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THE BIOLOGY AND BIOCHEMISTRY OF TERPENES IN ALCYONIIDAEAN CORALS
FROM THE CENTRAL REGION OF THE GREAT BARRIER REEF, AUSTRALIA

Thesis submitted by
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in October, 1986

for the degree of Doctor of Philosophy in Biochemistry
at James Cook University of North Queensland

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Leith Webb

October, 1986

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Leith Webb

October, 1986

PUBLICATIONS:

- WEBB, L. (1986) Studies On The Ingestion Of Sarcophytoxide By The Prosobranch Mollusc *Ovula Ovum*. *Comparative Biochemistry and Physiology* In Press.
- SULLIVAN, B., FAULKNER, D.J. & WEBB, L. (1983) Siphonodictidine, a metabolite of the burrowing sponge *Siphonodictyon* sp. that inhibits coral growth. *Science* 221:1175-1176
- WEBB, L. & COLL, J.C. (1983) Effects of Alcyonarian terpenes on Scleractinian coral photosynthesis and respiration. *Toxicon* Supplement 3:485-488
- COLL, J.C., TAPIOLAS, D.M., BOWDEN, B.F., WEBB, L. & MARSH, H. (1983) The fate of a terpene from a *Sarcophyton* species of soft coral (Cnidaria, Alcyonacea) after ingestion by the Egg Cowrie *Ovula ovum* (Mollusca, Prosobranchia). *Marine Biology* 74:35-40
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- WEBB, L. Animal Synthesis Of Sesqui- And Di-terpenes In Alcyoniidae Corals.

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ABSTRACT

This dissertation investigated an array of topical arguments on the function of sesqui- and di-terpenes in alcyonacean corals, and built a platform of knowledge on which to base later experiments. The following facts were established:-

- (1) Terpenes occur throughout the colony but are concentrated in the epidermis and in the gonads.
- (2) Sarcophytoxide is probably localised in osmiophilic vesicles within a special cell-type found on the outer edge of the *Sarcophyton boletiforme* polyp epithelium.
- (3) The cnidarian animal, not the algal symbiont, is responsible for terpene synthesis.
- (4) The terpenes sarcophytoxide and furanoquinol are synthesised all year round, with increased synthesis prior to spawning.
- (5) Stress reduces the rate of biosynthesis of sarcophytoxide and of furanoquinol.
- (6) Detoxification of sarcophytoxide to deoxysarcophytoxide was not observed in the egg cowrie, *Ovula ovum*, voluntarily ingesting sarcophytoxide containing *Sarcophyton* species.
- (7) Alcyoniidae terpenes induce a temporary increase, followed by a slow decline, in respiration associated with decreased photosynthesis in scleractinian corals, suggesting uncoupling of oxidative phosphorylation in mitochondria and chloroplasts. At moderate concentrations these effects are reversible.

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Drs J.T.Baker, D.J.Barnes, B.F.Bowden, B.E.Chalker, J.C.Coll and W.C.Dunlap.

SAMPLES

Coral colonies and *Ovula ovum* from Magnetic Island, Orpheus Island, Rib Reef and Brittomart Reef were collected by Drs B.F.Bowden, D.T.Tapiolas, R.Babcock, S.la Barre, M.Streamer or myself.

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LIST OF ABBREVIATIONS

2B	= a common name given to the first terpene depicted in Figure 15.
Bq	= Bequerels
Ci	= Curie
C.I.	= confidence interval
DCMU	= 3-[3,4-dichlorophenyl]-1,1-dimethyl-urea
DMSO	= dimethylsulphoxide
FN45	= a eudesmane diterpene isolated from colony with the Field Number of 45. The structure of the terpene is depicted in Figure 30.
g	= gram
G	= gravitational force
¹ H nmr	= proton nuclear magnetic resonance
HPLC	= high performance liquid chromatography
l	= litre
m	= metre
OCS	= organic counting scintillant
R _f	= retention front
S.D.	= standard deviation
S.E.M.	= standard error of the mean
TLC	= thin layer chromatography
UV	= ultraviolet

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CHAPTER 1

INTRODUCTION

Investigations into the role of terpenes in the tropical coral community requires knowledge of the biology of corals (Section 1.1), of the biochemistry of terpenes in other organisms (Section 1.2), of the biological role of terpenes in other communitites (Section 1.3), and of their role in defence (Sections 1.4 and 1.5). This chapter reviews the current literature on these topics.

1.1 THE BIOLOGY OF CORALS

1.1.1 The Histology Of Corals

Corals are the sessile adult form of Cnidarian (= Coelenterate) animals of the class Anthozoa (Figure 1). They are classified as either members of the subclass Hexacorallia or of the subclass Octocorallia (= Alcyonaria) depending in part on whether they have multiples of six or eight tentacles, respectively, surrounding the polyp mouth. Hexacorals include members of the order Scleractinia, the reef-building (hermatypic) or 'hard' corals. The octocorals include the two major families, the Gorgonacea and the Alcyonacea (Figure 1). Members of the Gorgonacea are called 'horny' corals while members of the Alcyonacea are referred to as the 'soft' corals.

FIGURE 1

THE PHYLOGENY OF RELEVANT CORALS

PHYLUM:	CNIDARIA		
CLASS:	HYDROZOA	SCYPHOZOA	ANTHOZOA
SUB CLASS:	HEXACORALLIA		OCTOCORALLIA
ORDER:	SCLERACTINIA	GORGONACEA	ALCYONACEA
TRIVIAL NAME:	Hard Corals	Horny Corals	Soft Corals

The coral polyp consists of a retractile blind tube containing a gastric cavity or coelenteron which is surmounted by a mouth surrounded with tentacles (Figure 2) (Plate 1). Below the mouth is a pharynx with one siphonoglyph. From the siphonoglyph, mesenteries extend into the gastric cavity running longitudinally down the tube. These mesenteries harbour nematocysts which harpoon prey when stimulated mechanically or chemically. A primitive nerve-net lies along the polyp tube (Grimmelikhuijzen, 1983). Many coral species form colonial organizations by vegetative division of a single founder organism. The individual polyps of the colony are connected to each other by continuous sheets of tissue termed ^{coenenchyme}. The exposed regions of the polyp retract into the ^{coenenchyme} when threatened (Plates 1, 2 and 3). The polyp has two layers of tissue, the epidermis (=ectoderm) and the gastrodermis (=endoderm), which are separated by an acellular collagenous mesoglea.

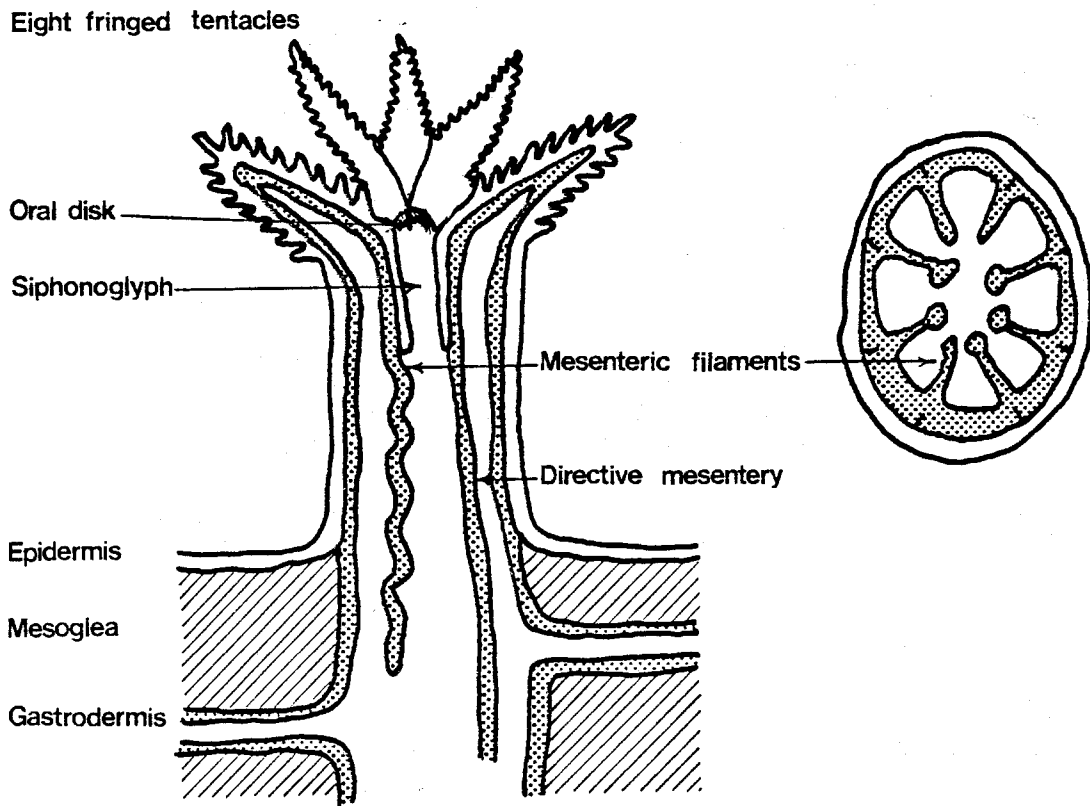
Coral reefs are primarily built from the calcium carbonate (aragonite) skeletons laid down by hermatypic scleractinian corals. Octocorals do not have an aragonitic skeleton. Instead, they have minute calcium carbonate (calcite) needles called spicules or sclerites (0.006 - 2.5 mm long) scattered throughout the octocorallian mesoglea, from the tentacles to the colony base (Plate 4) (Verseveldt, 1972; Kawaguti, 1974). The base of the alcyonacean colony is an acellular layer of fused spicules (la Barre & Coll, 1982). The proportion of spicules to tissue mass decreases towards the upper growing edge of alcyonacean colonies.

1.1.2 Reproduction In Corals

Sexual reproduction in octocorals is by the release of either unfertilised eggs or of planulae larvae (eggs fertilised in the gonads before spawning), depending on the species. For example, *Alcyonium siderium* Verrill releases planulae larvae (Sebens, 1983a,b); whereas *Paraerythropodium fulvum fulvum* Forskal releases unfertilised eggs which develop into larvae outside the polyp column (Benayahu & Loya, 1983). Most species releasing unfertilised eggs

FIGURE 2

A DIAGRAMATIC REPRESENTATION OF THE OCTOCORALLIAN POLYP



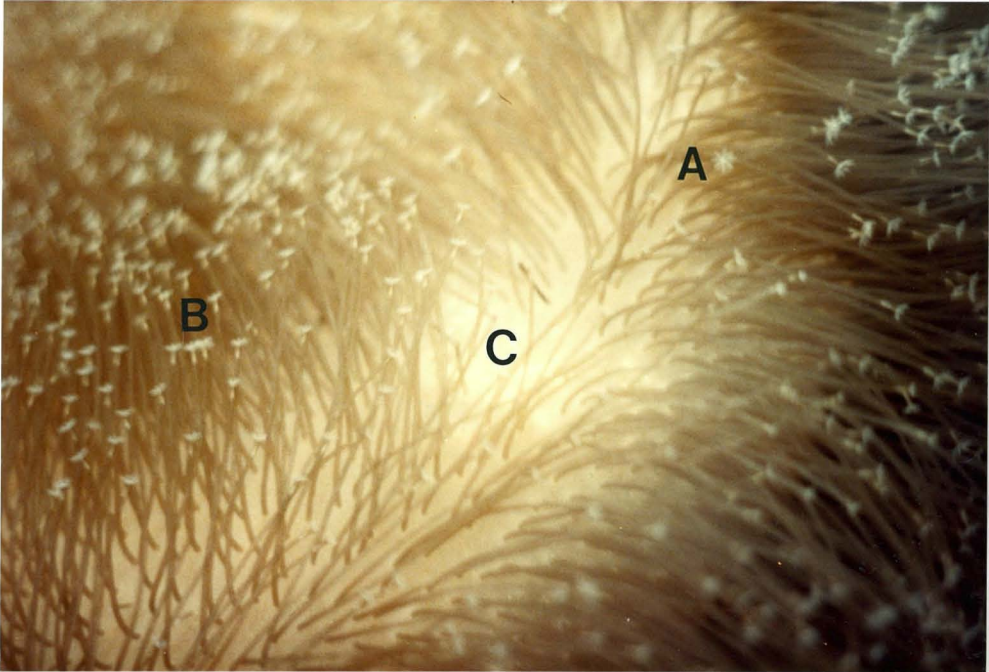
A. Longitudinal section through a colonial polyp.

B. Transverse section.

After Grimmelikhuijzen (1983).

PLATE 1

Sarcophyton infundibuliforme.



TOP Polyps extended.

a Eight tentacles surrounding mouth.

b Pharynx.

c Coenchyme.

BOTTOM One minute after the colony was disturbed.

produce dioecious colonies meaning that the colonies are either male or female. The gonads of all octocorals are endodermal in the mesenteries (Bayer, 1974) and the development of the products varies. The development of eggs in *Paraerythropodium fulvum fulvum* Forskal takes 10 - 11 months to develop (Benayahu & Loya, 1983), while the planula larvae of *Alcyonium siderium* Verrill is mature to the demersal stage in only eight months (Sebens, 1983b).

In spring, many scleractinian corals across the Great Barrier Reef release unfertilised eggs (Harrison, Babcock, Bull, Oliver, Wallace & Willis, 1984). At each reef, these corals release the eggs on a few successive nights, within a few hours of each other. This synchronised spawning may be due to the release of prostaglandins. Prostaglandins are fatty acid derivatives suggested to be spawning hormones (Morse, Duncan, Hooker & Morse, 1977). The synthesis of prostaglandins is induced by the addition of peroxide, and results in synchronous spawning in a variety of invertebrates.

1.1.3 Zooxanthellae: Plants Symbiotic In Corals

Many octocorals, along with hexacorals and other benthic invertebrates such as some sea anemones (Anthozoa) and clams (Mollusca), are symbiotic with dinoflagellate algae (Taylor, 1969). These algae are loosely termed zooxanthellae. Zooxanthellae can occupy a large proportion of the colonial mass, for example, 15 percent of the total biomass of *Zoanthus flos marinos* (DNA, RNA and protein) (von Holt & von Holt, 1968a). Three species names have been proposed for this alga: *Symbiodinium microadriaticum* Freudenthal, 1962; *Gymnodinium microadriaticum* Taylor, 1971; and *Zooxanthella microadriatica* Loeblich & Sherley, 1979.

The ultrastructure of the dinoflagellate endosymbiotic in sea anemones and in Scleractinia has been studied extensively (Kevin, Hall, McLaughlin & Zahl, 1969). Zooxanthellae have typical dinoflagellate nuclei, with dense, tightly coiled chromosomes. The chloroplast is large and ramose, filling most of the cell. The

PLATE 2

Sarcophyton spp.



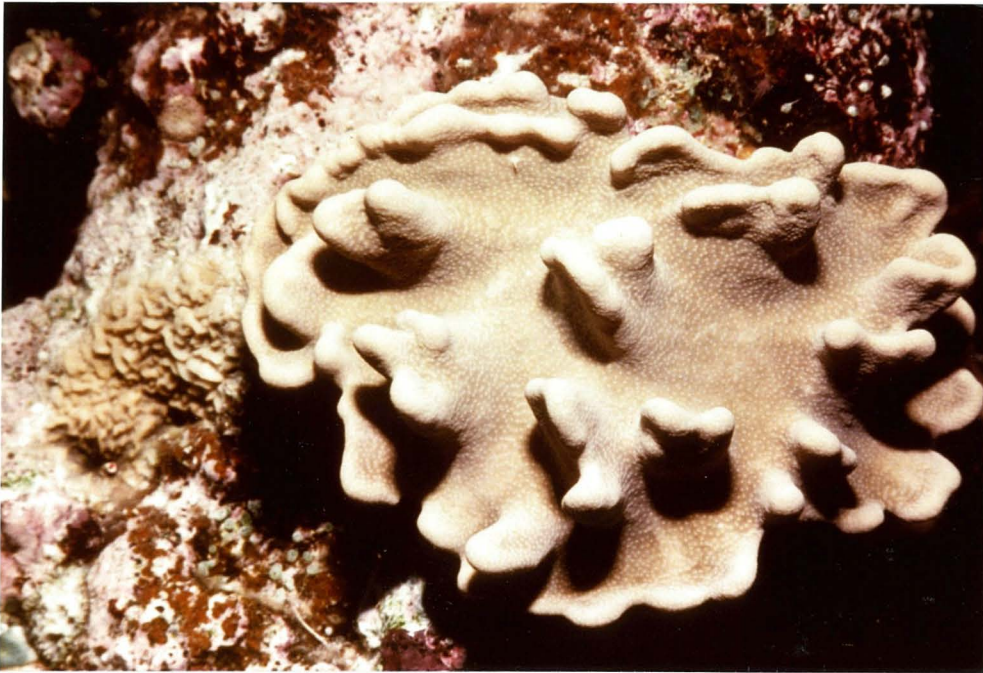
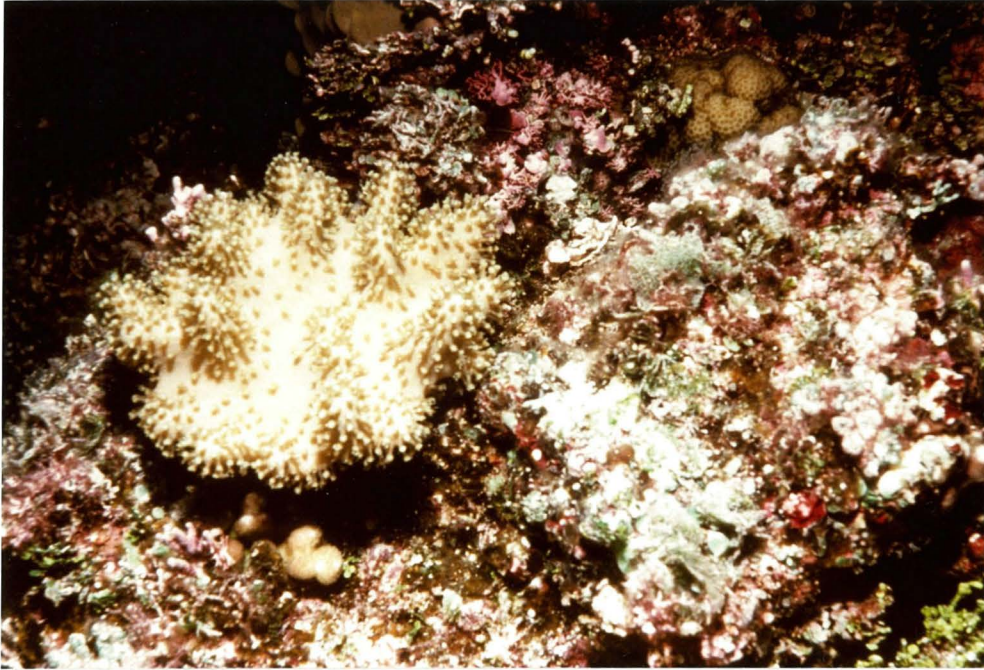
TOP Tentacles extended.

BOTTOM Tentacles retracted.

Photographs courtesy B.E.Chalker.

PLATE 3

Lobophytum spp.



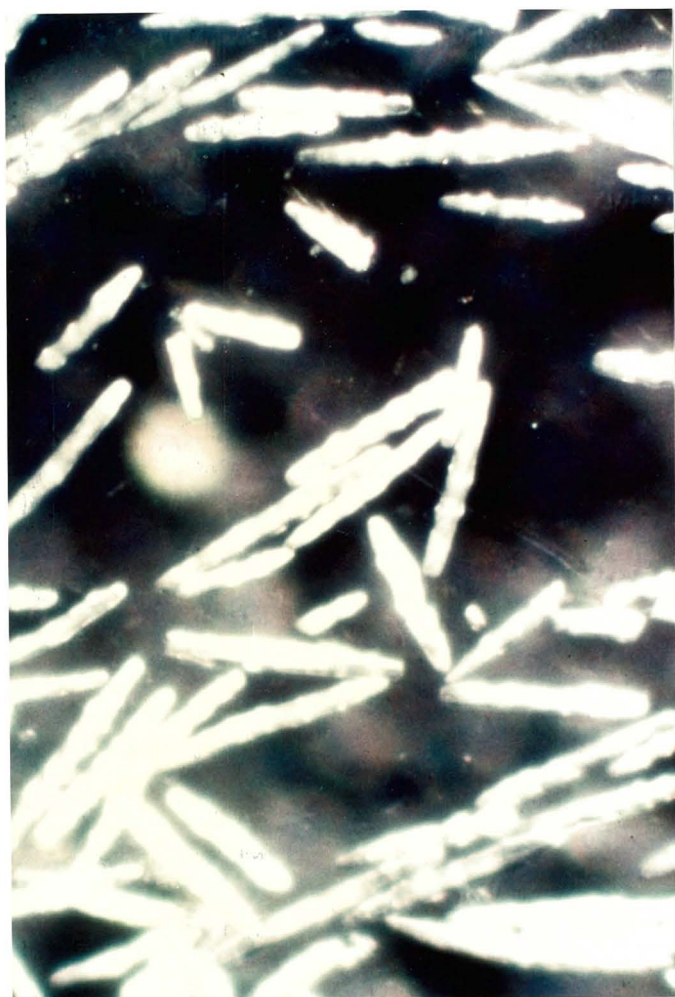
TOP Tentacles extended.

BOTTOM Tentacles retracted.

Photographs courtesy B.E.Chalker.

PLATE 4

SPICULES OF *Simularia* spp.



Photograph courtesy S.la Barre.

internal structure of the chloroplast consists of lamellae formed from three closely appressed thylakoids which traverse the length of the plastid. A single large, vase-shaped pyrenoid encapsulated with starch, projects from the inner surface of the plastid. There are no plastid lamellae within this structure. There is a second large structure termed the accumulation body. The cell boundary has a normal dinoflagellate arrangement, based on a series of five distinct structural layers (for reviews, see Taylor, 1973; Schoenberg & Trench, 1980b). Changes that occur when zooxanthellae are isolated from their hosts and are cultured, include the development of a pellicle and a complex theca (Schoenberg & Trench, 1979).

Rudman (1981b) presented light microscopical evidence of the localization of zooxanthellae in 'sacs' in the wall of the polyps of the alcyonacean *Xenia* spp. These sacs were remarkable, in that each sac was a pocket in the gastrodermis containing an estimated 500 zooxanthellae. Most of the zooxanthellae were free within the sac. All ultrastructural studies of the endosymbiont found in Alcyonacea indicate that this alga is the same species of dinoflagellate as that found in sea anemones and scleractinia (Kawaguti, 1974; Singh & Mercer, 1976; Trench, 1979), although this species occurs in several strains (Schoenberg & Trench, 1980a). Sea anemones of the genus *Aiptasia* treated experimentally to remove zooxanthellae were reinfected with zooxanthellae isolated from symbiotic sea anemones, Scleractinia and Gorgonacea (Kinzie & Chee, 1979; Schoenberg & Trench, 1980c).

Few studies on zooxanthellae feature those found in soft corals, although it is believed that they act in a manner identical with those symbiotic with other anthozoans (Kawaguti, 1974; Singh & Mercer, 1976; Kinzie & Chee, 1979; Trench, 1979; Schoenberg & Trench, 1980c). Discussed below are some of the properties of zooxanthellae symbiotic with scleractinian corals and anemones, and their effect of these zooxanthellae on their hosts.

Strains of zooxanthellae are characterised by the

electrophoretic patterns of the soluble proteins and isoenzymes; glucose phosphate isomerase, malate dehydrogenase, tetrazolium oxidase and esterase (Schoenberg & Trench 1980a). Zooxanthellae isolated from some gorgonians (*Gorgonia ventalina* and *Pseudopterogorgia bipinnata*) but not from all gorgonians (*Pseudopterogorgia acerosa* and *Pseudopterogorgia americana*) show electrophoretic patterns of isoenzymes and soluble proteins identical to that of the zooxanthellae isolated from the sea anemone *Aiptasia tagetes* (Schoenberg & Trench, 1980a). Within the zooxanthellae species, there is heterogeneity of morphology, rates of excretion of photosynthetic products, and isoenzyme patterns of zooxanthellae symbiotic with different species of sea anemone, Scleractinia and Gorgonacea show heterogeneity, suggesting that zooxanthellae from different hosts represent different strains (von Holt & von Holt, 1968a; Schoenberg & Trench, 1980a,b,c).

Occasionally, both octocorallian and scleractinian corals dispersed over large areas of the reef, expel their zooxanthellae leaving the colony white (for review, see Jaap, 1979). The normal brown colour of a colony is due to the photosynthetic pigments within the zooxanthellae chloroplasts; chlorophylls a and c_2 , β -carotene, the orange xanthophyll peridinin, and the yellow xanthophylls (Taylor, 1967; Jeffrey, 1972; Jeffrey & Humphrey, 1975; Kazlauskas, Murphy, Wells, Schonholzer & Coll, 1978).

These white or bleached colonies are often referred to as aposymbiotic (Vandermuelen, Davis & Muscatine, 1972; Kinzie & Chee, 1979; Schoenberg & Trench, 1980c). However, the term is also used to define corals which naturally do not have zooxanthellae. In this dissertation the term aposymbiotic describes corals which are normally symbiotic but which have lost the major part of their zooxanthellae population. Species which are never symbiotic with zooxanthellae are referred to as non-symbiotic or asymbiotic.

Stress such as abnormal salinity or temperature are suggested causes of the mass expulsion of zooxanthellae by hexacorallian and octocorallian corals (Jaap, 1979). Maintaining sea anemones

Aiptasia sp. in the dark, or exposing the anemones to the photosynthetic inhibitor 3-[3,4-dichlorophenyl]-1,1-dimethyl-urea (DCMU) (5×10^{-6} M) turns these normally brown organisms white within three weeks (Kinzie & Chee, 1979; Schoenberg & Trench 1980c). DCMU prevents incorporation of $^{14}\text{CO}_2$ both into photosynthetic products in the presence and in the absence of light (Legendre, Demers, Yentsch & Yentsch, 1983).

Zooxanthellae are photosynthetic organisms which supply nutrients to the animal host. There is substantial movement of photosynthetic products - up to 98 percent - from the algae to the animal tissues. This transfer occurs within 30 minutes of initiation of incubation with radiolabelled carbon dioxide (Trench 1971b). The products transferred to the scleractinian animal hosts such as *Pocillopora damicornis* and *Acropora formosa* include carbohydrates, amino acids and acetate (Muscantine, 1967; Muscantine & Cernichiari, 1969; Smith, Muscantine & Lewis, 1969; Lewis & Smith, 1971; Trench, 1971a; Muscantine, Pool & Cernichiari, 1972; Patton, Abraham & Benson, 1977; Bishop & Kenrick, 1980; Crossland, Barnes & Borowitzka, 1980; Crossland, Barnes, Cox & Devereux, 1980).

The animal tissues themselves seem to induce the excretion of photosynthetic products by the symbiotic algae. Trench (1971c) reported that cultures of zooxanthellae isolated from the sea anemone *Anthopleura elegantissima* excreted more organic compounds into the culture medium when incubated in the presence of a homogenate of the sea anemone animal tissue. Only homogenates of symbiotic animals effected an increase in excretion of photosynthetate. The longer the zooxanthellae were separated from the host, the less photosynthetate the cultures excreted when incubated with host tissues. The final values obtained approached those obtained for free-living dinoflagellate (Taylor, 1973).

Excretion of photosynthetate occurred in cultures of isolated zooxanthellae from several varieties of scleractinian corals (von Holt and von Holt, 1968b). The effect is non-specific as

homogenates of the clam *Tridacna crocea* (Mollusca) induced excretion by algae isolated from the scleractinian *Pocillopora damicornis* (Muscantine, 1967). Dinoflagellates isolated from the clam *Tridacna crocea* are generally considered conspecific with zooxanthellae of corals (Trench, 1979). The host factor which induces this excretion is heat labile and oxygen sensitive (Muscantine, 1967; Trench, 1971c; Muscatine, Pool & Cernichiaro, 1972). It also seems that the design of culture medium can inhibit the excretion (Hill & Ahmadjian, 1972). By comparing the pattern of radiolabels in intracellular and extracellular fluids of the zooxanthellae, the excretion was shown not to be due to lysis of the algae (Trench, 1971b).

Zooxanthellae from the sea anemone *Condylactis gigantea* extrude droplets of supercooled wax esters and triglycerides into the animal cytoplasm (Kellogg & Patton, 1983). ^{14}C -acetate was only incorporated into the lipids in the light. Zooxanthellae freshly isolated from *Stylophora pistillata* extruded triglyceride droplets with a circadian maximum occurring between 14:00 and 19:00 hours (Patton & Burris, 1983). *In vivo* studies on the scleractinian coral *Seriatopora hystrix* showed that zooxanthellae retained 75-82 percent of ^{14}C from $^{14}\text{CO}_2$ at 30 to 60 minutes after cessation of incubation (Black & Burris, 1983). As much as 45 percent of the newly fixed carbon moved from the zooxanthellae to the animal tissues each day although there was little net movement between these two organisms at night.

Photosynthetic products from symbiotic zooxanthellae are incorporated *in vivo* into the scleractinian skeletal matrix (Young, 1971; Young, O'Connor & Muscatine, 1971). Deposition of the scleractinian aragonitic skeleton is influenced by the presence of zooxanthellae. Calcification is markedly reduced when photosynthesis does not occur, either due to the use of inhibitors, to incubation in the dark, or to the use of aposymbiotic individuals (Vandermuelen, Davis & Muscatine, 1972; Chalker & Taylor 1975). This is a paradox as the prime area of calcification in branched corals, namely the growing tips, is free of zooxanthellae (Pearse &

Muscatine, 1971).

Several models have been proposed to explain the importance of photosynthesis to calcification. These hypotheses are divided into two groups: those that emphasise the translocation of reduced carbon compounds from the algae to the sites of calcification; and those that emphasise the removal of unwanted substances from the sites of calcification (for review, see Chalker, 1983). In the translocation models, the reduced organic carbon compounds produced by photosynthesis is used as a substrate for the formation of an organic matrix upon which crystal growth occurs, or as an energy source for ion transport. In the removal models, the interfering substances are assimilated by actively photosynthesizing zooxanthellae. Substances that may be removed according to these hypotheses include:

- (1) carbon dioxide;
- (2) general metabolic wastes;
- (3) phosphate which may poison the aragonite crystals;
- (4) ammonium ion.

Calcification in both scleractinian and octocorallian corals is not totally dependent on photosynthesis. Both classes of Anthozoa contain species which are not symbiotic with a photosynthesizing organism, yet these corals still produce calcium carbonate skeletons. It is not known whether the same mechanism of calcification is used.

The benefits of the symbiotic association to the zooxanthellae have also been studied. It has been suggested that the algae receive nitrogen in the form of ammonia wastes from the animal. Isolated zooxanthellae and symbiotic individuals of the scleractinian corals (*Madracis mirabilis*) take up ammonium ions from seawater whereas aposymbiotic individuals do not (Lewis & Smith, 1971; Muscatine, Masuda & Burnap, 1979). The value of zooxanthellae to soft corals is not known. Presumably, they too benefit from enhanced calcification and nutrient supply.

1.1.4 Defence In Octocorals

Although octocorals have small feeder nematocysts in the mesenteries, they do not have the defence nematocysts of hard corals (Bayer, 1974). Most octocorals are soft, fleshy organisms rich in lipids yet they are rarely eaten or fouled (Bernstein, Shmeuli, Zadock, Kashman & Neeman, 1974). Several chemicals elaborated by the corals inhibit the growth of fouling organisms, for example, homarine in gorgonian corals (Targett, Bishop, McConnell & Yoder, 1983). An octocorallian feature unique among the anthozoans is the presence of large quantities of sesqui- and di-terpenes (Tursch, 1976; Faulkner, 1984) with concentrations as high as 4.5 percent of the dry weight or 50 percent of the organic material in alcyoniidae corals (Kashman, Zadock & Neeman, 1974), and the lack of predation on octocorals is attributed to their terpene content (Neeman, Fishelson & Kashman, 1974; Bakus, 1981),

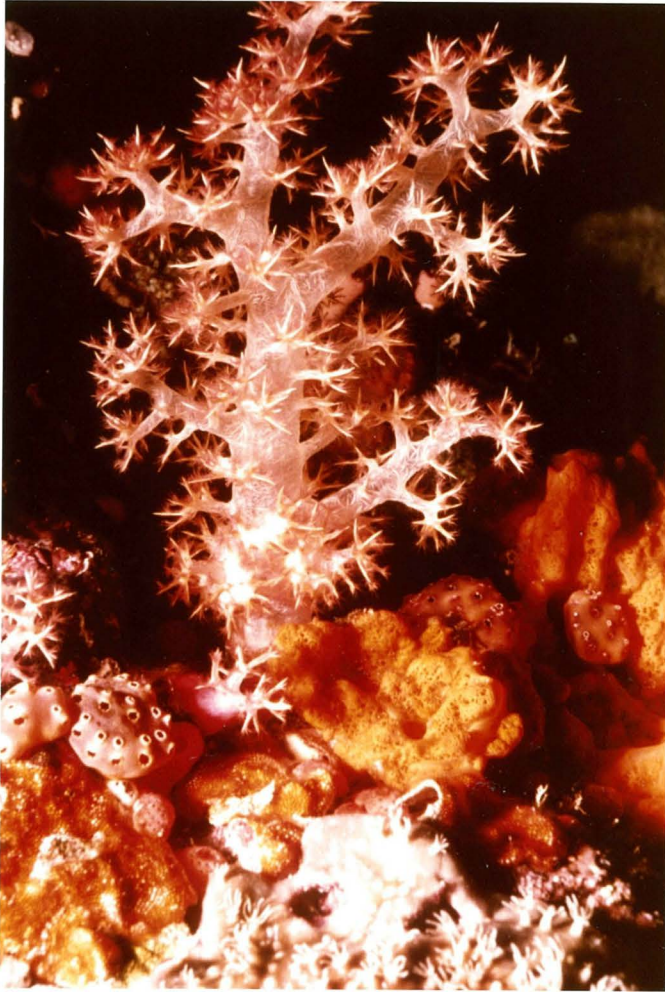
Despite repeated attempts, terpenes have not been isolated from the genus *Dendronephthea* (Tursch, 1976; Bowden, personal communication, 1982; Faulkner, 1984). La Barre (1983) suggested that the external location of calcareous spicules in *Dendronephthea* spp. (Plate 5) acts as a physical deterrent to predation, obviating the need for chemical protection. A comparable suggestion has been made for the dorid nudibranchs which have spicules located in their mantles (Thompson, 1960; Harris, 1974). Many octocorals slough a noncellular organic mucus sometimes embedded with calcareous spicules (Patton, 1972; Benayahu & Loya, 1983). This mucus may also act as a mechanical defence against fouling organisms

1.1.5 Corals Selected For Study

The corals studied in this dissertation belong to the class Anthozoa, subclass Octocorallia (= Alcyonaria), order Alcyonacea, family Alcyoniidae (Figure 3). The alcyoniidae family was chosen as it represents the most prolific octocoral in the central region of the Great Barrier Reef, and the terpenoid chemistry of this group

PLATE 5

Dendronephthea sp. SHOWING EXTERNAL LOCATION OF SPICULES.



Photograph courtesy B.E.Chalker.

FIGURE 3

THE TAXONOMIC CLASSIFICATION OF RELEVANT ALCYONACEA

FAMILY:	ALCYONIIDAE	NEPHTHEIDAE	NIDALIIDAE	XENIIDAE
GENUS:	<i>Alcyonium</i>	<i>Capnella</i>	<i>Siphonogorgia</i>	<i>Anthelia</i>
	<i>Bellonella</i>	<i>Lemnalia</i>		<i>Cespitularia</i>
	<i>Cladiella</i>	<i>Morchellana</i>		<i>Heteroxenia</i>
	<i>Parerythropodium</i>	<i>Paralemnalia</i>		<i>Xenia</i>
	<i>Lobophytum</i>	<i>Nephthea</i>		<i>Sympodium</i>
	<i>Sarcophyton</i>	<i>Dendronephthea</i>		<i>Efflatounaria</i>
	<i>Simularia</i>	<i>Stereonephthea</i>		
		<i>Roxasia</i>		
		<i>Spongodes</i>		
		<i>Umbellulifera</i>		

of corals has been studied extensively (for review, see Faulkner 1984). Alcyoniidae species are identified on the basis of colony morphology, and by the size and shape of the spicules (Verseveldt, 1982). Specimens were selected from the genera *Sarcophyton*, *Simularia* and *Lobophytum*. All the species used are normally symbiotic with zooxanthellae.

The genera *Sarcophyton* and *Lobophytum* both produce solitary colonies. The base of the stalk is either attached to dead scleractinian skeleton or is embedded in the loose coral fragments (sand) between patch reefs. The polyps or zooids of *Sarcophyton* spp. and *Lobophytum* spp. are dimorphic. Siphonozooids are specialised to provide hydrostatic turgor to the colony. They do not bear tentacles. Autozooids bear tentacles and have feeding and digestive functions.

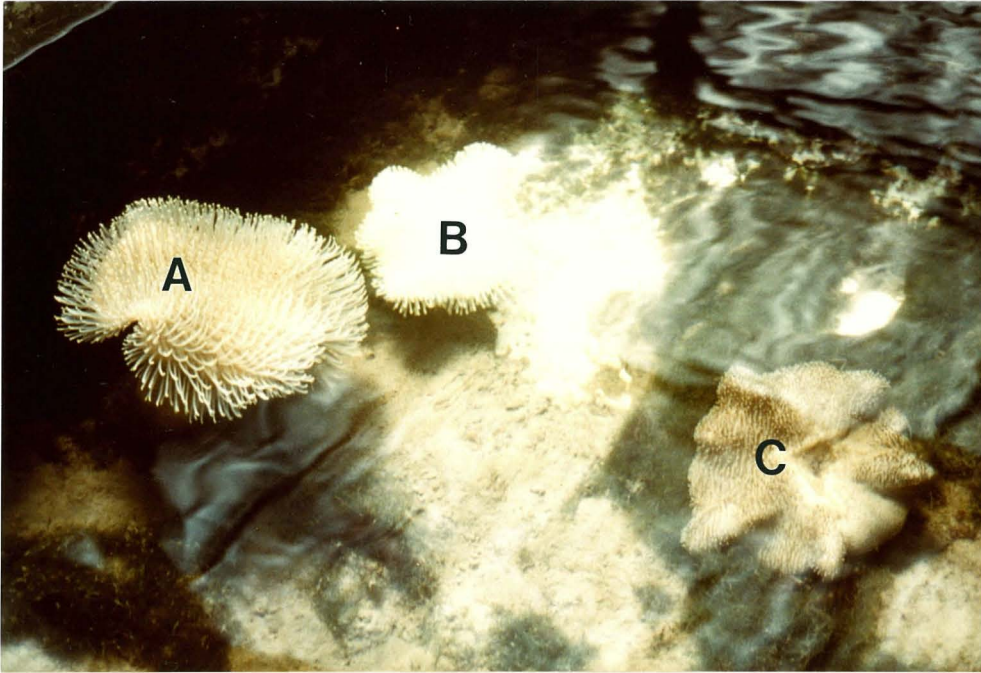
Members of the *Sarcophyton* and *Lobophytum* genera form massive (=nonbranching) colonies. *Sarcophyton* spp. are distinguished by a convex (Plate 6 A) or concave (Plates 1, 2 and 6 B) capitulum (cap) on a stalk. The edge of the capitulum may be folded (Plate 2). The texture is either soft and spongy, or coarse. Colonies of the genus *Sarcophyton* range in size from 3 cm high with a capitulum diameter of 2 cm, to colonies 30 cm high with a capitulum diameter of four meters.

The capitulum of the members of the genus *Lobophytum* have plate- or finger-like closed folds (Plates 3 and 6 C) (Verseveldt, 1982). The texture is usually coarse. The range in colony size of *Lobophytum* spp. is similar to that of the genus *Sarcophyton*. The similarity between these two genera is indicated by the reassignment of species from one genus to the other (Verseveldt, 1982).

Members of the *Sarcophyton* genus are not easily differentiated into species *in situ* because they are morphologically similar, particularly when young. It is often difficult to distinguish *Sarcophyton* spp. even with the aid of spicule identification (Verseveldt, 1982).

PLATE 6

SYMBIOTIC AND APOSYMBIOTIC ALCYONIIDAEAN SPECIES.



- A. *Sarcophyton infundibuliforme*.
- B. Aposymbiotic *Sarcophyton roseum*.
- C. *Lobophytum carnatum*.

Members of the genus *Simularia* form branching colonies. They have monomorphic polyps or zooids. *Simularia* spp. are distinguished by the presence of large spindle-shaped spicules in the stalk interior which can be more than 2 mm long (Verseveldt, 1982). Many *Simularia* spp. are encrusting, in that vegetative reproduction results in discrete colonies connected by coenchyme. Examples are *Simularia capillosa* and *Simularia leptocladys* (Plate 7). Other species form independent colonies attached to dead scleractinian skeleton or embedded in coral sand, for example, *Simularia flexibilis* (Plate 8). These colonies can grow as high as 0.5 meters. The two *Simularia* species used in this study were *S. capillosa* and *S. flexibilis*.

1.2 THE BIOCHEMISTRY OF TERPENES

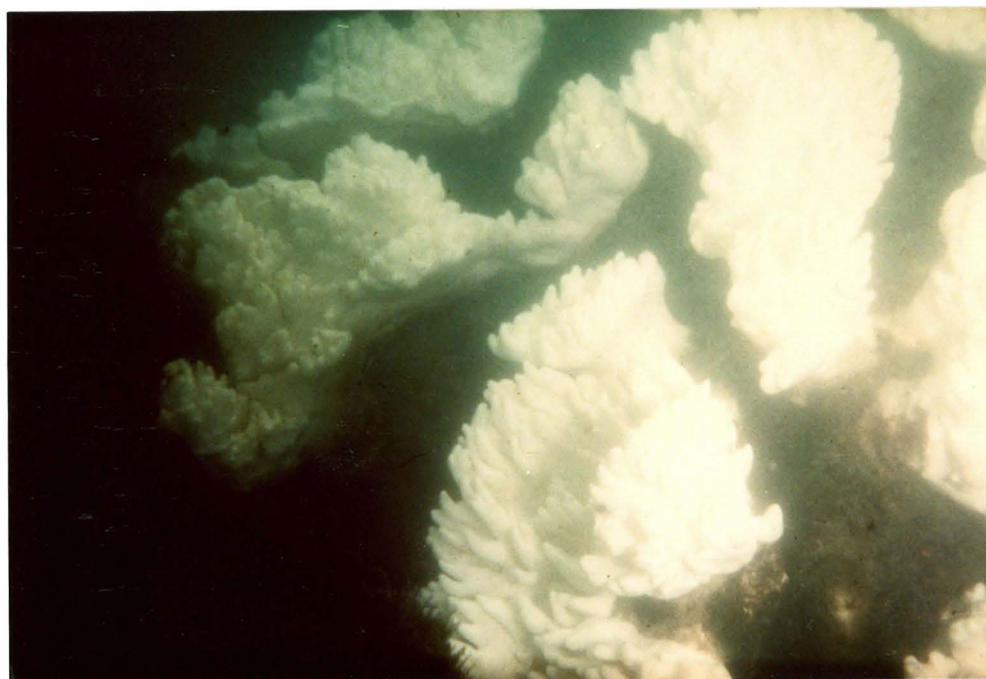
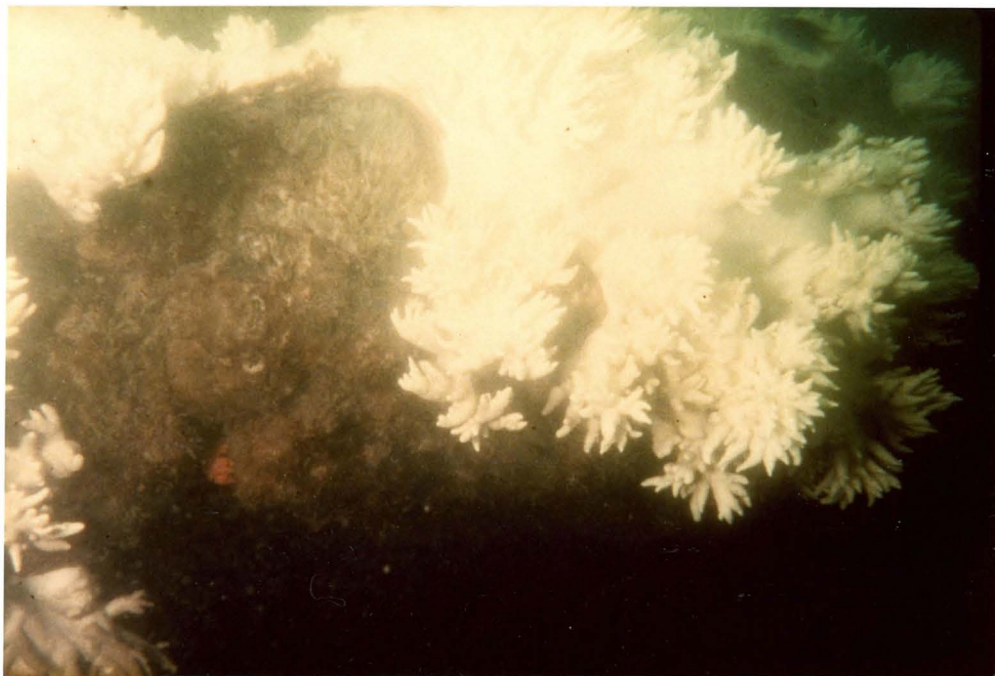
1.2.1 The Biosynthesis Of Terpenes

Terpenes belong to a group of compounds commonly termed secondary metabolites. The term secondary metabolites refers to their redundancy in the primary and intermediary metabolism of the producer species. Historically, they were considered to be the end products of primary metabolism, representing unexcreted wastes (for review, see Whittaker & Feeny, 1971; Seigler, 1977; Naylor, 1984). This belief was based on the observation that secondary compounds usually occur in large quantities, and early studies indicated that there was no turnover of these compounds. Later studies showed that these compounds are turned over slowly, with half-lives of months or years (Croteau & Martinkus, 1979).

Contrary to early beliefs, many secondary compounds possess powerful biological activities both in their natural environment, and in the hands of the pharmacologist. For example, the trichothecene (1) produced by the fungi *Fusarium sulphureum*, which infects potatoes, is implicated in oesophageal cancer in domestic animals and in humans (Steyn, Vleggaar, Rabie, Nicolaas, Kriek & Harington, 1978). In contrast, kaurenoid diterpenes are believed to

PLATE 7

BLEACHED *Simularia* sp. ENCRUSTING THE WRECK OF A PRAWN TRAWLER.



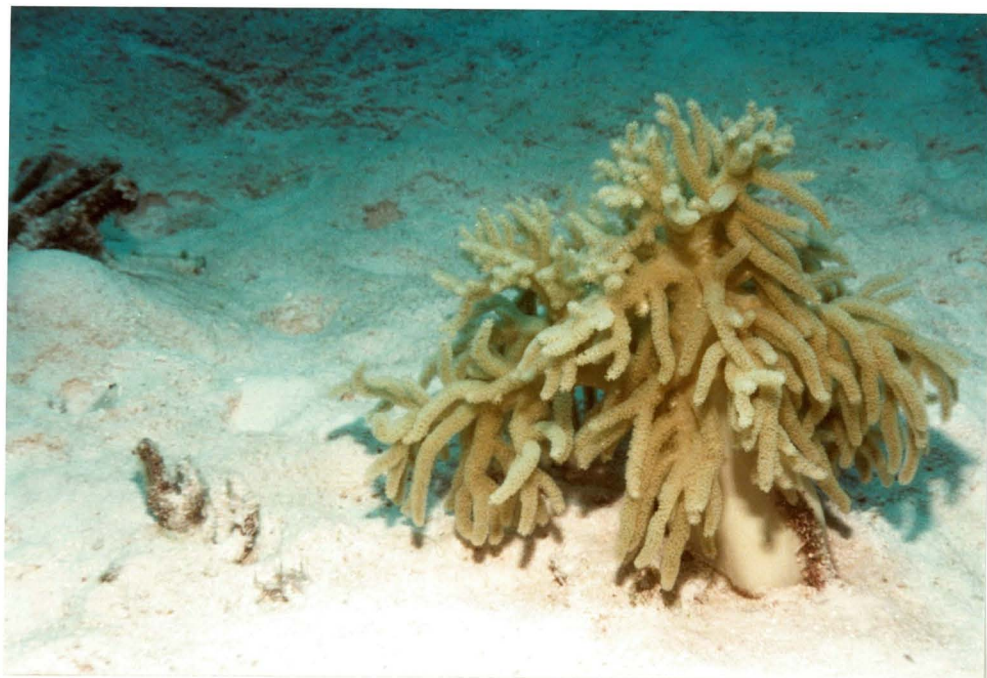
TOP *Simularia capillosa*.

BOTTOM *Simularia leptocladys*.

Photographs courtesy J.C.Coll.

PLATE 8

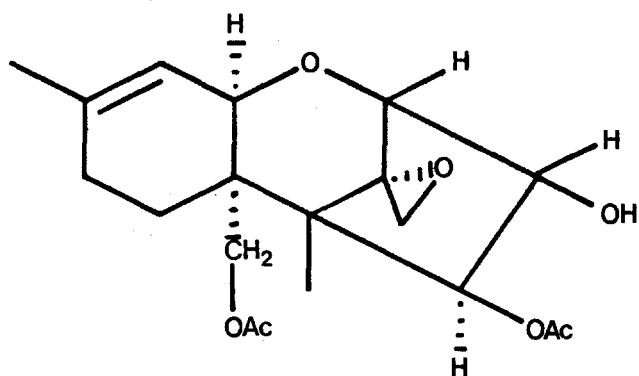
Simularia flexibilis in situ.



TOP Tentacles extended.
Photograph courtesy B.E.Chalker.

BOTTOM Tentacles retracted.
Photograph courtesy J.C.Coll.

FIGURE 4



3 α ,4 β ,15-TRIACETOXY-12,13-EPOXYTRICOTHEC-9-ENE

be the beneficial constituents of the medicinal herb *Stachys lanata* (Piozzi, Savona & Hanson, 1980). The pentacyclic triterpenoid (2) isolated from the aerial parts of the plant *Antidesma menasu* is a diuretic (Rizvi, Shoeb, Kapil & Popli, 1980), while two sesquiterpenes from the Indian weed *Parthenium hysterophorus* Linn. are responsible for dermatitis and pulmonary allergy in humans, which is sometimes fatal (Figure 6) (Khosla & Sobti, 1979).

Secondary metabolites function outside the cell in which they are synthesized (Naylor, 1984). Their action is to coordinate the primary and intermediary metabolism of different tissues (hormones), or of different organisms (pheromones). This coordination enables the organism or the species to respond to environmental phenomena in a manner favouring the survival of the organism or species. Chemical interactions between species are broadly termed allelopathy. The compounds effecting these interactions are termed allelochemicals. Allelochemicals can be divided into: allomones which confer an adaptive advantage on the producer species, such as antifeedants and growth inhibitors; and kairomones which are advantageous to the receiver species. If an animal "acquires" a toxic terpene from its diet and uses this terpene for its own defence, then the terpene is referred to as a kairomone.

Terpenes are polyisoprenoids built by successive additions of C_5 isoprene units (for review, see Beytia & Porter, 1976). Biosynthetically they are synthesized from mevalonic acid which in turn is synthesized from acetate (Figures 7 and 8) (Beytia & Porter, 1976). The early steps in terpenoid biosynthesis, up to and including isopentenyl pyrophosphate production, are considered to be identical in the biosynthesis of all terpenoids, including those elaborated by marine organisms (Fenical, 1982).

Monoterpenes are derived from geranyl pyrophosphate (Figure 8), sesquiterpenes are derived from farnesyl pyrophosphate, and diterpenes are derived from geranylgeranyl pyrophosphate. These molecules then undergo modifications producing a variety of terpenoid hydrocarbon skeletons or types, (see Figure 9 for

FIGURE 5

DIURETIC TRITERPENE

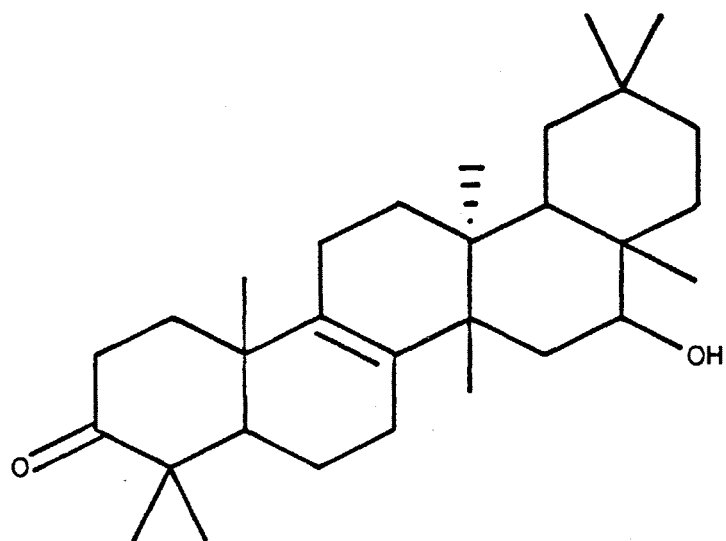
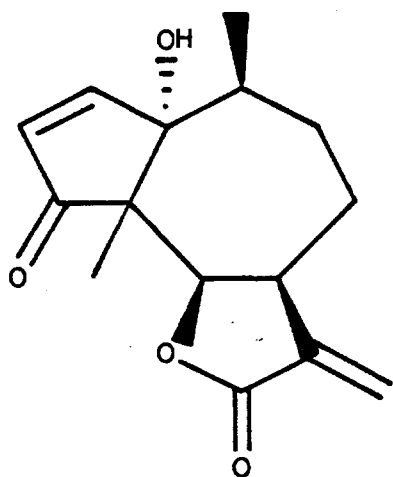


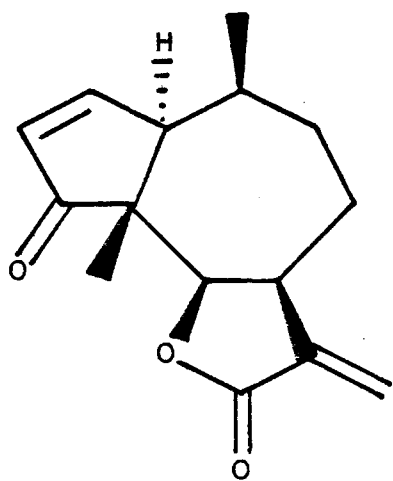
FIGURE 6

DERMATITIS - ELICITING TERPENES



PARTHENIN

3

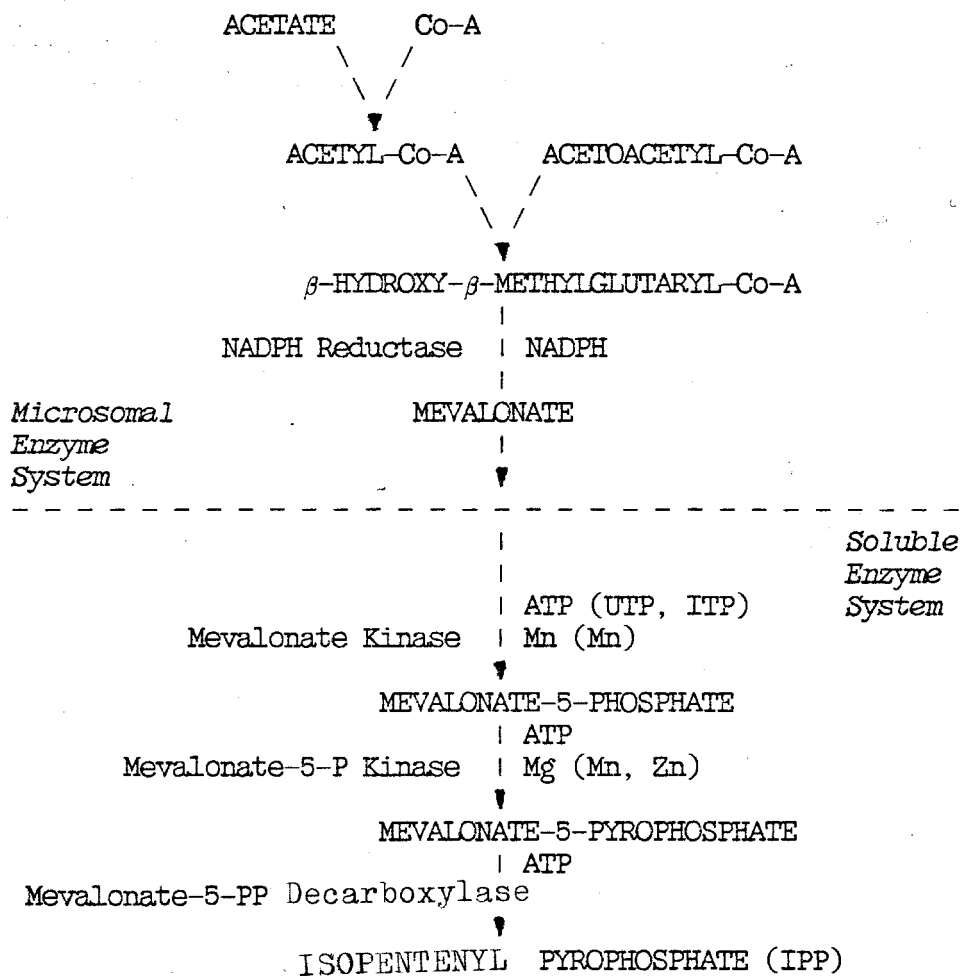


AMBROSIN

4

FIGURE 7

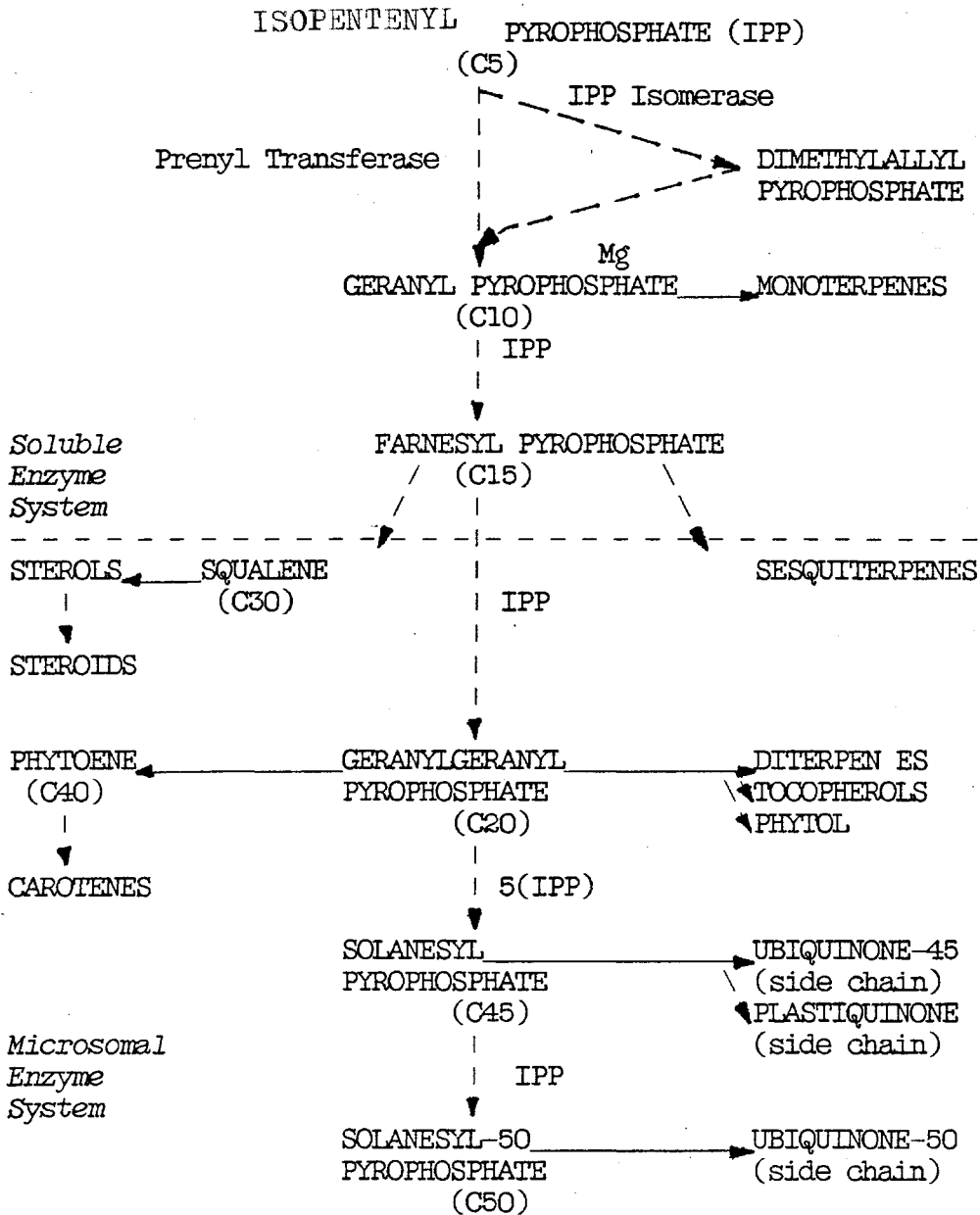
ISOPRENE SYNTHESIS



Adapted from Beytia & Porter (1976).

FIGURE 8

TERPENE SYNTHESIS

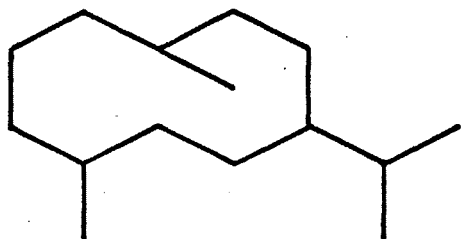


Adapted from Beytia & Porter (1976).

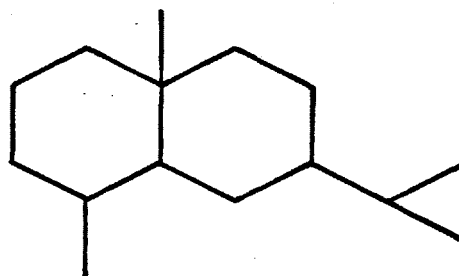
FIGURE 9

SOME COMMON TERPENE SKELETONS

SESQUITERPENES

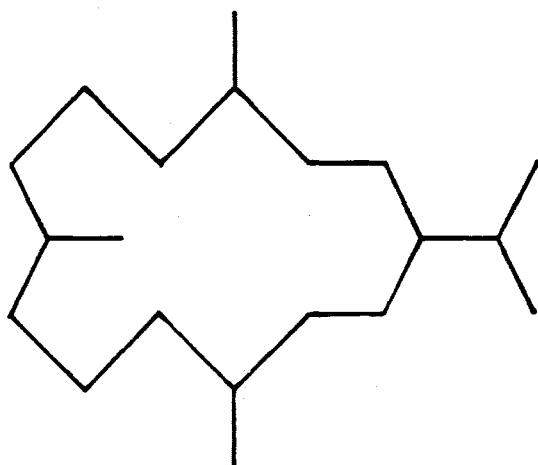


GERMACRANES

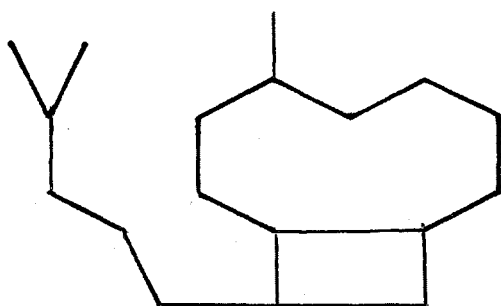


EUDESMANES

DITERPENES



CEMBRANES



CARYOPHYLLANES

examples), which are further modified to produce unique molecules.

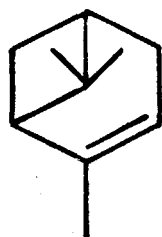
Modifications such as cyclizations (Figure 10), are enzymic conversions rather than spontaneous chemical transformations as demonstrated for both mono- and di-terpenes (Robinson & West, 1970b; Croteau & Karp, 1976; Croteau & Karp, 1979a,b; Portillo, Rojas, Chayet & Cori, 1982). Monoterpenes are cyclised from geraniol

(Figure 11) (Croteau & Karp, 1976). Minor modifications such as epoxidations may be spontaneous (Rodrigues, Garcia & Rabi, 1978). There are several thousand mono-, sesqui- and di-terpenes known to chemists, mostly of plant origin (Devon & Scott, 1972). Triterpenes and sterols are derived by tail to tail dimerization of the sesquiterpene precursor farnesyl pyrophosphate. Carotenes are tail to tail dimers of the diterpene precursor geranylgeranyl pyrophosphate.

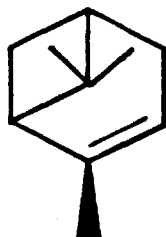
Carotenoids are isolated from many marine animals, including the molluscan nudibranchs and fish (Goodwin & Fox, 1955). The orange-yellow colour of the calcareous spicules of the fan coral, gorgonian *Eugorgia amplexa* is due to a carotenoid (Fox, Smith, Grigg & MacLeod, 1969), while the violet to yellow colouration of the calcareous skeletons of hydrocorals are due to the carotenoproteins (Ronnenberg, Fox & Liaaen-Jensen, 1979). The latter authors assumed the carotenoids to be of animal origin. This assumption is in contradiction to the widely held belief that all carotenoids are of plant or fungal origin (Bogorad, 1976).

Many marine organisms elaborate halogenated terpenes which is a rare occurrence in the terrestrial world and this implies substantial differences in the biosynthesis of terpenes in the two environments (Fenical, 1982). However, the terpenes studied in this thesis are not halogenated, and do not possess the rearranged skeletons which are typical of terpenes synthesized from halogenated intermediates (Fenical, 1982). In fact, halogenated terpenes are rare in alcyoniidae corals (Fenical, 1982; Faulkner, 1984).

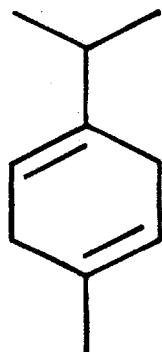
FIGURE 10
MONOTERPENES



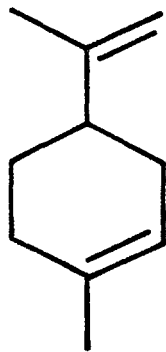
α -PINENE
5



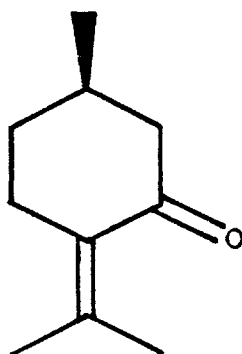
β -PINENE
6



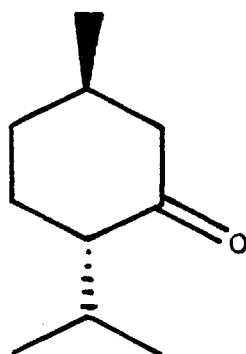
γ -TERPINENE
7



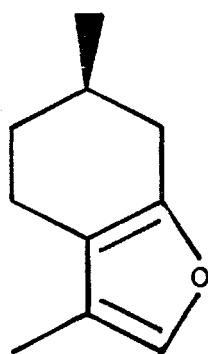
LIMONENE
8



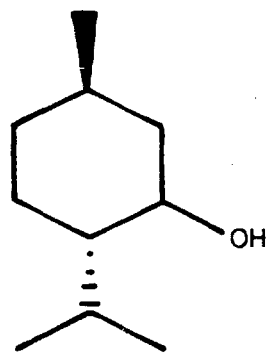
(+)-PULEGONE
9



(-)-MENTHONE
10



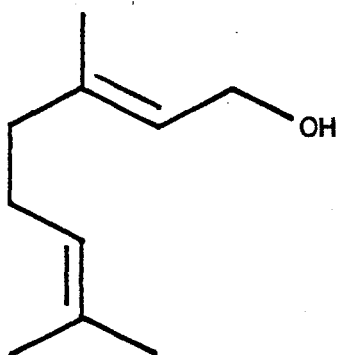
(+)-MENTHOFURAN
11



(-)-MENTHOL
12

FIGURE 11

PRECURSOR OF CYCLIC MONOTERPENES



GERANIOL

13

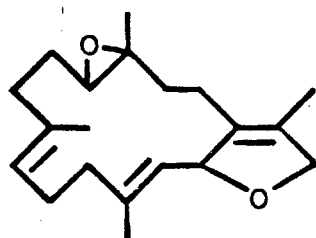
1.2.2 Terpenes In Octocorals

Most octocorals elaborate a variety of sesqui- and di-terpenes. Most specimens yield a series of biosynthetically related terpenes (Bowden, Coll, De Silva, De Costa, Djura, Mahendran & Tapiolas, 1983; Bowden, Coll & Mitchell, 1980). Cembranes (Figure 12) are the most common diterpenes in octocorals (Tursch 1976; Faulkner, 1984). In general, the ring system is unsaturated, and the isopropyl side chain often oxidised and cyclised onto the macrocyclic ring. Sometimes more than one type of hydrocarbon skeleton occurs in the same colony, for example, cembranes and caryophyllanes (Figure 9) in *Nephthea* spp. (Bowden, personal communication, 1981).

A particular terpene may occur widely throughout a genus. The cembranoid diterpene sarcophytoxide (15) is found in several *Sarcophyton* species (Bernstein, Shmeuli, Zadock, Kashman & Neeman, 1974; Kashman, Zadock & Neeman, 1974; Tursch, 1976; Bowden, Coll, Hicks, Kazlauskas & Mitchell, 1978a; Bowden, Breakman, Coll & Mitchell, 1980; Coll, Bowden, Tapiolas & Dunlap, 1982) and crosses the genus barrier into *Simularia flexibilis* (Bowden, personal communication, 1982). This terpene is often coisolated with the biogenetically related diterpene, sarcophine (17) (Bowden, Breakman, Coll & Mitchell, 1980).

In contrast, the linear quinolated sesquiterpenoid furanoquinol (18) has been isolated from only one colony of *Simularia*; a particular colony of *Simularia* encrusting the wreck of a prawn trawler (Coll, Hawes, Liyanage, Oberhansli & Wells, 1977; Coll, Liyanage, Stokie, Van Altena, Nemorin, Sternhell & Kazlauskas, 1978; Bowden, Coll, De Silva, De Costa, Djura, Mahendran & Tapiolas, 1983). This colony has been sampled repeatedly over a number of years and on each occasion it has yielded furanoquinol (Bowden, personal communication, 1983). The colony was initially identified as *Simularia gonatodes* (Coll, Hawes, Liyanage, Oberhansli & Wells, 1977), then as *Simularia lochmodes* (Coll, Liyanage, Stokie, Van Altena, Nemorin, Sternhell & Kazlauskas, 1978), and finally as

FIGURE 12
CEMBRANES FROM *Sarcophyton* spp.



ISOSARCOPHYTOXIDE

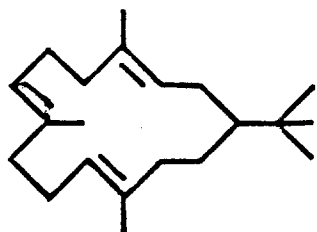
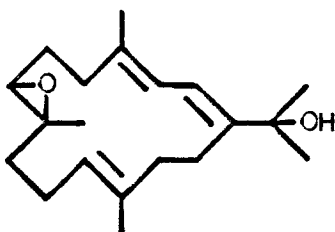
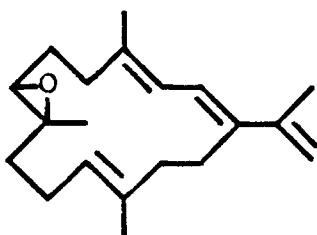
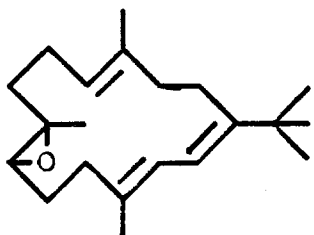
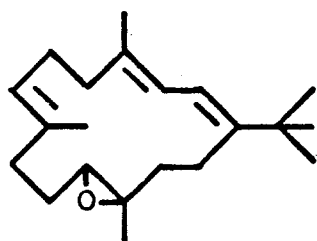
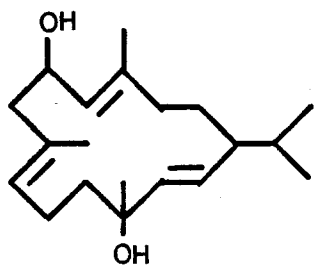
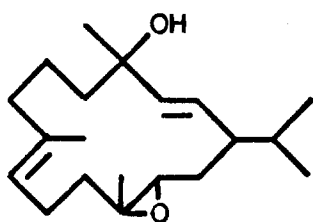
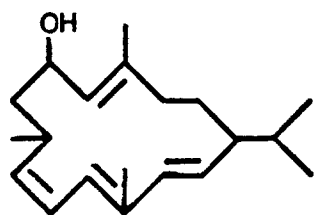
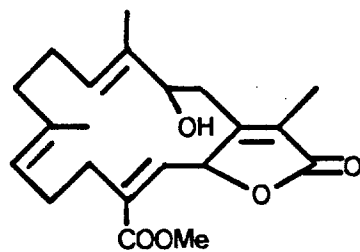
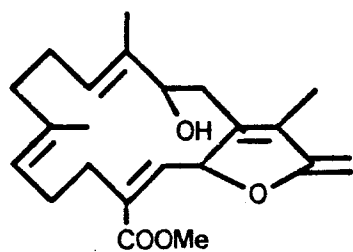
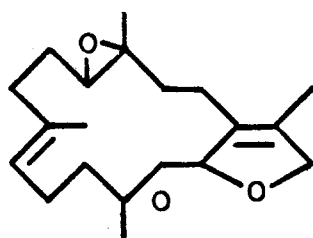
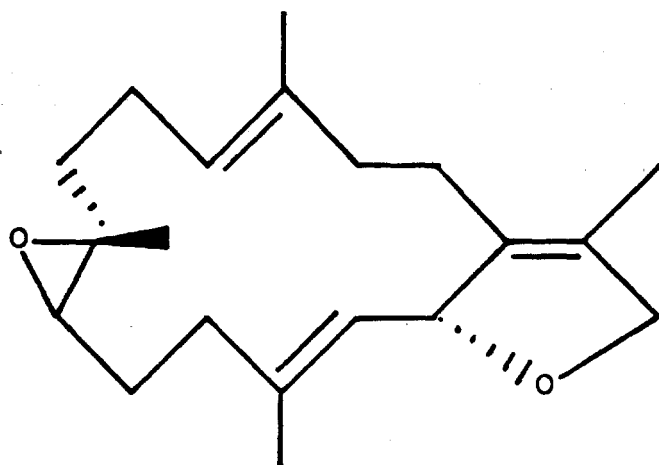


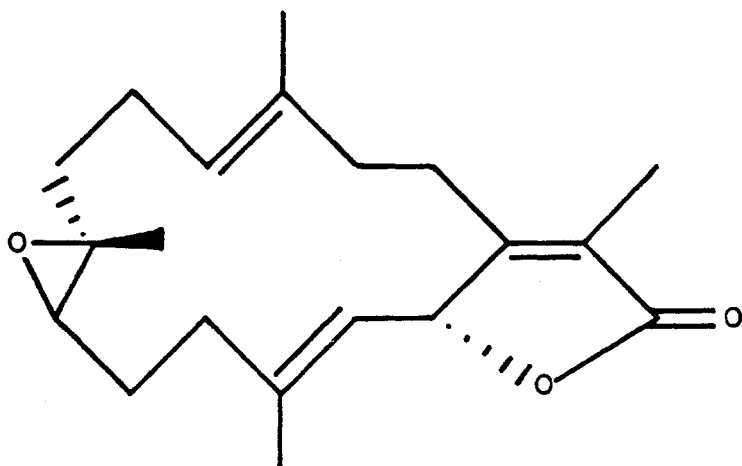
FIGURE 13

RELEVANT CEMBRANOID DITERPENES FROM *Sarcophyton* spp.



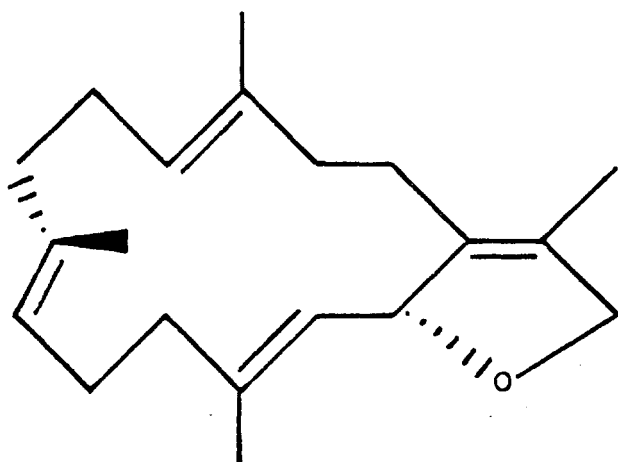
SARCOPHYTOXIDE

15



SARCOPHINE

16



DEOXSARCOPHYTOXIDE

17

Simularia capillosa (Bowden, Coll, De Silva, De Costa, Djura, Mahendran & Wells, 1983). Throughout this dissertation, the colony will be referred to as *Simularia capillosa*. It is the only colony of *Simularia capillosa* mentioned.

There is only one other discovery of a furanoterpene related to the furanoterpenes described in Figure 14. The terpene was isolated from a colony of *Simularia firma* found in Sri Lanka by Mahendran and coworkers (Bowden, Coll, De Silva, De Costa, Djura, Mahendran & Tapiolas, 1983). The colony did not contain furanoquinol nor furanoquinone. Instead it yielded large amounts of a compound suggested as the immediate precursor of furanoquinol but only found in small amounts in the colony of *Simularia capillosa*. Biosynthetic relationships between the various furanoterpenes found in these corals was suggested (Trott, 1980). The two *Simularia* species emphasize different sections of the pathway.

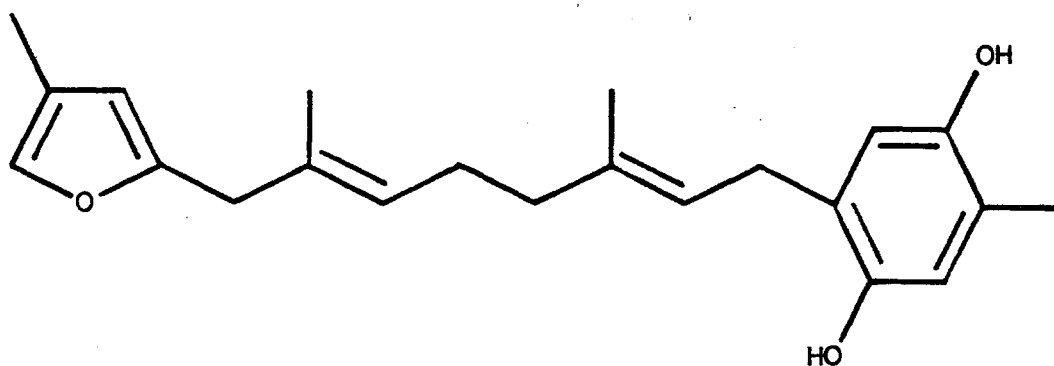
Alcyonacean corals sometimes contain terpenes found in terrestrial plants, for example, crassin acetate (21) in the gorgonian genus *Pseudoplexaura* (Ciereszko, 1962), or naphthenol from the alcyonacean genus *Nephtea* (Schmitz, Vanderah & Ciereszko, 1974). Large concentrations of mono-, sesqui- and di-terpenes are common in plants and their biosynthesis and their biology has been studied extensively (Stoessl, Stothers & Ward, 1978; Rodriguez, Mitchell & Towers, 1976). The biosynthesis and biology of terpenes in marine organisms has not been studied to the same degree.

1.2.3 Comparative Biosynthetic Studies In Plants

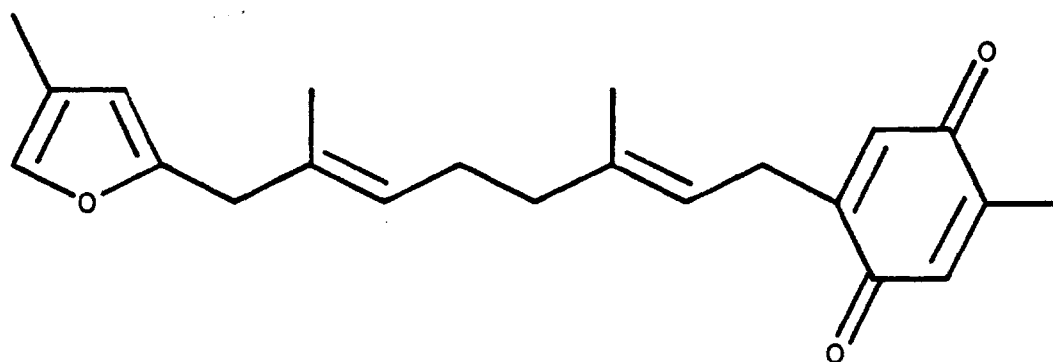
Radiolabels are incorporated into the mono-, sesqui- and di-terpenes of terrestrial plants by feeding labelled carbonate, acetate, mevalonate or pyrophosphorylated precursors (Robinson & West, 1970a; Croteau, Burbott & Loomis, 1971; Loomis & Burbott, 1969; Oba & Uritani 1979). For example, (+)- α -pinene (5) was synthesized from 2- 14 C-mevalonate *in vivo* by *Pinus attenuata*, *Pinus radiata* and *Pinus nigra* needles (Banthorpe & Le Patourel, 1972;

FIGURE 14

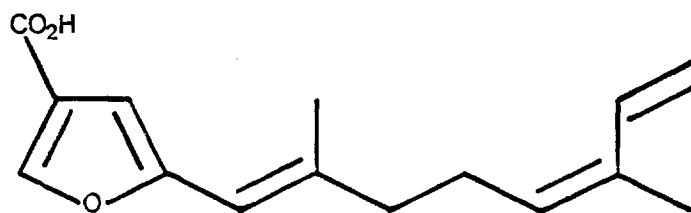
RELEVANT LINEAR FURANOSESQUITERPENES FROM *Sinularia capillosa*



FURANOQUINOL
18

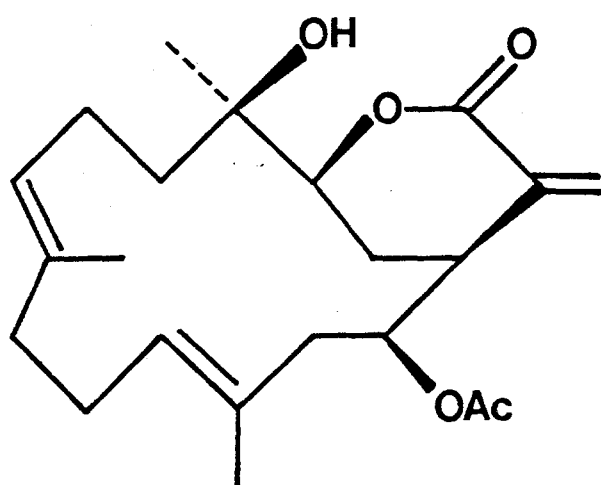


FURANOQUINONE
19



FURANOACID
20

FIGURE 15



CRASSIN ACETATE

21

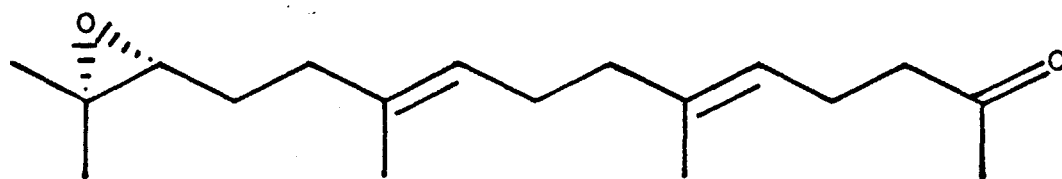
Stanley, 1958). The needles were incubated in water containing the radiolabelled precursor. Differential synthesis of mono- and sesqui-terpenes from radiolabelled carbon dioxide and from radiolabelled leucine was demonstrated *in vivo* in the fruit of *Poncirus trifoliata* and *Monarda fistulosa* (Heinrich, Schultze, Pfab & Bottger, 1983).

In the marine world, the biosynthesis of sesquiterpenes (22) from farnesylacetate was demonstrated in a marine alga (Gonzalez, Martin, Perez, Ramirez & Ravelo, 1981). In general, attempts to incorporate radiolabels into terpenes in algae and sponges have met with little success (Fenical, 1982; Barrow, personal communication, 1983; Garson, personal communication, 1985). However, radiolabels have been incorporated into other compounds in sponges and algae (for review, see Barrow, 1983). For example, the sea anemone *Metridium senile* did not incorporate 2-¹⁴C-mevalonate (5 μ Ci injected into each of 13 animals) into squalene or sterols but did incorporate the radiolabel into dolichol and ubiquinone (Walton & Pennock, 1972). The pathway for the synthesis of the C₉₀-terpene dolichol, and that for ubiquinone, branches from the pathway leading to cholesterol at the level of farnesyl pyrophosphate (Figure 7) (Potter, James & Kandutsch, 1981). Dolichol functions in glycosylating proteins (Allen, Kalin, Sack & Verizzo, 1978; Godelaine, Beaufay & Wibo, 1979; Godelaine, Beaufay, Wibo & Amar-Costesec, 1979).

Some of the enzymes involved in the biosynthesis of plant monoterpenes have been characterised, for example, bornyl pyrophosphate synthetase from *Salvia officinalis* (sage) shoot apices (Croteau & Karp, 1979a). Bornyl pyrophosphate hydrolase from the same plant was also characterised (Croteau & Karp, 1979b). These workers found geranyl pyrophosphate was the best precursor for cell-free synthesis of monoterpenes, producing product distribution comparable to that formed *in vivo* (Gambliel & Croteau, 1982).

Several studies on the synthesis of terpenes in terrestrial plants indicate that the terpenes found in plants are not parts of a

FIGURE 16



FARNESYLACETONE EPOXIDE

biosynthetic pathway but represent divergent pathways starting from a common precursor. For example, the sequence in which the relative amounts of the terpenes β -pinene (5) and limonene (8) are laid down in new shoots of the white spruce *Picea glauca* (Moench) Voss. was found not to be in keeping with accepted biosynthetic pathways for monoterpene biosynthesis (von Rudloff, 1972).

A more detailed study examined the synthesis of cyclic monoterpenes by leaves of the sage *Salvia officinalis* (Gambliel & Croteau, 1982). A cell-free system prepared from immature leaves catalysed the conversion of radiolabelled neryl pyrophosphate to cyclic monoterpenes, including 1,8-cineole, α -terpineol and limonene (8). Enzymes capable of forming 1,8-cineole were located primarily in the soluble fraction of the leaf homogenate, whereas activity responsible for α -terpineol formation, and phosphatase activity, was distributed among the soluble fraction and the 3 000 G particles. Exogenous 1,8-cineole, α -terpineol and limonene were not further metabolised indicating that these three terpenes are derived independently from neryl pyrophosphate, rather than as free intermediates of a common reaction sequence (Gambliel & Croteau, 1982).

The sesquiterpene germacrene D has a transitory appearance in young needles of Douglas Fir (Kepner, Ellison & Maarse, 1975). Although germacrene D is a likely biosynthetic intermediate, no obvious products of further metabolism were discovered. A progression of the presence of the various monoterpenes over the seasons suggested a synthetic pathway (ZimmermannFillon & Bernard-Dagan, 1977).

Although there is a wide knowledge of the chemical structures of terpenes in octocorals, very little is known of the biosynthesis of these terpenes. Only three studies have been reported to date: Rice, Papastephanou and Anderson (1970) successfully incorporated ^{14}C -acetate into the terpenoid moiety of crassin acetate (21) in the gorgonian *Pseudoplexaura porosa* *in vivo* in two hours and in the zooxanthellae isolated from the same species (Papastephanou &

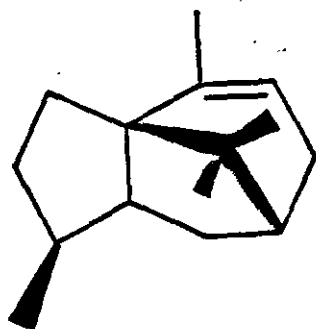
Anderson, 1982); and Trott (Coll, Bowden, Tapiolas, Willis, Djura, Sreamer & Trott, 1985) reported the *in vivo* biosynthesis of furanoquinol in 24 hours from 2-³H-mevalonic acid lactone by the alcyoniidæan *Simularia capillosa*. The radiolabel was incorporated into the terpenoid moiety. However, the terpene was not recrystallised to constant specific purity (Trott, personal communication, 1985).

1.2.4 The Cellular Location Of Terpenes

The major problem in locating terpenes histologically is that there are no specific stains for terpenes. Osmium is electron opaque and reacts with cross-linked proteins and unsaturated lipids reducing lipid loss during dehydration (Cope & Williams, 1967; Carde & Bernard-Dagan, 1975; Gleizes, Pauly, Carde, Marpeau & Bernard-Dagan, 1983). Lead attaches to the osmium-bound compounds, increasing contrast in the electron beam.

As terpenes are hydrocarbons displaying some degree of unsaturation, osmium binds strongly to mono- and sesqui-terpenes (Heinrich, 1970; Carde & Bernard-Dagan, 1975; Gleizes, Carde, Pauly & Bernard-Dagan, 1980). For example, the monoterpenes such as D-limonene (8), and sesquiterpenes of the fruit of *Poncirus trifoliata* and *Monardo fistulosa* were demonstrated to be synthesized by osmiophilic plastids (Heinrich, 1970; Heinrich, Schultze & Wegener, 1980; Heinrich, Schultze, Pfab & Bottger, 1983). Isolated leukoplast preparations from the exocarpium of young *Citrofortunella mitis* (Calamondin) fruits act as complete systems in which occur all the successive steps in monoterpene hydrocarbon elaboration from isopentenyl pyrophosphate occur (Gleizes, Pauly, Carde, Marpeau & Bernard-Dagan, 1983). These leukoplasts contain osmiophilic substances. Sesquiterpenes, including α -patchoulene (23), in *Pogostemon cablin* (Patchouli) also accumulate in osmiophilic vesicles in 'specialised internal accummulatory cells' and in external glandular trichomes (Henderson, Hart, How & Judge, 1970).

FIGURE 17



α -PATCHOULENE

Osmium staining has been used to study terpene biosynthesis in terrestrial plants. Infrastructural studies on the needles of the maritime pine *Pinus pinaster* showed an osmiophilic substance in the epithelial cells of the resin ducts at the base of the needles (Bernard-Dagan, Carde & Gleizes, 1979). The epithelial cells of the ducts were degenerated in the upper parts of the needles and the osmiophilic material was being discharged into the duct lumen.

Joel and Fahn (1980) concluded that the terpenes are produced primarily by plastids by following the synthesis of an osmiophilic substance in glands of the mango stem. The resin of mango stems consists mainly of terpenes. At the beginning of the secretion process, osmiophilic droplets were seen at the envelope of plastids, at the periplastidal endoplasmic reticulum, at Golgi bodies, and occasionally in association with mitochondria. The osmiophilic material eventually filled the well-developed endoplasmic reticulum. The authors concluded that the endoplasmic reticulum is involved in the transport of the secreted material towards the plasmalemma.

The extensive endoplasmic reticulum within the oil gland of leaves of *Citrus sinensis* L. was also suggested to be the site of terpene synthesis (Thomson, Platt-Aloia & Endress, 1976). The C₉₀-terpene dolichol was shown to be synthesized in the Golgi body of rat liver (Coolbear & Mookerjee, 1981). Previously, it had been suggested that animal dolichols may be derived from plant polyprenols (Mankowski, Jankowski, Chojnacki & Franke, 1976).

Osmiophilic droplets believed to be terpenoid, were observed in the plastids and the plastid envelope of *Pinus halepensis* seedlings (Benayoun & Ikan 1980). The terpenoid resin canal cells of another pine, *Pinus pinea*, contain numerous plastids (Wooding & Northcote, 1965). However, the material in the plastids of this species is not especially osmiophilic (Wooding & Northcote, 1965). Osmiophilicity may not be a character of all terpenes.

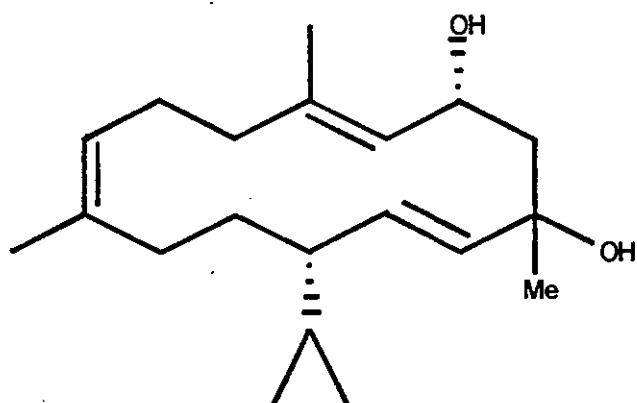
Rice, Papastephanou and Anderson (1970) described the presence of crassin acetate crystals in the gastric cavity and in the

epidermis of the gorgonian *Pseudoplexaura porosa*, extracellular to the zooxanthellae. The mono-, sesqui- and di-terpenes of terrestrial plants are located in glands on the leaves, inflorescences, involucre bracts, achenes, roots, wood, flowers, fruit and bark (McKey, 1979). Often, the different classes of terpenes co-occur in the ducts of the glands (Croteau, Burbott, & Loomis, 1971). Terpenes involved in defence and in aggression in terrestrial plants are usually concentrated in the tissues most exposed to the environment, for example, the epidermis (McKey, 1979). Terpenes are also concentrated in structures vulnerable to predation. For example, the leaves of *Monarda fistulosa* produce essential oils consisting mainly of thymol, whereas no thymol is found in the roots of this plant (Heinrich, Schultze, Pfab & Bottger, 1983). The oembranoid flavours of tobacco such as β -duvatatriene-1,3-diol (24), form a gummy exudate on the leaves (Takagi, Fujimori, Kaneko & Kato, 1980).

Zimmerman-Fillon & Bernard-Dagan (1977) found that the different tissues of the maritime pine *Pinus pinaster* had different terpene compositions; sesquiterpenes were elaborated only in the needles while monoterpenes occurred in the needles, wood and cortical tissues of young shoots. The distribution of the various monoterpenes was not equal in all tissues. The fruit of *Poncirus trifoliata* has glandular cell complexes in the exocarp which produce a volatile oil rich in monoterpenes but poor in sesquiterpenes (Heinrich, Schultze & Wegener, 1980). The juice vesicles of the endocarp contain mainly sesquiterpenes with only small amounts of monoterpenes. The cortex oil of Sitka spruce *Picea sitchensis* (Bong.) Carr. is essentially all monoterpene hydrocarbons while needle oil may be up to 50 percent oxygenated monoterpenes (Hrutfiord, Hopley & Gara, 1974).

Bernard-Dagan, Carde and Gleizes (1979), found that the preferential site of synthesis for the monoterpene hydrocarbons in maritime pine needles is in the epithelial cells of the resin ducts. The incorporation of $^{14}\text{CO}_2$ into monoterpenes was greater in the lower part of the needle and decreased along the needle in accord

FIGURE 18



β-DUVATRIENE-1,3-DIOL

with the degeneration of the resin duct epithelial cells. In contrast, sesquiterpenes were continually synthesized throughout the whole needle. $^{14}\text{CO}_2$ was incorporated into sesquiterpenes to a significantly greater extent than into monoterpenes. The authors concluded that sesquiterpene synthesis is not related to the activity of the resin ducts but occurs in permanent secretory structures located elsewhere in the leaves. In another report on their work, Gleizes, Carde, Pauly and Bernard-Dagan (1980) showed that sesquiterpene synthesis occurred *in vivo* along the entire length of the needle from any of the following radiolabelled precursors: carbonate, acetate, mevalonic acid and isopentenyl pyrophosphate; whereas monoterpenes were synthesized from carbonate and this synthesis only occurred in the epithelial cells of the resin ducts which were functional.

Firmage and Irving (1979) found significant differences in the quantity of monoterpenes in the leaves and flowers of *Pinus radiata* Don of different ages in the same plant, and between plants at different flowering stages. There are also qualitative differences in monoterpenes composition of the cortical tissue taken at different sites on the same tree. These differences were attributed to the different ages of the tissue (Zabkiewicz & Allen, 1975).

Members of terrestrial plant species often exhibit different terpenoid genotypes at different geographic locations. For example, the mono- and sesqui-terpene composition of the leaves of the *Lindera* genus allowed members of two species (*L. umbellata* and *L. sericea*) to be grouped into one species exhibiting four geographic races (Hayashi & Komae, 1974). The monoterpene composition in the Sitka Spruce families also showed geographical variation (Hrutfjord, Hopley & Gara, 1974). In contrast, von Rudloff & Lapp (1978) found no significant difference in the mono- and sesqui-terpene composition of the leaves of the western red cedar *Thuja plicata* due to age or to geography.

Fenical & Norris (1974) reported that the secondary metabolism of a single species of the red algal genus *Laurencia* was independent

of habitat, seasonal changes and reproductive state, although later, these workers found geographical variations in the secondary metabolite chemistry of one species, *Laurencia pacifica* Kylin (Fenical, 1976). In the only study of octocorals, the qualitative concentration of sarcophytoxide and of sarcophine in *Sarcophyton glaucum* was reported to vary with geographic location within the Red Sea (Bernstein, Shmeuli, Kashman & Neeman, 1974).

Studies on the terpenoid genotypes of terrestrial plants and liverworts indicate that the presence of a particular terpene can identify an order or even a genus, or rarely, a species (Asakawa, Toyota, Takemoto & Mues, 1981; Bohlmann, Grenz, Jakupovic, King & Robinson, 1983; Cambie, Cox, Croft & Sidwell, 1983; Waterman & Hussain, 1983). Terpenes have also been used in chemotaxonomic studies of marine organisms including sponges (Bergquist & Wells, 1983), marine algae (Erickson, 1983) and gorgonian corals (Gerhart, 1983). For example, one species of the red algal genus *Laurencia* was divided into different varieties on the basis of the chemistry of the sesquiterpenes elaborated by the algae (Fenical & Norris 1974; Howard, Fenical, Finer, Hirotsu & Clardy, 1977; Waraskiewicz, Sun, Erickson, Finer & Clardy, 1978). In another example, the chlorinated diterpenes, briantheins (Figure 19) were found to be restricted to the gorgonian genus of *Briareum* (Grode, James, Cardelline & Onan, 1983).

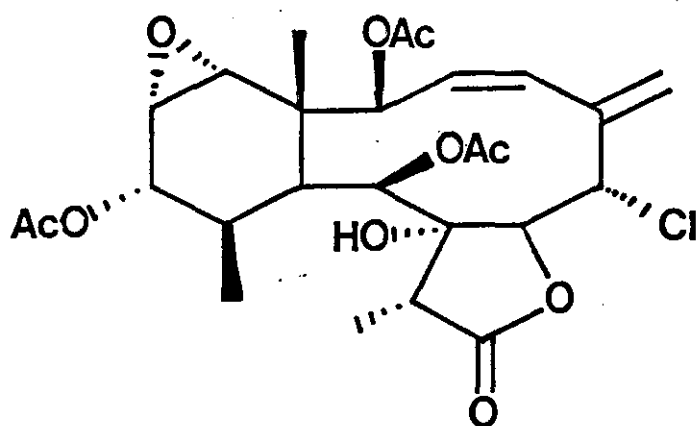
1.2.5 Factors Affecting Terpene Biosynthesis

Season

Phenotypic expression of terpenoid production in terrestrial plants often changes with season. For example, very wide seasonal variations occur in the needle oil of Sitka spruce *Picea sitchensis* (Bong.) Carr. (Hrutfiord, Hopley & Gara, 1974). At bud burst, the oil consists of more than 95 percent myrcene but by the end of the summer the concentration of myrcene has dropped to about 40 percent. During the summer, the oxygenated terpenes camphor and piperitone

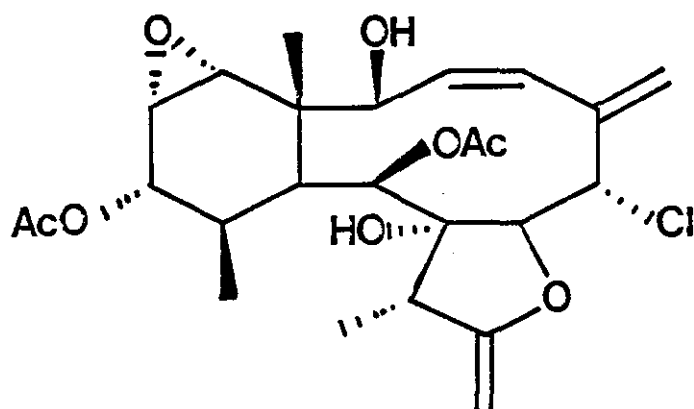
FIGURE 19

CHLORINATED DITERPENES FROM GORGONIAN *Briareum polyanthes*



BRIANTHEIN A

25



BRIANTHEIN B

26

develop to represent about 20 percent each of the needle oil at the end of summer.

Various species of spruce (*Picea glauca*, *Picea sitchensis* and *Picea mariana*) show seasonal change in foliage concentrations of mono- and sesqui-terpenes (von Rudloff, 1975; von Rudloff, 1972; Hrutfiord, Hopley & Gara, 1974). During spring and summer, the monoterpene concentration in the leaves decreases while the sesquiterpene concentration increases. Variations in plant terpene composition often seem to be due to growth rather than season.

von Rudloff (1972) reported major changes in the volatile terpene composition of new shoots of white spruce *Picea glauca* (Moench) Voss during the early part of summer. Minor changes were recorded for the older shoots during the same period. The relative amounts of β -pinene (6), limonene (8) and myrcene in the volatile oil of the buds changed significantly during autumn and winter. In contrast, the quantitative composition of the volatile oil of the leaves and twigs remained constant in during the late summer, autumn and winter. During spring, substantial amounts of sesquiterpenes were present in the new growth; but these disappeared as the summer progressed. The author suggested that the disappearance was due to further metabolism of the terpenes. The author also suggested that sampling at a time of the year when seasonal fluctuations are absent or at a minimum, would allow chemosystematic comparisons of different trees.

Studies on needles of the Douglas fir *Pseudotsuga menziesii* (Mirab.) Franco show an uncoordinated synthesis of monoterpenes (Maarse & Kepner, 1970). Acyclic oxygenated monoterpenes and *cis*-ocinene are absent from new tip growth. There is a gradual increase in concentrations of these components as the leaf matures. In contrast, cyclic oxygenated monoterpenes are present in the new growth in amounts equal to those in year old leaves, and show little seasonal variation in either young or old leaves. The authors suggest that there are separate synthetic pathways for the acyclic and the cyclic monoterpenes in the Douglas fir needles. The fact

that new growth is more palatable to browsers indicates that the acyclic oxygenated monoterpenes may be unpalatable.

Changes in terpene composition due to season are often linked to changes in the reproductive status of the plant. For example, cuttings of the peppermint *Mentha piperita* L. exhibit a steady increase in monoterpene concentration until floral initiation, then exhibit a rapid decrease in monoterpene content (Burbott & Loomis, 1969).

Seasonal variation in terpene composition has also been noted for marine organisms. The concentration of the terpenes sarcophine and sarcophytoxide in the octocoral *Sarcophyton glaucum* varied with season (Bernstein, Shmeuli, Kashman & Neeman, 1974) and the concentration of sterols in the gorgonian coral, *Muricea californica* varied substantially between summer and winter (Popov, Carlson & Djerassi, 1983). It is not known whether these changes are due to season, growth or reproduction. However, the sesquiterpenoid composition of members of the *Sarcophyton* and *Simularia* genera remained constant during different seasons of the year (Kashman, Loya, Bodner, Groweiss, Benayahu & Naveh, 1980).

Light And Temperature

Monoterpene synthesis in the leaves of peppermint *Mentha piperita* L. was influenced by temperature (Burbott & Loomis, 1967). Growing the seedlings at cooler temperatures enhanced the formation of menthone (10) and depressed the accumulation of pulegone (9) and menthofuran (11).

Burbott and Loomis (1967) found that the synthesis of monoterpenes in the leaves of the peppermint seedlings was not influenced by the intensity of the light available during incubation, nor by the duration of the photoperiod. Benayoun & Ikan (1980) also found that the synthesis of terpenes by *Pinus halepensis* seedlings proceeded as well in darkness as in light. Osmiophilic droplets were discovered in the plastids and in the plastid envelope

of the pine seedlings whether they were grown in the dark or the light.

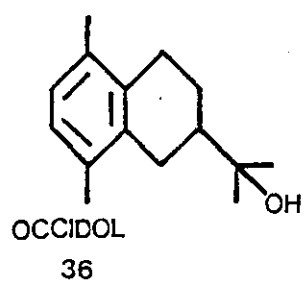
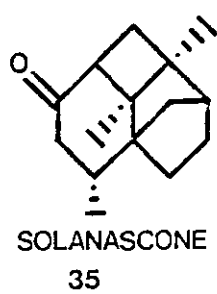
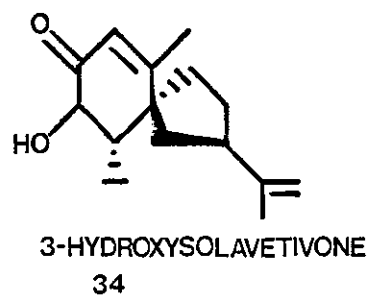
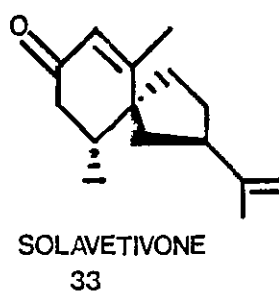
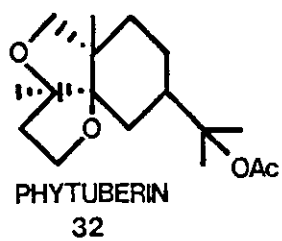
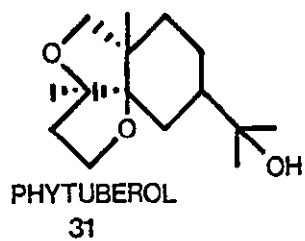
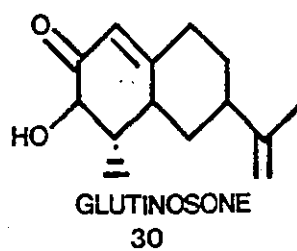
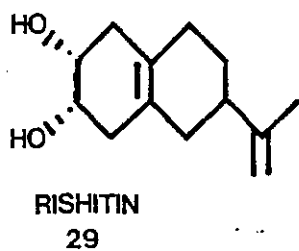
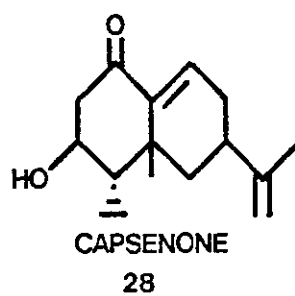
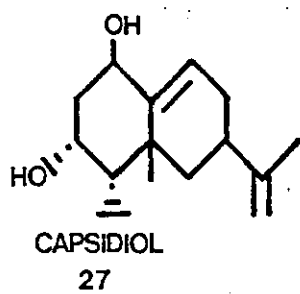
In contrast, the biosynthesis of the monoterpenes, mainly α - and β -pinene (5 and 6), in maritime pine *Pinus pinaster* Ait. seedling primary leaves did not occur in the dark but was strongly activated by light (Gleizes, Pauly, Bernard-Dagan & Jacques, 1980). Radiolabelled carbonate and acetate were incorporated into monoterpenes; mevalonic acid and isopentenyl pyrophosphate were not. Synthesis did not occur in damaged leaves, and only occurred in the parts of undamaged leaves where the epithelial cells of the resin ducts were functional. However, the sesquiterpene hydrocarbons caryophyllene and humulene were easily synthesized under light or darkness, in intact or fragmented leaves, from all the precursors mentioned above. Adams (1979) found a greater diurnal variation in terpene content of *Juniperus scopulorum* in summer than in winter; the latter season being the dormant growth season. Hopfinger, Kumamoto and Scora (1979) found that the diurnal variation in leaf oils of the Valencia orange *Citrus sinensis* (L.) Osbeck was due to changes in the total amount of oil rather than in a change in the composition of the terpenes present.

Stress

A major problem in using terpenes as chemotaxonomic markers is the possibility that the presence of terpenes is stress-induced. Injury to a plant cell sometimes leads to the production of terpenes (Figure 20) (Burdon, Bailey & Vincent, 1975). Generally the level of these compounds is low or absent in healthy plants but increases considerably on infection. These compounds can be completely absent from healthy tissues on the infected plant (Uegaki, Fujimori, Kaneko, Kubo & Kato, 1980a,b; Uegaki, Fujimori, Kubo & Kato, 1981).

These terpenes are known as stress compounds or phytoalexins (Stoessl, Stothers & Ward, 1978). They were originally termed phytoalexins as their synthesis was observed to occur in response to fungal invasion. It now appears that several kinds of injury or

FIGURE 20
STRESS TERPENES

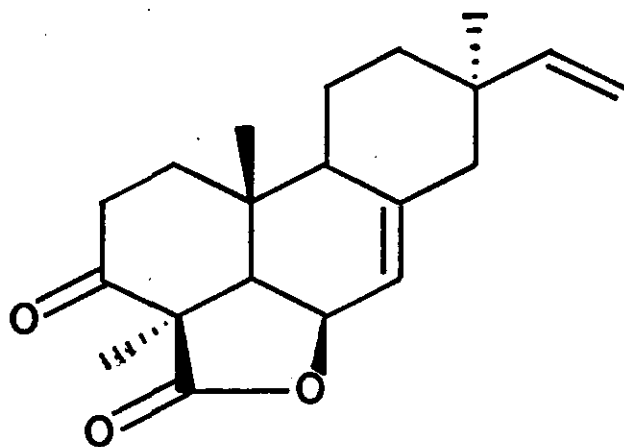


stress can induce their formation. For example, infection with fungi, bacteria and viruses, mechanical wounding, irradiation with ultraviolet light, dehydration, cold, or phytotoxic chemicals (for review, see Rodriguez, Mitchell & Towers, 1976). The terpenes capsidiol (27), solavetivone (33), rishitin (29) and glutinosone (30) are synthesized when leaves of the tobacco *Nicotiana tabacum* (cv Samsun NN) are inoculated with tobacco mosaic virus (Fuchs, Slobbe, Mol & Posthumus, 1983); whereas tobacco mosaic virus infection of the leaves of *Nicotiana rustica* induces synthesis of the tetrahydronaphthalene sesquiterpene occidol (36), terpenes biogenetically related to occidol, and phytuberin (32) (Uegaki, Fujimori, Kubo & Kato, 1983). Fuchs, Slobbe, Mol and Posthumus (1983) present a summary of the phytoalexin terpenes produced by the various species and cultivars of tobacco in response to different pathogens or abiotic inducers. These phytoalexin terpenes are synthesized *de novo* after injury from radiolabelled acetate and mevalonate, not from a nascent precursor (Hoyano, Stoessl & Stothers, 1980).

In another example, infection of rice crops by the fungus *Piricularia oryzae* (Rice Blast Disease) induces the synthesis of plant growth inhibitors, pimaradiene diterpenes momilactone A and B (Figure 21) (Cartwright, Langcake, Pryce, Leworthy & Ride, 1977). These workers suggested that the fungicide 2,2-dichloro-3,3-dimethyl cyclopropane carboxylic acid acts by increasing the phytoalexin synthetic capacity prior to infection.

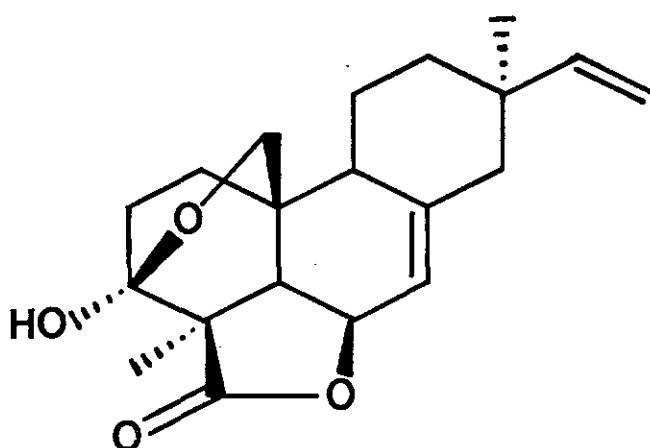
It is possible that octocorals produce terpenes after injury, particularly as the terpenes known to be phytoalexins in the plant family Solanaceae, include cembranoid diterpenes (Bailey, Burdon & Vincent, 1975; Fujimori, Uegaki, Takagi, Kubo & Kato, 1979; Takagi, Fujimori, Kaneko & Kato, 1980). Cembranoid diterpenes are the most common class of terpene found in octocorals (Faulkner, 1984). Experimental manipulation of the corals may actually induce terpene synthesis. This possibility should be taken into consideration when studying seasonal biosynthetic patterns.

FIGURE 21
PLANT GROWTH INHIBITORS FROM RICE PLANTS



MOMILACTONE A

37



MOMILACTONE B

38

1.2.6 Are The Terpenes Found In Octocorals An Animal Or An Algal Product?

Although octocorals are symbiotic with zooxanthellae, it is often stated that the coral animal is responsible for the synthesis of the terpenes (Coll, Hawes, Liyanage, Oberhansli & Wells, 1977; Bowden, Coll, Mitchell & Stokie, 1978; Coll, Liyanage, Stokie, Van Altena, Nemorin, Sternhell & Kazlauskas, 1978; Bowden, Breakman, Coll & Mitchell, 1980; Bowden, Coll, Mitchell, Nemorin & Sternhell, 1980; Kashman, Loya, Bodner, Groweiss, Benayahu & Naveh, 1980). However, this belief is contradictory to both observational and experimental studies:

- (1) Among marine animals the occurrence of large quantities of terpenes, excepting sterols and carotenes, is restricted to invertebrates; in particular sponges, molluscs and soft corals. Molluscs appear to obtain these terpenes from their algal or soft coral diets (Faulkner & Stallard, 1973; Bowden, Coll, Hicks, Kazlauskas & Mitchell, 1978a). As most soft corals are symbiotic with algae, and as algae often produce terpenes (Martin & Darias, 1978), the terpenes in soft coral symbioses may have an algal origin.
- (2) The diterpene crassin acetate was found in high concentration (8 percent of the dry weight) in zooxanthellae isolated from the gorgonian *Pseudoplexaura crassa* (Ciereszko, 1962), and the author concluded that the zooxanthellae were responsible for the synthesis of crassin acetate. It has since been pointed out that it is difficult to separate the zooxanthellae of this coral from the large volume of colourless crassin acetate microcrystals, using differential centrifugation (Rice, Papastephanou & Anderson, 1970). The latter workers observed the crassin acetate crystals to be external but adjacent to the zooxanthellae when the gorgonian *Pseudoplexaura porosa* was examined microscopically. *In vitro* studies using isolated zooxanthellae from *Pseudoplexaura porosa* were reported to be unsuccessful in 1970 (Rice, Papastephanou & Anderson, 1970),

but in 1982 it was reported that ^{14}C -acetate had been successfully incorporated into the terpenoid moiety of crassin acetate by zooxanthellae isolated from *Pseudoplexaura porosa* (Papastephanou & Anderson, 1982).

- (3) Trott (1980) demonstrated that the incorporation of radiolabelled mevalonate into furanoquinol was dose-dependently inhibited by the presence of the photosynthetic inhibitor DCMU.
- (4) The alcyonacean genus *Dendronephthea* (Plate 5) does not have zooxanthellae and does not contain mono-, sesqui- nor di-terpenes (Bowden, personal communication, 1983). Likewise, alcyoniidae Alcyonium *digitatum* has neither zooxanthellae nor terpenes (Tursch, 1976). The gorgonian *Eunicella stricta* elaborates the diterpene eunicelline when symbiotic with zooxanthellae but is devoid of the terpene when devoid of zooxanthellae, even though the chemical content of both forms are otherwise quite similar (Tursch, 1976). The gorgonian *Leptogorgia virgulata*, which is habitually devoid of zooxanthellae, contains terpenes (Fenical, personal communication, 1984). The alcyonacean *Nephthea chabrolii* is symbiotic with zooxanthellae and elaborates the diterpene germacrane-C in common with the clam *Tridacna maxima* (Mollusca) which also has symbiotic zooxanthellae (Bowden, Coll & Mitchell, 1980b). Sterols are an important chemotaxonomic marker for algal genera (Romeo & Tosacno, 1983). Novel sterols of the C_{27} , C_{28} , C_{29} and C_{30} skeletons were isolated from gorgonians and alcyonaceans (*Nephthea* sp. and *Lobophytum* sp.) and from their zooxanthellae but not from two *Nephthea* species which did not contain zooxanthellae (Ciereszko, Johnson, Schmidt & Koons, 1968).
- (5) Zooxanthellae isolated from gorgonians which produce sesquiterpenes, diterpenes and sterols, only synthesize sterols when cultured (Kokke, Fenical, Bohlin & Djerassi, 1981; Fenical, personal communication, 1984). It is noted, however, that symbiotic zooxanthellae isolated from other anthozoans

change their biochemical characteristics in a matter of hours (Trench, 1980).

It has been suggested that the zooxanthellae synthesize sesquiterpenes while the animals synthesize diterpenes (Tursch, 1976), and it is even possible that sesqui- and di-terpenes are synthesized in a collaborative process between the two symbionts (Rice, Papastephanou & Anderson, 1970). It is necessary to resolve whether the coral animal or its symbiotic algae are responsible for terpene biosynthesis before terpene presence can be used as a reliable chemotaxonomic marker for the animal species. It may be that each alcyoniidae coral species is associated with an algal species unique to that species (Trench, personal communication, 1985) but this has yet to be proved.

Gerhart (1983) overlooked this problem when studying the phylogenetic relationships of the various gorgonia genera based on the reported presence of specific terpenes. This oversight may not be a serious error as there is some evidence that different zooxanthellae strains are associated with different coral species (for review, see Trench, 1979). However, Ciereszko (1962) interpreted the presence of terpenes in a gorgonian coral in an entirely different light based on the belief that the zooxanthellae synthesize the terpenes. From the fact that *Pseudoplexaura crassa* contained two terpenes unrelated biogenetically, the sesquiterpene cadinene and the diterpene crassin acetate (21), the author inferred that the colony harboured two species of zooxanthellae. Interestingly, the observation of Ciereszko and coworkers (Ciereszko, Johnson, Schmidt & Koons, 1968) that gorgosterol is associated with the zooxanthellae of gorgonians and alcyonaceans but is not associated with the zooxanthellae of the sea anemone *Anthopleura elegantissima* and *Tridacna gigas* has recently been supported by the ultrastructural studies of Trench and coworkers (Trench, personal communication, 1985) where the ultrastructure of zooxanthellae from corals differs from that of *Cassiopea* sp. In addition, a study on the sesquiterpene composition of members of the *Sarcophyton* and *Simularia* genera of the Red Sea concluded that there

were species specific 'finger-prints' obtainable by gas-liquid chromatography which were independent of the zooxanthellae population (Kashman, Loya, Bodner, Groweiss, Benayahu & Naveh, 1980).

1.3 THE BIOLOGICAL ROLE OF TERPENES

There are many studies on the role of terpenes in plants. Several workers noted a loss of radiolabel from an intracellular pool of terpenes. Cuttings of the peppermint, *Mentha piperita* L., rapidly incorporated radiolabelled carbon dioxide into the monoterpenes menthone (10), menthol (12), menthofuran (11) and pulegone (9), then lost most of the label from the terpenes, without a corresponding change in the concentration of monoterpenes (Burbott & Loomis, 1969). The authors suggested that monoterpenes may serve as a substrate for energy metabolism after other stored substances have been depleted.

In another example, there was greater incorporation of radiolabel into α -pinene (5) from exogenous acetate or mevalonate in several pine genera at 2 hours than at 8 hours, which the authors suggested might be due to catabolism or further metabolism (Banthorpe & Le Patourel, 1972). Seigler (1977) also suggested that secondary compounds could be 'recycled' to primary metabolites. Benayoun and Ikan (1980) tested the hypothesis that terpenes represent stored energy reserves, and found that seedlings of *Pinus halepensis* do not metabolise accumulated terpenes when starved.

In a similar study, the scleractinian coral *Stylophora pistillata* was pulsed with $^{14}\text{CO}_2$ for 1.33 hours and the distribution and loss of label followed for 16 days (Patton, Battey, Rigler, Porter, Black & Burris, 1983). One third of the radioactivity was in a non-solvent extractable fraction which contained calcium carbonate and an organic fraction, which showed small, if any, decrease over the 16 days. The solvent-extractable fraction

decreased with a half-life of 2 days. There was persistence of a constant amount of radioactivity (18 percent) after four days. Although scleractinian corals do not contain mono-, sesqui- nor di-terpenes, they do contain other secondary metabolites (Chalker and Dunlap, personal communication, 1985). There is no information on the half-life of terpenes in octocorals.

1.3.1 Hormones

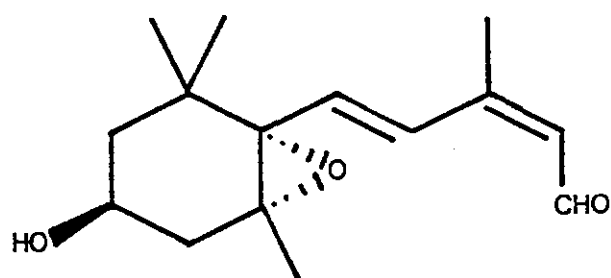
An example of plant hormones is afforded by the terpenes xanthoxin (39), abscisic acid and the gibberellins (40). Absciscic acid is contained in the mesophyll chloroplasts in the leaves of well-watered plants and is released when the water potential falls. The synthesis of abscisic acid is also induced by water stress (Mansfield, Wellburn & Moreira, 1978). Absciscic acid is also involved in the senescence of plants (Wareing, 1978). Xanthoxin is a precursor of abscisic acid, and regulates root growth (Dorffling, 1978). Interestingly, abscisic acid is reported to strongly and consistently inhibit accumulation of the stress metabolites, rishitin (29) and lubimin in the potato tuber slices treated with an elicitor prepared from cultures of *Phytophthora infestans* and *Cladosporium cucumerinum* (Henfling, Bostock & Kuc, 1979).

The gibberellins are a group of biogenetically related diterpenes which both inhibit and stimulate the growth of plants (Jusaitis, Paleg & Aspinall, 1981; Kapoor, 1981). Gibberellic acid₃ induces a two- to three-fold stimulation of RNAase synthesis and activity in cowpea seedlings and is involved in plant senescence (Kapoor, 1981). The same gibberellin acts as a growth hormone stimulating the growth of *Avena sativa* stem segments (Jusaitis, Paleg & Aspinall, 1981).

The potential commercial use of the gibberellins has prompted studies into their synthesis. A cell-free extract of the mycelia of the fungus *Gibberella fujikuroi* synthesized the diterpenes

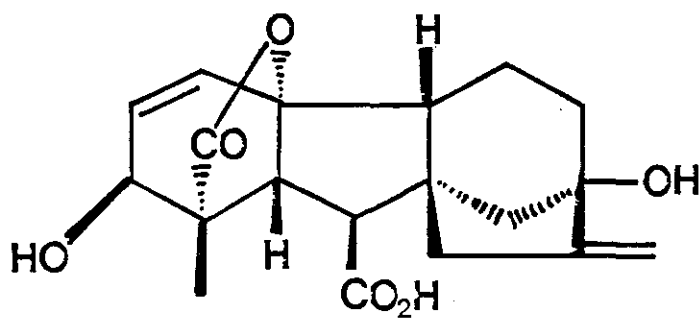
FIGURE 22

PLANT GROWTH HORMONES



XANTHOXIN

39



GIBBERELLIC ACID

40

(-)-copalol (41), (-)-kaurene (42), geranylgeraniol, and copalyl pyrophosphate from radiolabelled mevalonate (Shechter and West, 1969). Copalyl pyrophosphate was converted to (-)-kaurene by the same enzyme preparation, and by a similar preparation from the endosperm of wild cucumber *Echinocystis macrocarpa*, and to (-)-trachylobane (43) by an enzyme preparation from seedlings of the castor bean *Ricinus communis*. Further transformations of kaurene by *Gibberella fujikuroi* were studied by Fraga, Gonzalez, Hanson and Hernandez (1981), while Murofushi, Nagura and Takahashi (1979) studied the conversion by the same fungus of steviol (44) to several gibberellins.

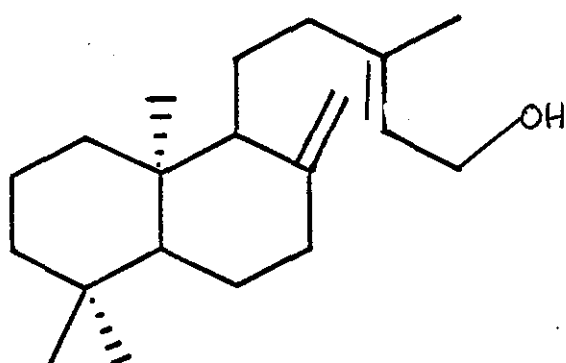
Studies on the inhibitors of gibberellin formation show that inhibitors of gibberellin synthesis such as secondary, tertiary and quaternary amines, also inhibit the synthesis of other terpenes such as lycopene, the chlorophylls and limonoid but enhance synthesis of carotenoids (Echols, Maier, Poling & Sterling, 1981). Gibberellin synthesis is under feedback control through endproduct inhibition by gibberellic acid (Dockerill & Hanson, 1981).

1.3.2 Pheromones

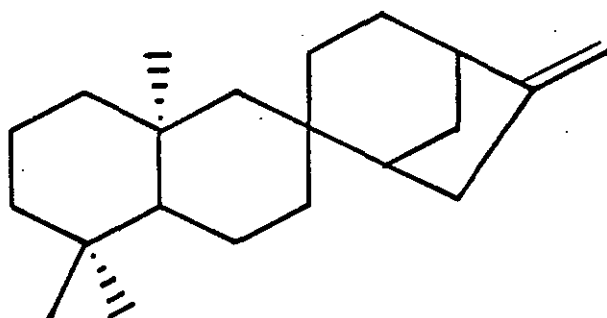
Frequently, a secondary metabolite has more than one function. Terpenes such as the steroidal sex hormones of mammals can also act as pheromones (White, Handler & Smith, 1973). Insects use mono- and sesqui-terpenes as pheromones, and as kairomones (Brown, Eisner & Whittaker, 1970). For example, bees secrete mono- and sesqui-terpenes from the mandibular and Dufour's gland (for review, see Wheeler, Avery, Birmingham & Duffield, 1984). These glandular secretions repel predators, and the differences in the composition of the secretions of the male and the female suggest a role in aggregating for mating. Fungi and plants use carotenes and steroids as growth hormones and as pheromones (Caglioti *et al.*, 1966; Guens, 1977).

Trisporic acid (45), the sex hormone of the Mucorale

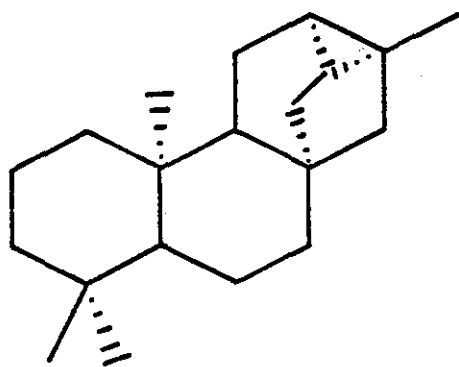
FIGURE 23
PRECURSORS OF GIBBERELLIC ACIDS



(-)-COPALOL
41

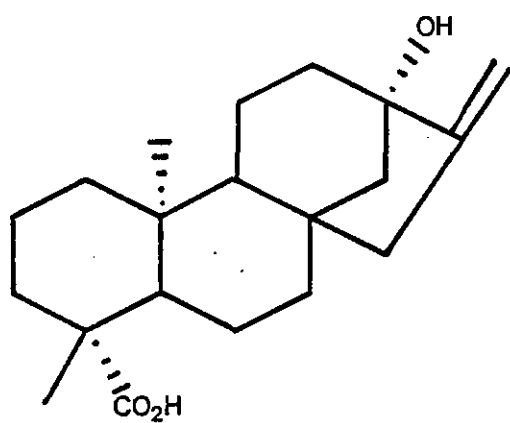


(-)-KAURENE
42



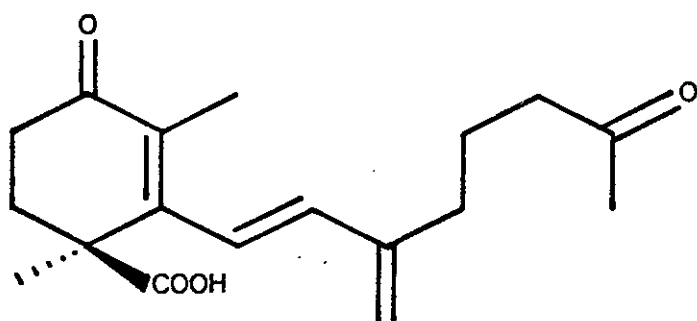
(-)-TRACHYLOBANE
43

FIGURE 24



STEVIOL
44

FIGURE 25



TRISPORIC ACID
45

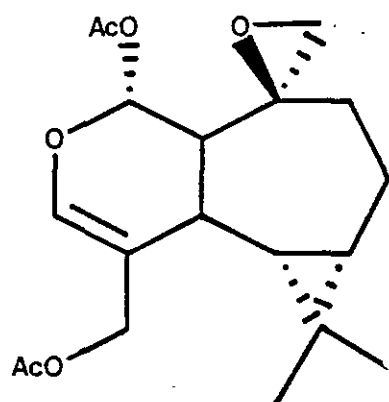
fungi elicits the formation of zygospores in (+) and (-) mycelia of *Mucor mucedo* by diffusing through the medium rather than by translocation through its hyphae (Gooday, 1978). The trisporic acid causes an increase in the content of β -carotene and ergosterol in recipient mycelium. Such increases are observed during mating.

1.3.3 Allomones

Two functions are ascribed to allelopathic terpenes: defence from predation (antifeedants), and the ability to deter rival plants from growing nearby in competition for space and nutrients (for review, see Rodriguez, Mitchell & Towers, 1976). An example of an antifeedant is the sesquiterpene hemiacetal plagiochiline A (46) which gives liverworts of the *Plagiochila* genus a characteristically pungent taste (Asakawa, Toyota, Takemoto, Kubo & Nakanishi, 1980). Plagiochiline A demonstrated strong antifeedant activity against the African army worm *Spodoptera exempta* when the worm was exposed for two hours to a concentration of plagiochiline A of 1-10 ng/ml.

Fungi and bacteria elaborate growth inhibitors of varying degrees of potency and specificity, some of which are in common medical use as antibiotics (Garb, 1961). Although plant growth inhibitors are often referred to as "toxic substances", they are usually not particularly toxic by pharmacological or toxicological standards (Garb, 1961). There is a wide gap between the concentration which inhibits the growth of sensitive plants and the concentration which actually kills those plants. An example of a plant inhibiting the growth of neighbouring plants is afforded by the demonstration that extracts of rapidly expanding tobacco leaves inhibit the growth of wheat coleoptiles (Cutler & Cole, 1974). This activity was not due to abscisic acid, and the authors suggested the stress diterpenes mentioned earlier were the active chemicals. Later, these authors showed that diterpenes such as sclareol (47) isolated from various tobacco species and cultivars, inhibit the growth of wheat coleoptiles (Cutler, Reid & Deletang, 1977).

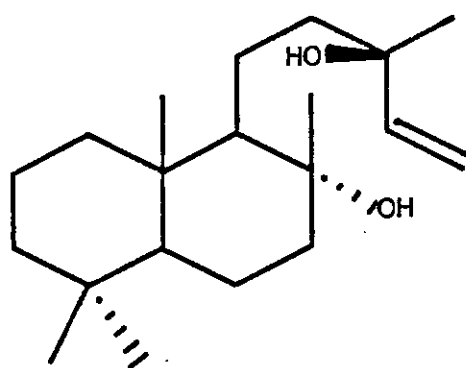
FIGURE 26



PLAGIOCHILINE A

46

FIGURE 27



SCLAREOL

47

Allelopathy against plants is noticeable at the organism level by such features as growth retardation, necrosis, or even absence of organisms adjacent to the producer species. For example, immediately adjacent to thickets of *Salvia leukophylla* are zones of bare soil 1-3 metres in width which merge into areas of inhibited herbs and finally undisturbed grassland at a distance of 3-9 metres (Muller, Muller & Haines, 1964). Of the various terpenes produced by *Salvia leukophylla*, cineole is the most inhibitory to the growth of herb seedlings (Muller & Muller, 1964). Cell elongation, cell division, and initiation of lateral roots are severely retarded by the presence of materials emanating from *Salvia leukophylla* (Muller, 1965; Muller & Hauge, 1967). The respiratory activity of seedlings and excised roots of annual herbs is also inhibited (Muller, Lorber & Haley, 1968).

In *in vitro* experiments, the volatile materials from the leaves of *Salvia leukophylla*, including the monoterpene cineole, reduce the oxygen uptake by mitochondria isolated from *Avena fatua* herb seedlings (Muller, Lorber, Haley & Johnson, 1969). In addition, the permeability of cell membranes is decreased on exposure to terpenes (Muller, Lorber, Haley & Johnson, 1969).

These physical effects are the cumulative result of subcellular pathologies. Ultrastructural changes that occurred in *Cucumis sativa* seedlings included accumulation of free lipids within cells. Widespread systemic disturbances resulted in a drastic reduction in a number of organelles, including mitochondria, and disruption of membranes surrounding nuclei, mitochondria and dictyosomes (Lorber & Muller, 1976). The authors suggested the free lipid that accumulated was from membranes of the degraded organelles.

Many plant growth inhibitors also inhibit the growth and/or multiplication of animal cells (Garb, 1961). Such terpenes interfere with the basic physiology of the cell. Terpenes bearing exocyclic α -methylene- γ -lactones elevate cyclic AMP levels, inhibit lysosomal activity, inhibit prostaglandin synthetase (Hall, Starnes, Lee & Waddell, 1980), and damage DNA templates (Jones, Kim &

Donnelly, 1981) by alkylating cellular DNA (Woynarowski, Beerman & Konopa, 1981; Woynarowski & Konopa, 1981). They also inhibit DNA and RNA synthesis (Klimek, Chmiel & Baer, 1981) and translation (Hladon & Twardowski, 1979) by binding to ribosomes (Cundliffe, Cannon & Davies, 1974; Cannon, Jiminez & Vazquez, 1976).

Aphidicolin inhibits protein synthesis by selectively inhibiting cytosolic DNA polymerase α (Ikegami, Taguchi, Ohashi, Ogura, Nagano & Mano, 1978; Ohashi, Taguchi & Ikegami, 1978; Seki, Oda & Ohashi, 1980). However, the major site of action is the mitochondria.

Terpenes with the α -methylene cyclopentenone rings decrease mitochondrial respiration at state 3 (Taniguchi, Yamaguchi, Kubo & Kubota, 1979).

The as yet unidentified inhibitor- β -complex from dormant potato peels was shown to stimulate oxygen uptake in potato discs and oat coleoptile sections at concentrations that inhibit coleoptile section growth (Marinos & Hemberg, 1960). Although this is in contrast to the effect of respiratory inhibitors on isolated mitochondria, the authors suggested that the growth inhibitory effect of the β -complex may still be mediated by uncoupling phosphorylation and thus deprive the tissue of the energy necessary for growth.

Uncoupling oxidative phosphorylation from electron transfer in mitochondria is induced by altering the proton permeability of the mitochondrial envelope. This occurs either by the uncoupling agent reacting with the protein moiety of the mitochondrial membrane (Fujita, 1966; Liberman, Topaly, Tsofina, Jusaitis & Skeluchev, 1969; Hanstein, 1976), or by the uncoupling agent dissolving in the lipid phase of the membrane and conducting protons across it, thus preventing formation of pH gradients during electron transport (Cunarro & Weiner, 1975).

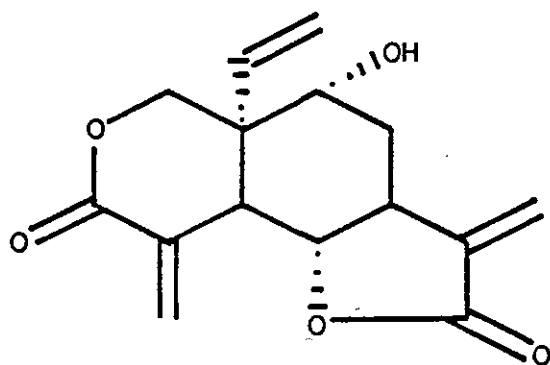
The most efficient phenol uncouplers are hydrophobic and there is a relationship between biological activity and uncoupling (Parker, 1965). Uncouplers such as 2,4-dinitrophenol increase the specific electrical conductance across bilayer phospholipid

membranes (Hopfer, Lehninger & Thompson, 1968). Experiments on isolated rat liver mitochondria indicate there is a direct correlation between proton-carrying and respiratory-releasing properties of a range of uncoupling agents (Cunarro & Weiner, 1975). Although experiments using bilayer lipid membranes show there is no simple correlation between the relative effectiveness of sulphydryl uncouplers, for example, the isothiocyanates, on mitochondrial respiratory control and their effects in increasing the specific conductance of bilayer lipid membranes, the uncoupling activity of sulphydryl uncouplers on isolated mitochondria is linearly associated with molecule lipophilicity (Miko & Chance, 1975).

One of the reactive groups known to act as an uncoupler is the exocyclic α -methylene- γ -lactone. Vernolepin (48) is an example of a terpene exhibiting this functional group (Kupchan, 1970). These uncouplers react with the sulphydryl groups on proteins to form thioesters (Smith, Larner, Thomas & Kupchan, 1972; Hanson, Lardy & Kupchan, 1970; Kupchan, Fessler, Eakin & Giacobbe, 1970). This is demonstrated on purified proteins such as glycogen synthetase from muscle or phosphofructokinase. It is extrapolated that the same reactive group would bind to proteins embedded in the mitochondrial membrane. Alkylating agents capable of binding to proteins in mitochondrial membranes uncouple oxidation from phosphorylation, thus altering the conformation of the membrane and uncoupling the electron transport chain (White, Handler & Smith, 1973).

The α -methylene- γ -lactone moiety is often found in sesquiterpenes, and a large body of literature exists examining the mechanism of action of these toxic compounds, for example, Kupchan, Fessler, Eakin and Giacobbe (1970). Of pharmacological interest is their association with activities against neoplasias, arthritis and inflammation. Although the cytotoxic activity of compounds bearing the α -methylene- γ -lactone moieties increases with increasing lipophilicity of the molecule, the *in vivo* antitumour activity of these molecules is not associated with lipophilicity (Kupchan, Eakin & Thomas, 1971). Instead, in addition to the α -methylene- γ -lactone, the possession of either an α -methylene- γ -lactone or a conjugated

FIGURE 28



VERNOLEPIN

48

side chain ester as the second functionality, and a hydroxyl or O-acyl group adjacent to the CH₂ of the γ -lactone, enhances the antitumour activity of these molecules.

Terpenes bearing the α -methylene- γ -lactone can be antineoplastic, antibacterial, and retard insect growth (Ganjan & Kubota, 1980; Taniguchi, Yamaguchi, Kubo & Kubota, 1979). This functional group is cytotoxic according to some workers (Lee, Meck, Piantodosi & Huang, 1973; Cassady, Ojima, Chang & McLaughlin, 1979; Lee, Ibuka, Wu & Geissman, 1977) but not to others (Rodriguez, 1980). Kim (1980) maintains that the toxicity of a lactone depends on the number of alkylating centres such as cyclopentenone, α -methylene- γ -lactone, or hemiacetal moieties in the molecule. Schroeder, Rhomer, Beck and Anton (1979) found that cytotoxic activity declined regularly with the increasing length of aliphatic side chains.

1.4 THE ALLELOPATHIC FUNCTION OF OCTOCORALLIAN TERPENES

1.4.1 Growth Inhibition

A growth inhibitory role for alcyonacean terpenes became apparent with the discovery that a colony of the scleractinian coral *Pavona cactus* was stunted in an arc downstream from the alcyonacean coral *Simularia flexibilis* (Plate 9) (Sammarco, Coll, La Barre & Willis, 1983). The cembranoid diterpene flexibilide (49) was isolated from the *Simularia flexibilis* colony and was believed to be the substance responsible for the observed stunting. In a laboratory experiment, flexibilide lysed the tissue from two scleractinian corals, *Pavona cactus* and *Porites andrewsii*, overnight at a concentration as low as 2 mg/l, as did the sesquiterpene FN45 (52) isolated from members of the alcyoniidae genus *Lobophytum* (Coll & Sammarco, 1983).

Dunlap designed a pumping system to push water from around an *in situ* colony of *Simularia flexibilis*, which also contained

PLATE 9

POSSIBLE *in situ* ALLELOPATHIC INTERACTIONS BETWEEN
ALCYONIIDAE AND SCLERACTINIA

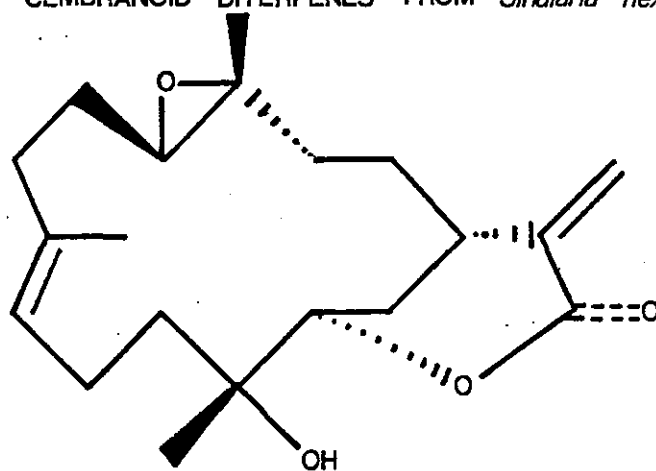


TOP *Simularia flexibilis* and *Pavona cactus*.
Photographs courtesy B. Willis.

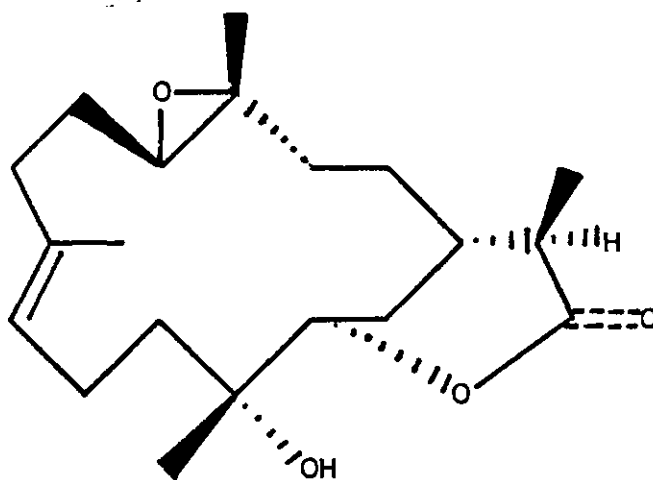
BOTTOM *Sarcophyton* sp. and *Pavona cactus*.
Photograph courtesy J.C. Coll.

FIGURE 29

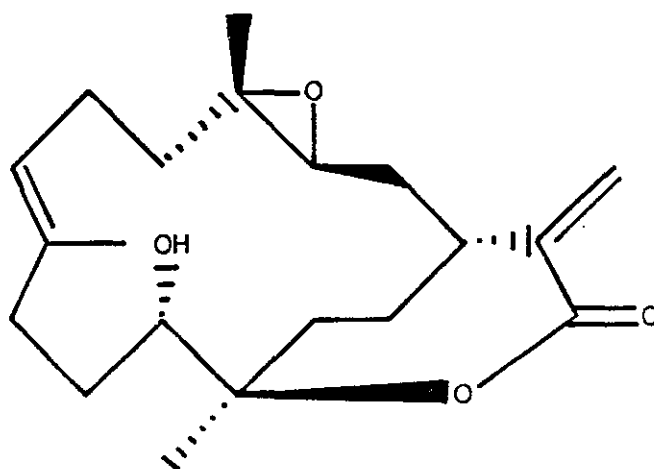
RELEVANT CEMBRANOID DITERPENES FROM *Sinularia flexibilis*



FLEXIBILIDE
49



DIHYDROFLEXIBILIDE
50

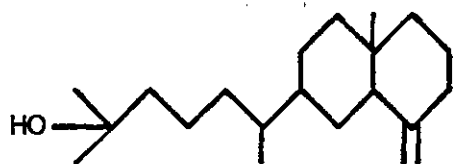


SINULARIOLIDE
51

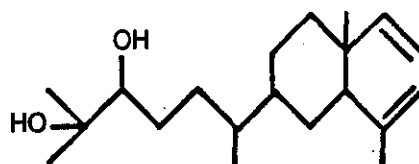
FIGURE 30

RELEVANT CARYOPHYLLANE

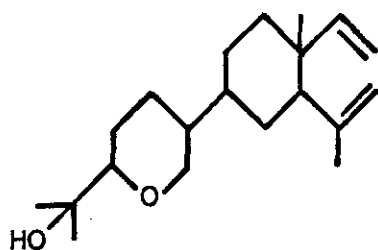
TERPENES ISOLATED FROM *Lobophytum* spp.



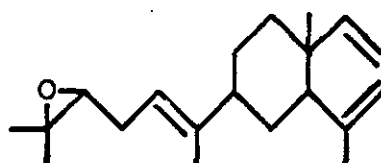
FN45
52



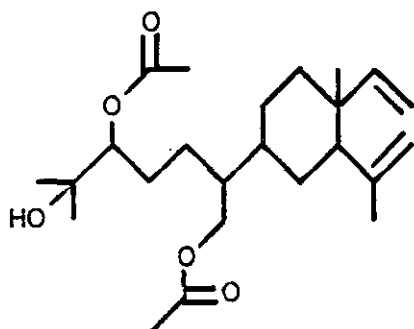
53



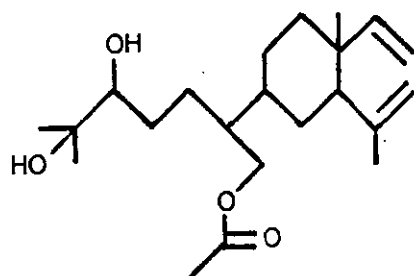
54



55



56



57

flexibilide, through reverse-phase silica columns (Coll, Tapiolas, Bowden & Dunlap, 1982). These columns retained flexibilide demonstrating that flexibilide was released from the coral into the surrounding water. Using the same apparatus, the cembranoid diterpenes, sarcophytoxide (15) and sarcophine (16) were detected in the water surrounding a colony of *Sarcophyton* sp. which contained sarcophytoxide and sarcophine.

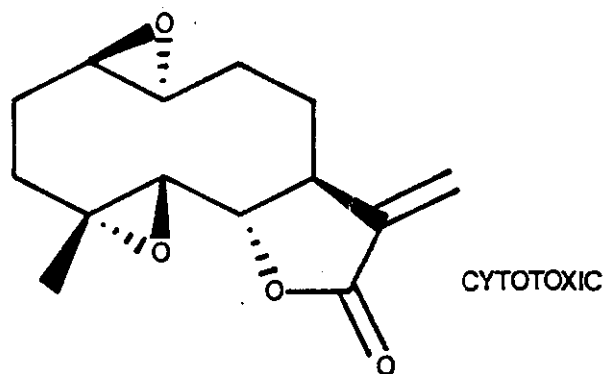
Flexibilide contains the exocyclic α -methylene- γ -lactone implicated as the functional group responsible for the properties of the growth inhibitory terpenes isolated from terrestrial plants, for example, michenolide (58) is cytotoxic whereas dihydroreynosin (59) is not (Kupchan, Fessler, Eakin & Giacobbe, 1970; Kupchan, Eakin & Thomas, 1971; Rodriguez, Mitchell & Towers, 1976; Cassady, Ojima, Chang & McLaughlin, 1979). Many of these terpenes bearing α -methylene- γ -lactone moieties are cytotoxic, for example, to Eagles' carcinoma of the nasopharynx cell culture system 9KB (Ogura, Cordell & Farnsworth, 1978; Cassady, Ojima, Chang & McLaughlin, 1979).

Lobophytum pauciflorum, *Simularia pavida* and *Xenia* sp. aff. *danae* all effected local tissue necrosis when placed within three cm of the scleractinian corals (Sammarco, Coll, la Barre & Willis, 1983). For an example, see Plate 10. The terpene content of these alcyonacean corals was not reported.

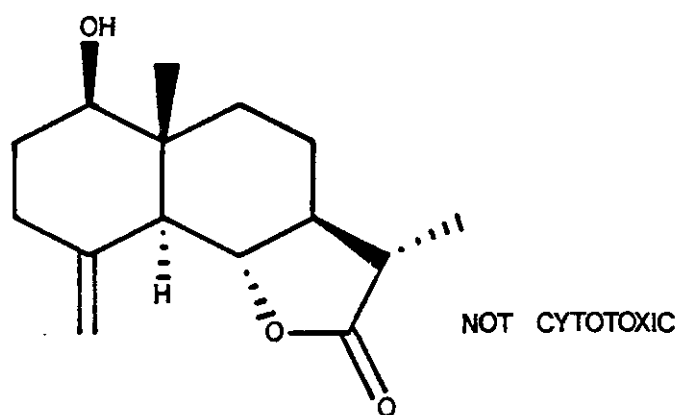
1.4.2 Defence

A dual role in defence and aggression is common in terpenes synthesized by terrestrial plants (McKey, 1979); and it is believed that, in addition to inhibiting the growth of rival corals, terpenes in octocorals have an antifeedant function (Ciereszko, 1962; Fenical, 1982; Bernstein, Shmouli, Zadock, Kashman & Neeman, 1983). Indeed, aqueous homogenates and ethanol extracts of several alcyonacean corals, including the genera *Sarcophyton*, *Lobophytum* and *Simularia*, are toxic to the fresh water fish *Gambusia affinis* (Coll,

FIGURE 31
TOXIC AND NONTOXIC TERPENES FROM COMPRESSAE



MICHELENOLIDE
58



DIHYDROREYNOSIN
59

PLATE 10

Lobophytum SPP. EFFECTING LOCAL TISSUE NECROSIS
ON ADJACENT SCLERACTINIAN CORAL.



Photograph courtesy S.la Barre.

La Barre, Sammarco, Williams & Bakus, 1982) and goldfish (Bakus, 1981).

A high proportion of extracts of marine organisms are cytotoxic, antibacterial, antifungal or antiviral (Rinehart *et al.*, 1981), and, in particular, terpenes isolated from octocorals possess antibacterial, cytotoxic, antifeedant and growth inhibitory properties (Rinehart *et al.*, 1981; Schmitz, Hollenbeak, Carter, Hossain & Van Der Helm, 1979; Jacobs, White & Wilson, 1981; Kinnel *et al.*, 1979; Burrenson, Scheuer, Finer & Clardy, 1975; Imamura & Ruveda, 1980; Schmitz, Hollenbeak, Carter, Hossain & Van der Helm, 1979).

An example is the sesquiterpene farnesylacetone epoxide (22), which is an analogue of juvenile hormone, inhibited respiration in isolated rat mitochondria at state 3 (Spence, Jamieson & Taylor, 1979). A 50 percent inhibition occurred at 5 μ M. The analogues of juvenile hormone found in plants are suggested to protect the plants from insects by instigating premature, and hence lethal, moulting (Herout, 1974).

Farnesylacetone epoxide (22) was isolated from the brown alga *Cystophora moniliformis* (Kazlauskas, Murphy & Wells, 1978). It displayed anticonvulsant activity in mice at nonsedative doses of 300mg/kg intraperitoneally (Spence, Jamieson & Taylor, 1979). At doses higher than 300 mg/kg, farnesylacetone epoxide initially caused hyperactivity which was followed by ataxia, and finally by a loss of the righting reflex. It caused a nonspecific block to agonists on isolated guinea-pig ileum and also inhibited the spontaneous beating of the isolated guinea-pig atria. The LD₅₀ was greater than 900 mg/kg administered intraperitoneally to mice. It was inactive orally. The caryophyllanoid sesquiterpene (53) biogenetically related to FN45 also isolated from the genus *Lobophytum*, likewise inhibited respiration in isolated rat liver mitochondria and also displayed anticonvulsant activity in mice (Baird-Lambert, Dunlop & Jamieson, 1980).

There are several studies indicating the toxicity of octocorals and their terpenes to potential predators and fouling organisms. Settlement of barnacle larvae *Balanus amphitrite amphitrite* in an aquarium was completely inhibited when either an aqueous homogenate or a dialysate of the homogenate of the gorgonian *Leptogorgia virgulata* was added to the aquarium water (Standing, Hooper & Costlow, 1984). The water ambient around the gorgonian also inhibited settlement of the larvae. Although the compound that inhibited settlement was not identified, the authors suggested that it might be a terpene. The only information on the compound is that it has a molecular weight of less than 20 000 daltons, and hence is not polymeric.

Studies on the toxicity of the diterpene crassin acetate (21) isolated from gorgonians of the *Pseudoplexaura* genus; *P. porosa*, *P. flagellosa*, *P. wagnaari*, *P. crucis* and *P. crassa* (Ciereszko, 1962; Jacobs, White & Wilson, 1975; Weinheimer & Matson, 1975; Hadfield & Ciereszko, 1978), indicate that this terpene is toxic to a wide range of potential predators. Seawater saturated with crassin acetate caused the velar cilia of the molluscan nudibranch larvae, *Phestilla sibogae* Bergh, to disappear immediately and the larvae to die overnight (Hadfield & Ciereszko, 1978). The same terpene is also toxic to the rotifer *Brachionus plicatilis* Muller, and to the amphipod *Parhyale hawainensis* Dana (Lee, Macko & Ciereszko, 1981); concentrations of crassin acetate of 1 mg/l in seawater resulted in loss of swimming activity or mortality in 48 hours.

At a concentration of 0.027 to 0.133 mM, crassin acetate increased the generation time, population density and motility, and caused the death of, the ciliated protozoa *Tetrahymena pyriformis* (Perkins & Ciereszko, 1973). Some strains of *Tetrahymena pyriformis*, however, are resistant to these toxic effects (Perkins & Ciereszko, 1970).

Crassin acetate has also demonstrated toxicity to *Endamoeba histolytica* (Ciereszko et al, 1960), the fertilised eggs of the sea

urchin *Lytichinus variegatus* (at 10 mg/l), and to parrot fish (Ciereszko, 1962), to juvenile barnacles (Faulkner, as reported by Weinheimer & Matson, 1975). This terpene was been shown to inhibit cell division in the fertilised eggs of the sea urchin *Strongylocentrotus purpuratus* but did not inhibit the *in vitro* polymerisation of microtubules from beef brain, as did other terpenes isolated from the gorgonian *Pseudopterogorgia rigida* and from marine algae and sponges (Jacobs, White & Wilson, 1981).

The pursuit of commercially useful drugs has generated literature describing biological properties of terpenes elaborated by corals which are obviously not exercised in nature. For example, gorgonians of the genus *Briareum* produce diterpenes (Figure 19) with insecticidal properties against the grasshopper *Melanopus bivattus* (Grode, James, Cardellina & Onan, 1983). Strangely, even though these terpenes are toxic to the grasshoppers, they do not act as antifeedants to the grasshoppers. The terpenes were toxic to bacteria but not mutagenic.

Lophotoxin, a cembranoid diterpene isolated from several gorgonians of the *Lophogorgia* genus, is toxic to mice; subcutaneous injection is lethal (50 percent lethal dose being 8.0 $\mu\text{g/g}$ body weight) (Fenical, Okuda, Bandurraga, Culver & Jacobs, 1981). At doses of lophotoxin as low as 8×10^{-8} M, rat phrenic nerve-hemidiaphragm preparations exhibit irreversible inhibition of nerve-stimulated contraction without affecting the contraction evoked by direct electrical stimulation.

An aqueous alcohol extract of a colony of *Simularia flexibilis* displayed antineoplastic activity in *in vivo* bioassays against lymphocytic leukemia (P-388, PS test), and the active components were determined to be the cembranoid diterpenes sinularin (=flexibilide) (49), dihydrosinularin (=dihydroflexibilide) (50) and sinulariolide (51) (Weinheimer, Matson, Hossain & van der Helm, 1977). Flexibilide was shown to have antitumour activity *in vitro* (human nasopharynx carcinoma, KB test) and *in vivo* antitumour activity (PS test) but dihydroflexibilide was shown to be relatively

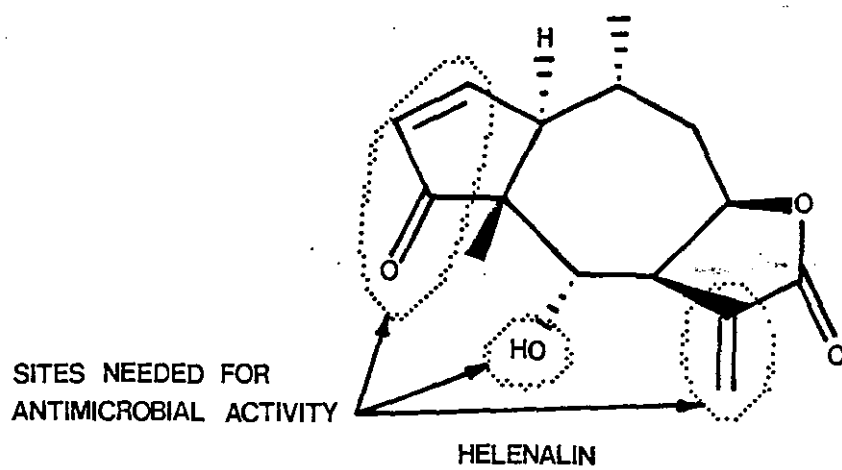
inactive (Schmitz, Prasad & Hollenbeak, 1978). Flexibilide has structural and stereochemical similarities with crassin acetate which is also antineoplastic in *in vivo* tests (P-388) and in *in vitro* tests (KB test) (Weinheimer & Matson, 1975).

However, flexibilide was shown to have low toxicity to mice when applied topically and when ingested, failing to cause ulceration of the gastrointestinal tract (Arrigoni-Martelli, 1981). These workers found flexibilide to have an anti-inflammatory effect on rat paw edema and granulomas due to embedded cotton pellets, and significantly reduced the development of primary and secondary lesions in adjuvant arthritic rats. These results were verified by Buckle, Baldo & Taylor (1980). Flexibilide had low toxicity to rats when administered orally and intraperitoneally, and was active after oral administration in both acute and chronic animal models of inflammation as ascertained by reduction of carrageenan-induced rat paw edema and cotton pellet granuloma.

Sarcophine contains a β -unsubstituted cyclopentenone ring which Lee, Ihuka, Wu and Geissman (1977) found to be more important in determining the antibacterial activity of the terpene than the presence of an α -methylene- γ -lactone ring, for example, helenalin (60) which also produces lethal damage to the DNA of the bacteria *Bacillus subtilis* (Jones, Kim & Donnelly, 1981). Sarcophine is reported to be calcium-antagonistic in rabbit aorta muscle (Kobayashi, Ohizumi, Nakamura, Yamakado, Matsuzaki & Hirata, 1983), and to be toxic to rats, mice, guinea pigs (Neeman, Fishelson & Kashman, 1974) and fish (Bernstein, Shmeuli, Zadock, Kashman, & Neeman, 1974; Kashman, Zadock & Neeman, 1974). Subcutaneous injection in mice at 10 mg sarcophine per kg body weight caused effects similar to those due to atropine: excitation, respiratory increase, paralytic effects, followed by coma and death (Neeman, Fishelson & Kashman, 1974). On the isolated guinea pig ileum, sarcophine at a concentration of 0.2 to 0.8 mg/l of solution, showed strong anti-acetylcholine activity (Neeman, Fishelson & Kashman, 1974). Sarcophine was also shown to be a competitive inhibitor of cholinesterase (Neeman, Fishelson & Kashman, 1974).

FIGURE 32

FUNCTIONAL GROUPS BELIEVED TO BE RESPONSIBLE FOR TOXICITY



Both sarcophine and sarcophytoxide are toxic to the fresh water fish, *Gambusia affinis* (Neeman, Fishelson & Kashman, 1974; Coll, Tapiolas, Bowden, Webb & Marsh, 1983). Tursch (1976) suggests fish would be deterred from eating corals if the corals contained toxic terpenes at a concentration of 0.001 percent or less. Tursch (1976) also found that the toxic terpenes from octocorals were potent inhibitors of algal growth.

1.5 MOLLUSCAN PREDATION ON ORGANISMS CONTAINING TERPENES

1.5.1 Predation On Octocorals

In spite of the possession of nematocysts, corals are preyed upon by a variety of organisms including fish, molluscs, echinoids and crustaceans (for review, see Vermeij, 1978), although the predators of octocorals are mostly molluscan (Patton, 1972; Harris, 1974; Rudman, 1981b). It is known why these animals are not deterred by the nematocysts nor by the toxic chemicals contained within the corals.

Patton (1972) examined 200 specimens of the gorgonian *Leptogorgia virgulata* and found several symbionts; the ovulid mollusc *Neosimnia uniplicata*, the nudibranch mollusc *Tritonia wellsi*, the shrimp *Neopontonides beaufortensis*, the bivalve *Ptereria colymbus*, the barnacle *Balanus galeatus*, and two unidentified copepods. None of these symbionts of *Leptogorgia virgulata* fed on the coral tissues.

However, many molluscs do feed on the tissues of octocorals. Seven species of aeolid opisthobranch molluscs belonging to the genus *Phyllodesmium* (Glaucidae; Favorininae) are specialised feeders on octocorals. There are also three species of aeolid opisthobranch and one arminid opisthobranch which feed on the scleractinian genus *Porites* (Rudman, 1981a). The octocorals known to be food sources for *Phyllodesmium* spp. are the alcyonacean genus *Xenia* and species of the octocorallian order Teleostacea. The

opisthobranchs have elaborate cerata which resemble the morphology of the corals on which they feed. The alcyonacean genus *Lobophytum* was suggested as a food source on the basis that the morphology of one species of *Phyllodesmium* resembles that of a *Lobophytum* spp. colony (Rudman 1981b). Crawling planula larvae released from the alcyonacean *Alcyonium siderium* are presumed to be eaten by the nudibranch *Coryphella verrucosa* Sars and by the sea urchin *Strongylocentrotus droebachiensis* Muller, both commonly seen browsing among the colonies of *Alcyonium siderium* (Sebens, 1983b).

Many molluscs feeding on corals, including some of the opisthobranchs mentioned above, develop a symbiosis with chloroplasts obtained from the zooxanthellae (Thompson, 1960; Trench, Colley & Fitt, 1981; Rudman, 1981b). The chloroplasts are retained in specialised pouches extending from the midgut. Photosynthesis occurs with the evolution of oxygen and the transfer of photosynthetate (Trench, Colley & Fitt, 1981).

Families of prosobranch gastropods living in symbioses with Cnidaria include members of the: Architectonicidae - feeding on zoanthids; Columbellidae - feeding on sea anemones; Epitoniidae - feeding on sea anemones, zoanthids and hard corals; Janthinidae - feeding on sea anemones, *Physalia* sp., *Velella* sp. and *Porpita* sp.; Corallophilidae - feeding on zoanthids, hard corals, Gorgonia and Alcyonacea (*Rapa*); and Ovulidae (=Amphiperatidae) - feeding on hard corals, Gorgonia, Alcyonacea, hydroids and hydrocorals (Robertson, 1965; 1967; Chalker, personal communication, 1985).

Ovulidae are a small family all of whose members live in association with Cnidaria (Robertson, 1970). Although *Jenmeria pustulata* accounts for about 80 percent of the biologically induced mortality of the scleractinian genus *Pocillopora* in Panamanian reefs (Glynn, Stewart & McCosker, 1972), the hosts of most Ovulidae genera are members of the Gorgonacea and the Alcyonacea. *Simnia spelta* feeds on the gorgonian *Eunicella stricta* coenchyme (Theodor 1967). *Simnia patula* feeds on the alcyonacean *Alcyonium digitatum* (Lebour, 1932; Fretter & Graham, 1962). *Pedicularia californica* feeds on

the purple stylasterine coral *Allopora californica* (Fox & Wilkie, 1970). *Cyphoma* sp. devour the gorgonian *Pseudopterogorgia* sp. (Berrill, 1966) and *Cyphoma gibbosum* graze on several gorgonians including *Gorgonia ventalina* (Birkeland & Gregory, 1975). The latter workers estimated that *Cyphoma gibbosum* accounts for four percent of mortality in *Gorgonia ventalina* in shallow water Panamanian reefs and 62.3 percent of tissue utilisation of this gorgonian species.

Several prosobranchs live on octocorals without ingesting the coral tissues as indicated by the observations that the tissues are undamaged after the prosobranch has passed over it. *Neosimnia uniplicata* feeds on surface debris and material shed by the gorgonian *Leptogorgia virgulata*, (Patton, 1972). *Cymbovula* spp. also live on gorgonian corals and have gorgonian spicules in their intestines and feces, yet do not damage the coral tissues (Birkeland & Gregory, 1975). These authors assume that *Cymbovula* spp. feed on the mucus and debris covering the coral as do juvenile forms of *Cyphoma* spp.

1.5.2 Use Of Ingested Terpenes As Defence Agents

Kairomones occur frequently in the marine world. Molluscs in particular retain for their own defence some of the toxic chemicals ingested from their sponge, algal or octocorallian diets for their own defence. For examples, see Faulkner and Stallard (1973) and Bowden, Coll, Hicks, Kazlauskas and Mitchell (1978a). Many novel chemicals first isolated from nudibranchs were later discovered in their respective food sources (Faulkner & Stallard, 1973; Stallard & Faulkner, 1974a; Burreson, Scheuer, Finer & Clardy, 1975; Hagadone, Burreson, Scheuer, Finer & Clardy, 1979; Kinnel et al., 1979; Schmitz, Hollenbeak, Carter, Hossain & Van Der Helm, 1979; Cimino, De Stefano, De Rosa, Sodano & Villani, 1980; Imamura & Ruveda, 1980).

Many species of opisthobranch lack a sturdy shell or mechanical

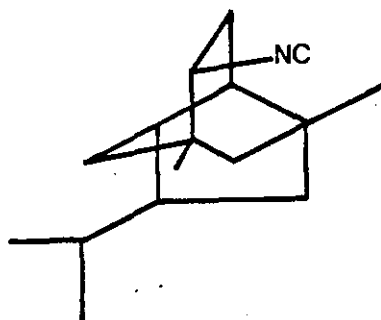
appendage to deter predators. Despite the lack of any obvious protection, these animals have few known predators. In one study, opisthobranch molluscs from 17 genera were refused as food by a variety of fish (Thompson, 1960). Their defences included possession of skin glands secreting strong acids, calcareous spicules in the skin, or sacs of nematocysts stored under the skin. Nematocysts were obtained from anthozoans which the opisthobranchs had ingested (Thompson & Bennett, 1969, Harris, 1974). Octocorallian-feeding opisthobranchs do not store nematocysts in the cerata (Rudman, 1981b).

The nudibranch *Phyllidia varicosa* Lamarck secretes as much as five mls of mucus within a few seconds of being disturbed (Johannes, 1963). This mucus is lethal to fish and crustaceans with death occurring within a half an hour to five hours after addition of the mucus (two percent). Interestingly, a crab *Metapograpsus messor* and another nudibranch *Placobranchus ianthobabus* were not affected after being exposed to the mucus for 24 hours. The active agent was hydrophobic, heat stable and volatile. This defensive secretion of the nudibranch *Phyllidia varicosa* contains isocyanopupukeanane (61) (Hagadone, Burreson, Scheuer, Finer & Clardy, 1979). This terpene is often isolated from the sponge *Hymeniacidon* sp. which is the preferred diet of *Phyllidia varicosa* (Burreson, Scheuer, Finer & Clardy, 1975). *Phyllidia varicosa* also accumulates debromoaplysiatoxin (62) from the blue-green alga *Lyngbya majuscula* (Scheuer, 1982).

The sea hare *Aplysia* spp. retains terpenes from its algal diet. For example, the digestive gland of *Aplysia californica* yielded a number of halogenated terpenes presumed to be retained from its algal diet (Stallard & Faulkner, 1974a); the digestive gland of *Aplysia kurodia* contained a brominated diterpene also presumed to be retained from its algal diet (Imamura & Ruveda, 1980); and the digestive gland of *Aplysia dactylomela* also contained sesquiterpenes from the algae *Laurencia nidifica* and *Laurencia filiformis*, in particular, aplysiastatin (63), 6 β -hydroxyaplysiastatin (64) and cyclohexadiene (65)

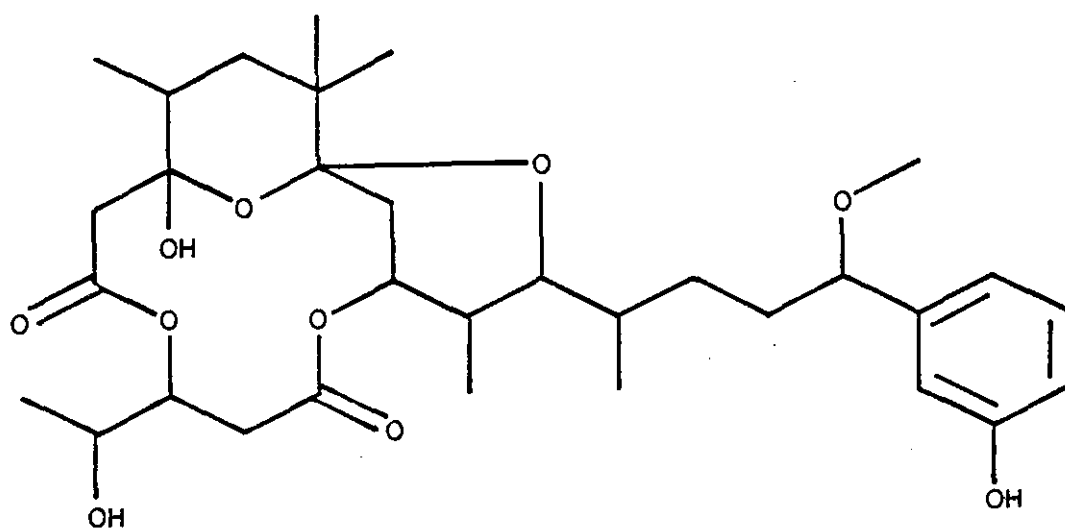
FIGURE 33

SOME COMPOUNDS ISOLATED FROM NUDIBRANCHS



9-ISOCYANOPUPUKEANE

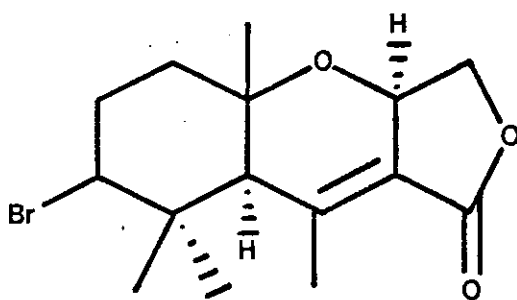
61



DEBROMOAPLYSIATOXIN

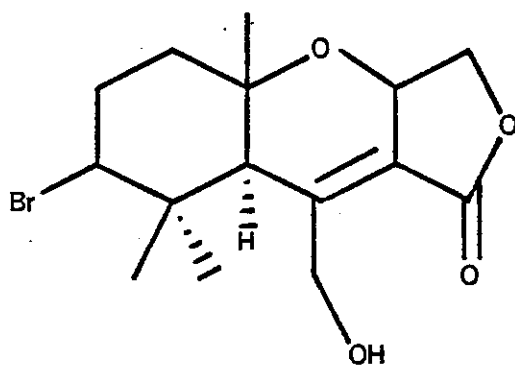
62

FIGURE 34
TERPENES ISOLATED FROM NUDIBRANCHS



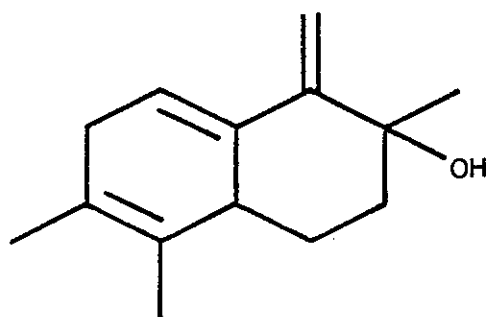
APLYSISTATIN

63



6β-HYDROXYAPLYSISTATIN

64



CYCLOHEXADIENE

65

(Capon, Ghisalberti, Jeffries, Skelton & White, 1981).

An example of *Aplysia brasiliiana* contained the algal sesquiterpenes brasilenyne and *cis*-dihydrorhodophytin in its body wall and digestive gland (Kinnel *et al.*, 1979). The body wall and the digestive gland of the *Aplysia brasiliiana* were unpalatable to sharks and small teleost fish.

Other nudibranchs that are known to store and concentrate secondary metabolites taken-up from their diet. These include *Dendrodoris grandiflora* which concentrates the terpene fasciculatin (66) from the sponge *Ircinia fasciculata*, and *Glossodoris gracilis* which concentrates the terpene longifolin (67) obtained from the sponge *Plerophysilla spinifera* (Cimino, De Stefano, De Rosa, Sodano & Villani, 1980).

The opisthobranchs that feed on octocorals have glands containing osmiophilic material scattered over the epidermis of the cerata (Rudman, 1981b). The cerata autotomise easily if the mollusc is disturbed. The autotomised cerata exude a sticky secretion and wriggle quite vigorously for some minutes. Rudman (1981b) presumes these autotomised ceratas stick to a predator's mouth and thus act as a deterrent. The author points out that there are no reports of predators on these molluscs, and suggests that camouflage is the greater defence. The composition of the sticky secretion and of the osmiophilic glands is not known although terpenes are generally osmiophilic (Heinrich, 1970).

1.5.3 Suggested Modification Of Alcyoniidacean Terpenes Ingested By A Predator

The ovulid egg cowrie, *Ovula ovum*, feeds on a variety of alcyonacean genera, including *Sarcophyton*, *Simularia* and *Nephthea* (Plates 11 and 12) (Bowden, Coll, Hicks, Kazlauskas & Mitchell, 1978a; Coll, Tapiolas, Bowden, Webb & Marsh, 1983). Two egg cowries feeding on an unidentified *Sarcophyton* colony (Plate 13) which contained the terpene sarcophytoxide, harboured large amounts

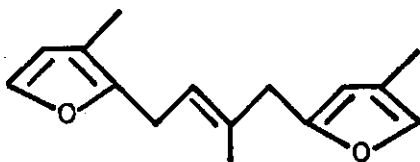
FIGURE 35

SPONGE TERPENES ISOLATED FROM NUDIBRANCHS



FASCICULATIN

66

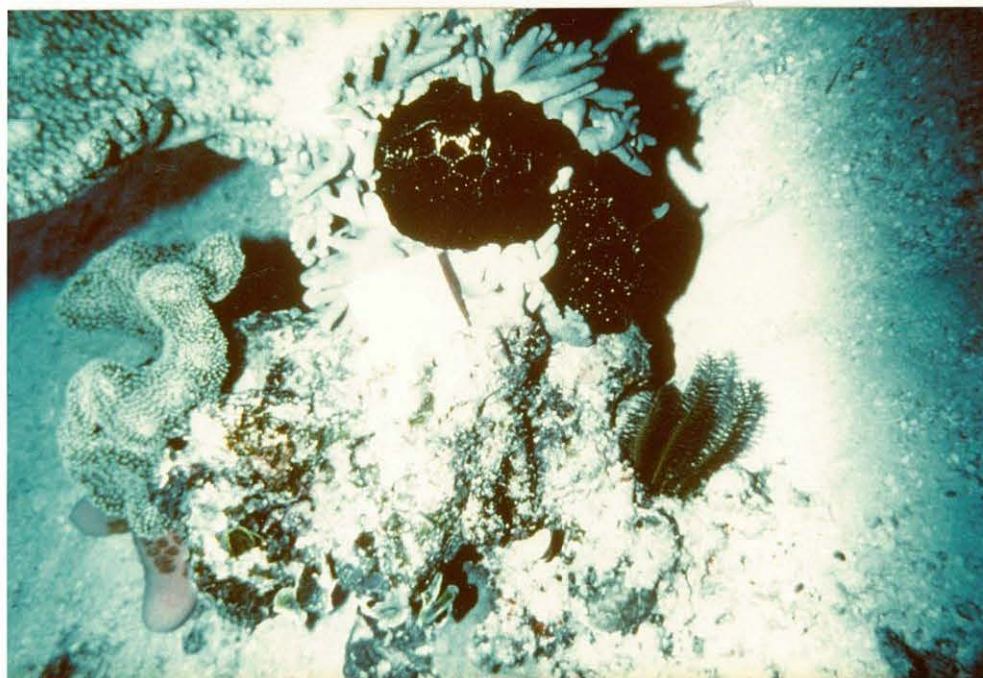


LONGIFOLIN

67

PLATE 11

TWO EXAMPLES OF *Ovula ovum* FEEDING IN PAIRS IN THE FIELD



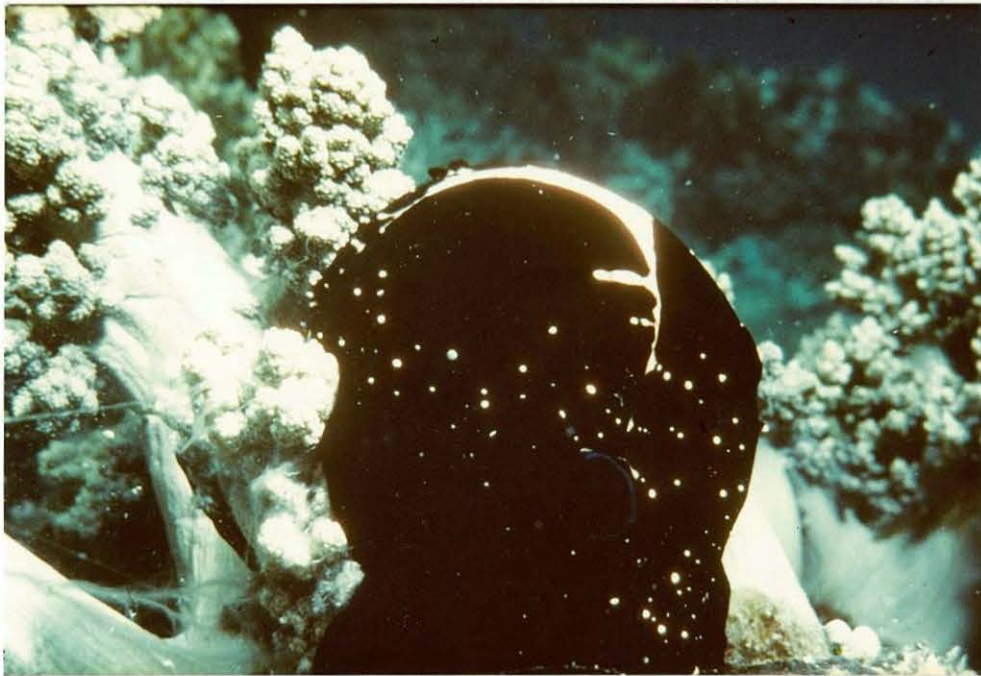
TOP These cowries feeding on *Sinularia* sp.
were found to contain epoxyisoneocembrane-A
(Bowden, Coll, Hicks, Kazlauskas, & Mitchell, 1978a).

BOTTOM Cowries 10 and 11 in Table 15, collected from John Brewer
Reef in November 1981.

Photographs courtesy J.C.Coll.

PLATE 12

Ovula ovum.

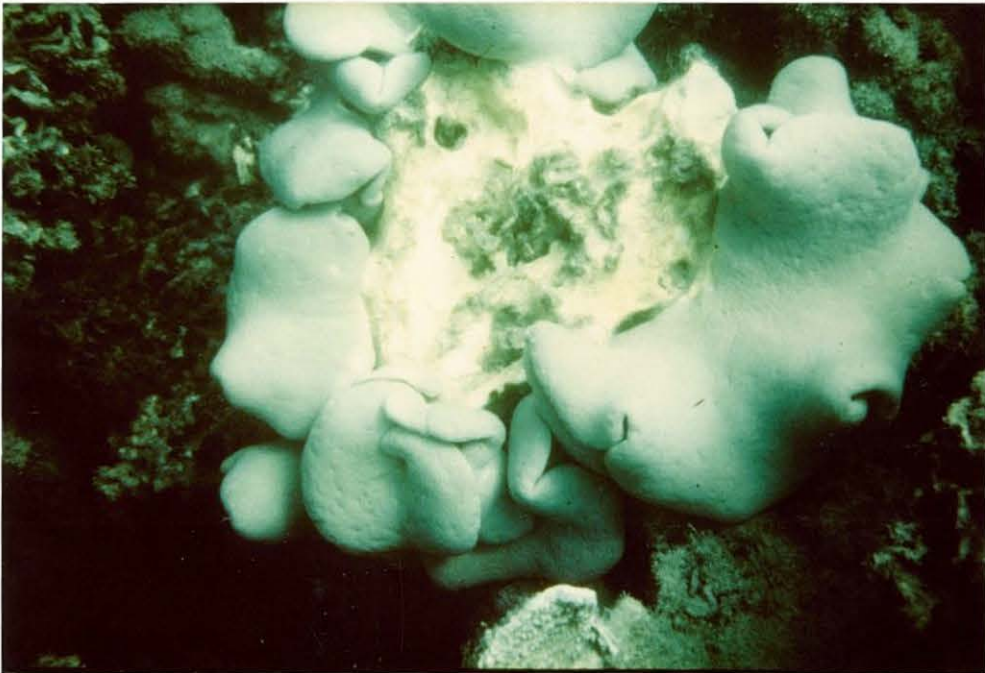


TOP Feeding on microalgae in the outdoor aquarium.

BOTTOM Feeding on *Nephthea brassica* *in situ* in the ocean.
Photograph courtesy S.la Barre.

PLATE 13

Sarcophyton stolidotum AFTER FEEDING BY *Ovula ovum* in situ.



Photograph courtesy J.C.Coll.

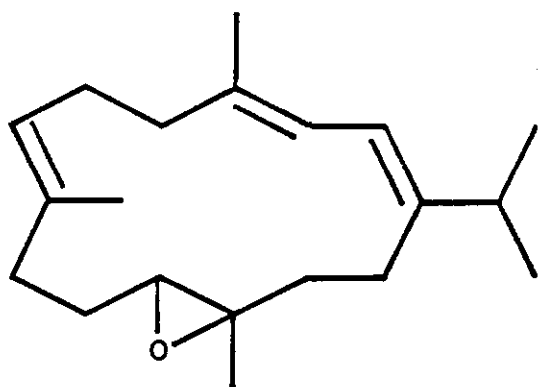
of deoxysarcophytoxide (18) in the digestive gland and feces yet no deoxysarcophytoxide was found in the coral (Coll, Tapiolas, Bowden, Webb & Marsh, 1983). Deoxysarcophytoxide was not toxic to the fresh water fish *Gambusia affinis* (0/6 fish in 24h at 2 mg/l) whereas sarcophytoxide was toxic (6/6 fish in 24h at 2 mg/l). The removal of the epoxide was shown to be neither spontaneous nor chemically trivial. The authors concluded *Ovula ovum* has the capacity to detoxify sarcophytoxide.

Ovula ovum is known to ingest terpenes from alcyoniidae corals and to retain them in their digestive glands unmodified, for example, epoxyisoneocembrene-A (68) (Bowden, Coll, Hicks, Kazlauskas & Mitchell, 1978a). However, Coll and coworkers (Coll, Bowden, Tapiolas, Webb & Marsh, 1983) clearly indicate their belief that neither the coral on which the cowries were originally photographed, nor the coral on which the cowries were later collected, contained the terpene deoxysarcophytoxide.

Arguing against the detoxification of sarcophytoxide by *Ovula ovum* is the observation by Ciereszko (1962) that the ovulid *Cyphoma gibbosum* habitually feeds on gorgonians containing toxin crassin acetate and is resistant to the toxic effects of crassin acetate. However, a precedent has been set for molluscs to modify terpenes. Stallard and Faulkner (1974b) demonstrated a 9 percent conversion *in vivo* of ³H-laurinterol (a sesquiterpene isolated from the red algae *Laurencia pacifica*) to aplysin by the seahare *Aplysia californica*. This conversion was believed to occur in the digestive gland. However, there^{are} arguments against this possibility:

- (1) The routine coisolation of the two terpenes from the algae and from the seahares may be an artefact of isolation, caused by rearrangement of the molecules on silica or on alumina. The authors admit decomposition of these compounds from extracts of *Aplysia californica* digestive glands when chromatographed on alumina (Stallard & Faulkner, 1974b). From the information presented in the paper it seems alumina was used in the final chromatography of radiolabelled aplysin. However, aplysin

FIGURE 36



EPOXYISONEOCEMBRENE - A

obtained from rearrangement of laurinterol should give a specific activity of greater than 9 percent of that of laurinterol. Degradation products of terpenes have specific activities identical to that of the mother terpene (Personal observation, 1985).

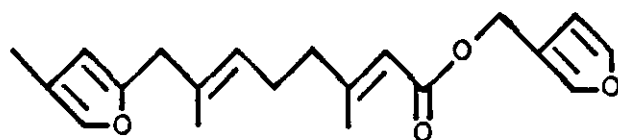
- (2) The organic extract of the *Aplysia californica* digestive gland was allowed "to stand" for one day before partitioning between ether and water. The authors were aware of the possibility that the conversion may be a result of experimental procedures, but felt that the harsh conditions needed to effect this conversion *in vitro* excluded this possibility.
- (3) The conversion from laurinterol to aplysin may be effected by the bacterial commensals of the digestive tract *in vivo*.
- (4) Laurinterol and aplysin are coisolated from several algae on which the seahare habitually feeds. There is no information on the relative retention times of these compounds in the seahare digestive gland.

Another nudibranch *Chromodoris marislae* contained the terpene marislin (70) which is similar to pleraplysillin (69) which is often isolated from sponges (Hochlowski & Faulkner, 1981). These authors also concluded that the nudibranch had modified the sponge metabolite. Recently, the *in vivo* synthesis of the terpene polygodial (71) from ^{14}C -mevalonate by another nudibranch, was reported by Cimino, De Rosa, De Stephano, Sodano and Villani (1983). Polygodial was found in the mantle of this shell-less mollusc. The digestive gland contained the terpene conjugated to acyl residues from several fatty acids (72). The latter terpenes were considered to be detoxification products.

A more decisive example of a predator modifying an ingested terpene is afforded by the larvae of the Douglas fir Tussock Moth which modified dietary monoterpenes obtained from the Douglas fir. The modifications were not effected by gut flora as: (a) most

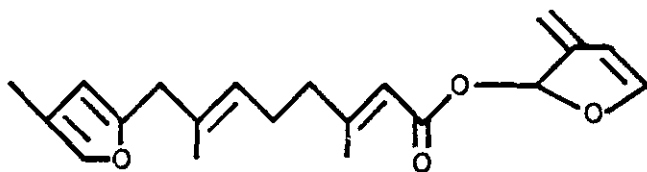
FIGURE 37

TERPENES ISOLATED FROM A SPONGE AND A NUDIBRANCH,
RESPECTIVELY



PLERAPLYSILLIN

69

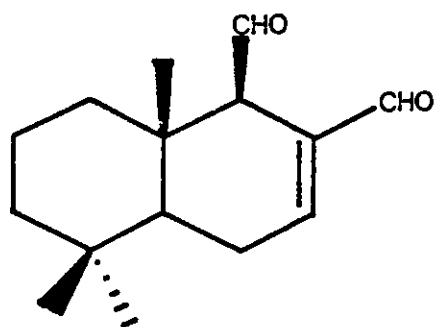


MARISILIN

70

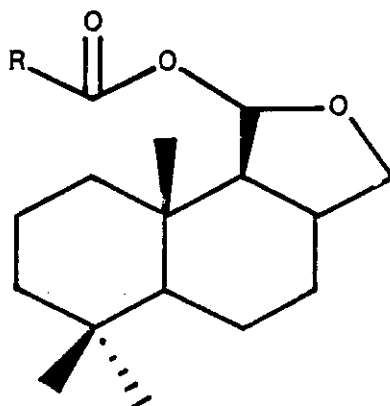
FIGURE 38

TERPENES SYNTHESIZED BY A NUDIBRANCH



POLYGODIAL

71



SESQUI-TERPENE ESTERS



Represents acyl residues from several fatty acids

72

midguts had no microbes, probably because of the toxic nature of the terpenes; and (b) larvae raised on antibiotics had the same composition of terpenes in the feces as those larvae not raised on antibiotics (Andrews & Spence, 1980).

The opisthobranch sea slug *Navanax inermis* is a voracious predator on other opisthobranch molluscs (Murray & Lewis, 1974), but does not appear to devour prosobranch molluscs. Blair and Seapy (1972) found in a field study, that, while 18 percent of the gastropods in the field were the prosobranch *Nassarius tegula*, it was absent from the diet of *Navanax inermis*. In fact, in laboratory studies, *Navanax inermis* did not follow the slime trail of *Nassarius tegula* as it did other molluscs which it overtook and ingested. Interestingly, *Navanax inermis* synthesizes its alarm pheromones, the navenones B and C (Figure 39), *de novo* from ^{14}C -acetate (Barrow, 1983). The navenones are not terpenoid.

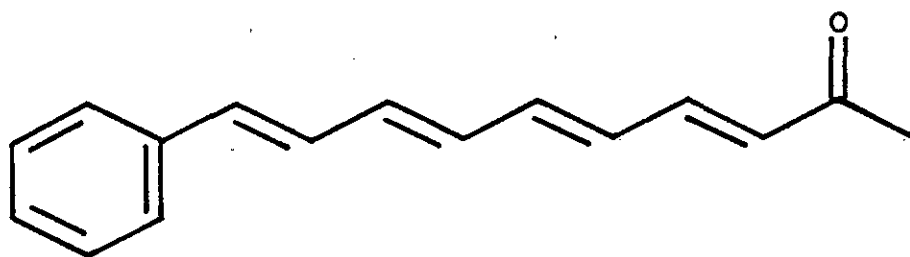
It is unlikely that *Ovula ovum* is susceptible to predation and hence uses terpenes obtained from the soft corals as a chemical defence, as its mechanical defence in the form of a thick shell would seem to be adequate. However, the ovulid *Jenmeria pustulata* (Solander) which preferentially feeds on the scleractinian genus *Pocillopora* appears to have natural predators as broken shells are found in the sediments near colonies on which they feed (Glynn, Stewart & McCosker, 1972). *Jenmeria pustulata* has a thinner shell than *Ovula ovum*, the largest of the prosobranch gastropods (Robertson, 1965). Birkeland & Gregory (1975) found no evidence of predation on *Cyphoma gibbosum* by the labrid fish *Lachnolaimus maximus*, nor by *Octopus* spp. or by crabs of the *Calappa* genus.

1.6 INTRODUCTION TO THIS STUDY

While there is a wealth of information on the chemistry of terpenes in alcyoniid corals (for review, see Faulkner, 1984), there is little information on their biological role (Tursch, 1976). Studies on the role of terpenes in alcyoniid corals are hampered

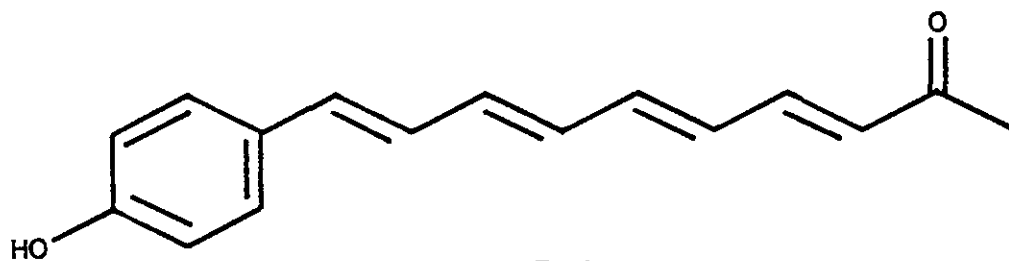
FIGURE 39

ALARM PHEROMONES



NAVENONE B

73



NAVENONE C

74

by the limited knowledge of both the biochemistry of the terpenes, and of the biology of living alcyoniidæan corals. The aim of this investigation was to elucidate a factual background pertaining to several key questions on the biochemistry and biology of these terpenes, rather than to conduct a detailed investigation of one topic.

To ensure this study detailed the biochemistry and biology of alcyoniidæan coral terpenes in general rather than in particular, linear and cyclic sesqui- and cyclic di-terpenes in both branching and massive species were examined. The terpenes were:-

- (1) A linear quinolated sesquiterpene (18) which will be referred to as 'furanquinol' throughout the dissertation. This terpene is isolated from the colony described above as *Simularia capillosa* (Coll, Hawes, Liyanage, Oberhansli & Wells, 1977; Coll, Liyanage, Stokie, Van Altena, Nemorin, Sternhell & Kazlauskas, 1978; Bowden, Coll, de Silva, de Costa, Djura, Mahendran & Tapiolas, 1983). The colony also elaborates several biosynthetically related terpenes (Figure 14). Trott (1980) proposed a pathway linking the various furanoterpenes found in *Simularia capillosa*.
- (2) A cyclic caryophyllanoid diterpene (52) originally isolated from a *Lobophytum hedleyi*, and referred to as FN45 throughout the dissertation (Bowden, Coll, Liyanage, Mitchell, Stokie & Van Altena, 1978b). FN is an abbreviation of field number. Other unidentified members of the *Lobophytum* genus elaborate the same terpene (Bowden, personal communication, 1981) and other biosynthetically related caryophyllenes (Figure 30) (Baird-Lambert, Dunlop & Jamieson, 1980).
- (3) The cyclic cembranoid diterpenes (15) and (16) which will be referred to as 'sarcophytoxide' and 'sarcophine', respectively, throughout the dissertation. These terpenes have been isolated from various *Sarcophyton* species, including *S. glaucum*, *S. trocheliophorum*, *S. ehrenbergi*, and *S. crassocaule* (Neeman,

Fishelson & Kashman, 1974; Bernstein, Shmeuli, Zadock, Kashman & Neeman, 1974; Tursch, 1976; Bowden, Coll, Hicks, Kazlauskas & Mitchell, 1978a; Bowden, Breakman, Coll & Mitchell, 1980; Coll, Bowden, Tapiolas & Dunlap, 1982). Bowden, Coll & Mitchell (1980a) suggested a biosynthetic pathway linking various cembranes isolated from *Sarcophyton crassocaule*.

- (4) A cyclic cembranoid diterpene (49) referred to as 'flexibilide' throughout this dissertation. This terpene has been isolated from several colonies of *Simularia flexibilis* (Weinheimer, Matson, Hossain & Van Der Helm, 1977; Kazlauskas, Murphy, Wells, Schonholzer & Coll, 1978; Buckle, Baldo & Taylor, 1980; Norton & Kazlauskas, 1980; Sammarco, Coll, la Barre & Willis, 1983), and from *Simularia conferta* (Schmitz, Prasad & Hollenbeak, 1978) and is sometimes referred to as simularin. Some biosynthetically related terpenes commonly isolated from *Simularia flexibilis* are presented in Figure 29.

This dissertation details studies on the incorporation of ³H-mevalonic acid lactone into the linear quinolated sesquiterpenoid furanoquinol by *Simularia capillosa*, and into the cyclic cembranoid diterpene sarcophytoxide by *Sarcophyton* species. The factors influencing terpene biosynthesis *in vivo* are investigated. The role of zooxanthellae in the biosynthesis of terpenes is studied using symbiotic and aposymbiotic colonies. Terpenes from several alcyoniid species are examined for toxicity to rival scleractinian corals and to the molluscan predator, *Ovula ovum*.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 CORAL BIOLOGY

2.1.1 Coral Collection

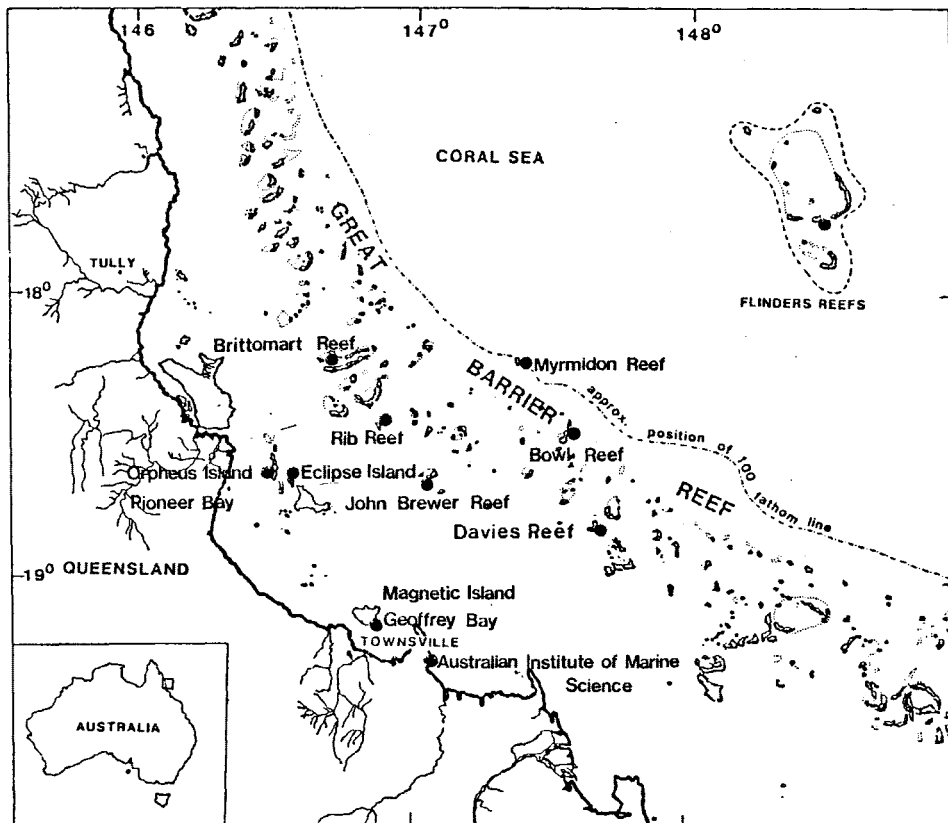
Alcyoniidæan soft corals of the *Sarcophyton*, *Simularia* and *Lobophytum* genera were collected by divers using SCUBA from several reefs in the central region of the Great Barrier Reef, Australia (Figure 40):

Bowl Reef (147°36'E, 18°31'S);
Brittomart Reef (146°42'E, 18°18'S);
Davies Reef (147°38'E, 18°51'S);
Eclipse Island (146°37'E, 18°32'S);
Flinders Reefs (148°27'E, 17°44'S).
Geoffrey Bay at Magnetic Island (146°49'E, 19°8'S);
John Brewer Reef (147°6'E, 18°41'S);
Lizard Island (145°28'E, 14°39'S);
Myrmidon Reef (147°23'E, 18°16'S);
Pioneer Bay at Orpheus Island (146°28'E, 18°32'S);
Rib Reef (146°53'E, 18°29'S).

Living colonies for biosynthetic experiments were initially collected either by peeling the stalk from the substrate, or by prying the substrate loose with a hammer and chisel. Corals harvested in this manner did not survive for long afterwards, although the polyps were often extended on the first few days. The best method of collecting soft corals was to select colonies attached to the aragonitic skeleton of dead scleractinian corals. The soft coral-rubble unit was transferred to a bucket underwater, and the bucket transferred by boat to the laboratory on shore.

FIGURE 40

COLLECTION SITES ON THE CENTRAL REGION OF
THE GREAT BARRIER REEF



2.1.2 Species Identification

Members of the *Sarcophyton* genus were identified to the species level by colony shape, distance between the autozooids, and the size and shape of spicules located in the stalk and in the capitulum (Verseveldt, 1982). *Lobophytum carnatum* was identified in a similar manner as per Verseveldt, 1982. Members of the *Simularia* genus were identified to the species level by Dr S.la Barre (personal communication, 1983).

2.1.3 Coral Husbandry

2.1.3.1 Introduction

Biosynthetic studies require organisms that are not only living but are thriving. A common practice is to collect a coral from the reef and subject it to experimentation on board-ship (Rice, Papastephanou & Anderson, 1970; Papastephanou & Anderson, 1982). This practice is not always convenient as ship time is limited and expensive. Where possible, biochemists maintain organisms in artificial support systems where the organisms can be continually monitored, and are at hand when required.

Attempts by several scientists to keep octocorals in aquaria have not been successful. Ciereszko and workers (Ciereszko, Johnson, Schmidt & Koons, 1968) reported the decomposition of the gorgonian *Pseudoplexaura porosa* due to poor light. Patton (1972) reported that the gorgonian *Leptogorgia virgulata* decomposed within a week in an indoor aquarium serviced with running seawater. The gorgonian survived better in aerated tanks of artificial seawater regularly stocked with brine shrimp. In the latter aquarium, the corals were reported to be in 'good' condition for a few weeks, and 'survived' for three months. The author points out that the polyps were not seen extended at any time.

2.1.3.2 Methods

For the studies reported in this dissertation two aquaria were used; an outdoor aquarium serviced by running seawater, and an indoor aquarium serviced by recirculated seawater. The outdoor aquarium system at the Australian Institute of Marine Science, Townsville, Australia (147°4'E, 19°17'S), is a series of holding tanks arranged in a cascade to aerate the water, and is shielded from ultraviolet light by a glass roof. The full glare of the sun is reduced to 10 percent with a shade cloth. Water in the aquarium is at oceanic temperature (20-29°C). This aquarium is referred to as the 'outdoor aquarium' throughout this dissertation.

Some colonies were transferred to an indoor aquarium at James Cook University of North Queensland, Townsville, Australia (146°31'E, 19°11'S). This aquarium uses recirculated seawater and is maintained at 15-20°C. Aeration is by airstone attached to a plastic tube conducting air from an outlet. Illumination is through fluorescent lights switched on for 12 hours per day. This aquarium is referred to as the 'indoor aquarium' throughout this dissertation.

2.1.3.3 Results And Discussion

Simularia capillosa and *Sarcophyton* spp. maintained in the indoor aquarium did not thrive. Symbiotic *Sarcophyton* spp. gradually expelled their zooxanthellae although they did not die. After several months the colonies were small, white and hard (Plate 14). None of the several batches of *Simularia capillosa* survived in this aquarium. On the first day after collection many of the polyps were extended. On the second day no polyps were extended. Many colonies had patches of black mucus. This black is presumably due to oxidation of some substance within the coral, as dissection of the colony produced a brown fluid which turned black within minutes. The epidermis also turned black during freeze-drying. After six days in the aquarium, the colonies were

PLATE 14

Sarcophyton roseum AND *Sarcophyton infundibuliforme*
IN THE INDOOR AQUARIUM.



coated in mucus and the epidermis was peeling (Plate 15). The colonies disintegrated into a pile of spicules. No other aquaria were used to house *Simularia capillosa*.

In contrast, members of the genera *Sarcophyton*, *Lobophytum*, *Simularia*, *Efflatounaria*, *Parerythropodium* and *Xenia* grew successfully in the outdoor aquarium over several years. These corals fed at night, and two of the *Sarcophyton stolidotum* colonies spawned. A *Sarcophyton* sp. juvenile settled on the aquarium floor and grew to a height of 1 cm. Other *Sarcophyton* spp. colonies divided, sometimes more than once. Two *Sarcophyton boletiforme* colonies which were connected by a common stalk when collected from the reef, slowly separated to form two independent colonies. Members of the *Efflatounaria* and *Xenia* genera sent stolens onto the capitulum of nearby *Sarcophyton* spp. colonies as previously observed by Dineson (^{personal} communication, 1982).

The colour of *Sarcophyton* spp. tentacles included white, yellow, green and brown. Sometimes a *Sarcophyton* spp. colony bore two types of autozooids with different coloured tentacles, for example, white and yellow in *Sarcophyton trocheliophorum*. The polyps of all colonies were extended on most days (Plate 16). *Simularia firma* displayed a retractable net extending from the tips of this branching coral, reminiscent of the mesenterial filaments of scleractinian corals. The net was only observed at night. A similar net was seen at night in the field extending from a colony of *Simularia flexibilis* (Bowden, personal communication, 1983).

The base of colonies of the *Sarcophyton*, *Lobophytum* and *Simularia* genera grew over the substrate on which they were collected, and adhered to the aquarium floor. Several members of the *Sarcophyton* and *Simularia* genera 'moved' a few centimeters by leaning the stalk onto the floor as described by la Barre and Coll (1982). When *Sarcophyton* spp. colonies were harvested by peeling from the substrate, the remaining colony stalk grew tentacles, and later, a capitulum. Such colonies were initially devoid of zooxanthellae though later they were colonised. None of these

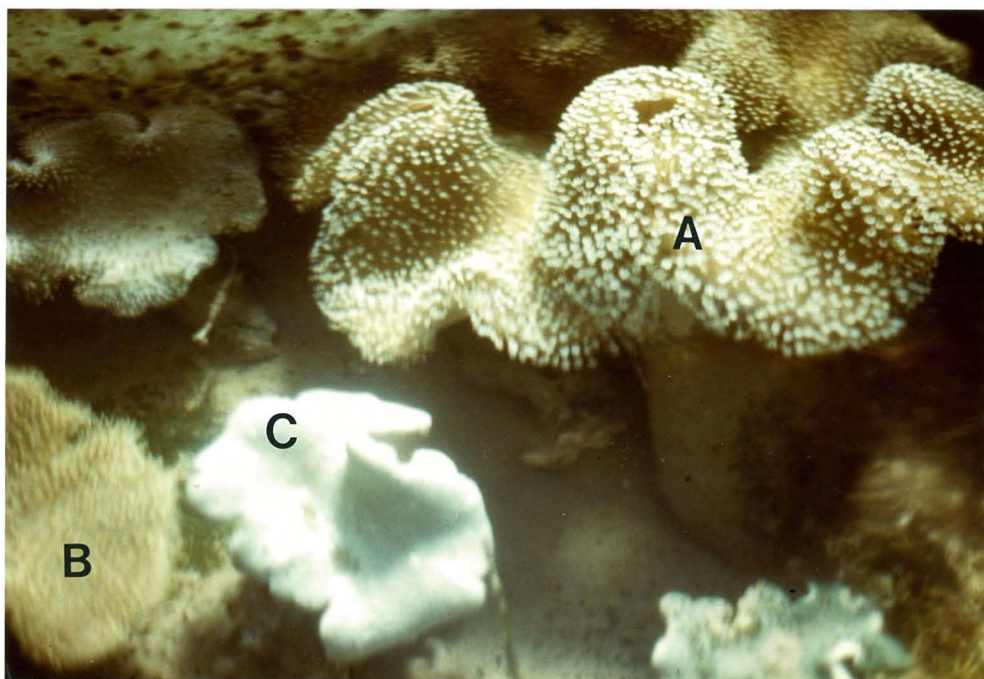
PLATE 15

Simularia capillosa IN THE INDOOR AQUARIUM.



PLATE 16

Sarcophyton spp. IN THE OUTDOOR AQUARIUM.



- A. *Sarcophyton auritum*.
- B. *Sarcophyton boletiforme*.
- C. *Sarcophyton roseum*.

latter colonies were subjected to experimentation.

Sampling living colonies of *Sarcophyton* spp. by taking "slithers" from the capitulum perimeter, as depicted in Figure 41, did not cause the colonies to contract. In contrast, samples (1 cm long by 1 cm wide by 0.2 cm deep) taken from the center of the capitulum of these same colonies of *Sarcophyton* spp., caused the colonies to droop for several weeks. With all sampling practices, the cut surface healed and showed polyp growth within three months.

Sampling *Simularia* spp. by cutting a growing tip (Figure 41) caused the colony to contract immediately, but the following day the colonies had recovered. The cut surface of a colony of *Simularia flexibilis* established in the outdoor aquarium showed polyp growth within two months, as did the cut surface of a colony of *Simularia firma* maintained in the same aquarium.

Colonies of *Acropora formosa* collected from Magnetic Island survived several years in the outdoor aquarium although the corals did not seem to grow noticeably. The white tips of the colony branches turned brown indicating growth had ceased (Oliver, personal communication, 1984).

2.2 TERPENE CHEMISTRY

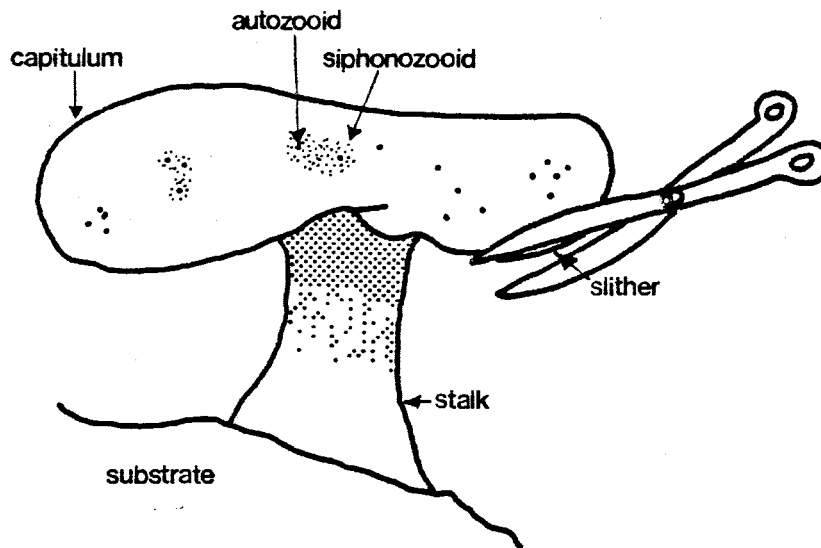
2.2.1 Solvent Preparation

Water, dichloromethane, petroleum ether and methanol were distilled using conventional methods. Diethyl ether, n-heptane and ethyl acetate were commercially prepared analytical solvents. Throughout this study, buffers, fixatives and incubation media are described as being constituted from seawater. The seawater was obtained from the ocean away from pollution by shipping and habitation. The seawater was filtered through cellulose membranes (Millipore, 0.22 μm) to remove bacteria and plankton.

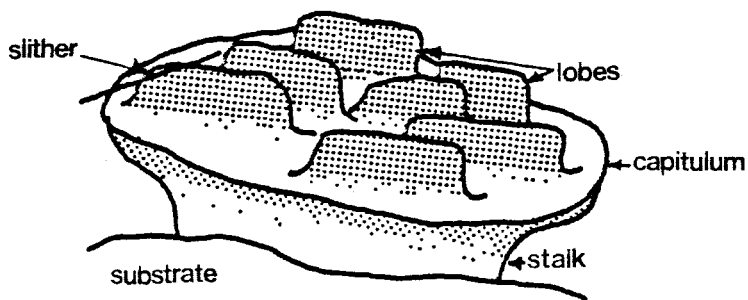
FIGURE 41

SITES ON THE COLONIES OF THREE GENERA USED IN REPETITIVE SAMPLING

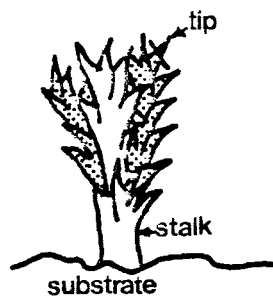
Sarcophyton



Lobophytum



Sinularia



2.2.2 Terpene Isolation And Identification

Colonies were harvested by peeling the stalk from the substrate. Colonies collected from the reef were placed in a nylon mesh bag underwater and transferred to a motorboat. The corals were frozen upon return to the mainland or research station (day trips), or to the mother ship. Corals previously established in aquaria were frozen immediately.

Tissue samples were freeze-dried, weighed, and extracted exhaustively in dichloromethane until the eluate was clear of pigmentation. The eluants were filtered through cotton wool or industrial grade tissue-paper, depending on the quantity of solvent, then concentrated under vacuum.

To check that all the organic material was extracted by this process, the weights of extract from tissues of various sizes were calculated as a percentage of the dry weight of tissue before extraction. These weights were constant as a percentage of the dry weight indicating that extraction was complete for all weights of tissue used in these studies (Table 1).

The crude extracts thus obtained were analysed by analytical thin layer chromatography (TLC), on commercially prepared silica plates (Merck Kieselgel 60 F₂₅₄, TLC grade). The plates were run in dichloromethane, or dichloromethane/diethyl ether (9:1). TLC has the value that different compounds produce characteristic colours when sprayed with vanillin/sulphuric acid and warmed. The compounds present in the extracts were identified by comparison of R_f and spot colour with those of known compounds.

Purified terpenes were obtained by repeated column chromatography on silica (Merck Kieselgel 60 H, column grade) using a step-wise gradient from petroleum ether (40-60°C) to diethyl ether. Each fraction was analysed by analytical TLC.

Compounds were identified by comparison of proton nuclear

TABLE 1

WEIGHT OF ORGANIC EXTRACT OF *Sarcophyton* SPECIES
AS A PERCENTAGE OF TISSUE WEIGHT

WEIGHT OF DRY TISSUE (mg)	WEIGHT OF EXTRACT/WEIGHT OF TISSUE (%)
------------------------------	---

Sarcophyton boletiforme

187.72	25.61
106.41	24.48
105.47	23.89
94.25	22.92
77.29	24.66
54.16	26.99
47.87	29.71

mean 25.47 S.D.=1.73, n=7

Sarcophyton boletiforme

104.21	16.75
96.73	17.39
81.25	13.94
74.24	15.02
73.20	15.19
73.02	16.82
72.77	14.10
72.57	18.93
61.12	13.69
57.07	13.58
51.95	14.03
51.88	18.22
49.19	14.74
43.81	17.83
38.98	17.01
34.39	14.77

mean 15.75 S.D.=1.79, n=16

TABLE 1 (Continued)

WEIGHT OF DRY TISSUE (mg)	WEIGHT OF EXTRACT/WEIGHT OF TISSUE (%)
------------------------------	---

Sarcophyton trocheliophorum

248.05	10.23
198.40	7.24
173.63	10.97
156.72	4.20
124.82	13.37
121.70	11.63
92.60	11.43
78.80	10.95
74.32	9.19
61.37	9.87
54.70	11.41
52.92	11.13
43.80	11.03
38.65	12.32

mean 10.83 S.D.=2.30, n=14

magnetic resonance ($^1\text{Hnmr}$) on a Jeol 90 MHz spectrophotometer with those of standard compounds. Samples were dissolved in either deuteriochloroform containing 1 percent tetramethylsilane as internal standard, or in deuteriochloroform in carbon tetrachloride. UV-visible spectral scans were conducted in ethanol using a Unicam SP800 spectrophotometer.

Repeated extraction of the freeze-dried tissue with dichloromethane at ambient temperature until the eluant was clear of pigments (approximately 12 hours for each extraction), completely extracted the terpenes of interest. Table 2 shows such an extraction for sarcophine. Sarcophine was the most polar compound examined in this dissertation. Neither ethyl acetate nor methanol further extracted the terpenes of interest after exhaustive extraction with dichloromethane, as assessed by TLC.

Sarcophytoxide crystallised from diethyl ether into large yellow rhombic crystals, and recrystallised from n-heptane as small white rhombic crystals. Sarcophine crystallised from diethyl ether as white needle crystals. Furanquinol and furanoacid crystallised and recrystallised from petroleum ether (40-60°C) as beige powders. Furanquinone crystallised from petroleum ether as orange rhombic crystals. Crystalline flexibilide and dihydroflexibilide, and an FN45 oil, were supplied by Drs B.F.Bowden and J.C.Coll, James Cook University of North Queensland, as were reference spectra of all the terpenes examined.

2.2.3 Terpene Quantification

2.2.3.1 Introduction

Dichloromethane extraction of soft corals yields a complex mixture containing compounds with a considerable range of polarities. Chromatography is traditionally used to analyse mixtures, and gas chromatography has been the method of choice for plant terpenes, for example, see von Rudloff (1965). This choice is

TABLE 2

EXTRACTION EFFICIENCY OF SARCOPHINE BY DICHLOROMETHANE

TIME (Hours)	AMOUNT (nmoles)
2.00	3.75
17.50	158.51
50.75	0.000

Time represents the interval at which the dichloromethane extractant was changed. The amounts represent the amount of sarcophine extracted into each batch of dichloromethane extractant as analysed by HPLC at 225 nm.

influenced by the volatile nature of the terpenoid constituents of plants. Kashman, Loya, Bodner, Groweiss, Beneyahu and Naveh (1980) developed a gas chromatographic method for screening alcyonacean corals from the Red Sea for the presence of sesquiterpenes. However, terpenes in alcyonacean corals are either crystalline or oils at room temperature (Faulkner, 1984). High performance liquid chromatography (HPLC) is an accepted method of determining the amount of a non-volatile terpene present in a mixture, for example, the C₉₀-terpene dolichol in extracts of various tissues yeast, mice and humans (Keenan, Rice & Quock, 1977; Reuvers, Boer & Hemming, 1978; Freeman, Rupar & Carroll, 1980). HPLC has the advantage over gas chromatography in that it is a non-destructive technique which enables the chemicals to be retrieved after analyses. It can be used as the last step in the purification of terpenes (Kazlauskas, Murphy, Wells, Schonolzer & Coll, 1978).

The presence of a compound in the eluate from HPLC is usually detected by either a change in the refractive index of the eluate, or by the absorption of light. The amount of change is a direct measure of the amount of the compound passing the detector at that moment. The amount of compound passing the detector at any one moment increases to the point of greatest density, then decreases to infinity. This change in amount of compound is recorded as a peak. The area under the peak is a measure of the total amount of the compound present in the mixture.

Detecting compounds by a change in refractive index has the advantage that the degree of change in refractive index is not dependent on the chemical nature of the compound. The amount of any compound in a mixture can be compared to the amount of another compound by comparing the relative peak areas. This method of detection can only be used with isocratic solvent systems as the detector is sensitive to the massive change in refractive index of the mobile phase used in gradient elution. The wide range of polarities of the compounds extracted from a soft coral by dichloromethane are not separated by isocratic elution.

When monitoring the HPLC eluate from a complex mixture by absorption of ultraviolet light, it must be remembered that different compounds absorb ultraviolet light to different degrees depending on the chemical nature of the compounds. As the absorption of a particular compound at a particular wavelength is a constant, the amount of each component present in each injection is the quotient of the area under the curve and the experimntally determined molar area factor.

2.2.3.2 Development Of A Method For Quantitating Sarcophytoxide

A colony of *Sarcophyton stolidotum* was collected from Magnetic Island in April 1983, and frozen upon return to the mainland. A 2 cm by 3 cm section of the capitulum perimeter was freeze-dried. This section was diced, then placed above a bed of column grade silica. The tissue was extracted with several volumes of petroleum ether.

The petroleum ether extracted compounds of the polarity of the polar carotenoids and chlorophyll c_2 , but these compounds were then retained on the bed of silica. The brief contact time of the petroleum ether with the silica also limited the elution of these polar compounds from the silica. Thus the final eluate contained mainly compounds of polarity between that of chlorophyll c_2 and chlorophyll a . The polarities of sarcophytoxide and sarcophine lie within this range.

The eluate was filtered through tissue-paper, air-dried, then dissolved in 200 μ l of the mobile phase (80 percent methanol in water). 50 μ l were injected (Waters U6K injector using an S.G.E. 100 μ l syringe) and chromatographed on a Waters C-18 μ Bondapak reverse-phase silica column, fitted with a silica (Whatmans Partisil) precolumn. Before use in chromatography, all the solvents were filtered and degassed under vacuum through teflon membranes (Millipore, 0.22 μ m). The mobile phase was pumped by a

single piston pump (Waters M 45 Solvent Delivery System) at a rate of 1.1 ml/min. The eluate was monitored by a UV detector (Waters Lambda Max Model 481 LC Spectrophotometer) then a refractive index detector (Waters Differential Refractometer R 401). A dual pen linear chart recorder (Servigor 120) recorded the respective responses with a 0.25 min lag between the two responses.

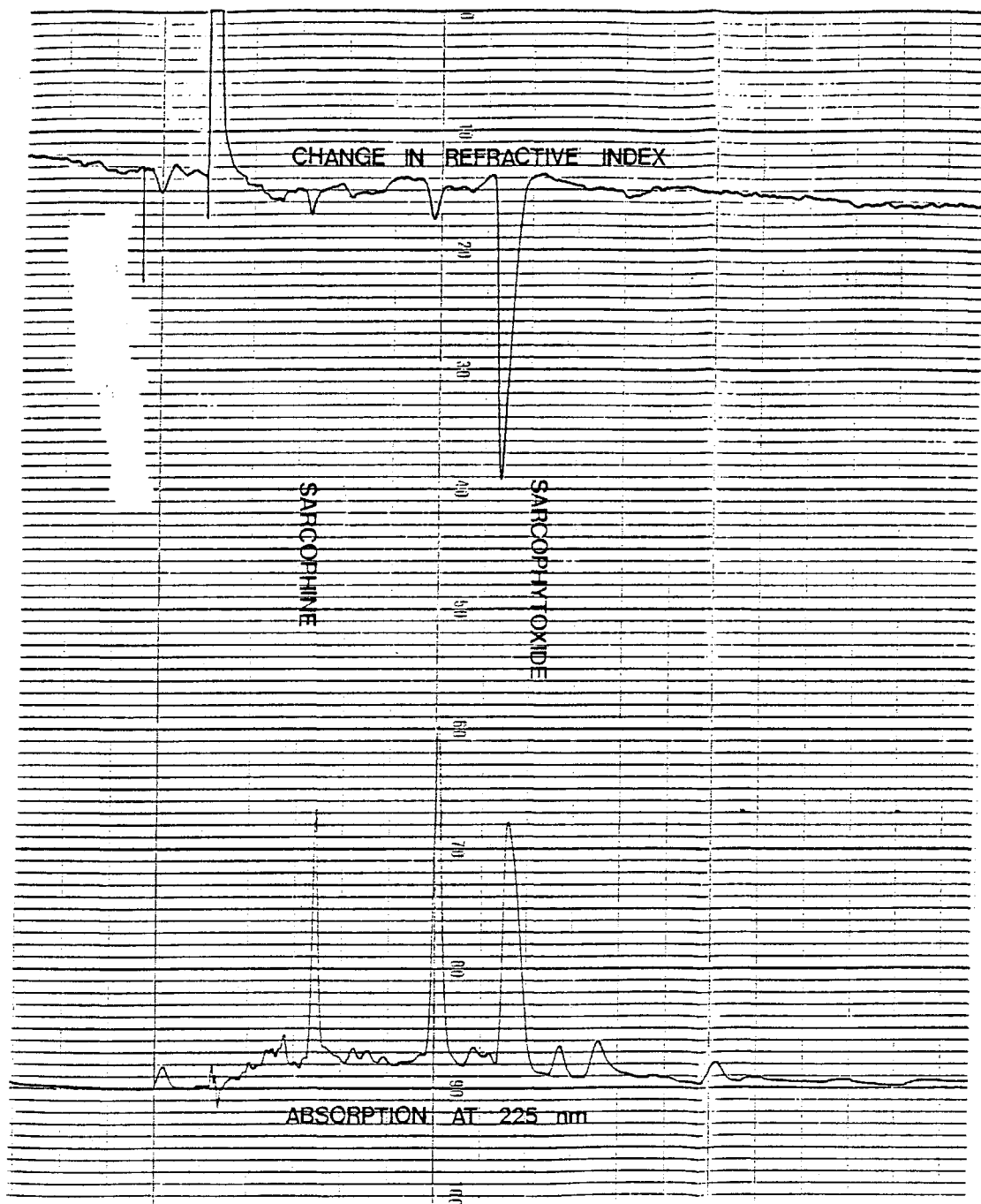
2.2.3.3 Results And Discussion

The sequential monitoring of the eluate by a UV detector, then by a refractive index detector, produced the trace shown in Figure 42. The refractive index detector recorded one dominant peak at 16 minutes and two minor peaks at 12 and 7 minutes. The UV detector also recorded these two peaks although the relative peak areas were different. Figure 43 shows the reference compounds sarcophytoxide and sarcophine assayed by the same HPLC technique. These compounds eluted at 16 and 7 minutes, respectively.

Analytical TLC analyses of the coral extract revealed a massive amount of sarcophytoxide and a much smaller amount of sarcophine, in a similar relative ratio to the peak areas of the peaks at 7 and 16 minutes recorded by the refractive index recorder. Before the TLC plate was developed with sulphuric acid and warmed, the plate was observed under a short-wave ultraviolet lamp. The spot later determined to contain sarcophytoxide did not quench ^{flourescence} at 254 nm. The spot later determined to be sarcophine did quench ^{flourescence} at 254 nm. This TLC analysis was repeated for each coral examined. These compounds were determined to be sarcophytoxide and sarcophine by isolation and characterisation of the compounds as described in Section 2.2.2

FIGURE 42

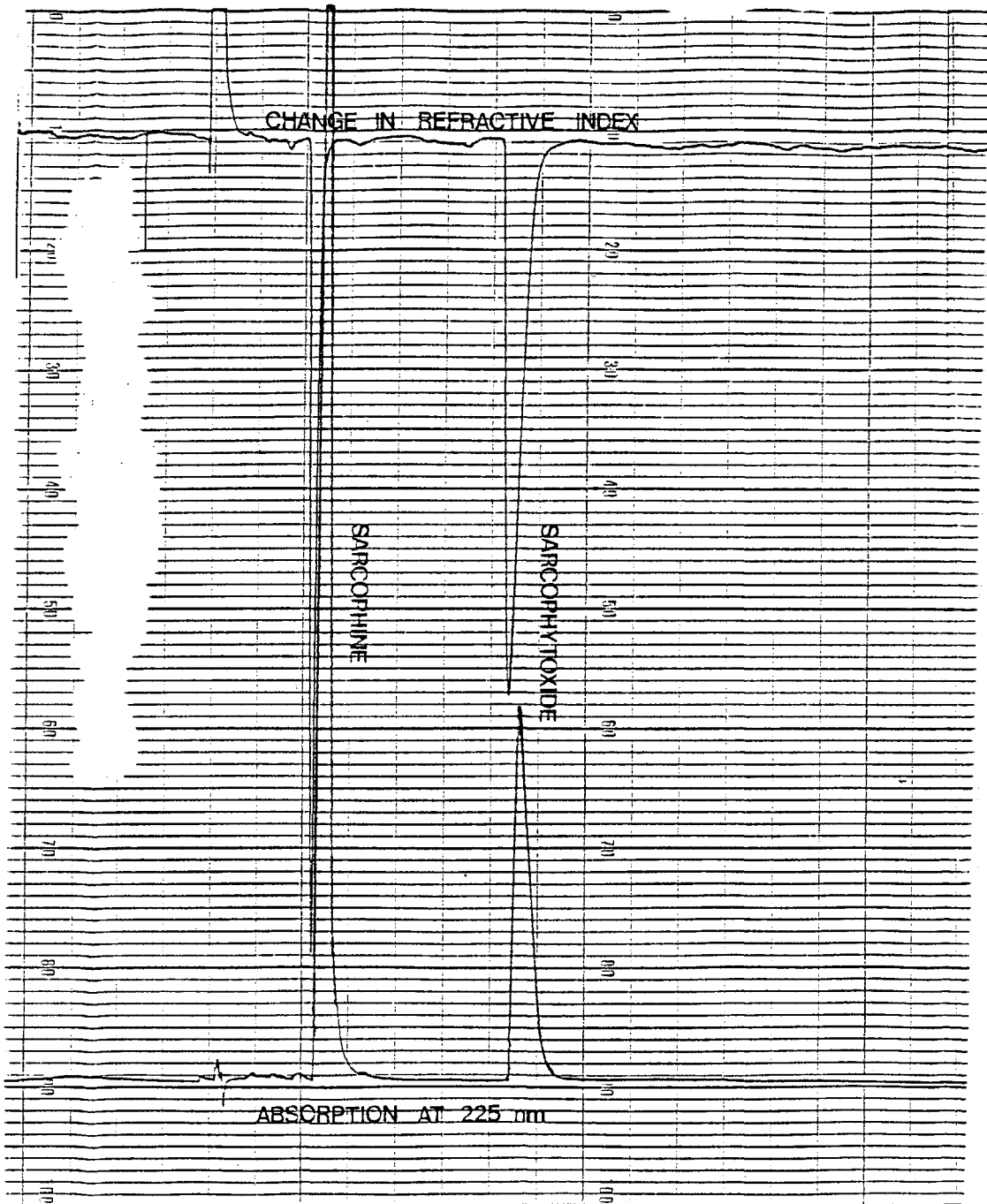
HPLC PROFILE OF ISOCRATIC ELUTION OF THE DICHLOROMETHANE EXTRACT OF A *Sarcophyton stolidotum* COLONY WHICH CONTAINED SARCOPHYTOXIDE AND SARCOPHINE



The eluate was monitored simultaneously by change in refractive index and change in absorbance at 225 nm. The vertical axis measures the degree of change. The upper trace records the change in refractive index. The lower trace records the change in absorbance at 225 nm. There is no scale on this axis as the output is adjusted to place the top of the tallest peak within the recorder span. The areas of the peaks are calculated by electronic integration and printed as a value at the end of the run. The horizontal axis represents time. The lower trace lags the upper one by 0.25 minutes.

FIGURE 43

HPLC PROFILES OF ISOCRATIC ELUTION OF SARCOPHYTOXIDE AND SARCOPHINE MONITORED SIMULTANEOUSLY BY CHANGE IN REFRACTIVE INDEX AND CHANGE IN ABSORBANCE AT 225 NM



The vertical axis measures the degree of change. The upper trace records the change in refractive index. The lower trace records the change in absorbance at 225 nm. There is no scale on this axis as the output is adjusted to place the top of the tallest peak within the recorder span. The areas of the peaks are calculated by electronic integration and printed as a value at the end of the run. The horizontal axis represents time. The lower trace lags the upper one by 0.25 minutes.

2.2.3.4 Method Used For Routine Analysis Of Sarcophytoxide Content Of *Sarcophyton* Species

The concentration of sarcophytoxide in the dichloromethane extracts from *Sarcophyton* spp. containing sarcophytoxide, were routinely determined by HPLC analysis.

Two HPLC instrument systems were used:-

- (1) Waters Associates U6K injector in conjunction with 25 and 100 μ l Hamilton syringes, coupled to a Spectra-Physics SP8700 solvent delivery system with a Spectra-Physics 8500 dynamic mixer, monitored by a Spectra-Physics 8400 UV/VIS detector and a Spectra-Physics 4100 computing integrator.
- (2) Waters Associates WISP 710 B automatic injector coupled to a Waters Associates 6000A solvent delivery system monitored by a Waters Associates 440 absorbance detector and Waters Associates Data Module computing integrator and system controller.

The mobile phase was a methanol:water gradient changing from 60 percent methanol to 100 percent methanol in 40 mins, followed by 20 mins in 100 percent methanol. The flow rate of the mobile phase was 1.1 ml/min.

Reverse-phase ^{octadodecyl} silica columns (RP18, Brownlee Laboratories and Alltech Australia) were used. These columns were preceded by a precolumn of ^{octadodecyl} silica (Brownlee, RP18 cartridge). The eluate was monitored for absorption of ultraviolet light at 225 nm which is optimal for sarcophine. Flexibilide and sarcophytoxide also absorb in this region.

The dichloromethane extracts were dried in air then resuspended in a known volume of mobile phase (200-1000 μ l), and a known amount injected into the HPLC system (5-50 μ l). Peak areas were measured by automatic electronic integration. The peaks of interest were identified by coinjection with a standard. The amount of terpene

injected was determined by calculation using the absorbance of freshly purified standards also analysed by this HPLC technique. The amount of terpene initially present in the tissue sample was calculated using the amount of terpene in the extract and the freeze-dried weight of the tissue before extraction. The concentration of sarcophytoxide expressed as a percentage of tissue weight was constant indicating that all the sarcophytoxide was extracted.

Results

The molar area factors of sarcophytoxide, deoxysarcophytoxide, sarcophine and flexibilide at 225 nm are presented in Table 3. The molar area factor at 225 nm for sarcophine is seven times greater than that for sarcophytoxide. This is attributable to the possession by sarcophine of the α -methylene- γ -lactone chromophore. Note that this chromophore also occurs on flexibilide, accounting for the comparable molar area factor of flexibilide at 225 nm. The loss of the epoxide from sarcophytoxide reduces the molar area factor by five orders of magnitude. The algal pigments chlorophyll *a*, *a'* and *c*₂, peridinin and neoperidinin, and yellow xanthophylls do not significantly absorb ultraviolet light at 225 nm. These pigments are monitored at 436 nm (Dunlap & Chalker, 1982). The same extract from a colony of *Sarcophyton boletiforme* was monitored at 225 nm and at 436 nm (Figure 44). It can be seen that the pigments do not elute in the region of sarcophytoxide or sarcophine, and their absorption at 225 nm is minimal.

The flexibilide concentration of dichloromethane extracts of *Simularia flexibilis* were determined using the same HPLC conditions as for sarcophytoxide. The strong absorption of flexibilide compared to the terpenes monitored in dichloromethane extracts from *Sarcophyton* spp., is demonstrated by the recording of only one major peak in the extract of a flexibilide containing *Simularia flexibilis*. Dihydroflexibilide was also present in the extract, eluting around 34 minutes as inferred from TLC, yet it does not

TABLE 3

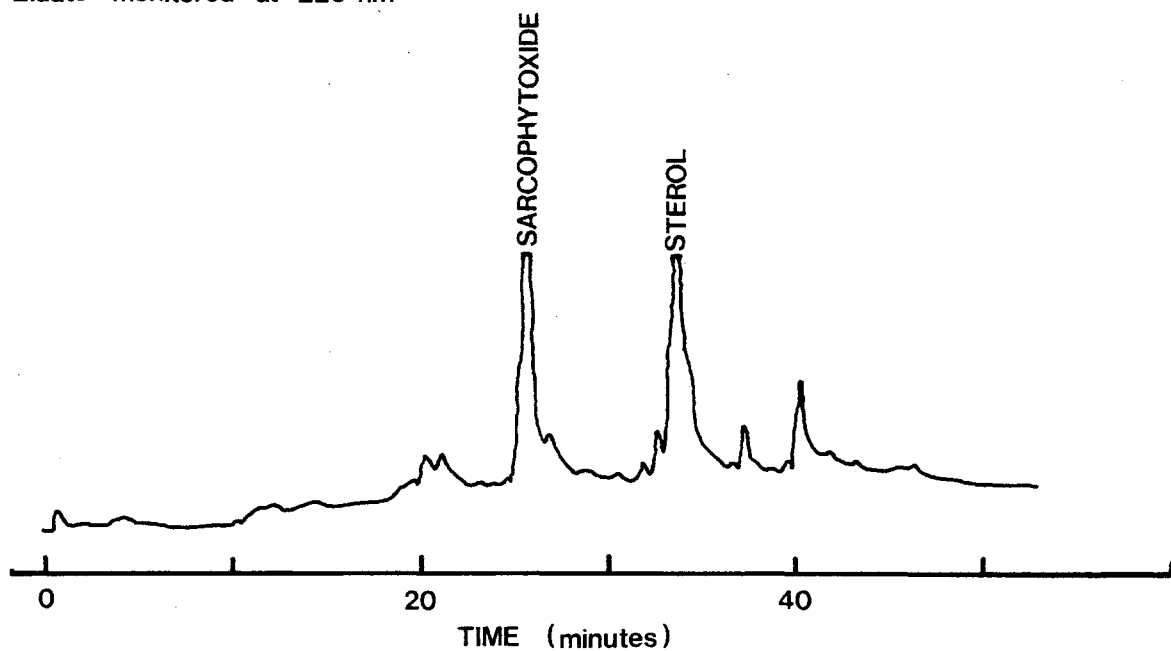
MOLAR AREA FACTORS FOR TERPENES QUANTITATED
BY ABSORPTION AT 225 NM.

TERPENE	MOLAR AREA FACTOR (absorption units/mole)
Sarcophine	4.918×10^{15}
Sarcophytoxide	7.422×10^{14}
Flexibilide	6.147×10^{17}
Deoxysarcophytoxide	2.027×10^9

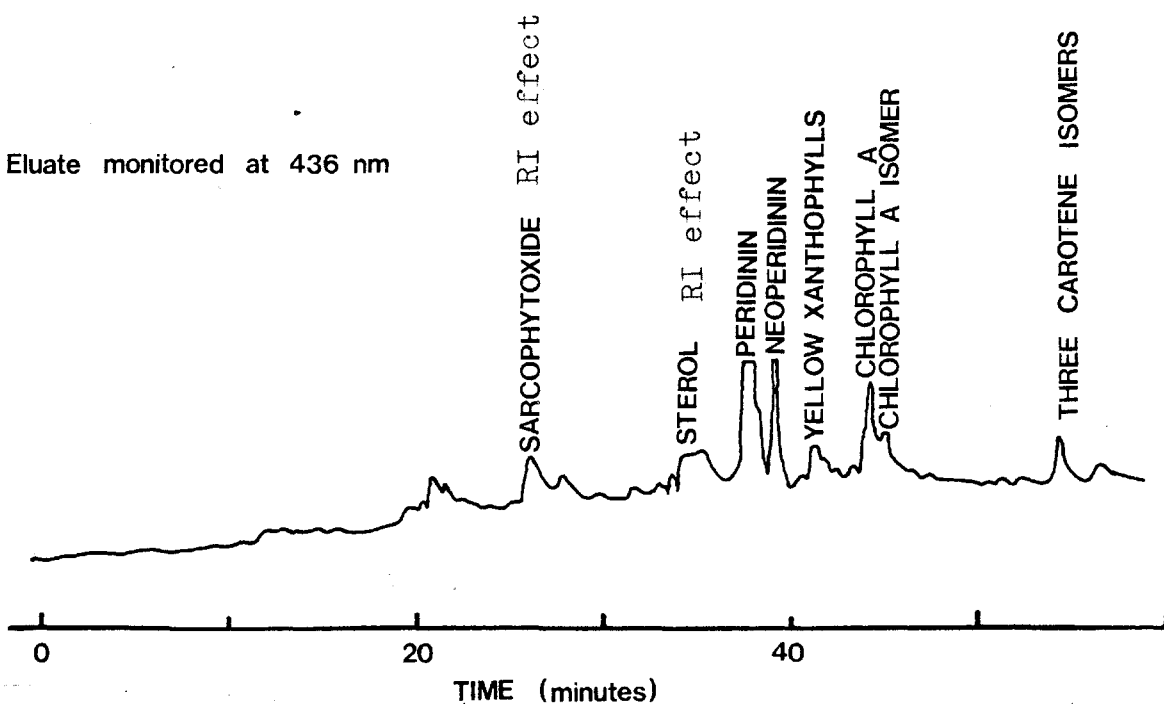
FIGURE 44

GRADIENT ELUTION OF AN ORGANIC EXTRACT OF *Sarcophyton boletiforme*
MONITORED BY ABSORBANCE OF ULTRAVIOLET LIGHT

Eluate monitored at 225 nm



Eluate monitored at 436 nm



appear in the trace as it does not absorb at 225 nm.

The same HPLC technique was used to determine the qualitative presence of FN45 in the extract from a *Lobophytum carnatum* colony. The peak was identified by coinjection with purified FN45, and by analysis of TLC plates.

CHAPTER 3

THE DISTRIBUTION OF TERPENES WITHIN ALCYONIIDAEAN COLONIES

Before studies on the biogeographic distribution and the biosynthesis of terpenes by corals can be initiated, the background level of terpenes in the various morphologically distinct structures of a colony needs to be known. Information on the concentration of terpenes within alcyoniidae corals is invariably presented as a yield obtained after extracting a whole colony, and typical concentrations are 0.5 to 5.0 percent of the dry weight of the colony (Kashman, Zadock & Neeman, 1974; Coll, personal communication, 1981). This chapter examines the pattern of distribution of terpenes within colonies, and the concentration of terpenes in coral eggs.

3.1 THE DISTRIBUTION OF SARCOPHYTOXIDE WITHIN A COLONY OF *Sarcophyton boletiforme*

3.1.1 Introduction

Rice, Papastephanou and Anderson (1970) reported that the concentration of crassin acetate was even, up and down the stipe of the colony, 4.4 percent dry weight of the cortex, or one percent of the total colony. Knowledge of the pattern of distribution of a terpene within a colony gives an indication of the biological role of that terpene. Terpenes involved in defence and in aggression in terrestrial plants are usually concentrated in the epidermis which is the tissue most exposed to the environment, or in tissues vulnerable to predation (McKey, 1979). It is not known where in the alcyoniidae coral colony the sesqui- and di-terpenes are concentrated. This section investigates the distribution of sarcophytoxide within a colony of *Sarcophyton boletiforme*.

3.1.2 Experimental

In September 1983, a colony of *Sarcophyton boletiforme* was sampled *in situ* in Pioneer Bay. The colony perimeter was sampled by taking slithers all the way around the colony, giving seven slithers. These samples were frozen, freeze-dried and extracted exhaustively with dichloromethane. The extracts were analyzed by TLC and HPLC.

A colony of *Sarcophyton boletiforme* was collected from Myrmidon Reef in June 1983, as one of a pair of colonies connected at the stalk. The colonies were maintained in the outdoor aquarium, and over several months, the two colonies separated to form independent colonies. The capitulum edge of each colony was sampled by taking slivers (Figure 41) periodically during these months. On each occasion both colonies contained sarcophytoxide.

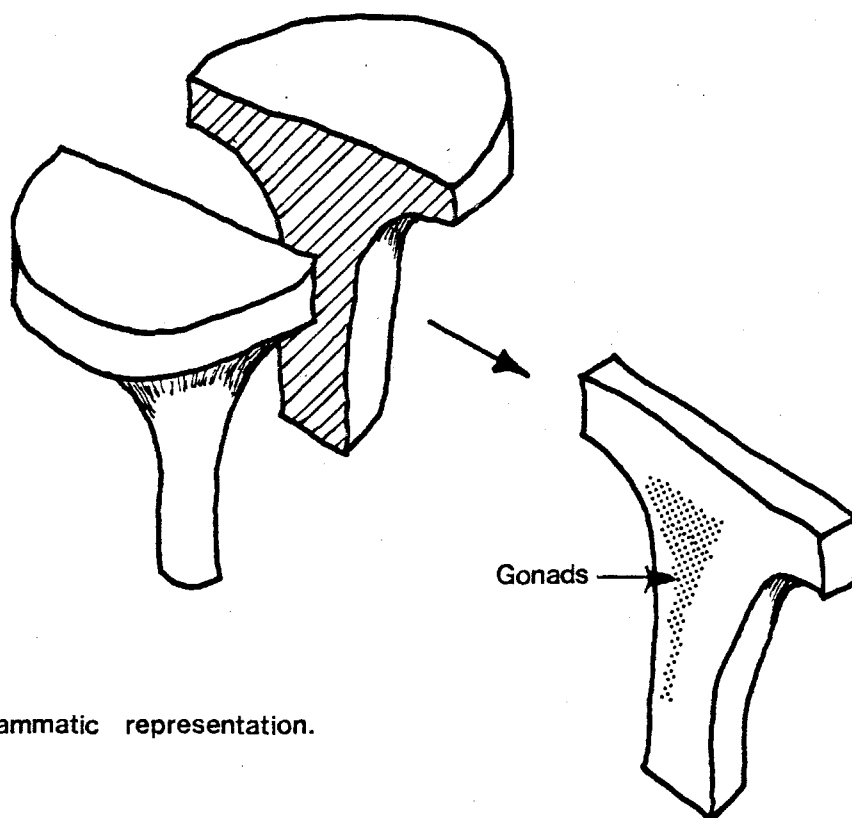
One of the colonies was harvested on February 16, 1984, by peeling the stalk from the substrate. The colony was frozen and freeze-dried. It weighed 4.0512 g. A vertical slab from the centre of the freeze-dried colony was cut with a razor as demonstrated in Figure 45. The slab did not include sections of the capitulum perimeter that had been previously sampled. Examination of the slab with a dissecting microscope revealed a coarse-grained region to one side of the stalk, extending into the lower part of the capitulum (Figure 45). This region is referred to as an 'L-shaped region' throughout this chapter. The slab was cut into 19 sections (Figure 45). Each section was weighed, then extracted exhaustively with dichloromethane. The extract was analysed by TLC and by HPLC, as described in the general methods section (2.2.3.4).

3.1.3 Results

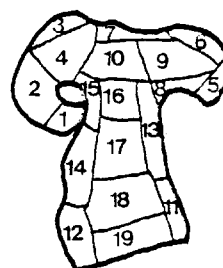
Slivers taken *in situ* from around the perimeter of a colony of *Sarcophyton boletiforme* in Pioneer Bay all contained sarcophytoxide, although there were substantial differences in the concentration of

FIGURE 45

SECTIONING A COLONY OF *Sarcophyton boletiforme*



Diagrammatic representation.



Actual size of
the slab.
Actual size of
sections
(numbered).

sarcophytoxide of the individual slithers (Table 4). In general, however, the amount of sarcophytoxide in each sample was directly proportional to the dry weight of the tissue sample ($r^2 = 0.656$).

The colony of *Sarcophyton boletiforme* collected from Myrmidon Reef and sectioned through the center of the colony, also demonstrated sarcophytoxide in all samples (Table 5, mean 50.64, S.D.=37.88 nmoles/mg dry tissue). The highest concentration of sarcophytoxide was found in sections taken from an L-shaped region (Figures 46 and 47; Table 5. The remaining sections of the capitulum contained a medium concentration of sarcophytoxide. There was no distribution pattern between the outer perimeter and the centre of the capitulum. Apart from the L-shaped region, the stalk had the lowest concentration of sarcophytoxide. There was no significant difference between the edge of the stalk and the interior of the stalk, excluding the L-shaped region (Student's t-test, $p > 0.05$). The concentration of sarcophytoxide in the capitulum perimeter in the colony of *Sarcophyton boletiforme* collected from Myrmidon Reef was significantly higher than the concentration of sarcophytoxide in the perimeter of the colony of *Sarcophyton boletiforme* sampled in Pioneer Bay (Student's t-test, $p < 0.05$).

3.1.4 Discussion

Sarcophytoxide occurred throughout a colony of *Sarcophyton boletiforme*, although the concentration of sarcophytoxide varied by an order of magnitude. The highest concentration was in an L-shaped region extending from one side of the stalk into the base of the capitulum. From the structure of the tissue and its position in the colony, it is concluded that the region contained gonadal tissue. The sarcophytoxide may be located in the ovaries as terpenes are found in the eggs of the brown alga *Fucus serratus* (Muller & Jaenicke, 1973) and of other soft corals (Bowden, 1984).

Apart from the L-shaped region, the stalk had low a

TABLE 4

VARIATION IN THE CONCENTRATION OF SARCOPHYTOXIDE AROUND THE PERIMETER
OF THE CAPITULUM OF A COLONY OF *Sarcophyton boletiforme*

WEIGHT OF TISSUE (mg)	CONCENTRATION OF SARCOPHYTOXIDE (nmoles/mg tissue)
187.72	2.954
106.41	3.766
105.47	1.972
94.25	1.740
77.29	3.895
54.16	0.792
47.87	3.053
mean 2.596 S.D.=1.138, n=7	

Colony sampled *in situ* in Pioneer Bay in September 1983.
These are the same tissues as the first colony presented in Table 1.

TABLE 5

CONCENTRATION OF SARCOPHYTOXIDE IN VARIOUS SECTIONS OF
Sarcophyton boletiforme

16 Feb 84

SECTION CONCENTRATION OF SARCOPHYTOXIDE
 (nmoles/mg dry tissue)

Sarcophytotoxide-Rich "L"

14	150.9	
15	101.9	
10	98.2	
mean	117.0	S.D.=29.4, n=3

Capitulum

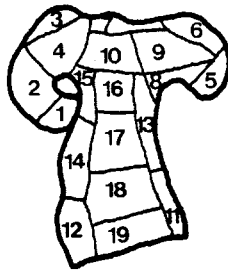
6	73.0	
3	66.3	
5	66.1	
1	60.8	
4	55.1	
2	51.3	
7	50.4	
8	43.2	
9	42.6	
mean	56.5	S.D.=10.7, n=9

Stalk

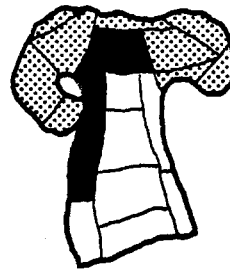
18	31.3	
17	18.4	
13	18.3	
12	18.0	
11	7.1	
16	6.9	
19	2.4	
mean	14.6	S.D.=9.9, n=7

FIGURE 46

DISTRIBUTION OF SARCOPHYTOXIDE IN A COLONY OF
Sarcophyton boletiforme



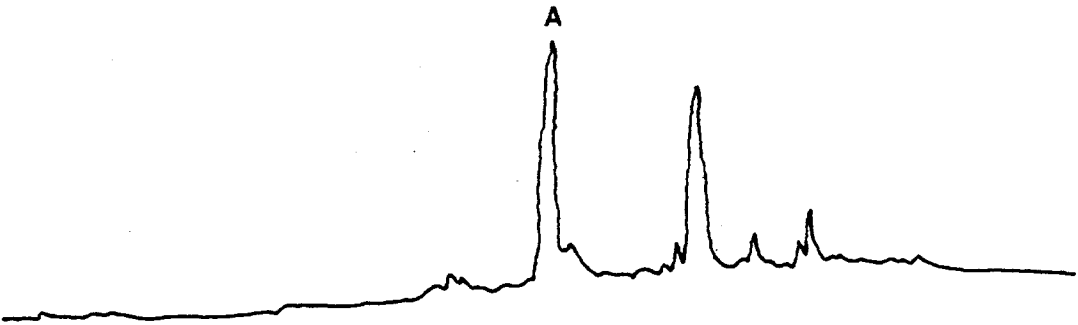
Section numbers.



Relative concentrations
of sarcophytoxide.

FIGURE 47
HPLC PROFILES OF SECTIONS OF A COLONY OF *Sarcophyton boletiforme*

12 September 1983



16 February 1984
Section 2



16 February 1984
Section 1



16 February 1984
Section 10



0 20 40
TIME (minutes)

A = SARCOPHYTOXIDE

concentration of terpene (one eighth that of the L-shaped region as determined by mean concentrations; 50.8 and 117.0 nmoles/mg dry tissue, respectively). The concentration of sarcophytoxide in the L-shaped region contrasts sharply with that of the rest of the stalk material.

The location of the gonads on only one side of the colony is not unusual as the location of developed eggs in the colonies of *Sarcophyton* spp. and of *Simularia* spp. in the field was asymmetric (personal observation, 1983). Another explanation of the asymmetrical localisation of sarcophytoxide is afforded by the recent separation of the colony, from another colony by vegetative division. Hence one side is still composed of material found in the interior of the stalk.

There was a moderate concentration of sarcophytoxide across the capitulum. The mean concentration was half that of the gonads (50.8, 117.0 nmoles/dry weight of tissue, respectively), although the higher concentrations were contiguous with those found in the L-shaped region, and the lower concentrations were contiguous with those found in the rest of the stalk.

The concentration of sarcophytoxide in the capitulum at both perimeters was similar to that of the rest of the capitulum. This indicates that slithers taken from the perimeter of the capitulum of *Sarcophyton boletiforme* do give representative concentrations of the terpenes present anywhere in the capitulum. This conclusion is supported by the sampling of a second colony *in situ* which also demonstrated limited variation in the concentration of sarcophytoxide around the perimeter of the capitulum.

Changes in the terpene concentration of the gonads in *Sarcophyton boletiforme* can not be monitored by sampling the capitulum edge as the gonads do not extend out to the perimeter of the capitulum. This conclusion is supported by field observations on the location of eggs deep in the stalks of *Sarcophyton* spp. and of *Simularia* spp. immediately before spawning.

The presence of sarcophytoxide in the capitulum supports an antifeedant role for sarcophytoxide. Antifeedants are often concentrated at or near the epidermal layers of plants (McKey, 1979). For example, the epidermis of sweet marjoram *Majorana hortensis* is the primary site of synthesis of monoterpenes (Croteau, 1977). A precedent has been set for the localisation of terpenes in octocorallian tissues vulnerable to predation. The terpene crassin acetate was located exclusively in the epidermis of the gorgonian *Pseudoplexaura porosa*, or more precisely, in the gastric cavity of its polyps (Rice, Papastephanou & Anderson, 1970). This terpene is toxic to potential parasites: larvae of the nudibranch *Phestilla sibogae* Bergh (Hadfield & Ciereszko, 1978); the rotifer *Brachionus plicatilis* Muller and the amphipod *Parhyale hawaiiensis* Dana (Lee, Macko & Ciereszko, 1981); the ciliated protozoa *Tetrahymena pyriformis* (Perkins & Ciereszko, 1970); the eggs of *Lytichinus variegatus* and of the parrot fish (Ciereszko, 1962).

Thus sarcophytoxide has two sites of accumulation: the growing edge of the capitulum where the polyp tentacles are located, and in the gonads. The next section further investigates the accumulation of terpenes in gonadal material.

3.2 THE PRESENCE OF TERPENES IN THE EGGS OF ALCYONITIDAEAN SPECIES

3.2.1 Introduction

To investigate whether the thesis proposed in Section 3.1.4 is valid; i.e. the high concentration of sarcophytoxide in the L-shaped region of the colony *Sarcophyton boletiforme* was due to the gonadal tissue contained in that region, the terpene composition of the following alcyoniidae tissues was investigated:

- (1) the egg-rich gonadal tissues of *Lobophytum carnatum* colony collected a month before the expected multispecific spawning by scleractinian corals at the same reef (Harrison, Babcock, Bull, Oliver, Wallace & Willis, 1984); and

- (2) the eggs released by a *Sarcophyton stolidotum* colony at spawning.

3.2.2 Experimental

3.2.2.1 *Lobophytum carnatum* Gonadal Tissue

A month before the mass spawning by corals at Magnetic Island in October 1983, a colony of *Lobophytum carnatum* containing purple eggs was collected from Magnetic Island. The colony of *Lobophytum carnatum* was sampled as follows: a tip of the lobe not containing eggs; an area deeper in the colony where some eggs were visible; and an egg-rich bed of stalk tissue. These samples were freeze-dried, extracted exhaustively with dichloromethane, and analysed by TLC.

3.2.2.2 Eggs Released From A Colony of *Sarcophyton stolidotum*

Twenty colonies of the members of the genus *Sarcophyton* were collected from Myrmidon reef in November 1983, prior to the expected mass spawning by scleractinian corals at a nearby reef in November 1983 (Harrison, personal communication, 1983). The colonies were acclimatised in the outdoor aquarium and one colony of *Sarcophyton stolidotum* spawned on the 24th and 25th November, 1983. A scleractinian colony of *Porites* sp. spawned on the same nights and these eggs were also collected.

All the eggs released from the above colony of *Sarcophyton stolidotum* were collected by pipette as they were released from the autozooids. The eggs were frozen at 4°C. A sliver was taken from the perimeter of the capitulum of the same colony of *Sarcophyton stolidotum* several months after spawning. The eggs and the sliver were freeze-dried, extracted exhaustively with dichloromethane, and the extract was analysed by TLC and by HPLC.

3.2.3 Results

The terpene FN45 was detected in each section of the colony of *Lobophytum carnatum*, but was relatively more concentrated in the eggs. The sample of the capitulum the colony of *Sarcophyton stolidotum* taken several months (23rd March, 1984) after the colony had spawned (24th November, 1983), contained sarcophine. The released eggs contained sarcophine at a similar though higher concentration (0.244 nmoles/mg dry weight of eggs compared to 0.156 nmoles/mg dry weight of the capitulum perimeter). The eggs of *Porites* sp. did not contain any organic material of the polarity of the terpenes investigated in this dissertation. The major component was a lipid-like material (TLC and HPLC).

3.2.4 Discussion

The eggs of alcyoniidae corals are a specific site of terpene accumulation. Egg-bearing tissue in *Lobophytum carnatum* contained FN45 and the eggs released by *Sarcophyton stolidotum* contained sarcophine. A sample of the capitulum perimeter of the colony *Sarcophyton stolidotum* taken after spawning, contained sarcophine as did the eggs released at spawning. Subsequent studies report that the eggs from a colony of *Lobophytum compactum* contained the diterpene thunbergol, while the eggs released from *Lobophytum crassum* were devoid of terpenes even though the parent colony contained the terpene 13-hydroxylobolide (Bowden, 1984)

Secondary metabolites are often concentrated in the ova of marine animals. For example, the brominated pyrroles of *Aplysina fistularis* are located in spherulous cells (Thompson, Barrow & Faulkner, 1983) which are included in the collagenous envelope around spawned oocytes of *Aplysina aerophoba* and *Aplysina cavernicola* (Gallissian & Vacelet, 1976). The eggs were released from the colony of *Sarcophyton stolidotum* were covered in a sheath of mucus. This mucus may contain the terpenes rather than the eggs themselves.

Two functions have been suggested for terpenes present in gametes: either to protect the gametes from predation, or to act as pheromones. Terpenes are known to act as pheromones in terrestrial insects, mammals and fungi (Caglioti, Cainelli, Camerino, Mondelli, Prieto, Quilico, Salvalori & Selva, 1966). In the marine environment, the eggs of the brown alga *Fucus serratus* release a sperm attractant, fucoserratene (Muller & Jaenicke, 1973). It is not known if sarcophytoxide has pheromonal activity; however, it is toxic to the fresh water fish *Gambusia affinis* (Coll, Tapiolas, Bowden, Webb & Marsh, 1983), which suggests at least a defence function for sarcophytoxide in the gametes after their release from the parent colony.

3.3 SUMMARY

The distribution of the terpene sarcophytoxide in a colony of *Sarcophyton boletiforme* was analysed. Sarcophytoxide was concentrated in the gonadal tissues and in the capitulum of a colony of *Sarcophyton boletiforme*, although the terpene was present throughout the colony. The gonadal location of terpenes was supported by detection of FN45 in the egg-bearing gonadal tissue of *Lobophytum carnatum*, and by the presence of sarcophine in the eggs released from a colony of *Sarcophyton stolidotum*.

3.4 FUTURE EXPERIMENTS

The localisation of flexibilide within the branching *Simularia flexibilis* colony should be investigated. Both sarcophytoxide and flexibilide are oembranoid diterpenes and it is logical that they would be synthesized by the same tissues, and that they would perform similar functions. However, the site of storage of terpenes may be different in massive and branching corals as different tissues are vulnerable to predation.

These results should be compared to the location of the cyclic

caryophyllanoid diterpene FN45 within the massive lobulated *Lobophytum* spp. colony, and to the location of the linear quinolated sesquiterpene furanoquinol in the branching and encrusting *Simularia capillosa*. The site of storage of sesquiterpenes may differ from that of diterpene storage, as mono- and sesqui-terpenes have different locations in plants that produce both classes of terpenes, and their syntheses are under different environmental controls (Bernard-Dagan, Carde & Gleizes, 1979; Gleizes, Carde, Pauly & Bernard-Dagan, 1980). From the results described above it is reasonable to expect that the concentrations of terpenes will be high in gonadal tissues, and moderately high in tissues exposed to predation or fouling.

CHAPTER 4

VARIATION OF TERPENE CONCENTRATION IN ALCYONIIDAEAN CORALS

Sarcophytoxide is the terpene most commonly isolated from the genus *Sarcophyton* (Coll, personal communication, 1981). This observation needs to be quantified: in what percentage of colonies of each species, in what range of concentrations, and how reliably from any given site?

This chapter examines the concentration of terpenes:

- (1) in the same colony through time,
- (2) in individuals of the same species on the same reef,
- (3) in individuals of the same species across the width of the Great Barrier Reef, and
- (4) in different species.

4.1 INTRA- AND INTER-SPECIFIC VARIATION OF SARCOPHYTOXIDE CONCENTRATION IN *Sarcophyton* SPECIES

4.1.1 Introduction

Before the change in concentration of a terpene within an organism can be monitored, a technique for repetitive sampling of living colonies must be developed. The sampling technique must not, in itself, induce an alteration in the concentration of the terpene being assayed. This is an important consideration for terpenes as some plants only produce terpenes in response to stress (Stoessl, Stothers & Ward, 1978), while others lower terpene production under stress (Gleizes, Pauly, Bernard-Dagan & Jacques, 1980).

The previous section established that the concentration of

sarcophytoxide in a *Sarcophyton boletiforme* capitulum can be ascertained without reference to the gonads, by taking tissue samples from anywhere across the capitulum. In this section, the concentration of sarcophytoxide in the capitulum perimeter of several colonies of *Sarcophyton boletiforme* is compared with that of *Sarcophyton stolidotum* and of an unidentified species of *Sarcophyton*.

4.1.2 Experimental

To determine the range of concentrations of sarcophytoxide found in the capitulum of *Sarcophyton boletiforme*, five identical colonies of *Sarcophyton boletiforme* were collected simultaneously from Myrmidon Reef in June 1983, and maintained in the outdoor aquarium. These colonies were sampled periodically at the capitulum perimeter over the next few months.

To determine if the concentration of sarcophytoxide in the capitulum of different species, or of examples of the same species found at different locations, was substantially different, two colonies of *Sarcophyton boletiforme* and a colony of *Sarcophyton stolidotum* were sampled *in situ* at Orpheus Island. In addition, a colony of *Sarcophyton* sp. was collected whole from Flinder's Reefs (Figure 40) and a tissue sample was taken from the capitulum perimeter after the colony had been freeze-dried.

The samples taken from the perimeter of the capitulum were slivers (1.5 cm long by 0.5 cm wide by 0.5 cm thick, the thickness of the colony edge) were taken from the capitulum perimeter of *Sarcophyton* spp. (Figure 41). All samples were freeze-dried, extracted exhaustively with dichloromethane, and analysed by TLC and by HPLC.

4.1.3 Results

The concentration of sarcophytoxide in the colonies presented in Table 6 ranged from 11.9 to 78.5 nmoles/mg dry tissue. The sarcophine concentration ranged from 0.0 to 5.4 nmoles/mg dry tissue. The qualitative compositions of the dichloromethane extracts of the four colonies of *Sarcophyton boletiforme* collected simultaneously from Myrmidon Reef were identical when analysed by TLC and by HPLC. However, the absolute concentrations of the compounds in the extracts were different (Table 6). The first two colonies were connected by a common stalk.

The concentration of sarcophytoxide in *Sarcophyton boletiforme* varied from 17.5 to 78.5 nmoles/mg dry tissue. All six colonies of *Sarcophyton boletiforme* were sampled in the June/July period of 1982 (Table 6). The first four colonies in the table were from the same reef. The concentration of sarcophytoxide in colonies 1 and 2 in Table 6, varied in a non-linear fashion over two months.

The two other species of *Sarcophyton* examined; *S. stolidotum* and an unidentified species of *Sarcophyton*, were sampled *in situ* and had low levels of sarcophytoxide (Table 6). These two colonies had sarcophine levels which were an order of magnitude greater than those of *Sarcophyton boletiforme*. Sarcophine levels in *Sarcophyton boletiforme* were low and sometimes absent, ranging from not detectable to 2.9 nmoles/mg dry tissue.

4.1.4 Discussion

Sarcophyton boletiforme had a higher concentration of sarcophytoxide and a lower concentration of sarcophine in both the capitulum and in the colony as a whole, than the colony of *Sarcophyton stolidotum* and the unidentified species of *Sarcophyton*. Sarcophine levels were not linked to the concentration of sarcophytoxide as demonstrated by the fact that colonies which contained no sarcophytoxide were often rich in sarcophine.

TABLE 6

TERPENE CONCENTRATIONS IN SYMBIOTIC *Sarcophyton* SPECIES

SPECIES	ORIGIN	DATE SAMPLED	CONCENTRATION (nmoles/mg dry tissue)	
			Sarcophytoxide	Sarcophine
† <i>S. boletiforme</i>	Myrmidon	14 Jul 1983	17.5	2.9
		25 Jul 1983	47.4	1.9
		22 Aug 1983	30.1	2.9
		12 Sep 1983	25.3	0.1
† <i>S. boletiforme</i>	Myrmidon	14 Jul 1983	43.0	1.1
		25 Jul 1983	38.2	1.4
† <i>S. boletiforme</i>	Myrmidon	22 Aug 1983	78.5	0.2
		12 Sep 1983	48.6	0.8
† <i>S. boletiforme</i>	Myrmidon	22 Aug 1983	33.3	0.9
		12 Sep 1983	45.4	1.0
† <i>S. boletiforme</i>	Myrmidon	22 Aug 1983	37.0	0.0
<i>S. boletiforme</i>	Orpheus Is	25 Sep 1983	54.7 *	0.4 *
<i>S. boletiforme</i>	Orpheus Is	25 Sep 1983	25.6 *	1.8 *
<i>S. stolidotum</i>	Orpheus Is	11 Feb 1981	19.6	5.4
<i>S. species</i>	Flinder's	7 Nov 1982	11.9	3.8

Samples taken from the capitulum perimeter except * when the whole colony was extracted.

† First five colonies were collected in June 1983, and established in the outdoor aquarium. The last four colonies were sampled *in situ*.

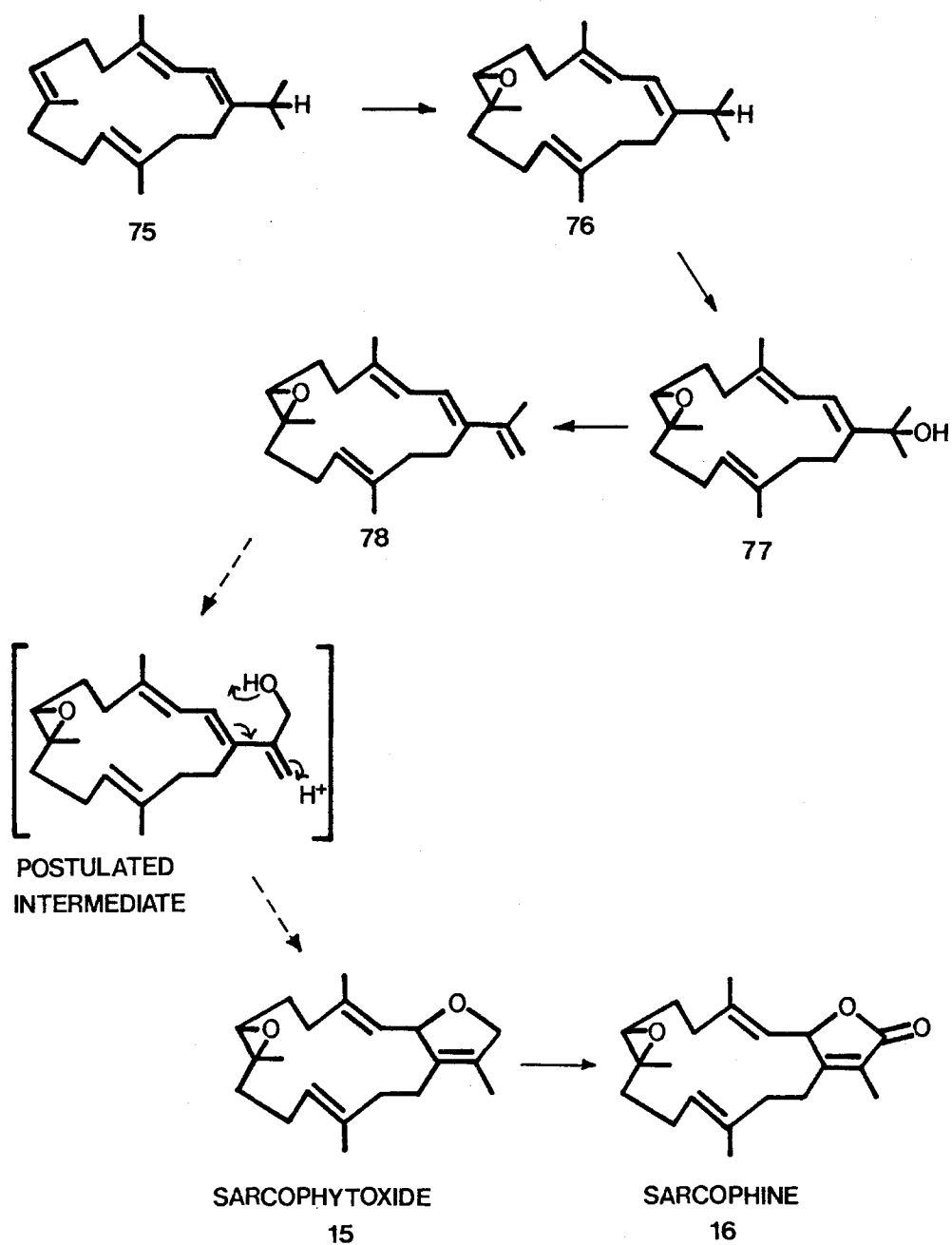
Robinson and West (1970a) found that each of five cyclic diterpenes in the castor oil bean was synthesised from one common precursor, rather than as part of a pathway linking the five compounds, as the authors had previously suggested (West & Robinson, 1969). By analogy, the structurally similar terpenes isolated from the one *Sarcophyton* colony, may not represent an array of precursors and products as suggested by Bowden, Coll and Mitchell (1980b) (Figure 48). Instead these structurally similar compounds may be the result of independent enzymic conversions from a common precursor, and the rates of synthesis of the various products would not necessarily be linked.

The colonies established in the outdoor aquarium did not have substantially different concentrations of sarcophytoxide nor of sarcophine in the capitulum perimeter than those sampled *in situ* in the ocean, indicating that living in an aquarium did not alter the concentration of these terpenes. Plants often react to stress by increasing the concentration of terpenes in the injured tissue (Stoessl, Stothers & Ward, 1978). The alcyoniidæan corals studied in this chapter did not show a consistent rise in terpene content after transfer to the outdoor aquarium (Table 6), nor during repetitive sampling of *Sarcophyton boletiforme* capitulum perimeter. This observation suggests that experimental manipulation does not alter the concentrations of the terpenes ^{sampled} in the tissues at the growing edges of alcyoniidæan corals.

FIGURE 48

SUGGESTED BIOSYNTHESIS OF SARCOPHYTOXIDE

(After Bowden, Breakman, Coll & Mitchell, 1980)



4.2 DISTRIBUTION OF SARCOPHYTOXIDE IN *Sarcophyton* SPECIES ACROSS THE CONTINENTAL SHELF

4.2.1 Introduction

The concentration of sarcophine and sarcophytoxide in *Sarcophyton glaucum* varied with geographic location in the Red Sea (Bernstein, Schmeuli, Zadock, Kashman & Neeman, 1974). When this dissertation was initiated, the method of collecting corals was to harvest approximately twenty colonies which were transported to the mainland. It was often found that perhaps only three of the colonies collected contained the terpene of interest. In this section, the presence of sarcophytoxide in *Sarcophyton* species across the central region of the Great Barrier Reef was investigated.

4.2.2 Experimental

Colonies of *Sarcophyton* spp. (>100) were collected from reefs spanning the central region of the Great Barrier Reef (Figure 40). Some colonies were frozen immediately, others were established in aquaria. Each colony was sampled at the perimeter of the capitulum, and the tissue samples freeze-dried, extracted exhaustively with dichloromethane, and analysed by TLC and then by HPLC for the presence of sarcophytoxide and of sarcophine.

4.2.3 Results

Sarcophytoxide and sarcophine were found in examples from each reef visited: Orpheus Island, Eclipse Island, Magnetic Island, Davies Reef, Rib Reef, Myrmidon Reef, Bowl Reef and Flinder's Reefs.

4.2.4 Discussion

Sarcophytoxide and sarcophine were found in colonies of

Sarcophyton spp. from reefs across the central region of the Great Barrier Reef. The presence of these two terpenes was not dictated by reef. There is no geographic localisation of sarcophytoxide producing *Sarcophyton* species on any reef, nor in any part of the central region of the Great Barrier Reef system.

This lack of variation in terpene composition with geographic location is contrary to the terpene composition of many other organisms. The mono- and sesqui-terpene composition of the leaves of shrubs of the *Lindera* genus allowed members of two species (*L. umbellata* and *L. sericea*) to be grouped into one species exhibiting four geographic races (Hayashi & Komae, 1974). The monoterpene composition in the Sitka Spruce families also showed geographical variation (Hrutfjord, Hopley & Gara, 1974). Fenical & Norris (1974) reported that the secondary metabolism of a single species of the red algal genus *Laurencia* was independent of habitat, seasonal changes and reproductive state, although later, these workers found geographical variations in the secondary metabolite chemistry of one species, *Laurencia paxifica* Kylin (Fenical, 1976). The dispersal of planulae larvae of alcyoniidae corals by oceanic currents probably accounts for the lack of geographic isolation of a particular gene pool, in this case the production of sarcophytoxide.

4.3 DISTRIBUTION OF SARCOPHYTOXIDE IN THE *Sarcophyton* GENUS

4.3.1 Introduction

Chemotaxonomic studies in terrestrial plants (Bohlmann, Grenz, Jakupovic, King & Robinson, 1983; Cambie, Cox, Croft & Sidwell, 1983; Waterman & Hussain, 1983), marine plants (Erickson, 1983) and sponges (Bergquist & Wells, 1983) have been conducted on the basis of their terpenoid content. Recently, the terpene content of gorgonians was used to draw phylogenetic relationships within the gorgonian family (Gerhart, 1983; Grode, James, Cardelline & Onan, 1983). This section examines whether there is a definite relationship between sarcophytoxide presence and a particular

Sarcophyton species.

4.3.2 Experimental

Data from the same colonies described in Section 4.2 was used.

4.3.3 Results

Six out of eight *Sarcophyton* species examined contained sarcophytoxide (Table 7). However, not all colonies of these six species contained sarcophytoxide. The two species *Sarcophyton auritum* and *Sarcophyton crassocaule*, did not yield sarcophytoxide on any occasion (0/6; 0/2 colonies). Most of the colonies that contained sarcophytoxide also contained sarcophine. When present, sarcophytoxide was the major compound in the extract. When sarcophine was the only terpene detected, it was the major compound in the organic extract.

4.3.4 Discussion

The presence and concentration of sarcophytoxide and sarcophine in the colonies of the genus *Sarcophyton*, was dictated by species. This is in agreement with the findings of a chemotaxonomic study of sixteen alcyonacean corals in which it was concluded that the species could be identified by their sesquiterpene composition (Kashman, Loya, Bodner, Groweiss, Beneyahu & Naveh, 1980).

Sarcophytoxide was isolated from six out of eight *Sarcophyton* species examined, but not from all colonies of these six species. The two species that did not yield sarcophytoxide on any occasion (*Sarcophyton auritum* and *Sarcophyton crassocaule*), have a coarser texture than the other species. They both have upright fingers and most nearly resemble the genus *Lobophytum* than the other species although they have the distinctive shape of the *Sarcophyton* genus.

TABLE 7

PRESENCE OF SARCOPHYTOXIDE AND SACOPHINE IN *Sarcophyton* SPECIES

SPECIES	SARCOPHYTOXIDE PRESENT	SARCOPHINE PRESENT
<i>S. boletiforme</i>	+	+
<i>S. infundibuliforme</i>	+	+
<i>S. roseum</i>	+	+
<i>S. spongiosum</i>	+	+
<i>S. stolidotum</i>	+	+
<i>S. trocheliophorum</i>	+	+
<i>S. auritum</i>	-	-
<i>S. crassocaule</i>	-	-

Dry tissue extracted with dichloromethane. Extracts assayed by TLC on silica gel against standard compounds and visualised by spraying with vanillin/sulphuric acid and warming.

In addition, the spicules of *Sarcophyton crassocaule* are similar to those of *Lobophytum* spp. (Verseveldt, 1982). The absence of sarcophytoxide from *S. auritum* and *S. crassocaule*, suggests that these two species may not belong to the genus *Sarcophyton*.

The observation that sarcophine only occurs in large quantities when sarcophytoxide is absent suggests that sarcophine is a precursor of sarcophytoxide rather a metabolite of sarcophytoxide, i.e. when the conversion of sarcophine to sarcophytoxide is blocked, sarcophine accumulates instead of sarcophytoxide. This disagrees with the biosynthetic pathway suggested by Bowden and co-workers (1980) (Figure 48). However, studies on the actual concentrations of sarcophine and sarcophytoxide in several colonies show that the concentrations of the two terpenes are not linked (see Section 4.1.4). The wide distribution of sarcophytoxide among the *Sarcophyton* species, coupled with the variability of the presence of sarcophytoxide in any one species, indicates terpene chemotaxonomy of the genus *Sarcophyton* may give different genetic relationships between individual colonies to that of conventional taxonomy. It is interesting that sarcophytoxide crosses the genus barrier and appears in *Simularia flexibilis* but not in the *Lobophytum* genus (Bowden, personal communication, 1982), which bears a closer morphological resemblance to the genus *Sarcophyton* than does *Simularia flexibilis*.

Thus it seems the synthesis of sarcophine is independent of sarcophytoxide synthesis. Several studies on the synthesis of terpenes in terrestrial plants have also indicated that terpenes found in plants are not parts of a biosynthetic pathway but sometimes result from divergent pathways starting from a common precursor (von Rudloff, 1972; Gambliel & Croteau, 1982). Fourteen cembrane diterpenes are known to occur in the *Sarcophyton* genus (Figures 12 and 13) (Tursch, 1976; Kashman, Zadock & Neeman, 1974; Bowden, Coll, Hicks, Kazlauskas & Mitchell, 1987a; Bowden, Coll, Mitchell & Stokie, 1979; Coll, Hawes, Liyanage, Oberhansli & Wells, 1977)

- * It is possible that a transient variation in terpene concentration occurred after each sampling, with a return to normal concentrations before the next sampling. However, this would not affect the concentration sampling results as the method was designed to record unstressed concentrations of the terpenes in the corals.

4.4 SUMMARY

A method of sampling living colonies repeatedly was developed which did not distress the colonies. Neither repetitive sampling of the capitulum perimeter, nor transfer of the colony from the reef to the outdoor aquarium, affected the concentration of sarcophytoxide in the capitulum perimeter.* This is in direct contrast to many plants where terpenes are induced by mechanical damage (Stoessl, Stothers & Ward, 1978).

By taking samples of the capitulum perimeter, the concentration of sarcophytoxide and of sarcophine in the capitulum of six *Sarcophyton boletiforme* colonies, one *Sarcophyton stolidotum* and one unidentified *Sarcophyton* sp. were compared. The observed differences were greater between species than between reefs, suggesting a genetic rather than an environmental cue for the level of sarcophytoxide found in the capitulum of *Sarcophyton* spp. However, the level of sarcophytoxide was not constant within a species.

Sarcophytoxide was found in examples of six out of eight species of *Sarcophyton*. The within species variation in presence and in concentration of sarcophytoxide rules out a species specific pathway. The absence of sarcophytoxide from two *Sarcophyton* species, *S. auritum* and *S. crassocaula*, suggests that these two species may not belong to the genus *Sarcophyton*. The variable presence of sarcophytoxide within a *Sarcophyton* species, and its tenuous association with the presence of sarcophine indicates the taxonomic association of terpenes with a species is not identical with that of morphological features.

The presence of sarcophytoxide in *Sarcophyton* spp. was not dependent on geographic location. Sarcophytoxide was detected in colonies collected from reefs spanning the continental shelf at the central region of the Great Barrier Reef.

4.5 FUTURE EXPERIMENTS

Quantitation of the concentration of each terpene of interest in both the gonads and the upper growing surface, should be monitored monthly for over a year. Differential distribution of the various terpenes within a colony, i.e. of sarcophine and sarcophytoxide, or of furanoquinol and furanoquinone, may reflect different roles in the survival of the colony. A seasonal variation in concentration of the terpenes at either site within the colonies, may allow an understanding of the role of the terpene in the biology of the colony.

CHAPTER 5

In vivo BIOSYNTHESIS OF TERPENES BY ALCYONIIDAEAN SPECIES

Although there is a wide knowledge of the chemical structures of the terpenes found in octocorals, very little is known of the biosynthesis of these terpenes, and nothing is known of the factors influencing their biosynthesis. This chapter outlines the conditions which alter the synthesis of terpenes in alcyoniidae corals *in vivo* (Section A), and to delineate which of the two symbionts is responsible for the biosynthesis (Section B). Both these questions need to be answered before pathway analysis can be attempted.

SECTION A: FACTORS AFFECTING TERPENE BIOSYNTHESIS IN
ALCYONIIDAEAN SPECIES

This section investigates:

- (1) the efficacy of a 24 hour incubation with 1 mCi/l of 2-³H-mevalonic acid lactone,
- (2) the effects of different incubation conditions on the level of incorporation of ³H-mevalonic acid lactone into terpenes in *Sarcophyton* spp.,
- (3) the seasonal variation in the concentration of terpenes in *Sarcophyton* spp. and in *Simularia capillosa*, and
- (4) the seasonal variation in incorporation of ³H-mevalonic acid lactone into terpenes by *Sarcophyton* spp. and by *Simularia capillosa*.

5.1 INCORPORATION OF ^3H -MEVALONATE INTO TERPENES BY ALCYONIIDAEAN CORALS *in vivo*

5.1.1 Introduction

The method used by Trott (Coll, Bowden, Tapiolas, Willis, Djura, Streamer & Trott, 1985) to incorporate radiolabel *in vivo* was developed to allow maximum incorporation of radiolabel into terpenes by several alcyoniidae genera.

5.1.2 Method

Whole colonies (3 to 15 cm diameter) were placed in glass beakers or jars containing enough seawater to amply cover each colony (100 to 1000 ml). $2\text{-}^3\text{H}$ -mevalonic acid lactone dissolved in toluene (500 Ci/mole, Amersham) was added to a final concentration of 1 mCi/l. Aquarium colonies were incubated in clear glass beakers with aeration. The beakers were placed near the aquarium in a running seawater bath and subjected to the same illumination regime as the aquarium itself. The aquarium colonies incubated on the same day were incubated in the same beaker. In the oceanic experiments, a glass jar with the base removed, was placed over an undisturbed colony and the radiolabel injected through a rubber septum covering the neck of the bottle. Colonies incubated on the same day in the ocean were incubated in separate vessels. The glass jars were either clear or dark brown in accordance with the nature of the experiment. These colonies were subjected to ambient sunlight.

All incubations were terminated after 24 hours by rinsing the colonies in fresh seawater, then freezing the colony. The colonies were freeze-dried and the terpenes extracted, purified and characterised. Either air-dried dichloromethane extract or chromatographically pure crystals were dissolved in 10 mls of OCS scintillant (Amersham). The radioactivity due to tritium was measured in a Wallac beta scintillation counter. Each vial was monitored till 10 000 disintegrations were recorded which gives a

standard deviation for the counting of 1 percent. Vials containing only OCS^{scintillant} were used as controls to measure the background radiation. This background radiation was subtracted from each sample vial. The specific activity was calculated from a quench curve for tritium, and is expressed in Bequerels(Bq)/mmole pure compound.

To determine whether 1 mCi/l of ^3H was sufficient to ensure that the coral still had access to exogenous mevalonate at the end of the 24 hour incubation, the level of radioactivity in the incubation medium during such an incubation, was determined.

5.1.3 Results

All colonies incubated incorporated ^3H -mevalonic acid into the dominant terpene elaborated by the coral. The level of radioactivity in the incubation medium over a 24 hour incubation period indicated that there was excess ^3H -mevalonate at the cessation of the experiment (Figure 49). The rate of loss of ^3H from the incubation medium dropped in the first hour with 8.4 percent being removed from the medium. 75.9 percent of the initial radioactivity remained in the medium at 24 hours.

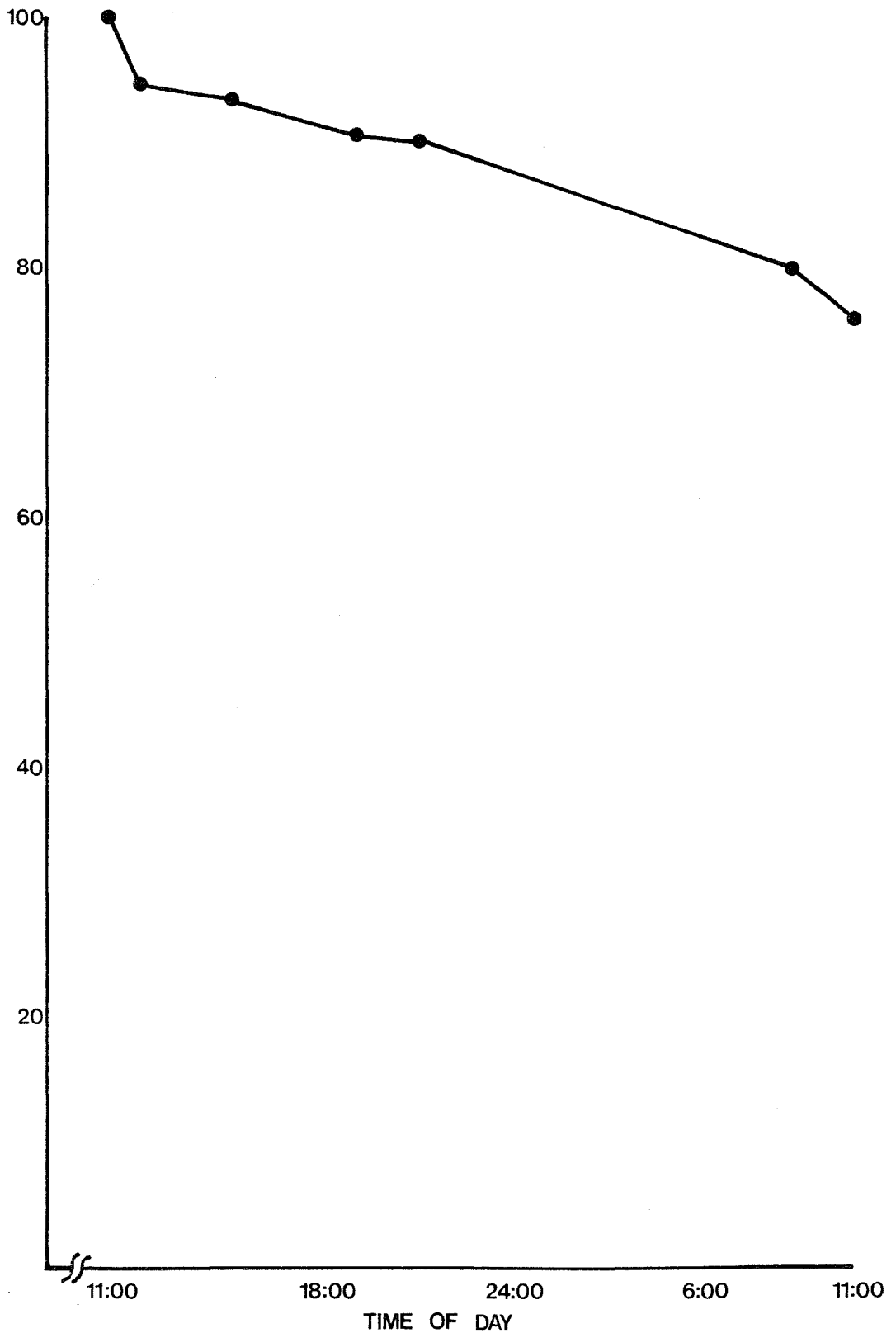
5.1.4 Discussion

The level of ^3H in the incubation medium during a 24 hour incubation steadily dropped with approximately 80 percent of the radioactivity remaining in the medium at the end of the experiment. This indicates that 1 mCi/l of ^3H -mevalonic acid is sufficient to ensure that the coral has access to exogenous mevalonate throughout the incubation period. The loss of ^3H is presumably due to three processes: initial rapid adsorption onto the glass beaker, coral mucus and debris; volatilisation of the mevalonate; and active uptake of ^3H -mevalonate by the coral.

FIGURE 49

RATE OF LOSS OF TRITIUM FROM THE INCUBATION MEDIUM OVER 24 HOURS

Rate of loss
(% of ^3H remaining)



Radiolabel incorporation into alcyoniidæan terpenes is a significant achievement in marine biochemistry as although both sponges and algae incorporate radiolabelled precursors such as acetate and amino acids into other secondary metabolites (Barrow, 1983), sponges (Garson, personal communication, 1985) and many algae (Barrow, personal communication, 1983) do not incorporate radiolabels into terpenes.

5.2 BIOSYNTHESIS OF SARCOPHYTOXIDE BY *Sarcophyton* SPECIES UNDER STRESS

5.2.1 Introduction

It is possible that terpenes are produced by octocorals after injury, particularly as the terpenes known to be phytoalexins in the plant family Solanaceae include cembranoid diterpenes (Bailey, Burdon & Vincent, 1975; Fujimori, Uegaki, Takagi, Kubo & Kato, 1979; Takagi, Fujimori, Kaneko & Kato, 1980). It is also possible that stress inhibits the synthesis of terpenes in corals as damaged pine needles reduce synthesis of monoterpenes (Gleizes, Pauly, Bernard-Dagan & Jacques, 1980). This section examines the level of ^3H -mevalonic acid incorporation into sarcophytoxide by *Sarcophyton* spp. incubated under stressful conditions.

5.2.2 Experimental

To understand the influence of stress on the biosynthesis of terpenes, and to determine the best pre-incubation procedure for the colonies, the synthesis of terpenes under four different incubation regimes was compared:-

- (A) Colonies incubated within hours of collection.
- (B) Colonies acclimatised to the indoor aquarium.
- (C) Colonies acclimatised to the outdoor aquarium.
- (D) Colonies incubated *in situ* in the ocean.

The colonies were incubated and the level of ^3H incorporation into the various terpenes determined as per Section 5.1.3.

5.2.3 Results

The amount of ^3H -mevalonic acid lactone incorporated into sarcophytoxide by *Sarcophyton* spp. in 24 hours under the four different regimes is shown in Table 8. Colonies in Group A were possibly still suffering from stress of handling and transportation. Colonies incubated within hours of collection and colonies acclimatised to the indoor aquarium (Groups A and B) had low levels of incorporation of ^3H into sarcophytoxide (Table 8). Colonies maintained in the indoor aquarium did not thrive. These colonies had low levels of incorporation of ^3H into sarcophytoxide (Table 8).

Sarcophyton spp. grew successfully in the outdoor aquarium over several years (Section 2.2). These colonies had comparatively high levels of ^3H -incorporation into sarcophytoxide (Group C, Table 8). Colonies incubated *in situ* in the ocean (Group D) also had high levels of ^3H -incorporation into sarcophytoxide.

Four *Sarcophyton* species were incubated under conditions C and D and they give an order of magnitude higher levels of ^3H incorporation into sarcophytoxide. Other examples of two of these species *Sarcophyton boletiforme* and *Sarcophyton stolidotum*, were also incubated under stressful conditions (Groups A and B), and gave comparatively low levels of incorporation of ^3H into sarcophytoxide. All the *Simularia capillosa* colonies were incubated under regime A and show relatively low levels of incorporation of ^3H -mevalonic acid lactone into furanoquinol (Table 9). However, all incubations yielded radiolabelled furanoquinol.

TABLE 8

INCORPORATION OF ^3H -MEVALONIC ACID LACTONE INTO TERPENES
BY *Sarcophyton* SPECIES

DATE INCUBATED	SPECIES INCUBATED	REEF COLLECTED FROM	SYMBIOSES	SPECIFIC ACTIVITY Sarcophytoxide Sarcophine (mCi/mole)	
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Group A. Colonies incubated within hours of collection.

28 Oct 81	<i>S. boletiforme</i>	Orpheus Is	Symbiotic	0.355 *	0.727
3 Apr 82	<i>S. infundibuliforme</i>	Magnetic Is	Symbiotic	0.047	0.001
	<i>S. boletiforme</i>	Magnetic Is	Aposymbiotic	0.043	0.001

Group B. Colonies incubated after weeks of acclimatisation in the indoor aquarium.

27 Aug 81	<i>S. infundibuliforme</i>	Magnetic Is	Symbiotic	0.391	-
16 Sep 81	<i>S. stolidotum</i>	Magnetic Is	Symbiotic	0.389	0.034

Group C. Colonies incubated after weeks of acclimatisation in the outdoor aquarium.

26 Jul 83	<i>S. trocheliophorum</i>	Davies Reef	Symbiotic	9.73	76.1
	<i>S. roseum</i>	Davies Reef	Aposymbiotic	2.22	31.6

Group D. Colonies incubated *in situ* in the ocean.

10 Feb 83	<i>S. stolidotum</i>	Magnetic Is	Symbiotic	8.52 *	-
25 Sep 83	<i>S. boletiforme</i>	Orpheus Is	Symbiotic	6.25	1.96
**	<i>S. boletiforme</i>	Orpheus Is	Symbiotic	1.39	0.0986

Incubated 24h in seawater containing ^3H -mevalonic acid lactone (1 mCi/l).

* Rechromatographed to constant specific activity.

** Incubated in reduced light.

TABLE 9

INCORPORATION OF ^3H -MEVALONIC ACID LACTONE INTO FURANOQUINOL
BY *Simularia capillosa*

DATE	SYMBIOSES	SPECIFIC ACTIVITY FURANOQUINOL (mCi/mole)
16 Mar 1982	Symbiotic	0.725
	Aposymbiotic	0.545
7 Apr 1982	Symbiotic	0.791
	Aposymbiotic	2.329
6 May 1982	Symbiotic	3.055 *
	Aposymbiotic	0.309
17 Sep 1982	Symbiotic	1.808 *
18 Oct 1982	Symbiotic	0.123 *
16 Feb 1983	Symbiotic	0.397 *

Incubated 24h in seawater containing ^3H -mevalonic acid lactone (1 mCi/l).

* Recrystallised to constant specific activity.

* Previously, it was demonstrated that injury due to sampling did not alter terpene concentrations (4.1.4, Table 6).

5.2.4 Discussion

The level of ^3H -incorporation into sarcophytoxide and into sarcophine was not dictated by species but by incubation conditions. The various incubations of *Sarcophyton boletiforme* yielded very different rates of incorporation into sarcophytoxide (Table 8).

The comparatively high levels of incorporation of ^3H -mevalonic acid lactone into terpenes under the conditions in Groups C and D indicates that the colonies were not stressed in the outdoor aquarium. In addition, colonies established in aquaria did not show any consistent change in terpene concentration away from the initial concentration (Table 4). Two of these species, *S. boletiforme* and *S. stolidotum*, were also incubated under conditions of stress (Groups A and B). On the latter occasions both these species incorporated relatively little ^3H -mevalonic acid lactone into either sarcophytoxide or sarcophine.

The low levels of incorporation of ^3H -mevalonic acid lactone into sarcophytoxide by colonies in Groups A and B (Table 8) indicates sarcophytoxide production is an order of magnitude lower when the colony is stressed. Colonies of both Group A and B appeared stressed presumably due to the recirculating seawater of high nitrogen content and low temperature and low levels of irradiation. The colonies in Group A had the additional stress of collection.*

The synthesis of sarcophytoxide was not induced under stress. In fact, there is evidence that the synthesis of sarcophytoxide and of sarcophine was inhibited by stress (Table 8). This is in direct contrast to synthesis of phytoalexin sesqui- and di-terpenes of the plant family Compressae which are induced under stress (Stoessl, Stothers & Ward, 1978), but is in keeping with the suppressed synthesis of monoterpenes in damaged pine needles (Gleizes, Pauly, Bernard-Dagan & Jacques, 1980).

Simularia capillosa incorporated low levels of radiolabel into

furanoquinol despite the obvious stress of the colonies incubated (Plate 17).

5.3 SEASONAL VARIATION IN TERPENE CONTENT OF ALCYONIIDAEAN SPECIES

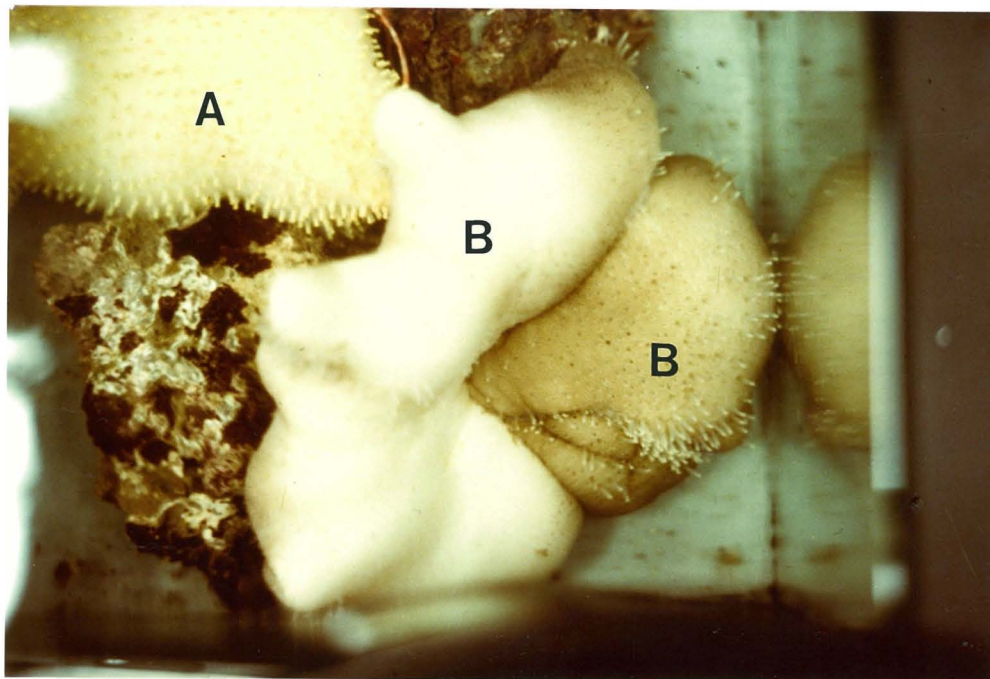
5.3.1 Introduction

The quantitative and qualitative variability of terpene composition is a problem when terpenes are to be used as chemotaxonomic markers. The concentration of sarcophine and of sarcophytoxide in *Sarcophyton glaucum* in the Red Sea changed with season (Bernstein, Shmeuli, Zadock, Kashman & Neeman, 1974). Seasonal and diurnal fluctuations often occur in the amount of essential oil of plants, for example, *Juniperus scopulorum* (Adams, 1979) and Valencia orange leaves (Hopfinger, Kumamoto & Scora, 1979). In contrast, a study on alcyoniidaeen corals in the Red Sea which covered several genera including *Sarcophyton* and *Simularia*, showed a constant sesquiterpene composition over different seasons (Kashman, Loya, Bodner, Groweiss, Beneyahu & Naveh, 1980).

The variability of the presence terpenes in terrestrial plants due to season is often due to the inter-related phenomenon, reproductive status (von Rudloff, 1972; Hrutfiord, Hopley & Gara, 1974; Zabkiewicz & Allen, 1975; Von Rudloff & Lapp, 1978; Firmage & Irving, 1979). The presence of terpenes in the gonads of members of the genera *Sarcophyton* and *Lobophytum* (Chapter 4), suggests that there will be changes in the concentration of terpenes in this organ as the colony goes through its annual reproductive cycle. The majority of scleractinian corals on the Great Barrier Reef spawn in November/December (Harrison, Babcock, Bull, Oliver, Wallace & Willis, 1984), and octocorals elsewhere spawn or release planula in July/August (Benayahu & Loya, 1983; Sebens, 1983a,b). The qualitative and quantitative changes in the terpenoid composition of whole colonies and of the growing edges of several alcyoniidaeen species with season was investigated.

PLATE 17

APOSYMBIOTIC *Sarcophyton* SPP. IN THE INDOOR AQUARIUM
IMMEDIATELY AFTER COLLECTION.



- A. The aposymbiotic *Sarcophyton boletiforme* incubated on 3 April 1982 (Table 8).
- B. A 'piebald' colony of *Sarcophyton spongiosum*.

5.3.2 Experimental

Whole colonies of *Simularia capillosa* were sampled monthly *in situ* at Magnetic Island for a year. The samples were freeze-dried and extracted exhaustively with dichloromethane. The extracts were examined by TLC only.

Sarcophyton spp. and a colony of *Simularia flexibilis* which were established in the outdoor aquarium, were sampled over several months. *Sarcophyton* spp. were sampled at the capitulum perimeter. The colony of *Simularia flexibilis* was sampled by cutting the terminal 4 cm portion of a branch. The samples were freeze-dried and extracted exhaustively with dichloromethane. The extracts were examined by both TLC and HPLC.

5.3.3 Results

In March 1982, the furanoacid (20) content of both the symbiotic and aposymbiotic colonies of *Simularia capillosa* was hardly noticeable by TLC analysis of the dichloromethane extracts. By September and October of the same year, the furanoacid was the dominant compound comprising more than 50 percent of the total dichloromethane extract.

In March 1982, there was a high concentration of furanoquinone in the dichloromethane extracts of *Simularia capillosa*; equal to that of furanoquinol. By the September of that year, the furanoquinone was barely detectable. Next February, the furanoquinone concentration had risen to approximately half that of furanoquinol. The concentration of furanoquinol declined during the year, to approximately a quarter of the concentration found at the start of the year.

There was little seasonal variation in the concentration of flexibilide in the tips of a colony of *Simularia flexibilis* over several months (Table 10), nor in the concentration of

TABLE 10

FLEXIBILIDE CONCENTRATION IN WHOLE TIPS FROM A COLONY OF
Simularia flexibilis

Date	Concentration of Flexibilide (nmoles/mg dry tissue)
12 Sep 1983	183.97
23 Mar 1984	163.89
19 Apr 1984	194.66

Colony collected from Rib Reef on 19 August 1983 and established in an aquarium.

sarcophytoxide at the perimeter of the capitulum of a colony of *Sarcophyton boletiforme* (Table 4).

5.3.4 Discussion

TLC observations on the furanoterpene composition of *Simularia capillosa* show a qualitative and quantitative change in the terpene composition with season, most noticeably in furanoquinol and furanoacid. This contrasts with a study of the sesquiterpene composition of several species of *Simularia* in the red Sea which showed a constant composition over different seasons (Kashman, Loya, Bodner, Groweiss, Beneyahu & Naveh, 1980).

The high level of furanoquinone in March 1982, coincided with large scale bleaching of the colony. If furanoquinone is a degradation product of furanoquinol (Bowden, personal communication, 1982), the presence of furanoquinone may have resulted from large scale degradation in the distressed colony. By September, the colony had completely regained its zooxanthellae. The following year the colony did not bleach but the effect of the earlier crisis was evident in that the majority of the colony had disappeared. The increase in concentration of furanoacid later in the year may be relative to the concentration of furanoquinol, or it may be associated with spawning which occurs annually in this species in October/November (Tapiolas, personal communication, 1985).

There was little variation in the concentration of the diterpene flexibilide recorded over several months in a colony of *Simularia flexibilis*, nor in the concentration of sarcophytoxide in a colony of *Sarcophyton boletiforme* in the months leading up to spawning. Presumably this lack of variation is due to the sampling method. The colonies of *Sarcophyton boletiforme* and *Simularia flexibilis* were sampled at their growing edges without reference to the gonads. The *Simularia capillosa* colonies were extracted whole and thus contained the gonads. The lack of change in terpene composition of the growing edges of a colony indicates that the

method of sampling both massive and branching colonies alcyoniidæan colonies at the growing edge can be used in chemotaxonomic studies but not in studies on the role of terpenes in reproduction.

Variations in plant terpene composition seems to be due to growth rather than season. In peppermint cuttings (*Mentha piperita*), there is a steady increase in monoterpene concentration until floral initiation and then a rapid decrease (Burbott & Loomis, 1969), but there is no change in concentration over a few days at a time when the cuttings are not flowering (Burbott & Loomis, 1972). It is likely that the terpene composition in *Simularia capillosa* is linked to reproductive status.

5.4 SEASONAL VARIATION IN TERPENE BIOSYNTHESIS BY ALCYONIIDÆAN SPECIES

5.4.1 Introduction

A seasonal variation in the terpene composition of alcyoniidæan corals has been noted (Bernstein, Shmeuli, Kashman & Neeman, 1974; Section 5.3.3). This section examines whether there is a seasonal pattern in the amount of terpene synthesized in *Simularia* and *Sarcophyton* species, and whether this pattern is linked to the reproductive status of the corals.

5.4.2 Experimental

The incubations described in Sections 5.2.3 spanned three years. The rates of radiolabel incorporation per 24 hours of the symbiotic colonies was plotted against time in months. No further incubations were conducted. A different scale of specific activity of the terpenes was used for the outdoor incubations of *Sarcophyton* spp. due to the high incorporation levels. Each point represents one whole symbiotic colony harvested after incubation, freeze-dried, and extracted exhaustively with dichloromethane. Spawning was

observed by Harrison (personal communication, 1981), by Tapiolas (personal communication, 1984), and by personal observation (1982, 1983).

5.4.3 Results

The terpenes examined were synthesized all year round (Tables 8 and 9). However, a plot of the levels of radiolabel incorporated into either furanoquinol, sarcophine or sarcophytoxide at different times of the year showed two phases of activity (Figure 50). From May to November the corals rapidly synthesized terpenes. There was a drop in synthetic activity of 50 to 90 percent in October and November. The level of incorporation remained low for the next six months.

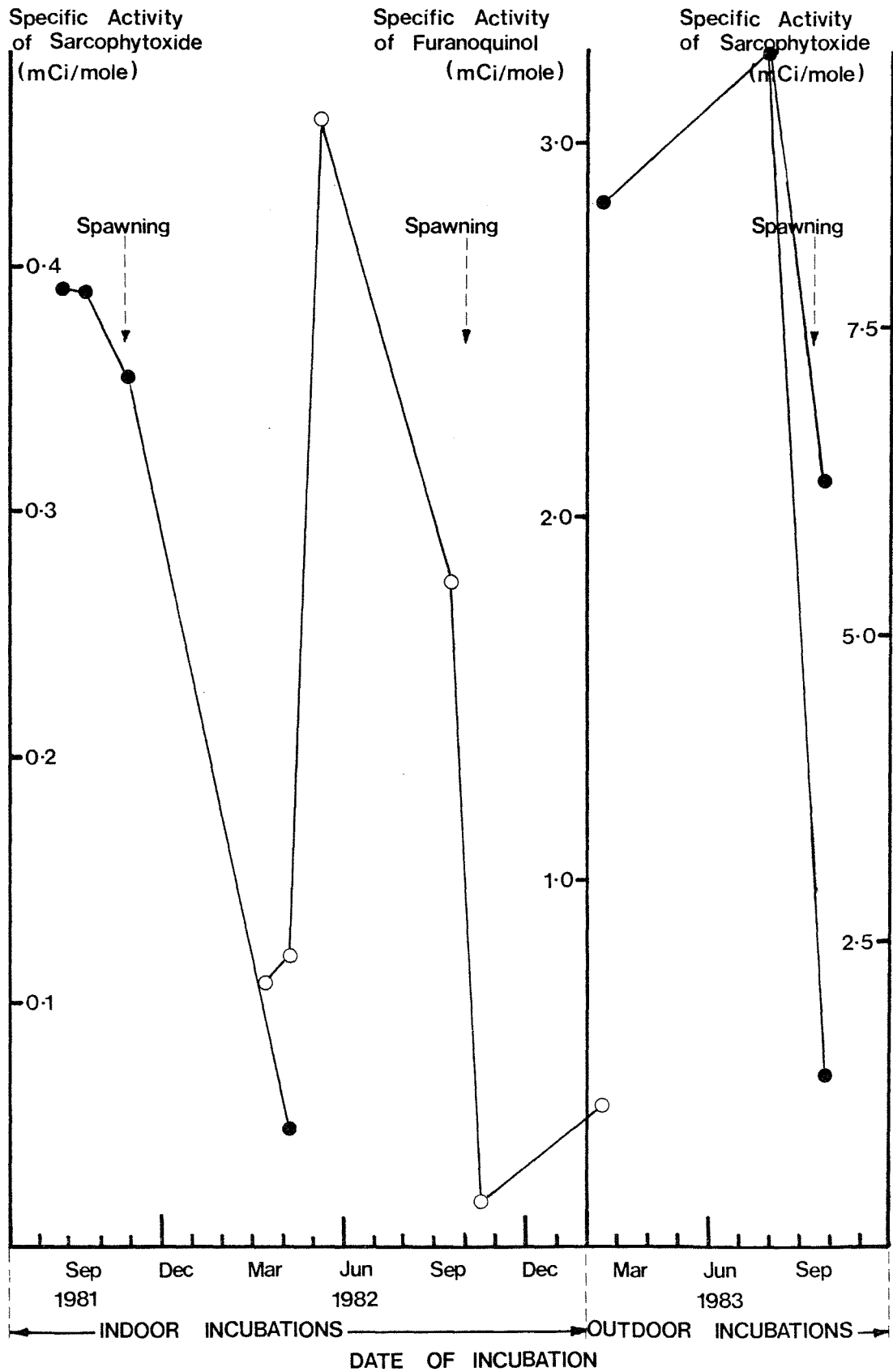
5.4.4 Discussion

For three years, the rate of radiolabel incorporation into furanoquinol by *Sinularia capillosa* and into sarcophytoxide by *Sarcophyton* spp. was maximal in the five months preceding spawning. Each year, there was a rapid drop of 50 to 90 percent in the biosynthetic rate after spawning.

The rate of biosynthesis of furanoquinol dropped suddenly when the furanoacid content was highest (October, 1982; Table 9). The following February, the biosynthetic rate of the furanoacid was almost double that of furanoquinol (0.66 mCi/mole). At that time the furanoacid concentration dropped back to the level of March 1982. The presence of large amounts of furanoacid in the months preceding spawning, coupled with the higher biosynthetic rate at spawning, suggests that the furanoacid may be located exclusively in the eggs, while the furanoquinol occurs in both the eggs and in the epidermis.

The drop in radiolabel incorporation into terpenes after

FIGURE 50
RATE OF INCORPORATION OF ^3H -MEVALONATE INTO TERPENES OVER
THREE SUCCESSIVE YEARS BY SYMBIOTIC COLONIES



spawning suggests that a large proportion of the terpenes are manufactured for the eggs. Sarcophine was found in the eggs released from a colony of *Sarcophyton stolidotum*, and FN45 was found in the eggs of a colony of *Lobophytum carnatum* (see Section 3.2.4). The possibility that terpenes in alcyoniidae coral eggs act as sperm attractants has not been substantiated by field trials (Tapiolas, personal communication, 1986). Both sarcophine and FN45 are toxic compounds (Neeman, Fishelson & Kashman, 1974; Coll & Sammarco, 1983). The terpenes in the eggs may act as antifeedants after release from the colony.

SECTION B: ARE TERPENES AN ANIMAL OR AN ALGAL PRODUCT?

As outlined in Section 1.2.6 there is observational and experimental evidence against algal synthesis of the terpenes found in octocorals. This section investigates the role of zooxanthellae in the production of terpenes in alcyoniidae corals, by examining:

- (1) the presence of terpenes in aposymbiotic colonies,
- (2) the incorporation of ^3H -mevalonic acid lactone into terpenes by aposymbiotic colonies, and
- (3) the effect of light on the incorporation of ^3H -mevalonic acid lactone into terpenes by symbiotic colonies.

5.5 THE PRESENCE OF TERPENES IN APOSYMBIOTIC ALCYONIIDAEAN SPECIES

5.5.1 Introduction

This section investigates the terpene content of aposymbiotic alcyoniidae corals.

5.5.2 Experimental

Symbiotic and aposymbiotic *Simularia capillosa* and *Sarcophyton boletiforme* were collected from Magnetic Island on March 11, 1982 and maintained in the indoor aquarium. Several symbiotic *Sarcophyton* colonies of various species were collected from Davies Reef in November 1982, and maintained in the outdoor aquarium for several months. In January 1983, one of these colonies, *Sarcophyton roseum*, became bleached. Another of these colonies, *Sarcophyton trocheliophorum*, did not bleach.

Tissue samples were taken from the growing edges of each of these colonies, the tips in the case of the *Simularia capillosa* and an edge of the capitulum perimeter in the case of the *Sarcophyton* species (Figure 41). The tissue samples were freeze-dried, extracted exhaustively with dichloromethane and analysed by TLC and by HPLC.

5.5.3 Results

In March 1982, most of the *Simularia capillosa* colonial mass was bleached. The terpenoid composition of the aposymbiotic and symbiotic colonies was identical (TLC) although the aposymbiotic colonies yielded half the organic extract. The difference in organic extract was due to the absence of zooxanthellae pigments and sterols. Both symbiotic and aposymbiotic *Simularia capillosa* contained the furanoterpene series presented in Figure 14.

Both symbiotic and aposymbiotic *Sarcophyton boletiforme* collected from Magnetic Island contained sarcophytoxide. All the nine aposymbiotic colonies of *Sarcophyton* spp. examined were rich in the terpenes sarcophine or sarcophytoxide (Table 11). There was approximately 50 percent more organic material in symbiotic colonies. This extra organic material was predominantly zooxanthellae pigments and sterols. Aposymbiotic colonies of *Sarcophyton* spp. contained large amounts of terpenes in similar relative ratios to that found in healthy brown colonies (TLC). The

TABLE 11

APOSYMBIOTIC *Sarcophyton* SPECIES CONTAINING TERPENES

SPECIES	SARCOPHYTOXIDE	SARCOPHINE
<i>S. spongiosum</i>	+	-
<i>S. boletiforme</i>	+	+
<i>S. roseum</i>	+	+
<i>S. infundibuliforme</i>	+	+
<i>S. infundibuliforme</i>	+	+
<i>S. infundibuliforme</i>	+	+
<i>S. infundibuliforme</i>	+	+
<i>S. infundibuliforme</i>	+	+
<i>S. trocheliophorum</i>	-	+

Dry tissue extracted with dichloromethane. Extracts assayed by TLC on silica gel against standard compounds and visualised by spraying with vanillin/sulphuric acid and warming.

terpene concentrations in the white colonies was as high, if not higher, than in the brown colonies. A piebald colony of *Sarcophyton spongiosum* was collected from Magnetic Island (Plate 17). One half was a light tan while the other half was white. The two halves had identical terpene composition, qualitatively and quantitatively (TLC). The aposymbiotic colony of *Sarcophyton roseum* (Plate 6 B) and the symbiotic colony of *Sarcophyton trocheliophorum* (Plate 6 A) had a similar composition of sarcophytoxide and sarcophine, on four sampling occasions (Table 12).

5.5.4 Discussion

All the aposymbiotic colonies examined belonging to the *Sarcophyton* and *Simularia* genera contained terpenes at comparable concentrations (TLC) to that of symbiotic colonies. This suggests the coral animals and not the zooxanthellae control terpene synthesis. However, the observation that bleached corals contain terpenes is not proof of animal synthesis of the terpenes for two reasons.

- (1) Bleached corals are not totally devoid of zooxanthellae. Zooxanthellae remain on the undersurface of corals and often in the polyps (Personal observation, 1983).
- (2) the turnover of terpenes in corals is not known. Terpenes found in plants can have a turnover of months or years (Loomis & Croteau, 1973).

The possibility that the terpenes in aposymbiotic corals are residual rather than newly synthesized is investigated in the next section.

TABLE 12

CONCENTRATION OF SARCOPHYTOXIDE IN A SYMBIOTIC COLONY OF
Sarcophyton trocheliophorum AND AN APOSYMBIOTIC COLONY OF
Sarcophyton roseum

DATE	COLONY COLOUR	CONCENTRATION (nmoles/mg dry tissue)	
		Sarcophytoxide	Sarcophine
9 Apr 1983	Symbiotic	27.18	0.594
	Aposymbiotic	34.72	0.000
24 Apr 1983	Symbiotic	96.92	0.408
	Aposymbiotic	98.91	0.360
5 Jul 1983	Symbiotic	14.25	0.753
	Aposymbiotic	13.04	0.369
26 Jul 1983	Symbiotic	16.30	0.564
	Aposymbiotic	21.64	0.748

Symbiotic colony = *S. trocheliophorum*

Aposymbiotic colony = *S. roseum*

Colonies collected from Davies Reef in November 1982, and established in the outdoor aquarium.

5.6 BIOSYNTHESIS OF TERPENES BY APOSYMBIOTIC ALCYONIIDAEAN SPECIES

5.6.1 Introduction

The above section demonstrated that aposymbiotic colonies do contain terpenes. If terpenes are a zooxanthellae product, then acclimatised aposymbiotic colonies will not incorporate ^3H -mevalonate into terpenes. Any terpenes present will be residual and will disappear in time. If terpene synthesis is under animal control, then there will be no difference in the level of radiolabel incorporation into terpenes by symbiotic and aposymbiotic colonies.

5.6.2 Experimental

Large areas of the previously described *Simularia capillosa* colony containing the furanoterpene series (Figure 14), became bleached in January 1982 (Plate 7). The bleached portions contained high concentrations of furanoquinol and furanoquinone (see Section 5.5.3). Symbiotic (brown) and aposymbiotic (white) colonies were collected from Magnetic Island and maintained alive in the indoor aquarium. These colonies were all incubated with ^3H -mevalonic acid lactone under regime A (see methods, Section 5.2.2). Each aposymbiotic colony was incubated simultaneously with a symbiotic colony, in the same beaker.

Various *Sarcophyton* species were collected from Magnetic and Orpheus Islands and were maintained either in the indoor aquarium or the outdoor aquarium. Aposymbiotic colonies of *Sarcophyton* spp. were collected whenever possible. These colonies were incubated with ^3H -mevalonic acid lactone under regimes A through D. Each aposymbiotic colony was incubated simultaneously with a symbiotic colony, in the same beaker.

5.6.3 Results

Table 8 shows the level of incorporation of ^3H into sarcophytoxide and sarcophine by various species of symbiotic and aposymbiotic *Sarcophyton* spp. The first five colonies were incubated in an indoor aquarium while the last four were incubated either in the ocean (February 10, 1983; September 25, 1983) or beside the outdoor aquarium (July 26, 1983).

The specific activity of the terpene furanoquinol synthesized by aposymbiotic and by symbiotic colonies of *Simularia capillosa* is presented in Table 9. The first aposymbiotic colony incubated yielded ^3H -furanoquinol of a similar though slightly higher specific activity than that of the symbiotic colony. The second aposymbiotic colony yielded ^3H -furanoquinol of a specific activity three-fold higher than that of the symbiotic colony. The third aposymbiotic colony yielded ^3H -furanoquinol of a specific activity ten times lower than that of the symbiotic colony. All incubations of *Simularia capillosa* were conducted beside the indoor aquarium.

The first symbiotic/aposymbiotic pair of *Sarcophyton* species incorporated similar amounts of ^3H into both sarcophytoxide and sarcophine, though both colonies had low incorporation levels. The second symbiotic/aposymbiotic pair showed widely different ^3H incorporation levels, with the symbiotic colony having a level of incorporation 4.4 times higher. In both colonies sarcophine was of a higher specific activity than sarcophytoxide, in contrast to the previous pair. Sarcophytoxide from the symbiotic colony was of a similar specific activity to that of oceanic incubations (10th February 1983; 25th September 1983).

5.6.4 Discussion

All the aposymbiotic colonies incubated were capable of terpene biosynthesis. The level of ^3H -mevalonic acid incorporation into terpenes in both aposymbiotic and symbiotic colonies of *Simularia*

capillosa and *Sarcophyton* spp. was quite high. The incorporation levels are comparable to those obtained later with symbiotic colonies.

One symbiotic colony acclimatised to life in the outdoor aquarium had a four-fold reduction in incorporation level. This may be due to a difference in species. This is echoed in a four-fold reduction in incorporation by a colony incubated with reduced light. In this case, the two colonies were of the same species. Even though several of the symbiotic/aposymbiotic pairs were incubated under conditions of stress, radiolabel was incorporated by aposymbiotic colonies. On occasion, they incorporated more radiolabel than the symbiotic partner.

5.7 BIOSYNTHESIS OF TERPENES BY SYMBIOTIC ALCYONIIDAEAN SPECIES IN THE PRESENCE AND IN THE ABSENCE OF LIGHT

5.7.1 Introduction

This section examines the effect of a reduction in the ambient light on the level of incorporation of ^3H -mevalonic acid lactone into sarcophytoxide by *Sarcophyton boletiforme*.

5.7.2 Experimental

Two colonies of *Sarcophyton boletiforme* were incubated simultaneously *in situ* in Pioneer Bay for 24 hours. One of the colonies was incubated in vessel made from a dark glass winchester while the other was incubated in a vessel made from a clear glass winchester.

5.7.3 Results

Incubation of a colony of *Sarcophyton boletiforme* in a dark glass vessel resulted in a decrease in the incorporation of ^3H into both sarcophytoxide and into sarcophine over a 24 period, compared to a conspecific colony incubated simultaneously (Table 8).

5.7.4 Discussion

Sudden loss of nutrients from zooxanthellae such as happens when photosynthesis is reduced by the use of chemical inhibitors, or by the restriction of light, may cause a metabolic crisis in the coral, resulting in a shut down of many non-essential activities. Symbiotic corals do adjust to the presence of zooxanthellae. For instance, by inducing the excretion of amino acids, carbohydrates and acetate from zooxanthellae (von Holt & von Holt, 1968b). Aposymbiotic animals could not induce cultures of zooxanthellae to excrete these compounds (Trench, 1971b). Colonies adjusted to the lack of subsidised energy and food, courtesy of the zooxanthellae, presumably gradually resume non-essential activities using a different energy budget.

In conclusion, terpene synthesis does occur in aposymbiotic colonies by alcyoniidæan corals. The decrease in the rate of terpene synthesis that occurs when there is a sudden loss of zooxanthellae in symbiotic colonies is presumably due to reliance of the animal on the zooxanthellae for supplementation of either energy or precursors used in synthesis of these terpenes.

5.8 SUMMARY

Sarcophytoxide and sarcophine were found in aposymbiotic colonies of *Sarcophyton* spp. in quantities comparable to the concentration of the same terpenes in symbiotic colonies. Furanoquinol occurred in large quantities in aposymbiotic *Simularia*

capillosa.

Repetitive sampling of the *Sarcophyton boletiforme* capitulum perimeter, or of the terminal branches of *Simularia flexibilis*, did not alter the concentration of sarcophytoxide or flexibilide, respectively. Thus repetitive sampling of the growing edges of alcyoniidae corals allows monitoring of the terpene concentration within those tissues. Incorporation of radiolabel into both furanoquinol and sarcophytoxide was suppressed in colonies incubated under stress.

Synthesis of both sarcophytoxide and furanoquinol occurred equally in both symbiotic and aposymbiotic colonies. However, sudden cessation of photosynthesis in symbiotic colonies caused an immediate decrease in the rate of terpene synthesis.

The terpene composition of the growing edges of *Sarcophyton boletiforme* and *Simularia flexibilis* did not alter with season or with reproductive status of the corals. However, the terpene composition of whole colonies of *Simularia capillosa* did vary with season in a manner linked to spawning. Thus, sampling a colony at the growing edge is a satisfactory method of obtaining samples for chemotaxonomic studies, and sampling the whole colony allows the changes in terpene composition of the gonads to be monitored. The seasonal appearance of furanoacid in whole colony extracts of *Simularia capillosa* coincided with spawning. The rates of biosynthesis of terpenes rose prior to spawning and dropped rapidly after spawning, supporting the thesis proposed in Chapter 3 that the terpenes are concentrated in the eggs.

5.9 FUTURE EXPERIMENTS

Analysis of *in vivo* synthesis using *in situ* colonies should be continued to determine whether there are circadian rhythms of terpene biosynthesis in alcyoniidae corals. The rates of synthesis of the various terpenes found in one colony may differ

between the gonads and the growing edge of the colony. The newly released eggs should be incubated with radiolabel to determine whether the eggs themselves are synthesizing the terpenes or whether they are simply storing the terpenes synthesized elsewhere in the colony.

CHAPTER 6

DISTRIBUTION OF TERPENES WITHIN THE POLYP

The location of terpenes within the alcyoniidæan polyp is not known. Microscopical examination of the gorgonian *Pseudoplexaura porosa* showed crystals of what was presumed to be crassin acetate to be external but adjacent to the zooxanthellae (Rice, Papastephanou & Anderson, 1970). These authors maintained that the association of the crystals with the zooxanthellae supported their belief that crassin acetate is an algal product. In this chapter, the location of terpenes in the alcyoniidæan polyp is investigated by differential centrifugation, and by electron microscopy. The location of the terpenes away from the zooxanthellae may further support the thesis that the terpenes in alcyoniidæan corals are produced by the coral animal, not by the zooxanthellae.

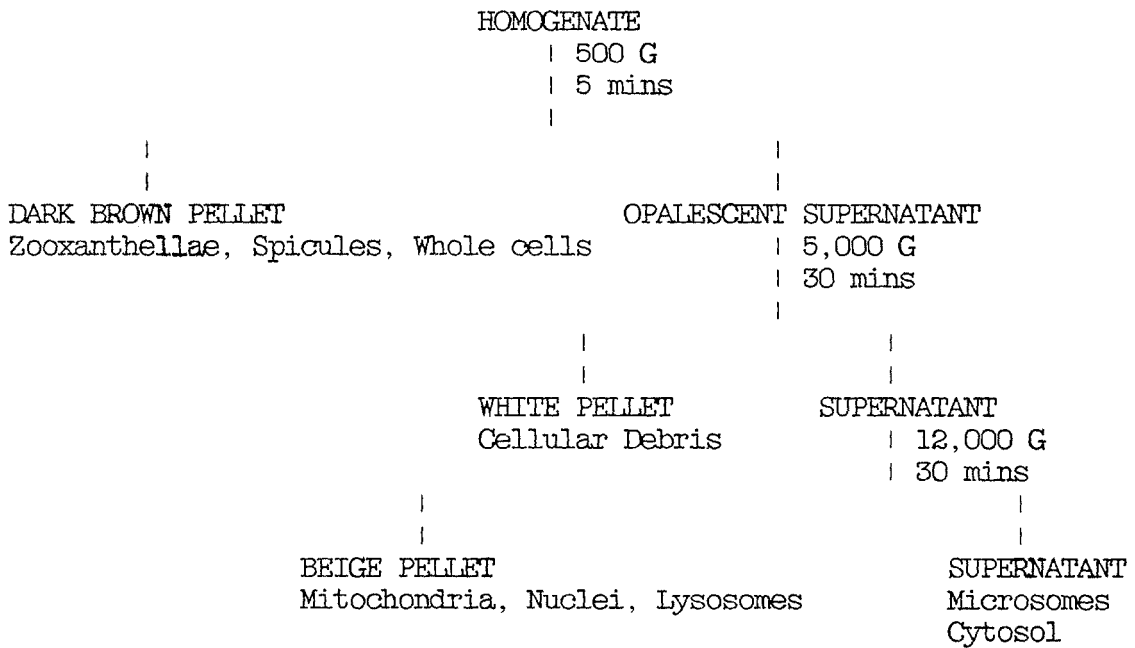
6.1 DISTRIBUTION OF FLEXIBILIDE WITHIN THE CELL

6.1.1 Introduction

The animal cells of coral colonies can be ruptured by homogenisation, leaving the tough algal cells intact (Muscantine, 1967; Crossland & Barnes, 1977; Kokke, Fenical, Bohlin & Djerassi, 1981). The algal pellet contains all the algal cytoplasm while the animal cytoplasm is distributed between the other centrifugal fractions. To determine whether the coral animal or the algal symbiont was the site of flexibilide storage in *Simularia flexibilis*, the coral and algal symbionts were separated by homogenisation, and the relative concentrations of the terpene in each symbiont compared.

FIGURE 51

FRACTIONATION OF *Simularia flexibilis* CELL COMPONENTS
BY DIFFERENTIAL CENTRIFUGATION



Buffer was Tris (0.01M, pH 7.4, containing 0.1M sucrose).
G = gravitational force.

6.1.2 Experimental

A colony of *Simularia flexibilis* was collected from Rib Reef on August 19, 1983 and maintained in the outdoor aquarium. Terminal lengths (4 cm long) (Figure 41) were collected on March 23, 1984 and April 19, 1983. These samples were freeze-dried, extracted exhaustively in dichloromethane. The extract was analysed by TLC and by HPLC.

Also on April 19, 1984, a 15 cm branch of the same colony of *Simularia flexibilis* (17.02 g wet weight) was homogenised in cold Tris buffer (Sigma) (0.01 M, pH 7.4 containing 0.1 M sucrose) and filtered through cotton. Differential centrifugation separated the cell components into five fractions (Figure 51). Each fraction was freeze-dried, extracted exhaustively with dichloromethane, and the extract was analysed by TLC and by HPLC.

6.1.3 Results

The colony of *Simularia flexibilis* collected from Rib Reef contained flexibilide. The concentration of flexibilide in the tips of the colony on two sampling occasions, including the day the larger segment was homogenised, is presented in Table 10. The concentration of flexibilide was constant over several months.

The amount of flexibilide in the various cellular fragments of *Simularia flexibilis* is presented in Table 13. Most of the terpene was in the microsomal cytosol (81.88 percent of the total amount of flexibilide present in the portion of coral homogenised). A small amount was in the cellular debris (8.31 percent of the total amount present in the homogenate) and in the zooxanthellae layer (7.77 percent of the total amount present in the homogenate). Negligible quantities were in the cell wall pellet (1.79 percent of the total amount present in the homogenate) and mitochondrial-lysosomal-nuclei pellet (0.24 percent of the total amount present in the homogenate). An oil formed on the air-water interface after centrifugation. This

TABLE 13

INTRACELLULAR DISTRIBUTION OF FLEXIBILIDE IN
Simularia flexibilis

CELL FRACTION	FLEXIBILIDE	
	(μ moles)	(% of total)
Microsomes, Cytosol	19.46	81.88
Animal cell walls	2.40	10.10
Zooxanthellae, Spicules, Intact animal cells	1.85	7.77
Mitochondria, Nuclei	0.06	0.24

Sample taken on 19 April 1984

Wet weight of sample = 17.02g

top layer of supernatant was removed, and was found to contain 7 percent of the total amount of flexibilide found in the segment of *Simularia flexibilis* homogenised.

6.1.4 Discussion

Virtually all the flexibilide in *Simularia flexibilis*, 81.88 percent, was in the animal microsomal-cytosolic fraction, demonstrating that the terpene is concentrated in the animal portion of the colony. The small amount of terpene in the zooxanthellae pellet is probably due to contamination with intact animal cells.

Microcrystals of the diterpene crassin acetate found in the gorgonian *Pseudoplexaura porosa* centrifuged in a layer below the zooxanthellae layer and above the spicular layer. The distribution of the crassin acetate within the different centrifugal layers differed so vastly from the distribution of flexibilide in the homogenate of *Simularia flexibilis*, that it can be concluded that flexibilide does not occur as a crystal *in vivo* in *Simularia flexibilis*. In fact the presence of flexibilide in the uppermost layer of the supernatant suggests that it is present as an oil, particularly as an oil was noticed on the surface of the supernatant.

6.2 THE PRESENCE OF OSMIOPHILIC MATERIAL IN THE POLYPS AND COENCHYMAL TISSUES OF *Sarcophyton boletiforme*

6.2.1 Introduction

Microscopy allows a visual comprehension of the spatial location of cell types, as well as a knowledge of the cellular location of biochemicals. There are no reports on ultrastructural studies on the site of terpene storage or synthesis in octocorals. The ultrastructure of octocorals themselves has not been extensively studied. Publications are limited to studies on a gorgonian

(Kawaguti, 1964), an alcyonacean, *Heteroxenia elisabethae* (Kawaguti, 1974) and the algal symbiont of alcyonacean *Sarcothelia edmundsoni* (Singh & Mercer, 1976).

Rice, Papastephanou and Anderson (1970) described the presence of crassin acetate crystals in the gastric cavity and in the epidermis of the gorgonian *Pseudoplexaura porosa*, as revealed by light microscopy. Terrestrial plants contain osmiophilic terpenoid material free in the cytoplasm in specialised gland cells (Heinrich, 1970; Carde & Bernard-Dagan, 1975; Gleizes, Carde, Pauly & Bernard-Dagan, 1980).

This section describes the ultrastructure of the polyp and coenenchymal epidermis of *Sarcophyton* spp., and of the zooxanthellae, in detail, and presents electronmicrographs of a putative terpene synthesising cell discovered in the epidermis of *Sarcophyton boletiforme*.

6.2.2 Experimental

In April 1982, a symbiotic and an aposymbiotic colony of *Sarcophyton boletiforme* was collected from Magnetic Island. The colonies were acclimatised in the indoor aquarium for a month. In November 1983, a symbiotic colony of *Sarcophyton boletiforme* was collected from Magnetic Island. Also in November 1983, a colony of *Sarcophyton infundibuliforme* was collected from Myrmidon Reef. These colonies were acclimatised in the outdoor aquaria for seven months.

Routine sampling by taking slithers of the capitulum, showed that all the colonies of *Sarcophyton boletiforme* contained sarcophytoxide. The colony of *Sarcophyton infundibuliforme* did not seem to contain any terpenes. These four colonies, and the colony of *Sarcophyton stolidotum* which released sperm in November 1983 in the outdoor aquarium (Chapter 3), were examined by electron microscopy.

The polypal tissue extending above the capitulum was excised. Each polyp collected was processed separately. The epithelial skin covering the coenchyme of the colonies collected in April 1982, was also sampled. The tissues were fixed, sectioned and stained using a modification of the methods used for zooxanthellae (Fitt & Trench, 1983), scleractinian corals (Harrison, 1985), and plant terpenes (Cope & Williams, 1967; Heinrich, 1970), as follows:

Fixed for 2h at 4C in 50 volumes of 2% glutaraldehyde in cacodylate buffered seawater (0.2 M, pH 7.2).

Postfixed in 1% osmium tetroxide for 2h.

Decalcified overnight in 0.1 M ascorbic acid and 0.1% NaCl.

Dehydrated in 50% ethanol in water for 2 minutes,

Dehydrated in 70% ethanol in water for 2 minutes,

Dehydrated in 85% ethanol in water for 2 minutes,

Dehydrated in 95% ethanol in water for 2 minutes,

Dehydrated in three washes in 100% ethanol for 10 minutes each.

Embedded in Spurr's epoxy resin in flat trays to allow orientation of the tissues for sectioning.

Ultrathin sections (5 μ m) cut with a glass knife on a LKB Ultratome III and mounted on copper grids (hexagonal mesh, 100). Most grids were stained with uranyl acetate (saturated solution in 50% ethanol) and Reynolds lead citrate. Some grids were left unstained. The grids were viewed under a Siemens transmission electron microscope and photographed by the same instrument.

6.2.3 Results

This chapter describes the ultrastructure of the polyp and coenchymal epidermis of *Sarcophyton* spp., and of the zooxanthellae, in detail (Section 6.2.3.1), and presents electronmicrographs of a putative terpene synthesing cell discovered in the epidermis of *Sarcophyton boletiforme* (Section 6.2.3.2).

6.2.3.1 *Sarcophyton* spp. Ultrastructure

The Gastrodermis

Digestive cells lined the gastrodermis along the length of the gastric cavity. The digestive cells of the tentacles and of the pharynx had extensive cytoplasmic extensions into the lumen. These extensions did not contain organelles. They contained only a granular ground material. These cells were loosely connected by semi-desmosomes.

The pharynx gastrodermal cells were ciliated. The cilia were often surrounded by cytoplasmic extensions from epithelial cells forming an eight or nine spoked wheel. The cilia appeared to be independent units with cell membranes between the epithelial extensions and the cilia. Microfibrils were visible. Upon contraction of the polyp, the cilia sometimes lay between the gastrodermal cells. The basal apparatus was not seen. Lower down the gastric cavity, the gastrodermal epithelial cells had microvilli. The microvilli were less numerous at the proximal end of the polyp tube.

The apical surface of the digestive cells showed digestive vacuoles containing fibrillar and lipid material. These vacuoles were associated with various vesicles, golgi bodies and mitochondria.

Solitary mucus cells were located behind the digestive cells. Mucus was seen in the lumen. An unidentified lipid cell was also observed.

The Mesoglea

The mesoglea was a collagenous matrix spanning the space between the gastrodermis and the epithelium, and even penetrated between the cells of these layers. Within the mesoglea was the

PLATE 18

Sarcophyton boletiforme WITH SARCOPHYTOXIDE.
POLYP EPIDERMIS AND MESOGLEA. MAGNIFICATION X 10 000.



v = osmiophilic vesicles, n = nucleus, m = mesoglea.

organic matrix remaining after the spicules were decalcified. Young scleroblasts were seen at the edges of the autozooid mesoglea. Larger scleroblasts occur deeper in the autozooid mesoglea and also in the coenchymal mesoglea. Nerve cells ^{occurred} at the mesoglea-gastrodermal interface. The mesogleal side of the nerve cell was bulky and rounded while the rest of the cell was elongate and threaded back between the other cell types.

The Epidermis

The epidermis of the polyp, tentacle and coenchrime was a thin layer of cells covered by short microvilli which were sparsely scattered on the cell surfaces. The outer cells of the epidermis were loosely attached by semi-desmosomes. There was less variety in the cell types than in the gastrodermis.

The epidermis of the three symbiotic and one aposymbiotic *Sarcophyton* spp. which contained sarcophytoxide, showed a cell type not seen in the symbiotic *Sarcophyton infundibuliforme* which did not seem to contain any terpenes at all. These cells contained numerous darkly staining vesicles are described in Section 6.2.3.2.

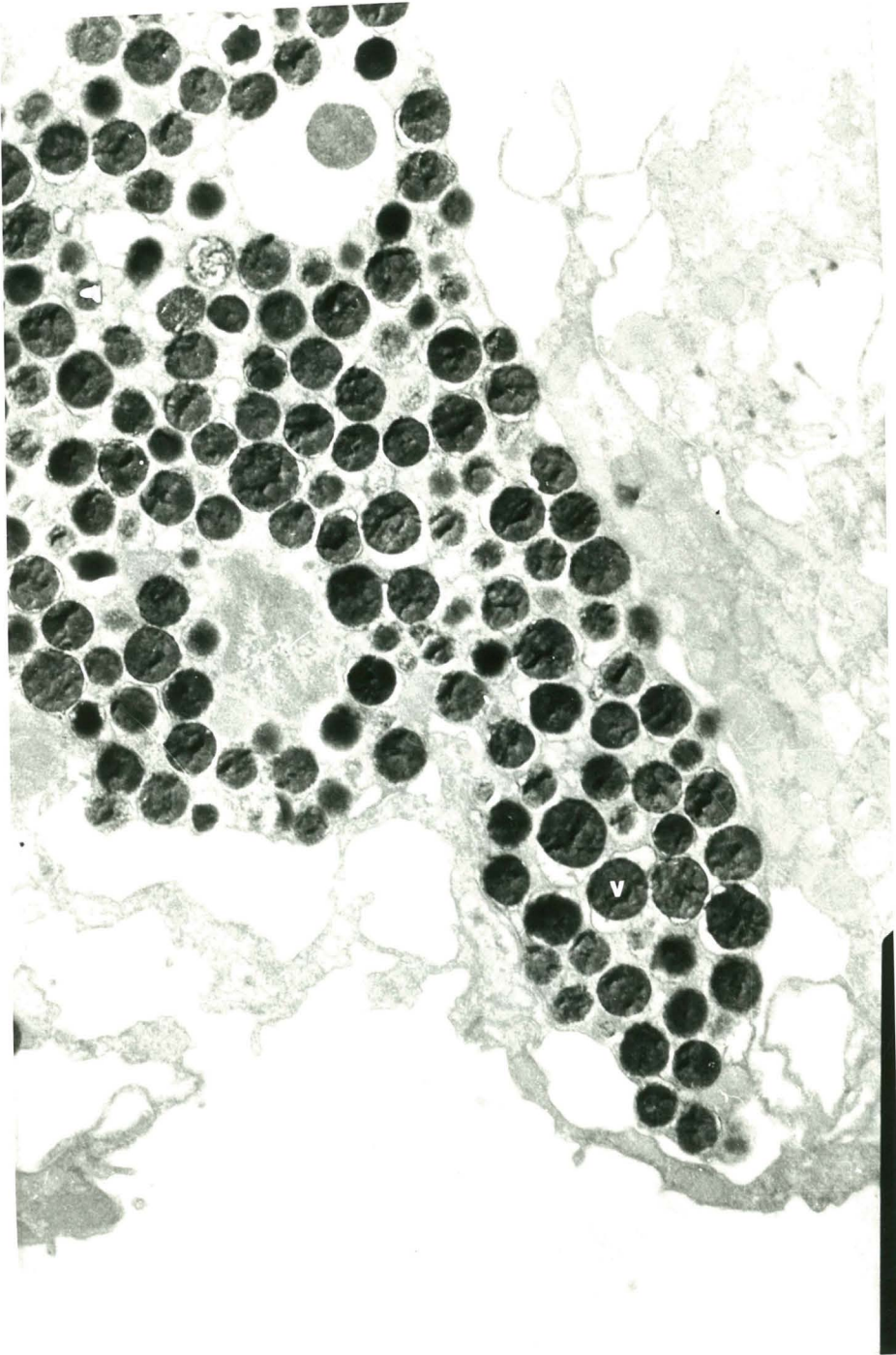
Solitary feeder nematocysts, in the form of spirocysts, occurred in the gastrodermis, and in the tentacle epidermis. The threads of the harpoon were clearly seen. No discharged nematocysts were seen.

The Zooxanthellae

The zooxanthellae were intercellular and were scattered throughout the epidermis and the gastrodermis. They often occurred in double lines. They contained peripheral chloroplasts with closely appressed thylakoids in groups of three, calcium oxalate crystals, starch sacs external to the chloroplasts, a pyrenoid, mitochondria with tubular cristae typical of plant rather than animal mitochondria, a nucleus containing dense chromosomes, and a

PLATE 19

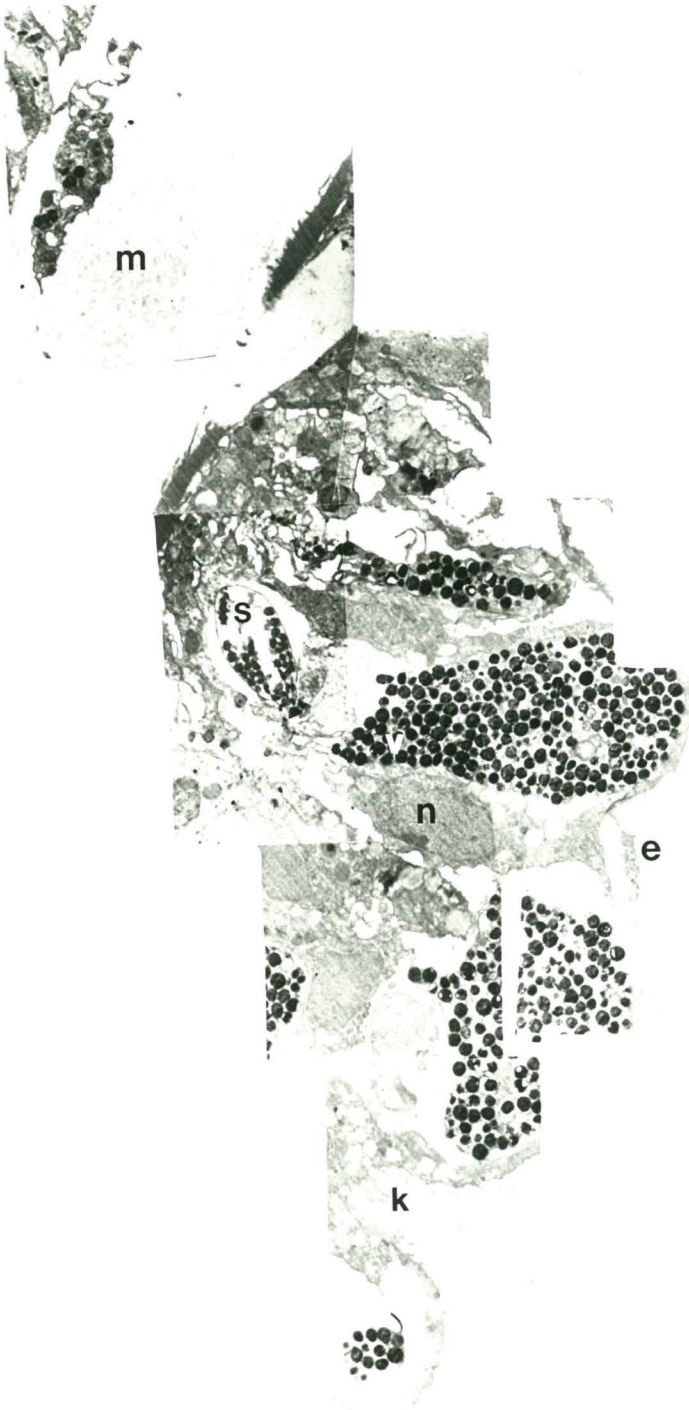
Sarcophyton boletiforme WITH SARCOPHYTOXIDE.
STAINED ONLY WITH OSMIUM TETROXIDE.
TENTACLE EPIDERMIS. OSMIOPHILIC VESICLES. MAGNIFICATION 30 000.



v = osmiophilic vesicles.

PLATE 20

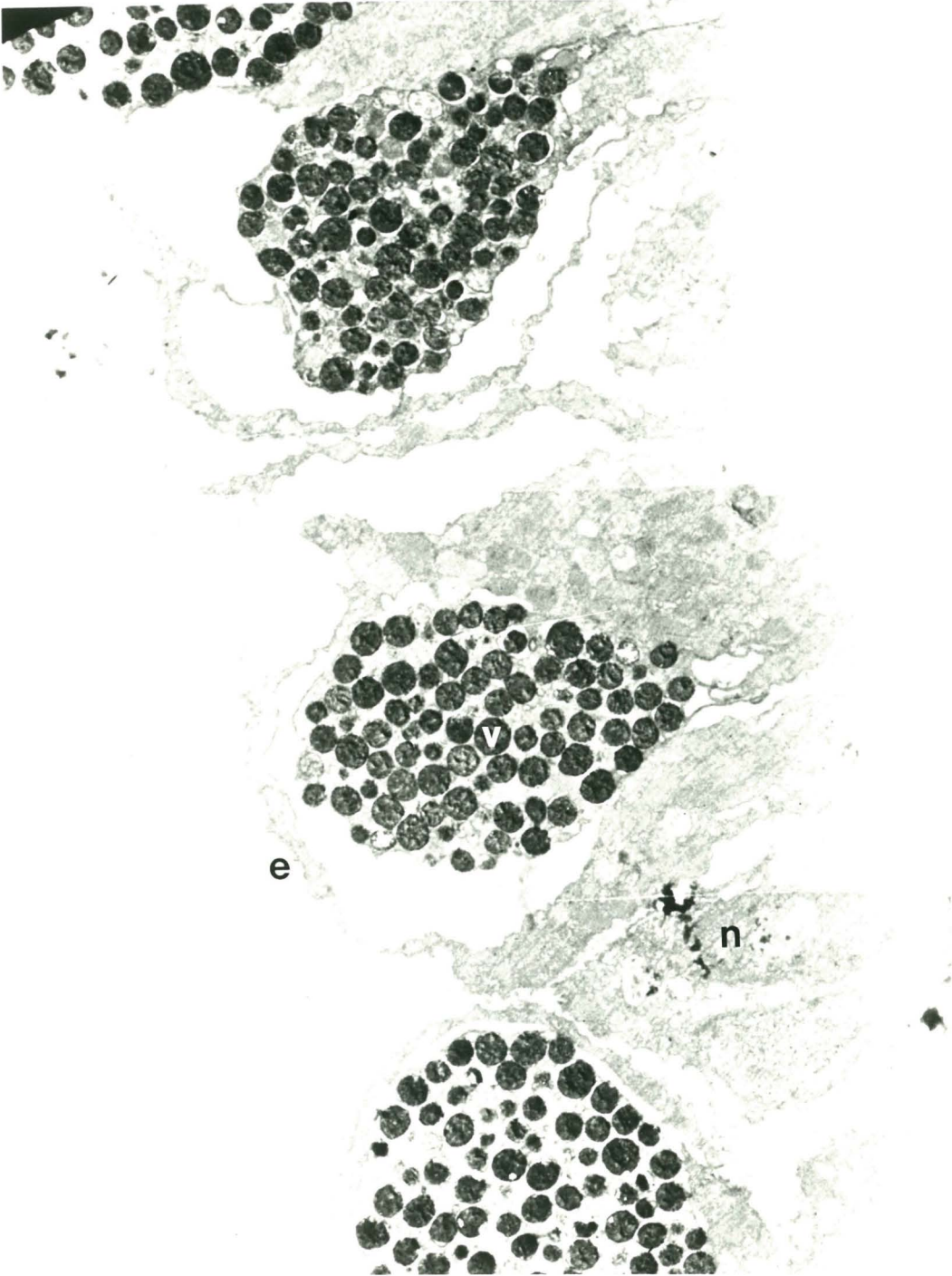
Sarcophyton boletiforme WITH SARCOPHYTOXIDE.
TENTACLE EPIDERMIS. OSMIOPHILIC VESICLES.
STAINED ONLY WITH OSMIUM TETROXIDE. MAGNIFICATION X 2 000.



v = osmiophilic vesicles, n = nucleus, m = mesoglea,
e = epidermis, s = spirocyst, k = microvilli.

PLATE 21

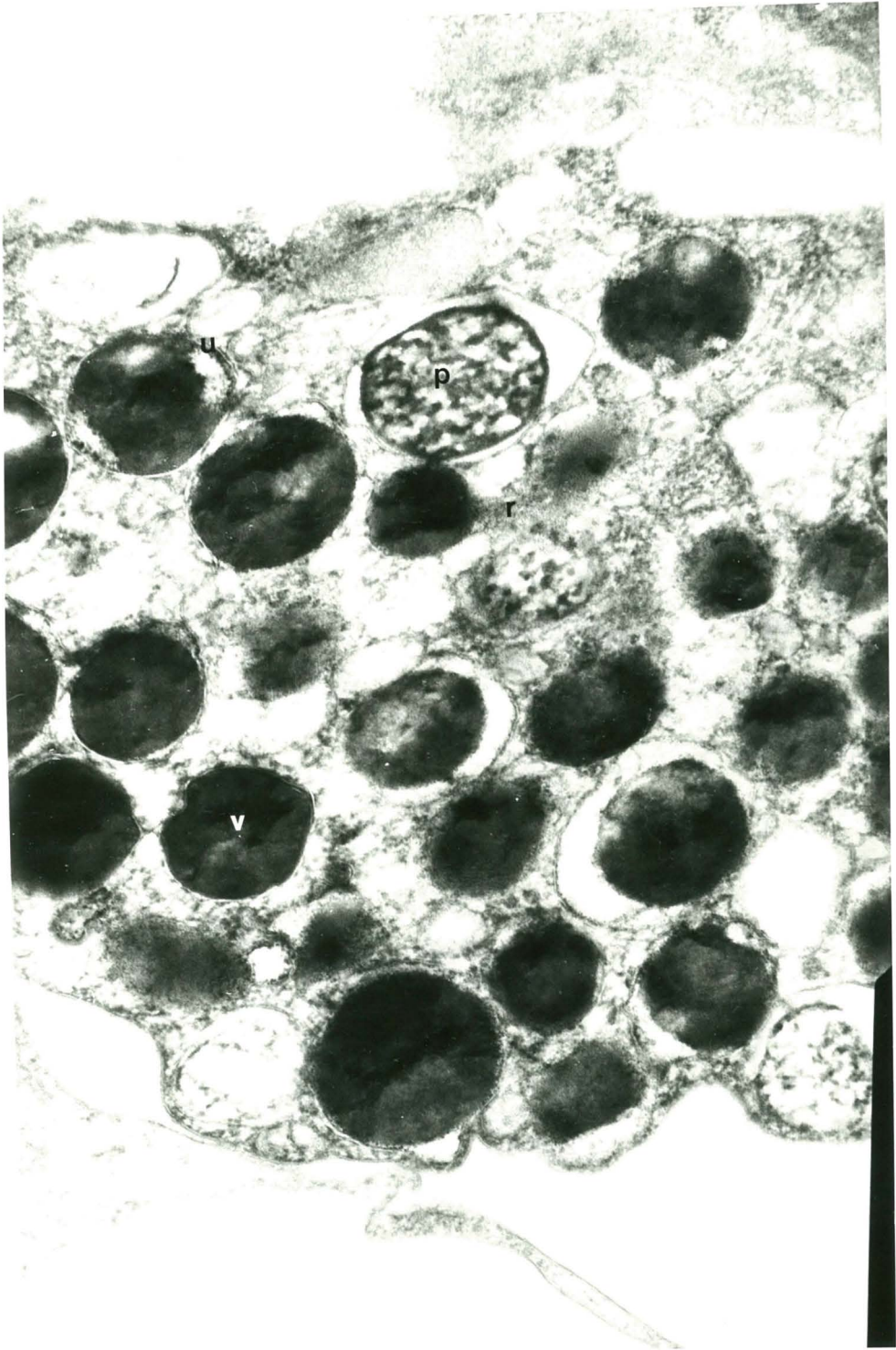
Sarcophyton boletiforme WITH SARCOPHYTOXIDE.
POLYP EPIDERMIS. STAINED ONLY WITH OSMIUM TETROXIDE.
MAGNIFICATION X 10 000.



v = osmiophilic vesicles, n = nucleus, e = epidermis.

PLATE 22

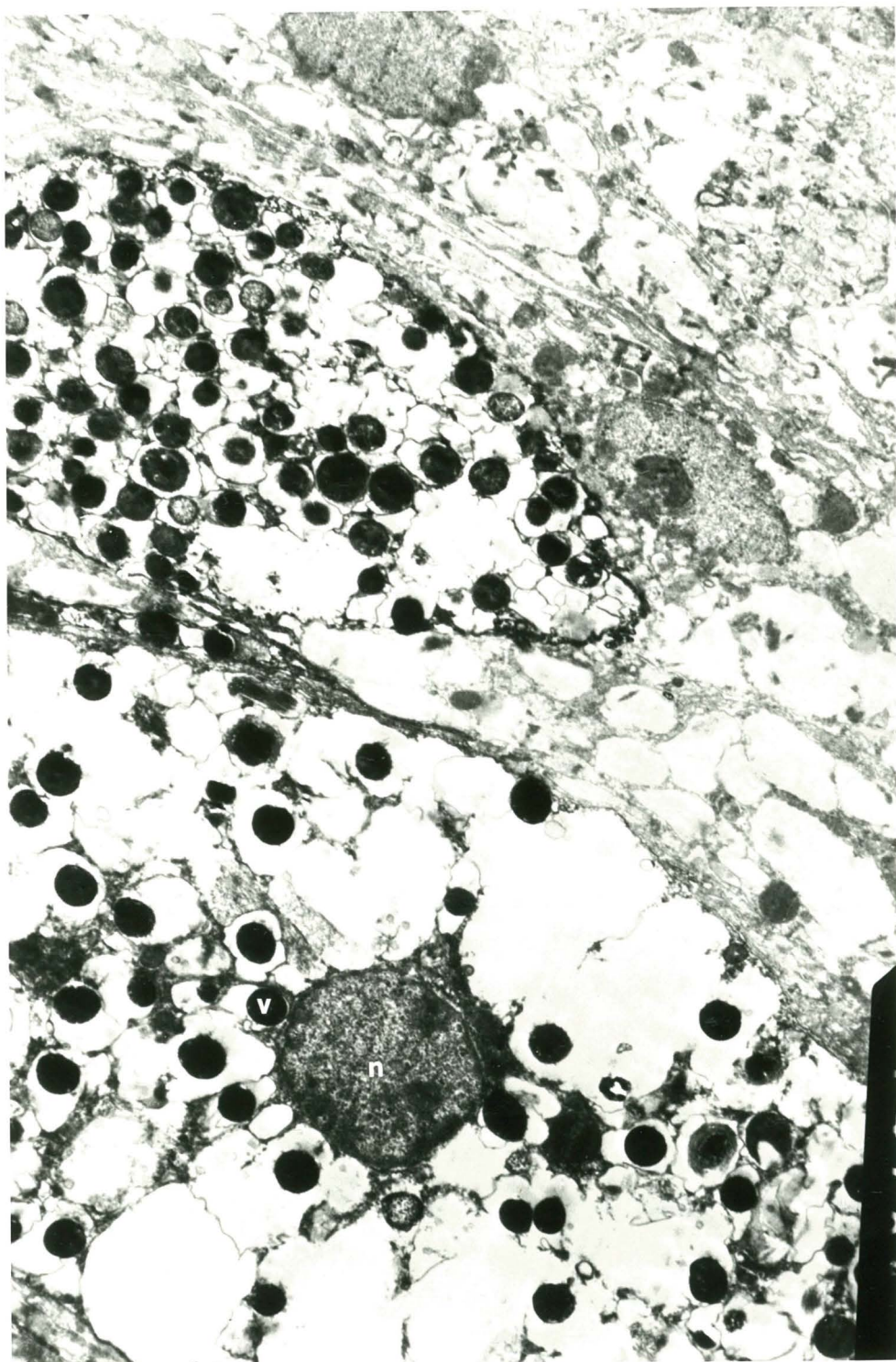
Sarcophyton boletiforme WITH SARCOPHYTOXIDE.
POLYP EPIDERMIS. STAINED ONLY WITH OSMIUM TETROXIDE.
OSMIOPHILIC VESICLES. MAGNIFICATION 80 000.



v = osmiophilic vesicles, p = precursor vesicles,
r = ribosomes, u = unit membrane.

PLATE 23

Sarcophyton stolidotum WITH SARCOPHYTOXIDE.
POLYP EPIDERMIS STAINED ONLY WITH OSMIUM TETROXIDE.
OSMIOPHILIC VESICLES. MAGNIFICATION 80 000.



v = osmiophilic vesicles, n = nucleus

cell wall. The animal cells surrounding the zooxanthellae sometimes showed whorls of membrane at the zooxanthellae-animal interface. The whorls often had a dark-staining spot near the center of the whorl. The animal cells showed golgi bodies and mitochondria adjacent the zooxanthellae starch sacs.

The aposymbiotic *Sarcophyton boletiforme* contained the degraded remnants of zooxanthellae. A few intact zooxanthellae were seen. The tissue in general was degraded with gaping holes suggesting the sites previously occupied by zooxanthellae.

6.2.3.2 Osmiophilic Vesicles

The epidermis of the three symbiotic and one aposymbiotic *Sarcophyton* spp. which contained sarcophytoxide, showed a cell type not seen in the symbiotic *Sarcophyton infundibuliforme* which did not seem to contain any terpenes at all. These cells contained numerous darkly staining vesicles (Plates 18 through to 23). The vesicles contained a heterogeneous osmiophilic material. This was particularly apparent in some vesicles (Plate 22). The more heterogeneous vesicles were interpreted as precursor vesicles (Plate 22). The only other organelle apparent was the nucleus (Plate 23). No mitochondria, golgi bodies, rough or smooth endoplasmic reticulum were seen. Ribosomes were seen free in the cytoplasm between the vesicles (Plate 22). The unit membrane was apparent around the vesicles (Plate 22) and evolved from the nuclear membrane (Plate 23).

The cells containing osmiophilic vesicles occurred only in the epidermis. They occurred across the span of the epidermis, from adjacent to the mesoglea to the outer epithelial layer, but were most concentrated along the epithelial surface where they formed a confluent layer (Plates 20 and 22). These cells were not particularly associated with the zooxanthellae though on rare occasions they were adjacent to the endosymbionts. The contents of these vesicles were not seen free in the cell, nor in the lumen.

There was no evidence of secreted osmiophilic substance except in the mucus outside the coenchymal epithelium of an aposymbiotic colony. Cells adjacent the mesoglea exhibited vesicles which had large areas of electron translucent material surrounding an osmiophilic core (Plates 18 and 23).

6.2.4 Discussion

The gastrodermal cells of autozooids of *Sarcophyton* spp. had digestive cells and mucus cells, resembling those of the sea anemone *Zoanthus sociathus* (Trench, 1974; Kevin, Hall, McLaughlin & Zahl, 1969). The pharynx had cilia which were attached to the gastrodermal cells by cytoplasmic extensions of the epithelial cells. The gastrodermal cells of the *Sarcophyton boletiforme* autozoid tube had microvilli but not cilia.

Kawaguti and Sato (1968) presented electron micrographs of the endodermal cells of *Acropora* sp. which exhibited cilia palisaded with as many as ten microvilli. The cilia in the pharynx of the sea anemone *Calliactis parasitica* appear to be free from adhesions to gastrodermal cells (Holley, 1984). Trench (1974) shows the same structures on the mesenterial epithelium of the sea anemone *Zoanthus sociathus*, and refers to them as flagellae. This description is fitting as the basal centriole is clearly visible in the accompanying electron micrographs. The cilia found on *Sarcophyton boletiforme* gastrodermal cells did not have centrioles. Trench did not report microvilli palisades around the flagellae, although microvilli were generally present on the gastrodermal epithelium.

Feeder nematocysts, in the form of spirocysts, ^{occurred} in the gastrodermis of the mid portion of the *Sarcophyton* spp. polyp tube, in agreement with the description of the alcyonacean *Heteroxenia elisabethae* (Kawaguti, 1964). Kawaguti and Sato (1968) describe nematocysts only in the epidermis of *Acropora* spp. whereas in *Sarcophyton* spp. they seemed to occur only in the gastrodermis. The nematocysts of *Sarcophyton* spp. do not resemble the holotrich

nematocysts of the sea anemone *Zoanthus sociathus* (Trench, 1974).

The epithelial cells of the gastrodermis of *Sarcophyton* spp. were loosely attached by semi-desmosomes. Digestive vacuoles were associated with various vesicles resembling the glycogen vesicles of the hydromedusan, *Sertularia pumila* (Taatzjes & Rivera, 1983). The presence of golgi bodies associated with these vesicles is also in agreement with the glycogen nature of these structures. The epithelial cells of the *Sarcophyton* spp. gastrodermis were hypertrophied as described by Trench (1974), and appear likewise to form a "digestive-excretory zone".

Structures at the gastrodermal-mesogleal interface agree with the structures which were referred to as nerve cells in *Acropora* spp. by Kawaguti and Sato (1968), and in sea anemones by Holley (1983). However, these same structures also match the description of the muscles found at the epidermal-mesogleal interface of the sea anemones *Stomphia coccinea* and *Aiptasia diaphana* (Amerongen & Peteya, 1980).

Scleroblasts and small spicules were embedded in the polyp mesoglea of *Sarcophyton* spp. The mesoglea of the polyps of *Sarcophyton* spp. was thicker than that seen in electron micrographs of *Acropora formosa* published by Kawaguti and Sato (1968). This difference may be due to the method of tissue collection; the polyps of *Sarcophyton* spp. were allowed to contract after being severed from the parent colony, while those of *Acropora* spp. were narcotised before experimentation and thus did not contract. Kawaguti and Sato also describe cells penetrating the mesoglea to the extent that, on occasion they reached the other side. No such cells were observed in the polyps of *Sarcophyton* spp.

The zooxanthellae closely resembled those found in the alcyonacean *Heteroxenia elisabethae* (Singh & Mercer, 1976), in the sea anemone *Zoanthus sociathus* (Trench, 1974; Kevin, Hall, McLaughlin & Zahl, 1969), and of those isolated from Cassiopeia spp. and grown in culture (Schoenberg & Trench, 1980b). The

zooxanthellae of *Sarcophyton* spp. were intercellular in both the gastrodermis and in the epidermis, and were most concentrated near the mesoglea. They often occurred in double lines suggesting intracolony replication. There was no suggestion of the sacs of zooxanthellae seen in the polyp walls of *Xenia* sp. by Rudman (1981b). Kawaguti and Sato (1968) found zooxanthellae only in the gastrodermis of *Acropora* spp. in contrast to the findings presented in this dissertation.

Trench (1974) published electron micrographs of the zooxanthellae of the sea anemone *Zoanthus sociatus* in which featured structures resembling the membranes 'whorls' of the zooxanthellae of *Sarcophyton boletiforme*. The difference is that those of *Zoanthus sociatus* were inside the zooxanthellae whereas those of *Sarcophyton boletiforme* were outside the zooxanthellae. Trench refers to these membrane structures as myelin figures, representing the *in situ* degradation of the zooxanthellae.

Overall, the description of the ultrastructure of *Sarcophyton boletiforme* and of *Sarcophyton infundibuliforme* are in agreement with the ultrastructural analysis of an alcyonacean coral, *Heteroxenia elisabethae* (Kawaguti, 1974), with the exception of the epidermal description. The difference is in the depth of the cellular layer and in the presence of osmiophilic vesicles (see Section 5. .3). The ultrastructure of the polyp and coenchymal epidermis of *Sarcophyton boletiforme* indicates that this species of alcyonacean coral does not differ in its ultrastructure from that of other cnidarians. The algal symbiont also did not differ from those inhabiting other cnidarians.

Osmiophilic Vesicles

The epidermis of colonies containing sarcophytoxide show cells full of osmiophilic vesicles. The vesicles range in size and are packed together in certain cells. This cell type occurs anywhere from adjacent the mesoglea to the outer epidermal surface, and

* The difference in amount of electron translucent material could also be due to lower concentrations of terpenes.

sometimes adjacent endosymbiotic zooxanthellae. The cells form a confluent layer on the outer epithelium. The presence of cells in the lower layers of the epidermis suggest exfoliation of the confluent layer with the cells containing the osmiophilic vesicles being continually replaced.

Osmium fixes and stains terpenes (Heinrich, 1970; Carde & Bernard-Dagan, 1975; Gleizes, Carde, Pauly & Bernard-Dagan, 1980a). Thus the osmiophilic vesicles found only in *Sarcophyton* spp. containing terpenes, most probably contain sarcophytoxide. The composition of the vesicles in the cells deeper in the epidermis may differ from that of the epithelial layer, as the cells adjacent^{to} the mesoglea exhibited a large amount of electron translucent material surrounding each osmiophilic globule. The deeper cells may contain precursors of the terpenes presumed to be present in the outer layers. *

The more heterogeneous vesicles were interpreted as precursor vesicles evolving from the nuclear membrane. The membrane surrounding the osmiophilic vesicles is probably the site of terpene synthesis. The hydrophobic nature of these terpenes would dictate a microsomal rather than cytosolic synthesis. Radiolabel incorporation studies (Croteau, Burbott & Loomis, 1971) and ultrastructural analyses (Henderson, Hart, How & Judge, 1970; Benayoun & Ikan, 1980; Heinrich, Schultze & Wegner, 1980; Joel & Fahn, 1980; Gleizes, Carde, Pauly & Bernard-Dagan, 1980) indicate the site of synthesis is cytosolic for monoterpenes and microsomal for sesquiterpenes (Figure 7 and 8). The corals presented in this section synthesized the diterpene sarcophytoxide.

The osmiophilic vesicles described here differ from the diffuse intracellular patches in electron micrographs of plants which synthesize both mono- and sesqui-terpenes; for example, pine needles (Bernard-Dagan, Carde & Gleizes, 1979; Benayoun & Ikan, 1980), *Poncirus trifoliata* (Heinrich, Schultze & Wegner, 1980; Heinrich, Schultze, Pfab & Bottger, 1983), and mangos (Joel & Fahn, 1980). The monoterpene hydrocarbons of *Citrofortunella mitis*

exocarp appear to be synthesized in leukoplasts containing osmiophilic material (Gleizes, Pauly, Carde, Marpeau & Bernard-Dagan, 1983). However, examination of the electron micrographs published by the latter workers shows that the vesicles do not contain the densely staining material found in *Sarcophyton* spp. polyps. The electron micrographs presented in this chapter are the first microscopical evidence of microsomal synthesis of diterpenes.

In plants, the contents of the terpene-containing cells are discharged into ducts, and then the synthesizing cells are entirely regenerated. There was no evidence of discharge from the osmiophilic vesicles in *Sarcophyton boletiforme* nor in *Sarcophyton stolidotum*, suggesting that the contents of the vesicles have a role in defence rather than in aggression.

Watson & Mariscal (1983) describe gland cells filled with electron-dense vesicles distributed at the epidermal surface of the sea anemone *Haliplanella* sp. Examination of the micrographs show these to be the threads of the harpoons of the feeder nematocysts, identical with those reported by Kawaguti (1964) for the gorgonian *Euplexaura erecta* Kükenthal, and seen in the electron micrographs examined in this dissertation. Kawaguti and Sato (1968) describe osmiophilic vesicles in the epidermis of *Acropora nasuta* which they believed contained the pigment responsible for the purple colour of this coral. The vesicles indicated in the electron micrographs accompanying the report by Kawaguti and Sato, are fewer and larger than the ones reported in this dissertation. Although no evidence of discharge of these 'pigment' vesicles was seen by Kawaguti and Sato, the cells containing the vesicles seemed to rise from the mesoglea. The planulae of the gorgonian *Euplexaura erecta* Kükenthal also contained osmiophilic vesicles in the ectodermis (Kawaguti, 1964). Again the author believed the vesicles to contain pigment.

Trench (1974) also described and published electron micrographs exhibiting cells containing osmiophilic vesicles. These vesicles occurred in the gastrodermis of the sea anemone *Zoanthus sociatus*

and the author referred to these as zymogen granules. These granules were homogeneous unlike the heterogeneous material contained in the vesicles in the epithelial cells of the epidermis of the polyps of *Sarcophyton boletiforme* and of *Sarcophyton stolidotum*. Obviously many biochemicals bind osmium strongly.

Tetrabrominated pyrroles in the marine sponge *Aplysina fistularis* are located in spherulous cells surrounding the excurrent canals. In an ultrastructural study, energy dispersive X-ray microanalysis located the bromine exclusively in osmiophilic vesicles (Thompson, Barrow & Faulkner, 1983). The contents of the vesicles of *Aplysina fistularis* are osmiophilic granules embedded in an electron translucent material. A few of the vesicles reported in this dissertation reminiscent of the sponge organelles but in general the contents are entirely osmiophilic, albeit inhomogeneously, suggestive of an oily mixture. Unfortunately, energy dispersive X-ray microanalysis cannot be used to locate the alcyoniidæan terpenes studied in this dissertation, as these terpenes do not have halogen substituents.

The greater surface area of the branched *Simularia flexibilis* would dictate a greater number of osmiophilic cells. This is supported by an order of magnitude higher concentration of flexibilide in *Simularia flexibilis* than the concentration of sarcophytoxide in *Sarcophyton* spp. (Chapter 4).

The presence of terpenes in the polyp suggests that the terpenes are protecting the exposed polyps from infection and attack. It is possible that the terpenes may be involved in prey capture. Crassin acetate in the gorgonian *Pseudoplexaura porosa* is restricted to the epidermis (Rice, Papastephanou & Anderson, 1970), and crassin acetate caused the feelers of copepod larvae to disappear (Hadfield & Ciereszko, 1978). Terpenes in the coral polyp might cause zooplankton to settle on the coral where they can be captured. However, the greatest number of putative terpenoid cells were in the outer layers of the polypal epithelium indicating an antifeedant, antifouling role.

6.3 SUMMARY

Differential centrifugation of a homogenate of *Simularia flexibilis* showed flexibilide was concentrated in the animal cytosol. The epidermis of the autozooids of both symbiotic and aposymbiotic *Sarcophyton boletiforme* and of symbiotic *Sarcophyton stolidotum* colonies that contained sarcophytoxide, exhibited a cells full of osmiophilic vesicles. These cells form a confluent layer along the outer epithelial edge, as well as being scattered throughout the epidermal tissues. No secreted osmiophilic substance was seen except in the mucus outside the coenchymal epithelium of an aposymbiotic colony. These vesicles were not observed in colonies in which sarcophytoxide nor any other terpene was detected. This is the first microscopical evidence of a ^{possible} microsomal synthesis of diterpenes.

6.4 FUTURE EXPERIMENTS

In vitro terpene biosynthesis using preparations of the osmiophilic vesicles. These should allow determination of the order of synthesis of the various terpenes found within a colony, and the genetic rather than the phenotypic rate of synthesis determined.

The nature of the osmiophilic vesicles described in sarcophytoxide containing *Sarcophyton boletiforme* should be determined by using antibodies to sarcophytoxide. There may be more active synthesis of terpenes in the vesicles in the deeper layers of the epidermis, as these cells may replace the outer cells as the tissue exfoliates. Autoradiography is not the method of choice as sterol of alcyoniidæan corals incorporates ³H from mevalonate to a greater extent than that of sarcophytoxide (personal observation, 1983). Presumably this sterol is located in membranes scattered throughout the cell.

Differential centrifugation of homogenates of alcyoniidæan corals should be refined to locate the terpenes within the animal

cytosol. Differential centrifugation should yield the osmiophilic vesicles intact. This has been achieved with liposomes of plant seeds using the simple principal that lipids are less dense than water, therefore lipid-filled vesicles centrifuge upwards in an aqueous milieu, forming a layer at the air-water interface (Jacks, Yatsu & Altschul, 1967; Allen, Good, Mollenhauer & Totten, 1971; Mollenhauer & Totten, 1971; Yatsu, Jacks, & Hensarling, 1971). These vesicles could be used in *in vitro* studies on the biosynthesis of terpenes in octocorals and in electron microscopical studies.

Comparison of the location of sesqui-terpenes in alcyoniidae corals may reveal a cytosolic site of synthesis in keeping with plant studies.

CHAPTER 7

STUDIES ON THE INGESTION OF SARCOPHYTOXIDE
BY THE PROSOBRANCH MOLLUSC *Ovula ovum*

7.1 INTRODUCTION

The ovulid egg cowrie (Plates 11 and 12) is known to ingest terpenes from alcyoniidaean corals and to retain them in the digestive gland without modification (Bowden, Coll, Hicks, Kazlauskas & Mitchell, 1978a). In 1981, two egg cowries were discovered feeding on a *Sarcophyton* sp. which contained sarcophytoxide. The digestive gland and feces of both these cowries contained large amounts of deoxysarcophytoxide (17) (Plates 24 and 25), yet no deoxysarcophytoxide was apparent in the coral. Since the postulated conversion from sarcophytoxide to deoxysarcophytoxide is neither spontaneous nor chemically trivial, Coll, Tapiolas, Bowden, Webb and Marsh (1983) concluded that the digestive gland of *Ovula ovum* has the capacity to modify sarcophytoxide by removing the epoxide group. This chapter re-examines that conclusion by examining this conversion in other *Ovula ovum*, and by examining for the presence of deoxysarcophytoxide in *Sarcophyton* spp.

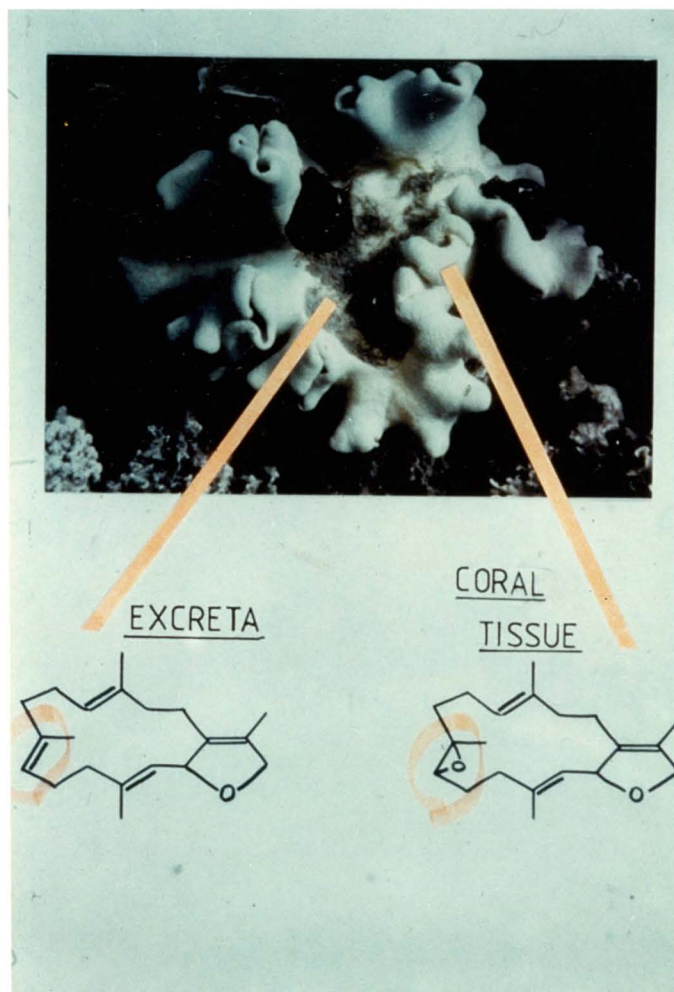
7.2 EXPERIMENTAL

7.2.1 Re-examination Of The Original Tissues

The original colony of *Sarcophyton* sp. (Coll, Tapiolas, Bowden, Webb & Marsh, 1983), and the digestive gland of one of the cowries found feeding on the coral were stored at 4°C (Tapiolas, personal communication, 1983). Samples from each tissue were extracted exhaustively with dichloromethane and analysed by both TLC and by HPLC.

PLATE 24

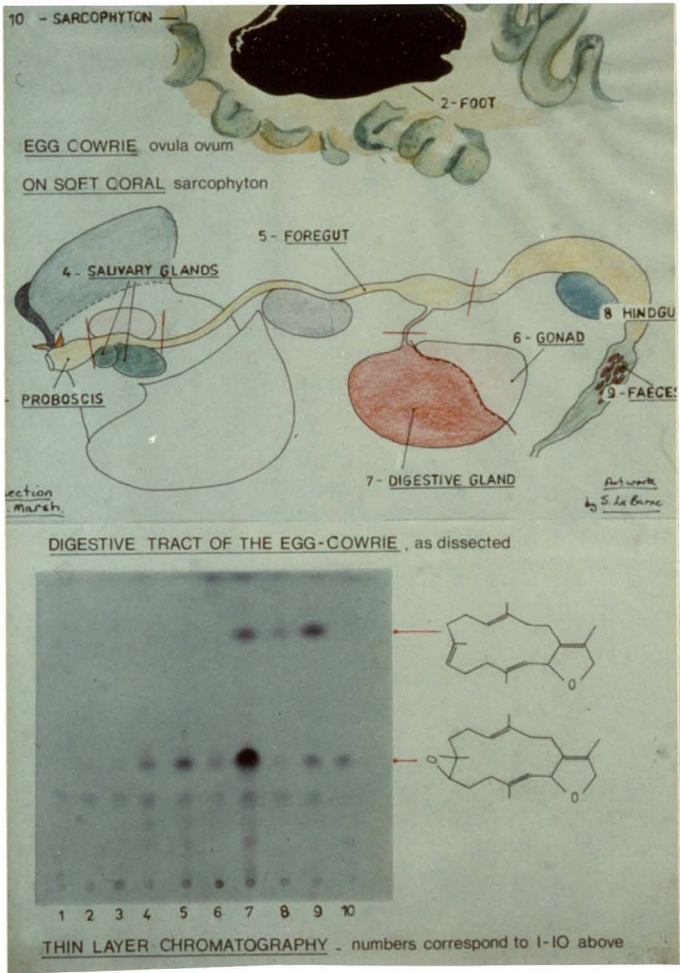
THREE *Ovula ovum* FEEDING *in situ* ON A COLONY OF
Sarcophyton stolidotum AT ECLIPSE ISLAND, IN NOVEMBER 1980.



Photograph courtesy J.C.Coll.

PLATE 25

THE ORIGINAL TLC PLATE OF EXTRACTS OF THE VARIOUS REGIONS OF
AN *Ovula ovum* SHOWING THE RELATIVE CONCENTRATIONS OF
DEOXYSARCOPHYTOXIDE AND SARCOPHYTOXIDE.



Photograph courtesy S.la Barre.

7.2.2 The Presence Of Deoxysarcophytoxide In A Colony Of *Sarcophyton boletiforme*

A colony of *Sarcophyton boletiforme* was collected from Magnetic Island and maintained in the outdoor aquarium for nine months. The coral was diced. The juice that ran freely from the coral was frozen separately from the solids. Both the tissue and the juice were freeze-dried and extracted exhaustively with dichloromethane. The terpenes were separated and purified by rapid column chromatography over silica gel as described in Section 2.2.1. The terpenes were identified by comparison of ¹Hnmr spectra with those of reference compounds.

7.2.3 In Vivo Conversion Of Sarcophytoxide To Deoxysarcophytoxide

Fifteen egg cowries were collected on an opportunistic basis over several years from various reefs on the Great Barrier Reef (Table 14). Two cowries were frozen immediately and the rest were transferred to aquaria. The corals on which they were feeding at the time of capture, were also collected. Both the indoor and the outdoor aquaria were used to house these molluscs successfully for several months.

Some of the cowries were forcibly starved as ingestion and catabolism often alternate in molluscs (Merdsoy & Farley, 1973; Mathers, Smith & Collins, 1978). Some of the cowries had previously eaten other soft coral genera, and some cowries had fed exclusively on *Sarcophyton* spp. which contained sarcophytoxide. One cowrie was allowed to feed for a week *in situ* in the ocean on a colony of *Sarcophyton stolidotum* which contained sarcophytoxide. Immediately before this experiment, the cowrie had fed on a colony of *Sarcophyton* sp. which also contained sarcophytoxide. After the *in situ* feeding experiment, the cowrie was sacrificed.

Eight of the cowries were sacrificed by crushing the shell in a vice. The digestive glands were dissected out and a portion

TABLE 14

DATA ON *Ovula ovum*

COWRIE	DATE COLLECTED	REEF COLLECTED	PREVIOUS DIET (GENUS)	AQUARIUM DIET (GENUS)	TIME IN AQUARIUM
1	Feb 83	Brittomart	<i>Cladiella</i>	<i>Sarcophyton</i>	2 weeks
2	July 82	Rib	<i>Simularia</i>	Refused to eat	2 weeks
3	July 82	Rib	<i>Simularia</i>	Refused to eat	4 weeks
4	July 82	Rib	Unknown	<i>Sarcophyton</i>	5 weeks
5	Aug 81	Eclipse	<i>Sarcophyton</i>	Frozen	-
6	Aug 81	Lizard	<i>Sarcophyton</i>	Denied food	2 weeks
7	Aug 81	Lizard	<i>Sarcophyton</i>	Denied food	6 weeks
8	Aug 83	Rib	<i>Sarcophyton</i>	<i>Sarcophyton</i>	4 weeks
9	Aug 83	Rib	<i>Sarcophyton</i>	<i>Sarcophyton</i>	5 weeks
10	Nov 81	John Brewer	<i>Nephthea</i>	<i>Sarcophyton</i>	2 weeks
11	Nov 81	John Brewer	<i>Nephthea</i>	<i>Sarcophyton</i>	5 weeks

freeze-dried, extracted exhaustively in dichloromethane, and analysed by TLC against standards. HPLC analysis was conducted on extracts from various sections of the digestive tract of one of these cowries.

7.2.4 *In Vitro* Conversion Of Sarcophytoxide To Deoxysarcophytoxide

Crude homogenates of the digestive glands (6 to 8 g) of seven of the cowries were enriched with exogenous sarcophytoxide (0.08 mM to 1.7 mM final concentration) which was added either as a solid or dissolved in ethanol or dimethylsulphoxide. The incubation mixtures were incubated in either phosphate buffer, seawater or Tris buffer (Table 15). The incubations were conducted at 20°C, 25°C or 37°C for periods lasting from 10 minutes to 46 hours. The incubations were stopped by the addition of diethyl ether, dichloromethane, or by freezing. On one occasion, homogenates of the oesophagus, stomach, hindgut, intestine, rectum, gland of Lieblen, and the salivary glands were incubated simultaneously with the homogenate of the digestive gland (Cowrie 9). The homogenate of the digestive gland of Cowrie 5 was incubated simultaneously with an homogenate of a rat liver (male Sprague Dawley) which was spiked with the same concentration of sarcophytoxide.

The incubation mixtures were extracted exhaustively by either diethyl ether or dichloromethane and these extracts dried; or the entire incubation mixture was freeze-dried and extracted exhaustively in dichloromethane (Cowrie 3 and 4). The organic extracts were examined by TLC.

7.3 RESULTS

7.3.1 Re-examination Of The Original Tissues

The colony of *Sarcophyton* on which the cowries were found in February 1981 was identified as *Sarcophyton stolidotum* as per

TABLE 15

DETAILS OF *IN VITRO* INCUBATIONS OF EXOGENOUS SARCOPHYTOXIDE WITH
VARIOUS SECTIONS OF THE DIGESTIVE TRACTS OF SEVERAL *Ovula ovum*

COWRIE *	MEDIUM	INCUBATION TEMPERATURE	INCUBATION TIME	SARCOPHYTOXIDE CONCENTRATION	SARCOPHYTOXIDE SOLVENT	REACTION STOPPED
1	Phosphate 0.05M, pH7.4	20°C	1, 10, 22h	0.0827mM	1.0 μ M ethanol	Frozen
3	Tris 0.01M, pH7.4	20°C	1, 10, 22h	0.6612mM	0.1 μ M ethanol	Frozen
4	Tris 0.01M, pH7.4	20°C	2:25, 10, 46h	0.0796mM	0.2 μ M ethanol	Frozen
5	Tris 0.01M, pH7.4	37°C	0.5, 3h	1.6531mM	0.1 μ M DMSO	Ether
6	Tris 0.01M, pH7.4	37°C	3.5h	0.8266mM	12 μ M DMSO	Ether
7	Tris 0.01M, pH7.4	37°C	5h	0.1653mM	0.2 μ M ethanol	Ether
9	Seawater	25°C	3:25h	0.6612mM	0.1 μ M ethanol	Ether

DMSO=Dimethylsulphoxide

The above conditions were used to incubate homogenates of the egg cowrie digestive glands.

* Numbers refer to the order the cowries were presented in Table 14.

Verseveldt (1982). Both TLC and HPLC analysis of the sample of the colony showed substantial amounts of deoxysarcophytoxide, approximately fifty percent of the quantity of sarcophytoxide. As this sample had been freeze-dried for two years before being assayed in this study, it is possible that the deoxysarcophytoxide represents a degradation product. There was substantial degradation in general of the compounds present in the organic extract. However, the concentration of the deoxysarcophytoxide present in the coral was of the same order of magnitude as that present in the cowrie digestive gland.

7.3.2 The Presence Of Deoxysarcophytoxide In A Colony Of *Sarcophyton boletiforme*

Examination of the dichloromethane extract of the colony of *Sarcophyton boletiforme* revealed the presence of deoxysarcophytoxide in both the juice and the solid tissue of the coral. Sarcophine and sarcophytoxide were also present.

7.3.3 *In Vivo* Conversion Of Sarcophytoxide To Deoxysarcophytoxide

The digestive glands of all cowries except those of the second and third cowries in Table 14, contained substantial amounts of sarcophytoxide, indicating that sarcophytoxide is retained in the digestive gland for at least six weeks (Cowrie 7). The digestive glands did not contain levels of deoxysarcophytoxide noticeable by TLC of the crude extracts. The digestive tract (Cowrie 1) examined by HPLC showed trace amounts of deoxysarcophytoxide throughout the digestive tract (Table 16). None of the feeding regimes induced removal of the epoxide from sarcophytoxide, even though the digestive glands were laden with sarcophytoxide.

Comparing the extracts of the various sections of the digestive tract of the cowrie 1, the rectum had the highest concentration of the terpenes sarcophytoxide, sarcophine and deoxysarcophytoxide. This indicates that these terpenes are not degraded by passage

TABLE 16

TERPENE CONCENTRATIONS IN THE DIGESTIVE TRACT OF AN *Ovula ovum*

SECTION OF DIGESTIVE TRACT	SARCOPHYTOXIDE	SARCOPHINE	DEOXY-SARCOPHYTOXIDE
	(pmoles/mg dry tissue)		
Oesophagus	28.9	0.13	1.9
Foregut	208.3	0.95	4.6
Digestive Gland	95.9	0.32	9.7
Intestines	3.3	0.03	0.3
Rectum	337.2	2.78	24.0
Fecal Pellets	23.1	0.73	2.2
<i>Sarcophyton</i> <i>stolidotum</i>	1015.0	19.66	35.9

through the digestive tract of *Ovula ovum*. The concentration of deoxysarcophytoxide relative to the concentration of sarcophytoxide is similar down the digestive tract. The ratio of the concentration of deoxysarcophytoxide to that of sarcophytoxide is highest in the fecal pellets, as is the ratio of the concentrations of sarcophine to sarcophytoxide (Table 17). Sarcophytoxide is 30 times more concentrated than deoxysarcophytoxide in the coral, and a similar ratio occurs in the digestive tract. There was no formation of deoxysarcophytoxide apparent in the digestive tract.

7.3.4 *In Vitro* Conversion Of Sarcophytoxide To Deoxysarcophytoxide

A variety of incubation media, incubation times, incubation temperatures, and doses of sarcophytoxide were used in *in vitro* incubations of homogenates of the digestive glands of several cowries (Table 15) and of a rat. All regimes were unsuccessful in converting sarcophytoxide to deoxysarcophytoxide.

7.4 DISCUSSION

Deoxysarcophytoxide was isolated from a colony of *Sarcophyton boletiforme*, and was detected in the colony originally determined to be free of deoxysarcophytoxide (Coll, Tapiolas, Bowden, Webb & Marsh, 1983). There is a precedent for trace amounts of a terpene to be found in a coral which at first seemed devoid of that terpene. After detailed analysis Weinheimer and Matson (1975) found trace amounts of the diterpene crassin acetate (?) in the gorgonian *Pseudoplexaura crucis* although the authors stated "no crassin acetate is detectable by routine TLC examination of the (dichloromethane) extract". In contrast, the same publication reported that crassin acetate was present in large quantities (1-2 percent) in three other species of *Pseudoplexaura*.

Deoxysarcophytoxide was found in the original coral. A major difference in the techniques used by Coll et al (1983) was in the

TABLE 17

THE RATIO ($\times 1000$) OF TERPENE CONCENTRATIONS IN THE DIGESTIVE TRACT
OF AN *Ovula ovum*

SECTION OF DIGESTIVE TRACT	SARCOPHYTOXIDE	SARCOPHINE	DEOXY-SARCOPHYTOXIDE
Oesophagus	1000	4.3	61
Foregut	1000	4.7	21
Digestive Gland	1000	3.6	97
Intestines	1000	5.0	80
Rectum	1000	8.6	68
Fecal Pellets	1000	33.1	91
<i>Sarcophyton</i> <i>stolidotum</i>	1000	20.3	34

duration of extraction of the tissues. The results reported in the paper were based on extraction times of 1 minute with dichloromethane of 1 g of material (Tapiolas, personal communication, 1985). In this dissertation, the tissues were extracted for 24 hours and three changes of dichloromethane were used, then pooled.

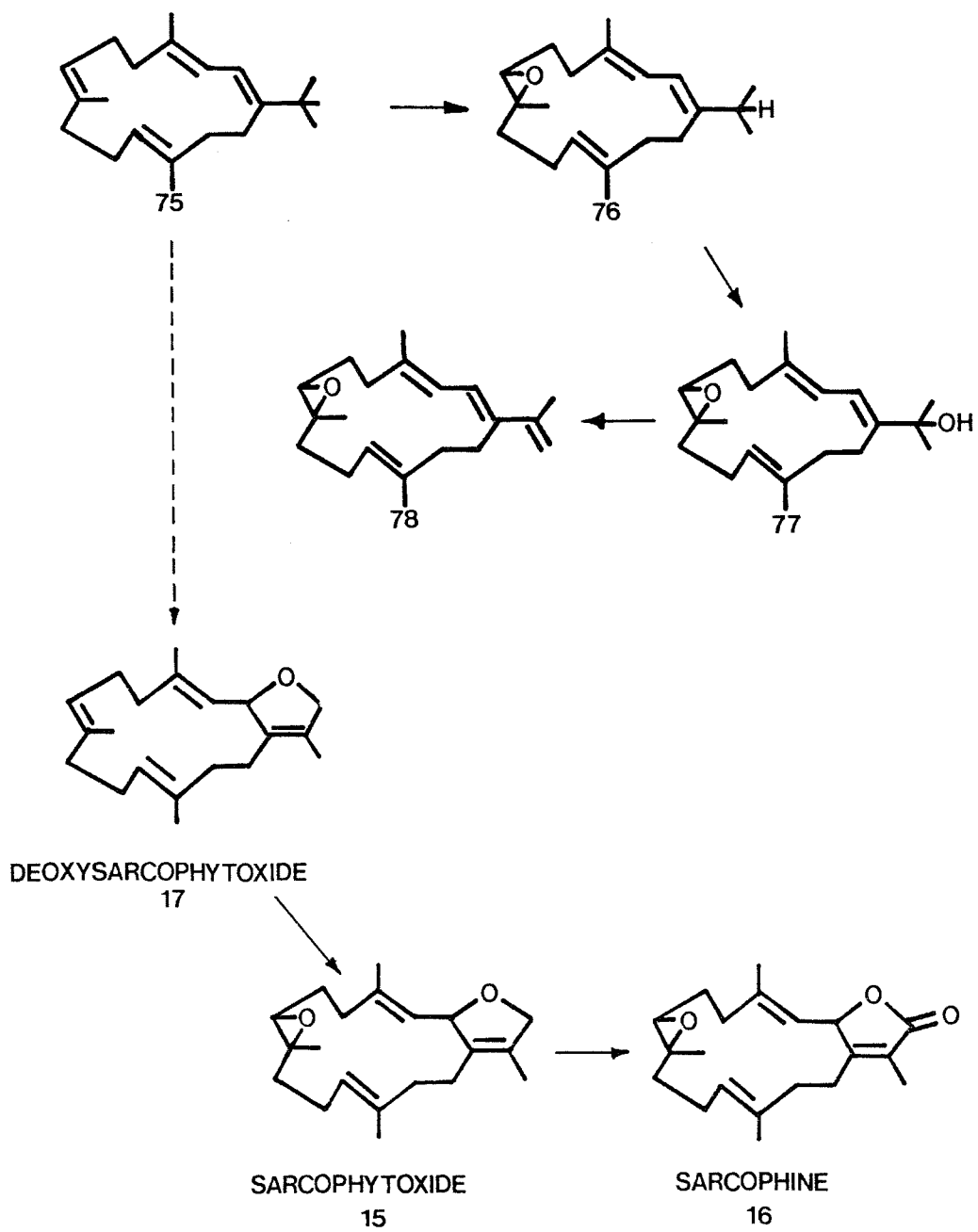
Deoxysarcophytoxide may be a precursor of sarcophytoxide, with the formation of the dihydrofuran ring preceding the formation of the epoxide (Figure 52) as sarcophine was found as a co-metabolite of deoxysarcophytoxide. This differs from the pathway proposed by Bowden, Coll and Mitchell (1980a) (Figure 48). Sarcophytoxide and sarcophine often co-occur while the other four terpenes in Figure 48 are found in only one species at one location. On only one occasion were the two sets of compounds isolated from the same specimen (Coll & Bowden, personal communications, 1981). The two colony types live in close proximity to each other (Bowden, personal communication, 1981; personal observation, 1983). It is possible that some colonies may contain genes from both parents through cross fertilisation of gametes as *Sarcophyton* spp. release gametes rather than planulae (Chapter 4). It is unlikely that the terpenes depicted in Figure 48 are biosynthetically linked in the manner as suggested by Bowden, Coll and Mitchell (1980a). It is also possible that each terpene is synthesized independently from a common precursor, as suggested in 4.3.4.

Several nudibranchs modify terpenes after ingestion (Stallard & Faulkner, 1974b; Hochlowski & Faulkner, 1981; Cimino, De Rosa, De Stephano, Sodano & Villini, 1983). However, sarcophytoxide passed unaltered through the digestive tract of all the *Ovula ovum* studied in this dissertation, and appeared in the feces. The enzymes postulated to effect this detoxification were not inducible. The conversion of sarcophytoxide to deoxysarcophytoxide was not effected by *in vivo* in any season, nor in *in vitro* preparations of digestive gland homogenate incubated under a variety of conditions.

The fecal pellets and midgut may contain material that differs

FIGURE 52

SUGGESTED PATHWAY FOR THE BIOSYNTHESIS OF CEMBRANOIDS
BY *Sarcophyton* spp.



from that which the cowrie is presently eating. The digestive stages of feeding, metabolism and excretion often co-occur in marine molluscs not influenced by the tides (Mathers, Smith & Collins, 1978). This means that while ingesting new food, old food is being released into the intestines. An *Ovula ovum* had spicules in the feces originating from a coral of a different genus to the coral on which the cowrie was found (Willis, personal communication, 1981). Deoxysarcophytoxide in the midgut and fecal pellets of the two cowries examined by Coll, Tapiolas, Bowden, Webb and Marsh (1983) may have come from a coral eaten earlier which was rich in deoxysarcophytoxide. This would apply to both cowries as cowries are usually found feeding in pairs on the same colony (observation, 1986).

When the cowries used in this dissertation were offered a selection of *Sarcophyton* spp., the cowries chose the nearest colony but only spent one or two days on it before moving onto the next colony. They often returned repeatedly to a colony but only for a few days at a time (observation, 1983). This may have happened on the reef with the cowries examined by Coll, Tapiolas, Bowden, Webb and Marsh (1983).

Terpenes from the previous coral could be flushed ahead of those from the new coral and hence have a higher relative concentration in the feces. This explanation presupposes a retention time of three months which may be possible, as after six weeks of starvation, the digestive gland of one cowrie in this study contained massive amounts of sarcophytoxide (Cowrie 7, Table 14). On one occasion a large amount of sarcophine from a previous coral was observed in the digestive gland (Cowrie 10) after the cowrie had subsequently eaten several corals not containing sarcophine.

In general, molluscs are not especially resistant to toxic chemicals. In one study, all parts of the terrestrial plant *Solanum nodiflorum* were toxic to the snails *Fossaria (Lymnaea) cubensis*, *Lymnaea columella*, *Biomphalaria glabrata* and *Physa cubensis* (Medina & Ritchie, 1980). The same plant was not toxic to the snails *Marisa*

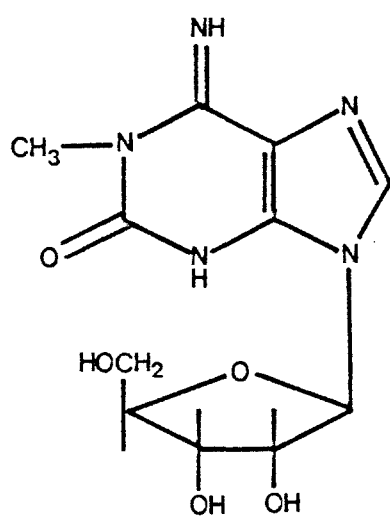
cornuarietis and *Tarebia granifera*. As discussed in Section 1.2.5, the genus *Solanum* is a rich source of terpenes (Stothers, Stoessl & Ward, 1978).

The fact that a compound is toxic to one animal does not mean that it is toxic to all animals. For example, the nudibranch *Anisodoris nobilis* harbours the nucleoside, 1-methylisoguanosine (79), obtained from the sponge *Tedania digitata* (Kim, Nachman, Pavelka, Mosher, Fuhrman & Fuhrman, 1981). This nucleoside elicits muscle relaxation, hypothermia, arterial hypotension and bradycardia in mammals (Fuhrman, Fuhrman, Kim, Pavelka & Mosher, 1980; Baird-Lambert, Marwood, Davies & Taylor, 1980), yet was not modified by or toxic to the nudibranch *Anisodoris nobilis*. Another marine mollusc, the sea hare, *Aplysia brasiliiana*, sequesters terpenes in the digestive gland. These terpenes are distasteful to fish (Dieter, Kinnel, Meinwald & Eisner, 1979), yet they cause no ill effects to the mollusc, nor are there any signs of detoxification (Kinnel, Duggan, Eisner & Meinwald, 1977).

There was selective retention of certain terpenes by the digestive gland of *Ovula ovum*. The amount of sarcophine from a previous coral that was retained in the digestive gland of one cowrie, suggests sarcophine is retained in preference to sarcophytoxide. Sarcophine is not toxic to *Acropora formosa* (Webb & Coll, 1983), but is to fish (Coll, Tapiolas, Bowden, Webb & Marsh, 1983), rats, mice and guinea pigs (Neeman, Fishelson & Kashman, 1974).

Contrary to popular belief, the Crown of Thorns Starfish, *Acanthaster planci* does devour soft corals during natural plagues (Plate 26) (Chalker, personal communication, 1984). The starfish feed on soft corals when local scleractinian coral has been destroyed. The Blue Starfish, *Linckia laevigata*, was found on *Xenia* sp. (Dunlap, personal communication, 1983), although it was not noted whether the stomach was extended. It is likely that each terpene in a soft coral has a limited toxicity to specific organisms, but the array of terpenes found in soft corals overall

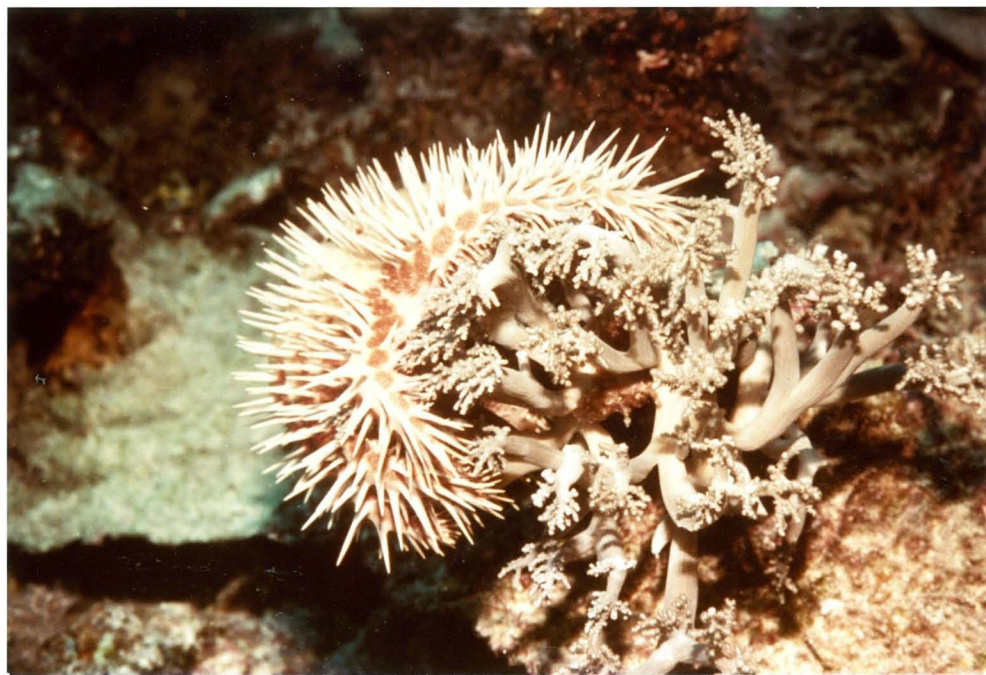
FIGURE 53



DORIDOSINE

PLATE 26

Acanthaster planci FEEDING *in situ* ON *Nephthea* SP.



Photograph courtesy B.E.Chalker.

present an effective screen against the range of organisms encountered.

Sarcophytoxide is mildly toxic to both *Acropora formosa* and *Porites andrewsii* (Webb & Coll, 1983). Epoxides can be toxic, reacting with DNA and histones (Smith & Bend, 1979; Smith, Plummer, Ball & Bend, 1980), and are implicated in carcinogenesis (Wood *et al.*, 1977). Conversion to a double bond, or to a diol removes this activity. In mammalian tissues, epoxide hydrase detoxifies epoxides by oxygenation to the trans-diol (Booth & Sims, 1974; Smith & Bend, 1979). However, under anaerobic conditions, cytochrome P₄₅₀ removes epoxides leaving a double bond (94% conversion) (Booth, Hewer, Keysell & Sims, 1975; Ivie, 1976; Kato, Iwasaki, Shiraga & Noguchi, 1976; Sugiura, Yamazoe, Kamataki & Kato, 1980).

The gut region of animals is anaerobic, harbouring obligate anaerobes such as *Bacteroides* species (Davis, Dulbecco, Eisen, Ginsberg & Wood, 1973). The conversion of sarcophytoxide to deoxysarcophytoxide may be spontaneous under anaerobic conditions. The chemical conversion conducted by Coll, Tapiolas, Bowden, Webb and Marsh (1983) was conducted under aerobic conditions. However, no such activity was seen in the later cowries which spontaneously fed on sarcophytoxide containing *Sarcophyton* spp.

In addition to sarcophytoxide, egg cowries ingest sarcophine (Personal observation, 1982) and epoxyisoneocembrane-A (68) (Bowden, Coll, Hicks, Kazlauskas & Mitchell, 1978a). Yet with neither of the two latter cembranes was the epoxide removed (Coll, personal communication, 1981; personal observation, 1982). This is important because all three epoxides on these planar molecules occur on the opposite side from the toxic exocyclic lactone of sarcophine (Figure 52) (Neeman, Fishelson & Kashman, 1974). It is not likely that the epoxide is a toxic principal when present in the sarcophytoxide molecule and is not toxic when present in the sarcophine molecule. Cowries survived several months on a diet rich in sarcophytoxide without effecting a conversion. Neither did they show any signs of distress due to toxicity. Ciereszko (1962)

observed that the ovulid *Cyphoma gibbosum* habitually feeds on gorgonians which contain toxin crassin acetate and is resistant to the toxic effects of crassin acetate as demonstrated on other marine organisms.

7.5 SUMMARY

Detoxification of sarcophytoxide to deoxysarcophytoxide as suggested by (Coll, Tapiolas, Bowden, Webb & Marsh, 1983), was not observed in the egg cowrie, *Ovula ovum*, after voluntary ingestion of *Sarcophyton* species which contained sarcophytoxide. Nor was it observed in *in vitro* incubations of various sections of the *Ovula ovum* digestive tract, including the digestive gland. Deoxysarcophytoxide was isolated from several sarcophytoxide containing *Sarcophyton* species and is concluded to be a biosynthetic precursor of sarcophytoxide.

7.6 FUTURE EXPERIMENTS

Pathway analyses of the synthesis of sarcophytoxide and of deoxysarcophytoxide in the *Sarcophyton* sp. *in vivo* should be conducted, followed by the development of *in vitro* biosynthesis of deoxysarcophytoxide, and of sarcophytoxide from various precursors. The technique should be repeated using an homogenate of *Ovula ovum* digestive gland instead of a *Sarcophyton* sp., to see if the cowrie is capable of independent modification of sarcophytoxide.

CHAPTER 8

THE EFFECT OF TERPENES FROM ALCYONIIDAEAN CORALS
ON THE RESPIRATION AND PHOTOSYNTHESIS OF SCLERACTINIAN CORALS

8.1 INTRODUCTION

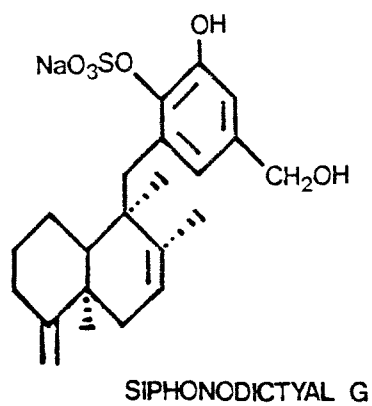
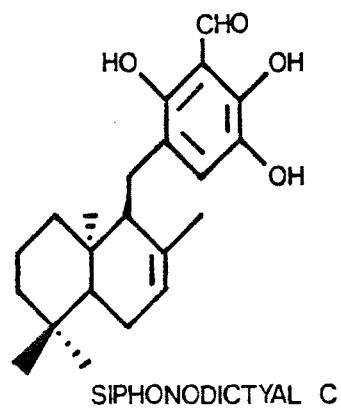
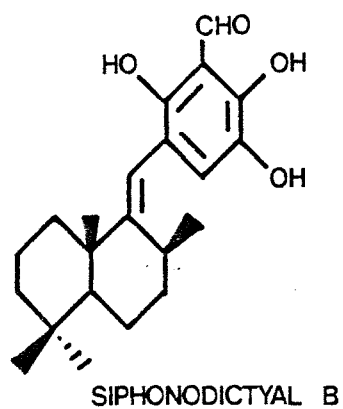
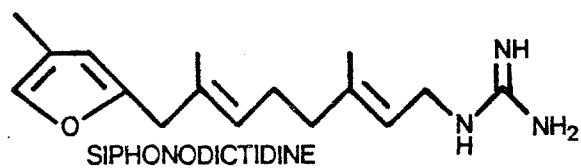
In the marine environment many organisms are known to excrete toxic terpenes to inhibit the growth of potential competitors for space or food (Bakus, 1981). An example is afforded by the *Siphonodictyon* sp sponges which burrow into the scleractinian corals *Montastrea annularis* (Jackson & Buss, 1975). A bare zone of dead coral skeleton appears around the sponge. The furanoterpene, siphonodictidine (Figure 54) is believed to be the agent responsible for this dead zone of coral (Sullivan, Faulkner & Webb, 1983).

Alcyonacean corals can have a marked physiological effect in the field on scleractinian corals, their natural rivals for space (Plates 9 and 10). Flexibilide was detected in the water column surrounding a colony of *Simularia flexibilis* which contained flexibilide (Coll, Bowden, Tapiolas & Dunlap, 1982). This compound was implicated in the growth inhibition of a colony of *Pavona cactus* which was an immediate neighbour of a colony of *Simularia flexibilis* which contained flexibilide (Sammarco, Coll, la Barre & Willis, 1983). Flexibilide features an exocyclic α -methylene- γ -lactone group implicated as the toxic principle in many growth inhibitory terpenes isolated from terrestrial plants (Kupchan, Eakin & Thomas, 1971; Rodriguez, Mitchell & Towers, 1976; Ogura, Cordell & Farnsworth, 1978; Cassady, Ojima, Chang & McLaughlin, 1979).

There is only one experimental study on the effects of alcyonacean terpenes on scleractinian corals. Flexibilide and the terpene isolated from *Lobophytum* spp., FN45, caused total lysis of living tissue from two scleractinian corals, *Pavona cactus* and *Acropora formosa*, in 12 hours at concentrations as low as 2 ppm (Coll & Sammarco, 1983).

FIGURE 54

TERPENES FROM *Siphonodictyon* sp.



A general property of uncouplers of respiratory chain phosphorylation is that they inhibit respiration if added at concentrations greater than necessary for maximal uncoupling. Toxicity is measured by the dose needed to produce maximal stimulation of oxidation. Uncoupling activity correlates well with biological activity such as toxicity to plants and bacteria (Miko & Chance, 1975). A less severe disturbance to respiration will severely alter the growth potential of that organism.

Energy flow through the transport chain in chloroplasts is similar to that in mitochondria and results in the generation of ATP, although coupling is not as tight as in the mitochondria. Nevertheless, uncoupling agents effect a substantial increase in the rate of photosynthetic oxygen formation (Gromet-Elhanan, 1968). Growth inhibitory stilbenoids (phenolic compounds) uncouple electron transport and photophosphorylation in isolated chloroplasts of *Spinacia oleracea* and inhibited CO_2 -dependent O_2 evolution and electron flow from water to methyl viologen in uncoupled chloroplasts (Gorham & Coughlan, 1980).

An alternative explanation to the growth inhibition/necrosis of scleractinian corals living adjacent Alcyoniidae, features the discovery of mesenterial filaments extended from the digits of *Simularia firma* (Personal observation, 1983), and *Simularia flexibilis* (Bowden, personal communication, 1983), as described in Section 2.1.3.3. These filaments were extended at night to a length of at least 20 cms. Alcyoniidae contain nematocysts in their mesenterial filaments (Plate 20), as do all cnidarians. It is possible that these filaments destroy the scleractinian polyps within their reach; particularly as a distinct arc is noticeable in Plate 9 A.

This chapter examines the hypothesis that terpenes abundant in alcyoniidae corals alter the respiration and photosynthesis of scleractinian corals in a manner which is specific to the terpene tested. The toxicity of the alcyoniidae terpenes is compared to the toxicity of several terpenes from *Siphonodictyon* sp. sponges

(Figure 54). These sponge terpenes were supplied by B.W.Sullivan, University of California, San Diego, USA.

8.2 EXPERIMENTAL

Acropora formosa and *Porites andrewsii* were used as the receiver species for alcyoniidæan terpenes, and *Acropora formosa* was used as the receiver species for the sponge terpenes. Both these corals are branching scleractinian colonies common on the Great Barrier Reef. Both species were used in previous assays for alcyoniidæan allelopathy (Coll & Sammarco, 1983; Sammarco, Coll, la Barre & Willis, 1983). *Acropora formosa* is used extensively in biochemical studies conducted in Townsville; for example, see Crossland, Barnes & Borowitzka, 1980; Crossland, Barnes, Cox & Devereux, 1980). *Pavona cactus*, the coral on which the allelopathic effect of a flexibilide containing *Simularia flexibilis* was observed *in situ*, does not afford itself to experimentation using this technique due to its leaflike skeleton.

8.2.1 Preparation Of Experimental Samples

Symbiotic *Acropora formosa* from Magnetic Island (2 m) aposymbiotic *Acropora formosa* from Rib Reef (15 m), and symbiotic *Porites andrewsii* (Plate 27) from Brittommart Reef (4 m), were kept in the outdoor aquarium. *Acropora formosa* was identified by Drs. M.Streamer and C.Wallace (James Cook University of North Queensland) and by Drs. B.E.Chalker and J.E.Veron (Australian Institute of Marine Science). *Porites andrewsii* was identified by Dr. J.E.N.Veron (Australian Institute of Marine Science).

Terminal lengths ("tips", 1.0 to 1.5 cm long) of these branching corals were cut with surgical bone forceps and placed in a plexiglass rack which allowed seawater to circulate freely around the tips (Plate 28). The tips were allowed 24 hours to recover from shock and cease mucus excretion before they were subjected to

PLATE 27

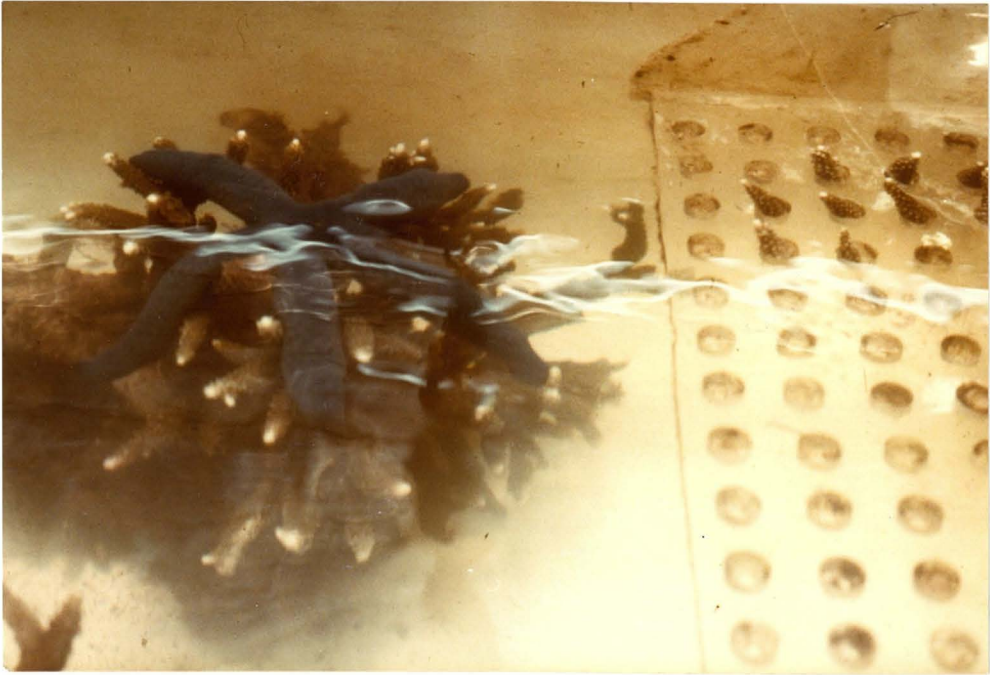
COLONY OF *Porites andrewsii*.



Photograph courtesy P.W. Sammarco.

PLATE 28

A COLONY OF *Acropora formosa* MAINTAINED IN THE OUTDOOR AQUARIUM,
AND CUT TIPS AWAITING INCUBATION.



experimentation. To ascertain the health of the coral tissue the tips were observed under a dissecting microscope before and after each experiment.

To isolate zooxanthellae from *Acropora formosa*, 10 cm lengths of coral were wrapped in aluminium foil and broken with a hammer. The resulting fragments were placed in an Erhlenmeyer flask containing membrane-filtered ($0.22\ \mu\text{m}$) seawater. The flask was shaken to allow the abrasive action of the skeletal chips to rupture the thin animal tissues leaving the tougher zooxanthellae intact and free.

The resultant suspension was filtered through cotton to remove the coarse skeletal chips and centrifuged at 500 G for one minute to remove the finer skeletal chips. The zooxanthellae were separated from the animal fraction by centrifugation at 1000 G for 5 mins. The zooxanthellae pellet was centrifugally washed five times at 750 G for 5 mins in seawater and resuspended in seawater. This suspension was used in tests of terpene toxicity on isolated zooxanthellae.

The growing tips of branching corals are usually devoid of zooxanthellae in the top few millimeters (Pearse & Muscatine, 1971). In colonies living at a depth of 15 m, this white tip extends several centimeters down the branch (Chalker, personal communication, 1982). The white terminal tips taken from deep water *Acropora formosa* were used as aposymbiotic test organisms.

8.2.2 Measurement Of Photosynthesis And Respiration

The rates of photosynthesis and respiration of zooxanthellae suspensions and of intact tips of *Acropora formosa* and *Porites andrewsii*, were measured by determining the rate of change in oxygen concentration in the seawater surrounding the organisms. The method was described in detail by Kanwisher and Wainwright (1967), Roffman (1968), Barnes and Taylor (1973), and by Crossland and Barnes

(1977).

Oxygen production and utilisation were determined with a Clark type polarographic oxygen electrode and chamber (Rank Bros., Cambridge, UK). This apparatus consisted of a cylindrical perspex chamber, 1.6 cm in diameter and 3.5 cm high with the electrode mounted at its base. A perspex plug was inserted into the top of the chamber. The distance the plug was inserted into the chamber determined the volume of the chamber. A 1 mm diameter vertical hole through the plug relieved any pressure within the chamber. A water jacket around the chamber was connected to a pumping water bath (Lauda, model 4KR) and maintained at 26°C which was the temperature of the water in the aquarium. The output from the electronics associated with the electrode was connected to a single pen recording potentiometer. The output had a range of 0 to 10 mV, consequently a high resolution recorder was required (Radiometer, REC 80 Servograph).

To calibrate the electrode, the potentiometer was adjusted to read 70 percent full span of the recorder, with seawater equilibrated with air (100 percent oxygen saturation). To saturate the water with oxygen, an Erhlenmeyer flask containing 20 mls of seawater was shaken in air for 2 to 3 mins. The electrode was assumed to have an output of 0 volts at 0 percent oxygen saturation. A stirring bar and magnetic stirrer was used to continually mix the water in the chamber throughout the experiments. The oxygen consumption by the electrode was negligible as determined in control experiments.

Coral tips were suspended by a cotton thread through the pressure relief hole in the plug, into the chamber which now contained 6 ml of filtered seawater. Alternatively, 6 ml of the zooxanthellae suspension was placed in the chamber, and the plug inserted. The chamber was alternately covered and illuminated by projector lamps at either side, at a light intensity which ensured maximal photosynthetic rate (Barnes, personal communication, 1981).

For each cycle the oxygen content of the chamber was monitored until a linear slope was attained, typically this required from 2 to 30 minutes. The slope of this linear section of the trace was used in the calculation of the rate of change in oxygen saturation. When reading the trace, a positive slope indicated net production of oxygen by the organism, and a negative slope indicated net oxygen consumption.

The oxygen consumed during the dark cycle is a measure of the uptake of oxygen from the seawater by the organisms for the purpose of respiration. This was measured in percent oxygen saturation per hour. Conversely, the oxygen production measured during the light cycle is a measure of the net amount of oxygen released from the coral. This amount is not the gross oxygen produced by the zooxanthellae during photosynthesis, but the amount of oxygen remaining after the organisms have used some of the oxygen in respiration. To calculate the gross photosynthetic rate, the respiration rate obtained during the dark cycle was added to the net rate of oxygen production, i.e.

Gross photosynthetic rate = Net rate of change in oxygen content +
the respiration rate in the dark.
(Change in % oxygen saturation per hour)

8.2.3 Addition Of Terpenes To The Incubation Medium

Photosynthesis and respiration were measured over several light/dark cycles to establish normal rates for these processes in each tip or algal suspension before the addition of terpenes. Several concentrations were prepared by dilution in ethanol (alcyoniidæan terpenes), or methanol (sponge terpenes). Each dose was administered by syringe through the pressure relief hole in the plug. After terpene addition, photosynthesis and respiration were again monitored. The rates of photosynthesis and respiration measured after the addition of the terpene, are presented as a percentage of the rates measured before the addition of the terpene.

These values are plotted against time. Each test was conducted on a fresh tip or zooxanthellae suspension.

The response of the coral tips or zooxanthellae to the terpenes was examined under two regimes:-

Progress Curves

To observe the progressive rather than the cumulative effects of the terpene, *Acropora formosa* tips were exposed to a single dose of a terpene at concentrations ranging from 10^{-10} to 10^1 M final chamber volume. The tips were monitored for several hours. This process was repeated with several concentrations of the same terpene, using fresh tips for each concentration.

Dose Response Curves

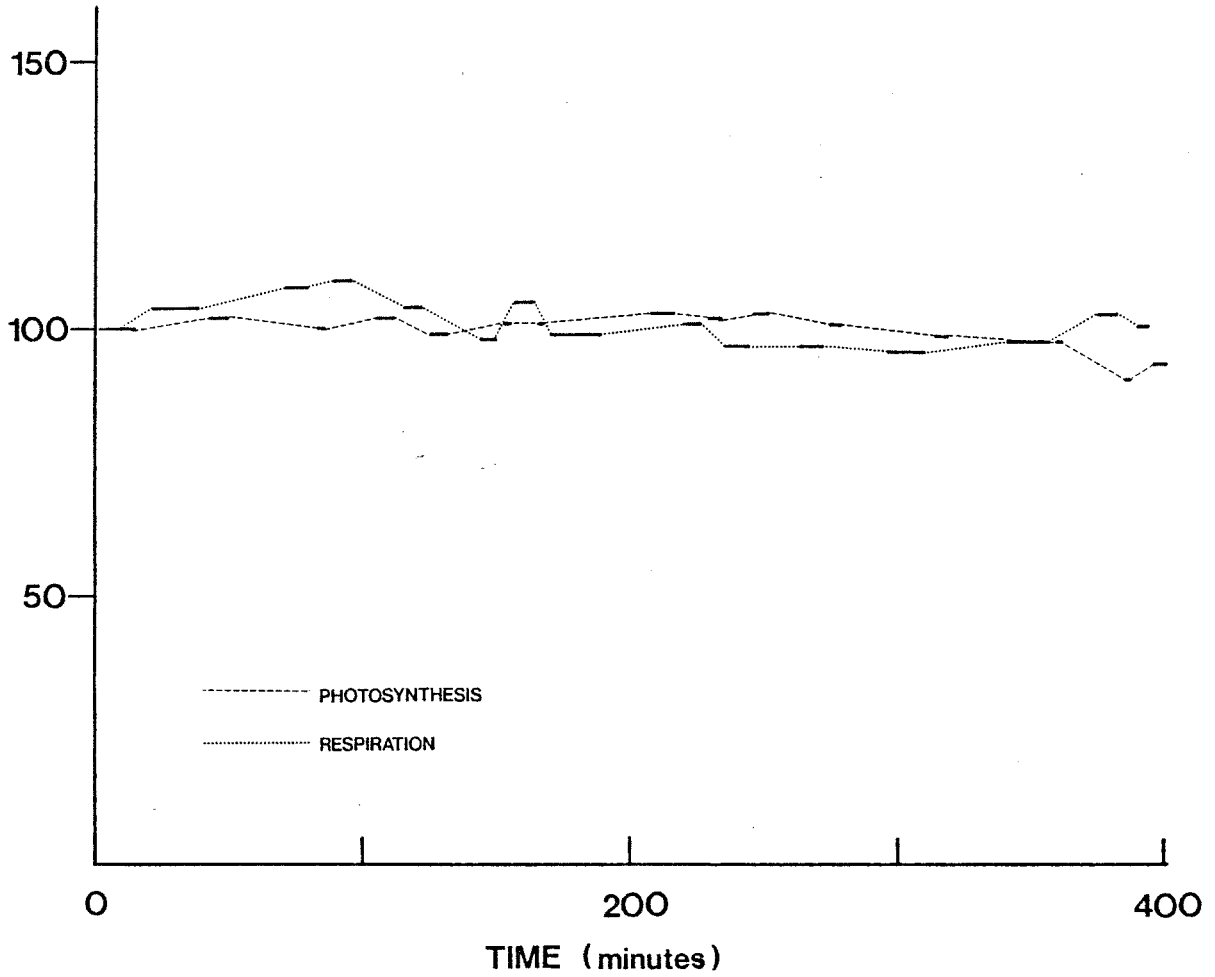
Terpenes were added in amounts which progressively increased the concentration within the chamber by an order of magnitude from 10^{-7} M to 10^{-2} M final chamber volume. The volume of ethanol added was between 0.1 μ l and 1.0 μ l. The rates of photosynthesis and of respiration in the organisms were monitored for several light/dark cycles and then a higher concentration of terpene was added.

With both single and multiple dose tests on coral tips, the tip was removed at the end of the run and the incubation medium alone monitored for respiration. This was to determine whether there was a bacterial bloom resulting from the death of the coral. On the occasions when bacterial respiration was measured, this was subtracted from the final three respiration measurements.

FIGURE 55

PROGRESS CURVE OF A CONTROL SYMBIOTIC *Acropora formosa*

% OF INITIAL RATE



8.3 RESULTS

8.3.1 Control Curves

Acropora formosa was maintained for eight hours in the chamber without significant alteration in the rates of photosynthesis and respiration. Figure 55 gives an example of *Acropora formosa* monitored for seven hours during which the coral was alternately illuminated and covered for approximately 10 minute intervals. The rates of respiration and net photosynthesis were plotted against time. There is no significant change in these rates with time (95% confidence interval, Student's t-test).

Concentrations of ethanol greater than 35 μ l per chamber cell significantly altered rates of photosynthesis and respiration in *Acropora formosa*. Concentrations used in these experiments were less than 20 μ l, and were most often 6 μ l.

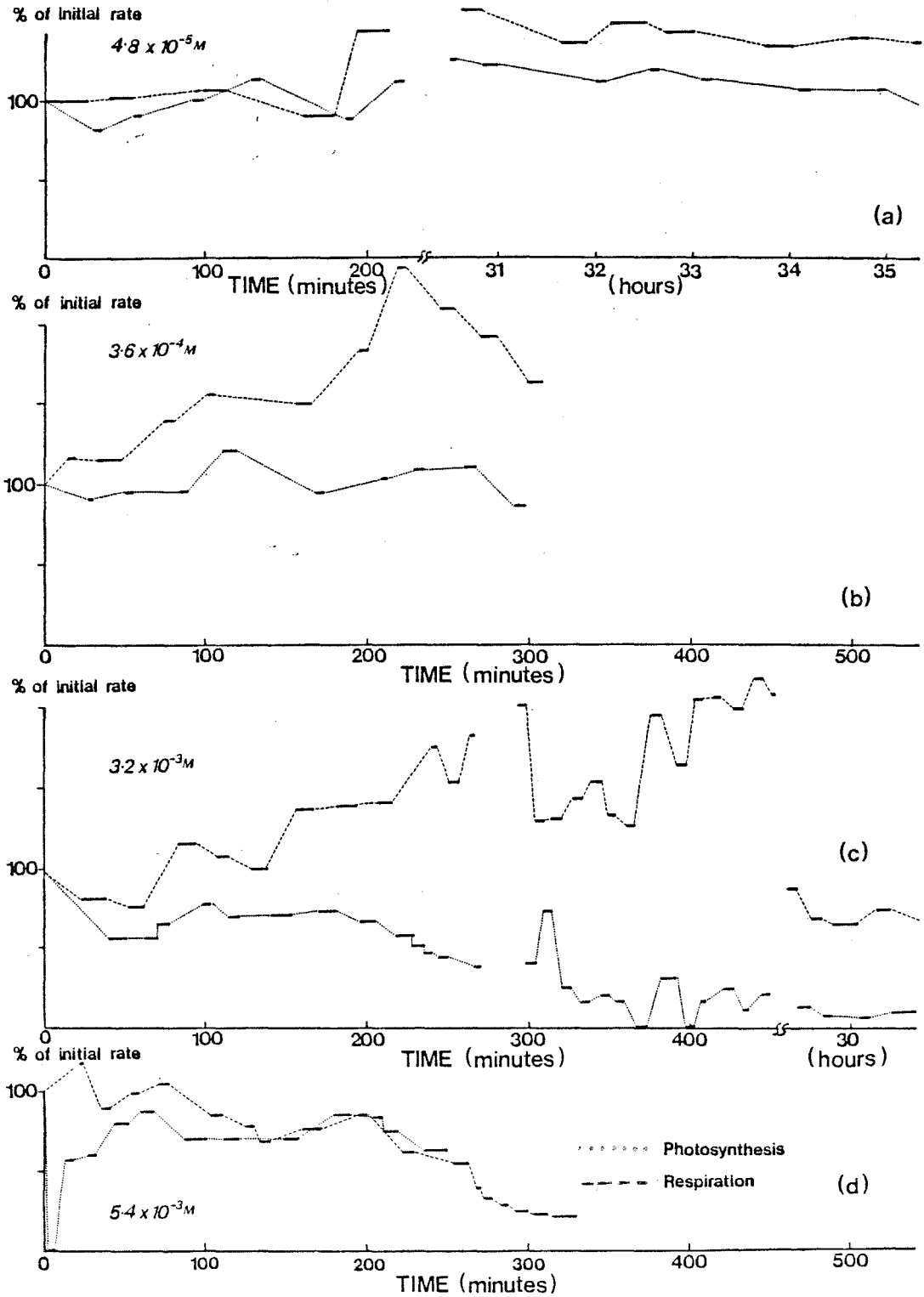
8.3.2 Progress Curves

In general, the toxic response was an initial increase in respiratory rate followed by a slow decline, concomitant with a depression in the rate of photosynthesis. With acute toxic responses, the coral was dead at the end of the run; the animal tissue had been lysed from the skeleton; and the zooxanthellae had been expelled. The incubation medium respired slightly at the end of such tests (<3 percent), indicating bacterial presence. In these cases the respiratory rate rapidly and markedly declined after the last addition of terpene, indicating death of the coral.

The tips exhibiting a mild to moderate response did not show loss of tissue and zooxanthellae. At the end of the run, the incubation medium did not respire. When these tips were returned to the aquarium, they did not die. When returned to the chamber a few days later, the photosynthetic and respiratory rates were the same as the initial rate before exposure to the terpene, suggesting the

FIGURE 56

PROGRESS CURVES OF THE EFFECT OF FN45 ON SYMBIOTIC *Acropora formosa*



tips had returned to normal.

The toxic effects of several of the terpenes was examined in more detail in a series of single dose experiments. The rates of photosynthesis and respiration in *Acropora formosa* tips monitored for several hours after one dose of terpene. The time interval during which photosynthesis or respiration was measured is marked by solid lines. Dashed (photosynthesis) or dotted (respiration) lines are used to interpolate between these intervals.

FN45

FN45 at a concentration of 4.8×10^{-5} M had no effect on the respiration of *Acropora formosa* for 180 mins (slope = 0.13, S.D. = 0.14, n = 5) (Figure 56 a). Then the respiration rate jumped to 143 percent of the initial rate. During this time there was a gradual rise in photosynthetic activity (slope = 0.10, S.D. = 0.07, n = 6). After being in the aquarium overnight, the respiration was still elevated (156 percent of initial rate) but slowly declined (slope = -0.04, S.D. = 0.02, n = 8). The rate of photosynthesis was higher the next day (125 percent of the initial rate), then it also declined (slope = -0.09, S.D. = 0.02, n = 8).

A dose of FN45 of 3.6×10^{-4} M had no effect on photosynthesis (slope = 0.02, S.D. = 0.04, n = 9) (Figure 56 b). In contrast, the respiratory rate rose (slope = 0.5, S.D = 0.2, n = 7) to a peak activity of 235 percent of the initial rate at 220 minutes, then the respiratory rate rapidly declined (slope = -0.84, S.D = 0.04, n = 4).

A dose of FN45 of 3.2×10^{-3} M caused an immediate decline of 20 percent in the respiratory rate followed by a gradual rise to 183 percent of the initial rate (slope = 0.41, S.D. = 0.05, n = 11) (Figure 56 c). The photosynthetic rate showed a decline of 40 percent immediately after terpene addition. The photosynthetic activity remained at a fairly steady level (slope = 0.0000, S.D = 0.0000, n = 13) until 200 mins, then declined steadily (slope =

-0.40, S.D. = 0.03, n = 8).

This tip was removed from the chamber and washed in clean filtered seawater, then returned to the chamber in clean filtered seawater which did not contain any terpene. The respiratory rate was higher for one reading, then plunged 70 percent. The respiratory rate then climbed steadily, with the last reading giving a rate of 220 percent of the initial rate. Similarly, the photosynthetic rate continued the trend apparent before the change in water. The tip was returned to the aquarium overnight then monitored the next day. The rate of respiration had returned to 90 percent of initial value. In contrast, the photosynthetic activity was still low, (10 percent of the initial value), and remained low.

A dose of 5.4×10^{-3} M FN45 caused an initial decline of 40 percent in the respiratory rate of *Acropora formosa* (Figure 56 d). The respiratory rate then remained fairly steady (slope = 0.0002, S.D. = 0.0001, n = 10) although there were two rises, at 60 mins, and at 180 mins. There was an initial rise in photosynthetic activity of 20 percent followed by a steady decline (slope = -0.21, S.D. = 0.04, n = 11).

Flexibilide

Flexibilide at a concentration of 3.3×10^{-6} M had little effect on the rate of photosynthesis (slope = 0.01, S.D. = 0.01, n = 11) and of respiration in *Acropora formosa* (slope = -0.06, S.D. = 0.01, n = 9) (Figure 57 a); photosynthesis (slope = 0.00, S.D. = 0.00, n = 12) and respiration (slope = -0.07, S.D. = 0.00, n = 13) (Figure 57 b). A concentration of 3.3×10^{-5} M also had little effect on the rate of photosynthesis (slope = 0.10, S.D. = 0.01, n = 19) and of respiration in *Acropora formosa* (slope = -0.11, S.D. = 0.16, n = 21), apart from a jump in the respiration rate at 70 minutes (Figure 57 c).

A dose of flexibilide of 8.3×10^{-5} M also induced a jump in

FIGURE 57

PROGRESS CURVE OF THE EFFECT OF FLEXIBILIDE ON SYMBIOTIC
Acropora formosa

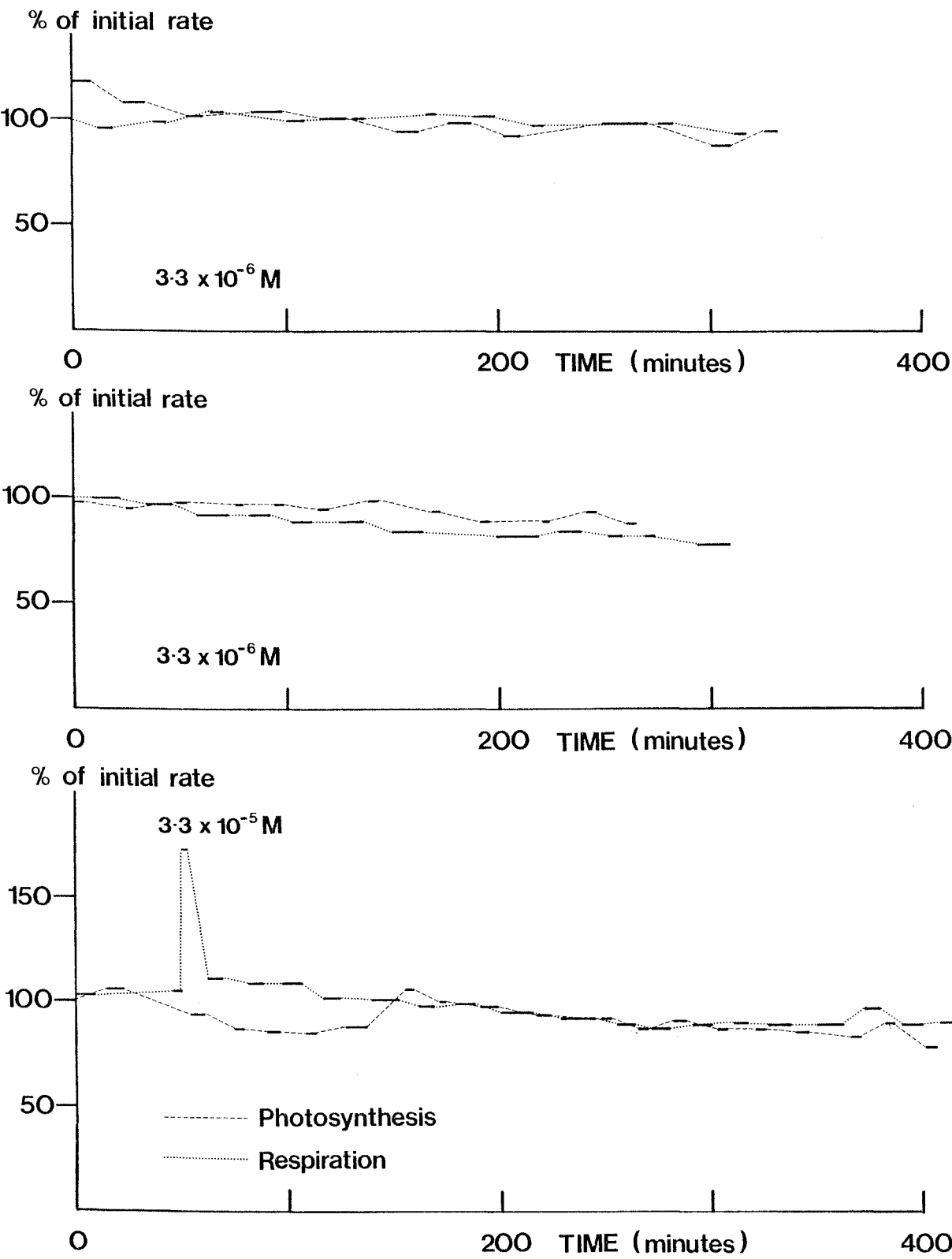
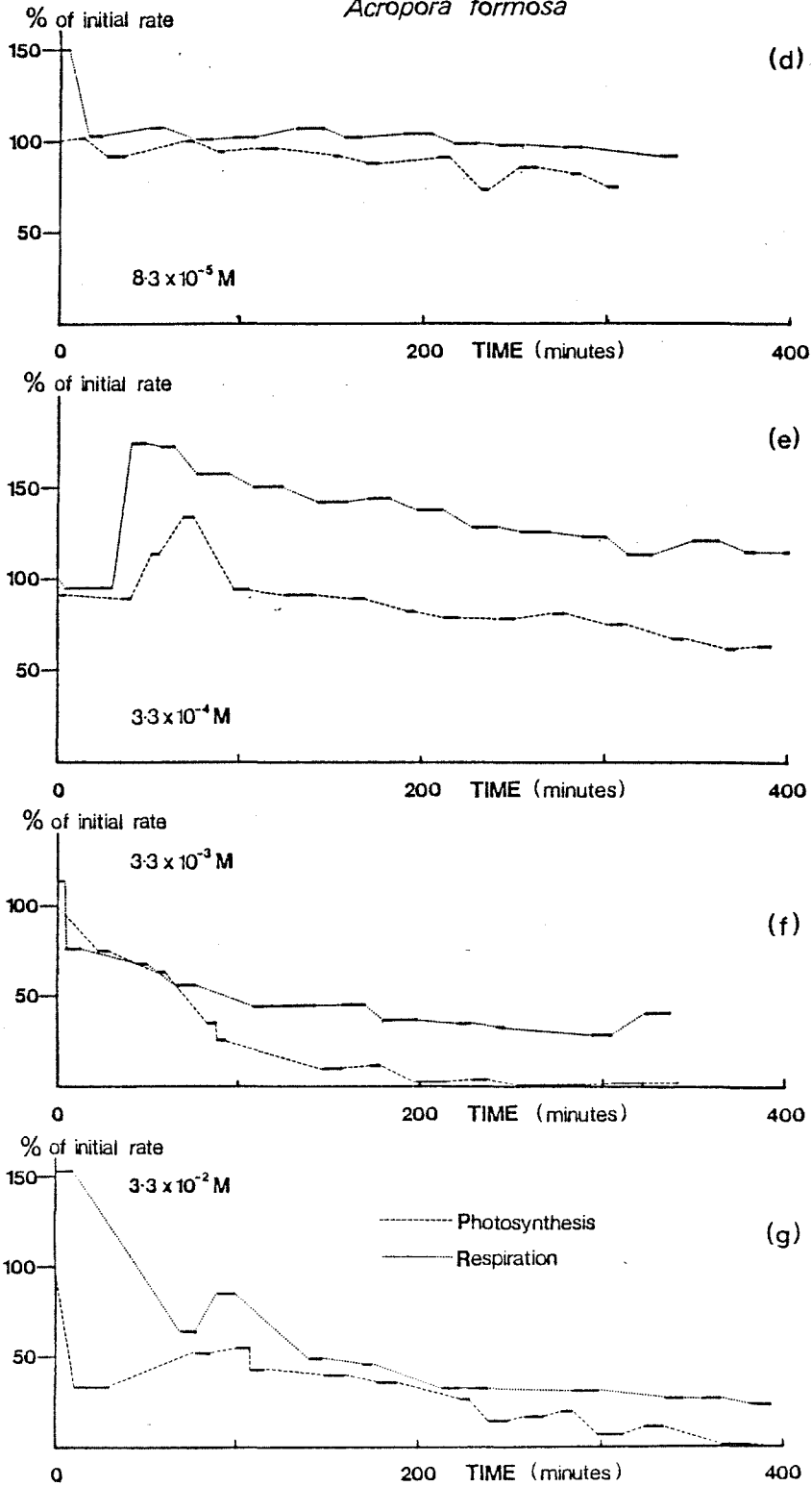


FIGURE 57 (Continued)

PROGRESS CURVES OF THE EFFECT OF FLEXIBILIDE ON SYMBIOTIC

Acropora formosa



respiratory rate. Both respiration and photosynthesis then declined linearly (respiration slope = -0.10 , S.D. = 0.02 , $n = 12$; photosynthesis slope = -0.07 , S.D. = 0.01 , $n = 12$) (Figure 57 d). A dose of flexibilide of 3.3×10^{-4} M likewise induced a jump in respiratory rate. This response was simultaneous with a temporary rise in photosynthetic rate. Both activities then declined linearly (respiration slope = -0.16 , S.D. = 0.02 ; photosynthesis slope = -0.13 , S.D. = 0.01) (Figure 57 e).

A concentration of 3.3×10^{-3} M flexibilide caused the respiratory rate of *Acropora formosa* to jump in the first reading, then both respiratory and photosynthetic rates declined. Photosynthesis declined at a faster rate (slope = -0.21 , S.D. = 0.06 , $n = 7$) compared to respiration (slope = -0.06 , S.D. = 0.13 , $n = 11$). By 200 mins the respiratory rate was steady at 50 percent of the initial rate. The photosynthetic activity was minimal (Figure 57 f).

A concentration of 3.3×10^{-2} M flexibilide also caused the respiratory rate of *Acropora formosa* to jump in the first reading, but the photosynthetic rate dropped temporarily. Both respiratory and photosynthetic rates then declined (photosynthesis slope = -0.27 , S.D. = 0.02 , $n = 9$) compared to respiration (slope = -0.09 , S.D. = 0.01 , $n = 9$) (Figure 57 g).

Furanoquinol

Furanoquinol at a concentration of 10^{-4} M did not disturb the photosynthesis (slope = -0.00 , S.D. = 0.00 , $n = 21$) nor the respiration (slope = -0.00 , S.D. = 0.01 , $n = 19$) of *Acropora formosa* (Figure 58 a). A higher concentration of furanoquinol (2.94×10^{-3} M) effected an immediate jump in the respiratory rate. The rate subsided to a rate comparable with that measured before terpene addition. The rate later declined further (slope = -0.14 , S.D. = 0.05 , $n = 13$). This gradual decline was echoed by a drop in the rate of photosynthesis (slope = -0.05 , S.D. = 0.02 , $n = 13$) (Figure 58 b).

FIGURE 58

PROGRESS CURVES OF THE EFFECT OF FURANOQUINOL ON SYMBIOTIC *Acropora formosa*

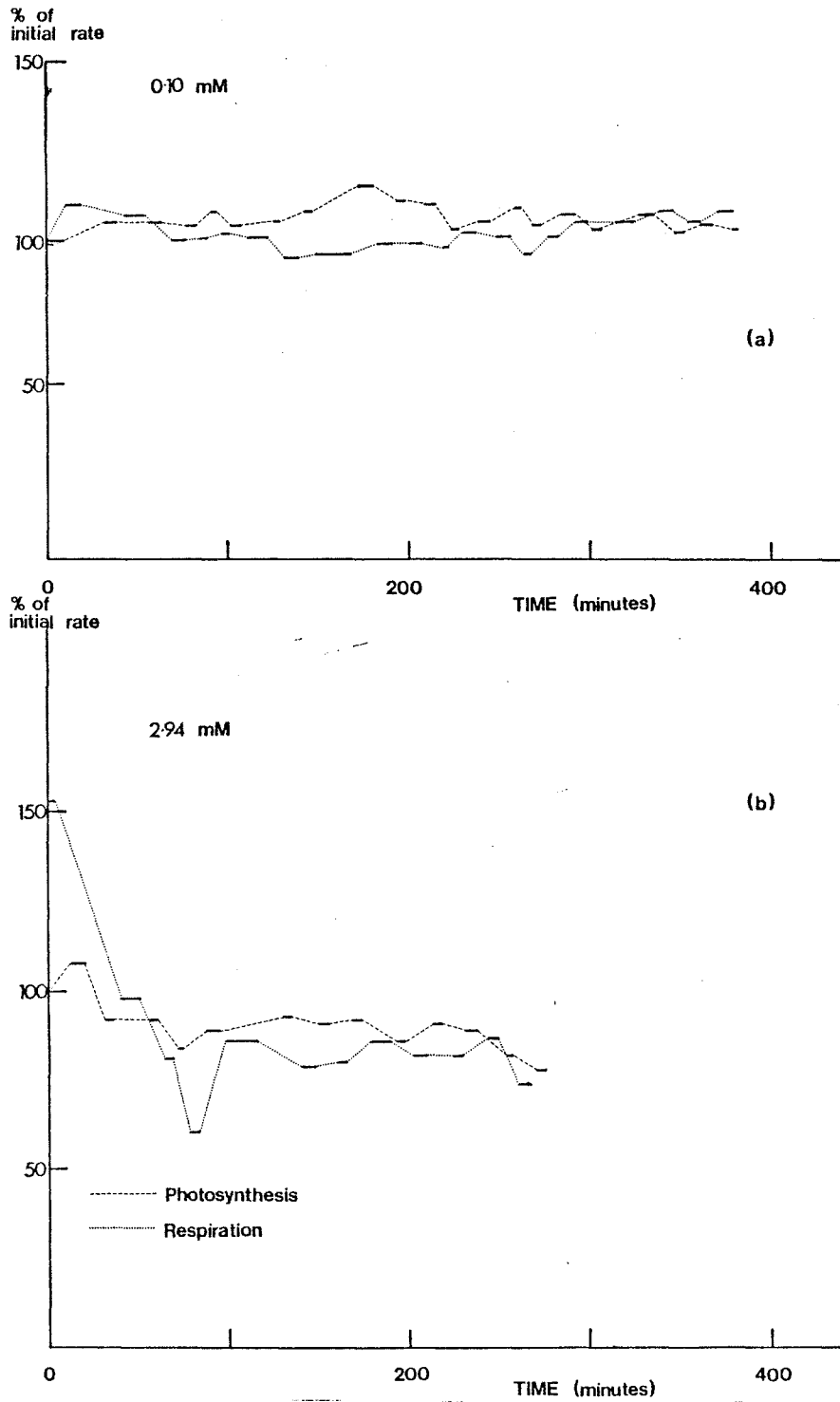
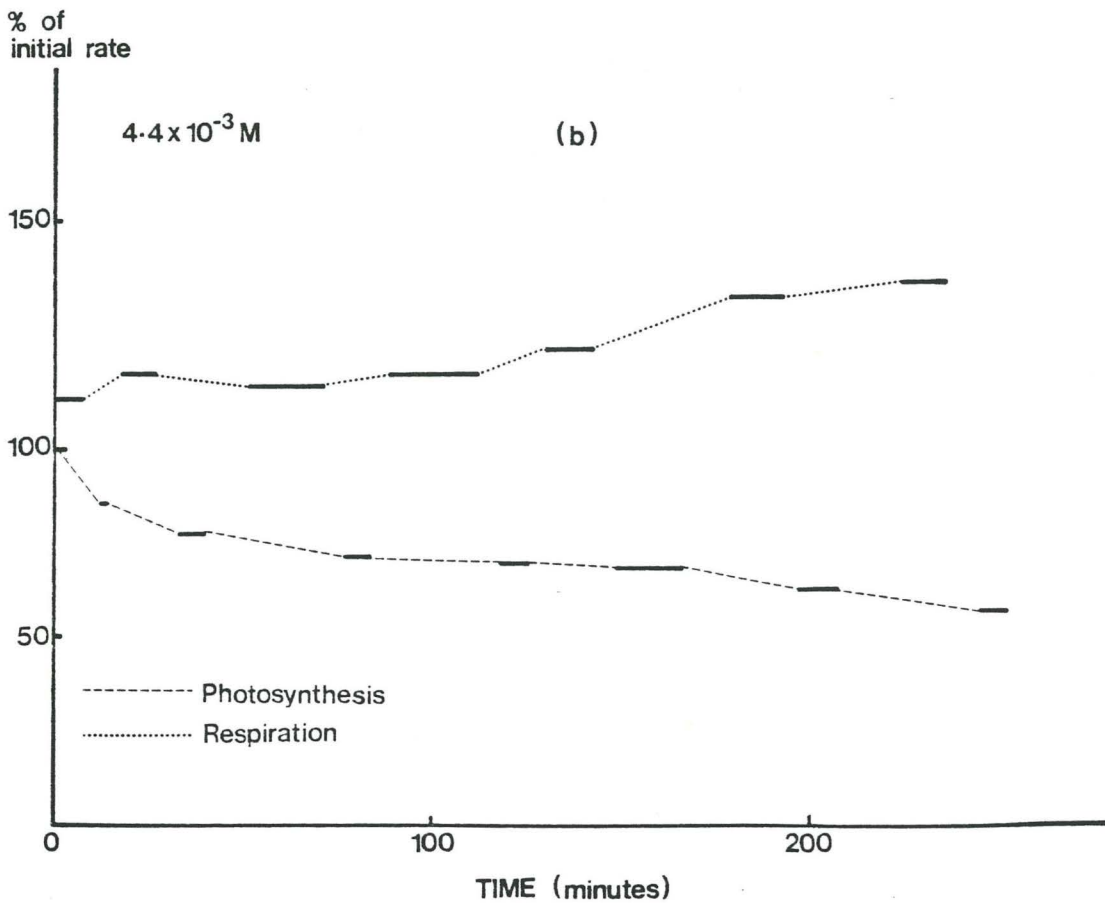
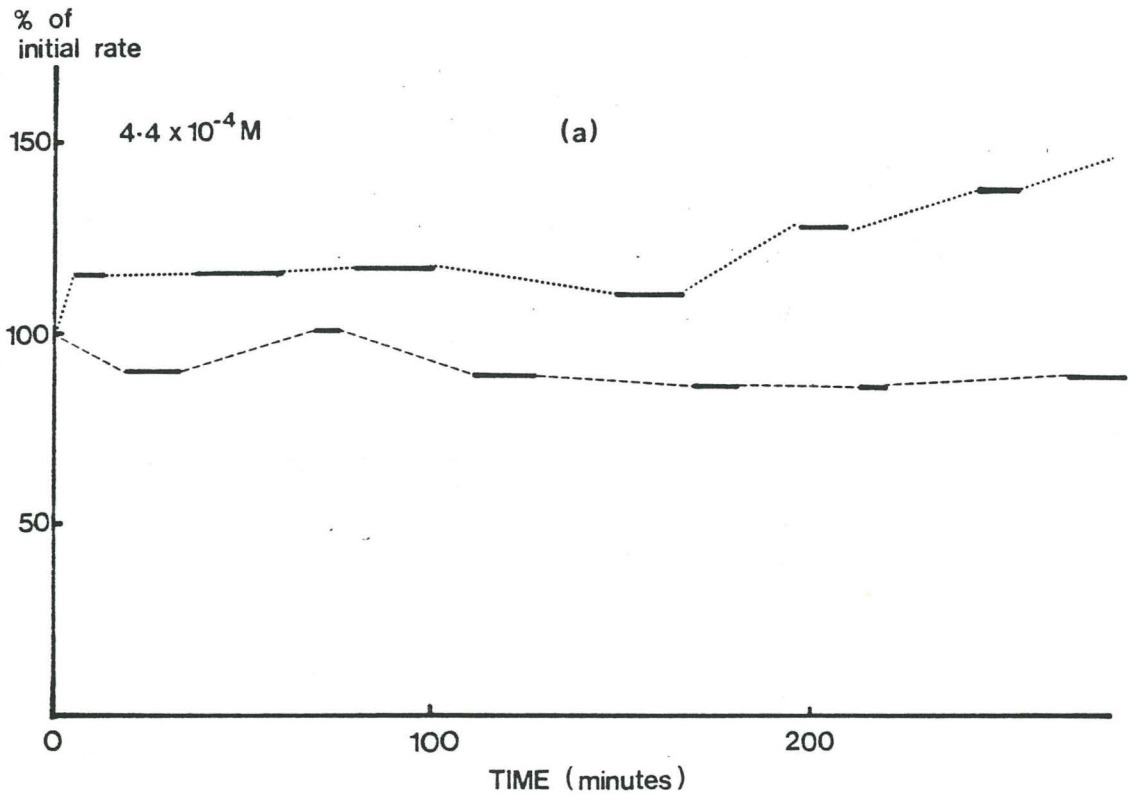


FIGURE 59

PROGRESS CURVE OF THE EFFECT OF SARCOPHYTOXIDE ON SYMBIOTIC
Acropora formosa



Sarcophytoxide

A concentration of sarcophytoxide of 4.4×10^{-4} M evoked a steady increase in the rate of respiration (slope = 0.11, S.D. = 0.03, n = 7), and a decline in the rate of photosynthesis (slope = -0.05, S.D. = 0.04, n = 6) (Figure 59 a). A concentration of 4.4×10^{-3} M sarcophytoxide also caused a steady increase in the rate of respiration (slope = 0.14, S.D. = 0.02, n = 7), and a decline in photosynthesis (slope = -0.10, S.D. = 0.00, n = 7) (Figure 59 b).

Sponge Terpenes

Siphonodictidine at a concentration of 3.6×10^{-10} M did not disturb the rates of photosynthesis (slope = -0.03, S.D. = 0.04, n = 6) or respiration (slope = -0.06, S.D. = 0.03, n = 5) over the 150 minutes the coral was monitored (Figure 60 a). A concentration of 3.6×10^{-6} M disturbed the respiratory rate of *Acropora formosa* by raising and maintaining the rate at 150 percent of the respiratory rate measured before terpene addition (slope = 0.00, S.D. = 0.04, n = 14). The photosynthetic rate also showed an increase to 115 percent of its previous rate, then levelled off before dropping steadily at 200 minutes (slope = -0.11, S.D. = 0.02, n = 12) (Figure 60 b).

A concentration of siphonodictyal B of 2.8×10^{-4} M immediately effected a decline in both photosynthesis (slope = -0.22, S.D. = 0.02, n = 4) and respiration (slope = -0.34, S.D. = 0.07, n = 4) which at 120 minutes levelled off to 60 percent of the initial rates (photosynthesis slope = 0.04, S.D. = 0.04, n = 4; respiration slope = 0.03, S.D. = 0.11, n = 4) (Figure 61).

A concentration of siphonodictyal C 2.8×10^{-4} M immediately caused a jump in the rate of respiration in *Acropora formosa* (Figure 62). Then the rate of respiration dropped to below the initial level (slope = -0.59, S.D. = 0.22, n = 5). After 150 minutes the rate of respiration rose (slope = 0.12, S.D. = 0.05, n = 4). The photosynthetic rate dropped after terpene addition (slope = -0.34,

FIGURE 60

PROGRESS CURVES OF THE EFFECT OF SIPHONODICTIDINE ON SYMBIOTIC

Acropora formosa

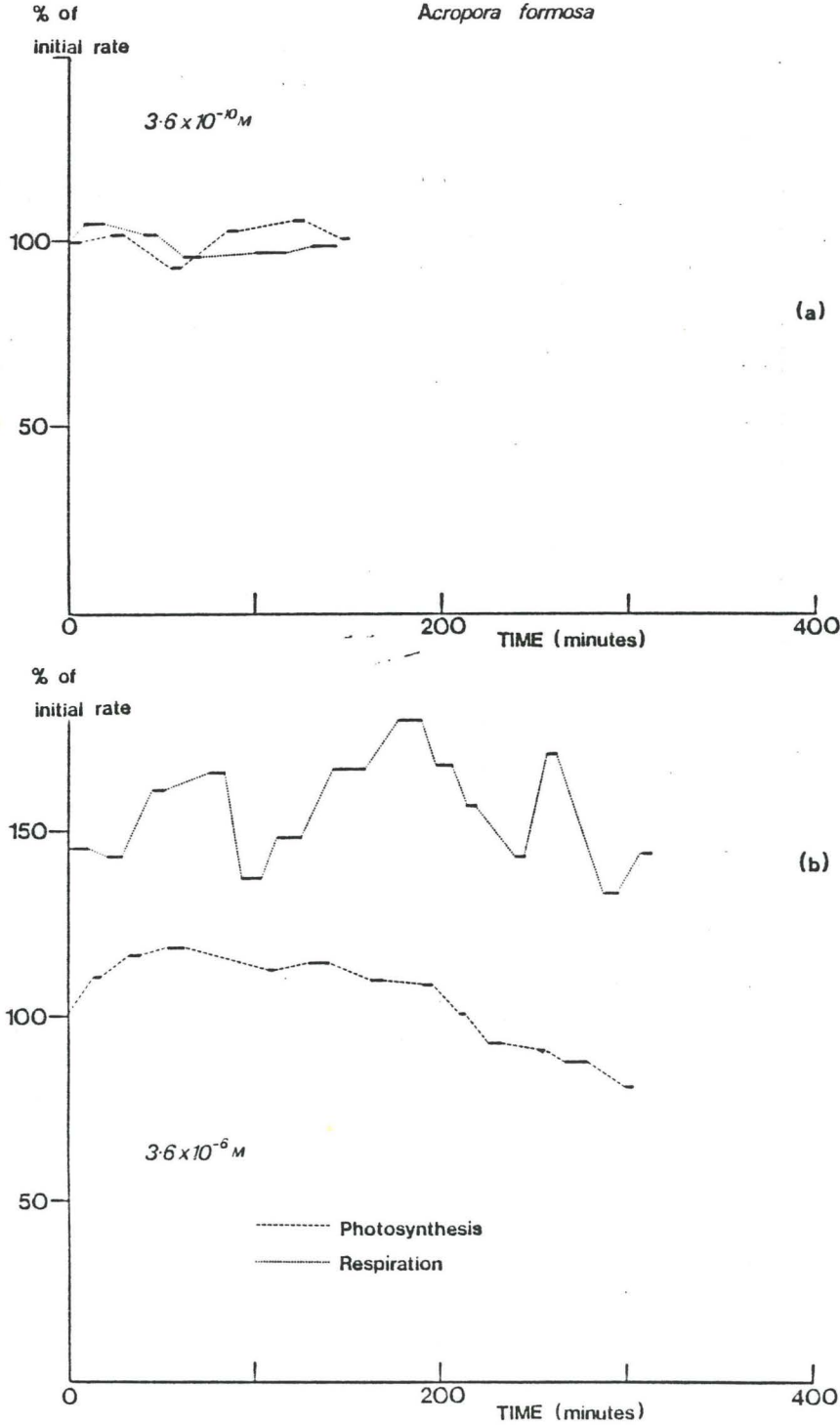


FIGURE 61

PROGRESS CURVE OF THE EFFECT OF SIPHONODICTYAL B
ON SYMBIOTIC *Acropora formosa*

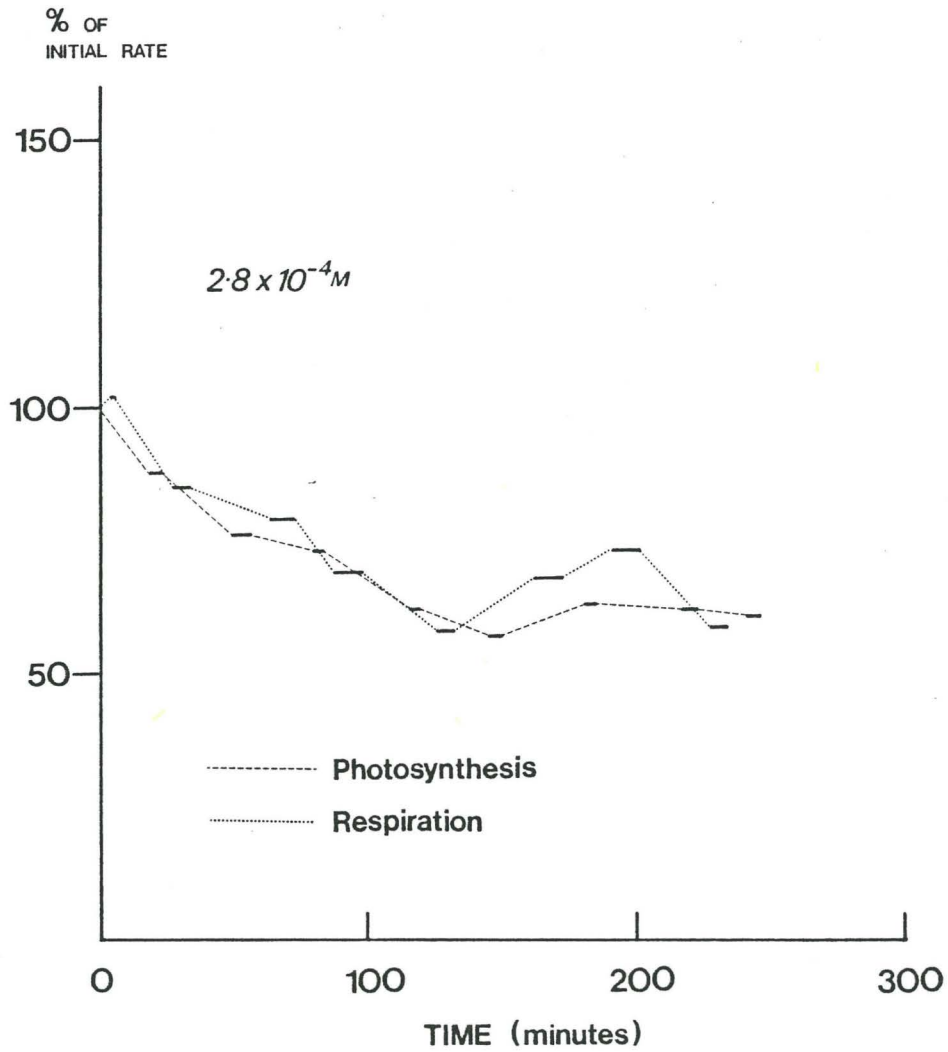


FIGURE 62

PROGRESS CURVE OF THE EFFECT OF SIPHONODICTYAL C
ON SYMBIOTIC *Acropora formosa*

% OF INITIAL RATE

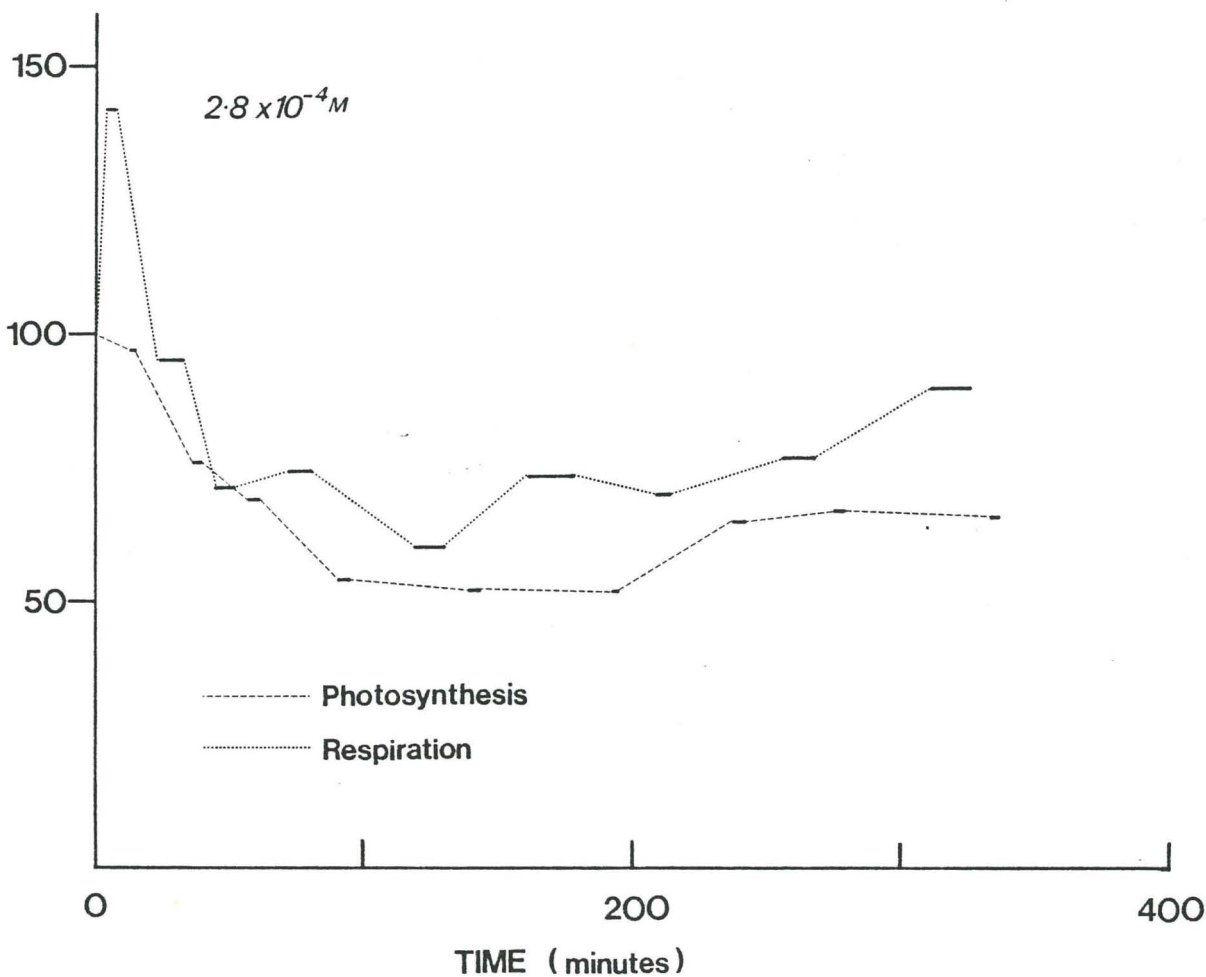
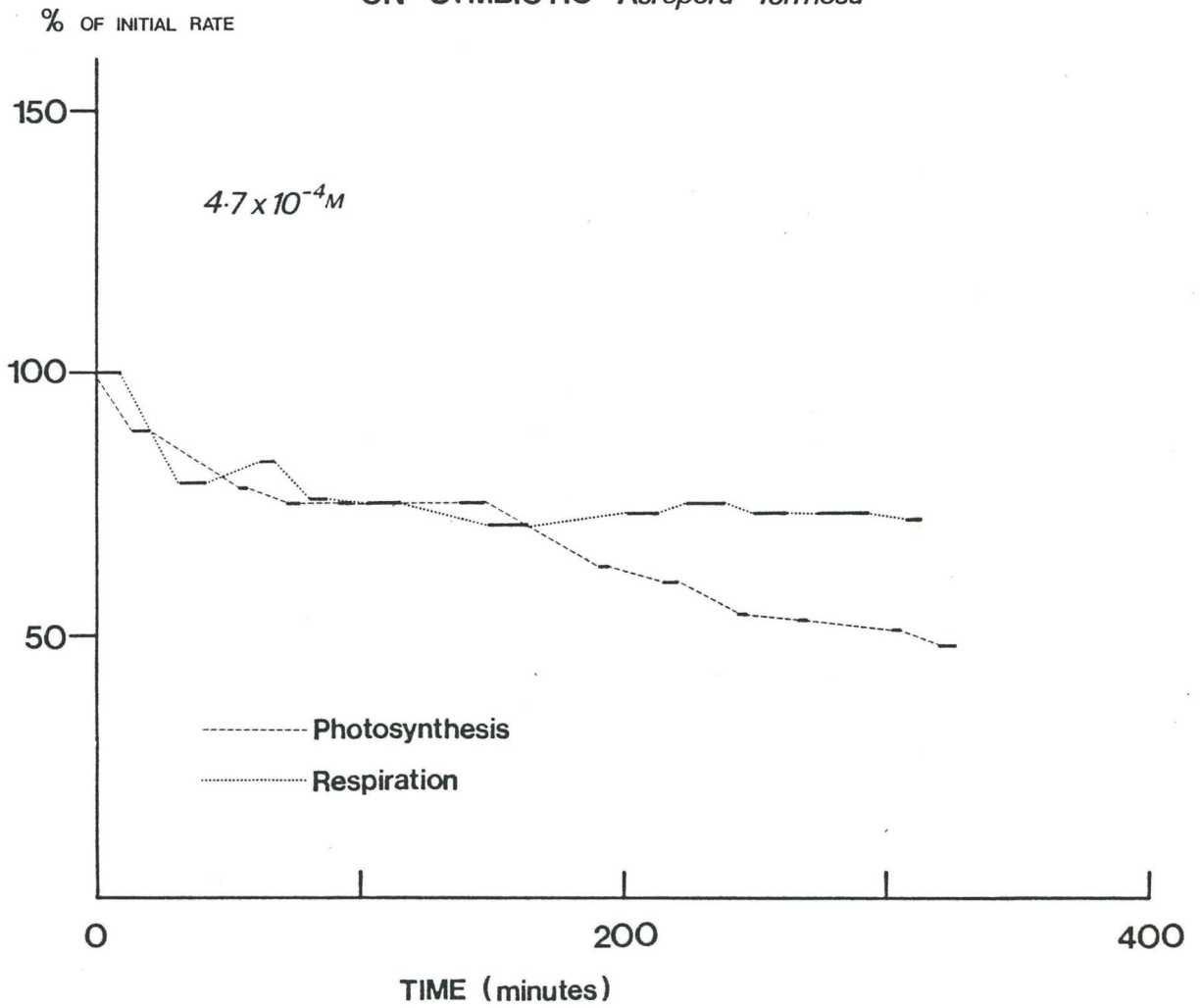


FIGURE 63

PROGRESS CURVE OF THE EFFECT OF SIPHONODICTYAL G
ON SYMBIOTIC *Acropora formosa*



S.D. = 0.08, n = 5). At 150 minutes the photosynthetic rate rose (slope = 0.09, S.D. = 0.05, n = 4).

Siphonodictyal G at a concentration of 4.7×10^{-4} M caused a steady decline in the rate of photosynthesis (slope = -0.13, S.D. = 0.11, n = 11) (Figure 63). The rate of respiration also dropped but levelled off after 100 minutes, at 75 percent of the initial rate (slope = -0.01, S.D. = 0.01, n = 11).

8.3.3 Dose Response Curves

The sensitivity of symbiotic *Acropora formosa* to terpene toxicity was compared to that of symbiotic *Porites andrewsii*. The influence of zooxanthellae on the sensitivity of *Acropora formosa* was examined by comparing the lowest concentration which elicited a response in symbiotic *Acropora formosa* to that of aposymbiotic *Acropora formosa* and of isolated zooxanthellae taken from symbiotic *Acropora formosa*.

The highest value obtained for changes in net photosynthetic and respiratory rates with each dose are plotted against terpene concentration. The open circles represent values obtained for respiration and closed circles represent the values obtained for photosynthesis.

Furanoquinol and Furanoquinone

The response of both scleractinian corals to increasing concentrations of furanoquinol was an increase in the respiratory rate followed by a decline (Figures 64 and 65). This was associated with a reduction in oxygen production. The lowest dose eliciting this response was 10^{-6} M for *Acropora formosa* (Figure 64). At doses higher than 10^{-10} M, *Porites andrewsii* also exhibited a gradual rise in the respiratory rate and a depression in the rate of oxygen production after a small rise (Figure 65). The degree of the response was greater on aposymbiotic *Acropora formosa* with the lowest dose evoking a

FIGURE 64

EFFECT OF FURANOQUINOL ON SYMBIOTIC *Acropora formosa*

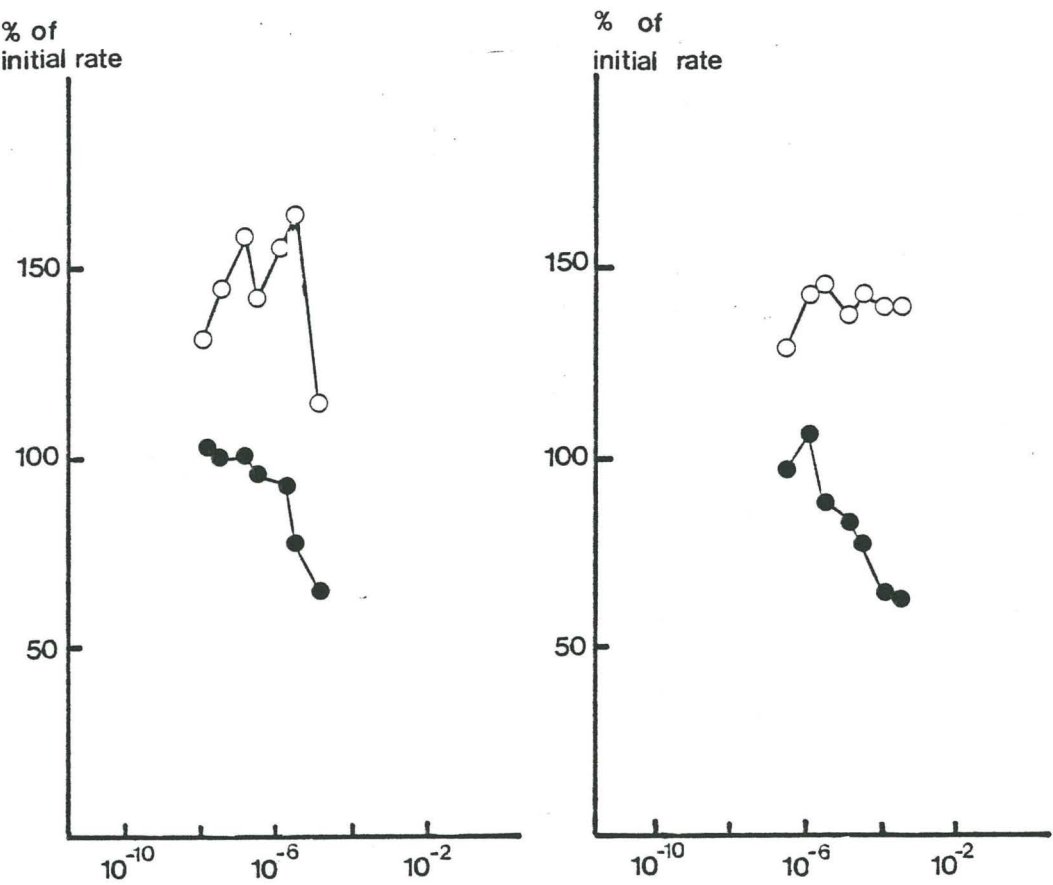


FIGURE 65

EFFECT OF FURANOQUINOL ON SYMBIOTIC *Porites andrewsii*

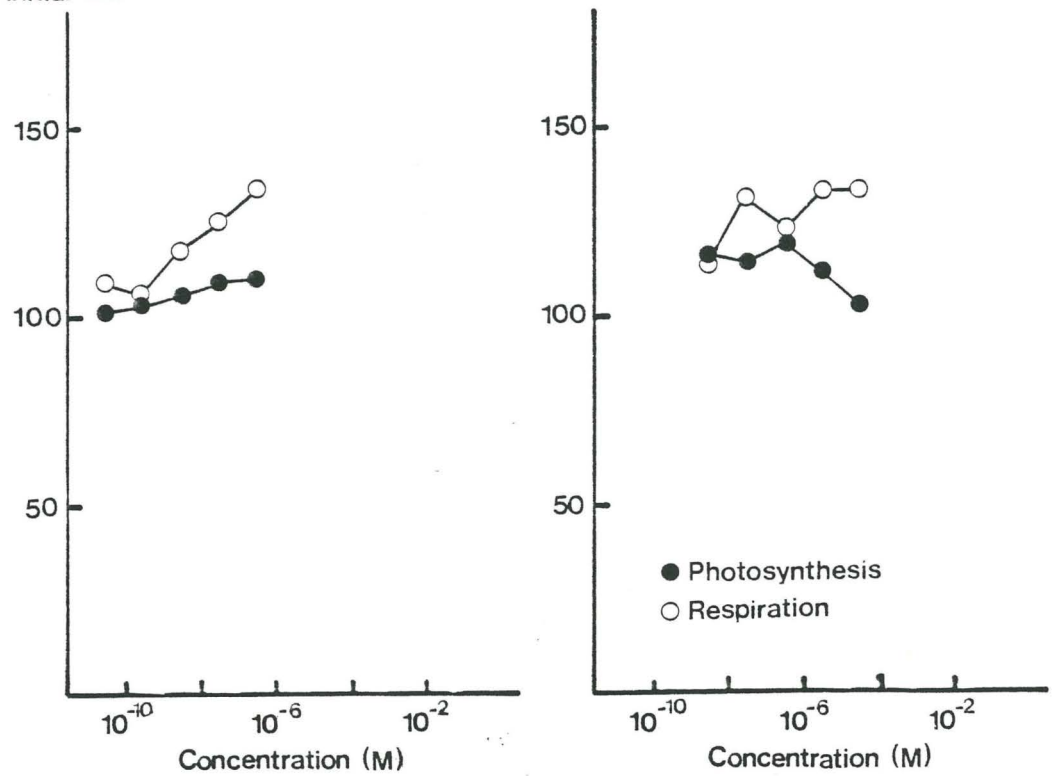


FIGURE 66
EFFECT OF FURANOQUINOL ON APOSYMBIOTIC *Acropora formosa*
% of
initial rate

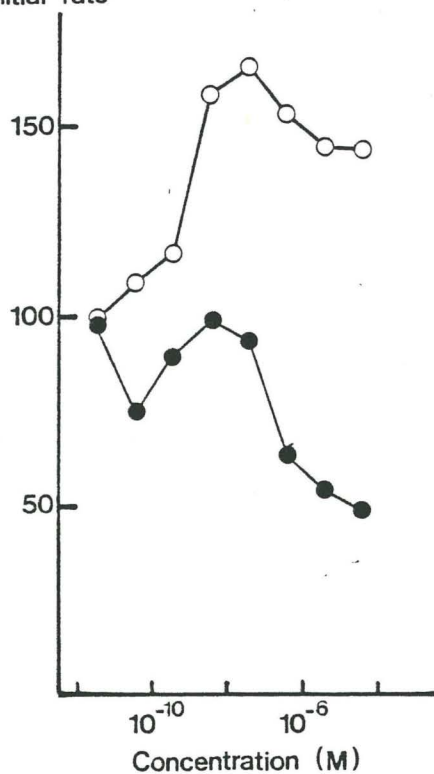


FIGURE 67
EFFECT OF FURANOQUINONE ON APOSYMBIOTIC *Acropora formosa*
% of
initial rate

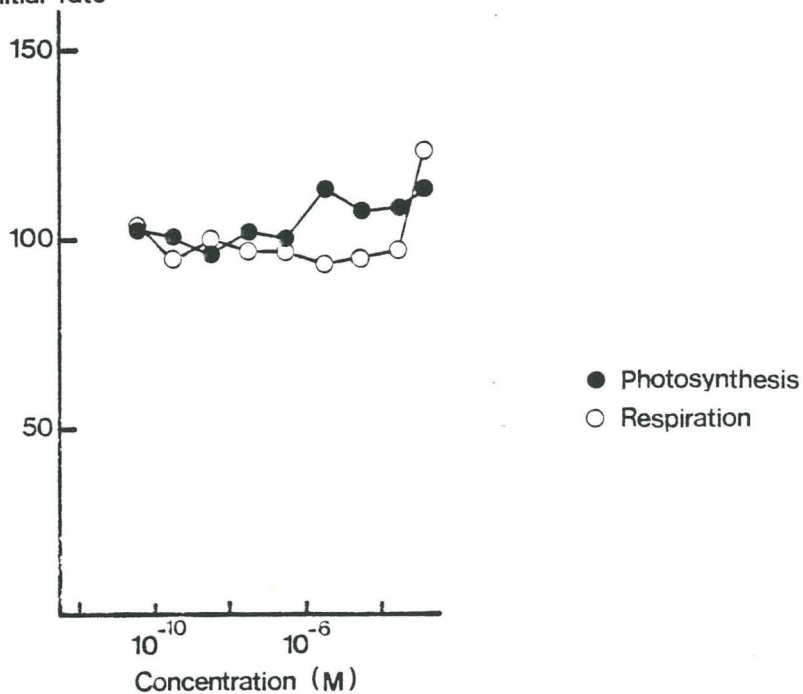


FIGURE 68
EFFECT OF FLEXIBILIDE ON SYMBIOTIC *Acropora formosa*

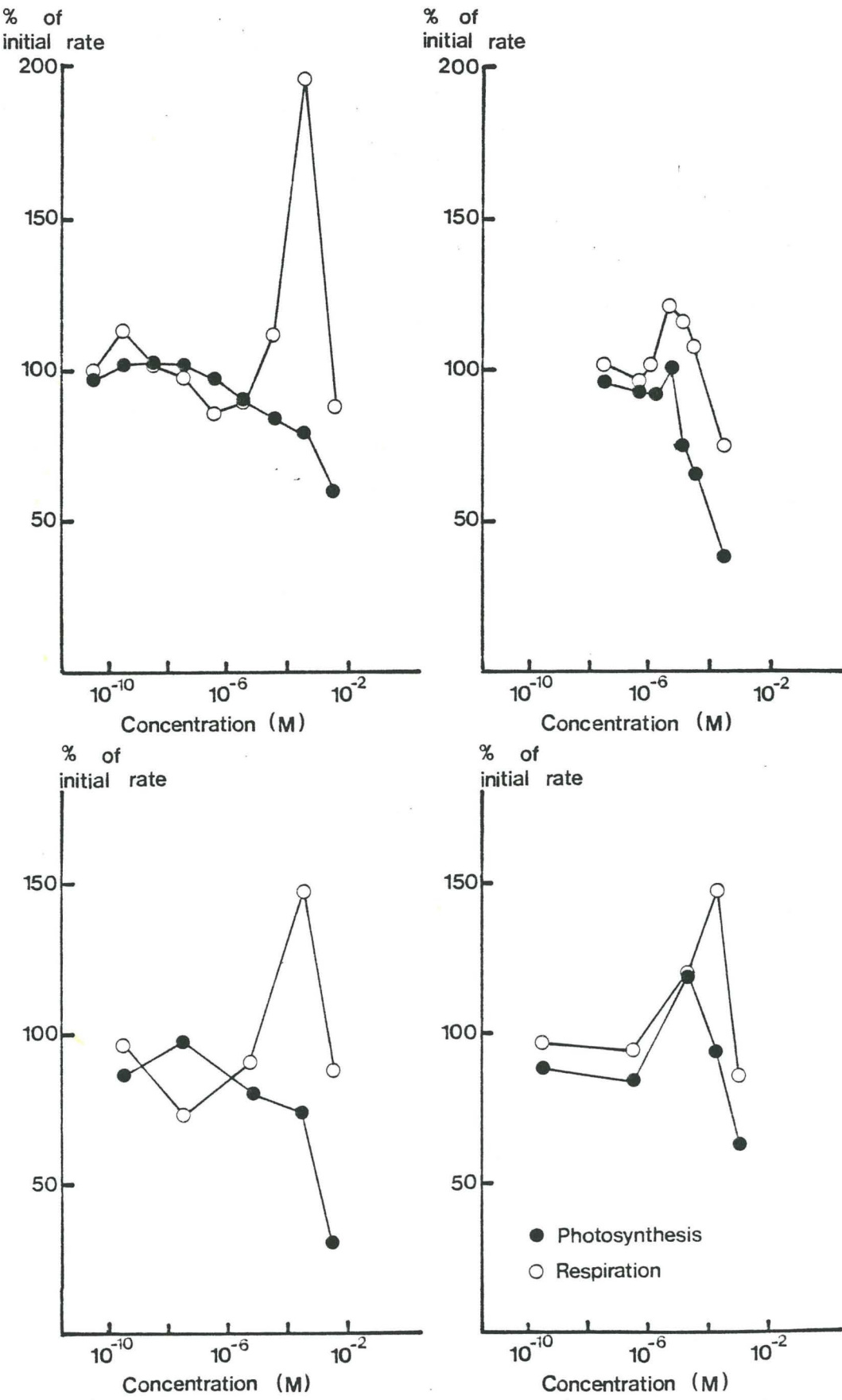


FIGURE 68

EFFECT OF FLEXIBILIDE ON SYMBIOTIC *Acropora formosa*

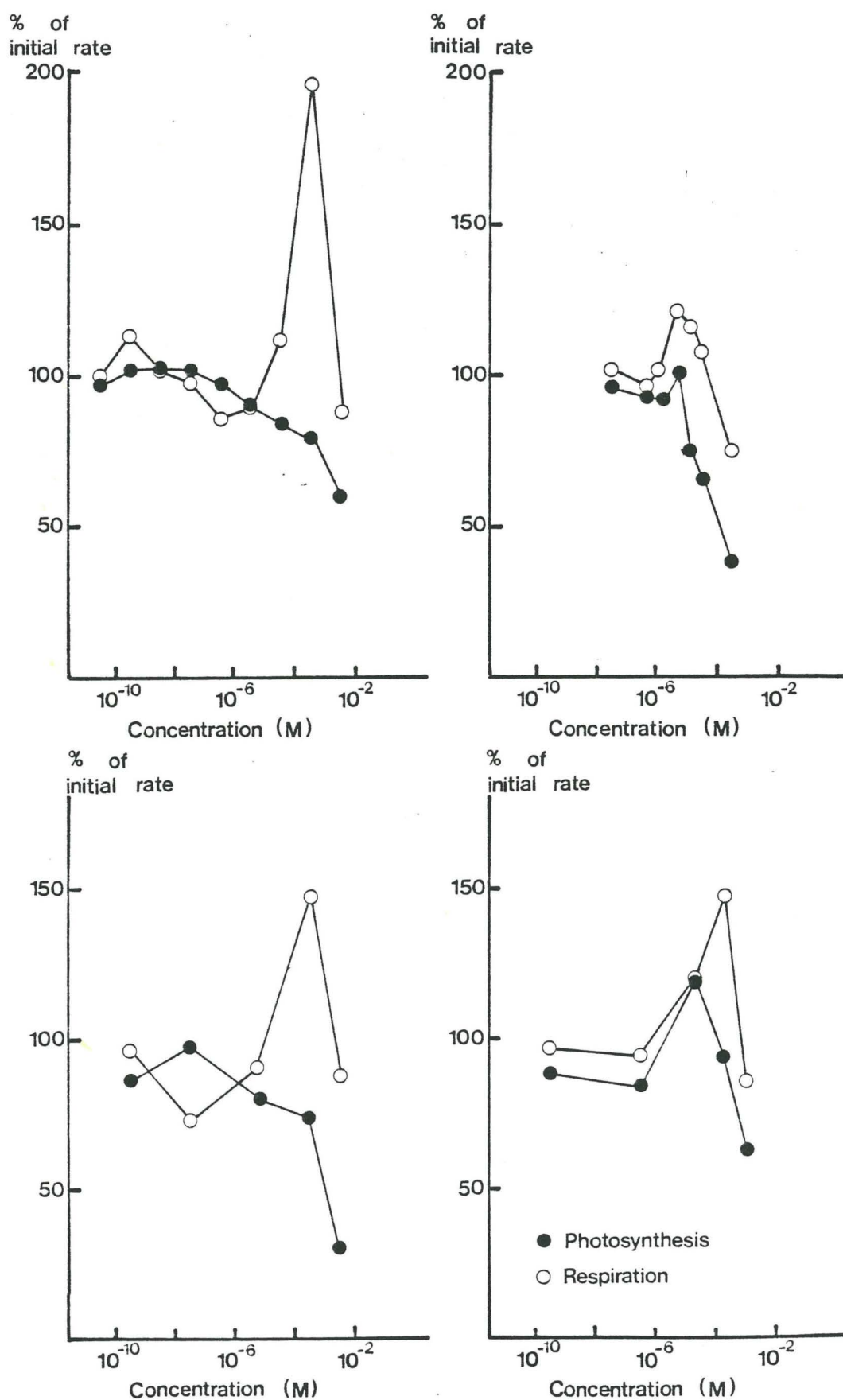


FIGURE 69

EFFECT OF FLEXIBILIDE ON SYMBIOTIC *Porites andrewsii*

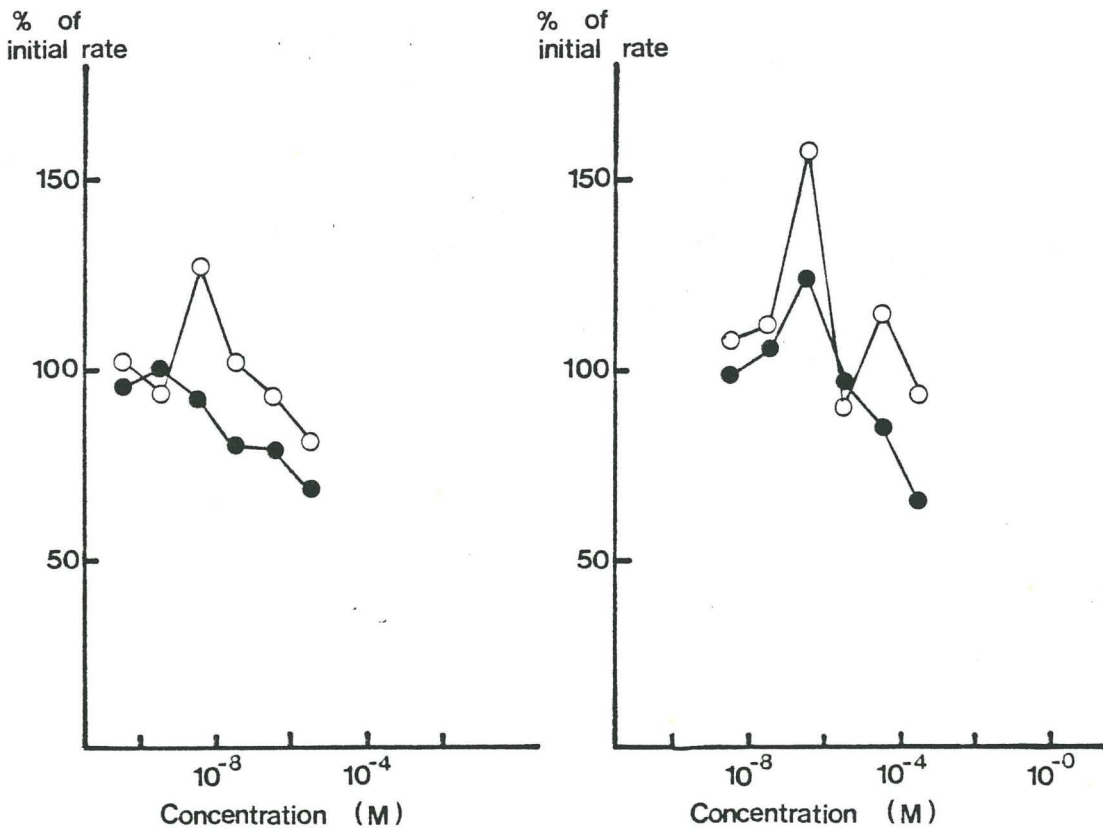


FIGURE 70

EFFECT OF FLEXIBILIDE ON APOSYMBIOTIC *Acropora formosa*

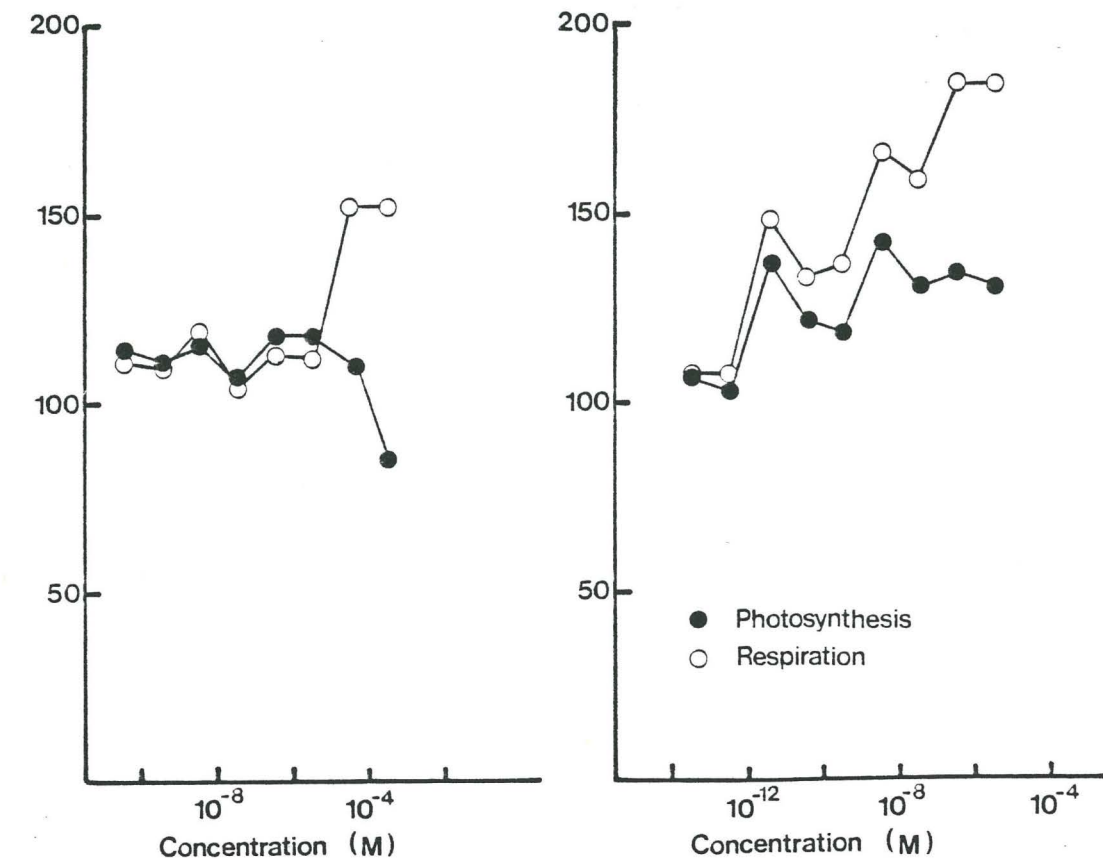
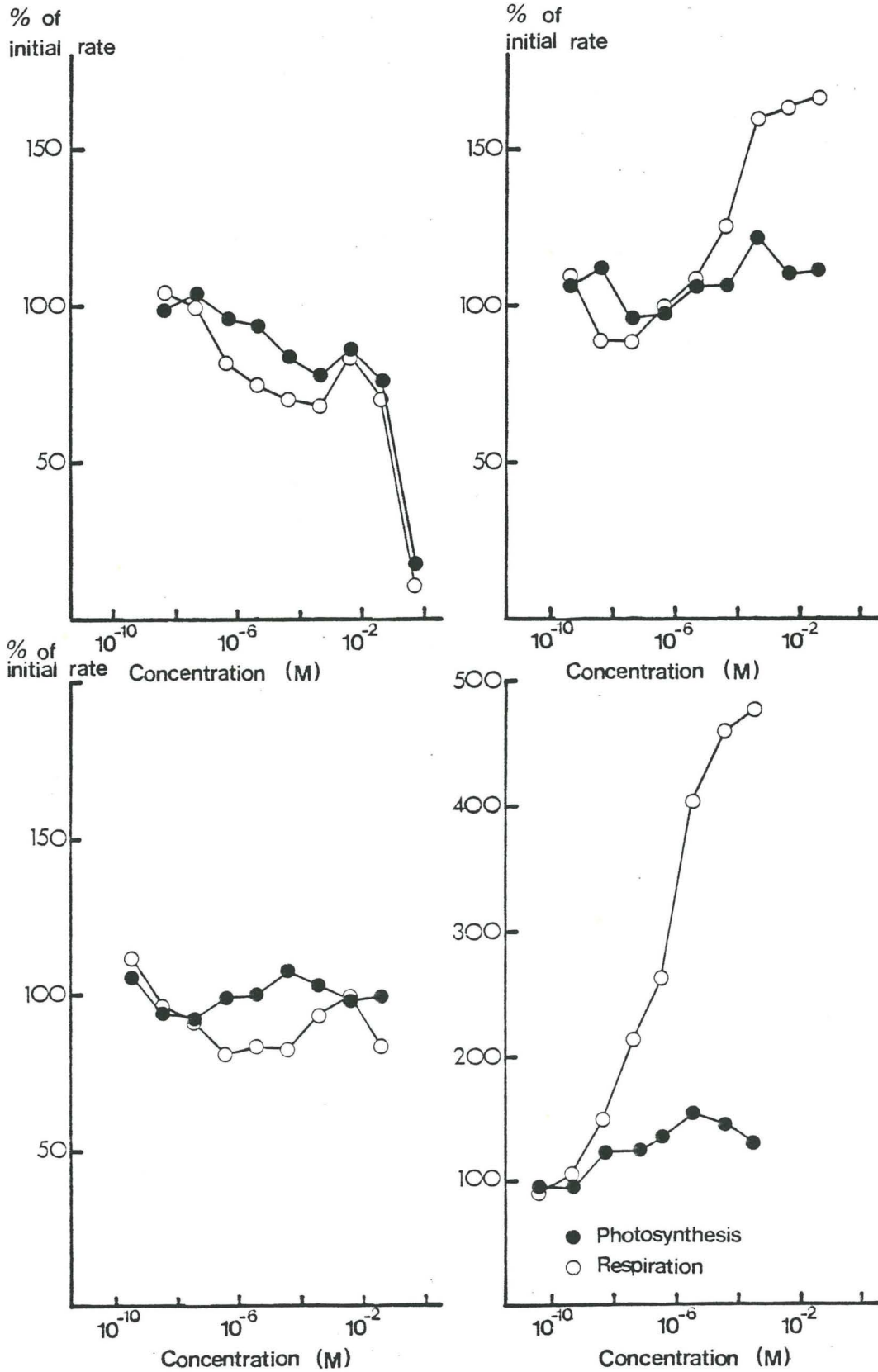


FIGURE 71

EFFECT OF FLEXIBILIDE ON ZOOXANTHELLAE ISOLATED FROM
Acropora formosa



response being 10^{-8} M (Figure 66). A slight rise in both the respiratory and oxygen production rates of aposymbiotic *Acropora formosa* occurred with doses of furanoquinone above 10^{-6} M (Figure 67).

Flexibilide And Dihydroflexibilide

Flexibilide caused a rise in the respiratory rate and a decline in the photosynthetic rate at doses as low as 10^{-5} M on *Acropora formosa* (Figure 68). A dose of 10^{-8} M caused a rise in the respiratory rate and a decline in the photosynthetic rate of symbiotic *Porites andrewsii* (Figure 69). At a dose of 10^{-6} M, the respiratory rate declined. Aposymbiotic *Acropora formosa* was more sensitive, responding at 10^{-8} M with a rise in the respiratory rate (Figure 70). In two cases out of four, flexibilide had little effect on the photosynthetic rate of isolated zooxanthellae from *Acropora formosa*, but caused a rise in the respiratory rate with doses above 10^{-8} M (Figure 71). The response of the zooxanthellae was variable, presumably due to the health of the zooxanthellae. Dihydroflexibilide caused a rise in the respiratory and photosynthetic rates at concentrations above 10^{-5} M (Figure 72). Sinulariolide was relatively inactive, eliciting a jump in the respiration rate with doses of above 10^{-2} M (Figure 73).

Sarcophytoxide And Sarcophine

Sarcophytoxide caused the respiratory rate of symbiotic *Acropora formosa* to increase when doses of 10^{-8} M were administered (Figure 74). Higher doses reduced the rate of respiration. The photosynthetic rate declined at doses above 10^{-6} M. Aposymbiotic *Acropora formosa* was less sensitive to the toxic effects of sarcophytoxide (Figure 75), first responding at doses of 10^{-6} M. Symbiotic *Porites andrewsii* gave a slight increase in the respiratory rate with high doses, and, on one run, there was a decline in the photosynthetic rate when a dose of 10^{-7} M was administered (Figure 75). At high doses (10^{-3} M), sarcophytoxide affected the respiration and photosynthesis of zooxanthellae

FIGURE 72

EFFECT OF DIHYDROFLEXIBILIDE ON SYMBIOTIC *Acropora formosa*

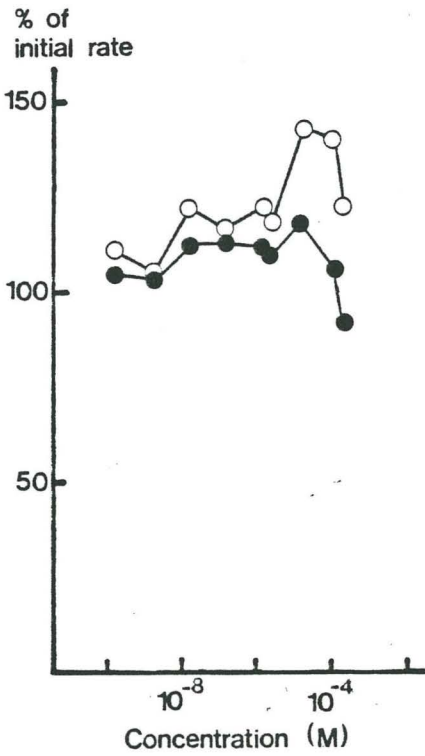


FIGURE 73

EFFECT OF SINULARIOLIDE ON APOSYMBIOTIC *Acropora formosa*

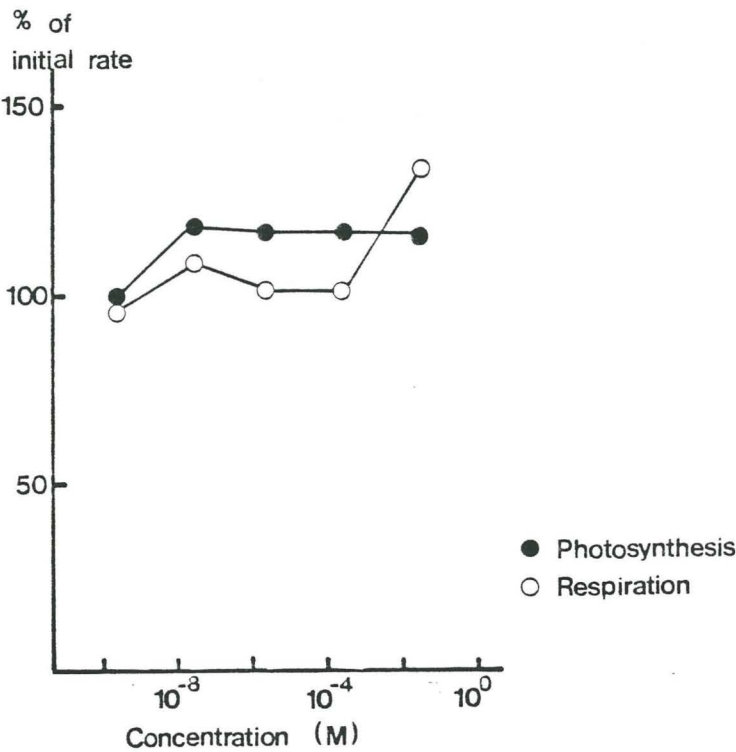


FIGURE 74

EFFECT OF SARCOPHYTOXIDE ON *Acropora formosa*

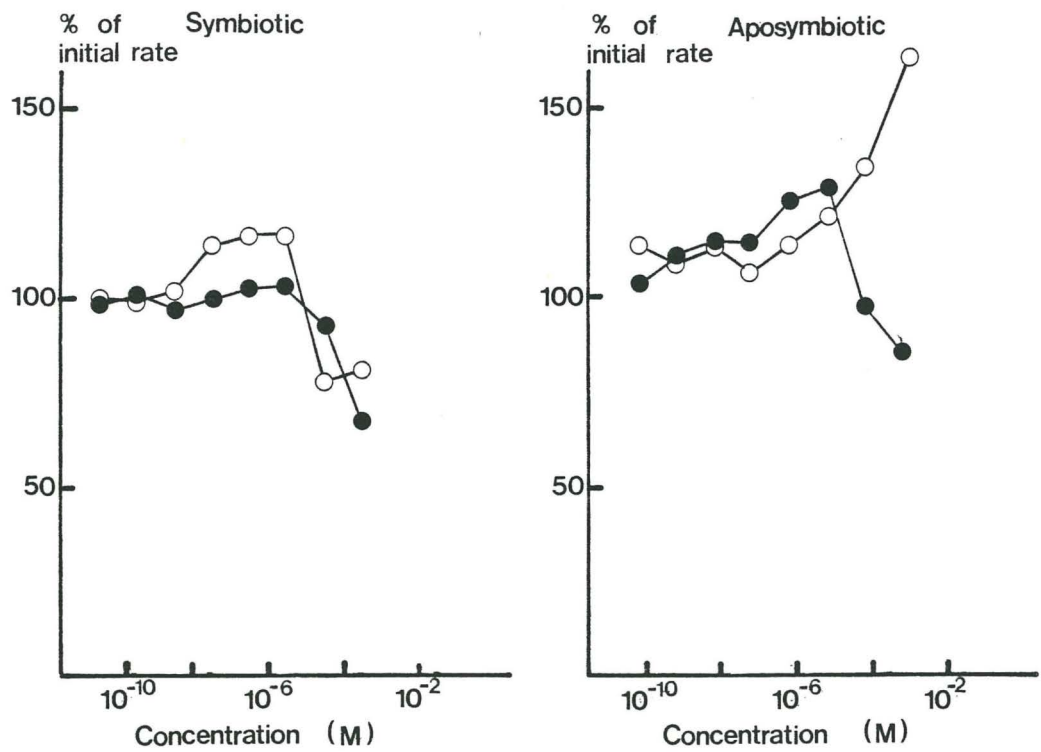


FIGURE 75

EFFECT OF SARCOPHYTOXIDE ON SYMBIOTIC *Porites andrewsii*

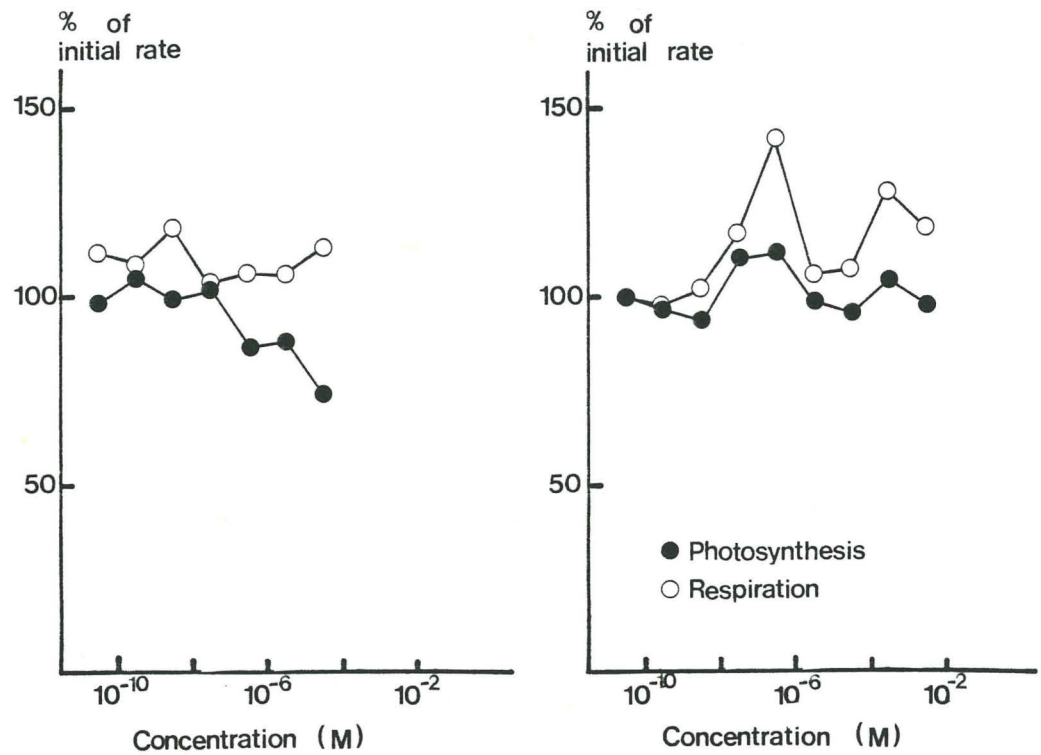


FIGURE 76

EFFECT OF SARCOPHYTOXIDE ON ZOOXANTHELLAE ISOLATED FROM
Sarcophyton sp.

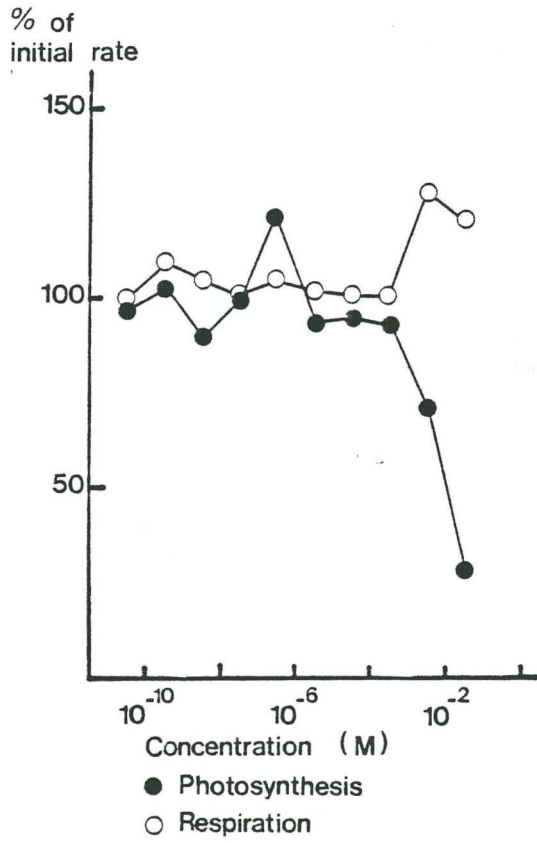


FIGURE 77

EFFECT OF SARCOPHINE ON SYMBIOTIC *Acropora formosa*

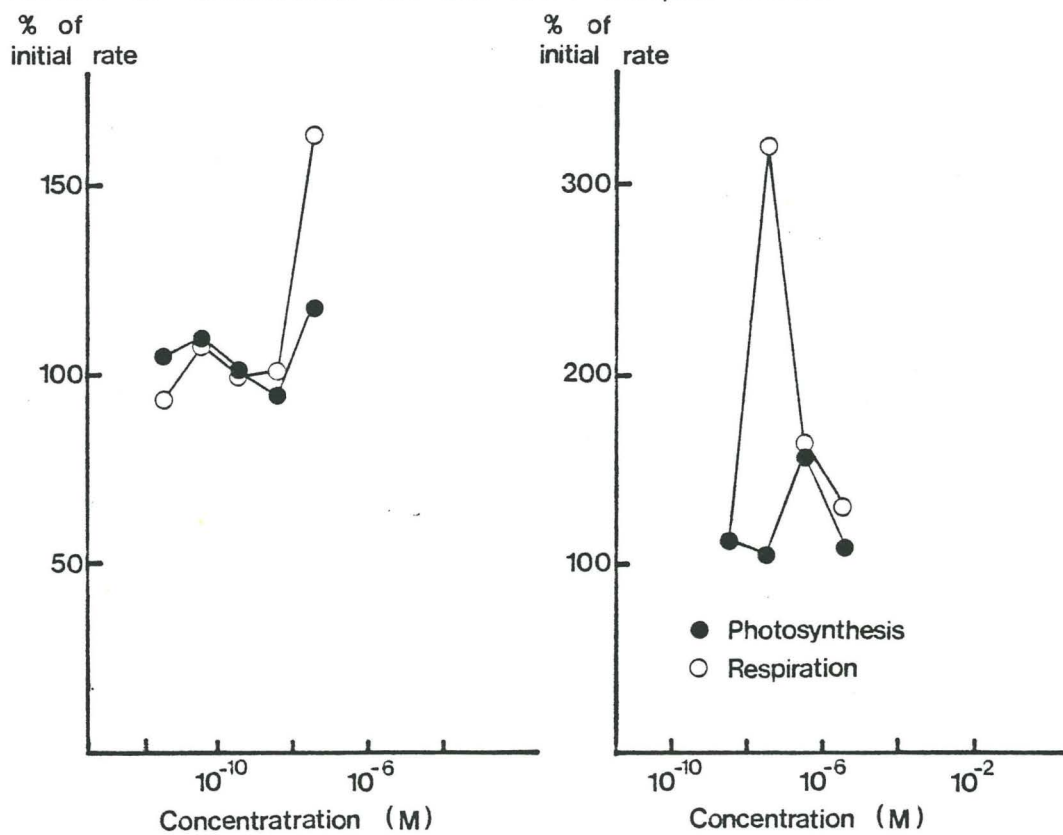


FIGURE 78

EFFECT OF FN45 ON SYMBIOTIC *Acropora formosa*

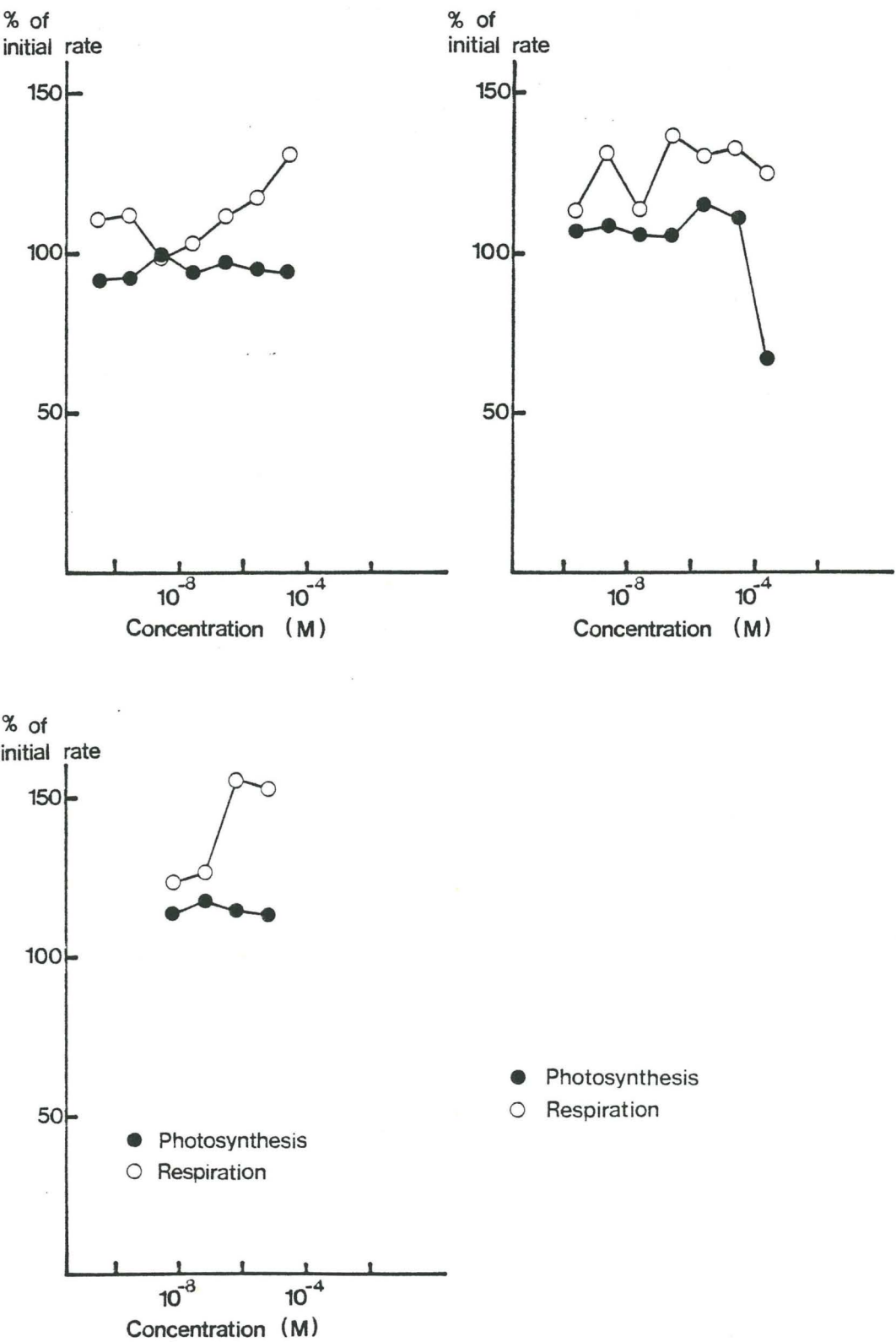


FIGURE 79

EFFECT OF FN45 ON SYMBIOTIC *Porites andrewsii*

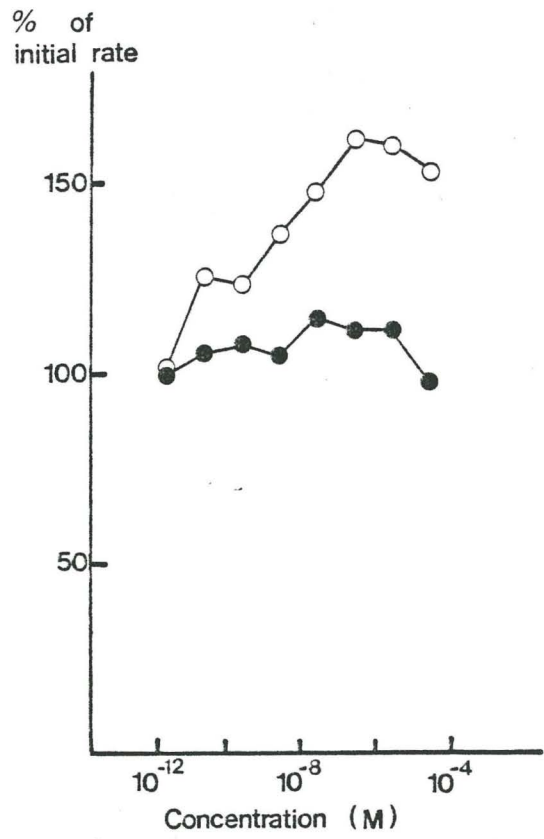
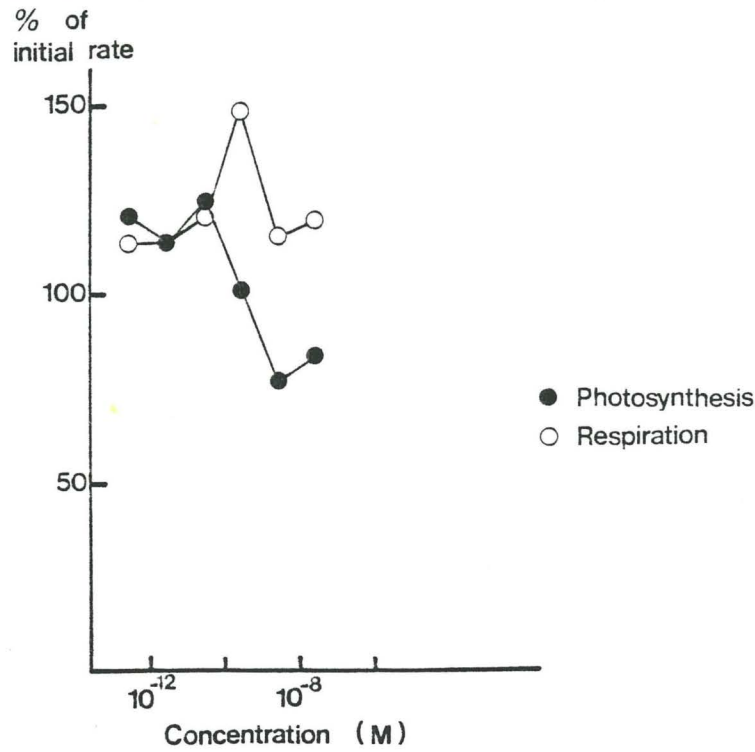


FIGURE 80

EFFECT OF FN45 ON APOSYMBIOTIC *Acropora formosa*



isolated from a *Sarcophyton* sp. containing sarcophytoxide (Figure 76). Sarcophine evoked a large increase in the rate of respiration at doses of 10^{-7} M in *Acropora formosa* (Figure 77).

FN45

FN45 raised the rate of respiration in symbiotic *Acropora formosa* at very low doses (10^{-10} M) (Figure 78). Photosynthesis was depressed at doses of 10^{-4} M. FN45 also caused the rate of respiration in symbiotic *Porites andrewsii* to rise at low doses (10^{-10} M), but did not affect the rate of photosynthesis with doses lower than 10^{-4} M (Figure 79). FN45 raised the rates of both photosynthesis and of respiration at low doses ($<10^{-10}$ M) in aposymbiotic *Acropora formosa* (Figure 80). FN45 evoked a strong rise in the rate of respiration at all concentrations. Doses above 10^{-10} M depressed the rate of photosynthesis.

2B

2B is a common name given to the terpene (75) which is biosynthetically related to sarcophytoxide and isolated from *Sarcophyton* spp. 2B evoked a strong rise in the rate of respiration in symbiotic *Acropora formosa* at doses of above 10^{-8} M (Figure 81). The rate of photosynthesis did not rise until doses above 10^{-4} M were applied.

Sponge Terpenes

Siphonodictidine evoked an increases in the rate of respiration in symbiotic *Acropora formosa* at doses above 10^{-6} M. Photosynthesis was depressed by doses above 10^{-4} M (Figure 82). Doses of siphonodictyal B of above 10^{-8} M caused a rise in the rates of both photosynthesis and respiration in symbiotic *Acropora formosa* (Figure 83). The rate of respiration declined at doses above 10^{-6} M. The rate of photosynthesis echoed the response of respiration. Doses of siphonodictyal C of above 10^{-6} M caused a rise in the rate of respiration of symbiotic *Acropora formosa* (Figure 84). The rate of

FIGURE 81

EFFECT OF 2B ON APOSYMBIOTIC *Acropora formosa*

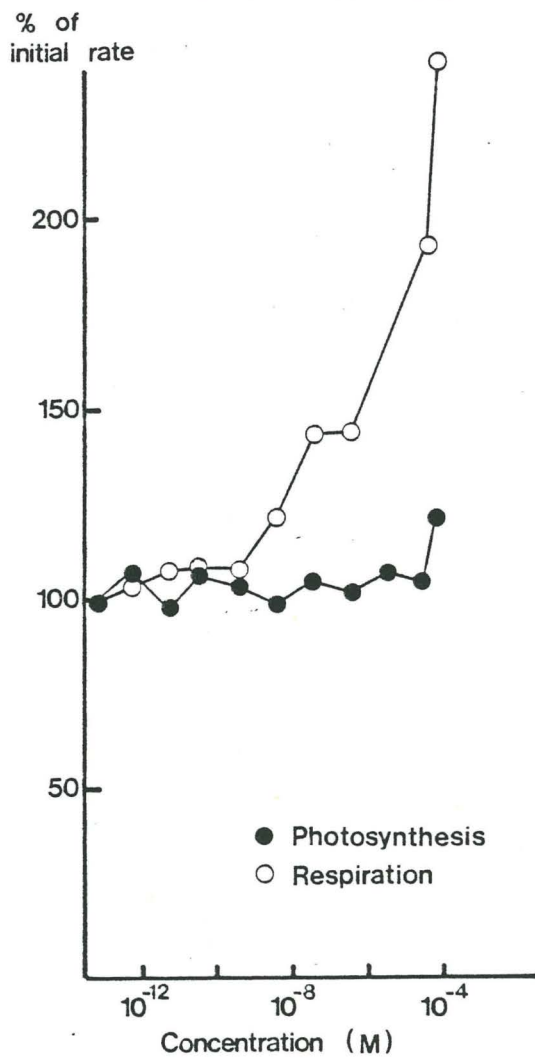


FIGURE 82

EFFECT OF SIPHONODICTIDINE ON SYMBIOTIC *Acropora formosa*

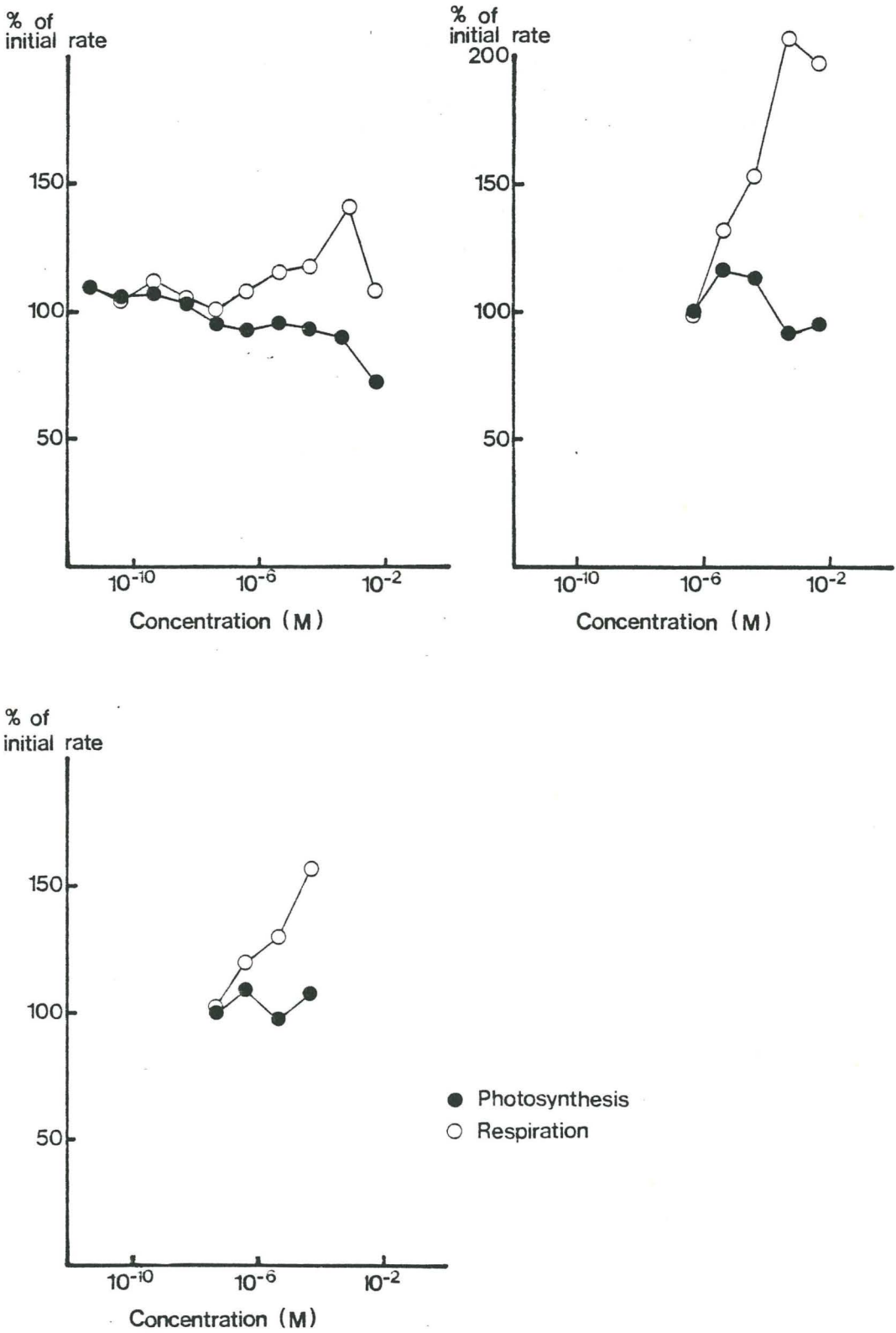


FIGURE 83

EFFECT OF SIPHONODICTYL B ON SYMBIOTIC *Acropora formosa*

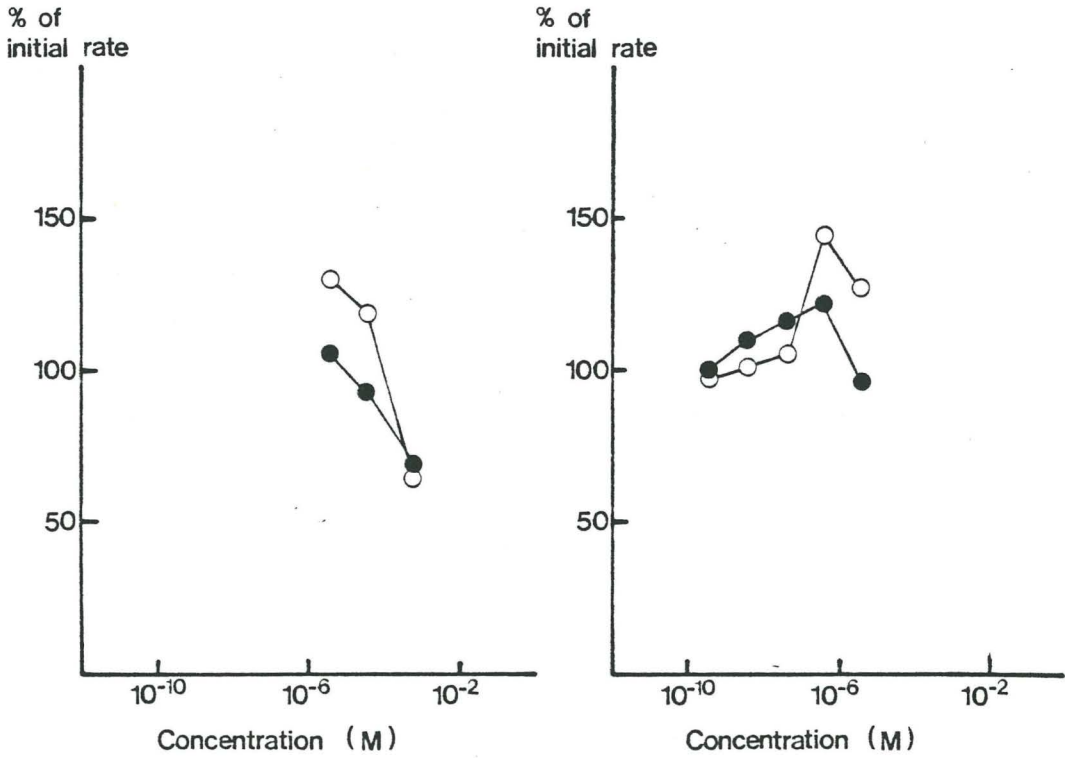
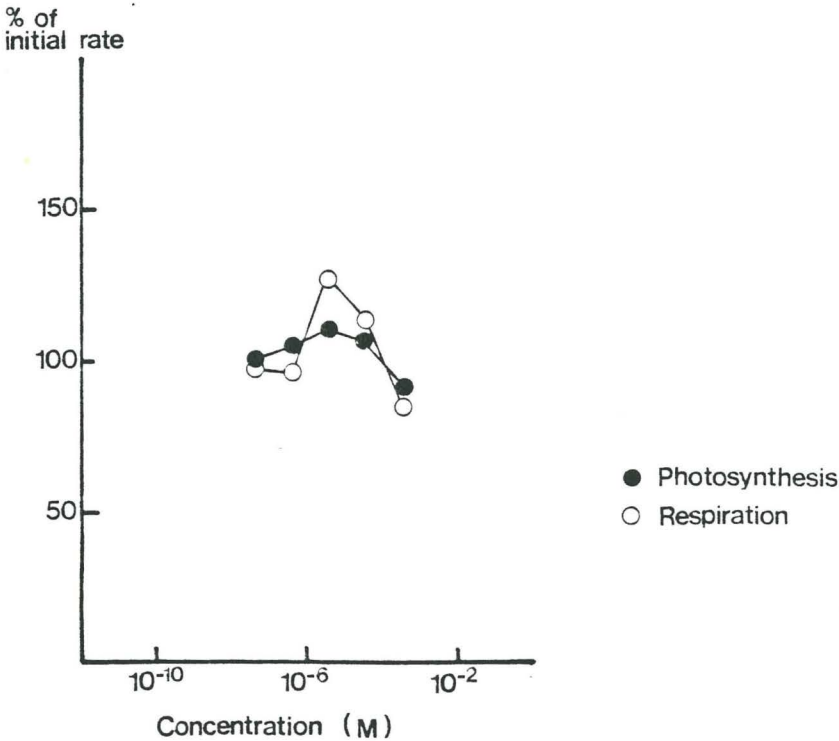


FIGURE 84

EFFECT OF SIPHONODICTYL C ON SYMBIOTIC *Acropora formosa*



respiration dropped with doses above 10^{-3} M. The rate of photosynthesis echoed this response. Doses of siphonodictyal G of above 10^{-7} M raised the rate of respiration, and slightly lowered the rates of photosynthesis (Figure 85).

8.4 DISCUSSION

8.4.1 The Physiological Response Of Scleractinian Corals To Acute Terpene Toxicity

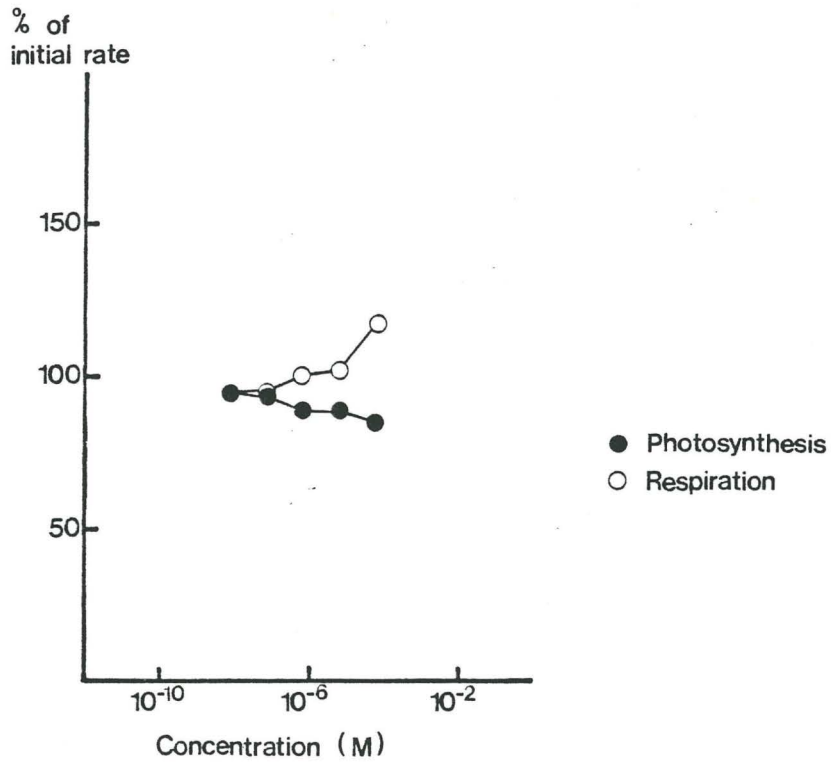
The time course for the physiological response of scleractinian corals to a pulse of each of a selection of marine terpenes was an initial increase in the respiratory rate followed by a slow decline. This change in respiration rate was ^{concomitant} with a slight rise in photosynthetic rate followed by a similar decline indicating that the two events were ^{occurring} together. The initial lag period is presumably due to the delay in reaching the site of action, i.e. the mitochondria. The duration of the lag period and the degree of increase in rate are proportional and inversely proportional, respectively, to the concentration of terpene added.

The initial elevation in respiratory rate suggests uncoupling of oxidative phosphorylation in mitochondria. This would allow oxidation of organic substrates to proceed at maximal velocity and result in an increase in oxygen consumption (White, Handler & Smith, 1973). Compounds capable of uncoupling oxidative phosphorylation are highly reactive, low molecular weight, lipophilic molecules (Parker, 1965; Miko & Chance, 1975), as are the terpenes studied in this Chapter. These physical properties would not permit distinction between uncouplers of mitochondria and of chloroplasts.

The effect on photosynthesis was similar to that observed for respiration. Although the initial rise was not as marked, it was simultaneous with the rise in the rate of respiration. The initial rise in the rates of respiration and photosynthesis were more pronounced in isolated zooxanthellae. Aposymbiotic *Acropora formosa*

FIGURE 85

EFFECT OF SIPHONODICTYAL G ON SYMBIOTIC *Acropora formosa*



were more sensitive than symbiotic *Acropora formosa*. This suggests that zooxanthellae may protect the coral from the effects of energy deprivation resulting from the uncoupling of oxidative phosphorylation. In general, the alteration of the rates of respiration and of photosynthesis is consistent with uncoupling of the mitochondria and of the chloroplasts.

Porites andrewsii was less sensitive than *Acropora formosa* to the terpenes tested here, as maximum alteration of photosynthetic and of respiratory rates were recorded at higher concentrations. *Porites andrewsii* responded with a more gradual rise in the respiratory rate and a more rapid decline in the photosynthetic rate than did *Acropora formosa*. Differences in scleractinian sensitivity to alcyonacean corals (Sammarco, Coll, la Barre & Willis, 1983) and to alcyonacean terpenes (Coll & Sammarco, 1983) has been reported. *Porites andrewsii* in general is more sensitive than *Pavona cactus*; the coral on which growth retardation due to an alcyonacean was first observed (Sammarco et al., 1983).

When tips that had been exposed to nonlethal doses of terpene were monitored the next day, the photosynthetic rate were low but the respiration rate had returned to near initial values. When returned to the chamber a few days later, the photosynthetic and respiratory rates in the tips that had been exposed to nonlethal doses of terpene, were identical to the rates before exposure to the terpene. This suggests that the effects of these terpenes on respiration and on photosynthesis are reversible, and the effects seen *in situ* are dependent on continual exposure. This implies that the terpenes are both antifeedants and allelopathic agents.

The property of terpenes to alter the respiration of a receiver species is fairly common in the terrestrial environment. In a study of terrestrial terpenes, the macerated leaves and sesquiterpene lactones from the sagebrush *Artemisia* sp. inhibited the growth and stimulated the respiration of *Cucumis sativis* (McCahon, Kelsey, Sheridan & Shafizahed, 1973). Growth inhibitory stilbenoids (phenolic compounds) uncoupled electron transport and

photophosphorylation in isolated chloroplasts of *Spinacia oleracea* and inhibited carbon dioxide dependent oxygen evolution and electron flow from water to methyl viologen in uncoupled chloroplasts (Gorham & Coughlan, 1980).

In another example, the sesquiterpene lactone, vernolepin (48) obtained from the plant *Vernonia hymenolepis* inhibited extension growth from 20 to 80 percent of wheat coleoptile sections when applied at a concentration of 5 to 50 $\mu\text{g/ml}$ (Sequeira, Hemingway & Kupchan, 1968). However, the inhibited tissues appeared to be normal and their respiration was unaffected.

8.4.2 Terpene Structure Related To Toxicity

Flexibilide

The progress curves show flexibilide to be more toxic than FN45 to *Acropora formosa* because the lag interval is shorter and the slope of the initial rise is greater. This results in the maximum response being recorded earlier and presenting a higher value. This pattern of response is echoed in the dose response curves. High concentrations of flexibilide evoke a strong alteration in the rate of respiration within minutes, while similar doses of FN45 induce only a mild response during the same interval.

The paradox is that flexibilide kills *Acropora formosa* and *Porites andrewsii* (Coll & Sammarco, 1983; Webb & Coll, 1983) and stunts *Pavona cactus*, yet has low toxicity to frogs and rats (Arrigoni-Martelli, 1981; Vickers, personal communication, 1983). Flexibilide may be an invertebrate toxin acting as an agent of aggression to rival corals and not possess antifeedant properties; although it has not been tested against fish.

In some pharmacological studies, flexibilide was shown to have a low toxicity to rats. It had antiinflammatory, antiarthritic properties and did not ulcerate the rat gastrointestinal tract

(Arrigoni-Martelli, 1981). Both dihydroflexibilide and sinulariolide were only mildly toxic to *Acropora formosa*. Schmitz, Prasad and Hollenbeak (1978) found flexibilide to be more active against leukemia than its cometabolite, dihydroflexibilide. Dihydroflexibilide, in turn, was more active than the biosynthetically related terpene, sinulariolide (51). Sinulariolide is elaborated by *Sinularia flexibilis* which do not synthesize flexibilide (Weinheimer, Matson, Hossain & Van Der Helm, 1977), and differs from flexibilide by the lack of a furan ring. Against *Acropora formosa*, both dihydroflexibilide and sinulariolide were inactive.

According to Tellez-Martinez, Taboada and Gonzalez-Diddi (1980), the presence of a furan ring increases the toxicity of the α -methylene- γ -lactone group to two different cell lines; the fibroblastoid cells L-929 from mice areolar tissue, and epithelial cells HEp-2 from human laryngeal carcinoma. Even though sinulariolide is the least active of the three compounds against neoplasias, it is toxic to fish and inhibits zooxanthellae growth (Tursch, 1976; Ogura, Cordell & Farnsworth, 1978; Lee, Ibuka, Wu & Geissman, 1977; Schmitz, Prasad & Hollenbeak, 1978).

Terpenes bearing exocyclic α -methylene- γ -lactones undergo Michael-type nucleophilic addition to sulphydryl groups when tested against phosphofructokinase (Hanson, Lardy & Kupchan, 1970; Kupchan, Fessler, Eakin & Giacobbe, 1970; Smith, Larner, Thomas & Kupchan, 1972). These workers showed that in a majority of cases, the loss of the methylene group removes biological activity as demonstrated in this chapter by the relative inactivity of dihydroflexibilide.

The α -methylene- γ -lactone group is known to be antiinflammatory and antiarthritic (Kim, 1980), antibacterial (Calzada, Ciccio & Echandi, 1980; Lee, Ibuka, Wu & Geissman, 1977), and antineoplastic (Ogura, Cordell & Farnsworth, 1978; Lee, Ibuka, Wu & Geissman, 1977; Schmitz, Prasad & Hollenbeak, 1978). Yet this functional group induces dermatitis (Rodriguez, 1980).

Alkylating agents capable of binding to proteins in mitochondrial membranes, uncouple oxidation from phosphorylation (Hanstein, 1976). Surprisingly, some workers conclude that the exocyclic α -methylene- γ -lactone group does not uncouple isolated mitochondria but acts elsewhere on the respiratory chain to inhibit respiration (Taniguchi, Yamaguchi, Kubo & Kubota, 1979). This is the opposite to the effect of flexibilide. However, growth inhibitory terpenes do increase respiration in whole seedlings and excised leaves (McCahon, Kelsey, Sheridan & Shafizadeh, 1973). More precisely there is an initial increase in respiration rate followed by a rapid decline (Marinos & Hemberg, 1960), in the same manner as the toxic terpenes studied here. From this it appears flexibilide may not induce the rise in respiration rate by uncoupling the mitochondria, but by some other process such as by stress from cell membrane damage.

The growth inhibition of the scleractinian coral *Pavona cactus* growing near a colony *Simularia flexibilis* (Sammarco, Coll, la Barre & Willis, 1983) can be explained by the presence^{of} flexibilide in the seawater (Coll, Bowden, Tapiolas & Dunlap, 1982) as this terpene uncouples mitochondria and chloroplasts, reducing the amount of energy available to the cell. The concentration of flexibilide in the seawater is presumably low as these terpenes have low solubility in water (Buckle, Baldo & Taylor, 1980; personal observation, 1981). Chronic exposure of the exposed (growing) edges to low levels of toxin would reduce the growth of the scleractinian colony to a degree dependent on the concentration of terpene. Hence the stunting occurs in an arc around the soft coral, being most extreme at the points closest to the colony.

FN45

FN45 is a moderately active in altering the rates of respiration and photosynthesis in *Acropora formosa*, suggesting uncoupling of mitochondria and chloroplasts. However, biosynthetically related terpenes isolated from the same genus, (Figure 30), inhibited respiration in isolated rat liver

mitochondria. The inhibition was not due to either uncoupling of or inhibition of the respiratory chain ($IC_{50} = 0.00007$ g/ml; Baird-Lambert, Dunlop & Jamieson, 1980). Thus FN45 may also not be an uncoupler but effect the alteration in respiration and photosynthesis rates in intact organisms through a different mechanism.

FN45 was found in the eggs of *Lobophytum carnatum* (Chapter 4) and is presumed to act as an antifeedant. It may also act as an allelopathic agent as in the field, the genus *Lobophytum* is toxic to scleractinia (Sammarco, Coll, la Barre & Willis, 1983). In laboratory experiments, FN45 killed *Pavona cactus* and *Porites andrewsii* overnight (Coll & Sammarco, 1983).

Sarcophine

Sarcophine did not alter the rates of respiration or photosynthesis in *Acropora formosa*. This is a puzzle as sarcophine possesses a β -unsubstituted cyclopentenone ring which is known to be toxic (Lee, Ibuka, Wu & Geissman, 1977) and to decrease mitochondrial respiration at state 3 (Taniguchi, Yamaguchi, Kubo & Kubota, 1979). Sarcophine is reported to be calcium antagonistic in rabbit aorta muscle (Kobayashi, Ohizumi, Nakamura, Yamakado, Matsuzaki & Hirata, 1983). It has also been shown to be toxic to rats, mice, guinea pigs (Neeman, Fishelson & Kashman, 1974) and fish (Kashman, Zadock & Neeman, 1974; Bernstein, Shmeuli, Zadock, Kashman, & Neeman, 1974; Coll, Tapiolas, Bowden, Webb & Marsh, 1983).

Diterpenes from the plant genus *Isodon* (Labiatae) strongly inhibited the oxidative phosphorylation in mitochondria isolated from silkworm midgut, and the α -methylene cyclopentenone moiety was essential for this activity (Taniguchi, Yamaguchi, Kubo & Kubota, 1979). These diterpenes also exhibited a relatively specific growth inhibitory activity against lepidopterous larvae. However, *Ovula ovum* showed no ill effects from voluntarily ingesting large quantities of sarcophytoxide (Chapter 7). Obviously, this toxicity

is not effected in a way which interferes with mitochondrial function. Sarcophine may be exclusively a vertebrate toxin. It was found in the eggs of *Sarcophyton stolidotum* (Chapter 4) which suggests an antifeedant role against fish.

Sarcophytoxide

Sarcophytoxide is often coisolated with sarcophine from *Sarcophyton* spp. (Bowden, Coll & Mitchell, 1980b; Coll, Bowden, Tapiolas & Dunlap, 1982). It is found throughout the colony and in the egg-bearing tissue of *Sarcophyton boletiforme*, suggesting an antifeedant role (Section 3.3.2). Sarcophytoxide does not contain a lactone yet is moderately toxic to *Acropora formosa*. It is also a fish toxin (Coll, Bowden, Tapiolas, Webb & Marsh, 1983). Like flexibilide and sarcophine, this compound contains an epoxide, and epoxides are toxic structures (Smith & Bend, 1979). They react with histones and nucleic acids (Wood *et al.*, 1977).

It was suggested that the egg cowrie, *Ovula ovum*, removes the epoxide as a detoxification measure (Coll, Bowden, Tapiolas, Webb & Marsh, 1983). However, sarcophine also has this epoxide in the same place. The only difference between the molecules is the absence of a lactone on the furan ring in sarcophytoxide. Thus, the furan ring itself may be the toxic functional group. Rodriguez (1980) found the possession of a furan ring sometimes gave cytotoxicity to a terpene. In Chapter 7 it was concluded that sarcophytoxide is not modified by passage through the digestive tract of *Ovula ovum*. Sarcophine has an epoxide in the same position of the planar molecule.

Many *Sarcophyton* spp. colonies yield both sarcophine and sarcophytoxide. Sarcophine is toxic to fish (Neeman, Fishelson & Kashman, 1974), whereas it is harmless to *Acropora formosa*. Sarcophytoxide demonstrates toxicity to both *Acropora formosa* and to fish (Coll, Bowden, Tapiolas, Webb & Marsh, 1983).

Furanoquinol

Both furanoquinol and furanoquinone occur in the same colony of *Simularia capillosa* (Coll, Mitchell & Stokie, 1977). Furanoquinol effected the same response as flexibilide and FN45 in both *Acropora formosa* and *Porites andrewsii*. Conversion of the quinol moiety to the quinone removed this toxicity to *Acropora formosa*, indicating the phenol moiety is the active structure. Phenols are known to uncouple oxidative phosphorylation by binding to the mitochondrial membrane (Hanstein, 1976). The furanoquinol is the more abundant of the two terpenes in *Simularia capillosa* (Personal observation, 1985).

Sponge Terpenes

All the sponge terpenes (Figure 41) tested in this chapter were toxic to symbiotic *Acropora formosa*. These terpenes contain either amino, imino, aldehyde or sulphated groups, all of which are intimated to be toxic. The effect of these sponge terpenes was similar to that of the terpenes isolated from alcyoniidae corals. Hence interference with respiration and photosynthesis is probably a common form of allelopathy effected on scleractinian corals by other sessile marine organisms.

Bakus (1981) made the observation that the Crown of Thorns Star Fish, *Acanthaster planci*, does not predate alcyonacean corals. However, the destruction of a colony of *Nephthea* sp. by *Acanthaster planci* was recorded by Chalker (personal communication, 1984) (Plate 26). Dunlap (personal communication, 1983) also noted the destruction of a *Xenia* sp. by the Blue Starfish, *Linckia lavigata*. Both the *Nephthea* and the *Xenia* genera are rich in sesqui- and di-terpenes (Tursch, 1976; Faulkner, 1984). Parrot fish and butter fish were seen pecking a colony of *Sarcophyton stolidotum* over three days (Tapiolas, personal communication, 1983; personal observation, 1983). This colony contained sarcophytoxide (Personal observation, 1983). Another *Xenia* sp. was found nestled in the branches of an *Acropora formosa* *in situ* (Plate 29). Studies on animals indicate

PLATE 29

Xenia SP. AND *Acropora formosa* in situ.



Photograph courtesy J.C.Coll.

none of the coral terpenes tested in this thesis are toxic to frogs or mice (Vickers, personal communication, 1983). Bowden (1984) commented that eggs released from a variety of alcyoniidæan corals were voraciously eaten *in situ* by fish. Eggs from some but not from all of the colonies were shown to contain terpenes (Bowden, 1985). Thus it seems that the presence of terpenes in alcyoniidæan corals does not necessarily render the corals immune from attack, nor injurious to their coralline neighbours.

8.5 SUMMARY

The response of the two scleractinian corals, *Acropora formosa* and *Porites andrewsii*, to toxic terpenes was an initial increase in the respiratory rate followed by a gradual decline. This might be due to uncoupling of oxidative phosphorylation in mitochondria as a result of the molecule binding to the mitochondrial membrane. Concomitant with this alteration in respiration rate was a similar alteration in the rate of photosynthesis. Toxicity was terpene dependent. Symbiotic *Acropora formosa* was more sensitive than symbiotic *Porites andrewsii*. Aposymbiotic *Acropora formosa* was even more sensitive suggesting the zooxanthellae offer a degree of protection from the toxic effects of terpenes. The effect of these terpenes was temporary, and reversible at low concentrations.

8.6 FUTURE EXPERIMENTS

Witt (1979) constructed a mathematical model to describe the changes in electric potential indicating carotenoid absorbance at 515 nm induced by flash illumination of chloroplasts. With a more extensive set of responses for each coral species to each of the test terpenes, it might be possible to apply this model to the change in rates of both respiration and photosynthesis recorded in scleractinian corals after a pulse of terpene.

The general observation that antiinflammatory drugs uncouple mitochondria has led to the suggestion that a screen for

antiinflammatory drugs be set up using isolated mitochondria (Noack, 1981). It might be that the terpenes from alcyoniidæan corals that were active in stimulating respiration in scleractinian corals, also have antiinflammatory properties. This is true for flexibilide (Arrigoni-Martelli, 1981).

SUMMARY

In response to the limited knowledge of the biochemistry of terpenes in alcyonacean corals, this dissertation examined a range of topics pertaining to the suggested biological role of these terpenes in the reef community. The following points were established:

(1) A method for determining the concentration of sarcophytoxide in a *Sarcophyton* spp. was developed using gradient-elution HPLC on a reverse-phase silica column to separate the components of a dichloromethane extract of the coral. Ultraviolet absorption was used to quantitate the amount of the selected terpene present. This technique was used to quantify sarcophytoxide, sarcophine and deoxysarcophytoxide in *Sarcophyton* spp., and flexibilide in *Simularia flexibilis*.

(2) Sarcophytoxide was concentrated in the gonadal tissues and in the capitulum of a colony of *Sarcophyton boletiforme*, although the terpene was present throughout the colony. The gonadal location of this terpene was supported by detection of FN45 in the egg-bearing gonadal tissue of *Lobophytum carnatum*, and by the presence of sarcophine in the released eggs of a *Sarcophyton stolidotum*. The seasonal appearance of furanoacid in whole colony extracts of *Simularia capillosa* coincided with the mass spawning of corals on the same reef, suggesting that furanoacid is located in and synthesised by the gonads of this coral.

(3) The terpene composition of the growing edges of *Sarcophyton boletiforme* and *Simularia flexibilis* did not alter with season or with reproductive status of the corals. Thus, sampling a colony at the growing edge is a satisfactory method of obtaining samples for chemotaxonomic studies, and sampling the whole colony allows the changes in terpene composition of the gonads to be monitored.

(4) Incorporation of ^3H from 2- ^3H -mevalonic acid into

furanoquinol and sarcophytoxide, sesqui- and di-terpenes respectively, was achieved *in vivo* under all incubation conditions. Incorporation of 2-³H-mevalonic acid into both furanoquinol and sarcophytoxide was not stressed induced; in fact it was suppressed in colonies incubated under stress. There was peak incorporation of 2-³H-mevalonic acid into the two terpenes in the months preceding spawning, and a 90 percent drop in the level of incorporation after spawning. This observation further supports the thesis that terpenes are concentrated in the eggs of alcyoniidae corals.

(5) Both sarcophytoxide and sarcophine were found in aposymbiotic colonies of *Sarcophyton* spp. in quantities comparable to the concentration of the same terpenes contained in symbiotic colonies. Likewise, furanoquinol occurred in large quantities in aposymbiotic *Simularia capillosa*. This suggests terpenes are a product of the coral animal. This thesis is supported by the observation that flexibilide was concentrated in the animal cytosol of *Simularia flexibilis*, and that 2-³H-mevalonic acid was incorporated into sarcophytoxide and into furanoquinol by both symbiotic and aposymbiotic colonies of *Sarcophyton* spp. and *Simularia capillosa*, respectively. However, sudden cessation of photosynthesis in symbiotic colonies caused an immediate decrease in the rate of terpene synthesis, further demonstrating the inhibition of terpene synthesis under stress.

(6) The epidermis of the autozooids of both symbiotic and aposymbiotic *Sarcophyton boletiforme* and *Sarcophyton stolidotum* colonies that contained sarcophytoxide, exhibited cells full of osmiophilic vesicles. These cells formed a confluent layer along the outer epithelial edge, and were also scattered throughout the epithelial tissues. No secreted osmiophilic substance was seen except in the mucus outside the coenchymal epithelium of an aposymbiotic colony. These vesicles were not observed in a *Sarcophyton infundibuliforme* in which neither sarcophytoxide nor any other terpene was detected. This is the first microscopical evidence of microsomal synthesis of diterpenes.

(7) Conversion of sarcophytoxide to deoxysarcophytoxide as suggested by (Coll, Tapiolas, Bowden, Webb & Marsh, 1983), was not observed in the egg cowrie, *Ovula ovum*, after voluntary ingestion of *Sarcophyton* species which contained sarcophytoxide, nor *in vitro* incubations of various sections of the *Ovula ovum* digestive tract, including the digestive gland. Neither sarcophine nor sarcophytoxide was toxic to the cowrie, but FN45 was implicated in the death of the only cowrie exposed to FN45. The various terpenes were retained by the cowrie digestive gland for different durations. Deoxysarcophytoxide was isolated from several sarcophytoxide containing *Sarcophyton* spp.

(8) The response of the two scleractinian corals, *Acropora formosa* and *Porites andrewsii*, to toxic terpenes was an initial increase in the respiratory rate followed by a gradual decline. This might be due to uncoupling of oxidative phosphorylation in mitochondria as a result of the molecule binding to the mitochondrial membrane. Concomitant with this alteration in respiration rate was a similar alteration in the rate of photosynthesis. Toxicity was terpene dependent. Symbiotic *Acropora formosa* was more sensitive than symbiotic *Porites andrewsii*. Aposymbiotic *Acropora formosa* was even more sensitive suggesting the zooxanthellae offer a degree of protection from the toxic effects of terpenes. The effect of these terpenes was temporary, and reversible at low concentrations.

This dissertation adds support to the thesis that the sesqui- and di-terpenes found in alcyoniidae corals are biologically active and are actively synthesized by the coral animal. The terpenes are not omnipotent as they do not always deter nor poison predating molluscs or fish, and are not always toxic to scleractinian corals. Possibly the terpenes are part of a multi-component defence system.

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