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Basic Organic Chemical Parameters of the Marine Alga *Nereocystis luetkeana* over the Growing Season

by

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and

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Basic Organic Chemical Parameters of the Marine Alga *Nereocystis luetkeana* Over the Growing Season

By J.N.C. Whyte and J.R. Englar

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ABSTRACT

Earlier studies on the marine alga Nereocystis luetkeana are considered in the general introduction to this presentation of the content and composition of the basic organic chemical parameters of the alga.

The seasonal variation in the gross chemical fabric of the alga, measured as the content of hydrophobic, hydrophilic and polymeric components, are presented together with the fluctuations observed for the content of nitrogen, protein, sodium alginate and mannitol in the fronds and stipes of the alga over the growing season.

Observations on the nature of the proteinaceous and polysaccharide constituents of Nereocystis are described and the commercial significance of the alginate component considered.

RESUME

L'introduction générale de cet exposé sur la composition chimique de base, qualitative et quantitative, de l'algue marine Nerocystis luetkeana fait appel à des études antérieures sur le sujet.

On donne un aperçu des variations saisonnières de la composition chimique générale, mesurées en fonction des constituants hydrophobes, hydrophiles et polymérisés, ainsi que celles des teneurs en azote, en protéines, en alginate de sodium et en mannitol des frondes et des stipes au cours de la saison de croissance.

Des examens portant sur la composition en protéines et en polysaccharides de Nereocystis sont décrits. On traite aussi de l'importance commerciale de l'alginate.

INTRODUCTION

Nereocystis luetkeana (Mertens) Postels and Ruprecht is indigenous only to the Pacific Ocean off the coast of North America and is the only species of the genus. The alga was first described by Mertens as Fucus lütkeanus (Mertens, 1829) although earlier descriptions of this floating seaweed were recorded in Maurelle's "Journal of a voyage in 1775 to explore the coast of America northward from California" (Setchell, 1908). The present name Nereocystis luetkeana was conferred on the alga when described by Postels and Ruprecht in 1840 (Postels et al, 1840).

Many of the early biological studies on this alga were performed at the Puget Sound Biological Station where observations on the growth and development of the alga were related to environmental habitat (Fallis, 1915; Sheldon, 1915; Rigg, 1915; Hurd, 1916; and Hartge, 1928), and the nature and physical parameters of the gases present in the pneumatocyst of the alga led to the discovery of carbon monoxide as a major component of the gas mixture (Zeller et al, 1915; Frye, 1915; Langdon, 1916; and Rigg et al, 1941).

The growth and development of Nereocystis luetkeana is of considerable importance to the commercial exploitation of this resource, especially since the alga as an annual plant relies on the yearly maturation and liberation of viable zoospores for its survival. More recent studies have demonstrated that the maximum rate of the elongation of stipes and fronds of Nereocystis in California waters was 12.7 cm and 8.8 cm per day respectively (Foreman, 1970), whereas in B.C. waters the elongation rate for the stipes was measured as 7.8 cm per day (Duncan, 1973). The observations

made in these studies indicated, however, that the growth rates of the plants were considerably influenced by environmental factors amongst which the depth of attachment and the quality and quantity of incident light were of paramount importance. The maximum elongation of the stipe and fronds of the alga were demonstrated to occur at the same stage of development, essentially just prior to the plant reaching the surface of the water; thereafter the elongation process was increasingly replaced by lateral enlargement of the pneumatocyst, together with the adjacent portion of the stipe and fronds, following further dichotomy, all resulting in the consequent rapid increase in biomass. The rapid appearance and maturation of the sori appeared to occur in the fronds two to three weeks after they had attained a surface location, at least in the waters of California (Foreman, 1970). A more complete study of the beds of _Nereocystis_ in the waters off the coast of British Columbia is currently being performed to assess the critical biological factors governing the regrowth of the beds following various harvesting procedures, thereby ensuring that judicious management of this renewable resource will be exercised in future utilization (Anon, 1975).

To complement these biological studies and to appreciate the full commercial potential of this resource a knowledge of the seasonal variations in the chemical nature of _Nereocystis_ was of considerable importance. As earlier chemical analyses of _Nereocystis_ were deficient in various parameters (Burd, 1915; Cameron, 1916; Karrer, 1916; Howard, 1921; Sager et al, 1946; Wort, 1955) a more complete and systematic evaluation of the alga was instigated and in earlier reports in this series the seasonal variations in the inorganic cationic and anionic components have been described (Whyte et al, 1974); Whyte et al 1975(a); Whyte et al 1975(b)). The present report

describes the seasonal variation in the basic organic constituents of the alga including the commercially significant alginate and mannitol components together with a preliminary examination of the nature of the nitrogenous and carbohydrate polymeric constituents.

EXPERIMENTAL

(a) Collection and Preparation of Specimens

Samples of *Nereocystis luetkeana* were collected from selected kelp beds off the north side of Stanley Park in Vancouver in the middle of the months April through October. This period was considered to be most representative of the active growing season since severe deterioration of the alga became evident in November. Only attached plants were collected, placed in plastic bags and transported to the laboratory in insulated coolers containing ice. The fronds and stipes (including pneumatocysts) were separated and freed from extraneous epiphytes and epifauna. The samples were blotted lightly with paper to remove excess surface water then packaged in zip-lock plastic bags and stored at -31°C. When necessary, subsequent freeze drying of the speciments afforded dry alga which was ground to 20 mesh size with a porcelain mortar and pestle and stored in the freeze dryer to ensure anhydrous conditions existed for the samples during the period of analyses. The samples analyzed for organic parameters were those examined for inorganic components in previous reports.

(b) Methods of Analysis

1. Extractive Fractionation

Fresh alga (200 g) was exhaustively extracted in a Waring Blendor (1 gal capacity) with the homogeneous solvent mixture, chloroform : methanol : water (1:2:0.5; v:v) until no further colouration was evident in the filtered

solvent extract. Normally, more methanol or less water was added to the solvent mixture in the first extract which compensated for the constituent water in the alga. To the combined solvent extract, chloroform: water (1:1; v:v) was added to afford a chloroform layer which was separated, concentrated to a syrup and weighed (hydrophobic components).

The remaining aqueous-methanol layer was evaporated by a cyclone evaporator followed by a rotary evaporator to yield a crystalline residue which was weighed (hydrophilic components). The residue from the extraction procedure was air dried on a Buchner funnel then dried thoroughly with a high vacuum pump prior to weighing (polymeric components).

2. Nitrogen Determinations

Dry samples were analysed by micro-Kjeldahl technique.

3. Sodium Alginate Determinations

Dry samples were analysed by acidic decarboxylation using a previously devised technique (Whyte et al, 1974(b)).

4. Mannitol Determinations

A sample of the aqueous-methanol residue (10 mg) weighed into a test tube was dissolved in dry pyridine (4 ml) then 1 ml of a solution of xylitol in pyridine (1mg/1 ml) was added followed by acetic anhydride (5 ml). The test tube was sealed and the contents heated at 100°C for 2 hrs. to effect acetylation. The reaction mixture was then poured into saturated sodium bicarbonate and extracted with chloroform. The combined extracts were subsequently washed with dilute hydrochloric acid, dilute aqueous sodium bicarbonate, dried with anhydrous sodium sulphate and evaporated to dryness. Distilled ethyl acetate (1 ml) was added to the residue and 1 μl

of the resulting solution injected into a gas-liquid chromatographic column (5 ft. x 0.25 in.) of 3% ECNSS-M on Gas Chrom Q (100/120 mesh) operating at $185^{o}C$. A comparison of the peak area generated by the mannitol and the standard xylitol peak, together with the established detector response factor of 0.95 provided the weight of mannitol in the sample taken which was correlated to the weight in the dry alga.

5. Viscosity Measurement

A 1% aqueous solution of the isolated sodium alginate was measured at $25^{o}C$ by a Brookfield viscometer using No. 3 spindle rotating at 12 rpm.

6. Protein Extraction Procedures

(i)(a)Phenol : Acetic acid : Water Extraction. Fresh *Nereocystis* frond (30g) was ground with sand and 30 ml of 10% trichloroacetic acid in a porcelain mortar. The mixture was filtered on a sintered glass funnel and the residue re-extracted four times with 15 ml of 5% trichloroacetic acid. The residue was ground successively with 20 ml acetone then 20 ml of a chloroform-methanol (2:1) solvent system before being air dried on a Buchner funnel. The pretreated material was then ground to a paste with 5 ml phenol : acetic acid : water (1:1:1; w/v/v)plus 0.5g. Hyflo Supercel and left overnight. The paste was then ground with a further 5 ml of extraction solvent and the extract obtained by filtration. Two further extractions were performed and the combined filtered extracts evaporated to small volume with the phenol and acetic acid being removed by co-distillation with water. To the resultant solution 1 volume of 10% trichloroacetic acid was added, followed by 7 volumes of acetone but no precipitate was formed.

(i)(b)The polymeric residue from the fronds (4 g, N = 3.77%) was exhaustively extracted at room temperature with phenol: acetic acid: water

(1:1:1) in an Omni-Mixer. The resultant extracts were centrifuged and the combined centrifugate concentrated to small volume. The addition of an equal volume of 10% aqueous trichloroacetic acid and 7 volumes of acetone failed to provide a protein precipitate. The above procedure was repeated with the extractions being performed at 40°C to provide a final polymeric precipitate (2 mg) which, however, afforded a strong carbohydrate Molisch test.

(ii) Calcium Hydroxide Extraction. The polymeric residue from the fronds (2 g) was ground to a paste with sand and 0.2% calcium hydroxide solution in a mortar. The resultant paste was then extracted by stirring with 300 ml water, centrifuged and the subsequently concentrated centrifugate treated with an equal volume of 10% trichloroacetic acid to afford a slight precipitate (1 mg) which produced a strong reaction to the carbohydrate Molisch test.

(iii) Formic Acid Extraction. The dried material from Nereocystis fronds (20 g, N = 2.43%) was successively extracted three times with 700 ml of 90% formic acid for six hr. with stirring. After centrifugation the combined centrifugates were evaporated to small volume and treated with an equal volume of 10% trichloroacetic acid and 7 volumes of acetone. The precipitate formed was washed with acetone and dried under vacuum to yield a polymer (1.62 g, N = 8.8%). The residual material (7.12 g. N = 2.79%) was obtained after washing with water and drying by solvent exchange.

(iv) Extractive Fractionation of Proteinaceous Material. The polymeric residue from the fronds of Nereocystis (8 g, N = 4.22%) was extracted with 700 ml of a 1% aqueous calcium chloride solution for three hr. with stirring at room temperature. The centrifugate obtained by centrifugation was concentrated to small volume and an equal volume of 10% trichloroacetic acid added. No precipitation occurred and the total nitrogen

in the supernatant was determined as 2.10 mg. The extraction procedure was repeated at 45°C and the resultant concentrated centrifugate (N total = 3.36 mg) on treatment with trichloroacetic acid also failed to produce a precipitate.

The residue from the above procedure was treated with 700 ml phenol: acetic acid: water (1:1:1) for three hrs. at room temperature. No protein was precipitated on addition of trichloroacetic acid to the concentrated extract (N total = 7 mg). The above extraction procedure was repeated at 45°C and the resultant concentrated centrifugate (N total = 8.12 mg) failed to produce a protein precipitate on addition of trichloroacetic acid.

Two successive extractions of the residue from the previous extraction with 700 ml of 0.2% calcium hydroxide at 45°C yielded no precipitable protein with trichloroacetic acid and the total nitrogen in the concentrated extract was 9.66 mg.

Cold 90% formic acid (700 ml) was added to the residue from the previous treatment and the mixture stirred at room temperature for six hr. then centrifuged and the centrifugate, together with water washings, was concentrated to small volume. A precipitate formed on addition of an equal volume of 10% trichloroacetic to the concentrate and following washings with 5% trichloroacetic acid was freeze dried to yield a polymeric material (35 mg, N = 7.48%); the supernatant solution was analyzed (N total = 12.32 mg).

The residue from the formic acid treatment was then extracted with 700 ml of 0.2% sodium hydroxide solution at 45°C and the extracts after centrifugation were concentrated and an excess of 10% trichloroacetic acid added to afford a gelatinous precipitate (570 mg, N = 0.73%) and a super-

natant solution (N total = 2.24 mg). The residue from the successive extraction procedures was washed thoroughly with water and freeze dried to yield residual polymeric material (7.85 g, N = 3.30%).

7. Carbohydrate Extraction Procedure

The polymeric residue from the fronds of *Nereocystis* (July sample, N = 3.77%, 20 g) was extracted with 500 ml of 1% aqueous calcium chloride for 0.5 hr. at 60°C and the resultant solution centrifuged. The residue was further extracted three times and the combined concentrated extracts treated with five volumes of ethanol to afford a crude "fucoidan" polysaccharide which was dried by solvent exchange (1 g). A sample of the crude polysaccharide was hydrolysed with 1 N sulphuric acid for 16 hr. at 100° C and the hydrolysate neutralized with barium carbonate, centrifuged and the centrifugate deionized with Rexyn 101 H^{+} resin. The resultant hydrolysate was evaporated to dryness and a sample analysed by paper chromatography while the remainder was reduced by the addition of sodium borohydride. Rexyn 101 H^{+} resin was added to the reduced hydrolysate to destroy the excess borohydride and the solution was evaporated to dryness. Removal of the boric acid by codistillation with methanol was followed by acetylation of the residue in acetic anhydride : pyridine (1:1; v:v) for six hours at 100° C. The acetylation mixture was poured into dilute sodium bicarbonate solution and extracted with chloroform. The combined extracts were washed with dilute hydrochloric acid, dilute sodium bicarbonate, dried over anhydrous sodium sulphate and evaporated to dryness. The resultant alditol acetates were chromatographed on a gas-liquid column (5 ft. x 0.25 in.) of 3% ECNSS-M on Gas Chrom Q (100/120 mesh) operating at 178° C. The composition of the neutral sugar fraction was 38% fucose, 32% mannose, 20% galactose, 5% glucose, 3% arabinose and 2% xylose. Paper

chromatography indicated the presence in the hydrolysate from the "fucoidan" material of a trace of the uronic acid, glucuronic acid.

The residue from the calcium chloride treatment was then exhaustively extracted with a 1% aqueous sodium carbonate solution at 60° C. The resultant concentrated dialysed extracts were precipitated by the addition of five volumes of ethanol and dried by solvent exchange to afford sodium alginate (7 g, N = 0.3%) which on hydrolysis yielded guluronic and mannuronic acids. The residue from the alkali treatment was dialysed against running water and dried by solvent exchange to afford a "cellulose" residue (10 g, N = 5.91%) which yielded only glucose on hydrolysis.

Similarly, the polymeric residue from the stipes of Nereocystis (July sample, 20 g, N = 2.44%) was extracted with 1% calcium chloride to yield "fucoidan" material (1 g) which on hydrolysis gave 36% fucose, 34% galactose, 20% mannose, 6% glucose, 5% xylose and traces of rhamnose and glucuronic acid. The residue from the above procedure was extracted with 1% sodium carbonate to yield sodium alginate (10 g, N = 0.12%) which gave guluronic and mannuronic acids on hydrolysis. The resultant residue (9 g, N = 3.89%) from the extraction procedures afforded only glucose on hydrolysis.

RESULTS AND DISCUSSION

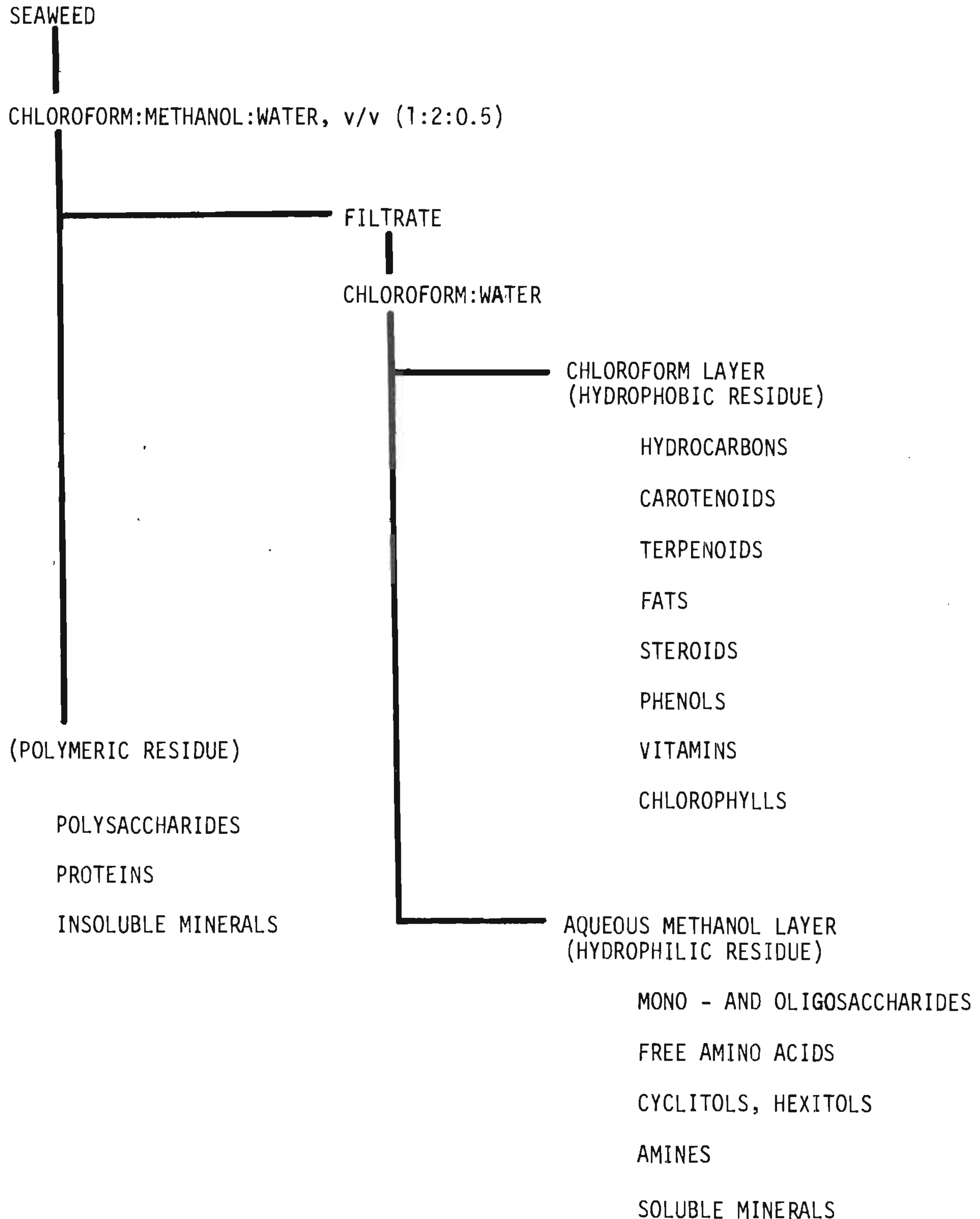
The average content of dry matter in the fronds and stipes of Nereocystis luetkeana, 8.40% and 8.57% respectively, was derived from a seasonal range in values of 5.89 to 9.63% and 7.53 to 9.60% respectively, Table 1. Virtually half the weight of this dry matter was in the form of inorganic minerals which yielded average ash contents of 40.2% and 51.3% for the fronds and stipes respectively, Table 1. Closer inspection of the mineral content indicated that the potassium and sodium ions constituted the bulk of the cations present while the chloride and sulphate ions accorded the major portion of the constituent anions (Whyte et al, 1974(a); Whyte et al, 1975(a); Whyte et al, 1975(b)).

In evaluating the overall chemical fabric of the alga use was made of an extractive fractionation technique (Whyte et al, 1970) which without multi-stage fractionations allowed for a convenient one step extractive separation of the algal components into distinctly separate chemical classes. The nature of the chemicals in algae which may be associated with the hydrophobic, hydrophilic and polymeric residues isolated from this extraction technique are outlined in the schematic illustration, Scheme 1.

The fronds of Nereocystis on extractive fractionation yielded an average content of 5.9% chloroform soluble hydrophobic residue which ranged throughout the season from 2.0 to 8.0% of the dry alga, Table 2. The low molecular weight water soluble components of the fronds forming the hydrophilic residue ranged from 37.5% to 49.8% from April to October to provide an average seasonal value of 44.5%. The residue remaining after the tri-solvent extraction procedure, containing essentially the nitrogenous and carbohydrate polymeric material, ranged from 44.2% to 56.0% of the dry weight

SCHEME 1

EXTRACTIVE FRACTIONATION OF SEAWEEDS



of the fronds and afforded an average seasonal 49.6% content of polymeric components. The lowest value for the hydrophobic residue, 2.0%, was provided by the June sample of fronds which, however, afforded the highest level of polymeric components,56.0%, Fig. 1. The high value,55.7%,for the polymeric residue in July compensated for the exceptionally low value, 37.5%, exhibited for the hydrophilic components for that month. Nevertheless, a more stable overall chemical composition was observed for the fronds collected after July towards the latter half of the growing season, Fig. 1.

The results of the extractive fractionation of the stipes of Nereocystis throughout the season are presented in Table 3 and graphically depicted in Fig. 2. The content of the chloroform soluble hydrophobic components remained quite stable throughout the season furnishing a 2.7% average content from a seasonal range of 2.1% to 3.3%. The divergencies in the content of polymeric components were automatically compensated by an adjusted content in the hydrophilic components. The low molecular weight water soluble components constituted 48.0% of the dry weight of the stipes and afforded a seasonal range in values from 39.8% to 52.1%. The variations in the content of polymeric components in the stipes, 45.2% to 57.3%, offered a seasonal average value of 49.3% which was practically identical to the average content of polymers, 49.6%, found in the fronds over the same period.

The nitrogen content of the dry alga together with the polymeric residue was determined by the Kjeldahl method and from these values the content of nitrogen in the hydrophilic residue was obtained, Tables 4 and 5. A value of 0.04%, measured for the nitrogen content of the hydrophobic residue from the August sample of fronds, equivalent to 0.002% of the dry weight of the frond, illustrated the negligible amount of nitrogen present

in the chloroform-soluble residues. This small percentage of nitrogen probably stemmed from the pyrrole nuclei of the chloroform soluble chlorophyll component.

The total nitrogen of the fronds, averaging 2.73% with a range of 2.43% to 2.91% over the season, was considerably higher than the 1.37% average value presented by the stipes which offered a range in total nitrogen content from 1.20% to 1.65% over the same period. Transposing the nitrogen contents of the polymeric residues, Tables 4 and 5, into corresponding values for the polymeric nitrogen in the whole alga seasonal averages of 1.98% and 1.06% content for the fronds and stipes respectively were obtained. The remaining nitrogen of the alga, averaging 0.75% for the fronds and 0.30% for the stipes, was contained in the hydrophilic residue, presumably as amines, free amino acids and oligopeptides. The total nitrogen contents of both segments of the alga attained the lowest levels in August principally because of the decline in the contents of polymeric nitrogen, Fig. 3. The subsequent increase in the total nitrogen content in both segments of the alga, from August, reflected an increase in the low molecular weight nitrogenous material, Fig. 3. These results would tend to suggest that the increase noted in the latter components towards the end of the season resulted from minor degradation of the nitrogenous polymeric material as the season progressed. Nevertheless, it was interesting to note from the values cited that from 73% to 77% of the nitrogen in the alga existed in a polymeric state.

To determine the protein content, Table 6, use was made of the 6.25 multiple and the nitrogen content of the polymeric residue which provided an accurate determination of the protein content of the separate algal segments, Table 6. The protein content of the fronds ranged from

10.9% to 14.4% over the season with an average value of 12.4%. The stipes contained half that content with only an average 6.6% protein from a seasonal range covering 5.6% to 8.5%. Removal of the hydrophobic and hydrophilic components by fractionation, Scheme 1, afforded the polymeric residues having seasonal protein averages of 25.1% and 13.6% for the fronds and stipes, respectively. The protein in the polymeric residue from the fronds ranged from a high value in April of 28.31% to the lowest value in June of 23.06% and thereafter increased only slightly towards the end of the season, Fig. 4. Conversely, more fluctuation was noted for the content of protein in the polymeric residue of the stipes with the highest level being attained in June at 17.44% and the lowest value in April at 11.81%, Fig. 4.

To determine the physical and chemical nature of the proteinaceous material in <u>Nereocystis</u> various extractive procedures were employed. The first, a mild isolation technique (Bagdasarian et al, 1964) was performed on the fresh frond by pretreating the tissue with aqueous trichloroacetic acid solutions followed by organic solvents then extracting the residual material with a phenol : acetic acid : water mixture at room temperature. No protein was precipitated on additon of aqueous trichloroacetic acid and acetone to the concentrated extracts. Similar negative results were obtained when the polymeric residue from the fronds was treated with the above extractant mixture in a Servall Omni-Mixer. Only a minor precipitate, 0.05%, was furnished by repetition of the above procedure at elevated temperatures, but it was noted that the isolated polymer contained considerable carbohydrate content. Under mild alkaline conditions using calcium hydroxide the polymeric residue from the fronds provided 0.05% yield of a trichloroacetic acid precipitable material which again presented a strong

positive reaction to carbohydrate qualitative tests. As a more drastic chemical means of isolating the proteinaceous material from the alga 90% formic acid was used as an extractant (Mazur et al, 1938). The dry frond, when extracted with this acid, yielded only 8% of a trichloroacetic acid precipitable polymer which was estimated to contain 55% protein. This procedure still proved to be ineffective in isolating all the nitrogenous material since the 35.6% residual polymer from this treatment still contained some 17% proteinaceous material.

A more systematic extractive fractionation technique was attempted by performing a series of successive extractions as outlined in Scheme 2. The nitrogen content associated with each extract, whether in the precipitate formed or in the remaining supernatant solution, was analyzed to afford a nitrogen balance for the complete procedure. After a sequence of seven extractions, Scheme 2, the majority of the nitrogen, 92%, still remained associated with the carbohydrate polymers in the final residue. It should be noted, however, that the amount of sodium hydroxide used in this extraction procedure was insufficient to effectively remove the majority of the alginate polysaccharide but removal of this polymer, as will be discussed later, had little effect on the dissolution of the bulk of the protein which appeared to be firmly associated with the cellulose fraction. Paper and thin layer chromatography of the hydrolysate from the residue of this fractionation procedure indicated at least fourteen amino acid fractions amongst which hydroxyproline was tentatively identified, by color reaction with ninhydrin, as an important constituent. No definitive characterization of the remaining amino acids was possible as a result of the interference to the chromatographic migration of the amino acids caused by the degradation products from the carbohydrate entities. Further,

SCHEME 2

FRACTIONATION OF PROTEINACEOUS MATERIAL

Polymeric Residue from Fronds (8 g; N= 4.22%) . . . . ($\equiv$337.6 mg N)

Cold $CaCl_2$ —— No ppt.
Supernatant (2.10 mg N) . . . . $\equiv$2.10 mg N

Hot $CaCl_2$ —— No ppt.
Supernatant (3.36 mg N) . . . . $\equiv$3.36 mg N

Cold PAW —— No ppt.
Supernatant (7.0 mg N) . . . . $\equiv$7.0 mg N

Hot PAW —— No ppt.
Supernatant (8.12 mg N) . . . . $\equiv$8.12 mg N

Hot $Ca(OH)_2$ —— No ppt.
Supernatant (9.66 mg N) . . . . $\equiv$9.66 mg N

Cold Formic Acid —— Ppt. (35 mg; N = 7.48%) . . . . $\equiv$2.62 mg N
Supernatant (12.32 mg N) . . . . $\equiv$12.32 mg N

Hot NaOH —— Ppt. (570 mg; N = 0.73%) . . . . $\equiv$4.16 mg N
Supernatant (2.24 mg) . . . . $\equiv$2.24 mg N

Residue (7.85 g; N = 3.3%) . . . . $\equiv$259.05 mg N

(Recovery . . . $\equiv$310.63 mg N)

Ppt. = precipitate;
PAW = Phenol: Acetic Acid: Water.

more specific isolation techniques will be necessary before the chemical nature of the proteinaceous material can be fully elucidated.

The polysaccharide components of the alga were extractively fractionated by the procedure outlined in Scheme 3. The July sample of fronds afforded 2.8% "fucoidan" material which contained 38% fucose, 32% mannose, 20% galactose, 5% glucose, 3% arabinose and 2% xylose as the sugar component. Fucoidan is a name given to a sulphated fucose polymer which, however, is invariably associated with other sugar residues in varying proportions (Percival et al, 1967) as was noted for the fucoidan polymeric material from Nereocystis. Although use has been made of this highly viscous polymer for the production of fucose and in the pharmaceutical industry as a blood anticoagulant and antithrombotic agent (Bernardi et al, 1962) the extremely variable composition of this polysaccharide limits its economic utilization.

The lack of significant amounts of glucose in the hydrolysate indicated the absence of the β (1 → 3) - linked glucan, laminaran (Whyte, 1971) in this alga in contrast to the relatively high percentages, up to 33%, which have been cited for other Laminariales (Black, 1950; Powell et al, 1964). Similarly, no significant amount of laminaran was detected in the "fucoidan" fraction isolated from the stipes as only 6% glucose was noted in the derived hydrolysate which contained in addition 36% fucose, 34% galactose, 20% mannose, 5% xylose and traces of rhamnose and glucuronic acid.

Alginic acid is a cell wall polysaccharide which is normally associated with brown algae although only a few species are used for commercial extraction. This linear copolymer is composed of block segments of β-D-(1 → 4)-linked mannopyranosyluronic acid residues, α-L-(1 → 4)-linked gulopyranosyluronic acid residues and alternating sequences of these

SCHEME 3

EXTRACTIVE FRACTIONATION OF POLYSACCHARIDES

Polymeric Residue (July sample)

extraction with
1% aqueous $CaCl_2$
60°, centrifuge

Residue

extraction with
1% aqueous Na_2CO_3
60°, centrifuge

Residue

"Cellulose" material

Fronds: 27.8% Dry Alga
Stipes: 20.6% Dry Alga
(Protein)

Centrifugate

(1) EtOH pptn.
(2) Dried by solvent exchange.

"Fucoidan" material

Fronds: 2.8% Dry Alga
Stipes: 2.3% Dry Alga

Centrifugate

(1) EtOH pptn.
(2) Dried by solvent exchange.

"Alginate" material

Fronds: 19.5% Dry Alga (η = 5200 cps)
Stipes: 22.9% Dry Alga (η = 6900 cps)

residues. The highly viscous nature of aqueous solutions of this polysaccharide coupled with the gel-forming abilities of these solutions when admixed with specific cations affords considerable scope for this polymer as an emulsifying, stabilizing, suspending, thickening, gel-forming and film forming agent. These physical characteristics make it useful in the pharmaceutical, cosmetic, soap, dental, food, dyeing, paint, building, electrode, pencil, filtration, fire protection, mining, fabric, foundry, wood preservation, rubber, ceramic, adhesive, leather, oil, paper, photographic, polish, pesticide, explosive and textile industries to name but a few.

The sodium alginate isolated from the July sample of the fronds and stipes of Nereocystis, Scheme 3, afforded 1% aqueous solutions with viscosities of 5,200 and 6,900 centipoise respectively, suggesting a degree of polymerisation of the polymer acceptable for commercial utilization since present high viscosity commercial alginate products range from 800 to 1,200 centipoise for 1% aqueous solution (Anon, 1961). Rather than isolate the sodium alginate from each monthly sample the content of this polysaccharide was determined by assessing the carbon dioxide evolved from acidic decarboxylation of the entire algal sample (Whyte et al, 1974(b)). Since only a trace of glucuronic acid, the only other uronic acid sugar detected in the alga, was present, the interferences to this in situ determination were considered to be negligible.

The fronds of Nereocystis contained from 18.6% to 23.3% of sodium alginate over the growing season and provided a seasonal average of 20.0%. A wider range of values 15.0% to 25.8% allowed for a higher seasonal average value of 22.4% for the content of alginate in the stipes. The alginate content in the samples collected after June was consistently higher in the

stipes than in the fronds of the alga although both segments attained peak levels of alginate inclusion in September, Fig. 5.

The residual material remaining after sodium carbonate extraction, Scheme 3, provided only glucose on hydrolysis and the polysaccharide component was considered to be the β (1 $\rightarrow$4)-linked glucan, cellulose. With the extraction procedure specified the residual material also contained 78% and 71% of the protein associated with the fronds and stipes, respectively. This indicated, in accord with the results of previous extraction procedures, that the proteinaceous material resisted chemical dissolution and appeared to be firmly associated with the cellulose component. Such intractability strongly suggests that chemical bonding exists between the hydroxyproline units of the protein and the sugar residues of the cellulose fibres forming the cell walls. Intractable complexes of this type have been observed in higher plants (Lamport, 1973).

The hexitol mannitol is one of the major products of photosynthesis in brown algae where its exact function in the cell sap is still ill-defined, although in periods of darkness it appears to be assimilated into the polysaccharide complex (Bidwell, 1967) and is used in the formation of proteins (Black, 1948(a)). The production of mannitol from seaweeds is conducted in the U.S.A., Great Britain, France and Japan where the recovery processes are protected by a number of patents (Levring, 1969). Use is made of this alditol in the medical profession as an osmotic diuretic, as a diluent for solids and liquids, as a test for kidney function and as a dusting powder; extensive use of this sugar alcohol is also made in the paint, varnish, leather, plastics, resins, paper, pyrotechnics, explosives and diabetic food industries.

The mannitol content of the fronds and stipes of Nereocystis was

determined by gas-liquid chromatographic analysis of the derived acetate and the results obtained are tabulated in Table 8. An average value of 6.50% from a seasonal range of 3.23% to 8.34% was afforded by the fronds while a lower seasonal content of 4.30% emerging from a range of 2.92% to 5.37% was provided by the stipes. A marked decline in the mannitol content of the fronds was observed for the month of June, however, subsequent to this month the mannitol inclusion increased significantly in both segments of the alga to attain maximum levels in August, Fig. 6.

It can be noted from Table 9 that almost half the chemical fabric of both segments of Nereocystis was polymeric in nature and that a 3% higher level of hydrophobic components in the fronds presented the most salient difference in the average gross composition between the fronds and stipes of this alga. On an average almost twice as much protein existed in the fronds than in the stipes of this alga. A narrower range in the protein content in Nereocystis occurs than in the Atlantic species of Laminariales which for L. cloustoni, L. digitata, and L. saccharina ranges from 9% to 17%, 5.5% to 14% and 4.5% to 15%, respectively, however, the resultant seasonal average values are quite similar (Black, 1948(a), (b) and (c)). The intractable nature of the protein from Nereocystis would suggest that processed kelp meal for animal feeding purposes could be more effectively utilized by ruminants, since they could degrade the associated cellulose portion of the meal thereby liberating the proteinaceous material for complete ingestion.

The seasonal range for the content of mannitol in Nereocystis, Table 9, was considerably narrower than the 4% to 27% covered by the three Laminaria species previously mentioned. Similarly, the content of sodium

alginate from Nereocystis exhibited a narrower range than the commercially exploited L. digitata fronds, 25% to 44%, L. digitata stipes, 35% to 47%, L. hyperborea fronds, 17% to 33%, and L. hyperborea stipes, 25% to 38%, yet was similar to the range afforded by Ascophyllum nodosum, 22% to 30% (Haug, 1964). The more constant values for the compositional parameters in Nereocystis are beneficial to commercial exploitation, and although the maximum contents exhibited by the littoral and sublittoral species mentioned above tend to be greater than that observed for Nereocystis, the ease of harvesting the latter floating kelp adequately compensates for this deficiency. Thus the compositional content and the physical characteristics of the extracted sodium alginate denote that Nereocystis luetkeana would be eminently suitable and equally as good as other commercially utilized seaweeds as a source of alginic acid from which the corresponding derivatives that find ready acceptance in a broad spectrum of industries could be prepared.

REFERENCES

Anon, 1961. Kelco algin, the story of its control over the water in products we use in our daily lives. Kelco Co., Calif., 13 pp.

Anon, 1975. Potential of kelp beds under study in B.C. Canadian Fisherman and Ocean Science, 61, 37.

BAGDASARIAN, M., MATHESON, N.A., SYNGE, R.L.M. and YOUNGSON, M.A., 1964. New procedures for isolating polypeptides and proteins from tissues. Biochem J., 91, 91.

BERNARDI, G. and SPRINGER, G.F., 1962. Properties of highly purified fucan. J. Biol. Chem., 237, 75.

BIDWELL, R.G.S., 1967. Photosynthesis and metabolism in marine algae. VII. Products of photosynthesis in fronds of Fucus vesiculosis and their use in respiration. Can. J. Bot. 45, 1557.

BLACK, W.A.P., 1948(a). The seasonal variation in chemical constitution of some of the sublittoral seaweeds common to Scotland. I. Laminaria cloustoni. J. Soc. Chem. Ind., 67, 165.

BLACK, W.A.P., 1948(b). The seasonal variation in chemical constitution of some of the sublittoral seaweeds common to Scotland. II. Laminaria digitata. J. Soc. Chem. Ind., 67, 169.

BLACK, W.A.P., 1948(c). The seasonal variation in chemical constitution of some of the sublittoral seaweeds common to Scotland. III. Laminaria saccharina and Saccorhiza bulbosa. J. Soc. Chem. Ind., 67, 172.

BLACK, W.A.P., 1950. The seasonal variation in weight and chemical composition of the common British Laminariaceae. J. Marine Biol. Assoc. of the U.K., 29, 45.

BURD, J.S., 1915. The economic value of Pacific Coast kelps. Univ. Calif. Coll. of Agric. Bull. 248.

CAMERON, A.T., 1916. The water and iodine contents of some Pacific coast kelps. Contrib. Canadian Biol., 1914-15, 169.

DUNCAN, M.J., 1973. In situ studies of growth and pigmentation of the phaeophycean Nereocystis luetkeana. Helgoländer wiss. Meeresunters, 24, 510.

FALLIS, A.L., 1915. Growth of fronds of Nereocystis. Pub. Puget Sound Biol. Station, 1, 1.

FOREMAN, R.E., 1970. Physiology, ecology and development of the brown alga Nereocystis luetkeana (Mertens) P. and R., Ph.D. Dissertation. Univ. Calif., Berkeley, Calif., 114 pp.

FRYE, T.C., 1915. Gas pressure in Nereocystis. Pub. Puget Sound Biol. Station, 1, 85.

HARTGE, L.A., 1928. Nereocystis. Pub. Puget Sound Biol. Station, 6, 207.

HAUG, A., 1964. Composition and properties of alginates. Norwegian Inst. Seaweed Res., Report No. 30, 123 pp.

HOWARD, G., 1921. Extraction and separation of the pigments of Nereocystis luetkeana. Pub. Puget Sound Biol. Station, 3, 71.

HURD, A.M., 1916. Factors influencing the growth and distribution of Nereocystis luetkeana. Pub. Puget Sound Biol. Station, 1, 185.

KARRER, J., 1916. Micrometabolism of Nereocystis. Pub. Puget Sound Biol. Station, 1, 227.

LAMPORT, D.T.A., 1973. Glycopeptide of extensin. o-D-galactosyl serine and o-L-arabinosyl hydroxyproline. Proc. Symp. on Biogenesis Plant Cell Wall Polysaccharides. Ed. F.A. Loewus, Acad. Press N.Y., p. 149.

LANGDON, S.C., 1916. Carbon monoxide in the pneumatocyst of Nereocystis. Pub. Puget Sound Biol. Station, 1, 237.

LEVRING, T., HOPPE, H.A. and SCHMID, O.J., 1969. Marine algae, a survey of research and utilization. Cram, De Gruyter and Co., p. 360.

MAZUR, A., and CLARKE, H.T., 1938. The amino acids of certain marine algae. J. Biol. Chem., 123, 729.

MERTENS, H., 1829. Fucus Lütkeanus. Linnaea, 48.

PERCIVAL, E. and McDOWELL, R.H., 1967. Chemistry and enzymology of marine algal polysaccharides. Acad. Press N.Y., 219 pp.

POSTELS, A. and RUPRECHT, F.J., 1840. Illustrationes algarum in Oceano Pacifico .... Collectarum, Petropoli.

POWELL, J.H. and MEEUSE, B.J.D., 1964. Laminarin in some Phaeophyta of the Pacific Coast. Econ. Bot., 18, 164.

RIGG, G.B., 1915. Seasonal development of the bladder kelp. Pub. Puget Sound Biol. Station, 1, 309.

RIGG, G.B. and SWAIN, L.A., 1941. Pressure-composition relationships of the gas in the marine brown alga, Nereocystis luetkeana. Plant Physiology 16, 361.

SAGER, R.W., ARRIGONI, L. and FISCHER, L., 1946. A phytochemical study of Nereocystis luetkeana. Amer. J. of Pharmacy, 118, 156.

SETCHELL, W.A., 1908. Nereocystis and Pelagophycus. Bot. Gaz. 45, 125.

SHELDON, S.M., 1915. Notes on the growth of the stipe of Nereocystis luetkeana. Pub. Puget Sound Biol. Station 1, 15.

WHYTE, J.N.C., 1971. Proton magnetic resonance and chemical studies on the permethylated derivatives of algal glucans. Proc. Int'l. Seaweed Symposium 7, 496.

WHYTE, J.N.C. and SOUTHCOTT, B.A., 1970. An extraction procedure for plants: extracts from the red alga Rhodomela larix. Phytochemistry 9, 1159.

WHYTE, J.N.C. and ENGLAR, J.R., 1974 (a). Elemental composition of the marine alga Nereocystis luetkeana over the growing season. Fisheries and Marine Service Technical Report No. 509. 29 pp.

WHYTE, J.N.C. and ENGLAR, J.R., 1974 (b). Determination of uronic acid content of saccharides by acidic decarboxylation: a simplified procedure. Analytical Biochemistry 59, 426.

WHYTE, J.N.C. and ENGLAR, J.R., 1975 (a). Estimation of the halogen content of the marine alga Nereocystis luetkeana over the growing season. Fisheries and Marine Service Technical Report No. 561. 23 pp.

WHYTE, J.N.C. and ENGLAR, J.R., 1975(b). Composition of the non-metallic inorganic components of the marine alga Nereocystis luetkeana over the growing season. Fisheries and Marine Service Technical Report No. 568, 34 pp.

WORT, D.J., 1955. The seasonal variation in chemical composition of Macrocystis integrifolia and Nereocystis luetkeana in British Columbia Coastal waters. Can. J. Bot., 33, 323.

ZELLER, S.M. and NEIKIRK, A. Gas exchange in the pneumatocyst of Nereocystis luetkeana (Mertens) P. and R. Pub. Puget Sound Biol. Station, 1, 25.

TABLE 1

Dry Weight (Freeze Dried) and Ash Content
of Nereocystis over the Growing Season

Month	Dry Weight (% Fresh Plant)		Ash (% Dry Weight)			
			Total		Insoluble	
	Fronds	Stipes	Fronds	Stipes	Fronds	Stipes
April	5.89	8.55	48.0	60.6	5.0	3.7
May	8.91	9.00	41.1	58.4	4.2	2.0
June	7.85	8.40	39.0	48.2	5.0	3.5
July	8.80	9.60	35.9	49.3	5.9	5.4
August	9.38	8.82	38.3	46.5	4.7	4.1
September	8.36	8.07	42.0	46.7	5.1	3.0
October	9.63	7.53	37.3	49.2	3.5	3.4

TABLE 2

EXTRACTIVE FRACTIONATION OF THE FRONDS OF
NEREOCYSTIS OVER THE GROWING SEASON *

Month	% Hydrophobic Residue	% Hydrophilic Residue	% Polymeric Residue
April	6.6	42.4	51.0
May	8.0	46.0	46.0
June	2.0	42.0	56.0
July	6.8	37.5	55.7
August	6.3	47.5	46.2
September	5.9	46.2	47.9
October	6.0	49.8	44.2

* % Dry Weight Basis.

TABLE 3

EXTRACTIVE FRACTIONATION OF THE STIPES OF

NEREOCYSTIS OVER THE GROWING SEASON *

Month	% Hydrophobic Residue	% Hydrophilic Residue	% Polymeric Residue
April	2.9	39.8	57.3
May	3.3	47.8	48.9
June	2.4	48.8	48.8
July	2.1	52.1	45.8
August	2.8	43.5	53.7
September	2.8	52.0	45.2
October	2.5	52.0	45.5

* % Dry Weight Basis

TABLE 4

NITROGEN COMPOSITION OF THE FRONDS OF

NEREOCYSTIS OVER THE GROWING SEASON *

Month	Total Nitrogen in Fronds, %	Nitrogen in Polymeric Residue, %	Polymeric Nitrogen in Fronds, %	Hydrophilic Nitrogen in Fronds, %
April	2.91	4.53	2.31	0.60
May	2.88	4.44	2.04	0.84
June	2.84	3.69	2.07	0.77
July	2.69	3.77	2.10	0.59
August	2.43	3.79	1.75	0.68
September	2.61	3.88	1.86	0.75
October	2.78	3.96	1.75	1.03

* Dry Weight Basis

TABLE 5

NITROGEN COMPOSITION OF THE STIPES OF

NEREOCYSTIS OVER THE GROWING SEASON *

Month	Total Nitrogen in Stipes, %	Nitrogen in Polymeric Residue, %	Polymeric Nitrogen in Stipes, %	Hydrophilic Nitrogen in Stipes, %
April	1.27	1.89	1.08	0.19
May	1.45	2.14	1.05	0.40
June	1.62	2.79	1.36	0.26
July	1.35	2.44	1.12	0.23
August	1.20	1.90	1.02	0.18
September	1.37	1.96	0.89	0.48
October	1.32	2.05	0.93	0.39

* Dry Weight Basis

TABLE 6

PROTEIN CONTENT OF *NEREOCYSTIS*

OVER THE GROWING SEASON

Month	Total Protein (% Dry Weight)		Protein in Polymeric Residue (% Dry Weight)	
	Fronds	Stipes	Fronds	Stipes
April	14.44	6.75	28.31	11.81
May	12.75	6.56	27.75	13.38
June	12.94	8.50	23.06	17.44
July	13.12	7.00	23.56	15.25
August	10.94	6.38	23.69	11.88
September	11.63	5.56	24.25	12.25
October	10.94	5.81	24.75	12.81

TABLE 7

SODIUM ALGINATE CONTENT OF *NEREOCYSTIS*
OVER THE GROWING SEASON

Month	Sodium Alginate (% Dry Weight)	
	Fronds	Stipes
April	19.2	15.0
May	20.4	18.7
June	18.6	25.3
July	19.5	22.9
August	20.4	25.0
September	23.3	25.8
October	19.0	24.4

TABLE 8

MANNITOL CONTENT OF *NEREOCYSTIS*
OVER THE GROWING SEASON

Month	Mannitol (% Dry Weight)	
	Fronds	Stipes
April	4.69	2.92
May	7.28	3.31
June	3.23	3.17
July	6.55	5.21
August	8.34	5.37
September	7.48	5.19
October	7.92	4.90

TABLE 9

MEAN, MINIMUM AND MAXIMUM LEVELS
OF ORGANIC CHEMICAL PARAMETERS IN
NEREOCYSTIS OVER THE GROWING SEASON *

Parameter	Fronds		Stipes	
	Mean	Range	Mean	Range
Hydrophobic Residue %	5.9	2.0 - 8.0	2.7	2.1 - 3.3
Hydrophilic Residue %	44.5	37.5 - 49.8	48.0	39.8 - 52.1
Polymeric Residue %	49.6	44.2 - 56.0	49.3	45.2 - 57.3
Total Nitrogen %	2.73	2.43 - 2.91	1.37	1.20 - 1.62
Polymeric Nitrogen %	1.98	1.75 - 2.31	1.06	0.89 - 1.36
Hydrophilic Nitrogen %	0.75	0.59 - 1.03	0.30	0.18 - 0.48
Total Protein %	12.4	10.9 - 14.4	6.6	5.6 - 8.5
Protein in Polymeric Residue %	25.1	23.1 - 28.3	13.6	11.81 - 17.4
Sodium Alginate %	20.0	18.6 - 23.3	22.4	15.0 - 25.8
Mannitol %	6.50	3.23 - 8.34	4.30	2.92 - 5.37

* Dry Weight Basis

Fig. 1

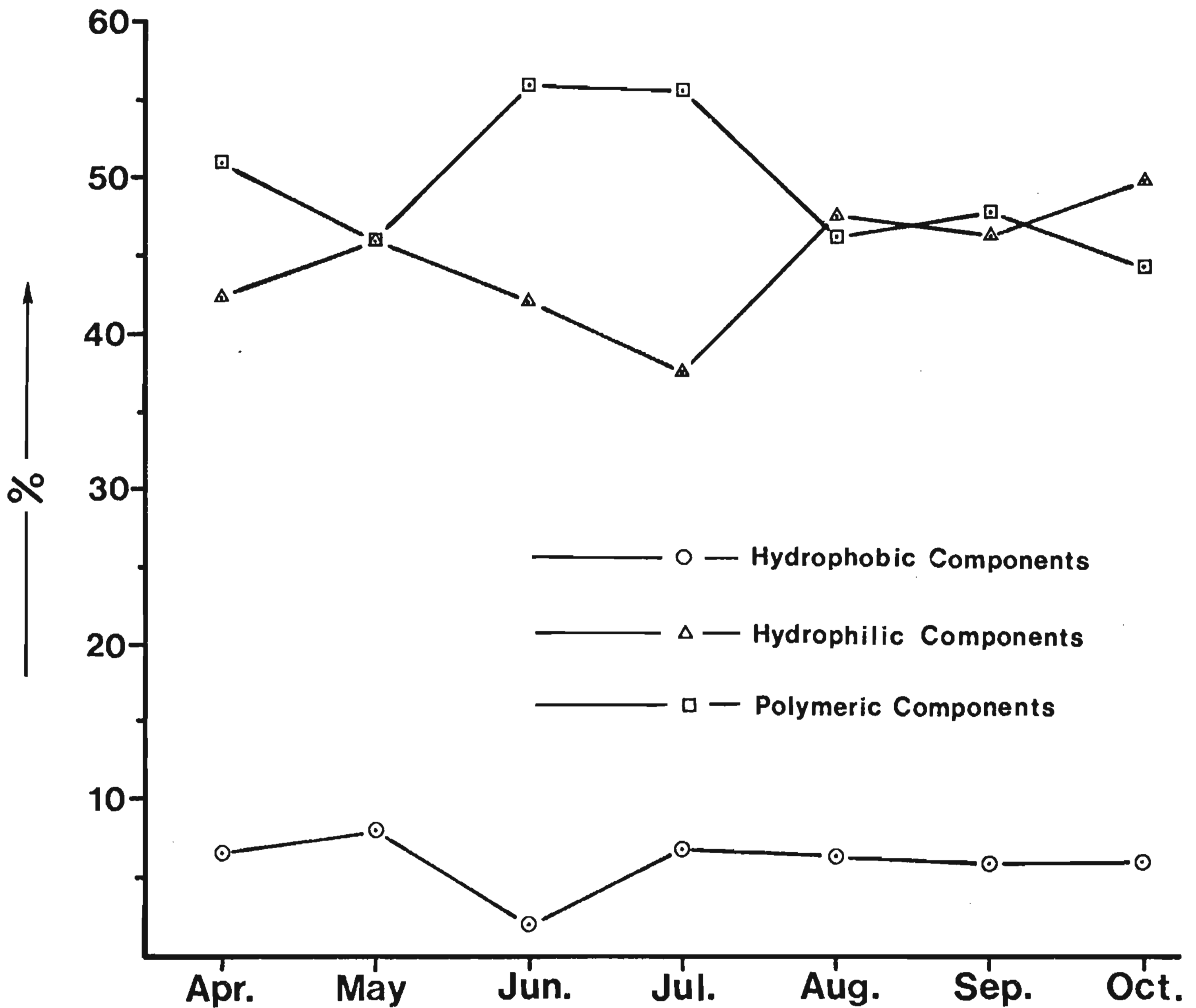


Fig. 2

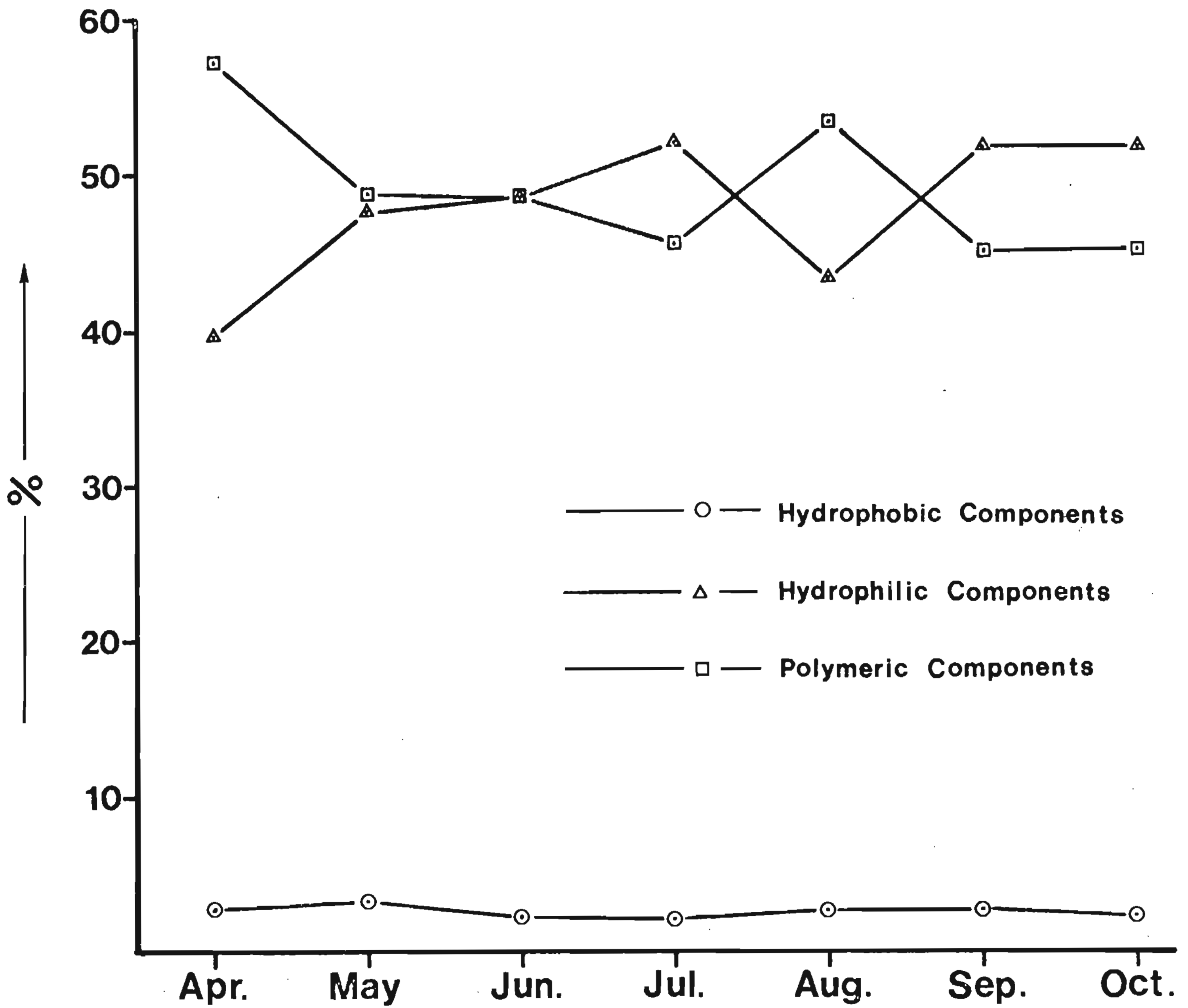


Fig. 3

Nitrogen Content

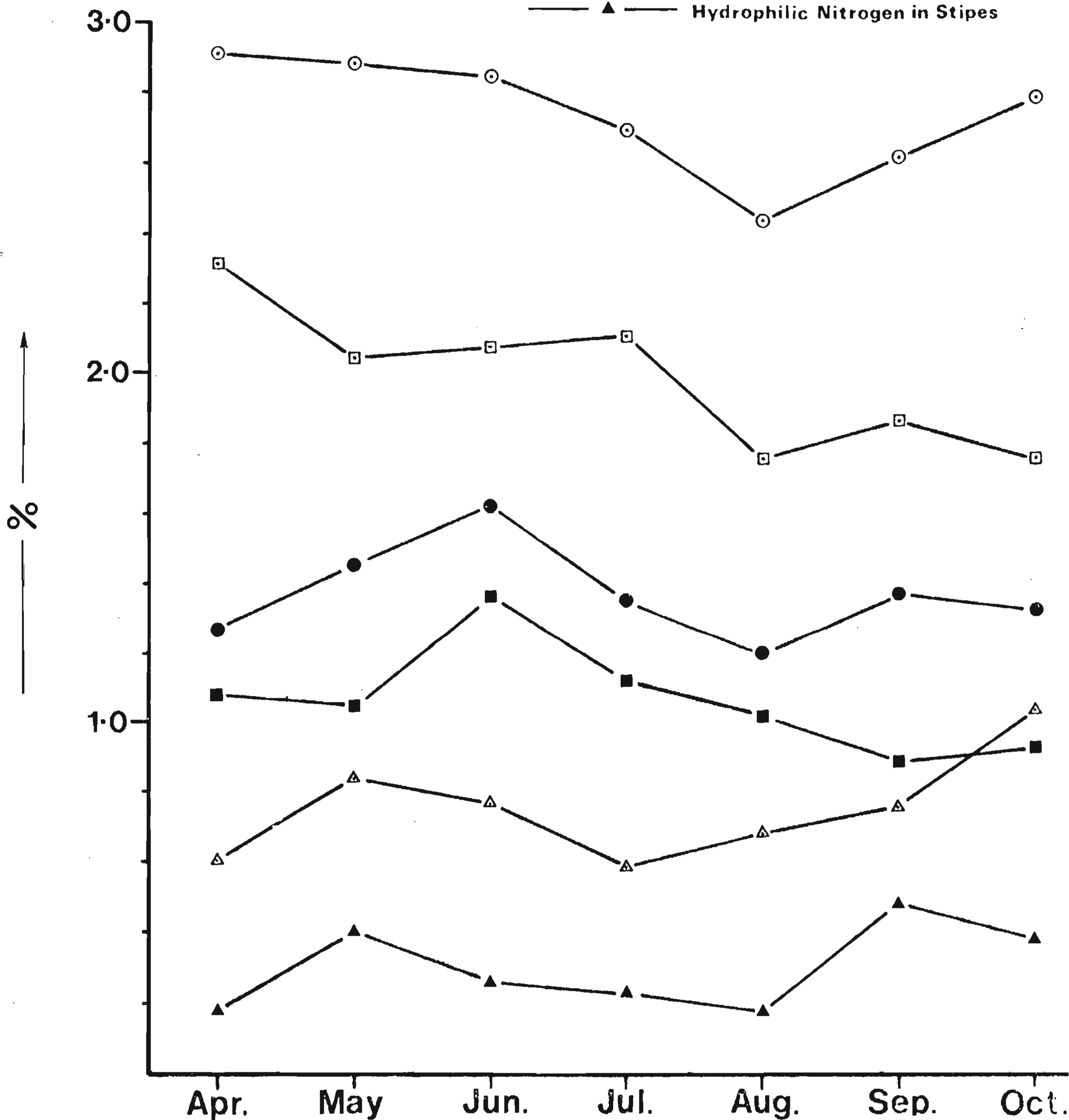


Fig.4

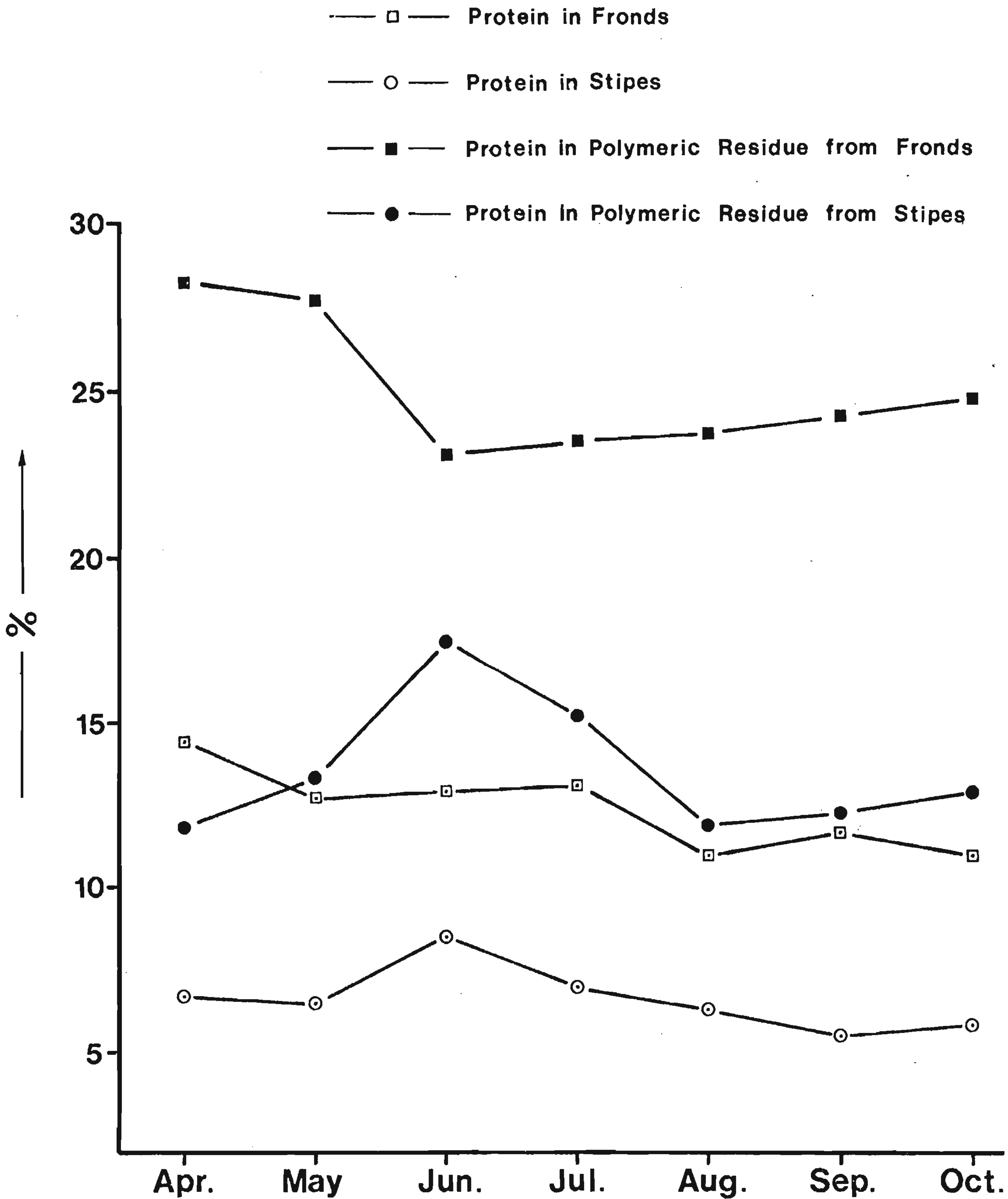
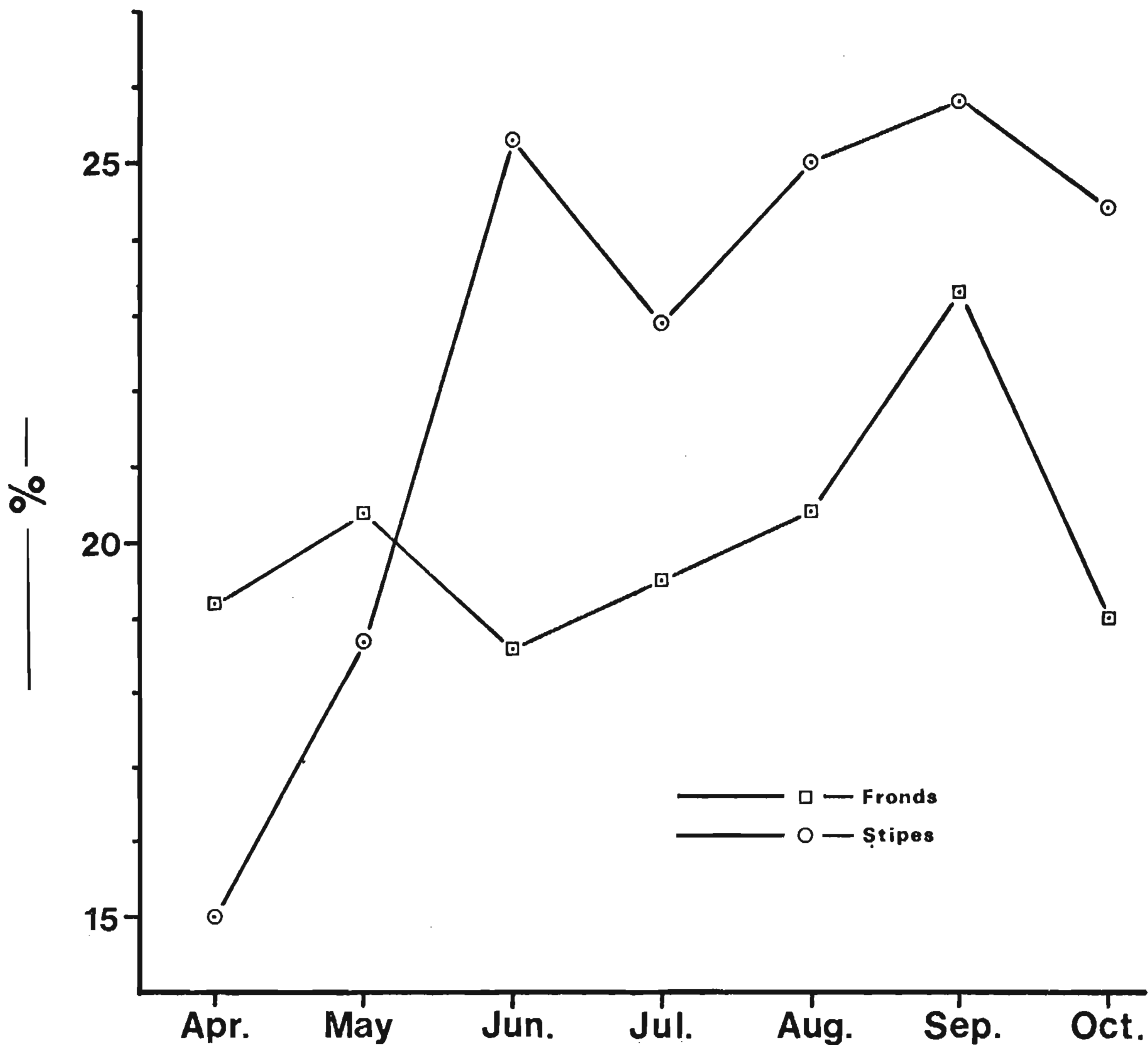


Fig. 5



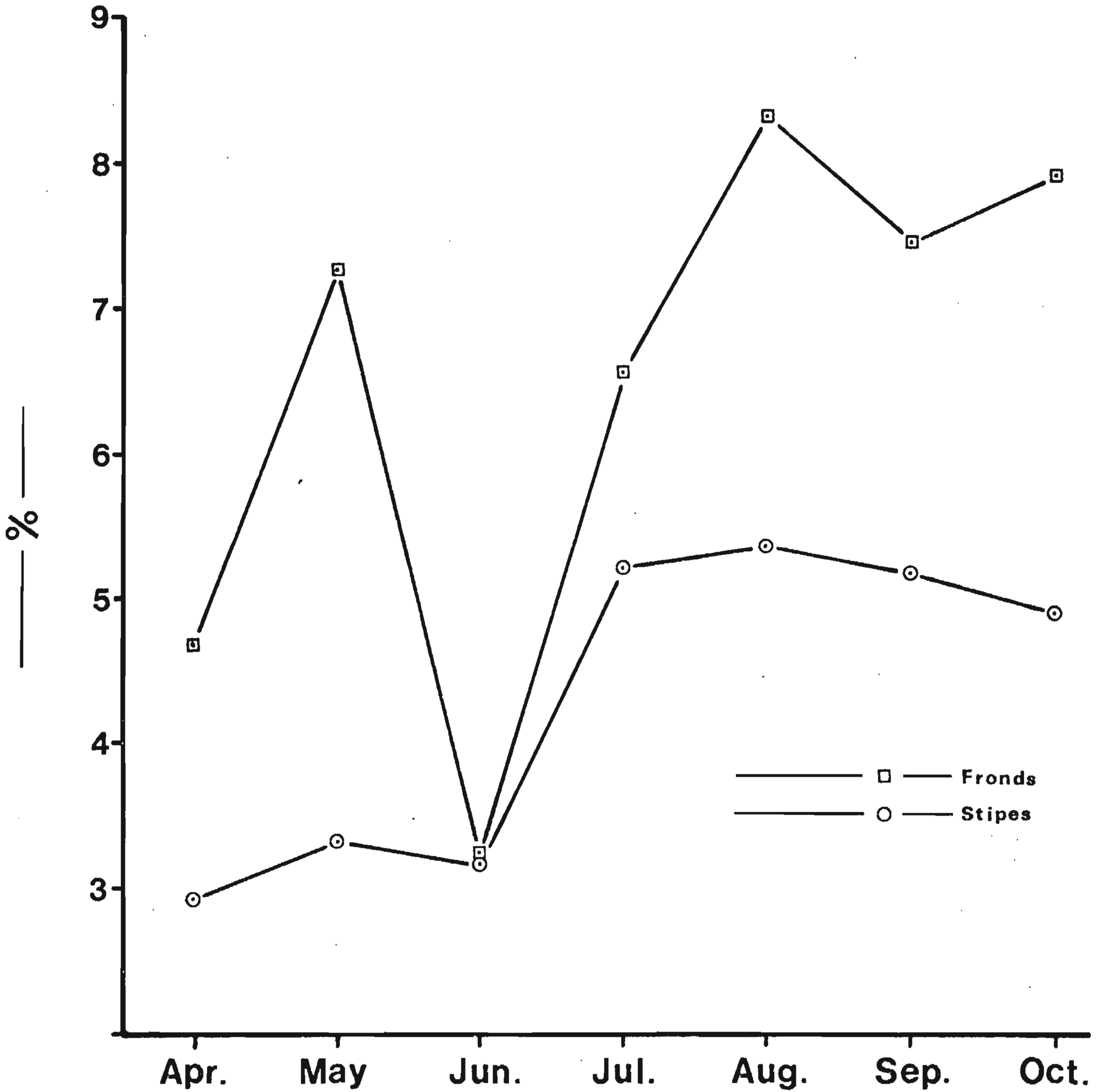


Fig.6
Mannitol Content
9
8
7
6
5
4
3
%
Fronds
Stipes
Apr.
May
Jun.
Jul.
Aug.
Sep.
Oct.