IONIC RELATIONS OF CELLS OF *CHARA AUSTRALIS*

II. THE INDIFFUSIBLE ANIONS OF THE CELL WALL

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[*Manuscript received February* 19, 1960]

Summary

Studies of isolated cell walls from *Chara australis* have been extended to measure the concentration of the endogenous anions of the wall and the pK of the acids which ionize to give these anions. The concentration of indiffusible anions in the wall is 0·8 equiv/l when the external concentration of cations is 20 mN but may be higher when it is greater than this. The mean pK of the acids from which the wall anions are derived is 2·2.

The number of indiffusible anions in wall segments of different thickness is proportional to thickness which indicates uniform addition of wall anions during growth and thickening.

The effect of some chemical treatments on the number of exchange sites in wall segments is consistent with their origin being carboxyl groups of polyuronic acids. Chemical analysis has subsequently shown that 15 per cent. of the dry weight of walls is uronic acid. This is mostly in the form of unmethylated polygalacturonic acid but some of the uronic acid is found associated with hemicellulose and cellulose.

I. INTRODUCTION

In an earlier paper (Dainty and Hope 1959) evidence was given showing that most of the quickly exchangeable cations in internodal cells of *Chara australis* R. Br. var. *nobilis* A. Br. are to be found in the cell wall.

The free space of the wall was measured using radioactive iodide and mannitol and the cation exchange using ^{22}Na and ^{45}Ca. The kinetics of the exchanges of both cations and anions, and the amounts involved, strongly suggested that the cell wall was a complex system of indiffusible anions and could be considered as a Donnan free space (D.F.S.) and a water free space (W.F.S.). The exchange was complex in that the kinetics did not correspond to "single compartment exchange", indicating various degrees of accessibility of the exchange sites.

It is of great interest to try to identify the endogenous anions of the cell wall (the Donnan ions) with a particular chemical constituent.

The present paper is concerned with measurements of the concentration of the indiffusible anions and of the pK of the acids from which they are derived. Measurements have also been made of the number of exchange sites in walls of different physiological age and thickness, and finally, the effect of certain chemical treatments on the number of wall anions.

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II. Experimental

Reference should be made to Dainty and Hope (1959) for details of the material used. In nearly all the present experiments, 1-cm segments of isolated cell walls were equilibrated in inactive solutions for several days followed by an equal period in a labelled solution of the same concentration. Then, after blotting, the wall segments were dried on standard planchettes and their radioactivity determined by direct counting. The specific activity of the solution with which the wall had been in equilibrium was then measured by counting an aliquot of the solution, usually 20 μl, which was dried in the centre of the planchette, together with a "dummy" wall segment. Then, assuming equality of specific activity, the amount of a cation in a segment could be calculated from its radioactivity.

Usually three to five segments from different walls were measured and the mean taken.

Table 1

AMOUNTS OF EXCHANGEABLE SODIUM IN CHARA AUSTRALIS WALL SEGMENTS IN EQUILIBRIUM WITH EXTERNAL SOLUTIONS OF THE COMPOSITION SHOWN

Sodium Chloride Concn. (mN)	Calcium Chloride Concn. (mN)	Exchangeable Sodium (μ-equiv/cm)	Sodium Chloride Concn. (mN)	Calcium Chloride Concn. (mN)	Exchangeable Sodium (μ-equiv/cm)
5·00	0	0·080	4·70	0·30	0·028
4·97	0·03	0·065	4·00	1·00	0·016
4·90	0·10	0·041			

III. Results

(a) *Concentration of Indiffusible Anions in the Wall*

An average value for this concentration of 0·6 equiv/l of water in the D.F.S. was found by Dainty and Hope (1959) from the amount of exchangeable calcium divided by the apparent volume of water in the D.F.S. The latter was calculated from the difference between the iodide free space and the total water space in the wall (measured as mannitol free space). Since all these measurements are subject to experimental error, the above value for the indiffusible anion concentration in the D.F.S. is approximate and another determination by a different method was considered desirable.

In the following experiments the indiffusible anion concentration was calculated from the amount of exchangeable sodium (or calcium) in walls which had been equilibrated with solutions containing various mixtures of sodium chloride and calcium chloride. Wall segments were soaked for several days in renewed solutions of the composition shown in Tables 1 and 2. They were then blotted and transferred to a solution of the same total concentrations but labelled with ^{22}Na.

After 2–3 days in the radioactive solution the segments were blotted, dried, and counted. Table 1 shows the amount of exchangeable sodium in a typical experiment. The amount of sodium in the W.F.S. is negligible in comparison with that in the D.F.S.

The progressive decrease in exchangeable sodium as external calcium concentration, $[Ca_o]$, is increased is qualitatively as expected from a Donnan system. Appendix I(*a*) gives the method used to calculate A, the effective concentration of the indiffusible anions, from such data. Table 2 summarizes the values of A so calculated for total (sodium+calcium) concentrations of 5, 10, and 20 mN. The approximate constancy of A at a given total external concentration is consistent with our assumption of a Donnan equilibrium. The fact that A is less when the total external concentration is 5 mN than when it is 10 or 20 mN could be due to

TABLE 2

VALUES FOR THE APPARENT CONCENTRATION A OF INDIFFUSIBLE ANIONS IN CHARA AUSTRALIS CELL WALLS WHICH ARE IN EQUILIBRIUM WITH EXTERNAL SOLUTIONS OF THE COMPOSITION SHOWN

Sodium Chloride Concn. (mN)	Calcium Chloride Concn. (mN)	A (equiv/l)	Sodium Chloride Concn. (mN)	Calcium Chloride Concn. (mN)	A (equiv/l)	Sodium Chloride Concn. (mN)	Calcium Chloride Concn. (mN)	A (equiv/l)
4·97	0·03	0·45	9·9	0·1	0·74	19·9	0·1	0·96
4·90	0·10	0·41	9·7	0·3	0·88	19	1	0·74
4·70	0·30	0·36	9·0	1·0	0·76	18	2	0·61
4·00	1·00	0·30	7·0	3·0	0·79	15	5	0·69

several causes which will be considered in Section IV. The values of A at 10 or 20 mN are not significantly different from each other and the mean value of A at either of these two concentrations can be taken as 0·8 equiv/l. There are indications that the apparent A is greater at external concentrations greater than 20 mN (Dainty and Hope, unpublished data).

(*b*) *p*K *of the Cell Wall Anions*

Wall segments were equilibrated in solutions of sodium chloride brought to pH values between 2·2 and 8 by addition of McIlvaine's buffer (citric acid+disodium hydrogen phosphate). The total concentration of sodium was kept at 20 mN in each solution. The segments were then transferred to labelled buffered solutions of the same chemical composition (labelled with ^{22}Na) and, after sufficient time to come to equilibrium (2–3 days), were blotted, dried, and the radioactivity

counted as before. Figure 1 gives the total exchangeable sodium (in μ-equiv/cm of wall) as a function of the pH of the buffered solution. The relation is consistent with a decreased ionization of the indiffusible anions as the external pH is decreased (i.e. the acidity increased) such that half-maximum ionization occurs at an external pH of about 3·7. The amount of the sodium in the W.F.S. in these experiments was less than 0·01 μ-equiv/cm and can be neglected in comparison with that in the D.F.S.

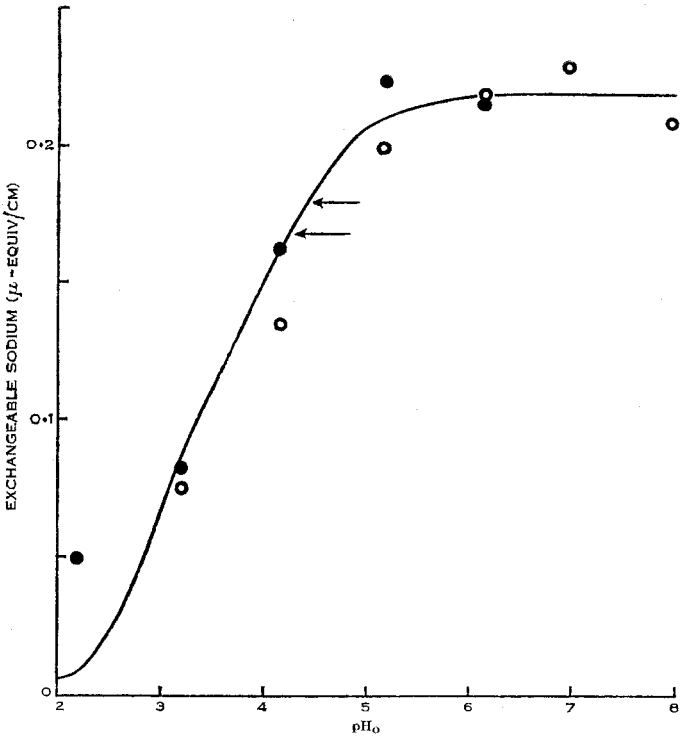

Fig. 1.—Amount of exchangeable sodium (in μ-equiv/cm of wall) plotted against the pH of buffer solutions, each containing 20 mN sodium (two experiments). The exchangeable sodium in wall segments in unbuffered 20 mN NaCl is indicated by the arrows. The line is the theoretical relation according to Appendix I(*b*), for a pK of 2·2 and a maximum concentration (a) of indiffusible anions equal to 0·8 equiv/l.

In Appendix I(*b*) a theoretical analysis is given of the amount of exchangeable sodium as a function of external pH (pH_o) on the assumption that all the anionic groups have, effectively, the same pK value. The curve shown in Figure 1 is the relation between the internal sodium concentration, [Na_i], and external pH assuming an indiffusible anion concentration (in the fully ionized state) of 0·8 equiv/l, a pK of 2·2, and an external sodium concentration, [Na_o], of 20 mN. Although this line is adjusted to give the same point of half-maximum ionization as the

experimental value, it is not a good fit at low pH. This could indicate that the wall contains a complex mixture of acids with different pK values.

(*c*) *Amount of Indiffusible Anions in Walls of Different Thickness*

The amounts of exchangeable calcium were measured in walls isolated from a strand of internodal cells of which the length and wall thickness increased progressively with distance from the apex. The thickness was calculated from the blotted weight, the density, and the area. The blotted weight and area were measured and the mean density of the walls was taken as 1·1 g/cm^3.

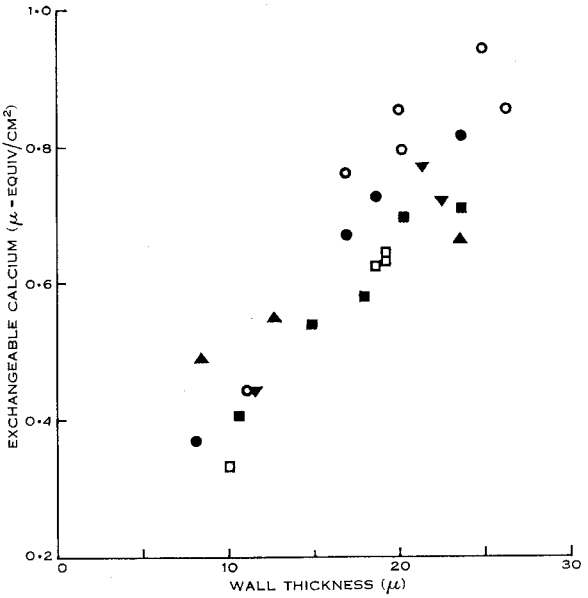

Fig. 2.—Amount of exchangeable calcium per unit area (in μ-equiv/cm^2) plotted against thickness of wall segments (μ). Each symbol represents a strand of cells from which the walls were isolated. In nearly all cases increase in wall thickness followed increase in cell length along the strand (from growing point downwards).

Figure 2 shows the exchangeable calcium per unit area plotted against the thickness of the wall. This gives a good measure of the number of indiffusible anions provided ionization is complete which is approximately true for [Ca_o] = 1 mN and p$K \leqq 2 \cdot 8$. There is an approximately linear relation between number of endogenous anions per unit area and thickness, which suggests that as a young cell elongates and thickens the new wall material laid down has the same composition as the older wall, as far as concentration of fixed anionic groups is concerned.

(d) Effect of Chemical Treatments on the Number of Wall Anions

The number of wall anions, as indicated by the total amount of exchangeable calcium, was measured in untreated wall segments 1 cm long and also in segments which had been given the following treatments:

(1) Boiled in distilled water for 12 hr. This treatment was designed to remove pectins of short chain length (Kertesz 1951).

(2) Segments treated with 0·05N HCl at 70°C for 1 hr then with 0·5 per cent ammonium oxalate at 70°C for 1 hr. This is a recognized procedure for dissolving protopectins and pectates (Ordin, Cleland, and Bonner 1955).

(3) Segments treated with 0·25 per cent. oxalic acid and 0·25 per cent. ammonium oxalate at 70°C for 1 hr. This treatment is supposedly more specific with regard to removing pectinic materials (Bishop, Bayley, and Setterfield 1958).

(4) Treatment (2) followed by 17·5 per cent. sodium hydroxide at 25°C for 4 hr. This treatment leaves only α-cellulose undissolved (Ordin, Cleland, and Bonner 1955), removing hemicelluloses as well as pectins.

TABLE 3

EFFECTS OF VARIOUS TREATMENTS ON THE TOTAL EXCHANGEABLE CALCIUM IN CHARA AUSTRALIS CELL WALLS

Results are expressed as percentage of exchangeable calcium remaining (untreated walls = 100 per cent.)

Treatment*	Exchangeable Calcium (% of untreated)	No. of Observations
Untreated	100	—
(1) Boiling water	92 ±2·5†	6
(2) HCl–ammonium oxalate	8·9±0·9	6
(3) Oxalic acid–ammonium oxalate	70·0±0·5	3
(4) HCl–ammonium oxalate–NaOH	4·4±1·2	6

* See p. 272 for further details.

† Standard error of the mean.

After these treatments the walls were rinsed and placed in calcium chloride solution for 2–3 days and then into labelled calcium chloride solution for the same time.

Table 3 gives the results of several such experiments. Treatment (1) had little effect on the amount of exchangeable calcium while treatment (2) removed more than 90 per cent. of the exchange sites with a 27 per cent. change in dry weight. Treatment (4) had a further small effect. Treatment (3) removed 30 per cent. of the exchange capacity of the walls. If treatment (3) is more specific than (2) in bringing pectinic materials into solution, it might be concluded from the

above experiments that the indiffusible anions of the wall are associated with long-chain protopectin materials (since treatment (1) was almost ineffective), and also with other hexose materials removed by the less specific treatment (2).

Any protein from cytoplasm remaining in the interstices of the wall would presumably have been denatured rather than removed by treatments (1), (2), and (3). Nevertheless treatment (2) removed 90 per cent. of the exchange capacity. In any case cytoplasm contributes very little to the quickly exchangeable cations (Dainty and Hope 1959; Diamond and Solomon 1959).

In addition to the measurements of exchange capacity after the above chemical treatments, control walls and wall material after treatment (2) were compared in respect of the amount of carbon dioxide evolved under mild hydrolysis (12 per cent. HCl at 130°C for 2 hr (Kertesz 1951, p. 36)). Untreated material produced carbon dioxide equivalent to 2 μ-equiv/mg dry wt., and walls after treatment (2) evolved 0·1 μ-equiv/mg dry wt. A further slow evolution of carbon dioxide occurred after the initial 2 hr, probably due to breakdown of hexoses (Kertesz 1951). The equivalent amounts of carbon dioxide, before and after treatment (2), are reasonably close to the exchange capacities of the walls measured directly, i.e. 1·3 μ-equiv/mg dry wt. for untreated walls and 0·12 μ-equiv/mg dry wt. after treatment (2). It is thus possible to conclude that the exchange capacity is derived from $-COO^-$ groups in the wall.

IV. Discussion

It is clear from the measurements of the amounts of exchangeable sodium in the cell wall as a function of the concentrations of sodium and calcium in the external solution that, at total external concentrations of 10–20 mN, the concentration A of indiffusible anions in the D.F.S. of the cell wall is about 0·8 equiv/l. This agrees quite well, considering the possible experimental errors, with the previous value of 0·6 equiv/l (Dainty and Hope 1959), obtained by a different method. Thus the analysis of the cell wall into a D.F.S. and a W.F.S. is confirmed, since the *average* concentration of indiffusible anions in the cell wall is about 0·3 equiv/l of total wall water.

With an external concentration of 5 mN, A was about 0·4 equiv/l. Several factors could give rise to the discrepancy between this and the value of 0·8 given above. The acids may not be completely ionized at an external salt concentration of 5 mN, and replacement of calcium by sodium, which is very slow (Dainty and Hope 1959), may not have proceeded to equilibrium; the latter is the more likely explanation since if as little as 0·02 μ-equiv. calcium per centimetre of wall was present in segments "equilibrated" in pure sodium chloride (5 mN), it can be shown that A would be raised to 0·65 equiv/l.

The chemical treatments (Table 3) and analysis suggest that the indiffusible anions of the cell wall are carboxyl groups of polyuronic acids, since the standard treatments to solubilize these acids resulted in the loss of about 90 per cent. of the cell wall exchange capacity. Further, the equivalent of the carbon dioxide evolved under mild hydrolysis (the standard method of estimation of uronic acid carboxyl groups) corresponded approximately to the observed exchange capacity

both in untreated and acid–oxalate-treated walls. Since this work was completed Anderson and King (personal communication) have kindly made a more detailed analysis of *C. australis* wall material supplied by the authors. Fifteen per cent. of the dry weight was uronic acid. This corresponds to 0·8 μ-equiv/mg dry wt., assuming an equivalent weight of 194. A reasonable correction for contamination by cytoplasm adhering to the wall preparation would raise this value by up to 40 per cent. Thus there is good quantitative agreement between the average concentration of uronic acids and the average concentration of indiffusible anions (measured by our ion-exchange studies) in the cell wall. The chemical analyses also indicate the complex nature of the uronic acids; they may be part of a pure galacturonic acid chain (pectin) or interpolated with sugar residues in a copolymer (hemicellulose); and a small number will be present in the so-called cellulose chains. None of the uronic acid groups are methylated.

The carboxyl groups may well be in different regions of accessibility and cause complex exchange kinetics (cf. Dainty and Hope 1959). This complexity is also suggested in the "titration" curve of Figure 1, which is not fitted altogether satisfactorily by a single pK value. However, the mean pK of 2·2 is reasonably close to the minimum quoted for pectic acid (2·8), considering the experimental errors.

The results shown in Figure 2 indicate that the cell wall of *C. australis* is built up uniformly in the sense that the uronic acid concentration remains constant throughout growth and thickening of the wall.

This work, then, supports the conclusions of the earlier paper (Dainty and Hope 1959) and underlines the complexity of the cell wall system. The indiffusible anions are predominantly ionized uronic acid carboxyl groups, some on polygalacturonic acids, some mixed with sugars in copolymers, and a few on the cellulose chains. Thus some of the groups will be close together and others relatively isolated.

One theoretical concept should be mentioned which will be published elsewhere. The terms D.F.S. and W.F.S. have quantitative meaning only at a single external solution concentration and depend on what kind of ions are in the external solution. For this reason the term "apparent free space" was first used by G. E. Briggs (unpublished data). The size of the D.F.S. is related to the width of the electric double layers near the fixed charges. This depends, in turn, on solution concentrations and to some extent on the valency of the ions. This is why it is stated that the D.F.S. concentration is 0·8 equiv/l at external solution concentrations of 10–20 mN. Only when the electric double layers overlap to a considerable extent can the D.F.S. be considered a homogeneous phase and only then is the use of classical Donnan considerations permissible.

V. Acknowledgments

Thanks are due to Dr. J. R. Vickery, Chief, Division of Food Preservation and Transport, C.S.I.R.O., and Professor R. L. Crocker, Botany School, University of Sydney, in whose Laboratories the work was carried out.

One of us (J.D.) gratefully acknowledges receipt of a Royal Society–Nuffield Commonwealth Bursary.

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Appendix I

(*a*) *Calculation of the Indiffusible Anion Concentration, A*

From data on the change in exchangeable sodium as the $[Ca_o]/[Na_o]$ ratio is changed we have, in the D.F.S., the Donnan distribution equation

$$\frac{Ca_i/v}{[Ca_o]} = \left(\frac{Na_i/v}{[Na_o]}\right)^2, \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where square-bracketed symbols represent concentrations and unbracketed symbols amounts, and v is the volume of the D.F.S. For consistency with earlier publications A is unbracketed.

Since the mobile anion concentration in the D.F.S. is negligible, then

$$A = [Ca_i]+[Na_i], \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

the concentrations being in equiv/l. Eliminating v it can be shown that

$$A = (Ca_i+Na_i).Ca_i.[Na_o]^2/(Na_i^2.[Ca_o]). \quad \ldots\ldots\ldots (3)$$

Since $[Na_o]$ and $[Ca_o]$ are known and Ca_i and Na_i are measured (i.e. Na_i directly, (Ca_i+Na_i) is taken as equal to Na_i when $[Ca_o] = 0$, and hence Ca_i by difference), A may be calculated. The sodium in the W.F.S. of the wall is negligible in comparison with that in the D.F.S.

(*b*) *Calculation of Exchangeable Sodium as a Function of External pH in Buffer Solutions*

The following equations enable $[Na_i]$, the concentration of sodium in the D.F.S., to be calculated in terms of known quantities:

$$[Na_i]/[Na_o] = [H_i]/[H_o], \quad \ldots\ldots\ldots\ldots\ldots (4)$$

$$A = [H_i]+[Na_i], \quad \ldots\ldots\ldots\ldots\ldots (5)$$

$$A+[HA] = a, \quad \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

$$[H_i].A/[HA] = k. \quad \ldots\ldots\ldots\ldots\ldots\ldots (7)$$

Equation (**4**) is the Donnan distribution equation, (**5**) expresses electrical neutrality in the D.F.S. (mobile anions can be neglected), (**6**) states that the maximum indiffusible anion concentration is a, while (**7**) expresses the ionization of the weak acid HA into H_i^+ and A^- with dissociation constant k.

It can be shown that

$$[\mathrm{Na}_i] = \tfrac{1}{2}\left\{\left[\frac{k^2[\mathrm{Na}_o]^2}{[\mathrm{H}_o]^2}+\frac{4k\alpha[\mathrm{Na}_o]^2}{[\mathrm{H}_o]([\mathrm{H}_o]+[\mathrm{Na}_o])}\right]^{\frac{1}{2}}-\frac{k[\mathrm{Na}_o]}{[\mathrm{H}_o]}\right\}. \quad \ldots.(8)$$

In Figure 1 the curve is the relation between $[\mathrm{Na}_i]$ and pH_o, assuming $\alpha = 0{\cdot}8$ equiv/l (Section II(α)), $k = 6{\cdot}3\times10^{-3}$ ($\mathrm{pK} = 2{\cdot}2$), and $[\mathrm{Na}_o] = 20$ mN (constant).