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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

I. STEROLS OF COELENTERATES

II. AMINO ACID COMPOSITION OF PEPTIDES FROM THE SPICULES OF *BRIAREUM ASBESTINUM*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

MARTIN STUART SILBERBERG

Norman, Oklahoma

1971

I. STEROLS OF COELENTERATES

II. AMINO ACID COMPOSITION OF PEPTIDES FROM THE SPICULES OF *BRIAREUM ASBESTINUM*

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PLEASE NOTE:

Some Pages have indistinct print. Filmed as received.

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DEDICATION

To my wife, Susan, who nagged me when I needed it, encouraged me when I wished for it, agonized over me far more than her share, yet somehow managed to love me through the whole ordeal.

ACKNOWLEDGEMENTS

In the course of five years, many people enter one's life and remain as part of one's total experience. It is difficult, if not impossible, to acknowledge them all. The attempt made here therefore is undoubtedly incomplete and, for the most part, refers only to those who helped, in one way or another, with the completion of this dissertation.

First, I would like to express my deepest appreciation to my major professor, Dr. Leon S. Ciereszko for his patience and encouragement. His keen intellect and incredible insight have been most inspiring; I only hope that some of it may have "rubbed off."

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advice and suggestions concerning the first draft of this manuscript.

I am indebted to Dr. Ralph A. Jacobson not only for his suggestions on the first draft of this dissertation, but also for his patience in putting up with my impatience during our tribulations with the amino acid analyzer.

I am very grateful to three people who helped with the collection of data: Mr. Keith Kinneberg, Department of Biochemistry, Oklahoma State University for the combined GC-MS runs; Dr. Robert Delaney, Department of Biochemistry, Oklahoma Medical Research Foundation for the Biochrom amino acid analyses and his valuable suggestions on their interpretation; and Dr. Tammanur Pattabhiraman for his advice and assistance with the gas chromatographic analyses; in retrospect even his own particular brand of wit and wisdom will be warmly remembered.

To my friends in the Department, I say "Thanks", and apologize for this inept expression of my deep affection for them.

My family has loved and supported me ever since I uttered my first scream. I have uttered countless numbers since then, yet their affection has never dwindled. I only hope they know how deeply I treasure their love.

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PART I

STEROLS OF COELENTERATES

INTRODUCTION

The comparative biochemistry of sterols had its beginnings when Charles Dorée's pioneering treatise, The Occurrence and Distribution of Cholesterol and Allied Bodies in the Animal Kingdom, appeared in 1909 (Dorée, 1909). Dorée speculated upon the vital importance of the sterols, suggesting that if cholesterol was not universally present in animals, some other "allied body" might take its place, perhaps even to the extent of a characteristic sterol for each major class of animal. He proceeded to isolate and characterize the sterols from a few representatives of each of the major phyla and found sterols present in every case, supporting his speculation about the importance of these compounds; but he did not find a sterol characteristic of each phylum. Nevertheless, Doree did show that in a sponge and a starfish, cholesterol was replaced by similar but not identical compounds.

However, for nearly a century prior to Dorée's work, ever since the discovery of "cholesterine" in gallstones by Chevreul in 1816, evidence had accumulated indicating the almost exclusive presence of cholesterol in vertebrates. The unfortunate extrapolation was apparently made that cholesterol was the sole animal sterol, and Dorée's exciting and potentially stimulating results seemed to have fallen on deaf ears. For the quarter century following his studies, still more reports of cholesterol in vertebrates continued to appear, serving only to entrench the idea of the ubiquity of this sterol.

Elucidation of the structure of cholesterol in 1932 at last provided the impetus for more intensive comparative studies until in 1949, drawing largely from his own work and from studies by Toyama in Japan, Bergmann published the only comprehensive survey on sterols from marine invertebrates, the source that had seemed so promising from Dorée's original investigations (Bergmann, 1949). It was becoming more and more obvious that the greatest diversity of sterols appeared in the lower animal forms and that, as had already been so often shown, cholesterol was preeminent among sterols from animals with a more recent evolutionary origin.

In 1962, Bergmann published a survey of the structures

and distribution of all known animal sterols and the picture became still clearer (Bergmann, 1962). A phenomenon of biochemical evolution seems to have occurred with the sterols: that is, the gradual disappearance of redundant or less efficient compounds with the appearance and eventual dominance of molecules more aptly suited to their biological roles.

Because of their unique position in evolutionary history, the coelenterates should occupy a critical position in any comparative survey of animal sterols. Present day coelenterates are the nearest living representatives of the ancestral stock of all other multicellular animals (Hill and Wells, 1963). Indeed, the fossil record shows some members of this phylum existing in Cambrian times almost 500 million years ago (Moore, 1963).

Bergmann (1962) lists the sterols of only twenty of the more than ten thousand members of this phylum. Undoubtedly, he would have been the first to suggest that a much greater effort was needed in studying the sterols of this important group of animals.

More recent work in this laboratory (Wolf, 1963; Ciereszko, et al., 1968), in other laboratories in this Department (Schmitz and Pattabhiraman, 1970; Gross, 1969), and elsewhere (Gupta and Scheuer, 1969) has resulted in a

significant increase in the number of species investigated. The diversity of sterols displayed is indeed remarkable. The structure of gorgosterol, the C_{30} sterol first seen by Bergmann, et al. (1943), has recently been elucidated (Hall, et al., 1970; Ling, et al., 1970). Its occurrence in coelenterate sterol mixtures has become almost commonplace, and has now been followed by its next-closest relative, 23-demethylgorgosterol (Schmitz and Pattabhiraman, 1970).

Many marine invertebrates that inhabit warm, shallow waters and all reef-building corals contain large numbers of unicellular algae, green zoochlorellae or brown zooxanthellae, living symbiotically within their tissues (McLaughlin and Zahl, 1964; Yonge, 1966). The question of their function within the animal has been the subject of much controversy. At present, it is thought that they are involved in the removal of excretory products from their host animals (Yonge, 1966), are of fundamental importance in the deposition of calcium carbonate and the growth of the reef (Goreau, 1963), and may protect their hosts by the secretion of toxic compounds (Ciereszko, et al., 1962). The occurrence of gorgosterol in several algae-bearing marine invertebrates and in their isolated algae is of particular interest here (Ciereszko, et al., 1968).

The aim of this present study is to increase our knowledge of the sterols from coelenterates and their symbiotic algae. It is hoped that these results may prove relevant to future studies on the evolutionary history of the sterols and on the biochemistry of algal symbiosis.

Species Examined

Table 1 below lists the animal sources of the sterol fractions analyzed in the following sections of this dissertation. Due to the absence of a complete classification for the symbiotic algae, they are not included.

TABLE 1

CLASSIFICATION OF ANIMAL STEROL S

Genus species (Authority)	Class (Subclass)	Order (Suborder)
Paragorgia arborea (Linnaeus)	Anthozoa (Alcyonaria)	Gorgonacea (Scleraxonia)
Clathraria rubrinodis (Gray)	Anthozoa (Alcyonaria)	Gorgonacea (Scleraxonia)
Primnoa resediformis (Gunnerus)	Anthozoa (Alcyonaria)	Gorgonacea (Holaxonia)
Eunicella verrucosa (Pallas)	Anthozoa (Alcyonaria)	Gorgonacea (Holaxonia)
Leptogorgia hebes (Verrill)	Anthozoa (Alcyonaria)	Gorgonacea (Holaxonia)
Leptogorgia setacea (Pallas	Anthozoa (Alcyonaria)	Gorgonacea (Holaxonia)
Virgularia presbytes (Bayer)	Anthozoa (Alcyonaria)	Pennatulacea
Heliopora coerulea (Pallas)	Anthozoa (Alcyonaria)	Coenothecalia
Palythoa mammillosa	Anthozoa (Zoantharia)	Zoanthidea
Metridium senile (Verrill)	Anthozoa (Zoantharia)	Actiniaria
Anthopleura elegantissima (Brandt)	Anthozoa (Zoantharia)	Actiniaria
Anthopleura xanthogrammica (Brandt)	Anthozoa (Zoantharia)	Actiniaria
Distichopora violacea (Pallas)	Hydrozoa	Stylasterina

TABLE 1

ATION OF ANIMAL STEROL SOURCES

Order (Suborder)	Family (Subfamily)	Reference
Gorgonacea (Scleraxonia)	Briareidae	Deichmann (1936), p. 87
Gorgonacea (Scleraxonia)	Melithaeidae	Bayer (1963), p. F200
Gorgonacea (Holaxonia)	Primnoidae (Primnoidae)	Deichmann (1936), p. 157
Gorgonacea (Holaxonia)	Plexauridae	Deichmann (1936), p. 92
Gorgonacea (Holaxonia)	Gorgoniidae	Deichmann (1936), p. 179; Bayer (1954), p. 280
Gorgonacea (Holaxonia)	Gorgoniidae	Deichmann (1936), p. 179; Bayer (1954), p. 280.
Pennatulacea	Virgulariidae	Bayer (1952)
Coenothecalia	Helioporidae	Wells (1954), p. 474
Zoanthidea		Attaway (1968)
Actiniaria	Sagartidae	Ricketts and Calvin, (1962) p. 321
Actiniaria	Cribrinidae	Ricketts and Calvin, (1962) p. 28
Actiniaria	Cribrinidae	Ricketts and Calvin, (1962) p. 51
Stylasterina	Stylasteridae	Wells (1954), p. 476

EXPERIMENTAL PROCEDURE

Sample Collection and Initial Preparation

Primnoa resediformis and *Paragorgia arborea* were collected by Dr. Roland L. Wigley aboard the research vessel Albatross IV of the United States Fish and Wildlife Service, Bureau of Commercial Fisheries, Biological Laboratory, Woods Hole, Massachusetts. The specimens were dredged during Cruise 66-1 at Station 120 on the southern margin of East Georges Bank in February, 1966. They were preserved for several days in 7% formaldehyde, rinsed in fresh water, and air-dried.

Eunicella verrucosa was collected off Plymouth, England in September, 1958 by the staff of the Marine Laboratory of the Marine Biological Association of the United Kingdom. The material was oven-dried.

Clathraria rubrinodis was collected by Professor Leon S. Ciereszko in the Red Sea off Al Ghardaqa, United Arab Republic in December, 1963. The air-dried sample was identified by Dr. F.M. Bayer, Associate Curator, Division of Marine Invertebrates, U.S. National Museum.

Heliopora coerulea and Distichopora violacea were collected and air-dried at Eniwetok by Dr. J.W. Mizelle in 1967 and Professor Ciereszko in August, 1969, respectively.

Virgularia presbytes was collected by Professor Ciereszko in March, 1970 on Padre Island, Texas. The specimens were washed up onto the beach while still alive and then allowed to air-dry.

Leptogorgia hebes and L. setacea were both collected by Professor Ciereszko on the south side of the south jetty at Port Aransas, Texas in June, 1969 and April, 1968 respectively. The animals were air-dried.

Metridium senile was collected by dredging in Puget Sound in May, 1964 by Professor Ciereszko. The samples were then oven-dried.

Palythoa mammillosa was collected off the south shore of Jamaica by Dr. D.H. Attaway in June 1967. Small pieces of rock and other debris were manually removed and the animal pieces stored in dry ice.

Brown and green Anthopleura elegantissima were collected near Light House, San Juan Island in August, 1965, and Anthopleura xanthogrammica was collected off Olympic Peninsula, Washington in May, 1964 by Professor Ciereszko. A. elegantissima specimens were preserved in 70% ethanol.

The algae from Anthopleura were isolated by Professor Ciereszko in the following way: the live sea anemones were cleaned by rinsing with sea water, and were then broken up in a Waring blender with filtered sea water. Blending was sufficient to break up the algae-containing tentacles, but was stopped before any significant homogenization of the animal tissue began. The suspension of algae and coarsely minced animal tissue was strained through a nylon kitchen sieve; the residue on the sieve was rinsed with filtered sea water, and the rinse combined with the suspension of algae which had passed through the sieve. The suspension was then strained through cheesecloth to remove any finer animal particles. The algae were concentrated by centrifugation, resuspended in water, the pH adjusted to 8, and a solution of 1:100 trypsin added. After standing overnight, the suspension was centrifuged to remove soluble material and the pellet washed by repeated suspensions in water and centrifugation until the supernatant was clear. The pellets of whole algae were frozen and later preserved in 70% ethanol.

The lipids of zoochlorellae from Anthopleura xanthogrammica were obtained by ether extraction of the alcohol wash followed by ether extraction of the alcohol-insoluble material. The ether extracts were combined, washed with

water, dried, and freed of solvent on a rotary evaporator.

Preparation of Lipid Extracts

All solvents used were re-distilled, except dimethyl sulfoxide (DMSO) (Analytical reagent; Mallinckrodt).

The specimens were extracted for total lipids by the following methods: samples of *Primnoa resediformis*, *Paragorgia arborea*, *Eunicella verrucosa*, *Clathraria rubrinodis*, *Leptogorgia hebes*, *Leptogorgia setacea*, and *Distichopora violacea* were broken into approximately one-half inch long pieces. All were extracted with hexane in a Soxhlet apparatus, with the exception of *D. violacea* which was extracted with chloroform:methanol (2:1, v/v). Every three days each pot was changed until the final extract was colorless (usually two weeks). The extracts were combined and the solvent removed under reduced pressure at 50°C on a rotary evaporator.

Benzene was added to remove trace amounts of water by codistillation on the rotary evaporator and the dried extract was weighed. Several additions of benzene and extended periods on the rotary evaporator were often needed to arrive at a constant weight. The various extracts ranged in color from yellow-brown to red-brown and exhibited only a faint blue or yellow-green flourescence when viewed under ultra-

violet light.

Brown Anthopleura elegantissima and green A. elegantissima were each filtered through a sintered-glass funnel with suction to remove the ethanol in which the specimens had been preserved. The solvent was removed on a rotary evaporator, chloroform was added to dissolve any lipids, and decanted, later to be combined with the remainder of the lipid extract. The animal pieces were transferred to a Waring blender, enough chloroform:methanol (2:1, v/v) was added to completely immerse all pieces (approximately 300 ml), and the mixture blended at high speed for two minutes. The finely ground pulp was transferred to a sintered-glass funnel and filtered with suction. Blending and filtration were repeated three more times with fresh solvent. The final filtrate was only very slightly colored. The filtrates were combined with the chloroform-soluble material from the ethanol wash, the solvent removed on the rotary evaporator, and benzene added and evaporated three times to remove residual water. In contrast to the previous extracts, these lipid fractions clearly showed a bright red fluorescence, characteristic of chlorophyll-containing mixtures, when viewed under ultraviolet light.

Lipid extracts from the zooxanthellae and zoochlorellae

of Anthopleura elegantissima were prepared by a similar procedure, except that the algae were extracted by refluxing in chloroform:methanol (2:1, v/v), instead of grinding in a blender. Refluxing was carried out for one hour, the mixture filtered, the filter cake returned to the flask with fresh solvent and the refluxing resumed. The final filtrate was colorless after four repeats of this procedure. The combined filtrates were treated as above on the rotary evaporator.

Heliopora coerulea lipids were extracted by Dr. J.W. Mizelle in a continuous percolator-type extraction apparatus with hexane (Ciereszko, 1966).

Virgularia presbytes lipids were extracted by Professor L.S. Ciereszko. The soft, fleshy outer portion of the animal was stripped off the central protein core and extracted in a Soxhlet apparatus with diethyl ether.

Preparation of the Unsaponifiable Fraction

After drying to constant weight, the lipid fractions were saponified by refluxing on a steam bath for one to one and one-half hours with ten parts of 10% potassium hydroxide in 75% ethanol to one part lipid residue (v/w). After saponification, two volumes of water were added and the mixture was extracted in a separatory funnel with chloroform

until the chloroform layer was colorless (usually five or six times with 30-40 milliliters of chloroform). Slowly separating emulsions were formed upon shaking with chloroform. The chloroform extracts that separated relatively quickly (less than two hours) were washed several times with water, freed of solvent on the rotary evaporator and of residual water by codistillation with benzene, and weighed. The weights of unsaponifiable fractions are reported only for those extracts.

In the case of saponification mixtures forming very slowly settling emulsions with chloroform, the bottom layer was separated, the solvent removed as before, and benzene added and codistilled until the residue was clear.

Preparation of the Sterol Fraction

The unsaponifiable fractions were dissolved in 50 milliliters of 90% ethanol by heating on a steam bath. Twenty-five ml of hot 1% digitonin in 90% ethanol were added, the mixture heated for a few minutes and then cooled in a refrigerator for one hour. The precipitated digitonide was collected on a sintered-glass funnel by vacuum filtration, the filtrate heated, 10 ml more of hot digitonin solution added, and the mixture again cooled in the refrigerator. This

procedure was repeated until no further precipitate was obtained. The digitonide was washed with cold 90% ethanol followed by diethyl ether, transferred to a small flask, and weighed.

The sterols were isolated from the digitonide as follows: twenty-five ml of DMSO were added to the digitonide and heated on a steam bath with stirring for 15 minutes (Issidorides, et al., 1962). Longer heating was often necessary to obtain complete solution. The solution was allowed to cool to room temperature and was extracted with small portions (15-20 ml) of hexane six or seven times. The hexane extracts were combined and washed with water to remove residual DMSO; the hexane was removed on the rotary evaporator, and the water removed with benzene. The weighed residue represented the isolated sterol fraction.

The sterol fraction of Metridium senile was isolated by Professor Ciereszko in the following way: the oven-dried animal was ground and extracted with hexane. After removing the hexane by distillation, the extract was saponified in methanolic potassium hydroxide, and the unsaponifiable material extracted with diethyl ether. The ether extract was treated with methanol and the precipitated sterols collected by filtration.

Palythoa mammillosa was being investigated in this laboratory by D.H. Attaway with respect to one of its polar constituents. Sterols were obtained from a methanol wash of the animal and therefore were probably not extracted completely. The isolation procedure was as follows: P. mammillosa, which had been preserved in dry ice since being collected, was extracted with three portions of methanol by standing overnight. The methanol extracts were combined and extracted with pentane in a continuous liquid-liquid extractor until the pentane extract was colorless. The combined pentane extracts were concentrated to approximately 500 ml on the rotary evaporator and stored in the refrigerator. After standing overnight, small clumps of crystals had appeared and were collected by vacuum filtration, washed with a small amount of cold hexane, and recrystallized twice from hot ethanol (melting point, 136-138°C). The white, flaky crystals gave positive Salkowski and Liebermann-Burchard tests attesting to their sterol nature.

The steryl acetate was prepared by refluxing 100 mg of crystals in 5 ml of acetic anhydride for two hours. The acetate crystals, which precipitated upon cooling, were collected on a sintered-glass funnel, washed with cold methanol, and recrystallized twice from chloroform/methanol (melting

point, 148-148.5°C). Only the free sterol mixture was analyzed further by gas chromatography or mass spectrometry.

Gas Chromatographic Analysis of Sterol Fractions

Each sterol mixture was analyzed on two different gas chromatographic columns: (a) five feet x 1/8 inch (O.D.) stainless steel column filled with 3% Silicone Gum Rubber SE-30 on 100-120 mesh Varaport #30; (b) five feet x 1/8 inch (O.D.) stainless steel column filled with 1.5% OV-17 on 80-100 mesh Gas Chrom Q (Applied Science Laboratories, Inc.) The SE-30 column was obtained from Varian Aerograph, Inc.

The OV-17 column packing was prepared according to Horning (1968). Briefly, the procedure used was the following: ten grams of Gas Chrom Q (80-100 mesh) were swirled for 20-25 minutes in 65 ml of a 1% solution of OV-17 in acetone in a side-arm filter flask. Suction was applied 3 or 4 times for a few seconds to dislodge entrapped air. After filtering the slurry on a Buchner funnel for 15 minutes, the material was air dried for 30 minutes and placed in a 110°C oven overnight. The column was filled by adding the oven-dried column packing in small increments while gently bouncing the metal tubing on the floor.

All samples were analyzed on a Varian Aerograph Series

1740 Gas Chromatograph. Conditions for analyses on the SE-30 column were: injection port: 270°C; column: 245°C; hydrogen-flame ionization detector: 265°C; helium carrier gas flow-rate (obtained with a bubble-type flowmeter): 28 ml/min. Conditions for analyses on the OV-17 column were: injection port: 270°C; column: 227 or 231°C; detector: 262°C; helium flow-rate: 25 ml/min. All fractions were dissolved in chloroform and injected by means of a 10 μl syringe (Glenco Scientific, Inc.), using the solvent flush technique.

Commercial samples of cholesterol, campesterol, desmosterol, stigmasterol and β-sitosterol were used to obtain known retention times. Cholestane was used as an internal standard with each sterol mixture. Retention times for 22-dehydrocholesterol, brassicasterol, and 23-demethylgorgosterol were obtained by chromatographing the sterol fraction from Gorgonia ventalina; the retention time for gorgosterol was obtained from the sterols of Pseudoplexaura porosa. Both sterol mixtures were kindly supplied by Dr. T.R. Pattabhiraman. β-sitostanol standard was prepared by hydrogenation. One hundred mg of β-sitosterol were mixed with 43 mg of platinum oxide in 15 ml of 10% absolute ethanol in hexane. The mixture was shaken under 50 psi of hydrogen for 24 hours. The product was isolated by gravity filtration and rotary

evaporation. The shiny, white crystals were recrystallized twice from hexane/methanol, and placed in a vacuum dessicator overnight.

Mass Spectrometric Analysis of Sterol Fractions

Each sterol fraction was analyzed on a Hitachi Perkin-Elmer RMU-7 Mass Spectrometer. Conditions for analyses were: ionizing voltage: 10 and 80 eV; sample inlet temperature: 165°C. Perfluorokerosene was used as an internal standard.

Relative intensities of the mass peaks were measured with a Gerber Variable Scale (Gerber Instruments, Inc.).

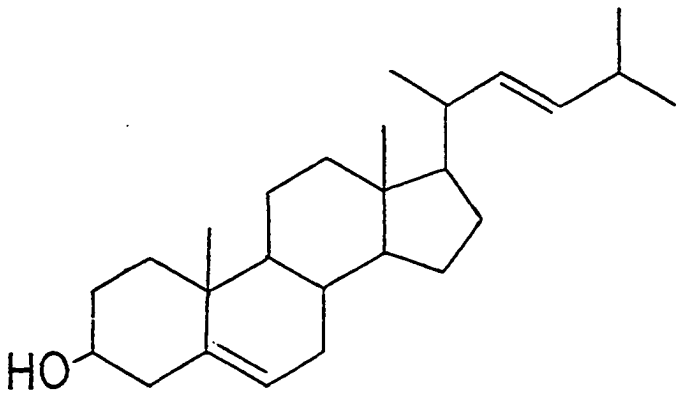
Combined Gas Chromatographic-Mass Spectrometric Analysis of Sterol Fractions

Sterol fractions from C. rubrinodis, E. verrucosa, V. presbytes, and zooxanthellae of brown A. elegantissima were analyzed on a LKB-9000 Gas Chromatograph-Mass Spectrometer (LKB Instruments, Inc.)

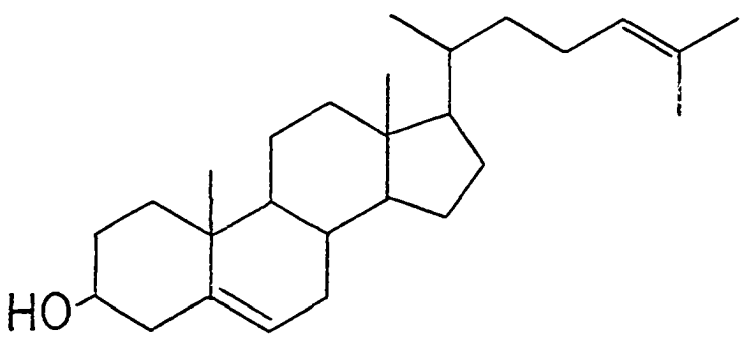
The column used was 1.5% OV-17 on 80-100 mesh Gas Chrom Q. Operating conditions were: injection port: 260°C; column: 215°C; molecular separators: 230°C; ion source: 240°C; ionizing voltage: 70 eV. Helium was used as the carrier gas with a flow-rate of 25 ml/min. The column effluent led directly into the ion source and the ion source current served

as the detector for the GLC trace. Mass spectral scans were taken at several positions along the tracing of the individual peaks.

Figure 1. Structures of Sterols

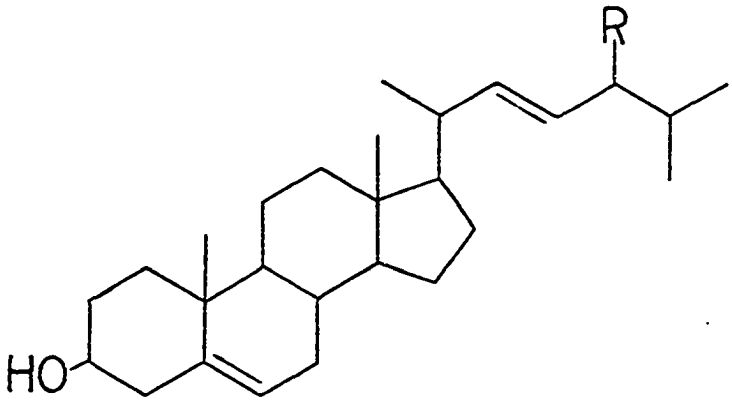


22-trans-24-norcholesta-5,22-diene-3β-ol
(22-dehydro-24-norcholesterol) - 370

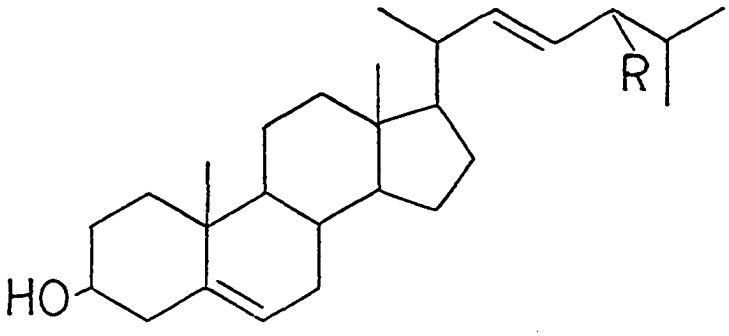


24-dehydrocholesterol (desmosterol) - 384-B

Figure 1 (continued)

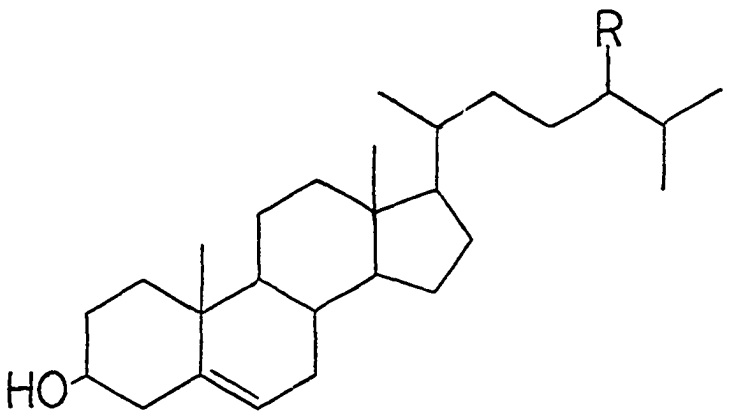


R=H: 22-dehydrocholesterol - 384-A
R=CH_3: 24β-methyl-22-dehydrocholesterol
(brassicasterol) - 398-A
R=CH_2CH_3: 24β-ethyl-22-dehydrocholesterol
(stigmasterol) - 412-A

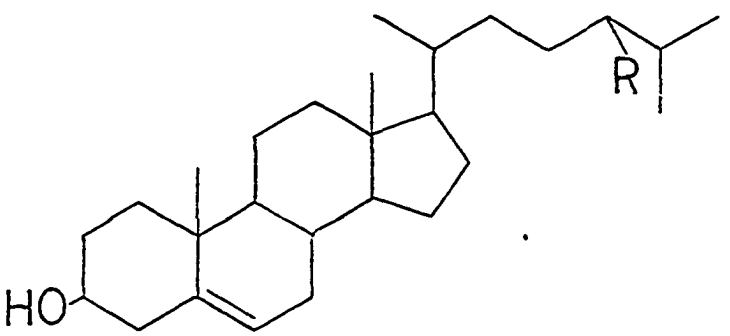


R=CH_3: 24α-methyl-22-dehydrocholesterol - 398-A
R=CH_2CH_3: 24α-ethyl-22-dehydrocholesterol
(poriferasterol) - 412-A

Figure 1 (continued)

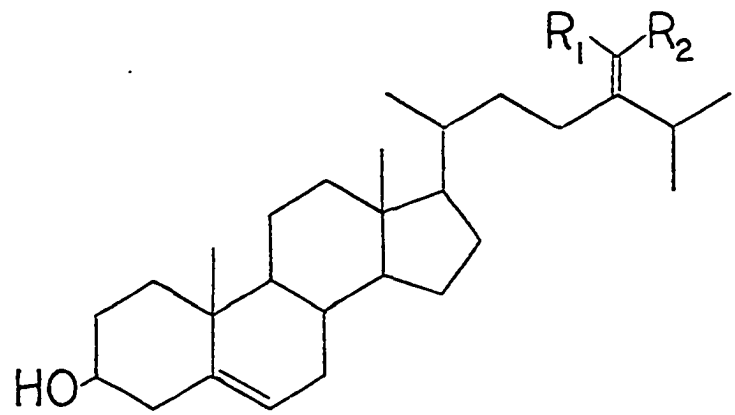


R=H: cholesterol - 386
R=CH_3: 24β-methylcholesterol
(22,23-dihydrobrassicasterol) - 400
R=CH_2CH_3: 24β-ethylcholesterol (β-sitosterol) - 414-B

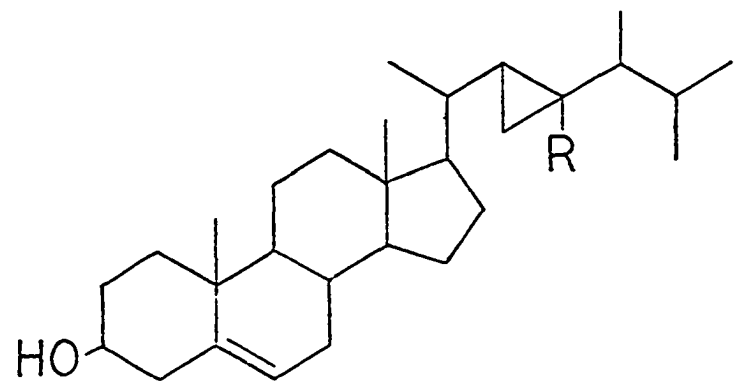


R=CH_3: 24α-methylcholesterol (campesterol) - 400
R=CH_2CH_3: 24α-ethylcholesterol (clionasterol) - 414-B

Figure 1 (continued)



R_1=R_2=H: 24-methylenecholesterol - 398-B
R_1=H, R_2=CH_3: 24-ethylidenecholesterol (fucosterol) - 412-C
R_1=CH_3, R_2=H: 24-ethylidenecholesterol (29-isofucosterol) - 412-C



R=H: 23-demethylgorgosterol - 412-B
R=CH_3: gorgosterol - 426

RESULTS AND DISCUSSION

Comparative Aspects of Lipid Content

Table 2 shows the percentage by weight of the various fractions obtained during the isolation of the sterol fractions. The percentage of unsaponifiable material correlates well with values for other coelenterates and symbiotic algae previously determined in this laboratory and elsewhere (Ciereszko, et al., 1968; Wolf, 1963; Bergmann, 1962). The amount of total lipids varies considerably from less than 1% to greater than 30% of the sample weight in the anemone, Anthopleura elegantissima. Since the anemones and their symbiotic algae were the only specimens not dried prior to extraction, the amount of lipids calculated, on a dry weight basis would no doubt be significantly higher. This difference in the amount of lipids present is due, at least in part, to the absence of any inorganic skeleton in the anemones, resulting necessarily in more organic matter per unit weight. In addition, several workers have seen greater than a twofold increase in lipid content among several species of marine

TABLE 2

FRACTIONS OBTAINED DURING STEROL ISOLATION[1,2]

Sample Name	Lipids in Sample	Sterols in Lipids	Unsap. in Lipids	Sterols in Unsap.
Paragorgia arborea	5.44	6.45		
Clathraria rubrinodis	0.357	6.73		
Primnoa resediformis	4.02	1.60		
Eunicella verrucosa	1.86	5.31		
Leptogorgia hebes	1.54	11.7	45.1	26.0
Leptogorgia setacea	4.59	25.2	54.1	46.5
Virgularia presbytes	8.21	2.92	31.9	9.15
Heliopora coerulea		1.80	37.2	4.83
Anthopleura elegantissima (brown)	33.6	1.43		
Zooxanthellae from Anthopleura elegantissima	29.8	5.02		
Anthopleura elegantissima (green)	9.04	12.3		
Zoochlorellae from Anthopleura elegantissima	30.8	2.89	26.6	10.9
Zoochlorellae from Anthopleura xanthogrammica		3.26	15.4	21.2
Distichopora violacea	0.425	1.95		

[1] Numbers are expressed as percentage by weight.

[2] Blank spaces refer to undetermined values.

algae collected in summer rather than winter months, or collected in shallow rather than deeper water (Idler and Wiseman, 1970; Black and Cornhill, 1951). The algae-containing anemones examined here were collected in very shallow water in the month of August.

Upon closer examination of Table 2, several interesting comparisons become more obvious. Leptogorgia hebes and L. setacea have identical classifications, differing only in species. The two animals were collected in very nearly the same location and extracted in identical ways; yet there is a surprisingly large difference in their lipid content. L. setacea not only has more lipids but a larger proportion of sterols in those lipids. And although unsaponifiable material makes up around 50% of the total lipids in both animals, almost one-half of those nonsaponifiable lipids are sterols in L. setacea, whereas in L. hebes only one-fourth are sterols. These differences might be explained in part if the two animals contained different sterols with slightly different functions. However, the gas chromatographic and mass spectrometric data discussed below answer this question and simultaneously pose more far-reaching ones in its stead. For it appears that the two animals contain the same sterols in nearly identical proportions. So we have a case where two

animals of the same genus, living in the same environment, contain the same sterols; yet one species has twice as much of these sterols as the other. A teleological explanation of these facts must await greater biochemical knowledge about the functions of animal sterols.

A somewhat different, but no less intriguing, comparison exists between the two color varieties of the anemone, Anthopleura elegantissima. These distinguishing colors arise from the unicellular algae living symbiotically within the animal's tissues - zoochlorellae in the green Anthopleura, zooxanthellae in the brown. But brown Anthopleura contains almost four times as much lipid as the green. Since the animal is presumably the same, the difference must be due to the algae. However, the zooxanthellae and zoochlorellae from A. elegantissima both contain the same amount of lipid; and therefore the explanation may be in the number of algae per unit volume of animal tissue. If more zooxanthellae inhabited an individual anemone than zoochlorellae inhabited another individual, the resulting brown anemone would contain more lipid than the resulting green one. According to this reasoning then, since the zooxanthellae contain a higher proportion of sterols than do the zoochlorellae, the brown anemone ought to contain more sterol than the green. This

is not the case however - 12% of 9 is more than twice as much as 1.4% of 34 (see Table 2) - and therefore the argument proposing more algae per animal is lacking.

Finally, a third comparison can be made. Anthopleura elegantissima is often considered to be a size and color variation of the giant green anemone, Anthopleura xanthogrammica (Ricketts and Calvin, 1962). The differences between the unsaponifiable content and the sterol content of the unsaponifiables in the values for the two zoochlorellae must then be due to 1) different species of zoochlorellae, synthesizing different amounts of lipid, inhabiting the same animal tissue, and/or 2) the differences in size and color of the anemones are concomitant with differences in lipid make-up of their tissue, which can then appear in their isolated algae. It will be seen below that these are both quantitative and qualitative differences.

Instrumental Analysis of the Isolated Sterol Fractions

Combined Gas Chromatographic-Mass Spectrometric (GC-MS) Analysis of the Sterols from Four Species

The sterol fractions from Eunicella verrucosa, Virgularia presbytes, zooxanthellae of brown Anthopleura elegantissima, and Clathraria rubrinodis were analyzed by combined

GC-MS. The results appear in Figures 2, 3, 4, and 7. Because of the relatively poor resolution in the gas chromatogram of the GC-MS, the figures shown are from the traces obtained with the Varian Gas Chromatograph. Numbers in the chromatograms refer to points at which a mass spectral scan was taken. The accompanying Tables 3 through 6 show the relative intensities of the sterol parent ions present in that particular scan, calculated in percentage of the sum of the parent ion peak heights.

Figure 2 and Table 3 show the results from combined GC-MS of the E. verrucosa sterols. Scan 1 showed no sterol material, but scans 2 through 4 show the occurrence of a sterol with a molecular weight of 370, corresponding to $C_{26}H_{42}O$. The mass spectrum of this sterol is identical with that of 22-trans-24-norcholesta-5,22-diene-3β-ol recently isolated by Idler, et al. (1970). A similar retention time to their reported value on SE-30 provides additional evidence for the equivalence of these two sterols.

In scans 5 through 7, a molecular ion appears at m/e 384, corresponding to a sterol with molecular formula $C_{27}H_{44}O$. These mass spectra show peaks at m/e 369 [M^+-15(CH_3)], 366 [M^+-18(H_2O)], 351 [M^+-(15+18)], 231 corresponding to loss of the side chain + 42 (ring D fragment), 213 (231-H_2O), 255

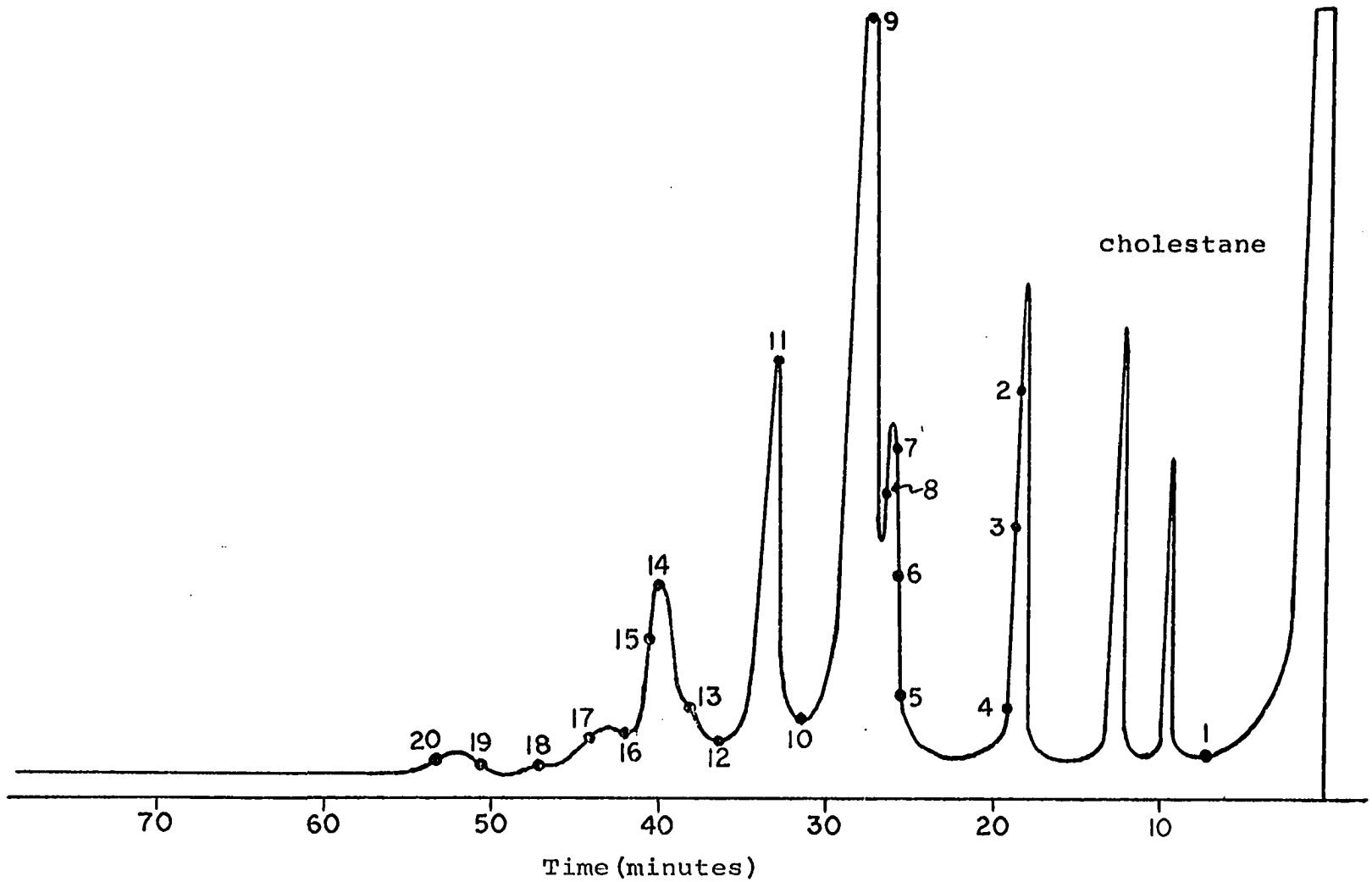


Figure 2. Gas Chromatogram from GC-MS of E. verrucosa Sterols

TABLE 3

MASS SPECTRA FROM GC-MS OF *E. VERRUCOSA* STEROLS

MS-Scan No.	Molecular ion, M^+							
	370	384	386	398	400	412	414	416
1								
2	100							
3	100							
4	100							
5		100						
6		100						
7		100						
8		71	29					
9		7	93					
10		13	50	37				
11			15	85				
12			35	49	16			
13				42	58			
14				62	38			
15				62	28	11		
16				43	23	34		
17				34	18	37		12
18				ns*		ns		
19					23		77	
20					25		75	

* not significantly above background

corresponding to loss of the side chain + H_2O, 271 indicating loss of the side chain + 2xH, and 300 corresponding to loss of C_{22} through C_{27} + 1xH. From these characteristic peaks (Knights, 1967; Wyllie and Djerassi, 1968), and from identical retention times on GC, this material will be tentatively identified as 22-dehydrocholesterol.

A peak at m/e 386 corresponding to $C_{27}H_{46}O$ first appears in scan 8 and then dominates scan 9. This mass spectrum shows intense peaks at m/e 371 (M^+-15), again corresponding to loss of a methyl radical, 368 (M^+-18), indicating loss of H_2O, and 353 (M^+-33), indicating loss of CH_3+H_2O, as well as peaks common to Δ^5-sterols with saturated side chains at m/e 213, 231, 255, and 273. Large mass peaks also occur at m/e 301 [M^+-(18+67[C_5H_7])], 275 (M^+-(18+93[C_7H_9])], and 247 [M^+-(18+121[C_9H_{13}])] (Knights, 1967). From this information as well as retention time and peak enhancement studies with standard cholesterol, the material with molecular weight 386 can be tentatively identified as cholesterol.

With scan 10, 386 begins to decline and a peak at m/e 398 ($C_{28}H_{46}O$) appears for the first time. In scan 11, it becomes the major parent ion with the further disappearance of 386. Mass peaks at m/e 355 [M^+-43(C_{25}-C_{27})] and at 337 [M^+-(43+H_2O)] indicate that this material is a $\Delta^{5,22}$-sterol

(Knights, 1967). Other peaks at m/e 255, 231, 213, 271 [M^+-(side chain + 2xH)], 253 (271-H_2O), and 300 [M^+-(C_{22}-C_{27}+1xH)], and a retention time similar to brassicasterol enables one to tentatively identify this sterol as brassicasterol, or its epimer at C_{24}, 24-α-methyl-22-dehydrocholesterol.

Scans 12 and 13 show the final disappearance of 386 and the appearance of a sterol of molecular weight 400 ($C_{28}H_{48}O$). Mass peaks at m/e 385 (M^+-15), 382 (M^+-H_2O), 367 [M^+-(15+H_2O)], 315 [M^+-(H_2O+67[C_5H_7])], 289 [M^+-(H_2O+93 [C_7H_9])], 261 [M^+-(H_2O+121[C_9H_{13}])] as well as the usual peaks for a Δ^5-sterol at m/e 273, 255, 231, and 213 indicate the presence of campesterol, or its C_{24} epimer, 22,23-dihydrobrassicasterol. Identical retention time and peak enhancement with standard campesterol confirm the mass spectral information.

Also in scan 13 a very intense peak at m/e 314 is seen. Scan 14 shows an increase in a parent ion at m/e 398 and a further increase in the peak at m/e 314. Other peaks at m/e 296 [M^+-(H_2O+C_{23}-C_{27}+1xH)], 281 [296-15(CH_3)], 253 [M^+-(H_2O+ side chain+2xH)], 211 [253-42(ring D fragment)], 255 and 213, strongly indicate the presence of 24-methylenecholesterol (Gupta and Scheuer, 1969; Knights, 1967). It is interesting

to note the transition from one 398 to the other. The combined GC-MS demonstrates this transition elegantly, heralding the latter 398 by its base peak at m/e 314. Even though the mass spectrum of another sterol, 24-methyldesmosterol, is identical to that of 24-methylenecholesterol (Wyllie and Djerassi, 1968), the latter was tentatively identified here for two reasons: 1) the present sterol behaves similarly to isolated 24-methylenecholesterol on GC (Jorque, 1971); and 2) 24-methyldesmosterol is unknown in nature (Lederer, 1969). From the gas chromatogram, 24-methylcholesterol and 24-methylenecholesterol appear together as one broad asymmetric peak. They are inseparable on both OV-17 and SE-30, but only on OV-17 does the peak even show any appreciable asymmetry. For this reason, accurate calculation of the areas for the separate compounds was extremely difficult and therefore when they are both present in the same sterol mixture, their values appearing in Table 10 incorporate this relatively large error in peak measurement.

In scan 15, an ion of molecular weight 412, corresponding to $C_{29}H_{48}O$ appears, and then finally dominates the spectrum in scan 17, while the peaks at m/e 398 and 400 simultaneously decrease in intensity. Mass peaks at m/e 369 [M^+-43(C_{25}-C_{27})], 351 [M^+-(43+H_2O)], and 300 [M^+-(C_{22}-C_{27}+1xH)]

indicate a $\Delta^{5,22}$-sterol. The presence of mass peaks characteristic of diunsaturated sterols at m/e 271-273, 253-255, 231, and 211-213, as well as identical retention times and peak enhancement with standard stigmasterol are strongly consistent with this material being stigmasterol or its C_{24} epimer, poriferasterol.

Also in scan 17, a peak appears at m/e 416, but since this ion occurs in much greater proportion in the sterol fraction from Clathraria rubrinodis, it will be discussed at that point.

Scan 18 shows only very small peaks at m/e 412 and 398, but scan 19 shows an intense parent ion at m/e 414 corresponding to $C_{29}H_{50}O$. Mass peaks characteristic of a Δ^5-sterol with a saturated side chain appear at m/e 329 [M^+-(H_2O+67 [C_5H_7])] and 303 [M^+-(H_2O+93[C_7H_9])] (Knights, 1967). Other peaks at m/e 273, 255, 231, and 213, as well as identical retention times and peak enhancement with standard β-sitosterol then allows the probable identification of this material as β-sitosterol or its C_{24} epimer, clionasterol.

In addition to the 414 ion, scans 19 and 20 show a peak at m/e 412. It is difficult to say definitely whether this represents a different compound of molecular weight 412 or merely some trace of stigmasterol discussed previously.

Whatever the case may be, there definitely is mass spectral evidence for a different parent ion at m/e 412 in the sterols of Virgularia presbytes discussed below.

Figure 3 and Table 4 show the results of the combined GC-MS analysis of the sterol fraction from the sea pen, Virgularia presbytes.

Scan 1 was taken before sample injection and therefore serves as a background scan. As previously, with the sterols from E. verrucosa, the first sterol material appears in scans 2 through 4 and has a molecular ion at m/e 370. Its mass spectrum is identical with the 370 sterol from E. verrucosa and therefore will be tentatively identified again as 22-trans-24-norcholesta-5,22-diene-3β-ol.

A molecular ion at m/e 384 (22-dehydrocholesterol) appears next and gradually diminishes until in scan 9 none is seen. At the same time, the ion at m/e 386 (cholesterol) appears and dominates scans 7 through 9. Also in scan 9, a molecular ion at m/e 398 (brassicasterol or its epimer) is first seen and in the next scan, taken at the top of the peak, it dominates the spectrum.

Scan 10 also shows the final disappearance of 386 and interestingly, a reappearance of a molecular ion at m/e 384. In scan 11, coinciding with the top of the shoulder in the

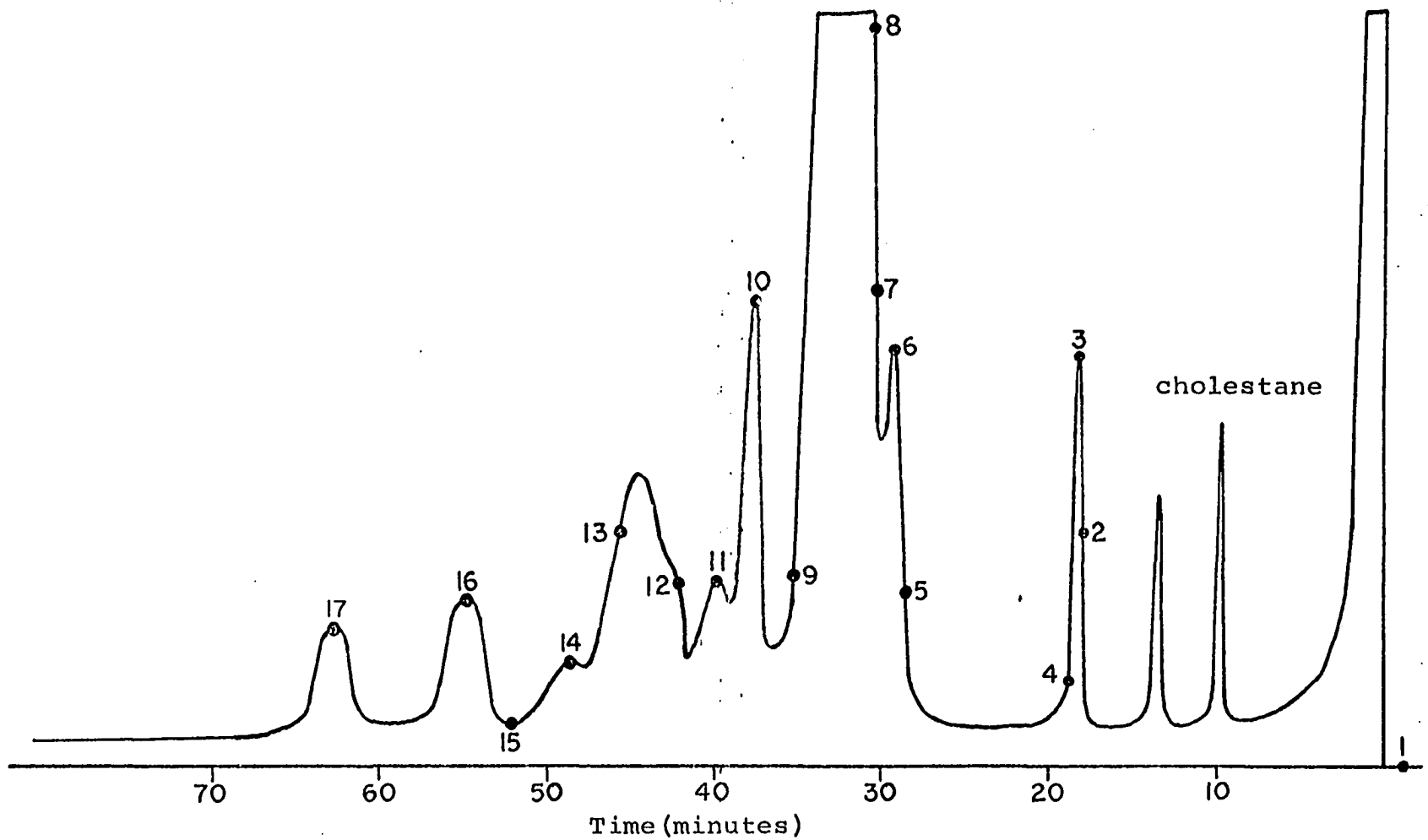


Figure 3. Gas Chromatogram from GC-MS of V. presbytes Sterols

TABLE 4

MASS SPECTRA FROM GC-MS OF *V. PRESBYTES* STEROLS

MS-Scan No.	Molecular ion, M^+						
	370	384	386	398	400	412	414
1							
2	100						
3	100						
4	100						
5		100					
6		59	41				
7		24	76				
8		9	91				
9			90	10			
10		8	10	82			
11		56		44			
12				23	77		
13				45	36	19	
14				16	12	72	
15				28		46	25
16						12	88
17						71	29

GC trace, 384 becomes the major parent ion. This mass spectrum shows peaks at m/e 369 (M^+-15), 366 (M^+-H_2O, 351 [M^+-(15+H_2O)], 273, 255, 253, 231, 213, and 211. These peaks also occur in the spectrum of ion 398 in scan 10 and although they are reported in the mass spectrum of desmosterol, a $\Delta^{5,24}$ C_{27} sterol with a molecular weight of 384 (Knights, 1967), one cannot conclusively assign these ions to 384 since a significant amount of molecular ion 398 still appears in scan 11. However, a base peak at m/e 271 [M^+-(side chain+2xH)] (Wyllie and Djerassi, 1968), and retention time and peak enhancement with standard desmosterol confirms its presence here.

Scan 12 is dominated by a molecular ion at m/e 400 previously identified as 24-methylcholesterol (campesterol or 22-dihydrobrassicasterol) and also shows the presence of some material with a molecular weight of 398. An intense peak at m/e 314 once again signifies the presence of 24-methylenecholesterol, part of the broad unresolved peak discussed previously. On the far side of the peak, scan 13 is taken, showing the dominance of 24-methylenecholesterol and the first appearance of a parent ion at m/e 412. Previously designated as the molecular ion of stigmasterol (or poriferasterol), this peak increases in scan 14, taken at the top of the shoulder, and then decreases in scans 15 and 16 with the

concomitant rise in intensity of the molecular ion of β-sitosterol (or clionasterol) at m/e 414.

A final peak is seen in the GC trace after β-sitosterol. Scan 17, taken of this peak, shows a reappearance of a parent ion at m/e 412. This mass peak, in addition to peaks at m/e 397 (M^+-15), 394 (M^+-18), 379 [M^+-(15+18)], 213, 231, and 255 strongly indicate a diunsaturated C_{29} sterol. A base peak at m/e 314 and a large peak at m/e 296 suggest several possible candidates. As mentioned previously in the discussion of 24-methylenecholesterol, the intense peak at m/e 314 occurs in sterols having a double bond in the $\Delta^{24(28)}$ position and has been rationalized in terms of a "McLafferty" type of rearrangement (Wyllie and Djerassi, 1968). The peak at m/e 296 then is presumably caused by a further loss of water. Four possible $\Delta^{24(28)}$-sterols could produce such peaks: fucosterol, 29-isofucosterol and their two Δ^7-isomers (Knights, 1965; Knights and Laurie, 1967). Recently, a new sterol with a molecular weight of 412 has been isolated from the purple sea fan, Gorgonia ventalina (Schmitz and Pattabhiraman, 1970). Interestingly enough, its side chain has been shown to contain the cyclopropyl moiety first seen in the side chain of gorgosterol (Hale, et al., 1970; Ling, et al., 1970). This new sterol, called 23-demethylgorgosterol,

also produces a mass spectrum with a base peak at m/e 314, caused by cleavage of the cyclopropane ring with the loss of carbons 23 through 29. However, 23-demethylgorgosterol also shows a peak at m/e 328 caused by a different cleavage of the cyclopropane ring with the loss of carbons 23 through 28. The absence of this peak in scan 17 as well as a different retention time on GC eliminates 23-demethylgorgosterol as a possibility.

In addition to having a large peak at m/e 314, the $\Delta^{7,24(28)}$-ethylidenecholesterols show a base peak at m/e 271 (M^+-side chain+2xH) (Knights and Laurie, 1967). The absence of a 271 peak of comparable relative intensity in scan 17 eliminates the Δ^7 compounds as possibilities also.

This material appearing as the major parent ion in scan 17 is therefore tentatively identified as fucosterol or its C_{29} isomer.

Figure 4 and Table 5 show the combined gas chromatogram-mass spectra of the sterol fraction from the zooxanthellae of brown Anthopleura elegantissima.

The first six scans show parent peaks at m/e 279, 299, 350, 366, 372, and 378. Although most show a peak corresponding to loss of methyl radical at M^+-15, none of these shows a peak at M^+-18 (loss of water) common to all sterols.

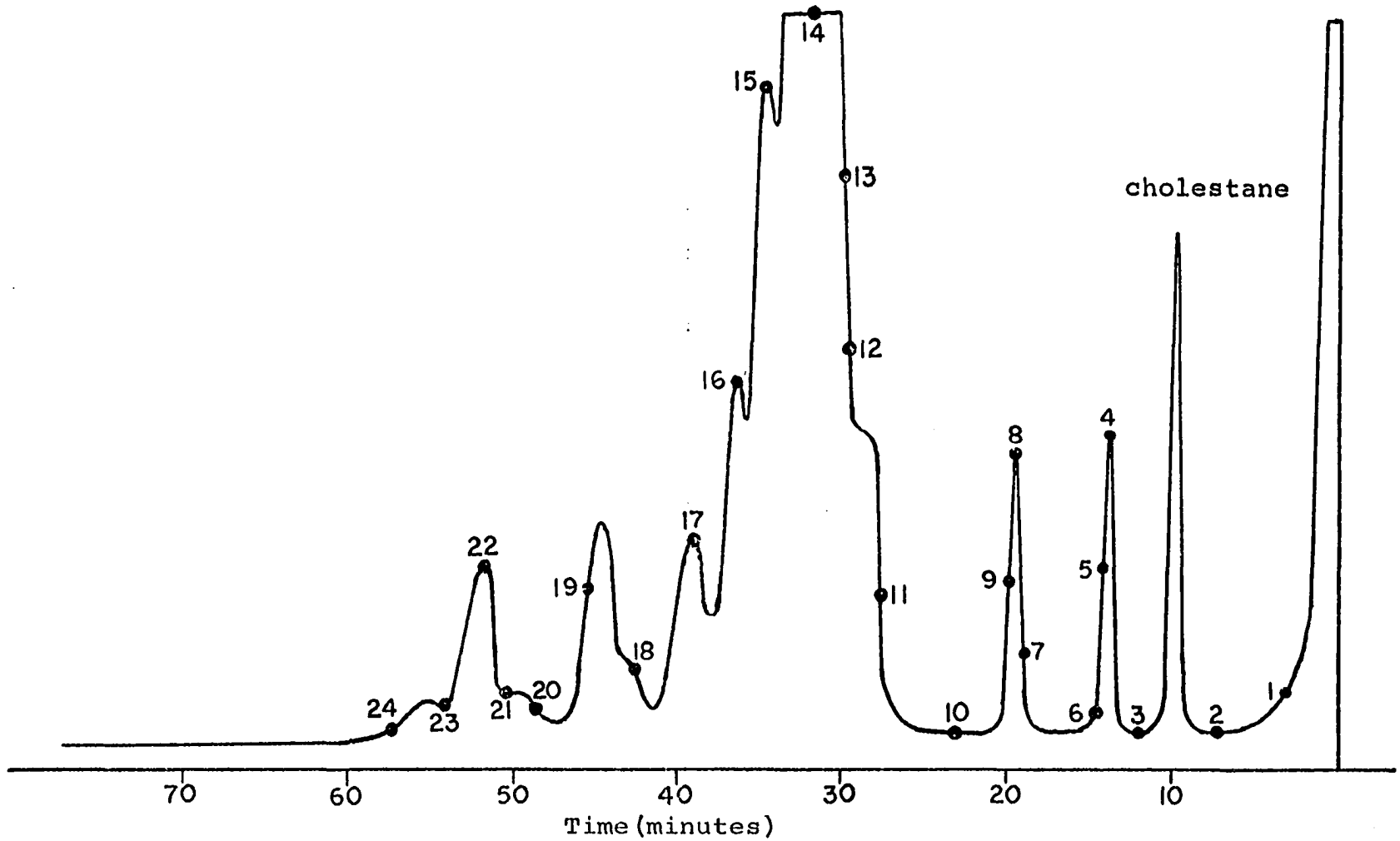


Figure 4. Gas Chromatogram from GC-MS of Sterols from Zooxanthellae of A. elegantissima

TABLE 5

MASS SPECTRA FROM GC-MS OF STEROLS FROM ZOOXANTHELLAE OF A. ELEGANTISSIMA

MS-Scan No.	Molecular ion, M^+									
	370	384	386	398	400	402	412	414	416	428
1 to 6										
7	100									
8	100									
9	100									
10										
11		100								
12		26	74							
13		15	85							
14		9	91							
15			14	34		52				
16			33	51		16				
17				18		11		71		
18				23	57			20		
19				42	25		18	15		
20				16			10	10	64	
21							7	12	61	20
22								13	14	72
23								76		24
24							21	66		13

Only the ion at m/e 366 which dominates scans 4 through 6 shows peaks of any significant intensity above m/e 200 and these at m/e 253 and m/e 247.

Once again the first sterol material to appear is 22-dehydro-24-norcholesterol which, after scan 10 taken between GC peaks, is followed as before by a molecular ion at m/e 384 (22-dehydrocholesterol) beginning in scan 11; this is followed in turn by the parent ion at m/e 386 (cholesterol) in scan 12. By scan 15 taken on the shoulder immediately following cholesterol, 384 has disappeared entirely, 386 has diminished greatly, and 398 (brassicasterol, or its C_{24} epimer) has made its first appearance. But the spectrum is dominated by a molecular ion, not seen previously, at m/e 402, possibly corresponding to a saturated sterol with molecular formula $C_{28}H_{50}O$ (hereafter referred to as Zx402). Parent ion 402 produces fragment ions at m/e 387 (M^+-15), 384 (M^+-18), and large peaks at m/e 229, 247, and 248. These peaks, coupled with relatively small peaks at mass values normally associated with saturated sterols, (e.g. m/e 215 and 233), may suggest an unusual steroid nucleus. Unfortunately, scan 15 also contains parent ions at m/e 398 and 386 and therefore assignment of fragment ions specifically to Zx402 cannot be done with certainty. These same ions at m/e 229, 247, and

248 also appear in the mass spectrum of one of the peaks in the gas chromatogram of Clathraria rubrinodis sterols and therefore will be discussed at that time with reference to parent ion 402.

In scan 16, the peak at m/e 402 has diminished in intensity and the molecular ion at m/e 398 (brassicasterol or its C_{24} epimer) becomes most prominent.

Although scan 17 was taken on the top of a peak whose retention time is identical with that for desmosterol, no parent ion appears at m/e 384. Instead the major molecular ion shows up at m/e 414. This material will be referred to below as Zx414. The largest peak in the spectrum above m/e 200 occurs at m/e 316. This could suggest a "McLafferty" type of rearrangement common to $\Delta^{24(28)}$-sterols, with the additional two mass units caused by a saturated steroid nucleus. However, the absence of significant peaks at m/e 215, 233, 257, and 275, and the presence of an intense peak at m/e 271, characteristic of a Δ^5-sterol with unsaturation in its side chain, make this possibility seem unlikely. Other large peaks occur at m/e 287, 288, 289, and 301. A very similar fragmentation appears in scan 22, discussed below. In that scan the parent ion is at m/e 428, but large peaks at m/e 271, 287, 301, and 316 point out the resemblance to Zx414 in

scan 17. Mass spectral scans 17 and 22 appear in Figures 5 and 6, respectively. The presence of an unusual side chain is being considered, and therefore, high-resolution mass spectrometry of the sterol mixture is currently underway in order to determine accurate molecular formulas.

In the next scan (18), m/e 400 (24-methylcholesterol) is the most abundant parent ion, and the large peak at m/e 314 indicates that the 398 parent ion present in this spectrum probably corresponds to 24-methylenecholesterol. The previous ion at m/e 414 decreases significantly in this scan and continues to do so in scan 19. As might be expected from the position of scan 19, 398 has risen to dominance and an ion at m/e 412 indicating as before the probable occurrence of stigmasterol or poriferasterol, appears and continues in only minor proportions throughout its duration into scan 21. It is important to note here that this 412 sterol was resolved in the mass spectra without any corresponding peak visible in the gas chromatogram. The reverse situation may also occur; that is, some compounds having relatively insignificant parent ion intensities may show up as quite important peaks in the gas chromatogram. These points will be discussed further below in reference to Table 8 with additional examples drawn from some of the other sterol mixtures.

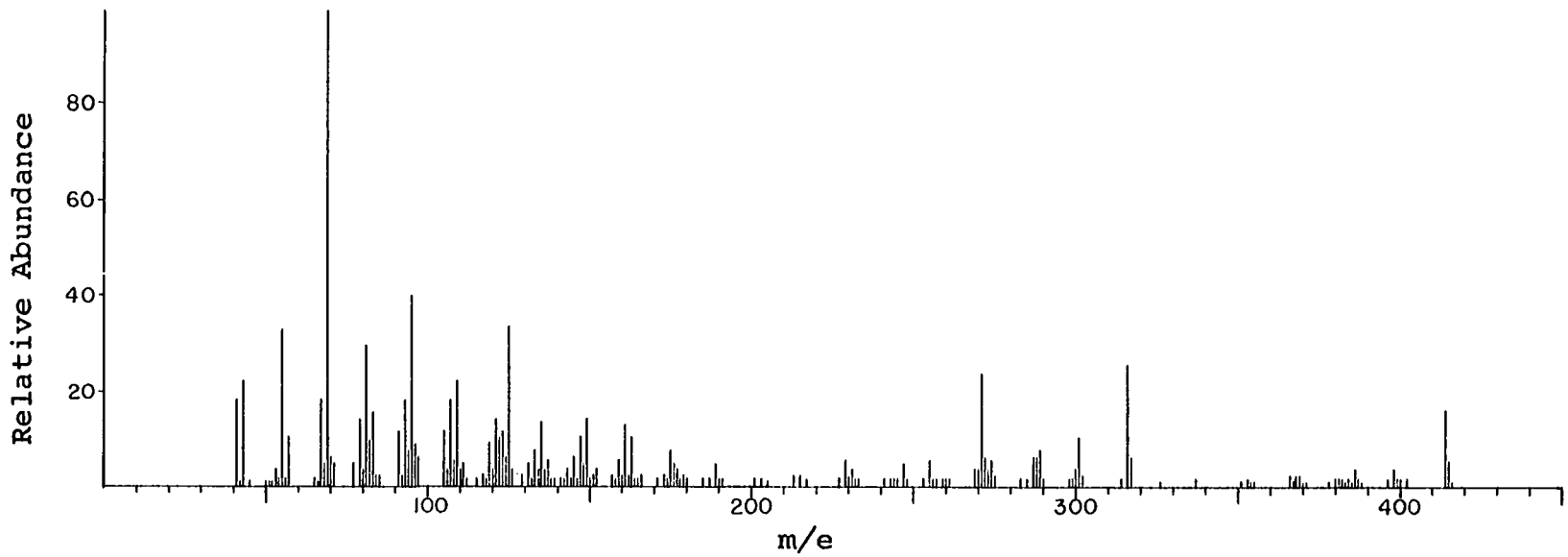


FIGURE 5. MASS SPECTRAL SCAN NO. 17

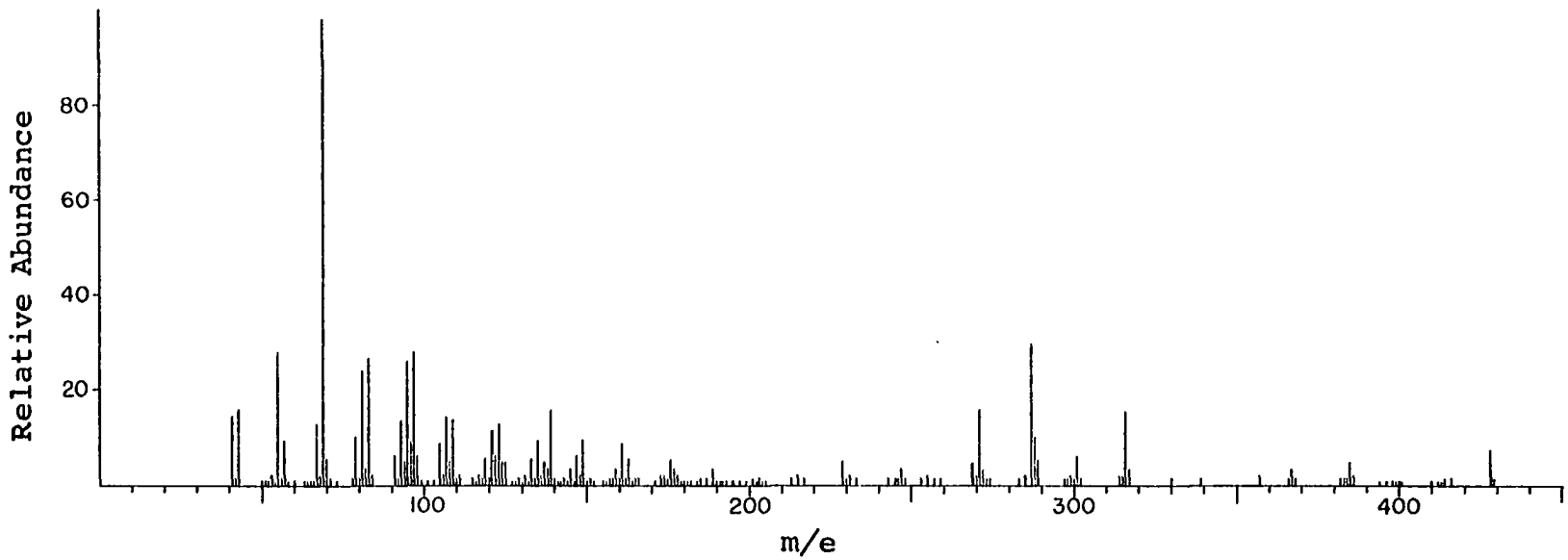


FIGURE 6. MASS SPECTRAL SCAN NO. 22

The last indication of 24-methylenecholesterol occurs in scan 20, taken at the base of a small peak whose parent ion at m/e 416 dominates both this and the next scan. This molecular ion was seen previously in the sterol fraction from E. verrucosa and will be described in detail in the discussion immediately following on the sterols from C. rubrinodis, in which this material is the major component.

Also in scan 21, a molecular ion at m/e 428 (hereafter referred to as Zx428) is seen for the first time. Scan 22, reproduced in Figure 6, shows this ion as the most intense parent ion present. As mentioned previously with respect to the ion at m/e 414 (Zx414) in scan 17 above, high-resolution mass-spectrometry is currently being used to help elucidate the structure of this material.

Scans 23 and 24 show the appearance of a different parent ion at m/e 414. Fragment ions at m/e 329 and 303 and the usual Δ^5-sterol peaks at m/e 273, 255, 231, and 213 signify, not unexpectedly from retention time data, β-sitosterol (or clionasterol).

A parent ion at m/e 412 also occurs in scan 24. The presence of a significant peak at m/e 314, which is absent from the mass spectra of either the 414 or 428 ions, may signify fucosterol (or 29-isofucosterol). If so, it is only

present in minute quantities as evidenced by the absence of any corresponding peak in the gas chromatogram.

The last sterol mixture analyzed by combined gas chromatography-mass spectrometry was isolated from Clathraria rubrinodis and the results of this analysis are shown in Figure 7 and Table 6.

Scan 1 shows the by now familiar appearance of a parent ion at m/e 384 already tentatively identified as 22-dehydrocholesterol from both mass spectral and gas chromatographic data.

The next scan is dominated by the m/e 386 ion as is scan 3, in which a less intense ion at m/e 398 also occurs. Scan 4 shows the 398 ion (brassicasterol, or its C_{24} epimer) in greatest proportion with cholesterol (386) dwindling sharply. In view of the small size of the 22-dehydrocholesterol peak in the GC trace, it is highly probable that the molecular ion appearing at m/e 384 in scan 4 belongs to a different compound, perhaps desmosterol, although due to its relatively low intensity, this cannot be verified mass spectrally.

Scan 5 shows the appearance of four parent ions at m/e 398, 412, 414, and the largest at 400. The GC peak corresponds in retention time to 24-methylcholesterol (400). This is a further example of peaks being present in the mass

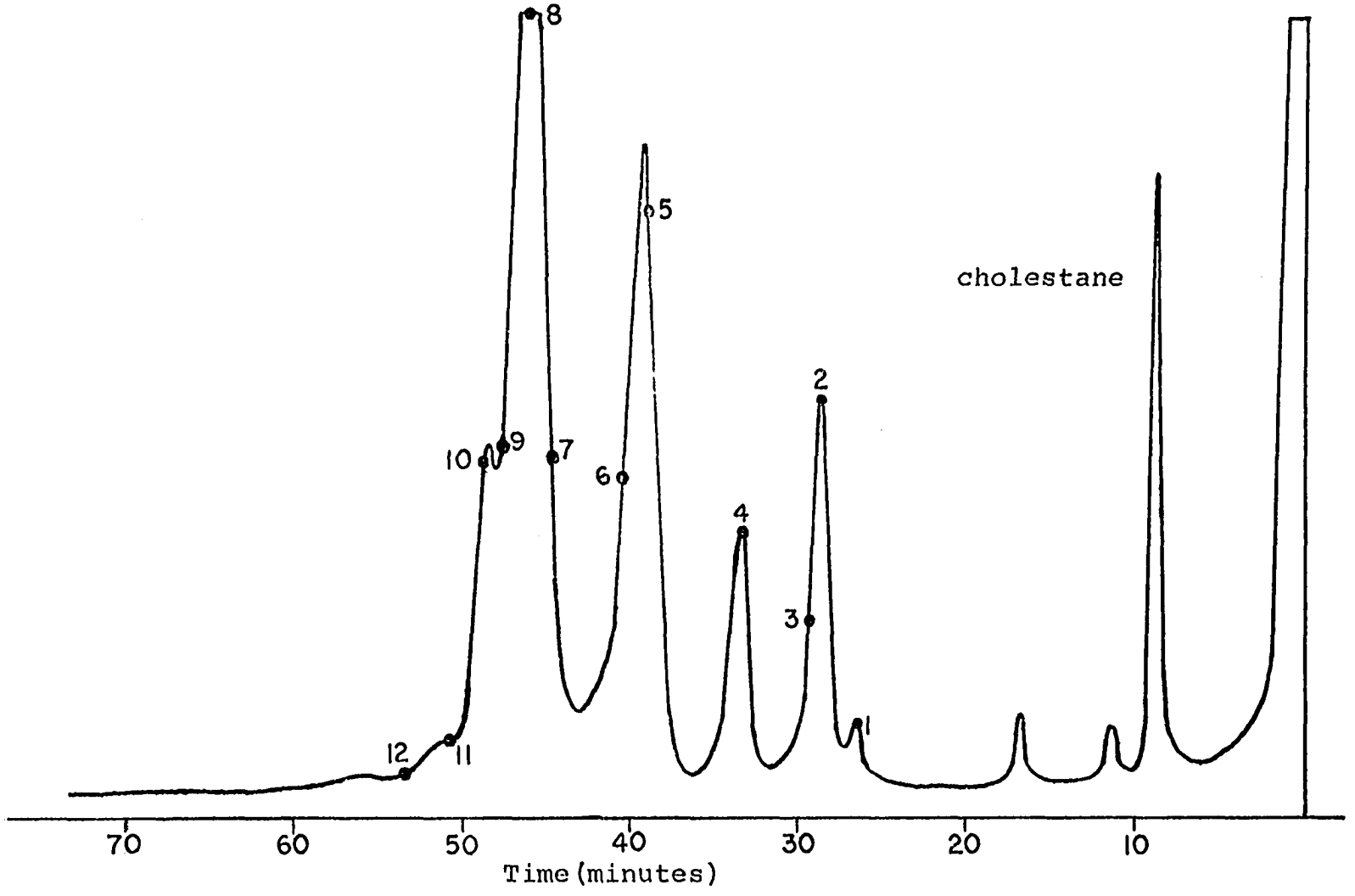


Figure 7. Gas Chromatogram from GC-MS of C. rubrinodis Sterols

TABLE 6

MASS SPECTRA FROM GC-MS OF C. RUBRINODIS STEROLS

MS-Scan No.	Molecular ion, M^+							
	384	386	398	400	412	414	426	428
1	100							
2	19	81						
3	18	77	5					
4	13	21	66					
5			12	61	3	23		
6			20	66	4	10		
7			13	11		10	66	
8						10	90	
9						18	74	9
10						21	62	17
11						34	50	16
12						35	41	16

spectrum but absent or hidden under a larger peak in the gas chromatogram. The occurrence of a peak at m/e 316 probably indicates the presence of Zx414 first encountered in the previous discussion on the sterols from the zooxanthellae of A. elegantissima.

Scan 6 is also dominated by the ion at m/e 400, but in scan 7, the appearance of an ion at m/e 416, seen previously in the sterol mixtures from E. verrucosa and from the zooxanthellae of A. elegantissima, occurs and is the major parent ion in all of the remaining spectra. According to the area in the GC trace, this material is the major component of the mixture. As determined from the relative intensity of the M^++1 peak, the compound has 29 carbon atoms, giving a molecular formula of $C_{29}H_{52}O$. Retention time and peak enhancement studies definitely show that this material is not β-sitostanol, the 5,6-dihydro derivative of β-sitosterol.

Table 7 below compares some of the principal ions above m/e 200 in the mass spectra of β-sitostanol and 416 from C. rubrinodis (Cr416). Relative abundances of the ions appear in parentheses. Also included are some of the mass peaks from the parent ion at m/e 402 seen previously in the combined GC-MS analysis of the sterols from zooxanthellae of A. elegantissima (Zx402). They are included because of the

TABLE 7

COMPARISON OF PRINCIPAL FRAGMENT IONS

FROM β-SITOSTANOL, Cr416, AND Zx402[1]

Fragmentation[2]	β-sitostanol	Cr416	Zx402
Molecular ion, M^+	416(100)	416(100)	412(100)
M^+-15	401(20)	401(30)	387(41)
M^+-H_2O	398(4)	398(12)	384(15)
M^+-15+H_2O	383(8)	383(20)	369(24)
M^+-side chain	275(3)	289(6)	289(8)
M^+-side chain+H_2O	257(6)	271(9)	271(41)
M^+-side chain+41	234(48)	248(46)	248(60)
M^+-side chain+42	233(52)	247(56)	247(68)
M^+-side chain+42+H_2O	215(47)	229(62)	229(74)
M^+-side chain+27	248(9)	262(14)	262(30)
M^+-side chain+27+HO	231(8)	245(14)	245(14)

[1] Principal ions from m/e 200 to the molecular ion.
[2] Possible fragmentations for the ions from Cr416 and Zx402.

similarity with the fragment ions of Cr416. Of course, it is not known that the mass peaks shown for Cr416 and Zx402 actually arise from the fragmentations indicated. However, mass peaks characteristic of a normal saturated steroid nucleus at m/e 215, 233, 234, 257, 275, and 231 in the spectrum of Cr416 are small or non-existent whereas the peaks shown are large and probably represent major fragmentations. It is interesting that the peaks in the mass spectra of Cr416 and Zx402, representing fragments that have lost the sterol side chain, are fourteen mass units higher than the corresponding peaks from β-sitostanol. There is even a surprisingly good correlation among the relative intensities of analogous peaks from the three sterols. (The rather high relative intensity for the peak at m/e 271 in the mass spectrum of Zx402 is due in large part to a small amount of the $\Delta^{5,22}$-sterol, brassicasterol, in whose mass spectrum m/e 271 is a major fragment.) This pattern is consistent with an additional methyl group somewhere in rings A, B, or C of the steroid nucleus. The peaks retaining the side chain suggest that Cr416 may be the next higher homologue of Zx402.

Assuming an additional nuclear methyl group, its exact position is far more difficult to ascertain. In this regard the most intense peak in the mass spectrum of Cr416 from m/e

300 to 380 is at m/e 330 (M^+-86). This peak may correspond to the loss of carbons 1 through 4 and their substituents (Ryhage and Stenhagen, 1960). Sterols having the usual steroid nucleus lose 72 mass units from this cleavage, whereas 4-methylsterols lose 86, and 4,4-dimethylsterols lose 100.

In scan 9, the ion at m/e 414 begins to increase in intensity signifying the presence of β-sitosterol (or its C_{24} epimer), and fragment ions at m/e 329 [M^+-(H_2O+67[C_5H_7])], 303 [M^+-(H_2O+93[C_7H_9])], 273, 255, 231, and 213 support this.

Also in scan 9, an ion at m/e 428 appears. Scan 10, taken on top of the shoulder, shows an increase in the intensity of the 428 ion and large peaks at m/e 271, 287 and 316 consistent with this material being the same as Zx428 seen previously.

Even though there is a greater abundance of a molecular ion at m/e 414 in scan 10, the retention time of this shoulder corresponds to Zx428 in the zooxanthellae sterols. Since the mass spectral scans were taken at 70eV, this phenomenon may simply reflect the relative stabilities of the 414 (24-ethylcholesterol) and 428 molecular ions. In support of this explanation, the molecular ion of β-sitosterol is the most abundant ion in its mass spectrum above m/e 210 (Knights, 1967), whereas this is certainly not the case with the

with the molecular ion of Zx428 (see Figure 6).

Scans 11 and 12 show the mass peaks discussed above that are characteristic of 24-ξ-ethylcholesterol, whose molecular ion appears in its greatest abundance in these scans. In addition, peak enhancement with standard β-sitosterol strongly indicates its presence.

Although no scans were taken of the GC peak around 56 minutes, its retention time is very similar to fucosterol (or 29-isofucosterol) seen previously in the sterol mixture from Virgularia presbytes.

Mass Spectrometric Analysis of Sterol Mixtures

Table 8 shows the intensities of sterol parent ions in the various sterol mixtures, relative to the most abundant molecular ion present. Except in the sterol mixtures from Clathraria rubrinodis and Palythoa mammillosa, this most abundant ion occurs at m/e 386, corresponding to the molecular ion of cholesterol. However, the gas chromatographic analyses in Table 10 below show that cholesterol is not the major component in the sterols of these two animals, nor in Heliopora coerulea or the zoochlorellae from Anthopleura xanthogrammica.

Apparent anomalies of this kind are common. They are caused in large part by differences in vapor pressure and in

TABLE 8

MASS SPECTROMETRIC ANALYSIS OF STEROL M

Sterol Source	368	370	384	386	398
Paragorgia arborea	35	6	59	100	11
Clathraria rubrinodis	16	4	29	44	59
Primnoa resediformis	65	12	58	100	24
Eunicella verrucosa	31	47	50	100	40
Leptogorgia hebes	21	9	22	100	20
Leptogorgia setacea	20	13	27	100	23
Virgularia presbytes	17	21	30	100	23
Heliopora coerulea	63	12	33	100	23
Palythoa mammillosa	-	-	3	4	25
Metridium senile	68	17	57	100	33
Anthopleura elegantissima (brown)	67	21	41	100	35
Zooxanthellae from *Anthopleura elegantissima*	20	8	14	100	15
Anthopleura elegantissima (green)	20	5	11	100	9
Zoochlorellae from *Anthopleura elegantissima*	21	7	12	100	20
Zoochlorellae from *A. xanthogrammica*	20	7	27	100	54
Distichopora violacea	63	10	55	100	38

[1] Ionizing voltage: 10eV
[2] Ion at m/e 368 (386-H_2O) is included for comparison to parent ion at m/e 370.

TABLE 8

METRIC ANALYSIS OF STEROL MIXTURES[1]

Sterol Parent Ion[2]										
0	384	386	398	400	402	412	414	416	426	428
5	59	100	11	28	12	2	3	1	1	1
4	29	44	59	78	24	16	47	100	3	14
2	58	100	24	59	27	5	19	7	2	2
7	50	100	40	12	5	4	6	4	4	-
9	22	100	20	11	2	6	9	2	-	-
3	27	100	23	12	2	8	9	2	1	1
1	30	100	23	8	1	6	7	1	1	1
2	33	100	23	17	3	42	4	1	1	2
-	3	4	25	100	6	9	2	-	21	2
7	57	100	33	35	16	9	17	4	2	2
1	41	100	35	39	11	5	12	3	2	3
8	14	100	15	7	6	2	6	2	-	3
5	11	100	9	6	2	12	4	-	-	-
7	12	100	20	22	4	6	23	3	2	1
7	27	100	54	61	4	13	70	4	1	1
0	55	100	38	34	11	10	34	8	1	2

to parent ion at m/e 370.

stability of the molecular ions formed. In other words, at a given ionizing voltage and inlet temperature, compounds with higher vapor pressures and more stable parent ions (or less stable fragment ions) will appear more abundant. On the other hand, the response of a flame-ionization detector in a gas chromatograph is not dependant upon the stability of ionic fragmentations as such, but on the amount of material combusted; therefore, for a series of similar compounds such as sterols, GC provides a much more reliable measure of the relative amounts of components in a mixture. Nevertheless, the mass spectrometer is invaluable for showing the presence of components that may not be in high enough concentration to be detected by the gas chromatograph (Djerassi, 1970). Several important examples of this are shown in Table 8. With few exceptions, ions appear at m/e 402, 416, 426, and 428 in every mixture examined, whereas these compounds appear much less frequently in the gas chromatograms of the mixtures. Admittedly, some compounds having these molecular weights may have retention times so different than the sterols that they might not appear in the GC traces under the operating conditions used; but this is not likely in every case. In this regard, Bergmann (1962) predicted the occurrence of stanols and methylsterols in small quantities in the complex sterol mixtures of

invertebrates.

Gas Chromatographic Analysis of Sterol Mixtures

Information obtained from the combined GC-MS analyses and from GC analysis of sterol standards resulted in the compilation of Tables 9 and 10. Table 9 shows the retention times of the various sterols on two different stationary phases relative to the internal cholestane standard. Table 10 shows the amounts of the individual sterols present in the mixture, both relative to the amount of cholesterol present and as the percentage of that sterol in the total amount of sterols.

It is apparent from Table 9 that the retention times for Zx414 and desmosterol are too similar to be separated on the OV-17 column. Therefore, the specific presence of one or the other is certain only in the specimens analyzed by combined GC-MS (i.e. zooxanthellae of brown A. elegantissima contain Zx414 and V. presbytes contains desmosterol). However, sterols from the brown A. elegantissima produced a GC trace so similar to the sterols from its zooxanthellae that Zx414, and not desmosterol, is probably present in that mixture also. The other assignments of these two sterols in Table 10 were done rather arbitrarily by observing relative retention times: those slightly less than 3.81 (e.g., 3.79 in zoochlorellae

TABLE 9

RELATIVE RETENTION TIMES OF STEROLS[1,2]

Sterol Name	Sterol Number	OV-17	SE-30
22-Dehydro-24-Norcholesterol	370	1.85	1.25(1.26)
22-Dehydro-Cholesterol	384A	2.85(2.86)	1.76(1.72)
Cholesterol	386	3.10(3.10)	1.94(1.94)
Zx402	402	3.37	
Brassicasterol, or C_{24} Epimer	398A	3.58(3.54)	2.20(2.13)
Zx414	414A	3.81	
Desmosterol	384B	3.87(3.88)	(2.18)
Campesterol, or C_{24} Epimer	400	4.23(4.21)	2.57(2.63)
24-Methylene-Cholesterol	398B	4.36	
Stigmasterol, or C_{24} Epimer	412A	4.68(4.72)	2.87(2.86)
Cr416	416	4.98	3.07
Zx428	428	5.12	
β-Sitosterol, or C_{24} Epimer	414B	5.44(5.42)	3.33(3.34)
23-Demethyl Gorgosterol	412B	5.75(5.75)	
Fucosterol, or C_{29} Isomer	412C	6.11	
Gorgosterol	426	8.04(7.94)	4.41(4.44)

[1] Relative to Cholestane: 9.5 minutes on OV-17
14.3 minutes on SE-30

[2] Numbers in parentheses refer to available standards.

TABLE 10

GAS CHROMATOGRAPHIC ANALYSIS OF STEROL

Sample Name								Stero
	370	384-A	384-B	386	398-A	398-B	400	402
Paragorgia arborea	2.3	27.7	13.1	100.0	3.4	-	5.3	-
	(1.5)	(17.7)	(8.4)	(63.8)	(2.2)	-	(3.4)	-
Clathraria rubrinodis	22.8	25.7	-	100.0	82.7	-	275.	-
	(2.2)	(2.4)	-	(9.4)	(7.8)	-	(25.9)	-
Primnoa resediformis	0.6	17.3	-	100.0	-	-	1.9	-
	(0.5)	(14.4)	-	(83.5)	-	-	(1.6)	-
Eunicella verrucosa	37.2	45.4	-	100.0	58.7	29.3	2.6	-
	(12.7)	(15.4)	-	(34.0)	(20.0)	(10.0)	(0.9)	-
Leptogorgia hebes	5.3	24.1	4.0	100.0	20.7	14.7	5.0	-
	(2.8)	(12.9)	(2.1)	(53.6)	(11.1)	(7.9)	(2.7)	-
Leptogorgia setacea	9.1	27.2	10.8	100.0	21.9	19.4	10.6	-
	(4.1)	(12.2)	(4.8)	(44.8)	(9.8)	(8.7)	(4.8)	-
Virgularia presbytes	14.3	36.0	7.6	100.0	26.8	14.8	7.2	-
	(6.3)	(15.9)	(3.4)	(44.2)	(11.8)	(6.5)	(3.2)	-
Heliopora coerulea	14.2	6.3	-	100.0	32.7	-	577.	-
	(1.8)	(0.8)	-	(12.5)	(4.1)	-	(72.3)	-
Palythoa mammillosa	17.3	-	-	100.0	570.	-	2465.	-
	(0.3)	-	-	(1.8)	(10.2)	-	(44.2)	-
Metridium senile	1.9	10.2	1.4	100.0	5.2	6.5	2.3	-
	(1.6)	(8.4)	(1.2)	(82.4)	(4.3)	(5.4)	(1.9)	-
Anthopleura elegantissima (brown)	2.3	6.1	-	100.0	8.6	8.2	1.5	7.0
	(1.6)	(4.3)	-	(70.7)	(6.1)	(5.8)	(1.1)	(4.9)
Zooxanthellae from A. elegantissima	2.4	7.1	-	100.0	8.2	5.0	1.4	10.5
	(1.7)	(4.9)	-	(69.0)	(5.7)	(3.4)	(1.0)	(7.2)
Anthopleura elegantissima (green)	4.0	8.3	-	100.0	4.9	9.1	3.4	-
	(3.0)	(6.3)	-	(75.3)	(3.7)	(6.9)	(2.6)	-
Zoochlorellae from A. elegantissima	5.4	11.5	-	100.0	7.0	30.6	20.2	7.8
	(2.7)	(5.7)	-	(49.5)	(3.6)	(15.1)	(10.0)	(3.9)
Zoochlorellae from Anthopleura xanthogrammica	5.8	13.0	-	100.0	8.0	125.	89.8	-
	(1.3)	(2.9)	-	(22.5)	(1.8)	(28.2)	(20.2)	-
Distichopora violacea	1.5	22.0	-	100.0	11.7	6.4	6.4	-
	(1.0)	(14.1)	-	(64.0)	(7.5)	(4.1)	(4.1)	-

*Top number shows amount relative to cholesterol (100.0) in sample.
Number in parentheses shows percent of total sterols in mixture (Area of Peak/To

TABLE 10

:RAPHIC ANALYSIS OF STEROL MIXTURES*

Sterol Number											
398-A	398-B	400	402	412-A	412-B	412-C	414-A	414-B	416	426	428
3.4	-	5.3	-	2.3	-	-	-	1.2	1.4	-	-
(2.2)	-	(3.4)	-	(1.5)	-	-	-	(0.8)	(0.9)	-	-
82.7	-	275.	-	-	-	8.9	-	30.5	372.	-	143.
(7.8)	-	(25.9)	-	-	-	(0.8)	-	(2.9)	(35.1)	-	(13.5)
-	-	1.9	-	-	-	-	-	-	-	-	-
-	-	(1.6)	-	-	-	-	-	-	-	-	-
58.7	29.3	2.6	-	10.4	-	-	-	7.7	-	-	-
(20.0)	(10.0)	(0.9)	-	(3.5)	-	-	-	(2.6)	-	-	-
20.7	14.7	5.0	-	4.7	-	2.2	-	6.0	-	-	-
(11.1)	(7.9)	(2.7)	-	(2.5)	-	(1.2)	-	(3.2)	-	-	-
21.9	19.4	10.6	-	4.4	-	10.2	-	9.5	-	-	-
(9.8)	(8.7)	(4.8)	-	(2.0)	-	(4.6)	-	(4.3)	-	-	-
26.8	14.8	7.2	-	4.0	-	7.8	-	8.2	-	-	-
(11.8)	(6.5)	(3.2)	-	(1.8)	-	(3.4)	-	(3.6)	-	-	-
32.7	-	577.	-	-	5.0	-	-	10.9	-	52.7	-
(4.1)	-	(72.3)	-	-	(0.6)	-	-	(1.4)	-	(6.6)	-
570.	-	2465.	-	273.	-	-	-	-	-	2158.	-
(10.2)	-	(44.2)	-	(4.9)	-	-	-	-	-	(38.6)	-
5.2	6.5	2.3	-	-	-	0.5	-	2.2	-	-	1.0
(4.3)	(5.4)	(1.9)	-	-	-	(0.4)	-	(1.8)	-	-	(0.8)
8.6	8.2	1.5	7.0	-	-	-	3.5	2.5	-	-	1.8
(6.1)	(5.8)	(1.1)	(4.9)	-	-	-	(2.5)	(1.8)	-	-	(1.3)
8.2	5.0	1.4	10.5	-	-	-	3.9	1.1	1.3	-	4.0
(5.7)	(3.4)	(1.0)	(7.2)	-	-	-	(2.7)	(0.8)	(0.9)	-	(2.8)
4.9	9.1	3.4	-	-	-	-	-	2.3	0.7	-	-
(3.7)	(6.9)	(2.6)	-	-	-	-	-	(1.7)	(0.5)	-	-
7.0	30.6	20.2	7.8	-	-	-	-	19.6	-	-	-
(3.6)	(15.1)	(10.0)	(3.9)	-	-	-	-	(9.7)	-	-	-
8.0	125.	89.8	-	-	-	-	13.4	88.6	-	-	-
(1.8)	(28.2)	(20.2)	-	-	-	-	(3.0)	(20.0)	-	-	-
11.7	6.4	6.4	-	1.8	-	-	-	6.5	-	-	-
(7.5)	(4.1)	(4.1)	-	(1.2)	-	-	-	(4.2)	-	-	-

in sample.
. mixture (Area of Peak/Total Area of Sterol Peaks).

from A. xanthogrammica) were designated Zx414, and those slightly greater than 3.87 (e.g., 3.94 in Leptogorgia hebes) were designated desmosterol.

Although the relative retention times for cholesterol and Zx402 are sufficiently different to effect separation, another factor may interfere and possibly account, in some cases, for the presence of a relatively abundant parent ion at m/e 402 in Table 8 but no corresponding peak in the gas chromatogram of the mixture (e.g., in the sterols of Metridium senile or Primnoa resediformis). That factor could be the overwhelming size of the cholesterol peak. Although the top of the peak may have a relative retention time of 3.10, the bottom of the peak will have a significantly greater one and could completely overlap small amounts of Zx402.

It is immediately obvious from Table 10 that the sterol mixtures are extremely complex; the one from A. elegantissima zooxanthellae containing as many as eleven sterols, most of the others containing eight to ten, and the mixture from P. resediformis being the exception with only four. According to Bergmann (1962), this amazing diversity of sterols is an indication of the "primitiveness" of the species, or in this case, phylum. For it seems that this diversity is most apparent in the sterols of sponges and coelenterates, but dis-

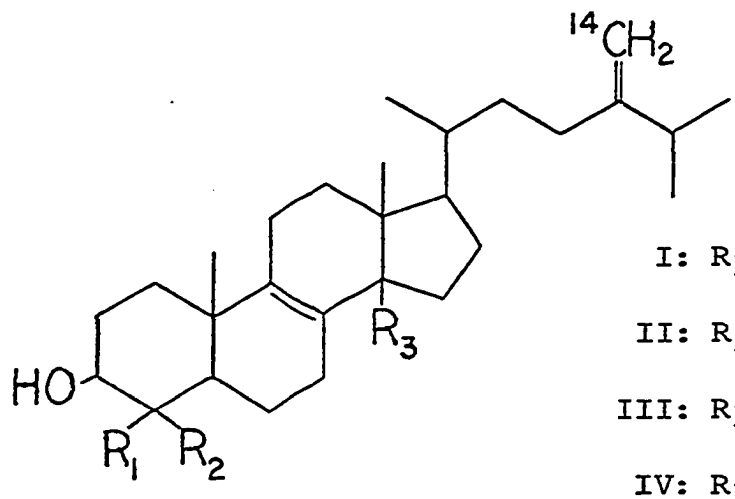
appears gradually through the various phyla until in the vertebrates, cholesterol is the principal, if not the only, sterol present. Although they are extremely primitive, the sponges are thought to lie on a separate evolutionary path; the coelenterates, on the other hand, are considered part of the main pathway leading to the higher animals. Perhaps for this reason, cholesterol is present in every mixture and is the major component in most.

Although complex, these sterol patterns clearly reveal numerous interesting relationships. With very few exceptions, 22-dehydrocholesterol (384-A), cholesterol (386), 24-methyl-22-dehydrocholesterol (398-A), 24- methyl-cholesterol (400), and 24-ethylcholesterol (414-B) are present in every mixture. Interestingly, 22-dehydro-24-norcholesterol (370) appears to be equally ubiquitous, as suggested by Idler et al. (1970). Next most widely distributed is 24-methylenecholesterol (398-B) followed by 24-ethyl-22-dehydrocholesterol (412-A) and 24-ethylidenecholesterol (412-C). Considering the almost total lack of knowledge about sterol biosynthesis in marine invertebrates or their symbiotic algae, it would be impossible to draw any conclusions concerning the origin of the side chains exhibited. In fact, it is not yet known whether or not the existence of sterols in coelenterates may be solely

of dietary origin. Indeed, by the very presence of such a great variety of similar compounds with presumably similar functions, it seems to this author that a valid a priori argument could be made for the inability of these animals to convert a relatively large number of dietary sterols to a relatively few important metabolic intermediates. It should be pointed out, however, that the ability to elaborate an homologous series of sterol side chains is common to many other "primitive" organisms and may require a series of relatively non-specific enzymes (Lederer, 1969). In this regard, Bergmann (1962) sees this diversity of sterols among lower animals not as a lack of biosynthetic prowess but, quite to the contrary, as a lack of biosynthetic selectivity, with the eventual "survival" of cholesterol as the "fittest" sterol finally occurring in the higher animals.

This seemingly low specificity among the enzymes responsible for side chain methylation as well as those catalyzing the demethylation of lanosterol (the methylated precursor of animal sterols), has caused much confusion concerning the "timing" of side chain alkylation; that is, whether it occurs prior to, during, or after lanosterol demethylation. From experiments with ^{14}C-labelled S-adenosylmethionine, it is now commonly accepted that this material is the

is the source of carbons 28 and 29 of the sterol side chain. Following incubation of an organism with this reagent, the isolation of compounds containing radioactivity in the alkyl groups attached to carbon 24 has been offered as evidence for the timing of side chain alkylations. Goulston, et al. (1967) suggested that isolated 24-^{14}C-sterols still retaining methyl groups at the 4α-, 4β -, or 14 positions indicated that side chain alkylation occurs prior to steroid nucleus demethylation. However, their results show that the largest amount of radioactivity incorporated appears in the completely demethylated product, 24-methylene-24,25-dihydrozymosterol (I), with the 4-methyl compound (II) having the next highest, the 4,4-dimethyl compound (III) next, and the 4,4,14-trimethyl compound, 24-methylene-dihydrolanosterol (IV), containing the least amount of radioactivity.



I: $R_1=R_2=R_3=H$

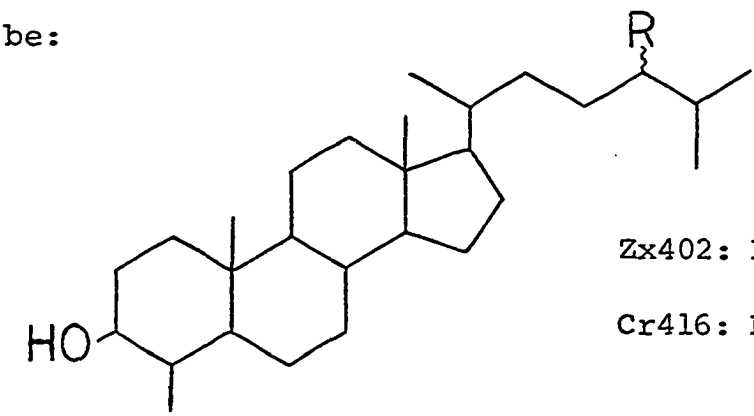
II: $R_1=CH_3$; $R_2=R_3=H$

III: $R_1=R_2=CH_3$; $R_3=H$

IV: $R_1=R_2=R_3=CH_3$

Contrary to the conclusions drawn, these results may show that zymosterol, the completely demethylated sterol, is the preferred substrate for a relatively non-specific enzyme (Lederer, 1969). Experiments on a recently isolated S-adenosylmethionine: Δ^{24} sterol methyltransferase from yeast support this latter conclusion (Moore and Gaylor, 1970). Enzyme activity, that is, addition of a methylene group to carbon 24, was greatest with zymosterol as the substrate and decreased more than 95% with either the mono-, di-, or trimethyl derivatives, strongly indicating that, if this enzyme is primarily responsible for C_{24}-methylation in yeast, lanosterol must be completely demethylated before side-chain alkylation can occur.

With these results in mind, an interesting biosynthetic pathway could be speculated for the origin of Zx402 and especially Cr416. If these sterols do in fact contain a methyl group at carbon 4 of ring A, possible structures for them could be:



Zx402: R=H

Cr416: R=CH_3

(Interestingly, Lenfant and Varenne report in unpublished data that a minor component of the sterol mixture from the slime mould, Dictyostelium discoideum, is a 4,24-dimethylcholestanol [Lederer, 1969]). If an enzyme system similar to that of yeast is responsible for side chain alkylation in C. rubrinodis, then addition of the methyl group at C_{24} must precede remethylation of C_4. Rearrangement of the 5,6 double bond to the 4,5 position probably would occur before remethylation, in view of the seemingly absolute requirement for the presence of unsaturation at a site undergoing alkylation (Lederer, 1969; Moore and Gaylor, 1970).

Aside from these biosynthetic considerations, the presence of Cr416 and Zx402 is of interest from a comparative point of view. The molecular weights 416 and 402 correspond to saturated sterols. In view of the occurrence of saturated sterols in sponges also (Bergmann, 1962), this may suggest a more common evolutionary development of these two phyla than is currently thought to be the case.

Some interesting comparisons also exist in the sterols isolated from different members of the same genus. In 1951, Bergmann et al. isolated a sterol, which they named palysterol, from Palythoa mammillosa collected near Bermuda. Gupta and Scheuer (1969) isolated a "palysterol-like" mixture, whose

physical properties closely resembled Bergmann's "palysterol," from P. tuberculosa, a Hawaiian species; whereas from a Tahitian species, Palythoa sp., they isolated 24-methylene-cholesterol as the only sterol present. The P. mammillosa studied here was collected in Jamaica. In Table 11 below, these various Palythoa sterol mixtures are compared.

TABLE 11

COMPARISON OF STEROL MIXTURES FROM PALYTHOA

Species	Location	Sterol M.P. Acetate M.P.	Sterol (%)
P. mammillosa	Bermuda	140-141°C 152.5°C	Palysterol (100)
Palythoa sp.	Tahiti		24-methylenecholesterol (100)
P. tuberculosa	Hawaii	139-141°C 144-145°C	24β-methylcholesterol (60) gorgosterol (20) cholesterol (10-15) 24β-methyl-22-dehydrocholesterol (1-2) 24β-ethylcholesterol (1-2)
P. mammillosa	Jamaica	136-138°C 148-148.5°C	24ξ-methylcholesterol (45) gorgosterol (40) cholesterol (2) 24ξ-methyl-22-dehydrocholesterol (10) 24ξ-ethyl-22-dehydrocholesterol (5)

Although the mixtures from P. mammillosa and P. tuberculosa are alike in that both contain high amounts of 24-methylcholesterol and gorgosterol relative to cholesterol, they differ in more subtle ways rather significantly. For example, the ratio of 24-methylcholesterol to gorgosterol is 3 in P. tuberculosa, but almost 1 in P. mammillosa. Also, the relative amounts of 24-methyl-22-dehydrocholesterol and cholesterol seem to be almost reversed in the two species. And further, the substitution of 24-ethylcholesterol in P. tuberculosa for its Δ^{22} analog in P. mammillosa may suggest the absence of a dehydrogenating enzyme in the former species. Of course, whether these differences are of dietary origin or actually reflect species-specific metabolic processes is not known at present.

A rather unusual comparison can be made between the Tahitian Palythoa sp. and the Jamaican P. mammillosa. Both species synthesize a very toxic compound called palytoxin, although the two toxins have not been shown to be identical (Attaway, 1968; Scheuer, 1969). Since this toxin is produced in significant quantities in P. mammillosa only during the summer months when sunlight is most intense, it has been suggested that the symbiotic zooxanthellae that inhabit these animals may somehow be involved in palytoxin biosynthesis

(Attaway, 1968). Gas chromatography of the ether-extractable material from a palytoxin hydrolyzate was shown to contain four major peaks, one of which had a retention time identical to cholesterol. The retention times of the three other peaks, relative to cholesterol, were 1.34, 1.48, and 2.66. The retention times relative to cholesterol for 24-methyl-22-dehydrocholesterol, 24-methylcholesterol, and gorgosterol analyzed in this study are 1.14, 1.36, and 2.29, respectively. In view of the fact that the two analyses were carried out on different SE-30 columns under different operating conditions, these discrepancies in retention time seem more understandable and therefore it is possible that these three other peaks were 24-methyl-22-dehydrocholesterol, 24-methylcholesterol, and gorgosterol. Indeed, Attaway speculates that palytoxin may actually be a family of toxins differing only in the nature of their sterol moieties. In view of the complete difference in sterol composition between the two toxic Palythoa species, it is tempting to speculate that the toxins produced would be different at least to the extent of the sterol moiety incorporated.

Of course these differences in sterol composition could be due to the particular species of algae living within the two animals. The variation in gorgosterol content between

P. tuberculosa and Jamaican P. mammillosa discussed above might also result from different species of algae. The possible relationship suggested by the frequent simultaneous appearance of gorgosterol and symbiotic algae was first observed by Ciereszko et al. (1964). They found gorgosterol present in all zooxanthellae-containing gorgonians studied and in the isolated algae from Pseudoplexaura porosa and Eunicea mammosa. Consistent with this is the fact that the two appearances of gorgosterol in the present work occur in P. mammillosa and Heliopora coerulea, both of which contain zooxanthellae (Attaway, 1968; Buchner, 1965). To date, gorgosterol has been found only in organisms containing symbiotic zooxanthellae, although it has not been found in all such organisms. Indeed, Ciereszko et al. (1964) also studied the sterol fraction from zooxanthellae of Anthopleura elegantissima and found it to contain no C_{30} sterols as evidenced by the lack of mass spectral parent ions at m/e 426 or 428. Although our work does show the presence of a sterol with a molecular weight of 428, all available evidence indicates that it is not dihydrogorgosterol (R.A. Gross, unpublished data).

The sterol fraction from the zoochlorellae of A. elegantissima, on the other hand, has never been studied and

although C_{30} sterols do not appear here either, the complete sterol pattern of these algae is somewhat different than the zooxanthellae pattern. Whereas in the zooxanthellae sterols 24-methylenecholesterol (398-B), 24-methylcholesterol (400), and 24-ethylcholesterol (414-B) occur in only minor proportions (3.4, 1.0, and 0.8% respectively), in the zoochlorellae sterols their proportions (15.1, 10.0, and 9.7% respectively) are exceeded only by cholesterol. In view of the similar patterns seen in the algae-bearing anemones themselves, it seems highly probable that the two algae synthesize sterols in differing amounts, and in the case of Zx414 and Zx428, may even synthesize different compounds.

Since it is not likely that animals in the same genus would synthesize significantly different sterols (Bergmann, 1962), a comparison between the sterols from zoochlorellae of A. elegantissima and A. xanthogrammica lend further support to the apparent biosynthetic ability of the algae. The compositions of these two mixtures are quite dissimilar even in the amount of cholesterol present. Although these relationships do not unequivocably show that the symbiotic algae synthesize their own sterols, they are certainly consistent with this possibility. Certainly the presence of complex mixtures of sterols in red, brown, green, and blue-green algae has been well documented (Patterson, 1971).

SUMMARY

The sterol fractions from thirteen species of the phylum Coelenterata and from the symbiotic unicellular algae of three species were isolated and analyzed by gas chromatography, mass spectrometry, and combined gas chromatography-mass spectrometry. The results show complex mixtures of C_{26}, C_{27}, C_{28}, C_{29}, and C_{30} sterols. Of the sixteen different sterols seen, four could not be tentatively identified with known compounds. From mass spectral data, two of these (Cr416 and Zx402) appear to be saturated and to contain an additional methyl group in the steroid nucleus, possibly at carbon 4.

Several lines of evidence indicate that the symbiotic algae may participate in sterol biosynthesis: 1) The sterols of Palythoa mammillosa were compared with other algae-bearing members of this genus. In some cases the mixtures differed markedly, suggesting that different species of algae may be present synthesizing different sterols; 2) the C_{30} sterol, gorgosterol, was found only in the sterol mixtures from two algae-containing animals, Heliopora coerulea and P. mammillosa,

further substantiating the occurrence of this sterol only from animals bearing algal symbionts; and 3) the sterols from three species of algae that inhabit the genus Anthopleura are significantly different in composition, again suggesting the possible involvement of the algae in sterol metabolism.

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PART II

AMINO ACID COMPOSITION OF PEPTIDES FROM THE SPICULES OF BRIAREUM ASBESTINUM

The terms "vertebrate" and "invertebrate" generally refer to those groups of animals that either contain or lack a vertebral, or spinal, column composed of a mineralized tissue called bone. Yet, of all living animal species containing mineralized tissue, which by definition includes all the vertebrates, two-thirds are invertebrates! In contrast to the mineralized tissues of the vertebrates, which in almost all cases consists of calcium and phosphate in the form of hydroxyapatite, the vast majority of invertebrate tissues consist of calcium and carbonate either as calcite or aragonite (Travis, et al., 1967).

These inorganic materials are, to varying degrees of complexity and intimacy, invariably associated with some type of organic matrix. Examples of this matrix in vertebrates are the familiar collagen of bone and the enamel protein of teeth. The organic matrices of the invertebrate mineralized tissues,

on the other hand, are far less familiar. Although invertebrate collagens have been known for some time in soft, non-mineralized tissues (Gross, et al., 1956; Gross and Piez, 1960), Piez (1963) states "that only among the vertebrates does collagen serve as the matrix on which a calcified tissue is constructed....Indeed, the fact that nature found a way to calcify collagen may well be one of the most important single events in the evolution of vertebrates."

However, Travis, et al. (1967) have shown by means of electron microscopy, X-ray diffraction, and amino acid analysis, that collagen exists as a minor component in the calcified tissues of a sponge, mussel, oyster, and quohog, and as the major component in the calcified plates of a sea-urchin. This latter finding is most exciting; sea urchins are echinoderms which as a group are closer to the vertebrates in both evolutionary and embryonic development than any other group of invertebrates. Therefore, one wonders if collagen may have, through the course of evolution, been gradually selected as the sole matrix for skeletal calcification. In other words, in primitive organisims, such as the sponge, collagen may be one of many similar biopolymers that make up the organic matrices of their mineralized tissues, whereas in animals of more recent origin (echinoderms) collagen is a major organic

component of these tissues. And finally, in the vertebrates, the organic matrix of bone is exclusively collagen.

If this is true, it is surprising that so little attention has been paid to the coelenterates. The primitiveness of the sponges is not questioned; but sponges are thought to be an evolutionary "dead-end", and therefore, from the point of view of biochemical evolution, discoveries from sponges may be of questionable comparative significance. On the other hand, the coelenterates are the "nearest living representatives of the ancestral stock of all other multicellular animals" (Hill and Wells, 1963). If for no other reason than this, any comparative study on mineralized tissue must include a representative of the coelenterates or be fundamentally incomplete.

The mineralized tissue of many coelenterates, as in the sponges, is in the form of spicules. Whereas sponge spicules may be either calcareous or siliceous, coelenterate spicules are always calcareous. Even though they exist in an incredible variety of shapes and sizes, there is uniformity within a species, and so coelenterate spicules have been a major taxonomic tool for more than a century. Nevertheless, little if anything is known of their physiological origin or chemical composition.

The situation is quite different, however, with respect to calcareous sponge spicules. Therefore, because it is relevant to further discussion, the origin of calcareous sponge spicules will be briefly discribed with specific reference to the development of a tract, or triradiate (a common spicular shape having three rays, or axons, emanating from a central junction and spaced approximately 120° from each other).

First, three scleroblasts associate to form a trio (Jones, 1970). Each then divides forming a sextet of sclerocytes, three outer cells superimposed upon three inner ones. Between each inner and outer cell a spicule ray is secreted, with each inner cell moving outward lengthening the ray as it moves. Each outer cell remains at the central junction usually in the angles between the rays. As the inner, or so-called "founder", cell completes its role in lengthening the ray and leaves its tip, the outer, or so-called "thickener", cell begins to move outward depositing additional inorganic material to thicken the ray. When the thickener cell reaches the end of the ray, it can either leave or remain on the tip depending upon the species of sponge and type of spicule formed. Additional rays, as in a tetraxon, are formed by one cell which completely and sometimes permanently envelopes the entire additional ray. Spicules are secreted by the sclerocytes in

the mesogloea, an amorphous region of wandering cells located between the cell layers that line the myriad, tiny channels and caverns of the sponge's body. Factors determining the specific position of the completed spicules, indeed the original orientation of the scleroblast trio, are poorly understood but are thought to be largely mechanical.

The spicule is composed of crystalline calcium carbonate surrounded by an organic sheath. This sheath is presumed to be a hardening or thickening of the mesogloeal "ground" substance (Jones, 1967). Although the presence of the enveloping sheath is well accepted, a controversy has raged, ever since the turn of the century, about whether or not the calcium carbonate is deposited <u>on</u> an organic template, or just <u>within</u> the organic sheath (Jones, 1967). The reason for this controversy is that a thin thread, or so-called axial filament, is visible running through the middle of each ray from the central junction to its tip. Upon igniting the spicule, this filament darkens but, in 1887, Von Ebner showed that this may be caused by formation of vacuoles, not carbon particles. From the absence of any residue left after acid corrosion, he concluded that the filament was not organic, but probably a region of less pure calcite. In 1901 however, Butchli did observe the presence of a shrivelled, twisted tube remaining

after acid corrosion, but said that natural calcite also left similar residue. In 1908, Minchin and Reid showed that the axial filament could be visualized with picronigrosin, a stain for protein; and Butchli determined that the spicules contained approximately 0.42% organic matter, although this value varied so much from sample to sample that he suspected organic impurities. Finally in 1955 and 1967, Jones showed that the stained axial filament seen by Minchin and Reid was probably due to part of the sheath which was shrivelled upon demineralization. Travis (1970) may have turned the controversy into somewhat of a semantic debate. Electron microscopy of the demineralized sponge spicule shows the sheath to be not a simple envelope but instead a latticework of "compartments and sub-compartments" within which the calcium carbonate crystals are deposited. Yet Jones (1970) states, in a recent monograph on sponges, that "no organic residue has been detected within the spicules."

One further finding is especially pertinent to the present work. Sea-urchin larvae spicules were obtained by incubating larvae with 0.2 M sodium hydroxide at 100°C for 30 minutes (Okazaki, 1960). The spicules isolated in this way had no covering whatsoever, whereas spicules not pretreated with alkali had a sheath similar to that of sponge

spicules. After decalcification with hydrochloric acid, a thin "cord" remained which stained with Nile blue sulfate, a non-specific stain for lipids. Okazaki concludes that spicule growth is not simply a physical crystallization of calcium carbonate within an organic envelope "but a successive arraying of calcareous elements onto an organic substratum."

The spicules studied in the present work are from Briareum asbestinum (Pallas). This animal is a member of the phylum Coelenterata, class Anthozoa, subclass Alcyonaria, order Gorgonacea, suborder Scleraxonia, family Briareidae. The gorgonians have two types of skeleton: calcareous spicule and hornlike axis. The axis is made of a protein called gorgonin, which will be discussed with respect to its amino acid composition in the discussion section below. In the scleraxonians, spicules often appear both in the fleshy outer portion (coenenchyne) and in the axis cemented together by varying amounts of gorgonin (Kaestner, 1967). Briareum is common to West Indian waters usually growing 30 cm high, with thick erect stems that are covered with polyps. When the polyps are expanded, the colony appears to be covered by a soft, brown fur.

The purpose of this work was to extend the study of mineralized tissues to the coelenterates, in order to determine

whether or not organic material existed within the spicules, and if so, to begin a study of any protein present by amino acid analysis.

EXPERIMENTAL PROCEDURE

Isolation of Spicules

Briareum asbestinum was collected near South Cay, Jamaica in October, 1966. The animal was air-dried, and ground to a powder in a Waring blender. The ground material was extracted by standing in hexane, then diethyl ether, and finally isopropanol. This partially extracted material was then extracted continuously in a percolator-type extractor (Ciereszko, 1966) with diethyl ether.

The lipid-extracted material (2920 gm) was stirred with two volumes (5 liters) of 30% potassium hydroxide; the mixture became dark brown and a strong "fishy" odor was immediately obvious. After standing overnight, the supernatant was decanted, and the residue washed with large quantities of water by stirring, allowing the spicules to settle, and carefully decanting. This procedure was continued until the supernatant was only slightly colored. Some of the smaller, less dense spicules are inevitably lost during decantation, but this loss is insignificant if enough time (10-12 minutes) is

allowed for settling.

The spicules were again treated with enough 30% potassium hydroxide (2 liters) to completely immerse them. After standing for five or six hours, the alkali-washed spicules were washed on a large sintered-glass funnel with tap water until the filtrate was neutral to pH paper (approximately 12-15 liters), and then with ten liters of distilled water.

The distilled water washings were tested for ninhydrin-positive material by collecting 200-300 ml of filtrate, concentrating on a rotary evaporator, spotting some of the concentrated filtrate on Whatman Number 1 paper, spraying with ninhydrin reagent, and heating in a 110°C oven for five minutes. This testing procedure was repeated at 2 liter wash intervals, concentrated filtrates that did not give a positive test with ninhydrin were also tested for biuret-positive material by the micro-biuret method (Itzhaki and Gill, 1964). Bovine albumin-Fraction V (Sigma Chemical Company) was used to prepare the protein concentration standard curve. The final washings showed no detectable ninhydrin-positive or biuret-positive material.

The spicules were then washed with one liter of methanol, one liter of diethyl ether, and finally air-dried.

Preparation of Intraspicular Material

The dried, purple-colored spicules weighed 1918 grams or 65.7% of the lipid-extracted material (w/w).

The spicules were added to enough water (about 2 volumes) to make a freely-flowing slurry. A Teflon stirring bar was added and the mixture placed on magnetic stirrer in a walk-in refrigerated room kept at around 4°C.

With constant, moderate stirring, 3175 ml of concentrated hydrochloric acid were added in 5-10 ml increments. The amount of HCl needed is determined by assuming the spicules to be pure calcium carbonate (MW=100.1). The number of "moles" of spicules is then easily calculated and twice that number of moles of HCl are needed to completely react with them. Slightly less than this amount of acid was used, thereby leaving a few spicules remaining so that the mixture never remains acidic for any significant length of time. A few drops of capryl alcohol were added when needed to reduce the large amounts of foam produced by the liberated carbon dioxide. The material released upon acid addition was brown and not completely water-soluble. In order to minimize contact between the acid and any released acid-labile material, at frequent intervals the brown suspension was poured off, more water added to the remaining spicules, and acid addition continued.

The total amount of brown suspension obtained was 7 liters. This was concentrated on a "cyclone" vacuum distilling apparatus at 35-40°C to a final volume of 2 liters. The concentrated suspension was extremely viscous due to the high amount of calcium chloride present. The material was transferred to a continuous-liquid-liquid extractor and extracted with diethyl ether for 10 days to remove capryl alcohol and any other ether-soluble organic material.

Approximately 100 ml of ether-extracted material was dialyzed in double-walled dialysis tubing against distilled water at 4°C to determine if dialysis could be used to remove dissolved salts. After four days, the liquid outside the dialysis bag was concentrated on a rotary evaporator at 30-35°C from 750 ml to approximately 25 ml. A negative ninhydrin test indicated the suitability of dialysis, and the remainder of the ether-extracted material was dialyzed against running tap water for 7 days.

Next, the dialyzed material was centrifuged in a Sorvall Superspeed RC2-B automatic refrigerated centrifuge at 16,300xg for 20 minutes at 0-5°C. The supernatant was decanted, the pellet resuspended in distilled water, and the centrifugation repeated under the same conditions. After repeating this washing procedure two more times, the last

supernatant gave a negative test for chloride ion upon the addition of a few drops of 1% silver nitrate solution. After centrifuging the pellet in acetone three times, the material was air-dried. The acetone washes were combined, the solvent removed on a rotary evaporator, and the acetone soluble material dissolved in water and combined with the aqueous supernatant.

The pale yellow supernatant was then ultrafiltered in an Amicon Model 401 ultrafiltration cell through a UM-05 Diaflo membrane (molecular weight cutoff=500) (Amicon Corporation). The ultrafiltration cell, containing the Diaflo membrane, was connected to an Amicon RF-25 Fiberglass Liquid Reservoir, which in turn was connected to a tank of nitrogen gas. The entire system, maintained at 4°C, was flushed with water, sterilized with 5% formaldehyde in water, and finally rinsed again with water. After filling the reservoir with the supernatant, the nitrogen supply was adjusted to 50 psi and the ultrafiltration begun. A flow-rate of 0.75 ml/min. was maintained and the entire volume of supernatant was ultrafiltered in 5 days. The ultrafiltrate appeared clear and colorless, while the ultraretentate was dark brown and viscous. The ultraretentate was washed on the membrane several times with water, and removed from the ultrafiltration cell with a

Pasteur pipette. Upon standing, a brown film settled to the bottom of the flask; this was probably material that was not obtained during centrifugation, and therefore after using a rotary evaporator to remove the 25 ml of water remaining, the material was combined with the air-dried brown solid obtained previously. This combined material weighed 17.3 gm, or 0.9% of the spicules (w/w), and represented the total ether-insoluble, non-dialyzable material obtained after decalcification of the spicules.

Isolation of Two Water-Soluble Peptide Fractions

In an attempt to extract biuret-positive material from the decalcified matrix, eight pilot batches of approximately 20 mg of this material each were divided into four groups of two tubes each. To the first group of two tubes were added 5 ml of distilled water; to the second group, 5 ml of 1% sodium chloride solution; to the third, 5 ml of 0.1% potassium hydroxide solution; and to the fourth, 5 ml of 75% ethanol in water. One member of each group was kept at 85°C in a hot water bath for 12 hours; the other member of the group was kept at room temperature (25°C). The contents of each tube were shaken vigorously several times during the 12 hour period. Each tube was then vacuum filtered, the filtrate

adjusted to 5 ml with the appropriate solvent, and tested by the micro-biuret method. The four hot solvents extracted varying amounts of biuret-positive material, whereas only potassium hydroxide solution was successful among the cold solvents. In decreasing order of material extracted, the results showed: hot KOH (most) > hot $H_2O \approx$ hot NaCl > hot EtOH > cold KOH (least).

From these results, hot water was chosen as the solvent to be used for a large-scale extraction in order to minimize hydrolysis of any peptide bonds present. Five grams of the brown material were extracted in a Soxhlet-type apparatus with water heated to boiling on a heating mantle. After two days, the clear, brown extract began to bump, and the pot was changed, and changed again after another week.

The combined extracts were centrifuged at 4°C in an International Centrifuge at high speed for one hour to remove any insoluble material and debris; an ultraviolet spectrum was taken of the supernatant on a Beckmann DB spectrophotometer from 320 nm to 200 nm. The extract was placed on a magnetic stirrer in the cold and solid ammonium sulfate was added in small increments with about 5-10 minutes of stirring between additions. Precipitation occurred at 58% and 72% of saturation. Each precipitate was centrifuged as before and dried

in a vacuum dessicator overnight. The precipitates, which still retained the brown coloration of the extract, were weighed, dissolved in a few milliliters of water, and their UV spectra taken. The precipitate which was obtained at 58% saturation weighed 35.4 mg; the precipitate which was obtained at 72% saturation weighed 12.3 mg. These were then each ultrafiltered to remove ammonium sulfate on a UM-2 Diaflo membrane (MW cutoff=1000) in a Model 52 Ultrafiltration cell under a nitrogen pressure of 40 psi. After washing the material on the membrane with 15 ml of water seven times, the UV spectra of the ultraretentates and ultrafiltrates were taken to obtain some information on the molecular weights of the UV-absorbing species.

Following this, a portion of each ultraretentate was chromatographed on a Bio-Gel P-10 column (MW cutoff=12,000) (100-150 mesh; Bio-Rad Laboratories) with water as the eluting solvent. Column bed dimensions were 52x1 cm. The column eluant was monitored for UV-absorbing material at 257 nm by passing through an Isco Model UA-2 Ultraviolet Analyzer, incorporating a fraction collector. Dextran Blue 2000 and thymidylic acid were used to determine the void volume and inclusion volume of the column, respectively. UV-absorbing material was eluted in the inclusion volume, indicating a

molecular weight less than or equal to 5000.

Another small portion of each ultraretentate was chromatographed on a Bio-Gel P-6 column (MW cutoff=4,600). Column bed dimensions were 67x1 cm. Distilled water again was the eluting solvent and the eluant was monitored as before. In this case, several overlapping peaks were seen in the eluant of each ultraretentate, with the largest peak in each case being eluted in the void volume, indicating a molecular weight greater than or equal to 5,000.

Amino Acid Analysis of the Peptide Fractions

The remaining portion of each ultraretentate was hydrolyzed using the following procedure: Ultraretentate 1 (UR-1) (7.0 mg) and ultraretentate 2 (UR-2) (7.0 mg) were each dissolved in 1.0 ml of water. One milliliter of concentrated hydrochloric acid (analytical grade; Mallinckrodt) was added and the contents mixed.

Hydrolysis tubes were made by sealing one end of a 10x0.48 cm (I.D.) piece of pyrex glass tubing. By means of a Pasteur pipette, each hydrolysis mixture was added to three hydrolysis tubes. The six tubes (3 from each ultraretentate) were each fitted with a small piece of rubber tubing with an attached screw clamp, and the contents were frozen by immersing

the tubes in a dry ice-isopropanol bath. By means of a two-way stopcock and the appropriate connections, each tube was evacuated and filled with dry nitrogen several times. After the final nitrogen addition, the screw clamp was closed and the tube sealed with a gas-oxygen torch. The sealed tubes were then placed in a $110\pm1^{\circ}C$ oven. After twenty-four hours, one UR-1 and one UR-2 tube were removed, the tubes broken, their contents emptied into tared flasks, and the solvent removed on a rotary evaporator at $50^{\circ}C$. Ten ml of water were added, the contents again taken to dryness, and weighed. Sodium citrate buffer (pH2.2) was added to a concentration of 1 mg/ml and the material called UR-1-24 or UR-2-24 (Ultraretentate 1 or 2 after 24 hour hydrolysis). This procedure was repeated after 48 hours and again after 72 hours to obtain samples UR-1-48, UR-2-48, UR-1-72 and UR-2-72.

Amino acid analyses were performed on either Beckmann-Spinco Model 120-B Automatic Amino Acid Analyzer (Beckmann Instrument Company, Spinco Division) or on a Biochrom Model BC-200 Automatic Amino Acid Analyzer (Bio-Cal Instrument Company). Details of the analyses on the Beckmann-Spinco analyzer were: column bed dimensions: 57x0.9 cm; operating temperature: $55^{\circ}C$. A one-column, three buffer program was employed with the following buffer changes: buffer pH 3.25:

0 to 70 minutes; buffer pH 4.25: 70 to 120 minutes; buffer pH 6.45: 120 to 250 minutes; 0.20N/sodium hydroxide: 250 to 280 minutes; buffer pH 3.25: 280 to 350 minutes. The last two changes are used to complete the elution of arginine followed by regeneration of the resin for subsequent runs. Buffer flow rate was 70 ml/hr; ninhydrin flow rate was 35 ml/hr.

Ninhydrin reagent was prepared according to Spackman et al., (1958). Reagents used were: ethylene glycol monomethyl ether (methyl Cellosolve) (peroxide-free, certified grade; Fischer Scientific Company); ninhydrin (Sigma Chemical Company); all other reagents were analytical reagent grade. Sample volume: 0.2 ml; followed by 1.0 ml buffer pH2.2 to wash all sample onto the resin. After sample addition, the program was begun. To standardize the amino acid elution pattern and to determine the exact program, Standard Amino Acid Calibration Mixture (Sigma Chemical Company) was used.

Details of the analyses on the Biochrom Analyzer were: two columns were employed: 1) for elution of acidic and neutral amino acids, the column bed was 51x0.9 cm; buffer pH 3.25: 0 to 60 minutes; buffer pH 4.25: 60 to 135 minutes; 2) for elution of basic amino acids, the column bed was 11x0.9 cm; buffer pH 5.28: 0 to 75 minutes. Both columns were regenerated by elution with 0.20 N/sodium hydroxide

followed by equilibration with either buffer pH 3.25 for the long column or buffer pH 5.28 for the short column. Operating temperature: 50°C. Buffer flow-rate: 100 ml/hr; ninhydrin flow-rate: 50 ml/hr. Ninhydrin reagent was prepared using ninhydrin and methyl Cellosolve obtained from Bio-Rad Laboratories. Sample volume for each column: 0.4 ml.

Table 1 below shows the composition per liter of the buffers used. All buffers and methyl Cellosolve reagent were filtered with suction through a Millipore filter (0.45μ pore size; Gelman Instrument Company) prior to pH determination. Buffer pH was checked on a Beckmann Zeromatic pH Meter (Beckmann Instrument Company) and, if necessary, adjusted with either concentrated hydrochloric acid or 50% sodium hydroxide solution.

Amino acid composition was determined from the areas of the peaks on the chromatograms by the height times width-at-one-half-height method, using dot counting.

TABLE 1

COMPOSITION OF BUFFERS USED IN AMINO ACID ANALYSIS

Reagent	Buffer pH				
	2.20 ± 0.03	3.25 ± 0.02	4.25 ± 0.02	5.28 ± 0.02	6.45 ± 0.02
Na^+ concentration	0.2N	0.2N	0.2N	0.35N	1.2N
Sodium citrate·$2H_2O$ (g)	19.6	19.6	19.6	34.3	19.6
Concentrated Hydrochloric acid (ml)	16.5	12.3	8.4	6.5	83.2
Thiodiglycol (ml)	20.0	5.0	-	-	-
Brij 35 solution (ml)	2.0	2.0	2.0	2.0	2.0
Caprylic acid (ml)	0.1	0.1	0.1	0.1	0.1
Ethanol abs. (ml)	-	40.0	-	-	-
Sodium hydroxide (g)	-	-	-	-	40.0

RESULTS AND DISCUSSION

Decalcification of the spicules with hydrochloric acid causes the release of a brown, amorphous solid. This material was not completely soluble in any of the following solvents: 1% HCl, 1% NaCl, 0.1% KOH, 75% ethanol, glacial acetic acid, distilled H_2O, or dimethyl sulfoxide. However, when heated with any of the solvents, some of the material did dissolve as indicated by the micro-biuret results discussed above.

The ultraviolet spectra taken of the ultraretentates showed a broad shoulder at 280-285 nm and a large peak beginning at 240 nm and rising steeply to 200 nm, the lower limit of the instrument. Aromatic amino acids absorb strongly in the region of the shoulder, although a contribution from the peptide bond is also present at those wavelengths. The major absorption of the peptide bond, however, occurs in the region from 200-220 nm with the wavelength of maximum absorption around 190 nm (Beaven and Holiday, 1952). The absorption spectra of the ultraretentates therefore are consistent with a polypeptide containing relatively few aromatic amino acid

residues. The UV spectra of the ultrafiltrates were identical to those of the ultraretentates, perhaps indicating that some smaller (less than 1000 MW) peptides passed through the ultrafilter membrane.

Gel chromatography of the ultraretentates (UR-1 and UR-2) on the Bio-Gel P6 column showed the presence of several distinct substances. In UR-1, three major peaks were present, having molecular weights of approximately 5000, 4200, and 1600. The molecular weights were estimated by comparing the volume needed to elute that substance to the elution volume of Dextran Blue 2000. In UR-2, three major peaks were present, having molecular weights of approximately 5000, 1800, and 1600. In both cases the material with molecular weight 5000 was present in largest amount (40% in UR-1, 75% in UR-2). It seems highly probable from these results as well as the absorption spectra of the ultrafiltrates discussed above, that some partial hydrolysis of any proteins present may have occurred. Although precautions were taken to minimize this, a small amount of hydrolysis, perhaps at highly susceptible peptide bonds involving serine or threonine, was seemingly inevitable. These gel chromatography results may explain the difference in solubilities of UR-1 (58% saturated) and UR-2 (72% saturated) during ammonium sulfate precipitation. The presence of more

than one component in varying proportions suggests that the interaction of these components could account for the differences in solubility encountered. Even if the 5000 MW components in UR-1 and UR-2 were identical, they could still appear to have slightly different solubilities due to different proportions of components in the aggregation.

Results of the amino acid analyses of UR-1 and UR-2 appear in Table 2 and Table 3. A comparison between UR-1 and UR-2 is most intriguing. The concentrations of only three residues differ significantly between them: ammonia, proline, and glycine. The relatively high amounts of ammonia present suggest that there may be large amounts of asparagine and glutamine residues in the peptides. However, insufficient removal of ammonium sulfate on the ultramembranes prior to hydrolysis may also be a factor. The differences in proline and glycine, on the other hand, could only have arisen from differences in UR-1 and UR-2. It is interesting that both proline and glycine increase in the same direction as the proportion of the 5000 molecular weight fragment increases. That is, UR-2, which contains a higher proportion of this fragment, also contains more glycine and proline. If the two 5000 MW fragments are similar, this suggests that the composition of the "pure" 5000 MW fragment may have still higher

TABLE 2

AMINO ACID COMPOSITION OF SOME STRUCTURAL PROTEINS COMPARED TO UR-1 AND UR-2*

Amino Acid	UR-1	UR-2	Sea Anemone Collagen[1]	Bovine Elastin[2]	Saw-Fly Silk Fibroin[3]	Wool Keratin[4]
Hydroxyproline	22	24	49	7	-	-
Aspartic Acid	72	83	81	7	22	64
Threonine	34	33	39	10	5	65
Serine	23	18	54	9	91	102
Glutamic Acid	62	63	95	17	363	119
Proline	129	160	63	125	0	59
Glycine	210	265	308	316	22	86
Alanine	74	80	113	213	382	53
Cystine(half)	0	0	3	0	0	105
Valine	56	59	34	134	19	55
Methionine	low	low	9	0	0	5
Isoleucine	27	24	23	27	19	31
Leucine	34	30	37	65	41	77
Tyrosine	14	13	8	6	21	40
Phenylalanine	26	26	12	34	0	29
Lysine	34	38	27	4	5	31
Histidine	low	low	5	1	6	9
Ammonia	172	53	71	-	350	-
Arginine	36	41	57	7	5	68

* Values expressed as amino acid residues/1000 amino acids.

1 Gross and Piez, 1960.

2 Franzblau and Lent, 1969.

3 Lucas and Rudall, 1968.

4 Bradbury, et al., 1968.

amounts of proline and glycine, perhaps even the requisite amount for a collagen sequence, in which every third amino acid must be glycine and the -Gly-Pro-X-Gly-sequence is common (Piez, 1960).

In Table 2, UR-1 and UR-2 are compared with four other structural proteins. Of the four, only elastin ever becomes mineralized, but this occurs as a pathological condition that increases with the age of the organism (Schiffman, et al., 1970). It is clear from Table 2 that the greatest similarity exists with the sea anemone collagen. The low amounts of sulfur-containing amino acids, the high amounts of imino acids, low histidine and tyrosine concentrations, and similar amounts of the aliphatic amino acids point out the similarity to the to this invertebrate collagen.

Elastin is next most similar to the ultraretentates. It too contains high imino acid and glycine concentrations, low sulfur amino acid presence, and similar amounts of the aromatic amino acids; but exceptionally high alanine and valine concentrations, and exceptionally low amounts of aspartic and glutamic acid are significant differences.

Silk fibroins vary considerably from species to species and, therefore, the obvious differences seen here may exaggerate the actual differences with the whole class of fibroins.

In general, however, the silk fibroins are low in imino acids. There also is a tendency in this group of proteins to have perhaps three or four amino acids account for almost the entire sequence. Although in UR-1 and UR-2 several amino acids stand out the composition in general is much more evenly distributed.

The keratins are rather unique among structural proteins in their high concentration of cystine. This, combined with relatively low amounts of imino acids and glycine, clearly illustrates the obvious differences between keratin and the ultrarententates.

In Table 3 UR-1 and UR-2 are compared to four other decalcified protein matrices. Enamel protein is least similar to the ultraretentates; its low glycine and high histidine concentrations are two of the more obvious differences.

The three other protein matrices shown in Table 3 all contain collagen, although to only a minor extent in the sponge spicule matrix (Travis, et al., 1967). The greatest differences between the ultraretentates and the sponge spicule matrix occur in imino acid, serine, and half-cystine concentrations, but the amounts of the other amino acids are quite well correlated. The sea urchin plate matrix differs most significantly in lysine, valine, phenylalanine, and serine content.

TABLE 3

AMINO ACID COMPOSITION OF SOME DEMINERALIZED ORGANIC MATRICES COMPARED TO UR-1 AND UR-2*

Amino Acid	UR-1	UR-2	Sponge Spicule[1]	Sea Urchin Plate[1]	Pig Enamel[2]	Human Bone[2]
Hydroxproline	22	24	0	73	0	99
Aspartic Acid	72	83	112	56	29	46
Threonine	34	33	53	33	37	17
Serine	23	18	86	88	46	33
Glutamic Acid	62	63	98	97	185	74
Proline	129	160	71	90	271	116
Glycine	210	265	221	339	49	329
Alanine	74	80	86	72	24	112
Cystine(half)	0	0	17	0	0	0
Valine	56	59	45	15	37	25
Methionine	low	low	4	23	47	5
Isoleucine	27	24	27	8	32	9
Leucine	34	30	44	25	94	24
Tyrosine	14	13	10	6	22	6
Phenylalanine	26	26	16	5	26	16
Lysine	34	38	42	4	11	22
Histidine	low	low	18	3	72	5
Ammonia	172	53	-	-	-	38
Arginine	36	41	38	57	6	52

* Values expressed as amino acid residues/1000 amino acids.

[1] Travis, et al., 1967.

[2] Piez, 1963.

Other than a rather high amount of imino acids, the collagen from human bone is remarkably similar in amino acid composition to the ultraretentates. In fact, aside from glycine content the composition of this collagen is more similar to the ultraretentates than to the collagen from sea-urchin plates. Considerable differences between the bone and plate collagen, in serine, methionine, and lysine content illustrate this. Of course, these differences may be somewhat irrelevant since the only absolute amino acid requirement for the collagen molecule is that one-third of its residues be glycine; nevertheless, the similarities between the ultraretentates and the collagens seen here do suggest that collagen molecules, or a collagen-like sequence as part of a larger protein, does exist in the organic matrix from gorgonian spicules.

Two other points should be made. One is by now obvious: if pretreatment with alkali removes any organic covering from the spicules as is the case with sea-urchin larvae spicules (Okazaki, 1960), then the material obtained after demineralization of coelenterate spicules with acid must have arisen from <u>within</u> the spicule.

The other point concerns the relation of the gorgonian spicule matrix to the horn-like protein of the central axis. Roche (1952) and Roche and Michel (1951) have studied the

gorgonins from several species of coelenterates. The most outstanding characteristic of these proteins is the presence, in significant amounts, of halogenated tyrosine residues. The complete lack of these in the ultraretentates in addition to higher amounts of tyrosine, the basic amino acids, and the common appearance of sulfur amino acids in the gorgonins testifies to the dissimilarity between these two types of skeletal protein.

SUMMARY

The spicules from the gorgonian Briareum asbestinum were isolated and the organic matrix obtained by decalcification with acid. Pretreatment of the spicules with alkali strongly indicates that the matrix arises at least in part from within the spicule. Because of probable partial hydrolysis during the isolation procedure, proteins were not isolated intact. Nevertheless, comparison of the amino acid composition of the peptides obtained with other decalcified organic matrices and several structural proteins suggests the presence of collagen in the organic matrix of Briareum spicules.

These results indicate that collagen may have been used as an organic substratum for calcification ever since the process of calcification appeared in evolutionary history.

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