STEROL BIOSYNTHESIS AND METABOLISM IN MARINE INVERTEBRATES

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Abstract - The present state of knowledge on the ability of marine
invertebrate animals to synthesise and metabolise sterols is reviewed.
While animals from some phyla have a limited ability to synthesise sterols
de novo other groups of invertebrates are apparently incapable of sterol
synthesis. It appears likely that much of the sterol of invertebrates
originates from algae either in the plankton or living as symbionts with
particular animals. The methods which can be employed to determine the
capacity of an invertebrate to synthesise its own sterol de novo are
critically discussed and possible problems considered. The known inter-
conversions of dietary sterols by marine invertebrates are described
and recent aspects of steroid hormone interconversions are briefly
outlined.

INTRODUCTION

After the early pioneering efforts of Bergman and his colleagues (Ref. 1) it was recognised
that marine animals and plants contain rich and diverse mixtures of sterols which present
challenging problems to tax the imaginations of natural products chemists and biochemists.
Since the resurgence of interest in marine sterols in the late 1960's there has been an
unabated flow of publications documenting the sterol compositions of species belonging to
most of the marine invertebrate phyla. The continuing refinement of analytical techniques,
particularly gas chromatography - mass spectrometry, NMR spectroscopy and, more recently,
high performance liquid chromatography, has permitted the isolation and structural elucid-
ation of many minor sterols. Some of these are helping to fill "biosynthetic gaps" but
others are presenting an almost bewildering array of side chain alkylation patterns, short-
ened side chains or modified ring systems. All but the most recent observations on marine
sterol structure and distribution have been described in recent reviews (Ref. 2-6). By
contrast, studies on the biosynthesis and metabolism of sterols by marine invertebrates have
not been so extensive with most attention being devoted to demonstrating the ability, or
otherwise, of animals to produce sterols de novo from simple precursors such as acetate or
mevalonate. However, some studies have also been initiated to investigate the metabolism of
dietary sterols and to establish steroid hormone interconversions which are essential
preludes to obtaining a full understanding of the role of sterols and steroids at the
structural or physiological level in marine invertebrate organisms. With the limited inform-
ation so far available there are clearly more unresolved questions, and possibly even
apparent contradictions, than there are firm answers. It is not the intention of this
article to present a detailed review of the literature on this topic but rather to outline
the present status of knowledge on marine sterol metabolism with an indication of some of
the challenging problems to be resolved and discuss some of the difficulties which may need
to be considered in such studies.

THE ABILITY OF MARINE INVERTEBRATES TO BIOSYNTHESISE THEIR OWN STEROLS

Although some animals such as crustaceans and cephalopod molluscs contain cholesterol (1)
as their predominant sterol (often more than 95% of the mixture) the animals from most other
phyla contain very complex mixtures with often twenty or more different sterols and in some
animals, such as many bivalve molluscs or sponges, a sterol other than cholesterol may be the
major constituent. The sterol composition of an invertebrate animal must result from the
balance established between several contributory sources of sterol. Factors to consider
are: (a) the spectrum of sterols encountered in the diet of the animal and the selectivity
which the animal displays for the absorption, or excretion, of any particular compound in
the mixture; (b) the assimilation by a host animal of sterols produced by symbiotic algae or
other associated organisms such as bacteria or fungi in the digestive tract; (c) the
capacity of an invertebrate to modify any absorbed dietary sterol; (d) the contribution, if
any, from de novo biosynthesis of sterol from simple precursors such as acetyl-CoA and
mevalonic acid. An assessment of the full significance of these various contributions to
the overall sterol pattern of any particular animal ideally calls for a collaboration between

natural products chemists providing analytical and biosynthetic data and marine biologists
to provide information on the animals feeding habits, its relationship to other organisms,
particularly microorganisms, in its environment and to isolate and culture symbionts or
other flora and fauna associated with the animals for metabolic studies.
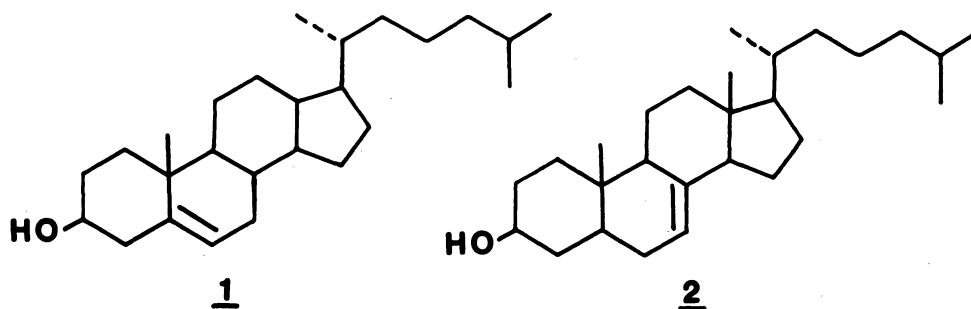
Many investigations have been conducted to determine the capacity of representative species
of all the major marine invertebrate phyla to biosynthesise sterol. Such studies have
employed $[^{14}C]$-labelled acetate, mevalonate or occasionally methionine injected into the
animal followed by incubations ranging from a few hours to several days before isolation
of sterols for radioassay (Table 1).

TABLE 1. Summary of the evidence for, or against, de novo sterol
           biosynthesis in marine invertebrate animals. The figures
           given indicate the reported results obtained from the
           incubation of marine animals with radioactive acetate or
           mevalonate. The references to the original publications
           will be found in reviews 3 and 7.

Phylum	Class	Sterol biosynthesis Positive	Negative
Porifera		6	5
Cnidaria	Anthozoa	5	7
	Scyphozoa	-	1
Nemertini		2	-
Annelida	Polychaeta	7	-
Arthropoda	Crustacea	-	16
Mollusca	Amphineura	3	-
	Gastropoda	27	2
	Bivalvia	6	11
	Cephalopoda	2	4
	Scaphopoda	-	1
Echinodermata	Crinoidea	1	-
	Asteroidea	8	-
	Holothuroidea	7	1
	Echinoidea	7	1
	Ophiuroidea	3	-
Urochordata		4	-

It is tempting, if not scientifically sound, to hope that all species within a particular
Class (or even phylum) may display roughly the same biochemical capabilities. With regard
to the ability to synthesise sterols such a generalisation does seem warranted to some
groups of animals. In the case of the crustaceans there is little doubt that these animals
are incapable of de novo sterol synthesis and in this respect they resemble the other major
class of the Arthropoda, the insects. The sterol requirements of these animals must there-
fore be met by an adequate supply of dietary sterols (Ref. 8). It also seems reasonable to
conclude that all five classes of echinoderm have the ability to synthesise sterols as do
worms (Nemertini and Annelida) and urochordates although in these cases more studies are
desirable to substantiate this view. The echinoderm classes show an interesting difference
in the identity of the final products of sterol synthesis. The crinoids, echinoids and
opiuroids contain predominantly Δ^5-sterols and cholesterol (1) is the sterol synthesised
(Ref. 3, 9-11). By contrast in the free sterols of asteroids and holothuroids Δ^7-sterols are

major constituents and in these aniamls cholest-7-en-3β-ol (2) is the product of sterol synthesis (Ref. 3, 9, 12-14).

The evidence for sterol synthesis in the sponges (Porifera) and coelenterates (Cnidaria) is ambivalent (Ref. 3, 7, 15). With the molluscs extensive investigations, particularly by Voogt and his colleagues, indicate that gastropods and chitons (Amphineura) probably have the capacity of limited sterol synthesis but the situation is again uncertain for the bivalves and cephalopods (Ref. 3, 7, 16). One reason for caution regarding the assignment of sterol synthesising capabilities to these groups of animals is the fact that in many cases the reported incorporation of precursor into sterol was low and individual labelled sterols have not often been isolated, purified and identified.

There may be a number of factors which can result in a low or inconclusive incorporation of a precursor into sterol. Both the physiological condition and basic metabolism of the animal under study and also the design of the experimental procedure are important consider- ations. The best means of administering a water soluble substrate (e.g. acetate mevalonate or methionine) to a marine animal presents an immediate problem. To maintain healthy animals for more than a few hours usually requires keeping the animal in a relatively large volume of preferably circulating and aerated sea water. Thus,if the radio- chemical is simply added to the water it will be excessively diluted and possibly exposed to chemical decomposition and only a very small proportion may be absorbed by the animal. Alternatively if the substrate is injected into the body mass of the animal it does not necessarily ensure a high concentration that will reach those tissues most active in sterol biosynthesis. Some may be used by other tissues for competing metabolic pathways and a large proportion may well pass out of the animal either passively, particularly with species such as sponges and coelenterates, or as the result of active excretory processes with more advanced animals. Such situations are obviously undesirable, not only because of the low and inconclusive incorporations into sterol which may ensue, but also because the high cost of radiochemicals and the possible radiochemical hazards mitigate against the use of very large amounts of labelled compounds to overcome these problems.

A further complication of whole animal incubations lies in the fact that it is very difficult, if not impossible at present, to obtain and maintain marine invertebrates which are free of other organisms, particularly microorganisms (algae, bacteria and fungi). Low incorporations, especially from incubations of long duration, are open to speculation that microorganisms are at least in part responsible. One possible solution to all these difficulties is the use of tissue slices or minces. Contamination with microorganisms may still be a problem, particularly with digestive tissues, but this can perhaps be minimised by short incubation times and use of suitable antibiotics to reduce any metabolic contri- butions made by a relatively small microorganism population. Indeed, isolated tissue preparations have been used successfully for investigations on marine invertebrate steroid hormone interconversions (Ref. 3) and also for studies on sterol biosynthesis in starfish (Ref. 13, 17).

The physiological condition of animals used for biosynthetic studies is a fact which requires more attention since age, sex, stage of sexual maturity and dietary status of the animal may all have a role in determining the animals sterol synthetic requirements. The length of time and conditions under which animals are maintained, both before and during incubations, could be significant since variations in sterol content of annelids have been reported which were dependent upon time and temperature (Ref. 18). Feeding could also be an important factor to consider since it is well established that fasting in mammals causes a marked reduction in the rate of sterol synthesis (Ref. 19) although of course, findings for mammals may not apply to invertebrates. The possible importance of seasonal fluctuations in the rate of sterol synthesis have been revealed by incorporation studies with a brittle star (Ophiocomina nigra) and an urchin (Psammechinus miliaris). With both these animals $[2-^{14}C]$- mevalonate incorporation during the late summer, autumn and winter was high into squalene and low into 4-demethylsterols. However, in the spring and early summer the situation was reversed with higher incorporation into 4-demethylsterols and lower labelling of squalene (Ref. 3, 9). This change may be related to gonad development and an increased requirement for membrane production and warrents a more detailed investigation with these echinoderms and with species from other phyla. Some marine invertebrates, such as bivalve molluscs, can survive anoxia for protracted periods and have developed efficient anaerobic metabolic pathways (Ref. 20). This may well explain the difficulty in demonstrating sterol synthesis in such animals since sterol biosynthesis has a requirement for molecular oxygen for squalene cyclisation, C-4 demethylation and Δ^5-bond introduction.

The choice of acetate or mevalonate can have an important bearing upon the results obtained and their interpretation for several reasons. $[^{14}C]$-Acetate first requires conversion to acetyl-CoA by a thiokinase which may be a rate limiting enzyme. The acetyl CoA will then be diluted by mixing with the endogenous pool of acetyl-CoA derived from pyruvate and fatty

acid metabolism. The result of dilution, coupled with rapid utilisation of acetyl-CoA for other metabolic purposes such as fatty acid biosynthesis and the citric acid cycle, may well minimise the incorporation into sterol to such an extent that it may be interpreted as a failure of the animal to synthesise sterol. This situation would be aggrevated in animals with a slow rate of sterol synthesis and most of the administered [^{14}C]-acetate may be rapidly depleted by competing pathways so rendering prolonged incubations of dubious value unless the [^{14}C]-acetate is replenished periodically. The possibility that the thiokinase may be limiting the utilisation of [^{14}C]-acetate has been monitored in some incubations by measuring incorporation into fatty acids or other polyisoprenoids such as ubiquinone (Ref. 7) and it does not appear to present a serious problem.

When using [^{14}C]-acetate a possible constraint on the incorporation of label into sterol may be imposed by the enzyme hydroxymethylglutaryl CoA (HMG-CoA) reductase which is responsible for mevalonic acid production. In mammals this enzyme is the principle regulatory enzyme in cholesterol biosynthesis and its activity shows a diurnal variation and can be greatly lowered by such conditions as fasting or feeding a sterol rich diet (Ref. 19). If HMG-CoA reductase were to play a similar regulatory role in marine animals it could well explain the difficulties in some instances of detecting sterol synthesis using [^{14}C]-acetate whereas [^{14}C]-mevalonate has given positive results (Ref. 3, 7, 21). Indeed, if it is only desired to demonstrate the capacity of an animal to synthesise sterol, rather than determine the actual rate of sterol synthesis, then [^{14}C]-mevalonate is the precursor of choice because its use will not only bye-pass the possible regulation at the HMG-CoA reductase step but it should also experience less dilution with any endogenous mevalonate pool as this should presumably be small in the presence of a tightly regulated HMG-CoA reductase step. While there may be secondary regulatory points after mevalonic acid in the biosynthetic sequence this is not necessarily a problem. In fact, it can be advantageous since the accumulation of identifiable labelled precursors, such as squalene and lanosterol which have been found in echinoderms incubated with mevalonate (Ref. 10, 13), gives confidence that the animal can synthesise sterols via established precursors and permits the preferred route to be investigated. In this respect it is notable that in the majority of reports on marine sterol synthesis there has been no attempt to identify radioactively labelled intermediates other than squalene.

The cyclisation product of squalene-2,3-oxide is known to be cycloartenol in photosynthetic organisms and lanosterol in animals and fungi (Ref. 22). Thus, identification of any labelled 4,4-dimethylsterols produced during an incubation as one or other of these compounds could help decide if incorporation of label is due to symbiotic algae. However, the identification of labelled lanosterol does not rule out a fungal origin and also it still remains for the squalene-2,3-oxide cyclisation product in diatoms and dinoflagellates to be established. It is also notable that although reports on the identification of 4-demethyl sterols occurring in only trace amounts continue to be published little is known of the 4,4-dimethyl and 4α-methyl sterols present in most classes of invertebrate. The potential value of identifying these latter sterols and demonstrating their labelling from [^{14}C]-mevalonate is illustrated by the studies on the starfish Asterias rubens which have permitted the establishment of the biosynthetic sequence shown in Fig. 1 (Ref. 13, 23).

Many of the studies on invertebrate sterol synthesis have been restricted to recording the incorporation of [^{14}C]-acetate or mevalonate into the total sterol recovered after chromatography, sometimes after digitonin precipitation, and often after several recrystallizations. However, as most of the animals investigated contain complex mixtures of C_{26}, C_{27}, C_{28}, C_{29} and C_{30}-sterols this method of study gives no information concerning which sterols are labelled and potentially valuable information may be lost. Indeed, erroneous conclusions may be made since the process of fractional crystallization can easily result in the loss of a labelled sterol with retention of unlabelled sterols of dietary origin as was reported in the case of the crystallizations of Asterias rubens sterols synthesised in the presence of [2-^{14}C]-mevalonic acid (Ref. 3, 13).

If it is accepted that the production of C_{28} and C_{29}-sterols with a C-24 alkyl group is the prerogative of algae then label in such sterols would be an indication of an involvement of symbiotic algae or other algae in the aquarium. However, label in C_{27}-sterol can not be taken as prima facie evidence that the sterol has been produced by the animal for two reasons. Firstly, C_{27}-sterols (cholesterol and desmosterol) are produced by some algae such as members of the Rhodophycea (Ref. 3) and more significantly by some dinoflagellates which are the class to which many zooxanthellae belong (Ref. 24-26). Secondly, C_{28} and C_{29}-sterols
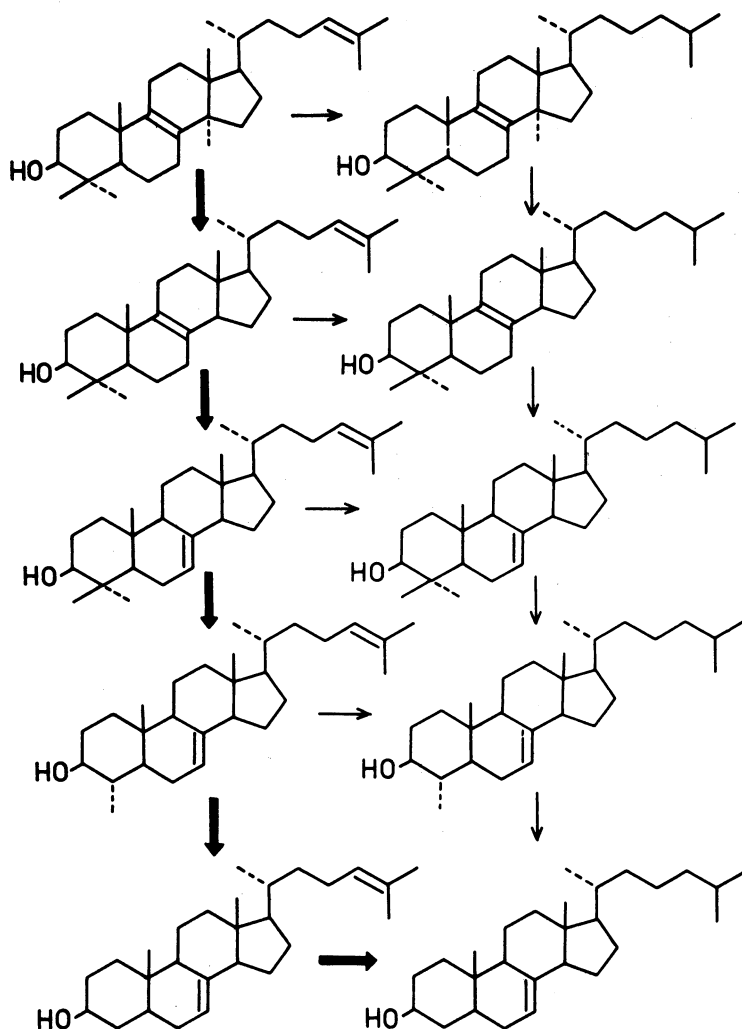
Fig. 1. Minor 4,4-dimethyl and 4α-methyl sterols identified in the star-
fish *Asterias rubens* and the probable routes for the biosynthesis of the
major sterol cholest-7-en-3β-ol.

produced by a symbiotic alga may, after assimilation by the animal host, be subjected to
C-24 dealkylation to produce a labelled C_{27}-sterol. Pertinent to the latter consideration
are the reports of dealkylation of C_{29}-sterols by an anemone and four gastropod molluscs
(Ref. 27-30). By contrast there are also reports implying that some bivalve molluscs and a
sea cucumber are unique among animals and capable of the transmethylation reaction to produce
C-24 alkyl sterols such as 24-methylenecholesterol (Ref. 31-34). The labelled C-24
alkylated sterols isolated in these studies may concievably have been produced by associated
algae or fungi and therefore it is very important that further experiments be conducted to
unambiguously establish the ability of these animals to produce C_{28} and C_{29}-sterols. It
would certainly be an extraordinary situation if animals of the same phylum (Mollusca) have
evolved the enzyme systems required for both C-24 alkylation and C-24 dealkylation
reactions.

THE ORIGIN AND METABOLISM OF DIETARY STEROLS

For the purpose of this discussion dietary sterols will be considered to include not only
sterol obtained from ingested food but also any assimilated from symbiotic algae and from
organisms present in the digestive tract of the animal.

The sponges contain the most diverse array of sterols found in any group of animals and many
of them are unique to this phylum. On balance, the present evidence does not favour de novo
biosynthesis by the sponge as the origin of these sterols, but reveals that the ring modified

sterols result from the transformation of exogenous sterols. Minale and his colleagues have shown that radioactive cholesterol (<u>1</u>) added as an ethanol solution to the aquarium water can be absorbed by the sponges and metabolised to produce either the A-nor stanol (<u>3</u>) by <u>Axinella verrucosa</u> or the 19-norstanol (<u>4</u>) by <u>A. polypoides</u> (Ref. 15, 35, 36). Some evidence for the mechanisms of production of these sterols has been obtained using dual labelled species of sterol. Feeding a mixture of $[4-^{14}C]$-cholesterol and $[7\alpha-^{3}H]$-cholestanol to <u>A. polypoides</u> resulted in the incorporation of ^{14}C only into <u>4</u> showing that a Δ^5-bond is an essential requirement for the reaction. It has also been shown that the mechanism results in partial loss of the C-3α hydrogen and retention of the C-4β and C-7 hydrogens   (Ref. 15, 37). Similarly a sterol with a Δ^5-bond is preferentially used by <u>A. verrucosa</u> for production of <u>3</u> but in this conversion the C-3α and C-4β hydrogens are completely lost (Ref. 36, 38) as they were in cholest-4-en-3-one (<u>5</u>) which was also isolated as a cholesterol metabolite from this sponge. 5α-Stanols (<u>6</u>) are found in considerable amounts in several sponges (Ref. 2, 3, 39-42) and the identification of <u>5</u> in some of these species (Ref. 40) and as a metabolite of cholesterol (Ref. 38) provides very strong evidence that they are produced from Δ^5-sterols by the oxidation-reduction sequence <u>1</u> ⟶ <u>5</u> ⟶ <u>6</u> which is well established in mammals and also in starfish (Ref. 3, 43). The prime question to be answered concerning the production of stanols <u>3</u>, <u>4</u> and <u>6</u> remains are they produced by the sponge itself or by the bacteria which may accumulate in large quantities in some sponges ? Certainly bacteria can reduce Δ^5-sterols to 5α-stanols and it is relevant that this conversion has recently been demonstrated by the microorganisms present in estuary sediments (Ref. 44).
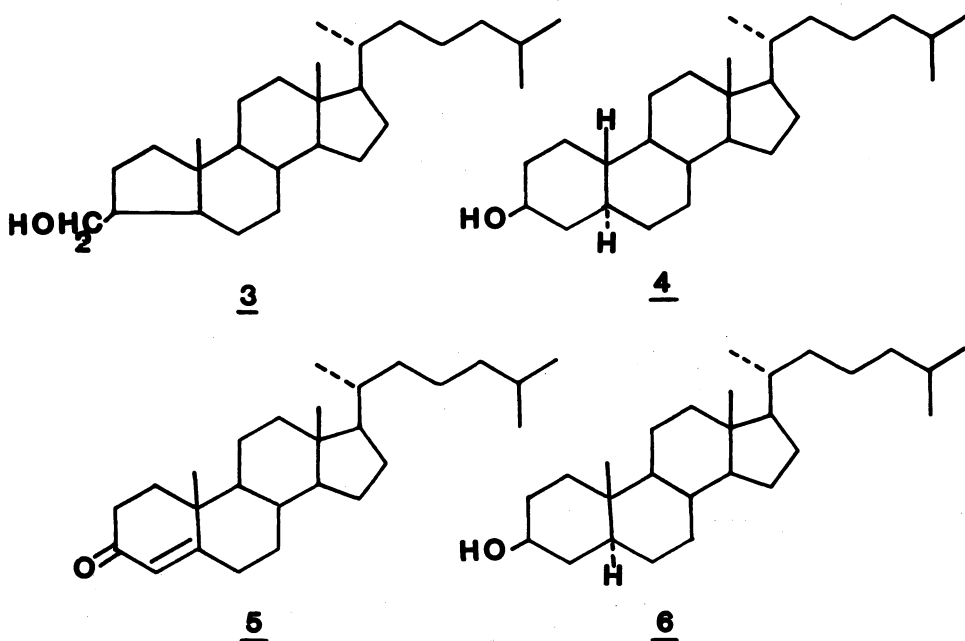
Some sponges contain appreciable quantities of $\Delta^{5,7}$-sterols (Ref. 3, 41) but there have been no investigations regarding their origin. They may conceivably be dietary since both fungi and some unicellular algae contain $\Delta^{5,7}$-sterols but the possibility should also be considered that the sponge can produce them from dietary Δ^5- or Δ^7-sterol which is an ability displayed by some unicellular protozoa such as <u>Tetrahymena pyriformis</u> (Ref. 22).

Sponges have now been analysed which present the fascinating array of modified sterol side chains shown in Fig. 2 (Ref. 2, 3, 15, 45-52). Nothing can be said at present regarding the origin of these unusual sterols and it remains an open question whether they are produced by algae and consumed by the sponges or are the result of modification of simpler sterols by further transmethylation reactions mediated by the sponge itself. An attempt to incorporate $[^{14}C]$-methionine into aplysterol by <u>Verongia aerophoba</u> was not successful (Ref. 15). Also, the incorporation of methionine into calysterol could not be shown but 2.7% conversion of tritiated fucosterol into calysterol was observed with the sponge <u>Calyx nicaenis</u> (Ref. 15). If these sterols are dietary the fact that some of these unique sterols represent the sole or major sterol in some species demonstrates a remarkable degree

of selectivity on the part of the sponge either for   particular dietary (or symbiotic)
algae or in their ability to absorb particular sterols from a mixture. Biosynthetic studies
on these sterols awaits the discovery of the prime source of these compounds but it is clear
that they can all be produced by an extension of the various transmethylation reactions
already established to occur in higher plants, algae and fungi (Ref. 53) and they should be
amenable to the experimental approach already adopted in the latter studies to investigate
the mechanisms and stereochemistry involved.

aplysterol   dehydroaplysterol   pterosterol

xestosterol   stelliferasterol   isostelliferasterol

strongylosterol   verongulasterol   calysterol

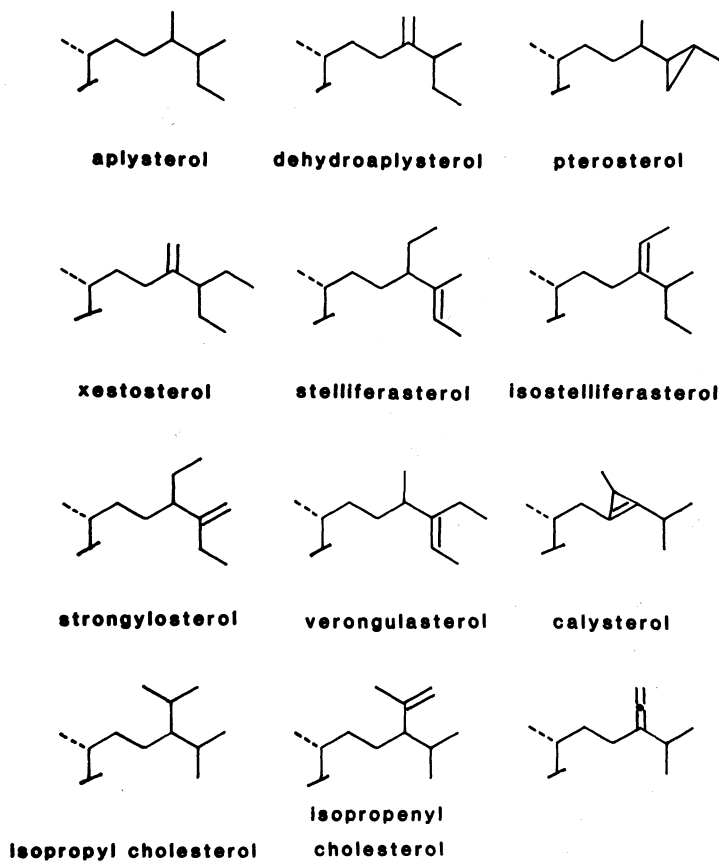isopropyl cholesterol   isopropenyl
cholesterol

Fig. 2. Some of the unusual alkylated sterol side chains found in sponges.

Recently several sterols containing the shortened side chains shown in Fig. 3 have been
detected in sponges and coelenterates (Ref. 54, 55) and sterols with the C_{21}-pregnane
skeleton seem most prevalent (Ref. 56-58). There is no experimental evidence indicating how
these sterols are produced but Carlson et al. (Ref. 55) have presented a very detailed argu-
ment that they may be produced by in vivo autoxidation processes from conventional
unsaturated side chain sterols in a manner analogous to the production of hydrocarbons in
other organisms (Ref. 59-61). Carlson et al. (Ref. 55) suggest that such reactions may be
particularly favoured in animals such as coelenterates which have large numbers of.
symbiotic zooxanthellae. These algae contain chlorophyll that could act as the photo-
sensitiser needed for the production of the singlet oxygen required for sterol side chain
hydroperoxide production prior to breakdown to give the short side chain sterols. Although
this is an attractive and plausible mechanism to account for the presence of these sterols
in marine animals an alternative route can be proposed for their elaboration.

The C_{21}-pregnane skeleton is readily generated from cholesterol by many organisms in the
production of the steroid hormones pregnenolone and progesterone. This reaction requires
hydroxylation at C-20 and it is notable that C-20 hydroxylated steroids with the cholestane
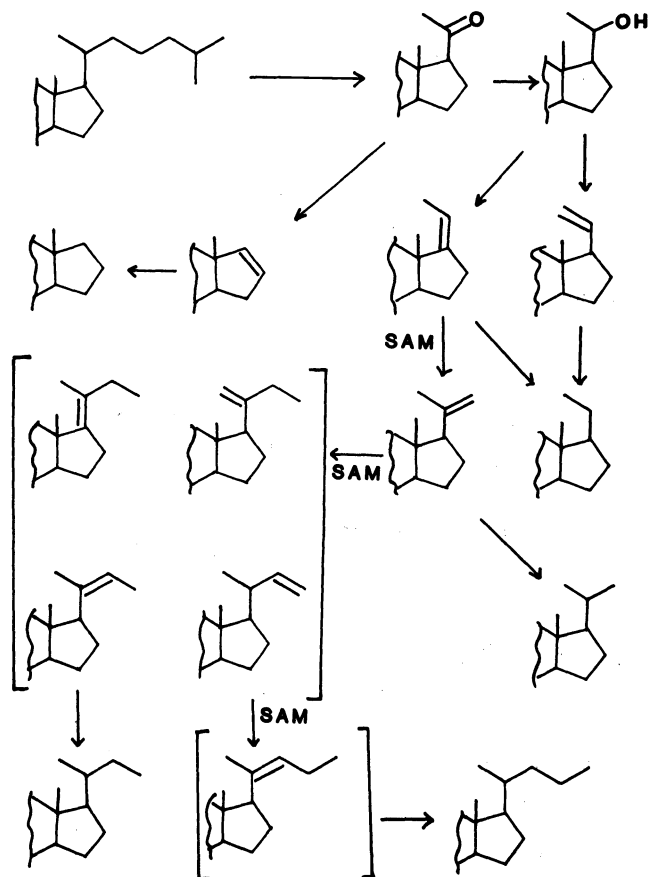
Fig. 3. The structures of the short side chain sterols identified in some
marine invertebrates and a hypothetical scheme for their biosynthesis by
successive transmethylation reactions from S-adenosylmethionine (SAM).
The compounds in square brackets have not yet been identified and are
postulated intermediates if this reaction sequence operates.

and lanostane skeleton are produced in marine animals, e.g. the ecdysones in crustaceans,
the asterosaponins in starfish and the holothurins in sea-cucumbers (Ref. 3). Moreover,
progesterone has been reported in a few marine invertebrates and, most significantly for the
present discussion, 3β-hydroxypregn-5-en-20-one (7) and $3\beta,20\alpha$- and $3\beta,20\beta$-dihydroxypregn-
5-ene (8 and 9) have been identified in the sponge Haliclona rubens (Ref. 62). Abstraction
of water from such a 20-hydroxy-steroid could generate a 3β-hydroxy C_{21}-sterol with an

olefinic 2-carbon side chain which may then be a suitable substrate for a transmethylation
reaction from S-adenosylmethionine (SAM) in a manner similar to the side chain alkylation
of phytosterols at C-24. As shown in Fig. 3 successive transmethylations and double bond
reductions would then build up the spectrum of short side chain sterols found in marine
animals. Indeed, if continued for a sufficient number of transmethylation steps such mech-
anisms could produce the C_{26}-sterol side chain and even restore the iso-octyl side chain of

cholesterol! If such a biosynthetic route is operative it predicts the production of some
unsaturated short side chain sterols which have not yet been found and it will be interest-
ing to see if these compounds can be detected in marine organisms.

7

8 20α

9 20β
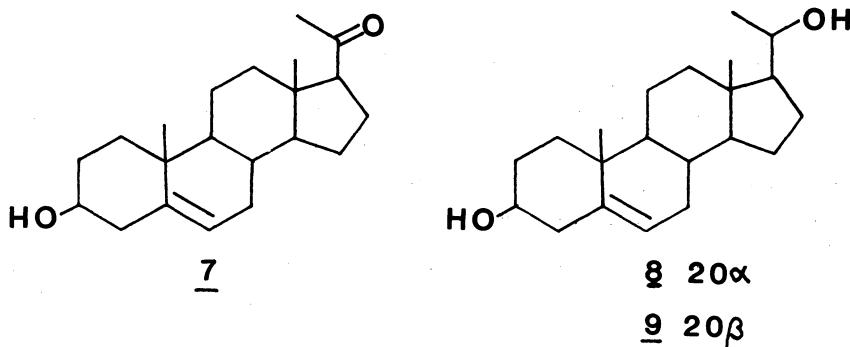
Regarding the mechanism of production of androsta-5,16-dien-3β-ol which is found at a very low concentration in sponges (Ref. 54,55) it is worth noting that this steroid is also produced in boar testis by a microsomal enzyme system requiring NADPH and oxygen for activity. 3β-Hydroxypregn-5-en-20-one is the substrate but 17α-hydroxylation does not appear to be involved in the reaction which thus differs from the C-17,20 lyase catalysed reaction involved in 3β-hydroxyandrost-5-en-17-one production in mammalian endocrine tissues (Ref. 63). Also evidence has been presented for the direct production of a C_{22}-sterol from acetate in mammalian tissue (Ref. 64).

While it seems likely that coelenterates can not synthesise their own sterols many of them contain cholesterol as the predominant sterol. This may be a reflection on the sterol content of the diet and in this respect the planktonic species are rich in cholesterol (Ref. 65,66). The preponderance of cholesterol could also result from a preferential absorption of C_{27}-sterols from more complex mixtures of dietary sterols while dealkylation of C_{29}-sterols has also been reported in one coelenterate species (Ref. 27). With those coelenterates which contain large quantities of unusual sterols such as gorgosterol the evidence is now pointing towards an origin in the dinoflagellate zooxanthellae since isolated cultures of these organisms and related free living forms of dinoflagellates contain 22,23-cyclopropane sterols related to gorgosterol or other sterols which can clearly be related as potential precursors (Ref. 67-69). However, the ability of the coelenterate host to modify these sterols either by further transmethylation reactions or ring structure modifications presents an interesting aspect to attract future studies. Also, there appear to have been no investigations on the biosynthesis of the polyhydroxylated sterols found in many coelenterates (Ref. 2, 3, 6).

Crustaceans must derive all their sterol from the diet since they are incapable of sterol biosynthesis. The preponderance of cholesterol (∼95% in most species) again suggests selectivity in sterol absorption but these animals are also clearly established to have the ability to dealkylate sterols such as ergosterol and sitosterol (Ref. 70). The mechanism of dealkylation in crustaceans remains to be established but it is likely to be similar to the pathway in the closely related Insecta. Certainly this route would explain the occurrance of desmosterol (cholesta-5,24-dien-3β-ol) in many crustaceans (Ref. 3) since this compound is produced as an intermediate in phytosterol dealkylation in insects. The reduction of desmosterol to produce cholesterol has also been reported in the crab Sesarma dehaani (Ref. 71).

Reports on the ability of gastropod molluscs to dealkylate C-24 phytosterol and of a few bivalve molluscs to apparently carry out the transmethylation reaction to produce C_{28}-sterols have been referred to above. The quantitative importance of these transformations needs to be established but they raise two intriguing questions regarding mollusc sterol balance. Firstly, does the ability of gastropod molluscs to dealkylate phytosterols produce adequate cholesterol for membrane requirements and therefore limit sterol biosynthesis to the very low, but apparently positive levels of synthesis reported? Secondly, since bivalves may well be unable to synthesise sterol why should they deplete the amount of available cholesterol further by C-24 alkylation of part of the dietary intake of C_{27}-sterol ?

A further point about mollusc sterols which awaits elucidation is the origin and significance of sterol-5,7-dienes which occur in appreciable quantities (10-20%) in the sterol mixture of many molluscs (Ref. 3). In gastropods the main component in this fraction seems to be cholesta-5,7-dien-3β-ol (10) and its presence could be adequately explained by de novo synthesis (Ref. 72, 73). By comparison, in several bivalves the major $\Delta^{5,7}$-sterol constituents have been identified as ergosterol (11) and ergosta-5,7,24(28)-trien-3β-ol (12) which suggests a dietary source from fungi or perhaps algae (Ref. 73). However, the presence in these bivalves of 24-nor-cholesta-5,7,22-trien-3β-ol (13), which has not previously been reported as a naturally occuring sterol, suggests that this and other $\Delta^{5,7}$-sterols, may be produced in the molluscs by conversion of the corresponding dietary Δ^5-sterol. In this connection the conversion of Δ^5-sterol to $\Delta^{5,7}$-sterol is already known to occur in some protozoa and insects (Ref. 22). The bioconversion of cholesterol to cholest-7-en-3β-ol by a chiton was reported and establishes the ability, at least of molluscs of the class Amphinura, to introduce a Δ^7-bond into the sterol nucleus (Ref. 74). Also, some incorporation of [4-^{14}C] sitosterol into the $\Delta^{5,7}$-sterol fraction of two molluscs was described by Teshima et al. (Ref. 30). Production of $\Delta^{5,7}$-sterol from dietary Δ^7-sterol also remains a possible route for which circumstantial evidence is provided by the demonstration of mainly Δ^5-sterols in the triton Charionia tritonis which feeds on the starfish Acanthaster plancii (Ref. 75). The triton therefore might be expected to derive a large proportion of Δ^7-sterol in its diet, and this could be metabolised by the "conventional" sterol biosynthetic sequence $\Delta^7 \longrightarrow \Delta^{5,7} \longrightarrow \Delta^5$.
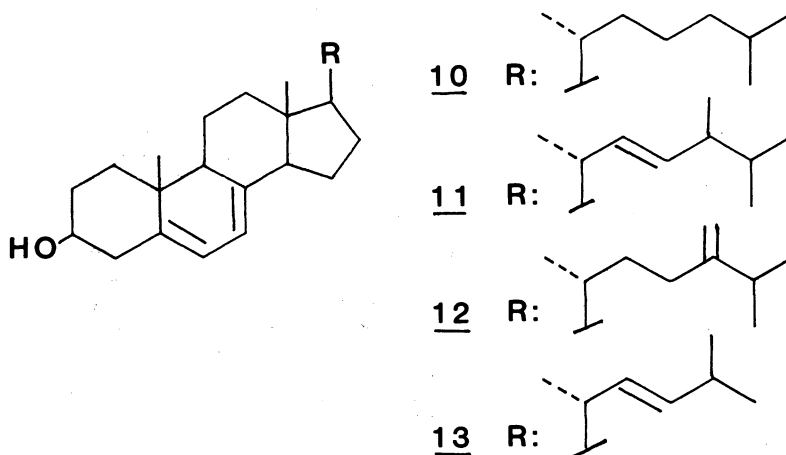
All five classes of echinoderm contain mixtures of C_{26} to C_{30}-sterols but they can only synthesise C_{27}-sterols and therefore the remainder are of dietary origin. So far no evidence has been presented for C-24 dealkylation in these animals (Ref. 10, 76). The expected preponderance of Δ^5-sterols in the diets of starfish and sea-cucumbers suggested that these animals were able to convert such compounds into their characteristic C_{26} to C_{30} Δ^7-sterols and this has been established experimentally (Ref. 3). It seems that two routes may be operating in starfish (Fig. 4). One pathway proceeds from the Δ^5-sterol through a steroid 4-en-3-one to the 5α-stanol and thence to the Δ^7-sterol and was elucidated by the conversion of [4-^{14}C]- and [3α-^{3}H, 4-^{14}C]-labelled sterols (Ref. 43, 77). This route adequately explains the presence of small amounts of 5α-stanols found in the free sterols of many starfish and also the occurrence of more substantial quantities in the sterol sulphates which are found in these animals (Ref. 78). The alternative route to Δ^7-sterol is believed to operate via production of a $\Delta^{5,7}$-sterol (Fig. 4) and was suggested by the results obtained from injections of [3α-^{3}H,4-^{14}C]-cholesterol into starfish. These experiments consistantly gave a very low ^{3}H:^{14}C ratio in the 5α-cholestanol as expected from the intermediacy of

cholest-4-en-3-one (Ref. 77) but the ^{3}H:^{14}C ratio of cholest-7-en-3β-ol was always substan-
ially higher and therefore was inconsistent with the 5α-cholestanol being the sole precursor
(Ref. 3, 79). Accordingly [3α-^{3}H,4-^{14}C]-cholesta-5,7-dien-3β-ol was synthesised and shown
to be converted by Asterias rubens into cholest-7-en-3β-ol without loss of tritium thus
providing evidence for the route $\Delta^5 \longrightarrow \Delta^{5,7} \longrightarrow \Delta^7$ but the conversion of cholesterol into
cholesta-5,7-dien-3β-ol remains to be demonstrated. Conversion of the $\Delta^{5,7}$-sterol to chole-
sterol could not be demonstrated (Ref. 79). The operation of this second route in starfish
also permits the $\Delta^{5,7}$-sterols obtained from molluscs in their diet to be utilised for Δ^7-
sterol production. In fact, it seems that metabolism of dietary $\Delta^{5,7}$-sterol by Asterias
rubens may be quite efficient as this animal contains only a trace of $\Delta^{5,7}$-sterol in its
sterol mixture (Ref. 73). A small amount of another sterol diene tentatively identified
as cholesta-7,9(11)-dien-3β-ol and produced as a metabolite of labelled cholesta-5,7-dien-
3β-ol, is present in A. rubens and it may perhaps be of relevance to asterosaponin product-
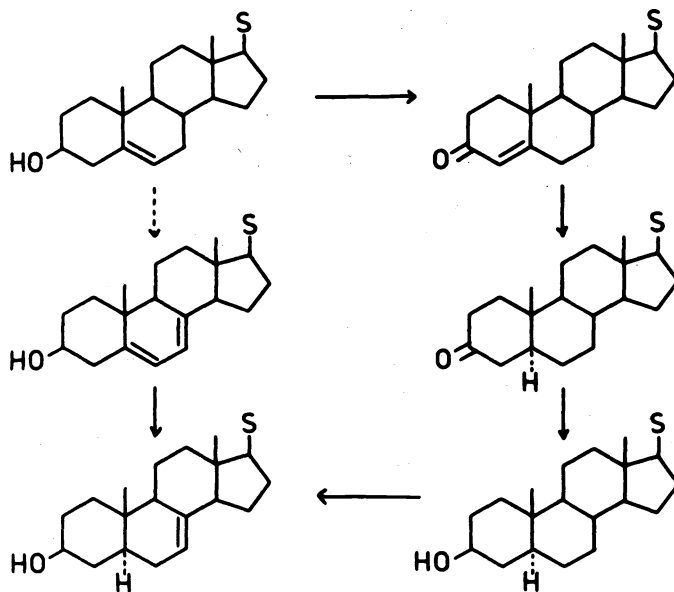ion (Ref. 73, 79).

Fig. 4. The two routes which appear to operate in starfish for the
conversion of dietary Δ^5-sterol into the Δ^7-sterols of these
animals.

Catabolism of [4-^{14}C]-cholesterol and reincorporation of the label into a wide range of
non-steroidal lipids by Asterias rubens has been reported (Ref. 76) but reinvestigation
seems warrented. Both steryl esters and sterol sulphates are appreciably labelled after
administration of [4-^{14}C]-cholesterol to A. rubens (Ref. 78, 80) and these have chromato-
graphic properties very similar to some of the lipid classes believed to be labelled in the
previous study (Ref. 76).

In summary, it seems likely that much of the sterol in the marine biomass may have originated
from the algal species present in the phytoplankton and as symbiotic partners with marine
animals and also from littoral and sub-littoral seaweeds. The way in which sterols can be
passed on through a group of organisms in a particular food-chain has been illustrated by
the analysis of coral reef populations (Ref. 81-83). Many preditor animals contain sterols
with a composition rather similar to the spectrum of sterols in the prey and tracing back
food chains may perhaps help to distinguish species from which sterols originate for use
in biosynthetic studies.

INTERCONVERSIONS OF STEROID HORMONES

The biochemistry and metabolism of steroid hormones in marine invertebrates described in the literature upto 1976-77 have been discussed elsewhere (Ref. 3, 84). Briefly, several C_{18}, C_{19} and C_{21}-steroid hormones have been identified, although in some cases rather tentatively. Also several ecdysones, the C_{27}-steroid hormones found in crustaceans, have been characterised. A range of enzyme activities (e.g. oxidase, reductase, 17α-hydroxylase, C-17,C-20 lyase) responsible for sterol interconversions have been demonstrated by incubation of radioactive steroids with isolated tissues (usually gonads or hepatopancreas) from animals of several phyla.

New reports have appeared describing the determination of progesterone and oestrone by radioimmunoassay in the ovaries and pyloric cacae of the starfish Asterias rubens (Ref. 85) and testosterone and oestradiol-17β by radioimmunoassay and GC-MS in the crustacean zooplankton Calanus sp. (Ref. 86). Studies on the biosynthesis of steroid hormones in molluscs have been extended to the cephalopod Sepia officinalis which has been shown by the use of suitable radioactively labelled steroids to contain in the gonads seven enzyme systems required for the metabolism of cholesterol to produce all the steroid intermediates through to testosterone (Ref. 87). The biosynthesis of oestrogens by the cuttlefish could not be demonstrated nor could oestrogens be detected in the plasma by radio-immunoassay although the presence of testosterone was indicated by this technique. Although oestrogens have been detected in several other marine animals (Ref. 3, 84, 86, 88) the biosynthesis of these steroids has never been demonstrated and their origin remains a perplexing problem.

Investigations have been conducted on the steroid hormone synthesising capacity of the starfish Asterias rubens and the possible correlation of steroid hormone production with the annual reproductive cycle discussed (Ref. 89-91). Homogenates of ovaries and pyloric caecae of females were reported to metabolise cholesterol to pregnenolone and progesterone while progesterone was converted into 17α-hydroxyprogesterone, androstenedione, testosterone, 20α-hydroxyprogesterone, 11-deoxycorticosterone and 5α-pregnan-3,20-dione. Androstenedione was reduced to testosterone and 5α-androstan-3,17-dione but again no evidence could be obtained for oestrogen biosynthesis. This is a wider range of metabolites than previously reported for cholesterol and progesterone metabolism by A. rubens (Ref. 92). In this study the metabolites from in vivo metabolism of cholesterol were identified as 5α-cholestanol, 5α-cholestane-3β,6α-diol (which may be pertinant to asterosaponin production), 3β-hydroxy-5α-pregnan-20-one and 5α-pregnane-3β,20ξ-diol. The various progesterone metabolites identified from progesterone metabolism in vivo with isolated gonads, or with gonad homogenates, were 5α-pregnane-3,20-dione, 3β-hydroxy-5α-pregnan-20-one, 5α-pregnane-3β,20ξ-diol and 3β,6α-dihydroxypregnan-20-one but androgens were not detected as metabolites. The incubation of [^{3}H]-androst-4-ene-3,17-dione with male and female gonads produced 5α-androstane-3,17-dione, 3β-hydroxy-5α-androstan-17-one, 5α-androstane-3β,17β-diol and testosterone as the main metabolites (Ref. 93). Incubation of labelled 17α-hydroxyprogesterone with A. rubens gonads yielded 17α-hydroxy-5α-pregnane-3,20-dione and 3β,17α-dihydroxy-5α-pregnan-20-one (Ref. 93). These latter studies all appear to show that in A. rubens the most active steroid transforming enzymes are the 5α- and 3-keto steroid reductases. However, in view of the seasonal variations in the activities of some of the steroid metabolising enzymes of A. rubens reported by Schoenmakers (Ref. 89, 90) the differences in metabolites obtained from these two sets of studies may perhaps be explained on this basis.

One striking difference in the metabolism of steroids by male and female gonads of A. rubens is the production in the male of appreciable amounts of fatty acyl esters of the 3β-hydroxy-5α-reduction products of progesterone, 17α-hydroxyprogesterone and androstenedione and of considerable amounts of the sulphate esters particularly in the female gonads (Ref. 92, 93). The production of similar steroid esters has not been reported in other marine animals and this would be worthy of study to see if it is a widespread phenomenon and to gain an insight to its possible physiological significance.

All the studies on marine sterol and steroid interconversions so far reported have utilised radioactively labelled compounds. It is well recognised that co-chromatography and co-crystallization of steroids can present potential problems to the unambiguous identification of labelled metabolites. The particular limitations of recrystallization for the positive identification of radioactive steroids has recently been emphasised (Ref. 94). An alternative to the use of radiochemicals is to utilise substrates labelled with deuterium followed by the identification of metabolites by gas chromatography - mass spectrometry (Ref. 95). The combined procedures of the initial TLC purification of the compound, coupled
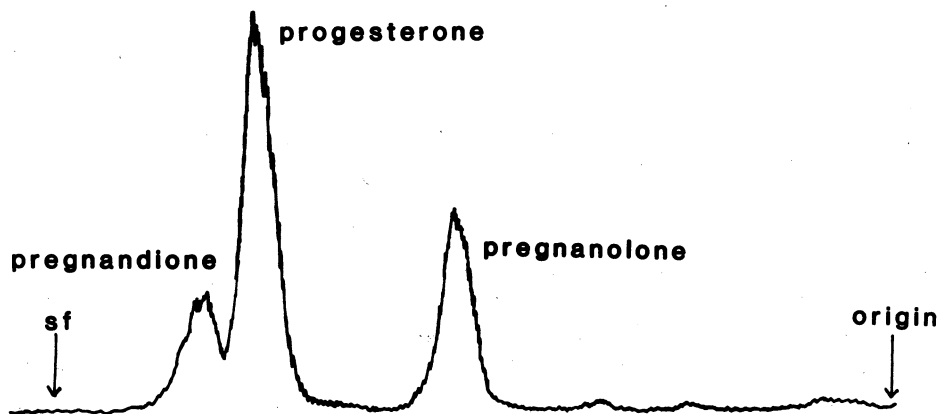
Fig. 5. TLC radioscan of the lipid recovered after incubation of $[4-^{14}C]$-progesterone and $[6,7,7,-^{2}H_3]$-progesterone with the ovaries of <u>Asterias rubens</u>. Progesterone (pregn-4-en-3-one), pregnandione (5α-pregnane-3,20-dione) and pregnanolone (3β-hydroxy-5α-pregnan-20-one) were added as markers and co-chromatographed with the radioactive zones indicated. TLC: silica gel (CHCl$_3$-acetone, 90:10).
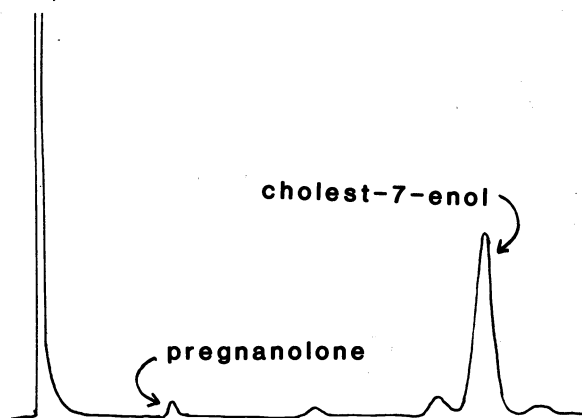

Fig. 6. Gas-chromatography of the pregnanolone zone recovered from the TLC separation shown in Fig.5. GC: 1% SE-30 at 240°.
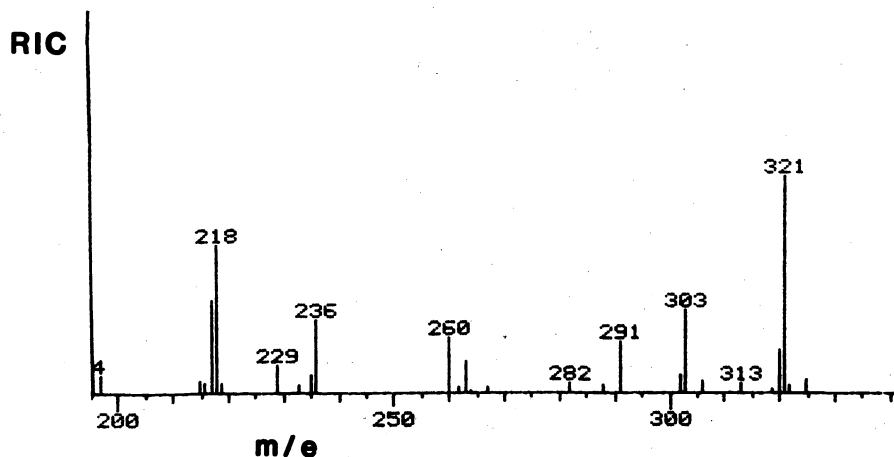

Fig. 7. The upper mass region of the mass spectrum of the pregnanolone peak shown on the GC trace in Fig. 6.

with its characteristic retention time on GC and its specific mass spectrum, suitably
displaced to higher mass by the appropriate deuterium content of the precursor, serve to
provide powerful criteria for the identification of a metabolite. The initial isolation of
of possible metabolites can be facilitated by mixing the deuterium labelled compound with
the [^{14}C]-labelled form prior to incubation followed by TLC radioscanning of the metabol-
ites. This method has been used to investigate mammalian steroidogenesis using mono-
deuterated pregnenolone as the substrate (Ref. 96) and we therefore decided to examine the
potential use of this technique for the investigation of steroid transformations by marine
invertebrates (Ref. 97).

[6,7,7-^{2}H$_3$]-Progesterone was synthesised, mixed with [4-^{14}C]-progesterone and incubated
with minced female gonad of A. rubens. TLC radioscanning of the recovered sterols
showed peaks for unchanged progesterone and two metabolites co-chromatographing with 5α-
pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one, respectively (Fig. 5). Elution of
the 3β-hydroxy-5α-pregnan-20-one band and GC-MS showed a very minor peak with the
retention time of this steroid and a large peak for contaminating cholest-7-en-3β-ol which
has a TLC mobility only slightly higher on the system used (Fig. 6). The identity of the
3β-hydroxy-5α-pregnan-20-one was finally confirmed by the mass spectrum which showed a
molecular ion and characteristic fragmentation ions for this steroid but all displaced by
three mass units as required by the incorporation of [6,7,7-^{2}H$_3$]-progesterone (Fig. 7).
Careful examination of the data processed by the on-line data system also revealed the
presence of traces of a second metabolite in this fraction which had a shorter retention
time and a mass spectrum (M$^+$ at m/e 321) indicating it to possibly be trideuterated
3β-hydroxy-5β-pregnan-20-one which had not been suspected from the previous studies using
[4-^{14}C]-progesterone alone (Ref. 92). GC-MS of the TLC band containing the suspected 5α-
pregnane-3,20-dione also confirmed the identity of this compound as a metabolite again with
a trace of a second metabolite thought to be the 5β-compound. These results demonstrate
the potential value of deuterium labelled steroids for invertebrate studies and an
extension of this technique should hopefully be useful to continue some of the invest-
igations referred to in this article.

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