, the taste receptors of the human tongue which are concerned in the sensation of sourness (*i.e.*, which are specifically stimulated by hydrogen ions) are not sensitive to most acids at a pH less acid than 3.0, but are excited even by the alkaline mixture of CO<sub>2</sub> and bicarbonate just mentioned.

These facts have been explained by the author as being due to a differential permeability, CO<sub>2</sub> being free to enter living cells while bicarbonates are not, or are to a far less extent. Consequently the equilibrium reached within the cell depends not on the mere pH of the surrounding medium but essentially on the amount of free CO<sub>2</sub> which the latter contains, as well as on the buffer content of the cell itself. An increase in intracellular acidity may be produced in such cases by a neutral or alkaline solution, while no change may occur with another solution, even though it shows a fairly high degree of acidity.

As far as acids are concerned, it appears that with the possible exception of H<sub>2</sub>S, on which investigations from this point of view have been begun, carbonic acid is a unique substance owing its peculiar characteristics to the combination of two properties: (*a*) its weakness as an acid which permits at neutrality or even at low degrees of alkalinity the presence of considerable amounts of the free acid and its anhydride, CO<sub>2</sub>; and (*b*) the extraordinary ease with which it (either as CO<sub>2</sub> or H<sub>2</sub>CO<sub>3</sub> or both) penetrates living cells, this ease of penetration being perhaps correlated with its free solubility in lipoids.

The facts which have been mentioned naturally suggest the question: Are there any substances of alkaline nature whose behavior is analogous to that of CO<sub>2</sub>—substances which from solutions having an acid reaction can produce intracellular alkalinity as CO<sub>2</sub> and from an alkaline solution can produce intracellular acidity? That there is at least one such substance is indicated by the experiments on ammonium salts which will now be described.

A solution of an ammonium salt, *e.g.* NH<sub>4</sub>Cl, in aqueous solution on account of the weakness of NH<sub>4</sub>OH as a base undergoes partial hydrolysis according to the equation:



Because of the greater degree of dissociation of HCl as compared with  $\text{NH}_4\text{OH}$ , the solution has an acid reaction. Now from the work of Warburg<sup>3</sup> and of Harvey<sup>4</sup> and others we know that  $\text{NH}_4\text{OH}$  (or  $\text{NH}_3$ ?) penetrates living cells with the greatest readiness. HCl, on the other hand, like other mineral acids, enters with difficulty. It would appear, therefore, on theoretical grounds that in a solution of an ammonium salt, even though the reaction of the solution be acid, a cell might readily become more alkaline. That this actually occurs, at least in certain cases, may be shown in a number of ways.

During the spring of 1922 it was found that the red flowers of a hybrid *Rhododendron* cultivated in the botanical gardens of the University of Pennsylvania contain a natural indicator which is extremely favorable for showing the entrance of  $\text{NH}_4\text{OH}$ . When small portions of the blossom are placed in an  $\text{N}/100$  or  $\text{N}/1,000$  or even a weaker solution of this substance, the color quickly changes along the torn edges to a violet and then to a beautiful clear blue. As seen with a hand lens, or even better with the compound microscope, the change in color is extremely striking. No more favorable material could be desired for following the penetration of ammonia from solutions of ammonium salts.

Preliminary experiments *in vitro* with the expressed filtered juice of some of the flowers, using phosphate buffer solutions to regulate the pH, showed that the indicator begins to change color from red to violet at about neutrality. At pH 7.0 the change is scarcely noticeable; at pH 8.0 it is very decided, though not complete. It may be mentioned that while the first change from red to violet is the same with the juice as with the living cells, the further change with increasing alkalinity in the former case is to a dirty green rather than to a clear blue.

Having found that the indicator is unaffected on the acid side of neutrality, an  $\text{M}/10$  solution of  $(\text{NH}_4)_2\text{SO}_4$  was brought to a pH of 6.2 by the addition of a trace of free ammonia and portions of the flowers were exposed to it. In every case there was a prompt change in the color of the cells, beginning at the cut and torn edges, to a

<sup>3</sup> Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305.

<sup>4</sup> Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507.

bright blue. Control experiments in which water alone or an M/10 solution of NaCl were used gave no such result. Also the ammonium sulfate solution when added to the extracted indicator caused no change in its red color. Finally, it was found that cells which had been sufficiently injured by heat or by a short exposure to water saturated with ether failed to turn blue. Evidently there is produced in the living cells an effect which is most reasonably explained by a differential penetration of  $\text{NH}_4\text{OH}$  from a solution in which it is present as one constituent, even though the reaction of the solution itself is on the acid side of neutrality.

The effect of pure  $\text{NH}_4\text{Cl}$  and  $(\text{NH}_4)_2\text{SO}_4$  (without the addition of  $\text{NH}_4\text{OH}$ ) was next tried. Solutions of both substances are considerably more acid than the one employed in the preceding experiment, and the free  $\text{NH}_4\text{OH}$  present is much less in amount. Nevertheless, distinct, even if less striking evidence than before was obtained of an increase in the alkalinity of the cells. The color change in some cases went only as far as a blue-violet, though in others a pure blue was obtained. The differences appeared to depend to some extent on the condition of the flowers, the least striking results being obtained with those which were old and somewhat wilted.

It would be expected that ammonium acetate, which is much more strongly hydrolyzed in aqueous solutions than either the sulfate or the chloride, would be more effective than they, and such is the case. Solutions of ammonium acetate of strengths varying between M/10 and M/2 to which no  $\text{NH}_4\text{OH}$  had been added were found to be as effective as the first solution used which was a mixture of the sulfate and the hydroxide. It may be mentioned that since the dissociation constants for acetic acid and for ammonium hydroxide are nearly equal, a solution of ammonium acetate has a reaction which is approximately neutral.

The results obtained with *Rhododendron* blossoms may be duplicated with animal cells. One simple, and at the same time striking experiment may be described which brings out not only the peculiar effect of ammonium salts but that of the  $\text{CO}_2$ -bicarbonate system as well.

Unfertilized starfish eggs were lightly stained in neutral red, concentrated by means of a centrifuge, and introduced with practically

no sea water into three Syracuse watch-glasses containing respectively the following solutions: (a)  $M/2$  NaCl brought to pH 7.2 by means of a trace of  $\text{NaHCO}_3$ ; (b)  $M/2$   $\text{NaHCO}_3$  brought to the same pH by saturating with  $\text{CO}_2$ ; and (c)  $M/2$   $\text{NH}_4\text{Cl}$  brought to the same pH by means of  $\text{NH}_4\text{OH}$ . The three solutions were identical in hydrogen ion concentration and in salt concentration, and according to the views tacitly accepted by many physiologists might have been expected to have the same effect on the intracellular reaction. Such was not the case, however. When the watch-glasses were gently agitated so as to collect the eggs in masses and these were viewed with the naked eye against a black background, the following appearances were clearly visible. The eggs in (a) had not changed their color; those in (b) had become bright pink, indicating an increased acidity; and those in (c) had become yellow, indicating an increased alkalinity. These effects were apparent within a few seconds; after standing for 15 minutes, as the cells began to be injured by the solutions (as shown by microscopic examination), the pink color of (b) and the yellow color of (a) began to be changed in the reverse directions until finally the three lots of eggs were practically indistinguishable in appearance.

That even a pure solution of  $M/2$   $\text{NH}_4\text{Cl}$  may cause increased intracellular alkalinity in starfish eggs may also be shown by the same method, though the color changes in this case are not so striking as those just described. Instead of becoming yellow in color, the eggs merely become orange-red. However, the difference between the treated eggs and the controls, when both are viewed in masses against a black background, is unmistakable.

Similar results may be obtained with animal material of a different sort. The author has found it convenient in certain studies, as yet unpublished, on the relative rates of penetration of various acids into living cells, to employ "artificial cells" which are constructed as follows: A piece of glass tubing 5 cm. long and 1 cm. in diameter is provided at one end with a lip. Over this end is gently stretched a piece of frog's skin, which is held in place with a rubber band. Within the "cell" is placed a solution of any desired buffer content and containing any desired indicator. Penetration of the acid may then be followed by changes in the color of the solution. Because the skin

of the frog is provided with glands and because there is a possibility of penetration between the cells rather than through them, the results obtained with this material would be expected to be more complicated than those secured where single cells are concerned; it appears, however, from a variety of experiments which need not here be detailed that the general behavior of the two kinds of material is in most respects surprisingly similar.

When "artificial cells," in which the "cell membrane" is composed of frog's skin are used with ammonium salts, very striking results may be obtained. With the skin turned *inside out*, an internal solution containing phenol red and neutral in reaction at the start may easily be carried beyond the range of the indicator on the alkaline side by dipping it into an M/10 solution of  $\text{NH}_4\text{Cl}$ , which on account of hydrolysis has a strongly acid reaction. That the alkalinity is due to ammonia rather than to some other substance secreted by the glands may be shown: (a) by the use of Nessler's reagent; and (b) by the fact that thorough aeration of the liquid greatly diminishes its alkalinity.

If the frog's skin is turned right side out instead of inside out, the results are much less striking. Clear evidence of selective penetration by  $\text{NH}_3$  has been obtained with this arrangement a number of times, though frequently it could not be seen at all, and in no case was it so striking as with the skin turned in the reverse direction. The reason for this difference is not altogether clear at present. Perhaps the glands in the skin are in some way concerned, though the facts that the characteristic result may at times be obtained with the skin turned in either direction and that it may be obtained with eel's skin which is said not to contain glands, as well as with single cells of both plant and animal origin, make it fairly certain that it is not primarily a glandular phenomenon.

The difference in the behavior, in the presence of living cells, of three solutions of the same pH and the same salt concentration but containing respectively  $\text{NaCl}$ ,  $\text{NH}_4\text{Cl}$  plus  $\text{NH}_4\text{OH}$ , and  $\text{NaHCO}_3$  plus  $\text{CO}_2$ , may be shown very clearly by the "artificial cell" method, using phenol red as the indicator and starting with all of the solutions concerned (both internal and external) at a pH of 7.2. At the end of a few minutes, if the internal solution is gently stirred, the colors

in the three tubes will be found to be orange, deep red, and lemon-yellow, respectively. The author has used this experiment with success as a class exercise which never fails to give striking results.

As to the significance of the observations which have been described, it may be mentioned that  $\text{NH}_3$ , like  $\text{CO}_2$ , is a substance probably formed by all living cells. That a differential permeability effect should at times be responsible for differences in reaction between body fluids and cells and even between different parts of the same cell is by no means unlikely. That ammonium salts may, as a matter of fact, behave in this way is indicated by the work of Haldane<sup>5</sup> who finds them to be extremely effective in producing in the human body the changes generally described by the term acidosis. In the same connection, the views of Mathews<sup>6</sup> as to the possible rôle of ammonium salts in the formation of HCl in the mammalian stomach may be mentioned. Probably other physiological peculiarities of ammonium salts may ultimately be found to be connected with their behavior as here described.

To state briefly the general conclusions to be drawn from these experiments, it appears that  $\text{NH}_4\text{OH}$  among bases occupies much the same position that carbonic acid does among acids. In both cases the intracellular pH depends not on the pH of the surrounding medium but rather on the absolute concentration of the free base or free acid as the case may be. It follows, therefore, that while the determination of the pH values of body fluids, culture media, etc. is of the greatest importance, it is necessary to keep in mind the fact that these values do not always enable a prediction to be made as to the effect of the medium on living cells. In certain cases the results may be the exact opposite of those which might be expected, namely, in the cases where an alkaline solution containing  $\text{CO}_2$  brings about intracellular acidity and where an acid solution containing  $\text{NH}_4\text{OH}$  brings about intracellular alkalinity.

<sup>5</sup> Haldane, J. B. S., *J. Physiol.*, 1921, lv, 265.

<sup>6</sup> Mathews, A. P., *Physiological chemistry*, New York, 1915, 374.

## SUMMARY.

1. It may be shown by means of cells of the flowers of a hybrid *Rhododendron* which contain a natural indicator, by means of starfish eggs stained with neutral red, and by means of an "artificial cell" in which living frog's skin is employed that increased intracellular alkalinity may be brought about by solutions of a decidedly acid reaction which contain ammonium salts.

2. These results are analogous to those previously obtained with the CO<sub>2</sub>-bicarbonate system, and depend on the facts: (a) that NH<sub>4</sub>OH is sufficiently weak as a base to permit a certain degree of hydrolysis of its salts; and (b) that living cells are freely permeable to NH<sub>4</sub>OH (or NH<sub>3</sub>?) and not to mineral and many organic acids, and presumably not at least to the same extent to ammonium salts as such.