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The Affinity of Trichomes of Blue-Green Algae for Calcium Ions[1]

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ABSTRACT: Cells of *Plectonema boryanum* and *Anabaena flos-aquae* obtained from large scale (to 15L) cultures were used to examine the cation exchange properties of killed algal cells.

Since *Microcoleus lyngbyaceus*, one of the most abundant species in our collections from marshes, could not be obtained in large-scale liquid cultures, it was collected in algal mats from tide-marsh pannes and separated from other algae, etc. by a combination of screening, air elutriation and density-gradient centrifugation. Characterization of the exchange properties of such preparations was pursued using ^{45}Ca for exchange measurements and Cd-EDTA or Cd-EGTA as indicators with the Cd-specific ion electrode for electrometric titration for total Ca^{++} and Mg^{++}, or Ca^{++}, respectively.

Algal cell preparations exhibited a capacity to exchange Ca^{++} readily with ambient aqueous solutions. K^+ and Na^+ could displace a substantial proportion of the Ca^{++} at concentrations about 100-fold greater than that of the ambient Ca^{++}. Mg^{++} was about 10 times as effective as the monovalent cations in displacing Ca^{++}. Cell walls isolated from rhizomes of *Spartina alterniflora* exhibited only about 1/10 the affinity for Ca^{++} as that found for the blue-green algal preparations. The intrinsic association constant and the total capacity for cation exchange indicated that in solutions of 2×10^{-4}M about 50% of the exchange sites are occupied.

The ligand for Ca^{++} could be extracted in part by dilute acid, but not by Triton X-100 or by EDTA, even in the presence of Triton X-100. The extracted material appeared to be a polysaccharide. Both the sheaths of *M. lyngbyaceus* by themselves and cells from which they were separated exhibited Ca-exchange properties.

Introduction

The purpose of this reasearch was to contribute to an understanding of tide marsh ecosystems. Blue-green algae were selected for study for a variety of reasons:

1. They were known to be ubiquitous inhabitants of tide marshes.
2. They have more-or-less unique attributes which suggest they might be important elements in marsh ecosystems, e.g.:
   a. Many species fix atmospheric N_2 (Holm-Hansen 1968; Schwimmer and Schwimmer 1968; Zarnecki 1968).
   b. Blooms of these algae are frequently reported in response to various pollution loads (Sawyer 1970).
   c. At least some species produce toxins which have deleterious effects upon other organisms (Holm-Hansen 1968).

Attention was focused upon possible role of these algae in Ca-cycling in tide marshes

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because: 1) The reported biochemical properties of blue-green algal cell walls and/or sheaths indicated that they might exhibit a high affinity for this ion. Specifically, staining with Ruthenium Red and Alcian Blue (Leak 1967; Osetrov, et al. 1967) indicated the presence of acidic polysaccharides and suggested the presence of pectin. 2) The high concentration of Na^+ in the sea water which periodically inundates tide marshes might be expected to largely displace Ca^{++} from exchange sites in marsh soils which might pose a problem with respect to Ca-nutrition of marsh grasses, etc.

Basically our rationale in testing this hypothesis was to identify what species of blue-green algae grow upon tide marshes, and their abundance (Ralph 1975), and to determine whether or not such algal cells did in fact have an intrinsic affinity for Ca^{++}, even after the cells were dead. (Unless they bound calcium reversibly after death they could hardly serve as a nutrient pool. Living algal cells would be expected to compete effectively with cells of other organisms for Ca^{++} by metabolic processes.) By quantifying and characterizing such intrinsic affinity and combining this information with the distribution and abundance of blue-green algae on the marsh, one might ultimately get some estimate of the size of the Ca^{++} pool provided by these organisms and an indication of whether or not such a nutrient pool could supply Ca^{++} to the environment.

The cell walls of higher plants contain pectins of which the —COOH groups (Carbon 6) are at least partially methoxylated (Bonner 1950; Dever, et al. 1968; Keegstra, et al. 1973). Ca^{++} exhibits very selective exchange equilibria with the free carboxyl groups (Deuel and Stutz 1958; Kohn and Furda 1967; Kohn 1968). Roots of plants have been demonstrated to exhibit Ca-exchange properties and this has been thought to be related to the affinity of pectin for Ca^{++} (Williams and Coleman 1950; Steward and Sutcliffe 1959; Bould and Hewitt 1963; Fried and Broeshart 1967).

With this background knowledge it seemed worthwhile to examine the Ca^{++} exchange properties of blue-green algal cells.

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Materials and Methods

Axenic cultures of *Plectonema boryanum* [=*Schizothrix calcicola* (Agardh) Gomont, Drouet 1968] were obtained from Miriam Shane and of *Anabaena flos-aquae* [=*Microcoleus vaginatus* (Vaucher) Gomont, Drouet 1968] from Diane Herson (Franklin). Field samples were collected from the Canary Creek Marsh near Lewes, Delaware.

Large scale cultures (to 15L) were grown in standard laboratory glassware in a modification of Chu's medium No. 10 (Chu 1942) and were aerated with a stream of air filtered through a bacteriological filter. Routine culturing in nutrient media demonstrated the continuing axenic condition of cultures used for chemical analyses. Stock cultures were maintained in 250 ml flasks (100 ml medium) and were transferred to fresh medium frequently to maintain them in an actively growing condition. Cultures were routinely grown in an illuminated room in which the temperature was mostly 25 to 27 C; however, during the summer months maxima of 30 to 36 C were recorded frequently. Reference cultures were maintained on agar slants in an illuminated refrigerator (3 C).

The algal cells grown in laboratory cultures were harvested by decanting the clear medium after the cells had settled, centrifuging the residue and washing the cells with water, methanol and acetone. The dried cells were stored in the dark over a desiccant.

Failure to obtain liquid cultures of algae isolated from the marshes and concern for physiological changes which might result from culturing under laboratory conditions

prompted consideration of an alternative approach. Thick mats containing various species of blue-green algae were peeled from the surface of soil in natural marsh pannes. By midsummer to early autumn these mats were sufficiently coherent to be harvested with minimal inorganic contamination, particularly if obtained from pannes in remote areas of a marsh. Such mats collected in 1972 from Canary Creek marsh were stored for several weeks to a few months in an illuminated refrigerator (3 C). Microscopic examination revealed no change in the condition of mat components during storage. From these, algal preparations were obtained for various chemical studies (Somers 1975). The raw mat material was washed in 70% methanol, was dried in methanol and acetone and ground in a mortar and pestle. The resulting powder was passed several times through a linear air elutriation apparatus (Somers 1975) consisting essentially of a series of flasks of increasing size. The algae could be made to accumulate in any of the several flasks by varying the air flow rate. The algae could be almost completely separated from other constituents of the mats in this manner. By varying the rate of flow in the final elutriation, it is possible to gain a rough separation of dominant species.

The preparations from the air elutriator were further purified by density gradient centrifugation using tetrabromoethane-methanol mixtures (Somers 1975). By taking care not to overload the gradients and with a suitable range in density, various species of algae could be separated. However, for the most part we used preparations which were representative of the mixture of species found naturally in the mat when stripped from the panne.

During the summer of 1973 (August 10) additional mats much freer from inorganic material were collected from more remote pannes on Canary Creek marsh. These were stored for 3 days in an illuminated refrigerator which maintained them in good condition. Then they were characterized microscopically and were stored for a few weeks to a few months at about −15 C. Subsequently clean algal trichomes, of *Microcoleus lyngbyaceus* (Kütz) Cr., i.e., unialgal and free from inorganic contamination, were obtained from such material by P. K. Nelson (Somers 1975). The technique involved washing in cold artificial sea water at low speed in a blender and subsequent washing of the trichomes while they adhered to solid support. The resulting algal trichomes were solely *M. lyngbyaceus* free of diatoms, inorganic material or other debris. No bacteria were evident by microscopic examination though no doubt some were present. Such cleaned algae were stored, without drying, at −15 C.

These washed samples of living *M. lyngbyaceus* were used to separate sheaths and cells. The trichomes could be fragmented and the contents discharged from the sheaths in two ways: (1) Shaking with sand in a high speed vibrator (Nossal). One gram algae (wet weight) was mixed with 8 g "standard" Ottowa sand (Matheson-Coleman-Bell) and 3 ml of 0.3 M NaCl. This mixture was shaken for 15 sec in a stainless steel chamber (18 ml). Sheaths could be washed from the sand and recovered by filtering with a 250 mesh screen (openings 58μ). (2) Homogenizing in 0.2 M NaCl with a Ten Broeck Tissue Grinder (Pyrex no. 7726) using ice in the inner chamber. Sheaths could be recovered from the homogenates by washing and screening using a 250 mesh screen and cells recovered by using a 325 mesh screen (openings 44μ). Material held on the screens was further washed and collected as pellets by centrifuging. These pellets were then frozen until a sufficient quantity had been accumulated. They were washed twice in 0.1 M NaCl at 4 C and then washed twice with methanol, transferred to a sintered glass filter, washed with acetone, dried at room temperature and stored in the dark over a desiccant. Aliquots were used to measure the affinity of separated sheaths and cells for Ca^{++} and the action of pectin esterase upon Ca^{++} binding.

Cell walls for comparison with algal cells were prepared from rhizomes of *Spartina alterniflora* Loisel., harvested from brackish Delaware marsh (St. Jones) in a manner similar to that used for onion cell walls (Somers 1973). This involved repeated grinding in ice water until all of the cells were broken, extraction with a cold 50% (w/v) aqueous urea solution, washing with

methanol and acetone and drying at room temperature.

Calcium content of trichome preparations was determined by chelometric titration with K_2EDTA (ethylenediaminotetraacetic acid) using the Cd-specific ion electrode and CdEDTA as an indicator in the presence of 0.2 M NH_4OH. With this titrant Ca^{++} and Mg^{++} were titrated. Using K_2EGTA [ethylene bis (oxyethylene nitrilo) tetraacetic acid] and CdEGTA indicator with this electrode, Ca^{++} was determined quantitatively, even in the presence of comparable concentrations of Mg^{++}. Gran's (1952) plotting techniques were used to determine endpoints. Titration of known solutions of these ions in concentrations comparable to those used in the algal studies gave expected values within 1 to 2%.

All of the Ca^{++} can be removed from onion cell wall preparations by washing them with acidified ethanol (60% ethanol containing 5%, v/v, concentrated HCl) (Somers 1973). However, repeated washing with such acid-alcohol did not remove all of the Ca^{++} from dried blue-green algal cells. As a result, the rather simplistic approach to the determination of an association constant used for onion cell walls (Somers 1973) could not be used with these algae. Hence, we had recourse to the analysis of multiple equilibria for the binding of small ions to proteins suggested by Klotz (1953; see also Steinhardt and Beychok 1964). In this:

$$\frac{1}{r} = \frac{1}{nK(A)} + \frac{1}{n} \qquad (1)$$

where r is the moles of bound cation, A, per mole of total protein; n is the total number of available binding sites and K is the intrinsic association constant. (A) is the activity of the cation in solution.

It was found, as expected, that added $^{45}Ca^{++}$ rapidly equilibrated with Ca^{++} bound to algal cells. ("Bound" here refers to that Ca^{++} which had not been removed by the repeared washings used in processing the cells. It does not influence the potential of a Ca-specific electrode.) Using this we could estimate the distribution of Ca^{++} between the solution and the algal cells and by graphic means could estimate K and n. Obviously we cannot speak of moles of Ca^{++} bound per mole of algae, but presumably the amount of ligand present is proportional to the weight of the cells. Hence, we computed K and n in terms of weight. The most reliable results were obtained (Somers 1975) when ^{45}Ca was counted in a cocktail containing a thixotropic gelling agent (Cab-O-Sil, Packard Inst. Co.). Ambient temperature scintillation counters were used throughout.

Results and Discussion

Characterization of Calcium Exchange Properties – Cultured Algae

Dried algal cells were used in a manner similar to that found suitable for onion cell walls (Somers 1973) to relate Ca^{++} uptake to free $—COO^-$ groups. For this purpose the cells were washed with acidified ethanol as recommended by Henglein (1955) to remove Ca^{++} from pectin. The excess acid was washed out and the cells dried once more before being used. The free acidic groups were titrated to pH 7 with KOH and the uptake of Ca^{++} was measured with the calcium-specific ion electrode as for onion cell walls. If one assumes that equation 1, above, is appropriate in this case, the following, by analogy with pectin, should apply (Kohn and Furda 1967; Kohn 1968):

$$\frac{1}{r} = \frac{1}{n^*K(A)} + \frac{1}{n^*}$$

where n^* is the number of binding sites per "repeated segment" of the Ca-ligand, and

r = moles bound Ca^{++}/total concentration of repeating segments.

Assume that for binding of Ca^{++} each effective repeating segment contains two —COOH groups. The total concentration of repeating segments then equals:

$$\frac{[-COOH]\,\text{total}}{2},$$

a value provided by titration of the acidified cells. Neglecting any calcium not removed

from the cells by acid washing the calcium electrode measures the $(Ca^{++})_{free}$ and that portion of the added Ca^{++} which is bound can be computed from the change in potential resulting when cells are added to a solution of known concentration. By adding several increments of cells and $CaCl_2$ several measures of these parameters can be obtained. By plotting 1/r versus $1/[Ca^{++}]$ one should obtain a straight line with intercept, n*.

As defined above for this case:

$$\frac{1}{r} = \frac{[-COOH]\,\text{total}}{2X\ \text{moles Ca bound}}$$

Such a relationship for acid-washed, laboratory-grown *Plectonema boryanum* cells which contained about 0.27 moles free —COOH per kilogram dry weight is shown in Fig. 1. While the data for the two samples apparently differ in slope, in both cases they extrapolate reasonably to n* = 1, i.e. two $-COO^-$ groups per Ca^{++} bound, for those sites which are free after acid washing. A similar value was found for onion cell walls (Somers 1973) and for acid washed pectin (Kohn and Furda 1967; Kohn 1968). However, we found that acid-washed algal cells still contained bound Ca^{++} which could

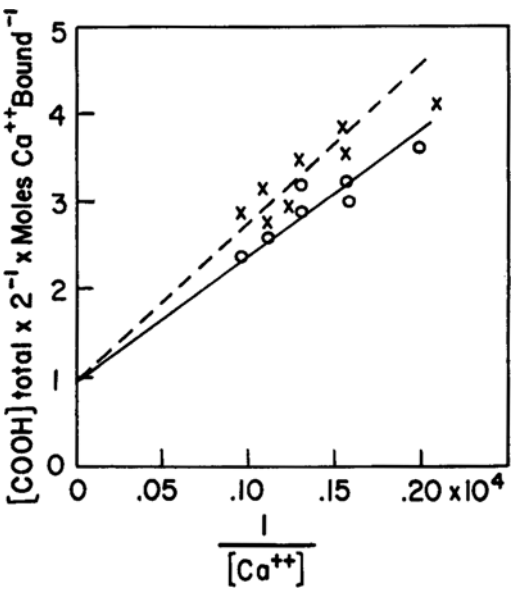

Fig. 1. Plot of 1/r vs 1/(A) for acid washed cells of *Plectonema boryanum* to test the hypothesis that each mole of Ca^{++} reacts with 2 moles of COOH.

TABLE 1. Composition of natural algal preparations as used after purification by air elutriation and centrifugation in density gradients (methanol-tetrabromoethane mixtures), based upon microscopic examination of random samples.

Preparation 72-1:

Species present	% of total number of cells[a]
Microcoleus lyngbyaceus	49
Schizothrix calcicola	15
S. Arenaria (Berk.) Gom.	10
Anacystis montana (Light f.) D&D	26
Spartina sp.	0

Preparation 72-2:

Species present	% of total volume of cells
Microcoleus lyngbyaceus	90
Schizothrix calcicola	0
S. arenaria	10
Anacystis montana	0.1
Spartina sp.	0.1

[a] Tends to underestimate *M. lyngbyaceus* about 10% on a volume basis and to overestimate the smaller-celled species by about 50% on a similar basis.

be removed by EDTA and could be detected by Eriochrome Black T. Pectin and onion cell walls do not. Obviously the algal cells fix Ca^{++} in such a manner that not all of it is removed by washing with acidified alcohol. Hence, computation of K from the slopes given in Fig. 1 is unreliable, at least insofar as *total* binding reactions are concerned. Moreover, the action of pectin esterase (pectin pectyl hydrolase, E.C. 3.1.11, Worthington Biochemical Corp., Freehold, N.J.) enhanced Ca^{++} binding in onion cell walls (Somers 1973). This enzyme had no effect upon Ca^{++} uptake by dried blue-green algal cells.

Characterization of Calcium Exchange Properties—Algae Harvested from Tide Marsh

For further characterization of the calcium-exchange properties, dried trichomes prepared from material collected from a panne in a tide marsh were used. The composition of two preparations used extensively for further studies is shown in Table 1.

Both preparations were largely *Microcoleus lyngbyaceus,* particularly on a volume basis. The trichomes of this species had

thick sheaths. When preparation 72-1 was titrated with EGTA using the Cd-specific ion electrode and CdEGTA indicator (for calcium content) and with EDTA with the same electrode and CdEDTA indicator (for calcium and magnesium content) the composition was found to be:

0.340 moles Mg/kg
0.107 moles Ca/kg.

Replicate titrations agreed closely (total range about ±5%) and recovery of added Ca^{++} and Mg^{++} was equally good, or better.

Solubility of Ca Ligand

Using preparation 72-1 it was found that the Ca-binding sites were not extracted by Triton X-100 (1.96% v/v) or K_2EDTA (pH 7, 0.005M). Cells were labeled in solution by exchange with ^{45}Ca at tracer levels. No additional Ca was added. The amount of ^{45}Ca exchanged was determined by centrifuging down the cells and counting aliquots of the supernatant. The Triton X-100 was then added and stirred with the cells for 10 min and ^{45}Ca in the solution was determined once again. Then K_2EDTA was added (Triton X-100 not removed) and the solution was again stirred for 10 min, centrifuged and aliquots counted once more. The Triton-X and EDTA were then washed out and the cells again allowed to exchange with ^{45}Ca as at the beginning of the experiment. The results of one experiment are shown in Table 2. This experiment was repeated twice more, doubling the length of exposure to Triton X-100 and K_2EDTA. Because of some problems in counting the isotope, a situation which was corrected in subsequent experiments by adding Cab-O-Sil, the results were somewhat erratic. In every case, however:

1. Triton X-100 extracted 5% or less of the counts.
2. The ^{45}Ca on the cells after exposure to EDTA *and* Triton X-100 was 1% or less of the original.
3. The uptake of ^{45}Ca by the cells after washing out the EDTA and Triton X-100 was from 97 to 130% (average = 113) of that of the cells at the start of the experiment.

TABLE 2. Effects of Triton X-100 and K_2EDTA upon the binding of ^{45}Ca by algal preparation 72-1 (see text for details).

Treatment	Bound cpm/mg algae
Original cells	248,000
Exposed to Triton X-100 for 10 min	238,000
Exposed to K_2EDTA and Triton X-100 for additional 10 min	1,600
K_2EDTA and Triton X-100 washed out and cells again allowed to exchange with ^{45}Ca	241,000

Some enhancement of Ca^{++} binding following EDTA treatment would be expected to result from the removal of Mg^{++} from the cells by this treatment since both of these cations would presumably have somewhat similar affinity for the same sites. This condition, in fact, was noted repeatedly. Thus it appears clear: (1) that 1.96% Triton X-100 does not extract the ligand; (2) that 0.005 M EDTA (pH 7) extracts all of the bound Ca; but (3) does not extract the ligand even in the presence of Triton X-100.

It was found, however, that a dilute aqueous acid solution could extract, irreversibly, a substantial proportion of the ligand. For this study 100 mg of preparation 72-1 were stirred in 10 ml of H_2O at about 4 C in the presence of ^{45}Ca. No cold Ca was added, except for that on the cells. Within 1 hr about 87% of the ^{45}Ca had been taken up by the cells and in 3 additional hours no more was absorbed. The labeled cells were washed with methanol, followed by acetone and allowed to dry at room temperature. The dried cells were stored in the dark in a desiccator at room temperature.

A 5 mg portion of the ^{45}Ca-labeled cells was stirred in 5 ml of H_2O for 10 min. The pH was measured, the cells were centrifuged down and aliquots of the supernatant were removed for scintillation counting. The cells were again suspended and serial aliquots of 1N HCl were added, followed by stirring for 10 min and subsequent counting of the supernatant, until the pH reached 2. Then 1N KOH was added in a similar fashion to pH 10. Finally an excess of K_2EDTA was added (pH 8 or 10) and aliquots of the supernatant were removed

for counting to measure the total ^{45}Ca remaining on the cells. These treatments released ^{45}Ca from the cells into the solution as acid was added. Only a small part of the ^{45}Ca was taken up again as the pH was increased (Table 3). The results of four other experiments were similar. Control studies demonstrated that neither evaporation of the test solution from the open tubes nor milling of the algal cells during mixing into particles not thrown down by centrifugation could explain these results.

The failure of the acid to remove all of the ^{45}Ca from the cells is consistent with the observation that acid-alcohol fails to remove all of the Ca from algal cells. The failure of the cells to adsorb all of the released ^{45}Ca when the pH is raised could be explained in either of two ways: (1) Some of the ligands have been extracted from the cells. (2) Competition of the K^+ (added as KOH) for the exchange sites. Actually, probably both factors were effective (see below). Acid does extract ligand material and K^+ does compete with Ca^{++} for exchange sites on the algal cells. However, only a small part of the failure of these cells to take up ^{45}Ca upon neutralization of the acid could be explained by competition by K^+ (Table 4).

Cation Competition

The competition of various cations for the Ca-exchange sites was measured using preparation 72-2. Each of several 5 mg portions was suspended in 15 ml centrifuge tubes in deionized water to which was added NaCl, KCl or $MgCl_2$ to produce 5 ml of the desired final concentration. ^{45}Ca was added in tracer amounts and the contents were mixed for at least 20 min before the cells were centrifuged down and aliquots of the supernatant were counted. Control aliquots were taken from similar tubes without algae or salts.

TABLE 3. Irreversible release of ^{45}Ca from labeled algal cells by acid.

1 pH	2 10^5 cpm/ml solution	3 10^5 cpm/mg cells	3/2
6.9[a]	2.01	10.3	5.12
6.6	2.11	10.2	4.83
4.2	3.81	8.41	2.21
2.1	6.63	5.36	0.81
2.4	7.23	4.67	0.64
2.8	7.23	4.65	0.64
3.1	7.89	3.94	0.50
4.7	5.98	5.95	0.99
5.4	6.68	5.20	0.78
6.4	5.93	5.98	1.01
7.0	6.17	5.73	0.93
7.1	5.74	6.17	1.07
8.1	6.25	5.63	0.90
9.3	5.64	6.24	1.11
9.9	6.0	5.83	0.97

[a] Initial pH of cell suspension.

TABLE 4. Means and standard errors of the Ca^{++} content of algal cells in the presence of various salts. The cells were suspended in various salt solutions at the concentrations shown (plus trace amounts of ^{45}Ca). After equilibration the cells were centrifuged down and aliquots of the supernatant were counted for ^{45}Ca content.

Salt	10^5 cpm/mg dry wt		
None	4.96 ± 0.07		
	10^{-3} M[a]	10^{-2} M	10^{-1} M
NaCl	5.01 ± 0.12	4.48 ± 0.14	1.54 ± 0.31
KCl	5.06 ± 0.05	4.18 ± 0.13	2.30 ± 0.47
	10^{-4} M	10^{-3} M	10^{-2} M
$MgCl_2$	4.87 ± 0.10	3.20 ± 0.22	0.91 ± 0.47

[a] Concentration of salt solution.

The results show clearly that in the absence of other cations the cells take up substantial ^{45}Ca (Table 4). The presence of Na^+, K^+ at 10^{-3} M and Mg^{++} at 10^{-4} M had no influence upon the amount of ^{45}Ca taken up. Even at 100-fold higher concentrations an appreciable portion of the Ca was not displaced. Mg^{++} was as effective as K^+ and Na^+ at 10-fold lower concentrations in displacing Ca^{++}. Note that 0.01M K^+ displaced only a small portion of the ^{45}Ca. This is about the concentration of K^+ which resulted from neutralizing the acid in the pH studies above. The total Ca^{++} and Mg^{++} content of this preparation was similar to that of 72-1.

The cation competition study was repeated using 10^{-3} M $CaCl_2$, instead of H_2O to suspend the cells, to which was added other salts as above. Separate measurements demonstrated that at this concentration the binding of Ca^{++} was proportional to the weight of the cells, a relationship which did not always apply at lower concentrations. The algal preparation used was 72-1. The cells became labeled with ^{45}Ca to

about the same extent as observed above. In the absence of competing ions, about 30% of the total Ca was removed from the solution by the cells. The results are presented in Table 5 (mean of 3 experiments) and are similar to those above in which the cells were suspended in H_2O instead of in 10^{-3} M $CaCl_2$. The Ca content of the cells was included in the calculations.

A similar experiment was conducted with cell walls prepared from rhizomes of *Spartina alterniflora*. Several 20 mg aliquots were suspended in 5 ml of 10^{-3} M $CaCl_2$ and cation competition studies were carried out using Na^+, K^+ and Mg^{++} as for the algal preparation 72-1, above. Higher speed centrifugation and special care to avoid floating particles (presumably cutinized epidermis) were required. The floating particles were removed with suction after centrifuging. The Ca content of the cell wall preparation which was 0.0253 moles/kg was included in the calculations. The data presented are the means of two experiments with one sample counted in duplicate for each salt concentration (Table 6). A comparison with the data for algal cells (Table 5) shows some interesting differences. The amount of Ca bound by the cell walls from rhizomes is only about $^1/_{10}$ that of the algal cells per unit dry weight. Moreover, 0.1M Na^+ and K^+ appear to be more effective in displacing Ca^{++} from the higher plant cell walls than from the algal cells. Mg^{++} also may be more effective in displacing Ca from the rhizome cell walls than from algal cells.

Pretreatment with $CaCl_2$

An apparent time dependence of the uptake of Ca^{++} by algal cells in some experiments suggested that perhaps $CaCl_2$ was somehow reacting with cell components to modify the exchange equilibrium or that the ultimate equilibrium was attained only slowly. To test this, six concentrations of $CaCl_2$ were used: 10^{-3}, 5×10^{-4}, 4×10^{-4}, 2×10^{-4}, 1.5×10^{4} and 10^{-4} M with the algal preparation 72-1. For the controls, 5 mg of algae were weighed into each of six 20 ml polyallomer centrifuge tubes, after which a 5 ml aliquot of $CaCl_2$ of the appropriate concentration, containing 2 μc ^{45}Ca, was added to each. Without delay the contents of each tube were mixed (Vortex) repeatedly in turn for 5 min. The tubes were then centrifuged at 7500 g for 20 min and an aliquot of the supernatant was removed for counting. The tube contents were again mixed for the desired time; centrifuging and aliquoting for counting were repeated. In this fashion the tube contents were mixed, centrifuged and sampled for various periods up to 300 min. In computing the results, adjustment was made, as usual,

TABLE 6. Ca^{++} content of cell walls of rhizomes of *Spartina alterniflora* in the presence of various salts. The cell walls were suspended in 10^{-3} M $CaCl_2$ (plus trace amounts of ^{45}Ca) to which was added other salts in various concentrations. After equilibration the cell walls were centrifuged down and aliquots of the supernatant were counted for ^{45}Ca content.

Salt	moles Ca^{++}/kg cell walls		
10^{-3} M $CaCl_2$	0.040		
	10^{-3} M[a]	10^{-2} M	10^{-1} M
10^{-3} M $CaCl_2$ + NaCl	0.039	0.023	0.009
10^{-3} M $CaCl_2$ + KCl	0.036	0.025	0.007
	10^{-4} M	10^{-3} M	10^{-2} M
10^{-3} M $CaCl_2$ + $MgCl_2$	0.039	0.027	0.013

[a] Concentration of salt other than $CaCl_2$.

TABLE 5. Means and standard errors of the Ca^{++} content of algal cells in the presence of $CaCl_2$ and various other salts. The cells were suspended in 10^{-3} M $CaCl_2$ (plus trace amounts of ^{45}Ca) to which was added other salts in various concentrations. After equilibration the cells were centrifuged down and aliquots of the supernatant were counted for ^{45}Ca content.

Salt	moles Ca/kg cells		
10^{-3} M $CaCl_2$	0.327 ± 0.007		
	10^{-3} M[a]	10^{-2} M	10^{-1} M
10^{-3} M $CaCl_2$ + NaCl	0.32 ± 0.007	0.307 ± 0.004	0.158 ± 0.007
10^{-3} M $CaCl_2$ + KCl	0.315 ± 0.016	0.318 ± 0.010	0.173 ± 0.009
	10^{-4} M	10^{-3} M	10^{-2} M
10^{-3} M $CaCl_2$ + $MgCl_2$	0.307 ± 0.040	0.271 ± 0.013	0.134 ± 0.029

[a] Concentration of salt other than $CaCl_2$.

TABLE 7. Effect of pretreatment in $CaCl_2$ upon the amount of Ca^{++} bound. Algal preparation 72-1 (mostly *Microcoleus lyngbyaceus*). The molar concentration $CaCl_2$ in which the cells were suspended is given; not the resulting concentration of the solution after equilibration.

Time (min) exposed to $CaCl_2$	Moles Ca^{++} bound per kg algae					
	10^{-3}[a]	5×10^{-4}	4×10^{-4}	2×10^{-4}	1.5×10^{-4}	10^{-4}
	cells exposed to $CaCl_2$ 2.5 hours prior to measuring uptake of ^{45}Ca					
5	0.51	0.34	0.31	0.21	0.19	0.16
20	0.54	0.35	0.31	0.22	0.19	0.16
35	0.51	0.34	0.31	0.21	0.19	0.16
50	0.49	0.34	0.30	0.21	0.19	0.16
180	0.53	0.35	0.32	0.22	0.19	0.16
$\bar{x}$	0.516	0.344	0.0310	0.214	0.190	0.160
±s.e.	0.0087	0.0024	0.006	0.024	0.000	0.000
	cells not exposed to $CaCl_2$ prior to measuring uptake of ^{45}Ca					
5	0.34[b]	0.28[b]	0.26	0.21	0.18	0.16
20	0.41	0.30	0.27	0.20	0.18	0.15
35	0.43	0.33	0.28	0.21	0.18	0.15
50	0.44	0.31	0.28	0.21	0.18	0.16
300	0.41	0.30	0.27	0.20	0.17	0.15
$\bar{x}$	0.423	0.310	0.27	0.206	0.178	0.154
±s.e.	0.0075	0.0055	0.0037	0.0024	0.002	0.0024

[a] concentration of $CaCl_2$ solution in which cells were suspended.
[b] value excluded from mean.

for ^{45}Ca removed with each counting assay.

Those algae pretreated with $CaCl_2$ were treated similarly, except that 5 mg portions were shaken for 2.5 hr at 3 to 4 C in 5 ml aliquots of $CaCl_2$ at the same concentrations used for the control before adding 2 μc of ^{45}Ca to each tube. The tubes were subsequently mixed, centrifuged and sampled at intervals as for the controls. The data are summarized in Table 7, from which some conclusions are apparent: The cells exposed to $CaCl_2$ for 2.5 hr before measuring ^{45}Ca uptake reached equilibrium values in 5 min. The first 5 min seem not to have been long enough to attain equilibrium in 10^{-3} M, and possibly not in 5×10^{-4} M, in the absence of pretreatment. The concentration of $CaCl_2$ used in pretreatment influenced the equilibrium attained at the higher concentrations, i.e. 0.516 vs. 0.423 moles/kg for 10^{-3} M, but not at the lower concentrations.

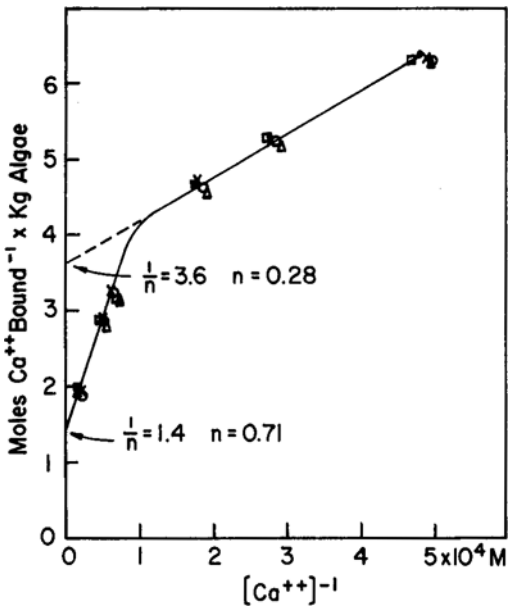

Fig. 2. Plot of 1/r vs 1/(A) for trichome fragments of *Microcoleus lyngbyaceus* pretreated with $CaCl_2$.

Total Binding Sites, n, and Intrinsic Association Constant, K

These data (Table 7) were used to compute K and n values using the Klotz relationship on a basis of weight. The results of the sample pretreated in $CaCl_2$ for 2.5 hr indicate that the relationship is not a simple one (Fig. 2). Only the data for those pretreated in $CaCl_2$ are presented, but in both the presence and absence of pretreatment an apparently linear relationship was obtained when $[Ca^{++}] \geq 10^{-4}$. With pretreated cells there appears to be also a linear rela-

tionship for $[Ca^{++}] \leq 10^{-4}$. For the higher concentrations with these cells the indicated value for n = 0.71 and for $K = 4.67 \times 10^3$; for the lower concentrations n = 0.28 and $K = 6.2 \times 10^4$. At the higher concentrations, for the cells which were not pretreated, the indicated value for n = 0.54 and for $K = 5.9 \times 10^3$. With these cells at the lower concentrations the plot of 1/r vs 1/A appeared to be curvilinear and values for n and K were not estimated.

From these results it is apparent that a prolonged exposure to 10^{-3} M $CaCl_2$ prior to measuring uptake resulted in more binding, but the data suggest that when uptake was measured promptly, equilibrium values were reached in less than 20 min at the higher concentrations and in less than 5 min at the lower concentrations. The reason for this paradox is not clear. In any case, the results of both treatments are in agreement with the interpretation that there is more than one class of ligands for Ca^{++}. There may be two as indicated by those cells pretreated in $CaCl_2$ for 2.5 hr. There may be more as indicated by the results with those cells not pretreated.

Characterization of Ca Ligands

In an attempt to further characterize the Ca binding material in the algal cells, two approaches were used: (1) extraction of the Ca-binding material with dilute acid (G. Clark, unpublished); and (2) the preparation of cell sheaths from living trichomes (P. K. Nelson, unpublished). In the first approach, 100 mg of cells similar to those used above were mixed at room temperature in 35 ml of HCl at pH 2 for 20 min and then removed by centrifugation. The supernatant was carefully neutralized to pH 7.0 and allowed to stir for 20 min. Centrifugation produced a gelatinous pellet. Ethanol was added to the supernatant to produce a final concentration of 75%. The small amount of additional precipitate was recovered by centrifuging and was added to that formed upon neutralization. The combined precipitate was washed in 80% ethanol until a negative test for Cl^- was obtained with $AgNO_3$. It was then washed in methanol, air dried and stored over anhydrous P_2O_5 at room temperature.

The acid-extracted cells were washed in deionized H_2O until Cl^--free, washed in methanol, air-dried and stored over anhydrous P_2O_5 at room temperature.

Calcium exchange conditions were measured using the material extracted by dilute acid, the cells which had been extracted with acid, and control cells from the original preparation. For this purpose 1 mg portions were suspended in 3 ml of 10^{-4} M $CaCl_2$ containing tracer amounts of ^{45}Ca. After being stirred for 10 min the suspension was centrifuged and an aliquot was removed for counting. Then sufficient 0.1 M $CaCl_2$ was added to increase the concentration of $CaCl_2$. This was followed by mixing, centrifuging, counting and further addition of $CaCl_2$ in sequence to produce test solutions as follows:

10^{-4}, 2.01×10^{-4}, 3.04×10^{-4}, 4.77×10^{-4}, 7.33×10^{-4}, 10.7×10^{-4} and 13.9×10^{-4} M.

The amount of Ca bound per unit dry weight for two separate extracts was in some cases erratic (Table 8). Hence, the absolute values obtained sometimes differed somewhat from one run to the next, but the overall results were as presented here. The material extracted by acid which precipitated upon neutralization (= acid extract) showed a greater affinity for Ca^{++} per unit dry weight than either the original cells or those which had been extracted with acid. The acid-extracted cells exhibited lower values for bound calcium than the original cells. In all cases it was clear that the acid extraction had removed only a portion of

TABLE 8. Relative Ca-binding capacity of material extracted from blue-green algal cells at pH2.

Extract no.	Sample	Relative amount of calcium bound per unit of dry wt		
		Original cell preparation	Acid extract	Acid-extracted cells
4	a	100	186	34
	b	100	131	46
	c	100	138	56
	$\bar{x}$	100	152	45
6	a	100	193	71
	b	100	196	86
	$\bar{x}$	100	195	79

the ligands for Ca^{++}. These measurements need to be repeated with larger amounts of material.

The substance extracted from the algal cells by dilute acid gave a positive test for carbohydrates with anthrone. Other tests for constituent components were not performed.

To further characterize the Ca-binding material, living trichomes of *M. lyngbyaceus* separated from a mat by washing with salt water were used to obtain sheaths and cells in separate preparations as described under Materials and Methods. The affinity of these for Ca was measured using the procedure outlined for the acid extracted material because here again only small quantities were available. Both sheaths and cells exhibited an appreciable capacity to bind Ca^{++}. When suspended in 10^{-3} M $CaCl_2$ sheaths bound 0.48 moles Ca^{++} per kg and the cells 0.34 moles per kg. Pectin esterase had no influence upon the amount of Ca^{++} bound to either preparation. For a control, the same enzyme solution, by hydrolyzing carboxymethyl groups of pectin, increased calcium binding of onion cell walls. Clearly then either the Ca^{++} binding constituent(s) of these blue-green algal cells and their sheaths is not pectin (which is likely), or it is pectin but the pectin is not methoxylated (unlikely).

Conclusions

It is clear from these results that dead trichomes of blue-green algae, both those grown in laboratory cultures and those harvested from a tide marsh, have a substantial capacity to bind Ca^{++}. Whatever the material is which removes Ca from solution it differs from pectin of higher plants in at least two ways: 1) Acidified alcohol does not remove all of the calcium. 2) Pectin esterase does not enhance Ca-binding. It can be at least partially extracted with dilute acid and gives reactions which suggest that it contains/is a polysaccharide. Apparently, the extraction is at least partially irreversible. It is not extracted by Triton X-100 nor by EDTA plus Triton X-100. At least to the extent that COOH groups are freed by acidified alcohol, they react with Ca^{++} in the expected ration of 2 moles COOH/mole Ca^{++}. Ca-binding is exhibited by both sheaths and cells. The influence of pretreatment in $CaCl_2$ upon the exchange promoters is puzzling. Why there is an increase in total binding sites and intrinsic association-constant is not clear. This should be examined further. In any case the exchange parameters indicate that there may be two classes of binding sites.

That the Ca-binding capacity in algae from a natural habitat exceeds that of the cell walls prepared from rhizomes of *Spartina alterniflora* with which they might be in competition in natural situations may be of ecological significance. At least in brackish waters (equal to 0.1 M NaCl) these algae cells retain a substantial portion of the Ca^{++} and this is exchanged readily with the ambient medium. Algal cells could then contribute to calcium cycling by exchange reactions and moribund or dead cells could serve as a temporary reservoir of this cation. A qualitative assessment based upon a visual estimate of their mass and seasonal abundance suggest that the blue-green algae may well contribute to the calcium cycling of a tide marsh by uptake during periods of rapid growth and release during their demise. But the total mass of these algae may not be great enough to make a substantial contribution to the calcium needs of a marsh community. Other algae which are abundant at seasons when the blue-greens are not conspicuous may play a similar role. Further work is needed to further quantify these relationships.

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