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Isolation and characterization of two sterols from the green alga, Selenastrum capricornutum

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ISOLATION AND CHARACTERIZATION OF TWO STEROLS FROM THE GREEN ALGA, SELENASTRUM CAPRICORNUTUM

by

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Title: Isolation and Characterization of Two Sterols From the Green Alga, Selanastrum capricornutum.

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The green alga, Selenastrum capricornutum, was cultured in artificial nutrient medium utilizing five-gallon carboys, each of which contained 16 l. of culture. The algal cells were separated from the nutrient medium by continuous-flow centrifugation at 7500 RPM, and were then lyophilized. The lyophilized cells were extracted by refluxing with ether, acetone, and chloroform:methanol (2:1). Free sterols and sterol esters were separated from the crude extract using preparative thin-layer chromatography. Sterol esters were saponified and both the sterols and the fatty acids were recovered.

Individual sterols were separated from the free sterol fraction using argentation thin-layer chromatography. Gas chromatograms, mass spectra, and ultraviolet spectra were obtained for these sterols. The free sterol fraction was found to contain approximately 40% 24-methylcholesta-5,7-dien-3β-ol and 60% 24-ethylcholesta-5,7-dien-3β-ol.

The sterol ester fraction also contained these two sterols; however, the composition and amount of esterified sterols varied as a function of culture age. Sterol ester content was higher for older cultures, and in older cultures the composition of the esterified sterols more closely resembled that of the free sterols. The fatty acids obtained from the saponification of the sterol esters were methylated and were analyzed using gas chromatography. Tentative identifications, based upon comparative retention times, were made for several of these acids.

Sterols were extracted from the nutrient medium after harvest of the algal cells. Extraction was accomplished by mixing large quantities of nutrient medium with ether for several days, or by shaking small aliquots with ether. 24-methylcholesta-5,7-dien -3β-ol and 24-ethylcholesta-5,7-dien-3β-ol were isolated from the nutrient medium in approximately the same relative amounts as from the algal cells. The concentration of sterols in the nutrient medium was approximately equal to the water-solubility of cholesterol (25-29μg./1.).

Extraction procedures which release sterols from water soluble complexes were carried out on extracted cells and on extracted nutrient medium. These procedures failed to yield measurable quantities of sterols.

Treatment of extracted cells with strong base and subsequent extraction showed that all sterols had been extracted without prior cell lysis or pretreatment. An extraction of algal cells was carried out using DMSO:ether as the extraction solvent. This extraction resulted in complete removal of sterols from the cells, and the sterols were accompanied by only small amounts of other lipid-soluble material.

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of Raymond Mark Owings presented August 4, 1975.

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INTRODUCTION

Mass spectrometry and gas and thin-layer chromatography are techniques which have been utilized for rapid separation and identification of specific organic compounds, particularly phytosterols, from mixtures of plant natural products. These techniques have been extensively applied in recent investigations of sterol composition of algae.

I. STEROLS

Sterols are one group of steroids which are lipids having in common the perhydrocyclopentanophenanthrene skeleton (Fig. 1). Sterols have been isolated from all major groups of living organisms. The occurence and biosynthesis

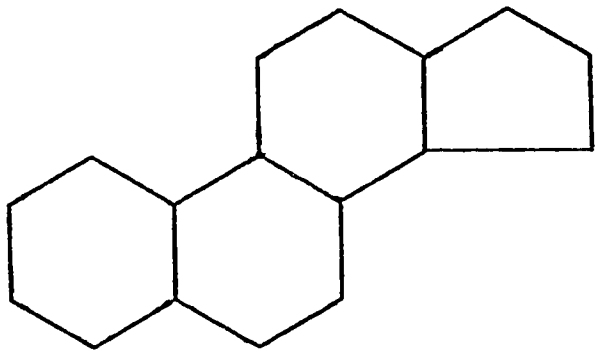


Figure 1. Perhydrocyclopentanophenanthrene.

of steroids in animals have been thoroughly investigated and are well understood. In higher animals cholesterol (Fig. 2) is the major sterol while cholesterol or mixtures of 27-, 28-, or 29-carbon sterols occur in lower animals (1). The 28- and 29-carbon sterols of lower animals appear to be derived from their diet (1,2).

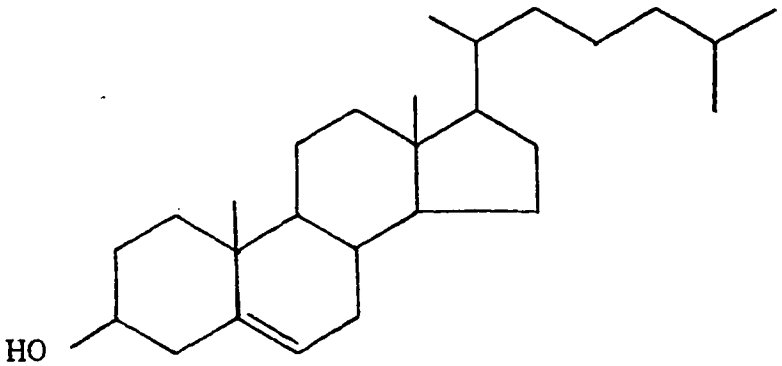


Figure 2. Cholesterol.

The majority of the phytosterols possess an alkyl side chain at C-24 and one or more double bonds in a ring or the side chain. In higher plants β-sitosterol is the major sterol in most species investigated and is often found together with varying amounts of stigmasterol and campesterol (Fig. 3). Cholesterol and other sterols have been reported in small amounts in higher plants; however, they are not common. Plant sterols have been assumed to be secondary metabolites of little importance to the organisms which synthesize them in contrast to the roles of steroids in animal metabolic processes. Results of recent studies of steroid metabolism in plants show, however, that this assumption may

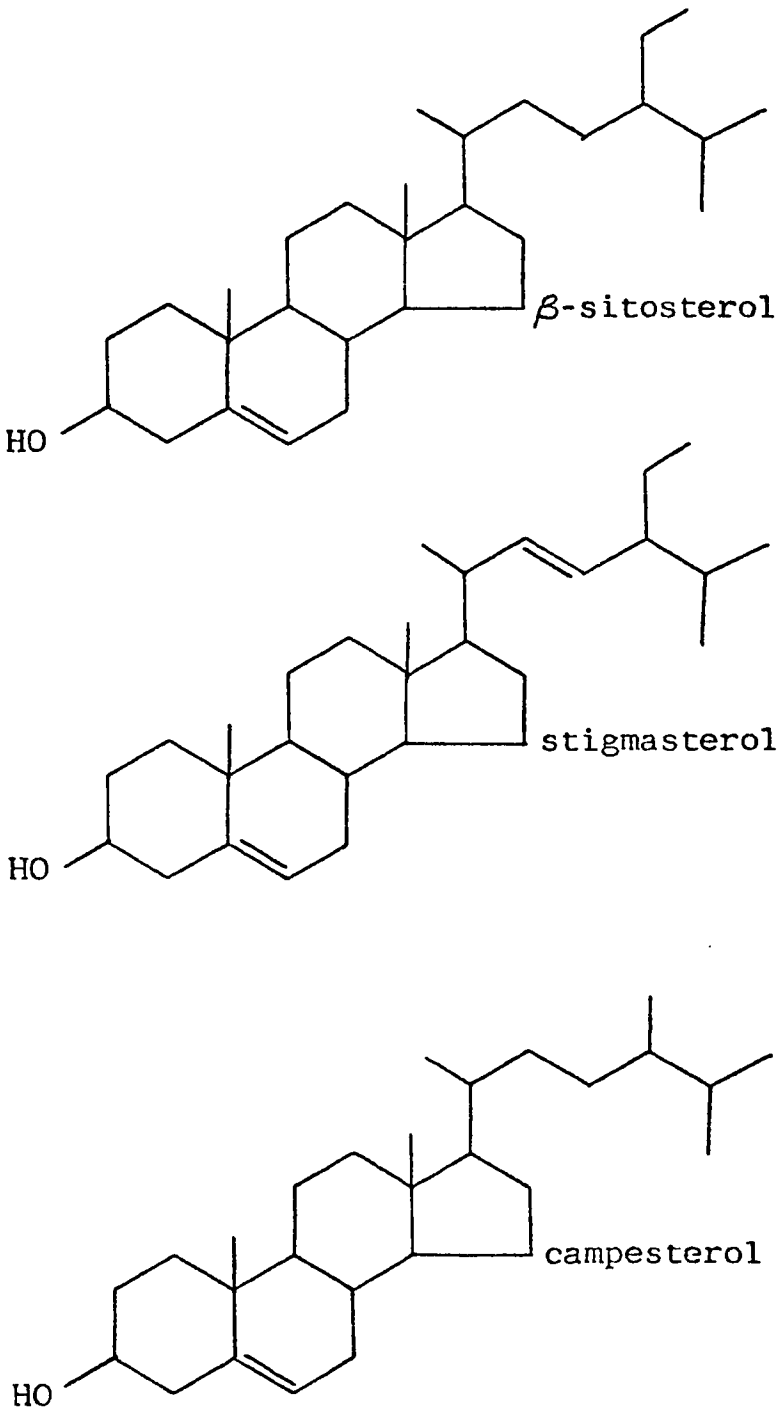


Figure 3. Sterols of higher plants.

not be correct. Much is being done to elucidate the possible roles of phytosterols in plant metabolism (1,3).

In contrast to animals and higher plants, the occurence of sterols in algae has not been thoroughly investigated, and generalizations as to sterol content can be made for only a few divisions of the algae. Until recently the analytical techniques available for study of algal sterols were not sufficiently advanced to allow definite characterization of small quantities of sterols isolated from complex mixtures. This factor, combined with a deficiency in the number of studies of algal sterols, has led to a confusion in the field of algal sterols which has yet to be fully resolved.

II. ALGAL STEROLS

Glenn W. Patterson, affiliated with the University of Maryland, reviewed the literature dealing with occurence of sterols in algae in 1971 (1). The brown algae (Phaeophyta) had been previously shown to contain fucosterol almost exclusively as the major sterol. Fucosterol was reported to occur together with lesser amounts of 24-methylenecholesterol in some species. The major sterol of most Rhodophyta varied from sample to sample (4,5). The sterols of Chlorophyta had been shown to be much less predictable with C-28 and C-29 sterols predominating and only a few species containing cholesterol as a major sterol. Few species of

other divisions had been investigated, and generalizations as to sterol content were not possible at the time of Patterson's review.

Sterols of Phaeophyta

Fucosterol is, without exception, the major sterol of all brown algae investigated (Fig. 4). Three species of Phaeophyta have been studied for sterols in recent years. Sargassum fluitans (Fucales) contains over ten sterols (6). Among these are fucosterol, cholesterol, 24-methylenecholesterol and trans-22-dehydrocholesterol. Approximately fifty percent of the total sterol is fucosterol. The occurence of 22-dehydrocholesterol as the major sterol of Hypnea japonica, a species of Rhodophyta, is interesting to note (7). Ascophyllum nodosum (Fucales), which had previously been shown to contain fucosterol (8,9,10), contains the following sterols: 90% fucosterol, 0.05% cholesterol, 1% brassicasterol, 2% 24-methylenecholesterol, 1% 24-ketocholesterol, and 6% saringosterol with sterol making up 0.10% of the algal dry weight (11). Laminaria saccharina (Laminariales) had also been shown to contain fucosterol (10). This species was reinvestigated and found to contain the following: 87% fucosterol, 11% 24-methylenecholesterol, 0.05% cholesterol, 0.05% 24-ketocholesterol, and 1.8% saringosterol with sterols making up 0.20% of the algal dry weight (11).

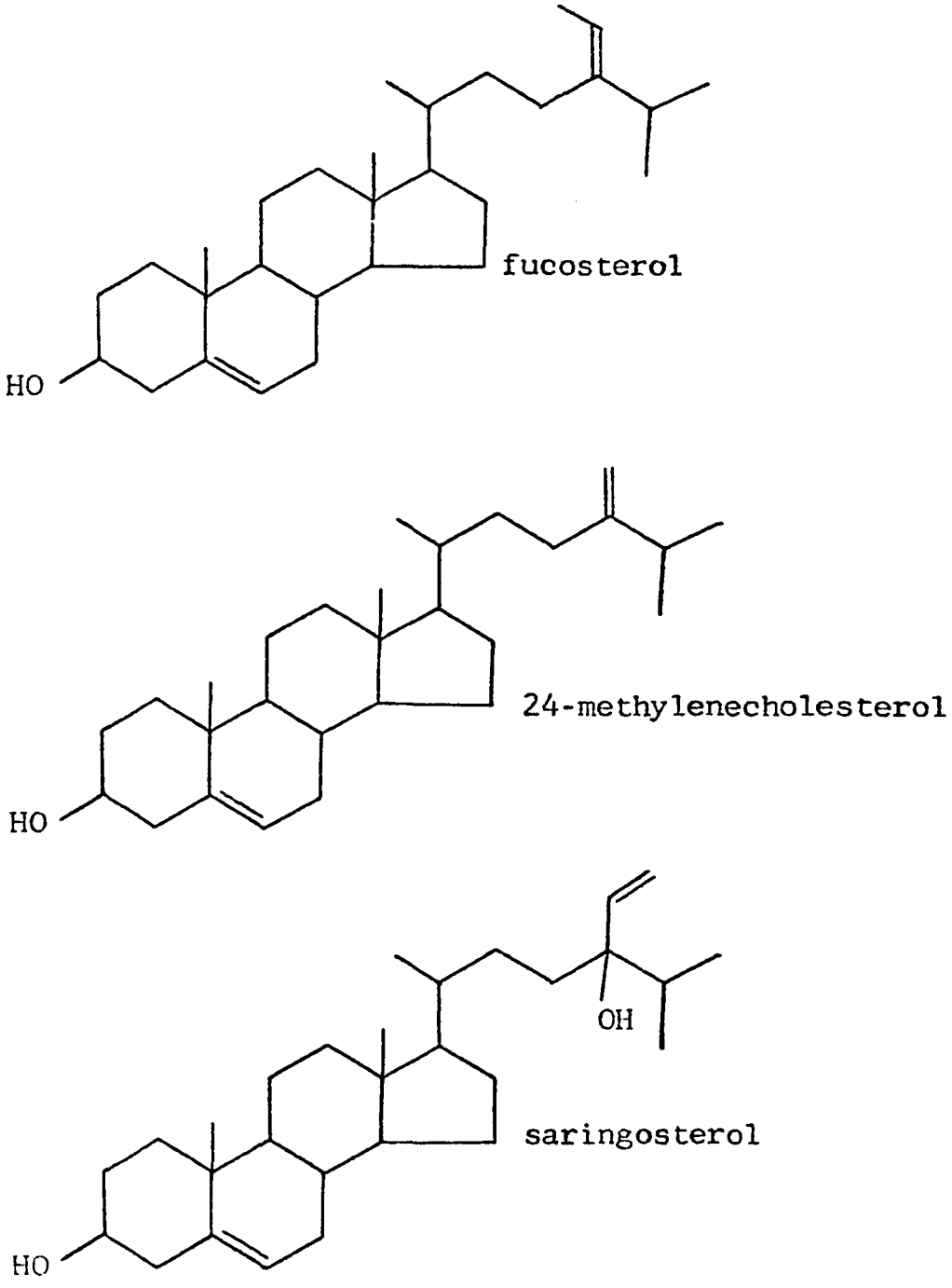


Figure 4. Sterols of Phaeophyta.

The presence of 24-methylenecholesterol in brown algae suggests the possibility that this sterol could serve as a biosynthetic precursor of fucosterol (1). Saringosterol has been identified in several species of Phaeophyta and could arise by air oxidation of fucosterol (12).

Sterols of Rhodophyta

The sterol composition of the red algae appears to be as predictable as that of brown algae (Table I). Most species investigated contain cholesterol alone or as the major sterol. Furcellaria fastigata contains 23% 24-methylenecholesterol, an unusual sterol in species of Rhodophyta (11). Of particular interest is the report of sterols in Porphyridium cruentum. This species had been reported as lacking sterols (13). Recent evidence indicates, however, that this alga contains 22-dehydrocholesterol (Fig. 5) as the major sterol along with cholesterol, desmosterol, ergosterol, and an unidentified C-29 sterol (14).

Patterson has suggested the possibility that sterols found in Rhodophyta might be absorbed by the algae from their environment (1). Several sterols including cholesterol, stigmasterol, and β-sitosterol have, in fact, been isolated from Gulf of Mexico waters (17). A probability exists, however, that if such absorption of sterols by the algae occurs, the sterol composition of the Rhodophyta would not be limited primarily to cholesterol. A more

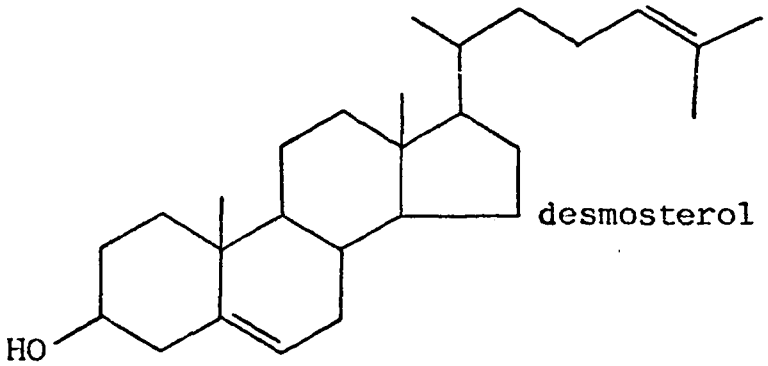
TABLE I

STEROLS OF RHODOPHYTA

Order	Species	Sterols[a]	% Sterols[b]	Ref.
Gigartenales	Gracilaria textorii	I	----	14
	G. verrucosa	I	0.22	15
	Meristotheca papulosa	I	----	14
	Furcellaria fastigata	I,II	0.004	11
	Phyllophora membranifolia	I	0.022	16
Porphyridiales	Porphyridium cruentum	I,III,IV,V,VI	----	14
Ceramiales	Polysiphonia subtilissima	I	0.18	15
	Chondria dasyphylla	I	0.20	15
	Ceramium rubrum	I	0.36	15
	Dasya pedicellata	I	0.17	15
	Grinnellia americana	I	0.18	15
	Caloglossa leprieurie	I	0.24	15
	Ptilota serrata	I	0.019	16

[a] I = cholesterol, II = 24-methylenecholesterol, III = 22-dehydrocholesterol, IV = desmosterol, V = ergosterol, VI = unknown C-29 sterol.

[b] % of dry weight.



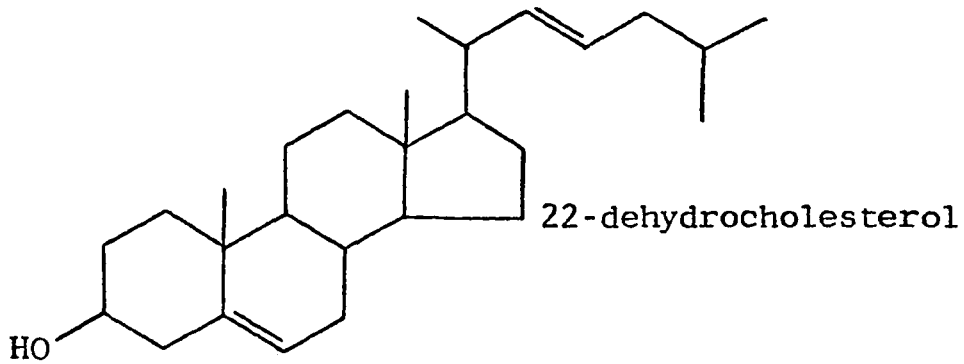


Figure 5. Sterols of Rhodophyta.

plausible explanation might be that algae and other marine organisms serve as sources for sterols isolated from marine waters. More experiments in this area are required before this question can be fully answered.

Sterols of Chlorophyta

The sterols of the Chlorophyta are much more varied than those of the Phaeophyta or Rhodophyta. Several species in seven orders of Chlorophyta have been investigated for sterols in recent years (Table II). In this division the

TABLE II

STEROLS OF CHLOROPHYTA

Classification	Sterols as % of total[a]															% Sterols	Ref.
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O		
Order Chlorococcales:																	
Chlorella ellipsoidea	6	22	65	7												0.32	18
C. pringsheimii	4	23	72	1												0.22	19
C. fusca								21	16	18						0.18	19
Scenedesmus obliquus								19	65	16						0.27	19
Order Cladophorales:																	
Cladophora flexuosa	1	2	2	6	45	22	21									0.06	19
Pithophora sp.	2		2	10	14	28	40								4	0.08	19
Order Zygnemetales:																	
Spirogyra sp.	6	12	32	39		11										0.19	19
Order Siphonales:																	
Halimeda incrassata		4		94		2										0.05	19
Codium fragile											94	6				----	20
Order Chlorosphaerales:																	
Coccomyxa elongata		48	19	33												0.27	19
Order Volvocales:																	
Chlamydomonas rheinhardi													44	56		0.38	19
Order Ulvales:																	
Enteromorpha intestinalis					90											0.01	15
E. plumosa					90											0.32	15
Ulva lactuca					90											0.12	15

[a] A = brassicasterol, B = Δ^5-ergostenol, C = poriferasterol, D = clionasterol, E = 28-isofucosterol, F = cholesterol, G = 24-methylenecholesterol, H = Δ^7-ergostenol, I = chondrillasterol, J = Δ^7-chondrillastenol, K = clerosterol, L = codisterol, M = ergosterol, N = 7-dehydroporiferasterol, O = unknown sterol.

most extensively studied genus is Chlorella. Species of Chlorella appear to contain Δ^5-sterols, Δ^7 sterols, or $\Delta^{5,7}$ sterols with none of these classes of sterols occuring together. Patterson (19) reported finding only Δ^7-ergostenol, chondrillasterol, and Δ^7-chondrillastenol (Fig. 6) in Scenedesmus obliquus in contrast to an earlier report that this species contained ergosterol and several additional sterols (21). With few exceptions the other orders of Chlorophyta are similar to the Chlorococcales. In the Ulvales 28-isofucosterol is the major sterol; this may be characteristic for this order. No other orders of Chlorophyta have been reported to contain 28-isofucosterol; however, this sterol has been isolated from higher plants (22). Patterson pointed out the possible importance of the relationship in view of the fact that Ulvales is thought to be a phylogenetically advanced order (15).

Codium fragile has been shown to contain the unusual 25-methylene sterols, clerosterol and codisterol (Fig. 7) (20). The Siphonales are thought to have been derived from the Chlorococcales (23), and the production of 24-ethyl and 24-methyl sterols in one species of Chlorococcales has been shown to proceed via 25-methylene intermediates (24,25). The lack of or low activity of a Δ^{25}-reductase has been proposed as the cause for the accumulation of clerosterol and codisterol in Codium fragile (20).

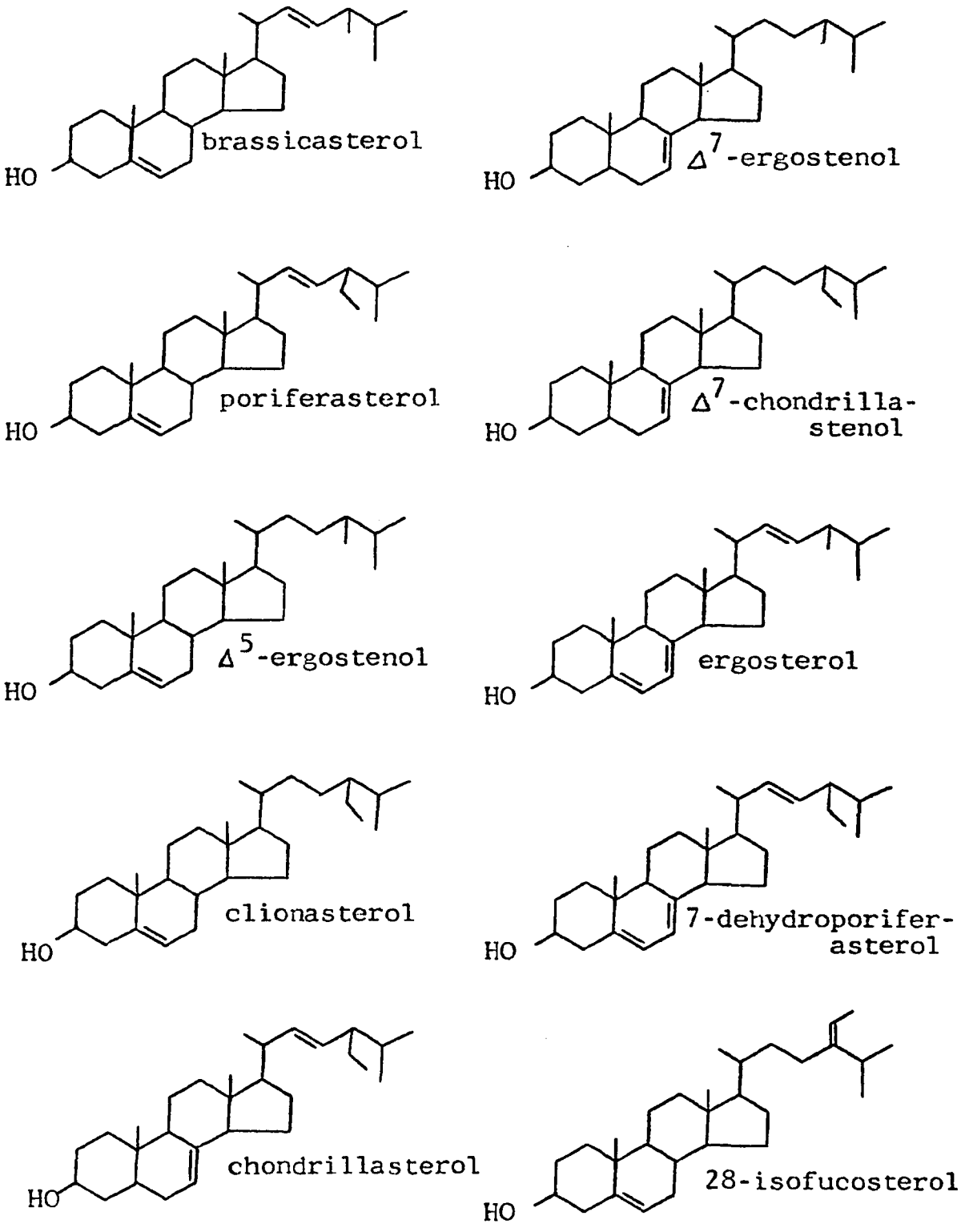


Figure 6. Sterols of Chlorophyta.

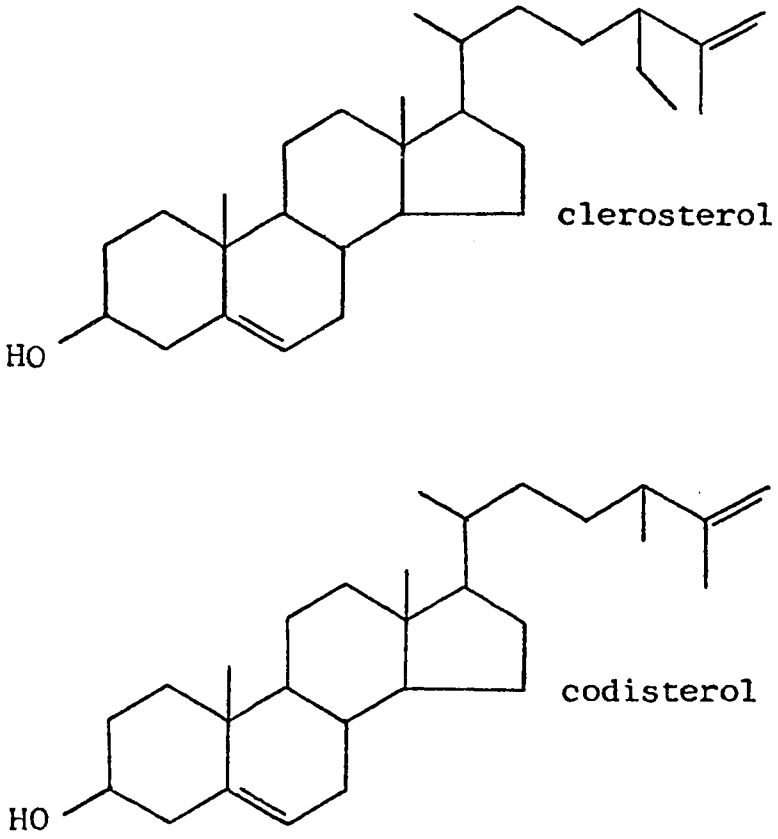


Figure 7. Sterols of Codium fragile.

Sterols of Chrysophyta

The earliest investigations of the sterol content of species of Chrysophyta concentrated on a few species of golden brown algae (Chrysophyceae). Since the time of Patterson's review, several species of Xanthophyceae and Bacillariophyceae have been investigated for sterol content (Table III). The marine diatom, Chaetoceros simplex calcitrans, was cultured in sea water containing 0.15 μg/ml.

TABLE III

STEROLS OF CHRYSOPHYTA

Class	Order	Species	Sterols[a]	% Sterols	Ref.
Bacillariophyceae	Centrales	Chaetoceros simplex calcitrans	I,II[b]	0.47	26
	Pennales	Nitzschia alba	III,IV		27
		N. closterium	V		28
		Phaeodactylum tricornutum	V		2
Xanthophyceae	Heterosiphonales	Botrydium granulatum	I,IV		29
	Heterotrichales	Tribonema aequale	I,IV		29
	Heterococcales	Monodus subterraneus	I,IV		29

[a]I = cholesterol, II = 24-methylenecholesterol, III = brassicasterol, IV = clionasterol, V = crinosterol.

[b]sterols in culture medium.

sterols, most of which was cholesterol (26). After harvest of the algae, the sea water was found to contain 0.18 μg/ml. sterols. Approximately 40% of the sterol mixture was cholesterol while 40% was 24-methylenecholesterol. Small amounts of β-sitosterol, campesterol, and stigmasterol were reported both before and after the growth of the algae in the water. This is one of few investigations of the culture medium for algal sterols, and it indicates both an uptake and release of sterols by the alga (26).

The diatoms Nitzschia closterium and Phaeodactylum tricornutum contain crinosterol which has opposite C-24 configuration to brassicasterol (2,28). The identification of this sterol in these species of Bacillariophyceae is the first well substantiated identification of a sterol with the 24-α configuration in algae. This sterol also occurs in esterified form in N. closterium. Sterols with the 24-β configuration have, however, been identified in the diatom, Nitzschia alba (27). Further investigations into the sterol content of species of Chrysophyta may provide useful phylogenetic information (2).

The sterol and sterol ester content have been determined for three species of Xanthophyceae (29). All three species contain cholesterol and clionasterol with these sterols present in approximately the same ratio in the free sterol and sterol ester forms.

Sterols of Charophyta, Euglenophyta, and Cyanophyta

Only a few species of these other algal divisions have recently been investigated for sterol content. Two species of Charophyta, Nitella flexilis and Chara vulgaris, contain clionasterol and 28-isofucosterol as the primary sterols (30). N. flexilis contains 58% clionasterol and 36% 28-isofucosterol while in C. vulgaris the ratio is reversed with clionasterol and 28-isofucosterol making up 39% and 54% of the total sterols. Both species contain traces of cholesterol and 24-methylenecholesterol.

Of the Euglenophyta, only Euglena gracilis has been thoroughly studied. This species had been reported to contain ergosterol (31,32); however, a reinvestigation has shown it to contain very little, if any, of this sterol (33). Sterols, sterol esters, and water soluble sterols were isolated from both light grown and dark grown E. gracilis. The free sterols of the light grown form are mostly Δ^7-sterols while those of the dark grown form are primarily a mixture of Δ^5- and Δ^7-sterols. In both types of E. gracilis only Δ^5-sterols occur in esterified and water soluble forms. Small quantities of $\Delta^{5,7}$-sterols were also identified in this species. E. gracilis appears to be one of only a few species of algae investigated which contains Δ^5-, Δ^7-, and $\Delta^{5,7}$-sterols simultaneously.

Two species of blue-green algae (Cyanophyta) have been recently investigated for sterols. Anabaena cylindrica con-

tains 90% brassicasterol, 8% cholesterol, and 1% Δ^5-ergostenol (34). Spirulina maxima was reported to contain cholesterol and β-sitosterol (35); however, the identification of β-sitosterol in this alga was based solely on thin layer chromatographic evidence. The configuration at C-24 has not been confirmed.

The brown algae contain fucosterol as the major sterol while cholesterol or desmosterol predominate in the red algae. The sterols of the green algae are more varied. Species of green algae contain either Δ^5-, Δ^7-, or $\Delta^{5,7}$-sterols, and the 24-alkyl sterols of these algae all appear to have the β configuration at C-24. With the exception of two species of diatoms, all 24-alkyl substituted algal sterols for which definitive data have been collected have been shown to have the 24-β configuration. This may be a generalization for most algal sterols as opposed to the 24-α configuration in higher plant sterols. Euglena gracilis contains Δ^5-, Δ^7-, and $\Delta^{5,7}$-sterols together. It is one of only a few algal species in which these are found simultaneously.

Sterol esters have been shown to occur in Euglena gracilis (Euglenophyta), Nitzschia closterium (Bacillariophyceae), and in three species of yellow-green algae (Xanthophyceae). Only in E. gracilis are the esterified sterols quantitatively and qualitatively different to any significant extent from the free sterols. Very little work

has been done toward clarification of the function of sterol esters in plants. Atallah and Nicholas (36) have examined the possible relationship between functions of sterol esters in plants and liquid crystalline properties of these esters. In spite of the lack of significant conclusions, the authors were able to show that microsomes and mitochondria of plant cells are able to esterify free sterol and to hydrolyze esters of sterols. Further investigations of this type may provide meaningful conclusions concerning the role of sterol esters in plant sterol metabolism.

The biosynthesis of algal sterols has attracted increasing attention in recent years (1). Cycloartenol replaces lanosterol as a precursor of sterols in several species of algae (18,37,38,39). Patterson and co-workers have utilized substances which inhibit certain steps in sterol biosynthesis to aid in isolation of sterol precursors from several species of Chlorella. The biosynthetic pathways proposed as a result of these experiments are, with the exception of the last few steps, similar to biosynthetic schemes proposed for higher plant sterols (41). The introduction of the side chain at C-24 probably proceeds by a similar mechanism in higher plants and in algae. The step of prime importance to the entire mechanism is alkylation at C-24 by S-adenosyl methionine (18, 39,40,41). This alkylation appears to be under strict

stereochemical control in all plants with the 24-α configuration predominating in higher plants and the opposite, 24-β configuration in algae. Few exceptions to this rule have been observed.

Knights (41) has reviewed several non-random metabolic processes involving plant sterols and has concluded from these processes and from the structural diversity of plant sterols that the idea that plant sterols are secondary metabolites of little importance to the plants which synthesize them is no longer acceptable. Further studies of the occurence and biosynthesis of algal sterols and of non-random metabolic processes involving sterols in algae may lead to a better understanding of the role of sterols in all plants. Since some algae serve as food for other organisms and some species have been shown to produce extracellular sterols (26,42), such studies may also lead to a better understanding of the role of algae in the biosphere.

III. WATER SOLUBLE STEROLS

Water soluble complexes of sterols have been studied in only a few species of plants since the discovery of a water soluble form of ergosterol in cell free extracts of yeast (43,44). Adams and Parks demonstrated that some complexing agent is present in yeast extracts which can form water soluble complexes with ergosterol and cholesterol

(43). Furthermore, the sterol can be released from the complex by several methods including treatment with acid, alkaline pyrogallol, dimethylsulfoxide, digitonin, or chromatography on silica gel (43,44,45). These authors concluded that the binding between the sterol and the complexing agent was non-covalent, and presented evidence that the complexing agent was a polysaccharide (44).

In Euglena gracilis, water soluble sterols were found to make up a substantial portion of the total sterols of both light and dark grown forms (33). Water soluble sterols were also found in the culture medium. Also, in E. gracilis, specific solubilization of cholesterol was observed (33,46). The water soluble sterols of E. gracilis which are released by acid treatment and by alkaline pyrogallol treatment differ in composition and the possibility exists that there are two types of water soluble complexes of sterols (46).

Water soluble sterols are known to occur in two species of higher plants, Kalanchoe blossfeldiana (47) and Zea mays (48). In these plants the water soluble sterols make up only a small portion of the total plant sterol.

Safe and co-workers (11) extracted water soluble sterols from three species of marine algae. Laminaria saccharina and Ascophyllum nodosum contained water soluble sterols equal in composition and amount to the free sterols while Furcellaria fastigata contained water soluble sterols

in about one-third the amount of free sterols.

Patterson and co-workers have investigated some species of Chlorophyta for water soluble sterol content; however, none were detected (49).

These discoveries of water soluble complexes of sterols in plants raise the following questions concerning plant sterol metabolism: What is the function of water soluble sterols in plants? What is the nature of the complex? Do these complexes occur in most green plants? Why is structural specificity observed in water soluble sterol complexes in some plants? Are there other chemical forms of sterols in plants which have not yet been discovered? Are water soluble and lipid soluble sterols present in different parts of the plant or of the cell? Do the water soluble sterols serve some purpose for organisms other than the plants which make them? This last question seems particularly appropriate for study in the algae since water soluble sterols are released by Euglena gracilis into the culture medium in which it is grown (42). The answers to these questions and others relating to water soluble sterols will be known only after further investigations into the occurence and structure of these complexes.

With the aid of modern analytical tools knowledge concerning structures and occurence of algal sterols is increasing at a rapid pace. Some of the most recent re-

search on algal sterols is expanding knowledge in areas such as mechanisms of biosynthesis of sterols. Bound forms of sterols including sterol esters and water soluble sterols may be of wide occurence in the algae and should be included in any study of algal sterols. Continued research into these areas is necessary to elucidate possible roles the algae may exert in the biosphere.

IV. SYSTEMATIC NAMES

The systematic names of sterols mentioned in this work are given below.

cholesterol = cholest-5-en-3β-ol

β-sitosterol = (24R)-24-ethylcholest-5-en-3β-ol

stigmasterol = (24S)-24-ethylcholesta-5,22-dien-3β-ol

campesterol = (24R)-24-methylcholest-5-en-3β-ol

fucosterol = E-24-ethylidenecholest-5-en-3β-ol

24-methylenecholesterol = 24-methylenecholest-5-en-3β-ol

desmosterol = cholesta-5,24-dien-3β-ol

22-dehydrocholesterol = cholesta-5,22-dien-3β-ol

brassicasterol = (24R)-24-methylcholesta-5,22-dien-3β-ol

24-ketocholesterol = cholest-5-en-3β-ol-24-one

ergosterol = (24R)-24-methylcholesta-5,7,22-trien-3β-ol

Δ^7-ergostenol = (24S)-24-methylcholest-7-en-3β-ol

Δ^5-ergostenol = (24S)-24-methylcholest-5-en-3β-ol

poriferasterol = (24R)-24-ethylcholesta-5,22-dien-3β-ol

clionasterol = (24S)-24-ethylcholest-5-en-3β-ol

28-isofucosterol = Z-24-ethylidenecholest-5-en-3β-ol

chondrillasterol = (24R)-24-ethylcholesta-7,22-dien-3β-ol

Δ^7-chondrillastenol = (24S)-24-ethylcholest-7-en-3β-ol

clerosterol = (24S)-24-ethylcholesta-5,25-dien-3β-ol

codisterol = (24S)-24-methylcholesta-5,25-dien-3β-ol

7-dehydroporiferasterol = (24R)-24-ethylcholesta-5,7,22-trien-3β-ol

crinosterol = (24S)-24-methylcholesta-5,22-dien-3β-ol

lanosterol = 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol

cycloartenol = 4,4,14α-trimethyl-9,19-cyclo-5α-cholest-24-en-3β-ol

In saturated side chains 24α-alkyl substituents are specified as (24R)- and 24β- substituents are specified as (24S)-; however, the presence of a Δ^{22}- double bond in the side chain reverses these specifications (29).

CHAPTER II

STATEMENT OF THE PROBLEM

With few exceptions recent investigations of algal sterols have been carried out using extraction and isolation methods which ignore sterols in any chemical form other than a free state or which fail to distinguish between free sterols and sterols in other chemical forms. Results from several species of higher plants and a few species of algae, however, have shown that esters and water soluble forms of sterols may make up a considerable percentage of the total plant sterols (11,29,33,42,47,48). In the case of Euglena gracilis the distribution of sterols in free and bound forms is quantitatively and qualitatively different between light and dark grown forms of the alga (33). The primary objective of the work described herein was to isolate and identify the sterols of the green alga, Selenastrum capricornutum, and to determine the distribution of these sterols among free and bound forms. Bound sterols include sterol esters and water soluble sterols.

Algae have received considerable attention in recent years in investigations involving water quality (50,51). Various algal species have been used extensively in bioassay studies of water quality whereby an alga is cultured

in artificial nutrient medium at varying concentrations of one or more of the constituents of the medium (51). The growth of the organism in these media is then compared with growth in samples of natural waters. Selenastrum capricornutum has been used for this purpose by several investigators and has been chosen by the United States Environmental Protection Agency as one of several test species for bioassay studies. Equally important, some species of algae have been shown to be contributors to water quality problems (52). Reports on algal extracellular products have been abundant for several classes of organic compounds; however, very little is known about release by algae of sterols into water. The wide range of compound types secreted into their environment by living algal cells has led to some speculation that algae may perform functions in the biosphere other than mere contribution to the carbon and energy cycle (53,54). The suggestion has been made, for example, that polypeptides secreted by some algal species may play a part in species relationships during algal blooms and may be involved in availing chemically "tied up" nutrients to algae and other planktonic organisms (53). Evidence is available to show that lipids secreted by some algae may constitute a part of the food supply for zooplankton (53).

The use of algae in bioassay studies is one method for studying the effects of human activity on our environ-

ment. In order to fully understand the interactions between the algae and their environment, however, more studies of the generation and fate of algal extracellular products are necessary. The sterols deserve attention because of their importance in animal metabolic processes and because of the evidence showing that they may function in plants in roles more important than those of secondary metabolites. A major objective of this work with S. capricornutum was to isolate and identify sterols released by the alga into the culture medium and to compare these results with those obtained from the algal cells.

In order to distinguish between free and bound sterols, extraction of S. capricornutum cells was carried out using no chemical pretreatment and no cell lysis. One problem which might arise from this approach is that extractions may be inefficient. In fact, isolated sterols may be from cell walls or membranes and may not be qualitatively or quantitatively identical to sterols contained in the cells. An objective of this project was to investigate this problem.

Dimethylsulfoxide has been used recently in studies of higher plant and fungal sterols (43,44,47). In addition to its usefulness for isolating sterols from water soluble complexes, the solvent properties of this compound may make it useful for extracting sterols from algal cells particularly in experiments involving extraction of untreated, intact

cells (55). This possible use of dimethylsulfoxide has been investigated.

Finally, one purpose for this work was to demonstrate the usefulness of preparative thin layer chromatography, gas chromatography, and mass spectrometry in isolating and identifying extremely small quantities of algal sterols, thus requiring only small amounts of harvested algal cells.

The specific objectives of the project may be outlined as follows:

1) To culture Selenastrum capricornutum in artificial nutrient medium and to obtain from these cultures sufficient algal cells and nutrient medium for extraction of sterols
2) To isolate and identify sterols of S. capricornutum obtained in the following forms:
    a) free sterols from algal cells
    b) sterol esters from algal cells
    c) water soluble sterols from algal cells by treatment with acid
    d) water soluble sterols from algal cells by treatment with alkaline pyrogallol
    e) water soluble sterols from algal cells by treatment with dimethylsulfoxide
    f) free sterols from the nutrient medium
    g) sterol esters from the nutrient medium
    h) water soluble sterols from the nutrient

medium by treatment with alkaline pyrogallol

i) water soluble sterols from the nutrient medium by treatment with dimethylsulfoxide

3) To determine the qualitative and quantitative differences between sterols extracted from algal cells before and after pretreatment of the cells with strong base.

4) To determine the usefulness of dimethylsulfoxide in extracting sterols from algal cells.

Preliminary chromatographic analysis of the fatty acids obtained from the sterol esters has been accomplished and tentative structural assignments have been made for some of these acids.

CHAPTER III

MATERIALS AND METHODS

I. CULTURE AND HARVEST PROCEDURES

The alga used in this project was Selenastrum capricornutum. The classification of this alga has been a matter of some controversy, and it has been given several names (56). The genus Selenastrum is in the Division Chlorophyta, Order Chlorococcales, and Family Oocystaceae (57). The morphology and growth characteristics of Selenastrum capricornutum have been described in detail (56).

Selenastrum capricornutum was chosen for this project for several reasons. First, the alga is readily available and is easy to culture in the nutrient medium used, and its growth characteristics in this medium are well known. Second, since the alga is used by many workers in bioassay studies, knowledge concerning the metabolism and extracellular products of the alga would be of value to these workers. Finally, a green alga was chosen because it is this division which contains the most diverse array of sterols among the algae, and it is the division over which much of the confusion in algal sterol studies has arisen.

All chemicals used in the preparation of the nutrient medium were Analytical Reagent grade. Weights were meas-

ured using a Mettler model H10 analytical balance. The water used was distilled and filtered through a charcoal filter purchased from the Gelman Instrument Company, Ann Arbor, Michigan, (model number 12510). Glassware used in the preparation of nutrient medium and cultures was cleaned according to the following procedure:

1) two detergent washes
2) sixty-second continuous rinse with tap water
3) two 10% HCl rinses
4) two-minute continuous rinse with tap water
5) three rinses with distilled water

Johnson, et al. found that six rinses were needed to remove acid wash from glassware under similar conditions (50).

During glassware cleaning and at all stages of the culturing and harvesting of the alga, procedures were strictly followed in order to prevent significant variation in the composition of the nutrient medium between batch cultures.

The nutrient medium was that described for use in the Provisional Algal Assay Procedure Bottle Test (51). The medium consisted of the following salts in aqueous solution. Macronutrients:

Salt	mg/l.	Element	mg/l.
$NaNO_3$	25.500	N	4.200
$K_2HPO_4 \cdot 3H_2O$	1.368	P	0.186
$MgCl_2 \cdot 6H_2O$	12.164	Mg	2.904

Salt	mg/l.	Element	mg/l.
$CaCl_2 \cdot 2H_2O$	4.410	Ca	2.143
$MgSO_4 \cdot 7H_2O$	14.700	S	1.911
$NaHCO_3$	15.000	C	1.202
		Na	11.001
		K	0.469

Micronutrients:

Salt	µg/l.	Element	µg/l.
H_3BO_3	185.520	B	32.460
$MnCl_2 \cdot 4H_2O$	415.447	Mn	115.374
$ZnCl_2$	32.709	Zn	15.691
$CoCl_2 \cdot 6H_2O$	1.428	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.011	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.260	Mo	2.878
$FeCl_3 \cdot 6H_2O$	159.882	Fe	33.051
$Na_2EDTA \cdot 2H_2O$	300.000		

Stock solutions of the individual macronutrient salts were prepared at one thousand times the final concentration. The micronutrients were prepared in a single stock solution at ten thousand times the final concentration. All stock solutions were stored in the dark at room temperature in one liter glass bottles stoppered with aluminum foil-wrapped rubber stoppers. The micronutrient stock solution was prepared fresh at two month intervals; however, preparation of fresh macronutrient stocks was unnecessary during the project.

The purpose of culturing Selenastrum capricornutum was to provide quantities of algal cells and supernatant sufficient for the experimental work while maintaining constant culture conditions between batches. This was accomplished by culturing the alga in batch cultures in five gallon carboys. The inoculum for the batch cultures was prepared from smaller stock cultures.

Stock cultures of S. capricornutum were grown in 100 ml. of sterile nutrient medium in 250 ml. erlenmeyer flasks stoppered with foam plugs. Stock cultures were incubated for seven days. Then aliquots were taken for inoculation into fresh nutrient medium. No culture older than seven days was used in preparing inoculum. Two or three stock cultures were kept at any one time.

The batch cultures used in the experimental work were prepared in five gallon Pyrex carboys containing 16 liters of sterile nutrient medium. The carboys were inoculated with aliquots from seven-day-old stock cultures after which they were incubated for 14 to 21 days prior to harvest.

Nutrient medium for stock S. capricornutum cultures was prepared by mixing 1.0 ml. each of the macronutrient stock solutions (measured by volumetric pipet) plus 0.1 ml. of micronutrient stock solution (measured by serological pipet) with distilled water in a one-liter volumetric flask. The prepared medium was dispensed by graduated cylinder into ten clean 250 ml. erlenmeyer flasks, each of which was

stoppered with a foam plug and autoclaved at 121 ^{o}C for 15 minutes. Following sterilization culture flasks were stored in the dark at room temperature until use, never more than six weeks.

Nutrient medium for each batch culture was prepared by mixing 16 ml. of each macronutrient stock solution (measrued by buret) plus 1.6 ml. of the micronutrient stock solution (measured by serological pipet) with 16 liters of distilled water in a clean five gallon carboy. The carboys were stoppered with rubber stoppers fitted with aeration connections (Fig. 8) and autoclaved for 90 minutes at 121 ^{o}C.

The aeration connections were installed in a #12 rubber stopper drilled with two holes. The air inlet consisted of a piece of 8 mm. glass tubing which extended a few inches above the stopper and terminated near the carboy bottom with a 0.5 inch fritted glass disk. The inlet tube was connected by means of 10 inch length of Tygon tubing to another piece of glass tubing which was enlarged at the inlet end to accomodate a foam plug. This enlarged section of tubing was fitted with a one-hole rubber stopper which served to connect the carboy air inlet to the air source (vide infra). The air outlet consisted of a piece of 8 mm. glass tubing terminating approximately two inches below the stopper and having the outside end enlarged to accomodate a foam plug. Following sterilization and cooling, the carboys were cov-

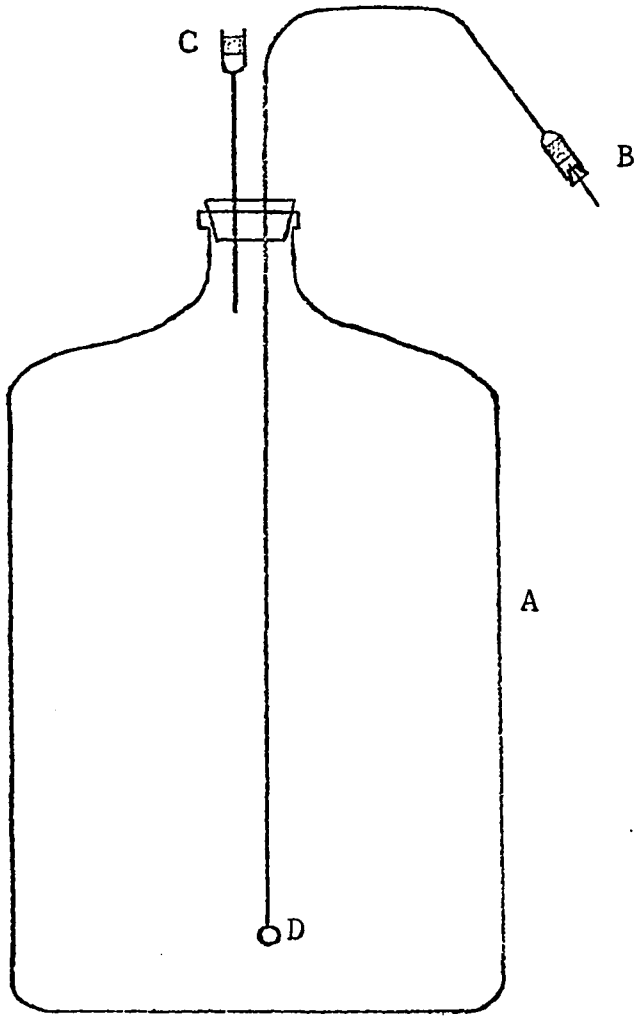


Figure 8. Apparatus for batch culture of Selenastrum capricornutum. A = five-gallon Pyrex carboy. B = air inlet. C = air outlet. D = fritted-glass disk.

ered with aluminum foil bags and stored at room temperature until use.

On the seventh day of incubation 30 ml. of culture was removed from a randomly chosen stock culture and placed in two sterile 15 ml. centrifuge tubes. The culture was centrifuged; the supernatant was discarded. The cells in each tube were resuspended in 15 ml. of sterile $NaHCO_3$ solution containing 15 mg/l. $NaHCO_3$. They were again centrifuged, and the supernatant was discarded. This wash procedure was repeated twice more, and the cells were finally suspended in 15 ml. of the sterile $NaHCO_3$ solution. By using a sterile disposable pipet a small aliquot was removed from one of the tubes, and the concentration of cells in cells/ml. was determined by direct counting in a Fuchs-Rosenthal hemacytometer. Direct counting is a convenient method for determination of cell concentrations in cultures of S. capricornutum. The cells fail to form aggregates or colonies and lack gelatinous sheaths (56).

An amount of the cell suspension was then transferred into each of the new stock culture flasks by using a sterile 1.0 ml. serological pipet. The volume of this transfer was calculated to result in an initial concentration of 1000 cells/ml. in each new stock culture.

Following inoculation, small amounts of each old stock culture and of the prepared inoculum were streaked onto nutrient agar plates. These plates were incubated in

the dark at room temperature for seven days after which they were visually checked for the presence of bacteria. The nutrient agar plates were prepared by mixing 4.6 grams of nutrient agar with 200 ml. of distilled water, heating the mixture to boiling and then autoclaving at 121 ^{o}C for 15 minutes. The sterile agar medium was then poured into sterile, disposable petri plates which were refrigerated until use.

When culturing of S. capricornutum was first undertaken, the new stock cultures were prepared by inoculation of fresh nutrient medium with 1.0 ml. of an old stock culture. The new stock culture was then incubated until used. The inoculation procedure outlined above was adopted for several reasons. First, the nutrient medium was totally inorganic, but the washing of the cells for the inoculum and the exclusive use of seven-day-old cultures were necessary in order to keep contamination of cultures by bacteria to a minimum (58). In spite of these precautions and the use of sterile techniques maintaining bacteria-free stock cultures proved to be impractical. No bacteria-free batch cultures were obtained. Second, it was desirable to record cell concentrations so that cultures differing greatly from average growth could be identified. Finally, a regular schedule of inoculating fresh stock cultures results in more viable cultures and more reproducible cell counts (59).

Inoculation of batch culture carboys was done by transferring the entire 100 ml. of a seven-day-old stock culture into each carboy. The concentration of cells in the stock culture was determined prior to inoculation, and the initial concentration in each carboy was calculated.

Initially S. capricornutum was cultured in a green house; however, for convenience the culturing operation was moved into the laboratory. The culture table consisted of a laboratory bench 30" X 15' fitted with a bank of ten pairs of vertically arranged, 24 inch cool-white fluorescent lights providing illumination of 600 foot-candles at a distance of six inches from the lights. Culture containers were placed in a line along the light bank with the edges of the containers nearest the lights lying on the 600 foot-candle marker (Fig. 9). Illumination was continuous. No special provision was made to maintain constant temperature in the room (Table IV). The pH of the nutrient medium was approximately seven both before and after sterilization. Two, and sometimes three, stock cultures were kept growing at any one time. Throughout the seven day incubation period the culture flasks were hand swirled at least twice daily at irregular intervals.

After inoculation the batch cultures were aerated continuously with Silent Giant air pumps fitted with dust traps (Fig. 10). In most cases the air bubbling provided sufficient mixing to maintain the cells in suspension; however,

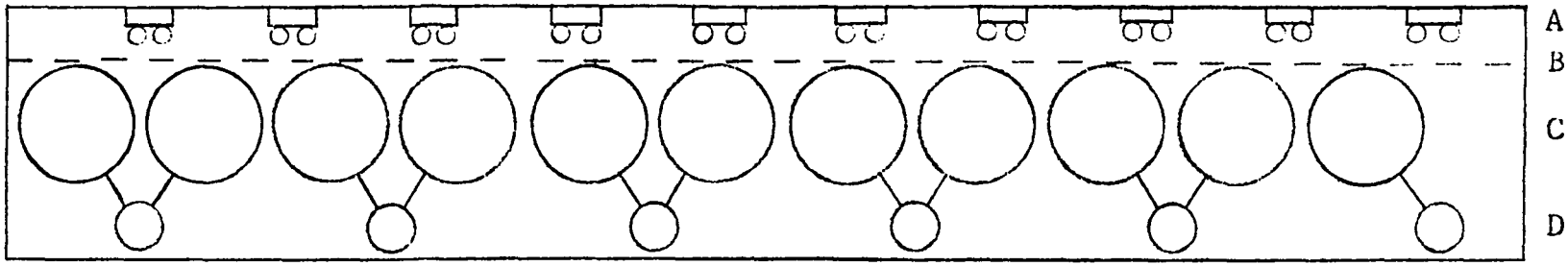


Figure 9. Laboratory bench with apparatus for batch cultures of Selenastrum capricornutum. A = light bank. B = 600 foot-candle marker. C = culture carboys. D = air pumps.

TABLE IV

MONTHLY TEMPERATURE EXTREMES IN THE CULTURE LABORATORY

Month	Temperature °C[a] Maximum	Minimum
March, 1974	24.0	19.5
April, 1974	24.5	20.0
May, 1974	24.5	19.0
June, 1974	27.0	22.0
July, 1974	26.5	25.0
August, 1974	25.0	21.5
September, 1974	22.0	21.0
October, 1974	23.5	18.5
November, 1974	21.5	19.0
December, 1974	21.0	14.0
January, 1975	20.0	18.0
February, 1975	21.0	19.0
March, 1975	20.5	19.0

[a]Temperatures were recorded three times daily.

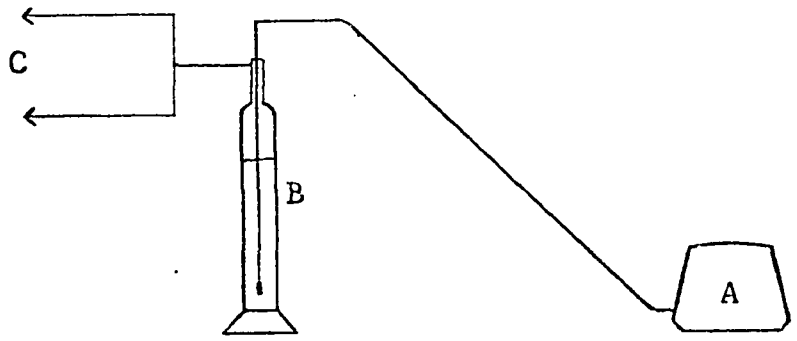


Figure 10. Air supply for batch cultures of Selenastrum capricornutum. A = Silent Giant air pump. B = water-filled dust trap. C = air supply for two culture carboys.

occasionally a layer of cells collected on the carboy bottom during the first few days of incubation. In these instances the carboys were hand swirled to resuspend the cells.

Initially batch cultures consisted of ten to twelve culture carboys per batch. In order to allow more efficient harvesting, batches were later reduced to three or four culture carboys each. Most batches included a control carboy treated in the same manner as the culture carboys except no inoculum was added.

Stock and batch cultures were labelled by sequential numbering with the number of each stock culture preceeded by the letter S and that of each batch culture preceeded by the letter B. Cell concentrations and other pertinent data recorded for batch cultures are presented in Table V.

After incubation for 14 to 21 days the aeration of batch cultures was stopped, and the carboys were immediately covered with aluminum foil bags. Separation of the algal cells from the nutrient medium was accomplished by continuous-flow centrifugation using a Sorvall RC2-B centrifuge at 7500 RPM (Fig. 11). The wet cells obtained were lyophilized, weighed, and stored in the freezer. The supernatant was then ready for extraction. If any delay was experienced between harvest and extraction, the carboys containing supernatant were kept covered with aluminum foil bags. Cells from the first four batches were lyophilized

TABLE V

pH AND CELL COUNTS OF BATCH CULTURES[a]

Batch #	Carboy #	Initial cells/ml.	Final cells/ml.	Final pH
B-13[b]	3	1.6×10^4	2.7×10^6	7.5[c]
	4	1.1×10^4	3.0×10^6	7.5
	6	control		7.5
	12	control		7.5
B-14[d]	1	1.3×10^4	4.4×10^6	7.2[c]
	5	8.1×10^3	3.2×10^6	7.3
	10	5.8×10^3	3.5×10^6	7.3
B-15[d]	2	2.4×10^4	3.0×10^6	6.8[e]
	7	1.6×10^4	2.4×10^6	6.8
	8	1.9×10^4	2.8×10^6	6.8
	9	control		6.9
B-16[b]	3	control		6.8[e]
	5	3.1×10^4	3.7×10^6	6.9
	12	3.3×10^4	4.2×10^6	6.9

[a]These represent only a select group of cultures for which full data were recorded. Throughout the project sixteen batch cultures with a total of eighty-nine carboys were grown, most of which were utilized to standardize culturing, harvesting, and extraction techniques.

[b]Incubation time 14 days.

[c]Determined with a Corning model 6 pH meter.

[d]Incubation time 21 days.

[e]Determined with a Photovolt-digicord pH meter.

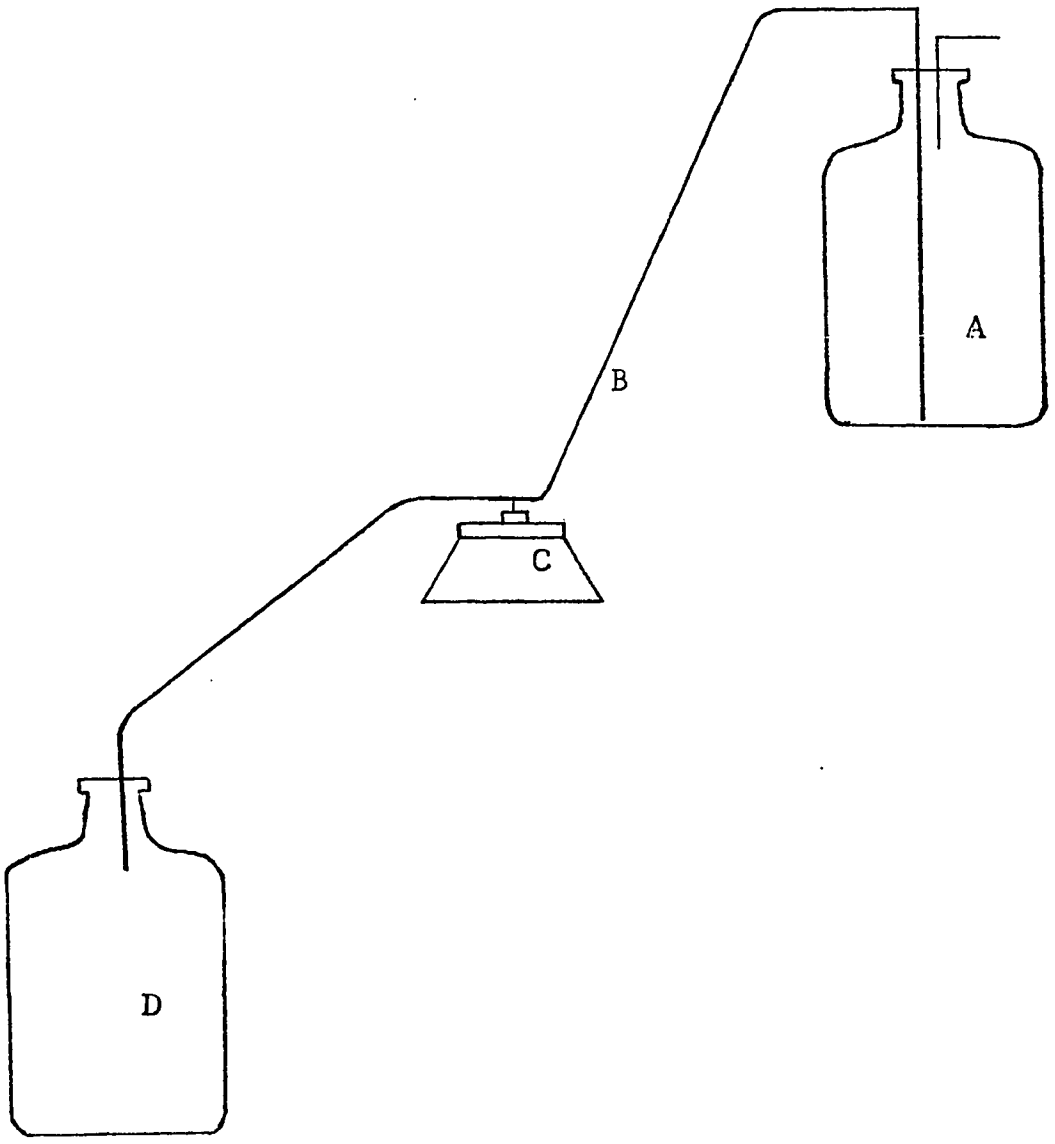


Figure 11. Apparatus for harvest of batch cultures of Selenastrum capricornutum. A = culture carboy. B = Latex-tubing siphon. C = continuous-flow attachment installed in a Sorvall RC2-B centrifuge. D = receiver for supernatant.

yielding 14.9, 9.1, 11.3, and 10.4 grams respectively of dry cells.

Prior to harvest, a small sample of culture from each carboy was streaked onto nutrient agar plates, and 100 ml. of culture was removed for pH measurements and cell counts. The nutrient agar plates were visually checked for the presence of bacteria after an incubation period of seven days in darkness and at room temperature.

II. CHROMATOGRAPHIC AND SPECTROMETRIC METHODS

Solvents

All solvents used for extractions and chromatography were Reagent grade and were redistilled just prior to use.

Thin Layer Chromatography

Thin layer chromatography was carried out using four different TLC systems. TLC system I consisted of 20 X 20 cm. glass plates pre-coated with a 2.0 mm. layer of silica gel F-254 (EM Laboratories, Inc., Elmsford, New York). The solvent for this system was benzene:ether (4:1). TLC system II consisted of 20 X 20 cm. glass plates pre-coated with a 0.5 mm. layer of silica gel F-254 with benzene:ether (4:1) as the solvent. The plates used in TLC system III were identical to those used in system II; however, in system III the solvent used was chloroform. Thin layer chromatography plates used for these TLC systems were kept in a desiccator and were activated at 110 ^{o}C for 30 minutes just prior to

use.

TLC system IV consisted of 20 X 20 cm. Baker-flex sheets coated with a 0.25 mm. layer of silica gel 1B-F (J.T. Baker Chemical Co., Phillipsburg, New Jersey) which was impregnated with silver nitrate. Impregnation was accomplished by dipping the pre-coated sheets in a 10% solution of $AgNO_3$ in ethanol:water (4:1) for one minute followed by drying at room temperature for one hour and activation at 110°C for 30 minutes. The solvent for TLC system IV was chloroform. The TLC systems are summarized in Table VI.

TABLE VI

TLC SYSTEMS

System	Adsorbent	Thickness	Solvent
I	silica gel F-254	2.0 mm.	benzene:ether (4:1)
II	silica gel F-254	0.5 mm.	benzene:ether (4:1)
III	silica gel F-254	0.5 mm.	chloroform
IV	silica gel $\frac{\text{1B-F}}{AgNO_3}$	0.25 mm.	chloroform

Gas Chromatography

All gas chromatograms were obtained using a Perkin-Elmer model 990 gas chromatograph fitted with dual flame ionization detectors which were operated at hydrogen and air pressures of 21 psi and 23 psi respectively. All injections were made into a glass lined injection port at 275 °C with the detectors also at 275 °C.

Gas chromatograms of sterols and sterol acetates were obtained using two GC systems. GC system I consisted of a 6' X 4 mm. I.D. glass column packed with 3% OV-1 on 80/100 mesh Anakrom Q. The column was operated isothermally at 275 ^{o}C with a helium flow rate of 30 ml./min. GC system II was a 6' X 4 mm. I.D. glass column packed with 3% OV-17 on 80/100 mesh Anakrom Q. It was also operated at 275 ^{o}C; however, the helium flow rate for this system was 60 ml./min.

GC system III employed the same column and carrier gas flow as GC system I, but in system III the temperature was programmed from 100 ^{o}C to 275 ^{o}C at an increase of 12 ^{o}C/min. after an initial hold time of four minutes after injection. GC system IV employed the same column and carrier gas flow as GC system II and the same temperature programming as system III. GC systems III and IV were used to obtain chromatograms of fatty acid methyl esters. The GC systems are summarized in Table VII.

Absorption Spectrometry

Ultraviolet absorption spectra were obtained with a Cary 14 spectrophotometer. Spectra were obtained for chloroform solutions of the samples.

Mass Spectrometry

Mass spectra were obtained using a Dupont 21-491B mass spectrometer. Samples were introduced through a di-

rect inlet heated to 150 °C. The ionizing voltage was 70 ev.

TABLE VII

GC SYSTEMS

System	Liquid phase	Temperature	Program rate	Carrier gas flow
I	3% OV-1	275 °C	isothermal	30 ml/min
II	3% OV-17	275 °C	isothermal	60 ml/min
III	3% OV-1	100-275 °C	12 °C/min[a]	30 ml/min
IV	3% OV-17	100-275 °C	12 °C/min[a]	60 ml/min

[a]After four minute initial hold.

III. EXTRACTION METHODS

Extraction of Lyophilized Cells

The general scheme for extraction of lyophilized cells of S. capricornutum involved extraction with a series of organic solvents followed by preparative thin layer chromatography of the extracts to separate sterols and sterol esters from the crude extract. The cell residue was then extracted with water to obtain water soluble sterols.

Extraction With Organic Solvents. A quantity of lyophilized S. capricornutum weighing 7.60 grams was extracted by refluxing for one hour with each of the following solvents:

300 ml. ether
300 ml. ether
300 ml. acetone
300 ml. acetone

300 ml. chloroform:methanol (2:1)
300 ml. chloroform:methanol (2:1)
300 ml. chloroform:methanol (2:1)
300 ml. ether

The organic extracts were combined, filtered, and evaporated to dryness in a rotary evaporator at reduced pressure. This procedure yielded 2.99 grams of a dark, green, oily residue which was then redissolved in 150 ml. of ether. A small portion of this solution was chromatographed on TLC system II along with a marker of authentic cholesterol, and sterols were visualized by spraying with 50% H_2SO_4 followed by heating for three minutes at 110 ^{o}C. The plate was examined under a long-wave ultraviolet lamp, and two spots were detected. One spot with Rf 0.17 to 0.23 corresponded to the cholesterol marker and was assumed to contain free sterols while the second spot with Rf of 0.70 to 0.84 was assumed to contain sterol esters. These assumptions were based on the visualization with 50% H_2SO_4 and on previously reported relative Rf values for sterols and sterol esters (60). The remainder of the organic extract was subjected to preparative thin layer chromatography on TLC system I. Several plates were required to accomplish this as no more than 300 mg. of sample was applied per plate. The plates were divided into five zones. The adsorbent in the zones of Rf range equal to those determined by analytical TLC to contain sterols and sterol esters was transferred into 250 ml. beakers and washed three times with 100 ml. of ether.

The ether extract of the adsorbent from the free sterol zone was allowed to evaporate to a small volume and was then subjected to a second preparative TLC separation on TLC system III. Also, a marker of authentic cholesterol was spotted at one edge of the plate. Following development, the marker region was sprayed with 50% H_2SO_4, the plate was heated at 110 ^{o}C for three minutes, and the marker region was examined under a long-wave ultraviolet lamp. The adsorbent from the zone having an Rf range equal to that of the marker spot (0.17-0.31) was transferred into a beaker and washed with 3 X 25 ml. of ether. The ether washings were combined and allowed to evaporate to dryness yielding 18.7 mg. of white crystalline residue which was redissolved in 5 ml. of ether and labelled fraction I. A second extraction of 10.5 grams of lyophilized S. capricornutum from a different batch culture yielded 28.5 mg. of fraction I.

The eluent from the sterol ester zone of the original separation was allowed to evaporate to dryness, and the residue was heated on a steam bath for two hours with 50 ml. of 10% KOH in methanol:water (9:1). After cooling to room temperature, this reaction mixture was poured into an equal volume of water and was shaken with three 25 ml. portions of ether. The combined ether extracts were dried over anhydrous $MgSO_4$ and evaporated to dryness under a gentle stream of nitrogen. The yield was 7.4 mg. of a white, crystalline residue. This residue, labelled fraction II, was redis-

solved in 5 ml. of ether and screened by thin layer chromatography on TLC system II. Visualization with iodine vapor revealed the presence of only one spot with Rf in the range 0.17 to 0.21. This Rf range corresponds to that observed for cholesterol on TLC system II.

The second extraction of lyophilized S. capricornutum (10.5 grams) yielded 18.0 mg. of fraction II.

The pH of the aqueous fraction from the saponification of the sterol esters was adjusted to 2.0 by addition of concentrated HCl. The acidified solution was shaken with three 25 ml. portions of ether, and the combined extracts were dried over anhydrous $MgSO_4$ and evaporated to dryness under a gentle stream of nitrogen. The brown, oily residue (100.0 mg.) obtained was redissolved in 5 ml. of ether and screened for fatty acid content on TLC system II. Visualization with iodine vapor indicated only one spot with an Rf range similar to that of authentic steric acid (Rf = 0.23-0.45). The solution was labelled as fraction III.

Extraction With Water. After the extraction with organic solvents the cell residue was allowed to air dry and was then extracted three times with water by refluxing for one hour with 300 ml. of water each time. The combined aqueous extracts were centrifuged to remove cellular debris, and the supernatant was shaken with three 50 ml. portions of ether. The purpose of the ether extraction was to remove any lipid soluble material from the aqueous extracts before

accomplishing the removal of water soluble sterols. These ether extracts were combined, dried over anhydrous $MgSO_4$ and the solvent allowed to evaporate. A residue weighing 3.3 mg. was obtained. An ether solution of this residue was screened on TLC system II and no sterols were detected.

The aqueous extract was split into two portions. One part was treated with acid using a modification of the procedure described by R. J. Pryce (47). This involved mixing with 450 ml. of concentrated HCl:water:methanol (1:2:3) and stirring this mixture for six days at room temperature. The resulting solution was shaken with 150 ml. and then three 50 ml. portions of ether. The ether extracts were combined, dried over anhydrous $MgSO_4$, and allowed to evaporate to dryness. This procedure yielded 71.6 mg. of a green, oily residue which was screened on TLC system II and found to contain no sterols. Acid treatment of aqueous extracts was not performed in subsequent extractions of lyophilized cells and supernatant.

The aqueous fraction remaining after the acid treatment was made basic by addition of solid potassium hydroxide and was then treated with pyrogallol using a method similar to that described by B. G. Adams and L. W. Parks (43). The alkaline solution was mixed with 150 ml. of 0.5% pyrogallol in methanol, 100 ml. of 60% aqueous potassium hydroxide, and 150 ml. of methanol. This mixture was refluxed for three hours, cooled, and then shaken with three 100 ml. por-

tions of ether. These ether extracts were combined, dried over anhydrous $MgSO_4$, and allowed to evaporate to dryness. A quantity of white, crystalline residue weighing 0.7 mg. was obtained by this method. The residue was redissolved in 5 ml. of ether and was labelled as fraction IV. The second extraction of S. capricornutum cells (10.5 grams) yielded 26.8 mg. of fraction IV from one half of the aqueous extract. The aqueous extract from the second extraction was evaporated to dryness in a rotary evaporator at 50 ^{o}C prior to treatment with alkaline pyrogallol.

The remaining 400 ml. of original aqueous extract was split into two 200 ml. portions. The first part was mixed with 150 ml. of dimethylsulfoxide (DMSO) and heated on a steam bath for three hours. The second part was treated in an identical manner with the exception that 20 grams of sodium carbonate was added to the mixture prior to heating. T. Santosusso and D. Swern reported that catalysis arising from in situ formation of strong acid in DMSO may be important in several reactions involving DMSO (61,62). Sodium carbonate was added to neutralize any strong acids which might be formed in this reaction and thus to prevent the isolation of products not directly resulting from the presence of DMSO (61). After cooling, each DMSO mixture was extracted with three 50 ml portions of ether. These ether extracts were washed with two 50 ml. portions of saturated sodium chloride solution and dried over anhydrous $MgSO_4$.

The solvent was removed by evaporation in a gentle stream of nitrogen; however, no weights were recorded for the residues obtained. One half of the aqueous extract of the second batch of S. capricornutum cells (10.5 grams) was evaporated to dryness in a rotary evaporator at 50 °C followed by identical treatment with DMSO, but without the sodium carbonate addition. The extraction of this reaction mixture yielded 10.9 mg. of a green, oily residue.

The three extracts from the DMSO treatments were screened using TLC system II. No sterols were detected in these samples.

Extraction of the Supernatant

Extraction With Organic Solvent. The extraction of large quantities of supernatant was accomplished by using two different methods. The first involved extraction of several aliquots of supernatant in three-liter separatory funnels followed by combination of the extracts from each aliquot. The second method involved simply stirring large quantities of supernatant with ether under nitrogen followed by removal of the ether with an all-glass siphon.

The first extraction method was used to extract 30 liters of supernatant from one batch culture. Aliquots of 2.5 liters of supernatant were first shaken with 300 ml. of ether and then with two 100 ml. portions of ether. The ether extracts were combined, dried over anhydrous $MgSO_4$,

and then concentrated to a small volume in a rotary evaporator. After the concentrate was allowed to evaporate to dryness, the yield obtained was 7.6 mg. of a light green, oily residue. This residue was redissolved in 5 ml. of ether and was screened using TLC system II. Visualization with iodine vapor revealed the presence of free sterols (Rf = 0.15-0.21); however, subsequent spraying with 50% H_2SO_4 and heating at 110 ^{o}C indicated that no sterol esters were present in this sample. The remainder of the extract was subjected to preparative TLC separation using TLC system II. Material in the free sterol zone was recovered from the plate by transferring the adsorbent in this zone into a 100 ml. beaker followed by washing with three 25 ml. portions of ether. The ether extracts were combined and filtered. Evaporation of the solvent yielded 1.0 mg. of a white crystalline material which was then dissolved in 5 ml. of ether and labelled as fraction V.

A total of 105 liters of supernatant from four different batch cultures was extracted with ether by using the second extraction method. Using a magnetic stirrer, fifteen liter aliquots were mixed under nitrogen with 2000 ml. of ether in a five-gallon carboy for 5 to 10 days. After mixing, the ether phase was removed using an all-glass siphon, and the aqueous phase was mixed with a second portion of 500 ml. of ether under the same conditions. Following removal of the second ether phase, the ether extracts were

combined, dried over anhydrous $MgSO_4$, and evaporated to a small volume in a rotary evaporator.

The extract from each of the eight aliquots was screened for sterol content using TLC system II. Visualization with iodine vapor revealed spots with Rf in the free sterol range (0.14-0.22) in all eight samples. No sterol esters were detected in these samples. The eight extracts were combined, and the solvent was allowed to evaporate leaving 21.4 mg. of a light green, oily residue. The entire residue was chromatographed on TLC system II, and compounds in the zone with Rf corresponding to that of free sterol were recovered by transferring and extracting the adsorbent as previously described. The material isolated by this procedure weighed 2.1 mg. and was also labelled as fraction V.

Thirty liters of nutrient medium from control carboys of two different batch cultures was extracted by using the second extraction method. Chromatographic screening on TLC system II showed that the extracts contained no compounds with Rf values in either the free sterol or sterol ester region.

Water Soluble Sterols. Sixteen liters of the supernatant which had been extracted by the first extraction method was placed in a 22 liter flask and mixed with 1000 ml. of 0.5% pyrogallol in methanol, 1000 ml. of 60% aqueous potassium hydroxide, and 1000 ml. of methanol. After re-

fluxing gently for two hours, this mixture was cooled, and a total of 17.5 liters, in 2.5 liter aliquots, was extracted with ether as described above for untreated supernatant. This procedure yielded 8.3 mg. of ether soluble material which was screened using TLC system II. Iodine vapor visualization revealed a spot with Rf in the free sterol region (0.18-0.23). A preparative TLC separation of the components of the residue using TLC system II and the methods described above for sample recovery gave 1.5 mg. of a white solid from the free sterol region of the plate. This sample was redissolved in 5 ml. of ether and was labelled as fraction VI.

Thirty liters of supernatant extracted by the second extraction method was divided into 15 liter aliquots. Each of these aliquots was treated with alkaline pyrogallol using the same conditions and quantities of added reagents as described in the preceding paragraph. The resulting mixtures were extracted with ether by using the second extraction method. The individual extracts were screened on TLC system II. Visualization with iodine vapor showed the same array of spots in each chromatogram, including a spot in the free sterol Rf region (0.14-0.19). These extracts were then combined, giving a total weight of 1.07 grams after removal of the solvent. Preparative TLC of the total extract on TLC system I resulted in recovery of 5.4 mg. of a green, oily residue from the free sterol region of the plate. The major

portion of the extract consisted of two unidentified compounds of high Rf. The recovered residue was redissolved in 5 ml. of ether and was also labelled as fraction VI.

As in the extraction of lyophilized algal cells, treatment with DMSO was utilized to check for water soluble sterols in the supernatant. Thirty liters of supernatant, extracted by using the second extraction method, was divided into two 15 liter aliquots. Each aliquot was mixed with 200 ml. of DMSO. The first was stirred under nitrogen for eight days and then extracted with ether directly in the carboy. The second aliquot was transferred to a 22 liter flask and was gently refluxed for one hour after which it was returned to a five-gallon carboy and extracted with ether. TLC screening of the two extracts obtained showed them to be similar in composition. A spot in each chromatogram in the range Rf = 0.13-0.23 indicated that free sterols were possible components of the samples. The extracts were combined, giving 7.7 mg. of residue from which the sterol-like components were removed by preparative TLC as previously described. A green, oily residue weighing 0.6 mg. was obtained and was labelled as fraction VII.

Summary

The extraction methods used to obtain sterols from lyophilized S. capricornutum cells and from the nutrient medium supernatant are summarized in Fig. 12-14.

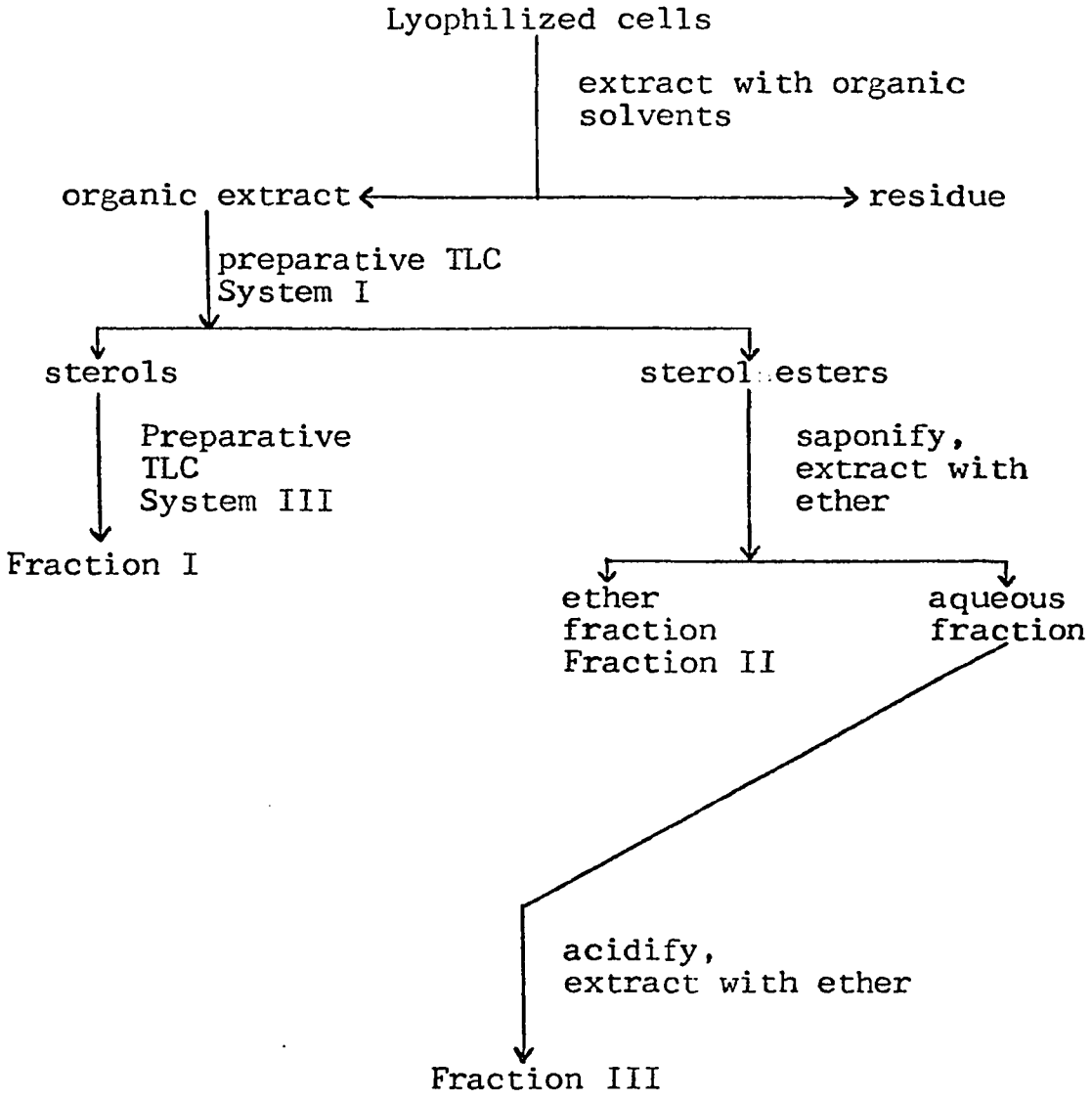


Figure 12. Isolation of free and esterified sterols from lyophilized Selenastrum capricornutum.

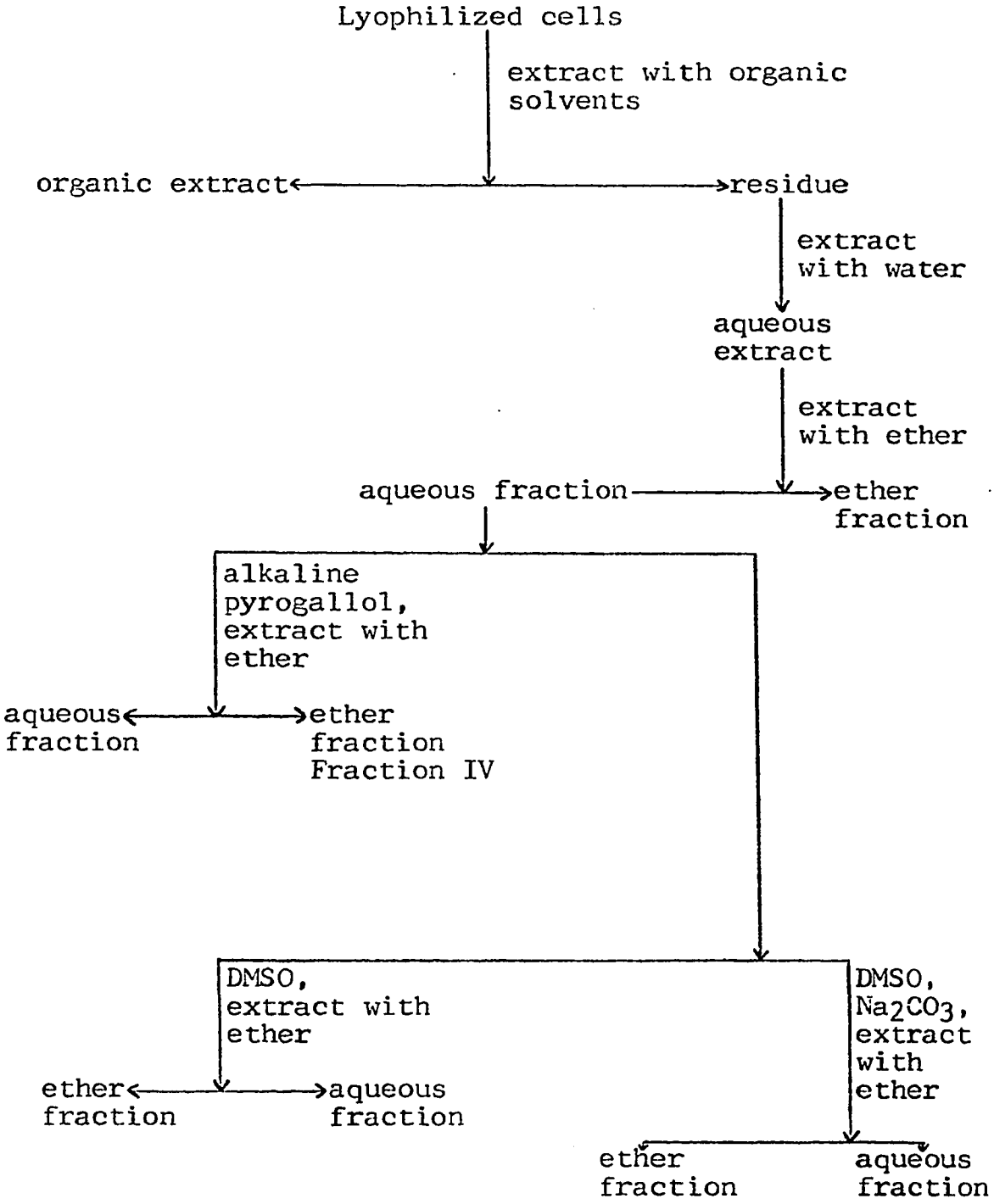


Figure 13. Treatment of the cell residue to release water-soluble sterols.

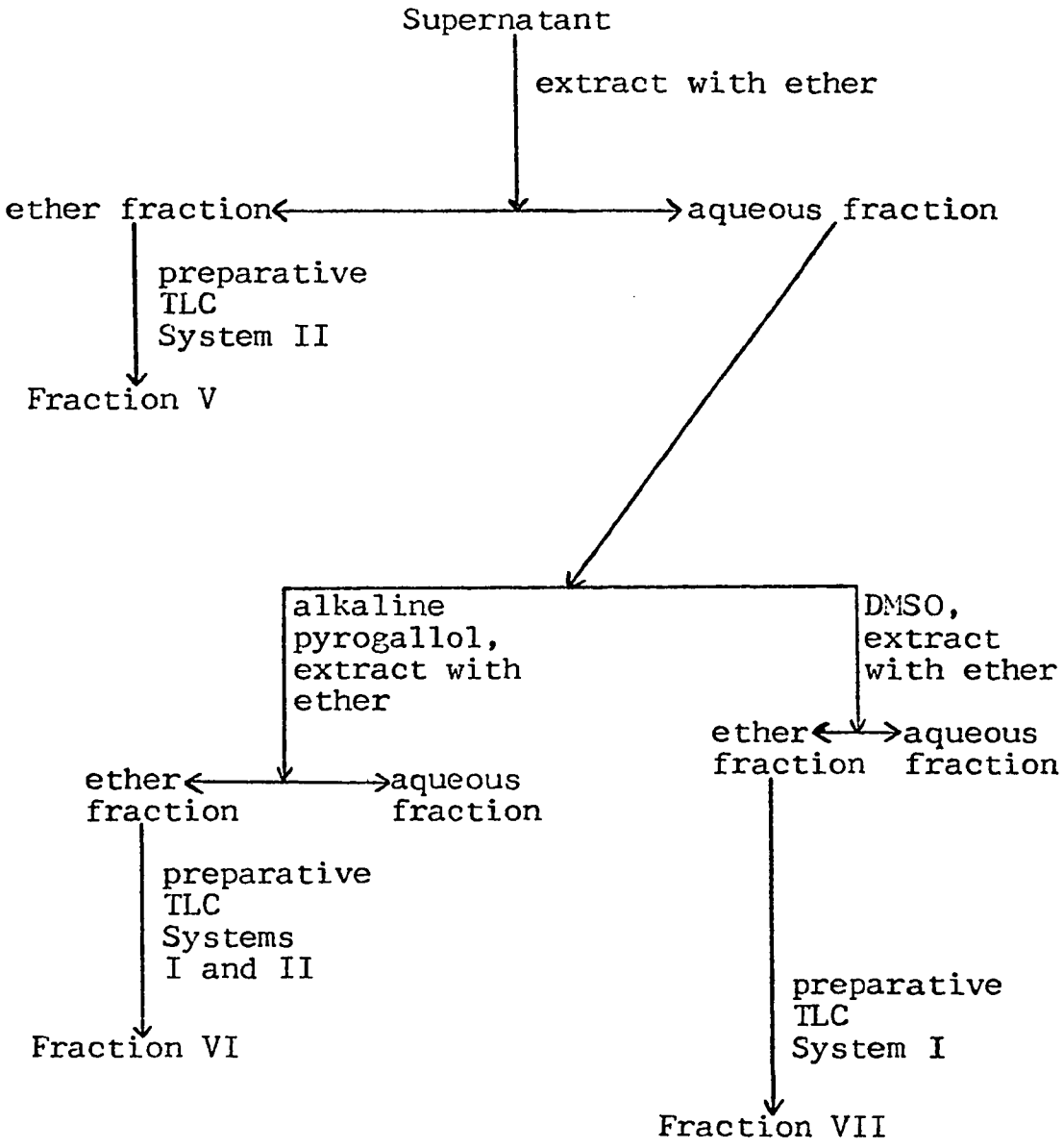


Figure 14. Isolation of free sterols from the supernatant and treatments for release of water-soluble sterols.

Pretreatment of Lvophilized Cells

A sample of lyophilized S. capricornutum was extracted by the methods previously described. The remaining cell residue was mixed with 150 ml. of water and 100 ml. of 20% potassium hydroxide in ethanol. This mixture was refluxed for two hours, cooled, and diluted with 100ml. of water. The final mixture was extracted by shaking with three 100 ml. portions of ether. A chromatographic screen of this extract on TLC system II showed that the treatment with potassium hydroxide failed to release sterols for extraction. Simple extraction with ether, acetone, chloroform: methanol, and water is, therefore, sufficient for removal of all sterols from lyophilized cells of S. capricornutum.

DMSO as a Solvent For Sterol Extraction

A sample of lyophilized S. capricornutum (9.38 g.) was extracted with ether:DMSO (8:1) by refluxing for two hours with three separate 500 ml. portions of the solvent. The extracts were combined and washed with three 500 ml. portions of saturated sodium chloride solution followed by one wash with 500 ml. of water. The resulting ether solution was dried over anhydrous $MgSO_4$. Removal of the solvent yielded 690 mg. of a dark green residue. This residue was screened on TLC system II and was found to contain a component with Rf in the free sterol region (Rf = 0.21-0.42). Preparative TLC separation of this component from the res-

idue resulted in recovery of 31.0 mg. of a light green residue which was labelled as fraction VIII.

Melting Points

Melting points were obtained using a Unitron melting point apparatus with a Kofler micro hot stage. Melting point values are uncorrected.

IV. METHODS FOR OBTAINING STEAM VOLATILE COMPOUNDS

An original objective of this project was to isolate and characterize any steam-volatile organic compounds produced by the cultured alga and subsequently released into the culture medium or retained within the algal cells. The experiments conducted in this regard yielded data which led to the decision to change from use of rubber tubing to Tygon tubing for making aeration connections.

Experiments designed to isolate steam-volatile compounds from the culture supernatant were conducted according to the following procedure. Fifteen liters of supernatant was transferred to a 22 l. round-bottom flask fitted with a condensor. This was heated and two liters of distillate was collected. This distillate was shaken in a separatory funnel with a 200 ml. portion and then three 100 ml. portions of ether. The ether fractions were combined, dried, filtered, and evaporated to dryness.

Two 15 l. samples of distilled water used for preparing the nutrient medium were treated as described for the

supernatant. After the original distillation of one of the distilled water samples, a three-feet-long piece of rubber tubing was placed in the distilling flask along with the remaining water. After three days the tubing was removed and an additional 2 l. of distillate was collected and extracted as described above.

A 5.4633 g. sample of lyophilized cells was mixed with 450 ml. of water in a 1000 ml. round-bottom flask fitted with a condensor. This mixture was heated and 200 ml. distillate was collected. The distillate was shaken with a 75 ml. portion and then three 50 ml. portions of ether. The ether fractions were combined and treated as described above.

All ether extractions obtained from steam-distillates were labelled as Fraction SD. The weights of the extracts are reported in Table VIII.

With the exception of sample number two, all samples in Fraction SD were screened by thin-layer chromatography on a single 20 cm. X 20 cm. plate coated with 0.25 mm. SilicAR TLC-7GF with benzene:ether (4:1) as the solvent. The results of this screening are presented in Table IX.

The results obtained in these experiments indicated that steam-volatile compounds isolated from culture supernatant and from lyophilyzed cells were also obtained in one or more of the control experiments. Furthermore, the small, variable amount of steam-volatile organic material obtained

TABLE VIII

WEIGHTS OF SAMPLES IN FRACTION SD

Sample	Source	Tubing type[a]	Weight(mg)
1	culture	rubber	2.0
2	culture	rubber	0.8
3	culture	rubber	12.0
4	culture	rubber	14.3
5	control	rubber	2.5
6	culture	rubber	0.2
7	culture	Tygon	0.9
8	control	rubber	0
9	control	Tygon	0
10	tubing[b]	rubber	3.7
11	distilled water	----	1.0
12	distilled water	----	0
13	lyophilyzed cells	rubber	4.8

[a]Refers to the type of tubing used in making the aeration connections.

[b]Refers to the experiment involving distilled water in contact with rubber tubing for three days.

from the cultures was apparently derived from the distilled water used in preparing nutrient medium and from the rubber tubing used in aeration connections. The latter was the more important source. Subsequent to the interpretation of these results, Tygon tubing was used in the aeration connections.

TABLE IX

TLC SCREEN OF SAMPLES IN FRACTION SD[a,b]

Spot #	Sample #								
	1	3	4	5	6	7	10	11	13
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2							0.04		
3		0.15	0.15		0.16			0.16	
4		0.30	0.28					0.31	
5		0.41		0.40					0.42
6	0.52			0.50			0.51	0.50	0.53
7		0.56		0.55					
8		0.63	0.63	0.61	0.59	0.58	0.60	0.58	
9				0.66					0.67
10		0.73	0.71	0.73			0.73		0.75
11	0.78	0.77	0.75		0.79	0.78		0.78	0.81

[a]See Table VIII for sample source and type of tubing.

[b]Numbers reported are Rf values of the spots in each chromatogram.

CHAPTER IV

RESULTS

I. FREE STEROLS

Sterols From Lyophilized Cells

Fraction I was analyzed by gas chromatography on GC systems I and II. Two peaks were observed and comparisons were made of the relative retention times of the peaks with those of several authentic sterols (Table X). The peak of shorter retention time (sterol A) was tentatively identified as representing a C-28 sterol. The second peak (sterol B) was identified as representing a C-29 sterol. Calculation of peak areas showed that fraction I was composed of 43% sterol A and 57% sterol B.

Relative retention times of sterols and sterol acetates were calculated relative to the retention times of cholesterol and cholesterol acetate, respectively. Cholestane is often used as a reference for these calculations; however, more reproducible values of relative retention times were obtained by using cholesterol and cholesterol acetate as references.

Sterols A and B were acetylated by treatment with 6 ml. of pyridine-acetic anhydride (1:1) for 16 hours at room temperature. After this time the reaction mixture was

TABLE X

RELATIVE RETENTION TIMES OF STEROLS AND STEROL ACETATES[a]

Sterol	Gas Chromatographic System		
	GC system I	GC system II	SE-30[b]
Sterol A	1.30(1.63)	1.24(1.47)	
Sterol B	1.56(1.97)	1.53(1.78)	
Cholesterol	1.00(1.00)	1.00(1.00)	1.00(1.00)
Brassicasterol	1.11	----	1.12(1.12)
Ergosterol	----	----	1.22(1.22)
Δ^5-Ergostenol	1.25	----	1.30(1.30)
$\Delta^{5,7}$-Ergostadienol	----	----	1.42(1.42)
Stigmasterol	1.40	1.37	1.42(1.42)
Poriferasterol	1.36	1.33(1.38)	1.42(1.42)
Δ^7-Ergostenol	1.24(1.38)	1.24(1.48)	1.46(1.46)
Chondrillasterol	1.33(1.48)	1.34(1.59)	1.58(1.58)
β-Sitosterol	1.50	1.50(1.55)	1.63(1.63)
Fucosterol	1.50	1.57(1.64)	1.63(1.63)
28-Isofucosterol	(1.84)	(1.70)	1.69(1.69)
$\Delta^{5,7}$-Stigmastadienol	----	----	1.78(1.78)
Δ^7-Chondrillastenol	1.49(1.67)	1.49(1.77)	1.83(1.83)

[a]Values in parentheses are for sterol acetates.

[b]Literature values for relative retention times on an SE-30 column (63).

diluted with 10 ml. of methanol and 50 ml. of water. The sterol acetates were recovered by extraction of the final mixture with ether, and they were analyzed by gas chromatography (Table X). Comparison of the relative retention times of these acetates with relative retention times of authentic sterol acetates confirmed the identification of sterols A and B as C-28 and C-29 sterols.

For purposes of obtaining mass spectra, small quantities of the acetates of sterols A and B were separated by using TLC system IV. The sterol acetate bands were located on the plate by visualization under a long-wave ultraviolet lamp, and the acetates were recovered by scraping the bands and extracting with ether as previously described. The major ions in the mass spectra of the acetates of sterols A and B are recorded in Table XI.

The mass spectrum of sterol A acetate had a molecular ion at m/e 440 indicating a C-28 sterol with two double bonds. The base peak at m/e 313 resulted from loss of a C_9H_{19} side chain, indicating that both double bonds were in the nucleus (29). The molecular ion at m/e 454 in the mass spectrum of sterol B acetate indicated that this was a C-29 sterol acetate with two double bonds. The base peak was at m/e 313 suggesting a $C_{10}H_{21}$ side chain with both double bonds in the nucleus. The similarity of the fragmentation patterns in the mass spectra showed that sterols A and B differed only in size of the side chain.

Table XI shows comparative mass spectral data for the acetates of sterols A and B, and the authentic sterols 24-methylcholesta-5,7-dien-3β-ol and 24-ethylcholesta-5,7-dien-3β-ol. The configuration at C-24 has no effect on the mass spectrum. The major differences between the mass spectra are due to loss of acetic acid from the acetates versus loss of water from the free sterols. The similarity of the frag-

TABLE XI

MAJOR IONS IN THE MASS SPECTRA OF THE ACETATES OF STEROLS A AND B[a]

Ion	A	C[b]	B	D	Fragmentation[c]
M^+	440(25)	398(35)	454(40)	412(51)	molecular ion
a	425(22)	383(9)	439(27)	397(12)	M^+-Me
b	380(11)	380(20)	394(11)	394(28)	M^+-HOAc(H_2O)
c	365(14)	365(58)	379(16)	379(78)	M^+- Me+HOAc(H_2O)
d	356(36)		356(11)		M^+-SCA
e	341(16)		341(16)		M^+-SCB
f		339(23)		353(28)	M^+-($C_1 \rightarrow C_3$+OH+6H)
g	313(100)	271(19)	313(100)	271(23)	M^+-SC
h	255(32)	255(10)	255(58)	255(6)	
i	253(20)	253(40)	253(14)	253(45)	M^+- SC+HOAc(H_2O)
j	229(18)	229(8)	229(26)	229(8)	
k	228(14)		228(11)		
l	227(23)	227(13)	227(26)	227(13)	
m	213(35)	213(13)	213(42)	213(14)	
n	211(15)	211(23)	211(11)	211(28)	
o	201(15)		201(14)		
p	200(10)	200(8)	200(8)	200(7)	
q	199(15)	199(30)	199(14)	199(32)	
r	197(13)	197(23)	197(8)	197(22)	
s	189(15)		189(8)		
t	187(15)	187(8)	187(13)	187(5)	
u	185(20)	185(18)	185(14)	185(19)	
v	183(10)	183(18)	183(10)	183(18)	
w	175(10)		175(11)		
x	173(15)	173(8)	173(15)	173(12)	
y	171(18)	171(25)	171(17)	171(26)	
z	161(30)	161(8)	161(28)	161(9)	
aa	160(10)	160(8)	160(15)	160(8)	
bb	159(20)	159(33)	159(22)	159(31)	
cc	158(10)	158(28)	158(8)	158(30)	
dd	157(20)	157(33)	157(17)	157(35)	

[a]Ion intensities are in parentheses.

[b]Columns C and D are major peaks in the mass spectra of (24R)-ergosta-5,7-dien-3β-ol and stigmasta-5,7-dien-3β-ol, provided by Dr. G. W. Patterson, Department of Botany, University of Maryland.

[c]SCA = C-23 through side chain end; SCB = C-22 through side chain end; SC = side chain.

mentation patterns of the sterol nucleus of the four compounds suggests that sterol A is 24-methylcholesta-5,7-dien-3β-ol and sterol B is 24-ethylcholesta-5,7-dien-3β-ol.

The melting point of the acetates of sterols A and B were 150-153 ^{o}C and 176-180 ^{o}C respectively. Values of 152-154 ^{o}C and 158 ^{o}C have been reported for the melting point of the acetate of 22-dihydroergosterol.

The ultraviolet spectrum of the sterol acetate mixture showed absorption at 284 nm., typical of $\Delta^{5,7}$-sterols (33). On the basis of this evidence sterol A was identified as 24-methylcholesta-5,7-dien-3β-ol, and sterol B was identified as 24-ethylcholesta-5,7-dien-3β-ol. Insufficient amounts of these sterols were isolated for determination of NMR spectra or of specific rotation, the only methods allowing assignment of configuration at C-24. By analogy with results obtained for most algal sterols these sterols were assumed to possess the β configuration at C-24. Sterol A was tentatively identified as (24S)-24-methylcholesta-5,7-dien-3β-ol, trivial name 22-dihydroergosterol, and sterol B was tentatively identified as (24S)-24-ethylcholesta-5,7-dien-3β-ol, trivial name $\Delta^{5,7}$-chondrillastenol (Fig. 15). The composition of the free sterols obtained by two extractions of lyophilized S. capricornutum are summarized in Table XII.

TABLE XII

COMPOSITION OF FREE STEROLS OF Selenastrum capricornutum

Sterol	% of free sterol first extraction[a]	second extraction[b]
A	43	32
B	57	68

[a]Culture age was 14 days.

[b]Culture age was 21 days.

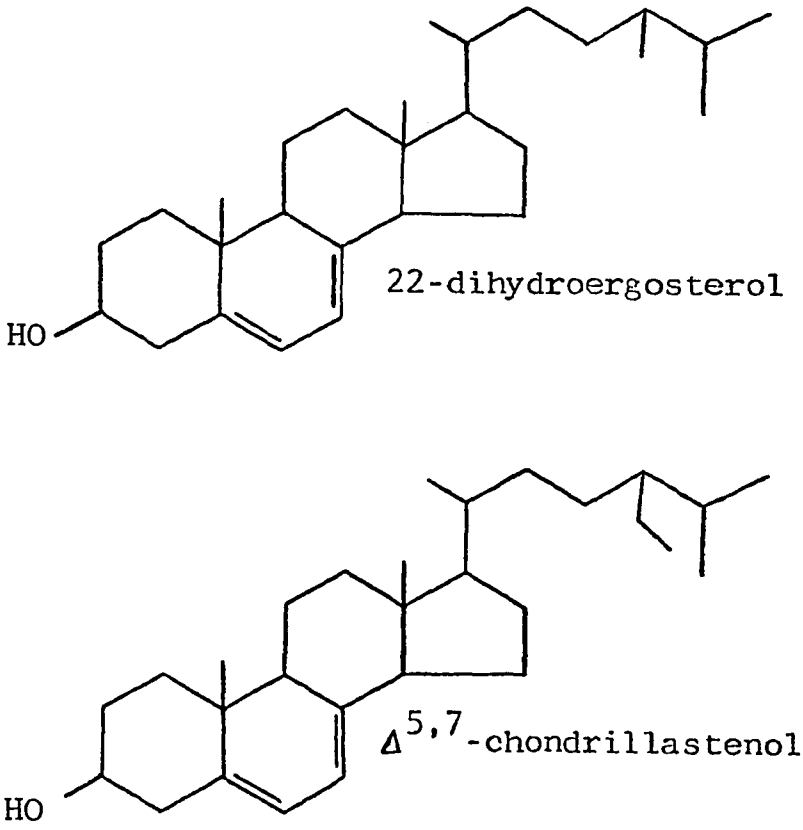


Figure 15. Sterols of Selenastrum capricornutum.

Sterols From the Supernatant

The two samples of fraction V were analyzed on GC system I. Both contained 24-methylcholesta-5,7-dien-3β-ol and 24-ethylcholesta-5,7-dien-3βol. The composition of sterols in these samples is given in Table XIII.

TABLE XIII

COMPOSITION OF STEROLS EXTRACTED FROM THE SUPERNATANT

Sterol	% of Sterol 1st extraction	2nd extraction
24-methylcholesta-5,7-dien-3β-ol	43	48
24-ethylcholesta-5,7-dien-3β-ol	57	52

The concentration of sterols in the first supernatant sample was 33μg/l., and in the second sample it was 20 μg/l. These data were obtained by two different extraction methods and the differences in sterol concentrations may be a result of differing efficiencies of extraction.

II. BOUND STEROLS

Sterols From Sterol Esters

Gas chromatographic analysis of the two samples of fraction II revealed that this fraction also contained 24-methylcholesta-5,7-dien-3β-ol and 24-ethylcholesta-5,7-dien-3β-ol. The relative amounts of the two sterols, however, were more variable in fraction II than in the free sterol

fraction. The composition of fraction II is given in Table XIV.

TABLE XIV

COMPOSITION OF STEROLS FROM STEROL ESTERS

Sterol	% of Esterified Sterol 1st extraction[a]	2nd extraction[b]
24-methylcholesta-5,7-dien-3β-ol	62	39
24-ethylcholesta-5,7-dien-3β-ol	38	61

[a]Culture age was 14 days.

[b]Culture age was 21 days.

Water Soluble Sterols

All samples of fractions IV, VI, and VII were analyzed on GC systems I and II. These samples were found to contain only minute quantities of sterols. Sterol peaks were too small for peak area measurements to be made, and in most cases the peaks were indistinct, making retention time measurements difficult. It is reasonable to conclude that sterols present in these fractions were lipid soluble sterols which were incompletely extracted by the organic solvents.

Sterols Extracted By DMSO:Ether

Analysis of fraction VIII on GC system I showed that this fraction was a mixture of 24-methylcholesta-5,7-dien-3β-ol and 24-ethylcholesta-5,7-dien-3β-ol. The composition

of the mixture, calculated by peak area measurements, was 41% 24-methylcholesta-5,7-dien-3β-ol and 59% 24-ethylcholesta-5,7-dien-3β-ol. The sterol in fraction VIII constituted 0.33% of the dry weight of the extracted algal cells.

Summary

The composition and quantities of sterols isolated from S. capricornutum are summarized in Table XV.

TABLE XV

COMPOSITION AND QUANTITIES OF STEROLS OF Selenastrum capricornutum

Fractions	First Extraction % sterol	First Extraction % dry weight	Second Extraction % sterol	Second Extraction % dry weight
1) free sterols from cells	72	0.25	61	0.27
2) esterified sterols	28	0.098	39	0.17
3) total sterols from cells		0.35		0.44
4) sterols from supernatant[a]		0.040[b]		0.024[b]

[a]Samples of supernatant were taken from batch cultures different from those used to obtain lyophilized cells.

[b]Based on average weight of dry cells obtained per liter of culture.

Fatty Acids From Sterol Esters

The fatty acids in fraction III were methylated by using BF_3 and methanol. Two ml. of $BF_3 \cdot O(CH_2CH_3)_2$ was mixed with 2 ml. of methanol and this mixture added to 50 mg. of

the fatty acid fraction. After heating in a boiling water bath for 3 minutes, the reaction mixture was allowed to cool to room temperature and was poured into 10 ml. of hot water. This final mixture was allowed to cool and the fatty acid methyl esters were extracted with three 15 ml. portions of ether. The extracts were combined, dried over anhydrous $MgSO_4$, and were analyzed on GC systems III and IV. The retention times of the components of this extract, along with the retention times of some authentic methyl esters of saturated fatty acids, are listed in Table XVI.

TABLE XVI

RETENTION TIMES OF FATTY ACID METHYL ESTERS[a]

Fatty Acid	RT of Authentic Ester	RT of Algal Acid Ester	% of Mixture[b]
unidentified	----	2.1	9.0
unidentified	----	3.2	2.1
C-10	8.3 (8.4)	8.4 (8.2)	2.1
C-12	10.8(10.9)	10.8(10.5)	2.1
C-14	12.9(13.1)	12.7(13.2)	1.6
C-16	14.7(14.8)	14.6(14.9)	9.0
unidentified	----	15.2(15.5)	21.0
C-18	16.3(16.5)	16.1(16.6)	12.2
unidentified	----	16.6(17.1)	40.9

[a]Retention times are in minutes. Values in parentheses are for GC system IV; others are for GC system III.

[b]Calculated from peak areas.

The quantity of fatty acids obtained in fraction III was several times the amount expected from the quantity of sterols in fraction II. The excess fatty acid most likely was obtained from sources in the cells other than sterol

esters. Therefore, the data presented in Table XVI do not necessarily reflect the actual distribution of fatty acids in the sterol esters.

CHAPTER V

DISCUSSION

Discussion of Results

The two sterols, 24-methylcholesta-5,7-dien-3β-ol and 24-ethylcholesta-5,7-dien-3β-ol, were the only sterols detected in extracts of Selenastrum capricornutum. By analogy with results obtained for most algal sterols these sterols were assumed to possess the β configuration at C-24. Although this is the first report of 24-ethylcholesta-5,7-dien-3β-ol in a species of Chlorophyta, this sterol was found in Euglena gracilis (33) and unidentified $\Delta^{5,7}$-sterols were reported in several Chlorella species (1). Further investigation of sterols identified from chromatographic evidence alone (64) may show that $\Delta^{5,7}$-chondrillastenol is of common occurence in the green algae. Extractions of S. capricornutum failed to yield Δ^{7}- or Δ^{5}-sterols, supporting the conclusion that the green algae contain either Δ^{5}- or Δ^{7}-, or $\Delta^{5,7}$-sterols and that the simultaneous occurence of these sterol types in algae is unusual.

The composition and quantities of sterols in sterol esters of S. capricornutum were determined; however, comparative data for other species of Chlorophyta are unavailable. Furthermore, insufficient data were obtained in all

aspects of this experiment to establish the precision of measured sterol concentrations and compositions. Although data reported are in line with results of other workers, the significance of small differences in concentration and composition cannot be established without further experiments. The two sterols found in esterified form were identical to those found in the free form. The composition of the esterified sterols varied markedly as a function of culture age, with the composition in older cultures being similar to that of the free sterol fraction. The composition and quantity of the free sterol fraction showed insignificant variation as a function of culture age. The total sterol content of S. capricornutum cells was shown to increase with culture age. This increase was a result of increased sterol ester content.

Several experiments designed to extract water soluble sterols from S. capricornutum cells and from the culture medium failed to extract measureable quantities of sterols. Water soluble sterols were found to make up only a small fraction of the total sterol content of two species of higher plants; however, other species of Chlorophyta have been found to lack water soluble sterols. In view of these reports, a reasonable conclusion is that the sterols isolated from aqueous extracts of S. capricornutum and from the nutrient medium after organic solvent extraction were lipid soluble sterols incompletely extracted by the organic

solvents.

The composition of the sterol fraction isolated from the nutrient medium was similar to that of the free sterol fraction from S. capricornutum cells. The concentration of sterols in the nutrient medium (20-33μg./l.) was approximately equal to the solubility of cholesterol (25-29 μg./l.) (17). Cell lysis and active secretion by the algal cells could serve as sources of the sterols found in the culture medium. Cell lysis could occur during incubation and during the harvest process.

Results of treatment of the algal cells with strong base prior to extraction have indicated that such pretreatment is unnecessary for complete extraction of sterols. Extraction of the algal cells with a DMSO:ether mixture resulted in recovery of a sterol fraction similar in composition and amount to those extracted by more commonly used solvents. DMSO did, however, prove to be useful, as lesser amounts of non-sterol material were extracted by DMSO:ether, making purification of the sterol fraction less complicated.

Recommendations

Of particular importance in future studies involving algal sterols, is the investigation of the composition and quantities of bound sterols. Results from higher plants and from algae have indicated that in some organisms water soluble complexes of sterols may be of major importance in metabolic processes involving sterols; however, such com-

plexes are absent in some organisms. A complete understanding of the role of water soluble sterol complexes in plant sterol metabolism will be possible only after thorough investigations into the occurence and structures of these complexes have been made. Sterol esters may also serve some purpose in sterol metabolism, possibly as a storage or transport form of sterols.

The mechanisms by which sterol biosynthesis is accomplished in algae have recently been investigated in a few algal species. With the diverse array of sterols produced by algae, particularly the green algae, further investigations of biosynthetic pathways will be necessary before algal sterol biosynthesis can be completely understood. Of particular interest in biosynthetic studies, is the mechanism and stereochemical control of side chain alkylation at C-24.

The compositions of both free and bound sterols of some algae appear to have value as guides to taxonomy and phylogeny. Further investigations of all algal divisions will make possible generalizations as to sterol content, and will determine the usefulness of these generalizations for taxonomic purposes.

Very little is known concerning the release by algae of sterols into their environment. Results of this work and of experiments with a few other algal species have shown that sterols are released by these algae into the culture

medium; however, the mechanism of the release is unknown. Elucidation of possible roles of algal sterols in the environment must await further investigations of the occurence of extracellular sterols and the mechanisms by which they are released.

CHAPTER VI

SUMMARY

The green alga, Selenastrum capricornutum, has been cultured in artificial nutrient medium in quantities sufficient for extraction of sterols from harvested algal cells and from the nutrient medium. The two sterols 24-methylcholesta-5,7-dien-3β-ol and 24-ethylcholesta-5,7-dien-3β-ol were the only sterols found in all sterol fractions isolated. The composition of the free sterol fraction from the algal cells showed insignificant variation as a function of culture age. The quantity of sterols in the sterol ester fraction increased as a function of culture age, and the composition of sterols in this fraction more closely resembled that of the free sterol fraction for older cultures.

The sterol fraction isolated from the nutrient medium was similar in composition to the free sterol fraction from the algal cells. The concentration of sterols in the nutrient medium was approximately equal to the solubility of cholesterol.

Extraction procedures which are known to release sterols from water soluble complexes were carried out on extracted nutrient medium. These procedures failed to yield measurable quantities of sterols.

Treatment of extracted algal cells with strong base

and subsequent extraction showed that all sterols had been extracted without prior cell lysis or pretreatment. An extraction of algal cells was carried out using DMSO:ether as the extraction solvent, and the usefulness of this solvent mixture for sterol extraction was demonstrated.

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