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Comparison of the Sterol-Synthesizing Ability in Some Marine Invertebrates

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Abstract

The present study deals with the *in vitro* biosynthesis of sterols from mevalonate-2-^{14}C in the liver or hepatopancreas extracts of rat, carp, mollusks (*Tapes philippinarum*, *Buccinum undatum*, and *Liolophura japonica*), and crustacean (*Ibacus ciliatus*). In all animals except the crustacean, mevalonate-2-^{14}C was incorporated into sterol fraction more or less. However, the percentage incorporation of mevalonate into sterol fraction in the mollusks examined was low as compared with those in the rat and carp.

In higher animals, sterols are generally synthesized from acetate via squalene etc. In lower animals such as marine invertebrates, however, sterols appear to be not always formed from lower units. For example, some species of mollusks[1-4], coelenterates[4,5], porifera[4], and echinoderms[1,6], and most crustaceans[7] have been supposed to lack the ability for sterol synthesis from acetate and mevalonate. Several workers have also assumed that most part of sterols occurring in some marine invertebrates may be derived from diets, because the incorporation rate of precursors into the sterol and squalene fractions after injection of radioactive acetate and mevalonate was extremely low[8-10]. The above facts suggest that the sterol-synthesizing ability in marine invertebrates varies from species to species.

In previous investigations, most workers have evidently deduced the sterol synthesizing ability of marine invertebrates on the basis of the results of *in vivo* tracer experiments. In this study, the authors examined the *in vitro* biosynthesis of sterols from mevalonate about the several invertebrates and vertebrates in order to compare the sterol-synthesizing ability among them.

Materials and Methods

Chemicals Cholesterol and squalene were purchased from Nakarai Chemical Co. Ltd. (Japan). Mevalonate-2-^{14}C (5–10 mCi/m mol) was obtained from Département des Radioéléments, Service des molécules marquées (Gif-Sur-Yvette, France).

Animals A rat was the Wister strain of male, 200 g in body weight. A carp, *Cyprinus carpio*, 800 g in body weight, was obtained from the commercial supplier.

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The Short-necked clam, *Tapes philippinarum* (class Pelecypoda), whelk, *Buccinum undatum* (class Gastropoda), and crustacean, *Ibacus ciliatus* (class Crustacea; Japanese name, Uchiwaebi) were obtained from a fishmonger in Kagoshima. A chiton, *Liolophura japonica* (Mollusca, class Polyplacophora), was harvested at the tide pool near Sakurajima in Kagoshima. These animals were kept alive until use.

Cell-free extracts From the animals, 5 g of the liver or hepatopancreas was excised out, cut into small pieces, and homogenized in 7 ml of phosphate buffer (pH 7.4) by using a glass homogenizer with Teflon pestle at 0–2°C. The homogenate was centrifuged at 3000 *g* for 15 min at 0–2°C, and then the volume of the supernatant (cell-free extract) so obtained was adjusted to 7.5 ml with phosphate buffer.

Incubation with mevalonate-2-^{14}C To 1.5 ml of the cell-free extract, equivalent to 1 g of wet liver or hepatopancreas, the cofactors, antibiotics, and 0.11 μCi of mevalonate-2-^{14}C in 1.5 ml of phosphate buffer were added, oxygenated, and then incubated in a 50 ml-flask covered with an aluminum foil with shaking at 22–23°C. Each incubation mixture contained the following cofactors[11] (μ mol) and antibiotics (mg): glucose-6-phosphate, 17; glutathione, 60; $MgCl_2$ $6H_2O$, 25; nicotinamide-adenine dinucleotide, 23; reduced nicotinamide-adenine dinucleotide, 7; reduced nicotinamide-adenine dinucleotide phosphate, 3; adenosine triphosphate, 13; nicotinamide, 200; glucose, 100; streptomycin, 0.1; penicillin G, 0.1.

Separation of lipid fractions To the incubation mixture, 12 ml of chloroform-methanol (1: 1) was added, homogenized with a Waring blender, and the chloroform phase was concentrated and saponified with 10% alcoholic potassium hydroxide at 80°C for 2 hr. The unsaponifiable matters were extracted from the saponification mixture with ether in the usual manner. An aliquot of the unsaponifiable matters was subjected to TLC on Kieselgel G with benzene-ethyl acetate (4: 1) followed by autoradiography. To the unsaponifiable matters, 2 mg of carrier cholesterol was added, and the sterols were isolated by the digitonin method[12]. The free sterols were obtained by decomposing the digitonide with dimethylsulfoxide followed by extraction with hexane. The squalene fraction was obtained from the unsaponifiable matters by TLC on Kieselgel G with acetone-benzene (4: 1). After TLC, the zone corresponding to carrier squalene was scraped off and squalene was eluted with chloroform-methanol (3: 1).

Check of radioactivity Radioactivity was determined with a Beckman scintillation counter, model LS–230, using a solution of 2, 5-diphenyloxazole (0.6%) as a scintillator. The efficiency of counting of radioactive substances was about 90 per cent. In autoradiography, thin-layer plate was covered with a Sakura X-ray film, and then the film was developed after 3 weeks.

Results

After the incubation of cell-free extracts prepared from the liver or hepatopancreas of several animals with mevalonate-2-^{14}C, the incorporation of mevalonate into the

unsaponifiable matter and sterol fractions was investigated. An aliquot of radioactive unsaponifiable matters from a 4 hr-incubation was subjected to TLC on Kieselgel G with benzene-ethyl acetate (4: 1) and autoradiographed. The results are shown in Fig. 1. In all animals except the crustacean, *I. ciliatus*, mevalonate-2-^{14}C was incor-

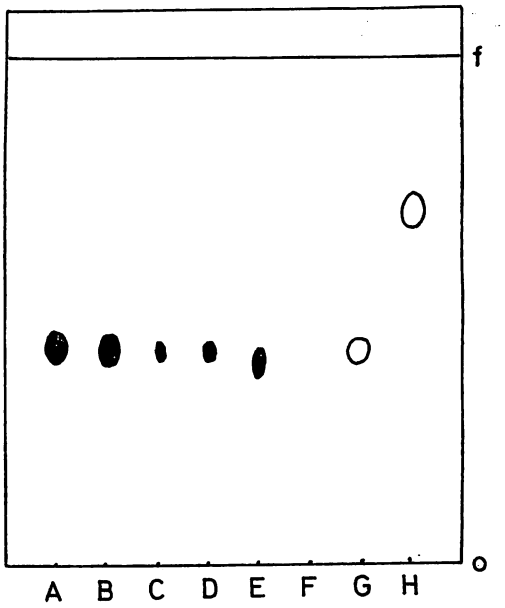

Fig. 1. Autoradiography of the sterols isolated from the 4 hr-incubation of cell-free extracts with mevalonate-2-^{14}C. The sterols were subjected to TLC on Kieselgel G and autoradiographed.
A, rat; B, carp; C, short-necked clam; D, whelk; E, chiton; F, crustacean (*I. ciliatus*); G, cholesterol; H, squalene.

Table 1. Incorporation of mevalonate-2-^{14}C into the unsaponifiable matter and sterol fractions in a 4 hr-incubation of the extracts of liver or hepatopancreas with mevalonate-2-^{14}C.

Animals	Unsaponifiable matters		Sterols	
	Radioactivity (cpm)	% Incorporation*	Radioactivity (cpm)	% Incorporation*
Rat	7,500	3.71	4,230	2.10
Carp	29,600	14.8	5,200	2.61
Short-necked clam	1,380	0.69	105	0.053
Whelk	5,850	2.93	431	0.22
Chiton	571	0.29	126	0.063
Crustacean, *I. ciliatus*	835	0.42	0	0

* The percentage incorporation was obtained by dividing the radioactivity recovered in the unsaponifiable matter and sterol fractions by the radioactivity of mevalonate-2-^{14}C added initially.

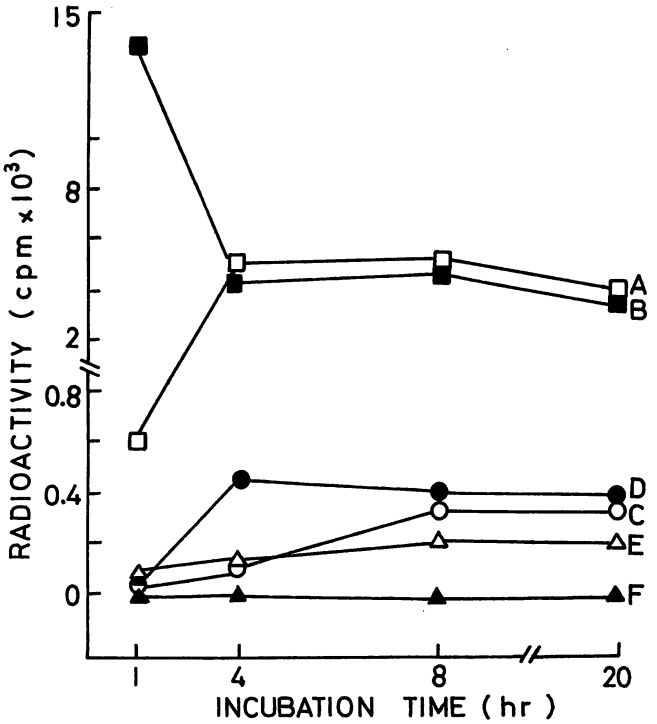

Fig. 2. Time-course of the incorporation of mevalonate-2-^{14}C into the sterols in the incubation of cell-free extracts of liver or hepatopancreas with mevalonate-2-^{14}C.
A, rat; B, carp; C, short-necked clam; D, whelk; E, chiton; F, *I. ciliatus*.

porated into sterol fraction, although a significant incorporation into squalene fraction was not detected. The results indicated that every animal except the crustacean is capable of synthesizing sterols from mevalonate. Table 1 shows the total radioactivity recovered in the unsaponifiable matter and sterol fractions of the 4 hr-incubation. Compared with the vertebrates, carp and rat, the incorporation of mevalonate into sterols was extremely low or not perceived in the marine invertebrates. In the gastropod, *B. undatum,* the radioactivity recovered in the unsaponifiable matters was high as well as in the rat, but the sterols gave very lower radioactivity than in the rat.

In order to study the time-course of the incorporation of mevalonate-2-^{14}C into the unsaponifiable matter and sterol fractions, each cell-free extract was incubated with 0.11 μCi of mevalonate-2-^{14}C for the period of 1 to 20 hr. The results are given in Fig. 2. In the carp and rat, the incorporation of mevalonate-2-^{14}C into the unsaponifiable matter and sterol fraction was rapid and reached its maximum within 1 hr and 4 hr, respectively. In the marine invertebrates except *B. undatum,* the incorporation of mevalonate was low throughout the incubation period, although there was a slight increase of incorporation. Also, in all mollusks examined, the incor-

poration of mevalonate into sterols was lower than in the vertebrates and reached its maximum after relatively longer incubation times to the carp, belonging to the same aquatic aminal; especially, no significant radioactivity was detected in the sterol fraction of the crustacean, *I. ciliatus,* throughout the incubation period. Compared with the maximum value of radioactivity recovered in the sterol fraction, the incorporation of mevalonate into sterols with the carp was 35–50 times greater than with the three species of marine mollusks under the experimental conditions adopted. If the pathway of sterol biosynthesis in the marine mollusks is the same as in the carp and rat, it may be postulated that in the mollusks the enzymatic activity for sterol biosynthesis is extremely low or the sterol biosynthesis takes place at extremely slow rate.

Discussion

Since ZANDEE's report[13] showing the absence of sterol synthesis from acetate in a crayfish, *Astacus astacus,* many workers have investigated the biosynthesis of sterols in marine invertebrates, and have demonstrated that some marine invertebrates[1-7] lack the sterol-synthesizing ability from acetate and mevalonate. Most investigations about sterol biosynthesis in marine invertebrates have been performed by the injection of radioactive precursors such as acetate-1-^{14}C and mevalonate-2-^{14}C into animals, and the conclusion has been drawn on the basis of the detection of radioactive squalene and sterols by means of measurement of radioactivity and autoradiography after separation of both the substances by chromatography, or constant specific activity (dis/min/mg) of sterols during repeated crystallizations. Number of workers have concluded about the sterol biosynthesis whether invertebrates examined possess the ability or else do not. However, due to the discrepancy in the dosage of radioactive precursors, the keeping period after administration of precursors, and the criterior for the incorporation of precursors into sterol and squalene fractions, the conflicting results have been occasionally obtained about the sterol-synthesizing ability even in the same species of animals.

In the present study, the authors intended to compare the sterol-synthesizing ability among the mollusks, crustacean, rat, and teleost by the incubation of cell-free extracts with mevalonate-2-^{14}C. All animals except the crustacean, *I. ciliatus,* was capable of incorporating mevalonate into sterol fraction more or less. However, the percentage incorporation of mevalonate-2-^{14}C into the sterol fraction in the mollusks, *T. philippinarum, L. japonica,* and *B. undatum,* was low as compared with those in the vertebrates, carp and rat. In the *in vivo* studies, it has been reported that the chiton, *L. japonica,*[14] and the mussel, *Mytilus edulis,*[15] belonging to the same Pelecypoda as the short-necked clam, *T. philippinarum,* are capable of synthesizing sterols from lower units. Regarding the whelk, *B. undatum,* the results obtained in the present study is contrast with that of the *in vivo* investigation reported by VOOGT.[2] He has concluded that the whelk is incapable of synthesizing sterols from acetate-1-^{14}C by the fact that the specific activity (cpm/mg) of the isolated sterols after injection of acetate-1-^{14}C was

not significant as contrast with the fatty acid fraction. The authors assume that the discrepancy may be due to that of experimental conditions such as cofactors, precursors, and incubation methods in addition to the physiological state of the whelk.

Sterols are recognized to be an important substance for maintenance of life, because all animals and plants without exception contain sterols in their tissues as constituents of lipids. Therefore, if marine invertebrates lacking sterol-synthesizing ability are maintained on a sterol-free diet, they would show certain abnormal aspects. In fact, KANAZAWA *et al.*[16] have demonstrated the culture of a prawn, *Penaeus japonicus*, lacking the ability for sterol synthesis from acetate, with the sterol-free diet caused the inhibition of growth and molting. Accordingly, it may be advisable that the conclusion obtained by tracer experiments using possible precursors for sterol biosynthesis is confirmed by nutritional experiments. But, unfortunately the culture of marine invertebrates except a few animals using the diets composed of chemically known substances has not been succeeded yet.

The authors postulate that marine invertebrates may be put into the three groups about sterol biosynthesis: (1) possessing the sterol-synthesizing ability enough for providing for almost need of sterols; (2) possessing the sterol-synthesizing ability but not enough for their requirements (In these animals, the sterol biosynthesis from lower units probably does not take on a significant role in providing for their sterols, and the large portion of sterols occurring in their tissues is of dietary origins.); (3) lacking the sterol-synthesizing ability. The present study suggests that the three mollusks are capable of synthesizing sterols from mevalonate but the biosynthesis appears to proceed at extremely slow rate, and also that the crustacean, *I. ciliatus*, probably lack the sterol-synthesizing ability. But, the slow rate of sterol biosynthesis in the mollusks does not always imply that they require a dietary source of sterols.

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