THE TAXONOMY, LIFE HISTORY AND CYTOLOGY OF BRYOPSIS

AND RELATED GENERA FROM SOUTHERN AUSTRALIA

by Graeme N. MacRaild

Thesis submitted for the degree of Doctor of Philosophy in the University of Melbourne, April 1974.
DECLARATION

I hereby declare that this thesis is my own work, except where stated to the contrary, and that it is not substantially the same as any thesis already submitted to any other University.

Gracene M. Beeslaaf
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ABSTRACT

MacRaild, G.N., The Taxonomy, Life History and Cytology of *Bryopsis* and Related Genera from Southern Australia.

Three genera, *Bryopsis*, *Derbesia* and *Pedobesia*, have been studied in the field, in culture and through carefully correlated light and electron microscopy. Three species of *Bryopsis* are recognised; *B. vestita*, *B. plumosa* and *B. gemellipara*. These are described by combinations of characters which can be easily determined in field populations. Additional descriptions are given of the cytology, life histories and interfertility of these species and the genera *Derbesia* and *Pedobesia*.

The study shows a close similarity between the three genera, which are classified within the Derbesiales.
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SECTION I

GENERAL BACKGROUND TO THE STUDY
I. 1 Introduction.

Greville (1830 p.187) commented that Bryopsis is "One of the most beautiful genera of the marine flora, and so perfectly natural that it is most difficult to define the species." Echoes of this statement have appeared numerous times in the algal literature (Harvey 1863, Chapman 1956, Womersley 1956), the two recent authors indicating the need for a general monograph of the genus. In the absence of such a monograph an attempt is made in this thesis to distinguish between the species of Bryopsis growing on the south eastern Australian coast and to develop a basis on which future taxonomic work on the genus may proceed.

The species concept has been described in a number of ways, the two most important elements of definition according to Davis and Heywood (1963 p.91) being; "(a) Taxonomic (embracing the orthodox, typological, morphological, morpho-geographical, etc.)

(b) Biological (including the biosystematic, genetical, cytogenetical, non-dimensional, multidimensional, etc.)."

In a morphologically simple genus the definition of species tends to rely on minor differences in one, or a small number of characteristics. In Bryopsis the morphological form of the alga is based on a simple siphonous organisation, with the complete thallus composed of a single tubular structure which may be variously branched. In consequence, most species of Bryopsis are described largely on the basis of their branching habit and size. The concept and description of species of Bryopsis has therefore not yet reached beyond the "taxonomic" level, and one may be excused for believing with John Locke (1689) that at least within Bryopsis, "genera and species... depend on such collections of ideas as men have made, and not on the real nature of things", and that "our distinct species are nothing but distinct complex ideas, with distinct names annexed to them" (quoted in Cowan 1962 p.434).

Both taxonomic and biological aspects of the species concept have been taken into account in describing the three species of Bryopsis which are now recognised from the south eastern Australian coast. While an attempt has been made to apply no undue weighting to any of the data obtained in this study, the species should where possible, be
units recognisable in the field by sets of morphological and ecological characteristics. It is hoped that the species described in this thesis will prove to be a more natural grouping of the morphological forms observed within the *Bryopsis* complex of this region.

1.2 The scope of the investigation.

A relatively small section of the southern Australian coast was chosen for detailed investigation. Frequent collections were made from the regions close to Melbourne, Victoria, and detailed in situ observations of populations of *Bryopsis* at Point Lonsdale, Werribee and Blackrock (Fig. I.1) were possible. Regular but less frequent visits were made to Cape Patterson, Sorrento, Peterborough, Yambuk, Nora Creina and Cape Lannes. From each of these localities (Fig. I.1) material has been collected and used for culture and cytological studies, and representative herbarium specimens are deposited in the herbarium of the Botany School, University of Melbourne (MELU).

![Fig. I.1 Map showing major collecting sites on the south eastern Australian coast.](image-url)
Examination of herbarium material of the southern Australian Bryopsis from ADU, MEL, MELU and several European herbaria extended the study to include specimens from a wider area of coastline. However, as only limited liquid preserved material, and no living specimens from Tasmania, Western Australia and the western region of South Australia were available, detailed descriptions are provided only for the species inhabiting the south eastern Australian coast.

Life history studies on species of Bryopsis other than those investigated here, have shown that one species, Bryopsis halymeniae Berthold alternates with a sporophytic generation which has previously been described as Derbesia neglecta Berthold (Hustede 1960). Other species also have a sporophytic generation, not typical of Derbesia, but producing Derbesia-like spores from which the succeeding gametophytic generation is derived (Rietema 1969-1972). It was therefore decided to undertake a life history study of the two species of Derbesia (one of which is now referred to Pedobesia MacRaild and Womersley 1974) and one species of Halicystis, all of which have been found at the localities described above, to determine their relationship with the species of Bryopsis. As unusual features were found within the Pedobesia life history many of the techniques employed in the study of Bryopsis were used to build up a detailed description of the new genus and its only known species, Pedobesia clavaeformis.

Observations of the genera Bryopsis, Derbesia, Halicystis and Pedobesia have been made from both natural populations growing in situ and specimens grown in the laboratory under controlled conditions. The descriptions of the cytological organisation of these plants has resulted from careful correlation of images of both living and fixed tissue obtained with the light microscope, and images from the transmission and scanning electron microscopes.

I. 3 Descriptions of the genera Bryopsis, Derbesia, Halicystis and Pedobesia.

I. 3-1 Bryopsis

The genus Bryopsis was erected by Lamouroux (1809) to accommodate the species Bryopsis arbuscula Lamouroux (1809 p.134,t.1,f.1),
Bryopsis cupressina Lamouroux (1809 p.135, t.1, f.3), Bryopsis hypnoides Lamouroux (1809 p.135, t.1, f.2), Bryopsis muscosa Lamouroux (1809 p.135, t.1, f.4) and Bryopsis pennata Lamouroux (1809 p.134, t.3, f.2). From these Egerod (1952) has established B.pennata as the lectotype of the genus. Plants referred to this genus have a coenocytic thallus consisting of an irregularly branched filamentous base variously described as a "creeping, filamentous and little branched rhi...om anchored by rhizoids..." (Fritsch 1945 p.375) or a rhizoidal system (Burr and West 1970) from which arise one to numerous erect main axes bearing lateral branches (ramuli). Egerod (1952 p.369) describes these erect parts of thallus as "axes beset with filiform pinnae in the upper part in a distichous or radial arrangement; axes and pinnae occasionally divided to second or third order; pinnae functioning as gametangia at maturity."

Internal septation of the tubular thallus occurs only at the points at which the individual ramuli leave the main axis, or occasionally where the thallus has been damaged. When first formed the ramuli are continuous with the main axis, there being only a slight constriction at their point of origin. However, the older ramuli on each plant progressively become isolated from the axis by formation of a basal septum at the point of constriction (Pringsheim 1871, Mirande 1913, Burr and West 1970). Isolated ramuli may then undergo gametogenesis with all the cytoplasm being used in the formation of male and female gametes. In a few species not all the isolated ramuli become gametangia but some retain their apical growth and form secondary branched axes attached to the main axis of the plant (Burr and West 1970). These secondary branched axes may have a simple rhizoidal system which generally becomes attached to the main axis.

All Bryopsis plants described are gametophytic, producing anisogamous male and female gametes. Most species are dioecious, however, monoecious species are known, with Bryopsis monica Funk producing male and female gametes in different gametangia on the same plant (Rietema 1971a) and Bryopsis hypnoides having both types of gamete in the one gametangium (Feldmann 1957).

I. 3-2 Derbesia

Solier (1847 p.157) established the genus Derbesia naming it in honour of the French algologist A.Derbès. It comprised two species
Derbesia marina (Lyngbye) Solier (=Vaucheria marina Lyngbye) and Derbesia lamourouxii (J. Agardh) Solier (=Bryopsis lamourouxii J. Agardh). The prior naming of one of these species of Derbesia as Bryopsis indicates the similarity between the simple siphonous structures of the two genera, and it is this that led to a lengthy discussion of the validity of the genus Derbesia by Agardh (1887).

Derbesia is characterised by plants which have a creeping, irregular rhizoidal base from which simple to sparingly branched tubular fronds arise. The degree of branching of the upright frond varies with the species but is generally dichotomous to sub-dichotomous. In this respect Derbesia differs from the much more regularly branched upright fronds of Bryopsis. Internal septation of the thallus occurs in some species at the point of branching, and in the production of a basal septum isolating the sporangia from the cytoplasm of the branch.

The only reproductive cells produced by Derbesia plants are multiflagellate spores which have a characteristic ring of flagella inserted into the hyaline apical end of the more or less spherical cell. Spores are produced in sporangia which are borne laterally on the tubular branches.

In comparing Bryopsis and Derbesia, Fritsch (1945 p.387) states "In its habit Derbesia shows some resemblance to Bryopsis, especially if the sporangia in accordance with several authorities are compared with the pinnae of the latter; other points of agreement are seen in the mode of septum-formation at the base of the sporangium and in the general character of the cell-contents. The zoospores are, however, unique...".

I. 3-3 Halicystis.

Halicystis, established by Areschoug (1850 p.446), includes plants which have the simplest morphological form of the siphonous organisation. The type species of the genus is Halicystis ovalis (Lyngbye) Areschoug (=Gastridium ovale Lyngbye). Individual plants are composed of small ovoid to spherical vesicles, which in some species may be up to 3cm in diameter. The rhizoidal parts of the plant are endophytic, only growing within the thallus of calcareous red algae. No internal septation of the thallus occurs, although areas in the cytoplasm which lines the cell wall become isolated by the production of a cytoplasmic membrane before gamete formation. Gametes are strictly anisogamous, and the male and female gametes show some similarity with those of Bryopsis.
They are produced within specialised regions of the cytoplasm, and no distinct reproductive organs are known. All species are dioecious.

I. 3-4 Pedobesia.

The genus Pedobesia was erected by MacRaild and Womersley (1974) and comprises only one species, Pedobesia clavaeformis (J. Agardh) MacRaild et Womersley (=Bryopsis clavaeformis J. Agardh), which grows as dense clusters of erect clavate fronds. These unbranched fronds have no internal septation and arise from a slender, much branched rhizoidal base, which in turn is produced by a small, multilayered, disc-like attachment stage. Spherical to ovoid sporangia are borne laterally on the clavate branches, and produce numerous stephanokont spores which are identical with those of Derbesia.

The most distinctive feature of the genus is the attachment stage which has strongly calcified cell walls and pillars running through the cytoplasm, joining the upper and lower walls. It is a coenocytic structure, which builds up successive layers of discs, finally appearing as a multilayered organism encrusting the rock surface.

I. 4 Historical background to the classification of the siphonous green algae.

Bory (1829) established the family Bryopsidaceae (corrected to Bryopsidaceae by De Toni 1889 p.247) to accommodate the genus Bryopsis which had been erected by Lamouroux (1809). In 1830, however, Greville placed Bryopsis with three other genera, Botrydium, Codium and Vaucheria in his "order" Siphoneae ("equivalent to families in the modern sense." Egerod 1952 p.326). Thus Greville (1830 p.183) is the first to recognise a taxonomic grouping within the green algae characterised by a "frond either composed of membranaceous, filiform, continuous, single or branched tubes, or formed of a combination of such tubes". Since this first recognition of the siphonous green algae as a taxonomic entity the classification of the siphonous organisms has been, and still is in a state of flux.

Both Greville (1830) and Harvey (1849) omitted Caulerpa from the Siphoneae, and it was not until 1858 that Harvey reconsidered the
position of this genus, placing it in the "order Siphonaceae". With this Harvey (1858) also subdivided the order into the Caulerpeae and Codieae, followed by Agardh (1887) placing *Bryopsis* in the Bryopsidaceae.

Although Wille (1890) suggested that the Siphoneae should be raised to the ordinal level, the first description of the order Siphonales was produced by Blackman and Tansley (1902). In this, these authors placed both the septate (Siphonocladeae Schmitz 1879) and the non-septate (Siphoneae), thus expanding the original concept of the order. In the Siphoneae, Blackman and Tansley (1902) recognised six families, including the Bryopsidaceae, Caulerpaceae, Codieae and Derbesiaceae.

It should be noted here that Blackman and Tansley (1902) accepted the proposal of Bohlin (1901) that *Vaucheria* should be removed from the Siphoneae and included in the "Heterokontan class as the type of a new series, the Vaucheriales." (Blackman and Tansley 1902 p.19). Although not accepted by other authors (Fritsch 1945, 1954, Oltmanns 1904), this was later vindicated by the observation that *Vaucheria* lacked chlorophyll b (Seybold, Egle and Hulsbruch 1941, Strain 1949), making its classification in the Chlorophyta (sensu Christensen 1962) untenable.

Oltmanns (1904) further refined the concept of the order Siphonales by removing the septate members referred to the Siphonocladeae (sensu Blackman and Tansley 1902). These he placed in the new order Siphonocladales (as Siphonocladales) based on the family Siphonocladeae of Schmitz (1879). In the Siphonales (sensu Oltmanns 1904) five families are recognised, the Bryopsidaceae, Caulerpaceae, Codieae, Derbesiaceae and the Vaucheriaceae.

This concept of the Siphonales has remained almost unchanged to the present and is used by a large number of algologists (Dawson 1966, Egerod 1952, Fritsch 1954, Harder et al. 1965, Taylor 1960, Womersley 1956) despite the revisions suggested by Feldmann (1946, 1954).

Setchell (1929) and Pascher (1931) suggested that on morphological grounds the Siphonales (sensu Oltmanns 1904) should be separated into two orders, the Caulerpales and Codiales. As these suggestions were not supported with descriptions of the orders, the proposals are invalid (Ducker 1967, Egerod 1952). However, Feldmann (1946) proposed a similar division of the Siphonales (sensu Oltmanns 1904), on the basis of the
distribution of two types of plastids, chloroplasts and leucoplasts (Czurda 1928, Chadeau 1941), and the composition of the cell wall (Mirende 1913). Feldmann (1946) considered that the heteroplastic condition found in the higher plants but no other group of the algae, was sufficient basis on which to form an order within the siphonous green algae, the Caulerpales. Into this order he placed the Caulerpaceae, Dichotomosiphonaceae and Udoteaceae, all of which are also characterised by the absence of cellulose in their cell walls. The second order comprising the homoplastic, non-septate, multinucleate siphonous green algae Feldmann (1946) called the Siphonales (or Eusiphonales, later changed to the Codiales, Feldmann 1954) in which were included the Bryopsidaceae, Codiaeaceae and Halicystidaceae. These were also characterised by the presence of cellulose in the cell walls (Mirende 1913).

Owing to the incomplete evidence of the nature of the plastids in the members of the family Derbesiaceae, Feldmann (1946) was unable to place this group within his scheme. However in 1954, Feldmann proposed that this grouping should be raised to the ordinal level, creating the Derbesiales which were characterised by the presence of only one type of plastid, cellulose in the cell walls and an alternation of generations with Halicystis (Kornmann 1938, Feldmann 1950).

Based on the reported alternation of generations between Derbesia neglecta and Bryopsis halymeniae (Hustede 1960, Rietema 1972) and the presence of a Derbesia-like sporophytic stage in the life history of other species of Bryopsis (Rietema 1969-1972), van den Hoek et al. (1972) suggest that the order Derbesiales is redundant, as Derbesia can now be considered within the Codiales. The value of this proposal will be discussed in detail later in this thesis.

The system of classification to be followed in this thesis is that proposed by Round (1971). This uses as its basis the proposals of Feldmann (1946, 1954) and takes into account more recent studies on the biochemical characteristics of the siphonous green algae (Miwa, Iriki and Suzuki 1961, Strain 1965, Kleinig 1969, Parker 1970). Under this system the siphonous green algae are grouped together in the class Bryopsidophyceae. Therefore Round (1963) accepts as valid, the nomenclatural change from the Siphonales (sensu Oltmanns 1904) to the Bryopsidales (Fott 1959) but elevates this order to a class. Included in the Bryopsidophyceae (Round 1963) are three series, the Hemisiphoniidae,
Cystosiphoniidae and the Eusiphoniidae. The series Eusiphoniidae is essentially the same as the Siphonales (sensu Oltmanns 1904) and includes five orders, Caulerpales, Codiales, Derbesiales, Dichotomosiphonales and Phillosiphonales.

Of the four genera which are studied in this thesis Bryopsis is classified within the Codiales, and Derbesia, Halicystis and Pedobesia within the Derbesiales.
SECTION II

MORPHOLOGICAL AND ECOLOGICAL CHARACTERISTICS OF

THE GROUPS WITHIN THE BRYOPSIS COMPLEX
II. 1 Introduction.

Thirteen species names describing the different forms of *Bryopsis* on the southern Australian coast can be found in the literature. Womersley (1956) accepted only eight of these stating that the herbarium records of the following species are either inadequate, cannot be located or should be referred to other species for which adequate records are available; *Bryopsis cypressoides* Lamouroux recorded from Kangaroo Is. (Womersley 1950 p.144), *Bryopsis hypnoides* Lamouroux recorded from Eaglehawk Neck, Tas. (Lucas 1912 p.168, 1928 p.9, Guiler 1952 p.74) and *Bryopsis vestita* J. Agardh recorded by Reinbold (1898 p.34) on the basis of a juvenile plant collected in the Lacepede Bay region, S.A.. Two further species have since been referred to other genera; *Bryopsis baculifera* = *Chlorodesmis baculifera* (J. Agardh) Ducker (1966) and *Bryopsis clavaeformis* = *Pedobesia clavaeformis* (J. Agardh) MacRaidt et Womersley (1974).

The species recognised by Womersley (1956) are shown in Table II.1 with the names applied to the corresponding taxa in the more recent accounts of MacRaidt (1970) and King et al. (1971). These latter publications follow extensive study of the type specimens of *Bryopsis* by the present author's supervisor, Mrs S.C. Ducker, and detailed examination by the present author of other herbarium specimens of southern Australian *Bryopsis* on loan from ADU, BM, C, HMB, L, and MEL.

Two species, *Bryopsis australis* Sonder and *Bryopsis foliosa* Sonder have been recorded only from the south western Australian coast. Examination of limited liquid-preserved material collected at the type locality for *B.australis* (MELU 21,753) suggests that this species is unlike anything collected from the south eastern Australian coast. However, without further in situ observations throughout the year and culture studies of this material and *B.foliosa*, the affinities of these species cannot be accurately determined.

II. 2 Morphological characteristics of the units within the *Bryopsis* complex.

It is commonly recognised that "At present, morphological and ecological circumscriptions are the two criteria of greatest value for the definition of species limits..." (Dixon 1966 p.61). All the *Bryopsis* material collected in this study of the south eastern Australian coast
has been assigned to six groupings which are described in the following sections with both morphological and ecological characteristics.

II. 2-1 Bryopsis vestita J. Agardh 1878 p.3.

This species was described from specimens collected from Warrington, Otago, New Zealand by Berggren. Reinbold (1898) records it from the Lacepede Bay region of S.A. and this appears to be the only early record of the species from the southern Australian coast. As indicated earlier, Womersley (1956) includes B. vestita in his "species of Bryopsis incorrectly recorded from southern Australia" as Reinbold's specimen has not been located and because of "the absence of any other collection of true B. vestita from the Lacepede Bay region of Reinbold's record" (Womersley 1956 p.364).

Specimens of Bryopsis collected from a number of localities along the south eastern Australian coast (Fig. II.1) fit the description of B. vestita (J. Agardh 1878 p.3, 1887 p.30) and mature specimens are identical with the type specimen (LD 15373, and the isotype from BM n.n. Fig. II.2). A study of the Bryopsis specimens in ADU show that this species has been referred to B. gemellipara by Womersley (1956).

As noted by J. Agardh (1887 p.30) B. vestita is one of the larger species of the genus and grows as dense clusters of erect fronds (up to 25cm) arising from an irregularly branched rhizoidal base (Fig. II.3). Erect fronds are branched, with ramuli appearing first tetrastichously (in four rows), then radially on the axis, the tetrastichous morphology persisting only until the plants have reached a length of approximately 5cm. The typical branching pattern of the mature plant is therefore radial. As the axes increase in length the lower ramuli become radially branched while not lengthening significantly and in the mature form, the overall shape of the branched axis is that of a cone with an acute apex.

II. 2-2 Bryopsis gemellipara J. Agardh 1887 p.25.

This species was first described from a specimen collected at Queenscliff, Vic. (J. Bracebridge Wilson, LD herb. Agardh No. 15150, Fig. II.4) with ramuli arranged apparently distichously but with two rows of ramuli on each side of the main axis (tetrastichous).
Fig. 11.1 *B. vestita* collected at Point Lonsdale. MELU 21,642.

Fig. 11.2 *B. vestita* type specimen. LD 15373.
Fig. II.3 Rhizoidal parts of *B. vestita* (Pt. Lonsdale MELU 21,493) showing irregularly branched base, producing numerous upright fronds.

Fig. II.4 *B. gemellipara* type specimen. LD 15150.
J. Agardh (1887 p.25) followed by Gepp and Gepp (1908 p.170) suggest that the ramuli are bifurcate at the base, but examination of the type specimen and collections of live material from the type locality, reveals no evidence of this. The lower ramuli of mature plants, may however, branch at their apex producing secondary ramuli which may be arranged radially or tetrastichously.

On the south eastern Australian coast, two apparently different algae fit the general description of the branching pattern of *B. gemellipara* (see also section II. 2-4, Figs II.5, II.10). Only one is found as an obligate epiphyte (Fig. II.6) and it is this form which corresponds with the type specimen. It is therefore thought that the description of *B. gemellipara* should be expanded to include its obligate epiphytic character. *B. gemellipara* can also be distinguished from the tetrastichous juvenile form of *B. vestita* by its simple rhizoidal base from which one to three branched axes arise (Fig. II.7).

The size of *B. gemellipara* is variable with most plants growing to 8cm long, but exceptionally large plants (to 25cm) have been observed. Like *B. vestita* the ramuli do not continue to elongate with the growth of the main axis, thus resulting in the production of a lanceolate plumule.

**II. 2-3 Bryopsis plumosa** (Hudson) C. Agardh 1823 p.448.

As stated by Womersley (1956) this species is almost cosmopolitan, but it is most commonly found in sheltered bays and inlets. Although the type of *B. plumosa* is apparently lost (Womersley 1956) the southern Australian material referred to this species compares with the early descriptions and drawings of European and British specimens (Fig. II.8, from Smith and Sowerby 1790 - 1814, as *Ulva plumosa* Hudson).

In the juvenile form the plant consists of a single, distichously branched axis arising from a small, irregularly branched rhizoidal base. As the axis continues to elongate, the distichous arrangement of the ramuli may become obscure, but is easily observed at the apices of the primary axis and the secondary axes formed through the continued growth and branching of the primary ramuli (Fig. II.9). As a result of this secondary growth and branching of the primary ramuli as the plant matures, it appears in nature as a more or less spherical cluster of branched axes (up to 10cm long) arising from a common axis. This is continuous with the rhizoidal base by which the plant is attached to the substrate.
Fig. II.5  *E. gemellipara* collected at Pt. Lonsdale. MELU 21,494.
Fig. II.6 *B. gemellipara*. Young plant growing on *Thuretia quercifolia* Pt. Lonsdale.
Fig. II.7 *B. gemellipara* collected at Little Dip, S.A. Dissected from host plant. MELU 21,537.
ULVA plumosa.

Feathered Laver.

CRYPTOGAMIA algae.

Gen. Char. Frond membranous or gelatinous. Seeds solitary, scattered throughout its substance, under the cuticle.

Spec. Char. Frond gelatinous, green, thread-shaped, somewhat compressed, branched; branches pinnate, with numerous, parallel, linear, shining segments.


Hudson discovered this elegant plant on submarine rocks and stones on the Devonshire coast. Mr. Woodward found it in little rocky pools, filled daily by the tide, at Cromer; and Mr. W. Borner gathered our specimens at Brighthelmstone in September last.

The whole frond is but two or three inches high. At first gathering, its hue is a bright uniform green, but the colouring matter soon subsides, or rather collects towards the skin, leaving the central part vacuous and pellucid. Several principal branches are sent off by the main stem, and these are elegantly pinnated, with thick-set linear segments, or leaflets, in their upper part; the lower being simple and naked, like the quill of the beautiful feather they thus compose. No. 7 is distinctly known of the fructification.

Fig. II.8 B. plumosa, figures and text from Smith and Sowerby (1790 to 1814).
II. 2-4 Large tetristichous form (Fig II.10).

Plants of this type have only been found at Cape Lannes, S.A., and frequent observations of this population at regular intervals through the growth season were not possible. The size and general growth form are similar to B. vestita, with many upright branched axes arising from an extensive, irregularly branched rhizoidal base. The branching pattern and narrow lanceolate shape of the upright fronds are indistinguishable from the large forms of B. gemellipara. The morphological features of this population of Bryopsis are therefore intermediate between those of B. vestita and B. gemellipara.

From careful dissection of the rhizoidal parts of the large tetristichous form it is thought that this plant grows attached to the rock surface, but the presence of a dense mat of Gelidium, and other red algae interwoven with the rhizoidal material may indicate an epiphytic nature. Early stages of growth of this alga must be studied in nature before a conclusion is reached.

II. 2-5 Small irregularly branched form.

Occasionally on the open coast, slender, irregularly branched Bryopsis plants are found. These may show radial or distichous branching patterns and it is possible that more than one grouping may be recognised. The radially branched members of this group have been referred to as B. minor Womersley (1955).

All branching forms within this group can be described as arising from a simple rhizoidal base, attached to rock or occasionally other algae. From the rhizoidal base, a single, upright, branched axis arises with ramuli which continue to elongate throughout the growth of the plant. In this sense the overall morphology of the mature plant resembles that of B. plumosa, while remaining consistently finer than this species.

II. 2-6 Large irregularly branched form.

Bryopsis plants collected from Werribee, Port Phillip Bay, Vic., represent a very large form in which the branching is generally radial to irregular, but at the apex frequently observed as distichous. This robust form shows the same general morphological features as B. plumosa, but is consistently larger than plants usually referred to this species. As the apices of this form show a basic distichous branching pattern, the radial to irregular branching lower down the axis is thought to be caused by disruption of the basic pattern by intercalary elongation of
Fig. II.9 *B. plumosa*. Young plant collected at Blackrock. Note elongation of lower ramuli.

Fig. II.10 Large tetrastichous form of *Bryopsis* from Cape Lannes.
the axis after initial branching has occurred.

As the lower ramuli elongate a reduced rhizoidal system may also develop at their base, with the rhizoidal filaments attached to the main axis and adjacent ramuli.

Bryopsis australis B. gemellipara (in part) south western Aust.
B. baculifera Chlorodesmis baculifera southern Aust.
B. foliosa --- south western Aust.
B. gemellipara B. vestita (in part) southern Aust.
B. gemellipara (in part)
B. indica --- southern Aust.
B. minor --- Kangaroo Is.
B. pennata --- Eyre Peninsula
B. plumosa B. plumosa (in part) southern Aust.
B. hypnoides (in part)

Table II.1 Species names applied to groups within the *Bryopsis* complex from the southern Australian coast.

II. 3 Ecological characteristics of the units with the *Bryopsis* complex.

II. 3-1 Introduction.

Each of the units recognised above can also be distinguished by their particular ecological habitat or distribution. *B. vestita*, *B. gemellipara* and *B. plumosa* have been recorded from a number of localities between Wilsons Promontory, Vic. and Rottnest Is., W.A. and around Tasmania (Fig. I.1). The first two species are typical of regions of maximal to submaximal wave action, while *B. plumosa* occurs most commonly in sheltered bays or estuaries. The other three forms described (sections II. 1-4 to II. 1-6) have only a very restricted distribution or are rarely collected in the intertidal region.
II. 3-2 Forms inhabiting the open ocean rocky coast.

_Bryopsis vestita_ grows in dense clusters of plants in the upper eulittoral zone. It is a seasonal alga, appearing between July and December on the eastern Victorian coast and through to late January at Nora Creina and Little Dip, South Australia. Unlike _B. gemellipara_ and the small irregularly branched form (section II. 1-5), no remnant populations have been found to persist in the intertidal region, nor has drift material of the species been collected at times of the year other than its particular growth season. This growth season coincides with minimal sea water temperatures at Point Lonsdale (King 1970) and is thought to continue until mid summer at Nora Creina and Little Dip, because of the upwelling phenomenon reported for that part of the southern Australian coast (Hynd and Robins 1967) maintaining lower summer sea water temperatures.

At Point Lonsdale where _B. vestita_ has been studied intensively, rocks between the Claneuse and Lighthouse reefs are seasonally colonised to the exclusion of almost all other algae, except for a mat of _Gelidium pusillum_ growing among the rhizoidal bases of the _Bryopsis_. Plants are also found on the two reefs, but only achieve the same dense distribution in channels where there is considerable backwash from waves breaking over the reefs. The isolated rocks most heavily colonised are covered with sand to a depth of 10 to 30cm during the summer months. This sand movement does not seem to be responsible for the seasonal disappearance of the alga, as the _Bryopsis_ population dies off six to eight weeks before the rocks are covered. However, the possible importance of the annual sand deposition to the alternate phase in the life history has yet to be determined.

_Bryopsis gemellipara_ is recorded as an epiphyte on _Gelidium australis_ in lower eulittoral rock pools (King et al. 1971). Although a wide range of host species is recognised (Table II.2), it is more commonly found on species of _Halopteris_. Plants of _B. gemellipara_ can be found throughout the year, but a distinct seasonal abundance is observed between June and September, when large numbers of plants can be seen at exceptionally low tides, in the upper sublittoral zone (sublittoral fringe) characterised by the presence of _Durvillea potatorum_ (King 1972 p.54).
Apjohnia laetevirens Harvey
Ballia scoparia (J.D. Hooker et Harvey) Harvey
Callipsygma wilsonii J. Agardh
Champia sp
Cladostephus verticillatus (Lightfoot) C. Agardh
Codium fragile (Suringar) Hariot
Corallina sp
Cystophora subfarcinata (Mertens) J. Agardh
Cystophora torulosa (R. Brown ex Turner) J. Agardh
Gelidium australis J.Agardh
Haloplegma preissii Sonder
Halopteris funicularis (Montagne) Sauvageau
Halopteris gracilesens (J. Agardh) Womersley
Halopteris pseudospicata Sauvageau
Jania sp
Laurencia elata (C. Agardh) Harvey
Laurencia tasmanica J.D. Hooker et Harvey
Lenormandi~ prolifera (C. Agardh) J. Agardh
Sphacellaria sp
Thuretia quercifolia Decaisne

Table II.2 List of species on which B.gemellipara has been found epiphytic.

The large tetrastichous form of Bryopsis has been found only at Cape Lannes, S.A., where it occurs in the mouth of a shallow cave at the base of near vertical cliffs. The population is subject to heavy wave action and backwash from waves breaking into the cave. Plants have been observed growing attached to rocks covered with 5 to 10cm of sand in February 1970 and 1972, and it is suggested that this area may be subject to a similar seasonal sand movement to that seen at Point Lonsdale. From personal observations, examination of herbarium specimens housed at ADU and the observations of Dr H.B.S. Womersley (personal communication) over many years, this population is also thought to show a similar seasonal distribution to the population of B.vestita found nearby at Nora Creina and Little Dip.
The small irregularly branched open ocean form of *Bryopsis* is found mainly throughout the summer months but may occur at almost any time of the year. Plants of this type have only been collected from Point Lonsdale, Flinders, Cape Patterson and Yambuk, Vic.. On the intertidal rock platforms at these localities, it occupies a position in the channels in the upper eulittoral and occasionally in the larger rock pools of the mid eulittoral. These plants have been found infrequently and little more can be said of their actual distribution on the south eastern Australian coast, or their habitat preferences.

On these open ocean forms of *Bryopsis*, green Nudibranchs are frequently observed and these are the only form of animal predation seen. Nudibranchs feed on the *Bryopsis* by puncturing the cell wall and removing the cytoplasm from large portions of the thallus. Owing to the large numbers found on the Point Lonsdale population of *B. vestita* towards the end of the growth season, Nudibranchs are thought to contribute to the rapid decline in the population during November and December.

Two different undescribed species of the Nudibranch genera *Ercolania* and *Stiliger* have been found on *B. vestita* and *B. gemellipara* at Point Lonsdale (Mr R. Burn, personal communication). The Nudibranchs found on other forms of *Bryopsis* have not been identified.

II. 3-3 Forms inhabiting sheltered bays.

Within the sheltered bays and inlets along the south eastern Australian coast, two forms of *Bryopsis* have been described. *B. plumosa* is a common species throughout the year in the mid to lower eulittoral, while the large irregularly branched form is restricted to the Werribee region of Port Phillip Bay, an area of high nutrient inflow from nearby sewerage treatment works. As this high level of nutrient inflow is reported to result in the growth of very large forms of other algal species (Spencer 1972) the affinities of this alga to other recognised forms of *Bryopsis* was studied.
SECTION III

LIFE HISTORY AND CULTURE STUDIES OF BRYOPSIS
III. 1 Introduction.

Plants required for experimental cultures were collected from the localities referred to in section I. 2. Specimens were carefully removed from the rock surface, or in the case of epiphytic species collected with their host, to ensure minimal damage to their continuous siphonous structure. These were then transported to the laboratory in cooled seawater, where unialgal cultures were established by one of the methods outlined in the following section. A list of collections from which cultures were established, with MELU numbers of corresponding herbarium records is given in Table III.1.

<table>
<thead>
<tr>
<th>Form</th>
<th>Locality</th>
<th>MELU No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. gemellipara</td>
<td>Point Lonsdale</td>
<td>21,643</td>
</tr>
<tr>
<td></td>
<td>Point Lonsdale</td>
<td>21,494</td>
</tr>
<tr>
<td></td>
<td>Little Dip</td>
<td>21,537</td>
</tr>
<tr>
<td></td>
<td>Nora Creina</td>
<td>21,613</td>
</tr>
<tr>
<td>B. plumosa</td>
<td>Robe</td>
<td>21,529</td>
</tr>
<tr>
<td></td>
<td>Blackrock</td>
<td>--------</td>
</tr>
<tr>
<td>B. vestita</td>
<td>Sorrento</td>
<td>20,602</td>
</tr>
<tr>
<td></td>
<td>Point Lonsdale</td>
<td>20,601</td>
</tr>
<tr>
<td></td>
<td>Point Lonsdale</td>
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</tr>
<tr>
<td></td>
<td>Point Lonsdale</td>
<td>21,493</td>
</tr>
<tr>
<td></td>
<td>Point Lonsdale</td>
<td>21,642</td>
</tr>
<tr>
<td></td>
<td>Peterborough</td>
<td>21,580</td>
</tr>
<tr>
<td></td>
<td>Little Dip</td>
<td>21,539</td>
</tr>
<tr>
<td>Large irregularly branched form</td>
<td>Kirk Point (Werribee)</td>
<td>21,115</td>
</tr>
<tr>
<td></td>
<td>Kirk Point (Werribee)</td>
<td>21,114</td>
</tr>
<tr>
<td></td>
<td>Werribee</td>
<td>4,933</td>
</tr>
<tr>
<td>Large tetrastichously branched form</td>
<td>Cape Lannes</td>
<td>20,836</td>
</tr>
<tr>
<td></td>
<td>Cape Lannes</td>
<td>21,611</td>
</tr>
<tr>
<td></td>
<td>Cape Lannes</td>
<td>21,656</td>
</tr>
<tr>
<td>Small irregularly branched form</td>
<td>Point Lonsdale</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td>Cape Patterson</td>
<td>-------</td>
</tr>
</tbody>
</table>

Table III.1 List of collections of *Bryopsis* from which cultures were established.
III. 2 Techniques.

III. 2-1 Methods for establishing unialgal cultures.

Male and female gametangia were excised intact from fertile plants collected in nature, or plants which became fertile while held in the laboratory. These were placed in small culture dishes with 10ml of medium, one male and one female gametangium per dish. Zygotes were isolated with a micropipette (section III. 2-4) four to five days after the release and fertilisation of gametes, washed several times in fresh culture medium and transferred to larger vessels. For experimental cultures two to four zygotes were placed in each dish, while dishes containing larger numbers of zygotes were maintained as stock cultures from which further isolates could be made if required.

When fertile plants were not available, cultures of *Bryopsis* were initiated from vegetative material by excision of individual ramuli, which had become isolated from the main axis by the production of a basal septum. Despite removal of all obvious epiphytes from this propagule with jewellers forceps and a soft camel hair brush, these cultures were frequently contaminated with other algal material. Where possible, contaminated cultures were maintained until the *Bryopsis* plants became fertile and unialgal cultures could be established by the isolation of zygotes as outlined above.

Vegetative protoplasts (MacRaid 1970, Tatewaki and Nagata 1970) were also used for establishing cultures. These were formed after cytoplasm of freshly collected plants was squeezed into culture medium in a small vessel. After approximately one hour, small spherical masses of cytoplasm (protoplasts) were observed which formed a cell wall within 24 to 48hrs. These were isolated from the initial culture after 48hrs and placed singly in separate culture vessels, after being washed in several changes of fresh medium. The protoplasts attached themselves to the base of the culture vessel and grew into complete new *Bryopsis* plants.

Three replicates were made of all experimental cultures and unless otherwise stated in the text, similar responses were obtained in each replicate.
III. 2-2 Culture media.

Seawater for the enriched seawater media used throughout this study was collected at Port Phillip Heads rather than at more easily accessible places within Port Phillip Bay, to ensure collection of uniform samples relatively free of industrial and human waste. It was filtered through Whatman no. 541 filter paper and stored in four gallon black plastic containers in a cool, dark room.

The enriched seawater media described below were used for the initial cultures of Bryopsis to determine optimum conditions for the development of natural growth forms in culture. Good results were obtained with the modified Erdschreiber medium (Rietema 1970), and unless otherwise stated in the text all further cultures were maintained in this medium.

(a) Modified Schreiber medium (von Stosch, personal communication).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>42.5 mg</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>10.75 mg</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.278 mg</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.0198 mg</td>
</tr>
<tr>
<td>Na₂EDTA·2H₂O</td>
<td>3.72 mg</td>
</tr>
<tr>
<td>Sterile seawater</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

(b) Modified Erdschreiber medium prepared using (a) above, with the addition of 25ml of soil extract.

(c) Modified Erdschreiber medium (Rietema 1970), containing a much lower phosphate concentration in the form of Na₂HPO₄·12H₂O, than in media (a) and (b).

(d) Modified Schreiber medium prepared according to Rietema (1970) with the exclusion of the soil extract.

(e) Modified Provasoli medium (Wynne 1969).

The soil extract used in the Erdschreiber media was prepared with 1kg of garden loam which had not been fertilised for at least two years, heated with 2 l. of tap water for 3hrs in a steam steriliser. The soil-water mixture was left standing overnight, then the aqueous extract decanted, snap frozen, thawed and filtered through Whatman no. 541 filter paper and then through 0.45μm Millipore membrane filters.
Freezing the solution before filtration tended to flocculate the colloidal clay particles, which could then be removed from the solution by the initial filtration, allowing faster and more efficient filtration and sterilisation through the millipore equipment.

As diatoms were a serious algal contaminant in many cultures, 7mg of germanium dioxide (GeO₂) was added to each litre of culture medium (Lewin 1966) for all first generation cultures, to limit diatom growth. With subculturing of zygotes from contaminated cultures, diatom-free cultures were established and GeO₂ was no longer required.

III. 2-3 Sterilisation procedures.

All glassware, including culture vessels, was prepared for use by washing thoroughly in hot tap water and rinsing twice in distilled water. Air-dried glassware was then wrapped in newsprint and sterilised in dry heat at 150°C for 3hrs. At no stage in the cleaning procedures were detergents or chemical cleaning agents used. Only Pyrex glass filter holders and storage vessels were used.

All enriched seawater media used for culturing the algae were "sterilised" by one of the following methods. As axenic cultures were not required the aim of "sterilisation" was the destruction or removal of all algal, fungal and animal contaminants in the complete media.

(a) Tyndallisation (Stein 1973). 1 l. aliquots of seawater were heated to 72°C in a steam steriliser on three consecutive days. To this "sterilised" seawater the nutrient additions were made with small aliquots of concentrated solutions of the required salts.

(b) Filtration of the complete medium through 0.45μm membrane filters (Millipore).

Of these two procedures the second was preferred as the complete medium was sterilised without precipitation of salts, a feature often associated with heat sterilisation of enriched seawater media. This second technique also resulted in considerable reduction in contamination of cultures by bacteria introduced in the fresh culture media.
III. 2-4 Maintenance of cultures.

Three types of Pyrex glass culture vessel were used for both experimental and stock cultures. Small petri dishes (5cm diameter, 2cm deep) were used for establishing young zygotes, sporelings and vegetative propagules. In general, plants were maintained in this size vessel for only four to five days as evaporation from such a small volume of culture medium in a relatively shallow container results in significant changes in the concentration of the medium over a short time. Most cultures were then maintained in 150ml crystallising dishes with glass lids. For larger plants and for specialised purposes such as aerated and moving cultures, 300ml crystallising dishes (no. 3250) were also used.

The medium of all experimental cultures was changed every two weeks even though this was not considered essential for continued growth. Cultures of plants of unknown life history were examined once every two to three days, while stock and other experimental cultures were examined less frequently. All cultures were observed with a Zeiss binocular dissecting microscope, and stages in the development of the plants recorded by means of camera lucida drawings or photographs.

All gametes, spores and small plants were transferred from cultures with a micropipette to minimise the risk of damage. These pipettes were made using fine capillary pipettes, drawn out to smaller diameter under the pilot flame of a Bunsen burner. They were attached to a length of rubber tubing and positive or negative pressure was applied, drawing the plant into or out of the capillary pipette by sucking and blowing through the rubber tube. Larger stages were transferred from culture with jewellers forceps and tungsten needles sharpened in molten sodium nitrite.

Cultures were maintained in constant temperature cabinets at 12°, 16° or 20°C (± 0.5°C), or in culture rooms at 13°C or 20°C with seasonal variations of ± 2.5°C. Fixed day length conditions were used for all cultures, providing 16hrs light and 8hrs dark. Light intensities between 1000 and 2700lx were provided with Phillips "cool white" fluorescent tubes. No additional incandescent lighting was used.

The majority of cultures were kept under still conditions.
However, for the experiments on the growth forms of these algae under different environmental conditions some cultures were maintained on a table rotating through a path of 3cm diameter at a rate of 130 cycles per minute. Some were also kept in vessels aerated by an aquarium pump supplying a constant stream of air bubbled through the medium. Unless otherwise stated in the text stationary, non-aerated culture conditions were used.

III. 3 Life histories of *Bryopsis*.

Observations on the life histories of *Bryopsis* began as gametophytic plants collected in nature became fertile. Gametogenesis occurs in ramuli which have become isolated from the axis by the production of a basal plug (Fig. III.1), and continues throughout the growth season of the alga. In young plants, only few of the lower ramuli become fertile, while in older plants at the end of the growth season, large numbers of ramuli and occasionally the whole plant, undergo gametogenesis. All the cytoplasm of the isolated organ is employed in the production of gametes and after the release of these the cell wall of the gametangium breaks away from its axis leaving a small scar.

Formation of the basal plug prior to gametangial development has been described in detail (Mirande 1913). The cytoplasm of the developing gametangium undergoes a series of rapid changes finally resulting in its segregation into individual gametes. Nuclei divide mitotically (Fig. III.2) with a resultant increase in number and decrease in size. As this occurs the chloroplasts divide rapidly, also with a resultant decrease in size. Following this there is a change in the distribution of the chloroplasts and nuclei to form the net stage in gametangial development (Figs. III.3, III.4) which has been recognised since the earliest studies of gametogenesis in *Bryopsis* (Pringsheim 1871). Stained preparations of this stage indicate that segregation of the cytoplasm has occurred (Fig. III.5). The reticulate pattern is only disrupted when the gametes become motile within the gametangium before release.

One feature of gametangial development observed by Pringsheim (1871) but not the more recent investigators (Neumann 1969a, Burr and West 1970), is the separation of layers of the cell wall and the apparent stretching of the outer layer to up to twice its original length.
Fig. III.1 LM *B. gemellipara*. Basal plug isolating ramuli from main axis.

Fig. III.2 LM Nuclei of developing gametangium of *B. vestita*. Stained with proprionic acid haematoxylin. Note metaphase nucleus (arrow).
Fig. III.3 LM *B. vestita* net stage in gametogenesis.

Fig. III.4 Net stage of gametogenesis in *B. gemellipara*. Note also separation and elongation of outer layer of cell wall.
(Figs. III.4, III.6). This phenomenon has been observed in the present study of both *Bryopsis vestita* and *Bryopsis gemellipara*. The gametes are first released through an apical or subapical pore, into the space between the outer stretched wall layer and the inner layer, which retains the original shape of the gametangium (Fig. III.6). Release to the external environment occurs through a pore which forms in the outer wall layer at the point at which the two layers meet, or more frequently, when the whole of the outer layer breaks away from the gametangium. It should be noted however, that motile gametes have been observed in gametangia of *B. vestita* without cell wall separation, but release of these gametes has not been observed.

A comparison of light and electron micrographs (Figs. III.6, IV.4) shows that the outer separated layer of the gametangial wall corresponds with an electron dense layer of the intact wall (see section IV. 3). The actual separation of this layer from the gametangial wall appears to begin by stretching (Fig. IV.4). This layer has been referred to by Burr and West (1970) as a cuticle similar to that seen in other green algae by Hanic and Craigie (1969).

As found by a number of other workers (Neumann 1969a, Burr and West 1970) release of gametes from the gametangium is associated with the onset of the light regime. In all species and forms studied here the gametes become motile within the gametangium and are released within 30 mins of the light in the culture cabinets being switched on. There is some evidence that the gametes may become motile within the gametangium even during the dark cycle. Under some conditions (high light intensity and temperature) gametes may be seen moving in some gametangia for up to 6 hrs after the onset of the light regime. However, close observation of a number of these abnormal gametangia has shown that release does not occur, the gametes stop moving and the whole gametangium begins to degenerate.

All forms of *Bryopsis* recognised from the south eastern Australian coast were found to be dioecious. However, one plant of the large distichous form collected from Werribee, Port Phillip Bay, has been seen with both male and female gametes in the same gametangium, a characteristic previously reported only for *B. hypnoides* (Feldmann 1957) and *B. plumosa* (West 1969). On the basis of this specimen (MELU 4933)
Fig. III.5 LM Net stage of gametogenesis in a female plant of *B. vestita*. Stained with proprionic acid haematoxylin.

Fig. III.6 LM Outer layer of wall material stretched away from mature gametangium. Release of gametes is occurring through apical pore, into space between wall layers.
both MacRaild (1970) and King et al. (1971) record the presence of *B. hypnoides* in Port Phillip Bay. After examination of a large number of other fertile specimens from the same locality no further monoecious plants have been found. Re-examination of the initial monoecious specimen shows it is morphologically indistinguishable from the other collections from this locality, which are generally much larger than *B. hypnoides* and show irregular to distichous branching. It is therefore suggested that the record of *B. hypnoides* in Port Phillip Bay should now be regarded as doubtful.

Male and female gametes have been observed in all forms of *Bryopsis* studied and are similar to those described by Pringsheim (1871). The female gametes (Figs. III.7, III.8) are pear shaped, 5 to 7 μm wide and 10 to 15 μm long, with two flagella inserted into a cytoplasmic papilla at the anterior end (see also Neumann 1969a, Burr and West 1970). In the posterior part of the gamete, two to four small rounded chloroplasts are found. One of these possesses an eye spot which is displayed laterally within the gamete. The pyrenoids which are present in all chloroplasts of the vegetative stage, are inconspicuous within the gametes, as the starch sheath appears to be either reduced or absent.

Remarkable similarity in size and general appearance is seen when comparison is made of the female gametes of the different species and forms of *Bryopsis* presently investigated. Although the average size of the gametes differs slightly, there is a wide range of variation within the forms and species. However, gametes of these algae are significantly different from those of *B. hypnoides* as illustrated by Neumann (1969a Fig. 6a), having fewer chloroplasts.

The male gametes of the south eastern Australian *Bryopsis* are similarly uniform. They differ from the female gamete in being much smaller, with a length of 6 to 10 μm and width of 2 to 4 μm and lacking a well defined chloroplast. In the reduced chloroplast no eye spot can be detected, even with phase contrast and differential interference contrast microscopy.

Evidence of fusion of the male and female gametes after release from the gametangia, was obtained by either direct observation under the light microscope, or from the results of germination tests in which
Fig. III.7  *B. vestita* male and female gametes.

Fig. III.8  Dark field LM Female gamete of *B. plumosa*. 
germination in cultures containing only male or female gametes was compared with that in cultures where both types of gametes were mixed.

After release from the gametangia the gametes move rapidly, the males tending to cluster around the females. Two fusing gametes are seen to approach with their flagella more or less intertwined. The male then moves so that it lies alongside the female and actual fusion of the two masses occurs laterally. This sequence has been observed only in B. vestita and B. plumosa, but is comparable with the process described by Pringsheim (1871 Fig.15, reproduced as Fig. III.9 of this thesis) and Neumann (1969a).

Numerous motile stages have been observed with four flagella. These would appear to be fertilised gametes which move about for some time before coming to rest, retracting or shedding their flagella and forming a cell wall.

Neumann (1969a) states that the difficulty in observing fusion of gametes, may result from the fact that very few gametes actually do fuse, thus also explaining the small number of germlings produced in his cultures of Helgoland material. Although stages of copulation have been observed only infrequently in the present study, large numbers of zygotes are produced in cultures in which only one male and one female gametangium are placed (see section III.5, Tables III.3 to 5). It therefore appears more likely that fusion is rarely observed, as while it occurs within 10mins of the first contact between gametes, this contact may not be made for up to 5hrs after release. Also, fusion occurs while the female gamete is actively moving, usually in rotation around its longitudinal axis.

Results of the germination tests which were run with all new isolates of gametes from plants collected in nature and from fertile cultures, indicate that parthenogenetic development of gametes does not occur. Non-fertilised female gametes do survive in culture for up to ten days, settling to the bottom of the vessel and enlarging in diameter by the production of a large vacuole. However, these do not produce a cell wall and the chloroplasts fail to divide. Stages interpreted as developing zygotes in MacRaild (1970) are in fact stages in the abortive development of the non-fertilised female gametes.

Developmental stages in the germination of the zygote are
essentially the same as those illustrated by Thuret (1850, Pl. 16, Figs. 1 to 6). The zygote is motile for some time after fertilisation, but eventually settles to the bottom of the culture vessel where it becomes spherical and produces a thin cell wall. Germination generally begins within 24hrs, with an increase in the diameter of the zygote and a unidirectional growth of a nonseptate tube. No resting stage has been observed in any of the forms of *Bryopsis* under study, at any of the temperature or light intensity conditions outlined. Limited irregular branching of the primary germination tube produced a protonemal growth, which resembles the rhizoidal material of the mature *Bryopsis* plant (Fig. III.10).

As found by Neumann (1969a), the nucleus of the protonema does not divide immediately, but begins to enlarge approximately 48hrs after germination, and continues enlarging for three to four weeks. The end product is a nucleus with a diameter up to 45 μm, approximately eight to ten times that of the vegetative nuclei of the normal *Bryopsis* thallus (See section IV. 4-3, Figs. IV.14 to 17). One third to one half the volume of this giant nucleus ("Riesenkern" Neumann 1969a) is occupied by the nucleolus, which becomes long and sinuous (Neumann 1969a).

Morphologically similar uninucleate protonema occur in the developmental history of all forms of *Bryopsis* studied. In all cases the giant nucleus divides after two to three months in culture and the cytoplasm of the protonema undergoes a reorganisation through a net stage similar to that observed in the developing gametangium (Figs. III.11, III.12). However, the cytoplasm cleaves into relatively large spherical masses (25 μm diameter) each with one small nucleus. These become motile and on release from the protonema are observed to possess a ring of flagella, as described by Rietema (1969 to 1971) from the protonemal stages of a number of European species of *Bryopsis*.

These stephanokont cells are motile for up to 24hrs before coming to rest and attaching to the base of the culture vessel where they begin to germinate. From these, a siphonous thallus develops firmly attached to the culture vessel and axes arising from this have the characteristic *Bryopsis* branching pattern and produce gametes in their ultimate branches.

As no fusion has been observed between the stephanokont motile
Fig. III.10  *B. vestita* uninucleate protonema grown in culture, 20°C, nonaerated.
Fig. III.11  *B. gemellipara* uninucleate protonema during spore production.

Fig. III.12  LM *B. plumosa* uninucleate protonema at net stage of spore formation.
stages, and through analogy with the spores of *Derbesia*, interpretation of these structures as spores (Rietema 1969) seems to be valid. However, Neumann (1970) and Bartlett and South (1973) have observed holocarpic division of the cytoplasm of the protonemal stage, with the production of gametes which are identical with those produced by the *Bryopsis* phase. This introduced the possibility that the spores might be reinterpreted as incompletely divided masses of gametes, but the presence of a regular ring of flagella and the highly coordinated spiral movement of the spore made such an interpretation unlikely.

The results of these culture studies therefore show that the life histories of all the forms of *Bryopsis* found on the south eastern Australian coast are identical, involving an heteromorphic alternation of sporophytic and gametophytic generations.

The discrepancy between the life history described here for *Bryopsis* and the classical diplontic life history (Thuret 1850, Neumann 1969a) is discussed by Rietema (1969 to 1972). He has found that species of *Bryopsis* from the European coast show variations in the type of life history expressed, depending on the locality from which the parent material is collected. For example a *B. plumosa* population from Roscoff had an heteromorphic life history, while the population of morphologically similar plants from Zeeland (Netherlands) produced protonemal stages which grew directly into the next generation of *Bryopsis* plants (Rietema 1969, 1970).

The life histories of the three recognised species of *Bryopsis* from south eastern Australia, *B. plumosa*, *B. vestita* and *B. gemellipara* were therefore studied using parent plants collected from a number of different localities. A list of these localities is given in Table III.1. The results of the investigations show that the life histories are quite uniform over this geographic range. However, as environmental conditions along this coast are expected to be relatively uniform, further investigations of species such as *B. vestita* from Tasmania, New Zealand and Western Australia are needed before any claim can be made for the absolute uniformity of their life histories.
III. 4 Morphological forms of *Bryopsis* in culture.

Dixon (1960 p.16) states "The present chaos that exists in algae taxonomy and nomenclature is the result, essentially, of the failure to recognise and interpret seasonal and environmental modifications of the external form of the thallus and of the failure by phycologists to refer back to original materials."

A survey of the descriptions of species of *Bryopsis* shows that the most widely used distinguishing characteristic has been the type of branching shown by the alga. In an attempt to determine the validity of this characteristic in distinguishing between true species from the south eastern Australian coast, a study was made of the morphological plasticity of the species under different culture conditions. The morphology of plants grown in culture was compared with the morphology of both the parent plants collected in nature, and other forms and species grown under identical conditions.

Upright fronds of *B.plumosa* develop a typical distichous branching pattern (Fig. III.13) under standard, nonaerated, nonmoving, 16°C culture conditions. The large irregularly branched form (section II. 2-6) and some plants fitting the small irregularly branched form (section II. 2-5) also revert to a distichous branching pattern, and their size in culture is similar to that of *B.plumosa* grown under the same conditions.

Variations in the light intensity and temperature under which these three forms were grown, produced only minor changes in the branching pattern of the upright frond. At a higher temperature (20°C) elongation of the main axis after branch initiation resulted in a lax arrangement of the ramuli, while at lower temperatures (16°C, 12°C) much more regularly spaced branches were formed. Aeration of the cultures of these algae produced very little change in the overall morphology.

Changes in the composition of the culture medium had a marked effect on the rate of growth and morphology of the cultured organism. In both the Provasoli and Modified Erdschreiber (von Stosch) media, the growth form of *B.plumosa* and the large irregularly branched form, resembles that of the latter growing in nature. Both these media have higher concentrations of added nutrients than the Erdschreiber (Rietema) but as the von Stosch medium differs from the Rietema medium
Fig. III.13 *B. plumosa* grown at 16°C under nonmoving, nonaerated culture conditions. Eight week old plant produced from spore developed in protonema from male and female gametes. Parent material collected at Blackrock.
only in the higher concentration of phosphate (as Na$_2$HPO$_4$·12H$_2$O) it is concluded that the morphology of these forms of *Bryopsis* is modified by the concentration of this ion.

Although some specimens of the small irregularly branched form show a similar response to culture conditions as *B.plumosa*, some radially branched plants retain this branching characteristic. As these plants are difficult to distinguish in nature from those which revert to the distichous branching habit and as few of this group were collected, only a preliminary investigation of the morphological characteristics was attempted. However, from the differing responses to the same culture conditions it is suggested that there may be two different genotypes represented within this group. Part of the group must be recognised as an open ocean form of *B.plumosa* (see section III. 5) while the radially branched material may possibly be referred to *B.minor* Womersley(1955). Whether *B.minor* remains as a distinct species or is referred to synonymy with an earlier described species, will depend on further study of naturally grown plants and culture collections.

The other three forms of *Bryopsis*, *B.vestita*, *B.gemellipara* and the large tetrastichous form (section II. 2-4) all show similar growth responses in culture. Under nonaerated conditions, they do not attain a typical morphology, but develop an exaggerated rhizoidal growth without the upright regularly branched fronds. These arise only with continuous aeration of the culture medium in which the plants are grown.

It is suggested from these observations, that the branching pattern expressed by these forms of *Bryopsis* under culture conditions is dependent on the concentration of the dissolved gases in the medium. The results of an experiment involving aeration of the culture medium during either the light or dark periods or both, are summarised in Table III.2. As the typical branched upright fronds of these algae are developed in cultures under constant aeration, or aeration during the dark period only, it is suggested that the concentration of oxygen in the medium during the dark is a limiting factor, and the production of these fronds is dependent on the rate of dark respiration. Further more sophisticated experiments to confirm these conclusions and to determine the biochemical control of the development of the branching patterns are obviously required, but are considered outside the scope of the present study.
Production of branched fronds

<table>
<thead>
<tr>
<th></th>
<th>B. vestita</th>
<th>B. gemellipara</th>
</tr>
</thead>
<tbody>
<tr>
<td>No aeration</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Constant aeration</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aeration during light</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aeration during dark</td>
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</tr>
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</table>

Table III.2 Effect of aeration of the culture medium on the production of upright branched axes (+/-) after 6 weeks growth from spores.

Even under aerated conditions, the growth form of B. vestita in culture does not exactly match that found in the field. As mentioned earlier (II. 2-1), the branching pattern of this species is first tetrastichous, then radial. Under culture conditions ramuli are produced only tetrastichously on the axes, and at no stage have cultured plants of B. vestita been seen to produce radially arranged ramuli (Fig. III.14). In this culture form B. vestita is indistinguishable from B. gemellipara and the large tetrastichous form.

From these studies of the morphological forms of Bryopsis grown under a range of culture conditions, two groups can be recognised as having close affinities. B. plumosa, the large irregularly branched form (section II. 2-6) and some plants referred to the small irregularly branched form (section II. 2-5), all produce in culture a distichously branched growth form. The second group comprising B. vestita, B. gemellipara and the large tetrastichously branched form (section II. 2-4) produce only a tetrastichous branching pattern under aerated culture conditions. These two groups are referred to in following sections as the B. plumosa complex and the B. gemellipara complex.

III. 5 Hybridisation studies.

Because of the similar morphological responses observed for the various forms of Bryopsis under a range of culture conditions, a study was undertaken to determine the possibility of hybridisation between these forms. The hybridisation study was begun initially within the two groups which show similar responses to the culture conditions (see section III. 4), and the results are shown in Tables III.3
and III.4. Hybridisation tests were done with gametes released from isolated gametangia of the two forms of *Bryopsis* under study, and all tests were compared with cultures in which gametangia of the same species were placed, and those in which only male or female gametangia were placed separately. This ensured that any germination of zygotes representing crosses between the morphological forms could be compared with the germination pattern of the natural species or forms, and also any possible parthenogenetic development of the gametes.

<table>
<thead>
<tr>
<th>Gametangia</th>
<th>Small irregularly branched form</th>
<th>Large irregularly branched form</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. plumosa</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>135*</td>
</tr>
<tr>
<td>Small irregularly branched form</td>
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<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>143</td>
</tr>
<tr>
<td>Large irregularly branched form</td>
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<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>195*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>346</td>
</tr>
</tbody>
</table>

Table III.3 Results of hybridisation studies between members of the *B. plumosa* complex. Figures=Average of number of zygotes produced in 6 dishes in which one ♀ and one ♂ gametangia were placed (* only one dish observed due to lack of fertile material).

<table>
<thead>
<tr>
<th>Gametangia</th>
<th>Large tetrastichous form</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. gemellipara</em></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>268</td>
</tr>
<tr>
<td><em>B. vestita</em></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>350</td>
</tr>
<tr>
<td>Large tetrastichous form</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>390</td>
</tr>
</tbody>
</table>

Table III.4 Results of hybridisation studies between members of the *B. gemellipara* complex. Figures=Average of number of zygotes produced in 6 dishes in which one ♀ and one ♂ gametangia were placed. +/− = presence or absence of hybrid zygotes.
As shown in Table III.3 the three members of the *B. plumosa* complex are interfertile, large numbers of germlings being produced in all crosses between these forms. A similar study was undertaken for the *B. gemellipara* complex and shows that *B. gemellipara* does not produce zygotes when crossed with the other two forms which are able to interbreed freely (Table III.4).

Figures shown in Tables III.3 and III.4 represent the average number of zygotes, one week after gamete release, in dishes in which one male and one female gametangium were placed. Where possible six dishes were set up for each cross, but as seen from the figures no distinction was made between the figures from $\sigma/\sigma$ and $\varnothing/\varnothing$ crosses. By comparison of the number of protonema found in dishes in which gametes of the same form were placed, with those representing crosses between forms, a qualitative appreciation of the hybrid viability, at least to the protonemal stage is possible.

In the results presented here, successful hybridisation was assessed as the production of a zygote after fusion between gametes of two different forms of *Bryopsis*. As this only represents the early development of the hybrid individual, some zygotes resulting from crosses were isolated and allowed to develop to maturity. In all successful crosses, hybrid zygotes are observed to develop into uninucleate protonema, producing stephanokont spores which reproduce the normal *Bryopsis* gametophyte. The branching patterns of the first generation hybrid plants are distichous for all crosses within the *B. plumosa* complex and tetrastichous for hybrids between *B. vestita* and the large tetrastichous form. Gametes of these hybrid plants have been crossed back where possible, with gametes of one of the pure forms. Where back crosses were not possible because of lack of fertile material of a pure strain, male and female gametes of the hybrids have been crossed and found to produce viable zygotes.

From these studies there appear to be three reproductive units within the *Bryopsis* complex and these can be referred to the species *B. plumosa*, *B. gemellipara* and *B. vestita*. The results of further studies between these three species are shown in Table III.5, indicating that each is a reproductively isolated unit.
B. gemellipara  B. vestita  B. plumosa

B. gemellipara + - -
B. vestita - + -
B. plumosa - - +

Table III.5 Results of hybridisation studies between B. gemellipara, B. vestita and B. plumosa. +/- = presence or absence of hybrid zygotes.

III. 6 Morphology and reproductive behaviour of the protonema.

The uninucleate protonema described by Neumann (1969a, 1970), Rietema (1969 to 1972) and Bartlett and South (1973) are all derived from culture studies. Although a detailed investigation has been made in the present study of the habitats of B. vestita and B. gemellipara, the protonema of these forms has not yet been found in nature. A study was therefore undertaken to investigate the morphological plasticity of this stage under a range of culture conditions. This was done in part to determine whether the sporophytic phase of the Bryopsis life history has previously been referred to another genus.

Typical protonema at various stages of development are shown in Figs. III.10, III.15. At maturity all protonema show the same very irregular form which resembles the rhizoidal growth of the Bryopsis plant. Under nonmoving, nonaerated culture conditions the protonema of all forms and species of Bryopsis have the same range of filamentous forms and the species cannot be determined on the basis of protonemal morphology.

Under aerated conditions the protonema are longer and the branching more regularly dichotomous (Fig. III.16). In this form they resemble small plants of the fine form of Derbesia found on the southern Australian coast, but have never been found to produce specialised external sporangia as seen in Derbesia. Culture studies on the fine Derbesia (section V. 4-1) show that this species is the alternate
Fig. III.14  *B. vestita* grown in 20°C, aerated culture from spore produced by uninucleate protonema. Parent material collected at Little Dip, S.A. Note tetrastichous branching typical also of culture form of *B. gemellipara*. Scale in cm.

Fig. III.15  *B. plumosa* uninucleate protonema grown in aerated culture, 20°C.
Fig. III.16 *B. gemellipara* uninucleate protonema grown at 16°C, aerated culture. Note the long, filamentous growth habit, with distichous branching.
generation of a Halicystis-like alga, and is not involved in the life history of any of the species of Bryopsis.

The only other southern Australian marine alga which is known to produce stephanokont spores, Pedobesia clavaeformis was also investigated to determine a possible relationship to the Bryopsis protonemal stage. However, from culture studies and in situ observations there appears to be no life history link between these algae (MacRaid and Womersley 1974).

As a result of this investigation it is thought that the protonemal stage in the life history of Bryopsis cannot be related to any of the previously described siphonous green algae of the southern Australian coast and that it occurs in quite different ecological habitats to the gametophytic Bryopsis plants.

Because of the different reproductive behaviour reported for the protonema of European and Northern American species of Bryopsis (Neumann 1969a, 1970, Rietema 1969 to 1972, Bartlett and South 1973) a study was undertaken to determine the reproductive behaviour of the protonema of B. vestita and B. plumosa under different temperature, day length and light intensity conditions. The day lengths available in culture cabinets attached to a time switch were fixed at 16hrs. Because of joint use of these facilities by several workers, modification of the daylength was achieved by daily covering the particular cultures with black glass covers.

Protonema were grown at temperatures of 12°C, 16°C and 20°C, and light intensities of 1000 and 2700lx. At each combination of temperature and light intensity, day lengths of 16 and 8hrs were used. Under no conditions were protonema found to produce gametes or develop directly into the new Bryopsis generation, the only reproductive structures observed being stephanokont spores. Table III.6 summarises the results of this experiment showing the conditions under which the protonema produce spores.
Table III.6 Protonemal spore production (+/-) in *B. vestita* and *B. plumosa* at various temperature, light intensity and day length conditions.

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>20</th>
<th>16</th>
<th>12</th>
<th>20</th>
<th>16</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.I. (lx)</td>
<td>2700</td>
<td>2700</td>
<td>2700</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>D.L. (hrs)</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>8</td>
</tr>
</tbody>
</table>

*B. vestita*  
- + - + - + + + - -  

*B. plumosa*  
+ - + - - + - + - - -
SECTION IV

THE CYTOLOGY OF BRYOPSIS
IV. 1 Introduction.

Initial cytological studies of Bryopsis were undertaken to determine the chromosome numbers of the different forms recognised in section II. 2. As this study progressed it became obvious that a detailed light and electron microscope study of the general cytoplasmic organisation of these organisms was needed to clarify some observations of the early workers (Pringsheim 1871, Küster 1899, Noll 1899). Comparisons are also made between the species recently studied by Neumann (1969a), Burr and West (1970, 1971a) and Urban (1969) and the forms recognised in the present thesis.

Techniques employed in this study and the later cytological studies of Derbesia and Pedobesia are detailed in the following section. A flow chart showing the preparation techniques and the types of microscopes used throughout these investigations is provided (Fig. IV.1).

IV. 2 Techniques.

IV. 2-1 Tissue preparation and techniques for light microscopy.

Zeiss light microscope equipment was used throughout this study. Both transmitted light and Nomarski differential interference contrast optics were used in the examination of whole plants, squash preparations and sections. The Nomarski system was found to be most useful for examination of the relatively thick preparations necessary when dealing with an intact siphonous organism, the comparatively minor interference from structures above and below the focal plane allowing observation of both the peripheral cytoplasm and the vacuole. Phase contrast, dark field and polarised light microscopy were also used for examination of specialised structures.

All light photomicrographs were produced with a Zeiss Ikon camera with microscope attachment equipment. Two different types of film were used depending on the contrast range of the subject to be photographed. Ilford "Microneg" and Kodak "Recordak" were used to produce negatives of high contrast and very fine grain. For specimens showing a greater range of contrast tones, Kodak "Plus-x pan" film was used.
Living plant tissue

1° fixation
2-3% glutaraldehyde

2° fixation
2% Osmic acid

Liquid N₂

Freeze drying

Dehydration
Acetone series

Dehydration
Acetone series

Infiltration
GMA

Infiltration
Spurr's low viscosity
embedding medium

First polymerization
Spurr's low viscosity
embedding medium

70°C 3-4hrs

Polymerization
50°C 12hrs

Polymerization
70°C 12hrs

Acetone wash

70°C 12hrs

Au coating

LIGHT MICROSCOPE

TRANSMISSION ELECTRON MICROSCOPE

SCANNING ELECTRON MICROSCOPE

Fig. IV.1 Flow chart showing tissue preparation procedures for light and electron microscopy.
Sections of the siphonous thalli were obtained after fixed tissues were embedded in glycolmethacrylate (GMA) according to the following procedure which is modified from Feder and O'Brien (1968).

(a) Fix for 16-24hrs in 2-3% glutaraldehyde in 95% seawater.
(b) Wash in 5 changes of regularly decreasing concentrations of seawater, ending in distilled water. 10mins each step.
(c) Cut the material into approximately 5mm lengths with a very sharp blade.
(d) Dehydrate through increasing concentrations of acetone, to absolute acetone (10% steps). 10mins each step.
(e) Infiltrate with GMA by single drop increases in the concentration of GMA in 2ml of acetone until sufficient is added to ensure that the plant material is covered when the acetone is evaporated off. After this, the tissue is left for at least one week in a fresh change of GMA to ensure adequate infiltration of the cell wall, cytoplasm and crystalline inclusions.
(f) Place infiltrated material in fresh GMA in gelatine capsules and polymerize overnight at 50°C. The tops of the capsules are indented to exclude air from the capsule as the presence of oxygen inhibits polymerization of GMA.

Following polymerization, sections 1-2µm thick were cut with a Cambridge Ultramicrotome using glass knives. Sections were removed from the dry knife edge with fine jewellers forceps and floated onto a drop of water on a clean microscope slide. As the water evaporated at room temperature the sections adhered to the glass surface and subsequent reaction and staining of the sectioned tissue was done on the slide.

A double staining procedure employing 0.0005% acid fuchsin in 1% acetic acid followed by 0.05% toluidine blue in benzoate buffer at pH 4.4 (Feder and O'Brien 1968) was used for routine examination of tissue. Other staining procedures and reactions used in the identification of specific cytochemical units are referred to in the text.

For nuclear studies two specific fixation and staining procedures were used. The first, following the acetocarmine staining and canada balsam
mounting procedures of von Stosch (1952), was found to give good general staining of the nuclei. However the high level of background staining even after hydrochloric acid (HCl) hydrolysis, made determination of the chromosome numbers difficult. A second method was tried using the fixation methods of Lu (1962), followed by staining in propionic acid haematoxylin (Henderson and Lu 1968).

The two different fixation and staining procedures are outlined below.

1. Acetocarmine technique.
   
   (a) Fix in methanol : formic acid (1 : 3). 8-12hrs.
   
   (b) Store fixed material in 2% dioxan in tertiary butanol.
   
   Note. Although primarily used as a storage fluid, 2% dioxan in tertiary butanol induces post fixation changes in the tissue, resulting in considerable reduction in background staining after 5-7 days.
   
   (c) Wash in glacial acetic acid, reswelling the tissue.
   
   (d) Wash in distilled water.
   
   (e) Hydrolyse RNA with 1N HCl at 60°C for 6-8 mins.
   
   Note. The time of hydrolysis has to be determined for each batch of material as insufficient time results in very heavy background staining while longer hydrolysis than necessary breaks down excessive amounts of DNA, thus reducing the staining intensity of the chromosomes.
   
   (f) Wash in distilled water followed by glacial acetic acid.
   
   (g) Mordant tissue with saturated solution of iron alum in glacial acetic acid.
   
   (h) Stain in iron acetocarmine for 2-4 hrs at room temperature followed by 7-8 mins at 100°C.
   
   (i) Wash in glacial acetic acid.
   
   (j) Wash in series of saturated phenol : acetic acid (1 : 3, 1 : 1, 3 : 1). 5 mins each solution.
   
   (k) Wash in saturated phenol, 2 changes. 10 mins each.
   
   (l) Mount material on a slide in a drop of phenol canada balsam.
2. Propionic acid haematoxylin technique.

(a) Fix in butanol : glacial acetic acid : 10% chromic acid
(9 : 6 : 2).

Note. The fixing fluid is also used for storing the material, generally at 3-4°C.

(b) Hydrolyse RNA in conc. HCl : ethanol (1 : 1) at 60°C for 1min.

(c) Wash in 50% propionic acid.

Note. The intensity of staining of the chromosomes is increased by the addition of a mordant at this stage. Usually 3 drops of a saturated solution of ferric acetate in 50% propionic acid are added to the washing solution.

(d) Place material in a drop of 0.5% (w/v) iron alum in 50% propionic acid on a microscope slide. One drop of a 2% (w/v) solution of aged haematoxylin in 50% propionic acid is added to this, and a cover glass added.

Note. The prepared material is examined in the staining solution and best results are obtained after 2-3hrs.

Comparison of the results obtained with these two staining procedures shows that much better definition of the chromosomes can be produced with propionic acid haematoxylin, partly because of the lighter background staining and partly because the nucleolus which is persistent through mitotic division of Bryopsis, is not as densely stained with the haematoxylin procedure, thus allowing the recognition of the chromosomes lying near to or on top of the nucleolus. One major disadvantage of the haematoxylin method is that preparations are not permanent, as in the acetocarmine technique. Attempts to devise a method of making these permanent have resulted in considerable loss of staining intensity and have therefore been considered unsuccessful.

IV. 2-2 Tissue preparation and techniques for transmission electron microscopy.

A Siemens Elmiskop 1, transmission electron microscope (accelerating voltage 80 or 100kV) was used in the study of thin sections of some of the developmental stages of the algae under investigation. All transmission electron micrographs were taken with Ilford "Special contrasty lantern
plates" developed in Ilford "Bromophen" bromide developer.

Standard fixation, dehydration and embedding procedures for transmission electron microscopy were followed with some slight modifications necessary because of the delicate structure of these plants. Most of the difficulties encountered in the treatment of the tissue were related to the retention of osmotic properties in fixed cells, causing collapse of the siphonous structure during dehydration and embedding. To overcome similar problems Burr and West (1970) embedded the fixed tissue in 2% Bactoagar before dehydration. In the present study this procedure was only partially successful for the larger species of *Bryopsis* (e.g. *B. vestita*) and much better results were obtained by dissection of the tubular thallus after adequate fixation, so that further dehydration and infiltration was done on open ended tubular elements 5-10mm long.

The following schedule for fixation and embedding of specimens for transmission electron microscopy summarises the techniques found to produce the best results with the siphonous green algae studied.

(a) Fix for 16-24hrs in 2-3% glutaraldehyde in 95% seawater.  
Note. 1. Better results were obtained using natural seawater as the buffer rather than cacodylate buffer made up in a salt or sucrose solution (Burr and West 1970). 2. Lower concentrations of glutaraldehyde in slightly diluted seawater resulted in least disruption of the cytoplasm.

(b) Wash in 5 changes of regularly decreasing concentrations of seawater, ending in distilled water. 10mins each step.

(c) Post fix in 2% osmium tetroxide in 0.1M sodium cacodylate buffer at pH 6.5 for 4-6hrs.

(d) Wash in 3 changes of distilled water. 20mins each step.

(e) Cut fixed plants into 5-10mm lengths with a sharp blade.

(f) Dehydrate through 10% steps of increasing acetone concentrations to absolute acetone which had previously been dried over molecular sieves. 10mins each step.

(g) Infiltrate with Spurr's standard low viscosity embedding medium (Spurr 1969) by single drop increases in the concentration of embedding medium in 2ml of acetone until sufficient is added to ensure that the plant material is covered when the acetone is evaporated off. 5mins allowed between addition of each drop.
(h) Adequate infiltration of the tissue is achieved in 3 days, after which the tissue is placed in fresh medium either in flat moulds or in gelatine capsules, and polymerized at 70°C for more than 10 hrs.

Note. Complete polymerization of Spurr's embedding medium depends on the removal of all water from the tissue. Inadequate dehydration due to too little time in the final dehydrating fluid, or water in the final solution results in the Spurr's within the tissue remaining liquid after polymerization while the rest of the block polymerizes normally.

Silver-grey sections (600-900Å thick) were cut on a Huxley Ultramicrotome (Cambridge Scientific Instruments Ltd) with glass knives. Sections were floated onto water, stretched with chloroform vapour and collected on 200 mesh grids. Image contrast was enhanced by treating the sections for 5 minutes with 2% uranyl acetate in distilled water followed by lead citrate (Reynolds 1963) for a further 5 minutes.

IV. 2-3 Tissue preparation and techniques for scanning electron microscopy.

Owing to the nature of the tissue, application of the scanning electron microscope to siphonous organisms is limited. A Stereoscan S 4-10 (Cambridge Scientific Instruments Ltd) was used to investigate the cytoplasm and vacuolar inclusions of Bryopsis.

All tissues studied were either freeze dried or partially embedded in Spurr's after glutaraldehyde fixation and acetone dehydration (Chambers and Hamilton 1973). Before the final polymerization of the embedding medium the siphonous thallus was cut and laid open with the cytoplasm uppermost on a scanning electron microscope stub. Before the prepared material was observed under the microscope it was coated with a thin layer of gold in a Speedivac coating unit (Edwards High Vacuum Ltd), to provide a good conducting surface.

IV. 3 General cytology.

Descriptions of the cytoplasmic organelles and their distribution in the siphonous thallus of Bryopsis date back to Thuret (1850) and Derbès and Solier (1856). In these and subsequent papers (Pringsheim 1871,
Küster 1899, Schussnig 1932, Zinnecker 1935, Burr and West 1970) the different species are shown to share a similar cytoplasmic organisation. In the present study, two species which have not previously been studied cytologically, *B. vestita* and *B. gemellipara*, and a third species *B. plumosa* were found to show uniform cytoplasmic organisation which is described in the following account.

The cytoplasm of *Bryopsis* lines the tubular thallus (Figs. IV.2, IV.3) and is bounded on the outside by the continuous cell wall which appears in electron microscope sections to be composed of fine granules or fibrils (Fig. IV.3 inset). A thin cuticle-like layer of electron dense material (compare with Hanic and Craigie 1969) is continuous over the cell wall (Fig. IV.3) and appears as a quite distinct layer when it separates from the remainder of the cell wall in the gametangia of *B. vestita* and *B. gemellipara* (section III.3, Figs. III.6, IV.4). Further differentiation of the cell wall into layers is not observed under the transmission electron microscope although layers showing differential staining reaction with toluidine blue are seen in light microscope sections of the cell wall of older parts of the thallus (Fig. IV.2).

Projections of the cell wall (trabeculae) into the cytoplasm are reported by Naegeli (1847), Printz (1927) and Fritsch (1945) accept this observation, and it is used by Fritsch (1945) as partial justification for retaining *Bryopsis* within the Caulerpaceae. However, in the present study, and that of Burr and West (1970) no such trabeculae have been observed, the cell wall being approximately the same thickness throughout and the inner layer of cell wall material smooth.

The cytoplasm forms a uniformly thin layer between the cell wall and the large central vacuole; except at the apex where a much thicker zone of cytoplasm occurs without plastids (Figs. IV.5, IV.6). As growth of the *Bryopsis* thallus is apical this specialised region of cytoplasm can be interpreted as homologous to the meristematic regions of cellular plants. A similar region in *Caulerpa* was termed "meristemplasm" by Janse (1910) and this has been adopted in the description of *Bryopsis* (Steinecke 1925, Burr and West 1970).
Fig. IV.2  LM B. vestita transverse section showing cytoplasm lining the cell wall. Chloroplasts occupy inner region lining the large central vacuole. Note proteinaceous structures in vacuole.

Fig. IV.3  TEM Cell wall, cytoplasm and chloroplasts of B. vestita.
Inset. High power TEM of cell wall.  
EM plate 27463
Inset 27471
Fig. IV.4 TEM Cell wall of gametangium of *B. vestita* showing separation of cuticle. Inset. region where separation is occurring.

EM plate 26698
Inset 26699

Fig. IV.5 TEM Region of cytoplasm (meristemplasm) at apex of the axis of *B. vestita*.

EM plate 27192
In *Bryopsis vestita* and *Bryopsis gemellipara* the ramuli are branches of limited growth (see sections II.2-1, II.2-2) and the typical meristemplasm disappears after the growth of the ramuli ceases. In these species the mature ramuli have blunt apices and the cytoplasm at the apex is no different either in thickness or composition from the normal cytoplasm lining the cell wall. However, in *Bryopsis plumosa* meristemplasm remains in all ramuli and allows continuous elongation and branching of these throughout the life of the plant.

The cytoplasmic organelles are unevenly distributed throughout the non-apical regions of the thallus. The outer cytoplasm contains nuclei, mitochondria, endoplasmic reticulum, ribosomes and dictyosomes, (Fig. IV.7) as seen in *Caulerpa* (Dawes and Barilotti 1969, Mishra 1969), and is continuous with the meristemplasm (Fig. IV.6). The plastids occupy the innermost layer of the cytoplasm, lining the vacuolar membrane (Figs. IV.3, IV.7). This cytoplasmic organisation was observed by Zinnecker (1935) and Burr and West (1970) in *B. plumosa* and *B. hypnoides*. Zinnecker (1935) considered the nuclei and other organelles lying external to the chloroplasts to be an arrangement atypical within the siphonous green algae, however, the same organisation is also noted in the present study of both *Derbesia* and *Pedobesia* (section V.5, VI.2-1).

Chloroplasts (Figs. IV.8, IV.9) are the only type of plastids found in the cytoplasm of *Bryopsis* and are regular lenticular organelles, each containing one and sometimes two pyrenoids surrounded by many starch plates. These pyrenoids are therefore referred to as polypyramidal (Chadefaud 1941). The chloroplasts are aligned within the younger parts of the thallus with their long axis parallel to the long axis of the branches. In all three species studied and the unusual forms described in sections II. 2-4 to II. 2-6, the chloroplasts have approximately the same dimensions, 12μm long and 4μm wide. More or less spherical chloroplasts are sometimes seen at the base of older plants and in plants where the tubular thallus has been damaged.

Cytoplasmic streaming is observed in the slow longitudinal movement of chloroplasts within the thallus. In all species studied streaming has been seen and the resultant distribution of chloroplasts follows a diurnal rhythm associated with the onset of the light regime.
Fig. IV.6 TEM *B. vestita* meristemplasm showing nuclei, mitochondria, dictyosomes and rough endoplasmic reticulum. EM plate 27069

Fig. IV.7 TEM Cytoplasm lining tubular thallus of *B. vestita*. Note nuclei, chloroplasts and rough endoplasmic reticulum. EM plate 27460
Fig. IV.8 TEM Chloroplast of B. vestita. Note polypymidal pyrenoid with perforated, branched thylakoid within pyrenoid matrix. Circular structure on plastid is an artefact arising from disruption of tonoplast.

EM plate 27488

Fig. IV.9 TEM Chloroplast of B. vestita. Note three thylakoid membranes within pyrenoid matrix, free starch grains and osmiophilic globules in stroma.

EM plate 27472
At night the chloroplasts accumulate in the rhizoids and lower parts of the thallus while during the day they appear more or less evenly distributed throughout the upright parts of the plant. Unlike Caulerpa (Sabnis and Jacobs 1967) no microtubules have been found associated with regions of streaming in the fixed cytoplasm of Bryopsis.

Electron micrographs of sections through well fixed chloroplasts (Figs. IV.8, IV.9) show them to be traversed by numerous bands of three (occasionally up to five) thylakoids, with some exchange of thylakoids between bands. One to three branched or unbranched thylakoids may also traverse the pyrenoid (Figs. IV.8, IV.9) and in all species studied the parts of the thylakoid within the pyrenoid are perforated with regular pores approximately 300Å diameter (Fig. IV.10). Osmiophilic globules and free starch grains are common within the stroma of the plastids (Fig. IV.8). In all respects this description corresponds with descriptions of chloroplasts from other species of *Bryopsis* (Hori and Ueda 1967, Burr and West 1970).

In a study of the fine structure of *B. hypnoides*, Burr and West (1970) note the presence of two types of organelles which they term "electron dense vesicles" and "schizogenous bodies". In a further study Burr and West (1971a) note that these structures are in fact different developmental stages of the same organelle, which they term a "protein body". In a survey of the three species *B. vestita*, *B. gemellipara* and *B. plumosa*, only in *B. plumosa* was any structure found that could be related to this organelle (Fig. IV.11). This would correspond with the early stage in the development of the protein body as described by Burr and West (1971a) but no further developmental stages corresponding with the schizogenous bodies have yet been found.

IV.4 Nuclear cytology.

IV. 4-1 Vegetative nuclei and chromosome numbers.

Large numbers of nuclei of varying sizes are found in the outer layer of cytoplasm of the vegetative *Bryopsis* plant (Fig. IV.7). They may range from 3 to 8μm in diameter, even within the same plant, and no consistent differences in size are observed between species.
Fig. IV.10 TEM Pyrenoid of *B. vestita* with section through thylakoid showing longitudinal section of pores. EM plate 27213

Fig. IV.11 TEM *B. plumosa* showing membrane bound protein body. EM plate 27228
Under the transmission electron microscope the nucleus is seen to be surrounded by a double membrane, the outer layer of which is covered with ribosomes (Figs. IV.6, IV.7). Pores, 750Å diameter, in the double membrane appear under some fixation procedures to be covered by a single layer of membrane-like material and have outside this membrane, a central electron dense granule, approximately 200Å diameter (Fig. IV.12). The structure of these pores appears to be unlike that of the nuclear pores of higher plants (Roberts and Northcote 1970), but more detailed information must be obtained from horizontal sections before an adequate description can be made.

The nuclei undergo asynchronous mitotic division and do not divide more actively at the apex where growth of the plant occurs. Throughout mitosis, the nucleolus and nuclear membrane are persistent and in this respect the three species studied here are similar to all other Bryopsis species for which the sequence of nuclear division is known (Zinnecker 1935, Neumann 1969a). The various stages in the normal mitotic division of B. vestita are shown (Fig. IV.13), both the other species having identical mitotic sequences.

Chromosome numbers for all the forms and species of Bryopsis from the south eastern Australian coast have been determined (Table IV.1). Although polar view of metaphase nuclei is rare, counts have been obtained from at least ten nuclei from all but the fine irregularly branched form. Figures given for this form have been obtained from plants which revert to the distichous branching habit in culture and no chromosome numbers are available from those which become radially branched. Where possible, the chromosome numbers of plants of the same species collected from different populations have been determined and are consistent with those given in Table IV.1.

From all the Bryopsis plants studied only three different chromosome numbers have been found. Those plants which are grouped together as a result of similar karyotypes (e.g. B. vestita and the large tetristichous form, B. plumosa and the large and small irregularly branched forms) are also those which are shown to be interfertile (section III.4). Three species are therefore recognised, B. gemellipara 2n?=16, B. plumosa 2n?=8 and B. vestita 2n?=12.
Fig. IV.12 TEM Nuclear pore of *B. vestita* showing covering membrane and central outer granule (arrow). EM plate 27467

Fig. IV.13 Stages in normal mitotic division sequence in *B. vestita*. 
1. \textit{B.\textit{vestita}} 12
2. \textit{B.\textit{gemellipara}} 16
3. \textit{B.\textit{plumosa}} 8
4. Large tetrasporangial form 12
5. Small irregularly branched form 8
6. Large irregularly branched form 8

Table IV.1 Chromosome numbers determined from the gametophytic stages of the forms of \textit{Bryopsis} from the south eastern Australian coast.

IV. 4-2 Gametangial nuclei and meiosis.

Zinnecker (1935), supported by Neumann (1969a), claims that meiosis occurs within the developing gametangia of \textit{Bryopsis}. However, since the discovery of an alternation of generations in the \textit{Bryopsis} life history, this has been questioned. Rietema (1969, 1970) and van den Hoek \textit{et al.} (1972) argue from indirect evidence that meiosis precedes spore formation within the protonemal sporophyte (for a summary and criticism of these arguments see section VII.2-2).

Present observations show that the nuclei of the developing gametangia undergo a series of rapid asynchronous mitotic divisions. In a detailed examination of a large number of gametangia from all three species, neither meiotic division stages nor reduction in chromosome number has been detected. However, as the reduction in size of the gametangial nuclei makes precise identification of division stages and chromosome numbers difficult, it would be premature to conclude with Rietema (1969, 1970) and van den Hoek \textit{et al.} (1972) that meiosis does not occur in the developing gametangia.

IV. 4-3 The zygote nucleus.

After fusion of the male and female gametes the zygote nucleus does not divide during germination but continues to enlarge for thirty to forty days, finally reaching a size eight to ten times that of the vegetative nuclei of the multinucleate stages (see also section III. 2). Both Neumann (1969a) and Burr and West (1971b) compare the giant nucleus of \textit{Bryopsis} with the giant nuclei of members of the Dasycladales.
Two techniques have been used in the investigation of the giant nuclei of the protonemal stages of the southern Australian Bryopsis. Some material has been fixed and stained according to the procedures outlined in section IV. 2-1, while living protonema have been studied under the Nomarski interference contrast microscope. As the images obtained with these two techniques are not directly comparable, particularly in the development of the nucleolus, the following description is made from direct observation of the living nuclei.

Soon after fusion of the gametes the zygote nucleus is approximately 5μm in diameter and the nucleolus is a small spherical mass (1-2μm in diameter) within this. Rapid enlargement of the nucleus and the nucleolus begins with the germination of the zygote, within 48hrs of fusion (Fig. IV.14). In ten to fifteen days the nucleus attains a diameter of 20 to 25μm, and the nucleolus appears as an ovoid structure with numerous small vacuoles (Fig. IV.15). As the protonema continues to grow the giant nucleus only enlarges slowly, reaching its maximum size (up to 45μm diameter) after thirty-five to forty days. The nucleolus at this stage is long and sinuous, often wound in a spiral, as described by Neumann (1969a) (Fig. IV.16). After this, the nucleolus begins to fragment and the mature nucleus commonly contains three to five nucleolar fragments, each of which may be vacuolate (Fig. IV.17). Although fragmentation of the nucleus is frequently seen in living nuclei it has not been observed in fixed, stained protonema. In these preparations, the fixation procedures are thought to aggregate the nucleolar material.

The mechanism of division of the giant nucleus has been reported by Neumann (1969a). The chromosomes form on a spindle on the side of the nuclear mass, and after they move to the poles of the spindle, two small nuclei are formed which give rise to the nuclei of the multinucleate thallus. The large mass of the giant nucleus which is left after the division, remains in the cytoplasm and gradually disintegrates. Neumann (1969a) claims that this division sequence is essentially mitotic and the chromosome number of the giant nucleus is diploid.

Attempts to study the division of the giant nucleus, from stained preparations of the protonema of the south eastern Australian Bryopsis have proved unsuccessful. Further culture studies are necessary
Figs IV.14 to IV.17 Stages in development of the protonemal giant nucleus of *B. gemellipara*. IV.14 early enlargement of nucleus with spherical nucleolus, four days old. IV.15 nucleus with ovoid nucleolus, twelve days old. IV.16 nucleus with spirally wound nucleolus, forty days old. IV.17 nucleus with fragmented nucleolus, seventy-five days old.
to determine the environmental conditions which initiate division, so that a detailed study of a large number of dividing nuclei can be undertaken.

However, some stained protonema have been observed after division of the giant nucleus with numerous small nuclei (4-7 μm in diameter) in the cytoplasm. Stages in division of these small nuclei have been rarely observed but are thought to be typical of the mitotic divisions seen in the gametophytic plant. Chromosome counts have not been obtained from these small protonemal nuclei.

Prior to the holocarpic division of the cytoplasm into spores, up to two hundred small nuclei are present in each protonema. After cytoplasmic division each spore mass contains only one nucleus at the centre of a spherical mass of cytoplasm, which is rich in chloroplasts. After release and during germination of these spores, the nucleus divides rapidly, reproducing the normal multinucleate state characteristic of the *Bryopsis* phase of the life history.

IV. 5 Vacuolar materials of *Bryopsis*.

In the early literature on *Bryopsis* (Berthold 1880, Küster 1899, Noll 1899) there are descriptions of fibrillar material, spherical structures which appear to have crystalline properties and regular protein crystalloids, all of which were found in the large central vacuole of this plant. Although these structures were thought to be associated with wounding, their origin and composition was unknown. Burr and West (1971a) and Burr and Evert (1972) report the only electron microscope studies of these structures yet published, with some details of origin, composition and wound healing function. They describe a process of formation of protein bodies in the cytoplasm, their release into the vacuole and subsequent breakdown into microtubular and granular-fibrillar materials.

In the three species of *Bryopsis*, *B. vestita*, *B. gemellipara* and *B. plumosa*, and particularly in the larger species *B. vestita* and *B. gemellipara*, masses of white material are found to accumulate at the base of the axis and the isolated ramuli (Fig. IV.18). Under the light microscope this material is seen in living specimens to be composed of granular, rope-like structures which are intertwined (Figs. IV.19 to 21). A similar picture is obtained with the scanning electron microscope from freeze dried or partially embedded specimens (Fig. IV.23).
Fig. IV.18 *E. vestita*. Note light green to white material at base of fronds on lower ramuli.
Figs IV.19 to IV.21 Vacuolar protein materials in *B. vestita*. IV.19 and IV.20 rope-like strands in living plants. IV.21 glutaraldehyde fixed vacuolar protein materials showing rope-like strands and spherical structures with radiating fibrils.
Smooth transparent spherical structures (up to 300 μm diameter) are found among the granular ropes (Figs. IV.21, IV.22). These spheres collapse under vacuum and are therefore seen as saucer shaped in the scanning electron micrograph (Fig. IV.24). They exhibit a complex form of anisotropy, producing a cross-like pattern when viewed under the polarizing light microscope, between crossed nicols (Fig. IV.25).

With the transmission electron microscope, accumulations of microtubular material are found in the vacuole (Fig. IV.26) and these correspond with the rope-like structures described from light and scanning electron micrographs. The individual microtubules are long and may be branched (sections up to 6 μm long have been obtained, Fig. IV.27). A central tubule 300 to 350 Å in diameter is surrounded by a layer of electron dense granular material up to 600 Å thick (Fig. IV.28).

Burr and West (1971a) describe short rod-like structures with central microtubules, with walls composed of twelve subunits arranged in a helix with a pitch of 38°. However, from the published electron micrographs they do not appear to be aggregated into complex masses as seen in B. vestita and B. gemellipara.

Burr and West (1971a) propose a developmental sequence for the microtubular elements, involving the breakdown of the protein bodies referred to in section IV. 3. In the present study, similar protein bodies have been seen in the cytoplasm only of B. plumosa, and have not been found in mature or decomposing state in the vacuole. It is therefore thought that the developmental sequence described for B. hypnoides (Burr and West 1971a) is unlikely to account for the accumulation of these vacuolar materials, at least in B. vestita and B. gemellipara.

As found by Burr and West (1971a) the microtubular elements are associated in the vacuole with a fine granular-fibrillar matrix (Fig. IV.29). This is seen as an unstructured mass and interpreted by Burr and West (1971a) as the results of breakdown of the microtubules and their associated granular-fibrillar coating. In the present study no evidence has been obtained for a similar breakdown of microtubules.

In the three species investigated, regularly organised structures are also found embedded with the microtubules in the granular-fibrillar matrix (Fig. IV.30). These correspond with the transparent spherical bodies mentioned earlier, which show paracrystalline
Fig. IV.22 LM Rope-like and spherical protein bodies in B. gemellipara.

Fig. IV.23 SEM Rope-like structures in vacuole of B. vestita.
Fig. IV.24 SEM B.vestita vacuolar protein bodies. Note rope-like structures and collapsed spheres (saucer-shaped).

SEM 2678

Fig. IV.25 Polarising LM of vacuolar material of B.vestita. Cross-like patterns show position of sphereocrystals.
Fig. IV.26 TEM Transverse section through rope-like vacuolar material.

EM plate 27433
Fig. IV.27 TEM Longitudinal section through microtubules forming rope-like structures in vacuole of *B. gemellipara*. Inset. Branched microtubule.
Fig. IV.28 TEM Transverse section through microtubules forming rope-like structures in B. gemellipara. Inset. High power view.

EM plate 27037
Inset 27449
Fig. IV.29 TEM Microtubular material of B. gemellipara embedded in granular-fibrillar matrix.

EM plate 27040
Fig. IV.30 TEM Glancing section through spherocrystal embedded in granular-fibrillar material. 

EM plate 27487
properties under polarized light, and which have therefore been termed "spherocrystals" by Noll (1899). Under the transmission electron microscope these spherocrystals appear to be composed of many microfibrils radiating from the centre of the sphere. Glancing sections through the spheres (Fig. IV.30) show little more than transverse sections of the radiating fibrils and this was seen but identified as unorganised granular-fibrillar matrix by Burr and Evert (1972, Fig.27). Sections taken close to the centre of the spheres show that the fibrils are in fact wavy and their regular waves give the impression of concentric lines in the structure (Figs. IV.31, IV.32). This microstructure accounts for the early observations of Golenkin (1894) and Noll (1899) and the light micrographs showing concentric and wavy radiating lines in some of the spheres observed (Figs. IV.2, IV.21). From figures published by Küster (1899, Figs. 1 to 3) and present observations it appears that spherocrystals divide within the vacuole by a process of fission (Fig. IV.33).

These spherocrystals show some superficial similarity with the "spherical bodies" described from the cytoplasm of Halimeda by Wilbur et al. (1969). However, as the spherocrystals have only been found as vacuolar inclusions in Bryopsis, it is suggested that these may be quite different structures. Also, without some evidence of the chemical composition of the spherical bodies of Halimeda, direct comparisons are premature. No other comparable vacuolar structures have yet been described in studies of the fine structure of other members of the siphonous green algae.

A third regular inclusion in the vacuole has been found in mature specimens of B. vestita. Among the microtubular material of the rope-like structures, sections of small triangular or tetragonal bodies are observed (Fig. IV.34) and their crystalline nature inferred from their regular shape and lattice structure seen under the transmission electron microscope (Fig. IV.35). As no electron diffraction pattern is produced by these crystalline structures it is thought that they may represent protein crystalloids and may be a stage in the development or breakdown of the microtubular material. Each crystal appears to be surrounded by granular-fibrillar material similar to the coating around the microtubules (Fig. IV.35). These crystals
Fig. IV.31 TEM Median section of spherocrystal showing radiating, wavy microfibrils. EM plate 27447

Fig. IV.32 TEM Magnified view of fine structure of spherocrystal shown above. EM plate 27442
Fig. IV.33 Spherocrystal from B. vestita apparently dividing.

Fig. IV.34 TEM Section through rope-like structures showing embedded crystals. EM plate 27489
are similar to the small octahedral crystals seen in the vacuole of the protonema of *B. gemellipara* (Fig. IV.36).

The different structures described in the vacuole of *B. vestita*, *B. gemellipara* and *B. plumosa*, all correspond with the range of inclusions described by the early investigators studying *Bryopsis* and *Derbesia*. Burr and West (1971a) and Burr and Evert (1972) in their study of *B. hypnoides*, have described only microtubular structures which as pointed out earlier differ only in minor respects from those described here. They appear to have failed to recognise the presence of spherocrystals, although presenting micrographs which are comparable with those presented here.

The developmental sequence proposed for the microtubular elements of *B. hypnoides* (Burr and West 1971a) has not been recognised in the present study of three other species, and the existence of two other structurally distinct bodies in the same complex of vacuolar materials, makes further comparison and interpretation of this sequence difficult.

The proteinaceous nature of the vacuolar materials has been shown by Burr and West (1971a) and Burr and Evert (1972). All the vacuolar materials described from the present study, stain with the protein specific stain Coomassie Brilliant Blue (Fisher 1968). However their solubility in dilute acids is quite different and suggests that the composition of all structures is not identical. The rope-like microtubular materials are soluble in cold, dilute HCl (IN) and acetic acid. The spherocrystals on the other hand, are quite unreactive in these acids, at least at room temperature. Paper chromatography of the acid solution of the microtubular material shows the presence of amino acid bands with the ninhydrin reaction.

On the basis of these preliminary observations the composition of the vacuolar material appears to be more complex than previously recognised (Burr and Evert 1972). As the cold acid-soluble fraction of the microtubular material is ninhydrin positive, it is suggested that the major constituent could be free polypeptides. The spherocrystals show a similar staining reaction to the microtubules, but are insoluble in dilute or concentrated acids at room temperatures and may be composed of protein.
Fig. IV.35 TEM Magnified image of crystals shown in Fig. IV.34, showing internal lattice structure. EM plate 27491

Fig. IV.36 LM B. gemellipara protonema showing large vacuolar crystal.
SECTION V

DERBESIA
V. 1 Introduction.

Despite attempts to culture species of Halicystis by Hollenberg (1935), it was not until Kornmann (1938) found that the spores of Derbesia marina produced a gametophytic stage that resembled Halicystis ovalis, that the heteromorphic nature of the Derbesia life history was first recognised. Several other studies with D. marina and other species of Derbesia have shown a similar alternation with species of Halicystis (Feldmann 1950, Ziegler and Kingsbury 1964, Page 1970, Sears and Wilce 1970). However, some studies have shown that direct reproduction is also possible within some populations of D. marina, the spores germinating to produce the new sporophytic generation (Kornmann 1966, 1970, Sears and Wilce 1970).

The discovery of an alternation of generations between Derbesia neglecta and Bryopsis halymeniae (Hustede 1960, Rietema 1972) indicated the close relationship between the two genera Bryopsis and Derbesia. However, as other species of Bryopsis were thought to have a monophasic, diplontic life history, Feldmann (1969) proposed that D. neglecta should be placed with its alternate Bryopsis stage in the new genus, Bryopsidella (nomen nudum).

Following this reported life history association between one species of Bryopsis and Derbesia, the discovery of a protonemal sporophyte producing stephanokont spores in the life history of some other species of Bryopsis (Rietema 1969 to 1972, present thesis), the study of the south eastern Australian Bryopsis was considered incomplete without an investigation of the life histories and cytology of the species of Derbesia found in this region.

V. 2 Identification of species of Derbesia.

The determination of the species of the fine, irregularly to dichotomously branched species of Derbesia has been the subject of considerable controversy for many years. As this is in part due to the frequent use of dried herbarium specimens for description and identification of species, it should be stressed that "The importance of the chloroplast and the reproductive morphology make it imperative that species determinations be based on living reproductive specimens. Identification of species of Derbesia should not be attempted from herbarium or
poorly fixed vegetative plants." (Sears and Wilce 1970 p.390).
Particularly on the northern American coast, confusion has arisen in
the distinction between the species *D. marina*, *Derbesia tenuissima*,
*D. lamourouxi*, *Derbesia osterhoutii* and *Derbesia vaucheriaeformis*,
because of intraspecific variation in filament diameter and sporangial
dimensions. (Sears and Wilce 1970).

The two species accepted from the southern Australian coast, are
*D. marina* (Lyngbye) Solier and *Derbesia clavaeformis* (J.Agardh) De Toni
(Womersley 1956). *D. clavaeformis* has since been removed to a new genus
Pedobesia (MacRaild and Womersley 1974) and this will be discussed in
detail in the following chapter.

Womersley (1956 p.372) states that the southern Australian material
of the fine, dichotomously branched *Derbesia*, referred by him to
*D. marina* "agrees in all respects with liquid-preserved and dried
specimens of Borgesen's from the Faroes (seen in C) and is very like
Lyngbye's figures." However, Womersley (1956) did not take into
consideration the presence and absence of pyrenoids in the plastids of
this alga (Womersley, personal communication), a characteristic which
has been used recently in distinguishing between *D. marina* and other

Collections of *Derbesia* from the south eastern Australian coast
fall into two groups, one group of specimens has pyrenoids in the plastids,
the other group lacks pyrenoids. However, as seen in Table V.1 there are
no consistent differences based on additional morphological characters
which could justify the separation of the two forms of *Derbesia* into
distinct species.

Sears and Wilce (1970 p.390) claim that "Gross differences in
sporangium morphology are taxonomically useful with the pyriform-clavate
sporangia of *Derbesia marina* being distinct from the globose sporangia
of *D. lamourouxi* and *D. osterhoutii*, and from the sausage shaped
sporangia of *D. tenuissima*." As shown in Fig. V.1, the shapes of the
sporangia of the south eastern Australian *Derbesia* range from clavate
to globose, there being no consistent differences between the plants
with and without pyrenoids. Both populations also have between fifteen
and forty-five spores in each sporangium.
<table>
<thead>
<tr>
<th>Species</th>
<th>Sporangium width</th>
<th>Sporangium length</th>
<th>Plug width</th>
<th>Plug length</th>
<th>Filament diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. Lonsdale (with pyrenoid)</td>
<td>116 (69-162)</td>
<td>207 (150-267)</td>
<td>24 (15-35)</td>
<td>26 (13-40)</td>
<td>57 (32-69)</td>
</tr>
<tr>
<td>Culture* (with pyrenoid)</td>
<td>115</td>
<td>182</td>
<td>21</td>
<td>19</td>
<td>44</td>
</tr>
<tr>
<td>Sorrento (with pyrenoid)</td>
<td>114</td>
<td>209</td>
<td>22</td>
<td>26</td>
<td>37</td>
</tr>
<tr>
<td>Mornington* (no pyrenoid)</td>
<td>117</td>
<td>173</td>
<td>21</td>
<td>23</td>
<td>49</td>
</tr>
<tr>
<td>Nora Creina* (no pyrenoid)</td>
<td>84</td>
<td>159</td>
<td>19</td>
<td>21</td>
<td>38</td>
</tr>
<tr>
<td>D. marina (type locality)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>D. marina (Womersley 1956)</td>
<td>75</td>
<td>160</td>
<td></td>
<td></td>
<td>54</td>
</tr>
<tr>
<td>D. novae-zealandiae (Chapman 1949)</td>
<td>95-102</td>
<td>120-150</td>
<td></td>
<td></td>
<td>37-52</td>
</tr>
</tbody>
</table>

Table V.1 Average measurements (µm) taken from Derbesia plants collected from the south eastern Australian coast, with comparative figures from other species of Derbesia.

+ Parent material, Halicystis collected at Point Lonsdale.

* Small sample size; less than 10 plants collected, with only 5 mature sporangia from Nora Creina and 15 from Mornington.
There is no difference in the ecological habitat or distribution of the two forms of Derbesia on the southern Australian coast. Plants with pyrenoids have been collected from Cape Patterson, Sorrento (ocean beach), Pt. Lonsdale, Nora Creina and Kangaroo Is., while material without pyrenoids has been collected from within Port Phillip Bay and from Nora Creina and Kangaroo Is.. At both Nora Creina (personal observation) and Kangaroo Is. (Womersley, personal communication) the two forms are growing in the same locality, and at Nora Creina, within the same rock pool.

The only form of Halicystis collected in nature has plastids with pyrenoids, and has been found growing in association with the fine Derbesia at Cape Patterson, Sorrento, Pt. Lonsdale and Nora Creina. These plants are pyriform to spherical, with an average diameter of 12mm (6 to 20mm).

The Halicystis plants have been referred to Halicystis ovalis (Lyngbye) Areschoug, by MacRaild and Womersley (1974), because of their small size. Typical H. ovalis, however, has no pyrenoids. In the absence of further information on the reproduction of the fine form of Derbesia without pyrenoids, no further comment can be made on the appropriate specific epithet for H. ovalis.

Chapman (1956) records D. marina and Derbesia novae-zelandiae (Chapman 1949) from the New Zealand coast, but makes no comment on the presence or absence of pyrenoids in their plastids. The difference between the species is based on differences in their size and small appendages by which the adjacent filaments are held together in D. novae-zelandiae. Chapman et al. (1964) also record the presence of a species of Halicystis in New Zealand and suggest without culture studies, that this is the alternate stage of one of the Derbesia species. This Halicystis is noted to have pyrenoids and may be related to the south eastern Australian species.

In the absence of detailed cytological, cross fertilisation and controlled environmental studies on the fine Derbesia, it is proposed here, to follow Womersley (1956) referring to a single species, D. marina. Two points must be stressed concerning this:

(a) The presence or absence of pyrenoids within the plastids of different collections of plants, suggests that a single species name...
is inadequate to describe the whole population. The southern Australian Derbesia may represent sympatric populations of two species which possibly correspond with D. marina, D. tenuissima or D. novae-zelandiae.

(b) The presence of a pyrenoid in the plastids of D. tenuissima and D. lamourouxii was reported by Ernst (1904) to be dependent on shading, and the depth at which the plants were growing. Although there is no more recent evidence of the environmental dependence of the presence and absence of a pyrenoid in Derbesia or any other green alga, the findings of Ernst (1904) have not yet been refuted. However, de nova formation of pyrenoids is reported by Hoffman (1968) in the zoospores of Oedogonium cardiacum (see also section V. 4-3).

V. 3 Ecological habitats of the south eastern Australian Derbesia and Halicystis.

Derbesia marina and its gametophyte Halicystis ovalis have been found at a number of different localities from the Eyre Peninsula, South Australia, to Cape Patterson, Victoria. Plants are frequently found in mid-eulittoral rock pools heavily shaded either by overhanging rocks or dense stands of other algae. The most common habitat is rock pools in shallow caves at the base of steep cliffs, undercut by wave action. These pools are always covered at high tide.

Halicystis has been found growing in nature on articulate or crustose coralline red algae. The rhizoidal parts of the thallus penetrate the coralline host and the vesicular Halicystis arises through a pore as illustrated by Kuckuck (1907) and Hollenberg (1935). These two authors also note that the rhizoidal part of this alga is a perennating structure which produces several vesicles in successive seasons. Large, well-developed rhizoidal stages have been dissected from crustose coralline red algae (Fig. V. 2) collected from Sorrento. This suggests that the rhizoids of the south eastern Australian populations may also act as perennating organs.

Derbesia on the other hand has been found growing on both rock and a number of different algae, not restricted to the coralline forms. The simple to irregularly branched rhizoids are attached to the
Fig. V.1  Sporangia of *D. marina* showing the range of size and shape within the Pt. Lonsdale population of plants with pyrenoids.

Fig. V.2  Rhizoidal portion of *H. ovalis* dissected from a crustose coralline red alga. Dotted line = reconstructed outline of the host alga.
surface of the substrate and produce numerous, upright dichotomously branched filaments. There is no evidence to suggest that the rhizoids of \textit{Derbesia} act as perennating organs.

V. 4 Culture studies.

V. 4-1 The life history of \textit{Derbesia}.

Unialgal cultures have been established from male and female gametes of fertile \textit{Halicystis} plants collected in nature (Fig. V.3), or from small pieces of the \textit{Derbesia} thallus dissected from the whole plant and placed in culture medium. The general techniques, culture media and conditions used are as outlined in section III. 2.

Growth of the \textit{Derbesia} phase of the life history occurs at both 16°C and 20°C and a light intensity of 1500lx. Sporangia are produced at both temperatures, but at 16°C many of these decompose without releasing their spores. At 20°C large numbers of spores are released and these move for up to 10hrs before coming to rest on the bottom of the culture vessel.

Cultures of \textit{Halicystis} were established by isolation into fresh culture medium of \textit{Derbesia} spores released from cultured material or fertile plants collected in nature. These were maintained at 13°C, 16°C or 20°C, where they develop through a typical germination pattern (Fig. V.4). The spores produce a fine germination tube which does not attach to the substrate but continues to elongate without branching until it reaches a length of 300µm (Fig. V.4). At this stage apical growth ceases and the tube begins to swell, producing a spherical to ovoid vesicle. A thin layer of cytoplasm lines its wall and surrounds the very large central vacuole.

\textit{Halicystis} plants grown in culture from \textit{Derbesia} spores become fertile at 20°C but not at 16°C or 15°C. Male and female gametes are produced in different plants by gametogenesis within specialised reproductive areas in the cytoplasm. The male and female plants are easily distinguished by the difference in colour of the gametangial areas, the female being dark green and the male light brown.
Fig. V.3 *H. ovalis* collected from Pt. Lonsdale. Fertile plants, three female and one male.
Fig. V.4 Germination stages of H. ovalis from spore (a), to mature vesicle (f).
The difference in colour of the reproductive areas is explained by the difference in structure of the two types of gametes. The female gamete is pear shaped (8um long and 10um wide) with the posterior part packed with numerous rounded chloroplasts (Figs. V.5, V.6) which give the gamete and the gametangium the dark green coloration. The anterior part is hyaline, with two flagella inserted into a cytoplasmic papilla, as seen in Bryopsis. The female gamete of the southern Australian Halicystis is easily distinguished from the female gametes of the species of Bryopsis which grow in the same area, by the very large number of chloroplasts and the absence of an eye spot.

In contrast, the male gamete of Halicystis is indistinguishable from that of Bryopsis, being much smaller than the female (10um long, 2um wide) and having only one (occasionally two) small brown chloroplast-like structures at its posterior end (Fig. V.6). The presence of these abnormal chloroplasts, and the complete absence of green chloroplasts in the male gametangium, is responsible for the light brown coloration of this structure. No eye spot has been detected with the light microscope. The male gametes are also biflagellate and like the female have the flagella inserted into a short papilla at the hyaline anterior end.

Gametes are released from the Halicystis plant through pores in the cell wall. Pores are seen before gamete release as small, circular, clear areas in the cytoplasm and one to several may be present within the one gametangial area (Fig. V.3). Gametes are forcefully discharged with the entire contents of each reproductive area released within 10 to 15secs. Not all potential pores are always used, but for the larger gametangial areas release occurs though more than one. As the gametes are released the crenate (dentate) margin of the gametangial area contracts towards the pores through which the gametes are ejected.

Hollenberg (1935) claims that the gametes of H.ovalis are ejected in a mass which settles to the bottom of the culture vessel before the individual gametes become motile. In the material observed here the gametes are not motile within the plant but do become motile very soon after release, generally breaking away from the cloud of gametes as they are ejected. After release from the plant the gametes move rapidly, males generally much faster than the females. Male gametes
Fig. V.5 Female gamete of *H. ovalis*. Note two flagella and numerous chloroplasts.

Fig. V.6 Male and female gametes of *H. ovalis*, with quadriflagellate motile zygote.
are only active for 2 to 3hrs while females have been observed to move for up to 24hrs.

Fusion of the gametes occurs in a manner similar to that described for Bryopsis. After initial contact with the flagella the male gamete comes to lie alongside the female and the two gamete masses fuse laterally. This type of gamete fusion corresponds with that described by Neumann (1969b) and has been observed frequently, soon after the male and female gametes are mixed. The mutual attraction of the male and female gametes in Halicystis appears to be much stronger than in Bryopsis (see section III. 3). Fusion of gametes occurs within half an hour of release from the gametangium.

Quadriflagellate motile stages (Fig. V.6) can be observed, but only in preparations where both male and female gametes are present. These are interpreted as motile zygotes seen immediately after fertilisation and prior to their settling to the base of the culture vessel. Once the zygote has settled, the flagella are either retracted or shed and the cytoplasmic mass rounds off and becomes enclosed by a cell wall within 12hrs. Germination begins within 24hrs of this with production of a germination tube which is firmly attached to the culture vessel. The initial growth corresponds with the rhizoidal parts of the plants found in nature and the upright dichotomously branched thallus arises from this.

It can be seen from this description of the life history that the south eastern Australian Derbesia is similar to that of European and North American coasts, showing an alternation of generations between a typical Derbesia sporophytic stage and a gametophyte which corresponds with the genus Halicystis.

V. 4-2 Germination tests.

As there are several reports indicating the possibility of direct reproduction within some populations of Derbesia (Feldmann 1950, 1952, Kornmann 1966, 1970, Sears and Wilce 1970) a study was undertaken of the germination pattern of more than 500 spores of Derbesia, produced in first to third generation cultures of material collected at Sorrento and Point Lonsdale, Victoria and Nora Creina, South Australia.
In order to check the influence of environmental conditions on germination, the cultures were divided into four groups maintained at 20°C, 16°C, and 13°C, with some of those at 16°C being kept under aerated and the others under non-aerated conditions. All viable spores (90% of the total number isolated) underwent the typical germination pattern described above, and at no stage were *Derbesia*-like plants produced directly from spores. No morphological differences were seen between the plants and except for the expected variations in growth rate, the only observable difference between cultures kept under these conditions was the germination percentage (Table V.2). As fertile plants were rarely observed at 16°C and never at 13°C the assessment of the germination percentage from the number of spores producing fertile *Halicystis* plants was not possible at all temperatures. Therefore spores were assessed as undergoing successful germination if they produced a vesicular plant of 5mm diameter.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Number of spores isolated</th>
<th>Number of spores germinating</th>
<th>Germination percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C</td>
<td>170</td>
<td>163</td>
<td>96</td>
</tr>
<tr>
<td>16°C non-aerated</td>
<td>170</td>
<td>159</td>
<td>93</td>
</tr>
<tr>
<td>16°C aerated</td>
<td>120</td>
<td>119</td>
<td>99</td>
</tr>
<tr>
<td>13°C</td>
<td>90</td>
<td>54</td>
<td>60</td>
</tr>
</tbody>
</table>

Table V.2 Germination of *Derbesia* spores under a range of culture conditions.

A full explanation of the differences in the germination percentages at the different temperatures cannot be attempted without further information of the growth and development of the *Halicystis* stage in nature.

At no stage in this study have any spores of the fine species of *Derbesia* been observed to produce a *Bryopsis*-like gametophyte. It is therefore concluded that there is no direct link between the life histories of *Derbesia marina* and the south eastern Australian species of *Bryopsis*.
As the formation of a calcified attachment stage in the germination of the spores of *Pedobesia clavaeformis* (MacRaild and Womersley 1974) occurred particularly when spores were maintained under constantly moving culture conditions, some spores of *Derbesia* were also grown under moving conditions. No similar calcified attachment stage develops from the *Derbesia* spores, all spores germinate to produce the typical *Halicystis*-like plant.

Germination studies were also undertaken to determine the possibility of parthenogenetic development of the gametes of *Halicystis*. Large numbers of both male and female gametes were isolated into culture dishes and their development followed. At no stage in this study were nonfertilised gametes observed to germinate. Female gametes may survive in culture for eight to ten days, but do not germinate even to the extent of the nonfertilised *Bryopsis* female gametes (see section III.3). Male gametes disintegrate within 24hrs of release from the gametangial area.

V. 4-3 Shading experiment.

Ernst (1904) claimed that the presence of a pyrenoid in the chloroplasts of *D. tenuissima* and *D. lamourouxi* was dependent on the degree of shading and the depth at which the plants were growing (see section V.2). While some evidence suggests that de novo formation of pyrenoids is possible in *Oedogonium* (Hoffman 1968), no recent work has either proved or disproved the possibility of environmental control of pyrenoid production. Therefore culture experiments were designed to investigate the importance of shading to pyrenoid production in the southern Australian species of *Derbesia*.

Young germlings (three days old) obtained from *Halicystis* gametes, were placed in culture dishes at 20°C under a range of light intensities from 1500 to 100lx. Very low light intensities were obtained by shading the culture vessels with layers of fibre-glass fly-wire screening. Three to four week old plants were also grown under the same conditions in an attempt to compare the responses of young and older plants.

At light intensities of 1000 to 1500lx, there was no evidence of change in either plastid morphology or the overall growth rate of the
plants. Below 1000lx the young plants showed only slow growth, with very little accumulation of starch in the plastids, either as free starch grains or as plates around the pyrenoid. After three to four weeks at this light intensity, older plants had only very small starch plates around the pyrenoid and no free starch grains in the chloroplasts.

Light intensities below 500lx resulted in the death of the young germlings after four weeks and negligible growth of the more mature plants, which did however, survive for up to ten weeks (termination of experiment) in an apparently healthy condition. Examination of these mature plants which had been shaded for ten weeks showed that the pyrenoid, although present within the chloroplasts, was relatively inconspicuous because of the absence of a starch sheath.

These observations indicate that the presence and absence of the pyrenoid in Derbesia marina is a characteristic which is not modified by environmental conditions. This supports the view that two sympatric species are found in the south eastern Australian region. However, without further cross-fertilisation studies and comparative cytological investigations which were outside the scope of the present thesis, this cannot be confirmed.

V. 5 Cytological investigation of Derbesia.

The cytoplasmic organisation of Derbesia is very similar to that of Bryopsis (see section IV.3), with chloroplasts occupying the inner region, lining the vacuolar membrane. The chloroplasts are lenticular, about 12μm long and 6μm wide, and possess one polypyrmaidal pyrenoid. The nuclei are numerous within the thallus and have several irregular nucleolar-like inclusions. Chromosomes at metaphase have been seen only once in the Derbesia stage and a tentative chromosome number of 2n=16 is given. No electron microscope investigation of Derbesia has been undertaken.

In summarizing the work of Rietema (1969 to 1972), van den Hoek et al (1972) suggest that the life histories of Derbesia and Bryopsis can be interpreted as showing a gradual reduction in the sporophytic generation. It is argued that the uninucleate protonema giving rise directly to the gametophytic Bryopsis plant (Neumann 1969a) is homologous with the sporophytic protonema of other Bryopsis life histories (Rietema 1969 to 1972) and the typical Derbesia-like sporophytes
of *Bryopsis halymeniae* and *Halicystis*. On this basis, van den Hoek *et al* (1972) also suggest that the order Derbesiales is no longer a useful taxonomic group and that *Derbesia* should be placed with *Bryopsis* in the Codiales.

In doing this, van den Hoek *et al* (1972) do not consider the important differences between the protonemal sporophyte of *Bryopsis*, and the typical *Derbesia*. The *Bryopsis* protonema is uninucleate for a large part of its life, possessing a giant nucleus (Neumann 1969a). The only other green algae which have a similar giant nucleus are *Pseudobryopsis* (Neumann 1970) and members of the Dasycladales (Puiseux-Dao 1966, Valet 1969). No typical *Derbesia* has yet been found with a uninucleate phase in the life history.

An additional study was undertaken of the south eastern Australian *Derbesia* in connection with the fate of the nuclei through the life history, to check the proposals of van den Hoek *et al* (1972). As initial studies with the differential interference microscope (Nomarski) failed to show the presence of a giant nucleus in the cytoplasm, both young sporophytic and gametophytic plants (one to two days after zygote formation or spore release) were fixed in B.A.C. fixative and stained with propionic acid haematoxylin as described in section IV.2-1.

The normal vegetative nuclei of *Derbesia* are spherical to spindle shaped (6 μm long and 4 μm wide) and have one to several densely staining regions in the nucleoplasm. These are interpreted as nucleoli and in this respect *Derbesia* nuclei are different from *Bryopsis*, which has only one, regular shaped nucleolus which divides during nuclear division. In *Derbesia* the nucleoli disappear through mitotic division but the nuclear membrane persists.

Davis (1908) describes the spores of *D. lamourouxii* as uninucleate at the time of release, while Neumann (1969b) studying *D. marina*, found them to be multinucleate. The nuclear state of the motile spores of southern Australian species of *Derbesia* has not been studied, but the spores have been observed to be multinucleate within 4hrs of settling to the bottom of the culture vessel, before germination.

As the spores germinate, the nuclei undergo rapid mitotic division, thus producing the typical multinucleate state of the *Halicystis* plant. The size of the nuclei of the developing gametophyte
is the same as described for the Derbesia plants. Unlike Bryopsis, the Derbesia zygote nucleus does not enlarge as the zygote germinates, but divides numerous times, producing a multinucleate thallus.

This pattern of nuclear activity in Derbesia is similar to that described by Neumann (1969b). No uninucleate stage with a giant nucleus is seen in the Derbesia life history. It is therefore suggested on cytological grounds that the typical Derbesia alternating with Halicystis may not be directly comparable to the protonemal sporophyte of the Bryopsis life history. However, it should be recognised that Rietema (1972) has undertaken a similar cytological study of the Bryopsis halymeniae life history and does not describe a uninucleate stage in this species. It therefore appears that while the B. halymeniae life history takes an important linking position in the morphological series of van den Heok et al (1972), its pattern of nuclear activity is much closer to the typical Derbesia-Halicystis life history, than to the species of Bryopsis which alternate with a uninucleate protonema.
SECTION VI

PEDOBESIA
VI. 1 Introduction.

The study of Pedobesia clavaeformis (J.Agardh) MacRaild et Womersley (Bryopsis clavaeformis J. Agardh 1887 p.20) was initially begun as part of the investigation of the Derbesia life histories and their association with Bryopsis. However, as the work progressed it became obvious that while P.clavaeformis was not involved directly with the Bryopsis life histories, its own life history required detailed investigation and description, being radically different from that of any other member of the Derbesiales or Codiales. For this reason the scope of this thesis was expanded to include a detailed life history and cytological study of P.clavaeformis.

Pedobesia clavaeformis was first described as a species of Bryopsis by J. Agardh (1887 p.20) and was later transferred to Derbesia by De Toni (1889 p.427). It is based on a specimen (LD herb. Agardh No. 14897) collected by J.B. Wilson from Western Port Bay, Victoria. A life history study of this species (MacRaild and Womersley 1974, included in this thesis as appendix 1) revealed certain characteristics which are unlike Derbesia, and it was proposed that a new genus Pedobesia be recognised, based on Bryopsis clavaeformis. A detailed description of this species and its life history is given in MacRaild and Womersley (1974).

Pedobesia is characterised by a distinctive calcified attachment stage, and a direct life history in which no gametophyte is known. The reproductive cells are stephanokont spores which are formed in large sporangia borne on the clavate branches of the thallus, a short distance from the apex.

With the production of Derbesia-like spores, the simple siphonous growth form of the clavate thallus and the presence of only one type of plastid (see section VI. 2-1), Pedobesia is considered to be closely related to both Derbesia and Bryopsis, and is retained within the Derbesiales (MacRaild and Womersley 1974).
VI. 2 The cytology of Pedobesia clavaeformis

The techniques used in this study of Pedobesia are as outlined in section IV. 2.

VI. 2-1 Cytology of the clavate thallus.

Pedobesia clavaeformis grows as dense clusters of elongate-clavate branches arising from the slender much branched rhizoidal base. The cell wall is smooth, 1.5 to 2.5μm thick, without projections (trabeculae) into the cytoplasm as seen in Caulerpa (Fritsch 1945). Good electron micrographs of the cell wall are difficult to obtain as sections of the wall material have, up to the time of writing, shown pronounced chatter, and after lead citrate staining appear very electron dense. Some unstained preparations have been used showing the cell wall to be composed of fibrillar to granular material. An outer region showing slightly higher electron density may be interpreted as a cuticle (Hanic and Craigie 1969) but this is not as distinct or as well developed as in Bryopsis (see section IV.3). No further differentiation of the wall material into layers has been observed in either light or electron microscope preparations.

The cytoplasm of the clavate branches occurs in a thin layer between the cell wall and the large central vacuole (Fig. VI.1) and the organisation within the cytoplasm is similar to that of Bryopsis (Burr and West 1970, present thesis). The chloroplasts occupy an internal position closest to the tonoplast, while the region of cytoplasm adjacent to the cell wall is occupied by mitochondria, nuclei, dictyosomes, ribosomes and endoplasmic reticulum. This organisation of the cytoplasm of Pedobesia refutes Zinnecker's (1935) claim that chloroplasts occupying a position internal to the other cytoplasmic organelles is an arrangement unique to Bryopsis.

When whole plants are viewed under a dissecting microscope, the chloroplasts are often seen to be concentrated in lines running parallel with the long axis of the branches. At higher magnification, these lines are seen to correspond to cytoplasmic streams which appear to maintain fixed polarity, adjacent streams often flowing in opposite directions. Transverse sections through well fixed specimens show that the lines representing cytoplasmic streams are regions in which
there may be more than one layer of chloroplasts while the other organelles are evenly distributed. From both light microscopy of living plants and the fine structural organisation within the cytoplasmic streams, it appears that as in Bryopsis (Burr and West 1970), streaming only occurs in the inner layer of cytoplasm containing chloroplasts. Microtubules seen in the cytoplasmic streams of Caulerpa (Sabnis and Jacobs 1967, Dawes and Barilotti 1969) have not yet been observed in Pedobesia, but preservation of these may depend on fixation under different conditions than have been used for the Pedobesia specimens.

The chloroplasts of Pedobesia are lenticular to ovoid, 2 to 5\(\mu\)m long and 1\(\mu\)m wide. They may have one to three starch grains and occasional osmiophilic globules in the stroma, but no pyrenoid (Fig. VI.2). From transverse sections of young filaments the chloroplasts appear to be regularly arranged with their long axes perpendicular to the cell wall (Fig. VI.3). However, in older parts of the thallus, and in all mature plants, the chloroplasts are realigned with their long axes parallel with the longitudinal axis of the thallus.

Individual chloroplasts are traversed by four to five bands of three to six thylakoids, each band stretching from one end of the chloroplast to the other (Fig. VI.2). At each end of the plastid the bands may become closely appressed, or remain as distinct entities. Interchange of thylakoids between bands and the splitting of bands is also noted. However, the number of thylakoids in each band is much more uniform than is observed in Bryopsis (see section IV.3).

MacRaild and Womersley (1974) describe a calcified attachment stage in the life history of P. clavaeformis. As other calcified members of the siphonous green algae belonging to the Caulerpales are heteroplastic (Feldmann 1954), a detailed electron microscope study was undertaken to determine whether Pedobesia is in fact homoplastic, as seen with the light microscope (MacRaild and Womersley 1974). In numerous sections of rhizoidal and clavate parts of plants collected from Pt. Lonsdale and Cape Patterson, and material grown through several generations in culture, there have been no structures identified which compare with leucoplasts as seen in members of the Caulerpales (Hori and Ueda 1967, Sabnis 1969).
Fig. VI.1  Transmission electron micrograph (TEM). Transverse section through clavate branch of *P. clavaeformis* showing cytoplasm lining cell wall. Tonoplast disrupted during fixation. Ingression of cell wall may have been caused by damage to the plant.

Fig. VI.2  TEM Chloroplasts of *P. clavaeformis*. Note prominent starch grains and osmiophilic globules.
The chloroplasts of *P. clavaeformis* are also noted to differ from those of the Dasycladales (Hori and Ueda 1967), the only other order containing calcified siphonous green algae. *Pedobesia* chloroplasts have neither grana-like accumulations nor evenly dispersed thylakoids, which are the only types of plastid organisation seen in that order. The absence of free starch grains from the cytoplasm of *Pedobesia* is also significant in comparison with the Dasycladales. The chloroplasts of *Pedobesia* compare most closely with those of *Derbesia lamourouxii*, the only member of the Derbesiales investigated in their survey of the fine structure of the plastids of the siphonous green algae, published by Hori and Ueda (1967).

The nuclei of *Pedobesia* are seen from light and electron microscopy, to be disc-shaped (Fig. VI.4), 2 to 3μm in diameter, and bounded by a double membrane, in which pores (500Å diameter) are seen. The outer layer of the membrane is covered with ribosomes, although these are not as heavily concentrated as in *Bryopsis*. When stained with acetocarmine or propionic acid haematoxylin (see section IV. 2-1), one to several irregular bodies are seen in the interphase nuclei (Fig. VI.4). Under the transmission electron microscope (Fig. VI.19) these appear to correspond to granular regions which are identical in structure with the single nucleolus of *Bryopsis* nuclei. The nuclei of *Pedobesia*, with several nucleoli, closely resemble those of *Derbesia*.

The nuclear membrane is persistent throughout the normal mitotic division sequence in the vegetative plant. As observed in *Bryopsis* and *Derbesia* the nuclei divide asynchronously throughout the plant, and division stages are not more frequently observed at the growing apex than elsewhere. The normal chromosome number seen from polar view of mitotic metaphase nuclei is 2n=10.

Neumann (1969b) found that meiosis occurs in the developing sporangium of *Derbesia marina*. A brief study was undertaken to determine the type of division occurring in the sporangium of *Pedobesia*. In a survey of twenty-five young sporangia at different stages of development between separation of the sporangial mass from the cytoplasm of the thallus and segregation of the cytoplasm into individual spores, no meiotic stages of nuclear division were seen. However, as no gametophytic stage has been discovered in the life history of *P. clavaeformis*.
Fig. VI.3 TEM Clavate branch of *P. clavaeformis*. Note chloroplasts perpendicular to cell wall and outer layer of cytoplasm with nucleus and mitochondria. EM plate 27378

Fig. VI.4 TEM Nucleus and mitochondria. Inset. Light micrograph (LM) Nuclei stained with propionic acid haematoxylin. Note densely staining nucleoli. EM plate 27380
a more complete examination of the nuclear activity is needed before
the life history is fully understood.

One major difference between the cytoplasm of *Pedobesia* and that
of the south eastern Australian *Bryopsis*, is the presence of an organelle
(fig. VI.5) which forms in the cytoplasm of *Pedobesia* and is released
into the vacuole where it appears to be involved in the development of
crystalline protein bodies (see section VI. 2-2). While in the cytoplasm,
these organelles are membrane bound and have a fine granular matrix
enclosing irregularly shaped areas of electron dense material. In
some transverse sections the main spherical body of the organelle (up to
0.5 μm diameter) is seen to have an electron dense cap attached to it,
within the membrane. Serial sections through a group of spherical bodies
have shown that each has a hemispherical cap which can only be observed
attached to the sphere when the complete organelle is cut in
longitudinal section.

These spherical bodies are unlike any other organelle described
for the siphonous green algae. In their sequence of development and release
into the vacuole they show some similarities with the protein bodies
described in *Bryopsis* by Burr and West (1971a). However, as their
structural organisation is quite different from those of *Bryopsis*, no
direct comparison can be made.

VI. 2-2 Vacuolar inclusions of the clavate thallus.

Ernst (1904) described a number of crystalline structures in the
vacuole of species of *Derbesia*. He also found a range of other vacuolar
inclusions all of which correspond with the proteinaceous materials
found in the present study of *Bryopsis* (see section IV. 5). In
*Pedobesia clavaeformis* the only vacuolar inclusions to have been
observed are octahedral crystals, the small spherical organelles
described above and fine granular to fibrillar material.

The crystals have been studied with conventional light and
electron microscope techniques (see section IV.2). However, valuable
information on the shape and symmetry of the crystals was obtained with
the scanning electron microscope, after tissues had been fixed in
glutaraldehyde and partially embedded in Spurr's low viscosity embedding
medium (Chambers and Hamilton 1973). Following the removal of excess
Spurr's from the tissue and before final polymerization, the cell wall
Fig. VI.5 TEM Membrane-bound spherical body in the cytoplasm of *P. clavaeformis*. Note uneven granular matrix and densely staining hemispherical cap. EM plate 27383
of the tubular plant material was cut open with a new razor blade and laid flat on the scanning electron microscope stub, with cytoplasm uppermost. The Spurr's was further polymerised for 24hrs at 70°C and the stub coated with a thin layer of gold. In this way a view was obtained of the vacuolar materials more or less in situ. If care is taken while cutting the cell wall little disruption of the vacuolar material occurs.

Large numbers of crystals are observed with sides ranging from 0.25 to 4μm long (Fig. VI.6). Analysis of numerous micrographs of the crystals shows that they can be referred to the tetragonal system of crystal symmetry, having two equal horizontal axes at right angles to one another and a third unequal vertical axis at right angles to the horizontal plane (Fig. VI.7). The system of symmetry can be summarised as follows: 5 planes of symmetry, 3 axial (1 horizontal, 2 vertical), and 2 diagonal vertical; 5 axes of symmetry, 1 axis of four-fold symmetry corresponding with the vertical crystallographic axis and 4 axes of two-fold symmetry corresponding with the horizontal crystallographic axes and the lines bisecting the angles between them.

Thin sections of *P. clavaeformis* for transmission electron microscopy show polymorphic, rectilinear, electron dense structures in the vacuole (Fig. VI.8). At high magnification, these are seen to possess a regular lattice structure with a point to point spacing of approximately 150Å. These centres forming the lattice appear to be spherical or microtubular (approximately 50Å diameter cross section, Fig. VI.8), with an electron translucent interior surrounded by electron dense material. As this fine structural organisation compares with proteinaceous crystalline materials found in other plant and animal tissues (Man and Meiners 1962, Inmann and Cooper 1963, Thornton and Thimann 1964, McBride and Cole 1972, Ootaki and Wolken 1973), it was concluded that these structures represent sections through the crystals seen with the light and scanning electron microscopes.

The crystals and their formation are intimately associated with the cytoplasmic organelle already described, which is released from the cytoplasm into the vacuole. In the vacuole these begin to lose their distinct outline and become surrounded by fine granular material and stain less intensely (Fig. VI.9). Large numbers of these are seen around groups of crystals (Figs. VI.10, VI.11) with both the scanning and transmission electron microscopes. The only other structures seen
Fig. VI.6 Scanning electron micrograph (SEM) Protein crystalloids in *P. clavaeformis*. Chloroplasts and cytoplasm seen in the background. SEM plate 2266

Fig. VI.7 Diagram of octahedral protein crystalloid shown in Fig. VI.6 showing intercepts on the crystallographic axes a, b, c.
Fig. VI.8 TEM Sections of crystals in the vacuole of the clavate branches of P. clavaeformis. Inset. Highly magnified view of crystal lattice structure showing small circular or tubular centres.

EM plate 27365
Inset 27407
Fig. VI.9 TEM Spherical bodies in the vacuole of the clavate branch of \textit{P. clavaeformis}. Note some breakdown, to produce fine, granular matrix. EM plate 27358

Fig. VI.10 TEM Centre at which crystallisation is occurring. Note numerous small crystal lattices at different angles and sections of spherical bodies. EM plate 27368
in the vacuole are rather dispersed masses of granular to fibrillar material, which is thought to be derived from the decomposition of the spherical structures. Both these and the decomposing spherical bodies are always found in close association with centres at which new crystal lattices are forming (Figs. VI.10, VI.12) and are often found enclosed within the crystalline structure (Fig. VI.13).

All crystals observed have small electron dense areas included in the lattice structure (Fig. VI.14). Similar inclusions are seen in the fibrillar-granular material, further indicating that this and the crystals are of similar composition, the latter perhaps being a more highly organised arrangement of the former.

As the crystals in the vacuole of P. clavaeformis stain intensely with the general cytological stains such as toluidine blue and the protein specific Coomassie Brilliant Blue (Fisher 1968), it is concluded that these are composed of proteinaceous material. This is supported by the removal of crystals from sections treated under controlled conditions with a 0.5% solution of Pronase at 50°C. It can also be assumed that the organelles from which the crystals are derived must also be of proteinaceous nature. These would then compare in composition with the protein bodies described by Burr and West (1971a) from Bryopsis, although their structural organisation is considerably different.

Ernst (1904) notes that the crystals found in the vacuoles of different species of Derbesia are not all proteinaceous. In Derbesia tenuissima he has found calcium oxalate crystals which are relatively large tetragonal prisms and combinations of tetragonal prisms and pyramids. As all attempts to obtain electron diffraction patterns from sections of the crystals of Pedobesia were unsuccessful, it is concluded that no crystals of mineral composition occur in this alga. This is also supported by the failure of sodium EDTA solution to affect sections of these crystals.

The function of the proteinaceous crystals is unknown. In Bryopsis there are protein materials which are involved in a complex wound healing mechanism (Burr and West 1971a, Burr and Evert 1972). These have microtubular or fibrillar fine structure and are thought to perform some clotting function at the site of wounding. Unless the crystalline protein of Pedobesia is dissolved rapidly when the plant
Fig. VI.11  SEM Protein crystalloid and associated spherical bodies.
SEM 2254

Fig. VI.12  TEM Small crystal with three spherical bodies associated with crystallisation.
EM plate 27369
Fig. VI.13 TEM Large protein crystalloid with spherical bodies and granular matrix incorporated within crystal structure.

EM plate 27432

Fig. VI.14 TEM Large crystal with densely staining bodies incorporated in lattice structure. Note similar bodies in surrounding granular matrix.

EM plate 27340
is damaged it is difficult to envisage a similar spontaneous wound healing function for these. It should be noted however, that a wound healing phenomenon is employed when vegetative cultures of this alga are established. Small pieces of the thallus can be cut from the plant and a new individual established from this propagule. The mechanism of this wound healing is unknown, but by analogy with *Bryopsis*, the vacuolar protein may be expected to be involved (see also section VII.2-3).

VI. 2-3 The spores of *Pedobesia clavaeformis*.

A general description of the sporangia and spores produced within these is given in MacRaild and Womersley (1974). Sporangia are produced in large numbers on each plant throughout the growth season, with up to three at different stages of development on each clavate branch. Mature sporangia are dark green to almost black in colour, having up to 500 spores, each with a large number of densely packed chloroplasts.

The sporangia begin as small buds (Fig. VI.15) which develop laterally on the clavate branches, a short distance below the apex. As they grow out from the branch they enlarge, becoming spherical to ovoid, with a diameter of 350 to 1000µm. The sporangia are not stalked, but there is a constriction at their point of divergence from the branch, and at this point a gelatinous septum develops approximately 70µm in diameter, isolating the cytoplasm of the sporangium (Fig. VI.16).

As the sporangium enlarges before the septum is formed the contents begin to appear a darker green and gradually become almost black. This is due to large amounts of cytoplasm migrating from the branch into the sporangium which appears to have no central vacuole. However, the cytoplasm of the young sporangium has numerous finger-like vacuolar projections (Fig. VI.17), which appear in serial section to be interconnected and derived from the vacuole of the clavate branch. These vacuolar ingressions are not seen in the mature sporangium and whether these can be considered as cleavage vesicles involved in the segregation of the cytoplasm into spores in unknown.

Nuclei which migrate from the cytoplasm of the clavate branch occupy a central position within the sporangium (Fig. VI.17). Here they
Fig. VI.15 LM Young sporangium forming on clavate branch.

Fig. VI.16 Mature sporangium with fully developed spores. Note septum separating sporangium from the clavate branch.
may undergo numerous mitotic divisions, increasing the number of potential spore nuclei. During nuclear division the chloroplasts tend to segregate towards the peripheral layers of cytoplasm.

The septum which forms isolating the sporangium from the branch is very similar to those which develop in the gametangium of *Bryopsis* and sporangium of *Derbesia*. Soon after this forms the cytoplasm begins to segregate into small spores (30 to 40μm in diameter, Fig. VI.18). Each spore has only one lobed nucleus which is found at the centre of the spore surrounded by a large number of chloroplasts, mitochondria and other organelles (Figs. VI.19, VI.20). Crystals, similar to those found in the clavate thallus have also been found in mature spores (Fig. VI.19).

Davis (1908) describes the spores of *Derbesia lamourouxii* as uninucleate at the time of release, while Neumann (1969b) describes those of *D. marina* as multinucleate. In this respect the spores of *P. clavaeformis* closely resemble those of *D. lamourouxii* as they become multinucleate only after germination.

The spores are typical of those of all species of *Derbesia*, having a characteristic ring of flagella. A hoop-shaped blepharoblast has been observed with the light microscope (Fig. VI.21), appearing as a double ring-shaped structure to which the flagella are attached in the depression between the two outer edges. This blepharoblast has been seen intact on disintegration of the spores in distilled water, and appears to be identical with those described by Davis (1908) and Neumann (1969b).

Attempts were made to observe this structure with the scanning electron microscope but proved unsuccessful. However, scanning electron microscopy of the sporangia broken open after infiltration and polymerization of the embedding medium (Chambers and Hamilton 1973), did show the relationship between the spores and their flagella when still contained within the sporangium (Fig. VI.22). In this state the flagella are attached in a ring (10 to 15μm diameter) and lie in an ordered spiral arrangement around the outside of the spore (Fig. VI.23). This description of the arrangement of flagella of spores within the sporangium is similar to that made by Neumann (1969b).
Fig. VI.17 TEM Young sporangium with numerous nuclei in central region of spore cytoplasm. Note very few chloroplasts and numerous vacuolar inclusions.

Fig. VI.18 Nomarski LM Mature sporangium. Note central nucleus in each spore, surrounded by numerous chloroplasts.
Fig. VI.19 TEM Mature sporangium, with spores surrounded by flagella. Note single nucleus and nucleoli and chloroplasts packed with starch grains. Knife marks through spore indicate direction of cutting and show little compression of tissue. Inset. Crystal seen included in spore. EM plate 27261 Inset 27265

Fig. VI.20 TEM Spore nucleus. Note double membrane with pores (arrow) and ribosomes attached to outer surface. EM plate 27220
Fig. VI.21 Drawing of the blepharoblast from spore of *P. clavaeformis* with attached flagella.

Fig. VI.22 SEM Broken sporangium with fully formed spores.
Fig. VI.23 SEM Single spore in sporangium. Note attached ring of flagella lying in ordered spiral arrangement around spore.

SEM 2159
Transmission electron micrographs of sporangia with fully developed spores, show that the flagella are typical of isokont motile cells, with the characteristic 9+2 arrangement of microfibrils and lacking flagellar appendages (Fig. VI.24). At the point at which the flagella emerge from the cell, the two central strands are not present, but their place is taken by a central structure which in transverse section appears as nine granules arranged in a circle of 350 to 390Å diameter, from which nine triangular units radiate with their apices pointing to the outer double fibres (Fig. VI.25). In longitudinal section this structure is seen as a row of six units stacked on top of each other, the lowest one at the level at which the flagellum enters the cell body (Fig. VI.26).

Once the nine double outer fibres enter the cell, their orientation changes so that they appear to be tilted radially, giving a type of spiral asymmetry. With this change in orientation, a third fibril is added to each of the nine double outer strands (Fig. VI.27), as described by Gibbons and Grimstone (1960). However, sections showing this pattern are not frequently obtained as this region of the flagellar base is embedded in an electron dense material, obscuring the fibrils in all but very thin sections.

At the base of the flagellar root a cartwheel-like structure is seen (Fig. VI.28). This has a short, longitudinal, tubular body with a central strand or row of granules. From the tube, nine straight radiating fibrils appear, attached at their outer end to an electron dense granule. There is no evidence of these fibrils attaching to the triplet strands of outer fibrils (compare with Gibbons and Grimstone 1960), but the presence of an electron dense outer matrix partially obscures the organisation of the fibrils at their extremities.

A diagrammatic representation of the flagella structure, showing both longitudinal and transverse sections is given in Fig. VI.31. From this it can be seen that the structure and organisation of the flagellar base of \textit{P.clavaeformis} is similar to that described by Gibbons and Grimstone (1960). However, where the flagella emerge from the \textit{P.clavaeformis} spore, a stellate structure is observed which was not reported by these authors, but which is considered typical within the green algae and possibly all plants containing chlorophyll b (Manton 1965).
Fig. VI.24 TEM Transverse section of flagella outside spore.  
EM plate 27160

Fig. VI.25 TEM Transverse section of flagella just above point of attachment to spore.  
EM plate 27162
Fig. VI.26 TEM Longitudinal section of a row of flagella. Note
(a) structure at point of attachment to spore (transverse
section Fig. VI.25). (b) structure at flagellar base, arrow (transverse
section Fig. VI.28). (c) dense structure between individual
flagellar bases.

EM plate 27279
Fig. VI.27 TEM flagellar base. Note nine triplet strands of microfibrils (arrow).

EM plate 27274

Fig. VI.28 TEM Lower portion of flagellar base, showing cartwheel structure.

EM plate 27238
In this transmission electron microscope study of the spores of *Pedobesia*, little information has been gathered on the structure and organisation of the blepharoblast. Hoffman and Manton (1962) have shown a complex structure in *Oedogonium*, comprising a fibrous band making contact with each flagellar base and a "gutter-shaped mass of apparently homogeneous dense material" (Hoffman and Manton 1962 p.446). The only similar material seen in *Pedobesia*, is a band of homogeneous material which is slightly more electron dense than the background cytoplasm. In transverse section (Fig. VI.29) this is seen on one side of the row of flagella, closest to the outer membrane of the spore. At some levels projections of this band are seen to penetrate the space between the flagellar bases. In longitudinal section of a row of flagella (Fig. VI.26) the electron dense band is seen between the outer edge of the spore and the end of the outer fibrils, and the projections between the flagellar bases are seen as small electron dense regions. Longitudinal section of a single flagellum taken at right angles to the row of flagella (Fig. VI.30) shows that this electron dense band is triangular in cross section and appears to be closely associated with the outer fibrils of the flagellar base. Further description of the structure of this ring of material at the base of the flagella, and comparison with *Oedogonium* will require the use of phosphotungstic acid as a stain for transmission electron microscopy (Hoffman and Manton 1962).

VI. 2-4 Detailed cytology of the attachment stage of *Pedobesia clavaeformis*. The attachment stage of *P. clavaeformis* has been described after a light microscope study of the surface and sections through the flattened, calcified thallus (MacRaild and Womersley 1974, Fig. VI.32). From this, a three dimensional picture of the complex structure has been developed. Scanning electron micrographs (Figs. VI.33, VI.34) confirm the structural organisation described, showing the multilayered growth form, the broad convex growing margin and the radiating lines representing apposition of adjacent segments of the thallus. Concentric lines resulting from undulations in the surface, are obvious and due to differences in calcification.
Fig. VI.29 TEM Glancing section through spore, showing transverse sections of flagella at various depths of penetration into spore. Note electron dense band of material between row of flagella and outer edge of spore. EM plate 27238

Fig. VI.30 TEM Longitudinal section of single flagellum, showing transverse section of blepharoblast region. Note triangular section of electron dense material between flagella base and outer edge of spore. EM plate 27240
Fig. VI.31 Diagrammatic picture of flagella, showing both longitudinal and transverse sections. Stippled area represents electron dense regions thought to be associated with the blepharblast.
Fig. VI. 32  LM Attachment stage of *P. clavaeformis*. Note regular pores in upper calcified wall.

Fig. VI. 33  SEM Part of a multilayered attachment stage. Note layers growing over each other, broad, convex growing margin and concentric undulations on the surface. SEM 2145
Fig. VI.34 SEM *P. clavaeformis* calcified attachment stage. Note pits on surface corresponding with pores seen on fractured face. Pillars join upper and lower surface. Disrupted cytoplasm still in structure. SEM 2150
High quality transmission electron micrographs showing details of the cytoplasm of the attachment stage are not frequently obtained. Fixation procedures used appear to disrupt the normal cytoplasmic organisation and the presence of calcium carbonate makes sectioning difficult. Some micrographs show no distinct cytoplasmic organisation within the attachment stage and the apparent absence of a large central vacuole (Fig. VI.35). The more typical organisation is thought to be that shown in Fig. VI.36, with the cell wall lined with a layer of cytoplasm without chloroplasts but containing nuclei, mitochondria, ribosomes, dictyosomes and endoplasmic reticulum. Chloroplasts are seen lining the vacuolar membrane. In this organisation the cytoplasm of the attachment stage is identical with the cytoplasm of the upright clavate branches.

A full range of cytoplasmic organelles is found in the attachment stage, with the exception of the small spherical bodies described in the cytoplasm and vacuole of the clavate branches. The chloroplasts are identical with those of other stages of the life history, and the cytoplasm is typically multinucleate. Although vacuolar crystals have been observed in the clavate branches, and similar crystals seen in the cytoplasm of the spores, none have been detected in the attachment stage. The absence of spherical bodies and crystals is probably related (see section VI. 2-2).

Under the scanning electron microscope the only surface structures seen on the attachment stage are regular pits (1 μm diameter) which are seen on fractured surfaces of the upper calcium carbonate layer, to be indentations of the cell wall overlying pores (Fig. VI.34). These pit-like structures form when the thin cell wall above the pores is deformed as the tissue is freeze dried or partially embedded. Indentations have not been seen in sections of embedded material where surface tension deformation is minimised.

The pores may be described as regular discontinuities in the calcium carbonate layer of the cell wall. They are lined with the inner layer of the polysaccharide wall material and all newly formed pores are continuous with the cytoplasm. The cell wall covering each pore is less than 1 μm thick and formed by the apposition of the cell wall layers surrounding the calcium carbonate (Fig. VI.37.). A thin electron dense layer of granular material is seen between these two layers.
Fig. VI.35 TEM Transverse section through multilayered attachment stage showing cytoplasm with nuclei and chloroplasts. Upper calcified layer of wall material with pores filled with electron dense plugs. Pillar, lower right hand corner.

TEM 27326
Fig. VI. 36 TEM Transverse section of flattened stage, showing regularly organised cytoplasm with central vacuole.

EM plate 27317

Fig. VI. 37 TEM Glancing section through pore.

EM plate 27311
With the transmission electron microscope a sequence has been observed in the development of pores, from the youngest with cytoplasm intruding into them (Fig. VI.38) to older ones isolated from the cytoplasm by a membrane (Fig. VI.39). After isolation, the organelles trapped in the pores lose their regular structure and U-shaped plugs of electron dense material fill the pores (Fig. VI.35). Under the light microscope, these plugs stain intensely with toluidine blue.

Where the thallus has been cut or broken after fixation (Fig. VI.34) large pillar-like structures are seen with the scanning electron microscope, running between the upper and lower wall of each layer within the organism. The pillar-like structures can be identified in transmission electron micrographs as inward projections of the upper cell wall. They are of similar construction to the calcified cell wall, with a central column of calcium carbonate surrounded by polysaccharide material. Unlike the pores they do not form at the growing margin, but at some distance back where small areas of the upper layer begin to grow inwards through the cytoplasm and finally join with the lower calcium carbonate layer.

Sections of the growing margin of an attachment stage, decalcified with uranyl acetate (Wilbur et al. 1969) before embedding, show that the calcium carbonate is laid down within the cell wall (Fig. VI.40). The cell wall, which is generally very thin (less than 1μm), is thickened at the margin by a wedge of granular material between an electron dense outer layer and the translucent inner layer. The developing margin of the calcium carbonate appears to grow into this. It would therefore seem that calcification is occurring in some organic matrix, but decalcified sections further back from the margin show only very small amounts of fibrillar material which may represent this matrix (Fig. VI.39).

Sections obtained with a diamond knife from material which has not been decalcified, show very small rounded or polygonal crystals (300 to 500μm) of calcium carbonate (Fig. VI.38). These are not as densely compacted as indicated from the scanning electron micrographs, but this may be accounted for by disruption of the layer of crystals during sectioning.

As recorded by MacRaild and Womersley (1974), the calcium carbonate deposited in the cell wall of the attachment stage of *P. clavaeformis* is in the form of highly purified aragonite. This finding was confirmed
Fig. VI.38  TEM Section through newly formed pore. Note sections of calcium carbonate crystals.  

EM plate 27328

Fig. VI.39  TEM Transverse section through pore. Note granular material in decalcified cell wall representing organic matrix in which crystallisation occurs.  

EM plate 27334
by X-ray diffraction analysis of the calcium carbonate after it had been separated from the cytoplasm and cell wall. Separation was achieved by grinding the plant material in a 1% solution of nonionic detergent (Triton X-100), allowing the heavier fraction to settle to the bottom of the grinding tube and decanting off the supernatant. After the material had been washed five times in the detergent solution, a small amount of the calcium carbonate was examined under a microscope (magnification X500) and found to be relatively free of cytoplasm and cell wall remnants. Some of the attachment stage was also reacted with snail stomach cytozyme (Chambers 1955) and an equally pure sample of calcium carbonate was produced.

The samples produced X-ray diffraction intensity peaks at 26.3°, 27.2°, 33.2° and 45.9° when rotated through 90° with respect to the incident X-ray beam. These correspond exactly with the intensity peaks obtained with a geological sample of aragonite. As calcite has a very intense peak at 29.6°, and no significant peak was obtained from the biological sample at this angle, it is concluded that very little or no calcite is present.

"The literature repeatedly refers to 'mucilage' as the medium in the algae with which the crystals are associated, but there appears to be little evidence as to how the calcium carbonate is laid down within this ill-defined matrix... Without ultrastructural evidence, however, it is impossible to decide to what extent the cell mediates the crystallization." (Arnott and Pautard 1970 p.435). A study of the calcification of Halimeda (order Caulerpales) by Wilbur et al. (1969) shows that crystals form in the spaces between utricles and filaments. These authors claim that there is no organic matrix within the calcified region while mentioning that the initial crystal formation occurs around fibrillar projections from the cell wall.

This development is different to that described for P. clavaeformis where crystal formation occurs within cell wall material which must be considered as an organic matrix. The crystals are never formed on or extruded to the outside of the cell wall. The structure of the calcified cell wall of the attachment stage of this alga is unlike that of any other calcified green alga. Gepp and Gepp (1911) refer to pores in the "calcarius sheath" of Penicillus and Udotea. These are described as
"minute spherical bubble-like chambers in the thickness of the calcareous layer...covered by a delicate calcified pellicle, in which is a minute ostiole." From the drawings of these pores (Gepp and Gepp 1911 Fig. 182) it is obvious that they are much larger structures than the pores of Pedobesia although it is claimed that the size varies greatly between species. The ostiole in the pores of Udotea and Penicillus has no corresponding structure in Pedobesia.

Gepp and Gepp (1911) suggested that the origin of the pores of Udotea and Penicillus is associated with bubbles of oxygen derived from photosynthesis. The distribution of pores "probably corresponds with that assumed by the green chromatophores inside the filaments." (Gepp and Gepp 1911 p.6). The deposition of calcium carbonate they claim is prevented at the numerous points at which the oxygen bubbles are evolved.

The structure and regular formation of pores of P. clavaeformis at the margin of the attachment stage appears to preclude this method of development.

As the pores are linked by lines in the calcium carbonate it is suggested that pore formation is a process involving calcification of finger-like processes at the growing margin. At different stages in growth they may be wide, with the sides of adjacent fingers closely appressed, or rather narrow, with the sides of the adjacent fingers separated by a space of up to 2μm in which the pores are formed. The physiological processes governing the width of the growing finger-like projections of calcium carbonate and consequently the formation of the pores, is unknown, but does appear to be associated with neither the frequency of light/dark cycles, or in culture, the degree of agitation or aeration of the medium.

Figure VI.41 shows an apparent plate-like structure within the calcified layer of cell wall. This view was obtained using Nomarski differential interference contrast microscopy, with the instrument focused below the top surface of the attachment stage but within the upper calcified wall. At this level the pores are not clearly seen. Each platelet has an irregular shape with one rounded point at which a pillar joins the upper calcified layer. From the composite picture of the surface view and the view described above (Figs. VI.42, VI.43) it can be seen that the pores and lines between pores cannot be explained as part of the overall platelet structure of the calcification.
Fig. VI.40  TEM Transverse section through growing margin of flattened stage, showing calcification into triangular thickening of polysaccharide cell wall.  EM plate 27308

Fig. VI.41  Nomarski LM Platelet structure in upper calcium carbonate layer.
Fig. VI.42 Composite drawing showing platelets and pores in the upper calcium carbonate layer.

Fig. VI.43 Diagrammatic representation of the structure of the calcified attachment stage of *P. clavaeformis*. 
However, as this platelet organisation has not yet been observed in either transmission electron microscope sections or whole mounts in the scanning electron microscope the overall interpretation is limited. Arnott and Pautard (1970 p.376) state "The word 'calcified' is usually absent from botanical texts, probably because most present-day botanists accept soluble calcium as an essential element while dismissing insoluble calcium as a waste product, or at best, an accidental, functionless inclusion in the tissue." However, as Lewin (1962) assumes that calcification of plants in the marine environment occurs as a result of photosynthesis shifting the equilibrium of the equation

\[ \text{Ca}^{++} + 2\text{HCO}_3^- \rightleftharpoons \text{CaCO}_3 + \text{H}_2\text{O} + \text{CO}_2 \]

to the right, "the problem is not so much why only certain algae deposit \( \text{CaCO}_3 \), but why certain seaweeds do not do so." (Lewin 1962 p.463). In Pedobesia only one stage in the life history is calcified, the attachment stage. As this calcification is both organised into a complex structure and present within the cell wall rather than at the cell surface, interpretation of this as an accidental chemical deposition resulting from photosynthesis is unacceptable.

The structural complexity and the deposition of pure aragonite, the least stable crystalline form of calcium carbonate, indicates that there may be some functional role in its production. With the limited knowledge of the ecology of the only known species of this genus, little indication is given of its functional role other than a possible structural element providing mechanical support for the attachment stage of the organism.
SECTION VII

DISCUSSION AND CONCLUSIONS
VII. 1 Taxonomy of the *Bryopsis* complex.

VII. 1-1 Introduction.

As the genus *Bryopsis* is characterised by species which have a relatively simple siphonous structure, the number of morphological characteristics which can be used to describe the various species is small. Most species are distinguished on the basis of the arrangement of ramuli on the axis, the overall size, general growth habit and ecological and geographic distribution. In the initial stages of this study, these criteria were used in the description of the recognisable units within the *Bryopsis* complex from the south eastern Australian coast and six units are described (see section II. 2).

Davis and Heywood (1963 p.119) describe "good" taxonomic characters as "those that (1) are not subject to wide variation within the samples being considered; (2) do not have a high intrinsic genetic variability; (3) are not easily susceptible to environmental modification; (4) show consistency, i.e. agree with the correlations of characters existing in a natural system of classification which was constructed without their use." The confusion surrounding the southern Australian *Bryopsis* (see table II.1) appears to have arisen because the characteristics used for species descriptions are unsatisfactory taxonomic criteria.

For example, branching patterns have been shown to be variable within both *Bryopsis vestita* and *Bryopsis plumosa*. *B. vestita* was described by J. Agardh (1878, 1887) as radially branched, but mature plants which agree with the type description and type specimen of this species have been found in the present study to develop through a tetrastichously branched juvenile form. Some local populations are known to retain this tetrastichous branching throughout their growth. This variability in the branching pattern of *B. vestita* appears to have led to the confusion between the species *B. vestita* and *B. gemellipara* (Womersley 1956).

It should also be recognised that the branching pattern is a difficult taxonomic character to use, especially in mature specimens, as it can frequently only be inferred from the arrangement of scars on the axes (see section III. 3).

The size of the plant is a characteristic similarly limited in taxonomic description. Detailed analyses of natural populations may be made to determine the range of size of mature plants, however,
two plants with the same genotype growing under different conditions may be significantly different in size. For example, the large form of *Bryopsis* (section II. 2-6) is shown through culture studies to be conspecific with *B. plumosa*, the two forms being interfertile, and under the same environmental conditions reverting to the same morphological form.

On the basis of the culture and cytological studies outlined in section III and IV, the six units within the *Bryopsis* complex can be reduced to three distinct species. These are now described in combinations of characteristics, some of which have not been previously recognised. From the descriptions and the key presented in this section, identification of all the species of *Bryopsis* commonly collected from the south eastern Australian coast should be possible. However, on the basis of experimental culture work (section III. 4) there may be one species which has not yet been adequately characterised. This very fine form, collected only rarely, is always radially branched and was included previously in the section II. 2-5. Unlike the other members of this unit it retains its radial branching pattern under all culture conditions, but insufficient material has been collected to allow hybridisation studies with the other species of *Bryopsis*. As previously indicated, it is possible that this material corresponds to *Bryopsis minor* Womersley (1955), or an earlier described species to which *B. minor* will be referred as a synonym.

VII. 1-2 *Bryopsis vestita* J. Agardh 1878 p.3


Plants epilithic, with numerous axes 12 to 25cm high, arising from an extensive branched rhizoidal system. Ramuli always tetrastichously arranged on the primary axis of the juvenile plant, while either radially or tetrastichously arranged in the mature plant. Ramuli may branch, with secondary ramuli arranged either tetrastichously or radially. Primary branched ramuli do not continue to elongate as the axis grows and are therefore interpreted as branches of limited growth.
Plants dioecious, producing male (10μm long and 4μm wide) and female (13μm long and 6μm wide) gametes. After fusion of the gametes the zygote has been shown in culture, to germinate into a uninucleate, protonemal, sporophytic plant producing stephanokont spores (25μm diameter), which in turn develop into the normal Bryopsis-like gametophyte.

Occurs only on the open ocean coast in areas subject to medium to high wave action. Growth of the normal Bryopsis plant is strictly seasonal (July to December). The sporophyte is unknown in nature.

**Type locality**  Warrington, Otago, New Zealand.

**Type specimen**  LD 15373 Berggren. Isotype BM (n.n.).
Both specimens on the type sheet are mature, radially branched plants, identical with radially branched plants from the southern Australian coast.

**Southern Australian distribution**  Wilsons Promontory, Vic., to Rottnest Is., W.A. and around Tasmania and some Bass Strait islands.

VII. 1-3  *Bryopsis gemellipara* J. Agardh 1887 p.25

Epiphytic, with one to three branched axes, up to 15cm long arising from a simple rhizoidal system by which the whole plant is attached to the host. Ramuli always arranged tetrastichously on the main axis. Lower ramuli may branch with the secondary ramuli arranged either tetrastichously or radially. Primary ramuli do not continue to elongate as the main axis grows and are therefore interpreted as branches of limited growth.

Plants dioecious, producing male (7μm long and 3μm wide) and female (12μm long and 9μm wide) gametes which, after fusion have been shown in culture to produce uninucleate protonemal sporophytes. Stephanokont spores (25μm diameter) germinate to produce the normal Bryopsis-like gametophyte.
Only found on open ocean coasts in areas subject to medium to high wave action. Growth of gametophyte is seasonal, but isolated plants can be found in rock pool habitats throughout the year. The sporophyte is unknown in nature.

**Type locality** Queenscliff, Victoria.

**Type specimen** LD. Herb. Agardh No. 15150. J. Bracebridge Wilson.

Isotype MEL 26489

Although not mentioned in the type description this specimen is epiphytic.

**Southern Australian distribution** Wilsons Promontory, Vic., to Rottnest Is., W.A. and around Tasmania and some Bass Strait islands.

VII. 1-4 *Bryopsis plumosa* (Hudson) C.Agardh 1823 p. 448.


Variable species with a distichous branching pattern, at least at the apex of the branched axes. Plants generally epilithic, although some have been observed growing on other algae. Young plants appear as single axes arising from a branched rhizoidal system. Primary ramuli distichously branched, and continue to elongate, sometimes overtopping the main axis. These are therefore interpreted as branches of unlimited growth. Mature plants appear as a spherical cluster of branched axes arising from a common axis. In sheltered bays, plants to 10cm tall are found, while on the open coast plants grow to 5cm. In areas of high inorganic nutrient inflow (e.g. sewerage treatment outfall areas) plants with apparently irregular branching, grow to 25cm.

Plants generally dioecious, producing male (8μm long and 3μm wide) and female (12μm long and 5μm wide) gametes which after fusion have been shown in culture to produce uninucleate, protonemal sporophytes. Stephanokont spores (25μm diameter) germinate to produce the normal gametophyte.

Most commonly found in sheltered bays and estuaries, although open coast forms have been found. Sporophyte unknown in nature.
Type locality Exmouth, England.
Type specimen Apparently lost (Womersley 1956).
Basionym Ulva plumosa Hudson 1798 p.571
Southern Australian distribution Recorded from numerous localities between Wilsons Promontory, Vic., and southern W.A.

VII. 1-5 Key to the south eastern Australian species of *Bryopsis*.

1. Branching tetrastichous..........................2
2. Branching distichous, radial, or irregular..........................2
2. Plants epiphytic, 1 to 3 axes arising from a simple rhizoidal system..................*B. gemellipara*
3. Branching radial.................................4
3. Branching distichous to irregular...........5
4. Many axes arising from an extensively branched rhizoidal system..................*B. vestita* (in part)
4. Single axis arising from a small, slightly branched rhizoidal system............*B. minor?*
5. Small plant to 10cm, branching distichous..........................*B. plumosa* (in part)
5. Large plant to 25cm, branching lax-irregular, sometimes distichous at apex..........................*B. plumosa* (in part)

VII. 2 Comparative cytology.

VII. 2-1 The general cytology of *Bryopsis*, *Derbesia* and *Pedobesia*.

The similarity in cytological organisation of the three genera *Bryopsis*, *Derbesia* and *Pedobesia* could perhaps be expected, given the similarity in the morphological forms of these algae. In each genus the cytoplasm forms a thin lining to the tubular thallus and occupies less than 10% of the total volume of the plant. The vacuole occupies the
remainder of the volume enclosed within the smooth cell wall, which has little internal differentiation. Within the cytoplasm, the chloroplasts are the only type of plastid observed, occupying a region lining the vacuolar membrane and bounded on the outside by the cytoplasm containing all other types of organelles.

The chloroplasts of the three genera are very similar, without distinct grana-like stacks of short thylakoid membranes. However, the thylakoids are grouped in bands which run the full length or part of the length of the plastid. In all species of *Bryopsis*, the chloroplasts have one (occasionally two) polypyrimalid pyrenoids traversed by one to three perforated thylakoid membranes. In *Derbesia*, species with and without pyrenoids have been described, but where pyrenoids have been observed, they appear to be typical of the polypyrimalid type. The pyrenoids of both *Derbesia* and *Bryopsis* appear to be a fixed characteristic of the species; presence or absence depends not on environmental conditions, but the genetic make-up of the plant. *Pedobesia* is observed without pyrenoids in the plastids, but has one to three starch grains in the stroma, a characteristic also seen in the other genera.

The nuclei of *Bryopsis*, *Derbesia* and *Pedobesia* also show some similarities, with the nuclear membrane persistent throughout mitotic division. Nuclei of *Derbesia* and *Pedobesia* are most alike with one to several small, irregular nucleoli. In *Bryopsis* only one nucleolus is seen in each nucleus and it is a regular, ovoid structure which is present throughout the division sequence of the nucleus. The nucleoli of *Bryopsis* divide during anaphase, while in the other two genera, they disintegrate and reform after nuclear division is complete. The chromosome numbers of all species studied have been found, but it is not known whether they are haploid or diploid numbers, as the place of meiosis has not yet been established.

In the sporophytic phases, the cytoplasmic organisation of *Bryopsis* is quite different from that of *Derbesia* and *Pedobesia*. The *Bryopsis* sporophytic stage is uninucleate until immediately prior to the production of spores and the giant nucleus of this stage cannot be compared with the small vegetative nuclei of the gametophytic phase or the *Derbesia* or *Pedobesia* sporophyte. This difference between the genera is considered to be most important and will be discussed further in section VII.4.
In summary, the cytoplasmic organisation of the three genera involved in this study shows essential similarities, but the presence of a uninucleate stage in *Bryopsis*, in which the nucleus enlarges to up to eight times the size of the nuclei of the gametophytic thallus, is considered to be a significant difference.

VII. 2-2 The place of meiosis in the *Bryopsis* life history.

The life history of *Bryopsis* was first described by Thuret (1850) and later by Schussnig (1932) and Zinnecker (1935), who showed that the gametes (or "zoospores" Thuret 1850) germinate under laboratory conditions to produce a fine, filamentous growth form. This filamentous form was thought to grow directly into the typical, upright, branched fronds, but the transition and development was not observed by any of the early workers. Neumann (1969a) confirmed the supposition of these authors when he grew *Bryopsis hypnoides* through several successive generations, finding a uninucleate protonemal stage (equivalent to the filamentous growth form) which in his populations gave rise directly to the next generation gametophyte.

Zinnecker (1935) first obtained evidence for the occurrence of meiosis in the series of nuclear divisions prior to gametogenesis, within the isolated ramuli of *Bryopsis*. Although this did not include observation of the reduction of the chromosome number, the division sequence of the nucleus was interpreted as meiotic. Neumann (1969a) observed reduction in the number of chromosomes in the developing gametangia of *B. hypnoides*, with a change from $2n=16$ to $n=8$, but showed no details of the division sequence leading to this. These results were consistent with the monophasic, diplontic life history thought to be typical of the genus *Bryopsis*.

Rietema, in a series of papers (1969 to 1972) has shown that the life history is more complex than this. In some populations he has observed that the uninucleate protonema produces stephanokont spores from which the next generation of gametophytic plants develop. However, other populations of the same species show only a monophasic life history, in which the uninucleate protonemal stage produces the gametophytic plant directly, by a process of budding as shown by Neumann (1969a).
Largely on the basis of the observation that spores produced by a single protonemal sporophyte of *Bryopsis* give rise to approximately equal numbers of male and female plants, Rietema (1969) followed by van den Hoek et al. (1972) suggest that meiosis must occur in the sporophyte. In supporting this van den Hoek et al. (1972) criticise the findings of Schussnig (1932) and Zinnecker (1935), claiming that although their drawings are characteristic of meiosis, it is unlikely that they could have observed such detail given the size of the nuclei and the number of other stained organelles present. Rietema (1972 p.446) concurs with van den Hoek et al. (1972) arguing also that "Neumann (1969a) recently adopted Zinnecker's point of view with regard to *B. hypnoides* without, however, providing new evidence." However, it must be noted here that Neumann (1969a p.53-54) states "Die Chromosomenzahl konnte in vegetativen Thallusspitzen bestimmt werden, wo zuweilen Metaphaseplatten in Polansicht zu finden sind, während sich sonst die Teilungsspindeln nur in der Längsrichtung der Thalli orientieren. Die gleiche Chromosomenzahl von 2n=16 lässt sich auch in prämeiotischen Mitosen vor der Gametenbildung in den Fiedern feststellen. Nach der Melose, die bei der grossen Menge der Kernteilungen in den Gametangien schwer zu identifizieren ist, weil sie nicht synchron verläuft, folgen weitere Mitosen mit n=8 Chromosomen."

The criticisms of van den Hoek et al. (1972) and Rietema (1972) are unfounded, as there is adequate karyological evidence for the existence of meiosis in the developing gametangia of some species or populations of *Bryopsis*. Evidence supporting the occurrence of meiosis in the sporophyte is indirect and relies on the assumption that the sex determining mechanism in *Bryopsis* involves a simple segregation of sex chromosomes at meiosis. This type of mechanism may be employed in the dioecious species, but the evidence of both monoecious and dioecious character in *B. plumosa* (West 1969), suggests that at least within this species, a much more complex form of sex determination occurs.

The difference in the life history of populations of European species of *Bryopsis* also suggests that meiosis and the accompanying sex determining mechanism may not occur in the protonema. Presumably, in populations of dioecious species showing a monophasic life history, sexuality is determined at the time of zygote formation and the nuclear
divisions which occur in the protonema and the developing branched axes will not affect this.

In further support of their hypothesis, Rietema (1972) and van den Hoek et al. (1972) argue by analogy with the genus Derbesia, that meiosis must occur in the sporophytic protonema of Bryopsis. In both D. marina, which alternates with H. ovalis (Neumann 1969b), and Derbesia neglecta which alternates with Bryopsis halymeniae (Rietema 1972), meiosis has been observed to occur in the developing sporangia. However, the analogy is based on an equivalence of the sporophytic stages, Derbesia and the Bryopsis protonema, that has yet to be shown convincingly. Although there is marked similarity in the type of spores produced, the phases of nuclear activity are quite dissimilar. No uninucleate stage with a giant nucleus has yet been seen in Derbesia.

The site of meiosis in the life history of Bryopsis therefore remains uncertain and the results of the present studies produced no further evidence to clarify the problem. The weight of the combined evidence of Schussnig (1932), Zinnecker (1935) and Neumann (1969a) obtained from karyological studies, tends to support the hypothesis that meiosis occurs during the sequence of nuclear divisions prior to gametogenesis. On the other hand, the indirect evidence of Rietema (1969, 1970, 1971b, 1972) indicates that this interpretation is only valid for some species or populations of Bryopsis.

VII. 2-3 The vacuolar protein bodies of Bryopsis, Derbesia and Pedobesia.

In the early literature on the genera Bryopsis and Derbesia, there are numerous reports of observations of vacuolar inclusions. (Klemm 1894, Golenkin 1894, Küster 1899, Noll 1899, Ernst 1904). The range of structures of these inclusions is comparable between the genera but not all forms were seen in all species. These vacuolar materials were described as calcium oxalate or protein crystalloids, protein spherocrystals and proteinaceous fibrous matrix. Ernst (1904) provides a detailed comparison of the various reports and discussed the origins of the protein structures. Küster (1899) and Klemm (1894) stated that they arose after the plant had been wounded, while Noll (1899) established their existence in the vacuole even before wounding had occurred.
In two recent accounts (Burr and West 1971a, Burr and Evert 1972) the fine structural organisation of these vacuolar inclusions, their composition and a sequence of development from cytoplasmic protein bodies are described. These studies are supplemented by the observations described in sections IV. 5 for *Bryopsis* and VI. 2-2 for *Pedobesia*. All the structures seen by the early workers have now been described from light, scanning and transmission electron microscopy and it is seen that the proteinaceous vacuolar materials are not produced by the breakdown of cytoplasm after wounding, but occur within the vacuole of healthy plants.

Ernst (1904) suggested that the proteins might be waste products or perhaps reserve materials. However, the highly organised fine structure and complex method of formation involving the release of cytoplasmic protein bodies into the vacuole, tends to preclude this. The wound healing function of these materials is well documented (Burr and West 1971a) and the survival value of the mechanism is not disputed.

The vacuolar protein structures observed in this group of plants may all be part of one complex system of wound healing materials which develop in one or several different ways. Burr and West (1971a) show a developmental sequence for the fibrillar-granular matrix of *Bryopsis hypnoides* but no similar pattern has been observed in the present study of *B. vestita* and *B. gemellipara*. In these species, additional elements in the protein complex are described (spherocrystals and crystalloids, section IV. 5), complicating the interpretation of the developmental sequence. It is suggested that the different proteinaceous inclusions of *B. vestita* and *B. gemellipara* are formed through at least two different pathways and are not all part of the sequential breakdown of one type of protein body as occurs in *B. hypnoides*. However, the development of the vacuolar protein crystals of *Pedobesia clavaeformis* as described in section VI. 2-2 shows superficial similarities with the mode of formation of fibrillar material of *B. hypnoides*.

The only other system of wound healing protein materials described in plants is the P-protein of the phloem tissues of higher plants. However, P-proteins are not present as definite structural bodies in healthy phloem tissue and the plugging of sieve elements by P-protein
is thought to be an artefact of fixation. In the plants investigated here the protein structures have been observed in the vacuoles of living plants and have been seen to develop in cultured plants derived from spores or zygotes and therefore known not to have incurred any damage which may induce the production of wound healing proteins. It may therefore be concluded that the proteins found in the vacuoles of these organisms are produced naturally by the plants before any wounding occurs, as a means of ensuring against permanent damage to the siphonous thallus. It is not known whether the vacuolar proteins are also involved in the process of protoplast formation.

VII. 3 A summary of the life histories of Bryopsis and related genera.

The validity of the generic distinctions between Bryopsis, Derbesia, Halicystis, Pedobesia, Pseudobryopsis, Bryopsidella and Bryobesia will now be considered. These genera are described according to their morphological form and with the exception of Bryopsidella no valid recognition is made of the pleomorphic life histories of Bryopsis, Derbesia and Halicystis. The two genera Pedobesia and Pseudobryopsis pose no problem at this stage as they are only known to have a monomorphic life history (Figs. VII. 2, VII.5).

The evidence for the existence of Bryobesia Weber-van Bosse (1911 p.26) is rather fragmentary (Taylor 1960), and an examination of the type specimen (Bryobesia johannae Weber-van Bosse) reveals little more information than is given in the type description. Bryobesia has been classified in the Derbesiales (Parker 1970) but there is no information of the life history of this genus. It is interesting to speculate however, that this naturally occurring alga may be related to the protonemal sporophyte of Bryopsis, especially given the production of spores (?) within the tubular thallus. The concept of the genus must rely on the type description and specimen, but it is stressed that life history studies on the genus Bryobesia are urgently needed.

Silva (1957) notes that there is no difficulty in choosing the correct generic name for the Derbesia-Halicystis alternation, as Derbesia (Solier 1847) predates Halicystis (Areschoug 1850). This means however that a Derbesia-like alga (sensu Solier 1847) appears in the life history of two genera Derbesia and Bryopsidella. As the current International Code of Botanical Nomenclature (1972, Article 62)
precludes the rejection of "A legitimate name...merely because it is inappropriate or disagreeable, or because another is preferable or better known, or because it has lost its original meaning."; the name Derbesia must be retained. Therefore the difficulty cannot be legally overcome by the use of the generic name Halicystis, even though this would simplify the nomenclatural and taxonomic picture.

If Bryopsidella is accepted as a valid generic concept, all the genera mentioned with the exception of Bryobesia can be described on the basis of their different life histories. These are given diagrammatically in figures VII.1 to VII.5, where the invalid generic names are used under the heading of another genus as simple descriptive terms, used strictly in the sense of their original descriptions.

Gametophyte
(Bryopsis-like 2n?)

Uninucleate
protonema (2n?)

Uninucleate protonemal
sporophyte (2n?)

Fig. VII.1 Bryopsis life history (After Neumann 1969a, Rietema 1969 to 1971b, Present thesis).

Gametophyte

Uninucleate
protonema

Fig. VII.2 Pseudobryopsis life history (After Neumann 1970).
Gametophyte
(Bryopsis-like n)

Sporophyte
(Derbesia-like 2n)

Fig. VII.3 Bryopsidella life history (After Hustede 1960, 1964, Rietema 1972).

Gametophyte
(Halicystis-like n)

Sporophyte
(Derbesia-like 2n)

Fig. VII.4 Derbesia life history (After Kornmann 1938, Feldmann 1950, Neumann 1967, 1969, Present thesis).

Fig. VII.5 Pedobesia life history (After MacRaild and Womersley 1974).
VII. 4 Classification of *Bryopsis* and related genera.

With the discovery of a sporophytic generation in the *Bryopsis* life history (Hustede 1960, Rietema 1969), the question of the relationship between the genera *Bryopsis* and *Derbesia* was first raised. In one species *B. halymeniae* the sporophyte was recognised as *D. neglecta* (Hustede 1960, 1964) while in all other species for which the life history is known, the sporophyte (if present) is a small protonemal growth which has not yet been seen in nature, or related to any other previously recognised species or genus. The direct relationship between *B. halymeniae* and *D. neglecta* prompted Feldmann (1969) to propose the genus *Bryopsidella* (nomen nudum) to separate those species of *Bryopsis* which alternate with *Derbesia* from those which were thought to possess a monophasic, diplontic life history.

It is now known that all species of *Bryopsis* studied with the exception of *B. halymeniae* produce a uninucleate protonemal stage which may act directly as a sporophytic generation, a gametophytic generation or give rise by a process of budding to the succeeding gametophyte. Using the *Bryopsidella* life history as a link between the typical *Derbesia* life history, showing an alternation of generations between *Derbesia* and *Halicystis*, and the *Bryopsis* life history, van den Hoek et al. (1972) show a gradual reduction in the sporophytic stages of these life histories. The protonemal stage which does not produce spores, is therefore interpreted as the ultimate reduction of the sporophyte. However, van den Hoek et al. (1972) claim that it is best not to use the term sporophyte for a stage that does not produce spores, but confuse their actual interpretation by describing the direct life history of *B. plumosa* from Zeeland as a "cycle diphasique heteromorphe..." (van den Hoek et al. 1972 p.57).

As corroborative evidence for their sporophytic reduction series van den Hoek et al. (1972) point out the similarities between *Derbesia* and the *Bryopsis* protonema on the one hand and *Halicystis* and *Bryopsis* on the other. Rietema (1970 to 1972) has shown from staining reactions with zinc-chlor-iodide and Congo red, and X-ray diffraction analysis of the cell walls, that the cell wall polysaccharide of the *Bryopsis* protonemal stage is composed of mannans, and the *Bryopsis* stage, xylan sub-units. These results are compared with those of
Percival and McDowell (1967) and Preston (1968) showing that Halicystis cell walls are composed of "1,3-linked xylan together with some cellulose, whereas the walls of Derbesia contain crystalline mannan, but neither cellulose nor xylan." (Rietema 1972 p.455).

On the basis of these results and their proposed sporophytic reduction series, van den Hoek et al. (1972) argue that the two genera Bryopsis and Derbesia should not be placed in separate orders (Codiales and Derbesiales respectively), but together in the Codiales. While the evidence for placing Bryopsis and Derbesia in the one order is reasonably strong, it remains unexplained why van den Hoek et al. (1972) choose to place Derbesia in the Codiales rather than placing Bryopsis in the Derbesiales. Members of the Derbesiales (sensu Feldmann 1954) are separated from the other homoplastic siphonous green algae in the Codiales (sensu Feldmann 1954) by the occurrence of an heteromorphic life history and the production of stephanokont spores by the sporophytic generation.

A character which has not yet been considered in relation to the classification of Bryopsis, is the presence of a uninucleate stage in the life history, in which the nucleus enlarges to eight times the size of the vegetative nuclei of the multinucleate thallus. Since the giant nucleus was first discovered in Bryopsis by Neumann (1969a), with the exception of the B. halymeniae life history (Rietema 1972), all the investigations on Bryopsis and Pseudobryopsis life histories have shown a protonemal stage with a giant nucleus.

The only other order within the green algae which has a similar giant nucleus is the Dasycladales. According to Round (1971 p.253) the Dasycladales "is perhaps the easiest group to define. All the genera have radial symmetry, the vegetative thallus is uninucleate with the massive nucleus in the rhizoidal portion, the multinucleate state occurs prior to reproduction, operculate cysts arise in specialised gametangia and these open to release the gametes...". The lack of radial symmetry and the vastly different mode of reproduction preclude the placing of Bryopsis in the Dasycladales (sensu Pascher 1931) despite the superficial similarity of the giant nucleus (Neumann 1969a, Burr and West 1971b).
With the production of stephanokont spores in the *Bryopsis* life history, and the similarity in cell wall biochemistry of the stages in the *Bryopsis* and *Derbesia* life histories, there appear to be reasons for placing *Bryopsis* in the Derbesiales. However, as there is no stage in the *Derbesia* life history which has a giant nucleus, *Bryopsis* and *Pseudobryopsis* may best be placed in a separate family of the Derbesiales, the Bryopsidaceae, with *Derbesia* in the Derbesiaceae. The absence of a giant nucleus in the sporophytic stage of *B. halymeniae* supports the proposal to isolate this species from *Bryopsis* by placing it in a new genus *Bryopsidella* (sensu Feldmann 1969). This would then be placed with *Derbesia*, and perhaps *Bryobesia* in the Derbesiaceae.

The other genus whose classification requires consideration, *Pedobesia*, is a homoplastic alga which produces *Derbesia*-like spores by which the sporophytic stage of the life history reproduces itself. No gametophyte is known. A calcified attachment stage, which is considered to be characteristic of this genus leads to the present problem about its classification. Several genera of calcified siphonous green algae are known and have in currently accepted classifications been placed either in the Dasycladales or Caulerpales. As the description of the Caulerpales relies largely on the heteroplastic condition, *Pedobesia* must be excluded from this group of algae on this basis. In section VI. 2-1 a comparison is made between the chloroplast fine structure of *Pedobesia* and the Dasycladales. It is noted that *Pedobesia* resembled none of the members of that order either in thylakoid distribution or the presence of free starch grains in the cytoplasm. Perhaps the most significant difference between the Dasycladales and *Pedobesia* is the absence of a giant nucleus in any stage of the *Pedobesia* life history. It therefore appears that the production of stephanokont spores and the absence of any characteristics which show affinities with the Caulerpales or Dasycladales, necessitates the placing of *Pedobesia* in the Derbesiales. However, it should be recognised that within this group it is the only member which produces a specialised accumulation of crystalline calcium carbonate in or on the cell wall.
VII.5 Summary.

Three genera, *Bryopsis*, *Derbesia* and *Pedobesia* have been studied both in the field and in culture. The life histories of all species inhabiting the south eastern Australian coast are described and cytological investigations of the stages in these life histories are included. Within species of *Bryopsis*, a detailed description of the giant nucleus of the protonemal stage has been made, although transition to the multinucleate stage has not been adequately studied. The wound healing substances from the gametophytic stage of *Bryopsis* have been observed in all three species from southern Australia and a detailed light and electron microscope study has extended the description of this material made by some of the earlier investigators of *Derbesia* and *Bryopsis*.

In the course of this study a new genus, *Pedobesia*, has been described and a detailed life history and cytological study of this alga is provided. In particular the flattened attachment stage is described from a correlation of light microscopy and transmission and scanning electron microscopy. A further investigation of the protein crystals in the clavate thallus of *Pedobesia* is also undertaken.

The fine species of *Derbesia* from southern Australia is shown, as in previous studies, to have an alternation of generations with a species of *Halicystis*. The correct specific epithet for this fine species of *Derbesia* is discussed but a conclusion is not reached.

Of the thirteen names that have been applied to the southern Australian species of *Bryopsis*, only three are used in the present study. These are *B. vestita*, *B. gemellipara* and *B. plumosa*, however, analysis of a wider area of coast may reveal further species. Detailed species descriptions are given and the circumscriptions include characters previously not recognised for these algae. The distinctness of these species has been determined from interfertility tests and controlled environmental studies on their morphological forms. As a result of these investigations, the characteristics traditionally used in the descriptions of species of *Bryopsis* are critically appraised.

In conclusion, the classification of *Bryopsis* and its related genera is reviewed and the genera studied here are included in the order Derbesiales.
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The morphology and reproduction of *Derbesia clavaeformis* (J. Agardh) De Toni (Chlorophyta)

G. N. MacRaid* and H. B. S. Womersley†

**Abstract**

Derbesia clavaeformis grows in heavily-shaded pools on rough-water coasts, from Kangaroo Island in South Australia to Cape Patterson in Victoria. Field and culture studies show that it reproduces by multilagellate zoospores which produce a prostrate pad, developing into a disc-like attachment stage characterized by an internal skeletal structure of calcium carbonate, deposited as aragonite. From this attachment stage, or directly from floating zoospores, fine, much-branched, non-septate filaments develop and here produce the adult, clavate, branches which bear sporangia. The life history is thus direct and sporophytic, and has been followed in culture through successive generations. In the field, the adult thalli with clavate branches occur in summer, and the discoid attachment stage in winter.

*D. clavaeformis* is retained within the Derbesiales, but recognized as a distinct genus, *Pedobesia* gen. nov., characterized by its direct life history and distinctive, calcified, attachment stage.

**Introduction**

Two species of *Derbesia*, *D. marina* (Lyngbya) Solier and *D. clavaeformis* (J. Agardh) De Toni, have been recognized on southern Australian coasts (Womersley, 1956). The sporophyte stage of *D. marina* is not uncommon, though easily overlooked, and its gametophyte, *Halicystis ovalis* (Lyngbya) Areschoug, has been found growing on both crustose and articulate coralline red algae in heavily shaded pools at Sorrento and Point Lonsdale, Victoria, and at Nora Creina, South Australia. The first record of *Halicystis* for Australia is a specimen from Sorrento, Victoria (S.C. Dicker, 28.x.1963; MELU, 944).

The second species, *D. clavaeformis*, was recorded by Womersley (1956) as 'only known from Port Phillip and Western Port, Victoria, and West Bay and Pennington Bay on Kangaroo Island.' It has now been found at Nora Creina in South Australia, Peterborough, Point Lonsdale, Sorrento and Cape Patterson in Victoria, and Hogan Island, Bass Strait. Nora Creina specimens comprise No. 14 (A37816) in 'Marine Algae of southern Australia', distributed to various herbaria from the Department of Botany, University of Adelaide.

*D. clavaeformis* is generally found growing submerged, in heavily shaded rock pools, or to 3 m below low tide level on steeply sloping faces of intertidal rock platforms on rough-water coasts. This habitat prevents easy collection except during extreme low tide conditions, and the species is probably more widespread than the above records might indicate. It has been collected between August (young plants) and May (old plants) from Nora Creina, and between November and June from Point Lonsdale and Cape Patterson. It appears to be a summer annual, since it has not been found during winter at Point Lonsdale despite careful checking.

The comparatively large size of *D. clavaeformis* (Fig. 1), the simple, clavate branches, and the absence of any septa such as those in *D. marina*, had raised doubts about the taxonomic status of this alga. During an Adelaide University summer school in marine plant biology in January, 1971, the alga was collected at Nora Creina and multilagellate zoospores were observed on release from the sporangium. Later collections from Point Lonsdale (by G. MacR.) and from Nora Creina (by H. B. S. W.) were maintained in culture and the life-history of this alga was followed independently, but collaboratively, at both Melbourne and Adelaide Universities.

The results are presented here, and will be followed by a paper by the first author, dealing with the detailed morphology and ultrastructure of the alga.

*There has been great interest in recent years in the life-history of species of *Derbesia*. Since Kornmann (1938) first showed that *D. marina* and *H. ovalis* are sporophytic and gametophytic phases of the one
species, numerous other studies have supported this, including our own (first by G. MacR.) on the Australian plants. A similar alternation of generations has been shown by Feldmann (1930) between Dicrateria tonduzae (De Notaris) Crouan and Crouan and Halicystis parvula Schmitz in Murray, and by Page (1970) between Dicrateria sp. and Halicystis osterhoutii Blinks and Blinks.

In addition to these observations, an alternation has been shown between Dicrateria neglecta Berthold and Bryopsis halymenoides Berthold by Hustede (1960). Kornmann (1966, 1970) has also reported the occurrence in culture of a mutant form of D. wimii which reproduces itself directly, without alternation with a gametophyte generation. Sears & Ilce (1970) have shown that this type of reproduction occurs within some natural populations of D. wimii.

Material from Nora Creina in South Australia and from Point Lonsdale and Cape Patterson in Victoria was transported in cool sea water to the respective laboratories, and cultures were established from oos which were liberated in abundance from bare sporangia. Provosal ES medium (Starr, ii) without vitamins, and a modified Erdmeister medium (Rietema, 1970) were used with success. Cultures were kept at 16° or 20°C with light intensity of 2100-2700 lx, under a 16 hr light/8 hr dark regime.

Cultures at Adelaide University were kept only in water, but some at Melbourne University were kept in rapidly moving water. Movement was even with a table rotating through a path of 6 cm diameter, at a rate of 130 cycles/min.

Sections (1–2 µm thick) of plants grown in situ or in culture were cut from material fixed in a mixture of acrocin and glutaraldehyde and embedded in glycol methacrylate (Feder & O’Brien, 1968).

Histochemical tests (Table 1), to determine the chemical composition of the skeletal structure of the attachment stage, were applied to both whole mounts and sections. X-ray diffraction analysis of the material forming this skeletal structure was carried out by Mr C. Kerr-Grant of the Geology School, University of Melbourne.

Structure and reproduction

**Morphology of the adult plants**

*Dicrateria clavaeformis* grows on rock, with dense clusters (Fig. 1) of 10–40(–60) elongate-clavate, undivided, branches arising from a slender, much branched, filamentous base. The clavate branches are mostly 3–4 cm high, sometimes to 6 cm, and 2–3 mm in diameter.

The erect branches are usually dark green in colour due to the densely packed peripheral layer of chloroplasts. Chloroplasts in the upper part of the branch are lenticular, 2–5 µm long and about 1 µm broad, while those towards the base are avoid to circular, 3–5 µm across. *Dicrateria clavaeformis* is homoplastic, and 1–2(–3) starch grains occur within each chloroplast.

**Reproduction**

Spherical to ovoid sporangia, 350–450(–1000) µm in diameter (Figs. 1 and 2) are formed laterally a short distance from the apex of the clavate branches and

| Test applied          | Specificity      | Reference                  | Result
|-----------------------|-----------------|----------------------------|--------|
| Periodic acid-Schiff stain | Polysaccharides | Feder & O’Brien, 1968       | –
| Lacmoid               | Callose         | Gifford & Esau, 1953       | –
| Bismarck Brown        | Cellulose and mucin | Conn, 1969               | –
| L/H2SO4               | C/µlulose       | Johansen, 1940             | –
| Oxalic acid           | Calcium         | Johansen, 1940             | +
| Fe** Metal substitution | Calcium       | McGeO-Russell, 1958        | +
| Fe** Metal substitution | Calcium       | McGeO-Russell, 1958        | +
| Haematoxylin pH >11.5 | Calcium        | McGeO-Russell, 1958        | +
| Fe/µl’s solution      | Aragonite       | Wolf et al., 1967          | + Aragonite |
are attached by a gelatinous septum. In the field, mature plants usually bear numerous sporangia, which are readily detached and leave scarcely any mark on the parent branch.

Under culture conditions, the sporangia take 5–9 days to mature, and zoospore release occurs in response to onset of the light regime, or to an increase in temperature of 3–5 °C. Release from mature sporangia can also be induced by transferring fertile plants to fresh culture medium. Zoospores become motile only after the sporangium wall ruptures, and no specialized release pore or tube has been observed.

Numerous zoospores (up to 500) are produced in each sporangium. They are spherical (Fig. 3), 30–40 μm in diameter, and bear a crown of 100–150 flagella (Fig. 4). Sporangia and zoospores are dark green to almost black in colour, the zoospores having densely packed chloroplasts and a single nucleus at maturity as in other species of *Derbesia* (Davis, 1908).

**Germination of zoospores**

After release, the zoospores move with a slow, spiralling motion for up to 4 h, finally coming to...
FIG. 5. Early germination stages of zoospores. (a) Zoospore producing a germination tube; (b) zoospore with an attachment-pad, showing finger-like processes at growing margin, larger circular clear areas corresponding to the pillars, and rows of smaller pores connected by lines (about 2 weeks old); (c) ditto, with branched attachment pad; (d) a later stage (3-4 weeks old, grown in moving water) with a semi-circular disc; (e) an almost circular disc showing its origin and the original germination tube; two secondary discs are present (6 weeks old, grown in moving water).

In view of the constantly agitated water in the natural habitat of the alga, it appears unlikely that this surface-floating stage is of any significance in the natural environment.

The germination tube of the zoospores which settle on a solid substrate becomes loosely attached, and at this early stage can be detached without damage. Within 3-4 days many germinating zoo-
spores produce a distinctive pad-like attachment (Fig. 5b, c) which adheres strongly to the substrate and cannot be detached without damage.

In still-water cultures, the loosely attached filaments continue to grow, branching subdichotomously or laterally, to reach a length of 3–4 μm (Figs. 12 and 13). In those plants where the germination tube produces an attachment pad, the non-septate branching filament arises from the opposite side of the zoospore, or laterally between the zoospore and the attachment pad. These fine, branched, filaments continue to grow for up to several months before further development occurs (see below).

**Development of the attachment stage**

When first formed either in still or moving water, the attachment pad is 20–50 μm across, ovoid with a broad, convex, growing apex (Fig. 5b, c) which under agitated conditions becomes semi-circular. The surface view shows circular transparent areas and minute pores connected by fine lines; short finger-like processes occur 3–5 μm behind the cell wall at the growing apex (Fig. 5b, c). The cytoplasm of this stage is continuous with that of the germination tube, and contains numerous chloroplasts and nuclei.

With constant water movement, growth continues from the convex margin of this flat, prostrate, attachment stage, forming a broadening thallus. This later becomes semi-circular (Fig. 5d) and often recurves to enclose the point of origin, forming a disc (Figs. 5e and 6). The size of the disc increases mostly by the lateral broadening of the margin, but the margin may also separate into convex segments which with continued growth show radial lines formed by apposition of wall material and calcium carbonate (see below) of adjacent sectors (Figs. 7 and 9). Growth of the disc may also be interrupted, resulting in concentric lines of development (Fig. 7), and in cases of active regrowth the growing margin (Fig. 8) may show structures identical with the earliest stage of the attachment pad. As well as these occasional concentric lines of regrowth, numerous regularly-spaced, concentric rings are apparent (Figs. 6 and 7), and these may be due to differences in the calcification. There is no septation within the disc.

Secondary discs, which overgrow older parts of the thallus (Fig. 6), often develop from the concentric lines of regrowth. Repetition of this forms a multi-layered encrusting structure.

From the margin of the disc, or the regrowth regions, fine, non-septate filaments may develop. If the cultures are transferred to still-water conditions, growth of the disc ceases and development of the filaments is stimulated.

Examples of the discoid attachment stage have been collected in situ at Point Lonsdale, both at the base of upright clavate thalli and also, between June and October (when adult plants are not found), as multi-layered encrusting structures on the rock surface. Young clavate thalli at Nora Creina in August also show the basal encrusting structures. Naturally occurring discs are usually much more irregular in form than those grown in culture, due to their irregular substratum. Fragments of these attachment discs, when growing in culture, develop fine filaments which give rise to the clavate branches (see below).

**Detailed structure of the attachment stage**

A detailed study of the ultrastructure of *D. clavaeformis* will be reported separately (by G. MacR.) and only preliminary light microscope observations, sufficient for description of the structure of the attachment stage, are given here.

The attachment stage has a distinctive surface pattern (Figs. 5b, 9 and 10), the most prominent feature being scattered, round, clear areas 2–3 μm in diameter. Over the whole surface also are much more numerous, smaller, round to slit-like pores, which are joined by fine lines running at right angles to the growing margin (Fig. 10); cross lines between the lines of pores are not present, though the lines may branch (Fig. 5b). New pores arise at the margin as gaps between the finger-like processes as they grow outwards, and the fine radial lines between the pores correspond to the closely adjacent sides of the processes before the next gap is formed. Growth, or at least calcification, at the apex thus appears to be regularly periodic, with the gap or pore being formed at the start of a growth cycle and calcification then closing in for the rest of the growth period. The number of pores may correspond to the age in days of the disc.

Transverse sections through the attachment stage show that the above features are part of a skeletal structure laid down inside the relatively thin polysaccharide cell wall (Fig. 11). This extends throughout the attachment stage, except very close
Fig. 6. Disc (cultured, 3 months old) in surface view, with two overlying secondary discs and mass of fine filaments. Fig. 7. Part of a disc showing three concentric regions of growth interruption (see arrow) and regularly spaced rings possibly due to differences in calcification; radiating lines between growth sectors are apparent, and the margin shows interruptions to the normally smooth periphery. Fig. 8. Periphery of disc showing regrowth of structures similar to the early attachment pads. Fig. 9. Several adjacent sectors of a disc showing apposition of cell walls, pillars (prominent circles) and pores (faint). Figs. 7, 8 and 9. From plants 5 months old, grown in moving water.
the growing margin. The upper layer of the skeletal structure is 3–6 μm thick, and is perforated by the pores. The lower part of the skeletal structure is an unbroken layer, joined to the upper layer by pillars of the skeletal material which traverse the continuous cytoplasm of the attachment stage (Fig. 11). These pillars are seen in surface view as the clear round areas (Figs. 9 and 10).

Unlike the cell wall of the attachment and filamentous stages, the skeletal material appears crystalline under polarized light. It reacts with hydrochloric and sulphuric acids, resulting in the evolution of a gas and disintegration of the structure. Table 1 lists histochemical tests carried out, indicating the presence of calcium. Feigl’s solution (Wolf, Easlon & Warne, 1967) and X-ray diffraction analysis show that the substance is approximately 90% aragonite. No calcite was detected.

Development of adult, clavate branches

After a variable time (usually one to several months) in still-water culture, the fine filaments, which are mostly prostrate on the substratum, produce upright clavate branches (Fig. 12) which closely resemble those of plants collected in the field. This development can be stimulated by a temperature change, e.g. fine-filament stages transferred from 20°C to 16°C for 3–4 weeks, and then returned to 20°C, produce clavate fronds then sporangia within another 4–5 weeks. It appears that clavate branches can arise from any part of the more or less prostrate filamentous stage (Figs. 12 and 13).

Similarly, the attachment discs produce fine filaments (see above) from which clavate branches later develop (Fig. 14). Examples of discs producing upright clavate branches have also been observed in situ, at Point Lonsdale and at Nora Creina.

Life history in culture and in natural habitats

*D. clavaeformis* has been taken through its reproductive cycle in culture twice at both Melbourne and Adelaide Universities. In each case mature clavate thalli produced sporangia and spores which passed through the attachment and filamentous
sages, to produce clavate branches, sporangia and spores from which the new generation developed. It appears that *D. clavaeformis* has only this direct life-history development, and is sporophytic; no gamete-bearing stage has been observed either in culture or in the field. This conclusion is supported by the seasonal occurrence of upright clavate thalli and of the attachment discs, as observed in the field. The life history is shown diagrammatically in Fig. 15.

The erect clavate thalli occur essentially during summer months, whereas the attachment stage alone is present during winter months. At Point Lonsdale, summer sea water temperatures are 18-20°C and winter 11-12°C (King, 1970). Thus changes in culture conditions which stimulate development of clavate thalli are in conformity with seasonal temperature variations at Point Lonsdale. The temperature regime at Nora Creina is not as well known; summer sea water temperatures are little if any higher than winter temperatures, due to upwelling of cold water on this coast (Hynd & Robins, 1967).

Under still-water conditions, vegetative reproduction occurs readily when fragments of the filamentous stage, or small groups of clavate thalli, attach themselves to the culture vessel. In old cultures, sporangia which fail to form spores may develop numerous clavate branches and become detached from the parent plant. A similar development has been shown in *D. tenissima* by Ziegler & Kingsbury (1964). Plants may also be propagated vegetatively by production of naked protoplasts, as recorded for *Bryopsis plumosa* by Tatewaki & Nagata (1970). The
importance in nature of these vegetative means of reproduction cannot be assessed, but the ability to produce new cell wall material in damaged regions may be important to the survival of the plants.

The taxonomic status of Derbesia clavaeformis

While *D. clavaeformis* has non-septate thalli and produces sporangia with multiflagellate zoospores such as characterize the order Derbesiales, it differs from typical *Derbesia* (as shown by the type species, *D. marina*) in having a direct life-cycle without the *Halicystis* gametophytic stage, and in lacking septa at points of branching. However, both Kornmann (1969, 1970) and Sears & Wilce (1970) have shown that direct reproduction of the sporophyte of *D. marina* can occur.

Apart from the above features, *D. clavaeformis* differs markedly from *D. marina* in the presence of the distinctive attachment stage and intermediate filamentous stage, before the clavate, sporangium-bearing, branches are produced. In particular the presence of calcium carbonate (as aragonite) in the attachment stage, and its characteristic pattern of deposition inside the cell wall, seems distinctive. Records of the presence of calcium carbonate within the non-septate siphonous green algae have previously been restricted to some members of the Caulerpales (Udoteaceae) and of the Dasycladales. However, as all developmental stages of *D. clavaeformis* are homoplastic, with storage of starch within the chloroplasts, it cannot be classified within the Caulerpales (*sensu* Feldmann, 1946) or Dasycladales (*sensu* Pascher, 1931).

Thus while *D. clavaeformis* appears to be best placed in the Derbesiales, especially on account of the characteristic multiflagellate zoospores, the differences from *D. marina* and other known species warrant its recognition as a distinct genus.

*Genus* Pedobesia gen. nov.

Adult thallus homoplast, non-septate, of numerous elongate-clavate branches arising from a branched filamentous stage, derived in turn from a prostrate
attachment pad or disc which has a distinctive skeletal structure of calcium carbonate (aragonite) deposited within the cell wall. Sporangia subterminal on the clavate branches, producing numerous multi-flagellate zoospores which give rise to the attachment or filamentous stages. Life history direct, sporophytic.

"Thallys maturus homoplasticus, non septatus, multorum rami et elongatorum et clavatorum ex ramosis filis ortorum compositus. Pes vel discus basalis ex quo fila orta sunt, propriam structuram calccaream (aragonite) intra parietem continet. Sporangia subapicalia in rami clavatis, zoosporas multas multiflagellares ex quibus discus vel fila evoluta efferentia. Sporophytic, gametophyta absentia."

**Type and only known species:** *Pedobesia clavaeformis* (J. Agardh) comb. nov.

*Basionym:* *Bryopsis clavaeformis* J. Agardh, 1887: 20.


Named from the characteristic foot-like early attachment pad and disc, and its relationship to *Derbesia*.

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