(Willd.) Hook. f., and berries of *Luvunga scandens* (Roxb.) Buch.-Ham. (family Rutaceae)[22,23]; and roots of *Foeniculum vulgare* Mill., *Selinum vaginatum* C.B. Clarke, and *Seseli sibiricum* Benth. (family Umbelliferae)[18,24,25]. Only in *L. scandens* and *C. lawsoniana* has the fenchol been reported to be optically active; the l-β-isomer has been definitely identified in *L. scandens*.

Fenchol has never before been reported in an animal product. Its occurrence in completely laboratory-reared male medflies was totally unexpected, since their rearing diet[26] contains no plant material other than wheat shorts. A steam distillate of wheat shorts showed no trace of fenchol by spectral analyses and consisted almost completely (92%) of 2-acetylpyrazine[3]. (−)-β-Fenchol was unattractive to either sex of medflies in laboratory tests and in the field on the infested islands of Maui and Hawaii. Combinations with MEN + (*E*)-6-nonen-1-ol + medfly acids in their natural proportions also failed to attract females in the field.

1 Mention of a proprietary product in this paper does not constitute a recommendation or an endorsement of the product by the US Department of Agriculture.

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Metabolism in Porifera XII. Further informations on the biosynthesis of 3β-hydroxymethyl-A-nor-steranes in the sponge *Axinella verrucosa*

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Summary. Direct incorporation and cold trap experiments suggest that cholest-4-en-3-one is an intermediate in the conversion of cholesterol into 3β-hydroxymethyl-A-nor-cholestane in the marine sponge *Axinella verrucosa*. Cholest-4-en-3-one is further transformed by the sponge into cholest-4-en-3β-ol, 5α-cholestan-3-one and 5α-cholestan-3β-ol; these compounds arise from side reactions, which are not part of the major metabolic route leading to 3β-hydroxymethyl-A-nor-steranes.

We have previously shown that the conversion[1] of cholesterol (**1**) into 3β-hydroxymethyl-A-nor-cholestane (**2**) by the sponge *Axinella verrucosa*, which contains as the sole sterol components a novel group of A-nor-stanols[2], involves the formation of a C-C linkage between the C-4 and C-2 of cholesterol, while the C-3 provides the hydroxymethyl carbon[3], and there is a loss of the 3α- and 4β-hydrogen atoms of cholesterol[4]. On these results a 3-oxo-Δ^4-steroid was also postulated[4] as an intermediate.

This paper presents the results of an investigation undertaken to examine the intermediacy of cholest-4-en-3-one (**3**) and other supposed intermediates in the above-mentioned conversion.

Materials and methods. [4-^{14}C]-Cholesterol (57 mCi/mmole) was supplied by the Radiochemical Centre, Amersham (Bucks., Great Britain). [4-^{14}C]-Cholest-4-en-3-one was prepared by an Oppenauer oxidation[5] of [4-^{14}C]-cholesterol and purified by preparative TLC.

Feeding experiments (10-day incubations) and the extraction of the metabolites were performed as previously described[1,3]. The weights of the extracts of each experiment are reported in the tables; from each g of the crude extracts 200–250 mg of 3β-hydroxymethyl-A-nor-steranes were recovered.

The extracts were chromatographed on a silica gel column (benzene-diethyl ether), after addition of the appropriate carrier material, and the metabolites were purified to a constant specific activity as follows. Cholesta-4-en-3-one (**3**), 5α-cholestan-3-one (**4**) and cholesterol (**1**) were purified by preparative TLC and crystallization. To 5α-cholestan-3β-ol (**5**) fractions 3-chloroperbenzoic acid was added to remove any unsaturated contaminant[6] and then 5α-cholestan-3β-ol (**5**) was recovered by preparative TLC (benzene-diethyl ether, 1:1) and purified by crystallization. 3β-Formyl-A-nor-steranes (partial structure **6**) were purified by preparative TLC and oxidation to the correspond-

ing 3β-carboxylic acids[2], which, in turn, were purified by preparative TLC.

3β-Hydroxymethyl-A-nor-steranes and cholest-4-en-3β-ol (7) were recovered unresolved from the silica gel column chromatography; the separation of these 2 compounds was performed after a chemical modification. The mixture was treated with MnO_2 in benzene to transform[7] cholest-4-en-3β-ol (7) into cholest-4-en-3-one (3) which was isolated by preparative TLC and crystallized, while the unaffected 3β-hydroxymethyl-A-nor-steranes were oxidized to the corresponding 3β-carboxylic acids[2] which were purified by preparative TLC.

Results and discussion. In a 1st experiment the sponge was fed with labelled cholest-4-en-3-one (3) (experiment 1) and the precursor was converted into 3β-hydroxymethyl-A-nor-steranes to a significant extent (table 1).

In this experiment the presence of a radioactive fraction of negligible mass was detected. This was shown to be 5α-cholestan-3β-ol (5) by co-chromatography and co-crystallization with a variety of standards.

In order to confirm the intermediacy of cholest-4-en-3-one (3) in the conversion of cholesterol into 3β-hydroxymethyl-A-nor-cholestane a cold trap experiment was performed, feeding the sponge with labelled cholesterol (1) and cold cholest-4-en-3-one (3).

The results (experiment 2, table 1) support the hypothesis that cholest-4-en-3-one (3) is an intermediate since the radioactivity recovered in the 3β-hydroxymethyl-A-nor-steranes fraction is about $^1/_{10}$ of that recovered in cholest-4-en-3-one, a behaviour expected from this kind of experiment.

Since in this experiment radioactive 5α-cholestan-3β-ol (5) was also isolated, the sponge was fed with labelled cholesterol (1) and cold 5α-cholestan-3β-ol (5) to investigate the role of the latter in the biosynthetic pathway. About the same amount of radioactivity was found to be associated with the 3β-hydroxymethyl-A-nor-steranes and the 5α-cholestan-3β-ol fraction (experiment 3, table 1), supporting the early hypothesis[3], based on comparative levels of incorporation in a competitive uptake experiment (cholesterol vs 5α-cholestan-3β-ol), that although 5α-cholestan-3β-ol (5) can act as precursor of 3β-hydroxymethyl-A-nor-cholestane (2), it does not seem to be an intermediate of the major pathway. In that case, in fact, a substantially smaller amount of radioactivity should be expected to be found associated with the 3β-hydroxymethyl-A-nor-steranes fraction, in analogy with experiment 2.

Having established the role of 5α-cholestan-3β-ol (5) as a side reaction product susceptible of transformation in to 3β-hydroxymethyl-A-nor-cholestane (2), our next problem was to follow the fate of cholest-4-en-3-one (3) in order to have additional information about the biosynthetic pathway. An attempt was therefore made to trap in the crude extract, after incubation with labelled cholesterol, compounds of negligible mass possibly related to cholest-4-en-3-one.

Since the transformation of this latter could occur via numerous pathways, we decided to follow its reduction products, namely 5α-cholestan-3-one (4) and cholest-4-en-3β-ol (7). In addition, the presence of a 3β-formyl moiety was investigated, to test the hypothesis that the mechanism of the ring contraction was reminiscent of that in gibberellin biosynthesis[8–10].

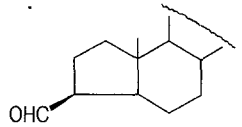

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Table 1. Incorporation data from experiments 1, 2 and 3

Administered substrates	Experiment 1 [4-^{14}C]-Cholest-4-en-3-one (3, 32 μCi; 57 mCi/mmole)		Experiment 2 [4-^{14}C]-Cholesterol (1, 50 μCi; 57 mCi/mmole) + Cholest-4-en-3-one (3, 5 mg)		Experiment 3 [4-^{14}C]-Cholesterol (1, 50 μCi; 57 mCi/mmole) + 5α-cholestan-3β-ol (5, 5 mg)	
Weight of the crude extract (g)	1.852		2.613		3.134	
Radioactivity recovered	Total dpm	%*	Total dpm	%*	Total dpm	%*
3β-Hydroxymethyl-A-nor-steranes	2.0×10^6	2.78	3.5×10^4	0.03	1.7×10^6	1.55
Cholest-4-en-3-one** (3)	2.1×10^7	29.2	2.4×10^5	0.22	3.2×10^5	0.29
5α-Cholestan-3β-ol** (5)	4.6×10^6	6.39	2.2×10^5	0.20	1.7×10^6	1.55
Cholesterol** (1)			2.5×10^7	22.7	2.5×10^7	22.7

* Percent of the administered radioactivity. ** Isolated after addition to the crude extract of 100 mg of the cold compound.

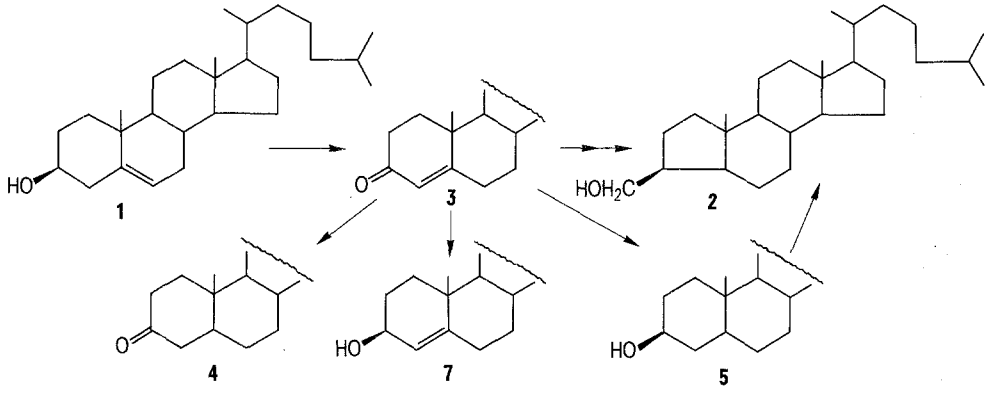
Table 2. Incorporation data from experiments 4, 5 and 6

Administered substrates	Experiment 4 [4-^{14}C]-Cholesterol (1, 50 μCi; 57 mCi/mmole)		Experiment 5 [4-^{14}C]-Cholesterol (1, 25 μCi; 57 mCi/mmole) + Cholest-4-en-3β-ol (6, 5 mg)		Experiment 6 [4-^{14}C]-Cholesterol (1, 25 μCi; 57 mCi/mmole) + 5α-Cholestan-3-one (4, 5 mg)	
Weight of the crude extract (g)	2.928		1.450		1.436	
Radioactivity recovered	Total dpm	%*	Total dpm	%*	Total dpm	%*
3β-Hydroxymethyl-A-nor-steranes	1.38×10^7	12.61	1.24×10^6	2.26	3.17×10^6	5.76
Cholest-4-en-3-one** (3)	1.58×10^6	1.44	2.60×10^5	0.43	3.20×10^5	0.58
5α-Cholestan-3β-ol** (5)	7.0×10^6	6.36	1.57×10^6	2.86	1.24×10^6	2.25
Cholest-4-en-3β-ol** (7)	2.52×10^6	2.29	2.60×10^5	0.06	5.80×10^4	0.11
5α-Cholestan-3-one** (4)	6.96×10^4	0.06	1.69×10^4	0.03	2.30×10^4	0.04
3β-Formyl-A-nor-steranes** (6)	-					
Cholesterol** (1)	2.01×10^7	18.3	6.86×10^6	12.47	2.85×10^6	5.18

* Percent of the administered radioactivity. ** Isolated after addition to the crude extract of 100 mg of the cold compound.

The failure to detect any radioactivity in the 3β-formyl-A-nor-steranes (part structure **6**) fraction (experiment 4, table 2) is convincing evidence that this aldehyde is not a product of the metabolism of cholesterol in the sponge, whereas different amounts of radioactivity were found associated with 5α-cholestan-3-one (**4**) and cholest-4-en-3β-ol (**7**) indicating that cholest-4-en-3-one (**3**) is reduced to these latter in the sponge.

In order to evaluate the role of these reduction products as intermediates in the conversion cholesterol/3β-hydroxymethyl-A-nor-cholestane 2 separate cold trap experiments were performed (experiments 5 and 6). The results (table 2), showing that low radioactivity is associated with the supposed intermediates, indicate that 5α-cholestan-3-one (**4**) and cholest-4-en-3β-ol (**7**) represent side reaction products of the metabolic fate of cholest-4-en-3-one (**3**). The results presented in this paper are summarized in the scheme.



Scheme. Intermediates and side products in the conversion of cholesterol into 3β-hydroxymethyl-A-nor-cholestane.

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Purification of canine myocardial mitochondrial creatine kinase[1]

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Summary. Mitochondrial creatine kinase, purified for the first time, is a dimeric molecule with a mol.wt of 82,000 and does not hybridize with M or B subunits or react to their specific antiserum. A specific antiserum to the mitochondrial form was developed which does not cross-react with the B or M subunits. Thus, the mitochondrial form is biochemically and immunologically unique.

Creatine kinase (EC 2.7.3.2) is known to exist in 3 molecular forms (isoenzymes) which based on their subunit composition are referred to as BB, MB and MM creatine kinase. These isoenzymes are present in the cytosol and based on their different net charge in an alkaline medium can be separated by electrophoresis. Mitochondria[3] are known to be rich in creatine kinase which on electrophoresis is positively charged exhibiting a distinctly different electrophoretic mobility from that of the cytosolic isoenzymes. However, skepticism remains since only 2 subunits are known. Attempts to purify mitochondrial creatine kinase have been unsuccessful due in part to its chemical lability and consistent contamination with MM creatine kinase[4,5]. In the present report, mitochondrial creatine kinase has been obtained in pure form and shown to be immunologically and biochemically distinct from that of cytosolic creatine kinase isoenzymes.

Material and methods. Creatine kinase activity was determined by the coupled enzyme system as previously described[6] and isoenzymes analyzed by electrophoresis[7]. Sodium-dodecyl-sulphate polyacrylamide gels were performed according to the method of Fairbanks et al.[8] and molecular weight assessed by comparison with markers of known molecular weight according to the method of Weber and Osborn[9]. Creatine kinase isoenzymes MM and MB were purified from dog heart and brain as previously described[10] and labelled with ^{125}I[11]. Mitochondrial CK was labelled with ^{125}I and specific antiserum developed according to the methods used for the development of antisera for cytosolic CK isoenzymes[12,13].

Canine hearts for purification of mitochondrial creatine kinase were removed immediately after sacrifice of the animal and minced with scissors and homogenized in a Potter-Elvehjem power homogenizer. Homogenizing medium (10 ml/g) contains sucrose (0.075 M), mannitol (0.225 M), EDTA (0.002 M) and MOPS (0.05 M) at a pH of 7.2. After homogenization, centrifugation was performed at 1000 × g (4 °C) for 10 min. The supernatant was centrifuged