Zoology Publications from Victoria University of Wellington

Nos. 42 to 46

Issued September, 1968

Distributed on an exchange basis from the Department of Zoology
VICTORIA UNIVERSITY OF WELLINGTON, NEW ZEALAND

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Notes on Lillie's (1945) Naphthol Green B Stain for Connective Tissue

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Zoology Publications from Victoria University of Wellington, No. 42

Issued September, 1968

INTRODUCTION

During preparation for a class in histology at Victoria University a triple stain was sought for the distinction of muscle and connective tissue that would give consistently good results using stains that are usually readily available in a department of zoology. The material to be stained was sections of vertebrate tissue, in particular that of mouse and chick embryo. A staining method giving green connective tissue, pinkish cytoplasm and muscle, and blue nuclei was wanted. Gurr (1962) gives the following stains for obtaining a very similar staining reaction, viz. Weigert's haematoxylin, eosin yellowish, and naphthol green B. His method is as follows:

1. Paraffin sections are mounted on the slide and brought down to distilled water.
2. Stain for 6 mins. in a freshly prepared mixture of equal volumes of Weigert's haematoxylin A and B.
3. Wash thoroughly in tap water; then stain for 3 mins. in 1% aqueous eosin y.
4. Wash in tap water; then immerse in a solution of 10% ferric chloride for 5 mins.
5. Rinse well in distilled water; then stain for 5 mins. in a 1% aqueous solution of naphthol green B.
6. Differentiate for 2-3 mins. in 1% acetic acid.
7. Drain well; then dehydrate with acetone, afterwards clearing in acetone-xylol; then mount.

The results are: connective tissue, green.
muscle and cytoplasm, pink.
nuclei, brown.

With the above procedure results were far from consistent and a clear differentiation of muscle and connective tissue was not obtained.

Testing the staining procedure consisted of working through the schedule using the times given by Gurr, and varying the times in the eosin, ferric chloride, naphthol green B, and acetic acid baths. Material used was F.A.A. fixed paraffin embedded sections of (a) mouse head, abdomen and duodenum, cut at 8μ on a rotary microtome, and (b) sections of frog stomach cut at 6μ on the same microtome. Staining procedures using alcoholic eosin as the plasma stain, and connective tissue stains of naphthol green B (George T. Gurr, 09506) in absolue alcohol and fast green FCF (Flatters and Garnett Ltd.) in absolute alcohol, clove oil, and cellosolve (ethylene glycol monoethyl ether) were also tried.

RESULTS AND DISCUSSION

The main fault in staining was loss of the eosin from all but the strongest eosinophil cells (for example, red blood cells) on dehydration prior to mounting. This resulted in greenish muscle and cytoplasm. The suggested dehydrant, acetone, seemed too vigorous. Even the minimum dehydration time permissible caused a loss of eosin from some muscle and cytoplasm, causing uneven staining of sections. An attempt to correct the fault by dehydrating through a series of graded alcohols was not more

successful. Increasing the time in the eosin bath showed that overstaining was impossible, and did not help to keep the eosin in the tissues during dehydration.

Cutting the time in the naphthol green B to as little as 15 secs. still allowed the green to colour muscle and cytoplasm (the eosin being removed in dehydration). An attempt to stain first in the naphthol green B followed by differentiation in 1% acetic acid, then in eosin y in alcohol (0.5% soln. in 90% alcohol) was, again, unsuccessful. The eosin would not stain muscle tissue or cell cytoplasm which had already been stained with naphthol green B. Lengthening the time in the acetic acid bath had little effect.

Gurr (1962) gives no information as to the purpose of the 10% ferric chloride solution, or the reason for dehydrating in acetone and clearing in 50-50 acetone-xylol. He gives a footnote that the original reference for the technique is Lillie (1945).

Lillie found that the staining method now under discussion was the most satisfactory using naphthol green B as a connective tissue stain. It was evolved during exploration for mordants for selective connective tissue staining, following a report that naphthol green B dyed wool green with an acid iron mordant. The ferric chloride bath in this staining method is described as a mordant by Lillie (p. 31), but it was found that omitting the ferric chloride prior to staining with naphthol green B resulted in deep green staining of all tissues. This suggests that the action of ferric chloride is not to mordant the tissues for the green dye, but to heighten the selectivity of the dye for connective tissue. Indeed, Lillie concludes in his paper that the selectivitiy of collagen stains is best at pH levels below 3.0, preferably below 2.0, and disappears at higher pH levels.

The best results using Lillie's triple stain were obtained by using the following adjusted staining schedule:

1. Sections are brought to water.
2. Stain in freshly prepared Weigert's haematoxylin 6 mins.
3. Wash in tap water, and stain in 1% aqueous eosin y for 5 mins.
4. Rinse in tap water, and immerse in a 10% solution of ferric chloride for 3 mins.
5. Rinse in distilled water and stain in naphthol green B (1% aqueous) for 15 seconds.
6. Differentiate in 1% acetic acid 2 mins.
7. Dehydrate as quickly as possible in acetone, clear in 50-50 xylol-acetone, and mount.

Gurr (1962) states that many acid dyes devoid of sulphonic groups ($-SO_3^-$) can be completely extracted from tissues by ordinary solvents such as water and alcohol. He suggests this is because tissue proteins, already possessing carboxyl groups identical with those of carboxylated (acid) dyes, have little tendency for exchange of ions with the acid dye. The dye ion is thus not in chemical combination with the tissue protein.

Eosin y is a carboxylated but not a sulphonated dye. It therefore seems reasonable to expect a better result with a sulphonated dye for the plasma stain in Lillie's triple stain. Most of the plasma stains used by Lillie in his investigations were sulphonated, e.g. acid fuchsin, Biebrich scarlet, aniline blue, and many others. Eosin, however, gives excellent contrast as a plasma stain. It was decided to use the eosin in 90% alcohol and to follow it with the connective tissue stain in absolute alcohol or a clearing agent, rather than substitute a sulphonated dye for the plasma stain.

Gurr (1962, p. 59) gives the solubility of naphthol green B in absolute alcohol as 3% at 15°C. I found, however, that it is insoluble in absolute isopropyl alcohol, and only soluble in absolute ethanol to 0.25%. This 0.25% soln. failed to give selective

connective tissue staining following a plasma stain of 0.5% eosin y in 90% alcohol. Therefore replacement of naphthol green B with some other green connective tissue stain seemed advisable.

Fast green FCF was used by Lillie (1945) as one of a number of dyes that gave good connective tissue staining. He used it in aqueous solution, but in the present instance the fast green FCF was used in clove oil, and with eosin y in 90% alcohol gave excellent differential staining of muscle and connective tissue. Weigert's haematoxylin was replaced with Delafield's to give the nuclear stain, thus eliminating a source of waste as Delafield's haematoxylin need not be freshly made every few days. (Histologists will attest to the fact that Delafield's haematoxylin becomes better with age.)

The staining procedure for the triple stain using Delafield's haematoxylin, eosin y and fast green FCF is as follows:

1. Bring paraffin sections to water.
2. Stain in Delafield's haematoxylin 6 mins. to give overstain.
3. Differentiate in acid/alcohol until nuclei only remain coloured.
4. Wash in tap water, and blue nuclei in tap water or in lithium carbonate solution if tap water is not sufficiently alkaline.
5. Dehydrate to 70% alcohol, and stain in 0.5% eosin y in 90% alcohol for 40 seconds.
6. Rinse in 95% alcohol, and differentiate in 100% alcohol 1 minute.
7. Stain in a saturated solution of fast green FCF in clove oil for 50 seconds-1 minute.
8. Wash off the excess stain thoroughly in xylol, and mount in DPX mountant.

Results: connective tissue, green.
muscle and cytoplasm, pink.
nuclei, deep blue.

The above staining schedule was found to be very effective for mouse embryo tissue, giving very good results each time. The same procedure was used for sections of frog stomach, and gave excellent results when the time in the fast green was increased to 3 minutes.

The fast green was also used in cellosolve and in absolute alcohol. With cellosolve as the dye solvent the best results for mouse embryo tissue were obtained with a 0.25% solution and a staining time of 12 seconds. The excess stain must be washed from the slides by moving them in cellosolve for a few seconds, prior to clearing in xylol and mounting. Washing the slides in alcohol or xylol does not remove the stain quickly enough, causing the staining time to be increased and resutling in some muscle being coloured green. Because of the short staining time when cellosolve is used as the dye solvent the method does not allow the degree of control of connective tissue staining that is possible when the fast green is dissolved in clove oil. A 0.25% solution of fast green in cellosolve gave excellent differential staining of muscle and connetcive tissue in sections of frog stomach with a staining time of 35 seconds. Again, excess stain was removed in cellosolve.

A 0.026% solution of fast green in ethyl alcohol was also successful as a connective tissue stain.* With mouse embryo sections the best staining time was 20 seconds, and with frog stomach 1 minute 50 seconds. In both cases excess stain was removed from slides by moving them for a few seconds in absolute alcohol.

The above method using Delafield's haematoxylin, eosin y in alcoholic solution and fast green FCF in clove oil, and its variations give reliable results. It could be described as being a derivative of the method of Lendrum (cited in Lillie, 1945).

*Gurr (1960, 1962, 1965) gives the solubility of fast green FCF in absolute alcohol as 9% at 15°C. Johansen (1940) states the solubility of the dye to be 0.33% in absolute alcohol. My own findings were more in line with those of Johansen.

He used a nuclear stain of alum haematoxylin followed by a plasma stain of either gallic acid-eosin-erythrosin-phloxine in alcoholic solution, or formol-eosin in alcoholic solution, followed by a connective tissue stain with tartrazine, fast green FCF, aniline blue, or methyl blue, dissolved in cellosolve.

SUMMARY

1. Lilie's triple stain of Weigert's haematoxylin, aqueous eosin y, naphthol green B is unreliable as a differential stain for connective tissue.
2. The main fault with the method is rapid loss of eosin from sections during dehydration.
3. Excellent differential connective tissue staining can be obtained using a plasma stain of eosin in 90% alcohol with the connective tissue stain in a clearing agent or in cellosolve.
4. The recommended technique employs Delafield's haematoxylin as nuclear stain, eosin y in 90% alcohol as plasma stain, and fast green FCF in clove oil or in cellosolve as the connective tissue stain.

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The Histology of a Species of *Solanderia* Duchassaing & Michelin, 1846

from Auckland Harbour, New Zealand, with Special Reference to the Internal Skeleton of the Solanderiidae (Coelenterata, Hydrozoa).

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Zoology Publications from Victoria University of Wellington, No. 43

Issued September, 1968.

ABSTRACT

Five infertile colonies, 4-12 cm tall, of the gymnoblastic hydroid, *Solanderia misakinensis* (Inaba, 1892), from Auckland Harbour, and two infertile colonies from Wellington Harbour, one 30 cm tall and the other 6 cm tall, are described. Comparison of growth features between the Auckland and Wellington specimens determines the Auckland specimens as young colonies. The relationships of *S. misakinensis* to other species of the genus, in particular those recorded from New Zealand and Australia, are reviewed. *Chitina ericopsis* Carter, 1873 is almost certainly conspecific with the present material. A specimen of *Solanderia fusca* (Gray, 1868) collected by Pennycuik is probably the species *Solanderia secunda* (Inaba, 1892).

A histological study of the Auckland Harbour material confirms Vervoort's findings (1966) that the chitinous skeleton is ectodermal in origin. The pattern of growth, and the formation of the skeleton is described for a branch tip of a colony.

INTRODUCTION

The athecate hydroids of the family Solanderiidae Marshall, 1892 were until recently (Vervoort, 1966) regarded as unique in possessing a chitinous skeleton in the form of a network of longitudinal and transverse connecting fibres that originated in the mesoglea (Rees, 1957; Vervoort, 1962). Vervoort clearly showed in his 1966 paper, however, that the lattice work skeleton is formed as are all other hydroid skeletons by ectodermal cells. Solanderid hydroids range from cushion-like forms to tall, much branched colonies. The polyps have capitate tentactles. Six species are recognised, and a further nine have been given the title "doubtful species" by Vervoort (1962). These doubtful species are those which have not been fully described, and although they are probably members of the recognised species they cannot, at present, be placed in them. The various species have been recorded from localities including the West Indies, Australia, Japan, South Africa, and from other subtropical and temperate waters. Until the present paper one species only has been recorded from New Zealand. This is *Chitina ericopsis* described from the skeleton by Carter in 1873. The collection locality is stated simply as New Zealand. The skeletons of several colonies were dredged from soft mud in Wellington Harbour some ten years ago, but the first colonies from New Zealand waters with identifiable soft parts

are those described here from Auckland, collected in 1960. This year (1967) the largest complete New Zealand colony was dredged from bottom mud at 8 fathoms in Wellington Harbour.

Early systematic studies were based mainly on the growth form of the colonies and on the structure of the skeleton, in particular the structure of the hydrophore or "supporting cup" which is found at the base of polyps in certain species.

MATERIALS

The material on which this study is based is a collection of five colonies preserved in formalin, from 4 to 12 cms high, taken from Auckland Harbour, N.Z., and two colonies, one 30 cms high and one 6 sms high, taken from Wellington Harbour, N.Z. Specimens of *Solanderia fusca* (Gray, 1868) from New South Wales and Queensland, Australia, and a specimen of *Chitina ericopsis* Carter, 1873 were studied to aid in identifying the Auckland and Wellington Harbour species which unfortunately were devoid of reproductive organs, so that descriptions of these organs were not made.

CLASSIFICATION OF THE NEW ZEALAND MATERIAL

The colonies from Auckland and Wellington Harbours are erect and have no hydrophore or supporting structure of any kind at the base of the polyps. This eliminates them from many of the species described by Vervoort (1962), but leaves them as possible members of the following genera and species.

(1) *Solanderia gracilis* Duchassaing and Michelin, 1846 (distribution West Indies).
(2) *Solanderia leuckarti* Marshall, 1892 (described as a "doubtful" species by Vervoort, distribution unknown, but probably the Pacific).
(3) *Chitina ericopsis* Carter, 1873 (a "doubtful" genus according to Vervoort, distribution New Zealand).
(4) *Solanderia misakinensis* (Inaba, 1892), found in Japanese waters.

The colony growth form of the Auckland specimens used in this study is identical with that of *S. gracilis* except that the polyps on the branch tips are borne in all planes, and not only in the plane of flattening of the colony. The skeleton of *S. gracilis* is purple coloured, but the skeletons of the Auckland and Wellington specimens preserved in formalin are a uniform light brown.

The present material lacks tubercles of fused fibres on fine branches such as are described for *S. leuckarti.*

A portion of a branch of *Chitina ericopsis* (dried material only) cannot be distinguished in structure from that seen in a branch of equivalent dimensions of the present material. *Chitina* may therefore be inseparable from *Solanderia,* as Vervoort suggests (1962, p. 537). However as no description of soft parts of *Chitina* exists, it is difficult to see how any living or well-preserved specimen can ever be assigned to this genus.

S. misakinensis, as described by Vervoort (1962) possesses a much closer, denser form of branching than the Auckland specimens, but is identical to the largest (30 cm high) Wellington Harbour colony. Growing from the same tangled root-like base of the Wellington material a little to the side of the large colony is a colony 6 cm high, and this smaller one has the same growth pattern as the Auckland Harbour specimens. There is little doubt that the Auckland and Wellington specimens are members of the species *S. misakinensis,* and that the Auckland material is at an earlier stage of development than that which is figured and described by Vervoort.

In the course of identifying the present material two specimens labelled as *Solanderia fusca* (Gray, 1868) were studied. One was collected in Sydney, Australia, (Australian Museum, No. Y.509) and the other from Queensland, collected by Dr. Pamela Pennycuik. The specimen from Sydney is undoubtedly *S. fusca* and agrees in every detail of skeletal structure with the specmien *Ceratella fusca* Gray, 1868 described by Spencer (1891). The specimen from Queensland, however, is certainly not *S. fusca.* The skeleton is very similar to that of *Solanderia secunda* (Inaba, 1892) with a flattened, thorn shaped projection either side of the polyp base and no hydrophore as in *S. fusca.* The colour of the Queensland specimen is dark brown as in *S. secunda,* and the method of branching is similar. As the distribution of *S. secunda* is tropical and subtropical regions of the Pacific Ocean (Vervoort, 1962) it seems very likely that the Queensland specimen is in fact *S. secunda.*

METHODS

Several tips of branches about 1 cm long were stained in Delafield's haematoxylin and embedded in wax. Some of these blocks were cut to give transverse serial sections from the stem tip, and others were cut to give longitudinal serial sections. The sections were placed on slides in series, over-stained in Delafield's haematoxylin and differentiated in acid alcohol, countersigned in eosin (0.5% soln. in 90% alcohol) and mounted in DPX mountant. Most sections were cut at 10μ on a rocker microtome, but one transverse series was cut at 5μ. A thicker piece of stem (1cm x .5mm x .4mm) was embedded, cut in transverse section at 10μ and stained and mounted as above.

In addition, a 1cm length of stem from the tip of a branch was denuded of soft tissues by dipping into "JANOLA" (a commercial solution of sodium hypochlorite), and the remaining skeleton was washed and stained in a 0.5% soln. of lignin pink in cellosolve for 48 hours. After this time the skeleton was mounted in DPX for observation. Three lengths of stem from the tip to the thicker parts of the branch were also rid of soft tissues by the same method and were stained in the lignin pink soln. for $\frac{1}{2}$ hour. After this time they were removed to absolute alcohol.

From one series of transverse sections of a branch tip, a model of the soft parts (endoderm only) was constructed to illustrate the method of branching of coenosarc tubes within a branch tip. (It was assumed that the growth pattern in a branch tip is indicative of the growth pattern of the colony as a whole).

MORPHOLOGY

External features: *The Colony* (Plate I, Fig. 1).

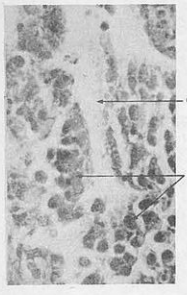
The colony is erect, arising from a flattened, root-like base from which is formed a strong stem. This stem branches irregularly forming large and small branches which, near the base of a colony, have the appearance of being formed of intertwined branches, and which may anastomose if they come into contact. Branching in small colonies (4-12cms high) is in one plane only, so that the colony has a flattened, fan-shaped appearance. Larger colonies have a more bushy appearance. The colour of the colony skeleton is an overall brown, but is paler on the small branch tips. The polyps (white in preservative) are borne on all branches, but do not arise directly from the tip of any of the branches. They are elongate (up to $2\frac{1}{2}$ mm in length in the preserved state) naked cylindrical structures with a conical hypostome. The base of the polyp is without hydrophore or supporting bract of any kind. Tentacles are given off throughout the length of the polyp and normally appear irregularly placed, but in a well extended polyp are seen to be arranged in a spiral at intervals along the polyp, except for an oral whorl of four tentacles.

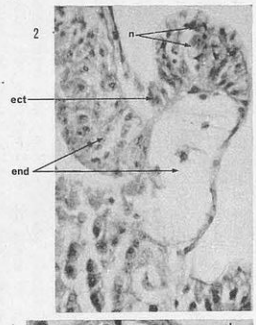
Plate 1

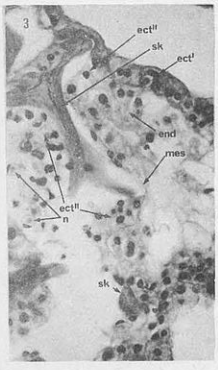
Fig. 1 The Colony X 3/5. Fan shaped, and flattened in the plane of branching.
Fig. 2 Longitudinal section of a polyp to show 3 distinct regions: Hypostome; glandular region; region of large endodermal cells.
Fig. 3 Cross section of a polyp in the hypostome region.
Fig. 4 The skeleton of a branch tip. Flattened, longitudinal fibres are joined by struts (transverse fibres).
co, coelenteron; ect, ectoderm; end, endoderm; gl, gland-cell region; hyp, hpostome; long fib, longitudinal fibre; trans fib, transverse fibre; t, tentacle.

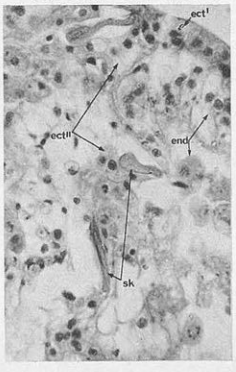
Plate 2

Fig. 1 The glandular region of a polyp. Cells packed with acidophil globules line the gastric cavity.
Fig. 2 Longitudinal section of a tentacle. It is capitate, and solid.
Figs. 3, 4 Cross section through a stem at a polyp base. The sections are 10μ apart. No skeletal supporting cup or hydrophore is present. The skeleton is lined with "inner" ectoderm which is continuous with the "outer" ectoderm.
co, coelenteron; ect, ectoderm; ect′, "outer" ectoderm; ect″, "inner" ectoderm; end, endoderm; gl, gland cells; mes, mesoglea; n, nematocysts; sk, skeleton.









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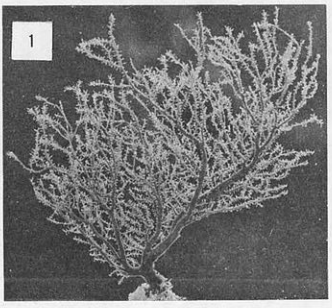
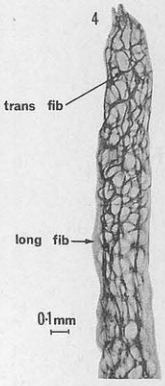
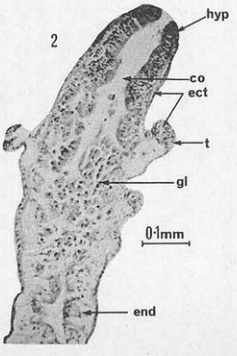
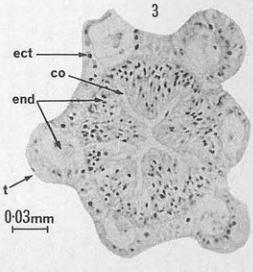








Internal structure: *The Polyp* (Plate I, Figs. 2, 3; Plate 2, Figs. 1, 2).

In longitudinal section (Plate 1, Fig. 2) the polyp has three distinct regions, namely (a)—the hypostome, in which the main feature is the very strongly developed endothelial muscular cells; (b)—a secretory region of from $\frac{1}{3}$ to $\frac{2}{3}$ the length of the polyp consisting of many cells opening into the coelenteron and its branches [these cells are packed with acidophilic globules (Plate 2, Fig. 1)]; and (c)—at the base of the polyp there is a region of large vacuolated endodermal cells which extends for approximately a third of its length. The nuclei and cytoplasm of these cells are distally placed, and each cell usually subtends, at its base, two or three ectodermal cells.

The ectoderm of the polyp is somewhat cuboidal and is continuous with that over the surface of the whole colony. In extended polyps it is drawn out into a very thin layer (Plate 1).

The mesoglea is prominent in retracted zooids, but is thin when the zooids are extended. It is light staining, and appears structureless. In retracted polyps a very large number of cut ends of myonemes from the ectodermal cells are seen embedded in the mesoglea, and myonemes can be seen in the ectodermal cells.

The tentacles have a solid core of vacuolated endodermal cells and are capitate. Batteries of nematocysts occur in their swollen "cap-like" distal ends (Plate 2, Fig. 2). Nematocysts are sometimes seen in the ectoderm towards the base of a polyp, but apart from this are confined to the tentacles. Only stenoteles (penetrating nematocysts) have been seen, and they are usually about 5μ long, but often may be as large as 10μ long.

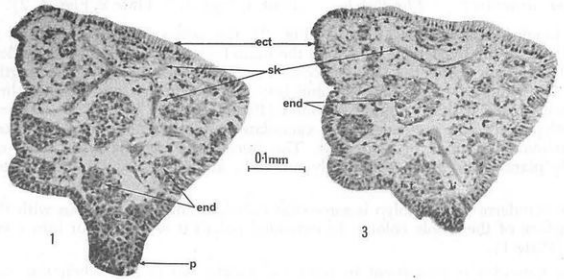
The Branch Tip (Plate 3, Figs. 1, 2, 3, 4).

Thicker branches of the colony give the appearance of being formed of intertwined smaller branches. The very tips of branches, although of small diameter show a similar structure when seen in transverse section.

In Plate 3, Figs. 1 and 3 are transverse sections through a typical branch tip and are 10μ apart. The stem or branch tip is seen to be composed of several coenosarc tubes. There is a central tube (that is endoderm, mesoglea, and ectoderm) and radially-placed coenosarc tubes.

Between these tubes are the skeletal elements. The skeleton is not formed around the whole circumference of the stem, but only between the inner ectoderm layer of adjacent coenosarc tubes (Plate 2). The whole stem is covered with a columnar epithelium.

The "inward facing" layer of the ectoderm of the outer coenosarc tubes, and the ectoderm of the central coenosarc tube are composed of vacuolate cells with the cytoplasm and nucleus distally placed. These ectodermal cells are clearly delimited from the endoderm of the tubes by a thin layer of structureless, lightly staining mesoglea. Small eosinophil particles are frequently present in the ectodermal cells, especially those cells which abut against the skeleton. The coelenteron of the coenosarc tubes appears as narrow branching channels (Plate 2). Gland cells packed with large eosinophil globules are seen in the endoderm of the tubes, especially in thicker stems, even though the coelenteron cannot be seen. In some transverse sections of a branch tip the coelenteron can be followed through the polyp to the endoderm of a coenosarc tube, but in the latter the coelenteron is not prominent. The "closing-over" of the coelenteron in the coenosarc tubes is probably due to contraction during preservation.



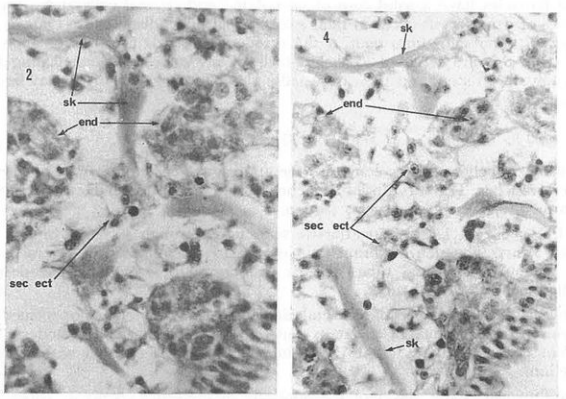


Plate 3

Fig. 1 Cross section through a branch tip. Note the columnar ectoderm enclosing skeletal elements and endoderm. The regular arrangement of coenosarc tubes, and therefore of skeletal elements, is clearly seen.

Fig. 2 An enlarged portion of Fig. 1. Secretory ectodermal cells are seen at the junction of 3 ribs of skeleton.

Fig. 3 Cross section of a branch tip 10μ from Fig. 1.

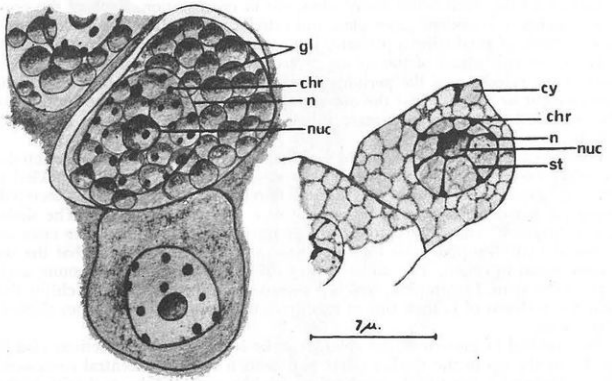
Fig. 4 The area of Fig. 3 corresponding to the area shown in Fig. 2. The skeletal structures are reduced, but in their place are secretory ectoderm cells which show a granular cytoplasm.

ect, ectoderm; end, endoderm; p, polyp base; sec ect, secretory ectoderm; sk, skeleton.

The ectodermal and endodermal cells are markedly different. Endodermal cells take stain more readily than do ectodermal cells; they also differ in the characteristics of their nuclei. Both the outer columnar and the inner secretory ectoderm cells have round nuclei of about 3μ diameter, while endodermal nuclei may be up to 6μ diameter and are often irregular in outline. The nucleus of the ectodermal cells (Text Fig. 1B) has a purplish staining nucleolus which is surrounded by about six small, darkly staining, chromatin bodies. From each of these small chromatin bodies there is a fine darkly staining radial process which connects with a small peripheral body. The nucleoplasm is irregularly granular. The nucleus of the endodermal cells (Text Fig 1A) has a nucleolus which stains a brighter red, and fewer darkly staining bodies. But these bodies are larger than in the ectodermal cell nucleus, and are found irregularly scattered throughout the nucleoplasm. The nucleoplasm of the endodermal cell nucleus is much less granular than that of the ectodermal cell nucleus.

Bipolar and multipolar nerve cells occur between endoderm and ectoderm of the coenosarc tubes. The nucleus of these cells occupies the greater part of the cell body and stains very darkly. The cytoplasm is also deeply stained, and is agranular.

Although the outer ectoderm appears to be free of nematocysts, except at the base of a polyp, nematocysts occur in the internal etcoderm. In the latter cell layer, they total about 40% of the ectodermal cell number in thicker branches (Plate 2, Figs. 3 and 4). Both large (10μ) and small (5μ) stenoteles are present, and many stages of development can be seen.



Text Fig. 1 Typical endodermal and secretory ectodermal cells. A, Endodermal cells. The cytoplasm contains large acidophil globules. The nucleus has a nucleolus and irregularly placed chromatin bodies. B, Secretory ectodermal cells. The cytoplasm is vacuolated. Fine chromatin strands radiate from the nucleolus to peripheral chromatin bodies in the nucleus.

chr, chromatin body; cy, cytoplasm; gl, globules; n, nucleus; nuc, nucleolus; st, chromatin strand.

The Skeleton (Plate 1, Fig. 4; Plate 2, Fig. 3).

The skeleton of a branch tip is a network of "chitinous" fibres (Plate 1, Fig. 4). There are major fibres running longitudinally which curve to meet at the tip of the branch. These main fibres are joined by struts which are flattened in the direction of the branch and which occur about 0.09 mm apart. Combining the information given in the accompanying Plates, the skeleton of a branch tip can be described as a network cylinder surrounding the central coenosarc tube, with radially arranged, longitudinal strips of chitinous material which are flattened laterally, and joined to the central cylinder at intervals by transverse struts. These separate the peripheral coenosarc tubes from each other. In a well preserved specimen the whole skeleton is enclosed by soft tissue.

The skeletal fibres are of lamellar construction (Plate 2, Fig. 3). A branch tip skeleton had to be left in a 0.5% solution of lignin pink for 48 hours before it stained evenly throughout its length. In three pieces of skeleton taken in series from a branch tip, the colour deepened greatly away from the tip of the branch when they were all stained for ½ hour. The tip stained very faintly, while the furthermost piece stained deeply.

DISCUSSION

The fact that the skeleton of the Solanderiidae is internal and is completely covered by a layer of epithelial cells in the living animal has undoubtedly led to the earlier belief that the skeleton is mesogleal in origin. However, it is evident from this study that each branch of a colony is composed of several coenosarc tubes. The appearance given in cross sections of a branch tip (Pl. 3, Figs. 1 and 3) is very similar to that of a cable containing many wires, cut in cross section. Each of the coenosarc tubes consists of endoderm, mesoglea, and ectoderm, and therefore could be expected to be capable of producing a perisarc, just as are other skelton-bearing hydroids.

The outer epithelium of the colony probably is formed by fusion of the superficial layer of the ectoderm of the peripheral coenosarc tubes in each branch. This idea is given weight by the fact that the outer epithelium can be seen to be continuous with "inner" ectoderm of the coenosarc tubes, especially at polyp bases (Pl. 2, Figs. 3 and 4).

The "inward facing" ectoderm of peripheral coenosarc tubes and the ectoderm of the other coenosarc tubes contained in a stem apparently become modified into a secretory tissue which forms the skeletal fibres. Skeletal material is secreted only where the ectoderm of two or more coenosarc tubes make contact. The skeleton is thus a "perisarc" contributed to by two or more coenosarc tubes. The cross sections shown in Plate 3 support this interpretation, and there is no doubt that the skeleton is ectodermal in origin. The skeleton may differ in composition in young and older parts of the stem. Lignin pink, which is considered a specific stain for chitin, does not stain the skeleton of branch tips as readily as it does the skeleton from thicker parts of the stem.

The method of growth of the colony can be seen from serial sections of a branch tip. From the tip to the thicker parts of a branch there is a central coenosarc tube, with peripheral tubes which anastomise with the central tube and with each other (but the central tube anastomoses far more frequently with the peripheral tubes than do the latter with each other). The polyps communicate with more than one coenosarc tube at their base (Plate 3, Fig. 1), and this base may be very large and extend over two or more skeletal "holes". (Plate 2, Figs. 3 and 4).

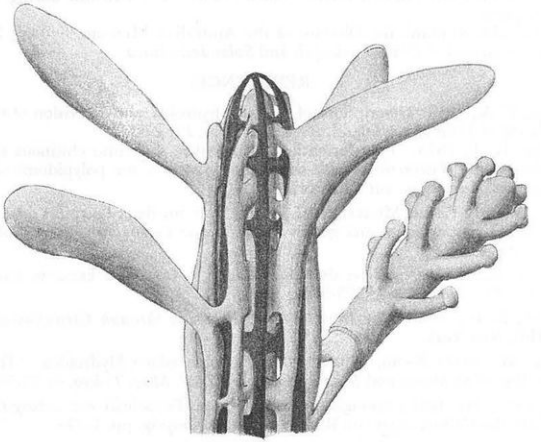
A serial reconstruction of the soft parts (endoderm only) (Text Fig. 2) shows these and other features of a stem tip. The central coenosarc tube has no terminal polyp. Some peripheral tubes may have a terminal polyp, but others do not. This type of anastomosing of coenosarc tubes is intermediate between that described for *Clathrozoon wilsoni* and *Plumularia procumbens* by Spencer in 1890, although there are fewer tubes in any one branch tip.

The skeleton shown in Text Fig. 2 was not reconstructed from serial sections. Skeletal rods were drawn between the peripheral endoderm tubes and then joined through the holes between the anastomosing tubes. This showed a skeleton of a branch tip similar to that described from serial cross sections as illustrated in Plate 3.

The stem is clearly a fasicled or bundled structure. But the status of each of the elements composing it is more difficult to define. The alternative possibilities are firstly, that the central coenosarc tube is equivalent to a hydrocaulus and is surrounded by its branches and stolons, which anastomose with it and with each other, and secondly, that the whole stem is a rhizocaulome, being composed of upright, branching and joining stolons.

Two features indicate that the first alternative is the more likely because (a) the stem tip clearly has a central coenosarc tube, and (b) the fact that this central tube anastomoses much more frequently with the peripheral tubes than the peripheral ones do with themselves. This suggests that the central tube is a primary structure, and the others are secondary to it.

The method of growth seems to be "secondary monopodial" (Hyman 1940, p. 406) in which the hydrocaulus and branches end in non-polyp bearing growing points. The growing points elongate and bud off laterally both polyps and new branches (this method is more typical of thecate hydroid colonies)—(Hyman 1940, p. 405). The picture is obscured somewhat, however, by the hydrorhizal elements of the stem.



Text Fig. 2 Diagrammatic reconstruction of the soft parts (endoderm only) of a branch tip from serial sections. One polyp is shown with ectoderm and tentacles. Note the central and peripheral endodermal tubes anastomosing freely; endodermal "feet" of the polyps. The skeleton (black) is formed by placing flattened longitudinal fibres between peripheral endodermal tubes and joining them through the holes left by anastomosing tubes.

SUMMARY

(1) The status of the New Zealand species of the family Solanderiidae is discussed, and material from Auckland and Wellington Harbour is recognized as *Solanderia misakinensis* (Inaba, 1892).

(2) It is concluded from a study of the internal morphology of the Auckland Harbour specimens that,
   (a) the skeleton is of ectodermal origin
   (b) the branches of the colony are fasicled structures consisting of a central coenosarc tube (corresponding to a hydrocaulus) which branches, and which anastomoses with its own branches and with hydrorhizal elements of the stem. These latter elements also branch and anastomose with each other.

(3) The method of growth of the colony is "secondary monopodial". That is, the hydrocaulus and its branches end in permanent growing points, and elongate by budding off polyps and other branches laterally.

ACKNOWLEDGEMENTS

I would like to acknowledge the assistance given by Dr. Patricia M. Ralph and Professor J. A. F. Garrick of the Zoology Department, Victoria University of Wellington. Dr. Ralph suggested this study and provided specimens to make it possible, and with Professor Garrick offered much constructive criticism during the writing of this paper.

I also wish to thank the Director of the Australian Museum, Sydney, N.S.W., for access to material of *Chitina ericopsis* and *Solanderia fusca*.

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Seasonal Changes in Growth of the Erect Stem of *Obelia geniculata* in Wellington Harbour, New Zealand

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Zoology Publications from Victoria University of Wellington, No. 44
Issued September, 1968

ABSTRACT

Seasonal growth changes shown by the erect stem of *Obelia* are described, based on the results of two series of field samples from Wellington Harbour, latitude 41.2°S, New Zealand. Endogenous and exogenous factors are involved in the form of growth expressed by the stem. Endogenous factors govern the sequential order in which the stem structures are formed. The feeding hydranths form first, then the reproductive gonangia. Exogenous factors, in the main temperature, affect the rate at which these growth sequences proceed. Warm temperatures (approximately 15°-20°C) give rapid metabolism and cold temperatures (approximately 9°-12.5°C) slow metabolism. Rapid metabolism shortens the time between sequences so that growth expressed in terms of gonangia production is attained when feeding hydranths are few (4-5) in number. Slow growth lengthens the time between the sequences. Maturity is then not attained until 8-10 feeding hydranths have been formed in comparison with rapid metabolic growth.

Seasonal growth forms can be distinguished within the annual temperature range. In winter, the stem is tall (20 mm), has up to 8 gonangia per stem, and from 1-4 branches on the hydrocaulus; in summer it is short, approximately half its winter height, with 1-2 gonangia per stem and no branches. Stems intermediate in growth form between these two extremes are found in spring and autumn. Production peak for gonangia is in the 9°-10°C winter temperature range in Wellington Harbour.

INTRODUCTION

The present work was commenced some six years ago by one of the authors (P.M.R.) as a follow-up study arising from an hypothesis (Ralph, 1956) that the temperature range of the three major water masses round the New Zealand coast affected the growth habit of *Obelia geniculata*. In the subtropical water mass of northern New Zealand, *O. geniculata* has short stems (5-6 mm) that lack branches, while in the intermediate zone of mixed waters the stems are longer (10-20 mm) and 8%-50% are branched. In the cold waters of the subantarctic zone the colonies are tall (up to 40 mm) and 80%-100% of the stems have branches.

The temperature in Wellington Harbour ranges from approximately 9°C in winter to approximately 20°C in summer. This range seemed sufficiently wide to further test the hypothesis that temperature was a major factor in the growth form attained by the erect stem. The first series of samples for the experiment were collected from Kau Bay (Fig. 1) in September 1961. Sampling continued through 1962 and terminatd in July 1963 as the senior author went on overseas leave. A partial assessment of the data made at this time was of sufficient interest to encourage further sampling if another opportunity arose for collection—particularly for collection in the autumn and winter seasons—as the records for this seasonal range were incomplete. Also,

as the original hypothesis formulated that the number of branches increased with decreasing temperature, it was very desirable to have as many samples from this temperature range as possible. The opportunity came to obtain more autumn-winter samples in 1967 and was undertaken by the junior author (H.G.T.) from March to July.

Hammett & Hammett (1945) recorded seasonal changes in colony composition for *Obelia geniculata* from Provincetown Harbour, Massachusetts, latitude 42.1°N. This is almost the same latitude in the northern hemisphere as the present study area is south of the Equator, namely, 41.2°S. The present study differs however from that of Hammett & Hammett in several aspects.

Firstly, the habitat of the hydroid colonies. The substrate in Wellington Harbour for *Obelia* is the broad, often longitudinally crinkled lamina of the southern kelp *Macrocystis pyrifera* (L.). The stolons are most frequently found in the grooves of the lamina, and the erect stems grow singly in rows along the stolons, Many of the laminae float at, or just below the surface of the water. Rarely is *O. geniculata* found growing on substrates other than *Macrocystis pyrifera* in New Zealand waters. In Provincetown Harbour, growing stems of *O. geniculata* were collected either from the shells of barnacles, or, when this habitat was depleted, from *Fucus*. The colonies grew in dense clusters on the barnacles, but on the *Fucus* singly "like trees in a grove".

Secondly, analysis of the Wellington Harbour material was made from formalin-preserved random samples. Hammett & Hamett selected living colonies (? the equivalent of our erect stems), "for desirable qualities"; this selection commenced at the time of collection and subsequently through to the setting up of the colonies for growth study in laboratory vessels. Sampling in Wellingon Harbour covered a full seasonal range for two and a half years, and a short "cold-water", 5 month, autumn-winter season. Sampling in Provincetown Harbour covered the "warm-water", 6 month, spring-summer season for seven consecutive years.

Thirdly, Hammett & Hammett regard endogenous chemical factors as solely responsible for seasonal changes in growth in *O. geniculata*. Our conclusion is that both endogenous and exogenous factors influence growth in this hydroid. The present study gives evidence that assessment of data for part of the seasonal range leads to a biased conclusion. Because of this conclusion, we have described the results of our short-term, autumn through winter sampling before that of the longer term annual data.

MATERIAL AND METHODS

(i) Collection and associated techniques

Colonies of *Obelia geniculata* were collected from two localities, namely Kau Bay (a weekly sample from September 1961 to July 1963) and from Point Haswell (a biweekly (= twice per week) sample from March to July 1967). The 1961-1963 samples, 48 in all, were subsidiary to a wider programme of harbour plankton collection (Fig. 1). The *Obelia* medusae from these collections were described by Wear (1965). The data given here for medusae are based on that of Wear.

Kau Bay is a relatively sheltered, wide, horse-shoe shaped bay, with the opening facing north-east. A narrow shingle beach is exposed at low tide. The kelp, *Macrocystis pyrifera* (L), on which the *Obelia* grows, occurs commonly along the rocky shoreline in Wellington Harbour at depths of 0.5 to 1.5 fathoms (Fig. 1). At Point Haswell a causeway has been built across the rocky foreshore to the small lighthouse beacon at the seaward extremity.

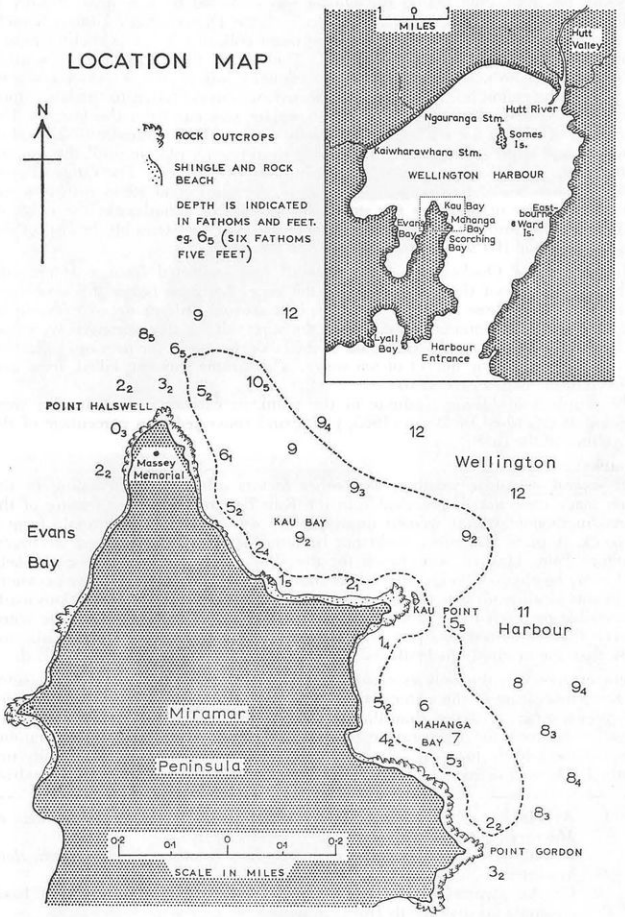


Fig. 1 Location map to show areas sampled in Wellington Harbour. (After Wear, 1966).

During the 1961-1963 period the *Obelia* was collected by a grapple thrown at random into the belt of kelp, from the deck of the Department's 16-foot launch. The surface temperature was recorded from water collected in a dip-bucket, using a centigrade thermometer graded to 0.2°C. The water, tide, and general weather conditions were also noted. One lamina, occasionally two, of *Macrocystis* was placed in a plastic one gallon bucket of fresh sea water for transportation to the laboratory. A random segment of approximately 2 cm square was cut from the lamina. The erect stems of *Obelia* were killed and fixed by placing the cut sample in 250 ml of sea water, and adding 40% formalin drop by drop from a pipette until the concentration of the formol-saline reached approximately five percent. The range of stem height; the number of feeding polyps per stem; the number of stems with terminal buds; the number of branches per stem; the number of gonothecae; the range of internode length, and the range of the distance apart of the stems on the hydrorhiza were recorded for 100 stems from each side of the lamina.

The colonies of *Obelia* from Point Haswell were collected from a *Macrocystis* lamina situated about the mid-portion of the stipe. Laminae below this area were usually damaged. Those near the growing point showed little or no colonization by *Obelia geniculata*. A lamina was cut from the stipe using a three-pronged wire rake attached to a long pole, and transported to the laboratory as in the previous collection series in a clean, plastic bucket of sea water. The sample was cut, killed, fixed and preserved in the manner noted above.

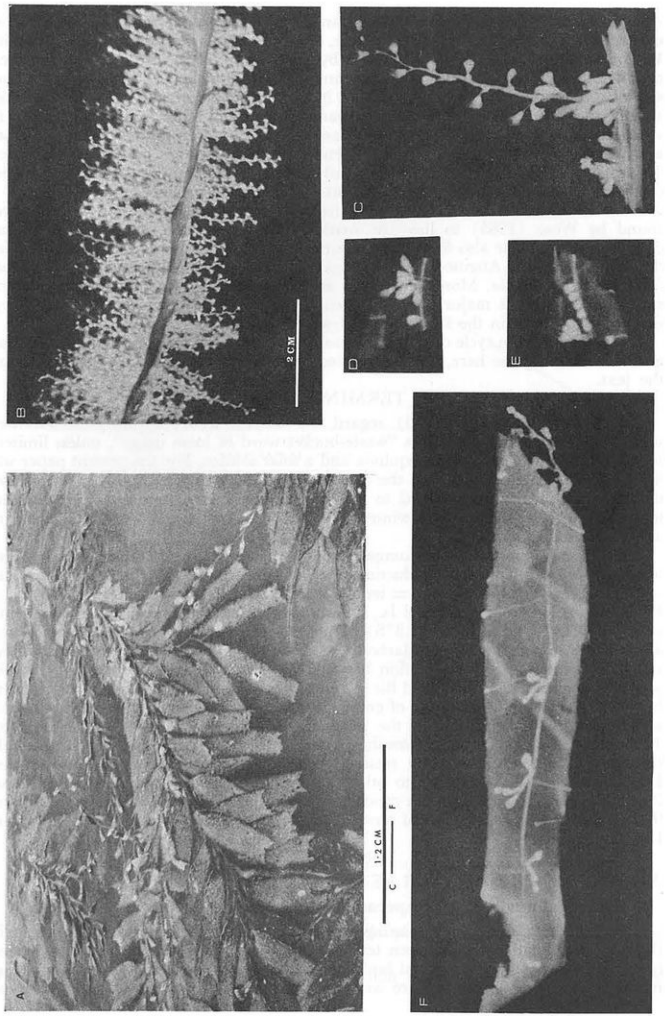
The numbers of *Obelia* medusae in the plankton catches from Kau Bay were estimated as described by Wear (1965, p. 2), and converted to a percentage of the total volume of the catch.

Remarks

On several occasions weather, and other factors affecting the running of the launch made collection impracticable in the Kau Bay area. Another feature of the original methodology that seemed unsatisfactory was collection by grapple from a boat deck. A piece of lamina could not be removed with any degree of accuracy. Therefore Point Haswell was chosen for the 1967 collection site because the kelp could easily be obtained in quantity under most weather conditions. On two occasions only in this locality, no samples were taken as high tides and stormy conditions made it impossible to reach the mid-sections of the stipes of the kelp. It should be noted however that combined analyses of both the short and long-term data indicated finally that the original "lucky-throw" method was the better collection method.

Temperature was the only external factor measured systematically. It is possible that the temperature of the water covering the surface laminae could be in the order of degrees warmer, or cooler, than that sampled in the bucket. The greatest degree of difference between the temperature of the water sample and that at the lamina surface is most likely to occur in calm weather, when the kelp floats virtually undisturbed. The well defined fluctuations that appear in the biweekly statistical analyses

Plate I A: Habitat of *O. geniculata* in Wellington Harbour, on the laminae of *Macrocystis pyrifera*.
B: Side view of *Macrocystis* lamina to show colonization by *O. geniculata* in winter.
C: An atypical erect stem taken in April showing a circlet of basal gonangia arising directly from the stolons.
D and E: Gonangia arising directly from the stolons. Sample collected in February.
F: Widely spaced summer stems showing decreasing order of vertical height.



(Figs. 4 and 5) possibly reflect sudden and short-lived temperature changes in the microclimate of surface laminae. However, calm conditions do not occur very often in Wellington Harbour. This is evidenced by meteorological records, and by the fact that of the 77 samples taken, only one sample had colonies of *O. geniculata* on one side of the lamina, and not on the other. No significant difference between colonization of the two surfaces of the lamina was found in any other sample. From this it seems reasonable to conclude that laminae have a fairly constant turnover by wind and water. Each side has an equal opportunity for colonization by settling planulae. It also indicates that with rare exceptions the water temperatures measured in the dip-bucket would closely approximate that at the lamina surfaces.

Fresh water influence, and pollution from the Ngauranga Freezing Works were found by Wear (1965) to have no marked effect upon the zoo-plankton in the collection area. Wear also found that the peak of the planktonic cycle occurs within the winter—May to August—period. Plankton is probably a major component of the food taken by *Obelia*. Moreover, it was anticipated that if these and other factors such as pH played a major role in influencing the growth rate of the hydroid, they would be indicated in the statistical analyses of the data.

Features of the life cycle of *O. geniculata* that bear on the interpretation of seasonal growth changes given here, but are masked in statistical analyses, are noted later in the text.

TERMINOLOGY

Hammett & Hammett (1945) regard the word 'season', applying to summer, winter, spring and autumn, as a "waste-bucket word of loose usage", unless limited to the interval between a solar equinox and a solar solstice. For the present paper we have not defined the word with the exactitude of Hammett & Hammett. But we do define it specifically with regard to Wellington Harbour in terms of approximate temperature range, as follows: winter, 9°C-12.5°C; summer, 15.5°C-20°C; autumn and spring, 12.5°C-15.5°C.

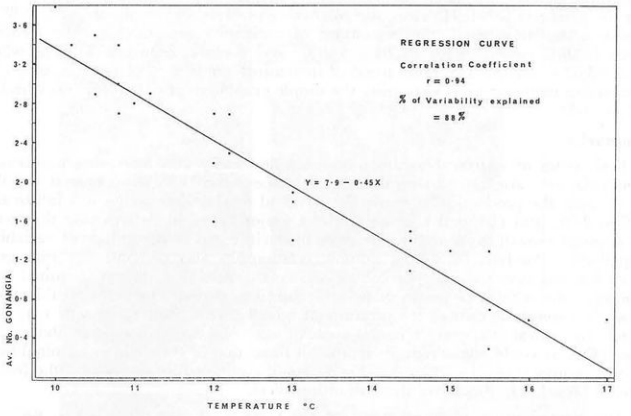
There is no doubt that loose usage of the words winter, summer, etc. can be misleading. For example, peak production of *Obelia* medusae in Wellington Harbour is winter, namely July-August (= temperature 9°C-12.5°C, latitude, 41.2°S). In Perseverance Harbour, Campbell Is., it is summer, namely February-March (= temperature 9°C-10°C, latitude 52.3°S). Thus, if peak production of medusae is not also defined for Perseverance Harbour in terms of temperature and/or latitudinal range, it could imply a correlation between the season of peak production in this southern hemisphere locality, and the season of peak production recorded for northern hemisphere localities. Formation of gonangia reaches a peak through increasing warm water (summer) conditions in the northern hemisphere (Hammett & Hammett, 1945: Russell, 1953). In the southern hemisphere the peak is reached through decreasing temperatures. When seasonal terminology is disregarded, Perseverance Harbour is seen to be similar to other southern hemisphere localities in the New Zealand region where maximum production occurs in cold water temperatures about 9°C. That is, winter, in seasonal terms of the lower latitudinal range of Wellington Harbour waters.

RESULTS

A. 1967 AUTUMN-WINTER SEASON Text-Figs. 2-5

Statistical analysis—Temperature and growth correlation

The data obtained from the samples were analysed (Figs. 2-5) to determine if there were a relationship between temperature and three characteristic stem structures, namely, gonangia, terminal buds and axillary branches. Figure 2 expresses the relationship between temperature and gonangia. The Regression Curve equation



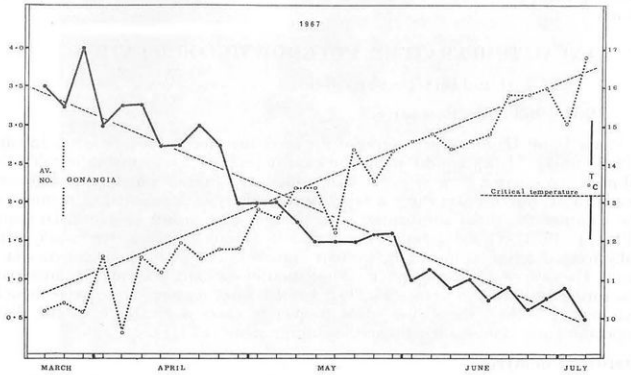


Fig. 2 1967 autumn-winter seasons. Gonangia plotted against temperature using the statistical method of least squares.

Fig. 3 1967 autumn-winter seasons. Correlation between the average number of gonangia per stem, and temperature.

using the method of Heine (1966) is: $Y = mX + c$. The value of $m = -0.45$ and the value of $c = 7.9$. Therefore, $Y = 7.9 - 0.45X$. The correlation coefficient (r) for the gonangia is —0.94. Thus, the percentage of variability explained (r^2) is 88%. Using a similar method, the percentage of variability explained for the terminal buds is 23% where $Y = 121.28 - 5.56X$, and for the branches 21.75% where $Y = 83.62 - 4.73X$. The same trend of increasing numbers of gongangia, etc., with decreasing temperature is seen using the simple graphic relationship for two variables (Figs. 3-5).

Remarks

Both series of statistical analyses demonstrate clearly that increasing numbers of gonangia, etc., are formed with decreasing temperature. Both series suggest (a) that in summer the production of gonangia, terminal buds and branches will fall to zero (Figs. 2-5), and (b) that temperature is a major factor in determining the trends of seasonal growth expressed by the erect stem (in terms of percentage of variability explained). Analysis by simple graphic relationship also demonstrates that great variation can occur in the percentage of stems possessing gonangia, terminal buds and branches within the weekly or monthly sampling period. Furthermore, this simple analysis suggests a critical temperature at which fluctuations in growth may take place. By critical temperature in this context, we mean that temperature above 13°C e.g., (Fig. 3) could either reduce or prohibit formation of gonangia or terminal buds. Temperatures above 11.5°C, e.g., (Fig 4) could either reduce or prohibit the formation of branches in the axil of the hydranth pedicel.

The annual range of morphology of the erect stem, described below, indicates however, that the relationship between stem growth and temperature is more complicated than is suggested by the results obtained from the autumn-winter seasons alone.

B. ANNUAL TEMPERATURE AND GROWTH CORRELATION

[Pls. I, II and III: Text-Figs. 6-9]

1961-1963 collection series

Plates I and II show the features of the erect stem for the four seasons. In winter (Pl. I, b: Pl. II, a) typical stems are close together, characteristically tall (15 to 20 mm), terminated by a hydranth bud, with 1 to 3 branches and up to 8 gonangia per stem. The stems also carry a fairly heavy epiphytic population of diatoms, and the chitin of the nodal annulations is medium to dark brown in colour. In summer (Pl. I, f: Pl. II, f) the stems are short (8.5 to 12 mm), wide apart, usually with a fully formed terminal hydranth, without branches, and with up to 2 gonangia per stem. They carry a heavy epiphytic population of diatoms. As could be anticipated, the spring and autumn stems (Pl. II, b and h) show features in between these two extremes. However, the spring stems frequently carry a greater epiphytic diatom population and more branches than the autumn stems (Pl III).

Statistical analysis

Figure 6 shows the pattern of increasing numbers of gonangia, terminal buds and branches with decreasing temperature, but does not indicate the well defined seasonal growth forms indicated for the erect stem in the visually selected examples of the stem described above. Figure 6 also demonstrates that gonangia and terminal buds may occur throughout the year. However, in late spring (November) or in the summer months (December, January, February), the percentage of stems with

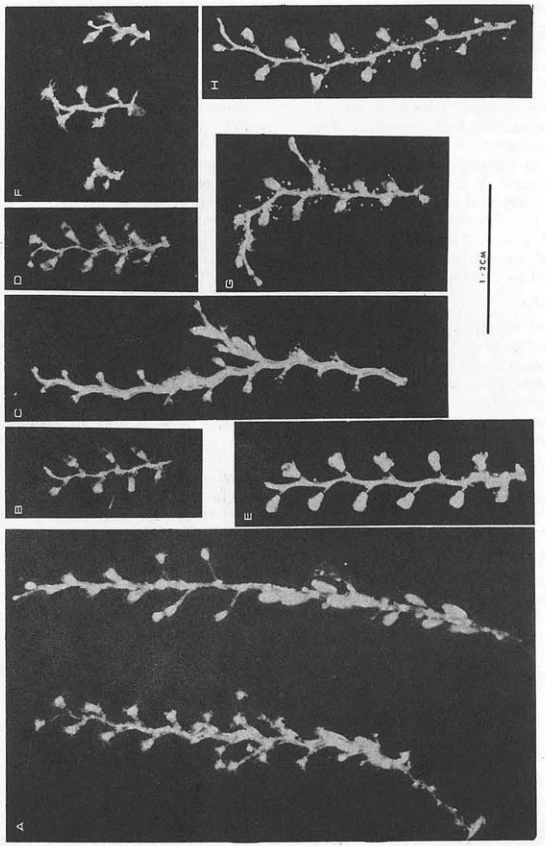


Plate II A: Tall, branched winter stems.
B and D: Short spring stems, with epiphytic diatoms.
C and E: Medium height spring stems.
F: Summer stems with epiphytic diatoms.
G and H: Autumn stems of *O. geniculata*. The epizooite is a species of *Epistylus* which is commonly attached to the erect stem at this season.

branches is very low. The percentage may even reach zero. Moreover, in the two late spring, summer seasons analysed, there is a well defined rise in the production of gonangia. This production rise of gonangia is more or less paralleled by increased numbers of medusae in the plankton.

Some of the data of the 1961-1963 collection series is shown statistically expressed using the method of least squares. The calculated percentage of variability explained for temperature and gonangia (Fig. 7) is 46.04%, where $y = 3.06 - 0.15X$. That is, at temperature 12°C, forty-six percent of the stems would have 1.2 gonangia per stem. For gonangia and branches (Fig. 8) the percentage of variability explained is 46.04% and $Y = 0.15 - 7.63X$. Also, the percentage of variability explained for temperature and branches and for temperature and feeding polyps was calculated, but is not figured. The percentage for temperature and branches is 27.5% and $y = 0.176 - 0.0148X$. In marked contrast is the percentage for the number of feeding hydranths on the hydrocaulus. The percentage explained is only 0.4% where $y = 5.53 - 0.024X$.

Remarks

Visual assessment (Pls. I and II) and graphic analysis (Fig. 6) demonstrates a changing pattern for growth of the erect stem during the year. The highest percentage of terminal buds, branches and gonangia occur in the autumn to winter season. All these stem characters are found with decreasing frequency on the stem as water temperatures rise in spring and summer. Branches rarely occur on stems when water temperatures approach the maximum. Gonangia in contrast rise in numbers with the approach of maximum temperature. All in all, these trends suggest a strong correlation between temperature and the seasonal form and maturity attained by the stem. However, when this correlation is tested utilizing the method of least squares, the assumption that temperature is the controlling factor in seasonal habit is less obvious. The percentage of variability explained ranges from non-significant for the feeding polyps (0.4%) to varying degrees of significance, for the other structures.
For the branches and terminal buds it is 27%.
For the gonangia it is nearly twice as high, namely 46%.

In brief, the analysis of the long-term series indicates that changes in stem form occur throughout the year. These are related to the four seasons and thus indirectly to temperature. The degree of correlation between temperature varies for the stem characters assessed. It is lowest and non-significant for the feeding polyps. It is highest and quite significant for the gonangia. Correlation for the terminal buds and branches is significant and in between the two extremes.

COMPARATIVE ACCOUNT OF THE AUTUMN-WINTER AND ANNUAL GROWTH TRENDS

The autumn-winter seasons of 1967 and the annual analyses of the 1961-1963 seasons give a correlation between temperature and growth expression for the branches, and terminal buds as 21% and 27% respectively. While this is not a high degree of correlation it is acceptable statistically as significant for biological material. Nonetheless, it indicates that factors other than temperature are operative in determining the changing pattern of these stem structures, and that these factors operate at varying levels throughout the year.

In contrast is the correlation between temperature and gonangia for the autumn-winter seasons of 1967 and for the similar seasons of the 1961-1963 series. The percentage of variability explained for the autumn-winter seasons is 88% as against 46% for the whole year. In the autumn-winter seasons, with r^2 —0.94, the probability of there being no correlation is less than 1%. Temperature then accounts for

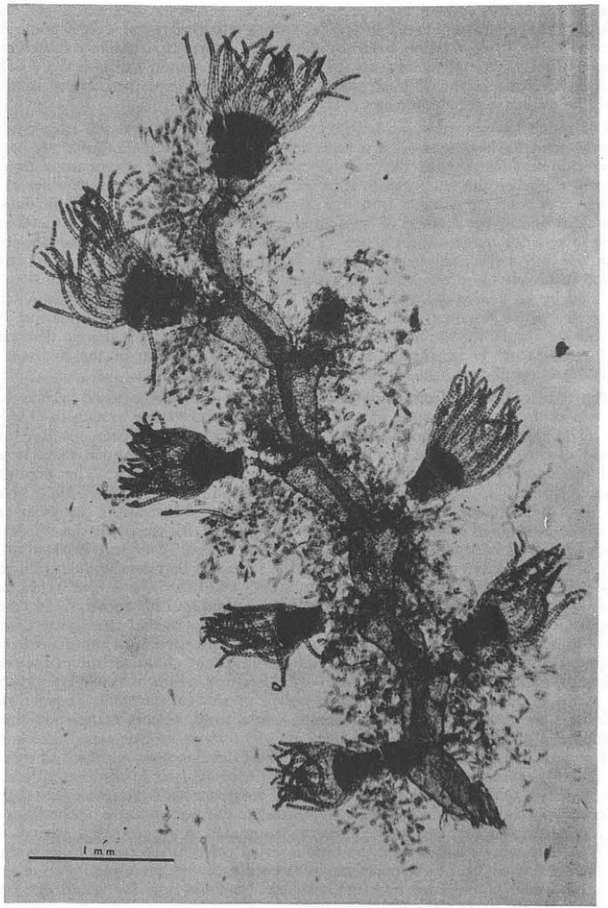


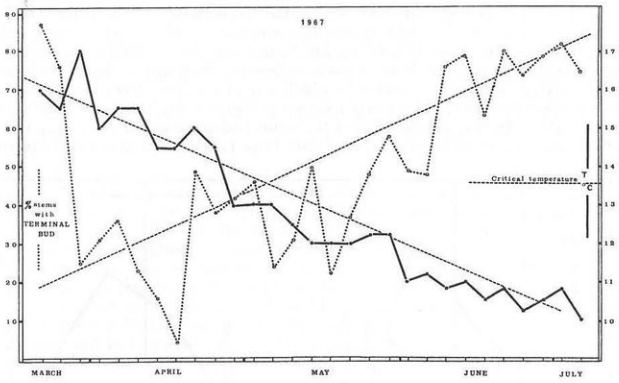
Plate III Photomicrograph of part of a late spring stem to show the epiphytic diatoms.

88% of all factors influencing the growth of the gonangia in autumn and winter. This is highly significant. In a biological system it is recognised that it is impossible to make a 100% correlation with any factor or factors. It is unlikely therefore that data on other external factors, e.g. pH, salinity, etc., would increase the coefficient by a significant amount. The margin of 0.006 left to be explained is very small, and could be an error in methodology.

The much lower correlation with temperature for the four season range, in comparison with the two seasons, is a reflection of two facts. Firstly, gonangia occur in small numbers throughout the year. Secondly, the numbers of gonangia produced rise sharply when the temperature approaches the annual maximum. Neither of these occurrences were predictable from the short-term results. The short-term data give a straight line by the method of least squares and suggest that gonangia numbers will drop to zero for temperatures above 17°C (Fig. 2). If the autumn-winter series had been the only series analysed, the result, as already noted, would have been strongly biased in favour of an exogenous factor being the controlling agent of the stem form attained by *O. geniculata*. Sampling over a whole year clearly shows that a sigmoid curve is a better representation for the seasonal trends in gonangia formation. Nonetheless, the results for the terminal buds, the branches and the gonangia demonstrate that temperature is significant in the overall growth pattern of the hydroid. It is particularly significant in the formation of the gonangia.

The results obtained from study of gonangial growth are recognised here as a more reliable guide than other stem features to the factors governing growth in *O. geniculata*. The reason is as follows. Where a series of samples are being utilized over a period of time, rather than observations on one colony, or one erect stem, it is best to have colonies at approximately the same stage of maturity. The presence of gonangia usually indicates that a colony is well established. Therefore their presence will also give a better indication than the number of hydranths per stem or the presence of terminal buds, etc. that a colony is mature. Moreover, hydranth development from bud initiation to senility is completed within 72 hours (Hammett, 1943, p. 350). Gonangial development including the initiation and development of medusae is not reached within 72 hours (Hammett, 1943) and may take several weeks. As the blastostyle in the gonangium reaches senility, it is shed and not replaced. In contrast, a hydranth may be produced from a site where a polyp had previously emerged, matured and regressed, and recurrent growth is integrated into the growth of the colony as a whole (Hammett, 1943). This growth cycle, characteristic of the feeding hydranth, undoubtedly contributes to the very low non-significant correlation between temperature and the number of feeding polyps present on the stem. Regeneration of hydranths in already present hydrothecae could mask almost completely the true relationship of temperature to this stem growth feature in long-term field samples. The gonangia therefore with their longer time of development and lack of regenerative powers are better stem structures for statistical analysis in the present instance. Coupled with the longer development time of the gonangia is the possibility that they do not respond to the same extent to minor day to day temperature fluctuations as do the hydranths and terminal buds. This is substantiated in the statistical analysis of the 1967 autumn-winter data where the time interval beween collections was the shortest for the present study. The fluctuations in successive biweekly samples (Figs. 4 and 5) are more clearly defined for terminal buds and branches than for gonangia (Fig. 3).

There are other features of the long-term series that bear on our interpretation of seasonal growth that have not yet been discussed. For example, gonangia and medusae occur throughout the year. It is probable therefore, that planulae settle and new colonies arise throughout the year. Thus, the monthly mean for any expression



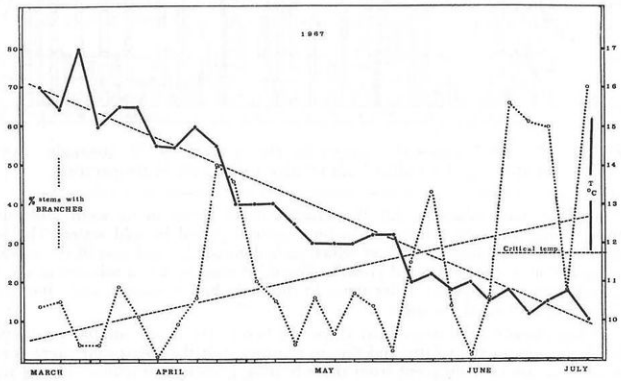


Fig. 4 1967 autumn-winter seasons. Correlation between the average number of branches per stem, and temperature.

Fig. 5 1967 autumn-winter seasons. Correlation between the percentage of terminal buds present and temperature.

of growth for the erect stem does not necessary represent the true average growth pattern of stems growing for one month at the mean monthly temperature. Individual samples for the month might represent a mixture of tall, mature stems; short immature stems, and stems of medium height and maturity depending on the time the stems have been growing from planula settlement. Alternately, the monthly average may represent individual samples in which one of the three stem types is dominant. The average percentage of gonangia shown in Figure 6 for November 1962 illustrates this situation. In one sample, 96% of the stems had gonangia present, were tall stems, with branches and with nodal rings of dark brown chitin. The stems and hydrothecae

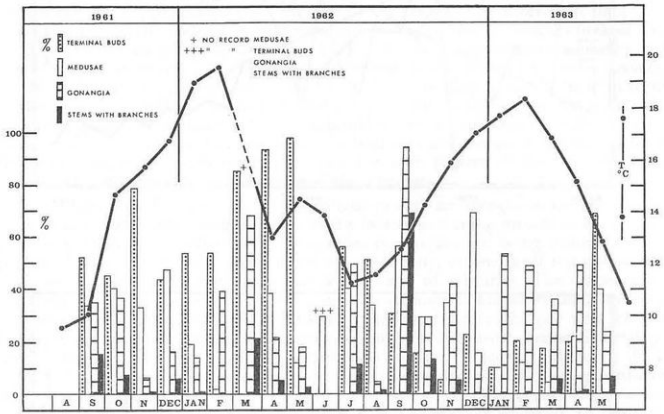


Fig. 6 1961-1963 seasonal changes in the percentage of gonangia, medusae, branches and terminal buds relative to changes in temperature.

were thick with diatoms. All the characters of these stems were indicative of approaching senescence following a long growth period in cold water. The second sample had small stems, 100% of which lacked gonangia, and overall the stems were indicative of a short period of growth. The third sample, was a mixture of the above growth forms, namely the taller stems in the main had gonangia, while the majority of short stems lacked gonangia.

It has already been noted that there are two peaks in the annual production of gonangia, one in the winter and one in summer. But the stems that carry gonangia in summer are very different from those bearing gonangia in winter. This is another example where the bare statistic does not fully represent the seasonal morphology of the animal. Direct observation of the samples for February shows that most stems at this time of year are very short, seldom have more than 4 hydranths, and that the distal end is terminated not by a bud, but by a fully formed hydranth. There may also be one or two gonangia present. However, other patterns of stem growth along the stolon have been observed. Plate I, f, shows a decreasing order of vertical height for stems along the stolon, and the "erect stem" may be represented only by a

gonangium arising directly from the stolon (Pl. I, d and e). The latter "stems" however were not included in the statistical analyses of the data.

A variation of this pattern is seen in Pl. I, c., where the erect stem growth expression is shown by a single stem surrounded by a circlet of gonangia arising directly from short, radially arranged stolons. This example is of interest as it comes from an autumn collection. The pattern of numerous gonangia on the stolons in this sample suggests a sharp upward fluctuation in temperature at a critical level, combined with calm weather, resulting in near lethal temperature conditions at the lamina surface. That is, summer conditions in autumn.

Figure 6 shows what we regard as another example of the effect of unseasonable climate. In the March-April period for 1962, temperature fell from a mean maximum of 19.5°C in February, to a mean of 12.8°C in April. The lowest temperature recorded for April was 10.4°C. The latter temperature approaches the annual minimum for Wellington Harbour. A temperature range of approximately 10°C to 13°C usually occurs from June to August not March to April. It is probable therefore, that this rapid, unseasonable temperature drop is responsible for the very high percentage of stems with terminal buds in March and April instead of later in the year as is suggested by analysis of other data obtained during the experiment. The more gradual fall in temperature from the February maximum in the 1963 March-April period shows an appreciably lower percentage of stems with terminal buds.

In brief, the above paragraphs indicate firstly, that assessment of part only of the seasonal data gives a biased result in favour of temperature as the prime factor in the seasonal growth form attained by the erect stem. Secondly, that some facets of stem growth are also obscured in statistical analyses of the annual data. But a combined assessment strongly suggests the same seasonal trend in changing growth form for the stem as that noted in direct visual observation of the two collection series.

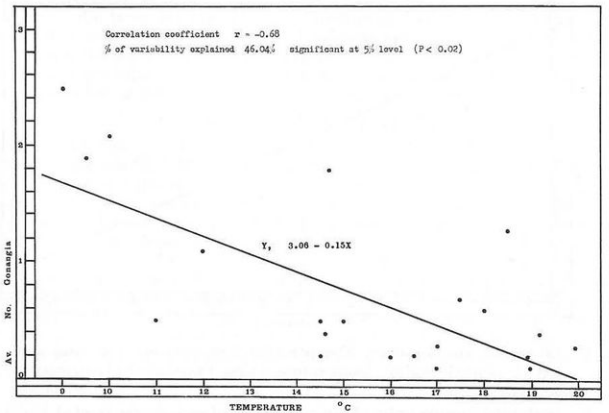
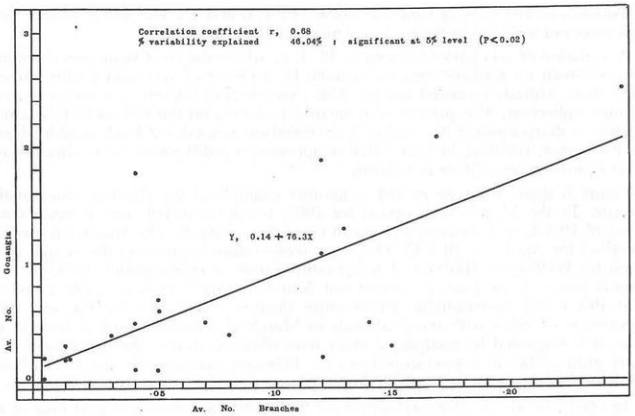


Fig. 7 1961-1963 sample series. Gonangia plotted against temperature using the statistical method of least squares.



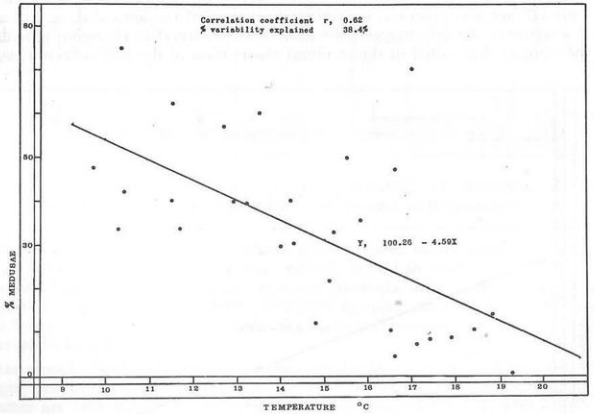


Fig. 8 1961-1963 sample series. The percentage of medusae per total volume of catch, plotted against temperature using the statistical method of least squares.

Fig. 9 1961-1963 sample series. Average number of gonothecae plotted against the average number of branches per stem using the statistical method of least squares.

SEASONAL OCCURRENCE OF *OBELIA* MEDUSAE IN WELLINGTON HARBOUR

So far, description has been of the colonial phase of the life cycle. Assessment of the seasonal occurrence of the medusa in the plankton has also a place in the final interpretation of stem growth. *Obelia* medusae are found all the year round in Wellington Harbour. Continuous occurrence is also recorded for the British Isles (Russell, 1953). Around British coasts, *Obelia* medusae are most abundant from spring to late autumn. In Wellington Harbour, they are most abundant from late autumn to winter. Numbers of medusae in the latter harbour do however also rise on occasion during summer (Fig. 6). But there is good evidence that this is due to aberrant growth causing the formation of gonangia on the hydrorhiza in place of the normal hydrocaulus. Thus, it is not a comparable rise in numbers to that found each winter. This aberrant summer rise is reflected in the annual low percentage of variability explained for the medusae per volume of catch when plotted against temperature. The percentage explained is 34.4%, where $Y = 100.26 - 4.59X$. (Fig. 8.)

Further evidence that there is a reversal in the southern hemisphere of the seasonal peak recorded for the northern hemisphere comes from records of *Obelia* from Perseverance Harbour, Campbell Island (latitude 52.3°S). Medusae are first recorded in January, when the sea-surface temperatures average approximately 10.0°C. A peak is reached in March (temperature average, 9.0°C). The medusae number is then between five and six thousand per 15 minute tow. By May, temperatures have dropped to about 7.0°C and the numbers of medusae per tow decrease, until by June no medusae were recorded (P. Roberts, Zoology Dept., V.U.W.—personal communication). The lowest temperature in this subantarctic area is approximately 5.0°C recorded in July-August.

The temperatures at which medusae have been recorded from Perseverance Harbour are comparable with those from Wellington Harbour in winter, where the lowest temperature is about 9.0°C. Wear (1965) found the peak in planktonic medusae in the latter area to occur in July-August. From both plankton studies it appears then that the largest numbers of medusae are obtained in the New Zealand region when the sea surface temperatures are around 9.0°C. It is also probable that there is a lower lethal temperature. This may occur in areas where the sea surface temperatures fall markedly below 9.0°C for any length of time.

INTERPRETATION OF RESULTS

Our interpretation of the overall growth sequence for *Obelia geniculata* from the settling of the planula, is nutrition, reproduction and regression. We regard this sequence as genetically determined, and not alterable by exogenous influences. But the rate at which the sequences proceed is materially affected by the season at which the planula settles and thus indirectly by temperature. Consequent on this conclusion, is the fact that the developmental growth form finally attained by the erect stem is variable from season to season and within the season, according to the length of time the stem has been growing within a particular range of temperature. This hypothesis is shown diagramatically in figures 10 and 11.

Support for the idea that exogenous factors such as temperature affect the growth processes of hydroid colonies comes from the work of Berrill (1948). Berrill found that growth in colonies of *Obelia* and other hydroids fluctuated greatly with temperature. They disappeared and reappeared respectively as the temperature rose and fell significantly above and below 20°C. One of the three species of *Obelia* studied by Berrill was *O. geniculata*. It is probable therefore in latitudes where the mean annual sea temperature is approximately 20°C that *O. geniculata* will be seasonal in its growth. Temperature is thus not a limiting factor in *Obelia* growth in Wellington

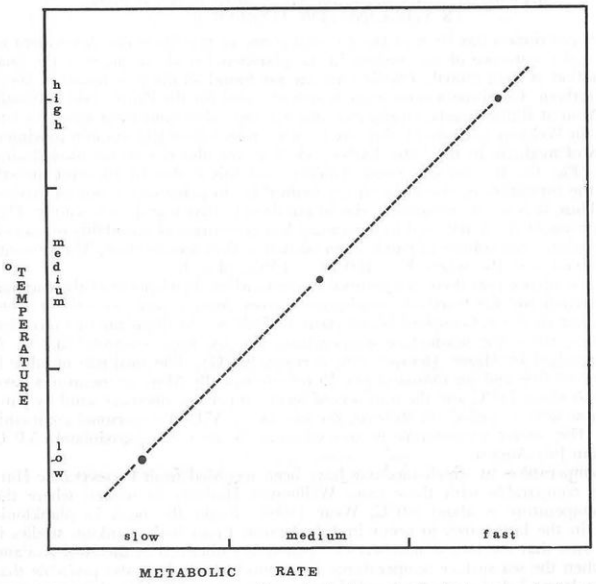


Fig. 10 Diagrammatic presentation of the hypothesis that seasonal changes in the exogenous factor temperature, is related to the metabolic rate and growth of the erect stem.

Harbour, except perhaps in February, when the microclimate of the surface laminae may exceed 20°C in days of calm hot weather. Also, growth is unlikely to be seasonal in latitudes in the New Zealand area higher than 40°S.

It is also concluded from the present study, that increasing temperature produces an increase in the metabolic rate of the colony, and that the endogenous sequences, nutrition, reproduction and regression, follow one another in rapid succession. Of significance for this conclusion is the decreasing order of stem height illustrated in Plate I, f., because as Manton (1940, p. 248) notes, budding in colonial hydroids takes place when growth is complete. This growth pattern of decreasing height along the stolon, also suggests that the rate of metabolism may be sufficiently rapid when temperatures approach the upper lethal limit for the two growth sequences, nutrition and reproduction, to be virtually telescoped one into the other. The "erect stem" then consists of a gonangium arising directly from the stolon (Pl. I, d and e). This type of "stem", is most likely to occur in Wellington Harbour if the planula settles in a period of high (18°-20°C) temperature. Nutrition for the developing gonangium would be provided by the hydranth-bearing stems already present on the stolon.

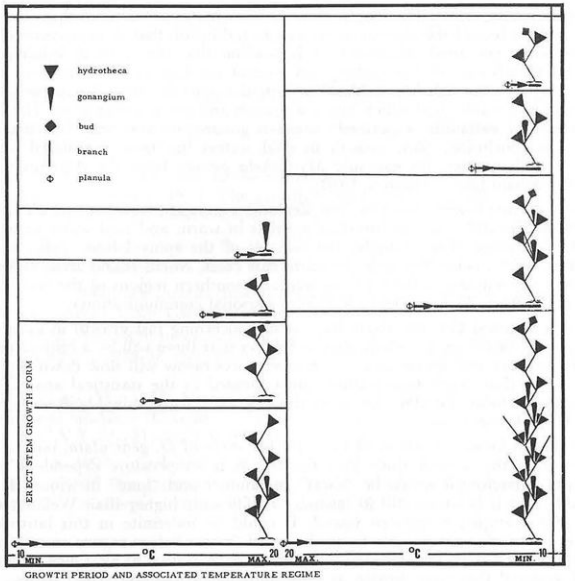


Fig. 11 Diagrammatic presentation of the hypothesis that the seasonal stem form attained, is related to the season at which the planula settles and the time available for growth within the seasonal temperature range.

If the planula settles earlier in the summer season, it will have a longer growing time in cooler water, and the stem will be able to produce feeding polyps in varying number, the number attained being dependent on the temperature when the planula settled and the subsequent growing time available before lethal temperature is reached.

In winter the metabolic rate slows with a decrease in temperature. The time period for the sequence nutrition, reproduction, regression, is lengthened. Before gonangia are produced the stem attains a vertical height approximately twice that attained by a stem in summer. If the planulae settle in a month of minimum temperature range the stems will give expression to growth, not only in vertical height, but also in the production of branches. Further evidence for this conclusion is given by the stem form of colonies collected in February from Kerguelen Island (Ralph, 1956). Mean annual sea temperature for Kerguelen Island is approximately 2°C below minimum winter temperature of 9°C in Wellington Harbour. No gonangia are present on these sub-antarctic colonies, but 80% of the stems have long branches. The erect stems without branches are very short, with terminal growing buds suggesting that

they were young stems. The length of some branches approximated that of the main stem, and we regard this growth pattern as an indication that absolute vertical height is genetically governed. Moreover, it is possible that this vertical height may be attained in advance of the endogenous control mechanism initiating reproduction when long slow growth is possible. The nutritive growth phase is expressed then in the form of an axial bud which forms a branch and not a gonangium. The correlation (46% of variability explained) between gonangium and branches further supports this conclusion. Slow growth in cold waters has been postulated for other hydroid coelenterates, for example *Myriothela penola* from the Argentine Islands area of Graham Land (Manton, 1940).

Other marine invertebrates in New Zealand waters are known in which there is a significant size difference in breeding animals in warm and cool water areas of the distribution range. For example, the females of the spiny lobster *Jasus edwardsii* (Hutton, 1875), come into berry in warm east coast, North Island areas at a smaller size than the females in the south-eastern and southern regions of the South Island. (Dr. R. B. Pike, Zoology Dept., V.U.W.—personal communication).

If it is assumed that the above hypothesis concerning fast growth in summer and slow growth in winter is correct, then it follows that there will be a critical temperature in autumn and spring at which temperatures below will slow down the overall stem metabolism. Such temperatures are indicated in the statistical analysis of the autumn winter data for 1967, i.e. approximately 13°C for terminal buds and gonangia and 11.5°C for branches.

We do not know the length of the stem life cycle of *O. geniculata,* but have concluded from the present study that the length is temperature dependent in large measure. Therefore it would be "short" in summer and "long" in winter. It is also probable that it is longer still in latitudes significantly higher than Wellington Harbour. For example, Kerguelen Island. It could be indefinite in this latitude. The feeding hydranths may be able to survive much longer before regression sets in. They may not have a cycle of regression followed by regeneration at all. Once fully formed and functional they may remain as such until the whole erect stem regresses. It is not inconceivable that regeneration of the blastostyle also occurs in such latitudes. Furthermore, it is possible that regression of the feeding hydranth in winter temperatures in Wellington Harbour is not followed by regeneration to any great extent, and that the reverse is the case in summer temperatures. For example in summer, the number of feeding polyps during the life of the stem could be the same number as in winter, if there was a very rapid succession of feeding polyps regenerating in already existing hydrothecae.

As is so often the case with studies of the present nature, while some questions may be answered, many more arise and cannot be answered from the data obtained in the originating experiment. Future laboratory studies could provide some answers to the questions posed by the present field work.

ACKNOWLEDGEMENTS

The authors wish to gratefully acknowledge the help given by the Internal Research Committee of the Victoria University of Wellington by a grant in aid of technical assistance for the 1961-1963 collection series; to Professor L. R. Richardson for his helpful advice during the 1961-63 collection series, and to Mrs. Helen Elliott and Mr. R. Buddle for efficiently processing and recording the data from this collection series; to Professor J. T. Salmon and Professor J. A. F. Garrick for helpful discussion; to Mr. H. S. Roberts of the Applied Mathematics Division of the Department of Scientific and Industrial Research for assistance with the statistical analysis of data, and to Mr. M. M. Loper, Technical Officer of the Zoology Department, V.U.W., for photographing the specimens.

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Plastic Embedding of Zoological Specimens

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Victoria University of Wellington

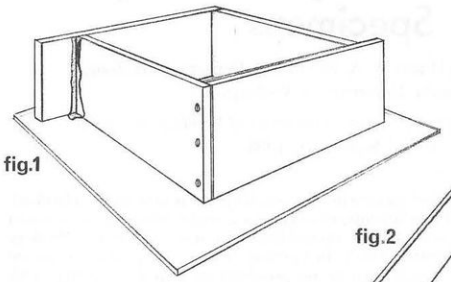
Zoology Publications from Victoria University of Wellington, No. 45
Issued September, 1968

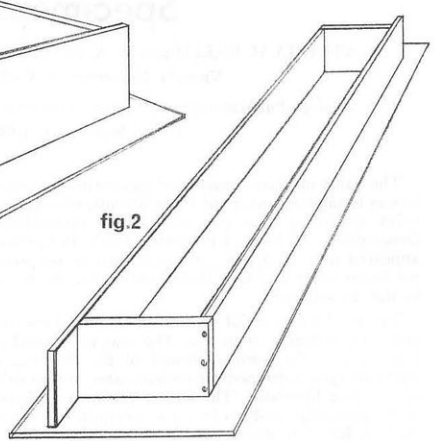
The value of plastic embedded specimens as a teaching aid is now well recognised. It was to take advantage of these benefits, especially as a replacement for wet-mount bulky specimens, that a programme of embedding was initiated in the Zoology Department, Victoria University. From instruction leaflets, etc., the technique appeared to be a simple one, with little or no problems to trap the unwary. This we found to be true to a limited extent for the range of teaching material required in the department.

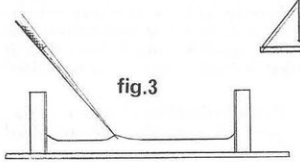
The teaching material we wished to embed covered a wide range both in surface and bulk texture and in size. The material varied from hard, barely porous shells and teeth to the open meshwork of the Porifera, through bone and cartilage to chitin-covered arthropods or coelenterates, and to delicate jellyfish or the early stages of the chick blastodisc. The largest specimen we have so far embedded is an eviscerated mammalian embryo 15 x 5 x 3.5 cm in which the skeleton had been stained with Alizarin Red S to distinguish the ossified tissue. The unpolished plastic block for this specimen measured 23 x 5 x 10 cm. The smallest individual specimens embedded were unstained copepods, 3-4 mm in length.

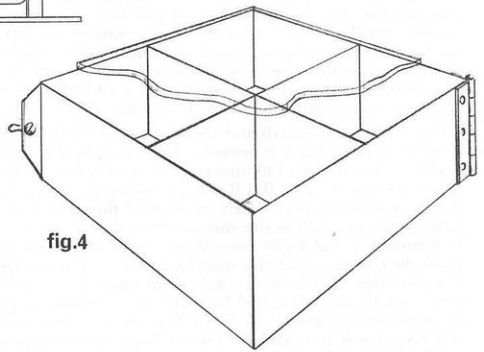
In the course of overcoming our difficulties with the embedding of such material, we also tried and found successful other procedures for sectioning the embedded material, and for block shaping and polishing. Comments on these procedures and on the embedding techniques we finally adopted we felt might be of interest and assistance to other workers contemplating plastic embedding of a similar range of zoological material. The quantities of material and the timing given for the embedding processes apply to polyester resin Au 8018C, styrine monomer and catalyst M.E.P.K., as manufactured by A. C. Hatrick (N.Z.) Ltd., Tawa, Wellington.

Basically, the chemicals and apparatus essential for embedding are the same if the embedding programme is extensive (for example, the skeletal material for an undergraduate class of some 150 students) or one of a few specimens for specialist teaching, or just general interest. But if the embedding programme to be undertaken is extensive, it is of value, particularly in terms of time saved and material used, if embedding moulds are built to size and can be used over and over again. The moulds shown in Figures 1, 2 and 4 also provide for easy release of the plastic block. Figure 4 illustrates the type of mould now most frequently used in the Department. Figures 1 and 2 show the type used in the experimental stage of our embedding programme. These latter moulds are well suited for a run of embedding requiring only a small number of specimens, say twenty or thirty. The pattern illustrated in Figure 1 is quick, easy and economical to make. We cut to shape formica topped bread boards, but "off-cuts" of any similar material obtainable from paint and glassware merchants would be equally satisfactory. Long wood screws fasten the two parts of the angle L-pieces together. The paired L-pieces of the mould can be adjusted to give a variety of shapes and sizes. However to obtain this flexibility of usage for a single pair of L-pieces, the joints and fit of the surface of the angles to the base plate must be leak









Figs. 1 & 2 Formica faced L-pieces placed to form a mould.
Fig. 3 Sectional view of mould to show the approximate degree of "tackiness" of the resin surface prior to the pouring of the next layer.
Fig. 4 Metal-sided, 4 in one mould showing also the glass cover plate.

proof. We attained this inexpensively by using plasticine and/or silicone wax in the manner illustrated in Figure 1. A variant of this type of mould we have found very useful for small specimens is one in which the wooden L-pieces are faced with glass instead of formica. We utilized 3″ x 1″ and 3″ x 2″ microscope slides for this purpose, and glued them to a wooden frame.

Flexibility of usage from a single "box" mould for large projects (Figure 4) is obtained by the provision of metal inserts, giving a one, two, three, or four-in-one box. Ease of removal of the blocks from this type of mould comes from the ability to open out the sides. The glass base and top plate give a surface to the hardened block that needs very little polishing. Virtually no presurfacing of the glass or metal with a releaser compound such as silicone wax is necessary as the plastic shrinks away from the mould during hardening.

Basically, the whole process of embedding can be summarized in four words: dehydrate, clear, embed the specimen and polish the plastic block. But before proceeding with a project, if you wish to admire your handiwork as a plastic block which has a high surface gloss, is almost crystal clear, and without bubbles, it is necessary to possess considerable patience and be aware of some of the problems that may be encountered in carrying out the four basic techniques noted above.

DEHYDRATION

This procedure varies according to the type of material to be embedded.

A. *Dry material*

It is very desirable to know something of the past history of the material to be embedded. This is particularly the case with specimens that have been stored dry such as insects, crabs, spiders, corals, teeth, etc., but may have been subjected to fixation and/or temporary storage in liquid. If the past history of the specimen is not known, dry material is best placed in acetone under vacuum until all the air is removed, and the specimen thoroughly penetrated. If the specimen is large, this may take upwards of an hour utilizing vacuum pressure at 20-minute intervals. The specimen can then be redried or placed in acetone for storage prior to dipping it in the styrine monomer at the next stage in the embedding procedure (see also p. 4 for arthropod material).

B. *Wet preserved specimens*

The material should be thoroughly washed free of preservative and then dehydrated through 50% to 100% acetone. The length of time in which the specimen remains in the two grades of acetone depends, as with alcoholic dehydration, on the size and density of the specimen. In general, however, specimens of a similar size need less time in an acetone dehydration series than in an alcohol series. Acetone has another advantage over alcohol for dehydration in that it retains the colour of a wider range of specimens, particularly arthropods, than does alcohol. Furthermore, it does not matter for plastic embedding if the specimen is considerably hardened in texture by the acetone. In fact it is advantageous with soft bodied material as such material is then better able to withstand the pressure and heat of the setting resin without distortion.

This two-stage dehydration series proved quite satisfactory for all the material we have so far embedded, with two exceptions. These exceptions are echinoderms, and material that has been macerated in potassium hydroxide. With echinoderm material, a test specimen should first be tried in acetone, as acetone decolourizes and to some extent decalcifies many echinoderms. We have found it better to fix in formalin, wash in water, and then oven dry the specimen. Specimens that have had the tissues

macerated in potassium hydroxide need a more gradual dehydration than is afforded by the two-stage series. An 80% acetone bath should also be included in the series. Moreover, it will already have been appreciated that there will also be considerable shrinkage of this material. However, we found that both the monomer and the uncatalyzed resin restore in large measure the lost tonicity of the tissues.

It is also very advisable during dehydration to remove any trapped air in the specimen. This procedure is necessary particularly for material with an open meshwork support, such as that found in the Porifera.

Any further preparation of the material prior to embedding now depends on the morphological features required for display. For example, with arthropods it is usually the exoskeletal features, but with soft-bodied animals it is more often the internal morphology. With the completion of dehydration in acetone, arthropods in which the exoskeletal features are required need only be dipped for a few minutes in the styrine monomer and they are ready for embedding in the catalyzed resin. Soft-bodied animals and any arthropods required for observation of internal morphology should be cleared to at least semi-transparency.

CLEARING THE SPECIMEN

The general procedure we adopted was to clear the tissues to semi-transparency by placing the specimen in the styrine monomer. (The catalyzed resin completes the clearing process). The specimens may again need to be placed under vacuum, and should be allowed not only to sink to the bottom of the container but to remain there for some hours longer. Overnight is satisfactory for small specimens of dimensions 7 x 5 x 3 cm. This ensures a thorough penetration of the monomer into the tissues.

We use, however, a slightly different technique for specimens that have been macerated in potassium hydroxide. With this material we have a further series of clearing baths. The first bath is a 1:1 mixture of monomer and polymer. The second a 1:3 mixture and the final bath a 1:6 monomer/polymer mixture. Again the specimens should remain in each grade of the series for two or three days at least after they have sunk to the bottom of the container. The mammalian embryo (noted above on page1) remained in the final stage of the clearing series for 14 days after it had sunk to the bottom. This technique not only assists materially in giving and retaining tonicity in the specimen, but it eliminates air bubbles that may remain even after vacuum treatment, beneath the skin and in the oral and nasal cavities.

Nonetheless, we have not had 100% success in eliminating air bubbles in the hardened block with this type of material (Fig. 12). This is particularly the case with young embryos 2 to 5 cm in length that were not eviscerated prior to maceration and in which the newly formed bone is very porous. Our main problem has been the prevention of very tiny air bubbles forming in the marrow cavities and lungs. This may happen even from one to three days after the block has set sufficiently hard to be removed from the mould, but not hard enough for polishing. The after-clearing method we have found most successful is given on p. 6 under the heading "Insertion of the specimen in the catalyzed resin".

EMBEDDING IN PLASTIC

(a) *Preparation of the mould:* We have found that moulds or parts of a mould made of metal formica or glass, need only be greased with a very thin film of releaser compound such as silicone wax. During the exothermic hardening process, the plastic contracts sufficiently from the walls of the mould for the completed block either to fall out, or be released by gentle leverage with a flat thin blade between the mould wall and the block. But we do use a thick layer of silicone wax or plasticine sealing

compound at the junctions of the L-piece moulds or the internal partitions and between the base of the sides of the mould and the base plate to give a tight seal to prevent loss of the plastic (Figures 1 and 2).

(b) *Preparation of the resin:* After several months of trial we have found the following procedure to be satisfactory. It has produced clear, hard blocks of plastic. We prewarm to 50°C in the oven the glass container for receiving the catalyst. (The hardening process once started is irreversible, so only sufficient resin should be mixed with the catalyst to give the desired layer depth. It is unwise to exceed a depth of half an inch for any one layer). Then add the correct amount of resin to the correct amount of catalyst in the container. With resin AU 8018 C it is 0.5 ml of catalyst for every 50 ml of resin. Stir gently with a glass rod until the resin and catalyst are thoroughly mixed. When the catalyst is stirred in, a distinct colour change takes place from the pale blue of the uncatalyzed resin to pale green. The recommended volume given in the instructions supplied with the catalyst is in general too great for biological material. With the recommended volume of hardener, the heat generated during the hardening process is such that the resin tends to "boil" and the fully matured block has a yellowish hue. This colour however may also be in part the result of substances released from the specimen as the resin heats up.

We next put the mixture of resin and catalyst, sufficient to give a layer $\frac{1}{4}$" in depth, into the oven at 50°C for 3-5 minutes. [The reason for this time range is that different batches of resin vary in the time taken to the commencement of setting. It is advisable to test run a small amount of mix with each new batch of resin. The batch number is marked on the container supplied by the manufacturer.] Now gently stir the warmed plastic with a glass rod. After stirring it is ready to pour into the mould. When pouring is completed, put the mould back into the oven for 10 to 15 minutes. No damage occurs to the caulking plasticine in this time. Return of the newly poured plastic to the oven for a short time brings stray bubbles to the surface to burst, hastens the hardening process and ensures a hard clear outer layer for the block. Further hardening should be continued out of the oven until only the surface is tacky (Figure 3). Cover the mould to prevent dust settling on the hardening plastic. Should the hardening process be inadvertently carried further and the surface become quite hard, we have found that the next molten layer keys on quite well if the surface is covered with a shallow layer of acetone for 2 to 3 minutes. This prepares the surface for a better take of the next resin layer.The junction between the layers is then only visible in side view.

The block is now ready for another pouring of a $\frac{1}{4}$ to $\frac{1}{2}$ inch layer of catalyzed resin, prepared as above. When the layer has been poured, any air bubbles can be surfaced with a mounted needle and moved to the edges and burst.

(c) *Insertion of the specimen in the catalyzed resin:* Take the specimen from the preparatory fluid in which it is held and place it on the surface of the newly poured layer. Let it sink into the layer under its own weight. As noted above, the mould can then be returned to the oven for up to 15 minutes. Remove any air bubbles that still remain. Then allow this layer to harden at air temperature, either till hardened right through if the specimen is adequately covered by at least $\frac{1}{4}$ inch of molten plastic or, if it is not completely covered, until the surface is tacky. If the specimen is not completely covered, continue layering with the resin.

With deep specimens that cannot be covered with one pour of the catalyzed resin, the specimen should, if possible, be positioned in the mould so that apertures such as the mouth and nostrils are completely covered when the specimen is first placed in the resin or alternately completely covered, during the pouring of a single layer.

Otherwise these apertures may allow a major ingress of air into the specimen during the hardening of the plastic. Internal air bubbles resulting from leaving these apertures exposed do not always become apparent until the block is too far advanced in the hardening process for them to be successfully removed.

We use a different method for specimens that have been stained to show the skeleton. We have attained our greatest success with this material by lengthening out the time taken for the catalyzed resin to set to a stiff gel. We have tried two methods. In the first, we proceed as above to the pouring of the layer in which the specimen is to be placed. Put the specimen in this layer and return the mould to the oven for not more than 3 to 7 minutes. The lower time range should be used for small specimens. Cover the mould and allow the plastic to harden at room temperature. Follow a similar procedure for any other layers that need to be poured. In the second method we proceed as above only as far as the lower protective layer. The succeeding layer to take the specimen is prepared without heating the vessel to contain the catalyzed resin, and the resin and catalyst are mixed and poured at room temperature. After inserting the specimen in this layer, the covered mould is placed in the refrigerator for not more than 30 minutes, and the layer further hardened at room temperature. The procedure for any succeeding layers is the same. All in all, the first method has so far given the best results. The plastic of the block is clear, not tinted, but a few tiny bubbles may appear in the marrow cavities. In the second method the hardened block is usually a pale straw colour, but tiny bubbles occur less frequently.

TRIMMING AND POLISHING THE HARDENED PLASTIC BLOCK

Block trimming is virtually unnecessary when moulds such as those described above are used. But should trimming be required, a hacksaw with a fine toothed blade can be used.

The procedure generally advocated for producing a high gloss surface to the block is to hand polish first on a graded series of emery paper, then jeweller's rouge and finally a metal polish such as Brasso. We found hand polishing so time consuming even for a small block (2 x 3 x 4 cm) that the prospect of using this method on blocked material of dogfish skeleton sufficient for a class of 150 students became very unattractive both from the point of view of economy and the sheer physical effort involved.

The answer to both problems came with the installation of a Linisher belt sanding machine. Various small modifications to this sander (Figure 5) allow the polishing process to go on virtually unattended. Also the addition of a vacuum suction tube to the apparatus permits the removal of troublesome plastic fluff and dust from the atmosphere. We used a coarse belt (No. 50), medium belts (No. 60 and 80) and a fine belt (No. 100). The blocks were then polished on plywood discs (Figure 5) to which hat felt had been glued. We soaked the felt on one disc with Brasso and the second with Silvo. The first polish was with the Brasso followed by the Silvo. A vigorous rub on a Dacron surface gives a high gloss finish, or the surface can be spray-coated with plastic.

REMARKS

The above paragraphs give our findings on general procedural techniques for pre-embedding and embedding a specimen in plastic. Two other techniques, a direct result of trying to correct some of our failures, may be of interest. The first concerns the removal of large air bubbles and cracks both from within and outside the specimen in the fully hardened block. These defects we found could be removed by drilling holes along the course of the cracks, and/or into the air bubbles and then filling these

Fig. 6 Chick embryo to show one method of storing embedded specimens in a plastic bag.

Fig. 7 Weta—*Hemideina thoracia* (Orthoptera).

Fig. 8 First embedded specimen of an alizarin preparation to show the skeleton of the flatfish *Rhombosolea retiaria* (alizarin preparation by J. Manikiam, Zoology Department, V.U.W.).

Fig. 9 *Peripatoides novae-zealandiae* (Onycophora).

Fig. 10 Cicada—*Melampsalta muta* (Homoptera).

Fig. 11 Hydrocoral.

Fig. 12 Enlarged view of *Rhombosolea retiaria*. ab = air bubbles.

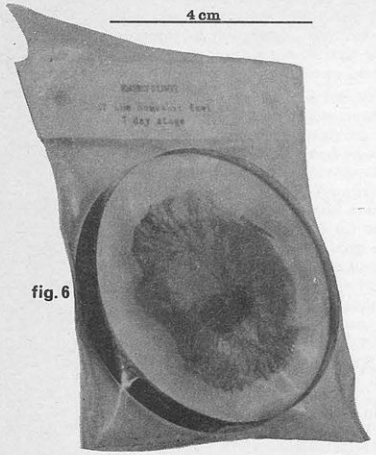
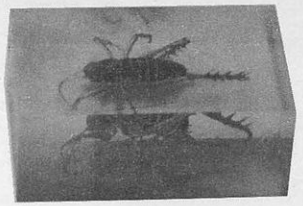


fig. 6



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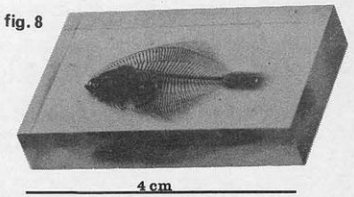

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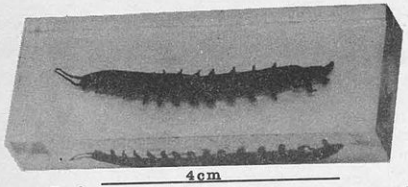

fig. 9

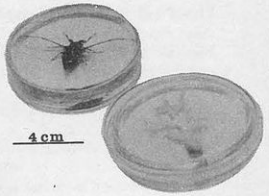

fig.10

fig.11

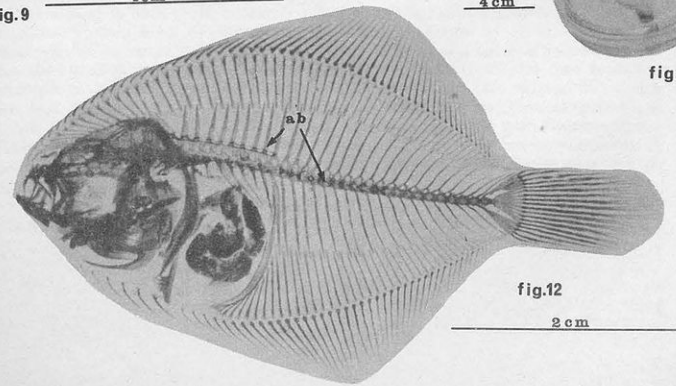

fig.12

2 cm

with new catalyzed resin. But before the new filling is made, the drilled areas should be cleaned out with acetone. It is frequently difficult to detect "the fill" from the original block texture.

Secondly, we also found that hardened plastic blocks turned very well on the metal lathe. This allows sections to be made of the specimen at various levels and through a variety of planes. We have not as yet experienced any difficulty in keying on an entirely new surface to the block or filling the cavities made at various levels through the specimen. Cavities, however, need careful cleaning out with acetone, and acetone should be allowed to remain in the cavities for 2-3 hours before the new plastic is poured into them. The junction lines between the original hardened block and the newly made pour can usually be detected, but they do not as a rule impair viewing of the specimen under the binocular microscope.

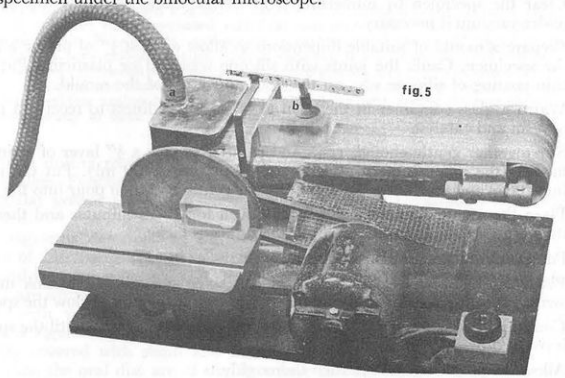

fig. 5

Fig. 5 Linisher belt sander, showing modifications for polishing the fully hardened plastic blocks. A = vacuum tube; B = rubber suction cup of lever arm to hold block firmly but lightly on the belt.

Another method of making sections is to grind down the block on the sander using the coarse belt. This is a particularly useful technique for sections of hard material such as teeth, bone, or corals. The block is polished in the usual way and either resurfaced with a very thin layer of plastic, or the block is surface sprayed with plastic. If the latter method is used it is possible to protect the viewing surface of small blocks from scratches by lowering a glass coverslip on to it while the spray layer is still liquid. We have used this method with success for small blocks containing sectioned teeth.

Other devices we have employed for protecting small specimens, or groups of specimens such as a developmental series of chick embryos, is to embed and retain the hardened block in a petri dish. This ensures, for small blocks that will receive a great deal of handling, microscopic viewing surfaces that are relatively free from scratches, and provides also for more or less dust-free storage. Our larger blocks are stored in plastic bags (Fig. 6).

We have found plastic letters pressed on to the surface of the block very suitable for labelling museum and special demonstration specimens. After labelling, the

surface is sprayed with plastic. Thin card labels sealed at one end of the plastic bag (Fig. 6) provide a more rapid and economical method for labelling specimens for a large class.

The paragraph below, outlining the procedures for embedding a small wet-preserved, soft-bodied specimen is given as a summary of the major techniques described above.

(1) Wash the specimen free from preservative.
(2) Dehydrate first in 50% acetone. Place the specimen under vacuum.
(3) Put the specimen in acetone under vacuum.
(4) Clear the specimen by immersing it in styrine monomer. Place the specimen under vacuum if necessary.
(5) Prepare a mould of suitable dimensions to allow at least $\frac{1}{4}''$ of plastic all round the specimen. Caulk the joints with silicone wax and/or plasticine. Put a very thin coating of silicone wax on the floor and walls of the mould.
(6) Warm a glass container in the oven at 50°C in readiness to receive a mixture of resin and catalyst.
(7) Stir together gently enough resin and catalyst to give a $\frac{1}{4}''$ layer of resin in the mould (the amount of catalyst to resin is 0.5 ml to 50 ml). Put the mixture into the oven at 50°C for 3-5 minutes. Stir gently and then pour into the mould.
(8) Place the mould and mixture into the oven for 10-15 minutes and then allow the plastic to harden in the air until the suface is tacky.
(9) Pour another layer of catalyzed resin prepared as in (7).
(10) Place the specimen on the surface of this layer and allow it to sink under its own weight into this layer. Be careful not to trap air bubbles below the specimen.
(11) Continue preparing, pouring, and hardening layers of plastic until the specimen is covered by $\frac{1}{4}''$ of plastic.
(12) Allow the plastic block to harden thoroughly.
(13) Polish the specimen with varying grades of carborundum, then buff it with Brasso and finally Silvo.

ACKNOWLEDGEMENTS

We wish to thank Mr. B. Perham, of A. R. Hatrick Ltd., for his helpful comments on plastic embedding techniques; and Mr. M. Loper, Technical Officer of the Zoology Department, V.U.W., for taking the photographs for the text-figures.

Pigments of the Sea-Anemone *Isactinia olivacea* (Hutton, 1878) (Coelenterata, Actinozoa)

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Zoology Publications from Victoria University of Wellington, No. 46
Issued September, 1968

ABSTRACT

Thin layer chromatography followed by spectrophotometry shows thirteen pigments in both the green and brown varieties of *Isactinia olivacea* (Hutton, 1878) collected from well illuminated, mid-tidal zone rock pools in the Island Bay area, Cook Strait. Phaeophytin, anthocyans, β-carotene and carotenoid pigments are found in both colour varieties. Colour variation is due to marked differences in density of carotenoid compounds. Two new pigments, an anthocyan and a flavone are described and named. The pigments have three functions; protective colouration, shielding against harmful effects of ultraviolet light, and in the case of flavin, contributing to basic metabolism.

INTRODUCTION

Research during recent years has revealed the importance to plants and animals of biochromes. Functions such as oxygen transport, the transfer of substances between the vascular system and the tissues, as well as animal colouration have long been recognized.

The pigments described in the present paper were extracted from two colour varieties of *Isactinia olivacea* collected at Island Bay, Wellington, from well illuminated and exposed rock pools. The rock pools receive both morning and afternoon sun and are situated between the low and high tide marks. The anemones are always covered with water and are sheltered from strong wave action by surrounding calcareous algae. Moreover, *I. olivacea* is well concealed in its natural habitat, as it is usually covered with stand and small stones, except for the oral disc. Tentacles surrounding the oral disc are of similar colour and appearance to adjacent sea weed, i.e. anemones living in rock pools in which olive-green algae predominate have olive-green tentacles, while those amongst brown sea weeds have brown tentacles. The young of the green variety do not change colour to brown when transferred to a rock pool where the brown animals are found, and vice versa. Many animals have been transferred for experimentation and after almost three years there was no change in coloration.

A. *Collection of material*

Specimens were collected when the water level just covered the animals. If they were not tightly adhered to the rock the anemones were gently removed by separating the base from the substratum with a blunt scalpel. Otherwise it was better to chip off a piece of rock wih the animal attached.

For transferring anemones from sea-shore to laboratory, 20 to 30 animals were placed in a glass screw-topped jar about $\frac{3}{4}$ full of sea water. The anemones were transferred to an aquarium within two hours of being brought back to the laboratory, and left overnight so that all undigested food could be ejected, leaving the coelenteron empty.

B. *Extraction of pigments*

Ten to twenty animals were removed from the aquarium, placed on a clean towel for about 5 minutes and gently squeezed a few times to dry out sea water from

coelenteron and body wall. They were then ground in a mortar with acid washed sand. Anhydrous sodium sulphate was then added until the mixture became fairly dry, and lastly the solvent of one part methanol to three parts petroleum ether (40-60°C m.p.) was added. The mixture is best left to soak overnight, but 30 minutes in the dark will give fairly good results. For the extraction of actiniochrome however, glycerine was used (with freshly ground material) as the pigment extraction medium.

The solvent extract was separated from the solid debris by suction filtration through two thicknesses of Whatman No. 1 on a Buchner funnel. The residue was re-extracted by adding more solvent. The filtered extract was collected in a separating funnel and allowed to stand for 15 minutes. Two distinct layers were obtained. The upper hydrocarbon layer was of an intense brown (epiphasic pigments) and the bottom layer was green (hypophasic pigments). The two layers were separated, placed in a dessicator in the dark and allowed to evaporate to dryness at room temperature. A few drops of petroleum ether or acetone were added to the dry residues to redissolve the pigments for thin layer chromatographic separation.

C. *Separation of pigments*

The condensed extract of each layer was spotted separately as a line, 2.5 cm from the edge of a 20 x 10 cm glass plate coated with an 0.50 mm thick layer of Merck Silica gel.

The plate was developed with a solvent containing one part methanol to five parts petroleum ether. The standard method of ascending thin-layer chromatography was used. The plates were air dried and the coloured bands then removed and eluted with either petroleum ether, methanol, ethanol, water or acetone, etc. . . ., depending on the solubility of the pigments. The pigments so obtained were stored in the dark because the coloured bands on the plates decolourize very rapidly in the light. The eluted pigments were chromatographed on other plates using different solvents in case they were not homogenous.

D. *Identification methods*

(i) *Absorption spectra*

Absorption curves were obtained using a Perkin Elmer Model 137 ultra-violet and visible spectrophotometer. The ultraviolet and visible regions are covered on separate quarto size charts, 190 to 390 and 350 to 750 millimicrons. Each range represents one drum revolution, and turning from one range to the other automatically switches the hydrogen and tungsten lamp sources.

(ii) *Rf values*

Rf. values for all the pigments were calculated and compared with those of known pigments.

The main criterion for identification was the value obtained for the absorption curves, as it is difficult to reproduce Rf. values exactly from one run to another in chromatography. No great reliance therefore was praced on the Rf. values obtained.

RESULTS

A. *Epiphasic pigments*

The upper brown hydrocarbon layer with thin-layer chromatography using five parts petroleum ether to one part methanol as solvent gave ten bands of pigments. Starting from the uppermost, these are referred to as follows: A yellow band (A) close to the front line; an orange band (A′) ; a yellow-pink band (A″) ; a light pink band (B) ; an intense violet-red band (B′) ; an orange band (C) ; a green band (D) ;

a pink band (E) ; a yellow band (E′) and a greeny-yellow band (F) .

Table 1 details the characteristics of these bands.

IDENTIFICATION OF THE EPIPHASIC PIGMENTS

Comparison of the absorption peaks obtained for A (450 and 440 millimicrons) as mentioned above and those given by Fox (1953) suggests that band A (fig. 2) is a flavin or lyochrome. The wavelength of the absorption peak of band A′ (fig. 7) suggests that this pigment is β-carotene. The nature of A″ (fig. 3) is still not known. However, the peaks between 450 and 500 millimicrons and the colour of the pigment suggest that it might be a carotenoid compound. The values given by Fox (1953) for cyanidin were 510 and 269 millimicrons. The characteristics of B′ (fig. 2) and its absorption peaks identify the pigment as cyanidin which is an anthocyan. Band B (fig. 1) only differs from B′ by the last peak. Band B is probably also an anthocyan compound. Further work is needed, however, to fully identify this pigment. The yellow-orange colour of pigment C (fig. 4) and the absorption peaks noted earlier when dissolved in ethanol suggest that band C contains a group of two or three flavones, but this needs to be verified by further separation, purification and chemical analysis.

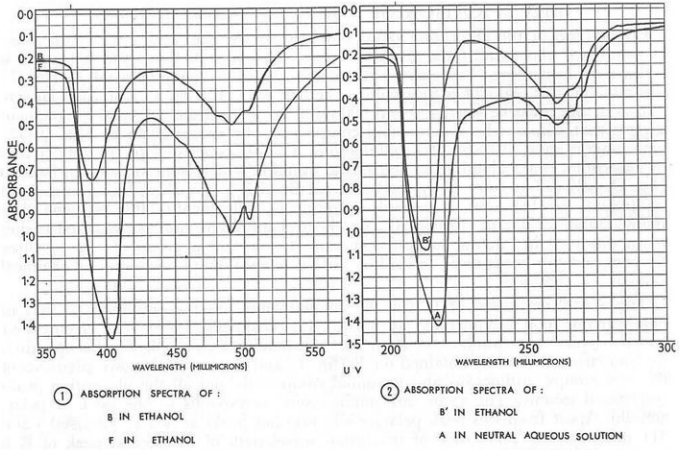
The absorption curve of D (fig. 6) in petroleum ether corresponds to that of phaeophytin, (660 millimicrons) as illustrated by Goodwin (1965). The green colour and the typical absorption curve show that this band is a chlorophyll (phaeophytin). By comparing the values obtained for E (fig. 4) and those of the known pigments of the two groups, anthocyans and flavonoid compounds, not all the absorption peaks correspond exactly. The value 267 millimicrons corresponds to one peak of pelargonidin. Apart from this peak pelargonidin also has peaks at 504.5; 450; 400.5 and 311 millimicrons. The range of maximum wavelength of the second peak of E is between 250-270 millimicrons. This corresponds to that of either flavones or flavonols. However the colour of the pigment, its solubility in water or cold methanol, its insolubility in fat solvents and its change of colour to green in the presence of ammonia shows that the pigment is more an anthocyan than a flavone. I have called this pigment "Isactinin" in the meantime. It could however be an incidental breakdown product of anthocyans accumulated as a result of the phytophagous nutrition of the animals.

Peaks of band E′ (fig. 2) suggest that this pigment is probably a flavone compound. The peak for chrysin is 266 millimicrons, and that of quercitin 258 millimicrons. Pigment F (fig. 1) has peaks at 267 and 255 which are very near to those of the above-mentioned pigments. However, the peaks of F do not correspond exactly to any of the known pigments. This unidentified pigment which has most of the properties of flavone and is similar to quercitin and chrysin is tentatively called "olivacitin" because of its faint greenish-yellow colour.

In all previous investigations of animal pigments, most workers had to identify the pigments tentatively on the basis of absorption spectra together with any available chemical and physical characteristics. The two new pigments of bands E and F do not have all the characteristics of known pigments. However some of their properties indicate the group of compounds to which they may belong.

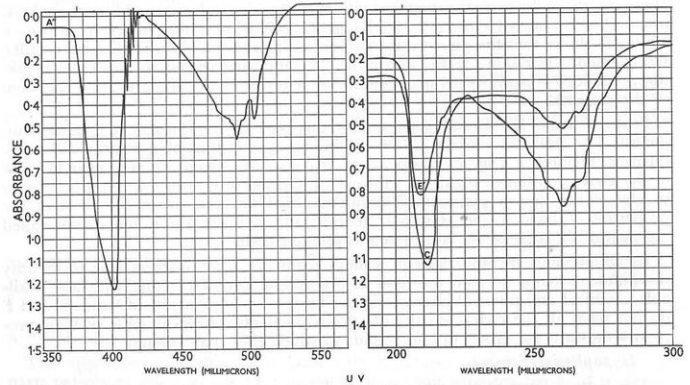
B. *Hypophasic Pigments*

The bottom layer of the extract when developed on a thin layer chromatogram showed three faint bands which decolorised very rapidly. (The solvent used was five parts petroleum ether to one part methanol). Nevertheless, one can distinguish three layers which did not move very far from the initial spot. This is to be expected



① ABSORPTION SPECTRA OF :
B IN ETHANOL
F IN ETHANOL

② ABSORPTION SPECTRA OF :
B' IN ETHANOL
A IN NEUTRAL AQUEOUS SOLUTION



③ ABSORPTION SPECTRUM OF
A'' IN METHANOL

④ ABSORPTION SPECTRA OF
C IN ETHANOL
E' IN METHANOL

because the mixture of pigments obtained was extracted in the aqueous-alcoholic layer. The three bands in ascending order are:

Band G — greenish-yellow
Band H — also greenish-yellow
Band I — yellow

Identification of the Hypophasic Pigments

The individual pigments of the hypophasic layer have yet to be identified conclusively. However, some of their constituents and physical properties suggest the presence of xanthophylls. In a crude extract, xanthophylls are preferentially soluble in 90% methanol. In the presence of two immiscible solvents, petroleum ether and 90% methanol shaken in a separating funnel, xanthophylls are expected to become concentrated in the lower aqueous-alcoholic layer. The colours obtained from this lower layer also agree with those obtained by other workers for xanthophylls. If the lower layer pigments are xanthophyll, the coloration of *Isactinia olivacea* agrees with Goodwin's (1962) statement that in coelenterates the predominant carotenoid pigments are xanthophyll and acidic carotenoids.

DISCUSSION

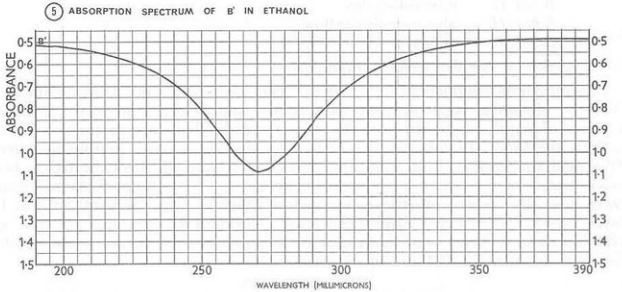
From the results of MacMunn (1885) and the present experiments it seems that in some actinians the respiratory requirements of the animal are provided by pigments found mainly in its symbiotic algae.

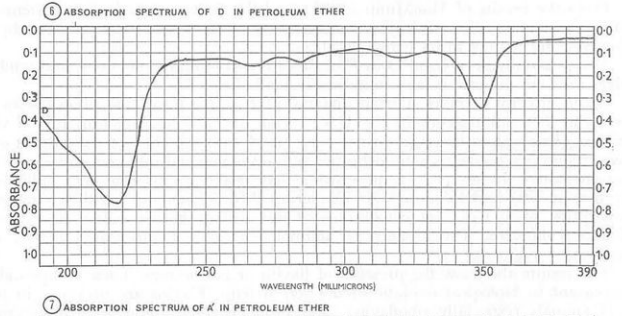
The phaeophytin of band D in *I. olivacea* is a chlorophyll pigment, and symbiotic algal cells are present in large numbers in epithelial cells.

Green glycerol extracts of fresh material of *Isactinia* have absorption curves with peaks at 211 millimicrons in the U.V. region and 397 millimicrons in the visible range. These values however do not correspond with the characteristic peaks of the two respiratory pigments actiniochrome and actiniohaematin known to be specific to sea-anemones. In glycerol, actiniochrome has peaks at 563 and 595 millimicrons (centre at 579) and 458 and 477 millimicrons. Actiniohaematin has the characteristic absorption of a, b_1, c, of cytochrome. These cannot be recognized as present in *Isactinia olivacea*. The absence of actiniohaematin is compensated for by other respiratory pigments.

The results also show the presence of flavins or lyochromes. These compounds are important in biological oxidation-reduction systems. Flavins are universal in plants and animals (especially riboflavin), and promote cell respiration, growth and fat absorption. In animals, flavins are derived either from symbiotic algae or from food. As Fox (1953) suggested, this class of biochrome plays an indispensable role in the basal metabolism of animals, thus explaining their presence in *I. olivacea*.

Band A′ and A″ were mentioned above as possible carotenoid compounds. Carotenoids have been found in various coelenterates by several previous workers. Therefore their presence in *I. olivacea* is not surprising. Extensive work on the pigments of *Actinia equina* by Abeloos-Parize (1926) and Fabre and Lederer (1934) demonstrated the presence of actinioerythrin, a- and b-carotenes and violerythrin. *Anemonia sulcata* was found to have carotenes (Elmhirst and Sharpe, 1919) mainly sulcatoxanthin. Several carotenoids were found in *Metridium senile* by Fox and Pantin (1941). They found ectodermal cells contained red fat droplets evenly distributed throughout the cell or nearer the cell's free surface. In *I. olivacea* sections do not show conspicuous fat droplets similarly placed. The presence of carotenoids in *I. olivacea* is here attributed to the presence of symbiotic algae. Goodwin (1962) emphasized that all carotenoids in animals are of dietary origin. He stated further that "animals", especially invertebrates, do not possess the ability to oxidize tertiary carotenoids to produce pigments which are often characteristic of the species".





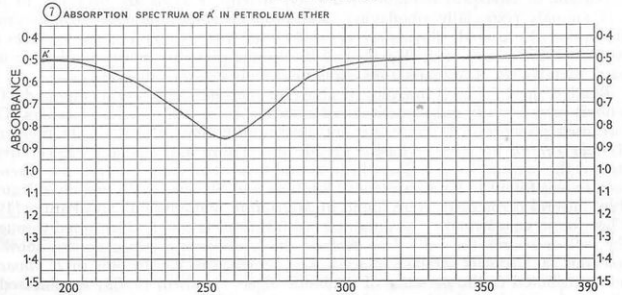


TABLE 1 CHARACTERISTICS OF EPIPHASIC PIGMENT BANDS

BAND	*FIG.*	*PEAKS IN ABSORPTION SPECTRUM* *U.V. (m)*	*VISIBLE (m)*	*SOLVENT*	*COLOUR*	*REMARKS*
A	2	217,261	440-450	neutral aqueous solution	Aqueous solution gives green-yellow fluorescence. Darkens to orange on crystallization.	Insoluble (or slightly soluble) in most organic fat solvents. Soluble in water & alcohols.
A'	7	none	483	petroleum ether	orange	
A''	3	215, 260, 265	450-500	methanol	yellow-pink	Soluble in methanol, ethanol, acetone.
B	1	210, 260, 267	500	ethanol	light-pink	
B'	2 & 5	213, 261, 267	511	ethanol	violet-red. Aqueous solution bleaches rapidly on heating.	Insoluble in ordinary fat solvents. Readily soluble in water & cold methanol.
C	4	major peaks 211, 261 minor peaks 255, 267	none	ethanol	orange	
D	6	none	two major peaks in range 400-410 minor peak 660	petroleum ether	green-decolorising rapidly in strong light.	Fluoresces under ultra-violet.
E	—	208, 255 261, 276	none	ethanol	Faint pink, decolorising on standing. Changes to green in presence of ammonia.	Band remains in contact when rechromatographed using acetone, ethanol/ water and petroleum ether/methanol as solvents. This suggest E to be pure pigment.
E'	4	major peak 210 minor peaks 255, 261, 267	none	methanol	yellow	Soluble in methanol & ethanol.
F	1	260, 267	none	ethanol	green-yellow. Colour intensifies on exposure to ammonia fumes.	Soluble in water, alcohols, dilute acids & alkalis. Insoluble in fat solvents.

It was suggested by Cheesman, Lee and Zagalsky (1967) that carotenoids function either in the stabilization of protein and carotenoid, or as protective coloration; they also suggested that carotenoids play an active role in electron transport and enzymatic activity.

In the case of *I. olivacea*, there is no evidence of carotenoids playing an important part in the stabilization of protein and carotenoid compounds. However, further research may isolate and identify carotenoid compounds similar to that of "metridene" or "astaxanthin". This is not impossible because band A′ which is not yet finally identified, could be one of these carotenoid compounds.

There is the further possibility that in *I. olivacea* carotenoids are used for protection against harmful effects of radiation. The anemones are found in well-illuminated rock pools, and in the laboratory they tend to move towards the light. They therefore need a layer of pigments which could form a shield and filter out harmful irradiations. With regard to the survival value of colour, *I. olivacea* may be classified as a predator. The close similarity of its colour to that of surrounding sea-weeds makes its presence less conspicuous to the crustaceans, worms and other small animals constituting its food. The density of the carotenoid pigments is important in this respect, because green and brown varieties of this species differ by the concentration of the two bands A′ and A″. For example, the green variety found in rock pools where olive-green sea-weeds are predominant, show only very faint traces of these pigments on the chromatogram. The brown variety however, has very concentrated bands of these pigments and they confer on the animal the intense brown colour that makes it virtually indistinguishable from its immediate environment.

Bands E, E′ and F, although not positively identified, do give some indication of their importance in being light sensitive because their absorption curves show peaks in the ultra-violet region and no peaks in the visible range. They absorb in the wave length from 210 to 267 millimicrons. These pigments could be of vital importance to *I. olivacea* because the animal lives in well illuminated rock pools and it therefore needs some device for filtering the ultra-violet wavelength. As Herring (1965) stated, "the ultra-violet wavelengths are generally considered as the most biologically harmful. . . ." It seems likely therefore that these pigments either protect the animal itself from excessive sunlight, or provide a filter for the protection of the symbiotic algal cells within the tissues of the animal.

The results obtained in this paper are very similar to those obtained by Strain, Manning and Hardin (1944). They made a thorough analysis of pigments of algal zooxanthellae which inhabit the tissues of the sea anemone *Cibrina xanthogammica*. An extract of pigments yielded, on chromatographic separation, no fewer than a dozen pigments, including chlorophyll a, a′, and c, phaeophytin a-carotene and several newly described xanthophylls.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Dr. P. M. Ralph, Zoology Department, V.U.W., for her kind encouragement and helpful discussions. I am also very grateful to Mr. M. Loper, Senior Technical Officer, and Mr. A. Hoverd for reproduction of text figures, to the Biochemistry Department for the use of the spectrophotometer, and to Mr. W. H. Johnston, Botany Department, V.U.W., for the use of the thin layer chromatography equipment. Mr. M. J. Williams, N.Z. Wildlife Service and Professor J. A. F. Garrick, V.U.W., made helpful criticisms of the manuscript and I thank them for their considerable effort.

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