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STEROLS AND α-GLYCERYL ETHERS IN THE UNSAPONIFIABLE

LIPIDS OF ECHINODERMS AND TUNICATES

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MORTON MEYER URIVETZKY

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STEROLS AND α-GLYCERYL ETHERS IN THE UNSAPONIFIABLE

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APPROVED BY

Simon H. Wender

O. J. Weinheimer

H. H. Rowley

J. C. Colbert

Harriet Harvey

DISSERTATION COMMITTEE

To Mom and Dad, Marcia, Sim Abbey,

and Lewis Mark

ACKNOWLEDGMENT

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LIPIDS OF ECHINODERMS AND TUNICATES

CHAPTER I

INTRODUCTION AND HISTORY

The investigations to be discussed in the present dis-
sertation have been carried out as part of a research project
devoted to a comparative study of substances of biochemical
interest in marine invertebrates and protochordates.

Sterols and α-Glyceryl Ethers

Poulletier, around 1769, described the isolation of a
colorless, crystalline compound from gallstones (1). Later,
during the course of his studies on the composition of fats,
Chevreuil observed that the unsaponifiable lipids of many
animals contained the compound first described by Poulletier
and, in recognition of the source from which it had first
been isolated, the substance was given the name cholesterine
(choleos=bile; stereos=solid) (2). Berthelot subsequently
found that the compound was a monovalent alcohol (3), where-
upon the name was changed to cholesterol in English publi-
cations. The original name, however, has been retained in

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the continental literature. Eventually other compounds,
similar to cholesterol, were discovered in the lipids of
plants, animals, and microorganisms and the name sterols was
adopted for all crystalline, high melting, unsaponifiable
alcohols, whose chemical nature resembled that of cholesterol.

The sterols as well as a great number of other naturally
derived substances, such as bile acids, saponins, alkaloids,
gonadal and adrenocortical hormones, are members of the ster-
oid family of compounds. By 1934 the structure of cholesterol
as well as the basic structure of steroids had been eluci-
dated. It was established that all steroids were derivatives
of perhydrocyclopentanophenanthrene. A detailed account of
the experimentation and reasoning which led to the formulation
of these structures may be found in the text, "Natural Pro-
ducts Related to Phenanthrene", by Fieser and Fieser (4).
Many compounds, which had been given the suffix-sterol were
eventually shown to belong to different classes of compounds,
such as triterpenes.

Prior to the report, in 1909, of the British biochemist
Dorée (5), who must be acknowledged as the father of compar-
ative sterol research in animals, all observations, with two
exceptions, had shown cholesterol to be a constant constituent
of animal tissues. The recorded investigations related
chiefly to man, a few of the more common mammals, birds, and
a few unidentified lower animals. Henze (6, 7) claimed that
he had isolated a new sterol from the Mediterranean sponge,

Suberites domuncula to which he assigned the name spongost-

erol (more recently shown to be a misture of cholestanol and

neospongosterol (8)). Menozzi and Moreschi (9), claimed the

isolation of a new sterol, bombicysterol, from the pupae of

Bombyx mori. Bombicysterol was later shown to be a mixture

of sterols consisting mainly of cholesterol (10). The object

of Dorée's studies has been expressed with admirable clarity

in the introductory paragraphs of his report:

> It is a remarkable fact that while cholesterol has been
> isolated uniformly from the tissues of mammals and birds,
> no other substance similar to it had, until quite re-
> cently, been discovered in animal protoplasm.

> If then, cholesterol is a body which is one of the primary
> constituents of animal protoplasm, we should expect to
> find it not only in the highly organized animal, but
> throughout the series from chordata to protozoa; or if
> cholesterol were not present its place should be filled
> by other closely related forms. In the latter case it
> might be found that each of the great classes of the
> animal kingdom was characterized by the presence of a
> different member of the cholesterol group. On the other
> hand, if cholesterol is not of primary importance to all
> forms of animal life, it is not impossible that animals
> might be found into the composition of whose protoplasm
> it did not enter.

Dorée, therefore, selected one or two species repre-

senting each of the most important phyla of the animal king-

dom, and from these he isolated and characterized the sterols

present. He did not succeed in finding a typical sterol for

each of the phyla, with the exception of the chordates, but

all animals which he had investigated contained either cho-

lesterol, or at least one other sterol. Cholesterol was

found to be dominant in the vertebrates, and widely distri-

buted in the invertebrates. In at least two species of marine
invertebrates, the sponge, Cliona celata, and the starfish,
Asterias rubens, there was evidence that cholesterol was
replaced by other sterols. In addition, there was isolated
from the unsaponifiable lipids of Asterias rubens a crude
wax-like substance, m.p. 55-57°, which we now know to be
composed largely of batyl alcohol.

Following the pioneer work of Dorée, well over a hun-
dred animal species of the major phyla and their subdivisions
have been investigated and, in several cases, reinvestigated.
Because of their great abundance and diversity, marine inver-
tebrates have thus far been the almost exclusive subjects
for comparative studies. Nevertheless, the field is far from
exhausted for even such a number of species is a small minor-
ity as far as marine invertebrates are concerned, and even
more so when one considers the vast number of all existing
invertebrates. As more and more species have been studied,
and greater care has been taken in the isolation and separa-
tion of sterols the general picture has undergone many modi-
fications. It has been found, as is the case with comparative
biochemistry in general, that it is dangerous and very often
misleading to base significant conclusions concerning com-
parative sterol chemistry on data derived from a study of
only a few representatives of a given phylum, subphylum, or
class. A few broad generalizations can, however, be made.
Cholesterol, although accompanied by trace amounts of 7-de-

hydrocholesterol, cholestanol, and a few other sterols, is the dominant sterol of vertebrates. On the other hand, invertebrates, especially the lower forms, i.e. sponges, and coelenterates, show a great diversity of sterols. As yet no animal devoid of sterols has been discovered. Anderson, et al. (11), however, demonstrated convincingly that sterols are absent in tubercle bacilli. Unfortunately, very little work has been done on protozoans, but one report on the parasitic Eimeria gadi, by Panzer (12), gives evidence for the presence of cholesterol which actually may have been assimilated from the host, the cod, Gadus virens.

Since we will be mainly concerned with the unsaponifiable constituents of echinoderms and tunicates, the following discussion will be devoted primarily to investigations related to the subject matter. An excellent survey, including exhaustive references, of comparative studies on the lipids of marine invertebrates up to 1949, may be found in an article by Werner Bergmann in the Journal of Marine Research (13). In addition a chapter by the same author in the first volume of the series, "Progress in the Chemistry of Fats and other Lipids", reviews structures, properties, and general information for almost all sterols, of both confirmed and uncertain identity, found in living organisms prior to 1952 (14).

In 1915, Kossel and Edelbacher (15), reported the isolation of a di-unsaturated sterol, m.p. 149-150° (acetate 176-

177°), of the probable formula $C_{27}H_{44}O$, from the unsaponifiable lipids of the starfish, Asterias rubens. In support of Dore's conclusions they found that the sterol was definitely not cholesterol and assigned the name of stellasterol to it. From the unsaponifiable fraction they also isolated a compound, m.p. 70-71°, which was presumably an alcohol but not a sterol, and called it astrol.

Page (16), in 1923, announced the presence of a sterol-like compound, m.p. 71° (acetate 97°), in the common New England starfish, Asterias forbesi. He assigned the name of asteriasterol to the supposedly homogeneous compound giving no details concerning analyses or purity.

A reinvestigation of the unsaponifiable lipids of Asterias forbesi by Bergmann in 1937 (17) revealed that Page's asteriasterol was a mixture of astrol and sterols. It was found that recrystallization of the unsaponifiable residues of the starfish would yield a substance with properties similar to asteriasterol. However, when the material was treated with an alcoholic solution of digitonin only about 60% was recovered as the insoluble digitonide. The sterols could then be recovered from the digitonide, but from the mother liquors of the digitonide a substance could be extracted which did not show the usual sterol reactions and which closely resembled astrol. The steryl acetates could then be separated into a less soluble fraction, m.p. 155-157°, resembling stellasterol and a more soluble acetate, m.p. 128-

130°.

In 1942 Bergmann (18) reported that he had isolated batyl alcohol from the gorgonian, *Plexuara flexuosa*. This was the first time that the alcohol had been shown to be present in invertebrates. In 1943 Bergmann (19) established the identity of astrol with batyl alcohol. Kossel and Edelbacher must, therefore, be credited as the discoverers of batyl alcohol in animals, for their investigation was reported seven years before that of Tsujimoto and Toyama (20) on the isolation of batyl alcohol from shark liver oils. The Japanese workers, however, were the first to assign the name of batyl alcohol to the compound. In addition to batyl alcohol, they isolated selachyl alcohol which is usually the major constituent of elasmobranch oils and which can be hydrogenated to give batyl alcohol. In subsequent studies, chimyl, selachyl, and batyl alcohols were isolated from a variety of shark, ray, and torpedo oils (21, 22, 23, 24). As a result of the work of the Japanese group, the investigations of Davies, Heilbron, et al. in England (25, 26, 27, 28), and the synthetic work of Baer and his group in Canada (29, 30, 31), it was established that the naturally occurring batyl, selachyl, and chimyl alcohols were the α-D-glyceryl ethers of n-octadecanol, n-9:10-cis-octadecenenol, and n-hexadecanol respectively.

Although the greatest concentrations of α-glyceryl ethers has thus far been encountered in the fats of elasmo-

branchs and starfish, their occurrence is by no means re-
stricted to these sources. Batyl alcohol has been isolated
from herring and whale oils (32), from a crustacean (Par-
alithodes camtchatica, a Japanese crab) (33), from arteri-
osclerotic human aortas (34), hog spleen (35), and the yellow
bone marrow of cattle (36). Chimyl alcohol has been obtained
from bull and swine testes (37).

There is a very striking difference in the unsaponifi-
able constituents of echinoderm lipids which should be men-
tioned at this point. The asteroids (starfish) and holothur-
(sea-cucumbers) which have been investigated have been found
to contain large amounts of batyl alcohol (15, 19, 38), and,
as yet, cholesterol has not been isolated from these classes
of echinoderms. The echinoids (sea-urchins) have been found
to contain cholesterol as the principal sterol (39, 40, 13)
and no batyl alcohol has yet been isolated from their lipids.
A recent report by Karnovsky (41) indicates the presence of
3.8% α-glyceryl ethers (calculated as batyl alcohol) in the
unsaponifiable lipids of the sea-urchin, Arbacia punctulata,
as compared to 61.8% for those of the pyloric caeca of
Asterias forbesi, and 11% for the sea-cucumber, Thyone bri-
areus (whole animal).

The α-glyceryl ethers are present as di-esters in the
lipid extracts of animals and upon saponification they are
converted to the di-hydroxy compounds (42). As esters they
may be isolated from the alcohol insoluble fractions of the

lipid extracts (17). Baer (29) has postulated that the bio-
genesis of α-glyceryl ethers might take place via the react-
ion of acetal phosphatides (plasmals), present in living
cells, and the corresponding alcohol, following which the
phosphate group is hydrolyzed off and the di-ester formed.
Toyama, in 1925 (24), had already indicated that there was
a definite relationship between the amounts of batyl, chimyl,
and selachyl alcohols present on the one hand, and of stearic,
palmitic and oleic acids on the other; the latter correspond-
ing to octadecyl, cetyl (hexadecyl), and oleyl alcohols.
Recently, Karnovsky (43) reported that he had found a direct
correlation between the titer of α-glyceryl ethers and acetals
in the unsaponifiable fraction of the pyloric caeca lipids
of **Asterias forbesi**. These observations lend support to
Baer's postulation of the biogenesis of the glyceryl ethers.

Returning to the discussion of sterols present in the
lipids of starfish, it has already been mentioned that in
1937 Bergmann had separated the sterols of **A. forbesi** into
a more soluble and a less soluble fraction. The melting
point of the latter indicated that it was identical with
stellasterol which had first been described by Kossel and
Edelbacher. It appeared to Bergmann that there were at least
two, and possibly more, sterols present in the starfish, and
upon reinvestigation of the species, several years after his
first report, Bergmann found further support for the com-
plexity of the sterol mixture (44). During the course of

twelve recrystallizations of the crude sterols the melting

point rose from 120-133° to 149-150°; the latter being the

melting point of stellasterol. The substance, however, was

not yet pure, for upon further recrystallizations the melt-

ing point increased. Similarly, recrystallization of the

steryl acetates gave a fraction, which like stellasteryl

acetate, melted at 177°, but after further recrystallizations

the melting point rose to 180°. It soon became evident that

a separation of the sterols or their derivatives, by fraction-

al crystallization, into pure compounds was not possible with

the amount of starting material available. In addition,

attempted separation of the mixture by column chromatography

(activated alumina) had to be abandoned due to the isomeri-

zation which accompanied the adsorption process. The chem-

ical and physical behavior of the sterols and their deri-

vatives, however, provided significant information concerning

their structure. Perbenzoic acid titrations indicated that

the various fractions contained a mixture of mono- and di-

unsaturated sterols. According to Kossel and Edelbacher,

stellasterol was a di-unsaturated compound, and in agreement

with their formulation the sterol fractions most closely

resembling stellasterol gave evidence for the predominance

of a di-unsaturated sterol. It was assumed, therefore, that

like the co-occurring poriferasterol and clionasterol of

sponges (45), the starfish sterols were also closely related

compounds differing only by the presence of a double bond in

the side chain of the di-unsaturated molecule. The name
stellasterol was retained to designate the di-unsaturated
sterol, and the mono-unsaturated sterol was given the name
stellastenol.

All fractions of the starfish sterols and their der-
ivatives were found to be slightly dextrorotatory. This
fact suggested the absence of a 5:6-ethenoid linkage which
is known to confer a strong negative contribution to the
rotation of steroids. In addition all sterol and steryl
ester fractions gave a green color when treated with bromine.
This reaction is typical of all steroids containing a double
bond joined to C-8 of the ring system and is known as the
Tortelli-Jaffe' reaction (46, 47, 48). It was then assumed
that both stellasterol and stellastenol possessed a double
bond in the γ-(7:8)-, δ-(8:9)-, or α-(8:14)- positions. It
had been established, by previous investigations (4), that
steroids containing double bonds in the γ-, or δ- positions
were stable towards hydrogenation with a platinum catalyst
in chloroform or other neutral solvents, but when hydrogenated
with the catalyst in glacial acetic acid they would rearrange
to the α-isomer. The latter was stable to hydrogenation in
either of the solvents mentioned, and the completely sat-
urated sterol could be obtained only after rearrangement of
the isomer to the β-(14:15)-stenol by the action of anhy-
drous hydrogen chloride.

Bergmann, therefore, subjected one of the isolated

steryl acetate fractions, m.p. 149-154°, (which indicated
that a mixture of mono- and di-unsaturated sterols were pre-
sent) to hydrogenation with platinum black in glacial acetic
acid. The reaction, carried out at room temperature and at
atmospheric pressure, stopped after the absorption of ap-
proximately 0.5 mole of hydrogen. A stenyl acetate, appar-
ently the α-(8:14)-isomer was recovered which after several
recrystallizations melted at 105-106°. The latter was dis-
solved in chloroform and upon treatment with anhydrous hy-
drogen chloride rearranged to the β-(14:15)-isomer, m.p.
94-96°, which after further hydrogenation gave the saturated
stellastanyl acetate. The evidence, therefore, indicated
the presence of a C-8 double bond in the sterols of the star-
fish but it did not establish whether the original sterol
mixture contained γ-, δ-, or α-double bonds or perhaps a
mixture of all three.

Later in a personal communication to Fieser (4), Barton,
who had made a complete survey of molecular rotations of
various sterols and their derivatives (49), suggested that
the starfish sterols isolated by Bergmann were most probably
γ-(7:8)-stenols. The study of the differences in the
molecular rotations of sterols and their derivatives, first
introduced by Bernstein (50), and later further developed
by Barton (49), has become a very important tool for estab-
lishing the structures and homogeneity of steroids.

The location of the double bond in the side chain, as

well as the length of the side chain, of both stellasterol
and stellastenol (side chain saturated) still required eluci-
dation. Bergmann found that, upon ozonolysis, the steryl
acetate mixture yielded an aldehyde, isolated as the 2,4-
dinitrophenylhydrazone derivative, which had the following
properties: m.p. 119-120°; $[\alpha]_D^{26} = +14.50$; analyses showed
that it was a derivative of an aldehyde of the formula
$C_5H_{11}CHO$. A mixture of the latter with a sample of the cor-
responding derivative of methylisopropylacetaldehyde, m.p.
124-124.5°, $[\alpha]_D^{20} = -27.7^C$, prepared from ergosterol melted
at 119-122.5° Since the only other optically active isomer
of the aldehyde $C_5H_{11}CHO$ is methyl-n-propylacetaldehyde,
which lacks the usual isopropyl structure of sterol side
chains, it was concluded that stellasterol was a $\Delta^{7,22}-$
sterol, of the order C_{28}, containing a methyl group at C-24
of the side chain with a configuration opposite to that of
the C-24 methyl group of ergosterol. Subsequent studies of
the hydrogenation products of stellasterol and of stellastenol,
including a comparison of the optical rotation and melting
point of stellastenol with those of ergostanol (C-24-b-
methylcholestanol), indicated that the starfish sterols con-
sisted principally of derivatives of campestanol with some
of the C-24-b-isomer also present (51, 52).

Thus stellasterol and stellastenol were the first ster-
ols isolated from animals which were shown to be devoid of
a double bond between carbon atoms 5 and 6 of the sterol

ring system. Zymosterol of yeast, and α-spinasterol isolated
from higher plants are also sterols which lack the 5,6-
ethenoid linkage. In addition, the starfish sterols were
the first principal sterols of the order C_{28} to be found in
animal tissues. Previous to the report of Bergmann, ergo-
sterol, a pro-vitamin D of the order C_{28}, was reported to
occur, in minor amounts, in snails (53), in the earthworm
(53), and in egg yolk (54).

In 1943, Matsumoto announced the isolation of a new
sterol, from the starfish, __Asterina__ __pectininfera__ and __Asterias__
__rollestoni__ (later renamed __Asteria__ __amurensis__ by Lutken) (55,
56). The sterol was given the name of hitodesterol and was
found to have a melting point of 167-168° (acetate 182-183°).
Two recent publications (57, 58) give convincing evidence
that hitodesterol is identical with α-spinasterol which pre-
viously had been isolated only from higher plants. Ozonolysis
of hitodesteryl acetate, isolated from both starfish, gave
ethylisopropylacetaldehyde (isolated as the 2,4-dinitro-
phenylhydrazone derivative) which was identical in pro-
perties, and showed no melting point depression with the
aldehyde obtained from stigmasterol, in which the ethyl group
at C-24 has the b-configuration. Mixed melting points of
hitodesteryl acetate and α-spinasteryl acetate showed no
depression.

One of the acetate fractions, m.p. 171-175°, was found
to yield a mixture of methyl and ethylisopropylacetaldehydes

and was probably a mixture of hitodesterol and stellasterol.

The occurrence in animals of sterols formerly isolated only from plants is no longer considered unusual. Brassicasterol, first isolated from rape-seed oil (59), has been found to occur in the oyster (60), clam (61), and mussel (62), and β-sitosterol has been isolated from aquatic invertebrates (63, 64), as well as from plants (65, 66). It should be mentioned in this connection that there is convincing evidence that, with the possible exception of those from parasitic forms, all sterols isolated from marine organisms are of endogenous origin (13).

Kuwata and Ban (67), in 1949, reported the isolation of a mono-unsaturated sterol, patiriasterol, from Asterina pectinifera. The sterol, m.p. 144-146° (acetate 160-162°), upon hydrogenation in glacial acetic acid (PtCl$_4$ as the catalyst) took up one mole of hydrogen to give patiriasterol, m.p. 122°. From optical rotation studies on patiriasterol and its derivatives, the authors concluded that it was a 22:23-ergostenol. As yet there have not been any confirmatory reports in the literature.

Several Japanese investigators found that there were large amounts of mono-unsaturated sterols present in the starfish, Luidia quinaria Von Martens, and Asterias amurensis (68, 69). From the latter species they have isolated a mono-unsaturated steryl acetate, m.p. 119-120°, which upon hydrogenation in glacial acetic acid rearranged to the 8:14-stenol,

m.p. 76-78°, without the absorption of any hydrogen. The
sterol was concluded to be identical with 7-cholestenol which
had previously been isolated from chiton (70, 71).

It is quite evident from the preceding discussion that
the sterol mixtures isolated from starfish are indeed complex
and that the story is far from complete. There have been
but two reports on the sterols of holothurians (19, 38) and
it appears that mixtures similar to those of the starfish
are present. As was pointed out earlier, cholesterol is
still to be demonstrated in the lipids of asteroids and holo-
thurians. All sterols isolated from starfish have been shown
to be 7-stenols with the exception of patiriasterol.

The story is more brief, but not necessarily uncompli-
cated, as far as the sterols of tunicates are concerned.
The only report on tunicates in the literature is that of an
investigation of **Styela** **plicata** by Bergmann(39) who found
that, except for the presence of about 7% $\triangle^{5,7}$-sterols (pro-
vitamins D), which were not identified, cholesterol was pre-
dominant. Only a few recrystallizations of the crude acetate
mixture were necessary for the isolation of cholesteryl
acetate.

During the course of our investigations of the sterols
of tunicates, we have found strong evidence that a more
extensive study would reveal the presence of other sterols
in tunicates. This does not by any means contradict the
results of Bergmann's study, for it is very possible that

in _Styela_ _plicata_, cholesterol is unaccompanied by any other sterols with the exception of the pro-vitamins D.

Karnovsky (41) reported that analyses of the unsaponifiable lipid fraction of the tunicate, _Ciona_ sp., indicated a content of 4.1% α-glyceryl ethers calculated as batyl alcohol. However, there are no reports concerning the nature of glyceryl ethers present in tunicates.

Table 1 summarizes the sterols previously found in echinoderms and tunicates.

During his stay at the Stazione Zoologica, in Naples, Dr. Ciereszko collected samples of the starfish, _Marthasterias_ _glacialis_ (L.), and the tunicates, _Phallusia_ _mamillata_ (Cuvier), _Microcosmus_ _sulcatus_ _polymorphus_ (Heller), and _Microcosmus_ _sulcatus_ _vulgaris_ (Heller). The starfish specimens were dissected and separated into the following parts: pyloric caeca, skin, ovaries, male gonads, and tube foot area. Lipid extracts were then prepared from each of the above fractions. In the cases of the tunicates the tests were removed, the stomachs and intestines flushed with water, to remove foreign matter, and lipid extracts prepared. The branchial basket of _P._ _mamillata_ was also removed because of infestation with copepods and other organisms.

Our primary object was to extend the study of sterols and α-glyceryl ethers to the above mentioned starfish and tunicates. In all previous investigations of starfish the lipid extracts were prepared either from the entire animals

TABLE 1

STEROLS REPORTED TO OCCUR IN ECHINODERMS AND TUNICATES

Phylum, Class, and Species	Sterols	References
Echinodermata		
Asteroidea		
Asterias rubens	Stellasterols	(15)
Asterias forbesi	Stellasterols	(44)
Asterina pectinifera	α-Spinasterol, Patiriasterol	(55, 58)(67)
Asterias amurensis Lutken	α-Spinasterol, 7-Cholestenol	(56, 57)(69)
Holothuroidea		
Holothuria princepo	Stellasterols	(39)
Cucumaria chronjhelmi	Stellasterols (?)	(38)
Echinoidea		
Tripneustes esculentus	Cholesterol	(39)
Centrichus antillarum	Cholesterol	(39)
Lytechinus variegatus	Cholesterol	(39)
Heliocidarus crassidus	Cholesterol	(40)
Arbacia punctulata	Cholesterol	(13)
Chordata **Sub-Phylum-Tunicate**		
Ascidiacea		
Styela plicata	Cholesterol	(39)

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or from the pyloric caeca. By working with the different parts of the body it appeared possible that we might encounter less difficultly separable mixtures, but it was not unreasonable to assume that the sterols present would be distributed throughout the animal.

Fluorides in the Sponge Dysidea Crawshayi

Dysidea crawshayi de Laubenfels (Class, Demospongiae; Order, Keratosa) is a sponge which is very abundant in Walshingham Pond, Bermuda. Bowen and Sutton (72), have reported that their analyses for silica in the sponge were complicated by an extraordinary fluorine content of about 1% of the dry sponge. Such a high fluorine content is apparently not only unique for sponges but for living organisms in general. The high concentration of fluorine in the sponge is readily detectable. For example, during a hydrolysis of the sponge residue with 20% hydrochloric acid, the flask and condenser were so badly etched by hydrogen fluoride that they could not be used again.

Our observations, in accord with that of Bowen and Sutton, indicate that the fluoride present is predominantly inorganic. It is apparent that the sponge is able to concentrate fluorine. We, therefore, have begun a study on the nature of fluorides present with an eye to the possible presence of organic fluorine compounds, particularly fluorinated amino acids. Our investigation is not complete as yet, but
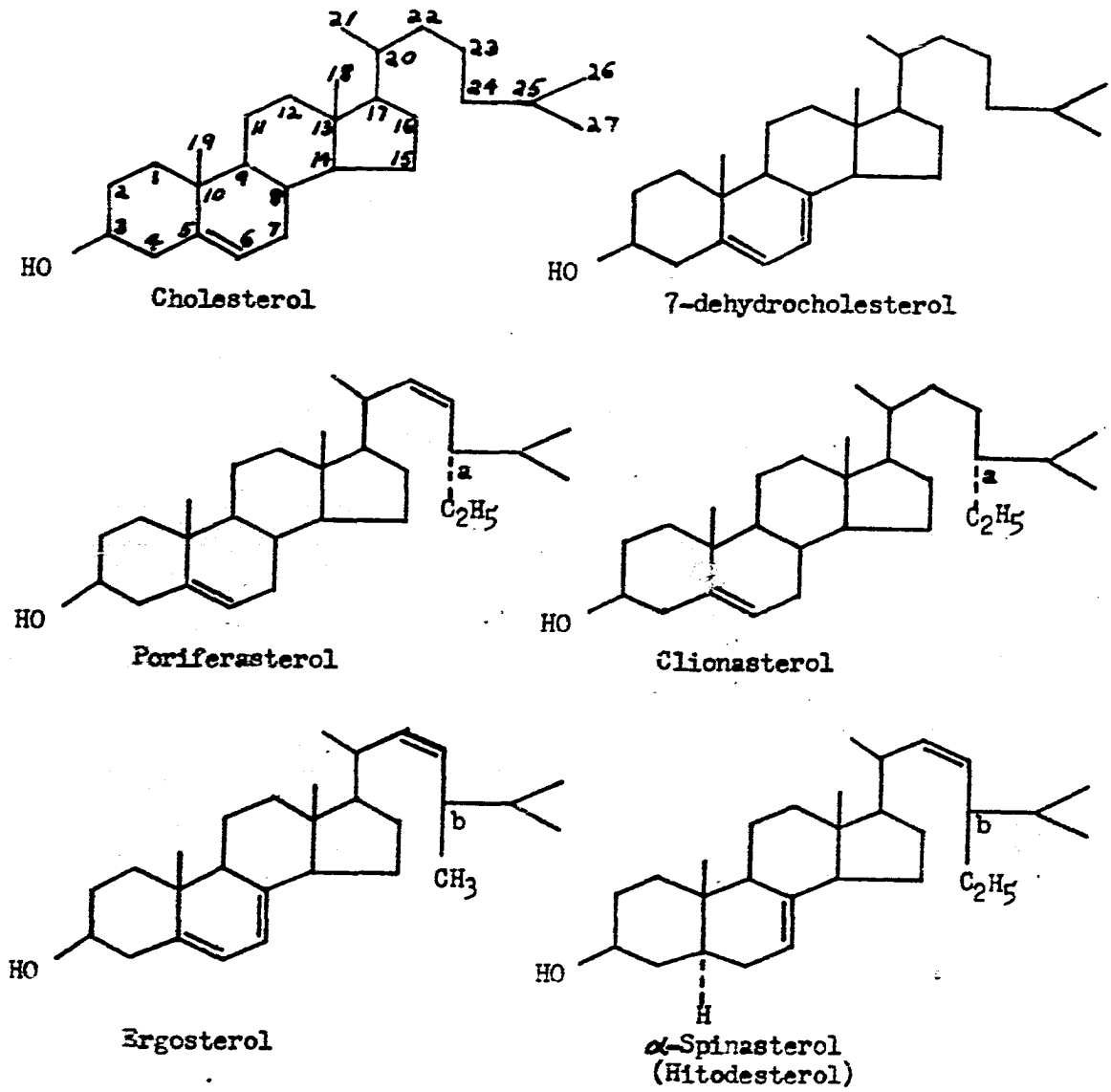
some significant results have been obtained.

FIGURE 1

STRUCTURES OF STEROLS AND α-GLYCERYL ETHERS

Cholesterol

7-dehydrocholesterol

Poriferasterol

Clionasterol

Ergosterol

α-Spinasterol
(Hitodesterol)

FIGURE 1-Continued

Stellasterol

(γ-stenols)

Stellastenol

$\Delta^{8:14}$-Stellastenol
(α-stenol)

$\Delta^{14:15}$-Stellastenol
(β-stenol)

HC—CH$_2$

HO—CH

RO—CH$_2$
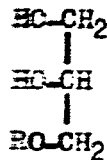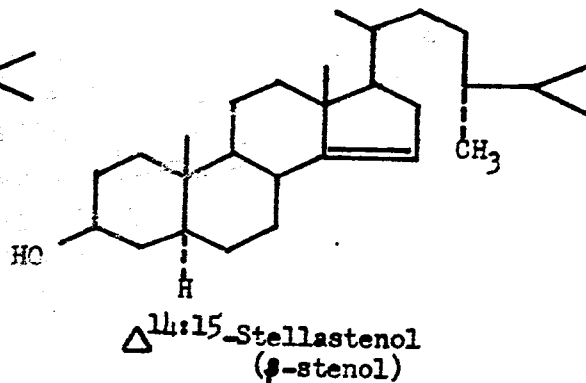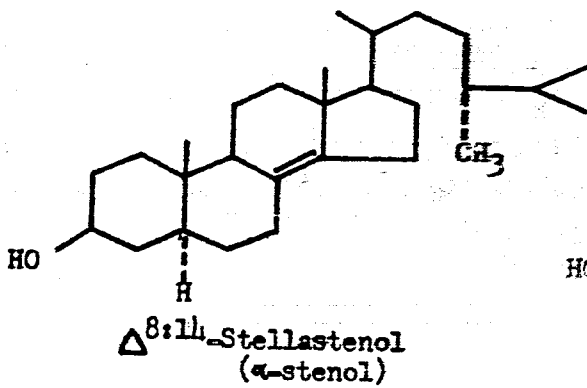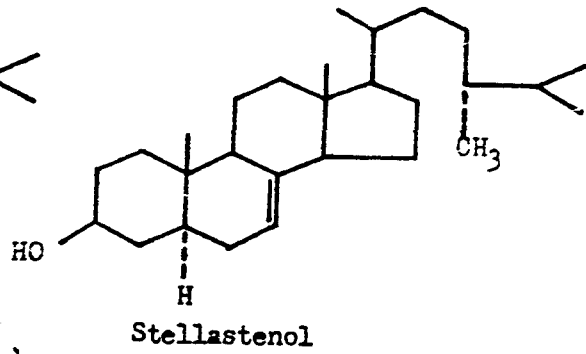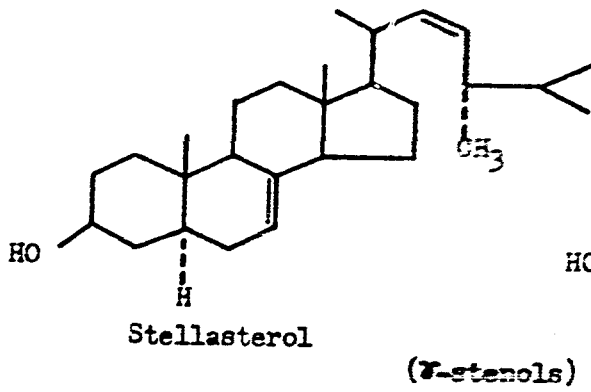
α-Glyceryl Ether (General Formula)

Batyl Alcohol, R=C$_{18}$H$_{37}$
Chimyl Alcohol, R=C$_{16}$H$_{33}$
Selachyl Alcohol, R=C$_9$H$_{18}$=C$_9$H$_{17}$

CHAPTER II

EXPERIMENTAL

Preparation of Animal Lipids

Live specimens of the starfish, _Marthasterias glacialis_ (L.), collected from the Gulf of Naples, were dissected and divided into the following parts: pyloric caeca, ovaries, male gonads, skin, and tube foot area.

The tunicates, _Phallusia mamillata_ (Cuvier), _Microcosmus sulcatus polymorphus_ (Heller), and _Microcosmus sulcatus vulgaris_ (Heller), were also collected from the Naples area. The tests were removed and the stomachs and intestines flushed with water. In the case of _P. mamillata_ the branchial baskets were also removed.

Lipid extracts were prepared in the following manner: The animal tissues were homogenized with acetone in a Waring Blendor (2x). The starfish skins could not be extracted in the blendor and instead they were cut up into small pieces which were then treated with acetone. The acetone extracts were filtered and the residues were extracted with acetone and then with ether in a Soxhlet apparatus. In each case the acetone solutions were combined, the solvent was removed _in_

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vacuo, and the remaining aqueous suspensions were extracted with ether. All ether extracts were combined and the lipid residues were obtained by drying with anhydrous sodium sulfate followed by removal of the solvent. In cases where much water was present, the ether was removed and the residue co-distilled with benzene, which was then evaporated off in vacuo.

The lipid residues were saponified by refluxing them on a steam bath, for one-half to one hour, with a 10% solution of potassium hydroxide in 75% ethanol (10 parts by volume to one part residue by weight). Two or three volumes of water were added to the saponified solution which was then extracted with several portions of ether. The combined ether extracts were washed with water and dried over anhydrous sodium sulfate. The filtered ether solutions were concentrated on a water bath and the final traces of ether removed in vacuo. The resulting material represented the unsaponifiable lipid residue.

Sterols

Procedure for the isolation and estimation of sterols.
The amount of sterols present in the unsaponifiable lipids was determined by dissolving a weighed quantity of the unsaponifiable fraction in hot 90% ethanol and treating the resulting solution with a hot 1% solution of digitonin in the same solvent. The precipitate, obtained upon cooling and

standing for several hours, was filtered off and washed with ethanol and ether. The filtrates were treated with the digitonin solution until no further precipitate appeared. The combined digitonides were dried to constant weight at 105°. Multiplication of the weight of the washed and dried digitonide by 0.25 gave the weight of sterols present in the sample (73).

In some instances, where the amount of sterols present was small or when the sterols were accompanied by large amounts of pigment and/or other alcohols, all of the sterol was isolated via the digitonide. In the remaining cases the unsaponifiable residues were dissolved in hot methanol, the crude sterol precipitate which formed on cooling was filtered off and washed with methanol. The methanol filtrates were combined, heated to boiling and treated with a hot 1% solution of digitonin in 90% ethanol. The resulting digitonides were washed and dried as described above. In order to isolate the sterols, the dried digitonides were dissolved in 10-20 volumes of dry pyridine and kept at 70-90° for one hour. The solvent was removed $\underline{in\ vacuo}$, the residues treated with anhydrous ether (2x) and ground to a fine powder. The powder was then extracted with ether in a Soxhlet, the ether extracts combined, filtered, and concentrated to dryness. The dry residue represented the sterols, and the ether insoluble material (digitonin) was treated with sufficient 90% ethanol to give a 1% solution (74).

For the estimation of $\triangle^{5,7}$-sterols (pro-vitamins D), portions of the crude sterols or their derivatives were dissolved in a 1% solution of chloroform in absolute ethanol. The resulting solutions were analyzed in a Beckman Model DU Spectrophotometer. Pro-vitamins D give characteristic absorption peaks at 262, 271, 282, and 292 mu. The amounts of $\triangle^{5,7}$-sterols present were estimated from the absorption at 282 mu based on the extinction coefficient for 7-dehydro-cholesterol at this wavelength (in the same solvent) (75).

Optical rotations were determined in a 0.2 ml. polarimeter tube, 1 dm. in length, with chloroform as the solvent.

The melting points are all corrected and were determined in melting point tubes heated in silicone (Dow 550) oil.

Marthasterias Glacialis

Sterols of pyloric caeca. From the saponification of 20 g. of pyloric caeca lipids, 1.94 g. of unsaponifiable matter was obtained, which was then taken up in hot methanol. The crude sterol precipitate, which formed upon cooling, was filtered off, washed with methanol and dried on the filter to give 114 mg. of sterols, m.p. 126-135°, $[\alpha]_D^{20}$= +8.2° (3.7 mg. in 0.5 ml., α= +0.06°), (Pc-I).

From the combined methanol filtrates 0.729 g. of digitonide was obtained and combined with 0.502 g. of digitonide

isolated from another batch of pyloric caeca unsaponifiable lipids. The resulting 1.231 g. of digitonides gave 290 mg. of sterols, m.p. 128-136°, (Pc-II).

Fraction Pc-I, 100 mg, was recrystallized from methanol with Norit, and methanol (2x) and 56 mg. of sterols, m.p. 139-146°, were isolated (Pc-III). The mother liquors were combined and taken to dryness _in vacuo_ giving 43 mg. of sterols. The sterol residue was recrystallized from absolute ethanol; 32 mg., m.p. 130-136°, (Pc-IV) was obtained as the first crop. Further concentration of the mother liquor gave, as a second crop, 6 mg. of sterols, m.p. 124-129°, $[\alpha]_D^{20} =$ +5.3° (3.8 mg. in 0.5 ml., $\alpha =$ +0.04°), (Pc-V).

The recrystallization of 50 mg. of sterol fraction Pc-II, from methanol (2x), ethanol (2x), and acetone, resulted in the isolation of 11 mg. of sterols, m.p. 147-152°, $[\alpha]_D^{20} =$ +6.5° (3.8 mg. in 0.5 ml., $\alpha =$ +0.05°), (Pc-VI).

Fifty milligrams of sterol fraction Pc-III (m.p. 139-146°) was dissolved in 0.7 ml. of pyridine and 0.2 ml. of benzoyl chloride was added. After standing at room temperature for 18 hours, the mixture was poured into 5 ml. of ice-cold 3N sulfuric acid. The precipitate was filtered off, washed with water and recrystallized twice from absolute ethanol giving 30 mg. of steryl benzoate m.p. 161-168°. Two more recrystallizations from ethanol and one from acetone gave 9 mg. of benzoate m.p. 179-184°, $[\alpha]_D^{20} =$ +9.3° (4.3 mg. in 0.5 ml., $\alpha =$ +0.08°).

Steryl acetates of pyloric caeca. From another batch of pyloric caeca unsaponifiable lipids, 50 mg. of sterols, m.p. 134-142, was isolated. The sterols were refluxed with acetic anhydride for one hour, the precipitate was filtered, washed with cold methanol, and recrystallized once from hot chloroform followed by the addition of methanol. Thirty milligrams of steryl acetate was thus obtained, m.p. 142-147°, (Pc-VII). During the recrystallization of Pc-VII from chloroform-methanol, ethanol (2x), and acetone, the melting point rose to 163-168°, 12 mg., $[\alpha]_D^{20} = +1.8°$ (5.4 mg. in 0.5 ml., $\alpha = +0.02°$) (Pc-VIII). The acetic anhydride mother liquor and methanol wash solutions were combined and concentrated. The resulting residue was recrystallized once from chloroform-methanol and 6 mg. of acetate, m.p. 115-123°, $[\alpha]_D^{20} = +6.0°$ (3.3 mg. in 0.5 ml., $\alpha = +0.04°$), was obtained (Pc-IX).

The crude acetate, obtained from the acetylation of 240 mg. of sterols Pc-II, was washed with methanol and recrystallized once from chloroform-methanol giving 197 mg. of steryl acetate, m.p. 141-148°, (Pc-X). Concentration of the chloroform-methanol mother liquor gave 12 mg. of acetate, m.p. 136-142°, (Pc-XI). The combined acetic anhydride mother liquor and methanol wash solutions were concentrated, and 21 mg. of acetate was recovered. Recrystallization of the acetate from chloroform-methanol yielded 6 mg. (1st crop), m.p. 124-131°, and 12 mg. of steryl acetate, m.p. 115-122°, $[\alpha]_D^{20} = +6.4°$ (3.9 mg. in 0.5 ml., $\alpha = +0.05°$).

After two recrystallizations of acetate Pc-X from chloroform-methanol and once from acetone, the melting point rose to 148-153°, $[\alpha]_D^{20}$= +2.8° (3.9 mg. in 0.5 ml., α= +0.03), 147 mg. (Pc-XII).

Isolation of 8:14-stellastenyl acetate. One hundred milligrams of Pc-XII was dissolved in 15 ml. of glacia acetic acid and hydrogenated in the presence of platinum-black at room temperature (28°) and atmospheric pressure (734 mm. Hg). Four milliliters of hydrogen was absorbed (0.72 mole/mole of acetate taken as stellasteryl acetate) over a period of about 90 minutes. The glacial acetic acid solution was filtered and concentrated to a small volume _in vacuo_. Methanol was then added to give a copious precipitate which was filtered off and recrystallized from ethanol (4x). An 8:14-stenyl acetate (α-stellastenyl acetate), 28 mg., was thus obtained, m.p. 104-105°, $[\alpha]_D^{20}$= +12.2° (5 mg. in 0.5 ml., α= +0.11°) (Pc-XIII). Further recrystallization of a portion of Pc-XIII did not raise the melting point. Concentration of the methanol-glacial acetic acid filtrate gave 17 mg. of acetate, m.p. 99-102°, which rose to 102-104° after three recrystallizations from ethanol.

All sterols and their derivatives obtained from the pyloric caeca of M. glacialis gave positive Tortelli-Jaffe' reactions.

Ovarian sterols. Ten grams of ovarian lipids gave 1.42 g. of unsaponifiable lipids from which 0.7420 g. of

digitonide was obtained. The split digitonide yielded 170 mg. of sterols, m.p. 126-134°, $[\alpha]_D^{20}$ = +8.5° (4.8 mg. in 0.5 ml., α= +0.08°) (O-I).

Ovarian steryl acetates. Sterol fraction O-I, 150 mg., was acetylated and the first crop after one recrystallization from chloroform-methanol gave 130 mg. of steryl acetate, m.p. 138-144°, (O-II). Concentration of the acetylation mother liquors gave 10 mg. of acetate which after one recrystallization from chloroform-methanol had a m.p. 119-124°, 6 mg., $[\alpha]_D^{20}$= +4.8° (4.2 mg. in 0.5 ml., α= +0.02°), (O-III).

Steryl acetate O-II was recrystallized from chloroform-methanol (2x), and acetone, giving 85 mg. of acetate m.p. 150-156°, $[\alpha]_D^{20}$= +2.1° (4.8 mg. in 0.5 ml., α= +0.02°) (O-IV).

Isolation of 8:14-stellastenyl acetate. Acetate O-IV, 80 mg., was hydrogenated using the procedure described above (T=29°, P=730 mm. Hg) and 3.8 ml. of hydrogen was absorbed (0.82 mole). The glacial acetic acid solution was filtered, concentrated to a small volume, and methanol added. Sixty-four milligrams of precipitate was recovered and after several recrystallizations from ethanol, 42 mg. of the 8:14-stenyl acetate was isolated (O-V). O-V (8:14-stellastenyl acetate) had a melting point of 104-105°, $[\alpha]_D^{20}$= +13.0° (5.4 mg. in 0.5 ml., α= +0.14°). Further concentration of the glacial acetic acid-methanol mother liquor gave 12 mg. of acetate which after two recrystallizations from ethanol had a melting point of 102-104°.

Isolation of 14:15-stellastenyl acetate. Twenty-five milligrams of 8:14-stenyl acetate O-V was dissolved in 10 ml. of chloroform, and dry hydrogen chloride was passed through the solution, kept at $0°$, for five hours. The resulting solution was washed with aqueous sodium bicarbonate, dried over potassium carbonate, filtered, and evaporated to dryness *in vacuo*. The oily residue was agititated with methanol and gave 15 mg. of acetate, which, after three recrystallizations from ethanol, melted at $93-95°$ (8 mg.), $[\alpha]_D^{20} = +19.6°$ (5.4 mg. in 0.5 ml., $\alpha = +0.21°$), (O-VI). O-VI is evidently the 14:15-stenyl acetate (β-stellastenyl acetate), and gives a negative Tortelli-Jaffe' reaction.

With the exception of O-VI, all sterols and their acetates obtained from the unsaponifiable lipids of the starfish ovaries gave positive Tortelli-Jaffe' reactions.

Male gonad sterols. From 0.8 g. of unsaponifiable lipids, 0.912 g. of digitonide was isolated. The digitonide yielded 200 mg. of sterols, m.p. $129-138°$, (M-I).

Male gonad steryl acetates. Sterol fraction M-I was acetylated, and the resulting crude acetate was filtered, washed with cold methanol, and recrystallized twice from chloroform-methanol. Steryl acetate M-II was thus obtained, 137 mg., m.p. $141-147°$. The combined acetic anhydride mother liquor and methanol wash solutions were concentrated, and an additional 22 mg. of acetate was recovered (M-III). M-III was recrystallized from chloroform-methanol giving 9 mg. (1st

crop) m.p. 129-136°, and 7 mg. of acetate (2nd crop) m.p. 120-125°.

Steryl acetate M-II was recrystallized from chloroform-methanol (2x), ethanol, and acetone, and 62 mg. of acetate, m.p. 156-161°, (M-IV) was isolated.

Isolation of 8:14-stellastenyl acetate. Hydrogenation of acetate M-IV (T=28°, P=729 mm. Hg) resulted in the absorption of 3.08 ml. of hydrogen (0.83 mole), and the isolation of 13 mg. of 8:14-stenyl acetate, m.p. 103-105°, $[\alpha]_D^{20}$= +12.5° (4.0 mg. in 0.5 ml., α= +C.10°), (M-V) (8:14-stellastenyl acetate).

Skin sterols. A sample of 1.7 g. of unsaponifiable lipids was dissolved in hot methanol. The crude sterol precipitate which formed upon cooling was filtered off, and washed with methanol giving 82 mg. of crude sterols. One recrystallization of this fraction, from methanol (Norit), resulted in the isolation of 61.5 mg., m.p. 137-146°, $[\alpha]_D^{20}$= +7.1° (4.2 mg. in 0.5 ml., α= +0.06°), (S-I).

The combined methanol solutions yielded 1.347 g. of digitonide from which 260 mg. of sterols, m.p. 129-138°, were recovered (S-II).

Fifty milligrams of sterols S-I was converted into the benzoate which after recrystallization from ethanol (5x), and acetone (2x), gave 6 mg. of benzoate m.p. 183-186°, $[\alpha]_D^{20}$= +8.8° (4 mg. in 0.5 ml., α= +0.07°).

Skin steryl acetates. Sterol fraction S-II was acety-

lated and the resulting crude acetate, after one recrystallization from chloroform-methanol, gave 210 mg., m.p. 129-137°, (S-III).

Concentration of the acetylation mother liquor gave an additional 25 mg. of acetate, m.p. 121-127°, (S-IV). Recrystallization of S-IV from chloroform-methanol gave 10 mg. (1st crop) m.p. 131-137°, 6 mg. (2nd crop) m.p. 123-129°, and 4 mg. of acetate (3rd crop) m.p. 115-121°, $[\alpha]_D^{20}$= +3.3° (4.5 mg. in 0.5 ml., α= +0.03°) (S-V).

Isolation of 8:14-stellastenyl acetate. Acetate S-V, 100 mg., was dissolved in glacial acetic acid and hydrogenated in the presence of platinum-black (T=28°, P=732 mm. Hg); 4.3 ml. of hydrogen was absorbed (0.74 mole). The solvent was filtered, concentrated, and methanol added to give a precipitate which after several recrystallizations from ethanol had a m.p. 103-105°, 42 mg., $[\alpha]_D^{20}$= +12.4° (4.5 mg. in 0.5 ml., α= +0.11°), (S-VI) (8:14-stellastenyl acetate). The acetates recovered from the mother liquors did not melt below 98°.

All sterols and their derivatives isolated from the skin gave a positive Tortelli-Jaffe' reaction.

Sterols from tube foot area. From 1.5 g. of unsaponifiable lipids, 0.792 g. of digitonide was obtained. The digitonide, when split, gave 168 mg. of sterols, m.p. 126-134°, (T-I).

Steryl acetates from tube foot area. Sterol fraction

T-I, 168 mg. was acetylated, the crude acetate was filtered off, washed with methanol, and recrystallized from chloroform-methanol, and 142 mg. of acetate, m.p. 136-147°, was obtained (T-II). The chloroform-methanol mother liquor was concentrated and an additional 14 mg. of acetate, m.p. 124-132°, was isolated (T-III).

The acetic anhydride mother liquor and methanol wash solutions were combined and concentrated giving 12 mg. of acetate which was recrystallized from chloroform-methanol. The first crop, resulting from the recrystallization, had a m.p. 115-123°, 6 mg., $[\alpha]_D^{20} = +6.3°$ (4.0 mg. in 0.5 ml., $\alpha = +0.05°$), (T-IV). The second crop, 3 mg., melted at 114-117°.

Acetate T-II was recrystallized from chloroform-methanol (2x), ethanol, and acetone yielding 40 mg., m.p. 152-157°. After four more recrystallizations from acetone the melting point rose to 175-178°, 5 mg., $[\alpha]_D^{20} = 0.0°$ (4.0 mg. in 0.5 ml., $\alpha = 0.00°$) (T-V).

All sterol and steryl acetate fractions gave positive Tortelli-Jaffe' reactions.

Spectrophotometric studies of sterols isolated from the pyloric caeca, skin, and ovaries of M. glacialis failed to reveal the presence of any pro-vitamins D.

Phallusia Mamillata

Sterols. A sample of 1.9 g. of unsaponifiable lipids of P. mamillata was taken up in hot methanol and the crude

sterol precipitate, which came out upon cooling, was filtered and washed with methanol. Five hundred milligrams of sterols, m.p. 124-129°, $[\alpha]_D^{20}=$ -63.3° (18 mg. in 1.0 ml., $\alpha=$ -1.14°), was thus obtained (PM-I).

The methanol filtrates were combined and treated with an alcoholic solution of digitonin, and 1.050 g. of digitonide was isolated. From the digitonide, 271.5 mg. of sterols, m.p. 124-130°, was recovered (PM-II).

Steryl acetates. Sterol fraction PM-I, 250 mg., was acetylated and the crude acetate, after treatment with cold methanol, and recrystallized once from chloroform-methanol. Acetate fraction PM-IV was thus obtained, 193.5 mg., m.p. 121-126°. Concentration of the chloroform-methanol mother liquor gave 10 mg. of acetate, m.p. 116-119° (PM-V). The acetic anhydride and methanol filtrates were combined, concentrated, and the residue recrystallized from chloroform-methanol. Fractionation of the chloroform-methanol solution gave 12 mg., m.p. 115-118°, (1st crop) (PM-VI), and 26 mg. (2nd crop), m.p. 112-115° (PM-VII). Acetate PM-VII was recrystallized from methanol and 20 mg. of acetate was isolated, m.p. 113-115°, $[\alpha]_D^{20}=$ -48.2° (11.0 mg. in 1.0 ml., $\alpha=$ -0.53°) (PM-VIII). The latter acetate did not give any melting point depression when mixed with authentic cholesteryl acetate, m.p. 114-115°, $[\alpha]_D^{20}=$ -48.0° (10 mg. in 1.0 ml., $\alpha=$ -0.48°).

Bromination of acetates and separation of the resulting bromides. Steryl acetate PM-III, 100 mg., was dissolved

in 0.5 ml. of anhydrous ether, and to the chilled solution, 0.9 ml. of a 5% solution of bromine in glacial acetic acid was added dropwise. A green coloration was evident during the addition of the first few drops of the bromine solution (Tortelli-Jaffe' reaction evidently due to the $\triangle^{5,7}$-sterols present) but the color was no longer apparent after all of the bromine had been added. The brominated solution was kept in the refrigerator (4°) overnight and the crystalline material which had separated was filtered off and washed with cold glacial acetic acid. Twenty-eight milligrams of acetate bromide, m.p. 172-176^{o}(d), was thus obtained. Concentration of the mother liquor gave an additional 3 mg. of bromide, m.p. 170-174^{o}(d), which was combined with the first crop (PM-IX, 31 mg.). PM-IX was then triturated with ether and there remained 21 mg. of ether insoluble bromide, m.p. 179-182^{o}(d), (PM-X). The ether solution (PM-EB) was saved. PM-X was recrystallized twice from ethyl acetate-ethanol giving 12 mg. of bromide, m.p. 183-186^{o}(d), (PM-XI).

Acetate PM-IV, 193.5 mg., was dissolved in 1 ml. of anhydrous ether, and the chilled solution was treated dropwise with 4.0 ml. of a 5% solution of bromine in glacial acetic acid. After standing overnight in the refrigerator, the precipitated material was filtered, washed with cold glacial acetic acid, and 46 mg. of acetate bromide was obtained. An additional 7 mg. was recovered from the concentrated mother liquor and combined with the first batch of

material (Pm-XII, 53 mg.). Bromide PM-XII was triturated
with ether leaving 34 mg. of insoluble bromide, m.p. 178-
181°(d), which after two recrystallizations from ethyl ace-
tate-ethanol had a m.p. 184-186°(d), 23 mg., (PM-XIII).

Isolation of cholesteryl acetate dibromide. The ether
solution was combined with that of the previous bromination
(PM-EB), and concentrated to a few drops. Methanol was
then added, giving 16 mg. of an acetate bromide, m.p. 133-
160°(d) (PM-XIV), and 5 mg. (2nd crop) m.p. 116-118°(d)
(PM-XV). PM-XV did not give a melting point depression
with authentic cholesteryl acetate dibromide.

PM-XIV was retriturated with ether giving 3 mg. of
insoluble bromide, m.p. 171-176°(d). Ten milligrams of
bromide, m.p. 149-153°(d), was recovered from the ether
solution. The latter was entirely soluble in ether and
upon recrystallization from methanol gave 8 mg. of bromide
m.p. 153-156°(d). The bromide was recrystallized two more
times from ether-methanol but the melting point remained
constant at 156-157°(d) (PM-XVI, 5 mg.). Due to the small
amount available it was not investigated any further.

Isolation of poriferasteryl acetate tetrabromide.
Acetate bromides PM-XIII (23 mg.) and PM-XI (12 mg.) were
combined and extracted with ethyl acetate. The ethyl ace-
tate solution was concentrated giving 22 mg. of bromide,
m.p. 189-191°(d), which after one recrystallization from
the same solvent had a melting point 191-192°(d), 14 mg.,

(PM-XVII) (poriferasteryl acetate tetrabromide).

Debromination of bromide mother liquor. The mother
liquor which resulted from the bromination of acetate PM-IV
was refluxed for three hours with zinc dust and the hot mix-
ture was then filtered. The filtrate was treated with water
until turbid and the resulting precipitate was filtered off
and washed with cold methanol. The material which remained
on the filter was dissolved in hot chloroform and upon the
addition of methanol a dark-brown tarry mass fell to the bot-
tom of the flask. The solution was quickly heated and the
supernatant poured off. Upon cooling, the precipitate which
formed was still contaminated with decomposition products
and the recrystallization procedure was repeated. Finally,
24 mg. of acetate, m.p. 127-131°, (PM-XVIII), was isolated
from the last chloroform-methanol recrystallization. Fur-
ther concentration of the mother liquor gave 14 mg. of ace-
tate, m.p. 121-125°(PM-XIX). The remaining solvent was then
removed in vacuo leaving 46 mg. of acetate, m.p. 114-119°,
(PM-XX).

Isolation of clionasteryl acetate. Acetate PM-XVIII
was recrystallized from chloroform-methanol (2x), and methanol
giving 7 mg. of acetate, m.p. 135-136°, $[\alpha]_D^{20}$= -42° (5.0 mg.
in 0.5 ml., α= -0.42°) (PM-XXI). When acetate PM-XXI was
mixed with an authentic sample of clionasteryl acetate, m.p.
137-138°, the melting point was 136-137.5°.

Isolation of cholesteryl acetate. Recrystallization

of acetate PM-XX, from methanol, gave 10 mg., m.p. 118-120°, and 31 mg. of acetate, m.p. 113-115°, $[\alpha]_D^{20}$= -47.0° (10 mg. in 1.0 ml., α= -0.47°). The latter acetate did not give any melting point depression when mixed with cholesteryl acetate.

Steryl benzoates. Sterol fraction PM-I, 100 mg., was dissolved in 1.2 ml. of dry pyridine and 0.3 ml. of benzoyl chloride was added. The mixture, which was allowed to stand for 24 hours, was poured into 8 ml. of ice-cold 3N sulfuric acid and the precipitate filtered off. After two recrystallizations, of the crude material, from ethanol 51 mg. of steryl benzoate was obtained, m.p. 140-143° (PM-XXII). Twenty-two milligrams of benzoate was recovered from the first ethanol mother liquor, m.p. 139-141° (PM-XXIII).

Isolation of cholesteryl benzoate. Benzoate PM-XXII was recrystallized from ethanol (3x), and acetone; 23 mg. of steryl benzoate, m.p. 144-145° (clearing at 174°) was isolated (PM-XXIV), $[\alpha]_D^{20}$= -13.4° (12 mg. in 1.0 ml., α= -0.16°). The latter did not give any melting point depression with cholesteryl benzoate, m.p. 145-146° (clearing at 175°).

Spectrophotometric analysis for pro-vitamins D. spectrophotometric analysis of sterol fraction PM-I indicated the presence of 3.1% $\triangle^{5,7}$-sterols. Analysis of steryl acetate fraction PM-III gave evidence for the presence of 3.5% $\triangle^{5,7}$-steryl acetates.

Microcosmus Sulcatus Polymorphus

Sterols. Seventy-nine milligrams of unsaponifiable lipids was dissolved in hot methanol and the crude sterol precipitate, which came out on cooling, was filtered off and washed with methanol. Sterol fraction MP-I was thus obtained, 273.5 mg., m.p. 123-127°, $[\alpha]_D^{20}$= -59.2° (12 mg. in 1.0 ml., α= -0.71°). From the combined methanol solutions 485 mg. of digitonide was isolated. The latter was combined with 275 mg. of digitonide obtained from a previous batch of M. polymorphus unsaponifiable lipids. The sterols recovered from the combined digitonide weighed 180 mg., m.p. 124-128°, (MP-II).

Steryl acetates. The precipitate which resulted from the acetylation of 250 mg. of sterol fraction MP-I was filtered, washed with cold methanol and recrystallized once from chloroform-methanol. The resulting acetate, 160 mg., had a melting point 122-126°, $[\alpha]_D^{20}$= -53.6° (11.0 mg. in 1.0 ml., α= -0.59°) (MP-III). The methanol wash solution and acetic anhydride mother liquor were combined and concentrated giving 42 mg. of acetate m.p. 117-123° (MP-IV).

Sterol fraction MP-II was acetylated and the crude acetate after one recrystallization from chloroform-methanol weighed 110 mg., m.p. 124-129° (MP-V). Concentration of the acetate mother liquors gave 40 mg. of acetate m.p. 118-121° (MP-VI).

Isolation of cholesteryl acetate. Acetate fractions

MP-IV and MP-VI were combined and recrystallized from methanol. Twenty milligrams, m.p. 117-121° (1st crop) and 12 mg. of acetate, m.p. 116-118° (2nd crop) were isolated. The remaining solution was concentrated in vacuo, and the residue recrystallized from methanol giving 25 mg. of steryl acetate, m.p. 114-116° (MP-VII), which gave no melting point depression with cholesteryl acetate.

Bromination of acetates and separation of the resulting bromides. Acetate fractions MP-III and MP-V were combined and 210 mg. of the acetate was dissolved in 1 ml. of anhydrous ether. To the chilled solution 4.0 ml. of 5% bromine solution in glacial acetic acid was added dropwise (a green coloration was again evident during the initial stages of the bromination). After standing overnight in the refrigerator, 47 mg. of insoluble bromide was filtered off. Concentration of the mother liquor gave an additional 8 mg. which was added to the first crop (MP-VIII, 55 mg.). Triturition of MP-VIII with ether left 30 mg. of insoluble bromide, m.p. 179-181°(d) (MP-IX). The ether solution was concentrated, methanol was added, and 18 mg. of bromide recovered, m.p. 140-159°(d). The latter was triturated with ether leaving 4 mg. of insoluble material, m.p. 169-174°(d). From the ether solution 12 mg. of acetate bromide was recovered which after one recrystallization from methanol had a m.p. 116-118°, 8 mg. (MP-X). Bromide MP-X gave no melting point depression with cholesteryl acetate dibromide.

Isolation of poriferasteryl acetate tetrabromide.

Bromide MP-IX was recrystallized from ethyl acetate-ethanol
(2x) and 24 mg. of bromide was obtained, m.p. 188-190°(d),
which was then extracted with ethyl acetate. The ethyl ace-
tate solution was concentrated; the precipitate recrystal-
lized from the same solvent gave 16 mg. of acetate bromide,
m.p. 190-192°(d) (MP-XI) (poriferasteryl acetate tetrabromide).

Isolation of poriferasteryl acetate.

Steryl acetate
bromides MP-XI (M. polymorphus) and PM-XVII (P. mamillata)
gave no mixed melting point depression. The above two fract-
ions (MP-XI, and PM-XVII) were combined, 26.5 mg., and de-
brominated with zinc in glacial acetic acid. The resulting
acetate after recrystallization from chloroform-methanol
(2x), and methanol (2x) had a melting point of 146-147°, 8
mg., $[\alpha]_D^{20}$= -51.0° (5.0 mg. in 0.5 ml., α= -0.51°) (MP-XII).
The latter acetate gave no melting point depression when
mixed with an authentic sample of poriferasteryl acetate,
m.p. 147-148°.

Isolation of cholesteryl acetate.

Debromination of
the glacial acetic acid mother liquor of the acetate bromides
prepared from M. polymorphus, followed by recrystallization
resulted in the isolation of 70 mg. of steryl acetate, m.p.
114-116° (cholesteryl acetate). No acetate fraction corres-
ponding to clionasteryl acetate was isolated from this tuni-
cate.

Spectrophotometric analysis for $\Delta^{5,7}$-sterols.

Steryl

acetate fraction MF-III gave evidence for the presence of 4.2% pro-vitamins D.

Microcosmus Sulcatus Vulgaris

Due to a lack of sufficient lipid material only the crude sterols were isolated via the digitonide, and the acetate was prepared.

The sterols had a m.p. 120-125°, $[\alpha]_D^{20}$= -61.7° (12 mg. in 1.0 ml., α= -0.74°). The acetate prepared from the sterols melted at 124-130°, $[\alpha]_D^{20}$= -54.0° (10 mg. in 1.0 ml., α= -0.54°). Spectrophotometric analysis of the acetate revealed the presence of 4.6% $\triangle^{5,7}$-steryl acetate.

α-Glyceryl Ethers

Methods and materials for the analyses of -glyceryl ethers. For the analysis of α-glyceryl ethers, the unsaponifiable lipid fractions were prepared by the method of the A.O.A.C. (76), using four ether extractions. The method used for the analyses is that of Karnovsky and Rapson (77).

The periodic acid solution was made up as follows: 1.97 g. of sodium periodate (L. Light and Co.) was dissolved in 15 ml. of water containing 0.25 ml. of sulfuric acid. To this was added 235 ml. of 96% ethanol. The white precipitate which formed was filtered off and the solution allowed to stand overnight before use.

A solution of sodium arsenite (ca 0.02 N) was prepared from sodium arsenite, analytical grade, dried to constant

weight. It contained 10 g. sodium bicarbonate per liter.

The arsenite solution was adjusted to give a back-titration figure of 3-5 ml. iodine (ca 0.02 N, standardized against arsenite) for 5 ml. periodic acid reagent in the blank determination (see under oxidation below).

The direct titre of the standard iodine solution against the arsenite solution was also determined.

The oxidations were carried out on solutions of unsaponifiable lipids in ethyl acetate, containing 30-50 mg. of lipid material per 5 ml. of solution.

An aliquot (5 ml.) of the solution of unsaponifiable lipids was pipetted into a 250 ml. Erlenmeyer flask and 5 ml. of periodic acid reagent was added. The solutions were shaken for three and a half hours and then allowed to stand for an additional half hour. At the end of this time 25 ml. of a saturated solution of sodium bicarbonate was added (graduate cylinder), and 25 ml. of sodium arsenite solution pipetted in. Potassium iodide solution (3 ml. of 10%) was then added, the mixture swirled thoroughly and allowed to stand for fifteen minutes. At the end of this time the flask was washed down with water, 1 ml. of starch solution was added, and the excess sodium arsenite titrated with standard iodine solution. Blank determinations (5 ml. of solvent replacing the solution of unsaponifiable matter) were carried out simultaneously. From the titre of periodic acid consumed, the content of glyceryl ether, as batyl alcohol, was cal-

culated.

The method was applied to commercial mannitol (Eastman) dried to constant weight at 105°. Two determinations on the commercial product gave values of 98.32% and 98.48% mannitol.

Table 2 summarizes the content of unsaponifiable lipids, α-glyceryl ethers, and sterols in the animals investigated. In addition to the animals mentioned above, analyses for α-glyceryl ethers were also carried out on the unsaponifiable lipids of the starfish, Oreaster reticulatus (L.) (Bimini), and on those of the sex-trees and viscera of the sea-cucumber, Stichopus badionotus Selenka (Bermuda).

Isolation of α-glyceryl ethers from M. glacialis. The unsaponifiable residue of 15 g. M. glacialis pyloric caeca lipids, from which the sterols had been removed by precipitation with digitonin, was dissolved in 10 ml. of a chloroform-benzene solution (1:10). The solution was poured onto 1.0 g. of urea moistened with 0.2 ml. of methanol (78). The mixture was stirred overnight on a magnetic stirrer and then allowed to stand for several more hours. The solid material was filtered off, washed with benzene, and dried on the filter. The urea adduct was then decomposed with water giving a turbid solution which was extracted with ether. The ether extract was washed with water and dried over anhydrous sodium sulfate. The filtered ether solution was then concentrated on a water bath and the last traces of solvent were removed in vacuo to give a wax-like residue, m.p. 60-63°, 141.5 mg. The material

TABLE 2

CONSTITUENTS OF THE UNSAPONIFIABLE LIPIDS OF THE

MARINE ORGANISMS INVESTIGATED

Animal	Source of Lipids	% Lipids Unsaponifiable	% Unsaponifiable Sterols	% Unsaponifiable Glyceryl Ethers as Batyl alcohol
Marthasterias glacialis	pyloric caeca	9.7	15.6	56.83, 56.40
	ovaries	14.0	13.6	32.25
	male gonads	29.3	36.0	10.27
	skin	14.0	27.0	2.75, 2.54
	tube foot area	14.2	39.6	12.86
Phallusia mamillata	whole animal	12.6	40.2	14.90
Microcosmus sulcatus polymorphus	whole animal	12.0	47.1	7.14
Microcosmus sulcatus vulgaris	whole animal	19.2	41.2	12.28
Oreaster reticulatus	pyloric caeca	9.6	----	20.12
Stichopus badionotus	sex-trees	3.2	----	3.95
	viscera	7.5	----	26.74

was sublimed <u>in</u> <u>vacuo</u> and 119.5 mg., m.p. 62-65° was re-
covered (B-I). Analysis of B-I by the periodate method gave
a value of 94.0% batyl alcohol. A portion of B-I was re-
crystallized from ethyl acetate (2x), dilute acetone, and
once again from ethyl acetate. During the course of the re-
crystallizations the melting point rose to 68-69° (Reported
m.p. of batyl alcohol 71-72°).

Fifty milligrams of B-I was dissolved in dry benzene
and refluxed with 0.2 ml. phenylisocyanate for three hours.
The solution was evaporated to dryness, <u>in</u> <u>vacuo</u>, and then
maintained at 100°, <u>in</u> <u>vacuo</u>, for one half hour. The residue
was recrystallized from benzene (2x), methanol (2x) and
ethanol (2x) giving 5 ml. of the diphenylurethan m.p. 99-101°
(reported melting point of batyl diphenylurethan 100.5-101.5°).

<u>Isolation of α-glyceryl ethers from P. mamillata</u>. The
unsaponifiable residue, less sterols, isolated from 15 g. of
<u>P</u>. <u>mamillata</u> lipids, was dissolved in a hot methanol solution
which had been saturated with urea at room temperature (79).
Upon cooling to room temperature, the precipitated urea adduct
was filtered off, washed with benzene and methanol, and dried
on the filter. The urea adduct was then recrystallized two
times from isopropyl alcohol, filtered, and decomposed with
water. The resulting turbid solution was extracted with
ether, the ether solution washed with water, dried and con-
centrated to give 17 mg. of wax-like material, m.p. 59-63°.
The latter analyzed as 96% α-glyceryl ether (calculated as

batyl alcohol). A portion of the substance was recrystal-
lized twice from ethyl acetate giving 3 mg. m.p. 62-66°.

Fluorides in the Sponge Dysidea
Crawshayi de Laubenfels

Fluoride was determined colorimetrically using "Ferrisal"
(ferric salicylate) as the colorimetric agent. The prepara-
tion of the Ferrisal reagent and its calibration was carried
out by the procedure described by Devonshire (80). The,
transmission-concentration, calibration curve was linear only
in the range of 0.25 to 1.50 mg. of fluoride. The reagent
required constant restandardization.

The residue of D. crawshayi (collected from Walsingham
Pond, Bermuda during the summer of 1954), which had been
extracted with methanol, acetone, and chloroform, had an ash
of 29.36%.

Analysis of sponge residue. The sponge was dried to
constant weight and 2 g. samples were removed. The samples
were placed in a platinum crucible, mixed with dry sodium
carbonate, and covered with a layer of the carbonate. The
mixture was then fused over a blast burner. After cooling
the melt was cautiously dissolved in dilute sulfuric acid,
transferred to a 250 ml. Claisen flask, 25 ml. of concentrated
sulfuric acid added, and the mixture steam distilled at
135 ± 2° (81). About 200 ml. of distillate was collected
and diluted in a volumetric flask to 250 ml. Aliquots were

then removed and analyzed colorimetrically with the Ferrisal reagent.

Fluoride in sponge residue = 1.04% (Avg. of three determinations).

Analysis of alkali treated sponge residue. Five grams of sponge residue was treated with a hot solution of 12% sodium hydroxide and the mixture was shaken overnight. The supernatant liquid was decanted and the residue washed by decantation with water and alcohol-water. The residue was then filtered on a Buchner funnel, washed with water-alcohol, alcohol, ether, and dried on the filter. A tan colored residue, 0.935 g., which was quite rich in iron, was thus obtained. Samples of the residue (ca 200 mg.) were then transferred to a 250 ml. Claisen flask, 25 ml. of sulfuric acid was added, and the mixture steam distilled as before. Aliquots of the distillate, which was diluted to 250 ml., were then taken for fluoride analyses.

Fluoride in the alkali insoluble residue = 4.80% (Avg. of two determinations).

Analysis of alkali treated, nitric acid digested sponge residue. Five grams of alkali insoluble sponge residue was digested with concentrated nitric acid. The nitric acid insoluble residue was filtered, washed with water, ethanol, and ether and dried to constant weight (yield-146 mg.). When analyzed for fluorine the residue gave evidence for the presence of less than 0.2% fluoride. The residue could be dis-

solved only with hydrofluoric acid and therefore, was evidently silica. Microscopic examination of the material revealed the presence of rod-like, pointed, spicules.

CHAPTER III

DISCUSSION AND CONCLUSIONS

Sterols

Marthasterias glacialis. Previous investigators, employing extracts of either the whole animal or the pyloric caeca, have found that a complex mixture of sterols was present in the unsaponifiable lipid residues of starfish. Our study of the sterols M. glacialis, for which there are no reports in the literature, indicates not only that a complex mixture of sterols is also present in this species, but that the mixture is distributed throughout the different tissues (pyloric caeca, ovaries, male gonads, skin and tube foot area) of the organism.

With the exception of steryl acetate fraction T-II (derived from the tube foot lipids), which showed no optical activity, all fractions of sterols and their derivatives, which were obtained from the starfish, were dextrorotatory. All of the fractions, with the exception of the 14:15-stenyl acetate O-VI, gave positive Tortelli-Jaffe' reactions. Finally, spectrophotometric analyses of the sterols isolated from the pyloric caeca, ovaries, and skin indicate the absence

of $\triangle^{5,7}$-sterols. We can therefore conclude that 5:6-sterols
(i.e. cholesterol) are absent in the unsaponifiable lipids of
M. glacialis. This is in agreement with the results of other
studies of starfish sterols.

Hydrogenation of the steryl acetate fractions Pc-II (py-
loric caeca), O-IV (ovaries), M-IV (male gonads), and S-V
(skin) resulted in the absorption of hydrogen in quantities
greater than 0.5 mole but less than 1 mole. The hydrogenated
acetates yielded 8:14-stenyl acetates whose melting points and
specific rotations compare favorably with those reported for
8:14-stellastenyl acetate (m.p. 105-106°, $[\alpha]_D^{23}$= +12.5°) (44).
The 8:14-stenyl acetates which we have isolated have the fol-
lowing properties: Pc-XIII, m.p. 104-105°, $[\alpha]_D^{20}$= +12.2°; O-V,
m.p. 104-105°, $[\alpha]_D^{20}$= +13.0°; M-V, m.p. 103-105°, $[\alpha]_D^{20}$= +12.5°;
S-VI, m.p. 103-105°, $[\alpha]_D^{20}$= +12.4°. The 14:15-stenyl acetate,
O-VI, obtained from O-V by the action of dry hydrogen chloride,
had a m.p. 93-95°, and a specific rotation value of 19.6°.
Both the melting point and the specific rotation agree closely
with those reported for 14:15-stellastenyl acetate, m.p. 94-
96°, $[\alpha]_D^{20}$= +19.0° (44). Hitodesteryl acetate (α-spinasteryl
acetate), when hydrogenated under the same conditions which
we employed, would be expected to yield an 8:14-stenyl acetate
melting at 115°, with a specific rotation value of +16.0° (82).
The 8:14-stenyl acetates which we were able to recover after
the hydrogenations did not melt above 105°, and specific
rotation values were never greater than 13°.

The steryl acetate fractions of the starfish, which we have subjected to hydrogenation, appear to be mixtures very similar to those of stellasterol and stellastenol. From the amounts of hydrogen absorbed by the acetate fractions, it appears that they contained better than 50% stellasterol (the di-unsaturated sterol).

Small amounts of low melting sterols and steryl acetates, whose melting points and specific rotation values approach those of 7:8-cholestenol (m.p. 122-123°, $[\alpha]_D^{15}$= +4.0°; acetate m.p. 119-120°, $[\alpha]_D^{20}$= +5.1° (69), were isolated from the various tissues of M. glacialis. However, the amounts which were obtained were too small to permit any further investigation. If 7:8-cholestenol, or a mono-unsaturated sterol similar in properties, exists in the lipids of M. glacialis it is present only in minor amounts.

Tunicates. As we have pointed out earlier, the only previous investigation concerned with the sterol constituents of tunicates indicated that cholesterol, accompanied by small amounts of pro-vitamins D, was the dominant sterol present, and no other sterol was isolated (39). During the early stages of our investigations of Phallusia mamillata, Microcosmus sulcatus polymorphus, and Microcosmus sulcatus vulgaris, it became apparent, from the chemical and physical properties of the sterols and their derivatives, that other sterols were present in sufficient quantities to obscure the presence of cholesterol.

Cholesteryl acetate is known to form an insoluble dibromide which permits it to be separated from other sterols. During our first attempts to brominate the steryl acetates obtained from P. mamillata and M. polymorphus, bromine was added in a slight excess of the amount which would have been theoretically required if all of the acetate present was that of cholesterol. A green coloration was evident during the initial stages of bromination which could be accounted for by presence of $\Delta^{5,7}$-sterols. However, very little or no precipitate at all resulted when the solutions were allowed to stand in the refrigerator. In subsequent experiments, larger amounts of bromine were added and precipitates did form. The precipitates, however, had melting points which were considerably higher than that reported for cholesteryl acetate dibromide. It, therefore, appeared that most of the bromine was being consumed by steryl acetates whose bromides were even less soluble than that of cholesteryl acetate. Several di-unsaturated steryl acetates (containing one double bond in the side chain) which yield high melting, ether insoluble, acetate tetrabromides, are reported in the literature (14). The tetrabromides can be separated from the more soluble dibromides by triturition with ether. The insoluble bromides which we had isolated, were, therefore, triturated with ether and, after several recrystallizations of the ether insoluble material, we obtained a bromide, m.p. 190-192°(d), from both P. mamillata and M. polymorphus. The

bromides appeared to be very similar to the acetate tetra-
bromide of poriferasterol. The latter sterol has previously
been isolated from sponges (45), and molluscs (61, 83).

Debromination of the compound suspected to be pori-
ferasteryl acetate tetrabromide gave an acetate, m.p. 146-
147°, $[\alpha]_D^{20}$= -51.0°. The acetate did not give any melting
point depression with authentic poriferasteryl acetate, m.p.
147-148° (furnished by Dr. W. Bergmann). The specific rota-
tion value for poriferasteryl acetate is reported to be -53°.

Our evidence shows that we have isolated poriferasterol
from the tunicates, P. mamillata, and M. polymorphus. This
is the first time that the sterol has been found to occur in
protochordates.

Fractionation of the acetates recovered from the de-
bromination of the bromide mother liquors of P. mamillata
gave an acetate, m.p. 135-136°, $[\alpha]_D^{20}$= -42°. The acetate
when mixed with an authentic sample of clionasteryl acetate,
m.p. 137-138° (furnished by Dr. W. Bergmann), melted at 136-
137.5°. The reported specific rotation value for clionasteryl
acetate is -40°. Clionasterol has been found to accompany
poriferasterol in other marine organisms (45, 83).

The amount of clionasterol, which we were able to re-
cover, was very small, and we were unable to isolate a similar
fraction from M. polymorphus. The process of bromination
and debromination is accompanied by the decomposition of
large amounts of sterol and it is very likely that this

accounts for our failure to isolate clionasterol from M. polymorphus, and for the low yield obtained from P. mamillata.

Fractionation of the acetates recovered from the de-bromination of the bromide mother liquors of both P. mamillata and M. polymorphus led to the isolation of large amounts of acetates which were found to be identical with cholesteryl acetate. In addition, from the ether soluble bromide fractions, small amounts of bromides corresponding to cholesteryl acetate dibromide were isolated.

Repeated fractionation of the steryl acetate mixtures of M. polymorphus and P. mamillata, and the steryl benzoates of the latter tunicate, yielded fractions of steryl esters whose properties were identical with those of the corresponding derivatives of cholesterol. Attempts to isolate the derivatives of poriferasterol and clionasterol by the method of fractional crystallization were not successful with the small amounts of material which we had available.

A few milligrams of an ether soluble bromide, m.p. 156-157°, was also recovered from P. mamillata (see PM-XVI). The bromide might be a derivative of still another steryl acetate but due to the small amount obtained it could not be investigated any further.

Spectrophotometric analyses of the crude sterols and their acetates, isolated from the tunicates, revealed the presence of from 3 to 4% pro-vitamins D. Due to the samll concentrations and the instability of such compounds we could

not hope to identify them.

We must conclude that although cholesterol is present in large amounts in the unsaponifiable lipids of the tunicates, which we have studied, it is also accompanied by significant amounts of other sterols. Further investigations on the same, as well as on different, species of protochordates are, therefore, necessary before the complete story is known.

Protochordates are theoretically the evolutionary link between vertebrates and invertebrates. The presence of large amounts of cholesterol would relate them to the vertebrates, whereas, the presence of such sterols as poriferasterol and clionasterol would indicate that they are also capable of synthesizing sterols which are characteristic of lower invertebrate organisms.

We have mentioned earlier that in the Phylum Echinodermata there is a very distinct difference in relation to the sterols present in the Class Echinoidea (sea-urchins), on the one hand, and the Classes Holothuroidea (sea-cucumbers), and Asteroidea (starfish) on the other. In the species of the latter two classes, which have been investigated, the sterols which have been isolated have been shown to be devoid of cholesterol and any other Δ^5-sterols (which has been borne out by our investigation of M. glacialis). Whereas, the sea-urchins, which have been investigated, have been found to contain cholesterol as the dominant sterol.

All echinoderms have hypothetically arisen from a

common ancestral form the dipleurula larva. Embryoclogical evidence favors a close relationship between holothurians and asteroids on the one side, and echinoids (as well as ophiuroids) on the other. According to the embryological evidence the different classes of echinoderms have evolved from two different larval forms of which the dipleurula larva was the ancestral predecessor. One larval form (pluteus) gave rise to the echinoids and ophiuroids, and the other (auricularia) to the asteroids and holothurians. Fell (84) concludes, however, that it would be impossible to accept the results of embryological evidence which he feels would lead to "a reductio ad absurdum in the case of echinoderms." However, the biochemical evidence, which has accumulated from the comparative studies of sterols and phosphagens (85), lends support to the embryological evidence.

α-Glyceryl Ethers

In accord with previous investigations, we have found that large amounts of α-glyceryl ethers are present in the tissues of starfish and sea-cucumbers. Different organs of the animals have, however, been found to contain varying amounts of the ethers. The pyloric caeca and ovaries of M. glacialis had the highest concentrations; the male gonads and tube foot area contained significantly smaller amounts; and very little was present in the unsaponifiable lipids of the skin. In the sea-cucumber, Stichopus badionotus, there

was only 3.95% of α-glyceryl ether present in the sex-tree lipids, but about eight times this amount was found in the unsaponifiable lipids of the viscera.

It should be pointed out that the values given in Table 2 are significant only so far as the specimens which have been used in our studies are concerned. The α-glyceryl ether content of starfish, for example, has been found to vary with the size and age of the animals (43).

The amounts of α-glyceryl ethers present in the unsaponifiable lipids of the tunicates were much smaller than those of the echinoderms, and were found to vary over a range of 7-14% for the different species.

From the unsaponifiable lipid residue of the pyloric caeca of M. glacialis, we have isolated batyl alcohol via its urea adduct. Although the melting point of our product and its diphenylurethan derivative are slightly less than those reported for batyl alcohol, we are quite certain that it is composed mainly of batyl alcohol. Mixtures of batyl, chimyl, and octadecyl alcohols (the latter two are also very probably present in starfish unsaponifiable lipids) are extremely difficult to separate (41, 86).

The α-glyceryl ether isolated from the unsaponifiable lipids of P. mamillata, via the urea adduct, is very likely a mixture of a solid α-glyceryl ether (batyl, chimyl or both) and an alcohol, such as octadecyl alcohol. This is the first time that any α-glyceryl ether has been isolated from pro-

tochordates.

Fluorides of the Sponge Dysidea Crawshayi

The analyses which have been carried out on the residue of D. crawshayi indicate that the major portion of the fluoride compound(s) present in the sponge is of an inorganic nature. The fluorides are for the most part insoluble in alkali, but are decomposed by hot nitric acid.

The methods which we have used thus far are not suitable for the detection of organic fluorides and the possibility still exists that such compounds may indeed be present in the sponge. The latter problem requires further investigation which we hope can be carried out in the near future.

CHAPTER IV

SUMMARY

Live specimens of the starfish, Marthasterias glacialis
(L.), and the tunicates, Phallusia mamillata (Cuvier),
Microcosmus sulcatus polymorphus (Heller), and Microcosmus
sulcatus vulgaris (Heller) were collected from the Gulf of
Naples. The pyloric caeca, ovaries, male gonads, skin, and
tube foot area were dissected from the starfish specimens.
The tests of all of the tunicates were removed and the stom-
achs and intestines flushed with water. In the case of P.
mamillata, the branchial baskets were also removed. Lipid
extracts were prepared from the tunicates and from the var-
ious organs of the starfish. The lipid residues obtained
from the animals were saponified and the unsaponifiable lipid
fractions were investigated for their contents of sterols
and α-glyceryl ethers.

The unsaponifiable lipids derived from each of the
different organs of M. glacialis were found to contain com-
plex mixtures of sterols. The sterols present were pre-
dominantly Δ^7-stenols, and no evidence could be found for

the presence of $\triangle^5$- or $\triangle^{5,7}$-sterols. Hydrogenation studies of acetate fractions obtained from the pyloric caeca, ovaries, male gonads, and skin indicate that the major portion of the sterols present in these tissues are very similar to mixtures of stellasterol (a $\triangle^{7,22}$-sterol of the order C_{28}) and stellastenol (a $\triangle^7$-sterol of the same order). The melting points and specific rotation values of the $\triangle^{8:14}$-stenyl acetates which were recovered from the hydrogenations, as well as those of the $\triangle^{14:15}$-stenyl acetate obtained by the induced isomerization of one of the 8:14-stenyl acetate fractions by the action of dry hydrogen chloride, agree quite closely with the melting points and specific rotation values which have been reported for the corresponding derivatives of mixtures of stellasterol and stellastenol.

The sterols isolated from the tunicates, P. mamillata and M. polymorphus, have been shown to contain mixtures of cholesterol and other sterols. Spectrophotometric analyses indicated the presence of 3 to 4% pro-vitamins D. Cholesterol was identified via its acetate, acetate dibromide, and benzoate.

From the tunicates we have isolated poriferasterol (P. mamillata and M. polymorphus) as the ether insoluble acetate tetrabromide. The tetrabromide was debrominated and the acetate which was recovered did not give any melting point depression with authentic poriferasteryl acetate.

From P. mamillata we have also isolated an acetate

which appears to be identical with clionasteryl acetate.

This is the first time that poriferasterol and cliona-steryl have been shown to occur in protochordates. The sterols have previously been isolated from sponges and mol-luscs.

The unsaponifiable lipids of the starfish and tuni-cates mentioned above, as well as those of the pyloric caeca of the starfish, Oreaster reticulatus (L.) (Bimini) and the sex-trees, and viscera of the sea-cucumber, Stichopus badi-onotus Selenka (Bermuda), were analyzed for their content of α-glyceryl ethers.

Batyl alcohol has been isolated, via its urea adduct, from the pyloric caeca unsaponifiable lipids of M. glacialis. A small amount of a solid α-glyceryl ether, m.p. 62-66°, was obtained from the unsaponifiable lipids of the tunicate, P. mamillata. The material is probably composed of batyl alcohol and a small amount of chimyl, octadecyl and/or hexadecyl alcohol.

A preliminary study has been made of the nature of fluorides in the sponge, Dysidea crawshayi de Laubenfels (Walsingham Pond, Bermuda). The major portion of the sponge fluoride is inorganic and constitutes 1.04% of the dry sponge residue. It is insoluble in alkali, but is decomposed by hot nitric acid. The possible presence of organic fluoride compounds must await further investigations.

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