

THE PENETRATION OF CO₂ INTO LIVING PROTOPLASM.*

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Experiments with H₂S on *Valonia*¹ indicate that under normal conditions it is chiefly if not exclusively undissociated molecules which enter the cell.² In view of the importance of this conclusion it seemed necessary to carry out comparable experiments with another substance. For this purpose CO₂ was chosen. It penetrates readily and is easily measured. Its importance in the organism renders its investigation particularly significant.

It might be supposed that the production of CO₂ by the cell would interfere with the measurements but it was found that under the conditions of the experiments this is so slight as to be negligible.

The experiments were carried out by the junior author, the methods being similar to those employed in the experiments on H₂S.² The CO₂, generated by the action of dilute HCl on Bermuda coral rock, passed through a column of cotton and bubbled through sea water until the latter had absorbed about one-fourth of its volume. The desired pH value was obtained by adding HCl or NaOH.

In each case 10 cells (the average volume of a cell being about 0.33 cc.) were placed in a bottle containing 125 cc. (with no air space above the solution) and tightly stoppered.

The process of penetration was practically complete in less than 2 hours but the cells were left for several hours in order to make sure that equilibrium had been attained. The solution was stirred during the exposure.

The sap was collected as described in the previous paper.¹ 1 cc. was used for each analysis. It was free from contamination by sea

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¹ *Valonia macrophysa*, Kütz, collected at Bermuda.

² Osterhout, W. J. V., *J. Gen. Physiol.*, 1925-26, viii, 131.

water as shown by the absence of SO₄. Sea water contains sufficient SO₄ to give a visible precipitate with acidified BaCl₂ when the sea water is diluted one hundred times. The absence of SO₄ also indicates that no injury has occurred and this is borne out by the fact that if cells which had been exposed to the experimental treatment were returned to sea water and kept under ordinary laboratory conditions they lived indefinitely.

The temperature ranged between 20° and 22°C. but did not vary more than 1° during any one experiment. The temperature coefficient (12.5° to 22.5°C.) of penetration and outward diffusion for living and dead cells is very low (less than 1.1).³

A Van Slyke apparatus⁴ was employed to determine the total CO₂.

The essential question is whether the total CO₂ in the sap corresponds to the undissociated fraction of the total CO₂ in the sea water or to the dissociated fraction. The total CO₂ includes the HCO₃⁻ and CO₃⁼ ions, the undissociated H₂CO₃, the undissociated carbonates and bicarbonates of all the metals present, the uncombined or free CO₂, and an unknown number of hydrates and complexes of any one or all of the above.

If the concentration of total CO₂ inside the cell depends on the outside concentration of one of these substances, and is independent of the concentration of others, we should expect the ratio of concentration inside to that outside to change when we alter the proportion of this particular outside substance. There is no obvious method of changing only one of these substances. Changing more than one will serve our purpose provided that we do not change in the same ratio a substance which penetrates and one which does not, in such a way as to make it impossible to decide which substance penetrates.

The most expedient way is to change the H⁺ ion concentration. An increase in the H⁺ ion concentration would decrease the proportion of HCO₃⁻ and CO₃⁼ ions, and of other substances which change with them, and would increase the proportion of undissociated H₂CO₃ and of free CO₂.

³ A low temperature coefficient for the absorption of pure gaseous CO₂ by solutions of K₂CO₃ was observed by Williamson, R. V., and Mathews, J. H., *Ind. and Eng. Chem.*, 1924, xvi, 1157.

⁴ Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Since we may assume that the relation between uncombined CO_2 and undissociated H_2CO_3 is approximately constant, we may for the purpose of the present paper (where relative values alone are considered) use the term undissociated H_2CO_3 to include the uncombined CO_2 (whose concentration is probably much greater than that of the undissociated acid). This course will be followed as a matter of convenience.

In order to ascertain the concentration of undissociated H_2CO_3 (including free CO_2) in the sea water at various pH values various means may be used. The method employed by McClendon is to determine the partial pressure of the CO_2 in the gas phase in equilibrium with the sea water (total CO_2 being constant). At low pH values, where the total CO_2 is undissociated, this partial pressure will reach its maximum value; as the pH value increases and H_2CO_3 begins to dissociate this value will fall in approximate proportion, *e.g.* when the total CO_2 is 50 per cent dissociated, the partial pressure will fall to about 50 per cent of its maximum value. The values of the partial pressures as determined by McClendon⁵ are shown in Fig. 1 by the symbol (Δ).

A somewhat different method (which gives similar results) was employed in the present investigation by circulating gas through sea water (at various pH values) and through artificial sap⁶ (containing no excess base). If the pH value of the artificial sap is low enough to prevent dissociation of H_2CO_3 , the amount of total CO_2 which passes over into the artificial sap corresponds to the undissociated H_2CO_3 (including free CO_2) in the sea water. For this purpose an apparatus was employed which consisted of two flasks fitted with tubes and an aspirator bulb so that the gas could be circulated and the two liquids brought to equilibrium with the same gas phase. The total CO_2 of the sea water and of the artificial sap was then determined with the Van Slyke apparatus. It was found that the lower the pH value of the sea water the higher was the relative total CO_2 of the artificial sap as compared with that of the sea water; this was true down to pH

⁵ McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Pub. 251*, 1917, 36. These values are for sea water of excess base 25 (at 20°C.).

⁶ This was made by mixing 86.24 cc. of KCl 0.6 M with 15.08 cc. of NaCl 0.6 M; *cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, v, 225.

3 below which lowering of the pH value produced no increase in the relative concentration in the artificial sap. The H₂CO₃ was therefore regarded as undissociated at pH values below 3. If the concentration of total CO₂ in the sea water is compared with that in the artificial sap, the latter is found to be about 16 per cent higher at pH values of 3 or lower, due to the fact that CO₂ is more soluble in the artificial sap. This is not surprising since CO₂ is less soluble in solutions containing sulfate.⁷

Let us suppose, for convenience, that the concentration of total CO₂ in the sea water is kept constant, for example at 100. Its concentration in the artificial sap (in equilibrium with the same gas phase) will be proportional to the concentration of undissociated H₂CO₃ (including free CO₂) in the sea water.⁸ Thus if the pH of the artificial sap is low enough to prevent dissociation of H₂CO₃ its concentration of total CO₂ will be 116 when the total CO₂ in the sea water is undissociated; it will be 58 when the total CO₂ of the sea water is 50 per cent undissociated. Hence it is evident that we can find the percentage of undissociated H₂CO₃ (including free CO₂) in sea water by expressing the concentration of total CO₂ in the artificial sap as per cent of the total CO₂ in sea water and multiplying these figures by 100 ÷ 116. Thus at pH 3 the per cent of undissociated H₂CO₃ (including free CO₂) in sea water is 100, at pH 5.8 it is 86 per cent, and so on.⁹ The results are expressed in Fig. 1 by the symbol (X).

These values may be compared with those which would be expected on a theoretical basis if the CO₂ were dissolved in distilled water. The undissociated H₂CO₃ (including free CO₂) expressed as per cent of total CO₂ may be calculated by means of the formula:¹⁰

$$\text{Per cent undissociated H}_2\text{CO}_3 \text{ (including free CO}_2\text{)} = \frac{100}{1 + \frac{K_1}{(H)} + \frac{K_1K_2}{(H)^2}}$$

⁷ Cf. Hildebrand, J. H., Solubility, New York, 1924, 140.

⁸ It is assumed that the apparent dissociation constant is the same in both.

⁹ These determinations are approximate. It is probable that if care had been taken to keep the pH value of the artificial sap (at equilibrium) low enough to suppress ionization of H₂CO₃ in all cases there would be less irregularity.

¹⁰ Cf. Michaelis, L., Die Wasserstoffionenkonzentration, 2nd edition, Berlin, 1922, 48.

in which $K_1 = 3.3 \times 10^{-7}$ and $K_2 = 6 \times 10^{-11}$. The values obtained by this calculation are expressed in Fig. 1 by the symbol (\square). The curve has the same general form as those already discussed but the latter are displaced somewhat to the left in the lower part. This is to be expected since E. J. Warburg¹¹ has shown that the presence of

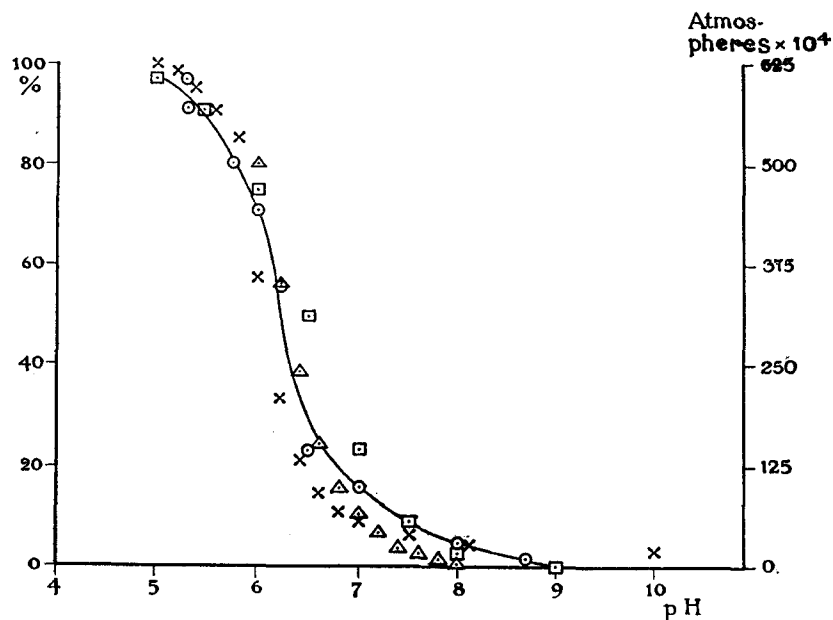


FIG. 1. Shows that the total CO_2 in the cell sap corresponds approximately to the undissociated H_2CO_3 (including free CO_2) in the sea water outside. The total CO_2 in the sap (\circ) is expressed as per cent of that in the sea water outside (the curve is drawn free-hand through the points to give an approximate fit). The per cent of undissociated H_2CO_3 (including free CO_2) as calculated from the dissociation constant is shown by the symbol (\square). The partial pressure of free CO_2 in the sea water as determined by McClendon is shown by the symbol (Δ). It is expressed as ten thousandths of a standard atmosphere as shown by the figures on the ordinate at the right. The concentration of H_2CO_3 (including free CO_2) in sea water, expressed as per cent of that found at pH 3 (where the H_2CO_3 is regarded as undissociated), is shown by the symbol (\times): it may be regarded as expressing the per cent of total CO_2 which is in the form of H_2CO_3 (including free CO_2). Each experimental point of symbols (\square) and (\times) represents one determination.

¹¹ Warburg, E. J., *Biochem. J.*, 1922, xvi, 153. See also Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.

salts lowers the value of the negative logarithm of the apparent first dissociation constant of H₂CO₃: that this would shift the curve to the left is obvious.¹⁰ The relation of this to the theory of Debye and Hückel has recently been discussed by Hastings and Sendroy.¹²

Let us now enquire what takes place inside the cell. If CO₂ acts in the same general way as H₂S and enters the cell only in the form of undissociated molecules we should expect the concentration of total CO₂ in the cell to correspond to the concentration of undissociated H₂CO₃ (including free CO₂) in the sea water. This expectation is realized as is evident from Fig. 1 in which the circles denote the concentration of total CO₂ found in the sap of living cells at various pH values after equilibrium is reached between the total CO₂ inside and that outside. The curve is drawn free-hand through these points to give an approximate fit. In order to facilitate comparison the concentration of total CO₂ inside is expressed as per cent of that outside: by this method the correspondence (or lack of it) between the total CO₂ inside and the undissociated H₂CO₃ outside can be made most clearly evident. If, for example, at a given pH value the undissociated H₂CO₃ (including free CO₂) in the sea water is about 50 per cent of the total CO₂ we shall expect the concentration of total CO₂ inside the living cell to be about 50 per cent of the total CO₂ in the sea water. Hence the curve for per cent of undissociated H₂CO₃ (including free CO₂) in the sea water should approximately coincide with that for total CO₂ in the sap (expressed as per cent of undissociated H₂CO₃, including free CO₂, in the sea water).

Fig. 1 shows that the correspondence between the total CO₂ in the living cell (○) and the undissociated H₂CO₃ (including free CO₂) as shown by the symbols (×) and (Δ) is fairly good. It cannot be expected to be exact for a number of reasons. In the first place, as already noted, CO₂ is more soluble in the sap than in the sea water. In the second place, if we assume that H₂CO₃ penetrates freely but that its ions cannot pass in or out, it follows that if it partly dissociates after entering the cell the ions so formed will be trapped: more undissociated H₂CO₃ will move in, until the concentration of undissociated H₂CO₃ is the same inside and outside. The total CO₂ inside, consist-

¹² Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, 1925, lxx, 445.

ing of undissociated H_2CO_3 (including free CO_2), plus ions, will therefore be greater than the undissociated H_2CO_3 (including free CO_2) outside. The amount of dissociation is not sufficient to make any great difference. If we regard the pH of the cell sap as approximately constant at 5.8,¹³ we may, for purposes of calculation, take the per cent of dissociation calculated from the dissociation constant of CO_2 dissolved in distilled water; *i.e.*, about 19 per cent of the total CO_2 . If the concentration of undissociated H_2CO_3 (including free CO_2) outside is 100 we should have the same concentration inside and this would be 81 per cent of the total CO_2 inside which would therefore amount to $(100 \div 81) 100 = 123.46$. The same relation would hold no matter what the outside pH or concentration of undissociated H_2CO_3 happened to be, *i.e.* we should always expect to find 23.46 per cent more total CO_2 inside than undissociated H_2CO_3 (including free CO_2) in the sea water outside. This excess will of course be less if the per cent of dissociation of H_2CO_3 in the sap is less.

Fig. 1 shows that at higher pH values there is more total CO_2 in the sap than undissociated H_2CO_3 (including free CO_2) in the sea water as calculated from the experimental data of McClendon and the writers. This would be expected on the grounds just mentioned (solubility and dissociation). At lower pH values, however, so much CO_2 might enter the cell as to lower the pH value, in which case the dissociation would be less and the total CO_2 of the sap would fall off somewhat. The curve indicates that this may be the case but at lower pH values the total CO_2 of the sap falls more than would be expected, becoming less than the undissociated H_2CO_3 (including free CO_2) of the sea water. For this no explanation is at present suggested.

Below pH 6.0 the curve for penetration becomes a little uncertain. The cells are soon injured at this pH and when they die the total CO_2 becomes the same inside and outside. Frequently we find that when the outside solution is at pH 5.8 or 6.0 the total CO_2 of the sap attains only 85 to 90 per cent of the outside concentration of total

¹³ The pH value of the sap varied but little except when lowered by the penetration of CO_2 and the difference in dissociation caused by this lowering may be neglected for our present purpose.

CO₂ even after standing 4 hours, as might be expected. Yet at other times we find practically 100 per cent with every evidence of normal condition of the cells. It is thought that this difference is not due to experimental error because there is no difficulty in getting consistent results with dead cells under these conditions.

In general it would seem that the concentration of total CO₂ inside the cell is approximately equal to the total undissociated H₂CO₃ (including free CO₂) outside. Possibly this would not be the case if the sap had much buffer action. The buffer action of the sap towards acids was found to be of a smaller order of magnitude than that of sea water. McClendon's excess base number¹⁴ for this sea water was 24.8¹⁶ for a typical sample and for the sap was less than 0.5.

Successive drops of 0.01 N HCl or Ba(OH)₂ were added to 1000 drops of sap, of distilled water, and of boiled KCl solution respectively, and the change in pH was measured by comparing with standard indicators. The sap has but little buffer action as is shown in Fig. 2. Its buffer action is of the same order of magnitude as that of a solution of KCl, of the same concentration of chloride from which most of the CO₂ had been driven off by boiling. According to the graph, 32 drops would change the sap from pH 8.0 to 6.2 and the sea water requires 200 drops for the same change.

We therefore seem justified in assuming that the total CO₂ inside the cell corresponds approximately to the total undissociated H₂CO₃ (including free CO₂) of the outside solution, and that the relations of the various ions in the sap are not complicated by the presence of any large amount of buffer.

If the facts found in the case of *Valonia* are generally valid we should expect that when the interior of any living cell is much more acid than the surrounding medium (the excess base being the same in both)

¹⁴ The excess base is determined by boiling off the CO₂ from the sea water and observing how much 0.01 N HCl must be added to 100 cc. of sea water to bring the pH back to the value it had before boiling: the number of cc. added is the excess base number. The excess base may be regarded as that part of the base which is not bound by strong acids and which in this case is largely bound by H₂CO₃. Cf. McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Pub. 251*, 1917, 31.

¹⁵ *I.e.* excess base = 0.00198 M.

the internal concentration of total CO_2 will be less than the external, providing the cell does not manufacture CO_2 rapidly enough to overcome the difference which would naturally exist at equilibrium. For example, in the case of *Valonia* we find that the total CO_2 content of the sap from cells in normal sea water (pH 8.2) is always less than that of the surrounding sea water. A typical analysis of cells that had

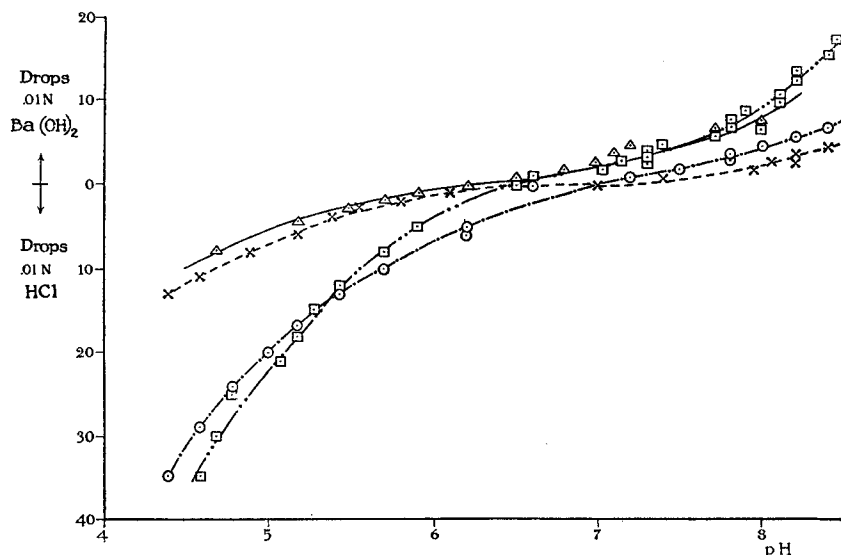


FIG. 2. Shows the buffer action of sap (\square), of 0.6 M KCl (\circ), of boiled distilled water (\times), and of unboiled distilled water from a wash bottle (\triangle). The ordinates give the number of drops (of 0.01 N HCl or 0.01 N $\text{Ba}(\text{OH})_2$) added to 1000 drops of the liquid. The curves are drawn free-hand to give an approximate fit. Each point represents one determination.

been 15 hours in a dark room showed that the sap had 0.009 cc. of total CO_2 per cc. of sap while the surrounding sea water had 0.038. A similar lot of cells after exposure to direct sunlight for 5 hours showed 0.004 cc. per cc. for the sap and 0.031 cc. per cc. for the sea water.

This has an important bearing on certain physiological problems . the study of which the assumption has frequently been made that the total CO_2 content of the cell at equilibrium is equal to that of the external medium.

Let us now consider another aspect of the penetration of CO₂ into *Valonia*. If we ignore the formation of CO₃⁼ ions and assume that some indiffusible ions are present so that a Donnan equilibrium is set up with H⁺ and HCO₃⁻ diffusing in and out freely, but not undissociated H₂CO₃ or CO₂ we might assume the relation

$$\frac{H^+ \text{ inside}}{H^+ \text{ outside}} = \frac{HCO_3^- \text{ outside}}{HCO_3^- \text{ inside}}$$

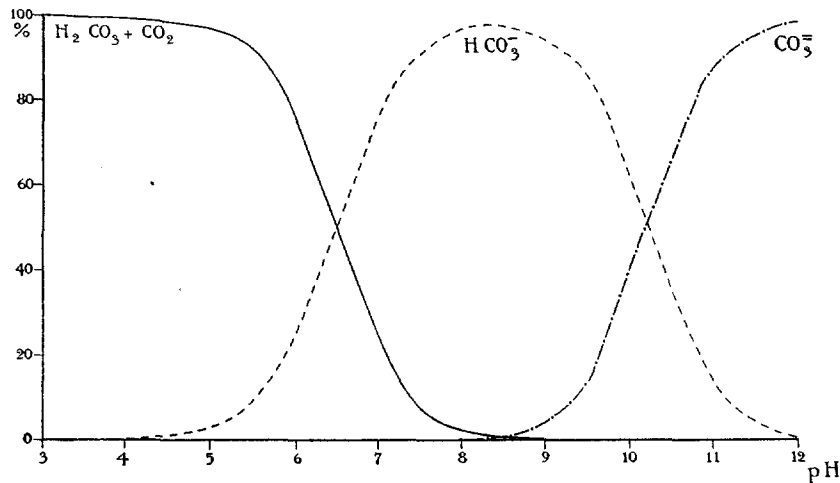


FIG. 3. Shows the per cent of undissociated H₂CO₃, including free CO₂, (—), of HCO₃⁻ (-----), and of CO₃⁼ (- · -) calculated from the formulæ:

$$\text{Per cent undissociated H}_2\text{CO}_3 \text{ (including free CO}_2\text{)} = \frac{100}{1 + \frac{K_1}{(H)} + \frac{K_1 K_2}{(H)^2}}$$

$$\text{Per cent HCO}_3^- = \frac{100}{1 + \frac{(H)}{K_1} + \frac{K_2}{(H)}}$$

$$\text{Per cent CO}_3^- = \frac{100}{1 + \frac{(H)}{K_2} + \frac{(H)^2}{K_1 K_2}}$$

in which $K_1 = 3.3 \times 10^{-7}$, and $K_2 = 6 \times 10^{-11}$.

All values are expressed as per cent of total CO₂.

Although this assumption may seem improbable,¹⁶ it may be interesting to ascertain to what values it would lead.

If we regard the internal pH value as practically constant at pH 5.8 we should have, when the outside pH value is 6,

$$\frac{H^+ \text{ inside}}{H^+ \text{ outside}} = \frac{10^{-5.8}}{10^{-6}} = 1.59.$$

TABLE I.

A Comparison of $\frac{H^+ \text{ Inside}}{H^+ \text{ Outside}}$ with $\frac{HCO_3^- \text{ Outside}}{HCO_3^- \text{ Inside}}$ at Various pH Values.

It is assumed that the inside pH is constant at 5.8, and that $HCO_3^- = 19$ per cent of total CO_2 in sap.

pH inside.	pH outside.	Total CO_2 in sap.	HCO_3^- in sap.	HCO_3^- in sea water.	$\frac{H^+ \text{ inside}}{H^+ \text{ outside}}$	$\frac{HCO_3^- \text{ outside}}{HCO_3^- \text{ inside}}$
5.8	5.8	77	$77(.19) = 14.63$	19	$\frac{10^{5.8}}{10^{5.8}} = 1$	$\frac{19}{14.63} = 1.299$
5.8	6.0	71	$71(.19) = 13.49$	25	$\frac{10^6}{10^{5.8}} = 1.585$	$\frac{25}{13.49} = 1.85$
5.8	6.55	24	$24(.19) = 4.56$	53	$\frac{10^{6.55}}{10^{5.8}} = 5.63$	$\frac{53}{4.56} = 11.62$
5.8	6.95	17	$17(.19) = 3.23$	74	$\frac{10^{6.95}}{10^{5.8}} = 14.13$	$\frac{74}{4.07} = 22.9$
5.8	8.0	5	$5(.19) = 0.950$	97.3	$\frac{10^{8.0}}{10^{5.8}} = 158.5$	$\frac{97.3}{0.950} = 102.4$

From Fig. 1 we find that the total CO_2 in the sap at pH 6 = 71 (regarding the total CO_2 in the sea water as constant at 100). For the purpose of comparing the HCO_3^- in sap and sea water at the pH values here considered we may assume that all the CO_2 is H_2CO_3 (in ionized or non-ionized form) and consider that 19 per cent of the total CO_2 in the sap (Fig. 3) is ionized at pH 5.8: we then have as the ionized portion $(71)(.19) = 13.49$. From Fig. 3 we learn that all of this may

¹⁶ Some objections to it have been stated in a previous paper.² The fact that the pH value of the sea water has been varied within wide limits (by adding HCl or NaOH) with little or no effect on the pH value of the sap indicates that H^+ ions do not diffuse in and out freely.

be regarded as HCO₃⁻. In the sea water we have at pH 6 (Fig. 3) HCO₃⁻ = 25. We therefore have

$$\frac{\text{HCO}_3^- \text{ outside}}{\text{HCO}_3^- \text{ inside}} = \frac{25}{13.49} = 1.85.$$

Proceeding in this manner we obtain the values given in Table I. The figures in the last two columns increase in somewhat the same fashion and the deviations are such as might result largely from experimental errors. The greatest discrepancy occurs where the total CO₂ inside deviates most from the values calculated from the dissociation constant (see Fig. 1).

It is evident that if it is the ions alone which enter, the rate of penetration will increase as the pH value of the external solution increases, while if it is only the undissociated molecules which enter, the rate of penetration will increase as the pH value of the sea water falls. The latter is found to be the case, and this would indicate that it is the undissociated molecules which enter unless for some reason the rate of penetration is proportional, not to the concentration of the penetrating substance in the sea water, but to its concentration at equilibrium in the sap, which appears improbable. It is of course possible that both ions and undissociated molecules enter but that the latter penetrate much more rapidly.

In order to arrive at the conclusion that ions enter, we are obliged to make improbable assumptions, and unpublished electrical experiments by L. R. Blinks make it difficult to believe that ions are able to penetrate. We may therefore conclude that little or no CO₂ enters except in the form of undissociated molecules.

In this connection it may be noted that the work of Loeb,¹⁷ Harvey,

¹⁷ Loeb, J., *Biochem. Z.*, 1909, xv, 255; 1910, xxiii, 95; *Arch. ges. Physiol.*, 1897-98, lxi, 1; 1898, lxxi, 457; Artificial parthenogenesis and fertilization, Chicago, 1913, 143; *J. Gen. Physiol.*, 1922-23, v, 231. Harvey, E. N., *Internat. Z. physik.-chem. Biol.*, 1914, i, 463; *Carnegie Institution of Washington, Pub. 212*, 1915. Crozier, W. J., *J. Gen. Physiol.*, 1922-23, v, 65, with references to earlier papers. Haas, A. R. C., *J. Biol. Chem.*, 1916, xxvii, 225. Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; 1920, liii, 457; *Biol. Bull.*, 1922, xliii, 14. Brooks, M. M., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 1449, 1470. Beerman, H., *J. Exp. Zool.*, 1924-25, xli, 33. Smith, H. W., and Clowes, G. H. A., *Am. J. Physiol.*, 1924, lxxviii, 183. Smith, H. W., *Am. J. Physiol.*, 1925, lxxii, 347.

Crozier, Haas, Jacobs, M. M. Brooks (dealing with *Valonia*), Beerman, Clowes, Smith, and others, on CO₂ and on various weak acids, indicates that undissociated molecules penetrate, although the methods employed do not enable us to decide positively whether ions enter or not. Those who have concluded that ions do not enter have done so on indirect grounds. This is also true to some extent where the opposite conclusion has been reached (*cf.* Smith and Clowes, and Van Slyke, Wu, and McLean).¹⁸

SUMMARY.

The experiments indicate that little or no CO₂ enters normal cells of *Valonia* except in the form of undissociated molecules.

Whenever the interior of a cell is more acid than the surrounding medium (excess base being the same in both) we may expect that at equilibrium the internal concentration of total CO₂ will be less than the external.

¹⁸ Smith, H. W., and Clowes, G. H. A., *Am. J. Physiol.*, 1924, lxviii, 183. Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.