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POLYSACCHARIDES IN DESERT RECLAMATION: COMPOSITIONS OF EXOCELLULAR PROTEOGLYCAN COMPLEXES PRODUCED BY FILAMENTOUS BLUE-GREEN AND UNICELLULAR GREEN EDAPHIC ALGAE

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ABSTRACT

The filamentous blue-green alga *Nostoc calcicola* Geitler, strain 79WA01, showed a peak production of ~70% of its biomass as a mixture of exocellular proteoglycan complexes, containing 3–30% of a polypeptide moiety and >70% of a complex glycuronoglycan. The former contained high proportions of Asp, Glu, Arg, and amido-NH_3, in addition to ~35% of "hydrophobic" amino-acids. The latter varied in composition in different fractions: GalA (2.5–10.3%), GlcA (4.7–11.5%), Glc (11.7–39.0%), Xyl (5.7–17.9%), Man (2.7–9.5%), Gal (5.7–9.5%), Fuc (1.5–11.1%), Ara (1.9–4.3%), and Rha (1.4–4.4%). None of the fractions showed a stoichiometric ratio of sugar residues.

Palmelloid cells of three unicellular green soil-algae of the genus *Chlamydomonas* yielded ~70% of their dry weight as capsular mucilage. About 50% of the sodium salt of this material was soluble in water, and contained 3–12% of polypeptide and 88–97% of glycuronoglycan (GlcA:Glc:Xyl = 1:1:3 for *C. humicola* Lucksch, and GlcA:Gal = 1:2 for *C. peterfii* Gerloff and *C. sajao* Lewin). These categorical differences in sugar composition, together with narrow composition distributions, suggested regular structures for the glycuronoglycans. The remainder of the mucilages contained essentially the same glycuronoglycan chains, but a higher proportion of polypeptide. These materials did not form true solutions in water, but dispersed as microgel particles.

INTRODUCTION

Algae are usually thought of as aquatic organisms, but they are also ubiquitous in soil and contribute importantly to its fertility[1–3]. Their contribution is especially significant in desert and semi-arid soils, because they are autotrophic and have no

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requirement for exogenous organic carbon. Many blue-green species also fix atmospheric nitrogen[4,5], which is the nutrient most commonly lacking in desert soil[5].

Blue-green algae[6] (Cyanophyta, also called cyanobacteria[7]) are well adapted for primary colonisation of arid environments because they have a remarkable ability to survive desiccation and extremes of temperature[8,9] and can grow actively at temperatures of up to 70°. They can also grow at high pH and salinity, which are other common characteristics of desert soils[4,5].

Although they are generally less tolerant of extreme environments, green algae (Chlorophyta) also occur commonly in semi-arid soils[10,11]. They cannot fix nitrogen, but may derive it from blue-green algae or heterotrophic nitrogen fixers such as *Azotobacter*[12].

The most successful algal colonisers of arid soil produce an abundance of sheath or capsular jelly[4,13,14]. This product is important in the *de novo* formation of soil, not only because it conserves moisture in the topsoil after rain, but also because it has adhesive properties which aggregate soil particles and resist erosion by water and wind[15,16]. It may also be utilised as a source of organic carbon and nitrogen by heterotrophic micro-organisms, thus providing a starting material for the production of other soil components[4].

The production and use of microalgal soil conditioners is an economically viable industry in the United States[17–20]. In order to facilitate the transfer of this technology to the drought-affected countries of sub-Saharan Africa and to provide a rational basis for the genetic improvement of selected strains, it is essential to learn more about the exocellular mucilages and their stability and biodegradation under field conditions.

There have been very few chemical studies of the extracellular mucilages of soil algae[21]. The most extensive modern studies have been carried out on the mucilage of *Porphyridium cruentum*, a unicellular red alga[22–27]. This material is a proteoglycan in which the glycan moiety is linked to residues of serine and threonine *via* D-xylopyranose residues[24–26]. However, *Porphyridium* is not a very typical soil alga*; it normally grows only on wet soil and is not mentioned in reviews on the microalgal flora of desert soils[4,5,10].

For preliminary studies, two genera were selected that are in use in the American field trials[17–20]. One of these genera was *Nostoc* (Order Nostocales, Family Nostocaceae), which are non-toxic, nitrogen-fixing, heterocystous, filamentous blue-green algae[13]. Their trichomes are enclosed in a copious gelatinous sheath which is bounded externally by a pellicle-like membrane[13]. The only previous investigation of the sheath material was a study of the sugar composition[29].

The other genus selected was *Chlamydomonas* (Order Volvocales, Family Chlamydomonadaceae), which are biflagellated, unicellular green algae, also re-

*The most important characteristics of a typical soil alga are that it can grow at a lower *water potential*[28] than a typical aquatic alga and remain viable when the water potential falls below the minimum required for growth.

garded by zoologists as protozoa (Order Volvocida)[13]. In their resting, "palmelloid" state, the cells (akinetes) of these organisms surround themselves with a gelatinous capsule, occupying many times their own volume[30]. The only previous chemical work on the capsular material was a qualitative study of the sugar composition[3].

EXPERIMENTAL

Materials. — Agar slants of *Nostoc calcicola* Geitler, strain 79WA01, were supplied by Dr. Blaine Metting (R & A Plant-Soil Inc., Pasco, WA, U.S.A.). Freeze-dried cells (with capsules) of *Nostoc commune* Vaucher, *Chlamydomonas humicola* Lucksch[31], *C. peterfii* Gerloff strain C-2, and *C. sajao* Lewin[32] strain M-18, were supplied by Professor Ralph A. Lewin (Scripps Institution of Oceanography, La Jolla, CF, U.S.A.). Axenic cultures of these four algae are deposited in the University of Texas Algal Culture Collection as Nos. 584, 225, 2400, and 2277, respectively. The cells were grown[33] in Märten's medium. A sample of exocellular proteoglycan from *Cyanospira capsulata* Florenzano[34,35], originally isolated at the Institute of Agricultural Microbiology, Florence (Italy), and further purified at the University of Trieste, was supplied by Professor Attilio Cesàro.

Methods. — Nitrogen was assayed with a Carlo Erba elemental analyzer (Model 1104). Free-boundary electrophoresis was carried out at 4° in a Perkin–Elmer Model 238 on 0.5% solutions in 0.05M buffers at pH 2 (sodium chloride–hydrochloric acid) or 7 (sodium hydrogenphosphate).

For qualitative sugar analysis, samples (20 mg) of proteoglycan were heated at 98° in 0.5M sulphuric acid (2 mL) in sealed tubes for 12 h. The hydrolysates were neutralised ($BaCO_3$), filtered, passed through Dowex 50 (H^+) resin, and concentrated to dryness. The residues were subjected to t.l.c. on cellulose (ethyl acetate–acetic acid–pyridine–water, 5:3:1:4; detection with 0.3% of *p*-anisidine hydrochloride in butan-1-ol[36]).

For quantitative sugar analysis, portions (100 mg) of aqueous 0.1% stock solutions together with aqueous 0.1% mannitol (60 mg) as an internal standard were concentrated to dryness over phosphorus pentaoxide *in vacuo* at 25°. Dry methanolic M hydrogen chloride (1 mL) was added to each sample, and the mixture was heated at 80° for 24 h in a capped vial. After cooling, each sample was dried in a stream of dry nitrogen, and residual hydrogen chloride was removed by repeated evaporation of dry methanol from the residue. After a final drying over phosphorus pentaoxide, the methyl glycosides were converted into trimethylsilyl derivatives by adding Supelco sylon HTP (0.2 mL) and injected into a Perkin–Elmer Sigma 2B gas chromatograph, equipped with a flame-ionisation detector and a fused-silica capillary column (DB-15, 30 m, J & W Scientific). The carrier gas was hydrogen and the temperature gradient was 140 →200°, at 2°/min. The output was analysed with a Hewlett–Packard 3390 integrator. Mixtures of sugars, representing an appropriate range of compositions, were used to calibrate the method.

For amino acid analysis, samples of proteoglycan were first enriched in the

polypeptide moiety by selective hydrolysis in 0.2M hydrochloric acid at 98° for 4 h. After neutralisation (Na_2CO_3), the solutions were dialysed against water, the solutions of non-dialysable material were concentrated to dryness, and the residues were hydrolysed in 6M hydrochloric acid at 110° for 24 h. The amino acids were analysed with a Biotronic LC-7000 instrument (Munich, F.R.G.) coupled to a Spectra Physics SP4200 (San José, CF, U.S.A.). The eluant was 0.067M lithium citrate buffer (pH 2.2).

The proteoglycan fractions were eluted from columns (30 × 3 cm) of DEAE-Sepharose CL-6B by using a linear gradient of sodium chloride (0 →4M in 2 L of eluant) in 25mM Tris buffer (pH 7) at 0.3 mL/min. Samples (20 mg) were applied to the column as solutions in Tris buffer (20 mL), after centrifugation at 30,000*g* for 30 min in order to remove particles of microgel. Fractions (7–9 mL) were collected and analysed. In column effluents (and in other solutions), total carbohydrate was determined by the phenol–sulphuric acid method[37] and polypeptide by the Coomassie Brilliant Blue G-250 method[38–40] (reagent supplied by Bio-Rad Laboratories).

Growth of Nostoc calcicola *in batch cultures.* — Stock cultures were maintained in WC medium[41] in 50-mL closed cultures at 20°. For large-scale cultivation, white-walled, conical fibreglass tanks (500 L, 1.5 m high, 1.0 m diameter at the top) were used. Light was supplied by eight 36-watt fluorescent tubes, enclosed in a Perspex cylinder, and immersed axially in the tank. Air containing 1% (w/v) of carbon dioxide was blown through the culture to cause turbulence and supply carbon and nitrogen. The temperature was 20°, 25°, or 31°. Smaller scale cultures were grown in 1-L glass tubes and 5-L glass bottles illuminated from the side, and in 20-L plastic buckets illuminated from above. Growth was monitored by taking replicate samples (50–100 mL) after thorough mixing to make them as representative as possible. The cells were collected in the centrifuge (5,000*g*, 30 min), freeze-dried, and weighed. The centrifugates were used to monitor the release of soluble carbohydrate and protein.

Isolation of proteoglycan fractions from Nostoc calcicola. — (*a*) *Cell-free centrifugate (CFC).* Cetyltrimethylammonium bromide (Cetavlon; 150 mg/L) was dissolved in the CFC. The precipitate was collected and washed with ethanol containing acetic acid (60 g/L). A suspension in 2M sodium chloride was dialysed against 2M sodium chloride until most of the particles had dissolved, then against water. The solution was centrifuged at 10,000*g* for 1 h and poured into 2 vol. of ethanol. The stringy precipitate was collected, and an aqueous 0.2% solution was passed through a Whatman glass microfibre filter (0.8 μm) and freeze-dried to give a creamy-white solid (PG_{cfc}).

(*b*) *EDTA extract.* The cells (20 g, dry weight) were stirred overnight with 0.1M ethylenediaminetetra-acetic acid (sodium salt, pH 7, 4 L) at 22°. The mixture was then centrifuged at 5000*g* for 30 min, and the centrifugate was passed successively through 2.7-, 1.6-, and 1.2-μm glass microfibre filters, and then poured into ethanol (8 L) containing sodium chloride (30 g/L). The precipitate was collected, and a solution in water (1 L) was poured with stirring into acetone (2 L). This step

was repeated three times in order to remove the purple phycocyanin–protein complex[7]. A solution of the final precipitate in water (500 mL) was centrifuged at 10,000*g* for 30 min, and then freeze-dried. The resulting solid was ground with 9:1 phenol–water (300 mL), and washed with ethanol. A solution in water (500 mL) was dialysed against water, centrifuged at 20,000*g* for 90 min, and freeze-dried to give a white solid (PG_{edta}).

(*c*) *Hot-water extract.* The residual cells, after extraction with EDTA, were heated for 2 h at 80° in water (2.5 L), then centrifuged down, and re-extracted twice more in the same way. The extracts were combined and passed successively through 2.7-, 1.6-, and 1.2-μm glass microfibre filters, concentrated *in vacuo* to 2.5 L, and poured into acetone (5 L). The precipitate was collected, redissolved in water (2.5 L), and reprecipitated with acetone (5 L) twice more, in order to remove pigments. A solution in water (1 L) was freeze-dried, and the resulting solid was ground with 9:1 phenol–water (400 mL), collected, and washed with ethanol. The pH of a solution in water (1 L) was adjusted to 7, and the solution was dialysed against water, centrifuged at 20,000*g* for 90 min, and freeze-dried to give a white solid (PG_{hw}). This material was divided into acid-soluble (PG_{hwas}) and acid-insoluble (PH_{hwai}) fractions by adjusting the pH of an aqueous 0.5% solution at 10° to 2, centrifuging at 10°, and neutralising the centrifugate and residue separately with aqueous sodium hydrogencarbonate. The separate fractions were isolated by dialysis and freeze-drying.

Isolation of proteoglycan fractions from Chlamydomonas *cells.* — The freeze-dried cells were milled to pass a 50-mesh (0.5 mm) sieve, then extracted exhaustively with boiling acetone, followed by boiling methanol. The resulting powder was ground three times with 9:1 phenol–water, collected, washed with ethanol, and air-dried. A portion (0.5 g) was shaken vigorously overnight at 22° with 0.1M ethylenediaminetetra-acetic acid (sodium salt, pH 7, 100 mL). The resulting slurry was dialysed three times against the same reagent (1 L), followed by water. Cellular debris was then removed by centrifugation for 30 min at 15,000*g*. The slimy supernatant liquid was decanted-off and freeze-dried. The resulting white solid (350 mg) was divided into water-soluble and microgel fractions by adjusting the pH of a 0.2% dispersion in water to 7, and centrifuging for 90 min at 35,000*g* and 15°. The subfractions were recovered by dialysis and freeze-drying.

RESULTS

Production of soluble carbohydrate and protein by Nostoc calcicola *in batch cultures.* — Fig. 1 shows the increase in biomass (dry weight of cells) with time at 31°. The organism grew at a constant exponential rate of ~1.4 doublings per day for 3–4 days, and the biomass levelled off at 132 ±8 mg/L on days 6–8. In the subsequent phosphate-starvation period, water evaporated from the culture, causing an increase in cell density, which reached 935 mg/L on day 22. A growth curve corrected for this is shown in Fig. 1. The culture remained viable throughout

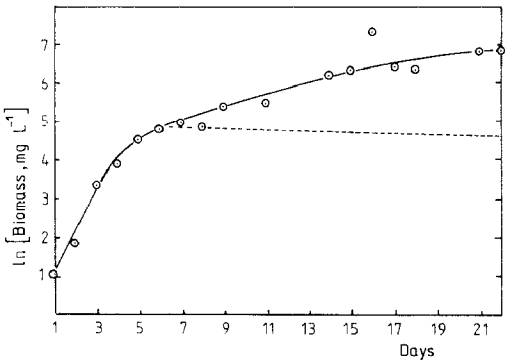

Fig. 1. Growth of *Nostoc calcicola* in a batch culture at 31°. The broken line represents the stationary phase of growth, after correction for evaporation.

the starvation period, thus illustrating the ability of this species to survive under extreme conditions.

The cell-free centrifugates, corresponding to the data points in Fig. 1, were analysed for total carbohydrate and polypeptide (expressed as equivalent amounts of D-glucose and bovine serum albumin, respectively). In Fig. 2, these quantities are expressed as percentages of the biomass (thus eliminating complications due to evaporation), and plotted against time. A comparison of Figs. 1 and 2 shows that there was insignificant release of soluble carbohydrate or polypeptide in the logarithmic phase of growth (days 0–3). Release commenced in the early stationary phase (day 4) and continued actively after the onset of phosphate starvation (day 6). The yield of soluble biopolymers passed through a maximum after 10–12 days. The reasons for the subsequent decline will be discussed elsewhere[42], but one of them was simply that the exopolymer started to precipitate from solution when its

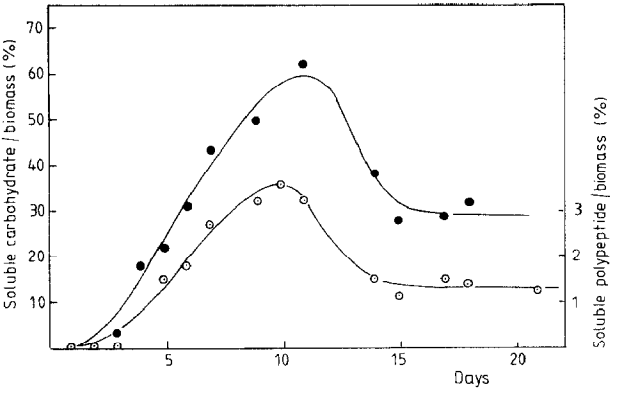

Fig. 2. Release of soluble carbohydrate (●) and polypeptide (○), expressed as a percentage of the biomass of *Nostoc calcicola* in batch culture at 31°. D-Glucose and bovine serum albumin were used as arbitrary standards for carbohydrate and polypeptide, respectively.

concentration became sufficiently high. The low solubility of the exo-polymer was due to the presence of calcium ions in the culture medium[41].

Isolation of proteoglycan complexes from Nostoc calcicola. — Addition of cetyltrimethylammonium bromide (Cetavlon) to the cell-free centrifugates precipitated >90% of the carbohydrate and polypeptide. This step avoided the need to concentrate large volumes of solution, and also made use of the bacteriostatic properties of Cetavlon. The yield of this fraction (PG_{cfc}) reached a peak after 10–11 days of growth (Fig. 2 and Table I).

Since the growth medium contained Ca^{2+} and traces of other essential multivalent cations[41], the cells, after harvesting, were extracted with aqueous EDTA (sodium salt). This solubilised more proteoglycan (PG_{edta}), and also converted any residual proteoglycan into its sodium salt,

Since the gelatinous sheath material surrounding the trichomes is enclosed in a pellicle-like membrane[13], the cells were extracted next with hot water, in the expectation that this would rupture the membrane by denaturing the protein. This treatment solubilised a substantial amount of a third fraction (PG_{hw}). Hough *et al.*[29] have previously reported that mucilage is extracted most readily from *Nostoc* by hot water.

It was usually necessary to freeze or freeze-dry the cells immediately after harvesting. This process ruptured a proportion of the cells, releasing cellular proteins into the EDTA and hot-water extracts. The most prominent of these proteins were the nitrogen-storage protein, cyanophycin[43,44], and the phycobiliproteins, with their covalently bound, blue and red chromophores (phycocyanin and phycoerythrin, respectively)[7]. The proportion of these products was highest in old cells, evidently because fixation of atmospheric nitrogen continued after the supply of nitrate and phosphate in the medium had been exhausted. These products were removed from PG_{edta} and PG_{hw} by extraction with aqueous 90% phenol and by repeated reprecipitation with acetone.

Although substantial manipulative losses were incurred during the isolation and purification procedures, Table I indicates roughly the variation in the yields of the three proteoglycan fractions as the culture aged. The best yield was obtained after 9–11 days of growth; purification became more lengthy and wasteful of material as the cells aged. The compositions of the three fractions are given in Tables II and III.

TABLE I

YIELDS OF PROTEOGLYCAN FRACTIONS FROM *Nostoc calcicola* EXPRESSED AS PERCENTAGES OF THE DRY WEIGHT OF CELLS

Age of culture (days)	9	10	23	28
Water-soluble (PG_{cfc})	31	n.d.[a]	13	9
EDTA-soluble (PG_{edta})	11	12	6	15
Hot-water soluble (PG_{hw})	n.d.	28	16	13

[a]Not determined.

TABLE II

COMPOSITIONS (%) OF EXOCELLULAR PROTEOGLYCAN COMPLEXES FROM *Nostoc calcicola* GEITLER, 79WA01

Fraction[a]	*Ara*	*Rha*	*Fuc*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*	*GlcA*	*Protein*[c]	*Total*[d]
PG^{20}_{cfc}	1.9	2.1	6.1	11.8	3.9	8.9	13.9	7.6	7.1	7.9	71.2
$PG^{20\ b}_{edta}$		4.2	10.7	17.4	5.6	6.4	14.3	9.5	11.2	5.0	84.2
$PG^{500\ b}_{edta}$		3.9	10.6	16.9	6.5	6.8	14.1	9.6	10.5	3.4	82.0
$PG^{20\ b}_{hw}$											
Whole	4.3	3.2	4.2	8.9	6.1	9.4	20.5	4.4	8.1	15.0	83.3
Centr.[e]	3.5	2.6	3.5	8.1	7.1	8.6	22.4	4.0	6.8	13.8	80.3
Peak B[f]	2.6	3.7	4.5	9.4	6.8	7.1	17.1	5.0	8.0	15.8	78.7
PG^{500b}											
Whole	3.2	1.6	4.5	8.4	3.7	9.5	14.8	3.1	5.3	25.6	79.6
PG_{hwas}	3.9	1.5	1.6	5.8	9.2	7.1	38.5	2.7	5.5	5.9	81.6
PG_{hwai}	3.0	1.5	4.7	8.7	2.8	9.4	11.9	3.4	4.8	30.0	80.0
N. commune[g]	2.8	2.0	3.3	23.0	2.9	14.1	4.4	9.3	15.6	16.7	94.1[i]
C. capsulata[h]	7.2		7.8		9.3		9.3	19.7		9.4	62.7

[a]Superscripts refer to different cultures (20 or 500 L). [b]Average of two parallels. [c]N(%) × 6.25. [d]Incomplete recovery due mainly to incomplete methanolysis of aldobiouronic acids (not determined). [e]Centrifuged at 35,000*g* to remove microgel particles. [f]See Fig. 3. [g]Included for comparison. The whole sample was extracted from freeze-dried cells with hot 0.1M EDTA. [h]Included for comparison. See refs. 34 and 35. [i]Enhanced recovery due to pre-hydrolysis with aqueous acid (see Experimental).

TABLE III

AMINO-ACID COMPOSITIONS (MOL%) OF SOME ALGAL PROTEOGLYCAN COMPLEXES[a]

Amino acid	*Nostoc* PG^{20}_{cfc}	*Nostoc* PG^{20}_{edta}	*Nostoc* PG^{20}_{hw}	Chlamydomonas		
				humicola	peterfii	sajao
Asp	8.1	10.0	22.6	9.8	9.9	8.8
Thr	4.5	5.2	4.5	6.5	5.4	5.5
Ser	10.8	9.1	4.9	8.7	14.3	17.3
Glu	13.5	10.2	8.1	14.8	12.1	15.6
Gly	13.4	10.4	6.3	10.8	14.0	16.8
Ala	7.4	8.3	6.4	11.3	10.0	10.5
Val	6.1	5.7	7.4	8.1	6.2	5.9
Met	10.3	0.9	0.3			
Cys				0.5		
Ile	2.6	3.5	3.5	3.7	2.2	2.5
Leu	5.1	6.1	6.4	8.3	4.9	5.1
Tyr		2.1	1.2	1.4		
Phe	4.3	3.6	4.2	3.9	3.7	3.5
Orn	3.8	2.5	0.3	2.5	6.5	
Lys	1.9	2.4	3.0	3.3	2.6	2.8
His	0.9	1.2		0.5	2.0	1.9
Arg	7.2	4.2	13.5	6.0	5.9	4.4
NH_3	n.d.	n.d.	13.3	n.d.	n.d.	n.d.

[a]The results are not corrected for selective losses occurring during acid hydrolysis. In order to minimise losses due to the reaction with reducing sugars and their dehydration products, a two-step hydrolysis procedure was employed (see Experimental).

TABLE IV

COMPOSITIONS OF EXOCELLULAR PROTEOGLYCAN COMPLEXES FROM *Chlamydomonas* SPECIES

Species	*Molar ratios*						*Protein (%)*
	Ara	*Xyl*	*Gal*	*Glc*	*GlcA*	*Rha*	
C. sajao							
Whole extract[a]	0.65	0.10	2.82	0.18	1.00	0.06	37.1
Water-soluble[a]	0.49	0.16	2.54	0.07	1.00	0.08	11.9
Water-soluble[b]	0.74		2.70	0.07	1.00		7.5
EtOH ppt.[bc]	0.47		2.45		1.00		3.6
Main peak[bd]	0.14		2.10	0.10	1.00		10.6
C. peterfii							
Whole extract[a]	0.57	0.18	2.48	0.08	1.00	0.12	39.1
Water-soluble[b]	0.70		2.40	0.12	1.00		8.8
Main peak[bd]	0.14		2.00		1.00		7.2
C. humicola							
Whole extract[a]	0.28	1.43	0.21	1.94	1.00	0.09	35.4
Water-soluble[b]	1.10	2.97	0.36	1.10	1.00	0.27	2.8
EtOH ppt.[bc]	0.79	2.88	0.29	1.08	1.00	0.18	3.2

[a]Sample isolated by K.A.H. and T.J.P. [b]Samples isolated by A.F. [c]Fraction (~70%) of water-soluble material precipitated from solution in 0.1M sodium chloride by adding 3 vol. of ethanol. [d]From resolution of water-soluble fraction on DEAE-Sepharose, as illustrated in Fig. 4.

Isolation of proteoglycan complexes from Chlamydomonas *palmelloids.* — A detailed study of the growth characteristics of *C. reinhardtii* Dang. under conditions of phosphorus limitation, and of the harvesting of its palmelloids, has been reported[30]. Preliminary experiments indicated that the capsular material was kept insoluble partly by divalent metal cations and partly by adventitious protein. After removal of the latter from the milled, solvent-extracted cells with aqueous 90% phenol, ~70% of the residue could be readily extracted with aqueous EDTA, to give a highly visco-elastic dispersion, from which the proteoglycan was recovered by dialysis and freeze-drying. The compositions of the products from three different *Chlamydomonas* species are given in Tables III and IV.

Sub-fractionation of Nostoc *proteoglycan complexes.* — Attempts to separate the glycuronoglycan and polypeptide components by physical methods were unsuccessful. Repeated extraction of the freeze-dried solids with aqueous 90% phenol failed to reduce the nitrogen content below a certain, characteristic value for each fraction (Table II), and, although there was a high proportion of arginine residues in the polypeptide (Table III), it could not be selectively precipitated from aqueous solution by trichloroacetic acid, ammonium molybdate, or sodium dodecyl sulphate. Upon addition of Cetavlon or calcium acetate to aqueous solutions, the polypeptide was quantitatively co-precipitated with the glycuronoglycan.

The behaviour of the fractions in acidic media was informative. Each of the three fractions contained hexuronic acid residues (Table II) and an arginine-rich polypeptide moiety (Table III). Fractions PG_{cfc} and PG_{edta} were soluble in acid, but

free-boundary electrophoresis at pH 2 did not reveal a component migrating towards the cathode. Fraction PG_{hw} could be separated into acid-soluble (PG_{hwas}, 30%) and acid-insoluble (PG_{hwai}, 70%) subfractions by adjusting the pH of an aqueous solution to 2. Contrary to the result expected for a physical mixture, however, PH_{hwai} contained five times as much polypeptide as PG_{hwas}. Moreover, PG_{hwai} contained about the same proportion of hexuronic acids as PG_{hwas}, and considerably less than PG_{cfc} or PG_{edta}, which were both soluble in acid (Table II).

The elution profiles of PG_{hw} on DEAE-Sepharose are shown in Fig. 3. There was complete co-elution of carbohydrate and polypeptide in the two main components (peaks B and D), and the proportionality between the two moieties in peak B was constant. It was established also that the Coomassie Brilliant Blue reagent reacted specifically with the polypeptide moiety in the two fractions. Although this reagent responds differently to different proteins[38–40], a reasonable correlation was found between the absorbances obtained with different PG fractions and their polypeptide contents calculated from nitrogen determinations (Table II). As expected, the dye showed no significant reactivity with nitrogen-free samples of pectate and C-6-oxycellulose, because the assay is carried out in strongly acidic solutions[38], in which ionisation of the hexuronic acid residues is suppressed. The polypeptide content of peak B was additionally established by analysing the isolated material for nitrogen (Table II).

Sub-fractionation of Chlamydomonas *proteoglycan complexes.* — The EDTA extracts appeared at first to be true solutions, but high-speed centrifugation revealed that a substantial part (~50% in *C. sajao*) of the material was dispersed as colloidal paticles of microgel. The colloidal material had the same glycuronoglycan

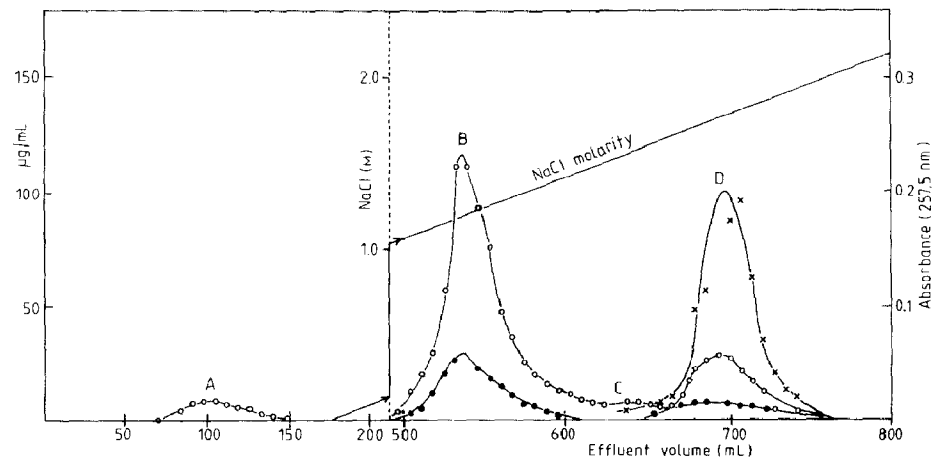

Fig. 3. Fractionation of the *Nostoc* proteoglycan complex PG^{20}_{hw} on DEAE-Sepharose CL-6B by gradient elution with sodium chloride in 25mM Tris buffer, pH 7. Both carbohydrate (○) and polypeptide (●) were calculated by using the unfractionated material as a standard. The intense absorbance at 257.5 nm (×) is tentatively ascribed to a polypeptide-linked sheath pigment[7].

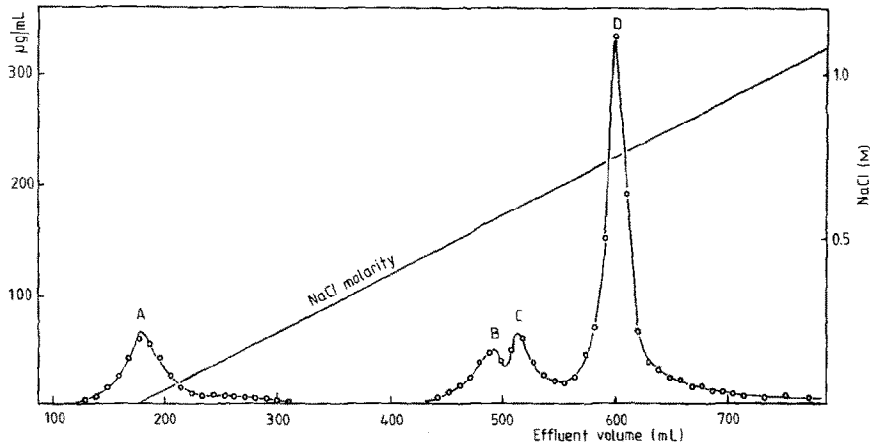

Fig. 4. Fractionation of the *Chlamydomonas sajao* mucilage on DEAE-Sepharose CL-6B by gradient elution with sodium chloride in 25mM Tris buffer, pH 7. The unfractionated material was used as a standard for the carbohydrate assays (○).

chains as the soluble part, but its polypeptide content was higher (Table IV). Hence, a fractionation based upon polypeptide content, similar to that brought about by low pH with the *Nostoc* mucilage, had occurred even at pH 7.

The elution profile of the soluble material on DEAE-Sepharose is shown in Fig. 4. Because of the low reactivity of the Coomassie Brilliant Blue dye with the polypeptide part, and a notable paucity of aromatic amino acids in the latter (Table III), it was possible to monitor the elution of the carbohydrate part only. However, the material in peak D, namely, that collected between the 550th and the 650th mL of effluent, was isolated and found to contain 57% of the total amount of eluted carbohydrate and 67% of the total amount of eluted polypeptide (N × 6.25). The monosaccharide composition of this material is shown in Table IV.

DISCUSSION

Carbohydrate-peptide bonding. — The constant ratio of carbohydrate to polypeptide released into the culture medium by *Nostoc calcicola* at the onset of the stationary phase (Fig. 2), together with the observation that they were co-precipitated almost quantitatively by Cetavlon, indicated their close association, and subsequent attempts to separate them failed. Precipitation with acid, which is usually an effective way of purifying glycuronans from adventitious protein, enhanced the nitrogen content of the precipitated gel (PG_{hwai}, Table II). This result, together with the co-elution of carbohydrate and polypeptide from DEAE-Sepharose columns (Fig. 3), indicated tenacious bonding of the two moieties.

Similar observations and conclusions apply to the mucilages from the three *Chlamydomonas* species, but there is an additional argument. This is because the monosaccharidic compositions of the glycuronoglycan moieties are similar in

soluble and insoluble fractions; the only significant difference between the two fractions involves the content of polypeptide (Table IV).

Even though the nature of the carbohydrate–peptide bonding has yet to be established, the term "proteoglycan complexes" is a valid description of these materials.

Functional significance of the polypeptide moiety. — The well-known tendency of *Nostoc* trichomes and *Chlamydomonas* palmelloids to adhere to the walls of the culturing tank reflects the properties of their exocellular polymers, whose significance in promoting soil aggregate stability has been noted repeatedly[14,17–20,45,46]. Whereas the sodium salts of protein-free glycuronoglycans may form highly viscous, and sometimes pseudoplastic, solutions, and their calcium salts may form rubbery gels, they are not notably adhesive unless substituted with hydrophobic groups[47].

In a study of a marine *Pseudomonas* species that is notorious for its ability to attach to solid surfaces, Christensen *et al.*[48,49] found that adhesion was associated with the elaboration of a "hydrophobic" polysaccharide, in which one-third of the sugar residues were *O*-acetylated. The polypeptide moieties of the proteoglycan complexes isolated in the present work were also "hydrophobic", since they diminished the solubilities of the glycuronoglycan chains to which they were attached. Additionally, they contained 35–40% of the "hydrophobic" amino-acid residues, Gly, Ala, Val, Leu, Ile, and Phe (Table III). Therefore, it is likely that the polypeptide moieties play a major role in binding the hydrophilic glycuronoglycan chains to the mineral components of soil.

Quest for homogeneous fractions and evidence of stoichiometry. — Completely regular structures are unusual in algal glycans, although regular "backbone" structures can be "masked" by an irregular pattern of substitution or other modifications[21]. Further complications arise in proteoglycans because glycan chains of different composition and structure can be attached in various proportions to a common protein core. Both kinds of irregularity manifest themselves as broad composition-distributions[21]. The *Porphyridium* mucilages are good examples of this kind of proteoglycan[21–27]. Different preparations display a wide array of compositions and structural features which seem to vary with conditions of growth, but nothing is known about the control mechanisms[21].

The *Nostoc* mucilages also appear to be proteoglycans of this type. Chromatography on DEAE-Sepharose (Fig. 3) revealed the presence of two discrete, main components (peaks B and D), but the composition of peak B was no simpler than that of the starting material (PG^{20}_{hw}), nor was there any indication of stoichiometry in the molar ratios of the nine different kinds of sugar residue (Table II). In this and in all the other fractions, the molar ratios of the sugars seem to vary in a continuous manner (Table II).

Generalisations about blue-green algae cannot be made on the basis of work on one strain of *Nostoc calcicola*. Analysis of one sample of mucilage from *Nostoc commune* gave no indication of stoichiometric sugar ratios, but a fraction from

Cyanospira capsulata contained residues of Ara, Fuc, Man, Glc, and GalA in the molar ratios 1:1:1:1:2, respectively (Table II).

Application of the same analytical methods to the *Chlamydomonas* mucilages indicated stoichiometry in the molar ratios of the principal monosaccharides (Table IV). After making allowance for errors arising from the incomplete release of monosaccharides, due to the resistance of hexuronosidic linkages to methanolysis, it is evident that the GlcA:Gal ratio is 1:2 in *C. peterfii* and *C. sajao*, and that the GlcA:Glc:Xyl ratios are 1:1:3 in *C. humicola* (Table IV). Such a result is not likely to be fortuitous. Purification is accompanied by a *simplification* in the pattern of monosaccharides, in that the proportion of arabinose residues is small in peak D. Moreover, the narrowness of peak D (Fig. 4) indicates a narrow composition distribution, such as would be expected for a regular structure. Finally, the different principal monosaccharides in the mucilages of different species (GlcA and Gal in *C. peterfii* and *C. sajao*, but GlcA, Glc, and Xyl in *C. humicola*) suggest that the enzymes involved in biosynthesis are specific.

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