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 interconversions 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 elucidation 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, shortened 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 information 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 93% 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 \(^{14}\text{C}\)-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.

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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 therefore 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 ophiuroids contain predominantly \(\Delta^5\)-sterols and cholesterol (1) is the sterol synthesised (Ref. 3, 9-11). By contrast in the free sterols of asteroids and holothuriods \(\Delta^7\)-sterols are
major constituents and in these animals cholest-7-en-3β-ol (2) is the product of sterol synthesis (Ref. 3, 9, 12-16).

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 considerations. 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 radiocal 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.

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 contributions 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 [2-14C]-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 echiuoderms 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-4 bond introduction.

The choice of acetate or mevalonate can have an important bearing upon the results obtained and their interpretation for several reasons. [14C]-Acetate first requires conversion to acetyl-CoA by a thiolokinase 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 aggravated in animals
with a slow rate of sterol synthesis and most of the administered [14C]-acetate may be
rapidly depleted by competing pathways so rendering prolonged incubations of dubious value
unless the [14C]-acetate is replenished periodically. The possibility that the thiol kinase
may be limiting the utilisation of [14C]-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 [14C]-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 [14C]-acetate whereas
[14C]-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 [14C]-mevalonate is the precursor of choice because its use will not only by-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
table-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
synthesize 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 photosyn-
thetic 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 ident-
ification 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 estab-
lished. 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 [14C]-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 [14C]-acetate or mevalonate into the total sterol recovered after chromato-
graphy, sometimes after digitonin precipitation, and often after several recrystallizations.
However, as most of the animals investigated contain complex mixtures of C26, C27, C28, C29
and C30-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
[2-14C]-mevalonic acid (Ref. 3, 13).

If it is accepted that the production of C28 and C29-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 C27-sterol can not be
taken as prima facie evidence that the sterol has been produced by the animal for two
reasons. Firstly, C27-sterols (cholesterol and desmosterol) are produced by some algae such
as members of the Rhodophyceae (Ref. 3) and more significantly by some dinoflagellates which
are the class to which many zooxanthellae belong (Ref. 24-26). Secondly, C28 and C29-sterols
Fig. 1. Minor 4,4-dimethyl and 4α-methyl sterols identified in the starfish 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<sub>27</sub>-sterol. Pertinent to the latter consideration are the reports of dealkylation of C<sub>29</sub>-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-methylencholesterol (Ref. 31-34). The labelled C-24 alkylated sterols isolated in these studies may conceivably 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<sub>28</sub> and C<sub>29</sub>-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 (1) added as an ethanol solution to the aquarium water can be absorbed by the sponges and metabolised to produce either the A-nor stanol (4) by Axinella verrucosa or the 19-norstanol (6) by A. polypoides (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 [4-¹⁴C]-cholesterol and [7α-³H]-cholestanol to A. polypoides resulted in the incorporation of ¹⁴C only into 4 showing that a Δ⁵-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 Δ⁵-bond is preferentially used by A. verrucosa for production of 3 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 (5) which was also isolated as a cholesterol metabolite from this sponge. Δ⁵-Stanols (6) are found in considerable amounts in several sponges (Ref. 2, 3, 39-42) and the identification of 5 in some of these species (Ref. 40) and as a metabolite of cholesterol (Ref. 38) provides very strong evidence that they are produced from Δ⁵-sterols by the oxidation-reduction sequence 1 → 5 → 6 which is well established in mammals and also in starfish (Ref. 3, 43). The prime question to be answered concerning the production of stanols 3, 4 and 6 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 Δ⁵-sterols to 3α-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 Δ⁵,7-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 Δ⁵,7-sterols but the possibility should also be considered that the sponge can produce them from dietary Δ⁵- or Δ⁷-sterol which is an ability displayed by some unicellular protozoa such as Tetrahymena pyriformis (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 tranmethylation reactions mediated by the sponge itself. An attempt to incorporate [¹⁴C]-methionine into aplysterol by Verongia aerophoba 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 Calyx nicamia (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.

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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 argument 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 3β,20α- and 3β,20β-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 mechanisms 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 interesting to see if these compounds can be detected in marine organisms.
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-Hydroxyprog-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 C22-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 C27-sterols from more complex mixtures of dietary sterols while dealkylation of C27-sterols has also been reported in one coelenterate species (Ref. 27). With those coelenterates which contain large quantities of unusual steroids 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 tranmethylation 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 occurrence 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 tranmethylation reaction to produce C28-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 C27-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 Δ(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 occurring sterol, suggests that this and other Δ(5,7)-sterols, may be produced in the molluscs by conversion of the corresponding dietary Δ(5,7)-sterol. In this connection the conversion of Δ(7)-sterol to Δ(5,7)-sterol is already known to occur in some protozoa and insects (Ref. 22). The bioconversion of cholesterol to cholest-5-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 [4-14C] sitosterol into the Δ(5,7)-sterol fraction of two molluscs was described by Teshima et al. (Ref. 30). Production of Δ(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 Charonia tritonis which feeds on the starfish Acanthaster plancii (Ref. 75). The triton therefore might be expected to derive a large proportion of Δ(5)-sterol in its diet, and this could be metabolised by the "conventional" sterol biosynthetic sequence Δ(7) → Δ(5,7) → Δ(5).

All five classes of echinoderm contain mixtures of C26 to C30-sterols but they can only synthesise C27-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 C26 to C30 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 [4-14C]- and [3α-3H, 4-14C]-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 Δ(5,7)-sterol (Fig. 4) and was suggested by the results obtained from injections of [3α-3H, 4-14C]-cholesterol into starfish. These experiments consistently gave a very low H: C ratio in the 5α-cholestanol as expected from the intermediacy of
cholest-4-en-3-one (Ref. 77) but the H: C ratio of cholest-7-en-3-ol was always substantially higher and therefore was inconsistent with the 5α-cholestanol being the sole precursor (Ref. 3, 79). Accordingly [3α-3H,4-14C]-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 \( \Delta^5 \rightarrow \Delta^{5,7} \rightarrow \Delta^7 \) but the conversion of cholesterol into cholesta-5,7-dien-3β-ol remains to be demonstrated. Conversion of the \( \Delta^{5,7} \)-sterol to cholesterol 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 \( \Delta^7 \)-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 \( \Delta \) rubens and it may perhaps be of relevance to asterosaponin production (Ref. 73, 79).

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

Catabolism of [4-14C]-cholesterol and reincorporation of the label into a wide range of non-sterol lipid by Asterias rubens has been reported (Ref. 76) but reinvestigation seems warranted. Both steryl esters and sterol sulphates are appreciably labelled after administration of [4-14C]-cholesterol to \( \Delta \) rubens (Ref. 78, 80) and these have chromatographic 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 up to 1976-77 have been discussed elsewhere (Ref. 3, 84). Briefly, several C18, C19 and C21-steroid hormones have been identified, although in some cases rather tentatively. Also several ecdysones, the C27-steroid hormones found in crustaceans, have been characterised. A range of enzyme activities (e.g. oxidase, reductase, 17α-hydroxylase, C17,C20 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 caeca 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 radioimmunoassay 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 caeca of females were reported to metabolise cholesterol to pregnenolone and progesterone while progesterone was converted into 17α-hydroxyprogesterone, androstenedione, testosterone, 20α-hydroxyprogesterone, 11-deoxy cortisolosterone and 5α-pregn-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-cholesterol, 5α-cholestan-3β,6α-diol (which may be pertinent to asterosaponin production), 3β-hydroxy-5α-pregn-20-one and 5α-pregn-3,20-dione. The various progesterone metabolites identified from progesterone metabolism in vivo with isolated gonads, or with gonad homogenates, were 3β-pregn-3,20-dione, 3β-hydroxy-5α-pregn-20-one, 5α-pregn-3,20-dione and 3β,6α-dihydroxypregnen-20-one but androgens were not detected as metabolites. The incubation of [3H]-androstan-4-ene-3,17-dione with male and female gonads produced 5α-androstan-3,17-dione, 3β-hydroxy-5α-androstan-17-one, 5α-androstan-3β,17β-diol and testosterone as the main metabolites (Ref. 93). Incubation of labelled 17α-hydroxyprogesterone with A. rubens gonads yielded 17α-hydroxy-5α-pregn-3,20-dione and 3β,17α-dihydroxy-5α-pregn-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
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[4-14C]-progesterone and [6,7,7,-2H3]-progesterone with the ovaries of Asterias rubens. Progesterone (pregn-1-ene-3-one), pregnandione (5α-pregnane-1,20-dione) and pregnanolone (3β-hydroxy-5α-pregn-20-one) were added as markers and co-chromatographed with the radioactive zones indicated. TLC: silica gel (CHCl3-acetone, 90:10).

Fig. 5. TLC radioscan of the lipid recovered after incubation of [4-14C]-progesterone and [6,7,7,-2H3]-progesterone with the ovaries of Asterias rubens. Progesterone (pregn-1-ene-3-one), pregnandione (5α-pregnane-1,20-dione) and pregnanolone (3β-hydroxy-5α-pregn-20-one) were added as markers and co-chromatographed with the radioactive zones indicated. TLC: silica gel (CHCl3-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 2400.

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 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 metabolites. This method has been used to investigate mammalian steroidogenesis using monodeuterated 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).

\[ \text{[6,7,7-}^2\text{H}^-] - \text{Progesterone was synthesised, mixed with [4-}^1\text{C}] \text{-progesterone and incubated with minced female gonad of } A. \text{ rubens. TLC radioscanning of the recovered steroids showed peaks for unchanged progesterone and two metabolites co-chromatographing with 5\alpha\text{-pregnane-3,20-dione and 3\beta-hydroxy-5\alpha\text{-pregnan-20-one, respectively (Fig. 5). Elution of the 3\beta-hydroxy-5\alpha\text{-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-3\beta-ol which has a TLC mobility only slightly higher on the system used (Fig. 6). The identity of the 3\beta-hydroxy-5\alpha\text{-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\text{H}^-] \text{-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/z at m/e 321) indicating it to possibly be trideuterated 3\beta-hydroxy-5\beta\text{-pregnane-20-one which had not been suspected from the previous studies using [4-}^1\text{C}] \text{-progesterone alone (Ref. 92). GC-MS of the TLC band containing the suspected 5\alpha\text{-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 5\beta\text{-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 investigations referred to in this article.}

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